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Toxicity and Effects of Tire Crumb Rubber in the Aquatic Environment

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TOXICITY AND EFFECTS OF TIRE CRUMB RUBBER PARTICLES IN THE AQUATIC ENVIRONMENT

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ABSTRACT

Plastic materials have provided innovative solutions to society's evolving needs and challenges. Due to their durability and resistance to degradation, plastics remain in the environment for long periods of time and can therefore be transported to many environmental compartments such as water, sediment, and biota. Microplastics (MPs) have been defined as synthetic plastic particles that have at least one dimension less than 5 mm and are insoluble in water. Microrubber (MR), a recently acknowledged sub-group of MPs, has been documented in environmental samples more recently, frequently comprising a large portion of total MPs of various samples. In South Carolina specifically, suspected tire wear particles (TWP) have been found abundantly in sediment and water in the Charleston Harbor area, potentially originating from road runoff near major highways and bridges. Recent studies on MR have demonstrated toxicity in a variety of organisms, but with mixed results due to the complexity of the composition of MR and differences in test conditions.

The goal of this research was to determine the toxicological impact of MR particles on aquatic organisms. To achieve this goal, the first objective addressed acute toxicity from exposure to MR particles and measured bile fluorescence as a biomarker for polycyclic aromatic hydrocarbon (PAH) absorption, metabolism, and biliary excretion, ethoxyresorufin-O-deethylase (EROD) activity as an indicator for cytochrome P450-1A (CYP1A) activity, and glutathione S-transferase (GST) activity as an indicator for oxidative stress. The second objective investigated toxicity under environmentally relevant conditions through a pulsed, chronic exposure. Immunohistochemistry (IHC)

was utilized to visualize CYP1A induction in various tissues in addition to biomarker analyses for DNA damage, membrane damage, and oxidative stress. Finally, the third objective was to understand the environmental impact and fate of MR by analyzing MP abundance in biota collected from stormwater ponds.

The results from acute toxicity tests revealed that bile fluorescence increased and CYP1A activity is induced as MR concentration increased, suggesting that PAHs are leaching from MR particles in the aquatic environment. Two fish species were utilized in acute exposures, the estuarine fish *Fundulus heteroclitus* (mummichog) and freshwater fish, *Pimephales promelas* (fathead minnow). Partial mortality was observed in *P. promelas* suggesting potential greater toxicity in freshwater conditions compared to estuarine or marine environments.

Chronic toxicity tests were the first to our knowledge that utilized whole MR particles in exposures at environmentally relevant concentrations (< 0.2 g/L) in fish. Immunohistochemistry of *F. heteroclitus* gill, intestine, and liver indicated strong induction of CYP1A in gill and liver cells and vasculature, with mild induction in intestinal cells and suggests that aqueous exposure to MR and thus MR leachate exerted a more prominent response compared to ingestion of particles themselves. Additionally, bile fluorescence increased as MR concentration increased as observed in acute exposures. Other biomarker tests indicated that antioxidant defenses were upregulated to prevent cellular damage as measured through an increase in the DNA damage byproduct 8-hydroxy-2'-deoxyguanosine (8-OHdG), a decrease in malondialdehyde (MDA)

production indicating less lipid peroxidation, and an increase in the antioxidant, glutathione (GSH) when MR concentration increased.

Finally, the abundance of MP and MR in field-collected organisms from coastal stormwater ponds was measured by visual microscopy of digested animals. Stormwater ponds are hot spots for environmental pollution, including MR from road runoff. The majority (>80%) of MP recovered from biota across all sampling sites were suspected tire particles. The average number of MP per individual ranged from 0.3 to 71 MP and the average number of suspected tire particles per individual ranged from 0 to 57.7 tire particles. There were significant differences observed in the number of MP per individual between sites and between species. A combination of factors such as availability of MPs based on surrounding land use, stormwater pond dynamics, organism size, and organism feeding habitat influenced the total MP observed.

Overall, these data indicate that MR particles and their associated compounds exert a toxic effect on aquatic species and that stormwater ponds serve as a sink for MR accumulation in the environment.

DEDICATION

I dedicate this dissertation to my family. Your support and encouragement made all the difference. Thank you for always believing in me and instilling in me a passion for the natural world and scientific curiosity.

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TABLE OF CONTENTS

Page

Table of Contents (Continued)

Page

Table of Contents (Continued)

Page

LIST OF TABLES

LIST OF FIGURES

List of Figures (Continued)

Figure Page

CHAPTER ONE

LITERATURE REVIEW

1.1 Introduction

Plastic materials have provided innovative solutions to society's evolving needs and challenges. Global plastic production in 2019 was approximately 370 million tonnes (PlasticsEurope, 2020). Plastic is a synthetic organic polymer that can be manufactured into different types for a variety of uses including packaging, building and construction, automotive, and electrical uses. Major classes of plastics include polyethylene (PE), polyethylene terephthalate (PET), polypropylene (PP), and polyvinyl chloride (PVC). In addition to these common thermoplastics, rubber is also considered a class of plastic (Kole et al., 2017). Global natural rubber production in 2019 was approximately 29 million tonnes (Malaysian Rubber Council, 2019) and the top three products produced were gloves, tires, and hoses.

In recent years there has been ample research on the abundance of plastic debris in the environment as well as its documented adverse effects on various terrestrial and aquatic organisms (Harrison & Hester, 2019). Due to their durability and resistance to degradation, plastics remain in the environment for long periods of time and can, therefore, be transported to many environmental compartments such as water, sediment, and biota. Microplastics (MPs) have been defined as synthetic plastic particles that have at least one dimension less than 5 mm and are insoluble in water (Andrady, 2011; Frias & Nash, 2019). Microplastic sampling methodologies and the identification of specific polymers have improved in recent years, allowing for researchers to determine

microplastic sources and investigate risk of microplastic exposure to a variety of organisms. Publications on microplastics have increased exponentially in the past decade but significant gaps in the assessment of organisms' response to microplastic exposure remain (Granek et al., 2020).

A recent survey of the presence of MPs in coastal South Carolina showed that the most abundant particles were black fragments (Gray et al., 2018). In a different study, black fragments that were found in the intertidal sediments, subtidal sediments, and sea surface microlayer in Charleston Harbor, South Carolina, were identified as crumb rubber particles and formed 17.1% of the total microplastics encountered (Leads & Weinstein, 2019). In recent years, the amount of crumb rubber particles in the environment has been documented worldwide at increasing amounts. The release of wear and tear from tires results from contact between the road surface and the tire which generates particles and fragments of different shapes and sizes which can then enter the environment (Kole et al., 2017). The amount and size of the particles released is dependent on multiple factors such as temperature, climate, tire composition, road surface, driving speed, and driving style (Kole et al., 2017). The estimated emissions of tire wear and tear for different countries has been calculated and ranges from 6,721 tonnes/year in Denmark to over 1.5 million tonnes/year in the USA (Kole et al., 2017). Other estimates reported include China with 756,240 tonnes/year, India with 292,674 tonnes/year, and Germany with 92,594 tonnes/year (Kole et al., 2017). These large estimates for emissions, coupled with the persistent nature of rubber and plastic polymer materials in the environment, could

mean that crumb rubber particles may represent a large percentage of total microplastics in the environment (Halle et al., 2020).

Research on the effects of MPs to marine organisms has demonstrated the ability of MPs to interact with a variety of organisms due to their small size. Studies have shown ingestion of microplastics in whales, fish, mussels, oysters, shrimps, and copepods (Cole et al., 2013; Gall & Thompson, 2015; Kuhn et al., 2015; Lusher et al., 2013). There have been mixed results in observed adverse effects from MP ingestion among organisms, but toxicity associated with MPs can generally be attributed to one or more of the following factors:

(a) Residual monomers from manufacture present in the plastic or toxic additives used in production may leach from ingested plastic (i.e. bisphenol A (BPA) or polybrominated diphenyl ethers (PBDE)) (Hermabessiere et al., 2017).

(b) Toxicity of intermediates from partial degradation of plastics.

(c) The persistent organic pollutants (POPs) present in the environment are absorbed and concentrated in microplastic fragments which can become bioavailable to organisms when ingested (i.e. polychlorinated biphenyls (PCBs) (Andrady, 2011; Engler, 2012).

The emergence of crumb rubber as a frequently encountered microplastic in aquatic systems is a relatively new breakthrough in microplastic research. Recent studies on crumb rubber particles have focused primarily on their generation, properties, occurrence, and detection in the environment (Kole et al., 2017; Wagner et al., 2018). Most toxicity studies thus far have investigated toxicity of crumb rubber or whole tire

leachates in aquatic organisms (Hartwell et al., 1998; Panko et al., 2013) and there is limited information on toxicity of crumb rubber particles to aquatic organisms. The purpose of this literature review is to examine the existing knowledge base on the effects of crumb rubber on aquatic organisms. Tire and rubber particle terminology is defined, the physical and chemical composition of particles are discussed, and environmental concentrations of MR are presented. Next, current knowledge on the acute and chronic toxicity of MR is evaluated. Finally, the knowledge gaps are identified and objectives for this dissertation are addressed.

1.2 Tire and rubber particle definitions

There are multiple definitions for crumb rubber and tire particles that are often used interchangeably in the literature. The three major tire-related terms commonly found in the literature include (1) tire-road-wear-particles (TRWP) which refers to a mixture of tire tread particles and road dust (Marwood et al. 2011), (2) tire wear particles (TWP) which describes particulates formed from tires undergoing friction with the road (Wagner et al. 2018), and (3) crumb rubber (CR) from end-of-life tires (ELT) which refers to worn tires that are repurposed for different applications such as rubber-modified asphalt, playgrounds, or sports fields (WBCSD, 2008). Microrubber (MR) has recently been used as an umbrella term to cover all rubber particles in the micrometer size-range (up to 1 mm) which encompasses TRWP, TWP, and CR particulates (Halle et al. 2020). Additionally, Halle et al. (2020) recently proposed the use of the three above mentioned tire-related terms but clarified the definitions of each as

(1) Tire-road-wear particles (TRWP): includes all particles abraded from tires used on the road.

(2) Tire wear particles (TWP): particles abraded from tires that do *not* include road wear (i.e. particles generated for laboratory experiments under manual abrasion or simulated road conditions).

(3) End-of-life-tires (ELT): includes worn tires that are repurposed for alternative applications (for example, artificial turf) and are ground into crumb rubber (CR) which can be further classified by size fraction.

TRWP are the likely particle form sampled from the environment as they represent particles abraded from driving on the road combined with other car emissions and road dust whereas TWP are unlikely to be sampled from the environment as they are generally generated under simulated conditions for laboratory experiments (Halle et al., 2020). In addition, 'tire crumb rubber' (TCR or CR) has been used recently to describe the fraction of recycled and shredded end-of-life tires (Hüffer et al., 2019). Hüffer et al. (2019) suggested that tire materials are introduced to the environment through two main pathways – from traffic non-exhaust emission caused by abrasion between the tire and road (entering as TRWP) and as tire crumb rubber (TCR) from ELTs that is commonly used as a construction material for turf fields, playgrounds, or embankment fill material. Therefore, the terminology adopted for this literature review follows

(1) Microrubber (MR): umbrella term for tire and rubber particulates that are less than 1 mm in size, which includes TRWP, TWP, and CR particles.

(2) Tire-and-road-wear particles (TRWP): particles generated from friction between tires and the road surface which may include mineral encrustations and are likely sampled from the environment.

(3) Tire wear particles (TWP): particles abraded from tires that do *not* include road wear (i.e. particles generated for laboratory experiments under manual abrasion or simulated road conditions).

(4) Crumb rubber (CR): particles generated from the fraction of recycled and shredded end-of-life tires that may be used in construction applications.

1.3 Microrubber composition

1.3.1 Tire composition

The composition of tires varies depending on the application of the tire. In general, tires are made mostly of a rubber polymer with fillers, reinforcing agents, processing aids, accelerators and retarders, adhesives, and activators potentially added. Rubber polymers are divided into either natural rubber or synthetic rubber. Natural rubber is derived mostly from the rubber tree (*Hevea brasiliensis*) which is presently the largest commercial source of natural latex (van Beilen & Poirier, 2007). Natural rubber is a liner polymer of isoprene (2-methyl butadiene) with high structural regularity, allowing for crystallization at low temperatures or when stretched but also elasticity and flexibility (Wagner et al., 2018). Sulfur vulcanization of natural rubber latex and the addition of carbon black filler results in the production of a strong, heat resistant material that is preferentially used in high-performance tires typically found on aircrafts, trucks, and buses (Wagner et al., 2018). Synthetic rubber types are petroleum-based rubbers and can

include styrene-butadiene, chloroprene, or nitrile rubbers (Ciesielski, 1999). Although a mixture of synthetic rubber types along with natural rubber are used in typical passenger car tires, styrene-butadiene rubber (SBR) is the primary synthetic rubber component of passenger car tires. Carbon black is the second most abundant material in passenger tires acting as a filler to make tires more resistant to wear as well as protection against ultraviolet radiation. Carbon black is a manufactured fine-particle product that are nearly pure elemental carbon in chemical composition (Long et al. 2013). Other chemicals can be incorporated to improve tire performance and durability such as crosslinking or vulcanization agents (i.e., sulfur), accelerators, zinc oxide, antioxidants, or plasticizers (Hirata et al., 2014). **Table 1.1** depicts typical tire composition with approximate percentages of different ingredients reported in the literature. A wide range of different chemicals may be used as protective agents and processing aids in the manufacture of tires, leading to an array of chemically different tires dependent upon manufacturer, tire type, and intended tire use.

1.3.2 Particle composition

Similarly, to plastics, microrubber can come in a variety of sizes and range from 0.001 μm for ultrafine particles to > 2 mm for coarse particles (Bowman et al., 1994; Wagner et al., 2018). In general, MPs can either be from primary or secondary origin. Primary MPs are intentionally manufactured to have micron-sized dimensions, whereas secondary MPs have fragmented from a larger plastic product, eventually reaching micron-sized dimensions (Weinstein et al., 2016). On the other hand, rubber and tire pieces are mainly from secondary origin where they are either particulates from tire

Table 1.1 – General composition of tires reported in various studies.

Category	Content (wt%)	Ingredients	Reference
Rubber	40 – 60	Synthetic and natural rubbers	Wik & Dave, 2009
Reinforcing agent or filler	22 – 40	Carbon black	Kole et al., 2017
	20 – 35	Carbon black, silica, silanes	Wik & Dave, 2009
Processing oil	12 – 15	Mineral oils	Wagner et al., 2018
	15 – 20	Mineral oils (high aromatic w/ high PAH* content)	Wik & Dave, 2009
Textile and metal	5 – 10		Wagner et al., 2018
Vulcanization agent	1 – 4	Sulfur	Kole et al., 2017
	1	Sulfur	Wik & Dave, 2009
	1	Zinc oxide	Kole et al., 2017
	1.5	Zinc oxide	Wik & Dave, 2009
	1	S, Se, Te, thiazoles, organic peroxides, nitro-compounds	Grigoratos & Martini, 2014
Additives	5 – 10	Preservatives, antioxidants, desiccants, plasticizers, processing aids	Wagner et al., 2018
	1	Antioxidants	Wik & Dave, 2009
	< 1	Plasticizers and softeners	Wik & Dave, 2009

* Polycyclic aromatic hydrocarbon (PAH)

abrasion (TRWP or TWP) or repurposed rubber granulate (CR) ground from whole worn tires (Halle et al., 2020).

Physical characteristics of microrubber particles typically include black in color, fragmented or irregular shape, elongated or cylindrical shape, partially or entirely covered with road dust, rough surface texture, and rubbery flexibility when manipulated with forceps (Leads & Weinstein, 2019; Parker et al., 2020). The density of particles ranges from 1.13 to ≥1.9 g/cm^3 depending on particle type (Klöckner et al., 2019; Rhodes et al., 2012; Unice et al., 2019). For example, TRWP, which contain road dust and mineral encrustations, may have an average density of 1.8 g/cm^3 (Klöckner et al., 2019; Unice et al., 2019) and TWP generated from recycled crumb rubber may have a density

between 1.13 – 1.16 g/cm^3 (Rhodes et al., 2012). Klöckner et al. (2019) found the greatest recovery of tire particles from sediment samples using separation solutions of >1.9 g/cm^3. Leads and Weinstein (2019) suggested that low-density TRWP (≤ 1.17 g/cm^3) may represent the mobile fraction of TRWP in the environment due to their distribution in various environmental samples including intertidal sediment, subtidal sediment, and the sea surface microlayer. Given that the average density of seawater is 1.025 g/mL, one would expect the majority of MR particulates to eventually be deposited in sediments; however, turbulence in the water column, inputs from rain, and road dust contributions could all influence the transport and distribution of MR particles in the environment. Factors such as pavement properties or composition, temperature, driving style (accelerating, breaking), vehicle characteristics (weight, engine power), and tire characteristics (size/width, composition, accumulated mileage) can influence MR emission, particle size, and particle morphology (Leads & Weinstein, 2019; Wagner, 2018; Wik & Dave, 2009). Therefore, it is difficult to predict the ultimate environmental fate of microrubber particulates.

The chemical composition of MR varies depending on the generation processes, size, and the initial composition of the source material. The general chemical composition of MR can include a mixture of the following components: polybutadiene, styrene-butadiene, neoprene isoprene, carbon black, and silica with additives such as mineral oils, thiazoles, organic peroxides, aromatic and aliphatic esters, ZnO, and S (Hüffer et al., 2019). The components of specific MR particles generally reflect the initial composition of the tire source material. However, MR particulates can also differ chemically from

their initial tire material under different environmental conditions and scenarios. Common components found in field-collected MR, such as TRWP or CR from recycled tires, that may originate from the environment include metals such as Fe, Ca, Sb, Zn, polycyclic aromatic hydrocarbons (PAHs), and benzothiazoles (Wagner et al., 2018).

1.3.3 Chemical sorption to MR particles

It has been suggested that MR acts as a vector for pollutants (Halle et al., 2020). The ratio of crystalline to amorphous regions in polymers can influence the sorption of organic contaminants to different polymers. Amorphous regions in polymers have greater flexibility and softness and are prone to interact with foreign substances contrary to crystalline regions which are more rigid (Fried, 2014; Halle et al., 2020). Elastomers such as rubber typically have more amorphous than crystalline regions as reflected by their rubbery consistency. Tire material has been shown to have high sorption capacities for organic molecules and has been suggested for use to remove organic contaminants from water, similar to activated carbon (Alamo-Nole et al., 2012). Because the composition of tire and rubber materials and thus MR particulates is dominated by styrene butadiene rubber (40 – 60%) and carbon black (20 – 35%), MR serves as a good sorbent material for organic chemicals (Wik & Dave, 2009). Hüffer et al. (2019) speculated that the mechanism for sorption of organic sorbate molecules (i.e., PAHs) to crumb rubber was a combination of adsorption onto carbon black surfaces that are exposed on crumb rubber surfaces and absorption into the rubber matrix and onto carbon black surfaces. Alamo-Nole et al. (2012) observed a similar mechanism when testing the removal of two PAHs by tire crumb rubber, carbon black, and styrene-butadiene polymer. In their study, the

sorption of PAHs by carbon black was slightly higher than the sorption by tire crumb rubber. The authors suggested that adsorption onto the carbon black surfaces was due to van der Waals forces and the absorption into the tire matrix was a physical process following partitioning with the hydrophobic organic molecules transported from the water to the hydrophobic sorbents. Sorption of nonpolar sorbates to tire material sorbents is primarily driven by the hydrophobicity of sorbates with stronger sorption observed for nonpolar compounds vs. polar compounds (Schwarzenbach et al., 2016).

1.4 Environmental concentrations of microrubber

Reported actual and estimated concentrations of microrubber particles in the environment are difficult to compare across studies. Methodology for determining particle concentrations from field sampling of different environmental compartments varies across the literature including differences in sample processing and sample analysis. Light microscopy of samples is often utilized to identify MR, but a secondary method of particle analysis is typically needed to confirm polymer and particle type. Microrubber is difficult to categorize by a single analytical method as the chemical composition varies greatly and the generation of MR particles can influence their properties – for example, TRWP may contain embedded minerals or chemicals picked up from the roadway whereas TWP generated in a laboratory may not. These differences could influence the density of particles and would need to be considered for processing field-collected samples. **Table 1.2** lists estimated and measured MR concentrations in various environmental compartments.

Table 1.2 – Estimated and observed microrubber concentrations from various environmental matrices.

Matrix or media type	Concentration	Location	Marker/Method	Reference
Road dust				
Road surface (mg kg^{-1})	72000	USA	Zn	Hopke et al., 1980
Road surface (mg kg^{-1})	35000	USA	BT	Rogge et al., 1993
Road surface (mg kg^{-1})	700	Denmark	SBR	Fauser et al., 1999
Soil				
0 m from road (mg kg^{-1})	600	Denmark	Extr. Org. Zn	Fauser et al., 2002
3 m from road (mg kg^{-1})	250	Denmark	Extr. Org. Zn	Fauser et al., 2002
10 m from road (mg kg^{-1})	100	Denmark	Extr. Org. Zn	Fauser et al., 2002
Road runoff (mg l^{-1})	93	Japan	24MoBT	Kumata et al., 1997
	97	Germany	BT	Baumann & Ismeier, 1998
	12	Japan	24MoBT	Kumata et al., 2000
	179	Japan	24MoBT	Kumata et al., 2000
	92	USA	24MoBT	Reddy & Quinn, 1997
River water (mg l^{-1})	1.6	USA	24MoBT	Reddy & Quinn, 1997
	3.6	Japan	NCBA	Kumata et al., 2000
Settling pond water (mg l^{-1})	2.3	USA	24MoBT	Reddy & Quinn, 1997
Settling pond sediment (mg kg^{-1})	350	USA	24MoBT	Reddy & Quinn, 1997
Sediment (mg kg^{-1} d.w.)	910	USA	Pyr GC/MS	Unice et al., 2013
	4500	France	Pyr GC/MS	Unice et al., 2013
	770	Japan	Pyr GC/MS	Unice et al., 2013
Air (μg m^{-3})				
	0.243	France	Pyr GC/MS	Panko et al., 2013
	0.102	Japan	Pyr GC/MS	Panko et al., 2013
	0.135	USA	Pyr GC/MS	Panko et al., 2013
Animal				
Marine fish	97 particles	USA	Microscopy, FTIR	Parker et al., 2020

1.4.1 Microrubber identification methods

There are a variety of environmental compartments in which MPs or MR may be found including water, sediment, and biota. After processing samples from any environmental media, the quickest and most inexpensive method for distinguishing MP types is typically by visual inspection using microscopy. Microplastics can be identified, counted, and classified by color and type (i.e., fragment, foam, fiber, MR). Secondary analysis of suspected MPs can involve a hot needle test to confirm plastic composition. A heated stainless-steel needle is held close to a suspected MP particle. Plastic particles will melt or curl when approached by the red-hot needle while organic or non-plastic material will not react (Barrows et al., 2017). This methodology can be useful for rapid and affordable identification of MP vs. non-plastic material in samples. However, it has been noted MR particles do not react to a hot needle test and are typically initially classified using stereomicroscopy based on physical characteristics like black color, elongated or cylindrical shape, partially or entirely covered with road dust, rough surface texture, and rubbery flexibility when manipulated with forceps (Leads and Weinstein, 2019; Parker et al., 2020). In addition, bitumen behaves differently than rubber and is commonly found among MR particles. Bitumen is a viscoelastic binding agent in asphalt that consists of highly heterogenous mixtures of hydrocarbons (Jarlskog et al., 2020). The density of bitumen from asphalt pavement has been reported between 0.9 – 1.1 g/cm^3 (Nynas, 2017) whereas the density of TRWP from pavement has been reported at an average of 1.8 g/cm^3 (Klöckner et al., 2019; Unice et al., 2019). Bitumen particles share similar visual properties as MR the black color and shape. Tactile manipulation can aid in

distinguishing between MR and bitumen as MR regains its shape after being squeezed with more of an elastic response whereas bitumen particles remain compressed and melting tests result in MR particles being morphologically unaffected after heating, but bitumen particles melt and expand in size upon heating (Jarlskog et al. 2020). Many studies do not distinguish between MR and bitumen particles in their reported estimates of MR concentrations (Kell, 2020; Leads & Weinstein, 2019).

For accurate identification of polymeric composition for either MP or MR particles, additional methods are needed. Fourier transform infrared (FTIR) spectroscopy, Raman spectroscopy, and pyrolysis- gas chromatography-mass spectrometry have been utilized for identification of MP types (Wang & Wang, 2018). Other suggested methods for MR identification are energy dispersive X-ray spectroscopy (EDXS) or simply visual characteristics (shape, surface characteristics, and structure) rather than polymer composition (Sommer et al., 2018).

Fourier transform infrared (FTIR) spectroscopy uses infrared radiation to obtain a spectrum from different chemical structures. When infrared radiation is passed through a sample, some radiation is absorbed by the sample and some passes through and is transmitted or reflected. The resulting signal from the detector represents the molecular spectral fingerprint, which is unique and can be used for identifying compounds. Some limitations of FTIR are that only particles with a size of > 10-20 μm can be identified, analysis is time consuming, and experience with instrumentation is necessary. Micro-FTIR can be utilized for analysis of smaller particles (10 - 20 μm). It can be difficult to identify MR using FTIR because of the variable composition of the particles depending

on factors like tire age and type. Leads and Weinstein (2019) analyzed commercially available CR fragments that were > 500 μm in size using attenuated total reflectance FTIR (ATR-FTIR) and compared their spectra to field-collected TRWP. ATR-FTIR was utilized for examining tire particles because the infrared light used in FTIR alone would be absorbed by the carbon black in tire particles, as carbon black absorbs the infrared radiation utilized in analysis. ATR-FTIR is more advantageous over other FTIR sampling modes because of its higher signal-to-noise ratio and reduced scattering and the penetration depth of IR light is independent of sample thickness (Bangaoil et al., 2020). Both the commercially available CR and the field collected TRWP generated a downward sloping spectrum that were consistent with the spectra of butyl rubber containing carbon black (Leads & Weinstein, 2019). It is possible to use FTIR for identification of MR particles when reference spectra are available although carbon black can mask the underlying spectra and variability among spectra should be expected in field-collected tire wear particles due to environmental degradation and variation in tire composition.

Raman spectroscopy is also commonly utilized to determine MP composition. In Raman spectroscopy, a monochromatic laser beam is irradiated onto a suspected sample, which results in a different frequency of backscattered light due to absorption, scatter, or reflection due to the sample's specific molecular structure and atomic composition (Wang & Wang, 2018). Similar to FTIR, a unique spectrum is produced for each polymer that allows for identification of microplastic composition. Raman spectroscopy has higher spatial resolution, wider spectral range, and lower sensitivity to water interference

than FTIR. Micro-Raman can identify MPs down to 1 μm in size. In complex samples (like MR particles), the Raman spectra can experience interference from the presence of additives, pigments, or other attached chemicals which may lead to inaccurate determination of composition. Raman spectroscopy may be useful to identify particles that are *not* MR.

Pyrolysis gas chromatography/mass spectrometry (pyr-GC/MS) has been used to quantify MP and MR particles in various matrices including air, soil, and sediment. In pyr-GC/MS, thermal energy is applied to split large molecules into smaller fragments and characteristic volatile pyrolysis fragments can be used to quantify the concentration of tire tread polymer and total tread particulate in environmental samples (Unice et al., 2012). Fragments generated by pyrolysis are separated by gas chromatography and identified by a mass selective detector for quantitative analysis of polymers. The major drawback from this analytical technique is that it is destructive and has mass-based method detection limits; however, it is useful in that it can provide identification of plastic additives present in samples as well as leading to more specific composition profiles (Zarfl, 2019).

Similar to the previously discussed techniques, energy dispersive x-ray spectroscopy (EDXS) also produces spectra that are used to identify a material when compared to a library database. EDXS uses x-ray excitation or electron beam excitation (for electron microscopes or scanning electron microscopes). The same limitations of MR particle complexity and spectral library matching apply to the EDXS identification technique as well (Sommer et al., 2018).

Advanced spectroscopic methods may be useful for more definite identification of MPs because their polymer types are likely less complex than that of MR and may produce a cleaner spectrum for matching, which can help determine which particles are *not* MR. Utilizing advanced spectroscopic methods for a subset of suspected MR particles may confirm identification when enough data and spectral signatures are available to compare to and when the size of particles is not an issue. Researchers recommend a combination of both visual identification based on physical properties (shape, surface characteristics, structure, color) and ATR-FTIR, Raman, pyr-GC/MS, or EDXS, if available, for identification of MR particles (Leads & Weinstein, 2019; Parker et al., 2020; Sommer et al., 2018).

1.4.2 Microrubber markers

Several researchers have suggested using certain markers to estimate tire and MR concentrations in the environment. Common markers utilized to date have included metals (mainly zinc), tire chemicals, and rubber polymers. MR particles are a major source of zinc in urban areas (Councell et al., 2004). Concentrations of Zn in finished tires can be up to 2% by weight (Rhodes et al., 2012). Fauser et al. (1999) developed a method for identifying and quantifying tire-tread particles in the environment by measuring extractable organic zinc using atomic absorption spectrometry (AAS). This method has been used by others as a marker for tire rubber in various environmental matrices (Adamiec et al., 2016; Legret & Pagotto, 1999). One limitation to using Zn as a marker for MR in the environment are the other sources of zinc in the environment, such

as galvanized road safety barriers, and could lead to overestimates of MR particulates in the environment if using Zn as a marker.

Tire chemicals used as markers for MR include benzothiazoles (BTs) including 24MoBT (2-(4-morpholinyl)benzothiazole), BT (benzothiazole), HOBT (2-hydroxybenzothiazole), and NCBA (N-cyclohexyl-2-benzothiazolamine) (Wik & Dave, 2009). The largest amounts of BTs are used as vulcanization accelerators for tires, but BTs have other uses such as corrosion inhibitors in antifreeze products, as pesticides, and as photosensitizers in photography (Brownlee et al., 1992). Many studies have utilized BTs as a tire marker (Knight et al. 2020; Kumata et al., 1997; Ni et al., 2008) to estimate MR concentrations or contributions of BTs to the environment from MR particles.

Rubber polymers, specifically styrene butadiene rubber (SBR), have been used as a tire indicator in environmental samples. Typical passenger car tires can contain up to 60% by weight of synthetic and natural rubbers with SBR concentrations ranging anywhere between 11% - 30% of the total rubber polymer used but little to no SBR is found in heavy duty vehicle tires (Eisentraut et al., 2018; Fauser et al., 1999; Wik & Dave, 2009). Infrared spectroscopy (IR) was utilized by Fauser et al. (1999) to identify SBR. Recent methods including pyrolysis gas chromatography coupled with mass spectrometry (Pyr-GC/MS) and thermal extraction desorption gas chromatography-mass spectrometry (TED-GC/MS) have been used to identify polymers like SBR and thus estimate MR in the environment (Eisentraut et al., 2018).

There are advantages and drawbacks of estimating MR amounts utilizing the described tire marker methods. Advantages include less sampling required and reduced

processing time. Disadvantages to estimating MR concentrations using tire markers are that some markers lack specificity for tires, have not been fully validated, there is variability in tire composition, and may not fully capture the extent of MR in the environment. Nevertheless, estimates using tire markers coupled with actual measured concentrations of MR in the environment can shed light on the overall concentration of MR in various environmental matrices.

1.5 Toxicity of rubber and microrubber

Toxicity of MR exposure has primarily been examined in the aquatic environment with many studies focusing on toxicity of the leachate fraction (Halle et al., 2020). Leachate is an aqueous sample where rubber, tire, or MR has been present but is then removed before use in toxicity tests (Rødland, 2019). Most early studies measured endpoints such as development or mortality in an effort to establish a half maximal effective concentration (EC50) or half maximal lethal concentration (LC50) for tire and rubber leachate while few studies have focused on sublethal effects or changes in biomarkers. There are several possible routes of uptake for exposure to MR including aqueous uptake of chemicals via leachate, ingestion or ventilation of particles, and trophic transfer (Khan et al., 2019; Parker et al., 2020; Stephensen et al., 2003).

Specific contaminants identified that are associated with the toxicity of MR include Zn, PAHs, and the recently identified transformation byproduct 6PPD-quinone from the tire-derived compound 6PPD (Stephensen et al., 2003; Tian et al., 2021; Turner & Rice, 2010). Zinc has been a contaminant of concern from MR and can be found at up to 1 - 2% by weight in finished tires (Fauser et al. 1999, Rhodes et al. 2012). The

dissolution of Zn from MR is influenced by pH, salinity, UV light, and particle size. In general, as salinity and pH increase, there is less Zn dissolution from MR and therefore less aqueous Zn (Degaffe & Turner, 2011; Hartwell et al., 2000; Rhodes et al., 2012). Dissolution of Zn from TWP was shown to increase in the presence of UV light and decrease in the dark (Degaffe & Turner, 2011). Rhodes et al. (2012) demonstrated that with smaller tire particle size, there was greater amounts of Zn leached due to the increase in both surface area and mass transfer rate for smaller particles. Excess Zn in the aquatic environment can lead to toxicity in aquatic organisms however, Turner and Rice (2010) found that phototoxicity of MR leachate was significantly lower compared to phototoxicity of equivalent Zn concentrations to the marine macroalga *Ulva lactuca*, and suggested that organic components of leachate (i.e., PAHs) are largely responsible for the overall toxicity of MR leachate in the species tested.

PAHs have been shown to be carcinogenic, mutagenic, and immunotoxic to a variety of species including aquatic organisms as reviewed by Honda and Suzuki (2020) and Patel et al. (2020). In MR leachate exposures, Stephensen et al. (2003) measured induction of CYP1A in exposed fish and hydroxylated PAHs in the bile of exposed fish which indicated uptake of PAH compounds by the fish.

For 6PPD-quinone, the mechanism of toxicity is unknown at present for aquatic organisms. Initial studies have found Coho salmon (*Oncorhynchus kisutch*) to be extremely sensitive to 6PPD-quinone and the proposed mode of action for toxicity is disruption of the blood-brain barrier (Blair et al., 2020; McIntyre et al., 2021). Other researchers have found no toxicity to 6PPD-quinone in other species of fish and

crustaceans (Hiki et al., 2021). A variety of contaminants associated with MR or combination of contaminants may exert a toxic effect in different species.

It is difficult to compare toxicity studies on MR as there are currently no standardized methods for measuring and reporting concentrations in various matrices. For example, some studies report concentrations in number of particles per liter whereas others report concentrations in grams per liter. A conversion between number of particles per unit to weight of particles per unit would likely vary depending upon particle size and type utilized in the study but it would be worth noting if possible for comparison across studies and reproducibility. This type of conversion is possible through counting the number of particles in a specified concentration of stock solution as described in Khan et al. (2015) but would likely need to be established for each individual study as particle size range used and particle type could influence the conversion.

Another issue in comparison of toxic effects across studies arises when considering the type of exposure evaluated. Studies may focus on the toxicity of MR leachate, spiked sediment, road runoff sediment, or whole particles (Gualtieri et al., 2005; LaPlaca & Van den Hurk, 2020; Marwood et al., 2011; Wik et al., 2009). Leachate can be generated in many ways under various conditions; currently there is no standardized method for producing MR leachate to compare across studies. There is a large body of research on MR leachate toxicity but limited studies on MR particle toxicity, especially on particle toxicity in the aquatic environment.

Finally, MR particles themselves may exhibit varying degrees of chemical toxicity depending on origin, initial chemical composition, particle type, particle size, or

a degree of other factors. For example, it has been noted that TRWP may contain chemicals picked up from the roadway whereas TWP from new tires generated under laboratory conditions would not have certain chemicals as they are not subjected to roadway influence or other abiotic factors (Halle et al., 2020). Physical effects from exposure to MR particles may occur such as tissue damage or inflammation which has been observed for other particle types (Happo et al., 2010) Therefore, the following assessment of the literature regarding the toxicity of rubber and MR aims to outline the current body of knowledge on MR toxicity under a variety of parameters. Short- and long-term effects from MR exposure are discussed for the two major types of studies frequently conducted, MR leachate and MR particulate toxicity tests. The major findings and experimental methods are discussed below with a summary of all studies (**Table 1.3**) following the discussion.

1.5.1 Short term effects and acute toxicity

Several short-term experiments, many of which focus on rubber leachate, have been conducted attempting to characterize rubber and tire toxicity. In one of the earliest studies, Day et al. (1993) examined acute toxicity (96-h) of whole tire leachate to rainbow trout (*Oncorhynchus mykiss*) and estimated the LC50 of leachate to range from 11.8 – 80.4 (%v/v). The LC50 values varied considerably based on the type of tire (breakwater, scrap, or new) and the amount of time allowed for leachate extraction (5 to 40 days). In general, leachates from used tires were more toxic than leachates generated from new tires for rainbow trout. Survival of *Daphnia magna* (48-h) and *Pimephales*

promelas (96-h) was not affected by exposure to leachates. The study demonstrates the variability in toxicity depending on tire type and tire age as well as species sensitivity.

Gualtieri et al. (2005) performed a series of leachate toxicity tests using algae (*Raphidocelis subcapitata*), daphnid (*Daphnia magna*), and frog embryos (*Xenopus laevis*) at two different concentrations of leachate, 50 g/L and 100 g/L TWP. The growth EC50 for *R. subcapitata* was 0.93% leachate for 50 g/L TWP and 1.64% leachate for 100 g/L TWP. The LC50 for *D. magna* after 24-h was 58.3% leachate for 50 g/L TWP and 53.3% leachate from 100 g/L TWP. The FETAX test showed that leachates were not embryolethal for *X. laevis* from 1 to 50% leachate while at 100% leachate, the mortality reached 80.2% for 50 g/L TWP and 26.8% for 100 g/L TWP. The authors suggested that the quantity of leached rubber components was related to the rubber exposed surface, not necessarily the concentration of rubber. The dilutions of leachate from 50 g/L TWP were more toxic than those produced from 100 g/L TWP as shown through *R. subcapitata, D. magna,* and *X. laevis* 50 g/L TWP leachate toxic values were two to three times greater than those measured from 100 g/L TWP leachate.

To characterize toxic effects among a wide variety of tires, Wik and Dave (2006) evaluated acute toxicity to *D. magna* at various dilutions of 10 g/L TWP leachate for 25 different tires. During leachate preparation, heat was applied (44°C for 72-h) during the leaching process to represent a "worst case scenario" and induce more rapid leaching at high temperature. The 24-hr EC50s for immobility ranged from 1.4 to >10.0 g/L and the 48-h EC50s for immobility ranged from 0.5 to >10.0 g/L TWP depending on the tire tested. In a follow up study, Wik et al. (2009) examined the acute toxicity of leachate at

0.001, 0.1, 1.0, and 10.0 g/L TWP concentrations generated from sequential leaching from different tires. They tested a variety of aquatic organisms and found the 72-h growth inhibition EC50 for *P. subcapitata* between 0.05 – 2.84 g/L TWP, 48-h immobility EC50 for *D. magna* between 0.37 – 7.45 g/L TWP, 9-d survival EC50 of *C. dubia* between 0.05 – 3.59 g/L TWP, and no mortality in *D. rerio* embryos.

These data together suggest large variation in acute toxic effects due to tire type, method of leachate preparation, and species tested. Many of the EC50 values in Wik and Dave (2006) and Wik et al. (2009) are likely much higher than what would be found in the environment. Maximum aqueous concentrations of MR are predicated well below 0.2 g/L however they tested concentrations up to 10 g/L TWP (**Table 1.3**).

Sediment elutriate toxicity tests have also been conducted. Rather than mixing particles in solution to obtain leachate, elutriate preparation involves mixing particles with sediment and solution to replicate sediment mobilization (Marwood et al. 2011). Marwood et al. (2011) observed no mortality for *D. magna* and *P. promelas* in 48-h and 96-h acute toxicity tests when exposed to MR elutriates. Elutriates were prepared by mixing sediment, TRWP generated from a road simulator, and water. The overlying water was collected for testing, which differs from leachate generation in that leachate is prepared by mixing TRWP and water only and collecting the overlying water or filtering to remove the TRWP. The researchers also exposed *D. magna* to leachate generated under different temperature conditions. They found that leachate where heat was applied during the leaching process was acutely toxic to *D. magna* compared to leachate prepared at room temperature. The authors observed no-effect concentrations (NOAEC) of >10

g/L TRWP for *P. subcapitata*, *P. promelas*, and *D. magna* when exposed to sediment elutriate. There was also a NOAEC of >10 g/L TRWP for *D. magna* when exposed to leachate or leachate + sediment at 21°C, representative of ambient environmental conditions, but NOAEC values of 1.25 and 2.50 g/L for *D. magna* when exposed to heated leachate (44°C) or heated leachate + sediment. In natural aquatic systems that contain sediment and suspended particulates, sediment acts as a sink for many chemicals with poor aqueous solubility and, as a result, the bioavailability of contaminants is greatly diminished (Marwood et al., 2011). This finding is supported through the authors' results showing the reduced toxicity of TRWP sediment elutriates compared to TRWP leachates.

The differences in toxicity values reported for Marwood et al. (2011) compared to Wik and colleagues (2006, 2009) may be attributed to the differences in particles utilized (i.e., TRWP vs TWP). Lower EC50 values were reported for *D. magna* in Wik and colleagues (2006, 2009) where TWP generated from used tires with a rasp were used in leachate generation compared to greater EC50 values reported in Marwood et al. (2011) where TRWP from a road simulator were used in leachate and elutriate generation. The conditions of leachate generation also varied with Wik et al. (2006) where heat was used (44°C) in all methods for leachate generation to represent a "worst case scenario" and simulate the road temperature on a hot summer day. It is likely that data presented in Marwood et al. (2011) are more representative of environmental conditions by taking into account sediment-particle dynamics at ambient temperatures.

Abiotic factors can influence the toxicity of MR in the environment. A series of two studies on the influence of salinity on scrap tire leachate found that the toxicity of

leachates decreased as salinity increased. Hartwell et al. (1998) observed almost 100% mortality in a 7-d larval sheepshead minnow (*Cyprinodon variegatus*) toxicity test for leachates at 5 ‰ salinity, 80% mortality at 15 ‰ salinity, and < 10% mortality at 25‰ salinity. This corresponds to a 96-h LC50 of 10% leachate at 5‰ salinity and 26% leachate at 15‰ salinity for fish. For juvenile daggerblade grass shrimp (*Palaemonetes pugio*), nearly 100% mortality was also observed for leachates at 5‰ salinity but no other significant mortality was observed in higher salinities tested. The 96-h LC50 at 5‰ salinity was 63% for grass shrimp. In this study, fish were more sensitive to leachate toxicity compared to the grass shrimp (Hartwell et al., 1998).

In a follow up study, Hartwell et al. (2000) assessed the impact of salinity on rubber leachate toxicity using Microtox® bacterial bioluminescence bioassays up to 25ppt. This study agreed with the authors' previous findings and found that toxicity of leachates decreased as salinity increased but it is unknown if this effect is due to an interaction between salts and toxic mode of action, or changes in release of toxicants from the rubber matrix under various salinities. Both studies provide evidence of the potential increased threat of MR to freshwater ecosystems compared to saltwater ecosystems.

More recently, Kolomijeca et al. (2020) assessed the impact of changes in four abiotic factors related to climate change on the toxicity of tire leachate to fathead minnow (*P. promelas*) embryos. Various TWP leachates were produced by differing temperature, mechanical stress (i.e., turbulence), UV light, and CO_2 levels followed by exposure to fathead minnow embryos. Endpoints assessed after exposure included: hatching success,

time to hatch, length, deformities, and heart rate. Hatching success and deformities were affected to the greatest extent, especially with increased temperature and increased mechanical stress of prepared leachates. For UV exposure, there was no significant correlation between change in UV exposure of leachate and leachate toxicity to fathead minnow. There were no significant trends in adverse effects from leachates prepared under variable CO_2 concentrations (i.e., pH). The authors demonstrate the influence of abiotic factors, especially temperature and turbulence, on toxicity of MR leachates which shows the potential for geographic variability of the impact of MR dependent upon local environmental conditions.

Sublethal effects such as induction of cytochrome P4501A1 (CYP1A1) and increased oxidative stress have been observed in organisms exposed to rubber leachates. Stephensen et al. (2003) exposed juvenile rainbow trout (*O. mykiss*) to leachate from two types of tires for 14 days – one that contained highly aromatic (HA) oil and one which had HA oil-free tread. Biomarker analyses indicated upregulation of CYP1A1 in exposed fish and increased antioxidant response as measured by elevated total glutathione (tGSH). There was no effect observed in other antioxidant enzymes such as superoxide dismutase and catalase and no effect on plasma vitellogenin concentration in exposed fish. The authors suggested that induction of CYP1A1 expression indicated that active chemicals leaching from the tires were aryl hydrocarbon receptor (AhR) agonists which agreed with chemical analysis of bile in the exposed fish where hydroxylated PAHs were identified. Additionally, they suggested that the upregulation of antioxidant biomarkers was caused by leakage of pro-oxidants from the rubber and identified several aromatic nitrogen

compounds (hydroxylated metabolites of diphenylamine (DPA), parent DPA, 1,2-dihydro-2,2,4-trimethylquinoline, N-(1-methylethyl)-N'-phenyl-1,4-benzenediamine) in the bile of exposed fish (Stephensen et al., 2003).

Fewer studies have focused on acute toxicity of MR particulates. Wik and Dave (2005) conducted a 24-h and 48-h toxicity test using *D. magna* and laboratory generated tire particles (TWP) in water using 12 different tires. The calculated EC50s for 24-h exposure ranged from 0.29 to 32 g/L and for 48-h exposure from 0.0625 to 2.41 g/L TWP. Particles from all 12 tires tested in this study were shown to be toxic to *D. magna* and showed greater toxicity after 48-h exposure compared to 24-h exposure. The authors suggested that the increase in toxicity with exposure time indicated that tires contain compounds that are bioconcentrating and variation in tire toxicity may be explained by variations in chemical composition, with different toxic modes of action. This study utilized particles still suspended in water, and therefore, provided an estimate for toxicity of leachate including particulates whereas previously discussed studies measure toxicity of leachate only.

Khan et al. (2019) found that in acute toxicity exposures of TWP (< 500 μm) dispersed in freshwater, *Hyallela azteca* were indiscriminately ingesting TWP and had a gut retention time of 24-48h. The estimated acute (48-h) toxicity LC50 was 3426 ± 172 particles mL^{-1} which corresponds to approximately 1 mg/mL or 1 g/L TWP. This estimated LC50 is within the range of calculated EC50s for *D. magna* described above from Wik & Dave (2005) for TWP particulates (0.0625 to 2.41 g/L). Khan et al. (2019) also acknowledged the impact of leachate in their study as both the MR particles and

their associated leachate influenced test organisms during exposure. When comparing the toxicity of TWP and TWP leachate, they found that at low concentrations leachate was more toxic than particles but at higher concentrations the particles were more toxic (for example, 40% mortality leachate/10% mortality TWP at 10^3 particles mL^{-1} and 50% mortality for leachate/90% mortality TWP at 10^4 particles mL^{-1}). It is unknown whether the more toxic effect of particles at higher concentrations was due to the physical impact of TWP in the digestive tract, enhanced chemical delivery by TWP, or something else but the study suggested MR particles may act as a 'Trojan Horse' for contaminant delivery. Interestingly, other studies have noted a decrease in toxicity for MR leachates as concentration increases (Gualtieri et al., 2005; Nelson et al., 1994) suggesting that the quantity of eluted chemicals was related to the rubber exposed surface and that aggregation of particles at higher concentrations may reduce the eluted components in leachates of higher concentration.

MR particulate toxicity has also been examined in sediment-dwelling terrestrial organisms such as the earthworm (*E. fetida*) (Pochron et al., 2018). For earthworms living in soil contaminated with crumb rubber, growth was reduced. Additionally, exposure to aged CR reduced earthworm survival time during a stress test relative to survival time for worms that lived in clean soil (Pochron et al., 2018).

In conclusion, there have been many acute toxicity tests conducted, especially using MR leachate. Fewer studies address acute toxicity of MR particles. Toxic effects vary among test organism, leachate preparation method, and concentration. More research is needed at environmentally relevant concentrations of MR as most previous

studies assessed concentrations in excess of those found in the environment. Additionally, sublethal endpoints such as changes in biomarkers or energy allocation should be investigated.

1.5.2 Long term effects and chronic toxicity

Few chronic and sub-chronic toxicity studies have been conducted using MR leachate and MR particles to determine potential effects from repeated exposure or continuous exposure over a considerable part of the lifetime of the test organism. For MR leachates, Wik et al. (2009) measured 9-d survival EC50 and 9-d reproduction EC50 for *C. dubia* from three different tire types after four sequential leachings. There was slight variation in toxicity from different tire types and in general, survival and reproduction were impacted to the greatest extent at initial leachate exposure with reduced toxicity after sequential leachings. The reproduction of *C. dubia* was the most sensitive endpoint tested with an EC50 of 0.01 g/L up to the third leaching of the most toxic tire. This EC50 is environmentally relevant compared to predicted environmental concentrations of 0.013 g/L (Wik & Dave, 2006).

Panko et al. (2013) conducted a series of chronic exposures to TRWP sediment mixtures for sediment-dwelling organisms and sediment elutriates for water-dwelling organisms. Sediment spiked with TRWP (< 150 µm) was prepared at a concentration of 10,000 mg/kg and used for whole sediment toxicity tests for *C. dilutus* and *H. azteca*. Additionally, the spiked sediment was used to prepare sediment elutriate for treatments with *C. dubia* and *P. promelas*. Results indicated little to no toxic effect in the organisms tested. For *C. dilutus*, there was a slight reduction in growth when exposed to TRWP-

spiked sediment although it was not significantly different from reference sediment. There was no effect on reproduction or survival. For *H. azteca* exposed to TRWP-spiked sediment, there was no effect on growth, reproduction, or survival indicating no significant effects from exposure (Panko et al., 2013). For both water-dwelling organisms *C. dubia* and *P. promelas*, exposure to TRWP-spiked sediment elutriate indicated no significant adverse effects on survival, growth, or reproduction although there was a slight reduction in survival of *P. promelas* when exposed to TRWP-elutriate. The authors concluded that given the high concentration of TRWP (10,000 mg/kg sediment) used in the study and the non-significant, minor effects on survival and reproduction of test organisms under chronic exposure conditions, biologically relevant impacts from TRWP in populations of aquatic plants or animals in the environment are unlikely. The concentration tested in this study exceeds predicted environmental concentrations in sediment by one to two orders of magnitude (Unice et al., 2013).

Few studies focused only on MR particle chronic toxicity; however, Khan et al. (2019) observed significant adverse effects in *H. azteca* when exposed to TWP (< 500 µm) at concentrations of 500 - 2000 particles/mL. Mortality, reproductive output, and net growth were negatively impacted following 21-d exposure. In this study, 500 particles/mL was equivalent to 0.145 g/L which is slightly below to the estimated maximum predicted concentration of TWP in road runoff (0.179 g/L) and well above the estimated maximum predicted concentration of TWP in river water (0.0036 g/L) (Kumata et al., 2000).

Other chronic toxicity studies supported the potentially low risk of MR in the environment. Freshwater macroinvertebrates exposed to TWP (10 - 586 µm) mixed with sediment showed no adverse effects on survival, growth, or feeding rate at concentrations up to 10% sediment dry weight indicating that neither the particles themselves nor any chemicals associated were toxic in the tested scenario (Redondo-Hasselerharm et al., 2018). At 10% TWP in sediment dry weight, a maximum of only 4 TWP were found in the body of *G. pulex*, and a maximum of 6 TWP in the feces. This study may resemble realistic environmental exposures of benthic macroinvertebrates to MR and suggested that TWP effects may be mild or absent at ecologically relevant conditions.

The few chronic toxicity studies available from the literature suggest that in general, the risk from MR exposure under typical environmental conditions poses a relatively low risk to organisms. Sublethal effects from chronic exposure to MR, such as upregulation of detoxification enzymes or DNA damage, have not yet been documented.

Table 1.3 – Summary of acute and chronic toxicity tests utilizing microrubber.

Acute toxicity			
Testing procedure	**Test organism**	**Toxicity or effect**	**Reference**
Whole tire leachate after 5 – 40 d leaching period	*D. magna* *P. promelas* *O. mykiss*	Nonlethal (48-h) Nonlethal (96-h) LC50 leachate 11.8% – 80.4%	Day et al., 1993
Laboratory generated TWP leachate at 50 g/L and 100 g/L TWP	*R. subcapitata* *D. magna* *X. laevis*	Growth EC50 0.93% - 1.64% 24-h LC50 58.3% - 53.3% Up to 80% mortality at 100% leachate	Gualtieri et al., 2005
14-d exposure to leachate from two types of tires, one with highly aromatic oil and one without	*O. mykiss*	Upregulation of CYP1A1, elevated tGSH, no effect on superoxide dismutase, catalase, or vitellogenin	Stephensen et al., 2003
7-d exposure to scrap tire chip (50 g/L) leachate generated at 5, 15, 25‰ salinity	*C. variegatus* *P. pugio*	96-h LC50 10% at 5‰, 26% at15‰ 96-h LC50 63% at 5‰	Hartwell et al., 1998
Microtox® bacterial bioassay using leachate generated from scrap tire chips (50 g/L) generated at 0, 5, 15, 20, 25‰ salinity	*V. fisherii*	Toxicity of leachates decreased with increasing salinity up to 15 ppt with no significant change at higher salinities.	Hartwell et al., 2000
Laboratory generated tire particles suspended in water from 12 different tires	*D. magna*	24-h immobility EC50 0.29 – 32 g/L TWP 48-h immobility EC50 0.0625 – 2.41 g/L	Wik & Dave, 2005
Acute toxicity to leachate at various dilutions (10 g/L) generated from laboratory generated rubber particles of 25 different tires. Leaching temperature of 44°C	*D. magna*	24-h immobility EC50s from 1.4 - >10.0 g/L 48-h immobility EC50s from 0.5 - >10 g/L	Wik & Dave, 2006

Acute toxicity			
Testing procedure	**Test organism**	**Toxicity or effect**	**Reference**
Acute toxicity of leachate (10, 1, 0.1, 0.001 g/L) generated from sequential leaching of tire rubber powder from 3 different tires	*P. subcapitata* *D. magna* *D. rerio*	72-h growth inhibition EC50, 0.05 – 2.84 g/L 48-h immobility EC50, 0.37 – 7.45 g/L Nonlethal 48-h	Wik et al., 2009
Acute toxicity of sediment elutriates compared to leachates for TRWP generated from a road simulator	*P. subcapitata* *D. magna* *P. promelas*	Elutriates – no mortality up to 10 g/L Elutriates – no mortality up to 10 g/L Leachate – NOAEC 1.25 and 2.50 g/L for heated leachate and heated leachate + sediment Elutriates – no mortality up to 10 g/L	Marwood et al., 2011
Acute toxicity of leachate prepared under various conditions generated from TWP. Hatching success and deformities were impacted to greatest extent.	*P. promelas*	Temperature ↑, toxicity ↑ Mechanical stress/turbulence ↑, toxicity ↑ UV light no effect CO_2 no effect	Kolomijeca et al., 2020
Acute and chronic toxicity of TWP dispersed in freshwater to *H. azteca*.	*H. azteca*	*H. azteca* indiscriminately ingest TWP with gut retention time of 24 – 48h. 48-h TWP toxicity for particles LC50 = 3426 ± 172 particles/mL (approx. 1 mg/mL or 1 g/L TWP). 21-d TWP toxicity – 92% mortality at 2000 particle/mL (0.58 g/L), significant reduction in reproduction at 1000 particles/mL (0.29 g/L), significant reduction in net growth at 500 (0.145 g/L) and 1000 particles/mL (0.29 g/L) concentrations.	Khan et al., 2019
Assessed earthworm survivorship and body weight after exposure to CR in sediment. Also quantified survival time during a stress test (exposure to bright light and high temperature) when exposed to aged and new CR.	*E. fetida*	Exposure to new CR reduced earthworm body weight. Exposure to aged CR negatively impacted earthworm resilience as measured by survival time of stress test. Exposure to aged CR did *not* reduce earthworm body weight.	Pochron et al., 2018

Chronic toxicity			
Testing procedure	**Test organism**	**Toxicity or effect**	**Reference**
Chronic (9-d) toxicity of leachate (10, 1, 0.1, 0.001 g/L) generated from sequential leaching of tire rubber powder from 3 different tires.	*C. dubia*	9-d survival EC50, 0.05 – 3.59 g/L 9-d reproduction EC50, 0.01 – 1.76 g/L	Wik et al., 2009
Measured chronic toxicity of TRWP at concentrations up to 10,000 mg/kg sediment or elutriates from spiked sediment using water and sediment-dwelling organisms. Concluded that under typical exposure conditions, TRWP in sediments pose low risk of toxicity to aquatic organisms.	*C. dilutus* (35-d) *H. azteca* (42-d) *C. dubia* (7-d) *P. promelas* (32-d)	Mild growth inhibition when exposed to spiked sediment No adverse effect on growth or reproduction No adverse effect on growth or reproduction Slightly diminished survival in larvae	Panko et al., 2013
Using freshwater benthic macroinvertebrates, 28-d exposure to TWP at concentrations 0, 0.1, 0.3, 1, 3, 10% sediment dry weight.	*G. pulex* *A. aquaticus* *L. variegatus* *Tubifex spp.*	No adverse effect on survival, growth, or feeding rate. Observed ingestion and egestion of TWP in *G. pulex.* Environmentally and ecologically relevant exposure indicated little to no adverse effect in test organisms.	Redondo-Hasselerharm et al., 2018

1.5.3 Knowledge gaps

Most toxicity studies regarding MR have thus far focused on acute effects of exposure to leachate. MR leachate may be prepared in numerous ways as no standardized method exists. Previous studies focused on elucidating an LC50 or endpoints such as growth or mortality and only one found for this literature review mentions sublethal effects such as alterations in organism's biomarkers and how they handle the stress of exposure to MR. In addition, there are few studies on chronic effects of MR exposure, especially sublethal effects in aquatic organisms.

Currently there are no studies on trophic transfer of MR. While some studies have inferred the possibility of trophic transfer of MR to be similar to that of MPs, it has not been specifically documented in the literature or tested under laboratory conditions. Additionally, other processes such as the influence of viruses and bacteria on MR interactions with organisms have yet to be addressed. Plastic materials in general represent an important and abundant environmental substrate for colonization of bacteria from the surrounding water column and MP materials have potential to act as long-distance transport mechanisms for human and animal pathogens (Bowley et al., 2021).

While environmental concentrations of MR have been predicted, sampling and analytical techniques make it difficult to accurately quantify MR in various environmental compartments. Additionally, it is not feasible to use field-collected MR for toxicity tests, so laboratory generated MR (TWP) or CR are generally used in toxicity tests. A wide array of concentrations has been tested for various organisms to assess toxicity; however, more environmentally relevant exposure scenarios are necessary, such

as a pulsed exposure to mimic storm events or a mesocosm approach to incorporate multiple biological processes and interactions in the environment. Finally, there is a lack of knowledge of environmental concentrations of MR in organisms collected from the field, making it difficult to relate laboratory observed toxicity to environmental outcomes.

1.6 Dissertation goals and objectives

The literature review above demonstrated knowledge thus far regarding the role of microrubber in the environment, specifically aquatic ecosystems. However, there were significant gaps in understanding potential adverse effects from MR exposure. This research aimed to fill gaps in microplastic research by identifying specific toxic responses of organisms to crumb rubber exposure. Based on my findings and supported by the literature, the overall assessment of the toxicity to crumb rubber particles in the aquatic environment was characterized and will be discussed in the chapters to follow.

The overarching goal of this dissertation was to determine the toxicological impact and risk of MR particles to fish in freshwater and marine environments. To achieve this goal, the first objective was to understand the acute toxicological effects from exposure to CR particles by understanding whether exposed fish were ingesting CR particles and determining the resulting toxicity as measured through three widely used biomarkers: bile fluorescence to indicate PAH absorption, metabolism, and biliary excretion; ethoxyresorufin-O-deethylase (EROD) activity as an indicator for cytochrome P450-1A activity, and glutathione S-transferase (GST) activity as an indicator for oxidative stress. The second objective was to investigate toxicity under environmentally

relevant conditions through a chronic, pulsed exposure. The third objective was to address the environmental impact and fate of MR by analyzing the digestive tracts of fish and macroinvertebrates collected from stormwater ponds and adjacent tidal creeks, assessing exposure using environmental observations.

The chapters herein elaborate on each objective and the methodology employed to accomplish each one. Each chapter contains information on exposure scenarios, biomarker analyses, and other methodology employed for each experiment. A discussion of observations and results is presented in each chapter and related to information presented in available literature. Further, limitations are discussed as well as general conclusions for each chapter. One publication to date has resulted from experiments conducted in support of this dissertation regarding the acute toxicity of MR to freshwater and marine fish species (LaPlaca & Van den Hurk, 2020). The final chapter provides overall conclusions and recommendations for future research.

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CHAPTER TWO

TOXICOLOGICAL EFFECTS OF MICRONIZED TIRE CRUMB RUBBER ON MUMMICHOG (FUNDULUS HETEROCLITUS) AND FATHEAD MINNOW (PIMEPHALES PROMELAS)

Stephanie B. LaPlaca and Peter van den Hurk

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2.1 Abstract

Recent studies on the distribution of microplastics in the Charleston Harbor, SC, USA revealed that a large part of the microplastic particles that are found in the intertidal sediments were tire wear particles. These particles originate from the wear of tire treads on roadways, and wash into the estuary during rain events. The abundance of these particles has raised questions about potential toxicity to aquatic organisms that ingest these particles. The synthetic rubber in car tires consists of a large variety of chemicals, which can vary between manufacturers, but usually contains styrene-butadiene rubber, carbon black and zinc. To investigate the potential toxicity of tire wear particles, both mummichogs (*Fundulus heteroclitus*) and fathead minnow (*Pimephales promelas*) were exposed to different concentrations of crumb rubber particles (38–355 μm) in a 7-day static renewal exposure. Dissection of the fish revealed that crumb rubber was ingested and accumulated in the intestinal tract. At the highest concentration tested (6 g/L) partial mortality (40%) was observed in the fathead minnow, which came close to the assumed LC50. To investigate if polycylic aromatic hydrocarbons (PAHs) were leaching from the particles, bile fluorescence was measured, together with potential induction of cytochrome P450-1A through the ethoxyresorufin-O-deethylase (EROD) assay. Elevated levels of 2-, 4-, and 5-, ring structures representative of PAHs were detected in the bile of exposed animals. Bile fluorescence indicated that 4-ring PAH compounds were the most bioavailable from the crumb rubber particles. Induction of EROD activity was observed in exposed animals at environmentally relevant concentrations of the crumb rubber particles (<1–2 g/L), and this elevated EROD activity indicated that PAH compounds

from the crumb rubber particles were being metabolized in both mummichogs and fathead minnow

2.2 Introduction

Plastic pollution is recognized as a worldwide environmental problem (Beaumont et al., 2019). The durability of plastic materials makes their decomposition in the environment difficult, but environmental conditions can reduce larger plastic objects to smaller particles known as microplastics (Weinstein et al., 2016). Microplastics can come in a variety of shapes but are generally defined as synthetic particles with size ranging from 1 μm to 5 mm which are insoluble in water (Frias & Nash, 2019). A recent survey of microplastic particle presence in coastal South Carolina showed that the most abundant particles were black fragments (Gray et al., 2018). In a follow-up study, black fragments that were found in the intertidal sediments, subtidal sediments, and sea surface microlayer in Charleston Harbor were identified as tire wear particles (TWPs) and formed 17.1% of the total microplastics encountered (Leads & Weinstein, 2019). Tire wear particles are defined as the particulates formed from tires undergoing friction on the road (Wagner et al., 2018). The prevalence of black fragments in South Carolina estuaries is considered unique as most surveys for MPs do not report black fragments or tire wear particles, although studies like those reported by Unice et al. (2019a, 2019b) which modeled tire and road wear particle release and transport in the Seine watershed (France), estimated that 18% of tire wear release was transported to freshwater and 2% exported to the estuary. The black fragments observed by Gray et al. (2018) were identified as tire wear particles based on general morphology and polymer composition, as described in Leads

and Weinstein (2019). Briefly, this classification as tire particles was based on physical criteria including black in color, elongated/cylindrical in shape, rough surface texture/encrustations, and rubbery consistency that maintained its shape when manipulated with forceps. In addition, Fourier transformed infrared spectroscopy (FTIR) demonstrated that these particles contain polybutadiene, styrene-butadiene, and carbon black, all components used in the tread of car tires (Leads & Weinstein, 2019).

Chemical properties of tire wear particles vary, with typical passenger car tire made up of mostly synthetic rubber polymer, reinforcing agents such as carbon black or silica, processing oils, and additives for vulcanization including zinc oxide and sulfur (Barbin & Rodgers, 1994). In addition, metals including aluminum, calcium, iron, sodium, potassium, and magnesium have been measured in elevated levels in tire wear particles but most likely originated from the pavement or road surface (Kreider et al., 2010).

Processing oils used in tire manufacturing typically have a high content of polycyclic aromatic hydrocarbons (PAHs). One study that analyzed compounds in recycled tire mulch pieces used on playgrounds found total PAH concentrations ranging from 1 μg/g to 200 μg/g in the mulch, and even higher concentrations of PAHs, up to 17000 μg/g, in recycled rubber tire tiles (Llompart et al., 2013). The most abundant PAH compounds detected in samples included pyrene, naphthalene, phenanthrene, fluoranthene, chrysene, and benzo[a]pyrene (Llompart et al., 2013).

Because tire wear fragments are prevalent in the estuaries of South Carolina, there is a need to assess the potential toxicity of these particles on estuarine organisms.

Previous studies have demonstrated that tire wear particles (size 38–355 μm) were not acutely toxic to grass shrimp (*Palaemonetes pugio*) at concentrations up to 100.0 g/L (Gray & Weinstein, 2017). However, a study of the toxicity of whole tire leachate (i.e. water containing dissolved and suspended material or chemicals from whole tires) in juvenile rainbow trout (*Oncorhyncus mykiss*) found both induction of CYP1A1 expression and hydroxylated PAHs in the bile of exposed fish, indicating release of PAHs from tires, followed by uptake and metabolism in the fish (Stephensen et al., 2003). Day et al. (1993) also observed toxicity in rainbow trout exposed to whole tire leachates, but no toxicity to the cladoceran, *Daphnia magna*, the nematode, *Panagrellus redivivus*, or fathead minnow, *Pimephales promelas*. Different studies assessing toxicity of tire particle elutriates (Panko et al., 2013) and leachates (Marwood et al., 2011) on fathead minnows found no significant adverse effects on fish growth or survival. There is a lack of knowledge on particle toxicity of microrubber (MR) particles themselves, as current studies focus primarily on the leachate of particles or whole tires. Leachate contains contaminants that are released from the particles themselves in selected media (sediment, water), and represents a passive form of uptake of contaminants into organisms. However, some contaminants may still be adsorbed to MR particles and only released under different environmental and intestinal conditions (i.e., salinity, pH) (Hartwell et al., 1998; Hüffer et al., 2019). Recent studies reporting the toxicity of tire wear particles have focused on invertebrates, for example: *Eisenia fetida* in Pochron et al. (2017), *Gammarus pulex* in Redondo-Hasselerharm et al. (2018a), and *Hyalella azteca* in Khan et al. (2019).

The goal of the current study was to assess if fish actively ingest crumb rubber, and what the toxicity was of these particles in both an estuarine fish species (*Fundulus heteroclitis*) and a freshwater fish species (*Pimephales promelas).* Toxicity was measured through three widely used biomarkers: (i) bile fluorescence as measure of PAH absorption, metabolism and biliary excretion; (ii) ethoxyresorufin-O-deethylase (EROD) assay as indicator for cytochrome P450-1A activity; and (iii) glutathione S-transferase (GST) activity with 1-chloro-2,4-dinitrobenzene (CDNB) as substrate.

2.3 Methods

2.3.1 Fish collection – Mummichogs (*Fundulus heteroclitus*)

Adult mummichogs were collected at Clam Bank in the North Inlet–Winyah Bay National Estuarine Research Reserve (33°20′02.3″N 79°11′;34.0″W), near Georgetown, SC. Both male and female mummichogs were used in the exposure experiments and ranged from 53 to 84 mm (total length) in size and 2.0 to 9.2 g in weight (wet weight). Animals were collected using frozen shrimp baited minnow traps around low tide, transported under constant aeration, and housed at the Clemson University Aquatic Animal Research Laboratory (AARL). Fish were acclimated for at least two weeks prior to exposure. Fish were housed in 20-gallon (75.7-L) tanks in a recirculating saltwater system. All fish were kept on a 16:8 light/dark cycle at 26 °C in 18ppt artificial saltwater (Instant Ocean, Spectrum Brands Inc.). Fish were fed a small amount of TetraMin tropical flake food once daily, before and during exposure.

2.3.2 Fish culture – Fathead minnows (*Pimephales promelas*)

Adult fathead minnows were cultured at the Clemson University Institute of Environmental Toxicology (CU ENTOX) in a static renewal system containing four troughs with moderately hard water artificially prepared using the US EPA recipe (USEPA, 1993). Both male and female fathead minnows were used in the exposure and ranged from 50 to 66 mm (fork length) in size and 1.2 to 3.5 g in weight (wet weight). Water hardness ranged from 80–100 mg/L as $CaCO_3$, alkalinity from 57–64 mg/L as $CaCO_3$ and pH = 8. Water turnover rate was five times per day. Fish were kept on a 16:8 light/dark photoperiod at 22 °C. Adult fish were fed TetraMin tropical flake fish food once daily before and during exposure.

2.3.3 Exposure set up

Commercially available micronized tire fragments (i.e., crumb rubber) were used as a reproducible source of particles similar in size, shape, and polymer composition as tire wear particles found in the environment. Crumb rubber was purchased from Edge Rubber (Chambersburg, PA, USA), which was produced from raw material derived from passenger and/or truck tires using wet-grind technology. The rubber produced contained the following compounds: up to 22% acetone extractables, 8% ash content, 38% carbon black, 35% natural rubber, and a minimum of 42% rubber hydrocarbon and particles had a specific gravity between 1.10–1.15 (Edge Rubber 2016). Crumb rubber was sieved to obtain fragments 38–355 μm in size.

Prior to crumb rubber exposure, nitrite (NO_3^-) and total ammonia (NH_3/NH_4^+) were monitored in three control tanks to determine required frequency of water renewals. Fish were placed in individual 4 L tanks with 2 L of aerated moderately hard water (for

fathead minnows) or artificial saltwater (mummichogs). Fish were fed TetraMin tropical flake food once daily. Temperature, pH, NO_3^-, and NH_3/NH_4^+ were recorded daily for three days. To keep ammonia levels within an acceptable range (<0.25 mg/L) (USEPA, 2013), it was determined that water renewals should occur every other day during exposures. Fish used in this pre-setup step were not used in crumb rubber exposures.

Mummichog exposures (Experiment A and Experiment B)

All exposures were performed according to the Clemson University Institutional Animal Care and Use Committee approved Animal Use Protocol 2015-067. Two separate exposures with different concentrations of crumb rubber were conducted using mummichogs, with identical exposure set up and methodology for both experiments. Concentrations of crumb rubber were based on experiences in previous experiments with grass shrimp (Gray & Weinstein, 2017) and included 0, 0.3, 1.9, and 6.0 g/L for the first exposure (Experiment A). Based on the results of Experiment A, a second experiment with lower concentrations was performed: 0, 0.1, 0.33, and 1.0 g/L (Experiment B) in an effort to capture partial responses for the measured biomarkers, which would enable the calculation of a traditional dose-response curve. Each of the four treatments had five independent, randomized replicates (n = 5 fish per treatment). All treatments were conducted in 4 L glass jars with aerated artificial saltwater (18ppt, Instant Ocean, Spectrum Brands Inc.) in a static-renewal set up, with water renewal every other day. Each of the five fish per treatment were exposed individually in a 4 L glass jar. A mixed population of both males and females was used in the exposure and distributed among treatments as fish were not spawning so no effects from sex were expected. Water

renewal also included renewal of crumb rubber to keep a constant concentration of particles. The exposure ran for seven days with the experiment ending on the eighth day. Fish were fed TetraMin tropical flake food daily during the first 6 days. During the exposure, the temperature, pH, NO_3^-, and NH_3/NH_4^+ were recorded daily in the control tanks. Temperature ranged from 22–28 °C, pH ranged from 7.5–7.8, NO_3^- remained below 5.0 mg/L, and NH_3/NH_4^+ remained below 2.0 mg/L. At the end of the experiment, animals were euthanized by a lethal dose of buffered tricaine methanosulfate (MS-222, 1 g/L). Standard length (mm) and wet weight (g) were recorded for each fish. Gallbladders were removed, placed in dark microcentrifuge vials, and frozen at −20 °C. Liver samples were dissected, wrapped in labeled aluminum foil, and frozen in liquid nitrogen until transferred to a −80 °C freezer.

Fathead minnow exposure (Experiment C)

One exposure with fathead minnows was conducted (Experiment C). Four treatments with five independent, randomized replicates (n = 5 fish per treatment) were included. Concentrations of crumb rubber included 0, 0.3, 1.9, and 6.0 g/L, as described above for mummichog. All treatments were conducted in 4 L glass jars with aerated moderately hard water (US EPA 1993), in a static-renewal set up, with water renewal every other day following the same procedure as experiment A. A mixed population of both males and females was used in the exposure and distributed among treatments, with fish not in spawning condition, so no effects from sex differences were expected. At the end of the experiment, animals were euthanized by lethal dose of buffered tricaine methanosulfate (MS-222, 1 g/L). Fork length (mm) and wet weight (g) were recorded for

each fish. Gallbladders were removed, placed in dark microcentrifuge vials, and frozen at −20 °C. Liver samples were dissected, wrapped in labeled aluminum foil, and frozen in liquid nitrogen until transfer to a −80 °C freezer.

2.3.4 Biomarkers

Bile Fluorescence

To measure absorption, metabolism and biliary excretion of PAHs released by the crumb rubber, bile samples were analyzed for fluorescence at excitation/emission wavelength pairs that are specific for 2-ring (290/335 nm), 4-ring (341/383 nm) and 5-ring (380/430 nm) PAHs (Aas et al., 2000a). Fluorescence properties vary between PAH compounds and are dependent on size, structure, and ring substitutes, allowing for categorization of compounds into 2-, 4-, and 5- ring structures by fixed wavelength fluorescence measurements (Aas et al., 2000a). Gall bladders were thawed, and bile was released into dark microcentrifuge tubes. Bile volume was measured and recorded, and if less than 50 µl, deionized water was added to bring the volume up to 50 µl. Three consecutive serial dilutions (1:100, 1:200, 1:500) were prepared in dark microcentrifuge tubes using a 50:50 methanol:water solution. Fluorescence of aromatic compounds (FACs) was then measured in three replicate aliquots from each dilution at 290/335, 341/383 and 380/430 nm on a BioTek Synergy H1 plate reader. Raw fluorescence data were plotted against dilution, and the values of the highest dilution not showing inner filter effects were used for further calculations. The FAC values were corrected using a methanol:water blank and normalized to bile volume (Van den Hurk 2006). Samples from experiment C were not normalized to bile volume due to limited total volume

collected from organism at the end of exposure. The FAC values for experiment C are presented as fluorescent units/bile sample.

Liver enzyme activity (CYP1A and GST)

Activities of two inducible enzymes that are involved in the metabolism of toxicants were measured in liver homogenates. Livers were individually homogenized with a glass Potter-Elvehjem homogenizer in 2 mL of chilled homogenization buffer (Van den Hurk, 2006). Liver homogenates were then centrifuged at 10,000 × g at 4 °C for 20 min, after which the supernatant (S9 fraction) was divided into three aliquots for later determination of ethoxyresorufin-Odeethylase (EROD) activity, glutathione S-transferase (GST) activity, and total protein concentration. The EROD and GST aliquots were stored in at −80 °C and the protein aliquot was stored at −20 °C prior to analysis. Protein concentrations in the S9 fractions were measured with a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL), using bovine serum albumin (BSA) to prepare the standard curve.

For the EROD assay, liver S9 fractions were diluted to 1.0 mg/mL total protein concentration, and 100 μL of these diluted S9 fractions (in duplicate) were added to a black 96-well plate. The reaction was started by adding 0.5 mM NADPH in 150 μL of reaction buffer (Tris buffer 0.1 M, pH 7.8, 0.2% BSA, 5 mM MgCl2, 2 μM ethoxyresorufin) to the assay wells (Schreiber et al., 2006). The fluorescence was then recorded at 530/585 nm in 5–10 min intervals over 30 min on a BioTek Synergy H1 plate reader. A 1 mg/mL BSA sample was used in duplicate as blank. A 7-step dilution series

in methanol of the enzymatic product resorufin was used to generate a standard curve ranging from 0–800 nM.

Activity of GST was measured as the conjugation of glutathione with 1-chloro-2,4-dinitrobenzene (CDNB) by cytosolic protein (Mierzejewski et al., 2014). The total reaction mixture of 250 μL contained 0.1M HEPES buffer (pH 7.6), 1 mM glutathione (GSH), and 25 μg S9 protein. The reaction was started by adding CDNB (1mM final concentration) where after formation of the CDNB conjugate was measured by taking absorption readings on a BioTek Synergy H1 plate reader at 20 s intervals for 2 min at 344 nm. This was quantified by using the molar absorptivity of 9.6 mM^{-1} for the enzymatic product.

2.3.5 Statistical analysis

All statistical analyses were performed using JMP Pro 14 (SAS Institute Inc.) statistical software. Data were checked for normality before analysis and log-transformed for analysis to better approximate normality when required. Samples of dead or morbid fish were excluded from analysis. Data were analyzed separately for each experiment (i.e., only Experiment A data were used for statistical analysis of Experiment A). A one-way analysis of variance (ANOVA) was conducted to assess differences in means between the various concentration groups in each experiment. When significant differences were observed, Dunnett's post hoc test was conducted to determine which treatment groups differed from the control group. A p value of <0.05 was considered significant.

2.4 Results

2.4.1 Experiment A – Mummichogs (0, 0.3, 1.9, 6.0 g/L crumb rubber concentrations)

All fish appeared healthy at the start of the exposure, and there was no mortality recorded during the exposure time. After dissection, crumb rubber was observed in the GI tract of the exposed fish, indicating that the mummichogs were actively ingesting the particles (**Figure 2.1**). For the bile fluorescence, there were significant differences between control and most exposed fish for 4- and 5-ring structures, but no differences between the individual particle concentrations among the exposed fish (**Figure 2.2A**) (For 0.3 g/L 4-ring bile fluorescence, $p = 0.0042$; 1.9 g/L 4-ring fluorescence, $p = 0.0426$; 6.0 g/L 4-ring fluorescence, $p = 0.0053$. For 0.3 g/L 5-ring bile fluorescence, $p = 0.0238$; 6.0 g/L 5-ring fluorescence, $p = 0.0039$). There was no difference in bile fluorescence for 2-ring structures between control and exposed animals, but when compared with experiment B there appears to be an unusually high amount of 2-ring fluorescence in the control animals of experiment A (**Figure 2.2A**). Although enzyme activity for CYP1A was slightly elevated in exposed fish, it was not significantly different between control and exposed fish (**Figure 2.3A**). There was no significant difference measured in GST activity between control and exposed fish (**Figure 2.4A**). There were no differences observed between males and females for any of the biomarkers tested.

2.4.2 Experiment B – Mummichogs (0, 0.1, 0.33, 1.0 g/L crumb rubber concentrations)

A second experiment was conducted at lower concentrations than in experiment A to capture partial responses at lower concentrations. All fish appeared healthy at the beginning of the exposure, but by the end of the exposure time, two fish had died in the highest concentration and one appeared morbid; these fish were excluded from further analysis. There were significant differences in bile fluorescence between control and exposed fish for 4- and 5-ring structures at most concentrations tested (For 0.1 g/L 4-ring bile fluorescence, $p = 0.0047$; 0.33 g/L 4-ring fluorescence, $p = 0.0380$; 1.0 g/L 4-ring fluorescence, $p = 0.0057$. For 0.1 g/L 5-ring bile fluorescence, $p = 0.0204$; 1.0 g/L 5-ring fluorescence, $p = 0.0188$) and significant differences for 2-ring structures between control and exposed animals for 0.1 g/L ($p = 0.0306$) and 1.0 g/L ($p = 0.0001$) crumb rubber (**Figure 2.2B**). Enzyme activity for CYP1A was significantly different compared to controls for 0.33 g/L ($p = 0.0018$) and1.0 g/L ($p = 0.0453$) crumb rubber concentrations (**Figure 2.3B**). There was no significant difference measured in GST activity between control and exposed fish (**Figure 2.4B**), although there appeared to be a slight increase in GST activity in the two highest doses, as was seen in the EROD assay. There were no differences observed between males and females for any of the biomarkers tested.

2.4.3 Experiment C – Fathead minnows (0, 0.3, 1.9, 6.0 g/L crumb rubber concentrations)

Fish appeared healthy at the beginning of the exposure, but there was 40% mortality recorded at the highest concentration (6 g/L) by the end of the seven day exposure. As with the mummichogs, crumb rubber was observed in the GI tract of the exposed fish, indicating that the fathead minnows were also actively ingesting the

particles. There were significant differences in bile fluorescence between control and exposed fish for 2- and 5-ring structures at all concentrations tested (For 0.3 g/L 2-ring fluorescence, p=0.0161; 1.9 g/L 2-ring fluorescence, p=0.0022; 6.0 g/L 2-ring fluorescence, p=0.0389 and for 0.3 g/L 5-ring fluorescence, p=0.0098; 1.9 g/L 5-ring fluorescence, p= <0.0001; 6.0 g/L 5-ring fluorescence, p=0.0332) and significant differences in bile fluorescence for 4-ring structures between control and exposed animals for 0.3 g/L (p=0.0012) and 1.9 g/L (p= <0.0001) crumb rubber concentrations (**Figure 2.2C**). The lower levels of fluorescent compounds at the highest exposure concentration may be attributed to the near morbidity of these fish. Enzyme activity for CYP1A was significantly elevated in exposed fish at all concentrations tested (For 0.3 g/L, p=0.0315; 1.9 g/L, p=0.0276; 6.0 g/L, p=0.0082) (**Figure 2.3C**). The GST activity was significantly higher in fathead minnows exposed to 1.9 g/L (p=0.0441) and 6 g/L (p =0.0015) crumb rubber (**Figure 2.4C**). There were no differences observed between males and females for any of the biomarkers tested.

2.5 Discussion

The goal of this study was to investigate if fish species ingest crumb rubber when exposed to this form of polymer debris, and if they show signs of toxicological responses, as measured through commonly used biomarkers. Previous studies on the toxicity of microrubber particulates have focused on growth and survival (Khan et al., 2019; Pochron et al., 2018) rather than sublethal effects as demonstrated in the present study. The exposure experiments showed that both mummichogs and fathead minnows do indeed ingest the particles, which may enhance the uptake of chemicals that can leach

from these particles. As a result of PAHs leaching from the particles, bile fluorescence was increased, indicating that PAHs were absorbed, metabolized, and excreted into the bile. The measured liver enzyme activity showed a significant increase for the EROD assay for both fish species, but for GST activity only an increase at the highest two exposure concentrations was found for the fathead minnows.

Based on our results, and others, it appears that PAHs leaching from the crumb rubber are toxicants of major concern, although other chemicals in tires may also contribute to observed effects (Llompart et al., 2013; Stephensen et al., 2003). PAHs are naturally fluorescent compounds that generally absorb UV light followed by emission of a longer wavelength light (Aas et al., 2000a; Rivera-Figueroa et al., 2004). Fish metabolize PAHs mainly in the liver and most of the metabolites produced are excreted into the bile, which is stored in the gallbladder until food enters the intestinal tract, and the bile is released to aid digestion (Varanasi, 1989). The detection of PAH metabolites in fish bile as fluorescent aromatic compounds (FACs) has been widely used in monitoring programs and other studies and demonstrate that fish were recently exposed to PAHs (Aas et al., 2000b; Otter et al., 2012; Van den Hurk & Haney, 2017b; Vuorinen et al., 2006). As such, bile fluorescence is a valuable biomarker of exposure, and demonstrates that the PAHs from the tire crumb were absorbed and excreted by the mummichogs and fathead minnows.

In this study, all experiments showed significant increases in bile fluorescence between control and exposed animals, indicating that fish were absorbing PAHs released by the crumb rubber. For experiment A and B (mummichogs), there were significant

increases in fluorescence for 4- and 5-ring structures in exposed fish. The 2-ring structures were elevated compared to controls, but because of relatively high levels of 2-ring fluorescence in the controls, not all differences with the crumb rubber treatments were statistically significant. Other studies have demonstrated as well that in general, 2-ring fluorescence is much higher in fish bile than 4-ring and 5-ring fluorescence (Mierzejewski et al., 2014; Van den Hurk & Haney, 2017b), and not necessarily correlated. Lakowicz (2006) attributed the high 2-ring fluorescence in bile to other fluorescent compounds, possibly fluorescent amino acids like phenylalanine, tyrosine, and tryptophan in peptides and proteins. Therefore, the high 2-ring fluorescence in the present study could be explained by the presence of other fluorescent compounds in the bile that are not PAHs (i.e. amino acids).

It is interesting to notice that for almost all treatments for both species, the ratio between 4-ring and 2-ring FACs is reversed compared to the controls. This indicates that the bioavailable PAH compounds from crumb rubber are mostly 4-ring structures. Chemical analysis of the crumb rubber used in this study indicated that they are composed of 80% 4-ring structures with pyrene found in the greatest abundance, followed by fluoranthene, phenanthrene, and benzo[a]pyrene (Beckingham, unpublished). Similarly, Kreider et al. (2010) also reported the majority of PAH compounds from roadway particles, tire wear particles, and tread particles were 4-ring structures. Future studies aimed at identifying specific compounds or PAH metabolites in bile of fish exposed to crumb rubber would be beneficial in determining which compounds are bioavailable from crumb rubber and could cause toxicological effects.

The observation that especially the 4-, and 5-ring PAHs were increased in the bile samples would predict that enzymes that are induced through activation of the Ah receptor should show an increased activity (Schlenk et al., 2008). The Ah receptor efficiently binds 4-, and 5-ring PAHs, and activation of the Ah receptor results in induction of cytochrome P450-1A, which is detected with the EROD assay. Indeed, we observed that in all treatments in experiment C (fathead minnow), and several treatments in experiment A and B (mummichogs), EROD activity was significantly increased. For mummichogs, the highest EROD activity was recorded at 0.33 and 1.0 g/L crumb rubber concentrations but decreased at concentrations greater than 1.0 g/L. This may suggest a suppressed induction or suppressed activity of the CYP1A enzyme at high concentrations of crumb rubber. Previous studies have found that certain PAHs or CYP1A-inducing compounds may co-occur in environmental samples with compounds that have inhibitory effects on CYP1A systems in teleosts; for example, in flounder (*Platichthys flesus*), and mummichog, suppression of the CYP1A induction response was observed in benzo[a]pyrene + cadmium treated fish (Beyer et al., 1997; Van den Hurk et al., 1998). While we do not expect cadmium to be present in significant amounts in crumb rubber, other metals that are present in synthetic tire rubber may result in comparable effects (Risso-de Faverney et al., 2009). This should be further investigated given the co-occurrence of trace metals in tire products. Likewise, organic compounds, such as nonplanar polychlorinated biphenyls (PCBs) and PCB mixtures, have been shown to inhibit CYP1A-mediated responses at high levels of exposure in fish (Boon et al., 1992; Melancon & Lech, 1983). While PCBs, and other chlorinated compounds like dioxins

and dibenzofurans may be associated with crumb rubber (Menichini et al., 2011), the concentrations released from the particles would be so low that we do not suspect PCBs to play a role in the present study. However, additional analysis of potentially toxic compounds in crumb rubber and which are bioavailable to organisms from the particles would help to further understand the induction or suppression response of CYP1A enzyme activity in these exposures.

EROD activity of exposed fish in experiment C (fathead minnow) was significantly higher compared to control fish (**Figure 2.3C**). Species differences between fathead minnow and mummichogs, as well as environmental differences (i.e., freshwater vs saltwater) are likely factors that resulted in greater expression of CYP1A activity in fathead minnows. Species differences in EROD activity were also observed in Cyprinid and Centrarchid species that were dosed with benzo[a]pyrene, indicating that phylogenetic differences can play a role in species sensitivity to environmental pollutants (Van den Hurk et al., 2017a). Also, it is well documented that increased salinities can reduce the solubility of PAHs (Ramachandran et al. 2006; Tremblay et al. 2005; Whitehouse 1984). Hartwell et al. (1998) demonstrated decreased toxicity of tire leachate with increasing salinity, further suggesting the reduced toxicity and solubility of crumb rubber or tire components at higher salinity. Future studies that assess the toxicity of tire wear particles and evaluate CYP1A activity under various salinities would be useful to identify if there is a certain threshold of salinity where tire wear particles begin to have a more or less toxic effect. Of particular interest would be to test at or near, above, and below salinities where mummichogs are iso-osmotic to seawater.

Overall, increased EROD activity in fish exposed to tire wear particles indicates induction of detoxification enzymes that metabolize PAHs. While the induction of CYP1A should be seen as an adaptive response to chemical stress, it does not necessarily mean that the overall health of the fish is affected. Because metabolism of 4-, and 5-ring PAHs by CYP1A can lead to the production of very toxic intermediates, further analysis of tissue damage or DNA damage would elucidate if overall health of the fish is affected by the exposure to crumb rubber.

In experiment A and B (mummichogs), GST activity was not significantly different between control and exposed fish. A high amount of oxidative stress is needed to detect a significant increase in GST activity (Ramachandran et al., 2006). One of the reasons for the relative insensitivity of the GST assay is that most researchers use CDNB as a substrate for the enzyme. CDNB is a substrate for most GST isoforms, which means that even if one or more isoforms are upregulated as a result of exposure to environmental pollutants, this signal may get lost in the overall variability of the entire GST isoform pool, which generally has a high constitutive expression already in most species (Schlenk et al., 2008). Our findings correspond to observations from other studies where GST activity was not affected after chemical exposure (Collier & Varanasi, 1991). For example, in mussels (*Mytilus edulis*) no correlation between GST activity and PAH pollution levels has been observed (Akcha et al., 2000). However, some studies in fish species have observed increases in GST activity as PAH concentrations increase (*Oreochromis mossambicus* in Shailaja & D'Silva, 2003; *Nocomis leptocephalus* and *Semotilus atromaculatus* in Van den Hurk et al., 2017a). Measurement of GST activity

has potential to indicate oxidative stress as a result of PAH exposure and metabolism but may not necessarily be the most effective biomarker of exposure. The GST activity in experiment C (fathead minnows) exposed to 1.9 and 6 g/L crumb rubber were the only concentrations that showed a significant increase compared to control group. The highest dose group (6 g/L) also had 40% mortality before the end of the exposure. It is possible that this high concentration crumb rubber in a freshwater environment allowed for increased bioavailability of PAHs which in turn, initiated an oxidative stress response as fathead minnows attempted to detoxify PAHs.

2.6 Conclusions

In conclusion, it is becoming more and more evident that crumb rubber is a serious environmental problem. Kole et al. (2017) reported an estimate of 152 × 108 kg per year of crumb rubber is released into the environment on average in the US alone. Combined with the observed response in the present study and reported by others (Day et al. 1993; Camponelli et al. 2009; Khan et al. 2019; Pochron et al. 2018; Stephensen et al. 2003), we suggest that more attention should be paid to management of road runoff. This study demonstrated toxicity responses from exposure to crumb rubber in two fish species, mummichogs (*F. heteroclitus*) and fathead minnow (*P. promelas*) when exposed to concentrations up to 6.0 g/L. Particles were ingested by these fish, and at environmentally relevant concentrations (1–2 g/L, Leads & Weinstein, 2019) the largest response in biomarkers was observed. Bile fluorescence measurements indicated that 4-ring PAH compounds (i.e. pyrene) were the most bioavailable of crumb rubber chemical transfer, which does corroborate with leachate studies (Beckingham, unpublished; Kreider et al.,

2010; Stephensen et al., 2003; Wik & Dave, 2006). Future studies to measure specific PAH compounds in the bile of fish, and the impact of salinity on crumb rubber toxicity will be helpful to further determine the long-term toxicity of exposure to crumb rubber.

2.7 Figures

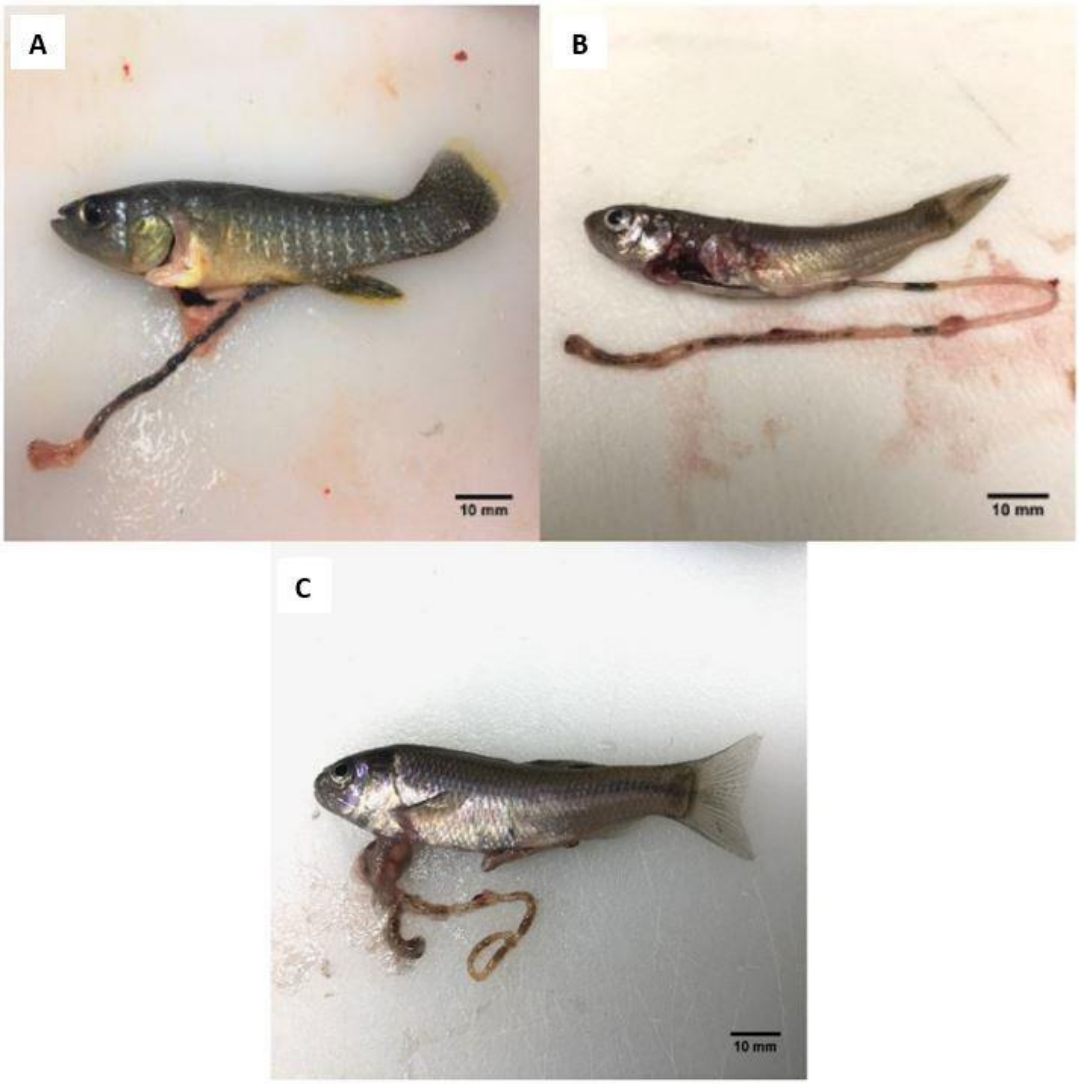
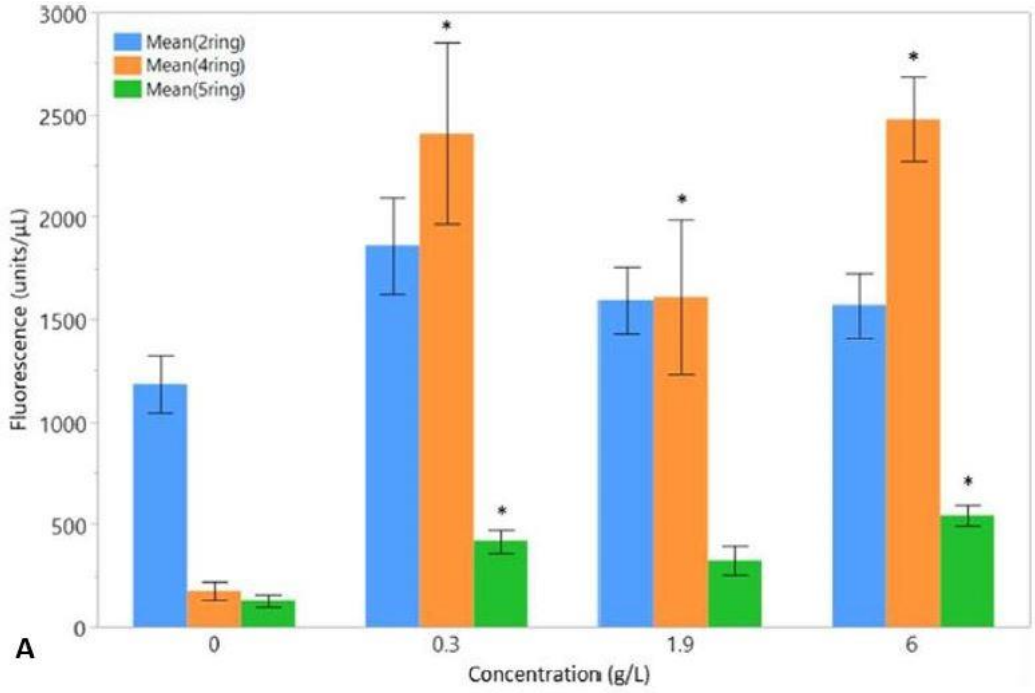


Figure 2.1 - Crumb rubber in the gastrointestinal tract of mummichog (A) and fathead minnow (B) at end of exposure. Mummichog (A) from experiment A at 0.1 g/L crumb rubber exposure, fathead minnow (B) from experiment C at 0.3 g/L crumb rubber exposure, and fathead minnow (C) from experiment C at 0 g/L crumb rubber shown as control. Fish were fed Tetramin® flake food daily during exposure until 1-day prior to experiment take down.


Mean(2ring)
Mean(4ring)
Mean(5ring)
Fluorescence (units/µL)
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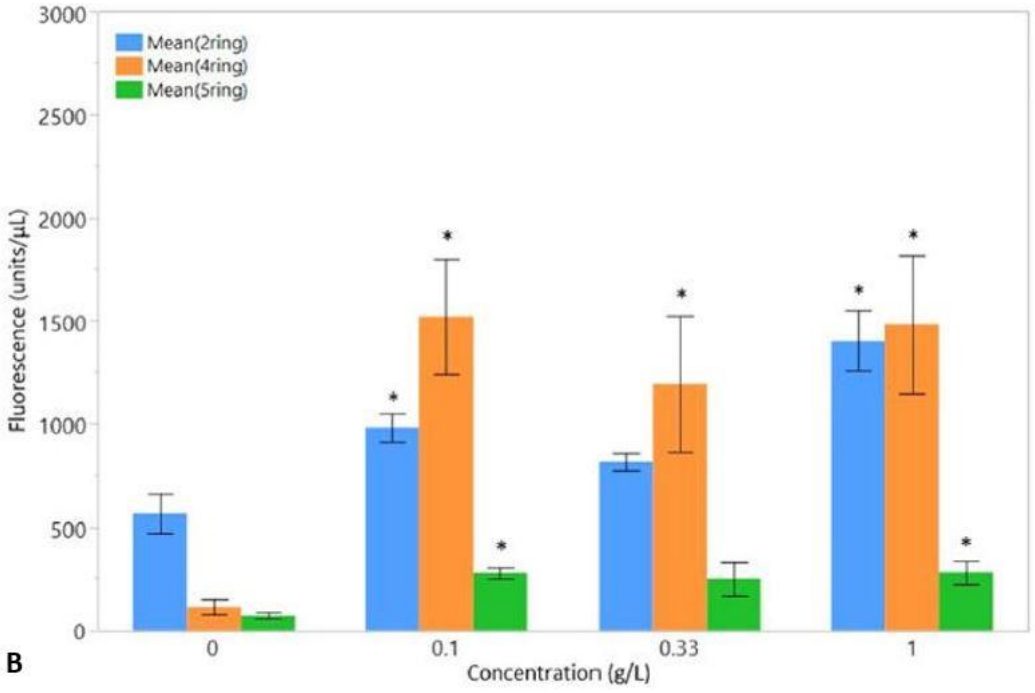
2500
2000
1500
1000
500
0
0
0.3
1.9
6
Concentration (g/L)
*

A


Mean(2ring)
Mean(4ring)
Mean(5ring)
Fluorescence (units/µL)
3000
2500
2000
1500
1000
500
0
0
0.1
0.33
1
Concentration (g/L)
*

B

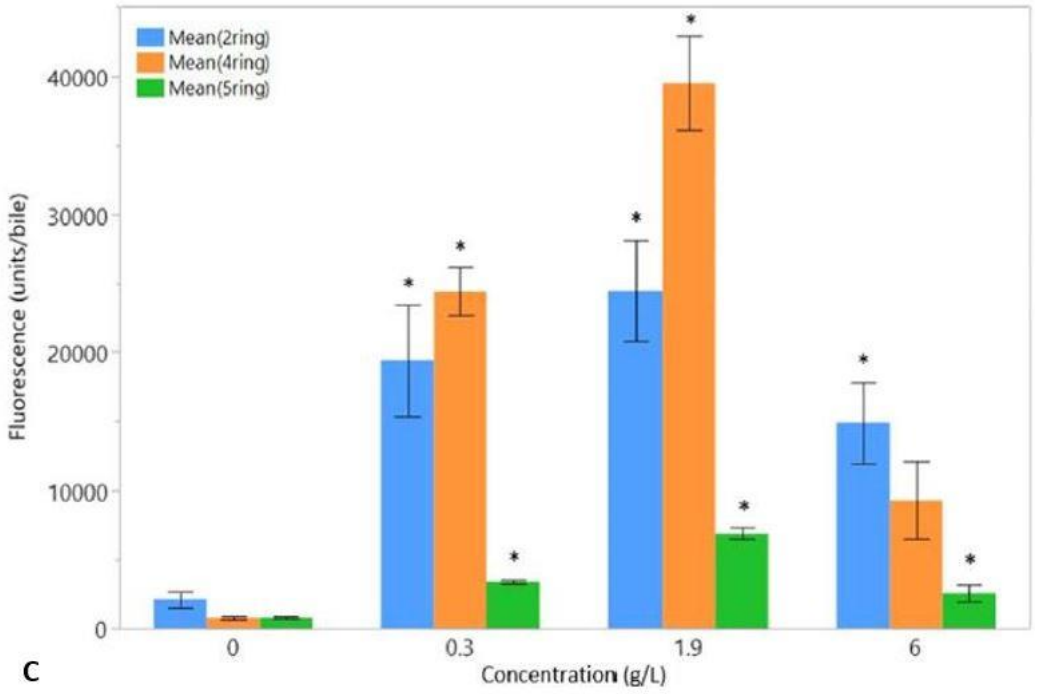


Figure 2.2 - Bile fluorescence in test organisms measured for 2-, 4-, and 5-ring structures for Experiment A (A), Experiment B (B), and Experiment C (C) after 7 d static renewal exposure of up to 6 g/L crumb rubber. Wavelength pairs used for measuring bile fluorescence of 2-ring structures were 290/335, 4-ring structures were 341/383, and 5-ring structures were 380/430 nm. Bile fluorescence units were normalized by bile volume for experiment A (A) and B (B). Bile fluorescence units were not normalized by volume for experiment C (C) due to limited bile produced in organisms at end of exposure. Bars indicate average fluorescence and standard error is shown. An asterisk above bars indicates significant difference from the control (p value < 0.05).

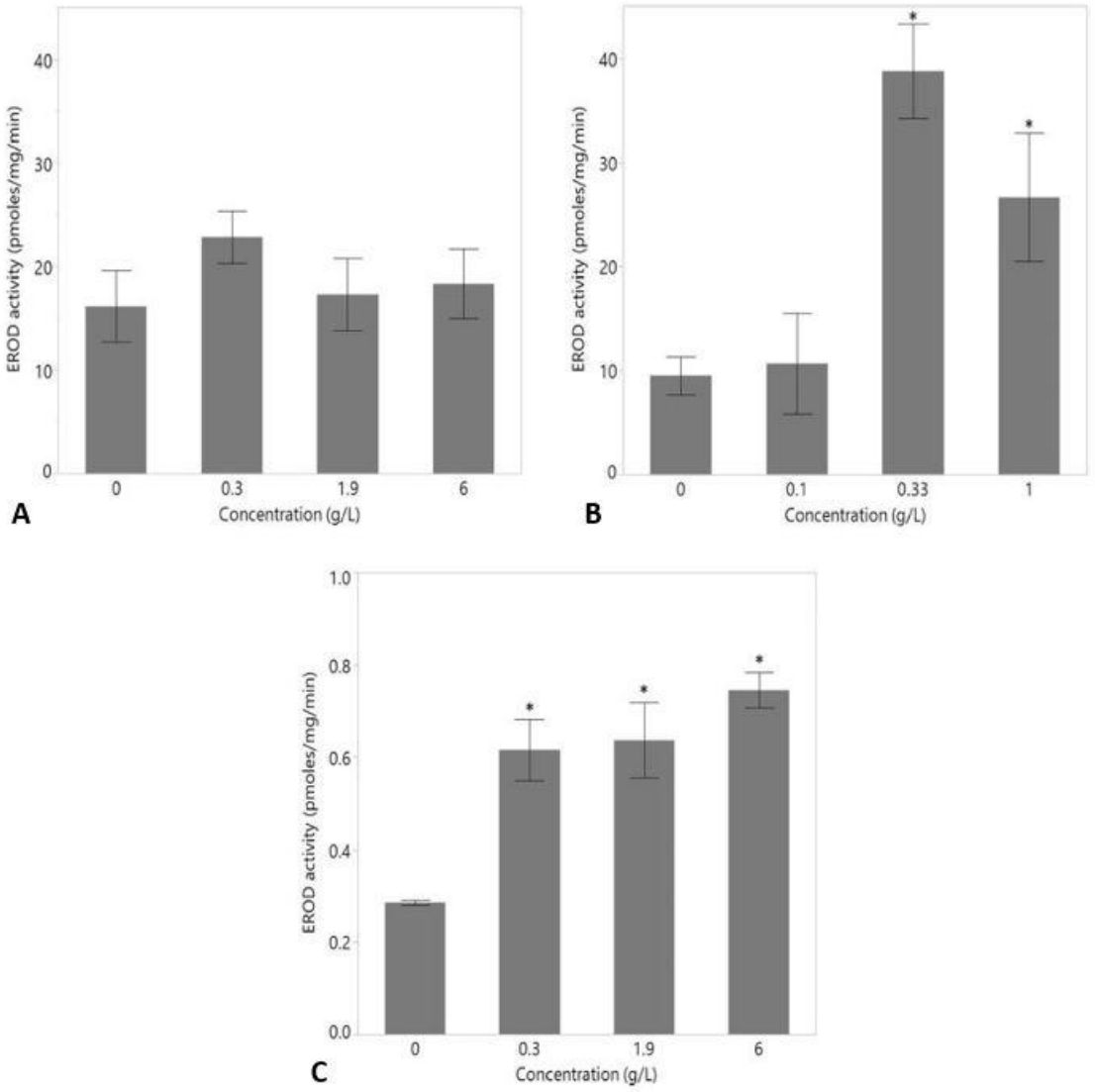


Figure 2.3 - Ethoxyresorufin-O-deethylase (EROD) activity for Experiment A (A), Experiment B (B), and Experiment C (C) after 7 d static renewal exposure of up to 6 g/L crumb rubber. EROD activity is indicative of cytochrome P450-1A enzyme activity. Bars indicate average EROD activity and standard error is shown. An asterisk above bars indicates significant difference from the control (p value < 0.05)

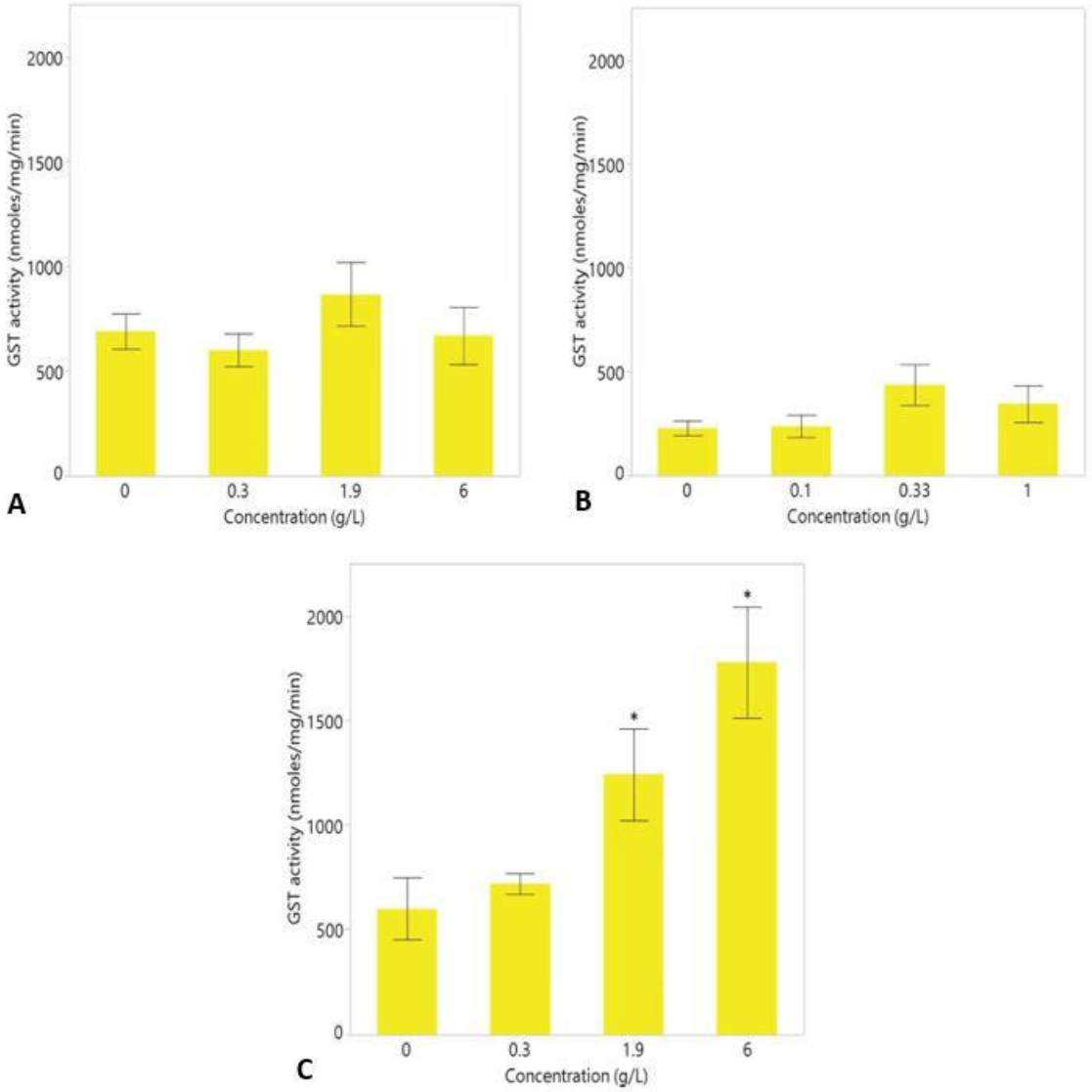


Figure 2.4 - Glutathione S-transferase (GST) activity for Experiment A (A), Experiment B (B), and Experiment C (C). Elevated GST activity is indicative of high amounts of oxidative stress. Bars indicate average GST activity and standard error is shown. An asterisk above bars indicates significant difference from the control (p value < 0.05)

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CHAPTER THREE

CHRONIC TOXICITY OF TIRE CRUMB RUBBER PARTICLES TO MUMMICHOG (*FUNDULUS HETEROCLITUS*) IN PULSED EXPOSURES

3.1 Abstract

Microrubber (MR) encompasses all tire-related particles in the micro-scale and has recently been recognized as a microplastic class. While tire particles have been entering the environment since the introduction of rubber tires for vehicles, the concern regarding tire wear particles (TWP) as an environmental contaminant is relatively new. Recent studies have examined physical and chemical toxicity of MR particles and leachates to a variety of organisms. However, there is a lack of information on the long-term effects of tire particle exposure under environmentally realistic conditions. The current study examines the chronic toxicity of crumb rubber (CR) particles to the estuarine fish species, mummichog (*Fundulus heteroclitus*), under a pulsed-dosing regime at environmentally relevant concentrations. Immunohistochemistry (IHC) of fish gill, intestine, and liver was performed to assess CYP1A activity in various organs. Bile fluorescence was measured as an indicator of exposure to polycyclic aromatic hydrocarbons (PAHs) from CR. DNA damage was measured through the formation of 8-hydroxy-2'-deoxyguanosine (8-OHdG) and oxidative stress was measured through the thiobarbituric acid reactive substance (TBARS) assay, free glutathione (GSH), and oxidized glutathione (GSSG). Upregulation of CYP1A in gill, intestine, and liver was observed in IHC from exposed organisms. Additionally, bile fluorescence increased as CR concentration increased, suggesting an increase in exposure to aromatic compounds from CR which agreed with previous findings from our lab and other studies

documenting the release of PAHs from CR. Data for DNA damage indicated greater 8-OHdG production at higher CR concentrations indicating DNA repair byproducts increased as organisms dealt with exogenous stress from CR. There was a decrease in malondialdehyde (MDA) production from the TBARS assay at higher concentrations of CR and an increase in GSH at higher concentrations of CR. Under long-term repeated dosing, it is possible that antioxidant systems in mummichog were upregulated in exposed organisms to deal with exogenous stressors as indicated by the higher measured 8-OHdG as a byproduct of oxidative repair, lower overall lipid peroxidation byproducts, and increased GSH at higher CR concentrations. Combined, these data suggest that fish exposed to tire crumb rubber particles illicit a mild biomarker response under environmentally relevant CR concentrations, and therefore, CR particles pose an overall low risk of chronic toxicity to *F. heteroclitus*.

3.2 Introduction

Microrubber (MR) is a ubiquitous pollutant found in various environmental compartments worldwide including air, sediment, water, and biota. MR is an umbrella term that encompasses all micronized rubber particles including tire and roadway particles (TRWP) which are generated from friction with the road surface and may include other components of road dust; tire wear particles (TWP) which are particles generated from new or used tires under laboratory conditions; and tire crumb rubber (TCR or CR) which are particles generated from the fractionation of recycled and shredded end-of-life tires that may be used in construction applications (Halle et al., 2020). Tire wear has been estimated to be one of the largest sources of microplastics

entering the aquatic environment with global emissions estimated at 0.81 kg/year/capita worldwide (Kole et al., 2017). Emission estimates in the U.S. are projected to be significantly higher at 4.7 kg/year/capita due to a higher number of cars per capita as well as longer average commutes than other countries (Kole et al., 2017; Sutton et al., 2019). Additionally, studies have found tire particles to represent anywhere from 17 to 50% of total microplastics encountered in environmental samples of sediments or stormwaters (Grbić et al. 2020; Leads & Weinstein, 2019; Sutton et al. 2019).

In recent years more specific data have become available regarding the distribution of MR in various environmental matrices. Measured concentrations of MR in surface water range from 9×10^{-5} to 6.4×10^{-3} g/L. Road runoff concentrations of MR determined by tire markers range from 3×10^{-4} to 0.179 g/L and concentrations of MR in surface water originating from road runoff is estimated to be one order of magnitude lower, between 3×10^{-5} to 1.79×10^{-2} g/L (Wik & Dave, 2009). Based on these estimates, road runoff may contain up to approximately 0.2 g/L MR. Organisms exposed to this highest estimated dose are likely to be exposed during a first flush effect during rain events. In the first flush period of a rain event, pollutant concentrations can be substantially higher compared to later stages of runoff due to the rapid displacement of pollutants that have accumulated on surfaces such as roadways (Poudyal et al., 2016).

The toxicity of MR and MR leachates varies greatly depending upon type of tire rubber tested, contaminant of interest, or method of leaching used for preparing MR extracts. Potential chemicals of concern identified from MR include Zn, PAHs, and the recently detected compound linked to Coho salmon die-offs, N-(1,3-dimethylbutyl)-N'-

phenyl-*p*-phenylenediamine-quinone (6PPD-quinone) (Klöckner et al., 2019; Peter et al., 2018; Stephensen et al. 2003; Tian et al., 2021). Data from acute exposures to MR suggest lethal and sublethal effects in various species tested. For example, Khan et al. (2019) observed an LC50 of approximately 1 g/L of TWP in *H. azteca*. Conversely, Marwood et al. (2011) observed no effect on mortality for *D. magna* and *P. promelas* when exposed to MR elutriates thus highlighting the differences seen in measured toxicity that depend on species, rubber type, or leachate preparation among other factors. Sublethal effects such as induction of cytochrome P4501A1 (CYP1A) and increased oxidative stress have been observed in organisms exposed to rubber leachates under acute exposure conditions (Stephensen et al., 2003; LaPlaca & Van den Hurk, 2020).

While an abundance of acute and short-term studies on MR toxicity exists, there is a lack of knowledge on the chronic and sub-chronic effects of MR leachate and MR particles to aquatic organisms, specifically information that demonstrates effects from repeated exposure or continuous exposure over a considerable part of the lifetime of the test organism. Within the handful of current studies on chronic and sub-chronic MR toxicity, Wik et al. (2009) found that in 9-d exposures to MR leachates, survival and reproduction of *C. dubia*, were impacted to the greatest extent in initial leachate exposure (5-d leaching period) and reduced toxicity was observed after sequential leachings (> 5-d leaching periods of the same MR refreshed with solution). This supports the theory that the first flush effect or initial runoff from rain events may be the most potent time of exposure in aquatic organism as both CR load and CR toxicity may be greatest at this time. The few chronic toxicity studies available from the literature suggest that the risk

from MR exposure under typical environmental conditions poses a relatively low risk to organisms (Panko et al., 2013; Redondo-Hasselerharm et al., 2018). Sublethal effects from chronic exposure to MR in fish, such as upregulation of detoxification enzymes or DNA damage, have not yet been documented.

The goal of the present study was to assess the effect of a pulsed, chronic exposure to CR particles in an estuarine fish species (*Fundulus heteroclitus*), simulating environmentally relevant conditions. Two pulsed exposure experiments were conducted with different batches of fish and are referred to as Experiment A and Experiment B. Fish from Experiment A were used for immunohistochemistry (IHC) to evaluate effects of CR exposure at the tissue level. For Experiment B, toxicity was measured through the following biomarkers: (i) bile fluorescence as an indicator of PAH absorption, metabolism and biliary excretion; (ii) concentrations of 8-hydroxy-2'-deoxyguanosine (8-OHdG) in blood plasma as evidence of oxidative DNA damage; (iii) thiobarbituric acid reactive substances (TBARS) assay as a measure of lipid peroxidation; and (iv) reduced (GSH) and oxidized glutathione (GSSG) concentrations as a measure of general oxidative stress.

3.3 Methods

3.3.1 Fish collection and maintenance

Adult mummichogs (*Fundulus heteroclitus*) were collected at Clam Bank in the North Inlet – Winyah Bay National Estuarine Research Reserve (33°20′02.3″N 79°11′;34.0″W), near Georgetown, SC, using baited minnow traps. Animals were collected using frozen shrimp baited minnow traps around low tide, transported under

constant aeration, and housed at the Clemson University Aquatic Animal Research Laboratory (AARL). Fish were acclimated for at least two weeks prior to exposure. Fish were housed in a 100-gallon (378.5-L) tank in a recirculating system. All fish used in the study were kept on a 16:8 light/dark cycle at 26 °C in 18ppt artificial saltwater (Instant Ocean, Spectrum Brands Inc.). Fish were fed spirulina flake food (Xtreme Aquatic Foods, Princeton, FL) once daily, before and during exposure. There were two experiments conducted using fish collected from the same location using the methodology described above. Fish used in Experiment A were collected in September 2020. Additional fish were collected in November 2020 to be used for Experiment B.

3.3.2 Exposure set up

Commercially available micronized tire fragments (i.e., crumb rubber) were used as a reproducible source of particles similar in size, shape, and polymer composition as tire wear particles found in the environment. Crumb rubber material used in chronic toxicity tests was the same material used in acute toxicity tests as described in LaPlaca and van den Hurk (2020). Crumb rubber was sieved to obtain fragments 38–355 μm in size for use in toxicity tests.

Concentrations of crumb rubber were selected based on predicted or measured MR concentrations in stormwater runoff and surface waters. Measured concentrations of MR in surface waters range from 9×10^{-5} to 0.0064 g/L with predicted concentrations in surface waters caused by road runoff ranging from 3×10^{-5} to 0.0179 g/L based on a one order of magnitude reduction from measured concentrations in road runoff (0.0003 to 0.179 g/L) (Wik & Dave, 2009). Previous research in our laboratory also informed the

decision for the CR concentrations selected for the present study (LaPlaca & Van den Hurk, 2020). Most concentrations utilized for Experiment A were higher than predicted for aqueous environments and represented a "worst-case" scenario. Concentrations tested in Experiment A included 0, 0.059, 0.585, 1.371, and 2.548 g/L crumb rubber. Concentrations selected for Experiment B were lower and more closely resembled the predicted and measured environmental concentrations in surface waters and road runoff and included 0, 0.01, 0.032, 0.10, and 0.25 g/L crumb rubber.

All exposures were performed according to the Clemson University Institutional Animal Care and Use Committee approved Animal Use Protocol 2019-030. For Experiment A, 40 fish were distributed among five 20-gallon (75.7-L) tanks in a recirculating saltwater system with eight fish per tank. The exposure for Experiment A was completed prior to initiating Experiment B. For Experiment B, 45 fish were distributed among five 20-gallon (75.7-L) tanks in a recirculating saltwater system with nine fish per tank. A mixed population of both males and females was used in both experiments and distributed randomly among treatments as fish were past spawning season so no effects from sex were expected.

Fish were maintained in this group housing set up with other fish of their respective exposure concentration before exposure to CR. For the actual exposure to CR, the fish were transferred to individual exposure vessels. The dosing regime for Experiment A consisted of individual exposure for 24-hr, return to group housing for six days, individual exposure for 24-hr on the 7^{th} day, etc. with this pattern repeated over 51 days for a total of eight, 24-hr pulsed exposures. The dosing regime for Experiment B

consisted of individual exposure for 24-hr, return to group housing for four days, individual exposure for 24-hr on the 5th day, etc. with repetitions of this pattern for 42 days for a total of nine, 24-hr pulsed exposures for Experiment B (**Table 3.1**). This dosing regime was chosen to represent a frequency of rain events therefore simulating environmental conditions of repeated storm events and was changed from every 7-days in Experiment A to every 5-days in Experiment B to allow for more frequent exposure to CR. During actual exposure for both experiments, fish were individually exposed to their respective concentration of CR by being transferred from the group housing tanks to individual 4 L glass jars with aerated artificial salt water (18ppt, Instant Ocean, Spectrum Brands Inc.) in a static-renewal set up. During the exposures, the temperature, NO_3^-, and NH_3/NH_4^+ were recorded daily in the control tanks. Temperature ranged from 22–28 °C, NO_3^- remained below 5.0 mg/L, and NH_3/NH_4^+ remained below 2.0 mg/L.

At the end of the Experiment A, animals were euthanized individually by a lethal dose of buffered tricaine methanosulfate (MS-222, 1g/L). Standard length (mm) and wet weight (g) were recorded for each fish. An incision was made along the ventral side of the fish to allow for fixative to penetrate internal organs. Whole fish from Experiment A were placed in 10% aqueous buffered zinc formalin (Z-fix, Anatech, Ltd., Battle Creek, MI, USA) for five days and the fixative replaced with 70% ethanol until further histological processing.

At the end of the experiment for Experiment B, animals were anesthetized individually with buffered MS-222. Standard length (mm) and wet weight (g) were recorded for each fish. Blood samples were collected in anesthetized fish by caudal

severance where the tail was severed posterior to the anal fin and a blood sample was collected from the caudal vein using a heparinized microhematocrit tube. After blood collection, fish were euthanized by a lethal dose of buffered MS-222 (1 g/L). Gallbladders were removed, placed in dark microcentrifuge vials, and frozen at −20 °C. Liver and intestinal samples were dissected, wrapped in labeled aluminum foil, and frozen in liquid nitrogen until transfer to a −80 °C freezer.

Table 3.1 – Dosing regime set up example. Fish were individually exposed every 7-days (Experiment A) or every 5-days (Experiment B). On days not exposed, fish were kept in group housing for their respective concentration of CR. A box with a 'X' indicates exposure for 24-hr

Experiment A	Day						
	M	T	W	Th	F	Sa	Su
Week 1	X						
Week 2	X						
Week 3	X						
Experiment B	Day						
	M	T	W	Th	F	Sa	Su
Week 1	X					X	
Week 2				X			
Week 3		X					X

3.3.3 Immunohistochemistry – Experiment A

Fish from Experiment A were used for immunohistochemistry (IHC) analysis. After fixing whole fish as described above, the gills, gastrointestinal tract, and liver were dissected from individual fish and placed in labelled cassettes. Tissues in cassettes were stored in 70% ethanol until processing. For processing, specimens were dehydrated in

alcohol, cleared using xylene, and paraffin added. Tissues were embedded in paraffin and sections were cut at 5-8 µm on a rotary microtome.

Immunohistochemistry staining protocol followed methodology as described in Margiotta et al. (2017) and Wojdylo et al. (2016). First, histological slides were deparaffinized and hydrated in a series of xylene, 100% EtOH, 95% EtOH, 75% EtOH, 50% EtOH, and water. Next, antigen retrieval was performed to unmask antigens by breaking cross-links formed during tissue fixation. A Tris-EDTA buffer (10 mM Tris base, 1mM EDTA solution, 0.05% Tween 20, pH 9.0) was utilized in a microwave antigen retrieval technique whereby slides were heated in buffer by a microwave on 100% power for 5 min followed by cooling for 5 min, followed by a final 5 min 100% power, and a final 30 min rest in the container. Samples were washed with PBS (0.01M, pH 7.4), endogenous peroxidase was quenched using 3% hydrogen peroxide in methanol and washed with PBS again. Tissues on slides were encircled with a Liquid Blocker Super mini pen to separate tissue slices. Samples were blocked with avidin solution for 15 min, washed in PBS, and blocked with biotin solution for 15 min (Vector Avidin/Biotin Blocking Kit, Vector Laboratories, Burlingame, CA, USA). Samples were blocked with 10% non-immune horse serum (VECTASTAIN® Elite® ABC Kit Peroxidase (HRP), Vector Laboratories, Burlingame, CA, USA) in PBS and incubated for 17 min in a humidified chamber. After incubation, samples were washed in PBS then slides were incubated with the primary antibody overnight at 4°C in a humidified chamber. Each slide with tissue slices contained one slice receiving secondary antibody only as a negative control, so PBS was added to this slice for overnight incubation. The

primary antibody used was CRC4 (obtained from Dr. Charles D. Rice, Clemson University) for detection of CYP1A induction.

After incubation, slides were washed in PBS and the secondary antibody was added (Horse anti-mouse IgG, biotinylated, BP-2000, Vector Laboratories, Burlingame, CA, USA) to each tissue and incubated for 30 min in a humidified chamber. After incubation, slides were washed in PBS and Vectastain ABC Reagent (PK-4000, Vector Laboratories, Burlingame, CA, USA) was applied to each section and incubated for 28 min in a humidified chamber. Slides were washed again in PBS. ImmPACT NovaRED (SK-4805, Vector Laboratories, Burlingame, CA, USA) was used to stain tissues. Tissues were stained for approximately 3 min and then counterstained with Hematoxylin QS (Vector Laboratories.) for approximately 30 seconds to 1 minute. After a wash in PBS, samples were dehydrated in a series of water, 50% EtOH, 75% EtOH, 95% EtOH, 100% EtOH, and xylene and coverslips added with PolyMount. Slides were examined under a microscope with digital camera attachment at 40x – 400x magnification for positive staining of CYP1A as indicated by dark red coloration.

3.3.4 Biomarkers – Experiment B

Bile fluorescence

Studies show that bile fluorescence in fish increases with recent exposure to polycyclic aromatic hydrocarbon (PAH)-containing compounds but quickly declines if the source of exposure is removed (LaPlaca & Van den Hurk, 2020; Struch et al., 2019; Vethaak et al., 2016). To measure the absorption, metabolism, and biliary excretion of PAHs released by the crumb rubber, bile samples were analyzed for fluorescence at

excitation/emission wavelength pairs that are specific for 2-ring (290/335 nm), 4-ring (341/383 nm) and 5-ring (380/430 nm) PAHs as described in Aas et al., (2000a) and LaPlaca and van den Hurk (2020).

DNA damage – blood plasma

At the cellular level, metabolism of chemical environmental stressors can result in the formation of reactive oxygen species (ROS). ROS are produced naturally during metabolism and their toxic effects are usually prevented by antioxidants. In conditions where the production of ROS is greater than the ability of cells to remove them, DNA damage may occur, leading to single or double strand breaks or modification of nucleotide bases (Negrato et al., 2013; Poljsak et al., 2013). Among numerous types of oxidative DNA damage, the formation of 8-hydroxy-2'-deoxyguanosine (8-OHdG) from deoxyguanosine is a ubiquitous marker of oxidative stress. 8-OHdG is formed and enhanced by chemical carcinogens. During the repair of damaged DNA *in vivo* by exonucleases, the resulting 8-OHdG is excreted without further metabolism into urine. While this measure of DNA damage is an indicator of current oxidative stress status and DNA repair mechanisms, high levels of 8-OHdG may indicate continuous DNA damage and repair reoccurring under chronic exposure scenarios, potentially resulting in carcinogenicity if left unchecked.

The 8-OHdG concentrations in blood serum samples were determined using the DetectX® DNA Damage ELISA kit (Arbor Assays, Ann Arbor, MI, USA). To obtain serum, whole blood samples were centrifuged at 3000 *g* for 10 minutes at 4°C. Serum samples were diluted 1:10 with diluted assay buffer supplied with the DetectX® DNA

Damage kit, according to the manufacturer's instruction. Samples were assayed in duplicate, and absorbance was read on a BioTek Synergy H1 plate reader at 450 nm. The standard curve was generated from 62.5 pg/ml to 2000 pg/ml, equivalent to 220.625 – 7,060 pM 8-OHdG. Data are presented in pM 8-OHdG.

Thiobarbituric acid reactive substances (TBARS) – liver samples

Liver homogenates were prepared utilizing a bead mill homogenizer. Briefly, liver samples were added to a bead mill tube with 1.4 mm ceramic beads (Fisher, Pittsburg, PA, USA) and 1 ml of ice-cold sodium phosphate buffer (0.1 M, pH 7). Samples were homogenized until tissue particulates were no longer visible. Samples were centrifuged at 14,000 rpm for 10 min at 4°C. Supernatant was collected and transferred to labelled tubes with an aliquot collected for protein determination.

Homogenates were diluted 1:10 by adding sample diluent, depending on protein concentration. Next, 10 µl of 12N HCl was added to each sample and vortexed to mix, resulting in an additional 1.0476-fold dilution. The assay followed protocol from Arbor Assay's TBARS/MDA Universal Colorimetric Detection Kit (K077-H1, Arbor Assays, Ann Arbor, MI, USA) with slight modifications. Malondialdehyde (MDA) standards were prepared according to kit instructions resulting in a standard curve from 1.563 to 100 µM MDA. Samples, standards, and blanks were pipetted in duplicate into a 96-well plate. TBA reagent (thiobarbituric acid) was added to all wells. The plate was incubated at 37°C and shaken every 10 min for 90 min. Absorbance was read on a BioTek Synergy H1 plate reader at 535 nm.

Liver glutathione (GSH) – free and oxidized

Liver homogenates were prepared utilizing a bead mill homogenizer as described above in the TBARS assay. Samples were prepared according to protocol from Arbor Assays Glutathione Fluorescent Detection Kit (K006-F1, Arbor Assays, Ann Arbor, MI, USA). An aliquot of sample was mixed with equal volume of cold 5% sulfosalicylic acid (SSA) and incubated for 10 min at 4°C. Samples were centrifuged at 14,000 rpm for 10 min at 4°C to remove precipitated protein. The supernatant was collected, diluted 1:2.5 with assay buffer, and further diluted 1:10 with sample diluent. Glutathione standards were prepared according to kit protocol ranging from 0.195 to 25 µM. A positive control was prepared in which complete conversion of a standardized amount of oxidized glutathione (GSSG) to glutathione (GSH) was measured. Samples, standards, controls, and blanks were pipetted into wells on a 96-well black plate, in duplicate. Next, 25 µl of ThioStar® thiol detection reagent was added to all wells and incubated at room temperature for 15 min. Fluorescence was read at 390 nm excitation and 510 nm emission to determine free GSH concentration. After, 25 µl of reaction mixture containing glutathione reductase, NADPH, and buffer was added to all wells and incubated for 15 min at room temperature. Fluorescence was read again at 390 nm excitation and 510 nm emission to determine total GSH concentration.

3.3.5 Statistical analysis

All statistical analyses were performed using JMP Pro 14 (SAS Institute Inc.) statistical software. Data were checked for normality before analysis and log-transformed for analysis to better approximate normality when required. Data from Experiment A and Experiment B were analyzed separately. Simple linear regression was used to test if

crumb rubber concentration significantly predicted each biomarker response. A p value of < 0.05 was considered significant.

3.4 Results

3.4.1 Experiment A

All fish appeared healthy at the start of exposure. Ammonia levels were relatively high in the recirculating system during the first two weeks of the experiment as the biological filter was established in the system. When ammonia levels were detected at > 1.0 mg/L, a water change and corrective action, such as adding ammonia conditioner to the water (AmmoLock, API, Chalfont, PA) was taken to reduce ammonia levels. The periodic high ammonia in the fish group housing (up to 2.0 mg/L) may have resulted in some mortality during the experiment. Mortality was attributed to high ammonia levels rather than exposure to CR. Mortality during the experiment was 10 fish total – one from the control group, three from 0.059 g/L concentration group, one from 0.585 g/L concentration group, two from 1.371 g/L concentration group, and two from 2.548 g/L concentration group. The total number of fish analyzed at the end of the exposure was 30. Average weight of the fish was 4.74 ± 2.25 g (wet weight) and average length was 67.27 ± 10.46 mm (standard length).

Immunohistochemistry

Immunohistochemistry staining for CYP1A in gill, intestine, and liver tissue of *F. heteroclitus* exposed to CR indicated upregulation of CYP1A expression in all tissues examined. Gills of exposed fish showed strong positive staining for CYP1A on secondary lamella (**Figure 3.1**). Control fish did not show positive staining in gill tissues. Intestinal

tissue of exposed fish indicated strong positive staining for CYP1A in the vascular endothelium of intestinal tissue and weak positive staining in mucosal epithelium cells (**Figure 3.2**). Control fish did not show positive staining for CYP1A in the intestinal tissue. Livers of exposed fish showed strong positive staining for CYP1A in hepatocytes and within vascular endothelium and sinusoids (**Figure 3.3**). Control fish did not show positive staining for CYP1A upregulation in liver tissue. Tissues receiving secondary antibody only as the negative control did not show any positive staining for CYP1A.

3.4.2 Experiment B

All fish appeared healthy at the start of exposure. There was one fish from the lowest concentration tested, 0.01 g/L, that appeared ill at the end of the exposure and was excluded from analysis. The total number of fish analyzed at the end of the exposure was 44. Average weight of the fish was 8.97 ± 2.48 g (wet weight) and average length was 84.02 ± 8.07 mm (standard length).

Bile Fluorescence

Bile fluorescence results indicated significant dose-response relationships for 2-ring, 4-ring, and 5-ring structures as crumb rubber concentration increased (**Figure 3.4**) Crumb rubber concentration predicted 4-ring fluorescence more strongly than predicting 2- or 5-ring fluorescence as indicated by the higher R^2 for the 4-ring linear regression (2-ring structures $R^2 = 0.15$, $F(1,34) = 6.19$, $p = 0.0179$; 4-ring structures $R^2 = 0.54$, $F(1,34) = 40.6$, $p < 0.0001$; 5-ring structures $R^2 = 0.35$, $F(1,34) = 18.14$, $p = 0.0002$).

DNA Damage

The concentration of 8-OHdG in blood plasma of fish increased as crumb rubber concentration increased (**Figure 3.5**). A linear regression between the concentration of CR and 8-OHdG (pM) in blood plasma showed a significant positive correlation (R^2 = 0.27, F(1,22) = 8.30, p = 0.0087) indicating there was more 8-OHdG produced in fish at higher exposure concentrations. Elevated 8-OHdG concentrations indicated greater DNA damage as more 8-OHdG was being produced in response to oxidative DNA stress.

Thiobarbituric acid reactive substances (TBARS)

The concentration of malondialdehyde (MDA) equivalents decreased slightly as the concentration of crumb rubber increased (**Figure 3.6**). The linear regression between the concentration of CR and MDA (μM) in liver tissue showed a significant negative correlation (R^2 = 0.21, F(1,41) = 11.12, p = 0.0018) indicating there was less MDA produced in fish at higher exposure concentrations. The slight reduction in MDA produced in the liver as CR increased may be due to upregulation of antioxidant defense mechanisms which prevented oxidative damage and thus prevented the formation of TBARS.

Glutathione – GSH & GSSG

Total glutathione (tGSH), free glutathione (GSH), and oxidized glutathione (GSSG) were determined in liver samples and the GSH:GSSG ratio was calculated. A linear regression between the concentration of CR and free glutathione (GSH) in the liver showed a significant positive correlation (R^2 = 0.15, F(1,30) = 5.25, p = 0.0291) indicating there was more GSH produced in fish at higher exposure concentrations. For oxidized glutathione (GSSG), the linear regression indicated a significant decrease in

GSSG as CR concentration increased (R^2 = 0.17, F(1,30) = 5.91, p = 0.0212) Typically, under oxidative stress or conditions characterized by increased ROS species, enhanced GSH may be required to maintain homeostasis and redox status. Under conditions where oxidative stress is prolonged or exceeds repair mechanisms, the amount of free GSH can decline as it is used more quickly than it can be synthesized, leading to cell degeneration and death (Aquilano et al., 2014). I observed the opposite, that GSH increased, possibly indicating an adaptive antioxidant response to prolonged exposure to stressors.

In general, a decrease in GSH:GSSG ratio typically is an indicator of oxidative stress as GSH is converted from its reduced form (GSH) to the oxidized form (GSSG) by glutathione reductase in the presence of electrophilic or oxidizing species. My results suggested however, a decrease in GSSG and an increase in GSH (**Figure 3.7**), which leads to an overall increase in GSH:GSSG ratio as more GSH was being produced and less GSSG was formed. It appears that the fish were effectively able to produce more GSH in response to oxidative stress thereby keeping GSH levels high or alternatively, changes to the activation of redox-sensitive signal pathways that enhanced GSH were occurring due to exposure to CR.

3.5 Discussion

3.5.1 Experiment A

Immunohistochemistry

To my knowledge, this is the first study to examine the effects of exposure to crumb rubber particles on tissues through histology and immunochemistry in fish. Exposure to crumb rubber particles in the aqueous environment is a combination of direct

exposure via interaction with particles themselves as well as indirect exposure from particle leachate. The gills are directly exposed to ambient water and filter large volumes of water to extract oxygen as part of respiration. Therefore, the gills are a major route of uptake and a typical target of toxicity for waterborne particulates and toxicants (Jönsson et al., 2009). Additionally, the first site of interaction between toxicant and fish is likely the gills, leading to the potential immediate activation of detoxification enzymes in the gills. For example, aqueous PAHs may be more rapidly metabolized by the gills compared to liver metabolism (Levine & Oris, 1999).

Immunohistochemistry of gill tissue from fish exposed to crumb rubber particles in the present study showed strong positive staining for CYP1A indicating upregulation of CYP1A in the gills (**Figure 3.1C**). Coupled with the bile fluorescence data from the present study for Experiment B, indicating significantly elevated fluorescent aromatic structures (i.e., PAHs) in the bile of fish exposed to CR, it is possible that upregulated gill CYP1A activity was caused by exposure to these xenobiotics being leached into the water from CR particles or directly through interaction with particles themselves. Previous research in our lab and others found significantly elevated aromatic compounds in the bile of fish exposed to CR or tire leachates (LaPlaca & Van den Hurk 2020; Stephensen et al., 2003). It is well documented that exposure to PAHs induces CYP1A activity in a variety of organisms. Additionally, aqueous exposure to PAHs has been shown to strongly induce CYP1A in gill pillar cells and gill epithelial cells (van Veld et al., 1997), which I also observed. The present study suggested that PAHs may leach from CR particles in the water column and induce CYP1A expression in fish gill tissues.

An additional route of exposure for crumb rubber particles in fish species is through ingestion as particles enter the gastrointestinal tract. Particles themselves are not absorbed into cells but are passed through the intestinal tract, similar to ingestion and egestion of other microplastic types (Leads et al. 2019; Redondo-Hasselerharm et al., 2018). Contaminants associated with the CR particles can therefore interact directly with intestinal cells as the particles pass through the gut. Depending on the clearance time of CR particles inside the gut and kinetics of chemical desorption from CR, there may or may not be sufficient time to elicit a biochemical response. The intestine is generally a major route of uptake of lipophilic compounds in fish with several biotransformation enzymes involved in detoxification and elimination of these compounds (Braunbeck et al., 1998). Immunohistochemistry from the present study indicated strong positive staining for CYP1A induction in vascular endothelium of intestinal tissue which agrees with observations from Woodin at al. (1997) and Wang et al. (2010) who also found CYP1A induction in vascular endothelium of intestinal tissue in *A. purpurescens* and *F. heteroclitus*, respectively when exposed to PAHs (**Figure 3.2C**). Additionally, the observed weak positive staining for CYP1A in mucosal epithelial cells in the present study is also corroborated by Husøy et al. (1994) who observed no CYP1A expression in mucosal cells in cod intraperitoneally injected with β-naphthoflavone and Van Veld et al. (1997) who observed low positive staining for CYP1A in *F. heteroclitus* after aqueous exposure to benzo[a]pyrene (BaP). Nevertheless, in the same study, Van Veld et al. (1997) also found that *F. heteroclitus* exposed to BaP via diet showed strong CYP1A staining in gut mucosal epithelium, indicating that route of exposure may play a

significant role in the metabolism for PAH-type compounds. Upregulation of CYP1A in intestinal vasculature but weaker staining in intestinal epithelium indicates that most contaminants (i.e., PAHs) from crumb rubber are potentially being absorbed in a different part of the body, such as the gills from leachate, and being transported via the bloodstream throughout the body. Although residence time of CR particles in the gut was not determined as part of the present study, weaker CYP1A induction in intestinal epithelium may also be attributed to a short residence time of CR particles in the gut.

The liver is an important site of detoxification for many xenobiotics. Previous acute toxicity tests in my lab with *F. heteroclitus* measured increased catalytic activity of CYP1A in the liver through the EROD assay on tissues from exposed fish (LaPlaca & Van den Hurk, 2020). It is well known that major CYP1A activity is found in the liver of fish and the prominent expression and induction of CYP1A in the liver is consistent with the role of this organ in xenobiotic metabolism and excretion (Sarasquete & Segner, 2000). Exposed fish from the present study showed strong IHC staining in the hepatocytes and endothelial cells lining the blood sinusoids of the liver (**Figure 3.3C**) which typically can be detected only after xenobiotic exposure (Husøy et al, 1996; Stegeman & Hahn, 1994;). Previous studies have shown induction of CYP1A in vascular endothelium in a variety of organs including the heart, liver, and gut (Guiney et al., 1997) suggesting that CYP1A induction in endothelium may be a sensitive biomarker for exposure to aryl-hydrocarbon receptor (AhR) agonists, like PAHs (Stegeman et al., 1991).

In the present study, the strong positive staining for CYP1A in the gills coupled with strong positive staining for CYP1A in intestinal vasculature, hepatocytes, and liver vasculature indicate the major site of toxicity is the gills via aqueous exposure before the toxicants were transported to other organs via the blood stream. Additionally, increased bile fluorescence with CR exposure as described below indicated that the metabolites were present in the bile of exposed fish and thus passed through the liver. The leachate produced by the particles suspended in the water column may potentially exert a more prominent effect on toxicity compared to the particles themselves as shown through the strong CYP1A induction in the gill and weaker CYP1A induction detected in the intestinal epithelium where the particles had direct contact once ingested.

3.5.2 Experiment B

Bile Fluorescence

Bile fluorescence measurements from the present study agreed with findings in previous studies (LaPlaca & Van den Hurk, 2020). Fluorescence for 2-ring, 4-ring, and 5-ring structures in the bile all demonstrated a significant dose-response relationship as crumb rubber concentration increased. These data provided evidence that fluorescent compounds (PAHs) from CR were metabolized in the liver and excreted into the bile.

In this chronic, pulsed exposure, 4-ring fluorescence showed the strongest dose-dependent increase in fluorescence as CR concentration increased (R^2 = 0.54). The most dominant PAH detected in CR is the 4-ring PAH pyrene (Aatmeeyata & Sharma, 2010; Kreider et al., 2010; Menichini et al., 2011; Stephensen et al., 2003) and these data

suggested that 4-ring fluorescence in the bile of fish may be a good indicator for exposure to CR or MR.

DNA Damage

Under normal physiological conditions in all aerobic organisms, antioxidants and endogenous oxidants cycle through a sequence of damage and repair. When imbalances occur, typically due to the introduction of excess exogenous oxidants, oxidative damage to DNA exceeds the ability of antioxidants to make repairs. Excessive DNA damage can lead to carcinogenesis (Marnett, 2000).

Analysis of 8-OHdG has been established as a biomarker to evaluate oxidative stress and determine risk of cancer after exposure to carcinogenic substances, environmental pollutants, or lifestyle factors in humans (Valavanidis et al., 2009). In many studies, urinary 8-OHdG has been evaluated as an indicator of current oxidative DNA stress condition in humans (Toraason et al., 2001; Yamauchi et al., 2004). For other species, Negrato et al. (2013) found elevated levels of 8-OHdG in liver samples from fish (*Zosterisessor ophiocephalus*) collected from an industrialized site contaminated with heavy metals, polychlorinated biphenyls (PCBs), and PAHs in the Venetian lagoon, Italy compared to a reference site. Additionally, 8-OHdG formation has been observed in murine models when exposed to a number of compounds including: N-nitroso compounds, benzo[a]pyrene, metals, and quinones (Klaunig et al., 2009).

The mechanism of action by which 8-OHdG is created occurs through the ability of ROS to interact with nucleobases. Specifically, the oxidation of guanine in the C8 position results in the formation of 8-OHdG. Subsequent mutations that may arise from

the formation of 8-OHdG involve GC → TA transversions (Valko et al., 2004). The 8-OHdG lesion is relatively easily formed and is mutagenic, thus is a useful indicator of DNA damage induced by a variety of pollutants.

Results from the present study indicate that the concentration of 8-OHdG in blood plasma of the test organisms increased as crumb rubber concentration increased. Elevated 8-OHdG concentrations indicate potentially greater DNA damage as more 8-OHdG is being produced in response to oxidative DNA stress. In a chronic scenario under repeated dosing to crumb rubber, it is possible that repeated toxic insult with crumb rubber and their associated chemicals may add an excess of exogenous oxidative stress to the fish and in turn, initiate carcinogenesis. Crumb rubber contains a variety of compounds known to be carcinogenic to aquatic organisms, such as phthalates, benzothiazole, PAHs, and possibly the newly identified 6PPD-quinone, which may contribute to the elevated levels of 8-OHdG measured in plasma of exposed fish (Ginsberg et al., 2011; Halsband et al., 2020; Logan, 2007; Vogelbein & Unger 2006). To my knowledge, these results are the first to demonstrate potential carcinogenicity of exposure to crumb rubber particles in aquatic organisms.

Thiobarbituric acid reactive substances (TBARS)

Lipid peroxidation occurs when oxidants such as free radicals or reactive oxygen species (ROS) attack lipids containing carbon-carbon double bond(s) (Ayala et al., 2014). Endogenous sources of ROS production are primarily the mitochondria, endoplasmic reticulum, and peroxisomes (Moldovan & Moldovan, 2004). In addition to these endogenous sources of oxidative stress, studies have demonstrated that exogenous

sources such as ultraviolet radiation, pathogen infections, or chemical exposure also stimulate ROS production that may lead to lipid peroxidation (Belló et al., 2000; Otitoloju & Olagoke, 2011). In a review of biomarker responses in fish exposed to PAHs specifically, Santana et al. (2018) observed an overall increase in lipid peroxidation in fish exposed to PAHs. Crumb rubber contains PAHs among other contaminants such as metals and the recently identified transformation byproduct 6PPD-quinone from the tire-derived compound 6PPD (Tian et al., 2021). Therefore, I anticipated increased lipid peroxidation as measured through MDA production in the TBARS assay when fish were exposed to crumb rubber.

Contrary to several studies that observed increased lipid peroxidation with environmental pollutant exposure (Otitoloju & Olagoke, 2011; Santana et al., 2018), I observed a slight decrease in lipid peroxidation as CR concentration increased in the chronic, periodic exposure to CR performed in the present study. For tire-related compounds specifically, this trend was also observed in mussels (*M. galloprovincialis*) in areas with artificial reefs made from tires where TBARS levels were lower in mussels caged near tire reefs compared to mussels caged in a reference location (Risso-de Faverney et al., 2010). Additionally, the review by Santana et al. (2018) determined that lipid peroxidation was negatively correlated with exposure time, indicating a potential adaptive antioxidant response in exposed fish. When other oxidative stress biomarkers are upregulated such as CYP1A, glutathione-s-transferases (GST) or glutathione (GSH), it is possible that cellular defenses are efficiently working to protect cells from damage to exogenous agents.

The reduction in TBARS levels from chronic exposure to CR may be explained by the antioxidative responses as demonstrated by the increase in measured 8-OHdG and increase in GSH. Additionally, previous EROD assays in acute exposures to CR from our lab demonstrated upregulation of CYP1A in the liver as crumb rubber concentration increased (LaPlaca & Van den Hurk, 2020). Further, the results from the IHC in the current study provides another line of evidence for induction of CYP1A in multiple tissues including gill, intestine, and liver. Although GST was not measured in fish from the present study, prior acute CR exposures in a previous study demonstrated a significant increase in GST activity in *P. promelas* but GST activity for *F. heteroclitus* was not significantly altered (LaPlaca & Van den Hurk, 2020). Under long-term repeated dosing, it is possible that these antioxidant systems were elevated in exposed organisms to successfully prevent cellular damage as demonstrated in the reduced amount of lipid peroxidation.

Glutathione (GSH and GSSG)

Cells produce glutathione (GSH) as an antioxidant to resist oxidative stress and cellular damage. Glutathione is involved in many other cellular functions such as nutrient metabolism and regulation of cellular metabolic functions such as gene expression, DNA and protein synthesis to signal transduction, cell proliferation, and apoptosis (Aquilano et al., 2014). Under typical physiological conditions, the antioxidant exists mostly in the reduced form, glutathione (GSH), with concentrations from 10 to 100-fold greater than the oxidized species (GSSG). High levels of GSH are maintained by *de novo* synthesis and by glutathione disulfide reductase (GR)-dependent recycling of GSSG back to GSH

(Zhu et al., 2013). GSSG is produced by the catalysis of GSH peroxidase (GPx) and from direct reactions of GSH with electrophilic compounds (Aquilano et al., 2014).

GSH is both a nucleophile and a reductant and can, therefore, react with electrophilic or oxidizing species, making them more soluble and excretable. The thiol moiety of GSH specifically serves as the antioxidant in the direct scavenge of radical species. Conjugation of GSH with electrophilic compounds is mediated by the Phase II detoxification enzymes, GSTs. Because of the sheer multiplicity of GSTs, they cannot be collectively inhibited or induced; therefore, the importance of glutathione conjugation (i.e., glutathionylation) in xenobiotic toxicity *in vivo* is often assessed by changes to levels of GSH. Changes to GSH can include 1) inhibition of GSH synthesis, 2) depletion of GSH, or 3) enhancement of GSH levels through Nrf2 activation (Parkinson et al., 2013).

Typically, under oxidative stress or conditions characterized by increased ROS species, enhanced GSH may be required to maintain homeostasis and redox status. The antioxidant system as a whole functions dynamically to avoid damage to biomolecules. My results indicate that GSH levels increased as CR increased, GSSG decreased as CR increased, and the overall ratio of GSH:GSSG increased as CR increased. Stephensen et al. (2003) also noted a significant increase in total GSH after 2 weeks in rainbow trout exposed to leachate with and without highly aromatic oil from car tire tread compared to controls. On the other hand, Santana et al. (2018) found no change to GSH levels as PAH concentration increased but observed an increase in GSH over time regardless of PAH concentration, but they were not specifically examining tire particles. Tire particles

contain other compounds such as Zn which has been shown to increase cellular GSH levels (Cortese et al., 2008).

Because I observed neither inhibition or depletion of GSH levels and instead, an increase in GSH levels as CR increased, one mechanism for this increase may be via Nrf2 activation. Pardo et al. (2020) suggested that exposure to particulate matter (PM) and its components (mostly metals and PAHs), which were similar to components found in CR, enhanced ROS formation which may lead to inflammation, disturbance of the redox homeostasis, and alteration of the activation of redox-sensitive signal pathways such as Nrf2 in addition to PAHs activating the AhR pathway leading to changes to CYP1A expression. Cortese et al. (2008) also found that Zn supplementation activates the transcription factor Nrf2 which enhances the *de novo* synthesis of GSH so it is possible that Zn associated with tire particles enhances antioxidant functioning.

For GSSG, results showed a weak correlation of a decrease in total GSSG as CR concentration increased ($R^2 = 0.17$). The decrease in GSSG may be explained by the efficiency of glutathione reductase (GR) in reverting GSSG to the reduced form GSH. However, Santana et al. (2018) found that GSSG increased across PAH concentration regardless of exposure time in fish. The influence of other contaminants in CR may partially explain the differences in my results compared to Santana et al. (2018). For example, Zn may exert an antioxidant effect by protecting thiol groups from oxidation via complexation or by inducing expression of proteins (i.e., metallothionein) with protective functions (Chung et al., 2005; Powell, 2000).

One other consideration to note is that a decreased GSH:GSSG ratio is typically indicative of oxidative stress as GSH is oxidized to GSSG. The antioxidant function of GSH involves glutathione peroxidase (GPx) and glutathione reductase (GR). GPx catalyzes the reduction of hydrogen peroxide, which is produced by superoxide dismutase (SOD) through the dismutation of superoxide anions or organic hydroperoxides. GSH and GSH-dependent enzymes work simultaneously to scavenge ROS and neutralize their effect (Zitka et al., 2012).

The GSH:GSSG ratio is typically high to maintain normal cellular function and prevent oxidative stress injury. Results from the present study indicated a further increase in GSH:GSSG ratio as CR concentration increased. This coupled with the results from the other biomarkers tests demonstrated that the antioxidant system in *F. heteroclitus* fluctuates dynamically to protect normal cellular functioning when exposed to exogenous stressors in CR and establishment of an adaptive equilibrium of GSH. GSH levels can be increased due to an adaptive mechanism to slight oxidative stress through an increase in GSH synthesis; however, severe oxidative stress may suppress GSH levels due to loss of adaptive mechanisms and the oxidation of GSH to GSSG (Zhang et al., 2004).

3.6 Conclusions

The goal of the present study was to assess the effect of a pulsed, chronic exposure to CR particles in an estuarine fish species (*Fundulus heteroclitus*), simulating environmentally relevant conditions. Two pulsed exposures were conducted with different batches of fish, the first focusing on immunohistochemistry and CYP1A induction in various organs and the second focusing on biomarker responses related to

detoxification of accumulated chemicals and/or metabolites associated with CR. I observed strong positive staining in IHC for CYP1A in the gill epithelium of exposed organisms, which was expected as the gills were in direct contact with both leachate and CR particles in the water column during exposures. Additionally, strong positive staining for CYP1A was detected in intestinal vasculature, hepatocytes, and liver vasculature and sinusoids, indicating uptake of contaminants from the gills to the blood stream and distribution to other organs, such as the liver for detoxification. Within the intestinal tissues examined, there was weak staining for CYP1A detected in intestinal epithelium indicating that particles ingested had a mild response in the gut. These data help shed light on the differences between particle and leachate toxicity and understanding the mechanism of toxicity between particle and leachate exposures under chronic conditions.

The other biomarkers measured indicated that antioxidant defenses were sufficiently functioning to prevent cellular damage. Measured 8-OHdG was significantly elevated in exposed organisms indicating that antioxidant defenses to prevent and repair oxidative DNA damage were occurring. Lipid peroxidation was significantly lower in exposed organisms indicating that antioxidant defense mechanisms were efficiently preventing adverse cellular membrane damage. GSH levels in exposed fish increased, possibly due to an adaptive mechanism to oxidative stress through an increase in GSH synthesis or it is possible that levels were increased due to Nrf2 activation by exogenous stressors.

Previous research demonstrated no mortality in *F. heteroclitus* up to 6 g/L CR (LaPlaca & Van den Hurk, 2020) therefore sublethal effects from exposure were

anticipated and th set of biomarkers was chosen as measurable endpoints for chronic toxicity. Mortality that could be attributed to CR exposure was not observed in the present study. There was overall low toxicity from exposure to CR particles in *F. heteroclitus* under these chronic conditions, with mild responses at the cellular level. Additional research on long term effects at the population level is needed to determine the overall risk characterization of the toxicity of crumb rubber particles to *F. heteroclitus.*

3.7 Figures

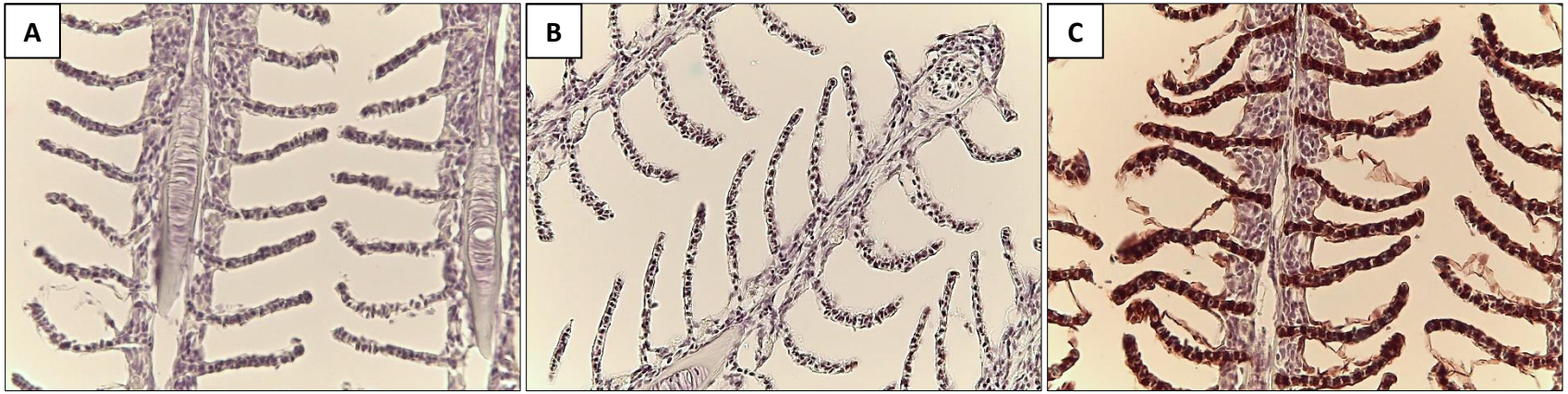


Figure 3.1 – Mummichog gill tissue. Immunohistochemical staining using secondary antibody only as negative control in fish exposed to CR (A). Immunohistochemical staining using primary antibody CRC4 in control fish (B). Immunohistochemical staining using primary antibody CRC4 in fish exposed to 2.548 g/L CR (C).

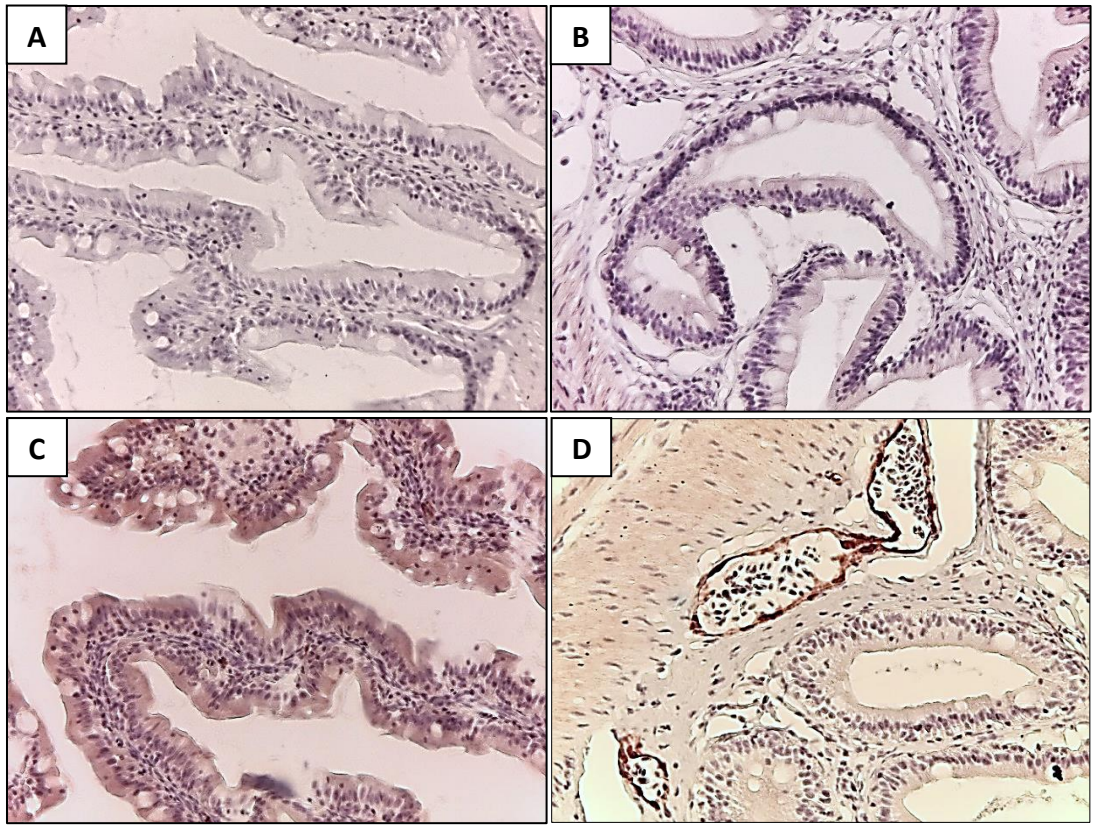


Figure 3.2 – Mummichog intestinal tissue. Immunohistochemical staining using secondary antibody only as negative control in fish exposed to CR (A). Immunohistochemical staining using primary antibody CRC4 in control fish (B). Immunohistochemical staining in intestinal epithelium using primary antibody CRC4 in fish exposed to 2.548 g/L CR (C). Immunohistochemical staining in intestinal vasculature using primary antibody CRC4 in fish exposed to 2.548 g/L CR (D).

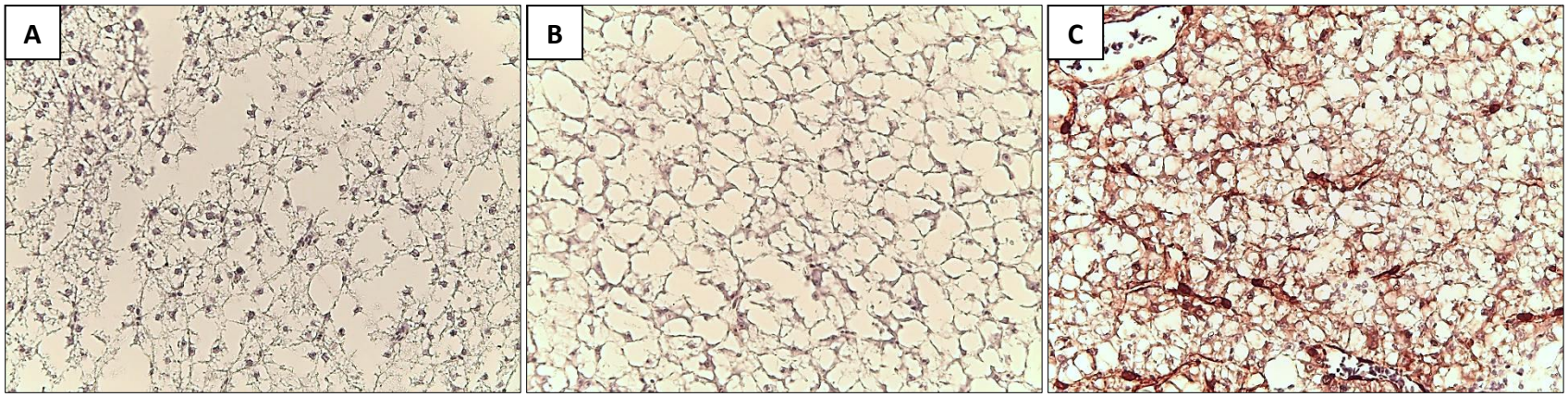
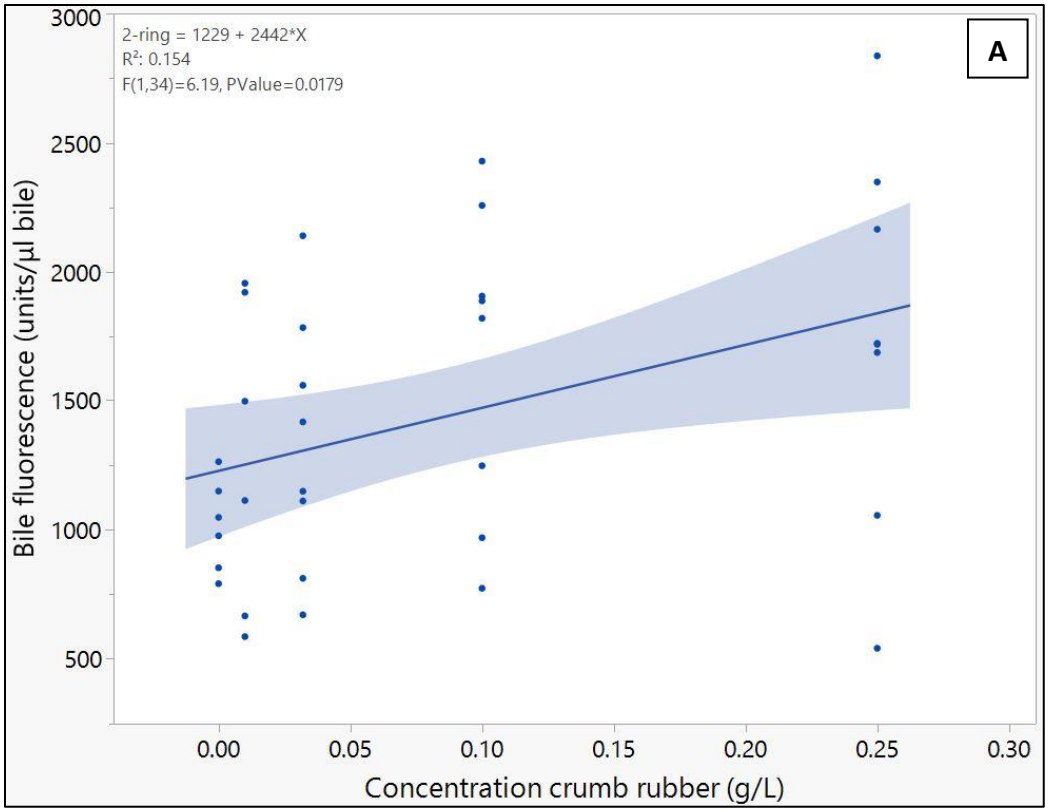
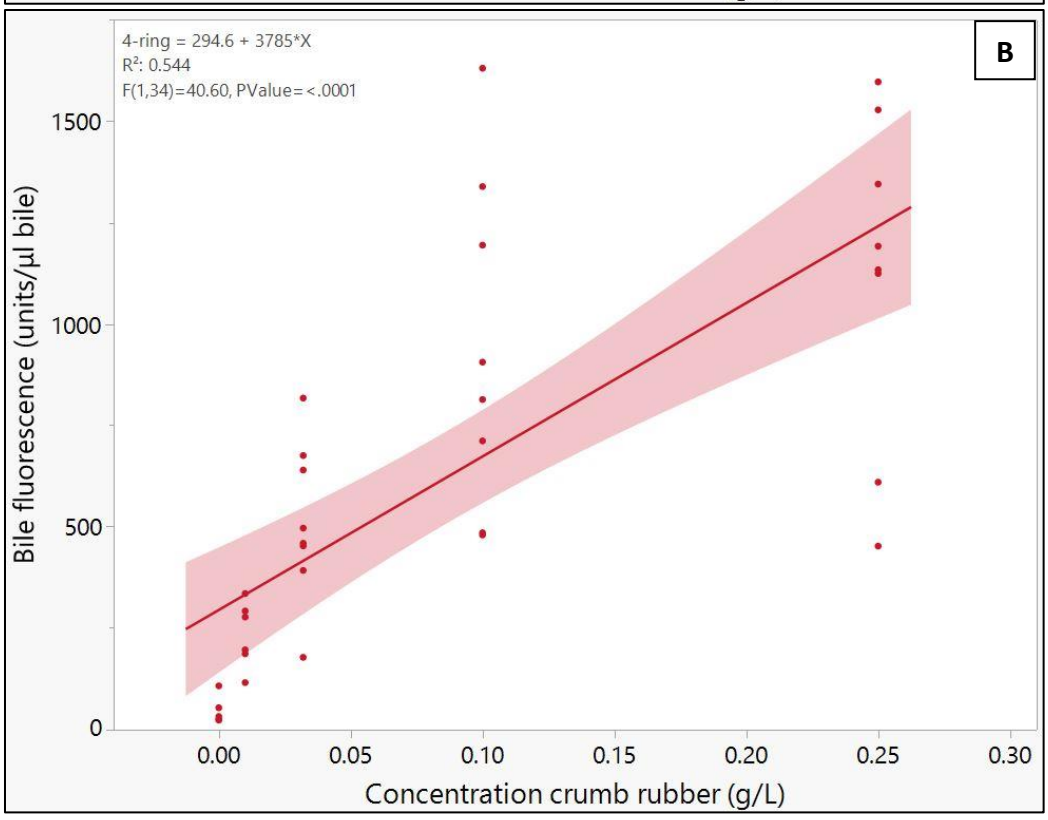


Figure 3.3 – Mummichog liver tissue. Immunohistochemical staining using secondary antibody only as negative control in fish exposed to CR (A). Immunohistochemical staining using primary antibody CRC4 in control fish (B). Immunohistochemical staining using primary antibody CRC4 in fish exposed to 2.548 g/L CR (C).


2-ring = 1229 + 2442*X
R²: 0.154
F(1,34)=6.19, PValue=0.0179
A
Bile fluorescence (units/µl bile)
3000
2500
2000
1500
1000
500
0.00
0.05
0.10
0.15
0.20
0.25
0.30
Concentration crumb rubber (g/L)


4-ring = 294.6 + 3785*X
R²: 0.544
F(1,34)=40.60, PValue=<.0001
B
Bile fluorescence (units/µl bile)
1500
1000
500
0
0.00
0.05
0.10
0.15
0.20
0.25
0.30
Concentration crumb rubber (g/L)

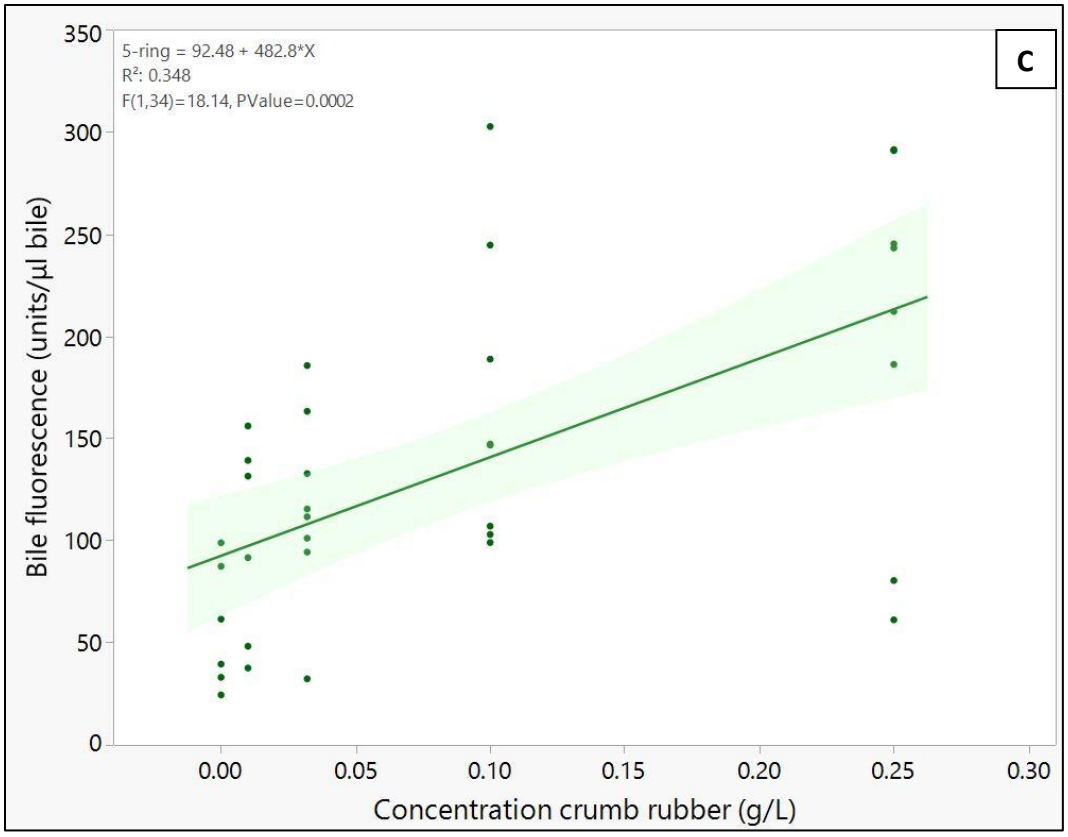


Figure 3.4 – Linear regression of bile fluorescence in mummichog (*F. heteroclitus*) measured for 2-, 4-, and 5-ring compounds (A, B, C, respectively) after 42 d pulsed exposure of up to 0.25 g/L crumb rubber. Note different scales for y-axis. Shaded area represents the 95% confidence interval.

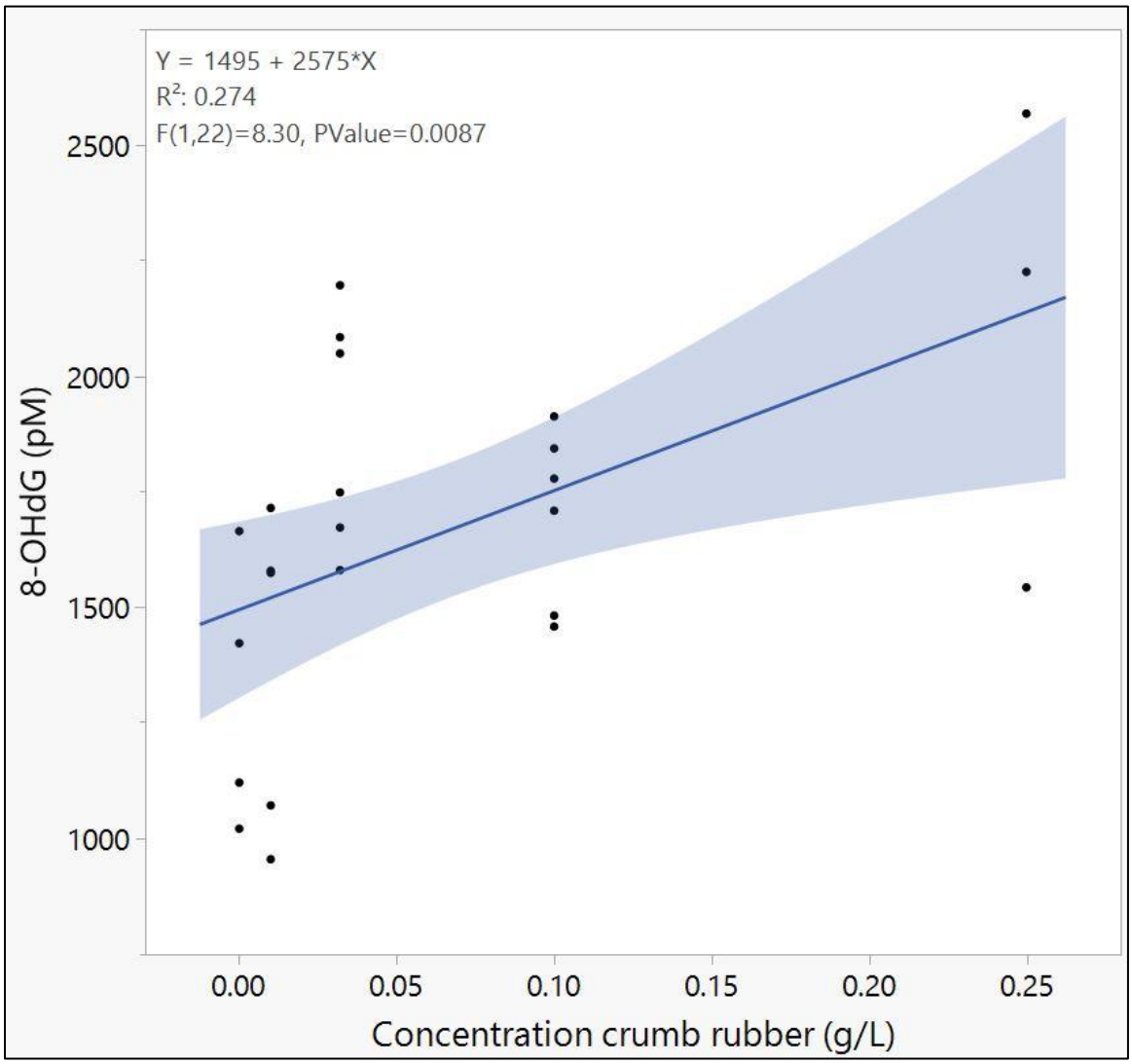


Figure 3.5 – Linear regression of the concentration of 8-OHdG from mummichog (*F. heteroclitus*) blood plasma after exposure to CR every 5-days for 42 days total. 8-OHdG is expressed in pM. Shaded area represents the 95% confidence interval.

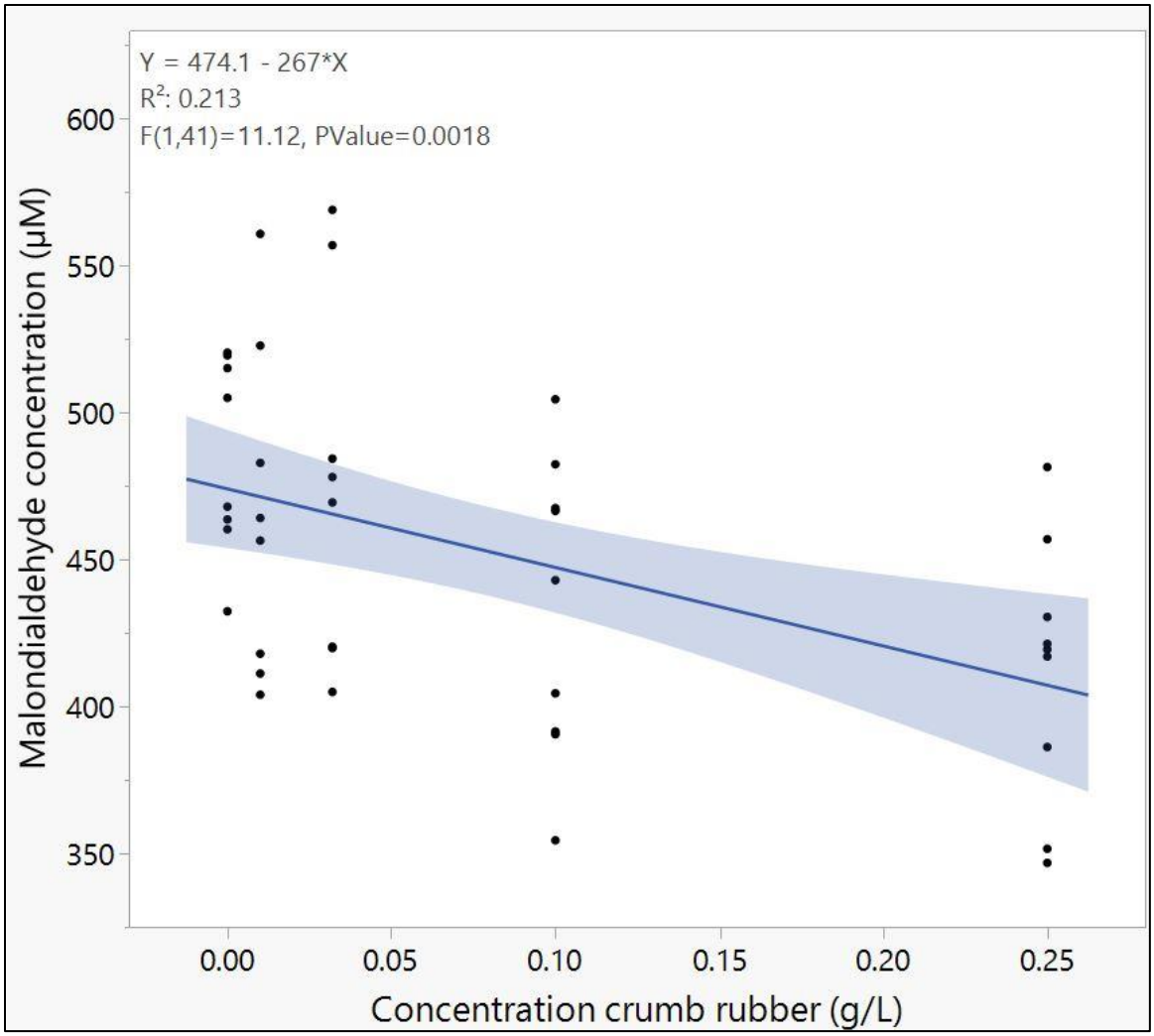


Figure 3.6 – Linear regression of the concentration of malondialdehyde (MDA) from mummichog (*F. heteroclitus*) liver after exposure to CR every 5-days for 42 days total. MDA is expressed in µM. Shaded area represents the 95% confidence interval.

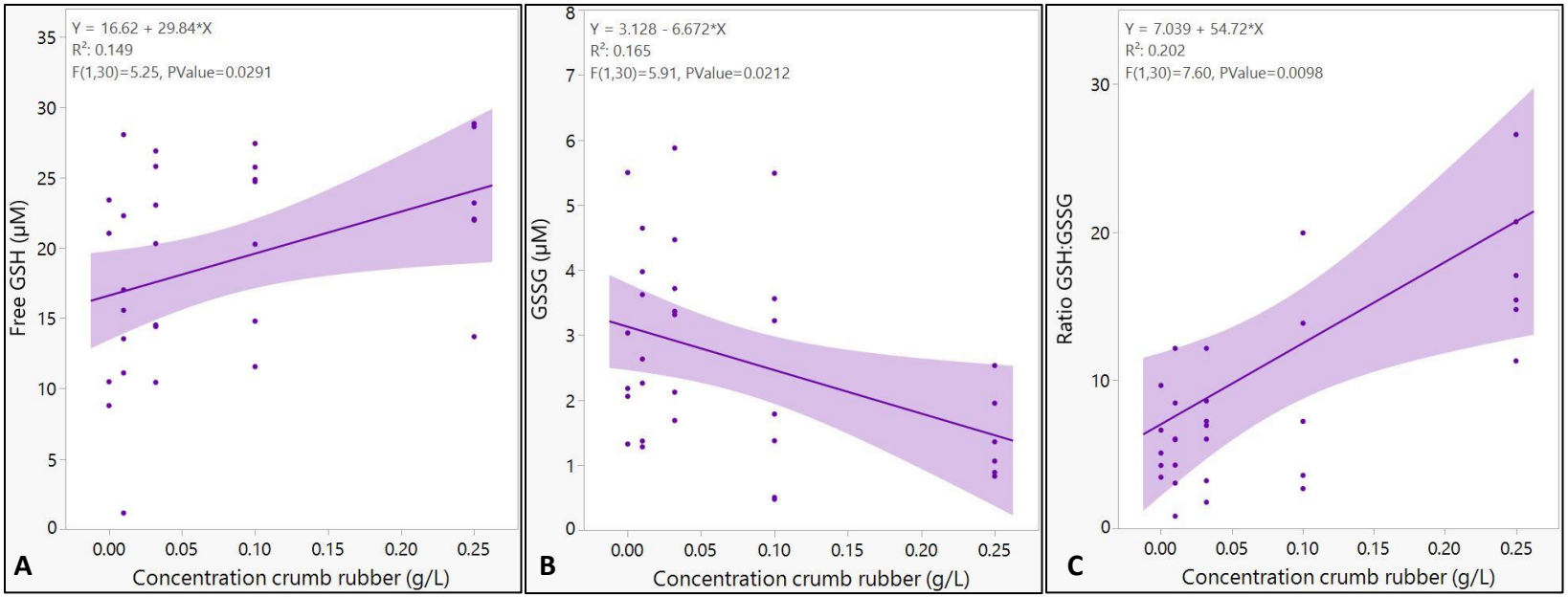


Figure 3.7 – Linear regression of glutathione (µM) as reduced GSH (A), oxidized GSSG (B) and ratio of GSH:GSSG (C) from mummichog (*F. heteroclitus*) liver after exposure to CR every 5-days for 42 days total. Note different scales for y-axis. Shaded area represents the 95% confidence interval.

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CHAPTER FOUR

ACCUMULATION OF MICRORUBBER PARTICLES IN STORMWATER POND BIOTA

4.1 Abstract

Stormwater ponds serve as effective stormwater runoff best management practices and trap or remove both physical and chemical pollutants before further discharge into receiving natural waterbodies. Stormwater ponds can therefore accumulate high levels of pollutants, such as microplastics (MP) and microrubber (MR) from road runoff. It was hypothesized that biota in stormwater ponds in proximity to roadways or those that receive large road runoff would contain high amounts of MP per individual, with high abundances of tire particles (TP). Fish and invertebrates were collected from stormwater ponds and their adjacent tidal creeks in Mt. Pleasant, SC, USA. Whole organisms were digested using KOH and digested contents were filtered and analyzed by visual microscopy to identify MPs.

The majority (>80%) of MP recovered from biota across all sites were suspected TP. The average number of MP per individual ranged from 0.3 to 71 MP and the average number of suspected tire particles per individual ranged from 0 to 57.7 tire particles. There were significant differences in MP per individual observed between sites and between species. A combination of factors such as availability of MPs based on surrounding land use, pond hydrodynamics, organism size, and organism feeding habitat likely influenced the total MP observed among the different sites and species analyzed. These data provide preliminary examination into the fate and transport of MP and MR in

stormwater ponds and an evaluation of MP and MR abundance in organisms from stormwater ponds the coastal zone.

4.2 Introduction

It is well known that microplastic (MP) particles have been found in air, sediment, and water samples worldwide (Eerkes-Medrano et al., 2015; Gasperi et al., 2018). Numerous studies cite MPs in various field-collected organisms including terrestrial and aquatic species (Foekema et al., 2013; He et al., 2013). More recently, microrubber (MR) has been identified as a secondary microplastic type of concern, entering the environment from tire wear under typical driving conditions. Studies have sampled MR from air, sediment, and water as well; however, fewer data on the abundance of MR particles in biota is known (Wik & Dave, 2009). Microplastic and microrubber particles are assumed to coincide in environmental samples and have similar transport mechanisms being synthetic polymer particles. Therefore, it is possible that MR particles may be as ubiquitous in biota as MP. Understanding the presence of MR in field-collected organisms is essential for characterizing the overall environmental risk of MR in the environment.

Land-based activities provide significant contributions to MPs in the environment with sources from industrial, commercial, and residential areas. More specifically, sources of MPs include textile washing, personal care and cosmetic products, tires, turfs, and road paints, packaging, littering, and the construction industry (Xu et al., 2020). In addition to transporting MPs, stormwater runoff is widely recognized as a major transporter of contaminants such as metals, organic pollutants, nutrients, and pathogens

from the overlying land to receiving waters (Hoffman et al., 1984; US EPA, 2012). For many pollutants including MPs, stormwater retention ponds serve as a hot spot for pollution and play a role in transport of MPs from land to the aquatic environment (Liu et al., 2019).

Almost 40% of the total U.S. population live in coastal counties yet the coast accounts for less than 10% of the nation's land mass (NOAA, 2018). South Carolina has experienced rapid coastal population growth rates over the last forty years in addition to an extraordinary increase in land development along its coast. From 1973 to 1994 in Charleston alone, the population increased by 40% while urban area increased by 240%, and that number is likely greater at present (Charleston Waterkeeper, 2014). Rapid urbanization of coastal regions presents stormwater management challenges as impervious surfaces and population increase in the local area. Stormwater ponds are the most widely used best management practice for mitigating stormwater flooding and pollution.

The Charleston Harbor is a major urban estuary located on the southeastern Atlantic coast of the U.S., with the City of Charleston residing on the peninsula between the Ashley River and the Cooper River. The Charleston Harbor is formed by the confluence of the Ashley River, Cooper River, and Wando River (Crist et al., 2019). According to the U.S. Census Bureau, the population in the Charleston area (including Goose Creek, Hanahan, North Charleston, Mount Pleasant, and Charleston) is approximately 422,000 individuals (U.S. Census Bureau, 2020). The Charleston metropolitan area has a strong economy due to the Port of Charleston, tourism, military

installations, medical facilities, and manufacturing. The port is ranked 6th in the U.S. in terms of cargo value (S.C. Ports Authority, 2020).

Utilizing satellite imagery, an estimated 21,594 ponds have been counted within the coastal zone of South Carolina associated with either rural or development-related land uses (Smith et al., 2020). Of those, 43% were determined to be associated with development including residential, golf course, or commercial uses. The Charleston area has approximately 4,000 ponds alone as of 2015, with nearly half of those associated with developments listed above (Smith et al., 2020). In the coastal region of South Carolina, wet detention ponds are the most common type of stormwater pond constructed (Crawford et al., 2010; Drescher et al., 2007). Stormwater ponds remove pollutants through physical, chemical, and biological processes such as sedimentation, photodegradation, or particulate trapping by vegetation, respectively (Beckingham et al., 2019). The densities of MR particles including tire wear, crumb rubber, bitumen particles range from 1.13 – 2.2 g/cm^3 and are likely to settle and accumulate in stormwater pond sediments; however, pond hydrodynamics (i.e., size, depth, shape, flow, hydraulic residence time) may influence their settlement (Degaffe & Turner, 2011; Kayhanian et al., 2012; Kell, 2020; Rhodes et al., 2012; Unice et al., 2019). Another factor related to pollutant and MR accumulation in stormwater ponds is the efficiency of particle capture on the landscape (e.g., by rain gardens), by stormsewer catch basins, and manufactured treatment devices (MTDs) (Werbowski et al., 2021). MTDs function as stormwater treatment devices before the stormwater is discharged off-site or to receiving water bodies through processes such as filtration, screening, or settling (SC DOT, 2015). MR

particles that are not effectively captured and sequestered along stormwater urnoff pathways may be resuspended and transported to adjacent receiving waters, and in this scenario, the adjacent marshes or tidal creeks found in the local area of study.

Settled MR particles may be ingested by benthic or bottom-feeding organisms in stormwater ponds or adjacent tidal creeks (Redondo-Hasselerharm et al., 2018). Parker et al. (2020) also observed MPs and suspected tire wear particles in the digestive tract of pelagic fish collected from an urban harbor, which suggests that suspension or resuspension of MR in stormwater ponds can lead to uptake by various species. Feeding ecology can potentially have an impact on MP exposure in aquatic organisms, specifically due to food preference, habitat, feeding mechanism, and selectivity although there have been mixed results on the impact of microplastic exposure among various species feeding traits (Parker et al., 2020; Pazos et al., 2017; Walkinshaw et al., 2020).

In an assessment of MPs in coastal South Carolina (Charleston Harbor, SC, USA), black fragments classified as tire wear particles (TWP) comprised the majority of total microplastics encountered in sediment samples (Gray et al., 2018). It is likely that hot spots of high MR levels occur in proximity to roads and bridges when considering the fate and transport of MR and evaluating data from previous studies that show high abundances of MR in proximity to roadways, (Järlskog et al., 2020). Additionally, stormwater ponds which function to collect stormwater runoff from roads are also a potential hot spot for MR particles. Kell (2020) measured levels of MP and TWP in stormwater ponds in the Charleston area of SC and found that levels decline in sediments from pond to the discharge point at tidal creek and finally downstream or upstream in

tidal creek indicating that stormwater ponds are a sink for MR particles within stormwater transport pathways yet are not perfectly effective at their capture.

The objective of the present study was to characterize the abundance of MPs found in aquatic biota from stormwater ponds and their adjacent tidal creeks, with an emphasis on MR particulates or tire particles (TP). These data will add to the growing body of knowledge on the efficiency of stormwater ponds in capturing and retaining MR particulates and provide an assessment of MP, and more specifically MR, in stormwater pond biota.

4.3 Methods

4.3.1 Sampling sites

Stormwater pond sites and adjacent tidal creeks were selected from Mount Pleasant, South Carolina, USA, within the Charleston Harbor watershed (**Appendix A, Figure 1**). Ponds selected represented a variety of drainage area land uses including residential, commercial, highway, and golf course areas. Detailed descriptions of pond location and receiving waterbody can be found in **Appendix A.** Five stormwater ponds (Whipple Road, Oak Marsh, Oyster Point, Tides Condos, Patriots Point) and their adjacent tidal creeks were targeted to sample for aquatic biota. The reference pond (Patriots Point) is located on a golf course and thus has little to no influence from vehicular traffic that would create MR particles, aside from golf carts.

4.3.2 Biota collection

Aquatic biota were captured from stormwater ponds and adjacent tidal creeks using a seine net (2m length, 5mm mesh). Nets were pulled through stormwater ponds as

depth allowed in proximity to the shoreline as needed to collect sufficient organisms. Nets were pulled through tidal creeks moving from downstream to upstream (near pond) 1-3 times as needed to collect sufficient organisms. Biota (n = 285) were sampled on April 27th and April 28th, 2021 from each of the five stormwater ponds and tidal creeks (**Appendix B**). However, organisms were not collected from Oak Marsh pond, Whipple Road pond, or the Tides Condos tidal creek as conditions did not allow for collection using the methods described (limited access, too much debris, etc.) Water quality parameters (salinity, pH, temperature, dissolved oxygen) were recorded at each pond and tidal creek site. Collected fish were euthanized using MS-222 solution, separated by species, wrapped in labelled aluminum foil, and placed on ice in the field. Invertebrates (i.e. crawdads and grass shrimp) were separated by species, wrapped in labelled aluminum foil, and placed on ice in the field. Immediately upon returning to the lab, all samples were transferred to a -20°C freezer for storage until further analysis.

4.3.3 Digestion of samples and isolation of particles

Biota were thawed, weighed (wet weight), and length measured individually so that results could be reported per individual fish by dividing total microplastic counts by the number of individuals assessed. Organisms were pooled by species into 3 to 12 individuals, depending on size, to be digested together due to their overall small size (< 100 mm). Whole organisms were placed in a glass beaker with 1 M KOH solution at approximately three times the volume of the organic biological material (Foekema et al., 2013; Lusher et al., 2017; Parker et al., 2020). Beakers were sealed with aluminum foil and digested for a period of three to five days, or until interfering tissue had been fully

dissolved, with heat applied for a portion of the digestion period (24-hr at 60°C in a water bath). Following digestion, samples were sieved through a series of stacked metallic brass sieves (500µm and 53µm). Contents from each sieve were washed into separate glass beakers with DI water and covered with aluminum foil until further processing.

4.3.4 Microplastic and microrubber quantification

Sieved digested samples were washed onto a mixed cellulose ester membrane (Whatman, sterile mixed cellulose ester membranes, color: green with black grid, size: 0.45 µm pore) over a glass vacuum filtration funnel to remove liquids. If samples contained a large amount of digested material, more than one filter was utilized and microplastic counts from all filters of the same sample and sieve size were totaled.

Microplastics were identified and counted under a dissecting microscope (20x-40x, Meiji EMT Tokyo, Japan). Microplastics were classified by type as fibers, suspected tire wear particles, or fragments and colors were noted. Other microplastics (foams, films, spheres) were noted, if observed. Microplastics were counted by reading the filter in a crystallizing dish in a serpentine pattern and analyzing each square on the gridded filter. Microplastics were identified using established criteria for microplastic morphology and confirmed using either the hot needle test or manipulation with a probe (Barrows et al., 2017; Lusher et al., 2017). For microplastics not including suspected tire wear particles, items were considered microplastic under the following criteria: homogeneously colored, no visible cellular or organic structure, equal thickness throughout for fibers, and does not crumble when manipulated with forceps. The hot needle test was used to help distinguish between microplastic pieces and organic matter as needed where, plastic

pieces would melt or curl in the presence of a very hot needle and biological or non-plastic materials would not (Barrows et al., 2017; De Witte et al., 2014). Suspected tire wear particles are more difficult to identify as they do not react to a hot needle test and have more variability in appearance and behavior depending on tire age, particle age, and particle structural integrity. In general, particles were classified as suspected tire wear particles under the following criteria: darkly colored (black), elongated, cylindrical, or irregular in shape (cuboidal/angular), partially or entirely covered with road dust (glittery sheen), rough surface texture, rubbery flexibility when manipulated with forceps (Kell, 2020; Kreider et al., 2010; Leads & Weinstein, 2019; Parker et al., 2020). Suspected tire wear particles that disintegrated or turned powdery when probed with a dissecting needle were not counted as part of the total suspected tire particles (TP).

4.3.5 Quality Assurance/Quality Control

To reduce potential contamination from MP, a number of measures were taken. After thawing field-collected samples, organisms were rinsed with deionized water after weighing and measuring to reduce contamination from any MPs potentially adhered to the outer surface of the organisms prior to digestion. In the laboratory, only stainless-steel or glass equipment was used, except for LDPE wash bottles and nitrile gloves. Samples were kept covered to help eliminate airborne contamination. Additionally, all equipment (i.e., beakers, sieves) was rinsed with deionized water three times before use and in between each sample.

Procedural blanks (without tissue) were processed with each batch of samples (n=10 total blanks). Briefly, DI water was poured through the rinsed nested sieves

similarly to sample processing, and DI water rinse was collected from the sieve in a glass beaker (approximately 200 mL). Blanks were poured over a gridded filter under a vacuum as described above. The number of microplastics on the gridded filter was enumerated for each blank by visual microscopy.

4.3.6 Statistical analysis

All statistical analyses were performed using JMP Pro 14 (SAS Institute Inc.) statistical software. Microplastic counts were normalized to the number of individuals examined in each batch processed. The percentage of microplastic types (fiber, suspected tire particle, and fragment) was determined for each site sampled. Data was log transformed to better approximate for normality when required. When the data was approximately normally distributed, an ANOVA or t-test was performed to test for significant differences. Tukey's post hoc analysis was performed if significant differences were found. Simple linear regression was used to assess relationships between variables, such as MP abundance and biota weight. A p-value of < 0.05 was considered significant.

4.4 Results

4.4.1 Background Contamination

Procedural blanks (n=10) contained an average of 1.1 ± 0.6 MP per blank. MP sample counts were adjusted to account for procedural blank contamination by subtracting the average counts in procedural blanks from the total MPs counted for each batch analyzed.

4.4.2 Microplastic counts

A total of 285 organisms were processed for total microplastics which included nine species of fish, one species of grass shrimp, and one species of crawdad. Microplastic counts were normalized to the number of individuals examined for analysis. Overall, 100% of the samples examined contained individuals with at least 1 MP, with an average of 9.5 ± 6.5 MP per individual across all organisms and all sites sampled (**Figure 4.1**). Because individuals were grouped into batches of 3-12 for processing, I am unable to deduce the exact number of MP per individual analyzed or the percentage of individuals examined overall that contained MP. A linear regression between microplastics per individual and the average length (mm) and weight (g) of biota was performed to determine if there were significant relationships between organism size and MP abundance. There was no correlation between the length of individual organisms and abundance of MPs (R^2 = 0.00, F(1,255) = 0.49, p = 0.4857) but the weight of individual organisms was significantly correlated to the number of MP per individual (R^2 = 0.07; F(1, 283) = 21.68, p < .0001) (**Figure 4.2**). Given the significant relationship observed between the number of MP per individual and weight, MP abundance was also analyzed in relation to organism weight for comparisons across sites and species described in sections 4.4.3 and 4.4.4 (**Figure 4.3**).

Microplastics were also classified by size fraction according to sieve and were either 53µm – 500µm or 500µm – 5mm in size. Of the 2,713 MP counted, 74.8% were between 53µm - 500µm and 25.1% were 500 µm – 5mm in size. There were significantly more MP in the size range from 53µm – 500µm (t(58) = -2.32, p = 0.0119). Microplastics were also categorized into three major types: fibers, suspected tire particles (TP) or

fragments. (**Figure 4.4**). The distribution of MP types encountered was 8.0% fibers, 89.9% suspected tire particles, and 2.1% fragments across all MPs counted. The focus of this study was understanding the occurrence of suspected tire particles in organisms from stormwater ponds and their adjacent tidal creeks; therefore, the microplastics were divided into two groups for further analysis, suspected tire particles (TP) and fibers + fragments. The distribution of MP types across sites indicated that suspected tire particles consistently made up the majority of total MP (**Figure 4.5**).

4.4.3 Site differences

Total microplastics (fibers, suspected tire particles, fragments) per individual were compared across all sites sampled (n = 7 sites, 3 sites were unable to be sampled), regardless of species or size fraction (**Figure 4.6, Table 4.1**). There was a significant difference in total MP per individual among sites ($F(6, 37) = 11.99$, p = <.0001). The number of MP per individual was significantly greater at the Whipple Road creek site (WR-C, 35.5 ± 11.2 MP per individual) site compared to Patriots Point pond (PP-P, 1.3 ± 0.5 MP per individual, and Patriots Point creek (PP-C, 0.9 ± 0.6 MP per individual), and Oyster Point pond (OP-P, 2.2 ± 0.9 MP per individual) (Tukey's HSD, p = 0.0003, p = < .0001, and p = 0.0003 respectively). There were also significantly more total MP per individual from the Oak Marsh creek site (OM-C, 15.1 ± 4.0 MP per individual) compared to Patriots Point creek, Patriots Point pond, and Oyster Point pond (Tukey's HSD, p = < .0001, p = 0.0033, and p = 0.0055, respectively). The Oyster Point creek site

Table 4.1 – Microplastic counts and types in each species across all sites sampled. Total microplastics includes fibers, fragments, and suspected tire particles. N = number of individuals collected at each site.

Site	n	Total microplastics per individual (mean ± SEM)	Tire particles per individual (mean ± SEM)
Whipple Road Creek (WR-C)			
Crawdad	3	71	57.7
Molly	19	48.3 ± 32.2	48.2 ± 32.2
Mosquitofish	36	23.3 ± 11.5	17.6 ± 8.4
Overall for site	*58*	*35.5 ± 11.2*	*30.3 ± 10.1*
Tides Condos Pond (TC-P)			
Mosquitofish	30	5.5 ± 1.5	5.0 ± 1.3
Overall for site	*30*	*5.5 ± 1.5*	*5.0 ± 1.3*
Patriots Point Pond (PP-P)			
Mosquitofish	30	1.3 ± 0.5	1.1 ± 0.4
Overall for site	*30*	*1.3 ± 0.5*	*1.1 ± 0.4*
Patriots Point Creek (PP-C)			
Grass shrimp	13	0.3	0.2
Molly	5	0.4	0
Sheepshead	10	0.3 ± 0.1	0
Silversides	15	2.0 ± 1.8	1.7 ± 1.6
Overall for site	*43*	*0.9 ± 0.6*	*0.6 ± 0.5*
Oyster Point Pond (OP-P)			
Bass	30	1.3 ± 0.6	1.0 ± 0.4
Silversides	20	1.6 ± 0.7	1.1 ± 0.7
Sunfish	8	7.8	6.8
Overall for site	*58*	*2.2 ± 0.9*	*1.8 ± 0.8*
Oyster Point Creek (OP-C)			
Mullet	21	17.9 ± 4.8	17.5 ± 4.6
Overall for site	*21*	*17.9 ± 4.8*	*17.5 ± 4.6*
Oak Marsh Creek (OM-C)			
Grass shrimp	12	6.6	4.58
Mummichog	13	19.6 ± 5.1	16.2 ± 5.4
Pinfish	20	12.1 ± 8.0	9.6 ± 6.7
Overall for site	*45*	*15.1 ± 4.0*	*12.3 ± 3.7*

(OP-C, 17.9 ± 4.8 MP per individual) had significantly more MP per individual when compared to the reference sites (Patriots Point creek and Patriots Point pond) and when compared to the pond site for the same location, Oyster Point pond (Tukey's HSD, p = 0.0003, p = 0.0058, and p = 0.0114, respectively). Lastly, the Tides Condos pond site (TC-P, 5.5 ± 1.5 MP per individual) had significantly more MP per individual compared to the reference creek site, Patriots Point creek (Tukey's HSD, p = 0.0172).

Data were also analyzed as total MP per weight (g) of organism. There was a significant difference in MP abundance per g among sites ($F(6,278) = 47.52$, p = < 0.0001). When normalized to weight of the organism, differences in MP abundance between sites were more apparent with the reference sites at Patriots Point having significantly fewer MP compared to the other sampling sites (**Figure 4.7).**

There were only two locations that had samples from both the pond and adjacent tidal creek, the reference site, Patriots Point, and Oyster Point. At Patriots Point, the total MPs per individual were not significantly different between the tidal creek (PP-C, 0.9 ± 0.6 MP per individual) and the pond (PP-P, 1.3 ± 0.5 MP per individual) ($t(8.4) = 0.907$, p = 0.3892) (**Figure 4.8A**). At Oyster Point, there were significantly more total MPs per individual from the creek (OP-C, 17.9 ± 4.8 MP per individual) compared to the pond (OP-P, 2.2 ± 0.9 MP per individual) ($t(9.2) = -4.325$, p = 0.0009) (**Figure 4.8B**). This was unexpected as I anticipated the pond to contain more MP in general and expected to find more MP in organisms collected from ponds compared to the creeks.

4.4.4 Species differences

To assess species differences in microplastic abundance in biota, microplastics per individual were compared across all organisms collected, regardless of site or size fraction (**Figure 4.9, Table 4.1**). There was a significant difference in total MP per individual among species ($F(10, 274) = 22.55$, $p = < 0.0001$). Tukey's HSD post-hoc analysis for comparisons of each pair showed there were significantly more MP per individual in crawdads (71 MP per individual) and mollies (48.3 ± 32.2 per individual) compared to pinfish, mosquitofish, grass shrimp, silversides, bass, and sheepshead. There were significantly more MP per individual in mummichogs (19.6 ± 5.1 MP per individual) and mullet (17.9 ± 4.8 MP per individual) compared to mosquitofish, grass shrimp, silversides, bass, and sheepshead. Sunfish (7.8 MP per individual), pinfish (12.1 ± 8.0 MP per individual), and mosquitofish (10.0 ± 3.3 MP per individual) had significantly more MP per individual than silversides, bass, and sheepshead. Sheepshead minnow total MP per individual (0.3 ± 0.1 MP per individual) were significantly lower than all species except silversides and bass.

Data were also analyzed as total MP per weight (g) of organism. There was a significant difference in MP abundance per g among species ($F(10,274) = 15.55$, $p = < 0.0001$) (**Figure 4.10**). When normalized to weight of the organism, differences in MP abundance between species seem to suggest other variables such as feeding habitat may be responsible for the observed differences. When making comparisons among species, it is important to acknowledge that not all species were collected at each site. For example, sheepshead were only collected from the reference site creek (PP-C) and therefore have the lowest MP abundance recorded.

4.5 Discussion

Microplastic counts – size fraction recovered and distribution of MP types recovered

Biota collected from stormwater pond and adjacent tidal creeks in Mount Pleasant, SC, USA were found to contain an average of 9.5 ± 6.5 MP per individual, which included fibers, suspected tire particles, and fragments. The organisms analyzed included nine species of fish, one species of grass shrimp, and one species of crawdad (**Appendix B**). Other studies on microplastic abundance in biota mostly focused on marine species or freshwater studies. The range of MPs reported in biota can vary depending on several factors including species, geographic location, habitat, anthropogenic influence and nearby land use, or seasonal weather patterns. The average number of MP per fish from the present study were within the range of MP concentrations observed in other studies; for example, 9.9 ± 13.4 particles per fish were found in organisms collected upstream and downstream from a sewage treatment plant in Korea (Park et al., 2020b). Additionally, other estimates of MP in various species and geographic locations include 0.19 to 1.63 MP per individual for *Lepomis* spp. from the Brazos River Basin, USA (Peters & Bratton, 2016), 0 to 18 MP per individual in *Carassius auratus* collected from a freshwater lake in China (Yuan et al., 2019), an average of 1.71 ± 2.27 MP per individual in the terrestrial crab *Cardisoma carnifex* from Vanuatu (Bakir et al., 2020), and an average of 1.8 ± 1.7 MP per individual in coastal and offshore fish from the Northeast Atlantic (Murphy et al., 2017). The lack of standard reporting for microplastic identification and sample processing of microplastics in biota made it difficult to compare studies as both microplastic identification methods and

sample processing methods (i.e., gut only vs. whole organism) can vary. Nevertheless, the reported ranges for MPs in biota from freshwater environments were typically greater than those observed in marine environments, and a general reduction in the abundance of MPs from land (i.e., freshwater) to nearshore to offshore (Bakir et al. 2020; Graca et al., 2017) has been observed suggesting that anthropogenic influence greatly impacts MP abundance in different environmental matrices.

Parker et al. (2020) assessed microplastics in estuarine fishes in the same geographical area (Charleston Harbor, SC, USA) and found an average of 26.9 ± 4.7 MP per fish. Fish processing methods (digestion of whole organism in KOH) and the size range of MPs analyzed (63 µm to 5mm) in Parker et al. (2020) were similar to the present study, making for good comparability between studies. They observed a much higher average number of MP per individual than was observed in the present study which may be attributed to the larger size of the fish collected and analyzed by Parker et al., who suggested along with others that MP abundance increases as fish size increases (Hossain et al., 2019; Hurt et al., 2020; Parker et al., 2020; Peters & Bratton, 2016). When normalized to the weight of fish, Parker et al. observed an average of 5.8 ± 1.6 MP per g fish and is comparable to the present study, which observed an average of 3.87 MP per g individual. For length of fish, Parker et al. reported an average length of 104 ± 6 (mm), therefore, an average of 0.25 MP per mm when normalized to MP per length of fish. My study found an average of 0.31 MP per mm fish (crawdads and grass shrimp not included).

Data available from studies that assess MP abundance in the water column, sediment, and biota suggested that in general, MPs accumulate the most in sediments followed by the water column, then biota although local differences can occur depending on environmental mixing and flow and species examined (Cera et al., 2020; Kazour et al., 2019; Yuan et al., 2019; Zhang et al., 2020). For stormwater ponds specifically, only one study to date has collected samples for MP analysis from the water column, sediment, and biota and found that MPs accumulated to the highest concentrations in sediments, followed by vertebrates analyzed from the pond, with the water column having the least number of MPs (Olesen et al., 2019). Although different water bodies were examined compared to the stormwater ponds examined in the present study, MP abundances in the Charleston Harbor estuary indicated that MPs in the water ranged from 3 to 36 MP/m^2 in water and 0 to 4,375 MP per kg wet weight in sediments (Leads & Weinstein, 2019). Additionally, for biota, Payton et al. (2020) observed 1.4% of zooplankton collected from the Cooper River front (adjacent to Mt. Pleasant, SC, USA) to contain microplastics. However, determining the MP abundance in stormwater ponds in the Charleston area is currently in its infancy. Kell (2020) reported greater MPs in stormwater pond water and sediment compared to MPs in adjacent discharge creek water and sediment for stormwater ponds also located on Mt. Pleasant, SC USA. My data reflects a different trend, that there were more MPs in biota from adjacent tidal creeks compared to MPs in biota from stormwater ponds, but is somewhat inconclusive as there was only one sampling location besides the reference site where organisms were collected from both the stormwater pond and tidal creek. For stormwater ponds globally, greater amounts of

MPs in stormwater pond sediment and water compared to discharge point samples are also observed (**Table 4.2)** where regardless of size fraction analyzed, stormwater pond sediments seem to act as significant sinks for microplastics. Additional data on the abundance of MP in the water, sediment, and adjacent tidal creek water and sediment from the stormwater ponds from the present study would assist in forming conclusions regarding the availability of MP to stormwater pond biota.

In the present study, total MPs were classified by two size fractions: 53µm – 500µm and >500 µm. There were significantly more MP collected from the smaller size range, which is corroborated by other studies that also found a greater abundance of the smaller sized MPs in surface waters, sediments, and biota (Bakir et al., 2020; Olesen et al., 2019; Park et al., 2020a; Park et al., 2020b; Ziajahromi et al., 2020). Smaller particles are potentially more prevalent in biota collected from stormwater ponds because i) they closely align with the size range of natural food items for the biota captured (Parker et al., 2020) and ii) larger particles may effectively be trapped or settled in sediments and less likely to be consumed (Besseling et al., 2017).

Additionally, the majority (>80%) of total MPs counted from individuals across all sites were suspected tire particles, highlighting the importance of stormwater ponds and their discharge points into tidal creeks as pathways and potential hot spots for MR in the environment. The majority of tire wear particles collected from the environment may range between 25 and 50 µm (Kreider et al.. 2010) which was below the threshold for the size sampled in this study (53 µm). Although the majority of total MP counted in the present study were suspected tire particles, difficulty with confirming polymer type for

Table 4.2 – Microplastic abundances in studies from Charleston Harbor, SC area and stormwater pond studies worldwide. Microplastics are reported as averages or min and max averages unless otherwise noted.

Sample type	Location	Size range	Microplastics (Average)	Study
Charleston Harbor, SC, USA studies				
Surface water	Charleston, SC USA	> 63 µm	3 – 36 MP L^{-1} (min – max)	Leads & Weinstein, 2019
Sediment	Charleston, SC USA	> 63 µm	0 – 4,375 MP kg^{-1} ww^{-1} (min – max)	Leads & Weinstein, 2019
Surface water	Charleston, SC USA	> 63 µm	3 – 11 MP L^{-1} (min – max)	Gray et al., 2018
Sediment	Charleston, SC USA	> 63 µm	42.2 – 11,195.7 MP m^{-2} (min – max)	Gray et al., 2018
Surface water	Charleston, SC USA	43 - 104µm	0.6 – 1.0 MP L^{-1}	Payton et al., 2020
Biota (zooplankton)	Charleston, SC USA	43 - 104µm	avg. 1.4% of individuals	Payton et al., 2020
Biota (fish)	Charleston, SC USA	43µm – 11.3mm	27 MP $individual^{-1}$	Parker et al., 2020
Stormwater pond studies				
Pond water	Charleston, SC USA	63 - 5000µm	1.6 – 132 MP L^{-1} (min – max)	Kell, 2020
Pond sediment	Charleston, SC USA	63 - 5000µm	1,379 - 10,557 MP kg^{-1} dw^{-1} (min – max)	Kell, 2020
Tidal creek sediment adjacent to stormwater pond	Charleston, SC USA	63 - 5000µm	100 – 10,000 MP kg^{-1} dw^{-1} (min – max)	Kell, 2020
Pond water	Denmark	10 - 500µm	270 MP L^{-1}	Olesen et al., 2019
Pond sediment	Denmark	10 - 500µm	9.5 x 10^5 MP kg^{-1} dw^{-1}	Olesen et al., 2019
Pond biota	Denmark	10 - 500µm	65 MP $individual^{-1}$	Olesen et al., 2019
Pond water	Denmark	10 - 2000µm	490 – 22,894 MP m^{-3} (min – max)	Liu et al., 2019
Pond sediment	Denmark	10 - 2000µm	1,115 – 128,732 MP kg^{-1} dw^{-1} (min – max)	Liu et al., 2019
Pond water	Sweden	20 - 300µm	977 – 4,267 MP L^{-1} (min – max)	Jönsson, 2016
Pond water	Australia	25 - 500µm	0.9 – 4.0 MP L^{-1} (min – max)	Ziajahromi et al., 2020
Pond sediment	Australia	25 - 500µm	320 – 595 MP kg^{-1} dw^{-1} (min – max)	Ziajahromi et al., 2020

suspected tire particles may have led to inaccurate counts. Nevertheless, other studies have documented suspected tire particles (25 - >500µm) in sediment samples, comprising up to 38% of total MPs, from both inlets and outlets of stormwater ponds, indicating they are available in stormwater ponds and at discharge points (Ziajahromi et al., 2020).

Additionally, Kell (2020) reported high abundances of TP in stormwater pond sediment and sediment of pond discharge points, up to 80% of total MP in pond sediments and up to 60% of total MP in discharge point sediments and more specifically, the majority were between 63 - 500µm in size. These data suggest that TP are accumulating in stormwater ponds from stormwater runoff and adjacent tidal creeks where ponds discharge and are available to biota in stormwater ponds and their adjacent tidal creeks.

Site and species differences

The average number of MP per individual was significantly different among the sampling sites. The reference tidal creek, Patriots Point creek, contained significantly fewer MP per individual (0.9 ± 0.6 MP per individual) compared to all other sites. The reference pond site, Patriots Point pond, also contained significantly fewer MP per individual (1.3 ± 0.5 MP per individual) than Whipple Road, Oyster Point, and Oak Marsh tidal creek sites. Interestingly, there was not a significant difference between the average number of MP per individual in Patriots Point pond compared to the other pond sites sampled, Oyster Point and Tides Condos ponds. Of those two pond sites, Oyster Point pond had significantly fewer MP per individual compared to its adjacent tidal creek (OP-C) as well as Oak Marsh creek and Whipple Road creek whereas Tides Condos pond

had significantly more MP per individual but only when compared to the reference tidal creek, PP-C.

Based on the high approximate daily traffic from traffic monitoring locations in proximity to the ponds and tidal creek sites (**Appendix A**), I hypothesized that Tides Condos pond (662,700 vehicles day^{-1}) would have the greatest number of MP per individual, especially suspected tire particles. However, this hypothesis was not supported in the present study, and instead, Whipple Road creek was found to contain the greatest amount of MP per individual collected. Whipple Road sites also experienced contributions from roadway pollution and although the area has lower daily traffic counts (11,500 vehicles day^{-1}) compared to other sites sampled, this site was directly adjacent to a major throughfare street, Whipple Road. Liu et al. (2019) evaluated MP abundance in the water phase from stormwater ponds that drained different land use areas including residential, industrial, commercial, and highway areas and found a greater abundance of MPs in stormwater ponds that drained industrial and commercial land areas compared to ponds serving highway and residential areas; however, their analysis did not include car tire rubber which would be a major contributor to stormwater MP in ponds draining highway runoff.

The lower MP collected per individual from Tides Condos pond may be due to the species heterogeneity observed at the Tides Condos pond where only one species (mosquitofish) was collected and analyzed. This may have led to an inaccurate characterization of the abundance of MP in biota from the pond because the sample was not representative of the entire pond population. Data for MP abundance in biota from

Whipple Road pond was absent for the present study but based on observations from Kell (2020) and others regarding MP abundance in sediments of stormwater ponds and discharge points, I suggest that the high abundance of MP in Whipple Road creek is due to a combination of site-specific attributes that allow for discharge of MP into the adjacent tidal creek of this pond. The MTD associated with the Whipple Road site serviced a large watershed catchment that included several neighborhoods, a large church parking lot, and the two-lane throughfare street, Whipple Road. Additionally, the MTD at this site was flawed in that it experienced back flow of water into the MTD during large storm events or very high tides. The MTD's outflow pipe was flush with the creek, allowing for a more direct connection between the pond and adjacent tidal creek.

The other two tidal creek sites sampled with relatively high abundances of MP were Oyster Point and Oak Mash creek. Both Oyster Point locations were representative of new (< 10 years old) residential areas. Surprisingly, Oyster Point pond contained less MP per individual compared to Oyster Point creek. Species diversity and heterogeneity of samples may have influenced MP abundances between these two sites where the sample from Oyster Point pond had bass, silversides, and sunfish and the sample from Oyster Point creek only had mullet. Differences in foraging behavior or preferred habitat between species collected may partially explain the differences in average MPs collected from the two sites. Juvenile bass, silversides, and sunfish are omnivorous feeders throughout the water column whereas mullet are primarily detritivores that feed closer to the bottom (Antonucci et al., 2014; Bester, 2017; Carlander, 1977; Miranda & Pugh, 1997). McNeish et al. (2018) argued that species traits can help explain microplastic

abundance and these are species dependent. They found that MP abundance was positively related to fish trophic fraction where zoobenthivores had greater MP abundance compared to omnivores.

Oak Marsh creek had the third highest average MP per individual of all sites sampled. Oak Marsh sites drained residential and highway areas, with daily traffic from traffic monitoring locations in proximity to the Oak Marsh sites estimated at 49,000 vehicles daily, which likely included airborne particulate contamination from the major highway, Intestate-526, adjacent to the Oak Marsh sites. Therefore, I hypothesized there would be a high amount of TP at the Oak Marsh sites, which I observed (79% of all MP at OM-C). Data on the abundance of MP in biota from Oak Marsh pond was unavailable for comparison. Species collected at Oak Marsh creek included grass shrimp, mummichogs, and pinfish and represented benthic omnivores, epibenthic omnivores, and carnivorous species, respectively (Abraham, 1985; Feinstein, 1975; Odum & Heald, 1972). The observed positive correlation between biota weight and MP abundance may also explain the high abundance of MP in biota from Oak Marsh creek as mummichogs were the second-largest species collected (behind crawdads) at 4.97 ± 2.15 g and pinfish were also one of the larger species collected at 1.57 ± 0.14 g. Oak Marsh creek specimens were also collected during a king tide with exceptionally high-water levels.. It is possible that the tidal influence caused resuspension of MPs from the pond and creek sediments which could have contributed to the overall greater MP per individual observed at this site.

As for species differences in MP abundance, there were significant differences in total MP per individual among species collected. Crawdads had the greatest MP per individual with an average of 71 MP per individual. Crawdads represent one of the only benthic organisms collected in this study. A greater number of MPs were expected from bottom-dwelling organisms that interact directly with sediment that may contain settled particles. In comparison, grass shrimp which are epibenthic feeders but also feed in floating vegetation, contained significantly less MP per individual (3.4 ± 3.1) than crawdads, but this difference may be attributed to the smaller size of grass shrimp and differences in size of natural food items typically ingested by each species.

As for fishes, there was a wide variability in MP abundance. As previously stated, size, trophic position, feeding strategy, or habitat may influence the distribution of MPs among species. Park et al. (2020a) found more MP per individual in bottom dwelling omnivorous fish (i.e. carp) compared to epibenthic and pelagic omnivorous and carnivorous fish (minnow and bass, respectively). While differences were observed between species in the present study, there were no clear relationships between MP per individual and species, likely due to a combination of site-specific availability of MP, organism size, and feeding habitat influence the total MP observed.

4.6 Conclusions

Stormwater ponds can function as effective stormwater runoff best management practices and trap or remove both physical and chemical pollutants before further discharge into receiving natural waterbodies. Stormwater ponds can therefore accumulate high levels of pollutants, such as microplastics and microrubber from road runoff. It was

hypothesized that biota in stormwater ponds in proximity to roadways or those that receive large road runoff would contain high amounts of MP per individual, with high abundances of TP. Indeed, the data indicated that the majority of MP recovered from biota across all sites were suspected TP.

There were significant differences in MP per individual observed between sites and between species. It seemed that a combination of factors such as availability of MP, organism size, and feeding habitat or behavior influenced the total MP observed. Indeed, I observed a significant positive correlation between MP per individual and organism weight, with larger individuals typically containing more MP. It appeared that species-specific feeding habits influence the total MP observed. For example, for benthic and sediment-dwelling organisms, sediments may contain more MP that were available for ingestion compared to pelagic dwelling organisms, although examining the detailed niche of each species in relation to MP abundance was not within the scope of the present study.

Comparisons between pond and adjacent tidal creek sites were somewhat inconclusive. There were only two locations in which both the pond and the tidal creek were sampled. Of these two, one was the reference site (Patriots Point) which had no significant difference between total MP per individual between the pond and creek. Additionally, MP abundance from Patriots Point creek was significantly lower compared to all other sites (except Patriots Point pond), making it a good reference site for MP abundance in biota. For the other sites, I hypothesized that there would be more MP, specifically more TP, in biota from pond sites compared to creek sites. The results are

inconclusive due to the small sample size and inability to collect organisms from all ponds and all tidal creeks. Completing the dataset by capturing biota from all ponds and all tidal creeks and analyzing sediment and water column samples for MPs will assist with making more accurate comparisons between the two and determining the efficiencies of stormwater ponds in retaining MP. Future work should attempt to collect and analyze the same species from both pond and tidal creeks or species with similar feeding mechanisms and habitat preferences for better comparability.

A major limitation of this study was in the identification of MP, specifically suspected tire wear particles. Additional analysis of a subset of MP and suspected TP using advanced spectroscopy such as micro-attenuated total reflectance (ATR) Fourier transform infrared (FTIR) spectroscopy for MPs (< 500µm) or scanning electron microscopy (SEM) for TPs would be beneficial to confirm suspected tire wear particle identity. Tire particles can be analyzed by ATR-FTIR and µATR-FTIR methods but the presence of filler materials such as carbon black confound analytical results and interfere with spectral signatures of TP (Leads & Weinstein, 2019). Instead, SEM and energy dispersive X-ray spectroscopy (EDX) or pyrolysis-GC/MS have been suggested as better methods for identifying suspected TP and can provide more accurate characterization of morphological properties in addition to chemical analysis of TP (Sommer et al., 2018; Unice et al., 2012).

4.7 Figures

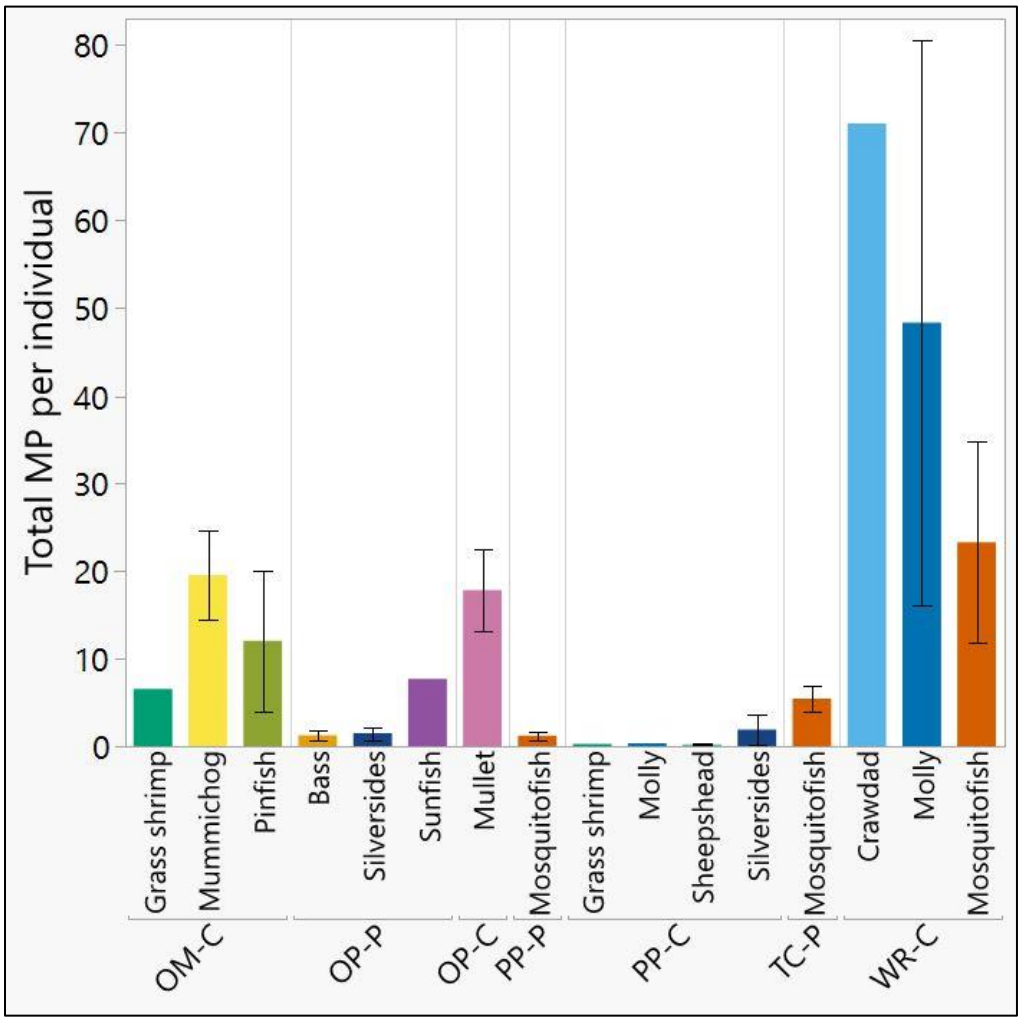

Figure 4.1 – Average total microplastics per individual by site and species. Total microplastics includes fibers, fragments, and suspected tire particles. Whiskers indicate standard error. The absence of whiskers indicates samples where standard error could not be calculated due to small sample size. OM-C is Oak Marsh creek, OP-P is Oyster Point pond, OP-C is Oyster Point creek, PP-P is Patriots Point pond, PP-C is Patriots Point creek, TC-P is Tides Condos pond, and WR-C is Whipple Road creek.

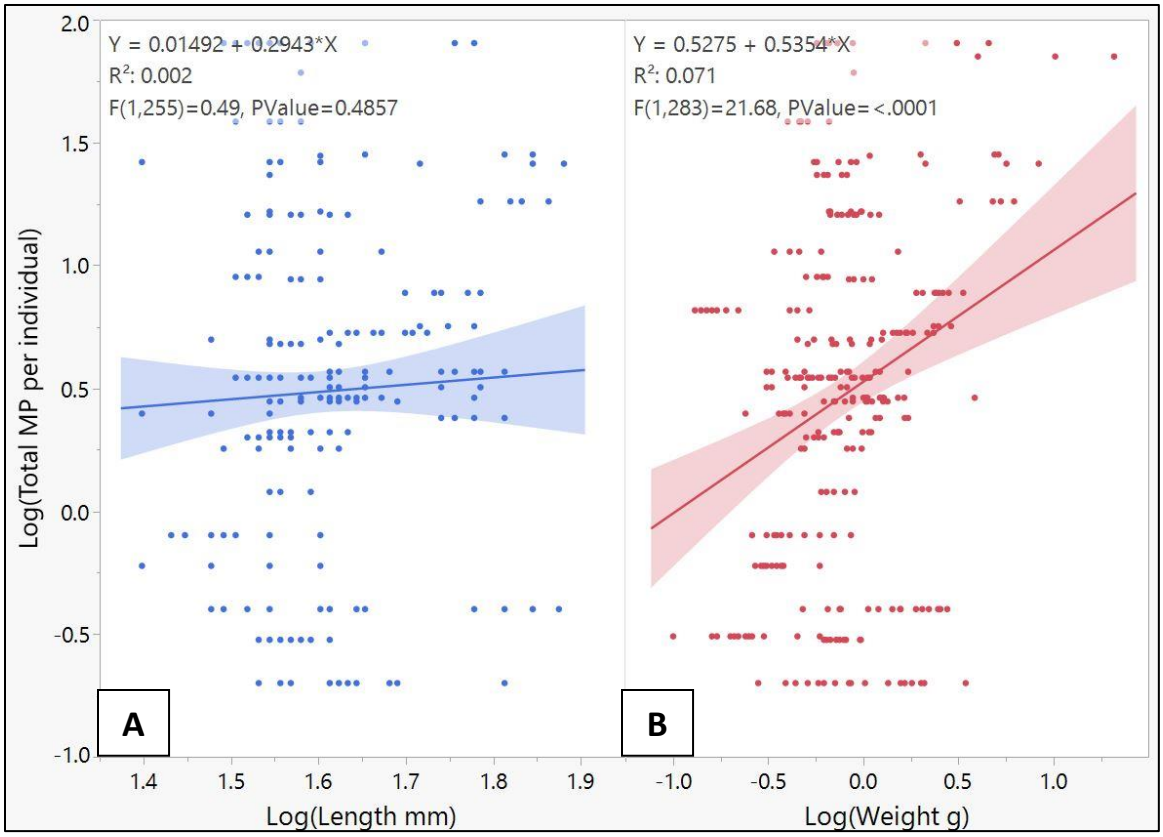

Figure 4.2 – Length in mm (A) and weight in g (B) of individual organisms and average microplastics (including fibers, suspected tire particles, and fragments) per individual. The shaded region represents the 95% confidence interval.

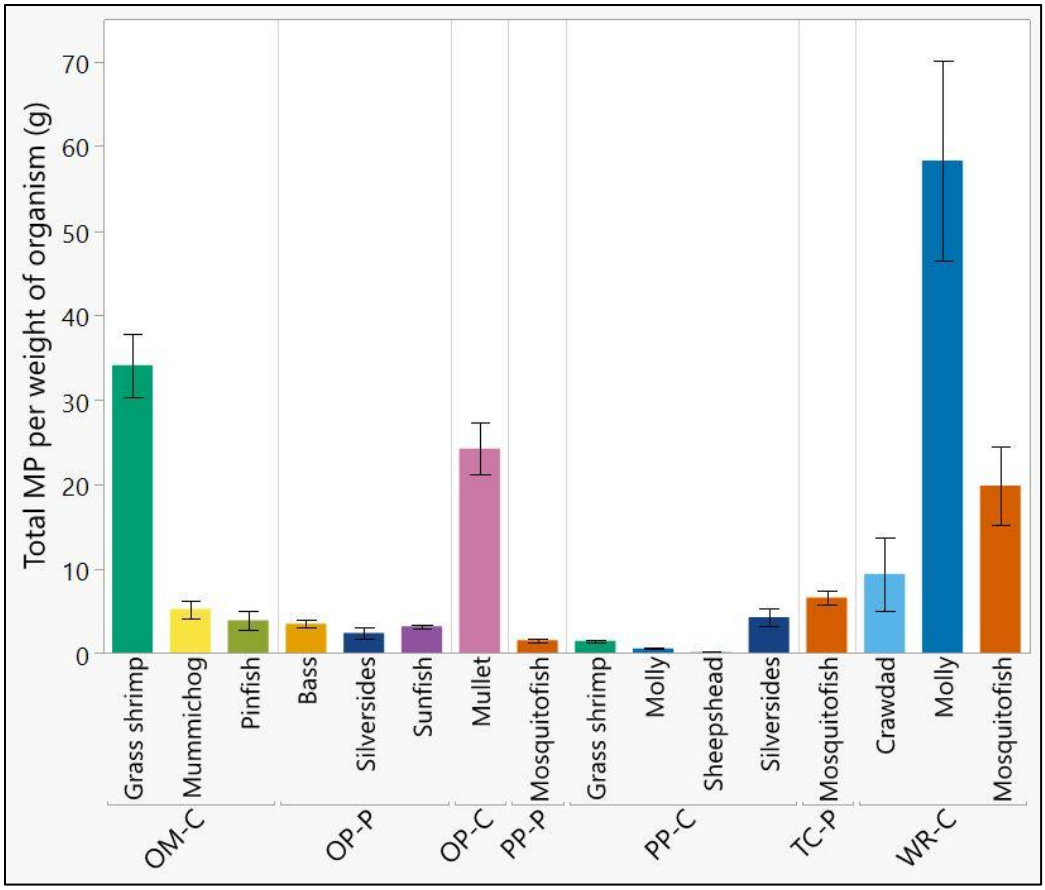

Figure 4.3 – Average total microplastics by weight (gram) of organism by site and species. Total microplastics includes fibers, fragments, and suspected tire particles. Whiskers indicate standard error. The absence of whiskers indicates samples where standard error could not be calculated due to small sample size. OM-C is Oak Marsh creek, OP-P is Oyster Point pond, OP-C is Oyster Point creek, PP-P is Patriots Point pond, PP-C is Patriots Point creek, TC-P is Tides Condos pond, and WR-C is Whipple Road creek.

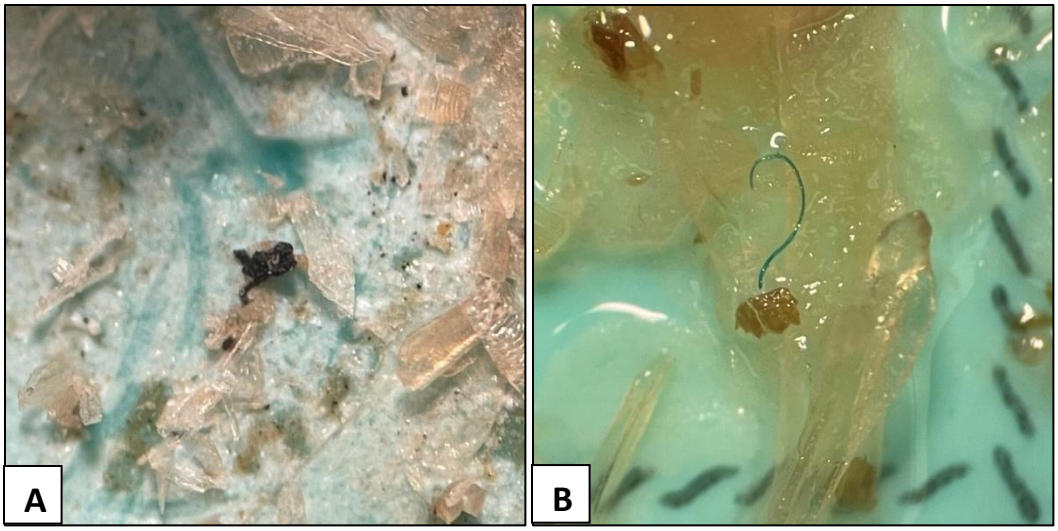

Figure 4.4 – Microplastic types in biota from stormwater pond and adjacent tidal creek sites. (A) A suspected tire particle and (B) a blue fiber.

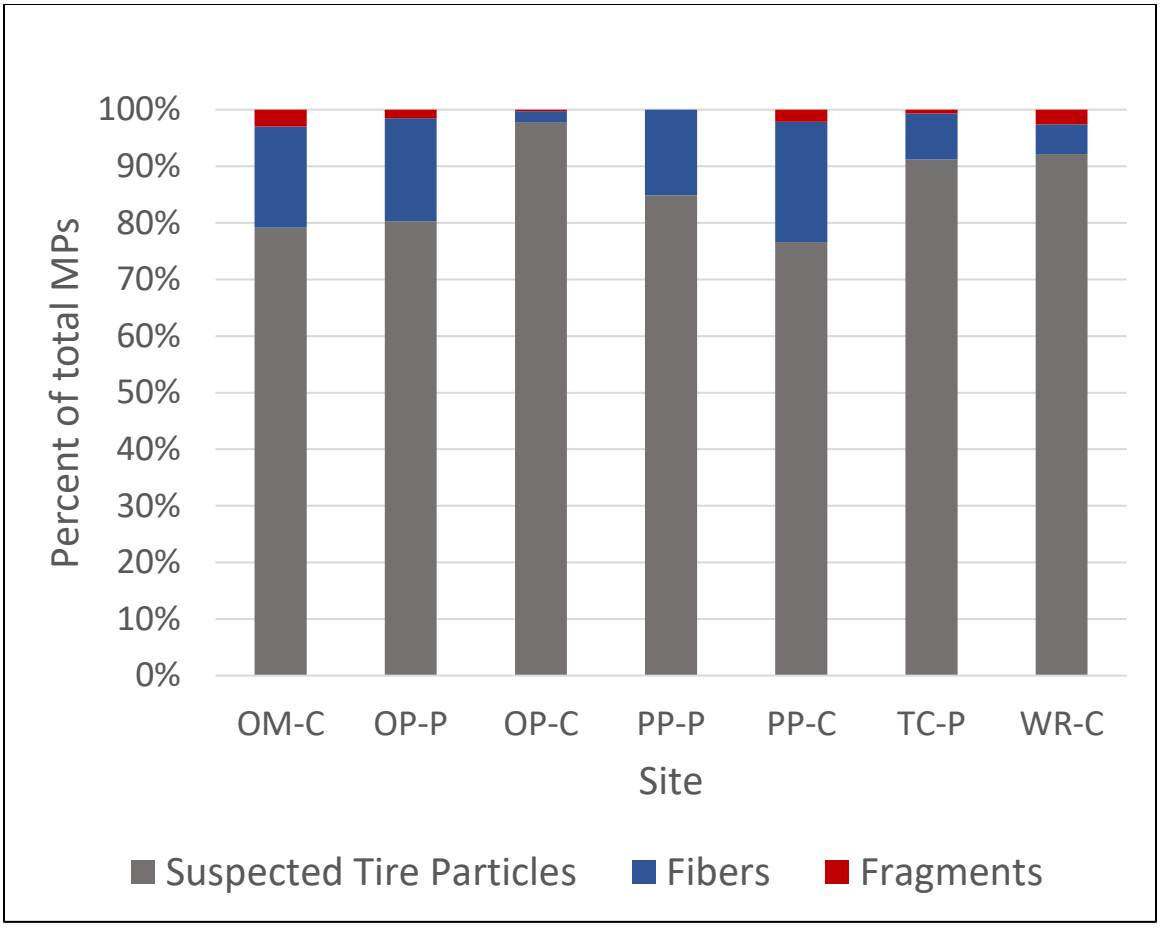

Figure 4.5 – Distribution of microplastic types in organisms collected from stormwater ponds and adjacent tidal creek sites. OM-C is Oak Marsh creek, OP-P is Oyster Point pond, OP-C is Oyster Point creek, PP-P is Patriots Point pond, PP-C is Patriots Point creek, TC-P is Tides Condos pond, and WR-C is Whipple Road creek.

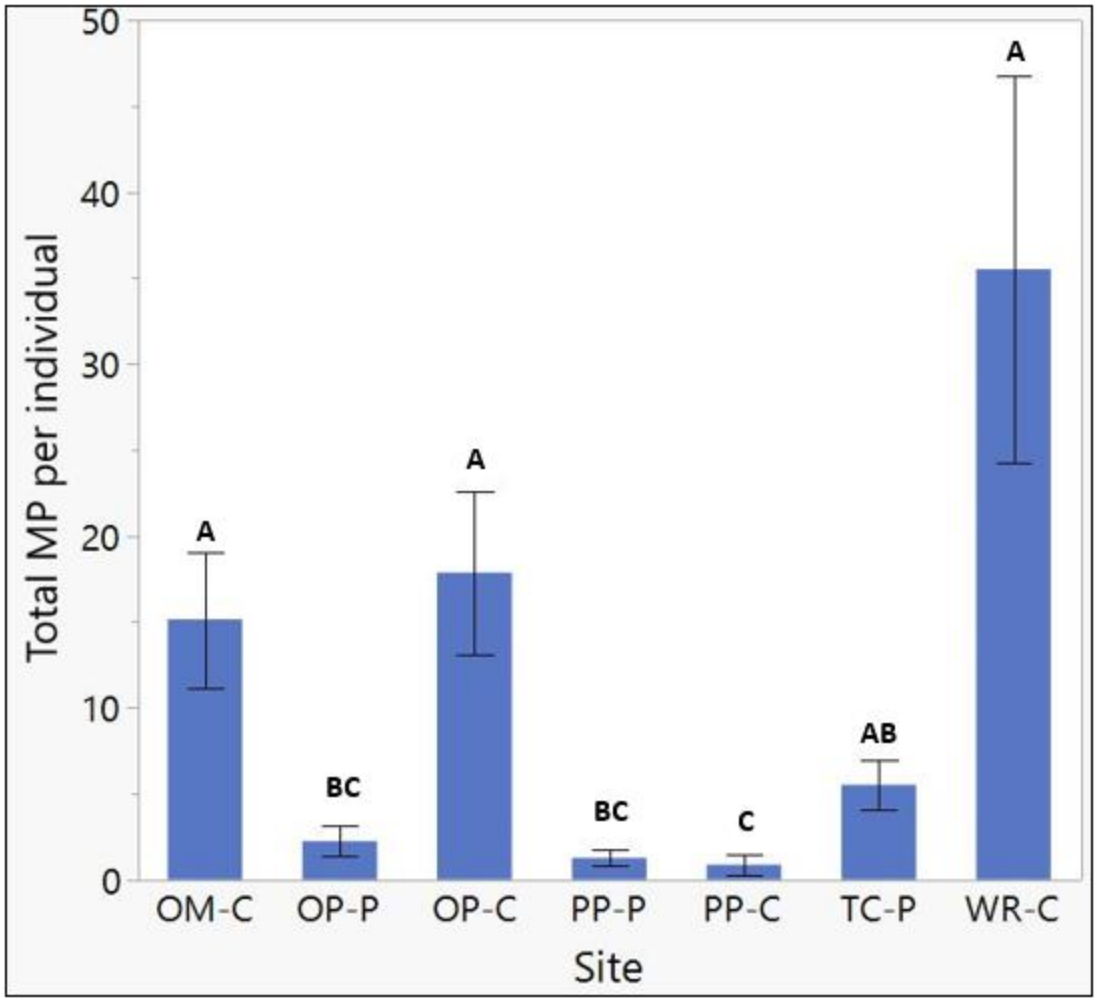

Figure 4.6 – Average total microplastics (fibers, fragments, suspected tire particles) per individual collected from stormwater ponds and adjacent tidal creek sites. Whiskers indicate standard error. Different letters indicate significant difference between sites ($p < 0.05$). OM-C is Oak Marsh creek, OP-P is Oyster Point pond, OP-C is Oyster Point creek, PP-P is Patriots Point pond, PP-C is Patriots Point creek, TC-P is Tides Condos pond, and WR-C is Whipple Road creek.

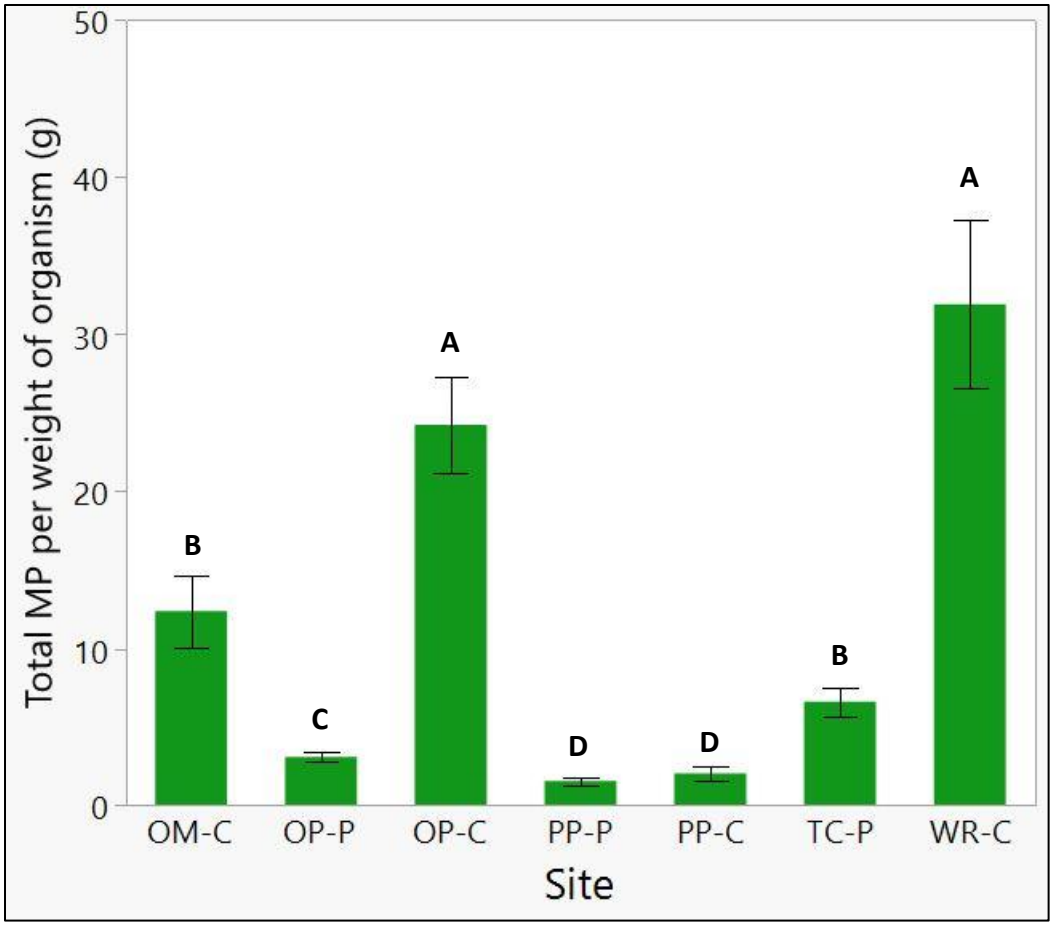

Figure 4.7 – Average total microplastics (fibers, fragments, suspected tire particles) by weight (g) in organisms collected from stormwater ponds and adjacent tidal creek sites. Whiskers indicate standard error. Different letters indicate significant difference between sites ($p < 0.05$). OM-C is Oak Marsh creek, OP-P is Oyster Point pond, OP-C is Oyster Point creek, PP-P is Patriots Point pond, PP-C is Patriots Point creek, TC-P is Tides Condos pond, and WR-C is Whipple Road creek.

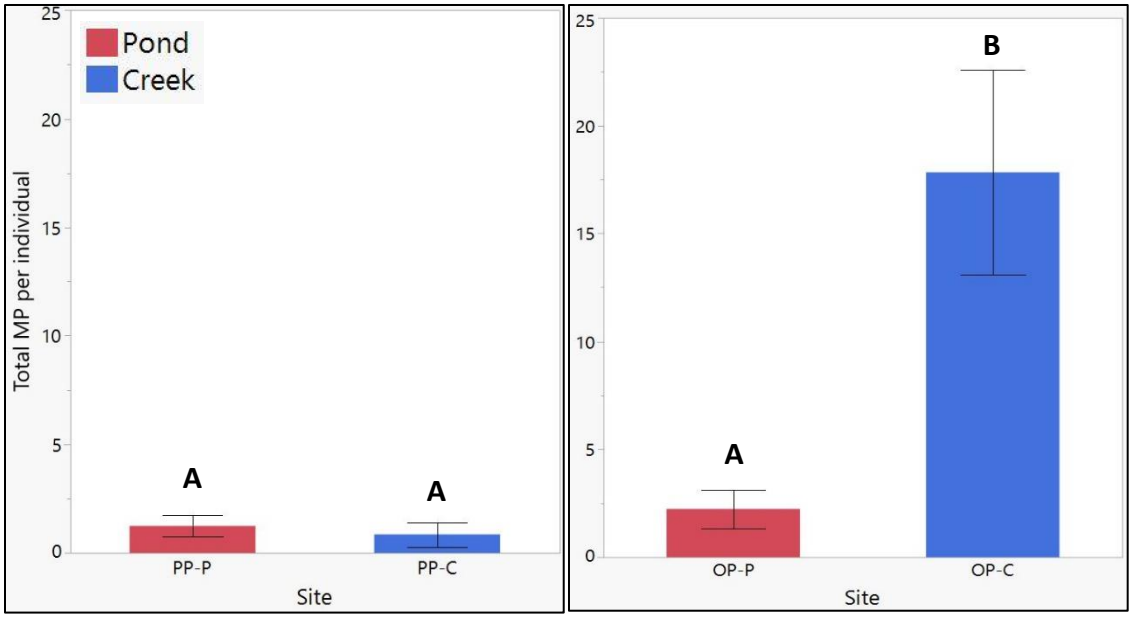

Figure 4.8 – Average total microplastics (fibers, fragments, suspected tire particles) per individual collected from stormwater ponds (P) and adjacent tidal creek (C) sites for Patriots Point (PP) and Oyster Point (OP) locations. Whiskers indicate standard error. Different letters indicate significant difference between sites ($p < 0.05$).

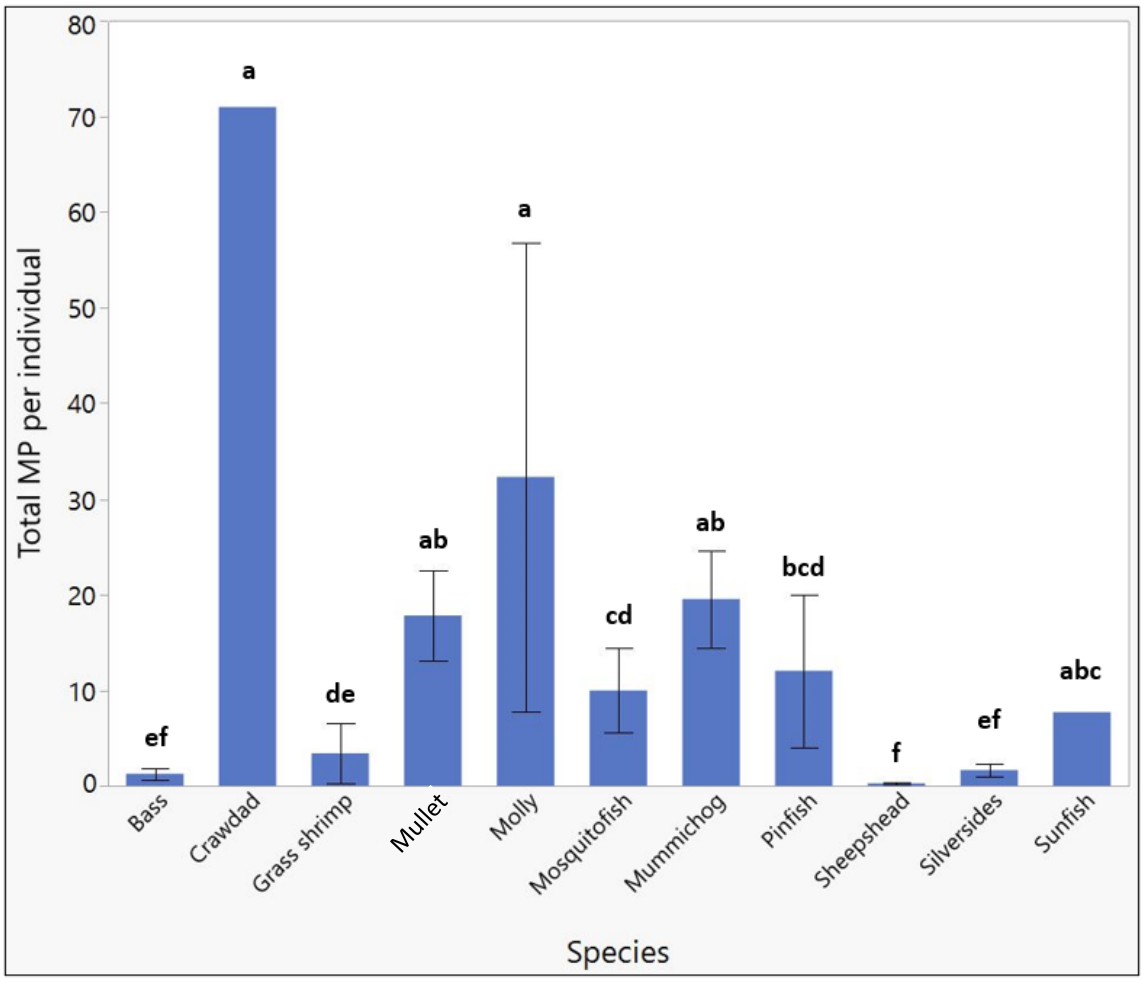

Figure 4.9 – Average total microplastics per individual collected from stormwater ponds and adjacent tidal creek sites for each species collected. Whiskers indicate standard error. The absence of whiskers indicates samples where standard error could not be calculated due to small sample size. Different letters indicate significant difference between species ($p < 0.05$).

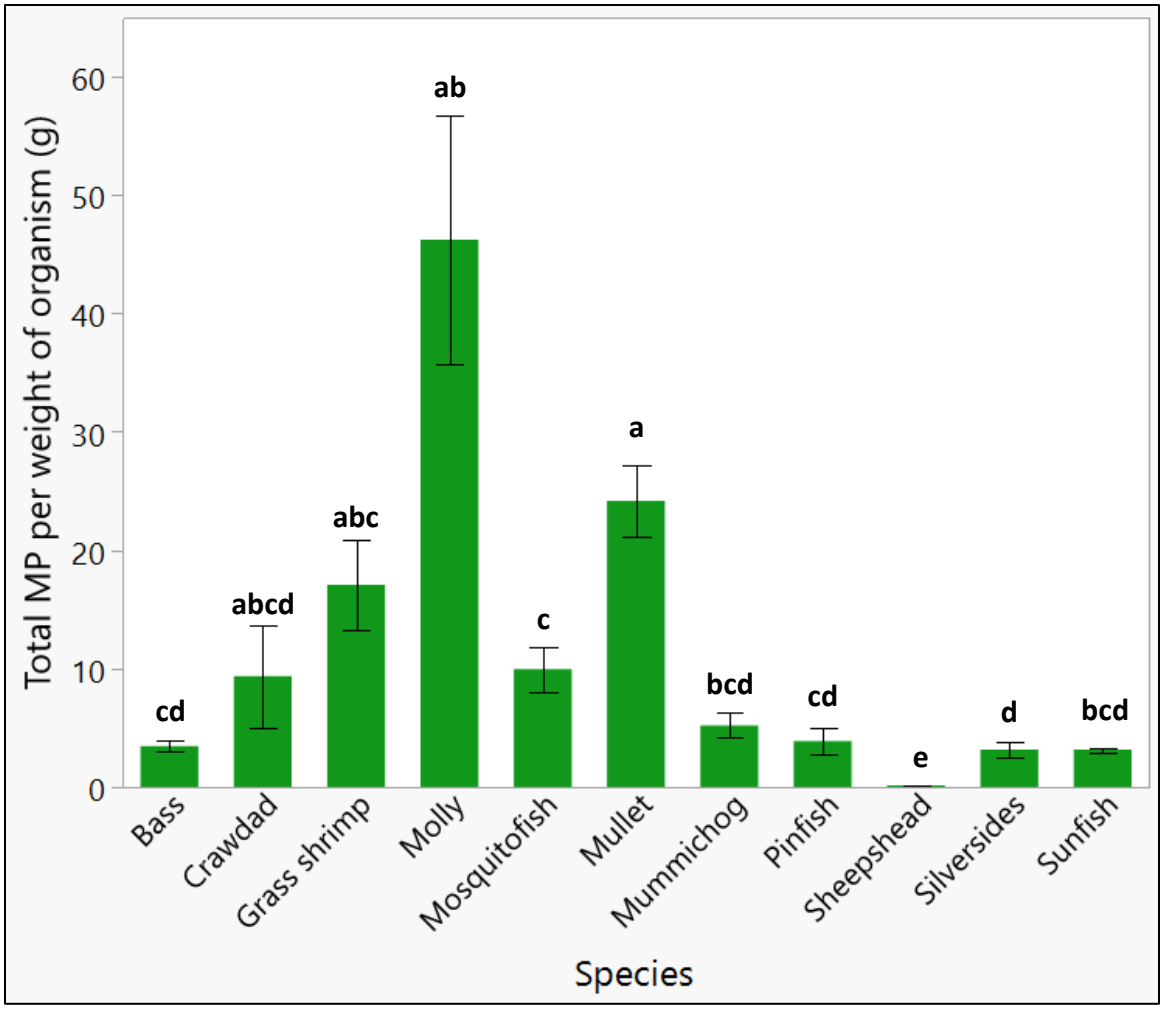

Figure 4.10 – Average total microplastics by weight (g) of organisms collected from stormwater ponds and adjacent tidal creek sites for each species collected. Whiskers indicate standard error. Different letters indicate significant difference between species ($p < 0.05$).

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CHAPTER FIVE

CONCLUSIONS

The emergence of microrubber as a frequently encountered microplastic in aquatic systems is a relatively new breakthrough in microplastic research. Recent studies on microrubber particles have focused primarily on their generation, properties, occurrence, and detection in the environment (Kole et al., 2017; Wagner et al., 2018). Most toxicity studies thus far have investigated toxicity of microrubber leachates or whole tires in aquatic organisms (Hartwell et al., 1998; Panko et al., 2013) and there is limited information on toxicity of MR particles themselves to aquatic organisms. This dissertation fills gaps in microrubber research through identification of specific toxic responses of organisms to crumb rubber particulate exposure. Acute and chronic laboratory toxicity tests were conducted using *P. promelas* (acute) and *F. heteroclitus* (acute and chronic) and field-sampling of aquatic biota from stormwater ponds and tidal creeks was performed to determine tire particle abundance in the environment.

The first objective for my dissertation was to understand the acute toxicological effects from exposure to CR particles by determining whether exposed fish were ingesting CR particles and measuring the resulting toxicity through biomarker analysis. Three 7-d static renewal exposures were conducted for both *P. promelas* and *F. heteroclitus* at CR concentrations up to 6 g/L. The results indicated that both species did indeed ingest CR particles as particles were visually observed in the intestinal tract of exposed organisms. Biomarker analysis showed an increase in bile fluorescence as the concentration of CR increased, especially for 4-ring and 5-ring chemicals. These results

were corroborated with other studies showing that the 4-ring PAH pyrene was often detected at high levels in MR samples. While a number of compounds may be detected in CR particles and CR leachate, these data coupled with the upregulation of CYP1A activity according to the EROD assay demonstrate that AhR receptor agonists (such as PAHs) were a potential contaminant influencing the response observed in both species. It appeared that *P. promelas* was more sensitive to CR exposure as partial mortality was observed at 6 g/L whereas no mortality occurred in *F. heteroclitus*. Based on this, species sensitivities or environment (i.e., freshwater versus estuarine or saltwater) may influence the toxicity of MR where freshwater environments may be more susceptible to toxic effects in comparison to estuarine or saltwater environments possibly due to leaching mechanisms of contaminants associated with MR (Hartwell et al., 2000). The results from the acute toxicity tests indicate that overall, CR exerts a potentially adverse effect, possibly depending on species and environment, but concentrations of 6 g/L were environmentally unrealistic and exceed what would likely be found in the environment at any given time.

The second objective was to investigate toxicity under environmentally relevant conditions through a chronic, pulsed exposure. Two pulsed exposure experiments were conducted, the first focusing on immunohistochemistry (IHC) and CYP1A induction in various organs and the second focusing on biomarker responses related to effects and detoxification of accumulated chemicals and/or metabolites associated with CR. Biomarkers measured included: bile fluorescence which measured PAH absorption, metabolism and biliary excretion; DNA damage as measured through the formation of 8-

hydroxy-2'-deoxyguanosine (8-OHdG) and oxidative stress as measured through the TBARS assay, free glutathione (GSH), and oxidized glutathione (GSSG). I observed strong positive staining in IHC for CYP1A in the gill epithelium of exposed organisms, which was expected as the gills were in direct contact with both leachate and CR particles in the water column during exposures. Additionally, strong positive staining for CYP1A was detected in intestinal vasculature, liver hepatocytes, and liver vasculature and sinusoids. There was weak positive staining for CYP1A in the intestinal epithelium of exposed fish. Strong induction of CYP1A in intestinal vasculature but weaker staining in intestinal epithelium indicated that the majority of contaminants (i.e., PAHs) from crumb rubber are potentially being absorbed in a different part of the body, such as the gills from leachate, and being transported via the blood stream throughout the body. Although residence time of CR particles in the gut was not determined as part of the present study, the lack of strong CYP1A induction in intestinal epithelium may be attributed to a short residence time of CR particles in the gut. These data helped shed light on the differences between leachate and particle toxicity and understanding the site of toxicity between leachate and particle exposure under chronic conditions.

Bile fluorescence for 2-, 4-, and 5- ring compounds increased as CR concentration increased in chronic exposures, which agreed with observations from the initial acute toxicity tests. The other biomarkers measured indicated that antioxidant defenses were sufficiently functioning to prevent cellular damage. Measured 8-OHdG was significantly greater in exposed organisms indicating that DNA damage was occurring in exposed fish, but biochemical defenses to repair DNA damage were occurring. Lipid peroxidation was

significantly lower in exposed organisms indicating that antioxidant defense mechanisms were upregulated, and thus efficiently preventing adverse cellular damage and reduced the amount of lipid peroxidation byproducts formed. There was a significant increase in the antioxidant, GSH, and a significant decrease in its oxidized form, GSSG, as CR concentration increased. Additionally, the ratio of GSH:GSSG increased significantly as CR increased. GSH levels can be increased due to an adaptive mechanism to slight oxidative stress through an increase in GSH synthesis or it is possible that they were increased due to Nrf2 activation by exogenous stressors (i.e., CR).

Mortality from CR exposure was not observed in the chronic study. These data indicated that there was overall low toxicity from exposure to CR particles in *F. heteroclitus* under these chronic conditions, with mild responses at the cellular level. The third objective was to address the environmental impact and fate of MR by analyzing the abundance of MP and MR in biota collected from stormwater ponds and adjacent tidal creeks, relating laboratory observed toxicity to environmental observations. There was an average of 9.5 ± 6.5 MP per individual across all organisms and all sites sampled, which included 11 species of organisms and seven sampling locations. The majority (>80%) of total MPs analyzed were suspected tire wear particles (TP), highlighting the importance of stormwater ponds and their discharge points into tidal creeks as pathways and potential hot spots for MR in the environment. There were significant differences in MP abundance observed between sites and between species. It seemed that a combination of factors such as availability of MP, organism size, and feeding habitat influenced the total MP observed. Availability of MP can be influenced by local land use in the

surrounding drainage area, pond structure and hydrodynamics, and local climate patterns. I observed a significant positive correlation between MP per individual and organism weight, with larger individuals typically containing more MP which has been corroborated by others (Hurt et al., 2020; Parker et al., 2020; Peters & Bratton, 2016). It appears that species-specific feeding habits influenced the total MP observed but examining the detailed niche of each species in relation to MP abundance was not within the scope of the present study.

5.1 Future research needs

Critical research needs in microrubber research have been highlighted elsewhere in the literature (Halle et al., 2020). However, suggestions in a few key areas that arise from the results of this dissertation include standardization of toxicity tests involving MR; elucidation of exposure pathways in organisms including information on gut retention of MR particles; and investigation of MR effects at the population level in biota.

A. Standardization of toxicity tests involving MR.

There are a variety of studies on the toxicity of MR to different biota (e.g., Kolomijeca et al., 2020; Turner & Rice, 2010). However, direct comparison of the effects from MR are difficult to make due to differences in type of MR used, leachate preparation or particle preparation, concentrations tested, and species examined, among other things. The data presented in this dissertation helped fill gaps in MR toxicity studies by addressing sublethal endpoints such as biomarker responses for both acute and chronic toxicity tests with MR. Future studies assessing sublethal endpoints such as growth, behavior, or gene expression are needed for other organisms in different environmental

matrices where MR is found. I acknowledge that MR comprises a complex suite of potential contaminants; therefore, different tires or different MR sources may vary in chemical formulations; however, standardization of leachate or MR preparation methods, toxicity tests, or endpoints measured will provide better comparability across studies. General improvements in chemical characterization of compounds associated with MR leachate and particulates will also determine compounds responsible for observed toxicity in laboratory experiments.

B. Exposure pathways in organisms including information on gut retention of MR particles.

The immunohistochemistry results from objective 2 of this dissertation demonstrated that MR leachate toxicity rather than MR particle toxicity is a more relevant exposure pathway in aqueous environments for *F. heteroclitus*. Exposure pathways in other organisms should be addressed and considered when determining the overall toxicity of MR. Dickens (2021) found greater toxicity to MR particles in larval silversides (*Menidia beryllina*) compared to leachate toxicity, highlighting that differences in species and life history stage may influence exposure and thus toxicity. Data on gut retention of MR particles will also help determine whether particle ingestion is an important route of exposure in organisms.

C. Investigation of MR effects at the population level in biota.

The results from this dissertation demonstrated sublethal stress effects from exposure to MR under acute and chronic experiments. There is only one very recent study to my knowledge that addresses population-level effects from exposure to MR

(Tian et al., 2021). In this case, a highly toxic quinone transformation product of the tire rubber antioxidant *N*-(1,3-dimethylbutyl)-*N'*-phenyl-p-phenylenediamine (6PPD) was identified and associated with mortality of adult salmon exposed to stormwater runoff in the U.S. Pacific Northwest. This study is one of the first to demonstrate potential environmental population-level effects from exposure to MR compounds suggesting that the toxic 6PPD-quinone, a reaction product from tire materials, was responsible for the observed mortality. It is unknown whether other species have similar sensitivities to this compound from rubber products, but initial studies have found that 6PPD-quinone did not exhibit acute lethal toxicity to *Danio rerio, Oryzias latipes, Daphnia magna,* or *Hyalella azteca* (Hiki et al., 2021). While there are a number of studies in different organisms evaluating different responses to MR particle and leachates, an overall risk assessment for MR has not been established.

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APPENDICES

Appendix A

Stormwater Pond Sampling Sites, Mt. Pleasant, SC, USA

Location	Latitude	Longitude	Type	Receiving Waterbody	Approx. avg daily traffic (# vehicles)[a]
Patriots Point Links (PP)	32°47'26.64"N	79°53'33.56"W	Reference, golf course	Branch of Shem Creek	225
Whipple Road (WR)	32°50'05.86"N	79°50'44.60"W	Residential	Branch of Hobcaw Creek	11,500
Oak Marsh Drive (OM)	32°49'53.27"N	79°51'15.90"W	Residential/Highway	Hobcaw Creek	49,000
Oyster Point (OP)	32°49'18.41"N	79°48'07.29"W	Residential (new)	Branch off Gray Bay	12,400
Tides Condos (TC)	32°48'09.03"N	79°54'02.33"W	Commercial/High Density Residential	Branch of Cooper River	62,700

[a] Approximate average daily traffic (# vehicles) data from South Carolina Department of Transportation (SC DOT) Traffic Counts (2020).

Table A-1: Site descriptions of stormwater pond sampling locations in Mt. Pleasant, SC, USA.

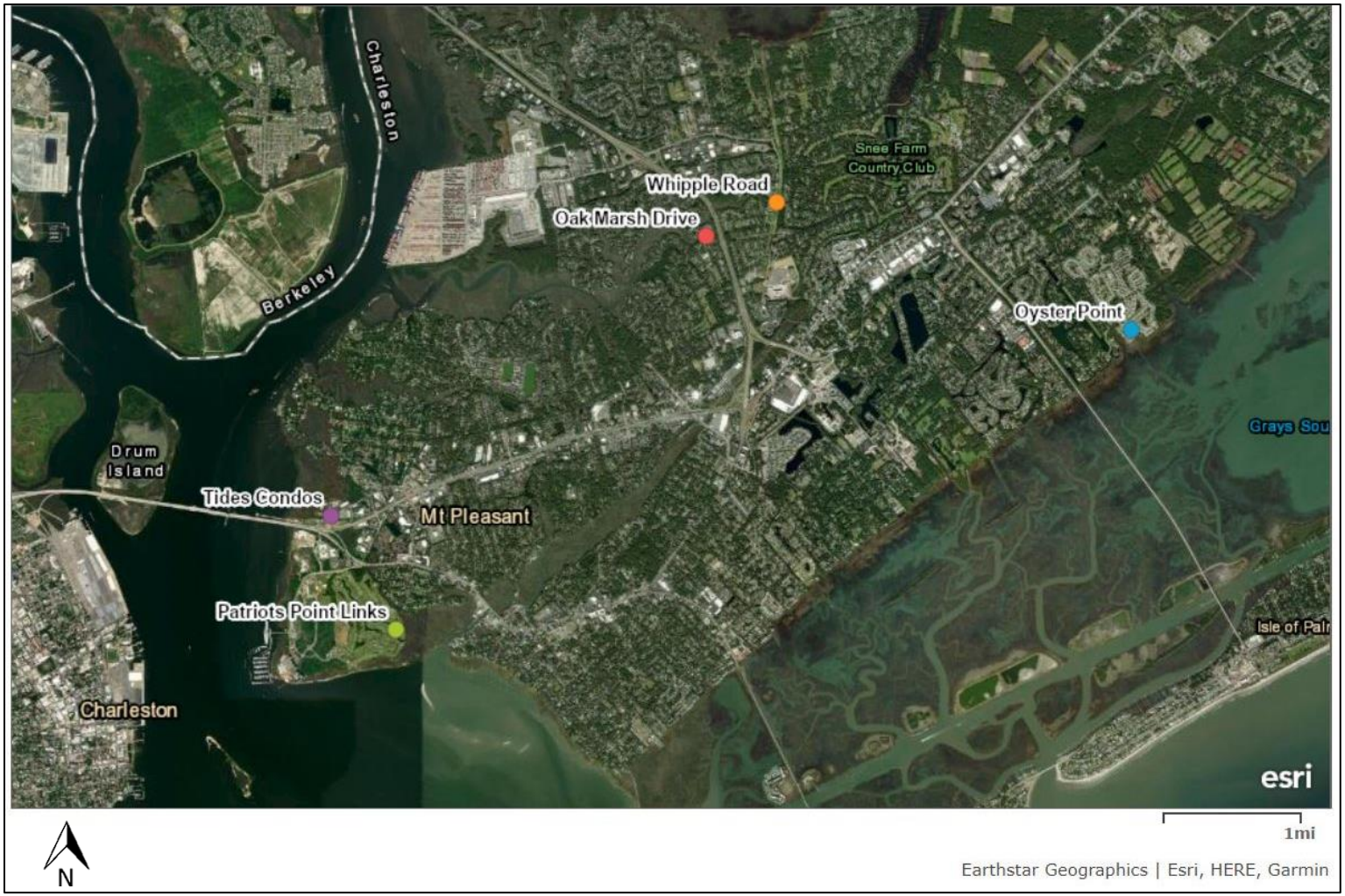

Figure A-1: Overview of stormwater pond sampling locations in Mt. Pleasant, SC, USA.

Figure A-2: Patriots Point Links (PP) pond and adjacent tidal creek. Star indicates adjacent tidal creek.

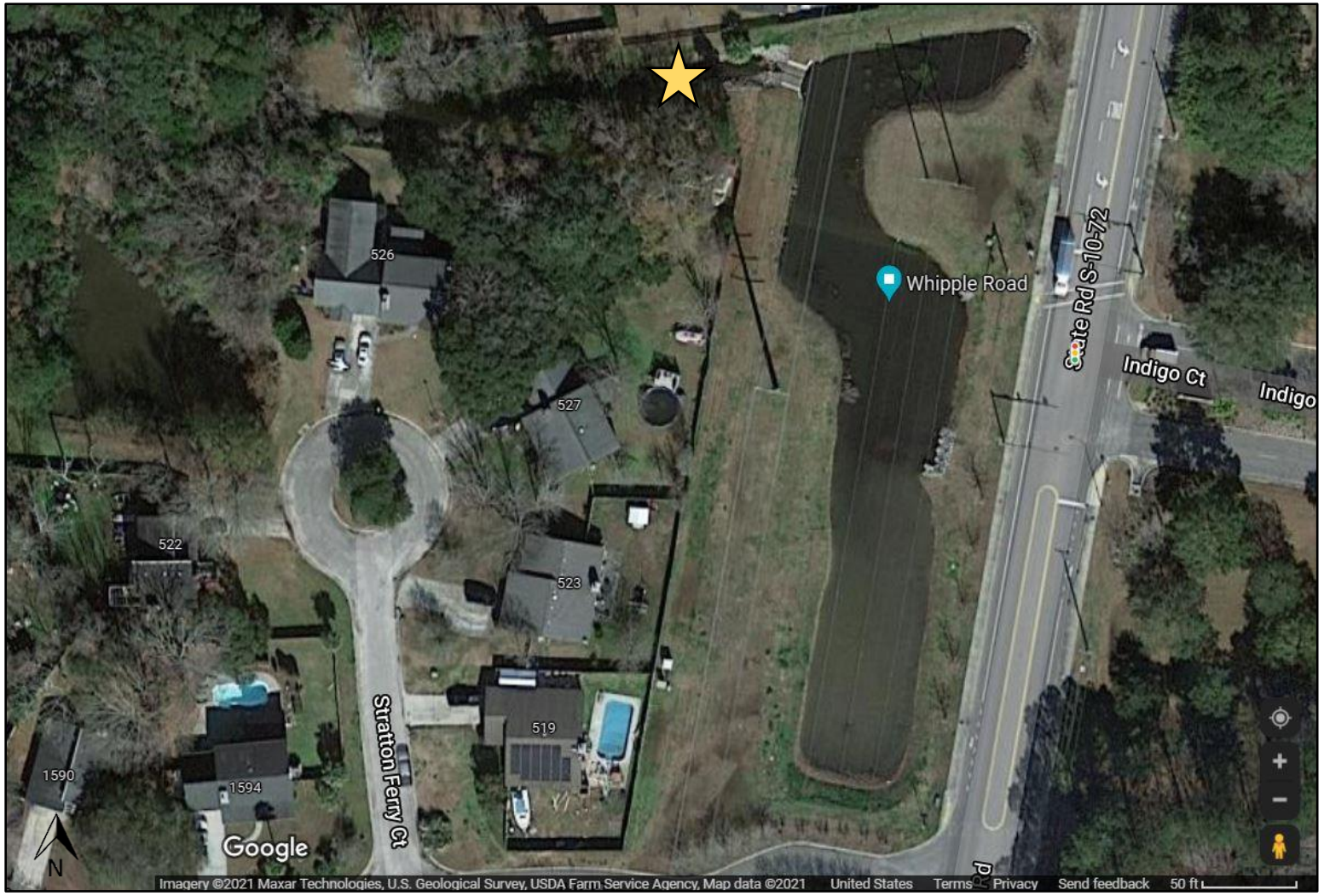

Figure A-3: Whipple Road (WR) pond and adjacent tidal creek. Star indicates adjacent tidal creek.

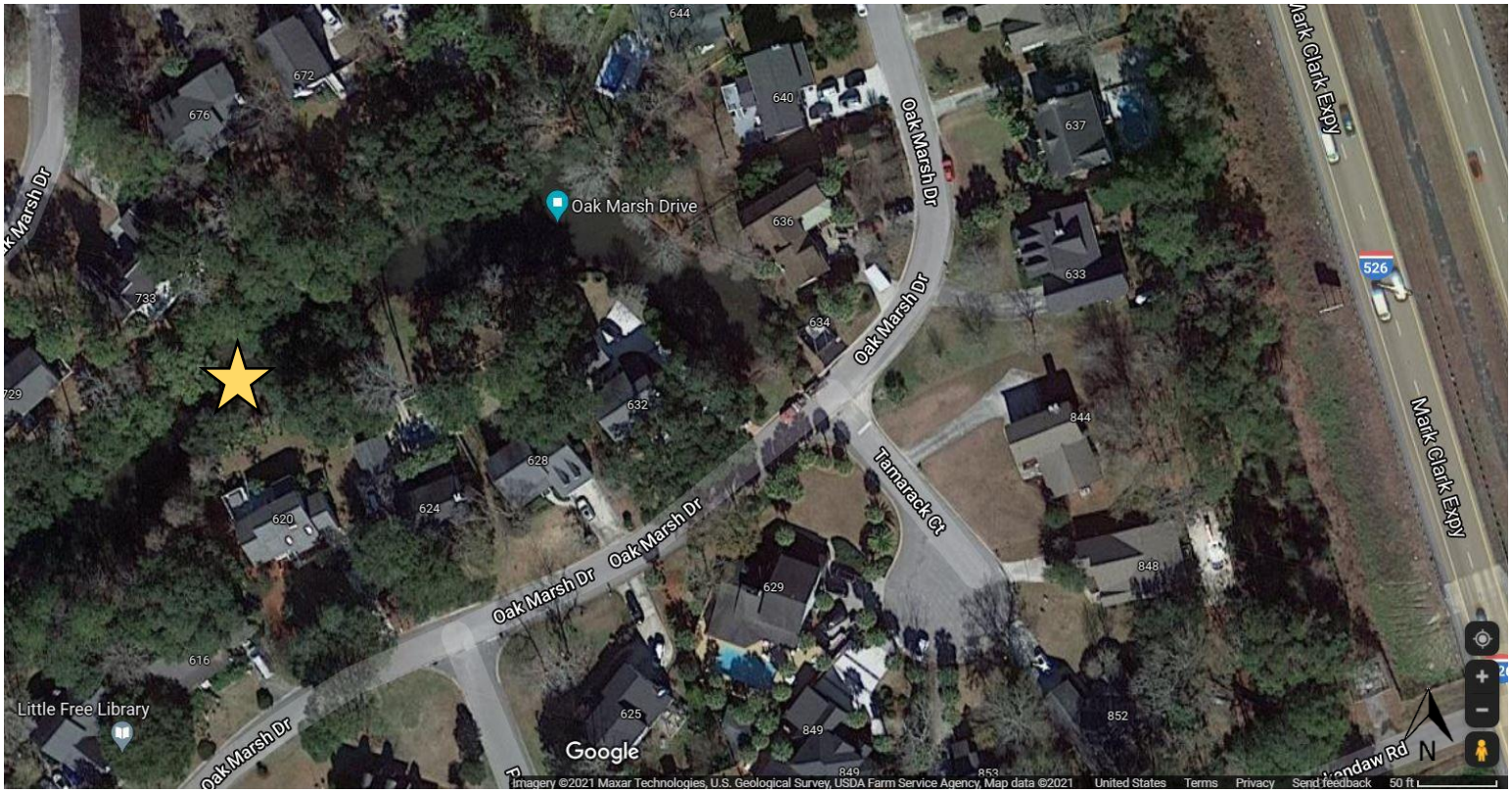

Figure A-4: Oak Marsh Drive (OM) pond and adjacent tidal creek. Star indicates adjacent tidal creek.

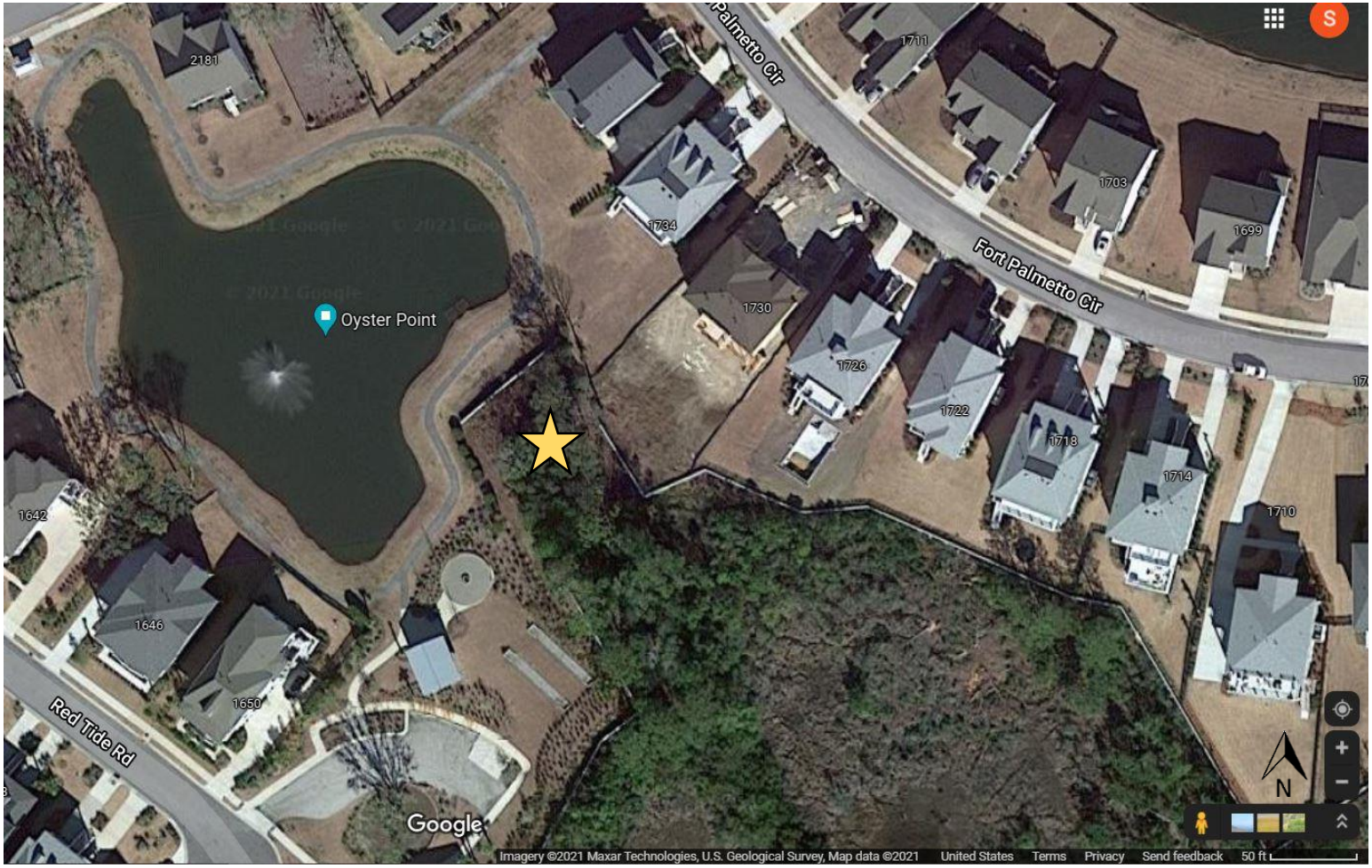

Figure A-5: Oyster Point (OP) pond and adjacent tidal creek. Star indicates adjacent tidal creek.

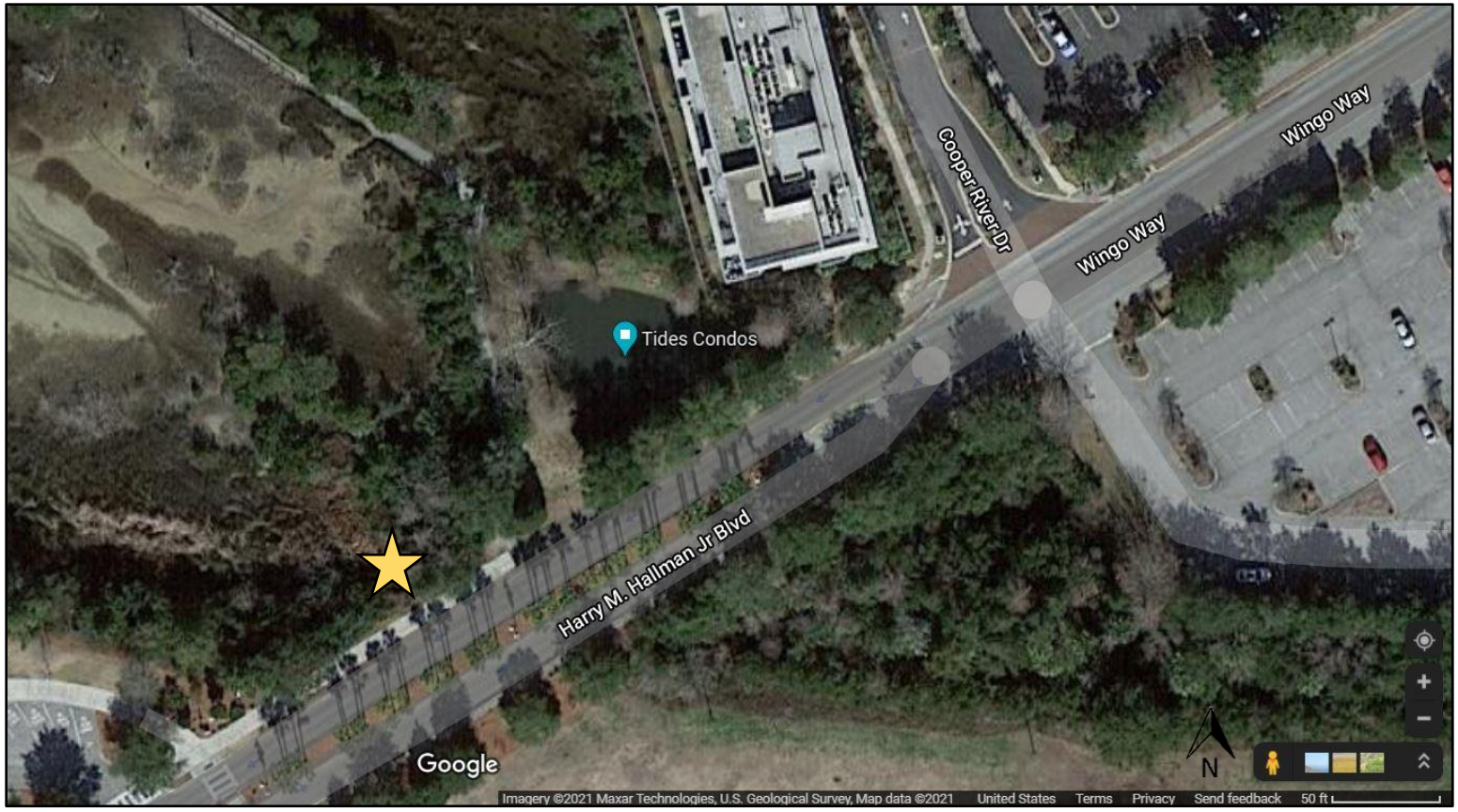

Figure A-6: Tides Condos (TC) pond and adjacent tidal creek. Star indicates adjacent tidal creek.

Appendix B

Species information from organisms collected from stormwater ponds

Species	Common name	Family	n	Feeding type	Feeding habitat	Main food	Wet weight (g)	Standard length (mm)
Micropterus salmoides	Bass	Centrarchidae	30	Omnivorous[*]	Surface, mid-water, epibenthic	Zooplankton, aquatic insects[a*]	0.40 ± 0.02	31.0 ± 0.58
Procambarus troglodytes	Crawdad	Cambaridae	3	Omnivorous, detritivores	Benthic, epibenthic	Aquatic vegetation, insect larvae, small fishes, detritus[b]	11.6 ± 4.0	n/a
Palaemonetes pugio	Grass shrimp	Palaemonidae	25	Omnivorous, detritivores	Benthic, epibenthic	Detritus, microalgae, mysids, nematodes[c]	0.25 ± 0.02	n/a
Mugil cephalus	Mullet	Mugildae	21	Detrivores	Benthic	Detritus, algae, microcrustaceans[d]	0.79 ± 0.04	36.4 ± 0.74
Poecilia latipinna	Molly	Poeciliidae	24	Planktivorous, Omnivorous	Surface, mid-water, epibenthic	Algae, plant material, aquatic insects[e]	1.05 ± 0.16	37.5 ± 1.32
Gambusia holbrooki	Mosquitofish	Poeciliidae	96	Omnivorous	Surface	Mosquito larvae, aquatic insects, plant material[f]	0.73 ± 0.02	36.9 ± 0.36
Fundulus heteroclitus	Mummichog	Fundulidae	13	Omnivorous	Surface, mid-water, epibenthic	Plant material, crustaceans, shrimps, insects, fish[g]	4.97 ± 2.15	65.4 ± 2.15
Lagodon rhomboides	Pinfish	Sparidae	12	Carnivorous[*]	Epibenthic	Shrimp, fish eggs, insect larvae, polychaete worms, amphipods[h]	1.57 ± 0.14	45.2 ± 1.07
Cyprinodon variegatus	Sheepshead minnow	Cyprinodontidae	10	Omnivorous, detritivore	Epibenthic	Shrimp, aquatic insects, detritus[i]	2.06 ± 0.22	41.4 ± 1.59

Continued								
Species	**Common name**	**Family**	**n**	**Feeding type**	**Feeding habitat**	**Main food**	**Wet weight (g)**	**Standard length (mm)**
Menidia menidia	Silversides	Atherinopsidae	40	Omnivorous	Pelagic	Algae, invertebrates, zooplankton, insects[j]	1.06 ± 0.14	56.15 ± 2.21
Lepomis spp.	Sunfish	Centrarchidae	8	Omnivorous	Epibenthic	Aquatic insects, zooplankton, plant material[k]	2.50 ± 0.14	54.87 ± 1.26

* Applies for juveniles

[a] Miranda & Pugh, 1997; [b] Skelton, 2012;[c] Odum & Heald, 1972; [d] Bester, 2017; [e] Rohde et al., 1994;[f] Rohde et al., 2009;[g] Abraham, 1985; [h] Feinstein, 1975; [i] Robertson & Van Tassell, 2019; [j] Antonucci et al., 2014; [k] Carlander, 1977

Figure B-1: Species, common name, family, total number of processed organisms (n) per species, feeding type, feeding habitat, food source. Arithmetic mean values (± standard error (SE)) for wet weight (g) and standard length (mm) for each species are shown. References listed can be found in section 4.8.