INVESTIGATING THE POTENTIAL EFFECTS OF POLLUTANTS ON THE RAINBOW TROUT (*ONCORHYNCHUS MYKISS*) GASTROINTESTINAL MICROBIOME

A Thesis Submitted to the College of
Graduate and Postdoctoral Studies
In Partial Fulfillment of the Requirements
For the Degree of Master of Science
In the Toxicology Graduate Program
University of Saskatchewan
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ABSTRACT

The gastrointestinal (GI) microbiome is an important aspect of organismal health with the ability to affect host nutrition, metabolic processes, and immune system development. Changes to the GI microbiome can affect any of these or other host functions and could result in negative effects on overall fish health. While it is known that low concentrations of pollutants can affect environmental microbes, little information is known about the effects of environmental contaminants on GI microbiota in fish. I conducted two studies to explore the potential effects of chemicals on the GI microbiome of fish. The pilot study aimed to develop a method to extract and analyse the microbiota within the GI tract of fish. It also compared the microbiomes of two phylogenetically distant fish species, lake sturgeon (*Acipenser fulvescens*) and rainbow trout (*Oncorhynchus mykiss*), under controlled conditions. The second study aimed to discover the effects of two common environmental contaminants, benzo(a)pyrene (BaP) and triclosan, on the GI microbiome in rainbow trout. In both experiments, DNA was extracted from intestinal contents of fish and GI microbiota were evaluated using next-generation sequencing techniques and downstream applications. The pilot study suggested GI microbial communities were more diverse in rainbow trout and were significantly different from those communities in lake sturgeon. Due to the similarities in rearing conditions and diet in the laboratory, microbial differences between the two species may indicate evolutionary differences. In the second study, the GI microbiomes of rainbow trout were compared after exposure to four different diets (solvent control, 5.09 mg/kg BaP, 40.7 mg/kg BaP and a mixture of 4.58 mg/kg BaP + 2.89 mg/kg triclosan) and sampled at each of three time points (exposure: one and 21 days; recovery: 28 days). Proteobacteria was dominant across all treatments and at all time points. Firmicutes, Tenericutes or Fusobacteria was the next most dominant phylum, depending on treatment and/or time. There were significant differences in both treatment and time. Composition was significantly different among treatment groups during each individual time, and time points differed for all treatments except the solvent control. Differences over time may be due to initial introduction of contaminants, followed by coping mechanisms, and recovery when exposure was removed.

Together, these results suggest that fish species, chemical exposure, and duration of exposure all contribute to differences in the GI microbial composition of fish. Thus, the

complexity of this system needs to be considered when identifying potential biomarkers of pollutant exposure in wild fish.

ACKNOWLEDGEMENTS

I would like to thank my graduate supervisors, Dr. Tim Jardine and Dr. Markus Hecker, for their continued guidance and support throughout my Masters. I would also like to thank my graduate committee, Dr. Steve Wiseman, Dr. Andrew van Kessel, and Dr. Lynn Weber, for their comments, support, and time. I would also like to extend thanks to my external examiner, Dr. Valerie Langlois.

I would like to express my gratitude to Dr. Song Tang, Dr. Tim Tse, Danielle Lemire, Dr. Markus Brinkmann and Dr. Hui Peng, along with all the other staff and students at the Toxicology Centre who provided assistance throughout my studies. I would also like to thank Sara Hanson and Tabata Bagatim for hiring me as a research assistant in 2014, providing opportunities that allowed me to gain valuable experience and begin my career as a toxicologist.

I would like to acknowledge my funding sources, the Natural Sciences and Engineering Research Council of Canada (NSERC) Collaborative Research and Training Experience (CREATE) program in Human and Ecological Risk Assessment (HERA), the Canada Research Chairs program, and the Toxicology Centre.

Thank you to my family and friends for their support throughout this journey. Most importantly, thank you to my husband, Travis Heath, for his love, support, and encouragement throughout the duration of my Masters. Finally, to my children, Kaia and Lincoln Heath, this is for you.

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LIST OF ABBREVIATIONS

°C - degrees Celsius

µg - microgram

µg/g - microgram per gram

µg/L - microgram per litre

AhR - aryl hydrocarbon receptor

ANOVA – analysis of variance

ATRF - Aquatic Toxicology Research Facility

BaP – benzo(a)pyrene

cm - centimetre

DNA – deoxyribonucleic acid

GI – gastrointestinal

g - gram

HSI – hepatosomatic index

IBD - inflammatory bowel disease

IBS - irritable bowel syndrome

IP – intraperitoneal

K – condition factor

L - litre

LC_{50} – lethal concentration 50%

LD_{50} – lethal dose 50%

LOEC – lowest observed effect concentration

mg/kg – milligram per kilogram

n – sample size

ng/L – nanogram per litre

NGS – next generation sequencing

NMDS - non-metric multi-dimensional scaling

OSI – organosomatic indices

OTU – operational taxonomic unit

PAH – polycyclic aromatic hydrocarbon

PCR – polymerase chain reaction

PERMANOVA – permutational multivariate analysis of variance

POP – persistent organic pollutant

PPCPs - pharmaceuticals and personal care products

rRNA – ribosomal ribonucleic acid

SSI – spleen somatic indices

PREFACE

This thesis is written in a manuscript style format. Chapter 1 of this thesis is a general introduction and literature review. Chapter 2 is an unpublished pilot study intended to inform methods used in Chapter 3. Chapter 3 is organized as a manuscript for publication in a peer-reviewed scientific journal. Chapter 4 is a general discussion of findings from both Chapters 2 and 3. There is some repetition between chapters.

CHAPTER 1: GENERAL INTRODUCTION

It has been estimated that more than 70,000 xenobiotics have the potential to harm humans and/or ecosystems (Hlavinek et al., 2008). With continual development of new chemicals and their appearance as contaminants in the environment, this number could continue to increase. Population growth, forestry, industrial development and production, technological innovation, and agricultural expansion have led to increases in energy demands, water use, nutrient loading, water, soil, and atmospheric pollution, and have ultimately led to deteriorating environmental quality (van der Perk, 2007; Twardowska et al., 2006). Not only is exposure to a single contaminant of concern, but exposure to multiple contaminants at a time is possible (van der Perk, 2007) and has the potential to pose an even greater risk. This contamination therefore threatens all organisms within ecosystems, but particularly those that are most sensitive.

1.1 Surface Water Contamination

Surface water contamination can occur due to point and diffuse sources such as urban runoff, effluent discharge from industrial or municipal wastewater, accidental spills, leaching from soil or groundwater, or as a result of atmospheric deposition (U.S. EPA, 2010; Environment Canada, 2010). Once a contaminant enters surface waters, it can lead to uptake by aquatic organisms and cause adverse effects such as minor lesions, behavioural changes, immunotoxicity, mutagenicity, genotoxicity, endocrine disruption, or even death (Hogan, 2014; Twardowska et al., 2006). These changes may be observed as impacts on a single organ, biological systems, or affect entire "ecosystems" within an organism, such as the gastrointestinal (GI) microbiome. Chemical exposure may also cause subtle effects that may affect the fitness of an organism but are not readily observed (van der Perk, 2007).

Two contributors to surface water contamination are industry and municipal wastewater effluents. Industry can produce contaminants such as hydrocarbons, organochlorines, and metals, while municipal wastewaters can release pharmaceuticals, personal care products, and antimicrobials (Pal et al., 2010). Industrial contaminants can enter the aquatic environment through seepage from wastewater impoundments, or directly through effluent discharge (Ritter et al., 2002). They can also enter the atmosphere through stack emissions, be deposited through wet or dry deposition, and enter surface waters through runoff (Ritter et al., 2002). When xenobiotics

enter surface waters, they can cause changes in water chemistry, deleterious effects to non-target organisms, reductions in biodiversity, and decline in ecosystem health (Hogan, 2014).

Seemingly small amounts of xenobiotics can have a great effect on organismal health, particularly in sensitive species. In the last few decades, freshwater fish populations have continued to decline due to water contamination, resulting in a global threat to aquatic diversity and food web impacts (van der Perk, 2007; Twardowska et al., 2006). In their natural habitat, fish are more likely to be exposed to mixtures of chemicals at any given time, as opposed to a single contaminant (Brown et al., 2004).

1.1.1 Polycyclic Aromatic Hydrocarbons

Polycyclic aromatic hydrocarbons (PAHs) are persistent organic pollutants (POPs) made up of two or more fused benzene rings. They are stable, ubiquitous, lipophilic solids and are colourless, or white to yellow-green colour. Due to their lipophilicity, they tend to bioaccumulate in organisms and persist in the environment (Clements et al., 1994; Lu et al., 1977; Twiss et al., 1999). There are over 10,000 possible PAHs (Logan, 2007), 16 of which are on the U.S. EPA's list of priority pollutants (U.S. EPA, 2014). Benzo(a)pyrene (BaP), known as a model PAH, is included on this list, and is often selected as a gross indicator for PAHs (Twardowska et al., 2006).

PAHs are formed during the incomplete combustion or pyrolysis of organic mater which can occur either naturally or anthropogenically; however, natural concentrations tend to be lower than anthropogenic inputs (Twardowska et al., 2006; van der Perk, 2007). Natural processes include forest fires, volcanic activity, and geological processes, while anthropogenic sources include input from industries such as oil refineries, aluminum smelting, resource extraction, and wood preservation (Hansen et al., 2005; Brown et. al., 2004; Ritter et al., 2002; van der Perk, 2007).

While PAH levels tend to be elevated in urban areas or around industrial sources, the 'Grasshopper Effect' can also transport them to remote areas, far from where they are produced (van der Perk, 2007). PAHs can be transported and distributed into surface waters via dry or wet deposition, through direct effluent release into water, or through urban runoff (Twardowska et al., 2006). Once PAHs enter surface waters, they can accumulate in organisms via exposure through water, sediments, or food (Brown et al., 2004). Concentrations of PAHs in fish

inhabiting industrial areas can reach levels of 20 µg/g (Wayland et al., 2008). The bioavailability of PAHs depends on their source, the media in which they are found, and their adsorption potential (Logan, 2007).

The rate at which PAHs are degraded, which can take weeks to months, has a large influence on their persistence in an environmental system (van der Perk, 2007). PAHs can be broken down by photo- and biodegradation, or a combination of the two; however, the ability of microorganisms to degrade PAHs may be decreased in waters where there is continual PAH loading into the system (Twardowska et al., 2006). PAHs can be metabolized by most vertebrates, a process in which the intestines are important as the first barrier after dietary uptake (Berntssen et al., 2015). PAHs are converted from non-polar lipophilic substances, to polar, hydrophilic substances, followed by detoxification and elimination (Berntssen et al., 2015; Hahn et al., 1994) through passive diffusion or biliary excretion after hepatic biotransformation (Logan, 2007). BaP, along with many other PAHs, is an AhR (aryl hydrocarbon receptor) agonist that causes the induction of cytochrome P450 1A and related phase I metabolic enzymes. BaP oxidative metabolism then results in the formation of 7,8-dihydrodiol-9,10-epoxide which leads to the formation of DNA adducts and results in genotoxicity (Gelboin, 1980). Reactive toxicants are then conjugated into intermediates through phase II metabolism (Schlenk, 2008).

Effects of PAHs in aquatic organisms (Table 1.1) can range from possible reproductive stimulation and oestrogen-like action to developmental and reproductive harm, decreased fitness (van der Perk 2007), and cardiovascular abnormalities (Ownby et al., 2002). More subtle effects can include the alteration of genes, such as cytochrome 1, 2, 3, and 4 in teleost fish (Randelli et al., 2011; Tseng et al., 2005; Wassmur et al., 2010; Williams et al., 2009; Zhang et al., 2012). Severity of effects can depend on species, life-stage, sex, genetics, exposure history, and diet (Deshpande et al., 2002; Nacci et al., 2002; Ownby et al., 2002; Aas et al., 2001). Tolerance to PAHs has also been demonstrated and can be heritable in some organisms such as mummichogs (Ownby et al., 2002).

1.1.2 Antimicrobials

Antimicrobials belong to a broad class of chemicals know as pharmaceuticals and personal care products (PPCPs), which have been detected in the environment since the early 1970s (Hlavinek et al., 2008). They are used in both personal hygiene and medicine to kill or

Table 1.1. Effects of PAHs and crude oil exposure on fish.

Effect	Species	Chemical	Dose	Reference
Cardiac Dysfunction Edema Spinal Curvature Craniofacial Abnormalities Mortality	Zebrafish *(Danio rerio)*	Naphthalene Fluorene Dibenzothiophene Phenanthrene Pyrene	10 mg/ml	Incardona et al., 2004
		Anthracene	5 mg/ml	
		Chrysene	1 mg/mL	
Decreased Growth	Inland silversides (*Menidia beryllina*) Grass shrimp (*Palaemonetes pugio*)	Crude oil	67-145 µg/L	Gundersen et al., 1996
		Partially combusted crude oil	4-12 µg/L	
	Zebrafish (*Danio rerio*)	Fluorene Dibenzothiophene Phenanthrene	10 mg/ml	Incardona et al., 2004
Fin Necrosis Hepatic Lesions Lower Condition Factor Increased Hepatosomatic Indices Higher Levels of External Parasite (*Crytocolyte lingua*) Lower levels of digestive tract parasite (*Steringophorous furcuger*)	Gray sole (*Glyptocephalus cynoglossus*) Yellowtail flounder (*Limanda ferruginea*) Winter flounder (*Pleuronectes americanus*)	PAH polluted site	Up to 50 µg/g	Khan, 2003

Compromised Immune System	Gray sole (*Glyptocephalus cynoglossus*) Yellowtail flounder (*Limanda ferruginea*) Winter flounder (*Pleuronectes americanus*)	PAH polluted site	Up to 50 µg/g	Khan, 2003
	Common carp (*Cyprinus carpio* L)	3-methylcholanthrene	20-200 µM	Reynaud et al., 2004
Antiestrogenic Activity	Flounder (*Platichthys flesus*)	Phenanthrene	0.5-2.5 nmol/g	Monteiro et al., 2000
		Chrysene	0.4 nmol/g	
	Rainbow trout (*Oncorhynchus mykiss*)	3-methylcholanthrene	0.39-1.56 µM	Navas and Segner, 2000
		b-naphthoflavone	0.024-6.25µM	
Hepatocarcinogenicity	Rainbow trout (*Oncorhynchus mykiss*)	Benzo(a)pyrene	500-1000 ppm	Hendricks et al., 1985

inhibit microorganisms either internally or externally (Lindberg et al., 2005; Murray et al., 2010). Their introduction into the environment is cause for concern because of the potential development of antibiotic resistance in naturally occurring bacterial populations (Miao et al., 2004; Halling-Sorensen, 2000; Wollenberger et al., 2000; Ritter et al., 2002). Antimicrobials can enter municipal wastewater where they may go untreated, be released into the aquatic environment, and result in unintentional ecological effects (Lindberg et al., 2005; Pal et al., 2010; Hlavinek et at., 2008; Ritter et al., 2002). Due to their relatively constant release into surface waters, they are known to be pseudopersistent (Pal et al., 2010; Murray et al., 2010). Concentrations of antimicrobials that have been recorded ranged from below detection limits of 0.0005 µg/L in wastewater or sewage treatment plant effluents, up to concentrations of 2.0 µg/L in freshwater rivers and canals, and as high as 56.7 µg/L in animal husbandry effluents (Pal et al., 2010).

Triclosan (2,4,4'-trichloro-2'-hydroxydiphenyl ether) is a broad-spectrum antimicrobial found in daily-use consumer products such as hand sanitizers (Bhargava and Leonard, 1996), detergents, toothpastes, and even socks (Adolfsson-Erici et al., 2002). It is hydrophobic, has low water solubility and has been recommended as one of the highest priority pollutants due to its high frequency of occurrence and potential for being a health hazard at currently occurring concentrations (Murray et al., 2010). It has also been demonstrated to have residual antimicrobial activity (Bhargava and Leonard, 1996; Lilly and Lowbury, 1974).

Triclosan enters surface waters as a component of municipal wastewater effluent after it is used in household and medical products. It is frequently detected in wastewater in concentrations ranging from 0.04 µg/L (Hua, 2005) to 2.3 µg/L (Kolpin et al., 2002). It can be removed from waters via microbial degradation, photodegradation, and absorption (Pal et al., 2010). Triclosan binds the enoyl-acyl carrier protein reductase enzyme which interferes with the final step of fatty acid synthesis and inhibits bacterial growth (McMurray et al., 1998; Levy et al., 1999, Gaulke et al., 2016). As a result of manufacturing, triclosan can form chlorinated dioxins upon incineration or sun exposure (Kanetoshi et al., 1988a; Kanetoshi et al., 1988b). It is readily absorbed in the GI tract and is excreted in urine, feces, and breastmilk (Gaulke et al., 2016). Triclosan has been detected in human milk samples, fish, wastewater, and sludge (Adolfsson-Erici et al., 2002).

Triclosan toxicity varies depending on the exposed species and route of administration (Bhargava and Leonard, 1996). Through an oral route of administration in mature mammals, the

acute LD_{50} of triclosan ranges from 3750 mg/kg to upwards of 5000 mg/kg with subchronic toxicity in the range of 150 mg/kg to 300 mg/kg, and a chronic exposure no effect level of 1000 ppm (Bhargava and Leonard, 1996). Fish species such as rainbow trout are more sensitive with an LC_{50} of 0.35 mg/L (Ciba 1998 referenced in Adolfsson-Erici et al., 2002). Effects of triclosan exposure have been shown in bacteria, invertebrate, and vertebrate species, including physical changes such as hepatic lesions to affects on metabolic processes and the GI microbiome (Table 1.2). Triclosan has also been associated with endocrine disruption and inflammatory disease (Gaulke et al., 2016; Pinto et al., 2013). Mechanisms of triclosan resistance have also been noted. They include mutations in triclosan target enzymes, increased enzyme expression, degradation of triclosan, and active efflux (Gaulke et al., 2016).

1.1.3 The Role of Microbes in the Degradation and Metabolism of Chemicals

Environmental microbes can degrade both PAHs and antimicrobials, as well as a host of other contaminants. This degradation process can play a role in the fate of contaminants in the environment. Microorganisms, including bacteria, obtain energy by oxidation. This typically occurs using organic matter, but organic pollutants made up of oxygen, nitrates, or sulphates can be used as well (van der Perk, 2007).

Bioremediation of crude oil involves microbes capable of mineralizing hydrocarbons to water and carbon dioxide (Twardowska et al., 2006). In winter flounder (*Pseudopheuronectes americanus*), the intestinal mucosa has the ability to metabolize PAHs similar to the liver (McElroy and Kleinow, 1992). In addition, estrogenic compounds can result from the metabolism of PAHs by human intestinal microbiota (Van de Wiele et al., 2005).

Heavy metals are immobilized by metal reducing bacteria or through sulfide precipitation by sulphate-reducing bacteria (Twardowska et al., 2006). Bacteria have been shown to bind heavy metals, such as mercury and chromium, and remove them from the environment or change valency to reduce toxicity (Twardowska et al., 2006). Microbes can mediate the transformation of metals such as the methylation of mercury to methylmercury, which increases its toxicity, or methylation of arsenic, which decreases its toxicity (Twardowska et al., 2006). Potentially toxic

Table 1.2. Possible effects of triclosan exposure in bacteria, invertebrate, and vertebrate species

Effect	Species	Dose	Reference
Hepatic lesions Thymic changes Kidney changes	Rat	125-300 mg/kg	Bhargava and Leonard, 1996
Inhibition of lipid biosynthesis Inhibition of fatty acid biosynthesis	*Escherichia coli*	0.2 µg/ml	McMurray et al., 1998
		2.5 µM	Levy et al., 1999
Alteration of kinase enzymes Compromised immune cells Redox balance interference	Mussels (*Mytilus galloprovincialis* Lam.)	2.9-29 ng/g	Canesi et al., 2006
Endocrine disruption	Long-Evans rats	1000 mg/kg/day	Crofton et al., 2007
	North American bullfrog (*Rana catesbeiana*)	0.03-0.15 µg/L	Veldhoen et al., 2006
	Western mosquitofish (*Gambusia affinis*)	101.3 mg/L	Raut and Angus, 2010
Changes in gastrointestinal microbiome populations	Fathead minnow (*Pimephales promelas*)	100-1000 ng/L	Narrowe et al., 2015

metabolites can also be created through the metabolism of dimethylarsine by *Escherichia coli* (Nicholson et al., 2005). The ability of microorganisms to degrade chemicals indicates the potential to be used as bioindicators, for improved monitoring, and for bioremediation (Harayama, 1997; Yergeau et al., 2012). Although our knowledge on the occurrence and fate of chemicals is growing, there are still many knowledge gaps, including the effects of chemicals on the GI microbiome in aquatic organisms and how these intestinal microbes may respond to such exposures.

1.2 The Gastrointestinal Microbiome

Bacteria are ubiquitous across all habitats that support life; however, the composition of these bacterial communities can vary greatly from one environment to another (Sullam et al., 2012). In the human gut alone, there are estimated to be over one thousand species of microorganisms (Nicholson et al., 2005), which may have some of the greatest influence on bacterial signalling in the host (McFall-Ngai et al., 2013). The GI microbiome is important in several aspects of organismal health including nutrition and digestion, regulation of fat storage, immunity and development of the immune system and prevention of pathogen colonization (Navarete et al., 2012; Sullam et al., 2012; Mansfield et al., 2010; Gaulke et al., 2016; Ley et al., 2008a). Examples of GI microbial activity include glucuronidase activity (Aura et al., 2002) and oxidative (Rumney et al., 1993) and aromatic hydroxylation reactions (Huycke and Moore, 2002).

The GI microbiome composition can change and recolonize throughout life, from birth to adulthood through varying influences (Yoshioka et al., 1983; Harmsen et al., 2000; Grolund et al., 1999; Ducluzeau, 1993; Mansfield et al., 2010; Nicholson et al., 2005), but there is thought to be a 'core microbiome' which remains relatively constant over time (Nelson et al., 2013b). Firmicutes and Bacteroidetes are typically the dominant phyla in the vertebrate gut (Ley et al., 2008b), including adult mammals (Sullam et al., 2012). Firmicutes includes several species of lactic acid bacteria, including those in orders *Carnobacterium, Lactobacillus, Streptococcus,* and *Weissella,* and are often found in carbohydrate rich feed (Liu et al., 2014). These bacteria are important in nutrient metabolism and thought to be beneficial to host health (Liu et al., 2014). Bacteroidetes are involved in the metabolism of proteins and sugars, inflammation, and neurologic disorders, and contain many antibiotic resistant species (Rajilić-Stojanović and de

Vos, 2014; Gibiino et al., 2018). Species such as *B. vulgatus, P. falsenii, B. fragilis*, and *P. copri* have been implicated in human disease (Gibiino et al., 2018). Interestingly, studies have also identified relationships between the gut microbiomes of seemingly unrelated species, such as some fish, mammals, and insects (Sullam et al., 2012).

Factors that influence the microbiome composition include evolution (Sullam et al., 2012; Ley et al., 2008a; McFall-Ngai et al., 2013), gut morphology and physiology (Nelson et al., 2013b; Turnbaugh et al., 2008; Sakata et al., 1978; Nelson et al., 2013a), illness, antibiotics, and the adaptive immune system (Ley et al., 2008b; Gaulke et al., 2016; Moore and Moore, 1995; O'Hara and Shanahan, 2006; Hopkins et al., 2001). Diet also influences GI microbiome composition (Gaulke et al., 2016; Sullam et al., 2012; Wong et al., 2013; Nelson et al., 2013b; Nelson et al., 2013a), with microbial diversity increasing from carnivorous animals to omnivores to herbivores (Ley et al., 2008a; Nelson et al., 2013a; Wong et al., 2013; Turnbaugh et al., 2008). Nelson et al. (2013b) found that species, age, sex, social interactions, and vertical transmission influenced the GI microbial composition in seals.

Environment has also been identified as playing a role in the GI microbiome composition in mammals (Nelson et al., 2013b; Uenishi et al., 2007; Villers et al., 2008; Nakamura et al., 2011), fish (Dhanasiri et al., 2011), and other non-mammalian species (Scupham et al., 2008; Xenoulis et al., 2010; Wienemann et al., 2011). Ley et al., (2008b) found that vertebrate gut microbial communities are unlike their associated 'free-living' communities while invertebrate gut microbial communities do cluster with 'free-living' microbes. GI microbial communities differ within the same species from different environments (Turnbaugh et al., 2008; McFall-Ngai et al., 2013). However, under similar circumstances, the same species have also been found to have the same stable core microbiome, suggesting co-evolution between a host and its GI microbial community (Sullam et al., 2012; Ley et al., 2008a; Nelson et al., 2013a). A study of 60 mammalian species found Firmicutes in all samples, while there were also OTUs (operational taxonomic units) unique to each host (Ley et al., 2008a).

Some studies have explored the effects of chemicals on the GI microbiome in animals including mice exposed to arsenic and cadmium. When exposed to arsenic, changes in Tenericutes and Firmicutes in the GI microbiome of mice were noted, and clusters were differentially formed with exposed mice and control mice (Lu et al., 2014). A study that exposed

mice to cadmium resulted in a significant decrease in Firmicutes and a significant increase in Bacteroidetes (Zhang et al., 2015).

There have been many studies conducted on the GI microbiomes in humans and other mammalian species, and while there is a growing amount of information available on the GI microbiome in fish, more research is still needed.

1.2.1 The Gastrointestinal Microbiome in Fish

Some similarities exist between the fish GI microbiome and that of other animals but there are also distinct differences, as several bacterial taxa have only been isolated in the fish gut (Sullam et al., 2012). Salinity, trophic level, diet, seasonality, ecological interactions, habitat, stress, developmental stage, and host phylogeny all play a role in shaping the GI microbiome in fish (Sullam et al., 2012; Mansfield et al., 2010). Freshwater fish tend to exhibit similarities to microbes in freshwater environments, while saltwater fish exhibit similarities to their saline environments (Sullam et al., 2012).

Like mammals, a 'core microbiome' has been noted in some fish species including rainbow trout (Wong et al., 2013), which is thought to be established from water and egg epibiota after the first feeding stages (Navarrette et al., 2012). This core microbiome is thought to be stable throughout life (Wong et al., 2013); however, individual variation is still observed (Kim et al., 2007; Spanggaard et al., 2000), even in genetically similar animals under the same rearing conditions (Desai et al., 2012). When zebrafish (*Danio rerio)* siblings were compared, microbial diversity varied between developmental stages, but a conserved core microbiome was still observed amongst more than ninety percent of individuals (Stephens et al., 2015). Wong and colleagues (2013) found a shared core microbiota in rainbow trout consisting of 52 common OTUs. This study noted that variations in diet and rearing density resulted in minor changes in the gut microbiome of rainbow trout, but the core microbiota remained despite changes in experimental diet (Wong et al., 2013). Navarete et al. (2012) found that even when reared in the same environment, individuals of different rainbow trout families harboured different GI microbial communities. In contrast, another study comparing wild and domesticated zebrafish demonstrated changes in their core microbiome from wild to captive, as well as between different facilities (Roeselers et al., 2011), leading to speculation about how stable this core community really is.

Fish gut bacteria play a role in digestion and metabolism, nutrition, nitrogen recycling, immunity and defence, intestinal development, and development of the gut and immune systems (Sullam et al., 2012; Nayak, 2010; Desai et al., 2012). Many fish species have lactic acid bacteria present in their guts, which are beneficial in a healthy gut lining (Desai et al., 2012). Most fish GI communities are dominated by microbes from the phylum Proteobacteria (Sullam et al., 2012).

Proteobacteria and Fusobacteria dominate the GI microbiome in zebrafish (Gaulke et al., 2016). When germ-free zebrafish guts were seeded with microbes from the mouse intestinal tract, over time, the zebrafish gut looked more like a typical zebrafish gut with Proteobacteria taking over Firmicutes and Bacteriodetes (Gaulke et al., 2016). This finding suggests symbioses between the host and its gut microbes that are adapted to the unique characteristics of the gut (e.g. pH, temperature), as opposed to simple colonization by microbes found in the immediate environment (Rawls et al., 2004). Exposure to triclosan has also caused microbial community shifts in the zebrafish gut. One study demonstrated that after an acute four day exposure to triclosan, the families Enterobacteriaceae and Aeromonadaceae were susceptible to exposure, while the genus *Pseudomonas*, family Rhodobacteraceae, and order Rhizobiales were more resilient. After a longer exposure of seven days, decreases in the genera Chitinilyticum were observed while family Aeromonadaceae and genus *Cetobacterium* were unaltered (Gaulke et al., 2016). In fathead minnow, GI microbiota involved in nitrogen cycling and triclosan metabolism were altered when exposed to triclosan (Narrowe et al., 2015). Microbiota of note included, order Pseudomonadales, which remained stable, and increases in classes *Flavobacterium, Chryseobacterium, Methylobacterium, Hydrogenophaga, Acidovorax,* and *Shewanella.* Changes in the GI microbiome have also been shown in rainbow trout when exposed to a variety of antimicrobials (Austin and Al-Zahrani, 1988).

Dominant rainbow trout OTUs are from phyla Proteobacteria and Firmicutes (Wong et al., 2013; Nayak, 2010; Roeselers et al., 2011; Sullam et al., 2012; Kim et al., 2007), followed by Actinobacteria and Bacteriodetes (Desai et al., 2012), then Fusobacteria (Navarete et al., 2012). Specifically, *Carnobacterium maltaromaticum* has been found to be the most abundant sequence in rainbow trout GI microbiomes (Mansfield et al., 2010). In a study by Navarete et al., (2012), Proteobacteria, Firmicutes, and Actinobacteria, were the only phyla present in all individuals, and a change in diet caused the disappearance of Bacteroidetes in some fish. In another dietary

experiment, common OTUs included *Weissella paramesenteroides, Acidovorax facilis, Citrobacter freundii, Enterobacter amnigenus, Alicycliphilus dentrificans,* and *Shewanella oneidensis* (Desai et al., 2012). In a third dietary study, a core GI community was found in rainbow trout that included classes Bacilli, Alphaproteobacteria, Gammaproteobacteria, Betaproteobacteria, and Clostridia and this core made up 81.8% to 89.8% of sequences in each treatment group (Wong et al., 2013). The same study also identified treatment specific communities based on diet and rearing density. Chemical exposure, such as a mixture of copper and zinc, also caused changes in rainbow trout gut bacterial populations (Syvokiene et al., 2006).

1.2.2 Effects of an Altered Gastrointestinal Microbiome

Microbial imbalances, also known as dysbiosis, can lead to disease development in humans (Round and Mazmanian, 2009) such as IBS (irritable bowel syndrome), IBD (inflammatory bowel disease), multi system organ failure, colon cancer (Guarner and Malagelada, 2003; Frank et al., 2007), diabetes, heart disease, arthritis, and malnutrition (Gaulke et al., 2016). An individual who is already predisposed to disease may be at an increased risk of disease development when their microbiome is altered (Round and Mazmanian, 2009). For example, Firmicutes are more highly represented in obese humans (Ley et al., 2008b) which could be evidence of a change in bacteria contributing to disease. Dysbiosis may also influence immune function (Shanahan, 2002; Round and Mazmanian, 2009) as it provides protection against foreign microbes (Guarner and Malagelada, 2003). This idea is demonstrated in germ free animals with compromised immunity, that have their immune function restored when microbes are reintroduced to their GI system (Umesaki et al., 1995). In vertebrate species, changes in the GI microbiome can also cause changes in dietary energy harvest and affect behaviour (Wong et al., 2013; Rawls et al., 2004; Turnbaugh et al., 2006; Nayak, 2010; Semova et al., 2012).

Heavy metals such as arsenic, cadmium, and lead can disrupt microbial community composition and metabolic profiles in mice and alter immune response and gut barrier function (Gaulke et al., 2016). GI microbial changes in goldfish (*Carassius auratus*) exposed to pentachlorophenol have been correlated with hepatic damage and organism growth. Results of this study identified an increase in Bacteroidetes and a decrease in Firmicutes (Kan et al., 2015). In addition, due to the role of microbes in the metabolism and biotransformation of chemicals,

changes to the GI microbiome may have the potential to alter such processes in the host and lead to changes in xenobiotic fate and toxicity.

Effects on the microbiome may differentially affect organisms depending on the life stage due to greater microbiome stability later in life (Gaulke et al., 2016). It is important to identify what can affect the GI microbiome and the extent of such effects in various species, life stages, and individuals, as it can lead to development of treatment and prevention of adverse effects (Gaulke et al., 2016). In theory, the abundance of resistant taxa would increase or remain unchanged due to exposure of a chemical, and susceptible microbes would decrease; therefore, community shifts could be used as biomarkers of exposure. These biomarkers could serve as early warning signs of exposure before an entire community is affected (Santos et al., 2017).

1.3 Objectives and Null Hypotheses

Xenobiotic chemicals are continuously entering surface waters and remain an environmental concern due to continuing anthropogenic activities. Contaminants of concern include PAHs from industrial activities and antimicrobials as a result of municipal wastewater effluent discharge into surface waters. These contaminants can affect organismal fitness, various organs, and organ systems, as well as the microbial communities within the GI tract of fish. These effects have the potential to extend beyond the individual and lead to impacts on biodiversity and ecosystem health. There is a wealth of knowledge on the effects of PAHs and antimicrobials on various endpoints in fish, but despite the potential impacts of chemicals on the GI microbiome in fish, there is little research available on this topic. Therefore, the purpose of my thesis was to evaluate the potential effects of ubiquitous contaminants, BaP and triclosan, on the GI microbiome in rainbow trout. Such research could lead to the identification of biomarker microbial taxa and provide insight into microbial metabolism of PAHs within fish and their ability to deal with contaminant exposure. Ideally, this information could lead to non-lethal field testing, such as a fecal swab, for identification of pollutant exposure.

Objectives:

The overall goal of this project was to characterize the effect of chemicals on the GI microbiome in fish and identify novel microbe-based biomarker(s) of exposure. Specific objectives and hypotheses were:

1. Use a pilot study to develop a method to extract and characterize the GI microbiome of two evolutionarily distant lab-reared fish species.

   $H_0$1: There will be no statistically significant difference in the GI microbiome between species.
2. Determine the effects of a PAH, benzo(a)pyrene, on the GI microbiome in rainbow trout.

   $H_0$2: Dietary exposure to benzo(a)pyrene will have no statistically significant effect on the GI microbiome composition in rainbow trout.
3. Determine the effects of a combination of benzo(a)pyrene and an antimicrobial, triclosan, on the GI microbiome in fish.

   $H_0$3: Dietary exposure to a combination of benzo(a)pyrene and triclosan will have no statistically significant effect on the GI microbiome in rainbow trout.
4. Determine if changes in the GI microbiome composition are accompanied by changes in fish condition when exposed to chemicals of interest.

   $H_0$4: There will be no statistically significant association between changes in the GI microbiome and rainbow trout body condition.
5. Identify novel biomarkers of pollutant exposure as a result of changes in the fish GI microbiome.

CHAPTER 2: MICROBIOTA INHABITING THE GASTROINTESTINAL TRACT OF TWO FISH SPECIES REARED UNDER SIMILAR CONDITIONS

2.1 Abstract

GI microbiota exist in symbiotic relationships with their host organism, and can influence a variety of processes, including immune function and metabolic processes. Microbial colonization in the gut can be influenced by a variety of factors, including environmental conditions, diet, and host phylogeny. The aim of this study was to use rainbow trout and lake sturgeon to develop a method to characterize the GI microbiome in fish and to characterize the GI microbiome in lake sturgeon. Results indicated that GI microbiomes in lake sturgeon were significantly different than the GI microbiomes in rainbow trout. Further studies should investigate the GI microbiome in fish closely related to lake sturgeon to discern if there is a conserved microbiome amongst ancient fish species.

2.2 Introduction

Microbiota inhabit various environments including land, water, and various parts of host organisms, including the gastrointestinal (GI) tract of animals. The GI microbiome is made up of microorganisms and their genetic material inhabiting the GI tract of an organism. These microbial communities can be highly diverse, encompassing thousands of different species (McFall-Ngai et al., 2013) and over sixty percent of solid faecal mass in humans (Stephen and Cummings, 1980). The gut microbiome plays a role in GI and metabolic processes and immunity and defense of the host (Sullam et al., 2012; O'Hara and Shanahan, 2006; Colombo et al., 2015; Round and Mazmanian, 2009; Turnbaugh et al., 2008; Guarner and Malagelada, 2003; Upreti et al., 2004; Xu and Gordon, 2003; Foligne et al., 2007).

Colonization of the microbiome is influenced by factors such as habitat, evolution, trophic level, and nutrition (Sullam et al., 2012). GI microorganisms live symbiotically within the gut, but a variety of factors can disturb this delicate balance and result in dysbiosis. Such factors include environmental conditions, diet, and chemical exposures (Desai et al., 2012; Wong et al., 2013; Kan et al., 2015; Narrowe et al., 2015). Observed microbial changes induced by dietary

changes range from general, such as an increased ratio of Firmicutes:Proteobacteria in rainbow trout fed plant-based diets when compared with fishmeal control diets (Desai et al., 2012) to more specific changes such as an increase in *Clostridia* observed in rainbow trout fed fishmeal and an increase in *Bacilli* in fish fed grain-based diets (Wong et al., 2013).

Disruption of a healthy GI microbial population may be a factor in negative health outcomes in humans such as IBS (irritable bowel syndrome), IBD (inflammatory bowel disease), multi system organ failure, and colon cancer (Guarner and Malagelada, 2003; Frank et al., 2007). It may also be linked to fat deposition and intestinal glucose uptake in mice (Backhed et al., 2004). In addition, microbes may also play a role in metabolism of toxins such as *Escherichia coli* metabolizing dimethylarsine to potentially toxic metabolites (Nicholson et al., 2005) or the metabolization of PAHs to estrogenic compounds by microbes in the human GI tract (Van de Wiele et al., 2005). Microbes have also been implicated in glucuronidase activity (Aura et al., 2002), oxidative reactions (Rumney et al., 1993), and aromatic hydroxylation reactions in the GI tracts (Huycke and Moore, 2002).

Microbiota change due to environmental and developmental changes is indicated by research showing decreasing diversity through zebrafish (*Danio rerio,* Stephens et al., 2015) and human development (Avershina et al., 2014; Palmer et al., 2007; Yatsunenko et al., 2012). However, when individual developmental stages are examined, their microbiome appears to be conserved (Stephens et al., 2015). This consistent community has come to be known as the ‘core microbiome’ in which individuals of the same species harbour a stable microbial community that is driven by evolution and conserved within a species, independent of external factors (Sullam et al., 2012). This has been demonstrated in seals (*Mirounga leonina*) with close phylogenetic relationship and social interactions (Nelson et al., 2013a) as well as related fish from varying origins, suggesting co-evolution between fish and their commensal gut microbiomes (Sullam et al., 2012).

Previous research has characterized the microbiota inhabiting the GI tract of fishes such as rainbow trout (Desai et al., 2012; Wong et al., 2013; Mansfield et al., 2012; Kim et al., 2007; Navarrete et al., 2012), fathead minnow (Narrowe et al., 2015), goldfish (Kan et al., 2015), and zebrafish (Semova et al., 2012; Stephens et al., 2015) but little to no information is available identifying the gut microbiome of ancient fishes such as lake sturgeon. This chapter includes methods development and results for the characterization of the GI microbiome in fishes,

specifically lake sturgeon and rainbow trout, using next generation sequencing. This study was intended to inform a follow-up study on how chemicals impact the GI microbiome in fishes.

2.3 Materials and Methods

2.3.1 Fish Species

Lake Sturgeon and rainbow trout were used to develop the methods for gut microbiome characterization. Both species are native to North America and can be found in the same drainage basins (e.g., Saskatchewan River) but they are only distantly related, both in superclass Actinopterygii and diverging from there. Rainbow trout are in class Teleostei and lake sturgeon in class Chondrostei. Unlike rainbow trout, lake sturgeon are bottom feeders. However, they do have similar diets in the wild, feeding on leeches, clams, crayfish, small fish, and aquatic insects. In addition, lake sturgeon will also eat plants and algae.

Rainbow trout are a widely used experimental model species and are easily cultured and maintained in the lab. Rainbow trout are relatively sensitive compared to other species and GI microbiome studies have previously been conducted using rainbow trout. Studies investigating effects of altered conditions such as diet and rearing density have concluded the core GI microbiome of rainbow trout is composed primarily of Firmicutes and Proteobacteria (Desai et al., 2012; Wong et al., 2013; Mansfield et al., 2010).

In the laboratory, the two species of fish were reared at approximately the same temperatures (12-14°C), fed the same food, and were on the same 12:12 light-dark schedule. An existing stock of both lake sturgeon and rainbow trout from the Aquatic Toxicology Research Facility (ATRF) at the University of Saskatchewan Toxicology Centre were used in these experiments. Rainbow trout were originally sourced from Trout Lodge in Sumner (WA, USA) and the lake sturgeon were reared from eggs acquired from the Wild Rose Hatchery (WI, USA). Fish husbandry and experiments were conducted following an animal use protocol (AUP #20160091) approved by the Animal Research Ethics Board at the University of Saskatchewan.

2.3.2 Experimental Set-up

Juvenile lake sturgeon (n=22) remained in an 890 L holding tank in the ATRF for approximately two years until they were sacrificed. They did not undergo any experimental

treatment. The holding tank was equipped with a flow through, aerated, dechlorinated water system and maintained at approximately 12-14°C, with a 12:12 light:dark cycle. Sturgeon were fed ad libitum five days per week until the time of sacrifice.

Rainbow trout (n=4) were separated into two 719 L tanks equipped with a flow through, aerated, dechlorinated water system maintained at 12-14°C at a 12:12 light:dark cycle. Rainbow trout were exposed via IP injection to 30 mg/kg benzo(a)pyrene (BaP) in corn oil (treatment, n=2), corn oil alone (solvent control, n=1), or unexposed (n=1) and sacrificed five days after being dosed. They were fed ad libitum until being dosed and then fasted until they were sacrificed.

2.3.3 Sample Collection

Rainbow trout and sturgeon were euthanized with an overdose of MS-222. Fork length and weight of each fish were recorded. Intestinal contents were collected from the last pyloric ceca to the vent (Figure 2.1) by squeezing the contents out through the vent with gentle and even pressure into a sterilized weigh boat and the mass of the contents was recorded. Each sample was then transferred to a sterile cryovial and stored on ice for no longer than thirty minutes at which time they were moved to and stored at -80°C until analysis. Water samples, blood, bile, liver, spleen, intestines, and muscle tissue were also collected and stored at -80°C for potential future analysis.

2.3.4 DNA Extraction and Next Generation Sequencing

Microbial DNA was extracted from a 0.25g wet weight intestinal sample using the PowerFecal® DNA Isolation Kit (Mo Bio Laboratories, Inc., USA) according to manufacturer's protocol. Where necessary, suggested steps were taken to concentrate DNA. Random samples were run on a gel agarose to determine if DNA isolation was successful prior to verification of DNA quality and quantity of all samples using a Nanodrop 1000 Spectrophotometer (Thermo-Fisher, USA). Isolated DNA was stored at -80°C until further analysis. The Metagenomic Sequencing Workflow was performed using the universal bacterial primers for the V3 and V4 variable regions of the 16S rRNA gene according to Illumina's 16S Metagenomic Sequencing Library Preparation (Part # 15044223 Rev. B). PCR products were confirmed using a Bioanalyzer DNA 1000 chip on the 2100 Bioanalyzer Instrument (Agilent Technologies,

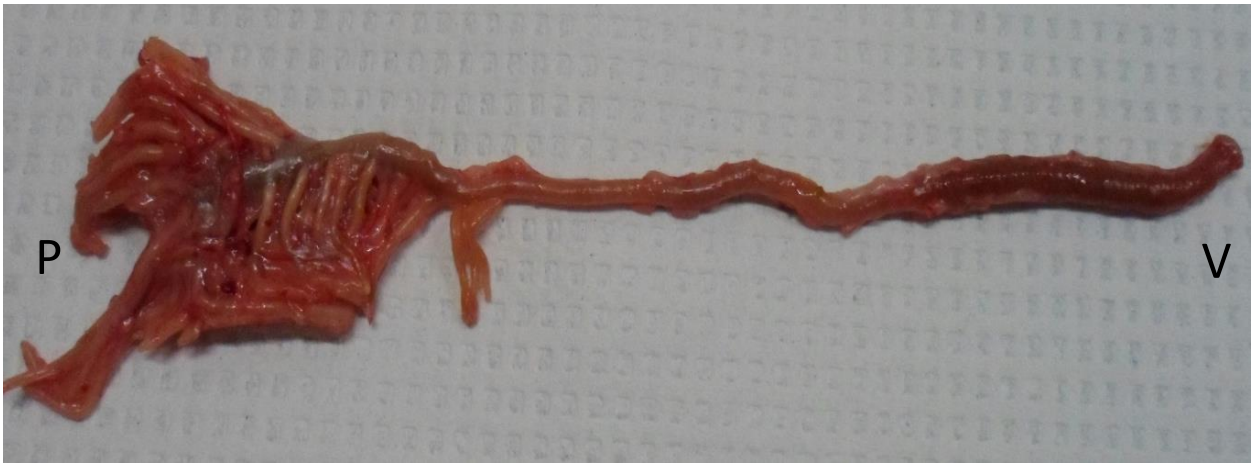

Figure 2.1. Section of the rainbow trout gastrointestinal tract from the pyloric ceca (P) to the vent (V).

California, USA). Library preparation was carried out with a limited cycle PCR, Illumina sequencing adapters, and dual indexed with the Nextera XT Index Kit. Next-generation sequencing was performed using a 2x300 base pair paired end protocol on an in-house (Toxicology Centre, University of Saskatchewan, Canada) Illumina MiSeq Desktop Sequencer (Illumina, California, USA) according to the manufacturer's protocol.

2.3.5 Bioinformatics and Statistical Analysis

The QIIME application in Illumina Basespace (https://basespace.illumina.com), a cloud-based NGS data analysis software system, was used to automatically demultiplex and quality filter reads, as well as taxonomically classify reads into OTUs using the Greengenes database based on 97% sequence similarity. Remaining analysis took place using R v.3.1.2. (R Core Team, 2014). OTUs not present in any samples and OTUs with zero reads were removed from the data set along with low abundance (<1% relative abundance) OTUs.

All statistical analyses were conducted in R v.3.1.2 (R Core Team, 2014). Alpha diversities (Shannon index and Chao 1) were calculated using the "phyloseq" package (McMurdie and Holmes 2013) and beta diversities (Bray-Curtis dissimilarity index and UniFrac distances) were calculated using "vegan" (Oksanen et al. 2020). Non-metric multi-dimensional scaling (NMDS) was conducted using the "phyloseq" package (McMurdie and Holmes 2013) followed by Simper analysis using "vegan". Statistical significance was calculated using PERMANOVA (permutational multivariate analysis of variance) (Anderson, 2017) after checking assumptions with a multivariate Levene's test (betadisper, Anderson, 2006). Homogeneity of dispersion between treatment types was not significant ($p=0.2405$), indicating there is no difference in dispersion between groups. Visual representations were produced using "ggplot" (Wickham 2016).

2.4 Results

2.4.1 Diversity Analysis

Sequence reads ranged from over 100,000 to over 500,000 reads per sample (Figure 2.2), from which 1603 OTUs were identified. Of the six distinct phyla present, only one phylum

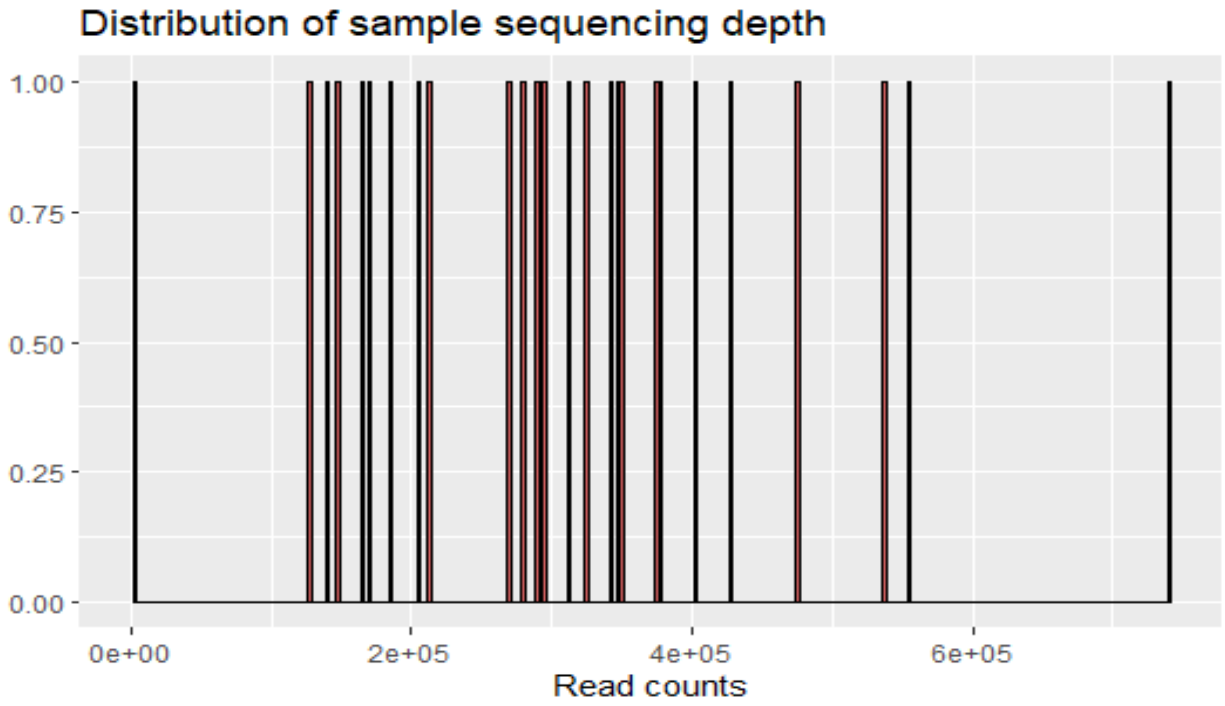

Figure 2.2. Histogram of total reads per sample.

occurred in all 26 samples, two were present in all but one sample, and two phyla were only present in rainbow trout samples. There were no bacterial species common in all 26 samples.

The rainbow trout GI microbiome was more diverse than that of lake sturgeon (Figure 2.3). The mean Chao1 value for lake sturgeon was 527.9 and rainbow trout was 876.6. The mean Shannon Diversity value for lake sturgeon was 2.3 and rainbow trout was 3.1. One fish, RTE6, had a lower diversity compared to the other rainbow trout samples and more closely resembled that of the lake sturgeon.

2.4.2 Composition of Microbial Communities

There were four phyla present in lake sturgeon samples and six in rainbow trout samples (greater than 1% abundance and excluding unclassified phyla) (Figure 2.4). The lake sturgeon GI microbiome was dominated by the phyla Fusobacteria (57%) and Proteobacteria (27%), while the rainbow trout microbiome was dominated by the phylum Firmicutes (78%). Lake sturgeon two (LSF2) and three (LSF3) more closely resembled the rainbow trout GI microbiome with Firmicutes being the more dominant phylum and lake sturgeon four (LSF4) and 18 (LSF18) were slight outliers with Proteobacteria being their most dominant phylum. There were no phyla unique to lake sturgeon. In contrast, Cyanobacteria and Tenericutes were only identified in rainbow trout.

The most dominant species in lake sturgeon was *Cetobacterium somerae* (2,353,286 reads) followed by *Tolumonas aurensis* (656,843 reads). Rainbow trout were dominated by *Carnobacterium maltaromaticum* (260,809 reads) followed by *Carnobacterium gallinarum* (80,596 reads). *Cetobacterium somerae* contributed to 23% of the differences between rainbow trout and lake sturgeon, with *Carnobacterium maltaromaticum* (13%) and *Tolumonas aurensis* (9%) being the next highest drivers of differences.

Overall, rainbow trout microbiomes clustered together but distinctly apart from those of lake sturgeon, with zero overlap between the two species (Figure 2.5). Rainbow trout controls and exposed rainbow trout grouped together, suggesting there are no distinct differences between individuals that were exposed to the BaP injection and those that were not. The rainbow trout GI microbiome was significantly different than the lake sturgeon GI microbiome ($p=0.0002$). Exposed rainbow trout and control rainbow trout were not significantly different from each other ($p=0.3480$).

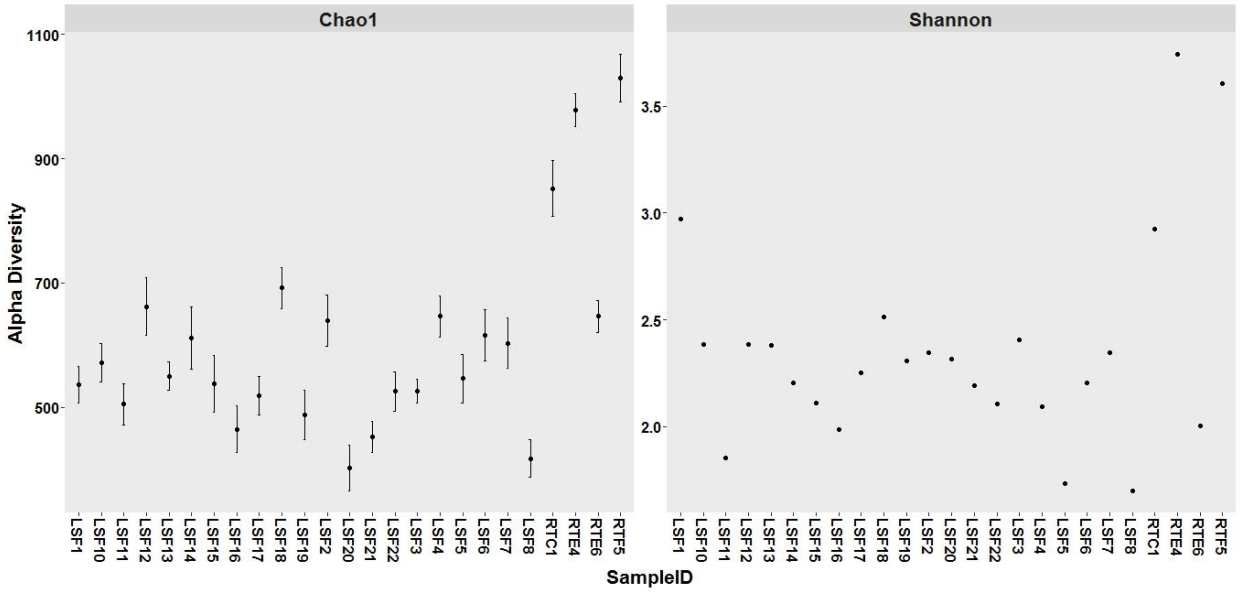

Figure 2.3. Alpha Diversity of gut microbiota in lake sturgeon (LS) and rainbow trout (RT) as represented by Chao1 and Shannon Diversity Indices.

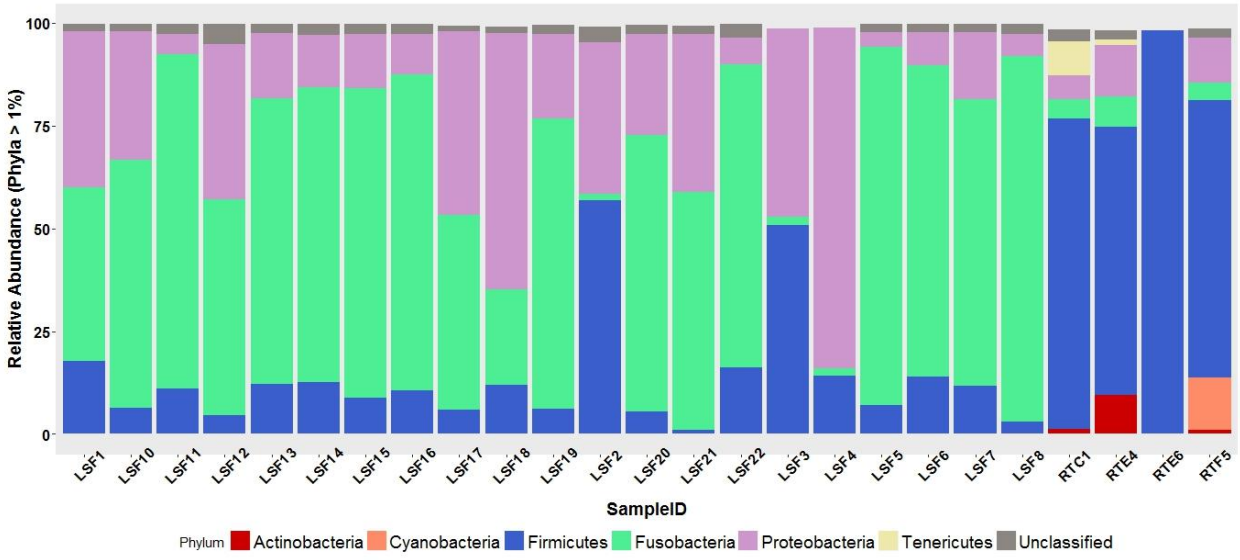

Figure 2.4. Relative abundance of bacterial phyla across 22 lake sturgeon (LS) and 4 rainbow trout (RT) gastrointestinal samples. RTE4 and RTE6 were exposed to BaP, RTC1 was a solvent control, and all other samples were untreated.

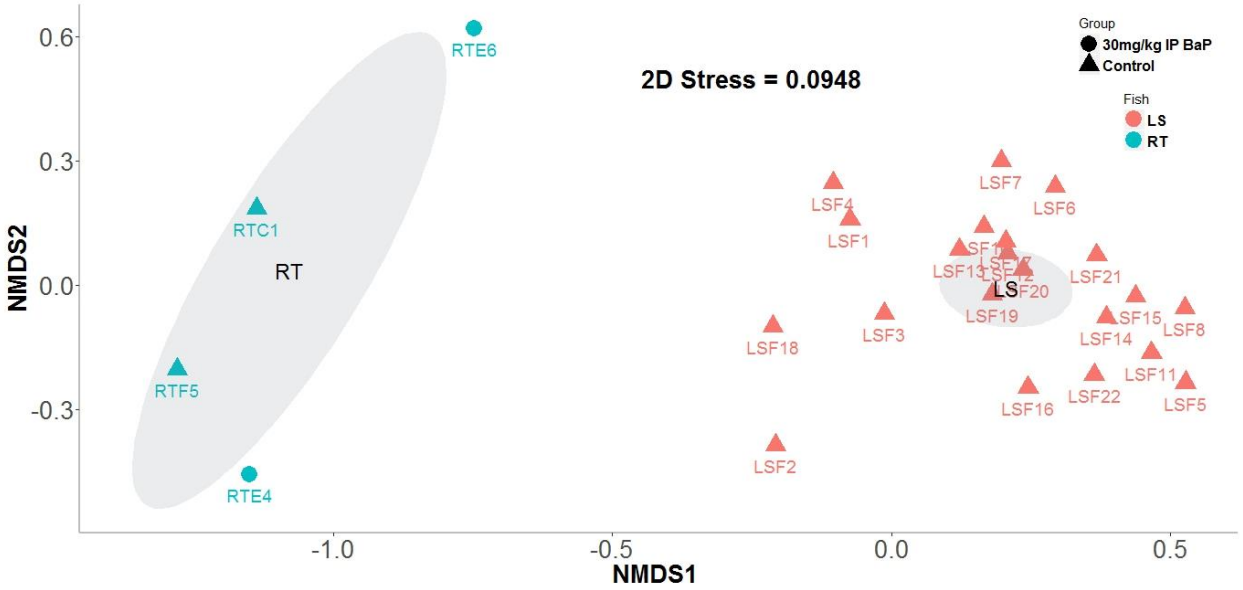

Figure 2.5. Ordination plot of the gut microbiome of rainbow trout (RT) and lake sturgeon (LS) reared together in the same facility on the same diet. Significant difference between species (p=0.0002***) but not between rainbow trout treatment types (p=0.3480). RTE4 and RTE6 were exposed to BaP, RTC1 was a solvent control, and all other samples were untreated.

2.5 Discussion

The lake sturgeon GI microbiome has been previously sequenced (Razak and Scribner 2020), and my study suggests a similar, but not identical, composition for this species. In a study exploring effects of rearing environment and ontogeny on the GI microbiome of lake sturgeon, eight dominant phyla accounted for 95% of all reads: Proteobacteria, Firmicutes, Bacteroidetes, Actinobacteria, Acidobacteria, Planctomycetes, Chloroflexi, and Fusobacteria (Razak and Scribner 2020). Prior to exogenous feeding, Proteobacteria were dominant, and composition was affected by water treatment. This shifted to a dominance by Firmicutes and decreased diversity after exogenous feeding began (Razak and Scribner 2020). In my study, Fusobacteria were dominant, followed by Proteobacteria and Firmicutes.

GI microbial studies have been previously conducted on rainbow trout with Proteobacteria and Firmicutes being most common and remaining highly abundant despite poor health and dietary alterations (Parshukov et al., 2019; Desai et al., 2012; Wong et al., 2013; Kim et al., 2007; Navarrete et al., 2012). Fusobacteria and Bacteroidetes are also frequently detected (Kim et al., 2007; Navarrete et al., 2012). Firmicutes were common in the present study; however, Proteobacteria and Fusobacteria were less frequently detected. Tenericutes and Actinobacteria were also detected, despite treatment type. Poor health in rainbow trout, according to physical indicators, increased abundance of Proteobacteria, Actinobacteria and Tenericutes, while Firmicutes was decreased (Parshukov et al., 2019). A decreased Firmicutes:Proteobacteria ratio was also identified in fish exposed to experimental diets (Desai et al., 2012). In contrast, Firmicutes increased in response to experimental diets along with significant differences in growth (Wong et al., 2013). One of my experimental samples also increased in Firmicutes, being the only phylum present over 1% abundance. One specific bacterium frequently identified in rainbow trout is *Carnobacterium maltromaticum* (Kim et al., 2007; Mansfield et al., 2010). This species was the most common one identified in the current study and identified as a top driver of the differences between the rainbow trout and lake sturgeon microbiomes.

Previous studies suggest diet and environment play a significant role in the GI microbiome composition (Di Maiuta et al., 2013; Sullam et al., 2012; Wong et al., 2013; Nelson et al., 2013a; Dhanasiri et al., 2011; McFall-Ngai et al., 2013; Larsen et al., 2014). However, the present study suggests species identity may play a greater role. Rainbow trout and lake sturgeon were fed the

same diet and were reared in the same laboratory conditions; however, their GI microbiomes were significantly different. The rainbow trout microbiome was dominated by Firmicutes, while Fusobacteria and Proteobacteria dominated that of lake sturgeon. Proteobacteria and Firmicutes are commonly found in high abundance in the fish gut (Sullam et al., 2012; Desai et al., 2012; Wong et al., 2013; Nayak, 2010; Roeselers et al., 2011; Huber et al., 2004; Spanggaard et al., 2000; Kim et al., 2007), and while Fusobacteria are often present, they are often less abundant.

Fusobacteria do, however, dominate the microbiome of the common carp (*Cyprinus carpio* L., van Kessel et al., 2011) and loricariid catfish (*Panaque sp.*, Di Maiuta et al., 2013). Fusobacteria, specifically *Cetobacterium somerae*, was highly abundant in bluegill (*Lepomis macrochirus*, 83%), largemouth bass (*Micropterus salmonides*, 91%), and channel catfish (*Ictalurus punctatus*, 95%), followed by Proteobacteria varying from 5-16% (Larsen et al., 2014). *Cetobacterium somerae* has been found in human feces and freshwater fish and produces cobalamin (Vitamin B_{12}). Therefore, the presence of *C. somerae* may indicate an increased requirement or decreased ability to acquire cobalamin in these fishes. However, with little research into the history and function of Fusobacteria, specifically in relation to the fish GI microbiome, it is difficult to draw conclusion about their presence and function in the GI microbiome of sturgeon.

2.6 Conclusion

The present study has demonstrated that species differences exist in the composition of the gut microbiome between evolutionary distant species of fishes, despite a common rearing environment and diet. However, the rainbow trout sample size was small, and likely insufficient to make generalizations. The GI microbiomes of sturgeon species have only rarely been studied; therefore, further studies to validate the present findings are warranted. Larger sample sizes of rainbow trout would also be useful to better elucidate difference between these two species. Studies conducted in a variety of environments, such as other laboratories and field studies, as well as repeated studies in the same environments, would also contribute to confirming these species differences.

CHAPTER 3: EFFECTS OF BENZO(A)PYRENE AND TRICLOSAN ON THE GASTROINTESTINAL MICROBIOME OF RAINBOW TROUT

3.1 Abstract

Xenobiotic exposure can adversely affect organisms in a variety of ways such as inducing hormonal imbalances, altering behaviour, or causing gross lesions or even mortality. Until recently, however, the effects of environmentally and ecologically relevant xenobiotics on the GI microbiome have been scarcely studied, with little focus on the GI microbiome in aquatic species such as fishes. This study aimed to characterize the GI microbiome in rainbow trout and how it changes in response to low level exposure to ubiquitous xenobiotics, benzo(a)pyrene and triclosan. Results indicated that GI microbiomes in rainbow trout were significantly different from each other as a result of chemical exposure to BaP and a mixture of triclosan and BaP over time. These results did not appear to be correlated with significant changes in condition factor or organosomatic indices. Further studies would be required to evaluate if these changes are linked to functional effects in fishes.

3.2 Introduction

Surface water pollution is a continuing concern due to industrial, agricultural, and other anthropogenic activities. Such pollution can be from point or diffuse sources, such as spills, industrial processes, or urban runoff (van der Perk 2007; Ritter et al., 2011) and can be the result of man-made contaminants or natural substances that have increased loads resulting from human activity (van der Perk 2007). Water can act as a dispersal system for pollutants, and the substrate and organisms within water bodies have the potential to bioaccumulate, biotransform, biomagnify, or even biodegrade potentially toxic substances (van der Perk 2007; Ritter et al., 2011). Classes of chemicals that can be detected in waters include pesticides, metals, organic compounds, pharmaceuticals, and personal care products (Ritter et al., 2011; Pal et al., 2010; Murray et al., 2010). Not only can contamination threaten individual organisms, but it has the potential to harm entire species, and ultimately ecosystem biodiversity (Santos 2017).

One main receptor of concern is fishes inhabiting receiving water bodies. Fish can be exposed to pollutants through contact with water, via gills, or through ingestion of food or water. Various effects of pollutants on organs and organ systems in several fish species have been

reported ranging from hepatic and liver damage, to changes in immune function and behaviour, to cancer and even death (van der Perk, 2007). However, there has been limited research done concerning the effects of environmental pollutants on the GI microbiome of fishes. The GI or gut microbiome includes the microorganisms and their genetic material found within the GI tract of a host organism. Microbiota have evolved with their hosts, be it environmentally or symbiotically, resulting in some bacteria inhabiting specific niches only found in a single species of host (McFall-Ngai et al., 2013). Microbial communities can change daily, or over longer time periods, lending them the ability to adapt and evolve with changes in their environment, and maintain homeostasis within the host (McFall-Ngai et al., 2013).

The GI microbiome influences several aspects of host health, including gut development (Nelson et al., 2013a; Mansfield et al., 2010), digestion, metabolism, energy extraction, and nutrient uptake from the host diet (Ley et al., 2008a; Mansfield et al., 2010; Nelson et al., 2013a; McFall-Ngai et al., 2013; Semova et al., 2012), and general wellbeing (Navarrette et al., 2012). As well, gut microbes play a role in immune system development and regulation (Nelson et al., 2013a; Colombo et al., 2015; Mansfield et al., 2010; Guarner and Malagelada, 2003; Nicholson et al., 2012), including pathogen exclusion (Mansfield et al., 2010; Guarner and Malagelada, 2003). Dysbiosis has the potential to impact the delicate relationship between the gut microbiota and immune system, which could lead to an altered immune response and result in disease (Round and Mazmanian, 2009). Disease could also result from the disruption of relationships, such as immune regulation and metabolic signalling, between the gut microbiota and other organs such as the gut, liver, muscle, or brain (Nicholson et al., 2012). Such relationships play a role in biotransformation and metabolism of xenobiotics, and disruption in these networks may result in host pathogenicity (Nicholson et al., 2005).

Chemicals have the potential to affect the GI microbiome in the host organism. In the few studies that have explored the effects of chemicals on GI bacteria, changes in composition have been noted. Microbial abundance and metabolic homeostasis was disturbed in mice exposed to arsenic in drinking water (Lu et al., 2014). Mice exposed to cadmium had altered gut microbiomes (structure and abundance) along with adverse effects on hepatic energy metabolism and decreased body weight (energy metabolism indicated by fewer Firmicutes and more Bacteroidetes, Zhang et al., 2015). Goldfish (*Carassius auratus*) exposed to pentachlorophenol experienced changes in the GI microbiome along with inhibited growth and hepatic damage

(Kan et al., 2015). Genera related to immunity changed with copper exposure in common carp (*Cyprinus carpio* L). More specifically, a decrease in bacteria related to probiotics and an increase in bacteria related to pathogens were observed (Meng et al., 2018). Lastly, cadmium exposure altered both composition and diversity in Nile tilapia (*Oreochromis niloticus*), but these effects were mitigated with the addition of probiotics (Zhai et al., 2017). With little research in this area, the effects and mechanisms of action are still largely unknown (Adamovsky et al. 2018).

Pharmaceuticals and personal care products (PPCPs) are a class of chemicals that include prescription and non-prescription drugs, and daily use products such as perfumes, shampoos, and household cleaners (Hlavinek et al., 2008). These substances primarily enter surface water through discharge from wastewater or sewage treatment plants (Hlavinek et al., 2008; Ritter et al., 2002), where these compounds could elicit adverse affects on non-target organisms in the receiving environment. One such PPCP, triclosan (2,4,4′-trichloro-2′-hydroxydiphenyl ether) is a broad-spectrum antimicrobial that is often found in wastewater and surface water. A study examining rainbow trout near wastewater treatment plants found triclosan in bile of rainbow trout ranging from <0.08 mg/kg up to 120 mg/kg (Aldolfsson-Erici et al., 2002). It has been classified as a high priority pollutant based upon occurrence and the hazard it presents at current environmental concentrations (Murray et al., 2010). The LC50 in rainbow trout is 350 µg/l (Ciba 1998 referenced in Adolfsson-Erici et al., 2002) and the LOEC in rainbow trout is 73.1 µg/L (Orvos et al., 2002). As an antimicrobial, triclosan also has the potential to alter the gut microbiome. Gaulke (2016) exposed adult zebrafish to triclosan spiked food which resulted in shifts in the structure and diversity of the microbiome. Fathead minnows (*Pimephales promelas*) exposed to 100 ng/L and 1000 ng/L triclosan showed time-dependant alterations in the GI microbiome and suggested changes in taxa involved in nitrogen cycling and metabolism of triclosan (Narrowe et al., 2015).

Another priority group of contaminants are polycyclic aromatic hydrocarbons (PAHs). PAHs are ubiquitous and persistent contaminants mainly produced through the incomplete combustion of organic matter (Twardowska 2006; van der Perk 2007). This can occur either naturally through volcanoes and forest fires (van der Perk 2007), or anthropogenically through industrial processes and resource extraction (Ritter et al., 2002; van der Perk, 2007). The resulting compounds can enter the environment though industrial wastewater effluent, urban

runoff, or atmospheric transport (Ritter et al., 2002; van der Perk 2007). The presence of PAHs in aquatic environments can result in exposure of aquatic organisms through water, sediment, or food, and result in potential adverse effects such as endocrine disruption, developmental and behavioural problems, and cancer (Twardowska 2006; Ritter et al., 2002; van der Perk 2007; Logan 2007). A model PAH, benzo(a)pyrene (BaP) (Twardowska 2006), is on the US EPA's Priority Pollutant List (US EPA 2014). The Canadian Water Quality Guideline for the protection of aquatic life for BaP is 0.015 µg/L in freshwater (CCME 2021). It can cause tumors, depressed growth rate, and carcinogenicity in rainbow trout (Hendricks et al., 1985). BaP exposure also elicits changes in the GI microbial composition of fathead minnows (DeBofsky et al., 2020), mosquitofish, zebrafish (Xie et al., 2020) and Atlantic cod (Bagi et al., 2018).

This study aimed to identify the GI microbiome in a model organism, rainbow trout, and to assess how two widely distributed chemicals, BaP and triclosan, may induce changes in the gut microbial composition. I hypothesized that chemical exposure, particularly to an antimicrobial, would cause a change in bacterial community composition in the GI tract and that changes would be more pronounced over time with continuing chemical exposure. Secondly, I hypothesized that microbial changes due to BaP exposure would be accompanied by decreased fish health. Lastly, I hypothesized that effects from BaP exposure would be exacerbated by co-exposure with triclosan, as this may reduce beneficial bacteria that may protect against adverse effects of BaP.

3.3 Materials and Methods

3.3.1 Fish Species

Rainbow trout (*Oncorhynchus mykiss*; n=90 triploid females) were acquired from an existing stock at the Aquatic Toxicology Research Facility (ATRF) at the University of Saskatchewan Toxicology Centre (originally sourced from Trout Lodge in Sumner, WA, USA). Trout were approximately two years old and housed in a holding tank using flow-through, dechlorinated, aerated water maintained at 12-14°C and on a 12:12 light:dark schedule. Trout were fed commercial fish feed (Martin Profishent^{TM} Classic Floating Fish Feed) both prior to and for the duration of the experiments. Rainbow trout are a common and relatively sensitive experimental species that have been used in previous microbiome studies (Desai et al., 2012; Sullam et al., 2012; Kim et al., 2007). Fish husbandry and experiments were conducted

following animal use protocol #20160091 with approval from the Animal Research Ethics Board at the University of Saskatchewan.

3.3.2 Experimental Set-up

Experimental feed was prepared in house using methods outlined in Ostrander (2005). Briefly, the amount of commercial fish feed needed for the duration of the experiment was weighed and added to a stainless-steel bowl. Dosing solutions for each treatment were solvent control (SC, nominal concentration = 0 mg/kg, measured <0.010 mg/kg), low BaP (LBaP, nominal = 5.0 mg/kg, measured = 5.09 mg/kg), high BaP (HBaP, nominal = 50.0 mg/kg, measured = 40.7 mg/kg), and Mixture (BaP: nominal = 5.0 mg/kg, measured = 4.58 mg/kg; triclosan: nominal = 6.0 mg/kg, measured = 2.89 mg/kg). All were prepared using dichloromethane as the solvent. This solution was poured over the pre-measured amount of food and mixed for one minute with a stainless-steel spoon. It was mixed again an hour later for one minute, and again after 24 hours. The mixtures were left to dry in a fume hood for one week, mixing daily. Feed was stored in a -20°C freezer until used.

Dosing was verified in house for triclosan and at a local laboratory (ALS Environmental, Saskatoon, SK) for BaP. Triclosan samples were extracted from fish feed then run on GC/MS. For the extraction, 0.1 g of feed was crushed and placed into 15 mL falcon tubes in triplicate. A stock solution (51 mg/L) of triclosan was dissolved in ethanol. Falcon tubes with crushed feed were then spiked with 20 µg stock solution, 5 mL ethyl acetate was added, and tubes were inverted to mix. Samples were then mechanically shaken for 30 minutes followed by 30 minutes of sonication, then centrifugation at 6000 rpm for ten minutes. Supernatant was then transferred to another 15 mL falcon tube. Beginning with the addition of ethyl acetate, this process was completed two more times. This was followed by nitrogen blowdown and reconstitution with 1 mL methanol and vortexed for 30 seconds. Lastly, the samples were transferred to amber vials and stored at -20°C until analysis. For BaP, analysis was carried out by ALS Environmental using procedures adapted from "Test Methods for Evaluating Solid Waste" SW-846, Methods 3570 & 8270 (US EPA). Mechanical shaking was used to extract a subsample of the feed with a 1:1 mixture of DCM-acetone, or Hexane-acetone. The extract was then solvent exchanged to toluene. The final extract was analyzed by capillary column gas chromatography with mass spectrometric detection (GC/MS). A BaP reference material yielded 90.5% recovery and a

method blank returned <0.010 mg/kg. Target doses for BaP were relatively successful; however, triclosan was approximately half the desired concentration (Table 3.1).

Twelve 719 L experimental tanks were set up with an aerated, dechlorinated, flow through water system. Tanks also required bio-filters which recirculated water to help keep ammonia levels under control. There were three replicate tanks set up for each of four treatments: Solvent Control, Low Benzo(a)Pyrene (5.0 µg/g), High Benzo(a)Pyrene (50.0 µg/g) and Mixture (5.0 µg/g BaP + 6.0 µg/g Triclosan). The Low BaP dose was chosen to be environmentally relevant (Wayland et al., 2008) and the High BaP dose was chosen as experimentally relevant and expected to elicit sublethal effects (Hendricks et al., 1985). The triclosan dose in the Mixture was chosen for environmental relevance (Leiker et al., 2009). Each tank was stocked with eight to nine fish which were then allowed to acclimate for two weeks prior to beginning experiments. Treatments were orally administered five days per week through food; no food aversion was observed at any point through the experimental process. Subsamples of intestinal content from eight fish per treatment were taken at predetermined time intervals of one (Time1), 21 (Time2) and 28 days (Time3). Experimental feed was ceased at 21 days and control feed was administered for the remaining seven days to assess recovery. There were insufficient numbers of fish to assess recovery in the Mixture treatment.

3.3.3 Sample Collection

Rainbow trout were killed using an overdose of buffered MS-222 (150 mg/L tricaine methanesulfonate) and fork length and weight of each fish were recorded. Intestinal contents were collected in two sections: mid gut and hind gut. For the purposes of this experiment, the mid gut was defined as the section from the last pyloric ceca until there was a visual change in intestinal diameter. Hind gut was defined from the end of the mid gut to the vent. A small section, approximately half a centimeter long, between the mid and hind gut, was excluded from sample collection to prevent sample overlap. Using sterilized gloves, contents were squeezed out of the intestine using gentle and even pressure into a sterilized weigh boat, mass was recorded, then contents were transferred into a cryovial and stored at -80°C. For the purposes of this thesis, only hind gut samples were analyzed.

Table 3.1. Targeted vs. Actual dose achieved in experimental diets.

Treatment	Targeted Dose (µg/g)	Actual Dose (mg/kg wwt)
Solvent Control for BaP	0	<0.010
Low BaP	5.00	5.09
High BaP	50.0	40.7
BaP in Mixture	5.00	4.58
Solvent Control for Triclosan	0	<0.010
Triclosan in Mixture	6.0	2.89

3.3.4 Fish Condition

Condition factor (K) and organosomatic indices (OSIs) are quantitative parameters used as gross indicators of fish health. Condition factor is calculated using total body weight (g) and fork length (cm; Eq.1) and is considered a general indicator of organismal fitness. Organosomatic indices calculated were hepatosomatic index (HSI) and spleen somatic index (SSI). Organosomatic indices indicate the size of a given organ relative to the total size of the individual. OSIs are calculated using organ weight and total body weight (Eq. 2).

$$\text{Eq. 1: } K = \frac{total\ body\ weight\ (g)}{fork\ length(cm)^3} \times 100 \quad (3.1)$$

$$\text{Eq. 2: } OSI = \frac{organ\ weight\ (g)}{total\ body\ weight\ (g)} \times 100 \quad (3.2)$$

3.3.5 DNA Extraction and Next Generation Sequencing

DNA extraction and Next Generation Sequencing (NGS) were carried out as described in Chapter 2 of this thesis. Briefly, microbial DNA was extracted from intestinal samples using the PowerFecal® DNA Isolation Kit (Mo Bio Laboratories, Inc., USA) according to the manufacturer's protocol. The V3 and V4 variable regions of the 16S rRNA gene were isolated according to Illumina's 16S Metagenomic Sequencing Library Preparation (Part # 15044223 Rev. B) and sequenced on an in-house (Toxicology Centre, University of Saskatchewan, Canada) Illumina MiSeq Desktop Sequencer (Illumina, California, USA) according to the manufacturer's protocols.

3.3.6 Bioinformatics and Statistical Analysis

The QIIME application in Illumina Basespace (https://basespace.illumina.com), a cloud-based NGS data analysis software system, was used to automatically demultiplex and quality filter reads, removing sequences below 1250 bp and with more than 50 wobble bases, as well as taxonomically classify reads into OTUs using the Greengenes database based on 97% sequence similarity. Remaining analysis took place using RStudio v.3.6.2 (R Core Team, 2019). OTUs not present in any samples and OTUs with zero reads were removed from the data set along with low abundance (<1% relative abundance) OTUs.

All statistical analyses were conducted in RStudio v.3.6.2 (R Core Team, 2019). Alpha diversities (Shannon index and Chao 1) were calculated using the "phyloseq" package (McMurdie and Holmes 2013) and beta diversities (Bray-Curtis dissimilarity index and UniFrac distances) were calculated using "vegan" (Oksanen et al., 2020). Non-metric multi-dimensional scaling (NMDS) was conducted using the "phyloseq" package (McMurdie and Holmes 2013) followed by Simper analysis using "vegan". Statistical significance was calculated using PERMANOVA (permutational multivariate analysis of variance) (Anderson 2001) after checking assumptions with a multivariate Levene's test (betadisper, Anderson 2006). Homogeneity of dispersion between treatment types was not significant (p=0.2405). Visual representations were produced using "ggplot" (Wickham 2016).

3.4 Results

3.4.1 Survival Rates, Condition Factor, and Organosomatic Indices

Mortality occurred throughout the experiment, including prior to experiments, upon commencing and during recovery. Survival was highest in the LBaP treatment (96%), intermediate in the SC (83%) and HBaP (77%) treatments and lowest in the Mixture (68%). It should be noted that, while months of recovery was allowed, this stock of fish had previously undergone multiple bouts of chemical stress due to high ammonia in the facility water prior to experimental use.

There were no significant differences in condition factor, HSI or SSI in any treatments in comparison to the solvent control (Figure 3.1). Condition factor increased over time across all treatments. There were significant differences in condition factor from Time1 to 2 ($p = 0.009^{**}$) and Time1 to 3 ($p = 0.002^{**}$), but not from Time2 to 3. HSI increased from Time1 to Time2, then decreased at Time3 (recovery) but remained higher than at Time1. There were significant differences between each set of time points ($p = 0.001^{**}$, $p = 0.013^{*}$, $p = 0.005^{**}$). There were no significant differences in SSI (Figure 3.2).

3.4.2 Diversity Analysis

There was a total of 3,996,836 raw sequence reads, with reads ranging from zero to over 400,000 reads per OTU. No treatment was distinctively more or less diverse than any other

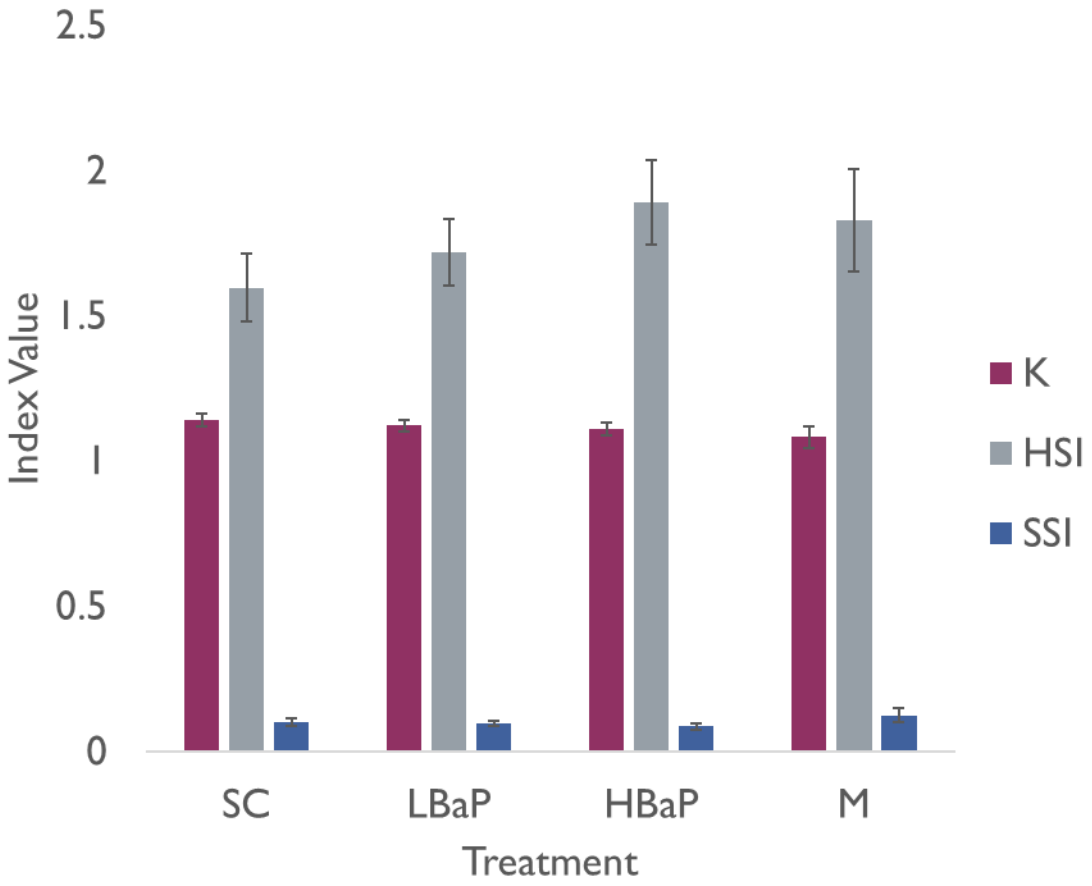

Figure 3.1. Average condition factor (K), hepatosomatic index (HSI), and spleen somatic index (SSI) of samples (±1 standard error) across treatments (SC = solvent control; LBaP = Low BaP; HBaP = High BaP; M = Mixture) for all time points. There were no significant differences in any of these indices when evaluated by treatment. Significant differences were detected using one-way ANOVA (analysis of variance) followed by Tukey's test ($p<0.05$).

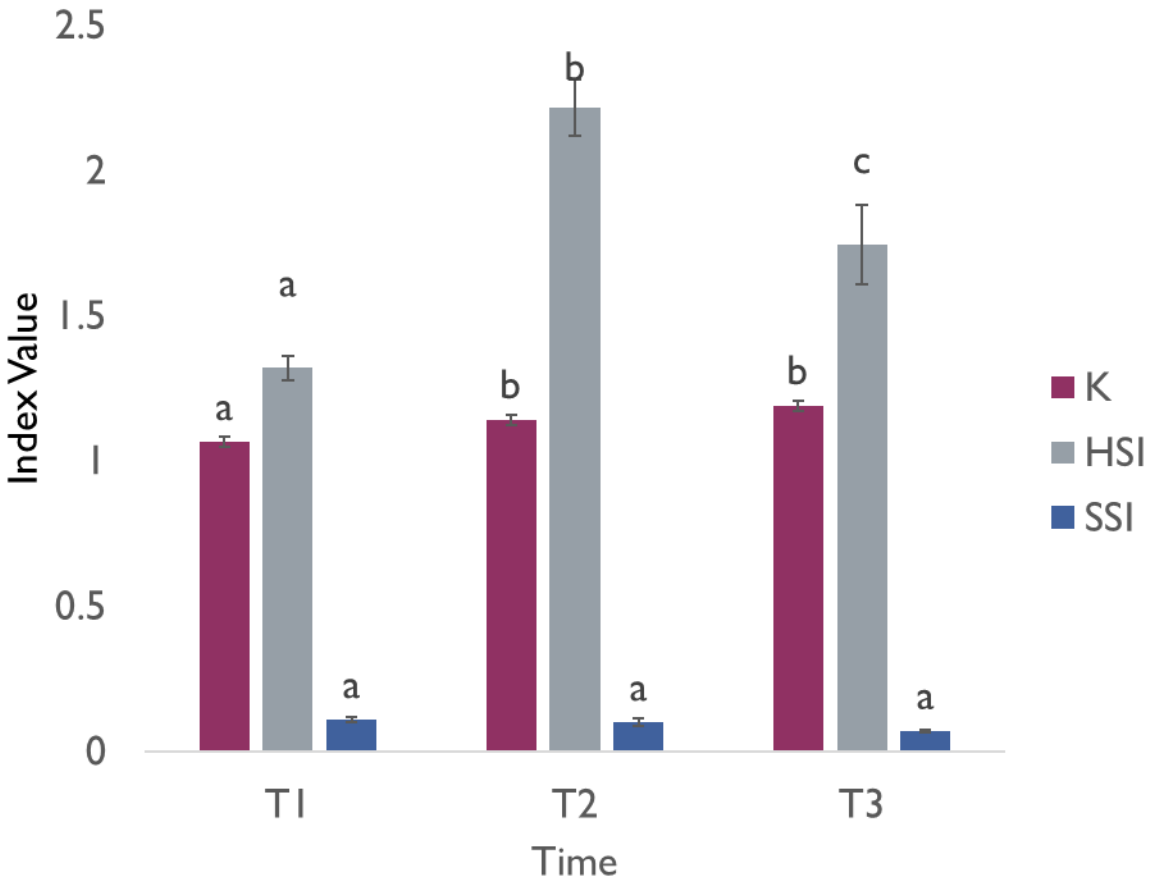

Figure 3.2. Average condition factor (±1 standard error), hepatosomatic index and spleen somatic index across time (T1 = Time1; T2 = Time2; T3 = Time3) for all treatments combined. Significant differences were detected using one-way ANOVA (analysis of variance) followed by Tukey's test ($p<0.05$). Significant differences are indicated by different letters.

treatment according to Chao1 or Shannon Diversity Indices (Figure 3.3). A total of 1144 unique OTUs were identified including 21 distinct phyla (22 including unclassified). Proteobacteria, Unclassified, Firmicutes, Cyanobacteria, Actinobacteria, Tenericutes, Planctomycetes, Fusobacteria made up the eight most common phyla. Of these, Proteobacteria, Firmicutes, Cyanobacteria, and Actinobacteria were common across all treatments and times. Planctomycetes were only present in Solvent Control and Mixture during Time3. 38 OTUs occurred in every sample, with only 14 being classified at a species level; 22 of these were from the phylum Proteobacteria.

3.4.3 Microbiome Data

Cetobacterium somerae and *Calothrix parietina* were two of the most common species amongst all treatments, especially at Time1 and Time3. An unclassified species from *Ureaplasma* genus was among the most common species in all exposure groups, and during Time2 and Time3. Proteobacteria was the dominant phylum in all treatments, with an abundance of 42% or more in each group, followed by Fusobacteria (>10%) in all groups except Mixture and Time2, in which Firmicutes (16.3%) and Tenericutes (17.9%), respectively, were second most abundant (Figure 3.4).

Microbiomes formed individual clusters according to treatment and time, with Solvent Control and Time3 clustering together most distinctly. Adonis tests indicated the interaction between time and treatment had a significant effect on community composition (Treatment p = 0.0233*; Time p = 0.0001***). Microbiomes were also analyzed during each individual time and results indicated community composition of each treatment was significantly different from each other during each period of time (Time1 p = 0.0123*; Time2 p = 0.0306*; Time3 p = 0.0132*). Treatment was also analyzed independent of time. There was no significant difference in the Solvent Control over time while adonis test indicated significant differences for all other exposure groups across time (LBaP p = 0.0007***; HBaP p = 0.0002***; Mixture p = 0.0085**) (Figure 3.5). There were no significant differences in homogeneity of dispersion when time and treatment were analyzed together or independently.

Relative abundance was calculated for the eight most abundant phyla identified across the experiment (Table 3.2). Notably, relative abundance of Proteobacteria increased with exposure

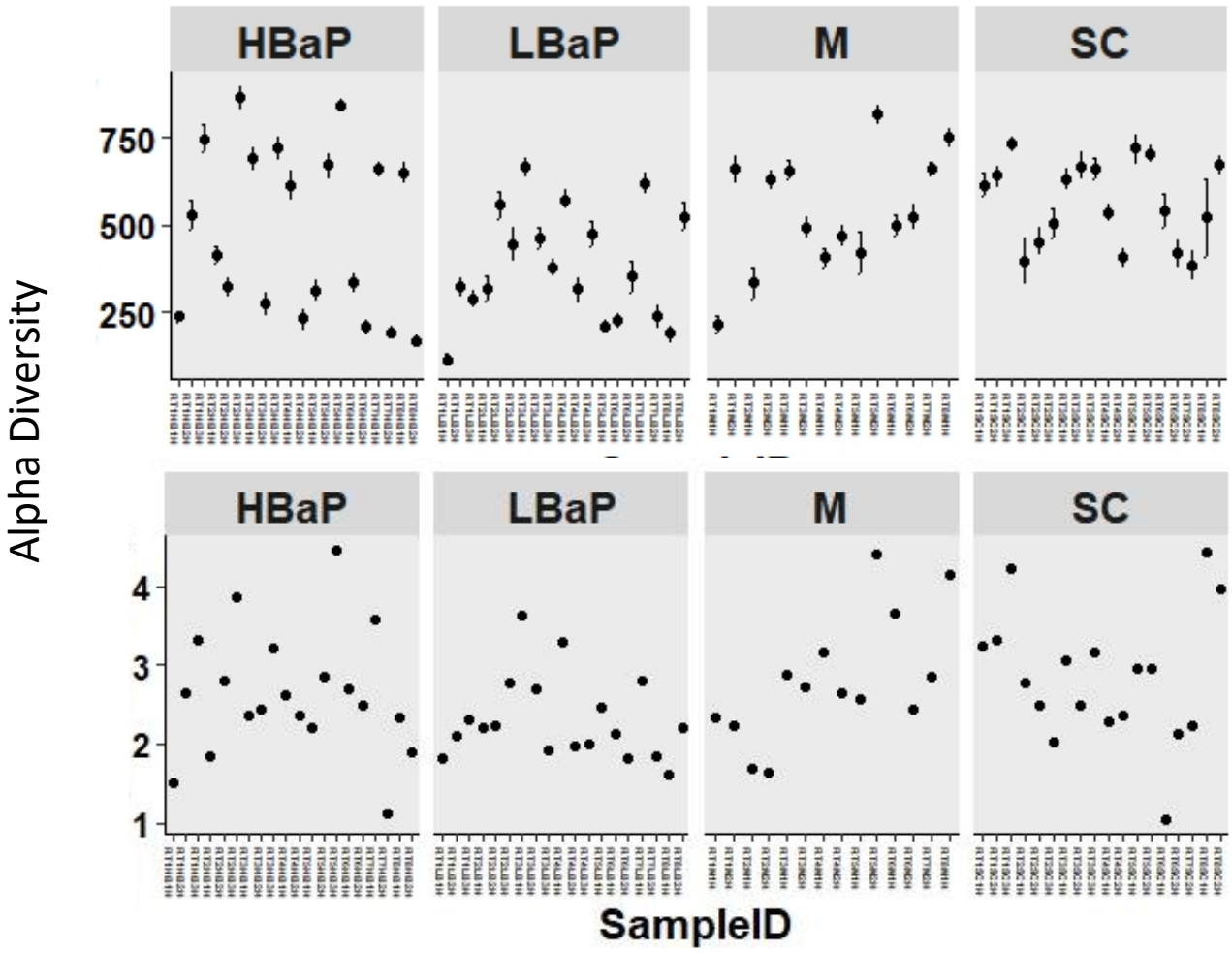

Figure 3.3. Alpha diversity of gut microbiota in rainbow trout (RT) as represented by Chao 1 (top) and Shannon Diversity (bottom) indices. Each dot represents an experimental sample and lines on the Chao1 index represent standard error.

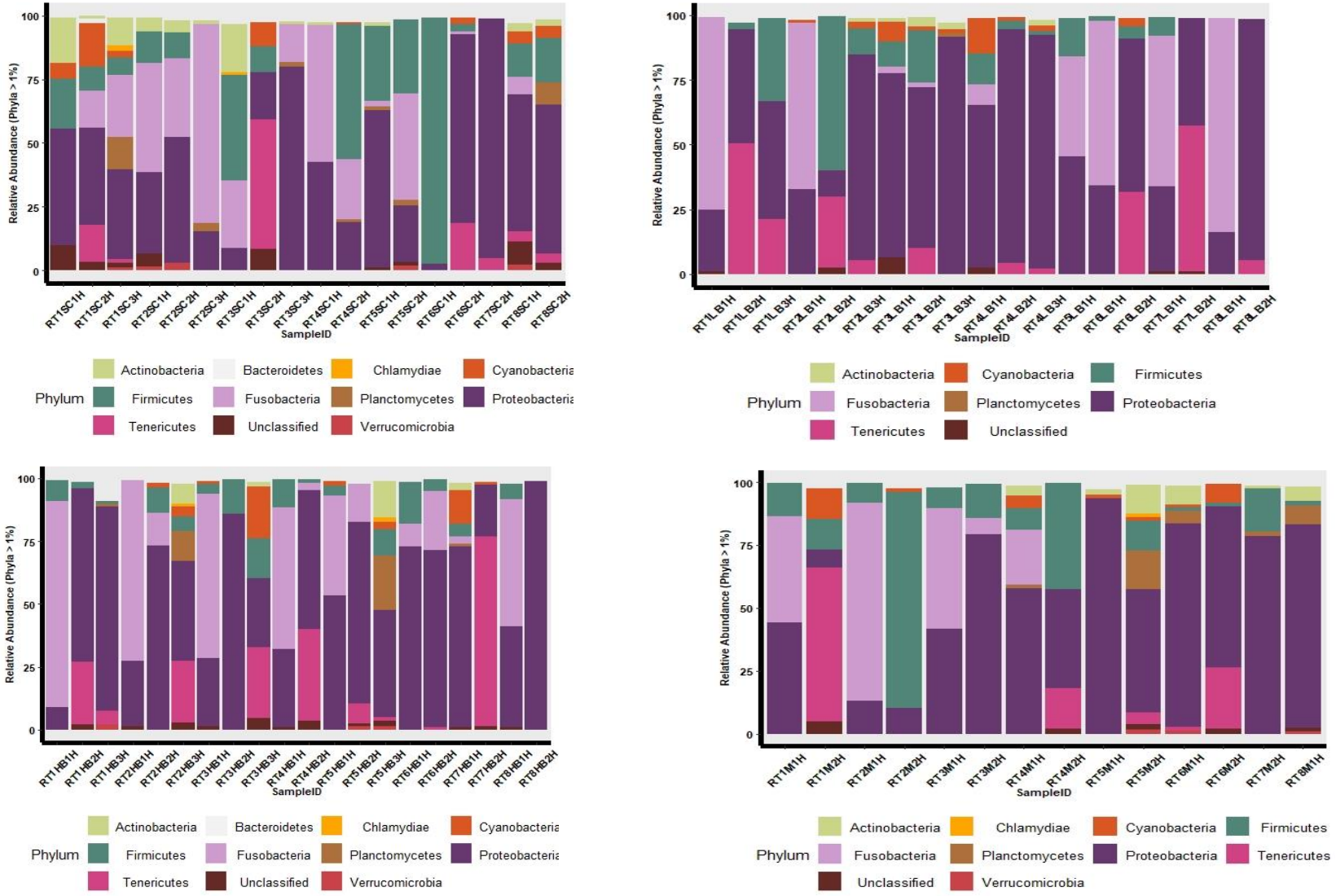

Figure 3.4. Percent relative abundance of phyla separated by treatment: solvent control (top left), low BaP (top right), high BaP (bottom left), and Mixture (bottom right).

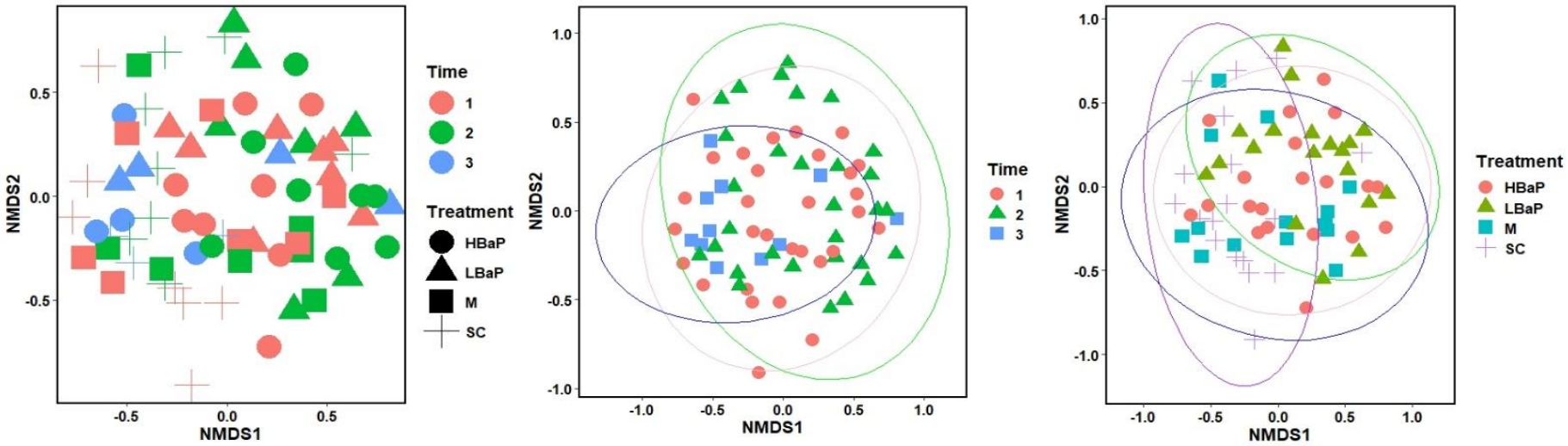

Figure 3.5. NMDS (non-metric multidimensional scaling) analysis of time and treatment (SC = solvent control; LBaP = Low BaP; HBaP = High BaP; M = Mixture) together (left; adonis: Treatment p = 0.0233*; Time p = 0.0001***), time alone (centre; adonis: Time1 p = 0.0123*; Time2 p = 0.0306*; Time3 p = 0.0132*), and treatment alone (right; adonis: LBaP p = 0.0007***; HBaP p = 0.0002***; Mixture p = 0.0085**). 95% confidence ellipses have been added to the time alone and treatment alone graphs.

Table 3.2. Percent relative abundance and percent standard deviation of the top eight phyla in the rainbow trout GI microbiome separated by treatment (SC = solvent control; LBaP = Low BaP; HBaP = High BaP; M = Mixture) and time.

Percent Relative Abundance ± Percent Standard Deviation

	Proteobacteria	Unclassified	Firmicutes	Cyanobacteria	Actinobacteria	Tenericutes	Planctomycetes	Fusobacteria
SC	42.48 ± 25.46	2.45 ± 3.01	19.87 ± 24.89	2.73 ± 5.08	3.95 ± 6.48	5.53 ± 17.49	1.75 ± 4.48	20.40 ± 22.00
LBaP	54.69 ± 26.45	0.80 ± 1.99	9.63 ± 16.21	1.80 ± 4.19	0.62 ± 0.93	11.42 ± 19.80	0.08 ± NA	20.96 ± 32.00
HBaP	54.16 ± 25.16	1.22 ± 0.87	6.10 ± 4.87	2.33 ± 7.14	1.39 ± 5.76	10.38 ± 23.57	1.81 ± 9.87	21.92 ± 28.52
Mixture	53.67 ± 28.57	0.86 ± 1.37	16.29 ± 23.11	2.20 ± 4.23	2.27 ± 3.81	7.80 ± 23.92	2.28 ± 5.69	14.27 ± 27.56
Time 1	44.30 ± 23.60	1.47 ± 3.09	11.97 ± 19.59	1.94 ± 4.67	2.41 ± 6.09	0.19 ± 1.92	0.55 ± 2.95	36.93 ± 26.98
Time 2	55.66 ± 27.47	1.31 ± 1.19	14.83 ± 21.30	2.23 ± 4.95	0.82 ± 3.81	17.89 ± 22.09	0.99 ± 6.12	5.90 ± 13.06
Time 3	58.22 ± 27.61	1.12 ± 0.63	7.80 ± 10.01	2.23 ± 6.80	4.14 ± 4.98	8.26 ± 11.36	4.98 ± 7.89	10.81 ± 34.15

and over time. Firmicutes decreased by approximately half with exposure to LBaP, nearly three quarters with HBaP, and approximately one quarter with the mixture. Actinobacteria also decreased with exposure. Tenericutes increased with exposure approximately twofold with LBaP and HBaP, but only slightly with the mixture. Fusobacteria remained steady across BaP treatments but decreased by approximately one quarter with exposure to the mixture. Proteobacteria and Planctomycetes increased over time. Firmicutes increased at Time2 and decreased at Time3. Tenericutes increased in Time2 and began to recover in Time3. Fusobacteria decreased in Time2 and began to return in Time3.

3.5 Discussion

Several fish studies have identified dominant bacteria in healthy fish similar to the present study. In farmed rainbow trout, these include Proteobacteria (41%), Firmicutes (39%), Bacteroidetes (11%), and Fusobacteria (16% in one group only) (Parshukov et al., 2019). Proteobacteria (>70%), were even more abundant in a study by Kim et al., (2007), with Fusobacteria and Bacteroidetes also being common phyla. Proteobacteria (43%), Firmicutes (20%), and Fusobacteria (20%) were also dominant in the present study. Dominant phyla in other fish species include Fusobacteria (0.2-95%), Firmicutes (13-28%), Proteobacteria (5-63%), Bacteroidetes (4-21%), Planctomycetes (12%), Actinobacteria (10%) and Cyanobacteria (7%) in northern pike (*Esox Lucius*, Reinhart et al., 2019), rainbow darter (*Etheostoma* caeruleum, Restivo et al., 2021), common carp (*Cyprinus carpio L*, van Kessel et al., 2011), paddlefish (*Polyodon spathula*, Yang et al., 2020), channel catfish (*Ictalurus punctatus*), largemouth bass (*Micropterus salmoides*), bluegill (*Lepomis macrochirus*) (Larsen, Mohammed & Arias 2014), tropical gar (*Atractosteus tropicus*, Mendez-Perez et al 2020), and fathead minnow (Debofsky et al., 2020). Additionally, *Cetobacterium*, a genera in the phylum Cyanobacteria, was highly abundant in northern pike (46%) (Reinhart et al., 2019), common carp (van Kessel et al., 2011), paddlefish (30%) (Yang et al., 2020), and tropical gar (42%) (*Atractosteus tropicus*) (Mendez-Perez et al 2020). Specifically, the species *Cetobacterium somerae* was abundant in channel catfish (*Ictalurus punctatus*), largemouth bass (*Micropterus salmoides*) and bluegill (*Lepomis macrochirus*) (Larsen, Mohammed & Arias 2014), as well as rainbow trout in the present study.

This study in combination with other fish gut microbiome studies yields inconclusive evidence of a 'core' microbiome in fish and specifically in rainbow trout. At the phylum level of classification, Proteobacteria were present in similar abundances in rainbow trout, as well as Firmicutes and Fusobacteria. Previous studies have suggested Proteobacteria and Firmicutes make up a core microbiome in rainbow trout (Desai et al., 2012; Sullam et al., 2012; Kim et al., 2007). However, this broad level of classification includes several species, of which abundances could vary greatly amongst studies. Rainbow trout also have several phyla in common with other fish species (Reinhart et al., 2019; Restivo et al., 2021; van Kessel et al., 2011; Yang et al., 2020; Larsen, Mohammed and Arias 2014; Mendez-Perez et al., 2020; Debofsky et al., 2020) but since abundances are highly variable among species, there does not appear to be a 'core' microbiome amongst fish in general. In contrast, specific genera and even species, such as *Cetobacterium* and *Cetobacterium somerae,* respectively, were highly abundant in the present study and fish in previous studies (Reinhart et al., 2019; van Kessel et al., 2011; Yang et al., 2020; Larsen, Mohammed and Arias 2014; Mendez-Perez et al., 2020), as well as across treatments, suggesting such species could comprise a 'core' microbiome.

This study demonstrated that the GI microbiome in rainbow trout may be affected by chemical exposure. These changes were evident in the differential abundance of microbial communities amongst control and exposure treatments, with shifts towards Proteobacteria and away from Firmicutes upon exposure to BaP and triclosan. These changes did not appear to be correlated with changes in measured organosomatic indices or condition factor, which differed little among treatments.

Changes to GI microbiomes can be elicited through altered conditions, such as environment, stress, diet, and chemical exposures. In unhealthy rainbow trout showing internal or external signs of poor health (lesions, fungi, necrosis, tissue discolouration, ulcers, etc.), dominant bacteria included Proteobacteria (54%), Firmicutes (23%), Actinobacteria (10% in one group only), and Tenericutes (32% in one group only; Parshukov et al., 2019). In Atlantic salmon (*Salmo salar*) Actinobacteria and Proteobacteria decreased, while Firmicutes increased when they migrated from fresh water to salt water (Rundi et al., 2018). Firmicutes and Proteobacteria were dominant in stressed Atlantic salmon, along with Tenericutes, Actinobacteria, and Planctomycetes (Webster et al., 2020). Tenericutes (6%) were also found in the present study but had not previously been

identified as a core microbe in healthy fish, suggesting that this taxon may be diagnostic of general stress.

Proteobacteria increased slightly with exposure in the present study and in rainbow darter downstream of a wastewater treatment plant (Restivo et al., 2021). The opposite occurred in common carp (*Cyprinus carpio* L) exposed to copper (Meng et al., 2018) and in Western mosquitofish (*Gambusia affinis*) and zebrafish (*Danio rerio*) following exposure to BaP (Xie et al., 2020). Restivo et al. (2021) suggests Proteobacteria as a potential indicator of stress, which could indicate our rainbow trout were stressed when exposed to all treatments.

Common carp exposed to copper (Meng et al., 2018), rainbow darter exposed to wastewater (Restivo et al., 2021), Atlantic cod (*Gadus morhua*) exposed to PAHs in the form of crude oil (Bagi et al., 2018), rainbow trout exposed to a pathogen (*Flavobacterium psychrophilum*, Valdez et al., 2020), and exposed rainbow trout in the present study all had lower proportions of Firmicutes relative to controls. Yet Firmicutes increased following exposure to BaP in Western mosquitofish and zebrafish (Xie et al., 2020), suggesting responses may be species specific. Decreases in Firmicutes have been implicated in effects on energy metabolism in mice exposed to cadmium (Zhang et al., 2015). Alternatively, an increase in Firmicutes in mice exposed to antibiotics increased adiposity (Cho et al., 2012). While Firmicutes in all treatment groups decreased from the solvent control in the current study, the abundance of Firmicutes in the mixture increased relative to both BaP treatments. Previous research has noted an increase in pathogenic staphylococcus (phylum Firmicutes) in fish after BaP exposure and suggested the link between the two could reduce immune function and increase susceptibility to infection (Xie et al., 2020). It is possible that triclosan in our mixture allowed the observed increase in Firmicutes over levels in BaP alone. The same pattern was observed for Actinobacteria, where relative abundance was higher in the solvent control and mixture, compared to both BaP exposures.

Bagi et al. (2018) studied Atlantic cod (*Gadus morhua*) exposed to PAHs in the form of crude oil and found Tenericutes decreased with exposure, opposite from what occurred in our study with rainbow trout, where relative abundance was lower in the solvent control and mixture and increased with low and high BaP exposures. Tenericutes having been previously associated with stress in fish (Webster et al., 2020) along with our results, suggest BaP induces stress in rainbow trout at environmentally relevant concentrations in food. In both Actinobacteria and

Tenericutes, triclosan in the mixture appeared to be counteracting or mitigating effects induced by BaP. As an antimicrobial, triclosan could have supressed bacteria such as Tenericutes that increased due to BaP exposure, or it was supressing bacteria that compete with Actinobacteria, allowing the Actinobacteria to return to levels resembling the control.

Fusobacteria decreased in Atlantic cod (*Gadus morhua*) exposed to PAHs (Bagi et al., 2018), while they increased in common carp when exposed to copper (Meng et al., 2018). In the present study, Fusobacteria remained relatively constant across the solvent control, low BaP, and high BaP, but decreased in the mixture by approximately 25%. This change could indicate triclosan had a direct effect on the abundance of Fusobacteria in the rainbow trout GI microbiome.

An increase in Cyanobacteria occurred in common carp exposed to copper (Meng et al., 2018) and in rainbow darter downstream of a wastewater treatment plant (Restivo et al., 2021). Very little change in this phylum occurred in the present study, with Cyanobacteria slightly decreasing in all exposure groups when compared to the solvent control. More studies with these rarer taxa are needed to understand when and why they might respond to host chemical exposure.

Fish condition and other endpoints have been correlated with microbial changes in previous studies. Cetobacterium negatively correlated with fish condition in tilapia (*Oreochromis niloticus*) when exposed to experimental diets (Parata et al., 2020). Diets were either a raw vegetable diet or a pellet diet with occasional supplementation of raw vegetables, with poorer condition being observed in vegetable fed fish. Goldfish (*Carassius auratus*) exposed to pentachlorophenol experienced hepatic damage and growth inhibition that was correlated with an increase in Bacteroidetes and a decrease in Firmicutes (Kan et al., 2015). The latter change was comparable to the decrease in Firmicutes we observed in rainbow trout upon chemical exposure. Due to the limited number of organs collected and evaluated, we are unable to identify correlations with other adverse outcomes that may have occurred in the exposed fish as a result of the present chemical exposures.

3.6 Conclusion

The composition of the GI microbiome in rainbow trout was significantly affected by both treatment and time; however, the functional roles of such bacteria in fish are not well known. Therefore, it is difficult to attribute bacterial changes to possible adverse affects. Future studies

should look more specifically at how the changes in key bacteria are affecting normal biological functions in fish. Additionally, in the present study, it is difficult to attribute any changes observed in the mixture to interaction effects or triclosan since triclosan alone was not able to be evaluated.

Changes have also been identified in other fish species and exposure scenarios; however, changes identified are highly variable among studies. There are many factors that may contribute to this variation, and many of these may be attributed to there being no standardized method of testing or reporting for microbial composition endpoints. Contributing factors may include varying bacterial regions (V1-V9) being isolated and sequenced, use of different sequencing platforms, sampling methods, culture conditions, regions of sampling, species variability, variability between fish of the same species, and level of bacterial classification being reported. These factors make comparisons among studies challenging.

CHAPTER 4: GENERAL DISCUSSION

4.1 General Findings

I used lab-reared fish under controlled conditions to explore the effects of two common chemicals on the GI microbiome in fish. First, I developed a method to extract and characterize the microbiome in the GI tract of fish using rainbow trout and lake sturgeon. Two rainbow trout were exposed to BaP via IP injection, one was untreated and exposed to corn oil (control) via IP injection, and one was not exposed to any experimental conditions. The lake sturgeon were not exposed to any experimental treatments. In this study, I found the composition of the GI microbiome may be influenced by species identity. Next, I used the same methods to characterize the GI microbiome in rainbow trout exposed to BaP, and a mixture of BaP and triclosan. I found both time and chemical exposure affect the GI microbiome in rainbow trout. To determine if these changes in the GI microbiome were accompanied by changes in general fish health, I compared body condition and organ sizes of fish in the different treatment groups. I found no significant changes, suggesting microbial changes may occur more readily than gross anatomical effects. Lastly, I identified five taxa (Proteobacteria, Firmicutes, Actinobacteria, Tenericutes, and Fusobacteria) that could be used as biomarkers of pollutant exposure, but the direction of changes in these taxa were often at odds with patterns observed in the literature.

Together with the increasing numbers of studies that confirm connections between changes in GI microbiomes and adverse health outcomes, the current findings suggest potential novel pathways for physiological disturbance associated with chemical exposure that is linked to gut flora dysbiosis. Effects of altered GI microbiomes on host health include inhibited growth and hepatic damage (Kan et al., 2015), decreased fish condition (Parata et al., 2020), and reduced immune function (Xie et al., 2020). While I did not observe changes occurring at these higher levels, they may have manifested if the experiment was carried out for a longer duration such as could occur under chronic exposures near industrial sites or downstream from wastewater treatment plants.

4.2 Comparison of Trial Study and Experimental Data

In the pilot study, 1603 OTUs were identified, with Fusobacteria (57%) and Proteobacteria (27%) dominating the lake sturgeon GI microbiome, while Firmicutes (78%) dominated the

rainbow trout microbiome. Cyanobacteria and Tenericutes were only identified in rainbow trout, while no phyla were specific to lake sturgeon. The most dominant species in lake sturgeon were *Cetobacterium somerae* and *Tolumonas aurensis*. Rainbow trout were dominated by *Carnobacterium maltaromaticum* and *Carnobacterium gallinarum*. *Cetobacterium somerae* contributed to 23% of the differences between rainbow trout and lake sturgeon, with *Carnobacterium maltaromaticum* (13%) and *Tolumonas aurensis* (9%) being the next highest drivers of differences.

In the dietary exposure study, 1144 OTUs were identified with 38 OTUs occurring in every sample. Proteobacteria (42-55%), Firmicutes (6-20%), Tenericutes (6-12%), and Fusobacteria (14-22%) were the most common phyla amongst rainbow trout. This contrasts with the results of the pilot experiment, where Firmicutes were more highly abundant (78%). However, this is consistent with other experimental studies in rainbow trout where Proteobacteria (41->70%) were also the dominant species in the GI tract (Parshukov et al., 2019; Kim et al., 2007). As experimental conditions were largely the same between my pilot and experimental study (temperature, facility, brand of fish food), this difference is likely driven by the small sample size of rainbow trout in the pilot study or fasting of the fish in the pilot study prior to sampling. Other contributing factors may have been the age of the fish, the way in which BaP was administered (IP injection vs. diet), or fish health. RTF5, a control fish in the pilot study, was sacrificed due to poor health as a result of unknown causes. The remaining fish in the pilot study appeared to be in good health and would not have undergone ammonia stress as many times as the fish in the experimental study.

Cetobacterium somerae was one of the most common species in both experiments; however, it was most common in lake sturgeon in the pilot experiment, not rainbow trout. Again, since other experimental conditions were similar, including experimental facility, temperature, and brand of fish feed, this similarity may have been due to sample size or other confounding stressors (i.e., fish used in the dietary exposure studies were of relatively poor general health). The lake sturgeon sample included 22 unexposed fish, while 90 rainbow trout were used in the experimental study, allowing for a more accurate representation of a common microbiome in each fish species.

4.3 Future Research

Future research in the field of GI microbial populations in fish should focus on expanding the general knowledge base and developing standardized experimental procedures for studies of

this nature. Currently there is no standardized method of testing or reporting and multiple ways to conduct and carryout downstream analysis of experimental data. Different sequencing platforms, sampling methods, regions of sampling, varying regions (V1-V9) of sequencing, species variability, variability between fish of the same species, etc. makes comparisons between studies difficult and inconsistent. Therefore, future research should focus on developing standardized methods for conducting both lab and field studies, extracting and identifying the GI microbiomes present, and evaluating and reporting data.

The pilot study indicates phylogeny may also contribute to differences in the GI microbiome. Despite the same laboratory rearing conditions and habitat in the wild, lake sturgeon and rainbow trout had distinct GI microbial communities. This could be explained by lake sturgeon being an ancient fish, and the two species being only distantly related. Future studies could explore this idea and delve deeper into phylogenetic relationship amongst fish and how they may contribute to gut microbial composition, particularly a 'core' microbiome, that may have evolved symbiotically with ancient fish such as the lake sturgeon.

There is still much research that needs to be conducted in the field of GI microbial studies, specifically in fish. Multi-omics approaches that link genomics with metabolomics, proteomics and transcriptomics could reveal stronger ties between changes in microbial populations and host function. Without these cause-effect relationships, microbial changes could have little ecological implications.

4.4 Conclusion

Increasing research on the GI microbiome in fishes is beginning to identify exposure situations in which adverse affects are occurring. The present studies illustrate the differences that can occur in fish of different species, as well as those of the same species, reared in the same facility under similar conditions, and fed the same diet. Factors contributing to these differences could include route of exposure, environmental stress, age, or evolutionary differences. I identified changes in Proteobacteria, Firmicutes, Actinobacteria, Tenericutes, and Fusobacteria, that all have potential use as biomarkers of exposure. However, there is currently either a lack of literature to support these findings, or studies with contrasting conclusions. This demonstrates the need for consistency in methodology when conducting such experiments, so studies can more easily and accurately be evaluated and compared. Once these procedures are established,

connections can begin to be made between adverse health outcomes and changes in the GI microbiome. Only then can researchers really begin to identify accurate and reliable biomarkers of exposure in fish, ideally using non-lethal sampling methods.

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