Pollution, Identification and Correction Program

Photo credit: Karleigh Gomez; Hans Daubenberger extracting water sample at Duckabush River flow wire below Collins Campground.

Final Report

2017

CFDA # 66.123 Puget Sound Action Agenda: Technical Investigations and Implementation Assistance Program DOH Contract N20718WDOH and Ecology Pathogens Prevention, Reduction, and Control PIC Programs Port Gamble Bay/Hood Canal PIC Program

Acknowledgements

This Pollution Identification and Correction (PIC) Program document was developed by staff of the Port Gamble S'Klallam Tribe (PGST).

PGST PIC Program was made possible through funding from the Washington State Department of Health (WDOH) and the United States Environmental Protection Agency (USEPA) National Estuary Program (NEP), with significant coordination with Washington State Department of Ecology (Ecology).

Photo Credit: Devon Hayes, PGST reservation shellfish opening during sampling event on June 19, 2015

Contents

Photo Credit: Courtney Ewing, Aurora Robles digging clams for the Port Gamble S'Klallam Early Childhood Program Yearly Celebration Clam Bake.

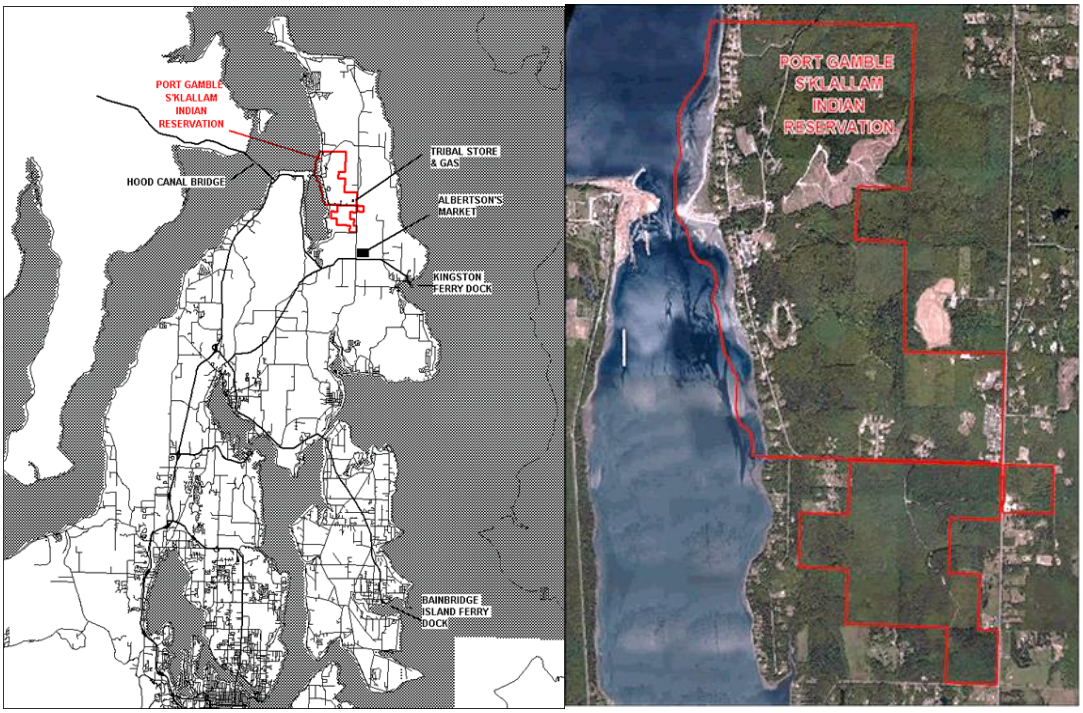
Acronyms and Abbreviations

autosampler	Hach Sigma SD 900 Portable Sampler
cfu	colony forming units
CDX	Central Data Exchange
EC	*Escherichia coli* bacteria
E. coli	*Escherichia coli* bacteria
Ecology	Washington State Department of Ecology
FC	fecal coliform bacteria
FEATS	Financial and Ecosystem Accounting Tracking System
GIS	Geographic Information System software
GMV	geometric mean value
GPS	Global Positioning System
HCCC	Hood Canal Coordinating Council
HCRPIC	Hood Canal Regional Pollution Identification and Control Program
KPHD	Kitsap Public Health District
MCPH	Mason County Public Health
NPDES	National Pollution Discharge Elimination System (permitting program)
NTR	National Toxics Rule
OSS	onsite sewage system
PGST	Port Gamble S'Klallam Tribe
PIC	Pollution Identification and Correction
PSAMP	Puget Sound Assessment and Monitoring Program
QA	Quality assurance
QAPP	Quality Assurance Project Plan
QC	Quality control
RPD	Relative percent difference
RSD	Relative standard deviation
SOP	Standard Operating Procedure
STORET	USEPA Storage and Retrieval database
TMDL	total maximum daily value
TOD	time of day
Tribe	Port Gamble S'Klallam Tribe
USEPA	U.S. Environmental Protection Agency
WAC	Washington Administrative Code
WDFW	Washington Department of Fish and Wildlife
WDOH	Washington Department of Health
WRIA	Water Resources Inventory Area
WSTMP	Washington State Toxics Monitoring Program
YSI	Yellow Springs Instruments

1 Introduction

The Port Gamble S'Klallam Tribe's reservation is home to more than 1,200 tribal members. Located near the northern end of the Kitsap Peninsula (Figure 1), the reservation lands rise from Admiralty Inlet and Port Gamble Bay. The reservation is mostly forested, contains approximately 2.5 miles of marine shoreline, and receives approximately 20 inches of rain per year. Port Gamble Bay is one of the largest and most productive marine areas open for commercial and recreational shellfish harvest in Kitsap County (see WA DOH shellfish harvest area classification map https://fortress.wa.gov/doh/eh/maps/biotoxin/biotoxin.html).

Figure 1. Overview maps of the Kitsap Peninsula and the PGST Reservation



Shellfish and other aquatic organisms along streams and shorelines within the PGST Usual and Accustomed Areas (U&A, Appendix A) have been negatively affected by nutrient and fecal pollution from failing onsite sewage systems (OSS) and other sources. Closure of shellfish beds due to fecal pollution, in particular, has prompted PGST and local governments to develop and implement marine and freshwater monitoring programs.

Pollution Identification and Control (PIC) programs in the Hood Canal region monitor marine and fresh water bodies, mainly for fecal coliform (FC) and/or *Escherichia coli* (EC) bacteria. Some of these programs also measure nutrient concentrations and ancillary environmental parameters such as temperature, salinity, specific conductance, pH, turbidity, and dissolved oxygen.

PGST is a member of the Hood Canal Coordinating Council (HCCC), whose mission is to protect and enhance the environmental and economic health of the Hood Canal and to support the Puget Sound

Action Agenda (PSP, 2014). The HCCC more recently developed the Hood Canal Regional Pollution, Identification and Control Program (HCRPIC) to monitor water quality. PGST serves on the HCRPIC Pilot Guidance Group to provide oversight, guidance and structure for consistent procedures and technical assistance for the HCRPIC program (Banigan, 2015).

Photo Credit: Katy Davis, Hans Daubenberger taking water sample at the marine sample stations outside the Duckabush River Estuary.

PGST's main role in the HCRPIC program is to research data gaps identified at HCRPIC meetings and during discussions of data submitted by its members. To do this, the PGST developed its own Pollution, Identification, and Correction program. This report describes PIC-related activities that have occurred at freshwater sites, both upland and near marine shorelines, within the reservation and throughout the Hood Canal including Water Resource Inventory Areas (WRIAs) 14-17. The study area and its surroundings are U&A harvest areas of the Port Gamble S'Klallam Tribe protected by the Point-No-Point Treaty of 1855.

Recent HCCC meetings and discussions about data shared by its members, identified issues that needed to be addressed by literature reviews or conducting "gap" analyses related to identifying sources of *E. coli* or fecal coliform bacteria.

This report and appendices describe PGST's PIC program development including wet and dry season sampling, literature reviews, a microbial source tracking (MST) study, and a temporal sampling investigation.

2 Regulatory Criteria Standards

Non-tribal lands

Washington State delegates the responsibility for identifying and correcting nonpoint pollution to local governments (KPHD, 2014a/b). The regulatory authorities in the Hood Canal region include Jefferson County Public Health (JCPH), Kitsap Public Health District (KPHD), and Mason County Public Health and Human Services (MCPH). These jurisdictions are given flexibility to implement water quality protection programs, e.g., PIC programs, using different tools. For example, these regulatory authorities may choose to measure EC, FC and/or nutrients as indicators of fecal pollution. FC is still a common analysis, but federal guidance suggests EC (a species of FC bacteria specific to humans and other warm-blooded animals) and enterococci are better indicators of health risk from water contact (USEPA, 2012).

Staff from local governments compared PIC monitoring results to current Washington State water quality standards to determine appropriate response actions. HCRPIC guidance and regional agreements

state that Hood Canal drainages with FC exceeding 200 colony forming units (cfu) per 100 milliliters (mL) or EC exceeding 100 cfu/100 mL, must be sampled at least two additional times for confirmation. The Geometric Mean Value (GMV) of the three (or more) sample results is then calculated. If the GMV for FC exceeds 500 cfu/100mL or 320 cfu/100mL for EC, further investigation is required.

PGST Tribal Lands

The Port Gamble S'Klallam Tribe is responsible for identifying and correcting nonpoint pollution on tribal lands. To this end, PGST collects and analyzes water samples from the reservation for EC concentrations. Collection and analysis methods are comparable to those used by other Hood Canal Regional PIC programs (Banigan, 2015). PGST staff compared its EC results to Tribal Water Quality Standards adopted to afford stringent levels of protection within the reservation (PGST, 2002):

Photo Credit: Courtney Ewing, Therron Sullivan digs cockles at Point Julia for Port Gamble S'Klallam Early Childhood Program's Yearly Celebration Clam Bake.

Waters designated for recreational and cultural use shall not contain concentrations of EC bacteria exceeding a 30-day GMV of 126 cfu / 100 mL (based on a minimum of 5 samples).

Water designated for shellfish and crustacean spawning, rearing, and harvesting shall not contain FC levels exceeding a GMV of 14 cfu / 100 mL and no more than 10 percent of the samples used to calculate the GMV shall contain 43 cfu / 100 mL.

Project Administration and Management

PGST Natural Resources staff was primarily responsible for managing and implementing the PGST PIC program. PGST prepared and submitted necessary documentation for planning and reporting, submitted semi-annual reports to the USEPA Puget Sound Financial and Ecosystem Tracking System (FEATS), and facilitated data reporting to USEPA's STOrage and RETrieval (STORET) website through the tribe's network node.

PGST administrated the PIC Program on the reservation and worked closely with Kitsap County Public Health District (KPHD) to plan and conduct Shoreline Surveys in the wet and dry seasons, as well as respond to elevated bacteria levels. PGST coordinated contracts with Spectra Laboratories – Kitsap, LLC for sample analysis and an environmental contractor for assistance with sampling and other program needs.

PGST led planning for regional gap studies based on available pollution trend analyses. PGST worked with county staff to prioritize sample locations for the temporal investigation, MST study, and the optical brightener and tryptophan evaluation.

3 Project Descriptions

The Port Gamble S'Klallam Tribe (PGST) has traditionally harvested shellfish for commercial, subsistence, and ceremonial purposes within areas that are currently prohibited and unclassified for harvest by State and Federal programs. PGST developed coordinated strategies to improve local PIC programs' ability to effectively protect shellfish beds within the tribal U&A harvest areas in Port Gamble Bay and the northern Hood Canal region. The following actions are the result of this undertaking.

Shoreline Surveys

PGST PIC Program conducted wet and dry season shoreline surveys on the PGST reservation in 2015. The wet season survey was conducted on February 24 and the dry season survey on September 25. KPHD was a critical partner for the shoreline surveys and provided valuable support to PGST personnel and contractors throughout the planning, field work, laboratory coordination, sample results review. PGST reservation shoreline survey results filled a lingering data gap in water quality records and allowed KPHD to assess and account for all shorelines in Kitsap County.

The wet season shoreline survey results led to one hotspot confirmation. PGST coordinated with KPHD on response. KPHD and PGST staff led a home visit and dye test in April 2015. The results of this dye test were negative, meaning the source of pollution remained unconfirmed, and subsequent monitoring results showed that water quality improved. Dry season shoreline survey bacteria results yielded no hotspot confirmation and required no investigation (See Appendix B).

A second hotspot investigation was initiated in response to a sewer overflow on the PGST reservation sewer system. PGST determined the source of the spill to be near Bud Purser Lane and began sampling streams in the vicinity. PGST worked with KPHD to confirm the hotspot and began a dye testing strategy for the neighborhood. As part of this ongoing investigation, PGST deployed charcoal filters in the impacted stream to test background conditions. After this, PGST deployed new charcoal filters and conducted dye tests in the lowest elevation houses. After the first dye test period, new charcoal filters were deployed and dye tests conducted on a new set of houses at the next higher elevation in the neighborhood. PGST then continued weekly sampling and coordinated with US Health and Human Services (USHHS). PGST closed the associated beach to shellfish harvest to protect tribal members until water quality improves sufficiently. As of March 2017, correcting this pollution source on the reservation is an ongoing effort.

Photo Credit: Devon Hayes, Due to elevated levels of FC and EC detected in Bud Purser Lane stream this popular shellfish harvest location was forced to close.

MST Literature Review and Study

To date, water quality monitoring and management practices have relied heavily on fecal indicator bacteria (FIB), including *Escherichia coli* and *Enterococcus,* which have low pathogenic potential but abundant presence in sewage and feces. FIB are therefore suggestive of pathogen presence. However, conventional indicators cannot discern between human and animal sources because FIB are present in the feces of most mammals and birds. It is important to distinguish between human and animal derived fecal pollution because of the heightened health risks associated with human sewage and the different remediation strategies for mitigating contamination from sewage versus surface runoff carrying animal waste. PGST conducted a literature review of published methods which have been used to identify microbial sources (see Appendix C). This resulted in the development of a DNA-based microbial source tracking study utilizing PCR and high throughput sequencing. Results of the microbial source tracking study are expected to be available Spring 2017.

Temporal Investigation

PGST coordinated sample collection of EC over a 24-hour period, to test for temporal variation in sample results. PGST collected water samples using a Hach Sigma SD 900 Portable Sampler (autosampler) which collected one sample per hour during the 24-hour sampling periods. Variability in results between samples was enough to warrant a second test, to determine the range of variability between split and replicate samples. A second collection period was coordinated with personnel collecting samples by hand four times a day over 72 hours.

Temporal studies were conducted on the PGST Reservation, and in Jefferson County at Irondale Creek and the Duckabush River. A time of day study was planned at Lofall Creek in Kitsap County during the wet season of 2015-2016 however was eventually canceled after excessive rainfall. Results showed that EC levels did vary significantly with time of day, beyond the variability found between split and replicate samples.

PGST recommends that future projects looking to utilize an autosampler should consider a model which can be easily dismantled and autoclaved. Additionally, unless the autosampler is going to be deployed regularly at a set location with proper infrastructure to house the equipment, using personnel to collect samples by hand is likely the more practical approach. For detailed methods and results of the temporal investigation, see Appendix D.

Tryptophan and Optical Brighteners

PGST used a Turner Designs Cyclops 7 Submersible Fluorometer with tryptophan and optical brightener sensors to determine if *in situ* measurements of tryptophan and optical brighteners are a useful proxy for identifying EC hotspots. Results showed no correlation between optical brighteners and EC at the three temporal investigation sites where the fluorometer was deployed. At one of the three sites, there was a weak correlation between EC and tryptophan (See Appendix D).

Implementation Summary

Table 1: Number of samples analyzed at each survey site

Shellfish Growing Area & County	Site	PGST Shoreline Survey	24-Hour EC Sampling	72-Hour EC Sampling	DNA	Total EC and FC
Port Gamble Bay, Kitsap	PGST Reservation	70	17	99	1	120
Port Townsend, Jefferson	Irondale Creek	-	14	-	1	16
Hood Canal 2, Kitsap	Lofall Creek	-	-	-	2	14
Hood Canal 3, Jefferson	Dosewallips River	-	-	-	8	14
Hood Canal 3 Jefferson	Duckabush River	-	-	104	12	122
Totals		70	31	203	24	286

4 Education and Outreach

Attending Puget Sound PIC workshops and regional meetings was valuable to PGST Natural Resources personnel to understand the broad program opportunities and educational options available. PGST maintained records of meeting agendas and notes in the Tribe's project files. With the understanding gained from these networking opportunities, PGST was able to consider the best ways to reach its audience.

PGST identified two priority audiences, tribal members, and regional policy-makers. To engage with these audiences, PGST developed its own outreach materials. This encouraged PGST to increase its field documentation, associated training and photographs of relevant field activities in U&A areas with actual PGST tribal members and personnel. PGST prepared outreach materials for social media, such as the PGST website and Facebook page. The materials are PowerPoint slides that stand alone to introduce PGST PIC priorities. Additionally, PGST created two PowerPoint presentations that are tailored to the tribal members and policy-makers.

PGST and KPHD conducted outreach and education with property owners and onlookers during field sampling events. Natural Resources Department staff offered presentations to college and grade school students on the reservation. The newly strengthened relationship with KPHD provided excellent networking opportunities for engaging with local policy-makers.

5 References

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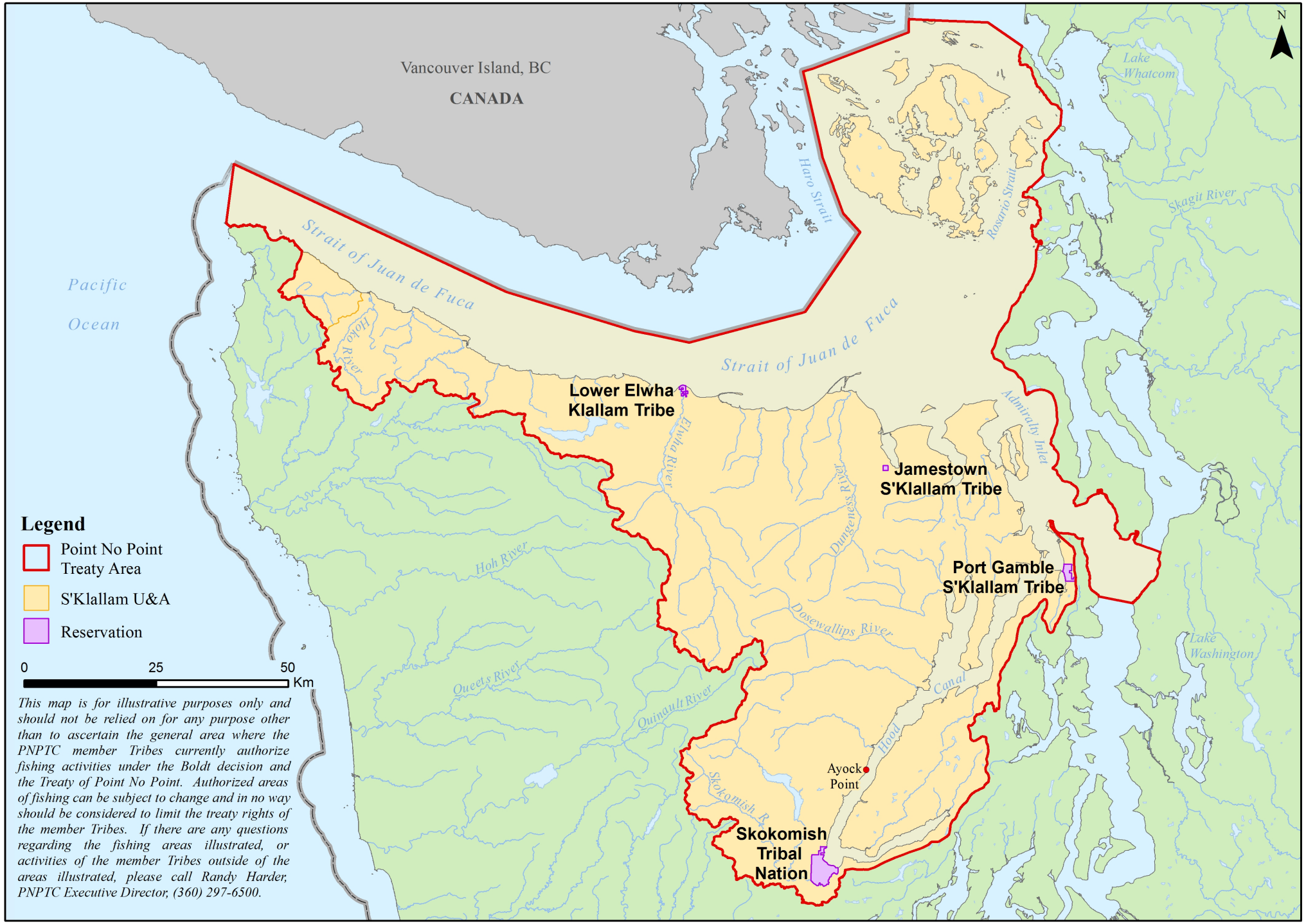
Available: http://water.epa.gov/type/rsl/monitoring/vms511.cfm.

Point No Point Treaty Area

PNPTC GIS



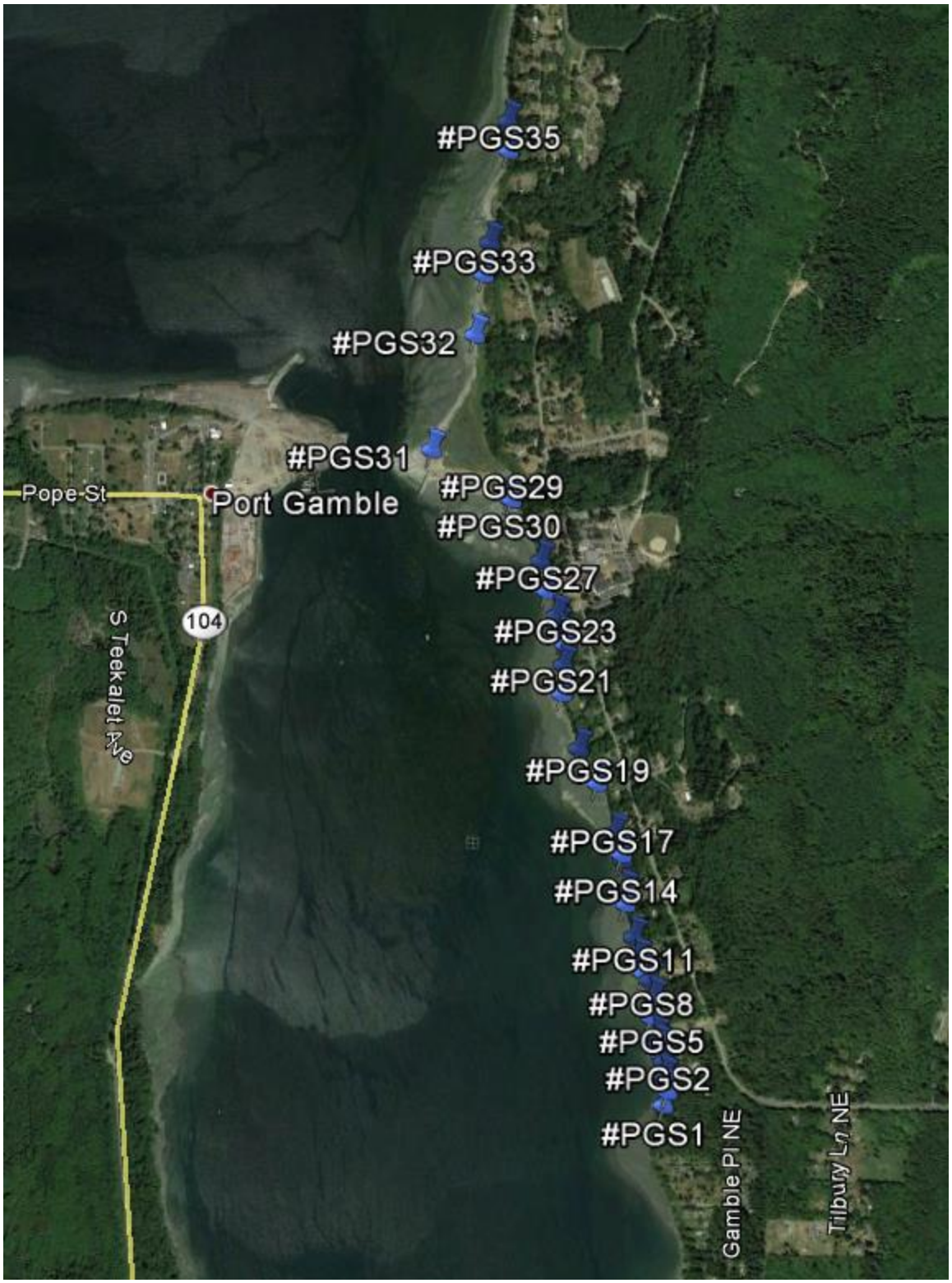
This map is for illustrative purposes only and should not be relied on for any purpose other than to ascertain the general area where the PNPTC member Tribes currently authorize fishing activities under the Boldt decision and the Treaty of Point No Point. Authorized areas of fishing can be subject to change and in no way should be considered to limit the treaty rights of the member Tribes. If there are any questions regarding the fishing areas illustrated, or activities of the member Tribes outside of the areas illustrated, please call Randy Harder, PNPTC Executive Director, (360) 297-6500.

Appendix B. Shorelines Surveys

Shoreline Monitoring Results and Rainfall Data

Duplicates Displayed (Use Ctrl-Shift-D to toggle)

Station	Date	Time	Rainfall totals			E.coli	Notes	Lat	Long
(units)			*inches*	*inches*	*inches*	*0ml*			
(Standard)			*24hr*	*48hr*	*72 hr*	*126.0*			
#PGS1	02/24/2015	11:56				1.0	Seep under clay bank, blackberries /	47.83886	-122.56671
#PGS1	09/25/2015	08:33				9.5	Seep under clay bank, blackberries /	47.83886	-122.56671
#PGS10	02/24/2015	12:46				11.0	Flow over alder roots off bank /	47.84202	-122.56722
#PGS10	02/24/2015	12:46				3.1	Flow over alder roots off bank /	47.84202	-122.56722
#PGS11	02/24/2015	12:51				1.0	Flow over clay bank, downed tree /	47.84221	-122.56745
#PGS11	09/25/2015	09:01				6.1	Flow over clay bank, downed tree /	47.84221	-122.56745
#PGS12	02/24/2015	12:56				64.4	Bank flow near tire /	47.84257	-122.56768
#PGS12	09/25/2015	09:09				12.0	Bank flow near tire /	47.84257	-122.56768
#PGS13	02/24/2015	13:05				4.1	Small stream, root ball. /	47.84310	-122.56783
#PGS13	09/25/2015	09:16				2.0	Small stream, root ball. /	47.84310	-122.56783
#PGS14	02/24/2015	13:14				0.5	Bank seep, alders above /	47.84395	-122.56814
#PGS15	02/24/2015	13:20				14.5	Flow over clay bank, algae /	47.84428	-122.56817
#PGS15	09/25/2015	09:30				31.3	Flow over clay bank, algae /	47.84428	-122.56817
#PGS16	02/24/2015	13:27				2.0	Composite (2)-Flow under alders & flow to west /	47.84506	-122.56823
#PGS16	09/25/2015	09:34				21.6	Composite (2)-Flow under alders & flow to west /	47.84506	-122.56823
#PGS17	02/24/2015	13:33				4.1	Flow down clay bank, cars above /	47.84520	-122.56827
#PGS17	09/25/2015	09:41				1.0	Flow down clay bank, cars above /	47.84520	-122.56827
#PGS18	02/24/2015	13:40				26.2	Flow under blackberries, black flex /	47.84572	-122.56856
#PGS19	02/24/2015	13:50				4.1	Small creek, downed trees /	47.84699	-122.56920
#PGS19	09/25/2015	09:56				10.8	Small creek, downed trees /	47.84699	-122.56920
#PGS19	09/25/2015	09:56				13.2	Small creek, downed trees /	47.84699	-122.56920
#PGS2	02/24/2015	12:03				2.0	Seep under clay bank, blue tarp above /	47.83921	-122.56658
#PGS20	02/24/2015	13:57				27.5	Small creek, stairs to west /	47.84787	-122.56989
#PGS21	02/24/2015	14:07				2.0	Small creek, flows east to west /	47.84926	-122.57054
#PGS21	09/25/2015	10:06				7.2	Small creek, flows east to west /	47.84926	-122.57054
#PGS22	02/24/2015	14:13				52.0	Flow under large, downed alder /	47.84998	-122.57047
#PGS22	09/25/2015	10:14				21.1	Flow under large, downed alder /	47.84998	-122.57047
#PGS23	02/24/2015	14:18				2.0	Seep from bank, alders /	47.85051	-122.57041
#PGS23	09/25/2015	10:21				3.0	Seep from bank, alders /	47.85051	-122.57041
#PGS24	02/24/2015	14:22				461.1	Bank flow under blackberries /	47.85069	-122.57050
#PGS24	03/26/2015	13:13				410.6	Bank flow under blackberries / 1st Confirmations	47.85069	-122.57050
#PGS24	03/26/2015	13:25				686.8	Bank flow under blackberries / 2nd Confirmation	47.85069	-122.57050
#PGS24	04/21/2015	13:11				4.1	Bank flow under blackberries /	47.85069	-122.57050
#PGS24	09/25/2015	10:26				48.9	Bank flow under blackberries /	47.85069	-122.57050
#PGS25	02/24/2015	14:27				2.0	Bank flow, blackberries, lots of wood /	47.85098	-122.57069
#PGS25	09/25/2015	10:31				5.2	Bank flow, blackberries, lots of wood /	47.85098	-122.57069
#PGS26	02/24/2015	14:30				9.6	Bank flow, trees above /	47.85123	-122.57074
#PGS26	09/25/2015	10:35				114.5	Bank flow, trees above /	47.85123	-122.57074
#PGS27	02/24/2015	14:39				74.8	Small flow next to large tree /	47.85123	-122.57074
#PGS27	09/25/2015	10:39				9.6	Small flow next to large tree /	47.85123	-122.57074
#PGS28	02/24/2015	14:42				7.4	Composite (2 flows)-Either side of cedar /	47.85261	-122.57131
#PGS28	09/25/2015	10:44				3.1	Composite (2 flows)-Either side of cedar /	47.85261	-122.57131
#PGS29	02/24/2015	14:49				2.0	Little Boston Creek /	47.85416	-122.57242
#PGS29	02/24/2015	14:49				5.2	Little Boston Creek /	47.85416	-122.57242
#PGS29	09/25/2015	10:50				34.1	Little Boston Creek /	47.85416	-122.57242
#PGS29	09/25/2015	10:50				59.1	Little Boston Creek /	47.85416	-122.57242
#PGS3	02/24/2015	12:12				3.1	Small flow near roots /	47.83959	-122.56668
#PGS30	02/24/2015	14:50				6.3	Composite-2 white PVC pipes /	47.85421	-122.57253
#PGS31	02/24/2015	15:00				1.0	Small creek at bridge /	47.85543	-122.57543
#PGS31	09/25/2015	11:02				2420.0	Small creek at bridge /	47.85543	-122.57543
#PGS32	02/24/2015	15:09				4.0	Wetland drain-salinity 35 / Salinity-35-Ran as marine water	47.85833	-122.57380
#PGS33	02/24/2015	15:14				4.0	Wetland drain-blue A-frame-salinity 21 / Salinity-21-Ran as marine water	47.85985	-122.57347
#PGS33	09/25/2015	11:15				10.0	Wetland drain-blue A-frame-salinity 21 /	47.85985	-122.57347
#PGS34	02/24/2015	15:18				1.0	Small creek, flowing north to south along beach /	47.86066	-122.57322
#PGS34	09/25/2015	11:21				7.3	Small creek, flowing north to south along beach /	47.86066	-122.57322
#PGS35	02/24/2015	15:24				0.5	Flow parallel to beach, cedars /	47.86298	-122.57254
#PGS35	09/25/2015	11:30				2.0	Flow parallel to beach, cedars /	47.86298	-122.57254
#PGS36	02/24/2015	15:27				0.5	Seep under alders /	47.86372	-122.57255
#PGS36	09/25/2015	11:36				9.7	Seep under alders /	47.86372	-122.57255
#PGS4	02/24/2015	12:18				3.1	Small seep, big stump, alders /	47.83996	-122.56669
#PGS5	02/24/2015	12:23				45.9	Small seep, roots, concrete blocks /	47.84015	-122.56680
#PGS6	02/24/2015	12:27				9.7	Hill flow & flow to west, either side of tree /	47.84038	-122.56692
#PGS7	02/24/2015	12:33				150.0	Large flow under blackberries /	47.84061	-122.56700
#PGS8	02/24/2015	12:38				3.1	Flow down bank, reeds, horsetail /	47.84109	-122.56719
#PGS9	02/24/2015	12:42				12.1	Middle Creek /	47.84157	-122.56708
#PGS9	09/25/2015	08:50				57.3	Middle Creek /	47.84157	-122.56708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#PGS19
#PGS17
#PGS14
#PGS11
#PGS8
#PGS5
#PGS2
#PGS1
Pope St
Port Gamble
104
S Teekalet Ave
Gamble Pl NE
Tilbury Ln NE

Station: #PGS1-N47.83886° W122.56671°

Description: Seep under clay bank, blackberries

Date	EC/100 ml	Sample type
2/24/2015	1	Routine

Station: #PGS2-N47.83921° W122.56658°

Description: Seep under clay bank, blue tarp above

Date	EC/100 ml	Sample type
2/24/2015	2	Routine

Station: #PGS3-N47.83959° W122.56668°

Description: Small flow near roots

Date	EC/100 ml	Sample type
2/24/2015	3.1	Routine

Station: #PGS4-N47.83996° W122.56669°

Description: Small seep, big stump, alders

Date	EC/100 ml	Sample type
2/24/2015	3.1	Routine

Station: #PGS5-N47.84015° W122.56680°

Description: Small seep, roots, concrete blocks

Date	EC/100 ml	Sample type
2/24/2015	45.9	Routine

Station: #PGS6-N47.84038° W122.56692°

Description:

Date	EC/100 ml	Sample type
2/24/2015	9.7	Routine

Station: #PGS7-N47.84061° W122.56700°

Description: Large flow under blackberries

Date	EC/100 ml	Sample type
2/24/2015	150	Routine

Station: #PGS8-N47.84109° W122.56719°

Description: Flow down bank, reeds, horsetail

Date	EC/100 ml	Sample type
2/24/2015	3.1	Routine

Station: #PGS9-N47.84157° W122.56708°

Description: Middle Creek

Date	EC/100 ml	Sample type
2/24/2015	12.1	Routine

Station: #PGS10-N47.84202° W122.56722°

NO PHOTO

Description: Flow over alder roots off bank

Date	EC/100 ml	Sample type
2/24/2015	11/3.1	Routine

Station: #PGS11-N47.84221° W122.56745°

Description: Flow over clay bank, downed tree

Date	EC/100 ml	Sample type
2/24/2015	1	Routine

Station: #PGS12-N47.84257° W122.56768°

Description: Bank flow near tire

Date	EC/100 ml	Sample type
2/24/2015	64.4	Routine

Station: #PGS13-N47.84310° W122.56783°

Description: Small stream, root ball

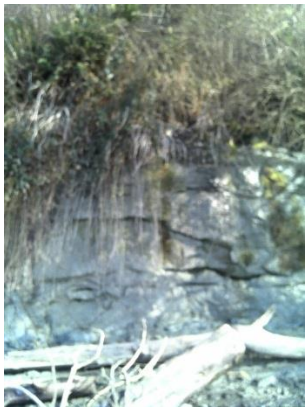
Date	EC/100 ml	Sample type
2/24/2015	4.1	Routine

Station: #PGS14-N47.84395° W122.56814°

Description: Bank seep, alders above

Date	EC/100 ml	Sample type
2/24/2015	<1	Routine

Station: #PGS15-N47.84428° W122.56817°



Description: Flow over clay bank, algae

Date	EC/100 ml	Sample type
2/24/2015	14.5	Routine

Station: #PGS16-N47.84506° W122.56823°

Description: Composite (2)-flow under alders & flow to west

Date	EC/100 ml	Sample type
2/24/2015	2	Routine

Station: #PGS17-N47.84520° W122.56827°

Description: Flow down clay bank, cars above

Date	EC/100 ml	Sample type
2/24/2015	4.1	Routine

Station: #PGS18-N47.84572° W122.56856°

NO PHOTO

Description: Flow under blackberries, black flex

Date	EC/100 ml	Sample type
2/24/2015	26.2	Routine

Station: #PGS19-N47.84699° W122.56920°

Description: Small creek, downed trees

Date	EC/100 ml	Sample type
2/24/2015	4.1	Routine

Station: #PGS20-N47.84787° W122.56989°

Description: Small creek, stairs to west

Date	EC/100 ml	Sample type
2/24/2015	27.5	Routine

Station: #PGS21-N47.84926° W122.57054°

Description: Small creek, flows east to west

Date	EC/100 ml	Sample type
2/24/2015	2	Routine

Station: #PGS22-N47.84998° W122.57047°

Description: Flow under large, downed cedar

Date	EC/100 ml	Sample type
2/24/2015	52	Routine

Station: #PGS23-N47.85051° W122.57041°

Description: Seep from bank, alders

Date	EC/100 ml	Sample type
2/24/2015	2	Routine

Station: #PGS24-N47.85069° W122.57050°

Description: Bank flow under blackberries

Date	EC/100 ml	Sample type
2/24/2015	461.1	Routine
3/26/2015	410.6	Confirmation
3/26/2015	686.8	Confirmation

Station: #PGS25-N47.85098° W122.57069°

Description: Bank flow, blackberries, lots of wood

Date	EC/100 ml	Sample type
2/24/2015	2	Routine

Station: #PGS26-N47.85123° W122.57074°

Description: Bank flow, trees above

Date	EC/100 ml	Sample type
2/24/2015	9.6	Routine

Station: #PGS27-N47.85123° W122.57074°

Description: Small flow next to large tree

Date	EC/100 ml	Sample type
2/24/2015	74.8	Routine

Station: #PGS28-N47.85261° W122.57131°

Description: Composite-2 flows-either side of cedar

Date	EC/100 ml	Sample type
2/24/2015	7.4	Routine

Station: #PGS29-N47.85416° W122.57242°

Description: Little Boston Creek

Date	EC/100 ml	Sample type
2/24/2015	2/5.2	Routine

Station: #PGS30-N47.85421° W122.57253°

Description: Composite 2 white PVC pipes

Date	EC/100 ml	Sample type
2/24/2015	6.3	Routine

Station: #PGS31-N47.85543° W122.57543°

Description: Small creek at bridge

Date	EC/100 ml	Sample type
2/24/2015	1	Routine

Station: #PGS32-N47.85833° W122.57380°

Description: Wetland drain-salinity 35

Date	EC/100 ml	Sample type
2/24/2015	<10	Routine

Station: #PGS33-N47.85985° W122.57347°

Description: Wet land drain-blue A-frame-salinity 21

Date	EC/100 ml	Sample type
2/24/2015	<10	Routine

Station: #PGS34-N47.86066° W122.57322°

Description: Small creek, flowing north to south along beach

Date	EC/100 ml	Sample type
2/24/2015	1	Routine

Station: #PGS35-N47.86298° W122.57254°

Description: Flow parallel to beach, cedars

Date	EC/100 ml	Sample type
2/24/2015	<1	Routine

Station: #PGS36-N47.86372° W122.57255°

Description: Seep under alders

Date	EC/100 ml	Sample type
2/24/2015	<1	Routine

2015 E.Coli Bacteria (EC) Hotspot at Station: #PGS24-N47.85069° W122.57050°

Date	EC/100 ml	Sample type
2/24/2015	461.1	Routine
3/26/2015	410.6	Confirmation
3/26/2015	686.8	Confirmation

Description: Bank flow under blackberries (looking east)

EC counts at or above100 EC/100 mL are resampled two times to confirm. A geometric mean value (GMV) of the three sample results over 320 EC/100mL leads to investigation.

GMV calculated by the Kitsap County database for PGS24 is 506. Sampling, photo, and GPS coordinates by Kitsap Public Health District: Kim Jones; Accompanied by Devon Hayes for PGST; EC Results by Twiss Analytical Laboratory, Poulsbo, WA, according to Hood Canal Regional PIC guidance.

Next Steps: Contact adjacent property owner(s) with information and obtain permission to access stream area and yard, Contact PGST Utilities for map of septic connections,

By 4/24/15: Sample site for EC and investigate area with Kitsap Public Health to identify potential fecal pollution sources including animal waste, septic system, or other.

4/27-29: Kitsap and PGST review lab results, determine if indoor dye test is needed, and plan next steps. “Dry Season” shoreline survey will occur before the end of Oct.

2016 *E. coli* Bacteria (EC) Hotspot at Station: #PGS19- N47.84699° W122.56920°

PIC Station 19 Stream Data

Date	EC/100 mL	Sample Type
2/24/15	4.1	Shoreline Survey
8/17/16	**218**	Stream-Response, >100EC/100mL
8/22/16	**1,483**	Stream-Confirmation 1/2, >100EC/100mL
9/7/16	**3,255**	Stream-Confirmation 2/2, >100EC/100mL
9/13/16 **Data Review**	**1,017**	**Stream-Geometric Mean Value** >320 EC/100mL = Investigation

Next: Request Utilities and Septic Map, Request access permission as needed, Sample upstream for EC within dry season, Inspect connections, Map Hotspot area with 200-foot buffer, Decide on coordination with KPH and dye testing needs.

References:

2015 Shoreline Survey by Kitsap Public Health, and Devon Hayes for PGST

2016 Sampling by PGST Natural Resources (SP, HD)

EC Results by SPECTRA Lab, Poulsbo, WA

Hood Canal Regional PIC Guidance: http://www.hccc.wa.gov/sites/default/files/resources/downloads/HCR_PIC_Program_Guidance_3-10-14_Final.pdf

Appendix C. Review of Methods and Markers for Microbial Source Tracking

Introduction

Fecal material containing pathogenic viruses, bacteria, and protozoa creates a public health risk in contaminated environmental water. Sources of fecal pollution in a watershed can be both point and non-point, from diverse human, agricultural and wildlife origins. The ability to track the fate and transport of fecal pollution and distinguish between sources is particularly important for mitigating and managing water quality and waterborne diseases. At present, monitoring for all waterborne pathogens is unrealistic due to the diversity present in sewage and the broad range of costly or challenging methods used to collect and identify pathogenic organisms in environmental samples.

To date, water quality monitoring and management practices have relied heavily on fecal indicator bacteria (FIB), including *Escherichia coli* and *Enterococcus,* which have low pathogenic potential but abundant presence in sewage and feces. FIB are therefore suggestive of pathogen presence. Studies have shown, however, that pathogen presence does not always co-vary strongly and consistently with FIB concentrations since the ecology and fate of FIB outside a host can vary widely (Anderson et al. 2005, Harwood et al. 2005, Colford et al. 2007, McQuaig et al. 2009). FIB can be native or adapted to stream, estuary, and bay habitats and some are shown to persist or even grow in association with aquatic sediments, aquatic vegetation, and terrestrial soils (Whitman et al. 2003, Ishii et al. 2006, Badgley et al. 2011). Furthermore, conventional indicators cannot discern between human and animal sources because FIB are present in the feces of most mammals and birds (Harwood et al., 1999; Souza et al., 1999; Leclerc et al., 2001). It is important to distinguish between human and animal derived fecal pollution because of the heightened health risks associated with human sewage and the different remediation strategies for mitigating contamination from sewage versus surface runoff carrying animal waste. As our knowledge of zoonotic disease potential increases, the need to identify specific sources of animal waste in contaminated water bodies also intensifies.

Despite the limitations of FIB methods, they continue to be broadly used because they offer fast, easy, inexpensive detection. Alternative indicators for waterborne fecal pollution also exist, including viruses, caffeine, and optical brighteners, and molecular markers (Noble et al. 2003, Buerge et al. 2003, Dixon et al. 2005; Glassmeyer et al. 2005, Hagedorn and Weisberg 2009). Using molecular markers to target DNA sequences from host-associated microorganisms or sequences derived directly from the

host offers an analytical approach with unprecedented specificity, sensitivity, and quantitative capacity. Differences in gut conditions such as temperature, diet, and type of digestive system shape the intestinal microbiota and select for microbial communities unique to their respective human or animal host (Sekelja et al. 2011, Shanks et al. 2011). Microbial source tracking (MST) relies on bacterial taxa or genetic markers that occur preferentially or exclusively in the intestinal system of a target host population and are excreted in high abundance through the host feces (Field and Samadpour, 2007). Ideally, MST markers are also directly correlated with public health risks and provide quantitative data for determining total daily maximum loads (TDML) of pollution in water bodies in accordance with state regulations and the federal Clean Water Act (US EPA CWA 303(d)). Advances in next-generation sequencing technologies and microbiome research have resulted in comprehensive inventories of microbial communities associated with a wide range of hosts and environments allowing rapid development and application of targeted genetic markers for microbial source tracking (MST) (Robinson et al. 2010, Lozupone et al. 2012, Quast et al. 2013, McLellan and Eren, 2014).

Overview of Methods

Techniques for MST can be generally divided into two categories, library-dependent and library-independent, with a focus on genetic or phenotypic traits. Phenotypic analyses measure expressed traits of microorganisms whereas genotypic methods detect a specific gene sequence or evaluate genetic polymorphisms (differences) in DNA.

Library-Dependent

Library-dependent techniques require a cultivation step to generate the library of known bacterial isolates from water sources and fecal samples, to which unknown bacterial isolates from environmental samples can be compared. The library of isolate bacteria is characterized by an identifying attribute such as genetic signature, antibiotic resistance or carbon source utilization (Hagedorn et al. 1999, Moore et al. 2005). Phenotypic analyses like antibiotic resistance and carbon source utilization assume that selective pressure alters the antibiotic resistance or metabolic profile of fecal bacteria from different animals and humans because they are likely exposed to different types of antibiotics or organic substrates. Therefore, antibiotic resistance or carbon utilization profiles of easily cultured FIB bacteria from known fecal samples can be used to classify unidentified environmental isolates based on profile similarity. Genotypic library-dependent analyses generally discriminate between *E. coli* or *Enterococcus spp*. based on the assumption that these organisms are uniquely adapted to their known host environment therefore differ genetically from other strains found in other host species.

Ribotyping and Pulsed Field Gel Electrophoresis (PFGE) are commonly used library-dependent genetic techniques (Carson et al. 2001, Stoeckel et al. 2004). Both methods use restriction enzymes to cut bacterial DNA into fragments that are separated by size and visualized as unique banding patterns, or fingerprints, that can be compared to a library of characterized DNA from known fecal bacteria. PFGE digests whole genomic DNA of bacterial isolates and visualizes large DNA fragments on a specialized gel. Ribotyping is based on differences in the genomic sequences within 16S ribosomal ribonucleic acid (rRNA) gene, a gene that is universally present in bacterial genomes and contains hyper-variable regions that are widely used for taxonomic classification of bacterial communities (Chakravorty et al. 2007).

In a comparison of phenotypic and genotypic library-dependent techniques using blind samples containing one to three of five possible fecal sources (human, dog, cattle, seagull, or sewage) all methods could correctly identify the dominant source in most samples (Harwood et al. 2003, Myoda et al. 2003). Overall, the genotypic library-based techniques performed better than the phenotypic techniques (Stoeckel and Harwood, 2007, Sargeant et al. 2011). While the phenotypic methods had high false positive rates (i.e. a source was identified when it was not actually present) the genotypic analyses showed variable sensitivity (Myoda et al. 2003). Issues with all methods were attributed to the statistical tests used to match patterns from blind sample isolates with the host library database and the limited representativeness of libraries (Stoeckel and Harwood, 2007, Sargeant et al. 2011). In order to establish a comprehensive library, observational knowledge of potential sources of fecal contamination is required and many representative fecal samples from target organisms across all geographic sites of interest must be collected. In general, the accuracy of with which environmental samples are classified into fecal source categories varies widely with library size and representativeness (Stoeckel and Harwood, 2007). The need to develop large site-specific libraries (>1000 isolates), that are both time and labor intensive, has decreased interest in using library-dependent approaches (Johnson et al. 2004, Santo Domingo et al. 2011).

Library-Independent

In contrast, library-independent techniques do not require the development of a source library database. These techniques rely on a species-specific genotype or characteristic detected within a mixed environmental sample. Nucleic acid replication via polymerase chain reaction (PCR) is an important genetic tool in library-independent approaches that can be applied to both laboratory-cultivated bacteria and DNA sequences obtained directly from environmental samples. PCR facilitates rapid, preferential amplification of specific nucleotide sequences from a mixture of non-target sequences. As a result, PCR allows detection and examination of gene targets that are strong indicators of fecal source DNA and only requires a small amount of starting

material from cultured bacterial cells or environmental DNA. PCR protocols that detect the presence or absence of a gene sequence are referred to as end-point PCR.

In one of the first library-independent studies, Bernhard and Field demonstrated the use of end-point PCR of the 16S rRNA gene of human-associated *Bacteroidales* to detect human fecal contamination (Bernhard and Field, 2000). This method served as a precursor for many other library-independent gene-specific PCR analyses (reviewed in Harwood et al. 2013). A common end-point PCR method for identifying human fecal pollution not based on the 16S rRNA gene, uses a culture step to enrich for target *Enterococcus faecium* cells and then amplifies and detects the enterococcal surface protein (esp) gene (Scott et al. 2005, Ahmed et al. 2008). Both methods have been shown to be highly sensitive and specific (>90%) (Ahmed et al. 2009, Boehm et al. 2013, Harwood et al. 2013) although additional studies have detected some level of *E. faecium* and human-associated *Bacteroidales* in the feces of animals (Kildare et al. 2007, Whitman et al. 2007; Layton et al., 2009; Boehm et al. 2013). In addition to human-associated microbial gene targets, many PCR methods have been developed to detect common animal sources including dogs, pigs, cows, poultry, gulls and other wild birds. These, and other gene-specific PCR targets discussed below, are adequate to determine the source of fecal microbial pollution in the environment, however, they cannot be used to quantify the amount the fecal pollution and evaluate associated public health risks.

Recently, quantitative real-time PCR (qPCR) assays which allow for more rapid detection of markers, as well as determination of their relative concentrations, have been developed (Dick and Field 2004, Seurinck et al. 2005). qPCR works much like end-point PCR but the accumulation of PCR products is quantified with each reaction cycle using a fluorescence detector. The strength of the fluorescent signal indicates the relative amount of a specific target DNA sequence in a sample (Walker, 2002) and thus can be used in TMDL analysis and subsequent management decisions. In many studies of human and animal-associated gene targets, qPCR methods have been found to more precisely correlate with pathogen presence compared to end-point PCR or other MST methods (Savichtcheva et al. 2007; Walters et al. 2009; Harwood et al. 2013). It should be noted that correlations between MST markers and pathogens have not been found in all studies yet the general conclusion in the field is that *Bacteroidales* markers have a comparable or better ability to predict pathogens compared with conventional FIB methods (Fremaux et al. 2009; Schriewer, et al. 2010).

Oligotyping is a recently introduced computational method that allows the identification of closely related but distinct bacterial strains that would normally be classified as one taxonomic unit. Variations within a single bacterial taxa can result in differential distribution patterns between geographically distinct host populations that can then be used to identify a source population. Eren and colleagues (2015) identified host-specific oligotypes of the bacterial taxon *Blautia* that occurred exclusively in fecal

samples of humans, swine, cows, deer or chickens. Oligotyping has also been used to distinguish between members of the taxon *Helicobacter* found in the gut and feces of wild and domestic animals including seabirds, marine mammals, and dogs (Oxley and McKay 2005).

Whole-community analysis based on bacterial 16S rRNA gene sequencing of fecal and environmental microbial communities demonstrate evidence of host patterns in entire bacterial assemblages. Early studies using whole bacterial communities demonstrated that the native microbial communities in water are changed by the addition of fecal contamination from bovine or equine sources (Cho and Kim 2000, Simpson et al., 2004). More recently, Newton and colleagues (2013) used community sequencing of bacterial 16S rRNA gene to describe three sewer infrastructure-associated bacterial genera and five fecal-associated bacterial families that served as signatures of sewer and fecal contamination in urban rivers and lakes. Other studies have found that microbial communities from the same fecal origin were highly similar and could be used to determine the dominant sources of fecal contamination in water samples (Lee et al. 2011, Cao et al. 2013).

Microarray technology provides high-throughput comprehensive screening of whole microbial communities or targeted MST markers. Microarray platforms contain thousands of short gene sequences for classes of markers specific to indicator organisms, pathogens, and source identifiers that hybridize with PCR products or whole genomic DNA in samples. Multiple microarrays have been designed and used to specifically detect waterborne bacterial pathogens (Miller et al. 2008, Gomes et al. 2015). Specifically for MST applications, the Phylochip microarray for 16S rRNA bacterial community analysis was modified by Dubinsky and colleagues (2016) to detect and distinguish fecal bacteria from humans, birds, ruminants, horses, pigs and dogs. Also, Li and colleagues (2015) developed a custom microarray targeting waterborne viral, bacterial, and protozoan pathogens, well-studied fecal indicator bacteria and markers, antibiotic resistance genes, as well as universal bacterial probes for whole community characterization. While microarray tests can be used to rapidly screen for multiple sources of fecal contamination and identify human health risks, they do not provide quantitative information about the identified sources that may be critical for environmental monitoring applications.

Microbial Targets

Recent MST research has focused on fecal anaerobe markers because of the unlikelihood that these organisms will successfully grow and reproduce outside their host. They are either specifically adapted to or selected for by the host gut, and consequently will be more tightly associated with fecal pathogen presence in the environment. Fecal anaerobes of the taxonomic order *Bacteroidales* have received the

majority of MST research effort (Bernhard and Field, 2000, and reviewed in Harwood et al. 2013); other potential indicators include members of *Clostridiales* and direct pathogen detection.

Bacteriodes

Selected for its high concentrations in feces and tendency to coevolve with its host, the Bacteroides-Prevotella taxon was one of the first targets of library-independent detection based on the HF183 end-point PCR of the 16S rRNA gene (Bernhard and Field, 2000). *Bacteroidales* are gram-negative, obligate anaerobes that occur in human and animal feces at concentrations from 10^9 to 10^{11} cells · g^{-1} and at concentrations of 10^9 cells · $100ml^{-1}$ in sewage (Holdeman et al. 1976, Wexler 2007) compared to traditional FIB that exist at orders of magnitude lower concentrations (10^6 to 10^7 CFU · $100ml^{-1}$ in sewage) (Harwood et al. 2005, Converse et al. 2009). Many studies have confirmed the high sensitivity and general specificity of HF183 and related *Bacteroides* markers for human and animal targets (Kildare et al., 2007, Harwood et al. 2013, Boehm et al. 2013).

Clostridiales

Obligate anaerobes of the phylum Firmicutes, members of the *Clostridiales* are commonly found in the gut of humans and animals. Within this group of organisms, MST focus has been on *Lachnospiraceae*, one of the most abundant groups of faecal bacteria in sewage (McLellan et al. 2013). A strong correlation was observed between between *Lachnospiraceae* and adenovirus, indicating a link between these markers and human pathogen presence (Newton et al. 2011). Members of *Clostridiales* have also been found in high abundance in avian and marine mammal hosts and feces and subsequently been developed as MST markers for these organisms (Oxley and McKay 2005, Green et al. 2012, Koskey et al. 2014).

Pathogens

Direct detection of pathogens in watersheds is beneficial for assessing public health risk. The Centers for Disease Control and Prevention (CDC) has found fecal pathogens shiga-toxin producing *E. coli, Shigella, Salmonella,* and *Campylobacter* as the dominant sources of fecal-associated waterborne disease (Lee et al. 2002). Among the fecal coliform bacteria strains of shiga-toxin *producing E. coli* O157:H7 and the pathogen *Shigella sonnei* both cause a range of intestinal illnesses. The *E. coli* O157 serotype and other pathogenic *E. coli* can be identified by the PCR detection of specific shiga-toxin genes and surface proteins (Maurer 1999, Osek 2003, Duris et al. 2009). Certain *E. coli* toxin genes can also distinguish between cattle and swine fecal pollution presence (Duris et al. 2011). Campylobacter is another leading cause of bacterial gastroenteritis in developed regions. Wild birds and poultry are recognized as sources

of the Campylobacter taxa, *C. jejuni, C. coli* and *C. lari*, frequently implicated in human illness (Butzler 2004). Campylobacter qPCR markers can discern between pathogenic and non-pathogenic strains and have been used to inform public health risk assessment from gull fecal pollution (Lu et al 2011). The issue with direct detection is pathogen strains are normally found in low densities in environmental water, and a cultivation step is required to increase the sensitivity of the assays (Duris et al. 2011).

Non-Bacterial Targets

Viruses

Monitoring for human viruses has been suggested as an alternate approach to assess human health risks in environmental waters. Viruses are generally highly host-specific and do not multiply in the environment or readily degrade under environmental stressors, such as UV irradiance and water treatment processes, unlike traditional FIBs. However, pathogenic viruses usually infect a small percentage of any given population, making them relatively rare targets (and thus more difficult to detect) (Pina et al. 1998). Certain non-pathogenic human viruses have a wider distribution in human populations than pathogenic viruses and their stable nature makes them ideal indicators of other viral pathogens, such as noroviruses and hepatitis A viruses, persistent in the environment (McQuaig et al. 2009). The human adenovirus (HAdV) and human polyomavirus (HPyV) are promising as human fecal indicators, as they are frequently excreted in the feces or urine of humans both with and without clinical symptoms and they are commonly detected in urban wastewater (Bofill-Mas et al., 2001). Certain adenoviruses exist that are specific to livestock as well providing distinction between human or animal-derived fecal pollution (Rusinol et al. 2014). Studies have demonstrated HPyV targets to be 100% specific, showing no cross-reactivity to animal fecal samples (Harwood et al. 2009, McQuaig et al. 2009, Ballesete et. al. 2010)

Archaea

Archaeal methanogens are commonly associated with the oral, vaginal, and intestinal mucosa of mammals (Belay et al. 1998, Belay et al. 1990, Miller et al. 1982, Miller et al. 1986). *Methanobrevibacter ruminantium* and *M. smithii* have been tested for possibilities as ruminant and human markers, respectively (Ufnar et al. 2006, Ufnar et al. 2007). *M. smithii* a methanogenic archaeon found exclusively and abundantly in the human gut and human fecal samples (Lin and Miller 1998, Dridi et al., 2009). Likewise, *M. ruminantium* is specific to the rumen of domesticated animals (Smith and Hungate 1958). The nifH gene is targeted in archaeal indicators because it is a predominantly methanogen-specific gene with sequence differences that can be used to discriminate between methanogen groups. The *M. ruminantium* nifH assay is shown to be successful at detecting cattle, sheep, and goat feces and contamination by agricultural lagoon

waste in environmental water samples (Ufnar et al. 2007). The *M. smithii* marker has high sensitivity against human sewage pollution especially in coastal waters but did show some cross-reactivity with bird feces (Ufnar et al. 2006, Johnston et al. 2010).

Direct source detection

The first fecal source tracking method based on a eukaryotic genetic marker was the end-point PCR assay targeting the human mitochondrial DNA (mtDNA) NADH dehydrogenase subunit (Martellini et al., 2005). mtDNA was proposed as a marker based upon the premise that it should be abundant in feces and especially host-specific. Other studies have used qPCR probes to target human, bovine, ovine and swine mtDNA for use as indicators in source tracking studies of shellfish harvesting areas (Baker-Austin et al. 2010). Developments in biodiversity monitoring using environmental DNA (eDNA), genetic material obtained directly from environmental samples from any organism, have also found application in fecal source tracking. Utilization and contamination of waterbodies by various wildlife, human, and domesticated animals can be detected through eDNA markers (Thomsen and Willerslev 2015).

Evaluation of Source Tracking Methods

Any satisfactory MST method must comply with a set of performance criteria (Stoeckel and Harwood, 2007). Some performance criteria are universally applicable while others depend on the objectives of a particular study (Santo Domingo et al. 2007). The key universal criteria are described here.

Sensitivity

The sensitivity of a MST method is defined by the percentage of true positive results detected. Sensitivity indicates the robustness of an assay provided that targets are present at or above detection levels. Samples spiked with fecal material or other known contaminated samples are used to directly test the number of positive controls correctly identified as positive by the assay. Physical or chemical properties of the water matrix or sample type may impair the sensitivity of certain methods (Siefring et al. 2008).

Specificity

The specificity of a MST marker is represented by the rate of false positive results or the percentage of negative results correctly ascribed to samples known to lack the host target in question (Stoeckel and Harwood, 2007). A highly-specific MST marker should not cross-react with unintended targets and accurately identify only target source species. It is desirable that a marker is tested against as many nontarget

fecal samples as possible to better constrain limitations of method specificity (Harwood and Stoeckel, 2011)

Stability

The stability criteria dictates that changes in environmental or biological conditions due to seasonal or regional differences should not affect the presence of MST targets in host feces. A stable marker does not vary in frequency or concentration over time at the population level, has consistent detection across all geographic regions of the host range, and exhibits predictable rates decay in all habitats and water matrices (Sargeant et al. 2011).

Challenges for stream, river, and estuarine systems

Understanding eDNA detection rates in lotic systems is critical for inclusion of eDNA analysis as a reliable survey method in fecal source tracking. The concentration of DNA in rivers and streams depends on dynamics between eDNA released into the water, downstream transport and losses to the system through physical, chemical and biological processes. The contribution and rate of production of eDNA by various organisms has been the focus of only a few studies (Pilliod et al. 2014, Thomsen et al. 2012, Klymus et al. 2015) and is likely influenced by the size, sex, health, and density of members in a population. Difficulty measuring the transport and residence time of eDNA in riverine systems also poses challenges to describing the geographic origin of eDNA and making spatial interference about the source organism(s). A study by Deiner and Altermatt (2014) observed movement of eDNA five to ten kilometers downstream of the source population within a 24hr sampling period, indicating that eDNA can persist over relatively large distances in a river system. It has been shown, however, that eDNA concentrations are generally localized and do not appear to accumulate downstream (Deiner and Altermatt 2014, Pilliod et al. 2013, Laramie et al. 2015). Dilution and removal processes such as settling and degradation, likely reduce the amount of detectable eDNA over time and as it travels downstream thereby limiting accumulation (Dejean et al. 2011, Jane et al. 2014).

Recommendations

In a review and critique of MST methods, the Washington State Department of Ecology highlighted the lack of standardized, validated, promulgated, and U.S. Environmental Protection Agency approved molecular MST methods. Sargeant and colleagues (2011) proposed the following quality assurance sampling for substantiation of results: 1) Field samples duplicated for reproducibility information; 2) Preliminary testing of source feces from the study area to confirm the source-specific MST indicator or marker is present; 3) Samples spiked with fecal material from each potential source per study as positive controls; 4) Samples from presumably uncontaminated sites as

field negative controls. The use of multiple MST techniques in parallel, was also recommended to overcome the experimental nature of fecal source tracking methods and to produce acceptable levels of accuracy, reproducibility, and investigation of numerous potential source types. Furthermore, library-independent methods are recommended over library-dependent methods because they typically have a lower cost and provide much faster results (Sargeant et al. 2011).

The Toolbox Approach

Because rivers, streams and estuaries can have considerable temporal and spatial variability in microbial water quality from a multitude of human and animal-derived sources, a monitoring strategy that captures data about all potential sources is optimal. No one marker has all the requisite performance qualities for identifying and quantifying the source and magnitude of fecal pollution in water. Thus, a toolbox approach using a suite of techniques and molecular markers, producing multiple lines of evidence, is considered important to effective microbial source tracking (Harwood et al. 2013).

Monitoring, mitigation, and management of fecal pollution can be costly to coastal communities, which depend on uncontaminated water bodies for tourism, recreation, and fisheries (Rabinovici et al. 2004). Most public advisories and closures in recreation areas and shellfisheries are posted without specific knowledge of the type and source of fecal contamination (NRDC, 2006). A better understanding and implementation of MST will facilitate targeted remediation, enhance protection of public health, and minimize economic costs associated with fecal pollution in water systems.

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Appendix D. Temporal Studies, Tryptophan, and Optical Brighteners

PGST coordinated sample collection of EC over a 24-hour period, to test if there are advantages to sampling at certain times of day. PGST collected water samples using a Hach Sigma SD 900 Portable Sampler (autosampler) which collected one sample per hour during the 24-hour sampling periods. Variability in results between samples was enough to warrant a second test, to determine the range of variability between split and replicate samples. A second collection period was coordinated with personnel collecting samples by hand four times a day over 72 hours.

Temporal studies were conducted on the PGST Reservation, and in Jefferson County at Irondale Creek and the Duckabush River. A temporal study was planned at Lofall Creek in Kitsap County during the wet season of 2015-2016 however was eventually canceled after excessive rainfall. Results showed that EC levels did vary significantly temporally, beyond the variability found between split and replicate samples.

PGST recommends that future projects looking to utilize an autosampler should consider a model which can be easily dismantled and autoclaved. Additionally, unless the autosampler is going to be deployed regularly at a set location with proper infrastructure to house the equipment, using personnel to collect samples by hand is likely the more practical approach.

Additionally, PGST used a Turner Designs Cyclops 7 Submersible Fluorometer with tryptophan and optical brightener sensors to determine if *in situ* measurements of tryptophan and optical brighteners are a useful proxy for identifying EC hotspots. Results showed no correlation between optical brighteners and EC at the three time of day study sites where the fluorometer was deployed. At one of the three sites, there was a weak correlation between EC and tryptophan.

24-Hour Sampling- Preliminary Temporal Study

Photo Credit: Devon Hayes, Hans Daubenberger deploying autosampler and optical brightener probe at Irondale Creek.

24-hour sampling was conducted between April 21 and 22nd 2015 on the PGST Reservation at Shoreline Survey Station PGS 24, which was a confirmed hotspot from the wet season survey. 24-hour sampling was also conducted in Jefferson County at the Irondale Creek PIC monitoring station PH028 between August 26 and 27th 2015.

PGST staff used a programmable Hach Sigma SD 900 Portable Sampler (autosampler), set up with a (24) 575mL Bottle Kit for automated sampling. The autosampler was programmed to automatically collect discrete water samples at preset or fixed-interval times over no more than a 24-hour period. These containers were cleaned and decontaminated between 24-hour sampling events. At the end of the 24-hour test, PGST personnel transferred water samples into 110 milliliter bottles

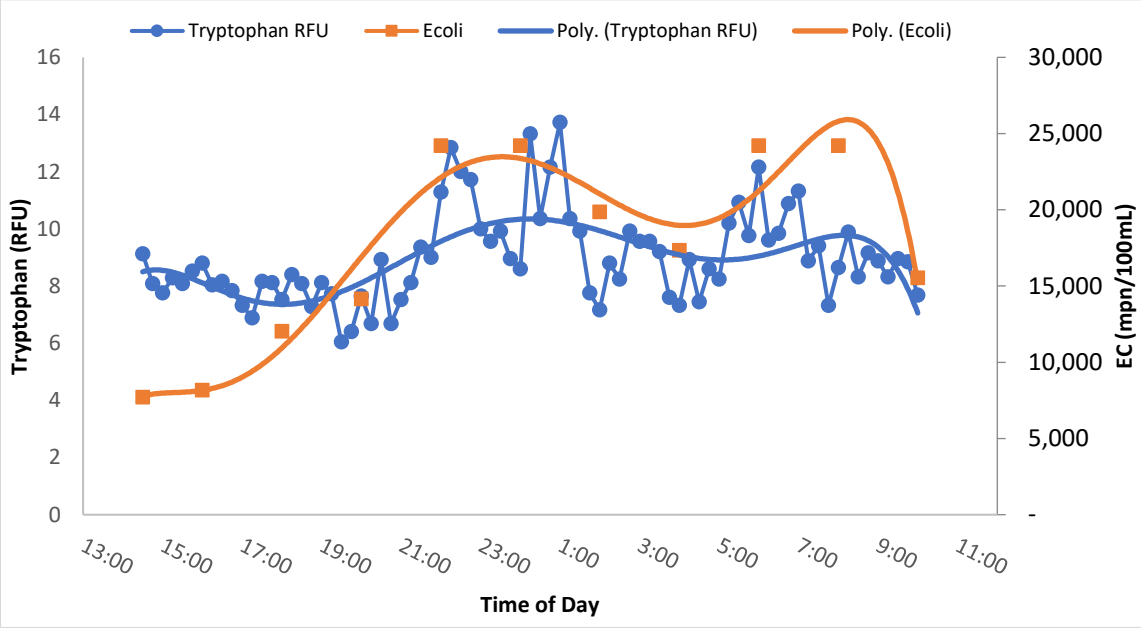
and immediately delivered them to the laboratory for analysis. Two to three blind split replicates were also delivered to the lab to examine variation. PGST staff programmed the autosampler to pump water through the tubing between samples to prevent bacteria growth and potential bias in analytical results.

PGST also collected discrete grab samples at the beginning before installing the autosampler and the end of the sampling period. This was to ensure samples were representative of the stream conditions at the time period and not potentially contaminated by bacteria from previous collection events, or otherwise influenced by the holding time or sampling system.

Results from the Reservation ranged between 1.0 and 248.1 mpn/100mL. The standard deviation was 63.7 and the coefficient of variation was 2.08, showing that results were disperse between samples taken at different times of day at this site. Irondale Creek yielded higher EC levels with a lower variation. Results ranged between 7701 and 24196 mpn/100mL with a standard deviation of 5750.1 and a coefficient of variation of 0.33.

During the 24hr sampling period of Irondale Creek, a Turner Designs Cyclops 7 Submersible Fluorometer was also deployed, to sample tryptophan and optical brightener levels at 15 minute intervals. Results showed a minor correlation between EC levels and tryptophan (r = 0.2453, Figure 1), but much less association with optical brighteners (r = 0.1662).

Figure 1. Tryptophan and EC levels at Irondale Creek



72-Hour Sampling

This sampling design was intended to determine variation between different temporal samples and single events (collected within 20 minutes). Greater variation between single event samples than the variation amongst different temporal samples would suggest there is no benefit to implementing temporal sampling, however if variation between single event samples is

significantly less than variation between temporal samples, then temporal sampling may improve hotspot identification.

72-hour sampling was conducted on the PGST Reservation upstream of Shoreline Survey station PGS 19, near Bud Purser Lane. The other 72-hour test location was at the Duckabush River tributary (also known as Pierce Creek) next to the Brinnon Volunteer Fire Station (42). The PGST Environmental Contractor (Devon Hayes) collected the water samples by hand during the 72hr test according to standard operating procedures for collection and handling EC samples.

Methods

Collection events took place at 8am, 12pm, 3pm, and 8pm on Oct. 31st-Nov. 2nd at the PGST Reservation site and on Nov. 14th-16th at the Duckabush River. Two 100 mL and three 50 mL samples were collected from the stream within 30 seconds of one another. Next, the first 100 mL sample was gently agitated and 50 mL was poured into the sixth sample bottle to produce a split replicate. This process was repeated for the second 100 mL sample to create a second split replicate, for a total of seven samples to be delivered to the lab.

At 3pm, three additional replicates were collected and held overnight, to determine whether longer holding time affected results of sample analysis. Field blank samples consisting of distilled water were submitted blind to the laboratory at a rate of one per sample batch. Salinity analysis was conducted on Duckabush River samples to test for effects of high tide on the site. Sampling on the PGST Reservation was located in a stream well above the high tide line and therefore did not require salinity analysis.

Samples were delivered to the laboratory immediately following collection at 8am, 12pm, and 3pm, excepting the three additional field replicates collected at 3pm. 8pm samples were submitted to the laboratory immediately following the 8am sample collection, with the 8am samples and the select replicates from 3pm the day before.

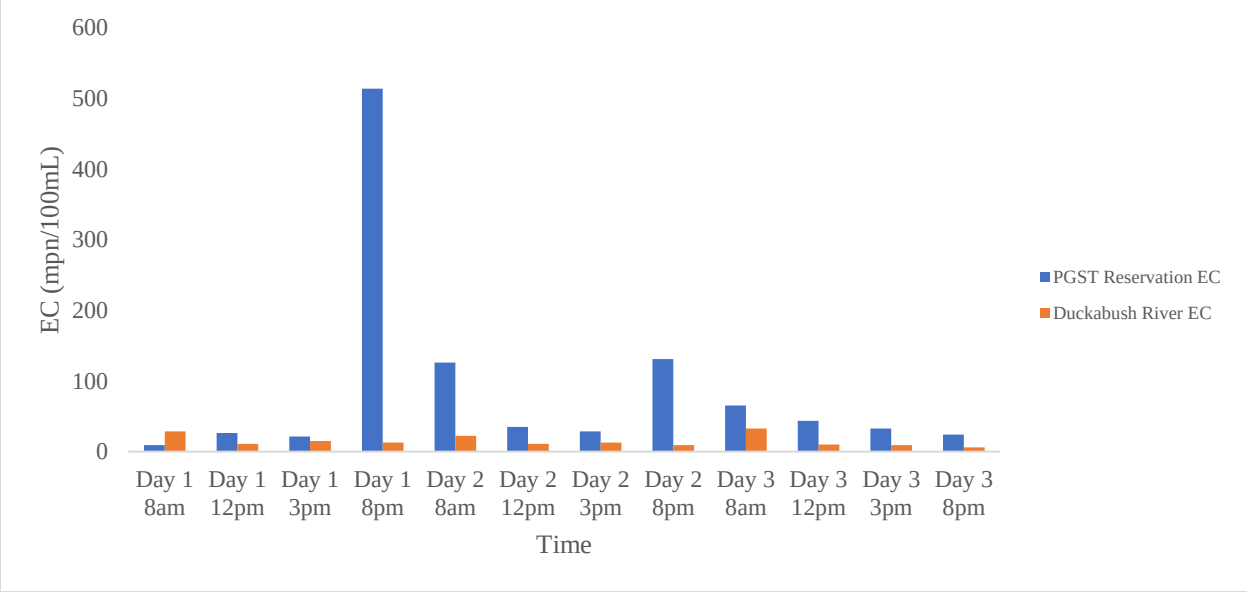
The Turner Designs Cyclops 7 Submersible Fluorometer was deployed at the PGST Reservation site just downstream of the EC sampling location on Oct. 30th and retrieved on Nov. 3rd. The fluorometer was deployed at the Duckabush River just after the 12pm measurement on Nov. 14th, and retrieved on Nov 18th. Optical brighteners and amino acid tryptophan measurements were set to be taken every 15min. The values recorded 15min before, during, and 15min after the EC sampling event times were averaged to give corresponding tryptophan and optical brightener values.

Results

A total of 99 water samples were collected over 72hrs for PGST Reservation EC analysis, including 6 blanks of distilled water. The first 7 samples were collected at the shoreline, and the rest upstream primarily for safe access, and a more controlled environment that adequately represented site conditions. Analytical results for EC ranged between less than 10 mpn/100mL to 783 mpn/100mL, which was detected at 8pm on 10/31/16 (Figure 2).

For the Duckabush River 72-hour test, 104 water samples were collected for EC analysis, including 12 distilled water blanks. 10 water samples were analyzed for salinity after the sample station was found inundated by king tides. EC results ranged from less than 10 mpn/100mL to 63 mpn/100mL, which was detected at 3pm on the third day (Figure 2).

Figure 2. Results of 72-hr E. coli test. Results of primary samples (n = 5 for each sampling event, no splits or blanks included) were averaged for each sampling event.



In the Duckabush River, E. coli values were highest at 8:00am on each of the three days sampled. At 8:00pm on October 31st, E. coli values at Bud Purser were approximately 4x higher than results from any other sampling event at that site during the 3 days studied. EC values varied significantly with time of day, in both systems, for each day sampled (Table 1). Samples did not vary notably within a single sampling event, however variance did increase as EC values increased.

Table 1. P-values for analysis of variance between EC sampling events (TOD) for Bud Purser and Duckabush (Single Factor Anova). *indicates a significant result

Bud Purser		Duckabush	
Date	P-value	Date	P-value
31-Oct	3.38E-15*	14-Nov	0.012226*
1-Nov	1.3E-05*	15-Nov	0.046141*
2-Nov	0.008859*	16-Nov	0.000164*

Split samples were not significantly different from their counterparts in either system (BP p = 0.8178, DB p = 0.7489, Anova: Two-Factor without Replication). Holding samples on ice for 6-17.5hrs before delivering them to the lab did not significantly alter results (BP p = 0.8025, DB p = 0.4770 Anova: Two-Factor without Replication).

At both sites Tryptophan and Optical Brightener values varied significantly between days (Tryptophan: DB p = 0.036616, BP p = 5.13E-07, Optical Brighteners: DB p = 4.62E-09, BP p =1.63E-15). Time of day had varied results (Tables 2 and 3).

Table 2. P-values for analysis of variance between Tryptophan sampling events for Bud Purser and Duckabush (Single Factor Anova, except 14-Nov which was a t-Test paired two sample for means). *indicates a significant result

Bud Purser		Duckabush	
Date	P-value	Date	P-value
31-Oct	5.87E-10*	14-Nov	0.97613
1-Nov	0.081139	15-Nov	0.065428
2-Nov	0.074953	16-Nov	0.177793

Table 3. P-values for analysis of variance between Optical Brighteners sampling events for Bud Purser and Duckabush (Single Factor Anova, except 14-Nov which was a t-Test paired two sample for means). *indicates a significant result

Bud Purser		Duckabush	
Date	P-value	Date	P-value
31-Oct	0.026521*	14-Nov	0.076448
1-Nov	0.001722*	15-Nov	1.01E-05*
2-Nov	0.349408	16-Nov	4.13E-07*

Despite the variability, both Tryptophan, and Optical Brighteners were significantly higher in Bud Purser than in the Duckabush River ($p = 4.49E-05$, $p = 1.38E-27$ respectively). Optical brighteners were highest at Bud Purser on Nov 1st, and Tryptophan was highest on October 31st (Figure 3, Figure 4). There was no correlation between Tryptophan or Optical Brightener values and E. coli levels, suggesting that neither can serve as a sufficient proxy for EC at the two sites tested.

Figure 3. Results of the 72-hr optical brighteners data collection. Values are an average of results collected within a 30-min time window of corresponding EC sampling event times (n=3 for each sampling event).

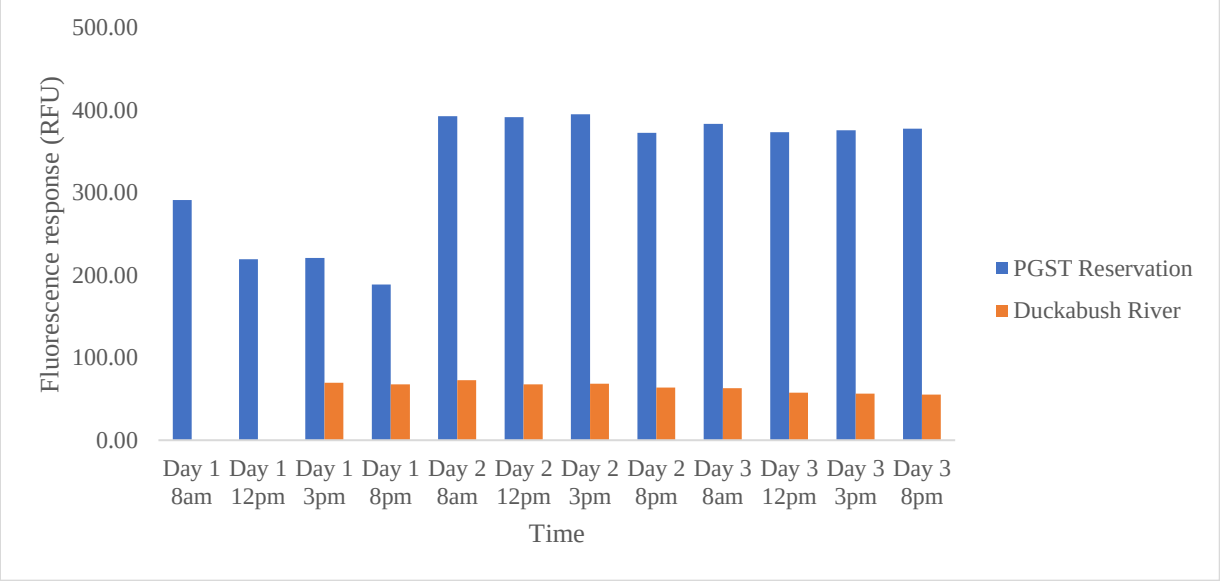
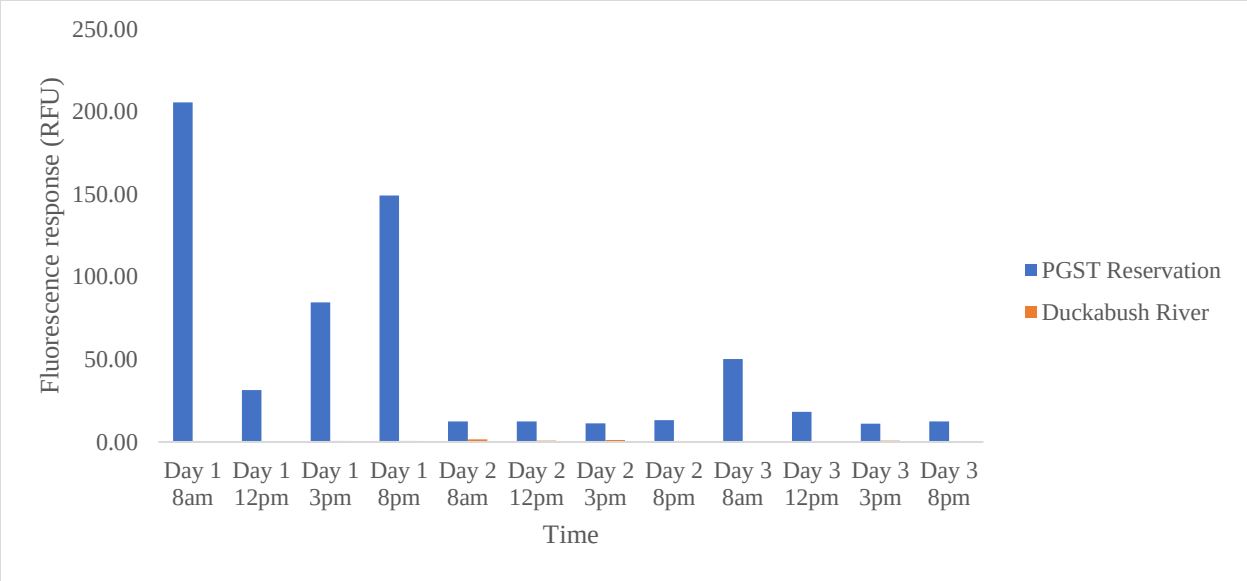


Figure 4. Results of the 72-hr tryptophan data collection. Values are an average of results collected within a 30-min time window of corresponding EC sampling event times (n = 3 for each sampling event).



Recommendations

Temporal sampling[1] in important shellfish harvest and recreational areas would likely reduce human exposure to fecal contamination.

Temporal sampling is valuable for increasing the probability of correctly identifying hotspots and reducing the risk to human health through exposure to fecal bacteria.

The temporal investigation results showed that variation was low between samples taken within a sampling event[2], but samples taken at different times of day produced results above and below threshold criteria for hotspot identification.

In situ tryptophan and optical brightener sampling is unlikely to be a good proxy for EC contamination. Tryptophan and optical brighteners may provide evidence of anthropogenic fecal contamination.

To evaluate the usefulness of tryptophan and optical brighteners for determining anthropogenic fecal contributions, an informative follow-up study could include the analysis of diluted samples from waste treatment facilities relative to environmental samples from systems with varying fecal concentrations.

[1] Temporal sampling in this report refers to collecting a minimum of 4 samples 3hrs apart over a 12hr interval.
[2] A sampling event in this study refers to a set of samples taken within 30 seconds of one another collectively representing one particular time of day.