TECHNICAL | MEMORANDUM

TO: Ian Dombroski, USEPA Region 1[1]

FROM: Laura Diemer, FB Environmental Associates (FBE)

SUBJECT: Task 3: PhyloChip® Prioritization & Results

DATE: December 31, 2019

CC: Jennifer Relstab & Richard Claytor, Horsley Witten Group (HWG); Forrest Bell, FBE

This memorandum summarizes two phases of the PhyloChip® analyses: 1) prioritization rationale for selecting a subset of samples for PhyloChip® DNA microarray analysis, and 2) assessment of PhyloChip® analysis results in a broader context of application in fecal source tracking. The Palmer River Water Quality Analysis Report (HWG & FBE, 2019a) includes the analysis of agricultural BMP efficacy, as well as PhyloChip® results in the context of specific fecal sources in the Palmer River watershed.

HWG and FBE were tasked with assisting the USEPA Region 1 with selecting a subset of 50[2] (out of 96) samples collected from twelve sites in 2017-18 by the Massachusetts Department of Environmental Protection (MassDEP), the Rhode Island Department of Environmental Management (RIDEM), and USEPA Region 1. The information used to make our recommendation was largely completed in Task 4 (Water Quality Analysis Report) and Task 6 (Land Use & Regulatory Analysis Report), the deliverables of which include documentation on sources and methodology (HWG & FBE, 2019a, 2019b). The rationale for sample prioritization was based on the decision matrix outlined in the project Quality Assurance Project Plan (QAPP) (HWG & FBE, 2019c) and presented in Figure 2. We assessed available information and provided recommendations for prioritizing (1) sites that met decision matrix metrics for water quality, agricultural BMPs, land use change, and pollutant sources and then (2) sample dates that met decision matrix metrics for seasonal and antecedent weather conditions.

Selected samples were sent to the Lawrence Berkeley National Laboratory for analysis. Following receipt of the quality controlled and validated PhyloChip® results, FBE assessed best practices when applying PhyloChip® in sampling plans for other watersheds impacted by pathogen contamination.

WATER QUALITY ANALYSIS

Since 2016, MassDEP, RIDEM, and USEPA Region 1 have collected monthly water quality samples at twelve fixed or "core" sites within the lower Palmer River watershed to help determine the effectiveness of remediation efforts with agricultural best management practice (BMP) installations (see section on Agricultural BMP Modeling). Refer to Figure 3 for a map of sample locations and sub-basin boundaries. The "core" sites included six saline and six freshwater stations, with three stations on Clear Run sampled for *E. coli*, three stations on the main stem sampled for enterococci, and the remaining six stations sampled for both parameters. All sites were also sampled for total suspended solids (TSS) and nutrients. Beginning in 2017, samples were collected for ribonucleic acid (RNA) microarray analysis using PhyloChip®; a subset of 50 samples were selected and analyzed based on the recommendation of this memorandum.

Sites and parameters with ten or more years of annual data were assessed for long-term trends using the Mann-Kendall trend test ($\alpha < 0.05$). Only six sites (CR01, CR02, CR03, PM31, PM30, and RR23) had 9-10 years of water quality data (and only for *E. coli*). A Mann-Kendall trend test[3] using the *rkt* package in R statistical programming was performed on the summarized data and no statistically significant trends were found ($\alpha < 0.05$).

Daily data for all twelve sites were summarized (median, average, minimum, and maximum) by site for application to state water quality criteria or natural background conditions. All sites exceeded state criteria for fecal indicator bacteria (*E. coli* and enterococci) for either geomean or single-sample or both (Attachment 1). Most sites also had elevated nutrient levels compared to natural background levels for the coastal ecoregion (USEPA, 2000).

[1] The views, thoughts and opinions expressed in this document may not necessarily represent EPA policies and positions. Mention of trade names or commercial products, services and enterprises in this presentation do not constitute EPA endorsement or recommendation for use.

[2] The USEPA Region 1 had funding for PhyloChip® analysis of 50 samples collected in 2017-18.

[3] Mann-Kendall trend test is a useful non-parametric, statistical test for monotonic trends in time series of environmental data.

Preliminary analysis showed no statistically significant difference in fecal indicator bacteria (*E. coli* and enterococci) or nutrients pre and post agricultural BMPs installation; however, a more in-depth analysis was included in the Palmer River Water Quality Analysis Report (HWG & FBE, 2019a). Assessing the annual variability of data for the years pre-2015, 2015, 2016, 2017, and 2018 showed average annual decreases in both fecal indicator bacteria (*E. coli* and enterococci) and nutrients in 2016 (corresponding with the implementation of most agricultural BMPs in the watershed) when compared to all data prior to 2016 (pre-2015); however, water quality parameters at some sites then showed continued decreases while others showed increases in 2017 and 2018 (Attachment 2). It was important to include sites with a variety of water quality responses in sub-basins with and without agricultural BMPs to establish study controls for weather or other external factors not associated with implementation efforts.

Based on water quality status, we recommended the following sites for PhyloChip® analysis (ordered from upstream to downstream): CR01 (control), CR03 (possible *E. coli* and nitrate-nitrite improvement, captures CR02), PM30 (possible *E. coli*, enterococci, and nitrate-nitrite improvement, significant degradation in water quality between PM31 and PM30), RR22 (possible *E. coli*, enterococci, and orthophosphate improvement, captures RR23), TC07 (possible *E. coli*, enterococci, and orthophosphate improvement, possible nitrate-nitrite degradation), TC08 (control), and PM43 (represents overall water quality, elevated orthophosphate from PM29 to PM43 may be from large gaggles of congregating geese).

AGRICULTURAL BMP MODELING

The Palmer River watershed was included in the National Water Quality Initiative (NWQI) to abate fecal contamination through the installation of agricultural BMPs. Through the NWQI, several successful agricultural BMPs have been installed in the Palmer River watershed since 2015 and more are ongoing or soon-to-be installed in the coming years.

To evaluate the efficacy of agricultural BMPs, FBE first identified the number and type of BMPs installed in each sub-basin and then modeled the pollutant reduction potential of those agricultural BMPs using the Spreadsheet Tool for Estimating Pollutant Load (STEPL); refer to HWG & FBE (2019a) for details on methodology. STEPL models the total load and total load reduction from implemented BMPs for total nitrogen, total phosphorus, and total sediment; load estimates for *E. coli* will be included in the next model version update. In the meantime, the range of estimated load reductions for nutrients and sediment can serve as a proxy for *E. coli*, especially sediment since *E. coli* can bind and be transported with sediment and may act more conservatively (i.e., not be as readily taken up or transformed) in the environment compared to nutrients that are more readily taken up or transformed through biochemical pathways.

From 2015-2018, 28 agricultural BMPs were successfully installed in the Palmer River watershed, 11 on cropland and 17 on pasture. Refer to Attachment 3 for a description of the agricultural BMP types based on general STEPL BMP types. These BMPs resulted in a total reduction of 528 lbs./yr in nitrogen, 149 lbs./yr in phosphorus, and 25 tons/yr in sediment (Table 1)[4]. The largest percent reduction of total load was for sediment in the TC07 sub-basin (13%); otherwise, most percent load reductions ranged from <1% to 3%. Refer to HWG & FBE (2019a) for a complete analysis of agricultural BMP efficacy in the Palmer River watershed.

Most of the agricultural BMP implementation work in the Palmer River watershed was completed in 2015-2016 with some additional work in 2017-2018 (Table 1). Implementation work completed in 2018 compared to prior implementation work in the sub-basins to PM31, PM44, RR22, and TC07 generated only modest additional reductions in estimated pollutant loads (Table 1). The pollutant reductions estimated for the sub-basin to CR02 more than doubled with the addition of 2017 BMPs; CR02 and/or CR03 were recommended for PhyloChip® analysis given the number and diversity of existing and planned BMPs in the sub-basins and the potential for achieving success (measured as improved water quality) because their small drainage areas allow for better management control. Additional agricultural BMPs are planned to be implemented in the direct sub-basins draining to the following sites: CR02, CR03, PM31, PM44, TC07, and PM29.

Based on the number and diversity of agricultural BMP types and the magnitude of pollutant reductions achieved prior to 2018, we recommended the following sites for PhyloChip® analysis: CR03 (includes BMPs to CR02), PM30 (includes BMPs to PM31), PM44, RR22 (includes BMPs to RR23), TC07, and PM43 (includes BMPs to PM29), plus CR01 and TC08 for control.

[4] Note that many of the sub-basins are nested, and any agricultural BMPs installed in the direct sub-basin draining to a given site has cumulative downstream effects on water quality. While general BMP types installed in the direct sub-basin draining to each site have been identified, Table 1 shows the total loads and cumulative load reductions for the total drainage area to each site. For example, general BMP types are described for the direct sub-basins to CR01 and CR02, but the total loads and load reductions for CR02 combine the loads from the sub-basins draining to both CR01 and CR02.

Table 1. Agricultural BMP types by sub-basin (non-cumulative) and total pollutant loads without BMPs and pollutant load reductions with BMPs by sub-basin (cumulative). N=nitrogen. P=phosphorus. Sed=sediment. Red=reduction. Dates in brackets [ ] indicate the year in which the BMP was installed.

Sub-basin	Agricultural BMP Types [implementation years]	N Load (lbs./yr)	P Load (lbs./yr)	Sed Load (tons/yr)	N Red (lbs./yr)	P Red (lbs./yr)	Sed Red (tons/yr)	N Red (%)	P Red (%)	Sed Red (%)
CR01	No BMPs	3,116	984	25	0	0	0	0%	0%	0%
CR02	Litter Storage and Management + Livestock Exclusion Fencing + Heavy Use Area Protection [2016, 2017]	7,028	1,866	68	46	4	0	1%	0%	1%
CR03	Litter Storage and Management [2016]	8,402	2,208	92	50	5	0	1%	0%	0%
PM31	Diverted Drainage + Grass Swale + Critical Area Planting + Litter Storage and Management [2016, 2018]	48,790	13,556	593	97	16	3	0%	0%	0%
PM30	Litter Storage and Management + Use Exclusion + Heavy Use Area Protection + Grass Swale [2017]	51,317	14,249	619	102	17	3	0%	0%	0%
PM44	Terrace + Conservation Tillage 2 + Prescribed Grazing [2015, 2016, 2018]	54,052	14,820	675	334	98	13	1%	1%	2%
RR23	Litter Storage and Management [2016]	11,662	3,374	134	1	0	0	0%	0%	0%
RR22	Livestock Exclusion Fencing + Grass Buffer + Prescribed Grazing + Use Exclusion [2016, 2018]	18,972	5,207	240	44	7	1	0%	0%	1%
TC07	Conservation Tillage 2 + Prescribed Grazing x2 + Critical Area Planting [2015, 2016, 2018]	4,776	1,252	78	114	42	10	2%	3%	13%
TC08	No BMPs	309	40	6	0	0	0	0%	0%	0%
PM29	Conservation Tillage 2 + Prescribed Grazing [2016]	79,009	21,462	1,012	528	149	25	1%	1%	3%
PM43	No BMPs	79,391	21,561	1,016	528	149	25	1%	1%	2%

LAND USE CHANGE ANALYSIS

Changing land use can impact water quality over time and is potentially a confounding factor that can mask any measurable water quality improvement resulting from watershed remediation efforts. To address this potential issue, FBE completed a land use change analysis using the 2003-2004 Rhode Island [Land_Use_and_Land_Cover_20032004] and 2005 Massachusetts [LANDUSE2005_POLY] land use layers as a baseline for comparing change in land use in the years 1995, 2001, 2011, 2015, and 2018.

Overall between 1995-2018, the PM43 sub-basin (which includes all sub-basins) experienced a decrease in forest (555 acres) and agriculture (139 acres) land uses and an increase in water/wetland (11 acres) and urban (683 acres) land uses (Table 2). More specifically, residential development largely replaced cropland and mixed forest (Figure 1). Increases in water/wetland areas were due to the installation of large stormwater retention ponds or the addition of farm ponds. All sub-basins experienced change in land use but most especially the total drainage areas to the following sites: CR02, PM31, RR23, RR22, TC07, and PM29. The sub-basins to CR01 and TC08 had the least amount of land use change in the study period. As a possible control for the confounding factor of land use change, we recommended CR01 and TC08 for PhyloChip® analysis.

Table 2. Percent area by land use type in 2018 and percent change in land use type from 1995-2018 for twelve sub-basins in the Palmer River watershed. Sub-basins include total drainage area to sites, with PM43 including all sub-basins. Grey- and red-highlighted values represent a decrease and increase in land use type from 1995 to 2018, respectively.

Year	Land Use	Data Type	CR01	CR02	CR03	PM31	PM30	PM44	RR23	RR22	TC07	TC08	PM29	PM43
2018	Forest	Percent Land Use Area	36%	32%	33%	67%	66%	65%	74%	71%	44%	25%	65%	65%
2018	Agriculture		2%	21%	25%	8%	9%	10%	5%	7%	18%	5%	10%	10%
2018	Water/Wetland		3%	4%	5%	4%	4%	4%	3%	2%	4%	5%	4%	4%
2018	Urban		59%	42%	37%	22%	22%	21%	19%	20%	33%	65%	22%	22%
1995-2018	Forest	Change in Land Use Area (Acres)	0.0	-11.3	-14.6	-337.1	-350.3	-350.3	-173.6	-196.3	-8.0	0.0	-554.5	-554.5
1995-2018	Agriculture		-1.2	-9.5	-11.6	-73.4	-73.4	-73.4	-37.3	-47.2	-16.1	-2.5	-139.2	-139.2
1995-2018	Water/Wetland		0.0	0.0	0.0	4.2	4.2	4.2	7.0	7.0	0.0	0.0	11.2	11.2
1995-2018	Urban		1.2	20.8	26.1	406.3	419.4	419.4	203.8	236.5	24.1	2.5	682.5	682.5

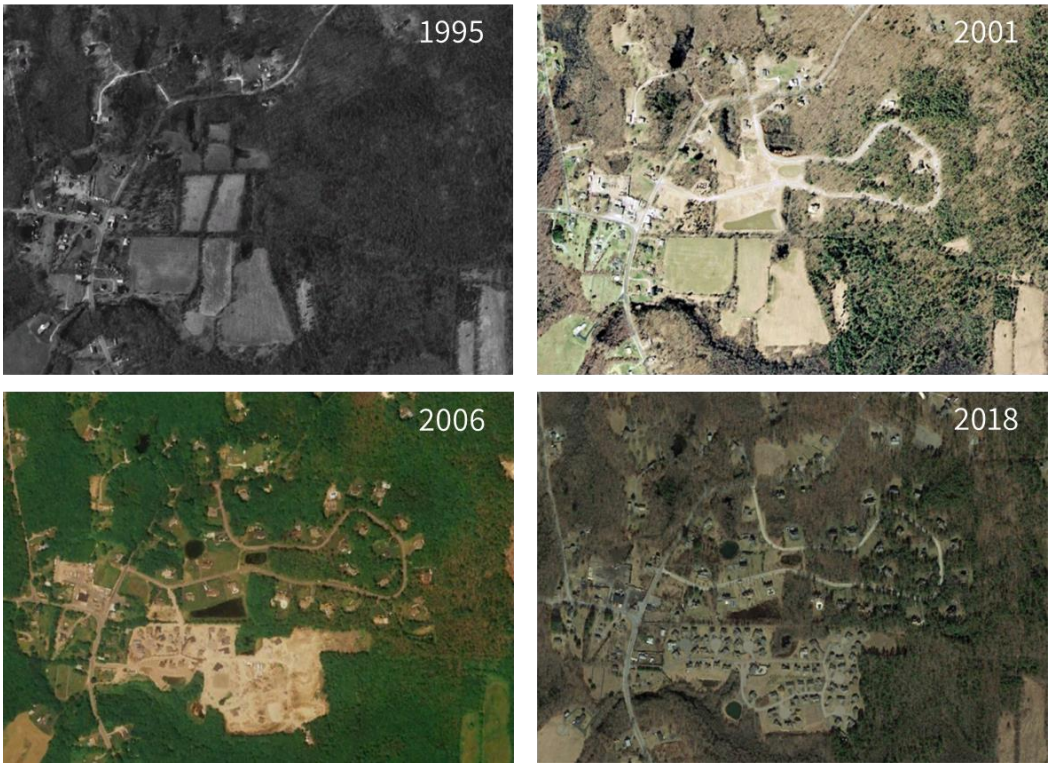


Figure 1. Example of land use change over time in the Palmer River watershed. From 1995-2001, forest and cropland were converted to residential development, which further developed and expanded to agricultural land to the south by 2006 and 2018.

POLLUTANT SOURCE ASSESSMENT

We identified potential nonpoint sources contributing to each site, including agriculture (percent agricultural land use, estimated total number of livestock, Microbial Source Tracking-DNA (MST-DNA) results from prior studies), stormwater (percent urban land use, percent MS4 urban census area), septic systems (number of people on systems, MST-DNA results), and wildlife (anecdotal information, percent forest and water/wetland land use, MST-DNA results) (Table 3). To include a variety of potential nonpoint sources, we recommended the following sites for PhyloChip® analysis: CR01 and TC08 for highly urban (stormwater), low-agriculture areas with septic systems; CR03, RR22, and TC07 for mixed-use urban and agriculture areas with septic systems and wildlife issues; and possibly PM29 or PM43 as all-encompassing areas with direct wildlife issues.

Table 3. Potential nonpoint sources of pollution by sub-basin (cumulative drainage areas). Livestock includes beef and dairy cattle, swine, sheep, horses, chickens, ducks, and goats based on local knowledge. If no information was provided in the MST-DNA Results, then the site was not included in the study. The ribotyping study data came from ESS Group Inc. (2003) and the Bacteroidetes data came from a prior study under the 2010-2015 Surface Water Monitoring & Assessment MassDEP Division of Watershed Management-Watershed Planning Program.

Sub-basin	% Agric	No. Livestock	% Urban	% MS4 Area	No. People on Septic	% Forest	MST-DNA Results	Other Notes
CR01	2%	0	59%	100%	766	39%		
CR02	21%	408	42%	86%	1,261	36%		
CR03	25%	468	37%	85%	1,396	39%	Cow, pig isolates from ribotyping study	
PM31	8%	551	22%	17%	8,360	70%		
PM30	9%	597	22%	16%	8,868	70%		
PM44	10%	617	21%	15%	8,952	69%		
RR23	5%	65	19%	11%	1,916	76%		
RR22	7%	105	20%	11%	2,842	73%	Cow, pig, horse, human, deer, rabbit, dog isolates from ribotyping study	Historic septic system failure at RR02 (upstream); remediated by 2015
TC07	18%	40	33%	68%	826	49%	Cow, pig isolates from ribotyping study	Waterfowl identified in 2004 MA TMDL
TC08	5%	0	65%	100%	8	31%	Weak human Bacteroidetes marker	
PM29	10%	782	22%	17%	12,673	69%		Major geese congregation
PM43	10%	782	22%	17%	12,740	69%		Major geese congregation

SAMPLE PRIORITIZATION RATIONALE

The strategy for determining the selection of priority samples for PhyloChip® analysis is presented as a decision matrix in Figure 2. The decision matrix is split between two major objectives: evaluation of agricultural BMP success (project-specific) and application of PhyloChip® in fecal source tracking (method-specific). The two objectives are further refined by priority goals.

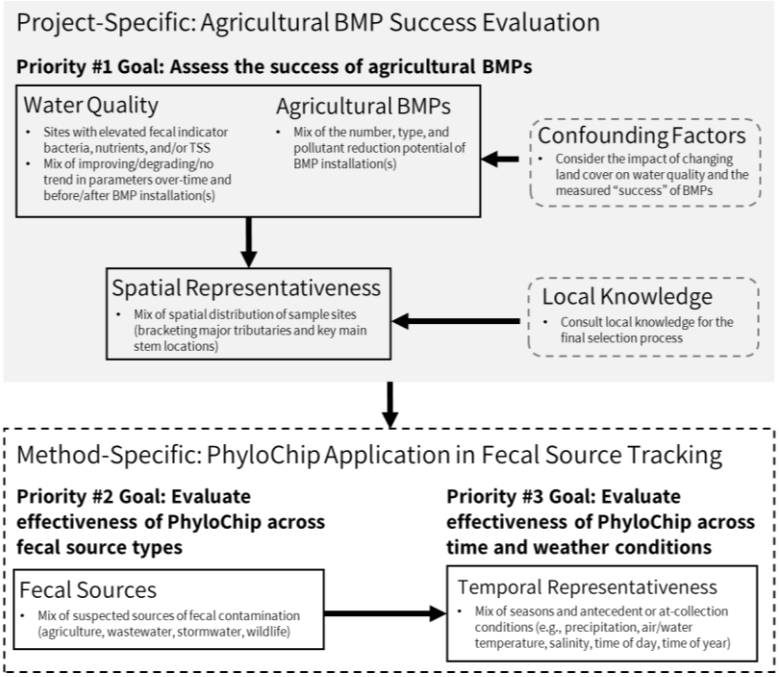


Figure 2. Decision matrix for prioritizing sample selection for PhyloChip® analysis.

The first priority goal for the project-specific objective was to assess the success of agricultural BMPs in the watershed. The first goal prioritizes samples based on a mix of water quality condition or status (e.g., trends or responses to implementation), agricultural BMPs (e.g., number, type, and modeled pollutant reduction), and spatial representativeness (e.g., tributary and main stem sites, Figure 3). Confounding factors such as the impact of changing land use on water quality were also considered, along with local knowledge. For the first goal, we recommended the following eight sites for PhyloChip® analysis: CR01, CR03, PM30, PM44, RR22, TC07, TC08, and PM43.

The second and third priority goals for the method-specific objective are to evaluate the effectiveness of PhyloChip® across fecal source types, as well as season and weather conditions, respectively. The eight sites recommended for PhyloChip® analysis in the first priority goal adequately cover a mix of suspected fecal sources (e.g., agriculture, stormwater, septic systems, and wildlife).

To further prioritize samples for seasonal and antecedent or at-collection conditions (e.g., precipitation, air/water temperature, salinity), we selected a subset of six samples collected in 2018 from the eight recommended sites[5]. Our selection was based on achieving a diversity of weather patterns across seasons that captured the most variability in water quality parameters. We performed a principal component analysis (PCA)[6] for complete cases for *E. coli* and enterococci, along with all other laboratory and field parameters (except orthophosphate due to missing values) and antecedent weather conditions presented in Table 4. Principal Component 1 (PC1) explained about 40% of the variability in both PCA runs, largely driven by air temperature and precipitation within the prior 1-2 days (Figure 4)[7]. Rainfall can have a dramatic influence on the flowpaths that deliver fecal waste to surface waters (e.g., surface runoff, shallow groundwater, deep groundwater). Human or agricultural sources of fecal pollution are activated during moderate precipitation events; thus, dry conditions would largely represent possible sources in baseflow from groundwater. Both can be important to understanding flowpaths of fecal sources.

Based on the above considerations to achieve a diversity of antecedent weather conditions, we recommended for PhyloChip® analysis the following sample dates: 4/24/2018 (spring (cool), dry sample), 6/7/2018 (early summer (cool-warm), dry sample), 7/9/2018 (summer (warm-hot), dry sample), 8/7/2018 (summer (hot), dry sample), 9/19/2018 (summer (hot), wet sample), and 11/5/2018 (late fall (cool), wet sample). We deferred to local knowledge, especially when related to any known activities in the

[5] Each of the twelve sites was sampled eight times in 2018 for a total of 96 samples. A maximum of six samples may be selected for each of the eight recommended sites for a total of 48 samples (up to 50 samples possible for PhyloChip® analysis).

[6] PCA is an exploratory data analysis tool that transforms and then groups variables into "principal components (PC)" with the first PC explaining the greatest variability in the data. This tool is useful for variables that may be highly correlated. In this case, our data set contained several correlated variables; we performed a PCA for complete cases of *E. coli* and enterococci to determine what variables helped explain the greatest variability in the data set. We then focused on achieving a diverse range of values for those variables to help prioritize sample dates for PhyloChip® analysis.

[7] Interestingly, individual linear regressions among all variables showed that *E. coli* and enterococci were not well predicted by antecedent weather conditions compared to nutrients.

watershed that might make one date preferable over the other, but no changes were suggested. Achieving a diversity in antecedent air temperature conditions can help determine possible impacts to DNA survivability with cold water temperatures.

Figure 3. Spatial location and/or extent of sample sites and sub-basins (drainage areas) to sample sites in the Palmer River watershed.

Table 4. Summary of antecedent weather conditions for 2018 sample dates in the Palmer River watershed. Wet weather was defined as precipitation greater than 0.25 inches in the prior day, or 0.5 inches in the prior two days, or 2 inches in the prior four days. Measurable rainfall was defined as greater than 0.25 inches in a day.

Date	Avg Air Temp (°C) on day	Max Air Temp (°C) on day	Min Air Temp (°C) on day	Avg Air Temp (°C) prior 7 days	Max Air Temp (°C) prior 7 days	Min Air Temp (°C) prior 7 days
4/24/2018	9.2	16.7	1.7	7.9	18.9	0.6
5/9/2018	15.0	21.7	8.3	19.0	32.8	8.3
6/7/2018	14.7	19.4	10.0	16.6	28.9	8.9
7/9/2018	23.4	31.7	15.0	24.5	33.9	12.8
8/7/2018	28.3	33.3	23.3	26.5	34.4	18.3
9/19/2018	17.5	20.6	14.4	21.3	27.8	15.6
10/18/2018	5.0	8.9	1.1	12.8	25.0	3.9
11/5/2018	7.3	10.6	3.9	10.8	20.0	-1.6

Date	Precip (in) prior 1 day	Precip (in) prior 2 days	Precip (in) prior 4 days	Precip (in) prior 7 days	Wet or Dry Weather	Days Since Measurable Rainfall
4/24/2018	0.00	0.00	0.00	0.46	Dry	4
5/9/2018	0.00	0.00	0.15	0.15	Dry	11
6/7/2018	0.00	0.16	0.69	0.84	Dry	2
7/9/2018	0.00	0.00	0.03	0.03	Dry	10
8/7/2018	0.00	0.00	1.62	1.63	Dry	2
9/19/2018	0.52	0.53	0.53	1.92	Wet	5
10/18/2018	0.00	0.09	0.13	2.25	Dry	5
11/5/2018	0.00	1.17	1.49	1.63	Wet	1

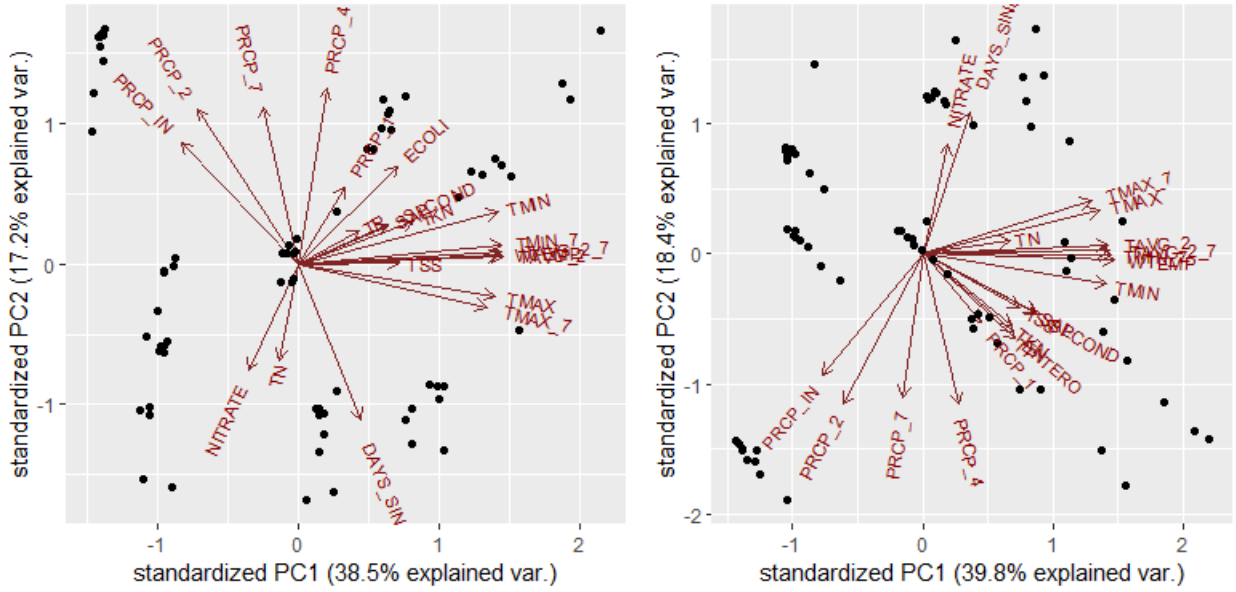


Figure 4. Principal component analysis (PCA) plots for complete cases for *E. coli* (left) and enterococci (right) with all other laboratory and field parameters (except orthophosphate due to missing values) and antecedent weather conditions (presented in Table 4). PC1 explained about 40% of the variability in the dataset, largely driven by temperature and precipitation within the prior 1-2 days or days since last measurable rainfall.

FINAL SAMPLE SELECTION & LABORATORY ANALYSIS

The recommended sites for PhyloChip® analysis were CR01, CR03, PM30, PM44, RR22, TC07, TC08, and PM43 for the dates of 4/24/2018, 6/7/2018, 7/9/2018, 8/7/2018, 9/19/2018, and 11/5/2018. These sites and sample dates were prioritized based on factors including water quality data, locations of agricultural BMPs, land-use changes, sources of pollutants, and seasonal antecedent weather, as listed in Table 5. We also recommended including two samples for TC07 (the sub-basin with the greatest potential for water quality improvement) on 6/20/2017 and 11/14/2017 to supplement the data set with more wet weather conditions under warm and cold temperature conditions.

USEPA Region 1, RIDEM, and MassDEP reviewed and approved the rationale for sample selection. A conference call was conducted on 4/24/2019 during which the project team discussed and provided feedback on the draft memorandum. FBE incorporated feedback and submitted an updated memorandum with a final recommendation for samples to be sent for PhyloChip® analysis.

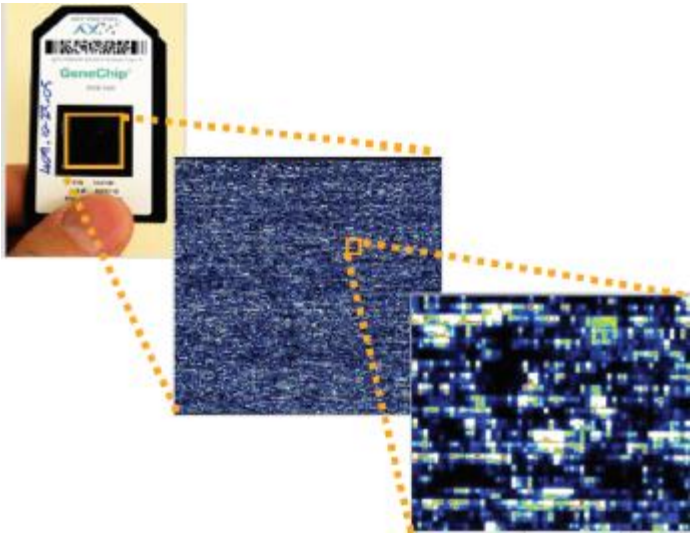
Selected samples were sent to the Lawrence Berkeley National Laboratory for analysis following protocols detailed in "July 25, 2016, Quality Assurance Project Plan (QAPP), 2016 US EPA Workforce Development Fund PhyloChip® Microbial Source Tracking (MST) Project, RFA 16126, US EPA Office of Environmental Measurement and Evaluation, North Chelmsford, MA & OECA" (US EPA, 2016).

Table 5. Summary of rationale for selecting samples for PhyloChip® analysis. Sites are ordered from upstream to downstream.

Site	Primary Purpose for Site Selection
CR01	No BMPs, no significant change in land use (control), highly urban, low agriculture with septics
CR02	*Not selected*
CR03	Mix of BMPs, possible *E. coli* and nitrate-nitrite improvement, change in land use, mixed urban and agriculture areas with septics and wildlife issues, captures CR02 drainage
PM31	*Not selected*
PM30	Mix of BMPs, possible *E. coli, e*nterococci, and nitrate-nitrite improvement, degradation in water quality between PM30 and PM31, change in land use, captures PM31 drainage
PM44	Mix of BMPs, otherwise similar to PM30
RR23	*Not selected*
RR22	Mix of BMPs, possible *E. coli* and enterococci improvement, change in land use, mixed urban and agriculture areas with septics and wildlife issues, captures RR23 drainage
TC07	Mix of BMPs, possible *E. coli* and enterococci improvement, nitrate-nitrite degradation, change in land use, mixed urban and agriculture areas with septics and wildlife issues
TC08	No BMPs, no significant change in land use (control), highly urban, low agriculture with septics
PM29	*Not selected*
PM43	All BMPs, represents overall water quality as a result of all changes in land use and all potential sources, including direct wildlife issues between PM29 and PM43

PHYLOCHIP® APPLICATION IN SOURCE TRACKING

We present here a subset of analyses that address the method-specific objective in determining application of PhyloChip® in fecal source tracking (refer to the decision matrix in Figure 2); additional PhyloChip® analysis results in the context of the Palmer River watershed were addressed in HWG & FBE (2019a). The following section is organized by a series of relevant questions for understanding PhyloChip® and its effective use.



Example of a PhyloChip® fluorescent image. Taken from Dubinsky et. al. (2012).

How does the PhyloChip® work and what do the laboratory analysis results mean?

Full method details are described in Hazen et. al. (2010). In summary, collected water samples are vacuum filtered and centrifuged for DNA extraction. The 16S rRNA gene is amplified using polymerase chain recreation (PCR) for 30 cycles. Microarrays are prepared, stained, and scanned as fluorescent images. Pixel intensities are background-corrected for a hybridization score, along with presence/absence determinations, for individual operational taxonomic units (OTUs)[8] to create a microbial community profile for use in subsequent statistical analyses. A subset of data from DNA probes that target fecal bacteria is run through the "SourceTracker program" to determine the probability that a source type (human, bird, dog, horse, pig, or cow) is present, based on comparison to reference samples from each source type.

The PhyloChip® analysis results were sent in Excel format by the Lawrence Berkeley National Laboratory. Relevant data tabs are described in more detail as follows:

> Fecal Source Results: "Result_" columns represent the probability that an individual source is present based on a subset of fecal-specific 16S rRNA gene sequences (or OTUs represented by distinct probe sets) known to be associated with an individual source. The stronger the signal, the more likely the source is present. The results do not represent true proportions of the total number of genes that belong to a source, so combining two or more source results would be inappropriate. Thresholds for signal strength may differ from other project results if calibration samples are reanalyzed with an updated set of diagnostic probes. Note that some correlation between signal strengths of the different source types may occur when low levels of signal are detected; this is because some DNA probes are shared among different mammalian sources. Most probes, however, are not shared among different sources and thus correlations due to shared probes are not expected to occur when signal strengths are high. For the Palmer River dataset, human and cow source types were positively correlated when signal strengths were high; conversely, human and bird source types were negatively correlated (refer to Attachment 4). We can reasonably justify these connections because humans inhabit the same areas that we also use to manage livestock, compared to wildlife areas where birds might be more prolific (though geese can be inadvertently attracted to large mowed areas adjacent to waterbodies).
>
> Diagnostic Probes: Shows presence/absence of diagnostic probe quartets used for the "SourceTracker program" analysis. The number of diagnostic probes is related to signal strength since more diagnostic probes that show positive will generally increase signal strength. Signal strength, however, is ultimately determined through a probabilistic approach that considers the frequency that each probe occurs in the different source types based on reference and background samples used to train the analysis.
>
> Community Binary: Shows the presence/absence (or taxonomic richness) of up to 59,959 OTUs that compose the microbial community for each sample. Determination of presence/absence is based on the scoring of individual probes (typically 15-30) that should collectively be present if the OTU is in the given sample. We can use these data to assess changes in

[8] OTUs are groups of closely related bacteria with similar DNA sequences. OTUs may not necessarily follow classic taxonomic classification. For instance, a species (lowest taxonomic classification) could have many OTUs.

community composition (or richness) in response to space, time, or other factors such as water quality or watershed characteristics.

Community Intensities: Shows the hybridization intensities (or fluorescence) of up to 59,959 OTUs that compose the microbial community for each sample. The ratio between hybridization intensities (fold change) for each detected OTU can be used to determine if an OTU is increasing or decreasing in relative abundance between samples. We can use these data to assess changes in community structure (or relative abundance) in response to space, time, or other factors such as water quality or watershed characteristics.

Where has PhyloChip® been successfully used?

In the last 10 years, PhyloChip® has been used in freshwater, estuarine, and marine environments for project objectives related to fecal source tracking in California and Hawaii, as well as documenting microbial community shifts following oil spills in the Gulf of Mexico. The approach has been pioneered by the Lawrence Berkeley National Laboratory in California. A summary of published studies is provided as follows:

- ***Hazen et al. (2010)*** – described the PhyloChip® methodology to generate a complete microbial community profile and is referenced by subsequent publications. The study methodology did not include running the microbial community profiles through Source Tracker to identify fecal source types because the study assessed oil-degrading bacteria in deep-sea oil plumes, similar to the study by Dubinsky et al. (2013).
- ***Dubinsky et al. (2012)*** – described the first application of PhyloChip® in fecal source tracking. The authors hypothesized that significant differences in bacterial taxa would be found among various source types, so that unique identifier taxa could be used to determine the presence or absence of fecal sources. A variety of human and animal wastes were collected at several locations throughout California and run through PhyloChip® to define the identifier taxa for major sources (human, bird, cow, horse, elk, and pinniped). Significant differences in taxa were found among human, bird, pinniped, and grazer sources. Specifically, Actinobacteria, Bacilli, and Gammaproteobacteria taxa distinguished bird from mammalian sources. Clostridia and Bacteroidetes taxa distinguished human, grazer, and pinniped sources. Application of the PhyloChip® in fecal source tracking at two sites showed that high fecal indicator bacteria from a large sewage spill in Richardson Bay in California were dominated by human source identifier taxa, while a nonpoint source impacted beach at Campbell Cove in California showed high fecal indicator bacteria were associated with a mix of identifier taxa dominated by gull feces.
- ***Cao et al. (2013)*** – evaluated the use of three microbial community analyses in detecting fecal sources from 64 blind, single- or dual source, challenge samples from 12 sources, including human, sewage, septage, dogs, pigs, deer, horses, cows, chickens, gulls, pigeons, and geese, collected within California. The three analyses included PhyloChip®, terminal restriction fragment polymorphism (TRFLP), and next generation (Illumina) sequencing. Overall, all three analyses did well with correctly identifying dominant sources in >90% of challenge samples (with minimal false negatives and excellent sensitivity and specificity).
- ***Boehm et al. (2013)*** – examined the sensitivity and specificity of 41 microbial source tracking methods by analyzing data generated by 27 different laboratories. The analyses targeted human, cow, ruminant, dog, gull, pig, horse, and sheep. Each laboratory was given 64 blind samples containing a single source or two sources, as well as diluted single samples to test sensitivity (same samples as Cao et al., 2013). PhyloChip® was identified as one of the most sensitive and specific assays based on an analysis of presence/absence in target and non-target fecal samples.
- ***Dubinsky & Andersen (2014)*** – described the application of PhyloChip® in fecal source tracking for the Russian River in California. The study aimed to determine the spatial and temporal variability of the microbial community in the river and at recreational beaches along the river and whether the microbial community varied with land use and/or onsite water treatment systems. The study found that wet periods strongly influenced microbial community composition and structure, regardless of land use. Human fecal signals with significant risk for pathogenic bacteria were detected at two beaches. Elevated fecal indicator bacteria were not associated with fecal bacterial taxa in the upper reaches of the river. Reference libraries were used from 80 different fecal sources collected by Dubinsky et al. (2012) and Cao et al. (2013).
- ***Dubinsky et al. (2016)*** – described the application of PhyloChip® in fecal source tracking for the Russian River in California, using it as example data to test two source discrimination tools: random forests and SourceTracker. Using the microbial community identified by PhyloChip®, along with the source reference library (Dubinsky et al., 2012; Cao et al., 2013), SourceTracker was found to produce better sensitivity and specificity for all source types compared to random forests. The study found that elevated fecal indicator bacteria counts in the upper reaches of the river during the wet period (with

corresponding no fecal signal) co-varied with bacteria associated with high nutrient and carbon loading, suggesting that elevated fecal indicator bacteria were due to growth within the environment and not from fecal sources.

- ***Dubinsky & Andersen (2019)*** – assessed fecal and environmental sources of fecal indicator bacteria in the Mahaulepu Valley on the island of Kauai, Hawaii and tracks human waste sources along coastal seeps impacted by wastewater injection wells. The study found no correlations between fecal indicator bacteria concentrations and the presence of fecal sources, suggesting that fecal indicator bacteria were largely derived from environmental sources and conditions. Strong human fecal signal was found in a coastal seep near a resort, indicating that injection wells may be contaminating the coastal area. Fecal samples from suspected animal sources were collected to serve as a more accurate reference library to run the PhyloChip® results through SourceTracker.

What are the limitations to using PhyloChip® in fecal source tracking?

As with any method, there are some limitations to its application in fecal source tracking:

- The ability to detect fecal sources is contingent on the availability of enough probes for detection of each source type (some source types may be underrepresented in the database and not used such as pinnipeds), as well as the applicability of a reference library to local conditions. For instance, the current reference library is trained to fecal samples collected from a variety of sources in California.
- The use of PCR (as with any method that uses PCR) can distort results and affect performance (and thus the method's ability to detect waterborne pathogens).
- At present, PhyloChip® can only detect the likelihood of the presence of an individual source and cannot apportion or quantify each source.
- Widespread and immediate use of the method is inhibited by the high cost of analysis and complexity of results.

Are fecal indicator bacteria reliable indicators for determining presence of fecal source types as identified by PhyloChip®?

As mandated by the USEPA and the US Food and Drug Administration (FDA), state water quality standards use fecal indicator bacteria (primarily *E. coli* in freshwater and enterococci in brackish water) as an estimate of the likelihood that harmful pathogens from fecal source types in the watershed are present in concentrations that make surface waters unsafe for drinking water, shellfish consumption, and/or recreational use. Yet, fecal indicator bacteria are limited in their use as indicators of pathogen contamination and thus public health risk in surface waters.

Previous studies of beaches impacted by point sources of sewage discharge found a significant correlation between fecal indicator bacteria and the probability of gastrointestinal (GI) illness in swimmers caused by bacterial or viral pathogens in the water (Wade et. al., 2003, 2010). However, subsequent studies of surface waters impacted by nonpoint sources of pollution found weaker or no correlation between fecal indicator bacteria and swimmer illness (Colford et. al., 2007; Young et. al., 2016). Studies have concluded that:

- Fecal indicator bacteria come not just from fecal sources but also non-fecal sources such as soils, sediment, algal wrack, decaying vegetation, and beach sands (Badgley et. al., 2010; Byappanahalli et. al., 2003; Hardina & Fujioka, 1991; Imamura et. al., 2011; Ishii et. al., 2006; Park et. al., 2017; Whitman et. al., 2014; Wu et. al., 2017; Yamahara et. al., 2007).
- Fecal indicator bacteria are highly variable and can proliferate or degrade in the environment depending on conditions such as temperature, sunlight, flow, salinity, among other factors (Boehm et. al., 2009; Boehm, 2007; Byappanahalli et. al., 2012; Nelson et. al., 2018; Pisciotta et. al., 2002). Bacterial and viral pathogens have been shown to react differently in the environment, so that external factors may influence the concentration of fecal indicator bacteria but not the viral pathogens of interest for protecting public health. This suggests that the magnitude of fecal indicator bacteria may not reflect a similar level of public health risk.
- Measuring fecal indicator bacteria in the laboratory can be challenging (and potentially confounding) as well due to variability in the ability of cultured specimens in each sample to grow. Because of this, laboratory and field duplicates can vary up to 200% or more, particularly at lower concentrations.

Because fecal indicator bacteria may come from non-fecal sources and/or proliferate/degrade in the environment, fecal indicator bacteria in waters impacted by nonpoint source pollution may not serve as the best proxy for fecal pathogen sources of interest to public health. This research highlights the need for further study into better indicators for pathogen contamination as it relates to public health risk.

To test this in the Palmer River watershed, we related culturable fecal indicator bacteria (*E. coli* and enterococci) with source signal strength determined by PhyloChip® for each sample (Table 6, Figure 5). Each sample was placed in one of four classifications based on primary contact recreation single-sample criteria (235 MPN/100mL for *E. coli* and 104 MPN/100mL for enterococci) and fecal source likelihood threshold of 0.1 set for the sample batch (summarized below)[9].

- False Negative: *E. coli* < 235 MPN/100mL or enterococci < 104 MPN/100mL and fecal source likelihood > 0.1 (likely).
- False Positive: *E. coli* > 235 MPN/100mL or enterococci > 104 MPN/100mL and fecal source likelihood < 0.1 (unlikely).
- True Negative: *E. coli* < 235 MPN/100mL or enterococci < 104 MPN/100mL and fecal source likelihood < 0.1 (unlikely).
- True Positive: *E. coli* > 235 MPN/100mL or enterococci > 104 MPN/100mL and fecal source likelihood > 0.1 (likely).

The goal for source tracking and management is to minimize the occurrence of false negatives and positives to have a reliable indicator of pathogen contamination as it relates to public health risk. Based on the limited data (n = 38 for *E. coli*-derived classification and n = 37 for enterococci-derived classification), we found that human and bird source types were roughly equally distributed among the four classifications (true positives/negatives were just as prominent as false positives/negatives) and that cow source type did poorly with both true and false positives. Dog, horse, and pig were not well detected in the sites monitored, so data were too limited to make any reasonable conclusions (note: results for those source types are presented in Table 6 but not in Figure 5 for this reason). These results indicate that fecal indicator bacteria were not a good indicator to determine the likely presence of human, bird, dog, horse, pig, or cow waste.

Table 6. Summary of samples (n, %) by source type (human, bird, dog, horse, pig, and cow) for *E. coli* and enterococci for the classifications of false negative, false positive, true negative, and true positive (refer to text and Figure 5 for determination criteria).

	Number of Samples by Source Type						**Portion of Samples by Source Type**					
	Human	Bird	Dog	Horse	Pig	Cow	Human	Bird	Dog	Horse	Pig	Cow
***E. coli* (235 MPN/100mL)**												
false negative	11	6	0	0	1	6	29%	16%	0%	0%	3%	16%
false positive	9	12	20	20	21	19	24%	32%	53%	53%	55%	50%
true negative	6	11	17	17	16	11	16%	29%	45%	45%	42%	29%
true positive	12	9	1	1	0	2	32%	24%	3%	3%	0%	5%
Enterococci (104 MPN/100mL)												
false negative	10	9	1	1	0	5	26%	24%	3%	3%	0%	13%
false positive	9	8	17	17	16	14	24%	21%	45%	45%	42%	37%
true negative	11	12	20	20	21	16	29%	32%	53%	53%	55%	42%
true positive	8	9	0	0	1	3	21%	24%	0%	0%	3%	8%

[9] To simplify this analysis, we assumed that PhyloChip® results were 100% accurate with no false negatives or positives. Previous studies have shown that PhyloChip® application in fecal source identification produces results with excellent sensitivity and specificity and minimal false negatives and no false positives (Cao et al., 2013; Boehm et al., 2013).

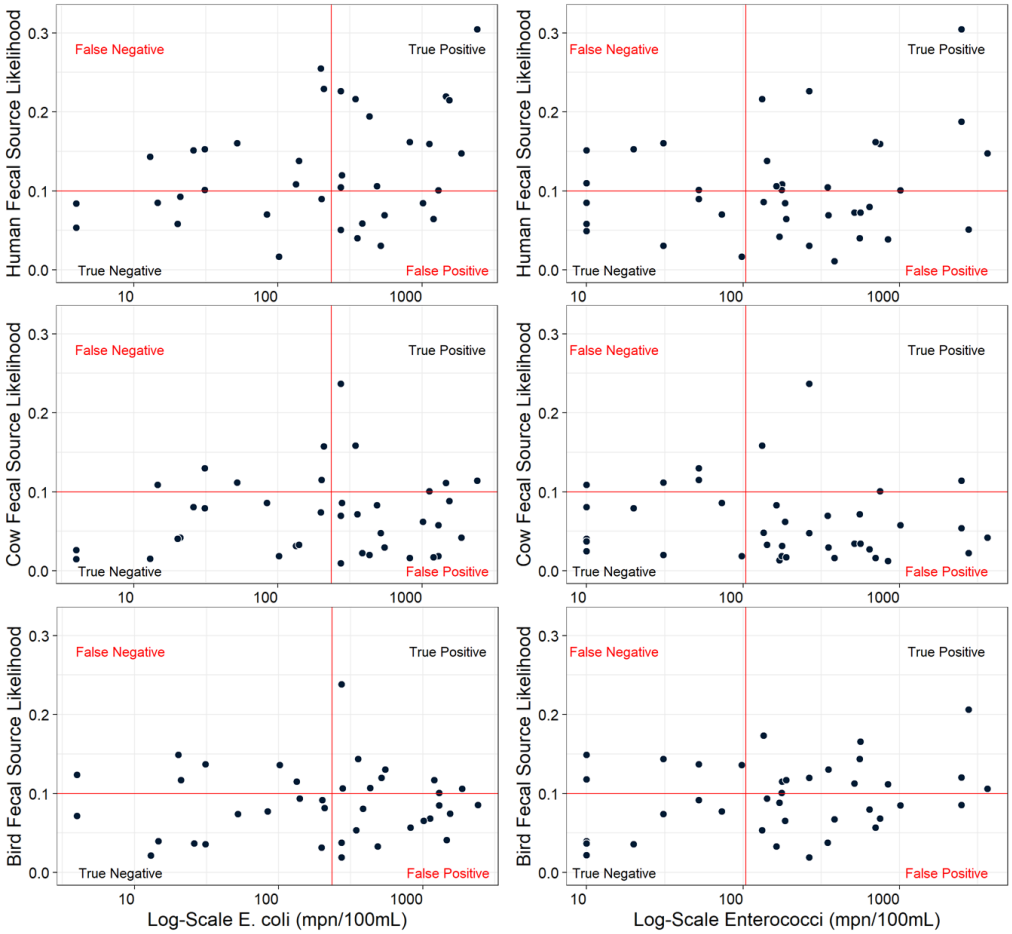


Figure 5. Human (top), cow (middle), and bird (bottom) fecal source likelihood from PhyloChip® analysis compared to culturable *E. coli* (left, $n = 38$) and enterococci (right, $n = 37$) for individual samples. Vertical red lines set the state single-sample criteria at 235 MPN/100mL for *E. coli* and 104 MPN/100mL for enterococci. Horizontal red lines set the signal strength threshold at 0.1, above which the presence of the fecal source type is likely and below which the presence of the fecal source type is unlikely. The resulting quadrants represent (moving clockwise from top left) false negative, true positive, false positive, and true negative. False negative and false positive are highlighted in red text because these are scenarios that we want to avoid.

Are there any factors that could help us to understand and possibly reduce the risk of false negatives and positives?

We analyzed a suite of 28 factors[10] representing available chemical and physical data related to the site (sample date conditions and watershed characteristics) to test significant differences among the four classifications (false negative, true positive, false positive, true negative). *E. coli* and enterococci were not included because their counts dictated the classification placement. Orthophosphate was included but contained several missing values due to tannins in the water samples interfering with the colorimetric analysis ($n = 36$ out of 50). The data matrix was limited to complete cases of fecal indicator bacteria results ($n = 38$ for both *E. coli*- and enterococci-derived classifications).

Analyses were performed in R (ver. 3.5.3) statistical programming using the *tidyr, dplyr, broom, data.table* and *PMCMRplus* packages. We tested for significant differences ($\alpha = 0.05$) among the four classifications with each of the 28 parameters using the non-parametric Kruskal-Wallis test and post-hoc Dunn's test (p-adjusted method = false discovery rate). We limited these tests to the human, bird, and cow source types because the other source types were not well detected in the sample set. Figures 7 and 8 show

[10] Factors included nitrate, total Kjeldahl nitrogen, total nitrogen, orthophosphate, total phosphorus, TSS, salinity, specific conductivity, and water temperature at sample collection, average, min, and max daily air temperature on the sample day, average, min, and max daily air temperature in the 7 days prior to the sample day, total precipitation on the sample day, total precipitation in the 1, 2, 3, 4, and 7 days prior to the sample day, days since last measurable precipitation event defined as 0.25 inches or greater, average areal-weighted discharge on sample day, drainage area, and cropland, pasture, urban, and forest area.

the distribution of values by classification for factors with statistically significant differences among the *E. coli*- and enterococci-derived classifications, respectively. Attachment 5 shows the summary statistics for those significant factors.

For the *E. coli*-derived classification matrix, five major factors showed statistically significant differences among the four classifications for human, bird, and cow source types. Multiple factors representing water and air temperature showed statistically significant differences among the four classifications. We selected water temperature as the representative factor for all temperature factors. For all three source types (human, bird, and cow), the *E. coli*-derived classifications showed statistically significant differences for total Kjeldahl nitrogen (TKN) and water temperature. Precipitation in the prior day or prior 7 days was statistically significantly different among the four classifications for human and bird source types. TSS was statistically significantly different among the four classifications for cow source type. We interpreted these results as follows:

- **TKN:** Generally, TKN was low for true negatives for the three source types and was statistically significantly different from true positives and false positives but not statistically significantly different from false negatives. This suggests that TKN was not an effective metric for reducing the risk of false negatives and positives, though there may be a divergence in the relationship between TKN and *E. coli* for samples with and without a strong source signal when TKN > 0.45 mg/L and *E. coli* > 500 MPN/100mL at an approximate rate increase of 1,000 MPN/100mL per 0.15 mg/L (Figure 6).
- **Water Temperature:** Generally, water temperature was cooler for true/false negatives and warmer for true/false positives, suggesting that fecal indicator bacteria may have proliferated in warmer weather and degraded in cooler weather without corresponding change in the presence of the DNA from fecal source types. Thus, based on human source type, water temperatures cooler than 11°C may increase the likelihood of false negatives (Figure 6). The warmest water temperatures (summer baseflow period) were dominated by human and cow source types (shown as true positives) but not bird source type (shown as false positives).
- **Prior Day Precipitation:** Precipitation in the prior day for human source type showed statistically significant differences between true positive-true negative and true positive-false negative. True positives occurred under a range of antecedent precipitation totals, while the other three classifications occurred generally during dry weather conditions, with some exceptions for false positive and negative, suggesting that storm events may trigger deep and shallow groundwater transport pathways to surface waters for human source type.

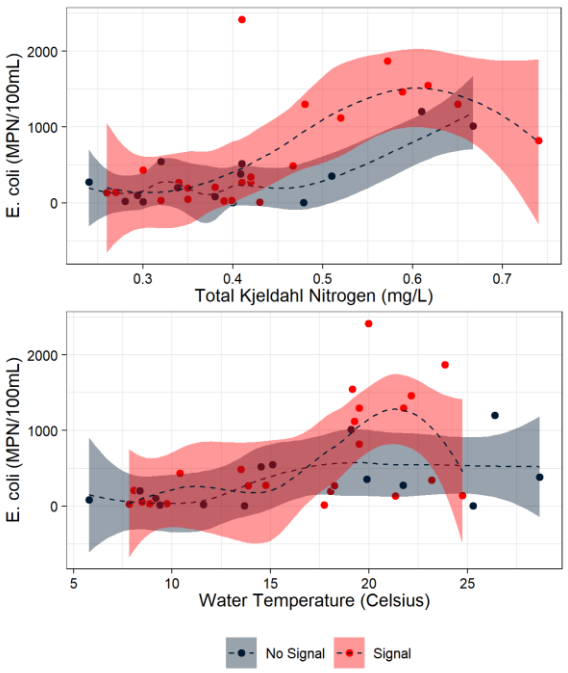


Figure 6. *E. coli* concentration compared to total Kjeldahl nitrogen concentration [TOP] and water temperature [BOTTOM]. Points are differentiated by no signal (gray) and signal (red) for human source type. Dotted lines represented the locally weighted smoothed regression line with 95% confidence intervals as shaded areas.

- **Prior 7-day Precipitation:** Precipitation in the prior seven days for bird source type showed statistically significant difference between true negative and false positive. False positives generally occurred during wet conditions (>1.6 inches), suggesting that directly deposited bird waste may have been flushed downstream. Bird waste can be both directly deposited to surface waters and indirectly transported to surface waters in runoff during storm events, depending on whether the birds are swimming in the water or congregating in grassy areas adjacent to the water.
- **TSS:** TSS for cow source type showed statistically significant differences between true negative-true positive and true negative-false positive, suggesting that true negatives corresponded to low TSS concentrations or less than detection (5 mg/L), but the likelihood of false positives and negatives was similar to true negatives. False positives were more likely with higher TSS concentrations (>10 mg/L). Given the different transport pathways of cow and human feces, it is reasonable that TSS (associated with surface runoff from precipitation events) would be a more significant indicator for cow than human (which typically comes from septic system sources in groundwater).

For the enterococci-derived classification matrix, three major factors showed statistically significant differences among the four classifications: total phosphorus for human, bird, and cow source types, percent urban and forest area for bird source type, and days since last measurable precipitation for cow source type. We interpreted these results as follows:

- **Total Phosphorus:** Generally, total phosphorus was higher for true/false positives and lower for true/false negatives, suggesting that total phosphorus and enterococci may be linked or have similar transport pathways in the environment, but the relationship is weak. Total phosphorus was not an effective metric for reducing the risk of false negatives and positives.
- **Days Since Measurable Precipitation:** Days since last measurable precipitation for cow source type showed statistically significant difference between false negative and true positive with drier conditions associated with true positives (signal, high enterococci) and wetter conditions associated with false negatives (signal, low enterococci). However, neither classification was well distinguished from true negatives nor false positives (the latter possibly from environmental bacteria resuspended during storm events). Days since last measurable precipitation was not an effective metric for reducing the risk of false negatives and positives.
- **Percent Urban and Forest Area:** Percent urban area for bird source type showed statistically significant difference between true and false negatives with false negatives more likely in drainages with smaller percent urban area (<22%). Similarly, percent forest area for bird source type showed statistically significant difference between true and false negatives with false negatives more likely in drainage areas with greater percent forest area (>69%). This suggests that drainage areas with small urban area, large forest area, but low enterococci counts may still be at risk for contamination by bird source type.

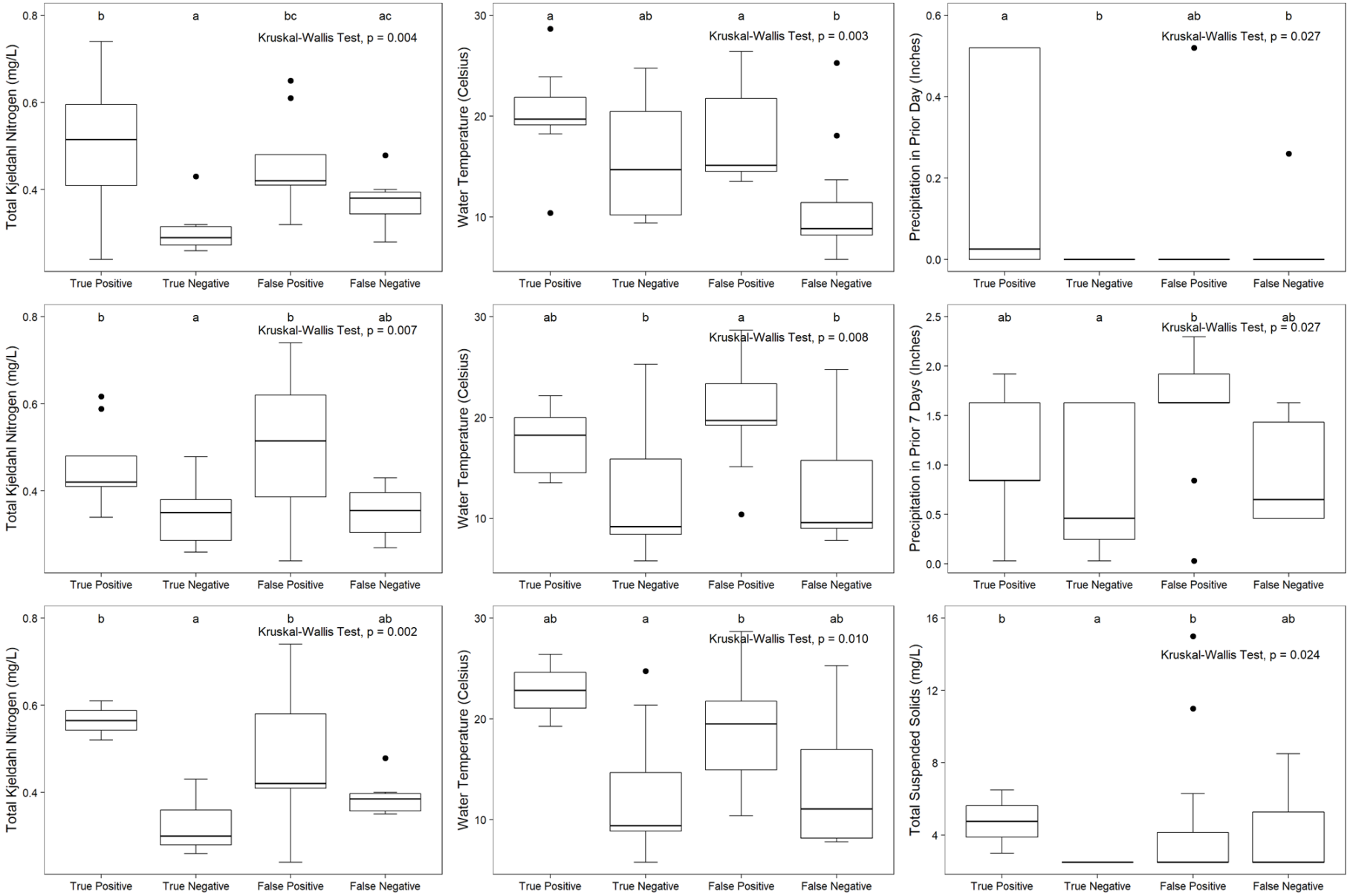


Figure 7. Boxplots of key factors with statistically significant differences among the four *E. coli*-derived classifications (true positive, true negative, false positive, and false negative) for three source types: human (top panels), bird (middle panels), and cow (bottom panels). Statistically significant differences were tested with the non-parametric Kruskal-Wallis test and the post-hoc Dunn's test (α = 0.05). Kruskal-Wallis test significance results are shown on the plots; Dunn's test significance results are shown as "a", "b", or "c" to indicate difference or no difference among classifications for each plot.

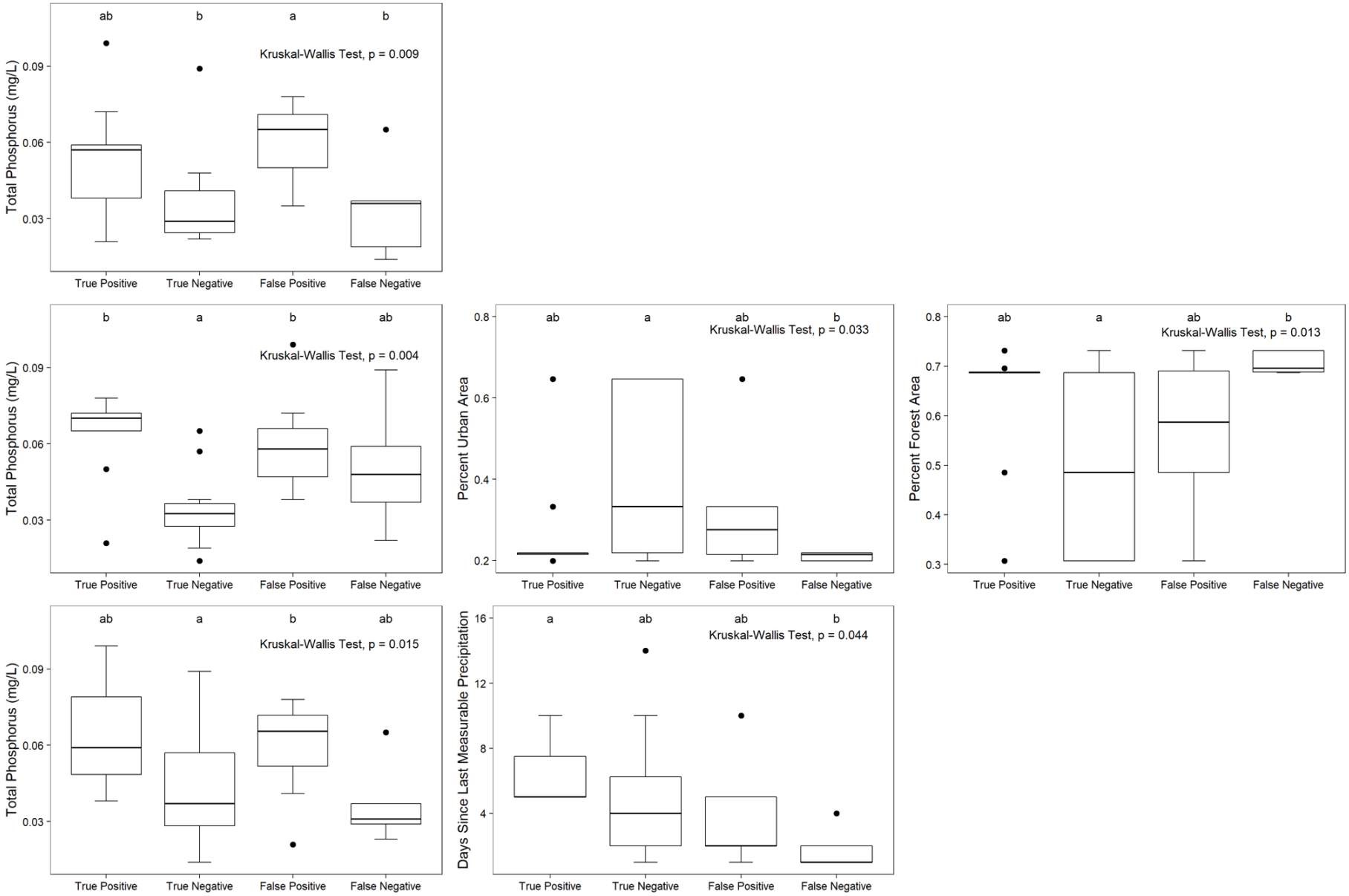


Figure 8. Boxplots of key factors with statistically significant differences among the four enterococci-derived classifications (true positive, true negative, false positive, and false negative) for three source types: human (top panels), bird (middle panels), and cow (bottom panels). Statistically significant differences were tested with the non-parametric Kruskal-Wallis test and the post-hoc Dunn's test ($\alpha = 0.05$). Kruskal-Wallis test significance results are shown on the plots; Dunn's test significance results are shown as "a", "b", or "c" to indicate difference or no difference among classifications for each plot.

Using Analysis of Similarity (ANOSIM) with Bray-Curtis distance metric, we then tested for significant differences (and degree of separation) in microbial community richness (summarized to genus level) among the *E. coli*- and enterococci-derived classifications for human, bird, and cow source types. Our intent was to determine if the presence or absence of certain genera accounted for differences in the four classifications (true/false positive/negative). Though the six tests were significant ($p < 0.05$) except for enterococci-derived classifications for cow source type ($p = 0.378$), the degree of separation was weak, with R ranging from 0.062 to 0.144. Similarity Percentage (SIMPER) analysis was then used to identify the top genera explaining differences among the *E. coli*- and enterococci-derived classifications for human, bird, and cow source types. Analyses were performed in R (ver. 3.5.3) statistical programming using the *vegan* and *dplyr* packages.

For *E. coli*-derived classification, samples with signal for human source type (true positives, false negatives) had greater counts of taxa present from the Firmicutes phylum (Clostridia, Bacilli), which are known to be associated with human gut bacteria (Table 7). Bacteria naturally occurring in the environment or associated with bird source type that dominated across most classifications included Actinobacteria and Proteobacteria. True negatives (no signal-low *E. coli*) had generally lower counts of all taxa, similar to what Dubinsky et al. (2016) found. True positive and false negative classifications were not substantially different, suggesting that the high or low *E. coli* measured were possibly the result of proliferation or degradation in the environment (that artificially increased or decreased the assumed relative risk to public health). Prior analyses in this section suggested that water temperature may play a part. False positive (no signal-high *E. coli*) had similarly elevated naturally occurring bacteria compared to samples with signal, suggesting that elevated *E. coli* were from non-human sources such as from bird or cow source types or were naturally occurring in the environment (in the case of Gammaproteobacteria, Actinobacteria, and Alphaproteobacteria).

Enterococci-derived classification for human source type was similar to *E. coli*-derived classification with some notable exceptions (Table 8). Differences in signal versus no signal groupings were not as well distinguished, suggesting that other source signals like bird and cow were present for all classifications. Naturally occurring bacteria were also more prevalent and dispersed among the four classifications, particularly for marine bacteria, but were generally lower in counts for false negative. Generally, the taxonomic richness of bacteria was greater for sites monitored for enterococci compared to *E. coli*, possibly because sites monitored for enterococci represented more downstream, tidally influenced areas and larger stream orders impacted by a potentially greater contribution of sources.

Overall based on this limited dataset, we found some possible factors to look out for when interpreting fecal indicator bacteria counts and reducing the risk of false positives and negatives (refer to Conclusion section), but the analysis would benefit from a significantly larger dataset across many different watersheds to provide more definitive results.

Table 7. Taxonomic richness of bacteria for *E. coli*-derived classifications (true positive, false negative, false positive, and true negative) for human source type. Values represent the number of detected OTUs for the top 10% of genera (summed to class here) that account for differences among the four classifications (determined by SIMPER analysis). OTUs are groups of closely related bacteria with similar DNA sequences. Taxa in families with more than 10 total OTUs are shown. The first four classes are associated with human sources of bacteria; the next five are generally associated with bird sources of bacteria; the remainder are known to occur naturally in the environment. Shading indicates the following: no shading (< 10 OTUs), light yellow (10-50 OTUs), yellow (51-100 OTUs), orange (101-150 OTUs), red (151-300 OTUs), and dark red (>300 OTUs).

	Signal-High FIB	Signal-Low FIB	No Signal-High FIB	No Signal-Low FIB
p_Phylum c_Class	**True Positive**	**False Negative**	**False Positive**	**True Negative**
p__Firmicutes c__Clostridia	111	142	93	66
p__Firmicutes c__Bacilli	283	183	124	124
p__Tenericutes c__Erysipelotrichi	20	27	10	10
p__Verrucomicrobia c__Verrucomicrobiae	19	15	24	13
p__Actinobacteria c__Actinobacteria	238	301	208	156
p__Proteobacteria c__Alphaproteobacteria	227	224	210	116
p__Proteobacteria c__Betaproteobacteria	134	153	105	112
p__Proteobacteria c__Gammaproteobacteria	576	425	491	238
p__Fusobacteria c__Fusobacteria	43	16	44	35
p__Acidobacteria c__Acidobacteria-5	26	16	9	12
p__Acidobacteria c__Chloracidobacteria	18	20	11	4
p__Bacteroidetes c__Flavobacteria	37	11	25	17
p__Bacteroidetes c__Sphingobacteria	99	43	40	15
p__Chlorobi c__Chlorobia	15	7	13	8
p__Chloroflexi c__Anaerolineae	7	10	4	7
p__Chloroflexi c__Caldilineae	15	22	25	8
p__Chloroflexi c__Dehalococcoidetes	48	41	28	29
p__Cyanobacteria c__Chloroplast	8	15	2	6
p__Cyanobacteria c__Nostocophycideae	22	11	36	20
p__Cyanobacteria c__Oscillatoriophycideae	6	10	9	12
p__Cyanobacteria c__Synechococcophycideae	3	9	13	8
p__Nitrospirae c__Nitrospira	15	21	8	13
p__Planctomycetes c__Planctomycea	70	59	59	15
p__Proteobacteria c__Deltaproteobacteria	121	103	104	32
p__Thermi c__Deinococci	32	30	17	37
p__Verrucomicrobia c__Opitutae	25	18	10	5
p__Verrucomicrobia c__Spartobacteria	9	13	17	7

Table 8. Taxonomic richness of bacteria for enterococci-derived classifications (true positive, false negative, false positive, and true negative) for human source type. Values represent the number of detected OTUs for the top 10% of genera (summed to class here) that account for differences among the four classifications (determined by SIMPER analysis). OTUs are groups of closely related bacteria with similar DNA sequences. Taxa in families with more than 10 total OTUs are shown. The first four classes are associated with human sources of bacteria; the next five are generally associated with bird sources of bacteria; the remainder are known to occur naturally in the environment. Shading indicates the following: no shading (< 10 OTUs), light yellow (10-50 OTUs), yellow (51-100 OTUs), orange (101-150 OTUs), red (151-300 OTUs), and dark red (>300 OTUs).

	Signal-High FIB	Signal-Low FIB	No Signal-High FIB	No Signal-Low FIB
p_Phylum c_Class	**True Positive**	**False Negative**	**False Positive**	**True Negative**
p__Firmicutes c__Bacilli	271	145	183	151
p__Firmicutes c__Clostridia	352	171	165	229
p__Tenericutes c__Erysipelotrichi	12	8	16	21
p__Verrucomicrobia c__Verrucomicrobiae	33	18	25	13
p__Actinobacteria c__Actinobacteria	219	91	278	178
p__Proteobacteria c__Alphaproteobacteria	225	163	223	181
p__Proteobacteria c__Betaproteobacteria	165	60	122	85
p__Proteobacteria c__Gammaproteobacteria	683	217	480	257
p__Fusobacteria c__Fusobacteria	81	9	31	12
p__Acidobacteria c__Acidobacteria-5	23	10	17	16
p__Acidobacteria c__Chloracidobacteria	93	20	45	63
p__Bacteroidetes c__Flavobacteria	45	11	31	23
p__Bacteroidetes c__Sphingobacteria	66	11	74	35
p__Chloroflexi c__Anaerolineae	26	35	28	37
p__Chloroflexi c__Dehalococcoidetes	46	33	31	21
p__Cyanobacteria c__4C0d-2	79	14	35	37
p__Cyanobacteria c__Chloroplast	41	18	17	30
p__Cyanobacteria c__Nostocophycideae	13	10	11	6
p__Cyanobacteria c__Synechococcophycideae	12	5	12	10
p__Elusimicrobia c__Elusimicrobia	27	17	17	20
p__Nitrospirae c__Nitrospira	23	21	10	23
p__Planctomycetes c__Planctomycea	74	32	108	61
p__Proteobacteria c__Deltaproteobacteria	174	70	100	85
p__Proteobacteria c__Epsilonproteobacteria	25	5	8	11
p__Verrucomicrobia c__Opitutae	23	4	7	5

What other indicators aside from fecal indicator bacteria can help predict the presence or absence of a fecal source type?

We analyzed six factors (nitrate, TKN, total nitrogen, orthophosphate, total phosphorus, and TSS) by testing for significant differences ($\alpha = 0.05$) between two groups (signal or no signal) for each source type (human, bird, and cow) as well as combined source types, using the non-parametric Kruskal-Wallis test. Only nitrate concentration showed a statistically significant difference between the presence and absence of combined source types (Figure 9). No statistically significant difference between the presence and absence of an individual source type was found. While nitrate may not serve as a good indicator for tracking a specific source type (such as human or livestock), it may be a good indicator of the likely presence of some source type in the sample when concentrations exceed 0.16 mg/L nitrate and serve as an alternative predictor if a monitoring program cannot include PhyloChip® analysis. Refer to Attachment 6 for summary statistics of significant factors.

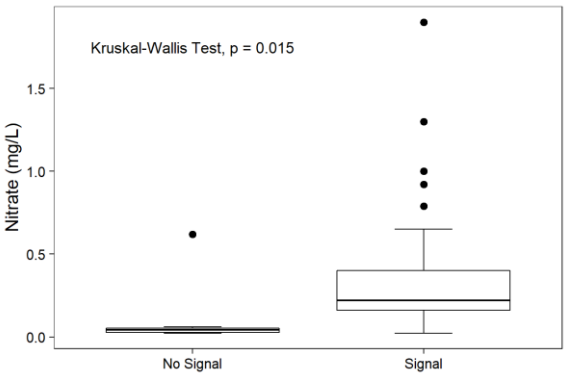


Figure 9. Nitrate concentrations differed significantly between samples with and without a fecal source signal from all types (human, bird, dog, horse, pig, and cow). Statistically significant differences were tested with the non-parametric Kruskal-Wallis test ($\alpha = 0.05$). Kruskal-Wallis test significance results are shown on the plot.

How can monitoring programs best prioritize resources for sampling efforts that efficiently capture possible source types?

We analyzed 18 factors[11] representing typical conditions considered in monitoring programs such as antecedent weather and flow conditions (including drainage area) by testing for significant differences ($\alpha = 0.05$) between two groups (signal or no signal) for each source type (human, bird, and cow) as well as combined source types, using the non-parametric Kruskal-Wallis test. Only drainage area showed a statistically significant difference between the presence and absence of human and bird source types (Figure 10). No statistically significant difference between the presence and absence of combined source types or individual source types for cow were found. Refer to Attachment 6 for summary statistics of significant factors.

Generally, human source type was more likely to be present in small contributing drainage areas. Conversely, bird source type was more likely to be present in large contributing drainage areas, which may be an artifact of known geese congregations at the downstream-most sites along the mainstem.

We also looked at how drainage area (though not statistically significant) may impact the four *E. coli*- and enterococci-derived classifications of true/false negative/positive described previously (Figure 11). We found that small drainage areas (302-1,341 acres) did well with true positives (signal, high *E. coli*) but were more at risk for false negatives (signal, low *E. coli*). Moderate sized drainage areas (1,341-6,794 acres) did poorly with false positives (no signal, high *E. coli*). Large sized drainage areas (PM43, 21,262 acres) and very small drainage areas (TC08, 68 acres) did poorly across all four classifications, indicating that *E. coli* was an unreliable indicator. Large sized drainage areas did best with true negatives (no signal, low *E. coli*).

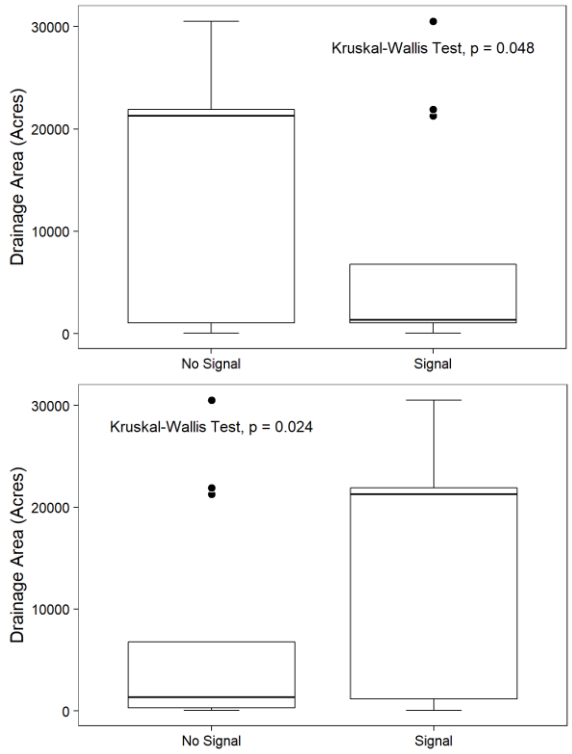


Figure 10. Drainage area differed significantly between samples with and without a fecal source signal from human (top) and bird (bottom). Statistically significant differences were tested with the non-parametric Kruskal-Wallis test ($\alpha = 0.05$). Kruskal-Wallis test significance results are shown on the plot.

[11] Factors included salinity, specific conductivity, and water temperature at sample collection, average, min, and max daily air temperature on the sample day, average, min, and max daily air temperature in the 7 days prior to the sample day, total precipitation on the sample day, total precipitation in the 1, 2, 3, 4, and 7 days prior to the sample day, days since last measurable precipitation event defined as 0.25 inches or greater, average areal-weighted discharge on sample day, and drainage area.

Given these results, it is likely that the drainage area factor is driven more so by proximal sources to the sample site, as well as moderate continuous flow through the sample site to reduce the risk of possible *E. coli* proliferation or degradation in warmer low flow conditions. Retention or degradation of sources upstream of the sample site would be more likely in larger drainage areas with greater stream networks.

A more robust dataset may reveal more definitive patterns in environmental conditions and the presence or absence of individual source types. We hypothesize that different source types may be controlled by different environmental conditions depending on the primary transport pathways, activities on the landscape such as timing of manure application to fields, and seasonal water table fluctuations.

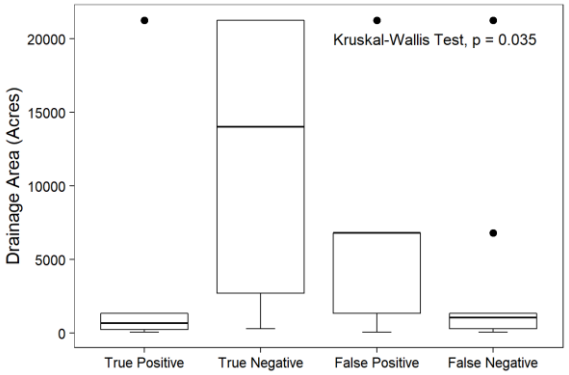


Figure 11. Boxplots showing drainage area among the four *E. coli*-derived classifications (true positive, true negative, false positive, and false negative) for human source type. Statistically significant differences were tested with the non-parametric Kruskal-Wallis test and the post-hoc Dunn's test ($\alpha = 0.05$). Kruskal-Wallis test significance results are shown on the plot; Dunn's test significance results failed to show differences between each classification.

How can monitoring programs use the PhyloChip® results to help answer questions about their study sites?

Possible evaluation steps and statistical approaches when analyzing the PhyloChip® results for a specific study area are summarized below.

1. **Summarize source types by site and compare to expected sources.** A table summarizing presence/absence of source types by sample site compared to any known or suspected sources can help confirm known or establish new sources of concern and describe the spatial extent of source types.
2. **Determine the dominant OTUs for each source type and how community composition and/or structure change in relation to space or time or in association with water quality or watershed characteristics or antecedent conditions.** Visualize differences in community composition and/or structure (non-metric multidimensional scaling or NMDS, Bray-Curtis distance metric), determine whether differences in community composition (richness, binary data) and/or structure (relative abundance, fold-change in hybridization intensity) between factors for each source group are significant (ANOSIM), and what OTUs largely comprise the factor-based differences for each source type (SIMPER). We can look at the entire community or individual taxa (such as pathogenic taxa) or co-occurrence among different taxa. OTUs can be searched by their accession numbers in GenBank (https://www.ncbi.nlm.nih.gov/genbank/) for additional information.
3. **Track water quality improvement over time as a result of remediation efforts.** We can use PhyloChip® to track changes in water quality over time. Setting up a sampling regime in year 1 (as a baseline) that can be replicated every few years can help determine if source signals are lessening or have become absent or if new source signals are popping up or if community composition and structure are changing. Refer to #2 for statistical approach. We were unable to perform a time series analysis with only one year of data.
4. **Identify factors that help predict presence of source types or best capture possible source types.** We looked at these questions in this report, but any study area can look at these questions with the PhyloChip® results at their sites to see if similar or different results are found. These are larger systemic questions that will require significantly larger datasets to help answer. Secondary indicators are needed to supplement fecal indicator bacteria, especially when making impairment determinations.

CONCLUSION

This memorandum provided 1) prioritization rationale for selecting a subset of 50 (out of 96) samples within the Palmer River watershed to be sent for PhyloChip® DNA microarray analysis and then 2) assessed the PhyloChip® results in a broader context of application in fecal source tracking to determine the most effective use of PhyloChip® in monitoring programs. The Palmer River Water Quality Analysis Report (HWG & FBE, 2019a) includes an analysis of PhyloChip® results in the context of specific fecal sources in the Palmer River watershed.

Based on analysis of the PhyloChip® results, we generally recommend that monitoring efforts within the New England area using PhyloChip® focus on collecting samples from smaller drainage areas in the summer months under both wet and dry weather conditions to best capture proximal sources and investigate manageable areas for pollutant source remediation. Signals for human, bird, and cow source types tended to be strongest in the summer months (July, August, and September) (HWG & FBE, 2019a);

otherwise, antecedent weather or flow conditions did not significantly control for variation in source signals for this dataset. When assessing change in microbial community composition and/or structure among sites and/or across time, it is recommended that a minimum of 20 samples be collected (E. Dubinsky, pers. comm.).

To account for possible local differences in the genetic makeup of fecal sources, we recommend that a reference library specific to New England be created. PhyloChip® results for New England could also be strengthened by adjusting for expected persistence of different bacterial taxa, depending on how long and where taxa have been in the environment; for instance, whether sources are nonpoint or from direct sewage spills. Future research should assess how fate and transport of bacterial taxa may influence detection rates using PhyloChip®.

When a monitoring program is unable to use PhyloChip®, be aware that small drainage areas with minimal urban area, large forest area, but low enterococci counts may still be at risk for contamination from bird sources. Monitoring programs should also include nutrient parameters such as nitrogen (TKN, nitrate) and phosphorus, both of which may serve as secondary indicators to fecal indicator bacteria. Managers should be careful with interpreting fecal indicator bacteria, especially *E. coli*, in cold water temperatures (tend to generate false negatives) and warm water temperatures (tend to generate false positives), as well as in very small drainage areas with low flow conditions, due to *E. coli*'s potential for degradation or proliferation in the environment. Our analysis showed that fecal indicator bacteria were not a good indicator for the presence of human, bird, dog, horse, pig, or cow waste because false negatives and positives (i.e., signal-low FIB, no signal-high FIB) were just as likely as true negatives and positives (i.e., no signal-low FIB, signal-high FIB) for all source types.

While our analyses helped define a few best practices for use of PhyloChip® in monitoring programs and revealed some possible secondary indicators for presence of fecal source types when PhyloChip® is unable to be used, our analyses were not conclusive and would require a significantly larger dataset across multiple watersheds and years in a variety of environments to better answer. More in-depth analysis of changes in microbial community profiles across seasons and/or years (or as remediation activities are implemented) would require multiple samples across several years. In the example of the Palmer River, the existing PhyloChip® dataset can serve as a baseline from which to compare to replicate PhyloChip® datasets collected every few years to assess change in water quality as more agricultural BMPs are put in place.

Overall, PhyloChip® has several beneficial uses in monitoring programs. It can be used as a one-time snapshot to determine presence/absence of fecal source types and/or microbial community taxa in a watershed. It can also be used to track change in the presence of fecal source types and/or microbial community taxa over time and/or space or in relation to changing environmental conditions such as land use or remediation efforts. We can also look at the entire microbial community or individual taxa (such as pathogenic strains or Cyanobacteria *spp.*) or co-occurrence among different taxa. PhyloChip® can also be used in monitoring programs for both local and regional evaluations of alternative or co-indicators to fecal indicator bacteria.

REFERENCES

Badgley, B.D., Thomas, F.I.M., Harwood, V.J. (2010). The effects of submerged aquatic vegetation on the persistence of environmental populations of Enterococcus spp. *Environ. Microbiol.* 12, 1271-1281. http://dx.doi.org/10.1111/j.1462-2920.2010.02169.x

Boehm, A. B. (2007). Enterococci Concentrations in Diverse Coastal Environments Exhibit Extreme Variability. *Environ. Sci. Technol.* 41 (24), 8227−8232.

Boehm, A. B., Yamahara, K. M., Love, D. C., Peterson, B. M., McNeill, K., Nelson, K. L. (2009). Covariation and Photoinactivation of Traditional and Novel Indicator Organisms and Human Viruses at a Sewage-Impacted Marine Beach. *Environ. Sci. Technol.* 43 (21), 8046−8052.

Boehm, A.B., Van De Werfhorst, L.C., Griffith, J.F., Holden, P.A., Jay, J.A., Shanks, O.C., Wang, D., Weisberg, S.B., 2013. Performance of forty-one microbial source tracking methods: a twenty-seven lab evaluation study. *Water Res.* 47, 6812e6828. http://dx.doi.org/10.1016/j.watres.2012.12.046

Byappanahalli, M., Fowler, M., Shively, D., Whitman, R. (2003). Ubiquity and persistence of *Escherichia coli* in a midwestern coastal stream. *Appl. Environ. Microbiol.* 69, 4549-4555. http://dx.doi.org/10.1128/AEM.69.8.4549-4555.2003

Byappanahalli, M.N., Nevers, M.B., Korajkic, A., Staley, Z.R., Harwood, V.J. (2012). Enterococci in the environment. *Microbiol. Mol. Biol. Rev.* 76, 685-706. http://dx.doi.org/10.1128/MMBR.00023-12

Cao, Y., Van De Werfhorst, L.C., Dubinsky, E.A., Badgley, B.D., Sadowsky, M.J., Andersen, G.L., Griffith, J.F., Holden, P.A. (2013). Evaluation of Molecular Community Analysis Methods for Discerning Fecal Sources and Human Waste, *Water Research*, doi: 10.1016/j.watres.2013.02.061.

Colford, J.M., Wade, T.J., Schiff, K.C., Wright, C.C., Griffith, J.F., Sandhu, S.K., Burns, S., Sobsey, M., Lovelace, G., Weisberg, S.B. (2007). Water Quality Indicators and the Risk of Illness at Beaches with Nonpoint Sources of Fecal Contamination. *Epidemiology*, 18: 27–35. DOI: 10.1097/01.ede.0000249425.32990.b9

Dubinsky, E.A., and Andersen, G. (2014). Russian River Human Impact Study PhyloChip Microbial Community Analysis. Final Report: May 1, 2014.

Dubinsky, E.A., and Andersen, G. (2019). Mahaulepu and Waikomo Watersheds PhyloChip Source Tracking Study, Hawaii. Final Report: May 22, 2019.

Dubinsky, E.A., Butkus, S.R., Andersen, G.L. (2016). Microbial source tracking in impaired watersheds using PhyloChip and machine-learning classifications. *Water Research* 105: 56-64. http://dx.doi.org/10.1016/j.watres.2016.08.035

Dubinsky, E.A., Conrad, M.E., Chakraborty, R., Bill, M., Borglin, S.E., Hollibaugh, J.T., Mason, O.U., Piceno, Y.M., Reid, F.C., Stringfellow, W.T., Tom, L.M., Hazen, T.C., and Andersen, G.L. (2013). Succession of Hydrocarbon-Degrading Bacteria in the Aftermath of the *Deepwater Horizon* Oil Spill in the Gulf of Mexico. *Environmental Science & Technology* 47, 10860-10867. dx.doi.org/10.1021/es401676y

Dubinsky, E.A., Esmaili, L., Hulls, J.R., Cao, Y., Griffith, J.F., Andersen, G.L. (2012). Application of Phylogenetic Microarray Analysis to Discriminate Sources of Fecal Pollution. *Environmental Science & Technology*, 46, 4340-4347. DOI: dx.doi.org/10.1021/es2040366

ESS Group Inc. (2003). Palmer River Microbial Source Tracking (MST) Study. Produced for Massachusetts Department of Environmental Protection. M252-000.11, May 16, 2003.

Hardina, C.M., Fujioka, R.S. (1991). Soil: the environmental source of *Escherichia coli* and enterococci in Hawaii's streams. *Environ. Toxicol. Water Qual.* 6, 185-195. http://dx.doi.org/10.1002/tox.2530060208

Hazen et. al. (2010). Deep-Sea Oil Plume Enriches Indigenous Oil-Degrading Bacteria. *Science*, 330 (6001), 204-208. DOI: 10.1126/science.1195979

HWG & FBE. (2019a). Palmer River Water Quality Analysis Report. Prepared for the US Environmental Protection Agency by Horsley Witten Group and FB Environmental Associates, December 2019.

HWG & FBE. (2019b). Palmer River Land Use & Regulatory Analysis Report. Prepared for the US Environmental Protection Agency by Horsley Witten Group and FB Environmental Associates, December 2019.

HWG & FBE. (2019c). Secondary Data Quality Assurance Project Plan (QAPP) for the Palmer River Source Tracking, Water Quality Trends Summary, and Watershed Plan. Prepared for the US Environmental Protection Agency by Horsley Witten Group and FB Environmental Associates, February 4, 2019.

Imamura, G.J., Thompson, R.S., Boehm, A.B., Jay, J.A. (2011). Wrack promotes the persistence of fecal indicator bacteria in marine sands and seawater. *FEMS Microbiol. Ecol.* 77, 40-49. http://dx.doi.org/10.1111/j.1574-6941.2011.01082.x

Ishii, S., Ksoll, W.B., Hicks, R.E., Sadowsky, M.J. (2006). Presence and growth of naturalized *Escherichia coli* in temperate soils from lake superior watersheds. *Appl. Environ. Microbiol.* 72, 612-621. http://dx.doi.org/10.1128/AEM.72.1.612- 621.2006

Nelson, K.L., Boehm, A.B., Davies-Colley, R.J., Dodd, M.C., Kohn, T., Linden, K.G., Liu, Y., Maraccini, P.A., McNeill, K., Mitch, W.A., Nguyen, T.H., Parker, K.M., Rodriguez, R.A., Sassoubre, L.M., Silverman, A.I., Wigginton, K.R., Zepp, R.G. (2018). Sunlight-mediated inactivation of health-relevant microorganisms in water: a review of mechanisms and modeling approaches. Environmental Science: Processes & Impacts, 20, 1089. DOI: 10.1039/c8em00047f

Park, Y., Pachespsky, Y., Hong, E-M., Shelton, D., Coppock, C. (2017). Escherichia coli Release from Streambed to Water Column during Baseflow Periods: A Modeling Study. *Journal of Environmental Quality,* 46, 219-226. doi:10.2134/jeq2016.03.0114

Pisciotta, et al. (2002). Marine bacteria cause false-positive results in the Colilert-18 rapid identification test for *Escherichia coli* in Florida waters. Applied and Environmental Microbiology, February 2002, 68 (2), p. 539-544.

Wade, T. J., Pai, N., Eisenberg, J. N. S., Colford, J. M. (2003). Do U. S. Environmental Protection Agency Water Quality Guidelines for Recreational Waters Prevent Gastrointestinal Illness? A Systematic Review and Meta-Analysis. *Environ. Health Perspect.* 111 (8), 1102−1109.

Wade, T.J., Sams, E., Brenner, K.P., Haugland, R., Chern, E., Beach, M., Wymer, L., Rankin, C.C., Love, D., Li, Q., Noble, R., Dufour, A.P. (2010). Rapidly measured indicators of recreational water quality and swimming-associated illness at marine beaches: a prospective cohort study. *Environmental Health*, 9 (66), 1-14. doi:10.1186/1476-069X-9-66

Whitman, R.L., Harwood, V.J., Edge, T.A., Nevers, M.B., Byappanahalli, M., Vijayavel, K., Brand~ao, J., Sadowsky, M.J., Alm, E.W., Crowe, A., Ferguson, D., Ge, Z., Halliday, E., Kinzelman, J., Kleinheinz, G., Przybyla-Kelly, K., Staley, C., Staley, Z., Solo-Gabriele, H.M. (2014). Microbes in beach sands: integrating environment, ecology and public health. *Rev. Environ. Sci. Biotechnol.* 13, 329-368. http://dx.doi.org/10.1007/s11157-014-9340-8

Wu, M.Z., O'Carroll, D.M., Vogel, L.J., Robinson, C.E. (2017). Effect of Low Energy Waves on the Accumulation and Transport of Fecal Indicator Bacteria in Sand and Pore Water at Freshwater Beaches. *Environ. Sci. Technol.* 51 (5), 2786-2794. https://doi.org/10.1021/acs.est.6b05985

Yamahara, K. M., Layton, B. A., Santoro, A. E., Boehm, A. B. (2007). Beach Sands along the California Coast Are Diffuse Sources of Fecal Bacteria to Coastal Waters. *Environ. Sci. Technol.* 41 (13), 4515−4521.

Young, N. (2016). The association between marine bathing and infectious diseases – a review. Journal of the Marine Biological Association of the United Kingdom, 96(1), 93-100. doi:10.1017/S0025315415002003

ATTACHMENT 1

Summary statistics (median, average, minimum, maximum, number of samples (after duplicate days averaged), number of years, start year, and end year) by site and parameter for twelve "core" sites monitored in the Palmer River watershed. Values exceeding state criteria or natural background conditions are displayed as bold red or orange, respectively. Refer to the end of the table for a list of applied thresholds and other assumptions. *E. coli* for saline sites were greyed out because *E. coli* has been shown to result in false positives in marine waters (Pisciotta et al., 2002) and thus is not the preferred indicator for saline sites.

Site	Parameter	Median	Average	Min	Max	*n* (samples)	*n* (years)	Start Year	End Year
CR01	E. COLI	55	57	2	**4884**	41	9	2001	2018
CR01	NITRATE + NITRITE	**0.330**	**0.703**	0.023	**2.900**	23	3	2016	2018
CR01	ORTHOPHOSPHATE	0.014	0.020	0.005	**0.087**	20	3	2016	2018
CR01	TOTAL KJELDAHL NITROGEN	**0.425**	**0.474**	0.240	**0.889**	22	3	2016	2018
CR01	TOTAL NITROGEN	**0.890**	**1.118**	0.370	**2.500**	23	3	2016	2018
CR01	TOTAL PHOSPHORUS	**0.071**	**0.092**	0.018	**0.240**	23	3	2016	2018
CR01	TOTAL SUSPENDED SOLIDS	3.4	5.6	2.5	18.0	24	3	2016	2018
CR02	E. COLI	**471**	**414**	18	**24196**	44	10	1999	2018
CR02	NITRATE + NITRITE	**1.200**	**1.206**	0.200	**2.100**	23	3	2016	2018
CR02	ORTHOPHOSPHATE	**0.065**	**0.084**	**0.027**	**0.220**	20	3	2016	2018
CR02	TOTAL KJELDAHL NITROGEN	0.300	**0.354**	0.010	**0.800**	23	3	2016	2018
CR02	TOTAL NITROGEN	**1.600**	**1.560**	**0.640**	**2.500**	23	3	2016	2018
CR02	TOTAL PHOSPHORUS	**0.150**	**0.164**	**0.060**	**0.450**	23	3	2016	2018
CR02	TOTAL SUSPENDED SOLIDS	2.7	6.0	2.5	31.0	24	3	2016	2018
CR03	E. COLI	**315**	**324**	12	**24196**	52	11	1999	2018
CR03	NITRATE + NITRITE	**0.460**	**0.533**	0.023	**1.400**	23	3	2016	2018
CR03	ORTHOPHOSPHATE	**0.100**	**0.116**	0.005	**0.270**	21	5	2001	2018
CR03	TOTAL KJELDAHL NITROGEN	**0.370**	**0.411**	0.210	**1.100**	25	5	2001	2018
CR03	TOTAL NITROGEN	**0.840**	**0.986**	0.450	**2.310**	30	6	2001	2018
CR03	TOTAL PHOSPHORUS	**0.215**	**0.266**	**0.080**	**1.500**	30	6	2001	2018
CR03	TOTAL SUSPENDED SOLIDS	4.1	9.7	2.0	48.0	26	5	2001	2018
PM31	E. COLI	31	33	2	**2420**	35	10	1999	2018
PM31	ENTEROCOCCI	14	23	2	**426**	26	4	2015	2018
PM31	NITRATE + NITRITE	0.145	0.197	0.012	**1.965**	34	5	1996	2018
PM31	ORTHOPHOSPHATE	0.008	0.012	0.001	**0.031**	45	7	1996	2018
PM31	TOTAL KJELDAHL NITROGEN	**0.330**	**0.347**	0.200	**0.840**	27	5	2001	2018
PM31	TOTAL NITROGEN	0.515	**0.594**	0.250	**2.132**	38	7	1996	2018
PM31	TOTAL PHOSPHORUS	**0.039**	**0.038**	0.011	**0.066**	38	7	1996	2018
PM31	TOTAL SUSPENDED SOLIDS	2.5	4.7	1.0	24.0	28	5	2001	2018
PM30	E. COLI	**136**	**169**	16	**2420**	36	10	1999	2018
PM30	ENTEROCOCCI	**142**	**125**	10	**2910**	27	5	2014	2018
PM30	NITRATE + NITRITE	0.280	**0.333**	0.026	**1.000**	23	3	2016	2018
PM30	ORTHOPHOSPHATE	0.012	0.018	0.005	**0.123**	30	4	2001	2018
PM30	TOTAL KJELDAHL NITROGEN	**0.301**	**0.325**	0.220	**0.700**	23	3	2016	2018
PM30	TOTAL NITROGEN	**0.590**	**0.658**	0.320	**1.700**	23	3	2016	2018
PM30	TOTAL PHOSPHORUS	**0.042**	**0.046**	0.018	**0.099**	23	3	2016	2018
PM30	TOTAL SUSPENDED SOLIDS	2.5	3.3	2.5	9.8	24	3	2016	2018
PM44	*E. COLI*	*1230*	*957*	*95*	*6328*	*5*	*3*	*2013*	*2016*
PM44	ENTEROCOCCI	**426**	**326**	10	**7701**	26	4	2015	2018
PM44	NITRATE + NITRITE	0.210	0.182	0.023	**0.380**	23	3	2016	2018
PM44	ORTHOPHOSPHATE	0.015	0.018	0.005	**0.044**	16	3	2016	2018
PM44	TOTAL KJELDAHL NITROGEN	**0.449**	**0.489**	0.300	**0.879**	23	3	2016	2018
PM44	TOTAL NITROGEN	**0.650**	**0.669**	0.400	**1.100**	23	3	2016	2018
PM44	TOTAL PHOSPHORUS	**0.057**	**0.062**	**0.026**	**0.110**	23	3	2016	2018
PM44	TOTAL SUSPENDED SOLIDS	5.0	8.5	2.5	45.0	24	3	2016	2018
RR23	E. COLI	**154**	126	4	**1099**	42	11	2001	2018
RR23	ENTEROCOCCI	**95**	**90**	10	**776**	25	4	2015	2018
RR23	NITRATE + NITRITE	0.170	0.222	0.005	**0.802**	34	5	1996	2018
RR23	ORTHOPHOSPHATE	0.016	0.021	0.000	**0.120**	41	6	1996	2018
RR23	TOTAL KJELDAHL NITROGEN	**0.382**	**0.437**	0.271	**0.990**	25	4	2001	2018

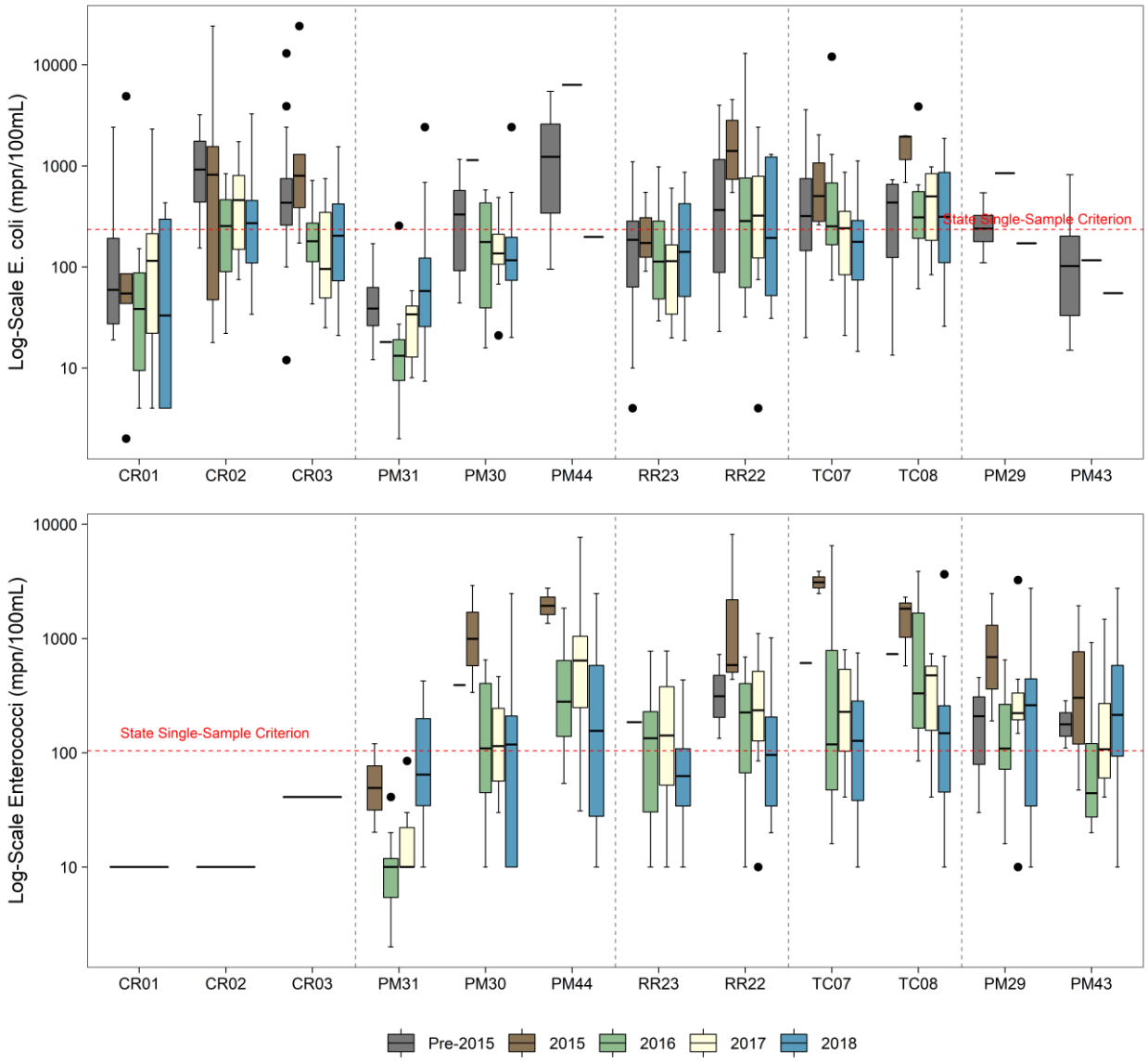
Site	Parameter	Median	Average	Min	Max	*n* (samples)	*n* (years)	Start Year	End Year
RR23	TOTAL NITROGEN	**0.655**	**0.769**	0.360	**1.424**	40	7	1996	2018
RR23	TOTAL PHOSPHORUS	**0.043**	**0.046**	0.010	**0.140**	41	7	1996	2018
RR23	TOTAL SUSPENDED SOLIDS	2.5	4.3	1.0	44.0	26	4	2001	2018
RR22	*E. COLI*	*365*	*336*	*4*	*12997*	*43*	*12*	*1999*	*2018*
RR22	ENTEROCOCCI	**201**	**192**	10	**8160**	29	6	2013	2018
RR22	NITRATE + NITRITE	0.180	0.215	0.023	**0.500**	23	3	2016	2018
RR22	ORTHOPHOSPHATE	0.014	0.021	0.005	**0.050**	17	5	2001	2018
RR22	TOTAL KJELDAHL NITROGEN	**0.490**	**0.571**	0.300	**1.020**	27	5	2001	2018
RR22	TOTAL NITROGEN	**0.810**	**0.808**	0.410	**1.400**	27	5	2001	2018
RR22	TOTAL PHOSPHORUS	**0.040**	**0.050**	0.018	**0.120**	27	5	2001	2018
RR22	TOTAL SUSPENDED SOLIDS	2.9	5.8	1.0	51.0	28	5	2001	2018
TC07	*E. COLI*	*272*	*266*	*15*	*12033*	*39*	*9*	*2001*	*2018*
TC07	ENTEROCOCCI	**206**	**211**	10	**6488**	26	5	2013	2018
TC07	NITRATE + NITRITE	**0.460**	**0.503**	0.054	**1.000**	23	3	2016	2018
TC07	ORTHOPHOSPHATE	0.010	0.015	0.005	**0.050**	24	5	2001	2018
TC07	TOTAL KJELDAHL NITROGEN	**0.360**	**0.414**	0.240	**0.900**	27	5	2001	2018
TC07	TOTAL NITROGEN	**0.940**	**0.993**	0.400	**1.930**	27	5	2001	2018
TC07	TOTAL PHOSPHORUS	**0.037**	**0.041**	0.020	**0.080**	27	5	2001	2018
TC07	TOTAL SUSPENDED SOLIDS	2.9	4.0	2.5	18.0	28	5	2001	2018
TC08	*E. COLI*	*487*	*348*	*13*	*3873*	*37*	*8*	*2002*	*2018*
TC08	ENTEROCOCCI	**475**	**326**	10	**3873**	27	5	2013	2018
TC08	NITRATE + NITRITE	0.081	0.084	0.023	0.260	23	3	2016	2018
TC08	ORTHOPHOSPHATE	0.005	0.008	0.005	**0.027**	19	3	2016	2018
TC08	TOTAL KJELDAHL NITROGEN	**0.559**	**0.569**	**0.339**	**0.919**	23	3	2016	2018
TC08	TOTAL NITROGEN	**0.610**	**0.650**	0.350	**1.000**	23	3	2016	2018
TC08	TOTAL PHOSPHORUS	**0.026**	**0.031**	0.014	**0.096**	23	3	2016	2018
TC08	TOTAL SUSPENDED SOLIDS	2.5	5.4	2.5	45.0	24	3	2016	2018
PM29	*E. COLI*	*239*	*281*	*110*	*846*	*6*	*4*	*2012*	*2016*
PM29	ENTEROCOCCI	**216**	**177**	10	**3255**	29	6	2013	2018
PM29	NITRATE + NITRITE	0.120	0.132	0.023	0.300	26	4	1998	2018
PM29	ORTHOPHOSPHATE	0.023	**0.025**	0.005	**0.056**	17	4	1998	2018
PM29	TOTAL KJELDAHL NITROGEN	**0.543**	**0.559**	0.290	**1.580**	26	4	1998	2018
PM29	TOTAL NITROGEN	**0.630**	**0.688**	0.370	**1.800**	26	4	1998	2018
PM29	TOTAL PHOSPHORUS	**0.054**	**0.060**	**0.027**	**0.130**	26	4	1998	2018
PM29	TOTAL SUSPENDED SOLIDS	7.0	8.5	2.5	33.7	27	4	1998	2018
PM43	*E. COLI*	*100*	*91*	*15*	*820*	*8*	*5*	*2001*	*2016*
PM43	ENTEROCOCCI	**121**	**142**	10	**2755**	28	6	2013	2018
PM43	NITRATE + NITRITE	0.079	0.106	0.023	0.240	23	3	2016	2018
PM43	ORTHOPHOSPHATE	**0.027**	**0.037**	0.007	**0.099**	27	5	2001	2018
PM43	TOTAL KJELDAHL NITROGEN	**0.554**	**0.616**	0.300	**1.430**	27	5	2001	2018
PM43	TOTAL NITROGEN	**0.730**	**0.742**	0.350	**1.600**	27	5	2001	2018
PM43	TOTAL PHOSPHORUS	**0.060**	**0.089**	**0.036**	**0.580**	27	5	2001	2018
PM43	TOTAL SUSPENDED SOLIDS	7.4	8.9	3.0	32.0	28	5	2001	2018

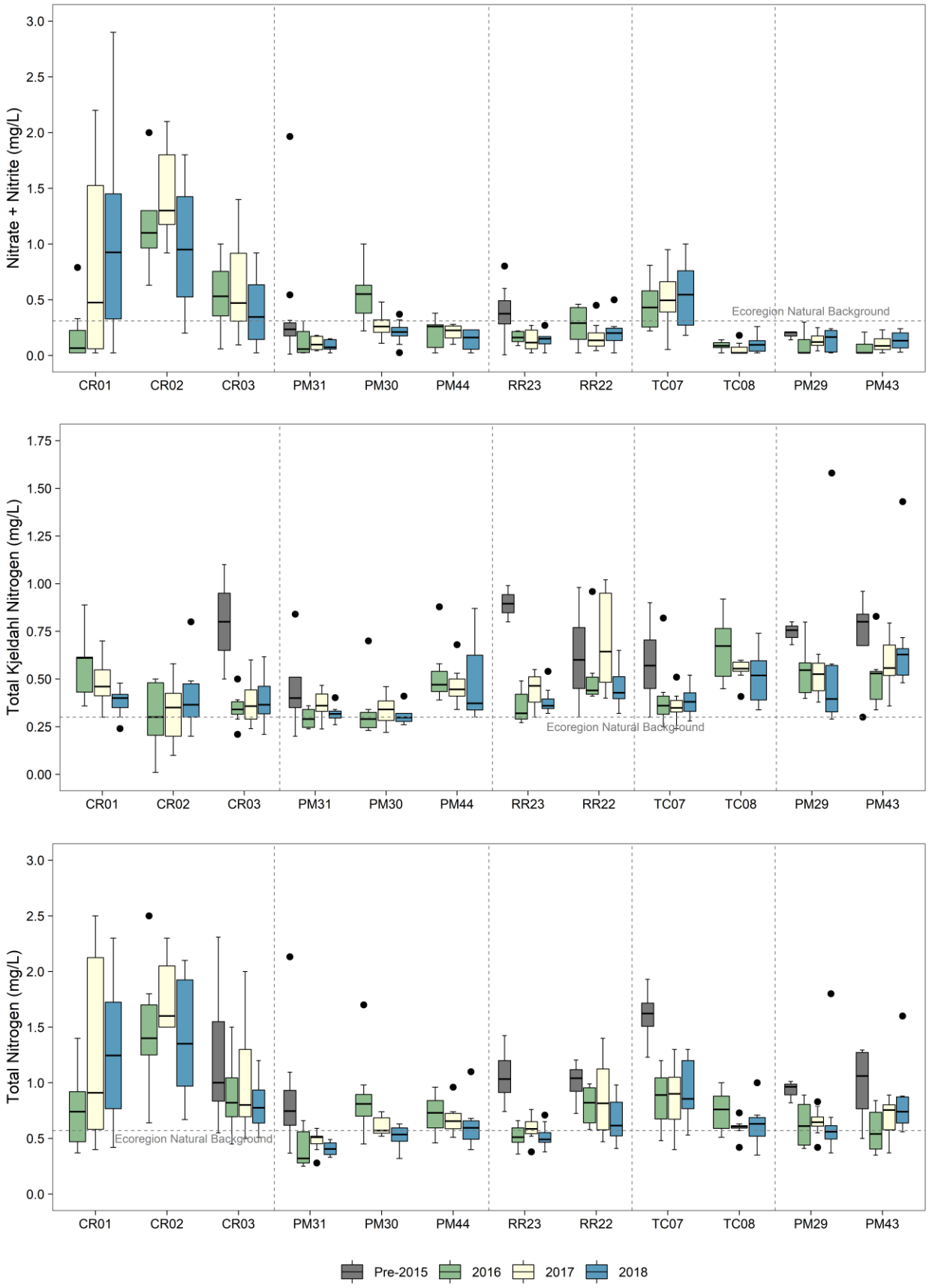
E. coli	*126 mpn/100mL (geomean); 235 mpn/100mL (single)*
Enterococci	*35 mpn/100mL (geomean); 104 mpn/100mL (single)*
Nitrate + Nitrite	*0.31 mg/L*
Total Kjeldahl nitrogen	*0.30 mg/L*
Total Nitrogen	*0.57 mg/L*
Orthophosphate	*0.024 mg/L (used Total Phosphorus Reference Condition)*
Total Phosphorus	*0.024 mg/L*
Total suspended solids	*30 mg/L (30-day average), 58 mg/L (daily max)*

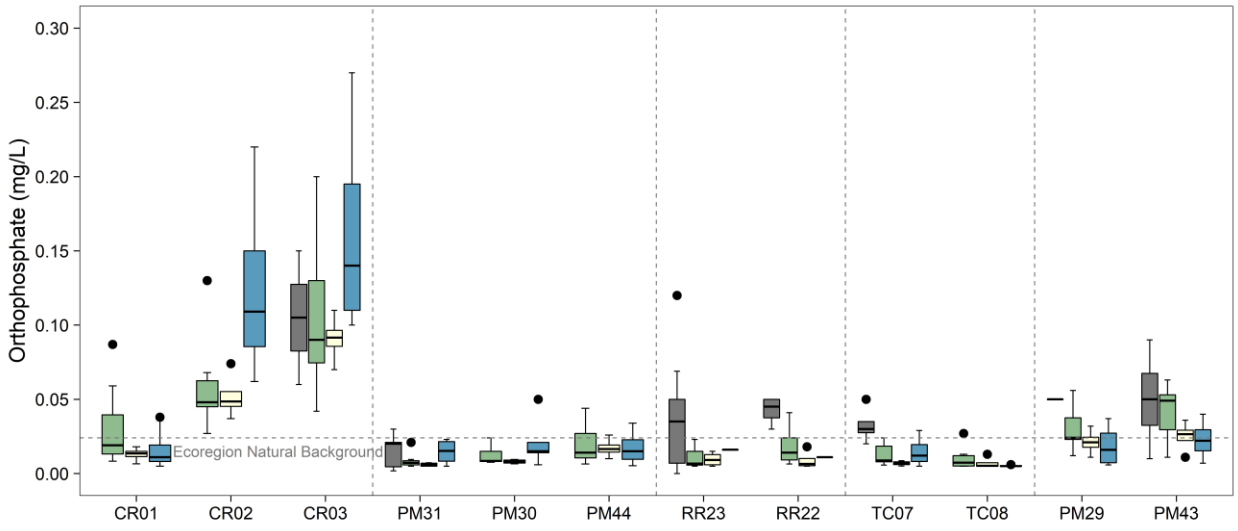
Note: both median and average *E. coli* and enterococci values were log-transformed before summarized (average represents true geomean)

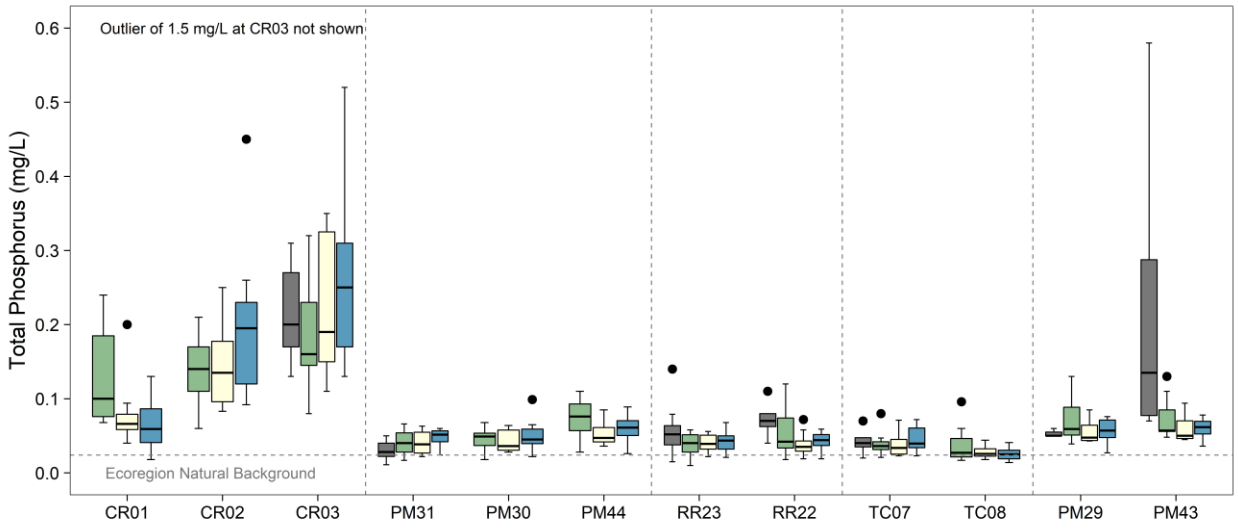
ATTACHMENT 2

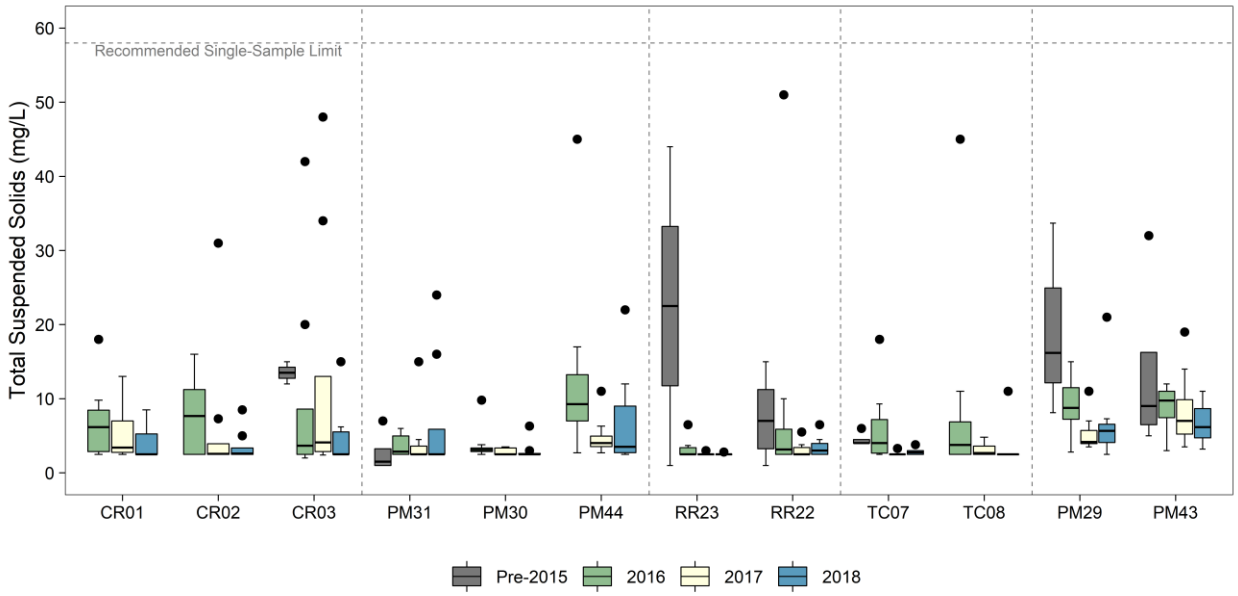
Summary of all data distribution by year (pre-2015, 2015, 2016, 2017, and 2018) for sites ordered from upstream to downstream (vertical gray dashed lines represent tributary inputs to the mainstem). The top and bottom of the box area in each boxplot represent the 75th and 25th percentiles of the data, respectively. The solid horizontal line in each box represents the median or 50th percentile of the data. The top and bottom whiskers represent the maximum and minimum non-outliers of the data, respectively. Any points above or below the whiskers are outliers, defined as 1.5 times the interquartile range (or the length of the box). Single horizontal lines represent only a single data point. Applicable criteria or natural background conditions are shown in red or grey horizontal dashed lines, respectively.




Nitrate + Nitrite (mg/L)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Ecoregion Natural Background
CR01
CR02
CR03
PM31
PM30
PM44
RR23
RR22
TC07
TC08
PM29
PM43
Total Kjeldahl Nitrogen (mg/L)
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
Ecoregion Natural Background
Total Nitrogen (mg/L)
Ecoregion Natural Background
Pre-2015
2016
2017
2018


Orthophosphate (mg/L)
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Ecoregion Natural Background
CR01
CR02
CR03
PM31
PM30
PM44
RR23
RR22
TC07
TC08
PM29
PM43


Total Phosphorus (mg/L)
Outlier of 1.5 mg/L at CR03 not shown
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Ecoregion Natural Background
CR01
CR02
CR03
PM31
PM30
PM44
RR23
RR22
TC07
TC08
PM29
PM43


Total Suspended Solids (mg/L)
Recommended Single-Sample Limit
60
50
40
30
20
10
0
CR01
CR02
CR03
PM31
PM30
PM44
RR23
RR22
TC07
TC08
PM29
PM43
Pre-2015
2016
2017
2018

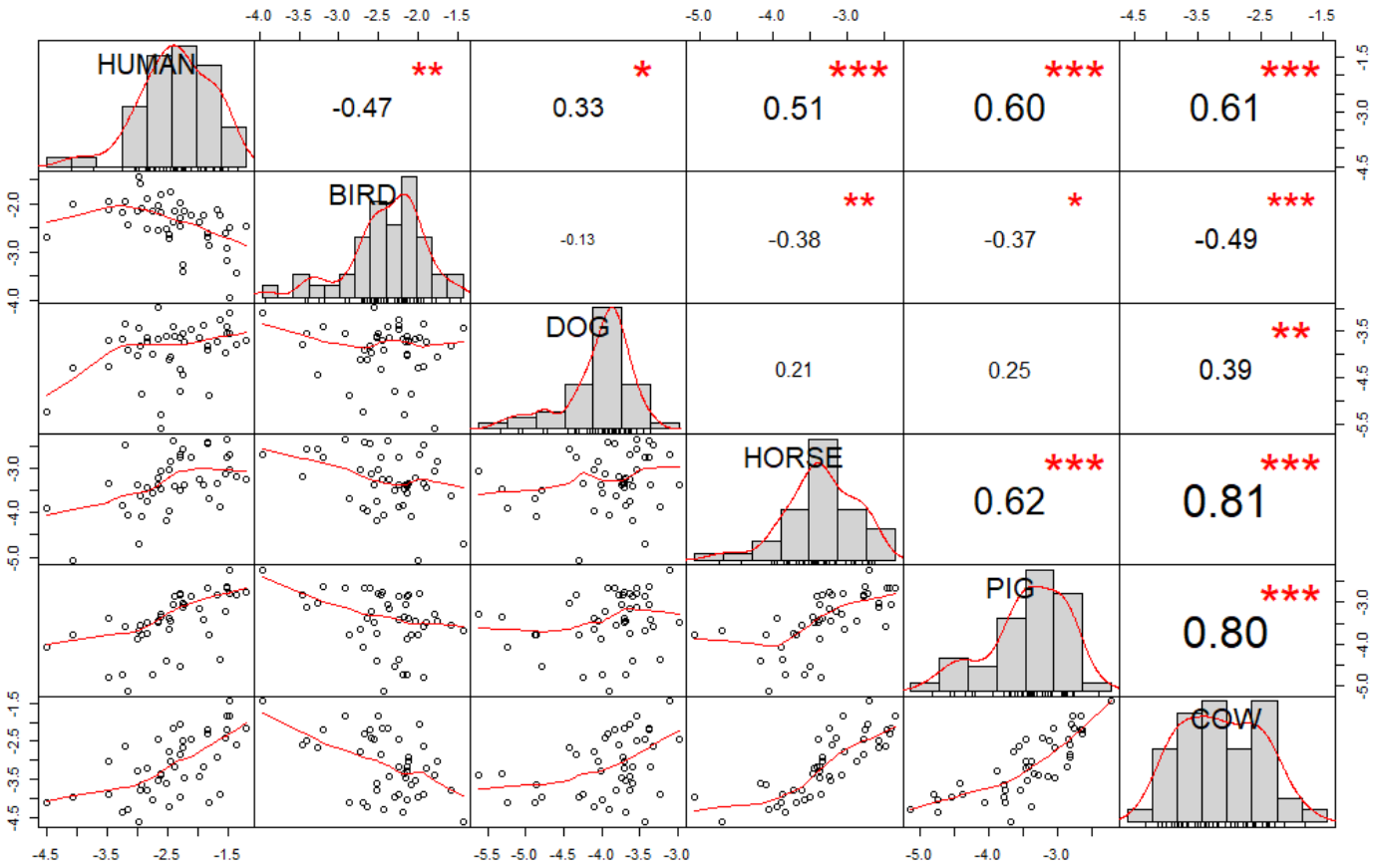
ATTACHMENT 3

Agricultural BMP definitions were adapted from STEPL 4.4 documentation.

BMP	Definition
Terrace	A terrace is an earth embankment, or a combination ridge and channel, constructed across the field slope to enable water to be stored temporarily to allow sediment deposition and water infiltration. This practice is applied as part of a management system to either reduce erosion and trap sediment or retain runoff for moisture conservation.
Prescribed Grazing	Prescribed grazing is the controlled harvest of vegetation with grazing or browsing animals, managed with the intent to maintain or improve water quality and quantity. For example, on grazed forest, native pasture, or rangeland, grazing is limited so that the grazing animals will consume no more than 50 percent (by weight) of the annual growth of high or medium preferred grazing species.
Critical Area Planting	Critical area planting is the planting of grasses, legumes, or other vegetation to stabilize slopes in small, severely eroding areas. The permanent vegetation stabilizes areas such as gullies, over-grazed hillsides and terraced backslopes. Although the primary goal is erosion control, the vegetation can also provide nesting cover for birds and small animals.
Conservation Tillage 2 (equal or more than 60% residue)	Limiting soil disturbance to manage the amount, orientation and distribution of crop and plant residue on the soil surface year-round. This will reduce sheet, rill and wind erosion and excessive sediment in surface waters; reduce tillage-induced particulate emissions; maintain or increase soil health and organic matter content; increase plant-available moisture; reduce energy use; and provide food and escape cover for wildlife.
Diverted Drainage	Capturing runoff from paved surfaces and diverting the flow away from agricultural fields.
Grass Swale	Grass swales are elongated depressions in the land surface that are at least seasonally wet, usually heavily vegetated, and normally without flowing water. Swales direct stormwater flows into primary drainage channels and allow some of the stormwater to infiltrate to the ground. Swales are vegetated with erosion resistant and flood tolerant grasses. Sometimes check dams are strategically placed in swales to moderate flow and an engineered soil mixture might underlie swales.
Litter Storage and Management	Can consist of a manure storage facility, bedded pack, manure composting, etc. Any practice which confines animal litter to an area designed to manage litter via confinement, treatment, or removal.
Livestock Exclusion Fencing	Fencing is used to restrict livestock access to streambanks because animal traffic erodes streambanks, increases sediment load, and contributes animal waste in and near the stream, impairing water quality.
Grass Buffer	A newly established area along a waterbody that intercepts overland flow and is used to maintain bank stabilization, reduce the impacts of upland sources of pollution by trapping, filtering, and converting sediments, nutrients, and other chemicals to supply food, cover and thermal protection to fish and other wildlife. To achieve these results, the recommended minimum width is 35 feet and should include native grass(es).
Use Exclusion	Pasteurized land no longer used for pasture. Land use converted away from pasture. All animals are sold, but the land is not necessarily retired from crop production, development, or regular mowing.
Heavy Use Area Protection	Heavy use area protection is used to stabilize ground surface that is frequently and intensively used by people, animals, or vehicles. Heavy use area protection is used to provide a stable, non-eroding surface and to protect or improve water quality.

ATTACHMENT 4

The correlation matrices shown below for the PhyloChip® pathogen source results (log-transformed to achieve normal distributions) were generated in R statistical programming using the package *PerformanceAnalytics.* The distribution of each variable is shown on the diagonal. The bivariate scatterplots with fitted lines are shown below the diagonal. The value and significance of the correlation are shown above the diagonal. Significance levels by symbol are as follows: "*" = <0.001, "**" = 0.001, "*" = 0.01, "-" = 0.05, " " = >0.05.**



ATTACHMENT 5

Summary statistics for the *E. coli*- and enterococci-derived classifications (true positive, true negative, false positive, and false negative, "TRUFAL") for each source type (human, bird, and cow, "SOURCE") by parameter ("PARA"). Parameters were limited to those that showed statistically significant difference (α = 0.05) among the four classifications and were representative of other similar parameters (e.g., water temperature). Summary statistics include number (n), minimum (min), 25th quantile (lower), mean (mean), median (median), 75th quantile (upper), maximum (max), and standard deviation (sd) of the data.

SOURCE	PARA	TRUFAL	n	min	lower	mean	median	upper	max	sd
ECOLI_BIRD	PRCP_7	False Negative	6	0.46	0.46	0.91	0.65	1.43	1.63	0.57
ECOLI_BIRD	PRCP_7	False Positive	12	0.03	1.63	1.58	1.63	1.92	2.30	0.60
ECOLI_BIRD	PRCP_7	True Negative	11	0.03	0.25	0.77	0.46	1.63	1.63	0.70
ECOLI_BIRD	PRCP_7	True Positive	9	0.03	0.84	0.99	0.84	1.63	1.92	0.71
ECOLI_BIRD	TKN	False Negative	6	0.27	0.31	0.35	0.36	0.40	0.43	0.06
ECOLI_BIRD	TKN	False Positive	12	0.24	0.39	0.50	0.52	0.62	0.74	0.16
ECOLI_BIRD	TKN	True Negative	11	0.26	0.29	0.34	0.35	0.38	0.48	0.06
ECOLI_BIRD	TKN	True Positive	9	0.34	0.41	0.46	0.42	0.48	0.62	0.09
ECOLI_BIRD	WTEMP	False Negative	6	7.82	8.99	13.05	9.56	15.73	24.74	6.75
ECOLI_BIRD	WTEMP	False Positive	12	10.41	19.22	20.55	19.70	23.35	28.67	4.85
ECOLI_BIRD	WTEMP	True Negative	11	5.79	8.42	12.61	9.17	15.87	25.29	6.30
ECOLI_BIRD	WTEMP	True Positive	9	13.51	14.52	17.55	18.25	19.99	22.15	3.44
ECOLI_COW	TKN	False Negative	6	0.35	0.36	0.39	0.39	0.40	0.48	0.05
ECOLI_COW	TKN	False Positive	19	0.24	0.41	0.47	0.42	0.58	0.74	0.14
ECOLI_COW	TKN	True Negative	11	0.26	0.28	0.32	0.30	0.36	0.43	0.06
ECOLI_COW	TKN	True Positive	2	0.52	0.54	0.57	0.57	0.59	0.61	0.06
ECOLI_COW	TSS	False Negative	6	2.50	2.50	4.12	2.50	5.28	8.50	2.61
ECOLI_COW	TSS	False Positive	19	2.50	2.50	4.21	2.50	4.15	15.00	3.33
ECOLI_COW	TSS	True Negative	11	2.50	2.50	2.50	2.50	2.50	2.50	0.00
ECOLI_COW	TSS	True Positive	2	3.00	3.88	4.75	4.75	5.63	6.50	2.47
ECOLI_COW	WTEMP	False Negative	6	7.82	8.17	13.57	11.08	16.97	25.29	7.03
ECOLI_COW	WTEMP	False Positive	19	10.41	14.94	18.89	19.50	21.76	28.67	4.38
ECOLI_COW	WTEMP	True Negative	11	5.79	8.86	12.32	9.39	14.67	24.74	6.11
ECOLI_COW	WTEMP	True Positive	2	19.26	21.05	22.83	22.83	24.62	26.40	5.05
ECOLI_HUMAN	PRCP_1	False Negative	11	0.00	0.00	0.02	0.00	0.00	0.26	0.08
ECOLI_HUMAN	PRCP_1	False Positive	9	0.00	0.00	0.06	0.00	0.00	0.52	0.17
ECOLI_HUMAN	PRCP_1	True Negative	6	0.00	0.00	0.00	0.00	0.00	0.00	0.00
ECOLI_HUMAN	PRCP_1	True Positive	12	0.00	0.00	0.22	0.03	0.52	0.52	0.26
ECOLI_HUMAN	TKN	False Negative	11	0.28	0.34	0.37	0.38	0.39	0.48	0.05
ECOLI_HUMAN	TKN	False Positive	9	0.32	0.41	0.46	0.42	0.48	0.65	0.11
ECOLI_HUMAN	TKN	True Negative	6	0.26	0.27	0.31	0.29	0.32	0.43	0.06
ECOLI_HUMAN	TKN	True Positive	12	0.24	0.41	0.50	0.52	0.60	0.74	0.15
ECOLI_HUMAN	WTEMP	False Negative	11	5.79	8.22	11.13	8.85	11.43	25.29	5.77
ECOLI_HUMAN	WTEMP	False Positive	9	13.51	14.52	18.07	15.11	21.77	26.40	4.77
ECOLI_HUMAN	WTEMP	True Negative	6	9.39	10.20	15.76	14.67	20.45	24.74	6.48
ECOLI_HUMAN	WTEMP	True Positive	12	10.41	19.14	20.17	19.70	21.84	28.67	4.21
ENTERO_BIRD	FOR_PER	False Negative	9	0.69	0.69	0.70	0.70	0.73	0.73	0.02
ENTERO_BIRD	FOR_PER	False Positive	8	0.31	0.49	0.57	0.59	0.69	0.73	0.15
ENTERO_BIRD	FOR_PER	True Negative	12	0.31	0.31	0.50	0.49	0.69	0.73	0.17
ENTERO_BIRD	FOR_PER	True Positive	9	0.31	0.69	0.63	0.69	0.69	0.73	0.14
ENTERO_BIRD	TP	False Negative	9	0.02	0.04	0.05	0.05	0.06	0.09	0.02
ENTERO_BIRD	TP	False Positive	8	0.04	0.05	0.06	0.06	0.07	0.10	0.02
ENTERO_BIRD	TP	True Negative	12	0.01	0.03	0.03	0.03	0.04	0.07	0.01
ENTERO_BIRD	TP	True Positive	9	0.02	0.07	0.06	0.07	0.07	0.08	0.02
ENTERO_BIRD	URB_PER	False Negative	9	0.20	0.20	0.21	0.21	0.22	0.22	0.01
ENTERO_BIRD	URB_PER	False Positive	8	0.20	0.21	0.31	0.28	0.33	0.65	0.15
ENTERO_BIRD	URB_PER	True Negative	12	0.20	0.22	0.40	0.33	0.65	0.65	0.19
ENTERO_BIRD	URB_PER	True Positive	9	0.20	0.21	0.28	0.22	0.22	0.65	0.14
ENTERO_COW	DAYS_SINCE	False Negative	5	1.00	1.00	1.80	1.00	2.00	4.00	1.30
ENTERO_COW	DAYS_SINCE	False Positive	14	1.00	2.00	3.79	2.00	5.00	10.00	2.94
ENTERO_COW	DAYS_SINCE	True Negative	16	1.00	2.00	4.94	4.00	6.25	14.00	3.91

SOURCE	PARA	TRUFAL	n	min	lower	mean	median	upper	max	sd
ENTERO_COW	DAYS_SINCE	True Positive	3	5.00	5.00	6.67	5.00	7.50	10.00	2.89
ENTERO_COW	TP	False Negative	5	0.02	0.03	0.04	0.03	0.04	0.07	0.02
ENTERO_COW	TP	False Positive	14	0.02	0.05	0.06	0.07	0.07	0.08	0.02
ENTERO_COW	TP	True Negative	16	0.01	0.03	0.04	0.04	0.06	0.09	0.02
ENTERO_COW	TP	True Positive	3	0.04	0.05	0.07	0.06	0.08	0.10	0.03
ENTERO_HUMAN	TP	False Negative	10	0.01	0.03	0.04	0.04	0.05	0.07	0.02
ENTERO_HUMAN	TP	False Positive	9	0.05	0.06	0.07	0.07	0.07	0.08	0.01
ENTERO_HUMAN	TP	True Negative	11	0.02	0.03	0.04	0.04	0.05	0.09	0.02
ENTERO_HUMAN	TP	True Positive	8	0.02	0.04	0.06	0.06	0.07	0.10	0.02

ATTACHMENT 6

Summary statistics for presence ("Signal") or absence ("No Signal") of a source type (human, bird, cow, or all, "SOURCE") by parameter ("PARA"). Parameters were limited to those that showed statistically significant difference ($\alpha = 0.05$) between signal and no signal. Summary statistics include number (n), minimum (min), 25th quantile (lower), mean (mean), median (median), 75th quantile (upper), maximum (max), and standard deviation (sd) of the data.

SOURCE	PARA	SIG	n	min	lower	mean	median	upper	max	sd
ALL	NITRATE	Signal	43	0.02	0.16	0.33	0.22	0.40	1.90	0.37
ALL	NITRATE	No Signal	7	0.02	0.03	0.12	0.04	0.05	0.62	0.22
HUMAN	AREA_AC	Signal	25	68	1,036	6,246	1,341	6,794	30,523	9,109
HUMAN	AREA_AC	No Signal	25	68	1,036	13,836	21,262	21,900	30,523	12,460
BIRD	AREA_AC	Signal	23	68	1,189	14,187	21,262	21,900	30,523	11,702
BIRD	AREA_AC	No Signal	27	68	302	6,509	1,341	6,794	30,523	10,176