THE EFFECTS OF WASTEWATER TREATMENT PLANT EFFLUENT ON THE GUT MICROBIOME OF AQUATIC AND RIPARIAN INVERTEBRATES IN THE GRAND RIVER, ON

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By ELISE MILLAR

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Supervisor:	Karen Kidd, Ph.D., Dept of Biology and School of Earth, Environment, and Society at McMaster University, and Canadian Rivers Institute
Examining Committee:	Patricia Gillis, Ph.D., Aquatic Contaminants Research Division, Environment and Climate Change Canada
	Michael Surette, Ph.D., Dept of Biochemistry and Biomedical Sciences, McMaster University

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TITLE: The Effects of Wastewater Treatment Plant Effluent on the Gut Microbiome of Aquatic and Riparian Invertebrates in the Grand River, ON

AUTHOR: Elise Millar, B.Sc. (Trent University)

SUPERVISOR: Professor K.A. Kidd

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ABSTRACT

The composition of gut microbes affects host weight, immune function, and disease status, and is sensitive to diet, environment, and pharmaceutical exposure. The gut microbiome modulates the toxicity and bioavailability of chemical stressors, however the effects of chemicals on the gut microbiome of aquatic biota are largely unknown. The Waterloo and Kitchener wastewater treatment plants (WWTPs) release effluents containing antibiotics, pharmaceuticals, and other contaminants into the Grand River (ON) that may negatively affect the gut microbiome of downstream organisms. In this study done in Fall 2018, I collected freshwater mussels (*Lasmigona costata*), several species of insect larvae, and riparian spiders (Tetragnathidae) from sites upstream and downstream of these WWTPs. The gut microbiome was analyzed following the extraction, PCR amplification, and sequencing of bacterial DNA using the V3-V4 hypervariable regions of the 16S rRNA genetic barcode. Changes in the relative abundance of major gut microbiome phyla were observed in all targeted aquatic organisms downstream of WWTPs except Hydropsychidae. Shannon alpha diversity, a measure of bacterial abundance and evenness, differed significantly among sites for mussels (one-way ANOVA: $F=7.894$, $p=0.001$), spiders ($F=4.788$, $p=0.01$), Perlidae ($F=3.1$, $p=0.0056$), Hydropsychidae ($F=3.674$, $p=0.0014$), and Heptageniidae ($F=2.715$, $p=0.0143$), but not for Baetidae and Ephemerellidae. In sites downstream of the Waterloo WWTP, alpha diversity decreased in spiders, while in sites downstream of the Kitchener WWTP diversity decreased in mussels and Perlidae, while increasing for spiders. Bray-Curtis beta diversity, a measure of dissimilarity between bacterial communities, was significantly dissimilar among sites in all invertebrate taxa (Permanova: $p<0.02$). Upstream sites differed from downstream Waterloo sites in spiders, Perlidae, and Hydropsychidae (Adonis pairwise: $p<0.05$), while upstream mussels, spiders, Perlidae, and Hydropsychidae differed from downstream Kitchener sites ($p<0.05$). Additionally, effluent-derived bacteria were found in the microbiomes of aquatic invertebrates downstream of the WWTPs and not upstream. Taxa was also a significant driver of

bacterial composition and diversity in invertebrates. These results indicate that the gut microbiome of downstream organisms differed from the bacterial composition observed in the same invertebrate taxa upstream of the WWTPs, potentially leading to altered host health. This adds to our understanding of how chemical stressors impact the gut microbiome of aquatic and riparian biota; however, future studies are needed to investigate linkages between the gut microbiome and health of these species.

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Table of Contents

List of Tables

List of Figures

The effects of wastewater treatment plant effluent on the gut microbiome of aquatic and riparian macroinvertebrates in the Grand River, ON

1. Introduction

1.1. General Introduction to the Gut Microbiome

The term microbiome describes the collection of microbes - bacteria, archaea, fungi, protozoa, and viruses - that live on and inside an organism (e.g. skin, gut, lung), or within an environmental niche (e.g. water, soil) (SETAC, 2020; Jandhyala et al., 2015; Thursby & Juge, 2017). It can also be described as the collective genome or genetic material of these microorganisms (Prakash et al., 2011). The host-associated microbiome is an ecological community of commensal and pathogenic microorganisms, the former of which are crucial for maintaining an organism's homeostasis (Prakash et al., 2011). In addition to the host's indigenous microbiome, transient microbes from dietary sources, e.g., may also be present.

Microbiome communities are crucial for the immunologic, hormonal, and metabolic homeostasis of their host (Turnbaugh et al., 2007). The gut microbiome, specifically, is the totality of microorganisms and their genetic material within the gastrointestinal tract (GIT). Most bacteria within the gut are commensal symbionts that live in harmony with the host. The role of gut bacteria has received increasing attention over the last decade, as a balanced and diverse gut microbial community is linked to improved overall health. The gut microbiome benefits the host in many ways, such as harvesting energy from otherwise inaccessible nutrients, metabolizing xenobiotics, protecting the host against pathogens, and supporting host immune function and gut integrity (Jandhyala et al., 2015; Thursby & Juge, 2017; Turnbaugh et al., 2007; Zoetendal et al., 2006). Most gut microbes use dietary carbohydrates to produce vitamins (Kho & Lal, 2018; Thursby & Juge, 2017) and metabolites such as short-chain fatty acids,

acetate, butyrate, propionate, as well as choline and bile metabolites (Jandhyala et al., 2015; Kho & Lal, 2018) that can provide energy for the host, regulate metabolic pathways such as lipid and glucose metabolism, play a role in gut immunity, inflammatory responses, signaling pathways, prevention of oxidative stress, and provide antimicrobial effects to fight off pathogens (Kho & Lal, 2018; Thursby & Juge, 2017). The latter occurs via the competition of shared niches and nutrients within the gut, as well as controlling host defense mechanisms (receptor signaling, antimicrobial peptides, immune cells) (Kho & Lal, 2018). Despite the abundance of bacteria in the GIT, there are a limited number of biochemical niches in the gut and this suggests a high degree of functional redundancy (Thursby & Juge, 2017; Turnbaugh et al., 2007).

Diet is considered an important determinant for the composition and diversity of the gut microbiome (Jandhyala et al., 2015). Organisms with a fibre-rich diet, such as fruits and vegetables, tend to have more rich and diverse gut microbiome communities. This diet requires bacteria capable of metabolising insoluble carbohydrates, such as the phylum Firmicutes (Walker et al., 2011). Animal-based diets on the other hand have shown decreases in Firmicutes and increases in bile-tolerant species of Bacteroidetes and Proteobacteria (Jandhyala et al., 2015).

1.2. Gut Microbiome of Aquatic Invertebrates and Riparian Spiders

1.2.1. Aquatic Macroinvertebrates

Aquatic benthic macroinvertebrates play essential roles in our ecosystems such as organic matter degradation, nutrient cycling, and the accumulation of nutrients for use by higher trophic levels, such as fish and birds (Ayayee et al., 2018). These invertebrates consist of the insects, crustaceans, and bivalves inhabiting the depths of rivers and lakes. Aquatic macroinvertebrates are commonly used in aquatic biomonitoring to assess water quality and are useful bioindicators of aquatic pollution. Taxa vary in their tolerance to environmental contaminants therefore, their presence or absence can indicate

anthropogenically impacted sites (Burdon et al., 2019; Holt and Miller, 2010). Invertebrate and microbial communities adapt quickly to changes in their environment (Dillon & Dillon, 2004; Pavlov & Ehrenberg, 2013). Therefore, the composition and diversity of the gut microbiome of macroinvertebrates may also be a useful biomarker of aquatic pollution.

Most studies involving the gut bacteria of invertebrates have focused on terrestrial insects (Jones et al., 2013; Zhu et al., 2018), with few having investigated the gut microbiomes of freshwater macroinvertebrates (Ayayee et al., 2018; Kroetsch et al. submitted). For those on freshwater species, the most common phyla found within the GIT include Proteobacteria, Bacteroidetes, Firmicutes, Tenericutes, and Actinobacteria (Ding et al., 2017; Ooi et al., 2017; Shoemaker & Moisander, 2017; Wang et al., 2011; Kroetsch et al. submitted). Kroestch et al. (submitted) also found that the gut microbiome of aquatic macroinvertebrates differed by taxa and sample year within the same habitat. Some aquatic insects have displayed a stable microbiome as 1-week-old adults with low susceptibility to colonization by other bacteria (Luxananil et al., 2001; McEwen & Leff, 2001). In addition, it is thought that insects with simple, straight digestive tracts, similar to that of omnivorous humans, possess less diverse microbial communities, while insects with complex digestive anatomy such as paunches, diverticula, and caeca possess various mutualistic microbes (Dillon & Dillon, 2004).

The diet of invertebrates, and the functional feeding group that they fall into, are thought to have substantial effects on their gut microbial communities (Tiede et al., 2017). Plant material is often low in essential amino acids, nitrogen, sterols, and B vitamins. Herbivorous insects rely on microorganisms, such as bacterial endosymbionts, to synthesize these dietary requirements (Dillon & Dillon, 2004). For example, herbivorous termites possess an enlarged paunch where acetogenic and methanogenic microbes, such as the phylum Spirochetes, provide their host with carbon, nitrogen, and energy via acetogenesis and nitrogen fixation (Dillon & Dillon, 2004). Additionally, the

pH within the herbivore gut, as well as secondary plant compounds, have antimicrobial qualities that select in favour of bacteria capable of detoxifying these compounds. For example, exposure to plant tannins via the herbivorous diet of the aquatic larval herbivore *Acentria ephemerella* shapes their gut bacterial composition (Dillon & Dillon, 2004). Other studies have found that crickets fed with a chow versus protein-based diet resulted in changes to the hindgut microbial composition and reduction in hydrogen and carbon dioxide production in the latter (Dillon & Dillon, 2004). Meanwhile, mosquitos fed a sugar versus blood-based diet showed decreased diversity and favoured enteric bacteria in the latter that are able to cope with oxidative and nitrosative stresses from blood catabolism, indicating a beneficial role of gut bacteria in redox homeostasis (Wang et al., 2011).

1.2.2. Mussels

Bivalves are an especially important group of benthic macroinvertebrates, as they play a substantial role in the movement of nutrients from pelagic to benthic zones. Mussels filter large amounts of phytoplankton, increasing primary production and nutrient cycling in water bodies via their waste, and as a result, indirectly impact the terrestrial ecosystem via increased insect emergence (Allen et al., 2012; Asmus & Asmus, 1991; Cadée & Hegeman, 2002). Freshwater mussels also provide an essential ecosystem service by filtering out large quantities of harmful algae and bacteria, as well as by accumulating heavy metals. They are commonly used to monitor the impact of anthropogenic activities on water quality due to their ability to accumulate contaminants (Craft et al., 2010). Despite their importance for healthy aquatic ecosystems, freshwater mussels are some of the most imperiled organisms in North America due to competition from invasive species, anthropogenic changes to hydrology, and contaminant exposure (Strayer et al., 2004). Freshwater mussels may therefore be useful tools for investigating the effects of contaminants on the gut microbiome of aquatic organisms.

There have been some studies on the gut microbiome of bivalves. Within freshwater bivalve species, common gut bacteria include Proteobacteria, Firmicutes, Bacteroidetes, Actinobacteria, Planctomycetes, Verrucomicrobia, and Fusobacteria (Aceves et al., 2018; King et al., 2012; Weingarten et al., 2019). The digestive gland microbiome of mussels tends to be more diverse and abundant than those of other areas, such as the gills and haemolymph (Vezzulli et al., 2018). Mussels also have distinct gut microbial communities from those of seston and the surrounding water column (Weingarten et al., 2019). This is likely because freshwater bivalves pull water into their inhalant siphon and over their mantle cavity where cilia and mucous sort its contents, with desirable particles, including bacteria, sent to the gut for digestion and others excreted via pseudofeces (Winters et al., 2011). Mussels also have a capacity to accumulate human pathogenic microbes from polluted water bodies (Burkhardt & Calci, 2000; Rippey, 1994). Bacterial communities within bivalves impact nutrient dynamics in coastal marine environments, as these bacteria are equipped with diverse enzymes that contribute to nitrogen cycling, such as ammonium assimilation, nitrate and nitrite ammonification, denitrification, and nitric oxide synthesis (Pfister, 2007). As filter feeders, mussel gut communities may be especially sensitive to environmental changes; they are constantly exposed to new microbes from their environment through both waterborne and sediment contaminants in their diet and habitat (Weingarten et al., 2019).

1.2.3. Riparian Spiders

Spiders of the family Tetragnathidae are riparian and obligate consumers of aquatic insects, receiving nearly 100% of their diet from aquatic sources (Allen and Wesner, 2012; Sanzone et al., 2017). These spiders spin horizontal webs over water to catch emerging aquatic insects, providing an ecological link between terrestrial and aquatic ecosystems. Tetragnathid spiders are therefore exposed to waterborne contaminants through their aquatic diet (Richmond et al., 2018). Walters et al. (2008) found polychlorobiphenyls (PCBs) in spiders consuming insects that have emerged from

a contaminated aquatic environment. More recently, Richmond et al. (2018) found pharmaceuticals from municipal wastewater outfalls in riparian spiders due to this same aquatic-terrestrial transfer. The microbiome of spiders may therefore be a useful indicator of wastewater effluent exposure in riparian and terrestrial invertebrates.

Spiders have been shown to contain an abundance of bacteria known as endosymbionts, which live symbiotically within the spider and require their host to survive (Hu et al., 2019). In general, microbial symbionts play critical roles in shaping the evolution of insects and their ecological interactions (Hammer et al., 2015), including sexual selection to further the transmission of endosymbiont bacteria to offspring (Lewis & Lizé, 2015). However, most functions of these bacteria are still unknown (Goodacre et al., 2006; Vanthournout & Hendrickx, 2015; Vanthournout & Swaegers, 2011; Zhang et al., 2018).

Most studies on spider-associated bacteria have focused on the dominant endosymbiont bacteria and little is known about their broader microbiomes. The bacterial communities of spiders, including in the gut, are dominated by Proteobacteria, with smaller proportions of Bacteroidetes, Tenericutes, and Actinobacteria (Hu et al., 2019; Zhang et al., 2017). Predatory invertebrates, such as riparian spiders, are additionally exposed to the extensive microbial communities on and within prey (Dillon & Dillon, 2004). Spiders have a unique feeding style in that they perform extra-oral digestion by expelling digestive fluid onto their prey and then suction the liquefied contents into their stomach (Zibaee et al., 2012). Only two studies have investigated the gut microbiome of spiders to date. Hu et al. (2019) found insect-associated bacteria in the hindgut of spiders, with high abundances of bacteria involved in amino acid, carbohydrate, and energy metabolism; meanwhile, the presence of endosymbionts may indicate a role in digestive function and immunity, or that the gut epithelium is a route of endosymbiont infection (Hu et al., 2019). Zhang et al. (2017) also found bacteria commonly found in the gut of insects in whole-body *Marpiss magister* spiders. Hu et al. (2019) suggest that the spider

gut microbiome is relatively stable, however a study of the microbial communities within spider excreta found very little bacterial growth compared to other areas of the spider body (Rivera et al., 2017). It is thought that spiders may contain antimicrobials in their venom, digestive fluid, and other body fluids leading to a lack of microbes in the excreta.

1.3. Disruptions of the Gut Microbiome

Disruptions to the gut microbiome can result in dysbiosis, an imbalance of healthy gut bacteria. When the gut microbiome is disturbed, declines in dominant commensal microbes occur, reducing the competition for resources in the gut and allowing for the invasion of pathogens (Kho & Lal, 2018). When the gut mucosal barrier is disrupted, the gut becomes permeable to commensal microbes and their metabolites which causes systemic inflammation associated with chronic diseases in mammalian hosts. Dysbiosis of gut bacteria in humans has been linked to an increased susceptibility to luminal diseases (inflammatory bowel disease, Crohn's disease, colorectal cancer), metabolic diseases (diabetes, obesity), and neurodevelopmental illnesses (Alzheimer's disease, autism spectrum disorder, depression) (Hawrelak & Myers, 2004; Jandhyala et al., 2015; Kho & Lal, 2018). In general, a lack of Proteobacteria and an abundance of the genera *Prevotella* (Bacteroidetes), *Bacteroides* (Bacteroidetes), and *Ruminococcus* (Firmicutes) have been associated with a healthy gut microbiome in humans (Jandhyala et al., 2015). In aquatic organisms, however, bacterial indicators of gut dysbiosis are unknown.

The composition of the gut microbiome can be influenced by factors such as age, diet, environmental stressors, and pharmaceutical use. Environmental stressors, such as habitat fragmentation, have resulted in less diverse microbiomes in wild animals (Bahrndorff et al., 2016). The gut microbiome is known to play a role in the tolerance of the host to environmental perturbations, which could have important implications in the field of conservation biology, especially for species with critical ecological functions (Bahrndorff et al., 2016). Studies now indicate that microbiome-host relationships can be modulated by chemical exposures (Jin et al., 2017). Early life exposure of organisms to

pollutants is thought to impact their gut bacterial composition into adulthood (Claus et al., 2016). Pollutant-induced alterations of the gut bacteria are also likely to contribute to the toxicity of these chemicals (Claus et al., 2016). Environmental chemicals have been linked to various health disorders. In humans, gut microbiome toxicity and resulting diseases are associated with changes in microbial metabolites, a loss of microbial diversity, and disruption of energy metabolism by microbes (Tu et al., 2020). Antibiotic exposure can reduce the competitive exclusion capabilities of healthy gut microbes, causing a decrease in bacterial diversity and richness within the gut, reducing the ability of the gut microbiome to fight off pathogens (Jandhyala et al., 2015; Thursby & Juge, 2017). Environmental pollutants such as methylmercury, chlorpyrifos, triclosan, artificial sweeteners, and phthalates have been shown to impact the composition of the gut microbiota (SETAC, 2020; Claus et al., 2016). In aquatic invertebrates, the herbicide glyphosate has caused dysbiosis in the Chinese mitten crab (Yang et al., 2019). However, an Eastern Mediterranean oil spill did not affect oyster-associated bacteria (Kassaify et al., 2009).

The microbiome can mediate the biotransformation of a variety of chemicals; therefore, these microbial communities may provide protection or influence toxicant properties, including dose and availability (SETAC, 2020; Adamovsky et al., 2018). The gut microbiome is equipped with a broad suite of enzymes, including those capable of metabolizing various environmental chemicals, which may increase or decrease their toxicity to their host (Claus et al., 2016). A variety of environmental pollutants such as polycyclic aromatic hydrocarbons (PAHs), pesticides, PCBs, metals, and azo dyes are metabolised by gut bacteria (Claus et al., 2016). These contaminants may be poorly absorbed by the gut post-ingestion, reaching the abundant bacteria towards the end of the GIT, and subsequently metabolised (Claus et al., 2016). These deconjugated and reduced metabolites are then easily reabsorbed by the gut, delaying the elimination of chemicals (Claus et al., 2016). Gut microbiota play a major role in xenobiotic and drug metabolism, affecting the efficacy of therapies for various diseases. Acetaminophen metabolism by the

liver is affected by gut microbial metabolites in humans (Clayton et al., 2009). The cardiac glycoside Digoxin is inactivated by a species of Actinobacteria, *Eggerthella lenta* (Saha et al., 1983). The anticancer drug, Irinotecan, can be deconjugated by microbial enzymes, contributing to its toxicities such as inflammation, diarrhea, and anorexia in humans (Wallace et al., 2011). Cleary et al. (2015) found microbe-associated metabolism of xenobiotics in aquatic invertebrates, as mussels near an urbanized area had an enrichment of bacterial xenobiotic degradation pathways as evidenced by functional pathway analyses.

For organisms in a highly contaminated environment, these changes in microbial structure and metabolism can have long term consequences on overall organismal health. Therefore, the gut microbiome may be a useful tool for monitoring the health of aquatic organisms in polluted environments. The composition of the microbiome may also serve as an important bioindicator for exposures to contaminants, acting as a microbial fingerprint (Adamovsky et al., 2018). There is also the opportunity to identify particular bacterial species that correlate with adverse conditions, such as a contaminated ecosystem, and using them as a biomarker or diagnostic tool (SETAC, 2020).

1.4. Wastewater and The Grand River Watershed

Municipal wastewater treatment plants (WWTP) are major contributors to aquatic ecosystem pollution, exposing aquatic organisms to diverse contaminants and causing biotic impairments such as decreased diversity and population size (Brown et al., 2011; Collins & Russell, 2009; Kidd et al., 2007; Gillis et al., 2017a). These effluents are typically complex mixtures of pharmaceuticals and personal care products (PPCPs) and other contaminants from domestic, municipal, and industrial sources from the population in which they serve (Holeton et al., 2011; Metcalfe et al., 2010; Servos et al., 2005). WWTP effluents add nutrients (phosphorus and nitrogen) and organic materials to surface waters, which can result in eutrophication and decreased oxygen during decomposition. Antibiotics and antimicrobials are common PPCPs found in municipal wastewaters and

they are continuously released into the aquatic environment with little understanding of their impacts on the natural bacterial communities of aquatic biota. There is also a major concern that these products contribute to the evolution of antibiotic-resistant strains of bacteria in the environment (Evariste et al., 2019).

There are three main levels of wastewater treatment used in Canada: primary, secondary, and advanced (or tertiary). Primary wastewater treatment involves using screens to remove large solids then holding the influent in a settling tank (or clarifier) where light (scum) and heavy (sludge) solids separate and can be removed (SDWF, 2017; Government of Canada, 2017). Secondary treatment is used to degrade the biological content of wastewater through processes such as biofiltration, aeration, or oxidation ponds. This step uses bacteria and oxygen to further digest the pollutants (SDWF, 2017; Government of Canada, 2017). Biofiltration uses sand, contact, or trickling filters to remove sediment from the wastewater. Aeration involves exposing wastewater to air to increase oxygen saturation and is usually a lengthy process. Oxidation ponds involve the use of lagoons where wastewater is retained for a set period. These processes allow for the reduction of common biodegradable contaminants such as organics (e.g. sugars, fats, food waste, soaps, detergent) to safe levels. Before discharge, the water is further disinfected with chlorine, ozone, or ultraviolet light, depending on the treatment plant. Additionally, tertiary (or advanced) treatment processes are used to further raise water quality for discharge into the environment based on Provincial Water Quality Standards. Dissolved substances such as dyes, metals, organics, and nutrients are removed (SDWF, 2017; Government of Canada, 2017). This step can include several physical, chemical, and biological treatment processes. One example is Biological Nutrient Removal where bacteria are used in bioreactors to remove nitrogen and phosphorus from water. Tertiary treatment can also involve the removal of pathogens. However, despite advanced treatment, several contaminants, both biotic and chemical are still found in low concentrations in aquatic environments that receive municipal wastewater.

The Grand River watershed in southern Ontario is the largest watershed that drains into Lake Erie (6965 km^2) and is surrounded by a population of close to 1 million people. The river receives effluent from 30 municipal WWTP outfalls as well as numerous agricultural inputs (Sonthiphand et al., 2013; Tanna et al., 2013). The central section of the Grand River includes two major WWTPs: Waterloo and Kitchener. The Waterloo WWTP serves a population of 139,527 people, while the Kitchener WWTP serves 242,626 people (Region of Waterloo, 2018). The Waterloo WWTP currently operates at partially nitrifying secondary treatment using conventional activated sludge and upgrades are underway (Region of Waterloo, 2018; Hicks et al., 2017). Major upgrades, such as the conversion from carbonaceous to nitrifying activated sludge, have been implemented at the Kitchener WWTP in the last eight years (since 2012) to improve overall treatment efficiency and effluent quality (Region of Waterloo, 2018; Hicks et al., 2017). Nitrification consists of two biological processes, ammonia and nitrite oxidation, and are performed by aerobic and anaerobic ammonia-oxidizing bacteria (Sonthiphand et al., 2013). Mussels collected in the urban area of this river prior to upgrades have shown lower condition factors, reduced mean age, and increased metal concentrations in their gills, as well as signs of increased immune activity compared to those collected upstream (Gillis, 2012). A complete loss of the mussel population had also been observed downstream from the Kitchener WWTP (Gillis et al., 2017b). Prior to the Kitchener WWTP upgrades, darter fish species had increased male intersex (testis-ova) occurring downstream of both WWTPs, resulting in delayed reproductive maturation, as well as lower sperm density and quality (Tanna et al., 2013; Tetreault et al., 2011). Since the upgrades, intersex rates have decreased as a result of reduced estrogenicity in waters downstream (Hicks et al., 2017). There were also changes in $\delta^{15}N$ values of organisms downstream of the Kitchener WWTP post upgrade to values that were more similar to those found in upstream benthic invertebrates and fish, suggesting improved nitrogen treatment (Hicks et al., 2017). In addition to the large Waterloo and Kitchener WWTPs, this section of the Grand River also receives inputs from the Conestogo River (St Jacobs WWTP), Canagagigue Creek (Elmira WWTP), and Speed River (Hespeler and Guelph

WWTPs) (Region of Waterloo, 2018). There are also other smaller WWTPs in this area that discharge directly into the Grand River, including the Fergus, Elora, Conestogo, Preston, and Galt WWTPs (Anderson, 2012; Region of Waterloo, 2018).

In addition to chemical inputs from WWTPs, there are also changes in downstream microbial communities associated with wastewater effluents. As examples, higher abundances of ammonia-oxidizing bacteria have been found in the sediment and water column downstream of the Waterloo WWTP outfall (Sonthiphand et al., 2013), and the nitrifying bacteria in an urbanized river in Paris, France, have been linked to wastewater effluents (Brion & Billen, 2000). Wastewater effluent reduces the abundance and diversity of benthic bacterial communities in urban and suburban rivers (Drury et al., 2013). Short-term changes in richness, diversity, and composition of bacterial communities have been observed in urban rivers, as the bacteria in discharged effluents changed the natural community composition (García-Armisen et al., 2014). Wastewater may also be a source of antibiotic-resistant bacteria in natural waterways, as Iwane et al. (2001) found increasing percentages of antibiotic resistance in an urbanized river downstream.

Municipal wastewater effluents also affect the bacterial communities within fish. Restivo et al. (submitted) found changes in the diversity and composition in the gut content microbiome of rainbow darter fish downstream of the Waterloo and Kitchener WWTPs. Lobb et al. (2020) found that the necrobiomes (microbiome post-mortem) of rainbow darters downstream of the Waterloo WWTP were enriched in pathogenic bacteria associated with human infections. Therefore, changes in the composition and diversity of the gut microbiome may also be observed in aquatic and riparian invertebrates downstream of WWTP outfalls.

1.5. Study Rationale

To date, little is known about the gut microbiome of aquatic and riparian macroinvertebrates as previous research has focused on terrestrial and marine invertebrates (Dillon & Dillon, 2004; Ding et al., 2017; Ooi et al., 2017; Shoemaker & Moisander, 2017; Tiede et al., 2017; Xia et al., 2017) and, until recently, there have been technological challenges. However, evidence suggests that the microbial community in the guts of both terrestrial and aquatic invertebrates is affected by diet, taxon, habitat and time (Shoemaker & Moisander, 2017; Tiede et al., 2017; Xia et al., 2017; Kroetsch et al. submitted). Recent studies have used high-throughput sequencing methods, such as the hypervariable regions of the bacterial 16S rRNA genetic barcode, as culturing methods are typically biased and unrepresentative of the overall bacterial community (Claus et al., 2016; Jandhyala et al., 2015; Zoetendal et al., 2006). These advances will undoubtedly allow for an improved understanding of the composition of the invertebrate microbiome and its links to organism health.

Furthermore, the effects of wastewater effluent exposure on the gut microbiome of aquatic and riparian macroinvertebrates have yet to be studied, despite its ecological relevance and the growing awareness of PPCPs in municipal wastewaters. Some investigations have employed single compound exposures and these were observed to cause compositional changes in the gut microbiome of aquatic organisms (SETAC, 2020; Adamovsky et al., 2018), such as the antimicrobial triclosan commonly found in wastewaters (Evariste et al., 2019). However, to date the focus has been on lab exposures and the results are not necessarily representative of the cumulative effects that the complex chemical mixtures present in wastewater effluents can have on downstream invertebrates.

Finally, riparian species are exposed to contaminants in municipal wastewaters via their diet of emerged aquatic insects (Walters et al., 2008), but it is unknown whether their microbiomes are impacted. Studies have demonstrated the transfer of waterborne

chemicals from water bodies to terrestrial ecosystems via predator-prey interactions, however research has yet to be conducted on the transfer of bacterial communities to riparian predators via their aquatic prey and whether a similar process is observed (Kraus et al., 2014; Raikow et al., 2011; Richmond et al., 2018). Similarly, the transfer of effluent-derived bacteria from receiving water bodies into riparian and terrestrial food webs and their species is currently unknown.

1.6. Study Objectives

In this study, I determined the effects of WWTP effluent exposure on the gut microbiome of benthic macroinvertebrates, as well as on the whole-body microbiome of riparian spiders in the Grand River, Ontario, Canada. I also characterized how the gut microbiome of benthic and riparian invertebrates differs among taxa. Based on previous literature, I hypothesize that there will be a change in microbial diversity and composition in invertebrates collected downstream of wastewater treatment plant outfalls in connection to effluent exposure. I also hypothesize that taxa will be a determining factor in the composition and diversity of the gut microbiome (Tiede et al., 2017; Weingarten et al., 2019; Kroetsch et al., submitted).

This study is the first to investigate and compare the gut or whole-body microbiomes of aquatic and riparian macroinvertebrates chronically exposed to wastewater effluent in the field. This project provides insight into the bacterial communities associated with benthic and riparian macroinvertebrates in the Grand River, as well as changes in bacterial diversity and composition in relation to WWTP outfalls along the river. This information will help facilitate future studies in determining the adverse health outcomes caused by effluent-associated changes in the microbiota of aquatic and riparian macroinvertebrates.

2. Methods

2.1. Field Collection

2.1.1. Flutedshell Mussels (*Lasmigona costata*)

Five sites used by Environment and Climate Change Canada (ECCC) for freshwater research along the Grand River in the Kitchener-Waterloo area, Ontario, Canada were sampled in Fall 2018 (Figure 1). These sites have been part of a long-term mussel research project by ECCC's Gillis lab. Flutedshell mussels (*Lasmigona costata*) were sampled from Oct 9-12th and 19th, 2018, with the help of ECCC during their mussel population surveys. Mussels at the sediment water interface were collected while wading using underwater viewers (plywood boxes with plexi-glass bottoms). Mussels were transferred to a mesh holding bag secured in shallow water until collection was complete, usually 1-2 hours later. Fifteen mussels were collected from each site except for site JN that yielded only three mussels after two full search days. After collection was completed at each site, mussels were transferred to a cooler containing river water and dissected in the field, usually 30 minutes to 3 hours after being placed in the cooler.

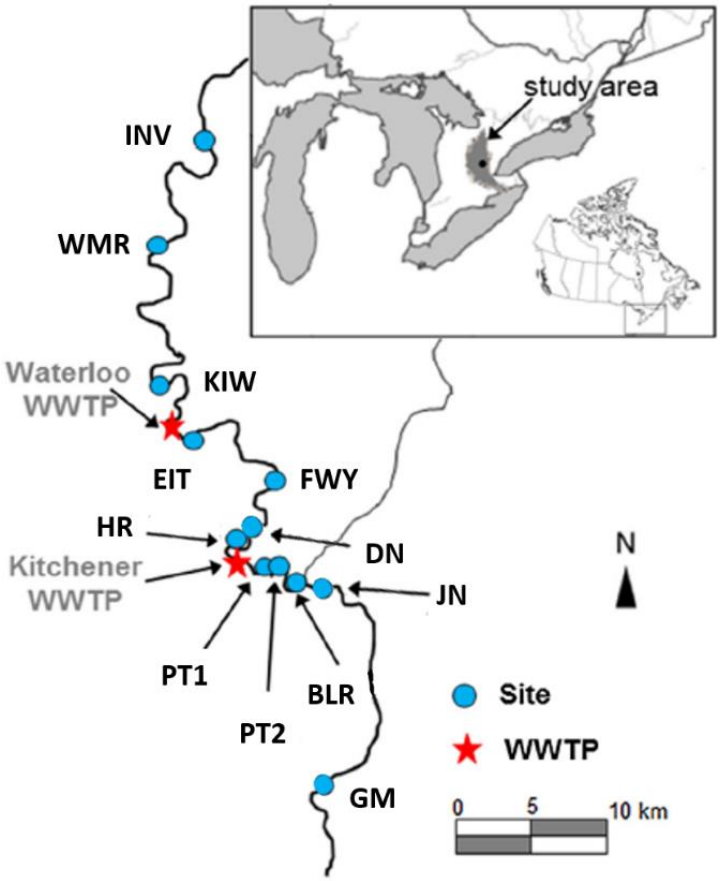

Figure 1. Map showing the locations of 12 sampling sites (blue circles) used for mussel, spider, and aquatic macroinvertebrate collections in Fall 2018. Red stars represent the locations of the Waterloo and Kitchener wastewater treatment plants. Mussels were collected from 5 sites (WMR, KIW, DN, JN, GM), while spiders and macroinvertebrates were collected from 10 sites (INV, WMR, KIW, EIT, FWY, HR, PT1, PT2, BLR, GM).

Prior to dissection, mussel shells were gently scrubbed and rinsed with double-distilled water to remove detritus, ensure accurate weighing, and to decrease any chances of contamination, then patted dry using Kimwipes (Kimtech Science™). All tools were sterilized between dissection steps by rinsing in 30% bleach (Clorox®), 70% ethanol (Commercial Alcohols, Greenfield Global), and UltraPure™ distilled water (Invitrogen), in that order. Tray surfaces were sterilized between dissections with 10% bleach and 95% ethanol. Mussels were given an identifying number and then weighed (± 0.01 g) and

measured for length (± 0.01 mm). The outer shell was wiped with 95% ethanol to decrease any chances of external contamination, and then placed on a new piece of aluminum foil. A section of the soft tissue containing the foot, gonads, and gut were removed and transferred onto a new piece of aluminum foil. The digestive gland was separated from the other tissues and the outer (connective) tissue was removed, until a marble-sized piece of digestive gland could be isolated and placed into a pre-weighed 2 mL screw top tube containing buffer (800 µl monobasic $NaPO_4$ at pH = 8, 100 µl of GES, 6 x 2.8 mm ceramic beads, 0.2 g 0.1 mm glass beads). Tubes were re-weighed to confirm the collection of 0.3-0.5 g mussel gut, then gently shaken to mix their contents. Tubes were stored on ice until they were transferred to a -20˚C freezer, typically 1-7 hours later.

2.1.2. Long-jawed Orb Weavers (Tetragnathidae) and Benthic Macroinvertebrates

Ten sites along the Grand River in the Kitchener-Waterloo area, Ontario, Canada, were used for spider and benthic invertebrate sampling in the Fall of 2018. These sites have been part of a long-term monitoring program by the Servos Lab at the University of Waterloo (Figure 1).

Long-jawed orb weaver spiders (of the family Tetragnathidae) were sampled after dark on September 27th, 30th, and Oct 3rd, 2018. From each site and using sterile nitrile gloves, fifteen spiders were collected along the shoreline from vegetation overhanging the river. Gloves were replaced in between each spider to prevent cross contamination. Spiders were placed into sterile 1.7 mL microcentrifuge tubes (Axygen®), and flash frozen in liquid nitrogen at the end of collections, usually 15-30 minutes later. The following morning spiders were transferred to a -80˚C freezer. Spider samples were sterilely transferred to 2 mL screw top tubes containing buffer (800 µl monobasic $NaPO_4$ at pH = 8, 100 µl of GES, 6 x 2.8 mm ceramic beads, 0.2 g 0.1 mm glass beads) prior to whole-body extractions of bacterial genomic DNA.

Aquatic macroinvertebrates were sampled from Oct 21st-Oct 27th at the same ten sites used for spider collections. Kick and sweep with dip nets (Wildco®) and

electroshocking were used to collect various invertebrate taxa from each site. Net contents were held in site water until they were sorted to family on site. To reduce chances of cross contamination, gloves were changed between sites and tweezers were rinsed in 30% bleach, 95% ethanol, and UltraPure™ distilled water between taxa within a site. To reduce the presence of external bacteria, invertebrates were rinsed with 95% ethanol and then placed into either 1.7 mL microcentrifuge tubes or 5 mL cryotubes depending on the size of the invertebrate. Although the presence of any remaining external bacteria was not quantified, previous studies indicate that the high bacterial biomass within the gut of invertebrates often masks that of residual environmental bacteria after surface rinsing (Hammer et al., 2015; Kroetsch et al., submitted). Tubes were then filled with 95% ethanol, labelled, and stored on ice until they were transferred to a -20˚C freezer, typically 1-7 hours later. Prior to bacterial genomic DNA extraction, invertebrate samples were rinsed with 95% ethanol and UltraPure™ water and then transferred to 2 mL screw top tubes containing buffer (800 µl monobasic NaPO4 at pH = 8, 100 µl of GES, 6 x 2.8 mm ceramic beads, 0.2 g 0.1 mm glass beads).

Invertebrates were selected for microbiome analysis to have representatives of each functional feeding group, and included: the filterer-collectors Hydropsychidae (n = 59), gatherer-collectors Baetidae (n = 32) and Ephemerellidae (n = 10) (one family was not present across all sites), predators Perlidae (n = 55), and scrapers Heptageniidae (n = 50). These individuals were chosen after DNA barcoding (see Section 2.2.1.).

2.1.3. Water Samples

In Fall 2019, water samples were collected from the same 12 sites that were used for mussel, spider, and macroinvertebrate collections in Fall 2018. Three replicates per site were collected from the left, center, and right-hand sides of the river facing upstream. Wearing sterile nitrile gloves, 50 mL falcon tubes were rinsed 3 times with river water and held upright underwater until all air bubbles had escaped. Tubes were then capped

underwater and placed on ice, typically 10-30 minutes later. Gloves were changed between samples.

2.1.3.1. Water Sample Filtration

Samples (n = 3 per site) were vacuum filtered through a sterile 0.45-micron cellulose filter paper (Whatman®, Cat. # 28297-734). The filter paper was then transferred using sterile techniques into a pre-labelled 2 mL screw top tube containing buffer (800 µl monobasic $NaPO_4$ at pH = 8, 100 µl of GES, 6 x 2.8 mm ceramic beads, 0.2 g 0.1 mm glass beads) and stored in a -20˚C freezer. Between replicates gloves were changed and all vacuum filtering equipment and forceps were rinsed using 95% ethanol and double distilled water.

2.2. Laboratory processing

2.2.1. DNA Barcoding of Aquatic Invertebrates

To identify genus and species of the invertebrates, a leg from each individual was run for DNA barcode analysis. Legs were placed into 96-well microplates, being careful not to cross-contaminate wells by rinsing forceps in 30% bleach, 95% ethanol, and UltraPure™ water between individuals. All plates were sent to the Canadian Centre for DNA Barcoding (CCDB) at the University of Guelph for analysis. A DNA extraction was performed, followed by PCR amplification using universal primers targeting the full barcode region (658 bp or similar length), followed by a PCR check (using Invitrogen E-gel® 96), bidirectional sequencing of the barcode region, sequence assembly and base calling (Ivanova et al., 2006). A single pass barcode analysis was conducted.

Purified DNA was amplified using the mitochondrial cytochrome c oxidase subunit I (COI) genetic barcode using 10% trehalose, 5U Taq, 10x buffer, 50 mM $MgCl_2$, 10 mM dNTPs, 100 µM primer stock, and 10 µM primer working solution for a single reaction. A primer cocktail of C_LepFolF (5' – ATTCAACCAATCATAAAGATATTGG – 3') and C_LepFolR (5' –

TAAACTTCTGGATGTCCAAAAAATCA – 3') was used (Hernández-Triana et al., 2014). Conditions for COI amplification included an initial denaturation at 94°C for 1 min, five cycles of 94°C for 30 sec, annealing at 45-50°C for 40 sec, and extension at 72°C for 1 min, followed by 30-35 cycles of 94°C for 30 sec, 51-54°C for 40 sec, and 72°C for 1 min, with a final extension at 72°C for 10 min, followed by an indefinite hold at 4°C (Ivanova et al., 2006). Invertebrate individuals that returned inconclusive DNA metabarcoding results were identified visually down to genus level by Dr. Paul Sibley at the University of Guelph, ON.

2.2.2. Extraction and Amplification of Bacterial Genomic DNA

Individual spider, mussel, and invertebrate samples were extracted for bacterial genomic DNA following the protocol developed by the Surette lab at McMaster University ("Isolation of DNA from Clinical Samples (Genomic Prep)").

Purified DNA was used to amplify the variable regions 3 and 4 (V3-V4) of the 16S rRNA gene using a two stage PCR approach. Initially the 8f (5' – AGAGTTTGATCCTGGCTCAG – 3') - 926r (5' – CCGTCAATTCCTTTRAGTTT – 3') region of the 16S gene was amplified using 100 ng of template with 1U of Taq, 1x buffer, 1.5 mM MgCl2, 0.4mg/mL BSA, 0.2 mM dNTPs, and 10 pmol of each primer. The reaction was carried out at 94˚C for 5 minutes, 15 cycles of 94˚C for 30 seconds, 56˚C for 30 seconds and 72˚C for 60 seconds, with a final extension of 72˚C for 10 minutes.

This reaction was then used as a template in the second stage of PCR. A volume of 3 µL from the first reaction was used with 1U of Taq, 1x buffer, 1.5 mM $MgCl_2$, 0.4 mg/mL BSA, 0.2 mM dNTPs, and 5 pmol of 341F (5' – CCTACGGGAGGCAGCAG – 3') and 806R (5' – GGACTACNVGGGTWTCTAAT – 3') Illumina adapted primers, as described in Bartram et al. (2011). The reaction was carried out at 94˚C for 5 minutes, 25 cycles of 94˚C for 30 seconds, 50˚C for 30 seconds and 72˚C for 30 seconds, with a final extension of 72˚C for 10 minutes. Resulting PCR products were visualized on a 1.5%

agarose gel. Positive amplicons were normalized using the SequalPrep normalization kit (ThermoFisher #A1051001) and sequenced with the Illumina MiSeq platform.

2.2.3. Read Processing with DADA2

Cutadapt was used to filter and trim adapter sequences and PCR primers from the raw reads with a minimum quality score of 30 and a minimum read length of 100 bp (Martin, 2011). Sequence variants were then resolved from the trimmed raw reads using DADA2 (Callahan et al., 2016). DNA sequences were filtered and trimmed based on the quality of the reads for each Illumina run separately, error rates were learned, and sequence variants were determined by DADA2. Sequence variant tables were merged to combine all information from separate Illumina runs. Bimeras (two-parent chimeras) were removed and taxonomy was assigned using the SILVA database version 1.3.2.

2.3. Data and Statistical Analyses

All statistical analyses were conducted in R (v. 3.5.2) and R Studio (v. 1.1.456) using the microbiome data analysis package, phyloseq (v. 1.26.1)(McMurdie & Holmes, 2013). The amplicon sequence variant (ASV), taxonomy, and metadata files were combined into a single phyloseq object. This object was subsidized to remove negatives and any unwanted samples present in the ASV table. The total number of assigned ASVs was determined.

Relative abundance values of each bacterial taxa were determined following the agglomeration, transformation, and pruning of the phyloseq object to remove taxa present in less than 2% of samples. The phyloseq object was then melted into a data frame and aggregated to determine the mean number of taxa, adding up to 100% and visualized using stacked bar plots. Relative abundance plots were constructed for Phylum, Class, Order, Family and Genus taxonomic ranks. Relative abundance values (%) of bacteria were calculated from the aggregated data for each site and taxon.

For each organism (mussels, spiders, and several invertebrates), the associated phyloseq object was rarified to a sequencing depth of the minimum sum of all sequence reads (1317 reads). This was followed by the statistical analysis and plotting of Shannon and Simpson alpha diversity by site. These indices were used to assess both the abundance and evenness of bacterial species present within the individual samples. Alpha diversity measures how evenly bacterial species are distributed in a sample. A one-way analysis of variance (ANOVA) test and linear model were used to examine differences among sites for both alpha diversity metrics (Shannon and Simpson). A Tukey honestly significant difference (HSD) pairwise test was used to determine which specific site pairs differed significantly from one another.

Beta diversity was assessed on non-rarified data to measure dissimilarity between the gut bacterial community composition of individual samples among sites. Beta diversity demonstrates the difference in taxonomic abundance profiles from different samples. The Bray-Curtis Dissimilarity measure calculates beta diversity using both presence/absence and abundance information of gut bacterial sequences and compares microbial community composition between individual samples. The phyloseq object was transformed to proportions, then ordinated for the principle coordinate analysis (PCoA) of site beta diversity. The transformed object was then used to calculate the Bray-Curtis distance matrix. Significant differences in beta diversity, as well as the effect size (R^2), were determined using the Adonis statistical test (similar to a Permanova), using 99999 permutations on the transformed data frame and Bray-Curtis distance matrix. A pairwise Adonis statistical test was then performed on the Bray-Curtis distance matrix to determine significantly different site pairs.

2.4. Dye Study of Mussel Gut Contents

To determine the passage of gut contents within the digestive system of the species and size of freshwater mussel employed in this study, a series of feeding

experiments with dyed food were conducted to improve dissection accuracy for future gut microbiome work.

2.4.1. Preliminary Trial

In a preliminary trial, flutedshell mussels were collected from a reference site on the Maitland River, ON (n=5) in May 2019, brought back to an ECCC lab at the Canadian Centre for Inland Waters (CCIW), and held at 11°C in dechlorinated, aerated, filtered and UV sterilized City of Burlington (ON) tap water. Freshwater mussels tend to feed on small particles between ~2-40 µm in size (Beck, 2001; Khan & Prezant, 2018; Martel et al., 2010), therefore two types of food in this size range were used for this study. *Saccharomyces cerevisiae*, commonly known as baker's yeast, which ranges from 5-10 µm in length (Duina et al., 2014) and an algal mixture commonly used to culture mussels (1:2:1 ratio of Shellfish Diet 1800®, Nanno 3600® [Instant Algae – Reed Mariculture], tap water), which ranges between 2 and 32 µm in size (Rahman et al., 2018; Throndsen & Zingone, 1997; Timmermans et al., 2001) were selected. Shellfish Diet 1800® is a concentrated microalgae mixture consisting of *Isochrysis*, *Pavlova*, *Tetraselmis*, *Chaetoceros calcitrans*, *Thalassiosira weissflogii*, and *Thalassiosira pseudonana*. Nanno 3600® contains the algae *Nannochloropsis* (Kandilian et al., 2013).

In this initial experiment, two treatments (algae and baker's yeast) and three sampling time points (30, 120, 240 minutes) were used. Yeast and algal mixtures were dyed for 20 minutes with Rose Bengal dye (Acros Organics) (Yeast Suspension: 0.4 g yeast, 10 mL freshwater, 0.2 mg Rose Bengal; Algal Suspension: 3 mL algae mixture, 3 mL freshwater, 8 mg Rose Bengal). Mussels were assigned to either the algae (n=3 at 30, 120, and 240 minutes) or yeast treatment (n=2 at 120, 240 minutes). Individual mussels were placed into beakers of 600 mL unfiltered dechlorinated water. A volume of 2.5 mL of the appropriate food source was added to each beaker at time = 0. Mussels were dissected at the specific time intervals to inspect the surfaces of the gills, labial palps, and crystalline style for the presence of dyed food particles. The digestive gland and its

contents were inspected for the presence and condition of ingested dyed food particles. The gonad was also inspected using cross sections of tissue because the lower digestive tract spans the length of the gonad tissue.

2.4.1. Laboratory Feeding Experiment

As in the preliminary experiment, flutedshell mussels (n = 25) were collected and held at CCIW. A yeast suspension was dyed for 20 minutes with Rose Bengal dye (Yeast Suspension: 1.6 g yeast, 60 mL freshwater, 12 mg Rose Bengal dye). Individual mussels were placed into beakers of 600 mL unfiltered freshwater (n = 5 mussels per treatment group) and allowed to acclimate for 20 minutes. A volume of 3 mL dyed yeast suspension was added to each beaker, mussels were then allowed to feed for either 0, 30, 60, 90, or 120 minutes prior to dissection. Tissues were examined for the presence of dye as described above for the preliminary experiment.

3. Results

3.1. Sequencing Results

A total of 13,032,136 16S rRNA V3-V4 high quality bacterial sequence reads were obtained from the freshwater mussel digestive glands and whole-body riparian spider and macroinvertebrate larvae samples (n=347), with individuals containing an average of 37,556 sequence reads and ranging from 1317 to 99,517 per sample. The reads clustered into a total of 32,443 unique bacterial amplicon sequence variants (ASVs), with a breakdown per taxa in Table 1.

Table 1. Total amplicon sequence variant (ASV) count per invertebrate taxa collected in Fall 2018 from the Grand River, ON (n=10-98/taxon).

Taxa	ASV Count
Mussels	10,934
Perlidae	8,847
Heptageniidae	7,533
Hydropsychidae	5,282
Baetidae	4,303
Spiders	3,857
Ephemerellidae	3,672

A total of 906,533 16S rRNA V3-V4 high quality bacterial sequence reads were obtained from river water and Waterloo and Kitchener WWTP effluent samples (n=42), with individual samples containing an average of 21,584 and ranging from 937 to 82,058. The reads clustered into a total of 7,740 unique bacterial amplicon sequence variants (ASVs), with a breakdown per site in Table 2.

Table 2. Total amplicon sequence variant (ASV) counts in river water and Waterloo (WAT) and Kitchener (KIT) WWTP effluent samples collected in Fall 2019 from the Grand River, ON (n=3/site). Sites are shown in Figure 1.

Taxa	ASV Count
INV	691
WMR	711
KIW	1353
WAT	1973
EIT	1663
FWY	947
DN	1005
HR	1703
KIT	262
PT1	1879
PT2	1350
BLR	1458
JN	1390
GM	1133

3.2. Bacterial Relative Abundance for All Taxa

A total of 46 bacterial phyla, 101 classes, 237 orders, 375 families, and 1113 genera were present among all macroinvertebrate samples (Table 3). Mussel samples contained the highest number of phyla, with spiders having the lowest. Spiders had the lowest number of taxa at each rank, except for Hydropsychidae at the family and genus level. Among all aquatic insect taxa, Hydropsychidae had the lowest number of bacterial taxa. Across all invertebrate taxa, 95.42% of the bacterial sequences were from only five bacterial phyla; Proteobacteria accounted for 52.59% of the detected sequences, while Bacteroidetes, Cyanobacteria, Firmicutes and Tenericutes accounted for 20.68%, 10.10%, 6.62% and 5.43%, respectively. Additional phyla in low relative abundances accounted for a combined 4.46% of the gut microbiota, while unassigned bacteria represented 0.13% of the detected bacterial sequences.

Table 3. Number of bacterial taxonomic ranks found within each taxon collected in Fall 2018 from the Grand River, ON (n=10-98/taxon).

Taxa	Phyla	Class	Order	Families	Genera
Mussels	41	85	192	303	728
Spiders	23	43	95	180	483
Perlidae	35	66	166	240	483
Heptageniidae	31	65	169	244	444
Hydropsychidae	25	50	130	177	297
Baetidae	32	57	151	233	451
Ephemerellidae	36	64	152	225	385

3.2.1. Mussels

A total of 41 bacterial phyla, 85 classes, 192 orders, 303 families, and 728 genera were present within the mussel digestive gland (Table 3). The top five phyla, classes, orders, families, and genera made up 92.54%, 83.33%, 60.39%, 30.98%, and 14.12% of the total bacterial sequences, respectively (Table 4). The number of phyla, orders, classes, families, and genera of bacteria within the gut microbiome of mussels declined downstream of the Waterloo (DN) and Kitchener (JN) WWTPs and then showed increases further downstream (GM) (Table 5).

Table 4. The top five bacterial taxa per rank along with their relative abundances within the digestive glands of freshwater mussels collected in Fall 2018 from the Grand River, ON (n=43).

Phylum	**Class**	**Order**	**Family**	**Genus**
Proteobacteria (53.05%)	Alphaproteobacteria (37.24%)	Rhizobiales (19.59%)	*Rhizobiales Incertae Sedis* (9.12%)	*Methylocystis* (7.33%)
Cyanobacteria (20.26%)	Oxyphotobacteria (22.72%)	Chloroplast (17.17%)	*Beijerinckiaceae* (8.61%)	*Mycoplasma* (2.45%)
Firmicutes (7.81%)	Gammaproteobacteria (14.46%)	Rickettsiales (11.70%)	*Mycoplasmataceae* (5.43%)	*Cyanobium PCC-6307* (1.68%)
Tenericutes (6.22%)	Mollicutes (6.22%)	Betaproteobacteriales (6.50%)	*Burkholderiaceae* (4.12%)	*Tabrizicola* (1.47%)
Bacteroidetes (5.19%)	Clostridia (5.19%)	Mycoplasmatales (5.43%)	*Rhodobacteraceae* (3.70%)	*Clostridium sensu stricto 1* (1.19%)
Other (6.76%)	Other (15.25%)	Other (33.25%)	Other (30.74%)	Other (26.62%)
Unassigned (0.70%)	Unassigned (1.42%)	Unassigned (6.36%)	Unassigned (38.27%)	Unassigned (59.24%)

Table 5. Number of bacterial taxonomic ranks per site found within the digestive glands of freshwater mussels collected in Fall 2018 from the Grand River, ON (n=10/site except JN with n=3). Vertical lines indicate WWTP outfalls that occur at some point in the river between the two listed sites. See Figure 1 for site locations.

	Site				
Rank	WMR	KIW	DN	JN	GM
Phylum	36	35	25	23	26
Order	66	70	52	42	53
Class	151	173	126	97	142
Family	225	262	186	129	208
Genus	434	535	330	196	417

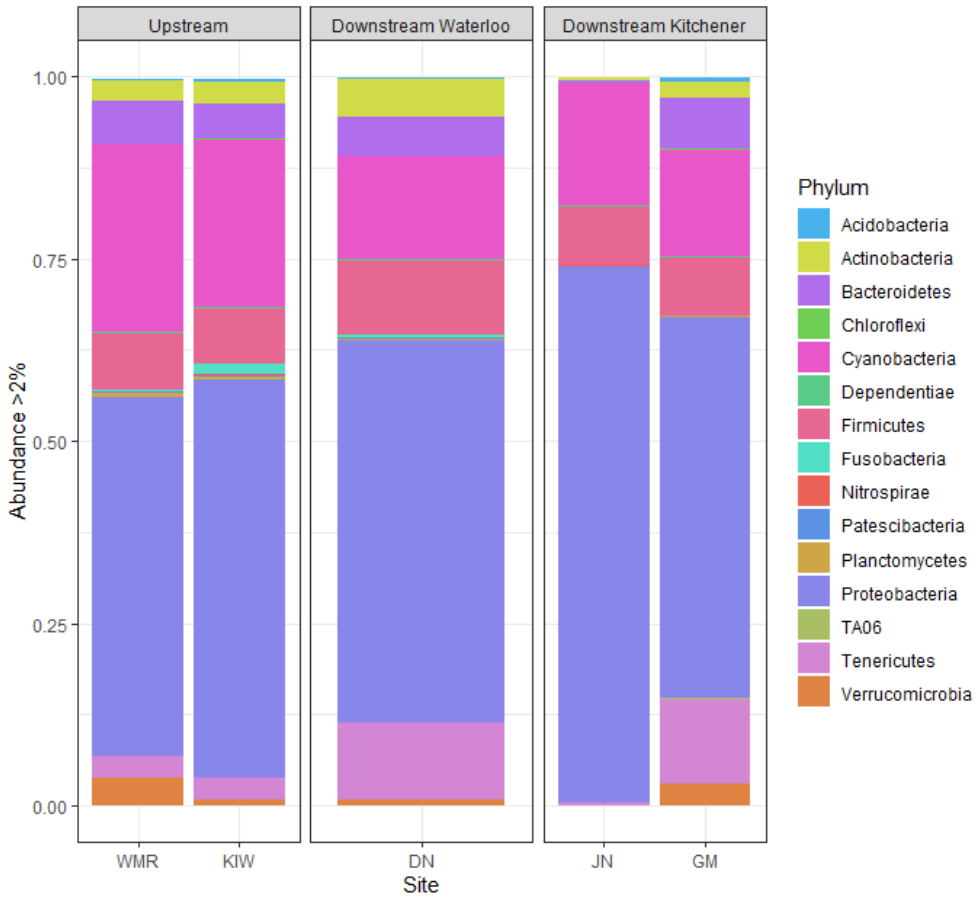

Figure 2. Abundance (>2%) of phylum-level bacteria across sites, from upstream to downstream within the digestive glands of mussels collected in Fall 2018 from the Grand River, ON (n=10/site except JN with n=3). Data was re-normalized based on relative abundance of bacterial taxa above 2%, while taxa below 2% ranged from 0-1.99% of the total. See Figure 1 for site locations.

The relative abundance of the dominant phyla in mussel digestive glands changed across sites (Figure 2, Table S1 in Appendix A). These phyla consisted of primarily Proteobacteria, Cyanobacteria, Firmicutes, and Tenericutes, as well as Bacteroidetes and Actinobacteria in smaller abundances. The proportion of Bacteroidetes and Actinobacteria increased past the Waterloo WWTP followed by a large decrease past Kitchener, and then at a site further downstream (GM), returned to levels similar to those observed in the mussels from the upstream region (WMR, KIW). Downstream of the

Kitchener WWTP (7.5 km, JN) there was a spike in Proteobacteria, as well as a decline in Actinobacteria, Bacteroidetes, and Tenericutes. Cyanobacteria decreased slightly past the Waterloo WWTP (DN), and then remained relatively consistent further downstream. There was also a slight increase in Firmicutes at this same site (DN), which diminished further downstream. Verrucomicrobia was found in similar proportions at the furthest upstream (WMR, 3.8%) and downstream sites (GM, 3.0%) and was low in abundance at all other sites (0.07-0.8%). Fusobacteria was only found in abundance at site KIW (1.5%).

3.2.1.1. Presence of Cyanobacteria

There were also spatial patterns in the relative abundance of Cyanobacteria genera, including ones known to produce cyanotoxins, in mussel digestive glands (Figure 3, Table S2 in Appendix A). The genus at the highest relative abundance, *Cyanobium PCC-6307*, increased in abundance downstream of both WWTPs (DN, JN), while the second most common, *Snowella 0TU37S04*, was lower in abundance at those sites relative to upstream. Bacteria known to produce cyanotoxins included *Planktothrix NIVA-CYA 15* (0-7.1%), *Microcystis PCC-7914* (0-8.6%), *Aphanizomenon MDT14a* (0-5.3%), and *Schizothrix LEGE 07164* (0-0.5%). *Planktothrix NIVA-CYA 15* decreased or was not detected at sites downstream of the WWTP outfalls (DN, JN), whereas *Merismopedia 0BB39S01* increased. Interestingly, two of these genera (*Aphanizomenon MDT14a*, *Microcystis PCC-7914*) were found solely or mainly at the two upstream sites (WMR, KIW). Abundances of *Pseudanabaena PCC-6802* decreased from upstream (WMR, KIW) to downstream.

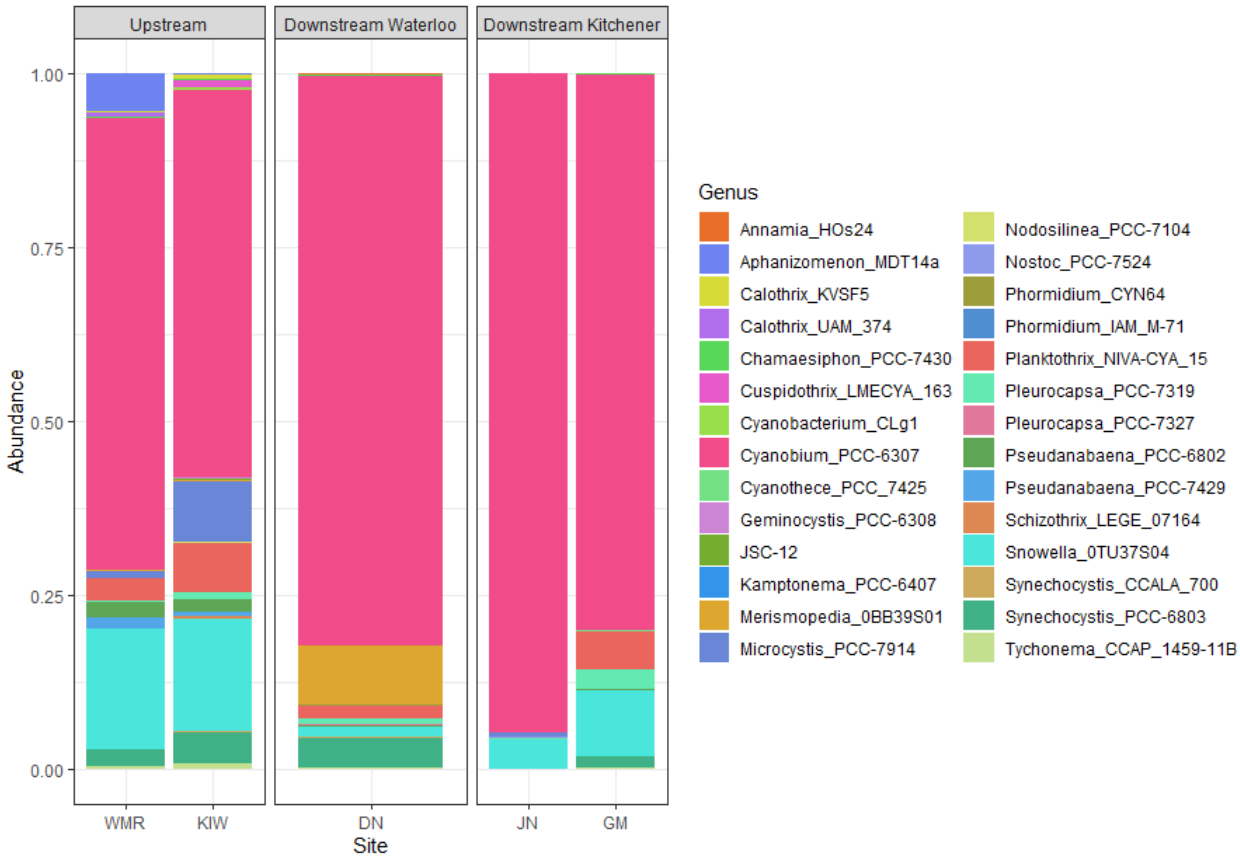

Figure 3. Proportional abundance of genera relative to total Cyanobacteria across sites, from upstream to downstream within the digestive glands of mussels collected in Fall 2018 from the Grand River, ON (n=10/site except JN with n=3. See Figure 1 for site locations. See Table S3 for overall percent relative abundance of Cyanobacteria according to site.

3.2.2. Spiders

A total of 23 bacterial phyla, 43 classes, 95 orders, 180 families, and 483 genera were present in the spiders (Table 3). The top five most abundant phyla, classes, orders, families, and genera accounted for 99.52%, 92.65%, 81.61%, 75.41%, and 74.63% of the total bacterial sequences, respectively (Table 6). Relative to upstream sites, the number of phyla, orders, classes, families, and genera of bacteria within spiders declined downstream of the Waterloo WWTP (EIT, FWY) and increased downstream of

the Kitchener WWTP (PT1, PT2), showing signs of recovery further downstream (GM) (Table 7).

Table 6. Summary of the top five bacterial taxa per rank along with their relative abundances within whole-body riparian spiders collected in Fall 2018 from the Grand River, ON (n=98).

Phylum	**Class**	**Order**	**Family**	**Genus**
Proteobacteria (66.65%)	Gammaproteobacteria (46.58%)	Diplorickettsiales (42.47%)	*Diplorickettsiaceae* (42.47%)	*Rickettsiella* (41.79%)
Bacteroidetes (15.86%)	Alphaproteobacteria (20.02%)	Rickettsiales (15.86%)	*Amoebophilaceae* (12.74%)	*Candidatus Cardinium* (12.71%)
Firmicutes (8.07%)	Bacteroidia (15.86%)	Cytophagales (14.25%)	*Rickettsiaceae* (11.99%)	*Rickettsia* (11.92%)
Tenericutes (5.52%)	Mollicutes (5.52%)	Clostridiales (4.67%)	*Spiroplasmataceae* (4.36%)	*Spiroplasma* (4.36%)
Actinobacteria (3.42%)	Clostridia (4.67%)	Entomoplasmatales (4.36%)	*Anaplasmataceae* (3.85%)	*Wolbachia* (3.85%)
Other (0.48%)	Other (7.32%)	Other (18.33%)	Other (24.13%)	Other (23.33%)
Unassigned (0.03%)	Unassigned (0.03%)	Unassigned (0.06%)	Unassigned (0.47%)	Unassigned (2.03%)

Table 7. Number of bacterial taxonomic ranks per site found within whole-body riparian spiders collected in Fall 2018 from the Grand River, ON (n=10/site except PT1 with n=8). See Figure 1 for site locations. Shaded columns indicate WWTP outfall sites.

	Site									
Rank	INV	WMR	KIW	EIT	FWY	HR	PT1	PT2	BLR	GM
Phylum	16	14	11	7	6	7	12	14	12	12
Class	29	24	19	12	12	12	20	24	18	22
Order	60	54	42	30	32	39	45	48	42	55
Family	111	96	72	48	53	63	80	83	74	119
Genus	247	203	119	78	71	111	142	147	151	239

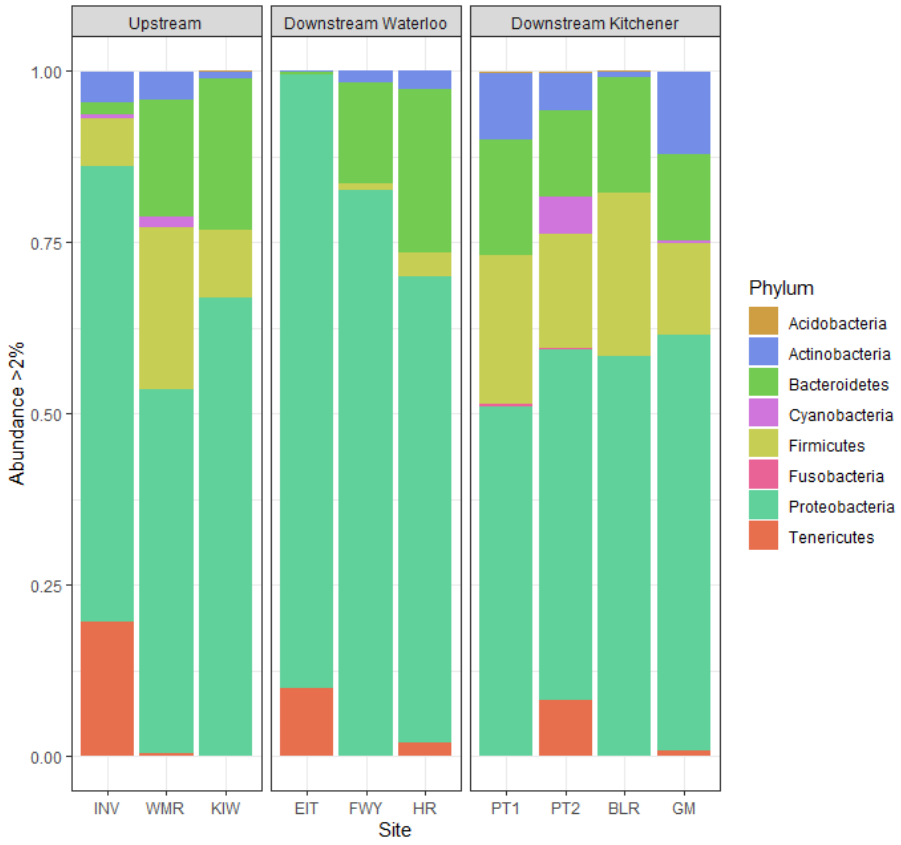

Figure 4. Abundance (>2%) of bacterial phyla across sites, from upstream to downstream within whole-body spiders collected in Fall 2018 from the Grand River, ON (n=10/site except PT1 with n=8). Data was re-normalized based on relative abundance of bacterial taxa above 2%, while taxa below 2% ranged from 0-1.95% of the total. See Figure 1 for site locations.

Although Proteobacteria dominated all spider samples, the relative abundance of the dominant phyla changed across sites (Figure 4, Table S3 in Appendix A). There was a spike in Proteobacteria downstream of the Waterloo WWTP outfall (EIT), which then diminished further downstream. There was also a decline in Bacteroidetes, Firmicutes, and Actinobacteria downstream of Waterloo (EIT), with proportions re-establishing further downstream. Spiders had a high relative abundance of Firmicutes at upstream site WMR as well as all sites past the Kitchener WWTP outfall.

3.2.2.1. Presence of Endosymbiont Bacteria

Endosymbiont bacteria may represent a core microbiome within the spider and looking at their compositional changes could better illustrate impacts of environmental exposures. These endosymbionts fall within the phyla Bacteroidetes, Proteobacteria, and Tenericutes, but spiders mainly contained endosymbionts of Proteobacteria (Figure 5a).

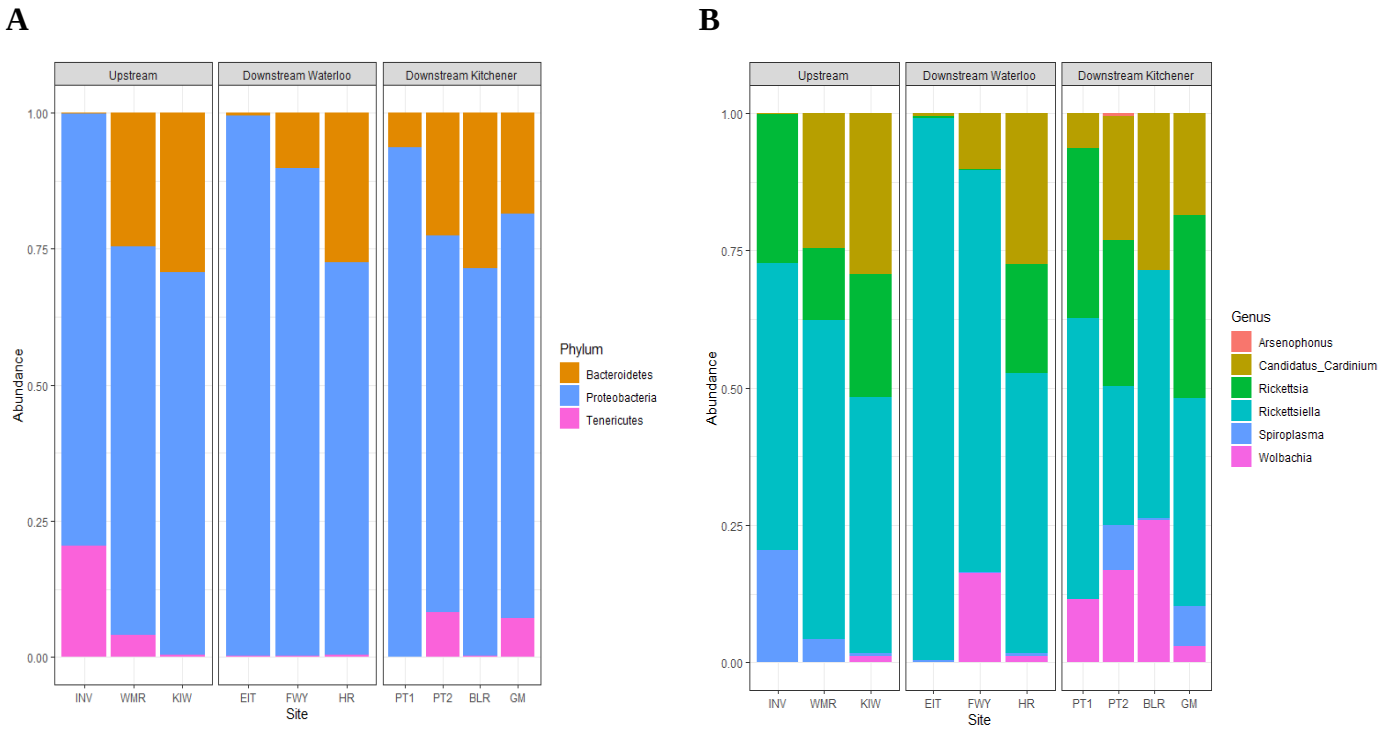

Figure 5. Abundance of phylum-level (A) and genus-level (B) endosymbiont bacteria across sites, from upstream to downstream within whole-body riparian spiders collected in Fall 2018 from the Grand River, ON (n=10/site except PT1 with n=8). See Figure 1 for site locations.

Of the endosymbiont genera observed in the spider microbiome, *Rickettsiella* dominated across sites but there were also some spatial patterns (Figure 5b, Table S4 in Appendix A). More specifically, *Rickettsiella* abundance was highest downstream of the Waterloo WWTP outfall (EIT, FWY), which then diminished further downstream. *Candidatus cardinium* was lowest at the furthest upstream (INV) site and the WWTP outfall sites (EIT, PT1). *Rickettsia* displayed low abundance at sites directly downstream

of the Waterloo outfall (EIT, FWY), while *Wolbachia* was highest in sites past the Kitchener outfall (PT1, PT2, BLR). *Spiroplasma* was highest in abundance at the most upstream site (INV); this site was dominated by three main endosymbionts, *Rickettsiella, Rickettsia*, and *Spiroplasma*. *Arsenophonus* could only be detected in small amounts at the PT2 site, downstream of the Kitchener WWTP.

3.2.2.2. Presence of Cyanobacteria

Spiders also contained small proportions of Cyanobacteria, including cyanotoxin-producing genera, but their prevalence was low within and among sites (5 of 10 sites) and the genera varied among individuals (Figure 6). Detections ranged from 10% (KIW, EIT, PT2, BLR) to 20% (WMR) of individuals from each site. At the second furthest upstream site (WMR), genus-level Cyanobacteria were detected in two individuals, with *Tychonema CCAP 1459-11B* found in one and *Planktothrix NIVA-CYA 15* in the other. At the third reference site (KIW), one individual had the genus *Potamolinea 1PC*. Downstream of the Waterloo outfall (EIT), one individual contained *Cyanobium PCC-6307*. Finally, downstream of the Kitchener outfall, genus-level Cyanobacteria was detected in one individual at each site, with *Cyanobium PCC-6307* at PT2 and *CENA359* at BLR.

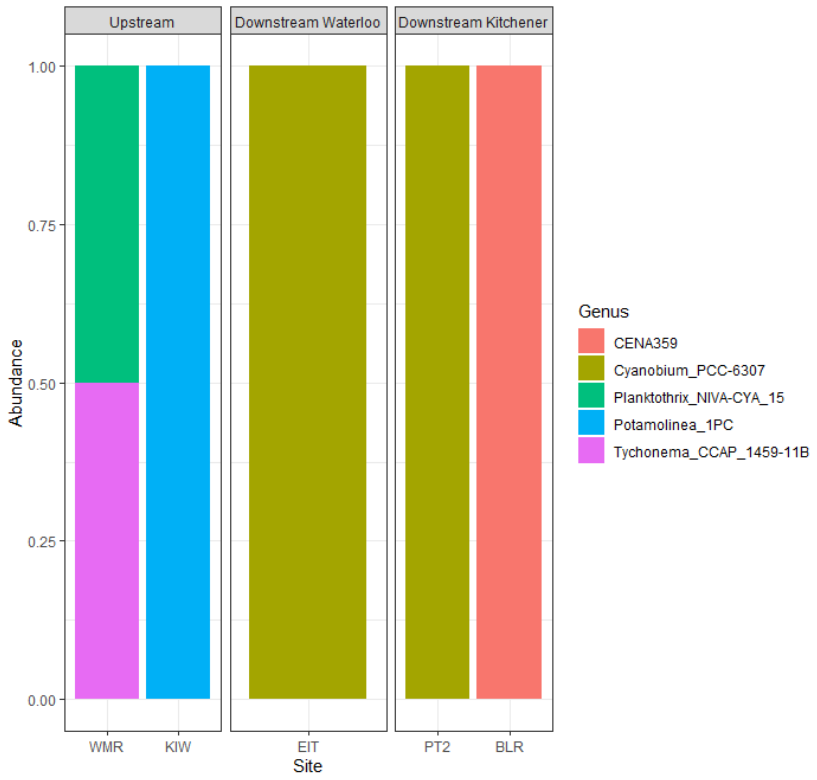

Figure 6. Proportional abundance of genera relative to total Cyanobacteria across sites, from upstream to downstream within whole-body spiders collected in Fall 2018 from the Grand River, ON (n=1-2/site). Genus-level Cyanobacteria were not found at sites INV, FWY, HR, PT1, GM. See Figure 1 for site locations. See Table S3 for overall percent relative abundance of Cyanobacteria according to site.

3.2.3. Aquatic Macroinvertebrates

A total of 41 bacterial phyla, 82 classes, 206 orders, 318 families, and 739 genera were present within the whole-body macroinvertebrate samples (Table 3). Relative to upstream sites, the number of phyla, orders, classes, families, and genera of bacteria declined downstream of the Waterloo WWTP (EIT, FWY) in both Perlidae and Hydropsychidae samples, and decreased and increased downstream of the Kitchener WWTP (PT1, PT2, BLR) in Perlidae and Hydropsychidae (PT1), respectively (Table 8).

Except for Hydropsychidae that were dominated by Bacteroidetes, Proteobacteria was the most dominant bacterial phylum across most invertebrate families (32.17-50.25%) (Table S5 in Appendix A). Other common phyla included Bacteroidetes (22.53-39.89%), Cyanobacteria (2.85-34.09%), and Tenericutes (2.25-18.72%). Bacteroidia was the most dominant bacterial class (22.47-39.24%), followed by Oxyphotobacteria (12.43-34.04%), Gammaproteobacteria (12.53-23.74%), and Alphaproteobacteria (10.34-27.66%). The bacterial order Betaproteobacteriales was most common across invertebrate samples (11.05-21.91%). Other abundant orders included Chloroplast (12.00-31.52%), Chitinophagales (7.42-12.12%), and Rhodobacterales (9.29-11.92%). The top bacterial families included *Burkholderiaceae* (10.12-14.88%), *Flavobacteriaceae* (5.01-12.50%), *Rhodobacteraceae* (9.29-11.92%), and *Sphingomonadaceae* (8.91-10.51%). Finally, the most common bacterial genera consisted of *Flavobacterium* (5.00-12.49%), *Sphingorhabdus* (5.18-7.49%), *Pseudorhodobacter* (3.56-6.88%), and *Rhodoferax* (3.64-5.59%).

Table 8. Number of bacterial taxonomic ranks found within whole-body benthic macroinvertebrates collected in Fall 2018 from each site on the Grand River, ON (n=1-11/site). Shaded columns indicate WWTP outfall sites. See Figure 1 for site locations. Blank cells indicate sites where the invertebrate taxa were unable to be collected.

Invertebrate Family	Rank	Site									
		INV	WMR	KIW	EIT	FWY	HR	PT1	PT2	BLR	GM
Perlidae	Phylum	24	27	26	13	16	24	18	16	15	27
	Class	49	51	46	23	28	42	30	35	30	50
	Order	115	129	122	61	70	108	86	88	72	113
	Family	160	178	167	82	97	151	121	119	97	154
	Genus	266	294	258	102	139	239	192	189	166	229
Hydrospychidae	Phylum	20	20	21	18	16	20	22	18	22	20
	Class	37	37	41	34	25	38	45	41	41	34
	Order	82	88	94	72	50	77	106	78	89	71
	Family	112	122	119	95	66	99	140	103	123	96
	Genus	164	148	155	132	87	140	205	145	169	125
Heptageniidae	Phylum	23	22	26	20	20	23	21	21	21	25
	Class	42	42	50	40	39	44	38	38	39	43
	Order	104	104	113	93	100	111	95	87	98	108
	Family	145	161	166	138	138	150	143	117	126	155
	Genus	211	226	250	219	197	229	196	178	183	205
Ephemerellidae	Phylum	18	36								
	Class	34	63								
	Order	85	147								
	Family	123	218								
	Genus	187	356								
Baetidae	Phylum			21	22	15		17	17	12	26
	Class			31	43	28		33	36	20	41
	Order			84	115	63		94	91	53	107
	Family			122	183	92		138	127	86	140
	Genus			171	289	121		210	190	137	206

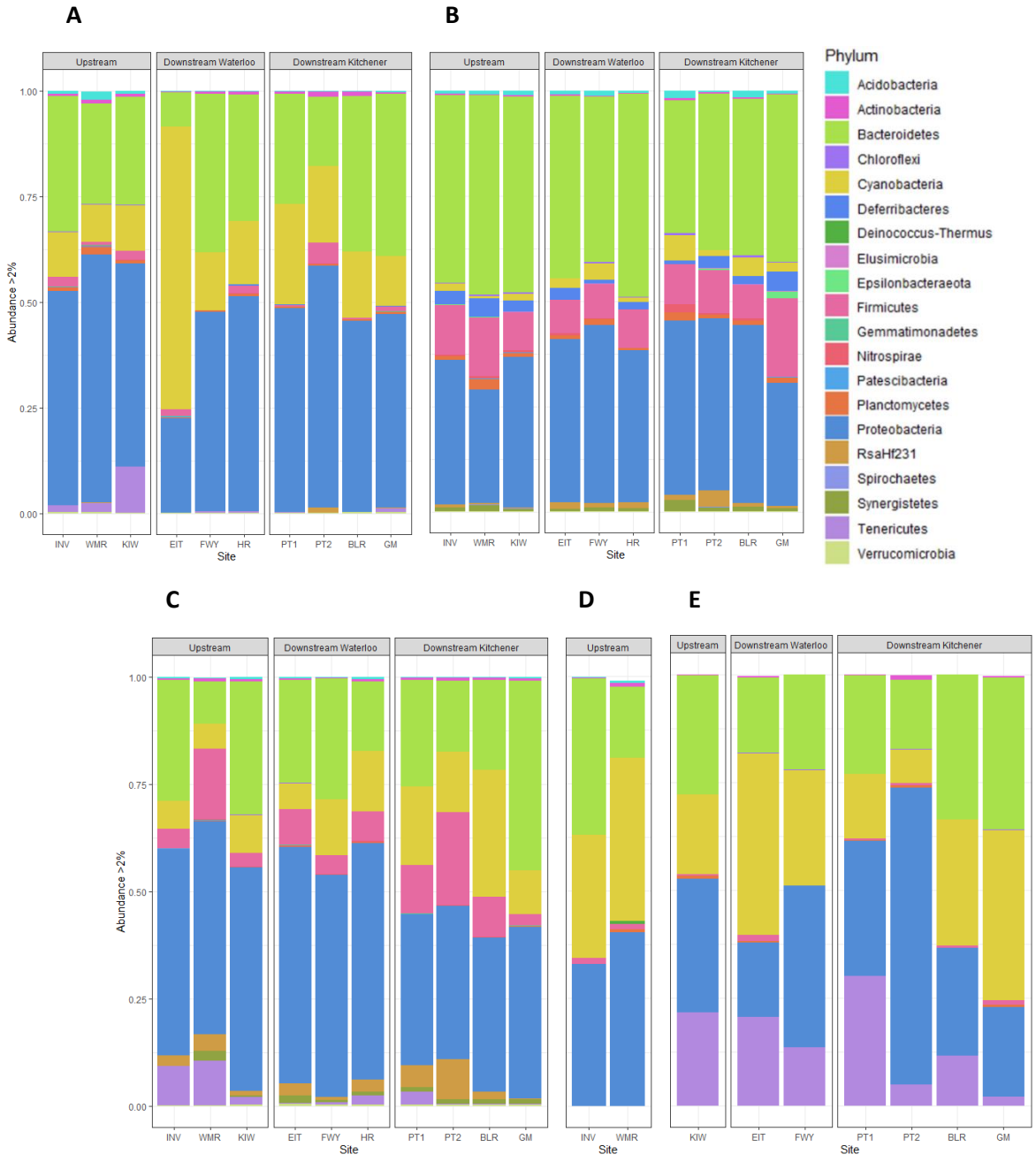

Figure 7. Abundance (>2%) of phylum-level bacteria across sites, from upstream to downstream, within whole-body benthic macroinvertebrates collected in Fall 2018 from the Grand River, ON: A) Perlidae (n=1-10/site); B) Hydropsychidae (n=3-11/site); C) Heptageniidae (n=5/site); D) Ephemerellidae (n=5/site); E) Baetidae (n=3-5/site). Data was re-normalized based on relative abundance of bacterial taxa above 2%, while taxa below 2% ranged from 0-1.99% of the total. See Figure 1 for site locations.

For almost all macroinvertebrate taxa examined herein, there were some shifts in the relative abundance of bacterial phyla among sites in the Grand River (Figure 7, Table S6 in Appendix A). In Perlidae, there was a spike in Cyanobacteria and a decrease in both Proteobacteria and Bacteroidetes just downstream of the Waterloo WWTP (EIT), while Tenericutes and Acidobacteria were higher in individuals from the upstream sites (Figure 7a, Table S6 in Appendix A). Hydropsychidae samples decreased in Bacteroidetes just past the Kitchener WWTP (PT1) (Figure 7b, Table S6 in Appendix A). In Heptageniidae, the relative abundance of Tenericutes decreased downstream of site WMR, while Cyanobacteria increased downstream of the Waterloo WWTP (EIT) and Proteobacteria decreased slightly downstream of the Kitchener WWTP (Figure 7c, Table S6 in Appendix A). Although no samples of Baetidae were collected from the two furthest upstream sites (INV, WMR), the highest relative abundance of Cyanobacteria in Baetidae occurred downstream of the Waterloo WWTP (EIT) and it decreased downstream of the Kitchener WWTP (PT1, PT2). Similarly, Tenericutes and Proteobacteria increased at these same sites (PT1 and PT2, respectively) (Figure 7d, Table S6 in Appendix A). Although both are classified as Gatherer-Collectors, the bacterial relative abundance of Ephemerellidae and Baetidae differed in composition, as there were no Tenericutes in the former (Figure 7e, Table S6 in Appendix A).

3.2.3.1. Presence of Cyanobacteria

Invertebrate taxa also contained several dominant Cyanobacteria genera; however, most were not toxin producing, and their prevalence and the dominant taxa varied among individual invertebrates and sites. Cyanobacteria at the genus-level were found in all taxa at all sites except for Hydropsychidae at FWY and this taxon showed the least variation in Cyanobacteria across sites (Figure 8b, Table S7 in Appendix A). Additionally, Cyanobacteria at the genus-level in insect taxa were not found consistently in all individuals (16.7-100%) (Table 9). In all invertebrates, *Cyanobium PCC-6307* increased in sites downstream of Waterloo and *Tychonema CCAP 1459-11B* increased in sites downstream of Kitchener. Perlidae and Heptageniidae samples showed similar

proportions of Cyanobacteria genera within sites, and both showed decreases in *Snowella 0TU37S04* and a large dominance of *Tychonema CCAP 1459-11B* downstream of Kitchener (Figure 8a, c, Table S7 in Appendix A). *Pleurocapsa PCC-7319* fluctuated across sites for all insects but was particularly abundant furthest downstream (GM).

Table 9. Percent of individuals at each site containing Cyanobacteria detected at the genus-level within whole-body benthic macroinvertebrates collected in Fall 2018 from the Grand River, ON. Blank cells indicate sites where the invertebrate taxa were unable to be collected. Shaded columns indicate WWTP outfall sites. Dashes indicate sites where genus-level Cyanobacteria were undetectable.

	Site (%)										
Taxa	INV	WMR	KIW	EIT	FWY	HR	PT1	PT2	BLR	GM	**N total**
Perlidae	100	60	50	100	40	75	80	60	100	80	55
Hydropsychidae	50	20	66.7	66.7	-	20	63.6	16.7	100	75	59
Heptageniidae	100	100	80	20	80	40	100	100	100	60	50
Ephemerellidae	100	100									10
Baetidae			100	80	60		100	50	100	66.7	32

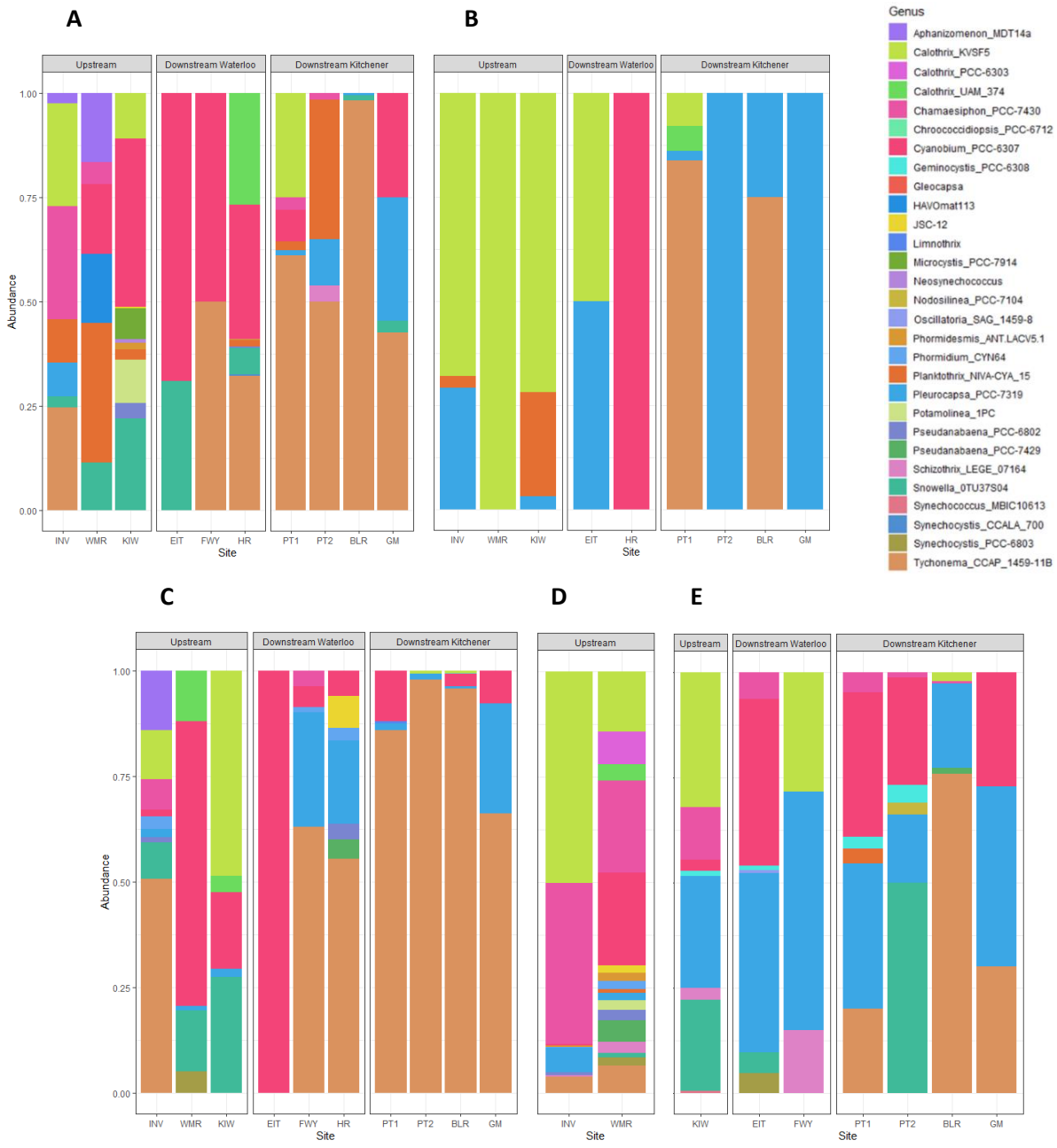

Figure 8. Proportional abundance of genera relative to total Cyanobacteria across sites, from upstream to downstream within whole-body benthic macroinvertebrates collected in Fall 2018 from the Grand River, ON: A) Perlidae; B) Hydropsychidae; C) Heptageniidae; D) Ephemerellidae; E) Baetidae. See Table 9 for n size and Figure 1 for site locations. See Table S6 for overall percent relative abundance of Cyanobacteria according to site.

3.2.4. Water and Wastewater Effluent Samples

A combined total of 40 bacterial phyla, 81 classes, 215 orders, 309 families, and 663 genera were present within the Grand River water samples collected from the study area and the Waterloo and Kitchener WWTP effluents (Table 2). Relative to upstream sites, the number of phyla, classes, orders, families, and genera of bacteria within river water samples increased at both outfall sites (EIT, PT1) and remained elevated in all downstream sites (Table 10).

The bacterial phyla of Proteobacteria dominated across all river water and wastewater effluent samples (50.60-59.94%) (Table 11). Other commonly shared phyla among all samples included Bacteroidetes (14.68-16.91%) and Epsilonbacteraeota (3.94-4.85%). Firmicutes was more common in the effluents (5.34-6.85%), while Cyanobacteria (17.89%) and Actinobacteria (7.05%) were more common in river water samples. Comparing the two WWTP effluents, Waterloo contained a higher abundance of Dependentiae (6.86%), while Kitchener contained more Patescibacteria (6.98%).

Gammaproteobacteria was the most dominant bacterial class across all samples (37.71-43.15%), followed by Bacteroidia (14.64 -16.70%) and Alphaproteobacteria (6.42-13.83%) (Table S8 in Appendix A). River water samples contained more Oxyphotobacteria (17.88%) and Actinobacteria (6.42%) compared to effluent samples. Interestingly, Waterloo WWTP effluent contained a larger proportion of Babeliae (6.86%) than both Kitchener effluent and river water samples.

Betaproteobacteriales was the dominant bacterial order across all samples (17.55-22.56%), while other common orders included Pseudomonadales (5.65-19.20%), Chitinophagales (6.18-7.88%), and Campylobacterales (4.74-4.85%) (Table S8 in Appendix A). Overall, river water samples contained a higher abundance of Chloroplast (14.94%), while Waterloo WWTP effluent contained more Babeliales (6.86%) and Kitchener WWTP effluent contained more Paracaedibacterales (6.31%).

The top bacterial families across all samples included *Moraxellaceae* (5.54-15.77%), *Burkholderiaceae* (5.82-18.55%), and *Arcobacteraceae* (3.94-4.76%). Greater proportions of *Rhodocyclaceae* (9.54-14.08%) were found in the effluents, while more *Flavobacteriaceae* (6.23%) and *Sporichthyaceae* (4.28%) were found in the river water samples (Table S8 in Appendix A).

Finally, the most common bacterial genera among all samples was *Arcobacter* (3.94-4.76%), while *Zoogloea* (7.68-7.78%) and *Sediminibacterium* (1.97-3.52%) had a greater relative abundance in effluent samples from both locations than in river water (Table S8 in Appendix A).

Table 10. Number of bacterial taxonomic ranks (richness) per site found within river water and Waterloo (WAT) and Kitchener (KIT) WWTP effluent samples (shaded in gray) collected in Fall 2019 from the Grand River, ON (n=3). See Figure 1 for site locations.

Rank	**Site**													
	INV	WMR	KIW	WAT	EIT	FWY	DN	HR	KIT	PT1	PT2	JN	BLR	GM
Phylum	23	21	21	35	27	19	22	25	14	24	27	27	25	23
Class	36	37	36	65	45	33	34	47	28	50	41	54	36	41
Order	81	82	82	154	114	83	83	114	64	123	107	123	98	94
Family	124	122	122	218	186	128	124	171	88	193	156	177	164	150
Genus	157	156	156	368	314	197	202	289	96	326	257	262	262	212

Table 11. Top five bacteria from each taxonomic rank and their relative abundances within river water and Waterloo and Kitchener WWTP effluent samples, collected in Fall 2019 from the Grand River, ON (n=3/site).

	Upstream (INV, WMR, KIW)	**Waterloo WWTP Effluent**	**Downstream Waterloo (EIT, FWY, DN, HR)**	**Kitchener WWTP Effluent**	**Downstream Kitchener (PT1, PT2, JN, BLR, GM)**
Phylum	Proteobacteria (48.55%)	Proteobacteria (51.08%)	Proteobacteria (51.10%)	Proteobacteria (59.94%)	Proteobacteria (50.92%)
	Cyanobacteria (19.14%)	Bacteroidetes (16.91%)	Cyanobacteria (17.47%)	Bacteroidetes (16.08%)	Cyanobacteria (17.78%)
	Actinobacteria (12.89%)	Dependentiae (6.86%)	Bacteroidetes (14.44%)	Patescibacteria (6.98%)	Bacteroidetes (16.05%)
	Bacteroidetes (11.00%)	Firmicutes (5.34%)	Epsilonbacteraeota (6.05%)	Firmicutes (6.85%)	Actinobacteria (6.16%)
	Epsilonbacteraeota (4.04%)	Epsilonbacteraeota (4.85%)	Actinobacteria (5.63%)	Epsilonbacteraeota (3.94%)	Epsilonbacteraeota (4.04%)
	Other (4.39%)	Other (14.59%)	Other (5.31%)	Other (4.99%)	Other (5.06%)
	Unassigned (0.05%)	Unassigned (0.37%)	Unassigned (0.01%)	Unassigned (1.22%)	Unassigned (0.01%)

Figure 9. Abundance (>2%) of phylum-level bacteria across sites, from upstream to downstream within river water samples as well as Waterloo (WAT) and Kitchener (KIT) WWTP effluent samples collected in Fall 2019 from the Grand River, ON (n=3/site). Data was re-normalized based on relative abundance of bacterial taxa above 2%, while taxa below 2% ranged from 0-1.99% of the total. See Figure 1 for site locations.

The relative abundance of the dominant phyla changed across sites, with the bacterial composition of upstream sites (INV, WMR) differing from sites further downstream (Figure 9, Table S9 in Appendix A). At site KIW onwards there was a decrease in Actinobacteria, increases in Epsilonbacteraeota and Firmicutes, and a slight increase in Proteobacteria. Past the Waterloo WWTP (EIT) there was also a slight increase in Patescibacteria. Overall bacterial composition remained relatively constant downstream of KIW onwards regardless of inputs from the two targeted WWTPs. Comparing the composition of the two effluents, Waterloo was more diverse in terms of bacterial phyla and had a higher abundance of Actinobacteria, Dependentiae, Nitrospirae,

and Planctomycetes compared to the Kitchener WWTP effluent. The relative abundance of Cyanobacteria was low in both effluents compared to river water samples.

3.2.4.1. Presence of Effluent Bacteria in River Water and Biota

Effluents from the Waterloo and Kitchener WWTPs contained 228 and 40 unique genera, respectively, that were not present in upstream river water samples. However, in river water from downstream of Waterloo and Kitchener WWTPs there were only 24 and 14 genera, respectively, unique to the effluents that were not found at the respective upstream sites.

River water samples from sites downstream of both WWTPs contained bacterial genera unique to Waterloo WWTP effluent in their microbiomes (Table 12). River water from sites downstream of the Waterloo and Kitchener WWTPs contained 24 and 11 bacterial genera, respectively, present in only Waterloo WWTP effluent. Samples from sites downstream of the Kitchener WWTP also contained three genera unique to both Waterloo and Kitchener WWTP effluent. Genera unique to Kitchener WWTP effluent were not found in the microbiomes of downstream river water samples.

Macroinvertebrates from sites downstream of both WWTPs contained bacterial genera unique to Waterloo WWTP effluent in their microbiomes (Table 13). Macroinvertebrates from sites downstream of the Waterloo and Kitchener WWTPs contained 7 and 11 bacterial genera, respectively, present only in the Waterloo effluent. Genera unique to Kitchener WWTP effluent were not found in the microbiomes of downstream macroinvertebrate taxa.

Table 12. Bacterial genera unique to Waterloo WWTP effluent found in the microbiome of river water samples from sites downstream of the Waterloo WWTP and Kitchener WWTP outfalls collected in Fall 2019 from the Grand River, ON.

Source	River Water Downstream of Waterloo WWTP	River Water Downstream of Kitchener WWTP
Waterloo WWTP Effluent	*Acetoanaerobium*	*Bact-08*
	Acidaminococcus	*BD1-7 clade*
	Aquimonas	*Candidatus Cloacimonas*
	AUTHM297	*Cloacibacterium*
	BD1-7 clade	*Desulfobacter*
	C1-B045	*Neochlamydia*
	Candidatus Cloacimonas	*Proteiniclasticum*
	Candidatus Paenicardinium	*Steroidobacter*
	Candidatus Protochlamydia	*Succinivibrio*
	Chiayiivirga	*Thermovirga*
	Cloacibacterium	*U29-B03*
	Lelliottia	
	Leptotrichia	
	Mesotoga	
	Neochlamydia	
	Planctopirus	
	Proteiniclasticum	
	SC103	
	Steroidobacter	
	SWB02	
	Thermovirga	
	Turneriella	
	U29-B03	
	XBB1006	
Waterloo or Kitchener WWTP Effluent		*Candidatus Protochlamydia*
		Chiayiivirga
		Tuneriella

Table 13. Bacterial genera unique to Waterloo WWTP effluent found in the microbiome of macroinvertebrate taxa from sites downstream of the Waterloo WWTP and Kitchener WWTP outfalls collected in Fall 2019 from the Grand River, ON.

Source	Invertebrates Downstream of Waterloo WWTP	Invertebrates Downstream of Kitchener WWTP
Waterloo WWTP Effluent	*Actinomyces* *BD1-7 clade* *Bifidobacterium* *Candidatus Paenicardinium* *Chiayiivirga* *Cloacibacterium* *Prevotellaceae UCG-004*	*Acidaminococcus* *Actinomyces* *Candidatus Paenicardinium* *Cloacibacterium* *Flavitalea* *Fusibacter* *Lelliottia* *Ochrobactrum* *Ottowia* *Steroidobacter* *SWB02*

3.2.4.2. Presence of Cyanobacteria

Cyanobacteria were found in river water and effluent samples from both WWTPs and the genera varied among sites for the former; Cyanobacteria were not identifiable to genus level in the effluent samples (Figure 10, Table S10 in Appendix A). From the first to second upstream site (INV to WMR), there was a decrease in *Microcystis PCC-7914* and *Tychonema CCAP 1459-11B*. In the river, the composition of the majora genera changed at site KIW onwards. More specifically, *Planktothrix NIVA-CYA 15* was undetectable at the two most upstream sites (INV, WMR) but represented the largest proportion of Cyanobacteria in sites further downstream. There were also smaller proportions of *Aphanizomenon MDT14a*, *Cyanobium PCC-6307*, *Pseudanabaena PCC-7429*, and *Snowella 0TU37S04* downstream of INV and WMR.

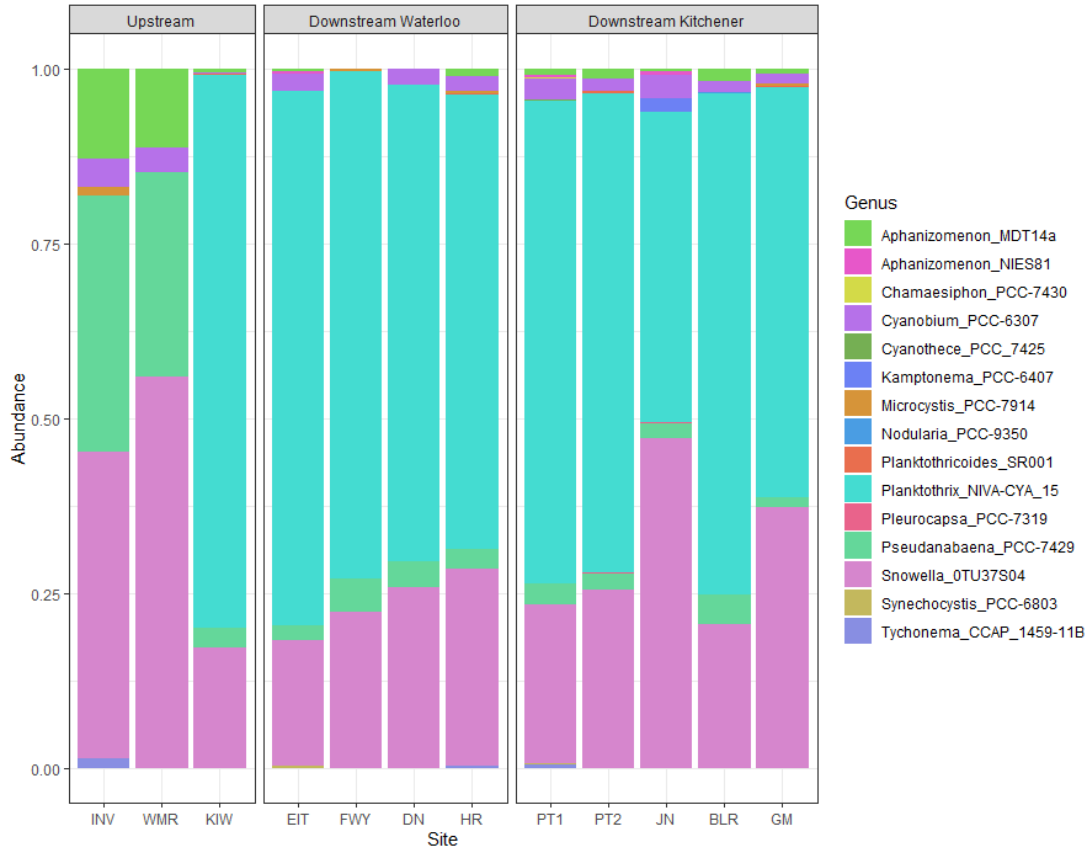

Figure 10. Proportional abundance of genera relative to total Cyanobacteria across sites, from upstream to downstream, within water samples collected in Fall 2019 from the Grand River, ON (n=3/site). See Figure 1 for site locations. See Table S9 for overall percent relative abundance of Cyanobacteria according to site.

3.3. Alpha Diversity

3.3.1. Mussels

Bacterial alpha diversity in the digestive gland of mussels tended to be lower at the downstream than upstream sites (Figure 11) and differed significantly among sites (one-way ANOVA: Shannon $F=8.015$, $df=4$, $p=<0.0001$; Simpson $F=11.04$, $df=4$, $p=<0.0001$). There was a particularly low diversity observed at the JN site (downstream of Kitchener WWTP and below the confluence of the Speed River) when compared to all

other sites, and it was significantly less diverse than all other sites (Table 14). In addition, the furthest downstream site (GM, downstream of Kitchener WWTP) was significantly less diverse than one site (KIW) that is upstream of both targeted WWTPs.

Table 14. Tukey HSD values from the one-way ANOVA test of Shannon and Simpson alpha diversity measures by site within the digestive glands of mussels collected in Fall 2018 from the Grand River, ON (n=10/site except JN with n=3). See Figure 1 for site locations. Significant values are depicted in red.

Factor	Pair	Shannon (H)		Simpson (D)	
		Diff	P adjusted	Diff	P adjusted
Site	JN-WMR	-2.400	0.003	-0.456	<0.0001
	JN-KIW	-3.214	<0.0001	-0.488	<0.0001
	JN-DN	-2.127	0.010	-0.464	<0.0001
	JN-GM	-1.756	0.046	-0.385	<0.0002
	GM-KIW	-1.457	0.009	-0.103	0.308

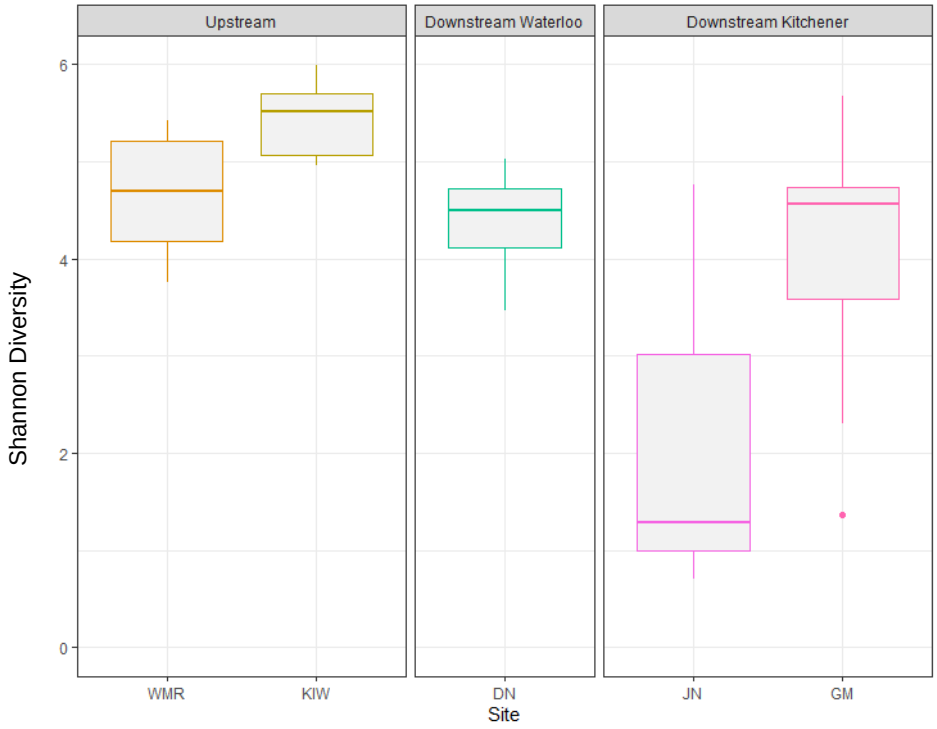

Figure 11. Bacterial alpha diversity (Shannon's Index) within the digestive glands of mussels, collected in Fall 2018 from sites upstream to downstream (left to right) in the Grand River, ON (n=10/site except JN with n=3). See Figure 1 for site locations.

3.3.2. Spiders

Bacterial alpha diversity in spiders tended to be lower than for other invertebrate taxa examined herein and, while this metric varied within and among sites, patterns were not consistently related to the outfall sites (Figure 12). There were low mean diversity values in spiders from upstream (INV, KIW) and downstream (EIT) of the Waterloo WWTP outfall but also further downstream from the Kitchener WWTP outfall (BLR). Alpha diversity of whole spiders differed among sites (one-way ANOVA: Shannon $F=3.336$, $df=9$, $p=0.0015$; Simpson $F=4.268$, $df=9$, $p=<0.0002$; Table 15). Site EIT (Downstream Waterloo) was significantly less diverse than upstream (WMR) and downstream of Kitchener (PT1, PT2) sites, while site PT1 (Downstream Kitchener) was

significantly more diverse than upstream (INV, KIW) and downstream Waterloo (EIT, FWY) sites.

Table 15. Tukey HSD values from the one-way ANOVA test of Shannon and Simpson alpha diversity measures by site within whole-body spiders collected in Fall 2018 from the Grand River, ON (n=10/site except PT1 with n=8). See Figure 1 for site locations. Significant values are depicted in red.

Factor	Pair	Shannon (H)		Simpson (D)	
		Diff	P adjusted	Diff	P adjusted
Site	EIT-WMR	-2.616	0.013	-0.632	0.011
	EIT-PT1	-3.096	0.003	-0.862	<0.001
	EIT-PT2	-1.950	0.166	-0.577	0.031
	PT1-INV	2.348	0.068	0.679	0.010
	PT1-KIW	2.362	0.064	0.675	0.010
	PT1-FWY	2.356	0.066	0.637	0.020

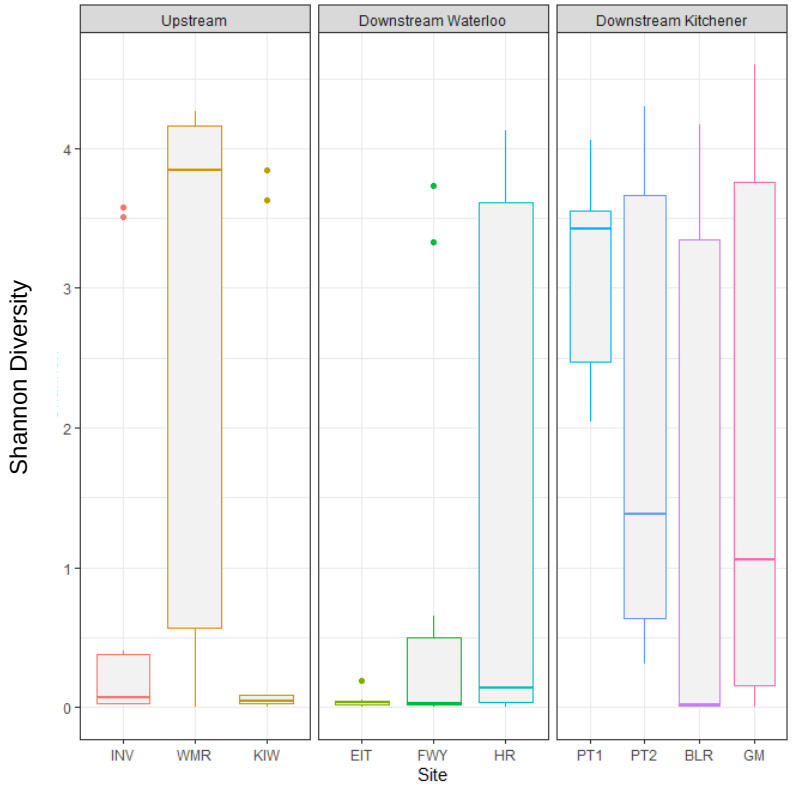

Figure 12. Bacterial alpha diversity (Shannon Index of Diversity) within individual, whole-body spiders, collected in Fall 2018 from sites upstream to downstream in the Grand River, ON (n=10/site except PT1 with n=8). See Figure 1 for site locations.

3.3.3. Aquatic Macroinvertebrates

In aquatic macroinvertebrates, alpha diversity values tended to be similar across taxa at these sites (exception was Baetidae with lower values), and diversity varied among sites within some (Perlidae, Hydropsychidae, Heptageniidae) but not all (Ephemerellidae, Baetidae) taxa (Figure 13). Ephemerellidae were only collected from the two most upstream sites (INV, WMR), while Baetidae were solely collected from the remaining eight sites. There were differences among sites in Shannon's, but not Simpson's, alpha diversity for Perlidae (one-way ANOVA: Shannon $F=3.1$, $df=9$, $p=0.0056$; Simpson $F=1.066$, $df=9$, $p=0.406$), Hydropsychidae (Shannon $F=3.674$, $df=9$, $p=0.0014$; Simpson $F=1.795$, $df=9$, $p=0.0931$), and Heptageniidae (Shannon $F=2.715$, $df=9$, $p=0.0143$;

Simpson F=1.703, df=9, p=0.12). In Perlidae, downstream Kitchener sites (PT1, PT2) were significantly less diverse than upstream sites (INV, WMR), respectively (Tukey HSD, Table 16). In Hydropsychidae, Shannon alpha diversity was significantly lower at site PT2 than upstream site KIW and all other downstream Kitchener sites (PT1, BLR, GM). Meanwhile, downstream Waterloo (FWY) was less diverse than downstream Kitchener (BLR).

Table 16. Tukey's HSD values from the one-way ANOVA test of Shannon and Simpson alpha diversity measures by site within whole-body benthic macroinvertebrates collected in Fall 2018 from the Grand River, ON (n=1-11/site). See Figure 1 for site locations. Significant values are depicted in red.

Invertebrate Family	Factor	Pair	Shannon (H)		Simpson (D)	
			Diff	P adjusted	Diff	P adjusted
Perlidae	Site	PT1-INV	-1.080	0.027	-0.025	0.999
		WMR-PT2	0.890	0.043	0.055	0.678
Hydropsychidae	Site	FWY-BLR	-0.634	0.030	-0.010	0.991
		PT2-BLR	-0.741	0.011	-0.027	0.271
		PT2-GM	-0.701	0.037	-0.034	0.092
		PT2-KIW	-0.607	0.049	-0.029	0.124
		PT2-PT1	-0.542	0.043	-0.023	0.229
Heptageniidae	Site	-	-	-	-	-
Ephemerellidae	Site	-	-	-	-	-
Baetidae	Site	-	-	-	-	-

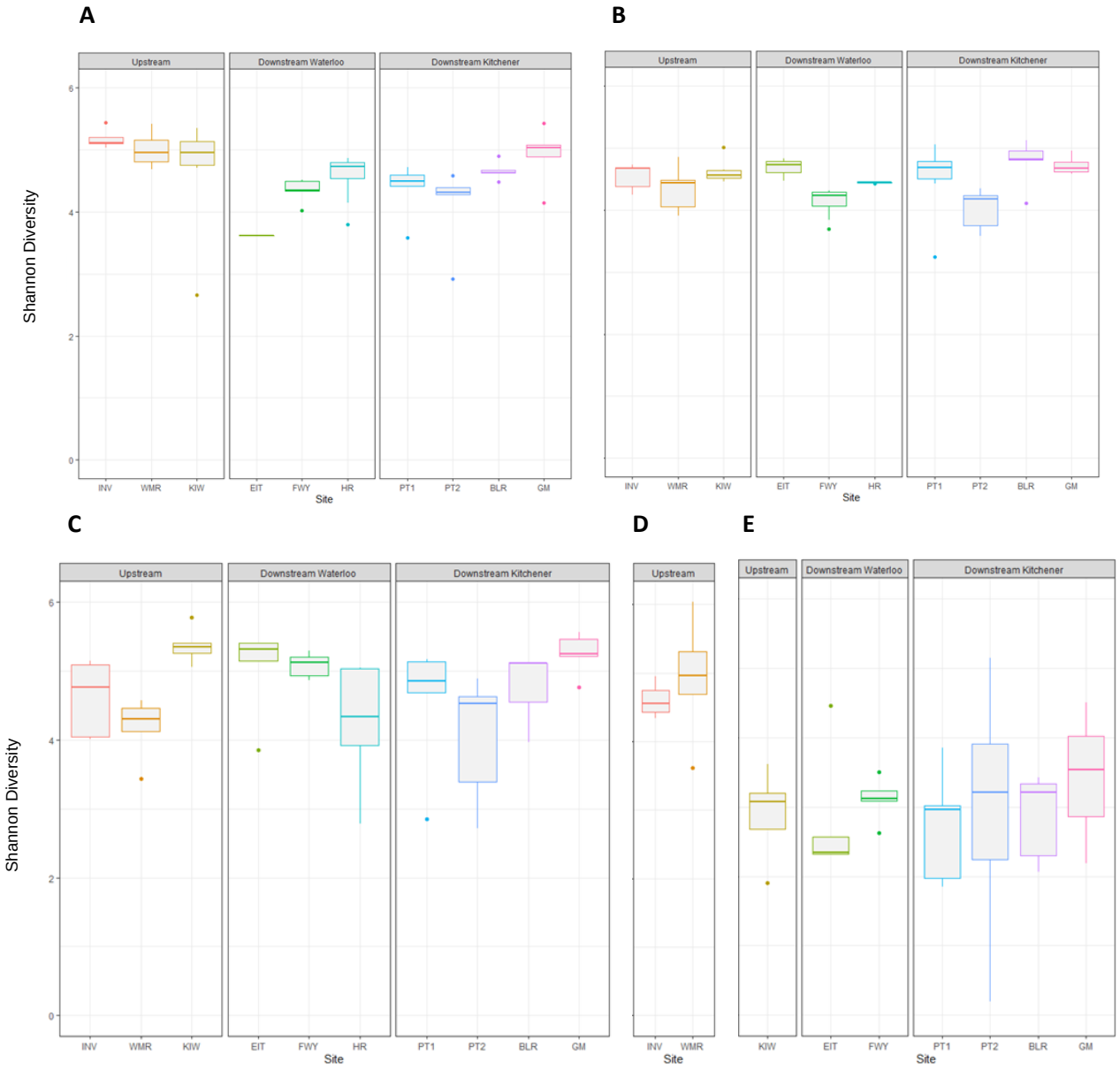

Figure 13. Bacterial alpha diversity (Shannon's Index) within whole-body benthic macroinvertebrates, collected in Fall 2018 from sites upstream to downstream in the Grand River, ON: A) Perlidae (n=1-10/site); B) Hydropsychidae (n=3-11/site); C) Heptageniidae (n=5/site); D) Ephemerellidae (n=5/site); E) Baetidae (n=3-5/site). See Figure 1 for site locations.

3.3.4. Water Samples

Water samples were similar in their level of bacterial diversity to mussel and invertebrate samples, and higher in diversity than spiders. As for the spider, Perlidae, and Baetidae samples, alpha diversity in river water was lowest downstream of the Waterloo outfall (EIT). Diversity was also low in these samples from sites WMR and KIW (upstream) (Figure 14). Alpha diversity in water from downstream of Waterloo (FWY, HR) and from all sites downstream of Kitchener were similar to that of the effluent samples from both WWTPs (WAT, KIT). However, the values in water differed among sites (one-way ANOVA: Shannon $F=17.2$, $df=13$, $p<0.0001$; Simpson $F=30.56$, $df=13$, $p<0.0001$). Upstream (INV), downstream Waterloo (FWY, DN, HR), and downstream Kitchener (PT2, BLR, GM) sites, as well as Waterloo WWTP effluent (WAT), were significantly more diverse than upstream sites WMR and KIW (Tukey HSD, Table S11 in Appendix A). Alpha diversity was significantly lower downstream of the Waterloo outfall (EIT) than all other sites, including effluent samples, except for the upstream site KIW. Meanwhile, Kitchener WWTP effluent (KIT) and downstream Kitchener sites (PT1, JN) were significantly more diverse than upstream site KIW.

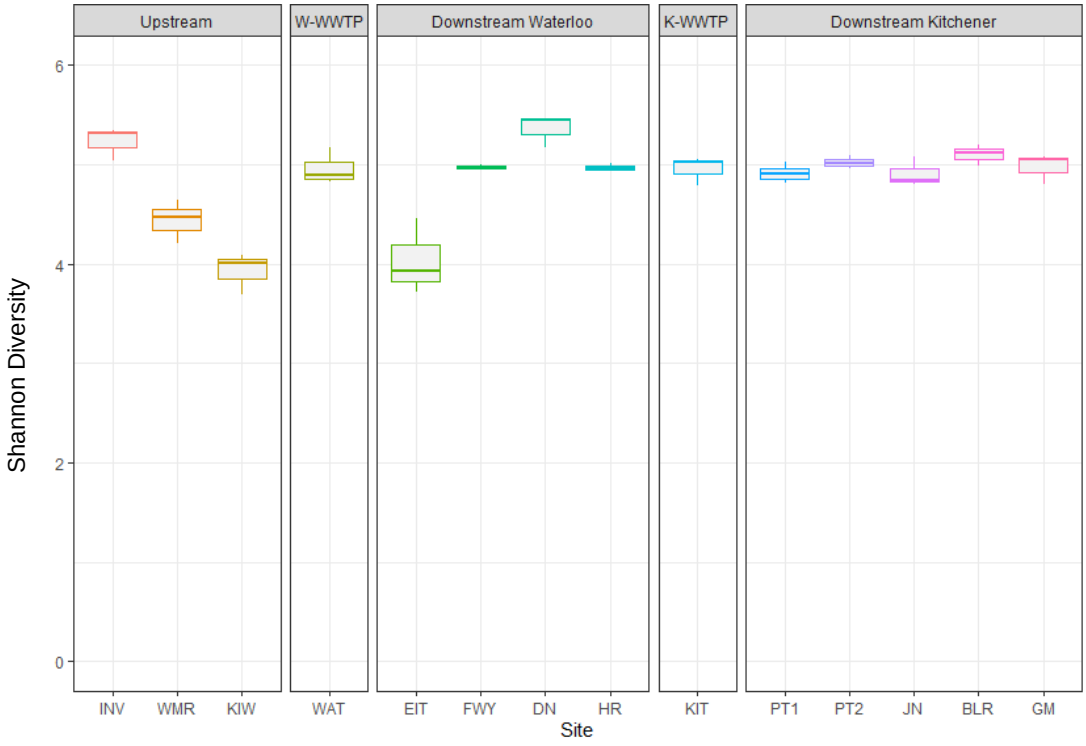

Figure 14. Bacterial alpha diversity (Shannon's Index) within river water samples as well as Waterloo (WAT) and Kitchener (KIT) WWTP effluent samples, collected in Fall 2019 from sites upstream to downstream in the Grand River, ON (n=3/site). See Figure 1 for site locations.

3.4. Bacterial Beta Diversity

3.4.1. Mussels

Beta diversity of bacteria in the digestive gland of mussels differed among sites (Permanova: df=4, Sum Sq=3.219, Mean Sq=0.805, F Model=3.241, $p=1x10^{-4}$), and accounted for 25.4% of the overall variation according to the effect size (R^2). The furthest downstream Kitchener site (GM) was significantly dissimilar from upstream (WMR, KIW) and downstream Waterloo (DN) sites (Adonis pairwise, Table 17). Upstream sites WMR and KIW were also significantly dissimilar from each other.

Table 17. Pairwise Adonis values from the Permanova analysis of Bray-Curtis beta diversity measures by site within the digestive glands of mussels collected in Fall 2018 from the Grand River, ON, using 99999 permutations. R^2 (effect size) values indicate how much of the overall variation in distances that can be explained by the factor being tested (n=10/site except JN with n=3). See Figure 1 for site locations. Significant values are depicted in red.

Factor	Pair	Bray-Curtis Dissimilarity	
		R^2	P Value
Site	WMR vs KIW	0.131	0.0003
	WMR vs GM	0.200	0.0003
	KIW vs GM	0.186	0.0002
	DN vs GM	0.136	0.0052

Individuals cluster by site (PCoA, Figure 15). Upstream (WMR, KIW) and downstream Waterloo (DN) sites tend to group together, while downstream Kitchener sites (JN, GM) overlap, indicating similarities in the bacterial composition of these sites. Greater dissimilarity among samples is observed within site GM, the furthest downstream site.

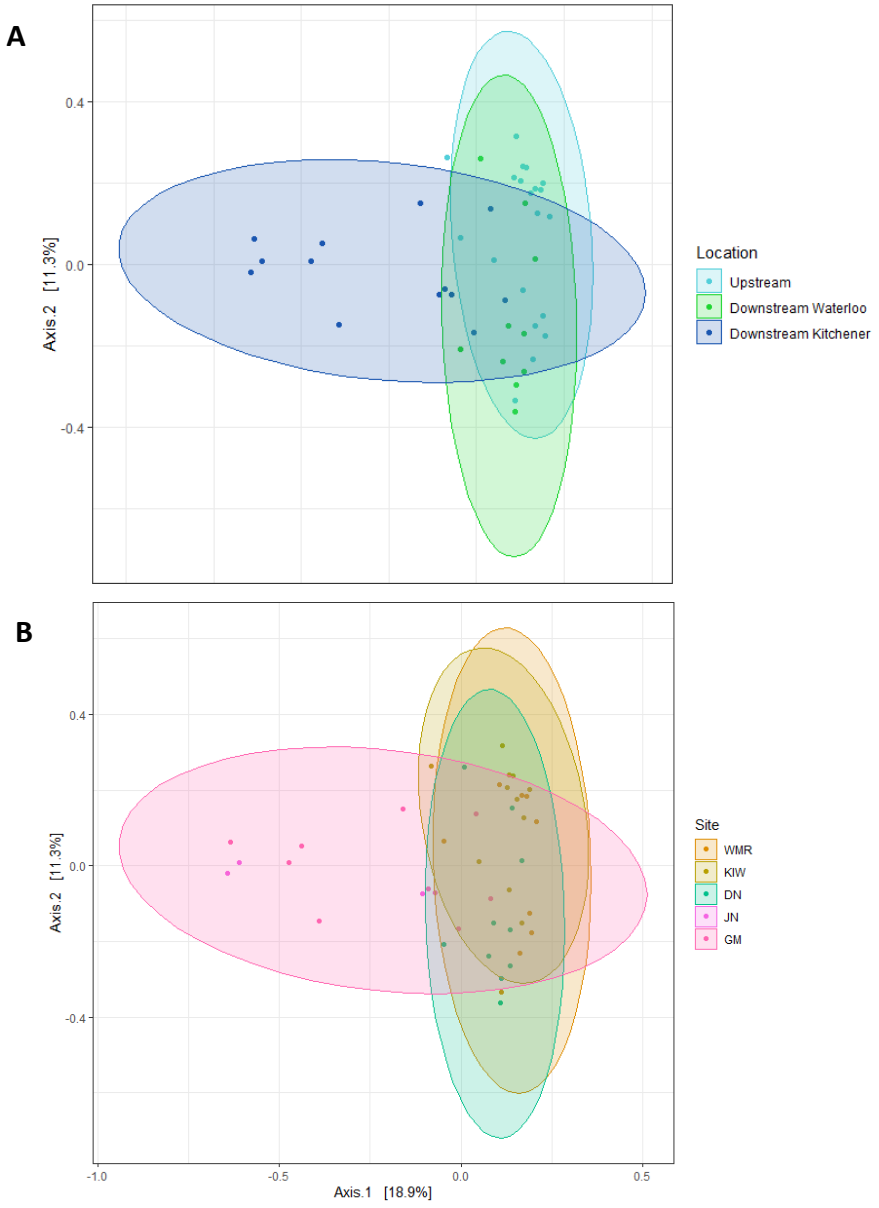

Figure 15. Principal coordinate analysis (PCoA) plots displaying the beta diversity between A) locations (all upstream sites (Upstream), all sites downstream of Waterloo WWTP (Downstream Waterloo) and all sites downstream of Kitchener WWTP (Downstream Kitchener) as well as B) sites within the digestive glands of mussels collected in Fall 2018 from the Grand River, ON. The Bray-Curtis Dissimilarity measure was used to construct the distance matrices used to generate this plot. Each coloured dot represents the microbiome of an individual sample (n=10/site except JN with n=3, hence no ellipse formed). See Figure 1 for site locations.

3.4.2. Spiders

Beta diversity differed among sites in whole-body spiders (Permanova: df=9, Sum Sq=6.776, Mean Sq=0.753, F Model=2.028, $p=1x10^{-4}$), and accounted for 17.2% of the overall variation according to the effect size. The Waterloo outfall site (EIT) was significantly dissimilar than upstream (WMR) and downstream Kitchener (PT1, PT2, GM) sites (Adonis pairwise, Table 18). Downstream Kitchener (PT1) was also significantly dissimilar from the upstream (INV) and downstream Waterloo (FWY) sites.

Table 18. Pairwise Adonis values from the Permanova analysis of Bray-Curtis beta diversity measures by Site within whole-body spiders collected in Fall 2018 from the Grand River, ON, using 99999 permutations. R^2 (effect size) values display how much of the overall variation in distances can be explained by the factor being tested (n=10/site except PT1 with n=8). See Figure 1 for site locations. Significant values are depicted in red.

Factor	Pair	Bray-Curtis Dissimilarity	
		R^2	P adjusted
Site	INV vs PT1	0.184	0.0410
	WMR vs EIT	0.273	0.0122
	EIT vs PT1	0.425	0.0018
	EIT vs PT2	0.291	0.0014
	EIT vs GM	0.218	0.0482
	FWY vs PT1	0.234	0.0499

Although beta diversity of individual spiders tended to cluster by site (PCoA, Figure 16), all sites tended to overlap, with upstream (INV), downstream Waterloo (EIT), and downstream Kitchener (PT1) samples displaying tight clustering, indicating a greater similarity in their bacterial composition. Within the remaining sites, there was more variability between individuals, indicating a greater dissimilarity between the bacterial composition of samples within those sites. Outliers in the data may be driving some of the variability observed within sites.

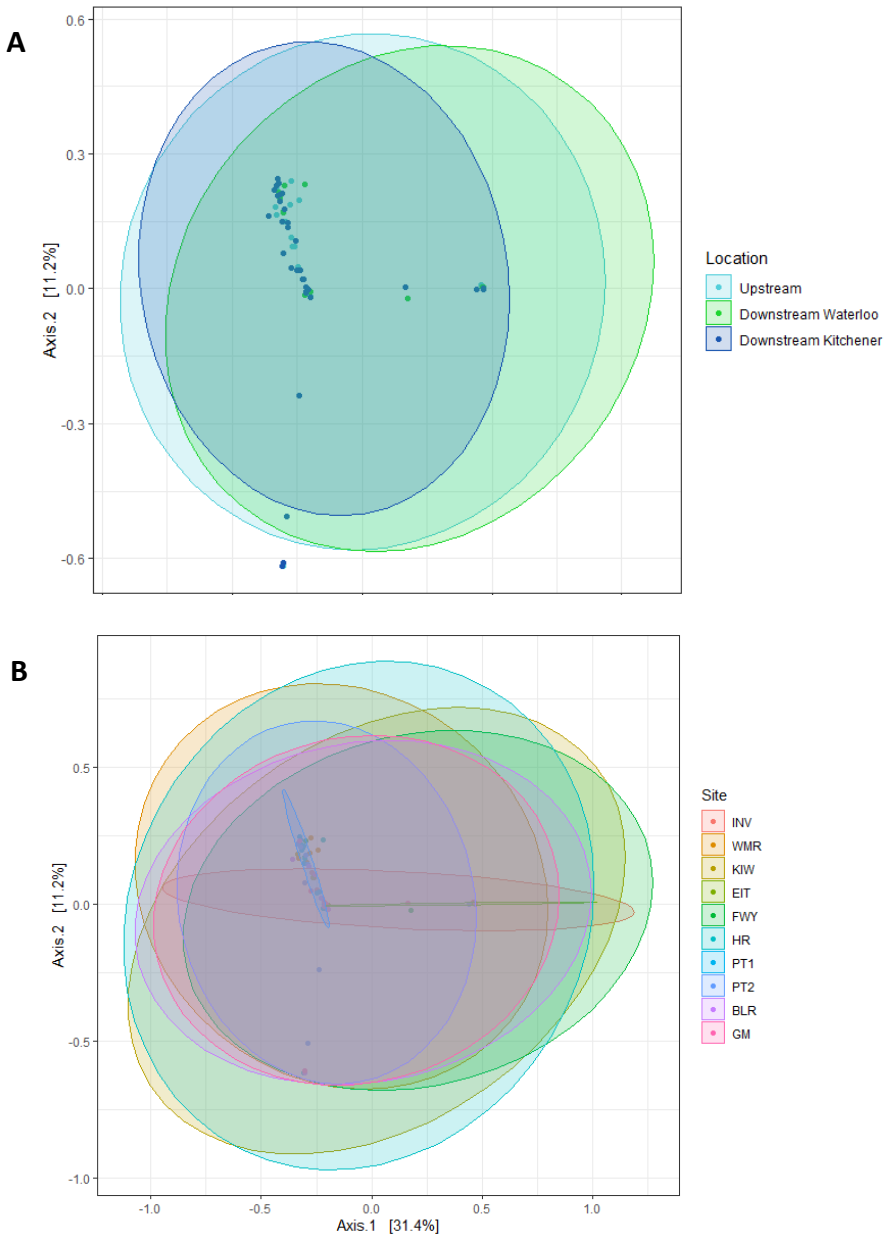

Figure 16. Principal coordinate analysis (PCoA) plots displaying the beta diversity between A) locations (all upstream sites (Upstream), all sites downstream of Waterloo (Downstream Waterloo) and all sites downstream of Kitchener (Downstream Kitchener) as well as B) sites within whole-body spiders collected in Fall 2018 from the Grand River, ON. The Bray-Curtis Dissimilarity measure was used to construct the distance matrices used to generate this plot. Each coloured dot represents the microbiome of an individual sample (n=10/site except PT1 with n=8). See Figure 1 for site locations.

3.4.3. Aquatic Macroinvertebrates

Within each taxon, the bacterial beta diversity of whole-body invertebrates differed by site and accounted for 27.4–44.4% of the overall variation according to the effect size (R^2) (Permanova, Table 19). In Perlidae, beta diversity of individuals from upstream (WMR) was significantly dissimilar from all other sites except downstream Waterloo (EIT) (Adonis pairwise, Table 20). Downstream Waterloo (HR) was also significantly dissimilar from upstream (INV) and downstream Kicthener (GM) sites in Perlidae. For Hydropsychidae, samples from downstream Kitchener (PT1) differed in beta diversity from upstream (INV, WMR, KIW) and downstream Waterloo (FWY, HR) sites, as well as site GM. Additionally, the beta diversity of downstream Waterloo (FWY) differed from all upstream sites (INV, WMR, KIW). Lastly, the two upstream sites with Ephemerellidae (INV, WMR) were significantly dissimilar.

Table 19. Values from the Adonis statistical test measuring beta diversity using the Bray-Curtis dissimilarity measure by site within whole-body benthic macroinvertebrates collected in Fall 2018 from the Grand River, ON, using 9999 permutations. R^2 (effect size) values display how much of the overall variation in distances can be explained by the factor being tested (n=10-59/taxa). Significant values are depicted in red.

Invertebrate Family	Factor	Df	Sum Sq	Mean Sq	F Model	R^2	P Value
Perlidae	Site	9	5.0185	0.55762	3.9976	0.444	<0.0001
Hydropsychidae	Site	9	4.3580	0.48423	2.7853	0.338	0.0001
Heptageniidae	Site	9	4.6385	0.51539	2.7709	0.384	0.0001
Ephemerellidae	Site	1	0.51569	0.51569	3.991	0.333	0.0069
Baetidae	Site	6	2.1963	0.36605	1.5754	0.274	0.0178

Table 20. Pairwise Adonis values from the Permanova analysis of Bray-Curtis beta diversity measures by site within whole-body benthic macroinvertebrates collected in Fall 2018 from the Grand River, ON, using 99999 permutations. R^2 (effect size) values display how much of the overall variation in distances can be explained by the factor being tested (n=1-11/site). Significant values are depicted in red.

Invertebrate Family	Factor	Pair	Bray-Curtis Dissimilarity	
			R^2	P adjusted
Perlidae	Site	WMR vs INV	0.266	0.0266
		WMR vs KIW	0.256	0.0108
		WMR vs HR	0.271	0.0023
		WMR vs FWY	0.373	0.0171
		WMR vs PT2	0.298	0.0149
		WMR vs BLR	0.395	0.0162
		WMR vs PT1	0.316	0.0144
		WMR vs GM	0.359	0.0140
		INV vs HR	0.329	0.0342
		HR vs GM	0.230	0.0356
Hydropsychidae	Site	WMR vs FWY	0.312	0.0383
		WMR vs PT1	0.282	0.0108
		INV vs FWY	0.217	0.0468
		INV vs PT1	0.205	0.0036
		KIW vs FWY	0.193	0.0320
		KIW vs PT1	0.186	0.0050
		HR vs PT1	0.191	0.0099
		FWY vs PT1	0.195	0.0009
		PT1 vs GM	0.205	0.0387
Heptageniidae	Site	-	-	-
Ephemerellidae	Site	WMR vs INV	0.333	0.0079
Baetidae	Site	-	-	-

The beta diversity within each macroinvertebrate taxa appeared to cluster by site (PCoA, Figure 18). Upstream sites (INV, WMR) diverged from other sites for Perlidae. Tight clustering was observed within individual sites INV, FWY, and BLR, and greater variability was observed at sites HR and PT1 (Figure 18a). Hydropsychidae sites tend to

overlap in beta diversity, with more clustering in upstream WMR and greater variability among remaining sites (Figure 18b). Greater dissimilarity between Heptageniidae samples was observed for sites WMR and HR, with clustering between samples from upstream site KIW and downstream Kitchener (PT2, GM). Upstream sites (INV, WMR, KIW) tended to overlap, while downstream Waterloo and Kitchener sites tended to cluster together (Figure 18c). Ephemerellidae displayed greater dissimilarity within upstream site WMR compared to INV (Figure 18d). For Baetidae, sites KIW and EIT overlapped, indicating a greater similarity, and there was tight clustering and overlap between sites FWY and BLR (Figure 18e).

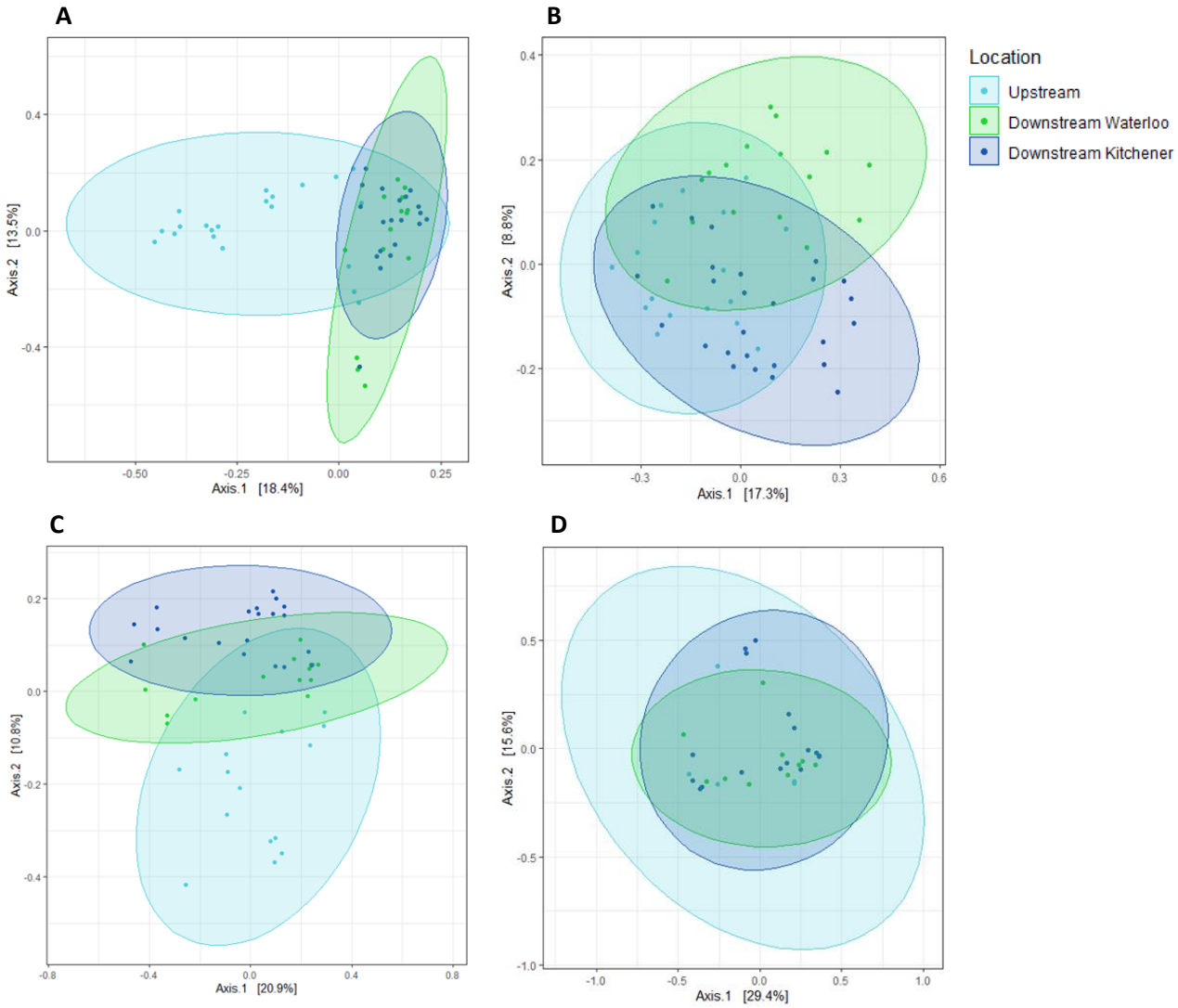

Figure 17. Principal coordinate analysis (PCoA) plots displaying the beta diversity between locations (all upstream sites (Upstream), all sites downstream of Waterloo (Downstream Waterloo) and all sites downstream of Kitchener (Downstream Kitchener) within whole-body benthic macroinvertebrates collected in Fall 2018 from the Grand River, ON: A) Perlidae (n=1-10/site); B) Hydropsychidae (n=3-11/site); C) Heptageniidae (n=5/site); D) Baetidae (n=3-5/site). The Bray-Curtis Dissimilarity measure was used to construct the distance matrices used to generate this plot. Each coloured dot represents the microbiome of an individual sample.

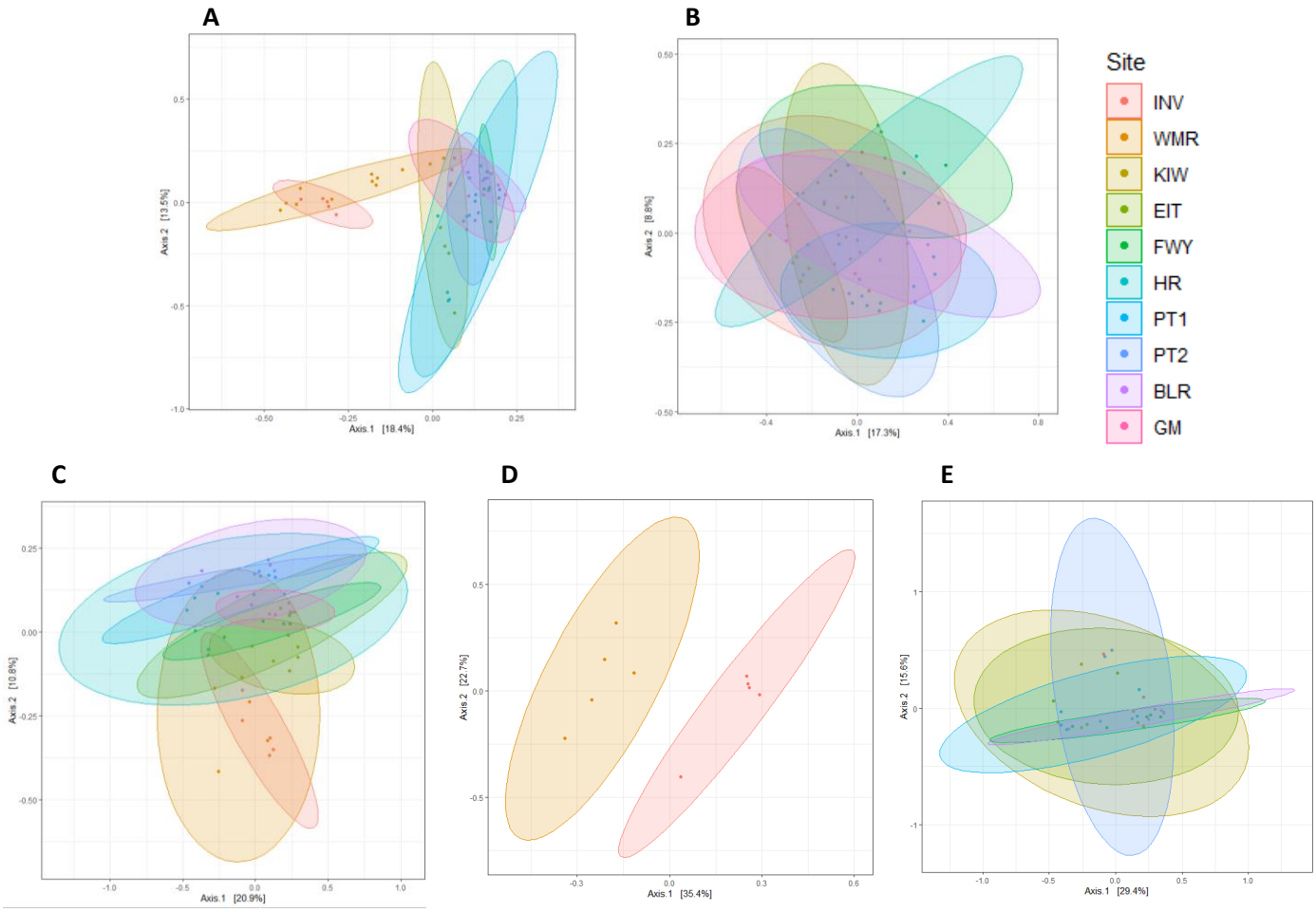

Figure 18. Principal coordinate analysis (PCoA) plots displaying the beta diversity between sites within whole-body benthic macroinvertebrates collected in Fall 2018 from the Grand River, ON: A) Perlidae (n=1-10/site); B) Hydropsychidae (n=3-11/site); C) Heptageniidae (n=5/site); D) Ephemerellidae (n=5/site); E) Baetidae (n=3-5/site). The Bray-Curtis Dissimilarity measure was used to construct the distance matrices used to generate this plot. Each coloured dot represents the microbiome of an individual sample. Note there were not enough samples to produce an ellipse for Perlidae (n=1) and Hydropsychidae (n=3) at site EIT, as well as for Baetidae (n=3) at site GM. See Figure 1 for site locations.

3.4.4. Water Samples

Beta diversity of the river water samples differed among sites (Permanova: df=13, Sum Sq=7.429, Mean Sq=0.571, F Model=12.468, $p=1x10^{-4}$), and accounted for 85.3% of the overall variation according to the effect size ($R^2=0.853$). However, no significant differences were found between sites using an Adonis pairwise test (P adjusted=1).

Beta diversity for the water samples tended to cluster by site (PCoA, Figure 19). The furthest upstream sites (INV, WMR) grouped together, with site WMR displaying greater dissimilarity between individual samples. Upstream KIW, downstream Waterloo (FWY, DN, HR), and downstream Kitchener (PT1, PT2, BLR) sites clustered together, indicating similarities between these sites. Site JN samples span a wider range of the PCoA, indicating greater dissimilarity between these samples. Sites EIT and GM are dissimilar from the other sites, with greater dissimilarity between individual samples at site EIT. Both WWTP effluents tended to cluster together and were dissimilar from the Grand River water samples.

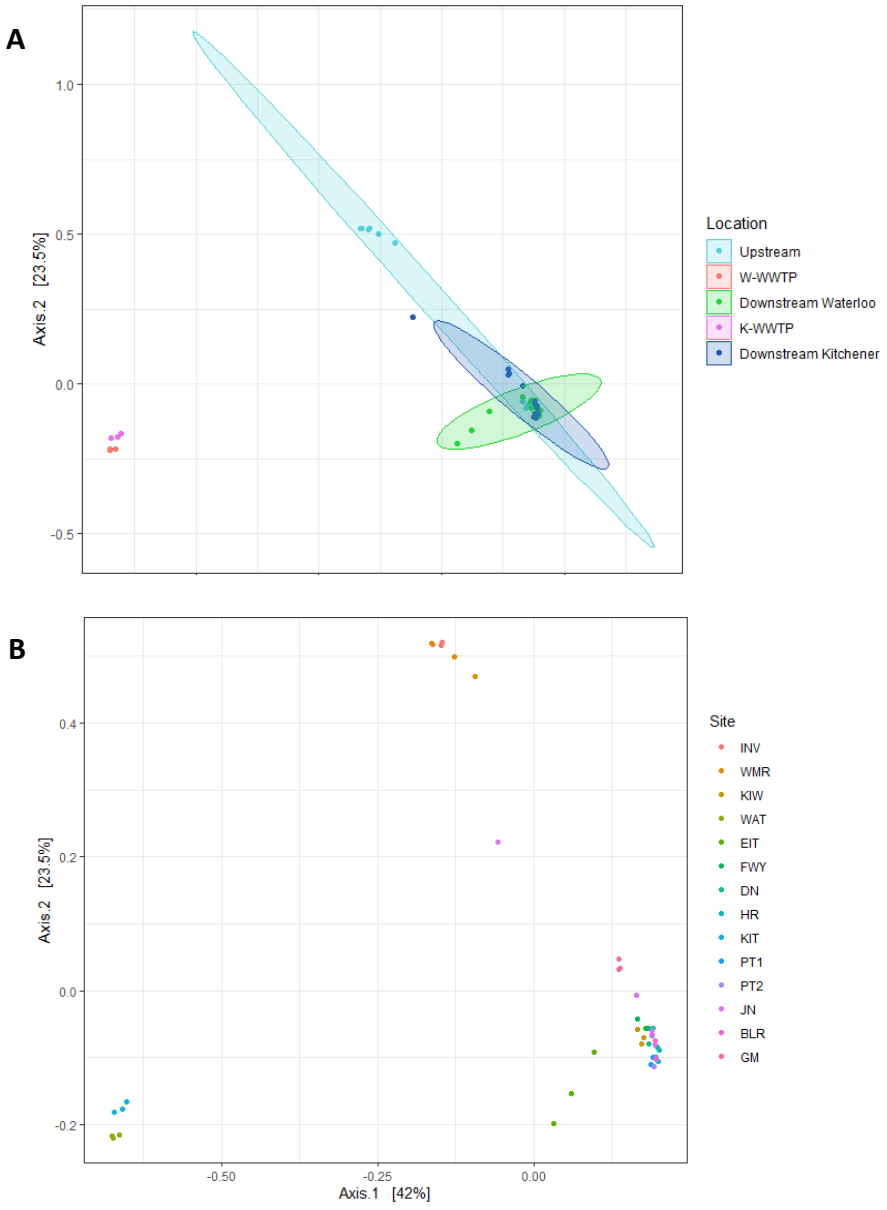

Figure 19. Principal coordinate analysis (PCoA) plots displaying the beta diversity between A) locations (all upstream sites (Upstream), all sites downstream of Waterloo (Downstream Waterloo) and all sites downstream of Kitchener (Downstream Kitchener) as well as B) sites within Grand River water and Waterloo (WAT) and Kitchener (KIT) WWTP effluent samples collected in Fall 2019, ON. The Bray-Curtis Dissimilarity measure was used to construct the distance matrices used to generate this plot. Each coloured dot represents the microbiome of an individual sample (n=3/site). See Figure 1 for site locations.

3.4.5. All Taxa and Water Samples

Beta diversity of individual samples appeared to cluster by sample type and sometimes among groups when all organisms and water data were combined (PCoA, Figure 20). Mussels, spiders, and effluent samples clustered together, indicating the greatest similarity between their bacterial compositions. All aquatic macroinvertebrates, except for Hydropsychidae, tended to group together; Hydropsychidae were more dissimilar from all other insect taxa, as well as mussels, despite their common filter-feeding strategy. Across all groups, spiders had the broadest range in beta diversity with both tight clustering for some samples and then also multiple outliers. Upstream river water samples were most similar to the beta diversity within mussel samples, with the downstream Waterloo and Kitchener river water samples clustered together and dissimilar from all invertebrate taxa and effluent samples.

Beta diversity differed between invertebrate taxa and water samples (Permanova: df=9, Sum Sq=68.439, Mean Sq=7.6043, F Model=28.099, $p=1x10^{-4}$), and accounted for 40.0% of the overall variation according to the effect size (R^2). All sample types were dissimilar from each other in terms of their bacterial compositions, with the exception of Ephemerellidae and both wastewater effluents, as well as between wastewater effluents (Adonis pairwise, Table S12 in Appendix A).

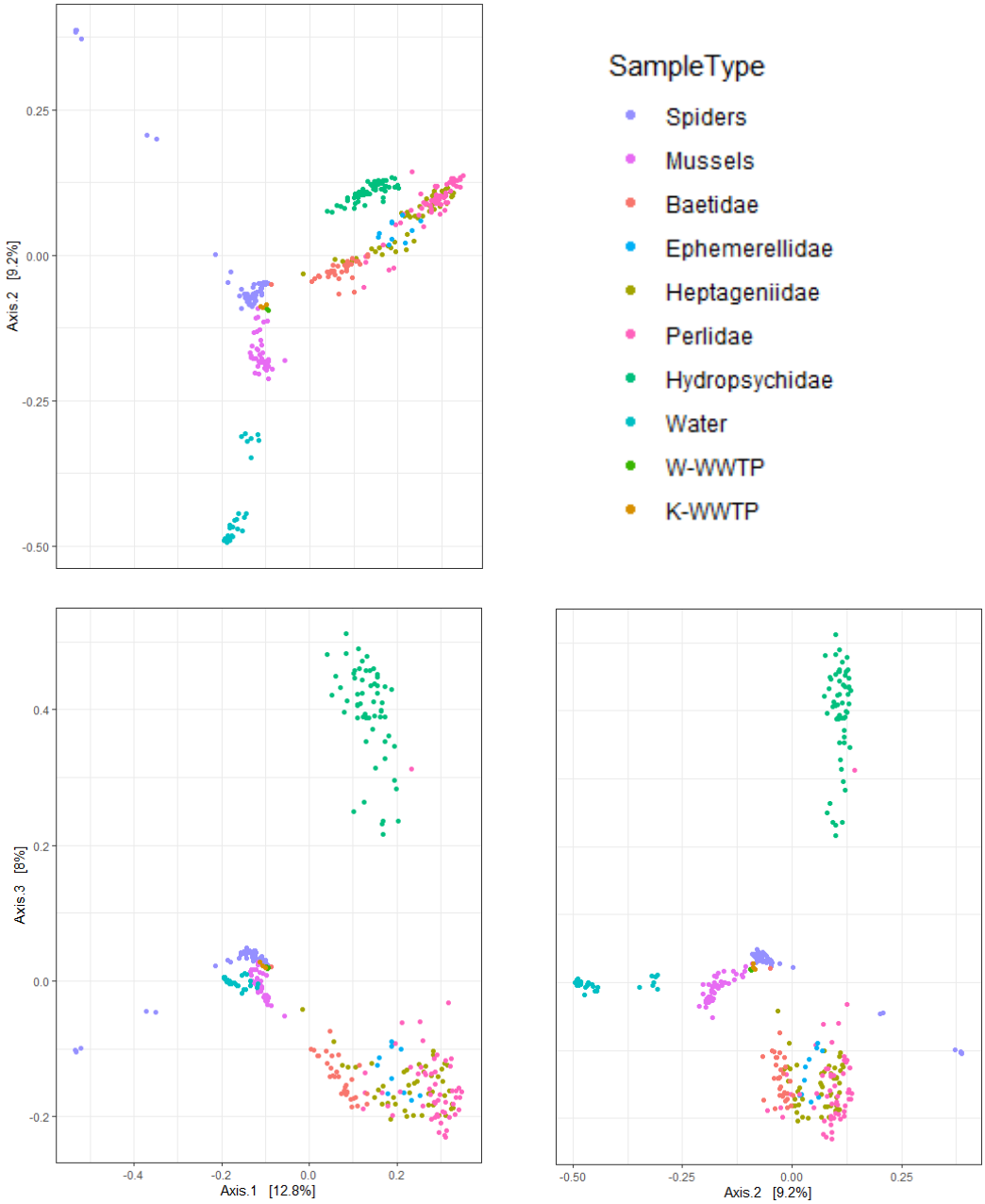

Figure 20. Principal coordinate analysis (PCoA) plot displaying the beta diversity between all taxa, water, and effluent samples across sites collected in Fall 2018 (taxa) and 2019 (water) from the Grand River, ON. The Bray-Curtis Dissimilarity measure was used to generate this plot. Each coloured dot represents the microbiome of an individual sample (n=389). Axes 1, 2, and 3 explain 12.8%, 9.2%, and 8% of the variation in the data, respectively.

4. Discussion

4.1. Effects of WWTP Effluent on Bacterial Composition and Diversity

The most common bacterial phyla within the microbiomes of samples from the Grand River were Proteobacteria, Bacteroidetes, and Cyanobacteria, followed by Firmicutes and Tenericutes within biota, and Actinobacteria and Epsilonbacteraeota within river water. In humans, gut microbes play a role in various functional pathways such as metabolism, biosynthesis, degradation, transport, and repair (Jandhyala et al., 2015; Kho & Lal, 2018), however, these roles are largely unknown in aquatic organisms. Looking at the changes in the abundance and diversity of bacteria responsible for these roles may give a better picture of how organisms are affected physiologically by wastewater exposure. Proteobacteria is often associated with poor health in human and mammalian studies, while Firmicutes and Bacteroidetes have been associated with the reverse (Jandhyala et al., 2015; Kho & Lal, 2018). While some bacteria are disease-causing (pathogenic), many are beneficial to organisms as they help with the metabolism and absorption of nutrients; an example is the nitrogen-fixing and denitrifying bacteria as they play important roles in nitrogen cycling within organisms (Lundberg et al., 2004).

4.1.1. Mussel Digestive Gland

Mussels collected from downstream of the Kitchener WWTP outfall had changes in bacterial relative abundance, number of bacterial taxa, alpha diversity, and beta diversity. Similarly, Pacific oysters in disturbed environments had decreased bacterial alpha diversity in their gill microbiome (Wegner et al., 2013). However, mussels at these sites on the Grand River are also exposed to inputs from additional WWTPs and urban areas from the Speed River which may be contributing to the changes in the bacterial communities, and it is unclear whether these shifts may affect mussel health.

As found herein, high proportions of Proteobacteria have been found in the gut of bivalves in previous studies (Weingarten et al., 2019). Several genera of Proteobacteria

increased downstream of the outfalls in the Grand River; *Tabrizicola* (family - *Rhodobacteraceae*) commonly associated with sulfur and carbon biogeochemical cycling (Chen et al., 2014), increased downstream of the Waterloo WWTP. *Rhizobiales Incertae Sedis* and *Beijerinckiaceae* increased past the Waterloo and decreased past the Kitchener WWTP outfalls; both are families of nitrogen-fixing bacteria from the order Rhizobiales, with the latter also being a methanotroph (Tamas et al., 2014). Mussels are commonly associated with increased nitrate and nitrite in aquatic ecosystems due to their consumption of algae from the water column, suggesting nitrifying activities by microbes in their gut (Pfister, 2007). *Legionella*, a human pathogen that causes Legionnaire's disease and Pontiac fever, pneumonia-type and flu-like illnesses (Diederen, 2008), increased downstream of the Kitchener outfall. Interestingly, in my study there were also increases in the arthropod and spider endosymbiont *Rickettsiella* (Zhang et al., 2018) in mussels downstream of Kitchener.

Within the phylum Tenericutes, the genus *Mycoplasma* increased in mussels downstream of the Waterloo WWTP. *Mycoplasma* species and members of the Mollicutes class are commonly found in mussels (Aceves et al., 2018; Cleary et al., 2015). King et al. (2012) found that oysters were dominated by Mollicutes and that these human pathogens may act as commensal microbes in the gut of molluscs. Mycoplasmas contain sialic acid lyase genes able to block pathogens from attaching to the stomach wall of deep-sea isopods, and genes for proteolysis and oligosaccharide degradation which may help the host survive when nutrients are low (Wang et al., 2016). Meanwhile, Pacific oysters in disturbed environments had increases in *Mycoplasma* in their gill microbiome (Wegner et al., 2013).

Within Firmicutes, *Clostridium sensu stricto 1*, a genus associated with the metabolism of carbohydrates, amino acids, alcohols, and purines (Alou et al., 2018), increased in mussels downstream of the Kitchener WWTP. The genus *Clostridium* includes many important pathogenic bacteria, and *Clostridium* species tend to accumulate

in marine and freshwater mussels, posing a threat towards terrestrial and other aquatic animals (Cleary et al., 2015; Weingarten et al., 2019; Winters et al., 2011).

4.1.2. Riparian Spiders

In spiders, the number of bacterial taxa, as well as bacterial alpha and beta diversity changed with respect to the WWTP outfalls. Downstream of the Waterloo WWTP outfall there were increases in the Proteobacteria genera *Rickettsiella* and *Diplorickettsia*, the latter being a human pathogen and a potential symbiont of the hard tick (*Ixodes ricinus*; Taylor et al., 2012). Within Tenericutes, *Mycoplasma,* a genus commonly known to cause infection in humans (Leblan, 2006), was only found in abundance at sites downstream of the Waterloo WWTP. Meanwhile, *Corynebacterium 1* (Actinobacteria), a genus known to cause disease in humans, with some considered zoonotic agents (Oliveira et al., 2017), increased downstream of the Kitchener WWTP.

4.1.3. Aquatic Macroinvertebrates

In aquatic macroinvertebrates, the number of bacterial taxa observed at each rank as well as alpha diversity decreased in Perlidae, Hydropsychidae, and Heptageniidae, with changes in beta diversity in Perlidae and Hydropsychidae with respect to WWTP outfalls. Studies have shown decreases in the bacterial diversity and richness of sediment downstream of WWTP outfalls most likely due to the homogenization of bacterial communities by toxic compounds (Drury et al., 2013; Lu & Lu, 2014). Therefore, similar trends may be observed in sediment-dwelling invertebrates downstream of WWTPs.

Several genera of the phylum Proteobacteria changed in abundance downstream of both WWTPs, and some of these bacteria have been linked to nutrient cycling, contaminant breakdown, or human and fish diseases. Several Proteobacteria genera increased in abundance downstream of one WWTP outfall in one or several macroinvertebrates, including *Rhodoferax,* a genus of phototropic and denitrifying bacteria (Waterloo WWTP only; Hiraishi et al., 1991). *Rhodobacter* and *Tabrizicola* are

genera from the family *Rhodobacteraceae*, commonly associated with sulfur and carbon biogeochemical cycling, with the second also being a phototrophic genus isolated from lakes and industrial effluents (Kitchener WWTP only; Chen et al., 2014; Ko et al., 2018; Tarhriz et al., 2019). *Sphingorabdus* is a genus of *Sphingomonadaceae*, a family known to degrade several PAHs and xenobiotics (Kitchener WWTP only; Glaeser & Kämpfer, 2014), and *Brachidontes* mussels from marine lakes near urban areas have increased abundances of these bacteria coupled with an increase in xenobiotic degradation pathways (Cleary et al., 2015). *Ideonella* contains species capable of breaking down polyethylene terephthalate plastic (Waterloo WWTP only; Palm et al., 2019), and *Aeromonas* is a genus that causes disease in humans and fish and is commonly found in freshwater ecosystems (Janda & Abbott, 2010). In contrast, some decreases were observed downstream of the Waterloo WWTP including *Pseudorhodobacter* associated with sulfur and carbon cycling, and *Sphingorhabdus* linked to PAH degradation (Chen et al., 2014; Glaeser & Kämpfer, 2014).

Within the phylum Bacteroidetes, *Flavobacterium* increased downstream of the Waterloo WWTP in Perlidae and Hydropsychidae. Several species of *Flavobacterium* are pathogenic and cause numerous diseases in freshwater fish (Bernardet & Bowman, 2006).

4.1.4. River Water

When compared to the two most upstream sites (INV, WMR), the number of different bacterial taxa at each rank increased while beta diversity and relative abundance changed in Grand River water at the third upstream site (KIW) onwards, and this was likely due to the shift from rural (INV, WMR) to urban and (large) municipal wastewater (KIW onwards) inputs. Above KIW, the Conestogo River and Canagagigue Creek join the Grand River, bringing discharges from the St Jacobs WWTP and Elmira WWTP, respectively, as well as agricultural inputs. With respect to the Waterloo WWTP, alpha diversity decreased at the outfall site (EIT), contrary to some studies that have shown

increases in bacterial diversity in the water column resulting from wastewater effluent (García-Armisen et al., 2014).

Bacterial alpha diversity in river water decreased downstream of the Waterloo WWTP outfall (EIT) and increased in sites downstream of Kitchener. The latter is in contrast to the decreases in alpha diversity observed in mussels, Perlidae, and Hydropsychidae, which may be related to changes in sediment bacterial community abundance and diversity, which is known to decrease as a result of wastewater exposure (Drury et al., 2013; Lu & Lu, 2014). Interestingly, Waterloo WWTP effluent contained almost 8-fold the number of ASVs found in Kitchener WWTP effluent, indicating a greater abundance of bacteria in the effluent of the Waterloo WWTP. This may be due to a difference in influent quality or wastewater treatment between the two WWTPs, as the Kitchener WWTP has recently been upgraded (2012-2017; Region of Waterloo, 2018; Hicks et al., 2017). In addition to looking at changes in bacterial diversity and composition, functional metagenomics are recommended to further our understanding of the specific functional microbial pathways within the microbiome that are affected by wastewater exposure.

4.1.5. Effluent-associated Bacteria in Taxa and River Water

Bacteria from Waterloo WWTP effluent were found in the microbiomes of taxa and river water samples downstream of both WWTPs and not in any upstream sites. Three genera were also found in river water samples collected downstream of the Kitchener WWTP, derived from either Waterloo or Kitchener WWTP effluents, and not found in upstream samples.

4.1.5.1. Bacteria Linked to Wastewater Treatment

Many of the effluent-associated genera found in both river water and biota at the wastewater-impacted sites have functional roles in wastewater treatment processes. Found in mussel, spider, and Baetidae samples downstream of the WWTPs, *Ochrobactrum* is

used to quantitatively reduce heavy metals in sewage sludge (Ozdemir et al., 2003). *Ottowia* was found in downstream Hydropsychidae samples and is an anaerobic digester commonly found in municipal wastewater and activated sludge (Spring et al., 2004). *Fusibacter* was found in downstream Baetidae samples and is used to treat wastewater in anaerobic sludge by reducing arsenate (Serrano et al., 2017). A cholesterol degrader able to tolerate high ammonia concentrations (Holert et al., 2018; Shao et al., 2019), *BD1-7 clade* was found in downstream river water, Baetidae, and mussel samples. Found in downstream river water and spiders, *Steroidobacter* degrades steroids, such as testosterone, with some species capable of denitrification and are commonly found in anoxic digested sludge (Fahrbach et al., 2008). The genus *Acidaminococcus* is capable of breaking nitrogen bonds in azo dye (Zhu et al., 2018) and was also found in downstream river water and spiders.

Some effluent bacteria found solely in downstream river water are also involved in the reduction and conversion of chemical compounds in wastewater. *Acetoanaerobium* can be found in anaerobic wastewater sludge and produces acetate from H_2 and CO_2, while *Candidatus Cloacimonas* is also an anaerobic digester commonly found in municipal wastewater and activated sludge (Yekta et al., 2019; Sleat et al., 1985). *Desulfobacter* is used to treat wastewater in anaerobic sludge by reducing sulfur (Ding et al., 2016). The sulfate-reducing genus *Thermovirga* is also capable of amino acid degradation, while the genus *AUTHM297* is thought to be a fermenter capable of converting sulfur (or thiosulfate) to H_2S (Briones et al., 2007; Dahle & Birkeland, 2006; Zhang et al., 2019). Meanwhile, *C1-B045* has been found in oil contaminated sites in China degrading PAHs (Liao et al., 2015; Peng et al., 2020). Bacteria such as *Aquimonas* are involved in the reduction of nitrate to nitrite in low oxygen conditions and is used in aerobic granular sludge (Cydzik-Kwiatkowska et al., 2017; Xiao et al., 2012). *BD1-7 clade* is known to tolerate high ammonia concentrations and raw lagoon supernatant percentages and is a cholesterol degrader (Holert et al., 2018; Shao et al., 2019). *Proteiniclasticum* has been noted for its role in degrading proteins and other carbon

sources, while *Succinivibrio* has been found to ferment glucose and produce hydrogen in anaerobic conditions (Amorim et al., 2018; Patterson & Hespell, 1985; Yin et al., 2017). Meanwhile, *U29-B03* can degrade complex organics resulting in sulfide generation (Baldwin et al., 2015). At the third upstream site (KIW) onwards, there were also increases in the sulfur-oxidizing phylum Epsilonbacteraota in river water (Waite et al., 2017).

Some effluent-associated genera have also been found in wastewater treatment processes by other studies, but their roles in wastewater are currently unknown. *Bifidobacterium*, only found in downstream spiders, are common indicators of human fecal pollution in treated wastewater (Wéry et al., 2010). Also only in downstream spiders, *Actinomyces* has been found in WWTP sewage and sludge (Valour et al., 2014). Genera commonly reported in municipal wastewater (Allen et al., 2006; Ji et al., 2019; Jia et al., 2020; Kim et al., 2014; Kong et al., 2019; Liu et al., 2016; Nesbø et al., 2019), *Cloacibacterium* was found in downstream river water, spiders, and mussels, *Chiayiivirga* was found in downstream river water and Perlidae, and *Lelliotia* was found in downstream river water and mussels. Meanwhile, the nematode endosymbiont *Candidatus Paenicardinium* (Noel & Atibalentja, 2006) was found in downstream river water, Heptageniidae and spider samples. Some effluent genera were only found in downstream river water samples. *Leptotrichia* has been found in WWTP sewage and sludge (Eribe & Olsen, 2008). The genera *Cloacibacterium, Chiayiivirga, Lelliotia, Planctopirus, SC103, Turneriella,* and *Mesotoga* have also been found in municipal wastewater (Allen et al., 2006; Ji et al., 2019; Jia et al., 2020; Kim et al., 2014; Kong et al., 2019; Liu et al., 2016; Nesbø et al., 2019). *Trichococchus* is found in urban sewage influent and bulking sludge (Scheff et al., 1984; Vandewalle et al., 2012). Finally, downstream river water also included common endosymbiont bacteria of amoeba, such as *Candidatus Protochlamydia* and *Neochlamydia* (Collingro et al., 2005; Horn et al., 2000).

4.1.2.2. Bacteria found in Humans and Other Animals

Many of the effluent-associated bacteria found in downstream river water and biota are also commonly found in the oral, digestive, and genital tracts of humans and ruminants (cattle, sheep, etc.). *Ruminococcus 1* and *Faecalibacterium* are considered important members of the core human gut microbiome (La Reau et al., 2016; Martín et al., 2018). *Faecalibacterium* increased in river water, while *Ruminococcus 1* increased in river water, mussels, and spiders downstream of the Waterloo outfall. *Bifidobacterium*, only found in downstream spider samples, are typically regarded as good gut bacteria (Wéry et al., 2010). Also found in downstream spiders, *Actinomyces* normally colonizes the mouth, digestive, and genital tracts of humans (Valour et al., 2014). *Prevotellaceae UCG-004* was found in downstream Baetidae samples and are a genus of *Prevotellaceae*, a family commonly found in the GI tracts of humans and ruminants and are important for carbohydrate and protein breakdown (Lopes et al., 2015; Thoetkiattikul et al., 2013). Found in downstream river water and spiders, *Acidaminococcus* commonly colonizes the human digestive tract (Ricaboni et al., 2017). *Romboutsia* is commonly found in the digestive tract of humans and increased in river water at the third reference site (KIW) onwards, as well as in Perlidae, Heptageniidae, and Baetidae samples downstream of the WWTPs (Gerritsen, 2015). *Turicibacter* has been found in human feces and increased in river water at the third upstream site (KIW) onwards, as well as in mussel, Perlidae, and Heptageniidae samples downstream of WWTPs (Auchtung et al., 2016). *Clostridium sensu stricto 1* is associated with the metabolism of carbohydrates, amino acids, alcohols, and purines in the human gut and increased in river water at upstream site KIW onwards, as well as in Perlidae, Heptageniidae, and Baetidae samples downstream of WWTPs (Alou et al., 2018).

There were also effluent-associated bacteria found solely in downstream river water related to the human and animal GIT. *Leptotrichia* was found in downstream river water and normally colonizes the mouth, digestive, and genital tracts of humans (Eribe & Olsen, 2008), while *Acidaminococcus* has been found in the human digestive tract

(Ricaboni et al., 2017; Zhu et al., 2018). Both ruminant bacteria, *Proteiniclasticum* has been noted for its role in degrading proteins and other carbon sources, while *Succinivibrio* has been found to ferment glucose and produce hydrogen in anaerobic conditions (Amorim et al., 2018; Patterson & Hespell, 1985; Yin et al., 2017). Meanwhile, *U29-B03* can degrade complex organics resulting in sulfide generation and is also found in the first stomach of ruminants (Baldwin et al., 2015; Zeng et al., 2020).

4.1.2.3. Pathogenic Bacteria Linked to Humans and Other Animals

Effluent genera also included common pathogenic bacteria that were found in the microbiomes of taxa and river water downstream of the WWTPs. Found in mussel, spider, and Baetidae samples, *Ochrobactrum* is a pathogen in humans (Ozdemir et al., 2003). Also found in spiders, *Actinomyces* can sometimes cause infections in humans called actinomycosis (Cyprowski et al., 2018). A common pathogen in humans (Nyaoke et al., 2020), *Paeniclostridium* increased in river water from the third upstream site (KIW) onwards, as well as in mussels downstream of the WWTPs. Another common pathogen, *Bacillus* increased in river water at the third upstream site (KIW) onwards, as well as in downstream mussels and Baetidae. Some species of *Bacillus* are used as a source of insecticidal toxins spread over fields from airplanes (Helgason et al., 2000). Lastly, in river water from the third upstream site (KIW) onwards, there were increases in the genus *Arcobacter*, an opportunistic pathogen of vertebrates (Waite et al., 2017).

4.2 Cyanobacteria

Cyanobacteria are a phylum of aquatic photosynthetic bacteria that often grow in colonies known as algal blooms. Cyanobacteria are important sources of oxygen in our atmosphere; however, their blooms in nutrient-enriched water bodies and subsequent decay can lead to oxygen depletion in many aquatic ecosystems (Huisman et al., 2018; Paerl et al., 2001). Cyanobacteria commonly enter the body via ingestion of algae and therefore pose a large risk to the digestive flora of aquatic organisms, and may be

transported from the GIT to other cells of the body, especially that of the liver (Bownik, 2016).

The activated sludge used in secondary wastewater treatment contains ideal conditions for the growth of Cyanobacteria due to the abundance of inorganic nutrients (nitrogen and phosphorus), light availability, and temperature. The presence and growth of Cyanobacteria in WWTPs may contribute to the development of blooms and cyanotoxins in receiving water bodies (Mur et al., 1999; Martins et al., 2011). In this study, Cyanobacteria were found in both effluent and river water samples. Secondary metabolites produced by Cyanobacteria may also negatively impact other microbes used to degrade organic matter, reducing wastewater treatment efficiency (Martins et al., 2011). At the tertiary level, some Cyanobacteria have proven effective in treating wastewater by removing nutrients, organics, and contaminants (Chevalier et al., 2000; Cuellar-Bermudez et al., 2017).

4.2.1 Mussel Digestive Glands

Mussels from the Grand River had lower amounts of overall Cyanobacteria, including multiple toxin-producing genera, in sites downstream of the wastewater treatment plants when compared to upstream (exception *Merismopedia 0BB39S01*). Bivalves absorb dissolved cyanotoxins from the water and from cyanobacterial cells, however, they are also thought to be efficient at expelling living toxic cells via pseudofeces, allowing them to better tolerate toxic algal blooms (Bownik, 2013). Cyanobacteria produce three main cyanotoxins: hepatotoxins, neurotoxins, and irritant toxins (dermatoxins). The most common hepatotoxin, microcystin (MC), is commonly found in freshwater, primarily affects the liver, kidneys, and reproductive system (Bownik, 2013), and causes elevated levels of the stress marker glutathione-S-transferase enzyme in the digestive gland, as well as changes in its activity in the gut of bivalves (Burmester et al., 2012; Vasconcelos et al., 2007). MCs can also change the expression of proteins involved in bivalve cytoskeleton assembly (Martins et al., 2009). Downstream of

WWTPs, MC-producing genera *Microcystis*, *Planktothrix*, and *Snowella* (Clercin, 2012; Dittmann et al., 2013; Oudra et al., 2002) decreased in mussel digestive glands. *Mycrocystis* spp. also produce the hepatotoxin, nodularin (Clercin, 2012). Another hepatotoxin, cylindrospermopsin, primarily affects the liver and kidneys (Zanchett & Oliveira-Filho, 2013), and the cylindrospermospin-producing genus *Aphanizomenon* decreased downstream of WWTPs in mussels. Saxitoxins are neurotoxins and known to cause Paralytic Shellfish Poisoning; saxitoxin-producing genera *Planktothrix* and *Aphanizomenon* (Dittmann et al., 2013; Zanchett & Oliveira-Filho, 2013) similarly decreased in mussels downstream of WWTPs. Another form of neurotoxin, anatoxins affect the central nervous system. Anatoxin-producing genera *Aphanizomemon*, *Microcystis*, *Snowella,* and *Planktothrix* (Clercin, 2012; Dittmann et al., 2013; Zanchett & Oliveira-Filho, 2013; WHO, 2003; US EPA 2020) all decreased downstream of WWTPs.

Despite the increased presence of nutrients at outfall sites (Figure S1 in Appendix A) typically associated with Cyanobacterial blooms (Mur et al., 1999), there were decreases in overall Cyanobacterial abundance in downstream mussel digestive glands. Higher abundances of Cyanobacteria in sites upstream of the WWTP outfalls could indicate poor mussel health due to increased cyanotoxin exposure, therefore future studies should be conducted to investigate the health of mussels at these sites in relation to cyanobacterial exposure.

4.2.2 Aquatic Macroinvertebrates

Cyanobacteria in general increased in abundance downstream of the Waterloo WWTP outfall (EIT) in Perlidae, Heptageniidae, and Baetidae insects and may be linked to increased nutrient load provided by the wastewater, causing blooms of Cyanobacteria in the river (O'Neil et al., 2012) (Figure S1 in Appendix A, nutrient data). Increases in Cyanobacteria in downstream insects but not in mussels may be due to a difference in diet source. Aquatic macroinvertebrates are more likely to consume Cyanobacteria from benthic algae, whereas mussels filter-feed algae from the water column. Therefore,

effluent-induced blooms of Cyanobacteria may be occurring in the benthic algae. The genus *Tychonema* is known to produce anatoxins, a form of neurotoxin that affects the central nervous system (Dittmann et al., 2013), and contrary to that observed in river water, all invertebrates increased in *Tychonema CCAP 1459-11B* downstream of the Kitchener WWTP. Interestingly, the MC-producing genera, *Snowella 0TU37S04* and *Planktothrix NIVA-CYA 15*, generally decreased downstream of WWTP outfalls. Some invertebrate species are thought to accumulate MCs, which may be then transferred up the food chain to higher predators, such as fish and spiders (Bownik, 2013). For example, the benthic macroinvertebrate, *Procambarus clarkii* (red swamp shrimp), has been shown to accumulate these cyanotoxins in their intestines (Tricarico et al., 2008). The effects of cyanotoxins on freshwater benthic macroinvertebrate health are currently unclear and further research, such as exposure studies, are required to further this understanding.

4.2.3 Riparian Spiders

Spiders also contained small proportions of Cyanobacteria, with cyanotoxin-producing and dominant genera varying according to individual and site. Two cyanotoxin-producing genera, *Planktothrix NIVA-CYA 15* and *Tychonema CCAP 1459-11B*, were present at upstream site WMR, potentially exposing spiders to both MCs and anatoxins. Similar to the aquatic macroinvertebrates, the presence of *Planktothrix NIVA-CYA 15* in spiders was restricted to upstream of the WWTPs.

The low relative abundances of Cyanobacteria in spiders collected herein is likely due to their diet. Tetragnathidae spiders are riparian obligate aquatic insect feeders, exposing them to benthic algae and Cyanobacteria through their diet of emerging insects, ecologically linking terrestrial and aquatic ecosystems (Collier et al., 2002; Tagwireyi & Sullivan, 2015). Spiders have also been shown to bioaccumulate MCs from their diet of aquatic insects, such as Chironomidae (Takahashi et al., 2014). Cyanobacteria has been shown to dominate in the larval and pupal stages of mosquitoes, with the transition into adulthood causing drastic changes in community structure, resulting in low or

undetectable levels in newly emerged adults (Wang et al., 2011). Therefore, the low overall abundance of Cyanobacteria in spiders compared to their aquatic insect prey may be due to insect metamorphosis, including the reabsorption of the gut, decreasing the presence of Cyanobacteria in emerging insects.

Unlike river water samples - mussels, spiders, and invertebrate (Perlidae, Heptageniidae, Baetidae) samples showed increases in *Cyanobium PCC-6307* in sites downstream of the Waterloo WWTP, potentially indicating sources of Cyanobacteria from WWTP effluent discharge. Information on the relative toxicity of this genera is lacking, however, all Cyanobacteria produce irritant toxins (inflammatory agents; skin, gastrointestinal irritants) called lipopolysaccharides (LPS) (Clercin, 2012; Dittmann et al., 2013; Zanchett & Oliveira-Filho, 2013). While *Cyanobium PCC-6307* and *Tychonema CCAP 1459-11B* were more abundant in downstream invertebrates, only small proportions of these genera were observed in river water samples. Therefore, WWTP effluents may be causing Cyanobacterial blooms primarily in benthic algae, rather than in the water column.

4.2.1.4. River Water

Contrary to invertebrates, river water samples in the third upstream site (KIW) and onward were dominated by *Planktothrix NIVA-CYA 15*, a cyanotoxin-producing genus, with decreases in all other Cyanobacteria genera. As previously mentioned, these compositional changes may be due to a shift in rural to urban geography, or due to WWTP or agricultural inputs from the Conestogo River and Canagagigue Creek which join the Grand River above upstream site KIW.

Although similar genera of Cyanobacteria were observed in both the water column and biota, their exact source in invertebrates is unknown, as these bacteria are also commonly found in benthic biofilms. Additionally, the number of river water and effluent samples collected was restricted to 3 replicates and sampled during a different time period than invertebrates, therefore their role as sources of Cyanobacteria in biota is

limited. Although Cyanobacteria in effluents could not be identified to the genus-level, *Cyanobium PCC-6307* and *Tychonema CCAP 1459-11B* may proliferate in the wastewater treatment process and subsequently cause benthic algal blooms, which would explain increases in downstream invertebrates.

4.3 Endosymbiont Bacteria

Riparian spiders from the Grand River had an unusually low number of ASVs and bacterial taxa at each rank compared to other invertebrates, which may be due to the presence of dominant endosymbiont bacteria. Endosymbionts are bacteria commonly found in arthropods and are maternally transmitted, allowing them to be maintained over generations of hosts (Vanthournout & Swaegers, 2011). This symbiotic relationship does not rely on environmentally acquired microbes and ensures stable vertical transmission and coevolution with the host insect, usually through manipulation of reproductive physiology and behaviour. Maternal transmission is necessary, as males represent evolutionary "dead ends" for endosymbiont bacteria. These bacteria have adapted by developing mechanisms in which they increase the number of resulting female offspring to further their transmission and increase the number of infection targets in the population by increasing male embryo mortality rates, feminizing male embryos, or causing parthenogenesis (Martin and Goodacre, 2009). Male-killing microbes have also been shown to reverse sex roles, causing typical male behaviours to be observed in females (Jiggins et al., 2000). Studies have noted the importance of looking at the hologenome, including the genomes of symbiotic bacteria in determining physiological, behavioural, and evolutionary processes (Lewis & Lizé, 2015). Therefore, looking at spatial changes in endosymbiont bacteria may provide a clearer picture on the effects of wastewater effluent exposure in riparian spiders along the Grand River.

All spiders in this study contained endosymbiont bacteria (commonly *Wolbachia, Rickettsia, Spiroplasma, Cardinium,* and *Rickettsiella*) and there were changes downstream of the WWTP outfalls including decreases in *Cardinium* and *Rickettsia* and

increases in *Rickettsiella* (Waterloo WWTP), and increases in *Wolbachia* (Kitchener WWTP). *Cardinium* is known to influence the oviposition choice of the parasitoid wasp *Encarsia pergandiella* while *Rickettsia* affects the long-term dispersal behaviour of the money spider *Erigone atra* (Goodacre et al., 2009; Kenyon & Hunter, 2007). *Rickettsiella* spp. have been known to infect invertebrates and vertebrates, including the respiratory tract of vertebrates, leading to chronical bronchopneumonia (Grabowski & Klein, 2017). *Wolbachia* increases mating rate and modifies host preferences in *Drosophila* species (De Crespigny et al., 2006; Miller et al., 2010). *Wolbachia* also causes cytoplasmic incompatibility to make gametes between infected males and uninfected females incompatible, allowing infected females to be more reproductively successful in the population, and furthering the transmission of the bacteria (Lewis & Lizé, 2015). *Spiroplasma*, found in spiders from upstream and downstream Kitchener sites, causes spider male fatality, and increases the rate of cannibalism by infected ladybird beetle hatchlings, promoting the survival of the bacteria (Nakamura et al., 2006). Overall, some endosymbionts, except for *Rickettsiella* and *Wolbachia*, decreased downstream of WWTPs for reasons that are unclear.

One potential explanation for the change in endosymbionts in spiders downstream of WWTP effluents is their exposure to wastewater-derived contaminants via emerging insects (Richmond et al., 2018), and this may have implications for the spider population. Vanthournout & Swaegers (2011) found that the solitary dwarf spider *Odeothorax gibbosus*, infected with *Wolbachia*, produced a significantly female-biased sex ratio. They also found that treatment of *Wolbachia*-infected females with the antibiotic tetracycline restored offspring sex ratios, therefore antibiotics in wastewater effluent may impact the presence of naturally occurring endosymbiont infections in riparian spiders, resulting in the production of more male offspring.

Endosymbiont bacteria (*Rickettsiella*, *Rickettsia, Spiroplasma*, *Wolbachia*) were also found in aquatic invertebrates from the Grand River, some invertebrate taxa (Perlidae, Heptageniidae, Baetidae, Ephemerellidae) had higher presence of them than

others (Hydropsychidae), and some endosymbionts were found in spiders but not macroinvertebrates. Unlike for spiders, *Candidatus Cardinium* and *Arsenophonus* were not found in any invertebrate samples, whereas *Rickettsiella* was the most common endosymbiont in both spider and invertebrate samples. As in spiders, endosymbiont bacteria play a similar role in insect host evolutionary processes due to their influence on the reproductive system. In *Nasonia* (parasitoid wasp) species, *Wolbachia* decreases mate discrimination while gut bacteria, in response, cause lethality in hybrids to avoid interspecific mating (Brucker & Bordenstein, 2013; Lewis & Lizé, 2015). This is potential evidence of the interaction between competing endosymbiont bacteria and gut bacteria, and the adaptation of the gut microbiome in response to endosymbiont manipulation.

4.3. Differences in Bacterial Communities among Components of the Food Web

4.3.1. Effect of Taxon on Bacterial Richness

The invertebrate taxa sampled from the Grand River varied considerably in bacterial richness, with mussels and spiders containing the highest and lowest numbers of taxa at each rank, respectively. Mussels may have more bacterial taxa present due to their role as filter-feeders and their residence within sediments, allowing them to suspension-feed from the water column and interstitial water within sediment, exposing them to bacteria within both media as well as concentrating bacteria from their environment (Cooke, 1976; Ripabelli et al., 1999; Selegean et al., 2001; Vaughn et al., 2008; Yeager et al., 1994). The low number of bacterial taxa present in the microbiome of whole-body spiders may be attributed to a dominance of endosymbiont bacteria resulting in increased competition for the establishment of microbes (Vanthournout & Hendrickx, 2015).

There were also differences in the richness of bacterial taxa within the benthic macroinvertebrates from the Grand River that may be due to their dietary habits. Perlidae contained the highest number of taxa at each rank, followed by Heptageniidae, Baetidae,

Ephemerellidae, and Hydropsychidae. Higher trophic level predators such as Perlidae require a diverse gut microbiota composition to digest prey, whereas scrapers like Heptageniidae feed primarily on algae, biofilms, and organic sediments where bacteria tend to concentrate (Cole, 1982; Lopez et al., 2010; Liu et al., 2016). Fine particulate organic matter (FPOM) is a substrate for growth and an important form of dissolved organic carbon for microbes (Cummins & Klug, 1979; Meyer, 1994). Gatherer collectors such as Baetidae and Ephemerellidae feed on FPOM from the river bottom where more bacteria are harboured in sediment, while Hydropsychidae are filterer-collectors feeding on FPOM from the water column where bacterial density and richness tend to be lower (Drake et al., 1998; García-Moyano et al., 2012; Luo et al., 2019). These factors may contribute to the differences in taxa richness between the functional feeding groups (Appendix B).

4.3.2. Effect of Taxon on Bacterial Diversity and Composition

Alpha diversity was lowest in spiders and Baetidae and tended to be higher in water samples and other insects, and some samples grouped together in their beta diversity (all insects except Hydropsychidae; mussels, spiders and effluents). Taxa in this study are linked via complex food web interactions which likely has implications for the transfer of bacteria among aquatic species as well as from aquatic to riparian species. For example, mussel biodiversity has been found to indirectly promote benthic macroinvertebrate diversity and abundance, even in polluted areas (Bially & MacIsaac, 2000; Chowdhury et al., 2016). This is likely because the nutrients excreted by mussels (inorganic nitrogen and phosphorus) increase algal production, which in turn increases aquatic insect grazing and emergence rates (Allen and Wesner, 2012; Pfister, 2007; Spooner & Vaughn, 2006; Spooner et al., 2012; Spooner, & Vaughn, 2008; Vaughn & Spooner, 2006; Vaughn et al., 2007). Tetragnathid spiders have been found to receive nearly 100% of their diet from aquatic sources, as they spin horizontal webs over water to catch aquatic emerging insects (Allen & Wesner, 2012; Sanzone et al., 2017). As a result,

increases in mussel species richness have been associated with increased spider standing crop biomass (Allen, 2011). These trophic interactions may help explain the similar diversity of some taxa examined herein.

Even though some invertebrate taxa examined herein have similar dietary habits, this was not reflected in similar bacterial communities of their guts. Both gatherer-collectors, Baetidae and Ephemerellidae were dissimilar in terms of their bacterial alpha and beta diversity, as well as relative abundance. Mussels and Hydropsychidae are both filter-collectors, filtering particles from the water column, yet they were also dissimilar in terms of bacterial diversity and composition. Although many studies have shown a strong influence of diet on the gut microbiome (Scott et al., 2013; Singh et al., 2017), the observed differences in diversity and composition herein may be attributable to taxonomic differences rather than feeding strategy. In a study by Kroetsch et al. (submitted), it was found that alpha and beta diversity, as well as operational taxonomic unit (OTU) relative abundance was significantly influenced by the genus, family, and order of aquatic benthic invertebrates, rather than their functional feeding group.

The gut bacteria of aquatic invertebrates can be affected by various conditions such as diet, habitat and season (Ding et al., 2017), but taxonomy is a key driver (Kroetsch et al. submitted) and appeared to play a considerable role in my study and in other types of organisms. There is a strong impact of host genetics on the gut microbiome community of human adults with varying levels of genetic relatedness, revealing little environmental effects on the host gut microbiome (Zoetendal et al., 2006). Species impacted bacterial richness and composition of lady beetles, and although all species had similar gut communities, there may be a group of core species-specific bacteria present driving differences (Tiede et al., 2017). Similarly, mussel species had a greater influence on microbiome composition than did site conditions and that species-specific selective retention of bacterial taxa by mussels occurs (Weingarten et al., 2019).

4.4. Potential Limitations

Limitations associated with my study include a lack of established and standardized protocols for microbiome sampling of aquatic and riparian invertebrates in the field. The methods used in this study were developed from suggestions provided by the Surette Lab at McMaster University (Hamilton, ON) and adapted from published studies. As such, there is potential to improve the field and laboratory methods to further prevent contamination and degradation of microbiome samples, and to better isolate the gut microbiome of samples. Rather than freezing spiders and aquatic macroinvertebrates for an extended period of time, individuals could be placed directly into genomic buffer solution after rinsing externally with ethanol and UltraPure water. There may also be ways to better isolate the gut of spiders and insects, such as sampling only the abdomen for microbiome analysis and discarding the rest of the body. For mussels, it could be advantageous to reduce the time between initial collection from the river and digestive gland sampling into genomic buffer solution. In a timed dissection study, mussel digestive glands were dissected immediately in the field or 5.75 hours later in the lab and their microbiomes compared (Figure S2 in Appendix A) (Millar, unpublished data). Mussels dissected later in the lab showed an increase in Verrucomicrobia and slight increase in Proteobacteria, with decreases in Cyanobacteria and Firmicutes. Therefore, the time between the collection and sampling of the organisms may influence the bacterial composition and diversity of the gut microbiome and should be minimized where possible.

The organisms I sampled are localized in their habitat use, therefore, I assumed that there was exposure of the biota to wastewater effluent at sites downstream of the two wastewater treatment plants; however, I do not have confirmation that this occurred. For future studies, species could be analysed for stable isotopes, as in Hicks et al. (2017), and run for pharmaceutical analysis to confirm wastewater exposures (Richmond et al., 2018). Effluents also dissipate as they travel further downstream of the outfall, resulting in lower exposures at downstream sites. There is also the possibility that observed effects on the

host-associated microbiomes are caused by other point sources of pollution in the river, such as agriculture, industry, or other anthropogenic activity. In future studies, the dilution of wastewater effluents and inputs from other contaminant sources should be quantified and/or considered.

The number and distance of sites from WWTPs may also influence the observed changes in the gut microbiome of taxa. Spiders and benthic macroinvertebrates were collected from more sites (10 sites) than mussels (5 sites). The number and location of sites used for spider and aquatic insect collection allowed for a tighter bracketing and closer proximity to the WWTP outfalls. Therefore, I have a higher confidence that changes observed in the gut microbiome of aquatic insects and riparian spiders at sites directly upstream (ex. KIW) and directly downstream (ex. EIT) of a WWTP (ex. Waterloo WWTP) are related to exposure to that WWTP's effluent. However, mussels were collected at fewer sites and at sites further apart (some >5 km). These sites were selected as they have been part of a long-term mussel research project by ECCC's Gillis lab. Therefore, changes observed in the digestive gland microbiome of mussels may be due to exposure to WWTP effluent and/or other urban and rural inputs in the Grand River or incoming rivers/creeks.

Although I focused my sampling on sites upstream and downstream of two large WWTPs (servicing >130,000), there are other anthropogenic inputs to these sites including from the rivers and creeks that discharge into the Grand River. The Speed River receives effluent from the Hespeler and Guelph WWTPs and joins the Grand River above sites JN and GM; both downstream of the Kitchener WWTP (Region of Waterloo, 2018). The Conestogo River contains the St Jacobs WWTP effluents and the Canagagigue Creek contains the Elmira WWTP effluents, both of which join the Grand River above upstream site KIW. There are also other smaller WWTPs that discharge directly into the Grand River, including the Fergus, Elora (above INV), Conestogo (above KIW), Preston, and Galt (above GM) WWTPs (Anderson, 2012; Region of Waterloo, 2018). Sampling at other sites on the Grand River and in upstream tributaries could be used to identify

whether these other municipal wastewater discharges are having similar effects as observed herein or contributing to responses observed at the sites I sampled. It would be interesting to investigate whether there are cumulative effects of exposure to multiple WWTP effluents at downstream sites, as well as if there are signs of recovery in the composition of gut microbial communities in samples collected further downstream.

There are also temporal limitations that may have influenced the comparisons I made among taxa. Spiders were sampled earlier in the year than mussels and benthic invertebrates. Spiders were sampled as late as possible in the field season to ensure the collection of matured adults. Around late September and early October, Tetragnathidae tend to die off as temperatures begin to drop. Mussels were also collected earlier than benthic invertebrates, as they tend to burrow down into the sediment for the winter by mid October as water temperatures drop, making their collection more difficult. To obtain mature benthic invertebrates, I chose to collect them later in the fall at the end of October. This also happened to be when our collaborators were conducting their field studies, allowing a comparison of the gut microbiome of fish and their benthic invertebrate prey. River hydrology and wastewater effluent discharge and composition vary over time, therefore exposure to effluent may not have been similar across all sampling periods. The diversity and composition of the gut microbiome may also vary with season and sampling period, as observed in a study of the gut microbiome of aquatic macroinvertebrates by Kroetsch et al. (submitted).

Lastly, I chose 5 families of benthic invertebrates to study the effect of functional feeding group on their gut microbiomes (Appendix B). However, recent research (Kroetsch, et al., submitted) and the results of this study indicate a strong influence of taxa in shaping the composition and diversity of the gut microbiome. Therefore, differences between insect gut microbiota may be due to taxonomic differences rather than diet; this would be better examined using taxonomically similar biota that vary in their dietary habits.

4.5. Conclusions and Future Directions

This study presents a first look at the potential effects of wastewater treatment plant effluent on the gut and whole-body microbiome of riparian and aquatic macroinvertebrates in the field, specifically exposure from Waterloo and Kitchener WWTP effluents in the Grand River, Ontario, Canada. Proteobacteria increased downstream of the Waterloo WWTP in spiders and downstream of the Kitchener WWTP in mussels and Baetidae. Tenericutes and Bacteroidetes tended to decrease downstream of the WWTPs in most taxa, while Cyanobacteria increased downstream of the Waterloo WWTP in most aquatic insects. An increase in the Cyanobacteria genera *Cyanobium PCC-6307* in most taxa and *Tychonema CCAP 1459-11B* in aquatic insects were associated with WWTP outfalls, while an increase in the arthropod endosymbiont *Rickettsiella* was associated with WWTP outfalls in mussels and spiders. A decrease in alpha diversity in spiders and river water was associated with the Waterloo WWTP outfall, while a decrease in bacterial alpha diversity in mussels and Perlidae was associated with the Kitchener WWTP. Beta diversity differed downstream of Kitchener in mussels and differed downstream of Waterloo in spiders. Additionally, I found effluent-derived bacteria established within the microbiomes of taxa and river water in sites downstream from both WWTPs, with the majority coming from Waterloo WWTP effluent.

My project establishes baselines for the composition and diversity of the digestive gland microbiome of flutedshell mussels, gut microbiome of aquatic benthic macroinvertebrates, and whole-body microbiome of Tetragnathid spiders impacted by wastewater effluent in the field. Future studies should include a controlled lab study to determine the direct effects of WWTP effluent on the gut microbiome of aquatic and riparian invertebrates. To better understand the physiological consequences of wastewater exposure on the gut microbiome, functional metagenomics should be conducted to determine if functional microbial pathways are affected, such as macronutrient

metabolism. There is also the need to investigate the core microbiome among invertebrate taxa and how these bacteria are impacted downstream of WWTPs.

Sampling of the sediment and other substrates for microbiome analysis may also be useful in comparing the bacterial communities in benthic habitats to those within the gut of benthic macroinvertebrates. For example, mussels are burrowed partially or entirely into the sediment for most of their lifetime, therefore porewater microbial communities may better reflect those within the mussel rather than communities from the water column (Weingarten et al., 2019). There is also a need to study the gut microbiome of different functional feeding groups without the influence of taxa. Therefore, choosing a lower taxonomic rank such as a single order or family of closely related benthic invertebrates may help reduce taxonomic influences shaping the gut microbiome when investigating functional feeding groups. There is also the potential to study changes in the gut microbiome of aquatic insects throughout metamorphosis, from larval stages to adulthood. This would be interesting as aquatic insects reabsorb their gut in the final stages of metamorphosis. This information would provide clarity regarding the bacteria that riparian spiders are exposed to via their diet of emerging insects. In addition, it would be useful to collect emergent insects from the webs of riparian spiders to identify their prey and whether they obtain their diet from more aquatic or terrestrial sources, as this could also impact the source of microbes within their microbiome. Finally, analyzing the eukaryotic DNA within gut microbiome samples may also give an idea of the prey and detrital sources being fed upon by the taxa in this study as a potential source of environmental bacteria.

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Appendix A

Table S1. Percent relative abundance of the top six bacterial phyla across sites, from upstream to downstream in mussel digestive glands collected in Fall 2018 from the Grand River, ON (n=10/site except JN with n=3). See Figure 1 for site locations. Vertical lines indicate WWTP outfalls occur at some point in the river between the two listed sites.

Bacterial Phyla	**Site (%)**				
	WMR	KIW	DN	JN	GM
Proteobacteria	49.1	54.6	52.4	73.7	52.2
Cyanobacteria	25.8	22.8	14.0	16.6	14.6
Firmicutes	7.7	7.6	10.2	8.1	7.9
Tenericutes	3.0	3.0	10.5	0.2	11.5
Bacteroidetes	5.9	4.9	5.4	0.3	7.0
Actinobacteria	2.8	2.8	5.1	0.4	2.2

Table S2. Percent abundance of the top six genera relative to total Cyanobacteria across sites, from upstream to downstream within the digestive glands of mussels collected in Fall 2018 from the Grand River, ON. Cyanotoxin-producing genera are coloured in red (n=10/site except JN with n=3). See Figure 1 for site locations. Vertical lines indicate WWTP outfalls occur at some point in the river between the two listed sites.

Cyanobacteria Genera	**Site (%)**				
	WMR	KIW	DN	JN	GM
Cyanobium PCC-6307	64.9	55.6	81.8	94.6	79.9
Snowella 0TU37S04	17.4	16.2	1.5	4.3	9.5
Planktothrix NIVA-CYA 15	3.2	7.1	1.8	0.0	5.5
Synechocystis PCC-6803	2.6	4.5	4.2	0.0	1.5
Microcystis PCC-7914	1.1	8.6	0.0	0.8	0.03
Aphanizomenon MDT14a	5.3	0.2	0.0	0.0	0.0

Table S3. Percent relative abundance of the top six bacterial phyla across sites, from upstream to downstream in whole-body spiders collected in Fall 2018 from the Grand River, ON (n=10/site except PT1 with n=8). See Figure 1 for site locations. Vertical lines indicate WWTP outfalls occur in the river between the two listed sites.

Bacterial	**Site (%)**									
Phyla	INV	WMR	KIW	EIT	FWY	HR	PT1	PT2	BLR	GM
Proteobacteria	66.5	53.2	67.0	89.5	82.5	68.0	51.0	51.1	58.4	60.6
Bacteroidetes	1.9	17.0	22.0	0.2	14.5	23.8	16.8	12.7	16.7	12.5
Firmicutes	6.9	23.5	9.8	0.2	1.0	3.5	21.7	16.6	23.9	13.3
Tenericutes	19.6	0.4	0.01	9.9	0.08	2.0	3.0E-5	8.2	1.0E-5	0.9
Actinobacteria	4.4	4.0	1.0	0.2	1.8	2.7	9.7	5.4	0.9	11.9
Cyanobacteria	0.6	1.7	6.1E-5	3.0E-5	0.2	0.1	0.06	5.5	0.05	0.5

Table S4. Percent abundance of genera relative to total endosymbiont bacteria across sites, from upstream to downstream in whole-body spiders collected in Fall 2018 from the Grand River, ON (n=10/site except PT1 with n=8). See Figure 1 for site locations. Vertical lines indicate WWTP outfalls occur in the river between the two listed sites.

Endosymbiont	**Site (%)**									
Genera	INV	WMR	KIW	EIT	FWY	HR	PT1	PT2	BLR	GM
Rickettsiella	52.2	58.0	46.5	98.5	73.2	50.8	51.1	25.3	45.1	37.9
Candidatus Cardinium	0.13	24.6	29.4	0.56	10.3	27.4	6.34	22.7	28.5	18.5
Rickettsia	27.2	13.1	22.4	0.42	0.02	20.0	31.0	26.6	0.03	33.3
Spiroplasma	20.4	4.2	0.52	0.31	0.29	0.52	5.1E-5	8.2	0.36	7.2
Wolbachia	0.03	0.16	1.2	0.17	16.3	1.3	11.6	16.8	26.0	3.1
Arsenophonus	-	-	-	-	-	-	-	0.46	-	-

Table S5. Top five bacteria from each taxonomic rank and their relative abundances within families of whole-body benthic macroinvertebrates collected from the Grand River, ON, in Fall 2018.

	Heptageniidae	**Perlidae**	**Hydropsychidae**	**Baetidae**	**Ephemerellidae**
Phylum	Proteobacteria (45.38%)	Proteobacteria (50.25%)	Bacteroidetes (39.89%)	Proteobacteria (32.17%)	Proteobacteria (36.65%)
	Bacteroidetes (24.34%)	Bacteroidetes (29.36%)	Proteobacteria (37.11%)	Cyanobacteria (25.03%)	Cyanobacteria (34.09%)
	Cyanobacteria (12.47%)	Cyanobacteria (13.73%)	Firmicutes (10.69%)	Bacteroidetes (22.53%)	Bacteroidetes (25.19%)
	Firmicutes (8.99%)	Tenericutes (2.25%)	Cyanobacteria (2.85%)	Tenericutes (18.72%)	Firmicutes (1.42%)
	Tenericutes (3.23%)	Firmicutes (1.56%)	Deferribacteres (2.49%)	Firmicutes (0.70%)	Actinobacteria (0.64%)
	Other (5.51%)	Other (2.83%)	Other (6.96%)	Other (0.85%)	Other (1.99%)
	Unassigned (0.09%)	Unassigned (0.03%)	Unassigned (0.01%)	Unassigned (0.004%)	Unassigned (0.01%)
Class	Bacteroidia (24.09%)	Bacteroidia (29.06%)	Bacteroidia (39.24%)	Oxyphotobacteria (25.03%)	Oxyphotobacteria (34.04%)
	Gammaproteobacteria (23.74%)	Alphaproteobacteria (27.66%)	Alphaproteobacteria (20.19%)	Bacteroidia (22.47%)	Bacteroidia (25.07%)
	Alphaproteobacteria (20.21%)	Gammaproteobacteria (21.73%)	Gammaproteobacteria (13.90%)	Gammaproteobacteria (21.57%)	Alphaproteobacteria (23.27%)
	Oxyphotobacteria (12.43%)	Oxyphotobacteria (13.70%)	Clostridia (10.00%)	Mollicutes (18.72%)	Gammaproteobacteria (12.53%)
	Clostridia (3.85%)	Mollicutes (2.25%)	Deltaproteobacteria (2.87%)	Alphaproteobacteria (10.34%)	Clostridia (1.02%)
	Other (8.52%)	Other (5.08%)	Other (12.20%)	Other (1.80%)	Other (3.94%)
	Unassigned (7.15%)	Unassigned (0.51%)	Unassigned (1.60%)	Unassigned (0.07%)	Unassigned (0.14%)
	Betaproteobacteriales (21.91%)	Betaproteobacteriales (16.34%)	Bacteroidales (25.26%)	Chloroplast (24.76%)	Chloroplast (31.52%)
	Chloroplast (12.00%)	Chloroplast (13.55%)	Betaproteobacteriales (13.44%)	Flavobacteriales (19.64%)	Betaproteobacteriales (11.05%)

Order	Rhodobacterales (9.29%)	Chitinophagales (12.12%)	Sphingomonadales (10.51%)	Mycoplasmatales (18.72%)	Chitinophagales (10.22%)
	Sphingomonadales (8.91%)	Rhodobacterales (11.92%)	Clostridiales (9.98%)	Betaproteobacteriales (13.39%)	Rhodobacterales (10.08%)
	Chitinophagales (8.45%)	Sphingomonadales (10.22%)	Chitinophagales (7.42%)	Aeromonadales (6.72%)	Sphingomonadales (9.88%)
	Other (29.86%)	Other (34.65%)	Other (28.95%)	Other (16.44%)	Other (25.50%)
	Unassigned (9.58%)	Unassigned (1.20%)	Unassigned (4.45%)	Unassigned (0.32%)	Unassigned (1.74%)
Family	*Burkholderiaceae* (14.84%)	*Burkholderiaceae* (14.88%)	*Rikenellaceae* (22.31%)	*Mycoplasmataceae* (18.72%)	*Burkholderiaceae* (10.12%)
	Rhodobacteraceae (9.29%)	*Rhodobacteraceae* (11.92%)	*Burkholderiaceae* (11.11%)	*Flavobacteriaceae* (12.50%)	*Rhodobacteraceae* (10.08%)
	Sphingomonadaceae (8.91%)	*Sphingomonadaceae* (10.22%)	*Sphingomonadaceae* (10.51%)	*Burkholderiaceae* (11.78%)	*Sphingomonadaceae* (9.88%)
	Saprospiraceae (6.80%)	*Saprospiraceae* (9.03%)	*Ruminococcaceae* (6.29%)	*Aeromonadaceae* (6.72%)	*Saprospiraceae* (8.19%)
	Flavobacteriaceae (5.01%)	*Flavobacteriaceae* (5.73%)	*Saprospiraceae* (4.75%)	*Weeksellaceae* (6.68%)	*Spirosomaceae* (6.16%)
	Other (28.61%)	Other (30.23%)	Other (31.28%)	Other (17.86%)	Other (19.52%)
	Unassigned (26.53%)	Unassigned (17.99%)	Unassigned (13.75%)	Unassigned (25.75%)	Unassigned (36.04%)
Genus	*Sphingorhabdus* (5.98%)	*Pseudorhodobacter* (6.88%)	*Mucinivorans* (12.10%)	*Cand. Bacilloplasma* (18.72%)	*Sphingorhabdus* (7.49%)
	Flavobacterium (5.00%)	*Flavobacterium* (5.72%)	*Sphingorhabdus* (7.29%)	*Flavobacterium* (12.49%)	*Pseudorhodobacter* (6.86%)
	Rhodoferax (4.28%)	*Sphingorhabdus* (5.18%)	*Alistipes* (6.42%)	*Aeromonas* (6.72%)	*Rhodoferax* (3.64%)
	Pseudorhodobacter (3.56%)	*Rhodobacter* (3.92%)	*Cand. Soleaferrea* (2.93%)	*Rhodoferax* (5.59%)	*Lacihabitans* (2.77%)
	Rhodobacter (3.42%)	*Ideonella* (3.33%)	*Rhizorhapis* (2.42%)	*Ideonella* (3.53%)	*Rhodobacter* (2.12%)
	Other (29.90%)	Other (32.48%)	Other (28.09%)	Other (12.54%)	Other (19.90%)
	Unassigned (48.86%)	Unassigned (42.50%)	Unassigned (40.74%)	Unassigned (40.41%)	Unassigned (57.22%)

Table S6. Percent relative abundance of the top six bacterial phyla across sites, from upstream to downstream within whole-body benthic macroinvertebrates collected in Fall 2018 from the Grand River, ON (n=1-11/site). Blank cells indicate sites where the particular invertebrate taxa were not collected. Vertical lines indicate WWTP outfalls occur in the river between the two listed sites. See Figure 1 for site locations.

Invertebrate Family	Bacterial Phyla	Site (%)									
		INV	WMR	KIW	EIT	FWY	HR	PT1	PT2	BLR	GM
Perlidae	Proteobacteria	50.7	58.8	48.0	22.4	47.3	50.9	48.3	57.4	45.3	45.7
	Bacteroidetes	32.2	23.7	25.6	8.1	37.4	30.0	26.1	16.4	36.8	38.4
	Cyanobacteria	10.5	8.8	10.8	66.9	13.7	15.0	23.8	18.1	15.7	11.7
	Tenericutes	1.7	2.1	10.9	0.0	0.4	0.2	0.04	0.0	0.0	0.9
	Firmicutes	2.4	0.7	2.1	1.7	0.08	1.8	0.4	4.9	0.2	1.2
	Planctomycetes	0.6	1.8	0.7	0.3	0.2	0.6	0.2	0.4	0.3	0.6
Hydrospychidae	Bacteroidetes	44.6	47.3	46.5	43.4	39.3	48.1	31.5	37.2	37.2	39.8
	Proteobacteria	34.4	26.8	35.7	38.8	42.2	36.0	41.5	40.8	42.2	29.1
	Firmicutes	11.8	14.1	8.9	7.9	8.2	9.1	9.4	10.4	7.9	18.8
	Cyanobacteria	1.7	0.5	1.7	2.3	3.9	1.0	5.9	1.3	4.4	2.2
	Deferribacteres	3.2	4.4	2.5	2.7	0.9	1.8	0.9	2.9	2.1	4.5
	RsaHf231	0.6	0.4	0.3	1.5	1.2	1.4	1.2	4.0	0.9	0.6
Heptageniidae	Proteobacteria	47.9	49.6	52.1	55.1	51.7	55.2	35.1	35.8	35.9	40.0
	Bacteroidetes	28.2	9.9	31.0	24.0	28.1	16.2	24.9	16.6	20.9	44.1
	Cyanobacteria	6.4	5.7	8.8	6.0	13.1	14.0	18.2	14.1	29.5	10.1
	Firmicutes	4.3	16.5	3.2	8.3	4.3	6.9	11.4	21.7	9.3	2.7
	Tenericutes	9.2	10.4	1.7	0.2	0.4	2.2	3.1	0.1	0.1	0.1
	RsaHf231	2.5	3.9	1.0	2.9	0.7	2.8	5.2	9.4	1.8	0.3
Ephemerellidae	Proteobacteria	33.0	40.4								
	Cyanobacteria	28.6	38.0								
	Bacteroidetes	36.4	16.5								
	Firmicutes	1.3	1.2								
	Actinobacteria	0.3	0.9								
	Acidobacteria	0.1	0.6								
Baetidae	Proteobacteria			31.0	17.3	37.5		31.2	68.9	25.0	20.7
	Cyanobacteria			18.5	42.0	26.8		15.0	7.7	29.3	39.4
	Bacteroidetes			27.7	17.5	22.1		22.9	16.1	33.6	35.3
	Tenericutes			21.6	20.5	13.4		30.1	4.8	11.5	2.1
	Firmicutes			0.3	1.5	0.08		0.4	0.6	0.6	1.0
	Planctomycetes			0.7	0.3	0.03		0.2	0.5	2.2E-5	0.6

Table S7. Percent abundance of the top six genera relative to total Cyanobacteria across sites, from upstream to downstream within whole-body benthic macroinvertebrates collected in Fall 2018 from the Grand River, ON. Cyanotoxin-producing genera are coloured in red. Blank cells indicate that taxa were unable to be collected at these sites. Vertical lines indicate WWTP outfalls occur in the river between the two listed sites. See Table 9 for n size and Figure 1 for site locations.

Invertebrate Family	**Cyanobacteria Genus**	**Site (%)**									
		INV	WMR	KIW	EIT	FWY	HR	PT1	PT2	BLR	GM
Perlidae	*Tychonema CCAP 1459-11B*	24.5	-	-	-	50.0	31.1	61.0	50.0	98.3	42.6
	Cyanobium PCC-6307	-	16.7	40.3	69.0	50.0	32.2	7.6	-	-	25.0
	Snowella 0TU37S04	2.7	11.5	21.9	31.0	-	6.3	-	-	1.1	2.7
	Planktothrix NIVA-CYA 15	10.5	33.3	2.3	-	-	1.5	2.1	33.3	-	-
	Pleurocapsa PCC-7319	8.1	-	-	-	-	-	1.3	11.3	0.6	29.7
	Calothrix KVSF5	24.8	-	11.0	-	-	-	25.0	-	-	-
Hydrospychidae	*Tychonema CCAP 1459-11B*	-	-	-	-	-	-	83.8	-	74.9	-
	Pleurocapsa PCC-7319	29.2	-	3.1	50.0	-	-	2.3	100	25.1	100
	Calothrix KVSF5	68.0	100	71.9	50.0	-	-	8.0	-	-	-
	Calothrix UAM 374	-	-	-	-	-	-	5.8	-	-	-
	Planktothrix NIVA-CYA 15	2.8	-	25.0	-	-	-	-	-	-	-
	Cyanobium PCC-6307	-	-	-	-	-	100	-	-	-	-
Heptageniidae	*Tychonema CCAP 1459-11B*	50.6	-	-	-	63.0	55.3	86.0	97.8	95.8	66.1
	Cyanobium PCC-6307	1.6	67.4	18.2	100	4.9	6.1	12.0	-	2.9	7.7
	Pleurocapsa PCC-7319	2.1	1.2	1.9	-	27.2	19.6	1.5	1.4	0.5	26.2

	Snowella 0TU37S04	8.6	14.4	27.5	-	-	-	-	-	-	-
	Calothrix KVSF5	11.6	-	48.7	-	-	-	-	0.8	0.6	-
	Chamaesiphon PCC-7430	7.2	-	-	-	3.7	-	-	-	-	-
Ephemerellidae	*Calothrix KVSF5*	50.2	14.3								
	Chamaesiphon PCC-7430	38.2	21.9								
	Pleurocapsa PCC-7319	5.9	1.9								
	Cyanobium PCC-6307	0.5	22.1								
	Tychonema CCAP 1459-11B	3.7	6.4								
	Calothrix PCC-6303	0.02	7.7								
Baetidae	*Pleurocapsa PCC-7319*			26.7	42.6	56.8		34.5	16.1	20.0	42.9
	Tychonema CCAP 1459-11B			-	-	-		20.0	-	75.9	30.0
	Cyanobium PCC-6307			2.6	39.6	-		34.4	25.5	-	27.1
	Snowella 0TU37S04			21.6	4.9	-		-	50.0	-	-
	Calothrix KVSF5			32.2	-	28.4		-	-	2.1	-
	Chamaesiphon PCC-7430			12.4	6.4	-		4.8	1.3	0.5	-

Table S8. Summary table of the top five bacteria from each taxonomic rank and their relative abundances within Grand River (ON) water and Waterloo and Kitchener WWTP effluent samples collected in Fall 2019.

	Grand River	**Waterloo WWTP**	**Kitchener WWTP**
Class	Gammaproteobacteria (43.15%)	Gammaproteobacteria (37.71%)	Gammaproteobacteria (42.29%)
	Oxyphotobacteria (17.88%)	Bacteroidia (16.70%)	Bacteroidia (15.83%)
	Bacteroidia (14.64%)	Alphaproteobacteria (10.53%)	Alphaproteobacteria (13.83%)
	Actinobacteria (6.75%)	Babeliae (6.86%)	Clostridia (6.05%)
	Alphaproteobacteria (6.42%)	Campylobacteria (4.85%)	Campylobacteria (3.94%)
	Other (10.75%)	Other (22.24%)	Other (16.47%)
	Unassigned (0.06%)	Unassigned (1.12%)	Unassigned (1.59%)
Order	Betaproteobacteriales (20.05%)	Betaproteobacteriales (22.56%)	Betaproteobacteriales (17.55%)
	Pseudomonadales (19.20%)	Babeliales (6.86%)	Pseudomonadales (12.97%)
	Chloroplast (14.94%)	Chitinophagales (6.18%)	Chitinophagales (7.88%)
	Flavobacteriales (8.03%)	Pseudomonadales (5.65%)	Paracaedibacterales (6.31%)
	Campylobacterales (4.74%)	Campylobacterales (4.85%)	Clostridiales (6.05%)
	Other (32.06%)	Other (49.37%)	Other (44.49%)
	Unassigned (0.53%)	Unassigned (4.52%)	Unassigned (4.75%)
Family	*Burkholderiaceae* (18.55%)	*Rhodocyclaceae* (14.08%)	*Moraxellaceae* (12.82%)
	Moraxellaceae (15.77%)	*Burkholderiaceae* (6.17%)	*Rhodocyclaceae* (9.54%)
	Flavobacteriaceae (6.23%)	*Moraxellaceae* (5.54%)	*Paracaedibacteraceae* (6.31%)
	Arcobacteraceae (4.65%)	*Arcobacteraceae* (4.76%)	*Burkholderiaceae* (5.82%)

	Sporichthyaceae (4.28%) Other (34.34%) Unassigned (16.18%)	*Chitinophagaceae* (3.31%) Other (48.60%) Unassigned (17.55%)	*Arcobacteraceae* (3.94%) Other (47.77%) Unassigned (13.78%)
Genus	*Acinetobacter* (15.26%) *Limnohabitans* (8.05%) *Flavobacterium* (6.20%) *Arcobacter* (4.65%) *hgcI clade* (3.89%) Other (37.51%) Unassigned (24.44%)	*Zoogloea* (7.68%) *Arcobacter* (4.76%) *Dechloromonas* (4.02%) *Nitrospira* (3.29%) *Sediminibacterium* (1.97%) Other (41.67%) Unassigned (36.61%)	*Zoogloea* (7.78%) *Cand. Paracaedibacter* (6.14%) *Arcobacter* (3.94%) *Sediminibacterium* (3.52%) *Acinetobacter* (2.62%) Other (39.94%) Unassigned (36.05%)

Table S9. Percent relative abundance of the top six bacterial phyla across sites, from upstream to downstream within river water samples, as well as Waterloo (WAT) and Kitchener (KIT) WWTP effluent samples (shaded in gray) collected in Fall 2019 from the Grand River, ON (n=3/site). Vertical lines indicate WWTP outfalls. See Figure 1 for site locations.

Bacterial Phyla	**Site (%)**													
	INV	WMR	KIW	WAT	EIT	FWY	DN	HR	KIT	PT1	PT2	JN	BLR	GM
Proteobacteria	38.9	44.2	54.8	52.8	48.2	50.6	51.2	52.9	60.9	51.9	53.9	48.2	52.8	45.0
Cyanobacteria	29.3	18.5	14.8	0.75	20.3	17.4	16.5	16.7	0.95	15.8	16.1	17.0	18.3	22.1
Bacteroidetes	8.6	11.6	11.8	17.6	13.8	14.1	14.9	14.5	15.4	15.0	13.5	20.2	15.7	18.4
Actinobacteria	19.8	21.7	6.5	0.85	4.8	6.5	6.3	5.8	0.25	6.1	5.8	7.9	5.4	8.1
Epsilonbacteraeota	0.31	0.61	7.0	4.7	6.3	7.0	5.9	5.5	4.2	5.5	5.1	2.1	3.8	2.1
Firmicutes	1.3	1.3	3.9	5.3	3.8	3.2	4.0	3.2	7.2	4.3	4.5	2.6	3.0	2.7

Table S10. Percent abundance of the top six genera relative to total Cyanobacteria across sites, from upstream to downstream within water samples from the Grand River, ON, collected in Fall 2019 (n=3/site). Cyanotoxin-producing genera are coloured in red. Vertical lines indicate WWTP outfalls occur in the river between the two listed sites. See Figure 1 for site locations.

Cyanobacteria	**Site (%)**											
Genera	INV	WMR	KIW	EIT	FWY	DN	HR	PT1	PT2	JN	BLR	GM
Planktothrix NIVA-CYA 15	0.0	0.0	79.1	76.4	72.6	68.2	65.1	69.1	68.6	44.4	71.8	58.7
Snowella 0TU37S04	43.8	55.9	17.2	18.0	22.3	25.8	28.3	22.7	25.4	47.1	20.6	37.3
Pseudanabaena PCC-7429	36.6	29.2	2.8	2.2	4.8	3.8	2.7	3.0	2.4	2.2	4.1	1.4
Cyanobium PCC-6307	4.0	3.5	0.14	2.4	0.0	2.3	2.1	3.0	1.7	3.5	1.6	1.4
Aphanizomenon MDT14a	13.0	11.4	0.61	0.44	0.0	0.0	1.1	1.0	1.5	0.41	1.8	0.73
Microcystis PCC-7914	1.2	0.0	0.0	0.0	0.30	0.0	0.38	0.0	0.009	0.0	0.0	0.37

Table S11. Tukey HSD values from the one-way ANOVA test of Shannon and Simpson Alpha Diversity measures by site within river water samples as well as Waterloo (WAT) and Kitchener (KIT) WWTP effluent samples collected in Fall 2019 from the Grand River, ON (n=3/site). See Figure 1 for site locations. For each pairwise comparison, the Shannon P adjusted value was <0.05 for all except EIT-WMR, which had a Simpson P adjusted value of <0.0001. Significant values are depicted in red.

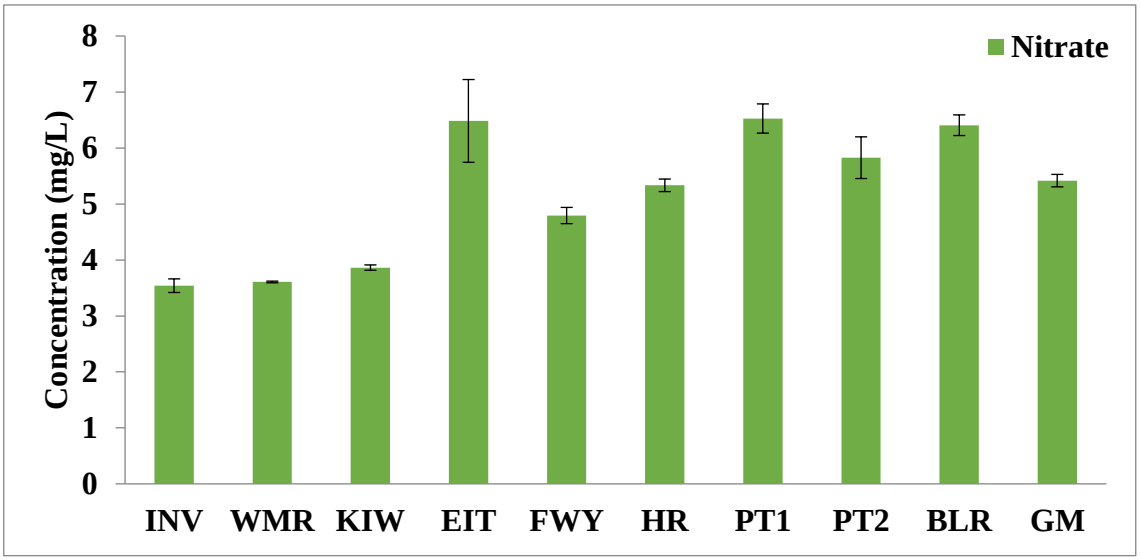
Factor	Pair	Shannon (H)		Simpson (D)	
		Diff	P adjusted	Diff	P adjusted
Site	EIT-INV	-1.194	<0.0001	-5.221E-2	<0.0001
	EIT-WMR	-0.403	0.264	-4.665E-2	<0.0001
	EIT-WAT	-0.930	<0.0001	-4.561E-2	<0.0001
	EIT-FWY	-0.942	<0.0001	-4.577E-2	<0.0001
	EIT-DN	-1.328	<0.0001	-5.415E-2	<0.0001
	EIT-HR	-0.939	<0.0001	-4.918E-2	<0.0001
	EIT-KIT	-0.923	<0.0001	-5.120E-2	<0.0001
	EIT-PT1	-0.878	<0.0001	-4.720E-2	<0.0001
	EIT-PT2	-0.989	<0.0001	-4.564E-2	<0.0001
	EIT-JN	-0.874	<0.0001	-4.553E-2	<0.0001
	EIT-BLR	-1.064	<0.0001	-4.979E-2	<0.0001
	EIT-GM	-0.941	<0.0001	-4.824E-2	<0.0001
	KIW-INV	-1.303	<0.0001	-6.113E-2	<0.0001
	KIW-WAT	-1.039	<0.0001	-5.454E-2	<0.0001
	KIW-FWY	-1.051	<0.0001	-5.469E-2	<0.0001
	KIW-DN	-1.436	<0.0001	-6.307E-2	<0.0001
	KIW-HR	-1.048	<0.0001	-5.810E-2	<0.0001
	KIW-KIT	-1.032	<0.0001	-6.012E-2	<0.0001
	KIW-PT1	-0.987	<0.0001	-5.612E-2	<0.0001
	KIW-PT2	-1.097	<0.0001	-5.457E-2	<0.0001
	KIW-JN	-0.983	<0.0001	-5.445E-2	<0.0001
	KIW-BLR	-1.173	<0.0001	-5.871E-2	<0.0001
	KIW-GM	-1.049	<0.0001	-5.717E-2	<0.0001
	WMR-INV	-0.791	0.0004	-5.556E-3	0.996
	WMR-WAT	-0.527	0.046	1.041E-3	1.000
	WMR-FWY	-0.538	0.038	8.839E-4	1.000
	WMR-HR	-0.536	0.040	-2.523E-3	1.000
	WMR-DN	-0.924	<0.0001	-7.496E-3	0.955
	WMR-PT2	-0.585	0.017	5.558E-2	<0.0001
	WMR-BLR	-0.661	0.004	-3.137E-3	1.000
	WMR-GM	-0.537	0.038	-1.589E-3	1.000

Table S12. Pairwise Adonis values from the Permanova analysis of Bray-Curtis beta diversity measures by SampleType collected in Fall 2018 (taxa) and 2019 (water) from the Grand River, ON, using 99999 permutations. R^2 (effect size) values display how much of the overall variation in distances can be explained by the factor being tested (n=389). The P adjusted values for significant pairs were <0.02. Significant values are depicted in red.

Factor	Pair	Bray-Curtis Dissimilarity	
		R^2	P Value
SampleType	Spiders vs Mussels	0.145	0.00045
	Spiders vs Heptageniidae	0.186	0.00045
	Spiders vs Hydropsychidae	0.211	0.00045
	Spiders vs Perlidae	0.213	0.00045
	Spiders vs Ephemerellidae	0.089	0.00045
	Spiders vs Baetidae	0.149	0.00045
	Spiders vs W-WWTP	0.039	0.00045
	Spiders vs K-WWTP	0.037	0.01710
	Spiders vs River Water	0.221	0.00045
	Mussels vs Heptageniidae	0.277	0.00045
	Mussels vs Hydropsychidae	0.314	0.00045
	Mussels vs Perlidae	0.307	0.00045
	Mussels vs Ephemerellidae	0.219	0.00045
	Mussels vs Baetidae	0.270	0.00045
	Mussels vs W-WWTP	0.124	0.00270
	Mussels vs K-WWTP	0.117	0.00360
	Mussels vs River Water	0.371	0.00045
	Heptageniidae vs Hydropsychidae	0.287	0.00045
	Heptageniidae vs Perlidae	0.156	0.00045
	Heptageniidae vs Ephemerellidae	0.129	0.00045
	Heptageniidae vs Baetidae	0.235	0.00045
	Heptageniidae vs W-WWTP	0.138	0.00135
	Heptageniidae vs K-WWTP	0.134	0.00225
	Heptageniidae vs River Water	0.425	0.00045
	Hydropsychidae vs Perlidae	0.305	0.00045
	Hydropsychidae vs Ephemerellidae	0.215	0.00045

	Hydropsychidae vs Baetidae	0.316	0.00045
	Hydropsychidae vs W-WWTP	0.139	0.00180
	Hydropsychidae vs K-WWTP	0.134	0.00090
	Hydropsychidae vs River Water	0.454	0.00045
	Perlidae vs Ephemerellidae	0.156	0.00045
	Perlidae vs Baetidae	0.266	0.00045
	Perlidae vs W-WWTP	0.154	0.00135
	Perlidae vs K-WWTP	0.149	0.00135
	Perlidae vs River Water	0.462	0.00045
	Ephemerellidae vs Baetidae	0.204	0.00045
	Ephemerellidae vs W-WWTP	0.524	0.14625
	Ephemerellidae vs K-WWTP	0.501	0.15300
	Ephemerellidae vs River Water	0.471	0.00045
	Baetidae vs W-WWTP	0.192	0.00675
	Baetidae vs K-WWTP	0.183	0.00900
	Baetidae vs River Water	0.446	0.00045
	W-WWTP vs K-WWTP	0.684	1.00000
	W-WWTP vs River Water	0.326	0.00540
	K-WWTP vs River Water	0.317	0.00315

A



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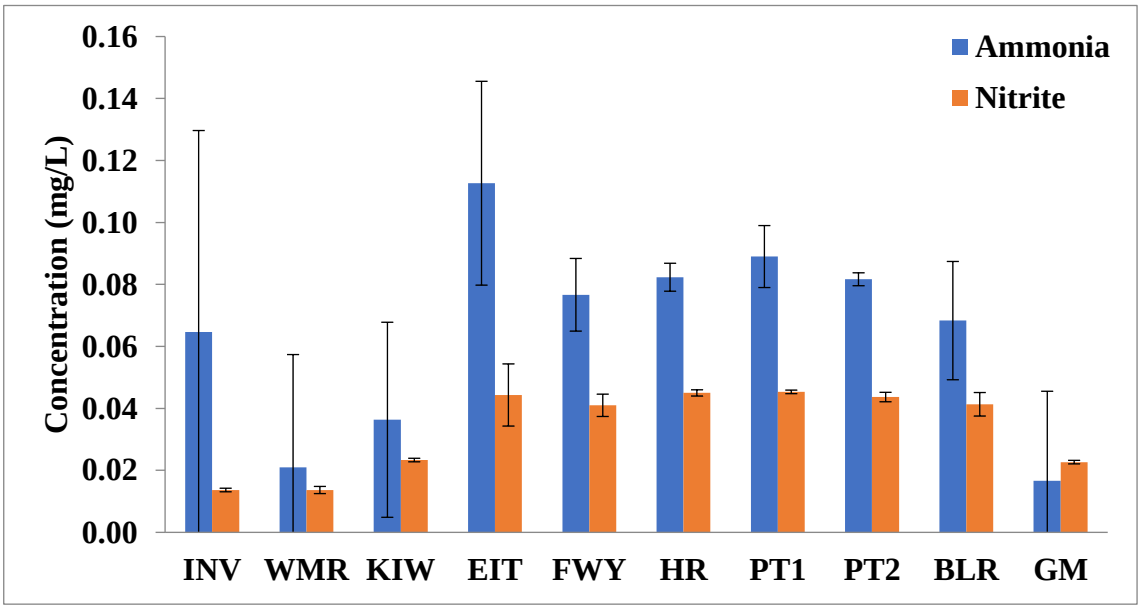

Figure S1. Concentration of A) nitrate, B) ammonia, and nitrite (mg/L) across sites, from upstream to downstream within river water samples collected in Fall 2018 from the Grand River, ON. See Figure 1 for site locations. Nutrients were not measured at sites DN and JN.

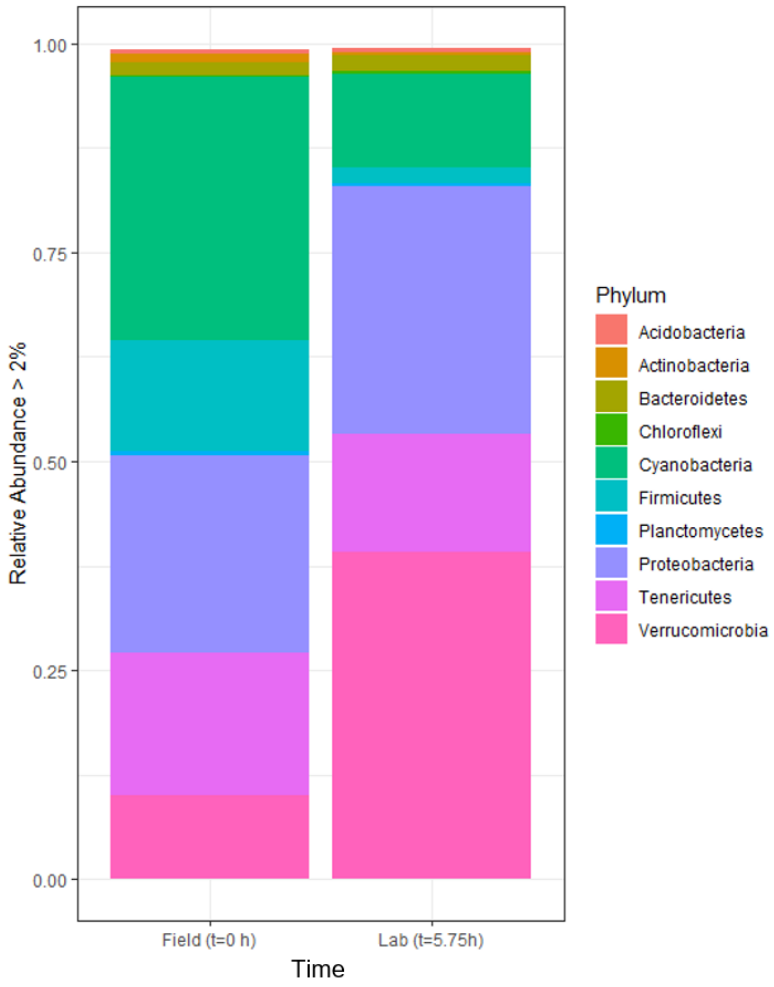

Figure S2. Abundance (>2%) of phylum-level bacteria in timed dissection groups within the digestive glands of mussels collected in Spring 2019 from the Grand River, ON (n=5/time group). Data was re-normalized based on relative abundance of bacterial taxa above 2%.

Appendix B

Differences between Functional Feeding Groups

Beta diversity differed between functional feeding groups (Permanova: df=3, Sum Sq=23.795, Mean Sq=7.932, F Model=33.207, $p=1x10^{-4}$), and accounted for 34.8% of the overall variation according to the effect size ($R^2=0.348$). All functional feeding groups were significantly dissimilar from each other in terms of their bacterial compositions (Adonis pairwise, Table S13).

Table S13. Pairwise Adonis values from the Permanova analysis of Bray-Curtis beta diversity measures by Functional Feeding Group (FFG) of various insect taxa in the Grand River, ON collected in Fall 2018, using 99999 permutations. R^2 (effect size) values display how much of the overall variation in distances can be explained by the factor being tested. Significant values are depicted in red.

Factor	Pair	Bray-Curtis Dissimilarity	
		R^2	P Value
FFG	Filterer vs Gatherer	0.30526	$6x10^{-5}$
	Filterer vs Gatherer	0.28614	$6x10^{-5}$
	Filterer vs Scraper	0.28748	$6x10^{-5}$
	Predator vs Gatherer	0.21787	$6x10^{-5}$
	Predator vs Scraper	0.15587	$6x10^{-5}$
	Gatherer vs Scraper	0.18533	$6x10^{-5}$

Individuals appear to cluster by Functional Feeding Group (PCoA, Figure S3). There appears to be some overlap between Predators and Scrapers, as well as between Scrapers and Gatherers. Filterers display a high degree of dissimilarity from all other functional feeding groups. However, when looking specifically at taxa, the Gatherer group separates between Ephemerellidae and Baetidae insect families. Ephemerellidae tend to overlap with Heptageniidae, while Baetidae display a greater dissimilarity from the other insect families.

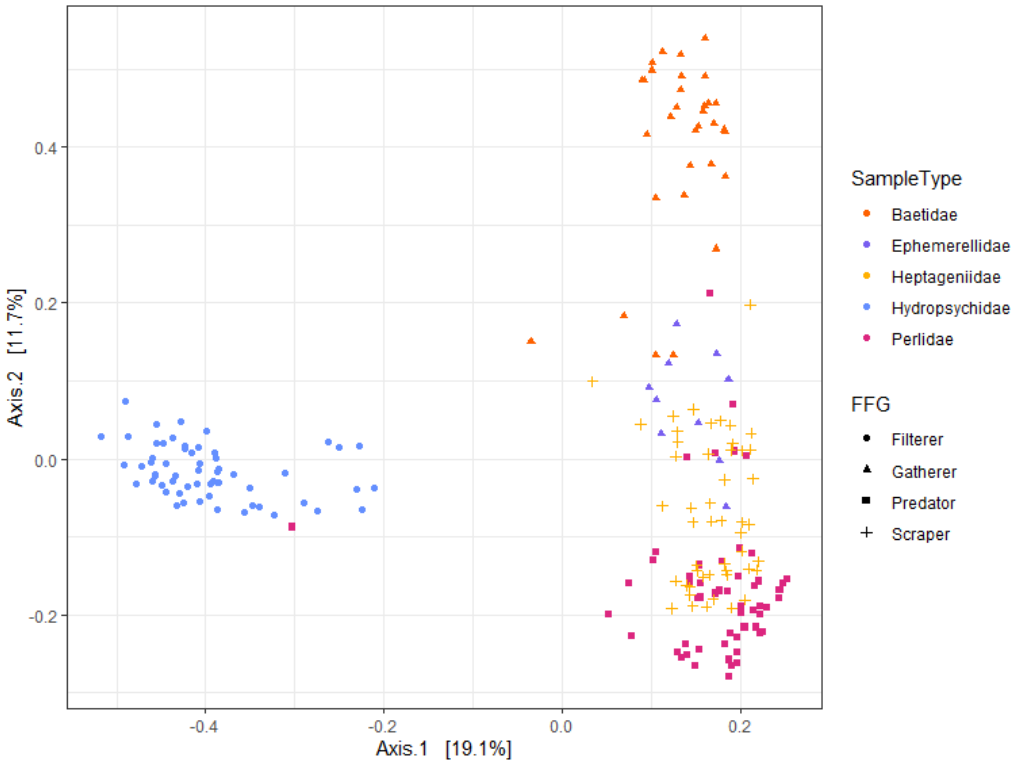

Figure S3. Principal coordinate analysis (PCoA) plot displaying the beta diversity between the functional feeding groups (FFG) and families of various insect taxa in the Grand River, ON collected in Fall 2018. The Bray-Curtis Dissimilarity measure was used to construct the distance matrices from which this plot was generated. Each coloured dot represents the gut microbiota of an individual sample.

Curriculum Vitae

Candidate's full name: Elise N. Millar

Universities attended:

Trent University, Honours Bachelor of Molecular Biology and Biochemistry, September 2014 – June 2018

Conference Presentations:

The gut microbiome of invertebrates upstream and downstream wastewater treatment plants on the Grand River. Millar, E.N., Kidd, K.A., Gillis, P.L., Surette, M.G. 21-Feb-19. Biology Graduate Research Day, Hamilton, ON.

The effects of wastewater treatment plant effluent on the gut microbiome of invertebrates on the Grand River. Millar, E.N., Kidd, K.A., Gillis, P.L., Surette, M.G. 07-Oct-19. Canadian Ecotoxicity Workshop, Québec City, QC.

The effects of wastewater treatment plant effluent on the gut microbiome of invertebrates on the Grand River. Millar, E.N., Kidd, K.A., Gillis, P.L., Surette, M.G. 01-Nov-19. McMaster Water Week, Hamilton, ON.

The gut microbiome of invertebrates upstream and downstream wastewater treatment plants on the Grand River. Millar, E.N., Kidd, K.A., Gillis, P.L., Surette, M.G. 05-Nov-19. Society of Environmental Toxicology and Chemistry North America 40th General Meeting, Toronto, ON.

The effects of urban inputs, including municipal wastewater treatment plant effluents on the gut microbiome of invertebrates in the Grand River. Millar, E.N., Kidd, K.A., Gillis, P.L., Surette, M.G. 03-Dec-19. 3rd Biennial Canadian Freshwater Mollusc Research Meeting, Burlington, ON.

The effects of wastewater treatment plant effluent on the gut microbiome of invertebrates on the Grand River. Millar, E.N., Kidd, K.A., Gillis, P.L., Surette, M.G. 04-Dec-19. Ecology, Evolution, and Behaviour Seminar, McMaster University, Hamilton, ON.

The effects of wastewater treatment plant effluent on the gut microbiome of invertebrates in the Grand River. Millar, E.N., Kidd, K.A., Gillis, P.L., Surette, M.G. 20-Feb-20. Biology Graduate Research Day, Hamilton, ON.