INVERTEBRATE MICROBIOMES DOWNSTREAM OF WASTEWATER DISCHARGES

MICROBIOMES OF FRESHWATER INSECTS AND RIPARIAN SPIDERS DOWNSTREAM OF MUNICIPAL WASTEWATER DISCHARGES IN THE BOW RIVER, AB

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Lay Abstract

Municipal wastewater treatment facilities release contaminants into aquatic ecosystems that may affect the health of exposed organisms, including their microbiome, which contains bacteria essential for host digestion and immune function. Alterations in microbiomes of contaminant-exposed aquatic insects are poorly characterized and such effects may be transferred to terrestrial ecosystems through emergent insects. This study evaluated microbiomes and food web dynamics of freshwater insects and riparian spiders upstream and downstream of wastewater treatment facilities in the Bow River, AB. Results indicate that microbiomes of some downstream aquatic insects had lower relative abundances of endosymbiont bacteria, shifts in bacterial diversities, and increases in abundances of effluent-associated bacteria than those collected at upstream sites, but no such changes were observed in the spider predators. This study improves our understanding of how freshwater insect microbiomes are altered by municipal wastewater effluents and suggests that directly effluent-exposed organisms are more at risk of dysbiosis.

Abstract

The host microbiome (mainly bacteria) is essential for host immune function, metabolism, and digestion. Alterations in these microbes, known as dysbiosis, generally results in adverse effects to the host, including diseases. Dysbiosis can be induced from exposures to various anthropogenic contaminants including constituents of municipal wastewater treatment effluents (MWWEs), namely, pharmaceuticals, antibiotics, and excess nutrients. Despite MWWEs being one of the largest dischargers to aquatic ecosystems, impacts of these contaminants on exposed organism microbiomes, especially in aquatic insects, is unclear. In addition, some aquatic contaminants may transfer to riparian habitats through predation on emergent insects that were exposed to contaminants as larvae, and subsequently alter microbiomes of terrestrial predators. Our study evaluated whether MWWEs altered microbiomes of freshwater larval and adult insects and their riparian spider predators using effluent-associated bacteria and stable nitrogen isotopes ($\delta^{15}N$) to confirm effluent exposure. We analyzed microbiome compositions through sequencing of the V3-V4 hypervariable region of the 16S rRNA gene and analyzed food web dynamics with stable carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) isotopes. We found that insects and spiders were enriched in $\delta^{15}N$ at one site downstream of wastewater outfalls, indicating exposure to effluents and transfer of nutrients to terrestrial ecosystems. Microbiomes of most larval and adult insects were altered downstream of wastewater outfalls and had lower relative abundances of endosymbiont bacteria, shifts in bacterial diversities, increases in abundances of effluent-associated bacteria, and downregulation of some biosynthesis pathways than those collected at upstream sites. However, spider microbiomes had little evidence of dysbiosis, and were distinct from those of adult insects, despite a close association in their isotopic signatures. Overall, this study provides evidence of biological impacts from MWWEs to exposed insects and suggests

that changes in microbial communities of invertebrates may be used as an effective indicator of effluent exposure as part of monitoring frameworks.

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List of Abbreviations

Abbreviation	Definition
ACWA	Advancing Canadian Wastewater Assets
ANOVA	Analysis of Variance
ASV	Amplicon Sequence Variant
MWWE	Municipal Wastewater Effluent
NMDS	Non-Metric Multidimensional Scaling
PERMANOVA	Permutational Multivariate Analysis of Variance
RDA	Redundancy Analysis
RPD	Relative Percent Difference
RSD	Relative Standard Deviation
SD	Standard Deviation
WWTP	Wastewater Treatment Plant
$\delta^{13}C$	Stable isotope ratios of carbon
$\delta^{15}N$	Stable isotope ratios of nitrogen

Declaration of Academic Achievement

This thesis was designed and written by Emilie Diesbourg under the supervision of Dr. Karen Kidd. Overall study and sampling design was created by Dr. Karen Kidd and Dr. Brittany Perrotta with help from collaborators at the University of Calgary including Dr. Fred Wrona and Dr. Kelly Munkittrick. Samples were collected by Emilie Diesbourg, Dr. Brittany Perrotta, Dr. Karen Kidd, and collaborators at the University of Calgary including members of the Wrona and Munkittrick Labs. Laboratory methods including DNA extractions, PCR amplification, and Illumina sequencing were designed and executed by Laura Rossi from the Surette lab at the Farncombe Institute (McMaster University). Data analysis, interpretation, and writing was completed by Emilie Diesbourg with advice from Dr. Brittany Perrotta, and editing from supervisor, Dr. Karen Kidd and committee members, Dr. Kelly Munkittrick and Dr. Joanna Wilson. I, Emilie Diesbourg, declare that this thesis is an original report of my research.

1.0 Introduction

1.1 The Host Microbiome

The host microbiome refers to the collection of symbiotic, commensal, and pathogenic microorganisms (i.e., bacteria, archaea, algae, fungi, protozoa, viruses) living on or inside a host organism (Berg et al., 2020). The host microbiome is dynamic and includes both resident and transient microbes, the latter of which are temporary colonizers, making it difficult to elucidate a stable or 'core' microbiome (Berg et al., 2020). Although several factors affect the microbiome including host phylogeny, age, life stage, etc., an organism's diet and environment seem to be pivotal components of shaping microbial communities (Turnbaugh et al., 2008; Voreades et al., 2014; Ayayee et al., 2022; Gohl et al., 2022). Symbiotic microbes have many beneficial functional roles within the host, which are shared across several animal species, including nutrient acquisition and digestion, metabolism of xenobiotics, and protection of the gut mucosal membrane against pathogens (Harris, 1993; Mendes and Raaijmakers, 2015; Muñoz-Benavent et al., 2021). Specifically, many gut bacteria have enzymes that aid in digestion and metabolism of food, specialized nitrogen fixing microbes to increase nitrogen acquisition of the host, and microbial metabolites and enzymes that can interfere with drug metabolism (Li et al., 2016).

Many studies have shown a strong positive association between alterations in the normal host microbial composition, known as dysbiosis, and the introduction of disease. Dysbiosis occurs when there is an imbalance in the normal host microflora composition or metabolic activities which typically results in loss of beneficial microorganisms, gain of potentially pathogenic microorganisms, and/or overall loss of bacterial diversity (Degruttola et al., 2016). Dysbiosis has been linked to inflammatory bowel disease, obesity, diabetes, heart issues, and

primary liver cancer in humans (Degruttola et al., 2016; Zhang et al., 2019), obesity and diabetes in mice (Turnbaugh et al., 2008), and intestinal inflammation in zebrafish (Orso et al., 2021). Some environmental contaminants are drivers of dysbiosis in aquatic organisms including pesticides (Narrowe et al., 2015; Gaulke et al., 2016), xenobiotics (Bertotto et al., 2020), pharmaceuticals (Pinto et al., 2022), municipal wastewater effluents (MWWEs; Restivo et al., 2021; Millar et al., 2022), and other wastewater treatment effluents (Wang et al., 2023). Endosymbionts, a group of intracellular bacterial associates of animals that have been linked to host reproduction and immune function in many insect and arachnid hosts (Moran and Baumann, 2000; Goodacre et al., 2006; Eleftherianos et al., 2013), are also sensitive to environmental contaminants including polychlorinated biphenyls (PCBs; Perrotta et al., 2022) and antibiotics (Vanthournout et al., 2011). However, consistent indicators of dysbiosis remain unclear, especially in aquatic biota, hence it is important to continue characterizing changes in bacterial composition in relation to environmental stressors to identify patterns that can be used to define specific contaminant exposures (Adamovsky et al., 2018).

1.2 Wastewater Treatment Effluent Contaminants

MWWEs commonly contain an assortment of antimicrobial agents, pesticides, pharmaceuticals, and excess nutrients (Holeton et al., 2011). Although usually present at low levels, effluent contaminants may have negative effects in downstream ecosystems including the introduction of disease causing or antibiotic resistant microbes to the environment (Akiyama and Savin, 2010), physiological or morphological changes in exposed organisms (Vajda et al., 2008; Tetreault et al., 2011), decreases in diversity and evenness of exposed fish communities (Brown et al., 2011), and alterations in nutrient cycling rates of exposed aquatic insects which is critical for primary

productivity in stream food webs (Fleeger et al., 2003; Van Dievel et al., 2020). Fortunately, enforcement of effluent regulatory standards and upgrades to wastewater treatment plant (WWTP) operational processes can decrease the prevalence of contaminants in effluents and reduce some impacts to exposed organisms (Hicks et al., 2017).

Some effluent-derived contaminants can be transferred to riparian ecosystems through emergent insects. Such contaminants include PCBs, heavy metals, pesticides, pharmaceuticals, and perfluorinated and polyfluorinated substances which can bioaccumulate in effluent-exposed aquatic insects, be retained in adult insects through metamorphosis, and be transferred to terrestrial predators through predation on emergent adult insects (Bundschuh et al., 2022). Effluent-associated bacteria have been observed in larval insects and riparian spiders downstream of effluent discharges, suggesting potential transfer and accumulation of effluent contaminants to terrestrial insectivore microbiomes (Millar et al., 2022). However, further research is required to determine whether emergent-mediated contaminant transfer influences overall microbiome bacterial community alterations from aquatic to riparian ecosystems.

1.3 Impacts of Wastewater Effluents in the Bow River Watershed

The Bow River is a major tributary of the Saskatchewan-Nelson River system, with a drainage area of ~25,000 km^2, and it originates from Bow Lake in the Canadian Rocky Mountains and passes through multiple municipalities including the City of Calgary (Alberta Government, 2014). The Bow River supplies ~60% of Calgary's freshwater and is recognized as a culturally and economically important watershed (Alberta Government, 2014). With a population of approximately 1.4 million people, about 430 million litres of municipal wastewater are treated in Calgary daily from three wastewater treatment facilities (Bonnybrook, Fish Creek, and Pine

Creek), and released as effluent into the Bow River (City of Calgary, 2014). While all three facilities use ultraviolet disinfection, an advanced treatment method, Bonnybrook uses biological nutrient removal while the others use chemical phosphorus removal, which may affect the forms and quantities of nutrients found in the final effluents (Chen et al., 2015). Bonnybrook also has a much greater treatment capacity than the other facilities, approximately 500,000 m^3 per day compared to 73,000 m^3 per day at Fish Creek and 100,000 m^3 per day at Pine Creek (Chen et al., 2015). Due to the high volumes of effluent discharged by the City of Calgary and minimal anthropogenic impacts above Calgary, the Bow River is an ideal system to monitor ecological impacts of effluent-derived contaminants on the freshwater environment.

A few studies have evaluated the impacts of effluent discharge on downstream communities in the Bow River. Wastewater treatment in Calgary is generally regarded as effective; however, as WWTPs are not designed to remove pharmaceuticals, some drugs including diclofenac, naproxen, ibuprofen, carbamazepine, trimethoprim, and caffeine are still found in the nano - microgram/L range downstream of WWTP outfalls (Chen et al., 2015; Arlos et al., 2023). Enteric viruses have also been reported in surface water downstream of Calgary, likely originating from wastewater outfalls (Pang et al., 2019), which may have implications for downstream microbial communities of exposed organisms. Historic increases in nutrient loads (specifically nitrogen and phosphorus) and primary production have been seen downstream of Calgary's WWTPs, decreasing dissolved oxygen levels and overall water quality (Alberta Government, 2014). Additionally, fathead minnow that were caged downstream of effluent outfalls displayed decreased gonadosomatic indices, decreased 17β-estradiol in females, and delayed spermatogenesis in males, suggesting impacts to fish reproduction (Patel, 2018). However, there is limited knowledge on the impacts of effluents on freshwater insect

communities in the Bow River (Sutherland, 2024) despite their critical role for aquatic-terrestrial energy subsidies and within stream nutrient recycling (Wallace and Webster, 1996).

1.4 Study Rationale and Objectives

This study contributes to a broader project, led at the University of Calgary, which assesses the fate and ecological impacts of municipal wastewater effluent discharges in the Bow River. The aim of this overarching project was to identify key indicators of wastewater exposure and new methods for determining impacts on exposed organisms and identifying areas of concern associated with wastewater discharge. This knowledge will be used to develop a monitoring framework to understand future spatial and temporal impacts in the Bow River and identify benefits of any future upgrades to the wastewater treatment plants to the downstream ecosystem. Alterations in host microbial communities may be a useful part of a monitoring framework to act as an early warning of higher-level impacts in organisms exposed to effluents and to identify potential areas of concern in the river.

Our study aims to characterize shifts in microbial communities of freshwater larval and adult insects and their riparian spider predators in relation to MWWEs by using effluent-derived bacteria and stable nitrogen isotopes as tracers of effluent exposure. We also examined whether there were differences in nutrient excretion of larval invertebrates from upstream and downstream sites on the Bow River and collected larval and emergent insects from experimental streams receiving different concentrations of municipal effluents. The methods and data from these studies are described in the appendices. To our knowledge, there has been no research assessing the impacts of MWWEs on freshwater insect microbiomes in Western Canada, nor

examination of microbiome alterations through aquatic insect metamorphosis and subsequent effects on their terrestrial predator microbiomes. This study will advance knowledge of aquatic insect and riparian spider microbiomes by comparing different life stages and taxa collected upstream and downstream of WWTP outfalls. We predict that aquatic insects from downstream, effluent-exposed sites will have altered microbiomes and increased effluent-associated bacteria and nitrogen compared to those from upstream, non-exposed sites. We also predict that larvae and adults of the same taxa will have dissimilar microbiomes since there is an extensive transition in their morphology during metamorphosis which tends to result in reorganization of the gut microbiome. Lastly, we predict that microbial communities of riparian spiders will closely reflect those of the emergent adult insects they eat since diet is a key driver of microbiome composition. Overall, this study may help identify biological impacts of wastewater exposure and potential areas of concern for use in monitoring of the Bow River for optimization and management of wastewater treatment in the City of Calgary.

2.0 Methods

2.1 Bow River Sample Collection

Biological samples were collected between the 7th -12th of July 2022, from five sites located along a stretch of ~75 km of the Bow River for microbiome and stable isotope analyses (Figure 1, Table A.1). These river sites were selected based on their position relative to three WWTP outfalls (three upstream reference sites and two effluent-exposed downstream sites) and four of the sites (excluding Graves Bridge) were part of the City of Calgary's water quality monitoring program, providing long-term data via monthly grab sampling (City of Calgary, 2020; Table

A.2). The following studies are included in the appendices. At 4 of the 5 sites on the Bow River (excluding Cushing Bridge), larval Hydropsychidae were collected in July 2022 to assess nutrient excretion rates (methods and results for nutrient excretion experiments are described in Appendix C). Microbiome and nutrient excretion experiments were also conducted in September 2022 at the Advancing Canadian Wastewater Assets (ACWA) facility located at the Pine Creek tertiary WWTP in Calgary, AB (methods, analysis, and interpretation of the microbiome and nutrient excretion studies are presented in Appendix B and C respectively).

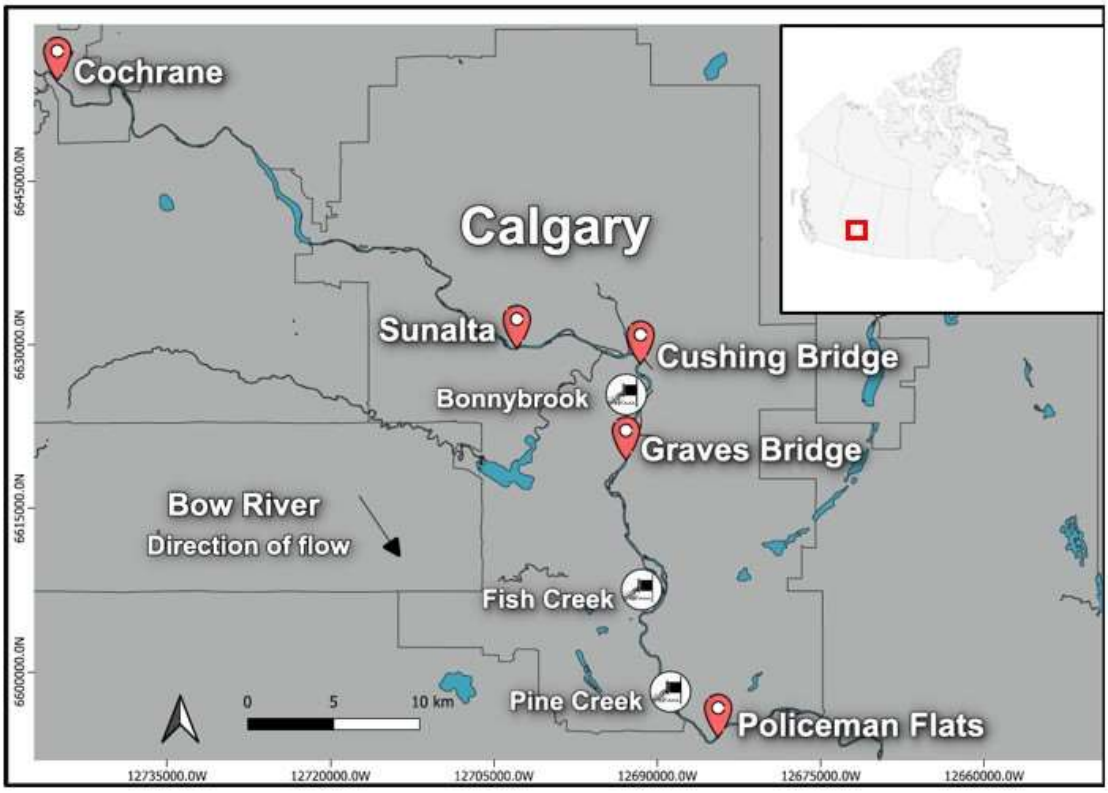

Figure 1. Map of sampling locations (red symbols) and municipal wastewater treatment plants (white symbols) along a ~75 km reach of the Bow River in Calgary, AB. See Table A.1 for site coordinates and distances from treatment plants.

2.1.1 Microbiome Samples

Larval freshwater insects were collected from the river during the day by flipping rocks or kick netting and then live sorted in an ice cube tray to family level. Individuals were identified to the most precise taxonomic level possible without using a microscope (to avoid laboratory contamination) as taxonomy is an important predictor of shaping aquatic insect microbiomes (Kroetsch et al., 2020). Larvae targeted for microbiome analyses were processed shortly after collection by lightly rinsing them with 95% ethanol to remove external microbiota, and aseptically transferring into individual 2.0 mL lysis buffer tubes containing 0.2 g of 2.8 mm ceramic beads (Mo Bio Laboratories, #13114-50) and 0.2 g of 0.1 mm glass beads (Mo Bio Laboratories, #13118-50) with 800 µL of monobasic $NaPO_4$ at pH = 8, and 100 µL of guanidine thiocyanate for lysing (Whelan et al., 2014). Samples were then stored on dry ice and then moved to a -80 °C freezer until DNA extractions could be completed. To avoid sample contamination, autoclaved forceps and new nitrile gloves were used for invertebrate processing at each site, and the forceps were rinsed with 30% bleach, 70% non-denatured ethanol, and DNA/RNase free UltraPure™ water, in that order, before and after each individual. Along with experimental samples, three negative control samples of the UltraPure water were taken by rubbing a sterile cotton swab on the inside of the Falcon tube containing the water and storing them in individual lysis buffer tubes at -80 °C. To validate the taxonomic classifications done in the field, three voucher samples of each invertebrate taxon were collected from each site and stored in 95% denatured ethanol for identification to family and genus level using a dissection microscope (Olympus SZX7) and following dichotomous keys by Merritt et al. (2008). The larval invertebrate taxa represented multiple families and functional feeding groups across all

sites: Hydropsychidae (n = 5-8/site, collector-filterers), Heptageniidae (n = 8/site, scrapers), Perlidae (n = 5-8/site, predators), and Chironomidae (n = 5-6/site, collector-gatherers).

Adult aquatic insects were caught after dark along the riverbank from the same sites and on the same day as larval collections using a moth sheet trap design with an ultraviolet light draped over an autoclaved bedsheet set up on the shoreline to attract the emerging insects from the river (Figure A.1). Before collection at each site, two negative control samples of the bedsheet were taken by rubbing sterile cotton swabs against it and placing them in individual microcentrifuge tubes, stored on dry ice. Up to eight individuals of each invertebrate taxon were identified to the order level and collected in individual sterile microcentrifuge tubes by holding them against the sheet to trap the insect inside and closing the lid while wearing sterile nitrile gloves. The samples were placed on dry ice and held at -80 °C until they could be aseptically transferred into individual lysis buffer tubes as described above. The collected insects represented three orders across sites: Trichoptera (n = 7-8/site), Ephemeroptera (n = 8/site), and Diptera (n = 6-8/site).

Lastly, orb weaver spiders (Araneidae, n = 7-8/site) and long-jawed orb weaver spiders (Tetragnathidae, n = 8/site) were collected after dark on the same day as the insect sampling from vegetation along the same shoreline sites while wearing sterile nitrile gloves. Spiders were caught directly in individual sterile Whirl-Pak® bags to avoid contamination, put on dry ice, and then stored at -80 °C until they could be aseptically transferred into individual lysis buffer tubes. In addition, three voucher samples of each type of spider were collected in microcentrifuge tubes filled with 95% denatured ethanol to validate the field taxonomic classification using a dissection microscope and Roth (1993)’s spider identification guide.

2.1.2 Stable Isotopes

Stable carbon and nitrogen isotopes were used to infer food source reliance of insects and spiders since consumers are enriched in nutrients compared to their prey (enriched in $\delta^{13}C$ by ~ 0.2 - 1 ‰ and $\delta^{15}N$ by ~ 3 – 5 ‰ in freshwater organisms; Jardine et al., 2003). Nitrogen isotopes were also used to quantify effluent exposure in downstream invertebrates because ^{15}N is more available to be assimilated by primary producers and subsequently, primary consumers, downstream of effluent discharges. More ^{14}N tends to be removed than ^{15}N during the wastewater treatment process due to a bacterial enzymatic preference for ^{14}N during nitrification and ammonification processes, increasing the ratio of ^{15}N:^{14}N entering receiving systems (Munksgaard et al., 2017; Loomer et al., 2015). Basal food sources (biofilm and riparian leaves), larval and adult aquatic insects, terrestrial adult insects, and spiders were collected for stable carbon and nitrogen isotope analysis at the same times and locations as the microbiome samples (total number of replicates per site in Table A.3). Biofilm was used as an indicator of autochthonous food sources within the river and was collected by scraping off several rocks with an ethanol cleaned knife into a Whirl-Pak bag. Riparian leaves were used as an indicator of allochthonous food sources in the river and were picked off shoreline shrubs, avoiding grasses, and put into Whirl-Pak bags. Several taxa of larval and adult insects and spiders were collected using a similar protocol to the microbiome collections, however, without aseptic techniques. Once collected, samples were put into 4 oz Whirl-Pak bags, grouped by their taxonomy, and stored at -40 °C. Collected invertebrate orders included Trichoptera, Ephemeroptera, Diptera, Plecoptera, Araneae, Coleoptera, and Lepidoptera, and functional feeding groups included collector-filterers, collector-gatherers, scrapers, piercers, and predators.

2.2 Laboratory Analysis

2.2.1 DNA Extraction, Amplification, and Sequencing

Whole individual insects and spiders, and field and laboratory blanks (n = 320 samples total, 303 experimental samples, 17 blanks) were extracted for bacterial DNA based on the protocol created by the Surette lab at McMaster University, "KingFisher Protocol for Low Biomass Samples", following DNA isolation methods as described by Stearns et al. (2015) with modifications. Plate preparations were done by hand using the MagMAX™ -96 DNA Multi-Sample Kit (applied biosystems), and further processing of the supernatant was done with a KingFisher Apex Benchtop Sample Prep machine (Thermo Scientific™). Modifications from the Multi-Sample kit included using the entire supernatant and eluting with 50 uL of elution buffer. Four extraction negative samples and one extraction positive sample (fecal material) were run on each 96-well plate, and two lysis buffer samples (with no sample) were run on the final extraction plate.

The extracted DNA was used to amplify the V3-V4 hypervariable region of the 16S rRNA gene using a nested PCR approach due to the presence of host DNA (L. Rossi, McMaster University, personal communication). The 8F (3' – AGAGTTTGATCCTGGCTCAG – 5') and 926R (5' – CCGTCAATTCCTTTRAGTTT – 3') region of the 16S gene was amplified at 94 °C for 5 min, followed by 15 cycles at 94 °C for 30 s. This reaction was used as the template for the second phase of PCR which used 341F (5' – CCTACGGGAGGCAGCAG – 3') and 806R (5' – GGACTACNVGGGTWTCTAAT – 3') Illumina adapted primers (IDT, Coralville, Iowa; Bartram et al., 2011) which underwent 5 min at 94 °C and 5 cycles at 94 °C, 47 °C, and 72 °C for 30 s each, followed by an additional 25 cycles at 94 °C, 50 °C, and 72 °C for 30 s each, with a final extension at 72 °C for 10 min. PCR products were visualized on a 1.5% agarose gel and

sequenced on the Illumina MiSeq platform (paired-end reads, 2 x 300 base pairs or bp) at the Farncombe Institute (McMaster University, Hamilton, Ontario).

2.2.2 Bacterial Read Processing

Cutadapt was used to filter and trim reads that had a minimum quality score of 30 and minimum read length of 100 bp as well as to remove adapter sequences and primers used in the nested PCR process (Martin, 2011). Amplicon sequence variants (ASVs) were generated from the filtered reads using the DADA2 pipeline in R (Callahan et al., 2016). DNA sequences were filtered to retain only good quality reads, error rates were estimated, and the forward and reverse reads were merged while maintaining an overlap of at least 12 bases to obtain the full denoised sequences which were converted into a sequence table. Chimeras and bimeras were removed and taxonomies were assigned using the SILVA database (version 1.3.2; Quast et al., 2013).

2.2.3 Stable Carbon and Nitrogen Isotopes

Prior to lyophilization, invertebrates were picked out of biofilm samples with ethanol cleaned forceps and invertebrate samples were identified and grouped to family level under a dissection microscope. Samples were then lyophilized for 72 hr, homogenized using either weigh paper (for very small organisms) or an ethanol cleaned glass rod (for larger organisms), and weighed into tin capsules (target mass for animals: ~1 mg, target mass for plants: ~3 mg) on a microbalance (Mettler Toledo XPR26). The weigh paper did not appear to affect carbon isotope values of the sample (relative percent difference or RPD between glass rod and weigh paper methods: 0.028 – 1.21%, n = 2). Samples were analyzed at the Canadian Rivers Institute, SINLAB in Fredericton,

New Brunswick, on a Thermo-Finnigan Delta V Mass Spectrometer for $^{13}C/^{12}C$ and $^{15}N/^{14}N$ isotopes. Duplicates were run randomly on ~10% of samples (n = 19) and had a mean ± standard deviation (SD) RPD within 0.79 ± 0.65% for carbon and 3.11 ± 6.44% for nitrogen. Three in house primary reference materials and USGS 61 standard reference material were run every 10-12 samples and had a relative standard deviation (RSD) ranging from 0.20 – 0.62% for carbon and 0.63 – 3.19% for nitrogen. Results were reported in delta (δ) notation as the deviation from standards in parts per thousand (‰).

2.3 Statistical Analysis

2.3.1 Microbiome Analysis

All statistical analyses were done in RStudio (v. 4.3.1; R Core Team, 2021), primarily using the phyloseq package (v. 1.44.0; McMurdie and Holmes, 2013), and a significance threshold of $\alpha = 0.05$. The ASV, taxonomy, and metadata tables were grouped into a single phyloseq object which then underwent further pre-processing steps before analysis. Due to laboratory contamination on one extraction plate, 8 samples (all adult Trichoptera from Policeman Flats) were removed from further analysis. Additionally, one larval chironomid was not amplified due to lack of sufficient DNA and was not included in downstream analysis. For all other samples, a PERMANOVA test indicated significant differences between bacterial beta diversity of field and extraction blanks and experimental samples (ADONIS: Pseudo-$F_{1,291} = 3.48$, $p < 0.001$; Figure A.2), therefore, no other samples were removed prior to data filtering steps. The remaining 294 samples were further filtered by the following steps: 1. Any non-target gene sequences such as eukaryotic organisms, archaea, mitochondria, and chloroplasts as well as phyla classified as “N/A” were

removed. 2. Any ASV whose sum of reads across all samples was less than 5 and was not present in at least 2 samples was removed. 3. Any samples with a total sum of reads less than 2000 were removed. Out of the 18 samples that were removed from further filtering, 9 were larval chironomids, 4 were adult chironomids, 4 were araneids, and 1 was an adult trichopteran (refer to Table A.4 for final sample sizes per site). Following the filtering steps, 276 samples remained for subsequent analyses.

The mean relative abundances of each bacterial taxa were determined by agglomeration of reads to a taxonomic rank, compositionally transforming the reads, and aggregating the reads to the phylum, family, or genus level using the mean abundance (class and order levels not shown). The phyloseq object was converted into a data frame and visualized as the mean percent relative abundance of each bacterial taxa from each collection site and host invertebrate type using stacked bar plots. Statistical differences in relative phyla abundance were tested across sites using non-parametric Kruskal-Wallis tests. Additionally, the phyloseq object was filtered for common municipal wastewater effluent-associated bacteria (see Appendix A for specific genera) and endosymbionts including *Buchnera, Candidatus Cardinium, Candidatus Hamiltonella, Cadidatus Megaira, Rickettsia, Spiroplasma,* and *Wolbachia* (Moran and Baumann, 2000; Goodacre et al., 2006; Eleftherianos et al., 2013) which were transformed to relative abundances for comparison to the whole microbiome across each collection site and invertebrate taxa.

Differentially abundant bacteria were statistically tested across individual invertebrate taxa and collection sites on non-rarefied absolute abundances using a parametric, modified geometric mean differential expression analysis with the package DESeq2 (Love et al., 2014). Pairwise comparisons were conducted across Bow River sites by using the furthest upstream site, Cochrane, as the reference site. Wald hypothesis tests ($\alpha = 0.001$) were used to determine

significant changes in bacterial abundance, corrected using the Benjamini-Hochberg method. Bacterial abundances were calculated and plotted as the logarithmic (base 2) fold change (log_2) against the reference variable.

Alpha and beta diversities, which measure the richness and evenness of bacterial composition within samples and the dissimilarity in bacterial composition between samples, respectively, were calculated and compared across sites and invertebrate taxa. There were no differences in statistical interpretations between rarefied and non-rarefied phyloseq objects, therefore the non-rarefied data were used for alpha and beta diversity analyses to avoid biasing the results. Alpha diversity was measured by the Shannon species diversity index (Shannon, 1948), which was calculated and visualized with boxplots, plotted across collection sites and invertebrate taxa. Pairwise differences between sites and taxa were determined using Kruskal-Wallis non-parametric and Dunn's multiple comparisons tests where applicable.

Prior to beta diversity analyses, the phyloseq object was transformed to relative abundances to standardize the data. Beta diversity was calculated with a Bray-Curtis dissimilarity distance matrix and ordinated with non-metric multidimensional scaling (NMDS) with a starting seed of 1. A redundancy analysis (RDA) was conducted to determine which explanatory factors (collection site or invertebrate taxonomy) explained more of the variation in bacterial beta diversity. Differences in beta diversity across sites and invertebrate families were tested using PERMANOVA with the 'adonis' function, and post hoc comparisons were done with pairwise adonis tests. Beta dispersion (a measure of homogeneity within the data) was also calculated with the function 'betadisper' which calculates the distances from each sample to the group centroid and compares them across groups. Environmental water quality data were obtained from the City of Calgary surface monitoring dataset measured between the 11^{th} -13^{th} of

July 2022 including variables such as water temperature (°C), dissolved oxygen (mg/L), conductivity (µS/cm), total phosphorus (PO_4-P) and nitrogen (NO_3-N; mg/L), etc. These values were correlated to the Bray-Curtis bacterial abundance distance matrix (excluding samples from Graves Bridge as water quality data was not taken from that site) using Mantel tests, measured with non-parametric Spearman correlations and 999 permutations.

Lastly, PICRUSt2 (Douglas, 2020) was used to predict differentially expressed metabolic functional pathways across collection sites and invertebrate taxa. The pipeline was run with the default minimum alignment threshold of 0.8 and 9 ASVs were removed from analysis due to poor alignment with the reference tree, leaving 7518 ASVs to be analyzed. Pathways were interpreted through MetaCyc pathway database classes (Caspi et al., 2020). Mean relative abundances of MetaCyc pathway classes were plotted across invertebrate type and collection site, and differentially expressed individual MetaCyc pathways were tested with DESeq2 following the same methods described for differential abundance analysis.

2.3.2 Stable Isotopes

$\delta^{13}C$ and $\delta^{15}N$ values were compared across sites, basal food sources, and invertebrate taxa groups using Kruskal-Wallis and ANOVA tests and Dunn/Tukey's post hoc multiple comparisons tests where applicable ($\alpha = 0.05$). Basal food source reliance of primary consumers was visualized on a scatterplot, comparing the mean $\delta^{13}C$ of the invertebrate relative to each food source. Shifts in isotopes across collection sites and invertebrates were visualized using boxplots.

3.0 Results

3.1 General Sequencing Results

A total of 8,547,820 bacterial sequence reads were obtained from whole body samples of freshwater macroinvertebrate larvae (n = 110), adults (n = 90), and riparian spiders (n = 76) from the Bow River. The number of reads per sample ranged from 2,010 – 222,648, with an average of 30,970, and in total, 7,527 unique ASVs were identified. At most sites, Heptageniidae larvae had the highest number of unique ASVs per taxonomic rank and Tetragnathidae spiders and Diptera adults had the lowest (Table A.4). The five most abundant phyla across all invertebrate samples, comprising ~98% of the total bacteria, were Proteobacteria (mean (± SD); 52.4 ± 30.4%), Bacteroidota (formally called Bacteroidetes; Oren and Garrity, 2021; 23.7 ± 23.7%), Firmicutes (18.0 ± 22.7%), Actinobacteriota (3.44 ± 6.59%), and Deferribacterota (0.49 ± 1.52%). The five most abundant families across all samples were Rickettsiaceae (12.6 ± 30.2%), Chitinophagaceae (9.06 ± 21.2%), Comamonadaceae (7.83 ± 11.4%), Mycoplasmataceae (5.82 ± 17.4%), and Anaplasmataceae (4.75 ± 19.3%) and the five most abundant genera were *Rickettsia* (13.7 ± 31.1%), *Vibrionimonas* (8.73 ± 23.2%), *Candidatus Bacilloplasma* (6.34 ± 18.5%), *Wolbachia* (4.77 ± 19.3%), and *Rhodoferax* (4.06 ± 6.57%), respectively.

3.2 Bacterial Relative and Absolute Abundance

3.2.1 Larval Aquatic Insects

Within individual larval taxa, there were few shifts in bacterial relative abundance at the phylum level across sites in the Bow River. Larval Hydropsychidae collected from the furthest upstream reference site (Cochrane) had a significantly higher proportion of Firmicutes than those from the

furthest downstream site (Policeman Flats; all percentages shown as mean ± SD: 19.3 ± 6.85% compared to 10.0 ± 4.09%; Kruskal-Wallis Dunn multiple comparison test: Z = 2.97, p = 0.03; Figure 2; Table A.5). However, there were no other significant shifts in the relative abundance of microbial phyla in hydropsychids, nor in any of the other larval taxa.

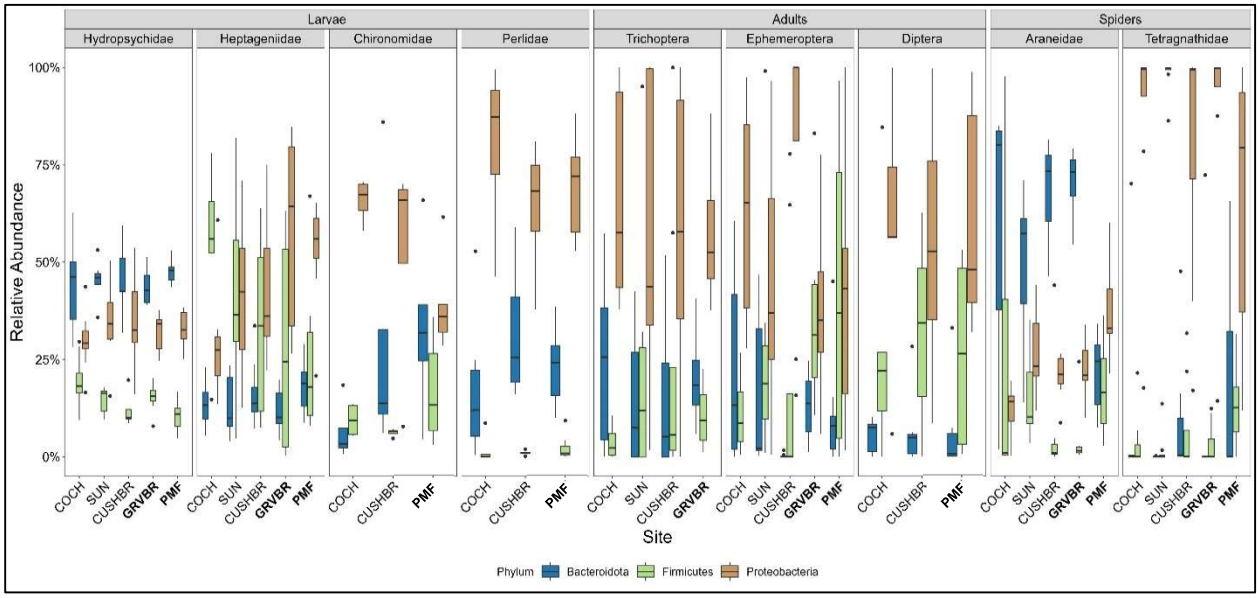

Figure 2. Boxplot of the three most abundant bacterial phyla shown in relative abundances (%) from all macroinvertebrate larvae families, adult orders, and spider families of the Bow River. Sites are arranged from upstream to downstream, where bolded sites are located downstream of wastewater outfalls.

There were some spatial shifts in relative abundances of bacterial genera across invertebrates (described in detail in Appendix A; Figure A.3), including shifts in endosymbiont bacteria (Figure 3, Table A.6). Within insect larvae, hydropsychids had the greatest abundance of endosymbiont bacteria relative to their total microbiome, comprised completely of *Rickettsia* and which decreased from upstream to downstream (Cochrane: 15.2 ± 13.7% compared to Policeman Flats: 0.41 ± 1.14%). Small proportions of *Rickettsia* and *Wolbachia* were found in larval

Heptageniidae and did not change in abundance from upstream to downstream, and low relative abundances of *Rickettsia* were found in chironomids only from the furthest upstream reference site, Cochrane, and were not present at the other two sites. *Candidatus Megaira* was also found at low relative abundances in perlids from Cochrane; however, there were no endosymbionts found in perlids from any other site. Additionally, using the furthest upstream site, Cochrane, for differential abundance analysis, the number of statistically differentially abundant bacterial genera increased from upstream to downstream, across most invertebrates (Table 1). When comparing both downstream sites combined (Graves Bridge, Policeman Flats) to all upstream sites (Cochrane, Sunalta, Cushing Bridge) combined, larval hydropsychids had the greatest number of differentially abundant bacteria and perlids had the least (Figure A.4).

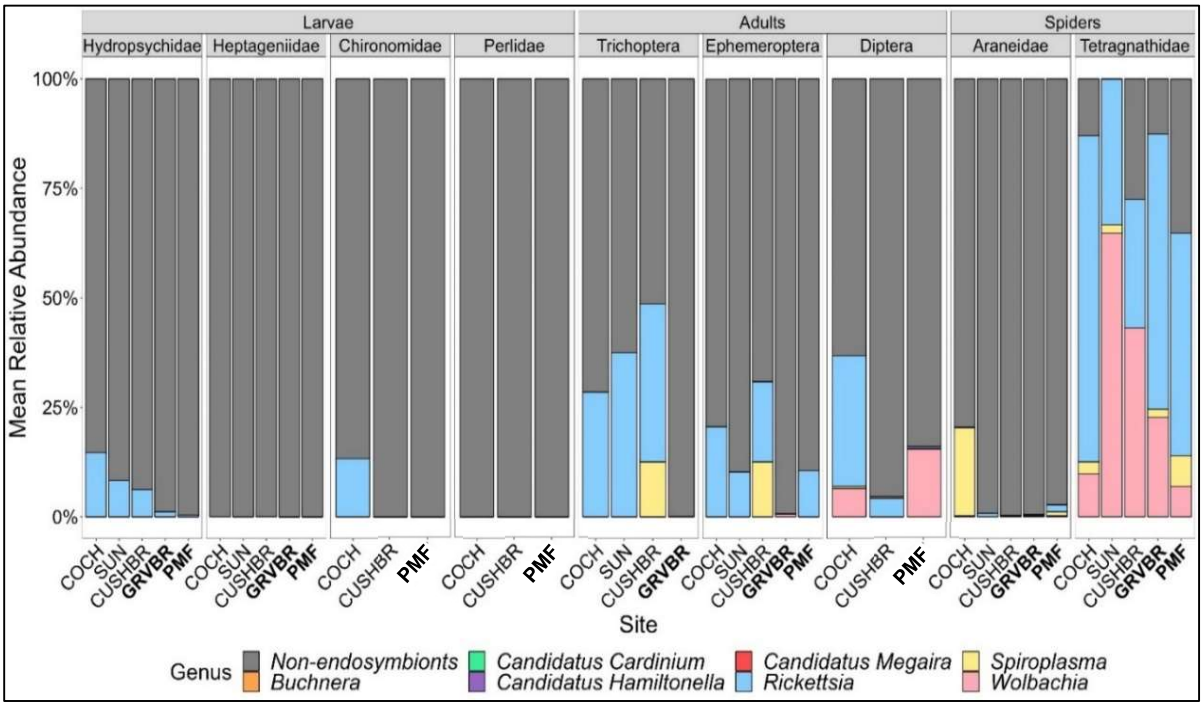

Figure 3. Mean relative abundance (%) of endosymbiont bacteria at the genus level relative to non-endosymbiont genera across families of larval insects, orders of adult insects, and families of spiders and sites in the Bow River. Sites are arranged from upstream to downstream, where bolded sites are located downstream of wastewater outfalls.

Table 1. Number of differentially abundant bacterial taxa at each collection site compared to the reference site, Cochrane, across invertebrates in the Bow River, based on DESeq2 ($\alpha = 0.001$). Bolded site names are located downstream of municipal wastewater effluent outfalls.

Life stage	Invertebrate taxon	Pairwise site comparison	Number of differentially abundant bacteria
Larvae	Hydropsychidae	Sunalta	7
		Cushing Bridge	12
		Graves Bridge	12
		Policeman Flats	134
	Heptageniidae	Sunalta	7
		Cushing Bridge	2
		Graves Bridge	18
		Policeman Flats	53
	Chironomidae	Cushing Bridge	22
		Policeman Flats	37
	Perlidae	Cushing Bridge	29
		Policeman Flats	9
Adults	Trichoptera	Sunalta	5
		Cushing Bridge	9
		Graves Bridge	26
	Ephemeroptera	Sunalta	19
		Cushing Bridge	15
		Graves Bridge	16
		Policeman Flats	10
	Diptera	Cushing Bridge	79
		Policeman Flats	162
Spiders	Araneidae	Sunalta	3
		Cushing Bridge	1
		Graves Bridge	0
		Policeman Flats	26
	Tetragnathidae	Sunalta	1
		Cushing Bridge	0
		Graves Bridge	0
		Policeman Flats	3

3.2.2 Adult Aquatic Insects

Adult aquatic insects had distinct relative abundances of bacterial phyla compared to the larvae of the same order/family (described in detail in Appendix A) but there were few spatial changes

within adult insect taxa along the Bow River. There were only significant shifts in the relative abundances of bacterial phyla for Ephemeroptera individuals from one of the upstream sites, Cushing Bridge. Specifically, ephemeropterans from Cushing Bridge had a significantly lower proportion of Bacteroidota (0.28 ± 0.60%) than the two other upstream sites (Cochrane: 22.1 ± 23.3% and Sunalta: 15.6 ± 20.0%), as well as the first effluent-exposed site (Graves Bridge: 12.9 ± 8.70%; chi-squared = 14.9, p = 0.0049; Figure 2, Table A.5). Trichoptera and Diptera individuals had slight, but non-significant shifts in bacterial phyla relative abundances across collection sites.

There were also differences between bacterial relative abundances at the genus level between adult and larval aquatic insects of the same taxa and some differences between upstream and downstream sites within the adult insects (described in Appendix A, Figure A.3). In general, adults had higher proportions of endosymbionts compared to the larvae and endosymbionts were predominantly composed of *Rickettsia, Spiroplasma,* and *Wolbachia* (Figure 3; Table A.6). Adults mostly decreased in endosymbiont proportions at sites located downstream of wastewater outfalls, especially at Graves Bridge in Trichoptera and Ephemeroptera individuals. Similar to the larvae, differentially abundant bacterial genera in adults increased as pairwise site comparisons moved from upstream to downstream, using Cochrane as the reference site, except for in Ephemeroptera (Table 1). When comparing the two downstream sites combined to the three upstream sites combined, Diptera adults had the greatest number of differentially abundant genera and Ephemeroptera had the least (Figure A.4).

3.2.3 Riparian Spiders

Spiders had variable relative abundances of bacterial phyla and genera across sites and had distinct microbiome compositions from those of larval and adult aquatic insects. Araneidae spiders were dominated by both Proteobacteria and Bacteroidota across most sites, while Tetragnathidae spiders were almost completely dominated by Proteobacteria at most sites (Figure 2). Araneids from the furthest downstream site (Policeman Flats) had a significantly lower mean proportion of Bacteroidota (21.8 ± 10.7%) compared to most sites, (Cochrane: 61.0 ± 31.7%, Cushing Bridge: 68.2 ± 14.4%, Graves Bridge: 70.6 ± 8.28%; chi-squared = 14.8, $p = 0.0051$), except for one upstream site, Sunalta. Araneids also had a significantly lower proportion of Proteobacteria at the furthest upstream reference site (Cochrane: 12.1 ± 6.23%) compared to one other upstream site (Sunalta: 27.1 ± 11.2%) as well as the furthest downstream site (Policeman Flats: 37.6 ± 13.6%; chi-squared = 18.5, $p < 0.001$). Tetragnathids had the greatest proportion of Proteobacteria at one upstream site, Sunalta (98.0 ± 4.76%), and the highest proportion of Firmicutes and Bacteroidota at the furthest downstream site, Policeman Flats (12.8 ± 10.5% and 16.3 ± 24.7% respectively), but these shifts were not significant.

Tetragnathids had a much greater proportion of endosymbiont bacteria compared to all other invertebrates and endosymbionts were mostly dominated by *Rickettsia* and *Wolbachia* (Figure 3). Tetragnathids from the furthest upstream site (Cochrane) had the greatest abundance of *Rickettsia* (74.5 ± 45.9%) while those from another upstream site (Sunalta) had the greatest abundance of *Wolbachia* (64.7 ± 45.4%); however, there were no consistent spatial patterns in endosymbiont relative abundance from upstream to downstream (Table A.6). Consistent with the insects, the number of differentially abundant bacterial genera was greatest between the furthest upstream to downstream site comparisons for both spider families (Table 1). Araneids and

tetragnathids both only had one genus that was differentially abundant when all upstream sites combined were compared to both downstream sites combined (Figure A.4).

3.2.4 Effluent-Associated Bacteria

Out of the 715 identified bacterial genera in microbiomes from invertebrates collected in the Bow River, 106 were commonly associated with sites downstream of municipal effluent discharges and comprised 10.1% of reads in larval insects, 16.6% in adult insects, and 2.17% in spiders (Table A.7-A.9 respectively). In total, with all taxa combined, larvae had the greatest mean proportion of effluent-associated bacteria in their microbiomes at the most downstream site (Policeman Flats, ~29.6%), and the lowest proportion at an upstream site, Sunalta (~7.8%; Table A.7). Adult aquatic insects had the greatest mean proportion at the first downstream site, Graves Bridge (~43.9%), and the lowest at the upstream reference site, Cochrane (~23.4%; Table A.8). Lastly, spiders had the greatest mean proportion at the most downstream site, Policeman Flats (~14.8%), and the lowest at upstream reference site, Cochrane (~2.2%; Table A.9).

When comparing absolute bacterial abundances in individuals collected from both downstream sites combined (Graves Bridge, Policeman Flats) to sites upstream combined (Cochrane, Sunalta, Cushing Bridge), there were several effluent-associated bacteria that were differentially abundant across invertebrates (Figure A.4). Within insect larvae, Hydropsychidae microbiomes increased in *Hydrogenophaga*, *Novosphingobium, Nocardiodes,* and *Nitrosomonas* at downstream sites by ~5-10 $\log_2$ fold, heptageniid microbiomes increased in *Fluviicola* at downstream sites by ~10 $\log_2$ fold, and perlid microbiomes increased in *Serratia* and decreased in *Runella* at downstream sites by ~20 $\log_2$ fold. Within adult aquatic insects, Trichoptera individuals increased in *Arthrobacter, Clostridium sensu stricto 1, Devosia, Rhodobacter,* and

Serratia by ~20 $\log_2$ fold downstream, and adult Diptera increased in *Aeromonas, Alcaligenes, Blautia, Cytophaga, Faecalibacterium, Ferruginibacter, Fluviicola, Klebsiella, Methylotenera, Nocardioides,* and *Serratia* by ~20-30 $\log_2$ fold downstream. Interestingly, *Staphylococcus* significantly decreased in abundance downstream compared to upstream in all adult insect taxa. Both spider families each only had one differentially abundant bacterium, neither of which was associated with wastewater effluents.

3.3 Alpha Diversity

Alpha (Shannon) diversity differed among sample types (larvae, adults, spiders), among individual invertebrate taxa, and across some collection sites within a few taxa. There were significant differences between alpha diversities of insect larvae and adults (Dunn test: $Z = -6.33$, $p < 0.001$), larval insects and spiders (Dunn test: $Z = 9.77$, $p < 0.001$), and adult insects and spiders (Dunn test: $Z = 3.58$, $p < 0.001$) with larvae having the greatest diversity (mean ± SD: 4.00 ± 1.07), then adult insects (2.49 ± 166), and spiders having the least (1.49 ± 1.31). At most sites, Hydropsychidae larvae had the highest mean alpha diversity of all the invertebrate taxa and Tetragnathidae had the lowest (Figure 4). Most larvae and spider taxa had the highest alpha diversity at the furthest downstream site (Policeman Flats; Table A.10). Hydropsychids had significantly higher Shannon diversity at both downstream sites compared to all three upstream sites (ANOVA: $F_{4,31} = 21.0$, $p < 0.001$) and Araneidae had higher diversity at the furthest downstream site compared to most upstream sites (Kruskal-Wallis: Chi-squared = 20.4, $p < 0.001$).

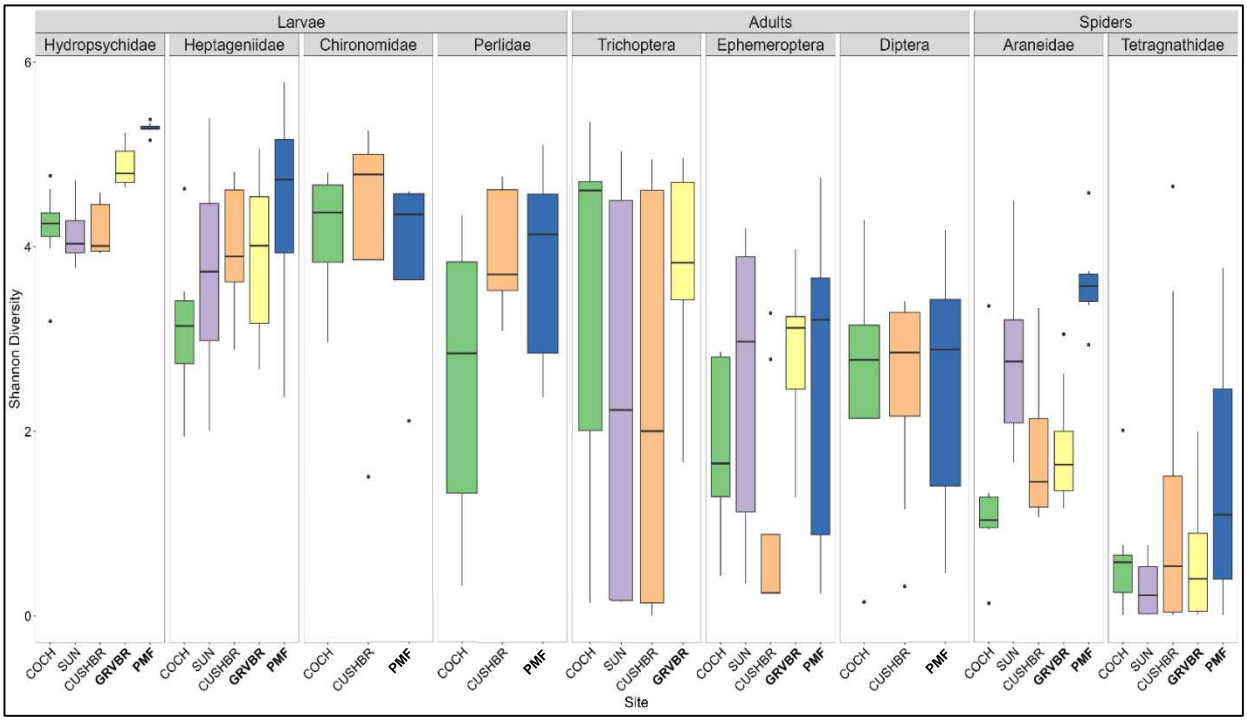

Figure 4. Boxplot of alpha diversity measured by the Shannon diversity index based on non-rarefied bacterial compositions within larval and adult aquatic insects and spiders across Bow River sites. Sites are arranged in order of upstream to downstream where bolded sites are located downstream of wastewater outfalls.

3.4 Beta Diversity

Beta diversity (community composition) varied by both invertebrate taxa type and collection site; however, the former was a more important predictor of community composition than site (RDA individual variance explained: 28.1% compared to 1.44%). With invertebrate type and sites analyzed separately, there were some taxa dependent spatial changes in beta diversity. All invertebrate taxa except for larval Chironomidae and Tetragnathidae spiders had at least one significant difference in beta diversity across collection sites (Figure A.5, Table A.11 for significant pairwise adonis comparisons). Within sites, there were significant differences between beta diversities of almost all invertebrate taxa (Figure 5); however, there was also significant beta dispersion within some groups, potentially influencing the PERMANOVA results. There were no significant correlations between any environmental water quality variable

and the microbial beta diversity. There was also no significant correlation between the microbiome community composition and collection site (Spearman correlation: r = 0.0053, p = 0.358).

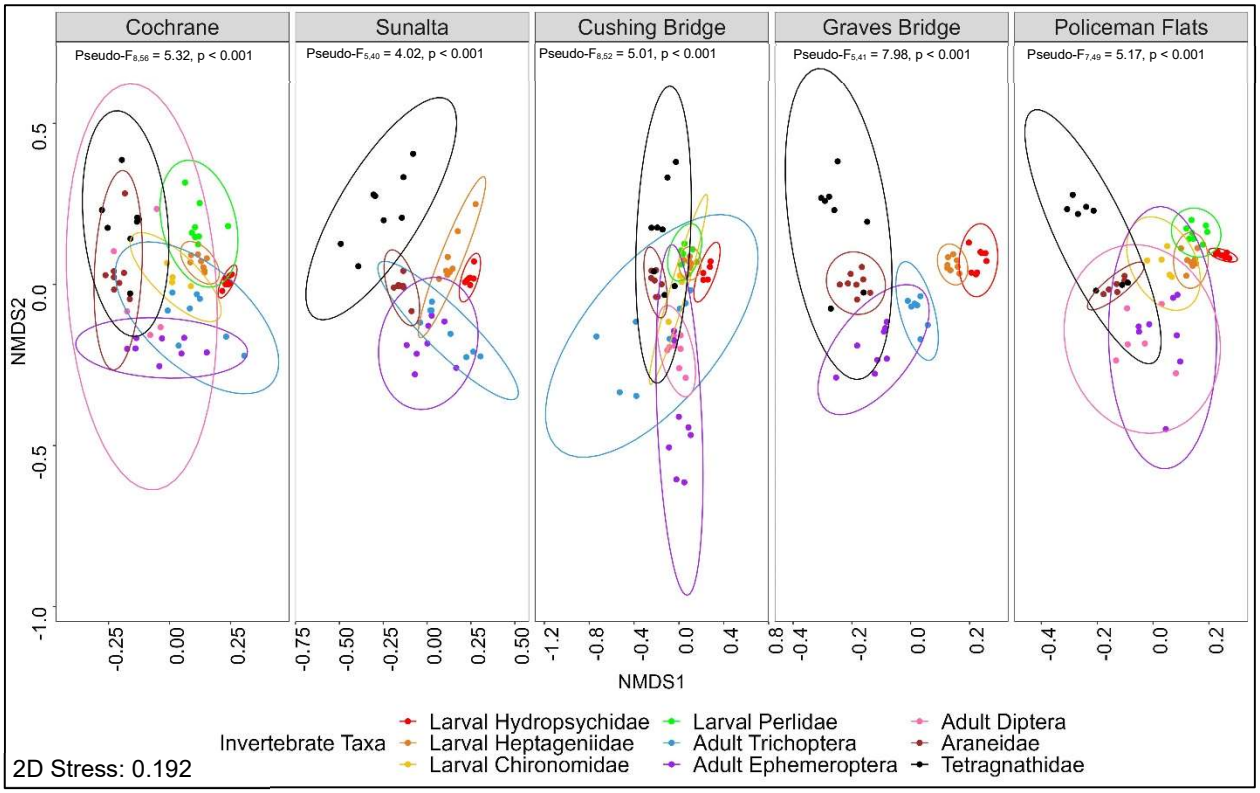

Figure 5. NMDS of bacterial beta diversity based on a Bray Curtis distance matrix across all Bow River invertebrate samples collected in July 2022, faceted by collection site. The colour of the symbol represents the invertebrate taxa type, surrounded by 95% confidence ellipses. Facets are arranged in order of upstream to downstream where Graves Bridge and Policeman Flats are located downstream of wastewater outfalls.

3.5 Predictive Functional Metabolic Pathways

The mean relative abundances of the top 25 most abundant predicted MetaCyc metabolic pathway classes did not change much across Bow River sites within invertebrate type (Figure A.6). Some invertebrates with notable shifts across sites included Trichoptera from Graves Bridge, Ephemeroptera from Cushing Bridge, and Araneidae from Policeman Flats. There were

several significantly different MetaCyc pathways across sites relative to the upstream reference site, Cochrane (Table A.12). Using Cochrane as a reference for differential abundance analysis, larval aquatic insects and araneid spiders had the greatest number of significantly different pathways between the furthest upstream to downstream site comparison (Cochrane and Policeman Flats), whereas adult aquatic insects and tetragnathid spiders had the most pathway differences between two of the upstream sites (Cochrane and Cushing Bridge).

When examining differentially abundant pathways with all upstream sites combined (Cochrane, Sunalta, Cushing Bridge) compared to both downstream sites combined (Graves Bridge, Policeman Flats), there were numerous significantly different pathways; however, the number of pathways observed differed among invertebrates (Figure A.7). Specifically, larval Hydropsychidae individuals had the greatest number of differentially abundant pathways (110) while Perlidae larvae, Diptera adults, and both spider families had none. The abundance of several biosynthesis pathways including amino acid, lipid, carbohydrate, cell structure, nucleotide, tetrapyrrole, and secondary metabolite biosynthesis decreased by ~1-15 $\log_2$ fold at downstream versus upstream sites in larval Hydropsychidae and adult Trichoptera and Ephemeroptera. Aromatic compound degradation was also enriched by ~ 1-5 $\log_2$ fold in larval Hydropsychidae and Heptageniidae exposed to effluents.

3.6 Stable Carbon and Nitrogen Isotopes

Shifts in $\delta^{13}C$ values across Bow River sites differed between basal food sources. Specifically, biofilm had significantly less ^{13}C at the furthest downstream site, Policeman Flats, compared to all upstream sites (ANOVA: $F_{4,10} = 7.59$, $p = 0.0045$). The mean ± SD $\delta^{13}C$ of

biofilm ranged from -8.47 ± 1.76 ‰ at Cushing Bridge to -25.7 ± 0.23 ‰ at Policeman Flats (Table 2). There was no difference in $\delta^{13}C$ of riparian leaves across sites ($F_{4,10} = 1.34$, $p = 0.323$) and means ranged from -28.8 ± 0.67 ‰ at Graves Bridge to -27.5 ± 1.51 ‰ at Policeman Flats.

Table 2. Mean (± standard deviation) $\delta^{13}C$ and $\delta^{15}N$ (‰) values across sample types and Bow River sites.

Sample type	Site	$\delta^{13}C$	$\delta^{15}N$
Biofilm (n = 3/site)	Cochrane	-14.6 ± 0.23	2.53 ± 0.35
	Sunalta	-13.2 ± 3.18	5.07 ± 0.21
	Cushing Bridge	-8.47 ± 1.76	5.10 ± 1.04
	Graves Bridge	-18.3 ± 8.30	7.40 ± 3.05
	Policeman Flats	-25.7 ± 0.23	2.75 ± 6.37
Riparian leaves (n = 3/site)	Cochrane	-29.1 ± 0.67	0.17 ± 1.58
	Sunalta	-28.4 ± 0.78	3.07 ± 2.29
	Cushing Bridge	-28.7 ± 0.51	2.70 ± 0.17
	Graves Bridge	-28.8 ± 0.67	6.67 ± 1.52
	Policeman Flats	-27.5 ± 1.51	5.90 ± 1.06
Aquatic larval insects	Cochrane (n = 5)	-32.0 ± 1.14	6.67 ± 0.69
	Sunalta (n = 14)	-29.2 ± 1.15	6.70 ± 1.19
	Cushing Bridge (n = 6)	-30.1 ± 1.03	6.35 ± 0.72
	Graves Bridge (n = 15)	-29.0 ± 1.11	9.97 ± 0.81
	Policeman Flats (n = 14)	-26.7 ± 2.62	6.38 ± 2.04
Aquatic adult insects	Cochrane (n = 9)	-29.3 ± 2.70	8.61 ± 1.25
	Sunalta (n = 11)	-29.8 ± 1.68	8.64 ± 1.28
	Cushing Bridge (n = 11)	-27.8 ± 1.49	9.10 ± 1.33
	Graves Bridge (n = 12)	-26.4 ± 1.34	11.4 ± 1.89
	Policeman Flats (n = 12)	-25.3 ± 2.34	7.70 ± 2.37
Terrestrial adult insects	Cochrane (n = 3)	-27.2 ± 1.79	6.57 ± 1.51
	Sunalta (n = 3)	-29.6 ± 1.10	6.37 ± 1.10
	Graves Bridge (n = 6)	-26.5 ± 1.24	5.95 ± 1.20
	Policeman Flats (n = 4)	-26.4 ± 1.01	3.23 ± 3.04
Spiders (n = 6/site)	Cochrane	-29.1 ± 0.60	8.53 ± 0.82
	Sunalta	-27.5 ± 0.82	8.53 ± 0.85
	Cushing Bridge	-26.6 ± 0.32	8.60 ± 0.48
	Graves Bridge	-24.8 ± 0.81	12.8 ± 0.56
	Policeman Flats	-25.1 ± 0.65	8.26 ± 0.66

Aquatic larval and adult insects and riparian spiders shifted in $\delta^{13}C$ at sites downstream of wastewater outfalls, but terrestrial insects did not. Within aquatic insects, larvae at upstream sites had $\delta^{13}C$ values that were closer to riparian leaves (lower in ^{13}C) compared to biofilm, suggesting a greater reliance on allochthonous food sources for their diet (Table 2, Figure 6, Figure A.8). At downstream sites, most larvae were significantly higher in ^{13}C (ANOVA: $F_{4,49} = 11.8$, $p < 0.001$) and had values closer to those of biofilm, especially at the furthest downstream site, Policeman Flats, potentially indicating a shift in diet towards more autochthonous food sources. Aquatic adults and riparian spiders showed similar trends to that of the larvae with significant shifts in $\delta^{13}C$ values, increasing in ^{13}C at sites downstream compared to most sites upstream ($F_{4,50} = 10.6$, $p < 0.001$ and $F_{4,25} = 43.3$, $p < 0.001$, respectively). Terrestrial insects had significantly lower ^{13}C at one upstream site, Sunalta, compared to both downstream sites ($F_{3,12} = 4.50$, $p = 0.025$), but did not have any shifts downstream compared to the furthest upstream reference site, suggesting no effect from effluent-exposure. Lastly, the $\delta^{13}C$ values of both spider families overlapped with those of aquatic adult and terrestrial insects at every site (means within 0.2 – 2.3 ‰ and 0.1 – 2.1 ‰, respectively), suggesting potential reliance on both aquatic and terrestrial insects as a food source ($F_{50,112} = 4.83$, $p < 0.001$).

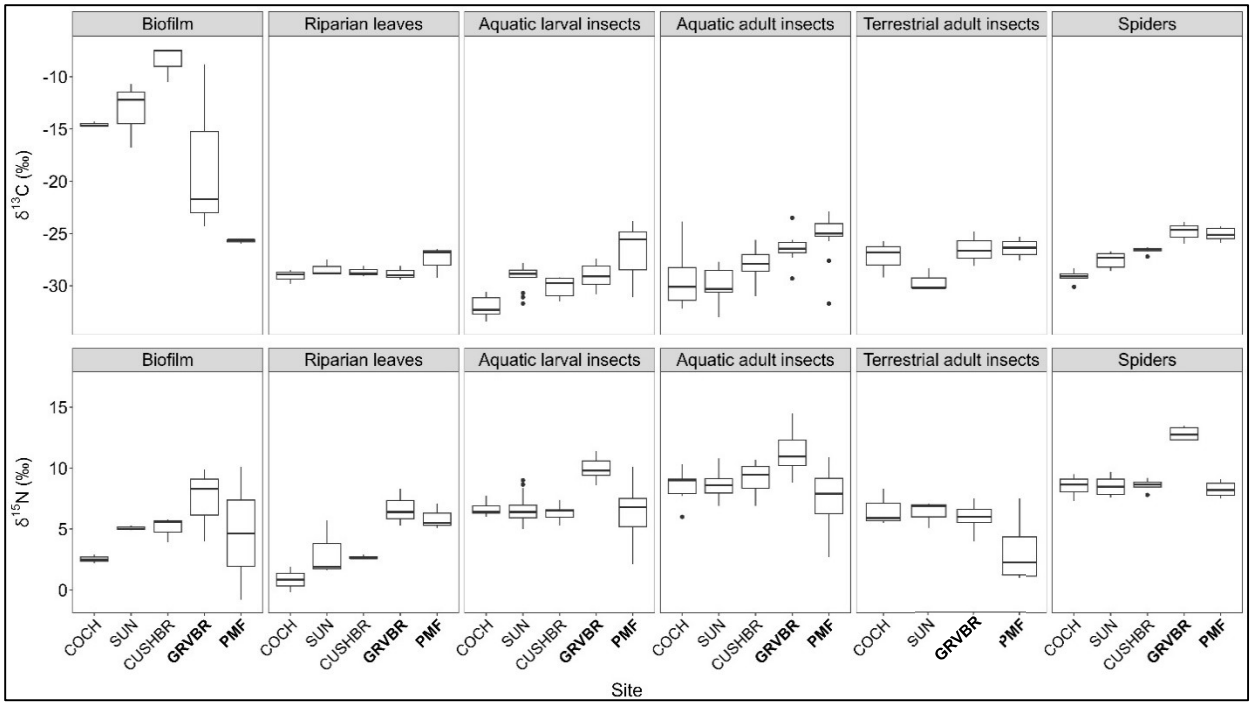

Figure 6. $\delta^{13}C$ and $\delta^{15}N$ across collection sites, faceted by sample groups. Sites arranged from upstream to downstream where bolded sites, GRVBR and PMF, are located downstream of effluent outfalls.

In addition to shifts in $\delta^{13}C$, $\delta^{15}N$ was enriched at downstream sites in many sample groups (Table 2, Figure 6). Riparian leaves from both downstream sites were enriched in $\delta^{15}N$ compared to the most upstream site (Cochrane; ANOVA, $F_{4,10} = 9.23$, $p = 0.0022$); although biofilm followed a similar trend, results were not statistically different across any sites ($F_{4,10} = 1.17$, $p = 0.379$). Despite riparian leaves being enriched in ^{15}N at both downstream sites, there was no such enrichment in terrestrial insects (orders Lepidoptera and Coleoptera; $F_{3,12} = 2.62$, $p = 0.099$). However, aquatic larval and adult insects from one downstream site, Graves Bridge, had higher $\delta^{15}N$ values compared to all other sites ($F_{4,49} = 18.5$, $p < 0.001$ and $F_{4,50} = 7.90$, $p < 0.001$, respectively). Similarly, riparian spiders had significantly higher $\delta^{15}N$ at Graves Bridge compared to all other sampling sites (Kruskal-Wallis, chi-squared = 14.7, $p = 0.0054$), suggesting some incorporation of effluent-derived nutrients at this site into primary and secondary

consumers and subsequent transfer to the terrestrial environment. Riparian spider predators had similar $\delta^{15}N$ values to aquatic adult invertebrates (Dunn comparison: $Z = -0.114$, $p_{adj} = 0.909$), but had significantly higher $\delta^{15}N$ compared to terrestrial invertebrates ($Z = 4.69$, $p_{adj} < 0.001$).

4.0 Discussion

The objective of this study was to identify potential microbial community indicators of municipal wastewater effluent impacts on freshwater larval and adult insects and riparian spiders of the Bow River for future potential use as part of a monitoring framework. Wastewater exposure was confirmed using the incorporation of effluent-associated bacteria and effluent-derived nitrogen ($\delta^{15}N$) into downstream invertebrates. Overall, the results presented herein provide some evidence of microbial dysbiosis in insects exposed to municipal wastewater effluents, supporting our prediction. There were decreases in endosymbiont abundance, shifts in alpha and beta diversities, higher abundances of effluent-associated bacteria, and downregulated biosynthesis pathways in most larval and adult insects from sites downstream of wastewater outfalls compared to those from upstream sites. Adult insects had higher endosymbiont relative abundances and lower alpha diversity compared to their larval forms at all sites, supporting our prediction that microbiomes are altered through metamorphosis. $\delta^{15}N$ was also enriched in all insects and spiders at one downstream site, providing evidence of the incorporation and transfer of effluent-derived nitrogen across the aquatic — riparian boundary. Although there was evidence of effluent-derived contaminants in riparian spiders, their microbiomes showed few spatial shifts downstream and little similarity to their assumed primary food source of adult aquatic insects, which contrasted with our prediction.

4.1 Shifts in Bacterial Communities in Relation to Effluent Exposure

There were a few changes in bacterial relative abundance at the phylum level in larval and adult insects and spiders across sites; however, the observed changes were not consistent across taxa at effluent-exposed sites and did not reflect ratios that have previously been associated with contaminant induced dysbiosis in animal models. These ratios include an increased proportion of Bacteroidota to Proteobacteria in fish (Legrand et al., 2018; Krotman et al., 2020) or a decreased proportion of Bacteroidota to Firmicutes in humans (Ley et al., 2006). In the current study, increased alpha diversity of the bacterial communities was observed in hydropsychids and araneids from effluent-exposed sites in the Bow River. This finding contradicts other studies that have found decreased alpha diversity in animal and sediment microbiomes exposed to municipal wastewater effluents (Drury et al., 2013; Millar et al., 2022) and other aquatic contaminants (Sarkar et al., 2015; Yang et al., 2019). However, a higher alpha diversity may also be linked to adverse outcomes, as higher diversity has been observed in human patients with various illnesses and may be indicative of a more unstable microbiome (Lozupone et al., 2013; Johnson and Burnet, 2016). In our study, there were also spatial shifts in beta diversities (community composition) in most effluent-exposed invertebrates, except for tetragnathid spiders and larval chironomids, which has been seen in other MWWE-exposed organisms (Drury et al., 2013; Restivo et al., 2021; Millar et al., 2022). It is well documented that many species of chironomids can tolerate some level of contaminant exposure and are typically the dominant insect taxa found in polluted waters (Armitage et al., 1995; Wright and Burgin, 2009; Sela and Halpern, 2022). The chironomid microbiome may have evolved to play an important role in detoxifying chemical contaminants, thereby increasing larval survival in these environments (Halpern and Senderovich, 2015; Sela and Halpern, 2020; Sela and Halpern, 2022) and potentially indicating

microbial resistance to contaminants, resulting in a lack of change in microbiome diversities in larval chironomids downstream of effluent discharges sites on the Bow River.

Within insect orders in our study, larvae and adults had dissimilar bacterial communities and shifts across sites. Specifically, adult insects had different spatial shifts in phyla and genera relative abundances, higher relative abundances of total endosymbiont bacteria at each site, and an overall lower alpha diversity compared to their larval forms. These results were not surprising since it has previously been observed that adult aquatic and terrestrial insects have distinct bacterial communities to that of their larval counterpart, thought to occur by changes from metamorphosis (Pechal and Benbow, 2016; Sela et al., 2020; Gohl et al., 2022; Kucuk et al., 2023). The gut microbiome is typically purged during metamorphosis and consequently forces the recolonization of the gut bacteria as adults through interacting with and sometimes feeding in a different environmental niche (Hammer and Moran, 2019; Kowallik and Mikheyev, 2021; Manthey et al., 2023), potentially explaining lower alpha diversity in adults. Some bacteria such as endosymbionts can be maternally transferred and retained in adults through metamorphosis (Maire et al., 2020) and may explain the higher relative abundances of endosymbiont bacteria in adults relative to the larvae since they may also be horizontally transferred through parasites in their new environment or mating (see section 4.4 for further discussion on endosymbionts).

4.2 Shifts in Metabolic Functional Predictions of Host Bacteria

Functional profiles indicated that the greatest number of significantly different metabolic pathways was observed between the furthest upstream to downstream site comparison across most invertebrate taxa. Several of these predicted pathways linked to the biosynthesis of essential

building blocks for host survival significantly decreased downstream, including amino acid, lipid, carbohydrate, cell structure, nucleotide, tetrapyrrole, and secondary metabolite biosynthesis. Although this analysis is presumptive (based on taxonomies), results suggest that there is a disruption to host metabolic processes when exposed to wastewater effluents (Deng et al., 2019). Contaminant exposure has previously been associated with changes in metabolic pathways, including a reduction in amino acid biosynthesis pathways in ground beetles exposed to pesticides (Giglio et al., 2021). However, contrary to our findings, some genes or pathways associated with metabolic processes such as lipogenesis genes or carbohydrate, lipid, and amino acid metabolism may be upregulated when exposed to environmental contaminants (Kalkhof et al., 2015; Tseng et al., 2023). To better understand and validate the predicted reduction in biosynthesis pathways observed herein, multi-omic tools such as metagenomics and metatranscriptomics should be used (see section 4.5 for further discussion on functional inferencing).

4.3 Evidence of Wastewater Exposure to Downstream Organisms

Total proportions of effluent-associated bacteria as well as differentially abundant effluent-associated genera increased in most larval and adult insects that were collected downstream of wastewater effluent outfalls compared to those from upstream sites, providing evidence of incorporation of effluent-derived bacteria. Previous studies have reported higher levels of some bacteria associated with the wastewater treatment process, opportunistic pathogens of fish and humans, and enteric bacteria linked to the digestive system of humans and fish in invertebrates exposed to wastewater effluents (Restivo et al., 2021; Millar et al., 2022; Wang et al., 2023). In our study, bacteria associated with the wastewater treatment process (including *Cytophaga*,

Hydrogenophaga, Methylotenera, Nitrosomonas, Nocardioides, Novospingobium, Rhodobacter) had higher abundances in many invertebrate taxa from effluent-exposed sites. *Cytophaga* has algicidal properties (Ye et al., 2016) which may contribute to sewage purification by polymer breakdown (Gude, 1980). *Methylotenera*, *Nitrosomonas, Nocardioides,* and *Novosphingobium* are nitrifying bacteria which convert ionized ammonia to nitrite (Gerardi, 2006; Mustakhimov et al., 2013), contributing to the removal of harmful nitrogen forms in the effluent. Additionally, some species of *Rhodobacter* have the potential for remediation of heavy metals (Li et al., 2017) and some are common nitrifiers and photosynthetic bacteria that are used to remove ammonia (Wen et al., 2016). Additionally, enteric bacteria (*Clostridium sensu stricto 1, Blautia*) and a potentially pathogenic bacterium (*Serratia*) had higher abundances in organisms collected downstream compared to upstream sites. *Serratia* is an opportunistic pathogen in humans (Mahlen, 2011), however, this genus may be part of the core microbiome in some aquatic insects and many species of this genus are not pathogenic to invertebrates (Castillo et al., 2020).

Stable nitrogen isotopes were used as a tracer of effluent-derived nitrogen uptake in effluent-exposed invertebrates. We found higher $\delta^{15}N$ in riparian leaves, aquatic larval and adult insects, and riparian spiders at one downstream site (Graves Bridge), providing evidence of effluent exposure and transfer of nutrients across the aquatic – riparian boundary. Stable carbon isotope ratios of riparian spiders had intermediate values between those of aquatic and terrestrial adult insects at most sites, suggesting that the spiders likely partially fed on both aquatic and terrestrial insects and had an exposure route of effluent-derived contaminants through their diet on exposed aquatic adult insects. Graves Bridge is located a few kilometers below the Bonnybrook WWTP that releases ~320,000 m^3 of treated effluent per day, about 3 times more discharge than the other two treatment plants (Fish Creek and Pine Creek) combined

(Government of Canada, 2024). Above all WWTPs, surface water quality is good and nutrient levels are low (total phosphorus between 0.008-0.009 mg/L, NO_3 between 0.085 – 0.104 mg/L). However, below all three wastewater inputs, near Policeman Flats, phosphorus and nitrate concentrations are higher (0.019 mg/L and 0.328 mg/L, respectively) and there were higher concentrations of pharmaceuticals (Arlos et al., 2023), indicating poorer water quality. Although there are no nutrient data for the Graves Bridge site, it is likely that majority of these increases in nutrients downstream is originating from the Bonnybrook plant due to its' large amount of discharge; this in turn, could explain the increase in the anthropogenic ^{15}N signal downstream of its' effluent outfall. Based on the unique treatment characteristics of each WWTP, the stable nitrogen isotope ratios or nitrogen dispersion rates likely differ and may also explain why we see different uptake patterns of primary producers and subsequent consumers at each site (Munksgaard et al., 2017).

4.4 Endosymbiont Bacteria

Most adult insects and spider microbiomes contained endosymbiont bacteria, and their endosymbionts were dominated by *Rickettsia, Spiroplasma,* and *Wolbachia*. Adults had a greater proportion of endosymbionts than larvae from the same order, which has also been observed in terrestrial weevils (Vigneron et al., 2014; Maire et al., 2020). A greater proportion of endosymbionts may be acquired in insect adults through horizontal transfer, in response to their rapidly changing environment after metamorphosis, with new bacteria, or through vertical (maternal) transfer, which usually inhabits areas of the body such as the hemolymph that remain more stable during metamorphosis (Hammer and Moran, 2019). Compared to other invertebrates, Tetragnathidae spiders had a much greater proportion of endosymbionts, more than

¾ of their entire microbiome as endosymbionts at some sites, and their endosymbionts were dominated by *Rickettsia, Wolbachia,* and *Spiroplasma*. These three bacterial genera are maternally transmitted and have been linked to cytoplasmic incompatibility and skewed sex ratios (female-biased) in offspring, which has implications for host reproduction and evolutionary histories (Wu et al., 2004; Goodacre et al., 2006; Eleftherianos et al., 2013). Endosymbionts may also prevent other bacteria from colonizing the host microbiome (Wang et al., 2023), which may explain the overall decreased alpha diversity and lack of differences in beta diversity in effluent-exposed tetragnathid spiders compared to other invertebrates in our study. In contrast, Araneidae spiders had a much lower proportion of endosymbionts compared to tetragnathids, comprising less than 5% their total microbiome at most sites and as such, had a slightly higher alpha diversity than tetragnathids at most sites. Tyagi et al. (2021) found a similar bacterial composition in tetragnathids to those in our study, with a high proportion of the microbiome composed of Proteobacteria and endosymbiont genera, *Rickettsia* and *Wolbachia* (although contrarily, no *Spiroplasma* was observed), and araneids contained a much lower proportion of endosymbionts compared to tetragnathids.

Relative abundances of *Rickettsia* decreased in larval and adult insects from wastewater exposed sites compared to reference sites, suggesting possible sensitivity of this bacterial genus to contaminants. However, riparian spiders did not exhibit the same decrease in *Rickettsia* at effluent-exposed sites, suggesting that spider endosymbionts are less influenced by or less exposed to effluent-associated contaminants from their diet. Other studies have found that spider endosymbionts are affected by contaminant exposures; more specifically, there was a reduction in the relative abundances of endosymbionts in riparian spiders exposed to PCBs (Perrotta et al., 2022), and *Wolbachia* infections in spiders exposed to antibiotics (Vanthournout et al., 2011).

Alterations in endosymbiont composition may have critical implications to host behaviour and immune function (Gupta and Nair, 2020); however, the mechanism by which contaminants affect endosymbionts remains unknown and would be beneficial to evaluate in future studies.

4.5 Limitations and Future Directions

Due to the dynamic spatial and temporal nature of the host microbiome from factors such as genetics, diet, environmental conditions, and host life history, it is challenging to characterize the 'normal' or 'core' bacterial composition of hosts (Phillips et al., 2012; Yun et al., 2014; Adamovsky et al., 2018; Neu et al., 2021). Results from our study may not be representative of the true core microbiome of these invertebrates since they were collected from one timepoint. Time and seasonality of sample collection can affect bacterial community composition (Kroetsch et al., 2020; Kodera et al., 2023), thus it may be beneficial to sample multiple timepoints to obtain a better understanding of temporal impacts of effluents on host microbiomes. Differences in taxonomy could have also influenced the variability in results since hosts with a similar phylogeny have microbiomes that are more closely related (Yun et al., 2014; Kroetsch et al., 2020; Mallott and Amato, 2021). Since our study grouped larvae to family level and adults to order level, adults may have inherently more variability compared to the larvae and this, in turn, may have affected some site differences (or lack thereof) or differences between larvae and adults. Currently, there is a lack of standardized approaches for microbiome studies in terms of sample collection, laboratory processing, and data analysis (Hornung et al., 2019; Berg et al., 2020; Neu et al., 2021); establishing standard approaches for sample collection, sample identification, storage conditions, laboratory processing and sequencing, and data analysis would help control for some of this variability, producing more reproducible results.

To counter the challenge of characterizing the composition of a core microbiome, researchers have suggested focusing more on the functional abilities of the microbes for their hosts (Adamovsky et al., 2018; Berg et al., 2020). In our study, we used PICRUSt2 analyses which are commonly used with DNA metabarcoding to predict metabolic functions of bacteria based on taxonomy (Douglas et al., 2020). Functional inference tools are highly valuable for predicting potential microbial functions at a lower cost than shotgun- based methods, which sequence the entire genome; however, this method also comes with limitations. Firstly, as metabolic pathways are inferred through taxonomy, the results only predict the functional potential of the bacteria as genes are not always present or expressed under all conditions. This analysis also does not capture genes that were acquired from lateral transfers such as transformation or transduction, potentially overlooking metabolic functions of the bacteria (Djemiel et al., 2022). Additionally, compared to human hosts, there is a lack of reference genomes for aquatic environments, which likely results in more rare metabolic pathways not being identified (Djemiel et al., 2022). PICRUSt2 may become more useful after further research characterizing the functions of environmental microbes using shotgun- based sequencing or metatranscriptomic analyses are added to the reference genomes.

The Bow River has other sources of anthropogenic contamination besides municipal wastewater effluent, including agricultural runoff, livestock management practices, and stormwater runoff, that were not evaluated in our study (Alberta Government, 2014). Nose Creek is historically contaminated with stormwater (Schonekess, 1981; Nose Creek Watershed Partnership, 2018) and flows into the Bow River directly above the Cushing Bridge site. We may have observed higher effluent-associated bacteria and a greater number of differentially expressed metabolic pathways in some insects collected from Cushing Bridge due to stormwater

or other anthropogenic pollution and effects of wastewater effluents may have been partially masked at downstream sites because of inputs from Nose Creek. Future studies could analyze effluent samples from all three WWTPs as well as inputs from Nose Creek and water or sediment samples at each site to better understand the types and sources of bacteria entering the receiving waters compared to the background composition from the river and compare it to the exposed invertebrates.

4.6 Conclusions

Overall, microbiomes of aquatic insects residing downstream of wastewater effluent outfalls were altered; however, some taxa appeared to be more resistant to perturbations than others. Although there was limited evidence for dysbiosis in riparian spiders, effluent-derived nitrogen was incorporated into all invertebrates at one wastewater impacted site and this may have implications for energy subsidies to terrestrial systems. It is evident that anthropogenic contaminant impacts are seen even at a microscopic level in receiving ecosystems, and monitoring microbial communities may be a useful tool for identifying sublethal organism stress from anthropogenic contaminants.

Results of our study should be compared with other projects assessing the impacts of effluents in the Bow River, to understand whether microbial responses correlate with organism-level or population-level impacts at the same sites. This can be done through linking the current results to, e.g., existing invertebrate community data (Sutherland, 2024) to understand whether impacts from effluents were consistently observed at downstream sites. While the microbiome may be a useful mechanism of identifying environmental stressors in affected organisms, a combination of more standardized approaches and understanding how alterations of microbiomes

are linked to physiological endpoints will be important when considering using this endpoint for a program monitoring the impacts of MMWEs on aquatic ecosystems and identifying sites that require further investigation and mitigation.

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Appendix A: Bow River Microbiome and Stable Isotope Supplementary Materials

Results

Relative Abundance Shifts of Bacterial Phyla and Genera

Larval aquatic insects from the Bow River had several spatial shifts in bacterial mean relative abundance at the genus level (although were not statistically tested). Specifically, hydropsychids increased in the proportion of *Tabrizicola* from upstream to downstream, heptageniids had a greater proportion of *Candidatus Bacilloplasma* at the highest upstream site and *ZOR0006* at the second highest upstream site compared to all other sites, chironomids decreased in *Tabrizicola* and increased in *Ideonella* and *Pseudorhodobacter* from upstream to downstream, and perlids decreased in *Rhodoferax* and *Sphingorhadbus* from upstream to downstream and had a greater proportion of *Emticicia, Ideonella,* and *Vibrionimonas* at one upstream site, Cushing Bridge (Figure A.3).

Adult aquatic insects had several shifts in bacterial phyla and genera compared to the larvae of the same order (not statistically tested, Table A.5, Figure A.3). Adult Trichoptera had an increased proportion of Proteobacteria and a decreased proportion of Bacteroidota at all sites compared to the larvae of the same order (Hydropsychidae). Adult Ephemeroptera had a much greater proportion of Proteobacteria at two upstream sites, Cochrane and Cushing Bridge, compared to the larvae of the same order (Heptageniidae), as well as increases in Firmicutes from upstream to downstream whereas the larvae decreased in Firmicutes from upstream to downstream. Adult Diptera had increased proportions of Firmicutes at the upstream sites compared to the larvae (Chironomidae) and decreased proportions of Bacteroidota at Cushing Bridge and Policeman Flats relative to the larvae. Shifts at the genus level included a greater

proportion of *Carnobacterium* at one upstream site (Sunalta) and a greater proportion of *Ideonella* and *Serratia* at one of the downstream sites (Graves Bridge) in adult Trichoptera compared to all other sites, a greater proportion of *Candidatus Bacilloplasma* at the furthest downstream site (Policeman Flats) and *Staphylococcus* at Cushing Bridge in adult Ephemeroptera, and decreased relative abundances of *Massilia* and increased relative abundances of *Flavobacterium, Serratia, Sphingorhadbus*, and *Staphylococcus* from upstream to downstream in adult Diptera.

Spiders were distinct from the larval and adult aquatic insects and had fewer shifts in their relative abundances at the genus level. The dominant genus among araneids was *Vibrionimonas*, comprising over 50% of the total mean abundance at 4 of 5 sites and had the lowest proportion of this genus at the furthest downstream site, Policeman Flats (Figure A.3). Araneids also had increased *Massilia* and *Rhodococcus* at the most downstream site (Policeman Flats) and an increased proportion of endosymbiont bacteria *Spiroplasma* at the highest upstream reference site (Cochrane) compared to all other sites. Tetragnathids were dominated by endosymbiont bacteria *Rickettsia, Wolbachia,* and *Spiroplasma* which did not have consistent shifts from upstream to downstream, however, *Spiroplasma* had a higher relative abundance at the furthest downstream site, Policeman Flats. Tetragnathids also had higher relative abundances of *Allorhizobium-Neorhizobium-Pararhizobium-Rhizobium*, *Rhodococcus* and *Vibrionimonas* at Policeman Flats.

Supplementary Tables

Table A.1. Locations and time periods of insect and spider sample collections for microbiome, stable isotope, and nutrient excretion analyses in the Bow River, Calgary, AB in 2022. Bolded site names are included in the City of Calgary watershed surface water quality monitoring program.

Site name	Site ID	~ Distance from nearest WWTP	Sampling month	Latitude	Longitude
Cochrane*	COCH	53 km upstream of Bonnybrook	July, September	51.185778	-114.490806
Sunalta*	SUN	13 km upstream of Bonnybrook	July, September	51.047070	-114.112000
Cushing Bridge	CUSHBR	4 km upstream of Bonnybrook	July	51.039244	-114.010721
Graves Bridge	GRVBR	2 km downstream of Bonnybrook	July	50.989310	-114.022000
Policeman Flats	PMF	3 km downstream of Pine Creek	July, September	50.845144	-113.946021

*Note the site name discrepancies listed in this study compared to the City of Calgary surface water quality monitoring program. “Cochrane” = “Bow River Highway 22 Bridge”, “Sunalta” = “Pumphouse”.

Table A.2. Water quality data across Bow River sites, reported as single grab samples measured by the City of Calgary in July 2022. Variables include water temperature (°C; Temp), dissolved oxygen (mg/L; DO), total phosphorus (mg/L; TP), total dissolved solids (mg/L; TDS), total organic carbon (mg/L; TOC), specific conductivity (µS/cm; Cond), ammonia (mg/L; NH_3), nitrate (mg/L; NO_3), pH, and phosphate (mg/L; PO_4). There are no data for the Graves Bridge site as it is not monitored by the City of Calgary.

Site	Date collected	Temp	DO	TP	TDS	TOC	Cond	NH_3	NO_3	pH	PO_4
Cochrane	July 11/22	12.5	10.3	0.008	142	1.00	257	< 0.05	0.104	8.3	< 0.005
Sunalta	July 13/22	14.7	9.8	0.008	145	1.00	265	< 0.05	0.085	8.4	< 0.005
Cushing Bridge	July 12/22	14.3	9.7	0.009	158	1.30	288	< 0.05	0.095	8.3	< 0.005
Policeman Flats	July 12/22	13.7	9.5	0.019	165	0.9	301	< 0.05	0.328	8.2	< 0.005

Table A.3. Sample sizes for stable isotope analyses organized by taxonomy, Functional Feeding Group (FFG), number of collection sites samples were collected from, and number of replicates per site, collected in July 2022 from the Bow River.

Sample type	Taxon	FFG	Number of collection sites	Number of replicates per site
Basal food source	Biofilm	-	5	3
Basal food source	Riparian leaves	-	5	3
Larval aquatic insect	Hydropsychidae	Filterer-collector	4	2-3
Adult aquatic insect			5	2-3
Larval aquatic insect	Chironomidae		3	1-3

Adult aquatic insect		Gatherer-collector	5	1-3
Larval aquatic insect	Chloroperlidae	Predator	3	3
Adult aquatic insect			2	1-3
Larval aquatic insect	Ephemerellidae	Gatherer-collector	3	3
Adult aquatic insect			4	1-3
Larval aquatic insect	Heptageniidae	Scraper	5	2-3
Adult aquatic insect			4	1
Adult aquatic insect	Leptoceridae	Gatherer-collector	2	1-3
Larval aquatic insect	Perlidae	Predator	2	3-4
Adult aquatic insect	Rhyacophilidae	Predator	5	1-3
Adult terrestrial insect	Coleoptera	Predator	2	1-3
Adult terrestrial insect	Lepidoptera	Piercer	4	3
Spider	Tetragnathidae	Predator	5	3
Spider	Araneidae	Predator	5	3

Table A.4. Total number of unique bacterial taxa (ASVs) and counts at each taxonomic level within whole-body invertebrate hosts and across sites in the Bow River, July 2022. Unclassified ASVs were not included in the taxonomic counts.

	Invertebrate Type	Unique ASVs	Phyla	Classes	Orders	Families	Genera
	Cochrane (n = 65)	**3637**	**33**	**71**	**171**	**245**	**485**
Larvae	Hydropsychidae (n = 8)	782	17	30	64	87	110
	Heptageniidae (n = 8)	1544	27	53	122	158	258
	Chironomidae (n = 4)	467	14	35	79	105	152
	Perlidae (n = 8)	639	15	25	62	89	125
Adults	Trichoptera (n = 8)	922	15	30	91	130	234
	Ephemeroptera (n = 8)	313	14	26	65	102	138
	Diptera (n = 5)	232	6	12	45	64	106
Spiders	Araneidae (n = 8)	324	10	18	52	78	132
	Tetragnathidae (n = 8)	210	9	16	45	73	109
	Sunalta (n = 46)	**2938**	**31**	**70**	**156**	**228**	**455**
Larvae	Hydropsychidae (n = 7)	634	18	26	48	63	81
	Heptageniidae (n = 8)	1594	25	54	118	163	275
Adults	Trichoptera (n = 8)	585	17	31	77	127	216
	Ephemeroptera (n = 8)	432	14	20	55	102	159
Spiders	Araneidae (n = 7)	457	12	24	60	88	172
	Tetragnathidae (n = 8)	63	4	5	21	27	32

	Cushing Bridge (n = 61)	**4043**	**35**	**74**	**176**	**268**	**533**
Larvae	Hydropsychidae (n = 5)	617	17	27	54	76	102
	Heptageniidae (n = 8)	1545	27	59	132	171	289
	Chironomidae (n = 4)	587	20	40	88	119	172
	Perlidae (n = 5)	1567	26	57	123	163	260
Adults	Trichoptera (n = 8)	824	19	33	80	138	230
	Ephemeroptera (n = 8)	190	8	15	46	68	95
	Diptera (n = 8)	244	8	13	39	74	101
Spiders	Araneidae (n = 7)	382	9	16	56	82	147
	Tetragnathidae (n = 8)	408	14	27	97	83	168
	Graves Bridge (n = 47)	**3468**	**28**	**62**	**157**	**241**	**484**
Larvae	Hydropsychidae (n = 8)	1245	22	40	80	102	145
	Heptageniidae (n = 8)	1576	22	49	109	153	145
Adults	Trichoptera (n = 7)	775	19	33	82	138	242
	Ephemeroptera (n = 8)	253	12	19	54	80	118
Spiders	Araneidae (n = 8)	429	11	21	63	97	169
	Tetragnathidae (n = 8)	134	8	14	37	52	80
	Policeman Flats (n = 57)	**4435**	**34**	**72**	**177**	**264**	**556**
Larvae	Hydropsychidae (n = 8)	1339	22	42	90	114	148
	Heptageniidae (n = 8)	1960	25	56	120	173	276
	Chironomidae (n = 5)	890	20	42	98	145	219
	Perlidae (n = 8)	860	18	32	77	103	149
Adults	Ephemeroptera (n = 8)	574	16	26	66	120	199
	Diptera (n = 6)	247	10	13	41	72	113
Spiders	Araneidae (n = 6)	591	15	26	71	106	215
	Tetragnathidae (n = 8)	427	13	21	62	99	193

Table A.5. Mean relative abundance ± standard deviation (%) of the average top three most abundant bacterial phyla across all sites within each invertebrate taxa of the Bow River.

	Invertebrate Taxa	**Phylum**	**Cochrane**	**Sunalta**	**Cushing Bridge**	**Graves Bridge**	**Policeman Flats**
Larvae	Hydropsychidae	Bacteroidota	44.9 ± 12.3	45.4 ± 5.19	45.5 ± 10.3	43.7 ± 4.55	47.6 ± 2.89
		Proteobacteria	29.8 ± 7.83	34.3 ± 10.8	34.8 ± 14.2	32.2 ± 4.69	32.9 ± 4.75
		Firmicutes	19.2 ± 6.85	14.4 ± 3.35	12.0 ± 4.49	15.4 ± 3.94	10.5 ± 4.09
		Other	6.17 ± 0.79	5.91 ± 1.19	7.66 ± 2.02	8.79 ± 0.72	9.03 ± 0.63
	Heptageniidae	Proteobacteria	28.7 ± 14.7	41.6 ± 19.9	43.6 ± 20.0	58.1 ± 25.4	52.6 ± 14.3
		Firmicutes	56.0 ± 19.6	40.1 ± 24.6	33.1 ± 22.1	28.0 ± 27.4	24.8 ± 20.0
		Bacteroidota	13.6 ± 5.98	13.0 ± 7.94	16.2 ± 8.59	11.9 ± 5.49	18.2 ± 6.82

		Other	1.63 ± 0.21	12.9 ± 2.46	7.10 ± 1.22	1.99 ± 0.34	4.40 ± 0.67
		Proteobacteria	65.9 ± 5.72	-	52.4 ± 29.9	-	39.5 ± 13.0
		Bacteroidota	6.42 ± 8.11	-	29.9 ± 37.6	-	33.2 ± 22.4
	Chironomidae	Firmicutes	9.48 ± 4.43	-	6.25 ± 1.05	-	17.2 ± 13.8
		Other	18.2 ± 3.83	-	11.4 ± 2.50	-	10.1 ± 3.23
		Proteobacteria	81.6 ± 18.0	-	64.0 ± 17.0	-	69.9 ± 13.1
		Bacteroidota	16.5 ± 17.1	-	32.1 ± 17.8	-	23.2 ± 10.0
	Perlidae	Firmicutes	1.28 ± 3.00	-	1.04 ± 0.64	-	2.31 ± 3.14
		Other	0.61 ± 0.27	-	2.81 ± 0.37	-	4.54 ± 0.77
		Proteobacteria	66.3 ± 26.7	57.0 ± 38.1	58.8 ± 36.4	57.4 ± 17.4	-
		Bacteroidota	24.1 ± 21.1	14.8 ± 17.6	15.6 ± 21.5	20.2 ± 11.8	-
	Trichoptera	Firmicutes	3.83 ± 4.38	22.2 ± 31.9	22.8 ± 36.5	10.5 ± 8.16	-
		Other	5.75 ± 1.38	5.98 ± 1.94	2.75 ± 1.07	11.9 ± 1.74	-
		Proteobacteria	63.5 ± 27.5	45.7 ± 32.8	80.1 ± 36.9	37.8 ± 24.3	42.1 ± 34.8
		Firmicutes	10.8 ± 9.42	27.1 ± 31.2	17.8 ± 33.2	35.6 ± 23.1	42.2 ± 39.5
Adults	Ephemeroptera	Bacteroidota	22.1 ± 23.3	15.6 ± 20.0	0.28 ± 0.60	12.9 ± 8.70	11.0 ± 14.6
		Other	3.68 ± 1.75	11.6 ± 5.70	1.80 ± 0.97	13.7 ± 3.62	4.59 ± 1.28
		Proteobacteria	58.6 ± 34.4	-	55.5 ± 32.6	-	60.8 ± 30.3
		Firmicutes	29.1 ± 32.7	-	31.5 ± 23.2	-	26.4 ± 25.0
	Diptera	Bacteroidota	5.49 ± 4.53	-	6.48 ± 9.20	-	7.06 ± 13.1
		Other	6.81 ± 4.52	-	6.55 ± 2.41	-	5.79 ± 2.77
		Bacteroidota	61.0 ± 31.7	49.1 ± 20.6	68.2 ± 14.4	70.6 ± 8.28	21.8 ± 10.7
		Proteobacteria	12.1 ± 6.23	27.1 ± 11.2	20.6 ± 6.19	22.5 ± 7.35	37.6 ± 13.6
	Araneidae	Firmicutes	24.3 ± 36.8	15.6 ± 11.6	7.57 ± 16.2	4.41 ± 8.15	17.7 ± 12.9
		Other	2.63 ± 1.98	8.25 ± 2.14	3.60 ± 2.77	2.44 ± 0.63	22.9 ± 6.37
Spiders		Proteobacteria	86.6 ± 28.8	98.0 ± 4.76	79.6 ± 32.7	87.4 ± 29.8	65.1 ± 37.0
		Bacteroidota	8.90 ± 24.8	0.08 ± 0.08	9.12 ± 16.6	9.11 ± 25.6	16.3 ± 24.7
	Tetragnathidae	Firmicutes	3.81 ± 7.54	1.94 ± 4.77	6.98 ± 12.6	3.25 ± 5.38	12.8 ± 10.5
		Other	0.71 ± 0.59	0.00 ± 0.00	4.25 ± 2.91	0.25 ± 0.19	5.75 ± 4.89

Table A.6. Mean relative abundance ± pooled standard deviation (%) of endosymbiont bacteria compared to non-endosymbiont genera across invertebrate taxa and collection sites of the Bow River.

		Site				
	Taxa Type	**Cochrane**	**Sunalta**	**Cushing Bridge**	**Graves Bridge**	**Policeman Flats**
Larvae	Hydropsychidae	*Rickettsia* (14.6 ± 12.7) Non-endosymbionts (85.4 ± 4.77)	*Rickettsia* (8.32 ± 6.59) Non-endosymbionts (91.7 ± 6.60)	*Rickettsia* (6.19 ± 8.53) Non-endosymbionts (93.8 ± 7.42)	*Rickettsia* (1.17 ± 1.69) Non-endosymbionts (98.8 ± 4.48)	*Rickettsia* (0.41 ± 1.13) Non-endosymbionts (99.6 ± 4.01)
	Heptageniidae	*Rickettsia* (0.012 ± 0.025) *Wolbachia* (0.021 ± 0.021) Non-endosymbionts (100 ± 8.04)	*Rickettsia* (0.018 ± 0.035) Non-endosymbionts (100 ± 14.5)	*Rickettsia* (0.082 ± 0.16) *Wolbachia* (0.040 ± 0.058) Non-endosymbionts (99.9 ± 10.3)	*Rickettsia* (0.002 ± 0.006) Non-endosymbionts (100 ± 9.41)	Non-endosymbionts (100 ± 9.48)
	Chironomidae	*Rickettsia* (13.3 ± 26.6) Non-endosymbionts (86.7 ± 7.85)	-	Non-endosymbionts (100 ± 18.2)	-	Non-endosymbionts (100 ± 16.4)
	Perlidae	*Candidatus Megaira* (0.007 ± 0.019) Non-endosymbionts (100 ± 17.1)	-	Non-endosymbionts (100 ± 12.7)	-	Non-endosymbionts (100 ± 12.1)
Adults	Trichoptera	*Rickettsia* (28.5 ± 44.7) Non-endosymbionts (71.5 ± 7.70)	*Rickettsia* (37.5 ± 51.7) Non-endosymbionts (62.5 ± 13.6)	*Buchnera* (0.004 ± 0.01) *Rickettsia* (36.1 ± 49.3) *Spiroplasma* (12.5 ± 35.4) *Wolbachia* (0.0004 ± 0.001) Non-endosymbionts (51.4 ± 8.62)	*Rickettsia* (0.13 ± 0.22) Non-endosymbionts (99.9 ± 14.4)	-
	Ephemeroptera	*Rickettsia* (20.6 ± 37.4) Non-endosymbionts (79.4 ± 13.2)	*Rickettsia* (10.3 ± 28.8) Non-endosymbionts (89.7 ± 15.7)	*Buchnera* (0.19 ± 0.53) *Rickettsia* (18.3 ± 35.0) *Spiroplasma* (12.5 ± 35.4) Non-endosymbionts (69.0 ± 20.0)	*Rickettsia* (0.14 ± 0.16) *Wolbachia* (0.60 ± 1.71) Non-endosymbionts (99.3 ± 13.2)	*Rickettsia* (10.5 ± 28.8) Non-endosymbionts (89.5 ± 18.8)
	Diptera	*Rickettsia* (29.8 ± 43.4) *Spiroplasma* (0.48 ± 1.01) *Wolbachia* (6.48 ± 13.5) Non-endosymbionts (63.2 ± 9.93)	-	*Buchnera* (0.38 ± 1.02) *Rickettsia* (4.23 ± 11.9) Non-endosymbionts (95.4 ± 15.2)	-	*Rickettsia* (0.45 ± 0.91) *Spiroplasma* (0.19 ± 0.46) *Wolbachia* (15.5 ± 37.9) Non-endosymbionts (83.9 ± 18.4)
Spiders	Araneidae	*Rickettsia* (0.20 ± 0.25) *Spiroplasma*	*Rickettsia* (0.71 ± 0.89)	*Rickettsia* (0.16 ± 0.11)	*Candidatus Hamiltonella* (0.007 ± 0.019)	*Candidatus Hamiltonella* (0.098 ± 0.24)

	(20.0 ± 38.1) *Wolbachia* (0.28 ± 0.42) Non-endosymbionts (79.5 ± 12.7)	*Spiroplasma* (0.027 ± 0.06) *Wolbachia* (0.08 ± 0.12) Non-endosymbionts (99.2 ± 12.0)	*Spiroplasma* (0.022 ± 0.043) *Wolbachia* (0.14 ± 0.15) Non-endosymbionts (99.7 ± 8.03)	*Rickettsia* (0.27 ± 0.32) *Spiroplasma* (0.054 ± 0.086) *Wolbachia* (0.27 ± 0.42) Non-endosymbionts (99.4 ± 5.61)	*Rickettsia* (1.54 ± 2.74) *Spiroplasma* (0.88 ± 0.97) *Wolbachia* (0.29 ± 0.26) Non-endosymbionts (97.2 ± 10.8)
Tetragnathidae	*Rickettsia* (74.5 ± 45.9) *Spiroplasma* (2.69 ± 7.62) *Wolbachia* (9.81 ± 27.7) Non-endosymbionts (13.0 ± 8.91)	*Candidatus Cardinium* (0.007 ± 0.01) *Rickettsia* (33.2 ± 42.6) *Spiroplasma* (1.92 ± 4.77) *Wolbachia* (64.7 ± 45.4) Non-endosymbionts (0.12 ± 0.032)	*Candidatus Cardinium* (0.0003 ± 0.001) *Rickettsia* (29.3 ± 45.0) *Wolbachia* (43.1 ± 47.3) Non-endosymbionts (27.6 ± 5.84)	*Rickettsia* (62.9 ± 45.4) *Spiroplasma* (1.84 ± 4.35) *Wolbachia* (22.7 ± 36.5) Non-endosymbionts (12.6 ± 9.09)	*Rickettsia* (50.8 ± 42.7) *Spiroplasma* (7.05 ± 10.6) *Wolbachia* (6.94 ± 12.6) Non-endosymbionts (35.3 ± 9.65)

Table A.7. Prevalence (Prev; %) and mean relative abundance ± standard deviation (RA; %) of common effluent-associated bacteria relative to the total microbiome at each collection site within aquatic insect larvae of the Bow River.

	Cochrane (n = 28)		Sunalta (n = 15)		Cushing Bridge (n = 22)		Graves Bridge (n = 16)		Policeman Flats (n = 29)	
Genus	Prev	RA	Prev	RA	Prev	RA	Prev	RA	Prev	RA
AAP99[12]	75.0	0.34 ± 0.49	80.0	0.55 ± 0.65	63.6	0.44 ± 0.73	93.8	1.36 ± 1.81	82.8	1.56 ± 2.00
Achromobacter[5]	21.4	0.07 ± 0.23	0	-	22.7	0.14 ± 0.36	0	-	10.3	0.02 ± 0.08
Acidovorax[33]	32.1	0.13 ± 0.29	6.67	0.01 ± 0.06	36.4	0.06 ± 0.12	37.5	0.06 ± 0.08	24.1	0.02 ± 0.05
Acinetobacter[16]	14.3	1.32 ± 3.64	0	-	4.55	0 ± 0.01	12.5	0 ± 0.01	27.6	0.95 ± 2.35
Aeromonas[16]	3.57	0 ± 0.01	20.0	0.01 ± 0.03	18.2	2.22 ± 6.34	18.8	0.10 ± 0.32	24.1	2.30 ± 8.22
Alcaligenes[16]	21.4	0.16 ± 0.59	13.3	0.01 ± 0.02	31.8	0.16 ± 0.34	0	-	10.3	0.02 ± 0.07
Amaricoccus[9],[28]	14.3	0.05 ± 0.18	13.3	0.01 ± 0.02	27.3	0.04 ± 0.11	12.5	0.01 ± 0.02	17.2	0.04 ± 0.15
Anaerovorax[22]	0	-	0	-	13.6	0.03 ± 0.11	0	-	17.2	0.01 ± 0.03
Arcticibacter[25]	0	-	0	-	4.55	0 ± 0.02	0	-	0	-
Aridibacter[42]	3.57	0 ± 0.01	0	-	13.6	0.01 ± 0.04	25.0	0.02 ± 0.04	13.8	0.01 ± 0.04
Arthrobacter[16]	32.1	0.18 ± 0.46	40.0	0.98 ± 2.38	68.2	0.38 ± 0.56	50.0	0.09 ± 0.14	20.7	0.03 ± 0.10
Asticcacaulis[6]	7.14	0 ± 0.01	0	-	4.55	0 ± 0.02	0	-	3.45	0.01 ± 0.06
Azoarcus[45]	0	-	0	-	4.55	0.01 ± 0.04	12.5	0 ± 0.01	0	-
Azospira[1]	0	-	6.67	0 ± 0.01	0	-	6.25	0	3.45	0 ± 0.01
Bacillus[16]	25.0	0.09 ± 0.28	33.3	0.05 ± 0.09	45.5	0.18 ± 0.28	37.5	0.03 ± 0.05	27.6	0.12 ± 0.33
Bacteroides[9],[16]	3.57	0 ± 0.02	6.67	0.06 ± 0.25	18.2	3.10 ± 14.4	6.25	0	3.45	0.02 ± 0.11
Bauldia[9]	3.57	0 ± 0.01	0	-	4.55	0 ± 0.02	0	-	3.45	0.01 ± 0.07
Bdellovibrio[15]	0	-	0	-	0	-	0	-	13.8	0.03 ± 0.09
Blastocatella[19]	32.1	0.02 ± 0.03	26.7	0.01 ± 0.02	18.2	0.02 ± 0.05	43.8	0.05 ± 0.11	65.5	0.20 ± 0.36
Blastomonas[31]	3.57	0	0	-	18.2	0.01 ± 0.03	6.25	0 ± 0.01	6.90	0
Blautia[9]	0	-	6.67	0.13 ± 0.49	0	-	6.25	0	0	-
Blvii28 wastewater-sludge group[24]	3.57	0	6.67	0	0	-	6.25	0	0	-
Bryobacter[41]	46.4	0.05 ± 0.14	26.7	0.02 ± 0.04	36.4	0.03 ± 0.05	43.8	0.06 ± 0.10	55.2	0.20 ± 0.32
Candidatus Accumulibacter[44]	3.57	0 ± 0.01	20.0	0.02 ± 0.06	27.3	0.07 ± 0.14	6.25	0 ± 0.01	3.45	0.01 ± 0.06
Candidatus Amoebophilus[1]	21.4	0.01 ± 0.03	20.0	0.02 ± 0.04	36.4	0.07 ± 0.16	75.0	0.03 ± 0.03	41.4	0.03 ± 0.05

Candidatus Anammoximicrobium[14]	3.57	0.01 ± 0.03	0	-	0	-	0	-	0	-
Candidatus Competibacter[40]	0	-	0	-	9.09	0 ± 0.01	0	-	3.45	0 ± 0.01
Candidatus Microthrix[29]	0	-	0	-	0	-	6.25	0	0	-
Candidatus Nitrotoga[26]	3.57	0	0	-	0	-	0	-	41.4	0.12 ± 0.21
Cetobacterium[38]	7.14	0 ± 0.01	20.0	0.01 ± 0.02	13.6	0.01 ± 0.02	6.25	0	6.90	0 ± 0.01
Chryseobacterium[9]	28.6	0.59 ± 1.75	20.0	0.03 ± 0.10	13.6	0.87 ± 3.97	18.8	0.01 ± 0.02	37.9	1.14 ± 4.95
Cloacibacterium[9]	7.14	0.02 ± 0.09	0	-	0	-	0	-	10.3	0.48 ± 2.31
Clostridium SS 1[16]	28.6	0.23 ± 0.84	33.3	0.04 ± 0.07	54.5	0.20 ± 0.33	31.3	0.01 ± 0.02	44.8	0.10 ± 0.21
Clostridium SS 5[16]	0	-	0	-	9.09	0.01 ± 0.05	0	-	24.1	0.02 ± 0.08
Clostridium SS 8[16]	0	-	0	-	0	-	0	-	3.45	0.01 ± 0.03
Clostridium SS 9[16]	0	-	6.67	0 ± 0.01	9.09	0.02 ± 0.07	0	-	0	-
Clostridium SS 10[16]	0	-	0	-	0	-	0	-	3.45	0.02 ± 0.13
Clostridium SS 13[16]	42.9	0.87 ± 2.44	46.7	0.31 ± 0.57	90.9	1.10 ± 1.48	56.3	0.09 ± 0.11	69.0	0.38 ± 0.96
Clostridium SS 14[16]	0	-	0	-	4.55	0 ± 0.01	0	-	3.45	0.02 ± 0.13
Comamonas[9]	0	-	6.67	0.01 ± 0.03	0	-	12.5	0 ± 0.01	3.45	0.02 ± 0.13
Corynebacterium[16]	17.9	0.03 ± 0.10	0	-	13.6	0.04 ± 0.13	12.5	0 ± 0.01	13.8	0.03 ± 0.08
Cytophaga[27]	21.4	0.01 ± 0.04	13.3	0.01 ± 0.02	27.3	0.02 ± 0.06	12.5	0 ± 0.01	17.2	0.01 ± 0.03
Dechloromonas[45]	35.7	0.36 ± 1.08	46.7	0.16 ± 0.25	77.3	1.56 ± 3.15	62.5	0.11 ± 0.12	69.0	0.67 ± 2.27
Defluviicoccus[7]	10.7	0.09 ± 0.42	0	-	36.4	0.11 ± 0.23	0	-	13.8	0.02 ± 0.05
Desulfovibrio[16]	28.6	0.10 ± 0.20	46.7	0.40 ± 0.60	22.7	0.09 ± 0.27	50.0	0.40 ± 0.56	27.6	0.15 ± 0.29
Devosia[9]	21.4	0.02 ± 0.05	26.7	0.06 ± 0.11	40.9	0.06 ± 0.10	43.8	0.03 ± 0.04	6.90	0 ± 0.02
Ensifer[9]	0	-	6.67	0 ± 0.01	22.7	0.02 ± 0.04	0	-	10.3	0.01 ± 0.07
Enterococcus[9],[27]	7.14	0.11 ± 0.56	6.67	0 ± 0.01	4.55	0.01 ± 0.07	6.25	0.02 ± 0.06	10.3	0.64 ± 3.00
Escherichia-Shigella[9],[16]	7.14	0 ± 0.01	6.67	0 ± 0.02	0	-	0	-	0	-
Faecalibacterium[9]	39.3	0.38 ± 1.10	13.3	0.08 ± 0.29	68.2	0.49 ± 1.04	25.0	0.01 ± 0.01	27.6	0.23 ± 0.72
Faecalitalea[34]	0	-	6.67	0.01 ± 0.02	4.55	0	0	-	0	-
Ferruginibacter[9]	78.6	0.62 ± 0.73	86.7	0.98 ± 1.57	86.4	0.61 ± 0.90	87.5	0.40 ± 0.45	86.2	0.90 ± 1.43
Flexibacter[16]	3.57	0	0	-	9.09	0.01 ± 0.02	12.5	0 ± 0.01	13.8	0.01 ± 0.02
Fluviicola[14]	71.4	0.20 ± 0.41	66.7	0.23 ± 0.32	63.6	0.12 ± 0.24	100	0.25 ± 0.32	96.6	0.61 ± 0.84
Gemmata[13]	7.14	0.07 ± 0.27	0	-	18.2	0.14 ± 0.42	0	-	13.8	0 ± 0.01
Gordonia[9]	0	-	0	-	0	-	0	-	6.90	0.03 ± 0.12
Haliscomenobacter[16],[45]	46.4	0.04 ± 0.06	66.7	0.15 ± 0.33	63.6	0.30 ± 0.72	81.3	0.10 ± 0.11	89.7	0.22 ± 0.31
Helicobacter[18]	0	-	0	-	4.55	0 ± 0.02	0	-	3.45	0 ± 0.01
Hydrogenophaga[9]	67.9	0.14 ± 0.18	73.3	0.44 ± 0.83	90.9	0.95 ± 0.75	100	1.66 ± 2.39	86.2	1.04 ± 0.86
Hyphomicrobium[16]	78.6	0.39 ± 0.48	93.3	0.71 ± 0.93	86.4	0.45 ± 0.48	100	0.46 ± 0.44	89.7	1.00 ± 1.48
Hyphomonas[9]	3.57	0 ± 0.01	0	-	0	-	6.25	0	10.3	0.01 ± 0.02
Ignavibacterium[9]	3.57	0 ± 0.01	0	-	22.7	0.03 ± 0.08	6.25	0 ± 0.01	0	-
Lachnospira[39]	0	-	6.67	0 ± 0.02	0	-	0	-	0	-
Legionella[10]	10.7	0.07 ± 0.30	6.67	0.01 ± 0.04	18.2	0.04 ± 0.11	0	-	3.45	0 ± 0.01
Methylotenera[42]	71.4	0.75 ± 1.72	80.0	0.16 ± 0.20	40.9	0.07 ± 0.15	93.8	0.16 ± 0.19	65.5	1.30 ± 2.46
Micrococcus[16]	3.57	0 ± 0.02	0	-	0	-	0	-	0	-
Mycobacterium[16]	10.7	0.02 ± 0.07	0	-	4.55	0 ± 0.01	0	-	31.0	0.17 ± 0.50
Nitrosomonas[16]	17.9	0.02 ± 0.07	33.3	0.03 ± 0.05	27.3	0.02 ± 0.03	31.3	0.06 ± 0.15	51.7	0.36 ± 0.46
Nitrospira[9],[16]	35.7	0.02 ± 0.04	33.3	0.03 ± 0.06	63.6	0.52 ± 1.02	50.0	0.04 ± 0.06	75.9	0.25 ± 0.42
Nocardioides[9]	35.7	0.09 ± 0.19	33.3	0.37 ± 0.73	31.8	0.09 ± 0.20	56.3	0.08 ± 0.14	69.0	0.15 ± 0.26
Novosphingobium[42]	78.6	0.64 ± 0.81	86.7	0.53 ± 0.35	86.4	0.61 ± 0.73	100	0.67 ± 0.38	86.2	0.88 ± 0.67
Paludibacter[38],[45]	21.4	0.02 ± 0.06	33.3	0.04 ± 0.08	50.0	0.17 ± 0.26	43.8	0.03 ± 0.04	10.3	0.01 ± 0.06
Paracoccus[16]	7.14	0 ± 0.02	46.7	0.23 ± 0.36	59.1	0.62 ± 1.04	75.0	0.12 ± 0.14	10.3	0.02 ± 0.07
Pseudomonas[16]	10.7	0.04 ± 0.18	0	-	0	-	6.25	0 ± 0.01	3.45	0.01 ± 0.03
Ralstonia[43]	0	-	6.67	0	4.55	0.01 ± 0.03	6.25	0.01 ± 0.03	0	-
Rhodobacter[9]	46.4	0.39 ± 0.99	73.3	0.51 ± 0.97	95.5	1.03 ± 1.32	100	0.98 ± 0.97	93.1	2.78 ± 2.53
Rhodococcus[9],[27]	0	-	6.67	0.01 ± 0.03	27.3	0.06 ± 0.16	31.3	0.02 ± 0.03	44.8	0.36 ± 1.08
Romboutsia[38]	35.7	0.08 ± 0.17	33.3	0.05 ± 0.10	18.2	0.06 ± 0.21	50.0	0.03 ± 0.04	51.7	0.56 ± 1.80

Runella[32]	53.6	0.21 ± 0.38	80.0	0.21 ± 0.36	59.1	0.85 ± 2.62	56.3	0.18 ± 0.23	72.4	0.48 ± 0.84
Serratia[16]	25.0	7.78 ± 24.0	0	-	13.6	0.23 ± 1.01	0	-	24.1	8.49 ± 19.4
Shinella[9]	3.57	0 ± 0.01	6.67	0	4.55	0.01 ± 0.03	6.25	0 ± 0.02	0	-
Staphylococcus[9],[38]	3.57	0 ± 0.01	13.3	0 ± 0.01	22.7	0.08 ± 0.20	0	-	17.2	0.13 ± 0.45
Stenotrophobacter[38]	7.14	0.01 ± 0.02	6.67	0 ± 0.01	18.2	0.03 ± 0.08	12.5	0 ± 0.01	24.1	0.01 ± 0.03
Stenotrophomonas[9]	3.57	0.04 ± 0.23	0	-	0	-	6.25	0 ± 0.01	0	-
Streptococcus[9],[16]	3.57	0	6.67	0.02 ± 0.07	13.6	0.16 ± 0.68	0	-	10.3	0 ± 0.01
Sulfuritalea[14]	10.7	0 ± 0.01	13.3	0.01 ± 0.02	9.09	0 ± 0.01	6.25	0 ± 0.01	3.45	0 ± 0.01
Syntrophobacter[17]	0	-	0	-	4.55	0.01 ± 0.06	0	-	0	-
Syntrophomonas[16]	0	-	6.67	0 ± 0.01	0	-	0	-	3.45	0
Syntrophus[22]	0	-	0	-	0	-	0	-	3.45	0 ± 0.01
Terrimonas[9],[42]	42.9	0.05 ± 0.10	26.7	0.04 ± 0.07	31.8	0.09 ± 0.26	62.5	0.07 ± 0.08	55.2	0.11 ± 0.16
Thermomonas[42]	3.57	0 ± 0.01	0	-	13.6	0.01 ± 0.03	0	-	13.8	0.02 ± 0.07
Trichococcus[29],[42]	3.57	0 ± 0.01	6.67	0 ± 0.02	22.7	0.08 ± 0.21	18.8	0.01 ± 0.02	3.45	0
Zoogloea[16]	3.57	0 ± 0.01	13.3	0 ± 0.01	4.55	0.04 ± 0.17	25.0	0.01 ± 0.02	6.90	0.01 ± 0.05
Non-effluent associated bacteria	-	83.0 ± 7.30	-	92.2 ± 9.88	-	80.9 ± 7.03	-	92.1 ± 8.01	-	70.4 ± 5.07

Table A.8. Prevalence (Prev; %) and mean relative abundance ± standard deviation (RA; %) of common effluent-associated bacteria relative to the total microbiome at each collection site within aquatic adult insects of the Bow River.

	Cochrane (n = 21)		Sunalta (n = 16)		Cushing Bridge (n = 24)		Graves Bridge (n = 15)		Policeman Flats (n = 14)	
Genus	Prev	RA	Prev	RA	Prev	RA	Prev	RA	Prev	RA
AAP99[12]	4.76	0.01 ± 0.05	6.25	0.05 ± 0.20	8.33	0.02 ± 0.08	0	-	14.3	0.08 ± 0.23
Achromobacter[5]	76.2	1.65 ± 3.06	50.0	0.65 ± 1.74	50.0	1.39 ± 2.60	53.3	0.69 ± 1.08	64.3	1.74 ± 2.52
Acidovorax[33]	28.6	1.53 ± 5.90	12.5	0.02 ± 0.06	12.5	0.02 ± 0.06	13.3	0.03 ± 0.08	14.3	0.07 ± 0.21
Acinetobacter[16]	0	-	6.25	0.01 ± 0.02	4.17	0.03 ± 0.13	6.67	0 ± 0.02	7.14	0.14 ± 0.54
Aeromonas[16]	0	-	0	-	4.17	0 ± 0.01	0	-	7.14	0.24 ± 0.91
Alcaligenes[16]	61.9	4.74 ± 9.97	62.5	3.37 ± 8.06	50.0	2.47 ± 4.81	66.7	2.45 ± 4.21	71.4	2.60 ± 3.75
Amaricoccus[9],[28]	14.3	0.32 ± 1.33	18.8	0.12 ± 0.33	8.33	0.02 ± 0.08	0	-	7.14	0 ± 0.02
Arcticibacter[25]	4.76	0.01 ± 0.03	0	-	0	-	0	-	0	-
Aridibacter[42]	9.52	0.07 ± 0.26	0	-	4.17	0.06 ± 0.30	20.0	0.11 ± 0.23	0	-
Arthrobacter[16]	38.1	0.21 ± 0.40	56.3	1.26 ± 1.95	29.2	0.34 ± 1.05	60.0	0.67 ± 1.01	35.7	0.42 ± 0.78
Asticcacaulis[6]	14.3	0.18 ± 0.65	0	-	0	-	0	-	0	-
Azospira[1]	4.76	0.01 ± 0.02	0	-	0	-	0	-	0	-
Bacillus[16]	47.6	0.71 ± 1.91	37.5	0.36 ± 0.74	16.7	0.06 ± 0.14	73.3	2.73 ± 3.64	50	0.48 ± 1.05
Bacteroides[9],[16]	28.6	0.58 ± 1.69	37.5	0.08 ± 0.19	29.2	0.16 ± 0.38	26.7	0.11 ± 0.24	35.7	0.08 ± 0.18
Blastocatella[19]	9.52	0.03 ± 0.09	18.8	0.17 ± 0.43	4.17	0 ± 0.01	20.0	0.11 ± 0.33	7.14	0.22 ± 0.81
Blastomonas[31]	9.52	0.02 ± 0.09	0	-	4.17	0.02 ± 0.08	6.67	0.09 ± 0.35	0	-
Blautia[9]	28.6	0.65 ± 1.81	25.0	0.07 ± 0.17	50.0	0.74 ± 1.35	40.0	0.54 ± 1.18	42.9	0.15 ± 0.33
Bordetella[9]	4.76	0 ± 0.01	0	-	0	-	0	-	21.4	0.01 ± 0.04
Brachybacterium[4]	0	-	0	-	0	-	6.67	0.01 ± 0.03	0	-
Bryobacter[41]	9.52	0.02 ± 0.06	6.25	0.02 ± 0.10	0	-	0	-	0	-
Candidatus Accumulibacter[44]	4.76	0.01 ± 0.05	0	-	0	-	0	-	0	-
Candidatus Anammoximicrobium[14]	4.76	0.01 ± 0.04	0	-	0	-	0	-	0	-
Candidatus Nitrotoga[26]	0	-	0	-	4.17	0.01 ± 0.07	0	-	0	-
Cetobacterium[38]	0	-	6.25	0 ± 0.01	4.17	0.03 ± 0.16	0	-	0	-
Chryseobacterium[9]	14.3	0.07 ± 0.28	6.25	0.62 ± 1.51	16.7	0.08 ± 0.25	46.7	0.39 ± 0.63	0	-
Citrobacter[16]	0	-	6.25	0.01 ± 0.02	8.33	0.01 ± 0.04	0	-	21.4	0.18 ± 0.60

Cloacibacterium[9]	4.76	0.01 ± 0.05	6.25	0.14 ± 0.56	4.17	0	0	-	7.14	0 ± 0.02
Clostridium SS 1[16]	14.3	0.19 ± 0.62	6.25	0.09 ± 0.35	16.7	0.29 ± 1.26	40.0	2.05 ± 4.98	35.7	0.45 ± 0.87
Clostridium SS 2[16]	0	-	0	-	4.17	0.01 ± 0.06	0	-	0	-
Clostridium SS 9[16]	0	-	6.25	0.02 ± 0.07	0	-	0	-	0	-
Clostridium SS 13[16]	42.9	0.63 ± 1.31	31.3	0.35 ± 0.85	20.8	0.17 ± 0.49	13.3	0.14 ± 0.42	21.4	0.16 ± 0.48
Comamonas[9]	4.76	0 ± 0.02	0	-	0	-	0	-	14.3	0.38 ± 1.21
Corynebacterium[16]	61.9	0.51 ± 0.89	75.0	2.45 ± 5.86	50.0	1.65 ± 3.26	80.0	2.00 ± 3.85	57.1	0.50 ± 0.83
Cytophaga[27]	14.3	0.11 ± 0.39	6.25	0.13 ± 0.52	4.17	0 ± 0.01	6.67	0.07 ± 0.25	7.14	0.10 ± 0.38
Dechloromonas[45]	4.76	0 ± 0.01	6.25	0.02 ± 0.08	12.5	0.04 ± 0.13	6.67	0.03 ± 0.11	0	-
Defluviicoccus[7]	0	-	6.25	0.03 ± 0.13	0	-	6.67	0.01 ± 0.03	0	-
Desulfovibrio[16]	0	-	0	-	4.17	0 ± 0.01	0	-	0	-
Devosia[9]	33.3	0.41 ± 0.83	25.0	0.48 ± 1.03	12.5	0.05 ± 0.22	46.7	1.12 ± 1.69	21.4	0.25 ± 0.67
Ensifer[9]	4.76	0.01 ± 0.03	0	-	0	-	0	-	0	-
Enterococcus[9],[27]	4.76	0.14 ± 0.64	6.25	0.02 ± 0.06	0	-	6.67	0.15 ± 0.57	0	-
Escherichia-Shigella[9],[16]	0	-	6.25	0	4.17	0 ± 0.02	0	-	0	-
Faecalibacterium[9]	19.0	0.27 ± 0.79	25.0	0.29 ± 0.68	29.2	0.61 ± 1.68	40.0	0.48 ± 1.15	50	0.88 ± 2.13
Ferruginibacter[9]	33.3	0.25 ± 0.57	31.3	0.40 ± 0.93	16.7	0.09 ± 0.23	40.0	0.20 ± 0.35	42.9	0.41 ± 0.80
Fluviicola[14]	33.3	0.86 ± 2.13	43.8	0.89 ± 1.43	25.0	0.45 ± 1.33	46.7	0.35 ± 0.56	35.7	0.32 ± 0.63
Gemmata[13]	0	-	12.5	0.04 ± 0.11	4.17	0.02 ± 0.09	0	-	0	-
Gordonia[9]	4.76	0.01 ± 0.04	0	-	0	-	0	-	0	-
Haliscomenobacter[16],[45]	9.52	0.07 ± 0.23	0	-	4.17	0.02 ± 0.08	0	-	0	-
Hydrogenophaga[9]	28.6	0.31 ± 0.70	25.0	0.12 ± 0.25	16.7	0.14 ± 0.39	33.3	1.42 ± 4.83	21.4	0.05 ± 0.13
Hyphomicrobium[16]	9.52	0.05 ± 0.18	12.5	0.04 ± 0.12	8.33	0.01 ± 0.04	6.67	0.01 ± 0.03	14.3	0.09 ± 0.25
Ignavibacterium[9]	4.76	0	0	-	0	-	0	-	0	-
Klebsiella[9],[16]	4.76	0.09 ± 0.41	0	-	0	-	0	-	7.14	0.29 ± 1.10
Lachnospira[39]	4.76	0.02 ± 0.07	6.25	0.07 ± 0.26	4.17	0.05 ± 0.25	0	-	7.14	0.02 ± 0.06
Legionella[10]	0	-	6.25	0 ± 0.02	0	-	0	-	0	-
Methylotenera[42]	4.76	0.01 ± 0.03	0	-	20.8	0.55 ± 2.08	13.3	0.07 ± 0.18	35.7	0.33 ± 0.59
Micrococcus[16]	28.6	0.32 ± 0.73	25.0	0.15 ± 0.33	37.5	0.21 ± 0.38	33.3	0.38 ± 1.14	28.6	0.25 ± 0.71
Moraxella[16],[45]	0	-	12.5	0.02 ± 0.09	33.3	0.37 ± 0.97	0	-	7.14	0.02 ± 0.07
Mycobacterium[16]	28.6	0.20 ± 0.56	43.8	0.11 ± 0.21	16.7	0.03 ± 0.09	53.3	0.33 ± 0.64	35.7	0.22 ± 0.53
Neisseria[16]	9.52	0.01 ± 0.02	25.0	0.21 ± 0.46	8.33	0.14 ± 0.65	20.0	0.33 ± 0.88	42.9	0.21 ± 0.50
Nitrobacter[16],[45]	0	-	6.25	0.10 ± 0.41	0	-	0	-	0	-
Nitrospira[9],[16]	4.76	0.04 ± 0.16	0	-	4.17	0	6.67	0.03 ± 0.12	0	-
Nocardioides[9]	33.3	0.18 ± 0.35	31.3	0.55 ± 1.44	20.8	0.18 ± 0.55	53.3	0.56 ± 0.89	57.1	0.44 ± 1.18
Novosphingobium[42]	19.0	0.35 ± 1.05	43.8	1.18 ± 2.24	12.5	0.06 ± 0.20	20.0	0.37 ± 1.25	14.3	0.07 ± 0.19
Paludibacter[38],[45]	14.3	0.28 ± 1.09	0	-	0	-	0	-	0	-
Pantoea[9],[34]	0	-	31.3	0.13 ± 0.27	8.33	0.08 ± 0.30	13.3	0.80 ± 2.54	7.14	0.01 ± 0.03
Parabacteroides[39]	14.3	0.11 ± 0.28	6.25	0.07 ± 0.26	4.17	0.01 ± 0.03	13.3	0.55 ± 2.00	7.14	0.01 ± 0.05
Paracoccus[16]	28.6	0.68 ± 2.82	31.3	0.56 ± 1.30	25.0	0.26 ± 0.58	60.0	1.49 ± 1.98	14.3	0.07 ± 0.22
Pseudomonas[16]	42.9	0.07 ± 0.15	37.5	0.13 ± 0.36	66.7	2.54 ± 5.69	13.3	0.06 ± 0.24	71.4	1.97 ± 3.00
Rhodobacter[9]	14.3	0.09 ± 0.28	6.25	0 ± 0.01	12.5	0.07 ± 0.21	33.3	1.28 ± 2.96	35.7	2.48 ± 7.31
Rhodococcus[9],[27]	33.3	0.21 ± 0.42	56.3	0.27 ± 0.46	20.8	0.19 ± 0.57	66.7	1.35 ± 1.94	28.6	0.11 ± 0.26
Romboutsia[38]	4.76	0.08 ± 0.38	31.3	0.62 ± 1.95	25.0	0.35 ± 0.97	33.3	0.67 ± 1.42	35.7	0.30 ± 0.80
Roseburia[9]	19.0	0.65 ± 1.42	6.25	0.05 ± 0.22	8.33	0.27 ± 0.91	20.0	0.20 ± 0.50	14.3	0.03 ± 0.09
Ruminococcus[9]	42.9	1.07 ± 2.70	31.3	0.44 ± 1.22	16.7	1.03 ± 4.20	20.0	0.08 ± 0.21	35.7	0.93 ± 2.96
Runella[32]	4.76	0.02 ± 0.09	0	-	0	-	0	-	0	-
Serratia[16]	9.52	0 ± 0.02	6.25	0 ± 0.02	29.2	6.51 ± 22.2	26.7	5.51 ± 19.94	42.9	8.59 ± 25.9
Shinella[9]	0	-	0	-	4.17	0.08 ± 0.40	0	-	0	-
Staphylococcus[9],[38]	71.4	2.94 ± 5.76	87.5	7.08 ± 8.44	66.7	19.82 ± 26.96	73.3	12.92 ± 21.80	85.7	14.6 ± 19.8
Stenotrophobacter[38]	0	-	6.25	0.01 ± 0.05	0	-	0	-	0	-
Stenotrophomonas[9]	14.3	0.07 ± 0.30	18.8	0.05 ± 0.17	16.7	0.05 ± 0.21	26.7	0.51 ± 1.23	14.3	0.05 ± 0.16

Streptococcus[9],[16]	47.6	1.03 ± 2.15	56.3	0.91 ± 1.80	37.5	1.07 ± 2.34	46.7	0.32 ± 0.64	50	0.90 ± 2.02
Sulfuritalea[14]	4.76	0.01 ± 0.03	0	-	0	-	0	-	0	-
Syntrophus[22]	0	-	6.25	0.01 ± 0.04	0	-	0	-	0	-
Terrimonas[9],[42]	14.3	0.02 ± 0.07	18.8	0.09 ± 0.21	4.17	0 ± 0.02	0	-	0	-
Thermomonas[42]	0	-	6.25	0.10 ± 0.42	4.17	0.04 ± 0.21	13.3	0.04 ± 0.13	7.14	0.01 ± 0.03
Trichococcus[29],[42]	4.76	0	0	-	0	-	0	-	7.14	0.03 ± 0.10
Xanthomonas[16]	0	-	0	-	0	-	20.0	0.09 ± 0.30	0	-
Zoogloea[16]	4.76	0.18 ± 0.84	0	-	4.17	0.01 ± 0.03	0	-	0	-
Non-effluent associated bacteria	-	76.6 ± 11.0	-	74.5 ± 14.4	-	57.0 ± 10.8	-	56.1 ± 5.72	-	58.0 ± 13.4

Table A.9. Prevalence (Prev; %) and mean relative abundance ± standard deviation (RA; %) of common effluent-associated bacteria relative to the total microbiome at each collection site within riparian spiders of the Bow River.

	Cochrane (n = 16)		Sunalta (n = 15)		Cushing Bridge (n = 15)		Graves Bridge (n = 16)		Policeman Flats (n = 14)	
Genus	Prev	RA	Prev	RA	Prev	RA	Prev	RA	Prev	RA
AAP99[12]	0	-	0	-	6.67	0.01 ± 0.03	0	-	0	-
Achromobacter[5]	37.5	0.08 ± 0.15	26.7	0.15 ± 0.28	20	0.04 ± 0.11	31.3	0.07 ± 0.12	28.6	0.12 ± 0.30
Acidovorax[33]	0	-	6.67	0.04 ± 0.14	0	-	6.25	0.11 ± 0.45	21.4	0.05 ± 0.12
Acinetobacter[16]	0	-	0	-	6.67	0	0	-	0	-
Alcaligenes[16]	56.3	0.08 ± 0.11	46.7	0.64 ± 1.13	46.7	0.16 ± 0.41	56.3	0.17 ± 0.27	50	0.21 ± 0.38
Amaricoccus[9],[28]	6.25	0 ± 0.01	6.67	0.04 ± 0.17	13.3	0.04 ± 0.11	0	-	28.6	0.09 ± 0.21
Arcticibacter[25]	0	-	0	-	0	-	6.25	0 ± 0.02	7.14	0.01 ± 0.03
Aridibacter[42]	0	-	0	-	6.67	0.01 ± 0.05	0	-	0	-
Arthrobacter[16]	31.3	0.02 ± 0.04	33.3	0.75 ± 1.51	26.7	0.18 ± 0.50	37.5	0.11 ± 0.24	57.1	0.28 ± 0.32
Bacillus[16]	50	0.06 ± 0.11	46.7	1.04 ± 2.40	33.3	0.11 ± 0.21	37.5	0.11 ± 0.19	50	0.38 ± 0.62
Bacteroides[9],[16]	25	0.17 ± 0.53	13.3	0.13 ± 0.36	6.67	0.35 ± 0.83	12.5	0.12 ± 0.35	35.7	0.34 ± 0.67
Bauldia[9]	0	-	0	-	6.67	0 ± 0.02	0	-	14.3	0.01 ± 0.04
Blastocatella[19]	12.5	0 ± 0.01	13.3	0.21 ± 0.70	0	-	12.5	0.02 ± 0.09	21.4	0.03 ± 0.10
Blautia[9]	25	0.23 ± 0.82	33.3	0.21 ± 0.58	33.3	0.51 ± 1.31	12.5	0.16 ± 0.49	42.9	0.56 ± 0.98
Brachybacterium[4]	0	-	0	-	6.67	0 ± 0.01	0	-	14.3	0.01 ± 0.03
Bryobacter[41]	37.5	0.09 ± 0.15	33.3	0.15 ± 0.32	66.7	0.15 ± 0.18	56.3	0.09 ± 0.12	28.6	0.06 ± 0.13
Chryseobacterium[9]	6.25	0.01 ± 0.02	20	0.22 ± 0.66	13.3	0.02 ± 0.05	6.25	0	57.1	0.49 ± 0.94
Clostridium SS 1[16]	18.8	0.07 ± 0.28	33.3	0.16 ± 0.38	33.3	0.25 ± 0.54	12.5	0.08 ± 0.21	71.4	0.45 ± 1.05
Clostridium SS 10[16]	0	-	0	-	6.67	0.01 ± 0.03	0	-	0	-
Clostridium SS 13[16]	6.25	0.01 ± 0.06	13.3	0.51 ± 1.43	40	0.67 ± 2.50	12.5	0.06 ± 0.18	42.9	0.17 ± 0.29
Comamonas[9]	0	-	6.67	0.02 ± 0.07	0	-	0	-	0	-
Corynebacterium[16]	31.3	0.04 ± 0.12	20	0.20 ± 0.46	33.3	0.03 ± 0.05	31.3	0.07 ± 0.13	28.6	0.08 ± 0.20
Cytophaga[27]	0	-	0	-	0	-	6.25	0 ± 0.01	7.14	0.01 ± 0.05
Defluviicoccus[7]	0	-	0	-	13.3	0.05 ± 0.17	0	-	7.14	0.01 ± 0.02
Devosia[9]	12.5	0.01 ± 0.03	20	0.27 ± 0.57	26.7	0.11 ± 0.28	25	0.20 ± 0.55	57.1	0.52 ± 0.78
Enterococcus[9],[27]	6.03	0 ± 0.02	0	-	6.67	0	0	-	7.14	0
Escherichia-Shigella[9],[16]	0	-	0	-	6.67	0	12.5	0	0	-
Faecalibacterium[9]	81.3	0.38 ± 0.68	53.3	1.95 ± 4.86	86.7	0.77 ± 1.22	56.3	0.59 ± 0.76	64.3	0.89 ± 1.28
Ferruginibacter[9]	6.25	0 ± 0.01	13.3	0.04 ± 0.12	13.3	0.11 ± 0.30	12.5	0.01 ± 0.04	35.7	0.14 ± 0.28
Fluviicola[14]	0	-	0	-	0	-	6.25	0.02 ± 0.07	0	-
Haliscomenobacter[16],[45]	0	-	0	-	0	-	0	-	7.14	0.02 ± 0.08
Hydrogenophaga[9]	6.25	0 ± 0.01	40	0.33 ± 0.68	13.3	0.05 ± 0.13	6.25	0.01 ± 0.04	14.3	0.04 ± 0.13
Hyphomicrobium[16]	0	-	0	-	6.67	0.02 ± 0.10	6.25	0.02 ± 0.08	14.3	0.03 ± 0.08
Lachnospira[39]	6.25	0.03 ± 0.11	13.3	0.09 ± 0.33	20	0.08 ± 0.20	18.8	0.01 ± 0.03	14.3	0.03 ± 0.07
Legionella[10]	6.25	0.03 ± 0.13	0	-	6.67	0.01 ± 0.05	0	-	0	-
Micrococcus[16]	18.8	0.05 ± 0.17	26.7	0.07 ± 0.14	20	0.03 ± 0.07	0	-	0	-
Moraxella[16],[45]	6.25	0 ± 0.01	0	-	0	-	6.25	0	0	-
Mycobacterium[16]	56.3	0.03 ± 0.04	33.3	0.09 ± 0.15	0	-	31.3	0.02 ± 0.04	28.6	0.06 ± 0.12
Nitrobacter[16],[45]	0	-	0	-	0	-	6.25	0 ± 0.02	0	-
Nocardioides[9]	6.25	0	6.67	0.04 ± 0.16	26.7	0.18 ± 0.57	12.5	0.01 ± 0.02	35.7	0.29 ± 0.72
Novosphingobium[42]	6.25	0 ± 0.02	20	0.03 ± 0.08	13.3	0.13 ± 0.44	6.25	0.04 ± 0.18	21.4	0.35 ± 1.06
Pantoea[9],[34]	0	-	0	-	0	-	0	-	7.14	0.04 ± 0.14
Parabacteroides[39]	12.5	0.02 ± 0.08	13.3	0.02 ± 0.10	20	0.04 ± 0.11	12.5	0.03 ± 0.07	21.4	0.06 ± 0.14
Paracoccus[16]	6.25	0.01 ± 0.02	40	0.57 ± 1.15	26.7	0.16 ± 0.59	31.3	0.12 ± 0.24	35.7	0.28 ± 0.57
Pseudomonas[16]	6.25	0.01 ± 0.02	6.67	0.01 ± 0.03	26.7	0.02 ± 0.04	6.25	0.01 ± 0.02	21.4	0.04 ± 0.10
Rhodobacter[9]	18.8	0.01 ± 0.03	0	-	0	-	0	-	14.3	0.02 ± 0.06
Rhodococcus[9],[27]	12.5	0.21 ± 0.84	20	0.49 ± 1.58	33.3	2.16 ± 5.78	25	0.08 ± 0.26	57.1	7.14 ± 11.68

Romboutsia[38]	12.5	0.08 ± 0.32	40	0.53 ± 1.66	46.7	0.20 ± 0.42	18.8	0.11 ± 0.27	57.1	0.36 ± 0.71
Roseburia[9]	12.5	0.10 ± 0.36	13.3	0.09 ± 0.34	33.3	0.24 ± 0.66	12.5	0.09 ± 0.25	50	0.37 ± 0.74
Ruminococcus[9]	25	0.21 ± 0.77	26.7	0.11 ± 0.30	53.3	0.43 ± 1.08	12.5	0.18 ± 0.49	50	0.39 ± 0.73
Serratia[16]	0	-	0	-	6.67	0 ± 0.01	0	-	0	-
Shinella[9]	0	-	0	-	6.67	0.04 ± 0.17	6.25	0 ± 0.01	7.14	0.03 ± 0.13
Staphylococcus[9],[38]	43.8	0.07 ± 0.12	20	0.19 ± 0.44	33.3	0.05 ± 0.12	18.8	0.04 ± 0.09	35.7	0.22 ± 0.63
Stenotrophobacter[38]	6.25	0.01 ± 0.03	6.67	0.05 ± 0.19	0	-	0	-	7.14	0 ± 0.02
Stenotrophomonas[9]	0	-	0	-	6.67	0 ± 0.02	12.5	0.01 ± 0.04	14.3	0.08 ± 0.21
Streptococcus[9],[16]	31.3	0.05 ± 0.14	33.3	0.16 ± 0.32	66.7	0.03 ± 0.09	25	0.02 ± 0.04	21.4	0.03 ± 0.06
Terrimonas[9],[42]	0	-	0	-	0	-	6.25	0.01 ± 0.05	7.14	0.02 ± 0.08
Thermomonas[42]	0	-	6.67	0.09 ± 0.34	0	-	6.25	0 ± 0.01	7.14	0.01 ± 0.03
Trichococcus[29],[42]	0	-	6.67	0.03 ± 0.12	0	-	0	-	0	-
Non-effluent associated bacteria	-	97.8 ± 18.3	-	90.3 ± 17.0	-	92.5 ± 17.0	-	97.2 ± 16.5	-	85.2 ± 13.0

Table A.10. Mean ± standard deviation of Shannon diversity within invertebrates collected across sites in the Bow River in July 2022. Bolded site names are located downstream of municipal effluent outfalls.

Life stage	Invertebrate taxon	Site	Shannon index
Larvae	Hydropsychidae	Cochrane	4.19 ± 0.475
		Sunalta	4.14 ± 0.319
		Cushing Bridge	4.19 ± 0.310
		Graves Bridge	4.87 ± 0.227
		Policeman Flats	5.29 ± 0.0654
	Heptageniidae	Cochrane	3.11 ± 0.828
		Sunalta	3.69 ± 1.20
		Cushing Bridge	3.98 ± 0.693
		Graves Bridge	3.89 ± 0.899
		Policeman Flats	4.41 ± 1.15
	Chironomidae	Cochrane	4.13 ± 0.826
		Cushing Bridge	4.08 ± 1.73
		Policeman Flats	3.86 ± 1.05
	Perlidae	Cochrane	2.55 ± 1.57
		Cushing Bridge	3.94 ± 0.721
		Policeman Flats	3.83 ± 1.03
Adults	Trichoptera	Cochrane	3.37 ± 2.12
		Sunalta	2.39 ± 2.30
		Cushing Bridge	2.33 ± 2.32
		Graves Bridge	3.81 ± 1.15
	Ephemeroptera	Cochrane	1.85 ± 0.905
		Sunalta	2.56 ± 1.56
		Cushing Bridge	0.944 ± 1.29

		Graves Bridge	2.82 ± 0.857
		Policeman Flats	2.57 ± 1.72
	Diptera	Cochrane	2.50 ± 1.53
		Cushing Bridge	2.46 ± 1.13
		Policeman Flats	2.49 ± 1.46
Spiders	Araneidae	Cochrane	1.26 ± 0.925
		Sunalta	2.79 ± 0.971
		Cushing Bridge	1.79 ± 0.857
		Graves Bridge	1.82 ± 0.674
		Policeman Flats	3.63 ± 0.544
	Tetragnathidae	Cochrane	0.621 ± 0.625
		Sunalta	0.296 ± 0.308
		Cushing Bridge	1.27 ± 1.79
		Graves Bridge	0.597 ± 0.687
		Policeman Flats	1.52 ± 1.40

Table A.11. Significant pairwise PERMANOVA differences between microbiome beta diversities based on a compositionally normalized Bray Curtis dissimilarity matrix across collection sites and invertebrate taxa of the Bow River ($\alpha = 0.05$).

Stage	**Invertebrate taxon**	**Site comparison**	**Pseudo-F**	**R^2**	**p-adj**
Larvae	Hydropsychidae	Cochrane – Cushing Bridge	2.12	0.161	0.003
		Cochrane - Sunalta	1.85	0.124	0.009
		Cochrane – Graves Bridge	4.16	0.229	<0.001
		Cochrane – Policeman Flats	9.72	0.410	<0.001
		Cushing Bridge – Graves Bridge	2.12	0.161	0.014
		Cushing Bridge – Policeman Flats	6.65	0.377	<0.001
		Sunalta – Graves Bridge	2.88	0.181	<0.001
		Sunalta – Policeman Flats	8.21	0.387	<0.001
		Graves Bridge – Policeman Flats	3.81	0.214	<0.001
	Heptageniidae	Cochrane – Cushing Bridge	2.56	0.154	0.016
		Cochrane - Sunalta	2.41	0.147	0.026
		Cochrane – Graves Bridge	5.84	0.294	0.004
		Cochrane – Policeman Flats	7.55	0.350	0.002
		Cushing Bridge – Graves Bridge	2.92	0.172	0.02
		Cushing Bridge – Policeman Flats	3.25	0.188	0.004

		Sunalta – Graves Bridge	2.64	0.159	0.021
		Sunalta – Policeman Flats	3.11	0.182	0.002
		Graves Bridge – Policeman Flats	3.25	0.188	0.019
	Perlidae	Cochrane – Cushing Bridge	2.68	0.196	<0.001
		Cochrane – Policeman Flats	2.80	0.167	<0.001
		Cushing Bridge – Policeman Flats	4.24	0.278	0.003
Adults	Trichoptera	Cochrane – Graves Bridge	1.69	0.115	0.003
		Graves Bridge - Sunalta	1.77	0.120	0.047
		Graves Bridge – Cushing Bridge	1.56	0.107	0.007
	Ephemeroptera	Cochrane – Graves Bridge	1.73	0.120	<0.001
		Cochrane – Cushing Bridge	5.01	0.263	<0.001
		Cochrane – Policeman Flats	1.66	0.106	0.01
		Graves Bridge - Sunalta	1.39	0.090	0.016
		Graves Bridge – Cushing Bridge	4.73	0.252	0.005
		Sunalta – Cushing Bridge	3.27	0.190	0.024
		Cushing Bridge – Policeman Flats	3.26	0.189	0.025
	Diptera	Cochrane – Cushing Bridge	1.48	0.118	0.022
		Cochrane – Policeman Flats	1.28	0.125	0.034
Spiders	Araneidae	Cochrane – Policeman Flats	3.64	0.233	0.004
		Graves Bridge - Sunalta	2.15	0.142	0.01
		Graves Bridge – Policeman Flats	7.31	0.378	<0.001
		Cushing Bridge – Policeman Flats	5.63	0.338	<0.001
		Sunalta – Policeman Flats	2.23	0.168	0.018

Table A.12. Number of differentially expressed MetaCyc pathways at each collection site compared to the reference, Cochrane, across invertebrate taxa in the Bow River, based on DESeq2 ($\alpha = 0.001$). Bolded site names are located downstream of municipal effluent outfalls.

Stage	Invertebrate taxon	Pairwise site comparison	Number of differentially expressed MetaCyc pathways
Larvae	Hydropsychidae	Sunalta	0
		Cushing Bridge	10
		Graves Bridge	79
		Policeman Flats	170
	Heptageniidae	Sunalta	0
		Cushing Bridge	15
		Graves Bridge	31
		Policeman Flats	62
	Chironomidae	Cushing Bridge	3
		Policeman Flats	10
	Perlidae	Cushing Bridge	4
		Policeman Flats	5
Adults	Trichoptera	Sunalta	1

		Cushing Bridge	2
		Graves Bridge	1
	Ephemeroptera	Sunalta	0
		Cushing Bridge	34
		Graves Bridge	4
		Policeman Flats	3
	Diptera	Cushing Bridge	50
		Policeman Flats	32
Spiders	Araneidae	Sunalta	27
		Cushing Bridge	0
		Graves Bridge	0
		Policeman Flats	176
	Tetragnathidae	Sunalta	2
		Cushing Bridge	19
		Graves Bridge	2
		Policeman Flats	0

Supplementary Figures

Figure A.1. Moth sheet trap used to collect emerging adult insects made with an autoclaved bedsheet and an ultraviolet light set up on the shoreline after dark.

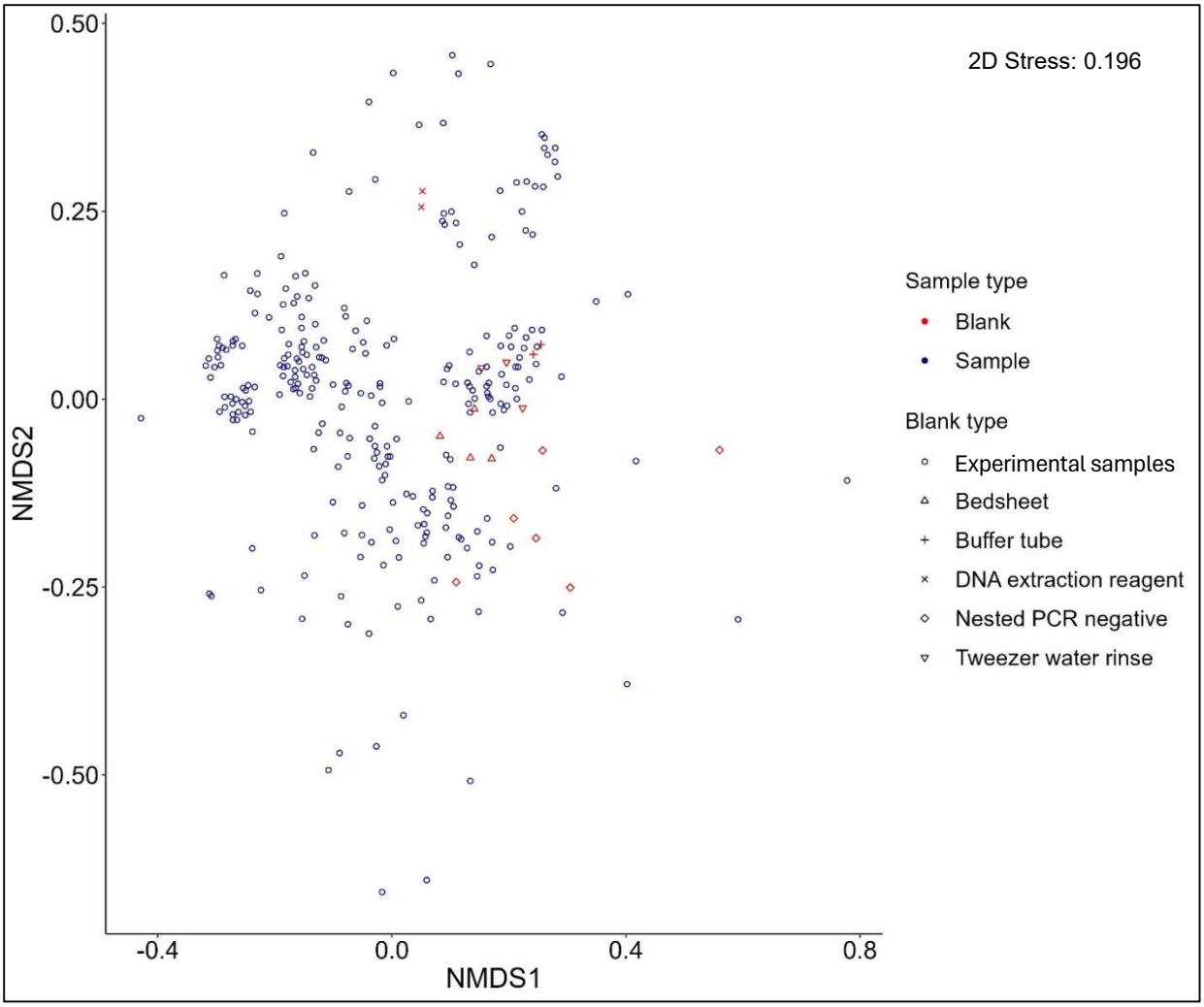

Figure A.2. Beta diversity composition of blank and experimental samples from the Bow River using Bray Curtis distance matrix and NDMS ordination. Sample type is denoted by the colours and the blank type is denoted by the shape of symbols.

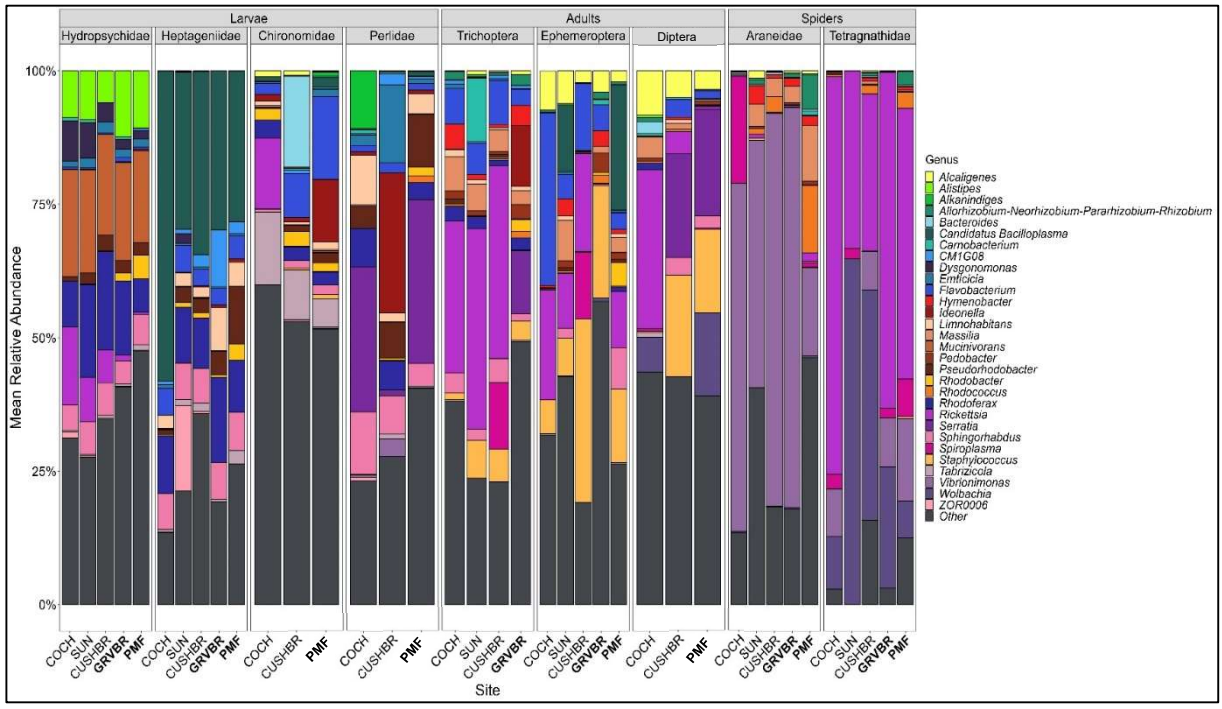

Figure A.3. Mean relative abundance (%) of the top 30 bacterial genera across all macroinvertebrate larvae families, adult orders, and spider families of the Bow River. Sites are arranged from upstream to downstream where bolded sites are located downstream of wastewater outfalls.

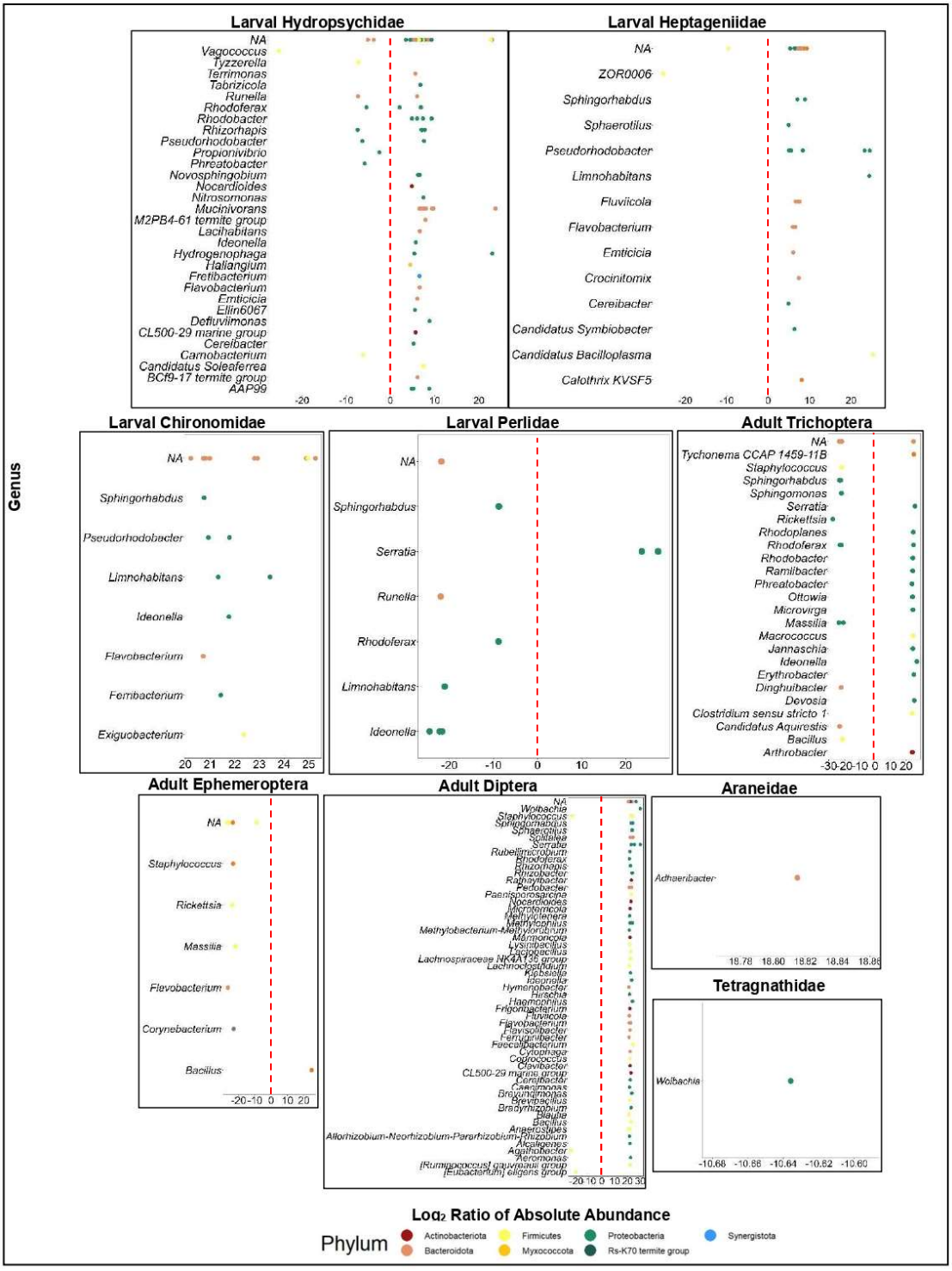

Figure A.4. Differentially abundant bacteria (log_2 ratio) from wastewater effluent-exposed sites (Graves Bridge/Policeman Flats) compared to non-exposed sites (Cochrane, Sunalta, Cushing Bridge) across invertebrate taxa, based on DESeq2 ($\alpha = 0.001$). The dashed red line indicates the zero-line, and the colour of the points represents the phylum of bacteria.

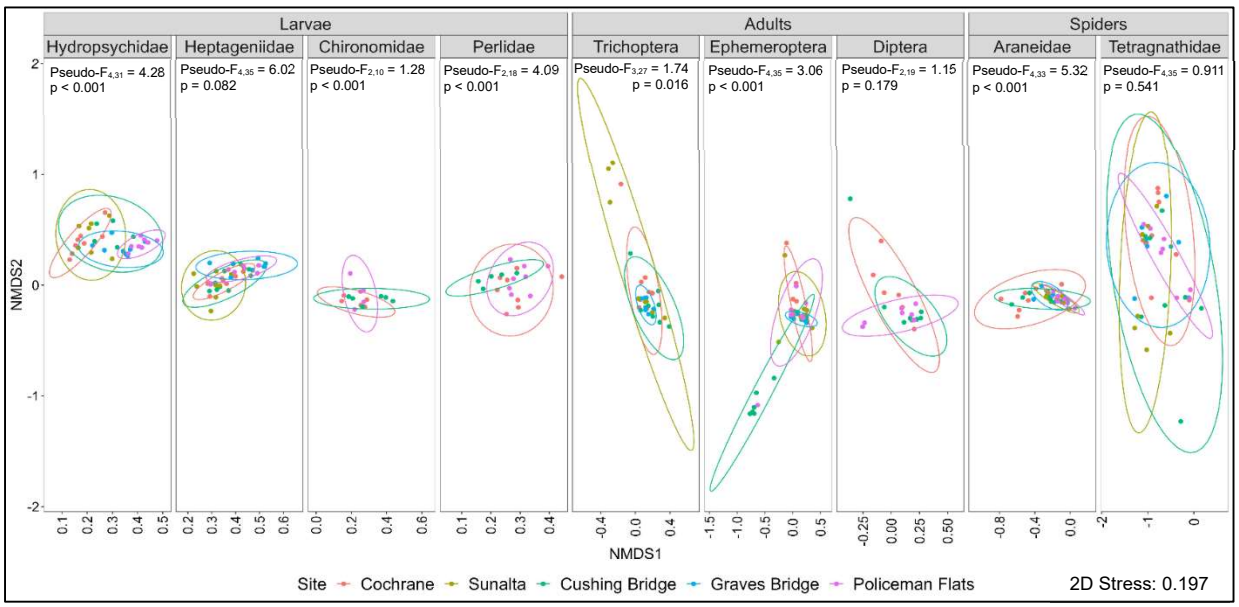

Figure A.5. NMDS of bacterial beta diversity based on a Bray Curtis distance matrix across all Bow River invertebrate samples collected in July 2022, faceted by invertebrate taxa type. The colour of the symbol represents the collection site, surrounded by 95% confidence ellipses.

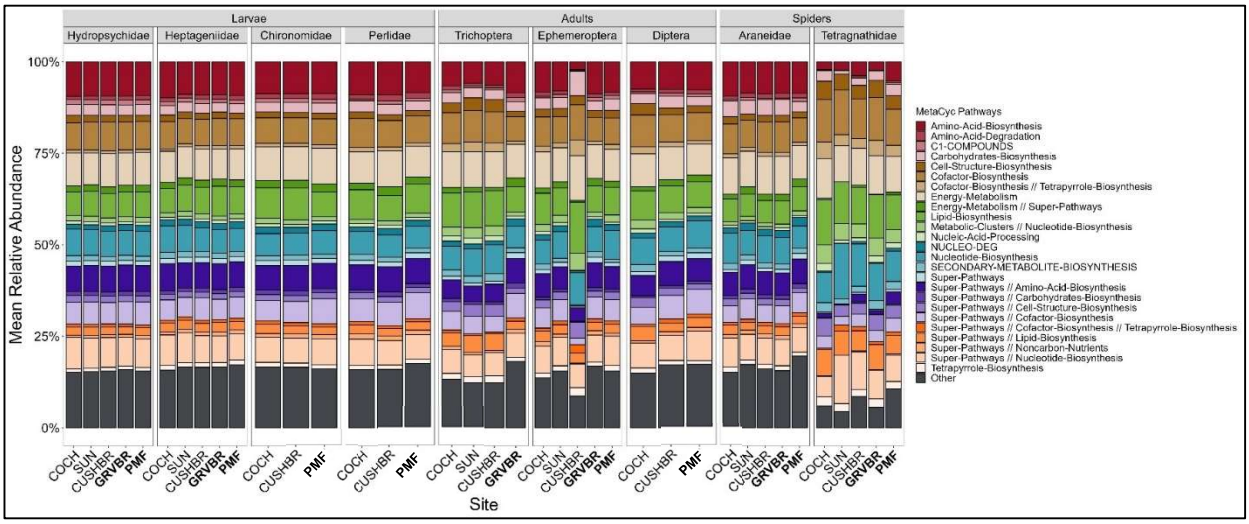

Figure A.6. Mean relative abundance of the top 25 most abundant MetaCyc functional pathway classes across invertebrate taxa and sites in the Bow River estimated using PICRUSt2.

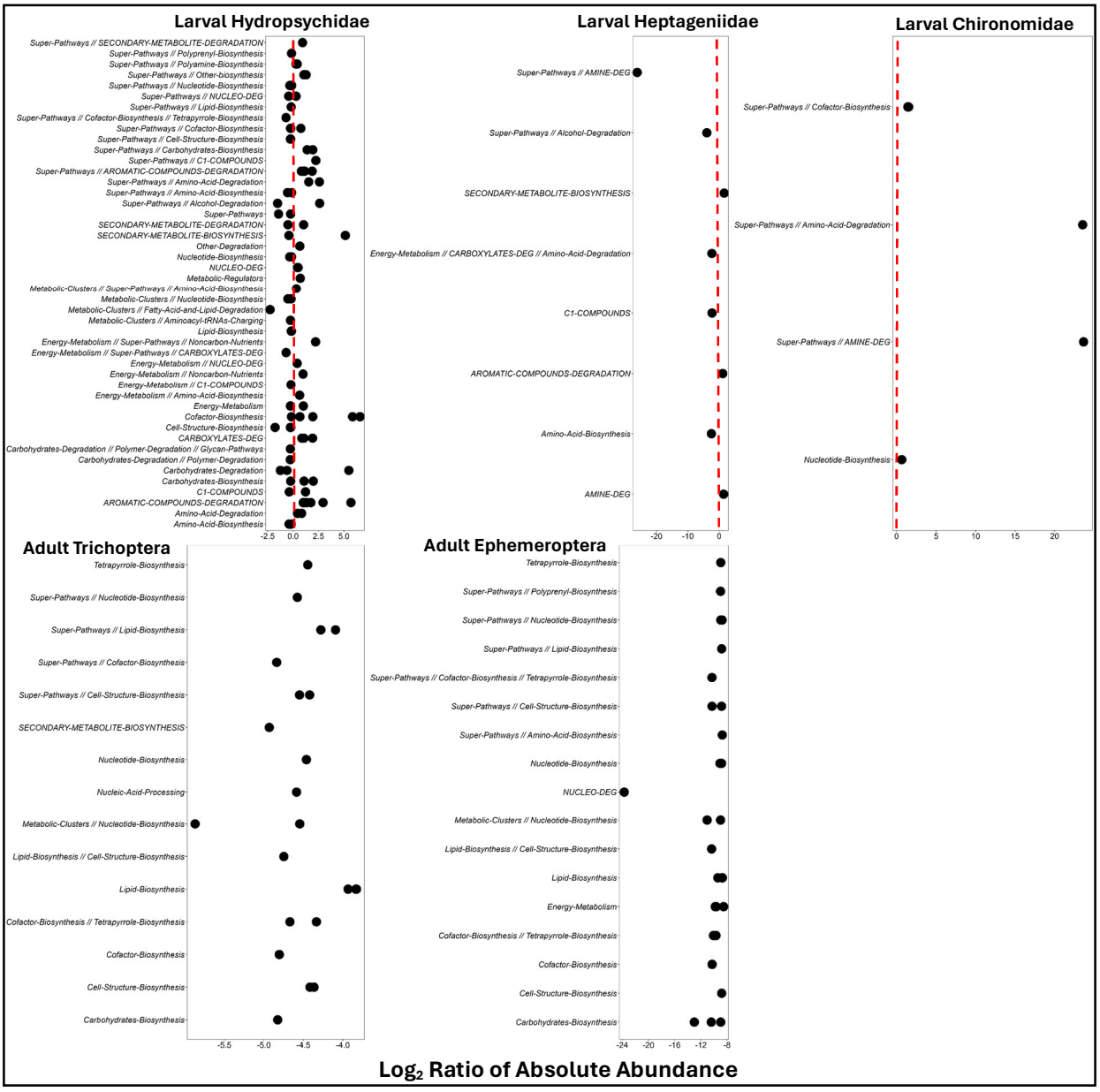

Figure A.7. Differentially expressed MetaCyc pathways (log_2 ratio) from wastewater effluent-exposed sites (Graves Bridge/Policeman Flats) compared to non-exposed sites (Cochrane, Sunalta, Cushing Bridge) combined across invertebrate taxa in the Bow River. Results based on DESeq2 ($\alpha = 0.001$).

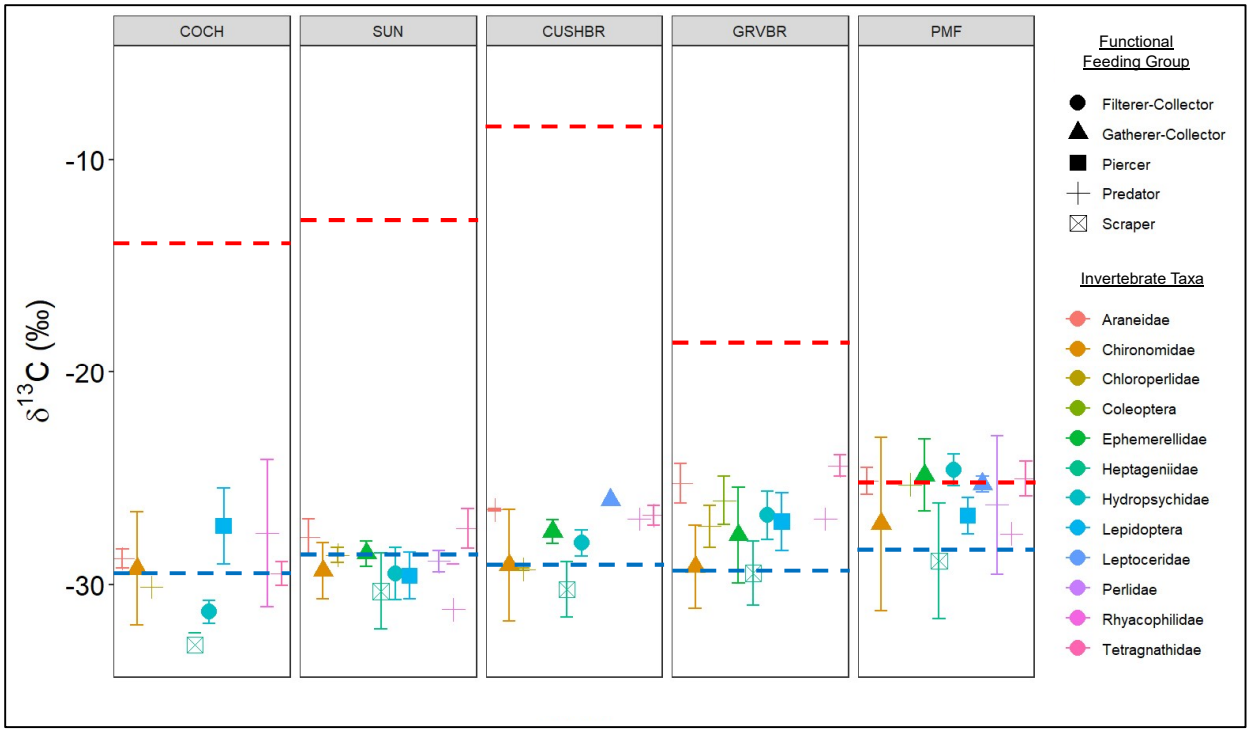

Figure A.8. Mean $\delta^{13}C$ values ± standard deviation (‰) of invertebrate taxa across sites of the Bow River where GRVBR and PMF are located downstream of wastewater outfalls. Dotted lines indicate the mean value of each basal food source where red is the mean $\delta^{13}C$ of biofilm and blue is the mean $\delta^{13}C$ of riparian leaves.

Appendix B: ACWA Experimental Streams Microbiome Analysis Supplementary Materials

Methods

Microbiome Sample Collection

The Advancing Canadian Wastewater Assets (ACWA) facility houses 12 experimental streams that are manipulated with differing effluent contribution rates of Pine Creek WWTP effluents to quantify ecological impacts from wastewater effluents in receiving waters. ACWA streams were 320 m long x 1 m wide x 0.1 m deep channels, lined with a clay pad to prevent drainage, and filled with gravel and rocks to create alternating 10 m long riffles and runs, attempting to recreate the natural conditions of Jumpingpound Creek, AB (Jackson, 2020). At the time of sample collection, the streams had constant flow rates of ~ 14.66 L/s, with water flowing in from a head pond filled with Bow River water, taken just above the location of the Pine Creek facility (approximate water source location: 50.861500, -113.985750). The head pond water was mixed with Pine Creek effluent from a post-biological nutrient removal secondary clarifier in different concentrations for each of the three treatments (REF: reference with no effluent addition, EFF5: 5% Pine Creek effluent addition, EFF15: 15% Pine Creek effluent addition; Table B.1) before draining into the experimental streams. It should be noted that the ACWA stream reference water, taken from the Bow River, is estimated to contain about 5-8% effluent, as it was taken downstream of both the Bonnybrook and Fish Creek WWTP outfalls (Vandenberg et al., 2005; Lazaro-Côte et al., 2018). For a complete and detailed description and diagram of stream setup and functionality, refer to Jackson et al. (2020).

Table B.1. Locations and names of experimental streams at ACWA, September 2022.

Site name	Site ID	Stream number	Latitude	Longitude
ACWA Reference (0% Pine Creek effluent addition)	REF	3	50.862517	-113.992838
ACWA Experimental 5% (5% Pine Creek effluent addition)	EFF5	2	50.862417	-113.992846
ACWA Experimental 15% (15% Pine Creek effluent addition)	EFF15	10	50.860840	-113.991562

Invertebrates were collected from the second riffle in each of the three streams for microbiome analysis. Larval caddisflies (family Hydropsychidae), larval mayflies (family Baetidae), and adult mayflies (order Ephemeroptera) were collected after ~3-4 weeks of Pine Creek effluent exposure between the 21st – 26th of September 2022, from the three ACWA streams. At all three streams, 8 Hydropsychidae larvae were aseptically collected and stored using the same methods described for the Bow River collections (section 2.2.1). Larval Baetidae were also collected using the same methods from the 5% (EFF5; n = 8) and 15% (EFF15; n = 7) effluent streams respectively, however, Baetidae were not found in the REF stream. In addition to the microbiome samples, three negative control samples of the UltraPure tweezer rinse water and three invertebrate samples for taxonomic validation were taken from each stream.

Adult Ephemeroptera were also collected from the two treatment streams (EFF5 and EFF15, n = 8/stream), respectively, however, none were caught in the REF stream. Adults were collected in six floating emergence traps (n = 3/stream) built based on methods from Cadmus et al. (2016) using polyvinyl chloride pipe, a plastic 500 mL Nalgene trapper bottle, and "No-See-Um" mesh fabric. While wearing sterile nitrile gloves, the emergence traps were disinfected by spraying the entire trap in 30% bleach, then in 70% non-denatured ethanol prior to being deployed in the streams and tied off to shoreline vegetation. A Nalgene bottle was attached to the

top of the trap and filled with ~200 mL of 95% non-denatured ethanol to catch the emergent insects. Adults were retrieved from the bottle daily and were aseptically transferred to individual 2.0 microcentrifuge tubes filled with 95% non-denatured ethanol, to match the storage conditions from inside the trap bottle. When an individual was collected from the bottle, three negative control samples of the traps were taken by rubbing a sterile cotton swab along the inside of the mesh put into tubes containing 95% non-denatured ethanol, and stored at -80 °C. Once the adult Ephemeroptera were collected from the bottle, the remaining ethanol and non-target insects were dumped out and the bottle was re-filled with 95% ethanol each day. No spiders for microbiome nor any isotope samples were collected from the ACWA streams. Grab samples of water quality parameters including temperature, dissolved oxygen, conductivity, total organic carbon, nitrate, etc. were also collected from each stream and reported as the mean ± SD (n = 3, Table B.2).

Table B.2. Water quality parameters across ACWA streams from September 2022 reported as either a single grab samples or the mean ± standard deviation of three replicates. Parameters include water temperature (°C; Temp), dissolved oxygen (mg/L; DO), total organic carbon (mg/L; TOC), specific conductivity (µS/cm; Cond), ammonia (mg/L; NH_3), nitrate (mg/L; NO_3), pH, and phosphate (mg/L; PO_4).

Site	Date collected	Temp	DO	TOC	Cond	NH_3	NO_3	pH	PO_4
REF	Sept 21/22	12.8 ± 0.33	12.3 ± 1.76	1.7	319 ± 3	0.091	5.4	8.4 ± 0.07	< 0.005
EFF5	Sept 22/22	13.0 ± 0.37	12.1 ± 2.83	1.4	373 ± 26	0.088	6.4	8.4 ± 0.19	0.0068
EFF15	Sept 22/22	13.4 ± 0.37	11.2 ± 1.70	2.3	390 ± 8	0.064	9.5	8.2 ± 0.31	0.012

Microbiome Laboratory and Data Analysis

Samples were processed and analyzed using the same methods described for the Bow River samples (sections 2.3.1, 2.3.2, 2.4.1) however, after read trimming and filtering, there were 71 total samples included in analysis (55 invertebrate samples and 16 blank samples; Table B.3). A PERMANOVA test indicated significant differences between bacterial beta diversity of field and

extraction blanks and experimental samples (ADONIS: Pseudo-$F_{1,69} = 4.92$, $p < 0.001$; Figure B.1), indicating no laboratory or field contamination in samples. For differential abundance analysis, the REF stream was used as the reference condition compared to the two treatment streams across invertebrates. Functional predictive pathways were analyzed the same way as the Bow River samples, using PICRUSt2, however, there were 3613 ASVs used in the analysis.

Table B.3. Total number of unique bacterial taxa (ASVs) and counts at each taxonomic level within whole-body invertebrate hosts and across sites in September 2022 from the ACWA streams. Unclassified ASVs were not included in the taxonomic counts.

	Invertebrate Type	Unique ASVs	Phyla	Classes	Orders	Families	Genera
	REF (n = 8)	**1472**	**23**	**50**	**117**	**137**	**187**
Larvae	Hydropsychidae (n = 8)	1472	23	50	117	137	187
	EFF5 (n = 24)	**2761**	**30**	**69**	**161**	**224**	**359**
Larvae	Hydropsychidae (n = 8)	1849	27	59	129	161	232
	Baetidae (n = 8)	1503	25	57	128	182	277
Adults	Ephemeroptera (n = 8)	193	12	21	46	65	86
	EFF15 (n = 23)	**2559**	**29**	**63**	**150**	**212**	**360**
Larvae	Hydropsychidae (n = 8)	1533	24	51	118	142	202
	Baetidae (n = 7)	1277	24	50	110	154	233
Adults	Ephemeroptera (n = 8)	265	15	25	64	96	145

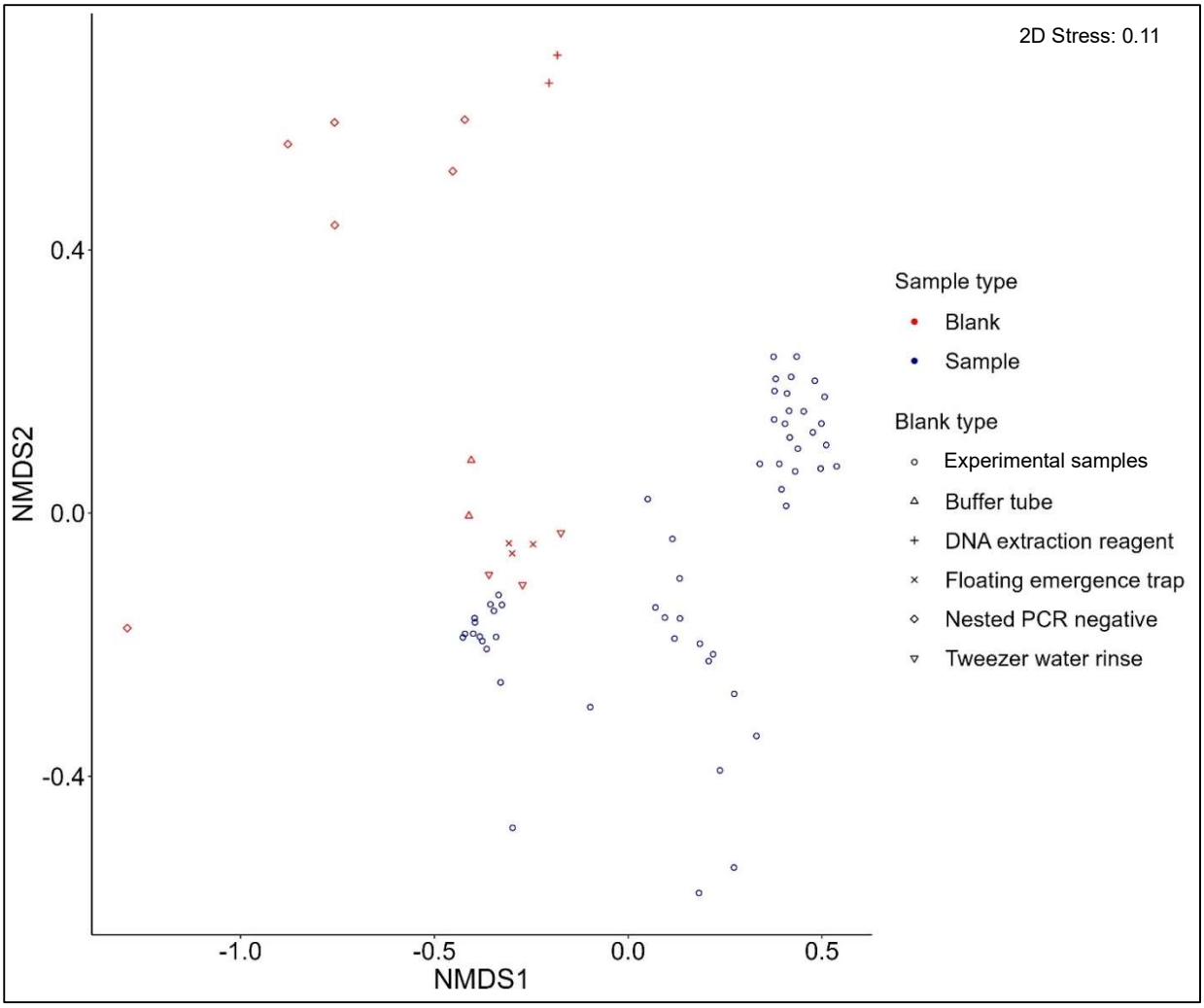

Figure B.1. Beta diversity composition of blank and experimental samples from the ACWA streams using Bray Curtis distance matrix and NDMS ordination. Sample type is denoted by the colours and the blank type is denoted by the shape of symbols.

Results

General Sequencing Results

There was a total of 2,015,542 sequence reads obtained from whole body larvae (n = 39) and adult (n = 16) macroinvertebrate samples in the ACWA streams. The number of reads per sample ranged from 8,982 – 90,569 with an average of 36,646 reads/sample and identified 3,613 unique

ASVs (Table B.3). The five most abundant phyla across all samples, comprising ~97% of the total bacteria, were Bacteroidota (mean ± SD; 45.5 ± 23.1%), Proteobacteria (43.7 ± 23.7%), Firmicutes (6.42 ± 8.23%), Deferribacterota (0.77 ± 1.65%), and Actinobacteriota (0.54 ± 0.57%). The five most abundant families across all samples were Chitinophagaceae (21.3 ± 31.1%), Comamonadaceae (13.3 ± 16.0%), Saprospiraceae (7.44 ± 9.29%), Rhodobacteraceae (6.89 ± 9.74%), and Spirosomaceae (6.47 ± 6.10%) and the five most abundant genera were *Vibrionimonas* (24.3 ± 37.8%), *Ideonella* (8.78 ± 16.4%), *Sphingorhabdus* (5.12 ± 7.49%), *Mucinivorans* (5.11 ± 8.04%), and *Pseudorhodobacter* (3.80 ± 4.25%), respectively.

Shifts in Bacterial Relative and Absolute Abundance

Bacterial phyla and genera relative abundances shifted mostly between invertebrate types rather than between experimental streams. The only spatial difference across all invertebrates was a significantly lower proportion of Firmicutes in adult Ephemeroptera collected from EFF5 compared those from EFF15 (Kruskal-Wallis Chi-square: 8.65, p = 0.0033; Table B.4, Figure B.2). There were more notable shifts in bacterial genera relative abundances in larval invertebrates compared to the adults (Figure B.3). Larval Hydropsychidae had an increase in the proportion of *Dysogonomonas* and a decrease in the proportion of *Sphingorhabdus, Rhodobacter,* and *Ferruginibacter* in both treatment streams relative to the reference. There was also an increase in the proportion of *Hydrogenophaga* and *Rhodoferax* in the EFF15 stream compared to the other two. In larval Baetidae, *Candidatus Bacilloplasma*, *Flavobacterium, Sphingorhabdus,* and *Lacihabitans* decreased and *Candidatus Neoehrlichia*, *Rhodobacter* and *Tabrizicola* increased in EFF15 compared to EFF5. Adult Ephemeroptera was almost entirely comprised of

Vibrionimonas, with slightly more at EFF15 compared to EFF5, and EFF5 had increased *Candidatus Neoehrlichia* and *Ideonella*.

Table B.4. Mean (± standard deviation) relative abundance (%) of the average three most abundant bacterial phyla across all sites within each invertebrate taxon of the ACWA streams.

	Taxa Type	Phylum	REF	EFF5	EFF15
Larvae	Hydropsychidae	Bacteroidota	45.3 ± 7.71	45.0 ± 7.95	53.3 ± 8.94
		Proteobacteria	39.4 ± 10.6	29.9 ± 9.06	34.4 ± 8.45
		Firmicutes	10.2 ± 4.65	15.5 ± 8.77	7.13 ± 2.63
		Other	5.16 ± 0.62	9.64 ± 1.76	5.11 ± 0.81
	Baetidae	Proteobacteria	-	65.5 ± 18.6	79.0 ± 8.09
		Bacteroidota	-	25.4 ± 17.2	14.3 ± 10.0
		Firmicutes	-	6.05 ± 15.2	1.59 ± 1.59
		Other	-	3.10 ± 0.42	5.15 ± 0.79
Adults	Ephemeroptera	Bacteroidota	-	56.2 ± 29.1	75.5 ± 1.95
		Proteobacteria	-	41.5 ± 30.4	20.5 ± 2.00
		Firmicutes	-	1.20 ± 0.86	2.59 ± 0.79
		Other	-	1.14 ± 0.30	1.43 ± 0.15

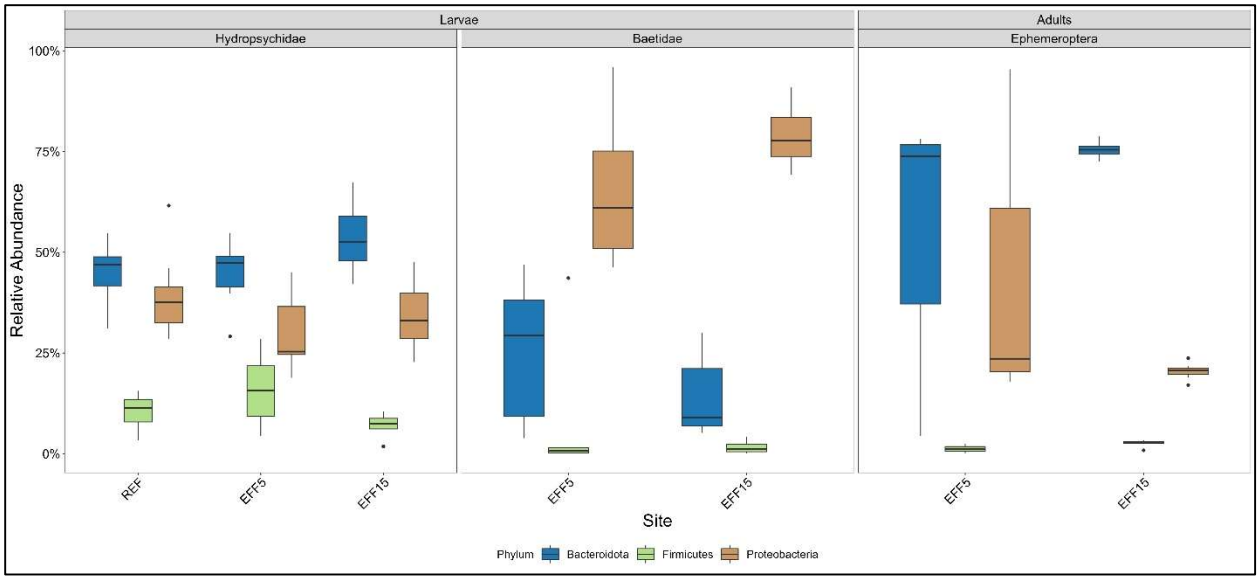

Figure B.2. Boxplot of the three most abundant bacterial phyla shown in relative abundances (%) from Hydropsychidae larvae, Baetidae larvae, and adult Ephemeroptera in the ACWA streams. Streams are arranged in order of increasing effluent additions.

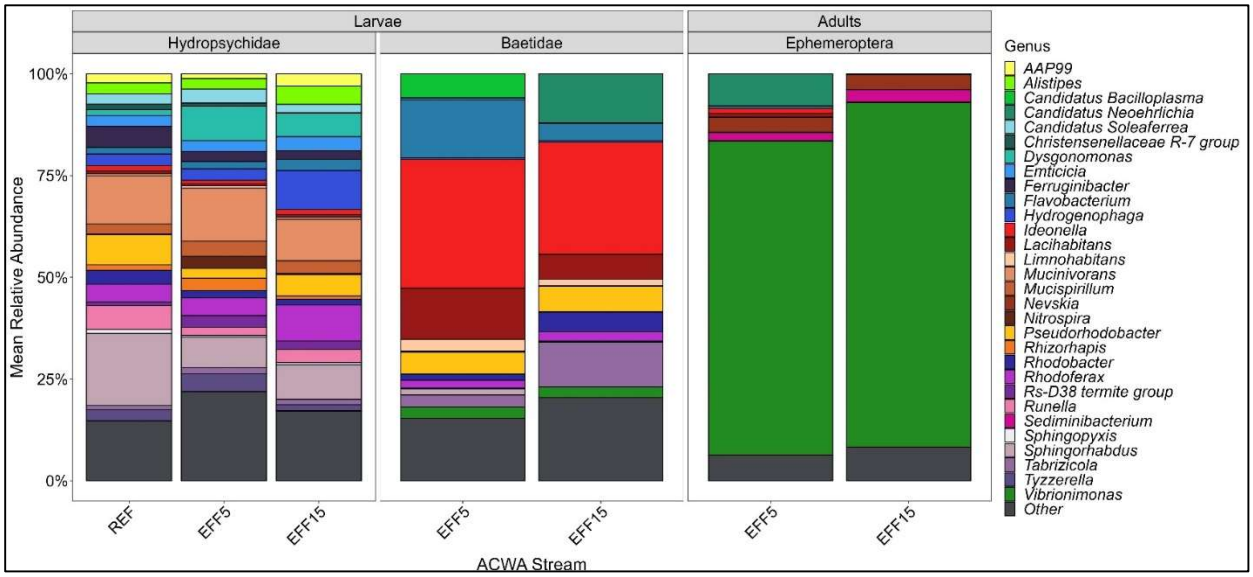

Figure B.3. Mean relative abundance (%) of the top 30 bacterial genera from invertebrates collected from the ACWA streams. Streams are arranged in order of increasing effluent additions (0% or REF, 5% or EFF5, 15% or EFF15).

There was a very small proportion of endosymbiont genera across invertebrates which included *Candidatus Megaira, Rickettsia*, and *Wolbachia*, but there were no consistent spatial

differences in their relative abundances (Table B.5). There were a few differentially abundant bacterial taxa between the ACWA streams across invertebrates, some of which may have been endosymbionts (belonging to the family Holosporaceae) and had the highest abundance at the EFF5 stream compared to EFF15 by ~25-30 $\log_2$ fold in larval baetids and adult Ephemeroptera (Table B.6). Hydropsychidae larvae had a greater abundance of *Alistipes* in the reference stream (REF) compared to EFF5, a greater abundance of *Mucinivorans* in individuals from the EFF15 stream compared to REF by ~23 $\log_2$ fold, and a higher abundance of unclassified Hungateiclostridiaceae in the EFF15 stream compared to EFF5 and REF by ~22-26 $\log_2$ fold.

Table B.5. Mean relative abundance (%) ± pooled standard deviation of endosymbiont bacteria compared to the total (non-endosymbionts) across invertebrate taxa and collection streams at ACWA.

Life Stage	Invertebrate Taxon	REF	EFF5	EFF15
Larvae	Hydropsychidae	*Rickettsia* (0.003 ± 0.010) Non-endosymbionts (100 ± 5.91)	*Candidatus Megaira* (0.080 ± 0.151) *Rickettsia* (0.020 ± 0.023) *Wolbachia* (0.016 ± 0.030) Non-endosymbionts (100 ± 7.40)	*Rickettsia* (0.074 ± 0.033) Non-endosymbionts (99.9 ± 6.81)
	Baetidae	-	*Candidatus Megaira* (0.003 ± 0.008) Non-endosymbionts (100 ± 9.06)	Non-endosymbionts (100 ± 16.6)
Adults	Ephemeroptera	-	*Rickettsia* (0.007 ± 0.019) Non-endosymbionts (100 ± 12.3)	*Rickettsia* (0.003 ± 0.010) *Wolbachia* (0.007 ± 0.019) Non-endosymbionts (100 ± 0.896)

Table B.6. Differentially abundant bacterial taxa within each invertebrate type across ACWA streams, based on DESeq2 ($\alpha = 0.001$). Log_2 fold differences are interpreted as the difference between the first listed site compared to the second.

Stage	Invertebrate Taxa	Stream comparison	Differentially abundant bacteria	Log_2 fold difference
Larvae	Hydropsychidae	REF – EFF5	*Alistipes* sp.	22.8
		REF – EFF15	*Mucinivorans* sp.	-22.6
			Hungateiclostridiaceae unclassified	23.5
		EFF5 – EFF15	Hungateiclostridiaceae unclassified	-26.8
	Baetidae	EFF5 – EFF15	*Ideonella*	-21.5
			Holosporaceae unclassified	25.4
			Aeromonas veronii	24.2
Adults	Ephemeroptera	EFF5 – EFF15	Holosporaceae unclassified	28.6
			Holosporaceae unclassified	23.5

Effluent-Associated Bacteria

Out of the 441 identified bacterial genera in the ACWA streams, 87 were associated with wastewater effluents, comprising 9.32% of all larvae and 1.87% of all adult insect reads. Larval Hydropsychidae had the greatest proportion of effluent-associated bacteria compared to the other invertebrate taxa, and adult Ephemeroptera had the least (Table B.7 - B.8); however, there were only slight shifts in the total proportion across streams for all invertebrate types. Specifically, in Hydropsychidae, the REF stream had the greatest mean total proportion of effluent-associated bacteria relative to the total microbiome (~24.6%) and the EFF5 stream had the least (~18.9). Larval Baetidae and adult Ephemeroptera also had a very slight increases in proportions of effluent-associated bacteria in the EFF15 stream compared to EFF5; however, these mean proportions were highly variable. Out of the differentially abundant bacteria across streams, *Aeromonas veronii*, was the only bacterium that was effluent-associated and potentially pathogenic to humans. This species had higher abundance at EFF5 compared to EFF15 in larval baetids (Table B.6).

Table B.7. Prevalence (Prev; %) and mean (± standard deviation) relative abundance (RA; %, rounded to 2 decimals) of common effluent-associated bacteria relative to the total microbiome within larval Hydropsychidae and Baetidae across ACWA collection streams.

	Hydropsychidae						Baetidae			
	REF (n = 8)		EFF5 (n = 8)		EFF15 (n = 8)		EFF5 (n = 8)		EFF15 (n = 7)	
Genus	Prev	RA	Prev	RA	Prev	RA	Prev	RA	Prev	RA
AAP99[12]	100	2.22 ± 1.76	100	1.16 ± 0.74	100	3.05 ± 2.81	37.5	0.01 ± 0.01	14.3	0
Acidovorax[33]	0	-	12.5	0	0	-	62.5	0.03 ± 0.06	14.3	0.02 ± 0.04
Acinetobacter[16]	0	-	0	-	0	-	100	0.19 ± 0.16	85.7	0.03 ± 0.03
Aeromonas[16]	12.5	0 ± 0.01	0	-	0	-	50	0.42 ± 0.77	0	-
Alcaligenes[16]	0	-	0	-	0	-	12.5	0 ± 0.01	28.6	0 ± 0.01
Amaricoccus[9],[28]	0	-	0	-	0	-	12.5	0 ± 0.01	14.3	0.01 ± 0.03
Anaerovorax[22]	50	0.05 ± 0.06	37.5	0.02 ± 0.03	25	0.04 ± 0.09	0	-	0	-
Aridibacter[42]	25	0.02 ± 0.04	50	0.07 ± 0.09	50	0.03 ± 0.07	0	-	14.3	0.01 ± 0.02
Arthrobacter[16]	0	-	0	-	12.5	0.01 ± 0.02	25	0.03 ± 0.07	28.6	0.01 ± 0.01
Bacillus[16]	0	-	12.5	0 ± 0.01	0	-	50	0.01 ± 0.02	42.9	0.02 ± 0.03
Bdellovibrio[15]	12.5	0 ± 0.01	12.5	0 ± 0.01	37.5	0.01 ± 0.02	0	-	0	-
Blastocatella[19]	100	0.42 ± 0.43	75	0.15 ± 0.14	100	0.16 ± 0.09	50	0.01 ± 0.01	0	-
Bryobacter[41]	87.5	0.42 ± 0.61	87.5	0.59 ± 0.84	62.5	0.09 ± 0.17	37.5	0.01 ± 0.02	42.9	0.01 ± 0.01
Candidatus Accumulibacter[44]	37.5	0.03 ± 0.05	87.5	0.11 ± 0.16	12.5	0.01 ± 0.02	75	0.06 ± 0.08	71.4	0.08 ± 0.11
Candidatus Amoebophilus[1]	87.5	0.07 ± 0.12	75	0.03 ± 0.03	37.5	0.01 ± 0.02	75	0.36 ± 0.40	28.6	0.01 ± 0.02
Candidatus Anammoximicrobium[14]	0	-	0	-	0	-	25	0 ± 0.01	0	-
Candidatus Competibacter[40]	25	0.01 ± 0.01	37.5	0.02 ± 0.04	0	-	37.5	0.01 ± 0.02	71.4	0.05 ± 0.07
Candidatus Microthrix[29]	0	-	0	-	75	0.04 ± 0.04	12.5	0	71.4	0.20 ± 0.25
Candidatus Nitrotoga[26]	12.5	0 ± 0.01	37.5	0.08 ± 0.12	0	-	12.5	0	0	-
Cetobacterium[38]	0	-	25	0 ± 0.01	0	-	50	0.03 ± 0.04	28.6	0.01 ± 0.02
Chryseobacterium[9]	75	0.10 ± 0.13	75	0.06 ± 0.08	75	0.15 ± 0.13	75	0.20 ± 0.17	28.6	0
Clostridium SS 1[16]	12.5	0.01 ± 0.03	87.5	0.07 ± 0.06	87.5	0.07 ± 0.04	100	0.15 ± 0.14	71.4	0.36 ± 0.36
Clostridium SS 3[16]	0	-	0	-	0	-	0	-	57.1	0.01 ± 0.01
Clostridium SS 8[16]	0	-	0	-	0	-	75	0	0	-
Clostridium SS 9[16]	0	-	0	-	12.5	0	12.5	0	42.9	0.01 ± 0.01
Clostridium SS 13[16]	37.5	0.03 ± 0.05	87.5	0.06 ± 0.04	62.5	0.03 ± 0.04	12.5	0.08 ± 0.07	0	-
Cytophaga[27]	0	-	0	-	0	-	12.5	0 ± 0.01	28.6	0
Dechloromonas[45]	100	0.33 ± 0.21	100	0.60 ± 0.57	100	0.42 ± 0.33	100	0.68 ± 0.42	14.3	1.63 ± 1.72
Defluviicoccus[7]	0	-	0	-	0	-	0	-	100	0.01 ± 0.02
Desulfatiglans[30]	0	-	0	-	0	-	12.5	0.01 ± 0.02	14.3	0
Desulfovibrio[16]	75	0.60 ± 0.54	62.5	0.40 ± 0.64	87.5	0.44 ± 0.34	0	-	0	-
Devosia[9]	0	-	12.5	0 ± 0.01	12.5	0 ± 0.01	12.5	0 ± 0.01	0	-
Enterococcus[9],[27]	25	0.01 ± 0.02	12.5	0.17 ± 0.47	50	0.06 ± 0.10	0	-	14.3	0 ± 0.01
Escherichia-Shigella[9],[16]	0	-	0	-	12.5	0 ± 0.01	0	-	14.3	0
Faecalibacterium[9]	0	-	0	-	0	-	50	0.02 ± 0.03	42.9	0.02 ± 0.03
Ferruginibacter[9]	100	5.14 ± 5.21	100	2.50 ± 2.87	100	2.17 ± 1.79	75	0.05 ± 0.06	42.9	0.01 ± 0.02
Flexibacter[16]	0	-	25	0 ± 0.01	0	-	0	-	0	-
Fluviicola[14]	100	0.17 ± 0.11	87.5	0.16 ± 0.10	100	0.24 ± 0.22	100	2.38 ± 1.70	100	0.60 ± 0.58
Gemmata[13]	12.5	0 ± 0.01	12.5	0.01 ± 0.02	25	0.01 ± 0.02	25	0.02 ± 0.05	42.9	0.02 ± 0.03
Gordonia[9]	0	-	0	-	0	-	0	-	14.3	0 ± 0.01
Haliscomenobacter[16],[45]	100	0.28 ± 0.20	87.5	0.37 ± 0.35	87.5	0.13 ± 0.13	50	0.03 ± 0.05	42.9	0.03 ± 0.07
Helicobacter[18]	0	-	0	-	0	-	12.5	0	0	-
Hydrogenophaga[9]	100	2.85 ± 1.63	100	2.81 ± 2.54	87.5	9.63 ± 9.50	100	0.37 ± 0.13	100	0.38 ± 0.33
Hyphomicrobium[16]	100	0.29 ± 0.31	87.5	0.50 ± 0.47	100	0.28 ± 0.18	75	0.21 ± 0.24	71.4	0.67 ± 0.81
Ignavibacterium[9]	0	-	25	0.01 ± 0.02	0	-	50	0.05 ± 0.09	14.3	0 ± 0.01
Isosphaera[13]	0	-	25	0.02 ± 0.04	0	-	0	-	0	-
Legionella[10]	12.5	0 ± 0.01	0	-	12.5	0 ± 0.01	0	-	28.6	0.01 ± 0.02
Methylocystis[9]	12.5	0 ± 0.01	25	0.02 ± 0.05	0	-	25	0.02 ± 0.03	57.1	0.06 ± 0.06
Methylotenera[42]	100	0.59 ± 0.37	100	0.52 ± 0.46	100	0.46 ± 0.40	37.5	0.02 ± 0.03	14.3	0.01 ± 0.03
Mycobacterium[16]	0	-	0	-	0	-	0	-	28.6	0.01 ± 0.01
Nitrosomonas[16]	100	0.27 ± 0.22	100	0.59 ± 0.76	100	0.38 ± 0.30	25	0.02 ± 0.04	14.3	0 ± 0.01
Nitrospira[9],[16]	62.5	0.11 ± 0.14	75	2.89 ± 5.81	87.5	0.23 ± 0.24	62.5	0.07 ± 0.13	42.9	0.05 ± 0.06

Nocardioides[9]	87.5	0.16 ± 0.19	75	0.13 ± 0.13	100	0.08 ± 0.06	0	-	42.9	0.01 ± 0.01
Novosphingobium[42]	100	0.85 ± 0.87	87.5	0.36 ± 0.37	87.5	0.30 ± 0.31	87.5	0.22 ± 0.19	57.1	0.03 ± 0.03
Paludibacter[38],[45]	0	-	62.5	0.02 ± 0.02	12.5	0.01 ± 0.02	50	0.07 ± 0.11	14.3	0 ± 0.01
Pseudomonas[16]	0	-	0	-	12.5	0 ± 0.01	0	-	0	-
Rhodobacter[9]	100	3.44 ± 2.15	100	1.83 ± 0.92	100	1.46 ± 0.77	87.5	1.45 ± 2.23	100	4.70 ± 4.25
Rhodococcus[9],[27]	0	-	0	-	0	-	12.5	0.01 ± 0.02	0	-
Romboutsia[38]	75	0.06 ± 0.05	100	0.15 ± 0.08	100	0.17 ± 0.05	87.5	0.34 ± 0.38	100	0.88 ± 0.89
Ruminococcus[9]	0	-	0	-	0	-	0	-	14.3	0 ± 0.01
Runella[32]	100	5.82 ± 3.23	100	1.91 ± 1.39	100	3.21 ± 1.19	12.5	0 ± 0.01	0	-
Shinella[9]	0	-	0	-	0	-	0	-	28.6	0.02 ± 0.04
Smithella[30]	0	-	12.5	0	0	-	37.5	0.01 ± 0.02	0	-
Staphylococcus[9],[38]	0	-	0	-	0	-	12.5	0 ± 0.01	14.3	0.01 ± 0.02
Stenotrophobacter[38]	12.5	0	62.5	0.13 ± 0.21	62.5	0.02 ± 0.03	0	-	14.3	0
Streptococcus[9],[16]	0	-	12.5	0	0	-	0	-	0	-
Sulfuritalea[14]	12.5	0 ± 0.01	37.5	0.02 ± 0.03	12.5	0 ± 0.01	50	0.04 ± 0.06	0	-
Syntrophobacter[17]	0	-	0	-	0	-	37.5	0.01 ± 0.01	42.9	0
Syntrophomonas[16]	0	-	0	-	0	-	12.5	0	0	-
Syntrophus[22]	0	-	0	-	0	-	12.5	0	14.3	0 ± 0.01
Terrimonas[9],[42]	62.5	0.20 ± 0.18	87.5	0.32 ± 0.33	100	0.40 ± 0.33	12.5	0 ± 0.01	28.6	0.01 ± 0.01
Thermomonas[42]	0	-	0	-	12.5	0 ± 0.01	37.5	0.02 ± 0.03	14.3	0.01 ± 0.02
Trichococcus[29],[42]	0	-	0	-	0	-	12.5	0	57.1	0.04 ± 0.04
Non-effluent associated genera	-	75.4 ± 5.36	-	81.1 ± 6.92	-	76.2 ± 5.77	-	92.3 ± 9.00	-	89.8 ± 16.5

Table B.8. Prevalence (Prev; %) and mean (± standard deviation) relative abundance (RA; %, rounded to 2 decimals) of common effluent-associated bacteria relative to the total microbiome within adult Ephemeroptera across ACWA collection streams.

	Ephemeroptera			
	EFF5 (n = 8)		EFF15 (n = 8)	
Genus	Prev	RA	Prev	RA
Achromobacter[5]	25	0.09 ± 0.23	25	0.03 ± 0.06
Aeromonas[16]	12.5	0.01 ± 0.04	0	-
Alcaligenes[16]	62.5	0.11 ± 0.14	75	0.11 ± 0.13
Arthrobacter[16]	12.5	0.01 ± 0.03	25	0.02 ± 0.04
Bacillus[16]	12.5	0.01 ± 0.04	50	0.07 ± 0.04
Bacteroides[9],[16]	0	-	50	0.12 ± 0.20
Blastocatella[19]	0	-	12.5	0.01 ± 0.03
Blautia[9]	0	-	25	0.06 ± 0.11
Bryobacter[41]	87.5	0.36 ± 0.27	87.5	0.22 ± 0.15
Candidatus Microthrix[29]	25	0.01 ± 0.03	25	0.02 ± 0.03
Chryseobacterium[9]	12.5	0 ± 0.01	12.5	0.01 ± 0.02
Cloacibacterium[9]	0	-	12.5	0 ± 0.01
Clostridium SS 1[16]	0	-	50	0.16 ± 0.23
Clostridium SS 2[16]	0	-	12.5	0.02 ± 0.07
Clostridium SS 13[16]	0	-	12.5	0.01 ± 0.02
Comamonas[9]	0	-	12.5	0 ± 0.01
Corynebacterium[16]	37.5	0.02 ± 0.03	62.5	0.07 ± 0.11

Dechloromonas[45]	25	0.02 ± 0.04	0	-
Faecalibacterium[9]	100	1.38 ± 1.00	100	0.83 ± 0.40
Ferruginibacter[9]	0	-	25	0.04 ± 0.07
Fluviicola[14]	12.5	0.07 ± 0.19	12.5	0 ± 0.01
Gordonia[9]	0	-	12.5	0 ± 0.01
Haliscomenobacter[16],[45]	0	-	12.5	0.01 ± 0.04
Hydrogenophaga[9]	12.5	0 ± 0.01	0	-
Hyphomicrobium[16]	0	-	12.5	0.01 ± 0.02
Lachnospira[39]	0	-	25	0.01 ± 0.02
Micrococcus[16]	12.5	0.08 ± 0.22	25	0.04 ± 0.07
Moraxella[16],[45]	12.5	0	0	-
Mycobacterium[16]	50	0.11 ± 0.17	62.5	0.02 ± 0.02
Neisseria[16]	0	-	12.5	0 ± 0.01
Nitrosomonas[16]	0	-	12.5	0 ± 0.01
Nitrospira[9],[16]	0	-	12.5	0 ± 0.01
Nocardioides[9]	0	-	37.5	0.02 ± 0.04
Parabacteroides[39]	0	-	12.5	0.01 ± 0.03
Paracoccus[16]	12.5	0.03 ± 0.07	25	0.02 ± 0.04
Rhodobacter[9]	12.5	0 ± 0.01	0	-
Romboutsia[38]	12.5	0.03 ± 0.09	75	0.62 ± 0.67
Roseburia[9]	0	-	37.5	0.04 ± 0.06
Ruminococcus[9]	0	-	37.5	0.04 ± 0.06
Staphylococcus[9],[38]	37.5	0.11 ± 0.19	62.5	0.23 ± 0.42
Stenotrophobacter[38]	0	-	12.5	0.01 ± 0.02
Streptococcus[9],[16]	37.5	0.13 ± 0.21	37.5	0.17 ± 0.41
Sulfuritalea[14]	0	-	12.5	0.01 ± 0.01
Non-effluent associated genera	-	97.4 ± 12.3	-	96.9 ± 0.81

Alpha Diversity

There were few differences in ACWA invertebrate alpha diversities. All invertebrate taxa were significantly different from each other (Kruskal-Wallis: df = 2, Chi-squared = 36.5, $p < 0.001$), with larval Hydropsychidae having the highest mean alpha diversity across all sites (4.78 ± 0.41), then larval Baetidae (3.43 ± 1.38), and adult Ephemeroptera with the lowest (1.30 ± 0.28; Figure B.4). Hydropsychidae larvae had the highest alpha diversity in the EFF5 stream whereas

larval Baetidae and adult Ephemeroptera had the highest diversity in the EFF15 stream (Table B.9); however, these differences were not significant (Hydropsychidae ANOVA: $F_{2,21} = 1.17$, $p = 0.331$; Baetidae t-test: $t = -0.47$, $p = 0.646$; Ephemeroptera Wilcox-test: $W = 16$, $p = 0.105$).

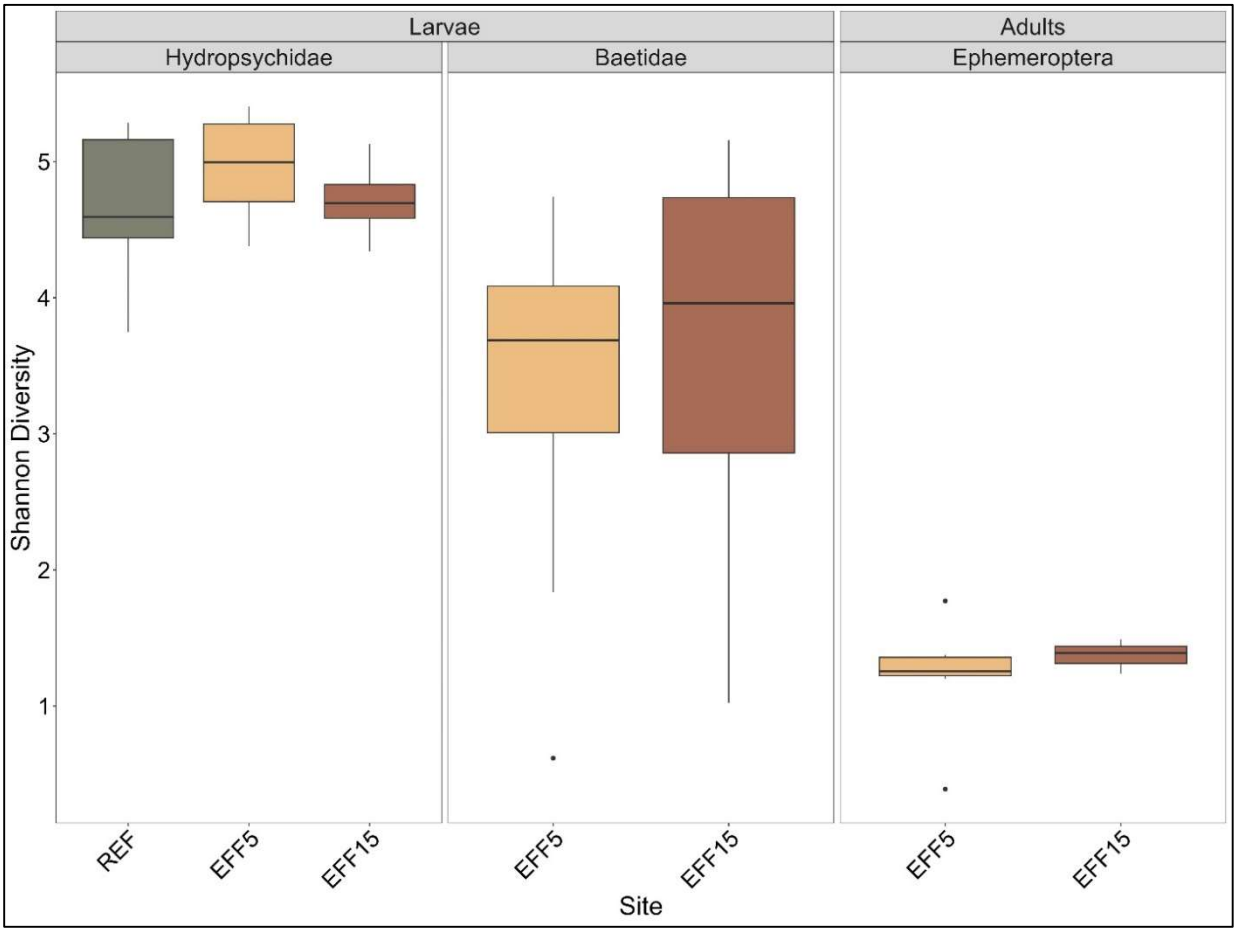

Figure B.4. Boxplot of alpha diversity measured by Shannon diversity index of non-rarefied bacterial composition within larval and adult invertebrates across ACWA streams. Sites are arranged in order of increasing wastewater effluent contribution (0, 5, 15%).

Table B.9. Mean ± standard deviation of Shannon diversity within invertebrates collected across ACWA streams from September 2022.

Life stage	Invertebrate taxon	Site	Shannon Index
Larvae	Hydropsychidae	REF	4.66 ± 0.527
		EFF5	4.96 ± 0.385

		EFF15	4.73 ± 0.271
	Baetidae	EFF5	3.27 ± 1.36
		EFF15	3.62 ± 1.49
Adults	Ephemeroptera	EFF5	1.23 ± 0.385
		EFF15	1.37 ± 0.0901

Beta Diversity

Microbial beta diversity varied across invertebrate type and between some experimental streams, however, the former was a more important predictor of community composition (RDA individual variation explained: 43.8% compared to 4.29%). Specifically, all invertebrate types had significantly different beta diversities (PERMANOVA: Pseudo-$F_{2,48}$ = 1.52, $p < 0.001$), and with invertebrate types analyzed individually across sites, Hydropsychidae community composition significantly differed between all three experimental streams (PERMANOVA: Pseudo-$F_{2,21}$ = 1.76, $p = 0.002$), while Baetidae and Ephemeroptera microbiomes did not differ between the two treatment streams (Pseudo-$F_{1,13}$ = 1.48, $p = 0.155$ and Pseudo-$F_{1,14}$ = 2.28, $p = 0.125$, respectively; Figure B.5). However, there was also significant beta dispersion between sites and invertebrate types (Pseudo-$F_{2,52}$ = 21.6, $p < 0.001$ and Pseudo-$F_{2,52}$ = 16.4, $p < 0.001$, respectively), which may have influenced the PERMANOVA results. Conductivity was a significant predictor of microbiome composition in the ACWA streams, however, had a very weak correlation to the abundance matrix (Cond: $r = 0.0712$, $p = 0.044$).

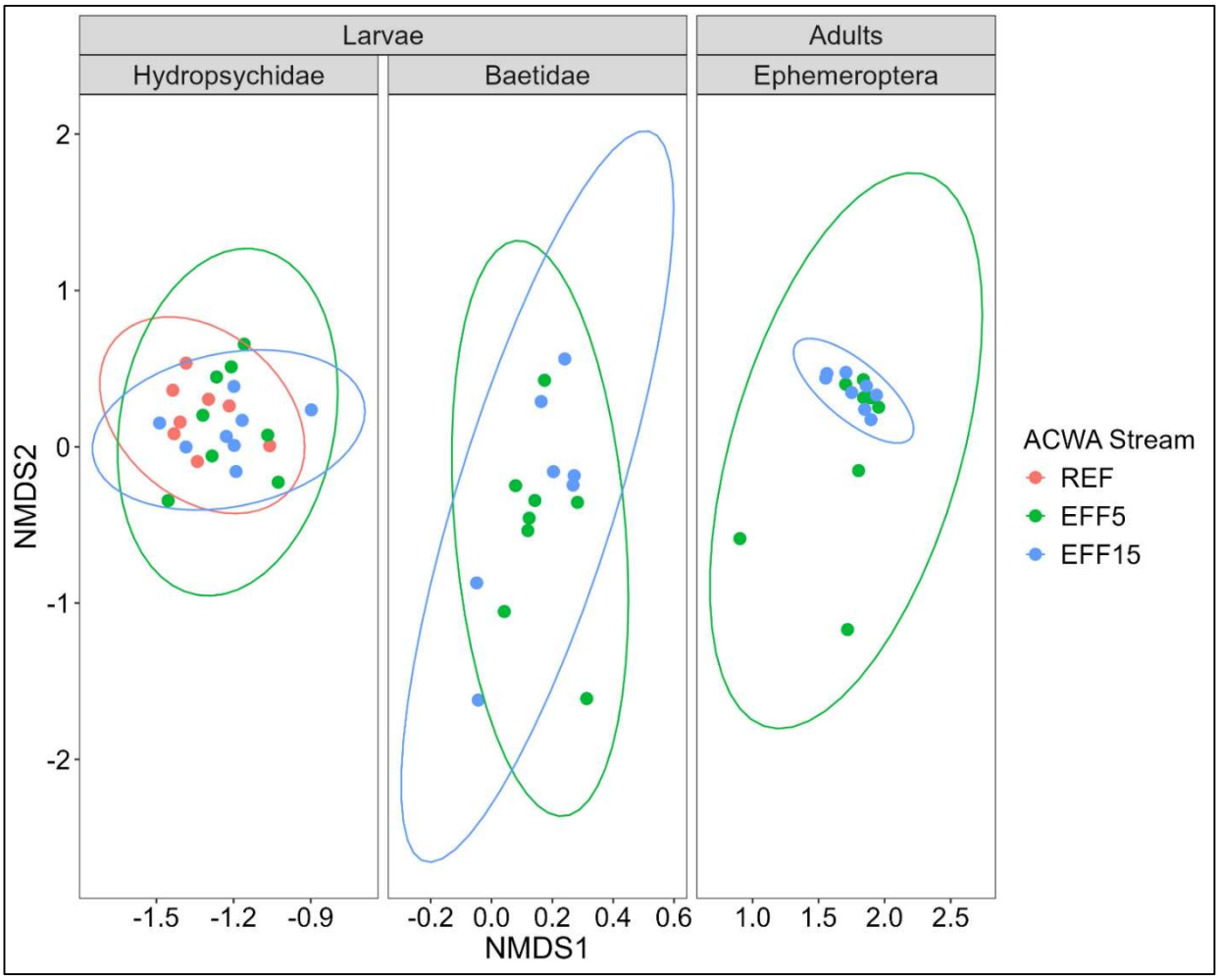

Figure B.5. NMDS of proportionally normalized bacterial beta diversity across ACWA streams, faceted by invertebrate taxa type collected in September 2022 (n = 55). The colour of the symbol represents the ACWA collection stream surrounded by 95% confidence ellipses.

Predictive Metabolic Pathways

The mean relative abundances of the top 25 most abundant MetaCyc metabolic pathway classes did not change much across ACWA streams or across invertebrate type (Figure B.6). There were a few differentially expressed pathways across ACWA streams, however, none had the same responses between invertebrate types (Figure B.7). In larval Hydropsychidae, secondary metabolite degradation was downregulated in the 5% effluent stream, EFF5, compared to the

reference by $\log_2$ fold, and tetrapyrrole and amino acid biosynthesis pathways were upregulated in EFF5 compared to the reference by ~ 1-5 $\log_2$ fold. In the 15% effluent stream, tetrapyrrole biosynthesis was actually downregulated compared to the reference by ~ $\log_2$ fold and cell structure biosynthesis was upregulated by ~10 $\log_2$ fold compared to the reference. In adult Ephemeroptera nucleotide biosynthesis was downregulated at the highest effluent contribution stream, EFF15, compared to EFF5 by ~5 $\log_2$ fold and an unidentified super-pathway was upregulated by $\log_2$ fold.

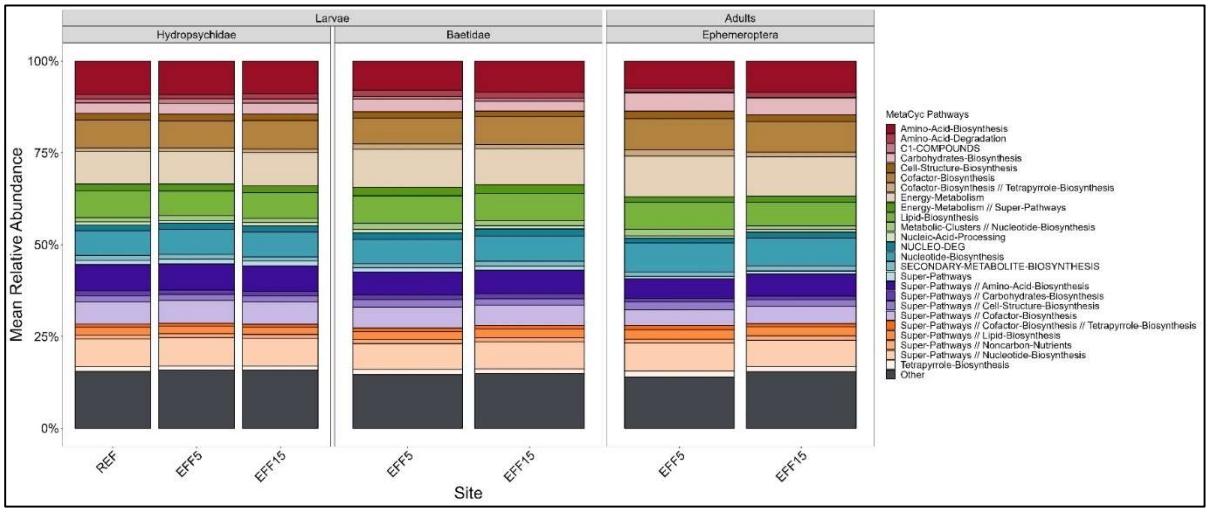

Figure B.6. Mean relative abundance of the top 25 most abundant MetaCyc functional pathway classes across invertebrate taxa in the ACWA streams, estimated using PICRUSt2.

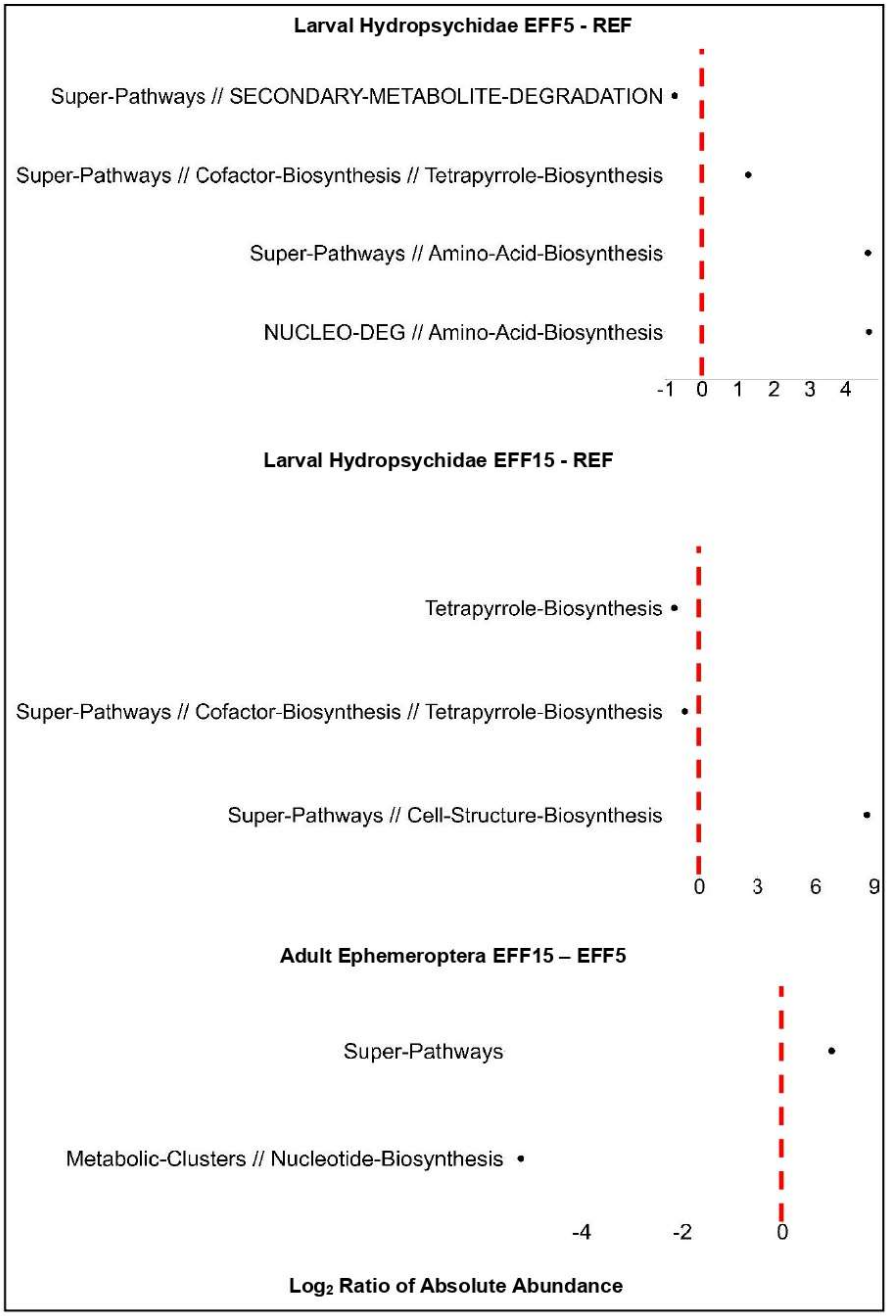

Figure B.7. Differentially expressed MetaCyc pathways (log_2 ratio) across invertebrate taxa and ACWA streams, based on DESeq2 ($\alpha = 0.001$). Red dotted line indicates the zero line.

Discussion

The experimental ACWA streams were used to measure the controlled, isolated effect of wastewater effluents on chronically exposed freshwater insects across a range of high environmentally relevant concentrations with the goal of comparing impacts to the microbiome of invertebrates from ACWA to those found in the natural conditions of the Bow River. Overall, there was no strong evidence to suggest dysbiosis due to effluent exposure in ACWA stream larval or adult insects. There were minor shifts in phylum and genus level relative abundances and no shifts in endosymbiont bacteria (contrary to observations from many Bow River invertebrates). There were a few differentially abundant bacteria between streams, however, only one seemed to be associated with effluents (*Aeromonas veronii*) and had a higher abundance in the lower effluent addition stream compared to the highest treatment stream in larval Baetidae. There were also no significant shifts in alpha diversity across the streams for any of the invertebrates; however, like the Bow River, larvae had higher alpha diversity compared to adults of the same order. Alpha diversities were also slightly lower in the ACWA streams compared to those observed in invertebrates from Policeman Flats. Community composition differed in larval hydropsychids across all ACWA streams, but larval baetids and adult ephemeropterans did not change between the two treatment streams.

Functional profiles revealed that there were some differentially abundant predicted metabolic pathways across experimental streams, but the pathways were not consistent across invertebrates nor across experimental streams. Some of the identified pathways were the same as those observed from the effluent-exposed sites compared to non-exposed sites in invertebrates of the Bow River, such as tetrapyrrole, amino acid, nucleotide, and cell structure biosynthesis; however, the pathways were not consistently downregulated in the ACWA stream insects as they

were in the Bow River. Overall, the functional profiles from the ACWA streams do not reflect similar metabolic impacts due to wastewater exposure as in the Bow River and suggests minimal impacts from effluent exposure.

Overall, there was not much accordance between microbiome responses from municipal wastewater exposure in the ACWA streams and in the Bow River. This may be due to several factors including variability in flow rates, water depth, water or sediment chemistry, and overall biogeography between the streams and the Bow River, which are major contributors to structuring species communities including species richness, composition, and biomass. For example, Baetidae was the only family of Ephemeroptera found at the second riffle in two of three ACWA streams (larvae were not found in the reference stream), whereas the Bow River was dominated by Heptageniidae at all sampling sites, suggesting a difference in their habitat conditions. As microbial assemblages reflect host taxonomy, diet, and geography (Ayayee et al., 2022), it may not be surprising that the microbiomes between these two systems differ. As there were no continuous water quality loggers or consistent nutrient sampling in the ACWA streams, it presents a challenge to compare the conditions observed in each watershed in attempt to parse out their individual contribution to the shifts in microbiomes. Continuous monitoring could be done in the future to build more confidence when making comparisons between stream systems.

Besides probable hydrological factors shaping the riverine environment, the Bow River has other sources of contamination draining into the river, including agriculture, livestock management, and stormwater runoff (Alberta Government, 2014). The shifts observed in the invertebrate microbiomes at Policeman Flats could in part be related to these other sources of contamination that were not individually evaluated in our study. Invertebrates from the ACWA streams were only exposed to effluents from the Pine Creek WWTP (besides background levels

in the reference water) which likely differs from Bonnybrook and Fish Creek WWTPs in terms of their bacteria composition in the effluents based on their influents and treatment processes (Kim et al., 2019). Future studies should consider evaluating the microbial compositions of the effluents themselves from each of the three WWTPs to determine how much influence each of these plants are having on downstream ecosystems.

Appendix C: Bow River and ACWA Nutrient Excretion Analysis Supplementary Materials

Methods

Field Methods

Nutrient excretion experiments were conducted at 4/5 Bow River sites (excluding Cushing Bridge) in July 2022, and at Cochrane, Sunalta, Policeman Flats and all three ACWA streams (details about ACWA stream design in Appendix B) in September 2022. Larval caddisfly belonging to the family Hydropsychidae were used as the representative test subject for these experiments as they were the most abundant macroinvertebrate across all sites, they are ubiquitous across North America, and they are commonly used as study organisms for environmental monitoring (Cain et al., 1992). To begin the experiments, up to 30 larvae were collected via rock flipping and held in an ice cube tray. Individuals were patted dry with a Kim Wipe to decrease nutrients and bacteria on their exterior and placed into a Falcon tube containing 8 mL of 0.2 μm (Nalgene™ 175Filter, Thermo Scientific, USA) pre-filtered water obtained from a site near Angel's Café (51.064451, -114.153344) in Calgary, AB with minimal nutrient input. This filtered water was used across all experiments to maintain a consistent background concentration of nutrients. Three individuals were placed into each Falcon tube with tweezers and the start time was recorded. Once all hydropsychids were placed in the Falcon tubes, they were held in a tube rack that was ¾ submerged in the river to maintain a constant incubation temperature (temperature range across sites: 11.0 °C - 14.7 °C at 15 – 20 cm depth). Organisms were left to excrete for ~1 hour (mean ± SD: 66 ± 7 min) before being transferred to individual microcentrifuge tubes and stored on ice for subsequent freezing and dry mass determinations. The solid excreta left in the water was carefully removed with a plastic pipette and the water

samples were stored at -20°C until nutrient analysis could be completed. The number of replicates of analyzed water and blank samples are described in Table C.1.

Laboratory analysis

Excretion water samples (total n = 87) and blanks (n = 10) were shipped on dry ice to Baylor University (Waco, TX) to be analyzed at the CRASR facility for nitrogen (NH_3-N) and phosphorus (PO_4-P). Once arrived, samples were thawed and filtered (0.45 µm Whatman Filter, Cytiva, USA) to remove any remaining feces prior to analysis. Ammonia was determined using the phenolate method (US EPA, 1993a) and phosphorus was determined using the molybdate colorimetric method (US EPA, 1993b) on a Lachat QuikChem 8500 flow-injection autoanalyzer. Method detection limits (MDLs) were calculated following US EPA methods with two replicates of seven spiked samples and resulted in 1.34 µg/L and 1.58 µg/L for NH_3-N and PO_4-P, respectively. All quality assurance/quality control values are reported in mean ± SD. Blanks were below the MDL in all cases, -0.33 ± 0.65 µg/L for PO_4-P, -1.43 ± 1.05 µg/L for NH_3-N. Duplicates had a relative percent difference within 4.07 ± 7.31% for PO_4-P (n = 11) and 4.13 ± 3.65% for NH_3-N (n = 9). Standards ranging in concentration from 5-500 µg/L (n = 12) were run for both nutrients and had a percent recovery within 101 ± 2.06% for phosphorus and 102.3 ± 4.25% for nitrogen. Mean percent recovery of spiked samples was within 104.1 ± 37% for PO_4-P (n = 12) and 83.5 ± 57.7% for NH_3-N (n = 12).

Statistical analysis

The mean background nitrogen and phosphorus concentrations from the pre-filtered blank water were subtracted from the total nutrient concentrations to determine the invertebrate excreted NH_3-N and PO_4-P concentrations. The excreted concentrations were then divided by the time elapsed and the sum of Hydropsychidae dry mass that was inside the tube during the experiment to obtain a rate of nutrient excretion measured in µg/L/mg/minute. For statistical analysis, values that were below the MDL were given a value of ½ the MDL (0.67 µg/L for nitrogen, n = 10, and 0.79 µg/L for phosphorus, n = 3). The excretion rates of each nutrient were modelled using one-way ANOVAs across sites of the Bow River and ACWA streams, analyzed separately. The mass normalized excretion rates of nitrogen and phosphorus were log transformed to meet the assumption of normality and Tukey's post hoc tests were used where applicable. There were 14 samples removed from the statistical analysis of nitrogen excretion (final samples sizes/site in Table C.1) due to negative excretion rates (i.e., the blank filtered water sample had a higher background concentration of ammonia than the test samples). This may have been due to contamination, a filtering error (failing to remove all biological activity in the water), or introduction of nutrient assimilating bacteria from the larvae (picked up through the environment) in the experimental tube.

Table C.1. Final nutrient excretion water sample sizes from the Bow River sites and ACWA streams after removing data with negative excretion rates.

Invertebrate taxon	Site	Replicates PO_4-P	Replicates NH_3-N
Larval Hydropsychidae	Cochrane	20	20
	Sunalta	14	8
	Graves Bridge	5	5
	Policeman Flats	18	10

REF	10	9
EFF5	10	9
EFF15	10	10

Results

Larval Hydropsychidae nutrient excretion rates in the Bow River did not seem to be influenced by chronic wastewater effluent exposure. The mean (± SD) phosphorus excretion rate ranged from 0.027 ± 0.018 µg/L/mg/min at Policeman Flats (n = 18) to 0.223 ± 0.236 µg/L/mg/min at Sunalta (n = 14) and the mean (± SD) nitrogen excretion rate ranged from 0.0043 ± 0.022 µg/L/mg/min at Sunalta (n = 14) to 0.185 ± 0.31 µg/L/mg /min at Cochrane (n = 20). There were a few significant differences in phosphorus and nitrogen excretion rates across Bow River sites and ACWA streams (Figure C.1). Hydropsychidae from Policeman Flats had a significantly lower mean phosphorus excretion rate than all other Bow River sites (ANOVA: $F_{3,53} = 12.3$, $p < 0.001$) and individuals from Sunalta had a significantly lower mean nitrogen excretion rate compared to Cochrane (Tukey HSD: diff. = -1.88, p = 0.0016). There were no differences in mean phosphorus excretion rates between any of the ACWA streams, but there was a significantly lower mean nitrogen excretion rate from individuals in EFF15 compared to EFF5 (Tukey HSD: diff. = -1.00, p = 0.037).

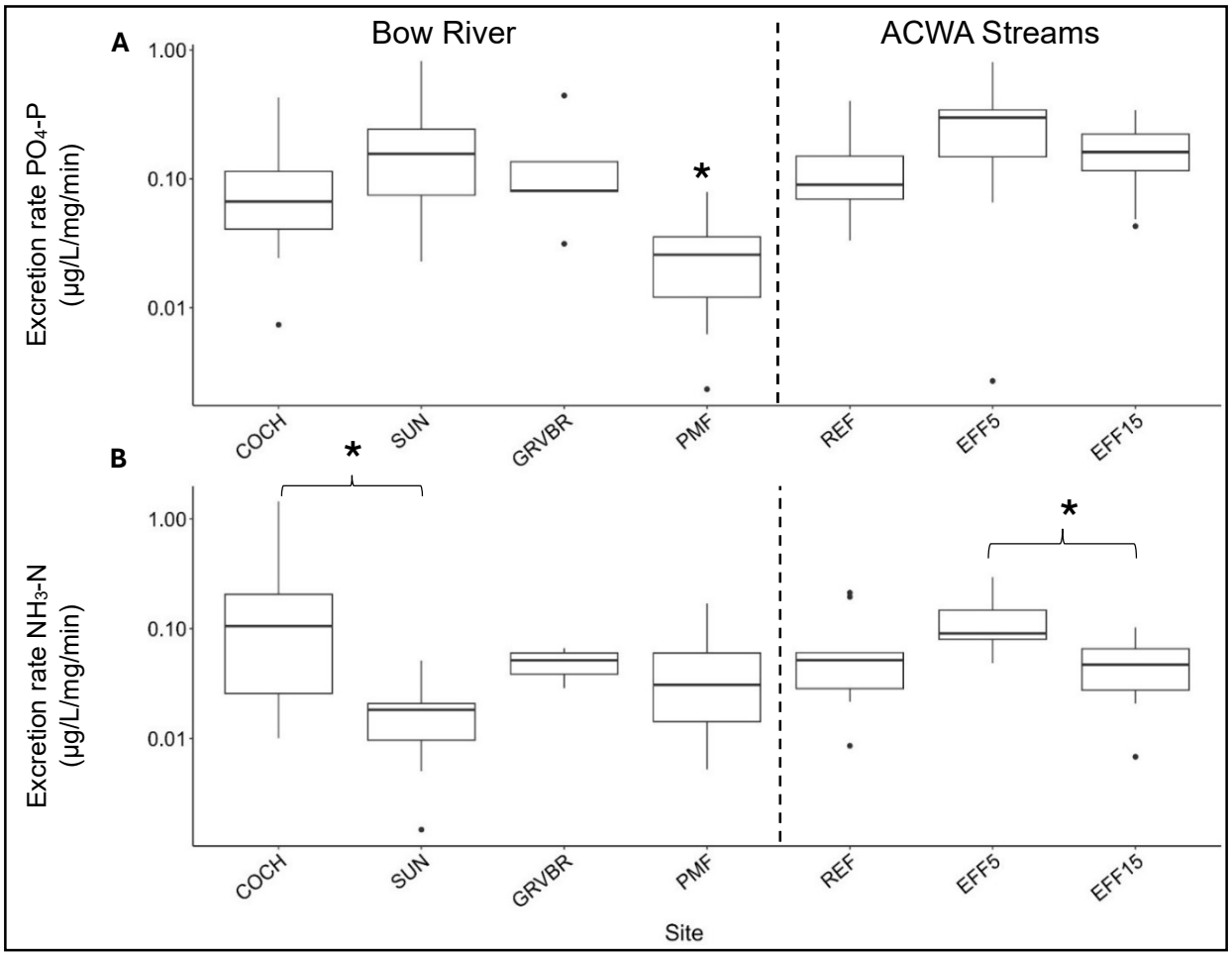

Figure C.1. Nutrient excretion rate (µg/L/mg hydropsychid/minute) of A) PO_4-P and B) NH_3-N across Bow River and ACWA stream sites. GRVBR and PMF are located downstream of wastewater outfalls in the Bow River and ACWA streams are arranged in order of increasing effluent contributions.

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