TOXICOLOGICAL EFFECTS OF MICRONIZED TIRE CRUMB RUBBER ON FATHEAD MINNOW (*Pimephales promelas)* AND MUMMICHOG (*Fundulus heteroclitus).*

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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 are 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 fathead minnow (*Pimephales promelas*) and mummichogs (*Fundulus heteroclitus*) 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 was observed in the fathead minnow, which is came close to the assumed LC_{50}. To investigate if polynuclear 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 tire particles,, and this elevated EROD activity indicated that PAH compounds from the crumb rubber particles were being metabolized in both fathead minnow and mummichogs.

Keywords: tyre/tire wear, PAHs , CYP1A, bile fluorescence, microplastics, microrubber

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 and 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 and 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, although studies like those reported by Unice at al. (2019a; 2019b) modeled tire and road wear particle release and transport in the Seine watershed (France) and estimated that 18% of tire wear release was transported to freshwater and 2% exported to the estuary. . Previous studies have demonstrated white (or related colors i.e. cream-white, pale yellow) to be the most common color of microplastic fragments in debris (Hidalgo-Ruz et al., 2012), or other colors like blue or green to be the most dominant colored fragments (Tsang et al., 2017, Lo et al., 2018). 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 (FT-IR) demonstrated that these particles contain polybutadiene, styrene-butadiene, and carbon black, all components used in the tread of car tires (Leads and Weinstein, 2019).

Chemical properties of tire wear particles vary, with typical passenger car tire which is 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 and 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 originate from the pavement or road surface (Kreider et al., 2010).

Processing oils used in tire manufacturing typically have a high content of 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.

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) are not acutely toxic to grass shrimp (*Palaemonetes pugio*) at concentrations up to 100.0 g/L Gray and Weinstein, 2017). However, a study of whole tire leachate (i.e. water containing dissolved and suspended material

or chemicals from whole tires) toxicity 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 (*Oncorhyncus mykiss*) exposed to whole tire leachates, but no toxicity to the cladoceran, *D. magna*, the nematode *P. redivivus*, or fathead minnow (*P. promelas)*. Different studies assessing toxicity of tire particle elutriates (Panko et al., 2013) and leachates (Marwood et al., 2011) on fathead minnows (*Pimephales promelas*) found no significant adverse effects on fish growth or survival. There is a lack of knowledge on particle toxicity of tire wear 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 tire wear 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: *E. fetida* in Pochron et al. (2017), *G. pulex* in Redondo-Hasselerharm et al. (2018a), and *H. azteca* in Khan et al. (2019). The goal of the current study was to assess if fish are actively ingesting crumb rubber, and what the resulting toxicity is of these particles in both an estuarine fish species (*Fundulus heteroclitis*) and a freshwater fish species (*Pimephales promelas*). To our knowledge, there have been no studies on the toxicity of crumb rubber particles to fish species. 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.

Methods - Fish collection

Mummichogs (Fundulus heteroclitis)

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 2-weeks prior to exposure. Fish were housed in 20-gallon (75.7-L) tanks in a recirculating saltwater system. All fish used in this study were kept on a 16:8 light/dark cycle at 26°C in 18psuartificial saltwater (Instant Ocean, Spectrum Brands Inc.). Fish were fed TetraMin tropical flake food once daily before and during exposure.

Fathead minnows (Pimephales promelas)

Adult fathead minnows (*P. promelas*) 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 US EPA recipe (US EPA, 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 5

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.

Methods – Exposure set up

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), it was determined that water renewals should occur every other day during TWP exposures. Fish used in this pre-setup step were not used in crumb rubber exposures.

Crumb rubber

. 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, U.S.), which . is produced from raw material derived from passenger and/or truck tires using wet-grind technology. The rubber produced contains 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 have a specific gravity between 1.10 – 1.15 (Edge Rubber, 2016). Crumb rubber was sieved to obtain fragments 38 – 355 µm in size.

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 (18psu, Instant Ocean) 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 were 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 7 days with the experiment ending on the 8th 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°C - 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 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 labelled aluminum foil, and frozen in liquid nitrogen until transfer 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 in this exposure. 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 labelled aluminum foil, and frozen in liquid nitrogen until transfer to a -80°C freezer.

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. 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 x g at 4° C for 20 min, after which the supernatant (S9 fraction) was divided into three aliquots for later determination of ethoxyresorufin-O-deethylase (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 in 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 $MgCl_2$, 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 0.1 M HEPES buffer (pH 7.6), 1 mM glutathione (GSH), and 25 µg S9 protein. The reaction was started by adding CDNB (1 mM 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.

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 was analyzed separately for each experiment (i.e. only Experiment A data was 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.

Results

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 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 (Figure 2i) (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 2i). Although enzyme activity for CYP1A was slightly elevated in exposed fish, it was not significantly different between control and exposed fish (Figure 3i). There was no significant difference measured in GST activity between control and exposed fish (Figure 4i). There were no differences observed between males and females for any of the biomarkers tested.

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, 2 fish had died in the highest concentration and 1 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 2ii). Enzyme activity for CYP1A was significantly different compared to controls for 0.33 g/L ($p = 0.0018$) and 1.0 g/L ($p = 0.0453$) crumb rubber concentrations (Figure 3ii). There was no significant difference measured in GST activity between control and exposed fish (Figure 4ii), 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.

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 in the highest concentration (6 g/L) after 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 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 2iii). 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 3iii). 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 4iii). There were no differences observed between males and females for any of the biomarkers tested.

Discussion

The goal of this study was to investigate if fish species ingest crumb rubber when exposed to this polymer debris, and if they show signs of toxicological responses, as measured through commonly used biomarkers. 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 out of these particles. As a result of PAHs leaching out of 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 in the highest exposure concentration was found for the fathead minnows.

Based on our results, and others, it appears that PAHs leaching out of the crumb rubber are toxicants of major concern, although other chemicals in tires may also contribute to observed effects (Stephensen et al., 2003; Llompart et al., 2013). 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 et al., 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, Vuorinen et al., 2006; Otter et al., 2012; Van den Hurk and Haney, 2017).As such, bile fluorescence is a valuable biomarker of exposure, and unequivocally demonstrates that the PAHs from the tire crumb were absorbed and excreted by the mummichogs and fathed 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 and Haney, 2017), 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 at 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. This should be further investigated given the co-occurrence of trace metals in tire products. Likewise, organic compounds, such as non-planar polychlorinated biphenyls (PCBs) and PCB mixtures, have been shown to inhibit CYP1A-mediated responses at high levels of exposure in fish (Melancon and Lech, 1983; Boon et al., 1992). 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) significantly compared to control fish (Figure 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 (Whitehouse, 1984; Tremblay et al., 2005; Ramachandran et al., 2006). 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 load 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 tire particles.

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 and 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 and 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 6 g/L crumb rubber was the only concentration that showed a significant increase compared to control group. This group 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.

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 x 10^8 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; Stephensen et al. 2003; Camponelli et al. 2009; Pochron et al. 2018; Khan et al. 2019), we suggest that more attention should be paid to management of road runoff.Our 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, Gray et all.,

2018) 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 (Stephensen et al., 2003; Wik & Dave, 2006; Kreider et al. 2010; Beckingham, unpublished). 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.

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Compliance with Ethical Standards

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Conflict of Interest: The authors declare that they have no conflict of interest.

Ethical approval: All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted (Clemson University Institutional Animal Care and Use Committee, Animal Use Protocol 2015-067). This article does not contain any studies with human participants performed by any of the authors.

Figures

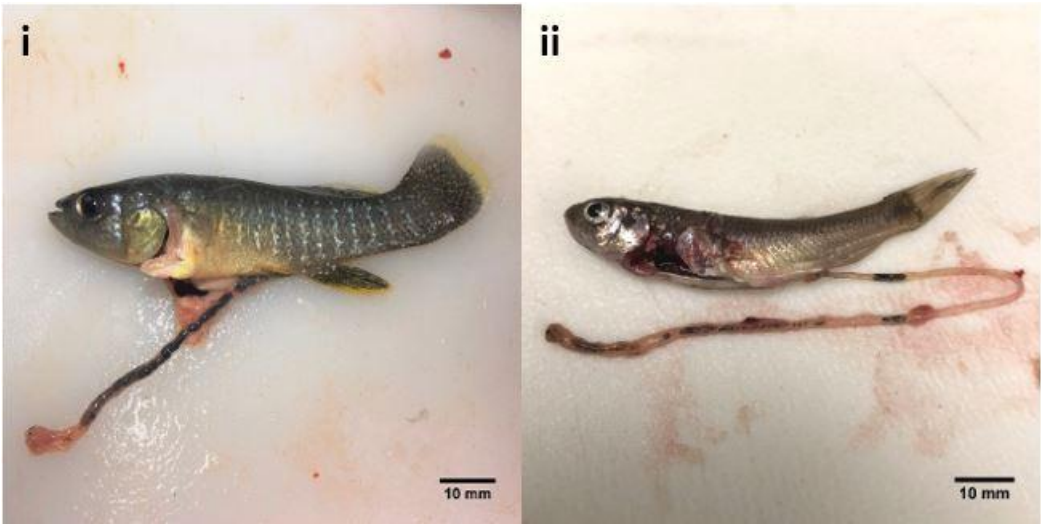
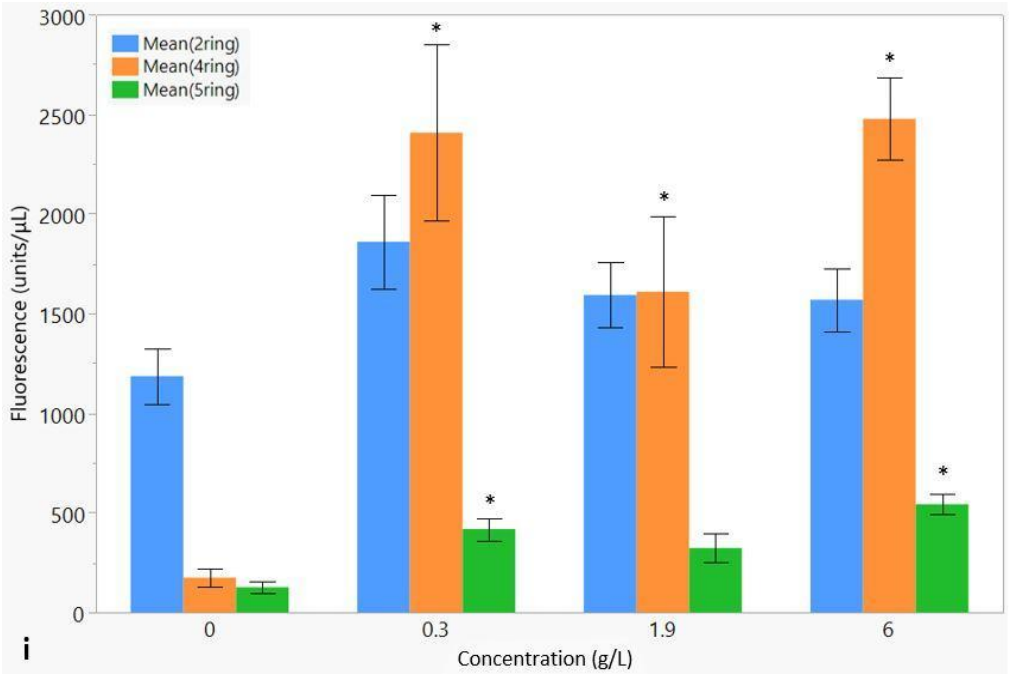


Fig. 1 Crumb rubber in the gastrointestinal tract of mummichog (i) and fathead minnow (ii) at end of exposure. Mummichog from experiment A at 0.1 g/L crumb rubber exposure and fathead minnow from experiment C at 0.3 g/L crumb rubber exposure. Fish were fed Tetramin flake food daily during exposure until 1-day prior to experiment take down.


Mean(2ring)
Mean(4ring)
Mean(5ring)
Fluorescence (units/µL)
3000
2500
2000
1500
1000
500
0
0
0.3
1.9
6
Concentration (g/L)
*
*
*
*
*
*
i

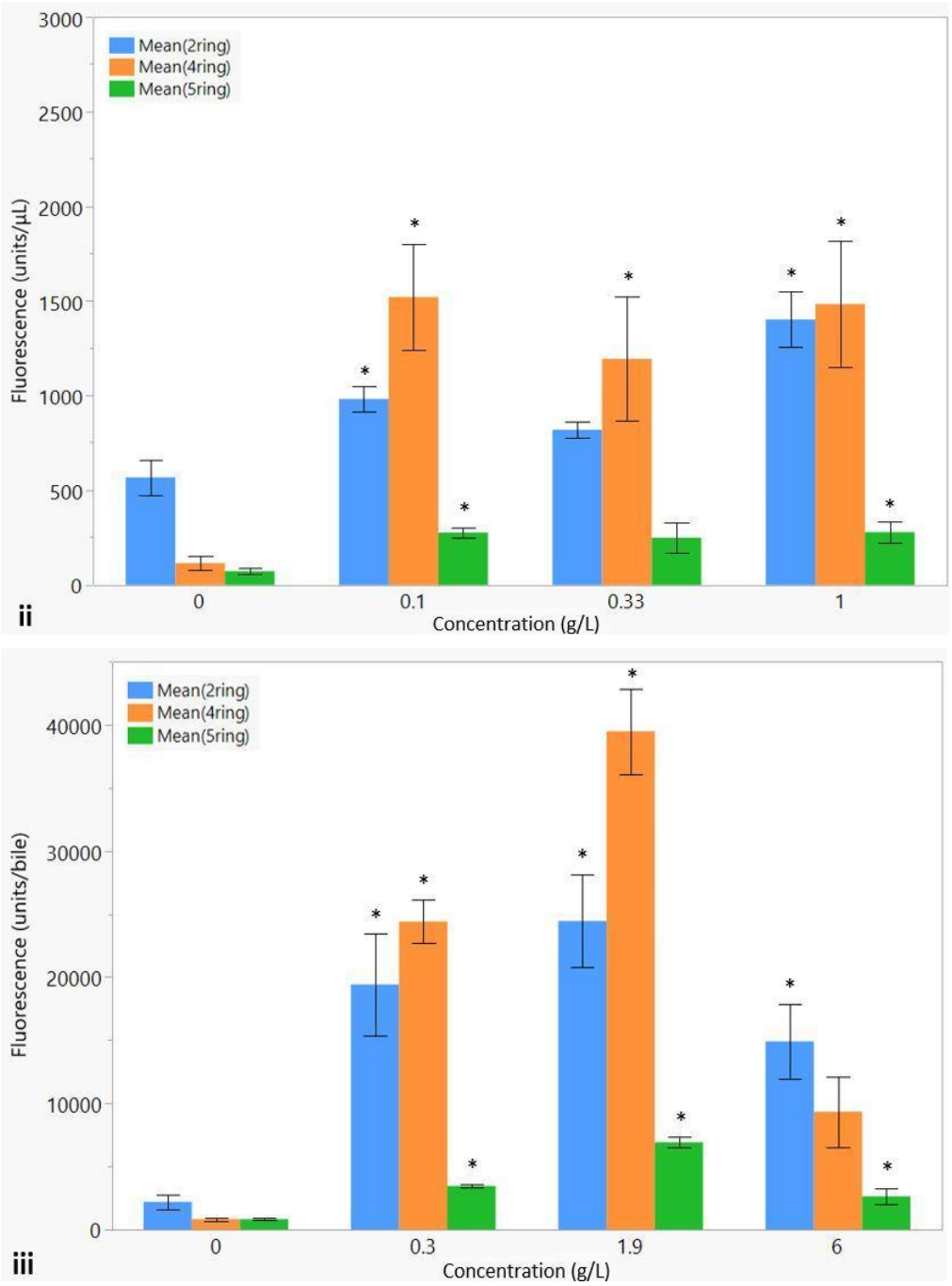


Fig. 2 Bile fluorescence in test organisms measured for 2-, 4-, and 5- ring structures for Experiment A (i), Experiment B (ii), and Experiment C (iii) after 7 d static renewal exposure of up to 6 g/L crumb rubber. Wavelength pairs used for measuring bile fluorescence of 2-ring structures were 290/335, 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 (i) and B (ii). Bile fluorescence units were not normalized by volume for experiment C (iii) due to limited bile

produced in organisms at end of exposure Bars indicate mean fluorescence and standard error is shown. Asterisks above bars indicate significant difference from the control ($p\text{-}value < 0.05$).

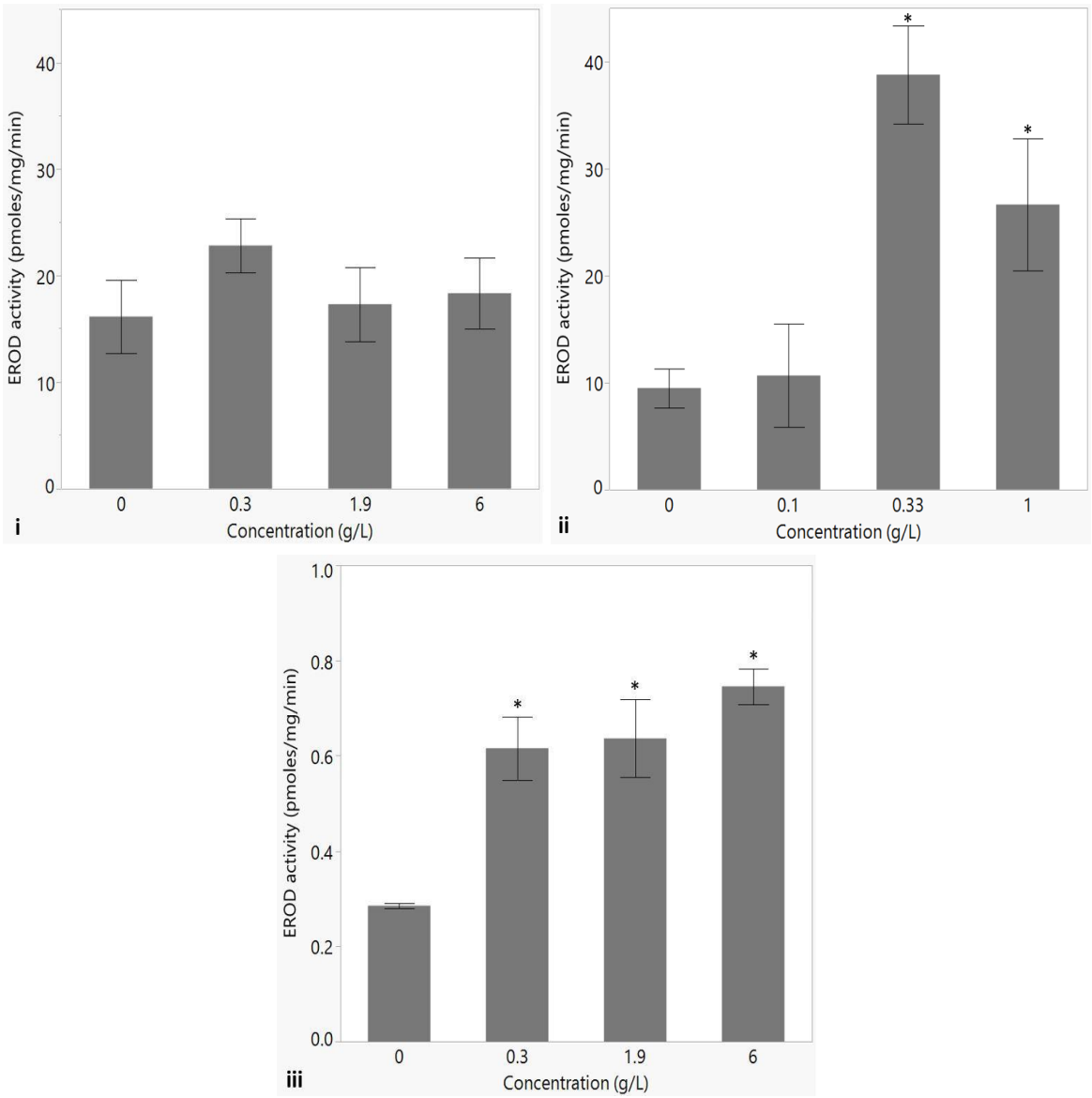


Fig. 3 Ethoxyresorufin-O-deethylase (EROD) activity for Experiment A (i), Experiment B (ii), and Experiment C (iii) after 7 d static renewal exposure of up to 6 g/L crumb rubber. EROD activity is indicative of cytochrome P450-1A enzyme activity. Bars indicate mean EROD activity and standard error is shown. Asterisks above bars indicate significant difference from the control ($p\text{-}value < 0.05$)

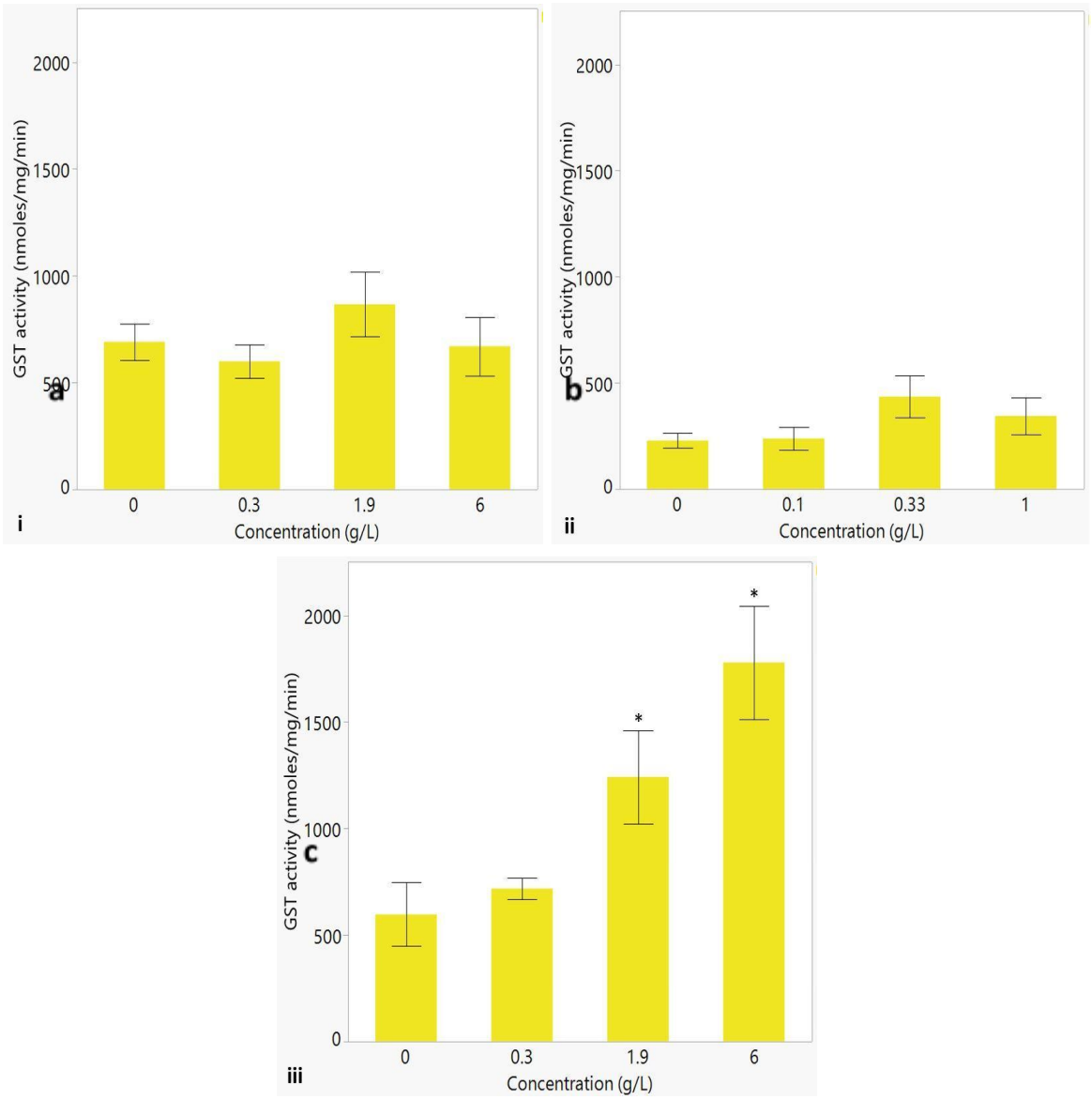


Fig. 4 Glutathione S-transferase (GST) activity for Experiment A (i), Experiment B (ii), and Experiment C (iii). Elevated GST activity is indicative of high amounts of oxidative stress. Bars indicate mean GST activity and standard error is shown. Asterisks above bars indicate significant difference from the control (*p-value* < 0.05)

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