Enteric Virus Surveillance and Microbial Source Tracking in Fresh and Marine Waters of the Seattle Area

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Abstract

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Introduction: (Part I.) Bacterial indicators, including fecal coliforms, *E. coli*, and Enterococci, serve as long-standing measures of fecal contamination. However, coliphages hold promise as viral indicators, which may be more representative of viral enteric pathogens. Filtration and concentration of large water sample volumes (i.e. 20 L) increases viral detection sensitivity. (Part II.) Poverty Bay spans 11 shoreline miles of Puget Sound west of Des Moines. In response to recent geoduck bed closures in the area, we performed a microbial source delineation study in 5 creeks upland from the Bay with the intent of discovering the source of fecal contamination.

Methods: (I.) Using the Bag-Mediated Filtration System (BMFS), followed by PEG precipitation and Vertrel XF extraction, we quantified levels of enteric viruses (AdV, NoV, EnV) and male-specific coliphage (MSC) at Green Lake, Lake Washington, and Puget Sound recreational beaches during June-November 2016. MSC was enumerated using the double agar layer, and enteric viruses were detected with qPCR. These data were compared to MPN estimates for traditional fecal indicator bacteria, using Colilert®-18 and Enterolert® substrates sealed in QuantiTrays®/2000. (II.) Coliscan Easygel ® kits were used during September 2016 – May 2017 to estimate *E. coli* levels in 5 streams upland of Poverty Bay during wet and dry weather conditions.

Results: (I.) Throughout the sampling period (n = 6-7 samples/site), detection of MSC occurred only following a wet weather event, at levels of 19 PFU/L (Matthews Beach) and 21 PFU/L (Carkeek Beach). No pathogenic enteric viruses were detected with qPCR throughout the sampling period. However, a number of indicator bacteria samples exceeded U.S. EPA and Washington State standards during the sampling period. (II.) *E. coli* levels across the Poverty Bay drainage basin led to identification of potential hotspots on the 5 upland creeks.

Significance: Unique to the Seattle Area, this study provides an overview of bacterial indicator, coliphage, and enteric virus levels in both fresh and marine waters. In addition, it contributes to the body of literature that evaluates the potential of coliphages to more accurately indicate fecal contamination than traditional bacterial indicators.

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Chapter 1:
Overview

Study Area: Fresh and Marine Waters of the Seattle Area

Located between the saltwater Puget Sound to the west and Lake Washington to the east, the Seattle Area provides residents with numerous opportunities for commercial and recreational water activities. However, chemical and microbiological sources of pollution present a constant problem in the region. Public health departments consistently monitor water bodies for fecal contamination, which may reach high levels in urban streams, stormwater runoff, and wastewater treatment plant outfalls. While the Washington State Department of Ecology and the King County Swimming Beach Monitoring Program measure fecal contamination at Puget Sound and Lake Washington recreational beaches, the Washington State Department of Health surveys shellfish growing areas (Ecology 2016; King County 2017; Swanson 2016).

Puget Sound

Puget Sound is unique among United States estuaries due to its fjord-like shape and form. The Sound encompasses approximately 2,330 km^2, including 4000 km of shoreline, fed by thousands of streams and rivers than drain a land area of 35,500 km^2. Home to numerous beaches, including four within Seattle, as well commercial and tribal salmon fisheries and shellfish beds, the Main Basin region stretches from Everett south to Tacoma and receives eighty percent of waste discharged from point sources into the estuary. The Washington Department of Natural Resources estimates that fifty-two percent of the shoreline in this area has been modified by human activities (Ruckelshaus and McClure 2007).

Lake Washington

With an area of 88 km^2, Lake Washington formed during the southward movement of the Cordilleran Ice Sheet near the end of the Late Pleistocene. It stretches for twenty-two miles and receives inflow from the Sammamish and Cedar Rivers. Lake Washington has undergone impressive cleanup efforts since the 1950s, when an estimated twenty million daily gallons of raw sewage entered the lake from Seattle and other surrounding communities. Between 1963 and 1968, one hundred miles of sewer trunk lines were laid to rely sewage to treatment plants; all effluent was eliminated in 1968, and the lake's transparency improved from thirty inches in 1964 to ten feet in 1968 (Ruckelshaus and McClure 2007).

Green Lake

A eutrophic lake in north Seattle that lacks natural surface inflows or outflows, Green Lake has an area of 4.1 x 10^5 m^3 and a mean depth of 4 m. It is fed by stormwater runoff, groundwater, and water released from Seattle's domestic water reservoirs. Green Lake is a popular year-round recreational area, with the entire lake shore developed as a park. Although monitoring data show low fecal coliform levels for twelve years running, large bird populations, dogs, and human bathers are all potential sources of fecal contamination (Scherer et al. 1995; King County 2017).

Poverty Bay

With a drainage area of approximately nineteen square miles, Poverty Bay spans eleven marine shoreline miles of Puget Sound in King and Pierce Counties, from Des Moines Creek south to Brown's Point. The area is characterized by the Des Moines Plain, an upland plateau with elevations that range from three hundred to four hundred feet above sea level, and is bounded to the east and west by steep bluffs. Due to this upland pattern, the drainage basin

flowing to the shellfish growing area is relatively small with creeks under two miles long. The land draining to Poverty Bay is urbanized and fully incorporated into the cities of Des Moines and Federal Way (Georgeson 2014).

There are five Wild Stock Commercial Geoduck Clam Fishery areas in the Poverty/Dumas Bay area, which are jointly managed by the Washington State Department of Fish and Wildlife and the Puyallup Tribe of Indians. As of September 2016, due to high fecal bacteria levels, the Washington State Department of Health downgraded 130 acres of shellfish beds to permit harvesting only during December – May each year. The downgrade also triggered the requirement that the King County council form a Shellfish Protection District and implement a shellfish protection program (Swanson 2016).

Enteric Pathogen Surveillance and Microbial Source Tracking

Microbial source tracking (MST) is a set of microbiological techniques used to determine sources of fecal pollution in the environment (Meschke and Boyle 2007). Point sources of pollution are single, identifiable sources (ex. wastewater treatment plant outfall) that have immediate adverse effects on the growing area. Conversely, nonpoint sources are diffuse waste discharges that reach the growing area in a roundabout way. They are more difficult to track and include leaking municipal wastewater conveyances, seepage from septic tanks, agricultural runoff, and stormwater runoff carrying pollutants from street surfaces, wooded areas, and construction sites (Booth et al. 2006). However, an understanding of an area's nonpoint sources proves necessary when striving to meet pollution control targets.

While MST narrows down pollution sources, fecal coliform monitoring does not discern whether bacteria are introduced to water bodies through human, wildlife, and/or domestic animal sources. *Bacteroides*, F+RNA coliphage typing, and tracer chemicals (ex. acetaminophen,

caffeine, and paraxanthine) are emerging species-specific techniques (Embrey 2001; Dick et al. 2005; Stewart-Pullaro et al 2006; James et al. 2016). Year-round monitoring provides additional pollution source information, as dry and wet weather sources may differ (Mitch et al. 2010).

Indicator Species

While specific enteric pathogens carried in human sewage and domestic animal waste are the true contaminants of concern, they are costly to detect, numerous, and present in small concentrations, rendering quantification difficult and expensive. As a result, indicator microorganisms are used to determine fecal contamination levels in growing waters or shellfish tissue. An ideal indicator species (a) is present whenever enteric pathogens are present; (b) should be useful for all water types; (c) should have a longer survival time than the dardiest enteric pathogens; (d) should not replicate in water; and (e) should be found in the intestines of warm-blooded animals. No indicator organism fits these criteria perfectly. Bacterial indicators, including fecal coliforms , *E. coli,* and Enterococci are most frequently used to monitor fecal contamination. Enterococci, a group of fecal streptococci bacteria, is monitored in both fresh and marine waters, while *E. coli*, a fecal coliform, is only monitored at freshwater sites (Maier et al. 2009).

While fecal indicator bacteria provide an inexpensive, quantitative means of determining fecal contamination and correlated with bacterial pathogens, they are less useful as predictors of viral pathogens, particularly in marine environments. A lack of correlation exists between indicator bacteria and virus growth and persistence in the environment, as coliform fate may differ biologically from the fate of viruses under various environmental conditions (Jiang et al. 2001; Leclerc et al. 2000). Thus, human enteric viruses may be present in water regardless of

fecal indicator bacteria densities. The presence of viral pathogens in Seattle Area water is understudied.

Study Specific Aims

- Using the Bag-Mediated Filtration System, a novel method for concentrating large water samples, we performed a preliminary surveillance study of adenovirus, norovirus, enteroviruses, and the bacteriophage MSC at Green Lake, Lake Washington, and Puget Sound recreational beaches from June 2016 – November 2016. Bacterial indicators, including fecal coliforms, *E. coli*, and Enterococci were evaluated as well. MSC was expected to more accurately reflect levels of enteric viruses (if present) than bacterial indicators.
- Through performing microbial source tracking with *E. coli* data between Fall 2016 and Spring 2017, we sought to identify sources of fecal contamination that may have affected the conditional closure of geoduck beds in Poverty Bay. Information gained in this project was expected to inform policymakers and the public, thereby supporting elimination of identified fecal coliform sources.

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Chapter 2:
Enteric Virus Surveillance at Seattle Recreational Beaches
June – November 2016

Introduction

Recreational Bathing, Enteric Viruses, and Gastrointestinal (GI) Illness

An increase in recreational waterborne disease outbreaks throughout the past 35 years implies a need for development and implementation of waterborne pathogen surveillance methods (Yoder et al. 2008). According to reports from state health departments and the Waterborne Disease and Outbreak Surveillance System report, viral outbreaks comprise an increasing proportion of recreational water outbreaks reported to the CDC (Sinclair et al. 2009). While 7 percent of outbreaks between 1971 and 2000 were definitely attributed to viral agents (Craun et al. 2005), viruses likely account for a large proportion of unknown etiological agents (Yoder et al. 2008). Epidemiological changes in viral and host epidemiology and behavior patterns, as well as improved viral detection methods, contribute to the increased incidence of viral outbreaks (Sinclair et al. 2009).

Since the 1950s, epidemiological studies have provided evidence for the association between bathing in recreational waters and elevated risk for gastrointestinal (GI) illness (PHLS 1953, Stevenson 1953). Of 55 recreational water outbreaks identified between 1951 and 2006, 40 percent were associated with lakes or ponds (Sinclair et al. 2009). Table 1 presents a summary of recent epidemiological studies relevant to risk factors for GI illness from recreational water exposure at relatively clean beaches with primarily non-point sources of pollution (Jaffer 2016). The studies assessed in this review suggest that although bathing may be associated with increased GI illness, odds ratios were not strikingly high. However, swallowing beach water and swimming on days when groundwater discharge is prevalent were found to most greatly increase an individual's risk of contracting a viral or bacterial infection (Arnold et al. 2013; Yau et al. 2014).

Table 1. Risk factors for GI illness following recreational water exposure (Jaffer 2016).

First Author, Year	Study Design	Water Type, Contamination Level	Study Population, Location, and Dates	N (completing follow-up)	Significant Findings (aOR's for risk of GI illness with 95% CI's) Comments
Arnold 2013	Prospective cohort	- Marine - 5 sample points, sampled at 8 am and 1 pm - Mean ENT 3 cfu/100 mL (Range 0.5-1740) - Avg *E. coli* 13 cfu/100 mL (Range 0.5-1000)	- Subjects recruited at beach - Malibu, CA - Non-point source pollution - 39 recruitment days - 5/23/09 – 9/20/09 - Followed up 10-19 days later, attn. given to illness w/in 3 days	5674 2559 bathers 1895 non-bathers	- 3-day results - Body immersion vs. non-bathers aOR = 1.90 (1.17-3.09) - Head immersion vs. non-bathers aOR = 1.91 (1.17-3.14) - Swallowed water vs. non-bathers aOR = 2.86 (1.64-4.97)
Cordero 2012	Prospective cohort	- Marine - 3 daily samples from 6 sites - Avg ENT 35 cfu/100mL	- Subjects recruited at beach - Luquillo, Puerto Rico - Non-point source pollution - Sundays 6/14/08 – 8/3/08 - Follow-up 10-12 days later	1299 921 bathers 378 non-bathers	- No statistically significant findings - aOR 0.88 (0.47-1.63) for bathers - Clean water conditions - Stratification by rainy/dry season insignificant
Fleisher 2010	Randomized control trial	- Marine - Bathers collected water sample from demarcated zone - Avg ENT 71 cfu/100mL	- Recruited local adult residents who regularly bathed in ocean - Randomized bather and non-bather assignment - Miami, FL - Non-point source pollution - 15 recruitment days - 12/15/07 – 6/21/08 - Follow-up within 7 days	1303 652 bathers 651 non-bathers	- No statistically significant findings - OR 1.79 (0.94-3.43); p = 0.07
Marion 2010	Prospective cohort	- Freshwater - Daily water sample - Avg *E.coli* 95.1cfu/100 mL	- Subjects recruited at beach - East Fork Lake, OH - Non-point source pollution - 26 recruitment days - 5/30/09 – 8/30/09 - 8-9 day follow-up	965	- Bathers vs. nonbathers aOR 3.2 (1.1-9.0) - Exposure to water with *E. coli* > 11.3-59 CFU/100 mL had significantly elevated GI illness aOR of 7.2 (1.3-39) (p = 0.022)
Sartorius 2007	- Norovirus outbreak - Age-matched case-control - clinic based cases - community based controls	- Freshwater - 1 sample NV+ (8/10/04) - 1 sample with 120 *E.coli*/100mL	- Vastra Gotaland County, Sweden - Delsjon and Aspen Lakes (12 km apart) - 8/11/04: first case reported to county med. Office - 400 sick individuals by 8/25/04	492 163 cases 329 controls	Risk factors for GI illness: Swallowing water (OR 4.7; 1.1-20.2; p = 0.041) Attendance at Delsjon Lake (25.5; 2.5-263.8; p = 0.02) Taking water from Delsjon Lake (17.3; 2.7-110.7; p = 0.003) Swimming < 20 m from shore (13.4; 2.0-90.2; p < 0.05)
Yau 2014	- Prospective cohort	- Marine - 3 daily samples; 4 locations - ENT range <2- >10,000 cfu/100 mL	- Subjects recruited at beach - Avalon Beach, CA - Point-pollution from sewerage discharge - 61 recruitment days - Summer months 2007-2008 - Follow-up 10-14 days, attn. given to illness w/in 2 days	6165	- Submerged groundwater discharge a significant effect modifier of water exposure/indicator density on GI illness. At >median SGD: - aOR for swallowed water vs. non-bathers 2.18 (1.22-3.89) - aOR for head submersion per log_{10} increase ENT density 1.36 (1.01, 1.84) - aOR for swallowed water per log_{10} increase ENT density 1.8 (1.06, 3.23)

Abbreviations: aOR – adjusted odds ratio; ENT – enterococcus

Spread through the fecal-oral transmission route, enteric viruses have received attention as emerging pathogens due to their low infectious doses, ability to survive in water, and health impacts (Sinclair et al. 2009; Xagoraraki et al. 2007). Although enteric viruses are primarily associated with diarrhea and self-limiting gastroenteritis, they may also cause respiratory infections, conjunctivitis, and hepatitis (Fong and Lipp 2005). Some enteric viruses are naturally present in aquatic environments and persist through attachment to suspended sediments (Muscillo et al. 2008). However, a majority are introduced through human-related sources, such as leaking sewage and septic systems, urban runoff, agricultural runoff, sewage outfalls, and wastewater discharge from vessels. Shed at levels of $10^5 – 10^{11}$ pathogens/g stool in infected individuals, enteric viruses infect and replicate in the host's gastrointestinal tract (Fong and Lipp 2005). Haas et al. (1993) estimate the risk of infection when consuming viruses in drinking water to be 10-10,000 fold greater than that for pathogenic bacteria at similar exposures. The human viruses most frequently associated with waterborne illnesses are noroviruses, adenoviruses, enteroviruses, rotaviruses, astroviruses, and Hepatitis E, with noroviruses responsible for a large majority of viral-based gastrointestinal illnesses (USEPA 2009).

Icosahedral DNA viruses in the family Adenoviridae and genus *Mastadenovirus*, adenoviruses (AdV) are the second most prevalent etiological agent of recreational water outbreaks, after noroviruses (Sinclair et al. 2009). Adenovirus was first isolated among military recruits in the 1950s, and the largest adenovirus outbreak to date occurred in 1991 when 595 people became ill after swimming in an inadequately chlorinated North Carolina pond (Hilleman and Werner 1954; Aslan et al. 2011). Highly stable under environmental conditions, adenoviruses are frequently present in higher concentrations than other enteric viruses (Enriquez et al. 1995). For instance, due to their double stranded nature, adenoviruses are 60 times more

resistant to UV radiation than RNA viruses, as undamaged DNA strands serve as templates for repair by host enzymes (Fong and Lipp 2005). Therefore, adenoviruses show potential for survival in recreational waters.

The etiologic agent of an estimated 23 million annual illness cases, noroviruses (family Calciviridae, genus *Norovirus*) are currently the most common etiological agent of gastroenteritis in the United States (Mead et al. 1999). Until the CDC adopted RT-PCR for routine testing in the 1990s, norovirus outbreaks were not frequently reported (CDC 2003). However, noroviruses were estimated to cause 45 percent of recreational water outbreaks, surpassing adenoviruses (24 percent) as the primary etiologic agent of GI illnesses (Sinclair et al. 2009). Noroviruses are single-stranded, nonenveloped RNA viruses associated with acute onset of projectile vomiting and diarrhea, low-grade fever, headache, and malaise. Of 25 recreational waterborne norovirus outbreaks occurring between 1977 and 2006, 56 percent resulted from lakes and 8 percent from rivers (Sinclair et al. 2009).

Enteroviruses (order Picornavirales, family Picornaviridae, genus *Enterovirus*) cause a wide spectrum of diseases in humans, whose clinical outcomes may extend beyond gastroenteritis, as some viruses travel from the intestinal tract to other organs (Fong and Lipp 2005). Recreational water infections in humans peak in the summer and early fall, coinciding with increased recreational water activities and water contact (Kocwa-Haluch 2001). Enteroviruses are single-stranded RNA viruses with icosahedral capsids, and 62 serotypes have been associated with human infections (Jimenez-Clavero et al. 2003). Unlike adenoviruses and noroviruses, which strongly associate with point sources of human fecal pollution, enteroviruses do not correlate closely with specific sources (Aslan et al. 2011).

Polymerase chain reaction (PCR) assays targeting multiple host-specific genetic markers are increasingly accessible, enabling rapid quantification of fecal contamination levels and assisting with identification of fecal contamination sources (Lee et al. 2014). PCR-based assays offer several advantages over cell culture assays. For instance, no *in vitro* norovirus assay exists, so detection relies upon molecular detection methods. PCR results can be obtained within 24 hours of sampling, and the technique is capable of differentiating specific viruses. Quantitative real-time PCR (qPCR) provides a numeric value (Cq value) that corresponds to the amount of template nucleic acid, enabling precise estimation of viral RNA and DNA. Primers can be designed to target whole-virus orders (ex. Picornovirales) or may be specific to a single virus type (ex. Adenovirus, strain 41) (Fong and Lipp 2005). Few studies have evaluated the presence of viral pathogens in recreational water with non-point source contamination from human inputs.

Bacterial Indicators and Recreational Water

The American Public Health Association's Committee on Bathing Beaches first discussed monitoring the microbiological quality of recreational waters in 1922 but found no compelling evidence to implicate beaches in the spread of disease until the 1970s when the first prospective cohort epidemiological studies were conducted at marine and freshwater beaches (Cabelli 1988; Dufour 1984). Enterococci, a group of fecal streptococci bacteria, is monitored in both fresh and marine waters, while *E. coli*, a fecal coliform, is only monitored at freshwater sites (Maier et al. 2009). In 1986 the EPA recommended Enterococci and *E. coli* criteria for recreational waters (USEPA 1986); the Beach Act of 2000 required adoption of these criteria as a minimum standard at the state level (USEPA 2016). As of 2012, the EPA criteria holds that the geometric mean of 5 samples taken at equal time intervals over a 30-day time period shall not exceed 35 Enterococci colony forming units per 100 mL (cfu/100 mL) in fresh and marine

waters (USEPA 2012) or 126 *E. coli* cfu/100 mL in fresh water. Washington State water criteria for extraordinary primary contact recreation hold that geometric mean fecal coliform levels should fall below 50 cfu/100 mL, with no more than 10 percent of samples exceeding 100 cfu/100 mL (WA Department of Ecology 2007).

Operated through the Washington State Department of Ecology, the Beach Environmental Assessment, Communication, and Health Program (BEACH Monitoring Program) measures weekly Enterococci levels at public Puget Sound beaches from Memorial Day through Labor Day. A report of 2016 Enterococci data is available on the WA Department of Ecology webpage (WA Dept. of Ecology 2016). Lake Washington beaches are monitored for fecal coliform and *E. coli* levels by the King County Swimming Beach Monitoring Program. The County's Water and Land Services webpage provides a record of fecal coliform and *E. coli* data and reports freshwater beach closures (King County 2016a).

While fecal indicator bacteria provide an inexpensive, quantitative means of determining fecal contamination and correlated with bacterial pathogens, they are less useful as predictors of viral pathogens, particularly in marine environments. A lack of correlation exists between indicator bacteria and virus growth and persistence in the environment, as coliform fate may differ biologically from the fate of viruses under various environmental conditions (Jiang et al. 2001; Leclerc et al. 2000). Thus, human enteric viruses may be present in water regardless of fecal indicator bacteria densities.

F+ Coliphage as a Potential Viral Indicator in Recreational Waters

Adoption of a viral indicator into EPA standards for water quality is currently being considered in monitoring programs. In April 2015, the EPA Office of Water published a comprehensive literature review that presented potential viral indicators of fecal contamination

(USEPA 2015). Advantages to viral indicators, as opposed to bacterial indicators, include their increased host specificity, resistance to conventional wastewater treatment processes, and ability to survive and remain infective in both seawater and freshwater (Fong and Lipp 2005). However, as with indicator bacteria, the efficacy of indicator viruses to prevent exposure to human pathogens may depend on the water type, fecal contamination sources, and indicator organism ecology. For instance, at beaches with point sources of sewage contamination, fecal indicator bacteria were found to correlate better with disease incidence among bathers than coliphages (Wade et al. 2010). Nonetheless, viral indicators hold promise as representations of viral pathogens than indicator bacteria. Bacteriophages, viruses that infect host bacteria, are attractive candidates as indicators of enteric viruses in groundwater, wastewater treatment, and recreational water.

Coliphages are bacteriophages that infect *E. coli* and other coliform bacteria. While somatic coliphages infect coliforms by attachment to the outer cell membrane, male-specific coliphages (MSC) attach only to the F-pilus of coliforms that carry the F+ plasmid and can produce F-pili (USEPA 2001a). Detected with simple, inexpensive methods, they have been proposed as reliable indicators of human viral pathogens associated with fecal contamination (Gerba 1987, Havelaar et al. 1993, Rose et al. 2004, Skraber et al. 2004). They are similar to enteric viruses in physical structure, composition, and survivability and are more persistent in the environment than fecal indicator bacteria (Grabow 2001, Nappier et al. 2006). Further, they originate almost exclusively from the feces of humans and other warm blooded animals and undergo limited multiplication in sewage.

Although there exists no ideal indicator organism, coliphages exhibit several positive attributes of an indicator species (USEPA 2015). They are nonpathogenic (Grabow 2001),

present in the intestinal tract of warm-blooded animals (Sobsey et al. 1995), present in greater numbers than pathogens (Leclerc et al. 2000), and detectable by rapid methods (< 1 day) (Havelaar 1993). Regional studies prove valuable in consideration of bacteriophages as an indicator organism. Three epidemiological studies have shown statistically significant associations between the risk of GI illness and density of F-specific RNA coliphages (Lee et al. 1997, Colford et al. 2007, Wade et al. 2010). Some microbiological studies report an association between the presence of coliphages and pathogenic viruses (Havelaar et al. 1993, Jiang et al. 2001, Ballester et al. 2005), while other studies find no correlation (Ibarluzea et al. 2007, Jiang et al. 2007, Boehm et al. 2009, Viau et al. 2011). Thus, formal adoption of bacteriophage as an indicator remains under consideration.

In 2001 the U.S. EPA approved two methods for monitoring coliphages in groundwater: the two-step enrichment assay (USEPA 2001a) and the single agar layer method (USEPA 2001b). While the single agar layer method is a plaque assay method used to enumerate coliphages in volumes up to 100 mL, the enrichment method is a liquid culture enrichment test developed for presence/absence analysis in volumes up to 1 L. While the two methods have been proven useful in surveillance efforts, but analyzing greater water volumes would enable more accurate pathogen detection, as enteric virus levels are often low in natural environments.

The Bag Mediated Filtration System (BMFS) was developed by the Meschke lab with the original goal of providing a simple in-field filtration sampling system for poliovirus recovery from environmental waters (Fagnant et al. 2014). The system yields a 2500 fold concentration factor for poliovirus when a 10 L water sample is filtered, eluted, and further reconcentrated to 4 mL with polyethylene glycol. Detection of poliovirus was found to be less than 1 virus/5 L (Fagnant et al. 2014). The system utilizes a ViroCap filter, a positively charged alumina

nanofiber filter that resists pH fluctuations and attracts negatively charged viruses (Bennett et al. 2010). The ViroCap filter effectively adsorbs the Group I male-specific coliphage MS2, as well as poliovirus (an enterovirus) and adenovirus. Due to its small size and low pI, MS2 represents a worst-case scenario for size-exclusion filtration. Taking this into account, the BMFS is expected to effectively concentrate enteroviruses and norovirues, two ss-RNA viruses with similar physical and biochemical properties to MS2. Adenovirus, a DNA virus, has already been tested (Bennett et al. 2010). Thus, the BMFS holds potential as a useful surveillance tool for a variety of viruses.

Purpose

The presence of viral pathogens and indicator species at Seattle Area recreational beaches is understudied. Using the BMFS, we performed a preliminary surveillance study of adenovirus, norovirus, enteroviruses, and the MSC at Green Lake, Lake Washington, and Puget Sound recreational beaches from June 2016 – November 2016. Bacterial indicators, including fecal coliforms, *E. coli*, and Enterococci were evaluated as well. MSC was expected to more accurately reflect levels of enteric viruses (if present) than bacterial indicators.

Methods

All recreational water field sampling was conducted between June and November of 2016. Enteric virus extraction and detection with RT-PCR was performed during April and May 2017. Analyses were carried out in the Environmental and Occupational Health Microbiology Laboratory at the University of Washington (4225 Roosevelt Way NE, Seattle, WA, 98105).

Sample Collection

Throughout June 2016 – November 2016, the BMFS was used to collect water samples from Green Lake, Matthew Beach Park, Magnusson Park, Carkeek Beach Park, and Golden Gardens Park. A map of sample locations and their descriptions are shown Figure 1 and Table 2. We used a polyurethane nylon bag to concentrate ~18-21 L (3 x 6-7 L water grabs) of water onto ViroCap filters. Samples were collected between 10:30 am and 2 pm, at approximately 1 m depths, where bather density was likely to be highest (Wymer 2007). Transects for the 3 bag grabs were at least 20 m apart within the defined bathing zones at each park and remained consistent at each site throughout the study. During each collection period, a chain of custody form was completed with the following information: investigator name, site, date, time, cloud cover, air temperature, and approximate number of bathers. Each site was visited on 6-7 days throughout the summer. A field log with dates and conditions is included in Appendix A.

Since the BMFS primarily relies upon gravity filtration, following each grab, the nylon sample bag was hung on a tripod. Because we sampled at low-turbidity sites, we did not encounter particulate matter that clogged the filter. Following filtration, we stored the filters on ice until returning to the laboratory (1-3 hours). Filters were eluted no more than 6 hours following filtration.

In addition to sampling with the BMFS, we collected a 1 L grab sample at each site for estimation of fecal coliforms, *E. coli*, and *Enterococcus* levels at each site. As with the BMFS, samples were taken from approximately 1 m depth in designated bathing areas.

Table 2. Characterization of field sites.

Sampling Site	Bather Load	Marina	Other Potential Fecal Contamination Sources
Matthews Beach (Lake Washington)	Seattle's largest freshwater bathing beach	N/A	Thornton Creek (S of bathing area)
Magnuson Beach (Lake Washington)	Moderate	South of bathing area	Wetland area and marsh outfall pond (W of bathing area)
Green Lake	High	N/A	Cement bathing pond drained into lake daily
Golden Gardens Park (Puget Sound)	Low	South of bathing area	Off-leash dog area, drainages off hill into water, marsh (N beach)
Carkeek Park (Puget Sound)	Low	N/A	Piper's Creek, North Beach CSO outfall, Carkeek CSO plant outfall

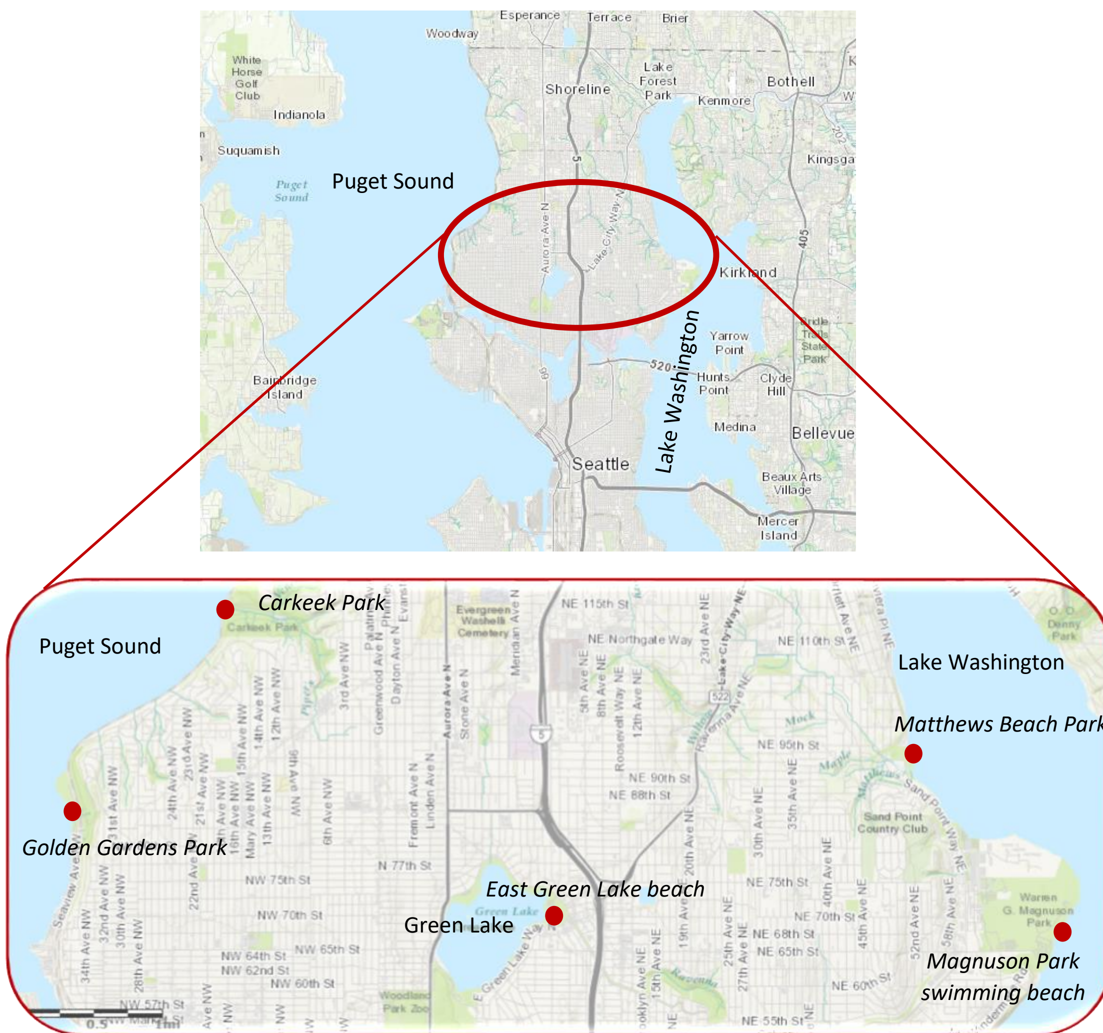


Figure 1. Sample sites for Summer 2016 surveillance sampling in the Seattle Area.

Analysis of Indicator Bacteria

Using Idexx Colilert-18® and Enterolert® with Quanti-Trays, we obtained most probable number (MPN) values for each indicator type. The Colilert-18 ® test uses nutrient indicators ONPG and MUG to detect coliforms and *E. coli*. Coliforms possess the β-galactosidase enzyme, which metabolizes ONPG to change it from colorless to yellow. *E. coli* use β-glucuronidase to metabolize MUG, which releases a fluorescent substrate (IDEXX 2017a). Likewise, the Enterolert® system relies upon the fact that Enterococci bacteria have a β-glucosidase enzyme that metabolizes a nutrient indicator and fluoresces (IDEXX 2017b).

Yellow or fluorescent wells were counted following an 18-22 hour incubation period at 41°C (*Enterococcus*) or 45°C (*E. coli* and fecal coliforms) and used to calculate most probable numbers (MPN) of bacteria. Three dilutions of each water sample were used for enumeration. To calculate MPN, the number of positive wells per dilution was used with the following formula (Blodgett 2006):

$$\sum_{j-1}^{k} \frac{g_j m_j}{1 - e^{(-\lambda m_j)}} = \sum_{j-1}^{k} t_j m_j$$

where K denotes the number of dilutions, g_j denotes the number of positive wells in the jth dilution, m_j denotes the amount of the original sample put into each well in the jth dilution, and t_j denotes the number of wells in the jth dilution.

IDEXX has an online program for rapid MPN calculation that was used throughout this project to estimate bacteria levels and their 95% confidence intervals.

Elution and Virus Concentration

Adsorption-elution methods such as the BMFS use pH changes to manipulate surface charges and maximize adsorption to charged filters (Fagnant et al. 2014; Bennett et al. 2010).

Upon return to the lab, 100 mL of a pH 9.50, 1.5% beef extract solution buffered with 0.05 M glycine was pumped into the filter and left to sit for 30 minutes. Eluate was then pumped out, measured, and immediately pH-adjusted to 7.0-7.5 with 1 M HCl.

Following elution, samples were precipitated overnight with 14 g/100 mL polyethylene glycol (PEG) and 0.2 M NaCl. Sample bottles were shaken overnight at 4°C, 200 rpm. In the morning, they were centrifuged in a Beckman Coulter Avanti J-20 XPI centrifuge at 6500 G, 4°C for 30 minutes (Lewis and Metcalf 1988; El-Senousy 2013). Supernatant was discarded, and 4 mL sterile 1X phosphate buffer solution (PBS) was added to centrifuge flasks, which were vortexed for 5-10 minutes at maximum speed to resuspend the pellet. A 2 mL aliquot was then set aside for subsequent MSC enumeration with the double agar layer overlay method.

To further purify the samples for RT-PCR analyses, Vertrel XF was added to 2.5 mL of the precipitated solutions to create a 1:5 Vertrel XF:Sample dilution. Conicals were vortexed for 5 minutes at maximum speed and centrifuged at 3000G, 4°C for 15 minutes (Mendez et al. 2000). Supernatant was collected and stored in 1.0 mL aliquots at -80°C for RT-PCR analysis.

MSC Enumeration

MSC was enumerated by the plaque assay with *E. coli* F_{amp} using the double-agar layer (DAL) method (Adams 1959). Bottom agar was prepared by autoclaving tryptic soy agar (TSA) and pouring the mixture onto 100x15 mm petri dishes. As specified in standard coliphage analysis methods (USEPA 2001a, USEPA2001b), all culture media in this procedure were autoclaved and supplemented with 1% streptomycin and ampicillin (final concentration 5 mL/L TSA) prior to cooling. Plates were stored at 4°C until use.

Streptomycin/ampicillin-resistant *E. coli* F_{amp} (ATCC #:700891) was used as the host for detection of F+ coliphages. Cultures to be used in the DAL were grown overnight at 37°C in Difco nutrient broth (8000 ppm) and shaken at 100 rpm.

The top agar layer consisted of 0.77% bactoagar and 0.55% NaCl. Molten bactoagar (~5.6 mL) was maintained at 37°C in a water bath and combined in glass tubes with 100 µL log phase *E. coli* F_{amp} and 300 µL PEG-precipitated sample. Two replicates of each sample were plated, resulting in analysis of 0.6 mL concentrated sample. In addition, 3 dilutions of MS2 stock (~10^9 PFU/mL) were plated each time the assay was run for titer and enumeration of sample MSC concentrations. Once the bactoagar solidified, plates were inverted and incubated at 37°C. After 16-20 hours, plaque-forming units (PFUs) were counted.

Quality Control MSC Seeding Experiments

In order to determine recovery efficiencies for the BMFS, elution, and PEG precipitation procedures, water from each site was collected in 20 L carboys on 3-4 different days and seeded with 5 mL volumes of 10^3 – 10^5 PFU/mL MS2 stocks upon return to the laboratory. Turbidity, pH, and salinity measurements were collected for each water sample. Samples were pumped through ViroCap filters with a peristaltic pump and sterilized silicone tubing at a rate of 2L/min. Filters were then eluted and processed as explained above. Percent recovery from the process was determined by comparison of the total virus spiked into the influent to the total virus present following PEG precipitation. Percent recovery was calculated with the following formula.

$$Percent\ Recovery\ = \frac{Actual\ MS2\ PFU}{Expected\ MS2\ PFU} \times 100,$$

where $Expected\ MS2\ PFU = titered\ MS2\ stock\ \left(\frac{PFU}{mL}\right) \times 0.6\ mL,$

and $Actual\ MS2\ PFU = \frac{sum\ recovered\ MS2\ plaques}{volume\ plated\ across\ dilutions}$

Nucleic Acid Extraction

Viral nucleic acids were extracted from the concentrated recreational water samples using a Qiagen Viral RNA kit (Qiagen, Valencia, CA). The spin protocol listed in the manufacturer's handbook was modified to extract 1 mL of sample, rather than 140 µL. As specified in the protocol, carrier RNA, lysis buffer (AVL), and EtOH volumes were increased proportionally with sample size. However, wash buffer (AW1 and AW2) volumes and elution buffer (AVE) volumes were not altered. A Qiagen vacuum manifold was used to facilitate extractions. All viral extracts were stored at -20 °C.

Prior to extracting samples, three validation experiments were performed to (a) determine which Qiagen kit to use; (b) to evaluate whether inhibitory effects resulted from recreational water matrices; and (c) to quantify whether nucleic acid quantity increased linearly with volume extracted.

(a) In order to simultaneously detect both ss-RNA and ds-DNA viruses in the samples, we evaluated whether both nucleic acid types could be extracted with a single kit. Although the Viral RNA kit is designed to extract viral RNA from cell-free fluids, previous laboratory experiments indicate that it also extracts DNA. Due to the high availability of Viral RNA columns in the laboratory, we verified the kit's ability to extract duplicate AdV 41 DNA relative to a DNA extraction kit (DNEasy) and a kit designed to extract all nucleic acids (MinElute). Calibration curves for duplicate Adenovirus 41 (AdV 41) extractions with each kit are shown in Table 3. Since PCR products ideally double with each cycle, a tenfold dilution should yield a slope of 3.32 (n = 3.32 Cq). Percent efficiency is calculated as $[(10^{-1/slope} - 1) \times 100]$. The MinElute and Viral RNA kits were slightly less efficient than the DNEasy kit, but Viral RNA kit efficiency was deemed high enough (81.0%) to proceed with the Viral RNA kit (Steward and Culley 2010).

Table 3. Calibration curves for Adenovirus 41 extractions.

Kit	Extraction 1	Extraction 2	Mean % Efficiency
DNEasy	$y = -3.45x + 14.6$ ($R^2 = 0.998$)	$y = -3.48x + 14.3$ ($R^2 = 0.998$)	94.4
MinElute	$y = -3.73x + 14.4$ ($R^2 = 0.999$)	$y = -3.62x + 13.4$ ($R^2 = 0.994$)	87.2
Viral RNA	$y = -3.85x + 14.8$ ($R^2 = 0.997$)	$y = -3.91x + 14.7$ ($R^2 = 0.998$)	81.0

We then verified the functionality of the Viral RNA kit relative to the MinElute kit with a stock of Poliovirus 1 (PV1). Calibration curves for PV1 extractions and the efficiencies for each kit are shown in Table 4.

Table 4. Calibration curves for PV1 extractions.

Kit	Extraction 1	Extraction 2	Mean % Efficiency
MinElute	$y = -3.47x + 15.6$ ($R^2 = 0.995$)	$y = -3.55x + 14.7$ ($R^2 = 0.990$)	92.7
Viral RNA	$y = -3.77x + 20.1$ ($R^2 = 0.963$)	$y = -3.70x + 19.0$ ($R^2 = 0.987$)	84.3

(b) To determine whether inhibition was present in the purified recreational water samples, we extracted 1 mL volumes of both 1X PBS and recreational water matrices spiked with approximately 10^9 AdV 41 particles / mL (Keim 2017; Song et al. 2012). Results are shown in Figure 2. Only slight inhibitory effects were detected for the recreational water matrix, as evidenced by the calibration curves minimal shifts and similar slopes. Therefore, only a 10-fold dilution of each sample was included in the PCR analyses to account for any inhibitory effects present in the recreational water matrices.

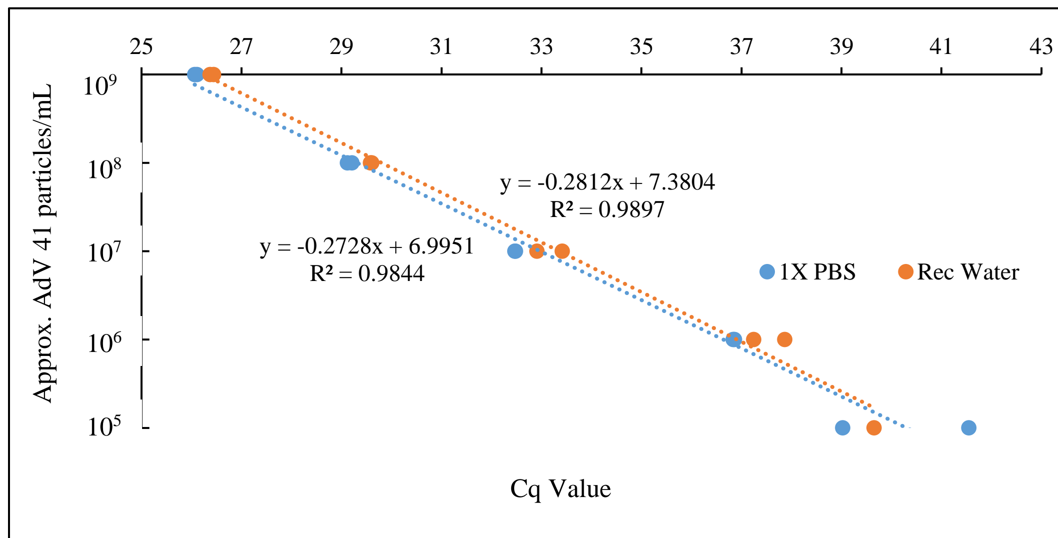


Figure 2. Calibration curves for Viral RNA extractions performed in 1X PBS buffer and recreational water matrices.

(c) Finally, because we expected low concentrations of viruses in the recreational water samples, we tested the Viral RNA kit's ability to extract increased sample volumes effectively (Figure 3). Matthews Beach water processed with the BMFS, PEG, and Vertrel methods was spiked with approximately 10^7 AdV 41 particles / mL, vortexed, and divided into 100 µL, 500 µL, and 1 mL aliquots. Quantitative PCR products ideally double with each cycle, so for a 10-fold dilution, $2^{Cq} = 10$; for a 2-fold dilution, $2^{Cq} = 2$. The following formula was used to compare extraction recoveries for the different extraction volumes: $2^{[(Cq,E1) - (C1,E2)]}$. At the 10^7 particles/mL AdV 41 concentration, the 1 mL and 500 µL extractions yielded 9.2 and 1.65 times as much DNA as the 100 µL extraction, respectively. As a result, we determined that extracting 1 mL volumes of the samples would enable surveillance of a greater recreational water volumes.

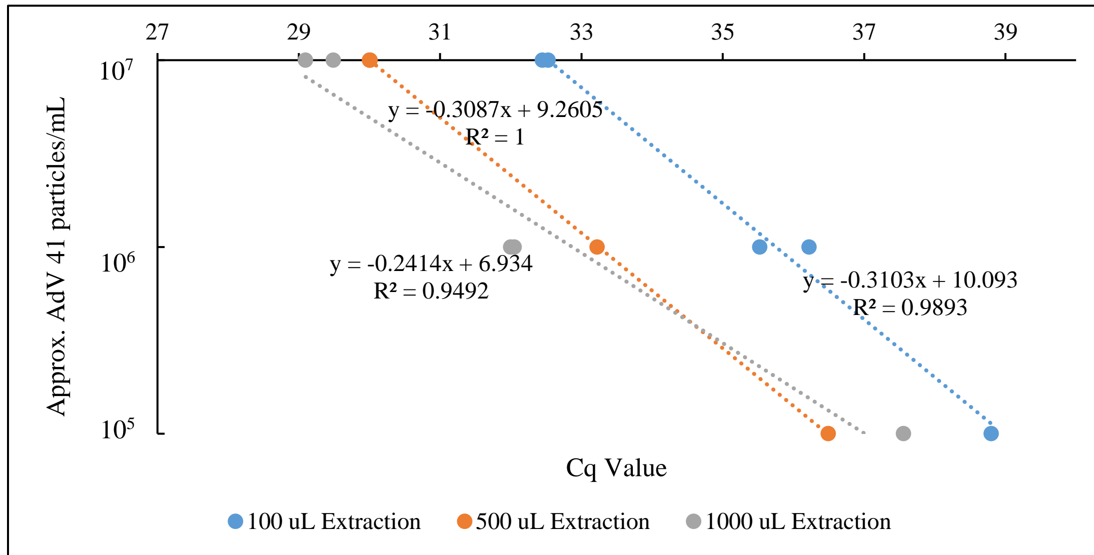


Figure 3. Calibration curves for AdV 41 extractions from 100 µL, 500 µL, and 1 mL samples.

Detection of Pathogenic Viruses: Quantitative (RT)-PCR

The primers and probes used for detection of AdV 41, NV GI and NV GII, and EV are listed in Table 5.

PCR assays were performed in a BIO-RAD C1000 Touch Thermal Cycler. The samples and standards were each run at least in duplicate. All PCR runs included a negative control reaction mixture (PCR-grade H_2O) and a positive control reaction mixture. The quantification cycle value (Cq) of each PCR was automatically determined with the BIO-RAD CFX Manager, version 3.1.

The positive control used for Adenovirus was the AdV 41 strain propagated in the UW Environmental Health Microbiology Lab (EHML). Quantitative PCR for the detection of AdV 41 with a DNA internal amplification control (IAC) was performed with a 20 µL reaction, which included 10 µL BIO-RAD iTaq Universal Probes Supermix, 2.38 µL primer mix, 1.13 µL probe mix, 2.5 µL PCR-grade H_2O, 3 µL sample DNA, and 1 µL IAC DNA. The primer concentrations for AdV 41 and the IAC were 400 nM and 75 nM, respectively. Probe concentrations were 300 nM and 150 nM, respectively. Thermal cycling was run with the following conditions: 2 min at 95 °C, followed by 45 cycles of 95°C for 3s, 53°C for 10s, and 65°C for 60 s (Heim et al. 2003; DePaula et al. 2010).

Norovirus Group I and Group II positive controls were synthetic RNA transcripts of norovirus acquired from ATCC. The 20 µL RT-PCR volume consisted of 10 µL 2x RT-PCR Reaction Mix for Probes, 2 µL primer mix, 1 µL probe mix, 3.5 µL PCR-grade H_2O, 0.5 µL iScript Reverse Transcriptase, 2 µL sample RNA, and 1 µL iacRNA. The 5' nuclease probe concentrations for NoV and the iac RNA target were 250 nM and 375 nM, respectively. Thermal cycling was run with the following conditions: 50°C for 3000 s, and 95°C for 900s, followed by 45 cycles of 95°C for 10 s, 53 °C for 25 s, and 62°C for 70s. Fluorescence was read at the end of the 62°C elongation step (Kageyama et al. 2003).

Poliovirus 1 propagated in the EHML served as the positive control for the pan-enterovirus primers and probe. RT-PCR was performed with a 20 µL reaction volume, consisting of 10 µL 2x RT-PCR Reaction Mix for Probes, 1 µL pan-E5', 1 µL pan-E3', 0.80 µL pan-E probe, 4.7 µL PCR-grade H_2O, 0.5 µL iScript Reverse Transcriptase, and 2 µL sample RNA. Thermal cycling was run with the following conditions: 50°C for 6000 s, 95°C for 300 s, followed by 40 cycles of 95°C for 10 s and 55°C for 30s (Schwab 1995).

Table 5. Primer and probe sequences used for detection of viral pathogens.

Target Organism	***Primer***	***Sequence (5' → 3')***	***Reference***
Adenovirus	ADVF (fwd)	GCCACGGTGGGGTTTCTAAACTT	Heim et al. 2003
	ADVR (rev)	GCCCCAGTGGTCTTACATGCACATC	Heim et al. 2003
	ADP (probe)	FAM-TGCACCAGACCCGGGCTCAGG	Heim et al. 2003
Norovirus GI	COG1F (fwd)	CGYTGGATGCGNTTYCATGA	Kageyama et al. 2003
	COG1R (rev)	CTTAGACGCCATCATCATTYAC	Kageyama et al. 2003
	COGP (probe)	Cy5-AGATYGCGATCYCCTGTCCA-IBRQ	Kageyama et al. 2003
	COGPb1 (probe)	Cy5-AGATCGCGGTCTCCTGTCCA-IBRQ	Kageyama et al. 2003
Norovirus GII	COG2F	CARGARBCNATGTTYAGRTGGATGAG	Kageyama et al. 2003
	COG2R	TCGACGCCATCTTCATTCACA	Kageyama et al. 2003
	COGP2	FAM-TGGGAGGGCGATCGCAATCT-IBRQ	Kageyama et al. 2003
Enterovirus	Pan-E5' (fwd)	CCTCCGGCCCCTGAATG	Schwab et al. 1991
	Pan-E3' (rev)	ACCGGATGGCCAATCCAA	Schwab et al. 1991
	Pan-E (probe)	TACTTTGGGTGTCCGTGTTTC	Schwab et al. 1991
Internal Control	IC46F	GACATCGATATGGGTGCCG	DePaola et al. 2010
	IC194R	AATATTCGCGAGACGATGCAG	DePaola et al. 2010
	IACP	TxR-TCTCATGCGTCTCCCTGGTGAATGTG	DePaola et al. 2010

Results and Discussion

Indicator bacteria at recreational water beaches

Fecal coliform, *E. coli*, and *Enterococcus* levels are shown in Tables 6-8, and dated data from saltwater and freshwater sites are portrayed in Figure 4 and Figure 5, respectively. Due to the limited resistance of *E. coli* in saltwater, *E. coli* data for Carkeek Park and Golden Gardens Park do not reflect fecal contamination to the same extent as at freshwater sites (Maier et al. 2009).

Matthews Beach Park and Carkeek Park had the highest fecal coliform and *Enterococcus* values throughout the sampling season (Tables 6-8; Figures 4-5). This may be explained by the discharges of Thornton Creek and Piper's Creek at these sites. The Thornton Creek watershed drains 7,400 acres and is Seattle's largest drainage basin. Over the past 30 years, fecal coliform readings have averaged 886 cfu/100 mL during routine monitoring events and 4793 cfu/100 mL during wet weather events (King County 2016b). The third largest watershed in Seattle, the Piper's Creek watershed drains 1,835 acres into Puget Sound, with average flows between 3 and 9 cfs. While Piper's Creek water quality is generally good, its fecal coliform levels have frequently exceeded the state standard. King County records indicate an average of 743 cfu/100 mL during 30 years of monitoring data, to date (King County 2016b).

Interestingly, fecal coliform, *E. coli*, and Enterococci levels fluctuated similarly at all sites except the East Green Lake swimming beach. Since *E. coli* and Enterococci are considered more specific to fecal material from humans and other warm-blooded animals, this may suggest that fecal contamination at Green Lake resulted from non-human sources, such as ducks and geese.

Table 6. Fecal coliform bacteria at beaches in the Seattle area, June 2016 – November 2016. The Washington State standard for primary contact recreation is 100 cfu/100 mL (Ecology 2017).

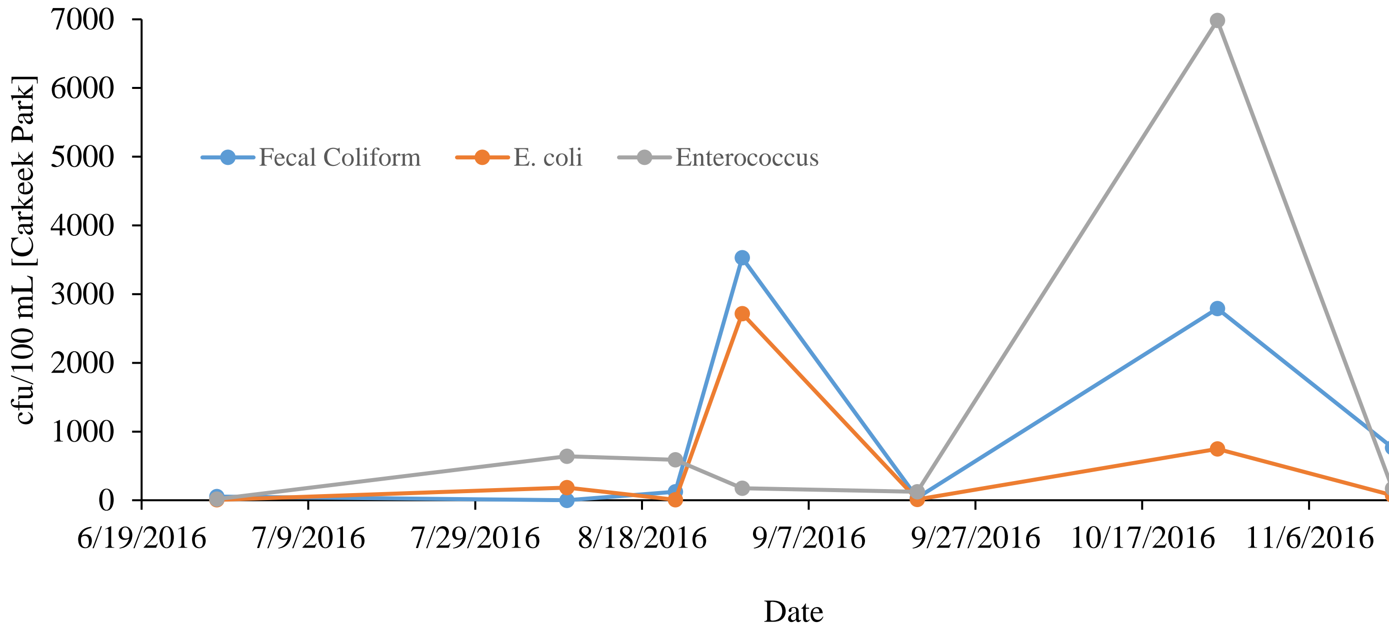
Site	***N***	***Geometric Mean (cfu/100 mL)***	***n > WA Standard***
Matthews Beach Park	6	791	6
Magnuson Park swimming beach	4	250	2
East Green Lake beach	5	334	3
Golden Gardens Park	7	54	3
Carkeek Park	6	342	4

Table 7. *E. coli* levels at beaches in the Seattle area, June 2016 – November 2016. The 2012 EPA standard for geometric mean *E. coli* in recreational waters is 126 cfu/100 mL (USEPA 2012).

Site	***N***	***Geometric Mean (cfu/100 mL)***	***n > EPA Standard***
Matthews Beach Park	6	367	4
Magnuson Park swimming beach	4	81	2
East Green Lake beach	7	12	0
Golden Gardens Park	7	11	2
Carkeek Park	7	74	3

Table 8. Enterococcus levels at beaches in the Seattle area, June 2016 – November 2016. The 2012 EPA standard for geometric mean Enterococcus in recreational waters is 35 cfu/100 mL (USEPA 2012).

Site	***N***	***Geometric Mean (cfu/100 mL)***	***n > EPA Standard***
Matthews Beach Park	6	305	5
Magnuson Park swimming beach	4	22	1
East Green Lake beach	7	6	1
Golden Gardens Park	7	117	5
Carkeek Park	7	286	6



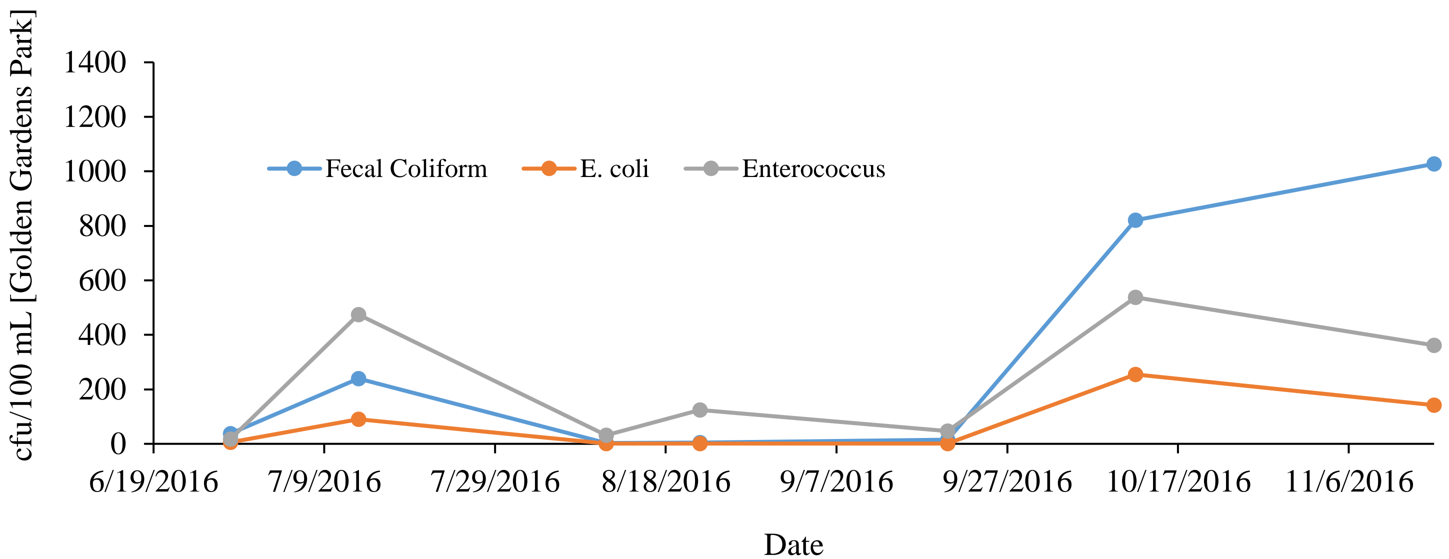
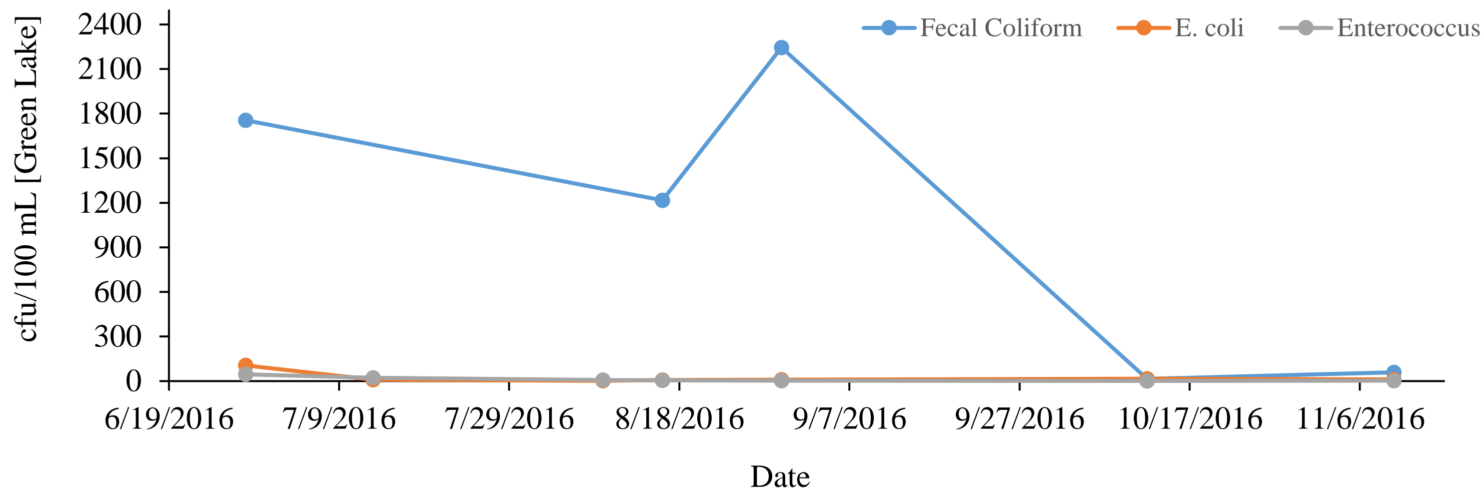
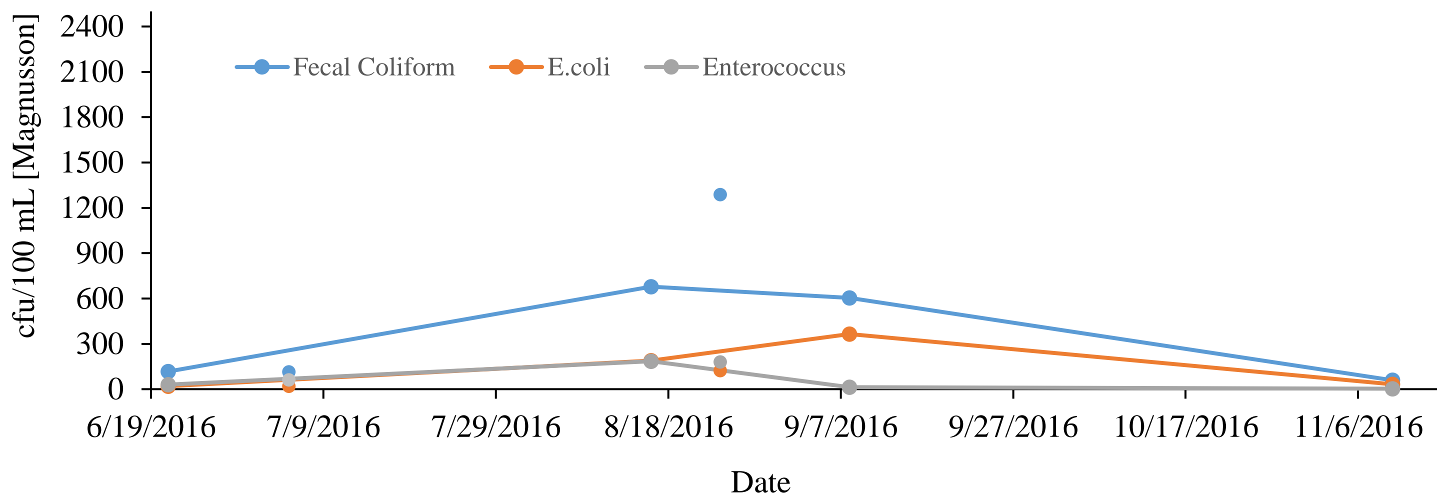


Figure 5. Fecal coliform, *E. coli*, and Enterococci levels on each sampling day throughout the 2016 sampling period at saltwater sites: Carkeek (top) and Golden Gardens Park (bottom).





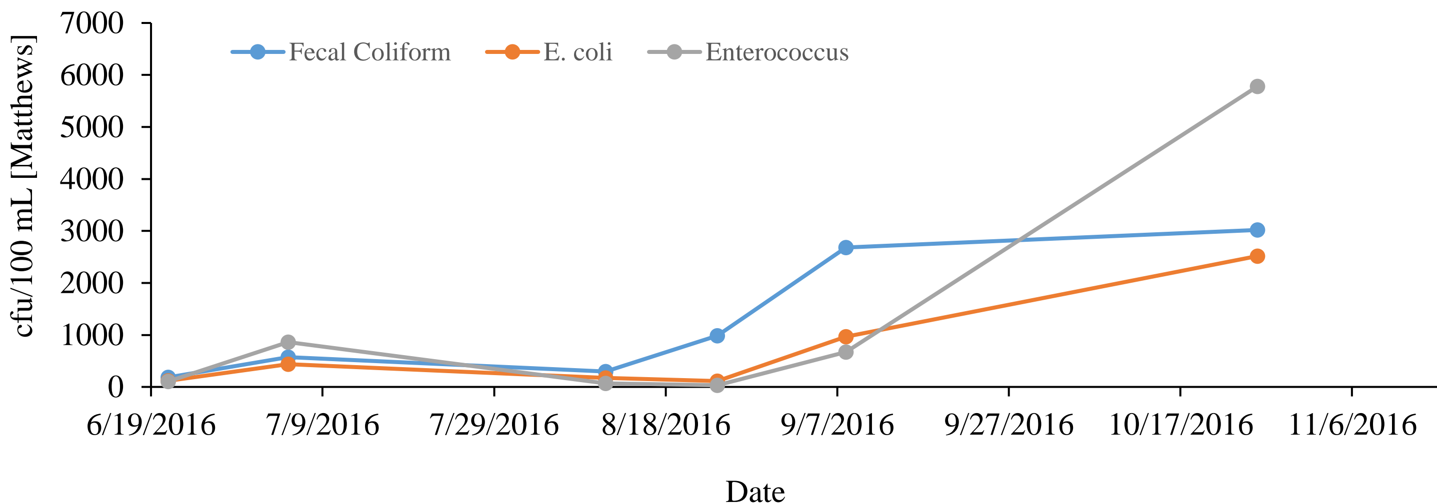


Figure 6. Fecal coliform, *E. coli*, and Enterococci levels on each sampling day throughout the 2016 sampling period at freshwater sites: East Green Lake (top), Magnuson (middle), and Matthews (bottom). Individual points on Magnuson chart represent samples taken at the Magnuson dog park.

MSC and enteric viruses at recreational water beaches

Throughout the sampling season, we detected MSC only twice, both during the 10/26/16 wet weather event, during which 1.66 inches of rain were recorded in the 24 hours prior to sampling. We sampled at Matthews Beach Park and Carkeek Park during the storm, and MSC levels were 19 PFU/L and 21 PFU/L, respectively.

No noroviruses, adenoviruses, or enteroviruses were detected with qPCR throughout the sampling season at any of the recreational beach sites. Given that bacterial indicator levels had occasional high hits, this lack of positive viral results suggests that indicator bacteria provide more conservative estimates of infection risk.

Recovery of MS2 from fresh and marine waters using the BMFS.

Bacteriophage recoveries from 20 L volumes of water from each field site are presented in Table 6. A seeding experiment was performed at least 4 times at each site, and seeded MS2 titers ranged from 9.5×10^2 PFU/20 L to 5.3×10^5 PFU/20 L (Table 9).

In certain cases, recoveries exceeded 100 percent. This may be attributed to the fact that MS2 tends to aggregate at concentrated seeding levels. The bacteriophage's RNA genome contributes van der Waals and hydrophobic forces that overwhelm electrostatic contributions, leading to undifferentiated aggregation behavior (Dika et al. 2013).

Median recoveries at freshwater sites were higher than those at saltwater sites (Table 6). Previous research suggests that electropositive filters may yield low recovery rates for viruses in marine waters, as the presence of salt negatively affects the adsorption abilities of charged viruses (Lipp et al. 2001). Alternatively, salt may affect MS2 resistance. Sinton et al. (2002) found both somatic and F-specific coliphages to have lower decay rates in river water than seawater. The presence of aquatic plant species in marine environments may enhance

rhizosphere bacterial populations, which increase coliphage inactivation due to the presence of metabolites or proteolytic substances released by saltwater microbes or plants (Karim et al. 2008).

However, Bennett (2009) found no difference in MS2 recovery in deionized water and simulated seawater created with Instant Ocean marine salts (1.002 – 1.029 d 20/20), suggesting that recovery differences may be attributed to differences in water turbidity. Throughout the sampling season, turbidity at Carkeek and Golden Gardens appeared lower than that at the fresh water sites. While fresh water sites typically yielded visible pellets during PEG precipitation, salt water sites did not. Turbidity was formally measured on the March 20, 2017 spiking event, and saltwater sites were indeed less turbid than the fresh water sites (Table 9). Viruses in the environment are often associated with particulate matter, affecting persistence and transport in the environment (Gerba 1987). Enteric viruses have been previously found to persist in sediment rather than in water during the dry season, resulting in isolation only during the wet season, when particles were suspended evenly in water samples (Ferguson et al. 1996). Therefore, more MS2 may have been recovered from spiked fresh water samples due to the coliphage's tendency to adsorb to sediment particles, which adhered to the ViroCap filter and were easily concentrated during the PEG precipitation process.

Table 9. Recovery of MS2 by ViroCap filtration, elution, and PEG precipitation.

Site	Date	Salinity	Turbidity	pH	Seeded Titer*	Recovery (%)
Matthews Beach Park	8.3.16	--	--	--	9.5×10^4	118
	8.3.17	--	--	--	9.5×10^2	67
	2.4.17	4 ‰	--	6.0 – 6.4	1.6×10^5	89
	3.20.17	0 ‰	22 NTRU	6.0	2.3×10^5	134
	3.29.17	3 ‰	--	6.0	5.3×10^5	70
					Median	**89**
					MAD**	**33**
Magnuson Park swimming beach	11.6.16	2 ‰	--	--	2.6×10^3	69
	2.4.17	3 ‰	--	6.4	1.6×10^5	79
	3.20.17	0 ‰	16 NTRU	6.0	2.3×10^5	150
	3.29.17	4 ‰	--	6.0	5.3×10^5	95
					Median	**87**
					MAD	**12**
East Green Lake beach	10.16.16	0 ‰	--	--	2.6×10^3	42
	2.4.17	4 ‰	--	6.4 – 6.7	1.6×10^5	101
	3.20.17	2 ‰	20 NTRU	6.0	2.3×10^5	139
	3.29.17	4 ‰	--	6.0	5.3×10^5	115
					Median	**108**
					MAD	**10**
Golden Gardens Park	10.16.16	22 ‰	--	--	2.6×10^3	18
	2.4.17	27 ‰	--	6.7 – 7.0	1.6×10^5	35
	3.20.17	20 ‰	5 NTRU	6.4 – 6.7	2.3×10^5	6
	3.29.17	32 ‰	--	6.7	5.3×10^5	76
					Median	**26.5**
					MAD	**13**
Carkeek Park	11.6.16	31 ‰	--	--	2.6×10^3	0
	2.4.17	37 ‰	--	6.7 – 7.0	1.6×10^5	60
	3.20.17	30 ‰	11 NTRU	6.4 – 6.7	2.3×10^5	32
	3.29.17	25 ‰	--	6.7 – 7.0	5.3×10^5	61
					Median	**46**
					MAD	**21**

***pfu/20 L recreational water** ****median absolute deviation**

Limitations

Because no MSC or pathogenic enteric viruses were not detected throughout the duration of the summer, this study does not contribute to the body of literature that investigates coliphages as potential indicators of fecal contamination, relative to current fecal indicators (USEPA 2015). Throughout the field sampling period, multiple investigators performed the elution, precipitation, and extraction procedures, leading to potential differences in technique and recoveries. Field samplers were limited to filtering water at only two sites on any given day, so neither the bacterial or viral dataset is particularly thorough for any of the recreational beach sites. We visited each site 6-7 times over a 5 month period. Conversely, when performing official recreational water monitoring, the WA Department of Ecology and King County sample for Enterococci and *E. coli* weekly between Memorial Day and Labor Day to obtain a running log of bacteria levels throughout the swimming season (Ecology 2016; King County 2016a). More data would be necessary to draw conclusions about bacterial and viral indicators at individual beaches.

The sensitivity of the double agar layer assay could have been increased if over 0.6 mL of each water sample had been plated. The present method resulted in concentration of 20 L recreational water to 5 mL 1X PBS buffer, of which only 0.6 mL was evaluated for MSC. Thus, the effective volume assayed was 2.4 L, resulting in a theoretical detection limit of 8.3 PFU/20 L sampled. This is still much higher than the single agar layer detection limit of 200 PFU/20 L. However, if 2 mL had been assayed with the double agar layer, the effective volume assayed would have increased to 8 L, and the theoretical detection limit of the procedure would have decreased to 2.5 PFU/20 L. Therefore, in order to maximize detection, future recreational water studies should assay larger water sample volumes with the double agar layer.

Conclusions

Adoption of a viral indicator, such as male-specific coliphage, into water quality standards is currently under consideration. We sought to provide regional recreational beach data on enteric pathogens, male-specific coliphage, and bacterial indicators. Throughout the summer, no pathogenic enteric viruses were detected at any of the Seattle Area recreational beaches. However, limited fecal indicator data showed surprisingly high fecal coliform, *E. coli,* and Enterococcus numbers, especially at Matthews Beach Park and Carkeek Park. This was likely attributed to the outfalls of Thornton and Piper's Creeks at these sites, rather than the presence of recreational bathers. MSC was detected at these sites only during an October rainstorm.

This study presents a novel method for assaying large volumes of recreational water, resulting in decreased detection limits for viruses. Bag-mediated filtration through a ViroCap filter and subsequent plating with the double agar layer results in lower detection limits than the EPA-suggested single agar layer (USEPA 2001b). Further characterization of the method with fresh and salt water samples with MSC seeding experiments should be performed prior to its formal use.

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Chapter 3:
Microbial Source Delineation in the Poverty Bay Region
September 2016 – May 2017

Introduction

Background

A large, burrowing hiatellid clam found in low intertidal and subtidal sediments of the Pacific Northwest, the Pacific geoduck (*Panopea generosa*, Gould, 1850) is currently one of the most economically important commercial shellfish species harvested for export (Dorfmeier et al. 2015). Shellfish production within Washington State represents 83 percent of total shellfish production by weight on the west coast of the United States (Booth et al. 2006). Harvesting over 86 million pounds of bivalve shellfish worth 16 million dollars annually, the state's shellfish industry is the largest in the U.S. (Meschke and Boyle 2007). The Department of Ecology estimates that 110 million adult geoducks are packed into Puget Sound, contributing to the highest geoduck density within the contiguous United States (WA Ecology A). Washington State accounts for 90 percent of global geoduck production (Shamshak and King 2015), producing over 6 million pounds of geoduck clams annually, 90 percent of which are sold to China (Garnick 2013).

In the 1850s Pacific Northwest Native American tribes signed the Treaty of Point No Point with the United States government in which they released titles to their lands but reserved the right to harvest shellfish in their "usual and accustomed" grounds (NWFSC 2002). However, when Washington State sold a majority of its tidelands to private owners long after the treaty was signed, the tribes were unable to exercise their shellfish harvesting rights. While the 1974 Boldt decision restored tribal fishing rights, shellfish rights were denied until 1989 when Western

Washington tribes filed a federal court case. Since this ruling, tribes are entitled to half the region's naturally occurring shellfish comanage these resources with the Washington Department of Fish and Wildlife, as well as Olympic National Park (NWFSC 2002).

Because they are filter feeders, shellfish accumulate waterborne pathogens to levels considerably higher than are found in the water column (Burkhardt and Calci 2000). In situ studies with bioaccumulation of viral indicators in oysters have shown that they can concentrate viruses up to 99 times higher than the surrounding water (Bellou et al. 2013; Westrel et al. 2010). Shellfish-borne infectious disease outbreaks have been found to peak in late spring and late fall (Rippey 1994) and are expected to rise with increases in extreme rainfall. A nationwide survey of states, tribes, and local governments found stormwater to be the greatest known source (21%) of beach closures and advisories in the nation (U.S. EPA 2003).

Epidemiological evidence suggests that human enteric viruses are the most common pathogens transmitted by shellfish (Baker et al. 2010). Of 76 million reported cases of foodborne illness in the U.S. each year, 6 percent are attributable to shellfish (Meschke and Boyle 2007). Molluscan shellfish have been documented as vectors of *Salmonella typhi*, *Vibrio parahaemolyticus, Vibrio vulnificus*, Hepatitis A virus, and Norovirus; the largest shellfish-related outbreak occurred in 1988 in Shanghai, when 290,000 people contracted Hepatitis A after eating clams, resulting in 47 fatalities (Bellou et al. 2012). Consumers are particularly vulnerable to viral illnesses, as viruses are removed more gradually from shellfish tissues than fecal coliforms or other bacteria (Meschke and Boyle 2007).

In 2003 Samish Bay shellfish growing areas underwent emergency closure due to a Norovirus outbreak (Booth et al. 2006), drawing attention to shellfish-related outbreaks in Washington State. Few studies have been conducted regarding parasite load, natural distribution

patterns, and epizootics specific to geoducks. Therefore, cautious monitoring is implemented to prevent infectious disease outbreaks.

Washington State Regulatory Framework for Shellfish Harvesting

While specific enteric pathogens carried in human sewage and domestic animal waste are the true contaminants of concern, they are costly to detect, numerous, and present in small concentrations, rendering quantification difficult and expensive. As a result, indicator microorganisms are used to determine fecal contamination levels in growing waters or shellfish tissue. An ideal indicator species (a) is present whenever enteric pathogens are present; (b) should be useful for all water types; (c) should have a longer survival time than the dardiest enteric pathogens; (d) should not replicate in water; and (e) should be found in the intestines of warm-blooded animals. No indicator organism fits these criteria perfectly. Bacterial indicators, namely fecal coliforms and *E. coli* are most frequently used to monitor fecal contamination (Maier et al. 2009). The Washington State Department of Health (DOH), Office of Environmental Health and Safety operates the state's water quality shellfish monitoring program. The DOH adheres to regulations set by the 2015 National Shellfish Sanitation Program (NSSP) Guide for the Control of Molluscan Shellfish, which stipulates that fecal coliform organism levels in individual samples must not exceed a geometric mean value of 14 colony forming units (cfu) per 100 mL water, with the 90th percentile below 43 cfu/100 mL(NSSP 2015). Consistent with NSSP protocols, DOH field samplers collect monthly grab samples from offshore Marine Quality Stations and use the most recent 30 samples at each station to classify areas for commercial shellfish harvesting (WAC Ch.173-201A).

Growing areas may be classified as *Approved, Conditionally Approved, Restricted, Conditionally Restricted,* or *Prohibited* following the completing of a formal Sanitary Survey by

the DOH. Water in *Approved* areas consistently meets the NSSP standards and receives no direct pollution discharges. *Conditionally Approved* areas may include Marine Quality Stations that approach or occasionally fail the NSSP standards for harvesting. However, if data in the Sanitary Survey identifies predictable, intermitant pollution sources, the area may remain open for harvesting during certain months. Intermitant sources may include potential bypasses from sewage treatment systems, seasonally used areas, land runoff, and freshwater flows. In *Restricted* areas, fecal pollution levels exceed standards but are low enough that shellfish may be made safe for human consumption by relaying, depuration, or low acid-canned food production. However, harvesting from *Restricted* beds is uncommon. Areas are classified as *Prohibited* when the Sanitary Survey determines that the growing area is adjacent to a sewage treatment plant outfall, or the area is undeniably contaminated (NSSP 2015).

Microbial Source Tracking

Microbial source tracking (MST) is a set of microbiological techniques used to determine sources of fecal pollution in the environment (Meschke and Boyle 2007). In shellfish pollution prevention, point sources of pollution are single, identifiable sources (ex. wastewater treatment plant outfall) that have immediate adverse effects on the growing area. Conversely, nonpoint sources are diffuse waste discharges that reach the growing area in a roundabout way. They are more difficult to track and include leaking municipal wastewater conveyances, seepage from septic tanks, agricultural runoff, and stormwater runoff carrying pollutants from street surfaces, wooded areas, and construction sites (Booth et al. 2006). However, an understanding of an area's nonpoint sources proves necessary when striving to meet pollution control targets. While monitoring with indicator species narrows down pollution sources, this does not discern whether bacteria are introduced to water bodies through human, wildlife, and/or domestic animal sources.

Bacteriodes, F+RNA coliphage typing, and tracer chemicals (ex. acetaminophen, caffeine, and paraxanthine) are emerging techniques thought to be more species-specific (Embrey 2001; Dick et al. 2005; Stewart-Pullaro et al 2006; James et al. 2016). Year-round monitoring provides additional pollution source information, as dry and wet weather sources may differ (Mitch et al. 2010).

Poverty Bay Shellfish Growing Area

With a drainage area of approximately nineteen square miles, Poverty Bay spans eleven marine shoreline miles of Puget Sound in King and Pierce Counties, from Des Moines Creek south to Brown's Point (Figure 1). The area is characterized by the Des Moines Plain, an upland plateau with elevations that range from three hundred to four hundred feet above sea level, and is bounded to the east and west by steep bluffs. Due to this upland pattern, the drainage basins flowing to the shellfish growing area are relatively small, with creeks under two miles long (Georgeson 2014). Appendix B includes drainage basin maps and flow estimates for each creek thought be directly relevant to the Poverty Bay area. The land draining to Poverty Bay is urbanized and fully incorporated into the cities of Des Moines and Federal Way. While the Midway and Lakehaven sewer districts service a majority of homes in the region, numerous homes continue to use on-site septic systems (Georgeson 2014). Figure 2 shows a detailed map of the creeks draining to Poverty Bay, septic parcels in the area, and stormwater outfalls (Klinka 2016).

There are five Wild Stock Commercial Geoduck Clam Fishery areas in the Poverty/Dumas Bay area, which are jointly managed by the Washington State Department of Fish and Wildlife and the Puyallup Tribe of Indians (Swanson 2016). Opened in 2008 following release of a DOH Sanitary Survey, the Poverty Bay geoduck tracts were the first commercial

shellfish beds on the east side of the Puget Sound between Tacoma and Everett (Schultz 2008). The Washington DOH monitors 21 Marine Quality Stations in the Poverty Bay shellfish growing area (Figure 3). The intertidal portion of the bay remains closed throughout the harvesting season. Shellfish harvesting was initially approved for the area between Station 857 and Station 705. Stations 703-705 and Stations 756/857 remain closed due to outfalls from the Lakehaven and Midway wastewater treatment plants and the presence of the Des Moines Marina. In addition, the areas around McSorley Creek and Woodmont Creek outfalls were classified as *Prohibited* due to high fecal coliform levels in the creeks.

During 2015-2016, Station 722 and Station 720 failed the NSSP standard for *Approved* classification. In response, the Washington DOH downgraded 130 acres between Stations 617 and 707 to *Conditionally Approved* in September 2016 (Figure 3). The area is closed to commercial harvest from June 1 through November 30 each year until geometric mean fecal coliform levels no longer approach the NSSP standard during the dry season and first flush rains (Swanson 2016). The Puyallup Tribe is currently licensed to harvest 178,000 pounds at these tracts in the 26D Redondo region within Poverty Bay between April and October 2017 and can send out between four and six boats each day (Puyallup Tribe of Indians 2017). The downgrade also triggered the requirement that the King County council form a Shellfish Protection District and implement a shellfish protection program. This project was completed to inform public education and cleanup efforts in the area.

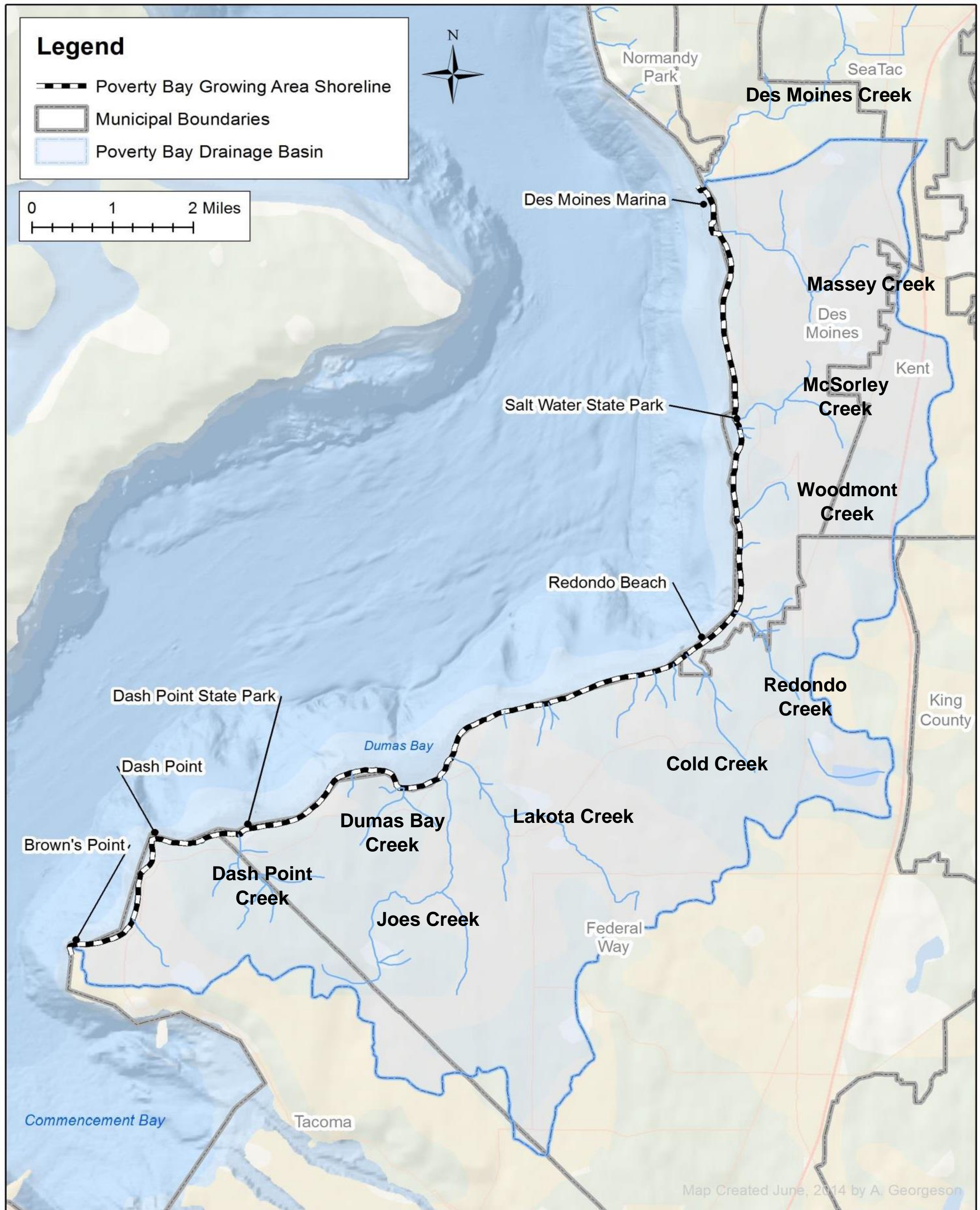


Figure 1. Poverty Bay drainage basin, municipalities, and upland creeks (Georgeson 2014).

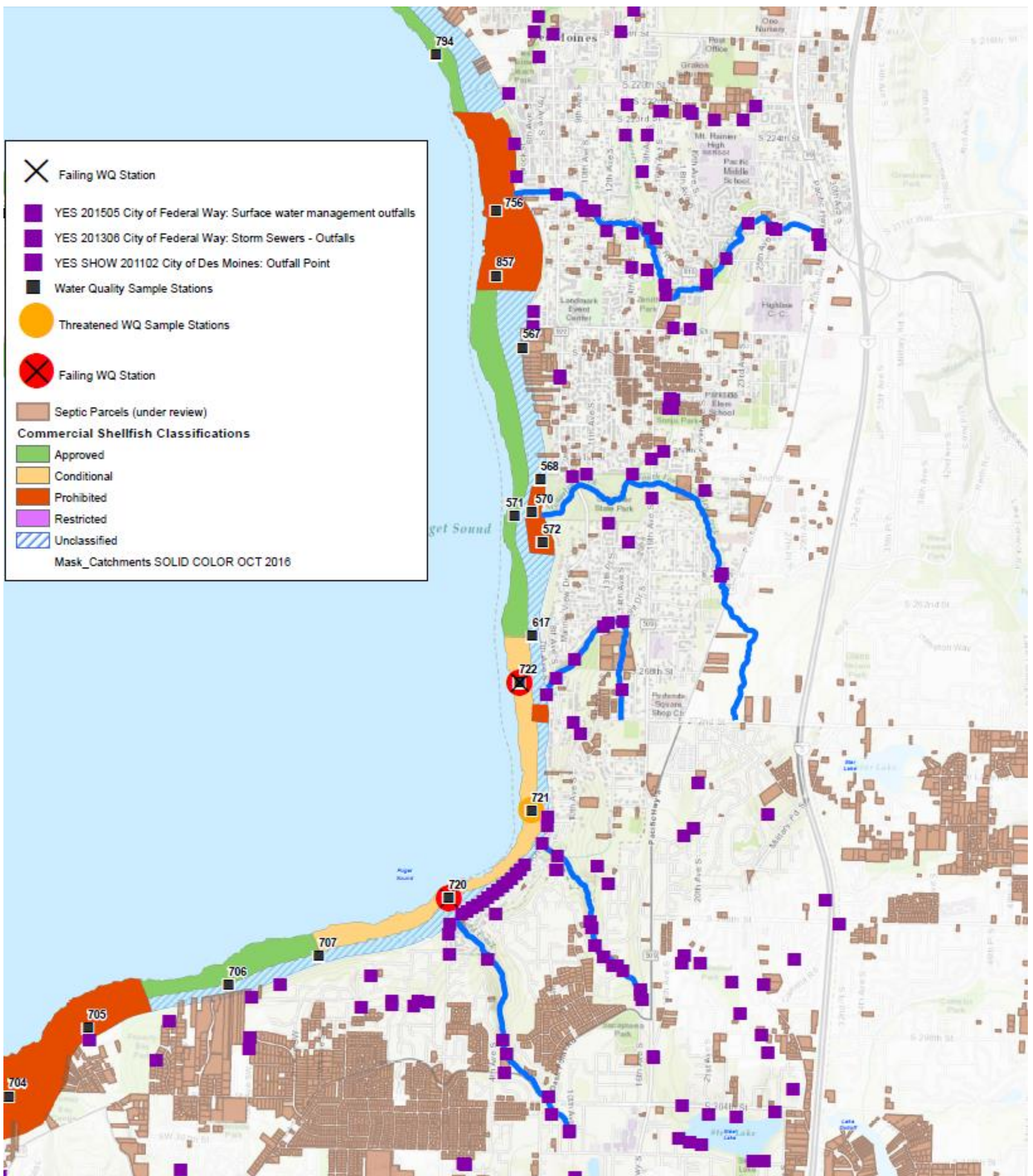


Figure 2. Detailed map of creeks in the Poverty Bay area. Septic parcels and stormwater outfalls are shown as well (Klinka 2016).

Figure 3. DOH marine monitoring locations along the Poverty Bay nearshore. Commercial shellfish classification as of September 14, 2016 (Swanson 2016).

Shoreline Monitoring in the Poverty Bay Area

The Washington Department of Health has compiled two surveys to characterize shoreline inputs of bacteria to the Poverty Bay shellfish growing area. In 2007 Seattle/King County Public Health characterized the Dash Point, Dumas Bay, and Redondo marine areas that included fecal coliform sampling at 41 discharge points. The most pertinent area to the current source tracking project was the Redondo Waterfront Park area. Figure 4 shows a map of the 20 sampled locations in the Redondo Waterfront Park Area and their fecal coliform levels, calculated as geometric mean values from seventeen sampling events. Eight of the 20 drainage discharge points (40 percent; sites 04, 05, 31, 53, 58, 60, and 79) had geometric means that exceeded 50 CFU/100 mL, the state surface water quality standard. The report concluded that certain homes along the Redondo Waterfront may have suspect on-site septic systems and suggested that the area be designated a Marine Recovery Area (Jenkins McLean et al. 2007).

In 2013-2014 the DOH completed another shoreline survey of the Poverty Bay Shellfish growing area that evaluated 97 drainage and discharge points and 59 developed parcels, as well as upland areas around the growing area. Of the drainages identified, 35 were monitored over 8 events between August 2013 and February 2014 (Georgeson 2014). Fecal coliform results from these sites are portrayed in Figure 4, alongside results from the 2007 survey. DOH collected 163 samples from this growing area, of which 37 (23%) did not meet the state freshwater quality standard for an individual fecal coliform sample (≥100 cfu/100 mL). Eleven sites had fecal coliform concentrations greater than 1600 cfu/100 mL; nine of the 11 sites were located in the Redondo Beach area.

Although the Poverty Bay shoreline was partially characterized by these reports, bacteria contributions from upland creeks remained understudied.

Purpose

Using microbial source delineation techniques, we sought to determine whether upland creeks in the Poverty Bay area are significant contributors of fecal contamination resulting in the *Conditionally Approved* status of Marine Quality Stations 720-722. Due to their proximity to the relevant stations, we initially expected Cold Creek, Redondo Creek, and Woodmont Creek to be the primary focus of this investigation. However, we included the larger creeks north of the immediate study area as well.

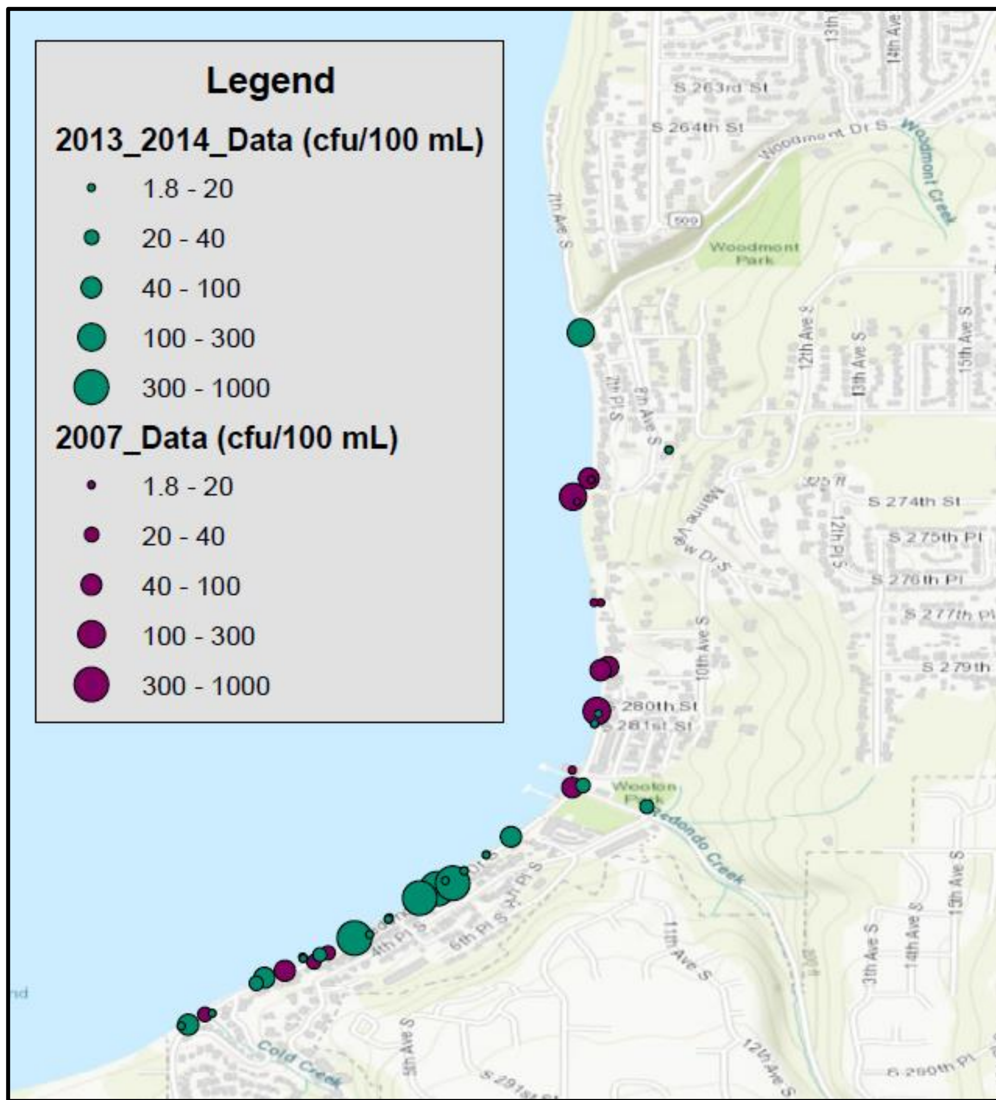


Figure 4. Data from 2007 and 2013-2014 Poverty Bay Shoreline Surveys. Chloropleth map created using ArcMap version 3.0.

Methods

Field Sampling: Coliscan Easygel ©

Fecal indicator indicator levels were determined using Coliscan Easygel © kits. Developed by Micrology Laboratories (Goshen, Indiana), the kits provide a low-cost aalternative to traditional methods for estimating indicator bacteria levels, such as membrane filtration. An enzyme substrate method is used to provide total coliform and *E. coli* bacterial counts present in 0.5 mL to 5 mL sample volumes. A sample is added to the medium, poured onto a petri dish, which is incubated at 35℃ for 36-48 h. General coliforms produce the enzyme galactosidase, and colonies that grow in the medium appear pink. *E. coli* produce both galactosidase and glucuronidase and grow as dark blue to purple colonies in the medium.

Data collected with the Coliscan Easygel © kits is not suitable for regulatory purposes, and the limited volumes assayed may not always accurately reflect environmental bacteria levels. However, previous research, including data collected by King County Stormwater Servivces, suggests that the technique presents a relatively accurate reflection of fecal coliform levels, especially when samples are considered in terms of rank-order (Chapman 2016, Chuang et al. 2011; Stepenuck et al. 2011). A study conducted in the Great Lakes states, in which volunteer-collected Coliscan data was compared to membrane filtration data showed a 16 percent risk of false positives, a 23 percent missed risk rate, and an 81 percent overall accuracy (Stepenuck et al. 2011). Coliscan Easygel ® has been found to be more accurate at elevated *E. coli* counts (Micrology Labs 2008; Chapman 2016). Figure 5 shows a log-linear comparison of Coliscan Easygel ® samples and fecal coliform by membrane filtration samples (Spectra Laboratories, Tacoma, WA) that we collected on 10/20/16 and 12/13/17 from sample locations in the Poverty Bay region.

For our purposes, grab samples were collected with a 500 mL collection cup, which was rinsed 3 times with stream water at each site. When necessary, a sampling extension pole was used to collect samples. Plastic 2 mL pipettes were used to transfer 5 mL water to the Easygel bottles. Ten percent of samples were replicated in the field, and a field blank was collected on each sampling day. Laboratory stocks of streptomycin/ampicillin-resistant *E. coli* F_{amp} (ATCC#:700891) were used as positive controls. Coliscan Easygel bottles were stored at -20°C until use, and samples were kept on ice until return to the laboratory when they were poured into pre-treated petri dishes, allowed to set for 45-60 minutes, and incubated at 35°C for 48 h.

Initial sampling locations were located on September 19. Surface water outfalls and storm sewer outfalls were considered when selecting sites. Upon identifying locations with high levels of fecal contamination, more specific bracketed sites were sampled as the project progressed. Figure 6 is a map of all locations sampled throughout the project, and a complete list with description of sampling locations is included in Appendix C. Table 2 shows sampling dates and rainfall totals the 3 days preceding sampling. A majority of first flush sampling events occurred prior to site identification and therefore only included the mouths of each of the five creeks. "First flush" days included September sampling dates; sampling days with < 1 in rain 3 days prior to sampling were considered "dry"; days with > 1 in rain 3 days prior to sampling were considered "wet."

<u>ArcGIS Analysis</u>

Using ArcMap version 3.0, we mapped *E. coli* levels in each of the Poverty Bay creek basins to facilitate source delineation. all sample site coordinates were documented in the field with the Esri Collector application. First flush, wet, and dry data were plotted to create chloropleth maps. Five manual breaks were assigned to *E. coli* data (1-60 cfu/100 mL; 60-180

cfu/100 mL; 180-300 cfu/100 mL; 300-1000 cfu/100 mL; 1000-5000 cfu/100 mL). The following data were included on each of the maps:

- DOH Shellfish_Growing_Areas_160203.
- Septic Parcels_King County.
- YES 201505. Surface water management outfalls. City of Federal Way.
- YES 201506. Storm Sewers – Outfalls. City of Federal Way.
- YES 201102. Outfall Point. City of Des Moines.
- Water Quality Sampling Stations
- Threatened Water Quality Sampling Stations
- Failing Water Quality Stations

A collective legend for the ArGIS maps is shown in Figure 7. All data files were obtained from King County.

Male-Specific (F^+) Coliphage by 2-Step Enrichment

To supplement bacterial fecal coliform data, we evaluated the presence/absence of male-specific coliphage (MSC) in creek water samples during 5 sampling events (Table 2). MSC has been proposed as a viral indicator organism and may provide a more accurate representation of the risk for gastrointestinal illness resulting from viral pathogens than do traditional bacterial indicators. EPA Method 1601 was performed following standard protocols using 100 mL sample volumes for F+ coliphages (USEPA 2001). This two-step enrichment procedure involves overnight enrichment of coliphage in a nutrient broth with *E. coli* F_{amp} bacteria, followed by spotting onto a lawn of host bacteria and assessment of lysis zone formation in the lawn.

Table 2. Fall 2016-Spring 2017 Poverty Bay field sampling events.

Date	3-Day Precip (in)	Classification	Sample Type
9.2.16	0.39	First Flush	Fecal Coliform (MF*)
9.6.16	0.43	First Flush	Fecal Coliform (MF)
9.8.16	0.49	First Flush	Fecal Coliform (MF)
9.19.16	0.22	First Flush	Coliscan
10.6.16	0.48	Dry	Coliscan
10.14.16	3.12	Wet	Coliscan
10.20.16	1.49	Wet	Coliscan
11.21.16	0.07	Dry	Coliscan
12.13.16	0.18	Dry	Coliscan/MSC
1.9.17	1.09	Wet	Coliscan/MSC
1.18.17	3.89	Wet	Coliscan/MSC
2.11.17	1.65	Wet	Coliscan/MSC
3.6.17	0.04	Dry	Coliscan
3.30.17	0.65	Dry	Coliscan/MSC
4.28.17	0.23	Dry	Coliscan
5.12.17	0.63	Wet	Coliscan
5.16.17	0.52	Wet	Coliscan

**MF: membrane filtration performed at Spectra Laboratories (Fife, WA).*

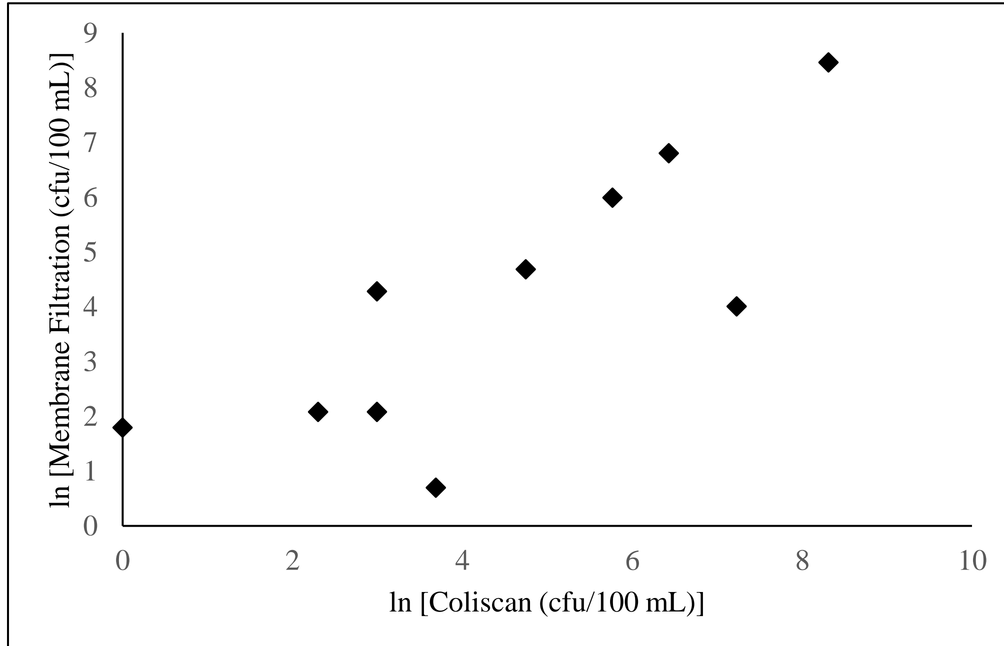


Figure 5. Log-linear comparison of Coliscan *E. coli numbers* and fecal coliform by MF (Spectra Laboratories, Tacoma, WA).

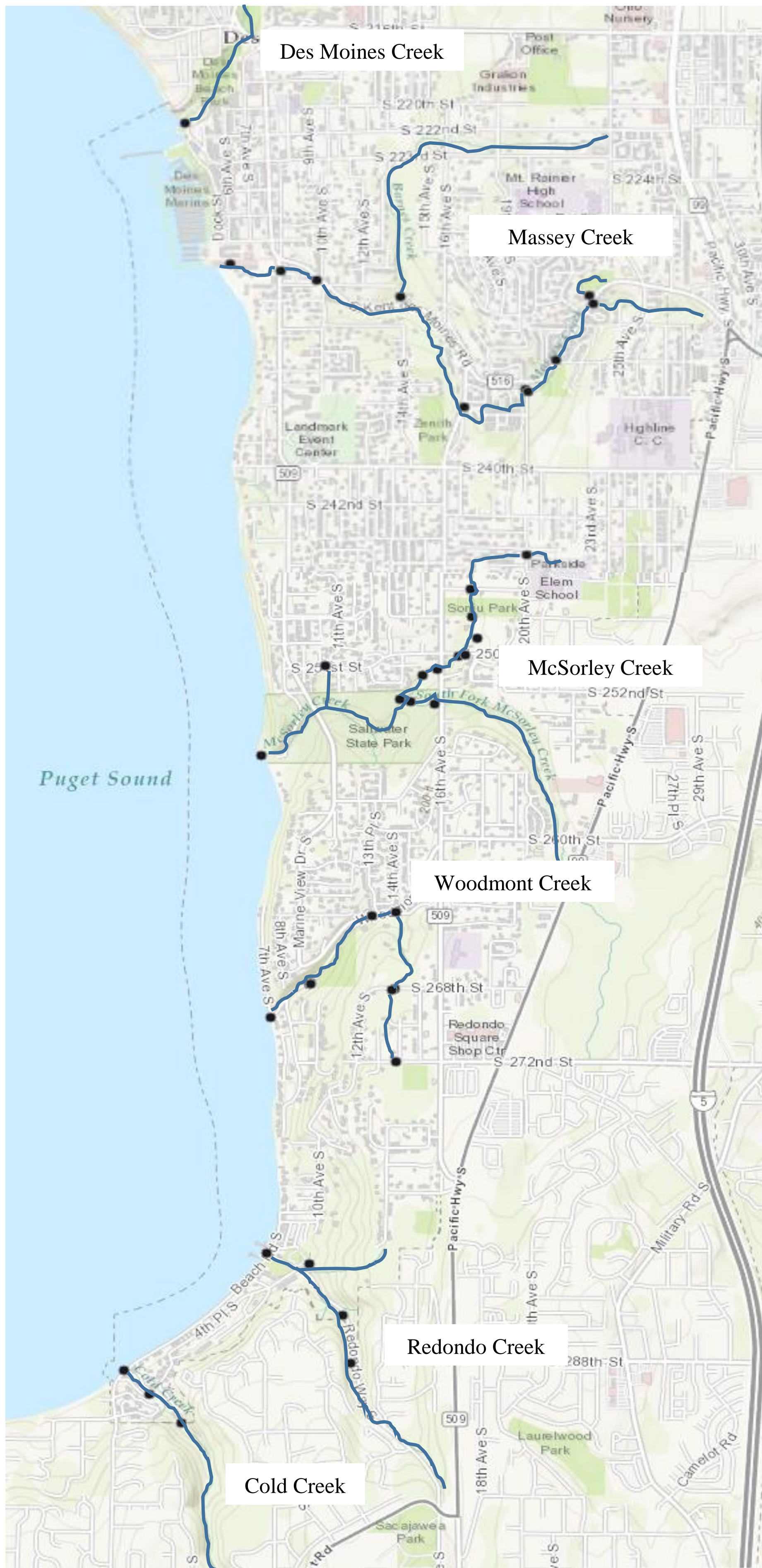


Figure 6. A map of all locations sampled with Coliscan Easygel © kits throughout the course of creek monitoring.

Results and Discussion

Overview

Creek water sampling in Des Moines was carried out between September 2016 and May 2017. Table 3 portrays geometric mean *E. coli* values for all sampling locations relative to the precipitation recorded 24 hours, 72 hours, and 10 days prior to sample collection. A complete data log of Coliscan *E. coli* estimates for each sampling date is included in Appendix D. In general, first flush and wet day *E. coli* values were higher than dry day values across all sample locations. Samples taken on October 14 and October 20, during the first large storm of the season, were particularly high, with composite geometric means of 2016 cfu/100 mL and 1576 cfu/100 mL, respectively (Table 3). While September "first flush" rains may have washed away fecal contamination from upland surfaces, perhaps the heavy October storm saturated soils extensively, flushing out fecal material that had accumulated deeper in the ground.

Although a majority of days with high overall *E. coli* levels occurred following during wet weather events, the 5/12/17 and 5/16/17 sampling dates did not follow this trend. Although < 1 inch of precipitation occurred preceeding these days, high numbers of exceedences were recorded (Table 3). It should be noted that only samples from Massey Creek and McSorley Creek were collected on these dates, after we had determined initial sites with high *E. coli* hits and performed further source tracking. Therefore, the presence of new sites on these dates may have contributed to high overall *E. coli* numbers. Additionally, creek volumes during the month of May were lower due to lower precipitation levels relative to the preceding winter and spring months. This may have resulted in higher overall fecal bacteria concentrations in creek water.

Based on the data presented in Table 3, high bacteria levels appear to be associated with rainfall events. In addition, low creek volumes may contribute to high *E. coli* numbers during moths with low rainfall (i.e. September 2016 and May 2017).

Figures 8-10 and Figure 12 show *E. coli* levels on first flush, wet, and dry sampling days for each of the creeks. The mouth of Des Moines Creek is not shown, but geometric mean *E. coli* values of 853 cfu/100 mL (n=4), 339 cfu/100 mL (n=5), and 236 cfu/100 mL (n=5) were recorded for first flush, wet, and dry days, respectively (Appendix D). Throughout the sampling season, the highest fecal coliform levels were detected in the mouth of the Des Moines Creek and along Massey Creek (Figure 8), McSorley Creek (Figure 9), and Woodmont Creek (Figure 10). Given that the downgraded Conditionally Approved area stretches from Station 707 (south of Cold Creek) to Station 617 (just north of Woodmont Creek), the Poverty Bay Technical Committed debated whether to include the creeks north of the Conditional Area (i.e. McSorley, Massey, and Des Moines Creeks) in source tracking efforts. Drift cells show a divergence zone between the mouths of Woodmont Creek and McSorley Creek, with northward flowing currents beginning just south Saltwater State park (McSorley Creek) (WA Ecology B). However, preliminary current mapping at Woodmont Beach and McSorley State Park suggests that currents in Poverty Bay may differ during flood and ebb tides , so southward transport of fecal contamination from McSorley Creek and Massey Creek toward Stations 720 – 722 should not be discredited (Ricker and James 2017).

Woodmont Creek

Despite the fact that Woodmont Creek is relatively short, with limited flow and a small drainage basin (Appendix B), its proximity to Marine Quality Station 722 makes it a promising target for source tracking. Woodmont Creek has previously been described as being heavily

impacted by stormwater (Toy 2012; Georgeson 2014). Located at the creek's lower reaches, WOOD_01, WOOD_02, and WOOD_03 showed high hits early on during wet weather events (Figure 10; Appendix D). All three points are located downstream from several stormwater outfalls (Figure 10). One identified area of concern was the stormwater discharge released directly above WOOD_02, where two closed conveyances channel water into the stream (Figure 11). We sampled additional sites on Woodmont Creek beginning in February 2017. The cement stormwater conveyance (WOOD_07; Figure 10) had one high *E. coli* value on 3/30/17 (a dry sampling day), but data is insufficient to draw conclusions. The Washington Department of Ecology took over Woodmont Creek source tracking efforts in April 2017.

McSorley Creek

Given that McSorley Creek has a large drainage basin and the uncertainty surrounding current flows in Poverty Bay, we decided to perform bracketing and further sampling on McSorley Creek following high *E. coli* hits during wet weather events. McSorley Creek discharges just north of Marine Quality Station 722, and its watershed is much larger than that of Woodmont Creek (Appendix B).

The base of the north branch of the creek (McS_02; 514 cfu/100 mL) had higher wet weather *E. coli* numbers than the south branch (McS_03; 167 cfu/100 mL) (Figure 9). During heavy rainfall, the Midway Sewer District occasionally experiences overflows and may bypass a portion of treatment plant flow. During bypasses, the plant discharges into the north fork of McSorley Creek, contributing to high fecal bacteria numbers in the creek and offshore in the Sound. Midway Sewer district overflows occurred on 2/9/17 and 2/16/17, with a bypass on 2/9/17, when ~660,000 gallons of wastewater received only primary treatment prior to disinfection (Public Health Seattle & King County 2017). Washington State DOH sampling on

2/9/17 showed a fecal coliform value of 540 cfu/100 mL at Station 722 on 2/9/17, but this value was later removed from the dataset (WADOH 2017). Our Coliscan sampling on 2/11/17 showed ~300 cfu *E. coli*/100 mL at the base of the north fork of the creek (Appendix D). This result was not particularly high, indicating that bacteria from bypass events may not persist in the creek for longer than 2 days. Despite the potential for bypasses, these events are rare.

As with Woodmont Creek, bacteria levels in McSorley Creek may be impacted by stormwater discharges. A majority of discharge locations are located on the north fork of the creek, which may have contributed to high hits at McS_02 during wet weather events (Figure 9). Further source delineation includes detailed sampling near stormwater discharges (Figure 9; see sites McS_05-McS_08; McS_11) but has thus far encompassed only dry days (Figure 9; Appendix D). However, as noted earlier, we observed high hits upstream on 5/12/17 and 5/16/17, following light precipitation. This may be a result of lower water volumes (higher bacteria concentrations) at sampling sites. Dry season sampling will help elucidate this pattern.

Massey Creek

Although it discharges north of the *Conditionally Approved* shellfish growing area (Figure 2), Massey Creek was determined to be a potential source of fecal bacteria contributing to high hits at monitoring stations. It is located adjacent to Station 756, at which harvesting is *Prohibited* due to the marina and wastewater treatment plant. However, Station 756 has consistently failed NSSP standards according to marine quality data (Swanson 2016). High hits during wet weather events (Appendix D; Figure 8) and a large drainage basin (Appendix B), make Massey Creek a likely pollution source in Poverty Bay, and there is potential for currents to carry fecal material toward Stations 720-722.

Located near the mouth of Massey Creek, MAS_01 and MAS_03 showed high bacteria levels during the first flush and wet weather events (Figure 8; Appendix D). Both points are located along urbanized sections of the creek that receive runoff from impervious surfaces. Urbanization results in a loss of water storage within the soil column. Processes such as vegetation clearing, soil compaction, ditching and draining, and the installation of impervious surfaces result in rapid conveyance of stormwater through gutters, drains, and storm sewers to stream channels (Booth et al. 2006). Fine sediment is moved into these channels throughout the year, but when coupled with land-cover changes, sediment loads can increase by many orders of magnitude, which is correlated with increased fecal contamination (Wolman and Schick 1967). The prevalence of impervious surfaces near the mouth of Massey Creek may therefore contribute to high bacteria levels, particulary during precipitation events.

It is worth noting that MAS_02, which is located at the base of the Barnes Creek tributary, also showed high bacteria levels throughout the sampling period (Figure 8). In contrast to MAS_01 and MAS_03, MAS_02 receives runoff from upstream wooded areas and may therefore may carry wildlife fecal material.

Upstream sampling began in May 2017 as a result of high hits downstream during first flush and wet days. Thus far, as discussed above, detailed sampling captured 5/12/17 and 5/16/17, two dry days when *E. coli* numbers were particularly high (Figure 8). Source delineation efforts have not yet encompassed Barnes Creek, but given the high *E. coli* hits at MAS_02, doing so may be worthwhile.

Redondo Creek and Cold Creek

There remains the question of what is causing DOH Marine Quality Stations 720 and 721 to fail. The stations are located offshore from the mouths of Cold Creek and Redondo Creek,

respectively and flow through areas where septic parcels are highly prevalent (Figure 12). However, neither creek showed particularly high *E. coli* data throughout the sampling period, despite the fact that Redondo Creek is 303(d) listed for fecal coliform (Georgeson 2014). Our sampling did not encompass the shoreline outfalls located between these creeks (Figure 12). However, 2007 and 2013-2014 data from the Shoreline Surveys completed by the Washington Department of Health suggested that they may be contributing pollution sources (Figure 4; Jenkins McLean et al. 2007; Georgeson 2014).

MSC Presence-Absence Data

Results from the two-step enrichment procedure are shown in Table 4. This method effectively detects low levels of MSC, as 100 mL water samples are incubated overnight with nutrient broth and *E. coli* host prior to the plaque assay (USEPA 2001). Our data set is limited to certain sites on 5 sampling dates; from these data, we discerned no clear trend between *E. coli* levels estimated with Coliscan Easygel © and MSC presence or absence. A more complete dataset could be used to more extensively compare the bacterial and viral indicator methods. The fact that the MSC presence-absence test detected coliphage at some, but not all, sties suggests that this method may provide useful source delineation information.

Limitations

Although sampling began to identify contamination hotspots, we did not identify distinct point sources of fecal contamination for pollution control efforts. Sample size limits this dataset; although we sampled on seventeen occasions, we divided the events into “first flush,” “wet,” and “dry” categories, therefore further decreases sample numbers. While we collected F+ specific coliphage data on 5 sampling days, datasets were incomplete and of limited usefulness to source tracking. Although this presence-absence data may prove useful if collected on a larger scale, the

procedure is time-consuming, and bacterial indicators provide equally useful information at the current stage of source delineation.

Although this study provides a start to fecal contamination source tracking efforts in the Poverty Bay region, *E. coli* data is limited by the fact that it is not species-specific. Protection of environmental health depends on distinguishing which contaminating sources impact a particular body of water. Analysis of the 16s rRNA gene in *Bacteroides* bacteria species shows promise for distinguishing between hosts, including human, bovine, pig, horse, dog, cat, gull, and elk (Dick et al. 2005). However, many sequences continue to show very close matches with multiple hosts. For example, dogs cohabitate with humans and share portions of their microbiota. One study estimated that 40 percent of human pathogens also infect domestic animal hosts (Cleaveland et al. 2001). Since then, newly developed SYBR Green TaqMan quantitative PCR assays for canine fecal source identification have become more species-specific (Green et al. 2014). Future source tracking would benefit from distinguishing between waterfowl, human, and dog contributors to fecal contamination.

Conclusions and Future Direction

A September 2016 downgrade of geoduck beds in the Poverty Bay region triggered King County to contract with the University of Washington to perform microbial source tracking in the area. Environmental monitoring in the creeks upland of Poverty Bay suggests that fecal coliform “high hits” at Marine Quality Stations 720, 721, and 722 may be attributed to stormwater runoff, as *E. coli* estimates were higher during wet weather events. This implicates nonpoint sources, such as runoff from urban areas, wooded areas, and faulty septic systems, as potential sources. Creek sampling in the area should be continued throughout the year to better distinguish temporal fecal contamination trends in the region.

Loctaed offshore from Station 722, Woodmont Creek was determined to be a likely source of fecal contamination, particularly during wet weather events. High hits were detected at sites located downstream from stormwater discharges (ex. WOOD_01, WOOD_02, and WOOD_03), prompting further source delineation and formal sampling by the Department of Ecology in the area. Contrary to our initial expectations, Redondo Creek and Cold Creek generally had low *E. coli* numbers, relative to the other creeks. As a result, future sampling should not focus on the creeks themselves, but instead on the outfalls located direcly along the shoreline on Marine View Drive S (Figure 12) to determine whether they contribute to occasional high fecal coliform hits at Marine Quality Station 720.

Preliminary evaluation of currents in Poverty Bay shows that contamination may be carried down to the north end of the *Conditionally Approved* area from creeks farther north, including Massey Creek and McSorley Creek. We began extensive sampling on these creeks during March-May 2017, and future sampling during the dry season will help elucidate (a) whether contributing sources exist upstream from our initial sampling locations and (b) whether fecal contamination is primarily associated with wet weather events.

While sampling with Coliscan Easygel © kits provided an inexpensive alternative to enumerating fecal coliform numbers, these data cannot be used for regulatory purposes. When clear hotspots are detected, creek samples should be taken to a laboratory accredited by the WA Department of Ecology for formal membrane filtration analysis. In addition, future source tracking should include species-specific techniques, including *Bacteriodes*, to determine whether the fecal loads at sites with high hits originate from human or animal sources. This assessment provides an initial overview of fecal contamination in the the creeks upland from Poverty Bay that should be used to inform future source delineation in the area.

Table 3. Coliscan Easygel © results summary and precipitation statistics by monitoring date. "First flush" dates are highlighted in red, "wet" dates in blue, and "dry" dates in green. Elevated samples were those that exceeded the Washington State Department of Ecology standard for individual surface water samples of 100 cfu/100 mL (WAC 173-201A).

Date	Composite Geomean	Elevated Samples/ Day	Total Samples/ Day	% Elevated/ Day	24 h Precip (in)	72 h Precip (in)	10 day Precip (in)	Monthly Precip	Avg Monthly Precip
9/2/2016	930	5	5	100%	0.05	0.39	0.39	0.99	1.5
9/6/2016	759	4	5	80%	0.42	0.43	0.82	0.99	1.5
9/8/2016	42	3	5	60%	0.03	0.49	0.89	0.99	1.5
9/19/2016	440	14	17	82%	0.08	0.22	0.48	0.99	1.5
10/6/2016	41	9	17	53%	0.31	0.48	0.55	10.05	3.48
10/14/2016	2061	6	6	100%	1.36	3.12	4.38	10.05	3.48
10/20/2016	1576	18	18	100%	1.19	1.49	6.07	10.05	3.48
11/21/2016	8	2	19	11%	0.02	0.07	2.00	6.48	6.57
12/13/2016	19	4	18	22%	0	0.18	1.21	3.86	5.35
1/9/2017	25	6	18	33%	0.05	1.09	1.09	3.91	5.57
1/18/2017	223	15	19	79%	1.21	2.95	3.52	3.91	5.57
2/11/2017	9	3	22	14%	0	1.65	4.61	8.85	3.5
3/6/2017	8	1	9	11%	0	0.04	0.75	7.05	2.72
3/30/2017	7	3	32	9%	0	0.65	1.93	7.05	2.72
4/28/2017	9	4	21	19%	0	0.23	1.55	4.21	2.71
5/12/2017*	378	22	23	96%	0.16	0.63	1.58	2.28	1.94
5/16/2017*	184	17	23	74%	0.15	0.52	1.20	2.28	1.94

Table 4. MSC presence-absence results from two-step enrichment test.

Site Name	12/13/2016		1/9/2017		1/18/2017		2/11/2017		3/30/2017	
	E. coli	MSC	*E. coli*	MSC	*E. coli*	MSC	*E. coli*	MSC	*E. coli*	MSC
DMC_01			180	+	260	+	80	+		
MAS_01			80	+	420	+	140	+		
MAS_02			300	+	700	+	140	+	20	+
MAS_03			420	+	680	+	60	-	20	+
MAS_04			100	-	440	+	--	-		
McS_01	8	+	180	-	420	+	300	+	1	+
McS_02	72	+	180	+	420	+	300	+	1	+
McS_03	8	+	20	+	400	+	60	+	40	-
McS_04							20	+		
McS_05									1	-
McS_06									1	-
McS_07									20	+
McS_08									1	+
McS_09									1	+
McS_10									80	+
McS_11									1	+
McS_12									20	-
McS_13										+
WOOD_01	102	-	70	+	400	+			40	-
WOOD_02	115	+	80	-	553	+				
WOOD_03	2	-	120	+	460	+	1	+	20	-
WOOD_04	6	-	1	-	60	+	1	-	1	-
WOOD_05							180	-	20	-
WOOD_06									1	-
WOOD_07									320	-
WOOD_08							60	-	20	-
WOOD_09							40	+	500	-
RED_01					60	-	1	+	1	+
RED_02	10	+	1	-	160	-	1	-	1	-
RED_03	20	-	1	-	80	+	1	+	1	+
RED_04	20	+	1	-	20	+	20	+	1	+
COLD_01			20	-	160	+	1	-	1	-
COLD_02			1	-	220	+	1	-	1	-
COLD_03			20	-	140	-			1	-

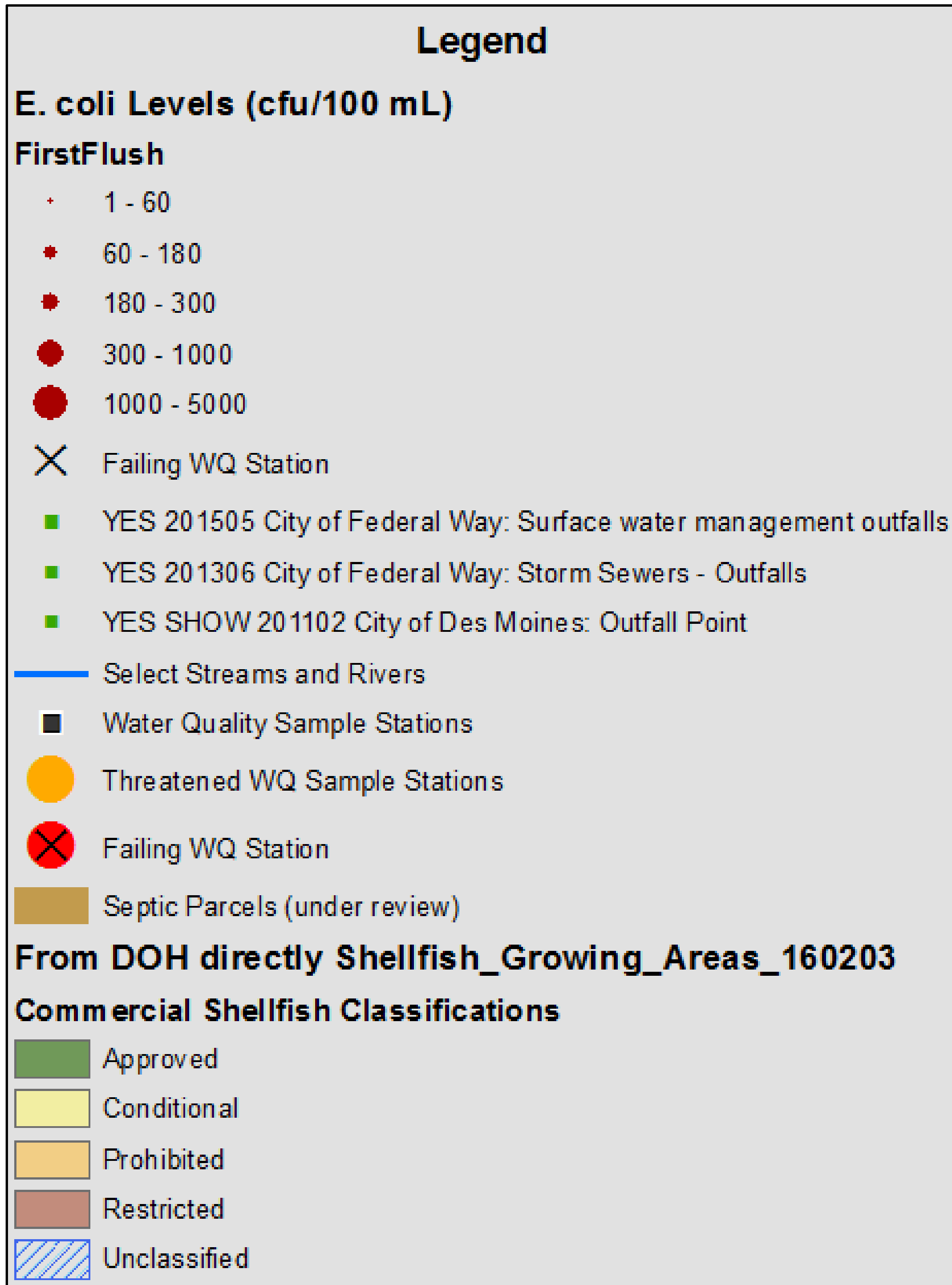
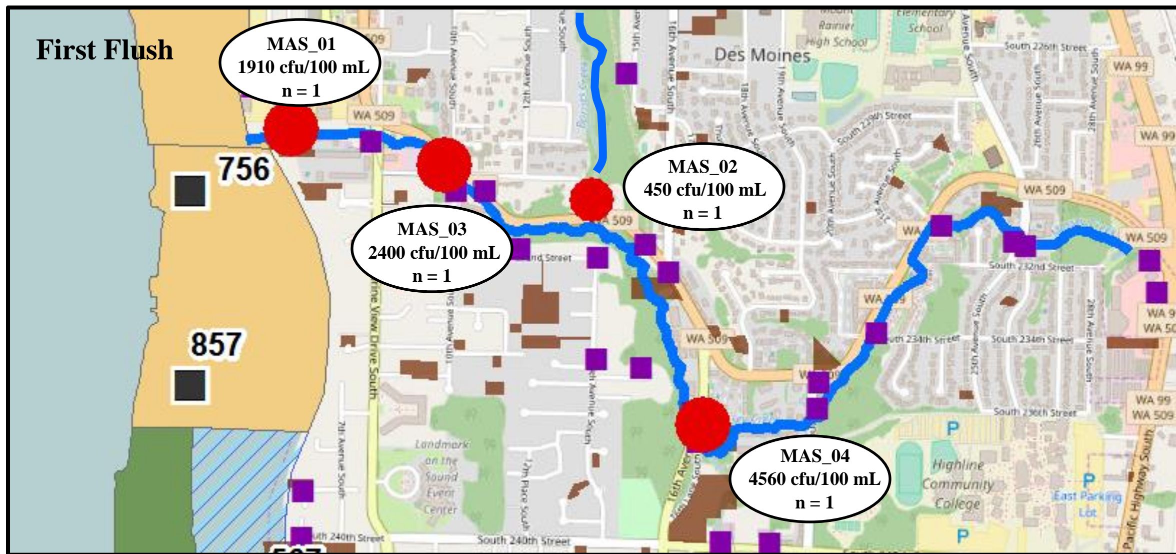
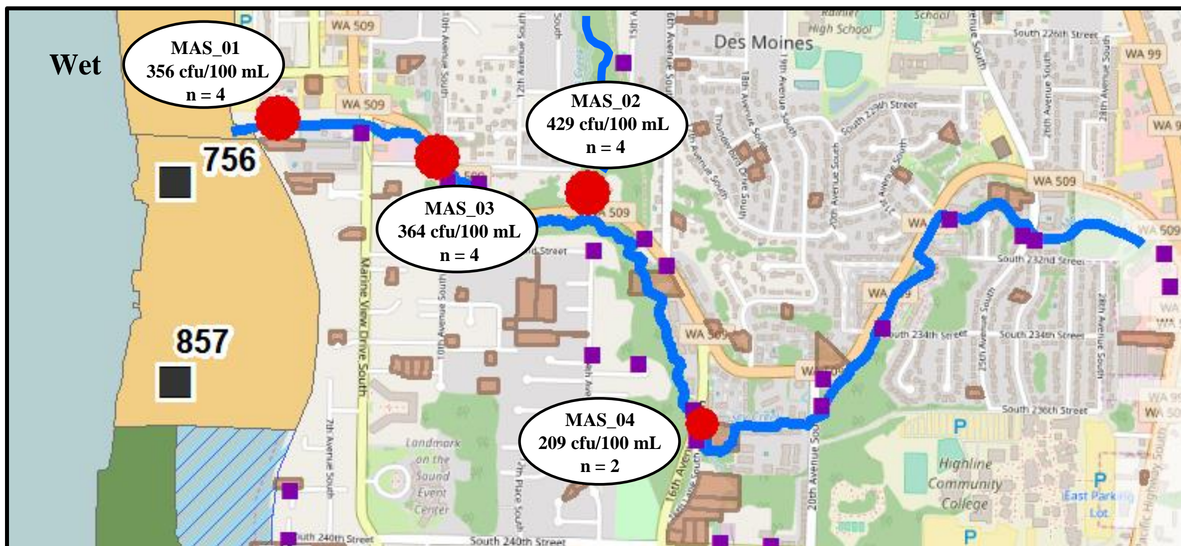


Figure 7. Legend for Figures 8-10 and Figure 12.





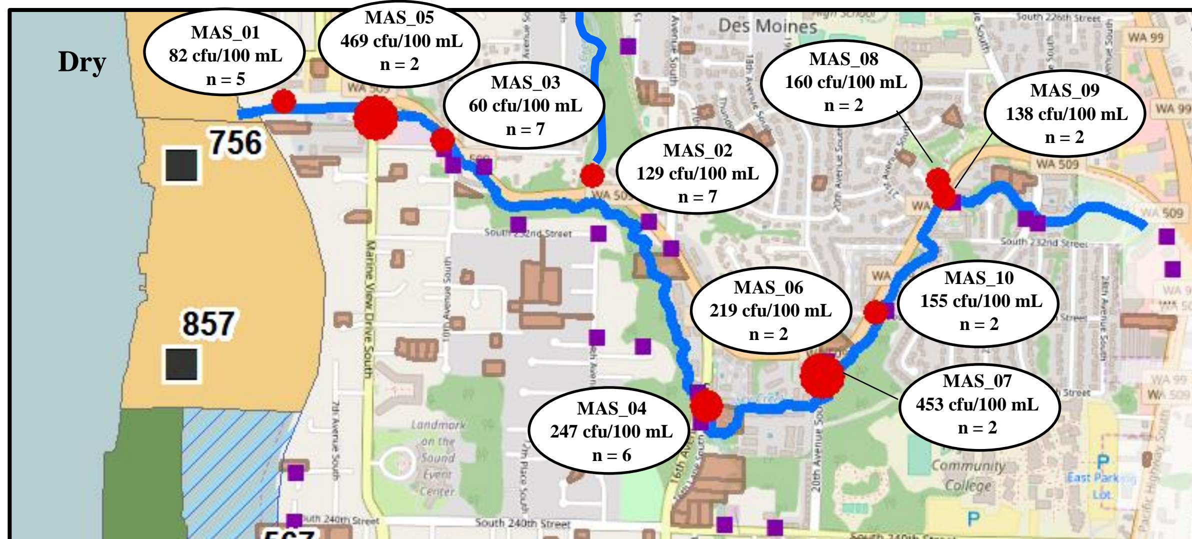
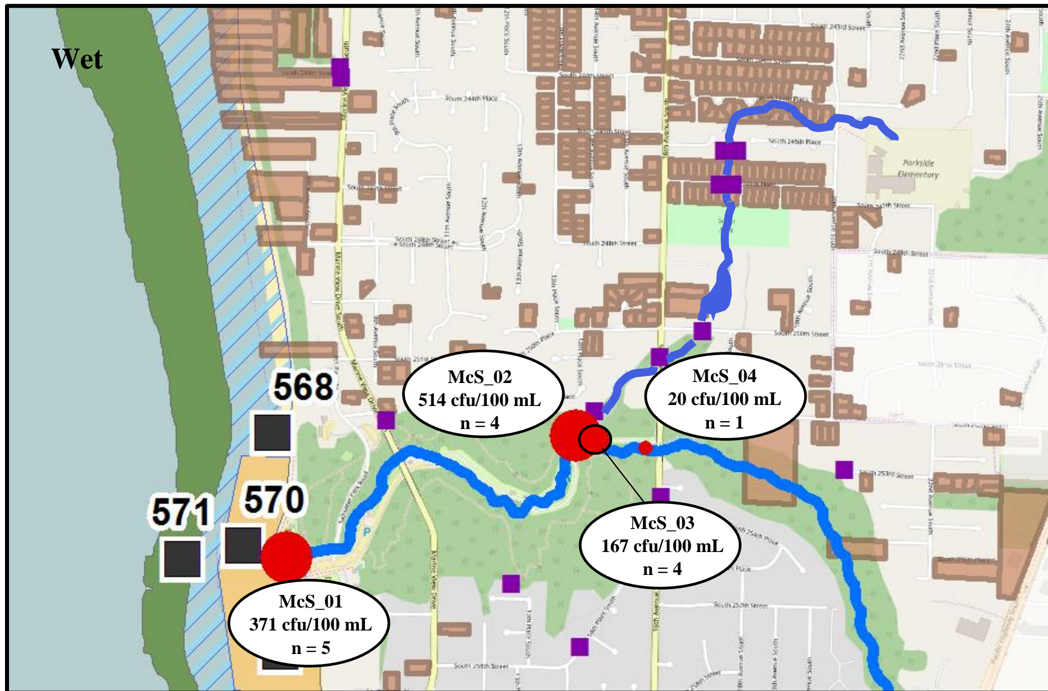


Figure 8. Geometric mean *E. coli* numbers in Massey Creek during first flush (top), wet (middle), and dry days (bottom).



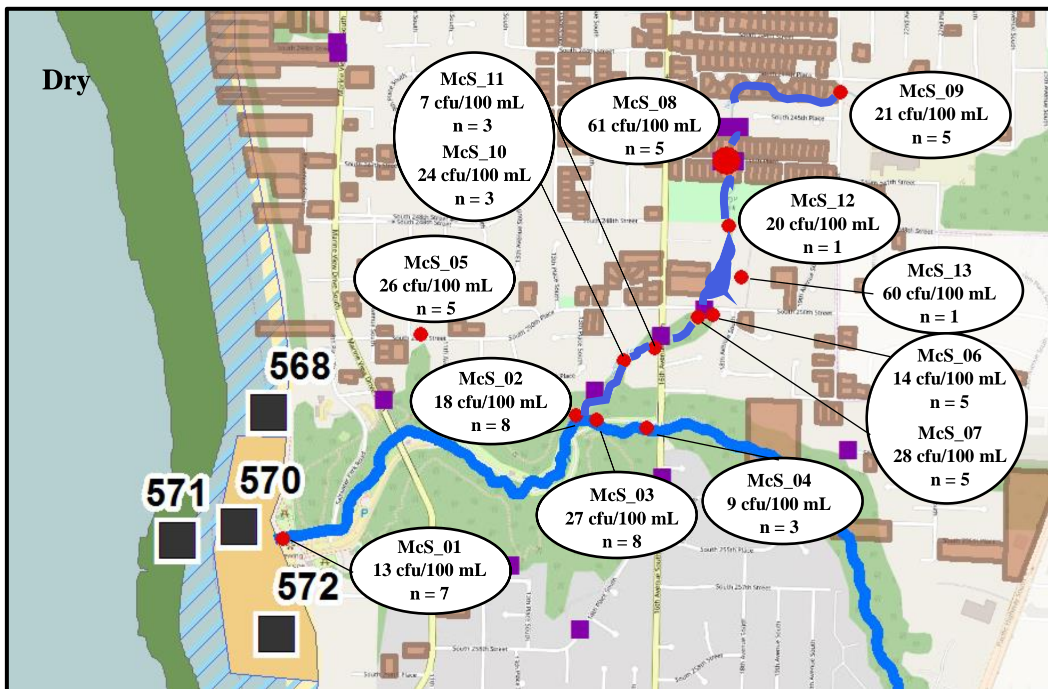
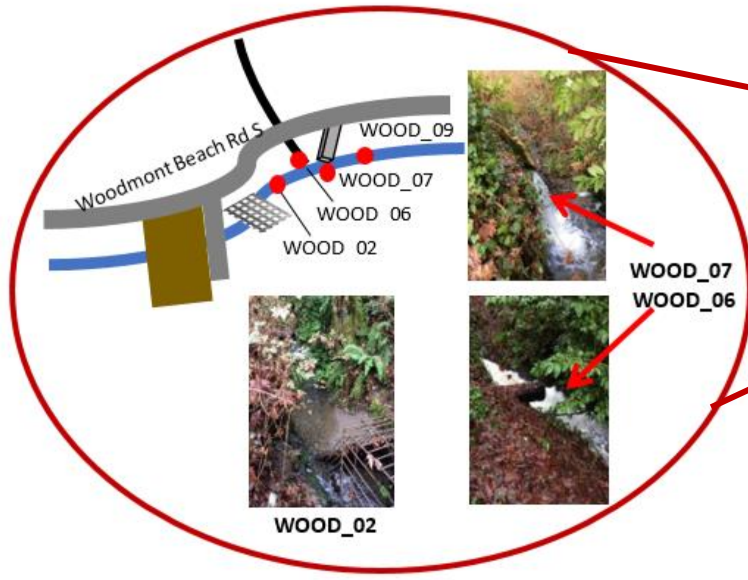


Figure 9. Geometric mean *E. coli* numbers in McSorley Creek during first flush (top), wet (middle), and dry days (bottom).



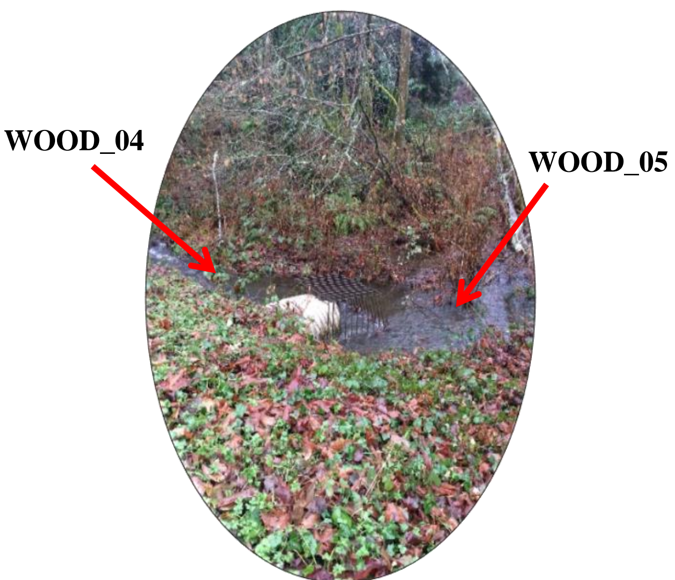


Figure 11. Source tracking in Woodmont Creek. WOOD_02 was expanded to include three new sampling locations (WOOD_06, WOOD_07, WOOD_09) (top). WOOD_04 and WOOD_05 were sampled from different branches of the creek (bottom).

First Flush

WOOD_02
1380 cfu/100 mL
n = 1

WOOD_03
1990 cfu/100 mL
n = 1

WOOD_04
1700 cfu/100 mL
n = 1

WOOD_01
416 cfu/100 mL
n = 4

617

722

Wet

WOOD_03
566 cfu/100 mL
n = 6

WOOD_08
60 cfu/100 mL
n = 1

WOOD_02
564 cfu/100 mL
n = 3
WOOD_06
1 cfu/100 mL
n = 1
WOOD_07
1 cfu/100 mL
n = 1
WOOD_09
40 cfu/100 mL
n = 1

WOOD_04
19 cfu/100 mL
n = 4
WOOD_05
180 cfu/100 mL
n = 1

WOOD_01
566 cfu/100 mL
n = 6

617

722

Dry

WOOD_03
28 cfu/100 mL
n = 4

WOOD_08
320 cfu/100 mL
n = 1

WOOD_02
96 cfu/100 mL
n = 3
WOOD_06
1 cfu/100 mL
n = 1
WOOD_07
320 cfu/100 mL
n = 1
WOOD_09
500 cfu/100 mL
n = 1

WOOD_04
2 cfu/100 mL
n = 4
WOOD_05
60 cfu/100 mL
n = 2

WOOD_01
32 cfu/100 mL
n = 4

617

722

Figure 10. Geometric mean *E. coli* numbers in Woodmont Creek during first flush (top), wet (middle), and dry days (bottom).

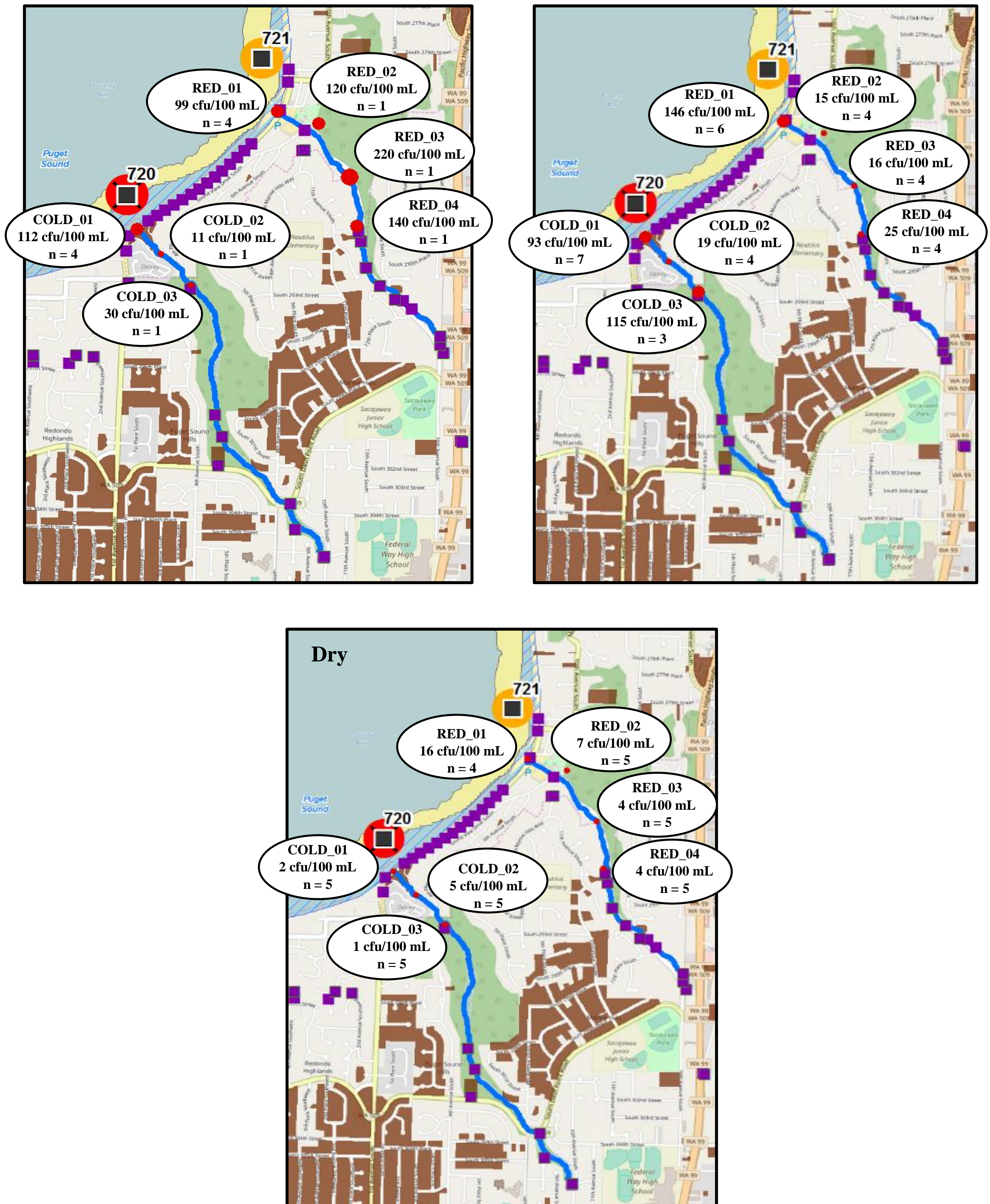


Figure 12. Geometric mean *E. coli* numbers in Redondo Creek and Cold Creek during first flush (top), wet (middle), and dry days (bottom).

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Chapter 4: Discussion

This project contributes to the literature on water quality monitoring of recreational beaches and commercial shellfish beds in Washington State. Recreational beaches provide people with opportunities for exercise and enjoyment. However, open waters, including lakes, estuaries, and oceans, potentially harbor enteric pathogens from fecal contamination. Monitored by the Washington State Department of Ecology, the shellfish industry is an important component of the state's economy; Washington produces 90 percent of the nation's geoduck (Shamshak and King 2015). As molluscan shellfish, geoduck are filter feeders with great potential to concentrate pathogens.

We had two specific aims. In the first, we used the Bag-Mediated Filtration System (BMFS) to perform a preliminary surveillance study of enteric viruses and male-specific coliphage at Green Lake, Lake Washington, and Puget Sound recreational beaches. Bacterial indicator data was collected as well. The second component of this research was an effort to delineate sources of fecal contamination in the Poverty Bay region using *E. coli* data from creeks upland of the Bay.

Overview of Findings

We encountered high levels of fecal indicator bacteria at Seattle Area recreational beaches on several sampling days, but we detected male-specific coliphage on only one sampling day in October, following a heavy rainstorm. Given that recreational swimmers typically do not enjoy Seattle beaches during fall rains, high levels of indicator pathogens during the first flush do not pose a risk to swimmers. However, high bacterial levels during the summer swimming season may be of concern.

Male-specific coliphage has several components of an ideal viral indicator, including its inability to replicate in the environment and its resemblance of human enteric viruses in terms of composition, structure, and size (Grabow 2004). Although coliphage presence does not guarantee the presence of enteric viruses, it is frequently found in situations of viral contamination and may be a more effective indicator than traditional bacterial indicators (ISCC 2014). This study did not contribute to the body of literature evaluating the effectiveness of male specific coliphage as an indicator species, as we did not encounter enteric viruses in our samples. However, we presented the BMFS as a novel surveillance method that can be used to assay large volumes ($\geq$ 20 L) of recreational water, as opposed to 100 mL volumes used in the established EPA standard operating procedures for coliphage monitoring (USEPA 2001a, USEPA 2001b). Through seeding 20 L volumes of water from each sampling location, we sought to provide information on method's effectiveness for detecting coliphage. In general, recoveries were lower at saltwater than freshwater sites; this may be attributed to differences in salinity and/or turbidity in Puget Sound and freshwater beaches. Overall, the BMFS holds potential for assaying large volumes of recreational water, thereby decreasing pathogen detection limits.

While we experimented with novel surveillance methods for detection of enteric pathogens at Seattle recreational beaches, we primarily used traditional methods to perform preliminary microbial source delineation in the Poverty Bay region. This study served as a first look at fecal pollution levels in creeks upland of geoduck harvesting tracts and used Coliscan Easygels kits to estimate *E. coli* levels and narrow down potential sources. Throughout the sampling season, spatial and temporal fecal contamination patterns began to emerge. At all sampling locations, *E. coli* levels were highest during September and October wet weather events. Woodmont Creek, McSorley Creek, Massey Creek, and the mouth of Des Moines Creek

had the highest *E. coli* levels. This contradicted our initial hypothesis that Cold Creek and Redondo Creek would be significant contributors to high hits at Marine Quality Stations 720 and 721. Further investigation into currents in the bay is pertinent, as fecal pollution may travel south from the creeks north of the Conditionally Approved area.

Project Impact

High levels of fecal bacteria in Seattle Area recreational waters imply the potential presence of enteric pathogens and may have negative implications for summer swimmers. Likewise, high fecal coliform measurements collected by the WA Department of Health at water quality stations may be associated with concentrated pathogen levels in molluscan shellfish.

Water quality regulatory jurisdictions in Washington State are responsible for beach and shellfish bed closures in the scenario of exceedances. Throughout Summer 2016, none of the recreational beaches investigated in this project were closed, implying that they were safe for swimming. Conversely, our limited bacterial indicator data would have resulted in a number of beach closures. Perhaps sampling times and locations affected our results. In addition, we presented the BMFS as a more sensitive alternative to traditional EPA methods for measuring coliphage levels recreational waters.

The Poverty Bay source delineation component of this thesis will be referenced when formation of a Shellfish Protection District in the Poverty Bay region is formally considered in June 2017 by King County and city governing bodies. Source delineation data from each of the creeks will prove useful when deciding on boundaries for the district (RCW 90.72.030).

This research used both traditional and novel fecal indicator methods to contribute to microbial surveillance and source delineation in recreational and commercial waters of the

Seattle Area. The BMFS holds potential for future enteric virus surveillance efforts. However, in scenarios where time and money must be conserved, traditional fecal indicators continue to prove useful.

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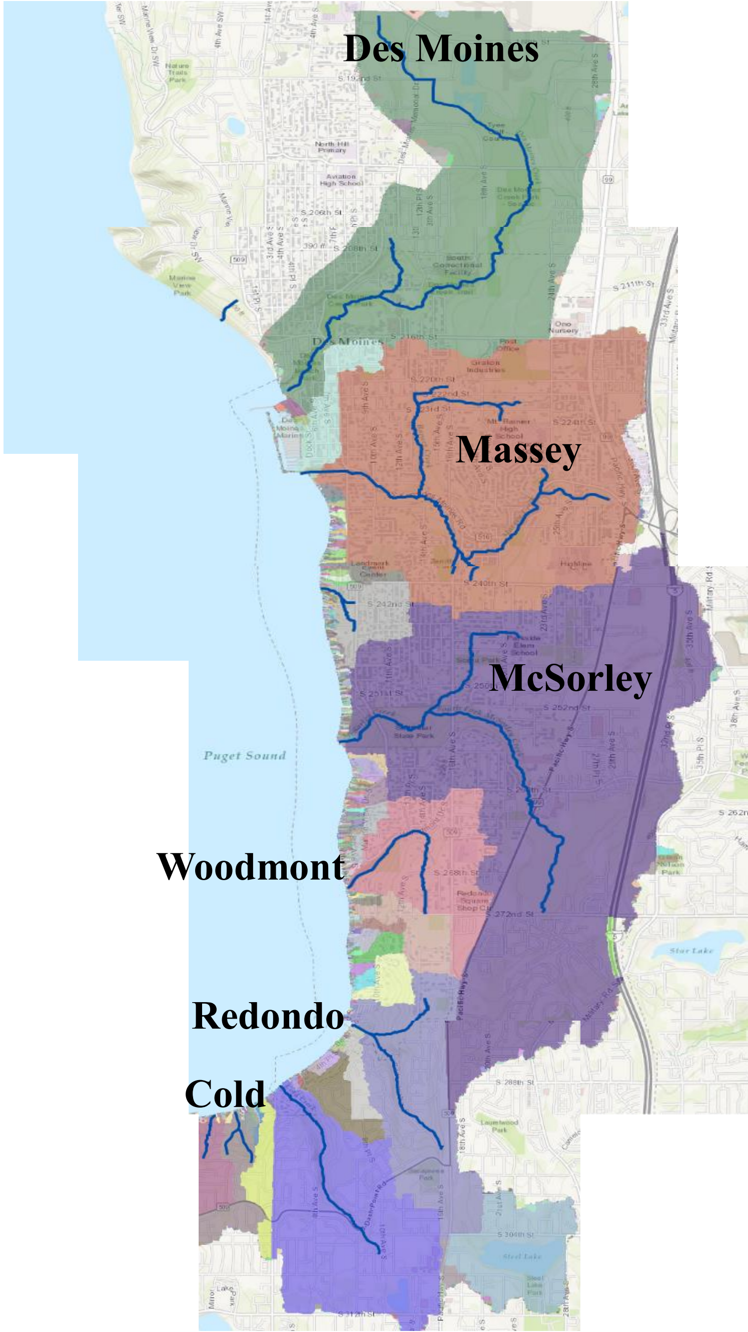
Appendix A:
Field Sampling Conditions at Seattle Recreational Water Sites

Site	Collection Date and Time	Air Temp (°F)	Notes
Matthews	6.21.16; 10:15 am	61	No swimmers, but lots of geese swimming
	7.5.16; 10:40 am	59	No swimmers, no duck/geese flocks
	8.11.16; 1:00 pm	71	~35-50 swimmers
	8.23.16; 12:15 pm	75-80	~35-50 swimmers; mostly kids
	9.9.16; 12:30 pm	64	3-4 swimmers; lots of seagulls/crows/geese
	10.12.16; 3:30 pm	54	Pouring and miserable.
Magnuson	6.21.16; 11:25 am	64	No swimmers; bathrooms 50 m uphill from beach.
	7.5.16; 12:38 pm	60	Dog park; ~15 dogs over course of BMFS
	8.16.16; 10:15 am	61	No bathers; some ducks
	8.24.16; 11:00 am	68-73	Dog park; ~10 dogs in water; 3-5 people as well
	9.9.16; 11:20 am	64	Sunny; no birds or swimmers except 1 skinny dipper right before leaving
Greenlake	6.28.16; 1:50 pm	72	~20 swimmers, boats, geese
	7.12.16; 12:20 pm	69	Some boats, no swimmers; dock area
	8.9.16; 12:10 pm	63	5-6 swimmers
	8.16.16; 11:50 am	64	
	8.30.16; 11:00 am	66	Overcast; no swimmers; ~5 ducks
	10.12.16; 2:15 pm	60	Swimming area closed; just a couple ducks in lake.
Carkeek	6.28.16; 9:30 am	61	Mouth of Pipers Creek; low tide at 6:21 am; high at 12:15 pm
	8.9.16; 10:35 am	62	No one swimming
	8.22.16; 11:58 am	61	Tide out; lots of seagulls/crows/geese and seaweed
	8.30.16; 12:20 pm	67	Tide out; lots of gulls/crows/geese; overcast/windy
	9.20.16; 11:00 am	59	Tons of seagulls at creek mouth
	10.26.16; 1:57 pm	54	Tide is in; up to creek. Pouring and miserable.
Golden Gardens	6..28.16; 12:00 pm	65 – 70	Lots of people out, 5 swimmers, boats, some geese
	7.12.16; 10:20 am	66	Not many people, time coming in
	8.11.16; 11:10 am	64	Some kids in water; primarily sailboats farther out
	8.22.16; 1:50 pm	68	Some boats, lots of seaweed, few swimmers
	9.20.16; 12:15 pm	61	No one in water, not even birds. Small creek emerges from sand and drains to sample area (first time we have noticed it).
	10.12.16; 3:30 pm	58	Creek mouth; nice afternoon, but no one in the water.
<u>Other</u>			
McSorley Creek	7.19.16; 10:40 am	68	Sample taken at creek mouth; no bathers but crows with diarrhea hanging out. Creek so shallow we used 1 L bottle to fill bag.
Redondo Creek	7.19.16; 11:50 am	70	Tide out. One swimmer, but doesn't look like people typically swim here. South of MAST.
	9.28.16; 12:55 pm	64	Tide coming in.
Des Moines Creek	8.3.16; 11:20 am	66	Low tide. Restroom nearby, some ducks and crows, sample taken near foot bridge.
Cold Creek	9.28.16; 1:45 pm	64	
Woodmont Creek	8.3.16; 12:50 pm	72	Creek low in volume. Filled bag with 1 L grabs. No swimmers, probably because this is a private beach.

Appendix B:
Poverty Bay Watersheds



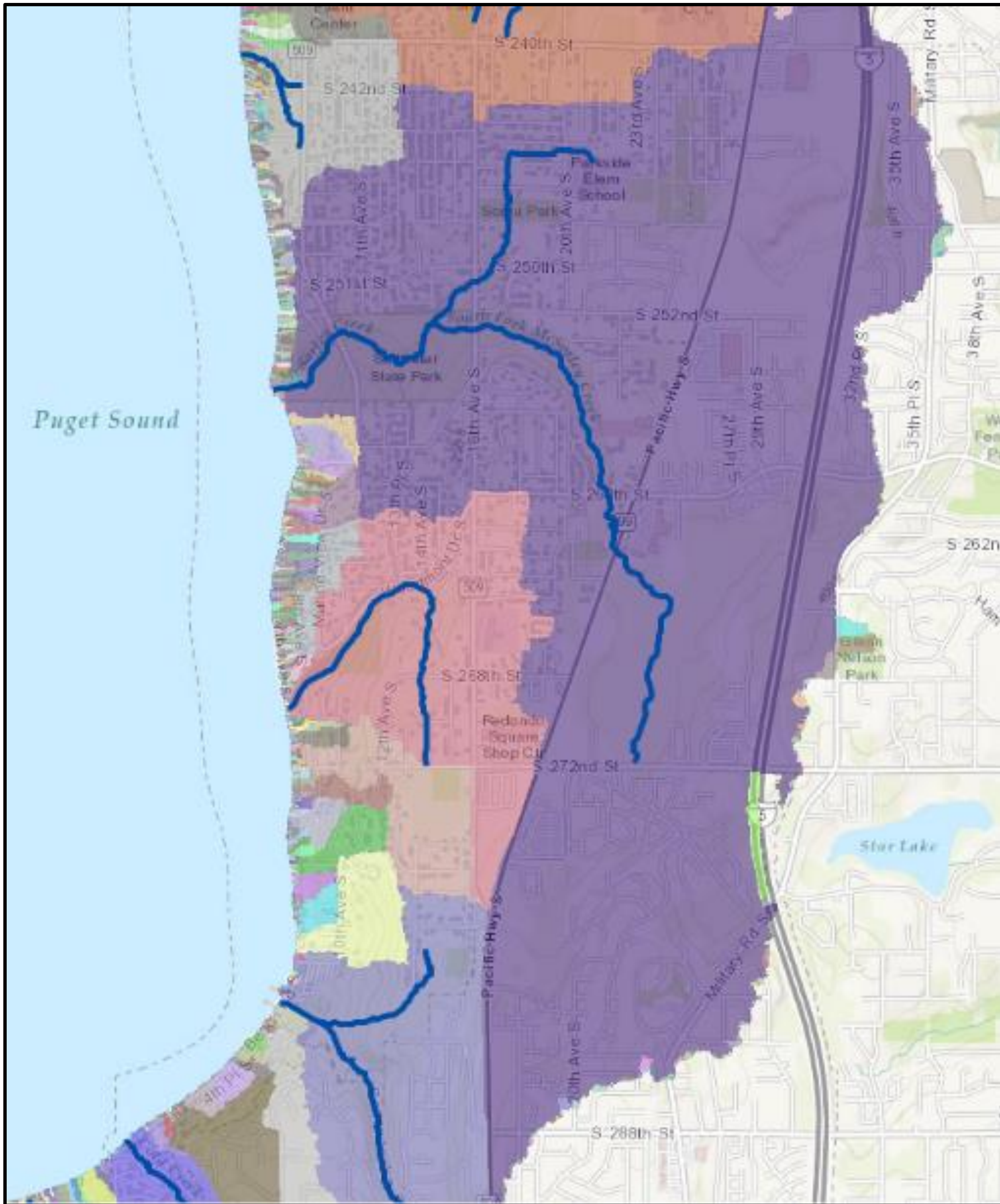
Des Moines Creek

Massey Creek

flow estimate: 2.7 cfs (Georgeson 2014)

McSorley Creek

flow estimates: 3.40 cfs (Schultz 2008); 6.7 cfs (Georgeson 2014)



Woodmont Creek

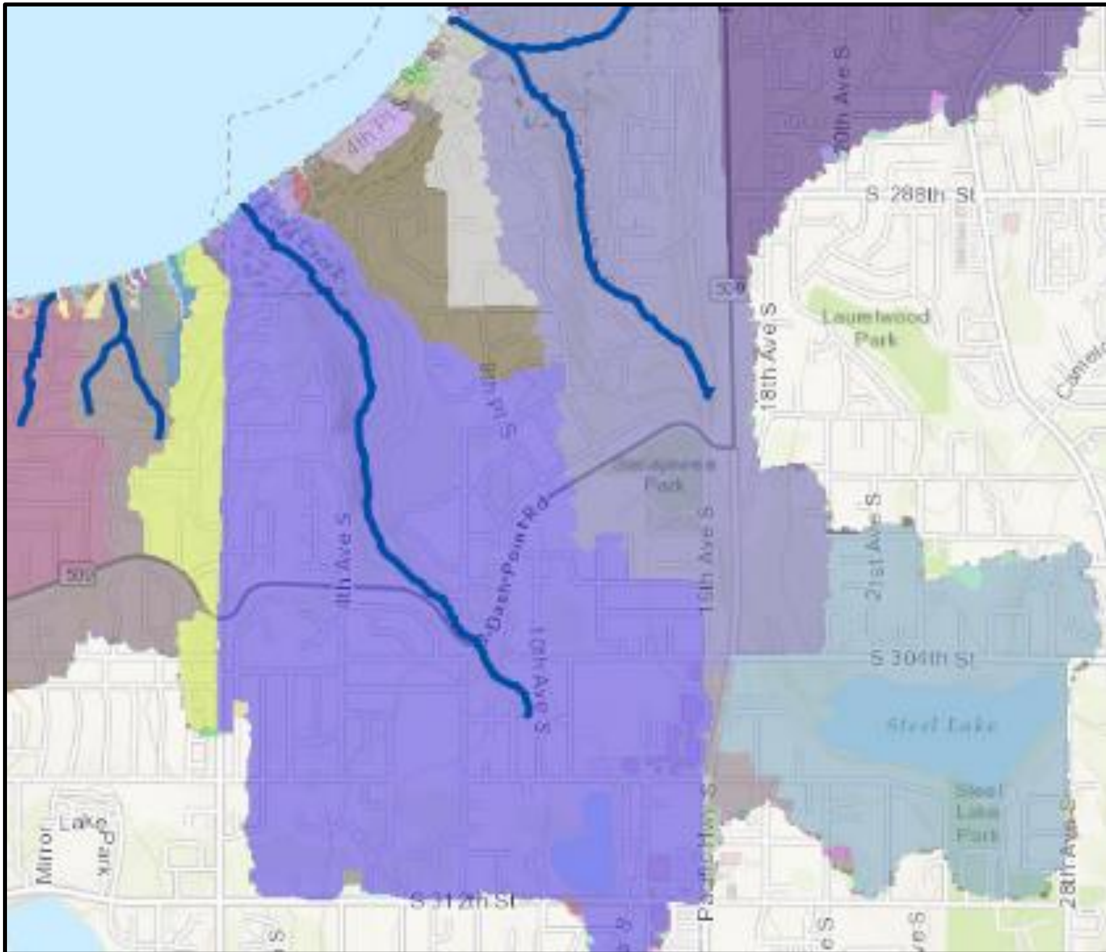
flow estimates: 0.78 cfs (Jaffer, August 2016); 0.48 cfs (Schultz 2008)

Redondo Creek

flow estimates: 1.08 (Jaffer, August 2016); 1.54 (Schultz 2008)

Cold Creek

flow estimates: 2.25 cfs (Jaffer, August 2016); 0.95 cfs (Schultz 2008)



Appendix C:
Poverty Bay Sampling Sites

Note: these sites are easiest to spot on the King County/Poverty Bay WQ sampling map through the Collector App. It displays streams and streets more accurately than Google Maps.

Des Moines Creek

- **DMC_01** (47.40371752, -122.32988801)
  Mouth of Des Moines Creek. Located north of the Des Moines Marina, in the Des Moines Park Conservation Area. We take the sample a bit downstream from the footbridge, unless the tide is in.

Massey Creek

- **MAS_01** (47.39675145, -122.32700397)
  Mouth of Massey Creek. Accessible through entrance to Des Moines Yacht Club via S 277th St. Must call Margret prior to sampling (1 day in advance or day of) because she has to open the gate. We take the sample downstream from yellow house. Margret can be reached at (206) 878-4441 or (206) 878-7220.

- **MAS_02** (47.39513441, -122.31602350)
  This one is actually Barnes Creek, right before it joins Massey Creek. Must stop on S. Kent Des Moines Road (going west) to take sample. Walk down slope to find creek north of road.

- **MAS_03** (47.39592629, -122.32136948)
  Located on S 230th Street. Take sample on east side of bridge over creek. Should be able to get it with the short pole. A little deceptive because MAS_03 is actually downstream from MAS_02.

- **MAS_04** (47.38965218, -122.31182140)
  16th Ave S, between 16th Pl S and 16th Ln S. Take sample on east side of bridge. Tricky because you'll have to walk down slope and potentially climb the chain-link fence depending how much water is in the stream. Long 48 foot pole required for this sample.

- **MAS_05** (47.395973, -122.321332)
  Established in May 2017 for further source delineation. Easiest to park at the Yardarm Pub and walk north on Marine View Dr S toward the creek. Take sample from east side of road. Long pole is helpful here.

- **MAS_06** (47.390453, -122.308071)
  Established in May 2017 for further source delineation. Turn right on 20th Ave S when going east on S Kent Des Moines Rd. Creek crosses under the road. MAS_06 is taken on west side of road.

- **MAS_07** (47.390279, -122.307728)
  Same general location as MAS_06 (off 20th Ave S). We take this sample on the east side of 20th Ave S; runoff from the side of the road that flows into the creek. I'm unsure of whether this flows year-round.

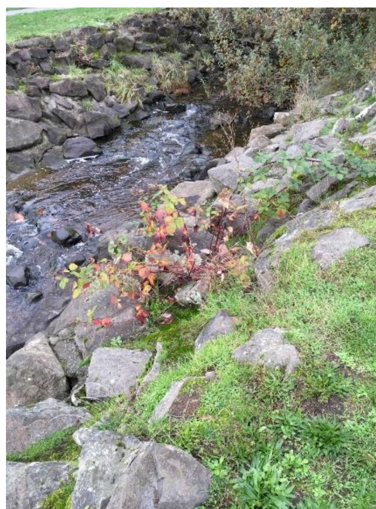
- **MAS_10** (47.392022, -122.30584)
  Makes sense to take MAS_10 after MAS_06/MAS_07. Drive up S Kent Des Moines Rd toward I5. Turn right on S 234th St. Creek is adjacent to S Kent Des Moines Rd. Take sample above Graceview Park.

- **MAS_08** (47.395218, -122.303737)
  Keep driving up S Kent Des Moines Rd. MAS_08 is taken from the City Park located off 22nd Pl S. Walk around fence and climb down to sample location. This one is not particularly accessible, and the long pole is helpful.

- **MAS_09** (47.394753, -122.303565)
  Same general location as MAS_08. Cross S Kent Des Moines Rd. Take sample from area behind Pinebrook Terrace Condos. Long pole is helpful, especially when flow is low.

McSorley Creek

- **McS_01** (47.37282945, -122.323471772)
  Mouth of McSorley Creek. Accessible via Saltwater State Park.



- **McS_02** (47.37552029, -122.31577129)
  North branch of McSorley Creek. Must drive or walk into campground to access this site. We take this sample from the footbridge over the creek.

- **McS_03** (47.37542700, -122.31513410)
  South branch of McSorley Creek. Must drive or walk into campground to access this site. This site is adjacent to a campsite.

- **McS_04** (47.375295, -122.313603)
  Farther up on south branch of McSorley Creek, at end of trail, almost to overpass bridge.

- **McS_05** (47.37714554, -122.32059759)
  South 251st St. This is an outfall that drains into north fork of the creek. Must climb into ravine to sample from outfall that runs under road.

- **McS_06** (47.37768073, -122.31164689)
  Another outfall adjacent to road (S 250th St.). Don't need to walk very far down the slope for this one, as the outfall is fairly high up.

- **McS_07** (47.37762080, -122.31207672)
  Same location as McS_06, but sample is taken from creek (downstream from the outfall that discharges adjacent to S 250th St.)

- **McS_08** (47.38089971, -122.31129359)
  Creek passes under S 246th Pl. Take sample from S side of road. This is just upstream from Sonju Park. Easiest to access creek from adjacent driveway – I talked to the resident, and she is ok with this. She said a family of ducks lives in this section of the creek.

- **McS_09** (47.38235565, -122.30783111)
  Sample taken from stormwater drain on 20th Ave S between S 244th Pl and S 245th Pl. The grate can be removed fairly easily. Throughout February and March, there has been a steady flow in this drain.

- **McS_10** (47.37667921, -122.31434167)
  Taken at pump station near 16th and 50th. Must contact Jace Layton (jace@midwaysewer.org) a couple days before taking sample, and they will unlock the station/gates to the creek. This sample should be taken downstream from the pump station.

- **McS_11** (47.37696940, -122.31339712)
  Also taken at pump station. This sample should be taken upstream from the pump station. (There are 2 gates that can be unlocked to access the creek.)

- **McS_12** (47.37954574, -122.31121162)
  Sample taken from Larry Grohs's property (1800 S 250th St.). Must call Larry the day prior to accessing McS_12 and McS_13 (206-372-9955). This sample is taken from the north end of his land, where the north fork of McSorley Creek runs under a footbridge.

- **McS_13** (47.37847487, -122.31080166)
  This site is also on Larry Grohs's property. Sample taken from private pond.

Woodmont Creek

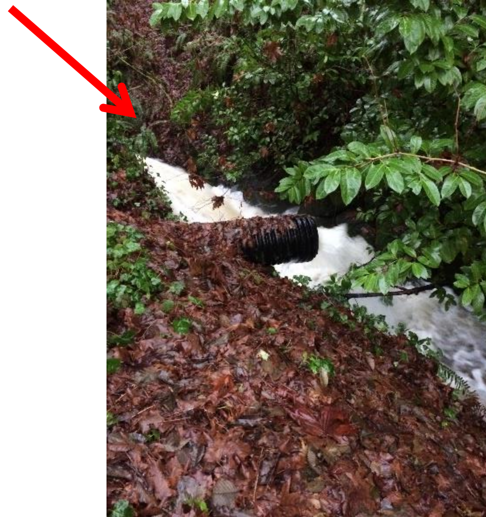
- **WOOD_01** (47.36011008, -122.32414934)
  Mouth of Woodmont Creek, must enter through Woodmont Country Club gate. (I've never seen it closed.) At the end of Woodmont Beach Road S. Rorie Zajac is our contact at the club (roriez@gmail.com), but no need to contact each time a sample is taken there. However, they would like to receive updates on the project.

- **WOOD_02** (47.36166861, -122.32199519) **NO LONGER SAMPLED – WAS DIVIDED INTO WOOD_06, WOOD_07, and WOOD_09 FOR SOURCE TRACKING PURPOSES.**
  Woodmont Beach Road S – samples were taken upstream of tan house (east of driveway), below large stormwater conveyances until Feb 2017. However, there is reason to believe that stormwater discharge from upland houses is influencing this sample spot. As a result, I added WOOD_06, WOOD_07, and WOOD_09 (see descriptions below).

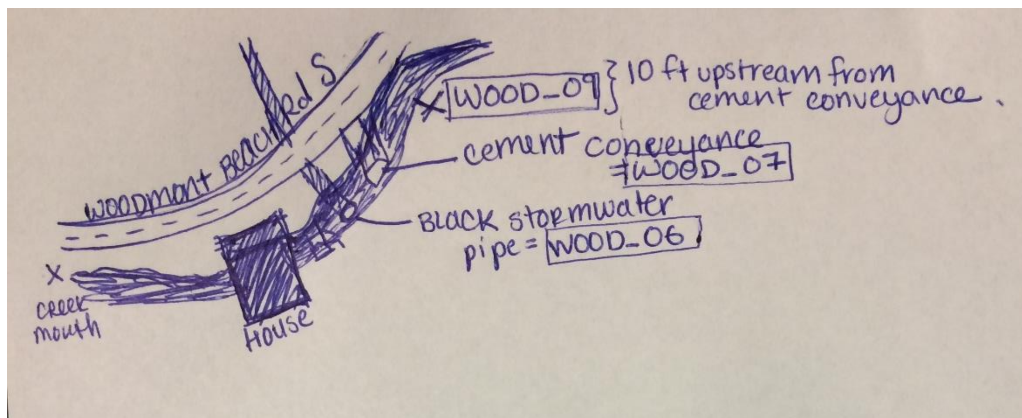
- **WOOD_06** (47.36170253, -122.32161988)
  Approx. same location as WOOD_02. However, this sample is to be taken directly from the BLACK stormwater conveyance that runs from upland houses N of Woodmont Beach Road. Note that water only comes out of this pipe during wet weather events.

- **WOOD_07** (47.36171524, -122.32159977)
  Approx. same location as WOOD_02. This sample is to be taken directly from the CEMENT stormwater conveyance (almost directly adjacent to WOOD_06). Note that water only comes out of this pipe during wet weather events.

- **WOOD_09** (47.36172796, -122.32154612)
  I created this sample to replace WOOD_02, which was taken below the two large stormwater outfalls in this location. WOOD_09 samples should be taken above the black and cement stormwater outfalls (~10 ft upstream). Accessing this sample involves climbing down the bank and a little ways up the stream.



WOOD_06, WOOD_07, WOOD_09



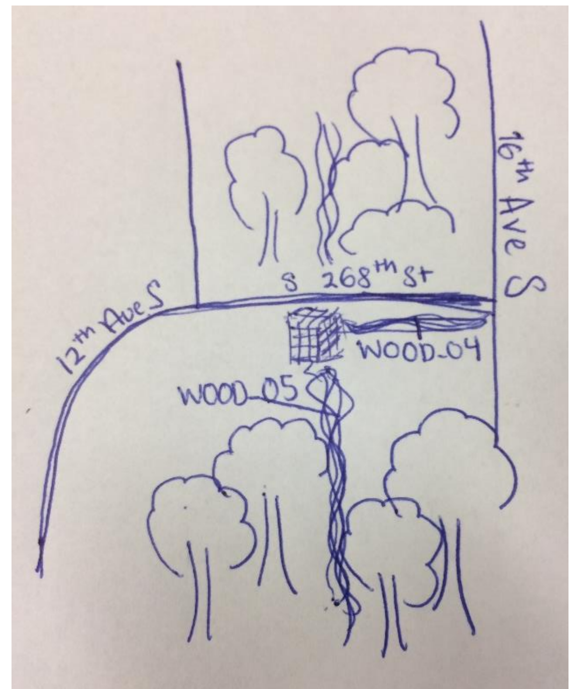
- **WOOD_03** (47.36502187, -122.31762411)
  Woodmont Drive S, just downstream from 13th Pl S. Sample taken from stream before it goes under road. Depending on how much water is in the stream, you might have to walk into house driveway and down steps to stream (The home owner gave us permission to do this._ Alternatively, you can use a 48-foot sampling pole for this spot and reach it from Woodmont Dr S. We have been doing this lately in order to avoid climbing around in this home's yard.

- **WOOD_08 (**47.36515833, -122.31607538)
  Uphill stormwater runoff into creek. Taken from S side of road, corner of Woodmont Dr S and S 263rd Pl. ***No picture available. There is not always water present in this spot – I first noticed it in January 2017, following a wet weather event.

- **WOOD_04** (47.36148798, -122.31615359)
  S 268th St, between 16th Ave S and 12th Ave S. Sometimes dry. Water flowing west from 16th Ave S. Take sample just upstream of culvert.

- **WOOD_05** (47.36140546, -122.31633761)
  Same location as WOOD_04, but this branch of creek flows north and originates in the wooded area south of S 268th St. I've only seen it flowing during the winter. Take sample upstream of culvert.

WOOD_04 and WOOD_05



<u>**Redondo Creek**</u>

- **RED_01** (47.34862616, -122.32437461)
  Mouth of Redondo Creek. Closed conveyance onto Redondo Beach. Located between Salty's and the boardwalk restroom. Take sample from pipe. **Check tides before sampling. This one is completely submerged when the tide comes in.

- **RED_02** (47.34809801, -122.32167961)
  Taken from north branch of Redondo Creek. Located across Redondo Way S from Redondo Heights Condos. Take sample above the grate pictured below.

- **RED_03** (47.34557452, -122.31953041)
  Up Redondo Way S, taken from main branch of creek. First daylight portion upstream.

- **RED_04** (47.34328458, -122.31898986)
  Taken right by Federal Way sign. Taken downstream from stormwater outfall and wood structure.

Cold Creek

- **COLD_01** (47.34292391, -122.33355593)
  Mouth of Cold Creek. Taken downstream from Redondo Beach Drive S, across from Redondo Shores community.

- **COLD_02** (47.34175895, -122.33195038)
  Taken from east end of Redondo Shores community. You can access the creek at east end of the loop.

- **COLD_03** (47.34035649, -122.32989128)
  Redondo Treatment Plant. Access via Sound View Drive S. Hit buzzer, and they'll open the gate. Our contact at the Redondo Treatment Plant is Brian Richardson. Don't have to contact him prior to sampling, but good to know if anyone asks. Sample taken from behind the plant. Walk to east end of treatment plant, then down to creek.

Appendix D:
Poverty Bay E. coli Data

Table i. Data summary for the mouth of Des Moines Creek.

First Flush					
9/2/2016 (0.39 in)	9/6/2016 (0.43 in)	9/8/2016 (0.49 in)	9/19/2016 (0.22 in)		**Geometric Mean**
2750	2000	310	310		**852.65**
Dry					
10/6/2016 (0.48 in)	11/21/2016 (0.07 in)	12/13/2016 (0.18 in)	3/30/2017 (0.65 in)	5/16/2017	**Geometric Mean**
160	520	340	260	100	**236.22**
Wet					
10/14/2016 (3.12 in)	10/20/2016 (1.49 in)	1/9/2017 (1.09 in)	1/18/2017 (3.89 in)	2/11/2017 (1.65 in)	**Geometric Mean**
870	1380	180	260	80	**339.27**

Table ii. Data summary for Massey Creek.

First Flush								
	9/19/2016 (0.22 in)							**Geometric Mean**
MAS_01	1910							**1910.00**
MAS_02	450							**450.00**
MAS_03	2400							**2400.00**
MAS_04	4560							**4560.00**
Dry								
	10/6/2016 (0.48 in)	11/21/2016 (0.07 in)	12/13/2016 (0.18 in)	3/30/2017 (0.65 in)	4/28/2017 (0.23 in)	5/12/2017	5/16/17	**Geometric Mean**
MAS_01	160	40	40	20			720	**81.91**
MAS_02	520	35	80	20	140	320	460	**129.17**
MAS_03	200	1	40	20	100	420	440	**60.47**
MAS_04		100	1000	20	280	640	640	**247.41**
MAS_05						440	500	**469.04**
MAS_06						480	100	**219.09**
MAS_07						1280	160	**452.55**
MAS_08						320	80	**160.00**
MAS_09						240	80	**138.56**
MAS_10						300	80	**154.92**
Wet								
	10/20/2016 (1.49 in)	1/9/2017 (1.09 in)	1/18/2017 (3.89 in)	2/11/2017 (1.65 in)				**Geometric Mean**
MAS_01	3400	80	420	140				**355.62**
MAS_02	2700	300	700	60				**429.47**
MAS_03	3080	420	680	20				**364.20**
MAS_04		100	440					**209.76**

Table iii. Data Summary for McSorley Creek

First Flush									
	9/2/2016 (0.39 in)	9/6/2016 (0.43 in)	9/8/2016 (0.49 in)	9/19/2016 (0.22 in)					**Geometric Mean**
McS_01	577.5	6000	160	2920					**1127.98**
Dry									
	10/6/2016 (0.48 in)	11/21/2016 (0.07 in)	12/13/2016 (0.18 in)	3/6/2017 (0.04 in)	3/30/2017 (0.65 in)	4/28/2017 (0.23 in)	5/12/2017	5/16/2017	**Geometric Mean**
McS_01	40	20	8		1	1	120	100	**13.38**
McS_02	140	1	72	20	1	1	280	180	**17.82**
McS_03	80	1	8	60	40	40	120	40	**27.15**
McS_04						1	40	20	**9.28**
McS_05				1	1	60	600	340	**26.16**
McS_06				1	1	1	1000	580	**14.21**
McS_07				1	20	1	2900	280	**27.68**
McS_08				20	1	100	860	480	**60.72**
McS_09				20	1	1	660	280	**20.58**
McS_10					80	1	180		**24.33**
McS_11					1	1	300		**6.69**
McS_12					20				**20.00**
McS_13					60				**60.00**
Wet									
	10/14/2016 (3.12 in)	10/20/2016 (1.49 in)	1/9/2017 (1.09 in)	1/18/2017 (3.89 in)	2/11/2017 (1.65 in)				**Geometric Mean**
McS_01	2050	3000	80	240	60				**371.59**
McS_02		3080	180	420	300				**514.10**
McS_03		1620	20	400	60				**166.99**
McS_04					20				**20.00**

Table iv. Data summary for Woodmont Creek.

					First Flush				
	9/2/2016 (0.39 in)	9/6/2016 (0.43 in)	9/8/2016 (0.49 in)	9/19/2016 (0.22 in)					**Geometric Mean**
WOOD_01	143	480	260	1680					**416.12**
WOOD_02				1380					**1380.00**
WOOD_03				1990					**1990.00**
WOOD_04				1700					**1700.00**
					Dry				
	10/6/2016 (0.48 in)	11/21/2016 (0.07 in)	12/13/2016 (0.18 in)	3/6/2017 (0.04 in)	3/30/17 (0.65 in)	4/28/17 (0.23 in)	5/12/17	5/16/17	**Geometric Mean**
WOOD_01	240	1	102		40		480	240	**69.51**
WOOD_02	260	30	115						**96.44**
WOOD_03	360	40	2		20				**27.55**
WOOD_04		1	6	1	1				**1.57**
WOOD_05				180	20				**60.00**
WOOD_06					1				**1.00**
WOOD_07					320				**320.00**
WOOD_08					20				**20.00**
WOOD_09					500				**500.00**
					Wet				
	10/14/2016 (3.12 in)	10/20/2016 (1.49 in)	1/9/2017 (1.09 in)	1/18/2017 (3.89 in)	2/11/2017 (1.65 in)				**Geometric Mean**
WOOD_01	2430	4200	70	400					**731.14**
WOOD_02		4060	80	553					**564.22**
WOOD_03		5120	120	460	1				**129.66**
WOOD_04		2400	1	60	1				**19.48**
WOOD_05					180				**180.00**
WOOD_06					1				**1.00**
WOOD_07					1				**1.00**
WOOD_08					60				**60.00**
WOOD_09					40				**40.00**

Table v. Data summary for Redondo Creek.

First Flush								
	9/2/16 (0.39 in)	9/6/16 (0.43 in)	9/8/16 (0.49 in)	9/19/2016 (0.22 in)				**Geometric Mean**
RED_01	532.5	485	1	370				**98.87**
RED_02				120				**120.00**
RED_03				220				**220.00**
RED_04				140				**140.00**
Dry								
	10/6/16 (0.48 in)	11/21/16 (0.07 in)	12/13/16 (0.18 in)	3/30/2017 (0.65 in)	4/28/17 (0.23 in)	5/12/17	5/16/17	**Geometric Mean**
RED_01	40	40		1	40	480	200	**42.80**
RED_02	100	20	10	1	1			**7.25**
RED_03	1	1	20	1	60			**4.13**
RED_04	1	1	20	1	80			**4.37**
Wet								
	10/14/16 (3.12 in)	10/20/16 (1.49 in)	1/9/17 (1.09 in)	1/18/2017 (3.89 in)	2/11/17 (1.65 in)			**Geometric Mean**
RED_01	2530	660		60	1			**100.05**
RED_02		320	1	160	1			**15.04**
RED_03		840	1	80	1			**16.10**
RED_04		980	1	20	20			**25.02**

Table vi. Data summary for Cold Creek.

First Flush								
	9/2/16 (0.39 in)	9/6/16 (0.43 in)	9/8/16 (0.49 in)	9/19/16 (0.22 in)				**Geometric Mean**
COLD_01	5750	90	10	30				**111.62**
COLD_02				10.5				**10.50**
COLD_03				30				**30.00**
Dry								
	10/6/16 (0.48 in)	11/21/16 (0.07 in)	12/13/16 (0.18 in)	3/30/17 (0.65 in)	4/28/17 (0.23 in)	5/12/17	5/16/17	**Geometric Mean**
COLD_01	1	20	1	1	1	180	60	**5.78**
COLD_02	20	10	1	1	20			**5.25**
COLD_03	1	1	1	1	1			**1.00**
Wet								
	10/14/16 (3.12 in)	10/20/16 (1.49 in)	1/9/17 (1.09 in)	1/18/17 (3.89 in)	2/11/17 (1.65 in)			**Geometric Mean**
COLD_01	3410	520	20	160	1			**89.29**
COLD_02		620	1	220	1			**19.22**
COLD_03		540	20	140				**114.78**