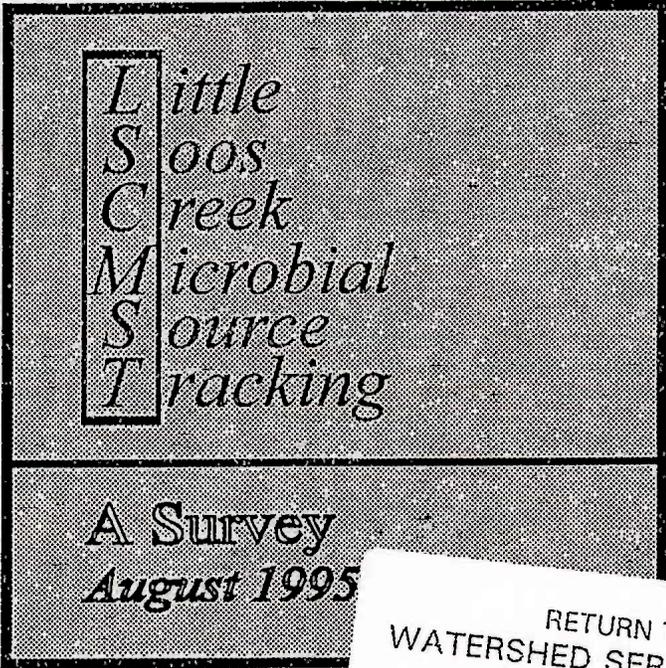




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LITTLE Soos Creek Microbial Source
King: a survey, 1995



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Prepared for:
**King County Department of Public Works
Surface Water Management Division**



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Water Verse

The stream, it tells a story of the land it passes through.
The land, it never stays the same for long.
The elements, creatures, and people, they change the land.
This change, it alters the stream-story to good or ill.
The people, they are able to read the water's verse
...and keep it sweet.

❧ *ACKNOWLEDGMENTS* ❧

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Dr. Mansour Samadpour* of the University of Washington Department of Environmental Health and Naomi Chechowitz of King County Surface Water Management are responsible for the study design, field sampling, and procedures carried out at the U.W. laboratory. They are the authors of this report.

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TABLE OF CONTENTS 

Acknowledgments..... i

Table of Contents..... ii

Executive Summary..... iv

1.0 Introduction..... 1

 1.1 Overview..... 1

 1.2 Background..... 1

 1.3 Little Soos Creek Watershed..... 3

 1.4 Study Goal and Objectives..... 5

2.0 Approach and Methods..... 7

 2.1 Conventional Analysis for Fecal Coliform Bacteria..... 7

 2.2 Microbial Source Tracking..... 8

 2.3 Sampling Procedures..... 10

 2.4 Bacterial Culture and Isolation..... 11

 2.5 DNA Isolation and Digestion..... 11

 2.6 Gel Electrophoresis and DNA Probing..... 12

 2.7 Ribotyping and Analysis..... 12

3.0 Results..... 15

 3.1 Receiving Water and Fecal Source Samples..... 15

 3.2 Fecal Coliform Enumeration..... 15

 3.3 Bacteria Isolates..... 19

 3.4 Ribotypes and Ribotype Diversity..... 19

 3.5 Water-to-Source Ribotype Matches..... 20

4.0 Discussion..... 27

 4.1 Ribotype Diversity..... 27

 4.2 Matching Efficiency..... 28

 4.3 Ribotype Confirmation and Database Development..... 28

 4.4 Source Contribution and Stream Distribution Mapping..... 30

5.0 Conclusions..... 33

6.0 Recommendations..... 35

References..... 36

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List of Figures

Figure 1. Land uses within the Covington MDP area, south King County, Washington. 2

Figure 2. Sampling locations within the Little Soos Creek watershed and vicinity. 6

Figure 3. Examples of ribotype autoradiograms from a group of *Escherichia coli* isolates. 13

List of Tables

Table 1. Summary of water data and fecal coliform bacteria concentrations in Little Soos Creek. 16

Table 2. Summary of source sample data from Little Soos Creek watershed and vicinity. 17

Table 3. Ribotype-to-isolate ratios as a coarse measure of diversity among water samples and locations..... 21

Table 4. Ribotype-to-isolate ratios as a coarse measure of diversity among source samples and types..... 22

Table 5. Cases of identical ribotypes appearing for two different source types. 23

Table 6. Distribution of *Escherichia coli* in Little Soos Creek (matched to sources by ribotypes)..... 24

Table 7. Summary of water-to-source ribotype and isolate matches for Little Soos Creek. 26

EXECUTIVE SUMMARY

*Purpose and Background**Introduction*

The Little Soos Creek Microbial Source Tracking survey was designed to help characterize the sources of fecal coliform bacterial contamination in Little Soos Creek. The study was conducted for King County Surface Water Management Division by investigators at the University of Washington Department of Environmental Health. It was begun in September 1993 and makes use of an innovative method for tracking microorganisms in the environment.

Little Soos Creek is located in the Covington area of southeast King County, Washington. In response to the impacts of existing and anticipated urban development in the area, King County Surface Water Management developed the Covington Master Drainage Plan (MDP) (King County, 1992). The purpose of the MDP is to address the management of water quality and quantity of the area's water resources and associated beneficial uses while accommodating development. The MDP was adopted by the King County Council in 1992. In 1993, the Covington Water Quality Monitoring Project (CWQMP) was begun to carry out specific monitoring activities recommended by the MDP. The Microbial Source Tracking study was initiated as part of the CWQMP in response to the recommendation that pollutant source monitoring for fecal coliform bacteria in Little Soos Creek be carried out.

Concentration of Fecal Coliform Bacteria

Little Soos Creek historically has been categorized as a Class A stream but violates fecal coliform standards for this classification. There are two water quality criteria for fecal coliform bacteria levels in Class A freshwater. The concentration of organisms is not to exceed a geometric mean value of 100 colonies (also, colony forming units or CFU) per 100 milliliters of water sampled, and not more than 10 percent of all samples obtained for calculating this mean can exceed 200 colonies per 100 milliliters.

The location on Little Soos Creek monitored as part of Metro's (King County Department of Metropolitan Services) ongoing freshwater monitoring program coincides with site eight of the CWQMP. The following are the results of samples taken at site eight from September 1993 to February 1995, the period of monitoring covered by the CWQMP:

- Metro Freshwater Monitoring Program: geometric mean = 187 CFU/100 mL
47% greater than 200 CFU/100 mL.
- CWQMP: geometric mean = 216 CFU/100 mL
38% greater than 200 CFU/100 mL.

These values represent both storm and base flow sampling in the stream. Individual sample concentrations have been in excess of 1000 organisms/100 mL.

Numerous human pathogens are spread by fecal contamination of water. These pathogens can be a risk to human health even at very low concentrations. Due to difficulties in the detection, identification, and enumeration of specific human pathogens in environmental samples, the concept of indicator organisms and related methodologies were developed and implemented in the late 1800's. Monitoring the level of indicator organisms, such as fecal coliforms, in water is used to assess the potential for the presence of pathogens.

The concept of indicator organisms is the principal component of regulatory microbiology. Each year millions of dollars are spent on fecal and total coliform assays to determine the extent of bacterial pollution in water environments, and to satisfy increasingly rigid regulatory requirements concerning microbiological quality of water. The utility of the indicator concept is limited by the lack of appropriate methodologies for tracking organisms associated with contamination to their potential sources. Knowing the sources rather than just monitoring the level of pollution enables water quality management efforts to be more effective by directing source control measures where the greatest problem is. Although there are human pathogens associated with fecal pollution of animal origin, the risk to human health would presumably be greater if contamination is caused primarily by human sources (mainly due to presence of human viruses). For these reasons, there is a need for the Microbial Source Tracking method to be used along with conventional analysis to more fully understand and address a bacterial pollution problem.

Microbial Source Tracking

The goal of the Microbial Source Tracking (MST) survey of Little Soos Creek was to help determine the contribution to contamination of the stream by two primary potential sources. These are: livestock on hobby farms and ranches adjacent to the stream and on-site septic systems close to the stream in highly permeable soils. Other animal sources were also included. This was done by performing a reasonably comprehensive sampling of both water from the stream and potential source fecal material from the watershed and vicinity from September 1993 through March 1995. These samples were then processed to establish collections of bacterial cultures, referred to as *isolates*, representative of the *Escherichia coli* (*E. coli*) population in each sample. Genetic fingerprinting (using ribosomal RNA typing) was performed on each *E. coli* isolate. These patterns or DNA types, referred to below as *ribotypes*, were then used to effectively match specific strains of *E. coli* from a contaminated site in the stream to its source. The intent was that the survey would provide information needed to support implementation of specific source controls.

MST has been applied to other studies in addition to Little Soos Creek. These include surveys of shellfish beds in Puget Sound, an urban watershed in northern Seattle, and a large primarily undeveloped watershed of a regional drinking water supply in King County. All of these studies demonstrate the usefulness of the methodology. Each one

helps to develop a regional database of *E. coli* isolates and ribotypes. The ability of the method to track contamination is only as good as the information in the database. As the database becomes more comprehensive and refined its effectiveness in helping to more fully characterize the nature of contamination at a site is improved.

Results and Conclusions

There were 1714 *E. coli* isolates available for the ribotype matching analysis. Of these 1714 isolates, 664 were water isolates with an adjusted total of 589. The remaining 1050 isolates were source type isolates, 227 isolated from septage and 823 isolated from animals.

The results of ribotype matching can be presented in two general ways. One is to consider the number or percentage of matches found among ribotypes. The other is to consider the number or percentage of total isolates associated with strains matched by ribotypes. The data is presented using both formats. Both of these aspects of matching and identification are significantly affected by sampling limitations. For this reason, it is most appropriate to use the data to form a qualitative understanding of the problem rather than interpreting the data as an exact quantitative analysis.

Matching efficiency refers to the extent that MST is able to identify potential sources of contamination at a specific location. There are two aspects of matching efficiency:

- the effectiveness of identifying strains found in water with strains found in sources by matching their ribotypes—percent of total water *ribotypes* matched.
- the effectiveness of the method to identify the sources of those strains present at the greatest frequency—percent of total water *isolates* matched.

In the Little Soos Creek study 57 of 171 (33%) ribotypes obtained from *E. coli* isolated from water were matched to source types other than water. Also, 421 of 589 (71%) water isolates belonged to the strains represented by these matched ribotypes. This suggests that, for the time period and stream locations sampled, MST identified the sources of approximately three-fourths of the fecal coliform contamination. When the two aspects of efficiency are taken together the matching efficiency of MST as applied to Little Soos Creek is very good.

The primary sources of contamination were determined to be cows, dogs, and horses. The greatest proportion of water-to-source ribotype matches were found to be water-to-cow and water-to-horse. However, the greatest proportion of water isolates belonging to strains associated with these matched ribotypes were water-to-cow and water-to-dog. This suggests that cows and dogs were the greatest contributors overall to the identified portion of the stream fecal coliform contamination.

Although septage was identified as a contributor to the contamination problem, it is not indicated as a major source. However, even low levels of contribution by septage suggest the potential for Little Soos Creek to harbor a number of human viral, bacterial, and parasitic pathogens associated with human sources. For this reason, further investigation of the contribution by septic systems and of human exposure (particularly children) to the stream may be warranted. It is possible that a portion of the unidentified water isolates are attributable to septage. Additional sampling of septic tanks in the watershed and vicinity or use of ribotype information from regional studies may provide additional water-to-septage matches.

The remaining unmatched ribotypes (66%) represent the smaller proportion of unidentified stream isolates (29%). A significant portion of water ribotypes not matched to sources may be attributable to unsampled source types. These include numerous wild animals and other domestic animals such as cats. These source types have been represented in other studies using MST. Source strains from these studies that are found to be regionally applicable can be used in this study to make potential additional matches or help confirm current matches.

Recommendations

The fecal bacterial contamination of Little Soos Creek could be addressed by efforts to:

- Encourage livestock owners to observe best management practices for pastures in general and particularly those with direct access to the stream and its tributaries. This involves fencing to restrict access, streamside vegetation effective at filtering pollutants, avoidance of overpasturing resulting in bare and/or compacted earth, collection and proper storage/disposal of animal wastes, and alternatives to direct stream watering of animals.
- Encourage dog owners to reduce the time their animals are allowed to freely roam unattended and make an effort to dispose of dog fecal material properly (away from streams). Dog owners who keep their animals in yards with direct access to the stream could be encouraged to tie the dogs away from the stream and its tributaries and provide for streamside vegetation.
- Further investigate the impact of on-site septic systems in the area of Little Soos Creek.

Further characterization of the fecal contamination in Little Soos Creek could be achieved as the regional database is developed and applied to this study. The cost of substantially increasing the ribotype matching efficiency of this study by additional sample processing from the Little Soos Creek watershed may outweigh additional achievable source control benefits. However, if necessary to better understand the problem, particularly if levels of contamination increase, additional sampling and analysis could be performed.

1.0 INTRODUCTION *CS*

1.1 *Overview*

In the summer of 1993 a study was begun to determine the sources of fecal contamination in Little Soos Creek. The stream is located in southeast King County, Washington. It is considered Class A freshwater, however, historically it has not met water quality criteria for fecal coliform bacteria levels for this classification. This study is part of a larger study of the water resources in the Covington area where Little Soos Creek is located and makes use of an innovative method for tracking microorganisms in the environment. This methodology is referred to here as Microbial Source Tracking (MST). It is based on the ability to identify and match microorganisms found at different locations in the environment with the sources, human or other animal, of those organisms by comparing genetic patterns. This was done by matching DNA patterns of fecal coliform bacteria isolated from the stream with patterns of fecal bacteria isolated from potential sources in the watershed. The study was conducted for King County Surface Water Management Division by investigators at the University of Washington Department of Environmental Health.

1.2 *Background*

The lower reaches of Little Soos and Jenkins Creeks are located in the Covington area of southeast King County (Figure 1.). The two streams are tributaries of Big Soos Creek. In addition, there is a shallow aquifer that is in direct hydrologic connection with the streams. The streams, their tributaries, wetlands, and the shallow aquifer together make up a system that provides important salmonid habitat. The streams also provide habitat for other animals. Direct human contact occurs through some recreational activities, particularly by children, and incidental uses as the streams pass through residential areas.

In 1985 a 1,237 acre (5 square kilometers) area of Covington was designated an urban activity center, the area identified for the regional location and concentration of high density residential, commercial, industrial, and employment activity. Development in the area has already resulted in measurable degradation of water quality (King County, 1992) particularly during and after storms when runoff washes accumulated pollutants off of rooftops, parking lots, roads, construction sites, pastures, and lawns. This jeopardizes the quality of stream habitat and the fisheries resource. It also poses a public health risk if the potential for the presence of human pathogens is high.

In response to the impacts of existing and anticipated urban development in the area, King County Surface Water Management developed the Covington Master Drainage Plan (MDP) (King County, 1992). The purpose of the MDP is to address the management of water quality and quantity of the area's water resources and associated beneficial uses

Little Soos Creek Microbial Source Tracking

Land Use

--- Covington Master Drainage Plan Area

Land use areas are approximate.

● High density on-site septic systems: predominantly residential

⊕ Sewered areas: residential, community, commercial

⊙ School, community

⊗ Commercial, office

⊘ Light industrial, commercial, office

Sections within the MDP without land use designations are forested or predominantly low density residential relying on septic.

These areas tend to have a high incidence of small animal farms and pasture land.

This description also applies to much of the watershed for the upper reach of Little Soos Creek.



1" = 0.48 mile

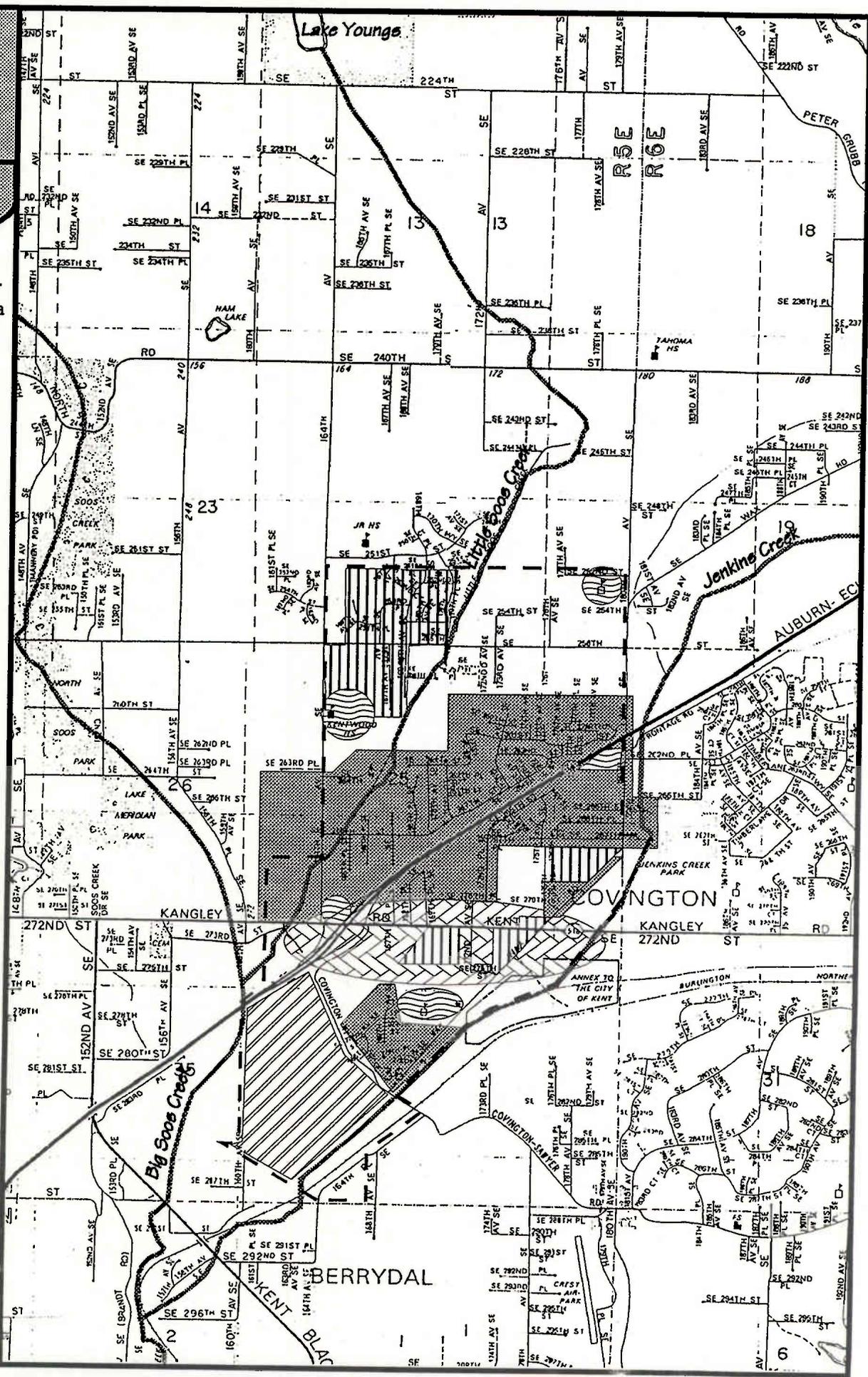


Figure 1. Land uses within the Covington MDP area, south King County, Washington.

while accommodating development. The MDP was adopted by the King County Council in 1992. In 1993 the Covington Water Quality Monitoring Project (CWQMP) was begun to carry out specific monitoring activities recommended by the MDP.

The Microbial Source Tracking study was initiated as part of the CWQMP in response to the recommendation that pollutant source monitoring for fecal coliform bacteria in Little Soos Creek be carried out. The stated objective of the suggested survey was to identify and document the extent and location of sources associated with fecal pollution of the stream.

This recommendation was meant to address violations of Class A fecal coliform standards (described below) at a downstream sampling station located close to the confluence with Big Soos Creek. This station has historically been monitored for fecal coliform bacteria as part of the freshwater monitoring program directed by the King County Department of Metropolitan Services (Metro).

The two potential major sources of contamination were considered to be uncontrolled livestock access to the stream and on-site septic system drainfields. Since MST is a sensitive method of tracking organisms genetically and is more effective than conventional methods alone, it was applied to this survey. The intent was that the survey would provide information needed to support implementation of specific source controls. For example, results may support the need for such nonpoint source control measures as fencing to restrict livestock from direct access to the stream. Also the need for sewerage certain areas currently served by on-site septic systems may be indicated.

1.3 *Little Soos Creek Watershed*

Location, Geology, and Hydrology

Little Soos Creek is approximately 4.75 miles (7.64 kilometers) long and originates at Lake Youngs, a regulating basin for the Cedar River water supply. It flows southeasterly through Section 13 Township 21 North, Range 5 East of the Willamette Meridian and then southwesterly through Sections 24 and 25 until its confluence with Big Soos Creek just south of Kent-Kangley Road (Route 516).

The elevation in the Little Soos Creek watershed ranges from approximately 315 to 480 feet (96 to 146 meters). The two major geologic materials found in the watershed are glacial till and glacial recessional outwash (King County, 1992). The outwash material consists of unconsolidated deposits of sand, gravel, and cobbles with variable amounts of silt. It is typically 10 to 20 feet (3 to 6 meters) thick and hosts the shallow aquifer. Highly permeable gravelly loam soils have formed on these deposits. They have low runoff potential. These materials are present through much of the southern portion of the watershed. The underlying glacial till was compacted by ice. It is a dense mixture of silt, sand, gravel, and clay with low permeability and occurs in much of the watershed often underlying the outwash materials. It is typically 35 to 50 feet (11 to 15 meters) thick. It

occurs at the surface in some areas such as the northwest corner of the MDP area along the west bank of Little Soos Creek. Moderately well drained gravelly sandy loam soils form in the till but have moderately high runoff potential because of the till below.

Stormwater runoff flowing directly into Little Soos Creek without any treatment contributes potentially high contaminant loads to the stream. This occurs where impermeable surfaces such as pavement and compacted pasture land occur near the stream channel. Because of the geology and soils in the watershed very little surface runoff occurs from undeveloped areas. Water reaches the stream mostly through direct precipitation, subsurface flow, and groundwater from the shallow aquifer (King County, 1992). The highly permeable outwash material and associated soils provide a better hydraulic connection for precipitation and stormwater runoff to enter the shallow aquifer than does the till. This permeability provides little treatment via sorption onto soil and thus contaminants typical of stormwater can be transported to the groundwater. Flow to the aquifer and potentially to the stream is also provided by on-site wastewater disposal systems. The high density of septic systems occurring in the outwash soils is another potential contributor of contaminants to the aquifer and hence to the stream.

Land Use

Land use has changed rapidly in the Little Soos Creek watershed as agricultural land and forested areas are converted to residential and commercial use. Much of the watershed north of the MDP area remains forested with residential and pasture land being the major uses. Figure 1. presents various land uses within the MDP area of the watershed. The northern portion of the area is predominantly high density residential with some forested tracts. The southern portion is high density residential, commercial, and light industrial. Several churches and schools are located throughout the watershed. Numerous small scale animal farms (hobby farms) are also located throughout the watershed, many have direct access to the stream. State Highway 18 intersects the area having an interchange near the stream.

Septic tank and drainfield systems are the dominant method of sanitary sewage disposal in the watershed. North of the MDP area it is the only method. Figure 1. shows the seweried sections within the MDP area and those sections with the highest density of septic systems, approximately greater than one system per acre (600 in Section 25) (King County, 1992). Much of this septic system density occurs in outwash material.

Water Quality

Little Soos Creek historically has been a Class A stream (King County, 1992) (Metro, 1994). This classification is defined as excellent water quality with characteristic uses of water supply, stock watering, fisheries habitat, wildlife habitat, recreation, commerce, and navigation (WAC 173-201A-030). There are two water quality criteria for fecal coliform bacteria levels in Class A freshwater. The concentration of organisms is not to exceed a geometric mean value of 100 colonies per 100 milliliters of water sampled, and not more than 10 percent of all samples obtained for calculating this mean can exceed 200 colonies per 100 milliliters.

The location on Little Soos Creek monitored as part of Metro's ongoing freshwater monitoring program is on the southern side of the stream's intersection with Kent-Kangley Road (Figure 2.). This location coincides with site eight of the CWQMP. Although most of the Class A water quality criteria have been met in Little Soos Creek (Metro, 1994), the stream at this location historically has not met the fecal coliform criteria. For the period 1990-1992 Metro reported the geometric mean for this site as 226 organisms/100 mL with 57% of 21 samples having concentrations greater than 200 organisms/100 mL. The current form of expressing the units for fecal coliform counts is: colony forming units (CFU)/100 mL. The following are the results of samples taken at site eight from September 1993 to February 1995, the period of monitoring covered by the CWQMP:

- Metro Freshwater Monitoring Program: geometric mean = 187 CFU/100 mL
47% greater than 200 CFU/100 mL
(Brenner, 1995).
- CWQMP: geometric mean = 216 CFU/100 mL
38% greater than 200 CFU/100 mL.

These values represent both storm and base flow sampling in the stream (one storm for Metro and three for CWQMP). Individual sample concentrations have been in excess of 1000 organisms/100 mL. Raw data for the CWQMP is shown in Table 1. Where there are duplicate results for the same date, the average was used in calculating the geometric mean. Any improvement seen between these results and past fecal coliform levels may be the result of lower precipitation during the sampling period or greater control of sources. It could also be due to the random nature of the organisms in the stream and the inability for monthly sampling to represent the complete picture. However, the persistent fecal coliform problem in Little Soos Creek has been attributed to the presence of numerous hobby farms and ranches adjacent to the stream and on-site septic systems in highly permeable soils close to the stream.

1.4 Study Goal and Objectives

The goal of the Microbial Source Tracking survey of Little Soos Creek was to help determine the contribution to contamination by two primary potential sources, livestock and septic systems. Other animal sources were also included. This was done by performing a reasonably comprehensive sampling of both water from the stream and potential source fecal material from the watershed and vicinity. These samples were then processed to establish collections of bacterial cultures representative of the *Escherichia coli* population in each sample. Genetic fingerprinting (using ribosomal RNA typing) was performed on each *E. coli* isolate. These patterns or DNA types, referred to below as ribotypes, were then used to effectively match specific bacteria from a contaminated site in the stream to its source.

Little Soos Creek Microbial Source Tracking

Sampling Locations

--- Covington Master Drainage Plan Area

Little Soos Creek

- 1 CVLS01 w/in fence at L. Youngs, v-notch wier
- 2 CVLS02 outlet at Shady Valley Ranch, S side of fence
- 3 CVLS03 int. w/ 172nd Ave. SE, W side of bridge
- 3a CVLS03a small tributary near CVLS03
- 4 CVLS04 int. w/ SE 240th St., S side of bridge
- 5 CVLS05 24453 180th Ave SE, int. w/ drive, W end of prop.
- 6 CVLS06 int. w/ SE 256th St., N side of bridge
- 7 CVLS07 end SE 264th St., at driveway bridge
- 8 CVLS08 int. w/ SR 516, S side of bridge

CH = chicken
 CO = cow
 DO = dog
 DU = duck
 FI = fish
 GO = goat
 HO = horse
 LL = llama
 PB = pig
 HU = septage



1" = 0.48 mile

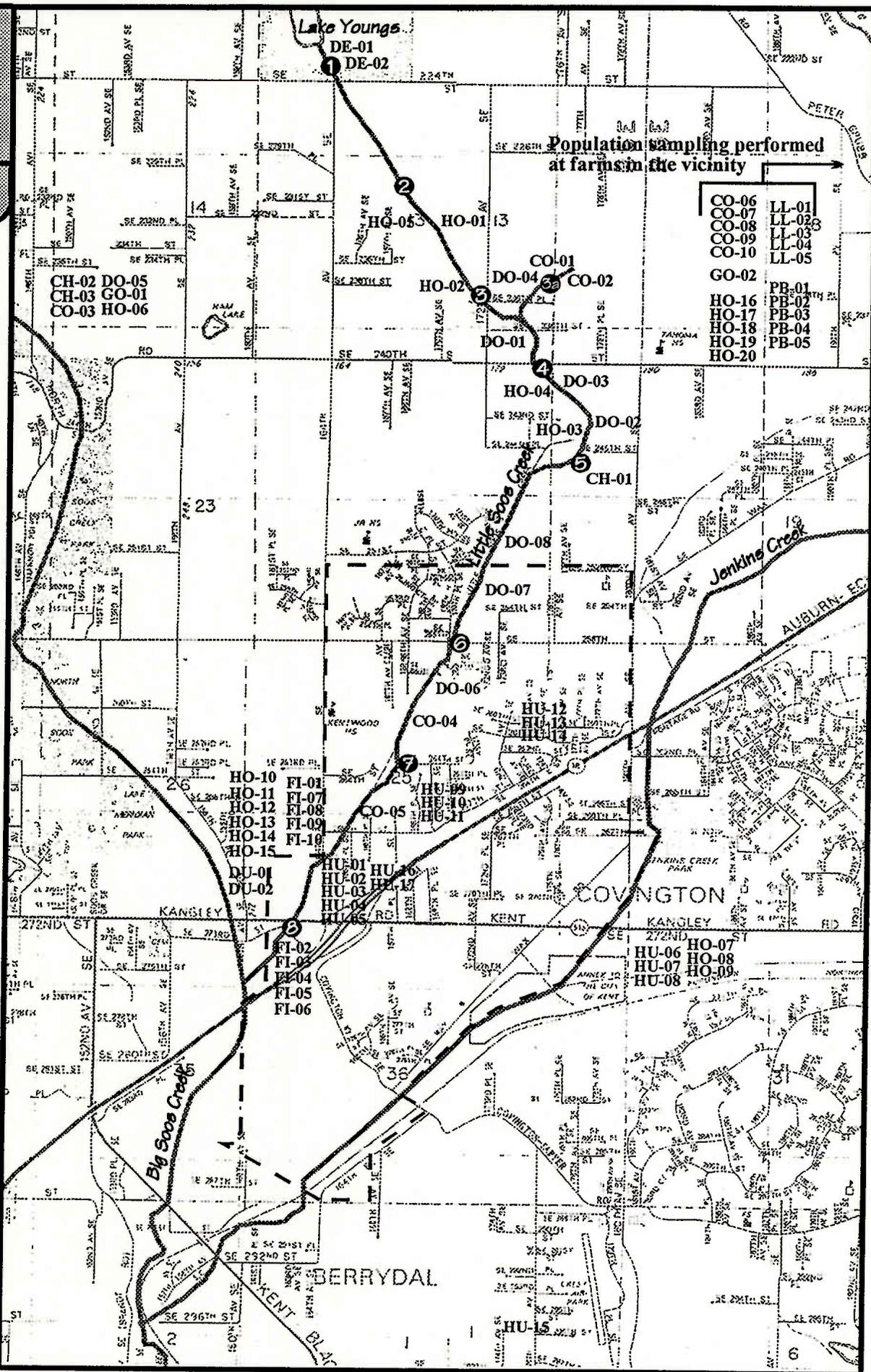


Figure 2. Sampling locations within the Little Soos Creek watershed and vicinity.

2.0 APPROACH AND METHODS

2.1 Conventional Analysis

Numerous human pathogens are spread by fecal contamination of water. Examples are *Vibrio cholera*, *Salmonella typhi*, *Giardia lamblia*, *Cryptosporidium parvum* and Hepatitis A. These pathogens can be a risk to human health even at very low concentrations. Due to difficulties in the detection, identification, and enumeration of specific human pathogens in environmental and food samples, the concept of indicator organisms and related methodologies were developed and implemented in the late 1800's. Indicator organisms are used to assess the potential for the presence of pathogens. These organisms must be prevalent in feces, found in higher concentrations than pathogens, be more resistant to disinfectants (more persistent in the environment), and easy to quantify. The group of bacteria referred to as fecal coliforms meet these criteria. A formal definition of this group is that they are facultatively anaerobic bacilli that ferment lactose with the production of gas within 48 hours at a temperature of 44.5°C. A prevalent and well studied member of this group is the species *Escherichia coli* (*E. Coli*).

The concept of indicator organisms is the principal component of regulatory microbiology. The major limitation of this concept is that it is an oversimplification of the complex dynamics of microbial ecology, physiology, and genetics. It is true that often the presence of indicators can be associated with fecal contamination. However, it is also true that in many instances there may be little or no correlation between the presence of indicator organisms and the presence of fecal contamination and human pathogens.

The utility of the indicator concept is further limited by the lack of appropriate methodologies for tracking organisms associated with contamination to their potential sources. Sources of water pollution can be divided into two general groups, point and nonpoint sources. Point sources of pollution have defined discharge points such as pipes—municipal and industrial wastewaters for example. Nonpoint sources of pollution do not have defined discharge points. Because of their diffuse nature, nonpoint sources are difficult to identify and control. Nonpoint sources of microbial pollution include wildlife, agricultural practices, on-site septic systems, commercial and recreational boating, aquaculture, and industrial practices. This impediment to the identification and control of sources of microbial pollution in water adversely affects the decision-making process of water quality and fisheries resources management.

Each year millions of dollars are spent on fecal and total coliform assays to determine the extent of bacterial pollution in water environments, and to satisfy increasingly rigid regulatory requirements concerning microbiological quality of water. Knowing the sources rather than just monitoring the level of pollution enables water quality management efforts to be more effective by directing source control measures where the greatest problem is. Although there are human pathogens associated with fecal pollution

of animal origin, the risk to human health would presumably be greater if contamination is caused primarily by human sources (mainly due to presence of human viruses). For these reasons, there is a need for the Microbial Source Tracking method described below to be used along with conventional analysis to more fully understand and address a bacterial pollution problem.

2.2 Microbial Source Tracking

Description

In response to limitations of conventional methods, the Microbial Source Tracking methodology was developed by Dr. Mansour Samadpour of the Department of Environmental Health at the University of Washington (Samadpour, 1990). MST can be summarized in two steps. The first step is the molecular characterization of strains of the study organism, in this case *E. coli*, by DNA fingerprinting, specifically referred to here as ribotyping. Secondly, ribotypes of *E. coli* strains isolated from potential sources are matched with the ribotypes of strains isolated from receiving water to determine the extent and distribution of each source's contribution to contamination.

The data resulting from an MST analysis can be used in:

- understanding the sources, distribution, and movement of microbial populations in the environment
- conducting risk and exposure assessment studies of the potential human effects associated with microbial pollution
- design and implementation of source controls
- studying the effects of control measures
- environmental litigation.

Definitions

An *isolate* is a pure culture of bacteria established from a source using sterile technique and appropriate growth media. The intent is that the culture originates from a single organism.

A *strain* is a classification of a group of organisms within a bacterial species based on relatedness resulting from *clonal descent*. A clone is defined as all the individuals (descendants) derived from a single individual (progenitor) by asexual reproduction (fission). The progenitor and descendants are genetically identical unless mutation occurs. A working definition of clone is: a group of bacterial cultures that have been isolated independently (from different sources, at different times, and in different places) and have so many genotypic and phenotypic characteristics in common that the most likely explanation for their relatedness is that they are of clonal origin.

A *ribotype* is a DNA pattern obtained from the DNA operon, or gene, that codes for ribosomal RNA (rRNA). This operon is highly conserved (not easily mutated) and can be

used to distinguish between bacterial strains of the same species over many generations in the environment (Atlas et al., 1992) (Selander et al., 1987). Thus, within a population of a given bacterial species there may be numerous isolates belonging to a single strain that can be distinguished from other strains of the same species by a unique ribotype.

MST makes use of the ability to classify organisms based on their genetic fingerprints into groups of clonal descent, or strains, as described above. The second concept forming the basis for the source tracking theory is that of *resident vs. transient strains* (Hartl and Dykhuizen, 1984). A bacterial strain that has adapted to a particular environment, or host (e.g. animal intestinal tract), is capable of colonizing that environment and competing favorably with members of the indigenous flora. These are called resident strains. Resident strains are usually shed over a long period of time from their host, thus providing a characteristic signature of their source. A transient strain is a bacterial strain that is introduced into a new environment, or host (e.g. into an animal by ingestion), but cannot colonize and persist in that environment. If the host is sampled over time for a given species of bacteria, a few resident strains are consistently observed in the system while a larger number of transient strains are seen passing through.

Rationale

Given that bacterial population structure is clonal and if within each species different clones have adapted to specialized environments, then it should be possible to:

- study a collection of bacterial isolates from a contaminated site (e.g. receiving water) and from possible sources of contamination
- divide the isolates into groups of clonal origin
- match the isolates from the contaminated site to the sources
- identify the contributing sources.

This requires the selection of an appropriate methodology for interstrain differentiation of bacteria. The method of choice needs to be sensitive enough to allow for dividing the species of interest into groups of clonal origin, and the results should be reproducible. The method should also be easy enough to perform, and the results should allow for comparing a large number of bacterial isolates. Ribosomal RNA typing with the use of appropriate restriction enzymes is the method of choice in MST studies of fecal coliforms. In special circumstances such as source tracking studies of *E. coli* O157:H7 (an *E. coli* strain associated with several food-related outbreaks), it has been necessary to develop and use other methods of differentiation.

Other Studies

MST has been applied to other studies in addition to Little Soos Creek. These include surveys of shellfish beds in Puget Sound, an urban watershed in northern Seattle, and a large primarily undeveloped watershed of a regional drinking water supply in King County. All of these studies demonstrate the usefulness of the methodology. Each one helps to develop a regional database of *E. coli* isolates and ribotypes. The ability of the method to track contamination is only as good as the information in the database. If

sources of contamination at a particular site have not been characterized, i.e. no source ribotype is available to match to an identified water ribotype, then a match cannot be made. As the database becomes more comprehensive and refined its effectiveness in helping to more fully characterize the nature of contamination at a site is improved.

2.3 *Sampling Procedures*

Little Soos Creek Water

Sampling of water from Little Soos Creek was performed from September 1993 through February 1995. The eight locations monitored for fecal coliform enumeration as part of the CWQMP are indicated on Figure 2. Both base and storm flows in the stream were sampled.

There were four primary locations monitored for fecal coliforms and also used in the MST survey. CVLS01 is located within the Lake Youngs (a drinking water reservoir) watershed, a protected area that is mostly forested. CVLS03 is downstream of a wetland and rural reach of the stream where there are a few large pastures. CVLS07 is within a high density residential neighborhood served solely by septic systems in outwash soils. The stream passes through a number of hobby farms prior to this site and several more downstream until the intersection of the stream with 164th Avenue SE. CVLS08 is located at the intersection of the stream and Kent-Kangley Road. Here it has passed through more residential, pasture land, commercial, and high motor traffic areas. A subset of samples taken at the other four sites were also used in the MST study.

All samples were grab samples taken in sterile containers. Standard quality assurance and quality control procedures were observed in the field including the collection of field and trip blanks. Samples were promptly placed on ice and delivered within one to two hours to the Metro environmental laboratory to be analyzed within the specified holding time.

Animal Fecal Samples and Septic Tanks

Fecal samples from various species of animals and septage from septic tanks were collected from the watershed and vicinity from September 1993 through March 1995. Sample locations are shown on Figure 2. The sampling focus was on the primary suspected sources, livestock and septic systems. Because of study limitations a more comprehensive sampling of domestic animals and wild animals was not done. The largest challenge to obtaining samples within the watershed was the lack of willingness, on the part of many private property owners, to participate in a study of this nature. Sample collection was also done in the vicinity of the watershed to obtain a greater number and diversity of samples. This also served the purpose of furthering the understanding of ribotype diversity within a population of animals of the same species living together and among members of the same species within a region.

All samples were collected with sterile implements and placed in sterile containers. They were promptly delivered to the laboratory of Dr. Samadpour at the University of Washington and immediately processed or refrigerated at 4°C until the following day.

2.4 Bacterial Culture and Isolation

The water samples were analyzed for fecal coliform enumeration by the Metro environmental laboratory according to the membrane filter method (APHA, 1992). After analysis, the plates were transported to the U.W. lab. Morphologically appropriate colonies (round, blue, and flat) were chosen from these plates and streaked for isolation onto MacConkey media and incubated at 37°C for 24 hours. The fecal and septage samples were transported directly to the U.W. lab. They were swabbed heavily onto MacConkey media plates and incubated at 37°C for 24 hours. Characteristic colonies (round, purplish-red, typically flat) were chosen from these plates to be streaked for isolation, again on MacConkey media.

Isolated colonies that fermented lactose on MacConkey were then restreaked onto Trypticase Soy Agar (TSA). An average of sixteen isolates were obtained from each water and fecal sample. Biochemical analysis was done to positively identify *E. coli*. This was done by inoculating each isolate into a tryptophane broth and onto a sodium citrate slant and incubating at 37°C for 24 hours. Isolates that were able to produce indole from tryptophane and not able to utilize sodium citrate as a sole source of carbon were positively identified as *E. coli*. These isolates were then assigned an isolate number and cultured again on TSA to obtain enough cells for storage in LB-15% glycerol freezing media at -70°C and for genomic (chromosomal) DNA isolation.

2.5 DNA Isolation and Digestion

Confluent growth of each isolate was scraped with a sterile flat-headed toothpick from TSA plates and suspended in Tris-EDTA buffer. The suspension was mixed well by pipetting up and down. To lyse the cells sodium dodecyl sulfate (SDS) and proteinase K (Pharmacia, Piscataway, N.J.) were added. These preparations were then incubated at 40°C for one hour. This was followed by phenol extraction to remove cellular material other than DNA. The preps were vortexed and then centrifuged for five minutes. The top aqueous layer containing DNA was removed and extracted with chloroform to further purify the DNA. DNA was precipitated out of solution by adding 2.5-3 times the prep volume of absolute ethanol. The DNA was spooled onto a glass capillary pipette, washed with absolute ethanol, dried, and resuspended in enough sterile distilled water (approximately 500 µL) to obtain a consistent DNA concentration among all preps.

Restriction endonuclease digestions of each DNA prep were done by using 10 units of appropriate restriction enzymes (Boehringer Mannheim, GmbH, Germany) as instructed by the manufacturer and 4 µL of DNA. Each 20 µL digestion prep was incubated at 37°C

overnight. The preps were then centrifuged and 3 μ L of stop dye were added to arrest the digestion reaction and prepare for loading into gels for electrophoresis.

2.6 Gel Electrophoresis and DNA Probing

The fragments of DNA produced by the enzyme digestion were resolved by agarose gel electrophoresis. The DNA fragments were then transferred from the gel by blotting onto a Nitran filter (Schleicher & Schuell, Keene, N.H.) in high salt solution (Maniatis et al., 1982) (Southern, 1975). These blots were baked at 80°C for one hour.

The blotted DNA was then hybridized with a radioactively labeled ribosomal RNA (rRNA) probe (Maniatis et al., 1982). The probe was labeled with [α -32P] dCTP, using random primers and incubated at 37°C for 30 minutes. The double stranded DNA molecules are denatured into single strands during the blotting process. During the hybridization reaction, the single stranded probe joins to single stranded DNA that contain segments of the ribosomal RNA operon. Hybridization of the probe to the blotted DNA was done under stringent conditions.

After hybridization the blots were washed, dried, and then exposed to X-ray film (Kodak, Rochester, N.Y.) with an intensifying screen at -70°C. Two to three different time exposures were done to ensure all DNA bands that hybridized with the probe would be visible on film. The X-ray image of the DNA banding produced in this way for each isolate is termed an autoradiogram. The actual banding pattern is a ribotype.

2.7 Ribotyping and Analysis

Figure 3. illustrates the autoradiograms from one gel or blot. Each row, or lane, of bands represents DNA from one *E. coli* isolate and is headed by a number. The isolates represented in lanes 7 and 8 have identical banding patterns, the same ribotype, and therefore belong to the same strain. This is also true for isolates represented in lanes 13, 14, 16, 17, and 19. The two groups belong to different strains determined by their unique ribotypes. Using an algorithm developed in the U.W. lab, the ribotypes were converted to an alphanumeric pattern.

The data for each isolate was entered into a computer database (using Microsoft Access 2.0). Isolates were sorted by ribotypes. Potential ribotype matches between isolates obtained from water and source samples, source samples of the same type, source samples of different types, and different water samples were confirmed by further inspection of the autoradiograms.

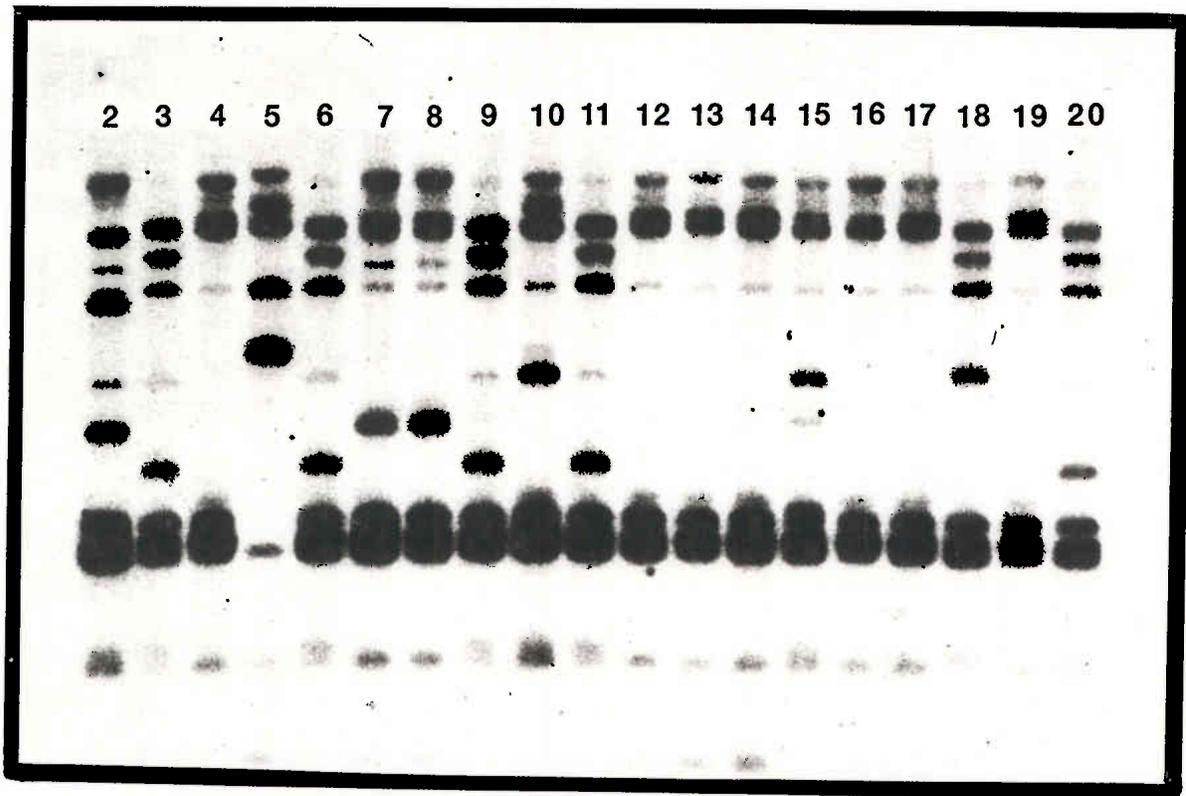


Figure 3. Examples of ribotype autoradiograms from a group of *Escherichia coli* isolates.

The data presented here for the Little Soos Creek survey are a result of this first confirmation process. A second confirmation is done by running side-by-side on the same gel the DNA from isolates representing a match between different sample types. The process from gel electrophoresis to analysis is the same as previously described. Although this confirmation step is not complete to date for this study, the results are not expected to significantly change, if at all, current findings.