**WRP Project:** Understanding the contribution of different microbial sources to surface water for informed management of waterborne pathogens in Wyoming

**2019-2022**

**PI:** Dr. Sarah Collins, UW Dept of Zoology and Physiology
**Co-PI:** Dr. Bledar Bisha, UW Dept of Animal Science

**Abstract:** Microbial pollution is a major contributor to water quality impairments in Wyoming rivers, and these impairments are identified using the fecal indicator bacteria *E. coli*. In this project, we evaluated methods for understanding what pollution sources contribute to impairments in two focal watersheds, the Laramie River in Albany County, and Fish & Flat Creeks in Teton County. During two summer field campaigns in the Laramie River (2020) and Teton County (2021), we collected a variety of samples that allowed us to compare methods for enumerating *E. coli*, evaluate the extent of antimicrobial resistance, apply next generation sequencing approaches to identify sources of fecal pollution, evaluate the utility of RNA coliphage approaches for microbial source tracking, quantify daily and seasonal fluctuations in bacterial loads, and compare microbial pollution and sources to watershed land use. Our results indicate that 1) different EPA approved *E. coli* enumeration methods can provide contrasting results, 2) wastewater is the primary source of fecal contamination to streams in Teton County, but wastewater contributions show strong seasonal variation that could be tied to hydrologic variability, 3) multiple animal sources seem to contribute to microbial pollution in Laramie River sites, and 4) different microbial source tracking methods provide consistent information about pollution sources. Our project trained three MS students and two undergraduate students. Finished products include two MS theses, one undergraduate thesis, seven conference presentations, two outreach presentations, and at least eight features in local news outlets. An additional MS thesis will be completed in 2023, and 1-3 papers will be submitted for publication in peer reviewed journals in the coming year.

## Students trained under this project and completed outputs

**Kelsey Ruehling**                    MS Zoology, August 2022

    Kelsey is currently a Natural Resource Analyst for WY DEQ based in Sheridan, WY

    Thesis: Understanding contributions of fecal bacterial sources in WY rivers to inform pollution management

**Harneel Kaur**                    MS Animal Science, August 2021

    Harneel is currently a lab technician in the WI Public Health Laboratory

    Thesis: Identification and characterization of antimicrobial resistance in indicator bacteria from the surface waters of WY

**Clara Bouley**  Undergraduate Assistant, 2020-2021
MS Animal Science, *in progress*, expected Spring 2023

After graduating, Clara plans to attend medical school

Thesis: Molecular characterization of select viruses from surface water and wastewater

**Emma Román**  Undergraduate Senior Thesis, Middlebury College, May 2022
Field and Lab Assistant, Summer 2021

Emma is currently a lab technician for the California Academy of Sciences and Stanford University and plans to pursue graduate studies in microbial ecology in the future.

Thesis: Diurnal fluctuations and effects of UV on *E. coli* in Jackson WY recreational streams

## Project Outputs:

## Conference Presentations:

Ruehling, K. et al. 2022. Agricultural land-use and flow variation influence bacterial loading in Wyoming streams. Front Range Microbiome Symposium, Fort Collins, CO. (Talk)

Ruehling, K., et al. 2022. Microbial Source Tracking (MST) study update. Wyoming Watershed Conference, Riverton, WY (Talk)

Bouley, C. et al. 2022. Source tracking via molecular characterization of FRNA coliphages. Front Range Microbiome Symposium, Fort Collins, CO. (Poster)

Román, E, K. Ruehling, S. Collins. 2022. The diurnal fluctuations and effects of UV on *E. coli* in Jackson, WY recreational streams. Middlebury College Undergraduate Spring Research Symposium (Poster)

Ruehling, K., N. Blouin, B. Bisha, S.M. Collins. 2022. Urban and hydrologic influences on bacterial communities and fecal pollution in Wyoming Snake River Tributaries. Joint Aquatic Science Meeting, Grand Rapids, MI. (Talk)

Kaur, H., K. Ruehling, S.M. Collins, B. Bisha. 2021. Characterization of antimicrobial resistance in indicator bacteria (*E. coli and Enterococcus* spp.) from surface waters of Wyoming. International Association for Food Production. Phoenix, AZ. (Talk)

Ruehling, K., H. Kaur, N. Blouin, B. Bisha, S.M. Collins. 2021. Understanding the contributions of microbial sources to surface water to inform management of waterborne pathogens in Wyoming. Society for Freshwater Science. Online. (Poster)
*Winner – best student poster in applied research

**Outreach Presentations:**

Collins, S. et al. Understanding the contribution of different microbial sources to surface water for informed management of waterborne pathogens in Wyoming. Wyoming Water Forum, September 2022

Ruehling, K. et al. Microbial source tracking study presentation. Teton Conservation District. May 2022

**News Features:**

"UW student tracking *E. coli* sources in 'impaired' Teton County waterways," Will Walkey, Jackson Hole Community Radio, August 27, 2021

"What's the Ruehling on Fish and Flat Creeks?" Teton Conservation District Blog, October 25, 2021

"What's behind fecal contamination in Fish and Flat Creeks?" Billy Arnold, Jackson Hole News & Guide, May 24, 2022

"UW research identifies sources of fecal contamination in Snake River tributaries," Caitlin Tan, Wyoming Public Radio, May 31, 2022

"Sewage is no. 1 source of identifiable fecal contamination in Fish and Flat Creeks," Billy Arnold, Jackson Hole News & Guide, June 2, 2022

"Human poop contaminating two Jackson-area creeks, says grad student," Ellen Fike, Cowboy State Daily, June 3, 2022

"Study determines human sewage as major fecal contaminant in select Teton County waterways," Will Walkey, Jackson Hole Community Radio, June 13, 2022

"Human waste in Jackson Hole's creeks sparks debate," Billy Arnold, Jackson Hole News & Guide, June 29, 2022

## Progress:

Methods and results for nearly all of our project activities are summarized in two published theses - Kelsey Ruehling's MS thesis and Harneel Kaur's MS thesis. Below, we provide highlights from each field campaign and associated lab results that are included in the theses, and indicate where in each thesis more details can be found about any of the methods or results. One piece of the project, using viral coliphages for Microbial Source Tracking, is the focus of Clara Bouley's incomplete thesis and is described below, but final results will be available when she defends her thesis in 2023.

Our project involved two field seasons: Summer 2020 sampling at sites in the Laramie River drainage and Summer 2021 sampling at sites on Fish and Flat Creeks. We had a slightly different focus for each field season with different types of data collected depending on project needs, local conservation district priorities, and personnel capacity.

**Laramie River 2020 data collection, laboratory methods, and results:**

We sampled seven sites with varying land cover in the Laramie River watershed in Summer 2020. The headwaters and small tributaries that feed the Laramie River are surrounded by forest and shrubland, while most of the area immediately around the main stem of the Laramie River is dominated by shrubland and cultivated land use, with some occasional development. We collected water at seven sites spatially distributed across the watershed to understand variability in bacterial communities within the watershed and across varying land use (Figure 1). We sampled at four locations on the Laramie River and two sites on the Little Laramie River. We also sampled Woods Creek, a tributary of the Laramie River near Jelm, WY. Two sites, one on the Laramie (LR4) and one on the Little Laramie (LL2) are located within river reaches that are listed as impaired by the Wyoming DEQ for high *E. coli* levels.
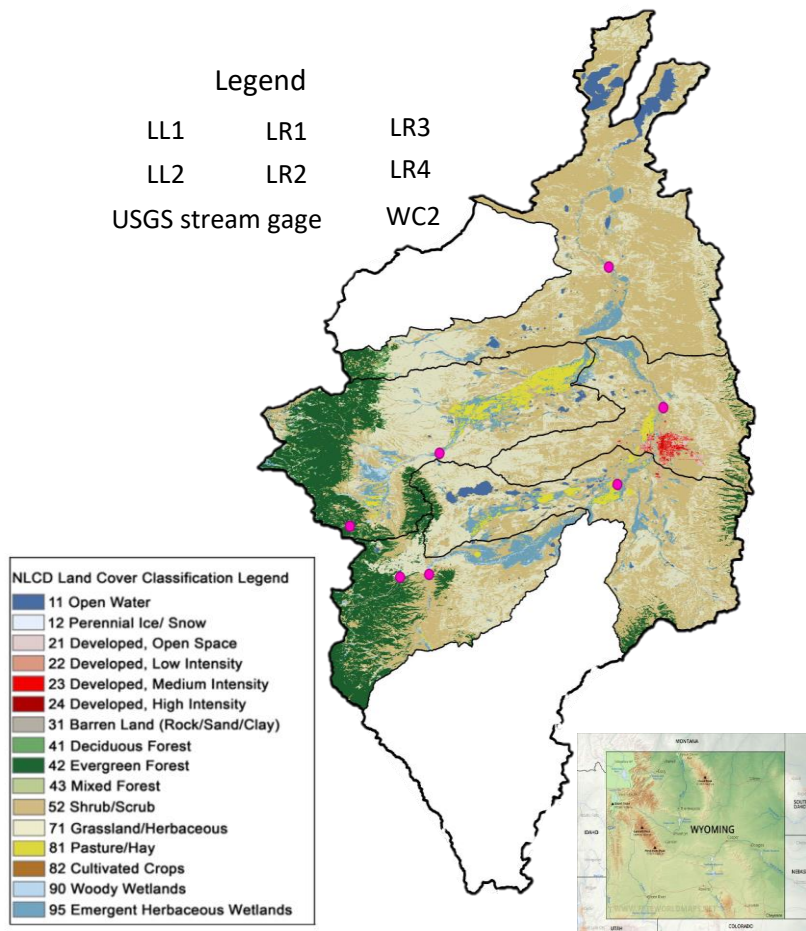


Figure 1. Map of Upper Laramie River watershed located in Southeastern Wyoming, land use on the map includes all NLCD classifications, but for land use analysis in this study categories were simplified into eight land cover types

In addition to spatial variation in land use, hydrology characteristic of snowmelt dominated ecosystems led to temporal variation in discharge from May – September 2020 at the USGS gage located on the norther edge of the city of Laramie between sites LR2 and LR3 (Figure 2). Peak flows occur during May and into mid-June during snow melt, then rapidly decrease with some variability throughout the summer.
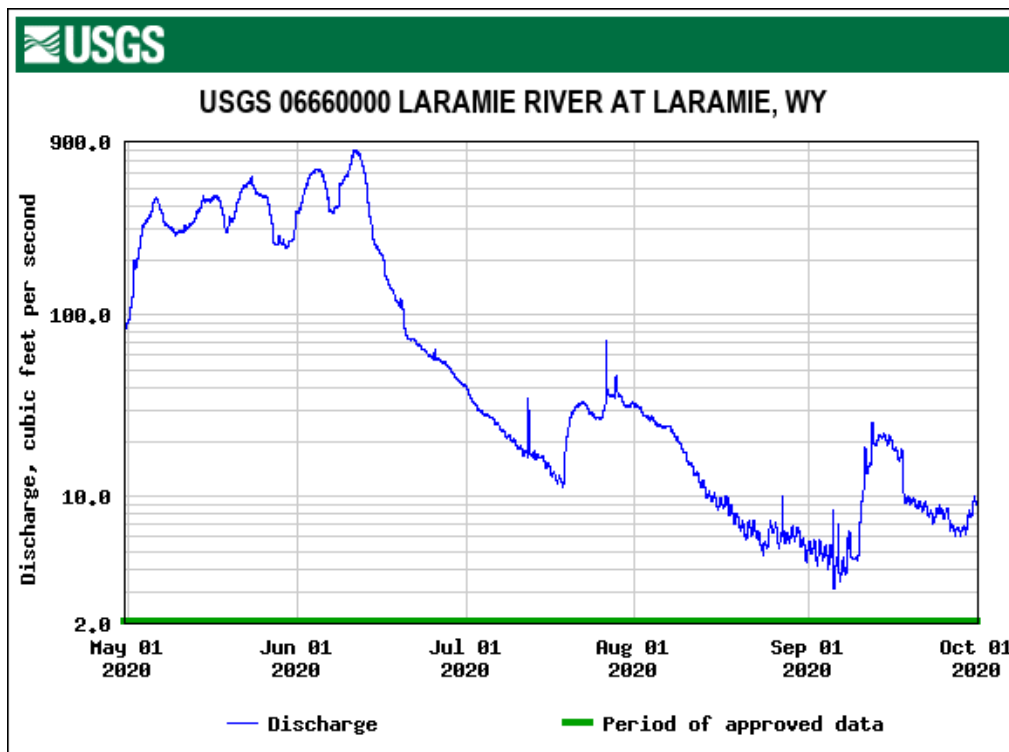


Figure 2: Summer 2020 discharge trends in the Laramie River. Graphic obtained from the U.S. Geological Survey (2022).

*Enumeration method comparison:* We quantified *E. coli* concentrations using two methods of enumeration, IDEXX Colilert-18 and EPA-1603 *E. coli* in water by membrane filtration technique using modified membrane-thermotolerant *Escherichia coli* agar (modified mTEC) (IDEXX, 2021; United States EPA, 2014). *E. coli* enumerations using the IDEXX Colilert technique were performed by the Wyoming Department of Agriculture Analytical laboratory (IDEXX, 2021). *E. coli* quantification using the EPA-1603 method was performed in the Bisha lab at the University of Wyoming (United States EPA, 2014). We processed field blanks using both methods but did not have positive and negative controls for the EPA-1603 method. However, 96% of 541 isolates collected via the EPA-1603 method were identified as *E. coli* via matrix assisted laser desorption ionization-time of flight mass spectrometry at the Colorado State University Bioanalysis and Omics laboratory.

No geometric means from the EPA-1603 method (circles and solid lines) exceeded the recreational standard of 126 MPN/100ml (indicated with the black dashed line), however geometric means for numerous sites exceeded the recreational standard based on IDEXX results (triangles and dashed lines) (Figure 3). The section of river that includes LL2 and LR4 are both already listed by Wyoming DEQ as impaired for high bacterial loads. However, LR2 and LR3 are not located within listed reaches. LR2 and LR3 are located on either end of the city of

Laramie, which has numerous public river access points that are popular recreation locations for fishing and swimming during the summer months.

Additional details about these methods and results can be found in Chapter 3 of Kelsey Ruehling's MS thesis.
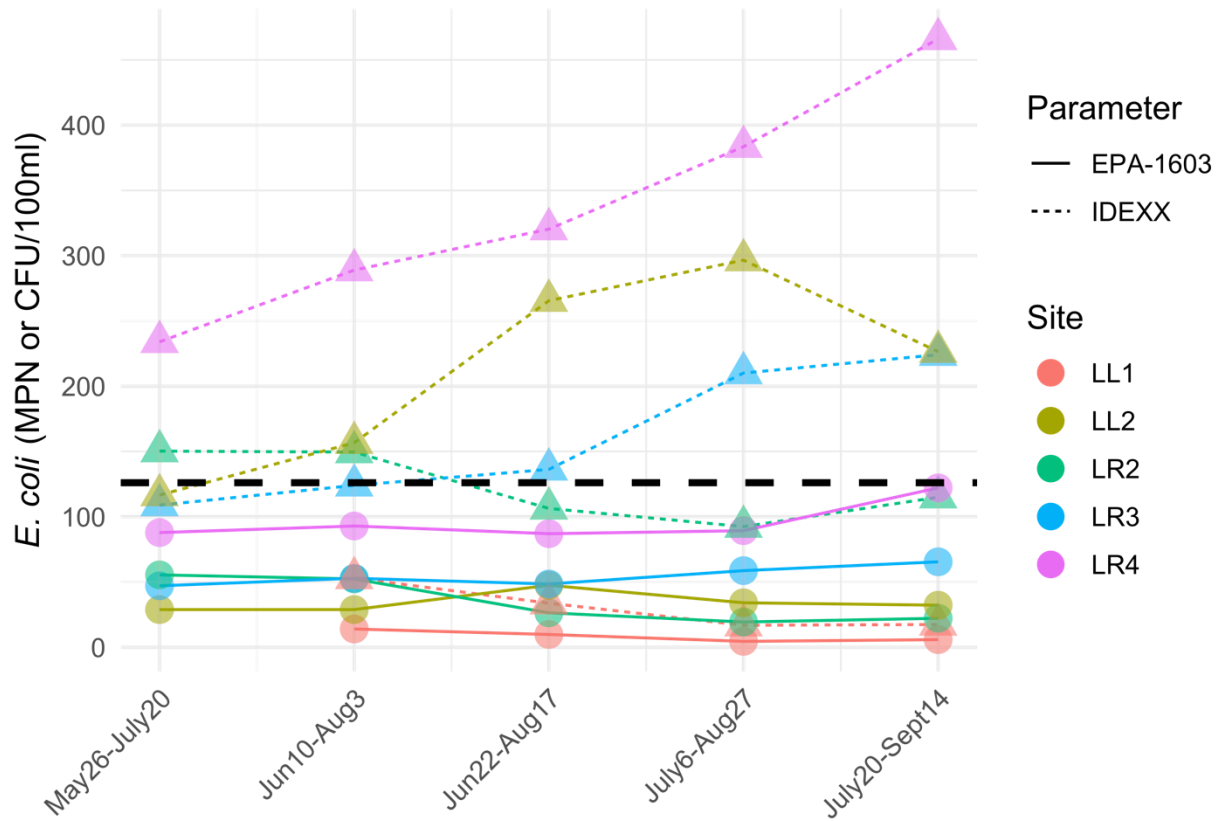


Figure 3: Enumeration data from the Laramie River for two different methods

*Microbial Source Tracking:* We characterized and analyzed the bacterial communities in water and fecal samples to evaluate spatial and temporal changes in aquatic communities and to identify likely inputs from fecal pollution. We sequenced the 16S rRNA gene to characterize the bacterial community in water and fecal samples. Polymerase chain reaction (PCR) and amplicon sequencing was performed by Novogene Corporation Inc. The V3V4 region of the prokaryotic 16S ribosomal RNA gene region was amplified using dual indexed primers 341F (5' CCTAYGGGRBGCASCAG- 3') and 806R (5'-GGACTACNNGGGTATCTAAT-3') (Caporaso et al., 2011). All PCR reactions were performed with the Phusion ® High-Fidelity Master Mix (New England Biolabs) and amplification processes followed Novogene proprietary methods.

Laramie water samples were compared to 190 fecal samples from 24 different host species collected from across Wyoming over a three-year period. We grouped samples into five different categories for visualization and analysis purposes. The domesticated livestock category includes cattle, horse, chicken, goat, donkey, pig, and sheep. Domesticated pets include cat and dog fecal samples. Wild ruminants include fecal samples from deer, moose, elk, pronghorn, and bighorn sheep. The wild animal category includes fecal samples from goose, bear, beaver,

raccoon, grouse, otter, small mammal, skunk, and wild horse. Fecal source contributions were evaluated using FEAST (Shenhav et al., 2019).

Our results suggest that domesticated livestock are the predominant contributor to fecal pollution across all sites, but that there is variation across sites and seasons, with pets, wastewater, wild animals, and wild ruminants also contributing in some sites at some times (Figure 4). The most significant sources of fecal bacteria to surface water were from domesticated livestock, these contributions were statistically higher than all other contributions (pairwise Wilcoxon test p- values < 0.001) except domesticated pet. Domesticated livestock proportions are primarily attributed to cattle and goat contributions, but also includes contributions from sheep, chicken, horse, and pig. There were no significant differences in domesticated animal contributions between sites in the watershed.

Wastewater contributions were significantly higher at LL2 and WC2, which are in rural regions of the watershed, compared to LR3, which is downstream of the City of Laramie's wastewater outfall. These high bacterial contributions from wastewater at LL2 may be the result of septic system usage, and WC2 may be the result of direct human defecation, as this site is located near a busy but undeveloped highway. Wild ruminant contributions were higher at WC2 compared to sites downstream. In addition to being located near a highway, WC2 is within USFS property and has high wild animal usage, particularly in the summer when ruminants are returning to higher elevations to forage.
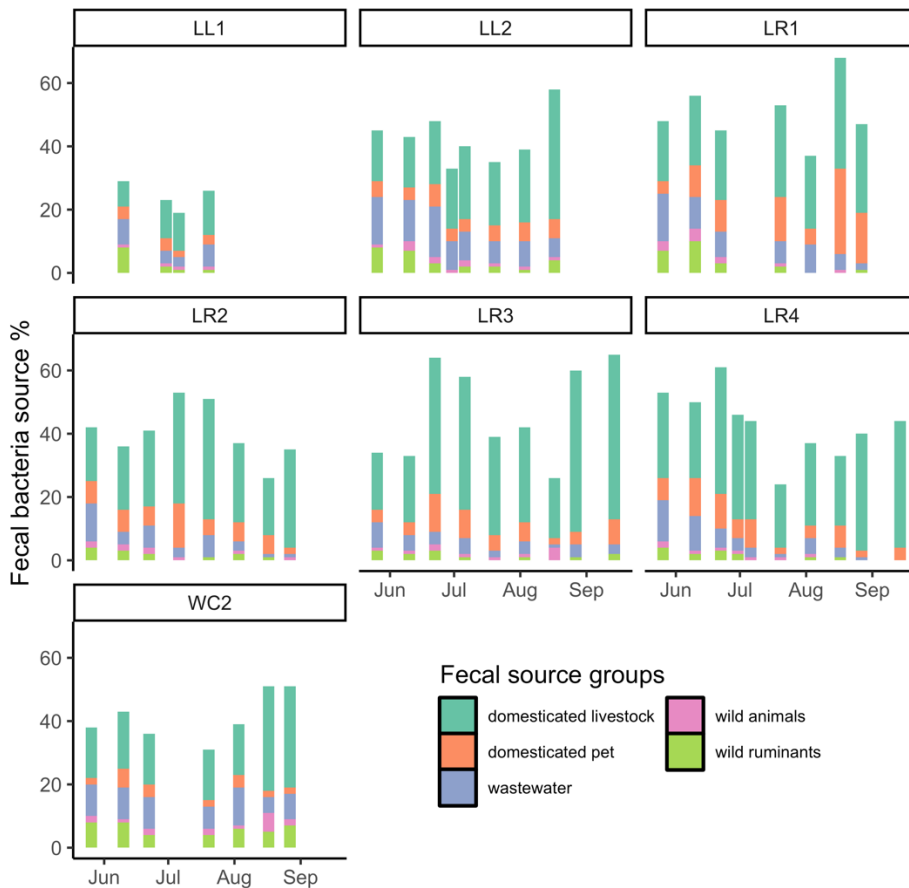


Figure 4: Microbial Source Tracking results for Laramie River sites

Additional details about these methods and results can be found in Chapter 3 of Kelsey Ruehling's MS thesis.

*Comparison of different indicator bacteria:* We compared enumeration data of two different fecal indicator bacteria. *E. coli* and *Enterococcus* spp. reside in gastrointestinal tract of warm-blooded animals and are frequently found in human feces and the environment. Higher concentrations of *E. coli* and *Enterococcus* spp. in environmental waters make them suitable indicators of fecal contamination for monitoring the water quality (US EPA, 2012).

We collected samples for both *E. coli and Enterococcus* spp. Enumeration during 5 sampling events Immediately after collection, water samples were placed on ice and transferred to the University of Wyoming's Food Microbiology Lab. The water samples were held at 4° C after reaching laboratory and processed within 24 hours from the time of collection. The bottles were shaken vigorously around 25 times to dispense the bacteria uniformly before filtering the water for bacterial isolation. *E. coli* was quantified using Method 1603 by United States Environmental Protection Agency (US EPA) (Method 1603, US EPA, 2002). Method 1600 provided by United States Environmental Protection Agency (US EPA) was used to detect Enterococcus in water samples based on the formation of colonies on the membrane filter surface (47 mm, 0.45 μm pore) (Method 1600, US EPA, 2002).

The US Environmental Protection Agency (EPA) and the US Food and Drug Administration (FDA) have both suggested utilizing common Escherichia coli and Enterococcus as fecal contamination indicators for recreational and irrigation water. The geometric mean of the indicator bacteria concentrations is used by both regulatory authorities (USEPA, 2018). The geometric means of *E. coli* and *Enterococcus* spp. were strongly correlated ($r2= 0.8902$), suggesting that both indicators had similar trends at all sampling sites.
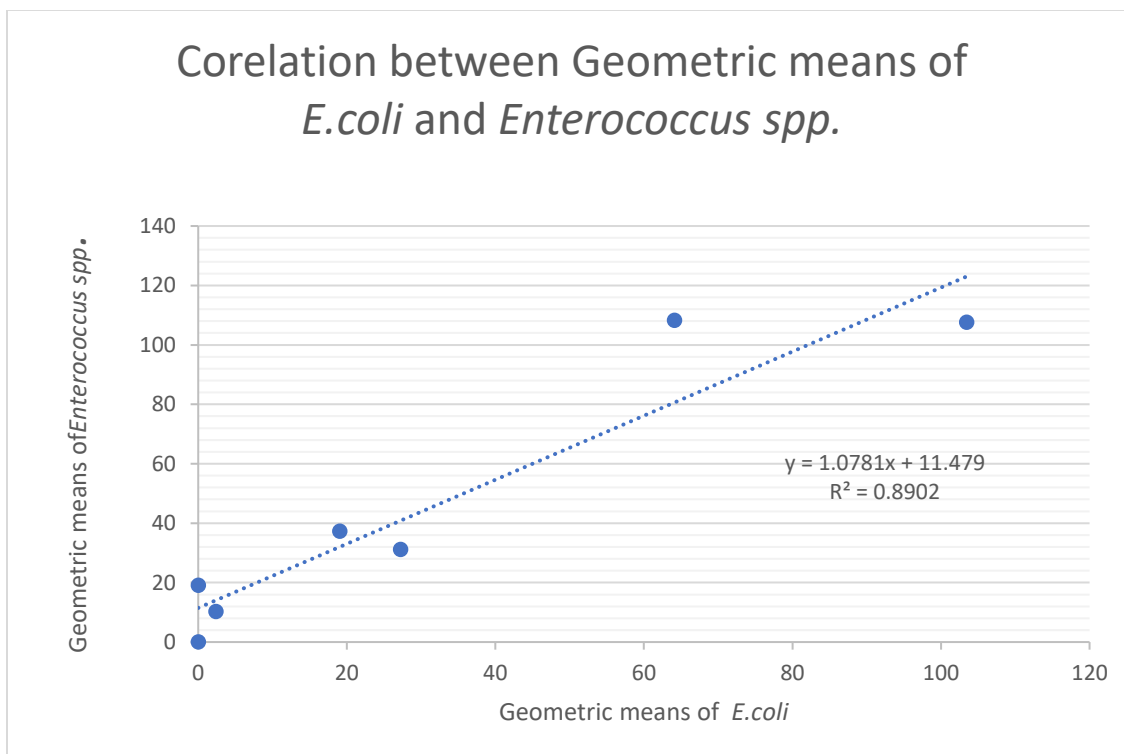


Figure 5: Comparison of two indicator bacteria

Additional details about these methods and results can be found in Chapter 2 of Harneel Kaur's MS thesis.

*Antimicrobial Resistance:* We determined antimicrobial susceptibility using broth microdilution (Sensititre) and the Gram Negative or Positive AST plate formats. Bacterial suspensions were prepared in demineralized water by transferring 3-5 colonies of pure cultures of isolates grown overnight on BHIA (BD, Franklin Lakes, NJ). The Sensititre nephelometer was then used to standardize bacterial suspensions to a 0.5 McFarland turbidity standard. After vortexing, ten µL of each solution were transferred to a tube of Cation Adjusted Mueller Hinton Broth (MHB) provided by the manufacturer, and the suspensions were vortexed once more. Sensititre Dosing Heads were used to replace the caps on MHB tubes. The Sensititre AIM™ Automated Inoculation Delivery System was used to load 50 µL of each Escherichia coli and Enterococcus spp. suspension into all wells of a Sensititre NARMS Gram negative (CMV1AGNF) and Gram-Positive (CMV3AGPF) plates (TREK Diagnostic Systems, Cleveland, OH) respectively and were examined for phenotypic antibiotic resistance. The Sensititre Gram negative plate (CMV1AGNF) each contained 14 different antimicrobial agents, i.e., ampicillin, amoxicillin-clavulanic acid, azithromycin, ceftriaxone, ciprofloxacin, cefoxitin, gentamicin, nalidixic acid, trimethoprim-sulfamethoxazole, ceftiofur, sulfisoxazole, streptomycin, tetracycline, and chloramphenicol. On the other hand, Sensititre Gram positive plate (CMV1AGPF) each contained 16 different antimicrobial agents i.e., tigecycline, tetracycline, chloramphenicol, daptomycin, streptomycin, tylosin (tartrate), quinupristin / dalfopristin, linezolid, nitrofurantoin, penicillin, kanamycin, erythromycin, ciprofloxacin, vancomycin, lincomycin, gentamicin. Each well of the Sensititre microtiter plate was inoculated according to the instructions of the manufacturer. The plates were then sealed with adhesive sealing and incubated at 37°C for 24 hours. With the use of a Sensititre Vizion Digital MIC Viewing System and the Sensititre Windows (SWIN) Software System (Version 3.3)., Minimum Inhibitory Concentrations (MIC) of isolates were calculated and recorded for each antibiotic after incubation.

We found spatial and temporal variation in antibiotic resistance patterns. Specific patterns for *E. coli* are summarized in Table 1. Additional details about these methods and results can be found in Chapter 2 of Harneel Kaur's MS thesis.

| Sampling Date | Isolate ID | AR Pattern |
|---|---|---|
| 5/26/2020 | LRLR (C) | Tri |
| | LRWC2 (A) | Tet |
| 6/10/2020 | LRLR4 (IU) | Tet |
| 6/22/2020 | LRLL1 (F) | Amp, Fox |
| | LRLR1 (I) | Amo, Amp, Fox |
| | LRLR2 (IZ) | Amo, Amp, Fox |
| | LRLR3 (IZ) | Amo, Amp, Tet, Tri |
| 6/30/2020 | LRLL2 (B) | Amo, Tet, Tri |
| | LRLR1 (IY) | Amo, Amp, Fox, Tet, Tri |
| | LRLR4 (IE) | Amo, Amp |
| 7/6/2020 | LRLL1 (D) | Amo, Amp, Fox, Tet, Tri |
| | LRLL1 (F) | Fox |
| | LRLR3 (D) | Amo, Fox, Tet, Tri |

| | | |
|---|---|---|
| | LRLR3 (G) | Amo, Amp, Fox |
| | LRLR4 (G) | Amo, Fox, Tet, Tri |
| | LRLR4 (V) | Amo, Amp, Fox, Tet, Tri |
| **7/20/2020** | LRLL2 (F) | Tet, Tri, Sul, Cip |
| | LRLL2 (I) | Tet, Tri |
| | LRLR1 (H) | Fox, Tet |
| | LRLR1 (I) | Tet, Tri, Sul |
| | LRLR2 (A) | Axo, Chl, Tet |
| | LRLR2 (C) | Chl, Axo, Tet, Tri |
| | LRLR3 (E) | Axo, Chl, Tet, Tri |
| | LRWC2 (A) | Chl, Tet, Tri |
| | LRWC2 (E) | Axo, Chl, Cip, Tet |
| **8/3/2020** | LRLR2 (C) | Tet |
| | LRLR3 (A) | Tet |
| | LRLR3 (F) | Tet |
| | LRLL2 (C) | Tet |
| | LRLR2 (B) | Tet |
| | LRLR4 (D) | Tet |
| | LRLR4 (E) | Tet |
| | LRLL2 (B) | Tet |
| | LRLL1 (A) | Tet, Amp, Chl |
| | LRLR1 (A) | Tet, Amp, Chl |
| | LRLL2 (A) | Tet |
| **8/17/2020** | LRLR4 (G) | Amp, Chl, Tet |
| | LRLL2 AT (C) | Amp, Chl, Tet |
| | LRLR4 (C) | Amp, Chl, Tet |
| | LRLR2 (A) | Tet |
| | LRLR1 (A) | Tet |
| | LRLL2 (I) | Amp, Chl, Tet |
| | LRLR2 (B) | Tet |
| | LRLR2 (C) | Tet |
| | LRLR3 (I) | Tet |
| | LRLR3 (H) | Tet |
| **8/27/2020** | LRLL1 (A) | Amp, Chl, Tet |
| | LRLL1 (B) | Chl, Tet |
| | LRLL2 (A) | Amp, Chl, Tet |
| | LRLL2 (B) | Amp, Chl, Tet |
| | LRLR1 (A) | Amp, Chl, Tet |
| | LRLR2 (G) | Amp, Chl, Tet |
| | LRLR2 (H) | Amp, Chl, Tet |
| | LRLR3 (H) | Amp, Chl, Tet |

| | LRLR3 (I) | Amp, Chl, Tet |
|---|---|---|
| **9/14/2020** | LRLL1 (E) | Tet |
| | LRLL2 (E) | Tet |
| | LRL3 (F) | Tet |
| | LRLR4 (I) | Tet |

Table 1: Antibiotic resistance patterns of *E. coli* isolates

**Teton County 2021 data collection, laboratory methods, and results:**

This study took place during the summer of 2021 at eleven sites with various land-use characteristics from across the Greys-Hoback watershed. Most sites were located on Fish and Flat Creeks, because sections of both of these streams had recently been listed as impaired. We collected water samples at four sampling locations on Fish Creek and five locations on Flat Creek, in addition to two tributaries of Flat Creek, Cache and Game Creeks. Sampling events occurred bimonthly from May 5, 2021 to September 26, 2021.
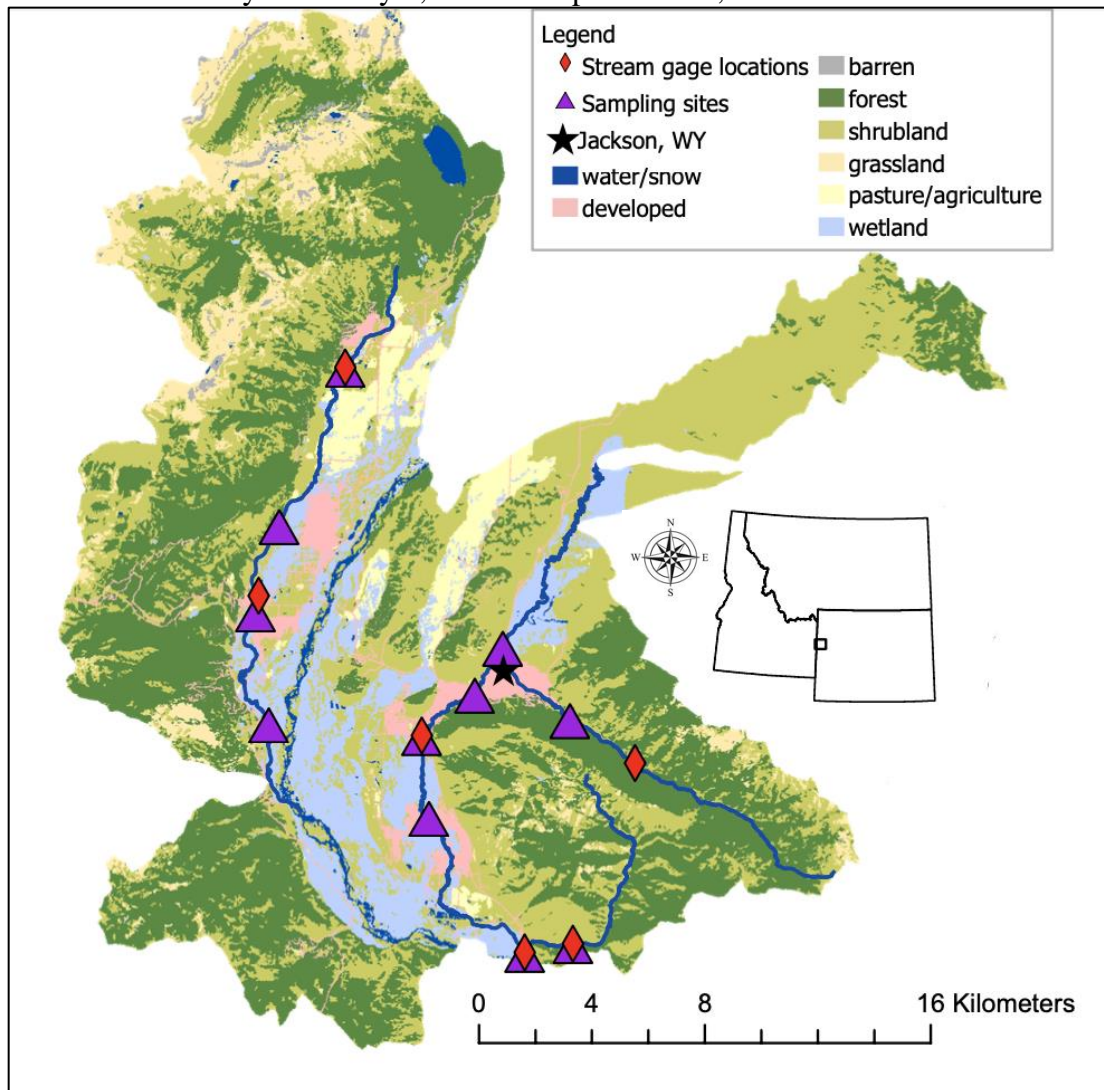


Figure 6: Map of sampling sites in Teton County

Sites includes in data collection included a variety of land cover and environmental conditions (Figure 7)
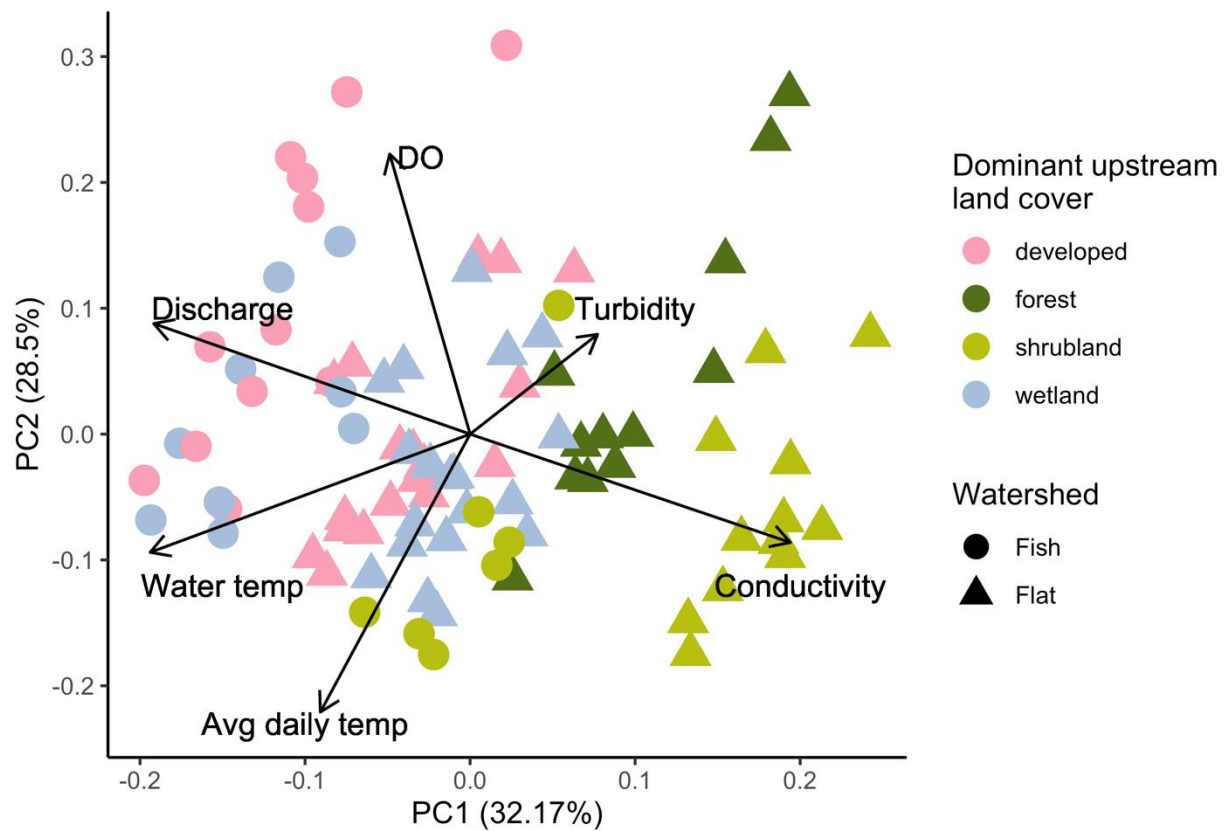


Figure 7. Principle Component Analysis of the environmental characteristics of samples collected at sites with different upstream land cover, denoted by point color, from the two watersheds, denoted by point shape.

*Enumeration:* Unlike in the Laramie River sites, we found that the two *E. coli* enumeration methods we compared produced extremely similar results in Teton County streams and used the IDEXX method for ease of data collection. *E. coli* concentrations varied over the course of the season and produced similar results to previous data from Wyoming DEQ (Figure 8).
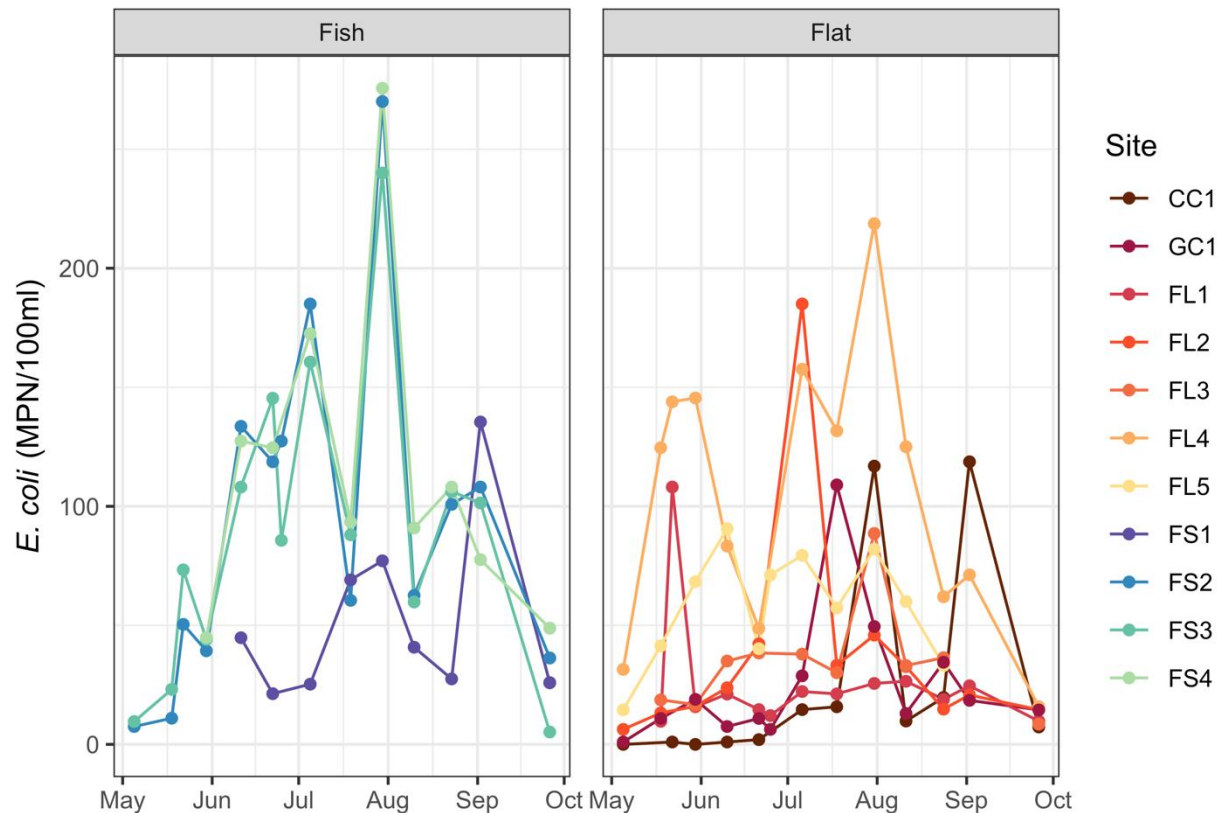
Figure 8. Temporal changes in aquatic *E. coli* concentrations at each sampling location on (A) Fish Creek and (B) Flat Creek using the IDEXX-Colilert method.

*Microbial Source Tracking:* We sequenced total DNA from surface water and fecal samples to understand changes in aquatic microbial communities and evaluate sources of fecal pollution. We filtered between 1 L and 2.5 L of water, depending on turbidity levels, on to a 0.45 µm sterile nitrocellulose filter (Pall corporation). We extracted the total DNA from the filters using the Qiagen DNEasy PowerWater kit according to manufacturer's instructions (QIAGEN, 2017). We stored genomic extractions at -40 to -80 °C prior to sequencing.

To generate a fecal reference library for evaluating pollution sources in our water samples we collected 104 fecal samples from 16 different animal hosts in Teton county during the 2021 sampling season. Samples were kept frozen at -60 °C until processing occurred. We extracted genomic material from fecal samples with DNEasy PowerSoil Pro kit according to manufacturer's instructions (Qiagen, 2017). We used wastewater influent samples (between 100-200 ml) from the Jackson and Aspen Pines (located near the town of Wilson and Fish Creek) wastewater treatment facilities as our representative sewage samples. These samples were filtered and kept frozen at -80 °C until processing. We extracted DNA from these wastewater samples following the same procedure as the surface water samples. We sequenced the 16S rRNA gene to characterize the bacterial community in water and fecal samples. Polymerase chain reaction (PCR) and amplicon sequencing was performed by Novogene Corporation Inc. The V3V4 region of the prokaryotic 16S ribosomal RNA gene region was amplified using dual indexed primers 341F (5' CCTAYGGGRBGCASCAG- 3') and 806R (5'-

GGACTACNNGGGTATCTAAT-3') (Caporaso et al., 2011). All PCR reactions were performed with the Phusion ® High-Fidelity Master Mix (New England Biolabs) and amplification processes followed Novogene proprietary methods.

We characterized and analyzed the bacterial communities in water and fecal samples to evaluate spatial and temporal changes in aquatic communities and to identify likely inputs from fecal pollution. Contributions from fecal source environments to surface water bacterial communities were evaluated using the 'Fast Expectation Maximization for Source Tracking' (FEAST, Shenhav et al., 2019) package in R. This model assumes the sink (surface water samples) are a convex mixture of known and unknown sources. FEAST assigns contributions for each source (fecal) environment in addition to determining the unknown portion of the sink environment.

We found strong seasonal patterns in fecal source contributions in both watersheds, that seem primarily driven by changes in hydrology. Sewage was the highest fecal bacterial contributor to aquatic bacterial communities, followed by cattle and canine feces (Figure 8). We found little variation in source contributions in our three replicate runs of FEAST. The highest proportions of sewage bacteria in surface water occurred during late May and early June (average wastewater bacterial contributions of 17%), coinciding with spring runoff in both Fish and Flat Creeks (Figure 10). There was a positive linear relationship between the sewage percentages and discharge in five sampling sites located along Flat Creek (adjusted $R2= 0.41$), with the highest discharge and source contributions occurring during May and June during the rising limb of the hydrograph. High sewage contributions in Fish Creek were not exclusive to May and June when run off was occurring. There were also instances of high contributions in August and September, though these high late summer contributions were not consistent throughout the watershed. Fecal contributions from other sources were relatively consistent and minor throughout the summer (Figure 9) and demonstrated no relationship to any of the climate or water quality parameters.

There were no significant differences in total fecal contributions between the four dominant upstream land cover groups. However, there were significant differences in fecal bacteria contributions between cover types for canine, goose, moose, and cattle. We found significantly higher contributions of canine fecal contributions in developed and wetland sites compared to forest and shrubland sites (pairwise Wilcoxon test, forest compared to wetland or developed $p < 0.01$, shrubland compared to wetland or developed $p < 0.001$). Goose contributions were significantly higher in shrubland than developed and wetland (both pairwise Wilcoxon tests, $p < 0.05$) and forested sites compared to wetland sites (pairwise Wilcoxon test, $p < 0.05$). Moose contributions followed a similar pattern, we found higher contributions in forested sites compared to wetland and developed sites (both pairwise Wilcoxon test, $p < 0.01$). There are some noticeable outliers in elk and deer fecal contributions, which appear to be skewed towards wetlands, these results seem logical given these ungulates behavior and summer foraging patterns. Cattle contributions were the highest in sites classified as wetland compared to forest and developed land use types (both pairwise Wilcoxon test, $p < 0.05$)..
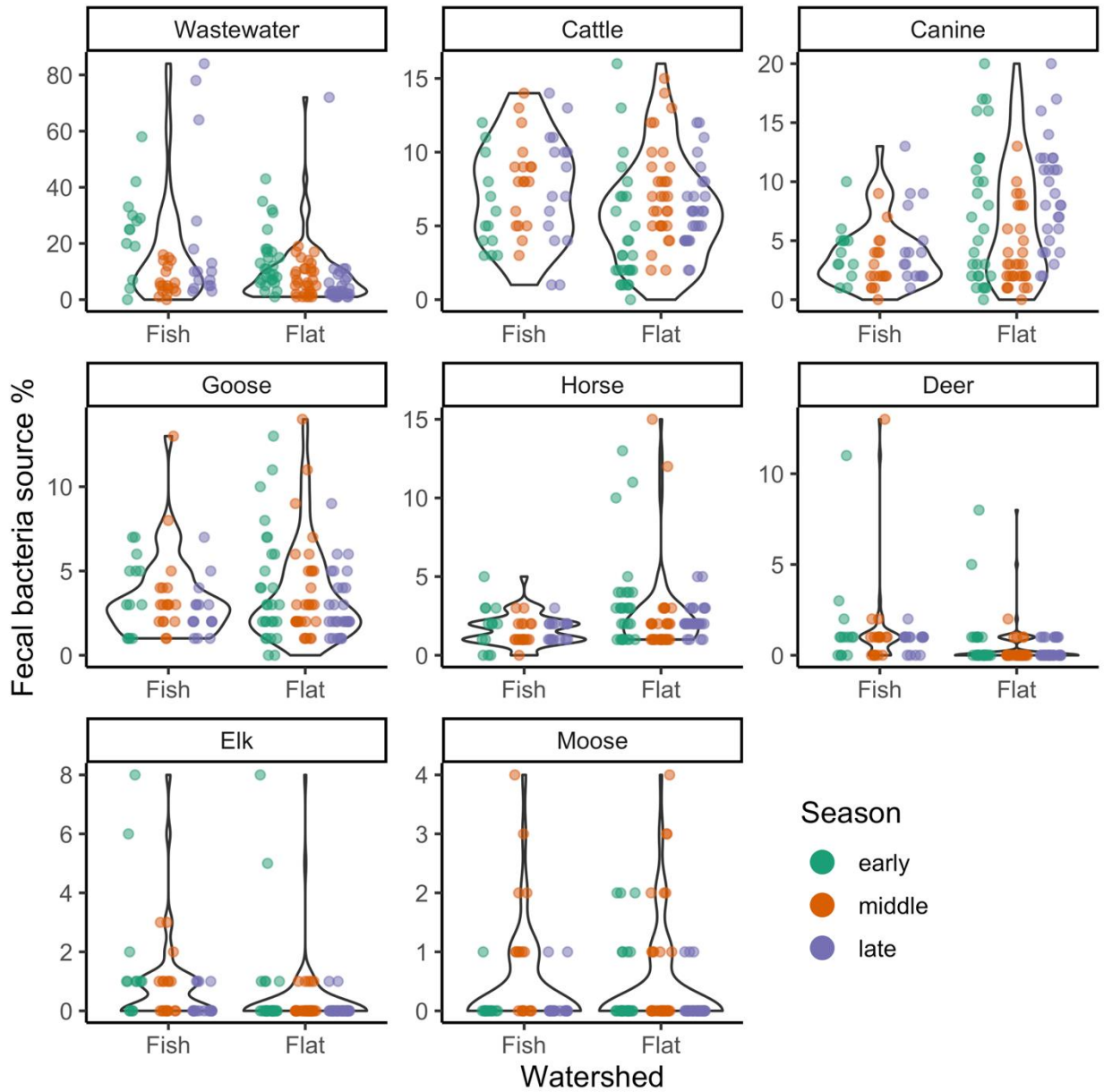
Figure 9. Violin plots showing the distribution of values for each fecal source's contribution to surface water bacterial communities in the two different watersheds. Panels are ordered based on relative contributions and the colors indicate the season in which samples were collected.
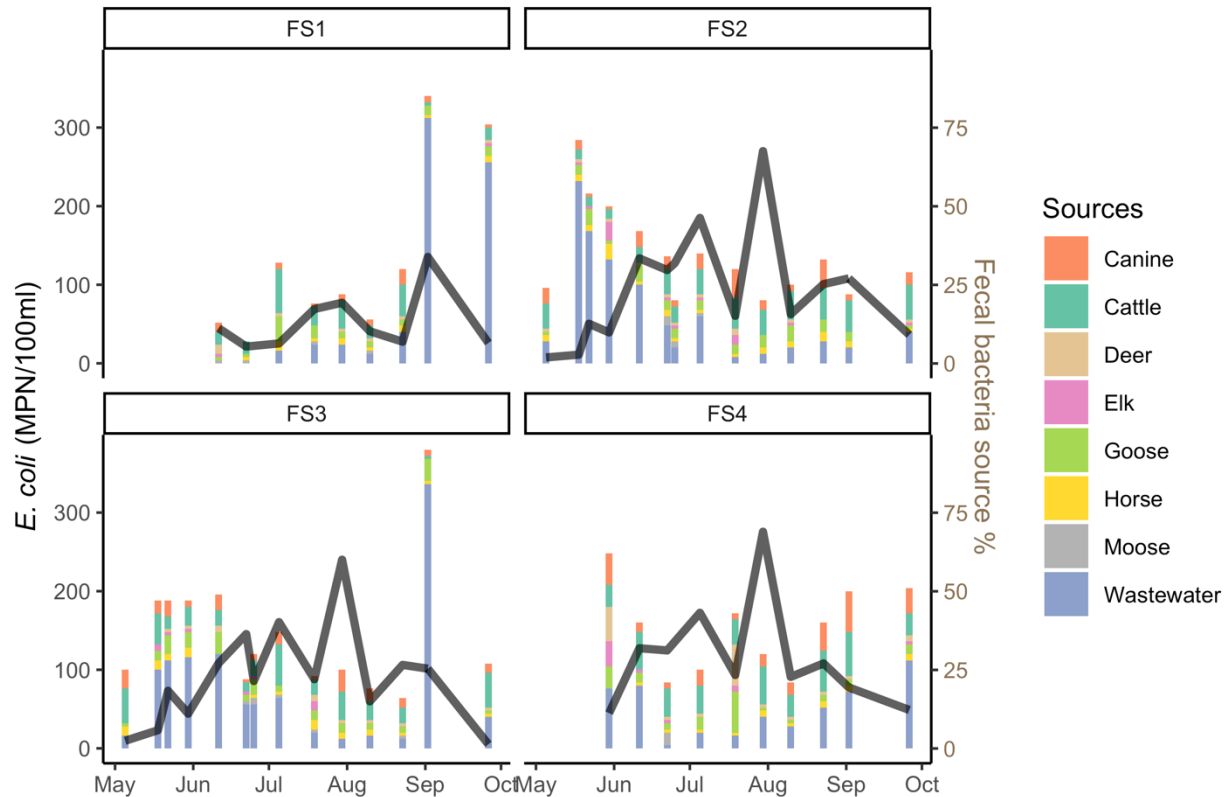
Figure 10: Seasonal Fish Creek *E. coli* concentration (left y-axis), indicated by the line, and fecal bacterial source contributions (right y-axis), indicated by the bars with colors representing different sources, at each sampling location.

Overall, this study serves as one of the few studies using MST and community microbial analysis to understand anthropogenic impacts to western headwater rivers at the wildland-urban interface. This study provides a unique juxtaposition of two geographically similar rivers, with differing human impacts, but forecasted to experience similar hydrology and temperatures shifts due to climate change. We found urbanization and water temperatures had a negative effect on aquatic bacterial diversity and were the primary drivers of bacterial community heterogeneity. These relationships are particularly concerning given this urbanizing region is poised to experience warming temperatures because of climate change. This present and potential future loss of microbial diversity could hinder natural ecosystem processes. We also found that high levels of wastewater bacteria in surface waters coincided with periods of hydrologic connectivity between groundwater and surface waters, suggesting that groundwater may be transporting potentially harmful bacteria into streams. This pattern was apparent in regions with high septic system density as well as centralized wastewater systems, indicating that both types of infrastructure may be contributing non-point source pollution to surface waters. This study found that even minor urbanization and anthropogenic influence on a landscape can decrease aquatic bacterial diversity as well as contribute significant quantities of potentially harmful fecal pollution to headwater streams.

Additional details about these methods and results can be found in Chapter 2 of Kelsey Ruehling's MS thesis.

*RNA Coliphage approaches to Source Tracking:*

We made use of male-specific (F+) Coliphage, a bacteriophage which is strongly associated with fecal material, to act as our marker for the MST approach. This group of bacteriophage is an apt marker for gauging fecal contamination presence and source through their abundance in feces and known associations with specific host types. This RNA virus comprises four genogroups, wherein GII and GIII are associated with mainly human and domestic sewage, GIV with animals and livestock, and GI with both human and animal feces. Environmental water samples from the Jackson, Wyoming area were tested for these four genogroups with digital PCR following concentration with an anion exchange resin concentration method and RNA extraction directly from the resin. Digital PCR allowed for absolute quantification for each of the four coliphage genogroups, and yielded detection mainly in genogroups I and III among the 22 pooled samples tested. As both of these genogroups have association with human fecal material, it is suggested to human sewage may have been a contributor to the elevated levels of coliforms, and potentially pathogens, in these waters.

These results are still preliminary and final results will be included in MS Student Clara Bouley's thesis. Her anticipated defense date is in Spring 2023. Clara assisted with field collections and used samples collected in the Teton County sites to extract RNA and develop methods to look at different coliphage groups that are associated with fecal sources. Optimizing the methods was complicated, Clara overcame numerous challenges, including insufficient RNA yield from 0.5L water samples and switching to digital PCR methods for detection in low concentration samples.

**Summary and Significance**

Overall, our project established and applied new methods for investigating the utility of fecal indicator bacteria and conducting microbial source tracking in Wyoming watersheds. Results may be useful to local and state agencies in mitigating microbial pollution. Source tracking methods may be useful for future studies in other parts of Wyoming where sources need to be identified to mitigate water quality impairments.