AN ABSTRACT OF THE THESIS OF

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We explain a new method of detecting non point source fecal contamination using a PCR based method called Touchdown Polymerase Chain Reaction (TD-PCR). Using genetic markers particular to general, ruminant and human *Bacteroides-Prevotella* genes, we identified presence in both fresh and salt water environments. Water samples from four sites were collected at approximate 2-week intervals for a year. Samples were analyzed for total coliforms, fecal coliforms, *E. coli*, and the presence of general, ruminant and human *Bacteroides-Prevotella* markers. We compared the odds of recovering each PCR marker between sites. We investigated the relationship between rainfall and recovery of PCR markers. Finally, we compared the sensitivity of the PCR methods to standard public health methods.

A Descriptive Analysis of the Waterways in Coos Bay Oregon on the Basis of General, Ruminant and Human *Bacteroides-Prevotella* 16S rDNA Markers

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Thomas E. Jones Jr., Author

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Chapter 1

A Descriptive Analysis of the Waterways in Coos Bay Oregon on the Basis of General, Ruminant and Human *Bacteroides-Prevotella* 16S rDNA Markers

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During the mid 1800's, severe cholera epidemics swept through London, killing thousands. John Snow, a London physician, suggested the cause was from water contaminated with vomit and stool from cholera patients. Snow performed an epidemiological study proving that water was the mode of transmission for cholera. It was previously hypothesized that diseases were spread by inhalation of vapors, but Snow discovered convincing evidence to the contrary (Snow 1936; Rosenberg 1962). Snow showed that cholera occurred more frequently in people who drew their water from the lower Thames, where it had become contaminated with sewage, whereas people receiving water from the upper Thames had little disease (Snow 1936; Rosenberg 1962). Many scientists found his study credible but it was not until John Snow detached the handle of the Broad Street water pump that government officials finally took notice. The cholera epidemic had become so severe in the neighborhood surrounding the corner of Cambridge Street and Broad Street that over five hundred people died in ten days. After accessibility to water from the Broad Street pump had been taken away, the epidemic ceased, lending validity to Snow's hypothesis (Snow 1936; Rosenberg 1962). If it were not for his great epidemiological triumph, thousands more could have died from cholera. His theory led to new ideas concerning how life-threatening organisms could spread through the life giving substance of water.

Today, contaminated water still poses a threat to the public health and welfare (USEPA 2002). Perhaps the greatest area of concern regarding disease in water is non-point source (NPS) pollutants (USEPA 2002). According to the United States Environmental Protection Agency (USEPA), NPS pollution occurs when rainfall,

snowmelt, or irrigation runs over land or through the ground, picks up pollutants, and deposits them into creeks and rivers. These NPS pollutants then are deposited into lakes, estuaries and coastal waters or introduced into the ground water. NPS pollution is widespread because it can occur any time activities disturb the land or water (USEPA 2002). This pollution is considered to be especially dangerous because of the difficulty in identifying its origin.

In general, NPS pollution encompasses a wide range of compounds, from fertilizers and pesticides to heavy metals and microorganisms. NPS pollution is the main reason that approximately 40 percent of the United States' rivers, lakes and estuaries are not clean enough to meet basic uses such as fishing or swimming (USEPA 2002). The latest National Water Quality Inventory (USEPA 2002) indicates that agriculture is the leading contributor to water quality impairments, degrading 60 percent of the impaired river miles and half of the impaired lake acreage surveyed by states, territories, and Native American tribes. Runoff from urban areas is the largest source of water quality impairments to surveyed estuaries (areas near the coast where seawater mixes with freshwater). Another area of concern is with bacterial NPS, with potential sources coming from agriculture, forestry, grazing, septic systems, recreational boating and urban runoff (USEPA 2002).

In the 1960's, researchers attempted to understand the relationship between water quality and microorganisms. Researchers from the National Technical Advisory Committee (NTAC) of the Department of the Interior studied the link between bacterial concentration in water and gastrointestinal illness (USEPA 1986). The

NTAC used a microbiological criterion that was based on a series of studies conducted during the late 1940s and early 1950s by the United States Public Health Service (USPHS) (USEPA 1986). Before this study, it was known that pollutants in water were harmful to both the environment and people, but a lack of published research made it difficult to determine quantitatively how the pollutants affected people.

The NTAC study was conducted at both saltwater and fresh water bathing beaches located on Lake Michigan at Chicago, Illinois; on the Ohio River at Dayton, Kentucky; on Long Island Sound at Mamaroneck; and New Rochelle, New York (USEPA 1986). Each site had two locations with differing water quality; one location had cleaner water than the other. These locations were chosen because of the large residential populations nearby, allowing the researchers contact with local everyday beachgoers. Gastrointestinal illness, respiratory difficulty and other symptoms such as skin irritations were recorded in conjunction with total coliform bacterial concentration at each site. The study concluded that in fresh water, there was a significantly greater illness rate in individuals who swam on the days when the total coliform density was 2300/100ml compared to those who swam when the coliform density was 43/100ml. The results from the saltwater swimming locations showed no significant association between illness and swimming in water containing between 398 and 815 coliforms per 100ml (USEPA 1986).

In the mid 1960's, the NTAC study was used as a basis for a fecal coliform index. This was done by using the ratio of fecal coliforms to total coliforms found at the location on the Ohio River, where the original study had been conducted. The

NTAC suggested this change because fecal coliforms were more indicative of fecal pollution and less subject to variation than total coliforms, thus decreasing the possibility for false positives in testing for fecal presence (USEPA 1986). For example, the bacteria *Klebsiella* is in the same family of bacteria, Enterobacteriaceae, as total coliforms. *Klebsiella* can be associated with paper mill effluents, textile processing plant effluents, cotton mill wastewater and sugar beet wastes (Nunez and Colmer 1968; Campbell, Micheals et al. 1976; Dufour and Cabelli 1976; Huntley, Jones et al. 1976; Caplenas, Kanarek et al. 1981). A positive indication for the presence of Klebsiella would not necessarily mean the presence of fecal matter. When the waters of the Ohio River were re-tested, where the original study had been conducted in 1949, it was found that approximately 18% of the total coliforms were fecal coliforms; thus the data were transformed using this criterion (USEPA 1986). Instead of the original cutoff of 2300 coliforms per 100ml of water, a new measure of 400 fecal coliforms per 100 ml of water was proposed (USEPA 1986). The NTAC went a step further and suggested that a detectable risk was undesirable, and therefore one-half of the density at which a health risk occurred, 200 fecal coliforms per 100 ml of water, should be used. The final recommended criterion for recreational waters from the NTAC was as follows:

> "Fecal Coliforms should be used as the indicator organism for evaluating the microbiological suitability of recreation waters. As determined by multiple tube fermentation or membrane filter procedures and based on a minimum of not less than five samples taken

over not more than a 30-day period, the fecal coliform content of primary contact recreation waters shall not exceed a log mean of 200/100ml, nor shall more than 10 percent of total samples during any 30-day period exceed 400/100ml." (USEPA 1986)

Many criticized the NTAC study based on "the poor quality of the database, the derivation of the specific limits and the indicator system used" (Henderson 1968). Others suggested that the term "swimming" was poorly defined because it was not known if individuals that participated in the study submerged their whole bodies in the water or simply waded in the shallow areas. Critics also objected to the lack of a beach going, non-swimming control group (Cabelli, Levin et al. 1975). In 1972, it was decided by the National Academy of Engineers Committee of the National Academy of Science that the water quality criterion presented by the NTAC could not be recommended because of the "paucity of epidemiological information available" (NTAC 1968).

Therefore, in that same year, the USEPA decided to perform a series of studies designed to correct any deficiencies of the NTAC study. As in the former study, the USEPA conducted a regiment of tests for both fresh and saltwater areas. Ten sites were tested; each site had two locations, one that had little known contamination and the other with water quality at barely acceptable levels. The membrane filter procedure was used for enumerating bacteria. Epidemiological surveys were performed on individuals who swam on days in which the water quality was measured. In doing so, researchers were able to show a true correlation between the water quality

measurements and illness rates (USEPA 1986). In both marine and fresh water locations, a statistically significant increase in swimming-associated gastroenteritis rates occurred at the areas with water quality at barely acceptable levels. Findings for those areas chosen as unpolluted showed no statistically significant increase in swimming-associated gastroenteritis rates (USEPA 1986). This finding was contrary to the earlier report given by the NTAC in which it was stated that fresh water sites had the only increase in gastroenteritis rates.

In the USEPA study, a wider range of indicator organisms was used. These organisms included enterococci, *E. coli*, *Klebsiella*, total coliforms, *C. perfringens, P. aeruginosa*, fecal coliforms, *A. hydrophila, V. parahemolyticus* and *Staphylococcus* for marine waters, and enterococci, *E.coli*, and fecal coliforms for fresh water. The Pearson Correlation Coefficient was used to determine the relationship between gastroenteritis rates and the various indicators of water quality used for both marine and fresh water (USEPA 1986).

For marine water, enterococci had the highest correlation coefficient to gastroenteritis (closest to plus one), followed by *E.coli* and then fecal coliforms. In the fresh water analysis, it was found that *E.coli* had the highest correlation coefficient to gastroenteritis followed enterococci and then fecal coliforms. After this second study, the recommended criteria for bathing, full body contact, in recreational waters for both fresh and marine waters were established. The USEPA stated that the geometric mean of the indicated bacterial densities should not exceed one or the other of the following:

1) Freshwater- *E.coli* 126 per 100ml or enterococci 33 per 100ml. No sample should exceed a one sided confidence limit of 75% for designated bathing beaches, 82% moderate bathing use, 90% light bathing use, and 95% infrequent bathing use (USEPA 1986).

2) Marine water- enterococci 35 per 100ml. No sample should exceed a one sided confidence limit of 75% for designated bathing beaches, 82% moderate bathing use, 90% light bathing use, and 95% infrequent bathing use (USEPA 1986).

Both fresh and marine water guidelines called for a statistically sufficient number of samples to be tested, meaning no fewer than five samples equally spaced over a thirty day period (USEPA 1986).

In the 1970s, public awareness of the dangers of water increased when several large shellfish bed closures, fish kills and gastrointestinal illness outbreaks were reported (Martin 1997). In 1971, the United States Army Corps of Engineers became involved. The Corps started issuing permits for discharging waste into navigable streams and their tributaries (Martin 1997). In 1972, Congress passed the Clean Water Act, designed to "protect our nation's waters, including lakes, rivers, aquifers and coastal areas" (EPA, 2002).

In 1976, the EPA included recommendations for both swimming and shellfish harvesting waters in the Quality Criterion for Water (QCW). The EPA recognized that in order to incorporate all the health risks associated with fecal contaminated water

into federal guidelines, marine waters used to grow food, such as shellfish, had to be tested.

Gastrointestinal illness among shellfish eaters is another indication of fecal contamination. Evidence showed that pathogens found in feces can be stored in shellfish (Fayer and Lewis 1999). Researchers found that oocysts from *Cryptosporidium parvum*, a zoonotic waterborne pathogen found in human feces, could be isolated from the gills and the hemolymph of bivalve molluscs living in contaminated water (Fayer and Lewis 1999). Researchers also discovered that oysters can remove *C. parvum* oocysts from artificially contaminated water and retain the oocysts in the hemocytes and gills for at least one month (Fayer and Lewis 1999). *Cryptosporidium* can enter waterways through leaking septic tanks, wastewater or recreational activities (Fayer and Lewis 1999). Other human pathogens besides *Cryptosporidium* are believed to be stored inside bivalves as well (Fayer and Lewis 1999).

Unfortunately, efforts by the USEPA to protect water were not enough, and outbreaks of gastrointestinal illness in both recreational and shell fishing waters still occurred. From 1989 to 1999, approximately 170 outbreaks associated with recreational water venues, such as swimming pools, water parks, fountains, hot tubs and spas, rivers, lakes and oceans, were reported, with almost half resulting in gastrointestinal illness (Minshew, Ward et al. 2000).

In Florida in 1995, the largest outbreak of oyster-associated gastroenteritis ever reported occurred when approximately 129 people became ill from eating oysters. It was determined that the oyster eaters who ate thoroughly cooked oysters were as likely to become ill as those who ate raw oysters (McDonnell, Kirkland et al. 1997). This observation disrupted the belief that those who ate raw oysters were more susceptible to illness than those who ate cooked oysters. Within a four-year period, 1993 to 1996, three outbreaks of gastrointestinal illness occurred in Louisiana (Berg, Kohn et al. 2000). Overboard disposal of sewage by oyster harvesters was blamed for the Norwalk-like virus (NLV) contamination of oysters (Berg, Kohn et al. 2000).

Most recently, the EPA started working on the "Implementation Guidance for Ambient Water Quality for Bacteria" (USEPA 2002). Started in 1999, this guide is designed to help governing bodies adopt and implement water quality criteria for protected waters. The guide is based upon the EPA's 1986 recommended water quality critieria for *E.coli* and fecal coliforms and should be complete by December 2002 (USEPA 2002).

Until the new guidelines are completed, current federal guidelines dictate the mandatory testing of waterways, including lakes, rivers and coastal waters. Today, the most common public health standards used are the same ones issued in 1986 by the EPA, quantifying fecal coliforms and *E.coli* in water samples (APHA 1995).

The two methods used most widely by public health officials for measuring fecal pollution are Heterotrophic Plate Count (HPC) and Multiple Tube Fermentation

(MTF) (APHA 1995). HPC is a method that estimates the number of live heterotrophic bacteria in a water sample. Colonies are grown on media specific to heterotrophs and incubated on pour plates at 35°C for 48 hours and measured as colony forming units (CFU). MTF tests are used to identify the presence of coliforms (of the family Enterobacteriaceae). Incubating within 48 hours at 35°C, the presence of gas and acid indicates that fermentation of lactose has taken place and thus that coliforms are present (APHA 1995). Results of the examination are reported in terms of the Most Probable Number (MPN) (APHA 1995).

Because many of these indicator organisms can survive outside of enteric habitats recent contamination events cannot be easily determined. (Flint 1987; Alkan, Ellioit et al. 1995; Davies, Long et al. 1995; Mezrioui, Baleux et al. 1995; Bogosian, Sammons et al. 1996; Stoddard, Coyne et al. 1998). Settling and growth patterns of fecal coliforms in the environment are not well characterized (Davies, Long et al. 1995) and so the validity of the public health standards has come into question. Researchers are trying to devise new ways to accurately identify fecal NPS.

In the 1980's and 1990's, advances in molecular technology provided opportunities for new approaches to Non Point Source identification. The Polymerase Chain Reaction (PCR) is an example of this new technology (Gibbs 2000). The PCR, which makes millions of copies of a specified gene or DNA sequence in a test tube, gave scientists the ability to identify living organisms in the environment based upon their DNA. The PCR technique mimics DNA replication that occurs naturally in living cells. There are three phases in a PCR reaction. In the first phase, called denaturation,

the template, or original piece of DNA, is heated to a temperature that ranges from 90° to 95 ° C for 30 seconds; this causes the strands to separate (Gibbs 2000). In the second phase, called annealing, the temperature is lowered to a temperature that is specific for primer-template binding. Primers are short strands of DNA, designed to be complementary to the template DNA at regions flanking the gene to be copied. This phase lasts roughly 30 seconds, allowing the two primers to bind to the separated DNA parent strands in the locations that they complement, dictated by their nucleotide composition (Gibbs 2000). When primers bind to a location, replication of the DNA can begin. In the third phase, called polymerization, the PCR mixture is brought to a temperature of about 72° C, a temperature at which the polymerase enzyme can replicate the DNA strand rapidly (Gibbs 2000). Limitations still existed with the PCR technique, such as non-specific binding of primers to template DNA; thus researchers began to refine the PCR.

By combining the high reproducibility of PCR technology with the ability to identify length variation in PCR products, discrimination among mixtures of bacterial gene sequences can be accomplished by detecting differences in the number of base pairs in a particular gene fragment (Avaniss-Aghajani, Jones et al. 1994). An example is Length Heterogeneity Polymerase Chain Reaction (LH-PCR) (Rappé, Suzuki et al. 1998). This technique enables researchers to detect different species of microorganisms from complex microbiological communities in a single analysis. This multi-step process requires selection and amplification of specific gene sequences using fluorescently labeled primers and the PCR. The gene amplified fragments are

separated by size on an automated DNA sequencer, and a fluor-detector registers the size of the fragments, measured by their electrophoretic mobility, and their relative abundance. Another method, Terminal Restriction Fragment Length Polymorphism (T-RFLP), a variation of LH-PCR, uses restriction endonucleases to cut PCR amplicons whereby only the labeled terminal fragments are detected. This technique allows researchers to characterize complex microbial communities both spatially and temporally (Bruce 1997; Liu, Marsh et al. 1997; Clement, Kehl et al. 1998; Moeseneder, Arrieta et al. 1999; Flynn, Loffler et al. 2000; Scala and Kerkhof 2000). Both the LH-PCR and T-RFLP techniques were used in developing the primers used in the study included in this paper (Bernhard and Field 2000B).

Still another development in PCR technology is Touchdown PCR (TD-PCR) (Hecker and Roux 1996). This method increases both the specificity of primer binding and the sensitivity of detecting small quantities of DNA. There are many steps and variables involved in PCR. TD-PCR focuses on one, the annealing temperature. Many times primers similar but not complementary to a DNA sequence can bind, allowing for amplification, and thereby producing false positives. In TD-PCR, the annealing temperature is raised to a level slightly higher than the melting temperature (T_m) of the PCR primers. At this higher temperature, only highly specific primer and template binding can occur, thus decreasing the chances of non-specific amplification. The annealing temperature is then decreased by 1° C for each subsequent cycle, ultimately reaching a point in which the desired product will have a geometric head start in

amplification. Another safeguard against nonspecific binding is the lack of remaining PCR resources available at this stage (Hecker and Roux 1996).

These molecular techniques have helped considerably with bacterial identification, but there is still a need for new indicator organisms. One of the problems with current public health indicators concerns their survivability outside the enteric habitat. Fecal coliforms are able to grow in sediment, potentially giving an inaccurate measure of recent contamination (Flint 1987; Sherer, Miner et al. 1988; Sherer, Miner et al. 1992; Alkan, Ellioit et al. 1995; Davies, Long et al. 1995; Mezrioui, Baleux et al. 1995; Bogosian, Sammons et al. 1996; Stoddard, Coyne et al. 1998). In the 1980's and 1990's, three microbial groups were proposed to take the place of fecal coliforms used as public health indicators. Anaerobic, fecal-specific, short-lived microorganisms such as *Clostridium*, *Bifidobacterium*, and *Bacteroides* were evaluated as indicators in various environments with varying degrees of success (Resnick and Levin 1981; Allsop and Stickler 1985; Carrillo, Estrada et al. 1985; Fiksdal, Make et al. 1985; Sorenson, Eberl et al. 1989; Kreader 1995; Straub and Dixon 1997; Bradley, Carter et al. 1999; Griffin, Gibson et al. 1999).

There were a number of problems with *Clostridium* that interfere with its use as an indicator organism. For example, *Clostridium* species are common in feces, but also are found in soil microflora; thus a positive test would not necessarily indicate fecal presence. Another concern was that *Clostridium* can form spores when faced with harsh environmental conditions, surviving many years in an environment (Madigan, Martinko et al. 1997). When conditions become optimal for growth,

Clostridium changes from a spore to a bacterium. Thus identifying recent fecal contamination would be difficult using this organism.

The *Bifidobacterium* species showed promise as an indicator of fecal contamination, but because they survive in the environment for only a few hours their use is restricted to identifying only recent contamination events (Resnick and Levin 1981; Carrillo, Estrada et al. 1985). Due to the fact that *Bacteroides* survives in the environment for several days (Avelar, Morales et al. 1998; Kreader 1998) and its abundance in feces is greater than that of coliforms (Savage 1977), *Bacteroides* seemed to be the most promising out of the three proposed new indicators. The *Bacteroides-Prevotella* group comprises anaerobic, gram negative, nonsporulating rod-shaped bacteria found in the gastrointestinal tract and mouth of animals.

In the late 90s Bernhard and Field experimented with two groups of bacteria as fecal source indicators, but one in particular, the *Bacteroides-Prevotella* group, was found to be more promising (Bernhard and Field 2000A). They hypothesized that by identifying regions within the *Bacteroides* 16S rDNA gene, both the presence of feces, and its source, could be identified (Bernhard and Field 2000A). LH-PCR and T-RFLP were used to characterize community profiles of *Bacteroides-Prevotella* in both cow and human fecal samples, using primers that specifically amplified 16S rDNAs from the *Bacteroides-Prevotella* group (Bernhard and Field 2000A). Two human and two ruminant specific markers were identified from the *Bacteroides-Prevotella* group and PCR primers specific to each marker were designed. The researchers demonstrated that the primers recognized only human and ruminant DNAs, and marker sequences

could be recovered from natural fresh and saltwater samples (Bernhard and Field 2000A). Using these primers, an experiment was performed to characterize Tillamook Watershed waterways based upon *Bacteroides-Prevotella* strains unique to ruminant, human and animal feces (Bernhard and Field 2000B). Thus they successfully identified the presence of fecal contamination in samples taken from Tillamook Watershed waterways as originating from a ruminant or human source with remarkable sensitivity (Bernhard and Field 2000B). This discovery brings a missing piece of the NPS puzzle to light. That piece being the link between fecal contamination at the site of detection and its origin, the animal that defecated it. The possibilities for using this technique are enormous.

NPS identification can help the public health industry tremendously, by protecting both the welfare and economy of communities. Hours of epidemiological analyses can be dramatically reduced by knowledge of the source of a pollutant. Money can be saved by proper allocation of funds where they are needed most. Perhaps most importantly, morbidity and even mortality can be reduced by immediate response to a contamination event at its source. The stringent testing of waterways that feed into the areas in which shellfish are grown and people swim and boat is important because of the adverse health consequences that can occur from fecal contamination. By identifying the non-point source of fecal contamination, governing bodies will be able to make a more informed decision on how to stop the contamination at its origin and stop the ever looming problem of fecal pollution in our water.

Chapter 2

A Descriptive Analysis of the Waterways in Coos Bay Oregon on the Basis of General, Ruminant and Human *Bacteroides-Prevotella* 16S rDNA Markers

Thomas E. Jones Jr.

2.1 ABSTRACT

We explain a new method of detecting non point source fecal contamination using a PCR based method called Touchdown Polymerase Chain Reaction (TD-PCR). Using genetic markers particular to general, ruminant and human *Bacteroides-Prevotella* genes, we identified presence in both fresh and salt water environments. Water samples from four sites were collected at approximate 2-week intervals for a year. Samples were analyzed for total coliforms, fecal coliforms, *E. coli*, and the presence of general, ruminant and human *Bacteroides-Prevotella* markers. We compared the odds of recovering each PCR marker between sites. We investigated the relationship between rainfall and recovery of PCR markers. Finally, we compared the sensitivity of the PCR methods to standard public health methods.

2.2 INTRODUCTION

Health risks associated with water encompass both recreational waters and waters used to grow food, such as shellfish. The inability to identify the source responsible for fecal contamination is partly to blame for the persistent problem of gastrointestinal illness due to fecal pollution found in coastal and inland waters. Existing methods aid in quantifying fecal pollution, but none quickly and accurately identify the human and animal sources. Bernhard and Field proposed PCR primers specific to fecal *Bacteroides-Prevotella* 16S rDNA to distinguish between human and

ruminant feces in water (Bernhard and Field 2000B). In this study, we tested this method in the Coos Bay, Oregon, watershed.

Four distinct locations in and around the cities of North Bend and Coos Bay, Oregon were chosen as test sites. These two cities are adjacent to each other, separated only by a few blocks. The North Bend/Coos Bay area has an important role in the economy and geography of the state. The bay itself is the largest estuary on the Oregon coast, and within the estuary, the largest concentration of oyster production in the state is found (CWA 1999). Due to the large shell fishing industry, influenced by strict public health standards for fecal contamination, the Coos Watershed Association reported that high levels of bacteria associated with feces continue to interfere with economic growth and the public safety of the Coos Estuary (CWA 1999). The association also stated that closures implemented by the current management plans have impacted the businesses of the shellfish growers and harvesters in the Coos Estuary. Approximately 60,000 bushels of oysters, worth $1.8 million dollars, are produced annually (CWA 1999). Over a five year period, 1995-1999, the South Slough growing area of Coos Bay had been closed an average of 64 days a year. The larger shellfish growing area of Upper Coos Bay had been closed an average of 33 days a year over the five year period (CWA 1999).

The land around North Bend/Coos Bay is approximately two-thirds forested with the rest predominately urban (10%), rural residential (7%), agriculture (6%) and coastal/estuary (8%) (CWA 1999). There are many potential transmitting routes for non-point source (NPS) fecal contamination. An understanding of how contamination

is associated with land use could help to identify patterns indicating the presence of fecal pollution specific to an animal source. It was hypothesized that the NPS fecal contamination would not be the same among the four sites because of their differing land usage. It was predicted that rural areas would be likely to have both ruminant and human associated *Bacteroides-Prevotella* due to the high ruminant population in those areas and the use of septic systems rather than sewers.

It was also hypothesized that *Bacteroides-Prevotella* fecal bacteria would be positively correlated with rainfall. Heavy rains can cause manure from cow pastures and septic systems to leach out into streams (CWA 1999). Overloads at sewage treatment plants can also cause contamination of waterways (CWA 1999).

The results of Touch Down PCR (TD-PCR) (Hecker and Roux 1996) of fecal *Bacteroides-Prevotella* markers was compared to concentrations of standard public health indicators (total and fecal coliforms and E.coli) in order to determine the applicability, reliability and sensitivity of this new test.

2.3 MATERIALS AND METHODS

2.3.1 Sample sites

Two sloughs, the Pony Creek and Coalbank, run through the cities of North Bend and Coos Bay (Figure 2.1). Two sites from each slough were chosen based upon different land use. The North Bend High School (NB) site, located on the Pony Creek

Slough, was chosen to represent an urban residential site (Figure 2.1). This site is situated in a residential area, in the north part of North Bend. The Coca-Cola Plant (CC) site, also located on the Pony Creek Slough, is positioned upstream and south from the NB site and represents a commercial or industrial site (Figure 2.1). Even though the CC site is located within the city of North Bend, it is surrounded predominately by other businesses and homes are not in close proximity to it.

The Coalbank Slough contains the other two sites. The Highway 101 (HW) site represents both a commercial and industrial site. The area is surrounded by small businesses and industry in the southern part of Coos Bay (Figure 2.1). The Shinglehouse Slough (SS) site represents a rural agriculture site because it is situated out of town, surrounded by small farms and open fields. This site was also chosen as a rural site because the people who live in and around Shinglehouse Slough have no access to city sewer services (Figure 2.1). The Shinglehouse Slough is up the Coalbank Slough from the HW site.

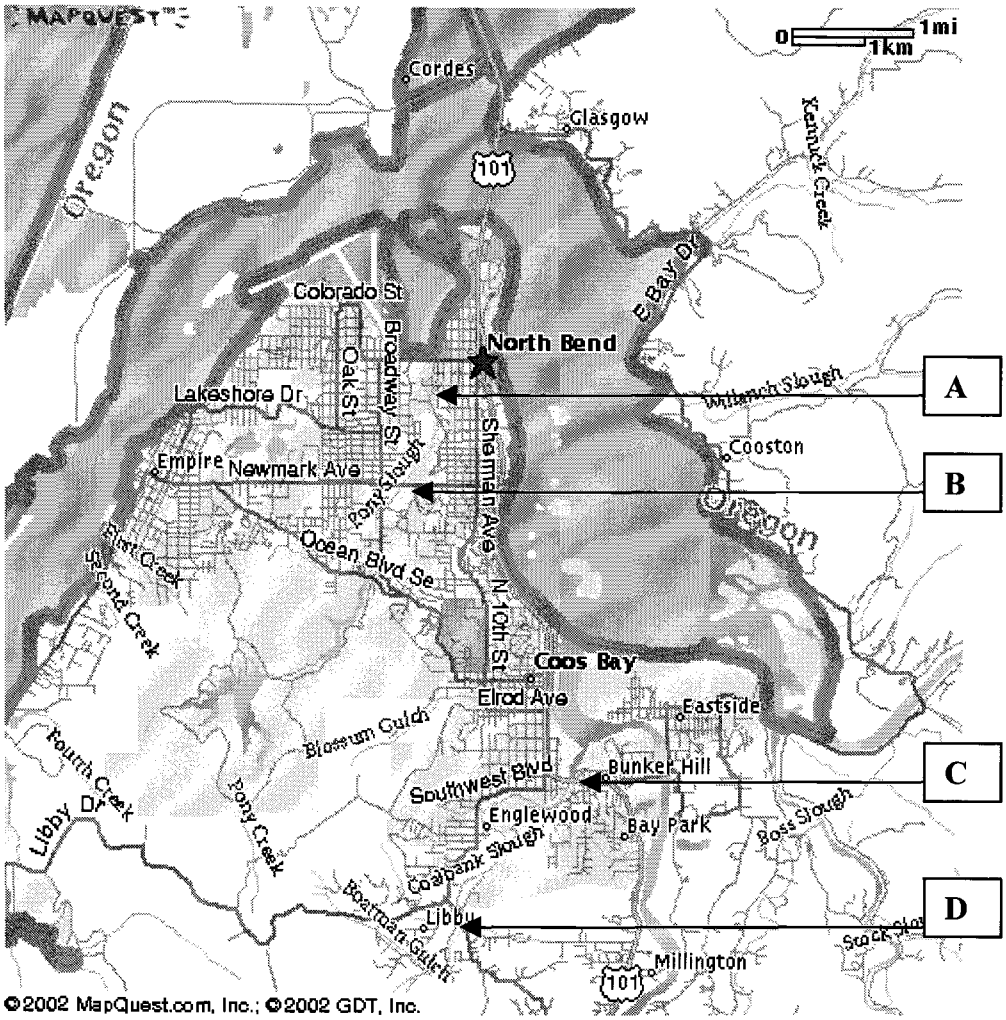

Figure 2.1 Map of North Bend and Coos Bay
A=NB site, B=CC site, C=HW site, D=SS site (MapQuest 2002)

2.3.2 Sampling

Water samples were collected bi-monthly, from March 2001 to March 2002. Separate, sterile, 500ml containers were used for each sample at each site. 100ml aliquots of each water sample were separated into three sterile containers for fecal

coliform, Colilert ™, and molecular analysis. Water samples were stored on ice during transport from the field.

2.3.3 Bacteriological tests

Fecal coliforms were quantified using the membrane filtration method (APHA 1995) at the Marshfield High School biology lab. Total coliforms and *E.coli* were measured using the Colilert ™ (IDEXX 2002) method at the South Slough National Estuarine Research Reserve lab within 24 hours of collection.

2.3.4 Sample filtration

Water filtration for molecular analysis was done at the South Slough National Estuarine Research Reserve lab. Supor-200, 0.2µm, 47mm filters (Pall Gelman, East Hills NY) were used in vacuum filtration of the 100 ml water sample. After filtration, filters were placed in 0.5ml guanidine isothiocyanate (GITC) buffer (4M GITC (Fisher Scientific, Fair Lawn NJ), 100mM EDTA and 0.5% Sarkosyl). The filters were stored at –20° C until transport to Oregon State University.

2.3.5 DNA extraction

DNAs were extracted with Qiagen DNeasy kits (Qiagen Inc., Valencia CA). Approximately 50µl of extract from each sample was stored at –20° C for preservation. These extracts were used as template in the five TD-PCR reactions.

2.3.6 PCR

PCR reactions were prepared in a negative-pressure PCR hood with UV light (Air Clean Systems, Raleigh NC). Approximately 3µl of template was used in each PCR reaction. A total of five PCRs were performed for each sample, one with general *Bacteroides-Prevotella* primers and four specific primers, two ruminant and two human. Primer sequences are given in table 2.1 (Bernhard and Field 2000B). Each 50µl PCR mixture contained: 1x PCR II buffer (ABI), 2.5mM MgCl, 200nM of each dNTP, 200nM of each primer, 0.1 units *Taq* DNA polymerase and 640ng/µl 0.4% BSA. The PCR Express Thermo Cycler (ThermoHybaid, Ashford, England) was used to perform TD-PCR on each sample.

Table 2.1 Primers used in this study

Primer	Sequence (5'-3')	Target
Bac 32F	AACGCTAGCTACAGGCTT	*Bacteroides-Prevotella**
Bac 708R	CAATCGGAGTTCTTCGTG	*Bacteroides-Prevotella**
CF 128F	CCAACYTTCCCGWTACTC	CF 123 cluster (Ruminant)
CF 193F	TATGAAAGCTCCGGCC	CF 151 cluster (Ruminant)
HF 134F	GCCGTCTACTCTTGGCC	HF 10 (Human)
HF 183F	ATCATGAGTTCACATGTCCG	HF 8 cluster and HF 74 (Human)

* Human, ruminant, pig, horse, chicken, gull, goose, duck, dog, cat and others

2.3.7 Gel electrophoresis and staining

10µl of PCR products were separated by electrophoresis on a 1.5% SeaKem LE agarose gel (BMA, Rockland ME) at 100 millivolts for 40 minutes. Two different staining techniques were used: (1) ethidium bromide (Fisher BioTech, Fair Lawn NJ)

stain was added to the gel immediately before pouring, or (2) a SYBR Gold stain (Molecular Probes, Eugene OR) was used to stain the gel for 40 minutes after electrophoresis. Gel images were captured and stored on a Transilluminator Image Store (UVP, Upland CA).

2.3.8 Quality control and quality assurance

Quality control and quality assurance (QA/QC) protocols were implemented throughout this study to eliminate PCR contamination and check for accuracy and reproducibility of results. Areas that the QA/QC protocols focused on were physical separation of reagents, sample filtration, PCR and gel electrophoresis, and inclusion of controls. This was accomplished by using four different laboratories, each having never been used for *Bacteroides-Prevotella* research prior to this study. A minimum of 1 negative and 1 positive control was used for each set of PCR reactions.

2.3.9 Statistical analysis

Logistic regression by means of the S-plus 2000 statistical package (Insightful, Seattle Wa) was used for both the between site analysis and the precipitation analysis. In this study data is given as the odds ratio, referring to the odds of finding *Bacteriodes-Prevotella* markers in one site as compared to another site. Data collected originated from samples taken bi-monthly for one year. Out of the 28 sampling dates, triplicate samples were taken at each site 17 times, or roughly 60%. It was decided to

treat each sample as independent, whether taken in triplicate or not. The samples, though not independent due to a possible spatial and temporal relationship, were treated as such because of the triplicate sampling technique used, the type of system sampled and the location of each site. For example triplicate sampling was performed at approximate 5 minute intervals, one after the other. The streams that sampling was done in were moving, non stagnant systems, and each sampling site was located in different geographical locations in and around the North Bend/Coos Bay area. Therefore we felt it permissible to treat them as independent.

Precipitation measurements were incorporated as a variable to the above analysis. Information regarding precipitation was obtained from local climatological data stored on the National Weather Service website for the North Bend Station (NWS 2002). Precipitation was measured from March of 2001, when sampling began, to the end of sampling in March of 2002. Precipitation was quantified by adding measurements together taken five days prior to sampling to the day of sampling. A measure of one inch of rainfall was used for this study because of the high precipitation observed in the North Bend/Coos Bay area.

We also looked at the probability of finding general, ruminant or human *Bacteroides-Prevotella* markers at each site. This was accomplished by using a simple equation; the number of samples testing positive for each marker divided by the number of total samples. When either one or both of the specific primers for ruminant or human gave amplification of *Bacteroides-Prevotella,* we ranked it as positive, and if no amplification was observed from both primers it was ranked as a negative.

2.4 RESULTS

281 samples were ranked for presence and absence of general, ruminant and human *Bacteroides-Prevotella* markers. Water samples from four sites were collected at approximate 2-week intervals for a year. Using data collected from the four sites, we determined if there were any differences between sites using two criteria: 1) the probability of finding general, ruminant or human *Bacteroides-Prevotella* markers, within and among sites, 2) the effect of precipitation on the probability of detecting general, ruminant or human *Bacteroides-Prevotella* markers. We also evaluated the sensitivity of the TD-PCR method used in this study compared with the public health standards, Colilert™ and HPC.

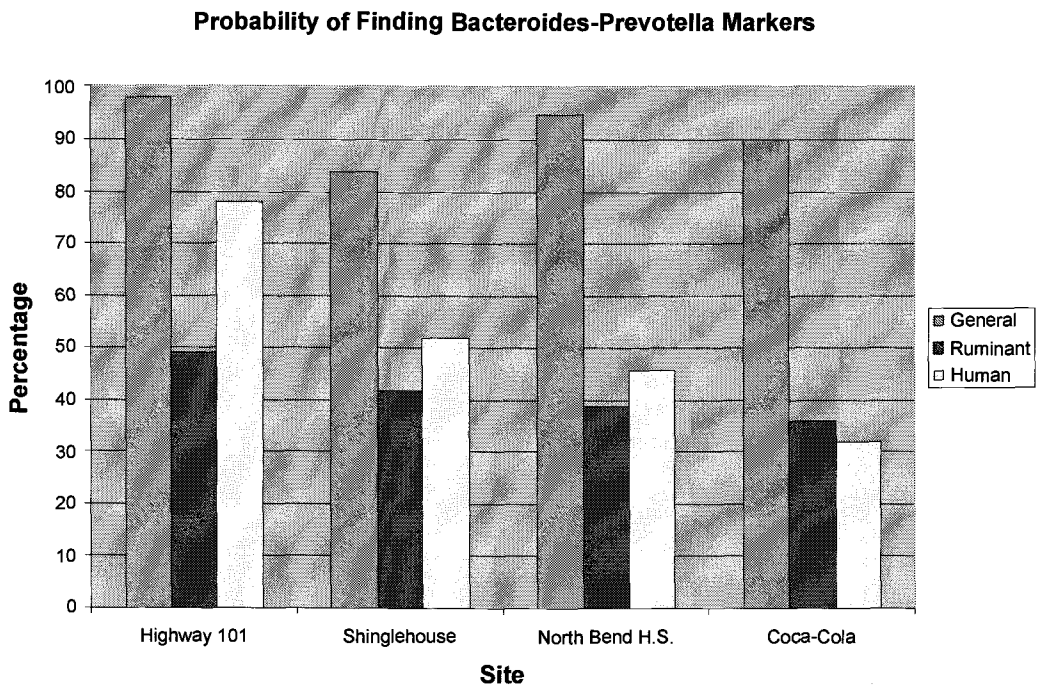

Figure 2.2 Probability of finding general, ruminant and human Bacteroides-Prevotella markers at each site.

2.4.1 General *Bacteroides-Prevotella* Markers

Site estimates: The probability of recovering the general *Bacteroides-Prevotella* marker was lowest (84.1%) at the Shinglehouse Slough and highest (98.4%) at the Highway 101 site (Table 2.2).

Table 2.2 Occurrence of General *Bacteroides-Prevotella* markers by site

Site	Est. Proportion	Standard Error	95% CI Lower Limit	95% CI Upper Limit
SS	0.841	0.044	0.754	0.927
CC	0.896	0.035	0.828	0.964
NB	0.955	0.025	0.906	1.00
HW	0.984	0.016	0.953	1.00

Site comparisons: Relationships between sites, within each slough, was estimated using logistic regression and odds ratios (Table 2.3). The odds of finding the general *Bacteroides-Prevotella* marker in the Shinglehouse Slough at the HW site was estimated to be 11.657 times the odds of finding it at the SS site (Table2.3). Similarly, in the Coalbank Slough, the odds of finding the general *Bacteroides-Prevotella* marker at the NB site was estimated to be 2.592 times the odds of finding it at the CC site (Table 2.3). For comparisons between sites, among the sloughs see table 2.3

Precipitation: Suggestive evidence of a relationship was found between the amount of precipitation five days prior to sampling and the odds of finding the general *Bacteroides-Prevotella* marker (p-value = 0.083). It was estimated that a one inch increase in rainfall was associated with a 2.120 times increase in the odds of finding the general *Bacteroides-Prevotella* marker (95% CI from 0.906 to 4.964) (Table 2.3).

Table 2.3 Between site estimates for General *Bacteroides-Prevotella* markers with rain

Site	Odds Ratio Estimate	95% CI* Lower Limit	95% CI* Upper Limit
CC/SS	1.590	0.211	11.986
NB/SS	4.122	0.269	63.250
HW/SS	11.657	0.165	823.271
NB/CC	2.592	0.154	43.600
HW/CC	7.331	0.098	549.326
HW/NB	2.828	0.026	307.971
RAIN	2.120	0.906	4.964

*Tukey's 95% CI used

2.4.2 Ruminant *Bacteroides-Prevotella* Markers

Site estimates: The probability of recovering one or both ruminant *Bacteroides-Prevotella* markers was lowest (36.4%) at the Coca-Cola site and highest (49.2%) at the Highway 101 site (Table 2.4).

Table 2.4 Occurrence of ruminant *Bacteroides-Prevotella* markers by site

Site	Est. Proportion	Standard Error	95% CI Lower Limit	95% CI Upper Limit
SS	0.420	0.059	0.304	0.537
CC	0.364	0.055	0.256	0.471
NB	0.388	0.060	0.271	0.505
HW	0.492	0.063	0.369	0.616

Site comparisons: Relationships between sites, within each slough, was estimated using logistic regression and odds ratios (Table 2.5). The odds of finding one or both ruminant *Bacteroides-Prevotella* markers in the Shinglehouse Slough at the HW site was estimated to be 1.30 times the odds of finding it at the SS site (Table2.5). Similarly, in the Coalbank Slough, the odds of finding one or both ruminant

Bacteroides-Prevotella markers at the NB site was estimated to be 1.15 times the odds of finding it at the CC site (Table 2.5). For comparisons between sites, among the sloughs see table 2.5

Precipitation: Suggestive evidence of a relationship was found between the amount of precipitation five days prior to sampling and the odds of finding one or both ruminant *Bacteroides-Prevotella* markers (p-value = 0.00001). A one inch increase in rainfall was associated with a 1.892 times increase in the odds of finding one or both ruminant *Bacteroides-Prevotella* markers (95% CI from 1.412 to 2.535) (Table 2.5).

Table 2.5 Between site estimates for ruminant *Bacteroides-Prevotella* markers with rain

Site	Odds Ratio Estimate	95% CI* Lower Limit	95% CI* Upper Limit
CC/SS	0.742	0.178	3.086
NB/SS	0.855	0.197	3.703
HW/SS	1.291	0.297	5.608
NB/CC	1.152	0.271	4.908
HW/CC	1.740	0.407	7.441
HW/NB	1.510	0.339	6.720
RAIN	1.892	1.412	2.535

*Tukey's 95% CI used

2.4.3 Human *Bacteroides-Prevotella* Markers

Site estimates: The probability of recovering both human *Bacteroides-Prevotella* markers was lowest (32.5%) at the Coca-Cola site and highest (77.8%) at the Highway 101 site (Table 2.6).

Table 2.6 Occurrence of Human *Bacteroides-Prevotella* markers by site

Site	Est. Proportion	Standard Error	95% CI Lower Limit	95% CI Upper Limit
SS	0.522	0.060	0.404	0.640
CC	0.325	0.053	0.220	0.429
NB	0.463	0.061	0.343	0.582
HW	0.778	0.052	0.675	0.880

Site comparisons: Relationships between sites, within each slough, was estimated using logistic regression and odds ratios (Table 2.7). The odds of finding one or both human *Bacteroides-Prevotella* markers in the Shinglehouse Slough at the HW site was estimated to be 3.179 times the odds of finding it at the SS site (Table 2.7). Comparing the two sites located within the Caolbank Slough, we discovered that the odds of finding one or both human *Bacteroides-Prevotella* markers at the NB site was estimated to be 1.812 times the odds of finding it at the CC site (Table 2.7). For comparisons between sites among the sloughs see table 2.7

Precipitation: Suggestive evidence of a relationship was found between the amount of precipitation five days prior to sampling and the odds of finding one or both human *Bacteroides-Prevotella* markers (p-value = 0.150). It was estimated that a one inch increase in rainfall was associated with a 1.195 times increase in the odds of finding one or both *Bacteroides-Prevotella* markers associated with humans (95% CI from 0.938 to 1.523) (Table 2.7).

Table 2.7 Between site estimates for Human *Bacteroides-Prevotella* markers with rain

Site	Odds Ratio Estimate	95% CI* Lower Limit	95% CI* Upper Limit
CC/SS	0.433	0.109	1.727
NB/SS	0.785	0.197	3.140
HW/SS	3.179	0.669	15.093
NB/CC	1.812	0.450	7.296
HW/CC	7.333	0.533	35.073
HW/NB	4.047	0.843	19.419
RAIN	1.195	0.938	1.523

*Tukey's 95% CI used

2.4.4 Sensitivity analysis

Sensitivity of the PCR method used in this study was determined by comparing results from Colilert™ and HPC to PCR results for each sample. The sensitivity of a test is the true positive rate, that is, it is a measure of the percent of general *Bacteroides* as determined by the *E.coli* and or Fecal Coliforms counts. Over 90% of the PCR tests indicated presence of the general *Bacteroides-Prevotella* marker when *E.coli* (Colilert™) was at or above EPA standards for bathing, shell fishing and recreational waters (30, 40 and 65 MPN/100ml respectively) for that same sample. Similar results were found for fecal coliforms (HPC), where over 90% of the PCR tests indicated presence of the general *Bacteroides-Prevotella* marker when fecal coliforms was at or above EPA standards for bathing, shell fishing and recreational waters (14, 35 and 200 CFU/100ml respectively) for that same sample.

2.4.5 Sample-blank filtrations

A sample-blank filtration experiment was conducted for two months to determine if cross contamination between filtrations was occurring. One hundred samples were analyzed, fifty-two field samples and forty-eight between samples filtrations (blanks). 100mls of distilled water were run through new Supor-200, 0.2µm, 47mm filters using the same vacuum apparatus as was used for the field samples. Each blank was run between field samples and then stored with the other samples in separate containers. DNA extraction and TD-PCR were performed on the blanks along with the field samples and gel electrophoresis determined if amplification occurred. 35 out of 48 blanks gave positive indication of *Bacteroides-Prevotella* bacteria for general animals. No pattern was observed between site and number of blanks. Each site had 3 blanks that had no cross contamination with the NB site having 4 blanks.

2.5 DISCUSSION

The site with the highest probability of finding general, ruminant and human *Bacteroides-Prevotella* markers was the Highway 101 site (Figure 2.2). The Highway 101 site is located near the mouth of the Coalbank Slough where it widens and becomes relatively shallow. In its entirety the Coalbank Slough runs through commercial, industrial, urban and rural areas. NPS pollutants from these places could travel in the waters of the Coalbank Slough and be deposited in and around the Highway 101 site. In addition, during high tides, the water from the bay invades the

Highway 101 site, allowing contaminants to enter this area. Taking all these factors into account, the HW site is a prime location for all types of fecal NPS pollution to collect.

The second most prevalent site for the presence of ruminant and human *Bacteroides-Prevotella* markers was the Shinglehouse Slough site (Figure 2.2). This was followed by the North Bend High School site and the Coca-Cola Plant site. The Shinglehouse Slough site is located in a rural area situated furthest south geographically in comparison to the other sites. The site is surrounded by farms, grazing land and creeks, like the Snedden. People living in this location, along with neighboring areas, have no access to city sewage services. Septic tanks and drain fields are their primary means for disposing of fecal waste. Researchers have found that fecal NPS pollution is associated with leaching septic tanks (Strittholt, Garono et al. 1998). Building upon this, Coos Bay has a high annual precipitation, averaging approximately 64 inches (NWS 2002). Due to land use and precipitation the Shinglehouse Slough site has the right setting for a general, ruminant and human fecal contamination event. Given this, an assessment of system conditions is necessary to determine if surrounding septic tanks are leaching out into the waterways, but the data from this study points in that direction.

Both the Coca-Cola site and the North Bend High School site are located with in the city of North Bend. The Pony Creek Slough runs through both of these sites. Less ruminant and human *Bacteroides-Prevotella* markers were observed at these sites compared to the sites within the Coalbank Slough (Figure 2.2). It is hypothesized that

the majority of the observed presence of *Bacteroides-Prevotella* bacteria associated with general, ruminant and human sources originated from areas outside the city and was carried in by the slough. The Pony Creek Slough begins at two rural areas, Lake Merritt and the Upper Pony Creek Reservoir. Both of these origins are prime locations for NPS pollution from animals and septic tanks to enter the slough and travel to the heart of North Bend.

Data from this study also showed that the North Bend High School site had a higher occurrence of the *Bacteroides-Prevotella* markers associated with humans than the *Bacteroides-Prevotella* markers associated with ruminants (Figure 2.1). Located further down the Pony Creek Slough, the North Bend High School site is positioned in the midst of a residential section of the city. The probability of human sewage leaking into the ground from cracked sewage lines and entering the slough is higher in this area due to the fact that the human population is greater around the North Bend High School area. General *Bacteroides-Prevotella* markers were observed more frequently at the NB site than any other site besides the HW site (Figure 2.2). One theory for this is that waste from domestic animals living within the dense neighborhoods surrounding the NB site could be running into the Pony Creek Slough in this area. Fecal waste associated with domestic animals would be detected by the general *Bacteroides-Prevotella* markers (Table 2.1). As time progresses primers for a large range animals will be discovered. Until then it is difficult to determine fecal presence from domestic animals specifically. As of today information is scarce on detailed animal influences in fecal contamination.

An interesting pattern was noticed when observing the occurrence of the general, ruminant and human *Bacteroides-Prevotella* markers for both the Coalbank Slough and the Pony Creek Slough. Each site located down stream, Highway 101 and North Bend High School, had a higher occurrence of the general, ruminant and human *Bacteroides-Prevotella* markers than their up stream counterparts, Shinglehouse Slough and Coca-Cola respectively (Table 2.3 general- HW/SS, NB/CC; Table 2.5 ruminant- HW/SS, NB/CC; Table 2.7 human- HW/SS, NB/CC). One possible explanation for this is that *Bacteroides-Prevotella* bacteria remain in water for the duration of time that would allow it to accumulate as it traveled, thereby gradually increasing in abundance.

Precipitation made a noticeable impact on fecal pollution: a one inch increase of rain fall was associated with the likelihood of finding general, ruminant and human *Bacteroides-Prevotella* markers. The data showed that one inch of rain within 5 days of sampling gave a 2.12 times increase in the odds of finding general *Bacteroides-Prevotella* markers (95% CI from 0.906 to 4.964), a 1.89 times increase in the odds of finding ruminant *Bacteroides-Prevotella* markers (95% CI from 1.412 to 2.535), and a 1.19 times increase in the odds of finding human *Bacteroides-Prevotella* markers (95% CI from 0.938 to 1.523). Since most fecal matter is generally deposited on the ground surface, it is not surprising that precipitation would play a role in the likelihood of observing general and ruminant *Bacteroides-Prevotella* markers. In this study feces from humans, for the most part, was contained underground, either in drain fields,

septic tanks or sewer lines. It would make sense that one inch of precipitation affects the occurrence of human fecal contamination less.

In order to appreciate the full effect of fecal contamination in the waterways of communities, an understanding of how much fecal contamination normally occurs in such waterways is needed. It can be assumed that in general wherever animals are fecal matter is also. Traces of fecal matter, existing as "background", from animals is a part of the natural environment. When fecal concentrations exist above background problems can arise. Unfortunately this is occurring in the rivers, lakes and estuaries of the United States. Approximately 40 percent of these areas are not clean enough to meet basic uses such as fishing or swimming and 60 percent of the impaired river miles and half of the impaired lake acreage surveyed are corrupted by agriculture runoff (USEPA 2002). Data on average fecal concentrations in waterways of the United States could help in indicating background levels, danger zones and patterns leading to contamination events. Unfortunately no such information was found in the databases of the Environmental Protection Agency. Compiling such information would be invaluable to understanding the dangers of Non-point source fecal contamination.

2.6 CONCLUSION

Contributions from this research include: (1) successful identification of three non-point sources associated with fecal pollution in the waterways in and around the

North Bend and Coos Bay area; (2) land use differences in regard to NPS prevalence; (3) evidence that precipitation plays a role in fecal prevalence. Further development of primers that target other animals would be necessary for a complete identification of all animals responsible for fecal contamination.

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