Thurston County Public Health
Environmental Health Division

Bacteriological Contamination Source Identification

Henderson Inlet
1999-2001

Final Report
January 2002

Prepared in conjunction with Dr. Mansour Samadpour
University of Washington

THURSTON COUNTY
WASHINGTON
Since 1852

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Executive Summary

In response to the worsening water quality of the marine shellfish growing waters of Henderson Inlet, Thurston County Environmental Health Division conducted this study to identify the sources of pollution that impact Henderson Inlet waters. This study was designed to find the difference between human and animal contributors, evaluate the impact of each source, and locate the point of impact of each of the sources. Water (marine and freshwater), marine sediment, and shellfish tissue were sampled at different locations within the watershed.

The method used to identify the sources of pollution is called Microbial Source Tracking (MST). The MST is based on the use of genetic fingerprinting of *Escherichia coli* (*E. coli*) bacteria strains isolated from water and source samples by a method called ribotyping. It is the DNA fingerprint of the *E. coli* that lives within the animal that is typed, not the DNA of the specific animal. Some of the *E. coli* bacteria found in different warm-blooded animal groups, including humans, have genetic differences. It is thought that these distinctions in bacteria occur because the intestinal environments differ between animal groups. It is in identifying these genetic differences that it is possible to associate bacteria with sources of fecal pollution.

In order to be successful with this method, it is necessary to build a library of fingerprints from known sources. It is these fingerprints from the database library of known sources that are matched with the environmental samples collected. The larger and more diverse the library, the more likely a match can be made. The library used for this study currently has approximately 65,000 fingerprints called source isolates. An additional 100 source isolates were added from local collection of fecal samples from a variety of domestic animals, wildlife, and human sewage.

The work to accomplish this project was a joint effort between Thurston County Environmental Health staff, Dr. Mansour Samadpour, the University of Washington, and volunteers, who collected the fecal samples in order to build this local library of *E. coli* DNA patterns. The County collected and cultured all of the samples, the environmental samples. Dr. Samadpour and the University of Washington cultured the fecal samples, and typed and matched the *E. coli.*

The study area was southern Henderson Inlet near Washington State Department of Health's marine monitoring station #5. This station's commercial shellfish status was downgraded to *prohibited* in October 2000. DNA typing was used to test for bacterial pollutants affecting the water, sediment, and shellfish tissue of marine station #5. Water from the 3 creeks most likely to influence station #5 was also tested.

A bacterial pathogen study was done on a set of environmental samples from the study area. Because fecal coliform and *E. coli* are used as indicator organisms, it is of interest to know if there are specific human bacterial pathogens present in the samples collected.

Sampling for this study began in February 2000 and was completed in May 2001. Four sampling events occurred in 2000 with the remainder being completed during wet weather in 2001. Sampling was done during saturated soil conditions when run-off of nonpoint pollution is most likely to occur. The study was designed to obtain 100 isolates each for sediment, shellfish tissue, Woodland creek, and small creeks entering the cove at Station #5. Two hundred (200) isolates were to be typed for the marine water. With the exception of the shellfish tissue, which had 89 isolates, all exceeded the designed number of isolates. A total of 943 isolates were typed. Matches were made for 85.7% of the isolates; only 14.3% of the isolates could not be matched to any fingerprint within the library. Woodland Creek had the highest percent of matches – 91.7%; the marine water had the highest percent of unmatched – 18.0%.

Overall, a total of 27 source types of fecal pollution were identified. They were avian, beaver, bovine, canine, cat, deer, dog, duck, duck-goose, feline, goose, horse, human, marine mammal, multiple species, muskrat, opossum, otter, porcupine, poultry, rabbit, raccoon, rodent, seagull, sea lion, seal, and unknown.

The raw data has been analyzed and is presented in a variety of formats:

- Total number of isolates
- Number of source types
- Frequency of bacterial source type occurrences
- Comparison of sampling during *conditional closure* conditions (wet weather) and *open* conditions (dry weather)
- Comparison of sampling under *ebb* vs *flood* tide sampling conditions.

A significant aspect of the study was the percent of matches that were made – 86%. The project proposal had estimated between 40 and 60%. The high number of matches is due both to the size of the library, which is around 65,000 isolate patterns, and to the rigor of the study design.

The primary observation of the data regardless of sampling site – this is nonpoint pollution. There is a bit of everything. However, in analyzing the three water bodies sampled, it can be determined that the Swayne creeks are of a rural nature and Woodland Creek is of an urban nature. The source types found in Swayne creek samples represent animals found in rural areas – birds, deer, canines, and rodents. The types in the Woodland Creek samples reflected the urbanization of the watershed with people and dogs being predominant source types.

This is a study of Henderson Inlet only. Though much can be learned from this body of work, it is a picture of Henderson Inlet. The specific results of this work can not, and must not, be transferred to another watershed.

This is a qualitative study. It was designed to differentiate between human and animal sources and evaluate the frequency of those sources. Each set of site-specific results was evaluated for its impact on, and relationship to,

the marine water quality and shellfish beds. The study was not designed to quantify the fecal loading of the sources. This is important to understand when reading the full report. One animal source may occur more frequently, but individually contribute less fecal matter. For example, one bird has much less fecal load contribution than one human. A study designed to calculate fecal loading contributions of individual sources would be more extensive and expensive.

The results of this study can help in development of continued remedial activity to improve water quality within Henderson Inlet. Of the predominant animal sources, birds are always present and everywhere – on land, on water, and in the air dropping their fecal matter at all sites. As a source, they are relatively beyond control other than to discourage grazing of migratory birds. Unknowns, until identified, are unmanageable. To identify these and reduce the number of unmatched isolates, more known *E. coli* fecal sources need to be typed and added to the library.

Human sources are the next most prevalent source. Human bacteria enters surface water, and subsequently sediment and tissue, through fecal contamination from failing septic systems, failing sewer lines, and direct deposition from humans and diapers. There are previously developed and proven programs that can be implemented that can find and correct many of these human sources.

This particular microbial source tracking (DNA ribotyping) method is considered by some professionals to be an experimental methodology. Studies and methods have not been subjected to rigorous peer review, and statistical evaluation has not been applied to the uncertainties and limitations of the method. Until this occurs, some environmental health professionals are reluctant to accept the results and conclusions of any study in which this methodology is used. To local environmental health professionals this methodology promises to be a valuable tool to use in order to prioritize remedial and preventative water quality work.

Specific conclusions from the study are --

- The percentage of matches with known sources was high – 86%.
- There must be an on-going effort to collect and type *E. coli* of fecal samples from known sources in order to increase the size of the library.
- Marine water samples had the greatest percentage of isolates without a match - 18%.
- This study represents only the Henderson Inlet study area.
- The study results are a 'picture' of nonpoint pollution. Twenty-seven (27) source types were identified.
- The results for the Swayne creeks were characteristic of its rural watershed.
- The results of Woodland creek were characteristic of its urban watershed.
- The results of the marine water were reflective of its complexity.
- Avian, human, canine, and unknown source types were found during more than half the water sampling events. These were the most frequently found source types.

- Overall, human, beaver, and bovine isolates were always more prevalent during wet sampling conditions. Feline, goose, and multiple species types were more prevalent during dry sampling conditions.
- Bovine source types were found in the marine water only during wet sampling events.
- On a flood tide and when the inlet is closed to commercial shellfish harvest, the marine water human source types were found more frequently than any other type at the marine station.
- Marine mammal source types were found more often during dry weather sampling under ebb tide conditions.
- Sediment samples had the least fingerprint 'uniqueness' of the sampling sites. There were more duplicate fingerprints within a given grab sample.
- The oyster sampling events produced such low number of isolates that no conclusions about the results can be made.
- The bacterial pathogen scan produced limited data. Human pathogens were found.
- This method shows promise as an effective tool for the complex task of identifying the sources of nonpoint pollution.
- The results of such studies can help prioritize local remedial efforts.

As a result of this study, the following recommendations are presented:

- State and local agency support is needed to fully develop the DNA ribotyping method for source identification of nonpoint pollution. Development of standard methods is needed in order that data can be shared, reviewed, and have professional support.
- State and local agencies in concert with researchers should develop a feces collection program in order to expand the DNA fingerprint library database.
- Due to the definite presence of human source types, the county and city should adopt a risk-based human waste assessment program that would include both evaluation of septic systems, as well as evaluation of the municipal sewer systems.
- Thurston Conservation District should continue development and management of conservation plans for watershed farmers and livestock owners so that best management practices that protect water quality are implemented and maintained.
- Area stormwater managers should discuss the possibility of conducting typing studies to categorize fecal sources within stormwater discharges.
- Advocate proper disposal of dog waste through state and local public education programs.
- Due to the limited data from sediment and oyster samples, future study designs should be modified to either omit these types of samples, increase the number of specimens collected, and/or add sampling events.
- Due to the limited data from the bacteriological pathogen scan, future studies having similar objectives should consider the value of the scan.

Terminology

Clonal type – a strain of bacteria that has so many identical characteristics (including their DNA fingerprints) that they have most likely originated from a common ancestral cell.

DNA – deoxyribonucleic acid; DNA is the chemical name for the genetic elements that all living cells have (their chromosomes).

DNA Fingerprint Library – a database of genetic fingerprints, in this instance an *E. coli* DNA Fingerprint Library.

Environmental samples – samples collected in the field as part of the study.

Escherichia coli – a fecal bacteria; commonly carried by humans and animals, most *E. coli* strains are not harmful to humans, a few, like *E. coli* O157:H7 are human pathogens. *E. coli* is used as an indicator organism for assessing the microbiological quality of water.

Isolate – a pure culture of bacteria that has been established in a laboratory.

Microbial Source Tracking (MST) – a methodology used to identify the sources of microbial pollution and to quantify their impact.

Transient clones – clonal types that are present in more than one different group or species of warm-blooded animals; referred to as 'transient' in the data tables.

Resident clones- clonal types that are shared by members of one source type; referred to as 'resident' in the data tables.

Prevalence – how often something is occurring.

Ribotyping – the specific methodology of genetic fingerprinting of bacteria.

Source types – the various types and groupings of warm-blooded animals that are sources of fecal nonpoint pollution.

Introduction and Background

Henderson Inlet is in south Puget Sound northeast of Olympia. The watershed is 45 square miles in area - 29,275 acres in size with Woodland and Woodard Creeks being the two major tributaries to the inlet. Both Woodard and Woodland Creeks originate in the upper watershed that includes much of the urban areas of Lacey and Olympia with both commercial and residential land uses. The unincorporated area in the northern part of the watershed closest to the marine water is zoned 1 unit per 5 acres, thereby maintaining a rural nature, including small-scale agricultural operations. Higher densities can be obtained through a density bonus for cluster developments. The Henderson Inlet shoreline is the least densely populated marine shoreline in the County with roughly 14 houses per shoreline mile. The watershed includes high-density commercial development and residential development with both municipal sewer service and on-site sewage systems. Fifty-five percent of the watershed is within the designated urban growth boundaries of the Cities of Lacey and Olympia. Approximately 15,000 residents have moved into the watershed in the past decade. The population of Henderson Inlet watershed was 38,000 in 1989. The 2000 census counted the population at approximately 53,000. The projected 2010 population is estimated to be 65,000. Therefore, long-term protection of water quality of the marine environment of Henderson Inlet and its tributaries to the level required for unrestricted shellfish harvest and consumption will be a challenge.

Shellfish

In 1984, 180 acres of shellfish growing area in the south end of Henderson Inlet were changed by Washington State Department of Health from *approved* to *conditionally approved.* In 1985, 120 acres of *conditionally approved* area were downgraded to *prohibited.* The reason for the downgrades was nonpoint pollution run-off. That was confirmed by a Thurston County water quality study in 1984 that showed a link between storm events and fecal pollution from nonpoint sources. Rainstorms of more than 0.75 inches in 24 hours closed the area to commercial harvest for 5 consecutive days. In 1996 this translated into 58 days closed to harvest, and in 1997 it was closed for 68 days. In a 1998 data review by Washington State Department of Health, it was noted that water quality standard violations were occurring under the 0.75 inch rainfall management condition. Consequently, in December 1998 State Department of Health, Office of Food Safety and Shellfish Programs, (DOH) changed the rainfall management condition to 0.50 inches of rain in a 24-hour period closes the area to commercial harvest for 5 days. In 1999 consequently, 134 days were closed to harvest – nearly twice what it had been in 1997. Despite control measures (voluntary implementation of farm management practices, search for failed on-site systems, updated standards for on-site sewage standards, land-use density limits, stormwater management, etc.), contamination levels have intensified. In October 2000, DOH expanded the *prohibited* area by adding nine acres. In June 2001, the Department of Health placed another 300 acres into the *conditionally approved* area. There are now a total of 128 acres in the *prohibited* area and 360 in the *conditionally approved* area.

The *conditionally approved* shellfish growing area now closes to harvest for five days after 0.5 inches of rain falls in a 24-hour period. This rapid deterioration of water quality with a relatively low amount of rainfall indicates that the travel time for contaminated runoff to reach the inlet is very short. It has been documented in the literature that bacteria are deposited in the sediments within storm drainage systems, as well as within the streambeds and the marine environment, and are resuspended during rain events. So it is contamination that has previously washed into the man-made and natural drainage systems, as well as "new" contamination, that effects the water quality during and after rain events.

In Henderson Inlet there are currently three commercial shellfish growers. In addition to the commercial harvest, there is considerable harvest of shellfish for personal use by private property owners along the Henderson Inlet shoreline. The Washington Department of Natural Resources manages the Woodard Bay Natural Resources Conservation Area on the west side of Henderson Inlet. The conservation area comprises approximately 600 acres of upland property along Woodard and Chapman Bays, as well as hundreds of acres of tidelands in the vicinity.

Within all of Puget Sound, Henderson Inlet is the last inlet that has a viable *conditionally approved* commercial shellfish growing area given its level of urbanization and population density. All other areas that have equivalent population and rainfall levels have either *restricted* or *prohibited* growing areas.

Nonpoint Pollution Factors

There are many factors that contribute to the nonpoint pollution dynamic within the watershed. An examination of the water circulation patterns in the southern portion of the inlet during ebb tide conditions revealed that floats placed at the mouth of Woodland Creek reached the recently *closed* marine station (station #5) within two hours of release. (Appendix A) Water quality measurements at that marine station showed that the water was influenced both by the creeks flowing into it (contributing freshwater) and the inlet water (saline) and the degree of mixing or influence is not consistent at Station #5. A freshwater lens is often visible on the water surface. Salinity varied from 4 parts per thousand (ppt) to 20 ppt on opposite sides of the boat a few yards west of Station #5. (In the midst of flood tide, the salinity readings range from 27 – 30 ppt.) Travel time estimates calculated for Woodland Creek during a 2-year return storm from the Martin Way crossing to Hawks Prairie bridge (mouth) is one hour and 24 minutes. (The velocities used were from a HEC-2 analysis computer model done during the HSPF modeling [Hydrologic Simulation Program – Fortran] and basin planning effort.) These estimates indicate that bacteria generated in the urban areas can easily reach the marine inlet and the shellfish growing area in a viable condition.

Stream segment sampling done along Woodland Creek in the early 1990's and again in the Spring of 2001 indicate that the stream segments downstream of Pacific Avenue to Martin Way, Martin Way to Draham Road, and Draham Road to Pleasant Glade Road appear to have significant bacterial loads entering the stream in those sections.

Stormwater from city and county road networks, as well as Interstate 5, discharge into the 2 main Henderson Inlet tributaries. While the City of Lacey has constructed regional stormwater treatment facilities for large storm sewer

networks to mitigate pollutant loads from these systems into Woodland Creek, many stormwater outfalls continue to discharge untreated runoff. A few of the remaining large discharges into Woodland Creek include: Interstate 5 runoff, College Street system, Tanglewilde at Martin Way system. The magnitude of bacterial pollution that can be expected from these outfalls is documented by some specific sampling data from these sites and studies in the literature. One study found that fecal coliform concentrations up to 24,000 Colony Forming Units (CFU)/100ml, but more typically 5,000 to 10,000 CFU/100ml, could be isolated by vacuuming 3' by 3' sections of dry pavement (Bannerman, Roger. 1992. Wisconsin DNR). So runoff from impervious surfaces that discharge to streams tributary to Henderson Inlet is a significant source of bacteria pollution.

Septic system testing, on a voluntary basis, has been done for 27 percent of the systems located along the marine shoreline. Of those tested, 14 percent were found to be failing - defined as retrieval of dye and a fecal coliform result of 200 or greater organisms/100 ml. (Those systems found failing have been repaired.) Another 25 percent were considered "suspect" - defined as the retrieval of dye OR bacteria. Failure of near shore septic systems can have an impact on the water quality in the immediate vicinity. Assuming the 14 percent failure rate represents the rate for both tested and untested systems, an additional 21 shoreline systems could be expected to be failing. A residential subdivision in the Woodland Creek reach between Draham and Pleasant Glade Roads has been suspected of having failing septic systems contributing to bacterial loading. Dye testing of creekside systems, in the mid-1990's, did not identify any failures. Upland systems were not tested. This subdivision generates much stormwater with high levels of fecal coliform bacteria.

In addition to the urban development and residential development occurring within the Henderson watershed, large tracts of land are being converted to smaller, non-commercial farms, often with higher animal densities than the original farms. The areas where this poses the greatest threat to water quality is in the northern part of the watershed where the predominant soil types are fine textured soils underlain by hardpan or relatively impervious clay. Poor farm management practices in these areas can result in manure-contaminated runoff reaching creeks, small tributaries, and the inlet.

Planning and Remedial Action Efforts

Water quality problems for the inlet have been identified as urban stormwater run-off into Woodland and Woodard Creeks, failing septic systems, and agricultural run-off. From 1987 to 1989, a watershed management committee, composed of citizen representative of various interests throughout the watershed, developed a watershed plan to address the nonpoint pollution problems in Henderson Inlet. Since that time many of the recommendations have been implemented. The Cities of Lacey and Olympia and Thurston County have responded to the stormwater problems through construction of regional stormwater facilities that provide both water quality treatment and reduce peak discharges to the creeks. A nonpoint pollution ordinance to address pollution from agricultural runoff and hazardous waste was adopted. The County established an operation and maintenance program for on-site sewage systems. This is a voluntary program for the majority of system types, though the more technical systems are required to have 3-year renewable certificate that lists maintenance requirements. These and other actions

have served to slow the deterioration of water quality in a rapidly urbanizing watershed. However, a recent evaluation of the marine water quality data by Washington Department of Health, Office of Food Safety and

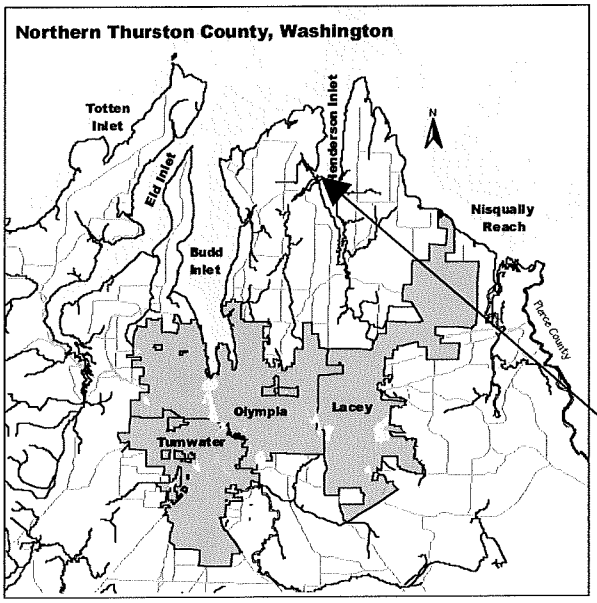
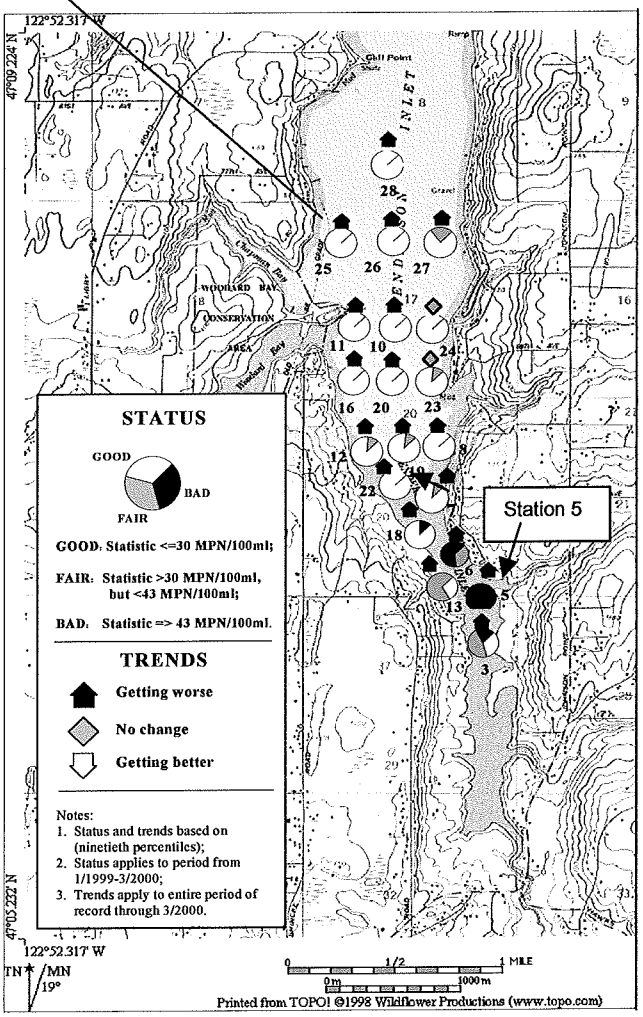


Figure 1. Status and trends of fecal pollution in Henderson Inlet through March 2000.



Shellfish Programs, shows an upward trend in the fecal coliform bacteria levels, a degradation of water quality. Eighteen of 20 stations have increased pollution over the entire period of record. (Determan, 2000). Figure 1

Purpose of Study

In response to the worsening water quality, Thurston County Environmental Health Division conducted the present study to identify the sources of microbial pollution that impact the Henderson Inlet. This study was designed to differentiate between human and animal contributors, discern the impact of each source, and locate the point of impact of each of the sources. The design included sampling different locations and different media, i.e. water, sediment, and tissue, as well as a bacterial pathogen scan. Figure 2 on page 6 shows the study area.

The methodology used to identify the sources of microbial pollution is called Microbial Source Tracking (MST). The specific method has been developed by Dr. Mansour Samadpour, at the University of Washington (Samadpour 1995, 2001). The MST method has been used in more than 80 studies around the country and in Canada. [Appendix E has further detail.]

The MST is based on the use of a genetic fingerprinting of *Escherichia coli* strains isolated from water and source samples by a method called ribotyping. It is the DNA fingerprint of the *E. coli* that lives within the animal that is typed-not the DNA of the specific animal. Though fecal coliform bacteria found in many animal species are very similar genetically, there are differences among members of the same species that have adapted to live in different host species. It is thought that these distinctions in bacteria occur because the intestinal environments differ between animals, including humans. It is in identifying these genetic differences that it is possible to associate bacteria with sources.

In order to be successful with any type of microbial source tracking methodology, it is necessary to build a library of isolates from known sources. Isolates from the database library of known sources are matched or associated with the environmental samples collected. The larger and more diverse the library, the more likely a match can be made. The library used for this study currently has approximately 65,000 source isolates.

It was the purpose of this study to differentiate the *E. coli* in the samples collected and associate those fingerprints with known source types. The study results provide a picture of the nonpoint pollution for each study site. These sites can then be compared with each other to determine the predominant source types affecting the area. It is hoped that analysis of results will help develop realistic implementation of remedial efforts to restore water quality.

The study was not designed to quantify the fecal loading of the sources. This is an important point to recognize. One animal source may occur more frequently, but it individually contributes less fecal matter. For example, one bird has much less fecal load contribution than one human.

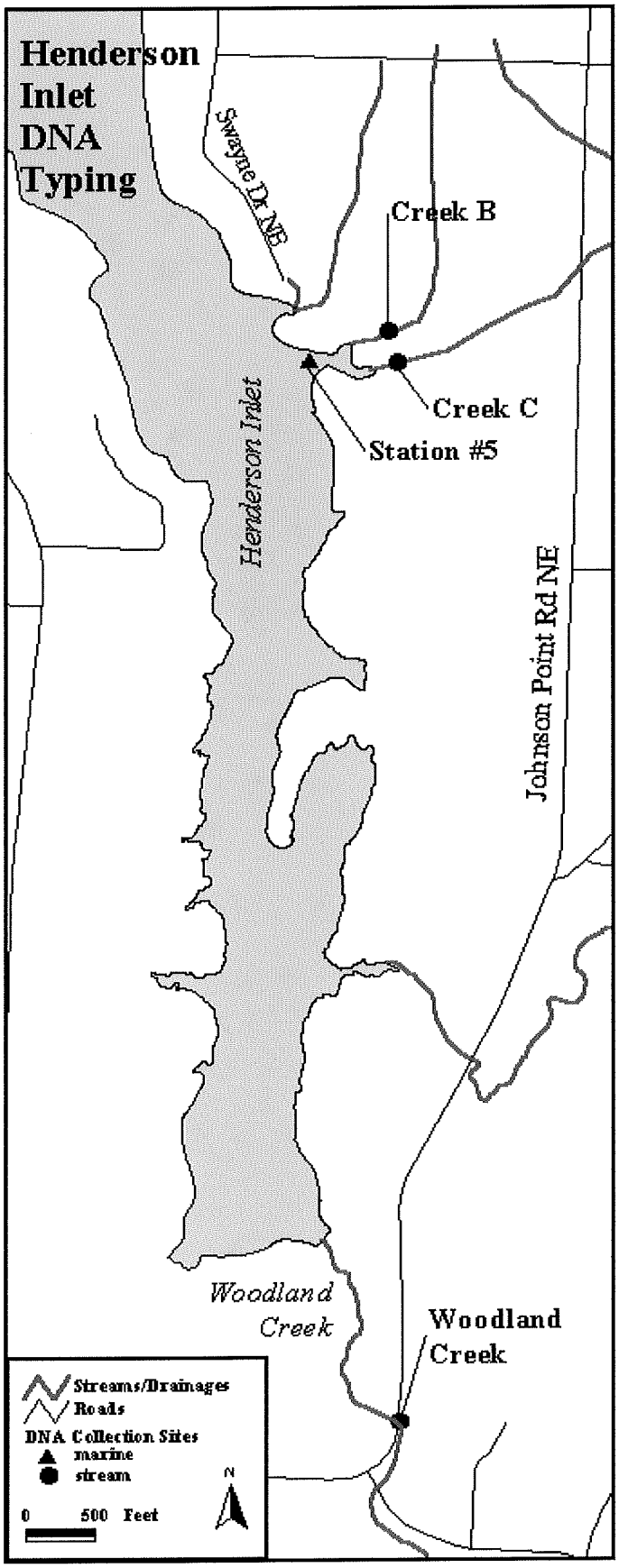


Figure 2

Study Area

The area of study was the head of Henderson Inlet near DOH (Washington State Department of Health) station #5 (now referred to as 152). In October 2000, this station's commercial shellfish status was downgraded to *prohibited*. Figure 2 shows the marine station, Woodland Creek at the head of the inlet, and the two Swayne creeks adjacent to station #5 (Creek B and C)

These sample sites were chosen based on information from the circulation study (Appendix A). The results of that study concluded that station #5 is directly in the flow pattern from Woodland Creek. Under certain tidal conditions freshwater from creek B and C are trapped within the cove at Station #5.

Study Elements

The work to accomplish this project was a joint effort between Thurston County, Dr. Mansour Samadpour, and the University of Washington.

The three elements in the study were as follows:

1. Develop the Thurston County DNA library;
2. Identify bacterial sources of contamination in the study area through DNA typing;
3. Conduct bacterial pathogen study.

Thurston County DNA Library

It was anticipated that the percentage of DNA matches with known sources would be between 40 and 60%. In order to improve the percentage of DNA matches between test samples and known source types, an inventory of local fecal samples was collected and typed.

Environmental Health staff and volunteers collected 82 fecal samples in order to build this local library of *E. coli* DNA patterns.

- Fecal samples were collected using sterile containers or whirl-pak bags. All containers were labeled with date of collection, location, and species.
- Staff collected dozens of fecal samples from the animals at the 2000 Thurston County fair.
- The residents along Swayne Drive near marine station #5 assisted by collecting domestic and wildlife feces.
- The Public Works staff for the City of Lacey assisted with collection of sewer effluent samples from three of the sewer lift stations.
- Septic system effluent samples were collected during routine septic system pumping.
- Samples were refrigerated until shipment on ice to Dr. Samadpour by United Parcel Service, Next Day Delivery.

Dr. Samadpour cultured and typed all samples submitted.

- Dr. Samadpour added these DNA patterns to his database.
- In the raw data (Appendix B) these local library patterns are identified with an "L".

DNA typing was used to evaluate the bacterial contaminants affecting the water, sediment, and shellfish tissue of marine station #5. In addition, water from the three creeks most likely to influence station #5 was tested. The county collected samples. The sampling matrix was as follows:

Table 1
Sample Collection Design

Sample location	Sample type	Number of sampling events	Number of samples / event	Desired number of isolates / sample	Desired total number of isolates
Marine station #5	water	20	5	2	200
	sediment	10	5	2	100
	shellfish tissue	2	25	2	100
Swayne creeks	water	10	5*	2	100
Woodland Creek: Head of Inlet	water	10	5	2	100
				Total	600

Environmental Health staff collected the water and sediment samples following Standard Methods.

- Stream samples were collected mid-channel and mid-depth.
- Marine samples were collected from a boat at the marker for station #5.
- Sediment samples were collected from station #5 using a pre-sterilized petite Ponar dredge.
- All sample bottles were marked with the site location, sample number, date, and time.
- Samples were stored on ice in a cooler until returning to the office. Samples were delivered to the Thurston County Health Lab upon returning from the field.

Shellfish samples were provided by Western Oyster Co. and were delivered to the University of Washington by Jerry Yamashita, owner of Western Oyster Co. UW did the culturing of the *E. coli* Isolates from the shellfish samples.

* Five samples were collected – three from one creek, two from the second creek.

The Environmental Health lab, accredited by Department of Ecology, prepared the *E. coli* isolates.

- The lab used the membrane filter method for the water samples.
- The most probable number method was used for the sediment samples.
- At least two isolates were produced from each sample.

Environmental Health staff shipped the plates to Dr. Samadpour's laboratory.

- Each shipment contained a chain of custody.

- Shipping was by United Parcel Service, Next Day Delivery.

Dr. Samadpour and his staff, using the DNA technique, tested the isolate cultures submitted and produced a DNA fingerprint of each *E. coli* isolate.

Dr. Samadpour matched as many study sample DNA patterns with known DNA isolates contained in the *E. coli* library (that includes the local library). A detailed record of each isolate, that includes the following, is in Appendix B:

- Whether a match was made - both known (matched) and unknown (no match with known sources found) isolates
- From which library the match was made
- What animal the match was from

Bacterial Pathogen Study

On May 23, 2001, Environmental Health staff collected 45 samples (15 each of water, shellfish tissue, and sediment) for a bacterial pathogen scan. These samples were delivered the same day on ice to the University of Washington. Dr. Samadpour and his staff completed the Polymerase Chain Reaction (PCR) pathogen scan and provided written results to the County. The detailed methodology can be found in Appendix C.

The bacterial pathogen study was undertaken to scan a set of environmental samples from the study area for the presence of pathogens. Since fecal coliform and *E. coli* are used as indicator organisms, it is of interest to determine if there are infective agents present in the samples collected. The testing was to determine the presence of seven specific bacterial pathogens: *Campylobacter, E. coli (enteropathogenic enteroinvasive, enterohemorrhagic), Salmonella, Shigella, Vibrio parahemolyticus, Vibrio vulnificus, and Yersinia spc.*

Methodology

Bacterial strains and culture conditions

Water grab samples were taken from three sites and processed by membrane filtration for fecal coliforms (Standard Methods). After incubation at 44.5°C for 24 hours they were read and sent to the University of Washington, Environmental Health laboratory. Based on morphological characteristics (round, blue, and flat), appropriate colonies were chosen and streaked for isolation on MacConkey media, then incubated at 37°C for 24 hours.

Fecal samples were collected from representative animal species in the watershed and were shipped to Dr. Samadpour's lab for processing. They were streaked on MacConkey plates and incubated at 37°C for 24 hours.

Non-mucoid colonies that fermented lactose on MacConkey were re-streaked on Tripticase Soy Agar (TSA) plates. Ten *E. coli* - type colonies per sample were isolated. Biochemical analysis was done to positively identify *E. coli*. These isolates were assigned an isolate number and stored in LB-15% glycerol freezing media at -70°C.

Genomic DNA isolation and restriction endonuclease digestion

Confluent growth was scraped with a sterile flat-headed toothpick and suspended in 200 µl 50mM Tris and 50mM EDTA (pH 8.0). An additional 600 µl of 50mM Tris and 50 mM EDTA were added, and the suspension was mixed well by pipetting up and down. Next 45 µl 20% sodium dodecyl sulfate (SDS) and then 10 µl proteinase K (20 µg/ml; Pharmacia, Piscataway, N.J.) were added. This was then incubated at 40° C for 1 hour. An equal volume of phenol was added to each tube. Samples were vortexed and centrifuged for 5 minutes. The top layer was extracted, and an equal volume chloroform was added. The preparation was vortexed again, centrifuged, and extracted. Two and a half volumes of absolute ethanol were added. The DNA was precipitated out and spooled onto a glass capillary pipette. The DNA was washed with a few drops of absolute ethanol, dried, and re-suspended in 50µl dH2O.

Using 2 µl DNA, restriction endonuclease digestion reactions were set up using *EcoR*1 and *Pvu*II, 10 u/µl (Boehringer Mannheim, GmbH, Germany) as instructed by the manufacturer. They were incubated at 37° C overnight. The samples were centrifuged and .5µl of enzyme was added. The samples were re-incubated at 37° C for a minimum of three hours. They were centrifuged again and 3 µl stop dye was added.

Gel electrophoresis and Southern hybridization

Samples were run on a 0.8% agarose gel in 1X Tris-borate-EDTA at 22 volts and 17 milliamps for 17 hours. λ HindIII was used as a size standard along with an *E. coli* isolate designated as 3915. The DNA fragments were then transferred to a Nitran filter (Schleicher & Schuell, Keene, N.H.), baked at 80° C for one hour, and probed with ^{32} P labeled copies of *E. coli* ribosomal RNA. These labeled copies were made by extension of random hexanucleotide primers using Avian Myeloblastosis Virus reverse transcriptase (Stratagene, La Jolla, CA) under conditions specified by the supplier. Hybridization was done in 5X SSC (1X SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% SDS, 1mM EDTA, and 50% formamide at room temperature overnight. Salmon sperm DNA and blocking reagent, (Boehringer Mannheim GmbH, Germany) were used to block non-specific binding. Three washes were done with a solution of 2X SSC and .1% S.D.S. - once at 25 °C for 20 minutes and twice at 65° C for 20 to wash off low-homology, non-specific binding. Blots were then exposed with an intensifying screen to X-ray film (Kodak, Rochester, N.Y.) for 24 hours at -70° C. Two to three exposures were done to ensure all possible bands would show up.

RFLP Analysis

Molecular characterization was then done on individual *E. coli* strains by assigning a numerical pattern to each ribotype based on how closely the bands were grouped and by size. If a band was within 3 mm of another band, then it was designated part of that set and not considered alone. If a band ran farther away than 3 mm, then it was

considered alone. The groups of numbers were then listed together. Each individual isolate ribotype pattern was then entered into a database and was compared to the rest of the database. Ribotype patterns that numerically appeared to be similar were compared next to each other visually.

Study Results

Sampling for this study began February 29, 2000 and was completed May 31, 2001. (The dates, rainfall, and tidal phase can be found in Table 4, p. 14.) A total of 943 isolates were typed. The breakdown of those isolates can be found in Table 2. The study was designed to obtain 100 isolates each for sediment, shellfish tissue, Woodland Creek, and two small creeks entering the cove at Station #5. Two hundred isolates (200) were to be typed for the marine water. With the exception of the shellfish tissue, which had 89 isolates, all exceeded the designed number of isolates. Matches were made for 85.7% of the isolates; only 14.3% of the isolates could not be matched to any pattern within the library. Woodland Creek had the highest percent of matches – 91.7%; the marine water had the highest percent of unmatched – 18%.

Table 2
Summary of Number of Isolates Identified and Matched

	Swayne creeks	Woodland Creek	Marine water	Sediment	Oyster	**Total**
Total # of isolates identified	153	168	323	210	89	**943**
No match made	22	14	58	33	8	**135**
% not matched	14.4	8.3	18	15.7	9.0	**14.3**

The raw data of the study can be found in Appendix B.

- The data is listed by sample site identifier: oyster, sediment, 833 (marine water), 832 (Woodland Creek), and 831(Swayne creeks).
- Each isolate was assigned a unique isolate number.
- Each isolate was matched with the library of DNA patterns and was given a number associated with a particular DNA pattern. (The 'note' column in the raw data.)
- Each particular note is described as to what type of creature it is – resident/transient. *Unknown* means that the pattern has not been found in any previous studies and does not exist in the library at this point in time. (It may in the future at which time these unknowns could be 'reprocessed' to determine if there is a match.) A notation of *transient,* identified as *multiple species* in the graphs and charts, means that the DNA pattern can be found in more than one source, e.g. the same pattern resides in cows, ducks, and cats.
- The matrix type is water = 1, sediment = 78, and tissue = 97.
- The last column, Provider Sample, identifies the grab sample for the particular sampling event.

Explanation of source terms

There were 27 source types found during this study. A complete list of sources (27) is as follows: avian, beaver, bovine, canine, cat, deer, dog, duck, duck-goose, feline, goose, horse, human, marine mammal, multiple species, muskrat, opossum, porcupine, poultry, rabbit, raccoon, rodent, seagull, sea lion, seal, and unknown. When a source type is identified, the isolate is specific to that source type. Most sources are clearly understood. However, avian, bovine, canine, feline, marine mammal, multiple species, and unknown need further explanation.

Each source type is a separate category and has its own exclusive set of patterns with corresponding numerical identifiers. These patterns (and identifiers) are not shared between source types. *Avian* is a composite of all kinds of birds and has its exclusive set of patterns. These *E. coli* patterns may be shared by different kinds of birds and not exclusive to a particular bird species. In this study there are 41 different clonal types for avian. *Duck, goose, seagull* are categories that contain patterns for just that group of bird, and each would have its set of exclusive identifiers. A seagull's fingerprints might be found in *avian*, and in that instance would have a numerical identifier that was part of the *avian* type, but would also mean that the same fingerprint could and would be found in another kind of bird. However, birds other than seagulls will not be found in *seagull*. *Bovine* refers to cows, cattle, and ruminant animals. *Canine* is a composite group that includes dogs, wolves, coyotes, etc., who share patterns. *Dog* contains only domestic dog isolates and would have its set of numerical identifiers. *Feline* is a composite for cats, cougars, etc. *Marine mammals* is a composite of seals, sea lions, otters, etc.

Multiple species, identified as 'transient' in the raw data, is an identifier that occurs when the fingerprint is found in more than one source type, e.g. found in deer, raccoon, and porcupine. *Unknown* refers to a fingerprint that has not yet been identified with a source. It is important to note the difference in the last two terms – multiple species and unknown. Though a multiple species isolate can not be matched to a specific source, its pattern has been identified and is known to reside in more than one source type. On the other hand, when an isolate is unknown, it has not been matched to any library source. The multiple species isolate narrows the possibility of sources though it cannot be specific which it is. For an unknown isolate, the possible source could be any animal.

Data Analysis

The raw data has been analyzed and is presented in a variety of formats and interpretations.

- Frequency of bacterial source occurrences

For water samples:

- Comparison of sampling during *conditional closure* conditions vs. *open* weather conditions

For marine water samples:

- Comparison of sampling during *ebb* tide vs *flood* tide conditions
- Number of clonal types – graph and chart
- Total number of isolates – graph and chart (Appendix D)

Samples were grabbed consecutively at random. The colonies to type were chosen at random. The randomness was an intentional part of the study design. As defined in the study design, during each sampling event, 5 samples were grabbed from each sample site. Oysters were sampled separately. Each sampling event had 20 samples – 5 each of marine water, Woodland Creek water, sediment, and 5 water samples from Swayne creeks. (Creeks B and C on the map – Figure 2) Each grab sample was to render at least 2 isolates. Most always had 2 isolates though up to 6 isolates from a single grab sample were typed.

Frequency of bacterial source occurrences: This analysis is to show the frequency that the bacterial source is occurring. In other words, of the total number of sampling events how often was the source present. It helps answer the question – Is this bacterial source always present or only occasionally so? The study design was set up for 10 visits to all sites; an additional 10 sets of marine water, plus 1 bacterial pathogen scan sampling. Therefore, there were 11 events for sediment, 14 for marine water, and 5 for oyster.

Comparison of sampling during *conditional closure* conditions and dry weather conditions: This analysis examines what isolate sources (animals) are present during the commercial shellfish harvest closure conditions (wet conditions) in the inlet. Rainfall data (Table 3) was used to identify those sampling events that occurred when the inlet was closed to shellfish harvest (0.5 inches in 24 hours closes harvesting for 5 days). The data was compiled to yield the number of clonal types for dry/open sampling and another set for wet/closed sampling events. The mean was calculated for each source data set. For example, for Swayne creeks there were 18 avian isolates identified during the 6 sampling events when the inlet was closed. The mean is '3' (18 isolates ÷ 6 events = 3). This manner of looking at the data prompts questions impossible to answer, or yet to be answered, about animal behavior, run-off patterns, location of fecal matter, proximity to drainages, etc.

Comparison of sampling during *ebb* tide vs *flood* tide conditions: This analysis was done only for the marine water data. There were 7 *flood* sampling events and 7 *ebb* events. (Table 3) The freshwater sample locations were not impacted by the tides and were therefore not included in the comparison. The bacteria in sediment and shellfish do not react quickly to tidal changes; the shellfish filter the surrounding water for food, ingest the bacteria, and then excrete the waste. This takes far longer than a tidal cycle. Likewise, sediment can adsorb bacteria and keep them viable for long periods of time before resuspending them into the water column.

Table 3
Rainfall and Tidal Phase Data for Sampling Events

Tidal phase	Sampling date	Rainfall in inches				
		Day of sampling	Day previous	2 days previous	3 days previous	4 days previous
Flood	2/29/00	0.69	0.37	0.21	.05	0.31
Ebb	3/8/00	0.14	0	0.02	0.01	0.63
Flood	3/15/00	0.15	0.06	0.66	T	0.1
Flood	3/29/00	T	0.01	0.18	0	0
Ebb	3/28/01	0.02	0.76	0.31	0.39	.011
Ebb	4/4/01	0	0.01	0.29	0.01	0.25
Ebb	4/10/01	0.54	T	T	0.01	0.04
Flood	4/17/01	0.39	0.16	0	0	0
Ebb	4/24/01	0	0.11	0.22	0.01	0
Flood	5/1/01	0.11	0.81	0.19	0.26	0.14
Additional Marine Samples						
Flood	5/15/01	0.48	0.83	0.01	0.01	0
Ebb	5/22/01	0	0	0	0	0.01
Ebb	5/23/01	0	0	0	0	0
Flood	5/31/01	0	0	0	0	0

Note: Those rows shaded are when the area was closed to commercial shellfish harvesting.

Number of clonal types: This analysis is a refinement of the total number of isolates. It was done to examine the number of times an *E. coli* pattern, clonal type, appeared within a grab sample. Within a grab sample of water, often (about half the time – see Table 4) there was more than one isolate that had the same pattern as another isolate. (See Appendix B: the raw data 'note' fields have the same identifying number for the DNA pattern.) In order to identify **clonal types**, the pattern was counted only once even though it may have appeared in 2 or more isolates from one grab sample. For example, if a grab sample yielded 4 isolates and they were as follows: avian (pattern #39), avian (pattern #162), unknown (pattern #191), and avian (pattern #162), it was counted as 3 clonal types: avian (39), avian (162), and unknown (191).

Table 4
Uniqueness of Clonal Types within Sample

Sample source	Number of samples where all isolates are unique	Number of samples where there is 1 isolate match within the sample	Number of samples where at least 2 isolates match	Total number of samples
Swayne	24	14	9	47
Woodland	25	19	6	50
Marine water	55	31	19	105
Marine sediment	14	11	37	62
Oysters	11	17	9	37
			Total	301

With this analysis the results do not overstate or exaggerate the isolate sources present. When the exact same isolate pattern appears within a single grab sample, it could be from a single animal. Or it might be from two or more animals. On the assumption that it might be from a single animal, the conservative option would be to report it only once. All discussion in this report uses the number of clonal types, not the total number of isolates.

Woodland Creek Results

Woodland Creek is the largest creek in this watershed, drains an urban area of county, and its mouth is at the head of the inlet. Its flow ranges from 2.6 cfs to 81.6 cfs. This creek has a major influence on the marine water at station #5. It was found during the DOH circulation study that on an ebb tide the water from the mouth of the creek reaches station #5 within 2 hours and can still be predominantly fresh water with very low salinities (1 – 10 ppt). Analysis of ambient water quality data showed that when station #5 was sampled on an ebb tide 2 hours after the onset of ebb, the fecal coliform levels most often exceeded standards. (12 of 14 sampling events)

Figure 3

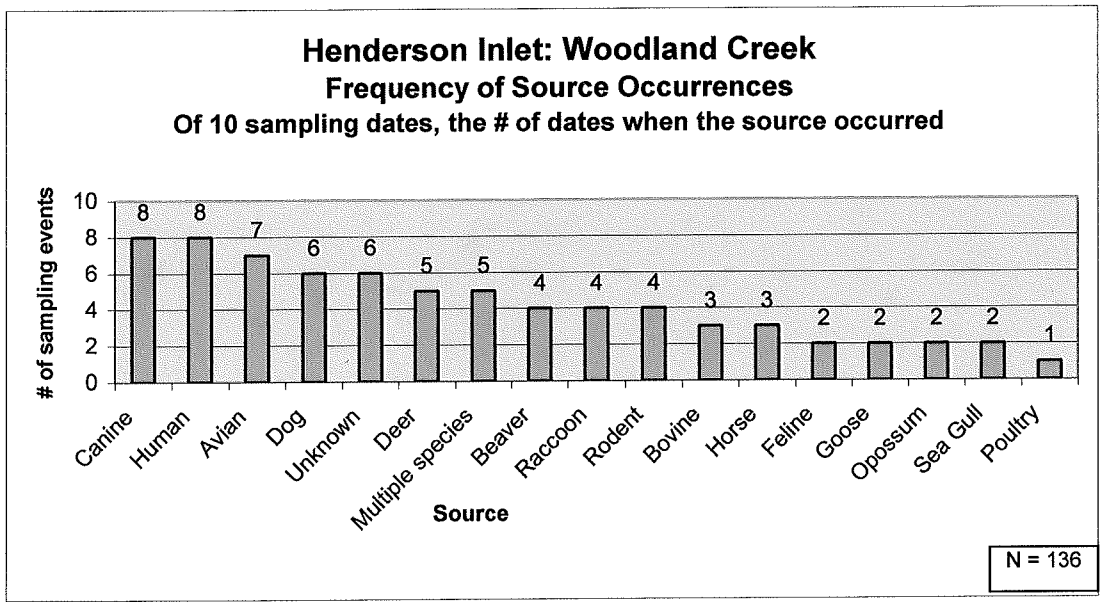


Figure 3 shows the frequency of source occurrence. Viewing the data this way helps to answer the question: How frequently is this source occurring? Is this an animal that is always present or just occasionally so? Woodland Creek was sampled 10 times; 50 samples were tested. Canine and human occurred in 80% of the events. Avian, canine, dog, human, and unknown occurred more than half the time.

Figure 4

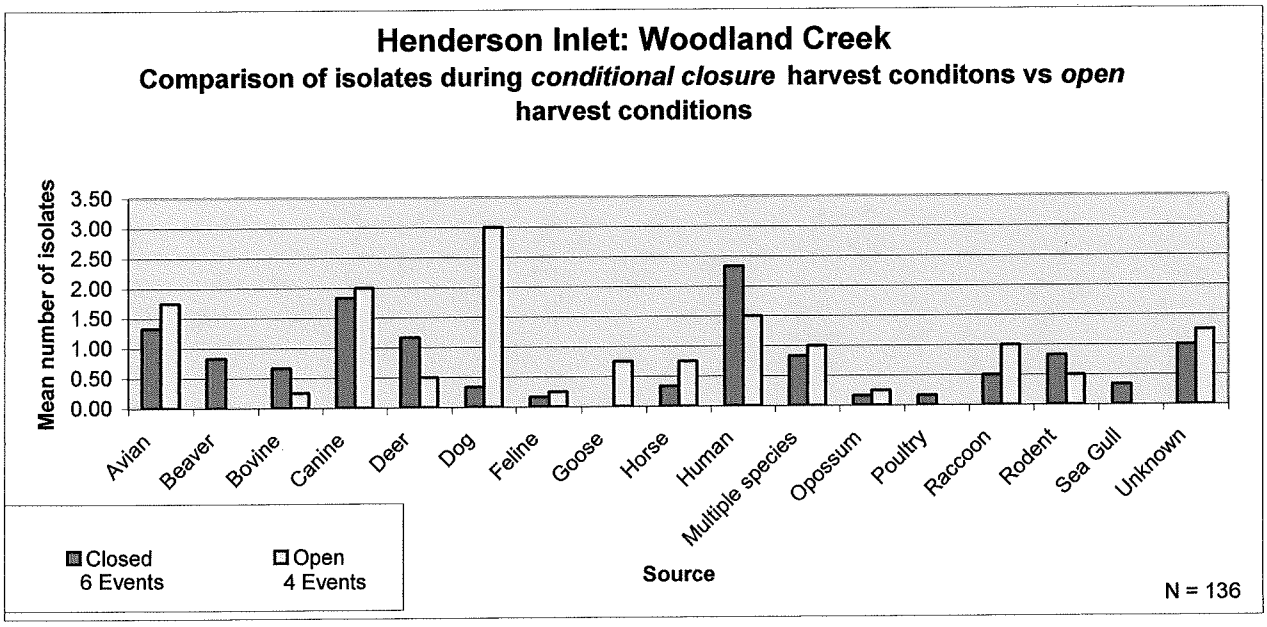


Figure 4 compares the number of clonal types during *closed* (6 events) and *open* sampling events (4). This analysis prompts the question - Are the sources of *E. coli* different under the different weather conditions? In a rural sub-basin, do birds, human, and unmatched isolates show up more during wet conditions? Deer and human sources have higher occurrence (mean difference greater than .5) during wet weather. In this data set, dog clonal types occur ten times more frequently during dry weather (0.3 vs 3.0).

Figures 5 and 6 show the 136 clonal types found in the Woodland Creek samples. Seventeen (17) source types were identified. The predominant isolate sources are human, canine, avian, and dogs. Human isolates represent the greatest number of clonal types. These results would seem to reinforce the characteristic nonpoint pollution sources of an urbanized basin.

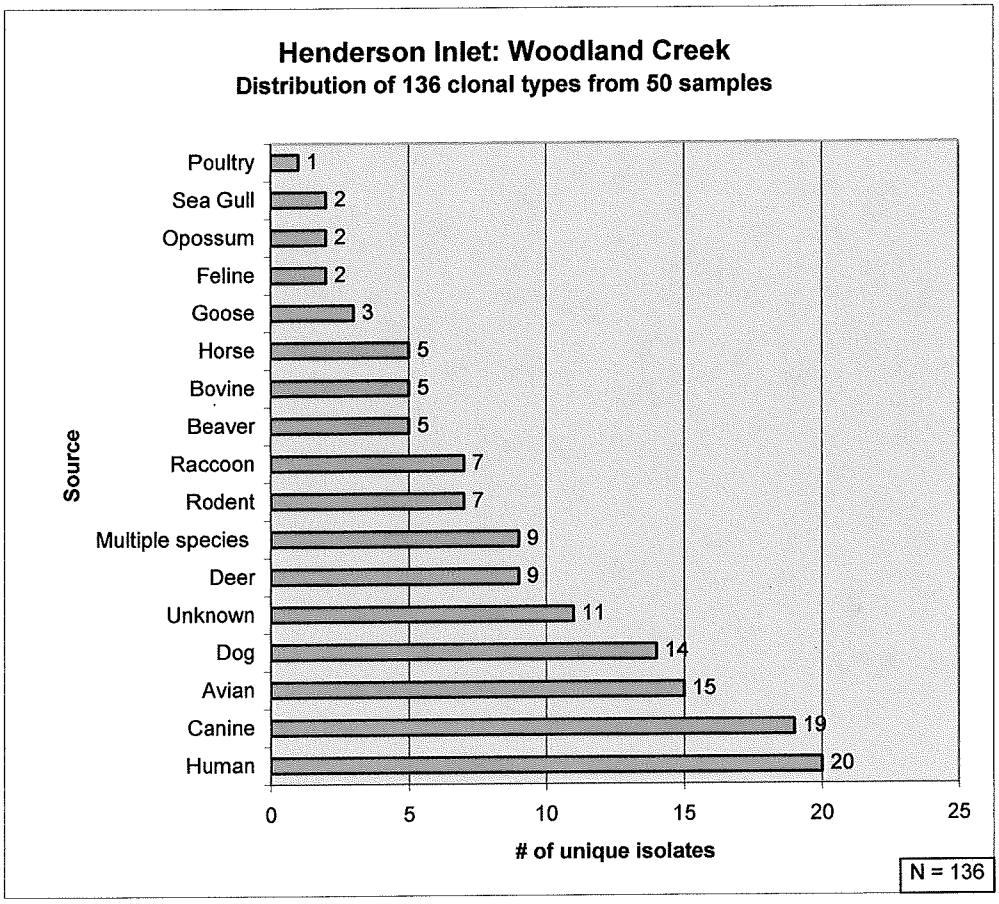
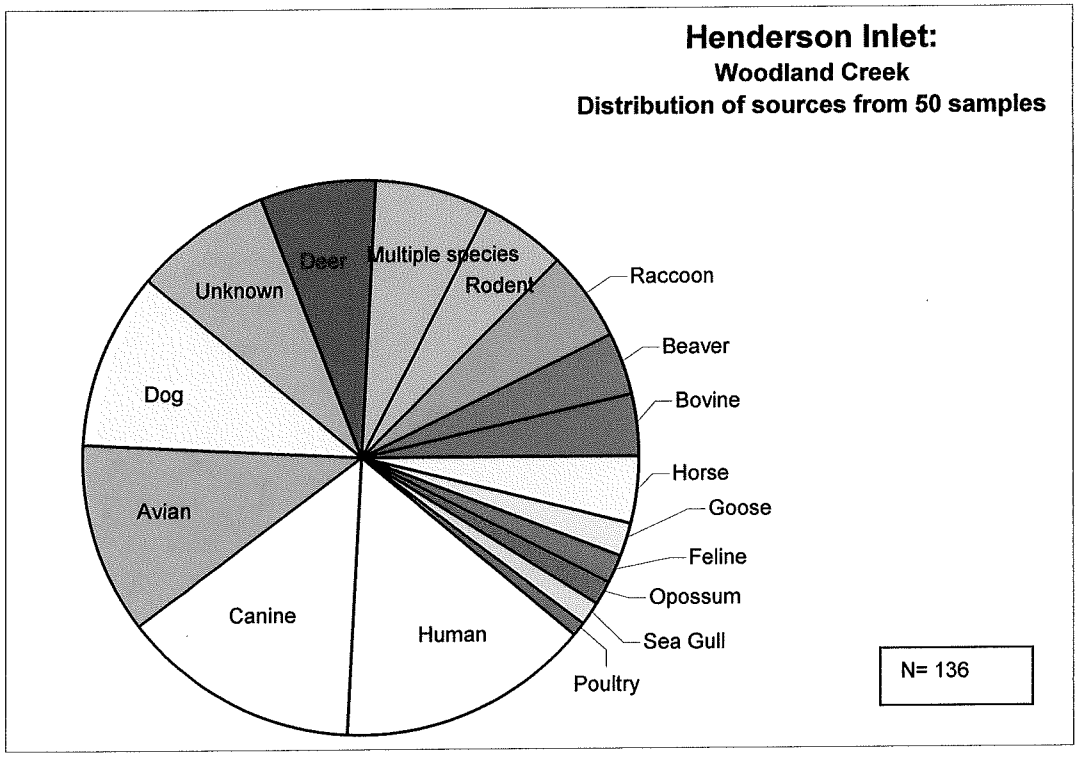


Figure 5

Figure 6



Swayne Creeks Results

These 2 small creeks, B & C on Figure 2, flow into the cove where marine station #5 is located. Their seasonal flows range from 0.02 to 11.09 cubic feet per second (cfs). In a sediment study that is part of another project, the fecal coliform levels have ranged from <5 to 2900 colonies / 100 mL. In a circulation study done by Washington Department of Health, Office of Food Safety and Shellfish Programs, (Appendix A), it was found that during the higher low tidal phase the water may not flush out of the cove, but remains there until the following lower low tidal phase. Therefore, the water flowing from these creeks can remain in the cove for an extended period of time – up to 18 hours.

Figure 7

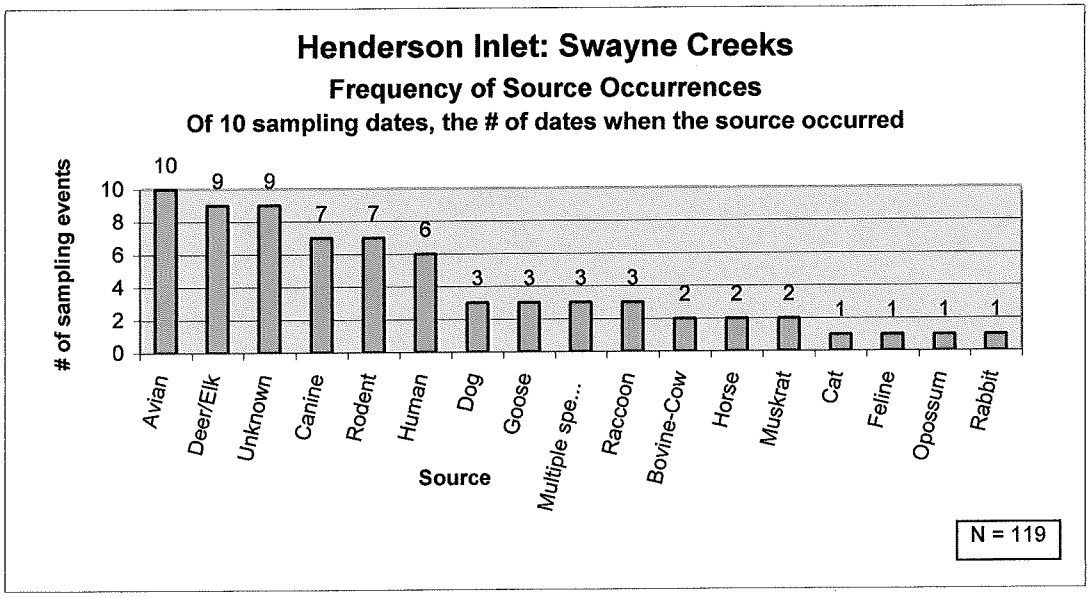


Figure 7 depicts the number of sampling dates when the source occurred. Swayne creeks were sampled 10 times. Over half the sampling events showed evidence of avian, deer, canine, rodents, humans, plus unknowns. The other sources together were seen less than 1/3 of the time.

Figure 8

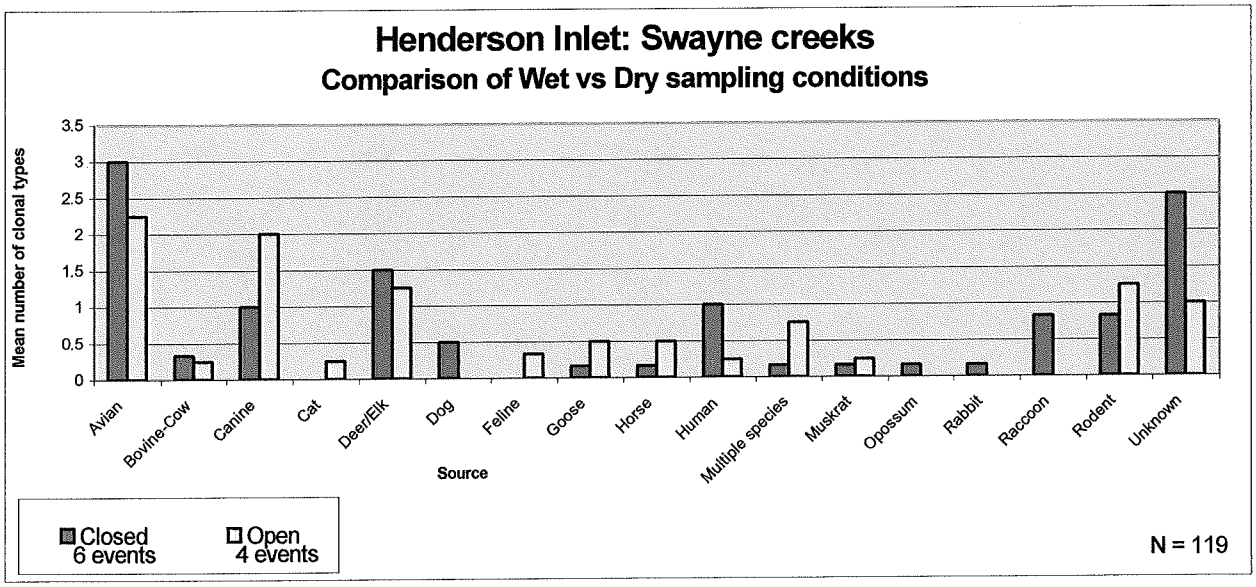
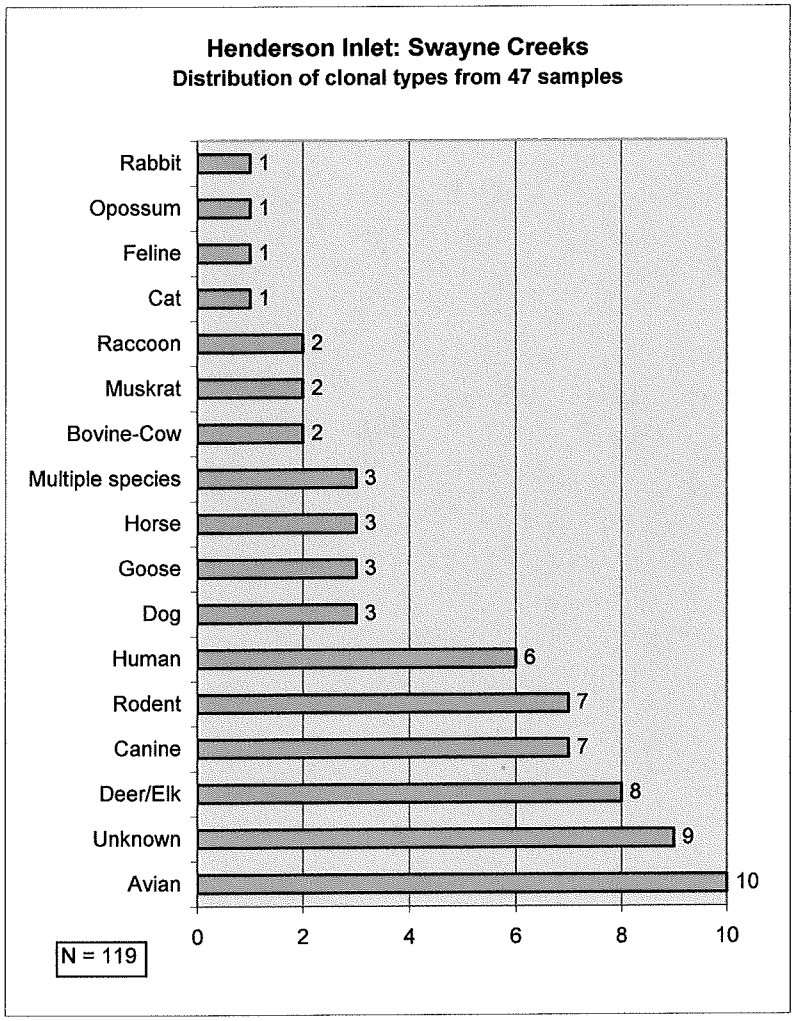


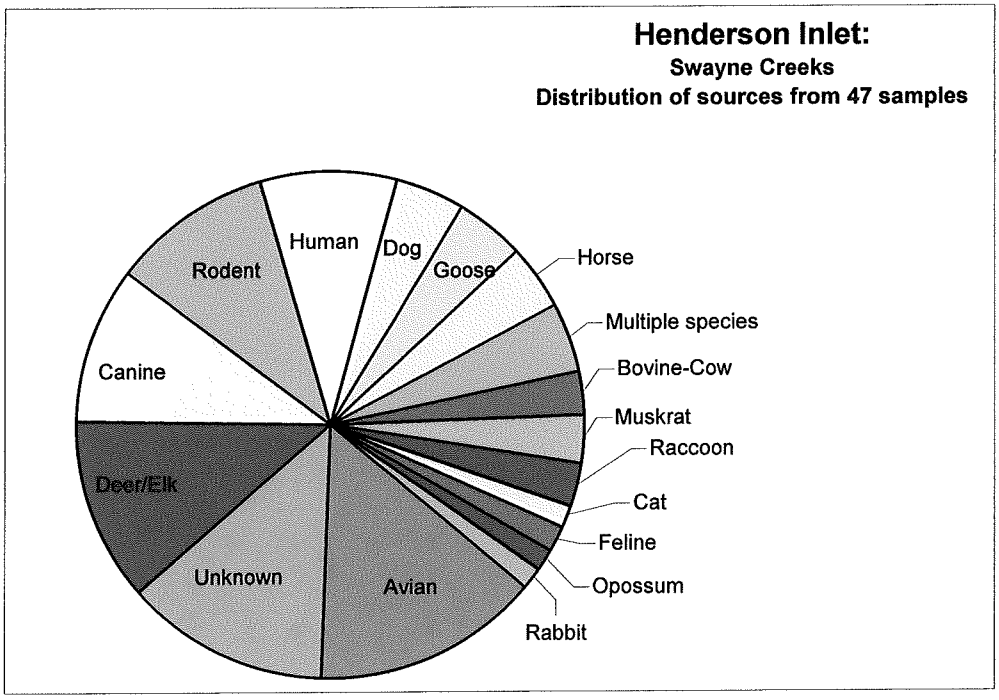
Figure 8 shows the comparison of results from sampling when the shellfish growing area is closed vs sampling under dry, *open* conditions. Are canines more prevalent under dry conditions? Some sources appeared only in wet or dry weather, but this is not significant given the low number of occurrences, i.e. cat, opossum, rabbit.

Figure 9



Figures 9 and 10 show the results for the number of clonal types from Swayne creeks. There were 119 clonal types; 17 different sources were found. The predominant sources (about 2/3) are birds, canines, deer, and rodents - those representative of a rural watershed area.

Figure 10



Marine station #5 results

This is the marine station that was downgraded to a *prohibited* shellfish harvesting status, October 2000. This site had water, sediment, and shellfish tissue sampled. There were 11 sediment collection events. Water samples were collected during 14 sampling events, 4 of which had 5 additional grab samples for a study total of 105 grab samples.

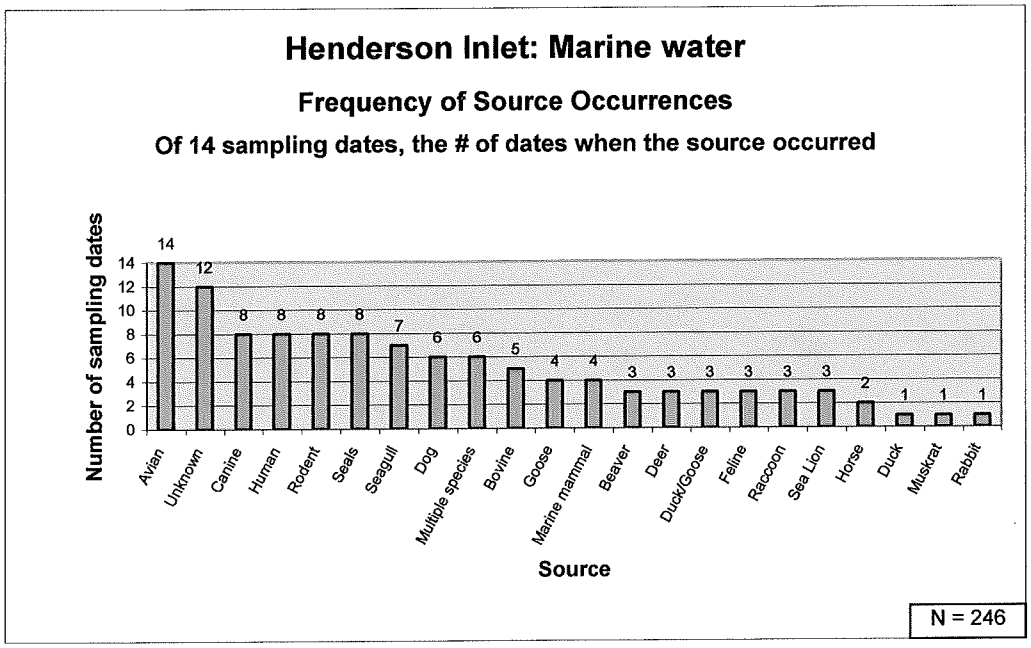


Figure 11

Figure 11 shows the frequency of occurrences for each of the 22 source types. Birds always occurred; they were present in each of the 14 sampling events. Unknowns were the next most frequent, showing up in 12 of the 14 events. Canine, human, rodent, and seals were found in more than half the sampling events.

Figure 12

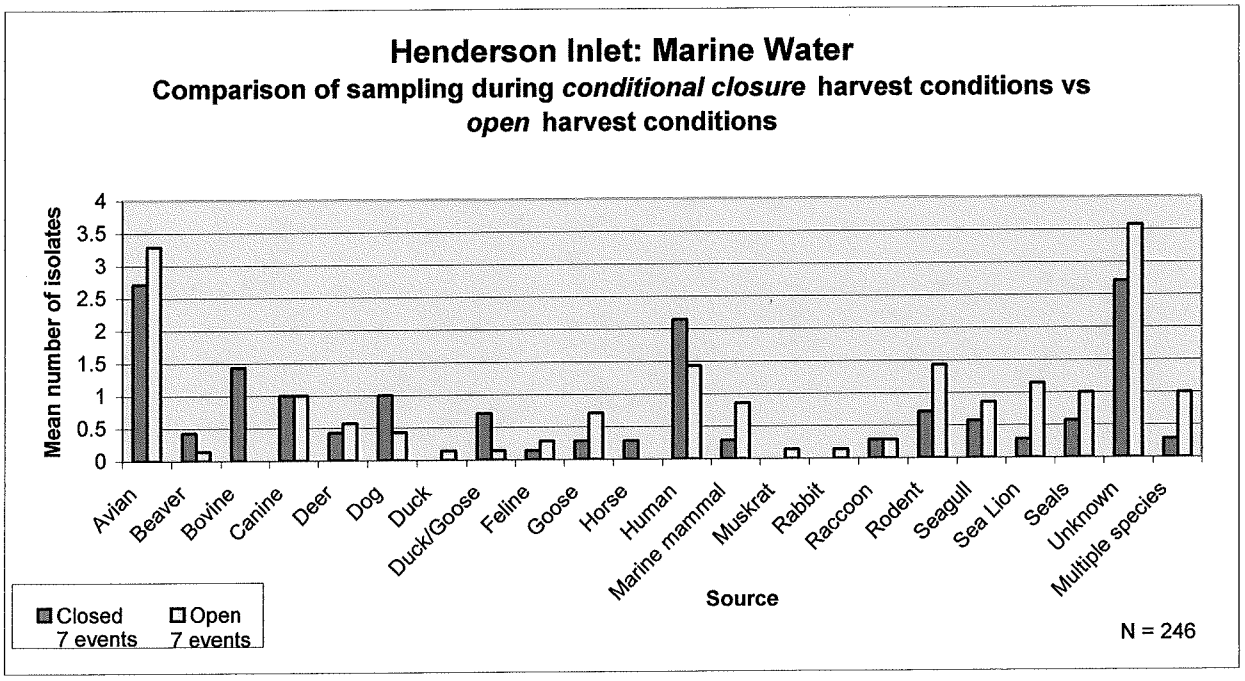


Figure 12 compares the data collected under wet and dry conditions without consideration of tidal influence. There are 5 source types that have the same configuration when comparing the graphs of the creeks with the marine water: bovine, feline, goose, human, and multiple species. Feline, goose, and multiple species have a higher mean during dry weather sampling. Human and bovine have a higher mean during wet weather. Marine animals means are higher during dry weather conditions.

Figure 13

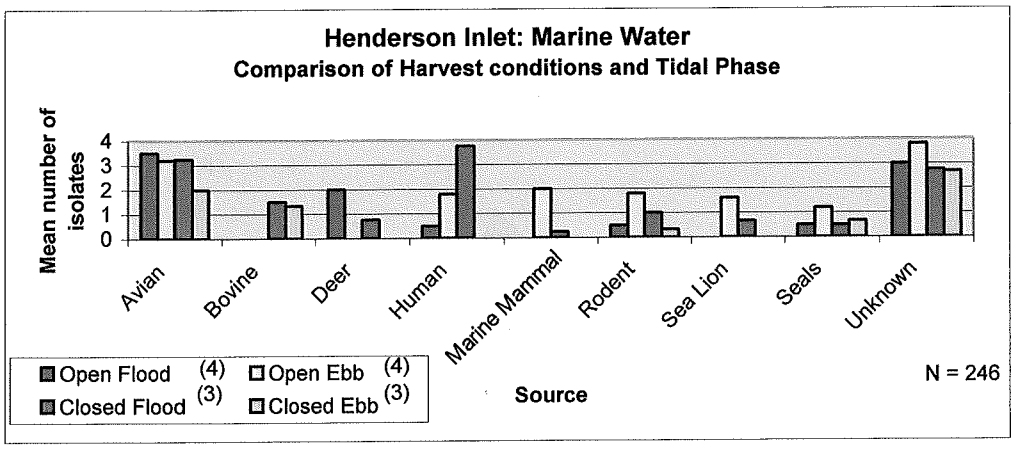
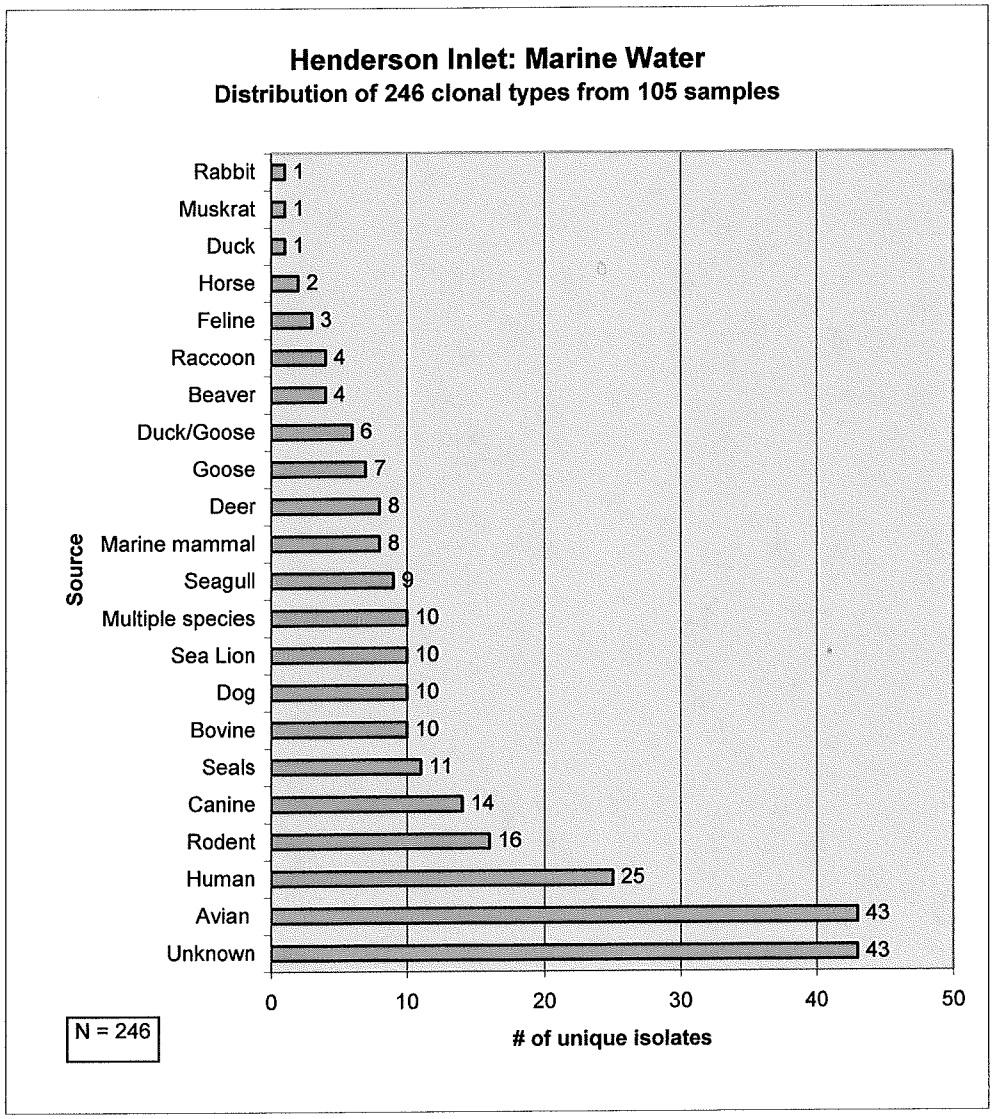


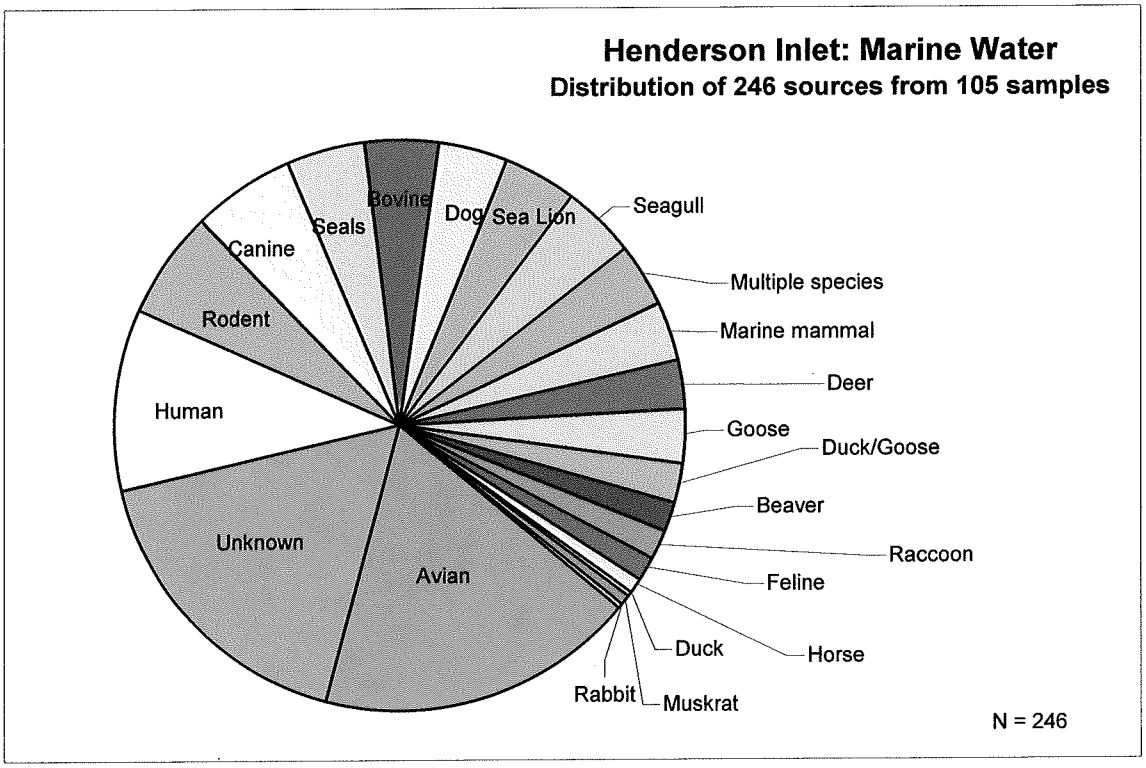
Figure 13 compares 9 of the predominant sources to see what differences there might be under the combination of tidal phase and wet / dry weather conditions. Bovine sources were found in the marine water only under closed conditions. Deer were found only under flood conditions. On a flood tide when the inlet is closed to commercial shellfish, human sources have the highest mean of isolates. Birds, rodents, seals, and unknowns were found under every condition.

Figure 14



Figures 14 and 15 show the distribution of the 246 clonal types for the 22 source types. Unknown, birds, and humans are the predominant source types.

Figure 15



Sediment results from station #5

Sediment was collected with a petite Ponar dredge at the same location and after the marine water was collected. This sequence was to assure the marine water was not contaminated with resuspended bacteria from the disturbed sediments. Figure 16 charts the frequency of source occurrence of the 17 source types found in sediment samples. Birds were present in all sampling events. Unmatched isolates showed up in 8 of the 11 events.

Figure 16

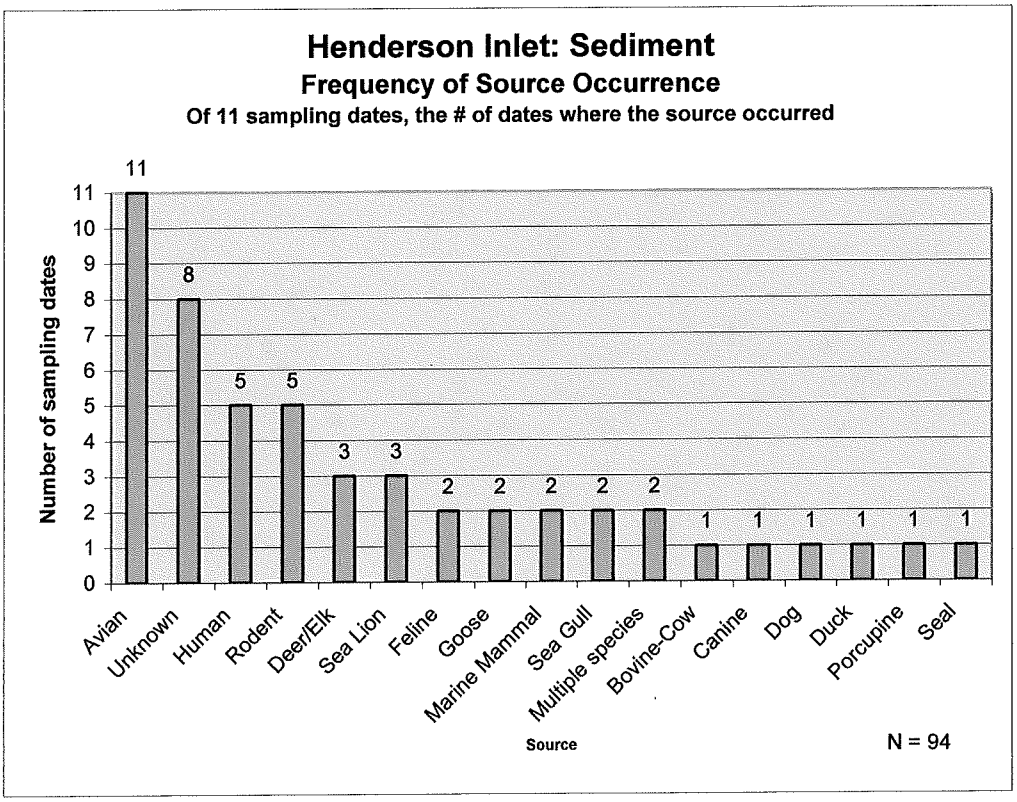
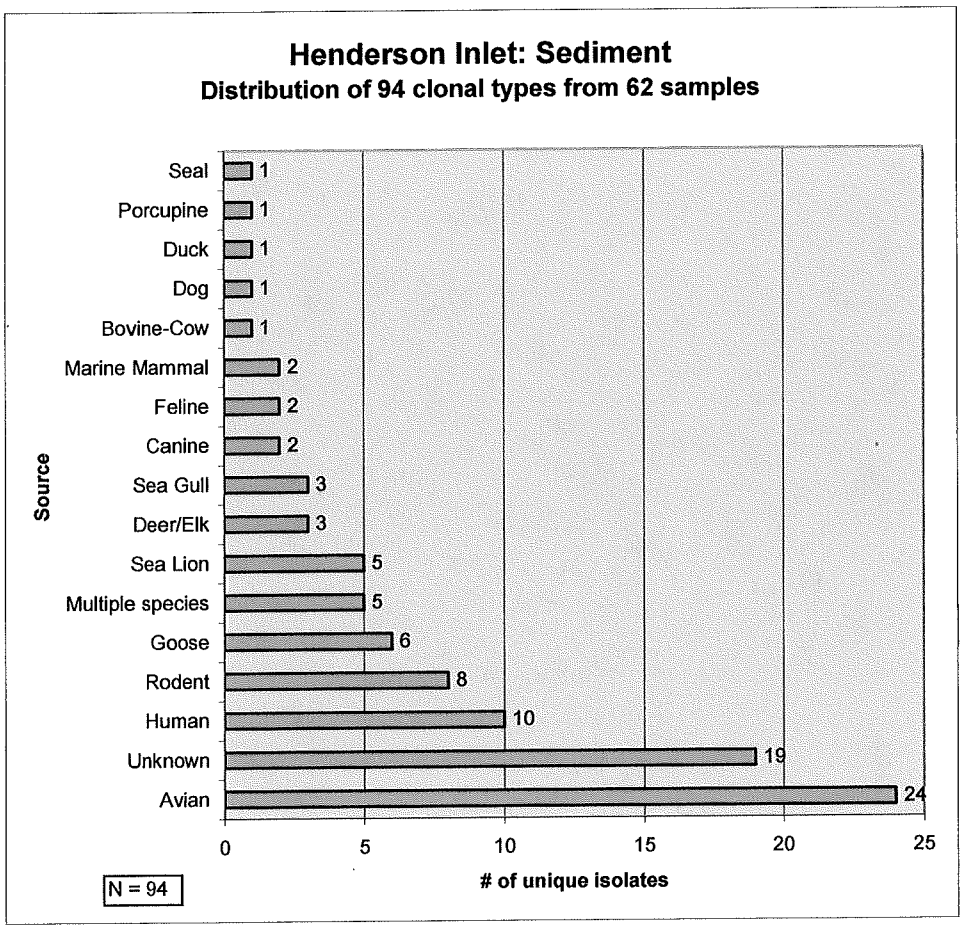


Figure 17



There were a total of 94 clonal types. Unmatched isolates made up 19% of these isolates. Figures 17 and 18 show the distribution of this data set.

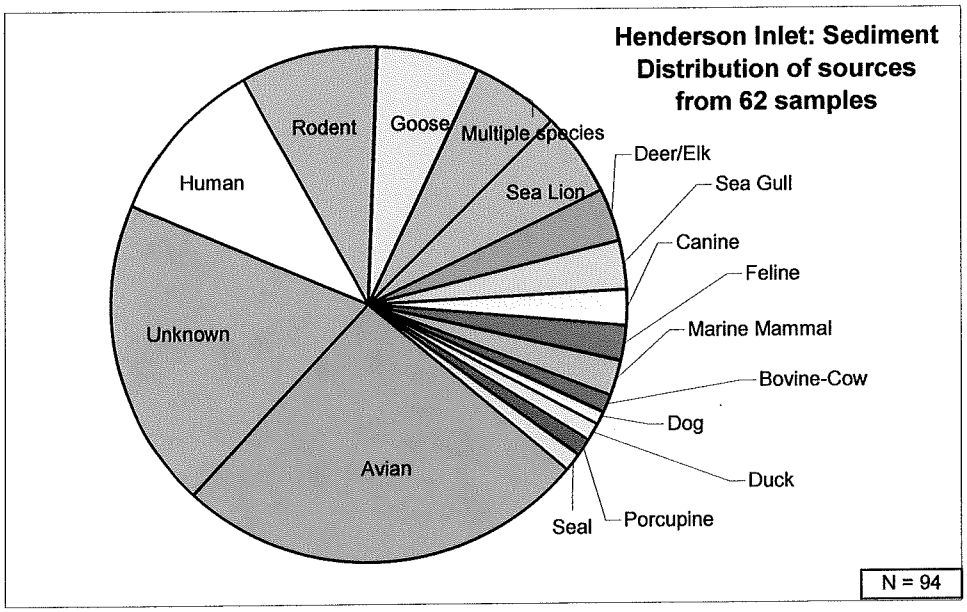


Figure 18

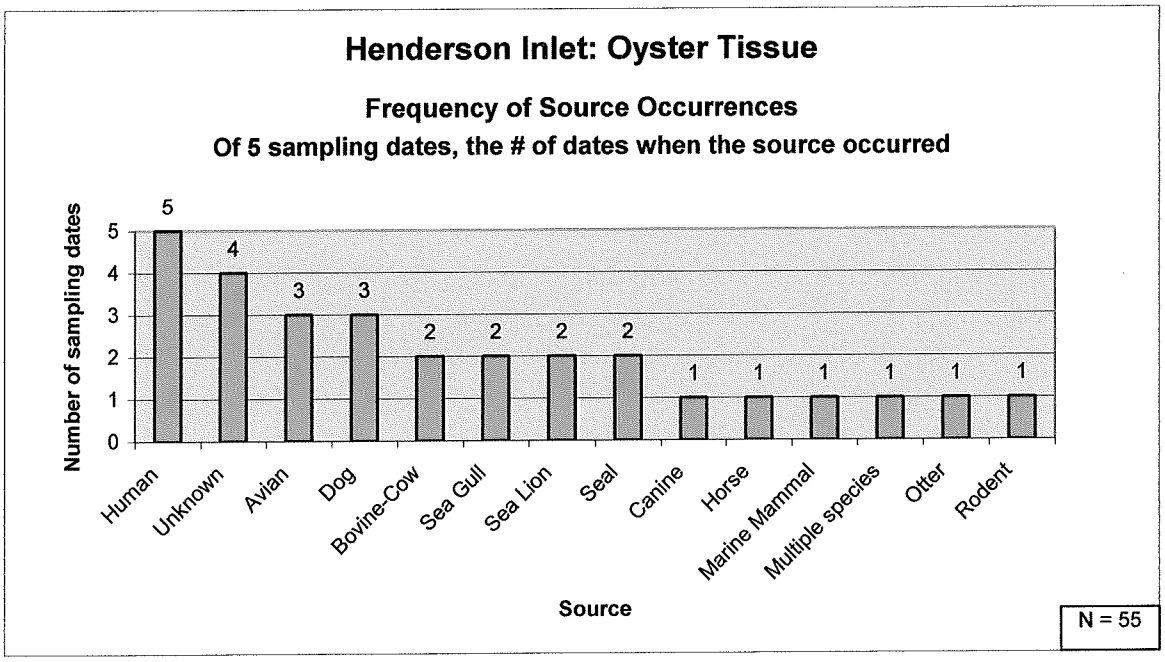
Oysters results

Oysters were sampled 5 times at two different locations. This sampling scheme was designed to look at shellfish tissue from a prohibited station and a conditionally approved and then move the product to the other location to see if there was an effect on the tissue. The original locations were sampled 10/30/00 and 11/10/00. The moved shellfish were sampled 5 months later on 4/6/01.

Sampling date	10/30/00	11/10/00	4/6/01	4/6/01	5/22/01
Sample site #	1200	1313	222	307	833
Sampling location	Station 5	Station 9	Station 5 – previously at station 9	Station 9 - previously at station 5	Station 9
No. of isolates identified	5	27	5	3	13

Figure 19 shows the frequency of source occurrence. Human, bird, dog, and unmatched isolates were found in more than half the sampling events.

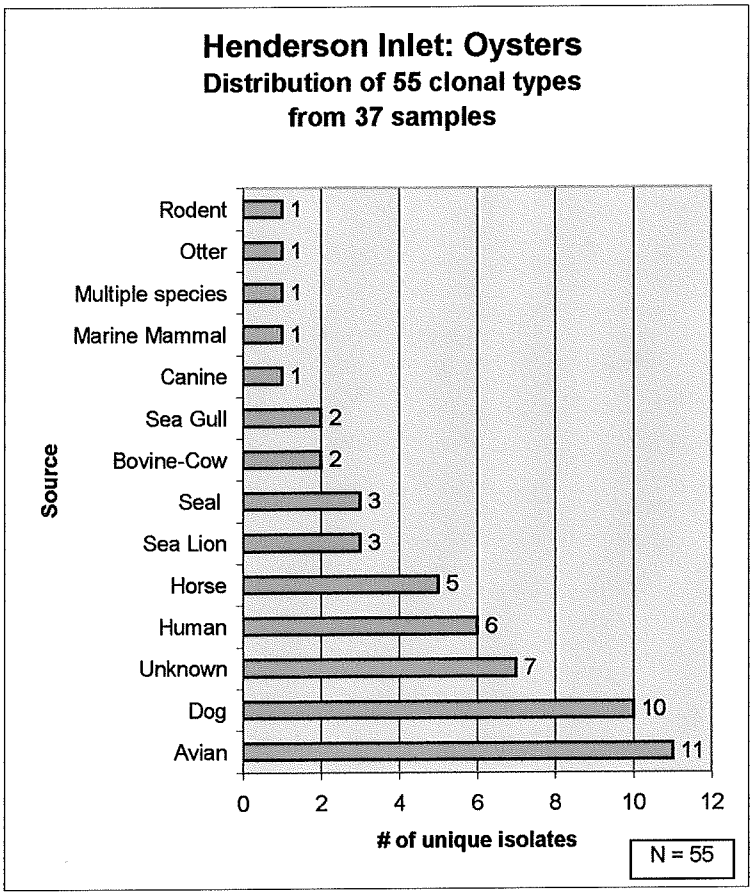
Figure 19



It was difficult to culture *E. coli* from the oyster tissue. There simply was not the abundance of colonies present as in the other media.

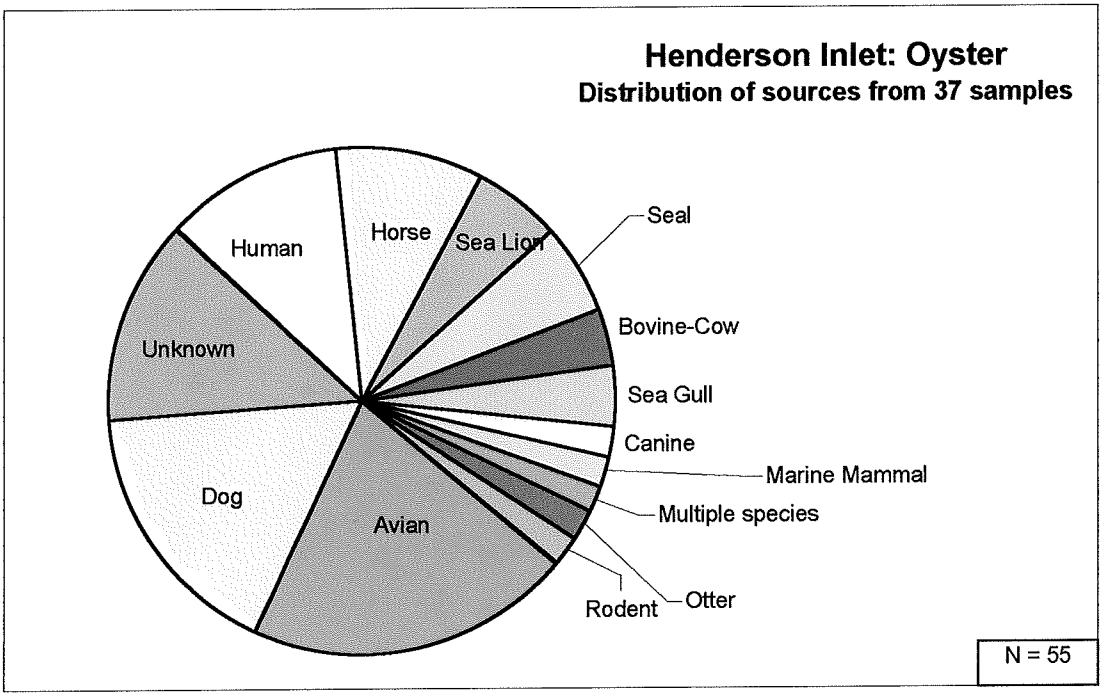
Because the number of isolates identified for each sampling event was so small, the data for oyster tissue is presented as a whole.

Figure 20



Figures 20 and 21 show the 55 clonal types. The predominant sources are birds, dogs, and unmatched.

Figure 21



Total project results

A total of 943 isolates were typed with a total of 650 clonal types. Figures 22 and 23 show the distribution and representation of the types from the entire project.

Figure 22

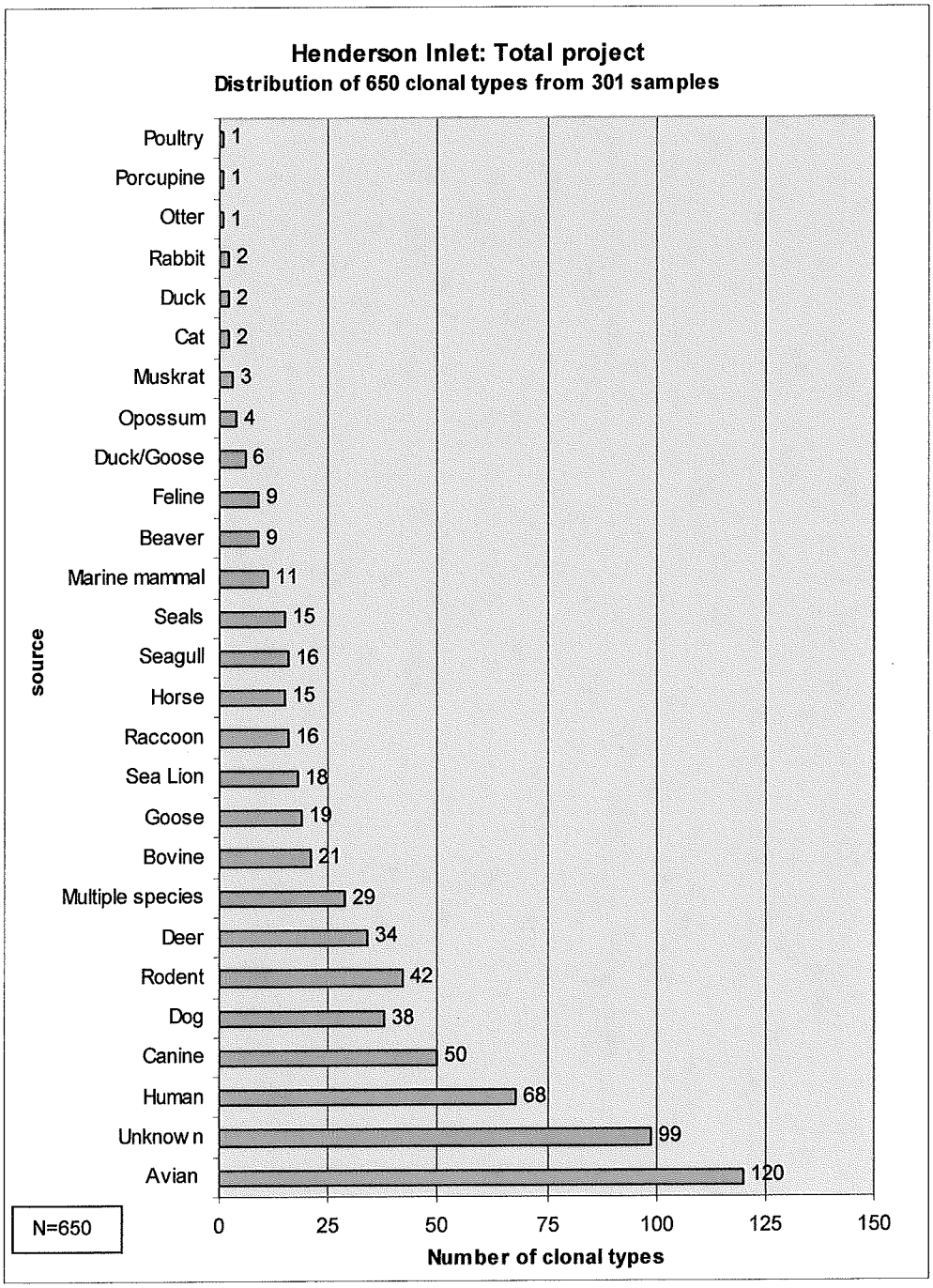


Figure 23

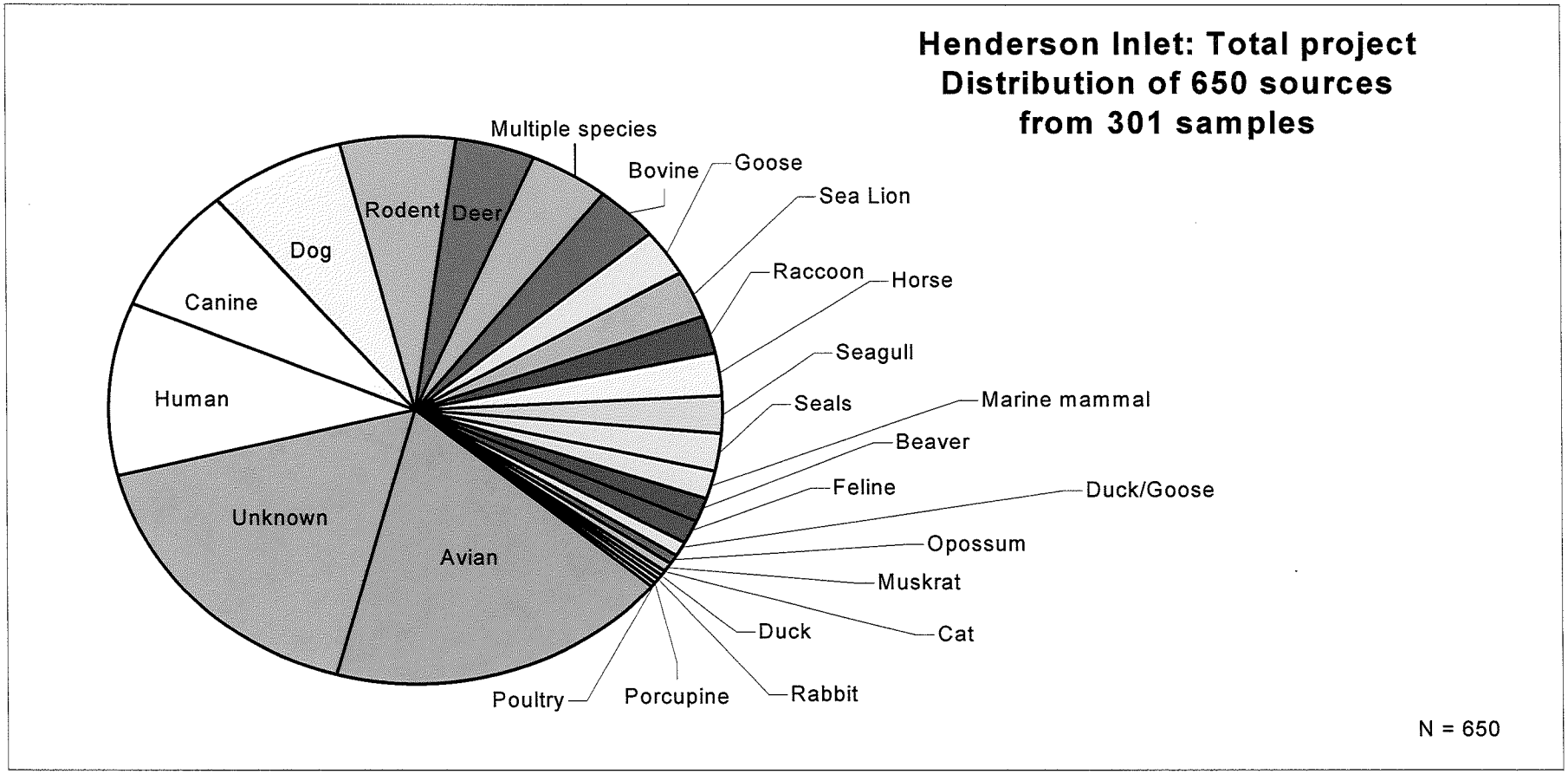


Figure 23 illustrates the 650 clonal types for the entire project. Birds, humans, canines and dogs, along with unknowns, are the predominant clonal types.

Table 5 lists all the source types of clonal types for each site/media. Avian species make up 18% of all clonal types, nearly 1/5. Humans represent 10% of the clonal types. Seventeen percent (17%) were unmatched.

Table 5

Totals for Clonal types

Source type	Marine	Woodland	Swayne	Sediment	Oyster	Total
avian	43	15	27	24	11	120
beaver	4	5	0	0	0	9
bovine	10	5	3	1	2	21
canine	14	19	14	2	1	50
cat	0	0	1	0	0	1
deer	8	9	14	3	0	34
dog	10	14	3	1	10	38
duck	1	0	0	1	0	2
duck - goose	6	0	0	0	0	6
feline	3	2	2	2	0	9
goose	7	3	3	6	0	19
horse	2	5	3	0	5	15
human	25	20	7	10	6	68
marine	8	0	0	2	1	11
multiple	10	9	4	5	1	29
muskrat	1	0	2	0	0	3
opossum	0	2	1	0	1	4
Otter	0	0	0	0	1	1
porcupine	0	0	0	1	0	1
poultry	0	1	0	0	0	1
rabbit	1	0	1	0	0	2
raccoon	4	7	5	0	0	16
rodent	16	7	10	8	1	42
seagull	9	2	0	3	2	16
sea lion	10	0	0	5	3	18
seal	11	0	0	1	3	15
unknown	43	11	19	19	7	99
Totals	246	136	119	94	55	650

Analysis of sample for the presence of human pathogens

A total of 75 oyster, water, and sediment samples were analyzed for the presence of human pathogens using polymerase chain reaction detection method. (Raw data in Appendix F) Fifteen of the total 75 samples (20%) tested positive for the presence of human pathogens, these include four of the 45 (9%) oysters samples, nine of the 15 (60%) sediment samples, and two of the 15 (13%) water samples. Of the four oyster samples that tested positive for the presence of human pathogens, two contained *V. vulnificus*, one was positive for *Campylobacter jujonie*, and the fourth one tested positive for *V. parahemolyticus*.

Of the nine sediment samples that contained human pathogens, four were positive for *V. vulnificus*, one for *Yersinia enterocolitica*, one for *Campylobacter jujonie*, two for enteropathogenic *E. coli*, and one for both enteropathogenic *E. coli* and *V. parahemolyticus*. Two water samples tested positive for the presence of enteropathogenic *E. coli*.

It is interesting to note that all of the 20 oyster samples, collected in November from the closed areas, tested negative for the presence of pathogens, while four of the 25 samples taken in April and May tested positive for pathogens. All of the water and sediment samples were taken in April and May.

Although the number of samples analyzed for the presence of human pathogens were too small to allow for reaching broad conclusions regarding the water and shellfish quality, the pathogen data, taken together with the source tracking results, support the State Health Department's decision for downgrading the shellfish beds in the area.

Discussion

Perhaps the most significant aspect of the study was the percent of matches that were made – 85.7%. The project proposal had estimated between 40 and 60%. The high number of matches is due both to the size of the library, which is around 65,000 isolate patterns, and to the rigor of the study design. Approximately 100 patterns were added from local source samplings.

The primary observation of the data regardless of sampling site – **this is nonpoint pollution**. There is a bit of everything. However, in analyzing the three water bodies sampled, it can be determined that the Swayne creeks are of a rural nature and Woodland Creek is of an urban nature. The source types found in Swayne creek samples represent animals found in rural areas – birds, deer, canines, and rodents. The types in the Woodland Creek samples reflected the urbanization of the watershed with people and dogs being predominant types.

This is a **study of Henderson Inlet only**. Though much can be learned from this body of work, it is a picture of Henderson Inlet. The specific results of this work can not, and must not, be transferred to another watershed.

The results of this study can assist in development of continued remedial activity to improve water quality within southern Henderson Inlet. Of the predominant animal sources, birds are always present and everywhere – on land, on water, and in the air dropping their fecal matter at all sites. As a source, they are relatively beyond control other than to discourage grazing of migratory birds. Unknowns, until identified, are unmanageable. To reduce the number of unmatched isolates, more known fecal sources need to be typed and added to the library.

Human sources are the next most prevalent source. Human bacteria enters surface water, and subsequently sediment and tissue, through fecal contamination from septic systems, failing sewer lines, and direct deposition from humans and diapers. There are previously developed and proven programs that can be implemented that can find and correct many of these sources.

Microbial source tracking, DNA ribotyping, is considered by some professionals to be an experimental methodology. Standard methods have not been developed; studies and methods have not been subjected to rigorous peer review; and statistical evaluation has not been applied to the uncertainties and limitations of the method. Until this occurs, some environmental health professionals are reluctant to accept the results and conclusions of any study in which this methodology is used. To local environmental health professionals this methodology promises to be a valuable tool to use in order to prioritize remedial and preventative water quality work.

Conclusions

- The percentage of matches of this study with known sources was high – 86%. This was due both to the size of the source library (65,000 isolate patterns) and to the rigor of the study.

- In order to increase the number of source matches, and thereby increase the percentage of matches, there must be an on-going effort to continue to collect and type fecal samples from known sources.

- Of all 5 sample sites, marine water samples had the greatest percentage of isolates without a match - 18%. Sediment had 16% unmatched, Swayne creeks 14%, oyster tissue 9%, and Woodland Creek 8%.

- This study represents only the Henderson Inlet study area. The results can not be used to describe other watershed areas.

- The study results are a 'picture' of nonpoint pollution. Twenty-seven (27) source types were identified in the total project – ranging from 14 types in oyster tissue to 22 types found in the marine water.

- The results for the Swayne creeks were characteristic of its rural watershed. In order of percent present in the samples, the source types were birds, canines, deer, and rodents.

- The results of Woodland creek were characteristic of its urban watershed. In order of percent present in the samples, the source types were human, canine, birds, and dog.

- The results of the marine water were reflective of its complexity – it receives water from a variety of sources, i.e. streams, shorelines, and the marine water itself. Factors such as dilution, die-off, and predation all impact the resulting water quality. In order of percent present, the source types for marine water were unknowns, avians, and humans. Birds were 17% of the matches and the predominant source type – not surprising because they were the most predominant clonal type for Swayne creeks, the fourth most predominant type for Woodland Creek, and are present on the marine water itself. Human clonal types, which were the predominant type for Woodland Creek, were 10% of the isolates for the marine water.

- When sampling water (Swayne creeks, Woodland Creek and the marine water), avian, human, canine, and unknown clonal types were found during more than half the sampling events. These types also had the greatest number of isolates. It can be concluded that in this study area these are the most frequently found source types.

- Overall, human, beaver, and bovine isolates were always more prevalent during *conditional closure* conditions than during dry weather sampling. Feline, goose, and multiple species clonal types were more prevalent during dry sampling conditions than during *conditional closure* conditions.

- Bovine clonal types were found in the marine water only during *conditional closure* sampling events. It may be that, in this watershed, bovine nonpoint pollution reaches surface waters only during rain events.

- On a flood tide and when the inlet is closed to commercial shellfish harvest, the marine water human clonal types were found more frequently than any other type at the marine station which may indicate that marine shoreline septic systems are also a contributing source.

- Marine mammal clonal types were found more often during dry weather sampling under ebb tide conditions. Possible explanations could be that there is less dilution during an ebb tide; fecal material left in intertidal areas that the animals use are picked up and carried out by the outgoing tide, or these animals exhibit different behavior depending on the weather / tide.

- Sediment samples had the least isolate 'uniqueness' of the sampling sites. There were more duplicate fingerprints within a given grab sample. There were 210 total isolates but only 51% (108) were different clonal types. [These percentages for other sites were as follows: Swayne creeks had 82%; Woodland Creek had 80%; marine water had 77%; and oysters had 60%.] The low percent for sediment is probably due to its solid, non-mixing state.

- The oyster sampling events produced such low number of isolates that no conclusions about the results can be made. However, it can be concluded that more tissue samples must be collected in order to assess shellfish tissue.

- The bacterial pathogen scan produced limited data. Human pathogens were found. The sample set was relatively small. More consideration must be given to the sample design in order to meet the study objectives.

- This method does show promise as an effective tool for the complex task of identifying the sources of nonpoint pollution. The results of such studies can help in prioritization of local remedial efforts.

- State and local agencies support is needed to fully develop the DNA ribotyping method for source identification of nonpoint pollution. Work toward development of standard methods so that data can be shared, reviewed, and have professional support.

- State and local agencies in concert with researchers should develop a feces collection program (perhaps throughout Puget Sound or even the state) in order to expand and refine the DNA fingerprint library database.

- Due to the definite presence of human clonal types, adopt a County and City risk-based human waste assessment program. This would include both evaluation of septic systems, as well as evaluation of the municipal sewer systems.

- Thurston Conservation District should continue development and management of conservation plans for watershed farmers and livestock owners so that best management practices that protect water quality are implemented and maintained.

- Discuss with stormwater managers the possibility of conducting typing studies to categorize fecal sources within stormwater discharges.

- Through state and local public education programs, advocate proper disposal of dog waste.

- Due to the limited data from sediment and oyster samples, future study designs should be modified to either omit these types of samples, increase the number of specimens collected, and/or add sampling events.

- Due to the limited data from the bacterial pathogen scan, future studies having similar objectives should consider the value of the scan.

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List of Appendices

Appendix A

Circulation Study

APPENDIX TO SOUTH HENDERSON INLET SANITARY SURVEY: DRAFT HYDROGRAPHIC STUDIES NEAR STATION #5

INTRODUCTION

From December 1999 through February 2000 the Department of Health (DOH) and the Thurston County Environmental Health Program (TCEH) conducted a series of hydrographic studies to help determine the source of pollution at Station #5 in south Henderson Inlet. This effort was prompted by the failure of Station #5 to meet the water quality standards for its shellfish classification. At the time of this report, shellfish harvesting in the area is closed when 0.5 inch or more of rainfall occurs within a 24-hour period. Identification of the general pollution source area(s) would be needed prior to remediation efforts, and to demonstrate the need for subsequent survey efforts in the identified watershed(s).

Prior to the hydrographic studies, an analysis of recent water quality data at Station #5 indicated three significant findings:

1. Most of the unsatisfactory water quality results occur during ebb tide cycles, and overall ebb tide water quality results are worse than samples collected on flood tides.
2. While rainfall is associated with many of the pollution events, several of the excessively high (i.e., greater than 43 FC/100 ml) fecal coliform results are obtained during relatively dry (< 0.5" rainfall in 24 hours) conditions.
3. Water quality at Stations 1 and 2, located to the south of Station #5 in the Prohibited area, is worse than at Station #5. However, the water quality at Station 3, located south and west of Station #5 in the Prohibited area, is better than at Station #5.

DOH and TCEH coordinated a series of studies in south Henderson Inlet to help explain the factors and reasons for these three findings.

RESULTS OF INVESTIGATIVE STUDIES

DECEMBER 9

The first intensive investigation by DOH and TCEH occurred on December 9th on an ebb tide with a tidal range ("swing") of 7.5 feet. The Henderson Inlet Conditionally Approved area was open to harvest on that day. The previous rainfall event with greater than 0.5"/24 hours (0.92") occurred on December 2nd. A high tide of 14.9 feet was at 7:28 AM and a low tide of 7.4 feet was at 12:56 PM (tide chart is attached). Two sets of fecal coliform samples were collected at five locations to observe any differences in results during different stages of ebb tide within and just outside the cove at Station #5. The samples collected just outside the cove were taken about ten yards north and south of the western shoreline boundary of the cove.

The first set of samples, taken at the start of ebb tide, indicated that the fecal coliform level at Station #5 (46 FC/100 ml) was higher than that of either Creek B (north creek branch) or Creek C (south creek branch, see map) entering the cove. These results were 33 and 17 FC/100 ml, respectively. However the water quality at the south entrance to the cove (from the inlet) was greater than Station #5 (49 FC/100 ml). Water at the north entrance to the cove was 17 FC/100 ml).

A set of three drogues and three surface floats were released at the mouth of both Creek A and Creek B at the beginning of ebb tide to observe their movements into the main area of the cove. The surface floats indicate movement in the upper 2-3 inches of the water column, whereas the drogues indicated water movement a foot below the surface. Surprisingly, none of the drogues or floats moved into the main body of the cove throughout this study day, and instead remained near the creek mouths.

Drogues, surface floats and dye (fluoresceine) were released at the southern entrance of the cove at mid-ebb tide. While the relative speeds of these three indicators varied, all three of these indicators entered the cove and passed directly through or by Station #5. These results explain how the fecal coliform level at the south entrance to the cove could impact the water quality at Station #5, even on ebb tide. The surface floats, drogues and dye passed through the Station #5 area, headed directly towards the cove's north shore, and either got beached there or circled around the main area of the cove in a continuous clockwise pattern.

A second set of fecal coliform samples was collected towards the end of this ebb tide cycle. Water quality at the south entrance to the cove still had the highest count of the series (79 FC/100 ml). Station #5 results had dropped to 13 FC/100 ml, both creek mouths had a level of 33 FC/100 ml, and the area at the north cove entrance showed 34 FC/100 ml.

The results obtained during December 9 show that fecal coliform results at Station #5 can vary within a single tidal cycle. In addition, on this day, the highest source of pollution to the cove and Station #5 appeared to be entering the cove from the south, from Henderson Inlet. Finally, polluted water entering the cove can remain in the cove for long periods due to the clockwise gyre evidenced during this ebb tide cycle.

DECEMBER 13

The second hydrographic study by DOH and TCEH occurred on December 13th, on an ebb tide with a range of 8.2 feet. The Henderson Inlet Conditionally Approved area was closed to harvest on that day, due to 1.26" of rain in the previous 24 hours. A high tide of 15.1 feet was at 9:55 AM and a low tide of 6.9 feet was at 4:08 PM (see attached tide chart). The main purpose of this investigation was to trace the flow of Woodland Creek through the south part of Henderson Inlet on a typical ebb tide. Drogues and floats were used as indicators of this flow. A 12-channel global positioning system (GPS) unit monitored their positions with time. In addition, a series of fecal coliform samples were collected within and just outside the cove at Station #5.

Prior to the drogue and float release, the cove at Station #5 was visited to observe if any of the eleven surface floats left in the cove from the December 9th study still remained (all drogues were retrieved at the end of the first study). Four surface floats still remained within the inner portion of the cove, again indicating that pollution can remain in the cove for long periods of time.

Surface floats and drogues were released at 10:17 AM at the southern end of Henderson Inlet, approximately 500 feet northwest of the Johnson Point Road bridge. The surface floats moved at a faster speed, but in the same direction as the drogues. On occasion the floats or drogues would get hung up on a branch or on a shoreline, at which time they would be retrieved and re-released nearby. The surface floats better represented the flow of Woodland Creek, as the salinity at the surface (3 ppt) was much less than at a one-foot depth (10 ppt) at the southern part of the inlet. At 10:58 AM the surface floats were 0.23 mile from the initial dropoff point, which is an average speed of 0.34 mph. This relatively slow speed may have been due to the release of drogues and

floats at the very start of ebb tide. The drogues did not arrive at this location until approximately 11:03 AM (average speed of 0.30 mph). The floats traveled 0.35 mile by 11:05 AM, but the drogues did not arrive at this approximate location until 11:28 AM. In order to not lose sight of the surface floats, the drogues were retrieved and placed near the surface floats.

At 11:15 the floats were near Dobbs Creek Cove. Surface salinity at this location was 11 ppt, and 22 ppt at a one-foot depth. At 11:30 AM the surface floats were due south of the mouth of the large unnamed cove, which faces to the south, at a distance of 0.58 mile to 0.63 mile from the release point (an average speed of 0.50 mph). At this location their trajectory abruptly but consistently changed from a northern direction to a WNW direction. The surface floats then all approached within 20-40 feet of the western shoreline for the next 0.3 mile. At 12:02 the floats had traveled 1.06 mile and started a trajectory directly for the Station #5 cove from the western shoreline. At this location the surface floats had achieved an overall average speed of 0.61 mph from their initial release location. The floats continued in a straight line until just south of the airplane runway, at which time the trajectory become parallel to the eastern shoreline. The floats passed west of Station #5 about 60 yards at 12:15. Therefore the surface floats traveled to the immediate area of Station #5 from the southern end of Henderson Inlet, a distance of 1.38 mile, in just less than two hours on a typical ebb tide. The average speed for their entire trip was 0.70 mph.

The path traveled by the surface floats is illustrated on the attached map. This route directly coincides with the streambed of Woodland Creek that meanders through Henderson Inlet. In addition, this route helps explain why the water quality at Station 3 is much better than at Stations 1, 2 or 5. I.e., Station 3 appears to be hydrographically isolated from the direct influence of Woodland Creek.

At 12:20 a series of surface floats was released in a transect perpendicular to the concrete boatramp near the airplane runway (near Station 34). This location is just over 100 yards south of the cove entrance. A light wind was coming from the south and southwest. Each of seven floats was released at an approximate 30-yard interval, starting 30 yards from shore, to observe which (if any) would enter the Station #5 cove. The float nearest to shore ran aground on the shoreline prior to the cove whereas the float released 60 yards from shore headed directly for Station #5. The float released 90 yards from shore passed by the mouth of the cove, and the other floats passed the cove further out (to the west). At 12:36 the float which passed close to Station #5 was drifting out of the cove.

Another series of float releases was conducted at 12:40. Six surface floats were released at 50-foot intervals, starting 50 feet from shoreline, in the same transect as the previous release. All of the floats released within 200 feet of the shoreline ran aground on the shoreline south of the cove. The float released 250 feet from shoreline entered the cove. The surface float released 300 feet from shoreline headed directly up the inlet and did not approach the cove.

A third float release was conducted at the immediate south of the cove at 1:17. The float released ten feet offshore entered the cove, whereas the float released 40 feet offshore avoided the cove, moving directly up the inlet. The results of these three series of float releases are similar, and help explain how fecal coliform levels in the water immediately south of the cove mouth can impact water quality at Station #5 on ebbing tides.

Fecal coliform samples and salinities were collected in or near the cove from 12:46 to 1:06. The results are as follows:

SAMPLE LOCATION	*FC/100 ML*	*SALINITY (ppt)*
South of cove mouth	23	20
Station #5	70	5
South (creek) branch	110	0
North (creek) branch	49	0
North of cove mouth	49	17

These results indicate, by the salinity readings, that the water at Station #5 was influenced during this sampling both by the creeks (contributing freshwater) and the (saline) inlet water. However, this degree of mixing or influence is not consistent at Station #5, even considering only ebb tide cycles. For example, during this investigation, the extent of a freshwater lens was visible on the water surface. Salinity varied from 4 ppt to 20 ppt on different sides of the boat a few yards west of Station #5. However, this delineation of mixing of inlet waters with creek waters in the cove has been observed at different locations, as will be described in the following paragraphs.

JANUARY 27

On January 27th DOH and TCEH collected water samples for fecal coliform analysis during a dry period, on a tide with a range of 11.4 feet. No more than 0.20" of rain had fallen during any day in the week previous to this sampling. A high tide of 14.7 feet was at 10:29 AM and a low tide of 3.3 feet was at 5:29 PM. Sampling occurred between 2:18 PM and 3:00 PM. Station 34 is located just over 100 yards south of the cove entrance near the private airstrip (see map). The results are listed below.

SAMPLE LOCATION	*FC/100 ML*	*SALINITY (ppt)*
400 yards south of Station #3	49	13
Station #34	9.3	13
100 yards east of Station #34	33	14
Station #5	13	9
Confluence of 2 creeks in cove	14	0
Station #6	13	9

Salinity readings (again) suggest that the water at Station #5 is partially mixed with freshwater from the creeks in the cove. In addition, DOH and TCEH observed a salinity divergence line (the boundary of the surface water lens) between Station #5 and the confluence of the creek mouths. However, a surface float released at Station #5 moved directly out of the cove during this later stage of ebb tide.

FEBRUARY 1

DOH and TCEH collected fecal coliform samples on February 1 following a very intensive rainfall event that dropped approximately two inches of rain in the previous 24 hours. A high tide of 12.4 feet was at 2:08 PM, and a low tide of 0.1 foot was at 9:46 PM. The results collected on this ebb tide (with a range of 12.3 feet) are listed below.

SAMPLE LOCATION	*FC/100 ML*	*SALINITY (ppt)*
400 yards south of Station #3	95	20
Station #34	540	10
100 yards east of Station #34	540	9
Station #5	540	4
South creek mouth	240	0
North creek mouth	350	0
Station #6	920	10

It is difficult to make conclusions from the data listed in the table, due to the extreme intensity of rainfall that fell immediately prior to this sampling. This area of Henderson Inlet is closed to shellfish harvesting when more than (only) 0.5" of rain falls in 24 hours. However, some mixing of inlet water with creek water is evident at Station #5, even though the entire cove appeared to be turbid. The salinity (4 ppt) at Station #5 was surprising since an even lower salinity was observed a few yards to the west of it. On closer inspection, DOH and TCEH observed a ribbon of clearer inlet water pushing through the turbidity on the south mouth of the cove directly to Station #5. The ribbon of inlet water helps explain the higher salinity observed at Station #5 on this day than at locations immediately to its east and west.

SUBSEQUENT HYDROGRAPHIC AND DATA EVALUATIONS

Following the field observations and studies, the TCHD sorted water quality data at Station #5 by several parameters to observe any correlations with degraded water quality. The parameters included rainfall, salinity, fecal coliform results, stage and phase of tide, wind speed and direction, and season. All water quality results collected from 1/23/96 through 10/4/99 were used in this assessment. The sorting evaluations include the following observations for Station #5:

1. Elevated water quality occurred 20 times during the four-year period. Eleven results were greater than 43 FC/100 ml, while nine had levels between 14 and 43 FC/100 ml.
2. Thirteen of the 24 samples collected during ebb tides occurred two hours or more after the start of ebb tide. During this period of ebb tide, six results were greater than 43 FC/100 ml and nine were greater than 14 FC/100 ml. None of the 12 samples collected during the first two hours of ebb tide were greater than 43 FC/100 ml.
3. No circulation patterns were studied in the field during flood tide cycles. Therefore it cannot be determined how long into the first part of the flood tide phase that the flow from the head of the inlet may continue to have an impact on water quality at Station #5.
4. A total of 58% of all samples were collected during tides of a moderate range, versus 42% during tides with a large range. However, 75% of the results greater than 14 FC/100 ml were collected on tides with a moderate range, while 25% occurred on tides with a large range. A total of 73% of the results greater than 43 FC/100 ml was collected on tides with a moderate range.
5. It was noted during the field studies that the Station #5 cove surface water does not leave the cove during large portions of ebb tides of moderate range. Flushing of surface waters in the cove appears limited, especially in the inner cove near the creek inlets. Several surface floats remained within the cove for four days in December. Water quality violations appear more likely to occur during moderate tidal swings when flushing of the cove is reduced.

6. Wind from a southerly (SE, S, or SW) direction is associated with water quality observed at Station #5. Nine of the 11 results greater than 43 FC/100 ml, and five of the nine results between 14 and 43 FC/100 ml occurred when the wind came from a southerly direction.
7. Winds from the south tend to have a greater speed and be accompanied by rain in comparison to winds from other directions. Only two of 18 sampling events collected during southerly winds had no associated rainfall.
8. Wind velocity ranged from 8.5 to 21 mph during collection of six of the 11 samples that had results greater than 43 FC/100 ml. Rain was associated with all these (11) events.
9. Elevated water quality at Station #5 is associated with rainfall. Twelve of the 20 samples with greater than 14 FC/100 ml were collected when the rainfall accumulation during the four days prior to sampling exceeded the criteria in the DOH management plan for Henderson Inlet. Nine of these 12 results were greater than 43 FC/100 ml. Only two results with greater than 43 FC/100 ml occurred when the four-day rainfall accumulation was less than the management plan criteria.
10. Three septic system failures were repaired in the fall of 1998 on the west shoreline approximately 400 yards south of Station #3. During the field study of 12/12/99, inlet water passing within a few feet of the shoreline of these properties reached the area of Station #5 in approximately 15 minutes. At the site of one of these failures a grab sample result of the water entering the inlet was 215,000 FC/100 ml on 12/30/97. Since these failures could account for elevated water quality at Station #5 before their repair, the trend of water quality at Station #5 after completion of these repairs should continue to be noted.

CONCLUSIONS

Empirical water quality results show that ebb tide is the adverse tidal condition for water quality at Station #5. The results of the December 13th floats and drogue study demonstrate that the waters of Woodland Creek traveled on a typical ebb tide from its mouth to the immediate vicinity of the Station #5 cove in approximately two hours. Other sampling results obtained in or near the cove demonstrate that inlet water enters the cove during many portions of ebb tide directly toward or through Station #5. Meteorological information indicates that winds from the south are associated with elevated water quality results at this station. In addition, a high proportion of samples collected two hours or more after the start of ebb tide have degraded water quality.

Salinities of water samples from the cove show that the area around Station #5 appears to be a zone of mixing of water from the creeks and marine water brought in from the inlet. Fecal coliform results also indicate that the results at Station #5 could be a combination of creek water and inlet water. It therefore appears that the creeks that discharge into the Station #5 cove as well as Woodland Creek and/or other southern inlet sources contribute pollution to Station #5.

Measures to reduce pollution in these watersheds are recommended in order to improve the water quality at Station #5, which currently fails water quality standards. An example of such measures includes the repair of three residential onsite systems in 1998. These systems are located very close to the main pathway of Woodland Creek through the inlet on ebb tides, and hence could have been pollution sources for water collected at Station #5. However, water quality at Stations 1 and 2 demonstrate that pollution sources still exist in Woodland Creek and/or the more southern portions of Henderson Inlet.

The improved water quality at Station 3 (as compared to Station #5) appears to be due to its relative isolation from the direct path of the plume of Woodland Creek. The main pathway of Woodland Creek through this portion of south Henderson Inlet during ebb tide appears to flow to

the east of Station 3 but very close to Station #5. It is therefore recommended that the southern border of the Henderson Inlet conditionally approved area on the western shoreline be extended to the south, to include Station 3.

Appendix B

Raw Data

The raw data of the study is as follow:

- The data is listed by sample site: oyster, sediment, marine water (833), Woodland Creek (832), and Swayne Creeks (831).
- Each isolate has a unique isolate number.
- The 'note' column number is associated witha particular DNA library pattern.
- Each particular note is described as to what type of creature it is – resident/transient. *Unknown* means that the pattern has not been found in any previous studies and does not exist in the library at this point in time. [It may in the future at which time these unknowns could be 'reprocessed' to determine if there is a match.] A notation of *transient,* identified as *multiple species* in the graphs and charts, means that the DNA pattern can be found in more than one source, e.g. the same pattern resides in cows, ducks, and cats.
- The matrix type is water = 1, sediment = 78, and tissue = 97.
- The last column, Provider Sample, identifies the grab sample for the particular sampling event.

Henderson Inlet: Swayne creeks
Raw Data

Sample Site	Isolate	Note	Resident/Transient	Source Type	Provider Sample .
831	27709	14	Avian	1	C1
831	27710	15	Deer-Elk	1	C1
831	27711	14	Avian	1	C1
831	27712	71	feline	1	C2
831	27713	25	Deer-Elk	1	C2
831	27714	271	Unknown	1	C2
831	27715	71	feline	1	C3
831	27716	257	Avian, L-D	1	C3
831	27717	271	Unknown	1	C3
831	27718	41	canine	1	C4
831	27719	50	goose	1	C4
831	27720	67	human	1	C5
831	27721	25	Deer-Elk	1	C5
831	27722	46	Avian	1	C5
831	27753	14	Avian	1	C1
831	27754	14	Avian	1	C1
831	27755	14	Avian	1	C1
831	27756	289	Deer-Elk	1	C2
831	27757	3	Bovine, L-D	1	C2
831	27758	109	Human, L	1	C2
831	27759	267	rodent	1	C3
831	27760	253	canine	1	C3
831	27761	289	Deer-Elk	1	C3
831	27762	67	human	1	C4
831	27763	253	canine	1	C4
831	27764	310	Unknown	1	C4
831	27765	267	rodent	1	C5
831	27766	27	dog	1	C5
831	27767	3	Bovine, L-D	1	C5
831	27834	196	Raccoon	1	C-1
831	27835	196	Raccoon	1	C-1
831	27836	196	Raccoon	1	C-1
831	27837	165	avian	1	C-2
831	27838	196	Raccoon	1	C-2
831	27839	196	Raccoon	1	C-2
831	27840	293	muskrat	1	C-3
831	27841	201	Unknown	1	C-3
831	27842	217	deer-elk	1	C-3
831	27843	271	Unknown	1	C-4
831	27844	89	Dog, L-D	1	C-4
831	27845	38	human	1	C-4
831	27846	267	rodent	1	c-5
831	27847	271	Unknown	1	c-5
831	27848	271	Unknown	1	c-5
831	28105	153	rodent	1	C-1
831	28106	308	Unknown	1	C-1
831	28107	308	Unknown	1	C-1
831	28108	204	rodent	1	C-2
831	28109	4	Horse	1	C-2
831	28110	257	Avian, L-D	1	C-2

Henderson Inlet: Swayne creeks
Raw Data

Sample Site	Isolate	Note	Resident/Transient	Source Type	Provider Sample .
831	28111	153	rodent	1	C-3
831	28112	273	avian	1	C-3
831	28113	197	horse	1	C-3
831	28114	36	Avian	1	C-4
831	28115	334	canine	1	C-4
831	28116	36	Avian	1	C-4
831	28117	36	Avian	1	C-5
831	28118	46	Avian	1	C-5
831	28119	36	Avian	1	C-5
831	51554	39	Avian	1	C-1
831	51555	39	Avian	1	C-1
831	51556	237	Unknown	1	C-2
831	51557	34	Deer, L-D	1	C-2
831	51558	34	Deer, L-D	1	C-2
831	51559	237	Unknown	1	C-3
831	51560	194	rabbit	1	C-3
831	51561	172	avian	1	C-3
831	51562	57	horse	1	C-4
831	51563	237	Unknown	1	C-4
831	51564	128	Avian	1	C-4
831	51565	128	Avian	1	C-5
831	51566	266	avian	1	C-5
831	51567	238	Unknown	1	C-5
831	51616	289	Deer-Elk	1	C-1
831	51617	289	Deer-Elk	1	C-1
831	51618	289	Deer-Elk	1	C-1
831	51619	143	avian	1	C-2
831	51620	237	Unknown	1	C-2
831	51621	150	Unknown	1	C-2 Transient
831	51622	110	Human, L	1	C-3
831	51623	294	muskrat	1	C-3
831	51624	267	rodent	1	C-4 (1)
831	51625	149	Unknown	1	C-4 (1) Transient
831	51626	267	rodent	1	C-4 (1)
831	51627	50	goose	1	C-4 (1)
831	51628	50	goose	1	C-4 (2)
831	51629	289	Deer-Elk	1	C-5
831	51630	289	Deer-Elk	1	C-5
831	51631	289	Deer-Elk	1	C-5
831	51927	39	Avian	1	C1
831	51928	162	avian	1	C1
831	51929	191	Unknown	1	C1
831	51930	162	avian	1	C1
831	51931	289	Deer-Elk	1	C2
831	51932	161	Unknown	1	C2
831	51933	18	Raccoon	1	C2
831	51934	161	Unknown	1	C2
831	51935	83	rodent	1	C3
831	51936	223	human	1	C3
831	51937	184	avian	1	C3

Henderson Inlet: Swayne creeks
Raw Data

Sample Site	Isolate	Note	Resident/Transient	Source Type	Provider Sample .	
831	51938	184	avian	1	C3	
831	51939	221	opossum	1	C4	
831	51940	317	Unknown	1	C4	
831	51941	262	avian	1	C4	
831	51942	41	canine	1	C4	
831	51943	128	Avian	1	C5	
831	51944	270	canine	1	C5	
831	51945	270	canine	1	C5	
831	51946	257	Avian, L-D	1	C5	
831	51999	270	canine	1	C1	
831	52000	270	canine	1	C1	
831	52001	270	canine	1	C1	
831	52002	188	Unknown	1	C1	
831	52003	270	canine	1	C2	
831	52004	270	canine	1	C2	
831	52005	270	canine	1	C2	
831	52006	270	canine	1	C2	
831	52007	270	canine	1	C3	
831	52008	270	canine	1	C3	
831	52009	270	canine	1	C3	
831	52010	270	canine	1	C3	
831	52011	270	canine	1	C4	
831	52012	208	avian	1	C4	
831	52013	208	avian	1	C4	
831	52014	178	Unknown	1	C4	
831	52015	6	Bovine, L	1	C5	
831	52016	34	Deer, L-D	1	C5	
831	52083	302	cat	1	C2	
831	52084	270	canine	1	C2	
831	52085	225	rodent	1	C2	
831	52086	269	goose	1	C2	
831	52087	199	Unknown	1	C3	Transient
831	52088	270	canine	1	C3	
831	52089	270	canine	1	C3	
831	52090	270	canine	1	C3	
831	52091	46	Avian	1	C4	
831	52092	46	Avian	1	C4	
831	52093	216	deer	1	C5	
831	52094	34	Deer, L-D	1	C5	
831	52095	209	avian	1	C5	
831	52096	270	canine	1	C5	
831	52158	94	dog	1	C1	
831	52159	267	rodent	1	C1	
831	52160	98	deer	1	C1	
831	52161	19	Unknown	1	C1	Transient
831	52162	47	Avian	1	C2	
831	52163	253	canine	1	C2	
831	52164	16	Raccoon	1	C2	
831	52165	18	Raccoon	1	C2	
831	52166	288	Unknown	1	C3	

Henderson Inlet: Swayne creeks
Raw Data

Sample Site	Isolate	Note	Resident/Transient	Source Type	Provider Sample .
831	52167	81	Unknown	1	C3
831	52168	79	human	1	C3
831	52169	84	avian	1	C3

Henderson Inlet: Woodland Creek
Raw Data

Sample Site	Isolate	Note	Resident/Transient	Source Type	rovider Sample
832	27723	180	Beaver	1	H1
832	27724	239	canine	1	H1
832	27725	110	Human, L	1	H1
832	27726	313	canine	1	H2
832	27727	110	Human, L	1	H2
832	27728	110	Human, L	1	H2
832	27729	110	Human, L	1	H3
832	27730	234	feline	1	H3
832	27731	175	human	1	H3
832	27732	311	sae gull	1	H4
832	27733	59	deer	1	H4
832	27734	110	Human, L	1	H4
832	27735	110	Human, L	1	H5
832	27736	110	Human, L	1	H5
832	27768	265	human	1	H1
832	27769	289	Deer-Elk	1	H1
832	27770	253	canine	1	H1
832	27771	300	canine	1	H2
832	27772	300	canine	1	H2
832	27773	202	beaver	1	H2
832	27774	300	canine	1	H3
832	27775	296	raccoon	1	H3
832	27776	296	raccoon	1	H3
832	27777	296	raccoon	1	H4
832	27778	296	raccoon	1	H4
832	27779	296	raccoon	1	H4
832	27780	314	avian	1	H5
832	27781	296	raccoon	1	H5
832	27782	253	canine	1	H5
832	27819	134	poultry	1	H-1
832	27820	2	Canine	1	H-1
832	27821	2	Canine	1	H-1
832	27822	271	Unknown	1	H-2
832	27823	177	Beaver	1	H-2
832	27824	177	Beaver	1	H-2
832	27825	311	sea gull	1	H-3
832	27826	311	sae gull	1	H-3
832	27827	177	Beaver	1	H-3
832	27828	267	rodent	1	H-4
832	27829	179	Unknown	1	H-4
832	27830	179	Unknown	1	H-4
832	27831	232	human	1	H-5
832	27832	152	human	1	H-5
832	27833	37	Avian	1	H-5
832	28057	176	avian	1	H-1
832	28058	160	Unknown	1	H-1
832	28059	125	goose	1	H-1
832	28060	289	Deer-Elk	1	H-2
832	28061	289	Deer-Elk	1	H-2
832	28062	138	Avian	1	H-2

Henderson Inlet: Woodland Creek
Raw Data

Sample Site	Isolate	Note	Resident/Transient	Source Type	rovider Sample
832	28063	16	Raccoon	1	H-3
832	28064	214	canine	1	H-3
832	28065	21	Unknown	1	H-3 Transient
832	28066	198	Unknown	1	H-4 Transient
832	28067	36	Avian	1	H-4
832	28068	55	horse, L	1	H-4
832	28069	65	dog	1	H-5
832	28070	65	dog	1	H-5
832	28071	276	feline	1	H-5
832	51568	289	Deer-Elk	1	H-1
832	51569	62	opossum	1	H-1
832	51570	225	rodent	1	H-1
832	51571	289	Deer-Elk	1	H-2
832	51572	267	rodent	1	H-2
832	51573	315	dog	1	H-2
832	51574	289	Deer-Elk	1	H-3
832	51575	289	Deer-Elk	1	H-3
832	51576	91	Unknown	1	H-3 Transient
832	51577	289	Deer-Elk	1	H-4
832	51578	6	Bovine, L	1	H-4
832	51579	48	Bovine, L	1	H-4
832	51580	267	rodent	1	H-5
832	51581	289	Deer-Elk	1	H-5
832	51582	289	Deer-Elk	1	H-5
832	51632	289	Deer-Elk	1	H-1
832	51633	256	raccoon	1	H-1
832	51634	40	rodent	1	H-1
832	51635	63	dog	1	H-1
832	51636	63	dog	1	H-2
832	51637	267	rodent	1	H-2
832	51638	256	raccoon	1	H-2
832	51639	175	human	1	H-3
832	51640	63	dog	1	H-4
832	51641	299	human	1	H-4
832	51642	63	dog	1	H-4
832	51643	61	dog	1	H-4
832	51644	61	dog	1	H-5
832	51645	38	human	1	H-5
832	51646	315	dog	1	H-5
832	51877	295	Unknown	1	H1
832	51878	89	Dog, L-D	1	H1
832	51879	91	Unknown	1	H1 Transient
832	51880	43	Unknown	1	H1 Transient
832	51881	187	avian	1	H2
832	51882	41	canine	1	H2
832	51883	187	avian	1	H2
832	51884	187	avian	1	H2
832	51885	266	avian	1	H3
832	51886	270	canine	1	H3
832	51887	43	Unknown	1	H3 Transient

Henderson Inlet: Woodland Creek
Raw Data

Sample Site	Isolate	Note	Resident/Transient	Source Type	rovider Sample	
832	51888	116	Rodent, L	1	H4	
832	51889	262	avian	1	H4	
832	51890	277	human	1	H4	
832	51891	70	Unknown	1	H4	
832	51892	70	Unknown	1	H5	
832	51893	262	avian	1	H5	
832	51894	70	Unknown	1	H5	
832	51895	70	Unknown	1	H5	
832	51947	278	dog	1	H1	
832	51948	257	Avian, L-D	1	H1	
832	51949	258	Unknown	1	H1	
832	51950	63	dog	1	H1	
832	51951	210	human	1	H2	
832	51952	175	human	1	H2	
832	51953	210	human	1	H2	
832	51954	41	canine	1	H2	
832	51955	257	Avian, L-D	1	H3	
832	51956	41	canine	1	H3	
832	51957	63	dog	1	H3	
832	51958	28	Bovine	1	H3	
832	51959	41	canine	1	H4	
832	51960	186	opossum	1	H4	
832	51961	272	Unknown	1	H4	
832	51962	278	dog	1	H4	
832	51963	220	canine	1	H5	
832	51964	2	Canine	1	H5	
832	51965	2	Canine	1	H5	
832	51966	298	avian	1	H5	
832	52017	5	Horse	1	H1	
832	52018	251	Unknown	1	H1	
832	52019	5	Horse	1	H1	
832	52020	268	goose	1	H1	
832	52021	53	horse	1	H2	
832	52022	277	human	1	H2	
832	52023	63	dog	1	H2	
832	52024	313	canine	1	H2	Transient
832	52025	176	avian	1	H3	
832	52026	18	Raccoon	1	H3	
832	52027	18	Raccoon	1	H3	
832	52028	176	avian	1	H3	
832	52029	21	Unknown	1	H4	Transient
832	52030	21	Unknown	1	H4	Transient
832	52031	70	Unknown	1	H4	
832	52032	91	Unknown	1	H4	Transient
832	52033	269	goose	1	H5	
832	52034	269	goose	1	H5	
832	52035	270	Canine	1	H5	
832	52036	270	canine	1	H5	
832	52097	56	human	1	H1	
832	52098	28	Bovine	1	H1	

Henderson Inlet: Woodland Creek
Raw Data

Sample Site	Isolate	Note	Resident/Transient	Source Type	rovider Sample	
832	52099	304	bovine	1	H1	
832	52100	250	avian	1	H1	
832	52101	110	Human, L	1	H2	
832	52102	202	beaver	1	H2	
832	52103	249	Unknown	1	H2	Transient
832	52104	249	Unknown	1	H2	Transient
832	52105	270	canine	1	H3	
832	52106	270	canine	1	H3	
832	52107	270	canine	1	H3	
832	52108	270	canine	1	H3	
832	52110	5	Horse	1	H4	
832	52111	270	canine	1	H4	
832	52112	270	canine	1	H4	
832	52113	223	human	1	H4	
832	52114	5	Horse	1	H5	
832	52115	223	human	1	H5	
832	52116	259	Unknown	1	H5	
832	52117	298	avian	1	H5	

Henderson Inlet: Marine Water
Raw Data

Sample Site	Isolate	Note	Resident/Transient	Source Type	Provider Sample
833	27748	14	Avian	1	W3
833	27749	108	marine mammal	1	W4
833	27750	9	Sea Gull	1	W5
833	27751	9	Sea Gull	1	W5
833	27752	7	Canine	1	W5
833	27792	56	human	1	W1
833	27793	31	avian	1	W1
833	27794	202	beaver	1	W1
833	27795	76	Bovine	1	W2
833	27796	2	Canine	1	W2
833	27797	2	Canine	1	W2
833	27798	271	Unknown	1	W3
833	27799	185	beaver	1	W3
833	27800	176	avian	1	W3
833	27801	236	feline	1	W4
833	27802	274	raccoon	1	W4
833	27803	153	rodent	1	W4
833	27804	67	human	1	W5
833	27805	253	canine	1	W5
833	27806	59	deer	1	W5
833	27849	46	Avian	1	W-1
833	27850	135	Sea Lion	1	W-1
833	27851	135	Sea Lion	1	W-1
833	27852	140	Unknown	1	W-2
833	27853	140	Unknown	1	W-2
833	27854	140	Unknown	1	W-2
833	27855	140	Unknown	1	W-3
833	27856	37	Avian	1	W-3
833	27857	147	Unknown	1	W-4
833	27858	151	dog	1	W-4
833	27859	207	sea gull	1	W-4
833	27860	46	Avian	1	W-5
833	27861	135	Sea Lion	1	W-5
833	27862	86	seal	1	W-5
833	28072	104	Deer-Elk	1	W1A
833	28073	104	Deer-Elk	1	W1A
833	28074	311	sea gull	1	W1A
833	28075	267	rodent	1	W1B
833	28076	267	rodent	1	W1B
833	28077	104	Deer-Elk	1	W1B
833	28078	106	Unknown	1	W2
833	28079	100	avian	1	W2
833	28080	25	Deer-Elk	1	W2
833	28081	270	canine	1	W3
833	28082	89	Dog, L-D	1	W3
833	28083	254	avian	1	W3
833	28084	311	sae gull	1	W4
833	28085	106	Unknown	1	W4
833	28086	106	Unknown	1	W4
833	28087	25	deer/elk	1	W-5

Henderson Inlet: Marine Water
Raw Data

Sample Site	Isolate	Note	Resident/Transient	Source Type	Provider Sample	
833	28088	170	avian	1	W-5	
833	28089	25	Deer-Elk	1	W-5	
833	51600	303	Unknown	1	W-1	
833	51601	266	avian	1	W-1	
833	51602	237	Unknown	1	W-1	
833	51603	227	avian	1	W-2	
833	51604	227	avian	1	W-2	
833	51605	267	rodent	1	W-2	
833	51606	226	duck-goose	1	W-2	
833	51607	227	avian	1	W-3	
833	51608	242	Unknown	1	W-3	
833	51609	288	Unknown	1	W-3	
833	51610	93	Bovine	1	W-4	
833	51611	267	rodent	1	W-4	
833	51612	246	Unknown	1	W-4	
833	51613	17	Unknown	1	W-5	Transient
833	51614	237	Unknown	1	W-5	
833	51615	156	sea gull	1	W-5	
833	51660	33	muskrat	1	W-1	
833	51661	267	rodent	1	W-2	
833	51662	82	human	1	W-3	
833	51663	39	Avian	1	W-4	
833	51664	267	rodent	1	2-W-3	
833	51896	270	canine	1	W1	
833	51897	270	canine	1	W1	
833	51898	270	canine	1	W2	
833	51899	262	Avian	1	W2	
833	51900	14	avian	1	W3	
833	51901	195	Bovine	1	W3	
833	51902	195	Bovine	1	W3	
833	51903	118	seal	1	W5	
833	51904	118	seal	1	W5	
833	51905	203	Unknown	1	2W1	
833	51906	101	Bovine	1	2W1	
833	51907	255	sea gull	1	2W3	
833	51908	28	Bovine	1	2W4	
833	51909	208	avian	1	2W5	
833	51910	208	avian	1	2W5	
833	51967	146	Seal, L	1	W1	
833	51968	266	avian	1	W2	
833	51969	298	avian	1	W3	
833	51970	1	Unknown	1	W3	
833	51971	219	Unknown	1	W3	
833	51972	316	Unknown	1	W4	
833	51973	316	Unknown	1	W4	
833	51974	231	dog	1	W4	
833	51975	52	rabbit	1	W5	
833	51976	266	avian	1	2W3	
833	51977	110	Human, L	1	2W3	
833	51978	189	duck-goose	1	2W4	

Henderson Inlet: Marine Water
Raw Data

Sample Site	Isolate	Note	Resident/Transient	Source Type	Provider Sample	
833	51979	298	avian	1	2W5	
833	51980	298	avian	1	2W5	
833	52037	146	Seal, L	1	W1	
833	52038	19	Unknown	1	W1	Transient
833	52039	309	Unknown	1	W1	
833	52040	309	Unknown	1	W1	
833	52041	257	Avian, L-D	1	W2	
833	52042	71	feline	1	W2	
833	52043	241	Unknown	1	W2	
833	52044	60	raccoon	1	W3	
833	52045	20	Avian	1	W3	
833	52046	41	canine	1	W3	
833	52047	20	Avian	1	W3	
833	52048	54	Human, L-D	1	W4	
833	52049	54	Human, L-D	1	W4	
833	52050	175	human	1	W4	
833	52051	35	rodent	1	W5	
833	52052	218	Unknown	1	W5	
833	52053	117	canine	1	W5	
833	52054	257	Avian, L-D	1	W5	
833	52055	164	rodent	1	2W1	
833	52056	164	rodent	1	2W1	
833	52057	245	Unknown	1	2W1	
833	52058	203	Unknown	1	2W1	
833	52059	243	marine Mammal	1	2W2	
833	52060	164	rodent	1	2W2	
833	52061	318	Unknown	1	2W2	
833	52062	257	Avian, L-D	1	2W2	
833	52063	58	rodent	1	2W3	
833	52064	107	marine mammal	1	2W3	
833	52065	243	marine Mammal	1	2W4	
833	52066	20	Avian	1	2W4	
833	52067	60	raccoon	1	2W5	
833	52068	17	Unknown	1	2W5	Transient
833	52069	309	Unknown	1	2W5	
833	52070	309	Unknown	1	2W5	
833	52109	146	Seal, L	1	W5	
833	52118	227	avian	1	W1	
833	52119	223	human	1	W1	
833	52120	223	human	1	W1	
833	52121	227	avian	1	W1	
833	52122	231	dog	1	W2	
833	52123	231	dog	1	W2	
833	52124	177	Beaver	1	W2	
833	52125	223	human	1	W2	
833	52126	223	human	1	W3	
833	52127	97	raccoon	1	W3	
833	52128	223	human	1	W3	
833	52129	223	human	1	W3	
833	52130	223	human	1	W4	

Henderson Inlet: Marine Water
Raw Data

Sample Site	Isolate	Note	Resident/Transient	Source Type	Provider Sample	
833	52131	190	Unknown	1	W4	
833	52132	227	avian	1	W4	
833	52133	266	avian	1	W4	
833	52134	266	avian	1	W5	
833	52135	316	Unknown	1	W5	
833	52136	305	Unknown	1	W5	
833	52137	77	avian	1	W5	
833	52138	210	human	1	2W1	
833	52139	223	human	1	2W1	
833	52140	5	Horse	1	2W1	
833	52141	41	canine	1	2W1	
833	52142	227	avian	1	2W2	
833	52143	223	human	1	2W2	
833	52144	87	human	1	2W2	
833	52145	261	dog	1	2W2	
833	52146	32	goose	1	2W3	
833	52147	223	human	1	2W3	
833	52148	41	canine	1	2W3	
833	52149	267	rodent	1	2W3	
833	52150	224	bovine	1	2W4	
833	52151	224	bovine	1	2W4	
833	52152	74	bovine	1	2W4	
833	52153	227	avian	1	2W4	
833	52154	224	bovine	1	2W5	
833	52155	43	Unknown	1	2W5	Transient
833	52156	206	human	1	2W5	
833	52157	228	dog	1	2W5	
833	52658	175	human	1	1	
833	52659	282	sea lion	1	1	
833	52660	175	human	1	1	
833	52661	282	sea lion	1	1	
833	52662	282	sea lion	1	1	
833	52663	102	Seal	1	2	
833	52664	102	Seal	1	2	
833	52665	102	Seal	1	2	
833	52666	102	Seal	1	2	
833	52667	252	Unknown	1	2	
833	52668	84	avian	1	3	
833	52669	84	avian	1	3	
833	52670	84	avian	1	3	
833	52671	84	avian	1	3	
833	52672	84	avian	1	3	
833	52673	270	canine	1	4	
833	52674	322	marine mammal	1	4	
833	52675	322	marine mammal	1	4	
833	52676	47	Avian	1	5	
833	52677	47	Avian	1	5	
833	52678	47	Avian	1	5	
833	52679	47	Avian	1	5	
833	52680	47	Avian	1	6	

Henderson Inlet: Marine Water
Raw Data

Sample Site	Isolate	Note	Resident/Transient	Source Type	Provider Sample	
833	52681	297	rodent	1	6	
833	52682	297	rodent	1	6	
833	52683	20	Avian	1	6	
833	52684	297	rodent	1	6	
833	52685	90	Goose, L-D	1	7	
833	52686	73	goose	1	7	
833	52687	297	rodent	1	7	
833	52688	90	Goose, L-D	1	7	
833	52689	90	Goose, L-D	1	7	
833	52690	175	human	1	8	
833	52691	269	goose	1	8	
833	52692	269	goose	1	8	
833	52693	45	Seal	1	8	
833	52694	182	Unknown	1	8	
833	52695	282	sea lion	1	9	
833	52696	154	avian	1	9	
833	52697	154	avian	1	9	
833	52698	282	sea lion	1	9	
833	52699	87	human	1	9	
833	52700	248	Unknown	1	10	Transient
833	52701	248	Unknown	1	10	Transient
833	52702	248	Unknown	1	10	Transient
833	52703	248	Unknown	1	10	Transient
833	52704	114	rodent	1	10	
833	52705	282	sea lion	1	11	
833	52706	132	Sea Gull	1	11	
833	52707	282	sea lion	1	11	
833	52708	132	Sea Gull	1	11	
833	52709	132	Sea Gull	1	11	
833	52710	322	marine mammal	1	12	
833	52711	323	marine mammal	1	12	
833	52712	322	marine mammal	1	12	
833	52713	320	Unknown	1	12	
833	52714	324	Unknown	1	12	Transient
833	52715	67	human	1	13	
833	52716	67	human	1	13	
833	52717	324	Unknown	1	13	Transient
833	52718	325	Unknown	1	13	Transient
833	52719	324	Unknown	1	13	Transient
833	52720	105	Sea Gull	1	14	
833	52721	105	Sea Gull	1	14	
833	52722	42	Human, L	1	14	
833	52723	42	Human, L	1	14	
833	52724	29	dog	1	14	
833	52725	85	duck	1	15	
833	52726	85	duck	1	15	
833	52727	85	duck	1	15	
833	52728	85	duck	1	15	
833	52729	85	duck	1	15	
833	53187	51	horse	1	w1	

Henderson Inlet: Marine Water
Raw Data

Sample Site	Isolate	Note	Resident/Transient	Source Type	Provider Sample	
833	53188	26	marine mammal	1	w1	
833	53189	215	duck-goose	1	w1	
833	53190	58	rodent	1	w2	
833	53191	215	duck-goose	1	w2	
833	53192	215	duck-goose	1	w2	
833	53193	56	human	1	w3	
833	53194	301	Unknown	1	w3	
833	53195	301	Unknown	1	w3	
833	53196	215	duck-goose	1	w4	
833	53197	89	Dog, L-D	1	w4	
833	53198	158	Unknown	1	w4	
833	53199	215	duck-goose	1	w5	
833	53200	158	Unknown	1	w5	
833	53201	158	Unknown	1	w5	
833	53202	110	Human, L	1	2w1	
833	53203	151	dog	1	2w1	
833	53204	116	Rodent, L	1	2w1	
833	53205	10	Unknown	1	2w2	Transient
833	53206	289	Deer-Elk	1	2w2	
833	53207	90	Goose, L-D	1	2w2	
833	53208	120	Deer-Elk	1	2w3	
833	53209	120	Deer-Elk	1	2w3	
833	53210	20	Avian	1	2w3	
833	53211	235	seal	1	2w4	
833	53212	235	seal	1	2w4	
833	53213	28	Bovine	1	2w4	
833	53214	228	dog	1	2w5	
833	53215	122	Bovine	1	2w5	
833	53216	54	Human, L-D	1	2w5	
833	53217	284	Unknown	1	w1	
833	53218	175	human	1	w1	
833	53219	284	Unknown	1	w1	
833	53220	284	Unknown	1	w2	
833	53221	284	Unknown	1	w2	
833	53222	284	Unknown	1	w2	
833	53223	272	Unknown	1	w3	
833	53224	248	Unknown	1	w3	Transient
833	53225	284	Unknown	1	w3	
833	53226	282	sea lion	1	w4	
833	53227	84	avian	1	w4	
833	53228	282	sea lion	1	w4	
833	53229	282	sea lion	1	w5	
833	53230	282	sea lion	1	w5	
833	53231	282	sea lion	1	w5	
833	53232	84	avian	1	2w1	
833	53233	282	sea lion	1	2w1	
833	53234	41	canine	1	2w1	
833	53235	129	Seal	1	2w2	
833	53236	282	sea lion	1	2w2	
833	53237	291	Unknown	1	2w2	

Henderson Inlet: Marine Water
Raw Data

Sample Site	Isolate	Note	Resident/Transient	Source Type	Provider Sample	
833	53238	282	sea lion	1	2w3	
833	53239	283	avian	1	2w3	
833	53240	275	feline	1	2w3	
833	53241	41	canine	1	2w4	
833	53242	177	Beaver	1	2w4	
833	53243	281	Unknown	1	2w4	
833	53244	281	Unknown	1	2w5	
833	53245	270	canine	1	2w5	
833	53246	84	avian	1	2w5	
833	53690	19	Unknown	1	W1	Transient
833	53691	269	goose	1	W1	
833	53692	269	goose	1	W1	
833	53693	1	Unknown	1	W2	
833	53694	146	Seal, L	1	W2	
833	53695	1	Unknown	1	W3	
833	53696	14	Avian	1	W3	
833	53697	146	Seal, L	1	W4	
833	53698	146	Seal, L	1	W4	
833	53699	105	Sea Gull	1	W4	
833	53700	131	Unknown	1	W5	
833	53701	131	Unknown	1	W5	
833	53702	131	Unknown	1	W5	
833	53703	50	goose	1	W5	

Henderson Inlet: Sediment
Raw Data

Sample Site	Isolate	Note	Resident/Transient	Source Type	Provider Sample	
833	27737	130	Sea Gull	78	S1	
833	27738	130	Sea Gull	78	S1	
833	27739	263	Unknown	78	S3	
833	27740	267	rodent	78	S3	
833	27741	267	rodent	78	S3	
833	27742	85	duck	78	S4	
833	27743	263	Unknown	78	S4	
833	27744	267	rodent	78	S4	
833	27745	14	avian	78	S5	
833	27746	14	avian	78	S5	
833	27747	12	Sea Gull	78	S5	
833	27783	169	avian	78	S1	
833	27784	169	avian	78	S1	
833	27785	169	avian	78	S1	
833	27786	289	Deer-Elk	78	S2	
833	27787	289	Deer-Elk	78	S2	
833	27788	13	Unknown-Transient	78	S3	Transient
833	27789	13	Unknown-Transient	78	S3	Transient
833	27790	13	Unknown-Transient	78	S3	Transient
833	27791	267	rodent	78	S4	
833	27863	271	Unknown	78	S-1	
833	27864	270	canine	78	S-1	
833	27865	271	Unknown	78	S-1	
833	27866	193	rodent	78	S-2	
833	27867	46	avian	78	S-2	
833	27868	46	avian	78	S-2	
833	27869	46	avian	78	S-3	
833	27870	307	feline	78	S-3	
833	27871	2	Canine	78	S-4	
833	27872	46	avian	78	S-5	
833	27873	3	Bovine, L-D	78	S-5	
833	27874	46	Avian	78	S-5	
833	28090	240	Unknown	78	S-1	
833	28091	139	porcine	78	S-1	
833	28092	271	Unknown	78	S-2A	
833	28093	271	Unknown	78	S-2A	
833	28094	271	Unknown	78	S-2A	
833	28095	271	Unknown	78	S-2B	
833	28096	271	Unknown	78	S-3A	
833	28097	271	Unknown	78	S-3A	
833	28098	271	Unknown	78	S-3A	
833	28099	312	Unknown	78	S-3B	
833	28100	314	avian	78	S-3B	
833	28101	11	Deer	78	S-4	
833	28102	11	Deer	78	S-4	
833	28103	108	marine mammal	78	S-5	
833	28104	54	Human, L-D	78	S-5	
833	51583	113	avian	1	S-1	
833	51584	135	Sea Lion	1	S-2	
833	51585	287	Unknown	1	S-2	

Henderson Inlet: Sediment
Raw Data

Sample Site	Isolate	Note	Resident/Transient	Source Type	Provider Sample
833	51586	266	avian	1	S-2
833	51587	266	avian	1	S-2
833	51588	266	avian	1	S-2
833	51589	266	avian	1	S-2
833	51590	237	Unknown	1	S-3
833	51591	237	Unknown	1	S-3
833	51592	237	Unknown	1	S-3
833	51593	237	Unknown	1	S-3
833	51594	46	Avian	1	S-4
833	51595	46	Avian	1	S-4
833	51596	46	Avian	1	S-4
833	51597	266	avian	1	S-5
833	51598	266	avian	1	S-5
833	51599	266	avian	1	S-5
833	51647	200	feline	1	S-1
833	51648	46	Avian	1	S-1
833	51649	46	Avian	1	S-1
833	51650	267	rodent	1	S-2
833	51651	40	rodent	1	S-2
833	51652	40	rodent	1	S-2
833	51653	46	Avian	1	S-3
833	51654	266	avian	1	S-3
833	51655	266	avian	1	S-3
833	51656	237	Unknown	1	S-3
833	51657	237	Unknown	1	S-4
833	51658	237	Unknown	1	S-5
833	51659	46	Avian	1	S-5
833	51911	280	Unknown	1	S-1
833	51912	113	avian	1	S-1
833	51913	280	Unknown	1	S-1
833	51914	280	Unknown	1	S-1
833	51915	257	Avian, L-D	1	S3
833	51916	30	Human	1	S3
833	51917	257	Avian, L-D	1	S3
833	51918	257	Avian, L-D	1	S3
833	51919	72	human	1	S4
833	51920	257	Avian, L-D	1	S4
833	51921	257	Avian, L-D	1	S4
833	51922	257	Avian, L-D	1	S4
833	51923	257	Avian, L-D	1	S-5
833	51924	257	Avian, L-D	1	S-5
833	51925	257	Avian, L-D	1	S-5
833	51926	54	Human, L-D	1	S-5
833	51981	208	avian	1	S1
833	51982	212	Unknown	1	S1
833	51983	267	rodent	1	S2
833	51984	267	rodent	1	S2
833	51985	267	rodent	1	S2
833	51986	267	rodent	1	S2
833	51987	125	goose	1	S3

Henderson Inlet: Sediment
Raw Data

Sample Site	Isolate	Note	Resident/Transient	Source Type	Provider Sample
833	51988	125	goose	1	S3
833	51989	125	goose	1	S3
833	51990	125	goose	1	S3
833	51991	260	avian	1	S4
833	51992	260	avian	1	S4
833	51993	260	avian	1	S4
833	51994	260	avian	1	S4
833	51995	267	rodent	1	S5
833	51996	267	rodent	1	S5
833	51997	267	rodent	1	S5
833	51998	267	rodent	1	S5
833	52071	257	Avian, L-D	1	S2
833	52072	257	Avian, L-D	1	S2
833	52073	257	Avian, L-D	1	S2
833	52074	257	Avian, L-D	1	S2
833	52075	181	seal	1	S4
833	52076	181	seal	1	S4
833	52077	181	seal	1	S4
833	52078	181	seal	1	S4
833	52079	174	human	1	S5
833	52080	174	human	1	S5
833	52081	174	human	1	S5
833	52082	174	human	1	S5
833	52584	182	Unknown	78	sed1
833	52585	182	Unknown	78	sed1
833	52586	182	Unknown	78	sed1
833	52587	182	Unknown	78	sed1
833	52588	182	Unknown	78	sed1
833	52589	282	Sea Lion	78	sed2
833	52590	44	Sea Lion	78	sed2
833	52591	44	Sea Lion	78	sed2
833	52592	44	Sea Lion	78	sed2
833	52593	321	Unknown	78	sed2
833	52594	44	Sea Lion	78	sed3
833	52595	44	Sea Lion	78	sed3
833	52596	44	Sea Lion	78	sed3
833	52597	43	Unknown	78	sed3 Transient
833	52598	43	Unknown	78	sed3 Transient
833	52599	54	Human, L-D	78	sed4
833	52600	54	Human, L-D	78	sed4
833	52601	54	Human, L-D	78	sed4
833	52602	54	Human, L-D	78	sed4
833	52603	54	Human, L-D	78	sed4
833	52604	43	Unknown	78	sed5 Transient
833	52605	43	Unknown	78	sed5 Transient
833	52606	43	Unknown	78	sed5 Transient
833	52607	43	Unknown	78	sed5 Transient
833	52608	43	Unknown	78	sed5 Transient
833	52609	269	goose	78	sed6
833	52610	112	Sea Gull	78	sed6

Henderson Inlet: Sediment
Raw Data

Sample Site	Isolate	Note	Resident/Transient	Source Type	Provider Sample	
833	52611	112	Sea Gull	78	sed6	
833	52612	112	Sea Gull	78	sed6	
833	52613	112	Sea Gull	78	sed6	
833	52614	26	marine mammal	78	sed7	
833	52615	26	marine mammal	78	sed7	
833	52616	26	marine mammal	78	sed7	
833	52617	26	marine mammal	78	sed7	
833	52618	26	marine mammal	78	sed7	
833	52619	111	avian	78	sed8	
833	52620	111	avian	78	sed8	
833	52621	111	avian	78	sed8	
833	52622	43	Unknown	78	sed8	Transient
833	52623	43	Unknown	78	sed8	Transient
833	52624	279	Unknown	78	sed9	
833	52625	126	Sea Lion	78	sed9	
833	52626	126	Sea Lion	78	sed9	
833	52627	126	Sea Lion	78	sed9	
833	52628	126	Sea Lion	78	sed9	
833	52629	43	Unknown	78	sed10	Transient
833	52630	43	Unknown	78	sed10	Transient
833	52631	269	goose	78	sed10	
833	52632	43	Unknown	78	sed10	Transient
833	52633	43	Unknown	78	sed10	Transient
833	52634	269	goose	78	sed11	
833	52635	269	goose	78	sed11	
833	52636	269	goose	78	sed11	
833	52637	269	goose	78	sed11	
833	52638	269	goose	78	sed11	
833	52639	269	goose	78	sed12	
833	52640	269	goose	78	sed12	
833	52641	269	goose	78	sed12	
833	52642	269	goose	78	sed12	
833	52643	269	goose	78	sed12	
833	52644	64	Unknown	78	sed13	
833	52645	269	goose	78	sed13	
833	52646	269	goose	78	sed13	
833	52647	269	goose	78	sed13	
833	52648	75	dog	78	sed14	
833	52649	75	dog	78	sed14	
833	52650	75	dog	78	sed14	
833	52651	75	dog	78	sed14	
833	52652	75	dog	78	sed14	
833	52653	175	human	78	sed15	
833	52654	175	human	78	sed15	
833	52655	175	human	78	sed15	
833	52656	175	human	78	sed15	
833	52657	175	human	78	sed15	
833	53174	289	Deer-Elk	1	s1	
833	53175	289	Deer-Elk	1	s1	
833	53176	289	Deer-Elk	1	s1	

Henderson Inlet: Sediment
Raw Data

Sample Site	Isolate	Note	Resident/Transient	Source Type	Provider Sample
833	53177	175	human	1	s2
833	53178	175	human	1	s2
833	53179	175	human	1	s2
833	53180	175	human	1	s3
833	53181	257	Avian, L-D	1	s4
833	53182	175	human	1	s4
833	53183	123	Sea Lion	1	s4
833	53184	257	Avian, L-D	1	s5
833	53185	257	Avian, L-D	1	s5
833	53186	257	Avian, L-D	1	s5

Henderson Inlet: Oyster tissue
Raw Data

Sample Site	Isolate	Note	esident/Transient	Source Type	Provider Sample
1200	38400	247	Unknown	97	Oyster 1
1200	38401	244	human	97	Oyster 3
1200	38402 by UW lab			97	Oyster 5
1200	38403	292	Unknown	97	Oyster 6
1200	38404	155	sea gull	97	Oyster 9
1200	38405	168	Unknown	97	Oyster 10
1313	39054	23	Seal	97	1
1313	39055	22	Horse, L	97	1
1313	39056	22	Horse, L	97	1
1313	39057	22	Horse, L	97	3
1313	39058	22	Horse, L	97	3
1313	39059	22	Horse, L	97	3
1313	39060	22	Horse, L	97	4
1313	39061	22	Horse, L	97	4
1313	39062	22	Horse, L	97	4
1313	39063	22	Horse, L	97	5
1313	39064	22	Horse, L	97	5
1313	39065	22	Horse, L	97	5
1313	39066	22	Horse, L	97	6
1313	39067	22	Horse, L	97	6
1313	39068	22	Horse, L	97	6
1313	39069	23	Seal	97	7
1313	39070	23	Seal	97	7
1313	39071	29	Dog	97	8
1313	39072	157	Unknown	97	8
1313	39073	41	canine	97	8
1313	39074	29	Dog	97	9
1313	39075	29	Dog	97	9
1313	39076	29	Dog	97	10
1313	39077	29	Dog	97	10
1313	39078	171	avian	97	11
1313	39079	29	Dog	97	11
1313	39080	171	avian	97	11
1313	39081	29	Dog	97	12
1313	39082	110	Human, L	97	12
1313	39083	29	Dog	97	12
1313	39084	29	Dog	97	13
1313	39085	29	Dog	97	14
1313	39086	29	Dog	97	14
1313	39087	29	Dog	97	14
1313	39088	138	Avian	97	15
1313	39089	138	Avian	97	15
1313	39090	126	Sea Lion	97	15
1313	39091	126	Sea Lion	97	16
1313	39092	49	Otter	97	16
1313	39093	24	Avian	97	16
1313	39094	24	Avian	97	17
1313	39095	24	Avian	97	17
1313	39096	24	Avian	97	17
1313	39097	24	Avian	97	18

Henderson Inlet: Oyster tissue
Raw Data

Sample Site	Isolate	Note	esident/Transient	Source Type	Provider Sample	
1313	39098	24	Avian	97	18	
1313	39099	122	Bovine	97	19	
1313	39101	319	Unknown	97	20	
1313	39102	24	Avian	97	20	
1313	39103	24	Avian	97	20	
222	51404	183	dog	97	oyster 1	
222	51405	183	dog	97	oyster 1	
222	51406	175	human	97	oyster 2	
222	51407	175	human	97	oyster 2	
222	51408	286	Unknown	97	oyster 3	
222	51409	286	Unknown	97	oyster 3	
222	51410	148	avian	97	oyster 9	
222	51411	148	avian	97	oyster 9	
222	51412	173	Unknown	97	oyster 10	Transient
222	51413	173	Unknown	97	oyster 10	Transient
307	51414	124	Marine Mammal	97	oyster 1	
307	51415	124	Marine Mammal	97	oyster 1	
307	51416	127	Human	97	oyster 2	
307	51417	144	seal	97	oyster 9	
307	51418	144	seal	97	oyster 9	
833	52564	228	dog	97	2	
833	52565	133	Sea Gull	97	2	
833	52566	133	Sea Gull	97	2	
833	52567	44	Sea Lion	97	2	
833	52568	133	Sea Gull	97	2	
833	52569	95	human	97	3	
833	52570	66	dog	97	3	
833	52571	258	Unknown	97	3	
833	52572	184	avian	97	3	
833	52573	184	avian	97	3	
833	52574	95	human	97	4	
833	52575	8	Bovine	97	4	
833	52576	184	avian	97	4	
833	52577	95	human	97	4	
833	52578	184	avian	97	4	
833	52579	184	avian	97	5	
833	52580	208	avian	97	5	
833	52581	208	avian	97	5	
833	52582	208	avian	97	5	
833	52583	192	rodent	97	5	

Appendix C

Pathogen Scan Protocol

PCR Pathogen Scan Protocol

A. Sample: Arrival
   1. Samples were shipped on wet ice in Styrofoam containers. At the time of arrival the shipment was checked for integrity and temperature.
   2. Sample logs were checked against the samples to ensure that there were no missing or improperly labeled samples.
   3. The chain of custody form was signed.
   4. The samples were logged into the laboratory's sample logbook, given laboratory ID numbers, and labeled with their laboratory IDs.
   5. Any damaged, leaking, or unacceptable samples were noted.

B. Sample: Short Term Storage
   Samples were stored at four degrees Celsius until they were processed. The samples were processed no longer than four hours after arrival.

C. Sample: Processing
   The samples were processed for bacterial enrichment in liquid media using aseptic technique.
   1. The work surface was wiped with 70% ethanol before and after each sample was processed.
   2. Any scalpels, scissors, or other tools that were used were soaked in ethanol and flamed prior to being used on a sample.
   3. The container that holds the sample was aseptically opened so that the technician does not contaminate the contents.
   4. A portion of the sample was removed using a sterile tool and placed into a labeled enrichment tube. If the sample was a shellfish, it was taken out of the container onto a surface that has been wiped with 70 percent ethanol and cracked open using knives that have been dipped in ethanol and flamed. The shellfish was then cut into portions using a sterile scissors so that it may be inoculated into several broths.
   5. The instruments used to handle the sample were dipped into ethanol and flamed.
   6. The sample container was closed and stored at 4 degrees Celsius until the results of the PCR reactions were obtained. Shellfish were disposed of immediately after processing.

D. Sample: Enrichment
   The bacteria that may be present in a sample were enriched using three liquid enrichment broths. Modified Tryptic Soya broth was used to enrich for gram-negative bacteria. Brain Heat Infusion broth was used to enrich for gram positive and fastidious microorganisms, particularly *Helicobacter pylori* and *Listeria monocytogenes*.

   1. The enrichment media was prepared in 400 milliliter to 800 milliliter volumes according to the manufacturer's directions or published recipes and was steam autoclaved using standard conditions.

2. The autoclaved media was stored at room temperature for several days. Bottles that have any signs of contamination were disposed of.
3. 22.5 milliliters of autoclaved media was aseptically transferred to 50-milliliter screw cap Sarstedt tubes. The caps were screwed on tightly.
4. The Sarstedt tubes were labeled with the laboratory ID of the samples and the date. One uninnoculated blank tube was labeled and prepared in the same way for every ten sample tubes.
5. The sample tubes were inoculated with the 2.5 milliliters or 2.5 grams of the appropriate sample. The caps were left slightly loose.
6. The sample tubes and blank tubes were incubated at 37 degrees and 150 rotations per minute in a shaking incubator overnight. The samples were disposed of if there was any growth in the blank tubes.
7. After incubation was complete, the tubes were placed in a fume hood until further processed. The processing occurs no more than four hours later.

E. Processing of Enrichment Products

The bacterial cells that have grown in the overnight enrichment were lysed for use in DNA amplification reactions. In addition, any cells that may have grown in the enrichment broth were frozen at – 80 degrees Celsius so that they may be recovered at some point in the future

Cell Lysis:

1. 100 microliters of cell suspension was pipetted from each sample enrichment tube and dispensed into a two milliliter tube labeled with the laboratory ID of the sample that contains 800 microliters of lysis buffer. For every fifth sample, a duplicate lysis tube was made.
2. The two milliliter tubes containing sample enrichments and lysis buffer were vortexed, then incubated at 55 degrees Celsius for one hour, followed by 95 degrees Celsius for 10 minutes, and then they were placed on ice or in a – 20 degree Celsius freezer.

Culture Freezing:

1. Following the preparation of the cell lysates, 7.5 milliliters of enrichment was pipetted from the sample enrichment tube and mixed with a labeled 12 milliliter tube containing 2.5 milliliters of freezing media, which consists of 40 % TSB and 60% glycerol.
2. The 12 milliliters tubes containing sample enrichments and freezing media were tightly capped, vortexed, placed in a labeled bag, and then put into a –80 degree Celsius freezer.

F. Amplification of DNA from Lysed Bacterial Cells

Amplification of DNA using primers specific for different strains of bacteria was performed using the Polymerase Chain Reaction (PCR) technique.

1. The theromoclycler was turned on at least 45 minutes prior to the beginning of a PCR reaction program so that it has time to warm up.
2. A cocktail was prepared with primers for the strain of bacteria whose DNA was amplified. The cocktail consists of all the reagents necessary except for the sample DNA and positive control DNA. The cocktail contains enough reagents

for reactions for all of the samples plus: one blank reaction for every seven samples, one positive control reaction for every fourteen samples, and one extra reaction for every ten reactions to be run. The blank reactions ensure that the reaction cocktail, the pipettor, and the pippetor tips were not contaminated with amplifiable DNA. The positive control reactions ensure that the reaction cocktail produces the desired product and allows for comparison between sample reaction products and the reaction product of a true positive.

3. Autoclaved 0.2 ml PCR tube strips were placed into a tube holder. The openings of the tubes were not touched while they were handled.
4. 40 microliters of the reaction cocktail was added to each tube using a pipettor that was only used during the preparation and dispension of the reaction cocktail
5. 10 microliters of sample lysate, ten microliters of autoclaved distilled water, or 1 microliter of control DNA and nine microliters of autoclaved distilled water were added to the appropriate sample, blank, or positive control tube using a pippetor that was only used to load samples, positive control DNA, and water.
6. Autoclaved caps were placed over the openings of the PCR tubes. The inner surfaces were not touched while the caps were handled.
7. The PCR tube holder was placed into the thermocycler and the appropriate program was started after reviewing the program log adjacent to the thermocycler. The technician monitors the thermocycler to ensure that the correct program has started and was running properly.
8. The location of the PCR tube where each sample, blank, and positive control has been loaded was recorded on a log sheet.
9. The PCR reaction products were loaded onto an agarose gel no longer than 24 hours after the reaction was started.

G. Agarose Gel Electrophoresis of DNA Amplification Products

The reaction products from the PCR reactions were separated using agarose gel electrophoresis to determine if bands of the correct sizes have been generated. The bands that samples generate were compared to the positive control and to a DNA size standard.

1. Mix the reagents for a 2 % agarose gel in 0.5 X TBE in a loosely covered Erlenmeyer flask and dissolve/boil the agarose using a microwave or flame. The mixture was shaken several times while being heated. It was removed from the microwave or flame if necessary to prevent overboiling. The mixture was ready to be cooled to approximately 50 degrees Celsius when large bubbles begin to form.
2. While the mixture was cooling, the ends of a gel mold were taped to prevent the mixture from leaking out after it has been poured. The mold was then leveled to ensure that the entire gel was the same thickness. After it has been leveled, combs were set into the gel at the appropriate height and distance from one another.
3. After the gel mixture has cooled to the appropriate temperature, it was poured into the gel mold. Any large bubbles that have formed were removed.
4. As the gel was cooling, 7.5 microliters of loading dye were added to each reaction tube using a pippetor that was only used to add loading dye. The loading dye that was loaded was transferred from the stock bottle to a microcentrifuge tube so that

the stock bottle would not be contaminated with amplification products. A new pippettor tip was used for each sample.

5. After the gel has solidified the combs and the tape on the ends were removed and it was placed into a gel box containing fresh 0.5 X TBE. The surface of the gel was completely covered by the 0.5 X TBE.
6. A gel sheet was prepared according to the following protocol: The size standard was loaded in the left-most lane of the gel. Then, seven sample reactions and one blank reaction were loaded, followed by another seven sample reactions and one blank reaction. The next lane was left empty. The following lane was loaded with the positive control.
7. The gel was loaded according to the gel sheet that has been prepared.
8. The gel was run at 210 volts for 40 minutes.
9. A container containing 200 microliter of 10 mg/mi of ethidium bromide in 200 ml of distilled water was prepared. After the gel was done running it was removed from the gel box and gel mold and placed into the casserole.
10. The gel was stained for thirty minutes, then destained for thirty minutes.
11. Using a UV transilluminator, the bands from the size standard were observed to ensure that the gel has run properly. Next, the bands from the positive control were observed to ensure that the PCR reaction has yielded the desired amplification products. Then, the lanes for the blank reactions were observed to ensure that no undesired reaction products have formed. If all the above conditions have been met, a picture of the gel was taken using a gel documentation system.
12. The results of the sample reactions were interpreted.

H. QA/QC Protocols enforced throughout the process.

1. Gloves were worn whenever handling samples, sample tubes, reaction tubes, and agarose gels.
2. The temperatures of the incubators were checked and written down in a log book before beginning incubation and after incubation was complete. If the temperature was greater than +/- 5 degrees of the desired value, the incubation was repeated with new aliquots of the sample.
3. The pipettors were cleaned on a weekly basis and calibrated on a monthly basis. Pipettors that dispense greater than +/- 5 % of the desired volume were labeled "DO NOT USE" and not used until they have been repaired.
4. All dirty materials, samples, and sample container were decontaminated and disposed of properly.

I. Supplies and Reagents:

Taq Polymerase: from Promega. 5 units per microliter. 1.5 units per reaction.
DNTPs: from Promega, stock solution was 100 millimolar. Working solution was 10 millimolar.
MgCl2: from Promega, 25 millimolar.
10 X magnesium free reaction buffer: from Promega.

Autoclaved deionized water.

10 millimolar Tris: from ICN, prepared from powder and autoclaved deionized water to a concentration of 10 millimolar.
0.5 X TBE
Agarose: from GIBCO
Achromopeptidase: from Sigma, prepared from powder and autoclaved deionized water to a concentration of 5 units per microliter.
Lysis Buffer: 62.5 units of Achromopeptidase per mililiter of 10 millimolar Tris

Autoclaved 1.5 ml tubes.
Autoclaved 2.0 ml tubes.
Autoclaved PCR tube strips
Autoclaved PCR cap strips
Autoclaved pippetor tips

Two 20 micro liter pipettes, one for preparing the PCR reaction, one for post reaction products
Three 200 micro liter pipette, one for preparing the cell lysates, one for preparing the PCR reaction, one for post reaction products
One 1000 micro liter pipette, one for preparing the cell lysates

Gel Mold and Combs
Ethidium Bromide
Gel Box
Incubator
Tube Racks
PCR strip holders
Thermocycler

Recipes for Liquid Enrichment Broths:

Modified Tryptic Soya Broth (mTSB):
30 g Tryptic Soya Broth
1.5 g Bile Salts No. 3
1.5 g Sodium Phosphate, Dibasic
1 milliliter of Novobiocin, at a concentration of 100 mg per ml, after the media has been autoclaved
1 liter of distilled water

Brain Heart Infusion - Difco 0037 (BHI):
Prepare the media per manufacturer's directions.

PCR Reaction Cocktail:
Each PCR reaction takes place in a total volume of 50 microliters consisting of 5 microliters of 10 X Magnesium Free Reaction Buffer (Promega Madison, WI), 4 microliters of 25 millimolar MgCl2 (Promega), one microliter of 10 micromolar working solutions of each of the four DNTPs, and 1.5 units of Promega Taq Polymerase, a volume of primer which yields the desired

reaction product, 10 microliters of sample or 1 microliter of positive control DNA, and a volume of sterile water to bring the reaction volume to 50 microliters.

PCR Primers and Thermal Conditions:

Primer sequences and thermal conditions for gene targets from pathogenic bacteria.

Organism (Reference)	Amplification Target (size, base pairs)	Forward Primer Reverse Primer	Thermal Conditions (Number of cycles)
Esherichia coli O157	*eae* gene (384)	5'-GACCCGGCACAAGCATAAGC-3' 5'-CCACCTGCAGCAACAAGAGG-3'	95 C/ 1m – 65 C/ 2m – 72 C/ 90s (10) 95 C/ 1m – 65>60 C/ 2m – 72 C/ 90s (5) 95 C/ 1m – 60 C/ 2m – 72 C/ 90s (10) 95 C/ 1m – 60 C/ 2m – 72 C/1.5>2m (10)
	hlyA gene (534)	5'-GCATCATCAAGCGTACGTTCC-3' 5'-AATGAGCCAAGCTGGTTAAGCT-3'	
	stx1 gene (180)	5'-ATAAATCGCCATTCGTTGACTAC-3' 5'-AGAACGCCCACTGAGATCATC-3'	
	stx2 gene (255)	5'-GGCACTGTCTGAAACTGCTCC-3' 5'-TCGCCAGTTATCTGACATTCTG-3'	
Listeria spp. and *Listeria monocytogenes*	Eubacteria 16s rRNA (408)	5'- CAG CMG CCG CGG TAA TWC-3' 5'-CCG TCA ATT CMT TTR AGT TT-3'	94 C/ 80 s – 50 C/ 90 s – 72 C/ 2 m (24) 72 C/ 10 m
	Listeria spp. 16s rRNA (938)	5'- CAG CMG CCG CGG TAA TWC-3' 5'- CTC CAT AAA GGT GAC CCT-3'	
	Listeria monocytogenes listeriolysin O (702)	5'- CCT AAG ACG CCA ATC GAA-3' 5'- AAG CGC TTG CAA CTG CTC-3'	
Salmonella spp.	Chromosome (429)	5'- AGC CAA CCA TTG CTA AAT TGG CGC A -3' 5'- TTT GCG ACT ATC AGG TTA CCG TGG -3'	94 C/ 1 m – 53 C/ 2 m – 72 C/ 3 m (35)
Helicobacter pylori	Urease gene A (411)	5'-GCCAATGGTAAATTAGTT-3' 5'-CTCCTTAATTGTTTTTAC-3'	95 C/ 5 m 94 C/ 1m – 45 C/ 1m – 72 C/ 1m (35) 72 C/ 4 m
Salmonella spp.	Chromosome (429)	5'- AGC CAA CCA TTG CTA AAT TGG CGC A -3' 5'- TTT GCG ACT ATC AGG TTA CCG TGG -3'	94 C/ 1 m – 53 C/ 2 m – 72 C/ 3 m (35)
Staphylococcus aureus	Nuclease gene (276)	5'- GCGATTGATGGTGATACGGTT-3' 5'-CAAGCCTTGACGAACTAAAGC-3'	94 C/3 s - 58 C/10 s - 72 C/35 s; 72 C/2 min
Vibrio parahemolyticus	*toxR* gene (~400)	5'-GTCTTCTGACGCAATCGTTG-3' 5'-ATACGAGTGGTTGCTGTCATG-3'	94 C/ 1 m – 63 C/ 90s – 72 C/ 90s (20)
Vibrio vulnificus	Cytolysin gene (383)	5'- CTCACTGGGGCAGTGGCT-3' 5'-CCAGCCGTTAACCGAACCA-3'	94 C/3 s - 58 C/10 s - 72 C/35 s; 72 C/2 min (35)

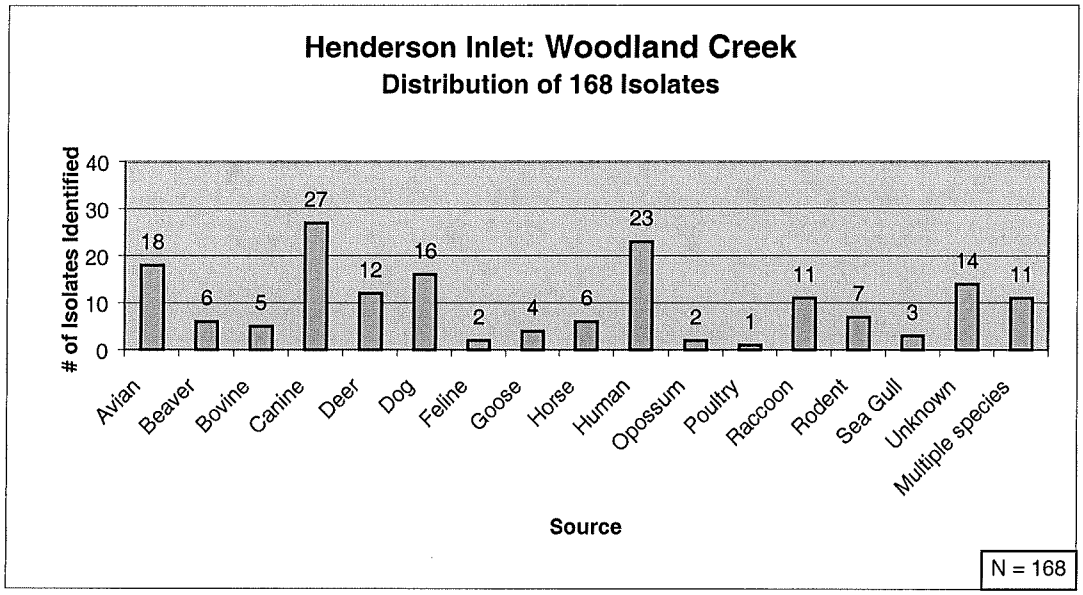
Appendix D

Total Number of Isolates

For each site
Woodland Creek
Swayne creeks
Marine water
Sediment
Oysters
Total project

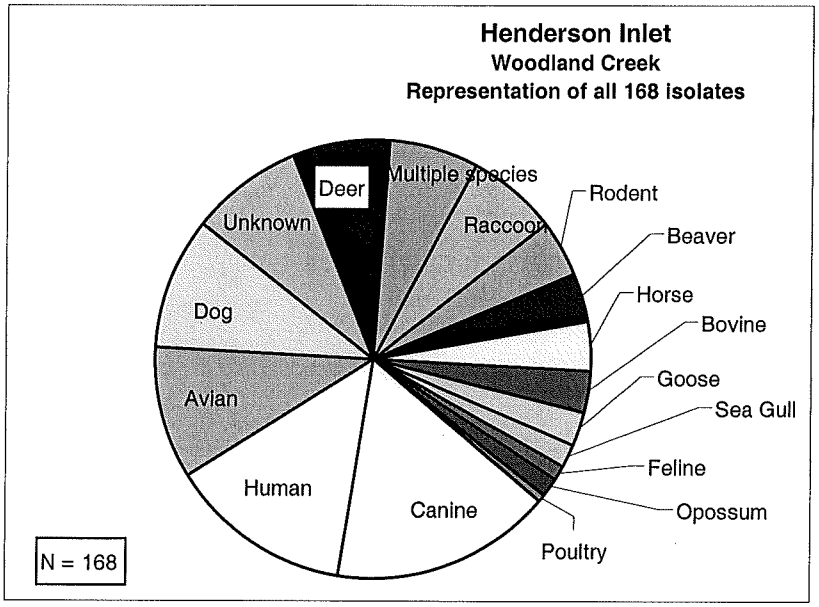
Total number of isolates: As defined in the study design, during each sampling event 5 samples were grabbed from each sample site. Oysters were sampled separately. Each sampling event had 20 samples – 5 each of marine water, Woodland Creek water, sediment, and 5 water samples from Swayne creeks. (Creeks B and C on the map – Figure 2) Each grab sample was to render at least 2 isolates. Most always had 2 isolates though up to 6 isolates from a single grab sample were typed. The following is a reporting of the total number of isolates typed.

Figure D1



Figures D1 and D2 show the total number of isolates (168) from Woodland Creek water samples. Figure D1 shows the distribution of the isolates for the 17 different sources. Figure D2 presents the same data in a pie chart format that illustrates the ranking of the various sources.

Figure D2



The pie chart shows that the predominant sources are canine, human, avian, and dogs – those sources that are characteristic of an urbanized area. The sources are in clockwise order, beginning with avian, from most clonal types to least.

Of all 5 sampling sites / media, Woodland Creek had the least number of unmatched isolates.

Figure D3

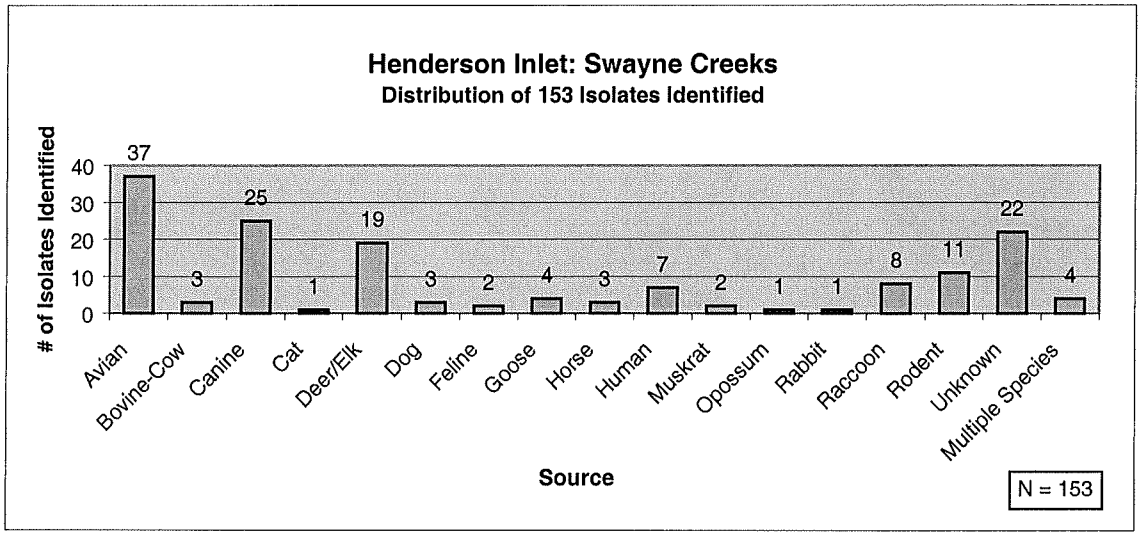
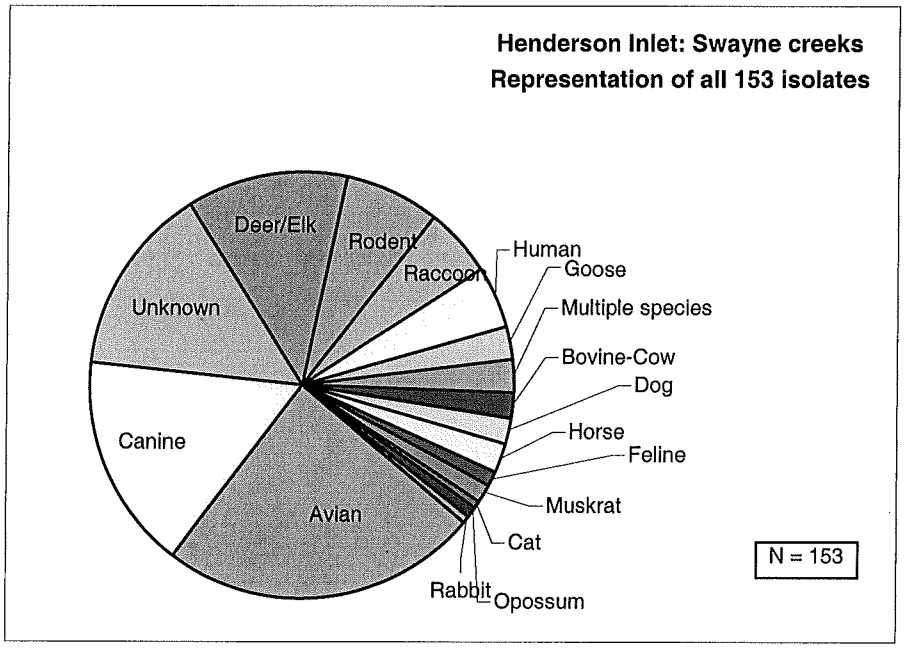


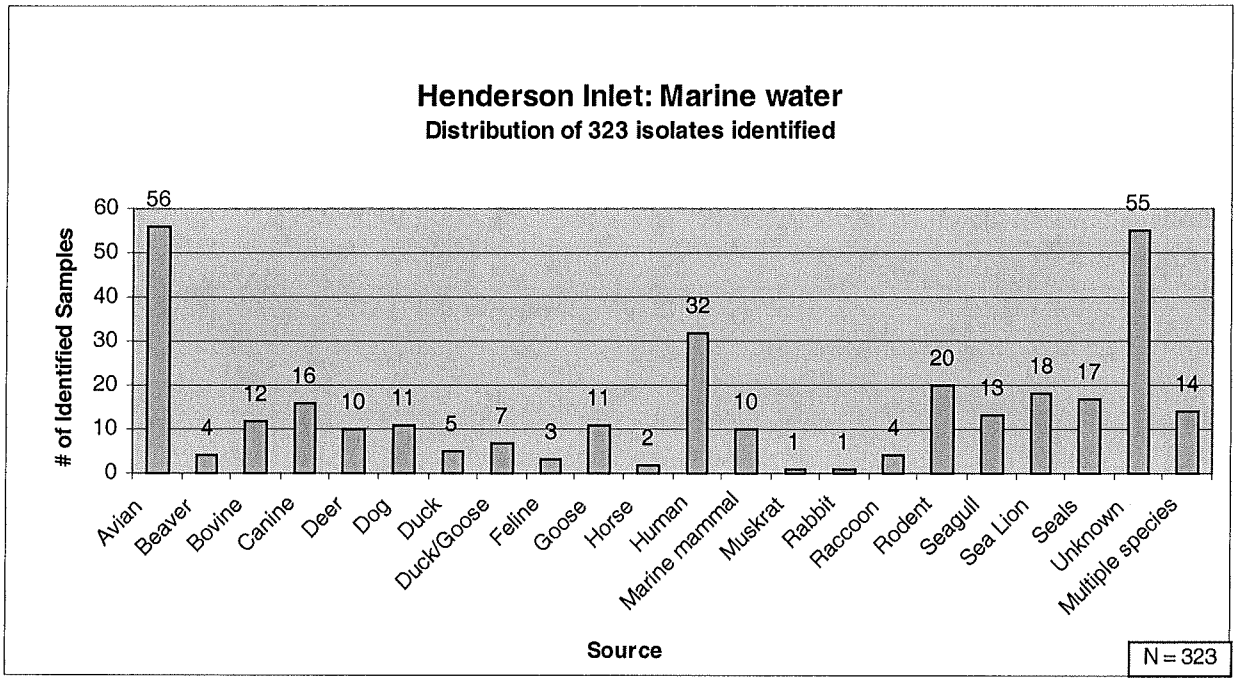
Figure D3 shows the distribution of the total number of isolates found in all Swayne creek water samples. There were a total of 153 isolates. [n = 153] Figure D4 presents this data in a pie chart.

Figure D4



The predominant number of *E. coli* isolates were from avian, canine, deer, and rodents. These sources would appear to be consistent with the rural characteristics of this sub-basin.

Figure D5



Marine water: A total of 323 isolates were identified from the 105 samples collected during the 14 sampling events. Figure D5 shows the distribution of the 22 sources. Birds and humans along with unknowns are the predominant sources.

Figure D6 represents how predominant birds and humans are.

Figure D6

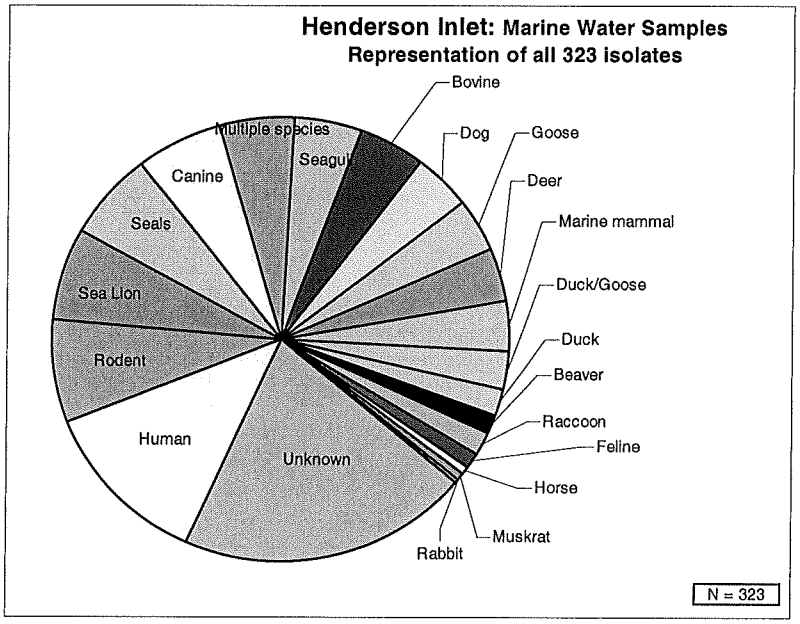


Figure D7

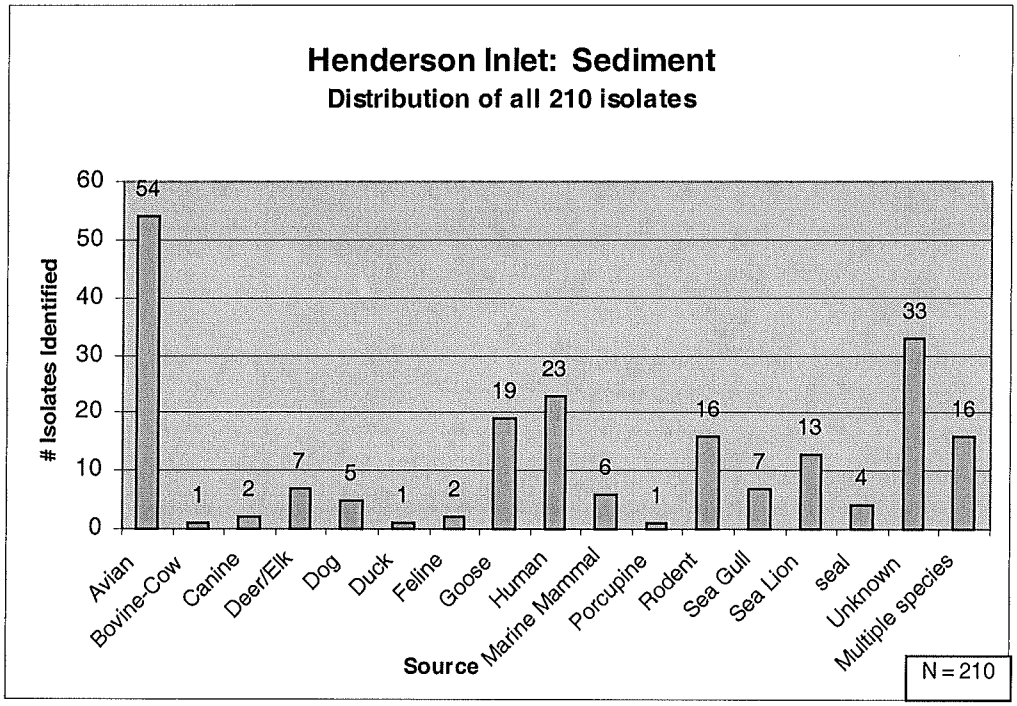


Figure D7 shows the distribution of the 210 sediment isolates. There are 16 different source types.

The predominant source types reflect the same pattern and sequence as the predominant source type of the marine water above it – avians, unknowns, and humans.

Figure D8

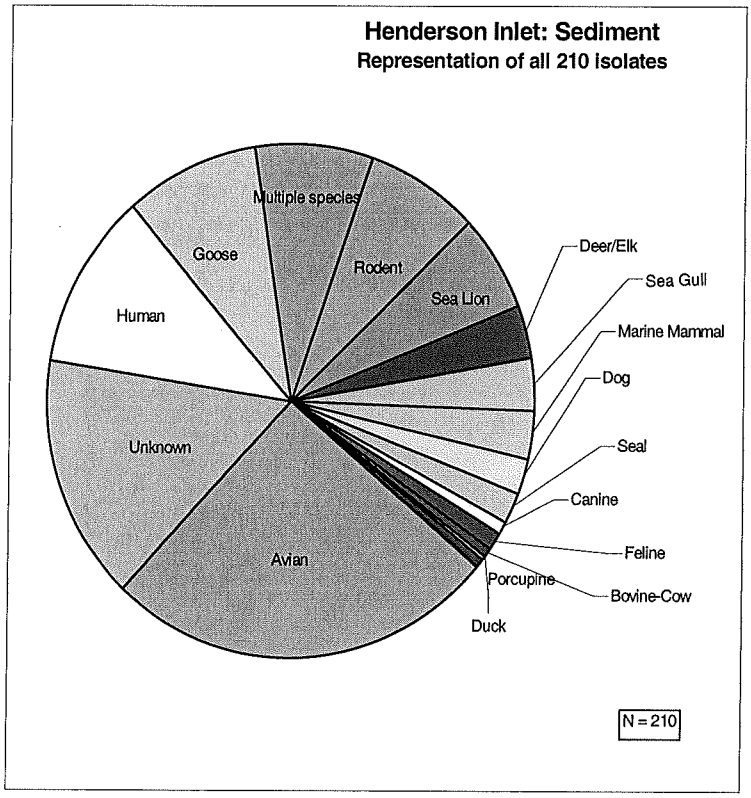
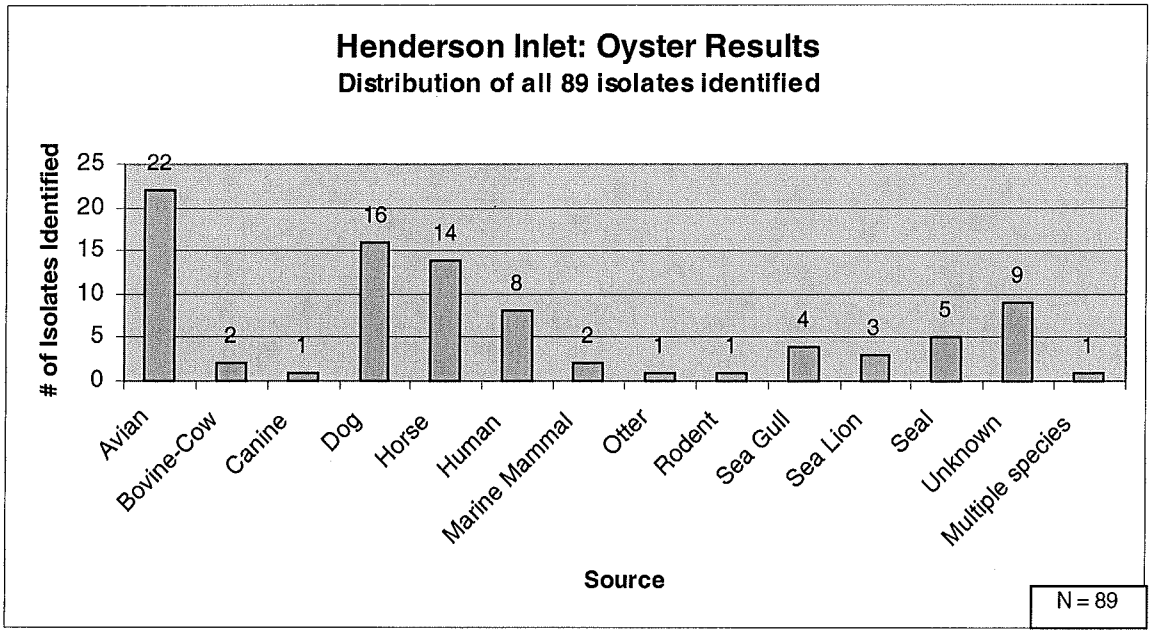
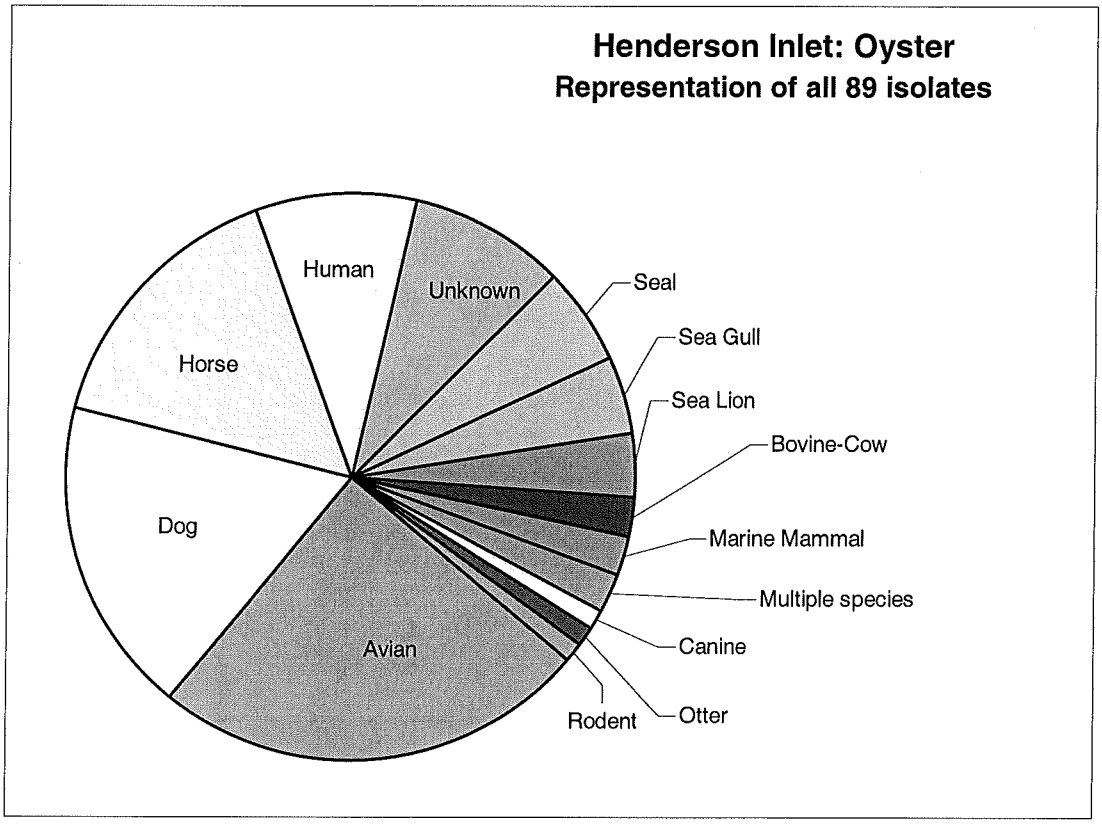


Figure D9



The target for the study was to collect 100 isolates. Though oysters were collected on 5 dates, only 89 isolates could be identified. Many samples did not produce an *E. coli* colony.

Figure D10



Fourteen source types were identified – the least for any of the 5 site / media. Birds again, as in sediment, are ¼ of the whole. Dogs comprise the next most number of isolates followed by horses and humans. In no other sites/media have horse and dog isolates been the predominant source.

Figures D11 and D12 shows the distribution for the total number of isolates for the entire project. Birds, unknowns, and humans have the most isolates.

Figure D11

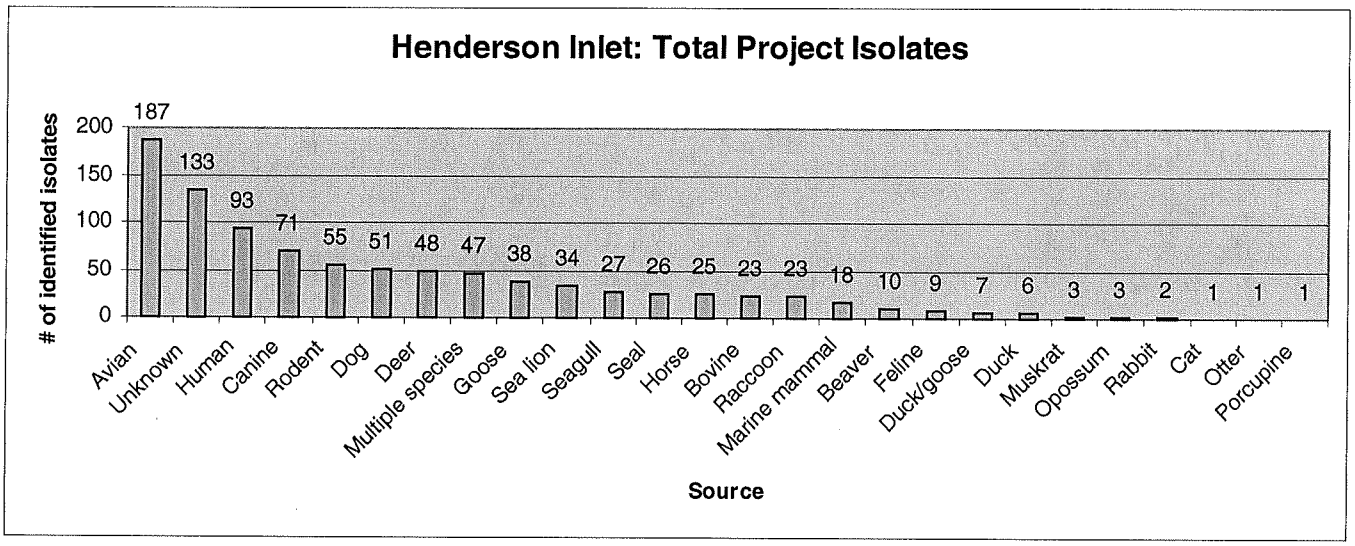


Figure D12

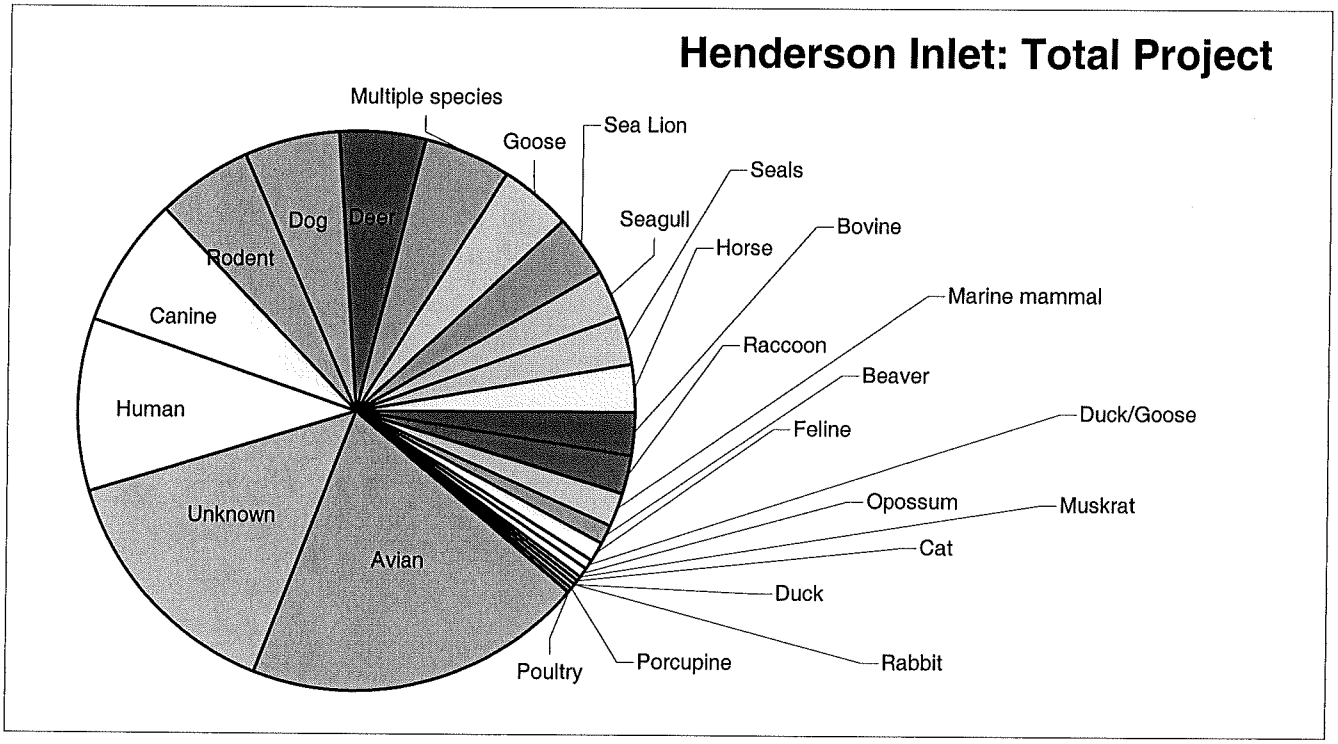


Table D1

Totals Isolates

	Marine	Woodland	Swayne	Sediment	Oyster	Total
avian	56	18	37	54	22	187
beaver	4	6	0	0	0	10
bovine	12	5	3	1	2	23
canine	16	27	25	2	1	71
cat	0	0	1	0	0	1
deer	10	12	19	7	0	48
dog	11	16	3	5	16	51
duck	5	0	0	1	0	6
duck - goose	7	0	0	0	0	7
feline	3	2	2	2	0	9
goose	11	4	4	19	0	38
horse	2	6	3	0	14	25
human	32	23	7	23	8	93
marine	10	0	0	6	2	18
multiple	15	11	4	16	1	47
muskrat	1	0	2	0	0	3
opossum	0	2	1	0	0	4
otter	0	0	0	0	1	1
poultry	0	1	0	0	0	1
rabbit	1	0	1	0	0	2
raccoon	4	11	8	0	0	23
rodent	20	7	11	16	1	55
seagull	13	3	0	7	4	27
sea lion	18	0	0	13	3	34
seal	17	0	0	4	5	26
unknown	55	14	22	33	9	133
Totals	**323**	**168**	**153**	**210**	**89**	**943**

Appendix E

Description of Microbial Source Tracking Methods

As developed by Dr. Mansour Samadpour

Microbial Source Tracking

Note: The following is excerpted from a project proposal by Dr. Mansour Samadpour:

During the past decade tremendous advances have been made in developing rapid sensitive microbial pathogen detection systems. Agencies such as the United States Department of Defense, Department of Energy, and Department of Agriculture have funded a large number of projects for rapid, automated detection of microbial pathogens and indicators in various matrices, including water, wastewater, and food. The biggest gap in knowledge and methodologies remains in the area of identification of the sources of microbial pollution. While the field of microbial source tracking is still in its infancy, advances in this field are needed to elevate the field of environmental microbiology to its next level and focus the efforts and resources toward control of sources of pollution.

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During the past decade several methods have been proposed for identification of the sources of microbial pollution in the environment. Currently there are several research groups that conduct source tracking and source identification studies, each using a different method and different target organisms. The methodologies that have been used to determine the sources of microbial contamination in the environment range from the use of phenotypic based methods such as antimicrobial resistance profiles (Wiggins, 1996), to genotypic based methods including ribotyping (Parveen et al. 2000), macrorestriction fingerprinting using pulsed field gel electrophoresis (Edberg et al, 1994), and polymerase chain reaction based methods (Dombek et al. 2000).

For the past eleven years we have worked on developing approaches and methods that would allow for identification of the sources of microbial contamination in the environment. The work has lead to development of the "Microbial Source Tracking" (MST) method. The MST method relies on a specific sampling plan designed on the basis of a sanitary survey of the watershed of interest, and the types of questions that are to be answered. The source identification portion of the method relies on generating genetic fingerprints of *Escherichia coli* strains isolated from the contaminated site(s) and comparing of the fingerprints to those of *E. coli* strains isolated from potential sources of pollution. The method that is currently in use, in our laboratory, for generating the genetic fingerprints of the isolates for the MST studies is ribosomal RNA typing using two restriction enzymes (Eco RI and Pvu II). To date we have subtyped more than 65,000 E. coli strains during the course of our studies.

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Background

Numerous human pathogens are spread by fecal contamination of water. Examples include *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 due to fecal contamination. These organisms must be prevalent in feces, found in higher concentrations than pathogens, be more resistant to disinfectants, more persistent in the environment, and they must be easy to quantify. The group of bacteria referred to as fecal coliforms meet these criteria. Fecal coliforms are facultative 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 *Escherichia 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. The utility of the indicator organism concept is further limited by its inability to track organisms associated with fecal contamination to their potential sources. Each year millions of dollars are spent on fecal and total coliform assays to determine the extent of bacterial and fecal pollution of aquatic environments and to satisfy increasingly rigid regulatory requirements concerning the microbiological quality of water. Knowing the sources rather than just monitoring the level of microbial pollution of source water enables water quality professionals and watershed managers to design and implement programs control pollution and protect source water.

The inability to conclusively identify the contributing sources of microbial contamination in watersheds has led to an over-reliance on treatment processes to insure a safe supply of drinking water. In many instances, the lack of effective source control programs has resulted in a deterioration of the microbiological quality of source waters, which in turn results in an increased likelihood of waterborne outbreaks of gastroenteritis in instances of treatment failure.

Until a few years ago, the identification of nonpoint sources of microbial pollution was an impossible task. However, advances in molecular biology and molecular epidemiology have resulted in the development of molecular subtyping methods that can be used to assess the impact of suspected sources of microbial pollution in rivers, lakes, and water reservoirs. Once the sources of microbial pollution are identified, appropriate control measures can be devised to reduce or eliminate their impact.

Principles of Microbial Source Tracking

Bodies of water are impacted by large numbers of sources of microbial pollution in their watersheds. In a given watershed, potential sources of microbial pollution include soil, vegetation, and the entire human and animal population residing in the watershed. Determination of the sources of microbial pollution in a watershed is not an easily accomplished task. It requires establishing a large collection of bacterial isolates of a specific species from the impacted body of water that is representative of the genetic diversity of that bacterial species in the watershed. Identification of the sources of the microbial pollution is then achieved by subtyping the water isolates and matching the subtypes to a collection of bacterial isolates of the same species from known sources, which include humans and various animal species.

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Our laboratory's MST method has been developed on the basis of the principles of microbiology, epidemiology, molecular epidemiology, microbial population genetics, sanitary engineering, and hydrogeology. There are several foundations on which the MST method is based. First, in any given pollution scenario there are multiple contributing animal sources of microbial pollution, each of which has its own unique clones of bacteria that constitute their normal flora. Second, collections of isolates from an appropriate bacterial species can be compiled from the polluted sites and the suspected animal sources of pollution, which are identified through a sanitary survey of the region surrounding the polluted site. Third, using an appropriate molecular subtyping method, the bacteria in the collection can be subtyped. Finally, the genetic fingerprints of the bacterial isolates

from the polluted site can be compared to those of the bacteria from the suspected animal sources. When a strain of bacteria with an identical genetic fingerprint is isolated from both a polluted site and a suspected animal source, the animal is implicated as a contributor of that specific clone of the bacteria to the polluted site.

Underlying Assumptions of Microbial Source Tracking

The MST method is based upon two principles. The first principle is that the bacterial population genetic structure is clonal. This is a well-established element of microbial genetics. Bacteria divide by binary fission. The two daughter cells that are generated as a result of this cell division are virtually identical in all aspects. All descendents of a common ancestral cell are genetically related to each other. Over time, members of a given clone may accumulate genetic changes, which will cause them to diverge from the main lineage and to form one or several new clonal groups. MST makes use of the clonal population structure of bacteria to classify organisms based on their genetic fingerprints into groups of clonal descent.

The second principle behind the MST methodology is the assumption that within a given species of bacteria, various members have adapted to living/environmental conditions in specific hosts/environments. As a result, there is a high degree of host specificity among bacterial strains that are seen in the environment. 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 its indigenous flora. Such a bacterial strain is called a resident strain. Resident strains are usually shed from their host over a long period of time, thus providing a characteristic signature of their source. A transient strain is a bacterial strain that is introduced into a new environment or host but cannot colonize and persist in that environment. If a host is sampled over time for a given species of bacteria, a few resident strains are consistently being shed while a large number of transient strains are shed for brief lengths of time. A study conducted by Hartl and Dykhuizen (1984) illustrates this point. Over a period of 11 months, 22 fecal samples were taken from a single individual. A total of 550 *E. coli* isolates were characterized, of which two were considered to be resident strains, appearing 252 times. We have accumulated considerable evidence to support this assertion for E. coli. Our data shows that using our subtyping method (ribosomal RNA typing using two restriction enzyme reactions) more than 96% of E. coli strains are seen in only one host species (or group of related species) (Mazengia, 1998).

Given that bacterial population structure is clonal and within each bacterial species different clones have adapted to specialized environments, 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 their sources
- Identify the contributing sources

Subtyping Methods Used in Source Tracking Studies

Another important factor in determination of clonality is the methodological issues. Our laboratory's ability to subtype microbes and divide them into groups of clonal origin largely rests upon the sensitivity of the methods that are used to subtype the organisms. For instance, consider a hypothetical collection of 100 E. coli strains isolated from 100 different source samples at 100 different times from 100 different sites which is to be analyzed with three methods representing

low, medium and high degrees of sensitivity. The first method, which has low sensitivity, may divide the 100 strains into 8 groups, while the second method divides them into 40 groups and the third method, with a high degree of sensitivity, divides them into 95 groups. A researcher using either of the first two methods may erroneously cluster unrelated strains of E. coli as members of the same clone. If this was a source tracking study, the practical implication is that a water isolate that is different from a bovine strain, but is seen by the subtyping method as being identical will be labeled as E. coli of bovine origin. However, this isolate may in reality have come from a source other than bovine. While insensitive subtyping methods are not suitable for use in MST studies, we have also found that very sensitive subtyping methods may not be as useful in source tracking studies as one would predict. The main reason is that highly sensitive subtyping methods can detect minute genetic changes that have occurred very recently, on the order of weeks to months. The practical implication of this is that the level of diversity seen by these methods is so high that the number of samples needed to achieve a sanitary survey of the study area which is representative of the population of a given species in a watershed would require the analysis of thousand of bacterial isolates, which would make the venture prohibitively expensive.

Ribotyping

The key methodological problem in tracing sources of bacterial contamination in the environment was the lack of a universal single-reagent typing scheme for bacteria. This has been overcome by the work of several investigators in fields of population genetics, molecular systematics, and molecular epidemiology. In 1986 Grimont, et al. showed that DNA probes corresponding to specific regions of the rRNA operon can be used to speciate bacteria. Stull, et al. (1988) and Lipuma, et al. (1988) used the rRNA operon to study the molecular epidemiology of several species of bacteria. In order to trace the indicator bacterium, *E. coli*, from the water to its specific source, the bacterial strain must first be uniquely identified. Populations of *E. coli*, like other bacteria, are composed essentially of a mixture of strains of clonal descent. Due to the relatively low rates of recombination, these clones remain more or less independent (Selander, et al 1987). These clones, or strains of bacteria, are uniquely adapted to their own specific environments. As a result, the *E. coli* strain that inhabits the intestines of one species is genetically different from the strain that might inhabit another.

Ribosomal ribonucleic acids (rRNA), which are integral to the machinery of all living cells, and tend to be very highly conserved, make an ideal choice of target in interstrain differentiation. Since the *E. coli* chromosome contains seven copies of the rRNA operon, a rDNA probe can be used as a definitive taxonomic tool (Grimont and Grimont 1986). That is, when digested with restriction enzymes, resolved by agarose gel electrophoresis, transferred to a membrane and hybridized with an rRNA probe, an *E. coli* chromosome will produce several bands to create a specific restriction fragment length polymorphism (RFLP) pattern that can be used to uniquely identify the bacterial strain.

The pattern of DNA fragments corresponding to the rRNA operon is referred to as the ribotype. Ribotyping has been useful in many studies to differentiate between bacterial strains that would have otherwise been difficult or impossible to distinguish. Fisher, et al. (1993) followed the transmission of *Pseudomonas cepia* from environmental sources to and between cystic fibrosis patients and discovered the majority of cases contracted cystic fibrosis from one of two treatment centers. Moyer, et al. (1992) used ribosomal RNA typing to identify the *Aeromonad* strains responsible for several waterborne gastroenteritis episodes in a community and was able to trace the contamination to specific locations in water treatment and distribution systems. Baloga and

Harlander (1991) compared several typing methods for distinguishing between strains of *Listeria moncytogenes* implicated in a food-borne illness and found that ribotyping was the preferred method due to its precision and reproducibility. Atlas and Sayler (1988) described the technology of ribotyping as applicable to the tracking of genetically engineered microorganisms (GEMs) in the environment.

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Ribotyping Using *Eco* R1 and Pvu II

Our initial source tracking studies were all conducted using a single enzyme (*Eco* R1) ribotyping protocol. The choice of *Eco* R1 was the result of a large scale screening of enzymes to determine the differentiative power of each of the available enzymes. *Eco* R1 showed the most differentiative power followed by *Pvu* II. Although in the beginning the single enzyme system was showing close to 100% residency among our source isolates, as our database grew and more isolates were studied we began to notice a sharp increase in the incidence of transients among the source isolates. At that time, we hypothesized that a single enzyme lacks the sensitivity to effectively separate the isolates into groups of clonal origin. We then conducted a study to test this hypothesis.

Table 1. Collection of *E. coli* strains which were used to evaluate the use of single versus double enzymes for ribotyping.

Source Type	No. of Isolates	Total Ribotypes Pvu II	Total Ribotypes Eco R1	Total Ribotypes Eco R1/ Pvu II
Human Sources[1]	813	265	348	381
Bovine	325	48	92	153
Horse/ Llama	342	43	66	104
Avian[2]	183	55	72	107
Canine[3]	194	38	53	72
Feline[4]	73	22	30	33
Deer/ Elk	53	18	14	21
Farm Animals[5]	100	25	36	41
Wild Animal[6]	59	13	21	25
TOTAL	2142	527	732	873

Table 2. Grouping of the 2142 *E. coli* strains by each of the two restriction enzymes and by the combination of the two enzymes.

ENZYME	Total Ribotypes	Source Specific Ribotypes	Source Related Ribotypes	Transient Ribotypes
PVU II	514	221 (43%)	31(6%)	262(51%)
ECO R1	723	368(51%)	38(5%)	317(44%)
PVU II and ECO R1	873	823(94%)	18(2%)	32(4%)

Pvu II divided the 2142 E. coli strains from various sources into 514 groups. 49% of the groups were resident clones and 51% were transient. *Eco* R1 divided the 2142 isolates into 723 groups, 56% of which were resident clones and 44% were transient. When we combined the results of the two enzymes we found that the 2142 isolates were divided into 873 clonal groups, 96% of which were resident clones and only 4% were transient. This was very convincing evidence that lead us to move towards a two enzyme ribotyping system. In order to increase the level of specificity of our source identification, we took an additional step of identifying and tagging the transient clones in the database. The transient clones are not used for source identification. The practical implication of a single versus double enzyme ribotyping protocol is shown in Table 3. While the 14 isolates from 14 different sources are seen as one with *Pvu* II, *Eco* R1 separates them into 14 different groups, allowing for their use in source identification.

Table 3. Illustration of the advantage of the use of a double enzyme system over single enzyme ribotyping. While *Pvu* II identifies the 14 isolates as transient, *Eco* R1 separates them into 14 resident clones.

Isolate #	PVU II	ECO RI	Source Type
20977	Z	A	BOVINE
21610	Z	B	HORSE
20699	Z	C	DOG
12069	Z	D	CAT
21696	Z	E	SEPTAGE
12805	Z	F	SANITARY SEWER
13076	Z	G	BOBCAT
8450	Z	H	DUCK
21464	Z	I	LLAMA
14328	Z	J	RAW SLUDGE
1601	Z	K	DEER
21438	Z	L	PIG
21641	Z	M	GOAT
14781	Z	N	BEAVER

MST Approach

We have developed the following approach in the MST studies conducted in our laboratory:

A. Interview with stakeholders, watershed managers, and local agencies that have been monitoring water quality of the study area
B. Sanitary Survey of the study area
C. Determine questions to be answered by the study
D. Design a sampling plan to answer the questions. The sampling plan is designed specifically to answer all the questions that are raised regarding the study site. The sampling plan is put together to reflect influences such as seasonality, storm events, landuse, recreational use, and regrowth. Another important element in sampling design is the total project budget.
E. Field Work (collecting water and source samples according to the sampling plan). This is often performed by collaborators at the site. The water samples are collected and processed locally (in certified laboratories). Source samples are shipped directly to our laboratory.
F. Processing of water samples (to determine levels of fecal coliforms/E. coli and isolate E. coli strains) by local laboratories with subsequent shipment of the plates containing the

organisms to our laboratory. The source samples are directly shipped to our laboratory. They are processed upon arrival to isolate E. coli strains.

G. Logging the samples and cultures drives from each sample in our sample and data logs (both hard copy and computer database). The samples and cultures are logged together with pertinent epidemiological information such as: Isolate number (our log number) study ID, provider sample ID, provider ID, sampling date, sampling site (complete address), and source type.
H. Establishing pure cultures of E. coli from primary water and source plates. Verification of speciation. Freezing the cultures in or permanent collection of isolates.
I. Subtyping of the isolate collection. We currently are using ribosomal RNA typing as using two restriction enzymes (Eco RI and Pvu II) as our subtyping method. On selective bases we use one or two additional restriction enzymes. Our long standing policy on the choice of subtyping method is that as soon as a better method becomes available we will subtype our collection with the new method and change our database accordingly with the results of the new method.

MST Data Analysis

The subtyping data for each isolate is analyzed (please refer to the materials and methods section for details), and entered into the database (Microsoft Access). Our MST Database contains detailed information regarding the E. coli strains in our collection. The source E. coli in the database are divided into two categories. Resident Clones (RC), and the Transient Clones (TC). The RCs are defined as clones that are unique to a particular host species (human, cow, etc.), or a group of closely related host species (dogs and coyotes), TCs are defined as clones which are seen in more than one unrelated host species. Only RCs are used for source tracking (assignment of source to water isolates). Using the current regiment of subtyping methods, more than 96% of all the host isolates fall into the RC category. The database is constantly updated to insure that the TCs are tagged and are not used for source identification purposes. We have found that when using additional enzymes we can eliminate more than half of the TCs and change them into RCs.

MST Utility

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

- Understanding the sources, distribution, and movement of microbial populations in watersheds, source waters, swimming beaches, fisheries resources, etc.
- Conducting risk and exposure assessment studies of the potential human health effects associated with the presence of microbial pollution in source waters
- Identifying human pathogens that have established reservoirs in watersheds
- Determining the impact of various types of land use on water quality
- Identification of the sources of microbial pollution and quantification of the impact of each source
- Designing and implementing source control programs
- Studying the effects of control measures
- Environmental litigation.

Materials and Methods

Bacterial strains and culture conditions. Water and sewage grab samples will be processed by membrane filtration for fecal coliforms (**Standard Methods for the Examination of Water and Wastewater, 20th ed.**). After incubation at 44.5°C for 24 hours they will be read. Appropriate colonies will be chosen morphologically (round, blue, and flat) and streaked for isolation on MacConkey media, then incubated at 37°C for 24 hours. Fecal samples will be collected from representative animal species in the Duwamish Watershed. They will be streaked on MacConkey plates and incubated at 37°C for 24 hours. Non-mucoid colonies that fermented lactose on MacConkey will be re-streaked on Trypticase Soy Agar (TSA) plates. Five *E. coli*-type colonies per sample will be isolated. Biochemical analysis will be done to positively identify *E. coli*. Two E. coli strains from each water sample and one E. coli strain from each source sample will be added to our study collection. These isolates will be assigned an isolate number and stored in LB-15% glycerol freezing media at -70°C.

Genomic DNA isolation and restriction endonuclease digestion. Confluent growth will be scraped with a sterile flat-headed toothpick and suspended in 200 µl 50mM Tris, 50mM EDTA (pH 8.0). Then another 600 µl 50mM Tris, 50 mM EDTA will be added and the suspension will be mixed well by pipetting up and down. Next, 45 µl 20% sodium dodecyl sulfate (SDS) followed by 10 µl proteinase K (20 µg/ml; Pharmacia, Piscataway, NJ) will be added. They will be incubated at 40°C for 1 hour. Equal volume of phenol will be added, samples will be vortexed, then centrifuged for 5 minutes. The top layer will be extracted and an equal volume of chloroform will be added. The prep will be vortexed again, centrifuged, and extracted. Two and a half volumes absolute ethanol will be added; the DNA will precipitate out and be spooled onto a sterile glass capillary pipette. The DNA will be washed with a few drops of absolute ethanol, dried, and re-suspended in 50 µl dH2O.

Restriction endonuclease digestions will be set up using *Eco* RI and *Pvu* II (in separate, individual reactions), 10 u/µl (Boehringer Mannheim, GmbH, Germany) as instructed by the manufacturer and 2 µgr DNA. They will be incubated at 37°C overnight. The samples will be centrifuged and .5µl pure enzyme will be added. The samples will be re-incubated at 37°C for a minimum of three hours. They will be centrifuged again and 3 µl stop dye will be added.

Gel electrophoresis and Southern Blot hybridization. Samples will be run on a 0.8% agarose gel in 1X Tris-borate-EDTA at 22 volts and 17 milliamps, for 17 hours. λ *Hind* III will be used as a size standard along with an *E. coli* isolate with a distinct ribotype pattern designated 3915. The DNA fragments will be then transferred to a Nitran filter (Schleicher & Schuell, Keene, NH), baked at 80°C for one hour and probed. P32 labeled copies of *E. coli* ribosomal RNA will be made by extension of random hexanucleotide primers (Finberg, et al.) using Avian Myeloblastosis Virus reverse transcriptase (Stratagene, La Jolla, Ca) under conditions specified by the supplier. Hybridization will be done in 5X SSC (1X SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% SDS, 1mM EDTA, and 50% formamide at room temperature overnight. Salmon sperm DNA and blocking reagent, (Boehringer Mannheim GmbH, Germany) will be used to block non-specific binding. Three washes will be done with a solution of 2X SSC and .1% S.D.S., once at 25°C for 20 minutes and twice at 65°C for 20 minutes to wash off low-homology, non-specific binding. Blots will then be exposed with an intensifying screen to X-ray film (Kodak, Rochester, N.Y.) for 24 hours at -70°C. Two to three exposures will be done to ensure detection of all possible bands.

QUALITY ASSURANCE: Measurement & Data Acquisition

Sampling QA/QC Water samples: Water samples will be collected using sterilized sampling bottles, by grab sampling method as described in **Standard Methods for the Examination of Water and Wastewater** (APHA, 1997). Samples will be delivered (on ice) to the Molecular Epidemiology Inc. Laboratory and analyzed within eight hours from collection. All the sample bottles will be labeled with sampling station identification number, sampling date and time, sample number, source identification number, and sampler's initials. All the sample information will be entered into the field log, and Chain of Custody forms. Both the sampler and the receiving laboratory will sign the Chain of Custody form.

Water samples will be analyzed by the mFC method (Standard Methods for Examination of Water and Wastewater, APHA, 1997). To ensure aseptic conditions, blank samples will be filtered to determine whether our filtering apparatus, dilution blanks, and other equipment are free of contamination by fecal coliforms. Prior to filtering each sample, a blank sample (containing only dilution water) will be filtered. This will allow testing of the sterility of our filter tower and dilution water. Following the filtering of the prescribed number of dilutions, a final blank sample will be filtered. This will allow us to determine whether our rinsing method between individual dilutions was adequate enough to prevent contamination from previous filtrations. After incubation the results will be entered into result forms, and the forms will be entered into a database. QC records will include positive and negative controls with each batch of water samples filtered, media preparation and performance characteristics documentation, incubator and waterbath temperature documentation. Method performance will be documented by performing duplicate samples and routinely confirming both positive and negative colonies at a frequency of one in twenty samples. Acceptable reproducibility range will be an RPD of 20% when cfu is in the countable range.

Source samples: Fresh animal fecal samples will be collected aseptically into sterile containers and delivered to the environmental microbiology laboratory, on ice. Animal fecal samples are only collected when they are positively identified as belonging to a given animal species. No more than three samples will be collected from the members of the same animal species from a given location. Only a single sample will be collected from an individual animal. All sample containers will be labeled with the following information: sample type, host species, sample date and time, sample location, and sampler's initials. All the sample information will be logged into the field log. After collection of the samples, samples are delivered to the lab where they will be given a sample number and will be logged into the permanent sample log.

MST QA/QC The goal of the MST project is to identify the sources of fecal coliforms that are present in water samples. Two types of samples will be received for this study: water and fecal samples. Our laboratory analysis includes:

a. Sample arrival, and logging.
b. Filtration and quantification of FC.
c. Isolation and purification of *E. coli* strains from water and fecal samples.
d. Growing pure cultures of *E. coli* strains for freezing (long term storage), and isolation of DNA.
e. Restriction enzyme digestion and Agarose gel electrophoresis of DNA samples.
f. Southern blot hybridization using radio labeled cDNA probe for rRNA genes.
g. Exposure of autoradiograms.
h. Analysis of the data.

Sample arrival and logging
All samples upon arrival are inspected for damage to sample containers or microbiological plates, and signs of contamination. Sample identifiers are also checked against the Chain of Custody forms. Samples are logged into our log book noting the provider's sample identification number, provider ID, sample type, study ID, sample site, sample collection date and sample arrival date. Compromised samples are noted and appropriately discarded.

Isolation and purification of *E. coli* strains from water and fecal samples
Fecal samples are plated on MacConkey agar and incubated at 35 ° C, overnight. The next day 3-5 lactose-fermenting, non-mucoid colonies are picked and replated on MacConkey agar for purification.
Five non-mucoid blue colonies picked from mFC plates corresponding to each water sample are plated on MacConkey agar for purification.
At this stage each of the colonies picked from a given sample bears the Sample ID number and an accession letter. A single, well isolated, non-mucoid colony is picked from each MacConkey plate and is plated on Tryptic Soy Agar. After overnight incubation at 35 ° C, each culture is tested by a spot indol test. Indol positive cultures are further tested for the ability to utilize citrate using Simmon's Citrate medium. Isolates with the combination of indologenesis, and citrate non-utilization are identified as *E. coli* and are given isolate numbers. Appropriate positive and negative controls are incorporated when testing the biochemical reactions.

Growing pure cultures of *E. coli* strains for freezing (long term storage)
A portion of each E. coli strain isolated, identified and retained from the samples will be stored at -80° C, in nutrient broth plus 15% glycerol.

Phenotypic and genotypic characterization of *E. coli* isolates
All methodologies for characterizing *E. coli* isolates will follow standardized protocols and will have the following QC documentation:

All record entries will include the analyst's initials, and the date.

All reagents, media and buffers are prepared according to written and approved SOPs. Each batch prepared is tested for sterility as appropriate and undergoes a performance test with positive and negative controls or with the previous batch prior to use in production. Commercially available sensidiscs impregnated with antibiotics will be tested against standard strains to ensure conformance with current designations of "sensitive, intermediate, and resistant" status.

Each batch of enzymatic reactions is performed with a positive control strain and is performance checked in an analytical procedure (eg, electrophoretic gel).

Incubation, electrophoresis, PCR conditions are all standardized for each method and documented for each run. Documentation includes: agarose gel concentration and volume, buffers, pH, mA, V, and run times. Each methodology will have its own logbook to track the isolates included in each run as well as the appropriate controls and their performance.

Any runs with control reactions out of normal response range will be noted and corrective action taken. Corrective action may include repeating the procedure and will be documented in a corrective action log.

All record books will be audited monthly for completeness, and technicians will be involved in the audit review.

Isolation of DNA, restriction enzyme digestion and agarose gel electrophoresis of DNA samples

Genomic DNA is isolated from each *E. coli* strain using a standard protocol. Every batch of restriction enzyme reaction contains two reactions with our positive control strain, which will be included on two lanes on each gel. Each agarose gel is assigned a number, and when more than one gel is run, the position of the first standard reference strain is changed in each gel (1^{st} lane on the first gel, to the Nth lane on the Nth gel). After electrophoresis, gels are stained in ethidium bromide; the two gels are each stained in a single container. One of the two gels is placed in the same container. The corner of the gel with the higher number is clipped and the label for each gel is also transferred to the staining container. Each gel is then photographed and a hard copy of the print is labeled with a gel sheet (containing the isolates' ID number loaded on each lane, the enzyme used to cut the DNA, as well as the date, gel number, voltage, mA, gel strength, buffer strength, and electrophoresis time). This information is kept in the gel book.

Southern blot hybridization using radio labeled cDNA probe for rRNA genes

Southern blotting is performed according to the protocol detailed in our SOP. After photography each gel is returned to the same staining container. Gels are denatured for Southern blotting in the same container. Each blotting apparatus is set in a separate container that is labeled with the gel number. Each membrane filter is labeled with the gel number, restriction enzyme designation, date, and technician's initials.

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Appendix F

Pathogen Scan
Raw Data

Pathogen Scan: Raw Data

Sample ID	type	Bed condition	Sample Collection	PCR No.	Sample Processing	E. C. Multi	Sal 2F/2R	Shi-1,2	YE-1,2	Campy-1,2	VPtoxR- Prelim	VV
1	Oyster	closed	10/30/2000	3430	11/8/2000	-	-	-	-	-	-	-
2	Oyster	closed	10/30/2000	3431	11/8/2000	-	-	-	-	-	-	-
3	Oyster	closed	10/30/2000	3432	11/8/2000	-	-	-	-	-	-	-
4	Oyster	closed	10/30/2000	3433	11/8/2000	-	-	-	-	-	-	-
5	Oyster	closed	10/30/2000	3434	11/8/2000	-	-	-	-	-	-	-
6	Oyster	closed	10/30/2000	3435	11/8/2000	-	-	-	-	-	-	-
7	Oyster	closed	10/30/2000	3436	11/8/2000	-	-	-	-	-	-	-
8	Oyster	closed	10/30/2000	3437	11/8/2000	-	-	-	-	-	-	-
9	Oyster	closed	10/30/2000	3438	11/8/2000	-	-	-	-	-	-	-
10	Oyster	closed	10/30/2000	3439	11/8/2000	-	-	-	-	-	-	-
11	Oyster	closed	10/30/2000	3440	11/8/2000	-	-	-	-	-	-	-
12	Oyster	closed	10/30/2000	3441	11/8/2000	-	-	-	-	-	-	-
13	Oyster	closed	10/30/2000	3442	11/8/2000	-	-	-	-	-	-	-
14	Oyster	closed	10/30/2000	3443	11/8/2000	-	-	-	-	-	-	-
15	Oyster	closed	10/30/2000	3444	11/8/2000	-	-	-	-	-	-	-
16	Oyster	closed	10/30/2000	3445	11/8/2000	-	-	-	-	-	-	-
17	Oyster	closed	10/30/2000	3446	11/8/2000	-	-	-	-	-	-	-
18	Oyster	closed	10/30/2000	3447	11/8/2000	-	-	-	-	-	-	-
19	Oyster	closed	10/30/2000	3448	11/8/2000	-	-	-	-	-	-	-
20	Oyster	closed	10/30/2000	3449	11/8/2000	-	-	-	-	-	-	-
1	Oyster	open to closed	4/6/2001	4428	4/6/2001	-	-	-	-	-	-	-
2	Oyster	open to closed	4/6/2001	4429	4/6/2001	-	-	-	-	-	-	-
3	Oyster	open to closed	4/6/2001	4430	4/6/2001	-	-	-	-	-	-	-
4	Oyster	open to closed	4/6/2001	4431	4/6/2001	-	-	-	-	-	-	-
5	Oyster	open to closed	4/6/2001	4432	4/6/2001	-	-	-	-	-	-	-
6	Oyster	open to closed	4/6/2001	4433	4/6/2001	-	-	-	-	-	-	+
7	Oyster	open to closed	4/6/2001	4434	4/6/2001	-	-	-	-	-	-	-
8	Oyster	open to closed	4/6/2001	4435	4/6/2001	-	-	-	-	-	-	-
9	Oyster	open to closed	4/6/2001	4436	4/6/2001	-	-	-	-	-	-	-
10	Oyster	open to closed	4/6/2001	4437	4/6/2001	-	-	-	-	-	+	-
1	Oyster	closed to open	4/6/2001	4438	4/6/2001	-	-	-	-	-	-	-
2	Oyster	closed to open	4/6/2001	4439	4/6/2001	-	-	-	-	-	-	-
3	Oyster	closed to open	4/6/2001	4440	4/6/2001	-	-	-	-	-	-	-
4	Oyster	closed to open	4/6/2001	4441	4/6/2001	-	-	-	-	-	-	-
5	Oyster	closed to open	4/6/2001	4442	4/6/2001	-	-	-	-	-	-	-
6	Oyster	closed to open	4/6/2001	4443	4/6/2001	-	-	-	-	-	-	-
7	Oyster	closed to open	4/6/2001	4444	4/6/2001	-	-	-	-	-	-	-
8	Oyster	closed to open	4/6/2001	4445	4/6/2001	-	-	-	-	+	-	-
9	Oyster	closed to open	4/6/2001	4446	4/6/2001	-	-	-	-	-	-	-

Pathogen Scan: Raw Data

Sample ID	type	Bed condition	Sample Collection	PCR No.	Sample Processing	E. C. Multi	Sal 2F/2R	Shi-1,2	YE-1,2	Campy-1,2	VPtoxR- Prelim	VV
10	Oyster	closed to open	4/6/2001	4447	4/6/2001	-	-	-	-	-	-	-
1	Oyster		5/22/2001	4778	5/22/2001	-	-	-	-	-	-	-
2	Oyster		5/22/2001	4779	5/22/2001	-	-	-	-	-	-	-
3	Oyster		5/22/2001	4780	5/22/2001	-	-	-	-	-	-	+
4	Oyster		5/22/2001	4781	5/22/2001	-	-	-	-	-	-	-
5	Oyster		5/22/2001	4782	5/22/2001	-	-	-	-	-	-	-
1	Sediment		5/22/2001	4783	5/22/2001	-	-	-	-	-	-	+
2	Sediment		5/22/2001	4784	5/22/2001	-	-	-	-	-	-	-
3	Sediment		5/22/2001	4785	5/22/2001	-	-	-	-	+	-	-
4	Sediment		5/22/2001	4786	5/22/2001	-	-	-	+	-	-	-
5	Sediment		5/22/2001	4787	5/22/2001	-	-	-	-	-	-	-
6	Sediment		5/22/2001	4788	5/22/2001	-	-	-	-	-	-	-
7	Sediment		5/22/2001	4789	5/22/2001	-	-	-	-	-	-	-
8	Sediment		5/22/2001	4790	5/22/2001	-	-	-	-	-	-	-
9	Sediment		5/22/2001	4791	5/22/2001	-	-	-	-	-	+	-
10	Sediment		5/22/2001	4792	5/22/2001	-	-	-	-	-	-	+
11	Sediment		5/22/2001	4793	5/22/2001	-	-	-	-	-	-	-
12	Sediment		5/22/2001	4794	5/22/2001	-	-	-	-	-	-	+
13	Sediment		5/22/2001	4795	5/22/2001	-	-	-	-	-	-	+
14	Sediment		5/22/2001	4796	5/22/2001	eae	-	-	-	-	-	-
15	Sediment		5/22/2001	4797	5/22/2001	eae	-	-	-	-	+	-
1	Water		5/22/2001	4798	5/22/2001	eae	-	-	-	-	-	-
2	Water		5/22/2001	4799	5/22/2001	-	-	-	-	-	-	-
3	Water		5/22/2001	4800	5/22/2001	-	-	-	-	-	-	-
4	Water		5/22/2001	4801	5/22/2001	-	-	-	-	-	-	-
5	Water		5/22/2001	4802	5/22/2001	-	-	-	-	-	-	-
6	Water		5/22/2001	4803	5/22/2001	-	-	-	-	-	-	-
7	Water		5/22/2001	4804	5/22/2001	-	-	-	-	-	-	-
8	Water		5/22/2001	4805	5/22/2001	-	-	-	-	-	-	-
9	Water		5/22/2001	4806	5/22/2001	-	-	-	-	-	-	-
10	Water		5/22/2001	4807	5/22/2001	-	-	-	-	-	-	-
11	Water		5/22/2001	4808	5/22/2001	-	-	-	-	-	-	-
12	Water		5/22/2001	4809	5/22/2001	eae	-	-	-	-	-	-
13	Water		5/22/2001	4810	5/22/2001	eae	-	-	-	-	-	-
14	Water		5/22/2001	4811	5/22/2001	-	-	-	-	-	-	-
15	Water		5/22/2001	4812	5/22/2001	-	-	-	-	-	-	-