EFFECT OF WASTEWATER ON THE GUT MICROBIOME OF RAINBOW DARTER

THE EFFECT OF WASTEWATER EFFLUENT ON THE GUT CONTENT MICROBIOME OF RAINBOW DARTER (*ETHEOSTOMA CAERULEUM*)

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A Thesis Submitted to the School of Graduate Studies in Partial Fulfillment of the Requirements for the Degree Master of Science

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TITLE: The Effect of Wastewater Effluent on the Gut Content Microbiome of Rainbow Darter (*Etheostoma caeruleum*)

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Lay Abstract

Wastewater is the largest source of pollution affecting Canada's aquatic ecosystems; effluents contain antibiotics and antimicrobials that can affect fish and other aquatic life. The gut microbiome of fish is influenced by host species, its diet, and the environment, and thus contaminants released via wastewater effluents may alter the gut microbiome of fishes in receiving waters. This study found that the gut microbiota of rainbow darter fish exposed to wastewater effluents in the central Grand River (Waterloo/Kitchener, Ontario) were dominated by Proteobacteria and had increased diversity. Wild fish transitioned to the lab were dominated by Firmicutes and had decreased bacterial diversity in the gut compared to those in the wild. Altogether, these results suggest that wild fish exposed to wastewater effluents had altered gut microbiomes; transitions to new environments and laboratory acclimation periods are important considerations when studying the fish gut microbiome.

Abstract

The microbiome plays an important role in host physiology and can be influenced by species, diet, and environment. Municipal wastewater effluent contains a mixture of chemicals including antibiotics and antimicrobials that may affect the gut microbiome of fish living downstream of these discharges. Thus, this study examines the effect of wastewater treatment plant (WWTP) effluent on the gut microbiome of wild rainbow darter (*Etheostoma cearuleum*), and examines how the gut microbiome of wild fish changes in the lab.

Fish were collected from sites upstream and downstream of 2 major WWTPs along the central Grand River and gut contents were aseptically sampled. After extracting gDNA, nested PCR of the V3-V4 region of the 16S rRNA gene, and Illumina sequencing were performed. The gut microbiome of exposed fish had increased bacterial diversity and was dominated by Proteobacteria, which has been linked to altered health outcomes in mammals.

Next, rainbow darters were collected from a reference site on the Grand River. Fish were sampled in the field, after a 14 day lab acclimation, and after a 28 day exposure to environmental stressors (WWTP effluent or triclosan, an antimicrobial found in WWTP effluent). Surprisingly, there were no changes in the microbiome after exposure to environmental stressors. Major changes were observed between the field and laboratory fish suggesting that environment and diet are important factors influencing the gut microbiome. Changes in the gut microbiome continued up to 42 days in the lab, indicating longer acclimation periods may be needed.

This study showed that effluents altered the gut microbiome of fish in the field, but not in the laboratory for unknown reasons. Laboratory studies indicated that transitioning to a new environment may require greater than 14 days before achieving a stable microbiome.

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List of Abbreviations

ASV	Amplicon Sequence Variant
°C	Degrees Celsius
µL	Microlitres
BHI	Brain Heart Infusion
cm	Centimetre
CFU	Colony Forming Units
DNA	Deoxyribose Nucleic Acid
DO	Dissolved Oxygen
EDTA	Ethylenediamine Tetra Acetic Acid
EtOH	Ethanol
g	Grams
g	Gravity
gDNA	Genomic Deoxyribose Nucleic Acid
GES	Guanidine Thiocynate
km	Kilometres
mL	Millilitres
mm	Millimetres
M	Moles
OTU	Operational Taxonomic Unit
PCR	Polymerase Chain Reaction
rpm	Revolutions Per Minute
WWTP	Wastewater Treatment Plant

Declaration of Academic Achievement

This thesis was designed and written with the help and supervision of Dr. Karen A. Kidd and Dr. Joanna Y. Wilson. Organization and design of field work was originally developed with the advice from the laboratory of Dr. Mark Servos (University of Waterloo). Design of experimental laboratory exposures had support and advice from committee member Dr. Carol Bucking. Field and laboratory experiments and sampling were conducted by Victoria Restivo, with assistance from members of the Servos, Kidd, and Wilson labs. Genomic DNA extraction, PCR, and Illumina sequencing were performed by Michelle Shah, Laura Rossi (Surette Lab, McMaster University) and the Farncombe Institute (McMaster University) under the supervision of committee member Dr. Michael Surette. Data analysis and interpretation was completed by Victoria Restivo with advice from all supervisory committee members.

I declare this thesis to be an original report of our research, except where indicated by referencing.

Chapter 1: General Introduction

Chapter 2: Rainbow darter exposed to wastewater effluent in the field have altered gut content microbiome composition and diversity

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Chapter 3: The gut microbiome of wild rainbow darter is altered in the lab

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Chapter 4: General Conclusion

Chapter 1: General Introduction

1.1 Wastewater Treatment Plant Effluent

Municipal wastewater is any water affected by domestic use including flush-toilets, sinks, dishwashers, laundry machines, and showers. In the case of combined sewer systems it also includes surface runoff and stormwater. Wastewater is pumped through municipal sewer systems to a wastewater treatment plant (WWTP) where it is treated before being released into an aquatic ecosystem.

Wastewater treatment methods in Canada vary, however they generally aim to remove organic matter before the effluents are released into the aquatic environment. Primary treatment includes the physical separation of solids, while secondary treatment adds aerobic bacteria to remove organic material and waste. Advanced treatment takes secondary treated water and removes specific compounds by various physical, chemical, and biological methods, for example carbonaceaous removal, nitrification, denitrification, phosphorus removal, and UV sterilization (Environment Canada, 2017). The remaining biosolids are often re-purposed for agricultural soils, burned for energy production, or disposed of by incineration or in a landfill (Environment Canada, 2017). After treatment, the remaining water has lower biological oxygen demand. At present, the Canadian *Wastewater System Effluent Regulations* (SOR/2012-139, passed under the Fisheries Act, R.S.C. 1985, c. F-14) has set standards for effluent quality that can be achieved using a minimum of secondary wastewater treatment. Although the percentage of Canadian municipalities using secondary or advanced treatment methods has increased from 40% in 1983 to 69% in 2009, wastewater is still considered to be the largest source of pollution, by volume, to Canadian waters (Environment Canada, 2017). The treatment methods are not designed to remove most contaminants; some persist in effluent and include: metals, legacy and emerging compounds, pesticides, pharmaceuticals and personal care product additives (Arlos et al., 2014).

The contaminants released in WWTP effluents can bioaccumulate in aquatic food webs. Mussels caged at 68 different locations along the coast of California accumulated several contaminants (e.g. alkylphenols, polybrominated diphenyl ethers, perfluorinated compounds, pharmaceuticals and personal care products) that were also found within the WWTP effluent, suggesting a high potential for biomagnification (Dodder et al., 2014). As well, pharmaceuticals and personal care products from WWTP effluent accumulated in fish fillet and liver tissue (Ramirez et al., 2009). Freshwater mussels and fish exposed to WWTP effluent in an urban Ontario river had increased contaminant load, including metals and anti-depressant compounds, in gill tissue when compared to upstream organisms (Gillis et al., 2012).

WWTP effluent affects the metabolism and stress responses of aquatic organisms in receiving waters. Freshwater mussels exposed to WWTP effluent in the field were found to have lower condition factors and life expectancy compared to upstream mussels (Gillis et al., 2012). Wild fish exposed to effluent had increased monosaturated fatty acids and decreased polyunsaturated fatty acids in the muscle, altering lipid metabolism (Giang et al., 2018). In a separate study, increased oxidative stress and metabolic energy demand were observed through "monocytosis, transaminase increase, antioxidant enzyme activation, lipid oxidative damage, and muscle glycogen depletion" in fish caged downstream of an effluent outfall (Cazenave et al., 2014). Similarly, effluent (100%) exposure resulted in increased plasma cortisol, lactate, and liver glucocorticoid receptor levels when compared to unexposed fish, indicating a chronic stress response (Ings, Servos & Vijayan, 2011).

Compounds found in municipal effluents may act as endocrine and reproductive disruptors in fish. In a whole-lake experiment, fathead minnow were exposed to concentrations of 17α-ethynylestradiol relevant to what is found in WWTP effluent, and this lead to the

feminization of males through the production of vitellogenin and impacts on gonadal development, as well as a decline in the population (Kidd et al., 2007). Increased vitellogenin has been found in male brown trout, largemouth bass, and rainbow darter collected in effluent-impacted systems (Giang et al., 2018, Barber et al., 2011, Fuzzen, 2016). Increased vitellogenin levels have also been found in laboratory fish (including early life-stage roach) exposed to various concentrations of effluent (Liney, 2006, Garcia-Reyero et al., 2011). Fish exposed to effluent have also been found to show reduced competition, nest-tending, and aggression when compared to controls (Garcia-Reyero et al., 2011). This can lead to decreased fecundity as well as altered community structure as a result of decreased reproductive success and reproductive behaviours (Fuzzen, 2016, Garcia-Reyero et al., 2011).

1.2 The Microbiome of Fish

The microbiota (bacteria, archaea, viruses, and eukaryotes) present on or in all organisms, is known as the microbiome. The microbiome is critical as it interacts with its host to maintain host biological functions and homeostasis, including roles in energy metabolism, immune responses, growth and development, as well as behaviour (Jin et al., 2017). Conversely, dysbiosis - altered microbial composition - has been correlated with many altered health outcomes in mammals including: obesity, inflammatory bowel disease, and asthma (Carding et al., 2015). In humans, dysbiosis of the gut can occur as a result of drugs, diet, and exposure to environmental pollutants (metals, persistent organic pollutants, pesticides, nanomaterials and food additives) and can be linked to issues with energy metabolism, nutrient absorption, and immune system function (Jin et al., 2017). A large body of research on the microbiome is available in mammalian models and humans; however there is limited, but increasing research surrounding the microbiome of fish due to the decreasing cost of sequencing (Figure 1.1).

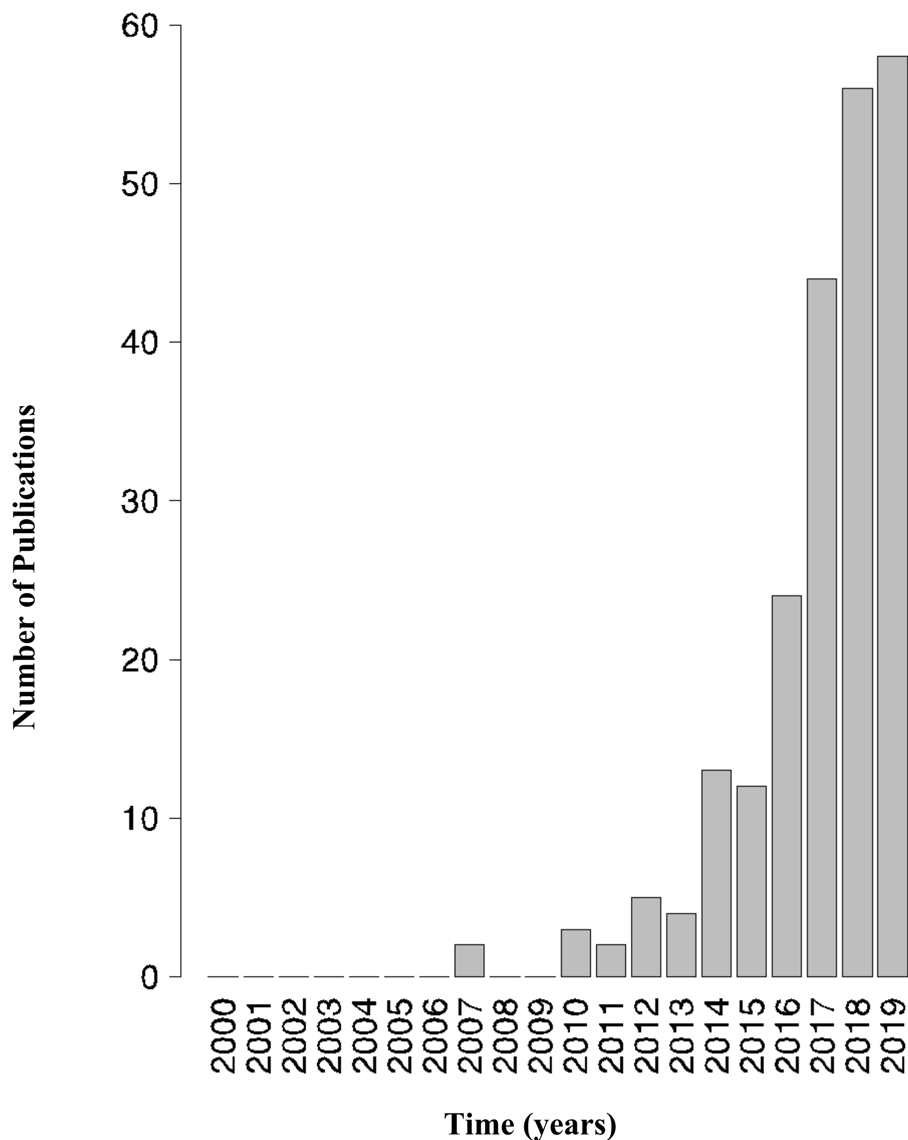

Figure 1.1 The number of journal publications available from 2000 to 2019 on Google Scholar (https://scholar.google.ca) after inputting the search term "fish gut microbiome" (search date: November 14, 2019).

The gut microbiome of fish varies based on the species, its diet, and its environment (Sullam et al., 2012, Tarnecki et al., 2017). The fecal microbiome of laboratory and wild carp share the same dominant phyla (Proteobacteria, Firmicutes, and Fusobacteria), suggesting the presence of a core microbiome (Eichmiller, 2016). In contrast, different fish species have a distinct gut microbiome; zebrafish, a common model species, is dominant in Proteobacteria and

Fusobacteria, while Asian seabass are dominant by Proteobacteria, Firmicutes, and Bacteriodetes (Roeselers et al., 2011, Xia et al., 2014). Since gut bacteria aid in digestion, carnivores and herbivores have distinct microbial communities (Sullam et al., 2012, Liu et al., 2016). When confronted with an altered diet, the gut bacterial community shifts to maintain the host's digestive physiology (Dhakal, 2017). For example, the gut microbiome of rainbow darter, a carnivorous freshwater fish, is typically dominant in Proteobacteria; after it was fed a commercial diet lower in protein there was an increased abundance of Bacteriodetes (Dhakal, 2017). The gut microbiome of laboratory fish are more similar to one another than to wild individuals of the same species; this suggests that environment also affects the microbiome (Eichmiller, 2016). Zebrafish sampled from different laboratories had significant variations in gut bacterial composition, which suggests that factors such as infrastructure, water chemistry, temperature, diet composition, feeding schedule, and/or antibiotic use affects fish gut bacterial development (Roeselers et al., 2011). Finally, the microbial composition of the fish gut is affected by season, with significant dissimilarities between samples collected at different water temperatures, and with different diet availabilities (Ray, 2016, Zarkasi et al., 2014).

1.3 Wastewater Treatment Plant Effluent and the Gut Microbiome of Fish

Some studies have found that WWTP effluent alters gut bacterial composition in fishes. The gut microbiome of wild brown trout downstream of a WWTP had increased abundances of *Enterobacteriaceae* and *Planctomycetaceae* compared to fish upstream (Giang et al., 2018). The genera *Nakamurellaceae*, *Oscillatoriales*, *Acidimicrobiales* were found exclusively in the downstream fish (Giang et al., 2018) and the first two genera have been isolated from activated sludge (Tice et al., 2010). In addition, the gut microbiome of common carp held in outdoor ponds

and exposed to WWTP effluent were abundant in Actinobacteria and Cyanobacteria, which have been found in WWTP sludge (Sakalli et al., 2018).

In addition to whole effluents, individual chemicals found in WWTP effluent can affect gut bacterial composition and diversity in fishes. Goldfish exposed to the pesticide and disinfectant pentachlorophenol had increased Bacteriodetes and decreased Firmicutes, which were believed to play a role in their decreased body and liver weight (Kan et al., 2015). Exposure of brown trout embryos to varying doses of the anti-diabetic drug metformin lead to an increase in Proteobacteria and a decrease in Firmicutes and Actinobacteria in the whole gut (Jacob et al., 2018). Mosquito fish exposed to the antibiotic rifampicin had altered gut microbiome composition and diversity; at the end of the exposure period, there was a complete change in the dominant operational taxonomic units (OTUs) and a 48% loss of diversity (Carlson, 2017). Acute exposure of zebrafish to triclosan, a widespread antimicrobial compound found in WWTP effluent (Arlos et al., 2014), resulted in decreased abundance of the bacterial family *Enterobacteriaceae*, while the genus *Pseudomonas* remained abundant (Gaulke et al., 2016).

1.4 The Grand River Watershed

The Grand River is located in southern Ontario, Canada, and flows south 280 km from its headwaters in Dufferin Highlands to Dunnville, where it empties into Lake Erie (Grand River Conservation Authority, 2014). This watershed is the largest in Ontario, and contains a population of more than 985,000, which includes several growing urban cities including Waterloo and Kitchener (Grand River Conservation Authority, 2014). The majority of the population is located around the central Grand River, at approximately 728.2 people per km^2 (Loomer & Cooke, 2011). As the population continues to grow, more pressure is placed on WWTPs to treat greater volumes of wastewater and to adopt increasingly advanced treatment

methods to remove more contaminants before they are released into the river. Furthermore, 70% of the watershed is used for agriculture (Grand River Conservation Authority, 2014). The river receives runoff from farms and urban stormwater, and is impacted by effluent from 30 different WWTPs (Figure 1.2).

Runoff and discharges from agriculture, industry, farming, and wastewater are known to affect the water quality of the Grand River, and monitoring of nutrients and dissolved oxygen (DO) is done at sites along the river to better understand and identify areas in need of improvement. The water quality in the river, around the towns of Elora and Fergus (upper-central Grand River), is typically classified as fair-good and is able to support a brown trout tailwater fishery (Loomer & Cooke, 2011). In general, several water quality measures fall below Ontario Provincial Water Quality Objectives including nitrate, ammonia, total phosphorus, and chloride, however as the river flows downstream these measures tend to increase. More specifically, phosphorous and nitrate concentrations increase as a result of inputs from the Irvine, Canagagigue and Conestogo rivers, which have catchments dominated by agricultural use (Loomer & Cooke, 2011). As the river flows through the urban city of Waterloo, high nitrogen and phosphorus in its waters have been attributed to WWTP effluent (Loomer & Cooke, 2011). In urban areas and downstream of WWTP outfalls, ammonia concentrations exceeding the Provincial Water Quality Objective of 20 µg/ L were reported at most sites, with Blair (a site downstream of the Kitchener WWTP) having the highest concentration (Loomer & Cooke, 2011). Between 2003-2007 at Glen Morris, a monitoring site downstream of the Waterloo and Kitchener WWTPs, the DO fell below the 4 mg/L provincial objective 25-30% of the time between June and September, while other locations hovered just above 4 mg/L (Loomer & Cooke, 2011). Urban road salt applications and WWTP effluents have also resulted in increased

chloride at urban locations (Loomer & Cooke, 2011). Modeling of the Grand River indicated that pharmaceuticals increase immediately downstream of WWTPs and decrease with distance due to dilution, degradation, biodegradation and photolysis (Arlos et al., 2014). Simulations have demonstrated that recent and future upgrades to both the Waterloo and Kitchener WWTPs will be successful in controlling the release of highly estrogenic effluents, even considering low flow in the river and the projected population growth (Arlos et al., 2018). Therefore, water quality in the Grand River is affected by the cumulative activities in the watershed, including WWTP discharge.

Several studies have demonstrated impacts of the Waterloo and Kitchener WWTP effluents on fish reproductive health and population dynamics. Fish downstream of effluent discharges have had altered gonadal development, high rates of intersex, reduced reproductive success, declines in previously dominant fish populations, and increases in tolerant fish species (Tetreault, 2012, Tanna et al., 2013, Fuzzen, 2016, Tetreault et al., 2013).

The rainbow darter *(Etheostoma caeruleum)* is a small, benthic, freshwater fish species, found most often in stream riffles throughout eastern North America (Paine et al., 1982, Ray et al., 2006). Furthermore, rainbow darters have been found in areas both upstream and downstream of WWTP outfalls on the Grand River (Brown, 2010). The habitat preference, reduced mobility, and abundance in the central Grand River make them an ideal sentinel species for a novel study exploring the effects of WWTP effluent on the fish gut microbiome.

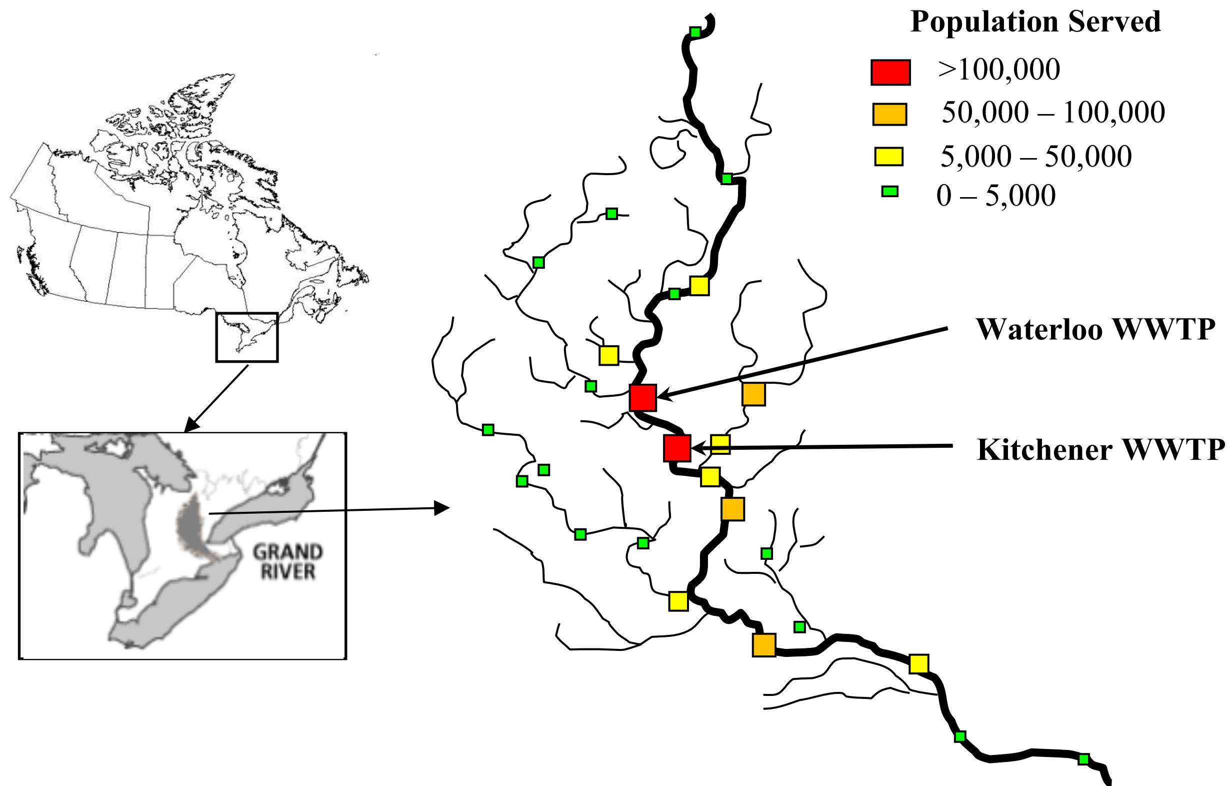

Figure 1.2 The location of WWTPs on the Grand River, as well as the population that they service (Grand River Conservation Authority, 2016, Fuzzen, 2016).

1.5 Objectives

Studies suggest that exposing fish to wastewater effluent alters the composition of their gut microbiome, and could potentially lead to altered fish health. Experiments assessing the effect of WWTP effluent in the field and in the lab are integral to enhance our understanding of the fish gut microbiome after exposure to an environmental stressor. The aim of this study was to examine the hypothesis that exposure to WWTP effluent will affect the diversity and composition of the gut content microbiome in rainbow darter of the central Grand River. Both field and laboratory studies were conducted to answer the following questions:

1. Does exposure to WWTP effluent in the field affect the composition and diversity of the rainbow darter gut content microbiome?

2. Does transition of wild fish to the lab, and 28 day exposure to environmental stressors (WWTP effluent and the antimicrobial triclosan), alter the composition and diversity of the gut microbiome of rainbow darter?

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Chapter 2: Rainbow darter exposed to wastewater effluent in the field have altered gut content microbiome composition and diversity

Abstract

The microbiome represents a community of bacteria integral in maintaining host health. At present little is known about the gut content microbiome of fish, but emerging research suggests that it is influenced by environmental stressors, diet, and habitat. This study evaluates how exposure to wastewater treatment plant (WWTP) effluent in the central Grand River, Ontario, Canada, affects the diversity and composition of the gut content microbiome of the rainbow darter (*Etheostoma caeruleum*), a sentinel species common in this system. In October 2018, gut content was sterilely collected from rainbow darter at 10 sites (n=15/site) located at varying distances upstream and downstream of two major WWTPs. Genomic DNA was extracted, and PCR amplification of the 16S rRNA gene V3-V4 region and Illumina sequencing were performed. Amplicon sequence variants (ASV) were mapped back to bacterial species using the SILVA database and DADA2 pipeline. Diversity of the gut content microbiome increased significantly downstream of both WWTP outfalls (Shannon Diversity, one-way ANOVA, F=2.575, p=0.09) when compared to upstream samples. Bray-Curtis beta diversity and principle coordinate analysis showed that individuals within and between sites were significantly dissimilar (PERMANOVA, F=2.9135, p=0.001). Dominance (Berger-Parker Dominance) decreased from upstream to downstream sites (one-way ANOVA, F=2.393, p=0.015). The dominant bacteria in upstream samples belonged to the phyla Proteobacteria and Firmicutes, whereas downstream samples had higher relative abundances of Proteobacteria and Cyanobacteria, and decreased abundances of Firmicutes. Mammalian literature suggests that an increased abundance of Proteobacteria is indicative of dysbiosis between the host and its gut microbiota and is linked to altered host health. However, much of the functionality of bacteria in

the fish gut is unknown and cannot yet be linked to fish health outcomes**.** This research suggests that the fish gut content microbiome is affected by exposure to the complex mixtures of emerging and legacy contaminants found in WWTP effluents.

2.1 Introduction

The fish microbiome is the collection of microorganisms found within the interior and on the exterior of an individual. Fish carry several different microbiomes including: mucosal, gill, intestinal, gut content, and fecal. Microbes from the surrounding environment and from feeding colonize the fish intestine resulting in a bacterial community important for maintenance of host physiology, nutrient supply, infection prevention, and mucosal maintenance (Tarnecki et al., 2017, Nayak, 2010). In mammalian studies, a change in bacterial composition, known as dysbiosis, has been linked to altered health outcomes (Ferrer et al., 2017, Antonopoulos et al., 2009). Altered health outcomes from microbiome dysbiosis may also occur in fish, but this is unknown.

Drugs including antibiotics and antimicrobials can disrupt the microbiome of humans and mice. In humans, the observed microbiome is dependent on the antibiotic used and its route of administration, but can result in lowered microbial diversity, altered microbial composition, and persistence of resistant bacteria (Ferrer et al., 2017). Similarly, mice treated with a mixture of ampicillin, metronidazole, and bismuth had altered microbial composition in the cecum and domination of the phyla Proteobacteria (from 1.1% in unexposed to 75.5% in exposed mice) (Antonopoulos et al., 2009). Antibiotics can deplete gut microbial diversity and cause adverse effects (inhibited mitochondrial gene expression and increased epithelial cell death) in mice (Morgun et al., 2015). Subinhibitory concentrations of antibiotics, antimicrobials and antibacterials can impact bacterial genes leading to DNA damage and ultimately affecting the gut microbiome (ciprofloxacin; Yim et al., 2011).

Environmental contaminants, including some found in WWTP effluent, affect the fish microbiome. The antimicrobial triclosan alters the microbial composition of fish guts, with the

family *Enterobacteriaceae* being susceptible to exposure and the genus *Pseudomonas* being more resilient (Gaulke et al., 2016). When compared to control fish, zebrafish exposed to the antibiotic oxytetracycline had increased Fusobacteria and decreased Proteobacteria and Planctomycetes (Zhou et al., 2018). Fish also had altered gut health as evidenced by increased metabolic rate and expression of inflammatory factors as well as decreased intestinal goblet cell numbers, alkaline phosphatase, acid phosphatase and anti-oxidant responses (Zhou et al., 2018). Zebrafish larvae exposed to low levels of streptomycin, used to treat aerobic, gram-negative bacterial infections in humans, agriculture and aquaculture, had reduced microbial diversity and reduction of the phyla Bacteriodetes, as well as increased early mortality and elevated expression of *int1* associated with antibiotic resistance genes (Pindling et al., 2018). Western mosquitofish exposed to the antibiotic rifampicin had lowered gut microbiome diversity and altered composition, with antibiotic-resistant bacteria persisting during a one-week recovery period (Carlson et al., 2015, Carlson et al., 2017). The microbiome of larval zebrafish exposed to arsenic had altered composition, structure, and diversity; in addition, high (100 ppb) level arsenic exposure resulted in increased expression of *int1* (Dahan et al., 2018). The fecal microbiome of Nile tilapia exposed to waterborne cadmium had lower diversity and altered composition (Zhai et al., 2017). Similarly, polycyclic aromatic hydrocarbons reduced the microbial diversity, altered bacterial abundance, and increased the bacterial order Deferribacterales in the guts of Atlantic cod (Bagi et al., 2018). To date, the effects of contaminant mixtures or complex effluents, on the gut microbiome of fish is limited.

WWTP effluent is a mixture of many contaminants including pesticides, pharmaceuticals, and metals, and few studies have investigated its effects on the fish gut microbiome. The gut microbiome of wastewater-exposed common carp had a high abundance of microbial species that

are associated with wastewater sludge, including the genera *Hyphomicrobium* (phyla – Proteobacteria), *Nakamurella* (phyla - Actinobacteria), *Phormidium* (phyla - Cyanobacteria), and *Pirellula* (phyla – Planctomycetes) when compared to unexposed fish (Sakalli et al., 2018). Similarly, wild brown trout exposed to wastewater effluent had an increased abundance of bacteria from the family *Enterobacteriaceae,* also associated with WWTP effluent (Giang et al., 2018). The fecal microbiome of wild-caught tropical fish exposed to wastewater discharges was dominated by Proteobacteria, followed by Firmicutes and Actinobacteria (Hennersdorf et al., 2016). Little is known about the effects of effluents on the gut microbiome of small-bodied freshwater species.

Rainbow darter (*Etheostoma caeruleum*), named for its bright colouration in males, is a small, benthic, carnivorous (insectivorous) fish typically found in riffles of small streams and rivers. They are abundant in the central Grand River (Ontario, Canada), and the effect of WWTP effluents on *Etheostoma* species has been well documented and includes reproductive and metabolic impairment (Tetreault et al., 2011, Tanna et al., 2013, Fuzzen et al., 2016). Rainbow darter collected downstream of WWTP outfalls along the Grand River had higher oxygen consumption and instances of intersex; females had increased cortisol levels, reduced hormone production, fertilization success, and embryo survival, and delayed spawning, and gonadal development (Mehdi et al., 2018, Tetreault et al., 2011, Fuzzen et al., 2015, Fuzzen, 2016, Tetreault et al., 2013). Rainbow darters have a small home range (median of 5 metres, Hicks et al., 2017), and thus are a good species for studying the effects of point-sources of pollutants. There is no current research on how or whether the composition of their microbial community responds to point-sources, making rainbow darter an ideal species for the present study.

This study aims to achieve a greater understanding of the microbiome of wild-caught freshwater fish by determining the effect of exposure to WWTP effluent on the gut content microbiome of rainbow darter inhabiting the central Grand River, Ontario, Canada. Female rainbow darters were sampled across 10 sites upstream and downstream of both the Waterloo and Kitchener WWTP effluent outfalls. It was predicted that gut content microbiome diversity would decline and the composition would be altered to include a greater abundance of Proteobacteria in wastewater exposed fish.

2.2 Materials and Methods

Site Characterization

This study was conducted in the Grand River watershed, which is located in southern Ontario and is the largest drainage basin flowing into Lake Erie. The Grand River watershed spans approximately 6800 km^2 and includes an abundant agricultural area, several cities, and 30 WWTPs (Mehdi et al., 2018). As such, it receives runoff from farms, aggregate extraction, urban storm waters, city roadways, and WWTP effluents. Ten different sites along the Grand River were selected and sampled over the course of one week (October 22-27) in the fall of 2018 (Figure 2.1). Sites spanned 60 km of the river and were chosen based on their location relative to two secondary-conventional activated sludge treatment plants, the Waterloo and Kitchener WWTPs (Figure 2.1, Table 2.1). Both WWTPs have been well studied in terms of their effects on the rainbow darter living downstream, including stress responses, metabolic performance, fertilization and reproductive success, and population surveys (Mehdi et al., 2018, Tetreault et al., 2011, Fuzzen et al., 2015, Fuzzen, 2016, Tetreault et al., 2013). The relative location of each sampling site, the distance from outfall, and GPS coordinates of the sites can be found in Table 2.1.

Water Quality Measurements and Water Sampling

Several water quality parameters were taken at the time of fish sampling including: temperature (°C), dissolved oxygen (mg/L), conductivity (μS/cm), total dissolved solids (ppm), pH, ammonium - $NH4^+$ (mg/L), and ammonia - NH3 (mg/L) using a YSI 556 multi-parameter sensor (Appendix A, Table A.1). Water quality measurements were obtained at each site at 5 separate points across the width of the river.

Water samples were collected in October 2019 at 3 locations (left, centre, right) within each site on the river, as well as from the Waterloo and Kitchener WWTP, for microbial analysis (Figure 2.1). Facing upstream and wearing sterile gloves, 50 mL of water was collected by inserting a sterile Falcon tube open side down until the tube was immersed, the tube was turned up and filled completely (no surface water), capped under water, and stored in a cooler with ice until returning to the lab (approximately 5 hours). Water was then filtered through a sterile 45 µm filter paper, and the filter paper was stored in buffer solution until gDNA was extracted (Materials & Methods, *Gut content extraction, 16s rRNA gDNA extraction*). Water samples were collected in fall 2019 to support the fish gut microbial analysis conducted in fall 2018; these water samples do not represent the conditions when the fish were collected.

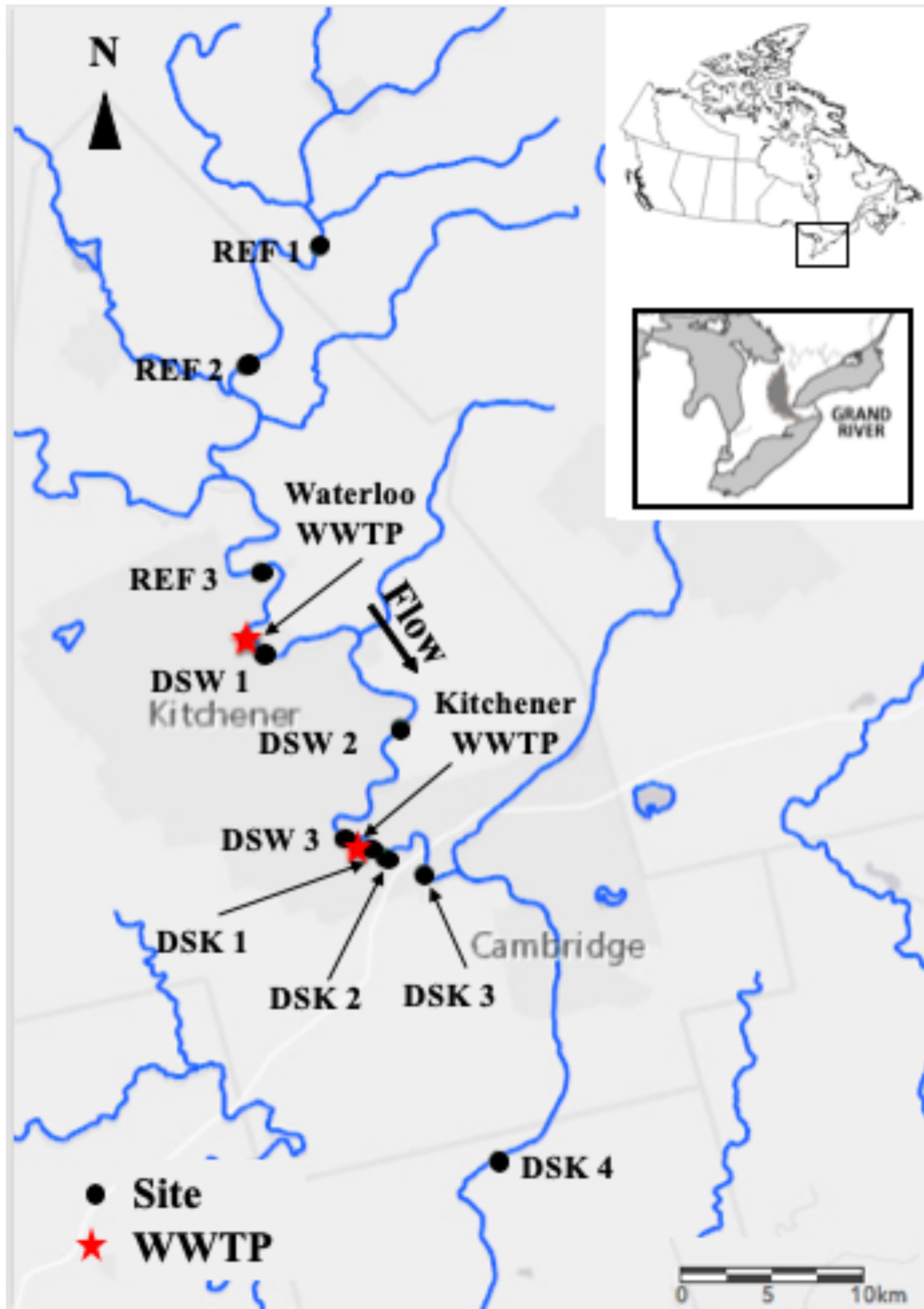

Figure 2.1 Rainbow darter gut content (October 2018) and water sampling sites (October 2019). Sites are indicated with a black circle and the Waterloo and Kitchener WWTPs are indicated with a red star.

Table 2.1 Site ID, relative location, distance from outfall, and GPS coordinates of sites sampled in October 2018 (fish) and 2019 (water).

Site Name (ID)	Relative Location	Distance from Outfall	GPS Location	
			Latitude	Longitude
Inverhaugh (REF 1)	Upstream of Waterloo WWTP	17.185 km from Waterloo	43.63120	-80.44286
West Montrose (REF 2)	Upstream of Waterloo WWTP	11.762 km from Waterloo	43.58515	-80.48230
Kiwanis (REF 3)	Upstream of Waterloo WWTP	2.936 km from Waterloo	43.50524	-80.47439
EIT (DSW 1)	Downstream Waterloo WWTP	0.987 km from Waterloo	43.47359	-80.47310
Fairway (DSW 2)	Downstream Waterloo WWTP	7.713 km from Waterloo	43.44495	-80.39948
Horse Ranch (DSW 3)	Downstream Waterloo WWTP	9.609 km from Waterloo	43.40202	-80.42977
PT1 (DSK 1)	Downstream Kitchener WWTP	0.605 km from Kitchener	43.39813	-80.41570
PT2 (DSK 2)	Downstream Kitchener WWTP	1.278 km from Kitchener	43.39483	-80.40833
Blair (DSK 3)	Downstream Kitchener WWTP	3.175 km from Kitchener	43.38821	-80.38671
Glen Morris (DSK 4)	Downstream Kitchener WWTP	14.884 km from Kitchener	43.27722	-80.34693

Collection of Rainbow Darter

Fifteen female rainbow darters greater than 4.5 cm in length were collected at each site using a backpack electrofisher (Smith Root, LR-20 and LR-24). Fish were stored live in aerated buckets and sampled up to 3-hours post-collection on site in a mobile lab. Fish lengths (± 1.0 mm) and weights (±0.01 g) were collected. All fish were collected and handled in accordance with MNR license #1092042, AUP #16-09-34 and Amendment #18-155 as approved by the Animal Research Ethics Board at McMaster University. Fish were first made unconscious by a blow to the head and then euthanized by spinal severance. Gut content was extracted using

aseptic methods as described in detail below. Time between euthanasia and gut content extraction was less than 5 minutes.

Gut Content Extraction

Prior to field sampling tubes were prepared with 900 µL of buffer (0.564 M guanidine thiocyanate, 0.01 M EDTA, 0.004 M N-lauroyl sarkosine and 177 mM of monobasic NaH_2PO_4) and 0.2 g of 0.1 mm glass beads (MoBio Laboratories, Carlsbad, CA, USA). All sampling was conducted aseptically. Prior to fish handling, the bench space was sterilized with 10% bleach and 70% EtOH, and then covered with aluminum foil. Forceps and scissors were sterilized in 30% bleach, 70% EtOH, and nanopure water (referred to as wash solutions), in that order, prior to dissection. Additionally, gloves were changed between each fish. First, fish were rolled in a Kim wipe pre-moistened with 70% EtOH to remove exterior bacteria. The ventral side of the fish was opened from anus to throat; the liver was removed then forceps and scissors were re-sterilized using the wash solutions. The intestinal tract was cut at the end nearest to the anus. Using forceps, the gut content was gently emptied from the intestine into a tube containing the NaH_2PO_4 and GES buffer solution and tubes were inverted to mix the contents. At each site, three blanks were collected by adding 0.5-1 mL of each wash solution (30% bleach, 70% EtOH, and nanopure water) to a buffer tube. Buffer tubes were stored at room temperature until the end of field sampling (6 days), when they were stored at -80ºC.

16S rRNA gDNA Extraction

Samples were thawed at room temperature and mechanically lysed using a bead beater at 3000 rpm for 3 minutes. A mixture of 50 µL of lysozyme (100 mg/mL; Sigma-Aldrich, Oakville, Ontario) and 10 µL of RNase A (10 mg/mL; Qiagen, Toronto, Ontario) was added to each sample, vortexed, and incubated at 37ºC for 1-1.5 hours. Next, 25 µL of 25% SDS, 25 µL of

proteinase K (30 units/mg; Sigma-Aldrich, Oakville, Ontario), and 62.5 µL of 5 M NaCl was combined with samples and incubated at 65ºC for 0.5-1.5 hours. The tubes were centrifuged at 13,500 *g* for 5 minutes, and the supernatant was pipetted into 900 µL of phenol-chloroform-isoamyl alcohol (Sigma-Aldrich, Oakville, Ontario), vortexed, and centrifuged at 13,000 *g* for 10 minutes. The aqueous-layer of the sample was pipetted into tubes filled with 200 µL of DNA binding buffer (Cedarlane Laboratories, Burlington, Ontario) and the solution was transferred to a DNA column (Cedarlane Laboratories, Burlington, Ontario). A vacuum manifold was used to move the solution through the DNA column, followed by wash buffer (DNA Clean and Concentrator-25, Cedarlane Laboratories, Burlington, Ontario). 50 µL of sterile DNase/RNase free water was added to each tube, and allowed to incubate at room temperature for 5 minutes. Finally, the DNA was eluted by centrifugation at 12,000 *g* for 30 seconds – 1 minute. Samples were then stored in the -80ºC until PCR.

Nested-PCR Amplification of the 16S rRNA V3 and V4 Regions

Nested PCR of the 16S rRNA V3 and V4 region was conducted. Fish and/or other eukaryotic DNA gave a high background with the V3-V4 primers, which was reduced with the nested protocol. In house testing of this method demonstrated that the protocol did not skew the bacterial profile (L. Rossi, personal communication). The 8F (AGAGTTTGATCCTGGCTCAG) to 926R (CCGTCAATTCCTTTRAGTTT) region of the 16S gene was first amplified using 1.5 µL of template with 1U of Taq, 1x buffer, 1.5 nM $MgCl_2$, 0.4 mg/mL BSA, 0.2 mM dNTPs, and 10 pmol of each primer. The PCR protocol was as follows: 5 mins at 94ºC, 30 seconds at 94ºC for 15 cycles, 30 seconds at 56ºC, 60 seconds at 72ºC, and 10 minutes at 72ºC. This reaction was used as the template for the second stage of PCR. 3 µL of the first reaction was used with 1U of Taq, 1x buffer, 1.5 nM $MgCl_2$, 0.4 mg/mL BSA, 0.2 mM dNTPs, and 5 pmol of 341F

(CCTACGGGNGGCWGCAG) and 806R (GGACTACNVGGGTWTCTAAT) Illumina adapted primers (IDT, Coralville, Iowa) (Bartram et al., 2011). The PCR protocol was as follows: 5 mins at 94ºC, 30 seconds at 94ºC for 5 cycles, 30 seconds at 47ºC, 30 seconds at 72ºC, 30 seconds at 94ºC for 25 cycles, 30 seconds at 50ºC, 30 seconds at 72ºC and a final extension of 10 minutes for 72ºC. PCR products were visualized on a 1.5% agarose gel. Positive amplicons were normalized using the SequalPrep normalization kit (ThermoFisher #A1051001, Mississauga, Ontario) and sequenced on the Illumina MiSeq platform (paired-end reads, 2 x 300 base pairs) at the Farncombe Institute (McMaster University, Hamilton, Ontario).

Read Processing

Cutadapt was used to filter and trim adapter sequences and PCR primers from the raw reads with a minimum quality of 30 and minimum read length of 100 bp (Martin, 2011). Reads were trimmed, sequences were filtered and trimmed based on quality, and sequence variants were determined using DADA2 (Callahan et al., 2016). Bimeras were removed and the amplicon sequence variants (ASVs) were assigned to taxonomy using the SILVA database (version 1.3.2).

Data Analysis

ASVs were analyzed using the Phyloseq package (version 1.30.0) in R (version 3.6.1). Samples belonged to one of three locations: upstream, downstream of Waterloo, and downstream of Kitchener. Each location included 3-4 sites: upstream – REF 1, REF 2, REF 3; downstream Waterloo – DSW 1, DSW 2, DSW 3; downstream Kitchener – DSK 1, DSK 2, DSK 3, DSK 4. Bacterial composition was determined by calculating relative abundance at various taxonomic levels (phyla, class, order, family, and genus) and patterns were determined between sites relative to the major WWTPs. Next alpha diversity was calculated at the ASV level; first samples were rarefied to minimum sample depth (3373 reads) and then alpha diversity was calculated

using the Shannon Diversity index and statistical significance was compared between sites using a one-way ANOVA and a Tukey's post-hoc test. Berger-Parker dominance was determined and statistical significance was compared between sites using a one-way ANOVA and Tukey's post-hoc test. Beta diversity was determined using a Bray Curtis with Principal Coordinate Analysis (PCoA) and statistical significance was determined using a permutational multivariate analysis of variance (PERMANOVA) using the function Adonis in the package Vegan (2.5.6). To understand data variability and error, a differential expression analysis (DESeq 2, version 1.22.2) was performed. Furthermore, redundancy analysis (RDA) and Pearson Correlation was performed between individual water quality parameters (temperature, DO, conductivity, total dissolved solids (TDS), pH, ionized (NH4) and unioinized (NH3) ammonia), fish length (cm), weight (g), condition factor (K), and distance from the WWTP. Fish condition was determined by calculating condition factor ($K = 100 \times \frac{weight\ (g)}{length^3\ (cm)}$). The condition factor analysis was computed on R studio using the package GGally. Lastly, site (distance from outfall) was plotted against condition factor (K) to determine if fish closer to the outfall showed signs of declining fish health.

2.3 Results

In total, there was 10,362,356 reads in all fish guts sampled; there was an average of 69,546 reads per sample (minimum of 3373 and maximum of 160,802 reads per sample). There was 36 phyla, 79 classes, 186 orders, 309 families, 839 genera, and 18,461 ASVs were identified in the gut content of all rainbow darter from the Grand River. Individual fish were assigned between 37 to 1196 ASVs, with a mean of 507 ASVs. From upstream to downstream, there was an average of 452, 422, 496, 656, 511, 541, 608, 458, 486, and 463 ASVs in the samples. Five bacterial phyla accounted for over 85% of all sequence reads: Proteobacteria (7411 reads, 40%), Firmicutes (5256, 28%), Actinobacteria (1759, 9.5%), Cyanobacteria (1323, 7.2%), and Bacteriodetes (688, 3.7%). Within Proteobacteria, all sequences belonged to the classes Alphaproteobacteria (3385, 46%), Gammaproteobacteria (3011, 41%), and Deltaproteobacteria (998, 13%). The most abundant genera across all samples were *Legionella* (phyla – Proteobacteria, Class – Gammaproteobacteria) (757 total reads) followed by *Bacillus* (phyla – Firmicutes, Class – Bacilli) (493 total reads).

Relative Abundance

Bacterial composition, using relative abundance of bacterial phyla in individuals, was highly variable, but most samples were dominated by Proteobacteria, Firmicutes, and Cyanobacteria, with lower abundances of Actinobacteria and Tenericutes (Figure 2.2, Figure 2.3). There were common patterns in the relative abundance of bacterial phyla, with Proteobacteria, Firmicutes, and Cyanobacteria dominating (Figure 2.4). The relative abundance of both Proteobacteria and Cyanobacteria increased downstream of both WWTPs, while Firmicutes decreased compared to the upstream locations (Table 2.2, Figure 2.3, Figure 2.4). Within the phyla Proteobacteria, the most abundant family was *Rhodobacteraceae* followed by

Rhizobiales (Figure 2.5). Within the phyla Cyanobacteria the most abundant family was *Cyanobiaceae,* followed by *Microcystaceae* (Figure 2.6). In the phyla Firmicutes, the most abundant family was *Clostridaceae* followed by *Rumincoccacceae* and *Erispelotrichaceae* (Figure 2.7). The relative abundance of bacterial phyla in the wash solutions used during sampling was also plotted (Appendix A, Figure A.1).

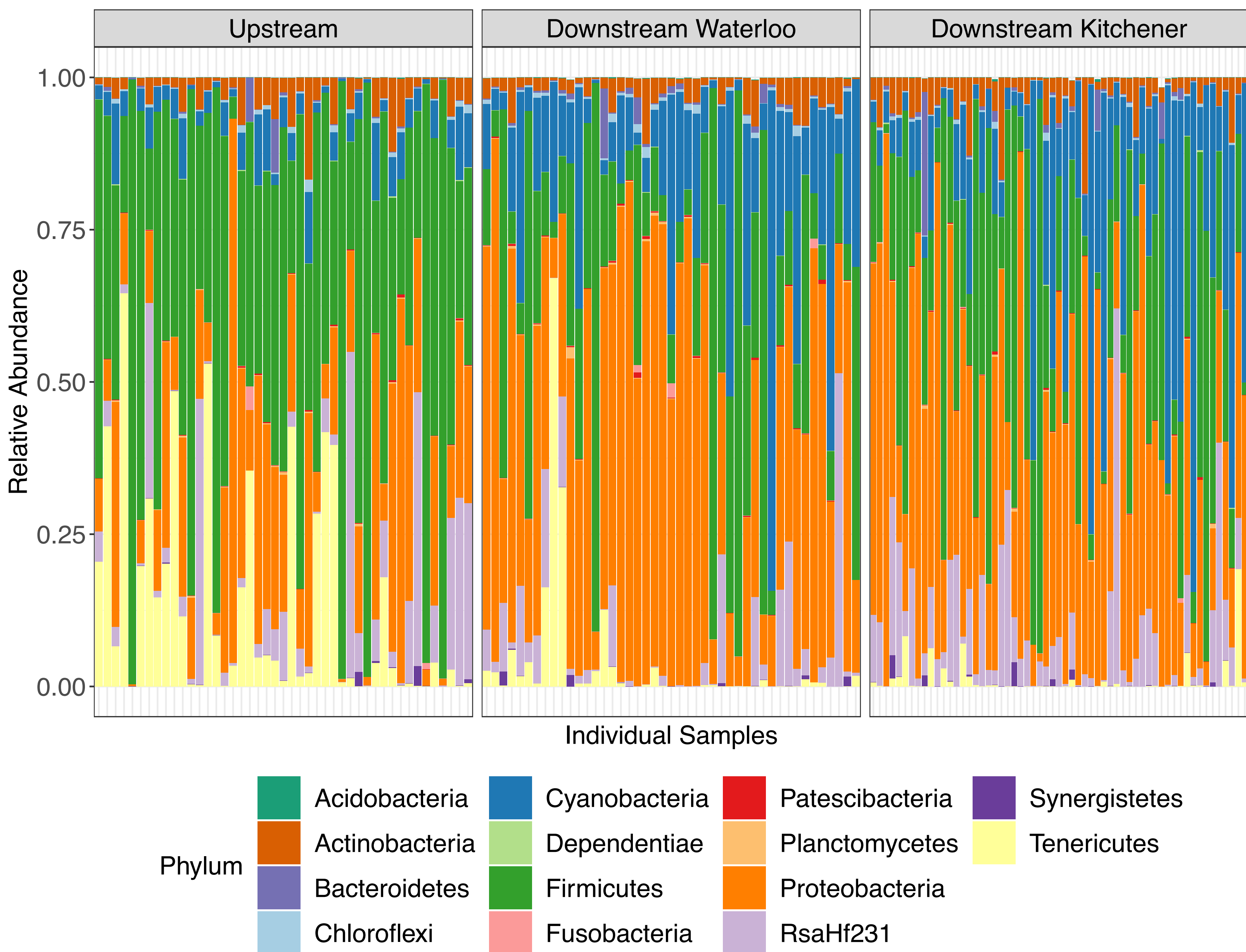

Figure 2.2 The mean relative abundance of gut bacteria at the phyla level in individual *E. caeruleum*. Only the phyla with > 2% of the total ASVs were included. The y-axis shows the proportion of ASVs within each included phyla from 0 to 1. The bars are ordered from upstream (REF) to downstream of the Waterloo (DSW) and Kitchener (DSK) wastewater treatment plants in order of flow (upstream – left, downstream - right).

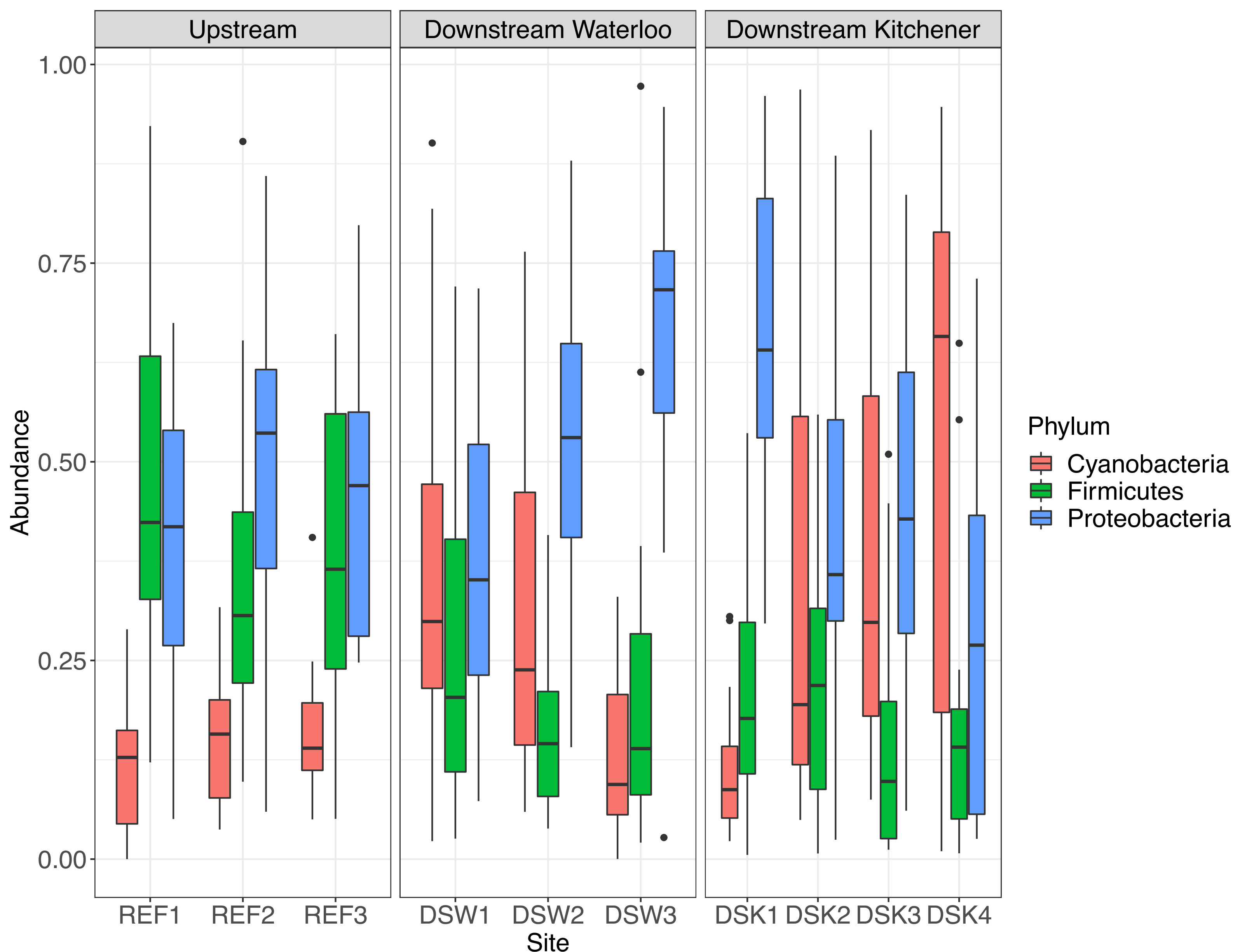

Figure 2.3 Boxplot showing the relative abundance of the most abundant phyla. The y-axis shows the proportion of ASVs within each included phyla from 0 to 1. Box and whiskers from upstream (REF) and downstream of the Waterloo (DSW) and Kitchener (DSK) wastewater treatment plants are in order of flow (upstream – left, downstream - right). The most abundant phyla, Cyanobacteria (red), Firmicutes (green), Proteobacteria (blue), are shown. Upper, middle, and lower lines represent first, second, and third quartiles; whiskers represent a 1.5 inter-quartile range.

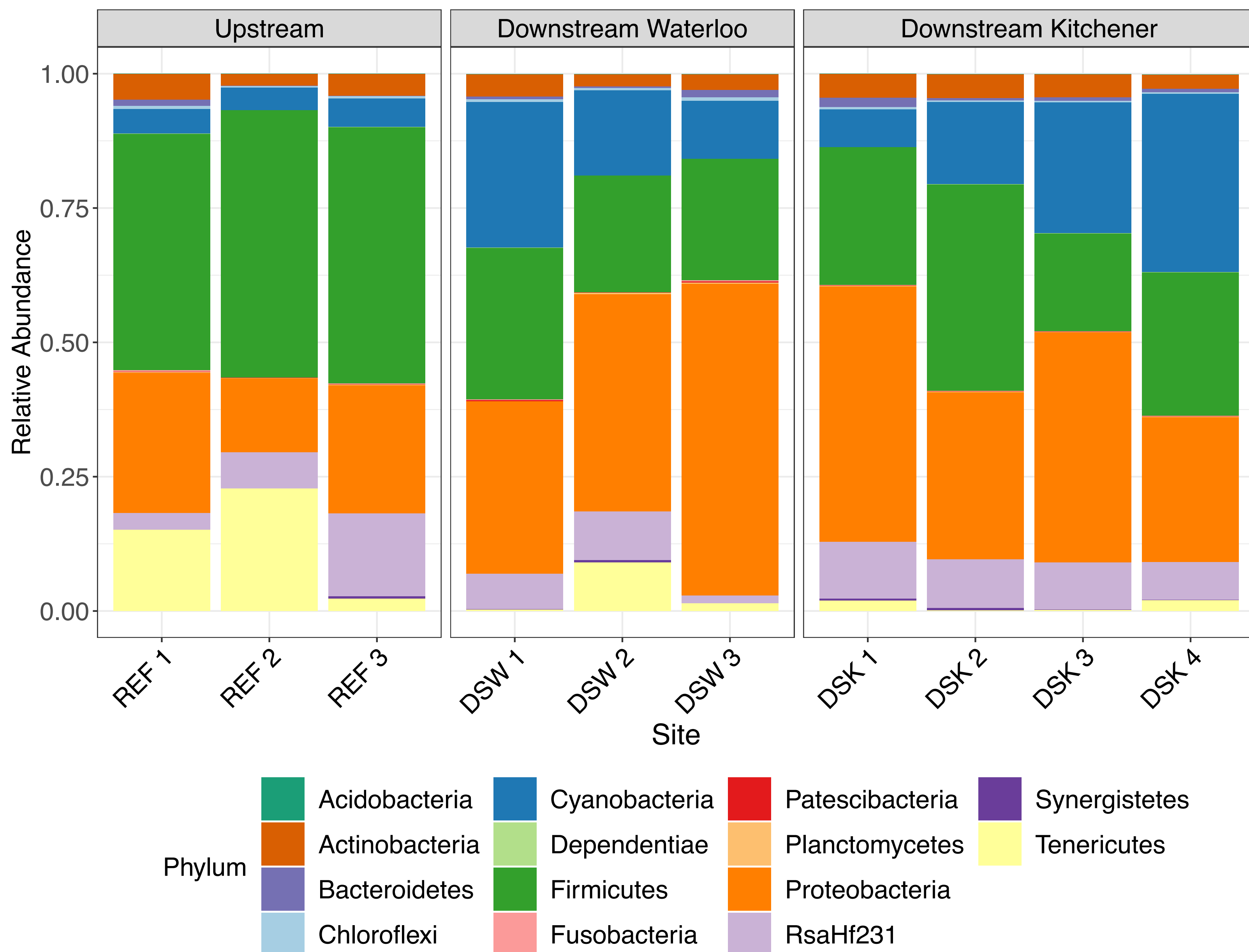

Figure 2.4 The mean relative abundance of gut bacteria at the phyla level in *E. caeruleum*. Only the phyla with > 2% of the total ASVs were included. The y-axis shows the proportion of ASVs within each included phyla from 0 to 1. The bars are ordered from upstream (REF) to downstream of the Waterloo (DSW) and Kitchener (DSK) wastewater treatment plants in order of flow (upstream – left, downstream - right). See appendix A for relative abundance plots at the class, order, family, and genus levels (Figure A.1, A.2, A.3, A.4).

Table 2.2 Relative abundance (%) of dominant phyla at sample sites.

	Upstream			Downstream Waterloo			Downstream Kitchener			
	REF1	REF2	REF3	DSW1	DSW2	DSW3	DSK1	DSK2	DSK3	DSK4
Proteobacteria	26.1	13.7	23.8	32.0	40.5	58.0	47.6	31.1	42.8	27.0
Cyanobacteria	4.56	4.16	5.27	27.1	15.8	10.7	7.05	15.3	24.4	33.2
Firmicutes	44.0	49.7	47.7	28.2	21.7	22.6	25.6	38.4	18.2	26.7

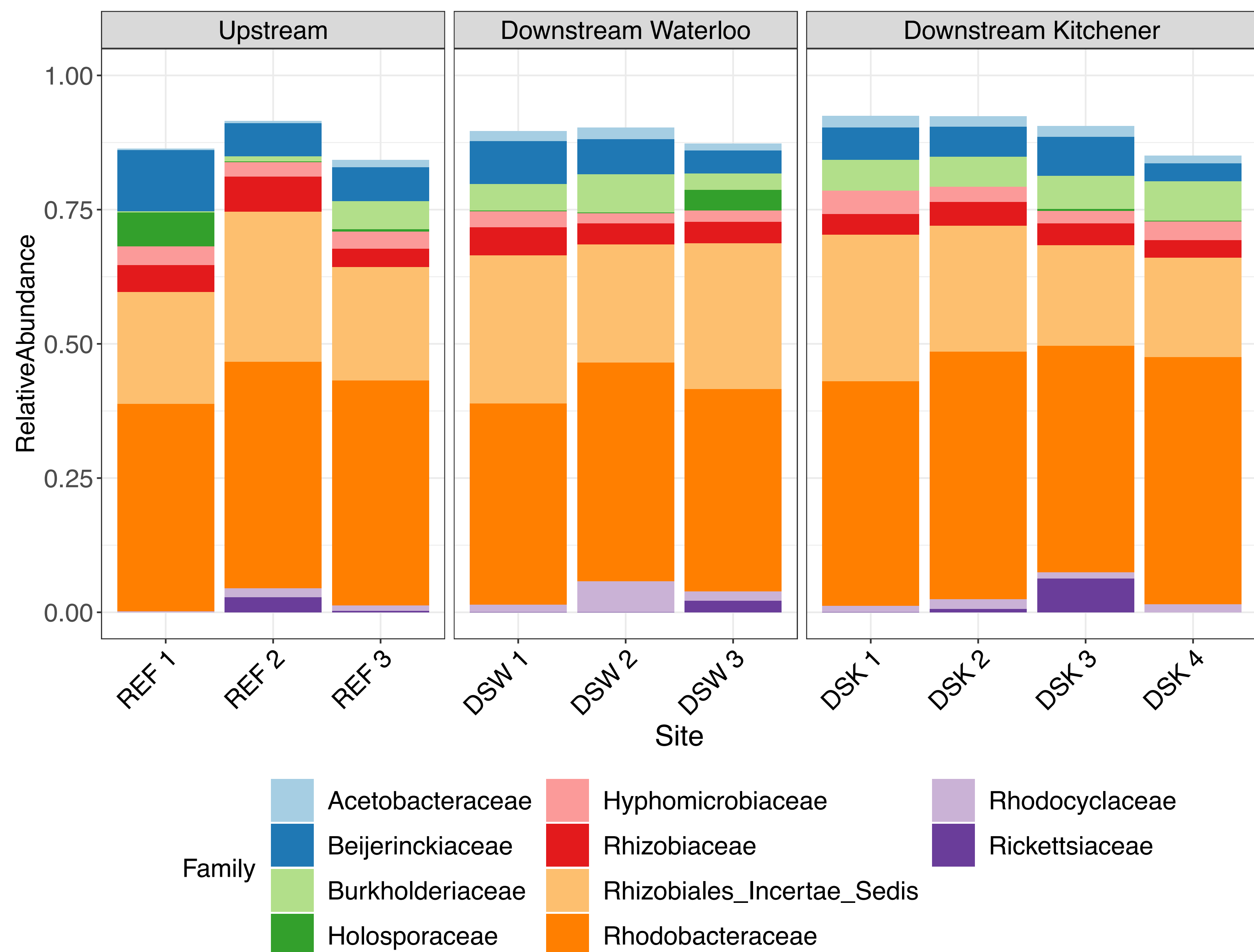

Figure 2.5 The mean relative abundance of top 10 families of Proteobacteria collected from the gut content of *E. caeruleum.* Only those with > 2% of the total ASVs were included. The y-axis shows the proportion of ASVs within each family from 0 to 1. The value of 1 on the y axis would include all families within Proteobacteria, but here only the top 10 are shown. The bars are ordered from upstream (REF) to downstream of the Waterloo (DSW) and Kitchener (DSK) wastewater treatment plants in order of flow (upstream – left, downstream - right).

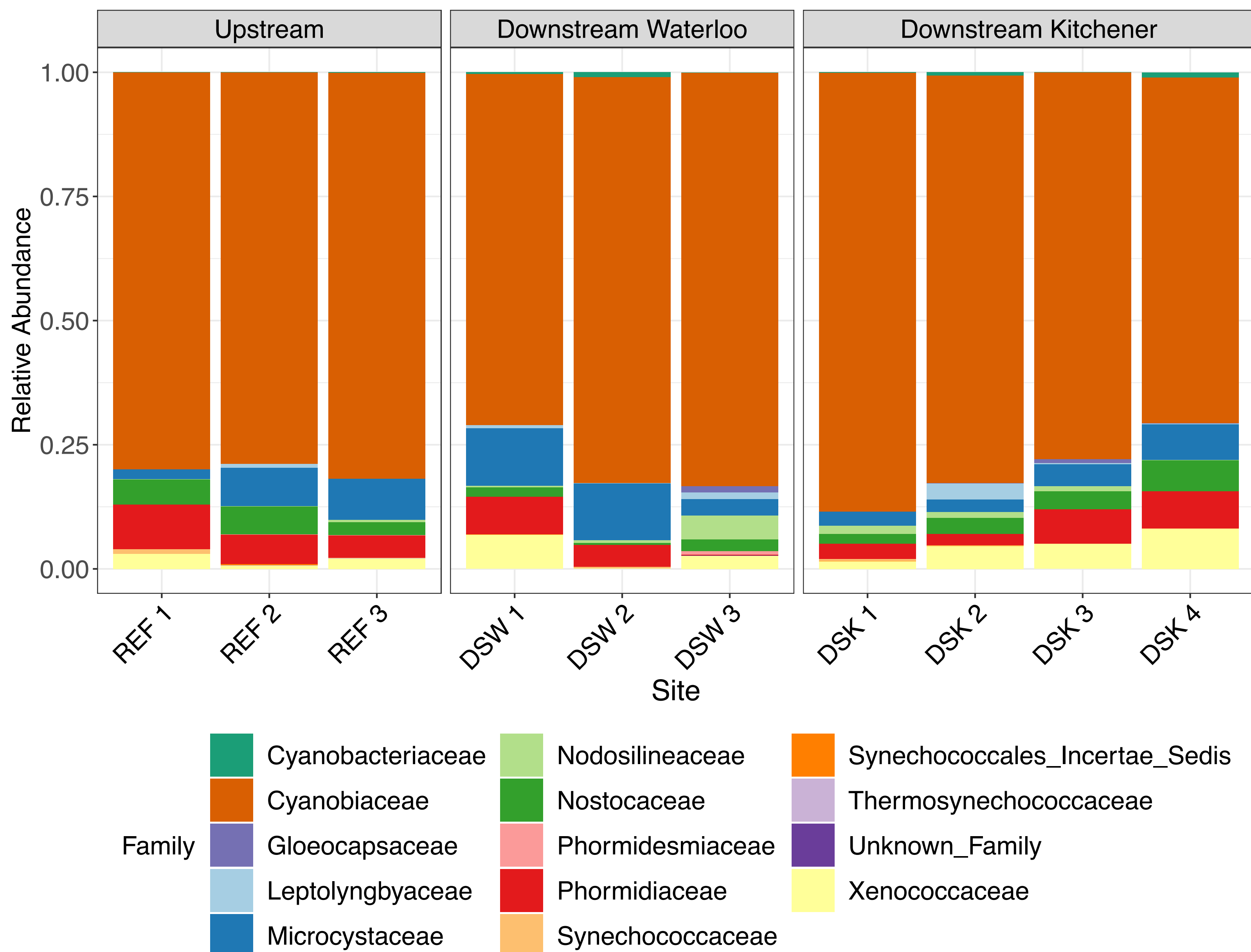

Figure 2.6 The mean relative abundance of Cyanobacteria collected from the gut contents of *E. caeruleum*. Only those with > 2% of the total ASVs were included. The y-axis shows the proportion of ASVs within each included phyla from 0 to 1. The bars ordered from upstream (REF) to downstream of the Waterloo (DSW) and Kitchener (DSK) wastewater treatment plants in order of flow (upstream – left, downstream - right).

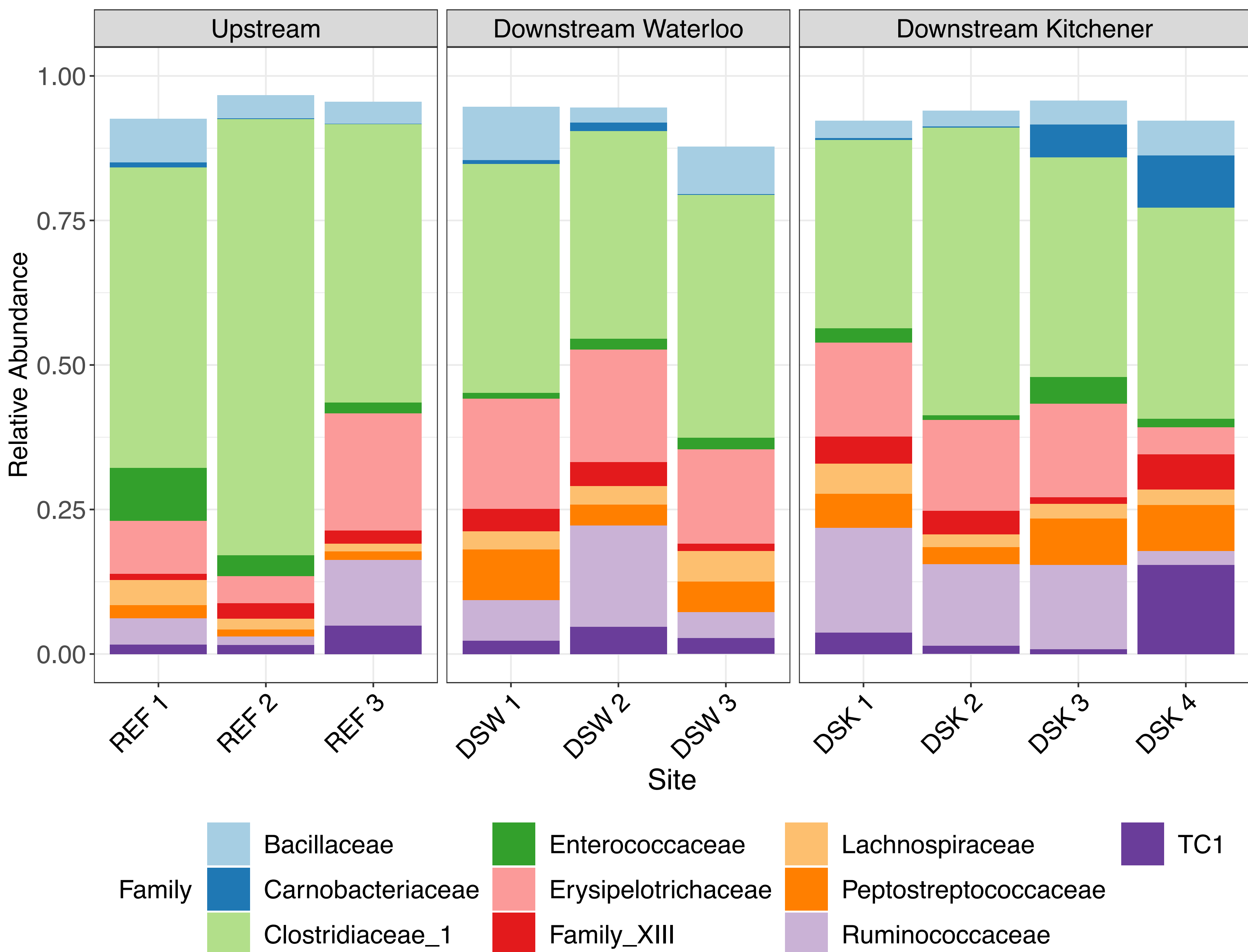

Figure 2.7 The mean relative abundance of top 10 families of Firmicutes collected from the gut content of *E. caeruleum.* Only those with > 2% of the total ASVs were included. The y-axis shows the proportion of ASVs within each family from 0 to 1. The value of 1 on the y axis would include all families within Firmicutes, but here only the top 10 are shown. The bars are ordered from upstream (REF) to downstream of the Waterloo (DSW) and Kitchener (DSK) wastewater treatment plants in order of flow (upstream – left, downstream - right).

Alpha Diversity

Shannon diversity was variable between sites, with medians ranging from 2.42 (REF 2) to 4.44 (DSW3). Alpha diversity differed significantly among sites (Figure 2.8; one-way ANOVA, Df=9, F=2.575, p=0.009). Samples from upstream sites (REF 1-3) were not significantly different from one another (Tukey HSD, p=0.31). In contrast, samples collected from REF 2 were significantly less diverse than those from DSW 3 (Tukey HSD, p=0.003) and DSK 1 (p=0.01). In general, samples collected from sites downstream of the WWTPs had increased diversity compared to those upstream. There was decreasing diversity at sites further downstream of the Kitchener WWTP, but this pattern was not seen downstream of the Waterloo WWTP.

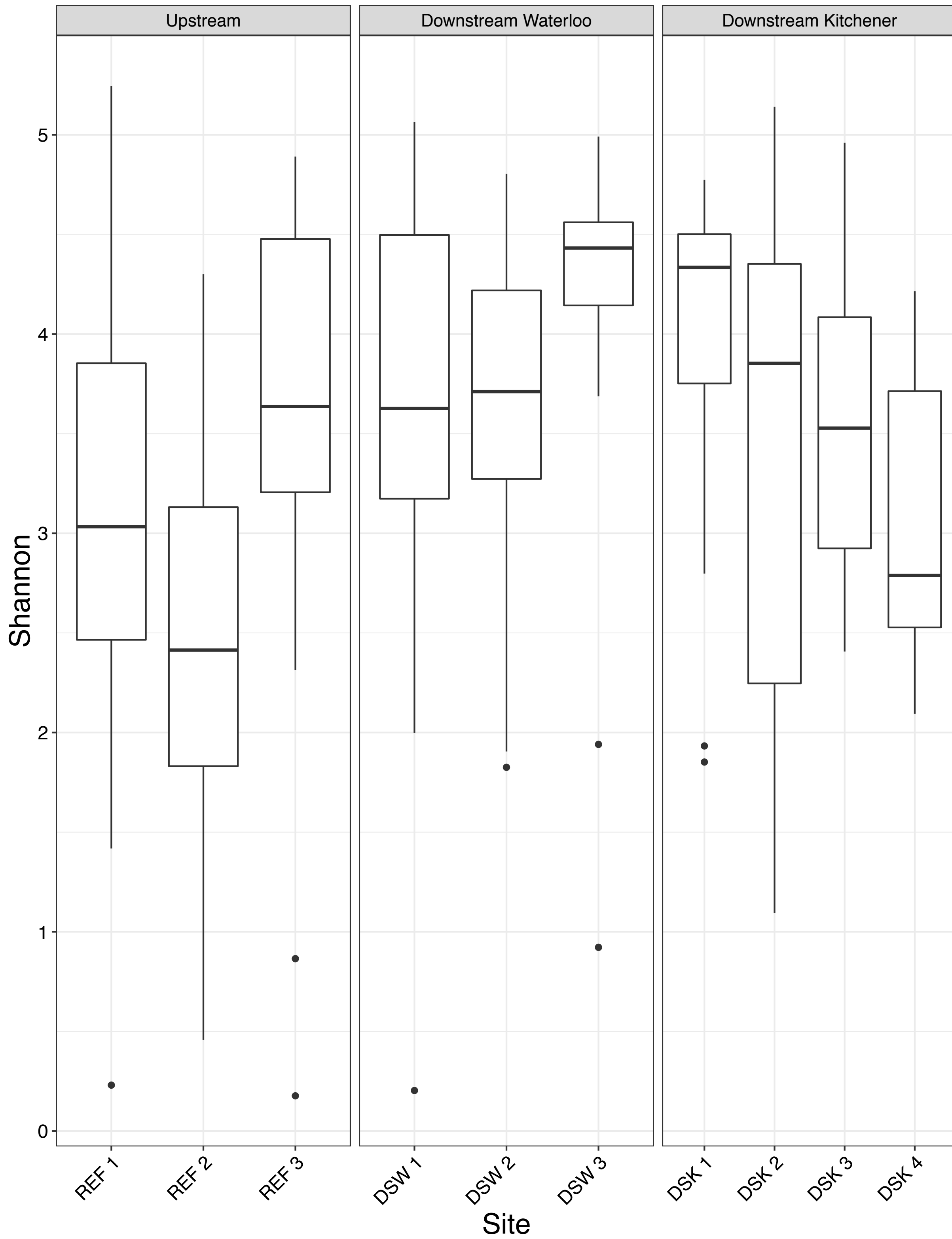

Figure 2.8 Shannon diversity of samples upstream (REF) to downstream of the Waterloo (DSW) and Kitchener (DSK) wastewater treatment plants in order of flow (upstream – left, downstream - right).

Berger-Parker dominance was variable within and between sites; samples from upstream sites had a higher abundance of dominant bacteria compared to those from downstream sites (Figure 2.9, one-way ANOVA, Df=9, F=2.393, p=0.015). REF 2 had the highest dominance and DSW 3 had the lowest. Samples collected from REF 2 had significantly higher dominance than those from DSW 2 (Tukey HSD, p=0.09), DSW 3 (p=0.006), DSK 1 (p=0.01), and DSK 3 (p=0.07). There were no other pairwise differences between sites (p>0.27).

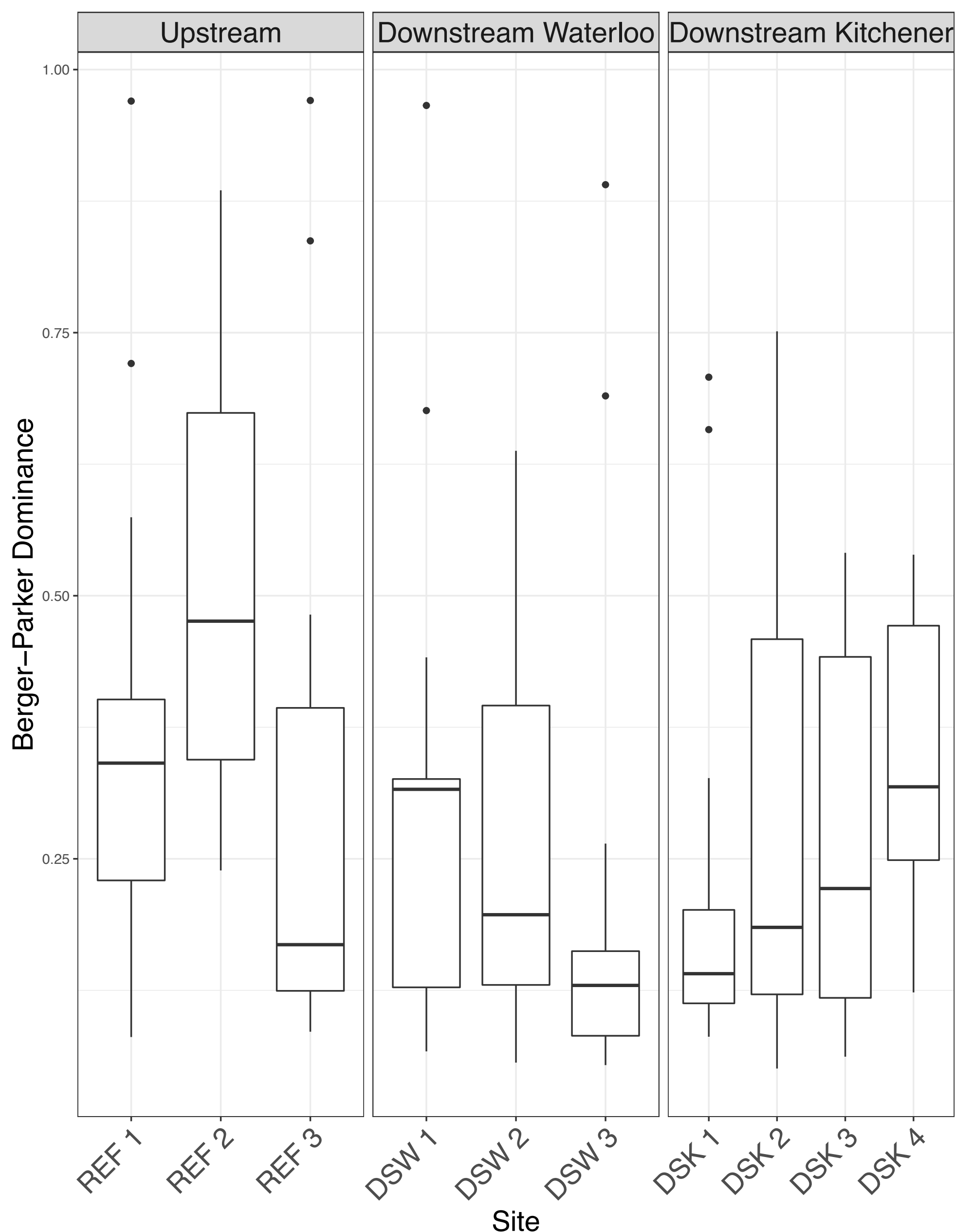

Figure 2.9 Berger-Parker dominance values of samples from upstream (REF) to downstream of the Waterloo (DSW) and Kitchener (DSK) wastewater treatment plants in order of flow (upstream – left, downstream - right).

Beta Diversity

Scree plots were generated to explore the proportion of total variability within the calculated Bray Curtis distance matrix, the largest variance was confirmed to be in axes 1 and 2 (Figure 2.10). Beta diversity was statistically dissimilar between sites (Figure 2.11, Adonis PERMANOVA, $F=2.9135$, $p=0.001$). Pairwise comparisons showed that most sites were significantly dissimilar from others ($0.03 < p > 0.001$) (Appendix A, Table A.2, Pairwise Adonis PERMANOVA). The exceptions were REF 1 - REF 2 ($p=0.125$), REF 1 – REF 3 ($p=0.06$), REF 3 – DSK 2 ($p=0.39$), DSW 1 - DSK 2 ($p=0.138$), DSW 1 - DSK 3 ($p=0.081$), DSW 2 – DSK 3 ($p=0.096$), DSK 1 – DSK 2 ($p=0.159$), DSK 2 – DSK 3 ($p=0.202$), which were not significantly different from one another.

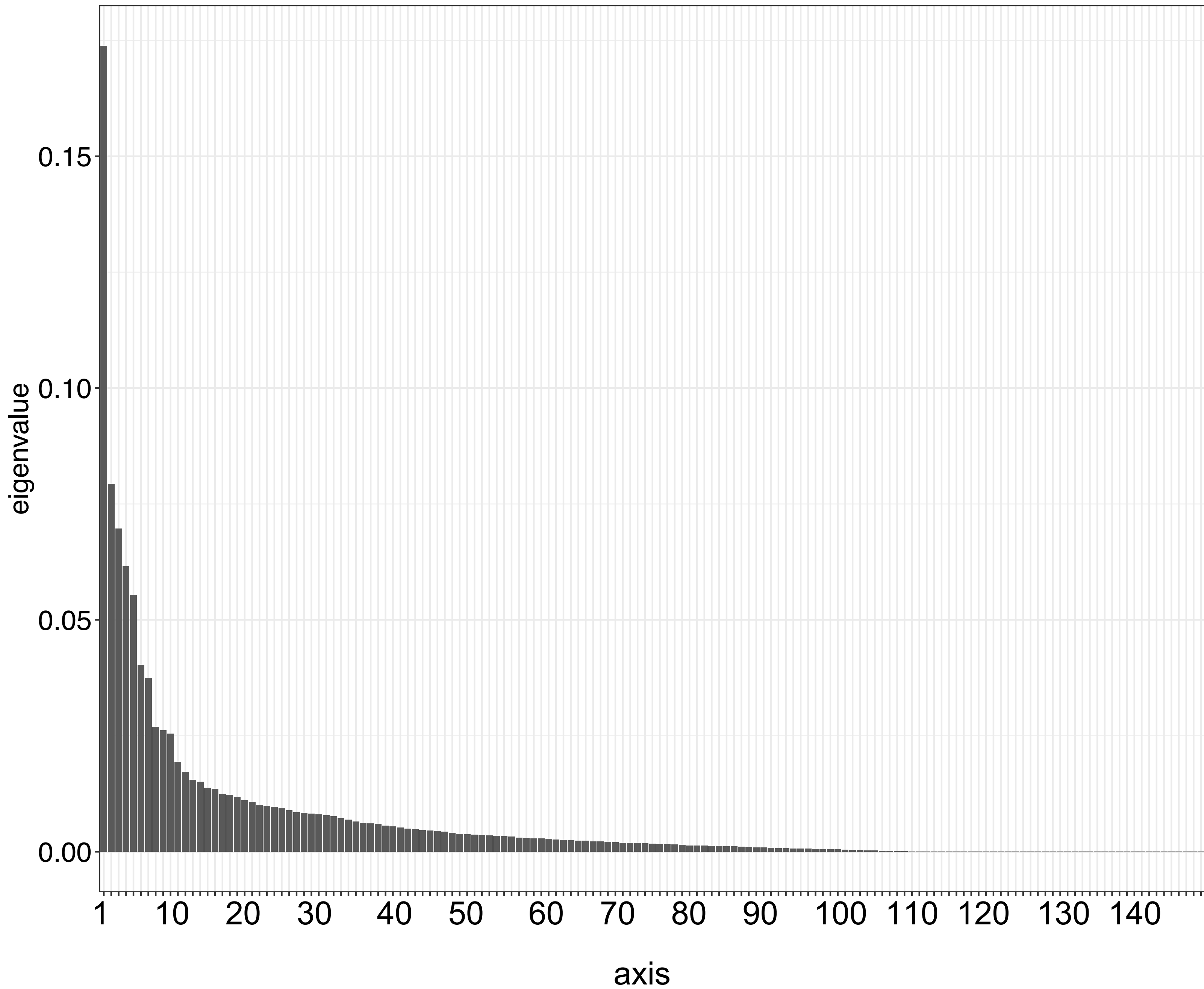

Figure 2.10 Scree plot generated using calculated Bray Curtis distance matrices and the Principal Coordinate Analysis. The y axis represents eigenvalues which indicate the variability within the distance matrix, while the x axis represents axes 1 – 149, with axis 1 representing the largest variance (17.4%) followed by axis 2 (7.9%).

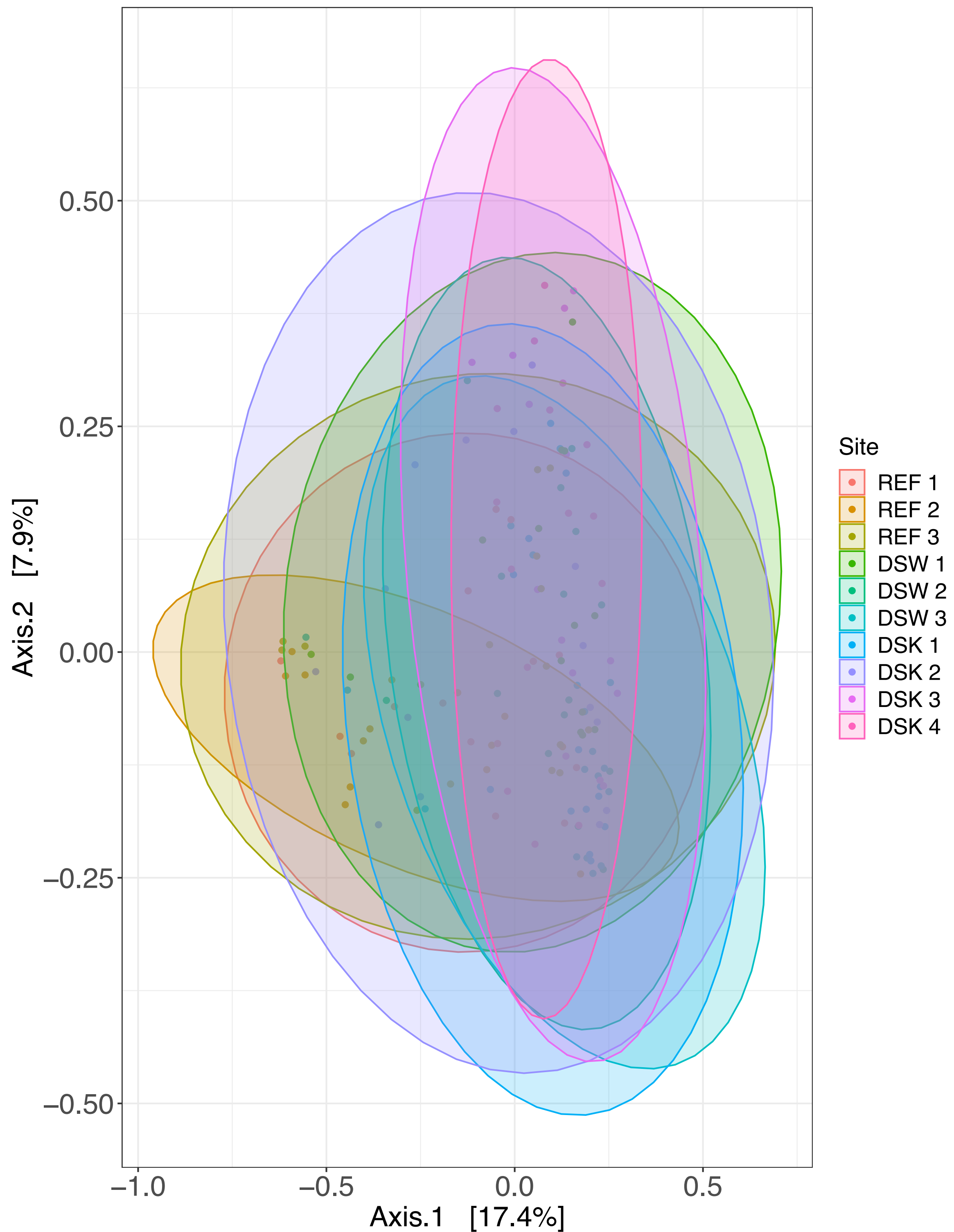

Figure 2.11 Bray-Curtis beta diversity with Principal Coordinate Analysis (PCoA) values from samples from upstream (REF) to downstream of the Waterloo (DSW) and Kitchener (DSK) wastewater treatment plants in order of flow (upstream – left, downstream – right; see Table 2.1 for site locations).

Abundance of Genera Differs by Location

Sites were pooled by location to compare changes in expression between upstream and downstream samples; more specifically, upstream included REF 1-3, downstream Waterloo included DSW 1-3, and downstream Kitchener included DSK 1-4. Between the upstream (REF 1-3) and downstream Waterloo (DSW 1-3) locations, there was an increase of 25 log2 fold of the genera *Breznakia, Alistipes, and Candidatus Bacilloplasma,* and between a 0-10 log2 fold increase in the genera *Vagococcus, Planktothrix*, *Ketogulonicigenium, Fictibacillus, Clostridium, Bacillus, Escherichia, Methylcycstis, Fodinicola, Rhodobacter, Rubellimicrobium,* and *Illumatobacter* (Figure 2.12). In contrast, there was a decrease in *Gemmobacter, Reyranella, Legionella, Intestinbacter,* and *Candidatus Xenohaliotis*. From upstream (REF 1-3) to downstream Kitchener (DSK1-4), there was an increase in the genera *Vagococcus, Breznakia, Alistipes, Pymaiobacter, Candidatus Bacilloplasma, Planktothrix, Clostridium, Ricketttsiella, Legionella, Bacillus, Enterococcus, Methylocytis, Illumatobacter, Fictibacillus, Fondinicola, Gaiella, Cyanobium, Ketogulonicigenium, Rhodobacter, Orthinibacter, Nitratireductor, Tabrizicola,* and *Rubellimicrobium* (Figure 2.13). In contrast, there was a decrease in *Gemmobacter, Roseomonas, Phreatobacter,* and *Afipia.*

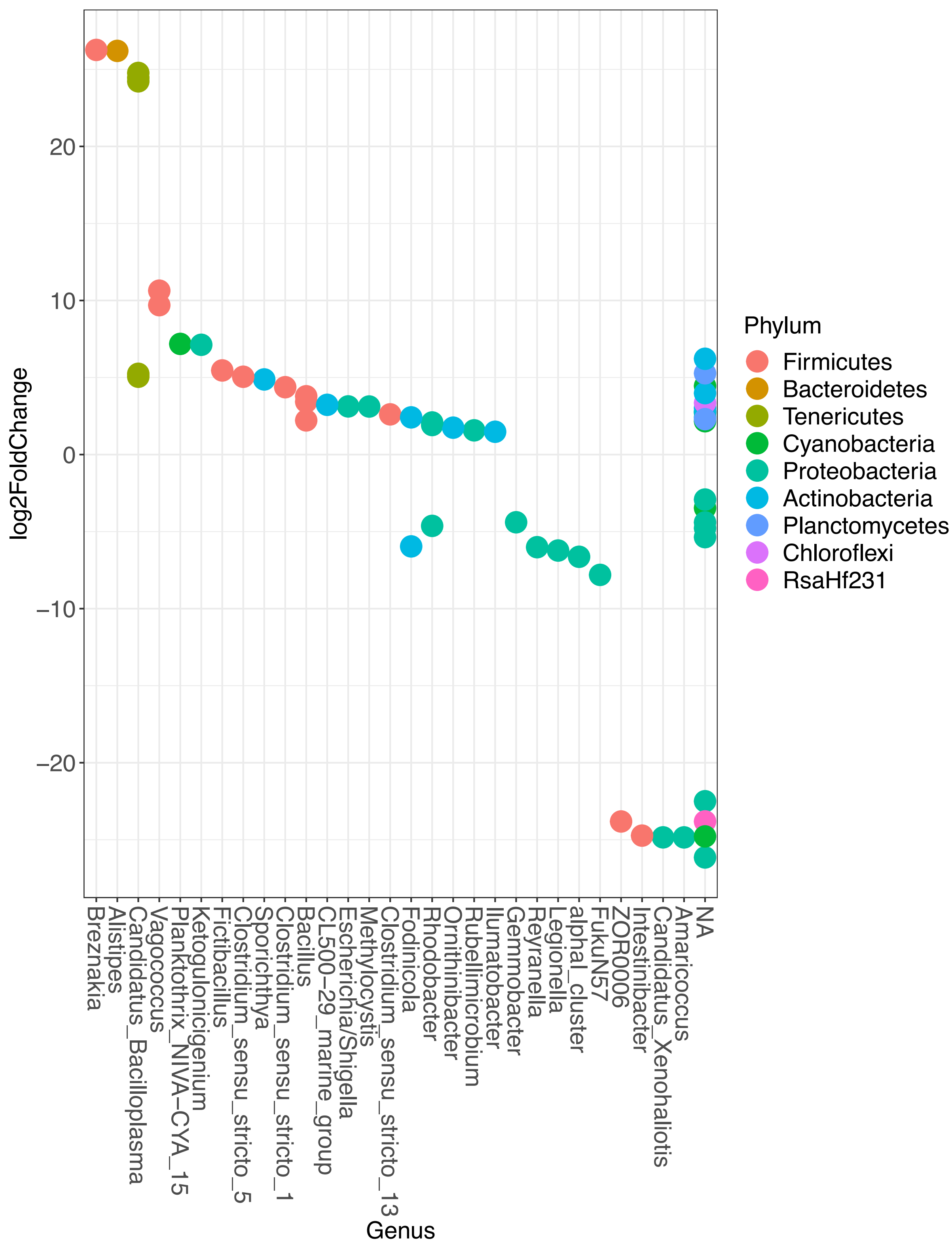

Figure 2.12 DESeq analysis comparing the change in bacterial abundance of fish gut samples from upstream (REF 1-3) and downstream of the Waterloo WWTP (DSW 1-3). Log 2 fold change is on the y-axis and bacterial genus is on the x-axis, points are coloured by phylum.

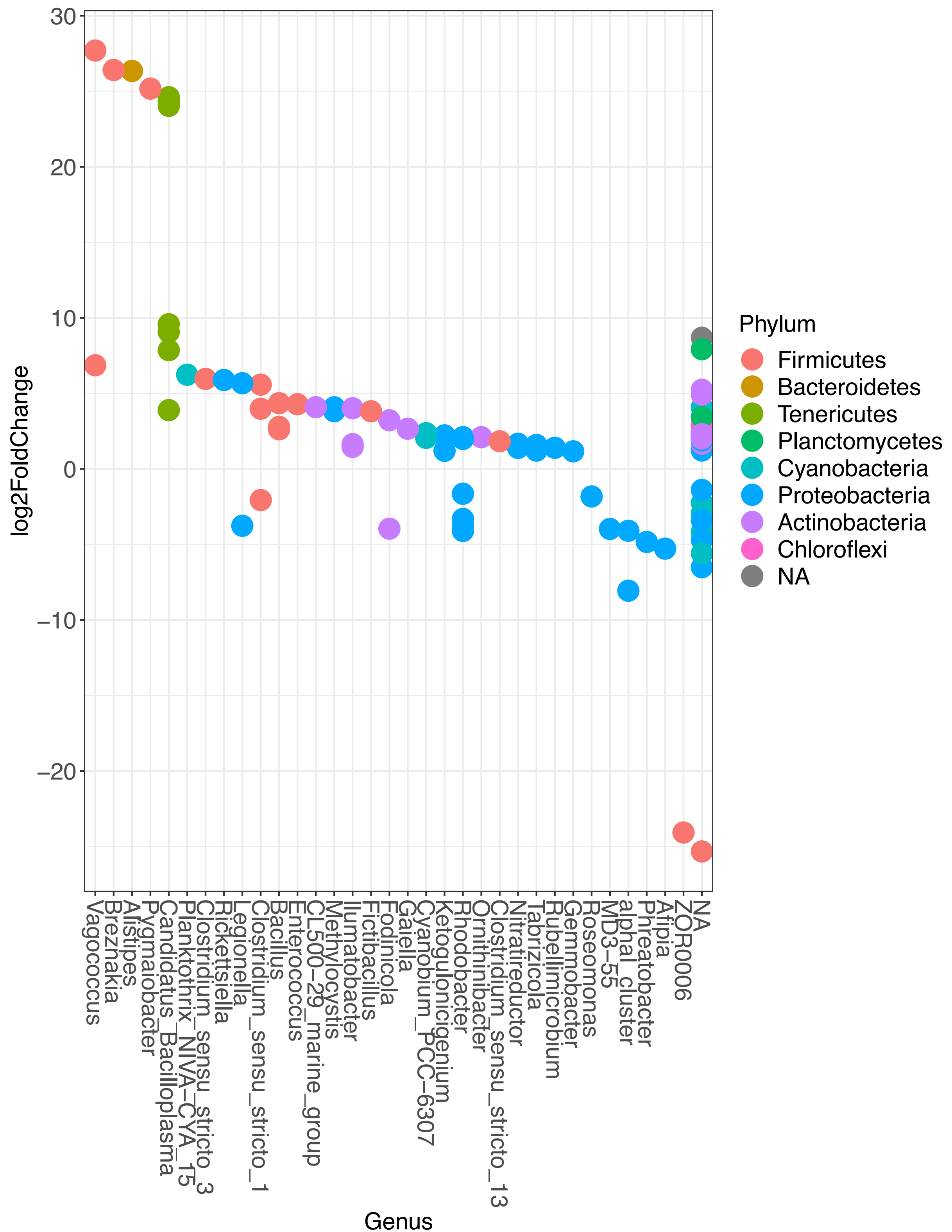

Figure 2.13 DESeq analysis comparing the change in bacterial abundance of fish gut samples from upstream (REF 1-3) and downstream of the Kitchener WWTP (DSK 1-4). Log 2 fold change is on the y-axis and bacterial genus is on the x-axis, points are coloured by phylum.

Weak Relationship Between Fish Condition and Distance from WWTP

The redundancy analysis indicated that was no observed effect between location and K. (Appendix A, Figure A.6). K ranged from 0.69 to 1.8 amongst all samples with an average of 1.2 across sites (Appendix A, Figure A.7) There was no correlation between water quality parameters and bacterial abundance, and weak correlations (Pearson Correlation coefficient cut off < 0.5) between site water quality measures and K (temperature = 0.3, DO = 0.2, conductivity = 0.1, TDS = 0.1, pH = -0.1; Figure 2.14). There was a weak positive correlation (0.2) between K and distance from both wastewater treatment plant outfalls.

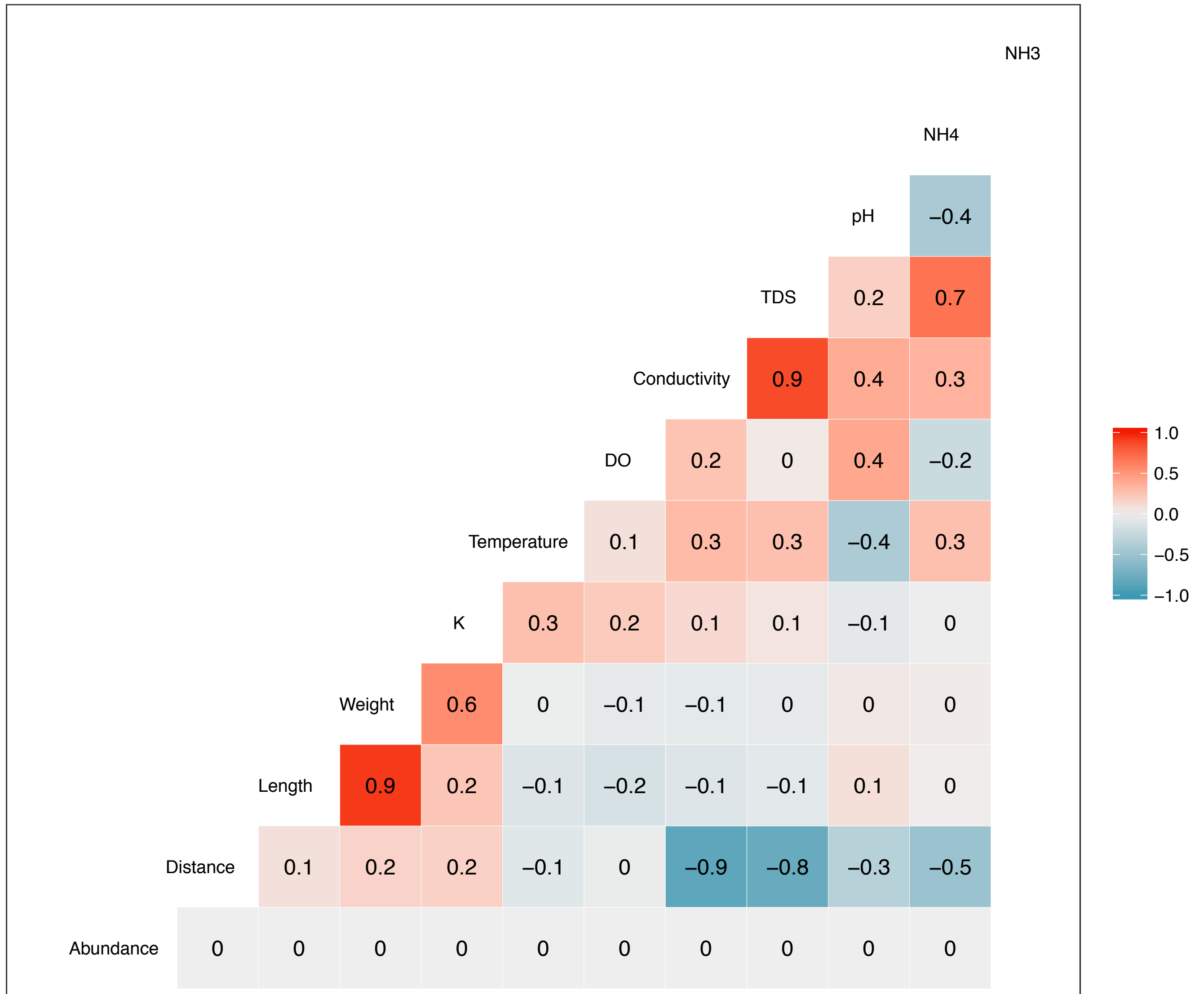

Figure 2.14 Pearson Correlation coefficients of the relationships among variables, including: bacterial relative abundance (Abundance), distance from the WWTP (Distance, km), fish length (cm), fish weight (g), condition factor (K), temperature (°C), dissolved oxygen (DO, mg/L), conductivity (µS/cm), total dissolved solids (mg/L), pH, and ionized ammonia (NH4, mg/L)*. *Unionized ammonia (NH3, mg/L) was 0 mg/L at each site and not included in these analyses.

Bacterial Composition and Diversity of Water

Water samples collected in 2019 had a total of 791,101 reads; there was an average of 21,975 reads per sample (minimum of 937 and maximum of 82,058 reads per sample). In total, samples had 40 phyla, 80 classes, 210 orders, 305 families, 646 genera, and 7202 ASVs. The top phyla accounted for 81% of all sequence reads: Proteobacteria (3277, 46%), Bacteriodetes (1826, 28%), Firmicutes (372, 5.1%), and Cyanobacteria (351, 4.8%). The relative abundance of bacterial phyla greater than 2% was dominated by Proteobacteria (Figure 2.15).

The alpha diversity (Shannon diversity index) varied significantly among sites; it was high at REF1 then decreased at REF 2, REF 3 and DSW 1 before increasing again from DSW 2 to DSK 4 (Figure 2.16, one-way ANOVA, $F = 20$, $p < 0.001$). The Waterloo and Kitchener WWTP effluents also had high alpha diversity. Generally, it appears that alpha diversity of river water was lower at upstream sites (REF 2-3), and then increased after exposure to WWTP effluents as indicated by the increased diversity at downstream sites.

Bray Curtis beta diversity was plotted and sites were very similar to one another, with the exception of REF 1 which clusters with REF 2 and the Waterloo and Kitchener WWTPs that cluster together (Figure 2.17, Adonis PERMANOVA, $F = 6.2196$, $p < 0.001$).

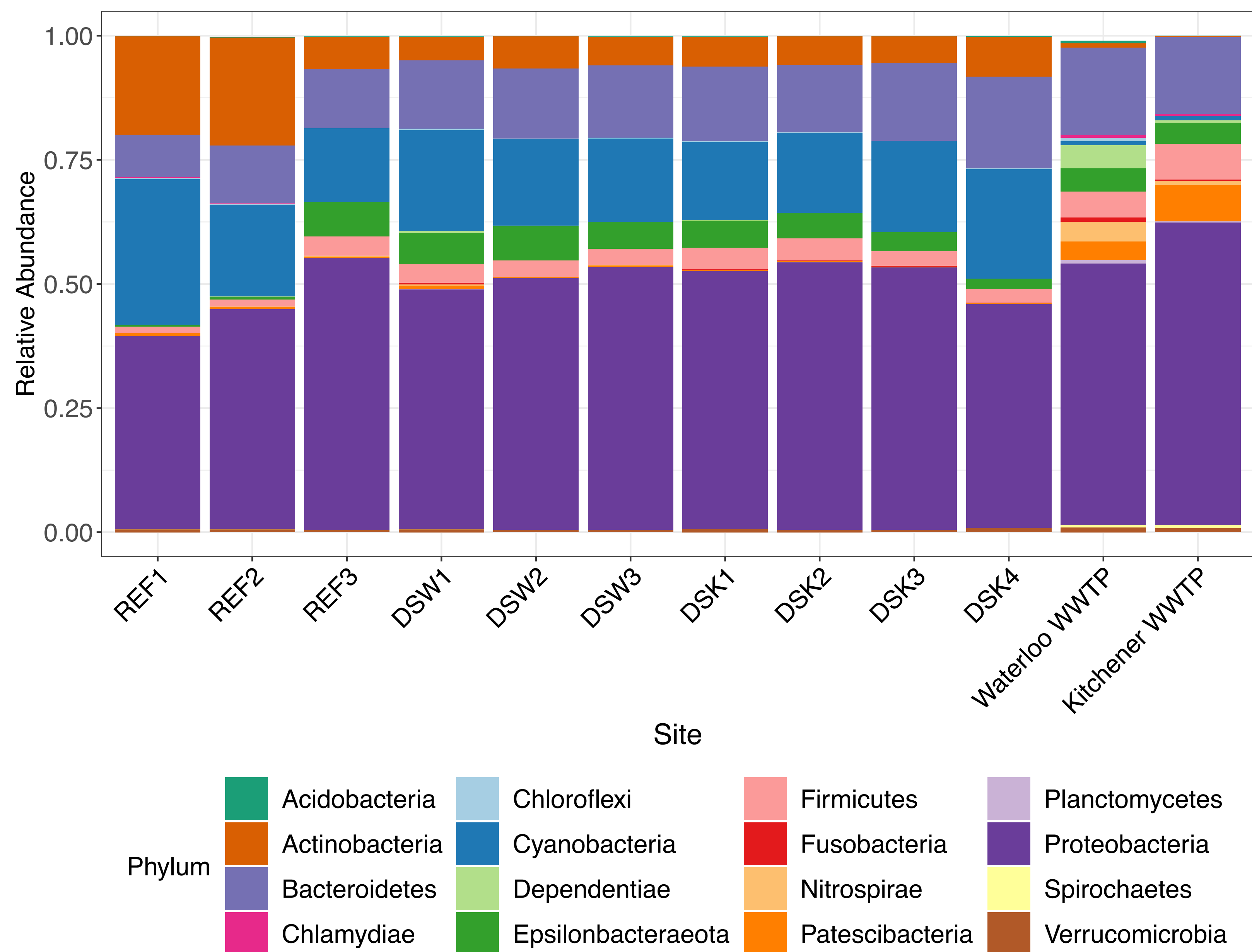

Figure 2.15 The mean relative abundance of bacteria from water samples. Only the phyla with > 2% of the total ASVs were included. The y-axis shows the proportion of ASVs within each included phyla from 0 to 1. The bars are ordered from upstream (REF) to downstream of the Waterloo (DSW) and Kitchener (DSK) wastewater treatment plants in order of flow (upstream – left, downstream - right).

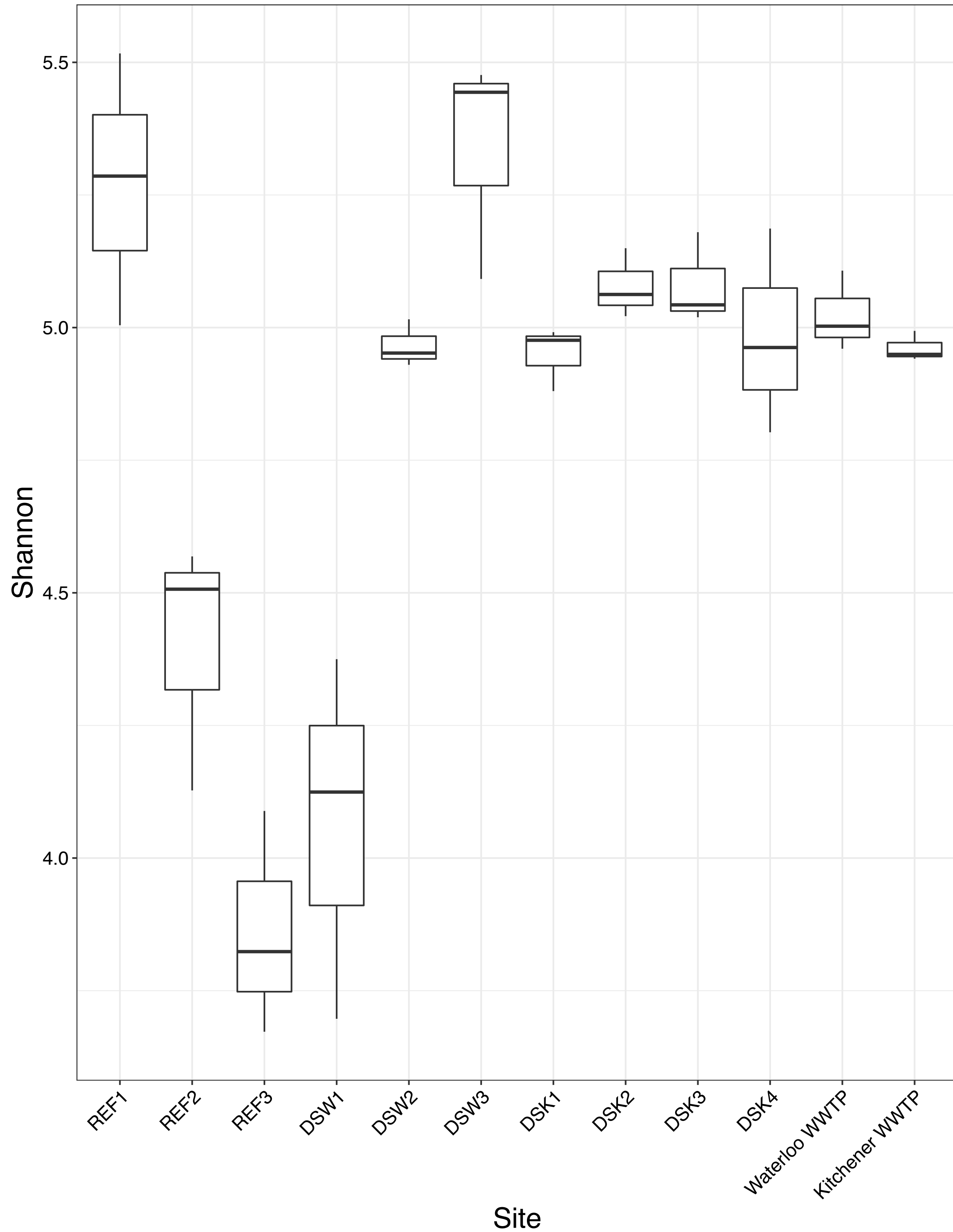

Figure 2.16 Shannon diversity of water samples ordered from upstream (REF) to downstream of the Waterloo (DSW) and Kitchener (DSK) wastewater treatment plants (WWTP) in order of flow (upstream – left, downstream - right), followed by the Waterloo and Kitchener WWTPs.

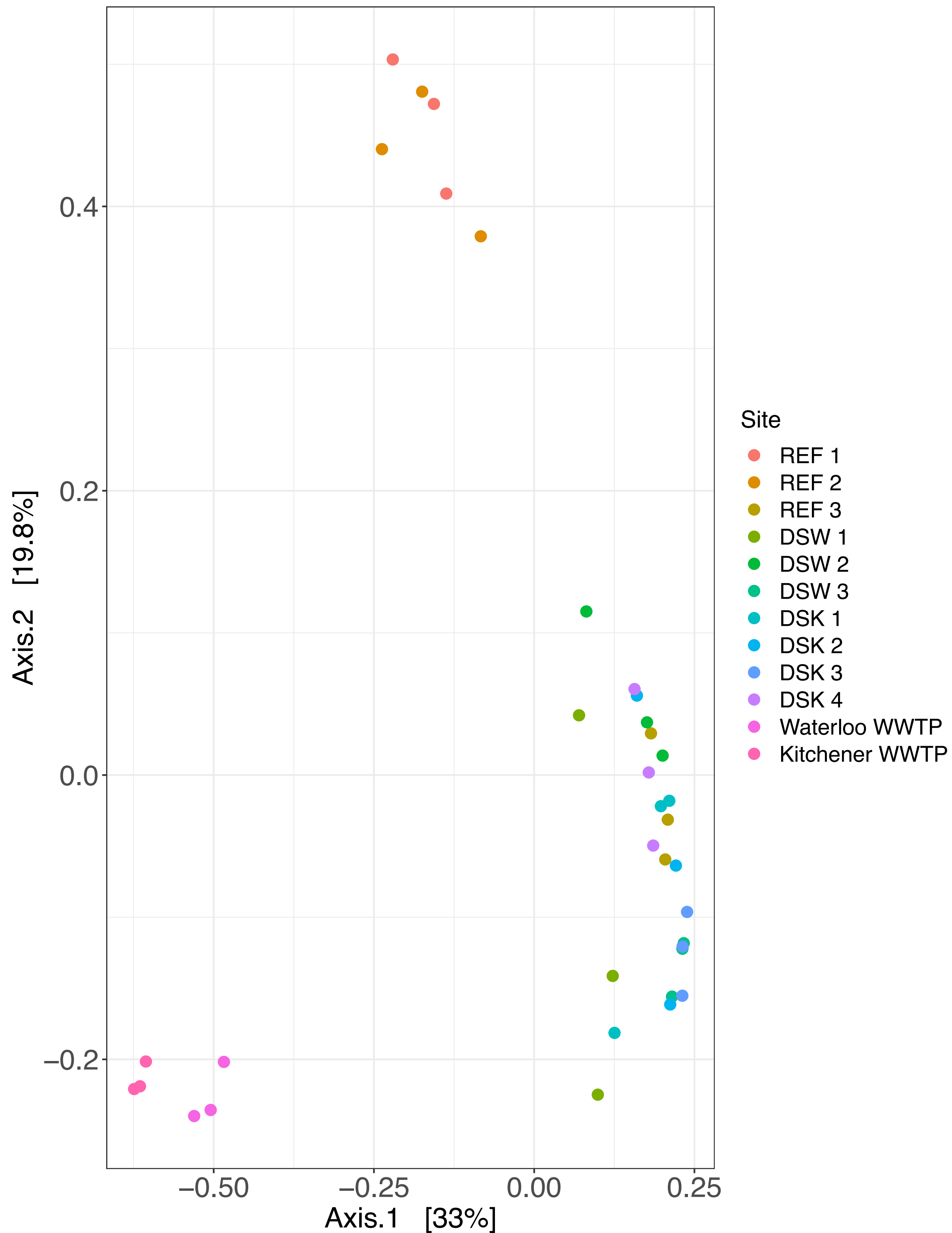

Figure 2.17 Bray-Curtis beta diversity with Principal Coordinate Analysis (PCoA) values from water samples, the locations are labelled as coloured points.

2.4 Discussion

Several studies have examined the effects of WWTP effluent on fish health, however there is limited literature on its effect on the fish gut content microbiome. This study investigated the effects of WWTP effluent in the Grand River, Ontario, on the microbiome composition and diversity of rainbow darter gut contents. At sites downstream of effluent outfalls, the microbial community shifted to include a greater relative abundance of Proteobacteria and Cyanobacteria, and lower relative abundance of Firmicutes. There was a significant increase in microbial diversity at sites downstream of the WWTP outfall, and these sites were also significantly dissimilar from sites located upstream of the Waterloo and Kitchener WWTPs. Distance from the outfall positively, but weakly, correlated with K, a measure of fish health. The results of this study help to fill this gap in knowledge and provide novel data on the effects of complex chemical mixtures on the fish microbiome.

Gut Content Microbiome Composition

The results of the current study showed that the microbiome of rainbow darter was dominated by the phyla Proteobacteria (39%), Firmicutes (29%), and Cyanobacteria (6.9%). In general, marine and freshwater fish gut microbiomes are dominated by Proteobacteria, Firmicutes, and Fusobacteria (Eichmiller et al., 2016, Tarnecki et al., 2017). The fecal bacterial communities of freshwater bighead carps were dominant in Proteobacteria, Firmicutes, and Fusobacteria, making up 76.7% of the total reads (Eichmiller et al., 2016). Diet is an important factor in the fish gut microbiome; herbivorous, carnivorous, omnivorous, and filter-feeding freshwater fish gut microbiomes are made up of 45.5%, 32.8%, 37.3%, and 38.1% Proteobacteria and 22.4%, 21.8%, 27.1%, 21.2% Firmicutes respectively (Liu et al., 2016). Fusobacteria made up 21.9% and 9.4% of carnivorous and filter-feeding freshwater fish gut

microbiomes, respectively (Liu et al., 2016). The rainbow darter in the current study is carnivorous (insectivorous) and appears to follow this trend with its high relative abundance of Proteobacteria (39%) and Firmicutes (29%), but it had a contrastingly lower relative abundance of Fusobacteria (0.15%) than other carnivorous species. Fusobacteria play a role in carbohydrate fermentation, producing butyrate as an end-product (Bennett & Eley, 1993); butyrate has been found in the gut of both herbivorous and omnivorous fish (Clements et al., 1994, Clements and Choat, 1995). However, because the rainbow darter is known to feed mainly on insects and not algae or plant material, the lower levels of Fusobacteria was not surprising.

Interestingly, samples collected from sites impacted by WWTP effluent on the Grand River had high relative abundances of Cyanobacteria. They are typically found in eutrophic waters caused by increased nutrient loading, e.g., fertilizer runoff or sewage discharge, but also dominate WWTP ponds at up to 99.8% of the total phytoplankton density (Vasconcelos & Periera, 2001). Cyanobacteria are released into receiving waters from these ponds and therefore WWTP was the likely source of Cyanobacteria found in the gut content of the rainbow darter in the current study. It is interesting to note that Cyanobacteria were also found in the guts of fish at the reference sites, which may be because of other point source pollutants upstream of the reference sites or because they are part of the rainbow darter's core microbiome.

The Cyanobacteria found in the guts of rainbow darter in the current study were dominated by the families *Cyanobiaceae* and *Microcystaceae*, and the genera *Cyanobium, Planktothrix,* and *Synechocytis*, which are known to produce cyanotoxins. The family *Cyanobiaceae* and genus *Synechocytis* (family *Microcystaceae*) have been observed in diseased corals (Rosales et al., 2019, Meyer et al., 2019), but their roles in freshwater ecosystems are unclear. Cyanobacteria species produce many types of toxins, which can bioaccumulate in the

gut, liver, kidney, and muscle of fish and are known to be toxic (Freitas de Magalhães et al., 2003, Mohamed et al., 2003). The genus *Planktothrix* produces neurotoxic saxitotoxins and this cyanobacteria has been found in the gut of whitefish exposed to algal blooms along with microcystin-protein adducts in liver homogenates and malformations in eggs and larvae were also observed (Ernst et al., 2001). Future work should investigate the impact of cyanobacteria on the gut microbiome, whether they have a functional role or are responsible for the production of toxins.

There was an increase in Proteobacteria classes (Alphaproteobacteria, Gammaproteobacteria, and Deltaproteobacteria) and the families *Rhodobacteraceae* and *Legionellaceae* in the gut content of rainbow darter at sites downstream of the WWTPs on the Grand River, which may be sourced from WWTPs. Species of the family *Rhodobacteraceae* (class – Alphaprotoebacteria) are found in wastewater samples (Rosenberg et al., 2013), while Betaproteobacteria and Gammaproteobacteria are used for enhanced biological phosphorus removal (Blackall et al., 2002). Genera in the phyla Proteobacteria, like *Pseudomonas, Shewanella, Bacillus, Arthrobacter,* and *Sphingobacterium,* are important for the degradation of pollutants in wastewater treatment (Hamilton et al., 2019). Overall, results from the current study suggest that fish ingest bacteria originating from WWTPs, but it is unclear if there is any functional role for them in the gut.

The observed increase in Proteobacteria in darters downstream of WWTPs suggests a departure in fish health. In the mammalian literature, the dominance of Proteobacteria has been linked to dysbiosis of the microbiome leading to declining host health (Shin et al., 2015, Salazar et al., 2018). Increased abundance of Proteobacteria may be a biomarker of disease as it has been linked to altered health outcomes in mice including malnutrition, obesity, diabetes,

inflammation, and cancer (Shin et al., 2015). Furthermore, exposure to environmental pollutants can result in impaired neurotransmission of the brain-gut-enteric microbiota axis, inflammation, oxidative stress, and dysbiosis, all of which can result in negative metabolic effects (Rhee et al., 2009, Snedeker & Hay, 2012, Chen et al., 2018). Although the increase in Proteobacteria observed herein may be indicative of a decline in fish health, metabolic tests or other direct observations of impaired fish gut health are recommended for future studies.

Gut Content Microbiome Diversity

Although alpha diversity of the gut microbiome in fish has tended to decrease after exposure to chemicals or stressors in some studies (Gaulke et al., 2016, Narrowe et al., 2015, Dahan et al., 2018), I found that microbial diversity increased at locations downstream of WWTPs. Additionally, there was a small increase in alpha diversity at REF 3, which may be due to urbanization surrounding this stretch of the Grand River or to the entry of the Conestogo River, which is made up of agricultural area and may be a source of increased nutrients and bacteria (M. Servos, personal communication). No other studies have examined how WWTP effluent affects the diversity of the gut content microbiome of rainbow darter, however the gut microbiome of fish exposed to high levels of arsenic resulted in increased alpha diversity compared to unexposed fish (Dahan, 2018). Additionally diseased coral microbiomes had an increase in microbial alpha diversity and dysbiosis compared to healthy coral tissue (Meyer et al., 2019). Thus, the increase in rainbow darter gut content diversity may indicate dysbiosis or a diseased state.

Beta diversity of the gut contents differed between all sites on the Grand River despite sampling the same fish species (and sex) during the same season. This suggests that microbial community dissimilarities are either due to changes in the fish's immediate external environment

as this species has a small home range (~5 m, Hicks et al., 2017) or to differences in dietary habits among sites. The beta diversity of wild three-spine stickleback and gizzard shad, species known to be localized in their habitat use, also clustered by location which was thought to be due to potential differences in diet at each site (Smith et al., 2015, Ye et al., 2016).

Fish Health Declines Downstream of WWTP Outfall

The Pearson Correlation analysis suggests that fish health (inferred from K) decreases with proximity to the WWTP. This, coupled with the increased abundance of Cyanobacteria and Proteobacteria in their gut microbiome, indicates gut dysbiosis and a possible departure from fish health. Further research is required to control for other point or non-point sources of pollution that may have affected the gut microbial composition in the sampled fish and confounded the effects of the WWTP effluent.

Water Samples

Microbiota in river water was similar in composition and diversity to fish gut contents. Similar to the rainbow darter gut samples, river water was dominant in Proteobacteria, Firmicutes, and Cyanobacteria and alpha diversity also increased downstream of the WWTPs. In contrast to the fish samples, the water samples were also dominant in the bacterial phyla Bacteriodetes. Furthermore, beta diversity PCoA plots indicated that samples from the WWTPs were similar to one another, and samples from REF 1 and REF 2 were also similar, in contrast to the samples collected at other field sites. The water samples were collected in 2019, one year after the rainbow darter were sampled. As such, the water samples cannot be directly compared to the rainbow darter gut microbiome samples, but they do provide insight into the environmental microbiome. The water samples were dominant in Proteobacteria, similar to the fish gut contents. This could indicate that the bacteria are colonizing the fish gut from the

external aquatic environment. Interestingly, the beta diversity analysis showed similar microbial compositions between the Waterloo and Kitchener WWTP. This suggests that similar bacteria may be used in the wastewater treatment process and then released into receiving waters. Future studies should collect water samples at the same time as fish gut contents to better determine similarities in the bacterial composition and diversity between the water and fish guts.

Limitations

Results of this study are difficult to compare with others as the methods used in the microbial analysis of fish guts have not yet been standardized. There are several methods used to analyze the fish gut microbiome including fluorescence *in situ* hybridization, targeted PCR amplification of regions unique to bacterial species, or culture-independent next generation sequencing (Tarnecki et al., 2017). In addition, different methods of sample preparation and storage, DNA extraction, PCR conditions, sequencing platforms, and data processing can affect results and interpretation (Tarnecki et al., 2017). As such, the variety in methodologies used for microbial research is a general issue in the field and needs standardizing to develop a broader understanding of the factors affecting fish microbiomes.

In this study, fish were only sampled once (fall 2018), thus I am limited to interpreting data that does not account for the dynamic nature of the fish gut microbiome. Several factors affect the gut microbiome of fishes (e.g. species, diet, and environmental surroundings), thus seasonal variation (as well as changes in WWTP effluent that occur with season) may affect the composition and diversity of the fish gut microbiome (Sullam et al., 2012, Roeselers et al., 2011, Tarnecki et al., 2017, Ray, 2016, Zarkasi et al., 2014). Future studies should return to resample these sites on the Grand River to better understand if gut content microbial composition or diversity varies between seasons or years. Interestingly, gut microbiomes of the three-spine

stickleback did not closely resemble water microbiota from the same sites, but did closely resemble those found in prey species collected from the same site (Smith et al., 2015). It would have been helpful to collect environmental samples for comparison to the sampled gut microbiome; however, microbiome analysis of invertebrate species from the same field sites and time (fall 2018) is being done, and will provide insight into whether prey species are associated with the rainbow darter gut microbiota (E. Millar, unpublished data).

Future Studies

Future studies should examine if changes in the gut microbiome can indicate altered fish health, exposure to stressful events, or variations in natural waters. Through targeted exposure studies, gut content may be sampled and various health indicators can also be measured to better understand the linkages between the gut microbiome and fish health. For example, fish respirometry can be used as a more precise measure of metabolic rate providing information on physiological health and aerobic scope. Additionally, this study found increased Proteobacteria in fish downstream of WWTP outfalls and it is known that Proteobacteria may be linked to stress in mammals, however this hasn't yet been established in fish. It would be interesting to study the response of the fish microbiome to stressful events in nature, such as predation. Furthermore, it is unknown whether daily water variations (for example temperature, pH, ammonia, or DO) cause rapid changes to the fish gut microbiome. Weather events, like heavy rainfall, can lead to increased flow and turbidity, as well as temperature fluctuations; it would be interesting to sample before and after these weather events to determine the resilience (or not) of the fish microbiome. Controlled laboratory exposures may be needed to confirm the impact of WWTP effluent on the fish gut content microbiome independent of confounding variables.

2.5 Conclusion

The effects of WWTP effluent exposure on the gut microbiome of wild fish was explored on the Grand River, Ontario, using rainbow darter as the species of interest. The microbial composition of the gut contents of rainbow darter shifted at sites downstream of the WWTP outfalls to include a greater relative abundance of Proteobacteria and Cyanobacteria, and a decreased relative abundance of Firmicutes. The increase of Proteobacteria could be an indicator of stress in fish, which may lead to a departure from good health although more research is required into the specific function of fish gut bacterial taxa. The increase in Cyanobacteria in the gut content from downstream sites is likely linked to increased nutrient loading from the WWTP effluent discharges. Upstream fish had increased dominance, while effluent-exposed fish had increased diversity, which may be a result of exposure to WWTP effluent; this cannot be stated definitively as environmental conditions like habitat, temperature, food availability, and seasonality have been shown to influence the composition of the fish gut microbiome. Finally, condition of fish decreased for individuals found closer to the WWTP outfall. This decline in fish health at locations nearest to the WWTP outfall could be partially due to the shift in microbial composition of the gut, but more research is required to determine causal links. Future studies should investigate the ability of the fish gut microbiome to act as a marker for fish health or a bioindicator for exposure to environmental pollutants.

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Chapter 3: The gut microbiome of wild rainbow darter is altered in the lab

Abstract

The fish gut microbiome is influenced by environmental stressors, diet, and habitat, however less is known about how the transition from the field to the laboratory affects the gut microbiome. This study determined how transition from field to laboratory, length of acclimation period, and exposure to environmental stressors affected the gut microbiome of wild rainbow darter (*Etheostoma caeruleum*). Fish were collected in June 2019 from a site along the Grand River (Waterloo, Ontario) and a subset was sampled (n=15) on site to establish a field baseline. Fish were transported to the laboratory and were sampled after a 14 day acclimation period (n=17) and after 42 days in the laboratory (n=20); the latter served as a control to the environmental stressor treatment groups. From days 15-42, fish were exposed to four different treatments: 10 (n=19), 20 (n=21), or 40% (n=21) wastewater treatment plant effluent, or 100 ng/L of triclosan (n=17). Genomic DNA was extracted from gut contents, and PCR amplification of the 16S rRNA gene V3-V4 region and Illumina sequencing were performed. Amplicon sequence variants were mapped to bacterial species using the SILVA database and filtered using DADA2. The gut microbiome of field fish had more phyla present (no single phyla dominant) compared to all laboratory fish; in contrast, lab fish were dominant in the phyla Firmicutes. Shannon diversity and bacterial communities shifted significantly from the field to the end of acclimation (14 days in the lab) and continued to change from acclimation to 42 days in the lab ($p<0.009$). The beta diversity of fish gut contents differed among treatments of environmental stressors ($p=0.001$) however, relative abundance of bacteria and alpha diversity was similar between treatments and controls. The results of this study indicate that the fish gut microbiome changes with the transition to new environments and continues changing over time; more time in a new setting may be needed before a stable microbiome is achieved in wild-caught fish.

3.1 Introduction

The fish gut microbiome is the community of microbiota found in the gut of fish, it plays a role in maintaining the host's physiological functions, and its composition differs both among and within fish species. For example, the gut bacteria of 12 wild fish species varied, with 7-15 phyla present (Givens et al., 2015). Even between related but distinct species, differences in the microbiome are observed; the fecal microbiome of laboratory reared common, silver, and bighead carp were compared and up to 20.5% of operational taxonomic units (OTUs) varied between species (Eichmiller et al., 2016). The gut microbiome of fish within a species share many of the same OTUs, yet their gut microbiome remains highly individualized (Givens et al., 2015). These differences in individual fish may be due in-part to changes in factors like diet and the aqueous environment.

Intestinal microbiota is either indigenous (colonizing the epithelial surface or microvilli) or transient (in the lumen, passing through the gut), and the latter is largely dependent on the diet (Ringo et al., 2015) as certain bacteria are required for the digestion of specific nutrients in foods (Ray et al., 2012). Fish with different dietary habits have distinct microbial communities; more specifically, herbivorous fish had increased Clostridiales, Bacteroidales, and Verrucomicrobiales, omnivorous fish had increased Rhizobiales, Fusobacteriales, and Planctomycetals, and both omnivorous and carnivorous fish had increased Desulfovibrionales and Aeromonadales (Sullam et al., 2012, Liu et al., 2016). The gut microbiome of stickleback resembled that of their invertebrate prey, suggesting that the bacteria provided by the diet make up at least part of the fish gut microbiome (Smith et al., 2015).

Changes in the gut microbiome have been observed when the diet of the fish is altered. Carnivorous wild-caught rainbow darter were fed a plant-based diet in the lab and the gut

microbiome changed to include a greater abundance of Bacteroidetes and decreased abundance of Firmicutes, which was thought to maintain the host's ability to absorb nutrients from their diet (Dhakal, 2017). In rainbow trout fed a plant-based diet there was an increased abundance of Firmicutes and lower abundance of Proteobacteria in the gut as well as distinct bacterial communities compared to control trout fed fish meal (Desai et al., 2012). Overall, a change in diet generally results in altered gut bacterial composition, which likely impacts the digestive physiology of the host.

The digestion of foods depends on the type of food ingested (specifically the proportion of protein, fat, and carbohydrate) and the activity of digestive enzymes; the latter is thought to be influenced by gut microbiota (Ray et al., 2012). Bacteria in the fish gut are known to produce exogenous digestive enzymes including amylase, cellulase, lipase, proteases, chitinases, and phytase (Ray et al., 2012). The guts of carnivorous fish are typically abundant in the bacterial phyla Proteobacteria, which are believed to have a role in the digestion and absorption of proteins (Ray et al., 2012). When fed a carnivorous diet, rainbow darter had increased abundance of Proteobacteria and increased activity of the enzyme trypsin (used to break down proteins) coupled with a decreased abundance of Bacteriodetes and decreased activity of the enzyme cellulase (Dhakal, 2017). Furthermore, when fed a low fat diet there was an increase in lipase activity, which was thought to be an attempt of the host fish to increase lipid absorption in response to the diet, although there were no increases in Firmicutes (associated with lipid metabolism; Dhakal, 2017). Changes in diet can result in changes in exogenous enzymes produced by bacteria which likely enhance the host's digestive physiology (Ringo et al., 2015).

Environmental conditions in both the field and the lab, including habitat type and water parameters, affects the fish gut microbiome. The fecal microbiome of common, silver, and

bighead carp clustered by capture location (lake or river); the diet of the carp was similar between locations, thus changes in the microbiome were attributed solely to environmental conditions and not diet (Eichmiller et al., 2016). The guts of anadromous Arctic charr collected from saltwater sites had increased presence of the phyla Firmicutes, Spirochaetes, and Tenericutes compared to charr collected from freshwater sites (Hamilton et al., 2019). Zebrafish held in different aquatic facilities had distinct gut microbial communities, perhaps due to differences in housing infrastructure, water source, temperature, diet, feeding schedule and/or antibiotic use (Roeselers et al., 2011). Finally, transporting and housing wild-caught fish into a lab can lead to changes in the gut microbial community. Wild common carp, mummichog, and zebrafish had distinct gut microbial communities and increased diversity than the same species held in the lab, likely due to the controlled diet and environment in the lab (Eichmiller et al., 2016, Givens et al., 2015, Roeselers et al., 2011).

Laboratory exposures to antibiotics, antimicrobials, and antibacterials, can alter the community and diversity of the fish gut microbiome. Fathead minnow larvae and zebrafish exposed to the antimicrobial compound triclosan had decreased microbial diversity and distinct gut bacterial communities compared to unexposed fish (Narrowe et al., 2015, Gaulke et al., 2016). Fish exposed to oxytetracycline, streptomycin, and rifampicin had lowered gut microbiome diversity and altered microbial composition (Zhou et al., 2018, Pindling et al., 2018, Carlson et al., 2015, Carlson et al., 2017). Wastewater treatment plant (WWTP) effluents are known to affect the microbiome of fishes and this may be due to the presence of antibiotics, antimicrobials, and antibacterials in the effluents. Gut bacteria of effluent-exposed fish had decreased alpha diversity, and increased abundance of bacteria (*Hyphomicrobium, Nakamurella* (phyla – Actinobacteria)*, Phormidium* (order – Oscillatoriales, phyla – Cyanobacteria)*,* and

Pirellula) associated with wastewater, when compared to upstream fish (Sakalli et al., 2018, Giang et al., 2018). In Chapter 2 of this thesis, wild fish exposed to WWTP effluent had increased gut bacteria alpha diversity, increased abundance of Proteobacteria, and distinct bacterial communities compared to unexposed fish. Studies examining how the gut microbiome of fish is impacted by exposure to whole WWTP effluent in the lab have not yet been conducted.

Rainbow darter (*Etheostoma caeruleum*) are a small-bodied, freshwater, benthic and carnivorous fish found in northeastern North America. The rainbow darter has been used to examine the effects of diet and WWTP effluents on the gut microbiome, making them an ideal species for the present study. The gut microbiome of wild-caught rainbow darters fed a bloodworm or plant based diet were studied in the lab; Proteobacteria dominated in the fish fed bloodworms, while fish fed a plant based diet had an increased abundance of Bacteroidetes and Firmicutes (Dhakal, 2017). In addition, rainbow darters downstream of WWTP outfalls had increased abundance of Proteobacteria and Cyanobacteria in their gut, increased alpha diversity, and distinct bacterial communities compared to upstream fish (Chapter 2).

The aim of this study is to determine how the gut microbiome of wild rainbow darter changes when fish are transitioned to a laboratory environment and diet, and are exposed to waterborne environmental stressors (WWTP effluent and triclosan). I hypothesized that when wild caught rainbow darter were transitioned to the laboratory their gut microbiome composition would change and predicted that bacterial diversity would decline. I hypothesized that with increased time in the laboratory the gut microbiome would stabilize, meaning there would be fewer changes in microbial composition and diversity over time. Finally, I predicted that when exposed to WWTP effluent and triclosan (TCS; 100 ng/L), the fish gut microbiome composition would be altered to include a greater abundance of the bacterial phyla Proteobacteria and have

altered bacterial diversity; WWTP effluent exposed fish would have increased diversity and TCS exposed fish would have decreased diversity compared to controls. The specific predictions were based on differences in fish downstream of WWTP plants (Chapter 2) and the above-mentioned studies.

3.2 Materials and Methods

Collection and Transport of Rainbow Darter

One hundred and thirty female *E. caeruleum* > 4.5 cm in length were collected from a reference site, REF 2 (West Montrose, Waterloo, Ontario; MNR license #1092042), located on the Grand River. Upstream of REF 2 there are several small municipal WWTPs and a flood control dam; the site is surrounded by agriculture and is currently used as a reference site for many studies conducted on the *E. caeruleum* population living in the Grand River (Tetreault et al., 2013, Fuzzen et al., 2015). Fifteen fish were sampled on-site as described below (*Gut Content Extraction*), while the remaining 115 fish were transported in two 45 L aerated coolers to the aquatic facility at McMaster University (Hamilton, Ontario).

Acclimation and Exposure

Upon arrival to the lab, fish were acclimated to holding tank temperatures (2°C cooler than transport conditions) over 2 hours. Fish were randomly assigned to eighteen tanks at a maximum density of 7 fish per 10 L tank with flow-through dechlorinated municipal tap water. Flow-through conditions were maintained by peristaltic pumps at 12 ± 1 mL/min. Fish were not fed for the first 12 hours after transfer from the field to laboratory. For the first 10 days of the acclimation period fish were offered a sterile adult zebrafish pellet (Zeigler, Pennsylvania), which they did not appear to eat. On day 11, fish were offered frozen bloodworms (San Francisco Bay Brand, California) and they began feeding immediately; fish were on this food source for the remaining time and were fed once daily *ad libidum*. Fish were acclimated for a total of 14 days. Tanks were randomly assigned in a block of 3 tanks to one of six treatments: acclimation (14 days holding), control (42 days holding), 10% effluent (low), 20% effluent (medium), 40% effluent (high), and 100 ng/L triclosan (TCS) (total of 18 tanks). Fish in

acclimation tanks were sampled on day 14, as described below, and the other tanks were exposed for an additional 28 days before sampling. Tanks were monitored daily throughout the acclimation and exposure period for temperature (18.70°C ± 0.8, mean ± SD), pH (7.32 ± 0.3), and dissolved oxygen (DO; 91.0% ± 4.7) using a handheld YSI 556 multi-parameter meter; general hardness (GH; 60-180 ppm, minimum-maximum), carbonate hardness (KH; 40-180 ppm), ammonia (0-3 ppm), nitrate (0-80 ppm), and nitrite (0-1 ppm) levels were measured using a test strips (API/Mars Fishcare, Pennsylvania). Individual tank water quality measurements including mean, standard deviation and the range (minimum-maximum) are provided in Appendix B, Table B.1. Fish were held under a 12:12-hour light:dark cycle and cared for according to McMaster's Animal Research Ethics Board (AUP #16-09-34, amendment 19-070).

Preparation of Treatment Solutions

A grab sample of 180 L of 100% WWTP effluent was collected twice weekly from the Waterloo WWTP and transported back to McMaster University (Hamilton, Ontario) in 20 L aquapaks. The Waterloo WWTP is located in southern Ontario, Canada and services the urban City of Waterloo and a small portion of the City of Kitchener. The plant is considered to be a medium-sized WWTP, servicing approximately 125,000 people (Fuzzen et al., 2015). It is a conventional activated sludge plant that provides year-round nitrification, as well as screening, grit removal, ferric sulfate addition (phosphorus removal), primary clarification, aeration, secondary clarification and UV disinfection prior to release (Waterloo Region, 2018). Upon arrival, effluent was stored in a dark, 4°C refrigerated room until used. Effluent was diluted and mixed to 10%, 20%, or 40% with de-chlorinated water; dilutions (52.5 L per treatment) were made once daily. Treatment reservoirs were filled in the morning (17.5 L) and evening (35 L); excess diluted effluent was stored in a dark, 4°C refrigerated room during the day.

For the triclosan treatment, 99% Alfa Aesar 5-chloro-2-(2,4-dichlorophenoxy)phenol (Fisher Scientific, Burlington, Ontario) was diluted to 5.25×10^{-4} g/mL in distilled water and aliquots were stored in the dark at -20°C. Each day, an aliquot was thawed and mixed with dechlorinated water to yield a final solution of 100 ng/L of triclosan (nominal concentrations).

Tank Water Analysis

Tank water (100 µL) was collected twice per week, 24 hours after each new batch of wastewater was brought to the lab. Two tanks were randomly selected and sampled at each collection for a total of 16 samples over the 28 day experiment. The tank water was used to create a 10x serial dilution in combination with brain heart infusion (BHI) broth to a final concentration of 1:100,000. Five µL of each dilution was dropped onto a BHI plate and then stored inverted at room temperature for 24 hours. The number of colonies was counted after 24 hours and colony forming units (CFU) were calculated ($CFU = \frac{number\ of\ colonies \times dilution\ factor}{volume\ of\ sample\ (mL)}$).

Gut Content Extraction and Bloodworm Sampling

Prior to sampling, tubes were prepared with 900 µL of buffer (0.564 M guanidine thiocyanate, 0.01 M EDTA, 0.004 M N-lauroyl sarkosine and 177 mM of monobasic NaH_2PO_4) and 0.2 g of 0.1 mm glass beads (MoBio Laboratories, Carlsbad, CA, USA). Fifteen wild fish were sampled at the time of collection to establish a field baseline (hereafter called "field fish"), 17 fish were sampled at the end of the 14 day acclimation to establish a laboratory baseline (hereafter called "acclimation fish"), and the remaining 98 fish were sampled at the end of the 28 day exposure (unexposed fish are hereafter referred to as "control fish"). Prior to fish handling, the bench space was sterilized with 10% bleach and 70% EtOH, and then covered with

aluminum foil. Forceps and scissors were sterilized in 30% bleach, 70% EtOH, and nanopure water (referred to as wash solutions), in that order, prior to dissection. Gloves were changed between each fish. Fish were first made unconscious by a blow to the head, euthanized by spinal severance, and then rolled in a Kim wipe pre-moistened with 70% EtOH to remove exterior bacteria. The ventral side of the fish was opened from anus to throat; the liver was removed then forceps and scissors were re-sterilized using the wash solutions. The intestinal tract was cut at the end nearest to the anus. Using forceps, the gut content was gently emptied from the intestine into a tube containing the buffer solution and tubes were inverted to mix the contents. Time between euthanasia and gut content extraction was less than 5 minutes. Three blanks were collected per treatment by adding 0.5-1 mL of each wash solution (30% bleach, 70% EtOH, and nanopure water) to buffer tubes. The laboratory diet was also sampled; three 200 mg samples of whole bloodworms (San Francisco Bay Brand, California) were collected and stored in buffer tubes. All buffer tubes were stored at room temperature until the end of daily sampling when they were stored at -80ºC.

16S rRNA gDNA Extraction

Samples were thawed at room temperature and mechanically lysed using a bead beater at 3000 rpm for 3 minutes. A mixture of 50 µL of lysozyme (100 mg/mL; Sigma-Aldrich, Oakville, Ontario) and 10 µL of RNase A (10 mg/mL; Qiagen, Toronto, Ontario) was added to each sample, vortexed, and incubated at 37ºC for 1-1.5 hours. Next, 25 µL of 25% SDS, 25 µL of proteinase K (30 units/mg; Sigma-Aldrich, Oakville, Ontario), and 62.5 µL of 5 M NaCl was combined with samples and incubated at 65ºC for 0.5-1.5 hours. The tubes were centrifuged at 13,500 *g* for 5 minutes, and the supernatant was pipetted into 900 µL of phenol-chloroform-isoamyl alcohol (Sigma-Aldrich, Oakville, Ontario), vortexed, and centrifuged at 13,000 *g* for 10

minutes. The aqueous-layer of the sample was pipetted into tubes filled with 200 μL of DNA binding buffer (Cedarlane Laboratories, Burlington, Ontario) and the solution was transferred to a DNA column (Cedarlane Laboratories, Burlington, Ontario). A vacuum manifold was used to move the solution through the DNA column, followed by wash buffer (DNA Clean and Concentrator-25, Cedarlane Laboratories, Burlington, Ontario). 50 μL of sterile DNase/RNase free water was added to each tube, and allowed to incubate at room temperature for 5 minutes. Finally, the DNA was eluted by centrifugation at 12,000 *g* for 30 seconds – 1 minute. Samples were then stored in the -80ºC until PCR.

Nested-PCR Amplification of the 16S rRNA V3 and V4 Regions

Nested PCR of the 16S rRNA V3 and V4 region was conducted. Nested PCR of the 16S rRNA V3 and V4 region was conducted. Fish and/or other eukaryotic DNA gave a high background with the V3-V4 primers, which was reduced with the nested protocol. In house testing of this method demonstrated that the protocol did not skew the bacterial profile (L. Rossi, personal communication). The 8F (AGAGTTTGATCCTGGCTCAG) to 926R (CCGTCAATTCCTTTRAGTTT) region of the 16S gene was first amplified using 1.5 μL of template with 1U of Taq, 1x buffer, 1.5 nM $MgCl_2$, 0.4 mg/mL BSA, 0.2 mM dNTPs, and 10 pmol of each primer. The PCR protocol was as follows: 5 mins at 94ºC, 30 seconds at 94ºC for 15 cycles, 30 seconds at 56ºC, 60 seconds at 72ºC, and 10 minutes at 72ºC. This reaction was used as the template for the second stage of PCR. 3 μL of the first reaction was used with 1U of Taq, 1x buffer, 1.5 nM $MgCl_2$, 0.4 mg/mL BSA, 0.2 mM dNTPs, and 5 pmol of 341F (CCTACGGGNGGCWGCAG) and 806R (GGACTACNVGGGTWTCTAAT) Illumina adapted primers (IDT, Coralville, Iowa) (Bartram et al., 2011). The PCR protocol was as follows: 5 mins at 94ºC, 30 seconds at 94ºC for 5 cycles, 30 seconds at 47ºC, 30 seconds at 72ºC, 30 seconds at

94ºC for 25 cycles, 30 seconds at 50ºC, 30 seconds at 72ºC and a final extension of 10 minutes for 72ºC. PCR products were visualized on a 1.5% agarose gel. Positive amplicons were normalized using the SequalPrep normalization kit (ThermoFisher #A1051001, Mississauga, Ontario) and sequenced on the Illumina MiSeq platform (paired-end reads, 2 x 300 base pairs) at the Farncombe Institute (McMaster University, Hamilton, Ontario).

Read Processing

Cutadapt was used to filter and trim adapter sequences and PCR primers from the raw reads with a minimum quality of 30 and minimum read length of 100 bp (Martin, 2011). Reads were trimmed, sequences were filtered and trimmed based on quality, and sequence variants were determined using DADA2 (Callahan et al., 2016). Bimeras were removed and the amplicon sequence variants (ASVs) were assigned to taxonomy using the SILVA database (version 1.3.2).

Data Analysis

Amplicon sequence variants (ASVs) were analyzed using the Phyloseq package (version 1.30.0) in R (version 3.6.1). Fish gut content samples were grouped by field baseline, acclimation baseline, or treatment group for subsequent analyses. Bacterial composition was determined by calculating relative abundance at various taxonomic levels (phyla, class, order, family, and genus) and patterns were examined between treatments. Next alpha diversity was calculated at the ASV level; first samples were rarefied to minimum sample depth (840 reads) and then alpha diversity was calculated using the Shannon Diversity index and statistical significance was compared between sites using a one-way ANOVA and a Tukey's post-hoc test. Beta diversity was determined using a Bray Curtis dissimilarity with Principal Coordinate Analysis (PCoA) and statistical significance was determined using a permutational multivariate analysis of variance (PERMANOVA) and a pairwise PERMANOVA using the Adonis function

in the package Vegan (2.5.6). Gonadosomatic index ($GSI = 100 \times \frac{gonad\ mass\ (g)}{total\ body\ mass\ (g)}$) was calculated to determine spawning condition (ggplot2, version 3.2.1). Fish condition (K) was calculated as follows ($K = 100 \times \frac{weight\ (g)}{length^3\ (cm)}$). GSI and K were plotted by treatment to determine if there were any changes among treatments.

3.3 Results

CFUs Found in Tank Water

Tank water samples ranged from 2.0×10^2 - 6.8×10^3 CFUs (Table 3.1; Appendix B, Table B.2) and there was no evidence of higher bacterial loads at greater effluent concentrations.

Table 3.1 Bacterial abundance (colony forming units (CFUs)/mL) of tank water sampled over the 28 day experiment. Two tanks were randomly selected 24 hours after each effluent collection (see Methods for details).

Treatment	Tank	CFU (per mL)
Control	16	$2.8 \times 10^3 – 2.0 \times 10^2$
Control	16	2.2×10^3
Control	14	6.0×10^3
Control	14	6.8×10^3
10% WWTP Effluent	15	1.0×10^3
10% WWTP Effluent	17	1.0×10^3
10% WWTP Effluent	18	1.0×10^3
20% WWTP Effluent	8	2.0×10^3
20% WWTP Effluent	3	2.4×10^3
20% WWTP Effluent	9	4.0×10^2
40% WWTP Effluent	12	1.0×10^3
40% WWTP Effluent	6	1.2×10^3
40% WWTP Effluent	12	$2.4 \times 10^3 – 2.0 \times 10^2$
40% WWTP Effluent	6	5.2×10^3
100 ng/L Triclosan	4	$1.0 \times 10^3 – 2.0 \times 10^2$
100 ng/L Triclosan	5	$2.2 \times 10^3 – 2.0 \times 10^2$

Relative Abundance

In total, there was 8,074,658 reads in all fish guts sampled; there was an average of 62,113 reads per sample (minimum of 840 and maximum of 109,477 reads per sample). Across all rainbow darter, 31 phyla, 70 classes, 170 orders, 292 families, 840 genera, and 11853 ASVs were identified in the gut contents. Individual samples were assigned between 19 and 1304 ASVs, with a mean of 285 ASVs. Generally, the field samples had greater ASVs per sample ranging from 423-1304, while the lab samples range from 19-685. The mean ASVs per sample were 4-fold higher in field baseline samples (813.5) compared to the 14 day acclimation (221.9),

control (42 days in the lab; 207.5) and treatment fish; the 10%, 20%, 40% WWTP effluent and 100 ng/L TCS had 227.0, 177.5, 225.6, 245.4 ASVs per sample respectively. Overall, five bacterial phyla accounted for over 85% of all ASVs and included: Firmicutes (4117, 35%), Proteobacteria (3397, 29%), Actinobacteria (1353, 11%), Planctomycetes (916, 7.7%), and Bacteriodetes (453, 3.8%).

The relative abundance of phyla was highly variable in individual field samples, whereas one phyla tended to dominate in laboratory fish at all times (Figure 3.1, Figure 3.2). The field baseline group was dominant in the phyla Proteobacteria and Firmicutes, while the gut contents of the lab fish (acclimation, control and exposed) were almost entirely dominant in the phyla Firmicutes (37-47%, Figure 3.3). In the phyla Firmicutes, field fish were most abundant in the family *Clostridaceae,* while lab fish were abundant in *Erysipelotrichaceae* (Figure 3.4). Within the phyla Proteobacteria, the family *Rhodobacteraceae* dominated the field samples, but did not dominate the lab fish (Figure 3.5).

The relative abundance of bacteria in field fish differed from laboratory fish; field fish had increased ASVs and a greater diversity of gut bacteria compared to lab fish. Field fish samples had a variety of bacteria present (total of 5581 ASVs across all samples), and the top phyla were Proteobacteria (1975 ASVs, 35%) and Firmicutes (1504 ASVs, 27%), followed by Planctomycetes (537 ASVs, 10%) and Actinobacteria (471 ASVs, 8%), Cyanobacteria (388 ASVs, 7%), and Bacteriodetes (239 ASVs, 4%). The most abundant families were *Legionellaceae* (292 ASVs, phyla – Proteobacteria), *Gemmataceae* (256 ASVs, phyla – Planctomycetes), *Rhodobacteraceae* (210 ASVs, phyla – Proteobacteria), and *Ruminococcaceae* (209 ASVs, phyla – Firmicutes). Acclimation fish samples had fewer bacteria present (2025 ASVs) compared to the field fish. The microbial community was dominated by Firmicutes (957

ASVs, 47%), followed by Proteobacteria (378 ASVs, 17%), and Actinobacteria (314 ASVs, 15%). The most abundant family was *Ruminococcaceae* (138 ASVs, phyla – Firmicutes). Control fish samples had only 1795 ASVs present, with high proportions of Firmicutes (782 ASVs, 43%), followed by Proteobacteria (431 ASVs, 24%) and Actinobacteria (225 ASVs, 12%). Similar microbial communities were found in the guts of fish exposed to 10% effluent (1935 ASVs; Firmicutes, 708 ASVs, 37%), 20% effluent (1588 ASVs; Firmicutes, 625 ASVs, 39%), 40% effluent (2098 ASVs; Firmicutes, 885 ASVs, 42%). Finally, the TCS exposed fish had 1636 ASVs, and again were dominated by Firmicutes (658 ASVs, 40%).

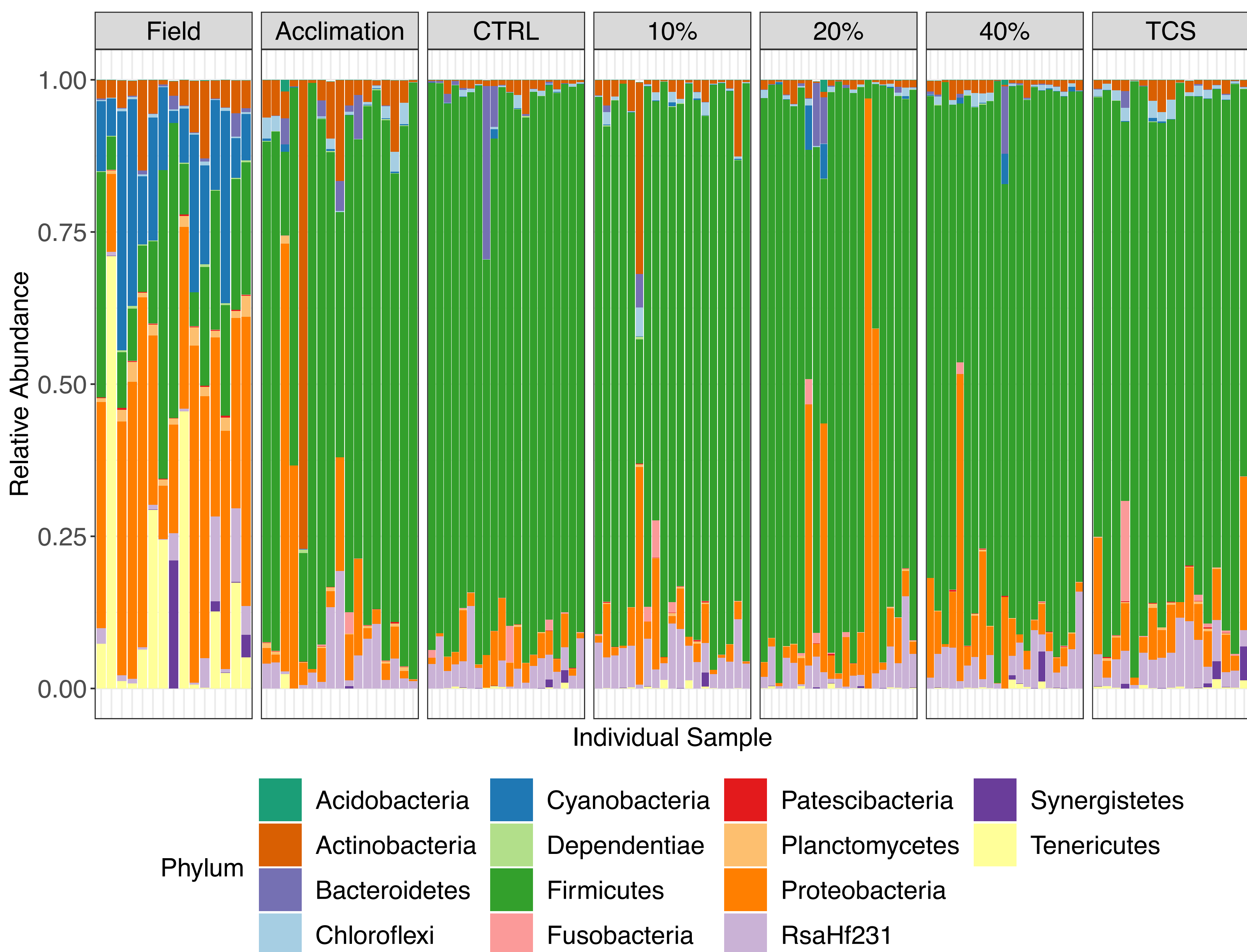

Figure 3.1 The mean relative abundance of gut bacteria at the phyla level in individual *E. caeruleum*. Only the phyla with > 2% of the total ASVs were included. The y-axis shows the proportion of ASVs within each included phyla from 0 to 1. The bars are ordered from left to right: field baseline (Field), acclimation, control (CTRL), 10% WWTP effluent (10%), 20% WWTP effluent (20%), 40% WWTP effluent (40%), and 100 ng/L of triclosan (TCS).

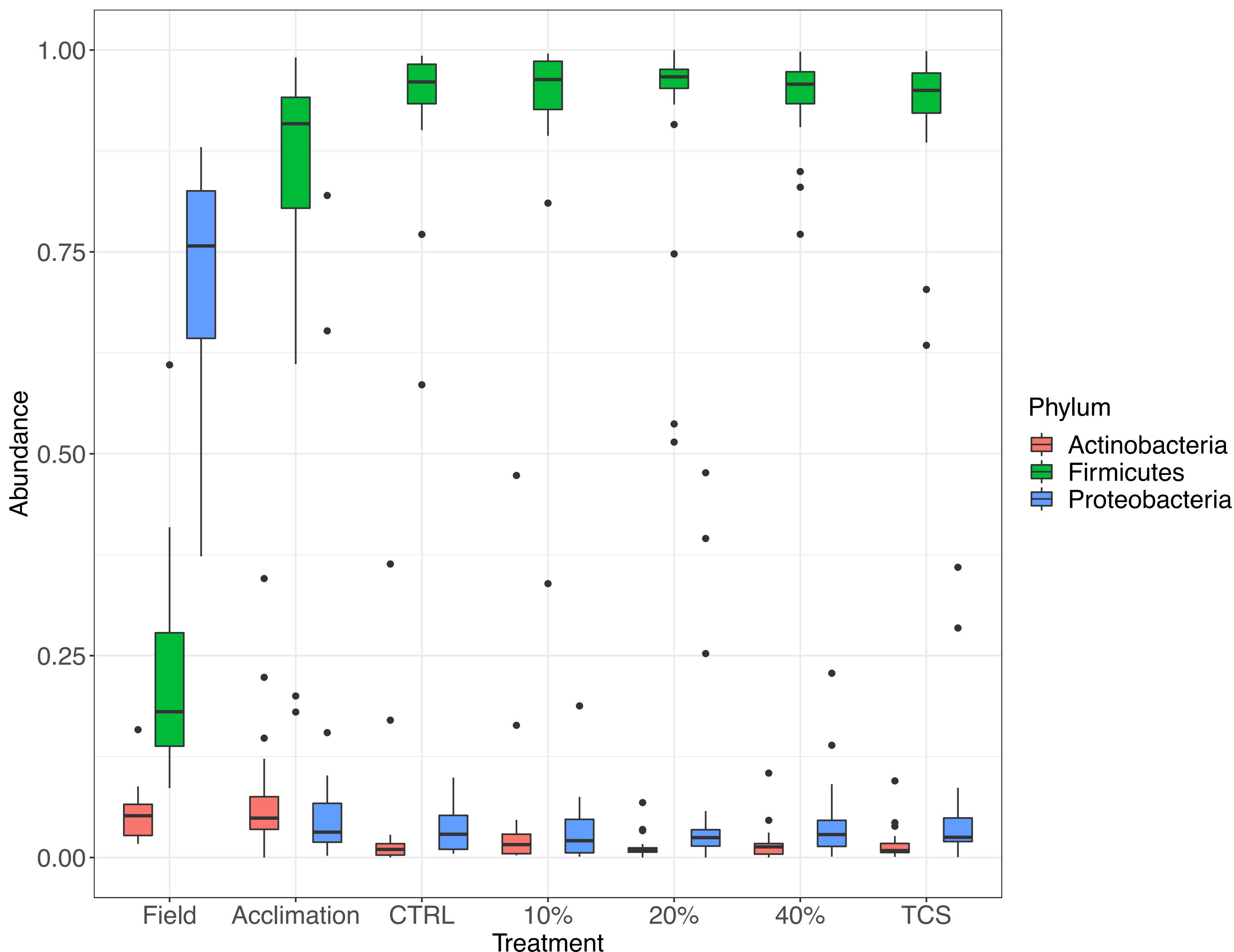

Figure 3.2 Boxplot showing the relative abundance of the most abundant phyla. The y-axis shows the proportion of ASVs within each included phyla from 0 to 1. The box and whiskers are ordered from left to right: field baseline (Field), acclimation, control (CTRL), 10% WWTP effluent (10%), 20% WWTP effluent (20%), 40% WWTP effluent (40%), and 100 ng/L of triclosan (TCS). The most abundant phyla, Actinobacteria (red), Firmicutes (green), Proteobacteria (blue), are shown. Upper, middle, and lower lines represent first, second, and third quartiles; whiskers represent a 1.5 inter-quartile range.

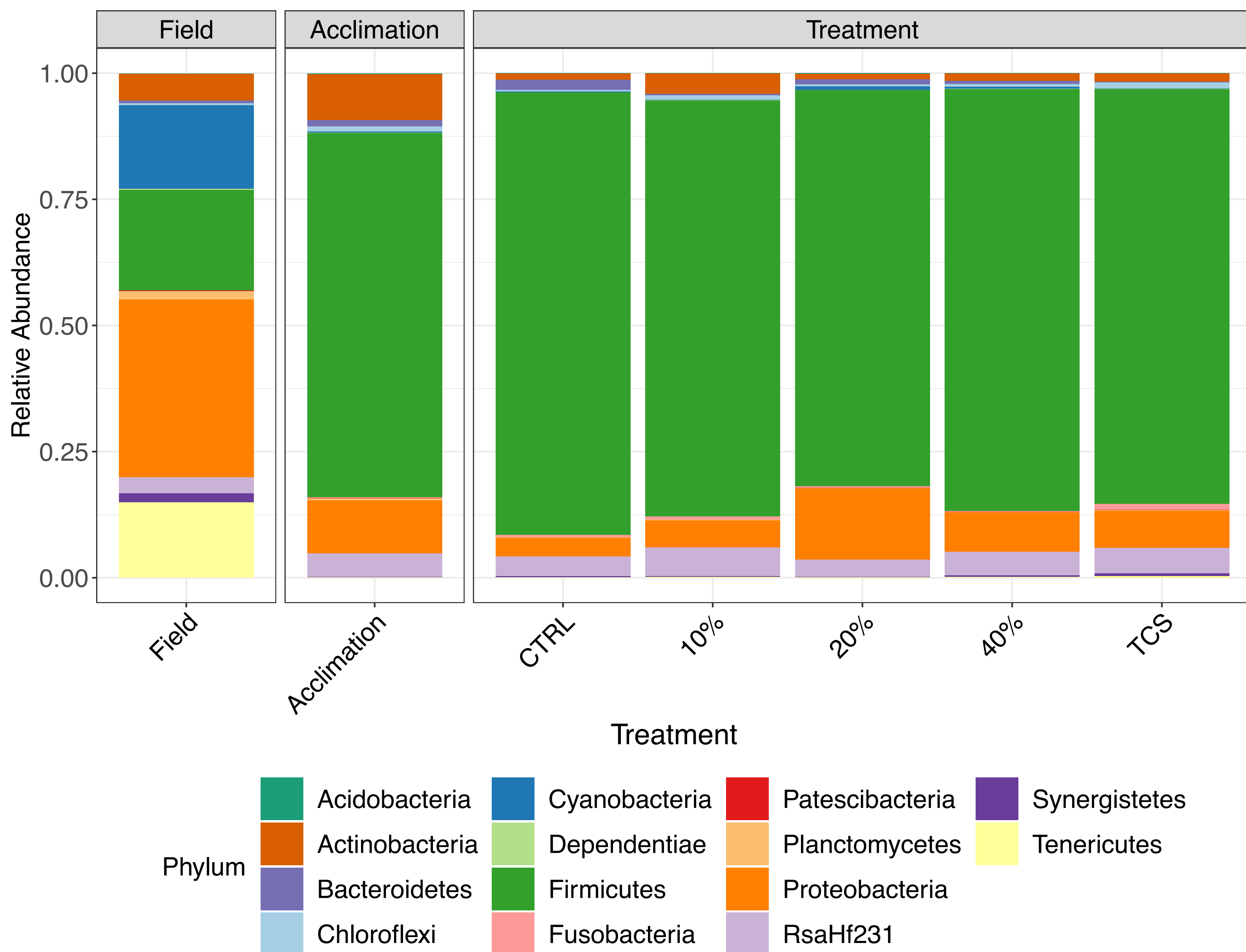

Figure 3.3 The mean relative abundance of gut bacteria at the phyla level in *E. caeruleum* by treatment group. Only the phyla with > 2% of the total ASVs were included. The bars are ordered from left to right: field baseline (Field), acclimation, control (CTRL), 10% WWTP effluent (10%), 20% WWTP effluent (20%), 40% WWTP effluent (40%), and 100 ng/L of triclosan (TCS). The y-axis shows the proportion of ASVs within each phyla from 0 to 1.

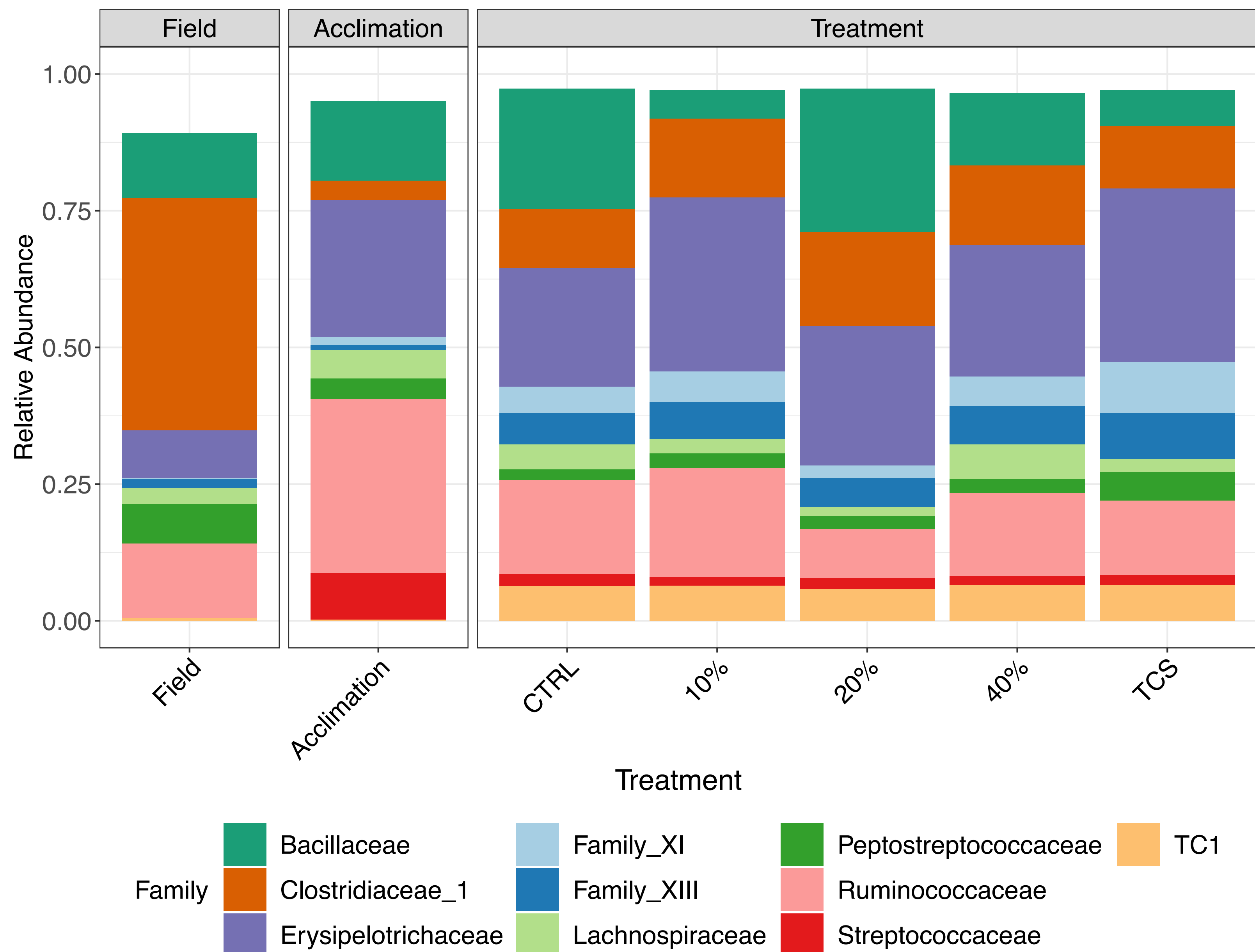

Figure 3.4 The mean relative abundance of top 10 families of Firmicutes collected from the gut content of *E. caeruleum*. Only those with > 2% of the total ASVs were included The y-axis shows the proportion of ASVs within each included family from 0 to 1. The value of 1 on the y axis would include all families within Firmicutes, but here only the top 10 are shown. The bars are ordered from left to right: field baseline (Field), acclimation, control (CTRL), 10% WWTP effluent (10%), 20% WWTP effluent (20%), 40% WWTP effluent (40%), and 100 ng/L of triclosan (TCS).

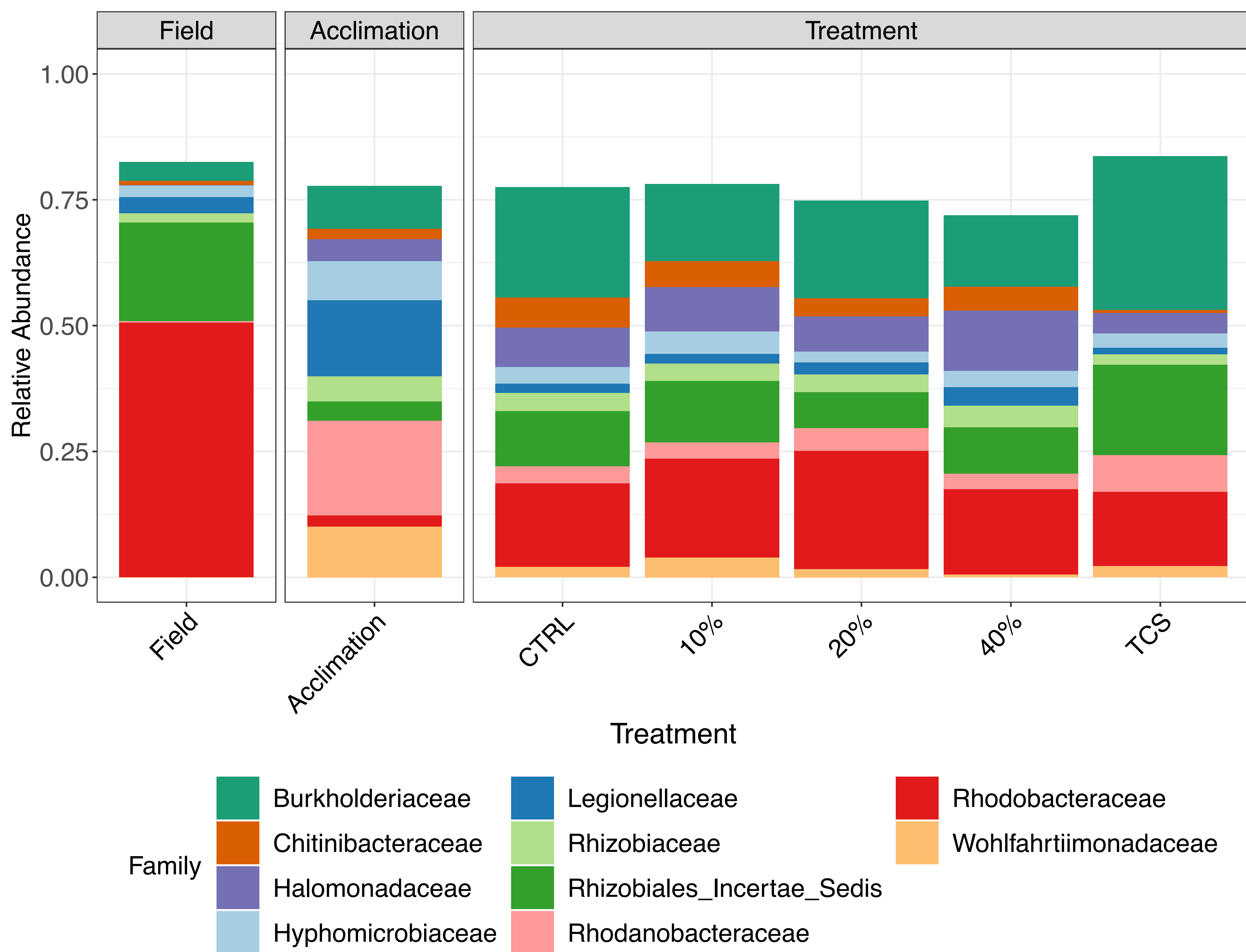

Figure 3.5 The mean relative abundance of top 10 families of Proteobacteria collected from the gut content of *E. caeruleum*. Only those with > 2% of the total ASVs were included. The y-axis shows the proportion of ASVs within each included family from 0 to 1. The value of 1 on the y axis would include all families within Proteobacteria, but here only the top 10 are shown. The bars are ordered from left to right: field baseline (Field), acclimation, control (CTRL), 10% WWTP effluent (10%), 20% WWTP effluent (20%), 40% WWTP effluent (40%), and 100 ng/L of triclosan (TCS).

Alpha Diversity

Alpha diversity was significantly different among treatment groups (Figure 3.6, one-way ANOVA, $F=8.744$, $p<0.0001$). Gut contents of the field fish had increased alpha diversity compared to the acclimation (Tukey HSD, $p<0.00001$), control ($p=0.015$), 20% WWTP effluent ($p=0.00001$), and 40% WWTP effluent ($p=0.008$) fish. The acclimation group had decreased alpha diversity compared to the control ($p=0.009$), 10% effluent ($p=0.003$), 40% effluent ($p=0.014$), and triclosan ($p=0.0007$) group.

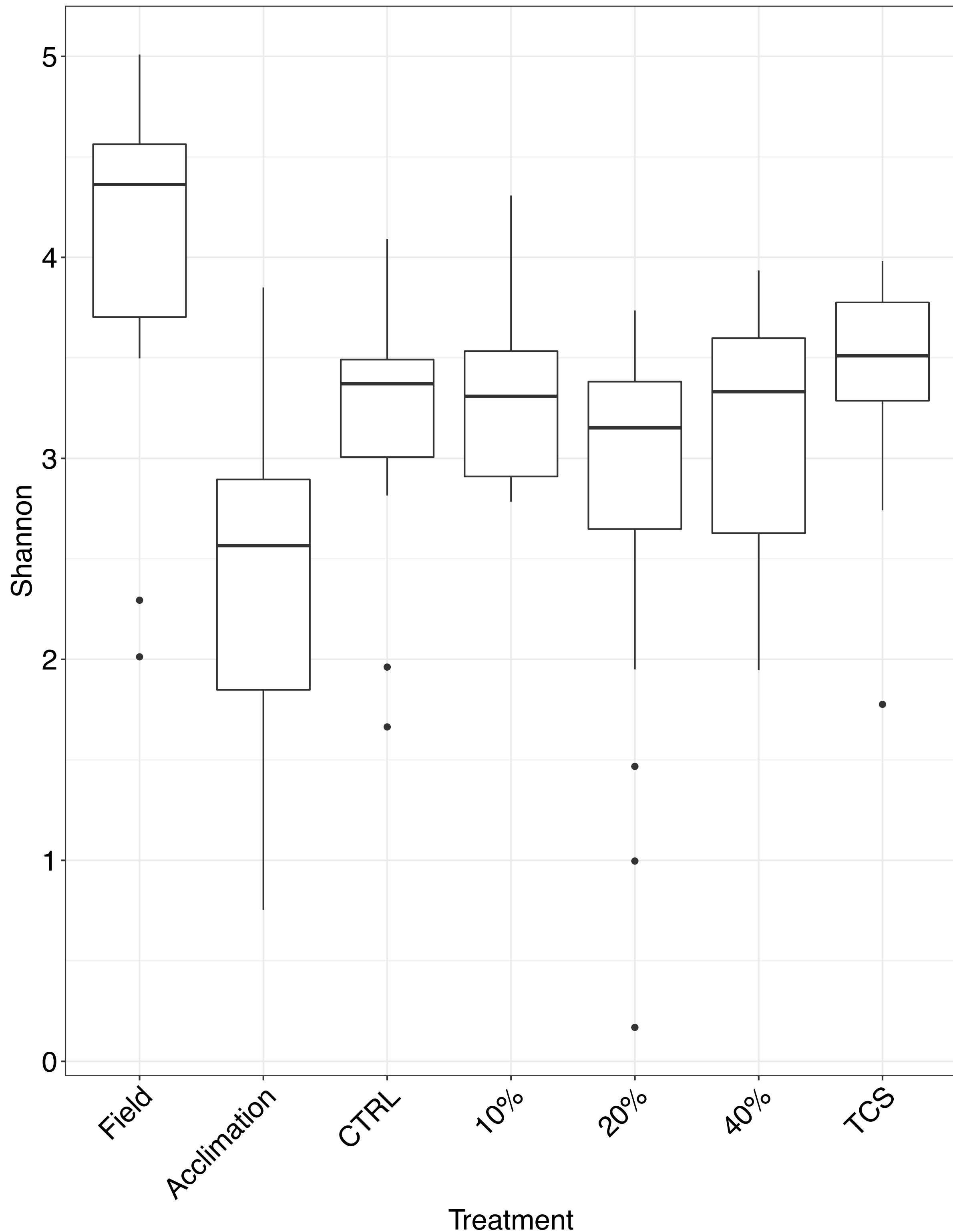

Figure 3.6 Shannon alpha diversity of the microbiome of rainbow darter gut contents collected from the field, after 14 days acclimation, and for the control (CTRL), 10% WWTP effluent (10%), 20% WWTP effluent (20%), 40% WWTP effluent (40%), and 100 ng/L of triclosan (TCS) exposure groups.

Beta Diversity

Scree plots were generated to explore the proportion of total variability within the calculated Bray Curtis distance matrix, the largest variance was confirmed to be in axes 1 and 2 (Figure 3.7). Beta diversity was statistically dissimilar among treatments (Figure 3.8, Adonis PERMANOVA, $F=6.6131$, $p=0.001$). The field group clustered tightly together and was significantly dissimilar from each of the lab groups (Appendix B, Table B.3, Pairwise Adonis PERMANOVA). The acclimation baseline group was also different from the exposure groups; the control was dissimilar from the 10% WWTP effluent and TCS groups; the 10% WWTP effluent was dissimilar from the 20% WWTP effluent and TCS groups; and the 20% WWTP effluent group was dissimilar from the TCS group.

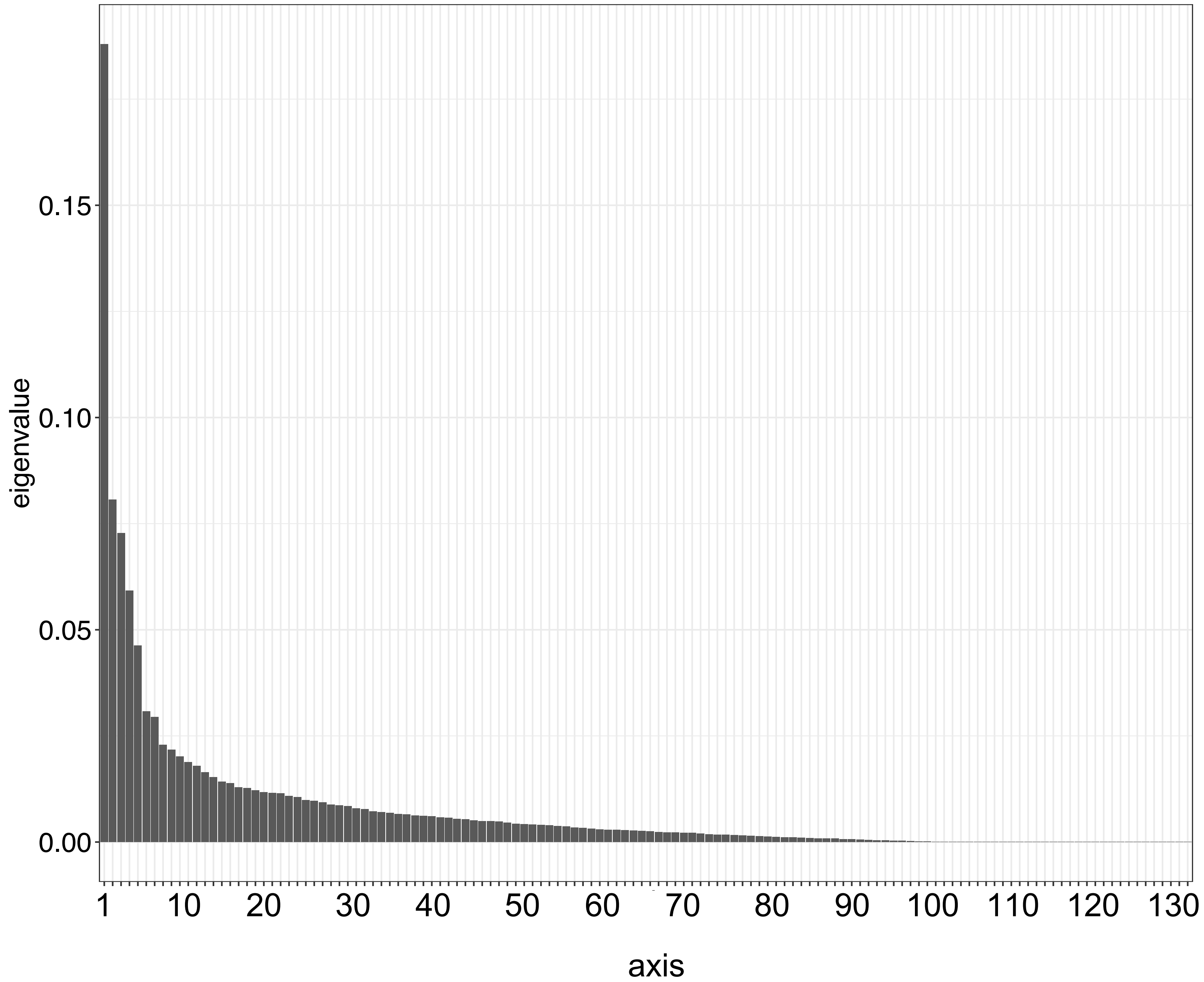

Figure 3.7 Scree plot generated using calculated Bray Curtis distance matrices and the Principal Coordinate Analysis. The y axis represents eigenvalues which indicate the variability within the distance matrix, while the x axis represents axes 1 – 130, with axis 1 representing the largest variance (18.8%) followed by axis 2 (8.1%).

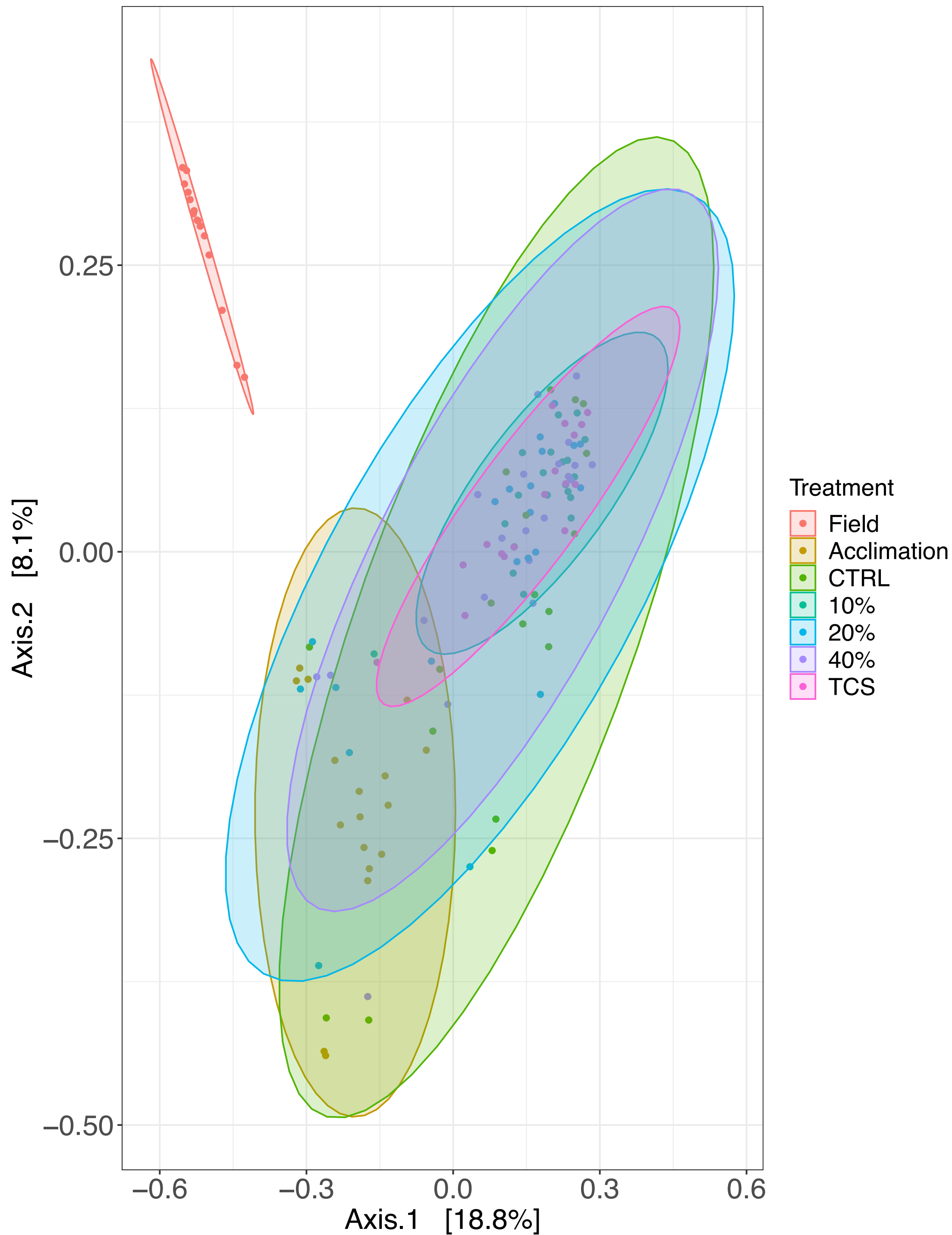

Figure 3.8 Bray-Curtis beta diversity with Principal Coordinate Analysis (PCoA) values from rainbow darter gut microbiome samples. The treatments – field, acclimation, control (CTRL), 10% WWTP effluent (10%), 20% WWTP effluent (20%), 40% WWTP effluent (40%), and 100 ng/L of triclosan (TCS) – are labelled as coloured points and grouped using ellipses. Axis 1 and 2 are labelled with the percent variation explained by treatment in square brackets.

Fish Condition

Gonad weight ranged between 0.009 g and 0.325 g and total fish weight ranged between 0.705 and 3.535 g. The median GSI ranged from 1.2% in the TCS treatment group to 6.8% in the field baseline (Figure 3.9). Gonad weight and total body weight were plotted and most fish were clearly non-spawning (criteria for non-spawning is a GSI below ~3%, Tetreault et al., 2014) when sampled in the laboratory. GSI differed significantly among all treatment groups (one-way ANOVA, $p=0.04$). The field fish had a higher GSI than the 40% WWTP effluent treatment (Tukey's HSD, $p=0.01$). All other treatments were not significantly different (Tukey's HSD, $0.99 < p > 0.056$). Condition factor (K) ranged from 0.69 to 2.2 amongst all samples, with an average K of 1.1 across all groups (Figure 3.10). K differed significantly among treatments (one-way ANOVA, $p<0.00001$). All treatments were different from one another (Tukey's HSD, $p<0.00001$), except for the 10% and 40% WWTP effluent treatments (Tukey's HSD, $p=0.99$). The field fish had an increased K compared to all lab fish, the acclimation fish had a higher K than all other lab fish, and the control had a higher K than all WWTP exposed fish. TCS exposed fish had a higher K than WWTP exposed and control fish, but lower than the acclimation and field fish.

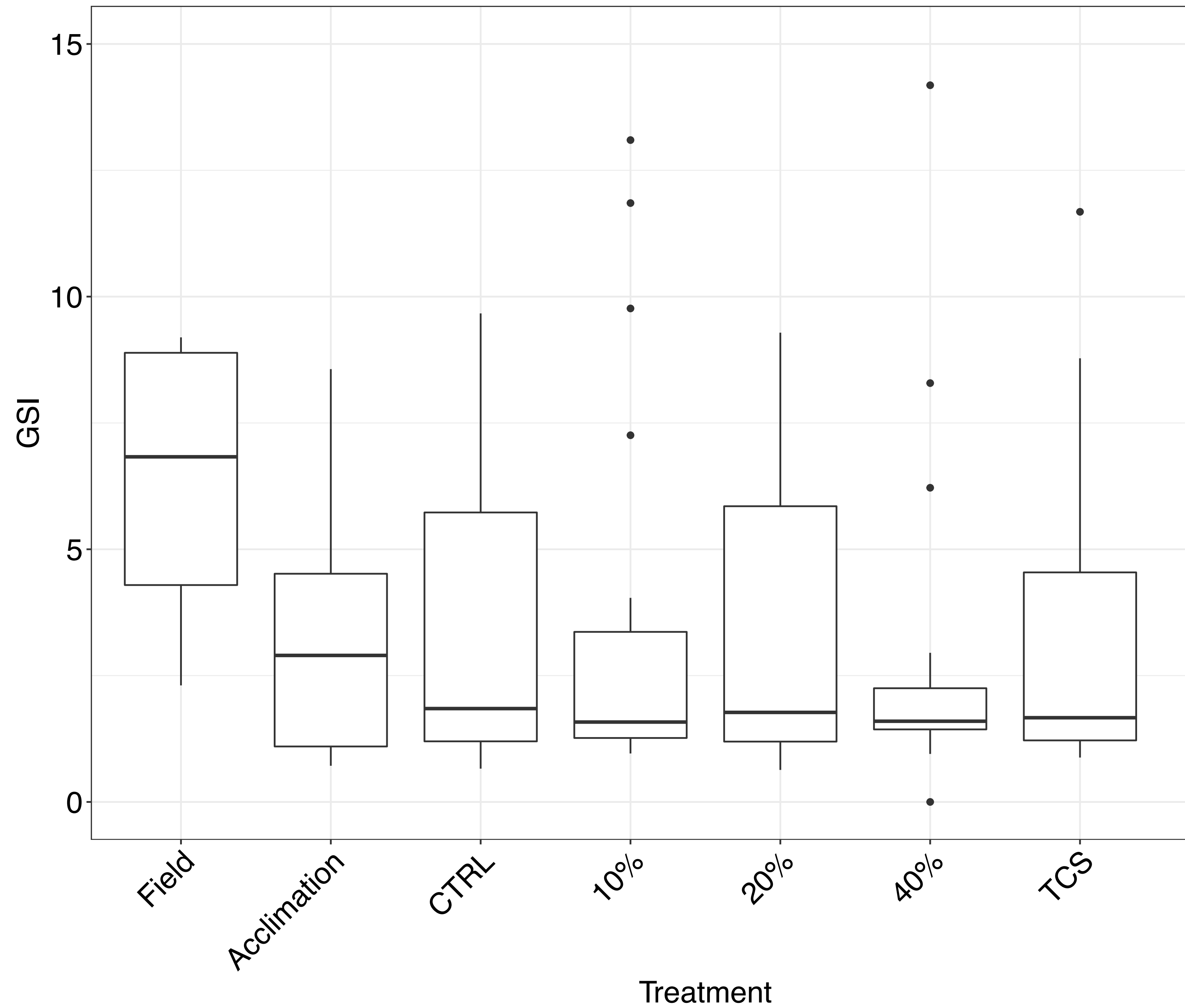

Figure 3.9 GSI of female rainbow darter by treatment – field baseline (Field), acclimation, control (CTRL), 10% WWTP effluent (10%), 20% WWTP effluent (20%), 40% WWTP effluent (40%), and 100 ng/L of triclosan (TCS).

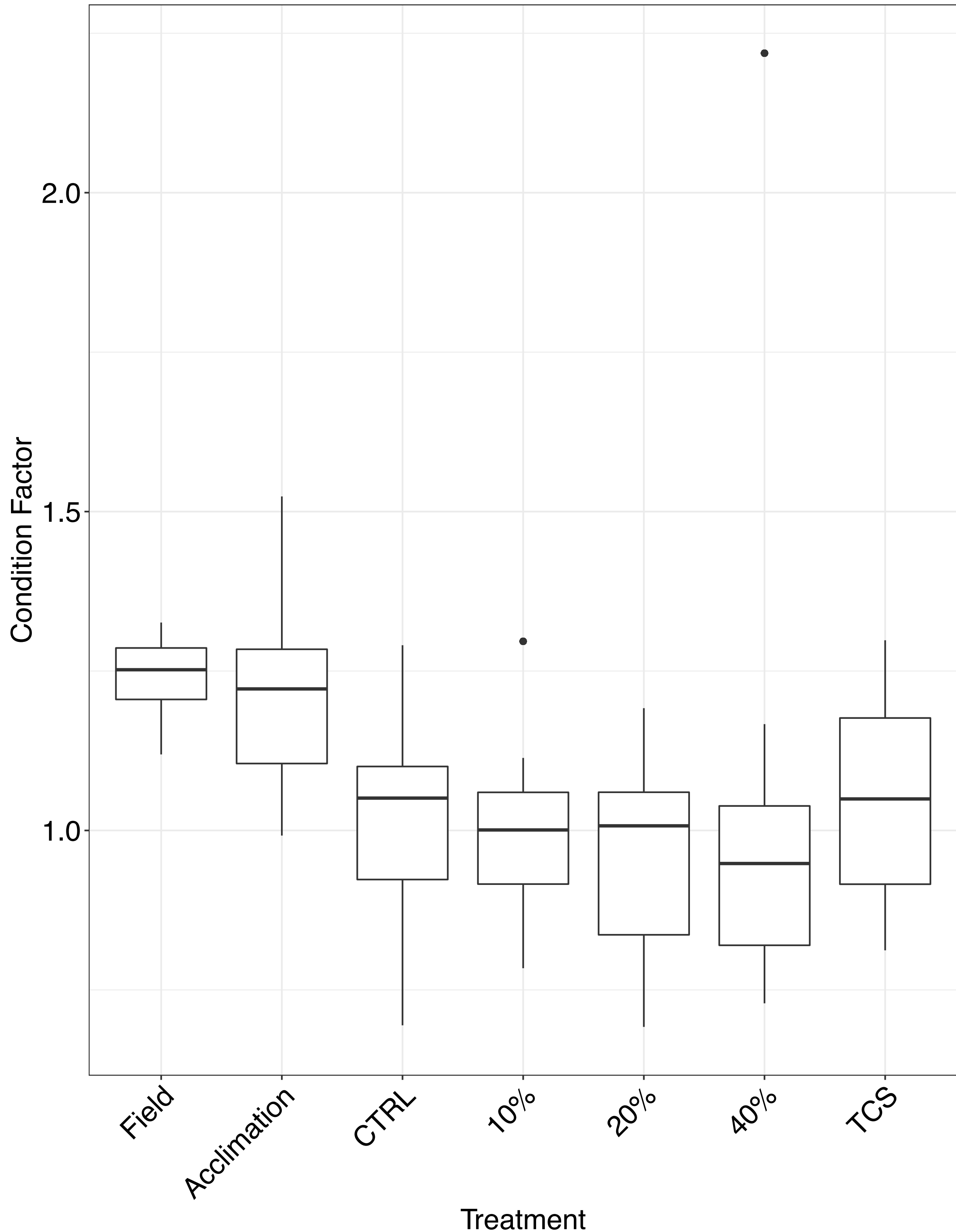

Figure 3.10 Fish condition (K) of female rainbow darter by treatment – field baseline (Field), acclimation, control (CTRL), 10% WWTP effluent (10%), 20% WWTP effluent (20%), 40% WWTP effluent (40%), and 100 ng/L of triclosan (TCS).

Microbiota Detected in the Bloodworm Diet

The bloodworm diet was variable between in bacterial composition; two of the three samples were abundant in Firmicutes and Proteobacteria, while the third was dominated by Fusobacteria (Figure 3.11). The bacterial community of the bloodworm diet was dissimilar from that of the rainbow darter gut contents (Figure 3.12, Adonis PERMANOVA, F=2.1376, p=0.016).

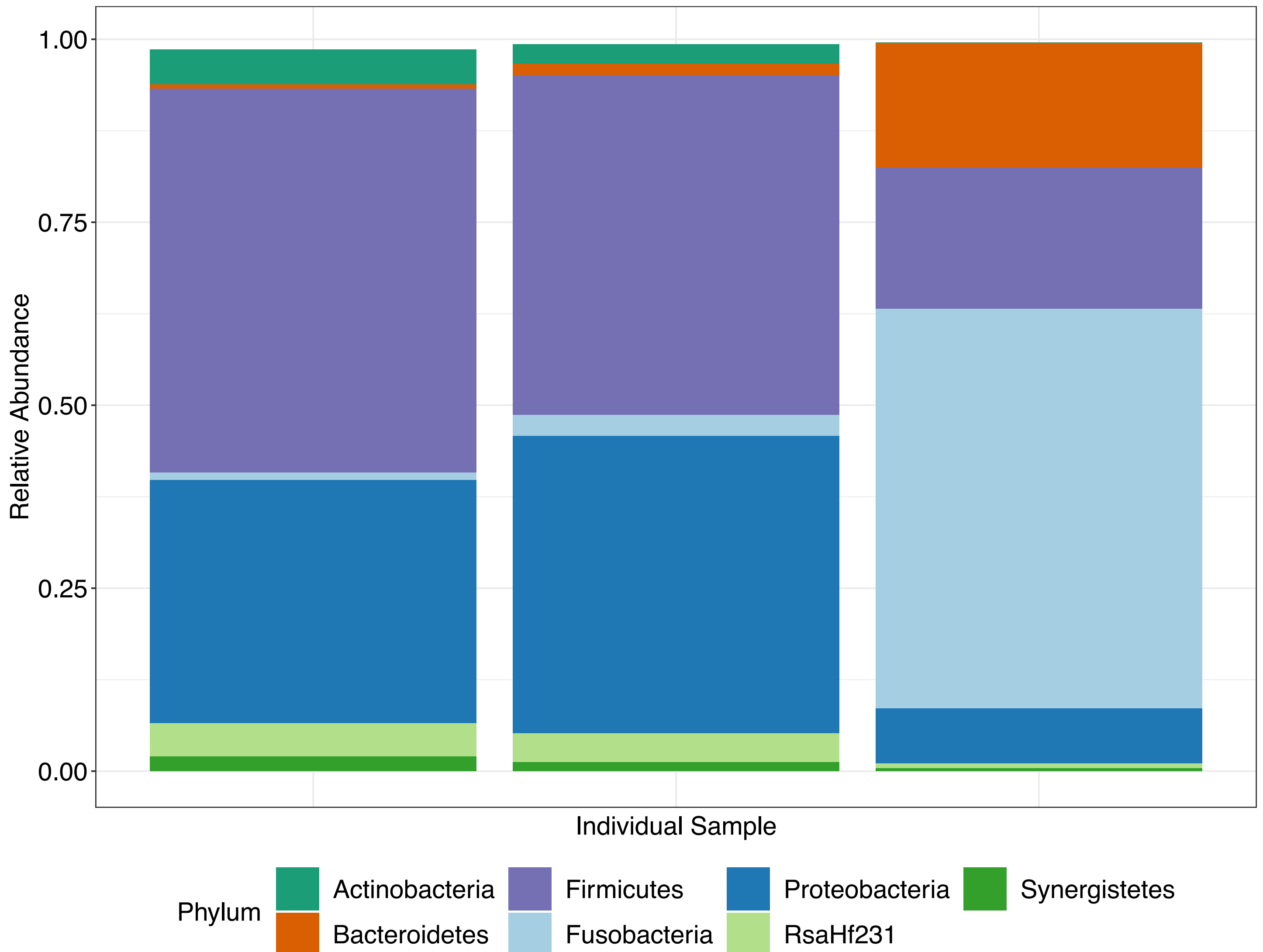

Figure 3.11 The mean relative abundance of phyla collected from the frozen bloodworm diet of *E. caeruleum.* Only those with > 2% of the total ASVs were included. The y-axis shows the proportion of ASVs within each family from 0 to 1. The bars indicate three individual bloodworm samples.

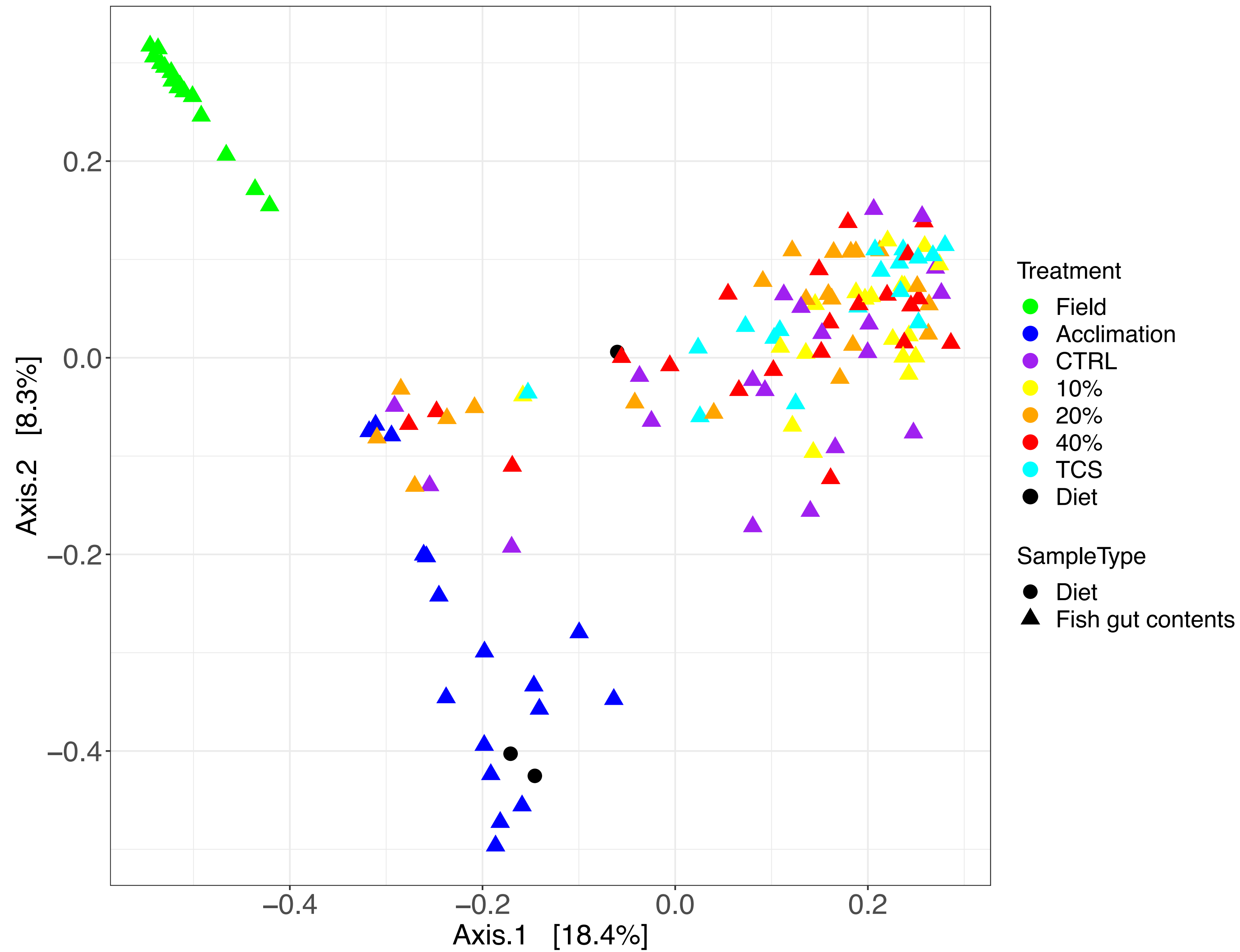

Figure 3.12 Bray-Curtis beta diversity with Principal Coordinate Analysis (PCoA) values from rainbow darter gut content and bloodworm diet samples. The treatments – field, acclimation, control (Ctrl), 10% WWTP effluent (10%), 20% WWTP effluent (20%), 40% WWTP effluent (40%), and 100 ng/L of triclosan (TCS) – are labelled as coloured triangles. The diet samples are black circles. Axis 1 and 2 are labelled with the percent variation explained by treatment in square brackets.

3.4 Discussion

The gut microbiome of wild rainbow darter declined in alpha diversity and had dissimilar microbial communities after being held in the lab for 14 or 42 days. These results indicate that moving wild caught fish into a laboratory holding environment results in a major shift in the gut content microbiome. The gut microbiome changed in diversity and community composition over time in the lab; when compared to samples collected after 14 days, the alpha diversity increased and had a distinct bacterial community after 42 days in the lab. Finally, while there were some differences in beta diversity, there was no effect of either WWTP effluent or TCS exposure on the rainbow darter gut microbiome alpha diversity or bacterial relative abundance. This was surprising as TCS exposure caused altered gut bacterial composition and declines in bacterial alpha diversity in zebrafish and fathead minnows previously (Narrowe et al., 2015, Gaulke et al., 2016).

The Gut Microbiome Changes After Transition to the Laboratory

The largest difference in gut content microbiome in this study was with the transition of wild fish into the laboratory, and this was likely due to differences in diets and aqueous environments. Darter collected in the field had a distinct microbial composition, higher alpha diversity and dissimilar beta diversity compared to acclimation and control fish. Similarly, when laboratory held fish were compared to wild fish, alpha diversity was lower and community composition differed in rainbow darters (Dhakal, 2017), invasive carp (Eichmiller et al., 2016), mummichog (Givens et al., 2015), and African cicchlids (Baldo et al., 2015) and these results were likely due to differences in diets. The gut content of wild fish species had differing Shannon diversity based on the trophic position of the fish (Liu et al., 2016), suggesting diet is a critical factor in the gut microbiome. Omnivorous fish had high bacterial diversity in the gut,

while carnivorous fish had lower diversity indicating that diet influences the alpha diversity of bacterial species in the fish gut (Liu et al., 2016). Furthermore, diet leads to differences in bacterial communities, with carnivorous, omnivorous, herbivorous, and filter-feeding fish clustering separately (Sullam et al., 2012, Liu et al., 2016). Laboratory held fish have lower diversity compared to wild fish, so do carnivorous fish when compared to omnivorous fish and this is because the former have less diverse diets compared to the latter. Wild fish are opportunistic feeders and often consume a variety of species whereas lab fish are maintained on a single diet; greater exposure to diverse microbial communities in the diets of wild fishes likely contributes to the higher diversity, as seen herein.

Wild rainbow darters are insectivorous, consuming a diversity of benthic invertebrates including Chironomidae, Trichoptera, Ephemeroptera, and Isopoda (Robinson et al., 2016), while the laboratory fish were fed bloodworms. Trichoptera (Hysropsychidae) and Ephemeroptera (Ephemerellidae) collected at the same site that was used in the present study, albeit from October 2018, were dominated by Proteobacteria (34%, 40%), Bacteriodetes (45%, 36%), Firmicutes (11.8%, 1.3%) and Cyanobacteria (2%, 29%), respectively (Millar, 2020), which was in contrast to the bloodworms used in this lab study (Firmicutes 35%; Proteobacteria 27%). Interestingly, the dominant phyla present in the diet was also dominant in the fish gut; natural prey species were dominant in Proteobacteria, similar to the field fish in this study, while bloodworms and laboratory fish were both dominant in Firmicutes. The greater diversity of bacterial phyla in the field-caught fish than the lab-held fish likely reflects the greater diversity of phyla in the wild macroinvertebrate prey than in the bloodworms.

The large effect of transition to the lab is not surprising and has been found in other taxa; the gut microbiome of wild caught mice (collected from 3 separate locations) and laboratory

mice (collected from 3 separate labs) clustered separately from one another, indicating their gut bacterial communities were dissimilar (Rosshart et al., 2017). In contrast, the gut contents of zebrafish recently collected from the wild (Shutunga River, India) were found to share 97% of OTUs with those reared in a lab facility (University of Oregon) and currently held in 5 separate aquatic facilities at the time of sampling (Roeselers et al., 2011). Yet, researchers did not sample fish directly in the field and instead the wild caught zebrafish were held in a static tank for 32 days in India, were transported to the US, and held in a 4 day quarantine prior to sampling (Roeselers et al., 2011). They had already experienced a major change in environment and diet and significant time in laboratory holding which suggests these data do not accurately represent the gut microbiome of wild fish transitioning to the laboratory. Collectively, the data suggest a major change in gut microbiome is associated with the transition to the laboratory, regardless of species.

Firmicutes are commonly found as part of the core phyla present in freshwater fish (Eichmiller et al., 2016, Tarnecki et al., 2017), however Firmicutes do not typically dominate the fish gut as in this laboratory study. In contrast to this study, rainbow darters fed bloodworms in the lab had decreased Firmicutes, and Bacteriodetes, and increased Proteobacteria (Dhakal, 2017). The dominance of Firmicutes has been observed in lab mice when compared to those collected in the field (Rosshart et al., 2017). The field to lab differences observed may be due to either the amount of food the fish consumed or the type of diet used herein. Increased abundance of Firmicutes has been associated with increased gut fullness in grass carp (Ni et al., 2014, Xia et al., 2014). During the acclimation period rainbow darter were fed bloodworms *ad libidum,* and it is possible that the higher food availability increased gut fullness and Firmicutes as a result. The bloodworm diet itself was dominant in Firmicutes, in 2 of the 3 samples collected, but not to the

same extent as the rainbow darter samples. The differences in bacterial communities between diet (bloodworms) and gut contents in this study coupled with the lack of increased Firmicutes in darters fed bloodworms in a separate study (Dhakal, 2017) suggest that the microbial composition of the food was not enough to explain the large increase in Firmicutes in the darters. In the transition to the lab, both *Erysipelotrichaceae* and *Ruminococcaceae* (family, phyla – Firmicutes) increased in abundance in the rainbow darter gut. In humans and mice, increased levels of Firmicutes have been found in the gut of obese individuals compared to those of normal weight/lean controls (Turnbaugh et al., 2006), in contrast lab fish in this study had increased Firmicutes coupled with decreased condition factor. In humans, increases in the *Erysipelotrichaceae* (family, phyla – Firmicutes) have been associated with inflammation-related gastrointestinal diseases and metabolic disorders like obesity (Kaakoush et al., 2015), however in contrast, mice with increased *Ruminococcaceae* (family, phyla – Firmicutes) had lower association with obesity (Menni et al., 2017). The relevance of a dominance of Firmicutes or increases in either of these bacterial classes for fish health has not been determined.

Time in the Lab Influences the Gut Microbiome

To my knowledge, no study has specifically addressed the effect of acclimation period on the gut microbiome in fish, however such studies have been conducted in mouse models. Two strains of mice from two different facilities were sampled before and after transport, upon arrival at 2, 5, and 7 days, and 9 weeks later to assess changes in the microbiome (Montonye et al., 2018). With few exceptions, the relative abundance of OTUs and beta diversity in the mouse gut changed over the entire 9 weeks (Montoye et al., 2018), similar to the changes in bacterial relative abundance observed over time in the present study. The gut microbiome of mice can change significantly after arriving in a new facility due to environmental changes and stressors

associated with transportation, suggesting that significant time in the lab is required before the gut microbiome stabilizes. Lab studies are conducted on fish species reared within the lab, transported to the lab from separate rearing facilities, or transported to the lab from the wild. For fish reared in the lab, acclimation periods are atypical, while fish transported into the lab from separate facilities or the wild are acclimated for periods of time that vary based on species and facility.

There is no standard acclimation period for fish microbiome studies, but the results of my study and that of others suggest that this needs serious consideration. Temporal sampling suggests that the microbiome changes over time, supporting the notion that acclimation to a new setting may take significant time. Similar to our study, wild-caught rainbow darter fed bloodworms in the lab had altered bacterial composition after 1 and 3 months (Dhakal, 2017). Lab reared fathead minnow larvae fed hatched brine shrimp had distinct bacterial communities after 7 and 14, but not 21 days in the lab, indicating the gut microbiome stabilized after 14 days (Narrowe et al., 2015). Similar results were found in a study conducted on juvenile goldfish (reared in a separate aquatic facility); gut microbiome communities were distinct after 14 days, but became similar after 21-35 days (Kan et al., 2015). Thus, with sufficient time, fish gut microbiomes will stabilize in the lab, but the length of time required may differ considerably between species. Based on the data from the current study and Dhakal (2017), rainbow darters appear to require a longer period, possibly on the order of months, to reach a stable microbiome in the laboratory environment. This longer acclimation time may be at least partially due to the animals being wild caught; the studies with shorter acclimation times involved fish raised in a lab environment.

Aqueous Environmental Stressors Did Not Cause Large Changes in the Gut Microbiome

Although there were some differences among treatments in beta diversity, it was surprising that alpha diversity and relative abundance of the gut microbiomes of the darter did not change after exposure to environmental stressors (WWTP effluent or TCS) and is in contrast to other studies. The gut microbiome of zebrafish fed a pellet diet comprised of 100 μg/g of TCS had an altered relative abundance (decreases in the family *Enterobacteriaceae,* phylum – Proteobacteria), dissimilarities in beta diversity, and a decrease in alpha diversity compared to unexposed fish (Gaulke et al., 2016). Furthermore, the gut microbiome of fathead minnow larvae exposed to waterborne TCS (100 or 1000 ng/L) for 7 days were found to have altered relative abundance, increased alpha diversity, and dissimilarities in beta diversity compared to controls (Narrowe et al., 2015). Interestingly, the Gaulke et al. (2016) and Narrowe et al. (2015) studies differed in route of exposure (diet vs. waterborne) and had different outcomes for alpha diversity; this suggests that dietary exposures affect the fish gut microbiome differently than waterborne exposures. Despite using the same route of TCS exposure and a similar nominal concentration as Narrowe et al. (2015), there were no changes in in relative abundance or alpha diversity in this study. Similarly, there was no change in alpha diversity or relative abundance in WWTP effluent exposed fish in the lab despite previous observations of rainbow darters living downstream of WWTP effluent discharges (Chapter 2) having increased abundance of Proteobacteria and differences in both alpha and beta diversity compared to upstream fish. Additional studies have found altered relative abundance, decreased alpha diversity, and dissimilarities in beta diversity in fish exposed to WWTP effluent in a river and outdoor exposed pond system (Giang et al., 2018, Sakalli et al., 2018). Thus our data is in contrast to most previous studies of TCS and WWTP effluent.

Although there were no effects on the relative abundance and alpha diversity of exposed fish in this study, other studies have shown that aqueous exposure to many environmental contaminants can alter the gut microbiome of fish. Zebrafish larvae exposed to waterborne arsenic resulted in differences in beta diversity between treatments, decreases in alpha diversity (between control, low, and medium exposures), and altered abundance of ASVs (Dahan et al., 2018). Goldfish exposed to waterborne pentachlorophenol (herbicide) had changes in the gut Bacteriodetes:Firmicutes ratio (Kan et al., 2015). Zebrafish exposed to waterborne titanium dioxide and bisphenol A had increased abundance of Bacteriodetes in the gut (Chen et al., 2018). Fathead minnow larvae exposed to waterborne triclosan had altered gut community composition and diversity (Narrowe et al., 2015). Western mosquito fish exposed to a solution of rifampicin (antibiotic) had a decline in gut bacterial diversity and altered communities (Carlson et al., 2015). Finally, zebrafish exposed to sulfamethoxazole and oxytetracycline (antibiotics) in water had distinct gut bacterial communities (Zhou et al., 2018). It is possible that diet may have contributed to the lack of observed effects; most studies that observed changes in the gut microbiome after waterborne chemical exposure were fed a dry diet (Kan et al., 2015, Carlson et al., 2015, Zhou et al., 2018) or a combination of a wet (e.g. brine shrimp, paramecium) and dry foods (e.g. flakes, pellets; Dahan et al., 2018, Chen et al., 2018). Fish fed a wet diet do not drink as much water compared to those fed dry food (Bucking & Wood, 2006), thus a wet diet may result in lower exposure to aqueous environmental stressors. This raises the potential that diet may be confounding in studies with waterborne chemical exposures. Yet, Narrowe et al. (2015) used a wet diet (brine shrimp) and found changes in the gut microbiome after TCS exposure. The potential interaction between diet and contaminant exposure route should be considered when designing future studies.

Limitations and Future Studies

This study observed differences in microbial communities and diversity over the entire period of the experiment and suggests that 14 days of acclimation to a new setting may not be long enough to stabilize the microbiome of wild rainbow darter. However, this finding is confounded by the fact that the fish did not eat the sterile commercial pellets they were initially offered and bloodworms were eventually offered as an alternative. In a study of intentionally starved Asian seabass, fish had increased Bacteriodetes and decreased Proteobacteria and Firmicutes compared to fed controls (Xia et al., 2014). Thus, the bacterial communities of acclimation fish may have been confounded by the initial lack of feeding. Some studies indicate that the gut microbial community of fish can change over time despite a controlled diet and aqueous environment (Dhakal, 2017). Careful attention will be needed to determine the distinct contributions of species and diet on acclimation periods. Future studies that include temporal sampling of fish will provide critical data to better understand the time required to reach a stable microbiome.

There was no effect of aqueous environmental stressors (WWTP or TCS) on the relative abundance or alpha diversity of the fish gut microbiome, but the exact reason for this finding is unclear. We have hypothesized that a wet diet could result in a potentially low exposure of aqueous environmental stressors to the fish gut. Measures of the uptake of waterborne chemicals and exposure to the gut are needed to demonstrate this clearly. Future studies should look at the effects of wet vs. dry diets during aqueous environmental exposures on the gut microbiome.

3.5 Conclusion

Moving wild caught fish into the laboratory altered the microbial composition, alpha diversity, and beta diversity of their gut contents when compared to wild fish, but we did not

observe consistent effects of environmental stressors (TCS or WWTP effluents) on these measures. This study found that the gut microbiome of fish changed between the wild and the lab, and is likely driven by changes in diet. Additionally, the gut microbiome of fish held in the lab between 14 and 42 days changed, which is longer than most laboratory studies suggest for the microbiome to stabilize; this may be because fish were wild-caught rather than lab reared. When designing laboratory studies on the gut microbiome of wild-caught fish, researchers should consider the need for a longer acclimation time to reach a stable microbiome as well as how the type of diet (wet vs. dry) may change exposures to waterborne contaminants.

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Chapter 4: General Conclusion

4.1 Study contributions

The fish gut microbiome is known to vary between individuals, species, diet, and environment. Environmental stressors such as antibiotics, antibacterials, and antimicrobials present in the aquatic ecosystem affect bacterial diversity and composition in the gut. The aim of this Master's thesis was to investigate the effect of wastewater treatment plant (WWTP) effluent on the bacterial composition and diversity of the gut contents of rainbow darter. In wild fish exposed to WWTP effluent the gut microbiome was dominated by Proteobacteria and had increased bacterial diversity, thought to be due to the increased nutrients present in effluents (Chapter 2). Surprisingly, laboratory exposures to environmental stressors, WWTP effluent and triclosan (TCS), in wild-caught rainbow darter had a limited effect on the gut microbiome (Chapter 3). However, the transition to the laboratory environment led to major changes in the gut microbiome, with increases in Firmicutes and decreases in bacterial diversity. These changes in the gut microbiome continued up to 42 days in the laboratory, suggesting that the microbiome changes with time and a longer laboratory acclimation period is necessary to reach a new, stable microbiome.

1. In Chapter 2 of this thesis, wild rainbow darter from 10 sites located varying distances upstream and downstream of two major municipal WWTP outfalls were collected and their gut content was sampled and analysed. Dysbiosis of the gut microbiome was observed with increases in the bacterial phyla Proteobacteria and Cyanobacteria at sites downstream from WWTP outfalls. In combination with decreased measures of Fulton's K, these changes could be linked to altered health outcomes. Additionally, increased alpha diversity was found in fish collected downstream of these WWTPs; the reasons for this shift are unknown but may be due to increased nutrient load in the effluents or to

other point sources (such as agricultural land use) near the sampling sites. These results contrast previous field WWTP effluent exposures, which observed decreases in the gut bacterial diversity of exposed fish (Sakalli et al., 2018, Giang et al., 2018).

2. In Chapter 3, wild fish were collected from a reference site and transported back to an aquatic facility where they were acclimated to the new setting for 14 days before being exposed to an aqueous environmental stressor (WWTP effluent or TCS) for 28 days. In contrast to the literature, there were no consistent effects of these environmental stressors on the fish gut microbiome. There were large changes (increased Firmicutes and decreased diversity) between the gut microbiomes of field and acclimation fish, which were likely driven by the change in environmental surroundings and diet. There were also changes observed between the fish held under the same control conditions between days 14 and 42, suggesting that the microbiome changes with time and an acclimation period greater than 14 days may be necessary to establish a new, stable gut microbiome.

This thesis contributed to a greater understanding of the fish gut microbiome, advances our understanding of the fish gut microbiome after exposure to environmental stressors, and indicated that a transition to new environments, in this case transition from the wild to the laboratory, results in changes in the fish gut microbiome that continue with time.

4.2 Can Laboratory Studies Inform Field Studies?

Laboratory studies have traditionally informed field studies, however they aren't interchangeable and there were major challenges involved in comparing field (Chapter 2) and lab (Chapter 3) study results in this thesis. While there was no major effect of WWTP effluent or TCS on the gut microbiome of laboratory rainbow darter there was an effect in wild-caught fish downstream of WWTP outfalls on the Grand River (Ontario, Canada). There were major

differences across Chapters in this thesis that need consideration; fish were collected in different seasons and years, there were differences in diet between wild and laboratory fish, and there were possible differences in route of exposure to environmental stressors between Chapter 2 and 3.

The fish in this thesis were collected in different seasons and years, which is known to lead to changes in the gut microbiome; field study (Chapter 2) fish were collected in October 2018, while lab study (Chapter 3) fish were collected in June 2019. Season impacts the gut microbiome of fish species with increased bacterial colony forming units (CFU) in autumn compared to summer and winter (Al-Harbi & Uddin, 2004). Farmed salmon had changes in fecal bacterial communities, with increases in the family *Vibrionaceae* in the summer, and clustering of communities on Principal Coordinate Analysis plots based on the month and season of sampling (Zarkasi et al., 2014). Fish gut content collected from REF 2 (Grand River, Ontario, Canada) in fall 2018 had altered bacterial composition and alpha diversity compared to those collected in spring 2019. Fish gut bacterial communities collected in fall 2018 were dominated by Firmicutes and followed by Tenericutes and Proteobacteria (Figure 2.4), while fish gut content collected in spring 2019 were dominant in Proteobacteria, followed by Firmicutes, Cyanobacteria, and Tenericutes (Figure 3.3). The Shannon diversity of fish gut bacteria collected in fall 2018 was lower (median=2.42, Figure 2.8) than those collected in spring 2019 (median=4.34, Figure 3.6). Fish gut contents collected in fall 2018 and spring 2019 from the same site were dissimilar indicating that their microbiomes cannot be easily compared between studies in this thesis.

Diet differed between the field and laboratory study; in the field darters eat insects native to the Grand River, while laboratory held darters were fed bloodworms. Wild-caught fish had a

more diverse gut microbiome, while fish held in the laboratory were dominant in Firmicutes. The effect of diet on the fish gut microbiome was further covered in Chapter 3; briefly, the presence and abundance of bacteria in the gut is largely due to host diet, and therefore it is most likely that differences in diet between the field and lab may have led to changes in the fish gut microbiome between these studies.

Route of exposure coupled with differences in diet may have contributed to the changes, or lack thereof, observed in the gut microbiome. It is possible that fish in the field had increased exposure to environmental stressors through their diet compared to fish in the lab. In the field, rainbow darter eat insects that bioaccumulate some contaminants from the aqueous environment, therefore fish were exposed to environmental stressors through both their insectivorous diet and the aqueous environment. In the lab, rainbow darters were fed bloodworms that were not pre-exposed to environmental stressors. Laboratory studies are crucial in removing confounding variables that exist in the field, such as seasonality and diet, however it is difficult to extrapolate the findings of laboratory studies to the field where season and diet variation is inevitable. In the future, lab studies could determine which route of exposure is most important to the gut microbiome by feeding the rainbow darter field-collected macroinvertebrates from WWTP exposed sites or exposing them to waterborne WWTP effluent.

4.3 Are There Sex Differences in the Fish Gut Microbiota?

This study did not address differences in the gut microbiome that exist between sexes, as female rainbow darter were studied exclusively throughout this thesis. Female fish were selected for both Chapters 2 and 3 to control for possible sex differences in the rainbow darter gut microbiome and to allow for comparison across chapters. However, in working with females it is possible that spawning season could have impacted their gut microbiota. Data from Chapter 3 is

susceptible to the impacts of spawning; fish collection was delayed to the end of spawning, and GSI data supports that this approach was largely successful. Differences in the gut microbiota of male and female mice have been noted (Chi et al., 2016), however several studies indicate that there are no effects of sex on the gut microbiome of fish (zebrafish – Stephens et al., 2015, Roeselers et al., 2011, stickleback – Smith et al., 2015, Arctic charr – Hamilton et al., 2019). Zebrafish spawn asynchronously and continuously, thus it would be difficult to determine the effect of spawning on their gut microbiota. In contrast, threespine stickleback spawn in the summer, thus it is possible that fish collected in the wild from June to July 2012 may have been spawning, but there were no differences in gut microbiota communities between sexes (Smith et al., 2015). Arctic charr were collected in freshwater between December and June and in open-water estuaries between August and September with no changes between male and female gut microbiota (Hamilton et al., 2019). Arctic charr spawn in freshwater in the fall, therefore the spawning season was not captured in the previous study and it is possible that spawning may lead to differences between male and female gut microbiota. Rainbow darter spawn asynchronously from April to June in southern Ontario (Fuzzen, 2016), which was during fish collection in Chapter 3, and females can have multiple clutches of eggs during that time (Fuller, 1998, Heins et al., 1996). Spawning condition of darters in this study was inferred from gonadosomatic index (GSI), and it was determined that some female fish may have been spawning when collected in the field in June 2019, but were no longer spawning while held in the laboratory. Differences in spawning condition of the fish collected in the wild and those transitioned to the lab (Chapter 3) may have contributed to the changes in gut microbiota. Furthermore, changes in gut microbiota between wild fish collected in the fall of 2018 (not-

spawning, Chapter 2) and those collected in spring 2019 (spawning, Chapter 3) may be due in part to spawning, therefore possible sex differences cannot be ruled out.

4.4 Dysbiosis of the Gut Microbiome Might Be Necessary for Host Homeostasis

Dysbiosis of the gut microbiota has been associated with irritable bowel syndrome (Tamboli et al., 2003), colorectal cancer (Sobhani et al., 2011), and other diseases in mammals, but it is also possible that changes in the gut microbial community may be beneficial and help to maintain host homeostasis. For example, rats exposed to the antipsychotic olanzapine and a cocktail of broad spectrum antibiotics had changes in the gut microbiome (decreased Firmicutes, increased Bacteriodetes), but there was no evidence of altered health outcomes typically seen after exposure to pharmaceuticals (no effects on weight gain, uterine fat deposition, or plasma free fatty acid levels) (Davey et al., 2013). Rainbow darter fed a protein or plant-based diet had distinct microbial communities; gut bacteria provide digestive enzymes required for nutrient acquisition and absorption from the diet and therefore compositional changes in the gut microbiome were thought to maintain digestive physiology (Dhakal, 2017). Anadromous Arctic charr had distinct microbial communities based on sampling location (saltwater or freshwater), which may be an effort to maintain physiology in varying salinities (Hamilton et al., 2019). It is more likely that changes in the abundance of specific (but not all) bacteria contributes to adverse health outcomes; bacteria thought to be associated with poor health outcomes can be understood further using functional profiling, metagenomics, metatranscriptomics, or direct manipulation (i.e. fecal transplanting) of the gut microbiome (Adamovsky et al., 2018, Legrand et al., 2020). In order to better understand if dysbiosis in the fish gut microbiome leads to poor health outcomes, independent of maintaining homeostasis, additional data should be collected to provide a greater perspective on fish health. For example, data on oxygen consumption would

indicate metabolic health and measurement of short chain fatty acids produced by gut bacteria would indicate alterations in digestive physiology.

Exposure to environmental stressors are known to alter the fish gut microbiome, with gut bacteria assisting in the biotransformation of many xenobiotics. Several drugs are metabolized by gut bacteria (Patterson & Turnbaugh, 2014, Claus et al., 2016), however little is known about the enzymes involved or the biotransformation processes. It is possible that the presence of certain bacteria in the gut after exposure to xenobiotics may be due in part to their ability to metabolize them. Therefore, specific bacteria working to transform drugs in the fish gut can further indicate exposure to certain environmental stressors and may act as an effective environmental biomonitoring tool (Adamovsky et al., 2018).

4.5 Technical Limitations That Exist in Fish Gut Microbiome Studies

One of the major limitations in comparing fish gut microbiome studies is the lack of standardized approaches; the variation in sampling, laboratory protocols, and analysis makes reproducibility and comparability across studies an issue. For example, sampling location along the gut (foregut, hindgut, gut intestinal tract), and sample choice (gut content, intestinal mucosa) have all been studied. For studies on the gut microbiome, what is the ideal sampling location? In mice and fish both intestinal mucosa and contents have been compared from the colon and the caecum; there was increased bacterial diversity in the contents compared to the mucosa, and both had distinct bacterial communities clustering separately on a Principal Coordinate Analysis (Li et al., 2015, Gajardo et al., 2016). Furthermore, the content collected from the proximal gut clustered separately from the mid and distal gut of Atlantic salmon (Gajardo et al., 2016). Not all fish have distinct regions in their gut (i.e. proximal, mid, and distal) and therefore standardization within fish species is required in order to compare between studies. The location of sampling

results in differences in gut bacterial composition and diversity, therefore it is important to understand the limitations of comparing studies with different sampling methods.

Laboratory protocols such as sample storage and genomic DNA extraction can impact the ability to compare between studies. Immediate processing of samples is better than freezing the sample at -20°C, but if immediate processing is not possible storage in RNAlater was preferred (Larsen et al., 2015). Furthermore, commercial stool kits (for example the QIAamp DNA Stool Mini Kit, Qiagen, CA) were better than tissue kits for DNA extraction. The samples stored in RNAlater and extracted using the stool kit clustered near those sampled fresh using the stool kit, while samples stored in the freezer and collected using the tissue kit did not (Larsen et al., 2015). This indicates that sample storage and extraction can have an impact on the observed bacterial community, indicating a need for standardized methods. In both Chapter 2 and 3 of this thesis fish guts were removed immediately upon euthanasia and stored in buffer (similar to RNAlater) until processing.

There is no best practice for selecting a hypervariable region(s) in fish studies (Table 4.1), although V4 is common (Tarnecki et al., 2017). Use of a mock bacterial community on three primer sets (V4-V5, V1-V2, and V1-V2 degenerate primers) lead to differences in relative abundance and richness (Fouhy et al., 2016). The Earth Microbiome Project has coordinated protocols and analytical methods for studying Earth's microbial communities. In their work, they have standardized DNA extraction and amplify the V4 region of the 16S rRNA gene (Thompson et al., 2017). The Earth Microbiome Project uses a reference-free method Deblur to determine taxonomy, as existing rRNA databases failed to map reads from plant-associated and free-living communities (Thompson et al., 2017). Taxonomy assignment using the Greengenes (version 13.8) and SILVA database (version 123) was compared; the SILVA database, used in this study,

had slightly higher reads mapped and higher alpha diversity metrics (Thompson et al., 2017). Although both Greengenes and SILVA are bias towards clinically relevant bacteria in humans; there was disagreement between databases (Greengenes, RDP, and SILVA) when carrying out taxonomic assignments with only 13% of sequences being similar between databases at the genus level (Pollock et al., 2018, Newton & Roeselers, 2012). So while sequencing 16S rRNA has provided an improved understanding of various biological bacterial communities compared to previously used culture-dependent methods, there are procedural variations that may introduce error or bias in studies. If consistent methods are used within a study or research group, this allows researchers to compare results of their own study accurately, however methods between studies are still not consistent so comparison is difficult and likely inaccurate. There is variation at every step in fish microbiome studies, therefore method standardization is necessary.

Table 4.1 A non-exhaustive list of hypervariable regions used in the analysis of 16S rRNA data in fish gut microbiome studies.

Species	Hypervariable region	Source
Nile tilapia (*Oreochromis niloticus L.*)	V1, V2, V3	Ray et al., 2017
Southern flounder (*Paralichthys lethostigma*)	V1, V2, V3	Bayha et al., 2017
Goldfish (*Carassius auratus*)	V1, V2, V3	Kan et al., 2015
African turquoise killifish (*Nothobranchius furzeri*)	V3, V4	Smith et al., 2017
Fathead minnow (*Pimephales promelas*)	V3, V4	Narrowe et al., 2015
Sea trout (*Salmo trutta trutta*), freshwater salmon (*Salmo salar*)	V3, V4	Skrodentya et al., 2008
Atlantic cod (*Gadus morhua*)	V3, V4, V5	Bagi et al, 2018
Central stonerollers (*Campostoma anomalum*), rainbow darter (*Etheostoma caeruleum*)	V3, V6	Dhakal, 2017
Zebrafish (*Danio rerio*)	V4	Gaulke et al., 2016

4.6 Future Studies

This study contributed to fish gut microbiome research by determining that 1) the fish gut microbiome is impacted by WWTP effluents in the field, 2) fish transitioning to a new environment have altered microbial composition and diversity, and 3) the microbiome of wild fish transitioned to the lab continue to change over time and longer acclimation periods may be required. Surprisingly, this study did not see a strong effect of environmental stressors in the laboratory; I have suggested that this might be due to the lack of fish drinking when fed a wet diet, which could reduce exposures to aqueous contaminants. It will be critical that studies look further at the use of a wet versus dry diet when the effects of contaminant exposures on the fish gut microbiome are being assessed. Researchers in this field should work to standardize fish gut microbiome sampling, storage, gDNA extraction, and hypervariable region and taxonomy database selection, which would aid in making studies reproducible, comparable, and advancing the field as a whole. Finally, further research is required to understand how changes in the gut microbiome of fish impact fish health overall.

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Appendix A

Appendix A contains supplementary information in support of thesis Chapter 2, "Rainbow darter exposed to wastewater effluent in the field have altered gut content microbiome composition and diversity".

Table A.1 Water quality measures from field sites on the Grand River, Ontario, Canada (no available data from Glen Morris (DSK 4) due to inclement weather during sampling).

Site Name (ID)	Relative Location	Date (m/d/y)	Temperature (°C)	Dissolved Oxygen (mg/L)	Conductivity (µS/cm)	Total Dissolved Solids (mg/L)	pH	NH4 (mg/L)	NH3 (mg/L)	Turbidity (NTUs)
Inverhaugh (REF 1)	Upstream of Waterloo WWTP	10/22/18	9.2	12.80	301.8	281.4	8.13	0.04	0.00	3.83
			9.1	12.84	301.5	280.8	8.08	0.05	0.00	3.36
			9.2	12.66	299.9	279.5	8.07	0.04	0.00	2.91
			9.2	12.64	299.4	278.9	8.06	0.04	0.00	2.75
			9.4	13.17	300.8	278.2	8.04	0.04	0.00	3.16
West Montrose (REF 2)	Upstream of Waterloo WWTP	10/22/18	6.3	12.09	280.7	284.1	7.97	0.08	0.00	1.55
			6.3	12.05	280.6	283.4	7.92	0.06	0.00	2.06
			6.5	12.14	281.4	282.8	7.92	0.05	0.00	1.63
			6.7	12.32	282.8	282.8	7.85	0.05	0.00	1.97
			7.0	12.66	287.7	285.4	7.83	0.05	0.00	1.90
Kiwanis (REF 3)	Upstream of Waterloo WWTP	10/23/18	6.9	12.57	512.0	332.8	8.81	0.06	0.00	2.02
			7.0	12.71	511.3	332.2	8.75	0.04	0.00	2.10
			7.0	12.71	511.4	332.8	8.73	0.04	0.00	2.09
			7.0	12.69	510.8	332.2	8.71	0.04	0.00	2.09
			7.0	12.69	511.1	332.2	8.71	0.04	0.00	2.50
EIT (DSW 1)	Downstream Waterloo WWTP	10/23/18	9.3	11.80	689.0	637.0	7.98	0.15	0.00	2.47
			9.3	11.15	668.0	617.5	7.94	0.13	0.00	2.48
			9.1	10.99	640.0	598.0	7.87	0.12	0.00	2.61
			8.8	11.05	615.0	578.5	7.84	0.11	0.00	2.75
			8.6	11.11	584.0	552.5	7.83	0.10	0.00	2.81

Table A.1 Continued

Site Name (ID)	Relative Location	Date (m/d/y)	Temperature (°C)	Dissolved Oxygen (mg/L)	Conductivity (µS/cm)	Total Dissolved Solids (mg/L)	pH	NH4 (mg/L)	NH3 (mg/L)	Turbidity (NTUs)
Fairway (DSW 2)	Downstream Waterloo WWTP	10/24/18	7.0	13.78	453.1	449.8	8.33	0.10	0.00	N/A
			6.8	13.83	444.9	443.3	8.39	0.11	0.00	N/A
			6.7	13.71	436.7	436.2	8.44	0.09	0.00	N/A
			6.6	13.52	431.4	431.6	8.46	0.08	0.00	N/A
			6.6	13.49	425.1	426.4	8.44	0.09	0.00	N/A
Horse Ranch (DSW 3)	Downstream Waterloo WWTP	10/24/18	6.5	12.89	447.5	444.0	8.83	0.06	0.01	1.71
			6.4	12.06	439.3	443.3	8.73	0.06	0.00	1.85
			6.2	11.76	432.8	438.8	8.64	0.05	0.00	2.10
			6.2	11.73	431.8	438.1	8.55	0.05	0.00	1.93
			6.2	11.83	433.0	439.4	8.54	0.05	0.00	2.09
PT1 (DSK 1)	Downstream Kitchener WWTP	10/26/18	8.0	13.56	720.7	468.7	8.48	0.07	0.00	N/A
			8.1	13.49	768.0	505.0	8.42	0.08	0.00	N/A
			8.6	12.92	864.0	559.0	8.36	0.09	0.00	N/A
			8.8	12.85	904.0	585.0	8.33	0.09	0.00	N/A
			8.8	12.91	895.0	585.0	8.34	0.08	0.00	N/A
PT2 (DSK 2)	Downstream Kitchener WWTP	10/26/18	7.2	12.77	885.0	578.5	8.63	0.06	0.00	1.17
			7.2	12.01	875.0	565.5	8.56	0.07	0.00	1.64
			7.3	11.74	873.0	565.5	8.49	0.07	0.00	1.76
			7.3	11.70	871.0	565.5	8.48	0.06	0.00	1.27
			7.3	11.71	871.0	565.5	8.44	0.07	0.00	1.14
Blair (DSK 3)	Downstream Kitchener WWTP	10/26/18	8.2	15.21	836.0	546.0	8.60	0.07	0.00	N/A
			8.2	15.23	837.0	546.0	8.59	0.07	0.00	N/A
			8.1	15.17	834.0	539.5	8.60	0.07	0.00	N/A
			8.1	15.17	828.0	539.5	8.60	0.06	0.00	N/A
			8.2	15.21	836.0	546.0	8.60	0.07	0.00	N/A

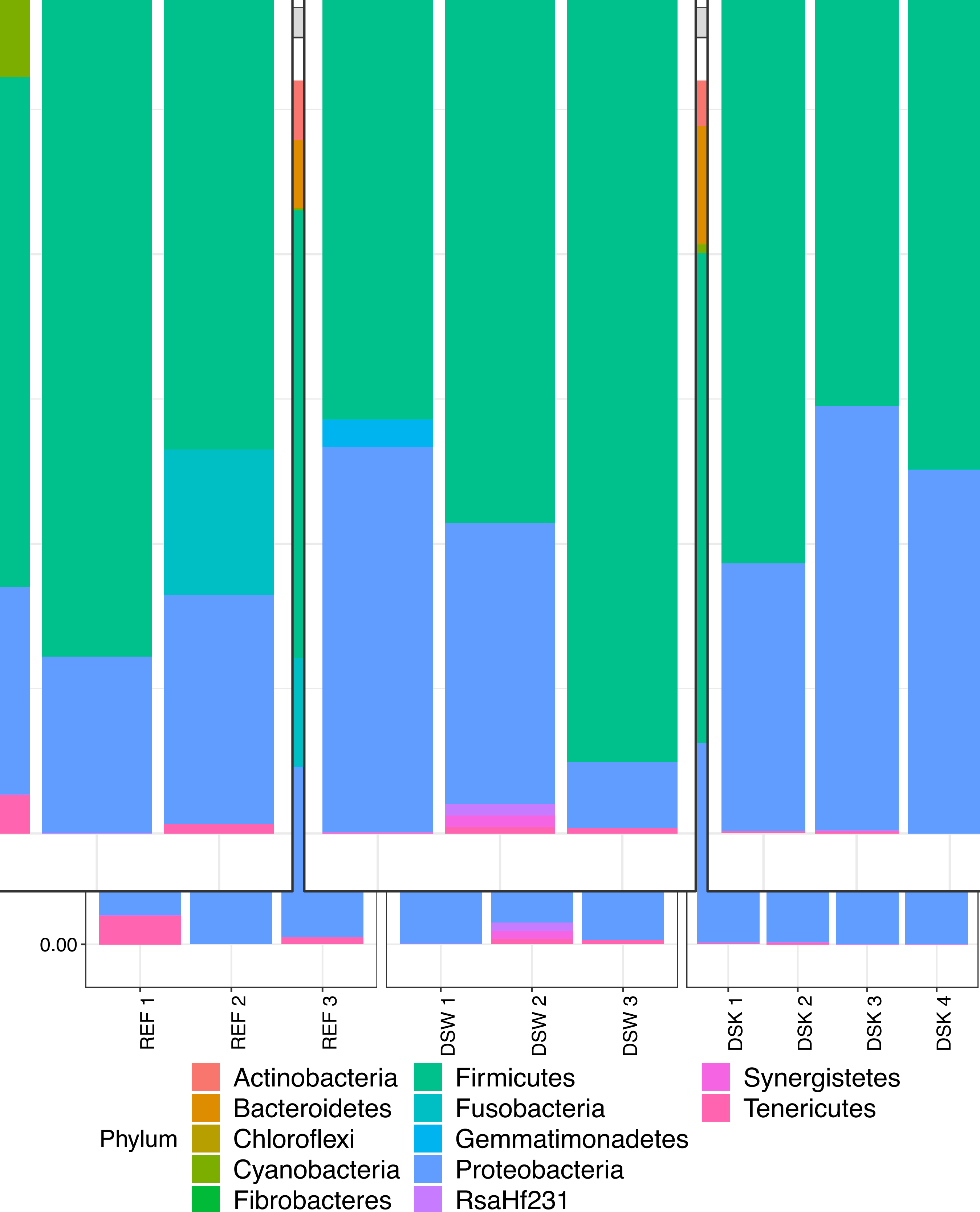


Figure A.1 The mean relative abundance of gut bacteria at the phyla level collected from wash solutions (bleach, ethanol, nanopore water) used to sterilize equipment. Only the phyla with > 2% of the total ASVs were included. The y-axis shows the proportion of ASVs within each included phyla from 0 to 1. The bars are ordered from upstream to downstream of the Waterloo and Kitchener WWTPs in order of flow (upstream – left, downstream - right).

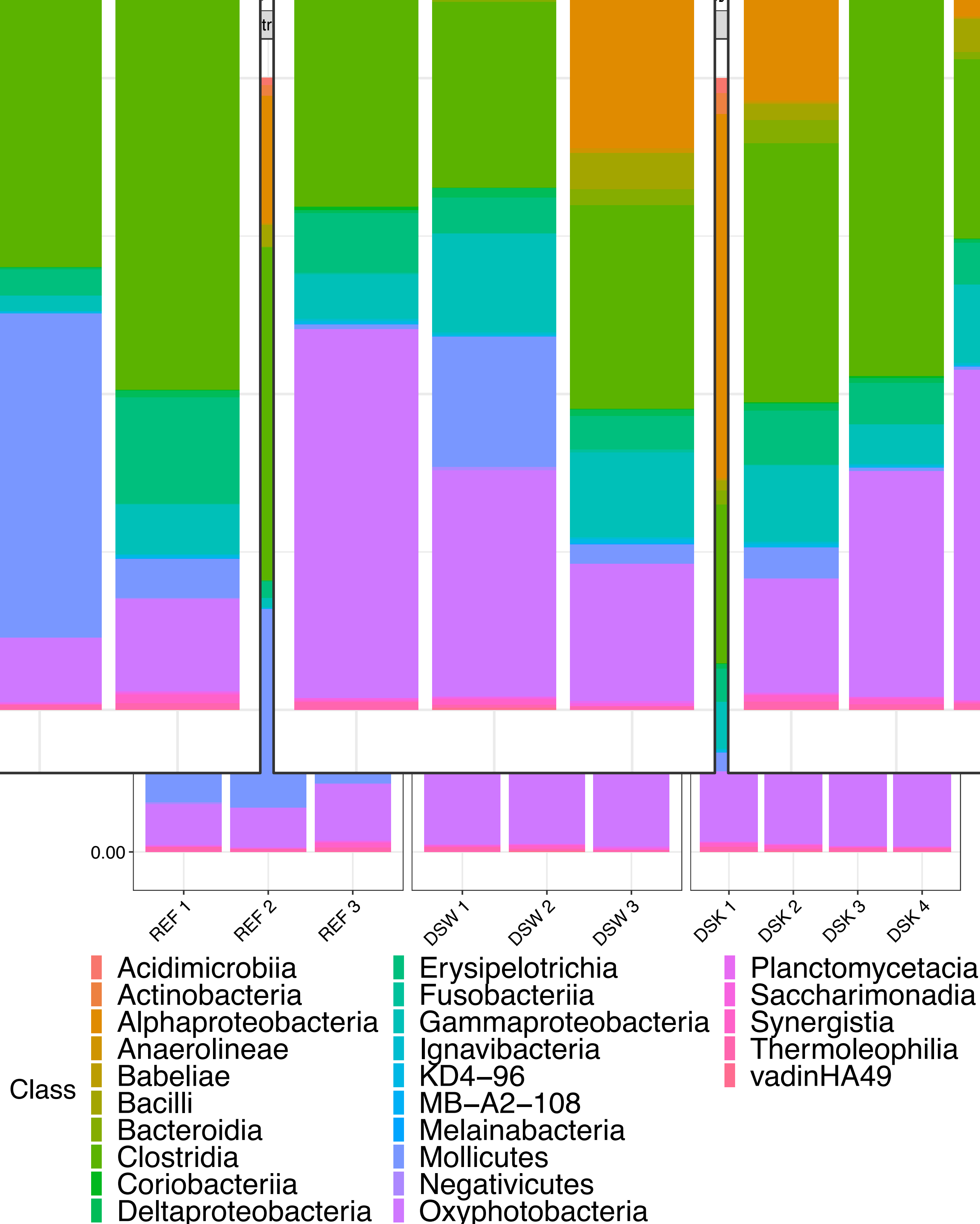


Figure A.2 The mean relative abundance of gut bacteria at the class level in *E. caeruleum*. Only the classes with > 2% of the total ASVs were included. The y-axis shows the proportion of ASVs within each included phyla from 0 to 1. The bars are ordered from upstream to downstream of the Waterloo and Kitchener WWTPs in order of flow (upstream – left, downstream - right).

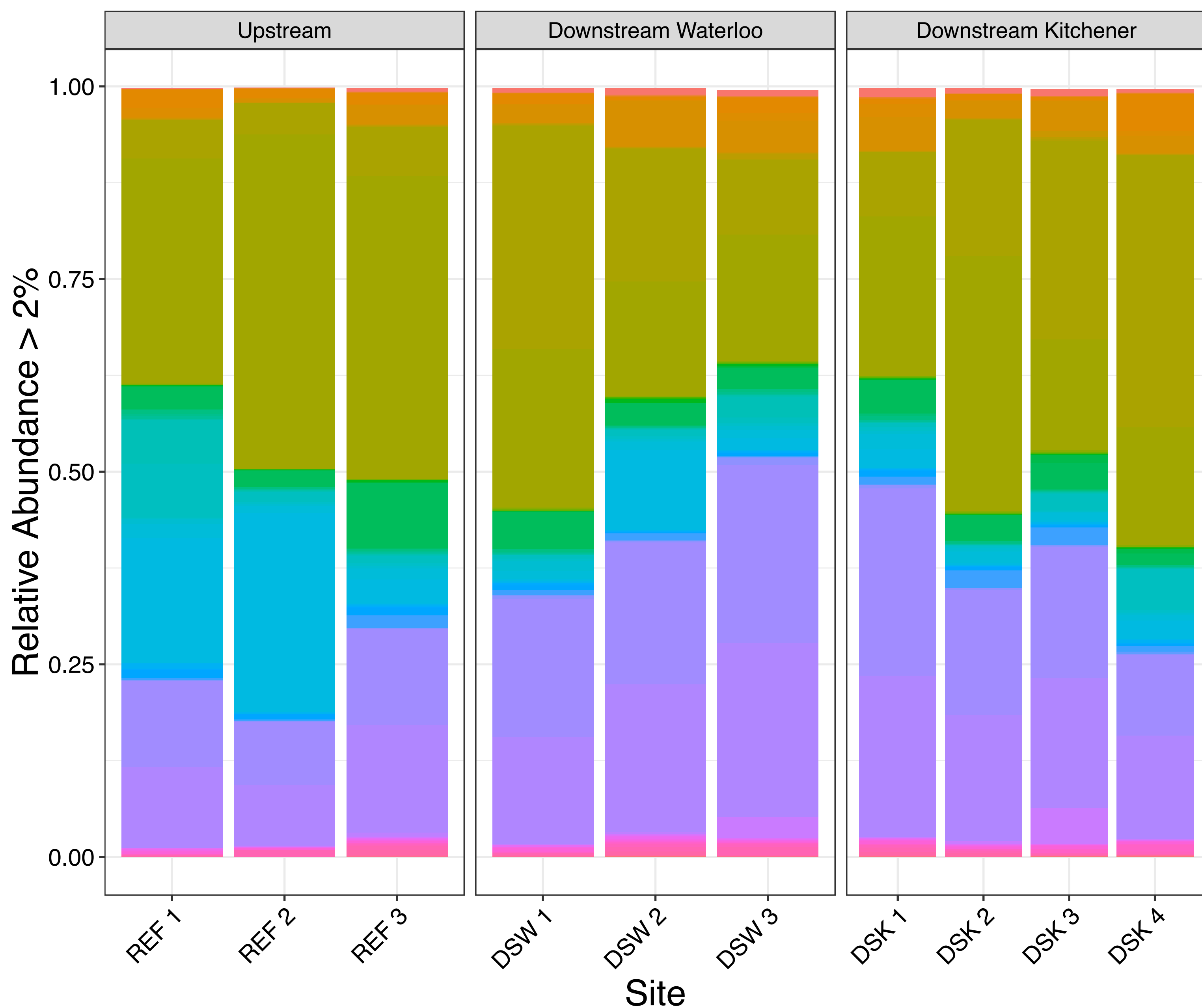


Figure A.3 The mean relative abundance of gut bacteria at the order level in *E. caeruleum*. Only the orders with > 2% of the total ASVs were included. The y-axis shows the proportion of ASVs within each included phyla from 0 to 1. The bars are ordered from upstream to downstream of the Waterloo and Kitchener WWTPs in order of flow (upstream – left, downstream - right).

Order

- Acetobacterales
- Aeromonadales
- Anaerolineales
- Arenicellales
- Babeliales
- Bacillales
- Bacteroidales
- Betaproteobacteriales
- Bifidobacteriales
- Caedibacterales
- Caldilineales
- Cardiobacteriales
- Chitinophagales
- Chloroplast
- Clostridiales
- Competibacterales
- Corynebacteriales
- Coxiellales
- Cytophagales
- Desulfarculales
- Desulfobacterales
- Desulfovibrionales
- Diplorickettsiales
- EC3
- Enterobacteriales
- Erysipelotrichales
- Flavobacteriales
- Francisellales
- Frankiales
- Fusobacteriales
- Gaiellales
- Gammaproteobacteria_Incertae_Sedis
- Gemmatales
- Holosporales
- Lactobacillales
- Legionellales
- Micrococcales
- Microtrichales
- Mycoplasmatales
- Myxococcales
- Nostocales
- Oceanospirillales
- Oligoflexales
- OPB41
- PeM15
- Propionibacteriales
- Pseudomonadales
- RBG−13−54−9
- Reyranellales
- Rhizobiales
- Rhodobacterales
- Rhodospirillales
- Rickettsiales
- Rs−K70_termite_group
- Run−SP154
- Saccharimonadales
- Selenomonadales
- SJA−15
- SJA−28
- Solirubrobacterales
- Sphingobacteriales
- Sphingomonadales
- Steroidobacterales
- Synechococcales
- Synergistales
- Syntrophobacterales
- SZB30
- Tistrellales
- Xanthomonadales

Figure A.3 Continued

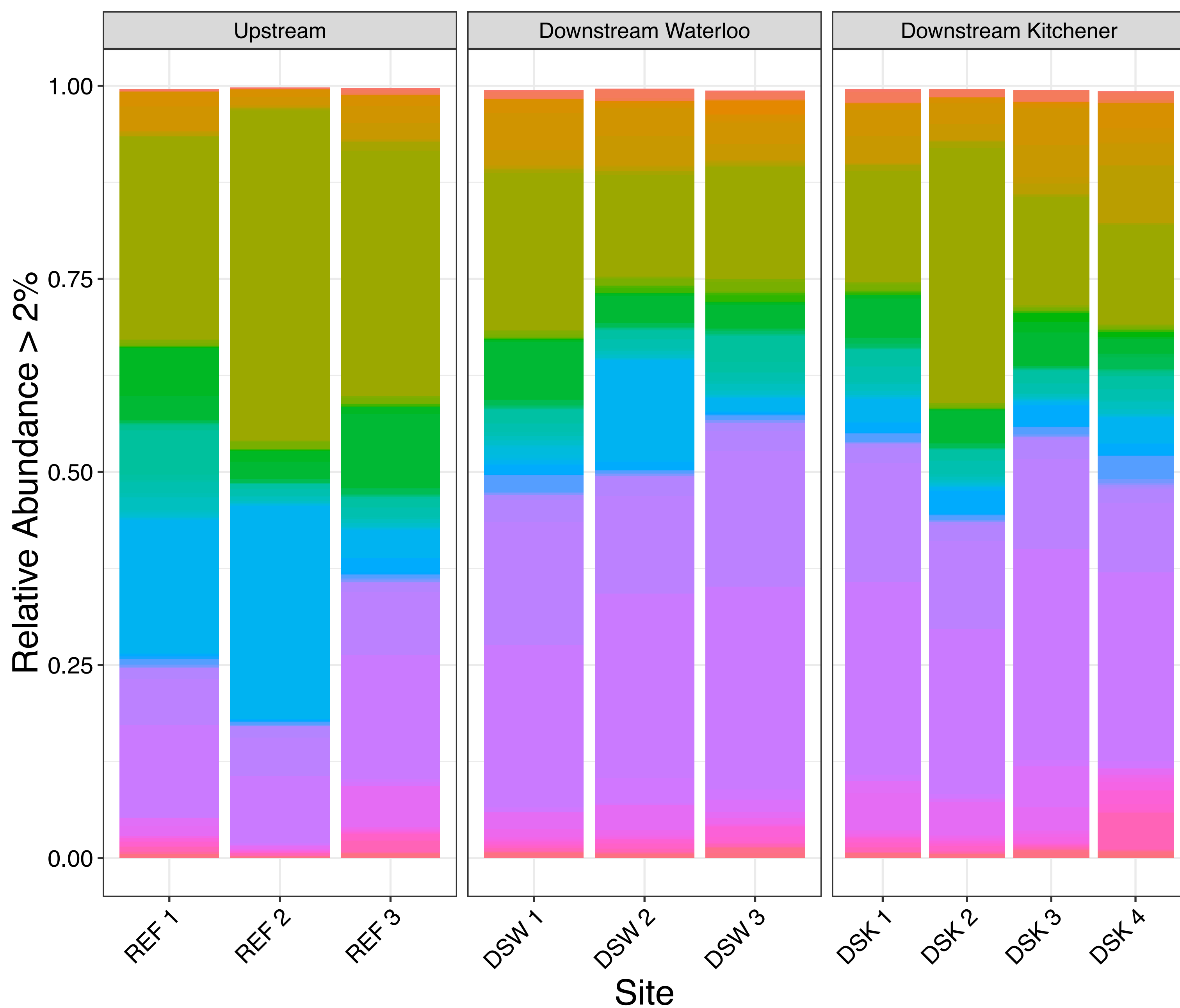


Figure A.4 The mean relative abundance of gut bacteria at the family level in *E. caeruleum*. Only the families with > 2% of the total ASVs were included. The y-axis shows the proportion of ASVs within each included phyla from 0 to 1. The bars are ordered from upstream to downstream of the Waterloo and Kitchener WWTPs in order of flow (upstream – left, downstream - right).

Family

0319−6G20
67−14
Acetobacteraceae
Acidaminococcaceae
Aeromonadaceae
Alicyclobacillaceae
Amoebophilaceae
Anaerolineaceae
Anaplasmataceae
Arenicellaceae
B1−7BS
Babeliaceae
Bacillaceae
Bacteroidaceae
Beijerinckiaceae
Bifidobacteriaceae
Burkholderiaceae
Caedibacteraceae
Caldilineaceae
Carnobacteriaceae
Chitinibacteraceae
Chitinimonadaceae
Chitinophagaceae
Christensenellaceae
Chromobacteriaceae
Clostridiaceae_1
Competibacteraceae
Corynebacteriaceae
Coxiellaceae
Cryptosporangiaceae
Cyanobiaceae
Desulfarculaceae
Desulfobacteraceae
Desulfobulbaceae
Desulfovibrionaceae
Diplorickettsiaceae
Dysgonomonadaceae
Enterobacteriaceae
Enterococcaceae
Erysipelotrichaceae
Family_XI
Family_XII
Family_XIII
Flavobacteriaceae
Francisellaceae
Fusobacteriaceae
Gaiellaceae
Geminicoccaceae
Gemmataceae
Halieaceae
Halomonadaceae
Heliobacteriaceae
Holosporaceae
Hyphomicrobiaceae
Iamiaceae
Ilumatobacteraceae
Intrasporangiaceae
JG30−KF−CM45
Lachnospiraceae
Lactobacillaceae
Legionellaceae
Leptotrichiaceae
Methyloligellaceae
Microbacteriaceae
Micrococcales_Incertae_Sedis
Microcystaceae
Microtrichaceae
Midichloriaceae
Mycobacteriaceae
Mycoplasmataceae
Neisseriaceae
Nitrosomonadaceae
Nocardiaceae
Nocardioidaceae
Nostocaceae
Oligoflexaceae
Paenibacillaceae
Peptococcaceae
Peptostreptococcaceae
Phormidiaceae
Planococcaceae
Pleomorphomonadaceae
Prevotellaceae
Propionibacteriaceae
Pseudomonadaceae
Reyranellaceae
Rhizobiaceae
Rhizobiales_Incertae_Sedis
Rhodanobacteraceae
Rhodobacteraceae
Rhodocyclaceae
Rhodospirillaceae
Rickettsiaceae
Rikenellaceae
Ruminococcaceae
Saprospiraceae
SC−I−84
Solirubrobacteraceae
Sphingomonadaceae
Spirosomaceae
Sporichthyaceae
Staphylococcaceae
Steroidobacteraceae
Streptococcaceae
Synergistaceae
Syntrophaceae
Syntrophobacteraceae
TC1
Thermoactinomycetaceae
Tsukamurellaceae
Unknown_Family
Veillonellaceae
Vermiphilaceae
Woeseiaceae
Wohlfahrtiimonadaceae
Xanthobacteraceae
Xanthomonadaceae
Xenococcaceae

Figure A.4 Continued

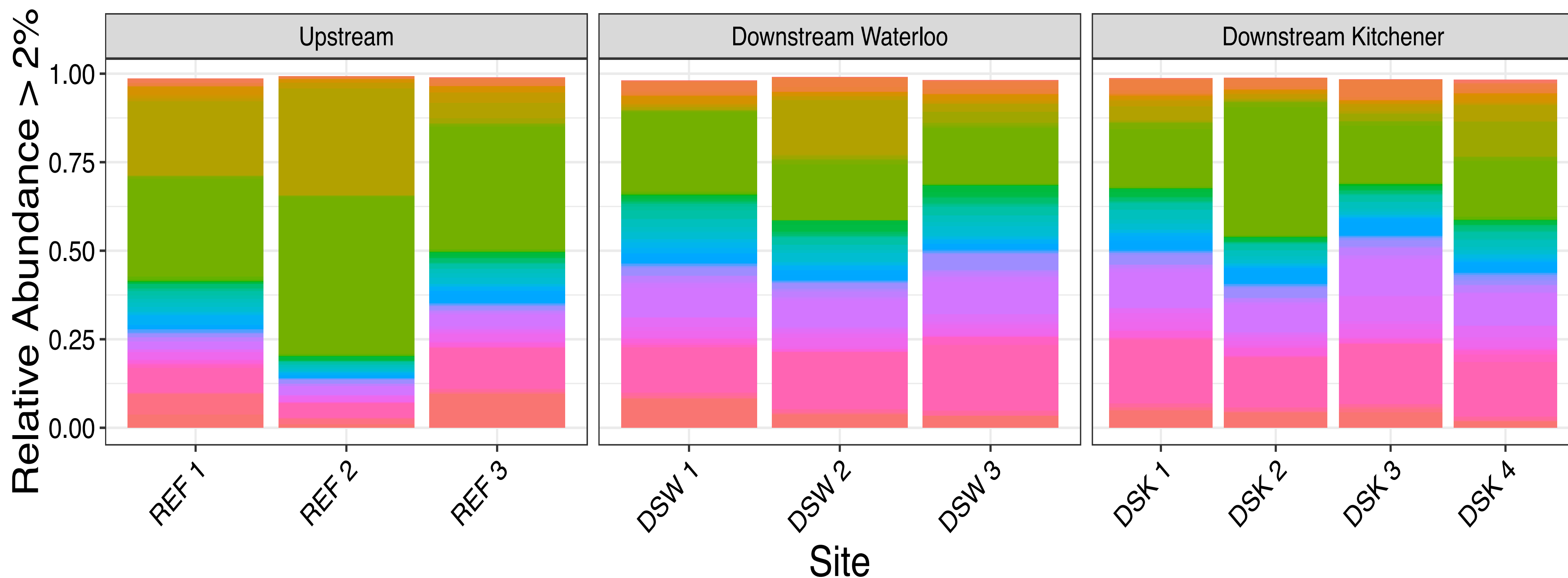


Figure A.5 The mean relative abundance of gut bacteria at the genus level in *E. caeruleum*. Only the genera with > 2% of the total ASVs were included. The y-axis shows the proportion of ASVs within each included phyla from 0 to 1. The bars are ordered from upstream to downstream of the Waterloo and Kitchener WWTPs in order of flow (upstream – left, downstream - right).

Genus

966–1
Achromobacter
Aeromonas
Afipia
Agathobacter
Alistipes
Alloprevotella
Allorhizobium–Neorhizobium–Pararhizobium–Rhizobium
alphaI_cluster
Alsobacter
Amaricoccus
Aminobacter
Anaerocella
Anaerofilum
Anaerosporobacter
Anaerostipes
Anaerovorax
Aquicella
Arenimonas
Asinibacterium
Aurantimicrobium
Aureimonas
Bacillus
Bacteroides
Bauldia
Bifidobacterium
Blautia
Bosea
Bradyrhizobium
Brevibacillus
Breznakia
Butyricicoccus
Caedibacter
Candidatus_Accumulibacter
Candidatus_Alysiosphaera
Candidatus_Amoebophilus
Candidatus_Bacilloplasma
Candidatus_Berkiella
Candidatus_Competibacter
Candidatus_Methylopumilus
Candidatus_Ovatusbacter
Candidatus_Soleaferrea
Candidatus_Xenohaliotis
Carnobacterium
Catabacter
Catellicoccus
Cellulosilyticum
Cetobacterium
Chitinimonas
Christensenellaceae_R–7_group
Chthonobacter
CL500–29_marine_group
Clostridioides
Clostridium_sensu_stricto_1
Clostridium_sensu_stricto_11
Clostridium_sensu_stricto_12
Clostridium_sensu_stricto_13
Clostridium_sensu_stricto_2
Clostridium_sensu_stricto_3
Clostridium_sensu_stricto_5
Clostridium_sensu_stricto_6
Clostridium_sensu_stricto_8
Clostridium_sensu_stricto_9
Conexibacter
Coprococcus_2
Corynebacterium_1
Coxiella
Crenobacter
Cryobacterium
Cyanobium_PCC–6307
Dechloromonas
Deefgea
Defluviimonas
Desulfobacca
Desulforhabdus
Desulfosporosinus
Desulfovibrio
Dorea
Dysgonomonas
Enterococcus
Epulopiscium
Erysipelotrichaceae_UCG–003
Escherichia/Shigella
Exiguobacterium
Faecalibacterium
Fictibacillus
Flavobacterium
Fodinicola
Francisella
Fretibacterium
FukuN57
Fusobacterium
Gaiella
Gemmobacter
Gordonia
Granulicatella
Halomonas
Herbinix
hgcI_clade
Hydrogenispora
Hyphomicrobium
Iamia
Ignatzschineria
Ilumatobacter
IMCC26207
Intestinibacter
Janthinobacterium
Ketogulonicigenium
Koukoulia
Labrys
Lachnospiraceae_UCG–010
Lactobacillus
Lactococcus
Leadbetterella
Legionella
Leptothrix
Leptotrichia
Leucobacter
Luteimicrobium
Lysinibacillus
MD3–55
Mesorhizobium
Methylobacterium
Methylocystis
Methylovirgula
Microvirga
Mucinivorans
Mycobacterium
Natranaerovirga
Nitratireductor
Nocardioides
Nodosilinea_PCC–7104
Nordella
Novosphingobium
OM60(NOR5)_clade
Ornithinibacter
Paenibacillus
Paeniclostridium
Paenisporosarcina
Paraclostridium
Paracoccus
Parvimonas
Patulibacter
Pedomicrobium
Peptoanaerobacter
Phreatobacter
Planktothrix_NIVA–CYA_15
Pleurocapsa_PCC–7319
Polymorphobacter
Polynucleobacter
Propionivibrio
Pseudomonas
Pseudorhodobacter
Pseudorhodoplanes
Pygmaiobacter
Qingshengfania
Reyranella
Rhodobacter
Rhodoferax
Rhodospirillum
Rhodovastum
Rickettsia
Rickettsiella
Romboutsia
Roseococcus
Roseomonas
Rs–D38_termite_group
Rubellimicrobium
Rubrivivax
Ruminiclostridium_1
Ruminococcaceae_UCG–013
Ruminococcaceae_UCG–014
Ruminococcus_1
Ruminococcus_2
Saccharofermentans
Salmonella
Sandaracinobacter
Snowella_0TU37S04
Sphingomonas
Sphingorhabdus
Sporichthya
Sporosarcina
Staphylococcus
Streptococcus
Subdoligranulum
Sva0081_sediment_group
Synechocystis_PCC–6803
Tabrizicola
Telmatospirillum
Terrisporobacter
Thermoactinomyces
Thioclava
Trichococcus
Tropicimonas
Tsukamurella
Tumebacillus
Turicibacter
Tychonema_CCAP_1459–11B
Tyzzerella_3
UBA1819
Vagococcus
Veillonella
Woeseia
Yonghaparkia
ZOR0006

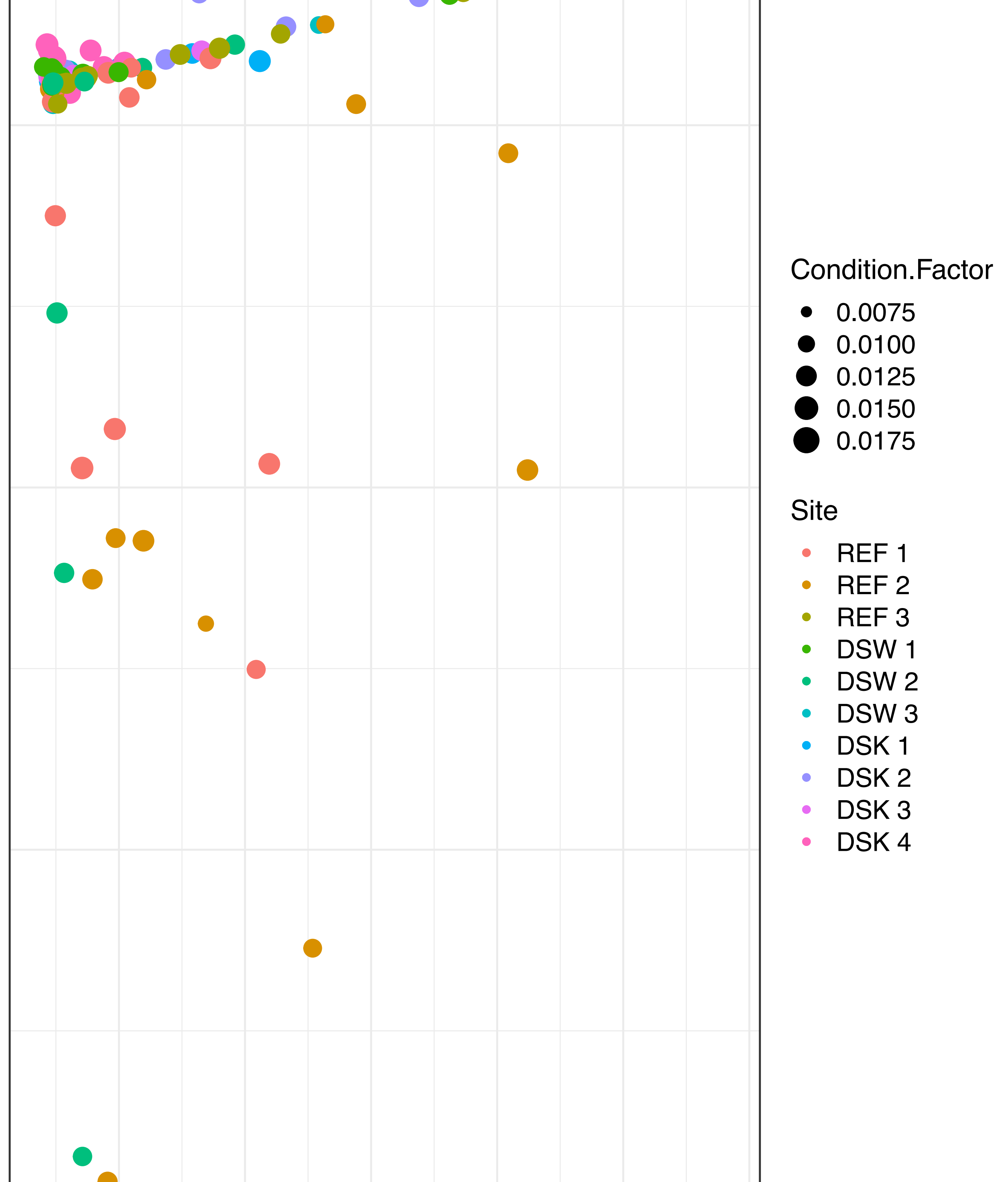
Figure A.5 Continued

Table A.2 Pairwise Adonis PERMANOVA calculated between sampled sites.

Site	Site	F statistic	P value
REF 1	REF 2	1.5196	0.125
	REF 3	1.6679	0.06
	DSW 1	3.2818	0.001
	DSW 2	3.1401	0.001
	DSW 3	4.1205	0.001
	DSK 1	3.086	0.001
	DSK 2	2.1791	0.007
	DSK 3	3.9511	0.001
	DSK 4	4.1487	0.001
REF 2	REF 3	2.9152	0.005
	DSW 1	5.2443	0.001
	DSW 2	5.0589	0.001
	DSW 3	6.5379	0.001
	DSK 1	5.2674	0.001
	DSK 2	3.4711	0.001
	DSK 3	6.5251	0.001
	DSK 4	6.3254	0.001
REF 3	DSW 1	2.3913	0.013
	DSW 2	2.5789	0.004
	DSW 3	3.6556	0.002
	DSK 1	1.8323	0.03
	DSK 2	1.0185	0.39
	DSK 3	2.5634	0.003
	DSK 4	3.635	0.001
DSW 1	DSW 2	1.9159	0.008
	DSW 3	3.1043	0.001
	DSK 1	2.534	0.001
	DSK 2	1.3865	0.138
	DSK 3	1.4576	0.081
	DSK 4	1.7969	0.018
DSW 2	DSW 3	2.4882	0.001
	DSK 1	1.9786	0.004
	DSK 2	1.8579	0.018
	DSK 3	1.3827	0.096
	DSK 4	2.9501	0.001
DSW 3	DSK 1	2.0243	0.006
	DSK 2	2.7314	0.005
	DSK 3	2.9925	0.001
	DSK 4	3.6787	0.002
DSK 1	DSK 2	1.2398	0.159
	DSK 3	1.8812	0.017
	DSK 4	3.2392	0.001

Table A.2 continued

Site	Site	F statistic	P value
DSK 2	DSK 3	1.2417	0.202
	DSK 4	2.0883	0.009
DSK 3	DSK 4	1.774	0.017



caeruleum to sample collection site (point colour) upstream and downstream of the Waterloo and Kitchener WWTP.

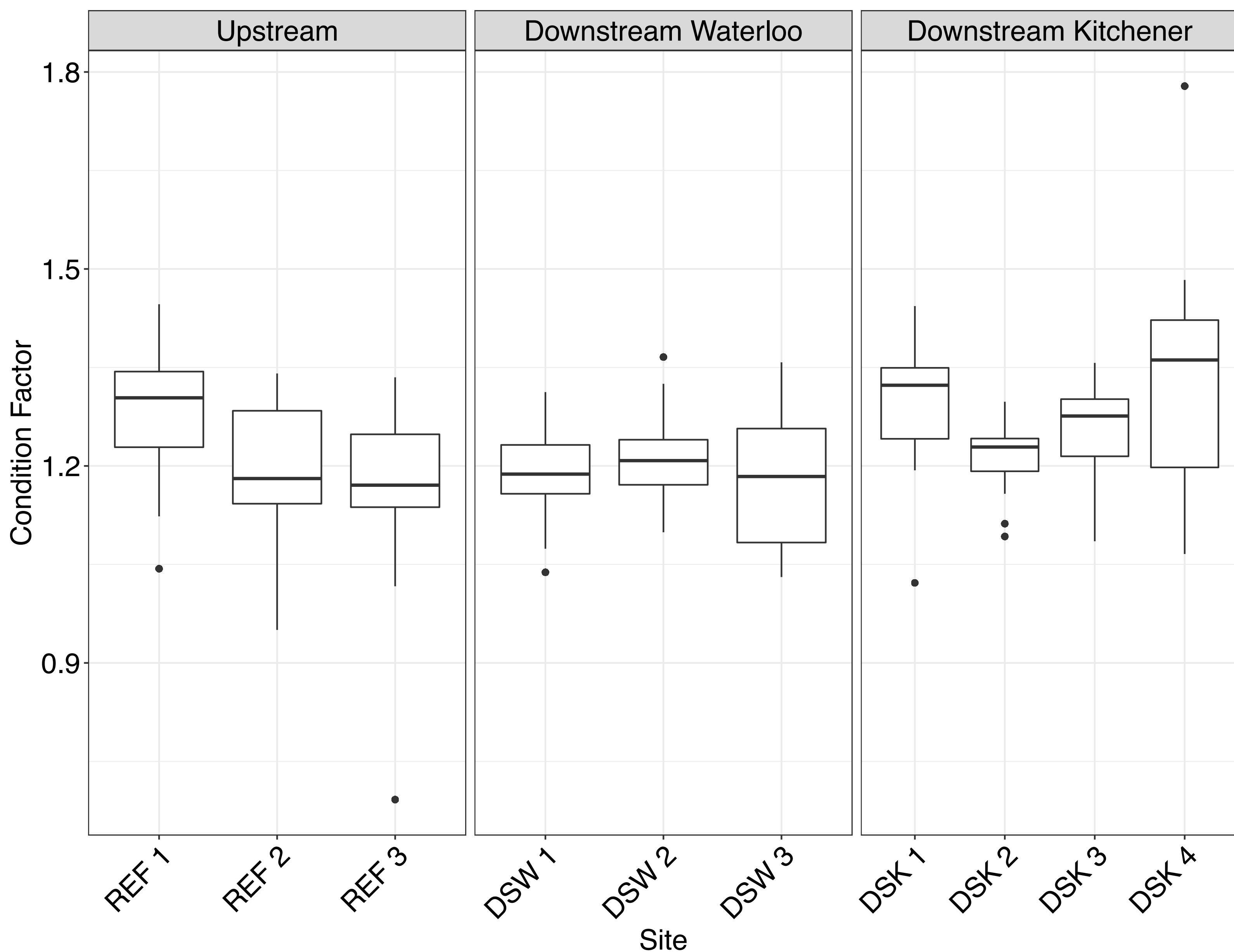


Fig. A.7 Condition factor (K) of female rainbow darter collected from upstream and downstream of the Waterloo and Kitchener WWTP in order of flow (upstream – left to downstream - right).

Appendix B

Appendix B contains supplementary information in support of thesis Chapter 3, "The gut microbiome of wild rainbow darter is altered in the lab".

Table B.1 Individual tank water quality measures by treatment group. Acclimation tanks were from days 1-14 in the laboratory; all other treatment groups were from days 1-42 in the laboratory. Acclimation and control were dechlorinated municipal water; exposure groups included dechlorinated municipal water and either wastewater treatment plant effluent (10%, 20%, or 40% WWTP effluent) or triclosan (100 ng/L; TCS). DO, pH, and temperature were measured using a YSI 556 multi-parameter meter. Nitrite, nitrate, carbonate hardness (KH), general hardness (GH), and ammonia were measured using a test strip (API/Mars Fishcare, Pennsylvania). Reported measures are calculated means ± standard deviation (minimum-maximum values).

Tank	Treatment (number of measurements)	DO (%)	pH	Temperature (°C)	Nitrite (ppm)*	Nitrate (ppm)*	KH (ppm)*	GH (ppm)*	Ammonia (ppm)*
1	Acclimation (n=14)	83.4 ± 4.7 (71.1-92.0)	7.1 (6.6-7.5)	19.3 ± 0.4 (18.4-19.7)	0	0	113.8 ± 15.0 (80-120)	170.8 ± 33.3 (60-180)	0.5 ± 0.4 (0-1)
2		88.7 ± 3.0 (84.6-94.2)	7.1 ± 0.2 (6.6-7.5)	19.3 ± 0.5 (18.4-19.8)	0	0	110.8 ± 17.5 (80-120)	175.4 ±16.6 (120-180)	0.7 ± 0.8 (0-3)
7		86.8 ± 3.5 (81.3-93.0)	7.2 ± 0.3 (6.8-7.8)	20.0 ± 2.8 (18.3-29.6)	0	0	113.8 ± 15.0 (80-120)	175.4 ± 16.6 (120-180)	0.5 ± 0.4 (0-1)
13	Control (n=42)	93.6 ± 2.9 (83.9-100.8)	7.3 ± 0.3 (6.4-7.9)	18.1 ± 0.7 (16.8-19.4)	0.02 ± 0.1 (0-0.5)	0.98 ± 4.4 (0-20)	112.2 ± 16.0 (80-120)	171.2 ± 21.5 (120-180)	0.2 ± 0.4 (0-1)
14		94.2 ± 4.8 (68.9-100.9)	7.3 ± 0.3 (6.4 – 7.8)	18.0 ± 0.7 (16.65-19.37)	0.01 ± 0.08 (0-0.5)	0.5 ± 3.1 (0-20)	110.2 ± 17.4 (80-120)	168.3 ± 24.1 (120-180)	0.2 ± 0.2 (0-1)
16		94.2 ± 2.6 (88.6-100.0)	7.3 ± 0.3 (6.5 – 7.9)	18.0 ± 0.7 (16.9-19.3)	0	0.5 ± 3.1 (0-20)	112.2 ± 16.0 (80-120)	169.8 ± 22.9 (120-180)	0.1 ± 0.3 (0-1)

Table B.1 Continued

Tank	Treatment (number of measurements)	DO (%)	pH	Temperature (°C)	Nitrite (ppm)*	Nitrate (ppm)*	KH (ppm)*	GH (ppm)*	Ammonia (ppm)*
15	10% WWTP Effluent (n=42)	95.0 ± 2.5 (90.2-101.7)	7.3 ± 0.3 (6.5-7.7)	18.2 ± 0.7 (17.1-19.5)	0	4.39 ± 14.5 (0-80)	112.2 ± 16 (80-120)	178.5 ± 9.4 (120-180)	0.2 ± 0.3 (0-1)
17		89.3 ± 3.6 (83.2-97.5)	7.3 ± 0.3 (6.4-7.7)	18.2 ± 0.8 (17.0 -19.7)	0	3.9 ± 10.2 (0-40)	111.7 ± 20.5 (80-180)	178.5 ± 9.4 (120-180)	0.2 ± 0.3 (0-1)
18		93.1 ± 2.6 (88.0-98.6)	7.3 ± 0.3 (6.5-7.7)	18.4 ± 0.7 (17.1-19.7)	0	3.4 ± 9.9 (0-40)	113.1 ± 15.2 (80-120)	177.1 ± 13 (80-120)	0.2 ± 0.3 (0-1)
3	20% WWTP Effluent (n=42)	92.8 ± 4.1 (80.0-99.9)	7.3 ± 0.3 (6.5-7.8)	18.9 ± 0.6 (17.3-19.6)	0.1 ± 0.3 (0-1)	13.7 ± 15.1 (0-40)	114.1 ± 14.3 (80-120)	175.6 ± 15.8 (120-180)	0.4 ± 0.7 (0-3)
8		85.3 ± 5.7 (65.0-93.3)	7.2 ± 0.2 (6.7-7.7)	19.3 ± 0.6 (17.7-20.1)	0	11.2 ± 16.8 (0-80)	115.1 ± 13.3 (80-120)	178.5 ± 9.4 (120-180)	0.2 ± 0.3 (0-1)
9		92.5 ± 2.5 (88.6-97.0)	7.3 ± 0.3 (6.7-7.7)	19.0 ± 0.6 (17.4-19.7)	0.02 ± 0.1 (0-0.5)	12.2 ±14.7 (0-40)	114.1 ± 14.3 (80-120)	177.1 ± 13.1 (120-180)	0.2 ± 0.3 (0-1)
6	40% WWTP Effluent (n=42)	90.4 ± 2.8 (84.7-95.4)	7.4 ± 0.3 (6.9-7.9)	19.04 ± 0.9 (17.0-19.9)	0.01 ± 0.08 (0-0.5)	19.5 ± 18.7 (0-80)	112.2 ± 18.4 (40-120)	178.5 ± 9.4 (120-180)	0.4 ± 0.7 (0-3)
11		87.1 ± 4.5 (77.7-94.8)	7.3 ± 0.22 (6.8-7.9)	19.03 ± 0.8 (17.3-19.9)	0.01 ± 0.08 (0-0.5)	20 ± 21.9 (0-80)	113.2 ± 15.2 (80-120)	174.1 ± 18.0 (120-180)	0.2 ± 0.3 (0-1)
12		87.2 ± 4.4 (75.1-96.5)	7.4 ± 0.2 (6.8-7.9)	19.2 ± 0.8 (17.3-20.1)	0	19.0 ± 20.0 (0-80)	115.1 ± 13.3 (80-120)	174.1 ± 18.0 (120-180)	0.3 ± 0.5 (0-3)

Table B.1 Continued

Tank	Treatment (number of measurements)	DO (%)	pH	Temperature (°C)	Nitrite (ppm)*	Nitrate (ppm)*	KH (ppm)*	GH (ppm)*	Ammonia (ppm)*
4	TCS (n=42)	91.0 ± 3.9 (84.6-100.8)	7.4 ± 0.3 (6.78-7.83)	19.0 ± 0.7 (17.1-19.8)	0	0.5 ± 3.1 (0-20)	105.4 ± 19.5 (80-120)	165.4 ± 26.1 (120-180)	0.2 ± 0.3 (0-1)
5		93.3 ± 3.3 (83.6-99.0)	7.4 ± 0.2 (6.7-7.8)	18.7 ± 0.7 (17.0-19.5)	0	0.5 ± 3.1 (0-20)	106.3 ± 19.2 (80-120)	171.2 ± 21.5 (120-180)	0.2 ± 0.3 (0-1)
10		91.1 ± 2.7 (85.7-96.9)	7.4 ± 0.3 (6.8-7.8)	19.0 ± 0.7 (17.2-19.8)	0	0.5 ± 3.1 (0-20)	108.3 ± 18.4 (80-120)	168.3 ± 24.1 (120-180)	0.2 ± 0.3 (0-1)

*Test strips provided discrete measures of water quality. Nitrite: 0, 0.5, 1, 3, 5, 10 ppm; Nitrate: 0, 20, 40, 80, 160, 200 ppm; KH: 0, 40, 80, 120, 180, 240 ppm; GH: 0, 30, 60, 120, 180 ppm; Ammonia: 0, 0.5, 1, 3, 6 ppm.

Table B.2 Randomly assigned collection of tank water for microbial and pharmaceutical analysis.

Date	Week	Tank	Treatment
July 4, 2019	1	6	40% WWTP Effluent
		12	40% WWTP Effluent
July 7, 2019	1	14	Control
		15	10% WWTP Effluent
July 11, 2019	2	17	10% WWTP Effluent
		18	10% WWTP Effluent
July 14, 2019	2	8	20% WWTP Effluent
		5	100 ng/L Triclosan
July 18, 2019	3	6	40% WWTP Effluent
		14	Control
July 21, 2019	3	16	Control
		4	100 ng/L Triclosan
July 25, 2019	4	16	Control
		3	20% WWTP Effluent
July 28, 2019	4	9	20% WWTP Effluent
		12	40% WWTP Effluent

Table B.3 The F statistic and adjusted P value for pairwise Adonis PERMANOVA calculated between treatments.

Treatment	Treatment	F statistic	P value
Field	Acclimation	11.765	0.001
	Control	16.194	0.001
	10%	21.071	0.001
	20%	13.793	0.001
	40%	15.652	0.001
	TCS	18.635	0.001
Acclimation	Control	6.589	0.001
	10%	9.2793	0.001
	20%	6.24	0.001
	40%	6.8795	0.001
	TCS	8.6634	0.001
Control	10%	1.9595	0.01
	20%	1.2961	0.157
	40%	1.2343	0.191
	TCS	1.8695	0.017
10%	20%	2.416	0.001
	40%	1.2768	0.153
	TCS	1.7127	0.023
20%	40%	0.82524	0.677
	TCS	1.6913	0.025
40%	TCS	1.356	0.111