9-19-2023

The Influence of Polystyrene Microplastics on Juvenile Steelhead Trout (*Oncorhynchus mykiss*)

Kaitlyn Marie Baker
Portland State University

The Influence of Polystyrene Microplastics on Juvenile Steelhead Trout

(*Oncorhynchus mykiss*)

by

Kaitlyn Marie Baker

A thesis submitted in partial fulfillment of the
requirements for the degree of

Master of Science
in
Biology

Thesis Committee:
Kim Brown, Chair
Bradley Buckley
Anne Thompson

Portland State University
2023

Abstract

Mass production of plastic within the past decade has led to over 100 billion tons of plastics being added to the world's oceans through rivers and effluent disposal and decomposition (Du, 2020). For marine environments, the sudden and constant growth of microplastics (plastics 1 μm to 5 mm in diameter), is of particular concern to top-predatory fish such as steelhead trout (*Oncorhynchus mykiss*), who passively or actively uptake microplastics while consuming prey (Baechler, 2020). Previous research has demonstrated clear detrimental impacts of microplastic accumulation in bivalves, seabirds, and zebrafish, including decreased diet, reproduction rates, and metabolism, as well as increased rates of mortality (Baechler, 2020; Brown, 2019; Faggio, 2019). However, research in large commercial fish such as steelhead trout is lacking (Brown, 2019; Du, 2020; Fackelmann, 2019). As steelhead trout are top-predatory anadromous fish, they are particularly susceptible to microplastic exposure through their interaction with widespread ecosystems and the bioaccumulation of microplastics during food consumption throughout their lifetime.

Here, we demonstrate how polystyrene microplastics affect juvenile steelhead trout behavior, intestinal tissues, and oxidative stress levels. Our study illustrates microplastic exposure in trout could lead to rapid die offs after acute periods of high exposure. Additionally, we found that even at low levels of polystyrene exposure (10μg/L) results in behavioral modifications, intestinal tissue damages, and increases in oxidative stress. More broadly, our results demonstrate that microplastic exposure causes negative responses in large commercial fish, with potential to alter species survivability and ecosystem dynamics in both wild and farmed populations.

Table of Contents

List of Figures

1. Background

Plastic has been a long-revered product due to its versatility, low cost, and widespread industrial and commercial use. Since their inception in 1946, plastics quickly became essential to society for their inexpensive and long-lasting durability. Plastic provides lightweight packaging in transportation, food, and service industries, as well as durability for extending shelf life, preventing disease and injury, and improving textiles. The ease of plastic disposability also improves hygiene and comfort for many across the world, with widely low and accessible prices to developing countries. Originally created from raw materials and refined into propane or ethane, monomers are then transformed into polymers followed by resin pellets, to eventually be molded into product for future intended use (Scheirs, 2003). Of these uses, the most prominent is packaging, with the greatest production in China, followed by North America, the remainder of Asia, and Europe (Gourmelon, 2015; Kumar, 2021).

While plastics demonstrate a unique and multidimensional resource, the lack of proper disposal methods has drawn worldwide attention in recent years. Combined with decomposition rates spanning 20 to 2,000 years, plastics are now significant to this geological era, being found from the North Seas of the Artic to the deep seas of the Southern Ocean (Foley, 2018; Walkinshaw et al., 2020). Prior to 2018, China received approximately 60% post-consumer content exports from over 43 different countries across the world (Ren, 2020). However, after the post-consumer content ban of 2018, solid waste exports shifted away from the country, resulting in worldwide recycling center overflows, shorter product lifespans, and decreased circular economy use (Ren, 2020). For countries without access to recycling services, waste is incinerated at rates as

high as 57% in Africa and 40% in Asia (Gourmelon, 2015), which can result in several adverse human health effects, including increased cancer and disease risk, reproductive dysfunction, and increased respiratory inflammation (Naidu, 2021; Tait, 2020). Furthermore, studies have shown global environmental injustice as impoverished areas are more likely to include pollution, suggesting that with increased consumption and improper waste disposal, minority groups will be at greater risk (Bell, 2012; Crowder, 2010; Samoli et al., 2019).

As plastics inevitably integrate into landfills and terrestrial systems, they also produce largely negative effects on the surrounding ecosystems and wildlife. Many of these effects are driven by microplastics (i.e., plastics 5 mm to 1 μm diameter), which are primarily sourced from direct production in cosmetic and textile industries (Fendall, 2009) and secondarily sourced from macroplastic decomposition (Browne, 2011; Collicut, 2019). Through littering, sewage disposal, industrial runoff, waste disposal, and production leakage, microplastics have readily integrated into ecosystems rather than their intended disposal target (Fig. 1). Once in the soil, microplastics directly interfere with biogeochemical processes such as the ability to sequester carbon, nitrogen and phosphorus uptake, and surface soil temperature and turnover regulation (Guo, 2020; Kumar, 2021; Rillig, 2021). Furthermore, plastics cause challenges for plant growth and induces a cascade of ecosystem effects, beginning at microbial composition within the soil (Zhao, 2021).

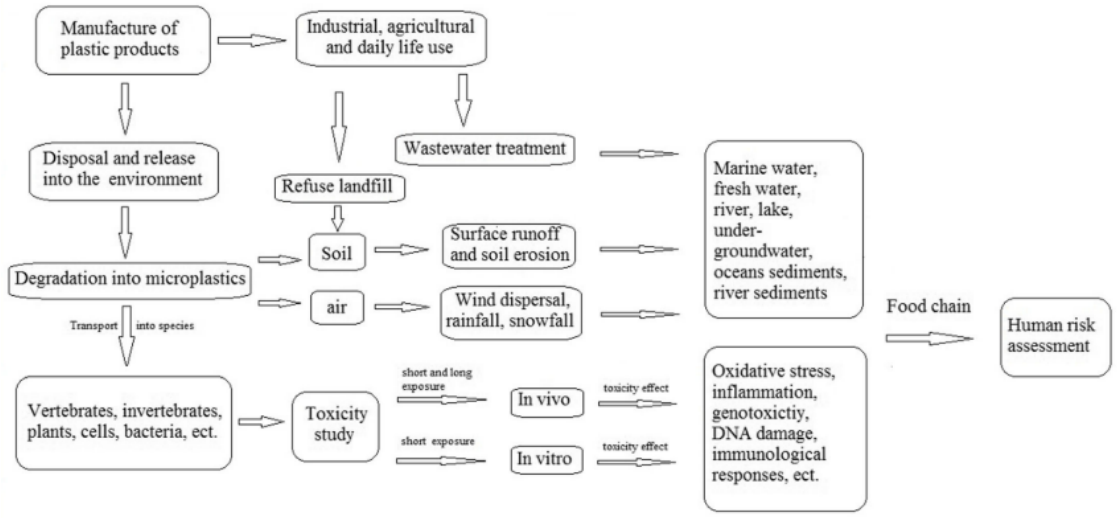

Figure 1. from Du, J. (2020): The dispersal of macroplastics into microplastics causes a cascade of effects that impact marine and freshwater systems, as well as pose a risk to humans during consumption (Beachler, 2020; Du, 2020). Microplastics can either be from a primary or secondary source and are 5 mm to 1 µm in length (Fendall, 2009). As plastic continues to degrade over time, microplastics become and persist as nanoplastics (plastic less than 1 µm in length) (Gigault, 2018).

Of the 79% of plastics that are landfilled, over 100 billion tons of plastic have been added to the world's oceans by rivers and effluent disposal and decomposition in the past decade (Du, 2020; Jambeck, 2015). According to Kumar 2021, the Yangtze River has the largest amount of plastic waste at 1.46 million tons, followed by the Indus River at 164 thousand tons (Kumar, 2021). In England, the River Tame demonstrated variations in sampling locations within rivers as well, with a 65% increase in urban tributaries when compared to rural locations (Tibbetts, 2018). Fluctuations of waste also vary per day and location, such as in Chicago USA, where the average river influx can be as high as 1.34 million particles per day (McCormick, 2016). As a result of these microplastic influxes, numerous freshwater organisms such as mayflies, caddisflies, mosquito larvae, rotifers, and amphipods have shown negative impacts, with effects such as increased oxidative stress, decreased reproductive rate, developmental delay, and increased mortality (Guimarães, 2021; Jeong, 2016; Malafaia, 2020; Silvia 2019; Silvia, 2020; Ziajahromi,

2018). Redondo-Hasselerharm et al. (2020) also found that the overall diversity index in freshwater rivers decreased due to microplastic exposure, further suggesting significant effects on freshwater invertebrate community composition.

As rivers integrate into marine environments, the sudden and constant accumulation of microplastics is of particular concern. Previous research has documented severe impacts of the ingestion, entanglement, blockage, and exposure of plastics to over 220 species of marine organisms, leading to the death of 1 million seabirds, 100,000 mammals, and several thousands of fish yearly (Baechler, 2020; Susanti, 2020). While plastic entanglement has been studied and observed prior to 1969, only recently have microplastic interactions come to light (Le Guen et al., 2019; Zantis, 2020). Ranging from 9-75% consumption per individual (Le Guen et al., 2019), the effects of microplastics in mammals remains largely unknown. However, in seabirds, research has shown largely negative effects due to ability of microplastics to reduce feeding capacity and digestion (Reynolds 2018; Susanti, 2019; Van Franker, 1985), reproductive growth, extracellular processes (Choy, 2013; Derraik, 2002; Susanti, 2019; Zhang, 2022), and increased induced stress (Choy, 2013). Studies have also shown a wide concentration of various microplastic ingestion by seabirds, penguins, raptors, passerines, and ducks through both environmental and trophic transfer (Clark, 2022; Le Guen, 2019; Reynolds, 2018; Susanti, 2020; Wang, 2021). Furthermore, it is estimated that by 2050, 99% of all seabird species will contain plastic (Wilcox, 2015).

Similarly, bivalves and fish are regularly affected by the influx of microplastics into the marine environment. In bivalves, the exposure of microplastic triggers the onset

of chronic diseases (Facklemann, 2019), alters gene capacity (Fackelmann, 2019), and decreases filtration activity (Zhang, 2022) and byssal thread growth (Sussarellu et al., 2016). Within fish, studies have shown altered hunting behavior and liver histology (Yin, 2019), as well as increased susceptibility to sorbed contaminants within the water column (Barboza, 2018; Granby, 2018; Karami, 2016). Further, a meta-analysis by Salerno et al. (2021) confirmed widely negative effects on a variety of fish species including decreased feeding, decreased growth, and altered behavior due to microplastic exposure (Salerno et al., 2021). As Salerno describes, the degree of effects varies uniquely to each fish species depending on life stage and feeding type. Overall, this suggests that some fish species are more susceptible to microplastic damage than others.

A final pressing concern is the rate and effect of plastic exposure in humans, which is likely through inhalation, contact, or ingestion via seafood (Amato-Lourenco et al., 2020; Lehner, 2019). In 2015 it was estimated that 17% of human animal protein consumption is from seafood intake, which serves as a major microplastic vector especially if seafood is consumed whole (Smith et al., 2018). Studies have reported microplastic levels in farmed mussels and both wild caught and farmed fish across the globe (Smith et al., 2018). While the human body is capable of filtering and disposing of most microplastic material, questions remain as to how exposure effects the gastrointestinal tract, as well as the translocation ability of nanoplastics (plastic diameter less than 1 μm) across cell membranes such as the placenta or the blood-brain barrier (Wright, 2017). With the potential to cross these barriers, plastics could pose serious threats to human welfare, especially in locations were seafood consumption or plastic disposal is irregularly high.

2. Introduction

In 1909, the first fully synthetic plastic was introduced to the public (Baekeland, 1909; Baekeland, 1911). Known as Bakelite, this synthetic polymer was created by Leo Baekeland in 1906 and was implemented into many commercially available products such as telephones, radios, and cameras as electrical insulation (Scheirs, 2003). Following its inception, the unique characteristics of Bakelite captured the attention of several chemical companies including Dow Chemical in the United States and Imperial Chemical Industries (ICI) in the United Kingdom, who began studying synthetic plastics in greater detail (Scheirs, 2003). With such studies and advancements, synthetic plastic became widely produced from readily available precursors of crude oil production into gasoline (Baekeland, 1911). One byproduct of particular interest was the use of ethylene gas and styrene to create polyethylene and polystyrene plastics, which were further revered for their light weight and durability. Finally, during World War II, ICI became the first company to mass produce and synthesize ethylene into plastic formation as radar cabling insulation (Scheirs, 2003). Shortly after, commercial applications followed with the advent of Tupperware, plastic bags, and many other products still used today.

Although polystyrene was initially discovered in 1839, it did not come into common use until over a century later (Scheirs, 2003). Today plastics including polystyrene are ubiquitous around the world, providing easy, inexpensive, and useful methods of transporting and storing everything from food and water to chemicals and waste products (Walkinshaw, 2020). Additionally, the versatility of these products has led to extensive uses and the highest production rates of plastic within the past decade (Lebreton, 2018). Yet despite many plastics being recyclable, lack of proper disposal

methods has resulted in relatively small amounts of plastic actually being recycled (Hopewell, 2009; Lebreton, 2018). It is estimated that within the past decade alone over 100 billion tons of plastics have been added to the world's oceans through rivers and effluent disposal (Du, 2020; Jambeck, 2015). For marine environments, the sudden and constant growth of microplastics (plastics 1 μm to 5 mm in diameter), is of particular concern to top-predatory fish such as steelhead trout (*Oncorhynchus mykiss*), who passively uptake microplastics while consuming prey (Beachler, 2020; Du, 2020). While previous research has shown negative impacts of microplastic accumulation in bivalves, seabirds, and zebrafish, research in anadromous, commercial fishes such as steelhead trout is lacking (Alimba, 2019; Baechler, 2020; Du, 2020; Facklemann, 2019; Van Franker, 1985).

Overall, microplastic ingestion and exposure is known to exhibit negative physiological effects in hundreds of marine organisms, including fish, shellfish, crustaceans, turtles, aquatic invertebrates, macroalgae, seabirds, and marine mammals (Baechler, 2020; Foley, 2018; Walkinshaw, 2020). In both bivalves and zebrafish, the exposure of microplastics triggers the onset of chronic diseases and limits gene functionality (Facklemann, 2019; Faggio, 2019). Additionally, microplastic exposure in zebrafish has also demonstrated numerous other health effects, including physiological tissue damage, cytotoxicity, decreased growth (Du, 2020), altered gene expression (Zhao, 2020), decreased metabolism (Lu, 2016; Qiao, 2020; Wan, 2019; Zhao, 2020) and increased oxidative stress (Du, 2020; Prokic, 2018; Qiao, 2020) in both larval and adult animals. However, findings from this small, warm-water, freshwater organism, may not

be analogous to predatory, warm-water or cold-water commercial fish where bioaccumulation may make them highly suspectable to microplastic exposure.

Multiple studies have already documented microplastics within the gastrointestinal tracts of large wild caught fish, including anchovy, tuna, and herring (Walkinshaw et al., 2020), yet physiological effects of microplastic ingestion in these commercially important fish remains widely understudied. In a study by Hyrenbach et al., (2021), both pole and line caught predatory fish such as Skipjack and Yellowfin Tuna had ingested marine plastic debris in O'ahu with varying polymer consumption types. Similarly, plastic debris have been found in fish of the North Seas (Foekema, 2013) and wild-caught North American finfish species (Baechler, 2020), that are regularly used in commercial fisheries for human consumption. With increased microplastic exposure, ingestion, and susceptibility, population dynamics of these wild-caught fishery species will likely be highly affected (Baechler, 2019; Du, 2020; Faraday, 2019). For example, as organisms ingest plastics, mistaking the particles for food, they inevitably accumulate as they move through trophic levels (Fig. 2) (Beachler, 2020; Du, 2020). Organisms further along the trophic process, such as top predatory fish, are particularly susceptible to the accumulation of microplastics through passive and active uptake, as they eat larger quantities of prey who have also bioaccumulated plastics (Beachler, 2020; Du, 2020). Although most fish are initially exposed through the digestive and respiratory systems, studies have shown microplastic ingestion could alter immune, cardiac, and endocrine system function as well (Brown, 2019; Faraday, 2019), further affecting growth and reproductive rates.

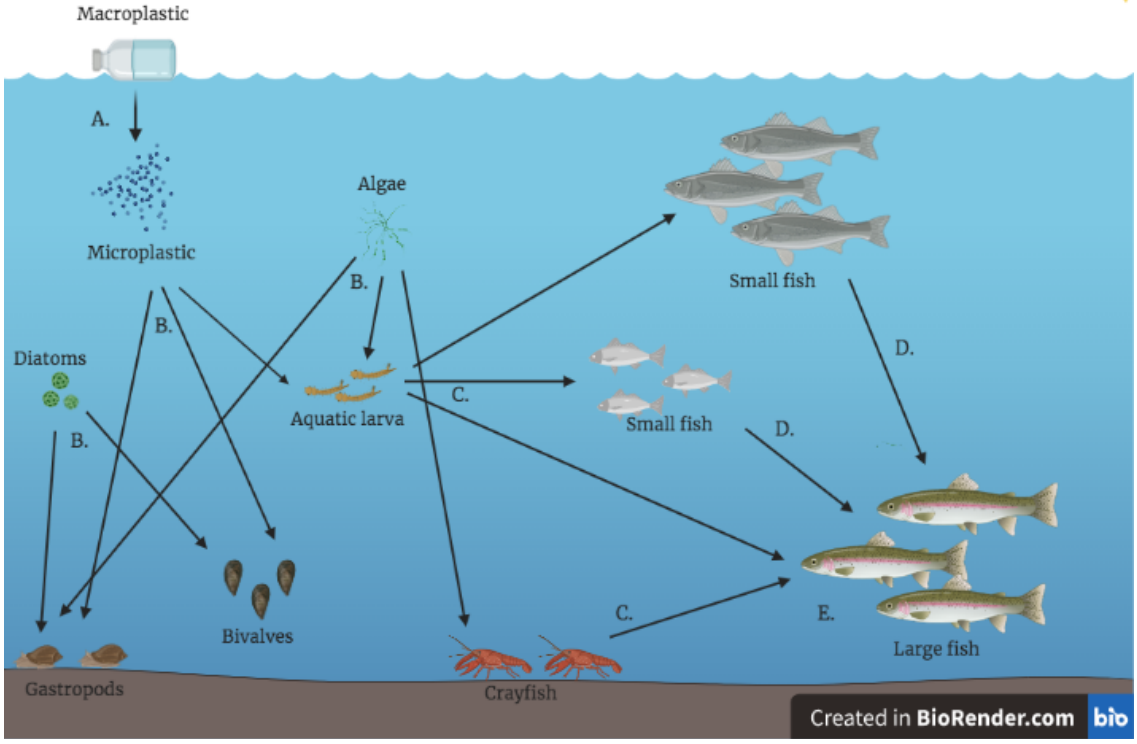

Figure 2. Bioaccumulation of Microplastics. The degradation of macroplastics leads to the accumulation of microplastics in trophic levels, reaching a climax in top predators. (A) Macroplastics such as plastic water bottles degrade break down into smaller particles, and eventually, microplastics at <5mm (Collicutt, 2019). Microplastics are often confused for food particles such as filamentous algae, diatoms, or phytoplankton (B). Primary consumers such as bivalves, insect and aquatic larvae, and crayfish consume microplastics as well as other small food particles (C). Microplastics continue to accumulate as small fish such as suckers and bass consume large numbers of primary and secondary consumers (D). Top predators, such as steelhead trout, consume small fish and accumulate large amounts of microplastics, creating potentially toxic health effects (E).

In rainbow trout specifically, microplastic exposures have been shown to result in increased oxidative stress, decreased intestinal microbiota diversity, and anatomical changes to the digestive system itself. In a study to assess susceptibility to *cold water disease,* a common aquaculture industry problem, Brown (2019) found microplastics increased oxidative stress that suggested a decrease in immune function leading to significant increases in cold water deaths. These findings suggest that microplastics have the ability to decrease immune function and increase disease susceptibility that could

affect population dynamics in both farm raised and wild populations (Brown, 2019). In addition to potential immunological effects, decreases in intestinal microbiota diversity from microplastic exposure has also been observed (Fackleman, 2019; Wan, 2019). Through direct mechanical disruption and indirect interruptions of bacterial-gut symbiotic relationships, growth rates are likely to decrease within both wild and commercial populations resulting in decreasing aquaculture production and harvest rates (Du, 2020). Finally, numerous sources have indicated distortion or inflammation of the digestive system after microplastic exposure (Du, 2020; Lu, 2016; Lu, 2018; Limonta, 2019; Peda; 2016; Qiao, 2019). As this distortion becomes chronic, it could lead to largely negative impacts to essential tissue function and reduced diet, inherently reducing overall fitness, reproductive success, and mass density of fish (Du, 2020; Liu, 2019; Ma, 2019).

Therefore, the primary purpose of this study is to analyze how the chronic exposure of an abundant microplastic, polystyrene, impacts the predatory fish steelhead trout (*Oncorhynchus mykiss*). First, we will examine the impacts of polystyrene on overall trout health and behavior. Next, we analyze histological tissue damage within the intestinal tract post exposure. Finally, we will determine how concentrations of polystyrene impacts oxidative stress levels, as well as overall liver function. By studying the implications of microplastic exposure in large commercial fish such as steelhead, we can then establish management techniques to mitigate exposure in waterways and limit fish population damage.

3. Methods

Seventy-five steelhead trout (*Oncorhynchus mykiss*) were obtained as stock from Island Spring Hatchery in Portland, OR. Three low-density tanks labeled A, B, and C were randomly populated with 25 fish/75.7 L tank, with a water turnover rate of 7.5 L/hour (Brown, 2019). Tanks were equipped with a standardized 12-hour light cycle, maintained at 11°C, and properly aerated (UV sterilized cultured water with pH value of 7.2 and dissolved oxygen content of 6.6 mg/L). Fish were acclimated to the tanks for 7 days prior to experimentation and fed Skretting® Classic Fry® (2.5 mm pellets) *ad libitum* every 12 hours.

Polystyrene microplastic particles were obtained from Uxcell (2.5 mm, model number: a20112400ux665) with two experimental group tanks and one control group. Tank B was exposed to microplastics at a low concentration of 10 µg/L, and tank C at a high concentration of 100 µg/L. Tank A remained unexposed. Both experimental concentration values were informed by representative microplastic influx to urban river environments across the Chicago metropolitan area (McCormick, 2016). Polystyrene particles were added every three days to represent a constant influx of microplastics into the environment. The non-experimental group, Tank A, was exposed to 100 mL sterilized, deionized water every three days. Tank water was cleaned and turned over every three days to remove any microspheres and feed not taken up by the fish (Liu, 2019). Exposure lasted for a total of 21 days, and water within each tank was continuously aerated to assure homogenous distribution of the microplastics throughout the exposure period. Standardized tank conditions remained constant throughout the

entirety of the experiment, and dead fish were removed, dissected, and frozen immediately upon discovery.

After exposure completion, all surviving fish were euthanized in 200 mgL^{-1} MS222 for 5 minutes and rinsed with deionized water to reduce external particle exposure. Euthanasia was conducted in accordance with an approved, Portland State University IACUC protocol consistent with all national standards. Photographs and morphological measurements for each fish were recorded, including weight, body depth, standard length, and total length in centimeters. Livers from each fish were immediately dissected, snap frozen, and stored at -80 °C for oxidative stress analysis (Qiao, 2020). The entire intestine of each fish was dissected into three 5 mm length pieces, treated with 10% formalin, and stored in 70% ethanol. Following fixation, intestinal tissues were embedded in paraffin wax, sliced into three 20 μm slices, and stained with hematoxylin and eosin for microplastic abundance and histology analysis (Qiao, 2020). Intestinal damage was then observed and scored using the matrix proposed by Zimmerli et al., 2007.

Liver samples were divided for superoxide dismutase (SOD) and glutothionine (GSH) oxidative stress analysis. Liver samples were deproteinated and total GSH concentration was analyzed using a commercial detection kit (Cayman Chemical, USA) according to manufacturer's protocols. End point method was used with plate measurements recorded using a 405-414 nm wavelength on a microplate spectrometer (Tecan Trading AG, Switzerland).

To determine total SOD activity, liver samples were homogenized in PBS solution and analyzed in triplicate using a commercial detection kit (Cayman Chemical,

USA) according to manufacturer's protocols. Plate measurements were read at 440-460 nm using a microplate reader (Tecan Trading AG, Switzerland). Total SOD activity and GSH concentration values were calculated according to manufacturer's protocols.

Calculation and Statistical Analysis

Premature death significance was calculated with a t-test assuming equal variances using exposure date and number of fish deaths within each tank. Morphological measurements among and between experimental tanks were analyzed using a single-factor ANOVA. Condition factor between tanks were analyzed using a t-test assuming equal variances, and intestinal tissue damage matrix scores were analyzed using a single-factor ANOVA (Peda, 2016). GSH and SOD concentration values were both analyzed using a t-test assuming equal variances.

Contamination Regulation

Cross contamination of air-borne and surrounding particles was maintained throughout the experiment. Gloves were changed between any consecutive fish handling and sampling, all experimental equipment was cleaned using sterilized water, and experimental equipment was restricted to glass and metal. Samples were tightly covered with aluminum foil and all extraction work was performed in a flow hood. Experimental personnel also wore cotton lab coats and natural fiber clothing to reduce textile fiber contamination.

4. Results

Survivability rate and behavior

Prior to experimental completion (exposure day 21), a significant number of experimental fish died prematurely (p= **4.41e^{-06}**), including 18.8% of control fish, 45.8% of low exposure fish, and 100% of high exposure fish. All high exposure fish died within 17 days of initial exposure, and low exposure fish did not begin dying until day 16 of exposure (Fig.3). No significant weight or size variation were found between tanks, however, within tanks increased weight and decreased length did have a significant effect on premature death risk (p= **0.00035 and 8.918 e^{-10}** respectively). The average condition factor 'K' of fish that died prematurely was 1.36 (Sd= 0.19), compared to the average condition factor of euthanized fish 0.89 (Sd=0.08), meaning that larger and shorter fish were significantly more likely to die prematurely than longer, skinner fish (Froese, 2006).

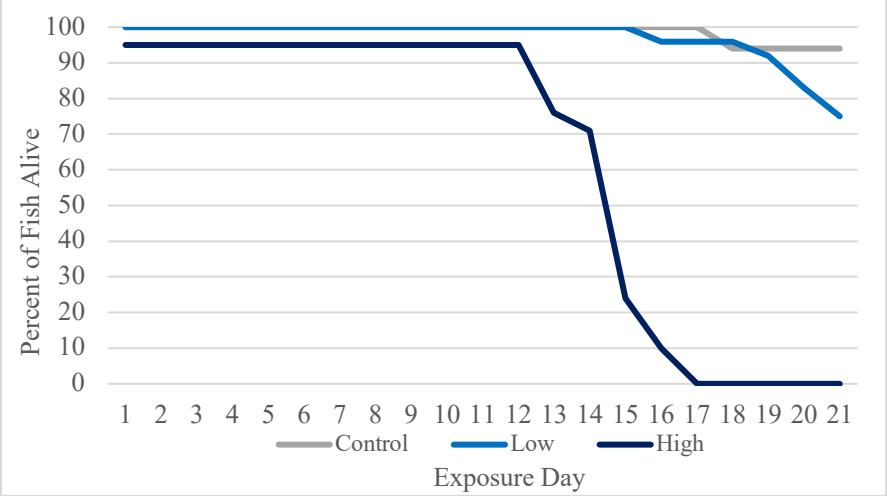

Figure 3. Exposure Fish Remaining. Percent of fish remaining during the exposure period. Day 1 illustrates the first day of microplastic exposure.

Behaviorally, fish exposed to microplastics illustrated signs of distress including swim bladder malfunction, lack of appetite, and erratic swimming prior to death. At day

two of exposure, fish in the high exposure system began to show loss of buoyance control, which resulted in fish showing difficultly maintaining an upright orientation and positional control within the water column (Fig.4). High exposure fish consistently stayed in the upper third quadrant of the water column, whereas control fish maintained regular orientations throughout the entire tank. No distress behaviors were observed in the control group throughout the experiment.

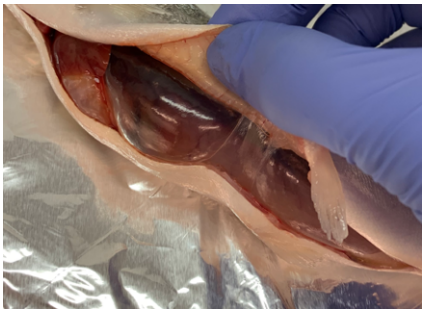

Figure 4. Swim Bladder Image. Example of swim bladder size in a low exposure fish that died prematurely. Fish demonstrated difficulty regulating swim bladder function immediately post exposure in high exposure fish (tank C), and following day 10 in low exposure fish (tank B)

Histological Analysis

High exposure samples and fish that died prior to experimental completion were excluded from histological analysis due to unknown postmortem intervals and possible tissue decomposition during premature death. During dissection, intestinal blockages were also visibly present within three of the experimental fish. In low exposure samples, 88.89% of samples demonstrated a complete detachment and lysis of muscularis mucosa, submucosa, and villi within the gastrointestinal layers, in addition to enlargement of goblet cells and detached epithelial layers (Fig. 4). In contrast, 92.86% of control samples demonstrated complete intestinal tracts, with no excessive goblet cells, inflammation, lysis, or tearing, with clear layers of epithelium, intestinal villi, muscularis mucosa, and submucosa (Fig. 4). ANOVA analysis of damage scoring results were highly significant between the two treatments (**$4.7312e^{-08}$**).

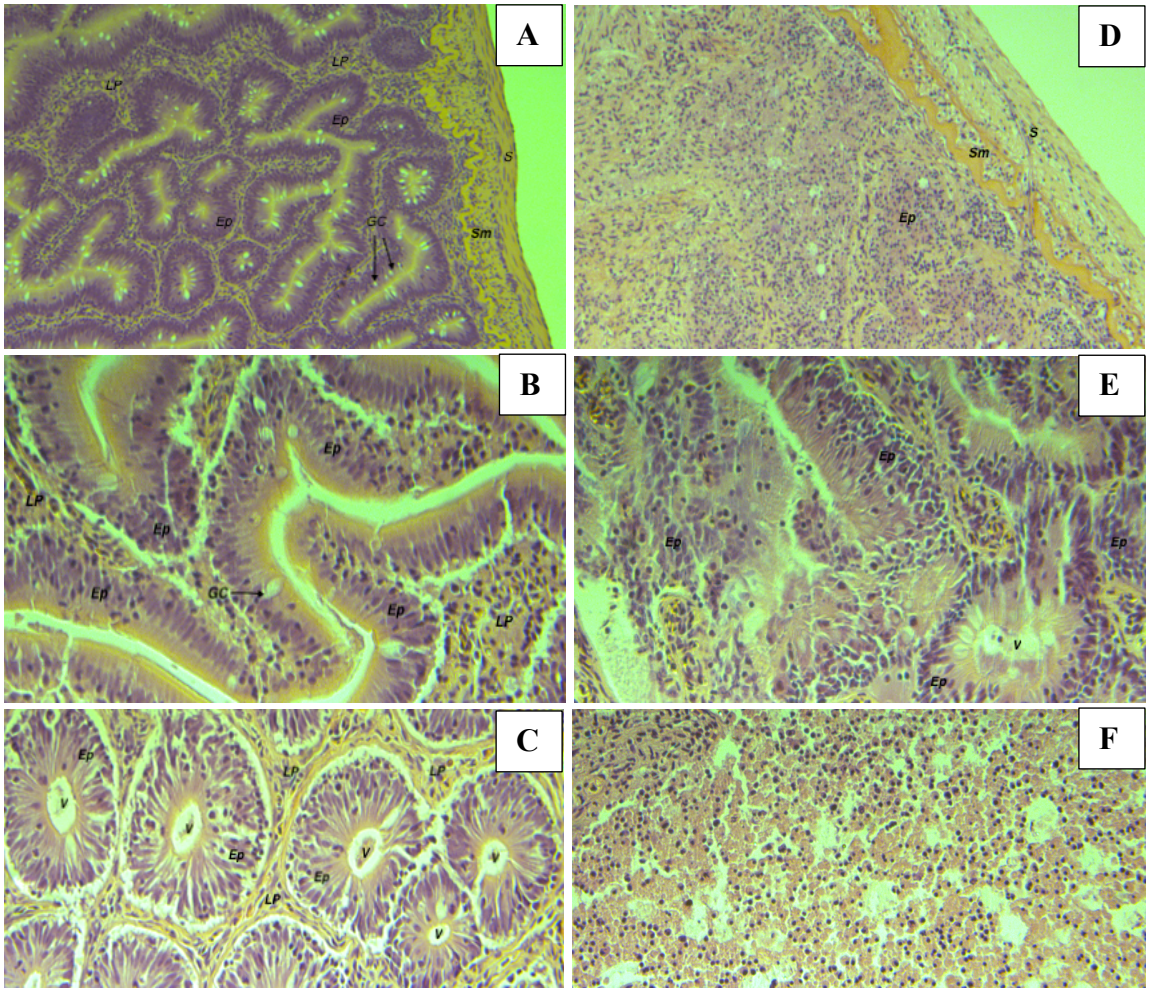

Figure 5. **Histology tissue imaging**. Imaging of intestinal tissues in control (A-C) and low exposure (10 µg/L) (D-F) systems. Image A from a control fish illustrates complete intestinal layers of epithelium, lamina propria, submucosa, and serosa, with intact goblet cells and organized arrangement (20x). Under the same magnification and location, image D from a fish exposed to 10 µg/L microplastics shows intact serosa and submucosal layers, but complete detachment of lysis of intestinal layers and cell types. Image B demonstrates a control fish with complete intestinal layers similar to image A, but at 40x magnification. Image E contrasts this, with lysis and remnants of epithelium and lamina propria from a fish exposed at 10 µg/L (40x). Finally, the control fish in image C is compared to the low exposed fish in F (40x), in which C shows intact intestinal villi, epithelial, and lamina propria layers, whereas F shows complete lysis and detachment of these features.

Liver Function Analysis

Total GSH concentration significantly increased in the low exposure liver samples relative to the control (**0.04508105**). Mean total concentration values for the control and low exposure were 25.43 µM and 29.54 µM respectively (Fig. 6A and C).

Total SOD activity likewise significantly increased in the low exposure group relative to the control (**$1.05e^{-64}$**). Mean total SOD activity values for the control and low exposure were 0.004 U/mL and 317.42 U/mL respectively (Fig. 6B and D).

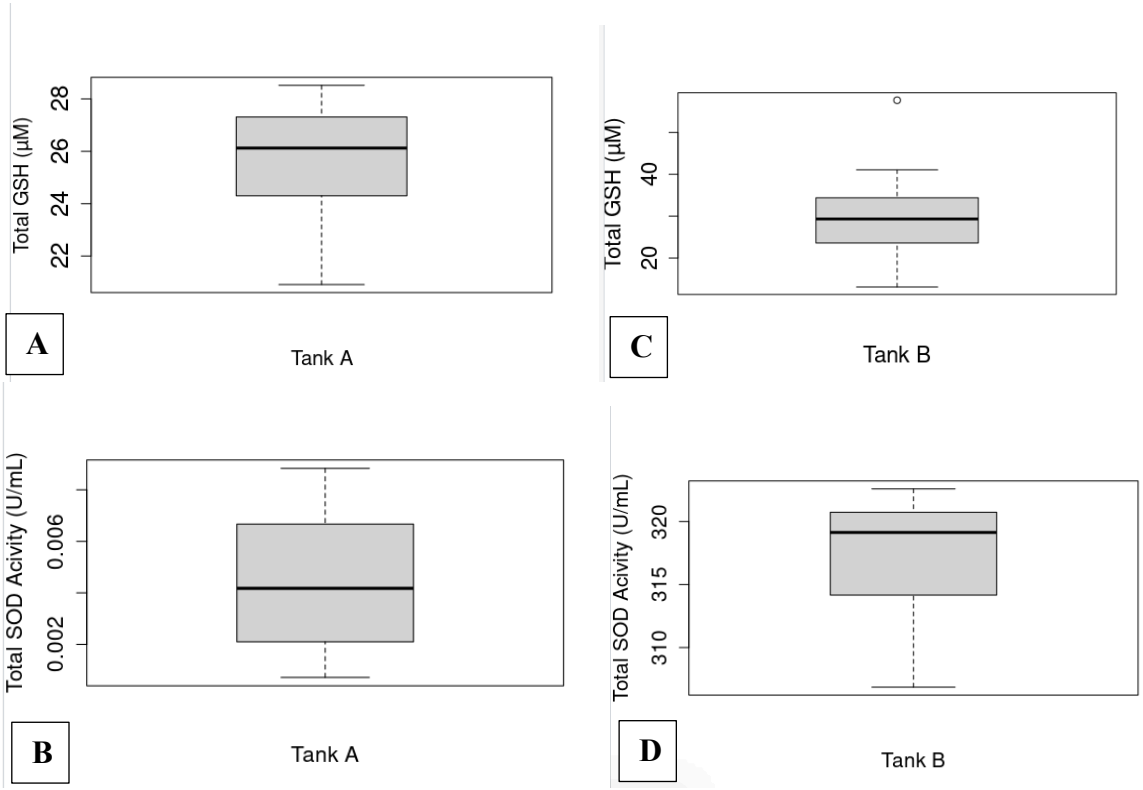

Figure 6. **Total GSH Concentration and SOD Activity Values.** Total GSH concentration in control (A) and low exposure system (C, 10 µg/L). Total SOD activity at control (B) and low exposure system (D,10 µg/L). Results for both GSH and SOD were significant (**0.04508105** and **$1.05e^{-64}$** respectively).

5. Discussion

Previous studies have investigated microplastic effects in small warm water fish or determined rates of wild caught plastic ingestion, however *in situ* lab research of commercial fishes has been lacking. Our findings suggest that despite size and life-strategy differences, many overarching themes regarding microplastic affects in smaller, warm water fish do transfer to large commercial fish as well. Our results also suggest that more aggressive and bold fish could be at higher risk of exposure, and that threshold values exist at which steelhead trout are no longer able to cope with microplastic ingestion.

Behavioral impacts

Microplastic consumption and digestion was high within the treatment groups, resulting in all fish within the high exposure group dying prematurely. Among deaths, those with increased weight and decreased length died earlier than longer and thinner fish. In the wild and farmed populations, salmonid species such as steelhead trout create aggressive feeding territories in which high competition results in unequal resource partitioning (Keeley, 2001). Due to this inequality, it is likely more aggressive fish consumed larger amounts of both food and microplastics, and therefore were at greater risk than smaller “shy” fish. Similarly, a study by Chen et al. (2020) found that zebrafish exposed to polystyrene plastics differed in ingestion capacity based on personality boldness, with “bolder” fish more likely to approach and ingest microplastics than “shy” fish (Chen et al, 2020). Studies have also shown that microplastic ingestion capacity in fish varies based on life stage (Salerno et al., 2021) and feeding mechanisms of fish, with

species that swallow food whole at greater risk than filter or sucking feeders (Li et al., 2021). As steelhead are swallow feeders and additionally have varying sizes throughout life stages, it is likely that these compounding factors increase microplastic susceptibility and risk, with our study suggesting steelhead remain susceptible at least through the juvenile stage.

Swimming behavior changes were also observed among microplastic exposed individuals that were determined to be a result of changes to swim bladder function. Difficultly in swim bladder function could be due to increased susceptibility to swim bladder disease, which can be contracted due to gastrointestinal issues such as inflammation (Conte, 2004; Du, 2020) or possible translocation of microplastics to the pneumatic duct (Yuji, 2020). Moreover, Yong (2020) reported difficultly in swim bladder formation following exposure to 51 nm nanoplastics in zebrafish larvae development (Yong, 2020), however, our results additionally suggest support for malfunction in swim bladder function past the developmental stage.

Researchers have also reported altered feeding behavior in the form of reduced food intake, altered hunting behavior, and overall reduced activity following microplastic exposure among fish species (Yin, 2018; Zhang, 2021). Using a marine Jacopever fish exposed to 15 μM polystyrene microplastics after 14 days, Yin and colleagues (2018) showed significant decreases in foraging and swimming behavior, along with increased shoaling behavior (Yin et al., 2018). Similarly, after day two of microplastic exposure, both of our experimental tanks demonstrated decreased foraging activity and reduced swimming behaviors. There are several possible explanations for these behavioral shifts, including increased damage to the digestive tract and therefore reduced metabolism

(Beachler, 2020; Du, 2020; Lei, 2018; Limonata, 2019; Qiao, 2020; Wan, 2019; Zhao, 2020), stress-induced behavioral changes (Lu, 2018; Yin et al., 2018), or neurological changes following high microplastic consumption (Anbumani, 2015; Salerno, 2021; Mattsson et al., 2017).

Several external morphological shifts were seen post-exposure, with high exposure experimental fish exhibiting spinal flexures and swollen abdomens consistent with previous studies in zebrafish exposed to 70 µM microplastics in (Lei, 2018) (Fig. 7). However, these changes were only consistent in the high exposure tank premature deaths, suggesting interior blockages or inflammation are only visible externally in high exposure scenarios. Additionally, considering swim bladder malfunction and significant size differences in fish that died prematurely, our results suggest increased microplastic susceptibility in larger, more aggressive fish that readily consume microplastics.

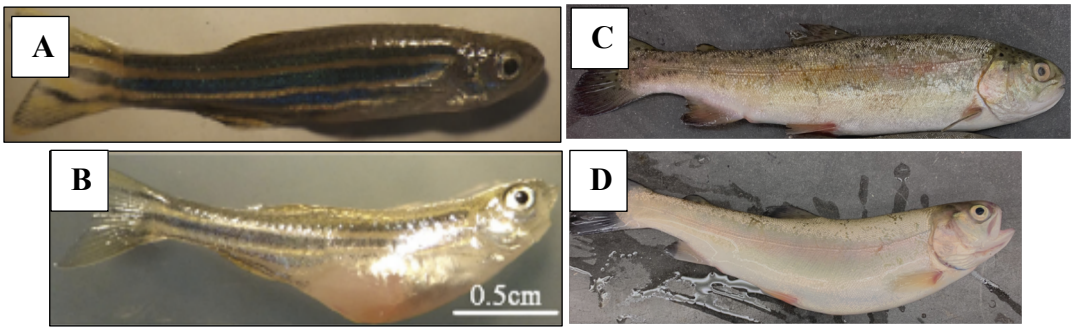

Figure 7. From Lei (2018)., A, B: Imaging of zebrafish after ten-day exposure to 70 µM microplastics (polyamides, polyethylene, polypropylene, polyvinyl chloride, and polystyrene) (Lei, 2018). A indicates control, B indicates dead fish after exposure. **C, D:** Imaging of steelhead fish from this study, C indicates control and D indicates high exposure fish (100 µg/L) after 13-days of polystyrene exposure.

Histology tissue damage

Histological analysis results emphasized complete dysfunction of digestive systems even in low exposure fish when compared to the controls. Three full intestinal blockages were visible in experimental fish that died prematurely, with one additional fish containing microplastics in portions of the esophagus (Fig.8). While intestinal blockages are recorded commonly in numerous marine organisms from birds to fish, (Choy, 2013; Du, 2020; Fackelmann, 2019; Lusher, 2022; Wright 2013), our results suggest that microplastics are capable of being passed by steelhead trout at the juvenile life stage and beyond to a threshold point. Several studies have reported damage from intestinal blockages, including reduced food intake and physical damage, eventually resulting in decreased nutrition, growth, and intestinal dysbiosis (Choy, 2013; Du, 2020; Fackelmann, 2019; Lusher, 2022; Wright 2013), and similarly, those that experienced blockages likely died immediately after obstruction. Furthermore, as fish are exposed to microplastic size variations (from 5 mm to 1μm) during the plastic decomposition process, it is likely that damages to the gastrointestinal tract vary for commercial fish; for example, macroplastics and large microplastics increase the likelihood of blockages, whereas nanoplastics increase the likelihood of translocation across cell membranes (Granby, 2018; Mattsson, 2017).

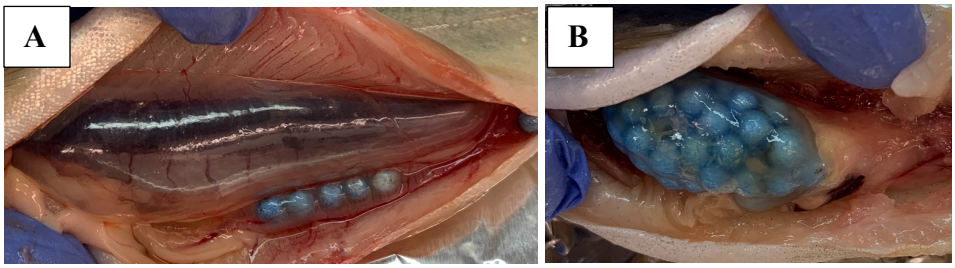

Figure 8. **Microplastics Visible in the Gastrointestinal Tract.** Low exposure fish (A), high exposure fish (B). Both fish died prior to the euthanization date.

Histological analysis of the gastrointestinal tract indicated significant severe damage from microplastics resulting in total disfunction and likely death in the low exposure fish (**4.7312 e^{-08}**). While damage is not uncommon from microplastic exposure, varying degrees of damage exist dependent on life stage, type of plastic, and relative size of fish exposed (Salerno et al., 2021). For example, several studies in zebrafish have indicated damage from exposure, including increased intestinal inflammation, tearing of tissues and intestinal villi, and proliferations of plastics into surrounding tissues (Du, 2020; Fackelmann, 2019; Lu, 2016; Lu, 2018; Lei, 2018; Qiao, 2020). Additionally, in a study by Peda et al. (2016) focusing on the larger European sea bass, severe intestinal damage was found to be consistent with our findings, including detachment and dysfunction in intestinal tissues, loss of tissue structure, and major cellular alterations. Our findings also found that juvenile fish began to die from low concentrations after day 16 of exposure, suggesting that fish may be able to repair intestinal damages from acute but not chronic periods of exposure.

Liver Function

Our study demonstrated significant increases in total GSH concentration in the low exposure group of 10μM/L relative to the control group, suggesting induction of oxidative stress and liver damage following plastic exposure. Total GSH concentration has been shown to increase in response to cell damage from unregulated reactive oxygen species (Srikanth, 2012). As reactive oxygen species and their products increase, enzymatic and nonenzymatic antioxidants such as glutathione and glutathione reductase promptly act to remove and prevent further cellular damage. However, with disfunction

of this coupling relationship, oxidative stress increases, cellular metabolism is reduced, cell death increases, and eventually organismal death occurs (Pena-Llopis, 2003; Srikanth, 2012). Similarly, our results suggest that microplastics likely alter cellular metabolism and coupling of GSH, dependent on rate of microplastic consumption. Studies investigating GSH shifts following metal exposure in fish found increases in total GSH concentration after exposure (Atli, 2008; Srikanth, 2012), and similarly, upregulation of GSH is successful in limiting damage from thermal stress in fishes (Do, 2019; Kim, 2019; Pena-Llopis, 2003; Srikanth, 2012). When exposed to various microplastics, GSH function has been shown to decrease significantly over time in fish as a result of diminished activity following increased ROS production (Lu, 2018; Wan, 2019; Yu, 2018). In contrast, one study by Wen, 2018 showed that exposing 32-40 μm polystyrene microplastics were exposed to blue discus (*Symphysodon aequifasciatus*), GSH concentration values increased significantly. Overall, this suggests that GSH concentration is dependent on exposure period and microplastic size, but also individual's capability of regulating the coupling of GSH and GSSG (Srikanth, 2012). Further, our results suggest that total GSH concentration varied following microplastic exposure likely due to microplastic size and capacity to cope with individual ingestion rates, as these values varied per organism.

With the increase of reactive oxygen species, total SOD activity also acts to prevent anoxic damage to cells and is often widely distributed to prevent disease including neurogenerative disorders (Mattsson, 2017). Found in the majority of animal and plant species, SOD regularly is used to identify oxidative stress levels in wild caught fish relative to pollutants such as metals and insecticides (Dawood, 2020; Naz, 2019;

Pena-Llopis, 2003; Wen, 2018), which increase SOD levels with increased exposure. Similar to these pollutants, our research demonstrates increases in total SOD activity levels due to microplastic exposure, indicating increases in oxidative stress and liver damage. Additionally, studies have shown polystyrene exposure in discus, zebrafish, and Chinese mitten crabs likewise increases total SOD activity following exposure (Lu, 2016; Lu, 2018; Wen, 2018; Yu, 2018). In a study by Lu et al. (2016), polystyrene exposure also increased SOD activity from 5 μm to greater than 70 nm, although larger microplastics also have the ability to decrease nutrient intake and further induce oxidative stress (Lu et al., 2016). Given our study fish additionally showed decreased feeding rates, it is probable that with decreased metabolism, oxidative stress would also increase, resulting in the extreme differences visible in our study.

Increases in oxidative stress have been shown to alter many features of digestion and metabolism, including gut dysbiosis (Fackelmann, 2019), increased inflammation and disease susceptibility, decreased metabolism, and cell apoptosis (Burton, 2011; Sies, 2017; Yu, 2018). As microplastics have illustrated increased damage to intestinal tracts and inflammation, inability to repair such damage from increased oxidative stress has potential to further compromise overall intestinal tissue function and integrity and reduce feeding capacity (Du, 2020). Additionally, increases in oxidative stress can further affect developmental and reproductive functions through the decrease of growth rates and reproductive output (Chowdhury, 2020; Lu, 2016), increase of heavy metal retention (Lu, 2018; Tourinho, 2019), and delay and increase of malformations during development (Chowdhury, 2020). Our study demonstrates that with polystyrene exposure, steelhead trout increase both total SOD activity and GSH concentration values, therefore,

indicating overall increases in oxidative stress as well. With the potential of chronic exposure from microplastics in wild caught and farmed populations, sustained damages from oxidative stress will further inhibit their ability to mitigate damage.

As steelhead trout populations migrate through waterways with various fluctuations of microplastic exposure levels, oxidative stress will also likely fluctuate synonymously. In large rivers, for example, microplastic concentrations vary based on many factors, including turbidity, river size, and distribution within the water column (Liedermann, 2018). With these variations, potential "hot spots" of high microplastic concentrations could rapidly increase oxidative stress within short regions or periods of time. As increases in oxidative stress have been shown to cause damages across metabolic, physiological, and developmental systems (Chowdhury, 2020; Fackelmann, 2019), repeated exposures throughout waterways will likely result in chronic liver damages and eventually in cell death (Chowdhury, 2020; Fackelmann, 2019). Finally, given oxidative stress damage has been shown to affect both farmed and fish populations (Chowdhury, 2020), both groups will likely sustain lasting effects from cellular damage even under acute microplastic exposure conditions.

6. Conclusion

Our study presents the findings that microplastics pose several threats to steelhead trout even at low concentrations of 10 µg/L. Both high and low concentrations of polystyrene within 21 days demonstrated significant die-offs prior to experimental completion and shifts in feeding and swimming behavior. Additionally, low exposure fish showed significant damage to the intestinal tract and increases in oxidative stress levels post exposure. While our research introduces the beginning of negative behavioral and physiological impacts in juvenile steelhead, further research is needed to investigate impacts of multiple plastic polymer types across numerous life stages in large commercial fish.

Seafood accounts for 17% of animal protein consumption worldwide (Smith et al., 2018), and will be critical to accommodate predicted increases in world population and consumption in the future. Our study suggests that high microplastic exposure within 21 days can induce severe intestinal physiological damages, and additional microplastic studies have shown reduced growth (Pena-Llopis, 2003; Srikanth, 2012) and increased disease susceptibility (Brown, 2019) in both wild caught and farmed fish populations. Without mitigation of microplastic exposure, sustained damage and minimal growth will likely result in reduced fish output and production in both farmed and wild caught populations, and further reduce seafood availability over time. As a result, future consumption rates will not be able to be maintained to support increased human population.

Not only are fish important to human consumption, but they are essential to maintaining ecosystem dynamics worldwide. Particularly, large anadromous fish such as

steelhead trout are highly susceptible to microplastic exposure as they interact in both freshwater and saltwater systems and complete annual migrations. Due to this widespread ecosystem interaction, steelhead and other salmonids often establish a critical role as a keystone species and heavily impact several food chains. Therefore, in order to reduce damaging risks from microplastic exposure during migration, microplastic concentrations must be determined across heavily utilized waterways to establish frameworks for management of high-risk areas. After understanding microplastic hotspots, we can limit exposure to these important keystone species throughout the migratory period and assure that surrounding food chains remain supported as well.

Finally, with proper disposal of plastics and additional waste, we can reduce ecosystem integration of plastics into waterways from the source. Through the creation of accessible recycling programs in areas with high human populations and single plastic use, longstanding methods can be established to minimize efflux of plastic into ecosystems. Additionally, with innovation and use of biodegradable plastics, microplastic decomposition rates will decrease and plastic accumulation will reduce over time. With centralized effort in recycling and reusing plastic products, we can limit risk and damage in highly concentrated areas and assure that commercial fish such as trout will be conserved well into the future.

References

Alimba, C. G., & Faggio, C. (2019). Microplastics in the marine environment: Current trends in environmental pollution and mechanisms of toxicological profile. *Environmental toxicology and pharmacology*, *68*, 61-74.

Amato-Lourenço, L. F., dos Santos Galvão, L., de Weger, L. A., Hiemstra, P. S., Vijver, M. G., & Mauad, T. (2020). An emerging class of air pollutants: potential effects of microplastics to respiratory human health?. *Science of the total environment*, *749*, 141676.

Anbumani, S., & Kakkar, P. (2018). Ecotoxicological effects of microplastics on biota: a review. *Environmental Science and Pollution Research*, *25*, 14373-14396.

Atli, G., & Canli, M. (2008). Responses of metallothionein and reduced glutathione in a freshwater fish Oreochromis niloticus following metal exposures. *Environmental toxicology and pharmacology*, *25*(1), 33-38.

Baechler, B. R., Granek, E. F., Hunter, M. V., & Conn, K. E. (2020). Microplastic concentrations in two Oregon bivalve species: Spatial, temporal, and species variability. *Limnology and Oceanography Letters*, *5*(1), 54-65.

Baechler, B. R., Stienbarger, C. D., Horn, D. A., Joseph, J., Taylor, A. R., Granek, E. F., & Brander, S. M. (2020). Microplastic occurrence and effects in commercially harvested North American finfish and shellfish: Current knowledge and future directions. *Limnology and Oceanography Letters*, *5*(1), 113-136.

Baekeland, LH, "The synthesis, constitution, and uses of Bakelite" (1909).). Journal of Industrial and Engineering Chemistiry-US 3, pp. 149-161.

Baekeland, LH, "Recent developments in Bakelite" (1911). Journal of Industrial and Engineering Chemistiry-US 3, pp. 932-938.

Barboza, L. G. A., Vieira, L. R., Branco, V., Figueiredo, N., Carvalho, F., Carvalho, C., & Guilhermino, L. (2018). Microplastics cause neurotoxicity, oxidative damage and energy-related changes and interact with the bioaccumulation of mercury in the European seabass, Dicentrarchus labrax (Linnaeus, 1758). *Aquatic toxicology*, *195*, 49-57.

Bell, M. L., & Ebisu, K. (2012). Environmental inequality in exposures to airborne particulate matter components in the United States. *Environmental health perspectives*, *120*(12), 1699-1704.

Brown, R. M., Wiens, G. D., & Salinas, I. (2019). Analysis of the gut and gill microbiome of resistant and susceptible lines of rainbow trout (Oncorhynchus mykiss). *Fish & shellfish immunology*, *86*, 497-506.

Browne, M.A., Crump, P., Niven, S.J., Teuten, E., Tonkin, A., Galloway, T., Thompson, R., (2011). Accumulation of microplastic on shorelines worldwide: sources and sinks. Environ. Sci. Technol. 45, 9175e9179. https://doi.org/10.1021/ es201811s.

Burton, G. J., & Jauniaux, E. (2011). Oxidative stress. *Best practice & research Clinical obstetrics & gynaecology*, *25*(3), 287-299.

Chen, Q., Lackmann, C., Wang, W., Seiler, T. B., Hollert, H., & Shi, H. (2020). Microplastics lead to hyperactive swimming behaviour in adult zebrafish. *Aquatic Toxicology*, *224*, 105521.

Chiba, R., Fujinuma, R., Yoshitomi, T., Shimizu, Y., & Kobayashi, M. (2023). Ingestion of rubber tips of artificial turf fields by goldfish. *Scientific Reports*, *13*(1), 1344.

Chowdhury, S., & Saikia, S. K. (2020). Oxidative stress in fish: a review. *Journal of Scientific Research*, *12*(1), 145-160.

Choy, C. A., & Drazen, J. C. (2013). Plastic for dinner? Observations of frequent debris ingestion by pelagic predatory fishes from the central North Pacific. *Marine Ecology Progress Series*, *485*, 155-163.

Clark, J. J., Renegar, D. E. A., & Kerstetter, D. (2022). Ubiquitous microplastics in the upper gastrointestinal tracts of Florida coastal seabirds. *Florida Scientist*, *85*(3-4), 91.

Collicutt, B., Juanes, F., & Dudas, S. E. (2019). Microplastics in juvenile Chinook salmon and their nearshore environments on the east coast of Vancouver Island. *Environmental Pollution*, *244*, 135-142.

Conte, F. S. (2004). Stress and the welfare of cultured fish. *Applied Animal Behaviour Science*, *86*(3-4), 205-223.

Crowder, K., & Downey, L. (2010). Interneighborhood migration, race, and environmental hazards: Modeling microlevel processes of environmental inequality. *American journal of sociology*, *115*(4), 1110-1149.

Dawood, M. A., Abdel-Tawwab, M., & Abdel-Latif, H. M. (2020). Lycopene reduces the impacts of aquatic environmental pollutants and physical stressors in fish. *Reviews in Aquaculture*, *12*(4), 2511-2526.

Derraik, J. G. (2002). The pollution of the marine environment by plastic debris: a review. *Marine pollution bulletin*, *44*(9), 842-852.

Do, T. D., Thi Mai, N., Duy Khoa, T. N., Abol-Munafi, A. B., Liew, H. J., Kim, C. B., & Wong, L. L. (2019). Molecular characterization and gene expression of glutathione peroxidase 1 in tor tambroides exposed to temperature stress. *Evolutionary Bioinformatics*, *15*, 1176934319853580.

Du, J., Xu, S., Zhou, Q., Li, H., Fu, L., Tang, J., ... & Du, X. (2020). A review of microplastics in the aquatic environmental: distribution, transport, ecotoxicology, and toxicological mechanisms. *Environmental Science and Pollution Research*, 1-12.

Fackelmann, G., & Sommer, S. (2019). Microplastics and the gut microbiome: how chronically exposed species may suffer from gut dysbiosis. *Marine pollution bulletin*, *143*, 193-203.

Fendall, L. S., & Sewell, M. A. (2009). Contributing to marine pollution by washing your face: microplastics in facial cleansers. *Marine pollution bulletin*, *58*(8), 1225-1228.

Foekema, E. M., De Gruijter, C., Mergia, M. T., van Franeker, J. A., Murk, A. J., & Koelmans, A. A. (2013). Plastic in north sea fish. *Environmental science & technology*, *47*(15), 8818-8824.

Foley, C. J., Feiner, Z. S., Malinich, T. D., & Höök, T. O. (2018). A meta-analysis of the effects of exposure to microplastics on fish and aquatic invertebrates. *Science of the total environment*, *631*, 550-559.

Granby, K., Rainieri, S., Rasmussen, R. R., Kotterman, M. J., Sloth, J. J., Cederberg, T. L., ... & Larsen, B. K. (2018). The influence of microplastics and halogenated contaminants in feed on toxicokinetics and gene expression in European seabass (Dicentrarchus labrax). *Environmental research*, *164*, 430-443.

Gigault, J., Ter Halle, A., Baudrimont, M., Pascal, P. Y., Gauffre, F., Phi, T. L., ... & Reynaud, S. (2018). Current opinion: what is a nanoplastic?. *Environmental pollution*, *235*, 1030-1034.

Gourmelon, G. (2015). Global plastic production rises, recycling lags. *Vital Signs*, *22*, 91-95.

Guimarães, A. T. B., de Lima Rodrigues, A. S., Pereira, P. S., Silva, F. G., & Malafaia, G. (2021). Toxicity of polystyrene nanoplastics in dragonfly larvae: An insight on how these pollutants can affect bentonic macroinvertebrates. *Science of The Total Environment*, *752*, 141936.

Guo, J. J., Huang, X. P., Xiang, L., Wang, Y. Z., Li, Y. W., Li, H., ... & Wong, M. H. (2020). Source, migration and toxicology of microplastics in soil. *Environment international*, *137*, 105263.

Hopewell, J., Dvorak, R., & Kosior, E. (2009). Plastics recycling: challenges and opportunities. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *364*(1526), 2115-2126.

Jambeck, J. R., Geyer, R., Wilcox, C., Siegler, T. R., Perryman, M., Andrady, A., ... & Law, K. L. (2015). Plastic waste inputs from land into the ocean. *Science*, *347*(6223), 768-771.

Jeong, C. B., Won, E. J., Kang, H. M., Lee, M. C., Hwang, D. S., Hwang, U. K., ... & Lee, J. S. (2016). Microplastic size-dependent toxicity, oxidative stress induction, and p-JNK and p-p38 activation in the monogonont rotifer (Brachionus koreanus). *Environmental science & technology*, *50*(16), 8849-8857.

Karami, A., Romano, N., Galloway, T., & Hamzah, H. (2016). Virgin microplastics cause toxicity and modulate the impacts of phenanthrene on biomarker responses in African catfish (Clarias gariepinus). *Environmental research*, *151*, 58-70.

Keeley, E. R. (2001). Demographic responses to food and space competition by juvenile steelhead trout. *Ecology*, *82*(5), 1247-1259.

Kim, S., Kim, A., Ma, S., Lee, W., Lee, S., Yoon, D., ... & Kim, S. (2019). Glutathione injection alleviates the fluctuation of metabolic response under thermal stress in olive flounder, Paralichthys olivaceus. *Metabolites*, *10*(1), 3.

Kumar, R., Verma, A., Shome, A., Sinha, R., Sinha, S., Jha, P. K., ... & Vara Prasad, P. V. (2021). Impacts of plastic pollution on ecosystem services, sustainable development goals, and need to focus on circular economy and policy interventions. *Sustainability*, *13*(17), 9963.

Lebreton, L., Slat, B., Ferrari, F., Sainte-Rose, B., Aitken, J., Marthouse, R., ... & Reisser, J. (2018). Evidence that the Great Pacific Garbage Patch is rapidly accumulating plastic. *Scientific reports*, *8*(1), 1-15.

Le Guen, C., Suaria, G., Sherley, R. B., Ryan, P. G., Aliani, S., Boehme, L., & Brierley, A. S. (2020). Microplastic study reveals the presence of natural and synthetic fibres in the diet of King Penguins (Aptenodytes patagonicus) foraging from South Georgia. *Environment international*, *134*, 105303.

Lehner, R., Weder, C., Petri-Fink, A., & Rothen-Rutishauser, B. (2019). Emergence of nanoplastic in the environment and possible impact on human health. *Environmental science & technology*, *53*(4), 1748-1765.

Lei, L., Wu, S., Lu, S., Liu, M., Song, Y., Fu, Z., ... & He, D. (2018). Microplastic particles cause intestinal damage and other adverse effects in zebrafish Danio rerio and nematode Caenorhabditis elegans. *Science of the Total Environment*, *619*, 1-8.

Li, B., Liang, W., Liu, Q. X., Fu, S., Ma, C., Chen, Q., ... & Shi, H. (2021). Fish ingest microplastics unintentionally. *Environmental Science & Technology*, *55*(15), 10471-10479.

Liedermann, M., Gmeiner, P., Pessenlehner, S., Haimann, M., Hohenblum, P., & Habersack, H. (2018). A methodology for measuring microplastic transport in large or medium rivers. *Water*, *10*(4), 414.

Limonta, G., Mancia, A., Benkhalqui, A., Bertolucci, C., Abelli, L., Fossi, M. C., & Panti, C. (2019). Microplastics induce transcriptional changes, immune response and behavioral alterations in adult zebrafish. *Scientific reports*, *9*(1), 1-11.

Lu, Y., Zhang, Y., Deng, Y., Jiang, W., Zhao, Y., Geng, J., ... & Ren, H. (2016). Uptake and accumulation of polystyrene microplastics in zebrafish (Danio rerio) and toxic effects in liver. *Environmental science & technology*, *50*(7), 4054-4060.

Lu, K., Qiao, R., An, H., & Zhang, Y. (2018). Influence of microplastics on the accumulation and chronic toxic effects of cadmium in zebrafish (Danio rerio). *Chemosphere*, *202*, 514-520.

Ma, P., Wei Wang, M., Liu, H., Feng Chen, Y., & Xia, J. (2019). Research on ecotoxicology of microplastics on freshwater aquatic organisms. *Environmental Pollutants and Bioavailability*, *31*(1), 131-137.

Malafaia, G., da Luz, T. M., Guimarães, A. T. B., & da Costa Araújo, A. P. (2020). Polyethylene microplastics are ingested and induce biochemical changes in Culex quinquefasciatus (Diptera: Culicidae) freshwater insect larvae. *Ecotoxicology and Environmental Contamination*, *15*(1), 79-89.

Mattsson, K., Johnson, E. V., Malmendal, A., Linse, S., Hansson, L. A., & Cedervall, T. (2017). Brain damage and behavioural disorders in fish induced by plastic nanoparticles delivered through the food chain. *Scientific reports*, *7*(1), 11452.

McCormick, A. R., Hoellein, T. J., London, M. G., Hittie, J., Scott, J. W., & Kelly, J. J. (2016). Microplastic in surface waters of urban rivers: concentration, sources, and associated bacterial assemblages. *Ecosphere*, *7*(11), e01556.

Naidu, R., Biswas, B., Willett, I. R., Cribb, J., Singh, B. K., Nathanail, C. P., ... & Aitken, R. J. (2021). Chemical pollution: A growing peril and potential catastrophic risk to humanity. *Environment International*, *156*, 106616.

Naz, H., Abdullah, S., Abbas, K., Hassan, W., Batool, M., Perveen, S., ... & Mushtaq, S. (2019). Toxic effect of insecticides mixtures on antioxidant enzymes in different organs of fish, Labeo rohita. *Pakistan Journal of Zoology*, *51*(4), 1355.

Peda, C., Caccamo, L., Fossi, M. C., Gai, F., Andaloro, F., Genovese, L., ... & Maricchiolo, G. (2016). Intestinal alterations in European sea bass Dicentrarchus labrax (Linnaeus, 1758) exposed to microplastics: preliminary results. *Environmental pollution*, *212*, 251-256.

Peña-Llopis, S., Ferrando, M. D., & Peña, J. B. (2003). Fish tolerance to organophosphate-induced oxidative stress is dependent on the glutathione metabolism and enhanced by N-acetylcysteine. *Aquatic Toxicology*, *65*(4), 337-360.

Qiao, R., Sheng, C., Lu, Y., Zhang, Y., Ren, H., & Lemos, B. (2019). Microplastics induce intestinal inflammation, oxidative stress, and disorders of metabolome and microbiome in zebrafish. *Science of the Total Environment*, *662*, 246-253.

Ren, Y., Shi, L., Bardow, A., Geyer, R., & Suh, S. (2020). Life-cycle environmental implications of China's ban on post-consumer plastics import. *Resources, Conservation and Recycling*, *156*, 104699.

Redondo-Hasselerharm, P. E., Gort, G., Peeters, E. T. H. M., & Koelmans, A. A. (2020). Nano-and microplastics affect the composition of freshwater benthic communities in the long term. *Science advances*, *6*(5), eaay4054.

Reynolds, C., & Ryan, P. G. (2018). Micro-plastic ingestion by waterbirds from contaminated wetlands in South Africa. *Marine pollution bulletin*, *126*, 330-333.

Rillig, M. C., Leifheit, E., & Lehmann, J. (2021). Microplastic effects on carbon cycling processes in soils. *PLoS Biology*, *19*(3), e3001130.

Salerno, M., Berlino, M., Mangano, M. C., & Sarà, G. (2021). Microplastics and the functional traits of fishes: A global meta-analysis. *Global Change Biology*, *27*(12), 2645-2655.

Samoli, E., Stergiopoulou, A., Santana, P., Rodopoulou, S., Mitsakou, C., Dimitroulopoulou, C., ... & Euro-Healthy Consortium. (2019). Spatial variability in air pollution exposure in relation to socioeconomic indicators in nine European metropolitan areas: A study on environmental inequality. *Environmental Pollution*, *249*, 345-353.

Scheirs, J. (2003). Historical overview of styrenic polymers. *Modern styrenic polymers: Polystyrenes and styrenic copolymers*, 1-24.

Sies, H., Berndt, C., & Jones, D. P. (2017). Oxidative stress. *Annual review of biochemistry*, *86*, 715-748.

Silva, C. J., Silva, A. L. P., Gravato, C., & Pestana, J. L. (2019). Ingestion of small-sized and irregularly shaped polyethylene microplastics affect Chironomus riparius life-history traits. *Science of the Total Environment*, *672*, 862-868.

Silva, C. J., Silva, A. L. P., Campos, D., Soares, A. M., Pestana, J. L., & Gravato, C. (2021). Lumbriculus variegatus (oligochaeta) exposed to polyethylene microplastics: biochemical, physiological and reproductive responses. *Ecotoxicology and Environmental Safety*, *207*, 111375.

Smith, M., Love, D. C., Rochman, C. M., & Neff, R. A. (2018). Microplastics in seafood and the implications for human health. *Current environmental health reports*, *5*, 375-386.

Srikanth, K., Pereira, E., Duarte, A. C., & Ahmad, I. (2013). Glutathione and its dependent enzymes' modulatory responses to toxic metals and metalloids in fish—a review. *Environmental Science and Pollution Research*, *20*, 2133-2149.

Susanti, N. K. Y., Mardiastuti, A., & Wardiatno, Y. (2020, July). Microplastics and the impact of plastic on wildlife: a literature review. In *IOP Conference Series: Earth and Environmental Science* (Vol. 528, No. 1, p. 012013). IOP Publishing.

Sussarellu, R., Suquet, M., Thomas, Y., Lambert, C., Fabioux, C., Pernet, M. E. J., ... & Huvet, A. (2016). Oyster reproduction is affected by exposure to polystyrene microplastics. *Proceedings of the national academy of sciences*, *113*(9), 2430-2435.

Tait, P. W., Brew, J., Che, A., Costanzo, A., Danyluk, A., Davis, M., ... & Bowles, D. (2020). The health impacts of waste incineration: a systematic review. *Australian and New Zealand journal of public health*, *44*(1), 40-48.

Tibbetts, J., Krause, S., Lynch, I., & Sambrook Smith, G. H. (2018). Abundance, distribution, and drivers of microplastic contamination in urban river environments. *Water*, *10*(11), 1597.

Tourinho, P. S., Kočí, V., Loureiro, S., & van Gestel, C. A. (2019). Partitioning of chemical contaminants to microplastics: Sorption mechanisms, environmental distribution and effects on toxicity and bioaccumulation. *Environmental Pollution*, *252*, 1246-1256.

Van Franeker, J. A. (1985). Plastic ingestion in the North Atlantic fulmar. *Marine Pollution Bulletin*, *16*(9), 367-369.

Walkinshaw, C., Lindeque, P. K., Thompson, R., Tolhurst, T., & Cole, M. (2020). Microplastics and seafood: lower trophic organisms at highest risk of contamination. *Ecotoxicology and Environmental Safety*, *190*, 110066.

Wan, Z., Wang, C., Zhou, J., Shen, M., Wang, X., Fu, Z., & Jin, Y. (2019). Effects of polystyrene microplastics on the composition of the microbiome and metabolism in larval zebrafish. *Chemosphere*, *217*, 646-658.

Wang, C., Zhang, D., Huang, H., Zhang, L., Zhang, C., & Shao, Q. (2018). Review of microplastics detection from marine organisms and their harms. *Journal of Food Safety and Quality*, *9*(11), 2678-2683.

Wang, L., Nabi, G., Yin, L., Wang, Y., Li, S., Hao, Z., & Li, D. (2021). Birds and plastic pollution: recent advances. *Avian Research*, *12*, 1-9.

Wilcox, C., Van Sebille, E., & Hardesty, B. D. (2015). Threat of plastic pollution to seabirds is global, pervasive, and increasing. *Proceedings of the national academy of sciences*, *112*(38), 11899-11904.

Wright, S. L., & Kelly, F. J. (2017). Plastic and human health: a micro issue?. *Environmental science & technology*, *51*(12), 6634-6647.

Yin, L., Jiang, C., Wen, X., Du, C., Zhong, W., Feng, Z., ... & Ma, Y. (2019). Microplastic pollution in surface water of urban lakes in Changsha, China. *International Journal of Environmental Research and Public Health*, *16*(9), 1650.

Yin, L., Liu, H., Cui, H., Chen, B., Li, L., & Wu, F. (2019). Impacts of polystyrene microplastics on the behavior and metabolism in a marine demersal teleost, black rockfish (Sebastes schlegelii). *Journal of hazardous materials*, *380*, 120861.

Yong, C. Q. Y., Valiyaveettil, S., & Tang, B. L. (2020). Toxicity of microplastics and nanoplastics in mammalian systems. *International Journal of Environmental Research and Public Health*, *17*(05), 1509.

Yuji Sado, R., Rodolfo Behr, E., René Eslava Mocha, P., Baldisserotto, B., Baldisserotto, B., Criscuolo Urbinati, E., & Cyrino, J. E. P. (2020). Chapter 2 - Anatomy of Teleosts and elasmobranchs. In F. Carlos de Souza (Ed.), *Biology and Physiology of Freshwater Neotropical Fish* (pp. 21–47). essay, Academic Press.

Zhang, T., Song, K., Meng, L., Tang, R., Song, T., Huang, W., & Feng, Z. (2022). Distribution and characteristics of microplastics in barnacles and wild bivalves on the coast of the Yellow Sea, China. *Frontiers in Marine Science*, *8*, 1988.

Zhang, Y., Wang, D., Yin, K., Zhao, H., Lu, H., Meng, X., ... & Xing, M. (2022). Endoplasmic reticulum stress-controlled autophagic pathway promotes polystyrene microplastics-induced myocardial dysplasia in birds. *Environmental Pollution*, *311*, 119963.

Zhao, T., Lozano, Y. M., & Rillig, M. C. (2021). Microplastics increase soil pH and decrease microbial activities as a function of microplastic shape, polymer type, and exposure time. *Frontiers in Environmental Science*, *9*, 675803.

Ziajahromi, S., Kumar, A., Neale, P. A., & Leusch, F. D. (2018). Environmentally relevant concentrations of polyethylene microplastics negatively impact the survival, growth and emergence of sediment-dwelling invertebrates. *Environmental Pollution*, *236*, 425-431.

Zantis, L. J., Carroll, E. L., Nelms, S. E., & Bosker, T. (2021). Marine mammals and microplastics: a systematic review and call for standardisation. *Environmental Pollution*, *269*, 116142.

Zimmerli, S., Bernet, D., Burkhardt-Holm, P., Schmidt-Posthaus, H., Vonlanthen, P., Wahli, T., Segner, H., 2007. Assessment of fish health status in four Swiss rivers showing a decline of brown trout catches. Aquat. Sci. 69, 11-25.