MICROBIAL SOURCE TRACKING IN THE CLACKAMAS RIVER

February 23, 2016

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This project has been funded in part by the United States Environmental Protection Agency under a federal grant issued under Section 319(h) of the Clean Water Act. The contents of this document do not necessarily reflect the view and polices of the Environmental Protection Agency, nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

Introduction

A 2006 Water Quality Assessment of the lower Clackamas River (Mm 0-15) conducted by the Oregon Department of Environmental Quality listed the overall status as impaired, citing *Escherichia coli* as the cause of impairment. Eight stream segments within the Clackamas River Sub-basin are listed on the 2002 303(d) list for violating the *E. coli* Total Maximum Daily Load (TMDL) standard.

Non-point sources, runoff from urban, rural residential and agricultural lands, failing septic systems, pet waste, wildlife waste, and livestock waste are likely sources of fecal bacteria in the Clackamas Sub-basin. Yet determining the actual source of non-point source fecal bacteria in the watershed to date has remained elusive.

In 2013 the Clackamas River Water Providers conducted GIS analysis to identify potential threats to drinking water quality from septic systems in the Clackamas River watershed. This included identifying geographic areas not served by municipal wastewater treatment facilities, and completing a high/low spatial cluster analysis identifying areas in the Clackamas River watershed where statistically significant groupings of septic systems are located. Through this analysis approximately 9,000 septic systems were identified in the Clackamas River watershed and of these 1,000 septic systems were ranked as high risk. The designation of a septic system as high risk, however, is not alone an indication of septic failure.

Water-borne human fecal contaminants harbor many pathogens, pose serious health risks to humans (Haile *et al*., 1999), cause economic losses and may disrupt aquatic ecosystems (van der Putten *et al*., 2007; Stewart *et al*., 2008). Pollution of water with human and animal feces can cause illnesses and have lethal consequences when enteric pathogens present in the fecal material

are transported into the water (Scott et al., 2002; Shanks et al., 2006; Walters et al.,2007). Therefore, our ability to identify and eliminate fecal contamination of water, now and in the future, is essential to reduce the risk of waterborne disease. Historically, fecal indicator bacteria (FIB), which include total and fecal coliforms, *Escherichia coli*, and fecal enterococci, have been used to measure fecal pollution and predict the risk of pathogenic microorganisms in aquatic environments. Elevated levels of these indicators suggest fecal pollution and possible association with enteric pathogens (Savichtcheva and Okabe, 2006; Roslev and Bukh, 2011).

The detection and enumeration of FIB does not discriminate among the sources of fecal contamination (Field et al., 2003). Still, fecal indicator bacteria (FIB) that normally reside in the gastrointestinal tracts of humans and animals are used throughout the world to assess the microbiological quality of drinking and recreational waters due to their easy detection and rapid growth in the laboratory setting. As a result, in the United States FIB are used to define bacterial water quality standards aimed at reducing health risks in recreational waters.

Many monitoring programs in the Clackamas River watershed and surrounding region have focused only on FIB measurements and do not test for pathogens. Over the past decade, however, the usefulness of using FIB data alone to assess fecal pollution has been challenged. A few limitations of using standard FIB to represent pathogens in water include the fact that FIB have been shown to multiply in the environment, that they are not host specific, and that the absence of FIB is not necessarily evidence of pathogen absence (Field and Samadpour, 2007; Santo Domingo et al., 2007; Savichtcheva and Okabe, 2006). Such limitations have led to the investigation of alternative indicators of fecal pollution.

In recent years, researchers have developed specific methods of fecal contaminant detection and identification using *Bacteroides* targeted polymerase chain reaction (PCR), and

sensitive and quantitative methods using quantitative real-time PCR (qPCR) (Converse et al., 2009; Dick and Field, 2004; Kildare et al., 2007; Layton et al., 2006; Shanks et al., 2009). Compared with culturing methods, qPCR offers advantages for estimating bacterial and viral concentrations, both because of its speed (same day results) and because it can detect difficult-to-cultivate organisms. Application of these methods could therefore reduce uncertainty in fecal source identification and associated risk assessment.

The primary purpose of this study was to conduct an examination of the impacts of potentially aging and failing septic systems in the lower Clackamas River. Our efforts resulted in scientifically quantified data that can aid in the determination of the efficacy of septic mitigation programs currently being established by a collaborative front consisting of the Clackamas River Water Providers, Water Environment Services (WES), the Clackamas County Soil and Water Conservation District (SWCD), and Clackamas Community College. To do so, molecular techniques employing quantitative polymerase chain reaction (qPCR) was used to identify relative quantities of human derived and animal derived fecal *Bacteroidetes* and human derived Enterococci at seven locations in the lower Clackamas River watershed identified as high risk for failing septic systems. Specific objectives of the study were:

1. Determine the influence of aging/failing septic systems on the microbial pollution load in the lower Clackamas River by examining the relative contribution of human fecal contamination in surface waters within high density clusters of aging and failing septics.

2. Determine the relative contribution of fecal matter derived from domestic animals (pets, livestock) to the microbial pollution load in the Clackamas River.

3. Establish baseline (current) levels of relative contribution of identified species to the microbial pollution load in the Clackamas River for which to base future evaluation of mitigation efforts.

4. Determine the efficacy of septic mitigation efforts in the Clackamas River watershed over time.

5. Provide a valuable data set for which to determine the efficacy of future mitigation efforts focused on the reduction of animal derived fecal contamination.

Methods

Field Sampling

Monitoring Plan

This study established a monitoring program that focused primarily on qPCR analysis to conduct molecular microbial source tracking to examine baseline contributions of septic systems and animals (domestic and/or livestock) to the microbial pollution load in the lower Clackamas River. Sample locations were identified using the Septic System layer in the CRWP GIS Risk Analysis Database developed by Herrera Environmental Consultants in 2012.

Initially 10 sampling sites were identified within clusters of high risk septic systems on the main-stem of the Clackamas River and associated tributaries. Due to logistical constraints, 3 sample locations were removed from the list. The remaining 7 sample locations consisted of 4 main-stem and 3 tributary sites. The tributary sites, Deep Creek, Clear Creek, and Rock Creek (Sites 4, 6, and 7, respectively) (Figure 1), like the main-stem, are all included on the 303(d) list for bacteria violations. Rock Creek is listed for bacteria violations in Winter, Fall, and Spring,

Deep Creek is listed for violations only in summer because insufficient data was available to evaluate bacterial concentrations in wetter months.

Shoreline samples were collected at each sample location once a month from April 2014 through March 2015. Water was filtered through a 0.2-µm pore-size Sterivex filter (PES, ESTAR, Millipore) using a peristaltic pump until the filter clogged. Samples were kept on dry ice while in the field and stored at −80°C in the lab at Clackamas Community College until

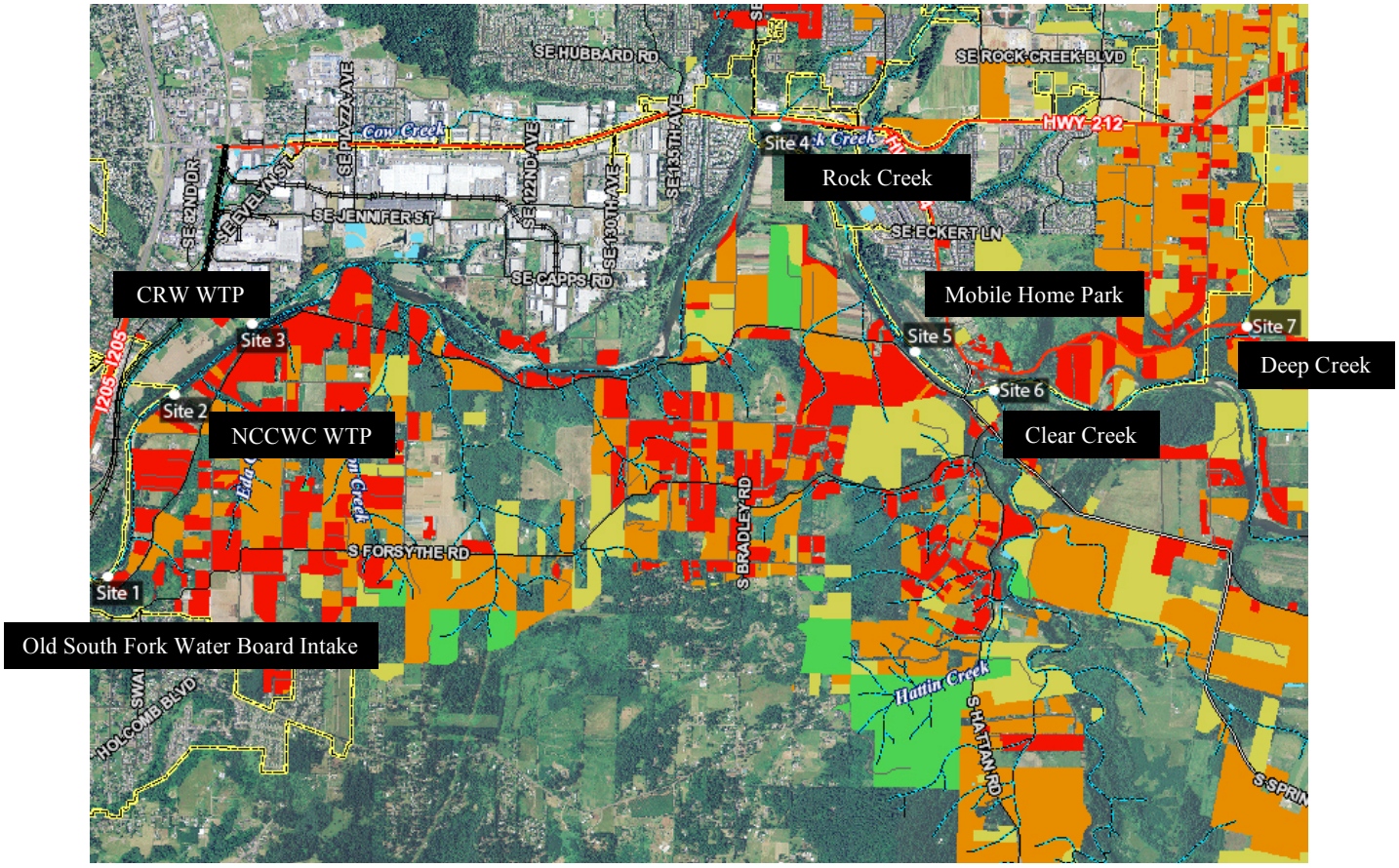

Figure 1. GIS map of septic systems in the Lower Clackamas River Watershed. Red indicates a high risk of failure, orange moderate to high, yellow moderate to low, and green low risk of failure.

processing.

Samples for Total Coliform (MPN) and *E. coli* (MPN) were collected in 150ml Colilert® sample bottles (IDEXX) and stored on ice. Samples were processed using the Quantitray®

method immediately upon arrival at the Clackamas River Water Drinking Water Quality Laboratory in Clackamas, Oregon.

Environmental Parameters

For each sample Temperature (^{o}C), pH, Dissolved Oxygen (mg/L), and Conductivity (µS/m) were collected using an ORION STAR Portable Multimeter. Total P (ppm) and Total N (ppm) were measured using LaMotte Low Range Field Test Kits.

Daily Mean Discharge (cu/ft/s) and Daily Mean Turbidity (fnu) were obtained from the USGS Water Quality Sensor near Oregon City, Oregon (14211010).

Precipitation recorded in the Prior 24 Hours (in) and daily Precipitation (in) were obtained from the Pleasant Valley School Rain Gage (Station #145) at 17625 SE. Foster Rd., Portland, Oregon. This rain gage is part of the City of Portland HYDRA Rainfall Network maintained by the USGS Oregon Water Science Center.

Laboratory Procedures

DNA Extractions

DNA extractions from the Sterivex filters were performed using a MoBio PowerFecal® DNA Isolation Kit per the manufacturers recommended protocol. DNA concentrations in the extracts were measured with a Thermo Scientific NanoDrop Lite Spectrophotometer.

qPCR

The qPCR primers used in this study were selected based upon previous studies and listed in Table 1. Modifications, in each case, were made to the internal primer sequence by replacing the TAMRA quencher with BHQ-1 to allow for better precision on the StepOne Plus Real-Time

PCR System. The PCR amplicon sizes range from 80-bp to 250-bp. The qPCR analysis was carried out by using the StepOne Plus Real-Time PCR System (Thermo Fisher). The qPCR reactions were done in triplicate. Briefly, the TaqMan® Fast Advanced Master Mix (Thermo Fisher) was used and qPCR reactions were carried out as follows: 50°C × 2min, 95°C × 20sec, 55°C for one cycle; 95°C × 1sec, 60°C × 20sec, followed by plate read, for 40 cycles. Serial dilutions of DNA samples were performed to determine amplification efficiency for each primer pairs. No template controls (NTCs) were used as negative controls.

Table 1. Taqman based qPCR primers used in this study

Target	Forward Primer	Forward Primer Sequence	Reverse Primer	Reverse Primer Sequence	Internal Primer Sequence	Reference
Total bacterial (16S)	EubF	TCCTACGGGAGGCAGCAGT	EubR	GGACTACCAGGGTATCTAATCCTGTT	FAM-CGTATTACCGCGGCTGCTGGCAC-BHQ-1	Nadkarni, 2002
All-Bacteroidales (16S)	AllBac296F	GAGAGGAAGGTCCCCCAC	AllBac467R	CGCTACTTGGCTGGTTCAG	FAM-CCATTGACCAATATTCCTCACTGCTGCT-BHQ-1	Mieszkin et al., 2009
Human Bacteroidetes	HF183F	ATCATGAGTTCACATGTCCG	BFDRev	CGTAGGAGTTTGGACCGTGT	FAM-CTGAGAGGAAGGTCCCCCACATTGGA-BHQ-1	Haugland et al., 2010
Enterococci	HEnteroF	GAGAAATTCCAAACGAACTTG	HEnteroR	CAGTGCTCTACCTCCATCATT	FAM-TGGTTCTCTCCGAAATAGCTTTAGGGCTA-BHQ-1	USEPA, 2012
Cow Bacteroidetes	CowM3F	CCTCTAATGGAAAATGGATGGTATCT	CowM3R	CCATACTTCGCCTGCTAATACCTT	FAM-TTATGCATTGAGCATCGAGGCC-BHQ-1	Shanks et al., 2007
Ruminant Bacteroidetes-Rum-2-Bac	BacB2-590F	ACAGCCCGCGATTGATACTGGTAA	Bac708Rm	CAATCGGAGTTCTTCGTGAT	FAM-ATGAGGTGGATGGAATTCGTGGTGT-BHQ-1	Mieszkin et al., 2009

Standard curves were generated for each probe and/or primer set with serial dilutions of a standard containing a known number of the target sequences. Standards for *Bacteroidetes*, Total Bacteria, *Enterococci*, and HF183 16S rDNA were generated from PCR amplicons cloned from environmental samples as follows. Target sequences were amplified with the same primer sets used for quantification, then purified by agarose gel electrophoresis. The PCR products were extracted with a QIAquick gel extraction kit (Qiagen, Valencia, CA), cloned using a TOPO TA cloning kit with TOP10 chemically competent cells (Invitrogen, Carlsbad, CA), then sequenced to verify specificity. A FastPlasmid Mini Kit (5Prime) was used to extract the plasmids. Linearized plasmids were produced from cloned amplicons by digestion with *EcoR1* restriction enzyme (Promega, Madison, WI), then run on an agarose gel. DNA concentrations in the extracts were measured with a Thermo Scientific NanoDrop Lite Spectrophotometer. Gene

abundance was calculated based on DNA concentration and plasmid plus insert sequence size. Dilution series ranging from 10^1 to 10^7 or 10^8 copies μl^{-1} were used for the standard curves.

Statistical Analysis

Spearman's rank correlation coefficient, a nonparametric measure of statistical dependence between two variables, was calculated for data and environmental variables using GraphPad Prism version 6.04 for Macintosh, GraphPad Software, La Jolla California USA, www.graphpad.com.

Results and Discussion

Microbial source tracking (MST) emerged at the end of the 20th century (Wiggins, 1996; Parveen et al., 1997; Hagedorn et al., 1999; Bernhard & Field, 2000a; Harwood et al., 2000) as an attempt to determine the dominant sources of fecal contamination in environmental waters (Scott et al., 2005; Field & Samadpour, 2007; Stoeckel & Harwood, 2007; Harwood et al., 2009). The impetus for emergence of this research area derives from (1) the effort to determine the extent to which fecal source (e.g. human, dog, cattle) influences human health risk from contact with water and (2) the desire to attribute FIB loading in water bodies to the correct fecal sources. The basic premise of MST is that certain fecal microorganisms are strongly associated with particular hosts and that identified attributes of these host-associated microorganisms can be used as markers for fecal contamination from the host.

The current project should be viewed as a Pilot Program regarding MST in the Clackamas River Watershed. An underlying goal was to determine if molecular MST methods could be applied to examine septic failure in the Clackamas River Watershed. It was also an

opportunity to strengthen collaborative and educational relationships with the Water Environment Technology program at Clackamas Community College by providing students with valuable, advanced field and laboratory training.

Objective 1: Determine the influence of aging/failing septic systems on the microbial pollution load in the lower Clackamas River by examining the relative contribution of human fecal contamination in surface waters within high density clusters of aging and failing septics.

To look at the influence of aging/failing septic systems on the microbial pollution load in the lower Clackamas River human fecal pollution was measured using human-associated qPCR methods. The first targeted a traditional fecal indicator bacteria (FIB), the 23S rDNA gene cluster of *Enterococci sp.* based on EPA protocol 1611 (USEPA, 2012). Enterococci are commonly found in the feces of humans. However, they can also be found in other warm-blooded animals and can be persistent in the environment. While not considered human-specific, these organisms are human-associated and the presence of enterococci in water is an indication of fecal pollution and the possible presence of enteric pathogens. The EPA has developed criteria for determining recreational water standards based on established relationships between the measured density of enterococci colony forming units (CFU) in the water by culture based methods and the risk of gastrointestinal illness (GI) associated with swimming in the water (USEPA, 2012). These criteria were used in the Clackamas River Watershed in the 303(d) listing of streams and river reaches sampled in this study. This target was chosen specifically to

look at the association between human-specific indicator bacteria and FIB detected through molecular methods.

Bacteroides, more specifically *Bacteroides dorei*, was chosen as the second target. This marker was initially identified in human fecal samples collected from the United States Pacific Northwest (Bernhard and Field, 2004). qPCR methods targeting the *Bacteroides* HF183 marker (Bernhard et al., 2003; Seurinck et al., 2005) allows for detection of sewage at much greater dilutions (five orders of magnitude) than *Enterococcus sp*.

Enterococcus sp. were identified in nearly all samples collected during this study. 23S rDNA copies/L varied slightly between locations and seasons, but no significant relationship of statistical significance was observed between the occurrence of *Enterococcus sp.* and sample location (main-stem shoreline or creek). However, *Enterococci* 23S rDNA copies/L were strongly at correlated to increases in water temperature (r^2- 0.4137, *P*- 0.0002). Not surprisingly, these data were also loosely correlated with Total Coliform MPN and *E. coli* MPN (r^2- 0.2364, *P*- 0.0398 and r^2- 0.2327, *P*- 0.0431 respectively).

Initially, we believed that these data would be complimentary and hypothesized that the occurrence and behavior of *Enterococcus sp.* bacteria would track the *Bacteroides* HF183 (human-associated) marker. This was not the case. In fact, *Bacteroides* HF183 (the human-specific marker), exhibited a different profile of occurrence with a slight negative correlation to Enterococcus 23S rDNA (r^2- -0.3018, *P*- <0.0281). As the HF183 marker increased in concentration it appears the occurrence of Enterococcus 23S rDNA decreased slightly (Figure 2).

The HF183 16S rDNA marker peaked during winter sampling events, although it was noticeably absent in January 2015 samples while Enterococci sp. 23S rDNA, the general fecal indicator bacteria, appeared significantly reduced compared to December 2014 (Figure 2). The December 2014 sampling date occurred when the Clackamas River was at flood stage following a storm event. River flows were 22,300 cu/ft/s, the highest observed during the study period (Table 2). This sample also exhibited the highest daily mean turbidity at 47 fnu (Table 2). In all creek samples HF183 16S rDNA peaks ranged from 2.39×10^9 to 4.68×10^{10} copies of DNA/L (Figure 2B and 4B).

The only sample location on the main-stem to show signs of human influence was sample location 1 (Figure 1) at the old South Fork Water Board Intake. This location is located near a small culvert that carries Eda Creek across Clackamas River Drive. This sample also represented the highest concentration of HF183 16S rDNA for all main-stem sites by nearly one order of magnitude with 1.57×10^{10} copies of DNA/L (Figure 2A and 4A).

HF183 was found to be negatively correlated with water temperature (r^2- -0.6441, *P*- <0.0001) and positively correlated with precipitation (in) (r^2- 0.6290, *P*- <0.0001) and turbidity (fnu) (r^2- 0.3892, *P*- 0.0036). These parameters suggest that there may be a significant correlation between the abundance of human-associated *Bacteroides* genetic markers and wet conditions. HF183 16S rDNA peaks were observed during wet winter months versus peaking of Enterococci sp. in the spring and summer months (Figure 2A and 2B). During this same period the concentration of general *Bacteroidetes* 16S rDNA

Table 2. Environmental parameters collected during DNA sampling events 4/10/14 through 3/23/15.

Date	Site	Location	Temperature (°C)	pH	DO (mg/L)	Conductivity (µS/m)	Total P (ppm)	Total N (ppm)	ml filtered -1	ml filtered-2	Total Coliform (MPN)	*E. coli* (MPN)	Daily Mean Discharge (cu/ft/s)	Daily Mean Turbidity (fnu)	Precipitation Prior 24 Hours (in)	Precipitation (in)
4/10/14	1	Old SFWB Intake	8.9	7.4	12.3	62.0	<0.1	<0.1	400	500	435.2	23.3	5270	1.6	0.12	0
4/10/14	2	NCCWC	8.6	7.6	12.7	43.5	0.8	<0.1	5000	4500	238.2	3.1	5270	1.6	0.12	0
4/10/14	3	CRW Intakes	11.9	7.1	12.5	42.2	<0.1	<0.1	3750	3500	128.1	8.4	5270	1.6	0.12	0
4/10/14	4	Rock Creek	12.8	7.7	11.1	133.1	0.2	<0.1	250	550	1203.3	65.7	5270	1.6	0.12	0
4/10/14	5	Clear Creek	10.7	7.0	11.9	45.8	<0.1	<0.1	1250	750	461.1	5.2	5270	1.6	0.12	0
4/10/14	6	Mobile Home Park	9.9	8.1	13.2	41.7	<0.1	<0.1	2500	2500	140.1	7.5	5270	1.6	0.12	0
4/10/14	7	Deep Creek	11.8	7.0	11.8	60.3	0.2	<0.1	1500	1500	686.7	13.2	5270	1.6	0.12	0
5/7/14	1	Old SFWB Intake	9.6	7.1	11.9	97.3	0.2	<0.1	750	500	387	13	4200	1.5	0	0
5/7/14	2	NCCWC	9.5	6.6	12.1	43.7	<0.1	<0.1	2000	1750	727	4	4200	1.5	0	0
5/7/14	3	CRW Intakes	10.9	7.4	12.6	92.9	<0.1	<0.1	2000	2000	727	19	4200	1.5	0	0
5/7/14	4	Rock Creek	14.1	7.0	10.2	140.3	<0.1	<0.1	500	1000	2419	77	4200	1.5	0	0
5/7/14	5	Clear Creek	11.0	7.1	11.6	49.2	<0.1	<0.1	1250	1000	2419	64	4200	1.5	0	0
5/7/14	6	Mobile Home Park	9.6	7.3	13.0	42.5	<0.1	<0.1	2000	1750	290	23	4200	1.5	0	0
5/7/14	7	Deep Creek	11.6	6.4	11.9	68.6	<0.1	<0.1	1300	1000	2419	119	4200	1.5	0	0
6/17/14	1	Old SFWB Intake	12.5	7.4	10.7	59.4	<0.1	<0.1	1800	1250	2419	30	2240	1.4	0.49	0.02
6/17/14	2	NCCWC	12.4	7.3	10.7	60.6	<0.1	<0.1	1000	1250	2419	19	2240	1.4	0.49	0.02
6/17/14	3	CRW Intakes	13.1	7.7	11.4	57.9	<0.1	<0.1	1250	1250	2419	30	2240	1.4	0.49	0.02
6/17/14	4	Rock Creek	14.7	7.6	10.2	150.1	0.2	<0.1	1250	1250	2419	21	2240	1.4	0.49	0.02
6/17/14	5	Clear Creek	12.7	7.6	11.1	60.0	<0.1	<0.1	1600	1800	2419	20	2240	1.4	0.49	0.02
6/17/14	6	Mobile Home Park	12.7	7.8	11.4	57.1	<0.1	<0.1	1750	1600	1300	33	2240	1.4	0.49	0.02
6/17/14	7	Deep Creek	12.4	7.7	11.1	92.3	0.4	<0.1	1000	750	1011	36	2240	1.4	0.49	0.02
7/10/14	1	Old SFWB Intake	17.5	7.5	10.0	95.3	0.2	<0.1	1600	750	2419	35	1170	0.7	0.01	0
7/10/14	2	NCCWC	17.6	7.2	10.0	77.4	<0.1	<0.1	2400	2500	2419	31	1170	0.7	0.01	0
7/10/14	3	CRW Intakes	21.1	7.8	10.2	60.0	<0.1	<0.1	2100	2100	1733	10	1170	0.7	0.01	0
7/10/14	4	Rock Creek	19.3	7.6	9.2	157.4	0.2	<0.1	2100	1750	2419	98	1170	0.7	0.01	0
7/10/14	5	Clear Creek	21.6	7.8	9.6	67.0	<0.1	<0.1	2600	2350	2419	67	1170	0.7	0.01	0
7/10/14	6	Mobile Home Park	21.2	7.9	10.2	77.4	<0.1	<0.1	2700	2900	2419	308	1170	0.7	0.01	0
7/10/14	7	Deep Creek	21.6	8.1	9.8	146.9	0.2	<0.1	950	2350	2419	34	1170	0.7	0.01	0
8/11/14	1	Old SFWB Intake	20.0	7.0	9.1	71.5	0.2	<0.1	3000.0	1500.0	2419	71.00	899	0.7	0	0
8/11/14	2	NCCWC	25.0	7.6	9.4	61.9	0.2	<0.1	3400.0	3250.0	2419	56.00	899	0.7	0	0
8/11/14	3	CRW Intakes	21.5	7.9	10.7	91.7	<0.1	<0.1	2750.0	3000.0	2419	22.00	899	0.7	0	0
8/11/14	4	Rock Creek	21.2	7.0	8.5	143.9	0.4	<0.1	1250.0	1100.0	2419	148.00	899	0.7	0	0
8/11/14	5	Clear Creek	22.5	8.1	10.0	122.8	0.2	<0.1	1600.0	3500.0	2419	150.00	899	0.7	0	0
8/11/14	6	Mobile Home Park	25.0	8.3	10.7	62.3	0.2	<0.1	1450.0	2250.0	2419	522.00	899	0.7	0	0
8/11/14	7	Deep Creek	20.7	8.7	11.3	164.6	0.2	<0.1	3100.0	3500.0	2419	108.00	899	0.7	0	0
9/9/14	1	Old SFWB Intake	16.6	7.4	10.0	155.0	<0.1	<0.1	300.0	500.0	2419	36.4	915	1.2	0	0
9/9/14	2	NCCWC	16.3	7.3	10.3	71.9	0.2	<0.1	2500.0	2500.0	2419	25.9	915	1.2	0	0
9/9/14	3	CRW Intakes	17.5	7.6	11.2	58.3	0.2	<0.1	2250.0	2000.0	2419	12.2	915	1.2	0	0
9/9/14	4	Rock Creek	16.8	7.7	9.3	165.5	0.2	<0.1	2250.0	2000.0	2419	50.4	915	1.2	0	0
9/9/14	5	Clear Creek	17.9	7.8	10.6	78.1	0.2	<0.1	4000.0	1300.0	2419	83.3	915	1.2	0	0
9/9/14	6	Mobile Home Park	18.1	8.3	11.9	67.4	0.2	<0.1	2000.0	1100.0	2419	214.3	915	1.2	0	0
9/9/14	7	Deep Creek	16.0	8.5	10.6	118.7	0.2	<0.1	2750.0	1100.0	2419	74.9	915	1.2	0	0
10/24/14	1	Old SFWB Intake	11.2	7.5	11.1	67.5	<0.1	<0.1	1250.0	1100.0	2419	127.4	2570	2.2	0.94	0.15
10/24/14	2	NCCWC	11.3	7.3	11.3	69.2	<0.1	<0.1	1000.0	1000.0	2419	142.1	2570	2.2	0.94	0.15
10/24/14	3	CRW Intakes	11.3	7.3	11.2	59.3	<0.1	<0.1	2250.0	2000.0	2419	78.5	2570	2.2	0.94	0.15
10/24/14	4	Rock Creek	12.1	7.6	9.9	220.0	<0.1	<0.1	1000.0	1000.0	2419	396.8	2570	2.2	0.94	0.15
10/24/14	5	Clear Creek	12.0	7.4	11.1	69.6	<0.1	<0.1	500.0	750.0	2419	1413.6	2570	2.2	0.94	0.15
10/24/14	6	Mobile Home Park	11.3	7.3	11.1	69.1	<0.1	<0.1	1250.0	1100.0	2419	131.3	2570	2.2	0.94	0.15
10/24/14	7	Deep Creek	12.2	7.6	10.9	99.5	<0.1	<0.1	500.0	750.0	2419	344.8	2570	2.2	0.94	0.15
11/14/14	1	Old SFWB Intake	9.5	7.47	12.02	36.2	0.2	<0.1	1750	2000	770	17	1750	0.9	0.05	0.24
11/14/14	2	NCCWC	9.6	7.33	11.99	55.1	<0.1	<0.1	2500	2500	727	17	1750	0.9	0.05	0.24
11/14/14	3	CRW	11.1	8.12	12.35	59.6	<0.1	<0.1	2250	2250	770	7	1750	0.9	0.05	0.24
11/14/14	4	Rock Creek	11.7	7.6	10.68	218.1	0.2	<0.1	2000	2000	2419	86	1750	0.9	0.05	0.24
11/14/14	5	Clear Creek	9.5	7.39	11.97	65.3	0.2	<0.1	2000	2000	2419	33	1750	0.9	0.05	0.24
11/14/14	6	Mobile Home Park	10.2	8.49	12.85	52.1	<0.1	<0.1	2750	1750	613	6	1750	0.9	0.05	0.24
11/14/14	7	Deep Creek	10.5	7.53	11.66	81	0.2	<0.1	750	1800	2419	14	1750	0.9	0.05	0.24
12/22/14	1	Old SFWB Intake	8	7.06	12.6	33.1	0.2	0.1	100	100	2419	1986	22300	47	0.01	0.04
12/22/14	2	NCCWC														
12/22/14	3	CRW	8.7	5.94	12.02	34.4	0.6	<0.1	100	100	2419	1413	22300	47	0.01	0.04
12/22/14	4	Rock Creek	10.9	7.55	11.29	165.9	0.4	<0.1	350	200	2419	224	22300	47	0.01	0.04
12/22/14	5	Clear Creek	9.9	6.62	11.59	47.2	<0.1	<0.1	100	100	1732	59	22300	47	0.01	0.04
12/22/14	6	Mobile Home Park	8.2	7.19	10.07	31.1	<0.1	<0.1	100	100	2419	16	22300	47	0.01	0.04
12/22/14	7	Deep Creek	9.9	7.13	11.93	65.8	<0.1	<0.1	200	300	2419	77	22300	47	0.01	0.04
1/29/15	1	Old SFWB Intake	7.3	7.1	12.67	55.3	<0.1	<0.1	2350	2550	308	7	2870	1	0.09	0
1/29/15	2	NCCWC	7	7.4	12.52	50.8	<0.1	<0.1	2950	2950	488	9	2870	1	0.09	0
1/29/15	3	CRW Intakes	6.9	8	13.14	55.5	<0.1	<0.1	3500	2500	1	0	2870	1	0.09	0
1/29/15	4	Rock Creek	9.5	7.9	11.52	162.1	0.2	<0.1	900	800	2419	54	2870	1	0.09	0
1/29/15	5	Clear Creek	8.5	7.4	12.2	51.6	<0.1	<0.1	1400	1350	1120	11	2870	1	0.09	0
1/29/15	6	Mobile Home Park	7.7	7.7	12.76	48.7	0.2	<0.1	1500	2150	248	3	2870	1	0.09	0
1/29/15	7	Deep Creek	8.5	7.3	12.14	71.1	0.5	<0.1	1650	1700	1203	19	2870	1	0.09	0
2/25/15	1	Old SFWB Intake	6.7	7.29	12.02	66.1	<0.1	<0.1	1250	1500	178.5	5.2	1830	0.3	0	0.01
2/25/15	2	NCCWC	6.8	7.31	12.01	59.6	<0.1	<0.1	4000	4000	435.2	7.2	1830	0.3	0	0.01
2/25/15	3	CRW Intakes	7.5	7.61	12.95	54.7	<0.1	<0.1	4000	4000	77.1	0	1830	0.3	0	0.01
2/25/15	4	Rock Creek	9	7.35	11.06	162.6	<0.1	<0.1	750	750	2419	66.3	1830	0.3	0	0.01
2/25/15	5	Clear Creek	6.5	7.29	12.29	57.7	<0.1	<0.1	2500	1250	1413.6	25.9	1830	0.3	0	0.01
2/25/15	6	Mobile Home Park	6.9	7.51	12.13	53.9	<0.1	<0.1	2750	4000	201.4	6.3	1830	0.3	0	0.01
2/25/15	7	Deep Creek	7.3	7.56	12.45	74.5	<0.1	<0.1	2500	2500	410.6	17.1	1830	0.3	0	0.01
3/23/15	1	Old SFWB Intake	8.5	7.45	11.18	54.41	<0.1	<0.1	600	750	2419	63.1	2810	3.2	0.12	0.74
3/23/15	2	NCCWC	8.6	7.31	11.3	56.62	<0.1	<0.1	500	500	2419	172.3	2810	3.2	0.12	0.74
3/23/15	3	CRW Intakes	8.9	7.35	10.99	53.89	<0.1	<0.1	1500	1400	1553.07	56.3	2810	3.2	0.12	0.74
3/23/15	4	Rock Creek	11.1	7.54	10.77	141	0.2	<0.1	450	250	2419	980.4	2810	3.2	0.12	0.74
3/23/15	5	Clear Creek	9.2	7.49	11.35	56	<0.1	<0.1	700	700	2419	517.2	2810	3.2	0.12	0.74
3/23/15	6	Mobile Home Park	9.3	7.79	11.88	51.94	<0.1	<0.1	1500	1250	2419	166.4	2810	3.2	0.12	0.74
3/23/15	7	Deep Creek	9.6	7.5	11.17	65.27	<0.1	<0.1	500	500	2419	135.4	2810	3.2	0.12	0.74

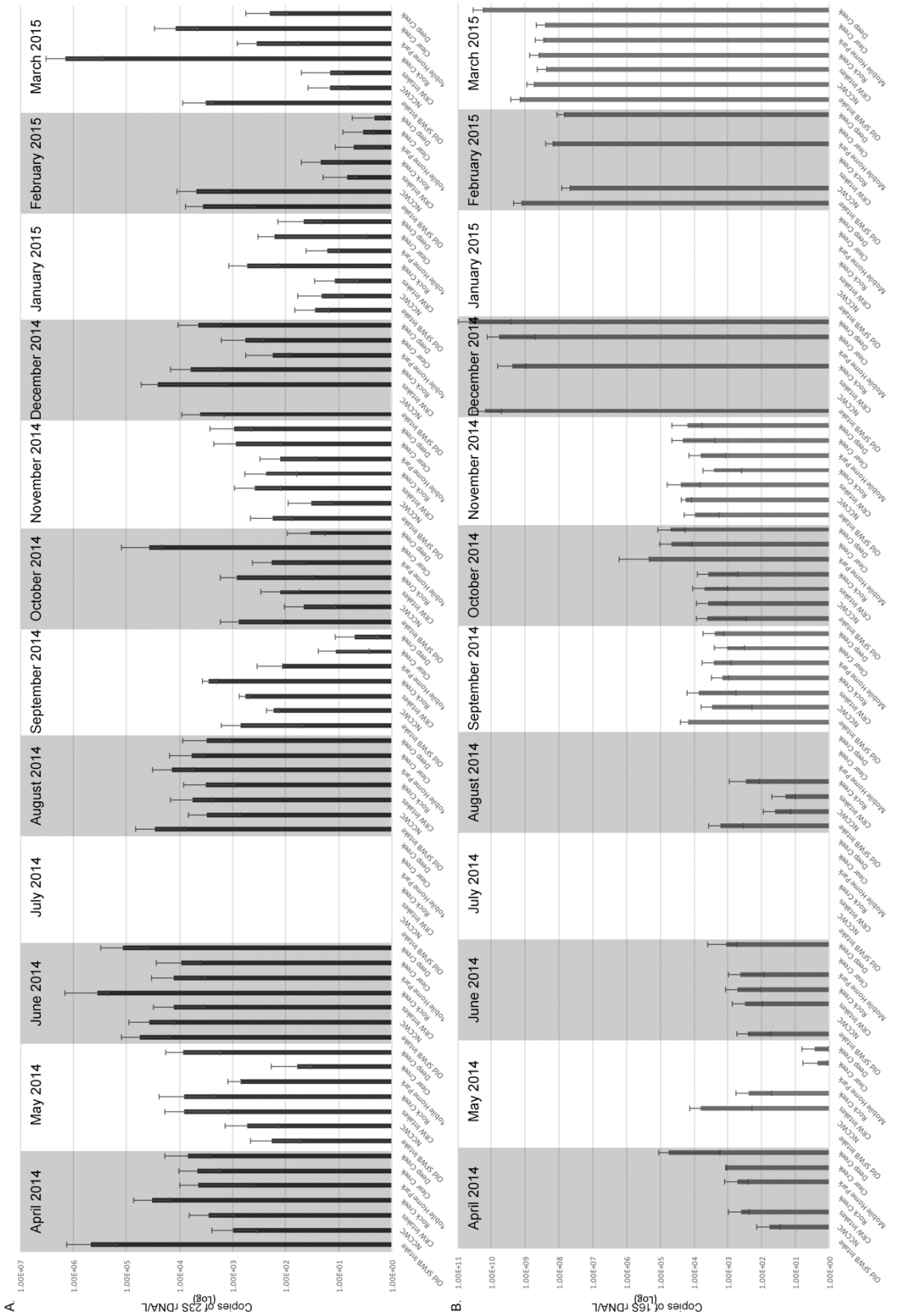

Figure 2. A. qPCR results for all samples collected and analyzed for Enterococci sp. 23S rDNA. B. qPCR results for all samples collected and analyzed for the HF183 marker 16S rDNA.

would rise in samples while Total Bacteria 16S rDNA (total bacterial DNA) never varied more than 2 orders of magnitude across sampling events (Figure 3). This observation suggests that while the total microbial load in the river may be *relatively* stable shifts in individual microbial populations could be significant and indicative of changing environmental influences, including but not limited to septic and wastewater influence, as well as rain water run-off. As microbial source tracking continues to evolve in the Clackamas River watershed and our baseline data grows it is likely that well be able to refine this wet/dry season analysis.

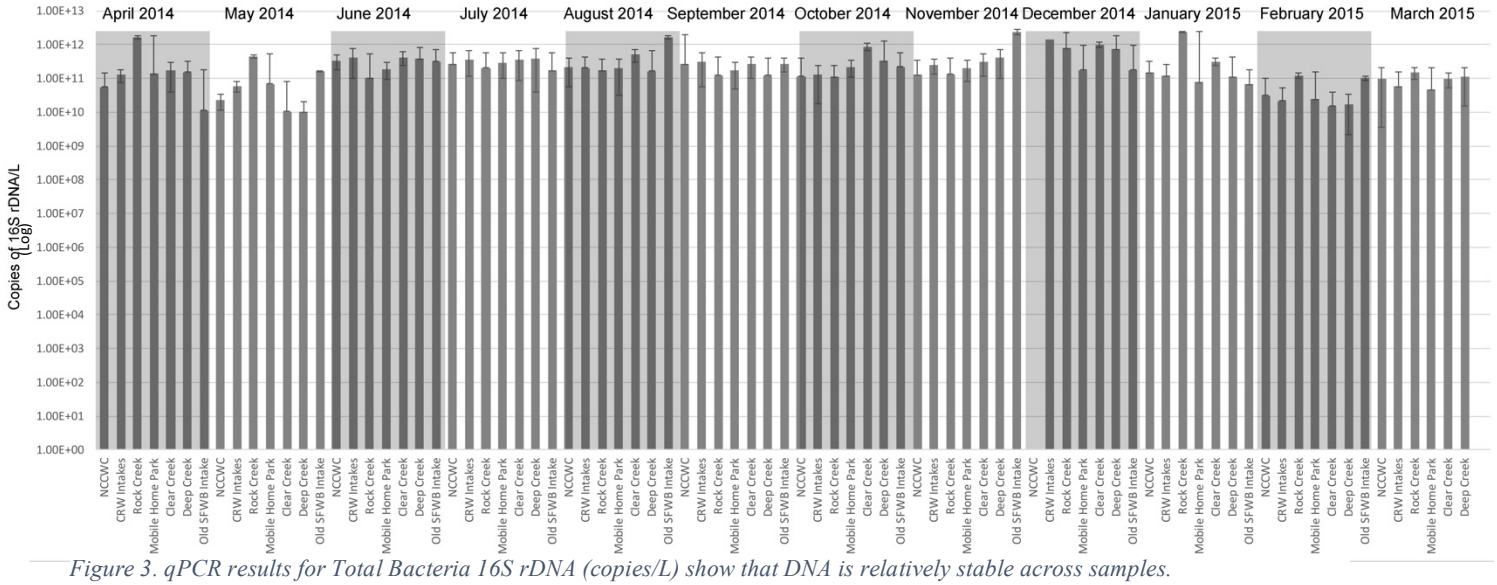

Figure 3. qPCR results for Total Bacteria 16S rDNA (copies/L) show that DNA is relatively stable across samples.

Fecal DNA indicators varied by location and sampling event. It is difficult to say that one sample site is more influenced by human fecal contamination than another as all sites exhibited peaking of fecal indicators at least once during the sampling period. Among creek sites, however Deep Creek showed more consistently elevated levels of the HF183 16S rDNA marker (6 out of 12 samples) than Clear Creek or Rock Creek (Figure

4B). Clear Creek appeared to be the least influenced by the human fecal marker, although peaking was still evident in December 2014 and March 2015.

Deep Creek enters the Clackamas River at RM 11.6, approximately midway between River Mill Dam and the uppermost (Clackamas River Water) of the series of 4 drinking water treatment plants in the lower 3.1 miles of river. As a source of human fecal pollution Deep Creek may be of particular concern. The lower portion of Deep Creek is an area flanked by septic systems identified as potentially high to moderate risk of failure (Figure 1), but the upper reaches carry treated effluent from the Boring WWTP (via North Fork Deep Creek) and seasonally from the Sandy WWTP (via Tickle Creek). The increase in human fecal contamination observed in during high flow months may be markedly influenced by the Boring WWTP, but more so by the seasonal release from the Sandy WWTP between the months of November and April. At present, it is difficult to determine the influence of the Sandy and Boring WWTPs versus potentially failing septic systems regarding human fecal contamination. Future studies may target Deep Creek alone with sample collections along the upper and lower reaches of the Creek and its tributaries, probing for human fecal DNA markers for bacteria as well as viruses.

It's important to note that our study design does not rule out possible influence from upstream sewage treatment plants on any level. If the HF183 marker was detected during summer, low-flow conditions, likely sources would include failing septic systems and/or cross connections between sanitary and storm sewer systems, but one could ever

disregard the influence of the WWTP on Deep Creek.

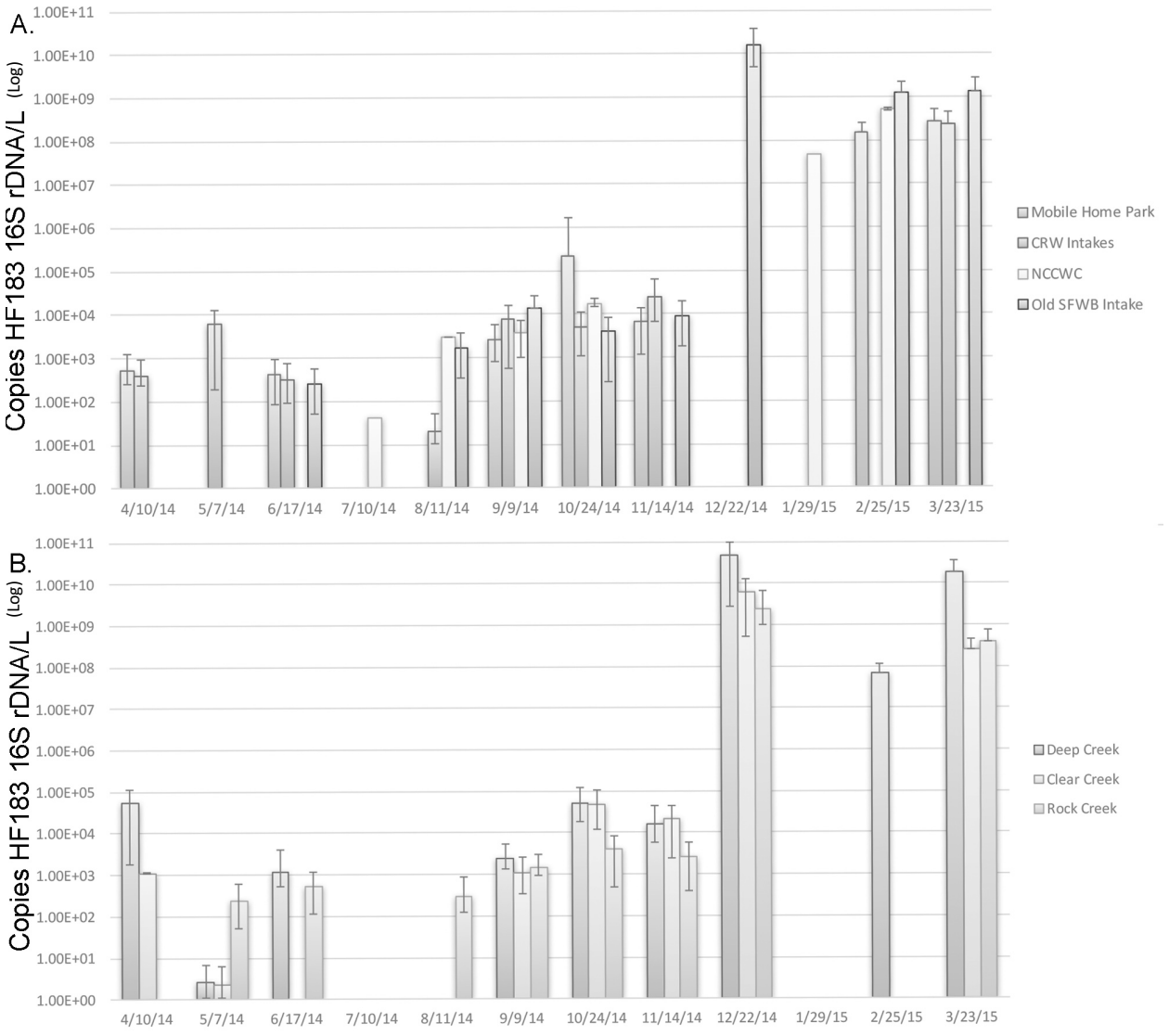

Figure 4. HF183 qPCR data (copies 16S rDNA/L) broken down by A. Main-stem sampling locations and B. Creek sampling locations.

With the present data it is difficult to determine if peaking of concentrations of HF183 DNA marker during higher flows and rainfall events are the result of increase influence of WWTP waste streams and/or sewer overflow during storm events. Consequently, when septic drain fields become waterlogged during rain events,

particularly from heavy rain or flooding, the septic system can malfunction causing contaminants from the partially treated wastewater can enter ground and surface waters.

In may be possible to evaluate the influence of WWTP discharge/storm overflow vs. septics by combining microbial source tracking with the detection of chemicals used in the wastewater treatment process such as sodium bisulfite, sodium metabisulfite, or vitamin C. Discussions for the development of a viable sampling plan began in late January, as the differentiation of these two sources of human fecal contamination are considered a priority for MST work in the Clackamas River Watershed moving forward.

So just how much do the human fecal markers contribute to the microbial pollution of the creeks and main-stem? To address this issue we used qPCR to assess the the relative quantity of 16S rDNA genes for *Bacteroidetes* and Total Bacteria. The ratio of the HF183 16S rDNA marker to total *Bacteroidetes* copies of DNA/L ranged between 0% and 0.2% while the ratio of the Enterococci sp. 23S rDNA to Total Bacteria never rose above 0%. Overall, our data suggests human fecal bacteria is widespread in the lower Clackamas River Watershed, but contributes only a fraction of DNA obtained from the microbial population.

2. *Determine the relative contribution of fecal matter derived from domestic animals (pets, livestock) to the microbial pollution load in the Clackamas River.*

Unfortunately, we were not able to determine the relative contribution of fecal matter derived from domestic animals during this study period. We focused heavily on the evaluation of ruminant DNA using qPCR primer sets BacB2-590F and 708Rm (REF#) and CowM3F and CowM3R (REF#). Use of these primer sets were fraught with

difficulty. Cow standards were amplifiable with endpoint PCR, but failed consistently to amplify during qPCR. This may be due to issue with the internal primer sequence of the TaqMan probe, but further research is needed to determine the cause of failure.

The ruminant primer set did amplify standards during qPCR, but amplification curves appeared late (Ct 25 or later). When amplification occurs later than expected it is an indication of poor amplification efficiency. Late amplification can be the result of poor primer design or poor template quality. Non-specific amplification was ruled out as no template curves showed no amplification. qPCR results built off of late amplification curves are likely to be inaccurate. However, we feel confident that qPCR results obtained from ruminant sampling runs are sufficient for consideration on a presence/absence basis as Ct values for samples considered positive were always below Ct 25. Positive samples would indicate that the sample location should be evaluated further.

Ruminant fecal contamination was not present consistently in our presence/absence analysis. In fact, only two locations showed indications of possible ruminant fecal contamination, Deep Creek and Clear Creek. Both creeks drain rural sub-watersheds. Ruminant fecal contamination was identified in Deep Creek samples collected in April, June, and October, and in October and March samples collected from Clear Creek (data not shown). It is our intent to continue the development of the ruminant primer set. Once standards and the protocol are established Deep Creek and Clear Creek should be considered top priorities for more in-depth qPCR analysis of ruminant fecal contamination.

3. *Establish baseline (current) levels of relative contribution of identified species to the microbial pollution load in the Clackamas River for which to base future evaluation of mitigation efforts.*

   One of the biggest challenges of evaluating the environmental impacts of mitigation efforts in any project is often attributed to a lack of baseline data. A goal of this project was to establish baseline levels of DNA for various species in the Clackamas River. While our efforts to obtain data for ruminant species and cows were unsuccessful during this study period, data for human *Bacteroides* marker HF183 and Enterococci sp. were obtained for a twelve-month period. Baseline data is collected over multiple years to refine natural variations and anomalies. These data serve to contribute to the overall establishment of a baseline to evaluate future mitigation efforts. It also serves as a foundation for evaluating target areas of study moving forward.

4. *Determine the efficacy of septic mitigation efforts in the Clackamas River watershed over time.*

   This goal will be addressed as more data is collected and future microbial source tracking studies in the Clackamas River Watershed evolve.

5. *Provide a valuable data set for which to determine the efficacy of future mitigation efforts focused on the reduction of animal derived fecal contamination.*

   Due to our failure to obtain viable qPCR data regarding Ruminant and Cow fecal contamination this goal will have to be addressed in future studies. Once these source tracking primers are functioning we will begin microbial source tracking at target areas identified through presence/absence results obtained in this study. In particular, we will focus on Clear Creek and Deep Creek.

Method Improvements

Total P (ppm) and Total N (ppm) were measured using LaMotte Low Range Field Test Kits, although the detection limit at 0.2ppm we still had difficulty detecting P and N in field samples. CRW recently acquired an Astoria-Pacific ChemWell T Nutrient Analyzer that has the ability to detect nutrients below 0.2ppm and will be used for nutrient analysis in future MST studies.

Clackamas Community College Student Engagement: Microbial Source Tracking (MST) and Development of Real-Time Quantitative PCR Methodology

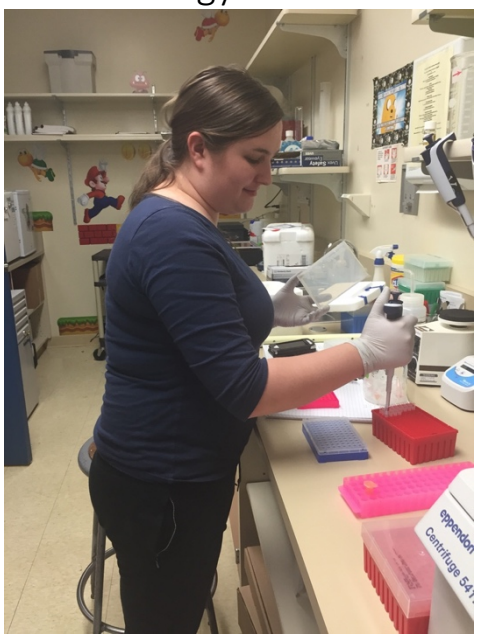

Figure 5. Intern Oksana Adamtsev extracts DNA in the MST Lab at CCC.

The Water & Environmental Technology (WET) program at Clackamas Community College (CCC) provides students the skills and knowledge to work in the water and wastewater industry. CCC offers a one-year certificate for students that have a prior degree and want to obtain the specific knowledge that will allow them to pass state level water and wastewater certifications, and a two-year associate of applied science (AAs) degree in Water & Environmental Technology (WET) for those who are looking to obtain a higher education degree. The CCC WET program is focused on providing students an experience that will give them the chance to obtain life long careers. One of the unique aspects of the program is the level of involvement that the WET program maintains with the water and wastewater industry in Oregon, Washington, and in Idaho. Through these engagements with industry, students are required to do two internships, one on the potable water industry and one with the wastewater industry.

The collaboration with Clackamas River Water (CRW) and Clackamas River Water Providers (CRWP) on this Microbial Source Tracking project has greatly impacted the WET program in several different ways:

Student Engagement through Research Based Internships

This project has given 5 WET students an opportunity to work with cutting edge technology on a project that is paramount to the water industry. These interns were able to work directly with an expert in the field of molecular microbiology. Typically, most internships last one term (11 weeks) and students are required to work a minimum of 120 hours. Three of the five interns for this project worked several terms and put in many more on the project. This dedication reflects how successful this internship collaboration was with CRWP. The interns were paid $1000 per term provided by the CRWP, and they were required to give a presentation at the end of each term. Several of the interns were also given the opportunity to present their results at either community events or plant meetings providing an opportunity for students to become engaged with people (possible future bosses) in the water industry.

One of the CCC WET interns actually left the program early because she obtained a job offer to manage a wastewater laboratory. The work that she conducted on the MST project with CRW and the CRWP and the abilities, knowledge and skills she obtained through the internship was a strong contributor to her job offer. In fact, this plant wants her to develop and obtain the same instrumentation and techniques that she was working on in this project. Another intern is working in the high purity water field and another is employed as a wastewater operator.

Student Engagement through WET Curriculum Development

The Elementary Microbiology and Aquatic Microbiology taught as part of the CCC WET program had been static for over 30 years; the material did not reflect the evolution of

microbiology and the techniques currently being used in the microbiological fields. This project offered CCC WET the opportunity to create an MST lab that contained novel modern laboratory equipment for identifying DNA. This lab was created out of a back storage closet in the CCC WET main classroom and was available to students/interns/industry whenever needed. The lab is used to showcase some of the work being done in the water industry and in lectures to get students excited about working with industry on various projects. According to Professor Jim Nurmi, "When students see things that they have only seen on TV shows like CSI and hear that they can do and learn how to do "Environmental CSI", this gets students excited about learning and engaged in the process. It has been a wonderful teaching tool."

Both the theory of microbial source tracking and new PCR related labs have been introduced into the Elementary Microbiology, Aquatic Microbiology, and Environmental Chemistry classes. Based on this project, the use of multi-plex PCR laboratories for several classes have been successfully implemented. Students like to see and use modern technologies and have them applied to what they are interested in. Students now participate in a lab once a year called, "Who pooped in my River?" which is basically what MST is all about. Students take samples from several important surface waters in the area and try to determine where the bacteria are coming from. The lab part has been boiled down into a week-long adventure. Recently some very disturbing results were obtained that could impact us in several different ways. This will be discussed below. Regardless, the WET curriculum has benefited from this project in that students are able to see and use modern technologies that could get them a career in the water, wastewater, or environmental fields.

Student and Industry Engagement through "DNA" Classes for Short Schools

The WET program through CCC offers two short schools during the year that provide industry and students the ability to learn about various topics in both the wastewater and water worlds and to obtain continuing education units (CEUs) needed to stay current in the water industry. These schools run for three days and are filled with various speakers from various industries. Through this collaboration, Suzanne DeLorenzo gives lectures about the MST project and now runs a DNA extraction lab during both the water and wastewater short school. The sessions have been offered for three years in the water short school and then last year in the wastewater school. Both of these sessions were extremely well attended (maximum attendance) and were considered one of the best sessions held during the schools. People love to get their hands on things and learn. Most of these industry operators have never seen DNA before and they were able to hold and touch their own DNA.

Student and Community Engagement through MST in our own Backyard: The Environmental Learning Center

CCC has a relatively large area on campus that is called the Environmental Learning Center (ELC) that was used for several programs on campus during the 1980's. These programs have dissolved over time and the ELC has been neglected. Within the ELC is at the headwaters of a local Creek (Newell) that runs into the Willamette River. This headwater is basically a stormwater catch basin for the area and is thus heavily contaminated with substances commonly found in storm water runoff (heavy metals, grease, gasoline derivatives, etc.). Through this collaboration and work with several of the students, it was found that the water in the ELC is also heavily contaminated with fecal coliform and *E. coli* at numbers larger than would be expected for environmental samples. The CCC WET students are currently applying the techniques and knowledge gained through the MST pilot project to try to figure out "Who

pooped in our ELC?". Students are now assisting with sewer inspections near the ELC during spring break this year. This is another example of how this project and collaboration has provided ample opportunities to get students engaged in their learning.

Future Student Engagement

This extremely fruitful project has created a great collaboration between the WET program and CRW and the CRWP. Several interns have obtained internships at CRW and CRWP that would not have happened without this collaboration. CCC WET is currently working on these projects and are planning on finding more funding to extend and go deeper into the MST project. CCC and CRW are also planning on several other projects that will include providing internship opportunities to many future students. This summer CCC with be implementing work with a new instrument the WET program has obtained to look for cyanotoxins and various pharmaceuticals in our local waterways. The goal is to correlate this data with data obtained by our CRW and CRWP partners and interns. By pushing the microbial source tracking project forward, learning new information about where contamination is coming from in our local watersheds, and using this to excite and engage current students, future water industry operators, and professionals CCC, CRW, and the CRWP have created successful, long-term collaboration.

Conclusion

During this study patterns of fecal indicator bacteria and human source markers were observed to vary, suggesting that the fecal contamination observed in the Clackamas River Watershed may be the result of a complex combination of sources of contamination. However,

human fecal contamination was widespread among samples as indicated by the HF183 marker and a clear contributor to the microbial pollution load. As we continue to develop MST in the Clackamas River Watershed an emphasis should be placed on developing and applying a suite of human fecal markers with long-term spatial and temporal sampling to refine temporal variation that may occur.

A major question arising from this work is the differentiation between sources of human fecal pollution resulting from WWTP effluent or failing septic systems. Currently a work plan to address WWTP effluent vs. septic systems in streams and the main-stem is underdevelopment, once again collaborating with CCC. Work is anticipated to begin on this project in or around January 2017.

As a pilot study Microbial Source Tracking in the Clackamas River Watershed was successful. We determined that molecular microbial source tracking is a viable technique for monitoring microbial pollution and can be integrated into our watershed management plan.

The relationship between CRW, CRWP, and CCC has benefited exponentially from this experience. This study helped the CCC WET program strengthen and evolve its environmental microbiology curriculum to teach students more cutting edge, modern microbial practices. It is the intent that CCC WET program interns continue to be involved in microbial source tracking providing experiential education opportunities unlike any program ever offered before at CCC.

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