AN ABSTRACT OF THE THESIS OF

Robert J. Bower for the degree of Master of Science in Bioresource Engineering presented May 2nd, 2000. Title: Source Identification of Fecal Pollution in the Tillamook Watershed: Antibiotic Discriminant Analysis.

Abstract approved: Redacted for privacy

James A. Moore

The identification of sources of point and nonpoint fecal pollution is difficult to determine. Understanding the sources of fecal organisms in quality limited waters could greatly enhance our ability to restore and protect the water quality and habitat of these systems.

Antibiotic resistance patterns of fecal streptococci bacteria were analyzed using discriminant analysis and these were in turn used to identify sources of fecal pollution in the Tillamook Bay watershed, Oregon. Antibiotic resistance patterns for humans, dairy cattle, and wild animals were established using a database of 830 isolates collected from known sources of feces in the Tillamook watershed. The average rate of correct classification (ARCC) for these three sources was determined to be 83%, with individual rates of 73% for human isolates, 88% for wild isolates, and 89% for dairy isolates. To test the application of this technique, water samples were collected for two independent studies. Samples were collected from a winter rainstorm event, as well as 9 samples over one year at the mouth of the five major rivers flowing into the Tillamook Bay. Results clearly demonstrate that monitoring bacterial sources is complex with results varying on a sample-by-sample, site-by-site, and river-by-river basis. Dairy and human sources contributed a majority of the fecal

bacteria in all samples collected while wild sources consistently contribute a small percentage.

Fecal coliform bacteria (FCB) and fecal streptococci bacteria (FSB) concentrations were also used in conjunction with source distributions to estimate the magnitude of individual samples and prioritize samples based on their water quality significance. These results demonstrate that antibiotic resistance profiles in fecal streptococci can determine sources of fecal pollution

Source Identification of Fecal Pollution in the Tillamook Watershed: Antibiotic Discriminant Analysis

by

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A THESIS

submitted to

Oregon State University

In partial fulfillment of
the requirements for the
degree of

Master of Science

Presented May 2nd, 2000

Commencement June, 2000

Master of Science thesis of Robert J. Bower presented on May 2nd, 2000

APPROVED:

Redacted for privacy

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I understand that my thesis will become part of the permanent collection at Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

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Robert J. Bower, Author

ACKNOWLEDGEMENT

This study was funded by the Tillamook Bay National Estuary Project, Oregon Department of Environmental Quality, and my wife Alison Luckett Bower, and I thank them for their support.

I would also like to thank my fellow graduate student Wendy Church, for her many hours of help in the laboratory, during the thesis editing process, and in preparation for my oral defense. Bob Sonn for his help early on in getting me up to speed on the use of antibiotics. To the SWAMP team members for being there for moral support during the my time in the Bioresource Engineering Department. To Bruce Wiggins for his support via email and telephone in helping us get our project off the ground here on the West Coast. To Jim Moore who envisioned this project and for his patience over the years despite my countless questions, was always there for support.

A special thanks to my wife Alison Luckett Bower for all the love and support over the last few years.

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To my father,

for my beginnings in Science.

The creeks overflow: a thousand rivulets run

'Twixt the roots of the sod, the blades of the marshgrass stir;

Passeth a hurrying sound of wings that westward whirr;

Passeth, and all is still; and the currents cease to run;

And the sea and the marsh are one.

Sidney Laniers "The Marshes of Glynn"

Source Identification of Fecal Pollution in the Tillamook Watershed: Antibiotic Discriminant Analysis

Introduction

Fecal pollution is a persistent problem in watersheds throughout the world due to the increased health risks it poses to drinking water, water contact from recreation, degradation of aquatic habitat and limitations on marine resources such as shellfish harvests. While points sources of fecal pollution have been monitored for years under the Clean Water Act, nonpoint sources of pollution pose a much more difficult challenge. Nonpoint sources vary in their contributions in numerous ways including the source and/or the amount contributed by a source. Examples of nonpoint sources of fecal pollution include pastured livestock such as dairy or beef cattle and/or wildlife such as elk or waterfowl. The amount of fecal pollution can be influenced by environmental conditions including changes in rainfall and/or river flow. While the quantity of fecal pollution can be measured by established techniques that obtain concentration values of these bacteria, source identification has proven much more elusive.

Currently techniques that isolate and enumerate fecal coliform and *Escherichia coli* bacteria are used in Oregon by the Department of Environmental Quality to quantify fecal pollution in natural waters (ODEQ, 1999). There have been several attempts in the past to use ratios of fecal coliform to fecal streptococci concentrations to differentiate between human and non-human sources (Feachem et. al., 1975). In 1995 it was deemed

unreliable and abandoned due to substantial variations in die off rates between the two bacterial groups (APHA, 1995). Recent advances in the fields of biotechnology and biochemistry have also provided alternatives to solving the identification problem. Promising new technologies such as the polymerize chain reaction, gene probes, and biochemical fingerprinting may provide researchers with definitive results (Asim, et. al., 1990), (Asim, et. al., 1991), (Kuhn, 1991). However, these techniques tend to require expensive and specialized laboratory equipment and are still in the development stage.

Antibiotic resistance in bacteria has been known for many years. Antibiotics have been widely used to control bacterial infections and diseases in domesticated animals and in humans, which can result in the occurrence of antibiotic resistant bacteria in those populations. The antibiotic patterns produced by the resistant bacteria that are passed through the feces have been shown to correlate from source to source indicating possible common antibiotic profiles (Kelch, 1978). Source identification using antibiotic patterns was developed further using several different types of enteric bacteria. *Escherichia coli* were indexed using antibiotics to determine sources and showed that urban waters harbored higher percentages of resistant bacteria than rural waters (Kaspar, et. al., 1990). Isolates from the enterococcal bacterial group were also isolated and screened with antibiotics to identify sources (Knudtson, et. al., 1993). A study in Tillamook, Oregon has shown that when multiple antibiotics resistance (MAR) is indexed for specific sources, wild animals are generally low while human and livestock sources are much higher (Krumperman, 1983). Other researchers have also

shown source patterns to be a distinguishable characteristic in other fecal bacteria (Parveen, 1997).

Wiggins improved the antibiotic resistance technique by using varying concentrations of each of the multiple antibiotic screenings (Wiggins[a], 1996). Wiggins effectively distinguish between nonpoint and point sources of fecal organisms by analyzing the antibiotic patterns with multivariate statistics. Further studies have shown the repeatability of this technique and its application as a water quality-monitoring tool for determining sources of fecal pollution (Wiggins[c], 1999), (Hagedorn, 1999).

This study was conducted in the Tillamook Bay watershed located on the Northwest Oregon coast of the Pacific Ocean. The Tillamook Bay watershed has been identified as having water quality limitations due to fecal contamination. The Tillamook Bay is home to a shellfish industry that is plagued by production loss due to fecal bacterial concentrations that exceed the water quality standard for shellfish harvest which results in Bay closures to harvest. The Tillamook watershed is home to substantial human, dairy and wildlife populations; all of which have the potential to contribute to the fecal pollution problem. High rainfall during the fall, winter and spring, transports large quantities of feces to the streams, rivers and eventually, the bay. The social and commercial impacts of fecal pollution have created a politically sensitive problem without a clear mechanism to determine and address the issue. The goal of this study is to provide such a mechanism. Funding for this project was provided by

the Tillamook Bay National Estuary Project (TBNEP) and Oregon's Department of Environmental Quality (DEQ).

Objectives

- To validate the reliability and repeatability of the antibiotic discriminant analysis (ADA) technique as a tool to identify the source of fecal pollution (Wiggins[a], 1996).
- To use the ADA method to monitor a winter storm event in a water quality limited watershed and coastal estuary.
- To use the ADA method to monitor the five rivers in a water quality limited watershed over one year.

Materials and Methods

Samples were collected in the Tillamook watershed and processed using the antibiotic discriminant analysis (ADA) technique described in the *Discriminant Analysis of Antibiotic Resistance Patterns in Fecal Streptococci Method* (Wiggins, 1996[a]). The specific laboratory protocols were followed according to an unpublished lab manual provided by Dr. Wiggins titled *How To Do It: Procedures for discriminant analysis of antibiotic resistance profiles of fecal streptococci* (Wiggins, 1996[b]).

Technique Overview

The overall purpose of this project was to characterize the major sources of fecal streptococci contamination in Tillamook Bay watershed by statistically analyzing antibiotic resistance patterns. The first step in the implementation of this technique is to identify the major potential fecal pollution contributors in the area of interest. In the Tillamook Bay watershed the primary sources were dairy cattle and humans. It was also deemed necessary to account for the potential contributions of wild animals (elk, beaver, birds, etc.) to establish the 'background' of fecal pollution in the watershed. Wild animal samples were analyzed as a potential 3rd source of fecal pollution.

The next step was to establish the antibiotic profile or 'fingerprint' for each of these fecal sources (human, dairy, wild) to be used in comparison to the unknown river samples. Samples were collected from wastewater treatment plants in Tillamook and Garibaldi, Oregon and dairy cattle at several dairies in the Tillamook watershed. 'Wild' fecal samples were collected from water in the forest-agriculture interface where upstream influences of both dairy cattle and human contamination were not expected to be present.

Source samples were processed by isolating and antibiotically screening fecal streptococci bacteria. Antibiotic 'screening' is a process that records the ability of isolated bacteria to grow on a culture inoculated with selected antibiotics. A 'profile' of each isolates response to the battery of antibiotics is established. The resistance profiles produced by each source group of isolates (wild, human, dairy) creates the group profiles or 'sources' needed for comparison with unknown samples.

Once the profiles or 'fingerprints' for the each source (wild, human, dairy) were established, river water samples collected during a winter storm event, as well as seasonal water samples for five rivers, over a one-year were screened. These samples contained fecal organisms of unknown origin. They were antibiotically screened in the same manner as the major source groups from which the group profiles were created.

The antibiotic discriminant analysis (ADA) consists of two distinctive yet consecutive processes. The first of these is conducted primarily in the laboratory and involves the identification, isolation and screening of antibiotics for the fecal streptococci bacteria from known and unknown sources. The second process is a statistical analysis of the data provided from the laboratory and an interpretation of the results. The specific steps and issues encountered for each of these two processes are discussed below.

Sample Collection

Source Sample Collection

Fecal samples used to establish the antibiotic resistant profiles for human, dairy, and wild sources were all collected from the Tillamook Bay watershed. Over a 3-month period, six samples were collected from the Garibaldi and Tillamook wastewater treatment plants (WWT). Samples of wastewater influent were collected in autoclave sterilized 500 ml Nalgene ™ bottles prior to primary treatment. These six samples produced a total of 309 isolates for classification

The 'dairy' fecal antibiotic resistance profile was established from eight samples collected over a 3-month period from three dairy farms in the Tillamook watershed. Sterile plastic bags were used to collect samples of dairy feces from several cows at each of the farms. These eight samples produced a total of 260 isolates for classification.

The 'wild' source antibiotic resistance profile was established by sampling the Trask and Tillamook rivers water at the transition zone between the agriculture and forested lands. On the Trask, the samples were collected just upstream from the Oregon Department of Fish and Wildlife's (ODFW) Trask River Fish Hatchery. On the Tillamook, samples were collected from the forest-agriculture interface at Beaver Creek, which is a tributary to the Tillamook River. A total of five wild samples were collected producing a total of 261 isolates for classification. The assumption was made that due to the low to non-existent human and dairy populations upstream of this point, samples would profile the 'wild' upper watershed animal populations. All 'wild' samples were collected in autoclaved sterilized 500 ml Nalgene ™ bottles. All samples were placed in a cooler (2-6 ^{0} C) immediately upon their arrival at the Water Quality Laboratory (WQL) at Oregon State University (OSU) and processed within 6 hours of collection.

Winter Storm Sample Collection

E&S Environmental Chemistry (E&S) collected the river water samples used for the winter storm study. Samples were collected by employees of E&S (Sullivan et. al., 1998) and analyzed for a number of water quality parameters including fecal coliform bacteria (FCB), total suspended solids (TSS), nutrients, and conductivity. For purposes of this study only the E&S FCB was used.

The staff from the Tillamook Bay National Estuary Project and E&S Environmental Chemistry determined sample site location as part of a larger water quality monitoring strategy. Some of the criteria used in site selection were; suspected or known point or non-point sources of fecal pollution, forest-agriculture interface locations, and logistical considerations such as bridge crossings. Sample sites were selected to avoid any strong influence by Tillamook Bay tides and to quantify tidal influence. On-site conductivity measurements were taken to ensure that bay water contamination was minimized (Sullivan, 1998).

The majority of the river water samples collected were from sites located on the Trask and Tillamook Rivers. These rivers were chosen due to previously recorded high levels of fecal contamination during peak flow winter storm events (Sullivan et. al., 1998). A total of four sites were chosen on the Tillamook River and 3 sites on the Trask River. One additional site was sampled on the Memaloose Slough. During the course of a five-day storm in 1998, a total of eight river water samples were collected at each location. Due to laboratory capacity limitations only two of the eight samples were used as part of this antibiotic resistance project.

Site characteristics determined sampling protocol at each of the river sites. Generally, sampling at bridge crossings was achieved by using a Van Dorn sampler or a weighted sterile bottle. Water was collected close to the middle of the stream current (at a depth of about 0.5 m) where rivers tend to be well mixed. Shallower sites or those without bridge crossings were sampled from shore using a pole to submerge a Nalgene bottle directly in the steam. Bottles were filled to minimize air bubbles and then placed in coolers on ice and transported to the lab (Sullivan, 1998). A few of the non-bridge location samples were collected from a boat using a weighted sterile bottle.

All river water samples were collected in autoclaved sterilized, 3.5 liter, screw top, Nalgene™ bottles. The bottles were sterilized using an autoclave for 20 minutes at 121 ^{0} C. Fecal coliform bacteria (FCB) analysis was performed by the Kilchis Analytical Laboratory in Bay City, Oregon. All antibiotics screening analysis was performed at WQL at OSU. Samples were immediately placed in a cooler maintained between 2-6 ^{0} C until the time of processing. All samples were processed within 6 hours of their arrival.

Sample Collection for River Mouth Study

River water samples were also collected from sites near the mouths of the Miami, Kilchis, Wilson, Trask and Tillamook Rivers. Samples were collected nine times from December 1997 through December 1998. Sampling locations were close to each river's confluence with the Tillamook Bay, as well as easily accessible from a roadway. River water was sampled from the riverbank in each location using an autoclaved 3.5-liter,

screw top Nalgene™ bottle. Upon the arrival of the samples at the WQL they were immediately placed in a cooler and maintained at 2-6 ° C until the time of processing. The processing of all river samples was begun within 6 hours of their arrival at the WQL.

Sample Filtration

Dairy Sources

Varying amounts of dairy feces (0.1 - 2.0 g) were suspended in 50 ml of saline (NaCl, 0.3 g of KH2PO4 and 0.6 g Na2HPO4 per liter [pH 7.3]) and filtered using 0.45-*u*m-pore-size filters (type GN-6; Gelman Sciences™). Filters were then transferred to 50-mm petri dishes containing absorbent pads soaked in 1.95 ml of the selective liquid media Enterococcosel broth (BBL™). Filters were then incubated for 48 hours at 37°C. Millipore ™ filtering equipment used in these procedures was autoclaved for at least 20 minutes at 121 °C prior to use.

Human Sources

Varying volumes of wastewater (0.1 - 20 ml) were filtered using 0.45-*u*m-pore-size filters (type GN-6; Gelman Sciences™). Filters were processed in the same manner as described for the dairy sources.

Wild Source and Unknown River Sources

Varying volumes of individual river samples were filtered using 0.45-*u*m-pore-size filters (type GN-6; Gelman Sciences™). Pre-filtering of the river samples as described in Wiggins[b] (1997) was not performed due to the relatively low turbidity of the samples. A majority of the samples (≤ 1000 ml) were filtered without excessive clogging of equipment. Filters were processed in the same manner as described for the dairy sources.

Isolate Identification

Following incubation of the plates, fecal streptococci isolates were selected with sterilized toothpicks and transferred to microwell plates with each well containing 0.2 ml of Enterococcosel broth (BBL™). Fecal streptococci isolates were selected from the filter plates by using the colony morphology as a selection criteria (Wiggins[b], 1996). Following selection, isolate-containing microwell plates were incubated in 37° C for at least 48 hours.

To confirm the identification of the isolated colonies, 180 isolates were randomly selected for further characterization. Physiological properties that distinguish them as a member in the fecal streptococci family were used including production of catalase, gram reaction, growth at 37° C in brain heart infusion broth (BBL™) containing 6.5% NaCl, and growth in brain heart infusion broth at 45° C.

Antibiotic Screening of Isolates

Nine discriminant antibiotics were selected because of their wide clinical use for bacterial infection control in animal and human populations. The nine antibiotics used to screen were Ampicillin (AMP), amoxicillin (AMX), cephaolahexin (CEP), chlorotetacycline hydrochloride (CTC), erythromycin (ERY), oxytetracycline hydrochloride (OTC), streptomycin sulfate (STR), tetracycline (TET,) and vancomycin (VAN). Stock solutions (10 mg/ml) were prepared and stored with the exception of OTC. OTC was found to precipitate out of solution when stored between screening sessions as a 10-mg/ml solution. Therefore OTC was newly prepared for each screening session (Wiggins[b], 1996).

Each antibiotic solution was filter sterilized and added to trypticase soy agar (BBL™). Agar containing incremental concentrations of each antibiotic was poured into 150-mm plastic petri dishes and allowed to gel. For the antibiotics AMP, ERY, CEP and TET, concentrations of 10, 15, 30, and 50 *u*g/ml were used. CTC, OTC, and STR were dispensed in 20, 40, 60, and 80 *u*g/ml concentrations while CTC, OTC and STR in 5 *u*g/ml increments of 5, 10, 15 and 20 *u*g/ml. Antibiotics AMX and VAN were dispensed in 5, 10, 15, and 30 *u*g/ml concentrations. These concentrations were identical to those used by Wiggins[b] (1996). The concentration range for each of the antibiotics were deemed appropriate if isolates from one source group were resistant at the strongest concentration while isolates from another source were sensitive (Wiggins[b], 1996).

Isolates were transferred to the antibiotic plates (4 concentrations per antibiotic x 9 antibiotics = 36 total plates) from the Enterococcosel broth microwell using a 96-prong replica-plater. An additional plate without antibiotic agar was inoculated and kept as a control for each sample set. All plates were then incubated in 37^0 C for at least 24 hours.

Following incubation, individual isolates were scored on growth/no growth responses to each antibiotic concentration. Isolates were scored by the highest concentration on which growth was recorded. When isolates failed to show growth on the control plates they were removed for the remainder of the analysis. 'Growth' was defined by the presence of a colony-forming unit evident to the naked eye.

Statistical Processes: Discriminant Function Analysis

The discriminant functional analysis procedure computes various discriminant functions for classifying observations into two or more groups on the basis of one or more quantitative variables. It can be simply thought as a procedure for identifying boundaries between groups, the boundaries being defined in terms of those variable characteristics which distinguish or discriminate the objects into the respective criterion groups (Tabachnick, 1983). For the purposes of our study the 'criterion groups' are our fecal sources (dairy, human, and wild) and the 'predictor variables' are the growth/no growth responses of the fecal streptococci bacteria isolates to the antibiotics (36 variables per isolate).

Discriminant function analysis was executed using the program DISCRIM[2] in the statistical computer program SAS (Windows Version 6.12: SAS Institute Inc.™). The DISCRIM procedure develops a discriminant criterion to classify each observation into one of the groups (dairy, human, wild sources). The data set that DISCRIM uses to derive the discriminant criterion is called the *calibration data set*. This calibration data set is used to discriminate the test data sets (unknown samples) into each of the criterion groups (dairy, human, wild).

The distribution for each source in the calibration data set (dairy, human, wild) was assumed to be multivariate normal which allows a parametric method to be used to develop the discriminant function. In a parametric method, the discriminant function is determined by a measure of generalized square distance of the predictor values. When the covariance matrix is pooled in a parametric method the discriminant function is linear. Finally, when the sources isolates (dairy, human, wild) are approximately the same, the prior probabilities are set as equal (**SAS**, 1987).

Discriminant function analysis can be evaluated for its performance in the classification of future observations. This is conducted by estimating a weighted average of the individual group-specific (dairy, human, wild) error-rate estimates, where the prior probabilities are used as the weights. The inverse of this weighted average error-rate estimate is the average rate of correct classification (ARCC) (**SAS, 1987**).

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[2]Specific settings: prior probabilities equal; covariance matrix pooled

The DISCRIM procedure produces a classification table, which calculates the percentages of misclassified isolates and determines the average rate of correct classification (ARCC). The table is a source-by-source matrix in which the numbers and percentages of correctly classified isolates are found on the diagonal axis. (**Wiggins, 1996**).

The DISCRIM analysis of individual isolates from each river sample is dependent on the source classification pattern to which they are compared. It was assumed that the majority of point and non-point sources of fecal pollution was from dairy cattle and humans and thus, deemed the primary fecal sources for this study.

General Storm Sampling Information

Precipitation (inches) and river flow data (cubic feet per second) was collected from February 28th - March 5th, 1998. The flow data was collected by the Oregon Water Resources Department (OWRD) using a monthly field logger that measures flow in 30-minute intervals. There were no major gaps in the data during the course of this storm (**OWRD, 1999**). The gage (OWRD # 14302480) is located above the confluence of Cedar Creek and the Trask River near the town of Tillamook, Oregon. Tillamook Bay precipitation data were provided by the Oregon Climate Service (OCS) from a gauge, OCS Station # 358494, located near the town of Tillamook (**OCS, 1999**).

General River Mouth Sampling Information

To understand the seasonal changes in fecal sources in the Tillamook Bay, samples were analyzed over a one-year time period. Samples were collected near the mouths of the Miami, Kilchis, Wilson, Trask and Tillamook Rivers at approximately six week intervals from December, 1997 through December, 1998. Samples were processed with the Antibiotic Discriminant Analysis (ADA) to record the changes in fecal source distributions on a seasonal basis. Estimates of magnitude of the source distributions were conducted using the fecal streptococci bacteria (FSB) filtered in the first step of the isolation process. These estimates were used to understand the magnitude of each distribution sample. Water samples were processed according to ADA protocol outlined in the materials and methods section of this document.

The Oregon Department of Environmental Quality (DEQ) has listed four of these five Tillamook watershed rivers, with the exception of the Trask, as being water quality limited for fecal bacterial contamination. The specific listings vary from river to river, however the Miami, Kilchis, Wilson and Tillamook have all shown a chronic fecal contamination problem (E&S, 1998).

Results and Discussion

Source Pattern Development

The nine antibiotics: Ampicillin (AMP), amoxicillian (AMX), cephaolahexin (CEP), chlorotetacycline hydrochloride (CTC;), erythromycin (ERY), oxytetracycline hydrochloride (OTC;), streptomycin sulfate (STR;), tetracycline (TET,) and vancomycin (VAN), were used to establish the antibiotic resistant patterns for all fecal pollution sources, i.e. dairy cow, human and wild. Isolates were measured by their growth on varied concentrations of each antibiotic. Responses were scored on a growth/no growth system that had five possible responses:

1. 'Conc 0' = no growth on lowest concentration plate,
2. 'Conc 1' = growth on the lowest concentration,
3. 'Conc 2' = growth on the first and second concentrations,
4. 'Conc 3' = growth on the first three concentrations,
5. 'Conc 4' = growth at all concentrations or 'resistant.'

The results show that sources were distinguishable by these antibiotic profiles growth and varied by group (**Table 1**).

Table 1. Source Isolates Antibiotic Resistance Responses

Sources	Source Isolates Growth Responses to Mulitiple Concentrations Antibiotics								
Dairy	AMP	AMX	CEP	CTC	ERY	OTC	STR	TET	VAN
% Growth (CONC 0)	99	88	37	53	90	60	18	73	95
% Growth (CONC 1)	0	0	3	16	1	3	27	1	2
% Growth (CONC 2)	0	6	4	7	1	2	22	2	0
% Growth (CONC 3)	1	5	3	3	0	1	18	2	0
% Growth (CONC4)	0	1	52	21	8	33	15	22	3
Total	100	100	100	100	100	100	100	100	100
Human	AMP	AMX	CEP	CTC	ERY	OTC	STR	TET	VAN
% Growth (CONC 0)	88	74	5	26	58	34	6	31	96
% Growth (CONC 1)	3	5	4	5	4	6	13	2	0
% Growth (CONC 2)	3	6	5	6	5	3	17	6	0
% Growth (CONC 3)	0	2	19	4	3	8	11	3	0
% Growth (CONC4)	6	13	68	60	30	49	53	58	4
Total	100	100	100	100	100	100	100	100	100
Wild	AMP	AMX	CEP	CTC	ERY	OTC	STR	TET	VAN
% Growth (CONC 0)	69	95	10	62	82	9	11	84	26
% Growth (CONC 1)	14	5	3	18	3	24	51	3	19
% Growth (CONC 2)	10	0	6	5	6	18	7	0	16
% Growth (CONC 3)	7	0	20	3	0	18	8	2	15
% Growth (CONC4)	0	0	61	12	8	31	23	11	24
Total	100	100	100	100	100	100	100	100	100

The 'dairy' profile was established by collecting eight samples, which produced 260 fecal streptococci isolates for classification. 'Dairy' isolates appeared weakly resistant to the antibiotics: AMP, AMX, ERY, and VAN (**Table 1**). However, 'dairy' isolates showed a varied resistant to the antibiotics CEP, CTC, OTC and STR. 'Dairy' isolates were distinguished from the other two source groups by exhibiting the least overall resistance to the nine antibiotics.

The 'dairy' antibiotic profile established for this study is consistent with other published findings. Hagedorn found that 'dairy' isolates sampled from dairy farms in Virginia, had low levels of resistance to the antibiotic ERY (**Hagedorn, 1999**). This is the case for the Tillmook 'dairy' isolates findings as well, in which over 90% showed no growth at the lowest concentration of ERY (**Figure 1**).

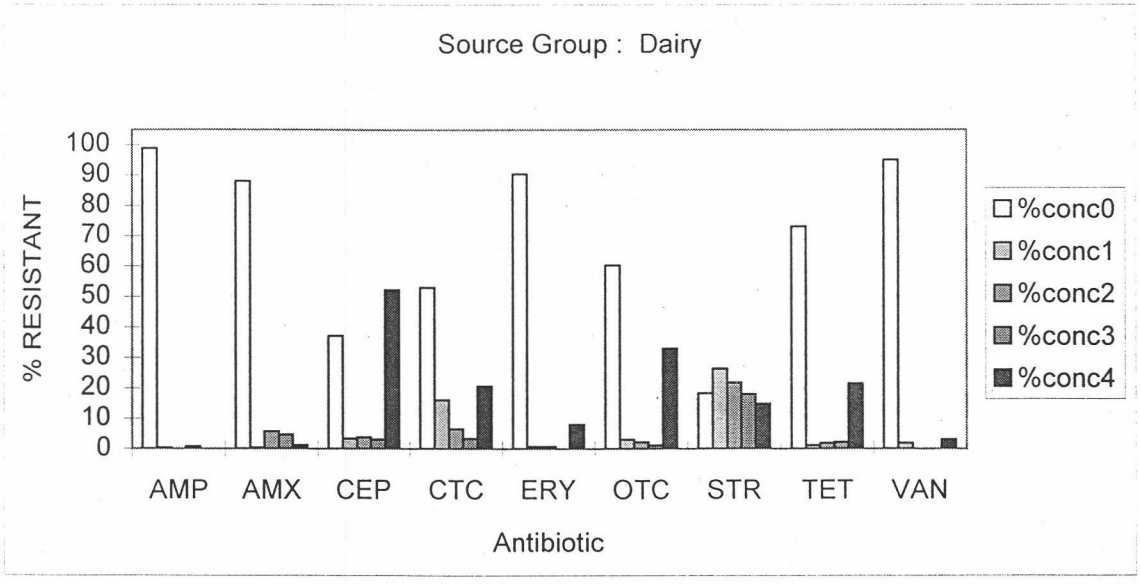

Figure 1. Dairy Source Isolates Antibiotic Resistance Responses

The 'human' profile was established from wastewater samples collected at municipal treatment facilities. Six samples were collected prior to primary treatment and provided 309 'human' isolates. The 'human' profile demonstrated a strong resistance to antibiotics CEP, CTC, OTC, STR and TET (**Figure 2**). 'Human' isolates showed weaker resistance to antibiotics: AMP, AMX, ERY and VAN. Of the three source groups, 'human' showed the strongest resistance to the antibiotics: CTC, STR, and TET. This is similar to findings reported by Wiggins (**1996**) in which 'human' isolates collected in Virginia showed strong levels of resistance to the antibiotics CTC and OTC.

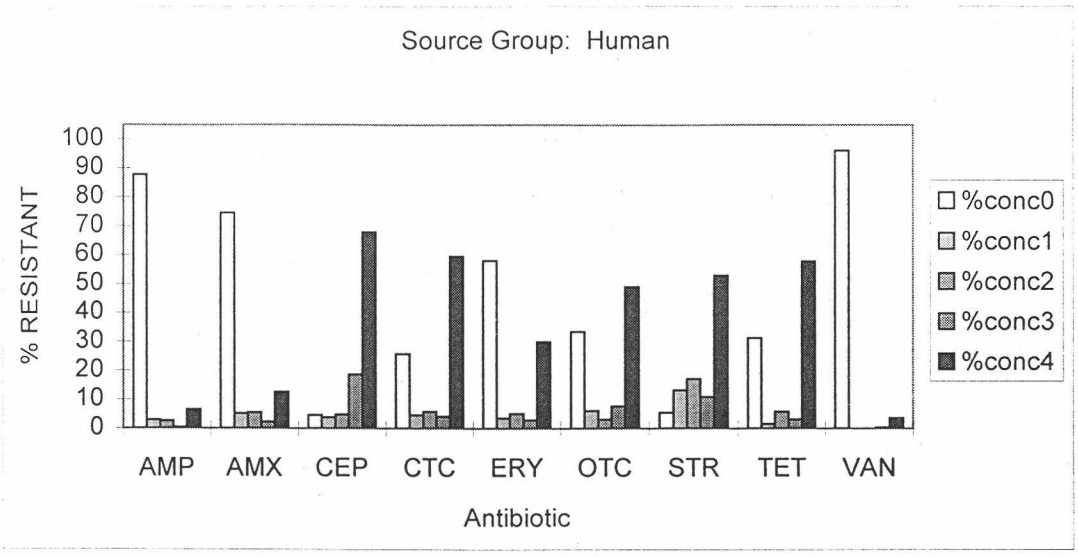

Figure 2. Human Source Isolates Antibiotic Resistance Responses

The 'wild' profile was established by collecting five river samples at the forest-agriculture interface on the Trask and Tillamook Rivers. These samples yielded a total of 261 'wild' isolates for profiling. These isolates showed weak overall resistance to antibiotics AMP, AMX, CTC and TET while showing a stronger response to CEP, OTC, STR, and VAN (**Figure 3**).

The 'wild' isolates were the only source that showed a substantial resistance to VAN. This response would be consistent with a finding published by Sternes (**1999**) in which resident populations of enterococcus bacteria sampled along the Rio Grande river were found to be resistant to the antibiotic VAN. Sterne showed that up to 30% of the bacteria displayed VAN resistance. This finding is of particular interest because VAN is generally reserved for use in humans as a last line of defense against bacteria that have become resistant to penicillin-type antibiotics. This supports the hypothesis that 'wild' bacteria can also be resistant to controlled use antibiotics.

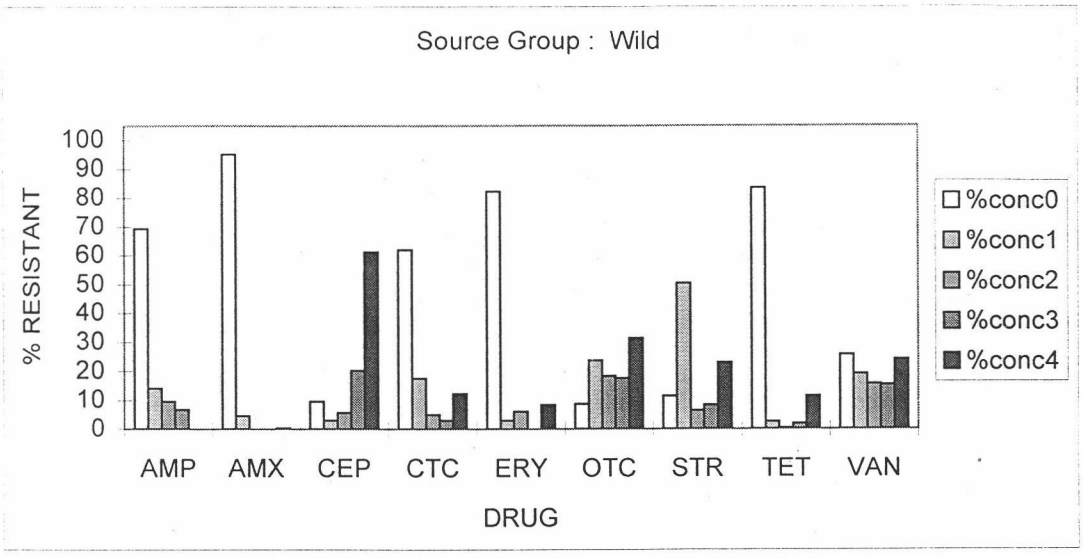

Figure 3. Wild Source Isolates Antibiotic Resistance Responses

The similarities between our source antibiotic profiles and those of other studies that used this technique suggest that antibiotic resistant responses to specific antibiotics may be similar across geographical regions. Additional comparisons between studies should be performed in the future to confirm this observation.

Average Rate of Correct Classification (Error Rate)

Discriminant statistical analysis averages the individual correct classification (CC) rates for each source (wild, human, dairy), which produces a value called the average rate of correct classification (ARCC), (Tabachnick, 1983). Discriminant statistical analysis conducted on the 830 source isolates collected from the three known sources (wild, human, dairy) revealed an average rate of correct classification (ARCC) of approximately 83.3% (Table 2).

The individual correct classification rates varied according to source group. The 'human' C.C. of 73% was the lowest of the three groups (Table 2). 'Wild' and 'dairy' sources were classified with C.C. rates of 88% and 89% respectively.

The results for 'human' CC rates is consistent with results from previous studies wherein the correct classification rates improved (from 73% to 92%) when samples were collected from raw residential septage rather than from WWT treatment facilities (Wiggins[a], 1996). It is hypothesized that municipal WWT effluent may be more prone to cross inoculation from other municipal sources of feces (e.g. cats, dogs, etc.) and from overland flow (Wiggins[d], 1996).

Misclassifications were most common between 'human' and 'dairy' sources. 'Human' isolates were misclassified as dairy 26% of the time, while 9 % of 'dairy' were misclassified 'human'. Wild isolates were predominantly misclassified (11%) as 'dairy' sources.

Table 2. Rates of Correct Classification for Sources Groups

Sources	Number of Isolates	Number Correctly Classified	Rate of Correct Classification (%)	Dominant Misclassification Source (%)
Human	309	225	73	Dairy (26)
Dairy	260	231	89	Human (9)
Wild	261	230	88	Dairy (11)
Totals	830	686		

Characterization of Fecal Streptococci Isolates

A total of 180 isolates were selected to carry out additional morphological tests to confirm that the bacteria selected for the screening processes were in the fecal streptococci bacterial family. All control isolates were screened for the ability to hydrolyze esculin, the production of catalase, gram positive cocci, growth at 37°C in brain heart infusion broth (BBL) containing 6.5% NaCl, and growth on bile esculin azide agar.

Table 3. Enterococcus Identification Tests

Isolate Characterization Results				
Source (Isolates Screened)	Catalase Negative	Gram Positive	Growth on Esculin	Growth on Bile Azide
Dairy (20)	95%	100%	95%	100%
Human (20)	100%	100%	100%	100%
Wild (20)	85%	100%	100%	90%
River (120)	84%	98%	92%	87%

'Dairy' and 'human' isolates generally had higher percentages of both catalase negative and esculin-positive responses than either of the river groups (Table 3). A majority of all the isolates screened were esculin-positive, catalase-negative, and gram positive, grew in brain/heart infusion broth at 37°C and grew on bile esculin azide agar. These results indicate that a majority of the bacteria isolated for antibiotic screening were correctly identified as fecal streptococci.

Storm Study Results

Environmental Data

Precipitation (inches) and river flow data (cubic feet per second) were collected from February 28th - March 5th, 1998 to be used for this study. **Figure 4** shows the precipitation and Trask River flow recorded during the late winter storm event. The predicted increase in river flow following a substantial increase in precipitation is shown on the hydrograph (**Figure 4**). A similar relationship is assumed to have occurred in the other rivers and sloughs sampled during this storm study.

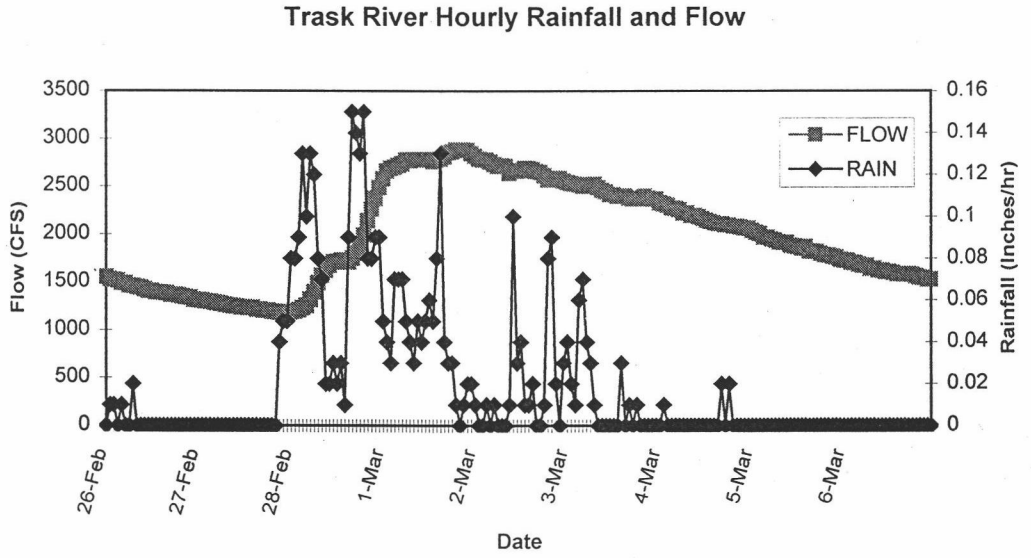

Figure 4. River Flow and Rainfall for Winter Storm

The storm period was preceded by a four-day no-rainfall period, which was attributed with decreasing the river flow (E&S, 1998). The storm was considered "moderate in size" but was atypical in that river discharge increased to relatively high

values during the first two days of the storm, and then remained relatively constant for a two to three day period (E&S, 1998). The cumulative rainfall collected during the storm was 4.3 inches.

E&S determined fecal coliform bacteria (FCB) concentrations from samples collected in conjunction with those samples analyzed for antibiotic resistance screening. Results indicated that increases in FCB concentrations generally coincided with the increase shown in river flow (**Figure 5**). Average peak FCB concentrations were 9 x 10^2 cfu/100 ml and 4 x 10^2 cfu/100 ml in the Trask and Tillamook rivers respectively. FCB loads in the Trask River were relatively high reaching levels of 1 x 10^6 cfu/100 ml, with several measurements during the storm in the range of 0.3 to 0.6 x 10^6 cfu/sec. Peak loads in the Tillamook River were considerably lower due to the river's flow being an order of magnitude lower than that of the Trask River (0.1 x 10^6 cfu/sec), (E&S, 1998).

Fecal Bacteria and Storm Dynamics

The times at which water samples were collected for the storm study are plotted against the Tillamook River's flow in **Figure 5.** Observe the relationship between the increase in flow with the consequent increases in FCB concentrations. It is assumed that this response of increased flow with increases in rainfall occurred in all of the rivers surveyed during the period of this storm study.

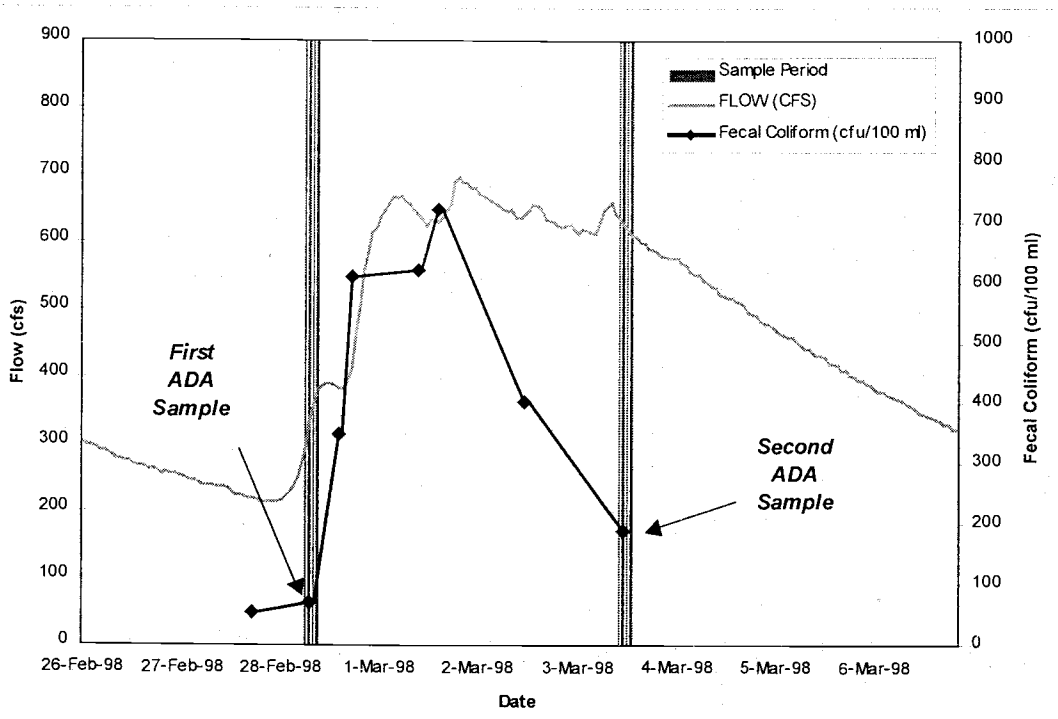

Figure 5. Trask River Hydrograph, FCB Concentrations

The first and second sample sets processed with the ADA technique were collected during the periods marked 'samplings' in Figure 5. A majority of the first and second ADA samples used in this study were collected during the rising and falling limbs of both the storm hydrograph and each sample site's FCB concentration curve. The FCB concentration values for eight samples collected nearest[3] the river flow gage on the Tillamook River are also plotted in Figure 5.

The relationship between increases in flow and increases in FCB concentrations can be typical of a watershed with a potential non-source pollution problem. The hypothetical scenario being that a large storm saturates the pastures and fields with rainfall. When the amount of rainfall exceeds the soil infiltration rate the excess begins

to travel down-slope across the pastures or 'overland' carrying bacteria and other particulates with it. The large amounts of feces now introduced to the system shows as an episodic peak in FCB concentrations. Specifically, identifying the source of these FCB peaks is critical to developing an effective water quality monitoring and restoration plan. This relationship between increases in FCB concentrations and increases in river flow was shown to occur at nearly all sites sampled during this storm study (E&S, et. al., 1998).

Storm Sample Locations

River samples processed with the ADA technique were collected twice during the storm in seven predetermined sites along the Trask and Tillamook Rivers with an additional site on the Memaloose Slough (**Figure 6**). **Table 4** shows the storm sample site codes, description and river mile. Trask River samples were collected in three locations; near a rural residential trailer park (TRA BTR2), at the 5th street boat ramp located upstream from the Tillamook WWT facility (TRA HOB 2). Tillamook River samples were collected at the following sites: Highway 101 Rest Stop (TIL RES), near Tillamook River Road (TIL TTR), near the Burton Road Bridge (TIL BUR) and near the Netarts Highway Bridge (TIL NET). Note that storm locations were select as a part of a much larger water quality monitoring plan and that these specific sites have no particular significance in relation to each other.

[4] FCB concentration values from samples collected at Netarts Bridge on the Tillamook River.

Table 4. Storm Sample Sites for Winter Storm Sampling

TBNEP Site Code	Description	River Mile
Tillamook River		
TIL-RES	Rest Area	8.1
TIL-TTR	Tillamook River Road	4.9
TIL-BUR	Burton Bridge	4.0
TIL-NET	Netarts Highway Bridge	0.9
Trask River		
TRA-BTR	Below Trailer Park	3.7
TRA-5th	5th Street Boat Ramp (Above WWT)	1.5
TRA-HOB	Hospital Bridge (Below WWT)	1.2
Tillamook Bay		
MEM-INL	Memaloose Boat Landing	-0.5

Distribution of Sources

Trask River Samples

The ADA technique sorts the unknown river water samples into each of the source groups (wild, human, dairy) as a percentage value. **Table 5** shows the distribution of sources by percentages for each sampling site on the Trask River. Note that the Average Rate of Correct Classification (ARCC) was established at 83% for the sources.

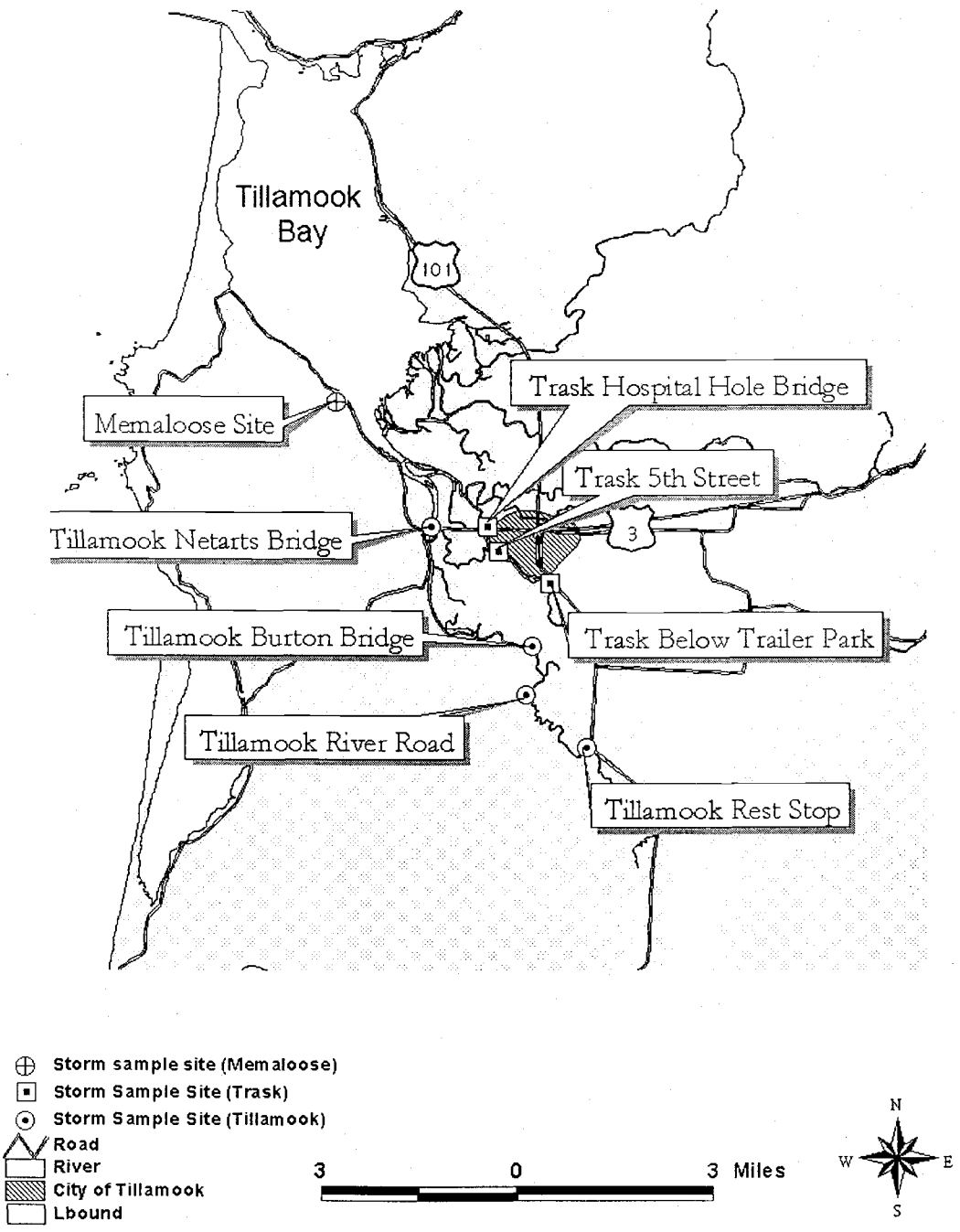

Figure 6. Map of Tillamook Bay Storm Sampling Locations

Table 5. Source Distribution for Trask River Sites

Sample Date	TBNEP ID Codes	River Mile	Dairy	Human	Wild	Isolates Screened
2/28/99	TRA BTR	3.7	69%	20%	11%	65
"	TRA 5th	1.5	73%	23%	4%	70
"	TRA HOB	1.2	63%	25%	12%	16
3/3/99	TRA BTR	3.7	22%	72%	6%	89
"	TRA 5th	1.5	37%	59%	4%	79
"	TRA HOB	1.2	62%	36%	2%	94

The number of isolates that were successfully isolated varied by sample location (**Table 5**). This was due to the inexperience of the processing staff and is not a reflection of problems with the ADA protocol. These storm event samples were the first 'unknown' samples processed by the WQL staff and problems with isolate selection and media plating were experienced during these initial samples. These problems were corrected and during the second storm sampling and isolate numbers were generally high for the remainder of the project.

The first Trask River sampling (2/28/98) shows a majority of isolates coming from 'dairy' sources (**Table 5**). 'Human' sources appear to be similar in all three-river locations with no notable change in distribution in relation to location. The percentage of 'wild' sources is the highest at both the TRA BTR and TRA HOB sites with distributions of 11% and 12% respectively.

The second set of samples (3/3/98) collected 4 days after the first set, show a complete reversal in fecal source distribution at both the TRA BTR and TRA 5th locations. 'Dairy' dominated distributions in the first sampling are replaced by 'human' sources. The TRA HOB location however, does show a consistent 'dairy' majority distribution. The number of 'wild' sources drops from the first to second sampling.

When we look at the different locations on the Trask River, there appears to be some differences in the source distributions based on when the samples were collected during the storm event. At the TRA BTR location the results show that the majority source group can changes significantly from the first to second sample **(Figure 7).** From the first sample (2/28/98) we see a majority of the isolates profiled as coming from 'dairy' sources. However, four days later the source distribution is reversed to a 'human' source majority (72%). These results indicate that the percentage of sources can change dramatically from sample to sample.

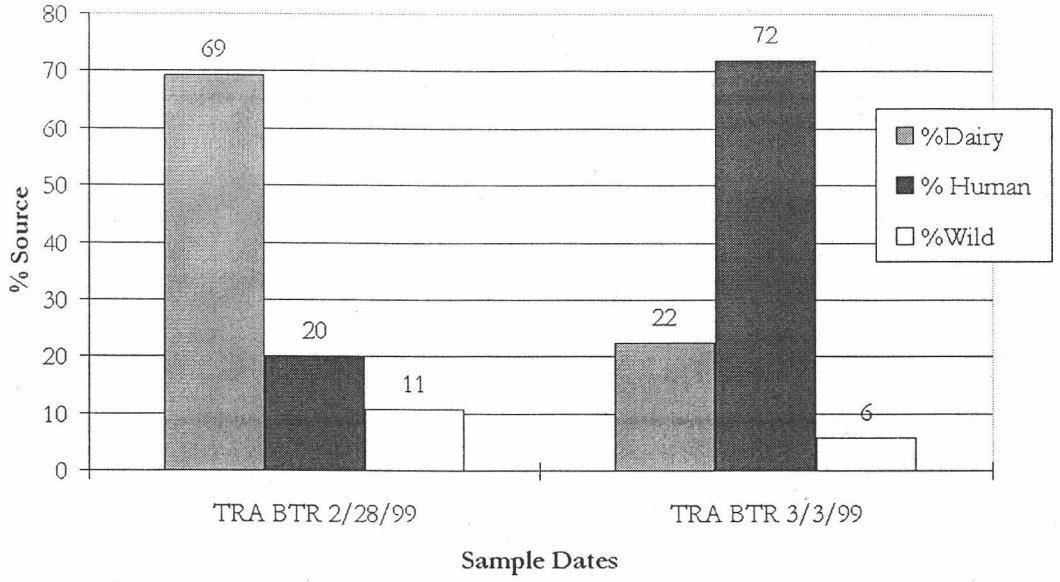

Figure 7. Source Distribution for TRA BTR Site

Tillamook River Samples

The ADA technique was used to process the samples collected on the Tillamook River (**Table 6**). It should be reiterated that the ARCC value was established at 83% for the source data used for this study.

Table 6. Source Distribution Data or Tillamook River Sample Sites

Sampling Date	Location	TBNEP Location Codes	River Mile	Dairy	Human	Wild	Isolates Screened
2/28/98	Tillamook River, HWY 101 Rest Stop	TIL RES	8.1	28%	50%	22%	18
2/28/98	Tillamook River, Trailer Park	TIL TTR	4.9	49%	40%	11%	65
2/28/98	Tillamook River, Burton Bridge	TIL BUR	4.0	38%	60%	2%	68
2/28/98	Tillamook River, Netarts Bridge	TIL NET	0.9	33%	67%	0%	30
3/3/98	Tillamook River, HWY 101 Rest Stop	TIL RES	8.1	17%	80%	3%	83
3/3/98	Tillamook River, Trailer Park	TIL TTR	4.9	58%	42%	0%	65
3/3/98	Tillamook River, Burton Bridge	TIL BUR	4.0	47%	52%	2%	64
3/3/98	Tillamook River, Netarts Bridge	TIL NET	0.9	66%	23%	11%	64

Samples processed from Tillamook River showed a complex picture in distribution relationships between sources. The first sampling (2/28/98) tended to show a majority of 'human' sources in three out of the four sample sites. 'Wild' sources contributed a higher percentage of the isolates at the TIL RES and TIL TTR locations. This may be related to the closer proximity of these locations to the agricultural-forest interface, where 'wild' sources were sampled.

Distributions for the 3/3/98 sampling revealed change in the distribution sources in each location. The TIL RES sample showed a strong 'human' isolate distribution along with a lower distribution of 'wild' (from 22% to 3%). The source type did not change in the samples collected at TIL RES, TIL TTR and TIL BUR but the value of

percentages did change between the first and second sampling. Only the TIL NET site showed a complete reversal in the source group changing from 'human' to 'dairy' dominated.

When we look at the data from the sites on Tillamook River we are shown another situation regarding source distributions. At the TIL RES location the results show that the major source (human) did not change from sample to sample **(Figure 8)**. The percentages of contribution did change significantly from 50% to 80% while both 'dairy' and 'wild dropped. These results indicate that sources may in fact remain a dominant in distribution from sample to sample at the same location.

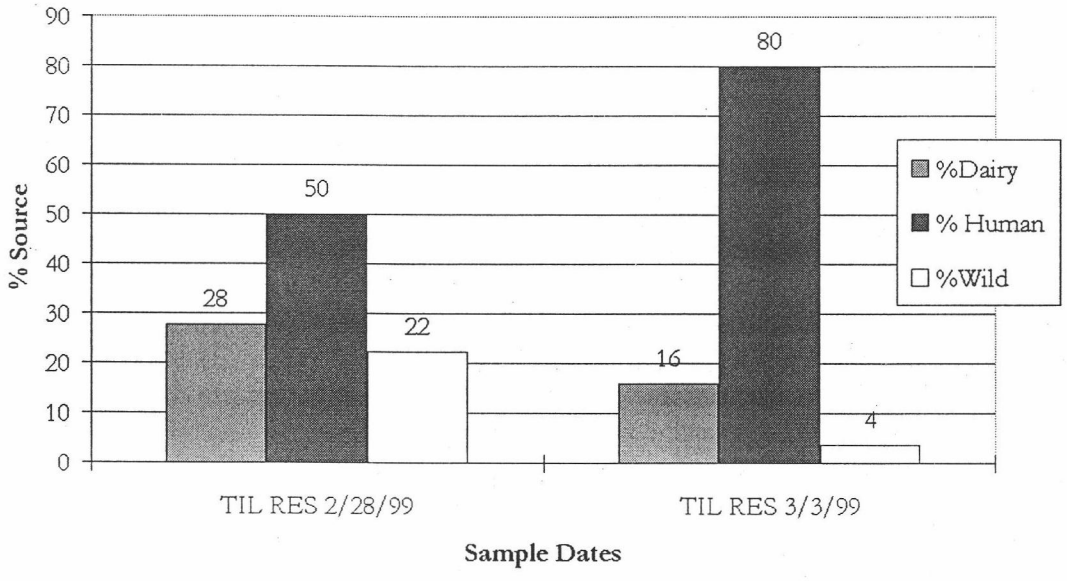

Figure 8. Source Distributions for TIL RES Site

River samples collected near a boat landing on the Memaloose Slough (MEM INL.) showed that source distribution for both sample times showed 'dairy' to consistently have the highest percentage of source distribution **(Table 7)**. Memaloose

slough shows results consistent with those recorded at the river sites in that, both 'dairy' and 'human' sources make up a majority. 'Wild' is consistently shown through out the storm study to play a small role in the overall distribution of sources. This may be partly explained by one of two factors. Firstly, that most of all the storm samples were collected much nearer the bay than the forested uplands. Secondly, that the populations of wildlife are smaller and therefore contributed less feces to the system than the larger 'human' and 'dairy' populations present in the watershed.

Table 7. Distribution for Memaloose Slough Storm Samples

Sampling Date	Location	TBNEP Location Code	River Mile	Dairy	Human	Wild	Isolates Screened
2/28/98	Memaloose Slough, Boat Landing	MEM INL	- 0.5	74%	21%	5%	19
3/3/98	Memaloose Slough, Boat Landing	MEM INL	- 0.5	68%	31%	1%	78

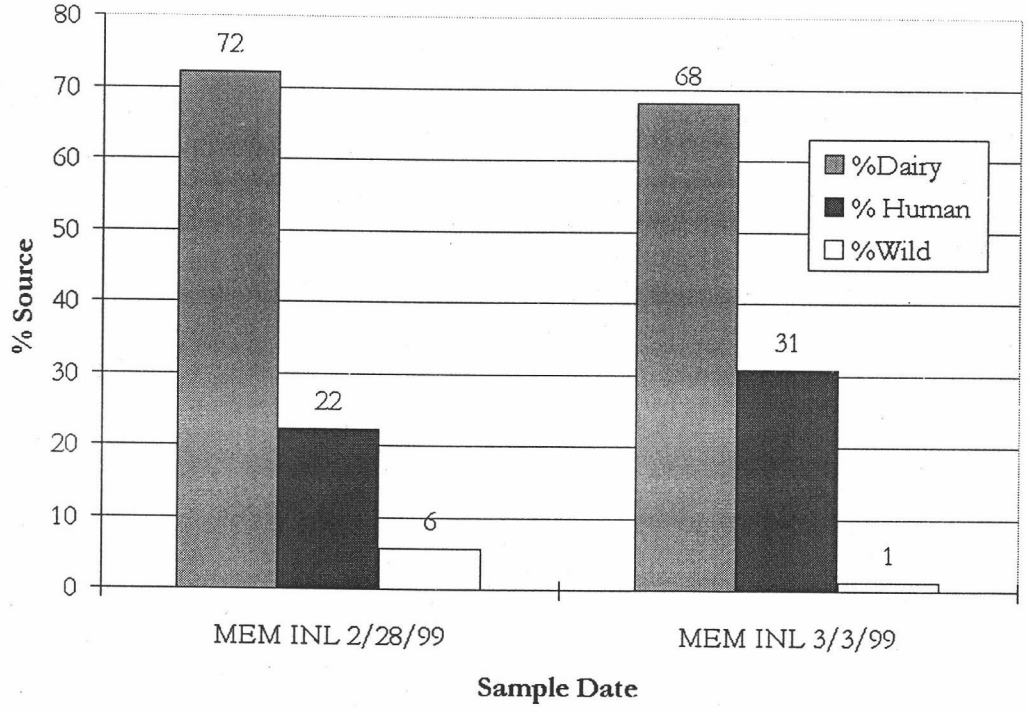

Figure 9. Source Distribution for MEM INL Site

When considering the distribution data for all of the storm locations it is difficult to identify any specific trends on either a site or river basin basis. This unpredictability in source distributions from site to site, sample to sample and watershed to watershed demonstrates the complex nature of developing a comprehensive fecal pollution-monitoring program. Without a clear understanding of what types fecal sources potentially influence a particular sample site, it is difficult, if not impossible, to infer trends or identify specific sources as a potential problem. However, by using a technique such as ADA in conjunction with a technique that quantifies the magnitude of each sample, (e.g. fecal coliform or E. coli testing), we may be able to better understand this pollution problem.

Estimating Magnitudes of Distributed Sources

The source distribution data shown in the last section raises a fundamental question about each unknown distribution sample: "How much is each source contributing?" For example, if a particular sample shows a human distribution majority of 98%, the significance of that information is linked to understanding of amount of fecal bacteria in the sample. If the sample represented a concentration well above water quality standards it would have much more water quality impact than if it consisted of just a few isolates. The ADA technique however reports results purely as distribution values and the statistical output does not give us an estimate of fecal bacteria concentrations.

To address the magnitude of each storm sample, water samples were collected in conjunction with the ADA processed samples to determine fecal coliform bacteria (FCB) concentrations. A total of eight fecal coliform samples were collected at each location in approximately 12-hour increments between February 27th, 1998 to March 3rd, 1998. Of these eight samples, two were collected to coincide with ADA processed samples.

Fecal coliform bacteria (FCB) are an accepted indicator of the presence of fecal pollution in water **(APHA, 1995)**. Concentration standards[5] have been established by Oregon's' Department of Environmental Quality with regards to water contact recreational purposes **(DEQ, 1999)**. Water samples are not to exceed 400 cfu/100ml for a single sample for the recreational uses of water. To utilize FCB concentration data as an estimate of the distribution data provided by the ADA selected fecal streptococci bacteria (FSB), a parallel representative relationship was assumed. This is based on the fact that both FCB and FSB groups are members of the enteric bacterial family[6] and can be considered bacterial indicators of fecal pollution. Prior studies raise questions about the ratio relationship between the groups and the potential differences in die-off rates (Feachem, 1975), **(APHA, 1995)**. For the purposes of this study it was assumed these factors would not effect the relationship shown in (1).

[5] Water Contact Recreation standards are a geometric mean of fecal coliform bacteria of 200 cfu/100 ml in more than 10 % of the samples and a minimum of at least two exceed, 400 cfu/100 ml for the season of interest.

Distribution x Fecal Coliform Bacteria Concentrations (1)

$$D_{d,h,w} \times FCB_{onc} = Q_d$$

$D_{d,h,w}$ = Source Distribution of 'dairy', 'human', 'wild' (%)
FCB_{onc} = Fecal coliform bacteria concentrations (cfu/100 ml)
Q_d = Quantified distributions (cfu/100 ml)

Using Equation 1, the magnitude of each ADA processed storm sample was estimated and results presented in Table 8.

Estimates of Fecal Bacteria Magnitude on the Trask River

The ADA processed samples provided the source distributions for each of the Trask River sample sites. Using the FCB concentrations in conjunction with these distributions the magnitude of each source distribution can be estimated from sample to sample, site to site and river to river. Table 8 shows the FCB concentrations, the source distributions and the product of multiplying these values at each storm sample location. Note that the samples of a water quality priority would be those that exceed the DEQ standard of 400 cfu/100 ml. The data shows a total of 5 samples that exceed the standard, one on the Tillamook River (TIL RES) and 4 on the Trask River (TRA 5th and TRA HOB). The data clearly shows that the 'dairy' or 'human' sources contribute a majority of the FCB isolates in all samples that exceed the water quality

[6] Originating from the intestinal tract of animals and past on through the feces.

Table 8. Source Distribution Quantified with Fecal Coliform Concentrations.

Location	TBNEP Code	Date	Distribution x FCB Concentrations cfu/100 ml (% Distribution)			Total Fecal Coliform (cfu/100 ml)
			Dairy	Human	Wild	
Tillamook River, HWY 101 Rest Stop	TIL RES	2/28/98	200 (28)	360 (50)	160 (22)	720
Tillamook River, HWY 101 Rest Stop	TIL RES	3/3/98	32 (17)	152 (80)	2 (3)	190
Tillamook River, River Road	TIL TTR	2/28/98	118 (49)	96 (40)	26 (11)	240
Tillamook River, River Road	TIL TTR	3/3/98	51 (58)	36 (42)	0 (0)	87
Tillamook River, Burton Bridge	TIL BUR	2/28/98	22 (38)	34 (60)	1 (2)	57
"Tillamook River, Burton Bridge	TIL BUR	3/3/98	70 (47)	77 (51)	3 (2)	150
Tillamook River, Netart HWY Bridge	TIL NET	2/28/98	23 (33)	47 (67)	0 (0)	70
Tillamook River, Netart HWY Bridge	TIL NET	3/3/98	125 (66)	45 (23)	21 (11)	190
Trask River, Trailer Park	TRA BTR	2/28/98	256 (69)	74 (25)	40 (6)	370
Trask River, Trailer Park	TRA BTR	3/3/98	13 (22)	41 (72)	3 (6)	57
Trask River, 5th Street	TRA 5th	2/28/98	525 (73)	165 (23)	31 (4)	720
Trask River, 5th Street	TRA 5th	3/3/98	220 (37)	357 (59)	23 (4)	600
Trask River, Hospital Hole	TRA HOB	2/28/98	484(63)	194 (25)	97 (12)	775
Trask River, Hospital Hole	TRA HOB	3/3/98	253 (62)	148 (36)	9 (2)	410
Memaloose Slough, Boat Landing	MEM INL	2/28/98	67 (74)	19 (21)	5 (5)	90
Memaloose Slough, Boat Landing	MEM INL	3/3/98	102 (68)	46 (31)	2 (1)	150

standard. The data also shows that neither source is solely 'responsible' for all of the FCB isolates at both the samples that exceed the standard as well as those that are not.

The data also shows us that 'wild' sources consistently contributed small amounts of FCB isolates in the storm sample locations **(Table 8)**. The only notable quantity of 'wild' isolates appears at the TIL RES location which may be explained by its proximity (RM 8.1) to the ag-forest interface on the Tillamook River.

The distribution data recorded at the TRA BTR location showed a majority of isolates in the first sample (2/28/98) as 'dairy' (69%) while the second (3/3/98) consists mainly of 'human' sources (72%) **(Table 8).** The concentration quantified source distributions for this location show that the 'dairy' majority first sample is much larger and has a stronger potential to be of water quality concern than that of the smaller second sample. The first sample of 370 FCB isolates is roughly 6 ½ times larger than the second (57 isolates) and is nearing the water quality standard (WQS) of 400 cfu/100 ml.

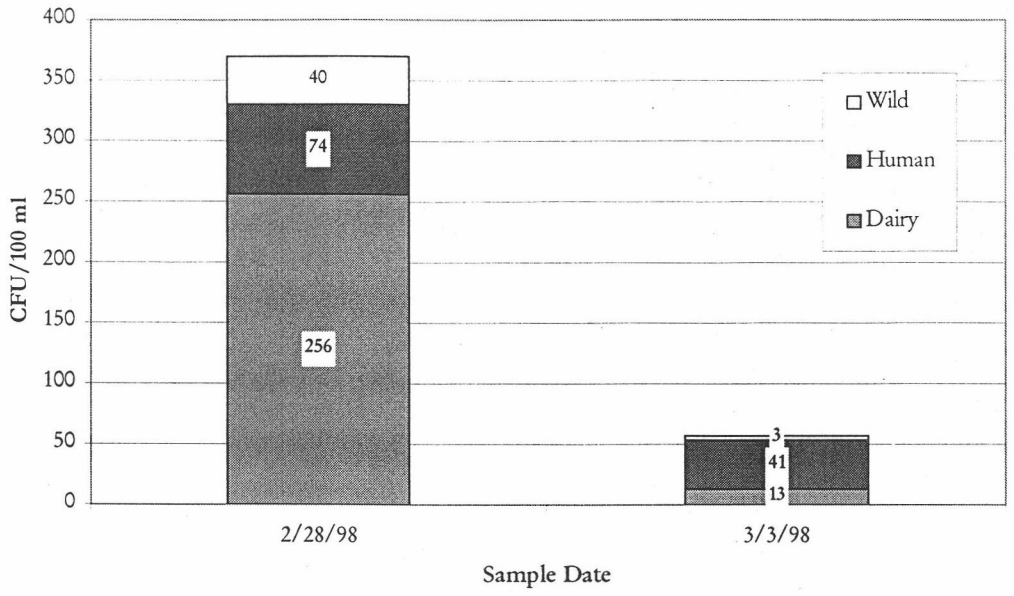

Figure 10. Quantified Distributions at TRA BTR Site

Figure 11 plots the eight FCB samples in conjunction with the two ADA processed samples in relation to the times they were collected during the storm. The quantified samples appeared to have been collected before and after the anticipated peak in FCB concentration.

Observing where the two samples are in relation to this spike, we can see that the first sample was collected during the rising limb of the FCB concentration curve. One possible explanation for 'dairy' sources showing, as a majority during this rising limb might be that they are a nonpoint source. Nonpoint sources can generally be expected to increase with parallel increases in the hydrograph. When rain falls on the ground, the soil can become saturated and the excessive rainfall is carried across the ground, termed 'overland flow'. When this flow passes through pastures with fecal organisms on them, they are washed into the streams and rivers. This causes a spike in the fecal

bacteria concentrations generally seen in conjunction with the initial increases in the hydrograph. Observe that 'wild' isolates, a non-point source, also appear to be high in the first sample corresponding to the increases in 'dairy'.

The second ADA sample appears to have been collected after the storm-driven FCB peak on 3/1/98 and may indicate a return to pre-storm FCB conditions. Observe that 'human' sources are in the majority in this smaller second sample. This would be consistent with the idea that 'human' sources of fecal pollution (e.g. WWT plants) might show up as more consistent FCB concentrations as points sources are generally monitored at the point of discharge.

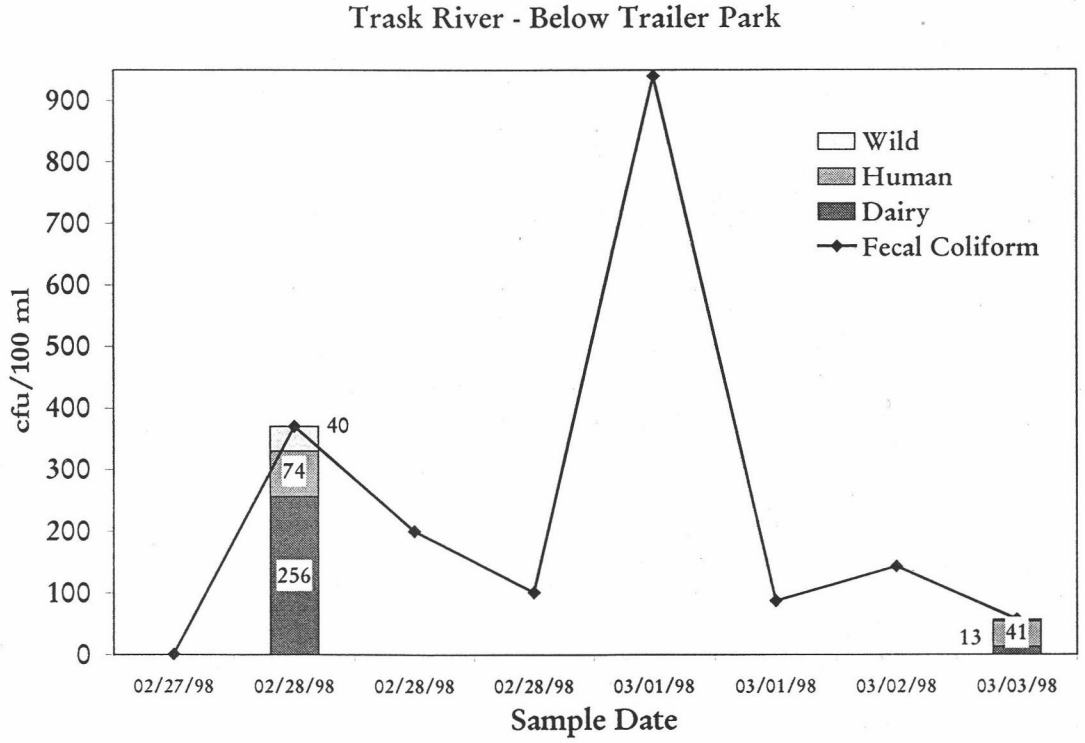

Figure 11. Quantified Distributions and FCB Data at TRA BTR Site

Further downstream on the Trask River, at the TRA 5th site, the concentrations of FCB increase to values above the water quality standard of 400 cfu/100 ml (**Figure 12**). Data from the first sample (2/28/98) shows 720 isolates of which 'dairy' sources make up a majority. Observe that the 525 'dairy' isolates in themselves exceed the 400 cfu/100 ml standard. The ability to distinguish which source contributes FCB concentrations that individually exceed the water quality standards may prove an important use of the ADA-FCB concentration technique.

Data from the second sample (3/3/98) shows 600 FCB isolates were made up of majority of 'human' isolates. Note that while both samples at this location were considered as exceeding the standard, the major source changed between samples from 'dairy' to 'human'. This ability to discern source contribution changes at a particular water quality monitoring location on a sample to sample basis is another promising tool offered by the ADA-FCB concentration technique. At this particular location it implies a complex upstream pollution problem that varies not only by major source but also by the time in the storm that the contributions are made.

It should be reiterated that all source groups aided in the samples exceeding the water quality standard. However, at this sample location 'wild' source contributions again appear of marginal concern as total contributions are relatively low.

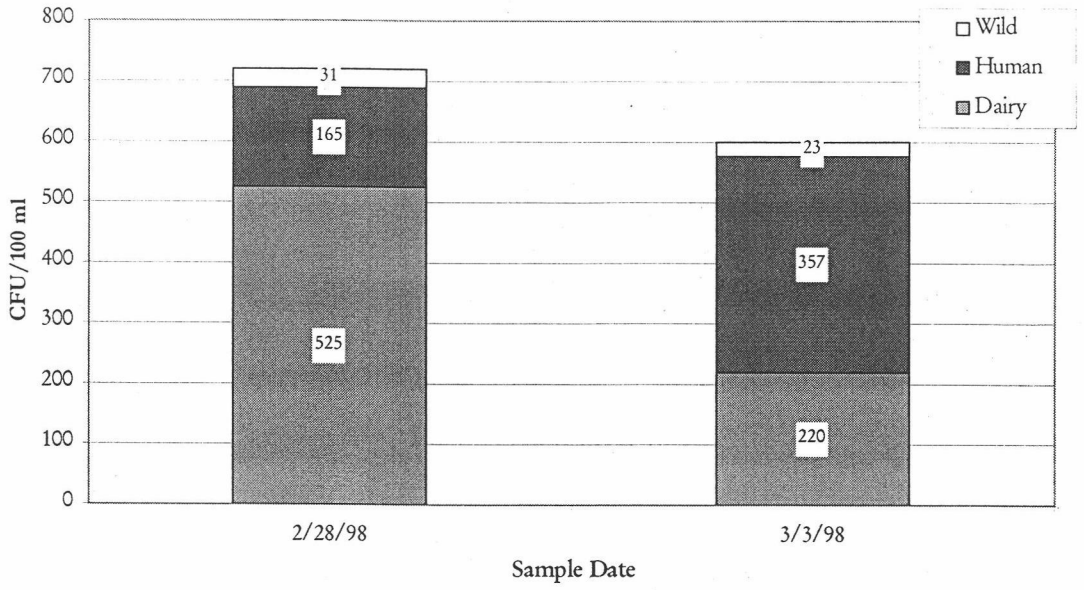

Figure 12. Quantified Distributions at TRA 5th Site

The ADA quantified data recorded at the TRA 5th location is plotted against all FCB values collected during the storm at that location (**Figure 13**). The quantified ADA-FCB concentration sample (2/28/98) appears to have been collected on the rising limb of the FCB concentration curve. Note that 'dairy' sources are the in the majority in this first sample which is consistent with the idea that increases non-point pollution may be linked to the rising limb of the FCB concentration peak.

The 3/3/98 sample was collected in what might be a late storm peak in FCB concentrations and shows a 'human' source majority. Following the highest concentration peak (900 isolates), the concentrations drop to values below the water quality standard for two consecutive samples (3/1/98 and 3/2/98). The 3/3/98 sample reports FCB concentrations that return to values above the standard. This second peak

may imply some type of secondary pulse of fecal pollution from an unidentified 'human' source upstream. That fact that it occurs after two subsequent below standard values supports a secondary pulse idea. Additionally, the sample shows a different source in the majority, which also lends credit to this theory.

This location is downstream from the City of Tillamook. It is possible that a pulse of fecal isolates may have been released from the storm water drain system and shown up as this secondary concentration peak.

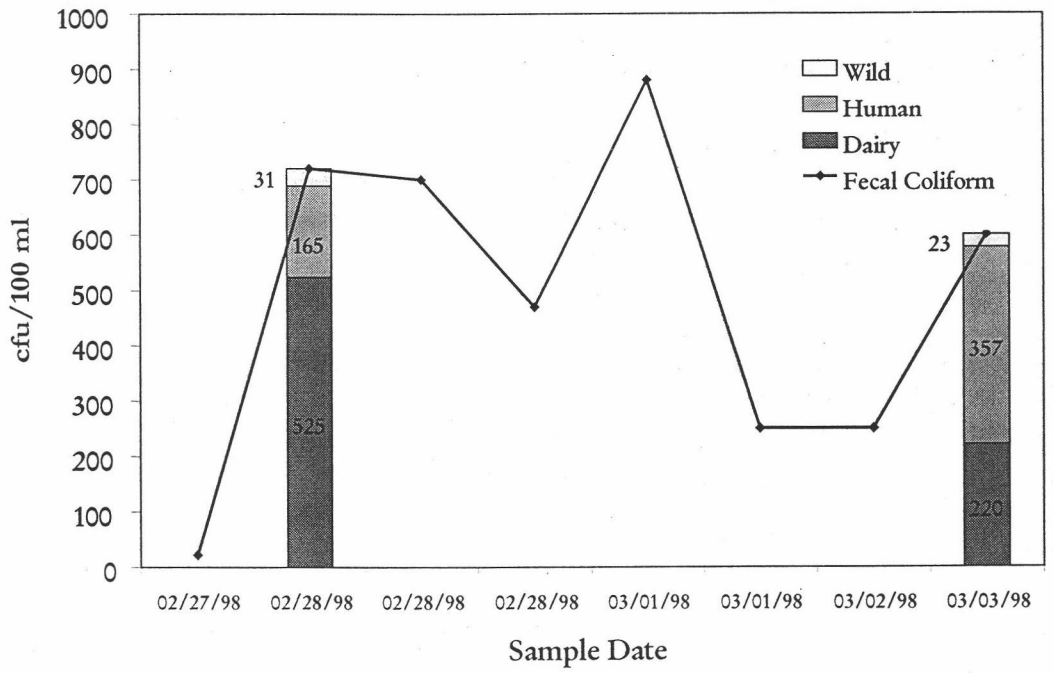

Figure 13. Quantified Distributions and FCB Data at TRA BTR Site

The TRA 5th sample site is located upstream from Tillamook's WWT facility while the TRA HOB site is located just downstream from this facilities' outfall. The

results shown in Figure 14 support the idea that increases in contributions of non-point sources may be linked to the rising limb of the hydrograph when FCB concentrations tend to increase. The first sample (2/28/98) collected on the rising limb of the FCB concentration curve shows an increase in both the 'dairy' and 'wild' sources, both of which are considered non-point sources.

Consistent with the TRA 5th sample location, there appears to be a late storm increase (3/3/98) in FCB concentration following the major concentration peaks recorded earlier in the storm. The 3/3/98 sample of 410 isolates exceeds the water quality standard while the two samples prior did not (3/1/98 and 3/2/98). Unlike the TRA 5th upstream location, the secondary peak shows a 'dairy' source majority. Note that 'human' sources still consist of a considerable portion (220 isolates) of the entire sample. 'Wild' isolates are again most prevalent during the initial FCB concentration peak.

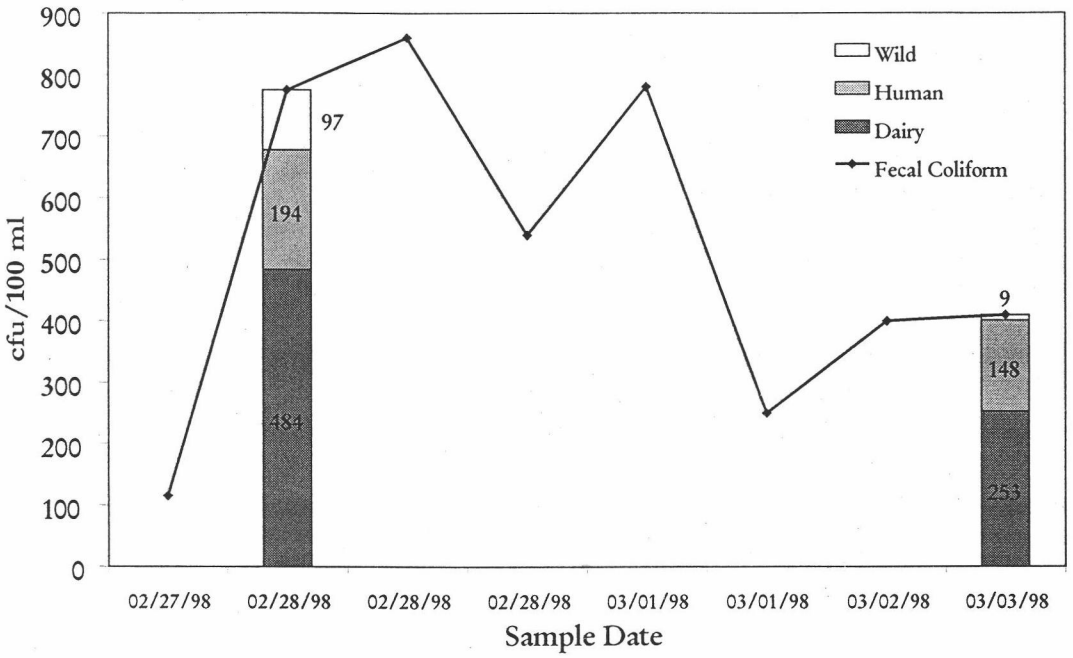

Figure 14. Quantified Distributions and FCB Data at TRA HOB Site

If we were to consider the upstream (TRA 5th) and downstream (TRA HOB) sample sites as monitoring stations of storm contributions made by the WWT plant the data appears inconclusive. The two ADA-FCB processed samples collected below the WWT plant (TRA HOB) show 'human' source concentrations (194 and 148 isolates) well below the water quality standard of 400 cfu/100 ml. These values appear to remain relatively constant when compared to the near doubling of the 'dairy' and the near 10-fold change in 'wild' sources. However with the 'human' source secondary peak at the upstream TRA 5th site, makes it difficult to clearly determine the influence, if any, of the treatment plants effluent.

Generally, in the Trask River samples, 'dairy' made up a majority of source contribution in all sample sites. 'Wild' sources also appeared most prevalent during the first of samplings. The data tended to support a non-point fecal source and rising limb correlation theory.

Tillamook River Samples Quantified

Consistent with the Trask River samples, a total of 8 water samples were collected and processed for FCB concentrations for each Tillamook River location in approximately 12-hour increments from February 27th, 1998 to March 3rd, 1998. Two of these 8 samples were collected in conjunction with water processed using the ADA technique.

Starting at the TIL RES site, the most upstream sample site on the Tillamook, a general pattern of increased FCB concentrations during the first sampling was observed **(Figure 15)**. This was similar to that shown in the Trask River. From the 2/28/98 sample there is a larger concentration of FCB isolates than recorded in the second 3/3/98 sample **(Figure 15)**. The 2/28/98 sample is above the water quality standard of 400 cfu/100 ml. Unlike the 'dairy' dominated majorities shown in these first samplings on the Trask River sites, this sample contained a 'human' majority.

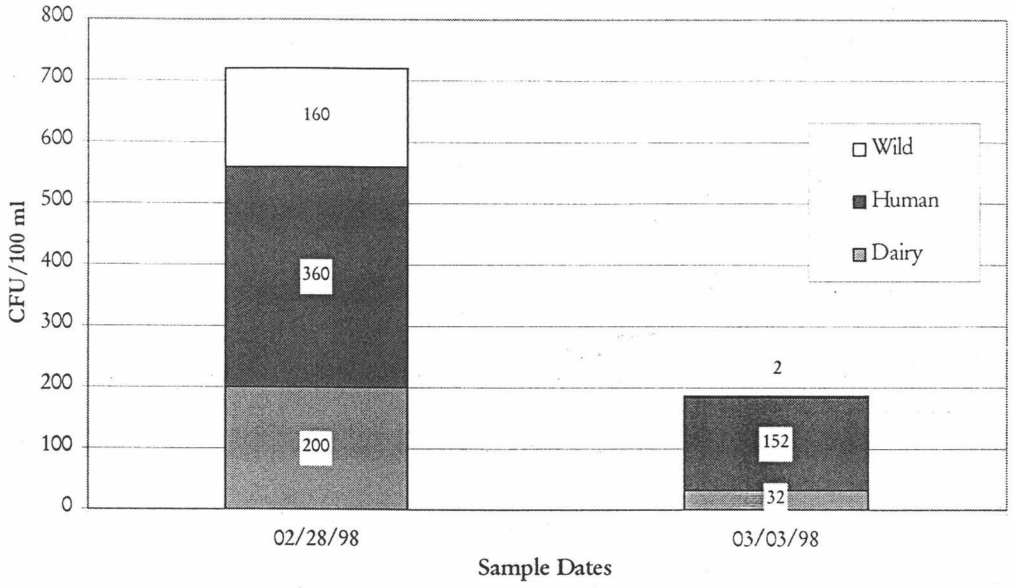

Figure 15. Quantified Distributions at TIL RES Site

The trend shown in the Trask River samples of 'wild' isolates being more numerous in the sample preceding the FCB peak is also seen in the Tillamook River sites. 'Dairy' sources are relatively low in both samples here, which may be explained by the sample site locations being high in the watershed (RM 8.1). Dairy farms are generally found in the lower watershed where grazing opportunities are better.

Figure 16 shows the two quantified distribution samples in relation to the FCB concentration curve recorded at this sample location. The 2/28/98 sample appears to have been collected during the highest FCB concentration peak recorded during the storm at this location. Ideally the best sample points to understand the quantity and source of fecal pollution would be collected during the highest peaks in FCB concentrations of a winter storm event. These samples would represent the worst case pollution scenario and help to suggest which source is contributing to these storm-

driven peaks. At this location 'human' (360 isolates) sources play the major role in this sample exceeding the water quality standard.

The second sample (3/3/98) still shows a 'human' source majority but is well below the water quality standard **(Figure 16)**. As this sample appears to follow the previous FCB concentration peaks, it potentially represents a return to non-storm levels of FCB concentrations at this location. As shown on the Trask, 'wild' isolates appear to be much less significant in during the second sampling.

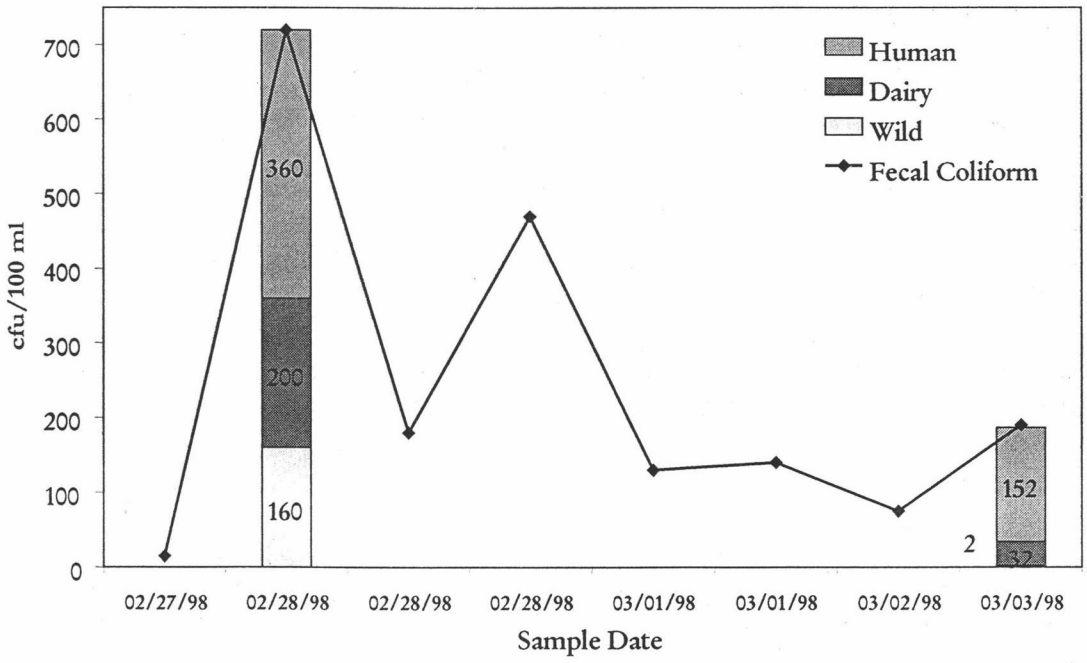

Figure 16. Quantified Distributions and FCB Data for TIL RES Site

While many of the sample locations show the ADA processed samples collected during the rising and falling limbs of the FCB concentration graph, not all data from

the Tillamook River fit this model. **Figure 17** shows the data recorded near the mouth of the Tillamook River (TIL NET) and was typical of the three remaining Tillamook sites. Observe that both ADA processed samples were collected before and after the peak in FCB concentrations while the previous data showed samples being collected during the storm driven increases in concentrations. While the peak in FCB concentrations were recorded as high as 700 cfu/100 ml, both of the ADA samples collected at this location were well below the water quality standard.

Also inconsistent with the previous results is that non-point sources ('dairy' and 'wild') appear to be slightly higher on the falling limb of the FCB concentration curve. This is most likely due to when ADA samples were collected relative to the peak in FCB concentrations. Considering that the second sample (3/3/98) was collected while the storm induced FCB concentrations were still higher than the (2/28/98) sample, this would be consistent with the theory that increases in non-point pollution is linked to increases in storm driven increases in FCB concentrations. Ideally, sampling more frequently during a storm would help to identify the sources during the storm peaks in FCB concentrations which would help us to better identify the sources of those peaks.

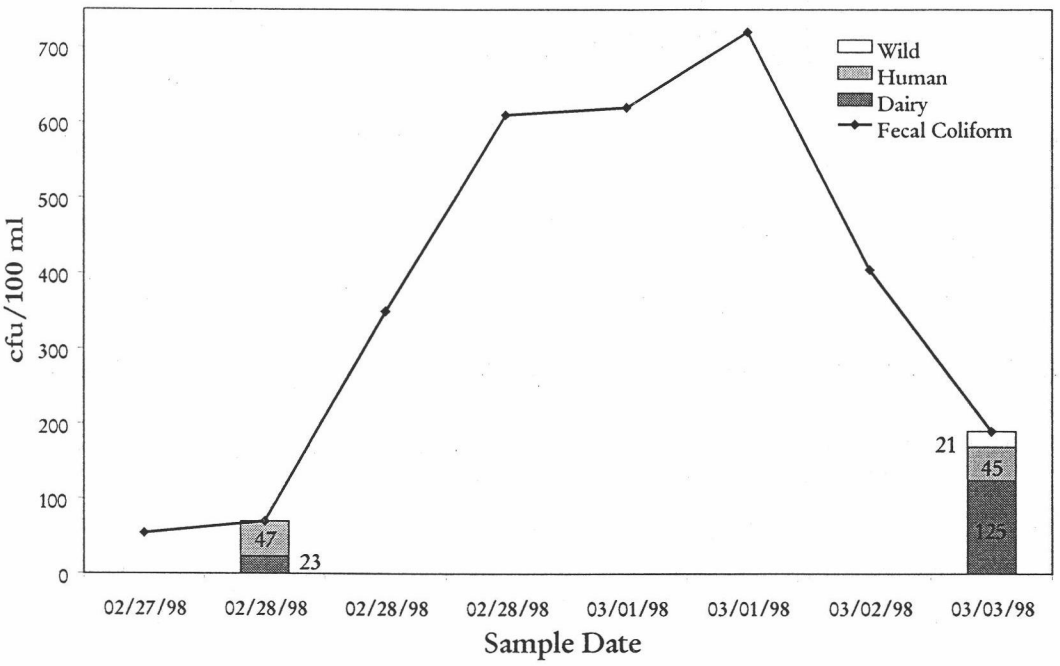

Figure 17. Quantified Distributions and FCB Data for TIL NET Site

Seasonal River Study

Samples were collected near the mouths of the Miami, Kilchis, Wilson, Trask and Tillamook Rivers in approximately six week intervals from December, 1997 through December, 1998. Samples were processed using the Antibiotic Discriminant Analysis (ADA) technique to record changes in each of the rivers' fecal source distributions on a seasonal basis. To estimate the magnitude of each samples' source distributions, the fecal streptococci bacteria (FSB) filtered in the first step of the isolation process (see Materials and Methods section) were enumerated to establish concentrations.

The samples were collected as individual samples near the mouth of each river. The original objective of this sampling was to search for river-by-river trends in the major contributing sources (**Figure 18**). However, after reviewing the data provided by the storm study chapter of this document, it is unlikely that a single sample collected near the mouth of a river would be representative of the composite contribution of that river to the Tillamook Bay. The storm sample results showed that the quantity and sources changed on sample-by-sample and site-by-site basis, making the assumption that single samples could indicate river basin wide contribution to the bay unlikely.

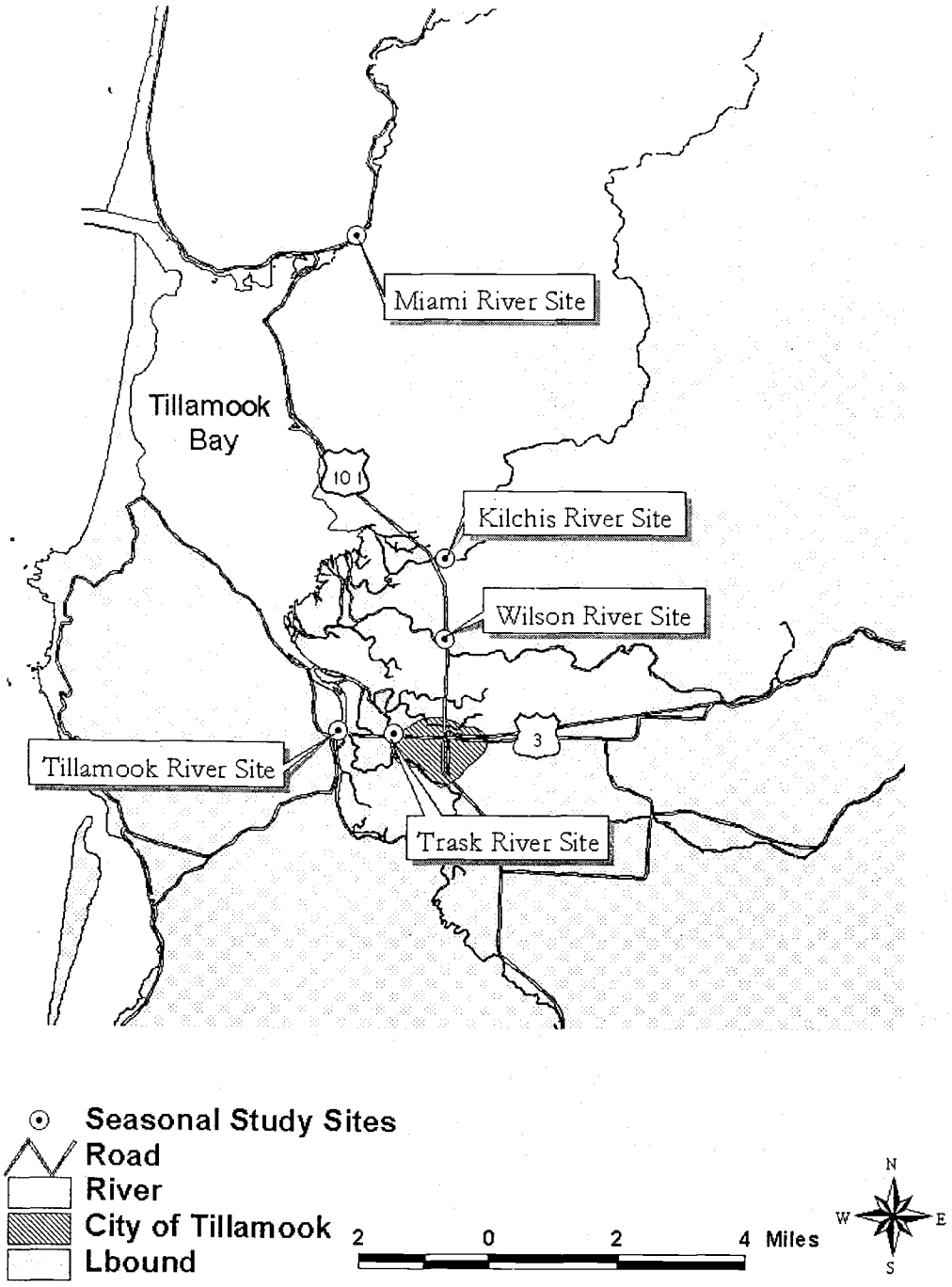

Figure 18. Map of Five-River Seasonal Sampling Locations

The seasonal data does however provide some interesting insights into the chronic fecal pollution problems that occur in the Tillamook watershed. There were similarities in the data when compared on a river to river basis and the Miami River is a representative example of the seasonal study data as a whole.

As shown in the storm study the distribution data generally showed that either 'dairy' or 'human's sources consistently were a majority of the source distributions. For example on the Miami, the 'human' sources made up the majority (6 of 8) of the samples (**Figure 19**). Also consistent with the storm study data was that 'wild' sources were consistently a low percentage of the samples collect in all of the Tillamook watershed rivers.

River samples also show source 'dairy' and 'human' source distributions that appear to change incrementally over the year study (**Figure 19**). The percentage of 'human' isolates decreases gradually (excluding Dec-97) from May through June and then gradually increases from July through December. This might give the impression that 'dairy' sources are most prevalent during the early summer months while 'human' sources are most prevalent in the fall and winter. However, as shown in the Storm section, the distribution samples are much more useful when quantified with concentration data.

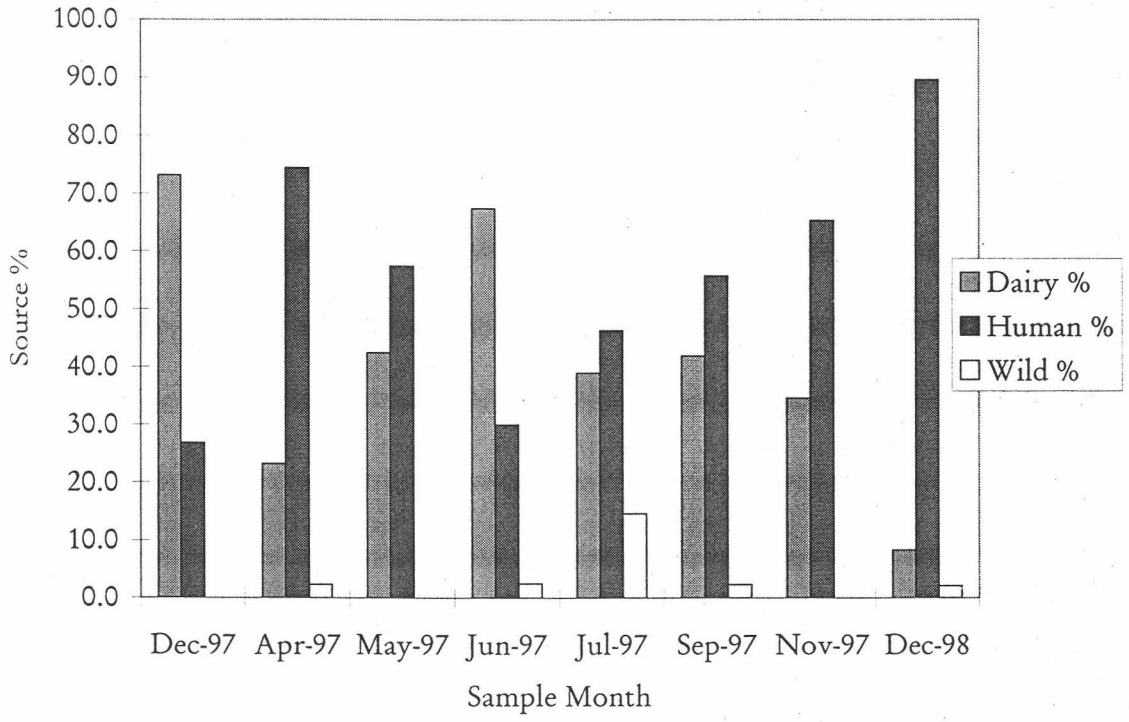

Figure 19. Source Distribution for Miami River Samples

To estimate the magnitude of each of the ADA distribution samples, an equation similar to that used in the storm study was established (2).

Distribution x Fecal Streptococci Bacteria Concentrations (2)

$$D_{d,h,w} \times Fs_{conc} = Q_d$$

$D_{d,h,w}$ = Source Distribution of 'dairy', 'human', 'wild' (%)
Fs_{conc} = Fecal streptococci bacteria concentrations (cfu/100 ml)
Q_d = Quantified distributions (cfu/100 ml)

Fecal streptococci concentrations were used to estimate each distribution on the Miami River (**Figure20**). Incremental trends that appeared significant in the

distribution data now appear much less prevalent as samples with larger concentrations (September and November) become the primary concern when considering the water quality issues.

The data showed that concentrations of all sources were generally low in the spring-summer and higher in late summer, and fall. In a majority of the rivers, there was a peak in FCB concentrations around the 'Sep-98' sampling. A majority of these 'Sep-98' samples showed a 'human' source majority with only the Tillamook showing 'dairy' as the majority. After this basin wide peak in 'human' source isolates was observed, Tillamook river flow and rainfall data was analyzed to determine any environmental causes (data not shown here). There were no notable changes in either the rainfall or river flow during the period that these samples were collected. The reason for this increase in FCB concentrations is unknown at this time.

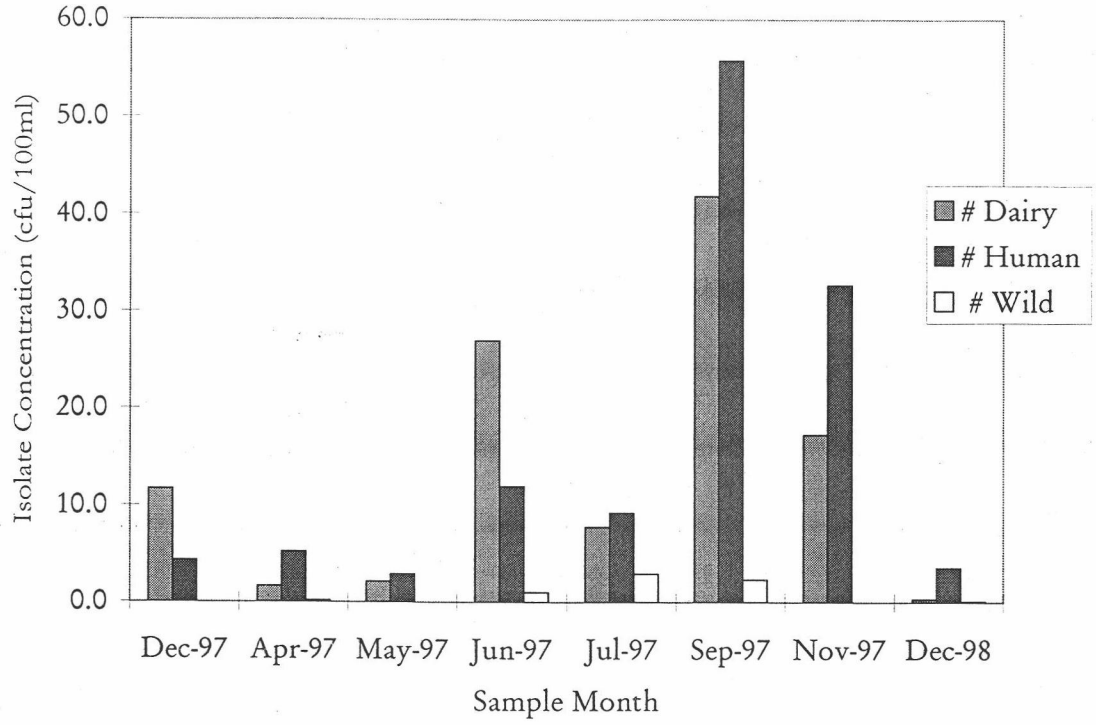

Figure 20. Miami River Source Distributions Quantified

As there are no known water quality standards for FSB it is difficult to assess these river samples in terms of water quality limitations. With nearly all of the concentration values well below 400 cfu/100 ml, it is most likely that nearly all the samples are inconsequential when compared to those values recorded during the storm study. Fecal coliform bacteria (FCB) concentrations collected in these same rivers have been reported to range as high as 1 x 10^6 cfu/ 100ml, which is well above the DEQ water quality standard being 400 cfu/100 ml **(E&S, 1998)**.

Conclusions

The repeatability of the antibiotic discriminant analysis (ADA) was validated by obtaining an average rate of correct classification (ARCC) of 83.3% for three known fecal pollution sources in the Tillamook Bay watershed. The antibiotic resistant profiles of individual sources also showed similarities in their response when compared to profiles from previous studies. The isolation and identification of the bacteria fecal streptococci was also successfully conducted with a significant majority of the isolates correctly identified

The ADA technique demonstrates a strong potential for use in water quality monitoring programs aimed at differentiating the sources of fecal pollution. Results obtained from a winter storm and seasonal five-river study of a water quality limited watershed supported this assertion. The ADA technique appeared to differentiate changes in the distribution of sources of fecal pollution on a site-by-site and sample-by-sample basis. When the ADA technique was used in conjunction with FCB and FSB concentration data, the magnitude and influence of specific samples was discerned in relation to their water quality impacts. The storm study data also appears to support a the hypothesis that non-point sources of fecal bacteria may increase with parallel increases in the hydrograph.

Specific water quality issues can also be addressed for the Tillamook Bay watershed. 'Dairy' and 'human' sources of fecal pollution were dominant in a majority

of samples from both storm and seasonal studies. 'Wild' sources played a marginal role in a majority of all samples collected. This is to be expected considering the watershed populations of the three sources of fecal pollution have relatively large 'dairy' and 'human' populations. It was also shown that while either 'dairy' or 'human' sources contributed a majority of isolates in a given sample that exceeded water quality standards, both sources contributed to that samples' value. The ADA technique demonstrates great promise as a definitive method for discerning sources of fecal pollution in natural waters.

Bibliography

American Public Health Association. (APHA) 1992. Standard methods for the examination of water and wastewater. 19th ed. American Public Health Associations, Inc., Washington, D.C.

Asim, K. B., J. L. DiCesare, L. Haff, and R. M. Atlas. 1991. Detection of *Escherichia coli* and *Shigella* spp. in water by using the Polymerase chain reaction and gene probes for *uid.* Appl. Environ. Microbiol. **57:**1013-1017.

Asim, K. B., R. J. Steffan, J. DiCesare, L. Haff, and R. M. Atlas. 1990. Detection of coliform bacteria in water by polymerase chain reaction and gene probes. Environ. Microbiol. **56:** 307-314.

E&S Environmental Chemistry, Inc. (E&S). 1998. Water Quality Monitoring in the Tillamook Watershed. Corvallis, OR.

Feachem, R., 1975. An improved role for faecal coliform to faecal streptococci ratios in the differentiation between human and nonhuman pollution sources. Water Res. 9:689-690.

Hagedorn, C., S. L. Robinson, J. R. Filtz, S.M. Grubbs, T. A. Angier, R. B. Reneau, Jr. 1999. Determining Sources of Fecal Pollution in a Rural Virginia Watershed with Antibiotic Resistance Patterns in Fecal Streptococci. Appl. Environ. Microbiol. **65:**5522-5531

Kaspar, C. W., J.L. Burgess, I. T. Knight, and R. R. Colwell. 1990. Antibiotic resistance indexing of *Escherichia coli* to identify sources of fecal contamination in water. Can. J. Microbiol. **36:**891-894.

Kelch, W. J., and J. S. Lee. 1978. Antibiotic resistance patterns of gram-negative bacteria isolated from environmental sources. Appl. Environ. Microbiol. **36**:450-456.

Knudtson, L. M. and P. A. Hartman. 1993. Antibiotic resistance among enterococcal isolates from environmental and clinical sources. J. Food Prot. **56**:489-492.

Krumperman, P. H. 1983. Multiple antibiotic resistance indexing of *Escherichia coli* to identify high-risk sources of fecal contamination of foods. Appl. Environ. Microbiol. **46**: 165-170.

Kuhn, I., G. Allestam, T. A. Stenstrom, and R. Mollby. 1991. Biochemical fingerprinting of water coliform bacteria, a new method for measuring phenotypic diversity and for comparing different bacterial populations. Appl. Environ. Microbiol. **57**:3171-3177.

Oregon Climate Service. Climate Data. http://www.ocs.orst.edu/

Oregon Department of Environmental Quality. (DEQ). 1999. Listing criteria for Oregon's 1998 303(d) list of water quality limited water bodies. Website: http://waterquality.deq.state.or.us/WQLData/ListingCriteria.htm

Oregon Water Resources Department. Stream Flow. http://www.wrd.state.or.us/surface_water/

Parveen, S., R. L. Murphee, L. Edminston, C. W. Kaspar, K. M. Portier, M. L. Tamplin. 1997. Association of Multiple-Antibiotic-Resistance profiles with point and nonpoint sources of Escherichia coli in Apalachicola Bay. Appl. Environ. Microbiol. **XX**:2607-2612.

SAS Institute Inc. *SAS/STAT™ Guide for Personal Computers, Version 6 Edition.* Cary, NC: SAS Institute Inc., 1987. pages 359-449.

Sternes, K. L., 1999. Vancomycin Resistance in natural waters of the Rio Grande. (Have to get this citation). Science News, 4/24/99 edition. P 268.

Sullivan, T. J., J. M. Bischoff, K. B. Vache. (E & S) 1998. Results of storm sampling in the Tillamook Bay watershed. E & S Environmental Chemistry Inc., Corvallis OR.

Tabachnick, B. G., and L. S. Fidell. 1983. Using multivariate statistics. Harper and Row, Publishers, Inc., New York.

Tillamook Bay National Estuary Project. (TBNEP). 1999. Tillamook Bay National Estuary Project GIS data layers. Website: http://www.orst.edu/dept/tbaynep/active.html.

Wiggins[a]. B. 1996. Discriminant analysis of antibiotic resistance patterns in fecal Streptococci, a method to differentiate human and animal sources of fecal pollution in natural waters. App. Envir. Micro. **62**:3997-4002.

Wiggins[b]. B. 1996. How To Do It: Procedures for discriminant analysis of antibiotic resistance profiles of fecal streptococci. James Madison University, Virginia.

Wiggins[c]. B. R. W. Andrews, R. A. Conway, C. L. Corr, E. J. Dobratz, D. P. Dougherty, J. R. Eppard, S. R. Knupp. M. C. Limjoco, J. M. Mettenburg, J. M. Rinehardt, J. Sonsino, R. L. Torrijos, and M. E. Zimmerman. 1999. Use of antibiotic resistance analysis to indentify nonpoint sources of fecal pollution. Appl. Environ. Microbiol. **65**:3483-3486.

Wiggins[d], B. R., 1997-1999. Personnel communications.