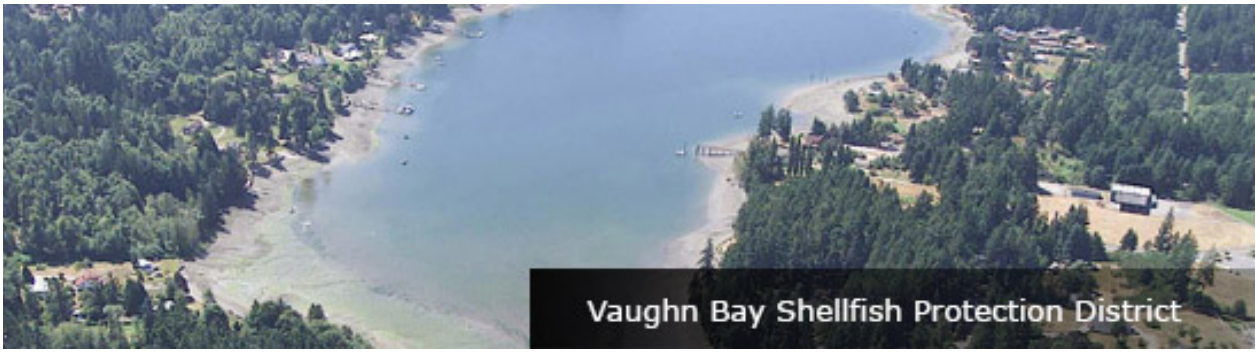
DATA ANALYSIS REPORT

QUANTITATIVE MICROBIAL SOURCE TRACKING DEMONSTRATION PROJECT



Prepared for
Washington State Conservation Commission

Prepared by
Herrera Environmental Consultants, Inc.

July 2019

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QUANTITATIVE MICROBIAL SOURCE TRACKING DEMONSTRATION PROJECT

Prepared for
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July 11, 2019

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EXECUTIVE SUMMARY

The Washington State Conservation Commission (WSCC) selected Herrera Environmental Consultants, Inc. (Herrera) to design and execute a quantitative microbial source tracking (MST) study to improve water quality management decision-making in Washington state. The goal of this study is to demonstrate the ability and cost-effectiveness of quantitative DNA-based MST technology to characterize pollution sources in surface waters with elevated fecal coliform bacteria levels in Washington state. The South Sound Shellfish Recovery Project and previous efforts have identified locations of high fecal bacteria concentrations in drainages to valuable shellfish protection areas, but has had limited success identifying and prioritizing effective control actions for sources from septic systems, livestock, and other animals in some areas where multiple sources appear to be present.

Herrera prepared a quality assurance project plan (QAPP) in August 2018 documenting procedures used for sample collection, field analysis, laboratory analysis, and data analysis to ensure high quality, scientifically defensible results. Vaughn Bay was selected as the study site, and the sample design included collection of water samples from August through December 2018 during eight separate events, which included dry weather base events and wet weather storm events. A QAPP addendum was prepared in December 2018 revising the sampling schedule and some analysis procedures in response to a loss of the MST samples that were initially collected for the study. Ultimately, water samples for MST analysis were collected from November 2018 through April 2019 during three storm events and four base events. Two of the storm events occurred during each of only two shellfish harvesting closures within the study period due to rainfall amounts exceeding 1 inch.

Historical fecal coliform bacteria and watershed data were evaluated to select 10 sampling stations, which included two large streams, two small streams, three problem drains (marine outfalls), and three marine stations in areas classified as conditional or restricted for shellfish harvesting. A total of 70 water samples were collected for analysis of fecal coliform bacteria and two MST methods. The primary MST method was quantitative polymerase chain reaction (PCR) by Source Molecular (Miami, Florida) using seven host-associated markers, which included human, cow, horse, ruminant, pig, dog, and bird. In addition, next generation sequencing (NGS) was conducted by the University of Minnesota BioTechnology Institute that is a library MST method matching DNA of the entire bacteria community in the water samples to that in fecal source samples collected from the study area.

Fecal source samples were collected from the study area for the NGS library and to validate accuracy of the selected qPCR host-associated markers. The validation results indicated that the qPCR results will have very few false positives or false negatives with the exception that the amount of horse and bird fecal DNA may be underestimated or not detected where present. All other measurement quality objectives established by the QAPP were met with minor exceptions.

The highest fecal coliform concentrations in Vaughn Bay and the freshwater discharges to the bay were observed during storm events. Geometric mean concentrations of fecal coliform bacteria were higher during storm events by a factor of 22 for marine stations and a factor of 5 for freshwater stations. Fecal coliform loading rates were generally lower in the small streams and drains compared to Vaughn Creek. Due to increased flow, fecal coliform bacteria loadings to Vaughn Bay were even higher during storm events than base flow events in the winter/spring months. High loadings were also observed during base flow events in the summer/fall months (pre-MST events) when concentrations were somewhat elevated in marine waters compared to winter/spring months (base events). Overall, the single sample criterion for fecal coliform bacteria in marine waters (43 MPN/100 mL) was exceeded in 33 percent (11 of 33) of the marine water samples, while the higher single sample criterion for freshwaters (200 CFU/100 mL) was exceeded in 18 percent (9 of 49) for the freshwater samples.

Moderate to high concentrations of the qPCR host-associated markers were primarily observed during storm events when fecal coliform bacteria concentrations were also highest. Moderate to high concentrations were observed on multiple occasions for bird at all three marine stations and stream stations 1 and 47, and for ruminant at stream station 1. Moderate concentrations were observed on one occasion for ruminant at stream station 47 and for bird at stream station 2. Maximum marker concentrations were much lower for human and dog. Cow and horse were rarely detected at low concentrations, and pig was never detected.

Average host-associated marker concentrations and loading rates clearly show the predominance of fecal sources in storm flow compared to base flow. For example, bird concentrations increased during storm events by a factor of 4.5 at the freshwater stations and a factor of 26 at the marine stations. Also, bird concentrations increased during high fecal coliform events (exceeding the single sample criterion) by a factor of 4.7 at the freshwater stations and a factor of 14 at the marine stations. The qPCR loading results clearly show the high importance of bird sources and moderate importance of ruminants in Vaughn Creek on the high fecal bacteria concentrations in Vaughn Bay. Fecal host loadings during base flow in the streams and during all flow from the drains were insignificant in comparison.

Normalizing qPCR concentrations for host-associated marker concentrations in the fecal source samples increases relative concentrations for human and decreases relative concentrations for ruminants Overall, the normalized qPCR results indicate that human and bird are the primary fecal sources present in the freshwaters and marine waters, followed by ruminant and dog.

The NGS results show that moderate to high amounts of bird (from gull and goose samples) were detected in all samples. Low to moderate amounts of human (from septic samples) were detected at all stations except only low amounts were detected at marine station 608. All other sources were typically not detected or only detected in very low amounts in a few samples. These other sources include livestock (ruminants from cow, sheep, goat, llama, and alpaca samples; horse from horse samples; and pig from pig samples); dog (from dog samples); and deer (from deer samples). The NGS results showed no consistent chronological patterns among the freshwater or marine stations and no differences between base and storm events, in contrast

to the higher fecal concentrations observed during storm events by the culture and qPCR methods.

The NGS results compliment the qPCR results by the high abundance of bird observed by both methods. The NGS method showed relatively higher amounts of human and lower amounts of ruminants than the qPCR method. The comparably low amounts of livestock and deer detected by NGS suggests that much of the ruminant detected by qPCR may have been deer rather than livestock.

The MST study results were used to identify appropriate source control actions to reduce fecal coliform concentrations in Vaughn Bay. Current practices include a wide variety of educational, financial incentive, and enforcement tools. Additional source control actions were recommended specifically for septic systems, farm animal keeping practices, and birds and dogs. It was also recommended to assess effectiveness of source control actions in approximately 5 years by additional fecal bacteria monitoring and quantitative MST.

Quantitative MST method protocols were developed for cost-effective application in other watersheds in Washington state. These protocols are based on the QAPP and report prepared for this MST study. Lessons learned from this MST study were also identified to anticipate and avoid potential problems with future projects in other watersheds.

INTRODUCTION

The Washington State Conservation Commission (WSCC) selected Herrera Environmental Consultants (Herrera) to design and execute a quantitative microbial source tracking (MST) study to improve water quality management decision-making in Washington state. The goal of this study is to demonstrate the ability and cost-effectiveness of quantitative DNA-based MST technology to characterize pollution sources in surface waters with elevated fecal coliform bacteria levels in Washington state.

To meet this study goal, Herrera teamed with public agencies and other private companies to develop and implement a scope of work that includes:

- Conducting a series of meetings with the project team and a technical oversight committee formed by WSCC.
- Designing a comprehensive and statistically-based MST demonstration project.
- Developing a Quality Assurance Project Plan (QAPP) that supplements ongoing monitoring activities with MST analysis using two types of advanced molecular tools including quantitative polymerase chain reaction (qPCR) and high throughput DNA sequencing (next generation sequencing [NGS]).
- Coordinating water sample collection and analysis to ensure high quality data are obtained.
- Applying a holistic approach by integrating molecular MST results with fecal bacteria concentrations and environmental factors using sophisticated geospatial statistical analyses.
- Preparing a detailed project report that presents key findings, identifies appropriate corrective actions, and provides MST study protocols for implementation in other watersheds.

Herrera prepared a QAPP in August 2018 (Herrera 2018a) to document procedures used for sample collection, field analysis, laboratory analysis, and data analysis to ensure high quality, scientifically defensible results. The QAPP was approved and distributed to all project team members, and includes the following information:

- Background Information – Background information about the study problem, objectives, and watersheds.
- Data Analysis – Analysis of fecal coliform bacteria and watershed data used in selection of sampling locations and fecal source analyses.

- Project Description – Project goals and objectives, and selected monitoring method to obtain the information required to meet the objectives.
- Organization and Schedule – Project roles and responsibilities, and the schedule for completing the work.
- Measurement Quality Objectives – Performance (or acceptance) thresholds for collected data.
- Experimental Design – The sampling process design for the study, including sample types, monitoring locations, analytical parameters, and sampling frequency.
- Field Procedures – A detailed description of sampling procedures and associated equipment requirements.
- Laboratory Procedures – Laboratory procedures that will be performed on collected samples.
- Quality Control – Quality control (QC) requirements for both laboratory and field measurements.
- Data Management Procedures – How data will be managed from field or laboratory recording to final use and archiving.
- Audits and Reports – The process that will be followed to ensure this QAPP is being implemented correctly and the quality of the data is acceptable.
- Data Verification and Validation – The data evaluation process, including the steps required for verification, validation and data quality assessment.
- Data Quality (Usability) Assessment – The procedures that will be used to determine if collected data are of the right type, quality, and quantity to meet project objectives.

Herrera prepared a QAPP addendum in December 2018 (Herrera 2018b) revising the sampling schedule and some analysis procedures in response to a loss of the MST samples that were initially collected for the study.

This report presents background information, summarizes methods and deviations from the QAPP, presents and discusses the project results, identifies source control actions, includes a summary of study findings, and provides MST study protocols for implementation in other watersheds.

BACKGROUND INFORMATION

More than $7.2 million in National Estuary Program (NEP) funds are supporting pollution identification and correction (PIC) programs in Puget Sound. PIC programs are identified in the Puget Sound Action Agenda as a key strategy to protect and restore shellfish beds. The South Sound Shellfish Recovery Project is designed to help address declining water quality in key Shellfish Protection Districts in South Puget Sound.

The Tacoma Pierce County Health Department (TPCHD) worked with the Pierce County Shellfish Partners to develop a strategic plan and an outreach plan with educational and marketing tools to guide their PIC work. They are working with advisory groups (water quality teams) to update and implement closure response plans for Burley Lagoon, Filucy Bay, Vaughn Bay, and Rocky Bay. TPCHD has conducted extensive sanitary surveys in watersheds of these four bays and are funded through 2019 to continue sampling freshwater sources in these Shellfish Protection Districts. They are working with landowners to find and correct bacterial pollution from onsite septic systems (OSS) and livestock, and evaluate their outreach efforts and the performance of professionals that do OSS inspections. Farm and municipal stormwater sources are being further investigated and controlled by their partners at Pierce Conservation District and Pierce County Surface Water Management.

PROBLEM STATEMENT

The South Sound Shellfish Recovery Project and previous efforts have identified locations of high fecal bacteria concentrations in drainages to valuable shellfish protection areas, but has had limited success identifying and prioritizing effective control actions for sources from OSS, livestock, and other animals in some areas where multiple sources appear to be present.

SITE DESCRIPTION

The initial proposal was to conduct an MST Demonstration Project focusing on two of the following four areas where the Washington State Department of Health (WDOH) has restricted shellfish harvest: Rocky Bay, Vaughn Bay, Filucy Bay, and Burley Lagoon (Figure 1). These four embayments in South Puget Sound were evaluated for the QAPP by analyzing historical data and Vaughn Bay was selected for the following reasons:

- It has the greatest amount of downgraded shellfish area (96 acres), which increases the potential benefit from source control (Figure 2 and Table 1).
- Tidal flushing is restricted by a long spit extending across the bay, which increases the importance of freshwater discharges to the shellfish harvesting status of marine waters.

- Watershed drainage is not complex, which allows for a simple sample design based on a limited number of monitoring locations targeting two primary streams draining most of the watershed and inclusion of inputs from problem drains to the bay (Figure 3).

- Rocky Bay has a relatively small downgraded shellfish area (30 acres) and the watershed is very large and primarily forested.

- Filucy Bay has complicated hydrology including several streams and separate embayments, including one embayment that is prohibited due to the presence of a marina.

- Burley Lagoon's watershed is primarily located in Kitsap County, who is responsible for source control and recently initiated an MST study in the watershed (KPHD 2017).

The Vaughn Bay watershed (Figure 3) is located on the northwest side of the Key Peninsula in western Pierce County, immediately south of Rocky Bay, and comprises approximately 3,600 acres. The upland portion of the Vaughn Bay Watershed includes approximately 43 farms and 530 registered OSS (Table 1). In August 2015, 55 acres of shellfish beds were downgraded from Approved to Conditionally Approved in Vaughn Bay. This led to the formation of the Vaughn Bay Shellfish Protection District in 2016. An additional 40 acres were downgraded from Approved to Conditionally Approved in July 2016. In 2016, 64 percent of the marine monitoring stations failed the 90th percentile shellfish harvesting criterion of 43 CFU/100 mL (without removing larger precipitation events of 0.5 or 1.0 inches in 24 hours). (see Table 1).

Table 1. MST Study Area Characteristics.

Watershed	Percent Failed Marine Stations in 2016[a]	Impacted Bay Area (acres)[b]		Watershed Area (acres)	2017 Shellfish Report	
		Restricted	Conditional		Number of Registered OSS	Number of Farms
Rocky Bay	30	12.3	17.6	12,000	641	114
Vaughn Bay	64	0	95.9	3,600	530	43
Filucy Bay	17	8.4	66.7	3,305	356	41
Burley Lagoon	7	8.4	233.3	10,000	547	13

[a] Percentage of marine monitoring stations failing the 90th percentile shellfish harvesting criterion of 43 CFU/100 mL in 2016 without removing larger precipitation events (0.5 or 1.0 inches in 24 hours).

[b] Bay area water quality does not support Approved Growing Area Classification.

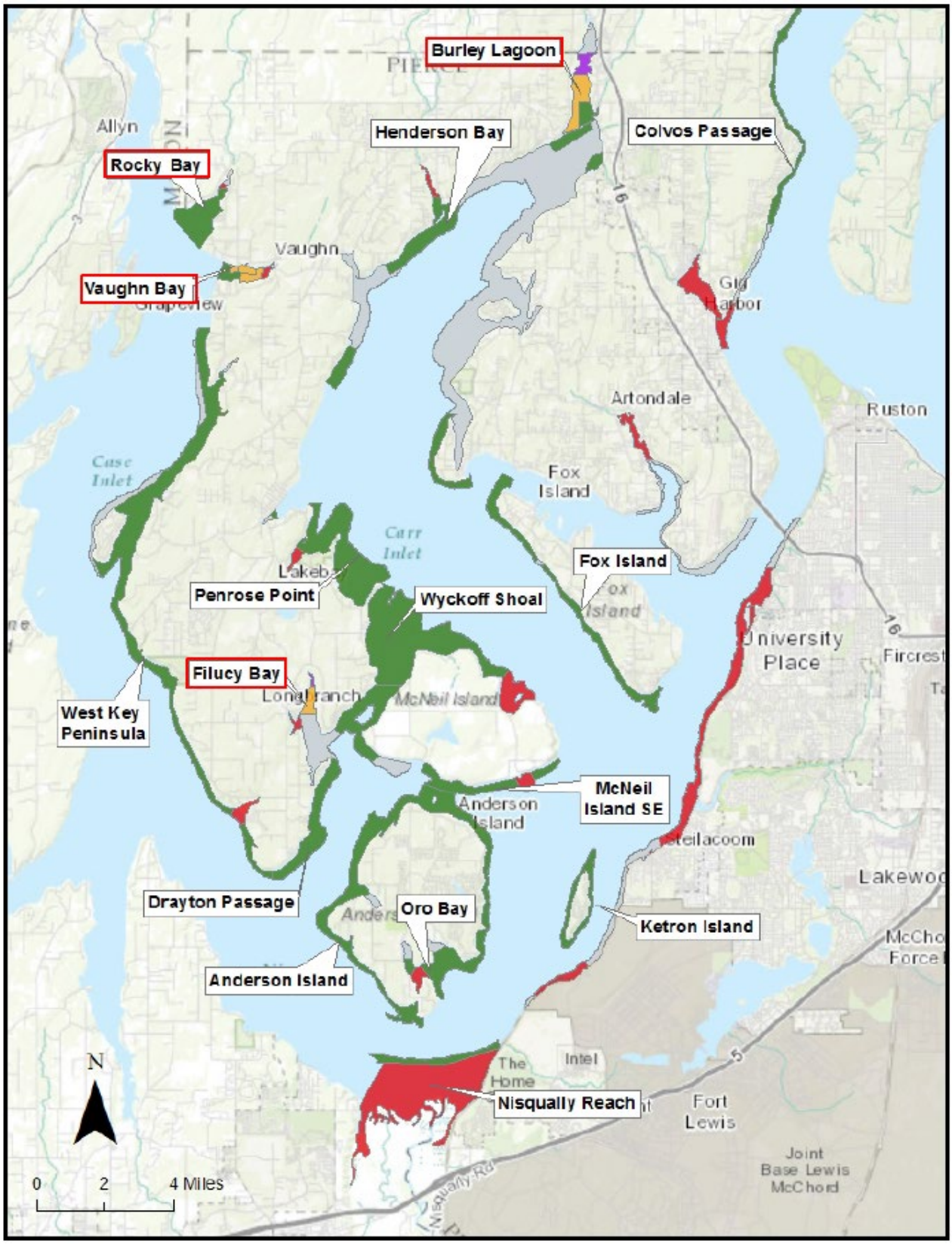


Figure 1. Pierce County Shellfish Growing Areas.

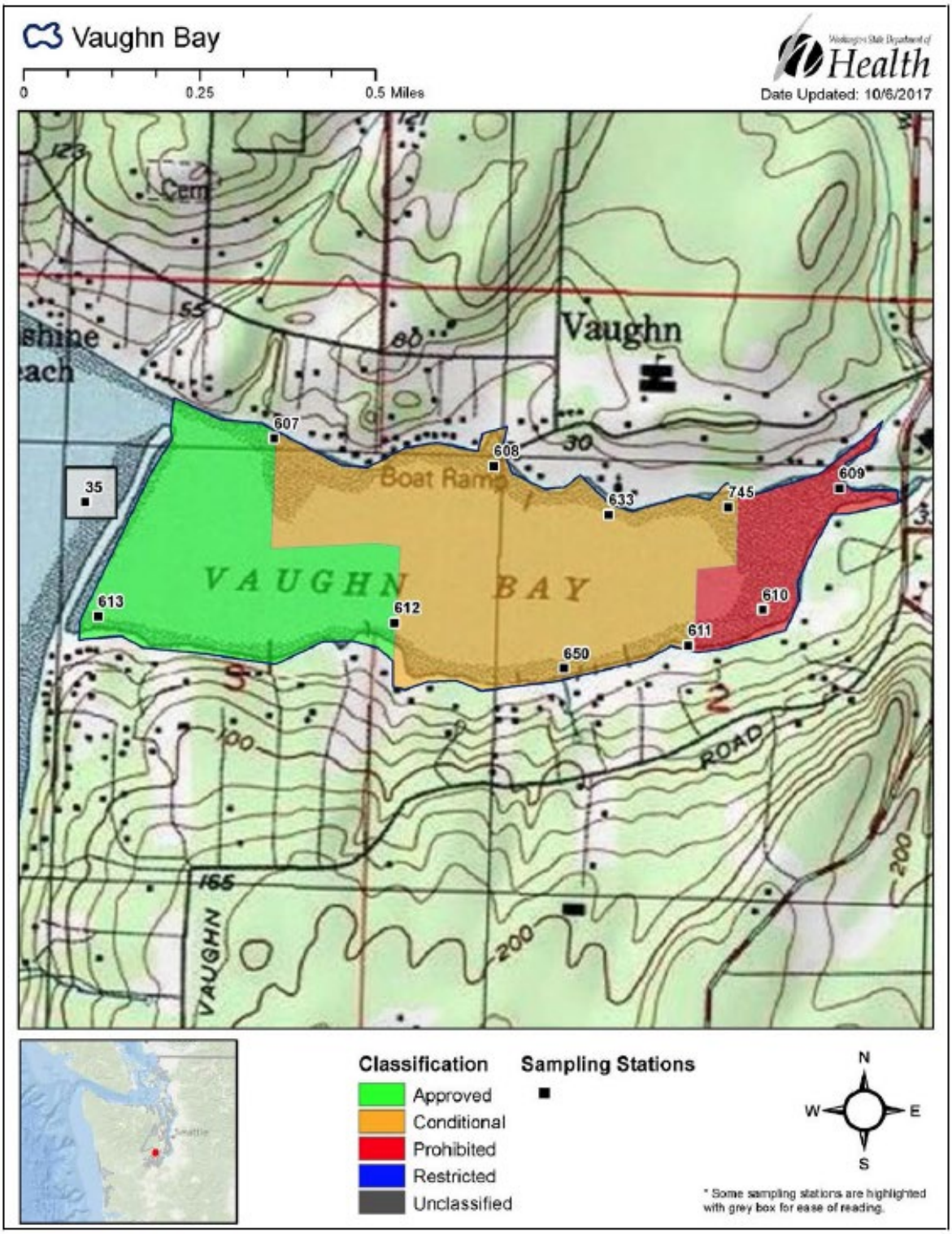
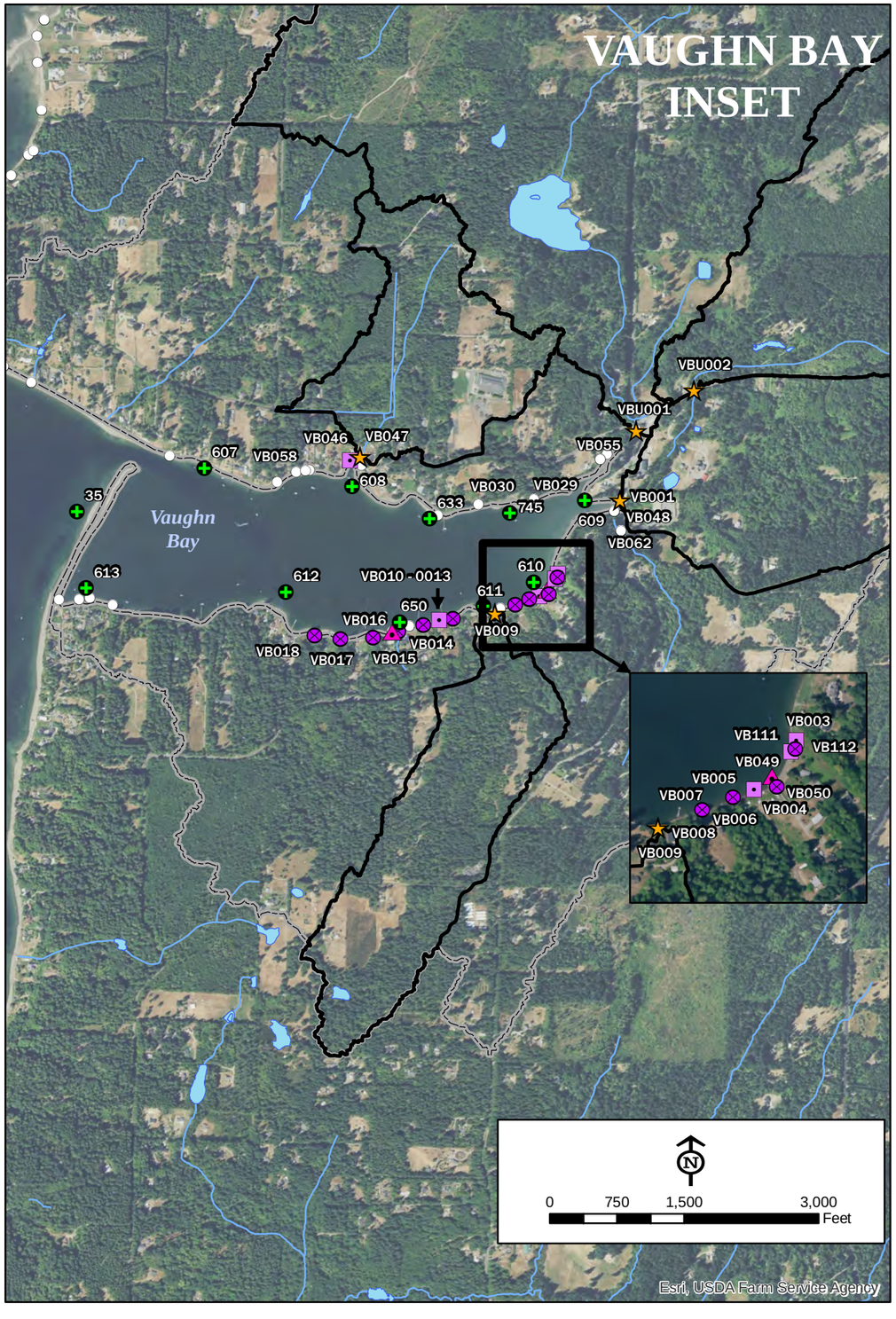


Figure 2. Vaughn Bay Shellfish Growing Area Classification, October 2017.



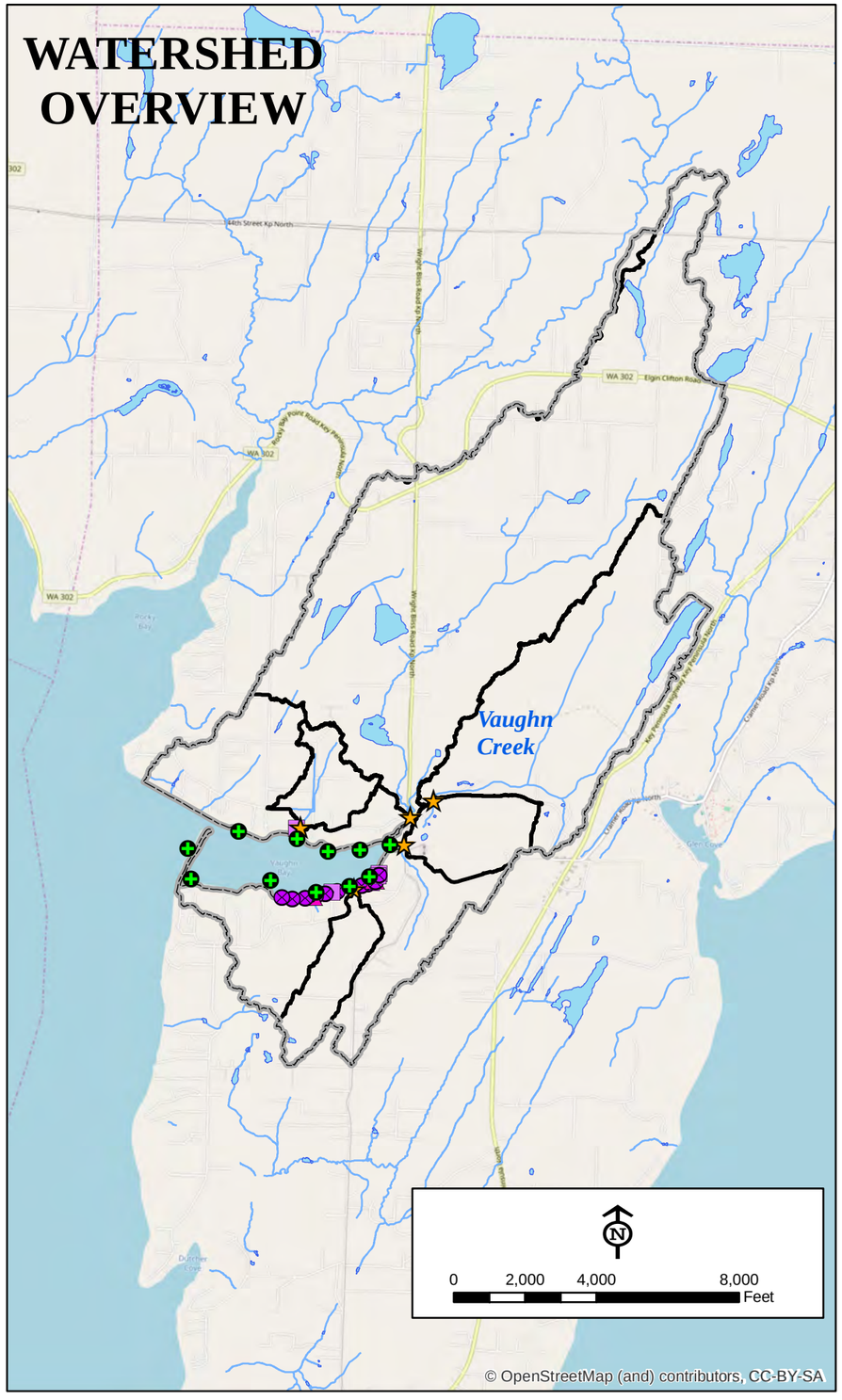


Figure 3.
Vaughn Bay Watershed and Sampling Stations.

Legend

- Marine Sampling Stations

Freshwater Sampling Stations

- Other
- Land Runoff
- Pipe
- River/Stream
- Stream station basin boundary
- Stations with <10 samples
- Vaughn Bay Watershed
- Highway
- Waterbodies
- Stream

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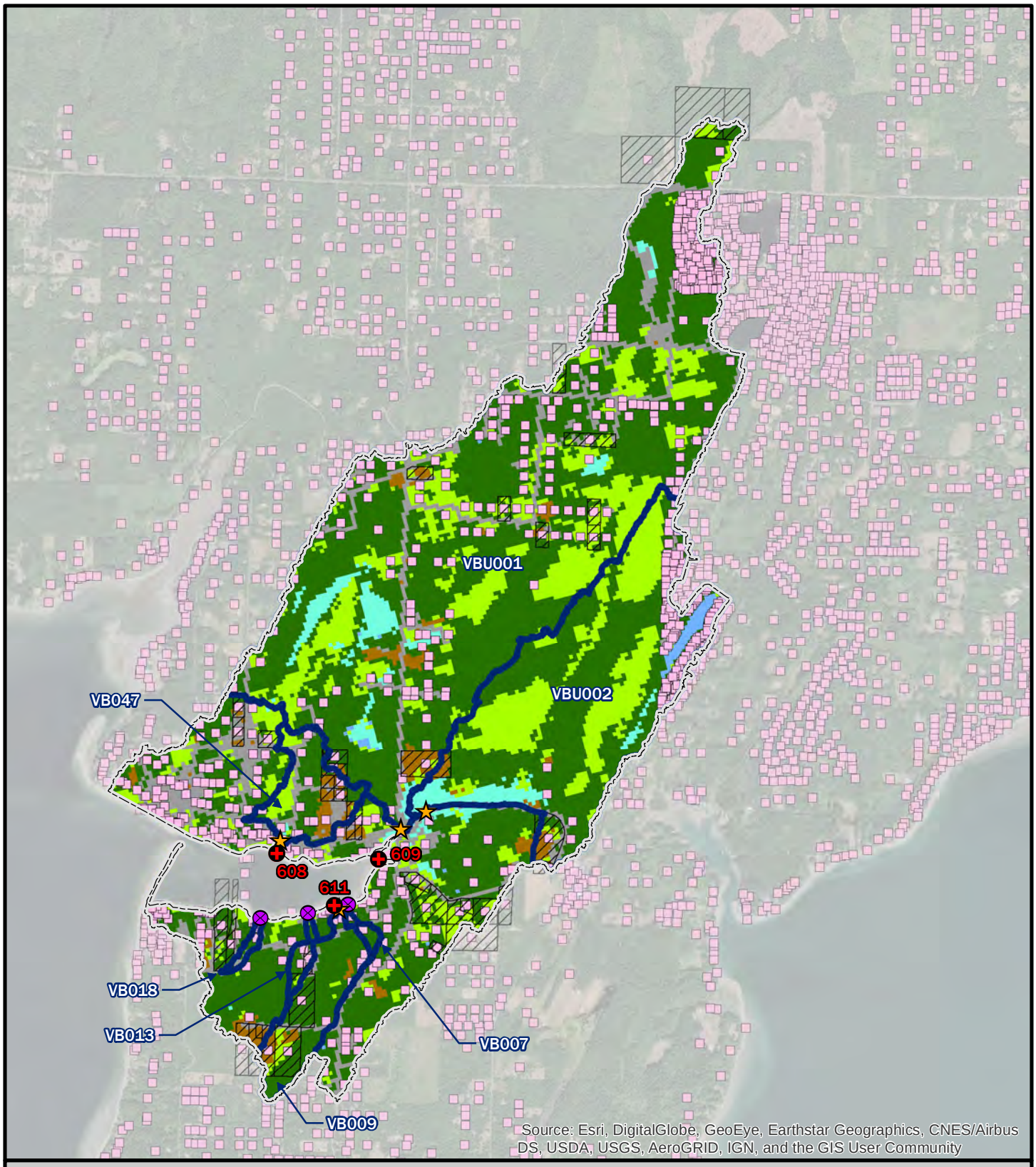
Background information on the Vaughn Bay watershed was used to develop the following assumptions of fecal sources likely present within the watershed for selection of sample station locations and qPCR host-associated marker analyses:

- The watershed is primarily forested and contains a variety of wildlife fecal sources, including birds, waterfowl (ducks, geese, and gulls), large animals (deer and bear), and small animals (raccoons and rodents).
- There are no large developments with municipal wastewater sewer systems or outfalls.
- There are no large dairies or other agricultural operations with large numbers of livestock.
- There are numerous homes and small farms with onsite septic systems that may include some failing systems and old systems with poorly functioning drain fields discharging sanitary waste to surface waters.
- There are numerous homes and small farms with pets, including dogs and cats.
- There are numerous small farms with a variety of farm animals, including cows, horses, pigs, llamas, geese, and chickens.
- Most of the watershed drains to the bay via two large streams (Vaughn Creek and an unnamed stream to the west, which is referred to as West Vaughn Creek in this report) and includes at least two small, unnamed streams.
- Numerous small drainages discharge freshwater to the bay from properties located near the bay shoreline.

Land cover in the Vaughn Bay watershed is presented in Figure 4. The predominant land cover is forest and closely followed by grassland/pasture. Among the monitored subbasins, the area draining to station VB047 contains the highest percent cover of developed and planted/cultivated land.

Septic system locations are also shown on Figure 4 and their density within each subbasin is presented in Table 2. Septic system density ranged from 0.09 to 0.24 per acre in the monitored subbasins. The unmonitored subbasin has a higher septic system density of 0.33 per acre, primarily due to dense systems located west of subbasin VB047. The lowest septic density is in subbasin VB009, which drains to the south unnamed stream. Dense clusters of septic systems are present in the upstream portions adjacent to the northeast boundaries of Vaughn Creek subbasin VBU002 and the unnamed, large stream subbasin VBU001, which is referred to as West Vaughn Creek for this study. Dense clusters of septic systems are not present in the other monitored subbasins.



Legend

- Vaughn Bay Watershed
- Monitored Subbasin
- Farm Parcels
- Marine Sampling Station
- Drain Sampling Station
- Stream Sampling Station
- Septic System

Land Cover (NCLD 2016)

- Developed
- Forest
- Grassland/Pasture
- Open Water
- Planted/Cultivated
- Wetlands

Figure 4.
Land Cover in the Vaughn Bay Watershed.

N

0 1,750 3,500 7,000 Feet

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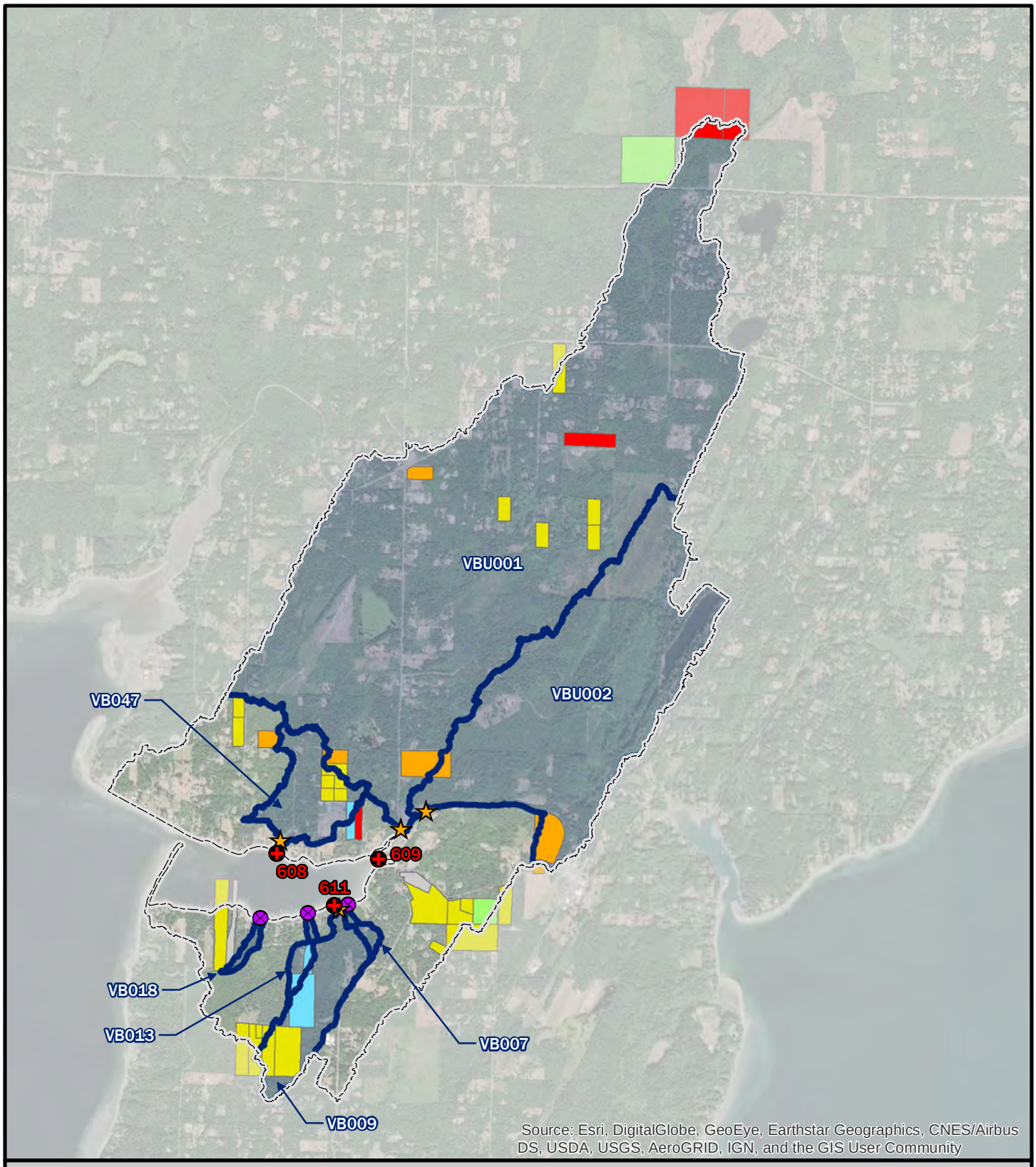
Table 2. Land Cover and Farm Ratings in Subbasins of the Vaughn Bay Watershed.						
Freshwater Station Subbasin	**Basin Area (acres)**	**Septic System Count[a]**	**Septic System Density (#/acre)**	**Farm Area (acres)**	**Farm Area Percentage**	**Average Pasture Condition Score (area weighted)**
VBU001	1956.0	304	0.16	73.4	3.8%	3.5
VBU002	721.5	129	0.18	22.9	3.2%	4.0
VB047	106.3	19	0.18	15.1	14.2%	2.5
VB009	126.1	11	0.09	46.2	36.6%	2.0
VB007	9.1	2	0.22	0.0	0.0%	NA
VB013	16.4	3	0.18	6.5	39.6%	1.5
VB018	8.3	2	0.24	0.7	8.9%	3.0
Unmonitored Area	614.3	200	0.33	98.1	16.0%	3.0

[a] Includes registered and potential unregistered septic systems.

Known farm parcels and pasture condition ratings for those farms inspected by the Pierce Conservation District are presented in Figure 5 and summarized in Table 2. Known farm area as a percentage of the subbasin area ranged from 0 to 40 percent. No known farms are in subbasin VB007, which drains only 9 acres to south shore. The West Vaughn Creek and Vaughn Creek subbasins VBU001 and VBU002, respectively, had the next lowest known farm cover at 3 to 4 percent of the subbasin area, but they also had the highest area-weighted pasture condition score of 3.5 to 4, indicating poor pasture conditions. The highest known farm densities of 37 to 40 are in subbasins VB009 and VB013, which drain to the south shore, and they had good average pasture ratings of 1.5 to 2. Thus, there is a tendency for large farm areas to have good pasture conditions and for small farm areas to have poor pasture conditions in the Vaughn Bay watershed.



Legend

- Vaughn Bay Watershed
- Monitored Subbasin
- Marine Sampling Station
- Drain Sampling Station
- Stream Sampling Station

Pasture Condition Ratings

- Not assigned
- 5 - Poor (visible)
- 4 - Poor (probable)
- 3 - Fair
- 2 - Good
- 1 - No Livestock

Figure 5.
Farm Ratings in the Vaughn Bay Watershed.

N

0 1,750 3,500 7,000 Feet

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PROJECT OBJECTIVES

Project objectives include:

- Leverage ongoing Shellfish Protection District efforts at collecting fecal bacteria samples in marine waters and freshwater drainages for collecting additional water samples at no cost to this project for MST analysis, with exception for a few strategically designed sampling events to fully meet the MST project objectives.
- Use two types of advanced molecular MST methods (single host-associated marker quantification by qPCR and whole community analysis by NGS) to characterize human and other animal sources at locations exhibiting high fecal bacteria concentrations in marine waters and freshwater drainages.
- Use a library independent, culture independent, EPA-preferred, and commercially available MST method consisting of quantitative polymerase reaction (qPCR) to quantify controllable fecal sources from humans and other animals (e.g., common livestock and pets) present in the collected water samples.
- Use a library dependent, culture independent, and microbial community-based MST method consisting of high-throughput DNA sequencing and advanced bioinformatics to identify bacteria sources present in the collected water samples.
- Supplement the existing community-based MST method library with at least 10 fecal samples each from humans (septic tanks) and other animals in the local area.
- Use the fecal source samples to assess the performance (sensitivity and specificity) of each host-associated marker analyzed by qPCR before analysis of the water samples.
- Evaluate relationships between the MST results and bacteria data, land use data, climate data, and waste management data using geospatial statistical analysis.
- Evaluate relationships between sources observed in marine waters and freshwater drainages to determine where and when marine water contamination is caused by freshwater discharges.
- Prioritize locations of the identified sources and identify appropriate methods for controlling high priority sources based on past experience.
- Evaluate the cost-effectiveness of the two MST methods for identifying high priority sources for control.
- Develop MST method protocols for cost-effective application in other watersheds based on the project findings.
- Involve key stakeholders throughout the project to enhance the project site understanding, experimental design, data interpretation, and source control.

PROJECT HYPOTHESES

The primary objective of this MST demonstration project is to explore the application of qPCR and community-based MST methods to aid management of shellfish protection areas to generate investigative leads providing actionable outcomes. For this study, criteria have been developed to help prioritize selection of a watershed, sampling stations, sampling schedule, and MST method to address the following hypotheses:

- H_1 – Fecal coliform bacteria concentrations in downgraded shellfish protection areas are primarily affected by loadings in freshwater discharges to those areas.
- H_2 – Fecal coliform bacteria concentrations in downgraded shellfish protection areas and freshwater drainages to those areas are higher during storm events than base flow events.
- H_3 – Fecal coliform bacteria concentrations in downgraded shellfish protection areas and freshwater drainages to those areas are highest during the seasonal first flush conditions in the fall and in the wettest years.
- H_4 – Sources of fecal coliform bacteria present in downgraded shellfish protection areas and watershed drainage will vary spatially, temporally, and hydrologically.
- H_5 – Sources of fecal coliform bacteria present in shellfish protection areas and watershed drainage may include humans from onsite septic systems and/or multiple types of farm animals, pets, and wildlife located in the watershed draining to those areas.
- H_6 – The qPCR and community-based MST methods will identify fecal sources present in the collected marine and freshwater samples.
- H_7 – Characterization of fecal sources in the collected samples will increase the ability to identify effective corrective actions for upgrading shellfish protection areas.

METHODS

MST METHOD SELECTION

Two state-of-the-art MST methodologies were selected for this study: single host-associated markers by qPCR and whole microbial community analysis by next generation sequencing (NGS). The qPCR method was the focus technology using Source Molecular, which is the nation's only accredited qPCR MST analysis lab, while NGS was performed at a very low cost to the project by leveraging resources available via collaboration with leading researchers in this field. The selected qPCR method is a non-library method where specific DNA fragments from bacterial groups associated with a specific fecal host (human or animal) source are quantified in extracts of filter residue of water samples without a need for matching those DNA patterns to a library of DNA from specific animal and human fecal material. The selected NGS method is a library method where DNA patterns of many different bacteria species are determined from extracts of filter residues from water samples and compared to DNA patterns in a library of DNA from specific animal and human fecal material.

The qPCR method was selected because this technology is currently the most mature and quantitative MST methodology. Comprehensive method evaluation has been published to confirm its high performance. Standardized protocols have been developed and are available for many fecal sources. The qPCR method protocols were either developed by EPA or recommended from a large method evaluation study in the California MST manual. Rigorous qPCR data acceptance criteria have also been published and are available to ensure highest MST data quality. Method diagnostic sensitivity and specificity will be reported, along with a range of measured marker concentrations and limit of quantification. The qPCR method is the most commonly used molecular MST method in Washington state, and is currently being used by the EPA Region 10 Manchester Laboratory and the King County Environmental Laboratory. Source Molecular's involvement in this study provided key advantages given they are a commercial laboratory offering high quality analysis and rapid data turnaround, and they would be available for use in future studies without having to obtain an agreement with a government or university laboratory.

The community-based NGS method selected for this project greatly complements the qPCR method because it is able to: 1) identify fecal sources that currently do not have a single marker qPCR assay, 2) distinguish subtypes of human fecal sources (septage vs. sewage) which is not possible by qPCR human marker assays, 3) estimate fecal contribution from different sources via sophisticated bioinformatics, and 4) provide an additional line of evidence along with qPCR results. The advantage of using the University of Minnesota BioTechnology Institute is that they are providing the analysis at a very low cost, have developed a fecal source genetic library with

access to libraries developed by others, and successfully used this MST method for various water bodies including Lake Superior embayments.

A key to success of the community-based NGS method is collection of local fecal sources to update the existing library of sources because of geographical differences in microbial DNA of fecal sources. Likely fecal sources in each study area were determined through review of previous investigations, land use data, and stakeholder knowledge. As described below, up to 10 individual samples from each identified fecal source were collected and used to validate and assess the performance of both MST methods in the local geographic region. Scientifically sound and published MST method validation approaches were followed.

Sample Station Selection

Sample stations were selected based on the following objectives:

- Include sample stations that have been monitored in the past and are planned for additional monitoring in the future as part of the South Sound Shellfish Recovery Project to:
    - Provide historical data for comparison to project data.
    - Leverage sampling and analysis resources for reducing project costs.
    - Ensure safe and reliable access.
- Include marine water stations located in conditionally approved or prohibited areas of the bay due to chronic fecal contamination.
- Include freshwater stream and drain stations representing most of fecal bacteria loading to the bay from the watershed.
- Exclude stream stations located upstream of human and farm animal sources, and where wildlife are the likely primary fecal sources that will not be managed for fecal source control.
- Exclude small drain stations that do not contribute high fecal bacteria loading to the bay from the watershed.

The following 10 stations were selected in the Vaughn Bay Watershed, which are listed in Table 3 and shown in Figure 6:

- Two marine stations (608 and 611) located in the conditionally approved area (where fecal coliform bacteria concentrations exceed shellfish harvesting criteria during large storm events). These stations are located on opposite shores (608 on north shore and 611 on south shore) and adjacent to stream sample stations to determine if fecal sources

causing the shellfish classification downgrade primarily originate from the adjacent streams.

- One marine station (609) located in the prohibited area (where fecal coliform bacteria concentrations exceed shellfish harvesting criteria during base and storm events). This station is located near the east shore adjacent to the mouth of the major streams (Vaughn Creek and West Vaughn Creek) discharging to the head of the bay.

- Four stream stations (VBU001, VBU002, VB047, and VB009) exhibiting high fecal coliform loadings to identify primary fecal sources from the majority of the watershed in West Vaughn Creek and Vaughn Creek (VBU001 and VBU002, respectively), one unnamed small stream draining to the north shore (VB047 adjacent to marine station 608), and one unnamed small stream draining to the south shore (VB009 adjacent to marine station 611). The stream station located at the mouth of Vaughn Creek (VB001) was not included for sampling because the additional drainage area below station VBU001 is small and sample collection at the mouth can be restricted at high tides.

- Three drain (land runoff or very small stream) stations (VB007, VB013, and VB018) to identify primary fecal sources from small catchments that drain directly to the bay, and have exhibited elevated fecal bacteria concentrations and loadings in the past.

Table 3. MST Study Water Sample Locations.

Station ID	Station Type	Station Location/Status	Coordinates	
			Latitude	Longitude
608	Marine	Conditional North Shore	47.34180	-122.77644
611	Marine	Conditional South Shore	47.33823	-122.77040
609	Marine	Prohibited East Shore	47.34155	-122.76595
VBU001	Stream	Prohibited East Discharge	47.343726	-122.763704
VBU002	Stream	Prohibited East Discharge	47.345014	-122.761137
VB047	Stream	Conditional North Discharge	47.342715	122.776089
VB009	Stream	Conditional South Discharge	47.338035	-122.769837
VB007	Drain	Conditional South Discharge	47.214206	-122.752377
VB013	Drain	Conditional South Discharge	47.337622	-122.773066
VB018	Drain	Conditional South Discharge	47.337207	-122.777947

MST qPCR Marker Selection

The initial number of host-associated fecal source markers analyzed by qPCR in the water samples was increased to seven from five originally proposed. This increase allows for monitoring of a broader range of pollution sources in response to the multiple potential sources identified by the background data evaluation. All seven markers were analyzed in all marine and freshwater samples regardless of whether they are detected in the initial samples because their presence could change as the study progresses.

The following seven high priority markers were selected for analysis:

1. Human marker HF183/BacR287 EPA (Green et al. 2014a). Other human markers are available, but the HF183/BacR287 EPA marker was selected because it has high sensitivity (low false negatives) and has been shown to be abundant in septic tanks in this region (Herrera 2017).

2. Cow marker CowM2 (Shanks et al. 2008). Other cow qPCR markers are available, but the CowM2 marker was selected because it can distinguish between cattle and other ruminants.

3. Horse marker HoF597F (Dick et al. 2005). This is the only horse qPCR marker available that has received wide method validation, and it was selected because horses are abundant in the watershed.

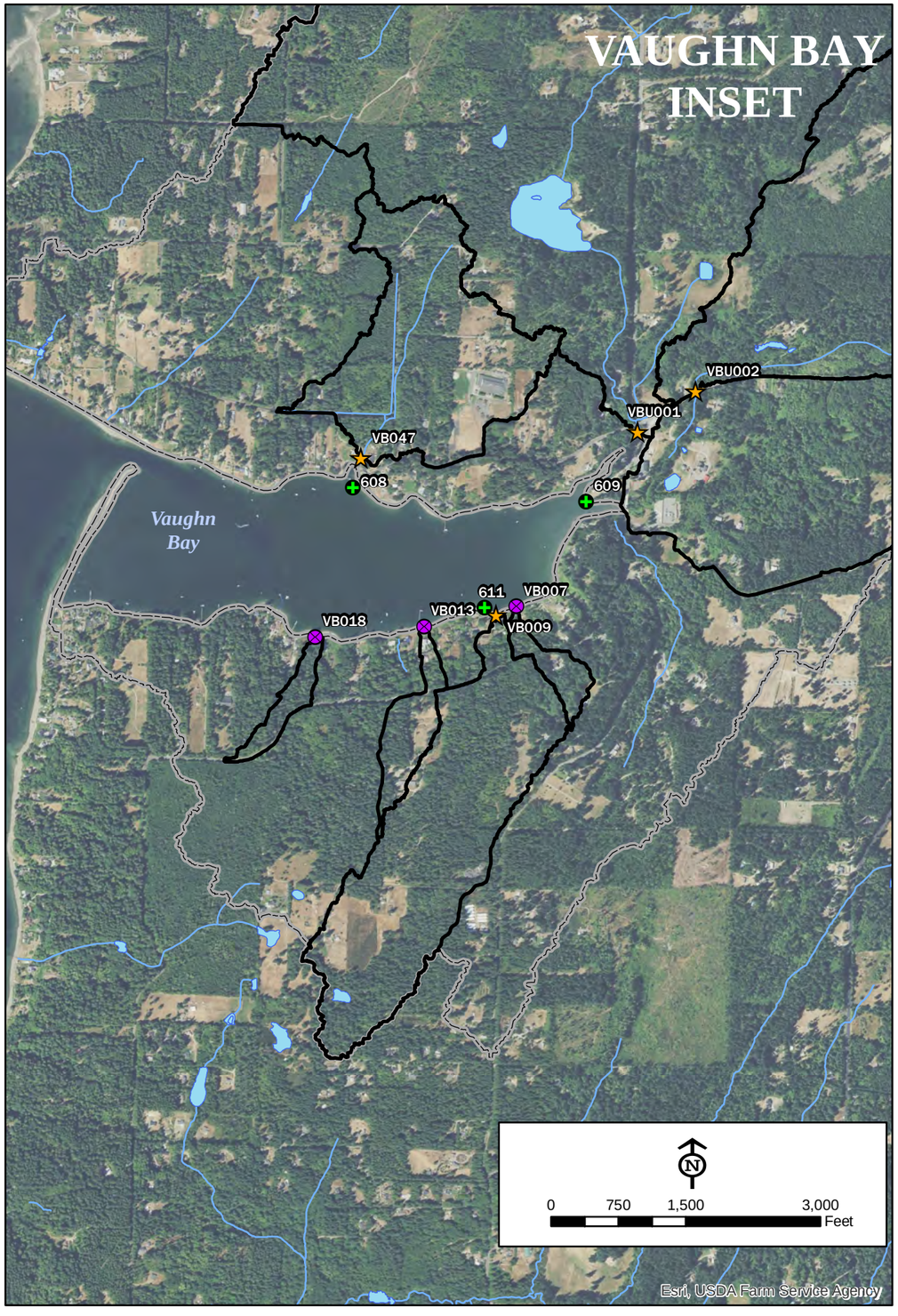
4. Ruminant marker Rum2Bac (Reischer et al. 2006). Other general ruminant qPCR markers and one elk host-associated qPCR marker are available, but the Rum2Bac marker was selected because it has a higher sensitivity for ruminants (sheep, goat, llama, and deer), which are all present in the watershed (Rene Skaggs, Pierce Conservation District, personal communication).

5. Pig marker Pig2Bac (Mieskin et al. 2009). This is the only pig qPCR marker available that has received most validation effort prior to this study, and pigs are present in the watershed.

6. Dog marker DG3 (Green et al. 2014b). Other dog qPCR markers are available, but the DG3 marker was selected because it has been successfully employed in other Pacific Northwest studies.

7. Bird marker GFD (Green et al. 2011). Other bird qPCR markers are available that are more specific to gulls, geese, chickens, and poultry litter. The GFD marker was selected because gulls, geese, and other birds are abundant in the watershed; and it is anticipated that differentiation between types of birds will not be needed for identifying fecal source controls.

These seven markers potentially would have been modified if the qPCR analysis of the fecal source sample analysis results were not acceptable, either because the source samples did not contain an acceptable amount of the targeted source marker or contained an unacceptable amount of a non-targeted source marker. The horse and bird markers did not meet all quality control objectives (see Data Quality Assessment). However, results of the fecal source sample analysis were found to be acceptable because better alternative markers were not available. Therefore, the list was not modified to include any substitute markers.



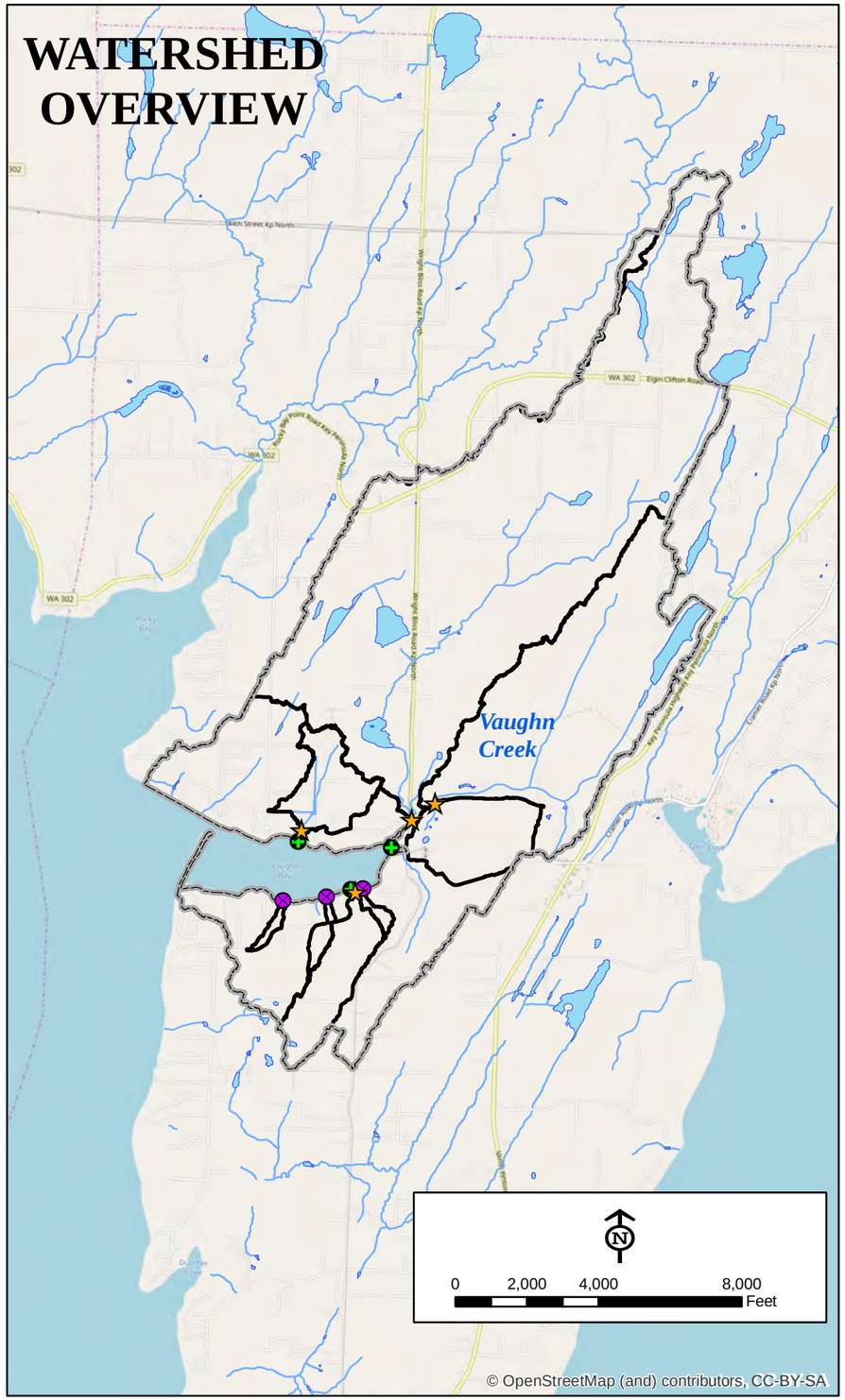


Figure 6.
MST Project Sampling Stations in Vaughn Bay Watershed.

Legend

- Marine Sampling Stations
- Drain Sampling Station
- Stream Sampling Station
- Sampling station basin boundary
- Vaughn Bay Watershed
- Highway
- Waterbodies
- Stream

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SAMPLING AND ANALYSIS

The sampling process design included collection of marine water samples in Vaughn Bay and freshwater samples (i.e., stream and drain) in the Vaughn Bay watershed. Fecal source samples were also be collected for quality control, as described below. Additional information about the experimental design is provided in the QAPP. The planned versus actual sampling and analysis is summarized in Table 4.

Sampling Schedule

Water samples were to be collected as grab samples at all 10 stations from August 2018 through January 2019 during five routine monthly events and three targeted storm events (defined as greater than 0.20 inches of rain within the previous 24 hours but targeting over 0.50 inches of rain). The five monthly sampling events were to coincide with the monthly marine water and PIC freshwater monitoring events for the Shellfish Recovery Project (without using project budget). The three targeted storm events were to be conducted in September through December 2018 specifically for the MST project (using project budget).

Water sampling proceded as specified by the QAPP through collection of the first four routine monthly events in August through November and one targeted storm sampling event in November 2018. The water samples were analyzed as planned for bacteria enumeration and filtered for MST analysis by the local bacteria lab (Centric Analytical Labs in Port Orchard, Washington). All frozen filters from all four events, including the duplicate samples, were shipped overnight with 2 pounds of dry ice to Source Molecular after the fourth event. Source Molecular observed that the dry ice had melted and the samples had thawed in transport. More importantly, the polycarbonate membrane portion of the filter had been removed and the filter funnels contained only the undelying cellulose support pad, which was unlikely to concentrate the fecal bacteria and DNA present in the water sample. Without notifying the project team, the bacteria lab removed the polycarbonate membrane to allow passage of water through the filter funnels. Thus, QAPP procedures were not followed and none of the water samples collected in Events 1 through 4 were deemed acceptable for MST analysis. Extra unfiltered water from samples collected during Events 2 through 4 had been stored in the refridgerator at the bacteria lab, but delayed filtration of those water samples was deemed unacceptable due to the likely degradation of fecal DNA.

In response to the loss of water samples collected for MST analysis during Events 1 thought 4, a QAPP addendum (Herrera 2018b) was prepared to revise the sampling schedule to add three routine monthly events from January through March 2018 for a total of seven events instead the the planned eight events. In addition, quality control procedures were implemented that included training bacteria lab technicians on proper MST sample filtration procedures specified in the QAPP, use of a more powerful filtration pump, sending only the primary sample filter with each shipment, and increasing the amount of dry ice to 5 pounds for each filter shipment.

Table 4. Original and Revised MST Project Sampling and Analysis Plan.

Water	Original Planned	Revised Actual
Sample Events	5 Monthly (Aug–Dec) 3 Storm (Sept–Dec) **8 Total**	4 Base (Dec–Apr) (+4 Bacteria only) 3 Storm (Nov–Mar) **7 Total**
Sample Stations	3 Marine 7 Freshwater **10 Total**	3 Marine 7 Freshwater **10 Total**
Number of Samples	24 Marine 56 Freshwater **80 Total**	21 Marine (+12 Bacteria only) 49 Freshwater (+28 Bacteria only) **70 Total (+40 Bacteria only)**
qPCR Marker Analysis	7/sample (human, cow, horse, ruminant, pig, dog, and bird) **560 Total**	7/sample (human, cow, horse, ruminant, pig, dog, and bird) **490 Total**
Fecal Source	**Original**	**Revised**
Fecal Source Samples	10 Septage (Human) 10 Cow (Cow) 10 Horse (Horse) 5 Sheep (Ruminant) 5 Goat (Ruminant) 5 Llama (Ruminant) 5 Pig (Pig) 10 Dog (Dog) 5 Canada Goose (Bird) 5 Gull (Bird) **70 Total**	11 Septage (Human) 10 Cow (Cow) 10 Horse (Horse) 5 Sheep (Ruminant) 5 Goat (Ruminant) 4 Llama (Ruminant) 5 Pig (Pig) 12 Dog (Dog) 7 Canada Goose (Bird) 12 Gull (Bird) 1 Alpaca (Ruminant) 6 Deer (Ruminant) **88 Total**
qPCR Marker Analysis	Discrete Source Samples: 10 Septage for Human (HF183 EPA) 10 Cow for Cow (CowM2) 10 Horse for Horse (HoF597F) 15 Sheep/Goat/Llama for Ruminant (Rum2Bac) 5 Pig for Pig (Pig2Bac) 10 Dog for Dog (DG3) 10 Goose/Gull for Bird (GFD) Composite Source Samples: 6 Non-human for Human (HF183 EPA) 6 Non-cow for Cow (CowM2) 6 Non-horse for Horse (HoF597F) 6 Non-ruminant for Ruminant (Rum2Bac) 6 Non-pig for Pig (Pig2Bac) 6 Non-dog for Dog (DG3) 6 Non-bird for Bird (GFD) **112 Total**	82* Discrete Source Samples for 8 Markers: Human (HF183 EPA) Cow (CowM2 and CowM3) Horse (HoF597F) Ruminant (Rum2Bac) Pig (Pig2Bac) Dog (DG3) Bird (GFD) **656 Total** *Insufficient DNA obtained from 6 source samples (1 Dog, 4 Gull, and 1 Goose)

The sampling schedule was revised again when the routine February event was cancelled due to a snow storm, and another routine event was added in April 2019. In addtion, the third targeted storm event was changed to a targeted base flow event in February 2019 to ensure that the sampling objective would be met to monitor a minimum of three base flow events and three storm flow events for evaluating effects of hydrologic conditions on fecal sources.

Table 5 presents the sampling schecule and event conditions for the four pre-MST events when only bacteria were enumerated in August through November 2018, the four base flow events January through April 2019, and three storm events from November 2018 through March 2019 when the preceding 24-hour rainfall total exceeded 0.20 inches.

Table 5. MST Study Sampling Schedule and Event Conditions.

Event Name	Event Type	Date	Marine Sample Time (hours)	Fresh-water Sample Time (hours)	Marine Tide Direction	Preceding Sample Rain (inches)	Storm Event Total Rain (inches)	Event Duration (hours)	Antecedent Dry Period (hours)
PreMST 1	Base (Routine)	8/15/18	1127–1150	1235–1428	Ebb	0.00	NA	87	NA
PreMST 2	Base (Routine)	9/20/18	1327–1340	1010–1240	Flood	0.00	NA	88	NA
PreMST 3	Base (Routine)	10/18/18	1242–1247	0955–1155	Flood	0.00	NA	217	NA
PreMST 4	Base (Routine)	11/1/18	1304–1316	0908–1050	Flood	0.00	NA	24	NA
Base 1	Base (Routine)	1/24/19	1053–1104	1228–1408	Ebb	0.08	NA	29	NA
Base 2	Base (Target)	2/21/19	0844–0918	1105–1245	Ebb	0.01	NA	116	NA
Base 3	Base (Routine)	3/7/19	0932–0944	1000–1230	Ebb	0.02	NA	13	NA
Base 4	Base (Routine)	4/9/19	1028–1039	1102–1327	Ebb	0.08	NA	15	NA
Storm 1	Storm (Target)	11/27/18	1105 – 1111	1106–1353	Ebb	1.96	1.97	31	67
Storm 2	Storm (Routine)	12/18/18	1251–1301	0840–1048	Flood	1.10	1.17	22	13
Storm 3	Storm (Target)	3/12/19	0950–1010	1140–1321	Ebb	0.62	0.62	12	74

[a] Pre-sample rainfall total: Amount of rain from beginning of event to time that sampling is completed.

[b] Event duration: Time elapsed during event with base event defined as <0.04 inches of rain in 6 hours and storm event defined as ≥ 0.04 inches in 6 hours.

[c] Antecedent Dry Period: Time elapsed for base flow event preceding a storm event.

NA not applicable

Storms 1 and 2 occurred during the only two occasions within the study period that the conditionally approved area of Vaughn Bay was closed because the rain amount exceeded 1 inch. The conditionally approved area was closed from November 27 through December 2, 2018, due to 1.82 inches of rainfall and from December 18 through 23, 2018, due to 1.02 inches of rainfall (J. Frost, WDOH, personal communication).

Fecal source sampling was to be completed in September 2018 before the final selection of markers for analysis in the first batch of water samples. Fecal source sampling was completed in October 2018 except for supplemental sampling of selected sources in January 2019. This delay did not impact the project because it was decided to analyze all MST water samples in one batch when sampling was complete in April 2019. Analysis of one batch instead of two batches reduces the potential for variance in analytical technique.

Water Sampling

Freshwater and marine water sampling was conducted on the same day for each event. TPCHD collected all freshwater samples, WDOH collected marine water samples for all routine events, and SIT collected marine water samples for the three targeted events (see Table 5). Samples were collected during daylight for safety. Sample times were adjusted as needed to access the freshwater stations during a low to moderate tide elevations and to access marine stations during a moderate to high tide elevation. WDOH identified the routine sample dates and TPCHD coordinated with SIT for the targeted sample dates. The marine water samples were transferred to TPCHD staff at the Vaughn Bay boat launch shortly after being collected. TPCHD delivered the samples to CAL for analyzing and processing, except that the bacteria samples collected for the targeted events were delivered to the WDOH Lab.

Field measurements included optical brightener and flow at the freshwater stations, and temperature and salinity at the marine water stations. Optical brightener fluorescence was measured with a Turner Designs Cyclops 7 probe and DataBank data logger. Flow was measured using a current meter or graduated bucket, or was visually estimated on occasion.

Water samples were collected for each event from all 10 stations (see Figure 6) for the following laboratory analyses:

- Fecal coliform bacteria in marine water samples analyzed by WDOH Lab (routine events) and CAL (targeted events)
- Fecal coliform bacteria and *E. coli* in freshwater samples analyzed by CAL
- Filtration of all MST water samples by CAL for freezing and shipping to Source Molecular
- DNA extraction and qPCR marker analysis of MST water samples by Source Molecular

- Community-based MST analysis by UMBTI from DNA extracts prepared by Source Molecular

- qPCR analysis of MST water samples by the King County Environmental Laboratory, only for the last sampling event in April 2019 (which was not planned but added during the study for interlaboratory comparison to Source Molecular qPCR results)

Each water sample was collected aseptically in a 1-liter, sterile plastic bottle. Separate aliquots were poured from this bottle into a bottle for fecal bacteria and *E. coli* analyses, and a bottle for MST sample filtration to ensure those aliquots originate from the same grab and contain the same fecal sources. Freshwater samples will be collected using the procedures described in Pierce County Pollution Identification and Correction Enhancement Project QAPP (TPCHD & WDOH 2013). Marine water samples will be collected from approximately 15 centimeters (cm) (6 inches) below the surface using the "U" scoop motion to address the fact that bacteria may be concentrated in the surface micro layer.

Samples were immediately placed in iced, insulated coolers. Routine marine bacteria samples were transported to the WDOH Public Health Laboratories in Seattle, Washington and processing began within 30 hours after collection. Targeted event marine bacteria samples and all freshwater samples were transported to CAL in Port Orchard, Washington for processing on the day of collection in most cases or within 24 hours after collection. Separate aliquots were poured from each sample bottle for fecal bacteria analysis and MST sample filtration to ensure those aliquots contained the same fecal sources.

Field sampling activities were recorded on field sampling sheets during the collection of water samples (see Appendix A). Station identification, location, sampling time, sampling date, weather, and the sample collector's name were recorded. Detailed observational data from each station was also recorded, including water appearance, biological activity, stream uses, unusual odors, specific sample information, and missing parameters or changes in procedures. The field sheets were scanned and emailed to Herrera following each sampling event.

A chain-of-custody form accompanied each set of samples (see Appendix B). The chain-of-custody form indicated the name of the collector of the samples, date and time of collection, number of containers, tests to be performed, shipper, receiver, and date and time of shipping and receiving.

All water samples for MST analysis were filtered by CAL using new 0.45-micron membrane filters. Two, 200 mL aliquots were filtered for each sample to provide one duplicate (split) sample for each water sample. A filter form was completed at the time of filtration and included with the sample shipment to Source Molecular (see Appendix B).

Once the MST samples had been filtered, the sample filters were placed in a labeled bead tube, which was placed in a Whirl-Pak bag and stored frozen at -20°C. The sample filters were shipped with dry ice to Source Molecular in three batches for DNA extraction and qPCR analysis. The DNA extracts were frozen at -20°C and shipped with dry ice to the University of Minnesota BioTechnology Institute in one batch for community-based MST analysis.

Fecal Source Sampling

Fecal source samples were collected to check the accuracy of the qPCR marker analyses and to supplement the existing community-based MST method library. Fecal source samples were collected aseptically from each of the following sources:

- Eleven septic tank wastewater samples from separate septic systems (three samples), one septage holding tank (three samples), and three septage pumping trucks (five samples) in the region
- Ten cow feces samples from separate fecal deposits at farms in the region
- Ten horse feces samples from separate fecal deposits at farms in the region
- Five sheep, five goat, and four llama, one alpaca, and six deer feces samples (representing ruminants) from separate fecal deposits at farms (or forest for deer) in the region
- Five pig feces samples from separate fecal deposits at farms in the region
- Twelve dog feces samples from separate fecal deposits at parks and private properties in the region
- Seven Canada geese feces samples from separate fecal deposits and 12 gull feces samples from multiple fecal deposits in the region

The septic tank and farm animal fecal samples were collected by TPCHD. The Canada geese, gull, and dog feces samples were collected by the SIT. The deer feces samples were collected by TPCHD and SIT. All fecal source samples were collected from the Key Peninsula area. The first batch of 77 samples were collected in September and October 2018, and the second batch of 11 samples were collected in February 2019. The second batch was collected to supplement the first batch with two dog, two goose, and seven gull feces samples because insufficient amounts of DNA were obtained from some of the first batch of samples.

Fecal source samples were immediately placed in a cooler with ice, transported to CAL, and stored frozen at -20°C. The frozen samples were shipped with dry ice for overnight delivery to Source Molecular in two batches that were received frozen and intact on October 30, 2018, and February 7, 2019.

The Canada Geese, gull, deer, and dog feces samples were collected along or near the shores of Vaughn Bay, with the majority of samples coming from the Vaughn Bay Sandspit at the mouth of the bay. For the septic tank (septage) samples, TPCHD contacted a number of septic pumpers to arrange sample collection. Due to the pumpers' busy schedules, this took much more effort than originally envisioned. TPCHD ended up working with two different companies to collect the samples. A total of eight samples were collected from two different pumper trucks, and three samples were collected directly from septic tanks. The samples were collected in sterile 500 mL bottles. The outside of each bottle was wiped with ethanol immediately after each sample was collected, and then wrapped with a paper towel and placed in a clean zip-lock bag to prevent contact with other fecal samples. The sample bottles were kept in a cooler with cold packs and delivered to CAL for freezing and shipping to Source Molecular.

To help find farmers to participate in the farm animal sampling, TPCHD developed a flyer that was then distributed by Pierce Conservation District. This led to the collection of several fecal samples but fell far short of the goal. TPCHD staff then called various farms and contacts within the area to arrange for additional sample collection. As with the septage sampling, identifying participating properties took significantly more time than expected.

For sample collection, staff wore disposable gloves and attempted to collect the freshest fecal material possible. Staff took pictures of the fecal material sampled and gathered information about the health status of the animals and their diet on field sheets (see Appendix A). Each animal fecal sample was collected using a sterile utensil, which was attached to the inside of a sterile plastic vial. Approximately a quarter-sized sample was collected from the interior of the fecal mass and placed into a plastic vial. The vial was then placed in a pre-labelled Whirl-Pak bag. The samples were kept in a cooler with cold packs and delivered the same day to CAL for freezing and shipping to Source Molecular.

A chain-of-custody form accompanied each set of samples (see Appendix A). The chain-of-custody form indicated the name of the collector of the samples, date and time of collection, number of containers, tests to be performed, shipper, receiver, and date and time of shipping and receiving. Photos, field sheets, and chain-of-custody forms were emailed to Herrera.

Laboratory Analysis

The analytical methods and reporting limits for laboratory analysis of the collected water samples were achieved as planned (Table 6). Exceptions include elevated reporting limits for three fecal coliform bacteria analyses (20 CFU/100 mL) and for all human qPCR analyses (34 copies/100 mL).

Table 6. Methods and Reporting Limits for Water Sample Analyses.				
Station-Event Type	**Parameter**	**Analytical Method**	**Method Number**	**Reporting Limit**
Marine-Monthly	Fecal coliform bacteria	Multiple tube	APHA A-1 modified	1.8 MPN/100 mL
Marine-Storm	Fecal coliform bacteria	Multiple tube	SM 9221 C/E	2 MPN/100 mL
Freshwater-All	Fecal coliform bacteria	Membrane filtration	SM 9222 D	1 CFU/100 mL
	E. coli	Quanti-Tray	SM 9223 B	1 MPN/100 mL
Marine-All Freshwater-All	qPCR markers	qPCR	EPA Method B modified	2–4 copies/ 100 mL
Marine-All Freshwater-All	Community-based MST	Laboratory	None	Not applicable

APHA = American Public Health Association (APHA 1970)

SM = Standard Methods (APHA et al. 2017)

EPA = EPA method (EPA 2010)

CFU = colony forming unit

mL = milliliter

MPN = most probably number

Laboratory responsibilities were as follows:

- Washington Department of Health (WDOH) Public Health Laboratories: – Fecal coliform bacteria analysis of marine monthly water samples
- Centric Analytical Laboratory (CAL) – Fecal coliform bacteria analysis of marine storm samples, fecal coliform bacteria and E. coli analysis of all freshwater samples, and filtration of all MST samples
- Source Molecular – DNA extraction of all fecal source and water samples, and qPCR marker analysis of all water samples
- University of Minnesota BioTechnology Institute (UMBTI) – Community-based MST analysis by next generation sequencing (NGS) of all fecal source and water samples
- King County Environmental Laboratory (KCEL) – qPCR analysis of all samples collected for the last sampling event in April 2019

CAL filtered all water samples for DNA extraction by Source Molecular. CAL prepared two filters of each sample (200 mL of sample water per filter) and stored the filters in a freezer at -20°C prior to overnight shipment with dry ice to Source Molecular for DNA extraction. The plan was to ship one duplicate filter and retain one filter for backup if needed. As noted above, both duplicate filters were shipped in one batch for the first four sampling events. One filter/sample was properly prepared, stored, and shipped for the remaining sampling events. One exception is that both duplicate filters for the last sampling event were shipped to Source Molecular to

provide enough DNA for additional qPCR analysis of those samples by KCEL. Source Molecular prepared all DNA extracts of all water samples and fecal source samples upon completion of all sampling for each matrix. Portions of the water and fecal source sample extracts were frozen and sent to UMBI. Extracts of the duplicate filters for the last sampling event were prepared, frozen, and shipped to KCEL.

Analytical methods and maximum holding times met recommendations by Standard Methods (APHA 1970, 2017) and EPA (2010, 2019). One exception is that the maximum holding time for fecal coliform bacteria and *E. coli* was been extended from the recommended 6 hours to 24 hours to allow for delivery of samples on the morning following a full-day sampling event.

WDOH Public Health Laboratories

WDOH Public Health Laboratories conducted fecal coliform bacteria analysis of marine monthly water samples using the multiple tube method (Method APHA A-1 modified in APHA 1970). This method is routinely used for marine water monitoring of all shellfish protection areas in Puget Sound, and is approved and required by the US Food and Drug Administration. Fecal coliform bacteria concentrations were determined by a five-tube decimal dilution test using the most probable number (MPN) with a detection limit of 1.8 MPN/100 mL. The method is similar to Method SM 9221 E (APHA et al. 2017), but uses A-1 broth and does not require prior enrichment in a presumptive medium.

Centric Analytical Labs

CAL conducted fecal coliform bacteria analysis of marine storm event water samples using the multiple tube method (Method SM 9222 C/E in APHA et al. 2017). This method is routinely used instead of the membrane filter method for marine water monitoring because the results are more reliable for turbid samples that are commonly collected from marine waters. CAL is certified by Ecology to use this method. Fecal coliform bacteria concentrations were determined by a five-tube decimal dilution test using the most probable number (MPN) with a detection limit of 2 MPN/100 mL. The method is a two-step process using lauryl tryptose broth as the coliform presumptive medium, followed by inoculation in EC broth and incubation at a higher temperature.

CAL conducted fecal coliform bacteria analysis of all freshwater samples using the membrane filter method (Method SM 9222 D in APHA et al. 2017). This method is commonly used for freshwater samples and has been used historically for the Pierce County PIC program. CAL is certified by Ecology to use this method. Fecal coliform bacteria concentrations were determined by filtering different sample volumes, adding the filters to the M-FC medium, incubation of the filters, counting colonies formed on the filters, and reporting results in colony forming units (CFU). The quality control objectives established for the fecal coliform membrane filter procedure are to filter a sample volume that yields an ideal range of 20 to 60 fecal coliform positive colonies on a culture plate to obtain statistically reliable results, and for not more than 200 colonies of all bacteria types to be present on a culture plate to ensure that the results are

not underestimated due to crowding (e.g., merged colonies or false negatives). Based on the expected range of concentrations, two sample volumes of 50 and 5 mL were analyzed to provide a detection range of 2 to 4,000 CFU/100 mL. The analysis method also provides guidance for calculation of fecal coliform density as follows:

- If one of the plate counts is between 20 and 60, then calculate the density for the sample volume yielding a plate count in this ideal range.
- If duplicate sample volumes were analyzed, then calculate the average density for both analyses.
- If all counts are outside the ideal range, then calculate the average density for all sample volumes analyzed, excluding counts greater than 200, by dividing the sum of the plate counts by the sum of the sample volumes, and multiplying by 100.
- If no plate counts less than 200 were obtained, but a plate had a total bacterial colony count greater than 200, then report the density as greater than the value associated with this plate count.

CAL conducted *E. coli* analysis of freshwater samples using the Quanti-Tray method, which is a semi-automated quantification method based on most probable number (Method SM 9223 B in APHA et al. 2017). This method is commonly used for freshwater samples and, in response to revised contact recreation criteria by Washington State Surface Water Quality Standards (WAC 173-201A), replaced fecal coliform bacteria analysis by the Pierce County PIC program in November 2017. CAL is certified by Ecology to use this method. *E. coli* bacteria concentrations were determined by inoculating a chromogenic substrate with 100 mL of sample, incubating the samples, and measuring fluorescence, and reporting results with a detection range of 2 to 2,400 MPN/100 mL.

CAL filtered two replicate aliquots of 200 mL using sterile techniques from the same 1-liter sample container used for fecal coliform and *E. coli* bacteria analyses. The filters were placed in sterile bead tubes, frozen at -20°C, and shipped to Source Molecular for DNA extraction. The filtration procedure, filter log, and shipping procedure are described in detail in the QAPP (Herrera 2018).

Source Molecular

Source Molecular performed extraction and qPCR analysis of all fecal source and water samples, as described separately below.

Water Sample Extraction and qPCR Analysis

The water sample filters received from CAL were homogenized for 1 minute and the DNA extracted using the GeneRite DNA-EZ ST1 extraction kit (GeneRite, New Jersey), as per the manufacturer's protocol. The extracted DNA samples proceed directly to qPCR analysis in

accordance with the laboratory standard operating procedures, which are proprietary and confidential but may be available upon request. A portion of the DNA extracts were frozen for storage and shipment to UMBTI for NGS analysis.

Host-associated marker analysis methods generally followed those described by Griffith et al. 2013 except for the bird marker analysis method, which generally follows Green et al. 2011. Amplifications to detect the target gene biomarker are run on an Applied Biosystems StepOnePlus real-time thermalcycler (Applied Biosystems, Foster City, California) in a final reaction volume of 20 microliter (µL) sample extract, forward primer, reverse primer, probe, and an optimized buffer. The final reaction volume was reduced from 25 µL prescribed by the method to reduce reagent cost, but the prescribed 2 µL DNA extract volume per reaction was used for all assays and increased to 4 µL for the CowM2 marker. All assays except human marker HF183 were run in duplicate. An assay is repeated if the duplicate results do not meet quality control objectives. Quantification is achieved by extrapolating target gene copy numbers from a standard curve generated from serial dilutions of known gene copy numbers. The human marker HF183 was run in triplicate in accordance with EPA Method 1696 (EPA 2019) (Appendix C).

For quality control purposes, a positive control and a negative control are run alongside the samples to ensure a properly functioning reaction and reveal any false negatives or false positives. Sample handling, equipment operation/maintenance, and consumable supplies are provided in accordance with the laboratory standard operating procedures.

Fecal Source Sample Analysis and Validation Testing

Fecal source samples were collected for qPCR marker validation and NGS library building. Fecal source sampling was completed in October 2018 and January 2019, providing a total of 88 fecal source samples compared to the planned total of 70 samples (see Table 4). A total of 82 fecal source samples were analyzed for qPCR markers because six of the samples did not contain a sufficient amount of DNA.

The original plan for fecal source sample analysis and validation of qPCR markers was to analyze each of 70 source samples for the targeted qPCR markers, and analyze one composite sample of the discrete source samples representing each of the seven targeted qPCR markers for the six non-targeted qPCR markers. Additional discrete sample analysis was to be performed if the composite sample exhibited cross-reactivity of a non-target source at a specificity less than 80 percent (except for septage samples that may contain waste from pets).

In accordance with the QAPP addendum (Herrera 2018b), all 82 fecal source samples were analyzed for all target and non-target qPCR markers. In addition, a second cow marker (Cow M3) was added, increasing the total number of markers from seven to eight. This modification increased the validation testing from a minimum of 112 marker analyses (plus additional non-target analysis as needed) to a total of 656 marker analyses (see Table 4). Thus, marker validation testing changed from an adaptive strategy for seven markers to a comprehensive strategy for calculating the specificity and sensitivity for eight markers.

DNA isolation and qPCR amplification procedures are described in Appendix C. A fecal source sample was deemed positive if the Cq value was greater than or equal to the lower limit of quantification (LLOQ), which changed from the original plan that a fecal source sample would be deemed positive if any of the qPCR replicates amplify above the reporting limit (practical quantitation level). For calculation of each marker's specificity, data from fecal source samples were entered as "Present" (Cq ≤ LLOQ) or "Absent" (Cq > LLOQ).

Measurement quality objectives for host-associated marker sensitivity and selectivity were revised in the QAPP addendum to account for the change from composite to discrete fecal source sample analysis. An assay was considered sensitive and specific if both metrics exceed 80 percent, which is equivalent to criteria established by Boehm (et al. 2013).

Sensitivity and specificity of each hose marker were calculated as follows:

- Sensitivity = Number of target individuals tested positive/total number of target individuals.
- Specificity = Number of non-target individuals tested negative/total number of non-target individuals.

A sample was deemed positive if any of the qPCR replicates amplify above the reporting limit (practical quantitation level). The Project technical committee used these performance metrics for final marker selection as described below in Quality Control.

University of Minnesota BioTechnology Institute

UMBTI analyzed the fecal source and water sample extracts for the community-based MST analysis by NGS according to methods described by Brown et al. 2017. The water sample results were compared to the fecal source results for this study. In the future, the fecal source results will be used to build on the existing source library, and the water sample results will be compared to the updated library data to evaluate the relative abundance of fecal sources in the water samples. Comparison to the updated library data was planned for this study, but the computer resources needed for this analysis were not available in time for this report due to the extended sampling schedule.

PCR and DNA sequencing were performed by the University of Minnesota Genomics Center (UMGC, Minneapolis, Minnesota) according to Gohl et al. 2016. The V5+V6 hypervariable regions of the 16S rRNA gene is amplified for bacteria and archaeabacteria using primers 784F (5′– RGGATTAGATACCC–3′) and 1064R (5′– CGACRRCCATGCANCACCT–3′). Samples are first amplified using the following cycling conditions: 95˚C for 5 minutes, followed by 25 cycles of 98˚C for 20 seconds, 55˚C for 15 seconds, and 72˚C for 1 minute. Then, the adapters and barcode are added for the additional 10 cycles of PCR. Amplicons are gel purified, pooled, and paired-end sequenced at a read length of 300 nucleotides on the Illumina MiSeq platform (Illumnia, Inc., San Diego, California) from UMGC.

All sequence data are conducted through mothur ver. 1.34.0 (Schloss et al. 2009). To remove low-quality regions at the ends of reads from the sequences, the first 150 nucleotides are trimmed. Then, fastq-join software will be used to paired-end join sequencing reads (Aronesty 2013). The joined sequencing reads are trimmed to maintain an average quality score greater than 35, homopolyer length greater than 8 nucleotides, sequences with more than two mismatches of primer sequences, and ambiguous bases. High quality sequencing reads are aligned on the basis of the SILVA database ver. 123, and subjected to a 2 percent pre-cluster step to remove possible sequence errors. UCHIME software is used to identify and remove the probable Chimeric sequences (Edgar et al. 2011). All of sequence data are rarefied to 10,000 sequence reads before the sustainable statistical analysis.

QUALITY CONTROL

To ensure the data quality objectives for this study were met, the project team implemented procedures specified in QAPP (Herrera 2018a). Field quality control procedures included aseptic techniques, field logbooks and data forms, custody procedures. Laboratory quality control procedures included method blanks for culture methods, extraction blanks for qPCR, positive controls, negative controls, and laboratory duplicates. The qPCR analyses included a laboratory duplicate and one diluted sample for every sample analyzed to assess matrix inhibition.

Data generated for the project were accurately entered into the project database and securely stored in a manner to facilitate data analysis in accordance with the QAPP (Herrera 2018a). Audits were conducted for field, laboratory, and data management activities within 1 week of receiving data. Deficiencies or nonconformances were tracked and corrective actions were taken as necessary.

All data obtained from field and laboratory measurements will be reviewed and verified for conformance to project requirements, and then validated against the data quality objectives that are listed in the QAPP (Herrera 2018a) for completeness, methodology, holding times, negative controls (blanks), positive controls, laboratory duplicates, and fecal coliform bacteria enumeration. Data quality review results are presented below in the Results section of this project report that includes summarizing quality control results, identifying when data quality objectives were not met, and discussing the resulting limitations (if any) on the use or interpretation of the data.

DATA QUALITY ASSESSMENT

FIELD DATA

Field procedures followed the project QAPP (Herrera 2018a) with the following exception:

- Optical brightener was not measured on April 9, 2019, at station VB013 due to low flow conditions.

Flow measurement procedures were not documented in the field notes except for notes that some flows at freshwater stations were estimated by field personnel.

FECAL BACTERIA

Laboratory procedures followed the project QAPP with the following exception:

- Water samples collected during four events between August 15 and November 1, 2019, were incorrectly filtered for MST analysis and qPCR analysis was not performed for these events.

Laboratory data were verified and validated to ensure that all data were consistent, correct, and complete, and that all required quality control information was provided. Values associated with minor quality control problems were considered estimates and assigned *J* qualifiers. Estimated values were used for evaluation purposes. The following laboratory quality control elements were reviewed for each sampling event:

- Completeness
- Methodology
- Holding times
- Blanks
- Control samples
- Laboratory duplicates
- Fecal coliform bacteria enumeration.

Based on the data validation, all reported results were considered acceptable for use as reported with the following exceptions:

- Several fecal coliform bacteria results were qualified as estimated due to plate counts outside the ideal range of 20 to 60.
- Five fecal coliform bacteria results were qualified as estimated due to laboratory duplicate criterion exceedances (i.e., relative percent difference greater than 35 percent).

QUANTITATIVE PCR

Method Validation

Method validation results for the qPCR fecal source sample analyses are presented in Appendix C. Marker sensitivity and selectivity results are presented in Table 7.

Table 7. Host-Associated Marker Sensitivity and Specificity from qPCR Analysis of Fecal Source Samples.

Host (Marker)	True Positive	Samples Containing Host Feces	Sensitivity	True Negative	Samples Not Containing Host Feces	Specificity
Treating DNQ as Negative						
Human (HF183/BacR287)	10	11	0.91	71	71	1.00
Cow (CowM2)	9	10	0.90	61	61	1.00
Cow (CowM3)	8	10	0.80	61	61	1.00
Horse (HoF597F)	5	10	**0.50**	61	61	1.00
Ruminant (Rum2Bac)	27	31	0.87	40	40	1.00
Pig (Pig2Bac)	5	5	1.00	66	66	1.00
Dog (DG3)	10	11	0.91	60	60	1.00
Bird (GFD)	8	14	**0.57**	55	57	0.96
Treating DNQ as Positive						
Human (HF183/BacR287)	10	11	0.91	71	71	1.00
Cow (CowM2)	10	10	1.00	61	61	1.00
Cow (CowM3)	8	10	0.80	61	61	1.00
Horse (HoF597F)	9	10	0.90	59	61	0.96
Ruminant (Rum2Bac)	29	31	0.94	40	40	1.00
Pig (Pig2Bac)	5	5	1.00	66	66	1.00
Dog (DG3)	10	11	0.91	60	60	1.00
Bird (GFD)	10	14	**0.71**	52	57	0.89

Bold values do not meet quality control objective of 80 percent for sensitivity or selectivity.

DNQ = Detected but not quantifiable

Validation tests were performed on 82 source samples for the seven specified markers and for a second cow marker (CowM3). Among the eight markers tested, specificity ranged from 96 to 100 percent, meeting the 80 percent specificity objective identified in the QAPP. Only the bird specificity was less than 100 percent, which was due to detectable amounts of bird in two of the four llama samples. Two markers did not meet the 80 percent sensitivity objective, including horse (HoF597F) at 50 percent and bird (GFD) at 57 percent. However, if detected but not quantifiable (DNQ) results are included in the sensitivity calculation, then the horse sensitivity increases to 90 percent and the bird sensitivity increases to 71 percent. The first set of method validation results excluding DNQ results below the level of quantitation is a more appropriate assessment of marker sensitivity for this project because this is a quantitative MST study and values assigned to DNQ for calculation purposes are considered to be estimates.

These results indicate that the project water test results will have very few false positives or false negatives with the exception that the amount of horse and bird fecal DNA may be underestimated or not detected where present. There is no alternative marker available for horse and horse is an important source to track in this watershed. Specific bird markers are available for gull and goose that may be more sensitive to those specific types of birds. However, both types of birds are important sources to track in the watershed and it was decided to not pick one specific marker of unknown sensitivity over the general bird marker. The CowM3 marker was less sensitive and equally selective than the CowM2 marker, so it was decided to not change from the planned analysis of CowM2.

Quality Control Data

Quality control data provided by Source Molecular are presented in Appendix C separately for the fecal source validation analyses and water sample analyses. Herrera reviewed the quality control data in comparison to the measurement quality objectives specified in the QAPP (Herrera 2018). All measurement quality objectives were met; and no problems were identified, as summarized below.

Fecal Source Validation Analyses

Between four and five calibration curves were generated from each fecal source sample assay. Efficiencies of the standard curves ranged from 81 to 105 percent and R^2 values were ≥0.987 (Table 8). All results meet measurement quality objectives of 80 to 110 percent efficiency and R^2 ≥0.98. Standards were used as positive controls that all met the measurement quality objective of positive detection because standard curves were re-run if any standard was not detected.

Three method extraction blanks (MEB) were included in each extraction batch. None of the extraction blanks generated positive signals, meeting the measurement quality objective. For all host-associated markers, three No Template Controls (NTC) were analyzed per qPCR plate as negative controls. None of the NTC generated positive signals, meeting the measurement quality objective.

For all host-associated assays performed on fecal source samples, inhibition effects were evaluated by comparing results for diluted and undiluted sample extracts where inhibition would be indicated if the 1:10 sample dilution yielded a lower Cq than the undiluted sample. Inhibition check concluded no inhibition was detected for any samples, meeting the measurement quality objective. All duplicate analyses met the measurement quality objective of being within 1.0 standard deviation of the mean Cq value unless the Cq value was ≥33.

Table 8. Standard Curve Summary of qPCR Analyses.

Host (marker)	Minimum Slope	Maximum Slope	Minimum Efficiency	Maximum Efficiency	Minimum R^2	Maximum R^2
Fecal Source Validation Samples						
Human (HF183/BacR287)	-3.400	-3.284	96.846	101.614	0.997	0.999
Cow (CowM2)	-3.418	-3.351	96.125	98.781	0.999	1.000
Cow (CowM3)	-3.468	-3.343	94.253	99.118	0.996	1.000
Horse (HoF597F)	-3.628	-3.211	88.653	104.856	0.995	0.999
Ruminant (Rum2Bac)	-3.456	-3.384	94.690	97.456	0.998	1.000
Pig (Pig2Bac)	-3.571	-3.325	90.552	99.863	0.994	0.999
Dog (DG3)	-3.876	-3.371	81.138	98.004	0.987	1.000
Bird (GFD)	-3.594	-3.447	89.789	95.026	0.995	0.999
Water Samples						
Human (HF183/BacR287)	-3.375	-3.279	97.820	101.831	0.996	0.998
Cow (CowM2)	-3.393	-3.314	97.127	100.321	0.999	1.000
Horse (HoF597F)	-3.681	-3.345	86.937	99.034	0.990	0.998
Ruminant (Rum2Bac)	-3.456	-3.277	94.697	101.929	0.996	0.999
Pig (Pig2Bac)	-3.557	-3.369	91.058	98.075	0.998	1.000
Dog (DG3)	-3.462	-3.340	94.455	99.236	0.997	1.000
Bird (GFD)	-3.538	-3.392	91.715	97.144	0.983	0.999

Water Sample Analyses

Between four and five calibration curves were generated from each assay. Efficiencies ranged from 87 to 102 percent and R^2 values were ≥0.990. (see Table 8). All results meet measurement quality objectives of 80 to 110 percent efficiency and R^2 ≥0.98. For all non-human host-associated markers, three NTC were analyzed per qPCR plate. For the human-associated marker, six NTC were analyzed per qPCR plate as negative controls. None of NTC generated positive signals, meeting the measurement quality objective.

For all non-human-associated assays performed on water samples, inhibition effect, indicated by 1:10 sample dilution yielding a lower Cq than undiluted sample, was observed in one sample for the bird (GFD) assay (sample VB018-040919) and several samples for the Horse (HoF597F) assay.

Therefore, the duplicated 1:10 dilution data was used for quantification on these samples. The sample processing control (SPC) test results (for target Sketa22) showed only one out of the 15 MEBs had a significant lower Cq than the others, and it was not included in the data analysis for HF183/BacR287 assay. All 74 water samples passed the SPC test, and 68 of them passed the SPC acceptance threshold (see Appendix C). All duplicate analyses met the measurement quality objective of being within 1.0 standard deviation of the mean Cq value unless the Cq value was ≥33.

Potential false-positive Horse (HoF597F) results were observed as MEB 1:10 dilutions also generated positive Cq signals. However, the melting curve of MEBs showed different melting temperatures (Tms) and patterns from the standards. Alignment of forward and reverse primers with salmon genomic DNA (RefSeq: GCF 000233375.1) suggested possible annealing sites. Therefore, Source Molecular applied a more stringent Tm qualification criterion. First, they compared the distribution of Tm1 (melting temperature of the major product) of horse standards, horse fecal samples from the validation study, MEB samples, and water samples. Then they set the acceptance range for true positive signals as ±1°C from median Tm1 of the standards, which has the least overlapping from the Tm1 for MEBs. Last, they carefully examined the melting curves of potential positive samples. Both replicates should have similar melting curve patterns to be considered as true positives. By applying these three stringent criteria, only one sample was designated as HoF597F positive (VB009-112718). It is recognized that these more stringent criteria could eliminate other potential positive samples.

Herrera requested values for results reported as either not detected (ND) or detected but not quantifiable (DNQ) to allow calculations and statistical analysis using all sample results. To include censored data in data analyses, it was agreed to use 50 percent of the lower limit of quantification (LLOQ) for DNQ values and 10 percent of the LLOQ for ND values. The LLOQ was defined as the lowest concentration run on the standard curve that displays a high level of precision (see Appendix C). It is common practice to use half the method detection limit for undetected chemistry values as a reasonable approximation of the actual concentration. The 10 percent of the LOQ was selected for ND values because of the log distribution of fecal bacteria populations and DNA.

Next Generation Sequencing

No quality control data were provided by UMBTI for the NGS analysis.

RESULTS

Results are presented and summarized separately below for field data, fecal bacteria, qPCR, and next generation sequencing. Freshwater station numbers have been truncated to include only the last two digits to ease discussion. The project database is presented in Appendix E; it is sorted by station and date for chronological comparison of all parameter values.

FIELD DATA

Flow and optical brighteners were measured at the freshwater stations. Mean values for the base and storm events are presented in Table 9 where bold base/storm paired values are significantly different based on a Mann Whitney U test (at alpha = 0.10). Completed field data forms for the freshwater samples are presented in Appendix A. Water temperature and salinity were measured and reported by WDOH for the marine stations but were not used for this study.

Freshwater Flow

Mean flow among the freshwater stations during the sampling events ranged 0.07 to 1.15 cfs. The Vaughn Creek stations exhibited the highest mean flow among the freshwater stations as would be expected by their larger subbasin size. However, flows were higher at station 2 than station 1 despite the much larger subbasin for station 1. Flows were generally higher at the small stream stations 47 and 9 than the three drains (7, 13, and 18) except that mean base flow was higher at drain station 13 than stream station 47. The mean flow for all freshwater stations was higher during storm events than base events, but only significantly higher at stations 47 and 7 discharging to the south shore.

Optical Brighteners

The mean fluorescence of optical brighteners among the freshwater stations ranged 63 to 267 RFUB. Vaughn Creek station 2 and drain station 7 exhibited the highest mean optical brighteners during base and storm events (see Table 9). The mean optical brighteners for all freshwater stations was higher during storm events than base events, but only significantly higher at stream station 47 discharging to the north shore and drain stations 9 and 13 discharging to the south shore.

Table 9. Average Parameter Values and Significant Differences Between Base and Storm Events.

Station	Event	Discharge Mean (cfs)	Optical Brighteners Mean (RFUB)	Fecal Coliform Geomean (CFU/100 mL)	*E. coli* Geomean (MPN/100 mL)	qPCR Marker Geomean (copies/100 mL)							
						Human	Cow	Horse	Rumin-ant	Pig	Dog	Bird	Total Host
1	Base	**0.19**	100	**3**	**5**	**48**	20	40	**40**	40	40	**343**	**605**
	Storm	**0.56**	199	**22**	**58**	**401**	61	40	**8,725**	40	117	**6,341**	**19,677**
2	Base	0.56	165	5	**7**	**48**	20	40	60	40	40	806	1,250
	Storm	1.15	242	21	**72**	**165**	34	40	312	40	117	4,184	5,477
47	Base	**0.07**	**79**	4	24	**32**	20	40	**60**	40	40	**347**	**624**
	Storm	**0.27**	**141**	48	144	**167**	20	40	**1,684**	40	202	**6,571**	**9,526**
9	Base	0.14	**94**	2	11	48	20	40	60	40	40	424	796
	Storm	0.25	**205**	10	27	34	20	40	117	40	68	768	1,134
7	Base	**0.03**	178	9	21	**32**	20	40	40	40	40	**134**	**364**
	Storm	**0.10**	267	67	87	**34**	20	40	40	40	40	**662**	**882**
13	Base	**0.10**	92	2	4	48	20	40	40	40	40	112	383
	Storm	**0.12**	192	9	9	98	20	40	40	40	192	158	800
18	Base	0.07	63	9	15	71	20	40	106	40	60	134	686
	Storm	0.12	152	10	24	130	20	40	200	40	40	200	725
609	Base	0	NA	7	NA	**47**	40	40	**40**	40	**40**	**380**	**698**
	Storm	**0**	NA	64	NA	**167**	40	68	**712**	40	**200**	**6,880**	**9,176**
608	Base	0	NA	**4**	NA	**32**	40	40	**40**	40	40	**89**	**343**
	Storm	**0**	NA	**132**	NA	**165**	40	40	**708**	40	117	**7,326**	**8,676**
611	Base	0	NA	**2**	NA	**31**	40	40	**60**	40	40	200	467
	Storm	0	NA	**74**	NA	**133**	54	40	**913**	40	243	2,441	4,393
All Fresh-water	Base	**0.17**	**111**	**4**	**10**	**45**	20	40	**55**	40	**42**	**260**	**622**
	Storm	**0.37**	**200**	**20**	**44**	**107**	25	40	**290**	40	**93**	**1,159**	**2,491**
	S/B Ratio	2.2	1.8	5.1	4.3	2.4	1.3	1.0	5.3	1.0	2.2	4.4	4.0
All Marine	Base	NA	NA	**4**	NA	**36**	40	40	**46**	40	**40**	**189**	**482**
	Storm	NA	NA	**86**	NA	**154**	44	48	**772**	40	**179**	**4,974**	**7,046**
	S/B Ratio	NA	NA	22.1	NA	4.3	1.1	1.2	16.9	1.0	4.5	26.3	14.6

Bold Base/Storm paired values are significantly different based on a Mann Whitney U test (alpha = 0.10).
NA = Not analyzed

Optical brighteners did not correlate with human marker concentrations because human markers were detected at a quantifiable value in only two samples of the 70 samples (Figure 7). Thus, optical brighteners are not a useful field measure for detecting septic system effluent in the Vaughn Bay watershed due to the low amount of septic system effluent in receiving waters. The low-level optical brightener meter used for this study has been useful for detecting moderate to high concentrations of human markers present in drainages that are more contaminated with septic system effluent (Herrera 2017).

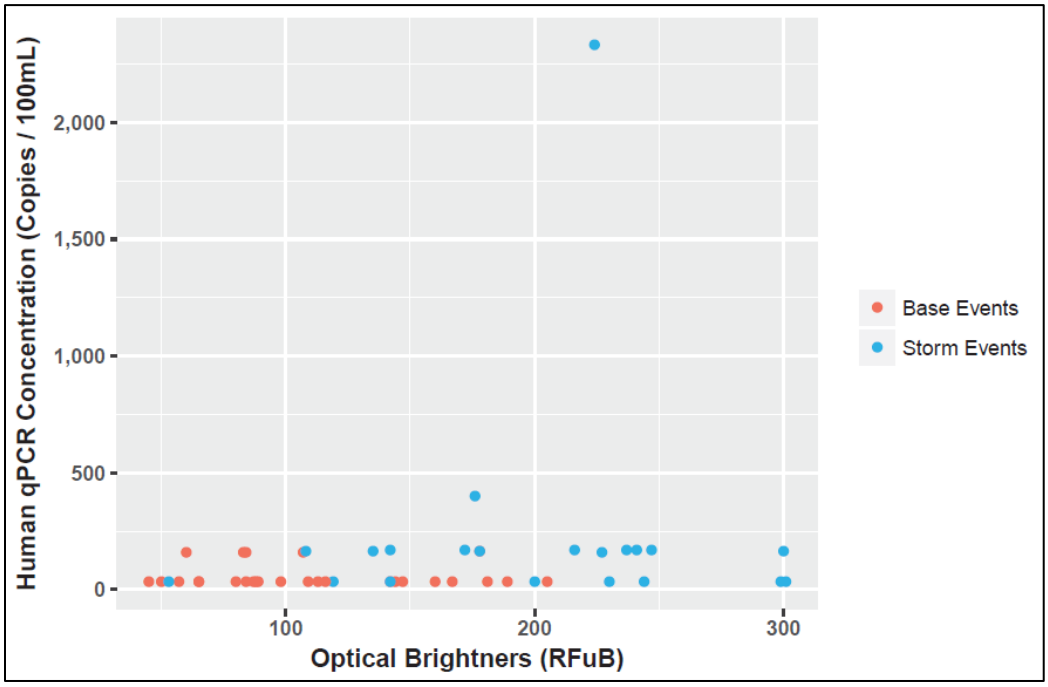

Figure 7. Optical Brighteners Versus Human qPCR Concentrations.

FECAL BACTERIA

Fecal Coliform Bacteria

Geomeans of fecal coliform bacteria concentrations at each station are also presented for base and storm events in Table 9. The range and geometric mean (geomean) of fecal coliform bacteria concentrations at each station are presented separately for pre-MST, base, and storm events in Figure 8. The range and geometric mean (geomean) of fecal coliform bacteria loading rates at each freshwater station are presented separately for pre-MST, base, and storm events in Figure 9.

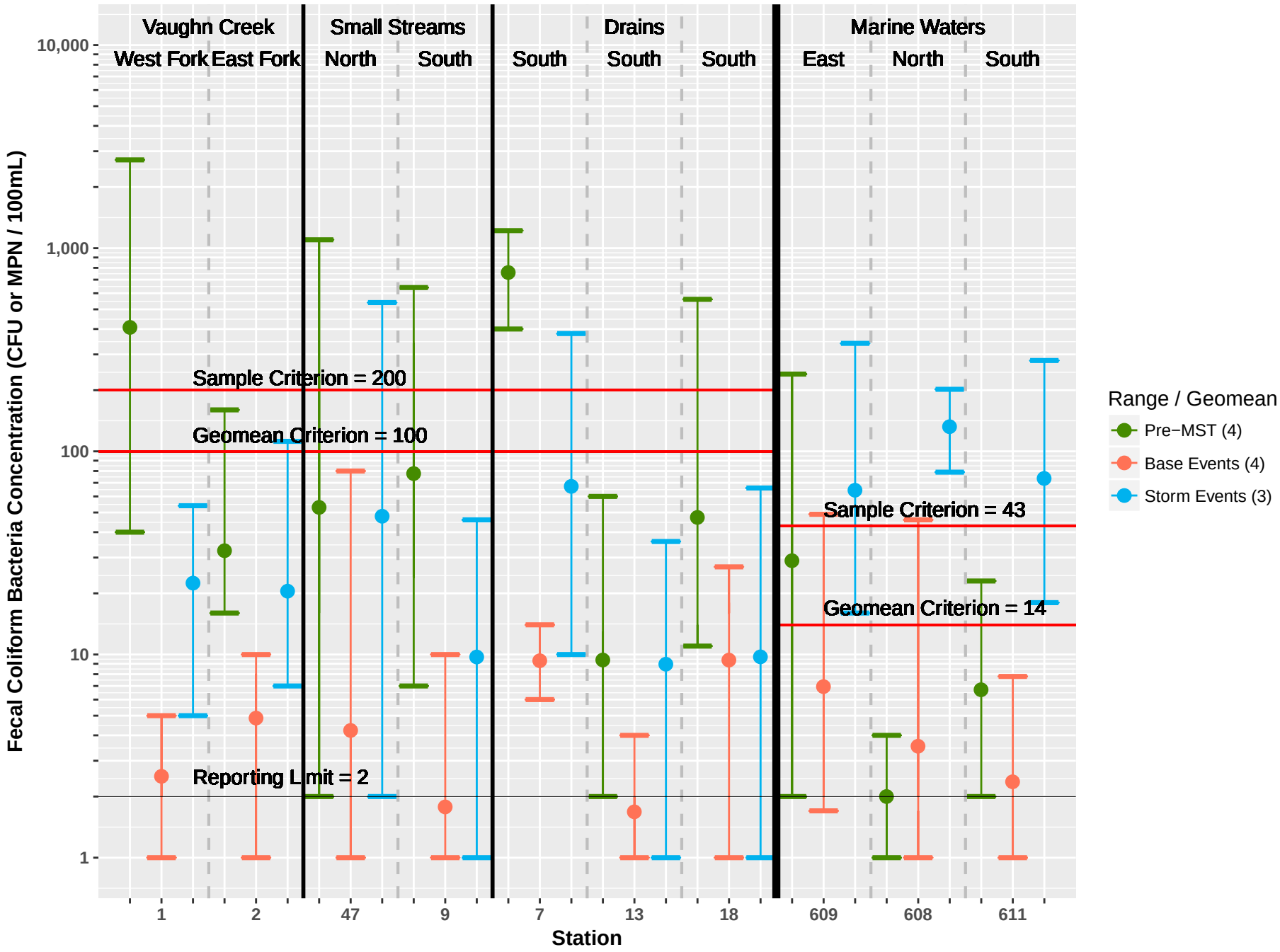

Figure 8. Fecal Coliform Bacteria Concentrations for Each Station by Event Type.

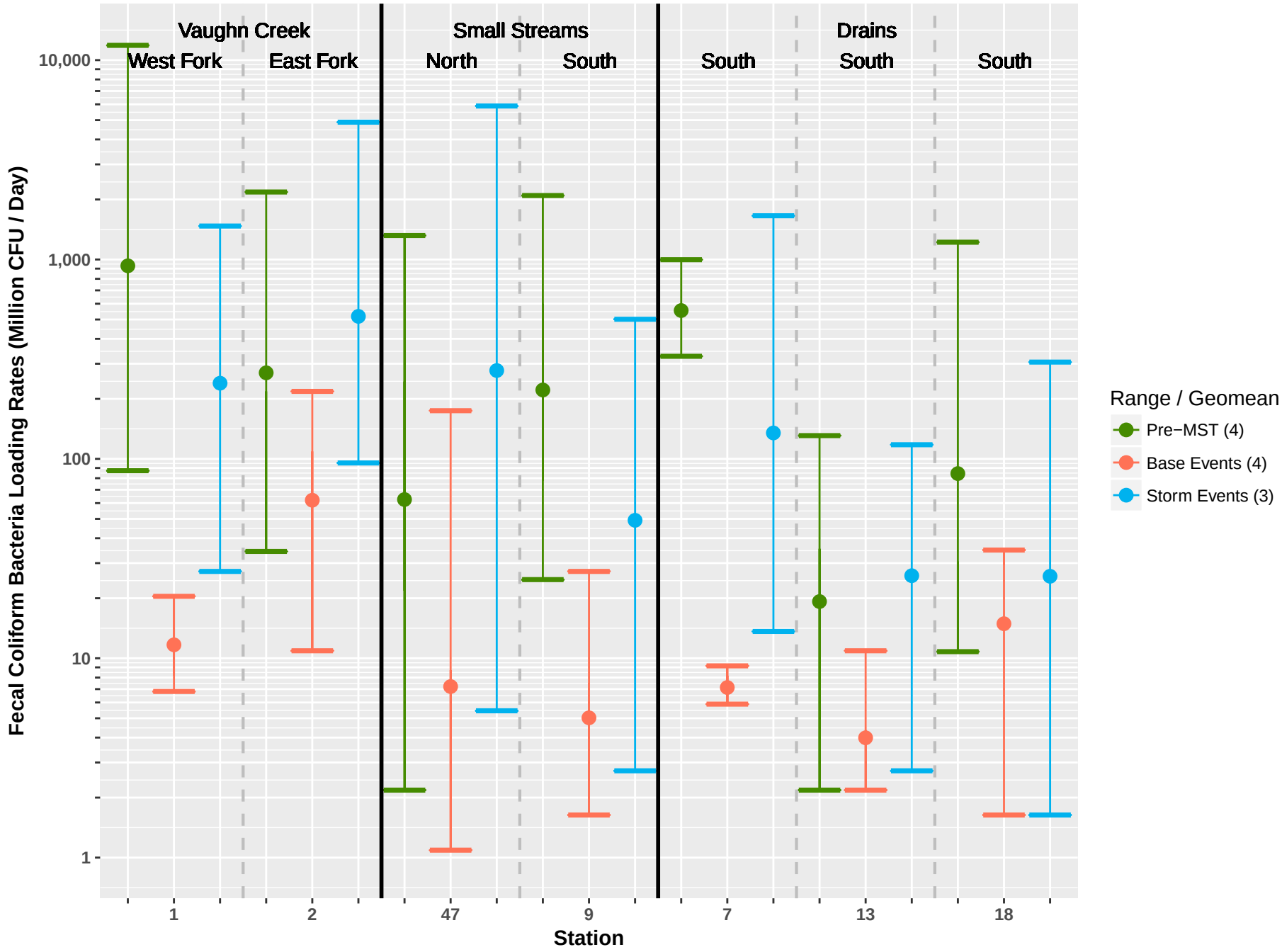

Figure 9. Fecal Coliform Bacteria Loading Rates for Each Freshwater Station by Event Type.

Fecal coliform concentrations were typically higher during the pre-MST and storm events than the base flow events at all freshwater and marine stations (Figure 8). Maximum concentrations at the freshwater stations were typically observed in the first sampling event in August 2018. The single sample criterion for freshwaters (200 CFU/100 mL) was exceeded at all freshwater stations during one or more of the three types of events except Vaughn Creek station 2, which exhibited the highest flow. The single sample criterion for freshwaters was exceeded in 18 percent (14 of 77) of all freshwater samples. The geometric mean criterion for freshwaters (100 CFU/100 mL) was only exceeded at stations 1 and 7 for the pre-MST events. These results show that fecal coliform concentrations in freshwaters were elevated on occasion during both base and storm flow conditions, and that base flow concentrations were much higher in the summer–fall season (pre-MST events) than the winter–spring season (base events).

Maximum fecal coliform concentrations at the marine stations were observed in the largest storm event in November 2018. The single sample criterion for marine waters (43 MPN/100 mL) was exceeded at all marine stations during storm events and base events except station 611 during base events. The single sample criterion for marine waters was exceeded in 33 percent of the marine water samples, compared to 18 percent (11 of 33) for the freshwater samples. The geometric mean criterion for marine waters (14 MPN/100 mL) was exceeded at all three marine stations during storm events but not during base events. The single sample and geomean criteria were only exceeded at station 609 during pre-MST events.

Fecal coliform concentrations at the marine stations were comparable to those at the freshwater stations during base flow, were even higher during storm flow, and exceeded criteria more often due to the lower criteria for marine waters. These results suggest that marine waters were contaminated by sources other than freshwater inflow considering tidal dilution. The abundance of bird sources observed at the marine stations suggests that the additional marine sources likely include seabirds. Monthly seabird counts at the Herron Island Ferry Terminal North, located approximately 5 miles south of Vaughn Bay, show an average of approximately 25 seabirds were counted each time from October 2018 through April 2019 (Seattle Audubon 2019). The surf scooter (duck) composed 80 percent of the total count and ranged from a low of 12 in December 2018 to a high of 51 in April 2019.

Fecal coliform loading rates (Figure 9) followed a similar pattern to concentrations because flow rates were much less variable than concentrations. Vaughn Creek station 2 exhibited higher base and storm loading rates than West Vaughn Creek station 1 because of its higher flow and similar concentrations. Fecal coliform loading rates were generally lower in the small streams and drains compared to West Vaughn Creek and Vaughn Creek.

Figure 10 presents a Kendall's tau correlation matrix of fecal coliform bacteria concentrations between sampling stations for base and storm events and not including pre-MST events. Of particular interest is whether concentrations at the marine stations relate to those in the adjacent stream (see boxed values in Figure 10). The only significant correlation (bold values) between adjacent stations was a positive correlation between Vaughn Creek station 1 and marine station 609 located near the mouths of West Vaughn Creek and Vaughn Creek (Figure 11). Significant positive correlations were also observed between drain station 13 and stream stations 2, 47, and 9. The lack of significant correlation at marine stations 608 and 611

with their adjacent small streams suggests they were affected by other marine water contamination sources, but the lack of correlation also may be due to the high variance among only seven pairs of samples.

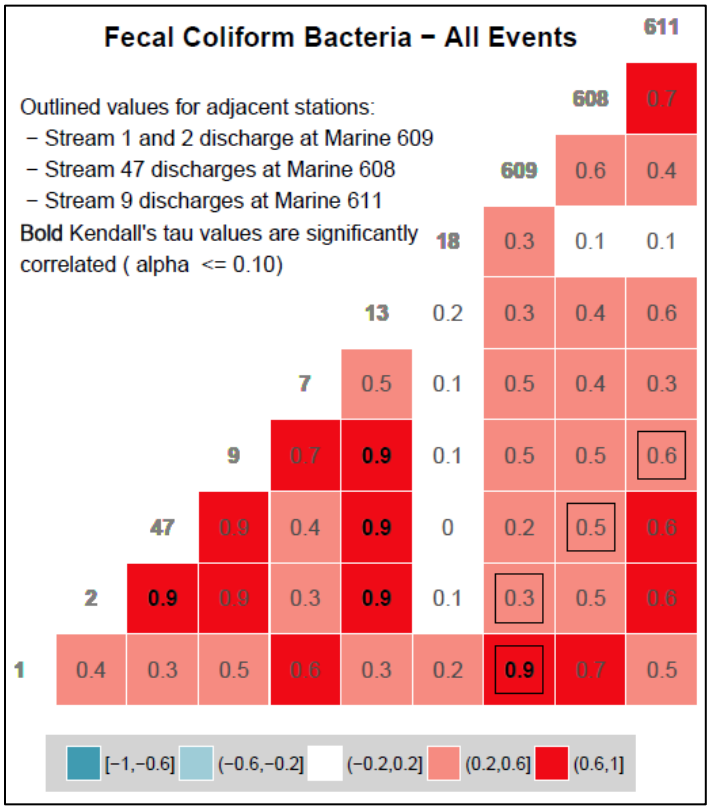

Figure 10. Fecal Coliform Bacteria Station Correlation Matrix.

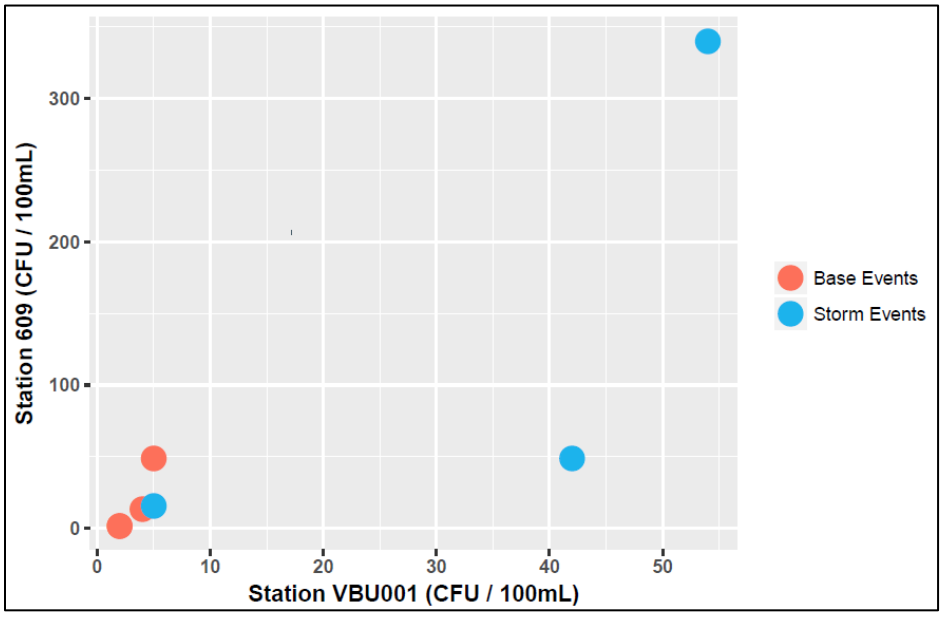

Figure 11. Fecal Coliform Bacteria Concentrations at Adjacent Stream Station VBU001 Versus Marine Station 609.

E. coli Bacteria

Geomeans of *E. coli* bacteria are presented for base and storm events in Table 9. Geomeans are not presented for the marine stations because only a few marine water samples were analyzed for *E. coli* bacteria, and the state water quality standards do not include *E. coli* criteria for marine waters. The geomean criterion for *E. coli* (100 MPN/100 mL) was only exceeded during storm events at station 47. The single sample criterion for *E. coli* in freshwaters (300 MPN/100 mL) was exceeded at all freshwater stations except station 13 during one to three of the four pre-MST events. The single sample criterion for *E. coli* was exceeded during one storm event at freshwater stations 2, 47, and 7 (see Appendix E), and was not exceeded at any station during any base event. The single sample criterion for *E. coli* in freshwaters was exceeded in 17 percent of the freshwater samples, which is similar to the 18 percent exceedance for the fecal coliform bacteria criterion.

E. coli (EC) concentrations were typically greater than fecal coliform (FC) concentrations in the freshwater samples (see Appendix E) with EC/FC ratios of geomeans for all freshwater stations exceeding 2 in base and storm events (see Table 9). EC concentrations would have been much lower and EC/FC ratios would not have exceeded 1 in any sample if the membrane filter method was used instead of the Quanti-Tray method for *E. coli* analysis because only positive FC are tested for EC with the membrane filter method. These results clearly show that the MPN value calculated by the Quanti-Tray method used for *E. coli* is much higher than the CFU value that would have been determined for *E. coli* if the membrane filter method was used instead of the Quanti-Tray method, and that *E. coli* results vary widely depending on the analysis method used.

QUANTITATIVE PCR

Detection Frequency

The number and frequency of host-associated marker detections at the marine and freshwater stations are presented in Table 10. Bird was most commonly detected at a frequency ranging from 57 to 100 percent among the stations. Human and ruminant were the next most commonly detected hosts at widely varying frequencies among the freshwater stations (0 to 57 percent) and similar frequencies among the marine stations (29 to 57 percent). Dog was detected at a lower frequency (less than 30 percent except for 43 percent at station 609) that did not exceed that for human or ruminant at any station. Cow and horse were rarely detected; cow was detected once (14 percent of seven samples) at the two major stream stations 1 and 2 and marine station 611, and horse was detected only once at marine station 609. Pig was not detected in any sample.

Table 10. Summary of qPCR Detections.								
Station	**Event**	**Human (HF183/ BacR287)**	**Cow (CowM2)**	**Horse (HoF597F)**	**Ruminant (Rum2Bac)**	**Pig (Pig2Bac)**	**Dog (DG3)**	**Bird (GFD)**
Number of Non-Detects (ND)/Detected Not Quantified (DNQ)/Detected Quantified								
VBU001	Base	3/1/0	4/0/0	4/0/0	4/0/0	4/0/0	4/0/0	0/2/2
	Storm	0/2/1	2/0/1	3/0/0	0/0/3	3/0/0	1/2/0	0/0/3
VBU002	Base	3/1/0	4/0/0	4/0/0	3/1/0	4/0/0	4/0/0	0/1/3
	Storm	0/3/0	2/1/0	3/0/0	1/0/2	3/0/0	1/2/0	0/0/3
VB047	Base	4/0/0	4/0/0	4/0/0	3/1/0	4/0/0	4/0/0	0/3/1
	Storm	0/3/0	3/0/0	3/0/0	0/0/3	3/0/0	1/1/1	0/0/3
VB009	Base	3/1/01	4/0/0	4/0/0	4/0/0	4/0/0	4/0/0	0/3/1
	Storm	3/0/0	3/0/0	3/0/0	2/1/0	3/0/0	2/1/0	0/0/3
VB007	Base	4/0/0	4/0/0	4/0/0	4/0/0	4/0/0	4/0/0	1/3/0
	Storm	3/0/0	3/0/0	3/0/0	3/0/0	3/0/0	3/0/0	0/0/3
VB013	Base	3/1/0	4/0/0	4/0/0	4/0/0	4/0/0	4/0/0	2/1/1
	Storm	1/2/0	3/0/0	3/0/0	1/1/1	3/0/0	1/1/1	1/1/1
VB018	Base	3/1/0	4/0/0	4/0/0	3/0/1	4/0/0	4/0/0	1/3/0
	Storm	1/1/1	3/0/0	3/0/0	0/3/0	3/0/0	2/1/0	0/3/0
609	Base	2/2/0	4/0/0	4/0/0	4/0/0	4/0/0	4/0/0	0/3/1
	Storm	0/3/0	3/0/0	2/1/0	0/1/2	3/0/0	0/3/0	0/0/3
608	Base	4/0/0	4/0/0	4/0/0	4/0/0	4/0/0	4/0/0	2/2/0
	Storm	0/3/0	3/0/0	3/0/0	0/0/3	3/0/0	1/2/0	0/0/3
611	Base	4/0/0	4/0/0	4/0/0	3/1/0	4/0/0	4/0/0	0/4/0
	Storm	1/1/1	2/1/0	3/0/0	0/1/2	3/0/0	1/0/2	0/1/2
Blank	Base	4/0/0	4/0/0	4/0/0	4/0/0	4/0/0	4/0/0	4/0/0
	Storm	3/0/0	3/0/0	3/0/0	3/0/0	3/0/0	3/0/0	3/0/0
Overall Frequency of Detections								
VBU001	All	57%	14%	0%	43%	0%	29%	100%
VBU002	All	57%	14%	0%	43%	0%	29%	100%
VB047	All	43%	0%	0%	57%	0%	29%	100%
VB009	All	14%	0%	0%	43%	0%	14%	100%
VB007	All	0%	0%	0%	0%	0%	0%	86%
VB013	All	43%	0%	0%	0%	0%	29%	57%
VB018	All	57%	0%	0%	57%	0%	14%	86%
609	All	57%	0%	14%	43%	0%	43%	100%
608	All	43%	0%	0%	43%	0%	29%	71%
611	All	29%	14%	0%	57%	0%	29%	100%
Blank	All	0%	0%	0%	0%	0%	0%	0%

Marker Concentration and Loading

Host-associated marker concentrations are not directly comparable because the amount of fecal DNA present in fecal sources varies considerably among types of hosts. To account for differences in the amount of host marker DNA in host feces, the average host marker DNA concentration was calculated from detectable concentrations of host markers reported for the fecal source samples associated with each host (e.g., using cow source sample results for the cow marker and with other ruminant source samples for the ruminant marker). Marker DNA concentrations in fecal source samples collected for this study are compared to fecal coliform bacteria concentrations reported by EPA (2001, 2002) in Table 11.

Table 11. Comparison of Host-Associated Marker and Fecal Coliform Bacteria Concentrations in Fecal Source Samples.

Host (marker)	Marker Concentration (10^9copies/gram)	Marker Concentration Sample Source	Fecal Coliform Concentration (million/gram)[a]	Fecal Coliform Concentration Sample Source[a]
Human (HF183/BacR287)	0.02	10 septage	400	residential wastewater
Cow (CowM2)	0.13	10 cow	3.4	dairy/beef cow
Horse (HoF597F)	0.28	10 horse	0.02	horse
Ruminant (Rum2Bac)	14.4	10 cow, 5 sheep, 5 goat, 4 llama, 1 alpaca, 4 deer	6.4	dairy cow, sheep
Pig (Pig2Bac)	11.7	5 pig	2.1	hog
Dog (DG3)	0.64	10 dog	18	dog
Bird (GFD)	0.35	5 gull, 5 goose	5.9	chicken, turkey, duck

[a] Average animal values reported from various sources and used as default variables in the BASINS model (EPA 2001). Average human value based on mid-point of ranges reported for fecal coliform bacteria (10^7 MPN/100 mL) and total suspended solids (250 mg/L) in residential wastewater (EPA 2002).

Host-associated marker concentrations in the water samples were then normalized for target marker concentrations in the fecal source samples by dividing the water sample DNA concentration (in copies/100 mL) by the average fecal source sample DNA concentration (copies/gram feces wet weight where 100 mL for septage samples were considered equivalent to 100 grams) to yield the proportion of host marker concentrations in the water relative to that in the fecal source. Each normalized value was then converted to log base 10 for comparison of logarithmic differences in the results.

Un-normalized, host-associated marker concentrations in each sample are presented chronologically in Table 12 with comparisons to fecal coliform bacteria concentrations and loading rates. The fecal source-normalized and log-transformed, host-associated marker concentrations in each sample are presented as a heat map in Table 13. The heat map colors detected results of increasing value from green (low) to yellow (moderate) to red (high) based on the range of detected results observed.

Table 12. Fecal qPCR Analysis Results Compared to Fecal Coliform Concentrations and Loadings.

Station	Month	Day	Year	Event	Fecal Coliform		qPCR Host-Associated Maker Concentration (copies/100 mL)						
					Conc. (no. /100 mL)	Loading (10^6/day)	Human	Cow	Horse	Ruminant	Pig	Dog	Bird
VBU001 Vaugn Ck W Fork	Nov	27	2018	Storm 1	54	1,472	**168**	**567**	40	**44,807**	40	**200**	**6,675**
	Dec	18	2018	Storm 2	42	343	**2,333**	20	40	**5,590**	40	**200**	**4,542**
	Jan	24	2019	Base 1	<2	<14	31	20	40	40	40	40	**200**
	Feb	2	2019	Base 2	4	16	**157**	20	40	40	40	40	**415**
	Mar	7	2019	Base 3	5	20	33	20	40	40	40	40	**200**
	Mar	12	2019	Storm 3	5	27	**165**	20	40	**2,651**	40	40	**8,412**
	Apr	9	2019	Base 4	2	8	33	20	40	40	40	40	**837**
VBU002 Vaughn Ck E Fork	Nov	27	2018	Storm 1	112	4,884	**162**	**100**	40	**1,695**	40	**200**	**3,033**
	Dec	18	2018	Storm 2	11	300	**168**	20	40	**447**	40	**200**	**15,284**
	Jan	24	2019	Base 1	8	109	**162**	20	40	**200**	40	40	**200**
	Feb	2	2019	Base 2	2	22	31	20	40	40	40	40	**1,548**
	Mar	7	2019	Base 3	7	57	33	20	40	40	40	40	**1,963**
	Mar	12	2019	Storm 3	7	95	**165**	20	40	40	40	40	**1,580**
	Apr	9	2019	Base 4	20	436	34	20	40	40	40	40	**695**
VB047 North Stream	Nov	27	2018	Storm 1	540	5,886	**168**	20	40	**5,783**	40	**1,037**	**6,596**
	Dec	18	2018	Storm 2	102	667	**168**	20	40	**1,012**	40	**200**	**11,611**
	Jan	24	2019	Base 1	4	9	31	20	40	40	40	40	**200**
	Feb	2	2019	Base 2	2	3	31	20	40	40	40	40	**200**
	Mar	7	2019	Base 3	2	2	33	20	40	40	40	40	**200**
	Mar	12	2019	Storm 3	2	5	**165**	20	40	**816**	40	40	**3,705**
	Apr	9	2019	Base 4	80	174	33	20	40	**200**	40	40	**1,805**
VB009 South Stream	Nov	27	2018	Storm 1	46	501	34	20	40	40	40	40	**1,264**
	Dec	18	2018	Storm 2	20	87	34	20	40	**200**	40	**200**	**739**
	Jan	24	2019	Base 1	<2	<15	31	20	40	40	40	40	**200**
	Feb	2	2019	Base 2	<2	<4	**157**	20	40	40	40	40	**200**
	Mar	7	2019	Base 3	<2	<3	33	20	40	40	40	40	**4,028**
	Mar	12	2019	Storm 3	<2	<5	33	20	40	40	40	40	**486**
	Apr	9	2019	Base 4	<20	<55	33	20	40	40	40	40	**200**
VB007 South Drain	Nov	27	2018	Storm 1	380	1,657	34	20	40	40	40	40	**727**
	Dec	18	2018	Storm 2	80	109	34	20	40	40	40	40	**893**
	Jan	24	2019	Base 1	6	6	31	20	40	40	40	40	**200**
	Feb	2	2019	Base 2	14	9	31	20	40	40	40	40	40
	Mar	7	2019	Base 3	9	6	33	20	40	40	40	40	**200**
	Mar	12	2019	Storm 3	<20	<27	33	20	40	40	40	40	**446**
	Apr	9	2019	Base 4	<20	<16	33	20	40	40	40	40	**200**
VB013 South Drain	Nov	27	2018	Storm 1	36	118	**168**	20	40	**200**	40	**200**	**494**
	Dec	18	2018	Storm 2	20	55	**168**	20	40	**888**	40	**888**	40
	Jan	24	2019	Base 1	2	4	31	20	40	40	40	40	40
	Feb	2	2019	Base 2	<2	<4	31	20	40	40	40	40	40
	Mar	7	2019	Base 3	<2	<5	33	20	40	40	40	40	**200**
	Mar	12	2019	Storm 3	<2	<5	33	20	40	40	40	40	**200**
	Apr	9	2019	Base 4	4	11	**165**	20	40	40	40	40	**486**
VB018 South Drain	Nov	27	2018	Storm 1	66	306	**400**	20	40	**200**	40	40	**200**
	Dec	18	2018	Storm 2	14	34	**157**	20	40	**200**	40	40	**200**
	Jan	24	2019	Base 1	18	29	**157**	20	40	40	40	**200**	**200**
	Feb	2	2019	Base 2	16	35	31	20	40	40	40	40	**200**
	Mar	7	2019	Base 3	27	29	33	20	40	**1,969**	40	40	40
	Mar	12	2019	Storm 3	<2	<3	31	20	40	**200**	40	40	**200**
	Apr	9	2019	Base 4	<2	<3	**157**	20	40	40	40	40	**200**
609 East Restricted	Nov	27	2018	Storm 1	340	NA	**168**	20	**200**	**3,653**	40	**200**	**4,779**
	Dec	18	2018	Storm 2	49	NA	**168**	20	40	**494**	40	**200**	**5,292**
	Jan	24	2019	Base 1	2	NA	31	20	40	40	40	40	**200**
	Feb	2	2019	Base 2	14	NA	**157**	20	40	40	40	40	**200**
	Mar	7	2019	Base 3	49	NA	**157**	20	40	40	40	40	**2,603**
	Mar	12	2019	Storm 3	16	NA	**165**	20	40	**200**	40	**200**	**12,875**
	Apr	9	2019	Base 4	2	NA	32	20	40	40	40	40	**200**
608 North Conditional	Nov	27	2018	Storm 1	202	NA	**168**	20	40	**1,195**	40	**200**	**5,648**
	Dec	18	2018	Storm 2	79	NA	**162**	20	40	**402**	40	**200**	**4,463**
	Jan	24	2019	Base 1	2	NA	34	20	40	40	40	40	40
	Feb	2	2019	Base 2	<2	NA	31	20	40	40	40	40	**200**
	Mar	7	2019	Base 3	46	NA	31	20	40	40	40	40	40
	Mar	12	2019	Storm 3	145	NA	**165**	20	40	**740**	40	40	**15,600**
	Apr	9	2019	Base 4	2	NA	32	20	40	40	40	40	**200**
611 South Conditional	Nov	27	2018	Storm 1	280	NA	**168**	**100**	40	**4,261**	40	**437**	**10,348**
	Dec	18	2018	Storm 2	79	NA	**423**	20	40	**892**	40	**824**	**7,030**
	Jan	24	2019	Base 1	8	NA	31	20	40	**200**	40	40	**200**
	Feb	2	2019	Base 2	<2	NA	31	20	40	40	40	40	**200**
	Mar	7	2019	Base 3	2	NA	31	20	40	40	40	40	**200**
	Mar	12	2019	Storm 3	18	NA	33	20	40	**200**	40	40	**200**
	Apr	9	2019	Base 4	2	NA	32	20	40	40	40	40	**200**

Underlined fecal coliform values exceed the single sample criterion for freshwaters (200 CFU/100 mL) or marine waters (43 MPN/100 mL).
Bold qPCR values are detected.
NA = not applicable

Table 13. Fecal qPCR Analysis Heat Map.

Station	Month	Day	Year	Event	Human	Cow	Horse	Ruminant	Pig	Dog	Bird
Mean Source Concentration (10^9 copies/100 g)[a]					0.02	0.13	0.28	14.40	11.70	0.64	0.35
					Sample value = log10 ([copies/100 mL] / [10^9 copies/100 g for target host in first row])						
VBU001 Vaugn Ck W Fork	Nov	27	2018	Storm 1	4.01	3.63	2.16	3.49	0.53	2.49	4.29
	Dec	18	2018	Storm 2	5.15	2.18	2.16	2.59	0.53	2.49	4.12
	Jan	24	2019	Base 1	3.27	2.18	2.16	0.44	0.53	1.79	2.76
	Feb	2	2019	Base 2	3.98	2.18	2.16	0.44	0.53	1.79	3.08
	Mar	7	2019	Base 3	3.30	2.18	2.16	0.44	0.53	1.79	2.76
	Mar	12	2019	Storm 3	4.00	2.18	2.16	2.27	0.53	1.79	4.39
	Apr	9	2019	Base 4	3.30	2.18	2.16	0.44	0.53	1.79	3.39
VBU002 Vaughn Ck E Fork	Nov	27	2018	Storm 1	3.99	2.88	2.16	2.07	0.53	2.49	3.94
	Dec	18	2018	Storm 2	4.01	2.18	2.16	1.49	0.53	2.49	4.65
	Jan	24	2019	Base 1	3.99	2.18	2.16	1.14	0.53	1.79	2.76
	Feb	2	2019	Base 2	3.27	2.18	2.16	0.44	0.53	1.79	3.65
	Mar	7	2019	Base 3	3.30	2.18	2.16	0.44	0.53	1.79	3.76
	Mar	12	2019	Storm 3	4.00	2.18	2.16	0.44	0.53	1.79	3.66
	Apr	9	2019	Base 4	3.31	2.18	2.16	0.44	0.53	1.79	3.30
VB047 North Stream	Nov	27	2018	Storm 1	4.01	2.18	2.16	2.60	0.53	3.21	4.28
	Dec	18	2018	Storm 2	4.01	2.18	2.16	1.85	0.53	2.49	4.53
	Jan	24	2019	Base 1	3.27	2.18	2.16	0.44	0.53	1.79	2.76
	Feb	2	2019	Base 2	3.27	2.18	2.16	0.44	0.53	1.79	2.76
	Mar	7	2019	Base 3	3.30	2.18	2.16	0.44	0.53	1.79	2.76
	Mar	12	2019	Storm 3	4.00	2.18	2.16	1.75	0.53	1.79	4.03
	Apr	9	2019	Base 4	3.30	2.18	2.16	1.14	0.53	1.79	3.72
VB009 South Stream	Nov	27	2018	Storm 1	3.31	2.18	2.16	0.44	0.53	1.79	3.56
	Dec	18	2018	Storm 2	3.31	2.18	2.16	1.14	0.53	2.49	3.33
	Jan	24	2019	Base 1	3.27	2.18	2.16	0.44	0.53	1.79	2.76
	Feb	2	2019	Base 2	3.98	2.18	2.16	0.44	0.53	1.79	2.76
	Mar	7	2019	Base 3	3.30	2.18	2.16	0.44	0.53	1.79	4.07
	Mar	12	2019	Storm 3	3.30	2.18	2.16	0.44	0.53	1.79	3.15
	Apr	9	2019	Base 4	3.30	2.18	2.16	0.44	0.53	1.79	2.76
VB007 South Drain	Nov	27	2018	Storm 1	3.31	2.18	2.16	0.44	0.53	1.79	3.32
	Dec	18	2018	Storm 2	3.31	2.18	2.16	0.44	0.53	1.79	3.41
	Jan	24	2019	Base 1	3.27	2.18	2.16	0.44	0.53	1.79	2.76
	Feb	2	2019	Base 2	3.27	2.18	2.16	0.44	0.53	1.79	2.06
	Mar	7	2019	Base 3	3.30	2.18	2.16	0.44	0.53	1.79	2.76
	Mar	12	2019	Storm 3	3.30	2.18	2.16	0.44	0.53	1.79	3.11
	Apr	9	2019	Base 4	3.30	2.18	2.16	0.44	0.53	1.79	2.76
VB013 South Drain	Nov	27	2018	Storm 1	4.01	2.18	2.16	1.14	0.53	2.49	3.16
	Dec	18	2018	Storm 2	4.01	2.18	2.16	1.79	0.53	3.14	2.06
	Jan	24	2019	Base 1	3.27	2.18	2.16	0.44	0.53	1.79	2.06
	Feb	2	2019	Base 2	3.27	2.18	2.16	0.44	0.53	1.79	2.06
	Mar	7	2019	Base 3	3.30	2.18	2.16	0.44	0.53	1.79	2.76
	Mar	12	2019	Storm 3	3.30	2.18	2.16	0.44	0.53	1.79	2.76
	Apr	9	2019	Base 4	4.00	2.18	2.16	0.44	0.53	1.79	3.15
VB018 South Drain	Nov	27	2018	Storm 1	4.38	2.18	2.16	1.14	0.53	1.79	2.76
	Dec	18	2018	Storm 2	3.98	2.18	2.16	1.14	0.53	1.79	2.76
	Jan	24	2019	Base 1	3.98	2.18	2.16	0.44	0.53	2.49	2.76
	Feb	2	2019	Base 2	3.27	2.18	2.16	0.44	0.53	1.79	2.76
	Mar	7	2019	Base 3	3.30	2.18	2.16	2.14	0.53	1.79	2.06
	Mar	12	2019	Storm 3	3.27	2.18	2.16	1.14	0.53	1.79	2.76
	Apr	9	2019	Base 4	3.98	2.18	2.16	0.44	0.53	1.79	2.76
609 East Restricted	Nov	27	2018	Storm 1	4.01	2.18	2.86	2.40	0.53	2.49	4.14
	Dec	18	2018	Storm 2	4.01	2.18	2.16	1.54	0.53	2.49	4.19
	Jan	24	2019	Base 1	3.27	2.18	2.16	0.44	0.53	1.79	2.76
	Feb	2	2019	Base 2	3.98	2.18	2.16	0.44	0.53	1.79	2.76
	Mar	7	2019	Base 3	3.98	2.18	2.16	0.44	0.53	1.79	3.88
	Mar	12	2019	Storm 3	4.00	2.18	2.16	1.14	0.53	2.49	4.57
	Apr	9	2019	Base 4	3.29	2.18	2.16	0.44	0.53	1.79	2.76
608 North Conditional	Nov	27	2018	Storm 1	4.01	2.18	2.16	1.92	0.53	2.49	4.21
	Dec	18	2018	Storm 2	3.99	2.18	2.16	1.45	0.53	2.49	4.11
	Jan	24	2019	Base 1	3.31	2.18	2.16	0.44	0.53	1.79	2.06
	Feb	2	2019	Base 2	3.27	2.18	2.16	0.44	0.53	1.79	2.76
	Mar	7	2019	Base 3	3.27	2.18	2.16	0.44	0.53	1.79	2.06
	Mar	12	2019	Storm 3	4.00	2.18	2.16	1.71	0.53	1.79	4.66
	Apr	9	2019	Base 4	3.29	2.18	2.16	0.44	0.53	1.79	2.76
611 South Conditional	Nov	27	2018	Storm 1	4.01	2.88	2.16	2.47	0.53	2.83	4.48
	Dec	18	2018	Storm 2	4.41	2.18	2.16	1.79	0.53	3.11	4.31
	Jan	24	2019	Base 1	3.27	2.18	2.16	1.14	0.53	1.79	2.76
	Feb	2	2019	Base 2	3.27	2.18	2.16	0.44	0.53	1.79	2.76
	Mar	7	2019	Base 3	3.27	2.18	2.16	0.44	0.53	1.79	2.76
	Mar	12	2019	Storm 3	3.30	2.18	2.16	1.14	0.53	1.79	2.76
	Apr	9	2019	Base 4	3.29	2.18	2.16	0.44	0.53	1.79	2.76

[a] Mean host-associated marker concentraion in fecal source samples collected for study, assuming 1 mL = 1 g for human septage.

Colored values are detected

0	None (non-detect minimum)
1	Low
2	Low-Moderate
3	Moderate
4	Moderate-High
5	High (source-weighted maximum)

Host Name: Marker (Source)
Human: HF183/BacR287 (Septage)
Cow: CowM2 (Cow)
Horse: HoF597F (Horse)
Ruminant: Rum2Bac (Cow, Goat, Sheep, Llama, Alpaca, Deer)
Pig: Pig2Bac (Pig)
Dog: DG3 (Dog)
Bird: GFD (Gull, Goose)

The un-normalized qPCR results (Table 12) show that moderate to high concentrations were primarily observed during storm events for each host-associated marker, as well as for fecal coliform bacteria concentrations and loading rates. Moderate to high marker concentrations (greater than 5,000 copies/100 mL) were observed on multiple occasions for ruminant at stream station 1, and for bird at stream stations 1 and 47 and all three marine stations. Moderate concentrations were observed on one occasion for ruminant at stream station 47 and for bird at stream station 2. Maximum concentrations were much lower for human (2,333 copies/100 mL at station 1), dog (1,037 copies/100 mL), and cow (567 copies/100 mL at station 1.

The normalized qPCR results (Table 13) increase relative concentrations for human and decrease relative concentrations for ruminants because of the relatively low human concentrations observed in septage (0.02 billion copies/100 grams) and relatively high ruminant concentrations observed in ruminant source samples (11.7 billion copies/100 grams). Detected human concentrations ranked moderate-high to high, while detected ruminant concentrations ranked low to moderate. Detected bird concentrations ranked moderate to high and detected dog concentrations ranked moderate. Overall, the normalized qPCR results indicate that human and bird are the primary fecal sources present in the freshwaters and marine waters, followed by ruminant and dog.

The geomean of host-associated marker concentrations (un-normalized) at each station are presented separately for base and storm events as stacked bar charts in Figure 12. The geomean of host-associated marker loading rates at each station are presented separately for base and storm events as stacked bar charts in Figure 13. These two charts more clearly show the predominance of fecal sources in storm flow compared to base flow. high concentrations. Total fecal host concentrations were highest during storm flow at streams 1, 2, and 47 and at all three marine stations. Bird dominated the analyzed hosts at each of these stations except for a high ruminant at station 1.

This same pattern is exhibited for stream loading rates in Figure 13 with the exception that station 2 had a much higher loading rate than stream station 47, which had a higher concentration. These results clearly show the high importance of bird sources and moderate importance of ruminants in Vaughn Creek on the high fecal bacteria concentrations in Vaughn Bay. Fecal host loadings during base flow in the streams and during all flow from the drains were insignificant in comparison.

The geomean of host-associated marker concentrations for all seven freshwater stations combined and all three marine stations combined are compared in Figure 14 for human, ruminant, dog, and bird. Cow, horse, and pig are not shown in this figure because they were detected in so few samples. This bar chart also compares the freshwater and marine geomeans for base and storm events and for low and high fecal coliform bacteria concentrations, where low concentrations meet single sample criteria and high concentrations exceed the single sample criteria.

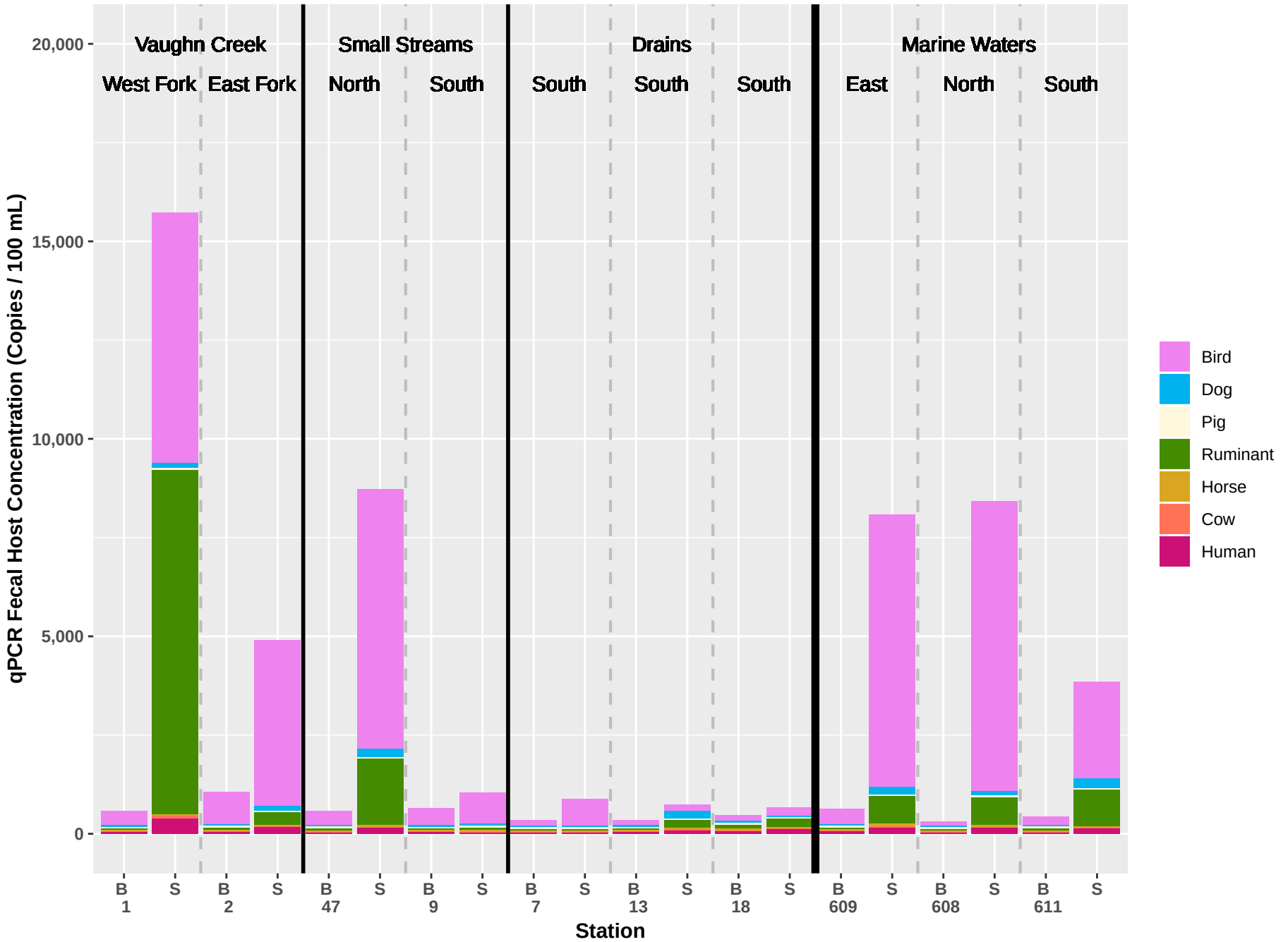

Figure 12. Host-Associated Marker qPCR Geomean Concentrations for Base (B) and Storm (S) Events.

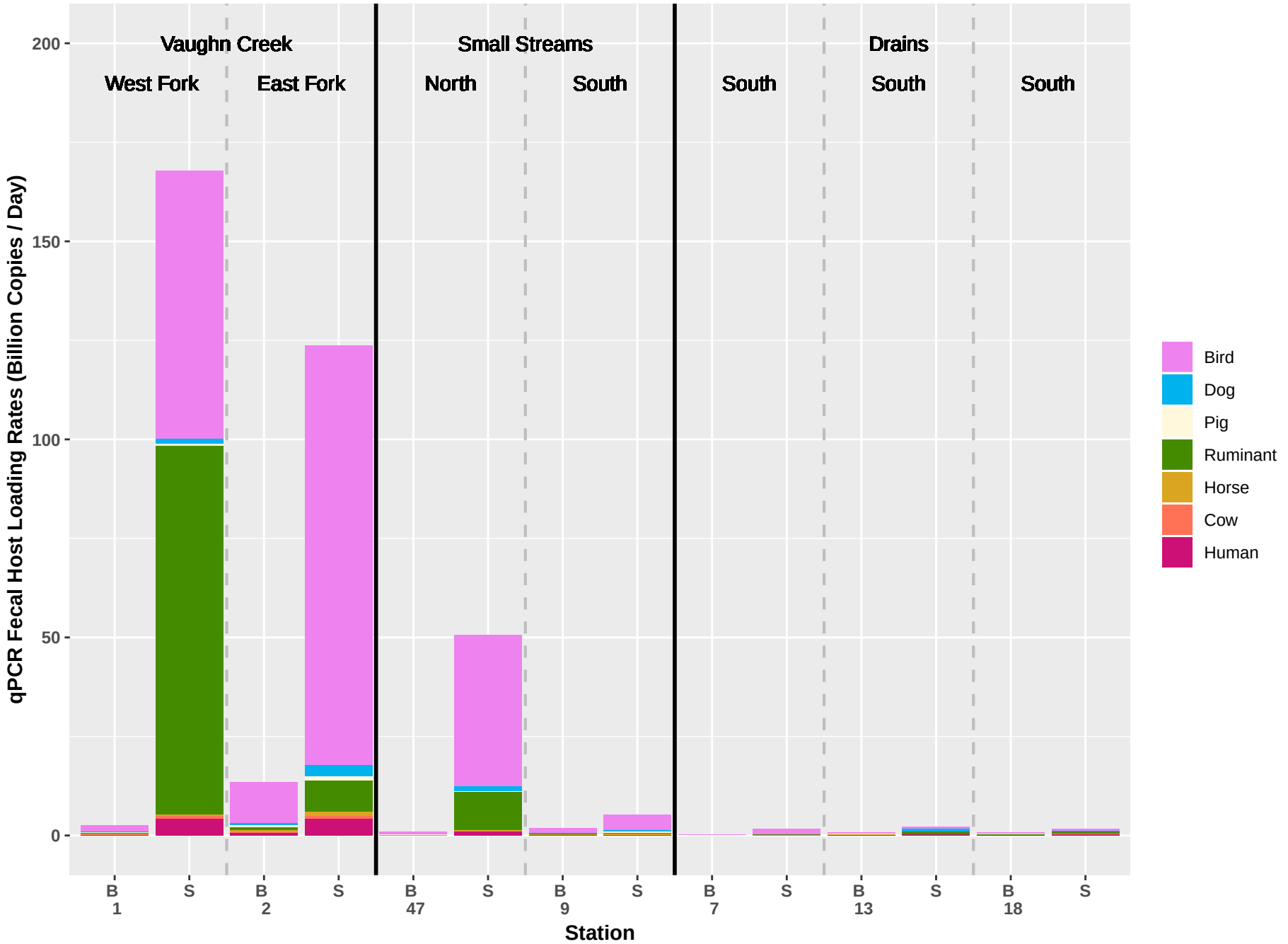

Figure 13. Host-Associated Marker qPCR Geomean Loading Rates for Base (B) and Storm (S) Events.

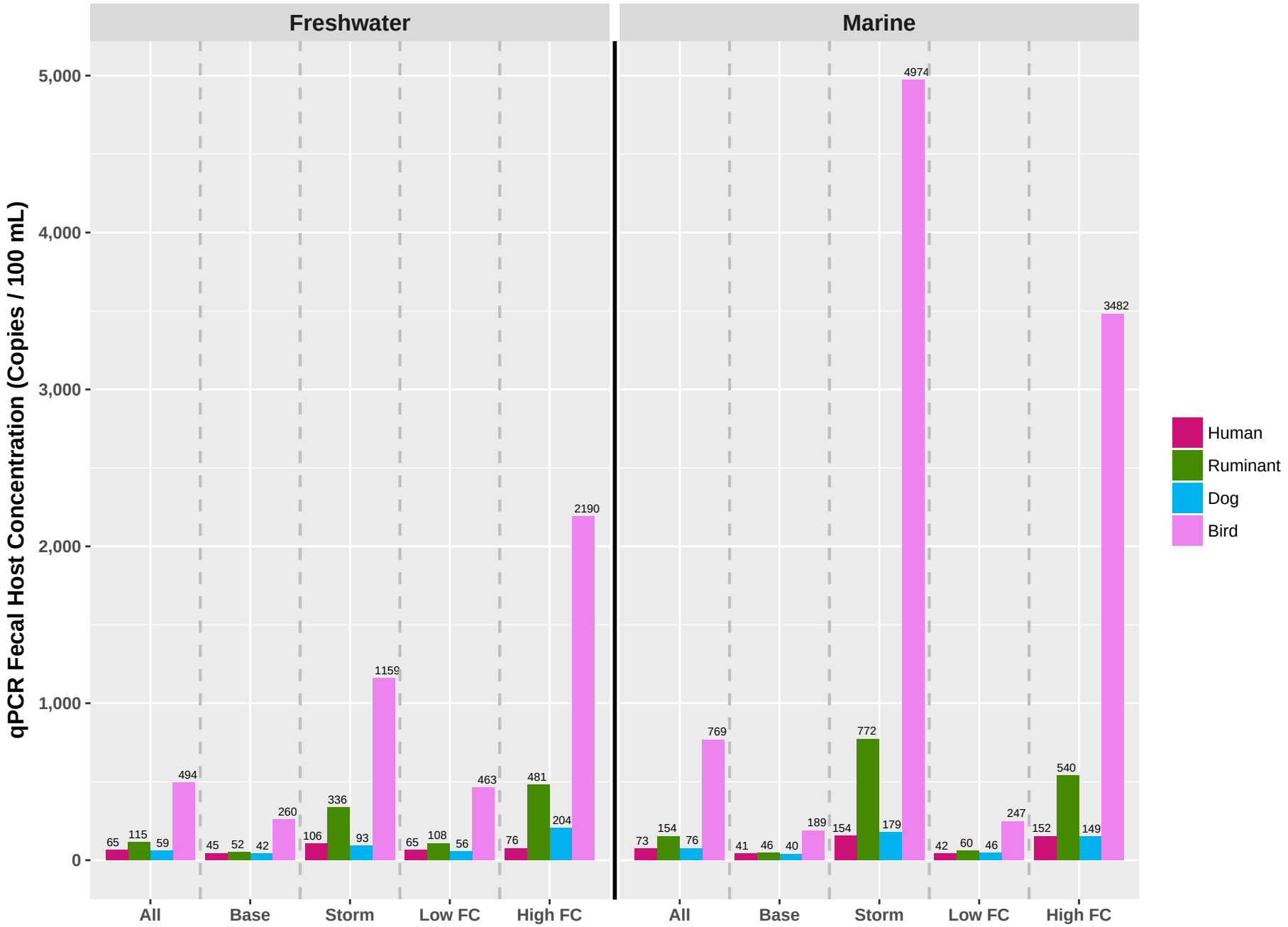

Figure 14. Host Marker qPCR Geomean Concentrations at All Freshwater and Marine Stations for Base/Storm Events and Low/High Fecal Coliform Concentrations.

Geomeans for all four hosts were substantially higher (more than double) for the sampled storm events than base events and for the low fecal coliform events than the high fecal coliform events at the freshwater and marine stations. One exception is that the human geomean was similarly low for the low and high fecal coliform events at the freshwater stations. The magnitude of these differences was much higher for the marine stations than the freshwater stations. For example, bird concentrations increased during storm events by a factor of 4.5 at the freshwater stations and a factor of 26 at the marine stations, and bird concentrations increased during high fecal coliform events by a factor of 4.7 at the freshwater stations and a factor of 14 at the marine stations. Combining host marker results for all events shows that marine stations were not substantially higher than the freshwater stations. These results clearly show the importance of evaluating spatial differences among fecal sources separately for base and storm events.

The qPCR results were not analyzed for spatial correlations among sampling stations due to the low number of samples (seven) and the high proportion of not detected or quantifiable values for most host-associated markers.

The sum of host-associated marker concentrations correlated poorly with fecal coliform bacteria (Figure 15). A lack of correlation between fecal coliform bacteria and DNA concentrations has been typically observed elsewhere and is not surprising given the differences in the populations tested and methods used for quantification.

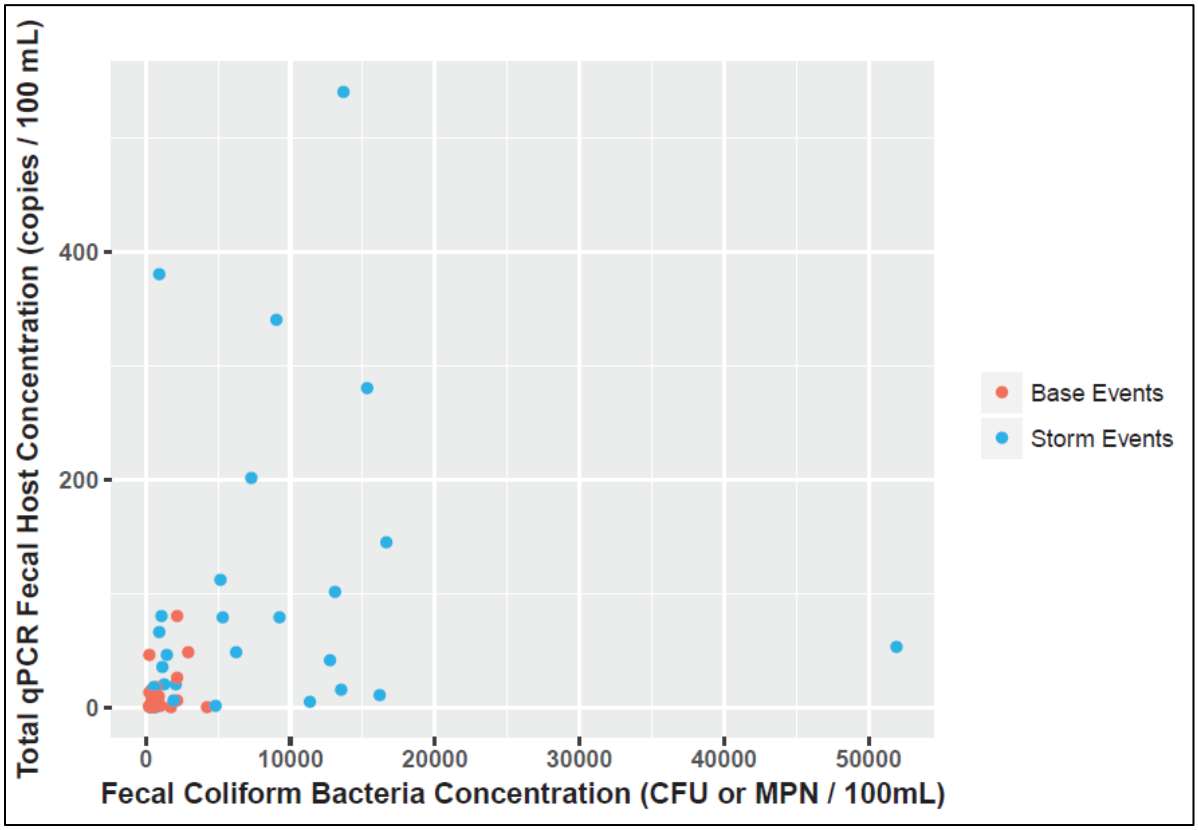

Figure 15. Fecal Coliform Bacteria Concentrations Versus Total qPCR Host Marker Concentrations.

Interlaboratory Comparison

The King County Environmental Laboratory was requested late in this study to participate gratis in an interlaboratory comparison of qPCR methods. KCEL analyzed all 10 samples collected for the last base flow event in April 2019 for all host-associated markers that they analyze, which include two human markers, two cow markers, one ruminant marker, and two dog markers. Source Molecular extracted the duplicate sample filters and shipped 50 microliters (half) of each sample extract to KCEL.

The qPCR results from KCEL are presented in Table 14. Only a trace amount of one human marker (Hu2) was detected in the samples from drains 13 and 18. These results agree well with the study results from Source Molecular that also detected trace amounts (detected but not quantifiable) of the human marker only in those same samples. No cow, ruminant, or dog were detected in the April samples by Source Molecular except for a trace amount (detected but not quantifiable) of ruminant in the sample from stream 47, which was given an estimated value of 200 copies/100 mL (see Table 12) and is well below the KCEL ruminant method detection limit of 11,320 copies/100 mL.

Table 14. King County qPCR Results for MST Project Samples Collected on April 9, 2019.

	Human (Hu2)	Human (Hu3)	Cow (Cow2)	Cow (Cow3)	Ruminant (Rum2)	Dog (Dog1)	Dog (Dog2)
	Method Detection Limit (MDL) (copies/100 mL)						
Station	700[a]	1,160[a]	480	180	11,320[a]	480	360
VBU001	<MDL[a]	<MDL[a]	<MDL	<MDL	<MDL[a]	<MDL	<MDL
VBU002	<MDL[a]	<MDL[a]	<MDL	<MDL	<MDL[a]	<MDL	<MDL
VB047	<MDL[a]	<MDL[a]	<MDL	<MDL	<MDL[a]	<MDL	<MDL
VB009	<MDL[a]	<MDL[a]	<MDL	<MDL	<MDL[a]	<MDL	<MDL
VB007	<MDL[a]	<MDL[a]	<MDL	<MDL	<MDL[a]	<MDL	<MDL
VB013	Trace[a]	<MDL[a]	<MDL	<MDL	<MDL[a]	<MDL	<MDL
VB018	Trace[a]	<MDL[a]	<MDL	<MDL	<MDL[a]	<MDL	<MDL
609	<MDL[a]	<MDL[a]	<MDL	<MDL	<MDL[a]	<MDL	<MDL
608	<MDL[a]	<MDL[a]	<MDL	<MDL	<MDL[a]	<MDL	<MDL
611	<MDL[a]	<MDL[a]	<MDL	<MDL	<MDL[a]	<MDL	<MDL

[a] MDL elevated because assay test volume was 40 percent of normal volume (5 microliters) due to low sample amount received.

Trace = detection but unquantifiable due to low sample volume.

Next Generation Sequencing

Next generation sequencing (NGS) results from UMBTI were based only on fecal source samples collected for this study. The results are presented as a heat map in Table 15. The water sample results are presented as the percent matching local fecal sources out of the entire bacterial community observed in the water samples. The unmatched percentage exceeded 50 percent in each sample because most fecal and non-fecal bacteria present in the water samples were not present in the local fecal source samples based on matching DNA patterns. NGS results based on the nationally fecal source library will be provided separately from this report when the analysis is complete.

Table 15. Next Generation Sequencing Community Analysis Heat Map.

Station	Month	Day	Year	Event	Human	Livestock	Deer	Dog	Bird
VBU001 Vaugn Ck W Fork	Nov	27	2018	Storm 1	4.60	0.32	0.09	0	11.99
	Dec	18	2018	Storm 2	3.25	0.02	0.12	0	11.26
	Jan	24	2019	Base 1	3.66	0	2.46	0	11.29
	Feb	2	2019	Base 2	7.05	0	0	0	14.13
	Mar	7	2019	Base 3	8.16	0	0	0	13.26
	Mar	12	2019	Storm 3	3.12	0	0	0	8.51
	Apr	9	2019	Base 4	3.56	0	0	0	5.84
VBU002 Vaughn Ck E Fork	Nov	27	2018	Storm 1	5.36	0	0	0	12.90
	Dec	18	2018	Storm 2	5.37	0	0	0	10.60
	Jan	24	2019	Base 1	7.31	0.04	0	0	10.94
	Feb	2	2019	Base 2	7.14	0	0	0	11.21
	Mar	7	2019	Base 3	6.87	0.01	0	0	9.07
	Mar	12	2019	Storm 3	6.47	0	0	0	15.53
	Apr	9	2019	Base 4	9.65	0	0	0	23.20
VB047 North Stream	Nov	27	2018	Storm 1	6.04	0.03	0	0	9.09
	Dec	18	2018	Storm 2	4.64	0.01	0	0	11.81
	Jan	24	2019	Base 1	3.57	0	0	0	11.19
	Feb	2	2019	Base 2	8.44	0	0.07	0	16.62
	Mar	7	2019	Base 3	6.72	0	0	0	19.93
	Mar	12	2019	Storm 3	4.75	0	0	0	10.51
	Apr	9	2019	Base 4	6.12	0.01	0	0	5.30
VB009 South Stream	Nov	27	2018	Storm 1	12.62	0	0	0	14.18
	Dec	18	2018	Storm 2	9.16	0	0	0	13.49
	Jan	24	2019	Base 1	9.14	0	0	0	18.84
	Feb	2	2019	Base 2	8.52	0	0	0	18.87
	Mar	7	2019	Base 3	3.72	0	0	0	10.73
	Mar	12	2019	Storm 3	5.83	0	0	0	18.74
	Apr	9	2019	Base 4	8.16	0	0	0	15.62
VB007 South Drain	Nov	27	2018	Storm 1	10.25	0	0	0	21.09
	Dec	18	2018	Storm 2	8.65	0	0	0	16.00
	Jan	24	2019	Base 1	6.84	0	0.02	0	23.90
	Feb	2	2019	Base 2	7.42	0	0.03	0	31.38
	Mar	7	2019	Base 3	8.89	0	0	0	22.73
	Mar	12	2019	Storm 3	7.14	0	0	0	20.39
	Apr	9	2019	Base 4	8.19	0	0	0	12.01
VB013 South Drain	Nov	27	2018	Storm 1	8.30	0	0.16	0.20	22.10
	Dec	18	2018	Storm 2	6.65	0	0	0.01	22.12
	Jan	24	2019	Base 1	3.94	0	0	0	30.12
	Feb	2	2019	Base 2	8.92	0	0.16	0	33.33
	Mar	7	2019	Base 3	5.49	0	0	0	26.41
	Mar	12	2019	Storm 3	4.34	0	0	0	17.06
	Apr	9	2019	Base 4	7.12	0.01	0	0	17.02
VB018 South Drain	Nov	27	2018	Storm 1	10.84	0	0	0	17.16
	Dec	18	2018	Storm 2	5.95	0	0	0	13.47
	Jan	24	2019	Base 1	6.80	0	0	0	23.30
	Feb	2	2019	Base 2	9.86	0	0	0	34.88
	Mar	7	2019	Base 3	10.39	0.01	0	0	26.59
	Mar	12	2019	Storm 3	10.12	0	0	0	23.85
	Apr	9	2019	Base 4	6.53	0	0	0	16.90
609 East Restricted	Nov	27	2018	Storm 1	7.36	0.01	0.03	0	19.36
	Dec	18	2018	Storm 2	3.52	0	0	0	11.43
	Jan	24	2019	Base 1	1.57	0	0	0	24.45
	Feb	2	2019	Base 2	6.56	0	0	0	21.31
	Mar	7	2019	Base 3	3.70	0	0	0	15.45
	Mar	12	2019	Storm 3	5.15	0	0	0	17.00
	Apr	9	2019	Base 4	2.89	0	0	0	26.88
608 North Conditional	Nov	27	2018	Storm 1	1.73	0	1.68	0	15.14
	Dec	18	2018	Storm 2	2.23	0	0.03	0.01	11.39
	Jan	24	2019	Base 1	0	0	0	0.01	24.46
	Feb	2	2019	Base 2	0.75	0	0	0	24.06
	Mar	7	2019	Base 3	0	0	0	0	21.18
	Mar	12	2019	Storm 3	2.59	0	0	0	12.96
	Apr	9	2019	Base 4	1.50	0	0	0	33.54
611 South Conditional	Nov	27	2018	Storm 1	8.02	0	0	0	13.67
	Dec	18	2018	Storm 2	3.03	0	0	0	9.39
	Jan	24	2019	Base 1	5.86	0	0	0.01	19.39
	Feb	2	2019	Base 2	0.16	0.04	0	0	32.65
	Mar	7	2019	Base 3	0.12	0	0	0	24.87
	Mar	12	2019	Storm 3	1.18	0	0	0	16.16
	Apr	9	2019	Base 4	1.84	0	0	0	28.30

0	None
0.01	Low
5	Mod. Low
15	Moderate
25	Mod. High
35	High

Values are percent of entire bacteria community matching fecal source samples:
Human: Septage
Livestock: Pig, Cow, Horse, Goat, Sheep, Llama, Alpaca
Deer: Deer
Dog: Dog
Bird: Goose, Gull

These results show that moderate to high amounts of bird (from gull and goose samples) were detected in all samples. Low to moderate amounts of human (from septic samples) were detected at all stations except only low amounts were detected at marine station 608. All other sources were typically not detected or only detected in very low amounts in a few samples. These other sources include livestock (ruminants from cow, sheep, goat, llama, and alpaca samples; horse from horse samples; and pig from pig samples), dog (from dog samples) and deer (from deer samples).

With a few minor exceptions, deer amounts were higher than livestock suggesting that deer were a major component of the ruminant detected by qPCR. Exceptions where livestock were detected in a low amount and no deer were detected in multiple samples include stream station 2 (two samples) and stream station 47 (three samples), but only two samples from station 47 had quantifiable ruminant concentrations by qPCR (compare Tables 12 and 15). Thus, it is possible that deer were a major component for the ruminant detected by qPCR at all stations.

Results for each of the 10 stations are presented as stacked bar charts in chronological order for samples collected from each station in Figures 16 through 25. Results are presented in percent matching local fecal sources with the total percent matched ranging from a low of 9 to 21 percent for West Vaughn Creek station 1 to a high of 19 to 45 for drain stations 7, 13, and 18. These results show no consistent chronological patterns among the freshwater or marine stations and no differences between base and storm events (Events 1, 2, and 6 of seven events in each chart), in contrast to the higher fecal concentrations observed during storm events by the culture and qPCR methods.

These NGS results suggest than septic systems are the major controllable fecal source in the Vaughn Bay watershed. It is possible that many more sources will be detected when the water samples are compared to the national fecal source library and the percent matched increases. However, it seems unlikely that livestock or dog will become a major source considering the numerous local livestock and dog feces samples used in the initial NGS analysis. It seems more likely that any new sources identified would primarily include other wildlife not sampled in the Vaughn Bay watershed.

The NGS results complement the qPCR results by the high abundance of bird observed by both methods. The NGS method showed relatively higher amounts of human and lower amounts of ruminants than the qPCR method. The comparably low amounts of livestock and deer detected by NGS suggests that much of the ruminant detected by qPCR may have been deer rather than livestock.

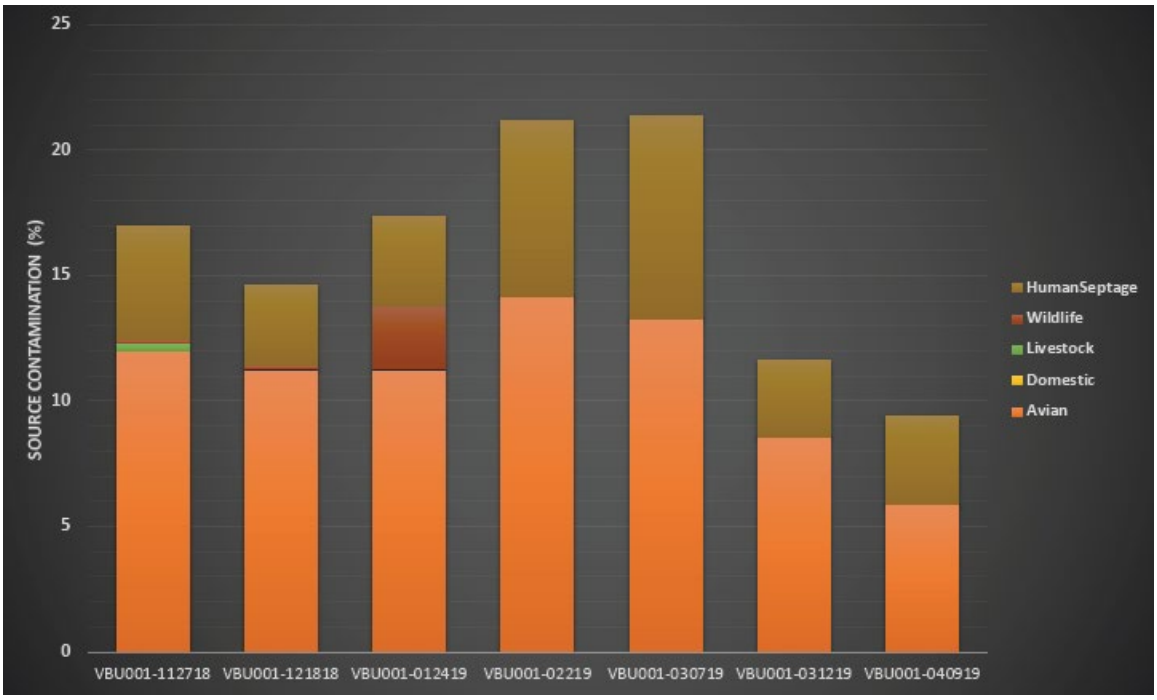

Figure 16. Next Generation Sequencing Results for Station VBU001.

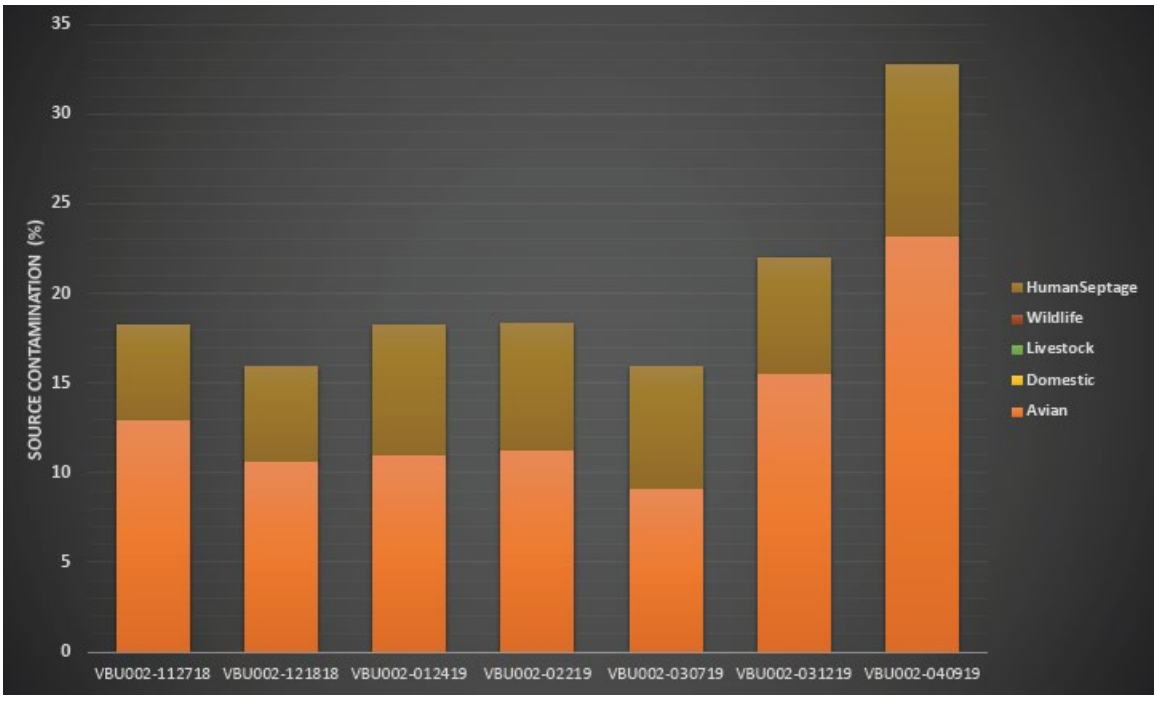

Figure 17. Next Generation Sequencing Results for Station VBU002.

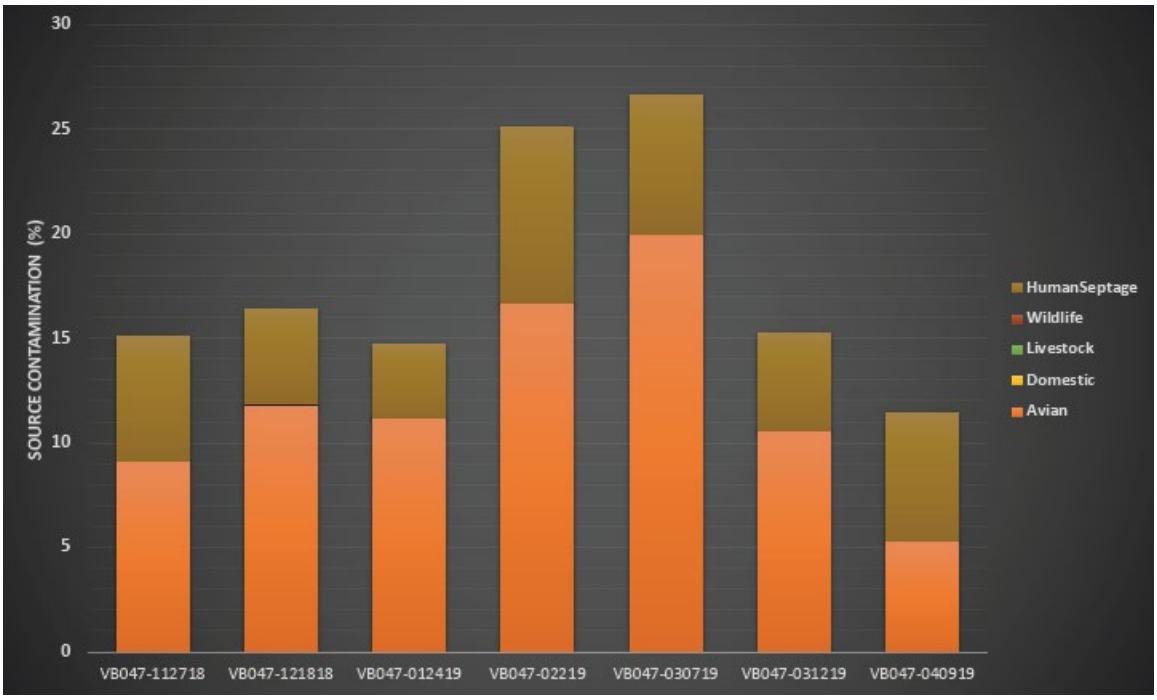

Figure 18. Next Generation Sequencing Results for Station VB047.

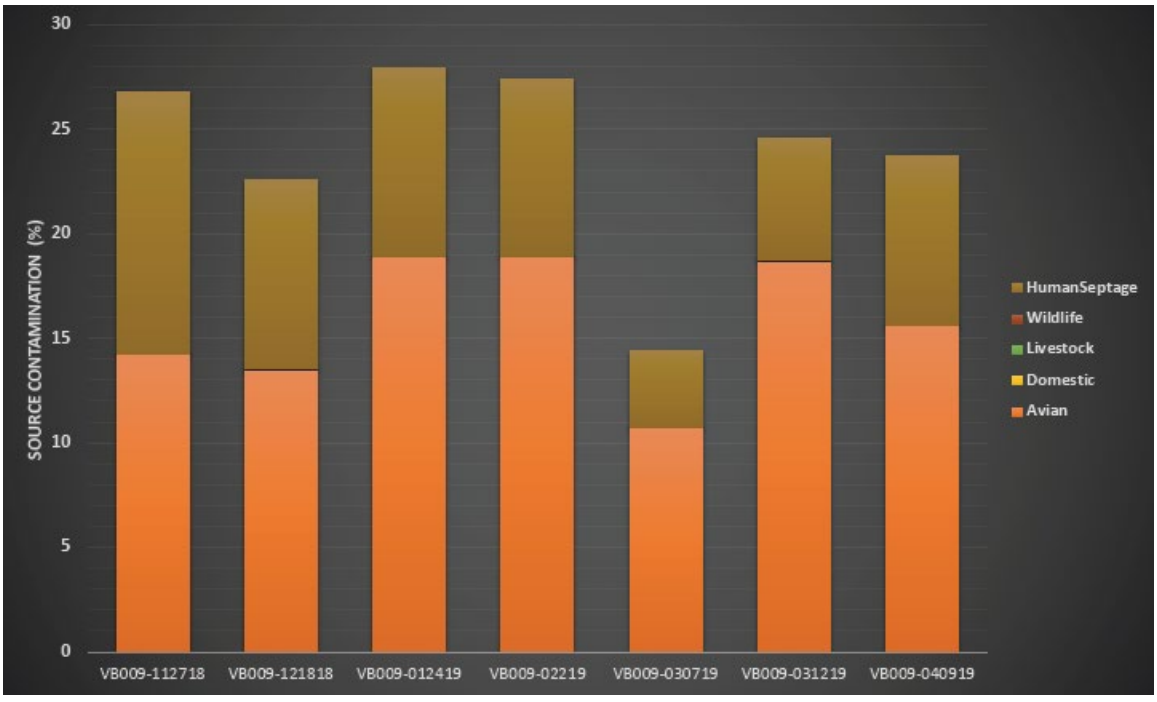

Figure 19. Next Generation Sequencing Results for Station VB009.

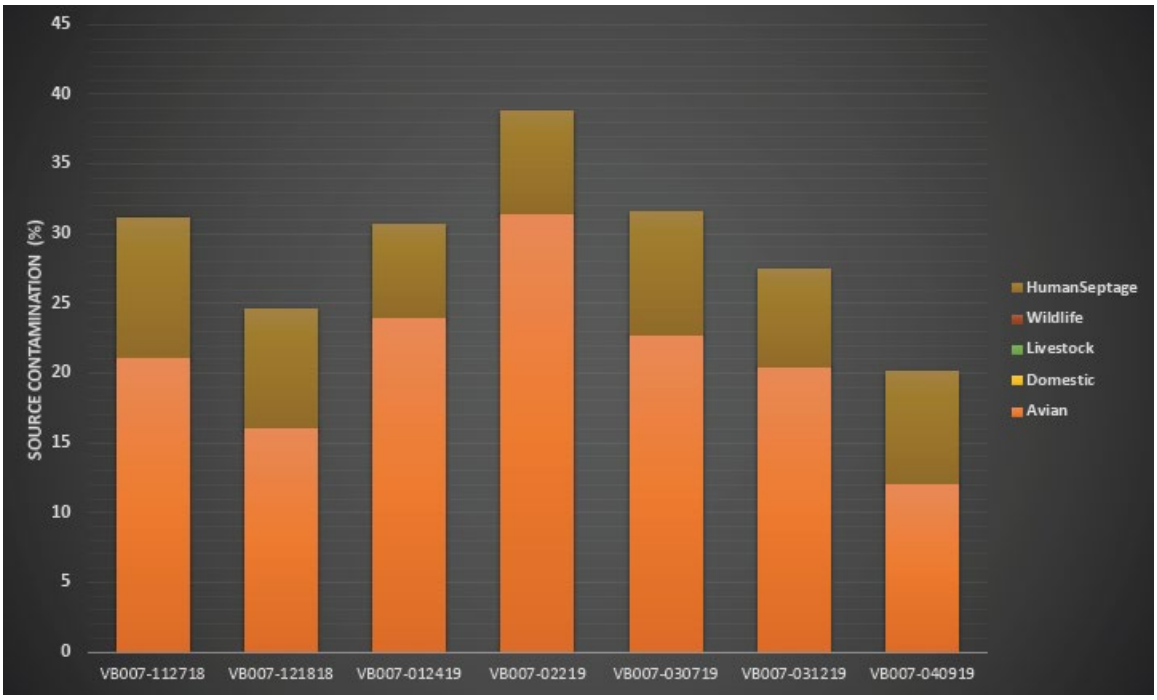

Figure 20. Next Generation Sequencing Results for Station VB007.

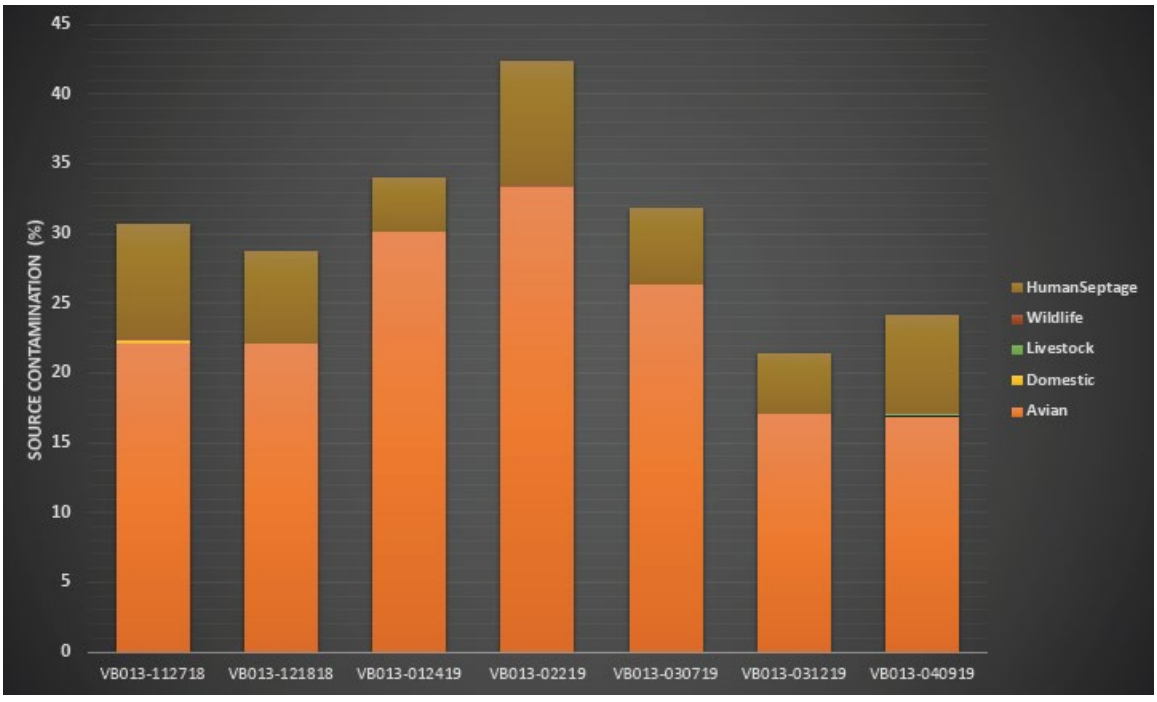

Figure 21. Next Generation Sequencing Results for Station VB013.

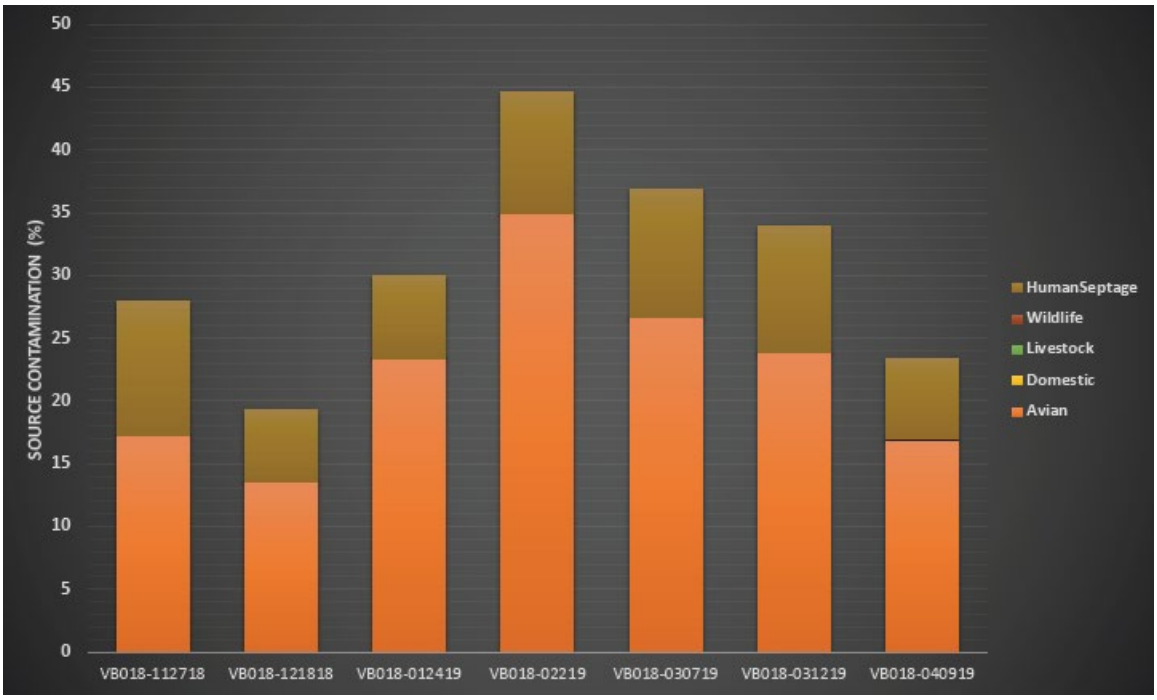

Figure 22. Next Generation Sequencing Results for Station VB018.

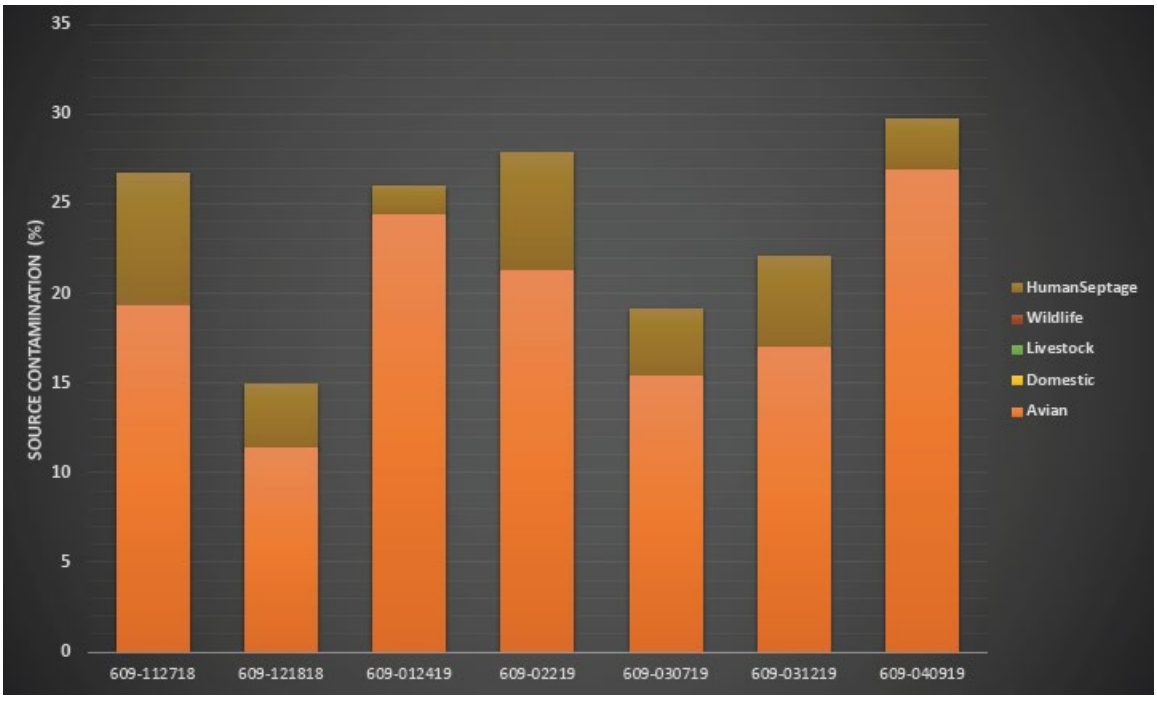

Figure 23. Next Generation Sequencing Results for Station 609.

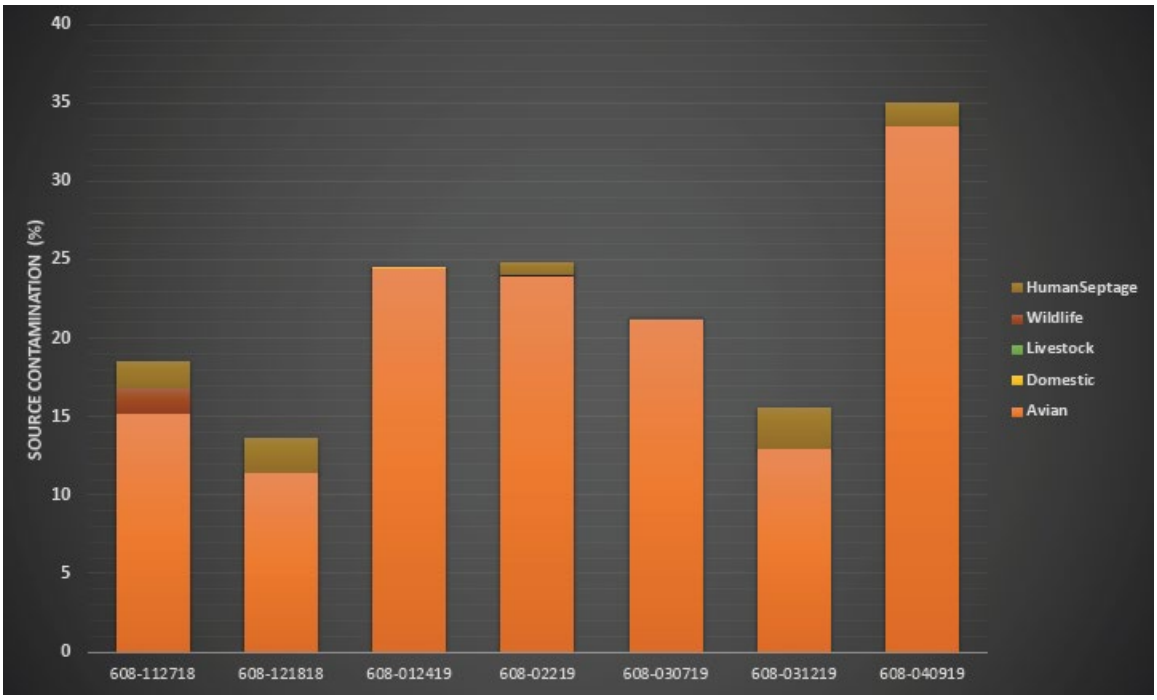

Figure 24. Next Generation Sequencing Results for Station 608.

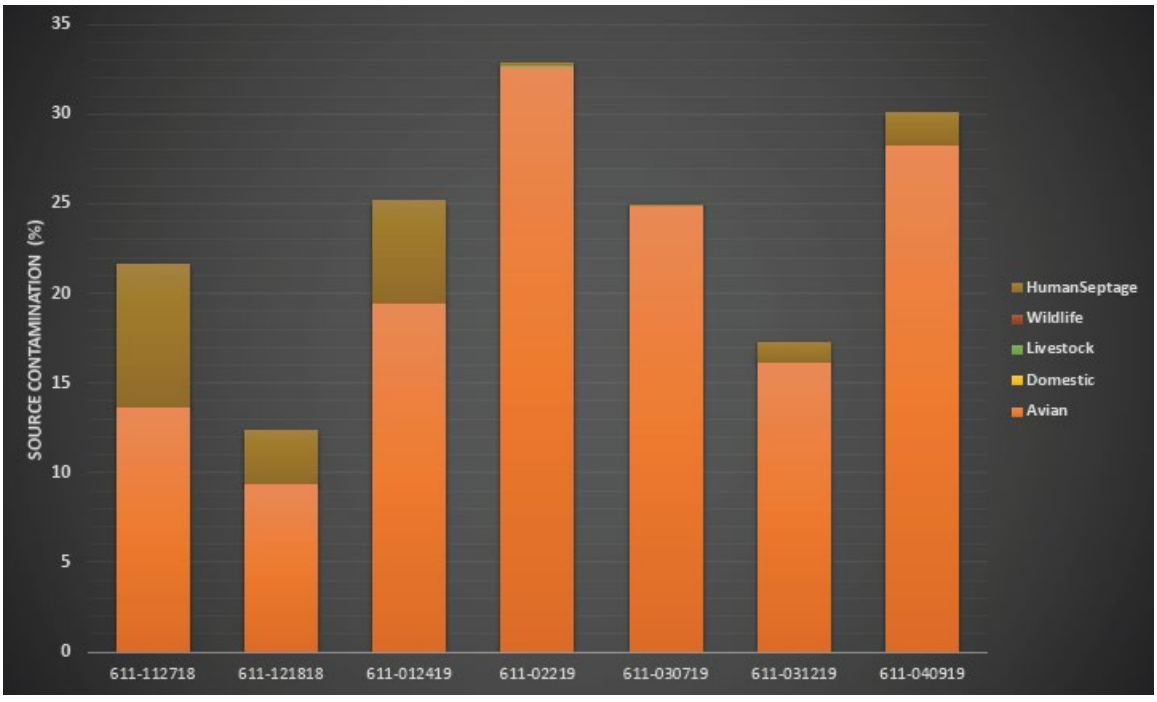

Figure 25. Next Generation Sequencing Results for Station 611.

SUMMARY OF FINDINGS

HYPOTHESIS FINDINGS

The quantitative MST demonstration project hypotheses are accepted, and the associated findings include:

- Accepted H_1 – *Fecal coliform bacteria concentrations in downgraded shellfish protection areas are primarily affected by loadings in freshwater discharges to those areas.* The highest fecal coliform concentrations in the conditional and restricted areas of Vaughn Bay were observed during storm events, which included two storm events in November/December 2018 when the conditional area was closed due to large storm events (greater than 1 inch in 24 hours) (see Appendix E and Figure 8). Fecal coliform bacteria loadings in freshwater discharges to Vaughn Bay were higher during storm events than base flow events in the winter/spring months (see Figure 9). High loadings were also observed during base flow events in the summer/fall months (pre-MST events) when concentrations were somewhat elevated in marine waters over winter/spring months (base events).

- Accepted H_2 – *Fecal coliform bacteria concentrations in downgraded shellfish protection areas and freshwater drainages to those areas are higher during storm events than base flow events.* Geometric mean concentrations of fecal coliform bacteria were higher during storm events than during base flow events by a factor of 22 for marine stations and a factor of 5 for freshwater stations (see Table 9).

- Accepted H_3 – *Fecal coliform bacteria concentrations in downgraded shellfish protection areas and freshwater drainages to those areas are highest during the seasonal first flush conditions in the fall and in the wettest years.* Historical data analysis showed these conditions to be true (Herrera 2018) and the MST study showed that base flow concentrations were higher in the late summer/fall (pre-MST events) than the winter/spring season (base events; see Figure 8), but the study design could not fully address this hypothesis because of the limited study period.

- Accepted H_4 – *Sources of fecal coliform bacteria present in downgraded shellfish protection areas and watershed drainage will vary spatially, temporally, and hydrologically.* Fecal coliform bacteria concentrations varied greatly among the stations, between base flow events in the summer/fall months (pre-MST events) and winter/spring months (base events), and between base and storm events (see Figure 8). Fecal coliform bacteria concentrations were significantly different between base and storm flow in the winter/spring at two of the three marine stations and at one of the seven freshwater drainage stations (see Table 9).

- Accepted H_5 – *Sources of fecal coliform bacteria present in shellfish protection areas and watershed drainage may include humans from onsite septic systems and/or multiple types of farm animals, pets, and wildlife located in the watershed draining to those areas.* The qPCR and NGS results showed that all of these fecal sources were present in at least one sample from every marine and freshwater station (see Tables 12 and 15) with a few exceptions (e.g., freshwater station VB007 did not detect farm animals or pets by either method).

- Accepted H_6 – *The qPCR and community-based MST methods will identify fecal sources present in the collected marine and freshwater samples.* Multiple fecal sources were positively identified by both methods in water samples collected from marine and freshwater stations (see Tables 12 and 15).

- Accepted H_7 – *Characterization of fecal sources in the collected samples will increase the ability to identify effective corrective actions for upgrading shellfish protection areas.* The detection of human sources at relatively low levels in both base and storm flow at most stations by qPCR (see Table 12), at moderately high levels when the qPCR results are normalized for human amounts observed in septage (see Table 13), and at low to moderate levels at all stations by NGS (see Table 15) identified that corrective actions should include septic system management throughout the watershed to include education about system maintenance to reduce ongoing discharges from properly functioning systems during base flow and inspection for failing systems during storm flow. The detection of ruminant sources by qPCR at higher levels during storm events than base events at all three marine stations and three of four major streams (stations 1, 2, and 47; see Table 10) identified that corrective actions should include farm maintenance to reduce stream contamination by farm runoff, particularly in the largest portion of the watershed located north of the bay where poor pasture conditions have been observed at small farms. However, detection of deer sources at comparably low levels to livestock (farm animals) by NGS (see Table 15) identified that farm animal source-control efforts may be less important than septic source control efforts. The detection of dog sources at low levels at most stations primarily during storm events by qPCR (see Table 12) that become moderate when normalized for dog amounts in source samples (see Table 13), and at low levels by NGS (see Table 15) identified that corrective actions should include education about pet waste disposal.

Data Quality Assessment

Data quality assessment findings include:

- Optical brightener fluorescence was not measured on April 9, 2019, at station VB013 due to low flow conditions.
- Water samples collected during four events between August 15 and November 1, 2019, were incorrectly filtered for MST analysis and qPCR analysis was not performed for these events. Three sampling events were added to supplement samples lost for these events.
- Several fecal coliform bacteria results were qualified as estimated due to plate counts outside the ideal range of 20 to 60 or due to laboratory duplicate criterion exceedances (i.e., relative percent difference greater than 35 percent).
- Validation tests were performed for qPCR analysis on 82 fecal source samples for the seven specified markers and for a second cow marker (CowM3) (see Table 7). Among the eight markers tested, specificity ranged from 96 to 100 percent, meeting the 80 percent specificity objective identified in the QAPP. Two markers did not meet the 80 percent sensitivity objective, including horse (HoF597F) at 50 percent and bird (GFD) at 57 percent. These results indicate that the project water test results will have very few false positives or false negatives with the exception that the amount of horse and bird fecal DNA may be underestimated or not detected where present.
- All quality control data for qPCR analyses met the measurement quality objectives.
- No quality control data were provided for the NGS analyses.

Field Data

Mean flow among the freshwater stations during the sampling events ranged 0.07 to 1.15 cfs. The West Vaughn Creek and Vaughn Creek stations exhibited the highest mean flow among the freshwater stations as would be expected by their larger subbasin size. The mean flow for all freshwater stations was higher during storm events than base events, but only significantly higher at stations 47 and 7 discharging to the south shore (see Table 9).

The mean fluorescence of optical brighteners among the freshwater stations ranged 63 to 267 RFUB. Vaughn Creek station 2 and drain station 7 exhibited the highest mean optical brighteners during base and storm events. The mean optical brighteners for all freshwater stations was higher during storm events than base events, but only significantly higher at stream station 47 discharging to the north shore and drain stations 9 and 13 discharging to the south shore (see Table 9).

Optical brighteners did not correlate with human marker concentrations (see Figure 7), suggesting that optical brighteners are not a useful field measure for detecting septic system effluent in the Vaughn Bay watershed. However, human markers values were low because they were rarely detected in a quantifiable amount, as summarized below. The low-level optical brightener meter used for this study has been shown to be useful for detecting moderate to high concentrations of human markers present in drainages that are more contaminated with septic system effluent.

Fecal Bacteria

Fecal coliform concentrations in freshwaters were elevated on occasion during both base and storm flow conditions, and base flow concentrations were much higher in the summer–fall season (pre-MST events) than the winter–spring season (base events) (see Appendix E). Fecal coliform concentrations were typically higher during the pre-MST and storm events than the base flow events at all freshwater and marine stations. The single sample criterion for freshwaters (200 CFU/100 mL) was exceeded in 18 percent (14 of 77) of all freshwater samples. The single sample criterion for freshwaters was exceeded at all freshwater stations during one or more of the three types of events except Vaughn Creek station 2, which exhibited the highest flow. The geometric mean criterion for freshwaters (100 CFU/100 mL) was only exceeded at stations 1 and 7 for the pre-MST events.

Fecal coliform concentrations at the marine stations were comparable to those at the freshwater stations during base flow, were even higher during storm flow, and exceeded criteria more often due to the lower criteria for marine waters (see Table 9). These results suggest that marine waters were contaminated by sources other than freshwater inflow considering tidal dilution. The greater abundance of bird sources observed at the marine stations (see Figure 14) suggests that the additional marine sources likely include seabirds.

Maximum fecal coliform concentrations at the marine stations were observed in the largest storm event in November 2018 when the conditional use area was closed due to high rainfall. The single sample criterion for marine waters (43 MPN/100 mL) was exceeded in 33 percent (11 of 33) of the marine water samples, compared to 18 percent (9 of 49) for the freshwater samples. The single sample criterion for marine waters was exceeded at all marine stations during storm events and base events except station 611 during base events. The geometric mean criterion for marine waters (14 MPN/100 mL) was exceeded at all three marine stations during storm events but not during base events. The single sample and geomean criteria were only exceeded at station 609 during pre-MST events.

Fecal coliform loading rates followed a similar pattern to concentrations because flow rates were much less variable than concentrations. Vaughn Creek station 2 exhibited higher base and storm loading rates than station 1 because of its higher flow and similar concentrations. Fecal coliform loading rates were generally lower in the small streams and drains compared to Vaughn Creek.

Fecal coliform bacteria concentrations at the marine stations typically did not relate to those in the adjacent stream station based on Kendall's tau correlation analysis. The only significant correlation (bold values in Figure 10) between adjacent stations was a positive correlation between West Vaughn Creek station 1 and marine station 609 located near the mouth of West Vaughn Creek. The lack of significant correlation at marine stations 608 and 611 with their adjacent small streams suggests they were affected by other marine water contamination sources, but the lack of correlation also may be due to the high variance among only seven pairs of samples.

E. coli concentrations were greater than fecal coliform concentrations for all events at all freshwater stations except station 13 with ratios varying widely from 0.4 to 7.9 among all stations. Considering *E. coli* are a component of fecal coliform bacteria, these results clearly show that the MPN value calculated by the Quanti-Tray method (used for *E. coli*) is much more sensitive (biased high) than the CFU value calculated by the membrane filter method (used for fecal coliform). Thus, lower *E. coli* results would have been obtained if the membrane filter method was used for this study.

The geomean criterion for *E. coli* (100 MPN/100 mL) was only exceeded during storm events at station 47 (see Table 9). The single sample criterion for *E. coli* in freshwaters (300 MPN/100 mL) was exceeded at all freshwater stations except station 13 during one to three of the four pre-MST events. The single sample criterion for *E. coli* was exceeded during one storm event at freshwater stations 2, 47, and 7 (see Appendix E), and was not exceeded at any station during any base event.

QUANTITATIVE PCR

Bird was most commonly detected in the water samples at a frequency ranging from 57 to 100 percent among the stations for the seven sampling events (see Table 10). Human and ruminant were the next most commonly detected hosts at widely varying frequencies among the freshwater stations (0 to 57 percent) and similar frequencies among the marine stations (29 to 57 percent). Dog was detected at a lower frequency (less than 30 percent except for 43 percent at station 609), which did not exceed the detection frequency for human or ruminant at any station. Cow and horse were rarely detected; cow was detected once (14 percent of seven samples) at the two Vaughn Creek stations 1 and 2 and marine station 611, and horse was detected only once at marine station 609. Pig was not detected in any sample.

Moderate to high fecal host concentrations were primarily observed during storm events when fecal coliform bacteria concentrations were also highest (see Table 12). Moderate to high concentrations (greater than 5,000 copies/100 mL) were observed on multiple occasions for ruminant at stream station 1, and for bird at stream stations 1 and 47 and all three marine stations. Moderate concentrations were observed on one occasion for ruminant at stream station 47 and for bird at stream station 2. Maximum concentrations were much lower for human (2,333 copies/100 mL at station 1), dog (1,037 copies/100 mL), and cow (567 copies/100 mL at station 1.

Normalizing qPCR concentrations for host-associated marker concentrations in the fecal source samples (see Table 13) increase relative concentrations for human and decrease relative concentrations for ruminants because of the relatively low human concentrations observed in septage (0.02 billion copies/100 grams) and relatively high ruminant concentrations observed in ruminant source samples (11.7 billion copies/100 grams). Detected human concentrations ranked moderate-high to high, while detected ruminant concentrations ranked low to moderate. Detected bird concentrations ranked moderate to high, and detected dog concentrations ranked moderate. Overall, the normalized qPCR results indicate that human and bird are the primary fecal sources present in the freshwaters and marine waters, followed by ruminant and dog.

The geomean of host-associated marker concentrations (see Figure 8) and loading rates (see Figure 9) at each station clearly show the predominance of fecal sources in storm flow compared to base flow. These results clearly show the high importance of bird sources and moderate importance of ruminants in Vaughn Creek on the high fecal bacteria concentrations in Vaughn Bay. Fecal host loadings during base flow in the streams and during all flow from the drains were insignificant in comparison.

Geomeans for the four most abundant hosts (bird, ruminant, human, and dog) were substantially higher (more than double) for the sampled storm events than base events and for the low fecal coliform events (meeting criteria) than the high fecal coliform events (exceeding criteria) at the freshwater and marine stations. One exception is that the human geomean was similarly low for the low and high fecal coliform events at the freshwater stations. The magnitude of these differences was much higher for the marine stations than the freshwater stations. For example, bird concentrations increased during storm events by a factor of 4.5 at the freshwater stations and a factor of 26 at the marine stations, and bird concentrations increased during high fecal coliform events by a factor of 4.7 at the freshwater stations and a factor of 14 at the marine stations. Combining host marker results for all events shows that marine stations were not substantially higher than the freshwater stations. These results clearly show the importance of evaluating spatial differences among fecal sources separately for base and storm events.

The qPCR results for individual host-associated markers were not analyzed for spatial correlations among sampling stations due to the low number of samples (seven) and the high proportion of not detected or quantifiable values for most host-associated markers. The sum of host-associated marker concentrations poorly correlated with fecal coliform bacteria (see Figure 15). A lack of correlation between fecal coliform bacteria and DNA concentrations has been typically observed elsewhere and is not surprising given the differences in the populations tested and methods used for quantification.

The qPCR interlaboratory comparison results from the King County Environmental Lab (KCEL) for all samples collected in April 2019 found that only a trace amount of one human marker (Hu2) was detected in the samples from drains 13 and 18 (see Table 14). These results agree well with the study results from Source Molecular that also detected trace amounts (detected but not quantifiable) of the human marker only in those same samples. No cow, ruminant, or dog were

detected in the April samples by Source Molecular except for a trace amount (detected but not quantifiable) of ruminant in the sample from stream 47, which was given an estimated value of 200 copies/100 mL and is well below the KCEL ruminant method detection limit of 11,320 copies/100 mL.

NEXT GENERATION SEQUENCING

Next generation sequencing (NGS) results from UMBTI were based only on fecal source samples collected for this study. NGS results based on the nationally fecal source library will be provided separate from this report when the analysis is complete. The unmatched percentage exceeded 50 percent in each sample because most fecal and non-fecal bacteria present in the water samples were not present in the local fecal source samples based on matching DNA patterns.

The NGS results show that moderate to high amounts of bird (from gull and goose samples) were detected in all samples (see Table 15). Low to moderate amounts of human (from septic samples) were detected at all stations except only low amounts were detected at marine station 608. All other sources were typically not detected or only detected in very low amounts in a few samples. These other sources include livestock (ruminants from cow, sheep, goat, llama, and alpaca samples; horse from horse samples; and pig from pig samples), dog (from dog samples) and deer (from deer samples).

With a few minor exceptions, deer amounts were higher than livestock suggesting that deer were a major component of the ruminant detected by qPCR.

The NGS results were reported as percent matching all bacteria DNA in local fecal source samples with the total percent matched ranging from a low of 9 to 21 percent for West Vaughn Creek station 1 to a high of 19 to 45 percent for drain stations 7, 13, and 18. These results show no consistent chronological patterns among the freshwater or marine stations and no differences between base and storm events (Events 1, 2, and 6 of seven events in each chart), in contrast to the higher fecal concentrations observed during storm events by the culture and qPCR methods.

The NGS results suggest than septic systems are the major controllable fecal source in the Vaughn Bay watershed. It is possible that many more sources will be detected when the water samples are compared to the national fecal source library and the percent matched increases. However, it seems unlikely that livestock or dog will become a major source considering the numerous local livestock and dog feces samples used in the initial NGS analysis. It seems more likely that any new sources identified would primarily include other wildlife not sampled in the Vaughn Bay watershed.

The NGS results complement the qPCR results by the high abundance of bird observed by both methods. The NGS method showed relatively higher amounts of human and lower amounts of ruminants than the qPCR method. The comparably low amounts of livestock and deer detected by NGS suggests that much of the ruminant detected by qPCR may have been deer rather than livestock.

SOURCE CONTROL EVALUATION

SOURCE CONTROL BASIS

The goal of fecal bacteria source control is to permanently open areas to shellfish harvesting by reducing fecal bacteria concentrations in marine waters. The principal objective of this source control evaluation is to prioritize source control actions using the MST study results for effectively achieving the source control goal. Many factors were considered for the prioritization, depending on which sources and environmental factors correlated best with fecal bacteria concentrations. Factors included high fecal bacteria loadings from freshwater sources exhibiting high flow and moderate to high fecal bacteria concentrations, and high fecal bacteria concentrations with moderate to high concentrations of controllable sources (humans/livestock) in relation to concentrations of uncontrollable sources (wildlife). Some wildlife sources are affected by human behavior and may be controllable (e.g., feeding birds or attracting rodents), but these types of sources are not expected to be significant in the Vaughn Bay watershed.

The project team identified appropriate methods for controlling high priority sources based on past experience and the project findings. By far, the most marker detections in the 70 collected water samples were for birds, (63 detections), followed by humans (29 detections) and ruminants (27 detections). Cow was detected in three samples, horse was detected in one sample, and pig was not detected in any samples. Hence, the predominant fecal sources appear to be birds (which includes both wild birds and domesticated birds such as chickens, geese, and turkeys), humans, and ruminants (which includes both domesticated ruminants such as cow, sheep, and goats, and wild ruminants such as deer). The fourth most detections were for dogs (17 detections). The source control evaluation focuses on these four sources.

Geomean concentrations of these four sources were much higher during wet weather storm events than dry weather base events at the freshwater stations (2 to 5 times higher) and even more so at the marine stations (4 to 26 times higher), and all differences were statistically significant (at alpha = 0.10; see Table 9). Similar or greater differences were observed between sampling events above and below single sample criteria for fecal coliform bacteria (see Figure 14). Geomean concentrations were higher at the marine stations than freshwater stations during storm events and above criteria events, but were similar during base events and below criteria events (see Table 9 and Figure 14).

During storm events, geomean concentrations of bird were highest at stream stations 1 and 47 (6,341 and 6,571 copies/100 mL, respectively) and at marine stations 609 and 608 (6,880 and 7,326 copies/100 mL, respectively) (see Table 9). Storm event loading rates for bird were higher at stream station 2 (102 billion copies/day) than stream stations 1 and 47 (68 and 39 billion copies/day), compared to negligible amounts (less than 5 billion copies/day) at the other

freshwater stations (see Figure 13). Stream stations 1 and 2 discharge to the east shore near marine station 609, and stream station 47 discharges to the north shore near marine station 608, showing a possible influence of freshwater discharges on adjacent marine areas.

Geomean concentrations of human during storm events were highest at stream stations 1, 2, and 47 (401, 165, and 167 copies/100 mL, respectively) and were similar among all three marine stations 609, 608, and 611 (167, 165, 133 copies/100 mL, respectively). Storm event loading rates for human were higher at stream stations 1 and 2 (4 billion copies/day) than stream station 47 (1 billion copies/day), compared to negligible amounts (less than 0.2 billion copies/day) at the other freshwater stations (see Figure 13).

Geomean concentrations of ruminant during storm events were highest at stream stations 1 and 47 (8,725 and 1,684 copies/100 mL, respectively) and were similar among all three marine stations 609, 608, and 611 (712, 708, and 913 copies/100 mL, respectively) (see Table 9). Storm event loading rates for ruminant were higher at stream station 1 (94 billion copies/day) than stream stations 2 and 47 (7 and 10 billion copies/day), compared to negligible amounts (less than 0.2 billion copies/day) at the other freshwater stations (see Figure 13).

Geomean concentrations of dog during storm events were highest at stream station 47 and drain station 13 (202 and 192 copies/100 mL, respectively) and at marine stations 609 and 611 (200 and 243 copies/100 mL, respectively (see Table 9). Storm event loading rates for dog were higher at stream station 2 (3 billion copies/day) than stream stations 1 and 47 (1 billion copies/day), compared to negligible amounts (less than 0.2 billion copies/day) at the other freshwater stations (see Figure 13).

Geomean concentrations of ruminant were higher than human during storm events by a factor of 3 at the freshwater stations and a factor of 5 at the marine stations, but were similarly low during base events (see Table 9). However, host-associated marker concentrations are not directly comparable because the DNA concentration and qPCR reactivity differ among host-associated feces. The normalized qPCR results (Table 13) increase relative concentrations for human and decrease relative concentrations for ruminants. As a result, detected human concentrations ranked moderate-high to high, while detected ruminant concentrations ranked low to moderate. Detected bird concentrations ranked moderate to high, and detected dog concentrations ranked moderate. Overall, the normalized qPCR results indicate that human and bird are the primary fecal sources present in the freshwaters and marine waters, followed by ruminant and dog.

Areas of Concern

Geographical Areas of Concern

Seven freshwater tributaries were sampled as part of this project. The tributaries with the most consistently elevated fecal coliform counts were initially selected for site investigation work. This group of tributaries included two major stream stations 1 and 2 (VBU001 and VBU002), two minor stream stations 47 and 9 (VB047 and VB009, and three drain stations 7, 13, and 18 (VB007, VB013, and VB018). These seven sampling stations drain 83 percent of the Vaughn Bay watershed, and the remaining 17 percent of the watershed drains to the bay or lower Vaughn Creek (below VBU002) via numerous small drainage outfalls (see Figure 3 and Table 2).

Fecal coliform concentrations in freshwaters were elevated on occasion during both base and storm flow conditions, and base flow concentrations were much higher in the summer–fall season (pre-MST events) than the winter–spring season (base events) (see Figure 7). Fecal coliform concentrations were typically higher during the pre-MST and storm events than the base flow events at all freshwater and marine stations. The single sample criterion for freshwaters (200 CFU/100 mL) was exceeded in 18 percent (14 of 77) of all freshwater samples.

Marine water concentrations are affected by fecal coliform loading rates more than concentrations. Fecal coliform loading rates were highest at the two major streams, with moderate rates at small stream station 47 and drain 7. Vaughn Creek station 2 exhibited higher base and storm loading rates than West Vaughn Creek station 1 because of its higher flow and similar concentrations. Thus, the geographical focus of source control is the area draining to stations 1, 2, 47, and 7 representing 78 percent of the watershed.

Fecal coliform concentrations at the marine stations were comparable to those at the freshwater stations during base flow, were higher than the freshwater stations during storm flow, and exceeded criteria more often than the freshwater stations due to the lower criteria for marine waters. These results suggest that marine waters were contaminated by sources other than freshwater inflow during base flow and storm flow considering tidal dilution. The abundance of bird sources observed at the marine stations suggests that the additional marine sources likely include seabirds.

Fecal Sources of Concern

The MST study has shown that the primary fecal sources of concern in the Vaughn Bay watershed include humans from septic systems, some farm animals, dogs, and birds. Farm animals of high concern likely include sheep, goat, and llama in the ruminant group, and chickens and turkeys in the bird group. Cow, horse, and pig were rarely observed in the collected samples and are considered farm animals of lower concern. Dog was observed more often than these farm animals of lower concern.

Bird was observed most often and at the highest concentrations of all host-associated markers tested at nearly all freshwater and marine stations. However, human ranked higher than bird when qPCR concentrations were normalized for the host marker amount in fecal source samples. Wild land birds and seabirds are expected to compose most of the bird detected because of its ubiquitous presence. However, chicken and turkey have been observed at a few farms in the watershed and, therefore, are considered a fecal source of concern.

Periods of Concern

Storm events are the primary period of concern for source control because storm runoff produced the highest concentrations of fecal coliform bacteria and DNA concentrations for all fecal sources of concern identified in the study area waters. Fecal coliform bacteria concentrations were also high at freshwater stations during base flow conditions in the late summer and early fall, but they resulted in only a small increase in concentrations at the marine stations due to the low flow rates and loading rates. Thus, late summer and early fall is a period of moderate concern for source control. Although fecal sources were not measured in samples collected during this period, it is expected that the sources of concern are similar to those identified in the samples collected in late fall through early spring.

Source Control Tools

Many tools are available to address sources of fecal contamination. These tools can be divided into three general categories, educational, incentive, and enforcement. All of these tools are currently being used by the Tacoma Pierce County Health Department (TPCHD), Pierce County Surface Water Management (PCSWM), and/or Pierce Conservation District (PCD) to improve water quality in Vaughn Bay, and the rest of the Key Peninsula. The three agencies are working together, along with local nonprofit organizations and community members as the South Sound Clean Water Partners.

Education is a common source control method that can be made more effective with MST information. For example, farm owner education can be modified when project results show which specific sources are primarily impacting water quality and shellfish harvesting. Herrera's Willapa River MST project (Herrera 2005) resulted in increased compliance by farmers when they were shown the high abundance of cow sources in the river samples.

Source control tools that are available for use in the Vaughn Bay watershed include:

Educational Tools:

- Technical Assistance:
    - Sanitary surveys and site visits of septic systems by TPCHD, which can also include assistance with livestock/manure management, pet waste management, and wildlife feeding or other attraction behaviors.

    - Farm visits by PCD.
    - Steps/actions community members can take to address wildlife. This information is provided in part by Washington Department of Fish and Wildlife.
- TPCHD Workshops:
    - Septic System Operation and Maintenance
    - Landscaping Around Your Septic System
    - Septic Socials
- PCD Workshops:
    - Build a Bin (manure bins for small farmers)
    - Starting or Growing Your Farm
    - Pasture Management
- Booths or displays at local events:
    - Key Peninsula Livable Community Fair
    - Key Peninsula Farm Tour
- Newspaper articles
- Newspaper advertisements
- Presentations to local community groups

Incentive Tools:

- Septic system incentives for inspections, tank pumpings, and/or installation of risers to make future inspections easier
- Financial assistance for septic repairs:
    - Craft3 Clean Water Loan
    - Pierce County Human Services
    - USDA Rural Development

- Cost-share assistance for farm BMPs:
    - Exclusion fencing
    - Buffer plantings
    - Waste storage solutions
    - Heavy use areas/paddocks
    - Drainage management
    - Off-stream stock watering

Enforcement Tools:

- Chapter 2 of the TPCHD's Code, which is conducted by the Code Enforcement Program to address failing septic systems.
- Pierce County Ordinance 11.05 is enforced by PCSWM to address pollution, including high fecal bacteria counts, impacting the municipal separate storm sewer system (MS4).
- RCW 90.48 is enforced by the Washington Department of Ecology (Ecology) to address water quality pollution, which includes addressing poor animal keeping practices that cause water pollution.

Source Control Actions

Source control actions are described separately for septic systems, farm animal keeping practices, and birds and dogs. Current practices and recommended actions are described for each of these source types. Proposed methods for assessing the effectiveness of source control actions are then summarized.

Septic Systems

Current Practices

The majority of Pierce County residents in the shellfish protection areas, including all residences in the Vaughn Bay Watershed, are served by septic systems. Educating homeowners about their systems and ways to protect the quality of Puget Sound is vital to protecting water quality. Proper septic system operation and maintenance (O&M) is instrumental in maintaining clean water. This includes regular inspections by a certified professional.

The Key Peninsula portion of the Key Peninsula-Gig Harbor-Islands (KGI) Watershed, along with the Burley Lagoon Watershed, was designated by TPCHD as a Marine Recovery Area (MRA) in 2007. The MRA covers approximately 63 square miles.

TPCHD has piloted septic system maintenance and water quality education and outreach campaigns in the MRA to raise awareness about septic operation and maintenance (O&M) regulations and evaluate effectiveness for possible replication in other Pierce County communities. Staff have, and continue to, work with residents to promote an understanding of the connection between a healthy environment, including water quality, and active septic O&M activities.

Additionally, education and outreach activities have been implemented in the MRA to raise the level of awareness and concern regarding water protection and to motivate homeowners to adopt behaviors that minimize human impact upon water quality. These activities include:

- Writing water quality articles for the Key Peninsula News newspaper
- Running water quality ads in the Key Peninsula News
- Staffing booths or tables at local events, including the Key Peninsula Livable Community Fair and the Key Peninsula Farm Tour
- Posting a water quality activities monthly schedule at TPCHD's Key Peninsula office
- Assisting PCSWM with developing a Water Quality Report for the Key Peninsula
- Holding septic landscaping workshops and septic system operation and maintenance workshops

To encourage residents to get their septic system inspected, the TPCHD has been able to provide financial assistance for the inspection of gravity and pressure distribution systems in the MRA. Permits require yearly inspections of more complicated systems and financial assistance for inspections isn't currently available for these types of systems. Through the Septic Care Incentive Program residents can receive $125 off the cost of a routine inspection, $200 off tank pumping, if needed, and $125 off riser installation, if desired. This program has been funded through several grants and has been highly successful, with more than 500 people taking advantage of this opportunity. At this point, it appears that funding for this program will end June 30, 2019.

When a failing septic system is identified, the site is referred to the TPCHD's On-Site Sewage Code Enforcement Program. The property owner is notified as soon as possible. Documentation is sent stating that the property's septic system was found to be out of compliance. The documentation also includes TPCHD's expectations regarding the repair timeline.

The property owner is encouraged to contact Craft3, a nonprofit, non-bank Community Development Financial Institution, for information on a possible low interest loan to help finance the repair. If there is a severe financial hardship, the property owner may be able to receive funding assistance for the repair from Pierce County Human Services. From 2007 through 2017, funding assistance was also available through the Pierce County Septic Repair Program, which was administered by PCSWM. This program was targeted at funding repairs that would result in a significant improvement to water quality. Several key repairs in shellfish growing areas were funded through this program, including one on Vaughn Bay. It is possible this program will be reinstated within the next few years.

The On-Site Sewage Code Enforcement program uses a multi-step and graduated approach to enforcement action that allows multiple opportunities for voluntary compliance and affords due process to the property owner. If the property owner is unable or unwilling to comply with TPCHD's requirements, a Violation Notice will first be issued. Continued non-compliance may result in a Certificate of Non-Compliance (CNC) being filed with the Pierce County Auditor's office, posting the property with a Notice to Vacate, or a Health Order to have water and/or electricity to the property shut off.

Recommended Actions

Funding of the Septic Care Incentive Program should be continued at a level equal to or greater than past amounts because it has proven to be successful for increasing inspections of gravity and pressure distribution systems in Vaughn Bay and other areas of the MRA. Septic system inspections are critical to identifying maintenance needs for reducing human sources of fecal bacterial contamination of streams and marine waters. In addition, program funds should be increased to provide financial assistance for inspections of more complicated systems that are required by the Permit to have annual inspections.

Based on the MST study finding that high bacteria counts and human fecal sources are most prevalent during wet weather storm events in the Vaughn Bay watershed, sanitary surveys and site visits of septic systems by TPCHD should target wet weather conditions when the systems are most prone for failure to adequately treat human waste. TPCHD should modify sanitary survey methodology to include qPCR analysis of human marker HF183/BacR287 for improved detection of septic system effluent in drainage waters.

The Pierce County Septic Repair Program administered by PCSWM should be reinstated as soon as possible. This program has funded septic system repairs that resulted in a significant improvement to water quality in shellfish growing areas, including one system on Vaughn Bay.

Technical assistance, workshops, local event displays, newsletter articles, and community presentations should include findings of this MST study that septic systems are a source of bacterial contamination of Vaughn Bay.

Farm Animal Keeping Practices

Current Practices

Poor animal keeping practices, whether ruminants, horses, or other types of livestock, are primarily addressed by the PCD through technical assistance and, when available, financial incentives. PCD's role is to promote proper management of farm resources, including the proper handling of livestock waste. PCD also assists landowners to develop farm plans, which include Best Management Practices (BMPs) to address water quality issues. If other agencies suspect that animal wastes are contributing to bacterial contamination of shellfish waters, then a referral is made to PCD through a referral protocol. In response, they will contact the property owner and offer technical assistance.

PCD may also conduct area wide farm and animal inventories. These inventories include identification of priority farms that may be the most likely sources of contamination. Landowners of priority farms are then invited to utilize PCD services. PCD does not have enforcement authority and avoids adversarial relationships with landowners. They encourage landowners to voluntarily adopt BMPs for management of animals and animal wastes. Funding assistance for agricultural BMPs is very limited and often has strict limitations on how the money can be used.

If a farmer doesn't address the problem causing a water quality impact, either on their own or with the assistance of PCD, the TPCHD requests assistance from Ecology. Ecology has the legal authority, through RCW 90.48 to ensure the problem is corrected.

Recommended Actions

PCD should continue inspections, technical assistance, and financial incentives for farms in the Vaughn Bay watershed to control the discharge of animal manure from the farms. TPCHD should include inspection of farm drainage during sanitary surveys and site visits, and provide recommendations for drainage improvements to the property owner and PCD. The farm inspections should target wet weather conditions when manure sources are most prevalent and it is easiest to observe drainage from the farm property. Based on the MST study findings, small farms with small ruminants (sheep, goat, and llama) and birds (chicken and turkey) should be prioritized over large farms with cow, horse, and pig.

Technical assistance, workshops, local event displays, newsletter articles, and community presentations should include findings of this MST study that poor animal keeping practices are a source of bacterial contamination of Vaughn Bay.

Birds and Dogs

Current Practices

There are very limited tools available, none of which include financial incentives or assistance, to address water quality impacts caused by wild birds (mainly Canada Geese and seabirds) or dogs. Domestic bird sources from chickens and turkeys are addressed above for farm animal keeping practices.

Technical assistance is provided by the Clean Water Partners by handing out dog waste brochures at local fairs and events. The Clean Water Partners are also planning to write a newspaper article on pet waste and wildlife impacts to water quality for an upcoming issue of the Key Peninsula News. Pet waste stations are a good option to remind pet owners to pick up and dispose of the waste properly. However, pet waste stations must be supported and maintained by the residents.

Under the Pierce County stormwater permit, PCSWM also has a legal obligation to address pollutants entering the County's storm drainage system. Pierce County Ordinance 11.05 gives PCSWM the authority to also address pollutants going to waters of the State. PCSWM has established a multi-agency team to determine how best to address pollution sources on a case by case basis. However, this team has very limited ability to reduce water quality impacts from wild birds and other wildlife.

Recommended Actions

Additional qPCR analysis should be conducted to determine the relative proportion of wild and domestic bird sources in marine waters during storm events. Water samples should be collected at the three marine stations during at least three storm events and analyzed for the general bird marker GFD and a chicken or poultry litter marker. In addition, approximately 10 chicken/turkey litter samples should be collected for validation of the sensitivity and selectivity of each qPCR marker.

Technical assistance, workshops, local event displays, newsletter articles, and community presentations should include findings of this MST study that dogs are a source of bacterial contamination of Vaughn Bay.

Source Control Assessment

Effectiveness of source control actions should be assessed by fecal bacteria monitoring and quantitative MST. The source control assessment should be conducted in approximately 5 years to allow sufficient time for actions to effectively reduce fecal bacteria concentrations in Vaughn Bay and to collect sufficient post-control data for statistical testing. A quality assurance project plan (QAPP) should be developed based on the following recommendations for fecal bacteria monitoring and quantitative MST.

Routine monthly fecal coliform bacteria monitoring of marine waters should be continued by WDOH. Storm events should be targeted for sampling whenever possible. The collected fecal coliform bacteria data should be evaluated for short-term (monthly) and long-term (annual) trends in concentration and criteria exceedance at each station and collectively for separate regions of the bay (restricted, conditional, and unrestricted).

Routine monthly fecal coliform bacteria monitoring of Vaughn Creek (station 2) should be continued by PCSWM. Two high-priority stream stations should be added to the routine monitoring program to include West Vaughn Creek (station 1) and the unnamed stream (station 47) draining to the north shore of Vaughn Bay. If routine indicator bacteria change from fecal coliform bacteria to *E. coli* for water quality standards compliance, then the collected samples should also be analyzed for fecal coliform bacteria using the membrane filtration method for direct comparison to marine monitoring data. Stream sampling dates should coincide with marine sampling dates and include targeted storm events whenever possible. Stream flow should be measured at each station during each sampling event.

Rainfall data should be used to designate monitoring events as either base events or storm events. Data should be presented graphically using box and whisker plots and tested for statistical differences between groups of samples collected before (pre-control) and after (post-control) implementation of source control actions. Source control effectiveness should be tested separately using appropriate non-parametric statistical methods for base and storm events at each station and for various groups of stations (e.g., all freshwater, all marine, and the three marine shellfish classifications).

Quantitative MST methods should be used for a limited number of post-control sampling events for comparison to pre-control data collected by this MST study. At a minimum, the post-control MST study should include of the same three marine stations, the three high-priority stream stations, four base events, and three storm events. Water samples collected for fecal coliform bacteria analysis should also be analyzed for quantitative MST following protocols recommended in Appendix D and summarized in the following chapter of this report. Study objectives and hypotheses should be carefully developed in the QAPP based on fecal bacteria trends and the implemented source control actions. MST methodology should then be selected and described in detail using information provided in this report and the QAPP developed for this quantitative MST study of Vaughn Bay (Herrera 2018).

LESSONS LEARNED AND RECOMMENDED MST PROTOCOLS

An objective of the Quantitative Microbial Source Tracking Demonstration Project is to develop MST method protocols for cost-effective application in other watersheds based on the project findings. Recommended MST protocols are presented in Appendix D that include the following sections:

- Problem Statement
- Historical Data Analysis
- Project Objectives
- Study Design
- Sampling and Analysis
- Quality Control
- Data Analysis

Lessons learned by the Quantitative Microbial Source Tracking Demonstration Project also should be considered to anticipate and avoid potential problems with future projects in other watersheds. Some of the lessons learned include:

- Collect and analyze watershed data for potential fecal sources to the greatest extent possible and allow sufficient time for that analysis before designing an MST study. Watershed information lacking for this MST study that would have been useful included:
    - Septic system construction and maintenance records
    - Farm locations, livestock counts, and observed pasture conditions
    - Wildlife observations and population estimates
- Design a flexible study schedule with enough time, staff, and budget resources to adapt to potential weather delays, tidal conditions, or lost data. Scheduling challenges encountered for this MST study include:
    - Correctly predicting the timing of acceptable storm events and having staff available to meet changing storm conditions.

- Limiting sampling to daylight hours on Monday through Thursday due to budget and sample holding time constraints.
- Limiting marine water sampling to a minimum tide elevation and freshwater sampling to a maximum tide elevation due to station access constraints.
- Cancelling a sampling event due to a snowstorm.
- Having enough sample bottles to add a sampling event with short notice.
- Adding one or more sampling events because samples were not properly processed or analyzed by the laboratory.

- Prepare agreements with laboratories specifying expectations for sample collection/delivery, sample processing/analysis, and quality control procedures/reporting, and include contingencies for laboratory errors.
- Prepare templates of chain-of-custody forms to be used for each type of sampling event and review every form to ensure the proper number of samples were collected and the analytical methods were requested.
- Coordinate with laboratories in a timely manner to notify anticipated sample delivery time, extend hours when possible to receive and process samples late on the day of collection, and ensure all samples were received intact and cold.
- Carefully consider the potential risks and benefits associated with having a local laboratory filter MST samples if they do not have previous MST sample processing experience. If a local laboratory is used to filter MST samples, then initiate the following protocols:
  - Provide filtration equipment and instructions, which include collection of duplicate filters for each sample.
  - Train laboratory staff on proper filtration and filter handling procedures before the first sampling event.
  - Audit laboratory procedures and freezer temperature for the first sampling event.
  - Ship the primary set of filters with enough dry ice to the MST laboratory after the first event, and make sure they were received intact and frozen.
- Do not freeze water samples collected for MST analysis.
- Collect fecal source samples from the study area to validate sensitivity and selectivity of selected qPCR host-associated markers, and to normalize qPCR results for the fecal bacteria DNA concentration in samples from each host. If the MST laboratory has not

performed marker validation in this region, then collect approximately 10 samples for each host-associated marker and analyze them for all study markers for thorough validation. If Source Molecular is used for MST analysis, then collect approximately 10 samples for each host-associated marker not tested in this MST study and collect additional samples from sources evaluated by this MST study to build on those validation results for high-priority sources in the study area.

- Provide additional project budget for fecal source sampling and work directly with residents to identify efficient sampling locations.

- Analyze the fecal source samples for concentrations of indicator bacteria used by the study to relate the amount of source DNA to viable indicator bacteria for evaluating the relative importance of controlling each source for meeting indicator bacteria criteria.

- Request, evaluate, and report specific quality control data from the MST laboratory to ensure high quality data are obtained.

- Provide additional time for reanalysis and reporting of MST data, and for data interpretation and reporting.

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APPENDIX A

Field Data

The contents of this appendix will be provided in a separate PDF.

APPENDIX B

Fecal Bacteria Lab Data Reports

The contents of this appendix will be provided in a separate PDF.

APPENDIX C

Source Molecular Lab Data Reports

The contents of this appendix will be provided in a separate PDF.

APPENDIX D

MST Study Protocols

The contents of this appendix will be provided in a separate PDF.

APPENDIX E

Project Database

The contents of this appendix will be provided in a separate PDF.