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Microbial Source Tracking in the Dungeness Watershed, Washington

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Richland, Washington 99352

Prepared for
Jamestown S'Klallam Tribe
in fulfillment of Task 1 (Microbial Source Tracking Study)
of the Dungeness River Watershed Final Workplan
for the EPA Targeted Watershed Grant Program (2004),
and a Washington State Department of Ecology Centennial Grant

September 2009

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Executive Summary

Two microbial source tracking (MST) studies were conducted in the lower Dungeness watershed and Dungeness Bay in order to determine the sources of fecal coliform pollution that have been impacting the water quality and shellfish harvesting activities for more than a decade. Between 2006 and 2009, two independent and sequential studies were conducted by Battelle – Pacific Northwest Division's Marine Sciences Laboratory in Sequim Washington under contract to the Jamestown S'Klallam Tribe. The first study (*Phase 1 – Ribotyping*) was implemented through an EPA Targeted Watershed grant awarded to the Tribe, and was aimed at determining predominant sources of bacterial contamination at selected stations in the lower watershed and Bay through a genotypic DNA-based ribotyping approach. The second study (*Phase 2 – Bacteroides Target-specific PCR*) was implemented through a Washington State Department of Ecology Centennial grant and designed to collect additional information regarding the presence of human and ruminant sources from an expanded number of stations in the freshwater and marine environment. The results of both studies are reported here.

The Phase 1 Ribotyping study was initiated in May 2006. Environmental samples were collected for ribotyping analysis from four freshwater stations (MAT0.1, MC0.3, GSS, BD-7) in the lower Dungeness watershed, two marine stations in Dungeness Bay (DOH-113, DOH-108) where water and underlying sediment were collected, and wrack (detrital algae) from the nearby shoreline. Sampling occurred once a month for 13 months and was completed in May 2007. The ribotyping methodology is a library-dependent approach, meaning that fragments of DNA from the bacteria *E. coli* are cultivated from an environmental sample such as water and are then matched to DNA fragments for *E. coli* cultivated from known fecal sources in a host reference library. During our study, additional fecal samples were collected from 45 known local animal and bird species and added to the preexisting reference library database managed by the Institute of Environmental Health, Inc.

There were 1164 *E. coli* isolates ribotyped during the Phase 1 study. Of those, the percentage of matched sources was relatively high (92%) with 34 species or groups identified in the watershed. While the Dungeness watershed contains typical non-point sources of bacterial contamination, it also contains diverse and unusual sources such as marine mammals and non-native game farm animals. The predominant sources of fecal coliform contamination in the Dungeness from all matrix types (e.g. water, sediment, wrack) in the freshwater and marine environments were, in rank order, avian (19.6%), gull (12.5%), waterfowl (9.7%), raccoon (9.2%), unknown (7.3%), human-derived (7.1%), rodent (6.3%) and dog (4.3%). When bird groups were combined, they represented in total about 42% of samples collected and analyzed throughout the study. They occurred from at least 85% of the sampling events at all freshwater and marine water stations and from at least 56% of marine sediment sampling station events.

When grouped together, wild mammal sources represented about 26% of isolates collected and included raccoons, rodents, deer, elk, beaver, otter, rabbit and marine mammals. Domestic animal and farm animal groups each represented about 7% of isolates. Source types representing animals from the Olympic Game Farm, Inc. were also found during the study, including bear, bison, burro, prairie dog and yak. Together, these sources accounted for about 2.5% of the total isolates collected. Game farm source types were present 46% of the time at MAT0.1, the freshwater station located on the Game Farm near the mouth of Matriotti Creek. These occurrences were detected throughout the year.

Human-derived sources included onsite sewage disposal systems, sewage treatment plant waste, and direct human sources. Human-derived sources were found at all freshwater stations, all marine water stations and one marine sediment station. These sources represented between 3 and 15% of the isolates for any given station. They were present throughout the year, occurring in 25 to 69% of the sampling events by station. Matriotti Creek (MAT0.1) had the highest frequency of occurrence of human sources, occurring during 9 out of 13 sampling events. Meadowbrook Creek (MC0.3) and a bluff ditch station (BD-7) also had human-derived sources present at least half of the time.

While there were no statistically significant trends through time during the study year, the proportion of wild mammals and game farm sources, when combined, increased slightly during the wet season (October through March). This trend may indicate the possible influence of surface runoff events. In general, freshwater source host organisms such as wild mammals, and domestic and farm animals were found consistently in marine water, sediment and wrack, indicating the conveyance of these sources into the marine environment.

The Phase 2 *Bacteroides* target-specific polymerase chain reaction (PCR) study was conducted over a shorter time period, between December 2008 and January 2009. This study had fewer sampling events (3 in total), however encompassed more stations in the lower watershed and Bay. A total of 21 stations were sampled during the study, with some sampled more than once. The *Bacteroides* methodology is a library-independent approach. This method amplifies known DNA biomarkers from *Bacteroides*, a fecal indicator bacteria, to match with *Bacteroides* DNA in water samples. The (PCR method is used to amplify the biomarker by replicating a target DNA sequence. The biomarkers available for this study included human and ruminant (e.g. cattle, bison, deer, goat).

A total of 42 samples were collected during Phase 2 and analyzed by EPA Region 10. Overall, there were relatively few samples that were identified as human or ruminant. Seventeen samples contained the general *Bacteroides*-only marker, indicating a fecal source other than human or ruminant. Two out of 42 samples were identified as ruminant-only and were collected from sites not sampled during Phase 1 (Bluff Seep-8 and Cooper Creek). Two additional samples were positive for human sources, from Meadowbrook Slough (MS0.3) and Meadowbrook Creek (MC0.2), also sites that were not included in the Phase 1 study. One sample from Cassalery Creek tested positive for both human and ruminant sources. The remaining 20 samples did not contain any *Bacteroides* fecal indicator bacteria. While the overall samples collected as part of Phase 2 of the study yielded few samples containing *Bacteroides*, the detection of human and/or ruminant-derived source bacteria occurring at sites not sampled during Phase 1 indicates a wider spread of these sources throughout the lower watershed.

The Phase 2 *Bacteroides* PCR study complemented the Phase 1 ribotyping study. The Phase 1 study identified a large variety of sources, while the Phase 2 study confirmed the presence of human and ruminant biomarkers from a larger region in the lower Dungeness watershed. Each of the microbial source tracking methods used in these studies had discrete strengths and weaknesses. While it is generally recognized that MST methods are still evolving and have not been standardized, both methodologies proved helpful in understanding the underlying sources of fecal contamination in the watershed. The ribotyping study (Phase 1) was comprehensive in nature and had a relatively high percentage match to identifiable sources (92%). However, it was more expensive and final test results were not available for close to a year after the last sampling event, making it an impractical technique for routine application. The *Bacteroides* study (Phase 2) was a smaller scaled study and less expensive on a per sample basis due to the library-independent approach and cultivation of the host organism is not

required. The results were accurate based on blind samples submitted, and the turnaround time for sample analysis and reporting was on the order of several months. However, only two primers (human and ruminant) were available for use in this study. In the future, the *Bacteroides* approach could be greatly enhanced by the incorporation of additional primers.

Overall, these methods were successful in providing empirical scientific evidence of the predominant sources of fecal contamination in the lower Dungeness watershed and Bay. While some of these contamination sources may be difficult to manage (i.e. birds and wild mammals), the microbial source tracking study also provided evidence of sources in the watershed that can be controlled or mitigated for, such as human-derived sources that can be considered a public health risk. Results of the combined studies provide the basis for continued education and public outreach regarding sources of bacterial contamination in the Dungeness watershed. For example, the scientific knowledge gained from this study provides the opportunity for resource managers to strengthen on-site septic system management programs and ensure they are aimed at reducing bacteria levels in the watershed. In addition, the results of the microbial source tracking study provide the basis for re-evaluation of the progress made toward achieving the goals of the *Clean Water Strategy and Water Cleanup Detailed Implementation Plan* and for making adjustments to current or future monitoring plans or cleanup strategies. While these results should not be extrapolated to other geographic regions, information gained from the overall approach, the application of these tools, and lessons learned can be applied to other watersheds.

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Acronyms and Abbreviations

CFU	colony forming unit
BMP	best management practice
DNA	deoxyribonucleic acid
DOE	Department of Ecology
DOH	Department of Health
EPA	Environmental Protection Agency
FC	fecal coliform
IEH	Institute of Environmental Health
MST	microbial source tracking
PCR	polymerase chain reaction
QAPP	Quality Assurance Project Plan
TMDL	Total Maximum Daily Load
TSS	total suspended sediments
TWG	Targeted Watershed grant

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1.0 Introduction

For the past several decades, the Dungeness River, its tributaries, and Dungeness Bay (collectively referred to as the Dungeness watershed) on the Olympic Peninsula in Washington State have experienced a decline in water quality. The decline in part, has included elevated levels of fecal coliform (FC) bacteria. This condition has placed the lower Dungeness River and several of its tributaries on Washington State's 303(d) list of impaired waters for bacteria violations, and resulted in downgrades in marine water quality classifications in Dungeness Bay that have led to the closure of shellfish harvest areas. A variety of responses and remedial actions have been undertaken by local agencies, Jamestown S'Klallam Tribe, and state and federal agencies in an effort to understand the sources of FC pollution and develop strategies to reduce the bacteria levels.

Between 2006 and 2008, researchers at Battelle—Pacific Northwest Division's Marine Sciences Laboratory in Sequim, Washington, under contract to the Jamestown S'Klallam Tribe conducted two independent microbial source tracking studies in order to determine what the predominant sources of fecal coliform pollution are in the Dungeness watershed. These studies were called out as recommendations in the *Clean Water Strategy for addressing Bacteria Pollution in Dungeness Bay and Watershed and Water Cleanup Detailed Implementation Plan* (Hempleman and Streeter 2004). They were implemented through an EPA Targeted Watershed grant awarded to the Tribe in 2004 and a Washington State Department of Ecology Centennial Grant awarded to the Tribe in 2006. The results of both studies are reported here.

1.1 Background

Since 1991, elevated levels of FC bacteria in Matriotti Creek, have been monitored and documented by the collective efforts of Clallam County, the Clallam Conservation District, and the Jamestown S'Klallam Tribe. In 1996, Matriotti Creek was placed on Washington's 303(d) list of impaired waters because of FC violations. In 1997, the Washington State Department of Health (DOH) reported increasing levels of FC bacteria in Dungeness Bay near the mouth of the Dungeness River (DOH 1998). In response to this, the Jamestown S'Klallam Tribe initiated water quality monitoring of several tributaries adjacent to the bay, hoping to find a definitive source that would explain the elevated bacteria levels. When it was determined that a number of tributaries were not meeting the water-quality standards for FC bacteria, it became evident that the poor water quality may be due to a variety of diffuse sources in the Dungeness River, its tributaries, and Dungeness Bay, exhibiting a classic case of nonpoint source pollution.

In 1998, the Washington State Department of Ecology (DOE) agreed to conduct a Total Maximum Daily Load (TMDL) study on Matriotti Creek and several other freshwater tributaries in the lower Dungeness watershed, in order to develop targeted reduction levels for FC and prioritize bacterial pollution control actions. In cooperation with the Jamestown S'Klallam Tribe and Clallam County, DOE began a year of monitoring in November 1999 in the Lower Dungeness River. As a result of that study, TMDL recommendations included a stringent FC target for the Dungeness River and bacteria loading reductions for the tributaries leading to Dungeness Bay (Sargeant 2002). The FC targets were more stringent than current freshwater standards because the intent was to achieve shellfish-harvestable FC results, which necessitate a stricter standard.

During the freshwater TMDL study, elevated FC levels in the bay continued to persist. In 2000, the high bacteria levels caused a reclassification by DOH of 300 acres of the bay near the mouth of the Dungeness River from *Approved for Shellfish Harvest* to *Prohibited for Shellfish Harvest*. An additional 100 acres were downgraded in 2001. In 2003, DOH changed the classification of the inner Bay to *Conditionally Approved for Shellfish Harvest*. This required the inner bay to be closed annually during the rainy season from November 1st through January 31st (Melvin 2003) (Figure 1.1). These closures are currently still in effect. As a result of the continued concern in the bay, a circulation study was conducted with particular emphasis placed on understanding FC sources and transport pathways (Rensel 2003).

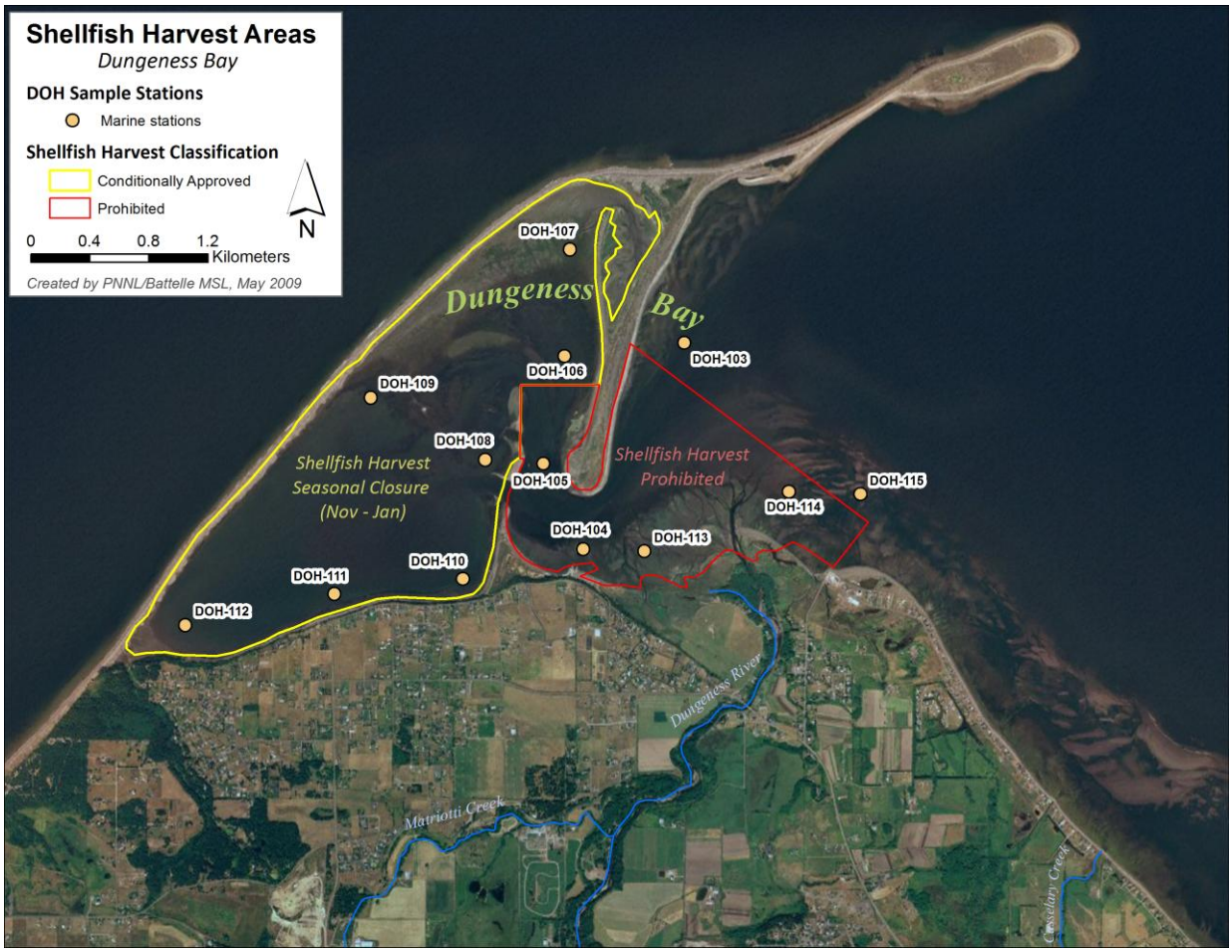

Figure 1.1 Shellfish Harvest Closure Areas and Department of Health Sampling Stations in Dungeness Bay

After the downgrade of the shellfish harvest areas, DOH initiated a closure response process. This included convening a Response Team, now called the Clean Water Workgroup that was formed in 2001, and developing a shellfish protection district (Clean Water District) by Clallam County. The Clean Water Workgroup developed a response plan, the *Clean Water Strategy for Addressing Bacterial Pollution in Dungeness Bay and Watershed* (Clean Water Workgroup 2002). In the *Clean Water Strategy*, a microbial source tracking study was identified as a high priority project. Based on that recommendation, a feasibility study was conducted in 2003 to review potential microbial source tracking methods that would

be applicable to the Dungeness watershed (Woodruff and Evans 2003). Two of the molecular methods included in that review were implemented and are the focus of this report.

1.2 Study Area and Land Use

The Dungeness watershed is located on the Olympic Peninsula near Puget Sound in Washington State (Figure 1.2). The major freshwater tributary to Dungeness Bay is the Dungeness River, which originates in the Olympic Mountains and flows 32 miles through wilderness, forested, agricultural, and residential areas into the bay. The upper two-thirds of the watershed are in the Olympic National Park and Olympic National Forest. The lower portion of the watershed flows predominantly through private land. The watershed lies in the rain shadow of the Olympic Mountains, where annual precipitation varies from 15 inches in the lower watershed to approximately 80 in. at the headwaters of the Dungeness River (Clallam County 1993).

Figure 1.2 Overview of Olympic Peninsula Showing Dungeness Watershed

The Dungeness River is the primary freshwater tributary flowing into Dungeness Bay. The bay is partly enclosed within a sand spit that extends approximately 5 miles eastward into the Strait of Juan de Fuca and is home to the Dungeness National Wildlife Refuge. The U.S. Fish and Wildlife Service manages approximately 131 hectares within the bay as a wildlife refuge that provides habitat for a multitude of migrating birds and marine mammals. Recreational enthusiasts enjoy kayaking, wind surfing, and bird watching in the bay. A limited fishery is used by multiple groups including Tribal,

commercial, and recreational fisherman. The bay has historically been used for shellfish harvesting by both Tribal and non-Tribal residents.

The lower Dungeness watershed has become increasingly dominated by residential land use with less emphasis on agricultural use of the land (Figure 1.3). Although the City of Sequim is on a sewer system, residences and commercial establishments in the unincorporated areas of the watershed use onsite sewage treatment systems. In addition, over 40% of homes in the area are located on or near a water body (e.g., Dungeness Bay, Dungeness River, Strait of Juan de Fuca, wetlands, creeks, and irrigation ditches) and many of those homes use onsite septic disposal systems. The Dungeness River is the source of water for an extensive irrigation network that serves agriculture, hobby farm, and residential community use. In the Dungeness basin, there are well over 100 miles of irrigation ditches, creating a complex network of surface water conveyance through the Dungeness valley. For over 20 years, local and regional institutions have worked collaboratively to maintain and restore ecosystem functions in the Dungeness watershed. However, as the area has been slowly converted from forest to agricultural and residential land uses, the challenges of maintaining healthy ecosystem function have increased.

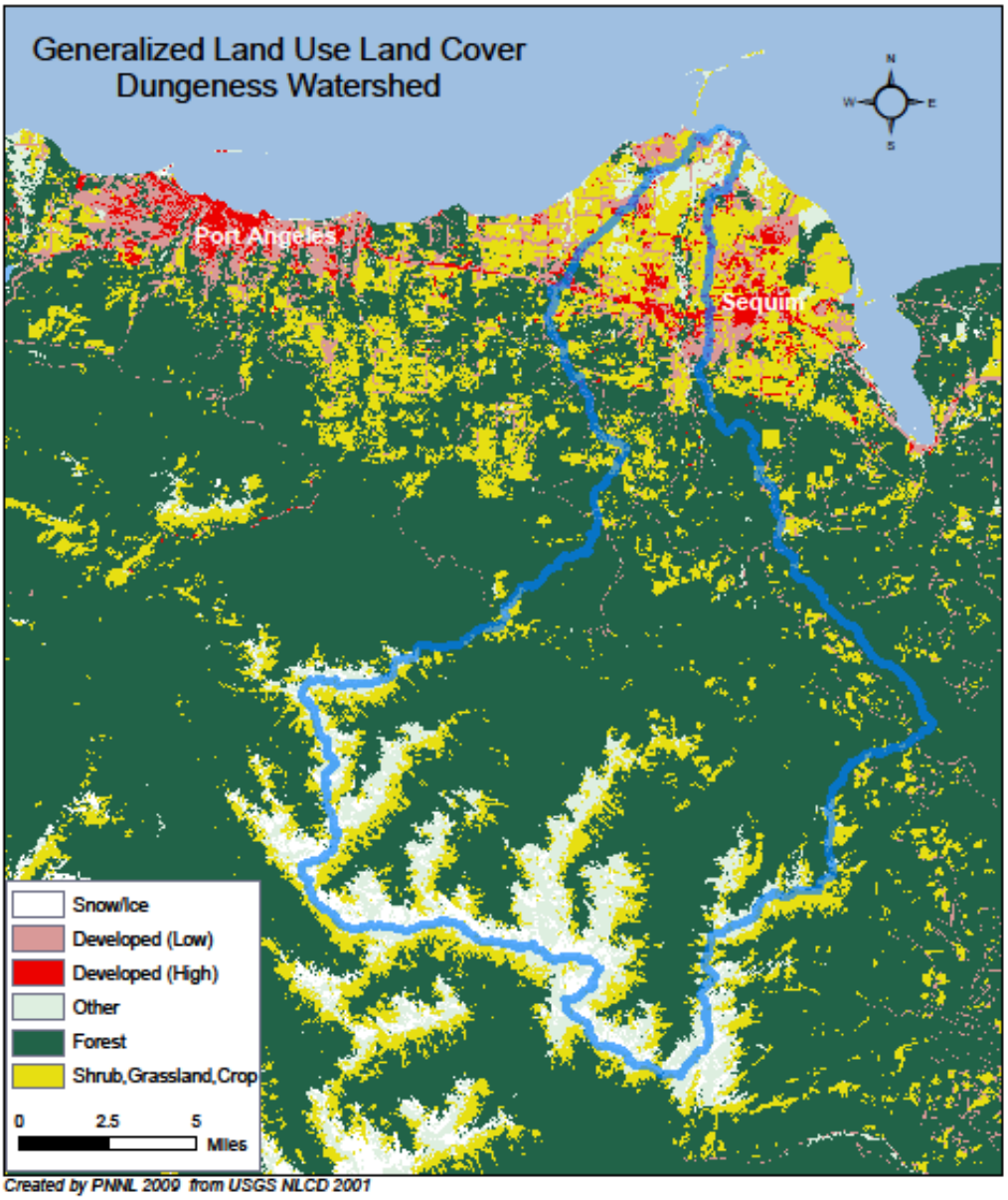

Figure 1.3 Land Use/Land Cover Attributes in the Dungeness Watershed

1.3 Potential Pollution Sources

In recent years, human-induced impacts have impaired the natural function of the river and bay. A variety of watershed health problems have ensued, resulting in the listing of salmonid species under the Endangered Species Act and closure of Dungeness Bay to shellfish harvesting beginning in 2000 (Sargeant- 2004) due to high levels of FC bacteria. As floodplain development continues in the region, issues of storm water runoff, failing septic systems, and impaired in-stream flows persist.

Within the Dungeness watershed, potential pollution sources of FC bacteria are primarily from diffuse (non-point) and diverse sources. These include but are not limited to the following, in alphabetical order:

- avian (waterfowl, gulls, etc…)
- failing septic systems
- farm animals (cattle, horses)
- farm-raised non-native wildlife (yak, bison, etc…)
- marine mammals (seals, sea lions)
- pets (dogs, cats, rabbits)
- recreational boaters
- wildlife (raccoon, deer, elk, rodents).

1.4 Microbial Source Tracking

Protection from pathogenic microbes is a concern in waters used for drinking water, recreation, and fish and shellfish harvesting. This is commonly assessed by monitoring for the presence of indicator bacteria such as fecal coliform, *E. coli* or fecal enterococci. These microorganisms are associated with fecal material from humans and other warm blooded animals, and their presence in water may indicate the presence of enteric (intestinal) pathogens. Detecting indicator bacteria and tracking them to their source is also of interest in light of current TMDL requirements. TMDL's are used to establish the maximum pollutant load that a water body can receive and still meet water quality standards. However nonpoint sources of fecal pollution from agricultural practices, wildlife, and storm water runoff are exceedingly difficult to track to source of origin. Without understanding the sources of non-point pollution and developing mitigation strategies where practical, it is extremely difficult to meet TMDL targets. In recent years microbial source tracking (MST) methods have been used to inform bacterial TMDL programs in order to track predominant source(s) of non-point fecal pollution impacting surface waters (Santo Domingo *et al*. 2007; Stoeckel and Harwood 2007; USEPA 2005).

Various MST approaches have been developed to associate specific types of animals, birds, or human sources with fecal pollution of natural waters. In general MST methods can be divided into two broad categories. The first are "cultivation-dependent" methods, meaning they rely on the culture of the target bacteria with further analysis based on some aspect of a bacteria's DNA sequence (genotypic or molecular approach), or some measure of a trait that is expressed (phenotypic or biochemical approach). These culture dependent methods usually rely on selective cultivation of indicator bacteria such as *E. coli* or *Enterococcus* sp. from water samples as well as from known fecal sources that are used to construct a host reference library of signature "fingerprints" for comparison. The second broad category of MST methods includes "cultivation-independent" methods which directly analyze samples from the environment using a molecular approach and don't require the culture of the target bacteria or the development of a source library. These methods are used with bacteria that are not easily cultivated in the laboratory, but are still considered common genera occurring in intestines of animals (e.g. *Bacteroides*, *Bifidobacterium*) (USEPA 2005).

In the Dungeness watershed we used two MST approaches to address recommendations provided in the *Clean water Strategy for Addressing Bacteria Pollution in Dungeness Bay and Watershed, and Water Cleanup Detailed Implementation Plan* (Streeter and Hempleman 2004). For the Phase 1 study we used a ribotyping method which is a cultivation-dependent, host-library dependent approach. For the follow-on Phase 2 study we used a *Bacteroides* target-specific method, which is a cultivation-independent approach that doesn't require the development of a host-reference library for comparison.

The ribotyping method used in the Phase 1 study is a genetic fingerprinting approach. It is based on the premise that the DNA fingerprint of the *E. coli* bacteria living within the gut or intestine of a particular species of animal, although genetically similar to other species, has certain unique differences in the DNA sequence that can be used to match to an *E. coli* bacteria from an unknown source (e.g. water or sediment sample). Hence this method requires a large database of known or typed source *E. coli* isolates that is used for comparison against unknown sources of *E. coli*. The ribotyping method is a multiple step process that involves restriction enzyme digestion of genomic DNA, separation of DNA fragments by gel electrophoresis, immobilization of fragments to a solid matrix and subsequent hybridization using a labeled probe of the *E. coli* rRNA genes. Fragments of ribosomal RNA from *E. coli* that are cultivated from an environmental sample such as water or sediment are then matched to RNA fragments from *E. coli* cultivated from known fecal sources in a host reference library.

The *Bacteroides* target-specific PCR approach used during the Phase 2 study is cultivation – independent method. This method amplifies known DNA biomarkers from *Bacteroides* to match with *Bacteroides* DNA in water samples. The polymerase chain reaction (PCR) method is used to amplify the biomarker by replicating a target DNA sequence such that it can be visualized after gel electrophoresis and staining. While a number of primers have been developed, only several are readily available. This study used primers that were available to the EPA Manchester Laboratory to identify human and ruminant sources of *Bacteroides.*

1.5 Project Objectives and Approach

This report focuses on the results of two independent and sequential studies aimed at determining the source (host organisms) of FC bacterial contamination in the Dungeness watershed and bay. The first study (referred to as *Phase 1 – Ribotyping Study* in this report) is part of a U.S. Environmental Protection Agency (EPA) Targeted Watershed grant awarded to the Jamestown S'Klallam Tribe and its partners in 2004. The second study (referred to as *Phase 2 – Bacteroides Target-specific PCR Study*) was funded through a Washington State Centennial grant awarded to the Jamestown S'Klallam Tribe in 2004. The Tribe contracted with Battelle – Pacific Northwest Division to conduct these studies.

The Phase1 ribotyping study was one of three tasks conducted under the EPA Targeted Watershed grant that focused efforts on surface water cleanup in the lower Dungeness watershed. The EPA Targeted Watershed grant program was established in 2003 to encourage innovative community-based approaches and management techniques to protect and restore clean water in the nation's watersheds. The Dungeness Targeted Watershed grant focused efforts between 2004 and 2009 on the following tasks as part of the Initiative that are ultimately related to surface water restoration activities in the watershed:

Task 1 a MST study to more precisely define pollutant sources in the lower Dungeness watershed and Dungeness Bay (the focus of this report);

Task 2 innovative best management practice (BMP) demonstrations (and market-based incentives for BMP implementation) related to water quality treatment including a mycoremediation treatment demonstration, septic system maintenance, and water conservation; and

Task 3 an Effectiveness Monitoring study, to compare the effectiveness of various BMP demonstrations within the watershed and examine the historic context within the watershed.

Task 1, under the Targeted Watershed grant, was a MST study aimed at more precisely defining FC bacteria sources in the watershed. This study used a ribotyping approach to address questions raised from earlier TMDL studies in the lower river (Sargeant 2002) and the bay (Sargeant 2004). Sites were selected in these two regions and sampled over a one-year time period to characterize the predominant sources (host animals) of *E. coli* bacteria and to assess the potential for mitigation. Specific objectives for Phase 1 included the following:

- Characterize the predominant sources of FC bacteria in the lower Dungeness watershed and Dungeness Bay.
- Determine the predominant sources of FC at key specific sites and examine the differences between sites.
- Determine if and/or what temporal trends may exist based on the time period sampled (1 year).
- Determine what sources may be controllable.
- Use results from this study for public outreach, mitigation where practical, and improvement of the design of future water-quality monitoring projects.

The second study (referred to as *Phase 2 – Bacteroides Target-Specific PCR* in this report) was funded through a Washington State Centennial grant awarded to the Jamestown S'Klallam Tribe. This study used a *Bacteroides* target-specific PCR (polymerase chain reaction) methodology to focus on confirming the presence/absence of human and ruminant sources at selected stations in the Dungeness watershed. Most of the stations sampled during the Phase 1 study were included in the Phase 2 sampling plan along with additional sites of concern or interest, based on the results of the first study. *Phase 2* was conducted approximately one and a half years after Phase 1 ended and was of shorter duration. Specific objectives for *Phase 2* included the following:

- Verify the presence or absence of human and ruminant sources at previous Phase 1 MST monitoring stations in the watershed.
- Determine the presence or absence of human and ruminant sources at additional selected monitoring stations in the watershed.
- Assess the spatial extent of human and /or ruminant sources in Dungeness Bay during the Shellfish Harvest Conditional Closure time period.
- Assess the presence of human and/or ruminant sources from freshwater seeps located along inner Dungeness Bay.
- Use the results of the study to target specific waste-reduction BMPs.
- Foster community education and outreach regarding sources of human fecal contamination in the Dungeness watershed.

1.6 Report Contents and Organization

The Phase 1 and Phase 2 methods (Section 2.0) and results (Section 3.0) are reported as independent studies. The discussion (Section 4.0), and conclusions and recommendations (Section 5.0) are based on the combined and collective knowledge gained from each independent study and reported as an integrated effort.

2.0 Study Design and Methodology

2.1 Phase 1 – Targeted Watershed Grant (Ribotyping Study)

The sampling stations, sampling approach, analytical methodology and quality assurance objectives for the Phase 1 - Targeted Watershed Grant Ribotyping Study are described below.

2.1.1 Sampling Sites

The results of TMDL studies conducted in the Dungeness River and Matriotti Creek (Sargeant 2002) and Dungeness Bay (Sargeant 2004) indicated that there were sources of elevated FC concentrations in the lower Dungeness watershed and in Dungeness Bay near the mouth of the Dungeness River. Based on these observations and criteria established in the Quality Assurance Project Plan (QAPP; Battelle 2005), six stations were selected (two marine and four freshwater) (Figure 2.1). Water was collected from all sites, and sediment and vegetation was collected from marine sites, as described below:

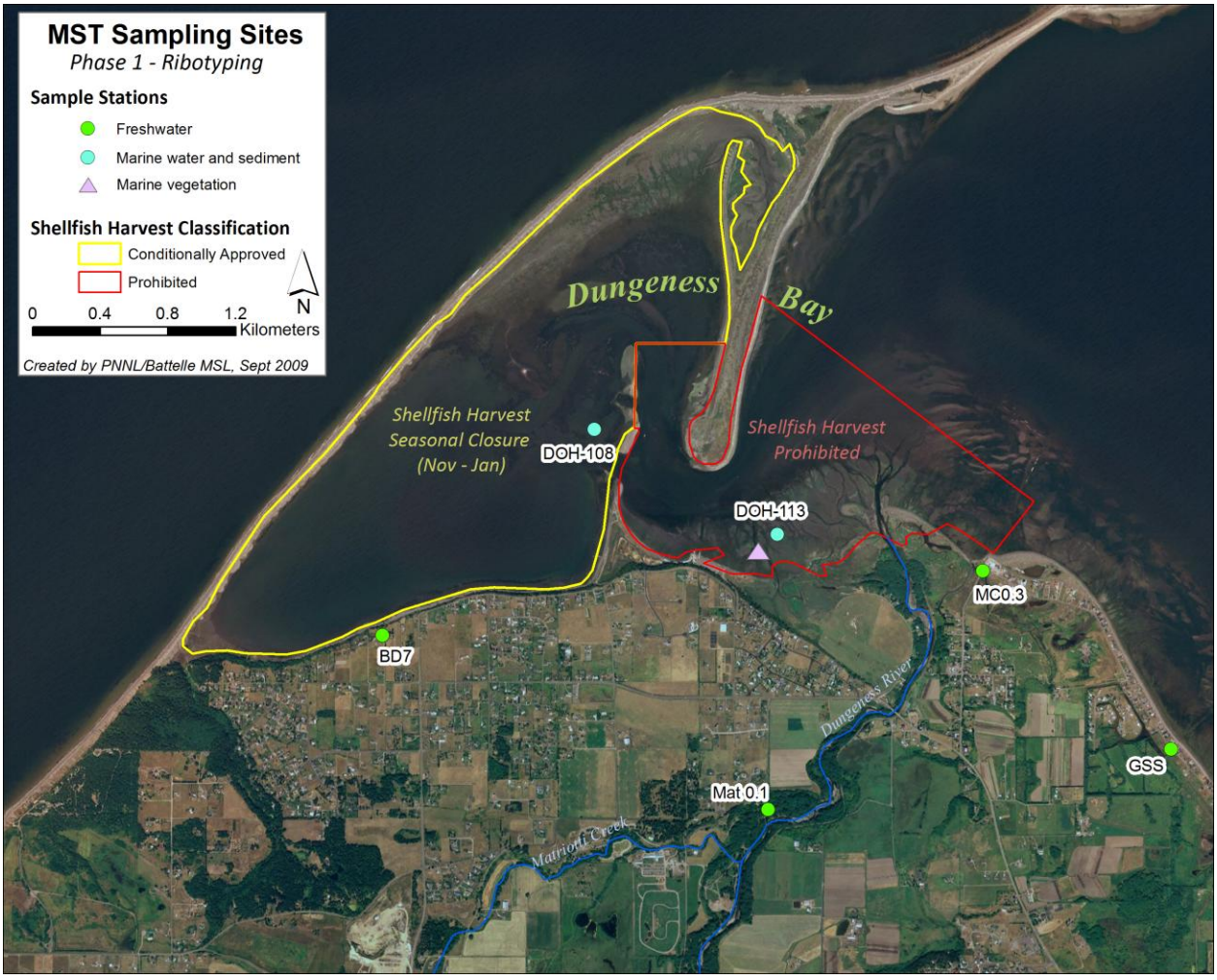

Figure 2.1 Microbial Source Tracking Stations Sampled During the Phase 1–Ribotyping Study.

- **Freshwater Stations**
  - MAT 0.1 (*Matriotti Creek, river mile 0.1)* – This sampling station was located on the Olympic Game Farm at the mouth of Matriotti Creek, just before the creek enters the Dungeness River. Land uses in the sub-basin include residential, commercial, cropland, and livestock, including beef and dairy cattle, horses and the Olympic Game Farm, which is a large wild animal park. Matriotti Creek was also used as a conveyance for the irrigation water in recent past, a practice that rarely occurs today. This site was included in the Lower Dungeness TMDL study (Sargeant 2002).
  - MC 0.3 (*Meadowbrook Creek, river mile 0.3)* – This sampling station was located near the mouth of Meadowbrook Creek before it enters Dungeness Bay. Land uses in the sub-basin include a horse farm near the mouth (no longer in use), a brackish tidal marsh used by birds, agricultural, residential, and some commercial use in the local community of Dungeness. All residences and commercial properties are on onsite sewage treatment systems. This site was included in the Lower Dungeness TMDL study (Sargeant 2002).
  - GSS (*Golden Sands Slough*) – This sampling station was located in a small drainage of constructed canals supporting a residential development close to Dungeness Bay. The canal is connected to the bay by an approximate 250-ft long concrete culvert under Three Crabs Road. Access to the bay is sometimes blocked by debris, creating flooding in some areas. The land use there is residential, with some lots served by septic systems and others using holding tanks. This site was included in the Lower Dungeness TMDL study (Sargeant 2002). The sampling location is just south of Three Crabs Road.
  - BD7 *(Bluff Ditch #7)* – This sampling station was in an irrigation ditch that empties into Dungeness Bay. The ditch is periodically dry, however historically it had more flow than other Marine Drive ditches in the same vicinity. The ditch occasionally conveys storm water during the rainy season. It was included in the Dungeness Bay TMDL study (Sargeant 2004).

- **Marine Stations**
  - DOH-113 water – This sampling station was located just off the mouth of the Dungeness River; it contains both marine and freshwater (Dungeness River) influences. This station is located in the Prohibited shellfish closure area. It was included in the Dungeness Bay TMDL study (Sargeant 2004).
  - DOH -113 sediment –The upper 3 cm of sediment were collected using a grab sampler, and was co-located with the DOH-113 water sample station.
  - DOH-113 vegetation – This sampling station was a wrack line of detrital vegetation (primarily the green alga *Ulva* sp. and eelgrass *Zostera marina*) located along the upper fringe of the intertidal region of outer Dungeness Bay. The collection was seasonal and location varied slightly over time within the vicinity of DOH-113, depending on the presence or absence of detrital wrack.
  - DOH-108 water – This sampling site was located on inner Dungeness Bay just west of Cline Spit. This site contains primarily marine influenced water. It is located within the *Conditional* shellfish closure area. It is included in the Dungeness Bay TMDL study (Sargeant 2004).

- DOH-108 sediment – The upper 3 cm of sediment were collected using a grab sampler, and was co-located with the DOH-108 water sample station.

2.1.2 Field Sampling Approach

The sampling design consisted of three primary field efforts that were conducted per an approved Quality Assurance Project Plan (Battelle 2005) during 2006 and 2007:

- *Collection of fecal source samples for addition to source library*. Samples representing the distribution and variety of animals, birds, marine mammals, and human sources that occur in the Dungeness watershed were collected. These samples were added to the DNA source library of the Institute of Environmental Health Laboratory (IEH) in Seattle. Collections occurred throughout the course of the overall study.
- *Collection of test samples (water, marine sediment and marine vegetation) for ribotyping analysis*. Test sampling of water, marine sediment and marine vegetation occurred on a monthly basis between May 2006 and May 2007, a total of 13 months, from each of the 6 study stations (Figure 2.1).
- *Collection of water samples for FC analysis*. Water sampling for FC occurred in conjunction with the test sample collection for ribotyping and was coordinated with the Effectiveness Monitoring component (Task 3) of the EPA Targeted Watershed grant.

2.1.2.1 Collection of Fecal Source Samples for the DNA Library

To improve the percentage of DNA matches between *E. coli* in test samples and *E. coli* of known source types, an inventory of fecal samples representing local species of birds, wildlife, domestic animals, marine mammals, and human sources (septic systems) from the Dungeness watershed and the greater Olympic Peninsula region was collected and shipped to IEH for inclusion in its extensive DNA library database.

Fresh fecal samples were collected aseptically in sterile containers and delivered the same day or shipped overnight on ice to IEH. Samples were collected only when they were positively identified as belonging to a known species. Only one sample was collected from each individual animal.

The collection of local source samples began in August 2006 and continued through September 2007, spanning the general timeframe of the target test sample collections (i.e., water, sediment, wrack/detrital marine vegetation). We anticipated collecting between 50 and 100 source samples during the study. We collected a total of 105 samples from 42 local species, representing a variety of host animals and birds present in the region and spanning the geographic range of the Dungeness watershed, and extending out to Protection Island. Table 2.1 lists the source samples collected from our study region.

Table 2.1 Source fecal samples collected from the Dungeness Watershed Study Area

Collection Date	Location	Species	No. Individuals Collected
Aug. 2006	Clallam County Fair	Horse	1
Aug. 2006	Clallam County Fair	Cow	4
Aug. 2006	Clallam County Fair	Llama	3
Aug. 2006	Clallam County Fair	Goat	3
Aug. 2006	Clallam County Fair	Hen	3
Aug. 2006	Clallam County Fair	Sheep	3
Aug. 2006	Clallam County Fair	Pig	3
Aug. 2006	Clallam County Fair	Rabbit	3
Jan. 2007	Dungeness watershed	Dog	2
Jan. 2007	Dungeness watershed	Cat	1
Jan. 2007	Graysmarsh Farm	Elk	5
Jan. 2007	Washington Harbor	American widgeon	1
Jan. 2007	Graysmarsh Farm	Gadwall	1
Jan. 2007	Graysmarsh Farm	Greenwing Teal	1
Jan. 2007	Graysmarsh Farm	Mallard	4
Jan. 2007	Graysmarsh Farm	Pintail	7
Jan. 2007	Graysmarsh Farm	Shoveler	1
Apr. 2007	Dungeness Spit	Canada Goose	1
May 2007	Washington Harbor	Crow	1
May 2007	Washington Harbor	Robin	1
May 2007	Cline Spit	Seagull	2
May 2007	Dungeness River Mouth	Otter	1
Jun. 2007	Northwest Raptor Center	Red Tailed Hawk	1
Jun. 2007	Northwest Raptor Center	Raccoon	1
Jun. 2007	Northwest Raptor Center	Raven	1
Jun. 2007	Northwest Raptor Center	Coyote	1

Table 2.1 (contd)

Collection Date	Location	Species	No. Individuals Collected
Jun. 2007	Northwest Raptor Center	Barred Owl	1
Jun. 2007	Northwest Raptor Center	Peregrine Falcon	1
Jun. 2007	Northwest Raptor Center	Bald Eagle	3
Jun. 2007	Northwest Raptor Center	Barn Owl	1
Jun. 2007	Northwest Raptor Center	Deer	1
Jun. 2007	Lower Dungeness	Human (On-site Septic System)	2
Jun. 2007	Olympic Game Farm	American Bison	7
Jun. 2007	Olympic Game Farm	Black Bear	3
Jun. 2007	Olympic Game Farm	Brown Bear	4
Jun. 2007	Olympic Game Farm	Burro/Donkey	4
Jun. 2007	Olympic Game Farm	Elk	4
Jun. 2007	Olympic Game Farm	Fallow Deer	4
Jun. 2007	Olympic Game Farm	Llama	3
Jun. 2007	Olympic Game Farm	Prairie Dog	1
Jun. 2007	Olympic Game Farm	Yak	3
Jun. 2007	Dungeness River Mouth	River otter	4
Aug. 2007	Protection Island	Pigeon	1
Sep. 2008	Protection Island	Caspian tern	1
Sep. 2008	Protection Island	Harbor seal	1
Total No. of Samples: 105		**Total No. of Species: 45**	

2.1.2.2 Collection of Environmental Test Samples for Ribotyping Analysis

Test samples for ribotyping (water, sediment, or wrack) were collected from the 6 study sites approximately once a month beginning in May 2006 and ending in May 2007 (13 sampling events). Water samples were collected every month at all stations with the exception of the bluff ditch station (BD7) which had intermittent flow and was therefore sampled less often. Sediment grab samples were collected from the 2 marine stations by boat; however, this depended on the weather and availability of sampling equipment. Detrital marine vegetation (wrack) samples were collected when present along the shore near DOH-113.

The water samples for ribotyping were collected in sterile sample bottles by the grab sampling method described in Standard Methods for Examination of Water and Wastewater (APHA 1998). At

each station, 5 independent grab samples (500 ml or 1 L) were collected approximately 2 minutes apart. Samples were placed on ice and delivered the same day or shipped via overnight delivery service to the IEH in accordance with the QAPP guidelines (Battelle 2005).

The surface sediment samples (~ upper 3 cm) were collected by boat at the 2 marine stations using a clean grab sampler or core sampler. Each sample was homogenized using a sterile spatula and portioned equally into 5 sterile containers. These were placed on ice and delivered to IEH for ribotyping in accordance with the QAPP guidelines (Battelle 2005).

Marine detrital wrack samples were collected on 9 dates throughout the course of the study near station DOH-113. The samples were primarily composed of either detrital *Zostera marina* (eelgrass) or *Ulva sp*. (green algae) that had washed up on shore and were decomposing. Up to 5 independent samples were collected per sampling event and placed in sterile containers. These were placed on ice and delivered to IEH for ribotyping analysis in accordance with the QAPP guidelines (Battelle 2005).

2.1.2.3 Collection of Water Samples for Fecal Coliform Analysis

At the time of sample collection for ribotyping analysis, additional surface water grab samples were collected from the 6 MST sites in sterile sample bottles using the standard grab sampling methods (APHA 1998). These samples were transported on ice to the Clallam County Environmental Health Laboratory and analyzed for FC concentration within 6 hours of their receipt in accordance with QAPP protocol (Battelle 2005).

2.1.3 Analytical Methodology

Laboratory analyses were performed per an approved QAPP (Battelle 2005), as described below.

2.1.3.1 *E. coli* Ribotyping Analysis

Water samples were well homogenized by shaking, then a portion was filtered and tested for FC using the membrane-filtration method described in Standard Methods for the Examination of Water and Wastewater (APHA 1998). Typical FC colonies were confirmed as *E. coli* by purifying on MacConkey agar and testing for typical indologenesis and the lack of citrate use. *E.coli*-confirmed isolates were then ribotyped using the procedure described below. For each water sample collected, the target goal for ribotyping was a minimum of 2 *E. coli* isolates. Hence for each sampling event and station, the goal was 10 *E. coli* isolates (5 samples X 2 *E. coli* per sample)

Sediment samples were homogenized by stirring them with a sterile tongue blade, then a portion was weighed and diluted for enrichment in FC selective media. Detrital vegetation samples were weighed and diluted for homogenization and enrichment in FC selective media. Cultures were tested for typical *E. coli* morphology on MacConkey agar. Selected colonies were positively identified as *E. coli* by biochemical testing for typical indologenesis and lack of citrate use. These isolates were then ribotyped following the MST ribotyping procedure. For each independent sample, the goal was selection of at least 2 *E. coli* isolates for ribotyping.

Confluent culture growth from *E. coli* isolates was collected in Tris/EDTA, lysed with sodium dodecyl sulfate followed by proteinase K, and extracted by phenol-chloroform. The DNA was precipitated with ethanol, washed and dried, and finally resuspended in distilled water. The extracted DNA samples were held at -20°C for batching purposes.

The extracted DNA was digested with the restriction endonucleases (*Eco*R1 and *Pvu*II) for 2 hours at 35°C. The resultant digestion products were separated by agarose gel electrophoresis, then DNA fragments were transferred for Southern Blotting. Blots were visualized by autoradiograms, and the resulting patterns recorded in an alphanumeric pattern for comparison to the IEH DNA source library.

2.1.3.2 Fecal Coliform Analysis

Samples were analyzed at the Clallam County Environmental Health Laboratory, an accredited Washington Department of Ecology laboratory, for FC bacteria using the membrane-filtration method (Standard Method 9222D; APHA 1998). The resulting data was used to determined effectiveness of the projects in the Targeted Watershed grant and complemented the information derived from the ribotyping analysis.

2.1.4 Quality Assurance

All field samples were collected and handled according to procedures outlined in the QAPP (Battelle 2005) including labeling of containers and logging of sample information on field logs. A Chain of Custody form accompanied all samples shipped or delivered to IEH. All samples were shipped on ice and were accepted in good condition, according to protocol. Samples were logged in upon arrival at IEH and given a unique sample number for identification purposes. All methodologies for characterizing *E. coli* isolates followed IEH standardized protocols and specified QAPP guidelines for this project (Battelle 2005).

2.2 Phase 2 – Centennial Grant (*Bacteroides* Target-Specific PCR study)

The sampling stations, field sampling approach, analytical methodology and quality assurance objectives for the Phase 2 – Centennial Grant (*Bacteroides* target-specific PCR study) are described below.

2.2.1 Sampling Stations

The objective of the Phase 2 study was to further define and confirm the results from the Phase 1 study and to expand the number of sites tested in the lower Dungeness watershed and Dungeness Bay. An alternative MST methodology was used for this phase. This *Bacteroides* target-specific PCR method used two biomarkers (human and ruminant), to further define locations of sources that could be potentially controlled. Sampling locations are shown in Figure 2.2.

2.2.2 Field Sampling Approach

During Phase 2, water samples for target-specific biomarker analysis were collected three times (twice in December 2008 and once during January 2009). This type of analysis does not require a source library collection as in Phase 1. To meet contractual obligations, the sampling window was shortened during this phase. Table 2.2 lists the sample stations, their descriptions, and when they were sampled.

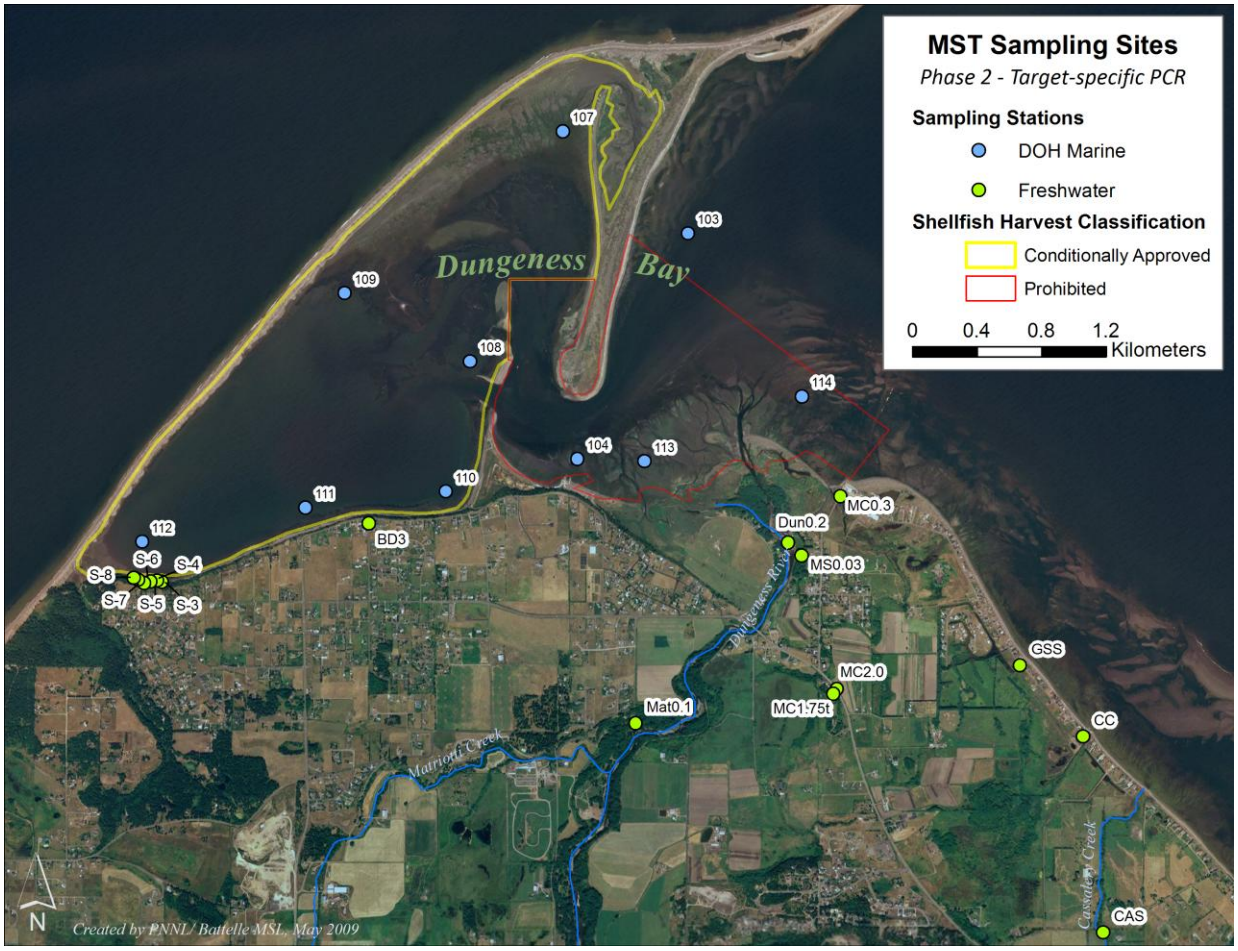

Figure 2.2 Microbial Source Tracking Stations Sampled During the Phase 2 – *Bacteroides*Target-Specific PCR Study

Table 2.2 Stations, locations, and dates of target-specific PCR samples for the Phase 2 sampling.

Sample Station Label.	Sampling Event			Coordinates		Location	Description
	12/2/08	12/11/08	01/06/09	Latitude	Longitude		
DOH 110	✓		✓	48.151167	-123.1535	Dungeness Bay	Marine, cond. approved
DOH 111	✓		✓	48.14955	-123.1658	Dungeness Bay	Marine, cond. approved
DOH 112	✓		✓	48.147417	-123.1797	Dungeness Bay	Marine, cond. approved
DOH 108	✓		✓	48.155483	-123.1528	Dungeness Bay	Marine, cond. approved
DOH 109	✓			48.161433	-123.1668	Dungeness Bay	Marine, cond approved
DOH 107	✓			48.172233	-123.1452	Dungeness Bay	Marine, cond. Approved
DOH 113	✓	✓	✓	48.155267	123.13597	River Mouth	Brackish water
DOH 104	✓			48.1534	123.14399	River Mouth	Brackish water
DOH 114	✓			48.15665	123.1231	Dung Bay (Outer)	Marine water
DOH 103	✓			48.16415	123.1359	Dung. Bay (Outer)	Marine water
DOH 102		✓		48.14765	123.104	Jamestown	Marine water
DOH 101		✓		48.13944	123.094	Jamestown	Marine water
DOH 182		✓		48.15441	123.116	Jamestown	Marine water
Seep-1	✓			48.147851	123.171	Inner Bay seeps	FW unregulated seep
Seep-2	✓			48.147337	123.172	Inner Bay seeps	FW unregulated seep
Seep-3	✓			48.14609	123.178	Inner Bay seeps	FW unregulated seep
Seep-4	✓			48.14615	123.17884	Inner Bay seeps	FW unregulated seep
Seep-5	✓			48.14611	123.179	Inner Bay seeps	FW unregulated seep
Seep-6	✓			48.14603	123.180	Inner Bay seeps	FW unregulated seep
CAS		✓	✓	48.12659	123.1000	Casselary Creek	Casselary Crk at Jamestown Rd
CC		✓	✓	48.13754	123.10128	Cooper Creek	at the end of 3 Crabs Rd.
MC 0.3		✓	✓	48.150946	-123.122	Meadowbrook Crk at 3 Crabs	Mixed ag., residential, wetland
GSS		✓	✓	48.141508	-123.1071	Golden Sands Slough off 3 Crab	Residential and vacation home-sites
MAT 0.1		✓	✓	48.13826	-123.1388	Matriotti Crk, at Game Farm	Exotic animal farm, ag., residential
DUN 0.2		✓	✓	48.148353	-123.1263	Dungeness R. mouth	Mixed ag. and residential
DUN 0.05			✓	48.152388	-123.1292	Dungeness R. Mouth	Mixed ag. and residential
MS 0.3		✓	✓	48.147637	-123.1252	Meadowbrook Slough	Upper reach – residential
MC 2.0		✓	✓	48.139911	-123.1226	Meadowbrook Creek	Mixed ag. and residential
MC 1.75t		✓	✓	48.140171	-123.1225	Meadowbrook trib.	roadside ditch, primarily ag
BD 3			✓	48.149374	-123.1614	Bluff Ditch-3	Thornton and Marine Drive – resid. area.

✓ - Denotes the station was sampled for PCR analysis.

2.2.2.1 Collection of Samples for Target-Specific PCR Analysis

Following the QAPP protocol (Battelle and EPA 2008), surface water samples were collected using a sampling wand or hand dipping following Standard Methods 9060A and 9060B (APHA 1998) procedures. Sterile pre-cleaned 250 ml containers were supplied by EPA Manchester Laboratory. Marine stations were sampled by boat. All other stations were sampled from shore. Seep samples were collected at the base of the bluff along inner Dungeness Bay. Care was taken to only sample freely flowing water and not contact the bluff sediment. Samples were collected on December 2, 2008 (10 marine stations, 6 seep stations), December 12, 2008 (9 freshwater stations), and January 6, 2009 (5 marine stations, 11 freshwater stations) (Table 2.2, Figure 2.2). Fourteen of these stations were sampled on two of the three dates. Samples were placed on ice and delivered to the EPA Manchester Laboratory on the same day they were collected. A temperature control was included in each transported cooler.

2.2.2.2 Collection of Water Samples for Fecal Coliform Analysis

At the time of sample collection for PCR analysis, additional surface water grab samples for FC analysis were collected in sterile containers from each site in the same manner as described above following the standard protocol (APHA 1998). The samples were transported on ice to the Clallam County Environmental Health Laboratory and analyzed for FC concentration within 6 hour of their receipt according to QAPP protocol (Battelle and USEPA 2008).

2.2.2.3 Collection of Total Suspended Sediment Samples

At each site, 500 ml of water was collected for analysis of total suspended sediments (TSS). These samples were collected from each station during all events. Samples were kept cold and transported to the Battelle Marine Sciences Laboratory for analysis. All TSS samples were collected after PCR and FC samples were taken, and care was taken to avoid disturbing the sample site or artificially creating turbid conditions. Salinity was measured at marine sites using a handheld refractometer. Temperature was measured at all sites.

2.2.3 Analytical Methodology

The laboratory methodology for the Phase 2 *Bacteroides* target-specific study is described below.

2.2.3.1 Target-Specific PCR Analysis

A *Bacteroides* target-specific polymerase chain reaction methodology (Field *et al*. 2003, Dick *et al*. 2005) was used to analyze all water samples from the Phase 2 study. The USEPA Manchester Laboratory followed procedures developed by the USEPA Office of Research and Development for the DNA extraction, PCR and gel electrophoresis. Human primers (HF183 and HF134) and ruminant primers (CF193 and CF128) were used for this study. The procedures are referenced in the QAPP (Battelle and USEPA 2008) and outlined below based on a Final Data Report Memorandum (USEPA 2009):

- Sample filtration within 8 hours of sample collection
- Filter placed in sterile tube; preservative added and frozen at -20°C
- DNA extraction/purification performed using FastDNA® kit

- Test each sample for the presence of the appropriate DNA target using master mix and primer sets specific to DNA segments associated with *Bacteroides* (general), human *Bacteroides* and ruminant *Bacteroides*. Five primer sets were utilized: 1 general, 2 human sets and 2 ruminant sets of target DNA sequences.
- Visualization of amplified DNA product using gel electrophoresis and ultraviolet trans-illumination. A sample was considered negative for the presence of *Bacteroides* if all five concentrations of the DNA extract from the sample (processed from previous steps) provided negative results. If at least one of the five concentrations of the DNA extract produced a positive result with one or both of the *Bacteroides* human primer sets, the sample was considered to be positive for human fecal contamination. If at least one of the five concentrations of the DNA extract produced a positive result with one or both of the *Bacteroides* ruminant primer sets, the sample was considered to be positive for ruminant fecal contamination.

2.2.3.2 Fecal Coliform Analysis

Water samples collected for FC analysis during December 2008 were analyzed at the Clallam County Environmental Health Laboratory using the membrane filtration method, (ASTM 9222D, APHA 1998). Samples collected during the January 2009 event were analyzed by Twiss Analytical, Inc. in Poulsbo, Washington, using the membrane-filtration method. In addition to using these data to better understand the overall concentrations of FC at a site, the data were used as a screening tool for PCR analysis. The initial target cutoff of 14 CFU/100 ml, below which PCR analysis would not be done, was modified during this study to include all samples (see results section).

2.2.3.3 Total Suspended Sediment Analysis

Total suspended sediment concentration was determined for all water samples at the Battelle Marine Sciences Laboratory based on the Standard Methods 2540C (APHA 1998) protocol for TSS. Approximately 500 ml of sample water was filtered through pre-tared Whatman GF/F filters. Filters were dried and weighed to obtain a concentration of sediment (mg/l).

2.2.4 Quality Assurance/Quality Control

All field samples were collected and handled according to procedures outlined in the QAPP (Battelle and USEPA 2008) including labeling of containers and logging of sample information on field logs. A Chain of Custody form accompanied all samples sent or delivered to respective laboratories. A temperature control sample accompanied samples delivered to the USEPA Manchester Lab. All samples were accepted with temperature controls below 10°C at the time of sample delivery. To ensure the use of the proper aseptic technique during sampling, a transfer blank was included in the delivery of samples to USEPA Manchester. To assess method accuracy, two "blind" water samples labeled as Source #1 and Source #2, containing fecal material from sources known only to the collector, were submitted to the Manchester laboratory.

In accordance with guidelines presented in the QAPP (Battelle and USEPA 2008), the following quality control activities were conducted during the PCR laboratory analysis: filtration controls, PCR amplification controls (positive and negative), replicate analysis (analyst and method precision), method accuracy (blind sample control), and specificity.

2.3 Data Analysis

The Phase 1 – ribotyping *E. coli* test samples were analyzed based on the proportion of total isolates typed. As such the data needs to be viewed qualitatively, with an understanding that the study was designed to determine what the predominant sources of fecal pollution were in the Dungeness watershed. It was not designed to quantify fecal loading of the sources. Sources were categorized into functional groups with similar characteristics (e.g., domestic animals) and proportions of isolates were analyzed based on sampling station and date. Non-parametric statistical tests were used to examine associations of source groups between stations, dates and sample types. Regression analysis was used to evaluate whether proportions were generally increasing or decreasing through time, and to compare proportions between the wet (October through March) and dry (April through September) seasons. The data was also examined based on frequency of occurrence during the 13 sampling events for a given functional group.

The Phase 2 - *Bacteroides* data were also presented as a proportion of matches based on the total samples analyzed. This included the proportion of samples that contained *Bacteroides*, the proportion of samples containing at least 1 human biomarker and the proportion of samples containing at least 1 ruminant marker. Although differences were noted between sample events, the data were not evaluated statistically due to the small number of samples collected over time.

3.0 Results

The results from each phase of the study are presented separately. Phase 1 results discuss the EPA Targeted Watershed ribotyping study. Phase 2 results discuss the Department of Ecology Centennial *Bacteroides* target-specific PCR study.

3.1 Phase 1 – Ribotyping

The Phase 1 – ribotyping *E. coli* test samples were analyzed based on the proportion of total isolates typed. As such the data needs to be viewed qualitatively, with an understanding that the study was designed to determine what the predominant sources of fecal pollution were in the Dungeness watershed. It was not designed to quantify fecal loading of the sources.

As discussed in the introduction, the ribotyping approach used in this study is based on matching the genetic fingerprint of *E. coli* bacteria strains isolated from environmental samples (e.g. water, sediment and wrack) to *E. coli* from fecal samples of known host source species. The DNA library-based approach is dependent on having an extensive library of *E. coli* ribotypes from strains that were isolated from the feces of known host species. The IEH *E. coli* DNA library contains over 120,000 isolates and included the addition of local source samples from 45 animal and bird species in the Dungeness watershed (Table 2.1). We analyzed the data in several different ways including:

- Number of isolates by source type
- Frequency of occurrence of source type
- Compositing of sources into functional groupings
- Temporal trends
- Human-derived source patterns

While this data was examined in several ways, it should be viewed overall as a qualitative characterization of the *E. coli* sources in the watershed. Because MST methodologies are still evolving, there are a number of assumptions and limitations inherent in these techniques. For this study in particular, it should be noted that while a known number of *E.coli* isolates were typed for each given sampling event (e.g. usually 10 to 15 isolates), it was not necessarily representative of the entire population of *E. coli* bacteria in a particular sample which could be range widely from sample to sample (e.g. several orders of magnitude).

3.1.1 Source types found

During the Phase 1 study a total of 472 environmental samples were collected (e.g. water, sediment, wrack) and 1164 *E. coli* isolates were ribotyped. Table 3.1 includes the number of isolates that were typed from each date and sample location. The target number of samples for each event was 5. These targets were met for all marine and freshwater sampling events, with the exception of the irrigation ditch station (BD-7) which was dry on occasion. Marine sediment and wrack were more difficult to collect, and wrack was not always present along the shoreline.

Table 3.1 Sample inventory by station and event from Phase 1 including number of environmental samples collected and number of isolates typed by IEH from each sampling event shown in parentheses ( ).

Location	**Freshwater**				**Marine Water**		**Marine Sediment**		**Wrack (Marine Vegetation)**
DATE	**MAT 0.1**	**BD-7**	**MC 0.3**	**GSS**	**DOH-108**	**DOH-113**	**DOH-108**	**DOH-113**	**DOH-113**
5/18/2006	5 (16)	5 (16)	5 (15)	5 (16)	5 (1)	5 (15)	0	0	0
6/21/2006	5 (16)	0	5 (10)	5 (1)	5	5 (2)	5 (21)	5 (26)	5 (12)
7/6/2006	5 (15)	5 (16)	5 (16)	5 (16)	5 (5)	5 (15)	0	5	5 (2)
8/29/2006	5 (16)	5 (16)	5 (16)	5 (16)	5 (1)	5 (15)	0	0	5
9/12/2006	5 (16)	5 (16)	5 (15)	5 (16)	5 (15)	5 (4)	5 (10)	0	5
10/30/2006	5 (15)	0	5 (16)	5 (16)	5 (7)	5 (10)	1	1	5
11/20/2006	5 (16)	0	5 (16)	5 (15)	5 (16)	5 (15)	0	1	5
12/4/2006	5 (16)	5 (14)	5 (15)	5 (16)	5 (16)	5 (15)	0	0	5
1/23/2007	5 (16)	5 (15)	5 (16)	5 (16)	5 (16)	5 (15)	2 (10)	2	0
2/8/2007	5 (16)	5 (15)	5 (16)	5 (16)	5 (16)	5 (16)	5 (26)	5 (21)	0
3/13/2007	5 (15)	5 (16)	5 (16)	5 (16)	5 (8)	5 (16)	5 (12)	5 (13)	4
4/23/2007	5 (16)	0	5 (16)	5 (15)	5 (6)	5 (15)	5 (7)	5 (4)	1
5/22/2007	5 (16)	0	5 (16)	5 (16)	5 (1)	5 (4)	5 (9)	5 (7)	0
Total	*65 (205)*	*40 (124)*	*65 (199)*	*65 (191)*	*65 (108)*	*65 (157)*	*33 (95)*	*34 (71)*	*40 (14)*

Of the 1164 isolates typed, matches were made for 1078 (92.6%) of the isolates; only 7.4% of the isolates were not matched to any known source (fingerprint) in the IEH library, or were sourced to multiple species (referred to as transient). The transients and unknowns were not distinguished from each other in the database and are referred to as a source category called unknown in our study. Detrital wrack samples had the lowest percent match to the source library at 78%, while marine sediments at station DOH-108 had the highest percent match at 100%. The percent match for other stations and sample types ranged between 91 and 96% (Table 3.2).

Table 3.2 Number of unidentified isolates and percent match from each station and matrix type**.**

Station	Matrix	Total No. of Isolates	No. of Unidentified Isolates	% Match
BD-7	water	124	6	95%
MAT 0.1	water	205	10	95%
MC 0.3	water	199	22	90%
GSS	water	191	21	90%
DOH 113	water	157	10	94%
DOH 113	sediment	71	7	91%
DOH 108	water	108	5	96%
DOH 108	sediment	95	0	100%
DOH 113	wrack	14	4	78%

There were 37 source types that were identified overall during the study. The complete list is shown in Table 3.3 with the number of isolates matched for each source from each station. While most source types are self explanatory, several categories need further explanation. Each source type has a unique set of genetic ribotypes or pattern identifiers (fingerprints) which are not shared between source types. For example, the source type avian has a fingerprint that is inclusive, and shared by all avians (birds) in that category, whereas a Canada goose source type, for example, contains a fingerprint that is exclusive only to Canada geese. Canine refers to a composite group made up of dogs, coyotes, and wolves that share a similar pattern, while dog refers to domestic dog. Feline is a composite of cats, cougars, bobcats, etc…. Bovine refers to a diverse set of medium to large-sized ungulates including domestic cattle, bison and yak. Marine mammal includes sea lions, seals, and otters. Several source types were linked to human-related sources including human, septage, and sewage. Human refers to a match with human fecal material. Septage refers to isolates matched to septic tank waste, and sewage refers to isolates matched from a wastewater treatment plant. For the purposes of our analysis, we have combined these three source types into a category called "human-derived".

Of the 1078 isolates that were matched to the overall source library at IEH, 5.1% (55 matches) were matched directly to the local Dungeness source library listed in Table 2.1. The identified samples that were matched to sources in our local database include waterfowl, bison, yak, llama, goat, deer, elk, human, prairie dog, river otter, raccoon, canine, sheep, and bear. Some of these matches included exotic species that were sampled at the Game Farm. All local source types were matched to samples collected at freshwater stations with the following exceptions; waterfowl was found in marine water and sediment in addition to freshwater, and bison was found only in marine sediment. Goat and river otter were found only in marine water, and raccoon was found in marine and freshwater.

Figure 3.1 shows the total distribution of isolates that were matched to the IEH database including our local contributions. Based on the proportion of total isolates typed, the predominant sources identified in our study area were, in ranked decreasing order, avian (19.6%), gull (12.5%), waterfowl (9.7%), raccoon (9.2%), unknown (7.3%), human-derived (7.1%), rodent (6.3%) and dog (4.3%).

Table 3.3 Number of isolates by source type, station and matrix (e.g. freshwater, marine water, sediment, wrack**)**

Source Types	Total No. of Isolates	Freshwater				Marine Water		Marine Sediment		
		MAT0.1	MC0.3	GSS	BD-7	St 113-water	St 108-water	St 113-sed	St 108-sed.	wrack
avian	228	34	26	39	24	48	21	12	22	2
bear	10	6	1		1	1		1		
beaver	16	1	4	11						
bison	7	1		1					5	
bovine	34	13	6	3	5	5	1		1	
burro	1						1			
canine	18	3	2	3	6	3	1			
chicken	3			3						
crow	2		1				1			
deer	33	7	2	8	3	3	2	3	3	2
deer/elk	15					3	2	10		
dog	50	12	8	8	9	4	3			6
elk	32	3	12	2		5	2	3	5	
equine	2			1				1		
feline	18		3	8	2	5				
goat	11	1	4		1	1	1		3	
goose	1					1				
gull	146	23	30	19	13	22	20	5	14	
horse	20	5	2	3			6	1	3	
human-derived	83	16	15	5	19	8	9		11	
llama	4	2	1	1						
marine mammal	16	2				7	6		1	
otter	8	3		2	1		2			
oyster	6						1		5	
pig	1	1								
porcine	3	1			1	1				
prairie dog	2				1		1			
rabbit	3	1	1		1					
raccoon	107	19	21	14	21	8	5	9	10	
rodent	73	22	13	7	8	7	8	6	2	
sheep	2		2							
swine	1			1						
unknown	85	10	22	21	6	10	5	8		4
waterfowl	113	15	21	30	3	15	7	13	9	
yak	9	4	2	3						

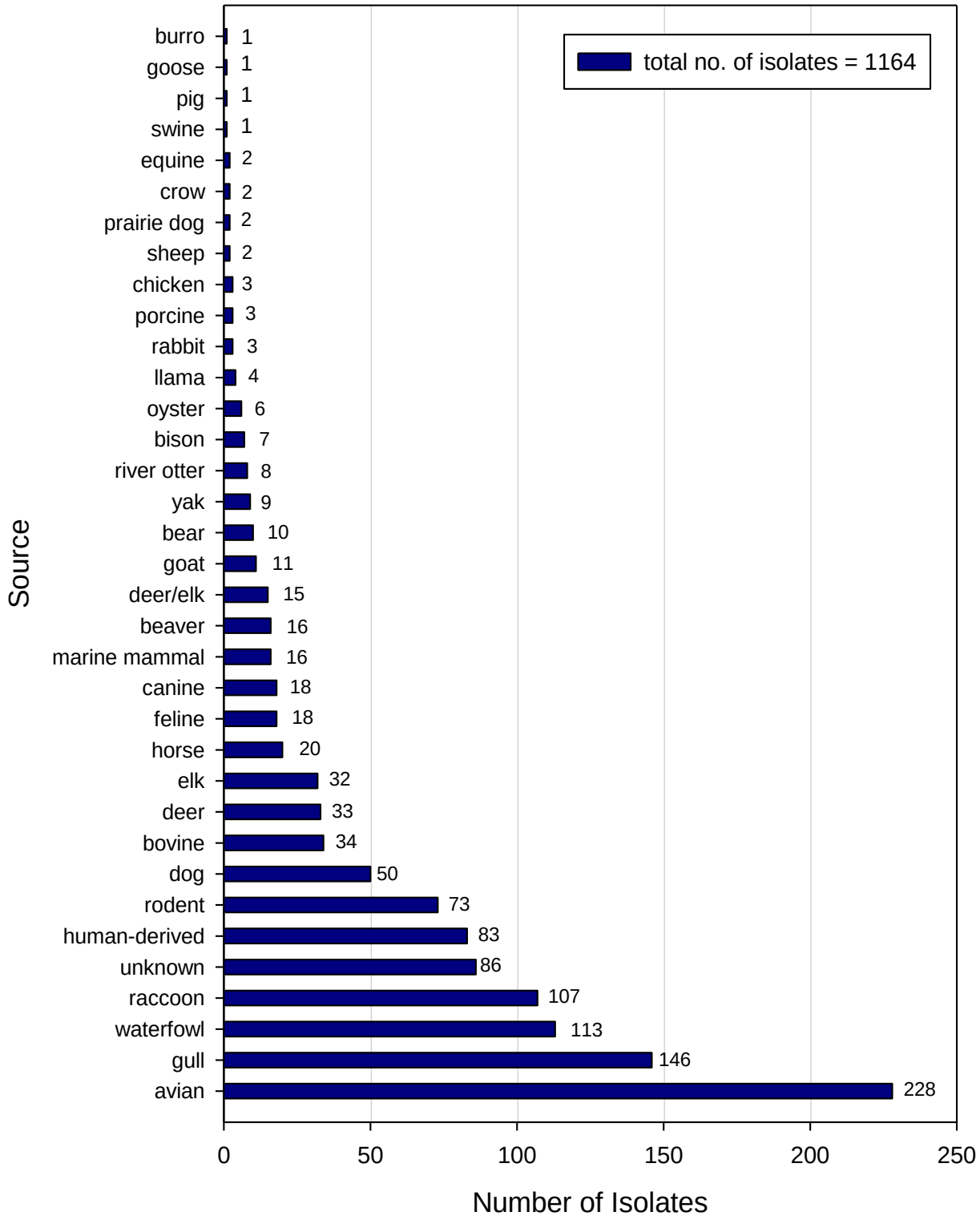

Figure 3.1 Distribution of total number of isolates from all stations based on source type.

3.1.2 Analysis by Functional Groups

To further analyze the data, we organized the source types into functional groups or guilds, in order to more easily assess the overall contributions. Table 3.4 represents the functional groups that were developed and the source types that make up those groups. These functional groups best represent the issues being addressed in the Dungeness watershed, and to a certain extent reflect groupings of sources that could be controlled (i.e. farm animals, domestic animals, game farm animals, and human) and those that are more difficult to control (i.e. birds and wild mammals). In some instances one source type could arguably fit into several functional groupings. For example the source type feline could fit into wild mammal, domestic animal or game farm functional groups, and the source type deer could fit into the wild mammal or game farm functional grouping. The grouping for the Game Farm includes source types that were found that had a reasonably high likelihood of only occurring from the Olympic Game Farm, located just upstream from MAT0.1.

Table 3.4 List of source types assigned to functional groups

Birds	Wild Mammals	Farm Animals	Domestic Animals	Game Farm	Human	Other	Unknown
avian	beaver	bovine	canine	bear	human	oyster	unidentified
crow	deer	chicken	dog	bison	septage		or transient
goose	deer/elk	equine	feline	burro	sewage		
gull	elk	horse	cat	prairie dog			
waterfowl	marine mammal	llama		yak			
	river otter	pig					
	rabbit	porcine					
	raccoon	sheep					
	rodent	swine					
		goat					

When the data are analyzed by functional group, the highest occurring proportion of isolates was birds (42%) present as 490 out of 1164 isolates (Figure 3.2), followed by wild mammals, present as 303 out of 1164 isolates (26%). Domestic animal, human-derived, farm animal, and unknown sources had similar proportions, about 7% each. The game farm proportion of isolates was 2.5%.

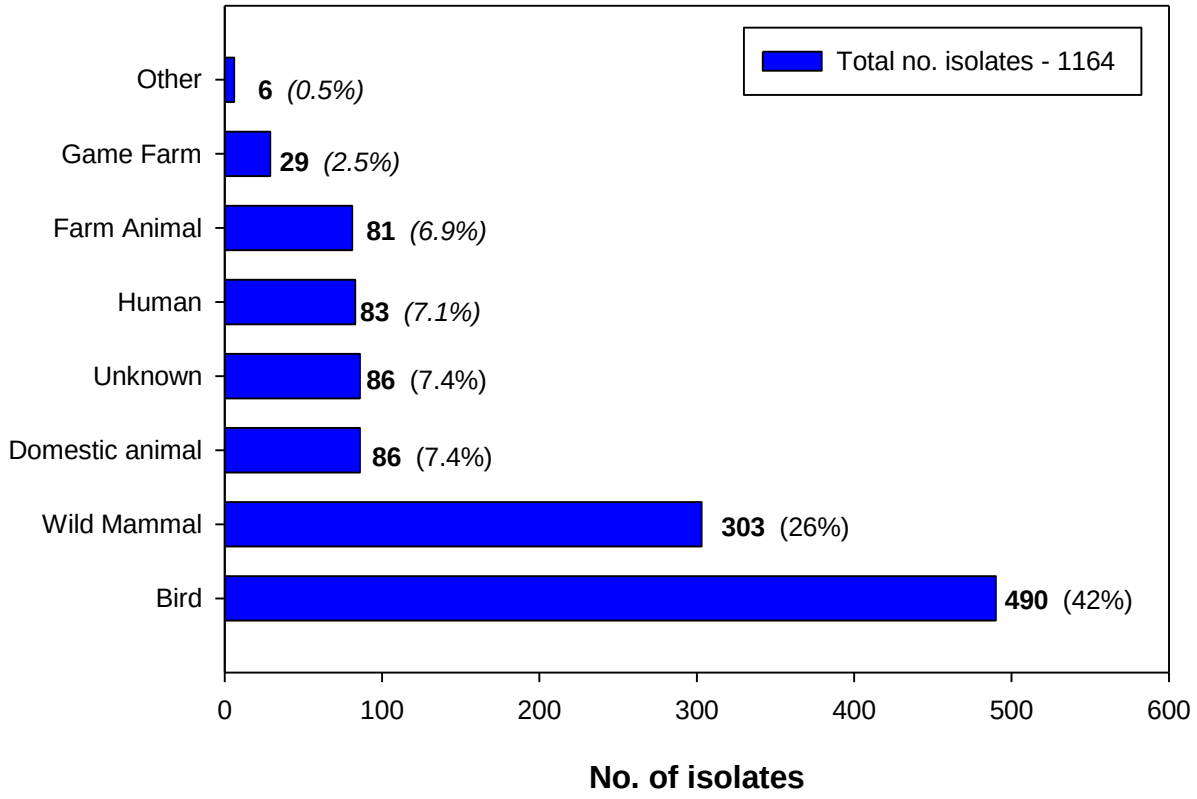

Figure 3.2 Distribution of total number of isolates from all stations based on functional groups.

3.1.3 Frequency of occurrence

We analyzed the data to determine how frequently a functional group source occurred during the length of the study (13 months) so that we could better understand if a source was frequently present or just occasionally present during our sampling events. This would help determine what mitigation strategy might be appropriate. Frequency of occurrence was examined by station and matrix type. The results are described as percent frequency of occurrence (Table 3.5). Birds were found at freshwater and marine water stations during most, if not all sampling events, with frequency of occurrence generally ranging between 85 and 100 percent of sampling events. Wild mammals were also found very frequently, in both freshwater (between 92 and 100 percent of the sampling events) and marine water (between 62 and 85 percent of sampling events). Farm animals and domestic animals were found frequently in freshwater and marine water, however more often in freshwater, occurring between 50 and 77 percent of the time. Human-derived sources were also found frequently in freshwater, and slightly less often, 38 percent of the time, in marine waters. Game farm animals occurred occasionally at freshwater stations, approximately 14 percent of the time, with the exception of MAT0.1 where the frequency of occurrence was greater (46%). Game farm sources were also found occasionally in marine water and marine sediment. Birds and wild mammals occurred frequently in marine sediments, with other functional groups occurring occasionally. The only exception was domestic animals which never occurred in sediment. Marine wrack samples showed only occasional presence of birds, wild mammals and domestic animals.

Table 3.5 Frequency of occurrence (percent) of functional groups by station based on the number of sampling events or possible occurrences

		Functional Groups							
Stations	**Possible Occurrences**	**Birds (%)**	**Wild Mammals (%)**	**Farm Animals (%)**	**Game Farm (%)**	**Domestic Animals (%)**	**Human Derived (%)**	**Unknown (%)**	**Other (%)**
Freshwater									
MAT0.1	13	100	100	77	46	69	69	62	0
MC0.3	13	100	100	69	15	69	54	62	0
GSS	13	92	92	54	15	69	38	62	0
BD-7	8	88	100	50	13	75	50	50	0
Marine Water									
DOH-113	13	85	85	46	13	62	38	46	0
DOH-108	13	85	62	31	23	23	38	23	13
Marine Sediment									
DOH-113	9	56	56	11	11	0	0	0	0
DOH-108	8	88	75	25	13	0	25	33	1
Marine Wrack	9	11	11	0	0	11	0	0	0

3.1.4 Freshwater stations

The total freshwater source isolate information was combined from MAT0.1, MC0.3, BD-7, and GSS to examine the distribution based on functional groups (Figure 3.3). Birds represented the greatest proportion of isolates present from the combined freshwater sites at 39% (278 out of 719). The wild mammal proportion contribution was 26% (189 out of 719). Each of the remaining functional group sources made up less than 10% of the proportion of freshwater isolates.

A Chi-square test of equal proportions for each of the identified sources was significantly different between stations ($p < 0.001$). The freshwater bluff ditch station (BD-7) had significantly greater human isolates present than other freshwater stations; GSS had significantly greater bird isolates and significantly fewer human-derived sources, and MAT0.1 had significantly greater game farm isolates than other stations. A regression analysis indicated there was no significant increase or decrease of any of the major functional groups over time for the duration of the study.

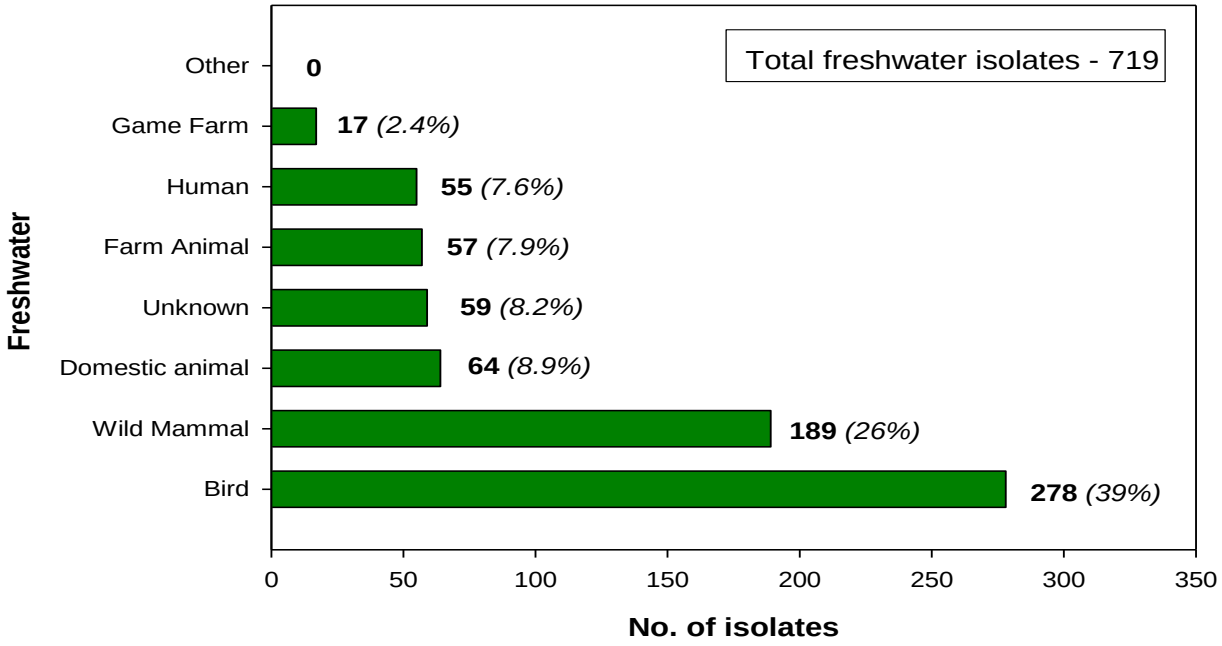

Figure 3.3 Distribution of the total number of isolates from freshwater stations based on functional groups**.**

3.1.5 Marine water stations

The marine water station isolate information was combined (DOH-113 and DOH-108) to examine the distribution by functional groups (Figure 3.4). Again, the greatest proportion of isolates present from the two marine water stations was bird, at 51% (135 out of 265). The wild mammal proportion was 23% (60 out of 265). All other functional group sources were each less than 10% of the proportion of marine isolates.

The proportion of bird isolates was significantly greater than any other functional group at the marine water sites. There was no significant increase or decrease of bird isolates over the duration of the study ($p>0.92$). The proportion of wild mammal isolates increased the most over this timeframe ($p=0.17$) and the proportion of farm animal isolates decreased the most over this timeframe ($p=0.06$), however both were highly variable.

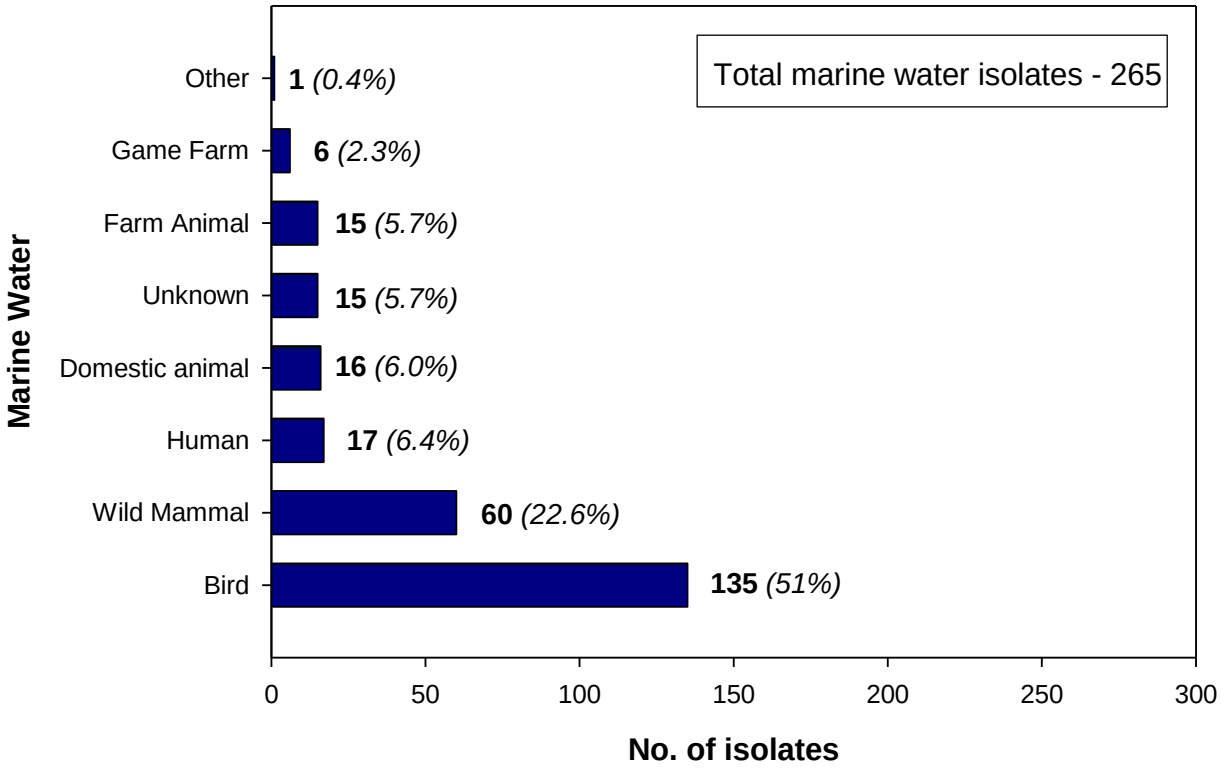

Figure 3.4 Distribution of the total number of isolates from marine water stations based on functional groups.

3.1.6 Marine sediment and wrack stations

The two marine sediment station isolates were combined (DOH-113 and DOH-108) to examine the distribution by functional groups (Figure 3.5). The greatest proportion of isolates present was from birds at 45% (75 out of 166). The proportion of isolates for wild mammals was 31%. All other groups were less than 10%. Functional group sources did not significantly increase or decrease over time.

Detrital marine vegetation or wrack was collected when present on shore closest to station DOH-113. It was found during 9 out of 13 sampling events (Table 3.2). However on only two of these events were *E.coli* detected, isolated and typed. Of the 14 isolates, 4 were typed as unknown, two as deer/elk and six as dog. Because so few *E.coli* were isolated, this data was not analyzed further.

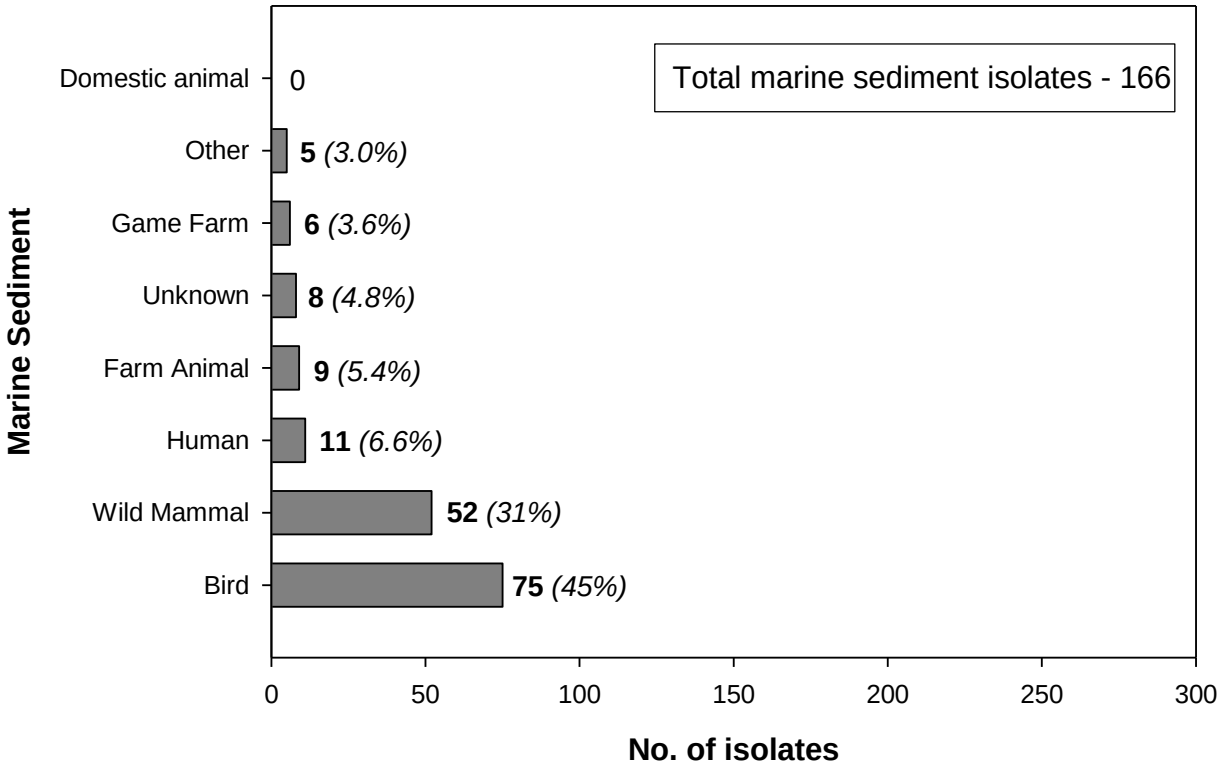

Figure 3.5 Distribution of the number of isolates from marine sediment stations based on functional groups.

3.1.7 Human-derived Sources

Human-derived sources, which are relatively controllable, are of particular concern from a public health perspective and protection from pathogens in waters used for recreation and shellfish harvesting. We examined human-derived sources in terms of the presence and proportion of typed isolates, the frequency of occurrence, and the timing of occurrences.

The number and proportion of human-derived isolates found at each station are shown in Table 3.6. The proportion of isolates ranges from 0 at the DOH-113 sediment station to 15.3 percent at freshwater station Bd-7. The majority of stations had between 5 and 10% human-derived sources.

The frequency of occurrence of human-derived isolates is shown in Table 3.5. The greatest frequency of occurrence occurred at MAT0.1 which contained human-derived sources on 9 out of 13 sampling events, or 69%. Figure 3.6 shows a time series of the number of human-derived isolates found from each of the freshwater stations. The frequent presence of human sources at MAT0.1 occurs from early summer through late winter. BD7 contained human-derived isolates during the winter months (December through February). Figure 3.7 shows the time series for the marine water and sediment stations. The marine water station DOH-108 also shows a presence of human-derived sources between November 2006 and March 2007, which coincides with the Conditional shellfish closure in the inner bay. These data combined with

the target-specific PCR data for presence of a human bio-marker (Section 3.2) provide a clearer picture of the influence of human sources in the Dungeness watershed, both from a temporal as well as spatial perspective.

Table 3.6 Number and proportion of human-derived isolates found at each station over the duration of the study.

Stations	No. of Human-derived Isolates Typed	Total No. of Isolates Typed	Proportion of Human-derived Isolates
Freshwater			
MAT0.1	16	205	7.8%
MC0.3	15	199	7.5%
BD-7	19	124	15.3%
GSS	5	191	2.6%
Marine Water			
DOH-113	8	157	5.1%
DOH-108	9	108	8.3%
Marine Sediment			
DOH-113	0	71	0
DOH-118	11	95	11.6%
Marine Wrack	0	14	0

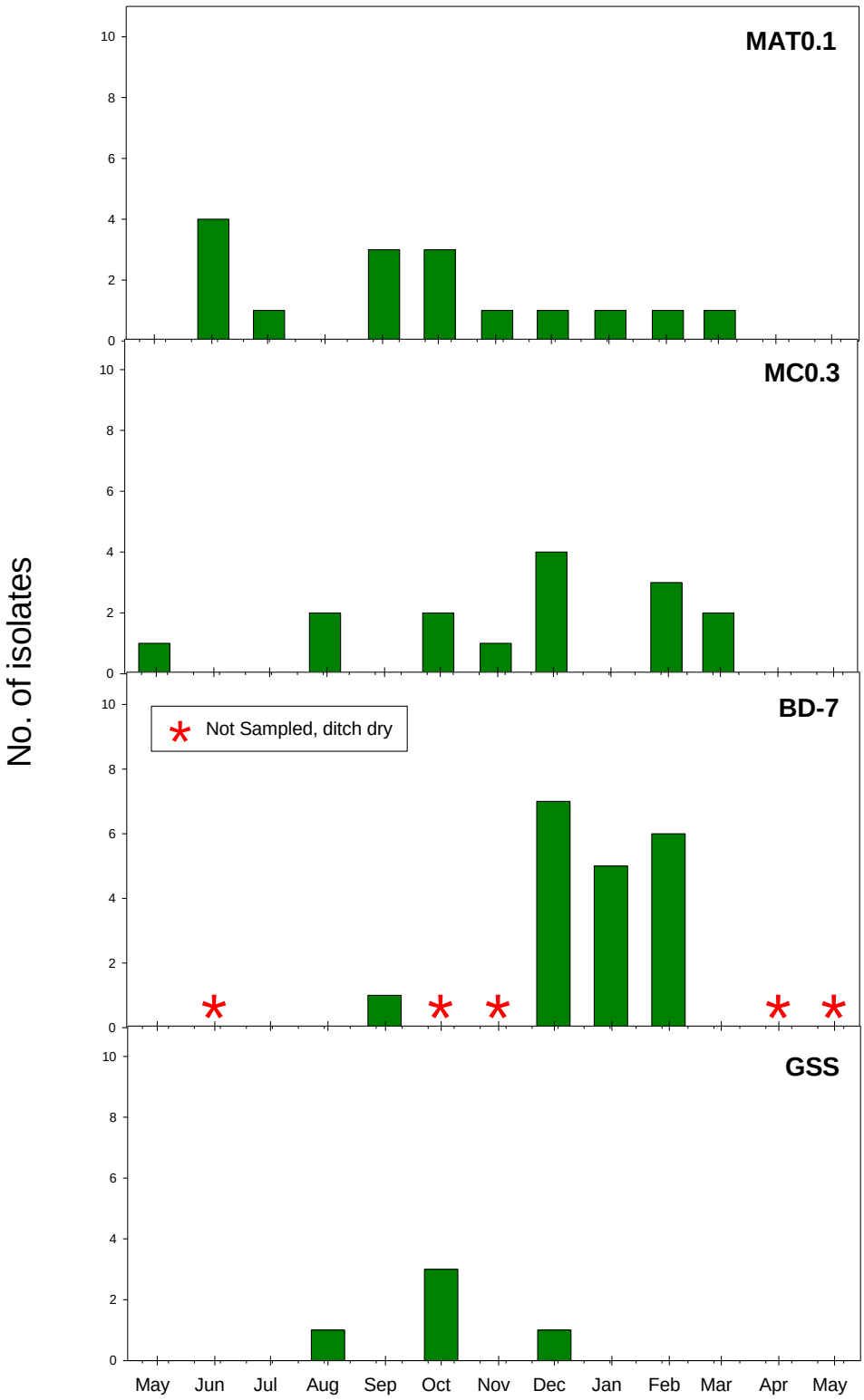

Figure 3.6 Human-derived source isolates combined (human, septage, sewage) between May 2006 and May 2007 for each freshwater station.

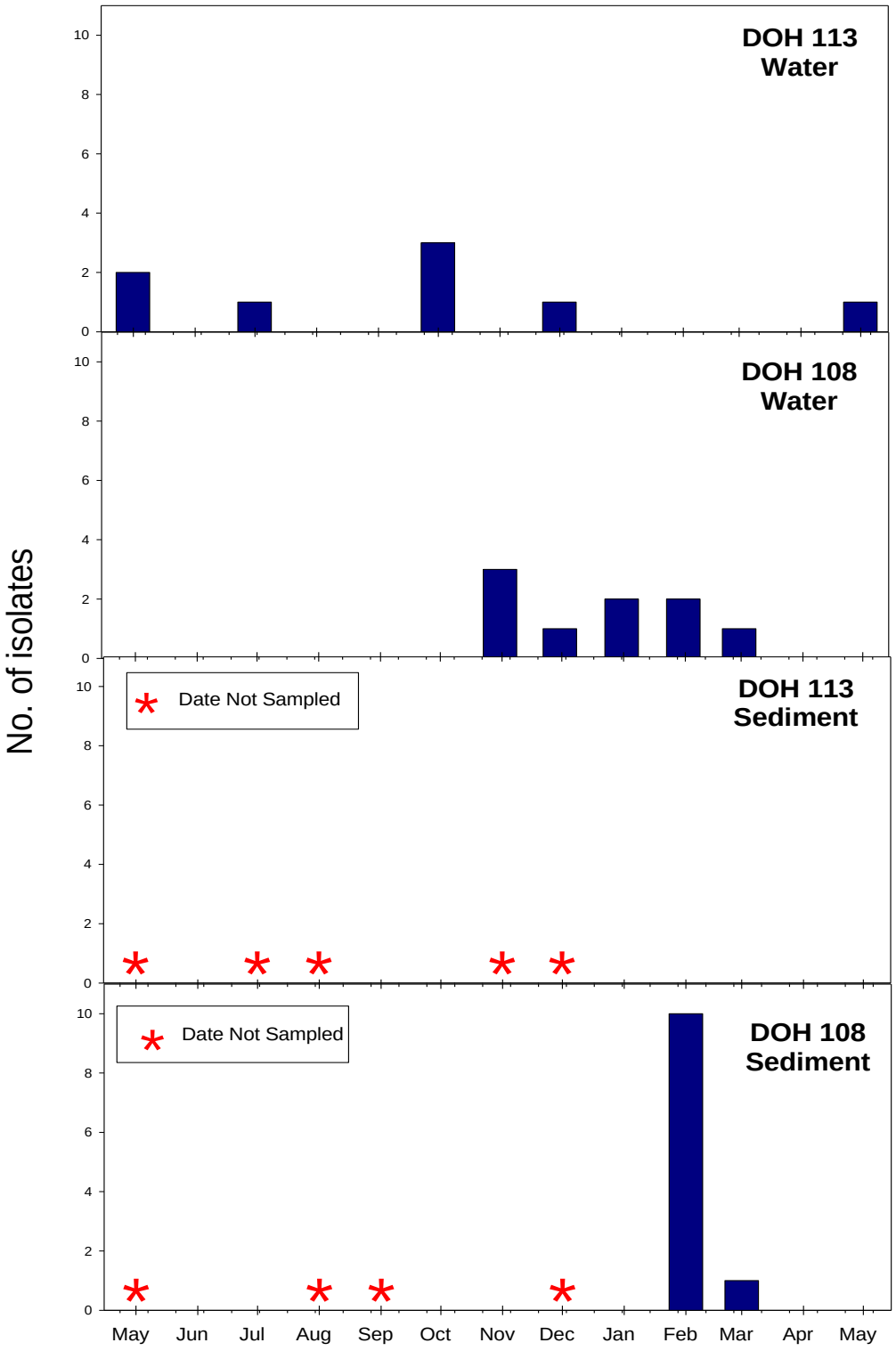

Figure 3.7 Human-derived source isolates between May 2006 and May 2007 for the two marine water stations (upper graphs) and the two sediment stations (lower graphs).

3.2 Phase 2 – *Bacteroides* Target-specific PCR

The target-specific PCR method used in the Phase 2 study is based on amplifying known DNA probes or markers from Bacteroides bacteria found in the gut of warm-blooded animals and matching those with Bacteroides DNA markers found in water samples. The two markers available for this study targeted human and ruminant. This approach did not require the development of a source library as in Phase 1 or require the culture of the indicator bacteria, *E. coli*, except as a screening tool.

This phase was a follow-up to Phase 1 and involved fewer sampling events, however a total of 27 stations were sampled during the three sampling events. Samples were collected on December 2, 2008 (10 marine stations, 6 seep stations), Dec. 12, 2008 (10 freshwater stations), and January 6, 2009 (5 marine stations, 11 freshwater stations) (Table 2.2, Figure 2.2). Sampling occurred at 14 of the 27 stations on at least 2 of the 3 dates, including a majority of the marine sites and some Phase 1 freshwater sampling stations.

3.2.1 Analysis of human and ruminant biomarkers

The species-specific primer sets that were used in this study were restricted to the identification of human and ruminant sources. The presence of general *Bacteroides* in a sample, combined with the absence of both human and ruminant target DNA in a sample indicated that the fecal contamination present was neither human nor ruminant, but was associated with another species of animal. The presence of general *Bacteroides* target DNA in a sample and the presence of the human target DNA indicated that the fecal contamination was human. Similarly, the presence of general *Bacteroides* target DNA in a sample and the presence of the ruminant target DNA indicated that the fecal contamination was from ruminant sources.

Table 3.7 provides the summary results of the PCR analysis for the human and ruminant primer sets presented by sampling date and sample station. The *Bacteroides* screening test or "*Bacteroides* present" column in the table indicates whether the presence of general *Bacteroides* was found in the sample. The columns for "human" and "ruminant" indicate whether the target DNA was present in the sample. The "other" column is checked if general *Bacteroides* was present but no human or ruminant DNA was present, meaning the presence of fecal contamination from a source other than human or ruminant.

A total of 42 samples were collected, not including blind or quality control samples, during three sampling events. Overall there were relatively few samples that were identified as a ruminant or human source. Twenty samples tested negative for the *Bacteroides* screening test (48%), and 17 samples (40%) contained the general *Bacteroides*-only marker indicating a source other than human or ruminant were present (Table 3.7 "Other").

Two out of 42 samples (4.7%) were identified as ruminant-only from the study. One of these samples was from a seep site (S-8) collected on Dec. 2, 1008, while the second sample was from Cooper Creek (Station CC) collected during the last event, Jan. 6, 2009. One sample from Casselary Creek (Station CAS) tested positive for human and ruminant sources and was collected during the last event. Two additional samples tested positive for human-only. These samples were collected during the last event from MS0.3 and MC0.2. The majority of samples with an identified human or ruminant source were collected during the January 2009 sampling event.

Table 3.7 Summary of Phase 2 analysis of FC concentration, total suspended sediments, salinity and target-specific PCR analysis for presence of *Bacteroides* human and ruminant biomarkers.

Sampling Date	Sample Station ID	Fecal Coliform (CFU/100 mL)	TSS (mg/L)	Salinity (psu)	*Bacteroides* PCR			
					Bacteroides Present	Human	Ruminant	Other
12/2/2008	DOH-114	4	8.81	29	No	---	---	---
12/2/2008	DOH-113	12	5.53	31	No	---	---	---
12/2/2008	DOH-103	2	7.22	32	No	---	---	---
12/2/2008	DOH-104	20	7.18	31	No	---	---	---
12/2/2008	DOH-107	<2	6.82	32	No	---	---	---
12/2/2008	DOH-108	12	7.79	32	No	---	---	---
12/2/2008	DOH-109	8	6.37	31	No	---	---	---
12/2/2008	DOH-110	32	7.83	31	No	---	---	---
12/2/2008	DOH-111	10	9.04	31	Yes	---	---	✓
12/2/2008	DOH-112	18	7.83	31	Yes	---	---	✓
12/2/2008	S-3	<2	26.5	NM	No	---	---	---
12/2/2008	S-4	8	38.9	NM	No	---	---	---
12/2/2008	S-5	<2	NS	NA	Yes	---	---	✓
12/2/2008	S-6	6	66.4	NM	No	---	---	---
12/2/2008	S-7	<2	NS	NA	No	---	---	---
12/2/2008	S-8	<2	NS	NA	Yes	---	✓	P
12/11/2008	MC0.3	18	1.09	0	No	---	---	---
12/11/2008	GSS	48	12.3	15	Yes	---	---	✓
12/11/2008	CC	290	2.16	0	Yes	---	---	✓
12/11/2008	CC (rep)	N/A	N/A	N/A	Yes	---	---	✓
12/11/2008	MS 0.3	82	1.69	0	No	---	---	---
12/11/2008	MC 2.0	66	1.53	0	No	---	---	---
12/11/2008	MC1.75t	38	3.47	0	No	---	---	---
12/11/2008	CAS	46	0.98	NM	Yes	---	---	✓
12/11/2008	MAT0.1	4	1.21	NM	No	---	---	---
12/11/2008	DR0.2	<2	0.58	NM	No	---	---	---
1/6/2009	DOH 108	47	7.93	30	Yes	---	---	✓
1/6/2009	DOH 110	74	10.0	30	Yes	---	---	✓
1/6/2009	DOH 111	53	< 1	30	Yes	---	---	✓
1/6/2009	DOH 112	39	5.43	30	Yes	---	---	✓
1/6/2009	DOH 113	32	14.9	25	Yes	---	---	✓
1/6/2009	CAS	TNTC	4.10	0	Yes	✓	✓	P
1/6/2009	CC	10	4.54	0	Yes	---	✓	P
1/6/2009	MC0.3	19	13.0	24	Yes	---	---	✓
1/6/2009	GSS	6	42.6	29	No	---	---	---
1/6/2009	MAT0.1	TNTC	4.45	0	No	---	---	---
1/6/2009	DR0.2	34	0.96	0	Yes	---	---	✓
1/6/2009	DR0.05	48	33.4	25.0	Yes	---	---	✓
1/6/2009	MS0.3	114	4.75	13	Yes	✓	---	P
1/6/2009	MC2.0	TNTC	5.24	NM	Yes	✓	---	P
1/6/2009	MC1.75t	14	7.49	NM	Yes	---	---	✓

Sampling Date	Sample Station ID	Fecal Coliform (CFU/100 mL)	TSS (mg/L)	Salinity (psu)	*Bacteroides* PCR			
					Bacteroides Present	Human	Ruminant	Other
1/6/2009	BD3	14	1.16	NM	Yes	---	---	✓
1/6/2009	Transfer Blank	NS	NA	NA	No	---	---	---
1/6/2009	Blind Source #1 (Cat)	NS	NA	NA	Yes	---	---	✓
1/6/2009	Blind Source #2 (Septic system)	NS	NA	NA	Yes	✓	---	P

TNTC - Too numerous to count
NS - Not sampled
NA - Not applicable
NM - Not measured
P - Possible
✓ presence of *Bacteroides* biomarker as indicated

Although the bulk of the samples where no *Bacteroides* were identified were associated with fecal coliform levels below 20cfu/100ml, the two were not always associated and there appears to be little relationship between the two. In some instances *Bacteroides* general or host-specific markers were identified in samples with low levels of fecal coliform and alternatively in several cases *Bacteroides* was not identified in the presence of higher levels of fecal coliform (>20cfu/100ml).

3.2.2 Quality Control Assessment

In accordance with the guidelines established in the QAPP for this project (Battelle and EPA, 2008), the following quality control tests were conducted as an integral part of the analyses:

Positive DNA Controls: These samples consisted of plasmid DNA containing the target sequence: A positive control was analyzed in conjunction with each set of amplifications and always provided an appropriate response for the data provided in this report.

Replicate Analyses: Filter replicates and sample replicates (10% of total samples) were analyzed. These did not always provide the same results on a qualitative basis for the data. Two of four replicate analyses provided qualitative identical results, however two did not. The discrepancy may be due to two possible factors: The level of extracted DNA present in the sample may have been below the detection limit for the sample that was negative. This is commonly seen in microbiological analyses when the sample contains the target at very low levels. The second possible explanation is that the level of background contaminants may have been high enough to interfere with the detection of DNA in one sample but not in the other.

Negative controls: Four negative controls were utilized, as described below.

Extraction Negative Controls: Each time a batch of samples was extracted a negative extraction control (DNA-free water used instead of sample) was extracted at the same time. These negative controls always provided an appropriate response.

PCR Negative Control (consisting of master mix and the appropriate primer set, but using water instead of sample): A negative PCR control was analyzed with each set of amplifications and always provided an appropriate response for the data.

Filtration Controls: This consisted of preparing an in-house filtration control and analyzing the resulting filter. The filtration controls analyzed were negative providing an appropriate response for each of the sample sets completed as part of this dataset.

Transfer Blank: A bottle of sterile water was provided by the Region 10 Laboratory and was transferred to a sterile bottle in the field by the sample collector. This was shipped and processed as a sample through the entire process. The transfer blank analyzed was negative for *Bacteroides* biomarkers.

Blind Samples: The sampling team provided the EPA Laboratory with two blind samples for the last sampling event (Table 3.7). The samples were collected using an EPA recommended collection protocol. The sample sources were known to the collectors, however unknown to the analyst. Both blind samples provided an appropriate response. The first blind sample source was from a domestic cat and was identified correctly as "other" by the Laboratory. The second blind sample was taken from an on-site septic system. This was identified correctly as *Bacteroides* positive and matched to a human biomarker (HF134).

3.2.3 Water Quality (fecal coliform and total suspended sediments)

Samples for fecal coliform analysis were collected and analyzed from each station during each event (42 samples). The results are presented in Table 3.7 and accompany the PCR results. Fecal coliform values ranged between the detection limit (<2 CFU/ml) to TNTC (too numerous to count). Coliform values during the first event (12/2/2008) were relatively low ranging between <2 to 32 CFU/100ml. All seep samples (S-3 through S-8) were extremely low ranging between <2 to 8 CFU/100ml. Samples from the second event which included only freshwater stations were somewhat higher ranging between <2 to 290 CFU/100ml. The last event included several stations with TNTC values including CAS, MAT0.1, and MC2.0. Interestingly, all samples with human source identification were from the last sampling event from stations with high FC values (114 CFU/100 ml to TNTC).

TSS samples were analyzed from each station and sampling event with the exception of several seep sites which were logistically difficult to sample (Table 3.7). TSS ranged from a low of <1 mg/L at DOH-111 on Jan. 6th, 2009 to a high of 66.4 mg/L TSS from a seep site (S-6) on December 2nd, 2008. The bluff seep stations had slightly higher turbidity values than most other stations. The turbidity data was collected in order to determine if there was a correlation with FC. In general the TSS values were relatively low and there was no apparent correlation with FC.

4.0 Discussion

The microbial source tracking study undertaken in the Dungeness watershed confirms that non-point source bacterial pollution is present on the landscape and is highly reflective of the complexity of the environment (i.e. multiple usages in a freshwater and estuarine/marine environment). Thirty-seven species or types of animals and birds were identified as probable sources of fecal contamination in this study. These sources represent classic inputs of non-point source pollution (e.g. birds, deer, raccoon, domestic pets, human-derived sources) as well as representing inhabitants and a usage that are somewhat unique but characteristic of this watershed and estuary (e.g. game farm animals, marine mammals).

The predominant source of fecal coliform pollution in the Dungeness watershed, both in the freshwater and marine environment is birds. This is evident when looking at the individual source type data (Section 3.1.1) from the overall Phase 1 study. Avian had the largest proportion of isolates (19.6%), followed by gull (12.5%), and waterfowl (9.7%). When these source types are grouped into functional guilds, birds as a functional group represented 42% of the distribution of isolates. The mean proportion of bird sources from marine water sampling events (50.9%) was somewhat greater than the freshwater station proportions of birds (38.6%). The predominant influence due to birds has been found at other study sites in Puget Sound as well. An MST ribotyping study in Henderson Inlet in south Puget Sound found that birds composed the highest proportion of isolates from the marine waters and a freshwater tributary of Henderson Inlet, Swayne Creeks (Thurston County, 2002). A recent study in Drayton Harbor and the adjacent California Creek watershed in Whatcom County, WA also found that avian fecal sources were the most frequently detected in the marine waters (Hirsch Consulting Services, 2008). The presence of waterfowl, in particular, is not unexpected in areas with larger bodies of water associated with the watershed. The Dungeness National Wildlife Refuge encompasses Dungeness Bay, and is home to more than 250 species of birds for some parts of the year. Migrating shorebirds are present in the spring and fall, and flocks of waterfowl are present during the winter months. The widespread influence of birds, particularly gulls, has been noted in other source tracking and fecal indicator studies as well, notably along coastal and lake waters (Edge et. al. 2007; Lu et al. 2008; Nelson et al. 2008).

Other individual sources that encompassed lesser proportions of isolates (between 4 and 9 % each) included raccoon, human-derived sources, rodent and dog. When these are grouped into functional guilds the most prevalent source after birds was wild mammals (including raccoon, rodent, deer, elk, beaver, otter, rabbit and marine mammals). Groups with somewhat less but similar proportions included domestic animal, human, and farm animal. Game farm animals represented a smaller proportion of isolates. This pattern was true for all station data that was grouped together, and was also true when the freshwater stations and the marine water and sediment stations were examined independently. When examining these trends spatially, there was a notable similarity between the proportion distribution of freshwater source type groups and marine water source type groups (Figures 3.3, 3.4, 3.5) indicating that at least a fair portion of freshwater sources, such as wild mammals, domestic and farm animals, are being conveyed into the marine environment, presumably as runoff directly into Dungeness Bay or by transport via the Dungeness River.

There was no statistically significant change in the partitioning of the proportion of isolates for the duration of the study; however several temporal trends were noted. In marine waters the proportion of wild mammals increased somewhat while the proportion of farm mammals decreased somewhat, however

both remained highly variable. In the marine waters, the proportion of combined wild mammals and game farm sources increased during the wet season, indicating the possible influence of runoff during this season.

The marine sediment isolate distribution was similar to freshwater and marine water stations; however sediment was not regularly sampled so a direct comparison could not be made. Although this study was not designed to determine whether sediment was acting as a reservoir of *E. coli*, the data do suggest that *E. coli* bacterial sources in the overlying marine water are similar to the *E. coli* sources in the upper few centimeters of sediment, and a number of those sources are land-based in origin (e.g. deer, elk, raccoon, rodent, horse). The MST study in Henderson inlet also found similar distributions between sediment samples collected and the overlying marine water (Thurston County, 2002).

Wrack samples (detrital algae) were collected when it was present along the shoreline near station DOH-113 (9 out of 13 sampling events). Because algal mats routinely form along the shoreline and tidal flats of Dungeness Bay, particularly in the summer, we were interested in understanding what sources might be present in this matrix type. Wrack has been noted as a potentially important reservoir of indicator bacteria in the nearshore areas of the Great Lakes and other areas (Whitman et al. 2003). Of the nine sampling events, the presence of *E. coli* was found only twice. From those 2 sampling events, the presence of dog, deer and avian were noted. These sources are not surprising, although the limited number of occurrences from this study would indicate that wrack may not be a major reservoir of *E. coli*. It should be noted however, that samples were not collected in areas of heavy algal accumulation, which usually occurs along the tide flats of outer Dungeness Bay.

One of the goals of the study was to examine the primary sources of fecal contamination at specific sites as well as determine any site specific differences. While the predominant source of bacterial contamination at all stations was birds, there were several site specific differences noted. BD-7 had a significantly higher proportion of human-derived isolates compared to other freshwater sites (Table 3.6) while MAT0.1 had a significantly higher proportion of game farm source types (including bear, bison and yak) compared to other freshwater stations. In addition, game farm animals occurred during 46% of sampling events throughout the year at this site. The MAT0.1 station was located on the Olympic Game Farm, close to the mouth of Matriotti Creek before it joins the Dungeness River. While a number of BMPs have been implemented on the game farm in the past, this evidence suggests that further mitigation efforts might be warranted. Although the game farm sources were not a predominant source at other freshwater sites, their occurrence was found at other freshwater, marine water and marine sediment sites, again pointing to the conveyance of land-based sources through the watershed and into the marine environment.

Human-derived sources occurred at all stations except the DOH-113 sediment station and the marine wrack station (Table 3.6), clearly indicating a source for concern. Although BD-7 had the highest proportion of human-derived isolates (15.3%) compared to other freshwater sites, these occurred primarily between December and February. This may be indicative of a septic drain field failure in relatively close proximity to the sampling site. This station was also dry during 5 out of the 13 sampling events, and was therefore not sampled every month. MAT0.1 and MC0.3 had the highest frequency of occurrence of human-derived sources, 69% and 54% respectively (Table 3.5, Figures 3.6 and 3.7). As tributaries to the Dungeness River, these two sources appear to represent a chronic and relatively constant source of human-derived sources. This has been suggested in previous studies (Sargeant 2002, Hempleman and Streeter, 2004; Rensel, 2003), and evidence from this MST study confirms those

findings. A number of failing septic systems have been documented in the lower Dungeness watershed of concern and at least 8 septics of concern and 53 neighboring septic of concern repairs or upgrades were completed since October 2004 under Task 2b - *Homeowner Sewage Management BMP Education and Training* of the Targeted Watershed grant. This study confirms that additional sources of human-derived input are still impacting the freshwater and marine waters. In the marine waters, another possible human-derived source is recreational boaters, although there are public restrooms available for day-use boaters at Cline Spit, and generally Dungeness Bay does not receive much usage from overnight boaters. While it is possible that recreational boaters are contributing FC sources directly to the Bay, it is more likely that the human-derived sources were transported from a freshwater source. These findings are consistent with the results of the FC studies conducted by Rensel et al. (2003).

The Phase 1 Ribotyping and Phase 2 *Bacteroides* PCR studies were designed as independent but sequential investigations in the same watershed. While the two studies cannot be compared directly, it was an opportunity to evaluate and contrast the two MST methodologies qualitatively. Results from the two studies led us to similar interpretations regarding fecal coliform sources in the Dungeness watershed. We concluded from the ribotyping study that birds were the dominant source of contamination in the freshwater and marine stations in the Dungeness watershed with numerous additional sources contributing smaller, but cumulatively significant inputs. Those smaller sources included human-derived sources, domestic, farm and game farm animal groups. In comparison, the Phase 2 study, initiated approximately 7 months after the Phase 1 sampling ended, focused on the presence of human and ruminant biomarkers exclusively. Those results confirmed the presence of both human and ruminant sources at several freshwater stations including additional stations that were not part of the Phase 1 study (i.e. Meadowbrook Creek 2.0, Meadowbrook Slough, Cooper Creek, Cassalery Creek, bluff seep #8). Overall the two methods provided complementary results. The Phase 1 study led to an understanding of the predominant sources in the watershed (i.e. birds and wild mammals). The Phase 2 study confirmed the Phase 1 study findings of the presence of human and ruminant sources, although it should be noted that the Phase 2 study was conducted in the winter only and may not be reflective of all seasons. The Phase 2 study also provided information suggesting the spatial widespread influence and spatial extent of human-derived sources in the Dungeness watershed.

Each of the methods employed here had strengths and weaknesses, and in general, MST methods are still evolving and improving. Currently there is no standard method that is appropriate to answer all MST questions. The Phase 1 ribotyping study was fairly comprehensive and answered questions about dominant sources and the variety of sources of fecal coliform pollution in the Dungeness watershed. However the collection of local source samples to add to the IEH library required additional time and resources. In addition the turnaround time for receipt of final results was close to ten months after the last samples were collected, which is impractical for most applications. The Phase 2 *Bacteroides* PCR study was smaller in scope in terms of the number of sampling events and samples collected. Since this was a library-independent approach, there was no need for source sample collection, and in general sample collection was relatively straight forward. The "blind" samples submitted to the EPA Manchester Lab for the study produced accurate results and the turn-around time was approximately 2 months for the study results. The analysis of samples submitted below the 14 CFU/100 ml cutoff value for sensitivity produced positive results indicating the usefulness of including these samples in future analyses. Only 2 primers (human and ruminant) were available for use by the laboratory. The usefulness of this method to resource managers could be greatly enhanced by the incorporation of additional primers.

Overall these methods were successful in providing credible scientific evidence of the predominant sources of fecal contamination in the lower Dungeness watershed and Bay. While some of these sources may be difficult to manage, such as birds and wild mammals, the study also provided evidence of sources in the watershed that can be controlled or mitigated for, such as human-derived sources that are a public health risk. This information will provide the basis for continued education and public outreach regarding sources of bacterial contamination in the watershed. The scientific information gained from this study will allow the opportunity for resource managers to re-evaluate and modify current on-site septic system management programs to specifically target resources toward actions that will be most effective at reducing bacteria levels in the watershed. In addition, it should also provide the basis for re-evaluation of the progress made toward achieving the goals of the *Clean Water Strategy and Water Cleanup Detailed Implementation Plan* (Hempleman and Streeter, 2004), and for making adjustments to monitoring plans or cleanup strategies as necessary. While these results should not be extrapolated to other geographic regions, information gained from the use of these tools, the overall approach and lessons learned can be applied to other watersheds.

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5.0 Conclusions and Recommendations

The following conclusions have been drawn from this study for the Dungeness watershed and estuary:

- The percentage of isolates matched to known sources during the ribotyping study was relatively high indicating a majority of fecal bacteria sources in the watershed were identified. Approximately 92% of the collective isolates from water, sediment, and wrack were identified and matched to a source. Of those, 5% were matched directly to the local database.

- The predominant sources of fecal coliform contamination identified in the Dungeness study area were, in ranked decreasing order of presence: avian (19.6%), gull (12.5%), waterfowl (9.7%), raccoon (9.2%), unknown (7.3%), human-derived (7.1%), rodent (6.3%) and dog (4.3%).

- Birds, in total, represented the largest source group, accounting for approximately 42% of the isolates collected and analyzed throughout the study. They also occurred most frequently throughout the course of the study. They were the dominant presence in Dungeness Bay and all freshwater stations in the lower watershed (i.e. Matriotti Creek, Meadowbrook Creek, and Golden Sands Slough) with the exception of BD7 where wild mammals occurred more frequently.

- Wild mammal sources represented about 26% of the isolates sampled and included raccoons, rodents, deer, elk, beaver, river otter, rabbit and marine mammals.

- Domestic animals represented about 7% of the isolates sampled and included dogs and cats.

- Farm animals also represented about 7% of the isolates sampled and included bovine, chicken, horse, llama, pig, sheep and goat.

- Human-derived sources, primarily from on-site septic systems, were present at all freshwater and marine water stations and one sediment station (DOH-108). These sources represented about 7% of isolates on average, and between 3 and 11% of the isolates at any given station. They were present throughout the year, and for each station that contained human-derived isolates, and occurred between 25 and 69% of the time of total sampling events. MAT0.1 (Matriotti Creek) had the highest frequency of occurrence, with human-derived sources occurring on 9 out of 13 sampling events. MC0.3 (Meadowbrook creek), BD-7 (bluff ditch), and Golden Sands Slough (GSS) also contained human-derived sources.

- Source types representing probable game farm animals (bear, bison, burro, prairie dog and yak) represented about 2.5% of the isolates sampled. Game farm animals were present at 46% of sampled events at MAT0.1, located on the Game Farm at the mouth of Matriotti Creek. Their presence occurred regularly throughout the year. Game farm animal types were also found at other freshwater sites as well as in the marine environment.

- While there were no statistically significant temporal trends, when combined, the proportion of wild mammals and game farm animal sources increased slightly during the wet season (October through March), indicating the possible influence of surface runoff events.

- The Phase 2 *Bacteroides* PCR study complemented the Phase 1 ribotyping approach. Although the study was of short duration, limited to the winter months, the PCR study identified human and ruminant sources over a larger geographic expanse in the lower Dungeness watershed, emphasizing the importance of increasing mitigation efforts of these sources.

- Once of the most important findings of the study was the presence of land-based sources (e.g. e.g. wild mammals, farm animals, domestic animals, game farm animals, human-derived sources) found in marine waters, sediment and wrack. This provides direct scientific evidence of the conveyance of these sources across the landscape into the marine environment.

- Based on the proportion of isolates sampled, approximately 24% of fecal coliform bacteria are from controllable sources (i.e. human-derived, domestic animals, farm animals, and game farm animals). If raccoons are included (categorized as wild mammal in this study), then approximately 33% or 1/3 of bacterial fecal contamination are from controllable sources.

Recommendations for the future include:

- Secure resources/funding to implement necessary improvements in management programs and enforcement mechanisms that will mitigate the public health risk by reducing human-derived sources and other readily controllable sources of fecal contamination, including:
    - On-Site Septic Systems
        - Ongoing homeowner education regarding septic system maintenance and homeowner inspections of septic systems
        - Investigate, identify, and repair or replace problem septic systems in the lower Dungeness watershed
        - Effective enforcement process
    - Game Farm Animals - Implementing additional BMP measures for reducing access to open waterways by game farm animals,
    - Domestic Pet Waste - Education and outreach to homeowners regarding proper disposal of domestic pet waste,
    - Urban Wildlife Populations – Education and outreach to homeowners about practices that discourage attraction of urban wildlife, particularly raccoons.
- Improve storm water management programs, including the promotion and implementation of Low Impact Development (LID) principles and practices such as the reduction of effective

impervious surfaces, dispersion of storm water runoff to vegetated areas, and Best Management Practices that are appropriate to the site specific conditions.

- Use the results from this study to evaluate current on-site septic system management programs and water quality monitoring plans in the Dungeness watershed. Re-examine implementation strategies and modify is necessary to achieve long-term water quality objectives.

- Continue emphasis on improving MST methodologies, including efforts that will encourage accessibility and use of these tools in a streamlined and cost-effective manner.

- Continue outreach (including dissemination of related study results) to the public about nonpoint source pollutant sources and steps that can be taken to mitigate those sources that are human-derived and controllable through improved management programs and enforcement mechanisms that will benefit ecosystem and public health.

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