A Multi-Tracer Approach to the Investigation of Human Wastewater Contamination in Bozeman and Matthew Bird Creeks

Summary Report

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1.0 Executive Summary

In the fall of 2018 and spring of 2019, Montana State University faculty and staff from the Gallatin County Environmental Health Department and the Gallatin Local Water Quality District undertook a project to further the understanding of potential fecal contamination in Bozeman and Matthew Bird Creeks and other streams flowing through the urban areas of Bozeman, Montana.

Previous studies indicated the presence of human-sourced fecal contamination in these streams on a reach-wide scale, but did not employ sampling designs sufficient to connect results to potential sources. Sampling locations for this project bracketed areas along both creeks served by individual septic systems that had no associated septic permits or were reaching the end of the typical 25-30 year lifespan.

In light of the diagnostic limitations of *E. coli* enumeration and the high costs of microbial source tracking methods used in previous studies, this project evaluated methods that detect other human wastewater tracers. Optical brighteners (OBs) are a common additive to laundry detergents. They adhere to cotton and fluoresce when viewed under ultraviolet light. Deploying cotton pads in-stream and viewing them under ultraviolet light provides a simple and low-cost way to screen for domestic wastewater. OBs can also be discerned from other fluorescing compounds via their unique rate of photo decay using spectrophotometry, which was also employed. Grab samples were also analyzed by mass spectrometry for OBs, caffeine, and other compounds indicative of human wastewater.

This report details the results of efforts to determine if concentrations of *E. coli*, OBs, and other potential tracers as identified through mass spectrometry correlate spatially with suspected areas of septic contamination. Samples were collected from sites bracketing these areas of suspected contamination with the intention of comparing the results with septic permitting records on file with the Environmental Health Division of the Gallatin City County Health Department.

Results showed that the methods used were not successful in detecting conclusive evidence of human wastewater influence. Mass spectrometry analysis found elevated concentrations of bile salt compounds that indicate human wastewater influence is likely, but did not point to a probable source. Dried OB pads warrant further testing using a wider range of time-integrated sampling and a deployment apparatus that protects them from photo decay.

With the exception of one stormwater outfall, *E. coli* concentrations did not exceed the higher numeric standard set by the Montana Department of Environmental Quality (DEQ) for colder months (November – March) when primary contact activities, such as swimming, are assumed to be minimal. Results suggest that both creeks likely exceed the lower concentrations outlined in the April-October standards, but *E. coli* enumeration is not recommended for use in detecting septic influence in streams and rivers, due to the natural variability of *E. coli* concentrations in surface water systems, and the non-specificity of this bacteria to human sources.

2.0 Introduction

2.1 Project Background

Bozeman Creek, also known as Sourdough Creek, originates in the mountains south of the City of Bozeman in Gallatin County, Montana. Flowing north through a rural area of larger (30-40 acre) parcels, the creek crosses the city limits into the urban area of Bozeman where it merges with Limestone, Nash

Spring, Matthew Bird, and other small creeks before flowing into the East Gallatin River north of town **(Figure 1)**. Although the creek is flanked by parks and open space, many stretches are adjacent to homes with aging septic systems and areas where culverts and bank armoring constrict the channel. The creek is also subject to the effects of limited riparian vegetation and inputs of untreated stormwater runoff.

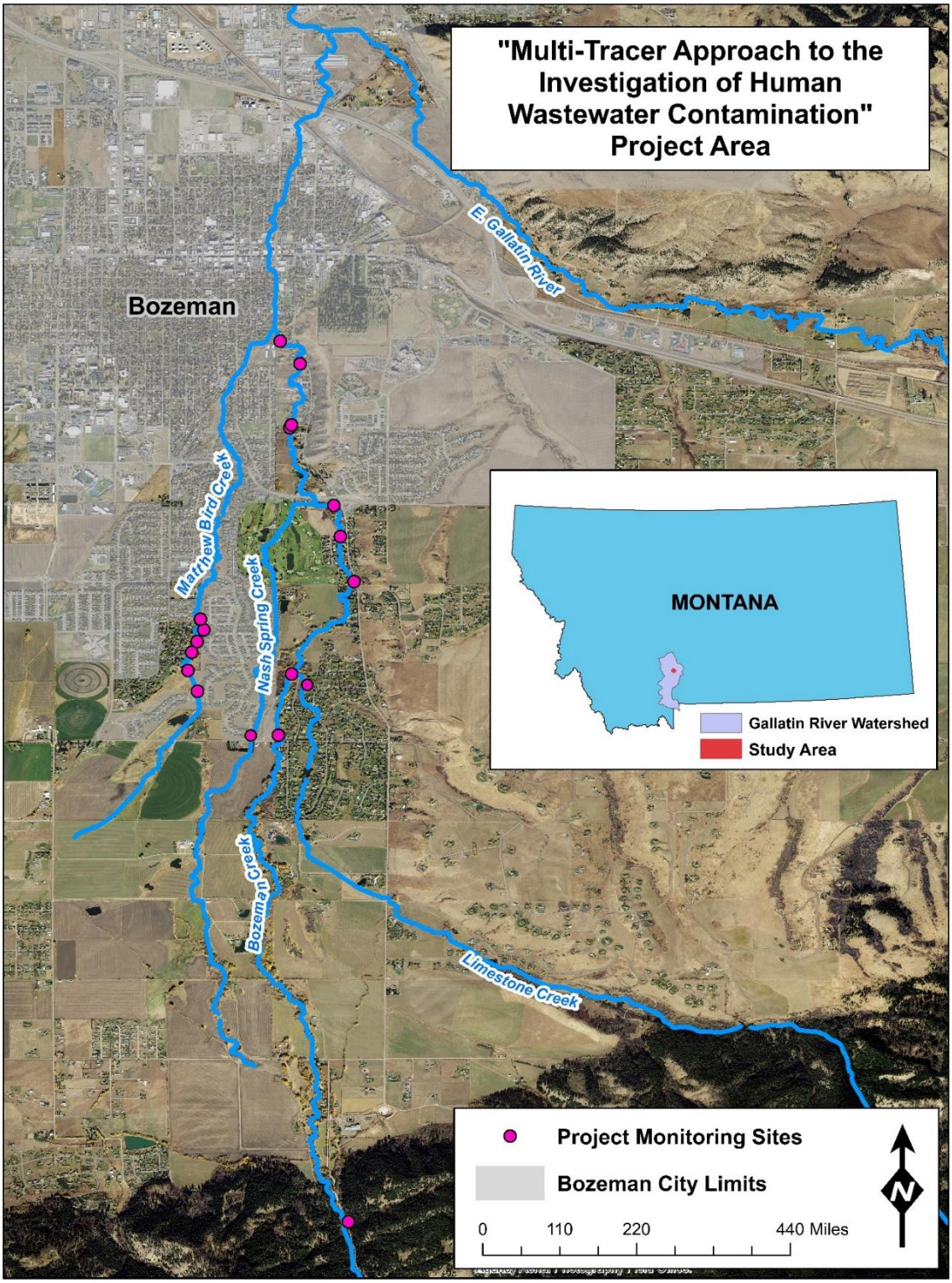

Figure 1. Map of the project study area.

Bozeman Creek is on DEQ's 303(d) list of impaired streams for nitrogen, sedimentation, and *E. coli* bacteria, meaning the results of sampling for these pollutants were found to exceed numeric standards based on the stream's known and anticipated uses. *E. coli* is of concern because its presence indicates fecal contamination, and also suggests that other pathogenic microorganisms might be present. The numeric *E. coli* standard for Bozeman Creek set by DEQ varies seasonally. From April 1 through October 31, the geometric mean may not exceed 126 colony forming units (cfu) per 100 milliliters and 10 percent of the total samples may not exceed 252 cfu per 100 milliliters during any 30-day period. From November 1 through March 31, the geometric mean may not exceed 630 cfu per 100 milliliters and 10 percent of the samples may not exceed 1,260 cfu per 100 milliliters during any 30-day period. Sources of *E. coli* contamination in Bozeman Creek identified by DEQ include stormwater inputs, pet and livestock waste, and septic systems.

Since the *E. coli* impairment listing by DEQ in 2006, several studies by Montana State University (MSU) Extension Water Quality, the City of Bozeman Stormwater Division, and the Gallatin Local Water Quality District (GLWQD) have sought to determine the sources of fecal contamination in Bozeman Creek (MSUEWQ, 2013), (Crone, 2014), (Wilson, 2015). These efforts employed *E. coli* monitoring, and in 2014 and 2015, microbial source tracking (MST) was also utilized. MST uses comparison to known host-specific bacterial gene sequences to identify the source of microbial contamination (human, dog, etc.). These studies confirmed *E. coli* impairment in Bozeman Creek. They also identified Matthew Bird Creek, a tributary to Bozeman Creek, as one likely source of *E. coli* loading to Bozeman Creek, and determined that human sources are a major contributor of fecal contamination in both stream systems.

While both methods are effective in identifying fecal contamination, both have associated limitations. *E. coli* sampling alone has no diagnostic power to identify the source of fecal contamination. Further, the natural variability in concentrations makes comparisons between individual grab samples or to the water quality standard challenging (Muirhead and Meenken, 2018). While MST has utility for source identification, use of the method at the spatial scale necessary to pinpoint sources can be cost-prohibitive. The results of both methods can be affected by external influences on a dynamic stream system, such as rainfall and stormwater runoff (Gilfillan et. al., 2018).

The results of the 2014 MST analysis drew attention to the potential human sources of contamination in Bozeman Creek and its tributary, Matthew Bird Creek. Of particular interest were areas of modest-sized (approximately 1 acre) lots along both creek corridors that are currently outside the city limits of Bozeman and are served by individual septic systems (ISS Areas). The Gallatin City-County Health Department Environmental Health Services (EHS) conducted an inventory of septic permits for lots within 30 meters of both creeks and found that about 13% of lots had no associated septic permits, indicating the systems were installed prior to 1966 or have otherwise not been verified to meet state standards (Welsh et. al., 2018). In addition, they found that 67% of permitted systems were installed before 1992, indicating these systems are at or near the end of the typical 25-30 year lifespan, and therefore less likely to be treating waste properly.

In 2018, MSU faculty, and staff from EHS and GLWQD met to plan a collaborative project to further the understanding of potential septic contamination in Bozeman and Matthew Bird Creeks. In light of the diagnostic and cost limitations of the methods used in previous studies, evaluation of methods that detect other indicators of human wastewater (tracers) was identified as a priority.

Optical brighteners (OBs) are a class of white fluorescent compounds that are common additives to laundry detergents and household paper products to enhance whitening. OBs are not naturally found in the environment and decompose slowly, except through photo decay, so they are useful as indicators of wastewater contamination in the environment (Burres, 2011). OBs have been used by state and local agencies, universities, and citizen science groups to detect sewer cross-connections to storm drains, leaking sewer pipes, and failing septic systems. The materials required to test for OBs via absorption to simple cotton pads are easily acquired, simple to use, and inexpensive. However, detection is a semi-quantitative process of comparing samples to "standards" made by soaking identical pads in solutions of known OB concentration. This method does not allow for OBs to be discerned from other fluorescing compounds naturally occurring in stream systems, such as leaf tannins. However, spectrophotometry can be used to distinguish OBs from other fluorescing compounds via unique rates of decay under UV exposure (Cao et al., 2009).

OBs can also be detected at lower levels in water samples via spectrophotometry. The primary limitation of this method is its use of instantaneous grab sampling, which doesn't allow for accumulation to detectable levels in stream systems with low OB concentrations. The appropriate equipment for spectrophotometry was available in the MSU Center for Biofilm Engineering Laboratory. A Big Sky Watershed Corps member and an EHS intern, under the supervision of EHS and GLWQD staff and MSU Microbiology and Immunology faculty, were assigned to this aspect of the project.

Mass spectrometry (mass spec) can be used to detect multiple human wastewater-specific tracers in stream water. This method involves analysis using a meter that sorts the molecules present in the sample based on their unique electrical and magnetic signatures, and then provides a readout that shows the relative abundance of molecules of different masses. The molecules are then identified through comparison to reference values. Spectrometry-based untargeted metabolomics was used for this study. An untargeted approach attempts to detect any compounds unique to human wastewater, rather than focusing on a specific contaminant. Untargeted mass spec has advantages when exploratory detection of a broad category of compounds is the goal. Untargeted mass spec is often used as a precursor to targeted analysis, which can be used to detect predetermined compounds at lower concentrations. Previous applications of mass spec have detected human wastewater-specific compounds such as caffeine and its metabolites, as well as common food additives and pharmaceuticals in surface water. A Montana IDeA Network of Biomedical Research Excellence (INBRE) grant provided support for the cost of untargeted analysis, and an INBRE intern, under the supervision of MSU Proteomics, Metabolomics and Mass Spec Laboratory faculty, was assigned to this aspect of the project.

2.2 Project Goals

The project had the following goals:

1. To determine if tracer (*E. coli*, OBs, mass spec indicators [e.g. caffeine]) concentrations correlate spatially with suspected areas of septic contamination identified in the EHS septic inventory.
2. To encourage student engagement with MSU's Environmental Health and INBRE programs.
3. To use results to inform Education & Outreach and/or potential regulatory decisions related to human wastewater contamination in Bozeman and Matthew Bird Creeks.

3.0 Sampling Design

3.1 Site Selection

In Sampling Event 1, thirteen initial monitoring sites on Bozeman and Matthew Bird Creeks were chosen to bracket the ISS Areas along both creek corridors. One site on Bozeman Creek in Sourdough Canyon, upstream of all development, was monitored as a control **(Figures 2 & 3, Table 1)**. In Sampling Event 2, two more sites (StormDrain-EL and MTWB-Trib) were added after they were discovered during Sampling Event 1. The results of Sampling Events 1 and 2 led to questions about inputs from Nash Spring and Limestone Creeks **(Figure 4).** Sampling was limited to bacterial sampling and OB pad deployment on these tributaries and the control site by a single student for Sampling Events 3 and 4.

Table 1. Project site names, descriptions, and locations.

	Site Name	Site Description	LAT	LONG
Bozeman Ck.	BC-Canyon	Bozeman Creek Canyon, Control Site	45.590734	-111.025367
	BOZMC03	Tuckerman Park @ Goldenstein	45.635337	-111.031792
	BOZMC03a	Gardner Park @ footbridge	45.640947	-111.030582
	BOZMC-MDWLK4	Upstream Meadowlark	45.649431	-111.024849
	BOZMC-MDWLK2	Midstream Meadow lark	45.653557	-111.026104
	BOZMC-KagyPark	Between Kagy and Candy Lane	45.656401	-111.026699
	StormDrain-EL*	E. Lincoln Storm Drain	45.663524	-111.030747
	BOZMC-ELCTS3	E. Lincoln Street	45.663787	-111.030593
	BOZMC-IPRD	Ice Pond Road	45.669401	-111.029801
	BOZMC-ELCTHTS1	S of Gallagator Trail	45.671472	-111.031617
Matthew Bird Ck.	MTWBC02	Trailhead @ Peacepipe Drive	45.639375	-111.039213
	MTWB-xing	Trail @ creek crossing	45.641273	-111.040097
	MTWB-SD3322	3322 Sundance Drive	45.642941	-111.039739
	MTWB-SD3318	3318 Sundance Drive	45.643938	-111.039246
	MTWB-Trib*	Tributary to MTWB @ Culvert	45.645006	-111.038633
	MTWB-SW106	Downstream of Sourdough Creek Subdivision	45.645982	-111.038938
Other	LIMC-GPARK	Limestone Creek near mouth @ Gardner Park	45.641259	-111.030270
	SPRNGC-TUCK	Nash Spring Creek @ Tuckerman Park	45.635115	-111.033850

* = sites discovered during first monitoring event and added for second event.

3.2 Sampling Schedule

OBs have a very strong affinity for cotton, therefore all of the OBs that have passed through a cotton pad (see Methods section below) at the time of pad removal from the stream should have adsorbed to the pad. Because of this, a time-integrated schedule of 3-, 7-, and 10-day deployment periods was used in Sampling Events 1 and 2 to allow for more sensitive detection of OB levels, and semi-quantitative fluorescence observations (i.e. 'Absent', 'Present', 'Highly Present') were used for assessment (see **Appendix A**). This time-integrated deployment schedule was also anticipated to help determine the appropriate deployment period for the method if it was used in future work on streams in this area. Based on the results of Sampling Events 1 and 2, a time-integrated schedule of 9-, 14 or 21-, and 23-day deployment periods was used in Sampling Events 3 and 4.

Wastewater inputs are often associated with higher temperature, conductivity, total dissolved solids and a lower dissolved oxygen in a cold stream system. Although large deviations indicative of concentrated wastewater contamination were unlikely to occur, these data were deemed potentially useful in interpretation of the other parameters' results and collected at each site visit during Sampling Events 1 and 2, and once per event during Sampling Events 3 and 4.

Figure 2. Map of sampling sites on Bozeman Creek.

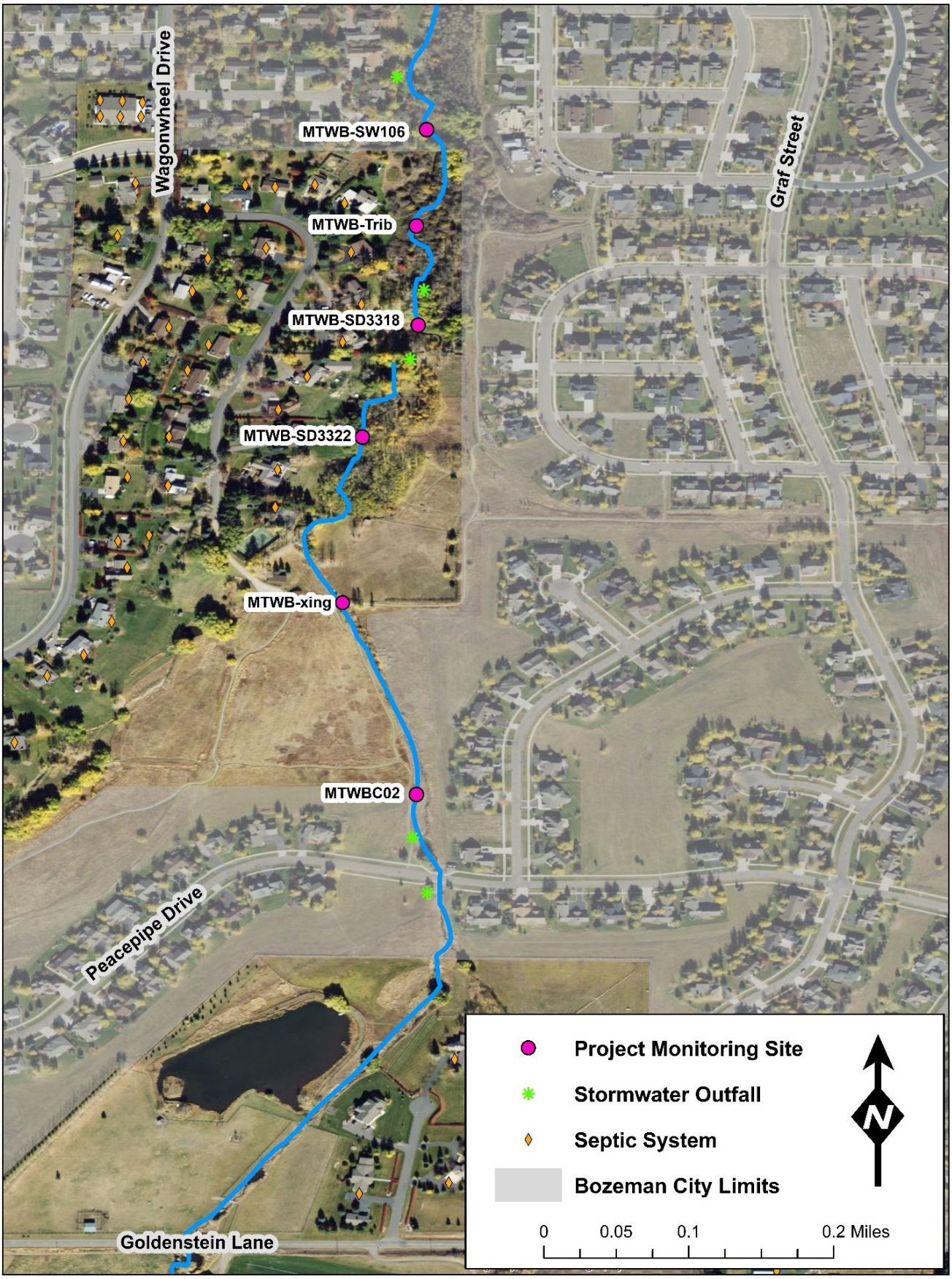

Figure 3. Map of sampling sites on Matthew Bird Creek.

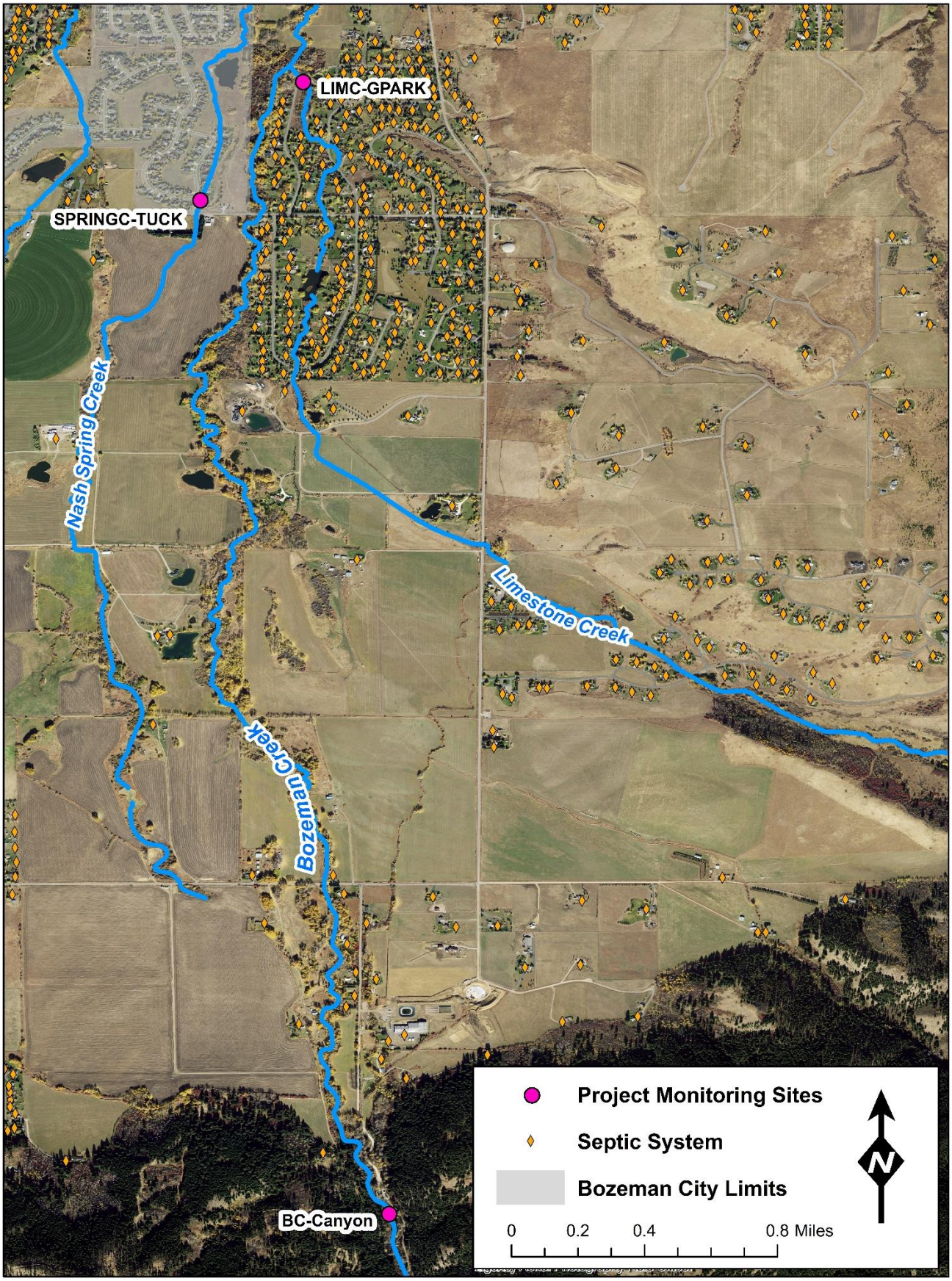

Figure 4. Map of sampling sites for Sampling Events 3 and 4.

Sampling Events 1 and 2 were 10-day sampling events consisting of four field days, occurring September 22-October 2, 2018 and October 20-30, 2018. The sampling schedule for these events is outlined in **Table 2**. Sampling Events 3 and 4 were limited to bacteria grab sampling and 21-day OB-pad deployment events consisting of four field days, occurring February 2-21, 2019 and February 28-March 14, 2019. The sampling schedule for these events is outlined in **Table 3**.

Table 2. Schedule for Sampling Events 1 and 2.

Field Day	Actions
Day 0	Deploy three sets of OB pads; collect instream field parameters; complete site visit form
Day 3	Retrieve first OB pad; collect instream field parameters; collect OB water grab sample; measure stream flow*; complete site visit form
Day 7	Retrieve second OB pad; collect instream field parameters; collect OB water grab sample; collect bacteria grab sample; collect wastewater tracers grab sample; measure stream flow*; complete site visit form
Day 10	Retrieve third OB pad; collect instream field parameters; collect OB water grab sample; measure stream flow*; complete site visit form

*= Flow measured only as indicated in Section 5.4.

Table 3. Schedule for Sampling Events 3 and 4.

Field Day	Actions
Day 0	Deploy three sets of OB pads; complete field notebook
Day 9	Retrieve first OB pad; complete field notebook
Day 14 (Event 3) or Day 21 (Event 4)	Retrieve second OB pad; collect instream field parameters; collect bacteria grab sample; complete field notebook
Day 23	Retrieve third OB pad; complete field notebook

4.0 Methods

The Standard Operating Procedures in **Appendix A** provide a detailed outline all of the methods used for data collection in the field and laboratory associated with this project. Deviations from these procedures are outlined below **(Tables 4 & 5)**.

4.1 Optical Brighteners – Absorption

Mesh bags containing three OB pads tied to metal stakes and deployed at all project sites **(Figure 5)**. One pad was collected from each site, taking care to minimize UV exposure, according to the schedule for that sampling event **(Tables 2 and 3)**. Wet pads were photographed, compared under ultraviolet light to pads exposed to OBs of known concentration, and recorded as “absent”, “present” and “highly-present” **(Figure 6)**. Pads were then allowed to dry at room temperature before being photographed and compared to standards again. Pads deployed during Sampling Event 1 were placed in the poly-net bag and zip tied to the stake **(right photo, Figure 5)**. When dried OB pads were found to fluoresce only at their edges **(Figure 6)**, pads were stretched over the stake to optimize flow through them for Sampling Events 2, 3 and 4 **(left photo, Figure 5)**. However, this method change did not produce different fluorescence patterns.

Figure 5. OB pad deployment setup for Sampling Event 1 (left) and as modified for Sampling Event 2 (right).

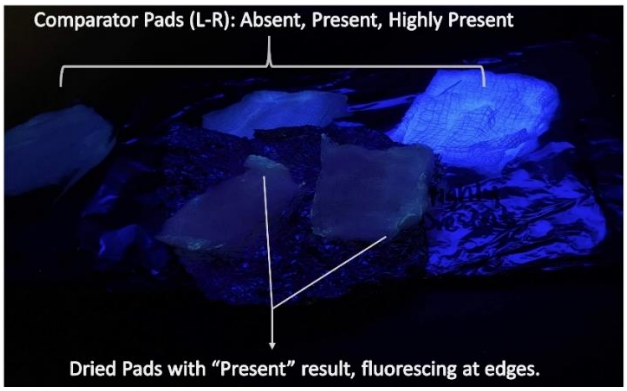

Figure 6. Dried OB spectrophotometry comparator pads (top) and retrieved pads fluorescing at edges (bottom).

4.2 Optical Brighteners – Spectrophotometry

Spectrophotometry samples were collected through grab sampling. The grab samples were processed within a day of sample collection using a Synergy Hybrid Reader. The reader was calibrated with a dilution series of four standards that spanned the range of concentrations expected at project sites. Equipment trays were loaded with 200µl of sample in each of three wells per site and results for each replicate were recorded. Sample trays were then exposed to UV light and read again, as the decay rate of fluorescence is unique to OBs.

4.3 *E. coli* Bacteria

E. coli bacteria samples were collected through grab sampling. Grab samples were processed using IDEXX's Colilert Quanti-Tray 2000 system, an EPA-approved method for estimating 'most probable number' (mpn) of coliform bacteria and *E. coli* in water samples, which is comparable to the colony forming units (cfu) used in DEQ standards. 100ml grab samples were mixed with a packet of IDEXX colilert media powder, poured into IDEXX trays, sealed, and incubated at 35°C incubator for 24 hours. Results were read after incubation by counting the wells in each tray that were yellow (indicating the presence of coliform bacteria), then by counting the wells in each try that fluoresced under UV light (indicating the presence of *E. coli* bacteria). Well counts are compared to a reference chart to obtain the mpn result.

4.4 Streamflow

Streamflow was measured using the velocity-area method with a Marsh-McBirney FloMate meter and topsetting rod. A measuring tape was strung across the channel and used to determine wetted width. This width was then divided into a minimum of 20 sections, and the topsetting rod used to determine the depth in each section. Section width (via tape reading), depth and velocity measurements were recorded on a data sheet, which was later transferred to a spreadsheet that uses these numbers to calculate streamflow in cubic feet per second (cfs).

4.5 Instream Field Parameters (YSI)

Instream field parameters; pH, dissolved oxygen (DO), water temperature, and conductivity; were measured using a YSI 556 multimeter probe, calibrated by GLWQD staff prior to daily use per the manufacturer's instructions. The meter was placed in the stream above other activities that might cause disturbance, and allowed to equilibrate before data were recorded.

4.6 Mass Spectrometry

The wastewater tracer grab samples were processed and analyzed at Montana State University's Mass Spectrometry Facility. Following sample collection in 1L amber glass bottles, samples were preserved with 1mL of 1% formic acid and pre-concentrated using solid phase extraction. Concentrated samples were then analyzed using liquid chromatography–mass spectrometry.

4.7 Deviations from Field Methods

Deviations from the field methods outlined in the Standard Operating Procedures (Appendix A) are summarized in **Table 4**. It is important to note that a simplified monitoring schedule was adopted for Sampling Events 3 and 4, but a new SOP was not created, and those modifications are reflected as deviations for those events.

*Table 4. Summary of field method deviations from those in the Standard Operating Procedures (Appendix A). *A simplified monitoring schedule was adopted for Sampling Events 3 and 4, but a new SOP was not created. Therefore, those modifications are reflected as deviations in this table.*

	DATE	SITE	DEVIATION
Sampling Event 1	DAY 0 - 9/22/2018	BOZMC-SRDGH1	Site Visit Form completed - site not on list
	DAY 0 - 9/22/2018	BOZMC-SRDGH2	Site Visit Form completed - site not on list
	DAY 3 - 9/25/2018	BOZMC-ELCTS3	Flow not measured - insufficient time
	DAY 3 - 9/25/2018	BOZMC-ELCTHTS1	OB pad apparatus was not found
Sampling Event 2	DAY 0 - 10/20/2018	All Sites	Duplicate pads deployed stretched
	DAY 7 - 10/27/2018	BC-Canyon	OB pad apparatus was not found
	DAY 7 - 10/27/2018	MTWB-Trib	Mass spec and bacteria samples not collected
	DAY 10 - 10/30/2018	BC-Canyon	OB pad apparatus was not found
Sampling Event 3*	DAYS 3+	All Sites	OB pad deployment schedule extended to 9-23 days
Sampling Event 4*	DAYS 3+	All Sites	OB pad deployment schedule extended to 9-23 days

4.8 Deviations from Laboratory Methods

Deviations from the laboratory methods outlined in the Standard Operating Procedures (Appendix A) are summarized in **Table 5**.

Table 5. Summary of lab method deviations from those in the Standard Operating Procedures (Appendix A).

DATE	SITE	METHOD	DEVIATION
10/29/2018 (Day 7, Event 1)	BOZMC-S (803 HOUSE)	OB Pad	Not a site but lab data recorded - determined to be BOZMC-ELCTHTS1
10/2/2018 (Day 10, Event 1)	BOZMC-ELCTHTS1	OB Pad	No data was recorded
10/30/2018 (Day 10, Event 2)	BOZMC-ELCTHTS1	OB Pad	No data was recorded

5.0 Results

5.1 Optical Brighteners – Absorption

No OBs were found to be present on wet pads in Sampling Events 1 or 2 **(Table 6)**. The dried pads from Days 3 and 10 of Sampling Event 1 indicated the presence of OBs at all sites on Bozeman Creek except BOZMC-ELCTS3, and from a single site (MTWB-SD3318) on Matthew Bird Creek. The dried pads from Day 10 of Sampling Event 2 indicated the presence of OBs at five sites on Bozeman Creek and from three sites on Matthew Bird Creek. Detections appeared as fluorescence at the edges of the pads, rather than throughout the pad fabric **(Figure 6)**.

Because no OBs were found to be present on wet pads in Sampling Events 1 or 2, only dry pads were analyzed during Sampling Events 3 and 4 **(Table 7)**. The dried pads from all days indicated the presence of OBs at all sites, including the control site.

Table 6. Optical brightener (OB) results from the absorption method for Sampling Events 1 and 2. Sites are shown upstream to downstream for each creek.

		Sampling Event 1						Sampling Event 2					
		DAY 3 - 9/25/2018		DAY 7 - 9/29/2018		DAY 10 - 10/2/2018		DAY 3 - 10/23/2018		DAY 7 - 10/27/2018		DAY 10 - 10/30/2018	
	Site Name	WET	DRY	WET	DRY	WET	DRY	WET	DRY	WET	DRY	WET	DRY
Bozeman Ck.	BC-Canyon	ABSENT	PRESENT	ABSENT	ABSENT	ABSENT	PRESENT	ABSENT	ABSENT	PAD LOST	PAD LOST	PAD LOST	PAD LOST
	BOZMC03	ABSENT	PRESENT	ABSENT	ABSENT	ABSENT	PRESENT	ABSENT	ABSENT	ABSENT	ABSENT	ABSENT	ABSENT
	BOZMC03a	ABSENT	PRESENT	ABSENT	ABSENT	ABSENT	PRESENT	ABSENT	ABSENT	ABSENT	ABSENT	ABSENT	PRESENT
	BOZMC-MDWLK4	ABSENT	PRESENT	ABSENT	ABSENT	ABSENT	PRESENT	ABSENT	ABSENT	ABSENT	ABSENT	ABSENT	PRESENT
	BOZMC-MDWLK2	ABSENT	PRESENT	ABSENT	ABSENT	ABSENT	PRESENT	ABSENT	ABSENT	ABSENT	ABSENT	ABSENT	PRESENT
	BOZMC-KagyPark	ABSENT	PRESENT	ABSENT	ABSENT	ABSENT	PRESENT	ABSENT	ABSENT	ABSENT	ABSENT	ABSENT	ABSENT
	StormDrain-EL	NOT SAMPLED						ABSENT	ABSENT	ABSENT	ABSENT	ABSENT	PRESENT
	BOZMC-ELCTS3	ABSENT	ABSENT	ABSENT	ABSENT	ABSENT	ABSENT	ABSENT	ABSENT	ABSENT	ABSENT	ABSENT	ABSENT
	BOZMC-IPRD	ABSENT	PRESENT	ABSENT	ABSENT	ABSENT	PRESENT	ABSENT	ABSENT	ABSENT	ABSENT	ABSENT	PRESENT
	BOZMC-ELCTHTS1	PAD LOST	PAD LOST	ABSENT	ABSENT	NO DATA	NO DATA	ABSENT	ABSENT	ABSENT	ABSENT	NO DATA	NO DATA
Matthew Bird Ck.	MTWBC02	ABSENT	ABSENT	ABSENT	ABSENT	ABSENT	ABSENT	ABSENT	ABSENT	ABSENT	ABSENT	ABSENT	PRESENT
	MTWB-xing	ABSENT	ABSENT	ABSENT	ABSENT	ABSENT	ABSENT	ABSENT	ABSENT	ABSENT	ABSENT	ABSENT	ABSENT
	MTWB-SD3322	ABSENT	ABSENT	ABSENT	ABSENT	ABSENT	ABSENT	ABSENT	ABSENT	ABSENT	ABSENT	ABSENT	ABSENT
	MTWB-SD3318	ABSENT	PRESENT	ABSENT	ABSENT	ABSENT	PRESENT	ABSENT	ABSENT	ABSENT	ABSENT	ABSENT	PRESENT
	MTWB-Trib	NOT SAMPLED						ABSENT	ABSENT	ABSENT	ABSENT	ABSENT	ABSENT
	MTWB-SW106	ABSENT	ABSENT	ABSENT	ABSENT	ABSENT	ABSENT	ABSENT	ABSENT	ABSENT	ABSENT	ABSENT	PRESENT

Table 7. Optical brightener (OB) results from the absorption method for Sampling Events 3 and 4. Sites are shown upstream to downstream for each creek.

	Sampling Event 3			Sampling Event 4		
	DAY 9 - 2/7/2019	DAY 14 - 2/12/2019	DAY 23 - 2/21/2019	DAY 9 - 2/28/2019	DAY 21 - 3/12/2019	DAY 23 - 3/14/2019
	DRY	DRY	DRY	DRY	DRY	DRY
BC-Canyon	PRESENT	PRESENT	PRESENT	PRESENT	PRESENT	PRESENT
LIMC-GPARK	PRESENT	PRESENT	PRESENT	PRESENT	PRESENT	PRESENT
SPRINGC-TUCK	PRESENT	PRESENT	PRESENT	PRESENT	PRESENT	PRESENT

5.2 Optical Brighteners – Spectrophotometry

No positive OB spectrophotometry results were found in Sampling Event 1 **(Table 8)**. A single positive result was found during Sampling Event 2 at the most downstream site on Bozeman Creek (BOZMC-ELCTHTS1) on Day 3. A single negative result was found during Sampling Event 2 at the East Lincoln storm drain site on Bozeman Creek (StormDrain-EL) on Day 3 because the sample fluoresced, but its decay rate did not indicate an OB source **(Appendix A, Section 2.2, Sample Analysis Step 8)**. Several Day 3 results from Sampling Event 2 were found to be inconclusive because two of three sample replicates detected fluorescence equivalent to 5µl/L of OBs, the concentration chosen to represent a "Positive" result. Spectrophotometry analyses were not continued in Sampling Events 3 and 4.

Table 8. Optical brightener (OB) results from spectrophotometry. Sites are shown upstream to downstream for each creek.

	Site Name	Sampling Event 1			Sampling Event 2		
		DAY 3 - 9/25/2018	DAY 7 - 9/29/2018	DAY 10 - 10/2/2018	DAY 3 - 10/23/2018	DAY 7 - 10/27/2018	DAY 10 - 10/30/2018
Bozeman Ck.	BC-Canyon	ABSENT	ABSENT	ABSENT	ABSENT	ABSENT	ABSENT
	BOZMC03	ABSENT	ABSENT	ABSENT	ABSENT	ABSENT	ABSENT
	BOZMC03a	ABSENT	ABSENT	ABSENT	ABSENT	ABSENT	ABSENT
	BOZMC-MDWLK4	ABSENT	ABSENT	ABSENT	INCONCLUSIVE	ABSENT	ABSENT
	BOZMC-MDWLK2	ABSENT	ABSENT	ABSENT	ABSENT	ABSENT	ABSENT
	BOZMC-KagyPark	ABSENT	ABSENT	ABSENT	ABSENT	ABSENT	ABSENT
	StormDrain-EL	NOT SAMPLED			NEGATIVE	ABSENT	ABSENT
	BOZMC-ELCTS3	ABSENT	ABSENT	ABSENT	ABSENT	ABSENT	ABSENT
	BOZMC-IPRD	ABSENT	ABSENT	ABSENT	ABSENT	ABSENT	ABSENT
	BOZMC-ELCTHTS1	ABSENT	ABSENT	ABSENT	POSITIVE	ABSENT	ABSENT
Matthew Bird Ck.	MTWBC02	ABSENT	ABSENT	ABSENT	INCONCLUSIVE	ABSENT	ABSENT
	MTWB-xing	ABSENT	ABSENT	ABSENT	ABSENT	ABSENT	ABSENT
	MTWB-SD3322	ABSENT	ABSENT	ABSENT	ABSENT	ABSENT	ABSENT
	MTWB-SD3318	ABSENT	ABSENT	ABSENT	INCONCLUSIVE	ABSENT	ABSENT
	MTWB-Trib	NOT SAMPLED			ABSENT	ABSENT	ABSENT
	MTWB-SW106	ABSENT	ABSENT	ABSENT	INCONCLUSIVE	ABSENT	ABSENT

5.3 *E. coli* Bacteria

With the exception of the lone StormDrain-EL sample, *E. coli* concentrations did not exceed the November-March single sample threshold of 1,260 cfu/100 ml **(Tables 9 and 10, Figures 7 and 8)**, or correspond to upstream proximity of stormwater inputs on either stream **(Figures 2 and 3)**. *E. coli* concentrations at sites on Bozeman Creek generally increased down the creek continuum during Sampling Event 1. Bozeman Creek results from this event were also generally higher than from Sampling Event 2. Concentrations were notably greater from the StormDrain-EL site, which was sampled only during Sampling Event 2.

Results from Matthew Bird Creek did not exhibit a clear pattern along the stream continuum for either sampling event. However, results were more variable during Sampling Event 1.

E. coli concentrations at all sites were very low during Sampling Events 3 and 4 **(Table 10)**.

Table 9. E. coli concentrations from Sampling Events 1 & 2 in most probable number /100mL (mpn). MPN is comparable to the colony forming units (cfu)/100mL in which the single sample threshold is expressed. Approximate distance to the closest upstream (u/s) storm drain is also shown.

	Site Name	Closest u/s stormdrain (miles)	Sampling Event 1 DAY 7 - 9/29/2018	Sampling Event 2 DAY 7 - 10/27/2018
Bozeman Ck.	BC-Canyon	none	12.2	67
	BOZMC03	none	33.1	7.5
	BOZMC03a	none	33.6	32.1
	BOZMC-MDWLK4	none	158.5	15.6
	BOZMC-MDWLK2	none	325.5	9.8
	BOZMC-KagyPark	0.07	249.5	18.9
	StormDrain-EL	*	NOT SAMPLED	1732.9
	BOZMC-ELCTS3	0.10	419.8	98.4
	BOZMC-IPRD	0.75	292.4	29.2
	BOZMC-ELCTHTS1	0.10	248.9	28.5
Matthew Bird Ck.	MTWBC02	0.03	NO DATA	17.3
	MTWB-xing	0.20	244.5	14.5
	MTWB-SD3322	0.35	38.9	83.6
	MTWB-SD3318	0.03	79.8	49.5
	MTWB-Trib	none	NOT SAMPLED	NOT SAMPLED
	MTWB-SW106	0.10	209.8	34.5

* = site is a storm drain that was sampled directly.

Table 10. E. coli concentrations from Sampling Events 3 & 4 in mpn/100mL. Most probable number/100 mL is comparable to the colony forming unit (cfu)/100mL in which the single sample threshold is expressed. All sites are upstream of City of Bozeman stormwater infrastructure.

	Sampling Event 3 DAY 14 - 2/12/2019	Sampling Event 4 DAY 14 - 3/5/2019
BC-Canyon-A	1	2
BC-Canyon-B	2	<1
LIMC-GPARK-A	2	2
LIMC-GPARK-B	1	2
SPRINGC-TUCK-A	3.1	9.7
SPRINGC-TUCK-B	2	10.8

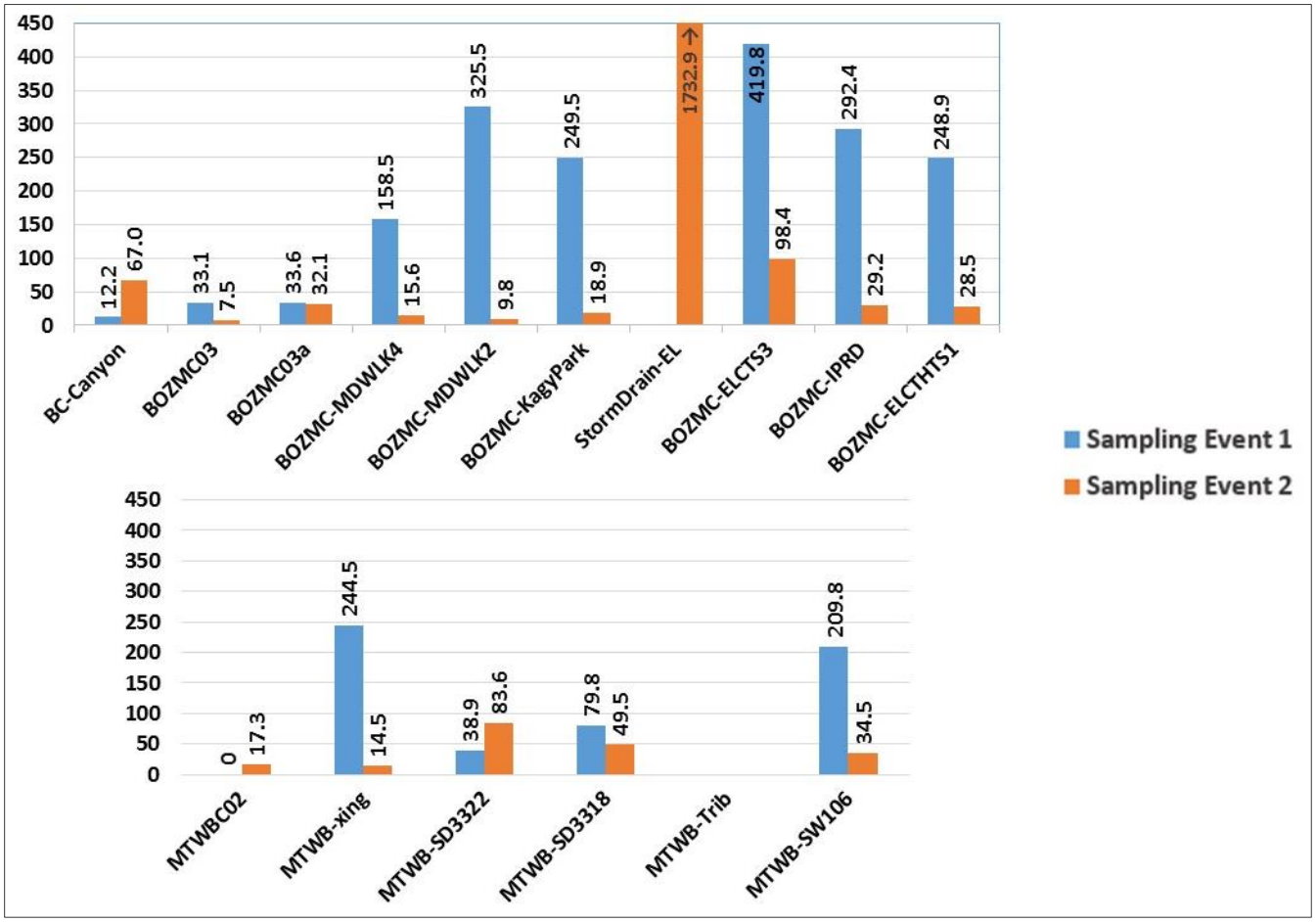

Figure 7. E. coli concentrations in mpn/100ml for Sampling Events 1 (9/25/18 – 10/2/18) & 2 (10/23/18 – 10/30/18). Most probable number/100 mL is comparable to the colony forming units (cfu)/100mL in which the single sample threshold is expressed. Only the sample from StormDrain-EL exceeded the November-March single sample threshold of 1,260 cfu/100 ml.

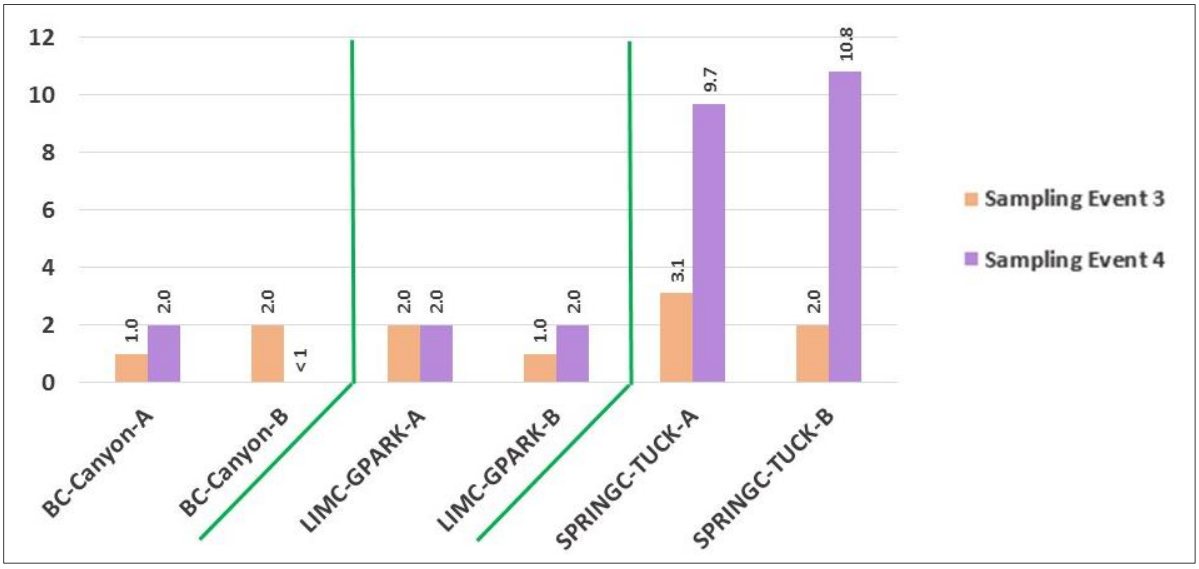

Figure 8. E. coli concentrations in mpn/100ml for Sampling Events 3 & 4. Replicates (-A or -B) were collected at all sites. Green lines separate results by site. Note the difference in scale on the Y-axis between Figures 7 and 8.

5.4 Streamflow

Streamflow generally increased down the creek continuum of Bozeman Creek during all monitoring days of Sampling Events 1 and 2 **(Table 11, Figures 9 and 10)**. Flows on Bozeman Creek were higher during Sampling Event 2. Flow was estimated at ≤ 1 cublic feet per second (cfs) from the StormDrain-EL site at all site visits.

Streamflow was generally similar at the upstream and downstream sites on Matthew Bird Creek during all monitoring days for both sampling events. Flows on Matthew Bird Creek at all sites during Sampling Event 2 were approximately one third of Sampling Event 1 flow measurements. Flow was estimated at ≤1 cfs from the MTWB-Trib site at all site visits. Streamflow was not measured during Sampling Events 3 and 4.

Table 11. Streamflow measurements shown in cubic feet per second (cfs).

	Site Name	Sampling Event 1			Sampling Event 2		
		DAY 3 - 9/25/2018	DAY 7 - 9/29/2018	DAY 10 - 10/2/2018	DAY 3 - 10/23/2018	DAY 7 - 10/27/2018	DAY 10 - 10/30/2018
Bozeman Ck.	BC-Canyon	4.62	5.45	4.55	5.42	10.09	5.38
	BOZMC03	8.67	10.41	8.99	11.16	18.65	12.41
	BOZMC03a	9.99	9.94	10.05	14.55	12.39	16.41
	BOZMC-MDWLK4	10.4	10.52	10.83	20.98	21.67	17.54
	BOZMC-MDWLK2	FLOW NOT MEASURED AT THIS SITE					
	BOZMC-KagyPark	11.96	10.78	11.6	14.19	17.23	13.7
	StormDrain-EL	FLOW NOT MEASURED AT THIS SITE					
	BOZMC-ELCTS3	NOT MEASURED	18.08	15.17	21.48	22.18	22.81
	BOZMC-IPRD	19.49	15.52	15.99	20.42	18.52	19.49
	BOZMC-ELCTHTS1	FLOW NOT MEASURED AT THIS SITE					
Matthew Bird Ck.	MTWBC02	3.69	4.16	4.37	1.31	0.68	1.93
	MTWB-xing	FLOW NOT MEASURED AT THESE SITES					
	MTWB-SD3322						
	MTWB-SD3318						
	MTWB-Trib						
	MTWB-SW106	3.15	3.31	3.01	1.27	1.23	1.67

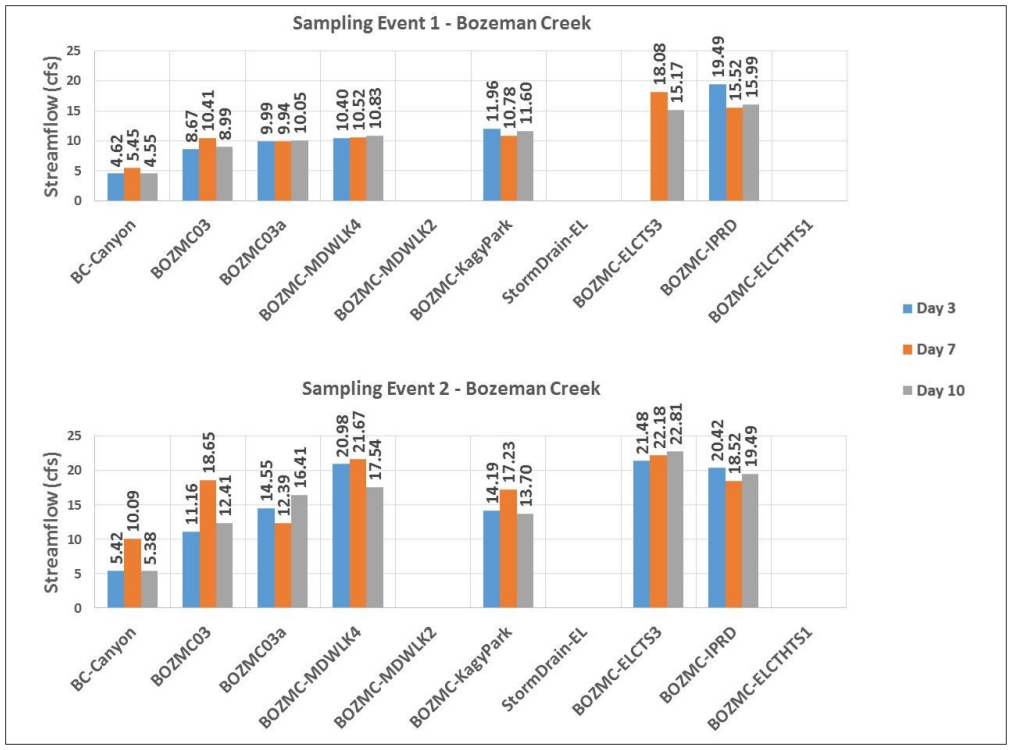

Figure 9. Bozeman Creek streamflows in cubic feet per second from Sampling Events 1 (9/25/18 – 10/2/18) and 2 (10/23/18 – 10/30/18).

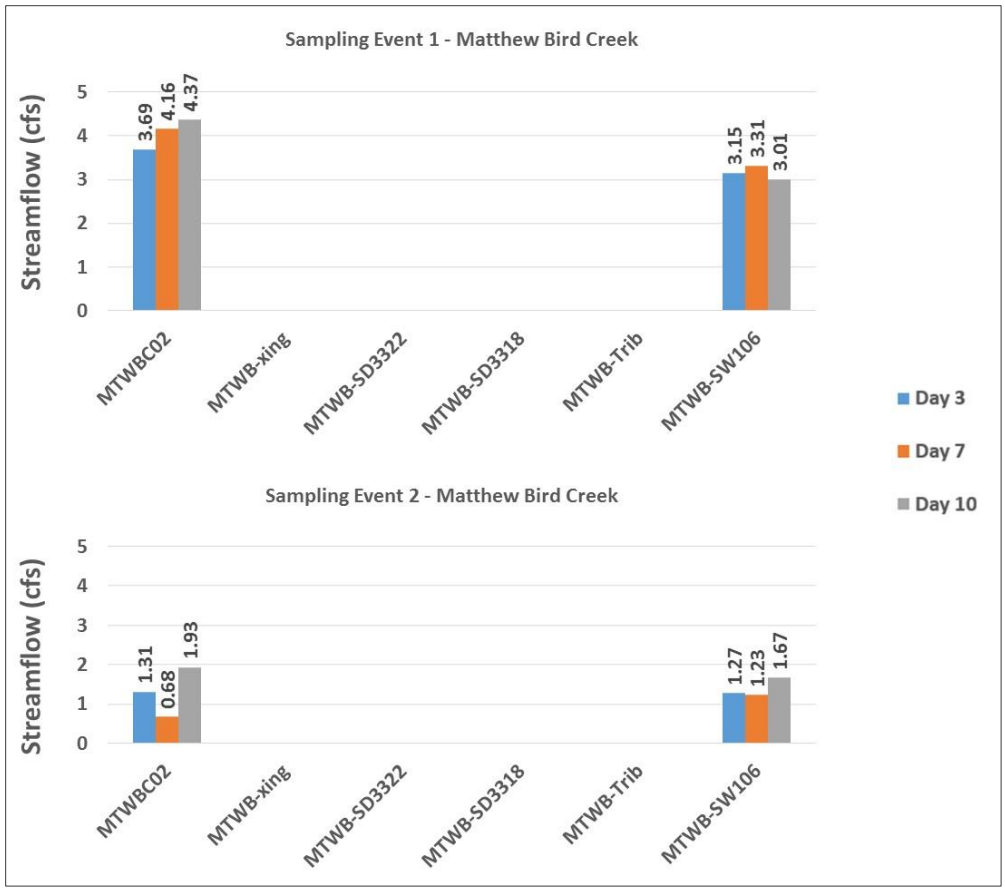

Figure 10. Matthew Bird Creek streamflows in cubic feet per second from Sampling Events 1 (9/25/18 – 10/2/18) and 2 (10/23/18 – 10/30/18).

5.5 Instream Field Parameters (YSI)

Bozeman Creek – Sampling Events 1 and 2

Mean water temperatures downstream of the BC-Canyon control site were fairly consistent during both sampling events **(Figures 11 and 12, top left)**. An increase of more than a degree was recorded below the StormDrain-EL site during both sampling events. Mean specific conductivity (SC) downstream of the BC-Canyon control site generally increased moving downstream during both sampling events **(Figures 11 and 12, top right)**. As with water temperature, an increase in SC was recorded below the StormDrain-EL site during both sampling events.

pH and Dissolved Oxygen (DO) did not exhibit an upstream-downstream trend during either sampling event **(Figures 11 and 12, bottom left and right)**. While a clear increase in pH was not seen below the StormDrain-EL site during Sampling Event 1, data from Sampling Event 2 indicate that this drain is contributing inputs with higher pH to the stream. DO was consistently highest on Day 3 of Sampling Event 1. The high DO at the BOZMC-MDWLK4 site on Day 3 of Sampling Event 2 is a suspected recording error on the site visit form.

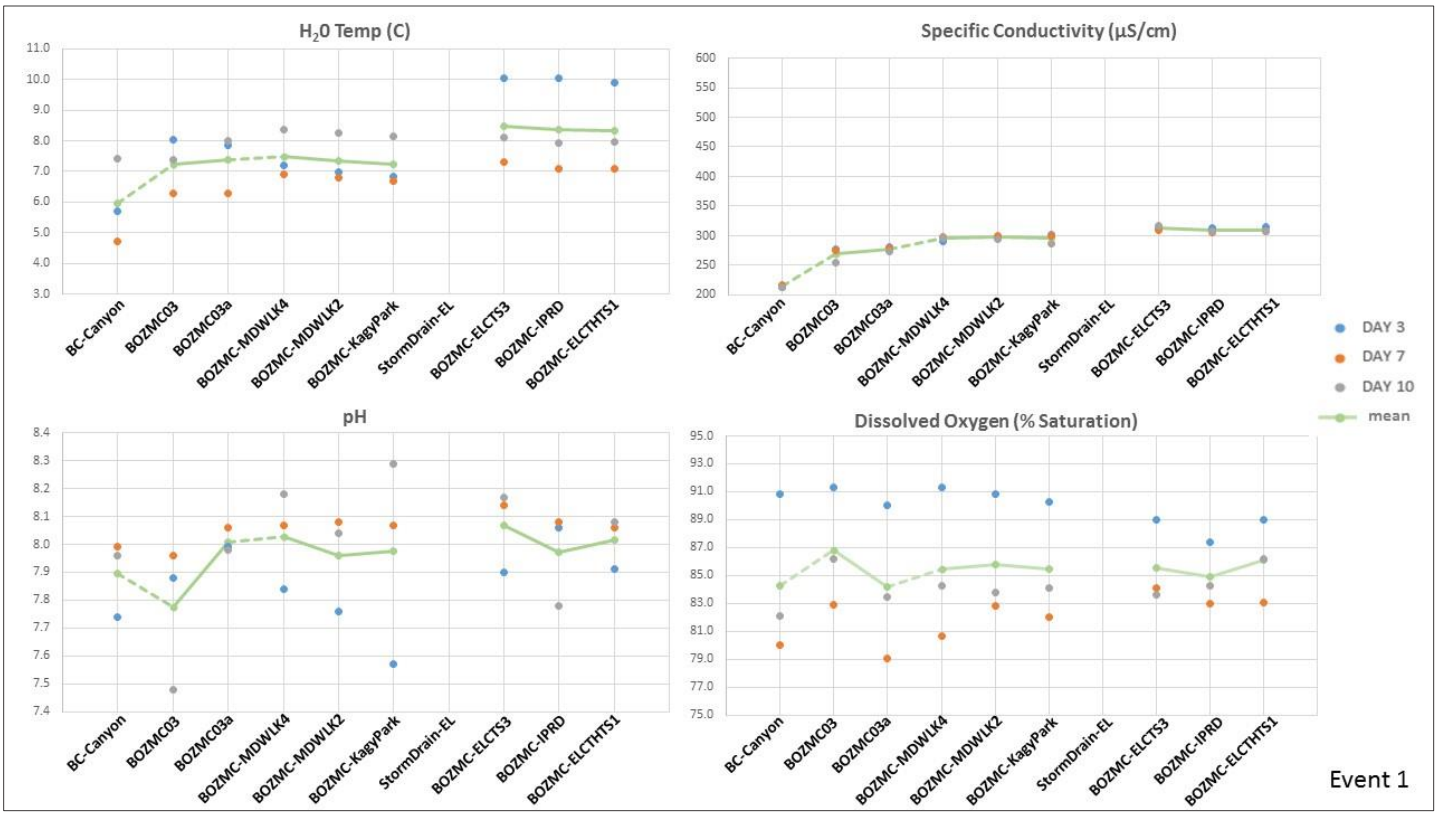

Figure 11. Instream field parameter results for Bozeman Creek for Sampling Event 1. Sites shown upstream to downstream. Line connecting event averages is solid between sites that bracket an ISS Area.

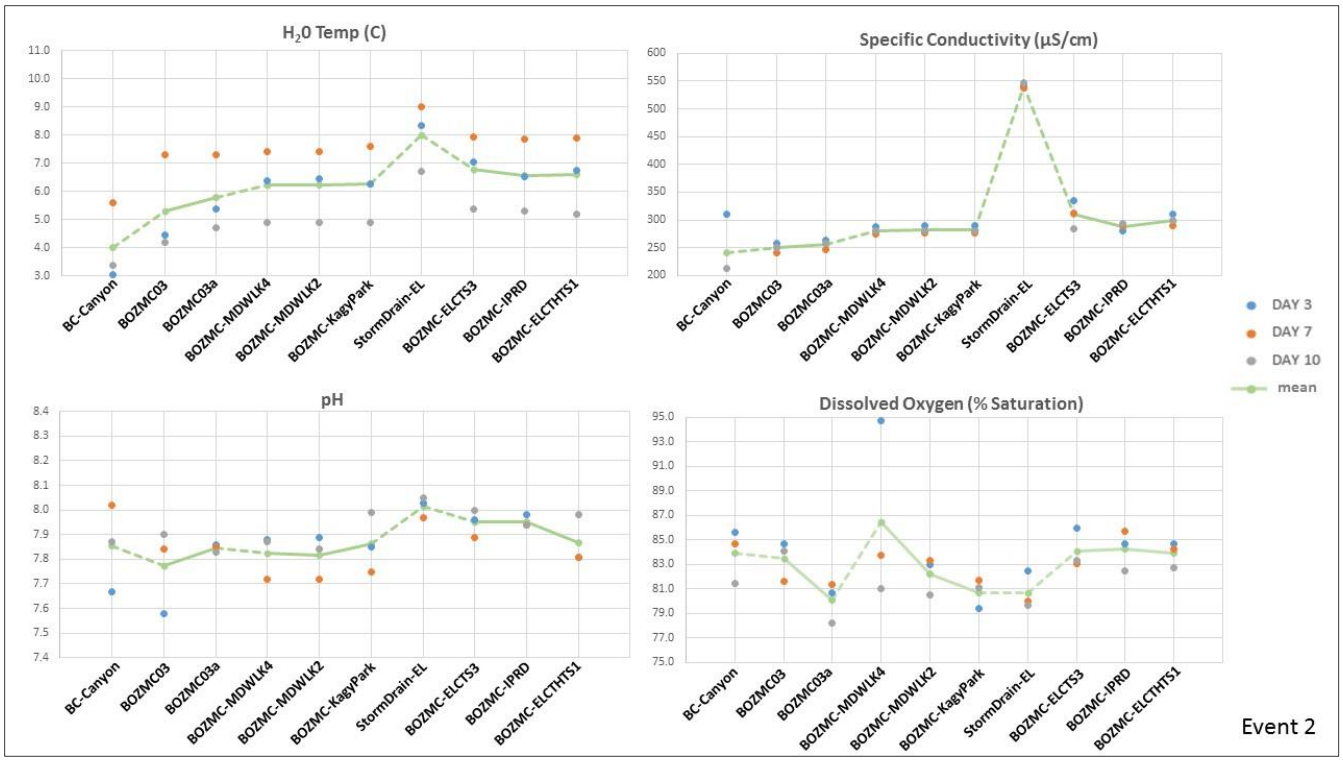

Figure 12. Instream field parameter results for Bozeman Creek for Sampling Event 2. Sites shown upstream to downstream. Line connecting event averages is solid between sites that bracket an ISS Area. The high DO at the BOZMC-MDWLK4 site on Day 3 of Sampling Event 2 is a suspected recording error on the site visit form.

Matthew Bird Creek – Sampling Events 1 and 2

Water temperatures were consistent down the stream continuum during both sampling events **(Figures 13 and 14, top left)**. SC values during Sampling Event 2 were notably higher than those from Sampling Event 1, with Sampling Event 1 averages in the range of 230-240 µS/cm and Sampling Event 2 averages in the range of 470-530 µS/cm **(Figures 13 and 14, top right)**. While a clear increase in SC was not seen below the MTWB-Trib site during Sampling Event 1, values from Sampling Event 2 indicate that this tributary is contributing inputs with higher SC to the stream, although the notably high SC at the MTWB-SW106 site on Day 10 of Sampling Event 2 is a suspected recording error on the site visit form.

The low pH at the MTWB-xing site on Day 3 of Sampling Event 1 is also a suspected recording error. If this point is omitted, average pH generally increased along the stream continuum at sites above the MTWB-Trib site. Values from Sampling Event 2 indicate that this tributary might be contributing inputs with lower pH to the stream **(Figure 14, bottom left)**. Similar to Bozeman Creek, DO was consistently highest on Day 3 of Sampling Event 1. While a decrease in DO was not seen below the MTWB-Trib site during either sampling event **(Figures 13 and 14, bottom right)**, values from Sampling Event 2 indicate that this drain is contributing inputs with lower pH to the stream **(Figures 13 and 14, bottom left)**.

Sampling Events 3 and 4

The timing of these sampling events during January and February are reflected in water temperatures that did not exceed 3.5 °C **(Figure 15, top left)**. SC values were nearly identical for Sampling Events 3 and 4, with notably different values at all three sites **(Figure 15, top right)**. pH and DO varied at all sites between sampling events, but all values fell within typical ranges **(Figure 15, bottom left and right)**.

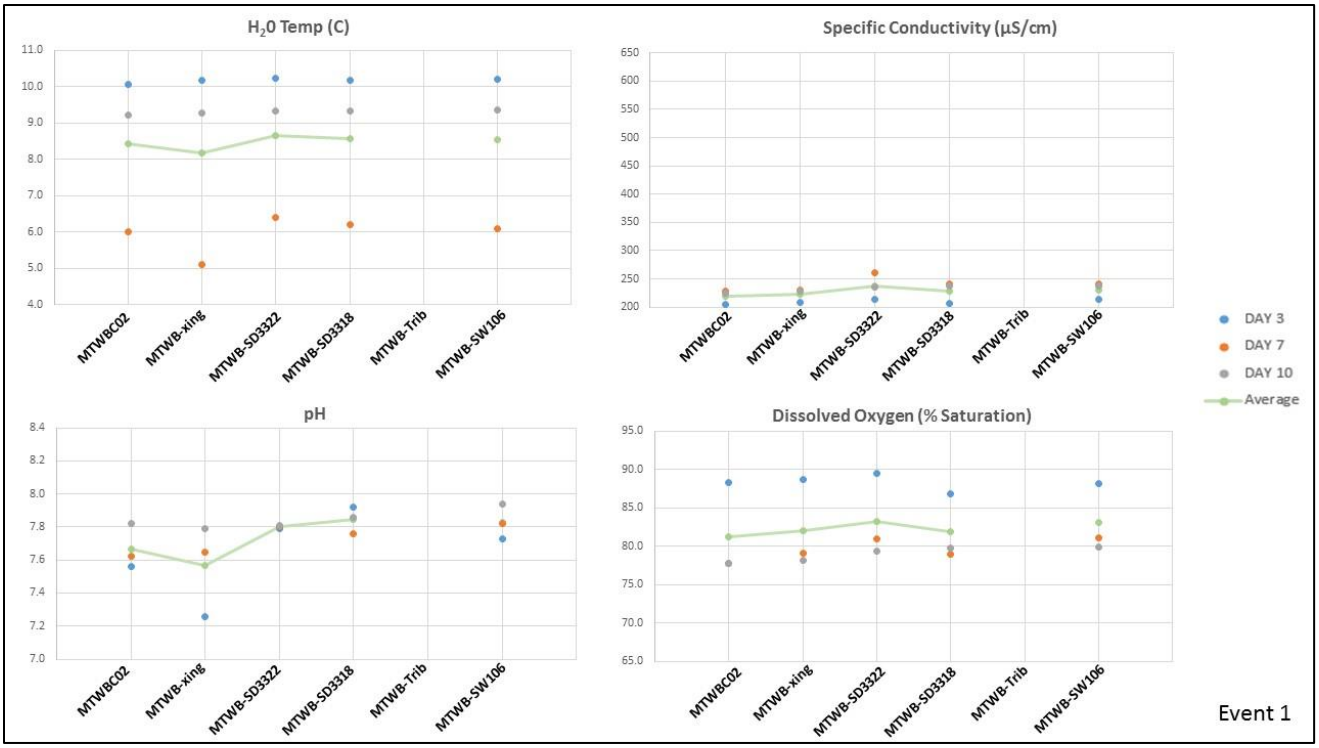

Figure 13. Instream field parameter results for Matthew Bird Creek for Sampling Event 1. Sites shown upstream to downstream. Solid line connecting event averages indicates all sites are located within a single ISS Area.

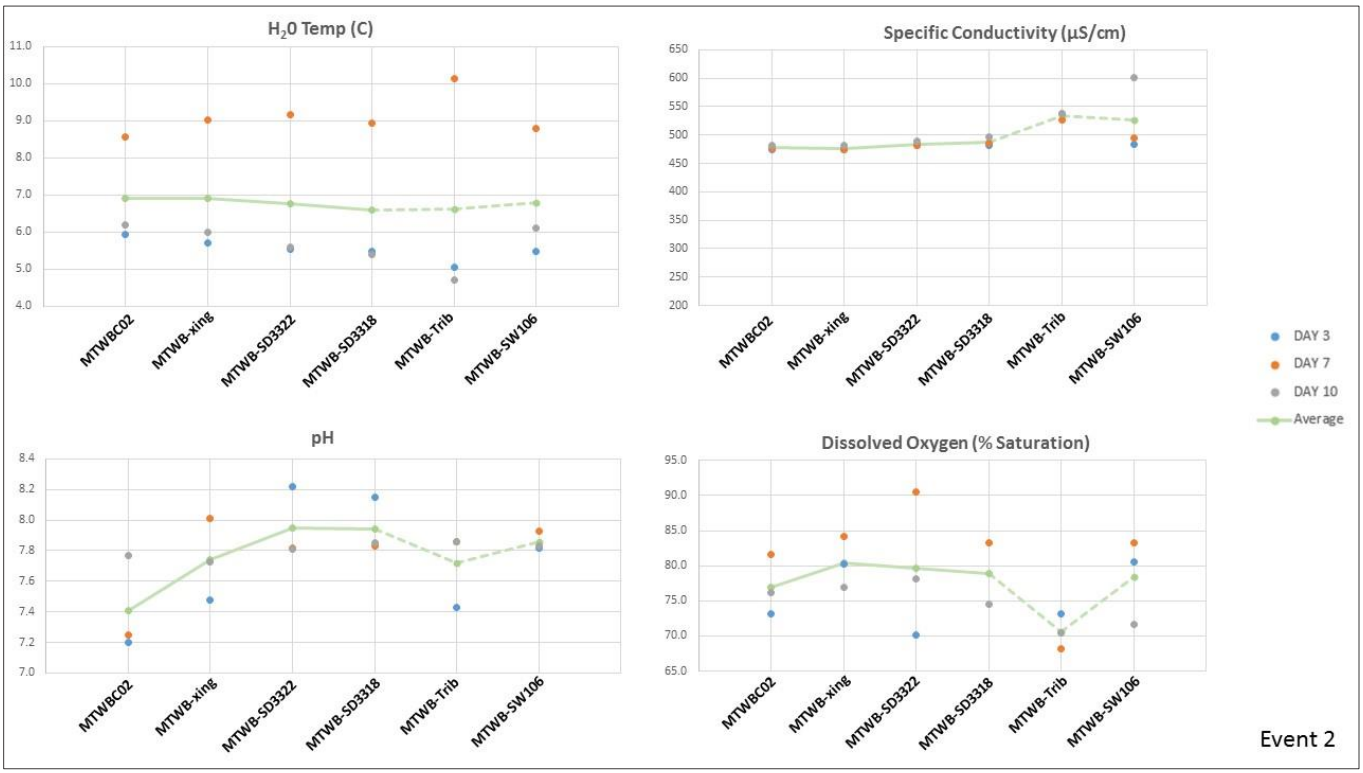

Figure 14. Instream field parameter results for Matthew Bird Creek for Sampling Event 2. Sites shown upstream to downstream. Dashed portions of line connecting event averages indicates tributary inputs to a single ISS Area.

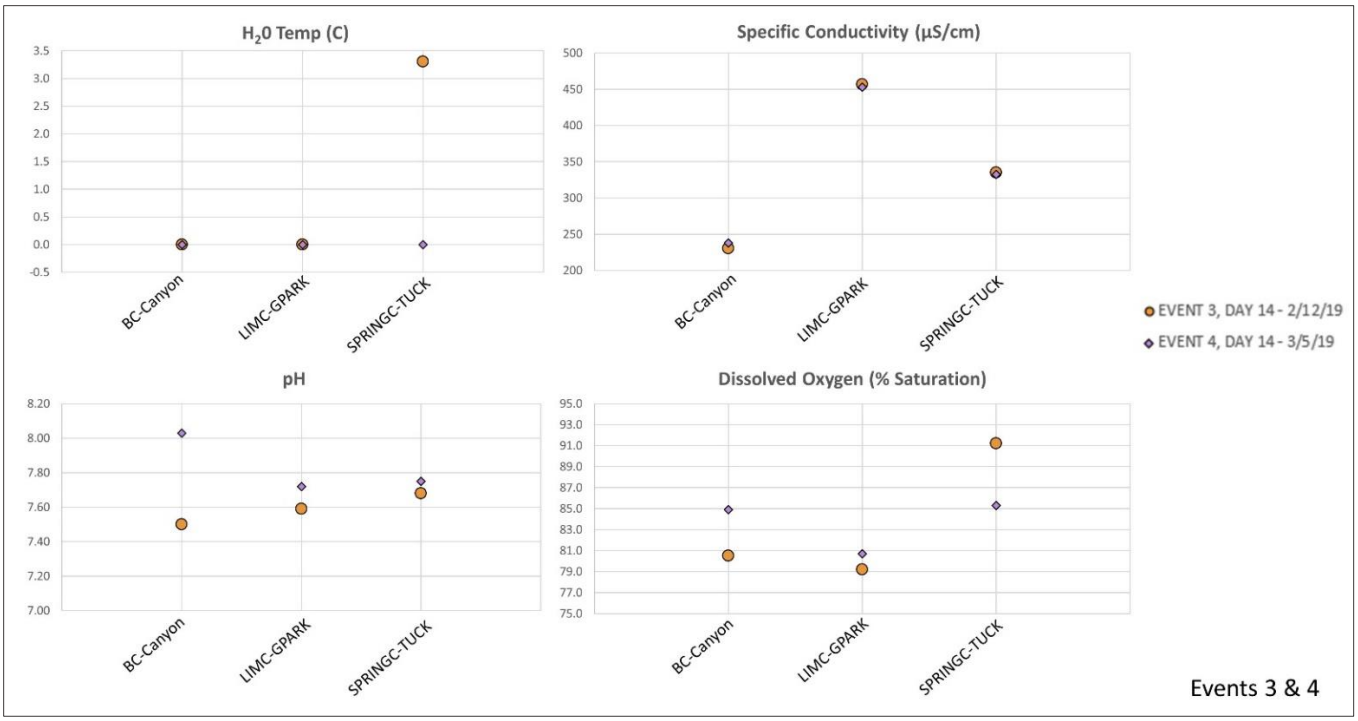

Figure 15. Instream field parameter results for all sites for Sampling Events 3 and 4. These sites are on three different creeks.

5.6 Mass Spectrometry

Due to faculty changes at the MSU Mass Spec Facility during the course of this project, a formal report of mass spec findings was not completed. A summary of mass spec results and some interpretation from all sampling events were communicated via phone by the former manager of the facility (Balasubramanian, 2020). Dr. Balasubramanian's interpretations are included in Section 6.5.

Spectrometry-based untargeted metabolomics analysis was completed for samples collected on a single day of Sampling Events 1 and 2. Human wastewater-specific compounds were not detected, although higher concentrations are required to definitively indicate their presence using this exploratory approach.

A broad class of common mammalian biomarkers called bile acids were found at concentrations that are considered elevated for surface water at some sites on both streams. While the specific bile acids detected are produced by most mammals, their role in digestion of dietary fats indicates that human wastewater is likely present. Although the specific sites at which elevated concentrations were detected were not known by Dr. Balasubramanian at the time of communication, he confirmed that their relative concentrations did not show an up- to downstream pattern or remain consistent between sampling events.

6.0 Discussion

6.1 Optical Brighteners - Absorption

An OB pad apparatus was lost during both Sampling Events 1 and 2. It is unclear whether the loss was due to an increase of flow between sampling events or to human/animal disturbance. These losses indicate the need to deploy the pads using a method that will better withstand increased velocities and provide better camouflage.

Sampling Events 1 and 2

Fluorescence, indicative of OBs and other compounds, was not present on any wet cotton OB pads, regardless of length of deployment. Wet pads were also noted to be saturated with sediment and "muddy looking", which could have masked fluorescence by the UV light. An emphasis was put on rinsing pads with stream water upon collection for the October series, but rinsing did not lead to detections. Since OBs are known to decay with UV exposure, it's possible that ongoing exposure to sunlight during deployment caused decay of low-level OBs present.

Day 3 detections on dried pads during Sampling Event 1 were not seen on pads collected after 7-day deployments at the same sites, but were detected again at the same sites on pads collected after 10-day deployments. This could be due to decay from sun exposure during the Day 4 to Day 6 period, or due to semi-quantitative results recorded by different student observers.

Fluorescence was detected on eight of thirteen dried pads collected after ten days in Sampling Event 1, and on eight of fourteen dried pads collected after ten days in Sampling Event 2. Fluorescence on the pad from the BC-Canyon (control) site was noted to be as present as it was at downstream sites during Sampling Event 1, and the pad apparatus was lost during Sampling Event 2, preventing observations after Day 3. This was not expected, as this location in Bozeman Creek is above any theoretical human influence. This indicates the possibility that the fluorescence detected in Bozeman Creek is from a non-OB source, such as the tannins released as leaves decay instream.

Since the upstream site on Matthew Bird Creek (MTWBC02) was not located in an area above human influence, the Day 10 "Present" result from Sampling Event 2 at this site doesn't necessarily indicate that fluorescence in this creek is from a non-OB source. However, spectrophotometry results that might have confirmed the source were either "Inconclusive" (inconsistent results from multiple sample replicates) or "Absent" (no fluorescence above the detection limit) at all sites on Matthew Bird Creek. This indicates that fluorescing compounds, regardless of their source, are likely very dilute. The location of fluorescence only at the pad edges supports this.

Sampling Events 3 and 4

Based on the results of Sampling Events 1 and 2, longer deployment lengths were chosen for Sampling Events 3 and 4. The presence of fluorescence on all dried pads deployed during this sampling event indicates that further experiments with this method warrant a wider range of deployment periods. However, consistent detections at the BC-Canyon (control) site is additional evidence that the fluorescence detected in Bozeman Creek is from a non-OB source.

6.2 Optical Brighteners - Spectrophotometry

In contrast to the OB absorption method, the use of grab samples in spectrophotometry doesn't allow for accumulation of OBs to detectable levels in stream systems with diffuse OB concentrations. The general lack of positive results indicates that this method is not sensitive enough to OBs at the concentrations present in Bozeman and Matthew Bird Creeks during late fall/winter conditions. However, the single positive result from the downstream-most site on Bozeman Creek (BOZMC-ELCTHTS1) could indicate the potential for concentrations to reach detectable levels at sites below all septic influence.

6.3 *E. coli* Bacteria

Sampling Events 1 and 2

The general increase in *E. coli* concentrations when moving downstream on Bozeman Creek is likely due to the cumulative effects of multiple storm drain inputs and other anthropogenic sources. The numeric standards for *E. coli* set by DEQ vary seasonally, with April-October standards considerably lower in response to increased primary contact in warmer months. However, *E. coli* concentrations generally decrease during colder months when bacteria survival is reduced by colder water temperatures (Blaustein et al., 2013). While only the StormDrain-EL site exceeded the single-sample threshold of 1260 cfu/100 mL in effect during the study period, concentrations at most of the sites in the lower portion of Bozeman Creek were near or exceeded the single-sample threshold of 252 cfu/100 mL in effect April-October. This suggests that Bozeman Creek continues to exceed the water quality standard, as has been found in previous studies.

No clear up- to downstream trend in *E. coli* concentrations is seen on Matthew Bird Creek. However, flows at the upstream and downstream sites were very similar for all six measurements, and higher at the upstream site (MTWBC02) in all but one measurement. This suggests that the study reach on Matthew Bird Creek is a losing reach, and that stormwater and anthropogenic inputs were minimal during the study period.

E. coli concentrations in urban streams have also been shown to respond to precipitation or snowmelt events (Chen and Chang, 2014). Concentrations typically spike in response to the first flush of stormwater and overland inputs to the stream, and then decrease due to continuing inputs of relatively cleaner water. Lower *E. coli* concentrations in Bozeman Creek corresponded with higher measured flows (except at the control site), while *E. coli* concentrations in Matthew Bird Creek were higher when measured flows were higher.

These disparate responses to precipitation reflect characteristic differences in the Bozeman Creek and Matthew Bird Creek watersheds. A significant rain event was noted on September 28, one day prior to *E. coli* sample collection for Sampling Event 1. Grab samples with higher *E. coli* concentrations collected from both streams the following day likely reflect an influx of runoff from the urban areas immediately adjacent to both creeks, carrying *E. coli* from stormwater runoff, shallow groundwater with septic effluent, pet waste from nearby trails, or other potential sources. Rain or snow events were also noted on October 19, 20, 21 and 24, three or more days prior to *E. coli* sample collection for Sampling Event 2 on October 27. The lower *E. coli* concentrations from both streams likely reflect that they were collected after the first flush of runoff from urban areas had subsided. However, flows likely remained high in Bozeman Creek during sampling Event 2 due to a gradual influx of water from Bozeman Creek's forested upper watershed, while flows dropped more quickly in Matthew Bird – a spring creek with a much smaller watershed.

Evidence of the stormwater system as a likely source of *E. coli* can be seen in the results from the StormDrain-EL site, with concentrations more than three times higher than any other samples collected on Bozeman Creek. Further investigation of the eight additional storm drains within the ISS Areas on Bozeman Creek and Matthew Bird Creeks would aid in understanding the storm system as an *E. coli* source.

Sampling Events 3 and 4

E. coli survival rates have been shown to be dependent on temperature (Blaustein et al., 2013). *E. coli* concentrations at all sites were very low during these two sampling events, likely due to the very cold temperatures experienced during the sample periods. Site Visit Form comments included a note that samplers had to use a hammer to break through surface ice in order to collect samples during both sampling events.

6.4 Instream Field Parameters (YSI)

Sampling Events 1 and 2

While every effort was made to collect data from all sites on a single sampling day, sites were not sampled synoptically. Water temperature, pH and DO are all known to exhibit diel fluctuation, and differences in these parameters among main stem Bozeman Creek sites did not exceed the natural variation expected among sites that were not sampled synoptically, particularly when data points that were identified as likely recording errors are excluded.

While the StormDrain-EL site is contributing higher temperature inputs to the stream, the estimated 1 cfs of flow from this drain is likely only one of several storm drain inputs contributing to the increase in flow seen at the sites below it **(Figure 2)**. This is supported by the general increase in flow seen through the downstream reach.

The higher DO values seen at all sites on Day 3 of Sampling Event 1 do not correspond with higher flows or lower water temperatures. The consistent nature of the high values indicates the possibility of calibration error on that day.

The higher mean SC levels at Matthew Bird Creek sites during Sampling Event 2 correspond with flows that were roughly one third of those measured during Sampling Event 1. Higher SC levels also corresponded with fluorescence observed on Day 7 dried OB pads from Matthew Bird Creek in Sampling Event 2. This might indicate dilution of pollutants with high conductivity occurring during Sampling Event 1. However, corresponding differences were not seen in other instream field parameters. Additional data is needed to find an explanation for these differences.

Notable differences in the mean values for many instream field parameters from the StormDrain-EL and MTWB-Trib sites indicate that additional research into the origins of these sources is warranted.

Sampling Events 3 and 4

Interpretation of instream field parameter data from is limited by the once per event frequency of data collection. However, the SC results indicate that both Limestone Creek and Nash Spring Creek are sources of higher conductivity flow to Bozeman Creek.

6.5 Mass Spectrometry

The elevated levels of bile acids detected at many of the sites indicate that human wastewater is likely present on both streams. However, because the relative concentrations of these molecules did not show an increasing or decreasing trend along these streams, neither simple dilution from a single point of origin, nor accumulation along the either steam's continuum is indicated. Further, relative concentrations did not remain consistent at any site between sampling events. This would suggest that if human wastewater sources are reaching these streams, they are not present consistently, or at concentrations that allow for the detection of specific points of entry using these compounds. Dr.

Balasubramanian suggested that a subset of the broader class of bile acids is specific to humans, warranting use of a targeted mass spec approach to assess these compounds, as this method can detect specific, predetermined compounds at lower concentrations.

7.0 Conclusions and Recommendations

The primary goal of this project was to determine if tracer concentrations correlate spatially with ISS Areas by comparing project results with the EHS septic system inventory. Since the results of this project suggest that OBs are not present in Bozeman or Matthew Bird Creeks at concentrations that can be reliably detected by the absorption or spectrophotometry methods used, comparisons to septic records are not warranted at this time. However, results from dried OB pads indicate that additional experimentation might be warranted to aid in further evaluation of the method at a wider range of deployment duration.

Because *E. coli* are not specific to human wastewater, efforts to pinpoint septic inputs require the method's use in conjunction with other, human-specific tracer methodology. In Sampling Event 1, the general increase in *E. coli* concentrations moving downstream suggests that cumulative anthropogenic inputs from diverse sources such as stormwater runoff, shallow groundwater with septic effluent, or animal waste from nearby trails and pastures are entering the stream. However, the inconclusive results from the other wastewater tracer methods tested could not be used to learn more about the specific source of the *E. coli* in Sampling Event 1. *E. coli* results from Sampling Events 3 and 4 indicate that concentrations are affected by sampling during cold winter weather.

The absence of human wastewater-specific tracers (caffeine, pharmaceuticals) at concentrations detectable by non-targeted mass spec indicates that these compounds are very low or absent in this system. While many bile acids are not human-specific, total bile acid concentrations were elevated enough to indicate the potential presence of human wastewater contamination. Further mass spec analyses employing the targeted approach might prove useful, as the subset of bile acids specific to humans might be present at levels that could be detected with this more sensitive method.

The following are suggestions for improved sampling design for continuing work toward this goal:

- Timing an OB study to coincide with the falling tail of the hydrograph (estimated to be approximately mid-June) could capture wastewater carried into the stream when it is most concentrated: after dilution by runoff has decreased, but while the stream remains connected to septic influence via shallow groundwater.
- Focusing efforts on methods other than *E. coli* is recommended, as project results and relevant literature both suggest that the natural variability typical in *E.coli* concentrations limits their utility in detecting the nonpoint septic sources likely present in these stream systems.
- Deploying OB pads for a wider range of time-integrated sampling, in a way that protects them from photodecay by ultraviolet light, could allow for further evaluation of this method.
- Maintaining a grab sampling and YSI measurement schedule in which all sites associated with an ISS Area are sampled synoptically could allow for improved comparison of these parameters above, below, and within the bracketed area.
- Further monitoring of all tributaries and stormwater outfalls within ISS Areas could inform our understanding of these potential sources of pollutants to Bozeman and Matthew Bird Creeks.
- Systematic dye testing of the septics of homes within ISS Areas, focused on homes with unpermitted systems, could provide more definitive source tracking.

- Conducting targeted mass spec analyses for human-specific bile acids could confirm their presence at low concentrations. In particular, analysis of bile acids in conjunction with fecal sterols has been used to successfully determine the origins of fecal pollution from stream sediments in mixed use watersheds (Sanez, et al., 2016)

The second goal of this project was to encourage student engagement and connection with MSU's Environmental Health and INBRE programs. This project was successful at meeting this goal, as evidenced by the following:

- Participation in data collection and analysis by twelve undergraduate microbiology or environmental health majors. Each had to put in a minimum of 20 hours on the project.
- Overall positive comments about the project provided in course evaluations.
- Four participating seniors have now graduated in Environmental Health, and two are headed to graduate school in the Environmental Health field.
- Two participating juniors subsequently won national level public health fellowships to conduct other environmental health research.
- At least three students were accepted into the highly competitive Medical Laboratory Sciences upper division curriculum. All of these students cited the experience with this project in their applications.
- One student majoring in microbiology became interested in water and wastewater treatment and expressed an intent to pursue a career in this area.

However, interagency collaboration, and the coordination and supervision of student scientists through a multi-semester project also created challenges in maintaining the scientific rigor of project data and the efficiency of workflow through multiple partners.

The following are suggestions for improving confidence in the quality of data collected by student scientist:

- Providing additional training for students in data collection and analysis roles, highlighting the importance of accurate and consistent sample labeling and maintaining field and laboratory notebooks for recording deviations from project methods at the time they occur.
- Making student training events a collaboration between those responsible for student safety and educational outcomes and those responsible for data analysis and presentation outcomes will help ensure the data collection experience is successful from multiple perspectives.
- Tailoring project scope to the nature of the class in which student participants are enrolled. For example, future projects with similar scope would be more appropriate as stand-alone capstone research classes for undergraduate upperclassmen, focusing their participation on the collection of high-quality data.

The following are suggestions for improving the efficiency of workflow for projects with multiple partners:

- Creating a schedule for students to check-in with project supervisors as part of project design would help ensure desired outcomes for student engagement and data quality are being achieved. This is particularly important when projects span multiple semesters so that agreed-upon deliverables are complete to a degree appropriate for passing on to those responsible for taking the project forward.

- Utilizing contracts or Memorandums of Understanding to ensure analysis will be completed by the responsible department in the event of staff turnover.

The third goal of this project was to use the results to inform Education & Outreach and/or potential regulatory decisions related to human wastewater contamination in Bozeman and Matthew Bird Creeks. While the results of this project did not allow for spatial correlation between human wastewater-specific tracers via mass spectrometry or OBs and suspected areas of septic contamination, *E. coli* results, as in previous studies, continue to exceed DEQ surface water standards. As noted in similar studies, "The search for an indicator that reliably predicts human health risks and indicates potential sources is an ongoing effort." (Gilfillan, 2018 JEQ). Until such an indicator is found, more general education and outreach efforts to homeowners with individual septic systems is still warranted.

The following are education and outreach suggestions:

- Additional promotion of the City of Bozeman's Adopt-a-Storm Drain program by project partners could reduce the impact of the stormwater system as a source of pollution to Bozeman and Matthew Bird Creek.
- GLWQD Well & Septic Awareness courses targeted at homes within ISS Areas, possibly organized and promoted through the associated Homeowners Associations, could provide the owners of lots within ISS Areas with additional knowledge on the upkeep and lifespan of their septic systems.
- A harmful algal bloom (HAB) in a pond in the study area during the summer of 2020 garnered attention and concern about its causes from area residents. This focus could be leveraged in outreach events by including the potential contributions from septic inputs to HABs.
- A pilot program for mandatory scheduled septic maintenance, targeted at homes within ISS Areas, could lead to a reduction in the number of unpermitted and/or failing septic systems along Bozeman and Matthew Bird Creeks.
- Dissemination of these findings and those from previous related research could assist decision-makers in evaluating the potential improvements to water quality associated with the connection of homes in ISS zones to municipal wastewater treatment infrastructure.

8.0 References

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10.0 Appendices

A. Standard Operating Procedures
B. Instream Field Parameter Data

A Multi-Tracer Approach to the Investigation of Human Wastewater Contamination in Bozeman and Matthew Bird Creeks

Appendix A - Standard Operating Procedures

Prepared by:
Michelle Pond, Big Sky Watershed Corps Member
Gallatin Local Water Quality District and Gallatin City-County Health Department
August 2018

Contents

GLWQD

1. Field Protocols

1.1 Optical Brightener Time-Integrated Sampling Protocol

Cotton gauze pads will be deployed at each sampling site on Day 0. One set will be collected and analyzed with each following field day, after being deployed 3, 7 and 10 days. Nitrile gloves will be worn at each step.

Deployment:

Note: Before deployment, pads will be checked to ensure that they do not fluoresce under black light.

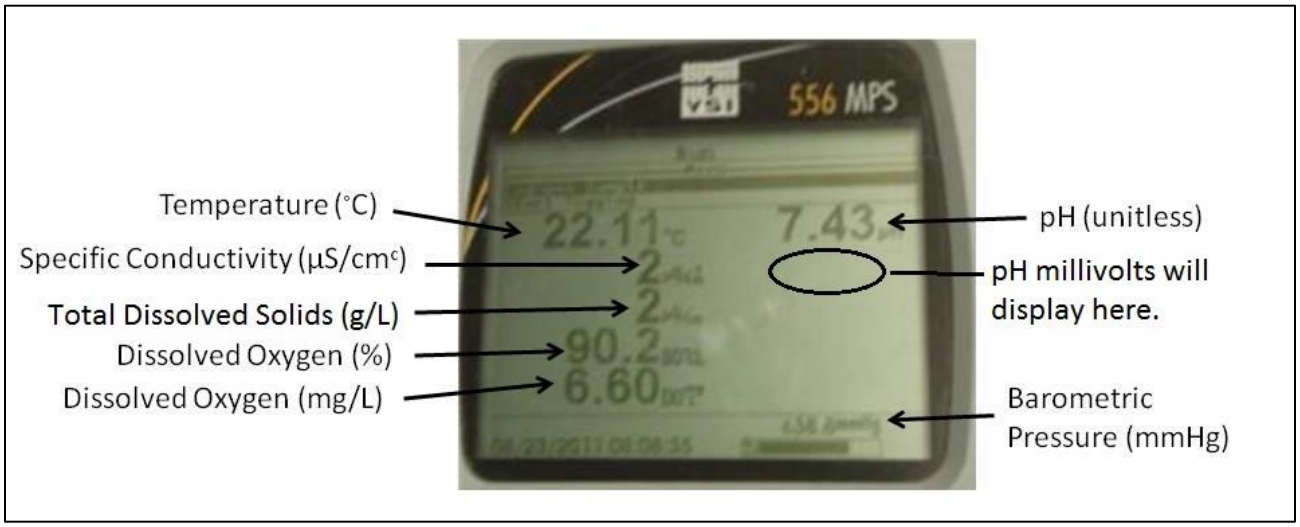
1. Place 2 gauze pads in polynet bag and close the bag. Repeat to make two more samples (three total).
2. Identify where in the creek the samples will be deployed. It should have full flow and protection from the sun. At public sites, it should be somewhere it will not attract too much attention, if possible.
3. Use rebar, rocks, wire and zip ties to secure the three samples at the site. Ensure that they are as shaded as possible. Do not wire samples flat against a rock – there should be ample flow around/through the pads.

Retrieval:

1. Remove one sample bag from the creek. Approach from downstream to prevent covering sample in extra kicked-up sediment.
2. Working in the shade, clean the gauze pads with stream water to remove sediment, debris, aquatic organisms, etc. A squirt bottle of stream water may be used.
3. Gently wring excess water from the pads.
4. Wrap both pads in one tin foil packet. Label with sample code. Place in a small pre-labeled ziplock bag.
5. Store in the cooler on ice.

1.2 Instream Field Parameters Collection Protocol

Instream field parameters include water temperature, pH, specific conductivity (SC), dissolved oxygen (DO) in milligrams per liter (mg/L) and percent saturation (% sat), and total dissolved solids (TDS). These parameters are "single point in time" measurements and only relate to the sampling point (water column) at the instant the reading is taken. These will be collected at all sites, during each field day, using a YSI 556 multiprobe meter.



Example of what the display looks like on the YSI meter.

Note: All parameters must be calibrated each day before use and checked for drift at the end of the field day.

1. Keep probe attached to the meter after calibrating.
2. Install probe sensor guard in place of the calibration/transport cup.
3. Press the **On/Off** key and select **Run** from the Main Menu to display the run screen.
4. Carefully enter the stream moving out as close to the center of the stream as is safely possible.
5. While facing upstream, place probe in the water. Completely immerse all the sensors.
6. If flow doesn't seem sufficient to move water through the sensor guard, gently yet rapidly move probe through the stream (provides fresh sample to DO sensor).
7. When the readings on the display stabilize, record them on the Site Visit Form.
8. Turn off meter, rinse probe. Place about 1/8 inch of tap water or stream water in the calibration/transport cup. Replace sensor guard with the calibration/transport cup. (Do not store the probe sensors in DI water).

1.3 Grab Sample Collection Protocol

OB grab samples will be collected on days 3, 7 and 10 at all sites using the 20mL vials. Take care to minimize sun exposure the OB samples. On day 7, Bacteria and wastewater tracers grab samples will be collected at all sites. The 100mL containers are for bacteria and the 1" amber glass bottles are for the wastewater tracers. Nitrile gloves must be worn for all grab sampling.

1. Fill out the sample bottle labels using a waterproof marker (Parameter, Sample ID, and Collection Date & Time).
2. Stand facing upstream in the center of flow. Be sure you are upstream from *any disturbances* to avoid contaminating the sample.
3. For wastewater tracers and OB grab samples, rinse sample bottles 3 times with the stream water by partially filling the bottle, placing the lid over the bottle, and shaking several times. Then pour the water out downstream (behind you). Note: **DO NOT** triple rinse the bacteria bottles.
4. Fill the bottles with stream water by completely submerging the sample bottle into the water upside down and righting it under water, as close to the streambed as possible without disturbing the sediment. Raise the bottle up through the water column as it fills, at a rate that allows the bottle to fill completely before it reaches the water surface. Recap tightly.

Collecting a water grab sample.

5. Be sure lids are tight and that no leaking will occur.
6. Keep bacteria and wastewater tracer samples in a cooler on ice. The ice should be stored in 1-gallon Ziploc freezer bags. Store OB grab samples at room temperature in a dark container.
7. Record the water chemistry sampling event on the Site Visit Form.

1.4 Stream Flow Measurement Protocol

Stream Flow will be measured days 3, 7 and 10 at the nine sites indicated in the work plan. The process consists of several water depth and water velocity measurements which are used to calculate stream discharge.

1. Select a good cross section of the stream. Things to consider:
   - Channel is relatively straight (not on a bend)
   - Waters is as smooth as possible (not turbulent or in a riffle)
   - Water is moving downstream across the entire width (no backwater areas)
   - There are no undercut banks or section with obstacles like large rocks or debris, if possible

2. String the measuring tape across the stream perpendicular to the flow with the zero end of the tape at the left bank (looking downstream). Secure the tape with bank pins or objects on the bank. Make the tape tight enough so that it doesn't sag in the middle.

3. Record the measurement on the tape at the left and right edges of water (wetted edge). Measurements should be in tenths of a foot. Use these numbers to determine the wetted width of the stream channel. Leave the tape in place.

   Example:
   Left bank tape reads 0.5 ft. Right bank tape reads 11 ft. 11 – 0.5 = 10.5 ft wetted width).

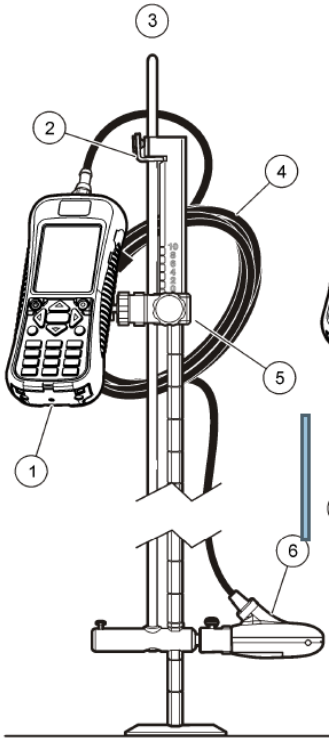
4. Based on the wetted width of the channel, determine the distance between measuring points. You will need a minimum of 20 measurements.
   Example:
   10.5 ft ÷ 20 = 0.525 ft. Round down to 0.5. Take measurements every 0.5 ft.

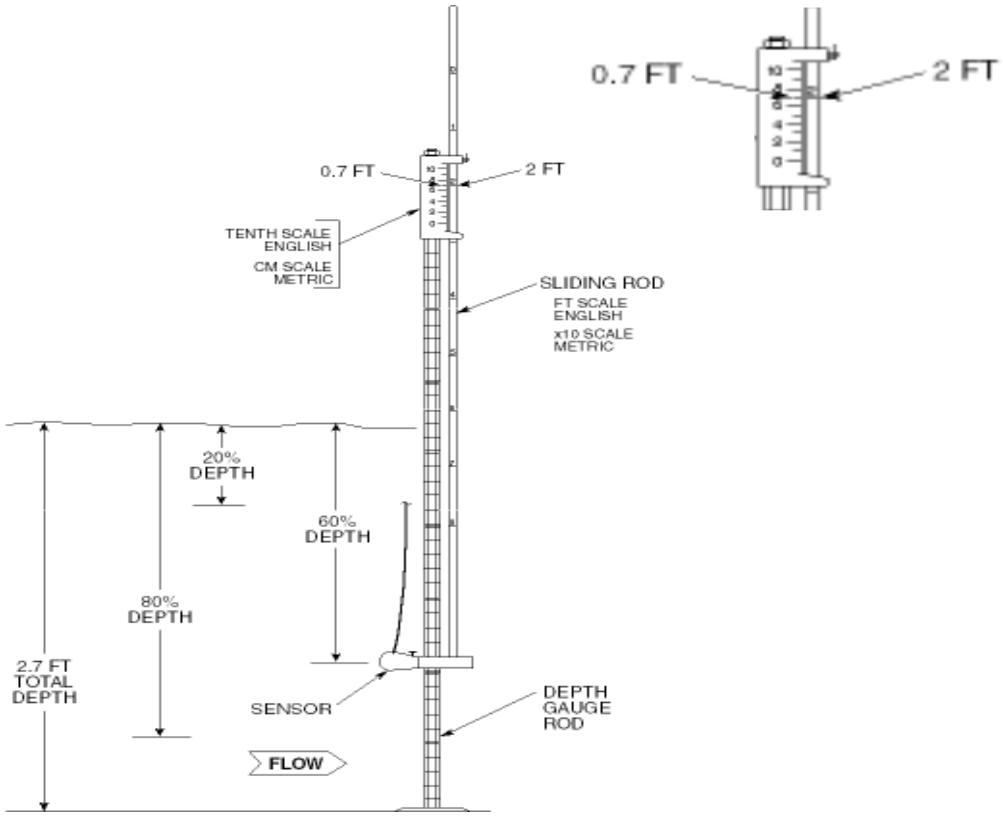
5. Start at the wetted edge of the left bank and record the tape measurement at that location with a "zero" depth on the field sheet. Work your way across the stream to the right bank, recording tape distance ("Distance from Initial Point" column) and measuring water depth and velocity with the OTT meter at each interval. (Instructions for specific use of the OTT meter on next page). Record the water depth and velocity in the appropriate columns on the field form. You should finish on the right bank wetted edge with a zero measurement and should have at least 20 depth measurements.

Operating the OTT Meter

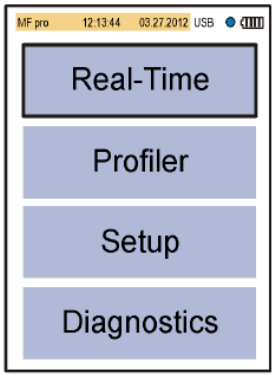
1. Attach the sensor to the top setting rod. Make sure the meter is vertical, so that the cord comes out the top of the meter. Make sure the thumb nut that attaches the sensor to the top setting rod is very tight.

2. Push the **Meter Power** button (1) until an audible beep is heard. The meter does a self-test and the display shows the results. If the meter fails the self-test, the display shows FAIL next to the failed parameter.
3. When the self-test is complete, push **OK** to go to the Main Menu.
4. Set the rod at the first interval determined by the stream width measurement. Note the height of the water at the depth gage rod.
5. Each single mark represents 0.10 foot, each double mark represents 0.50 foot, and each triple mark represents 1.00 foot. If the height of water is between to marks, estimate to the nearest .05 foot.
6. Line up the foot scale on the sliding rod with the tenth scale on the top of the depth gage rod. If, for example, the total depth of the water is 2.7 feet, then line up the 2 on the foot scale with the 7 on the tenth scale. This will set the sensor at 60% of the stream depth.





7. Push **OK** when **Real Time** is highlighted. A screen will appear which shows a graph of velocity. The graph will record the velocity for 20 seconds. At the end of 20 seconds, the velocity will be displayed in ft/sec on the screen. Enter this number in the stream discharge field form in the "Velocity" column.



8. When the last station is complete, press (1) the **Power Meter** button once then **Right Arrow**, **yes**, **OK** to turn off the meter.
9. Remove the sensor from the top setting rod (make sure the thumb screw is snug tight on the sensor, unplug the meter and place both the meter and sensor into the carrying bag.

STREAM DISCHARGE FORM

Modified 8/11/13 GLW QD

Stream Name: Bozeman Creek			**Station ID:** BOZMC.03	
Site Description: Downstream Goldenstein Ln			**Date:** 8/10/12 **Time:** 10:15	
Personnel/Team Members: Santa Claus, Tooth Fairy, Easter Bunny				
Staff Gage at Site? NO	**Staff Gage Reading:** —		**Confirm the Flow Meter is in Feet/Second?** YES	
Tape at Left Wetted Edge: 2.0	**Tape at Right Wetted Edge:** 6.0		**Stream Width (wetted edge - wetted edge):** 4.0 ft	
Section # (Start at Left)	**Tape Reading (Feet)**	**Water Depth (Feet)**	**Velocity (Feet/second)**	**Notes**
1 (Left Bank)	2.0	0	0	Left bank wetted edge
2	2.2	0.32	0.51	
3	2.4	0.71	0.62	
4	2.6	0.80	0.71	
5	2.8	0.45	1.21	
6	3.0	0.57	1.07	
7	3.2	0.41	1.13	
8	3.4	0.63	0.89	
9	3.6	0.57	0.72	
10	3.8	0.60	0.64	
11	4.0	0.55	0.73	
12	4.2	0.72	0.51	
13	4.4	0.81	0.83	
14	4.6	0.79	0.94	
15	4.8	0.73	0.97	
16	5.0	0.74	1.01	
17	5.2	0.52	0.81	
18	5.4	0.41	0.72	
19	5.6	0.32	0.43	
20	5.8	0.29	0.27	
21	6.0	0	0	Right bank wetted edge
22				
23				
24				
25				

NOTE:

Begin measurements from the left bank (determine left bank while looking downstream).
Initial point is often the tape reading of the waterline (wetted edge) & has no depth or velocity to measure.
At points where there is stagnant water or backflow effects, begin and end measurements at the edge of where positive flow can be measured.
Read depths on wading rod ignoring the "pile-up" effect of water on the rod.
Velocity is measured at six-tenths (0.60) depth from the water surface by moving the probe support so that the foot indicator marks align with the proper scale reading (in tenths of a foot).
20 cross-sections are adequate to reduce the level of error.
Space sections so none contain more than 10% of the flow. Ideal measurements have less than 5% in a section.

2. Lab Protocols

2.1 Optical Brighteners (Time-Integrated)

The OB pads will be processed according to the schedule in **Table 1**. Nitrile gloves should be worn for all steps and care taken not to cross contaminate samples.
Note: Minimize all exposure to light at each step because OBs photodegrade.

Prepare Standards:

This method is semi-quantitative. Samples will be compared to standards – pads exposed to OBs of known concentration.

1. Saturate one pad in DI water.
2. Create two solutions of laundry detergent in DI water (concentrations still TBD – this may require some trial and error). Update: 50pmm and 100ppm were used.
3. Saturate one pad in each solution.
4. Wring each pad out and set aside to dry in a dark space.
5. Once the pads are dry, staple to a piece of cardboard from lowest concentration to highest. Label them “absent”, “present” and “highly-present” on the cardboard below the corresponding pad.

Initial Check:

After field days 3, 7 and 10, compare the newly collected pads to prepared standards.

1. Set up the black light in a dark room.
2. For each site, there are two pads unless one was lost or damaged in the field. If both are in usable condition, use the brighter of the two. Discard the extra.
3. Compare the sample pad to the prepared standards.
4. Record “absent,” “present,” or “highly-present” accordingly in the “Wet” column of the data sheet.
5. Photograph the pad under the black light. Record the image number on the data sheet.

Note: if there are only flecks of fluorescence, it is likely due to contamination. Make note on the data sheet.

Drying:

After the initial check, all pads will be dried at room temperature in the dark. Method will depend on the storage space available.

Analysis of Dried Pads:

After all the pads are fully dry:

1. Set up the black light in a dark room.
2. Compare each sample pad to the prepared standards.
3. Record “absent,” “present,” or “highly-present” accordingly in the “Dry” column of the data sheet.

   Note: if there are only flecks of fluorescence, it is likely due to contamination. Make note on the data sheet.

4. Photograph each pad under the black light. Record the image number on the data sheet.
5. Wrap each pad securely in tin foil. Label the outside of the packet with the sample ID. These will be given to Dr. Bala for potential future use.

2.2 Optical Brighteners (Fluorometry)

The OB grab samples will be processed within a day of sample collection following an adaption of the California Water Board's protocol for "Measuring Optic Brighteners in Ambient Water Samples Using a Fluorometer" (https://www.waterboards.ca.gov/water_issues/programs/swamp/docs/cwt/guidance/3414.pdf). Rather than using a standard fluorometer and cuvettes, a Synergy Hybrid Reader HI and analysis trays will be used. The machine will be set to emit light at 360nm and to record light at 420nm.

Tray Preparation:
Note: Take care not to touch the bottom of the trays. This will impact results.

1. Load the first well, A1, with 200ul of DI water as a blank.
2. Load the following wells in the first row with a series of standard solution prepared with laundry detergent (record what type used). Use at least four standards that cover the expected range of concentrations.
3. Starting at B1 and working across rows, load the wells with 200ul of sample. Load three wells per site. Be sure to record what is in each well.

Sample Analysis:

1. Open Gen5 2.09 on the desktop
2. Open BIOM210 template. The Synergy Hybrid Reader is already programed to function at our specified wavelengths. The tray deck will open on the reader.
3. Load the tray, with the lid off. Make sure A1 on the tray aligns with A1 on the tray deck.
4. Press OK. The tray deck will close and run the samples.
5. Save the experiment in the BIOM210 folder.
6. Use the dropdown menu to switch to the blanked version of the data table.
7. Export to Excel by clicking the Excel logo and save to the BIOM210 folder and thumb drive.
8. Expose to UV and repeat steps 3-7 as needed:
    a. Expose samples directly to UV light for 5 minutes and then measure fluorescence again. Calculate the % reduction in fluorescence after 5 minutes of UV exposure. If < 8%, conclude the sample is negative for optical brighteners. If >30%, conclude the sample is positive for optical brighteners. If between 8-30%, continue to Step 8b
    b. Expose samples directly to UV light for 5 more minutes and then measure fluorescence again. If the ratio is ≥1.5, conclude that the sample is negative for optical brighteners. If the ratio is <1.5, conclude that the sample is positive for optical brighteners.
9. Log off
10. Record the machine use time in the notebook next to the reader. Leave the reader on.

2.3 E. coli and Coliform Bacteria

We will be using IDEXX's Colilert Quanti-Tray 2000 procedure, supplies and equipment. This is an EPA approved method for estimating Most Probable Number of coliform bacteria and *E. coli* in water samples. The following instructions and photos are adapted from https://idexxcom-live-b02da1e51e754c9cb292133b-9c56c33.aldryn-media.com/filer_public/d5/f8/d5f81805-8ceb-4893-b0b7-28b95db8ffab/colilert-procedure-en.pdf.

Introduction

Colilert simultaneously detects total coliforms and *E. coli* in water. It is based on IDEXX's proprietary Defined Substrate Technology. When total coliforms metabolize Colilert's DST nutrient-indicator, ONPG, the sample turns yellow. When E. coli metabolize Colilert's DST nutrient-indicator, MUG, the sample also fluoresces. Colilert can simultaneously detect these bacteria at 1 cfu/100 mL within 24 hours even with as many as 2 million heterotrophic bacteria per 100 mL present.

Storage

Store the Colilert supplies at 2–30°C away from light.

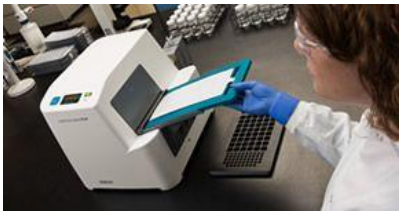
Quanti-Tray Enumeration Procedure

1. Wearing gloves, lab coat and eye guards, separate a single nutrient pack, point top of pack away from you and bend back the top to crack open the pack. (Avoid breathing the dust.)
2. Unscrew the lid of a 100 mL water sample and keeping the lid sterile, add contents of one nutrient pack to a 100 mL water sample in a sterile vessel.
3. Cap vessel and gently shake (without creating air bubbles) until dissolved.
4. Pour sample/reagent mixture into a Quanti-Tray*/2000 and seal in an IDEXX Quanti-Tray Sealer.
5. Use a sharpie to label the back of the tray with the sample date, time and place of collection and replicate number. Write along the top edge of the tray, so you aren't writing over any cells.
6. Place the sealed tray in a 35±0.5°C incubator for 24 hours.
7. Read results according to the Result Interpretation table below, using the Comparator provided to assess whether cells are sufficiently yellow to be positive. Count the number of large positive wells (including the top largest cell) and the number of small positive wells and record the results.
8. Load the tray into the UV light box (with light turned OFF) and be sure the black fabric flap is down. Turn light on and read the tray through the protective eye guard. Record the results, turn off light and then remove tray from box. *Never look directly at the UV light (without the protective eye guard).*
9. Refer to the MPN table provided with the trays to obtain a Most Probable Number of coliform and of E. coli. Record the results.
10. Still wearing gloves, bag the trays in an autoclave bag, rest in an autoclave tray and autoclave at liquid setting for 20 minutes. Dispose of in accordance with laboratory's practice (with other autoclaved waste).

Result Interpretation

Appearance	Result
Less yellow than comparator	negative for Total coliform and *E. coli*
Yellow ≥ comparator	positive for Total coliform
Yellow & fluorescence ≥ comparator	positive for E. coli

Step 4a: Pour into Quanti-Tray 2000



Step 4b: Seal using a Quanti-Tray Sealer

Step 8: Read the *E. coli* results (fluorescence shown here)

Notes

• Colilert results are to be read after 24 hours of incubation.
• However, if the results are ambiguous to the analyst based on the initial reading, incubate up to an additional four hours (but not to exceed 28 hours total) to allow the color and/or fluorescence to intensify.
• Positives for both total coliforms and E. coli observed before 24 hours and negatives observed after 28 hours are also valid.
• In addition, laboratories may incubate samples for additional time (up to 28 hours total) for their convenience.

Procedural Notes

• This insert may not reflect your local regulations. For compliance testing, be sure to follow appropriate regulatory procedures. For example, samples run in other countries are incubated at 36±2°C for 24–28 hours.
• If a water sample has some background color, compare inoculated Colilert sample to a control blank of the same water sample.
• If sample dilutions are made, multiply the MPN value by the dilution factor to obtain the proper quantitative result. Use only sterile, nonbuffered, oxidant-free water for dilutions.
• Colilert is a primary water test. Colilert performance characteristics do not apply to samples altered by any pre-enrichment or concentration.

• In samples with excessive chlorine, a blue flash may be seen when adding Colilert. If this is seen, consider sample invalid and discontinue testing.
• Aseptic technique should always be followed when using Colilert. Dispose of in accordance with Good Laboratory Practices.

2.4 Mass Spectrophotometry

The wastewater tracer grab samples will be processed and analyzed by Michelle Pond and Dr. Bala's lab on Day 7, following sample collection. An outline of the protocol is below:

Sample pretreatment:

1. Prior to sample preconcentration, to the water samples collected, add 1mL of 1% formic acid in water solution and mix well.

Sample preconcentration:

1. Attach the 1L glass bottles containing the acidified water to one end of the Solid Phase Extraction setup.
2. Fresh Oasis HLB cartridges should be used for each sample.
3. After passing the entire contents of the sample bottle through the SPE cartridge using vacuum suction, remove the cartridge and place it in solid phase extraction manifold.
4. Elute the cartridge with 8 mL of 0.1% formic acid in water: acetonitrile (0:80).
5. Evaporate the SPE eluate in a vacuum centrifuge at 45°C.
6. To the residue add 100µL of water: acetonitrile (50:50)

LC-MS analysis:

1. Transfer the reconstituted samples from the last step into clean LC-MS vials
2. Place the vials in Agilent 6538 MS equipped with 1290 UPLC.
3. LC-MS conditions:
4. Column: Waters HSS-T3
5. Mobile phase: 0.1% formic acid in water and acetonitrile

		Sampling Event 1														
		DAY 3 - 9/25/2018					DAY 7 - 9/29/2018					DAY 10 - 10/2/2018				
		H_20 temp	pH	SC	DO	DO	H_20 temp	pH	SC	DO	DO	H_20 temp	pH	SC	DO	DO
	Site Name	C		uS/cm	%	mg/L	C		uS/cm	%	mg/L	C		uS/cm	%	mg/L
Bozeman Ck.	BC-Canyon	5.7	7.74	214	90.8	11.4	4.7	7.99	217	80.0	10.3	7.4	7.96	213	82.1	9.9
	BOZMC03	8.0	7.88	277	91.3	10.8	6.3	7.96	275	82.9	10.2	7.4	7.48	255	86.2	10.4
	BOZMC03a	7.9	7.99	281	90.0	10.7	6.3	8.06	279	79.1	9.8	8.0	7.98	274	83.5	9.9
	BOZMC-MDWLK4	7.2	7.84	291	91.3	11.0	6.9	8.07	299	80.7	9.8	8.4	8.18	296	84.3	9.9
	BOZMC-MDWLK2	7.0	7.76	297	90.8	11.0	6.8	8.08	300	82.8	10.1	8.3	8.04	294	83.8	9.8
	BOZMC-KagyPark	6.8	7.57	301	90.3	11.0	6.7	8.07	301	82.0	10.0	8.2	8.29	287	84.1	9.9
	StormDrain-EL	NOT MEASURED					NOT MEASURED					NOT MEASURED				
	BOZMC-ELCTS3	10.0	7.90	312	89.0	10.0	7.3	8.14	309	84.1	10.8	8.1	8.17	316	83.6	9.9
	BOZMC-IPRD	10.0	8.06	313	87.4	9.9	7.1	8.08	306	83.0	10.0	7.9	7.78	307	84.3	10.0
	BOZMC-ELCTHTS1	9.9	7.91	315	89.0	10.1	7.1	8.06	307	83.1	10.1	8.0	8.08	307	86.2	10.2
Matthew Bird Ck.	MTWBC02	10.1	7.56	205	88.3	10.0	6.0	7.62	228	77.8	9.7	9.2	7.82	225	77.7	8.9
	MTWB-xing	10.2	7.26	208	88.7	10.0	5.1	7.65	231	79.1	9.8	9.3	7.79	228	78.1	9.0
	MTWB-SD3322	10.2	7.79	213	89.5	10.1	6.4	7.80	261	80.9	9.9	9.3	7.81	236	79.4	9.1
	MTWB-SD3318	10.2	7.92	206	86.8	9.8	6.2	7.76	242	79.0	9.8	9.3	7.86	237	79.8	9.1
	MTWB-Trib	NOT MEASURED					NOT MEASURED					NOT MEASURED				
	MTWB-SW106	10.2	7.73	214	88.1	9.9	6.1	7.82	241	81.1	10.1	9.4	7.94	238	79.9	9.1

		Sampling Event 2														
		DAY 3 - 10/23/2018					DAY 7 - 10/27/2018					DAY 10 - 10/30/2018				
		H_20 temp	pH	SC	DO	DO	H_20 temp	pH	SC	DO	DO	H_20 temp	pH	SC	DO	DO
	Site Name	C		uS/cm	%	mg/L	C		uS/cm	%	mg/L	C		uS/cm	%	mg/L
Bozeman Ck.	BC-Canyon	3.1	7.67	311	85.6	11.5	5.6	8.02	199	84.7	10.6	3.4	7.87	213	81.5	10.9
	BOZMC03	4.5	7.58	258	84.7	11.0	7.3	7.84	241	81.6	9.8	4.2	7.90	250	84.1	11.0
	BOZMC03a	5.4	7.86	263	80.7	10.2	7.3	7.85	247	81.4	9.8	4.7	7.83	258	78.2	10.1
	BOZMC-MDWLK4	6.4	7.88	288	94.7	11.7	7.4	7.72	275	83.8	10.1	4.9	7.87	280	81.0	10.4
	BOZMC-MDWLK2	6.5	7.89	290	83.0	10.2	7.4	7.72	276	83.3	10.0	4.9	7.84	281	80.5	10.3
	BOZMC-KagyPark	6.3	7.85	290	79.4	9.8	7.6	7.75	277	81.7	9.8	4.9	7.99	281	81.1	10.4
	StormDrain-EL	8.3	8.03	541	82.5	9.7	9.0	7.97	538	80.0	9.2	6.7	8.05	547	79.7	9.7
	BOZMC-ELCTS3	7.0	7.96	335	86.0	10.4	7.9	7.89	312	83.1	9.8	5.4	8.00	284	83.3	10.5
	BOZMC-IPRD	6.5	7.98	280	84.7	10.4	7.9	7.94	289	85.7	10.2	5.3	7.94	293	82.5	10.5
	BOZMC-ELCTHTS1	6.8	7.81	310	84.7	10.4	7.9	7.81	289	84.3	10.0	5.2	7.98	300	82.7	10.5
Matthew Bird Ck.	MTWBC02	5.9	7.20	474	73.2	9.1	8.6	7.25	477	81.6	9.5	6.2	7.77	483	76.2	9.5
	MTWB-xing	5.7	7.48	474	80.2	10.0	9.0	8.01	474	84.2	9.7	6.0	7.73	481	76.9	9.6
	MTWB-SD3322	5.6	8.22	481	70.1	9.0	9.2	7.82	482	90.5	10.4	5.6	7.81	489	78.2	9.8
	MTWB-SD3318	5.5	8.15	481	ND	ND	8.9	7.83	486	83.3	9.6	5.4	7.85	496	74.5	9.4
	MTWB-Trib	5.1	7.43	538	73.1	9.3	10.1	7.86	526	68.1	7.7	4.7	7.86	538	70.4	9.0
	MTWB-SW106	5.5	7.82	484	80.5	10.1	8.8	7.93	495	83.2	9.7	6.1	7.83	601	71.7	9.2

	Sampling Event 3, DAY 14 - 2/12/2019				
	H_20 temp	pH	SC	DO	DO
	C		uS/cm	%	mg/L
BC-Canyon	0.0	7.50	230.2	80.5	11.8
LIMC-GPARK	0.0	7.59	456.7	11.6	79.2
SPRINGC-TUCK	3.3	7.68	334.8	91.2	12.2

	Sampling Event 4, DAY 14 - 3/5/2019				
	H_20 temp	pH	SC	DO	DO
	C		uS/cm	%	mg/L
BC-Canyon	0.0	8.03	237.9	84.9	12.3
LIMC-GPARK	0.0	7.72	452.8	80.7	11.8
SPRINGC-TUCK	0.0	7.75	332.8	85.3	12.2