Bioaccumulative and Toxic Effects of Ingested Clean and PBT-Saturated Microplastics on Oryzias latipes (Japanese Medaka Fish): Method Development towards Physiological and Chemical Analysis

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by

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_____ **(Thesis Reader)**

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1. INTRODUCTION

1.1. MARINE PLASTIC POLLUTION

In recent decades, plastic has become the magic bullet of industrial production, highly valued for its durability, cost-effective synthesis, and utility as a packaging material (Gregory 2009; Teuten *et al.* 2009; Andrady 2011). Ever since the development of the first fully-synthetic plastic by Leo Baekeland in 1907 (Baekeland 1909) and its subsequent chemical refinement, plastic has come to fulfill myriad industrial, technological, textile, and packaging functions, used in everything from food containers and automobiles to medical procedures and children's toys. As a synthetic organic polymer derived from petrochemicals, plastic is a particularly resilient building material and has come to displace many traditional materials such as wood, stone, glass, leather, metal, and ceramic due to its malleability, ease of manufacture, and resistance to water (Thompson *et al.* 2009). This strength and impermeability also renders it a particularly valuable material in food and drinking containers, shopping bags, architectural siding, and even as scrubbing exfoliants in certain facial cleansers (Thompson *et al.* 2009). Furthermore, plastic mixes well with other chemical derivatives, resulting in an ever-expanding diversity of industrial and production uses (Thompson *et al.* 2009; Wu *et al.* 2013).

This proliferation of plastic production along with a greater understanding of its chemical impermeability has given rise to increasing environmental concerns regarding its slow decomposition and its tendency to be discarded in landfills in large quantities. Indeed, the birth of the recycling movement was largely in response to scientific and public recognition of plastic as an environmental contaminant, facilitating a public attempt to reduce the amount of plastic waste being generated (Wu *et al.* 2013). Nonetheless, recent plastic optimization techniques and higher product demand have rapidly accelerated global plastic production to nearly 300 million metric tons annually (Moore 2008). Of the plastic that is discarded, only about 6.5% is recycled into new materials and only 7.7% is used to generate electrical and heat energy; an estimated 30 million metric tons are projected to end up in landfills or leak directly into water systems (Moore 2008; Andrady 2011). According to environmental scientists at Columbia University, low recycling rates are likely due to inefficiencies within the recycling process (exacerbated by plastic waste contamination and confusing coding designations by the Society of Plastic Industries) and public ignorance of correct recycling practices (Thompson *et al.* 2009; Wu *et al.* 2013).

The extent of plastic waste has particular implications for aquatic and marine environments, which constitutes the second largest sink for discarded plastic. While most plastic debris is disposed of in landfills, about 10% is ultimately deposited in global marine and aquatic ecosystems via secondary leakage in water systems or directly into the marine and beach environment (Moore 2008; Cole *et al.* 2011). Indeed, plastic debris is now so ubiquitous it is thought to contaminate almost every aquatic system on earth, accumulating along coastal regions and in high-nutrient current swells where it becomes available

for ingestion by marine organisms (Andrady 2011; Ivar do Sul and Costa 2014). Empirical evidence of this contamination may be observed in the Pacific trash vortex, also described as the Great Pacific Garbage Patch, which constitutes a gyre of marine debris composed largely of small plastic particulates suspended just below the surface and covering an estimated area larger than the size of Texas (700,000 - 15,000,000 km^2) (Rios *et al.* 2010). These plastic particles are drawn and concentrated by ocean currents from the North Pacific Ocean off the coasts of the United States and Japan, indicating the significant transporting role that oceanic movement patterns play in the distribution of plastic waste (Ivar do Sul and Costa 2014). Also important to note is the drastic difference in relative abundances of larger plastic flotsam and smaller plastic particles comprising the Pacific trash vortex, complicating common public perceptions of marine plastic pollution. Rather than grocery bags and coke-can rings, it is the high concentration of small plastic particulates – called *microplastics* – floating in the neustonic zone that comprises the vast majority of the Garbage Patch and offers evidence of their critical significance as a marine contaminant. Microplastics and their hazardous environmental effects form the subject of this dissertation.

1.2. MICROPLASTICS AS OCEAN CONTAMINANTS

1.2.1. Discovery of Microplastic in the Oceans

As evidenced by the Pacific trash vortex, the class of contaminants called microplastics are major components of global accumulation of plastic debris (Ivar do Sul and Costa 2014). In spite of this, their characterization and identification has occurred only relatively recently within the scientific and environmental communities. Microplastics as ocean contaminants were first characterized by Carpenter and Smith in 1972, who observed large quantities of small plastic particulate floating in the neustonic zone of the North Atlantic Ocean (Ivar do Sul and Costa 2014). Frequent observations of microplastics in marine environments were made subsequently, prompting Thompson *et al.* (2004) to attempt a comprehensive categorization of different types of microplastic by type. According to their findings, one third of non-natural particulate matter collected off the coast of Plymouth, UK was identified as synthetic polymers, most of which were fragments derived from clothing, packaging, and rope, providing among the first evidence of plastic particulate breakdown in the ocean (Thompson *et al.* 2004). However, in spite of a growing number of studies observing small plastic debris in marine environments, microplastic was not recognized as a distinct class of pollution until 2008, when it was subdivided into macro (>50 mm in diameter), meso (~50-5 mm), and micro (<5 mm) pieces by Arthur *et al.* (2009). This formal designation – along with a host of subsequent studies examining its toxic effects on marine habitat and biota – rapidly hastened the examination of microplastic as an environmental pollutant and remains its defining characteristic.

1.2.2. Current Definitions and Characterizations

In light of Arthur's designation, the National Oceanic and Atmospheric Administration now formally recognizes microplastics as small plastic particles <5 mm in diameter (NOAA 2016); however, further characterization has diversified them in terms of anthropogenic origin, shape, size, density, and type, making them particularly difficult to study in the marine environment (Ivar do Sul and Costa 2014). Microplastics may be derived from a variety of industrial and postconsumer sources, including but not limited to exfoliating cosmetic products, chemical run-off from processing facilities, and most significantly, *in-situ* degradation of larger plastic pieces deposited along shorelines and at sea (Cole *et al.* 2011; Andrady 2011). Those particles which exist and are deposited in their original microplastic form (e.g., scrubbing beads in facial cleansers and pre-industrial "nurdles" used as thermoplastic processing feedstocks) are given the designation "primary microplastics" while those created by the weathering and photodegradation of larger plastic pieces are designated "secondary microplastics" (Moore 2008; Cole *et al.* 2011). Furthermore, microplastics may assume a variety of physical shapes (such as fragmented shards or shavings, filaments, pellets, and spherical beads) and sizes (anywhere from between 5 mm and <1 mm in diameter), depending on the source of plastic production (Hidalgo-Ruz *et al*. 2014). According to a recent review conducted by Ivar do Sul and Costa (2014), a majority of secondary plastic particles appear as filaments (derived from clothing, ropes, fishing lines, and other sources of synthetic fibers) and fragments degraded from larger macroplastic sources. Additionally, synthetic polymers vary based on their density to water; while most are buoyant enough to float on the surface, those that are denser than seawater tend to sink into lower ocean strata and even the seabed, making them even more difficult to track and identify in the ocean environment (Thompson *et al*. 2009; Andrady 2011; Ivar do Sul and Costa 2014).

Plastic materials exist in a variety of types, including highly-versatile polymers used for packaging and preindustrial building materials such as polyethylene (PE), polypropylene (PP), and polyvinyl chloride (PVC) that respectively constitute 38%, 24%, and 19% of global plastic production (Andrady 2011; Figure 1). Polyethylene in particular is a critical and increasing source of microplastic contamination; with over 80 million tons of polyethylene generated each year, it is by far the most common plastic used in industrial and packaging production (Andrady and Neal 2009). In its unreactive form, polyethylene is a white waxy plastic composed of a series of nonpolar saturated hydrocarbon chains, rendering it particularly stable, hydrophobic, and resistant to chemical and physical degradation (Andrady 2011; Cole *et al.* 2011; Figure 2). It also polymerizes at mild temperatures and pressures, making it an attractive plastic for industrial use due to its ease of manufacturing (Andrady 2011). For these reasons, polyethylene has emerged as a target contaminant for microplastic studies in the marine environment.
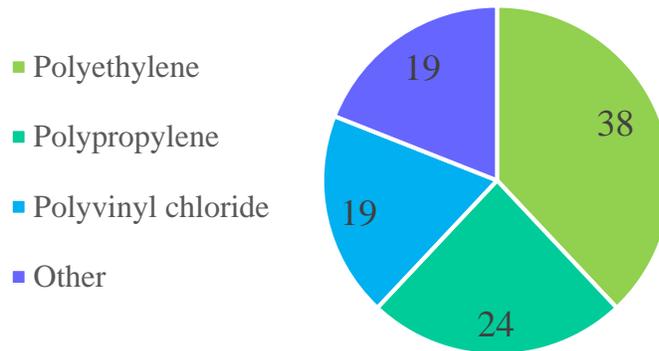
Figure 1. Global plastic production by plastic type. Numbers represent percentage of the total comprised by each plastic type.
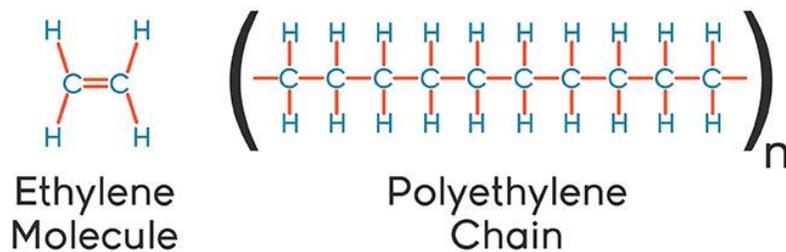
Figure 2. Ethylene molecule and polyethylene chain with repeating subunits, *n* (obtained from learner.org).

Microplastics evidently comprise a diverse assemblage of plastic particulates that vary widely in origin, shape, size, density, and chemical composition; subsequently, they are very difficult to identify within the marine environment (Hidalgo-Ruz *et al.* 2012). Additionally, microplastics have a widespread geographic distribution given their diversity in origin (industrial facilities, water systems, litter deposits) and the far-reaching influence of oceanic currents, factors further complicating their accurate quantification (Hidalgo-Ruz *et al.* 2012). Therefore, a multidisciplinary approach to their observation and quantification is necessary in attempting to understand the scope of their contamination and subsequent environmental effects.

1.2.3. Entrance into the Marine Environment

Microplastics enter the marine environment via a variety of different processes and pathways. Primary microplastics (those already in microplastic form) often enter the marine environment via transport by public water systems, direct runoff from industrial processing facilities, and spills from

shipping containers; these usually occur in the form of postconsumer facial cleansing scrubbing beads and pellets used in the production of manufactured plastic products (Hidalgo-Ruz *et al.* 2012). Microplastics from secondary sources – those derived from the degradation of larger discarded plastic pieces – often occur as fragments and filaments shaped from the inconsistent nature of weathering processes (Hidalgo-Ruz *et al*. 2012). Larger beached plastics are exposed to a variety of environmental forces that facilitate their degradation into smaller plastic particles. In particular, direct exposure to sunlight, high oxygen availability, and constant tidal action of beached plastics corrupt their structural and chemical integrity and render them susceptible to fragmentation (Cole *et al.* 2011; Teuten *et al*. 2009). Additionally, inadvertent plastic deposition from marine vessels contributes to microplastic pollution; everything from the synthetic fishing line used in small-scale fishing activities to the displaced cargo and litter from large container ships may directly enter the marine system and become subjected to the processes of photodegradation and physical weathering by ocean movement (Thompson *et al.* 2009).

The resulting small plastic particulates are then carried into ocean systems and concentrate in the neustonic zone in the upper water column, where they may easily enter the food chain via contamination of or ingestion by marine organisms (Wright *et al.* 2013; Cole *et al.* 2011; Andrady 2011). Denser microplastics may sink deeper below the surface, where they are more accessible to a greater variety of marine life and trophic levels; according recent estimates, a little more than half of all primary microplastics sink in seawater (Andrady 2011; Moore 2008). Collectively, these microplastic particles are projected to contaminate a majority of the world's oceans at a few fragments per hectare of ocean surface (with greater concentrations in areas of higher anthropogenic pollution and presence) (Barnes *et al.* 2009; Ivar do Sul and Costa 2014). Indeed, recent marine surveys in Puget Sound have found microplastic contamination in benthic communities such as forage fish and mussel populations, indicating the prevalence of microplastics throughout the water column and marine environment (von Moos *et al.* 2012; Oliveira *et al*. 2013; Lyon 2014; Mitchell 2015). This prevalence is especially concerning given microplastic's recent identification as a transference vector for environmental toxins into marine biotic tissue (Mato *et al.* 2001; Yamashita *et al.* 2011; Rochman *et al.* 2013).

1.2.4. Hazards for Marine Environments and Biota

Microplastics in the marine environment pose a host of serious environmental hazards for ecosystem and organismal health. Due to their light weight, microplastics are often subject to the movements of ocean currents and may be transported thousands of miles away from their entrance point, resulting in widespread contamination globally as well as significant changes in marine substrate locally (Moore 2008; Barnes et al. 2009). This widespread transport of microplastics also has been implicated in the spread of invasive species; a study conducted by Goldstein *et al*. (2012) found that increased

abundance of the insect species *Halobates sericeus* in the North Pacific Ocean was significantly correlated with microplastic contamination, implicating microplastic as a possible vector for egg oviposition (Goldstein *et al*. 2012; Majer *et al*. 2012; Ivar do Sul and Costa 2014). Therefore, patterns of microplastic contamination may increase the population density and mobility of species in areas where they were not previously, possibly disrupting predation and resource allocation in localized food webs. Furthermore, photodegradation of microplastic may have significant environmental consequences through the release of primary constituent chemicals (used to enhance plastic quality and durability), which may alter ocean chemistry and render conditions unsuitable for resident marine biota (Andrady 2011).

Microplastics also impact marine organisms directly through ingestion and subsequent blockage and contamination of internal systems (Teuten *et al.* 2009). Microplastics may remain suspended in the upper water column for long periods of time, where they may be directly ingested by marine predators or inadvertently consumed by filter-feeding organisms (Cole *et al*. 2011; Ivar do Sul and Costa 2014). Furthermore, most secondary microplastics assume a variety of different shapes and colors, making them attractive food items for marine predators such as fish and seabirds (Oliveira *et al*. 2013). Indeed, numerous studies have found evidence of microplastic consumption by vertebrates and invertebrates alike, particularly predator species that pursue prey along the surface and filter-feeders that filter nutrients at lower depths (Ivar do Sul and Costa 2014). University of Puget Sound student Bonnie Wirth (2014) found evidence of plastic in the stomachs of all eight species of forage fish studied from Washington State, confirming the presence of plastic via ingestion, a process that potentially disrupts digestive function. Similarly, a study conducted by University of Puget Sound student Olivia Feinstein (2013) found higher levels of brightly-colored high-density polyethylene in northern fulmar stomachs than any other plastic type, indicating its attractiveness as a target prey item. Microplastic also has been identified in a number of filter-feeding organisms, namely mussels and oysters (Lyon 2014; Mitchell 2015; Sassarulu *et al*. 2016). The sheer number of published studies dedicated to quantifying microplastic ingestion in marine organisms further speaks to the rate at which it is consumed (26 vertebrate studies and 11 invertebrate studies in 2013) (Ivar do Sul and Costa 2014).

After ingestion, microplastics may harm marine organisms by physical blockage or damage of the respiratory, digestive, reproductive, hepatic, and other internal systems, resulting in physiological stress, permanent injury, and death (Andrady 2011). Recently-discovered chemical hazards associated with microplastic ingestion pose a more subtle but just as potent threat to organismal and ecosystem health. These hazards include 1) the leaching of primary chemical constituents into biotic tissue post-digestion and 2) accumulation of adsorbed environmental chemicals from the surrounding ocean matrix in biotic tissue and organ systems (Nliml and Oliver 1989; Cole *et al*. 2011; Oliveira *et al*. 2013; Tanaka *et al*. 2013). These latter two mechanisms – leaching of primary raw chemicals from "clean" microplastics and

deposition of pollutant chemicals by contaminated microplastics – are the two most implicated but often overlooked pathways of chemical transference.

1.2.5. Vectors for Persistent Bioaccumulative Toxins

Direct leaching of existing primary chemical constituents into biotic tissue is an inherent risk associated with microplastic consumption. If microplastics remain and accumulate within internal systems for long periods of time, enzymatic activity may induce chemical deposition of constituents from within the microplastic chemical structure into tissues (Voparil *et al*. 2004; Oliveira *et al*. 2013). In contrast, secondary leaching of adsorbed chemicals only recently has been characterized in the wake of microplastic's identification as a potent vector of environmental pollutants (Mato *et al.* 2001; Yamashita *et al.* 2011; Rochman *et al.* 2013). Preliminary research on the chemical-adsorbing properties of microplastics has identified them as effective transport mechanisms for environmental toxins. This characteristic was first observed by Mato *et al*. (2001), who found high concentrations of anthropogenic chemicals coated on marine plastic along Japanese shorelines; a host of subsequent studies found similar correlations between environmental toxins and presence on microplastics (Rios *et al*. 2007; Ryan *et al*. 2012; Rochman *et al*. 2013). Given their physical durability, chemical stability, and hydrophobicity due to their nonpolar composition, microplastics are ideal carriers of persistent bioaccumulative toxins (PBTs), anthropogenically-derived environmental toxins that exist in the marine environment. These uncombined monomers and persistent organic pollutants adsorb onto plastic via a chemical process called partitioning, in which the toxins effectively coat the surface of microplastic fragments (Kubota *et al.* 2004; Andrady 2011). Like plastic particulates, PBTs exhibit strong hydrophobicity in water, facilitating their accumulation or "partitioning" from the surrounding seawater onto the microplastic particles (Wurl and Obbard 2004). Additionally, common microplastic shapes (flat fragments, spherical beads, filaments) have an increased surface area to volume ratio that optimizes the accumulation of environmental toxins (Hidalgo-Ruz *et al*. 2012). A study by Rios *et al*. (2007) demonstrated their effectiveness as chemical sinks when pollutant levels in microplastics were found to be comparable to those in sediment concentrations of the same chemical compounds, effectively implicating microplastic particles as PBT vectors. Thus, there is strong evidence pointing to microplastic particles as conduits for PBTs, partitioning chemicals from the water and carrying them through the ocean in small but highly-concentrated doses.

Ingestion of ocean-contaminated microplastics by marine organisms may deposit high concentrations of toxic and bioaccumulative chemicals into biotic tissue and the larger food web. Voparil *et al*. (2004) and Koelmans *et al.* (2014) provided evidence for the bioavailability of polycyclic aromatic hydrocarbons in microplastic particles in the marine lugworm digestive tract, discovering that gut surfactants of benthic organisms concentrate ingested PBTs. Chemical transference from microplastic

particles also has been identified in larger marine predator species such as the Great and Flesh-footed shearwater, indicating the ecological extent of microplastic contamination (Rodriguez *et al.* 2012; Yamashita *et al*. 2011). Additionally, University of Puget Sound students Emilie Kurth and Brad Heusinkveld examined the effects of phthalate-contaminated microplastic ingestion on Northern Fulmar and Sooty Shearwater neurological and reproductive function, finding evidence of endocrine disrupting compounds (Kurth 2015; Heusinkveld 2015). Chemical transference of PBTs by microplastic ingestion is of particular and increasing concern to the health of marine species and the ecological integrity of marine ecosystems; chemical leaching of environmental pollutants within the digestive system may be absorbed in biotic tissue and induce adverse and lethal physiological effects in marine organisms (Mato *et al.* 2001; Andrady 2011; Oliveira *et al.* 2013).

       One class of PBTs, polychlorinated biphenyls (PCBs) has been particularly implicated as a harmful marine contaminant and bioaccumulant in organisms. Indeed, many studies on the partitioning chemistry of microplastics have observed high concentrations of environmentally-adsorbed PCBs within microplastic fragments (Mato *et al.* 2001; Teuten *et al.* 2009; Andrady 2011; Rochman *et al.* 2013). Polychlorinated biphenyls are nonpolar and exhibit strong hydrophobicity in water, facilitating their easy adsorption onto microplastic particles (Rios *et al.* 2007; Andrady 2011; Figure 3). Furthermore, PCBs have extremely high toxicity and durability, allowing them to remain in and move through the water matrix for long periods of time without being degraded. This high mobility in water allows PCBs to bioaccumulate easily within marine organisms, infiltrating local food webs and contaminating higher trophic levels via biomagnification, a process by which toxins in prey tissue are transferred via ingestion and concentrate within dominant predator species (Nliml and Oliver 1989; Teuten *et al.* 2009; Rochman *et al.* 2013; Wright *et al*. 2013). Ingestion and accumulation of environmentally-contaminated microplastics by secondary consumers (such as fish) therefore has a disproportional effect on tertiary and quaternary consumers whose diet consists primarily of lower trophic-level species.
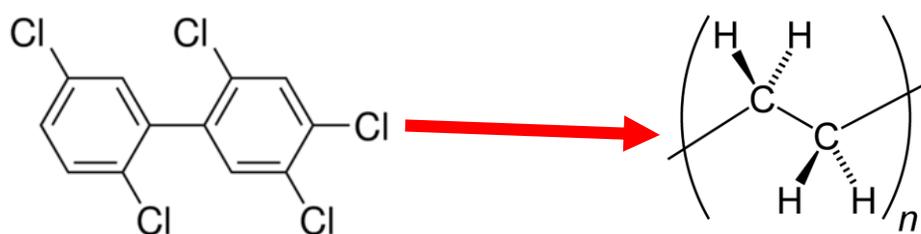
Figure 3. Polychlorinated biphenyl adsorbs onto polyethylene fragment.

2. CURRENT STATE OF MICROPLASTICS STUDIES

2.1. CURRENT TRENDS AND KNOWLEDGE GAPS

While the adverse environmental effects of larger plastic debris on marine organisms (strangulation, nutritional deprivation, etc.) have been heavily studied and characterized, microplastic research is a relatively young field of study given its recent identification as a marine contaminant by Thompson *et al*. (2004). Subsequently, several studies have been conducted in recent years attempting to fill critical knowledge gaps in microplastic's characterization as a separate class of marine pollutant and its effect on organismal and ecosystem health. However, in an attempt to provide some measure of scale to the level of microplastic pollution, many of these studies have focused on mapping microplastic contamination in the marine environment and quantification within marine fauna. While these are worthwhile pursuits of study as they provide critical baseline knowledge on how microplastics enter, move through, and accumulate within the marine environment, fewer studies have been conducted on the physiological effects of microplastic contamination on marine fauna and the transference pathways through which environmental toxins may be deposited in biotic tissue. Therefore, there is a current need for a quantitative analytic method assessing the transference of environmental chemicals from plastic vector to marine biotic tissue. The development of a methodology towards and findings derived from such a study would also contribute valuable physiological and quantitative knowledge to an existing wealth of environmentally-focused microplastic studies at the University of Puget Sound (Lyon 2014; Heusinkveld 2015; Kurth 2015; Mitchell 2015).

2.2. QUANTITATIVE ANALYSIS OF MICROPLASTIC TRANSFERENCE

2.2.1. Rochman et al. 2013

In addition to the lug-worm study by Voparil *et al.* (2004), one of the only current laboratory studies identifying specific transference mechanisms from microplastic vectors to marine biotic tissue was conducted in 2013 by Rochman *et al*. This baseline study found that mixtures of polyethylene and chemical pollutants adsorbed from the marine environment bioaccumulate in fish tissue, inducing liver toxicity and hepatic stress. Rochman *et al*. (2013) also directly implicated microplastics as potent vectors for PBT-transference to biotic tissue, reporting significantly higher transference levels in fish exposed to marine-microplastic conditions (PBT-exposed) than those exposed to virgin-microplastic and control conditions. The Rochman *et al.* (2013) study represents one of the only experiments directly testing the bioaccumulative effects of microplastics on marine physiology. Therefore, there is a need for intensive research on the bioavailability of different microplastic vectors to determine pathways for chemical transference, especially in lower-trophic level organisms that may easily ingest, bioaccumulate, and introduce microplastic particles into the ecological food web (Voparil *et al*. 2004; Rochman *et al*. 2013).

Previous studies therefore highlight the need for research identifying potent microplastic vectors of PBTs, transference mechanisms by which PBTs bioaccumulate, and physiological effects of bioaccumulated microplastics on marine organisms. In spite of several findings implicating microplastics as a transference vector for environmental chemicals, very few laboratory studies have been conducted assessing the specific bioavailability of absorbed chemicals to marine organisms via ingestion, with a majority of studies assessing environmental contamination rather than closed-system exposure. Thus, the biological risks of the plastic and pollutant chemical "cocktails" found in marine environments on biotic tissue and related health effects are largely unknown.

2.2.2. Proposed Study

This study is an attempt to assess the mechanism of PCB bioaccumulation (previously found in contaminated fish and seabird tissue) and its physiological effects on an aquatic vertebrate species, using polyethylene (PE) as a possible vector. By comparing "clean" (unexposed) and "contaminated" (PBT-exposed) microplastic effects across a range of plastic vectors, we may begin to prioritize potent microplastics and chemical pollutants that pose a critical threat to marine organisms and environments. This research will contribute valuable direct-transference data to the growing field of assessing microplastic contamination, enhancing current understanding of pollution transfer in lower-trophic organisms and thus informing future conservation and ocean management strategies.

3. STUDY OBJECTIVES, HYPOTHESES, AND JUSTIFICATION

3.1 OBJECTIVES

This study is a multi-pronged attempt to advance current understanding of chemical transference along the microplastic-biotic pathway, characterize physiological effects of microplastic exposure and accumulation, and develop a cost-effective and simplified methodology for approaching microplastic studies at the undergraduate level. To this end, the following objectives were identified and addressed:

3.1.1. Literature Review

In spite of increasing attention being directed towards marine microplastic contamination in the scientific literature, there exist few comprehensive literature reviews to compile the growing number of studies being conducted. While this literature review is by no means comprehensive and barely touches the surface of existing research, it highlights certain areas of microplastic studies that are less-studied but may provide critical knowledge of microplastic contamination and influence the direction of future studies. A brief literature review also is used to position this study within the larger framework of microplastic research in the hopes of identifying and prioritizing avenues of further study that may be worth pursuing.

Furthermore, given the current interdisciplinary emphasis on microplastic research within the University of Puget Sound scientific community, this brief consolidation of current and relevant literature may serve as a resource for students pursuing microplastic studies.

3.1.2. Methodology Development

This study was an attempt to develop a methodology based on Rochman *et al.*'s (2013) that could be conducted in more simplified, cost-efficient, and effective manner using a model organism. While existing research assessing and quantifying microplastic contamination is undertaken largely at the graduate level or higher, with little regard to cost and resources, undergraduate scientific research often is far more limited in scope. One of the primary objectives of this study was to simplify and make an existing study more cost-effective without sacrificing accuracy, depth, and significance of research. Given the current direction of microplastic research at the University of Puget Sound, the methodology thus derived serves as a continuing project for refining the identification and observation of contaminant studies at the undergraduate level. A viable analytic method for quantifying plastic and PBT-accumulation in biotic tissue will provide a template which future students may use to assess chemical transference across a wide variety of contaminants and microplastic types and observe contamination within a variety of tissue types.

3.1.3. Assess Chemical Transference Capacity of Polyethylene in Vertebrate Tissue

The primary objective of this study was to assess the chemical transference of PBTs via a microplastic vector and examine subsequent physiological effects of microplastic contamination within an aquatic vertebrate species, Japanese medaka fish (*Oryzias latipes*). While a majority of other studies have focused on mapping distributions of microplastic contamination within geographic ranges or quantifying microplastic contamination within marine species, there are no closed-system microplastic studies directly examining microplastic ingestion and chemical transference. Therefore, this study bridges the current knowledge gap between theoretical understanding of how microplastic enters biotic systems and the physiological effects that have been observed in previous studies. Freshwater Japanese medaka fish are a good model organisms for assessing the transference mechanisms at the plastic-PBT-biotissue interface, allowing identification of specific physiological effects and extrapolation of bioaccumulation rates to forage fish in natural marine environments (Mato *et al*. 2001; Rochman *et al*. 2013; Lavers *et al*. 2014).While statistical analyses are limited by a small sample size (based on the availability of medaka fish), the findings from this study contribute to a growing understanding of how microplastics enter marine ecosystems and deposit environmental pollutants at the plastic-toxin-tissue interface. This report details the development of a method to quantitatively measure polyethylene accumulation within Japanese medaka

fish and qualitatively characterize the physiological health of fish exposed to a range of plastic and pollutant conditions.

3.2. HYPOTHESES

By exposing Japanese medaka fish to three different treatment groups (control, clean-plastic, and PCB-plastic), I hoped to characterize polyethylene as a transference vector for PCB and measure plastic and PCB accumulation in fish tissue. I also hoped to monitor the physiological effects of microplastic and PCB contamination through live and postmortem observations, using mortality, activity level, morphology, reproduction, and internal integrity as measures of fish health. To this end, my hypotheses for this study were as follows: 1) polyethylene microplastic vector will bioaccumulate within fish via ingestion; 2) fish will have differential physiological responses in control, clean-plastic, and PCB-plastic conditions; 3) fish exposed to PCB-microplastic will exhibit higher PCB concentrations in tissues; and 4) concentrations of PCB contamination in fish tissue will reflect corresponding levels of microplastic contamination. This dissertation will concern itself with assessing the validity of the first two hypotheses (microplastic accumulation and physiological health)

3.3. JUSTIFICATION

3.3.1. Environmental Concerns

In light of rapid and increasing accumulation of plastic debris and chemical pollutants in marine environments, the transfer of toxic chemicals to biota via microplastic ingestion is of significant concern (Rochman and Browne 2013; Cole *et al.* 2011). This research is especially important in benthic organisms and forage fish that pass on bioaccumulated pollutants to higher trophic level predators, subsequently infiltrating larger ecological systems. Medaka fish are a good model organism in which to observe chemical transfer; they are capable of ingesting microplastic and are highly representative of contaminated prey species upon which vertebrate marine predators feed (Ivar do Sul and Costa 2014). While preliminary research strongly implicates microplastic fragments as vectors of PBT transfer, fundamental questions about how pollutants are transferred to biotic tissue remain unresolved. Inconsistent and complex sampling methodologies diminish the accuracy and comparative value of quantitative field studies to determine distribution. Enhanced laboratory studies are thus needed to identify mechanisms of pollutant transfer and consequences of microplastic bioaccumulation to inform more effective field sampling strategies. Comparing bioavailability and toxicity of chemical pollutants across a range of microplastic vectors may improve current understanding of pollutant transfer and aid in prioritizing critical microplastics for environmental hazard reclassification and discontinuation in industrial processes (Rochman and Browne 2013).

Furthermore, microplastics have been identified in the tissues of several different marine organisms in Puget Sound, including Northern fulmars, a variety of forage fish, and blue mussels (Feinstein 2013; Wirth 2014; Lyon 2014; Mitchell 2015). As one of the busiest ports in the Pacific Northwest and given its history of marine environmental pollution, Commencement Bay also is host to a variety of chemical contaminants from high vessel traffic and groundwater runoff. Therefore, further research investigating the chemical transference capacity of microplastics has local and regional implications, and may help project how certain plastic and pollutant conditions affect organism and ecosystem health within Puget Sound.

3.3.2. Human Health Concerns

While plastic in its inert form is not especially dangerous to humans, its degradation in the marine environment poses significant potential hazards to human health. Oceanic weathering of plastic via photodegradation and mechanical abrasion may release constituent monomers, additives, and chemicals present within microplastic particles, contaminating marine environments used by humans for food and recreation (Galloway 2015). Perhaps more significantly, microplastics may accumulate within lower-trophic level organisms, biomagnify through the food chain, and deposit high concentrations of plastic and adsorbed toxins in upper trophic consumers (Cole *et al*. 2013). As all trophic-level organisms may then be contaminated by microplastic to some degree, any organisms upon which human populations depend for food may be contaminated, from primary consumers (i.e., mussels) to secondary and tertiary predators (i.e., tuna). Subsequent ingestion of contaminated fish tissue may induce slight physiological effects in human systems; however, it is important to note that these concentrations are often too low to detect after seafood processing and preparation and effects are often negligible or nonexistent (Galloway 2015). Additionally, microplastics often accumulate within the organism's gut, which is not a popular food item for human consumption; therefore, transference of microplastic particles from seafood to humans largely depends on translocation of microplastics through the organism's system to target tissues of consumption (Galloway 2015; Cole *et al.* 2013). However, some organisms are an exception; the whole-body consumption of filter-feeding molluscs is a potential concern, especially given the rapid growth of the mussel and clam industries and recent identification of microplastic accumulation in mussels and oysters (Lyon 2014; Mitchell 2015; Sassarellu *et al*. 2016). Furthermore, internal leaching of toxins from gut-accumulated microplastic has been documented in a number of marine species and may pose a health concern for human consumption on contaminated tissues (Yamashita *et al*. 2011; Cole *et al*. 2013; Rochman *et al*. 2013; Kurth 2015; Heusinkveld 2015). More studies tracing the translocation of ingested microplastic to specific systems and tissues should be conducted to form a greater understanding of how plastic accumulates and moves within marine biota.

3.3.3. Policy Implications

Research on microplastic contamination is beginning to have policy implications, as evidenced by President Obama's ban on the production of scrubbing beads in facial cleansers in December 2015. While a positive step towards the reduction of plastic waste, this will eliminate only a fraction of what otherwise would have been deposited into aquatic systems, especially considering that exfoliating beads only constitute a small fraction of all microplastics (the majority being plastic pellets from industrial runoff and degradation of plastic flotsam; Hidalgo-Ruz *et al.* 2012). Further research characterizing the chemical hazards of microplastics may eventually shift policy from more specific plastic types towards the wholesale reduction and replacement of certain plastic materials in largescale industrial processes.

4. MATERIALS AND METHODS

Methodology for chemical exposure of Japanese medaka fish (*O. latipes)* and measurements of plastic bioaccumulation and PCB-toxicity was adapted from Rochman *et al.* (2013) and Heusinkveld (2015).

4.1. PLASTIC PREPARATION

Low-density polyethylene (PE) pellets (catalogue number 9002-88-4) were purchased from Sigma-Aldrich (St. Louis, MO). A polychlorinated biphenyl congener mix of 2,4,4'-Trichlorobiphenyl, 2,2',5,5'-Tetrachlorobiphenyl, 2,2',4,5,5'-Pentachlorobiphenyl, 2,2',3,4,4',5'-Hexachlorobiphenyl, 2,2',4,4',5,5'-Hexachlorobiphenyl, and 2,2',3,4,4',5,5'-Heptachlorobiphenyl was obtained from Sigma-Aldrich (catalogue number 47330-U). These congeners were selected based on Rochman *et al.*'s (2013) findings (high accumulation of these PCB congeners found on marine-exposed microplastic fragments). Individual reagent grade PCB congeners were purchased from Sigma-Aldrich as internal standards on recommendation from EPA procedures (U.S. EPA 2007). A schematic of these six PCB congeners (comprising experimental compound mixture and individual standards) is presented in Figure 4.
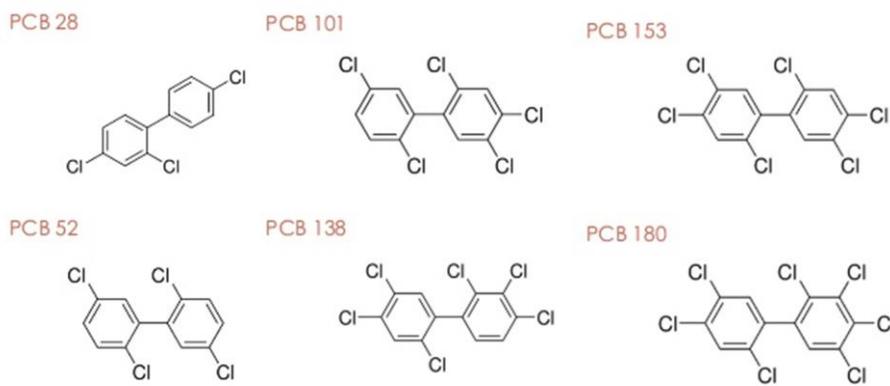
Figure 4. Six PCB congeners commonly found in marine environments.

Before preparation, all pellets, equipment, and glassware were rinsed in 70% ethanol solution to remove residual chemicals. Low-density PE plastic particles were received in the form of small pellets (nurdles); however, they were too large to directly expose to fish subjects and had to be broken down. To simulate mechanical abrasion processes, the pellets were blended in a Waring commercial blender for 5 minutes. Subsequent plastic particulate was poured through a 2.379 mm sieve system to collect particles smaller than 5 mm. Rochman *et al.* (2013) fed fish a diet consisting of 10% plastic by weight; therefore, 3.0 g of plastic in total were weighed, collected, dried in a drying oven, and divided into thirds (1.0 g) for each tank treatment. One third was placed in a volumetric flask and exposed to 0.04 µL PCB-congener mixture in 200 mL DI water; the flask was placed on a magnetic plate with a stirring rod to simulate water movement and facilitate partitioning. This plastic was left for 31 days prior to fish exposure to maximize chemical partitioning (Rochman *et al.* 2013). (All PE microplastic and PCB dosages were environmentally-relevant given Rochman *et al.*'s (2013) findings from San Diego Bay water analysis; however, it is important to note these concentrations assume areas of high-pollution and were selected to optimize PCB-plastic partitioning).
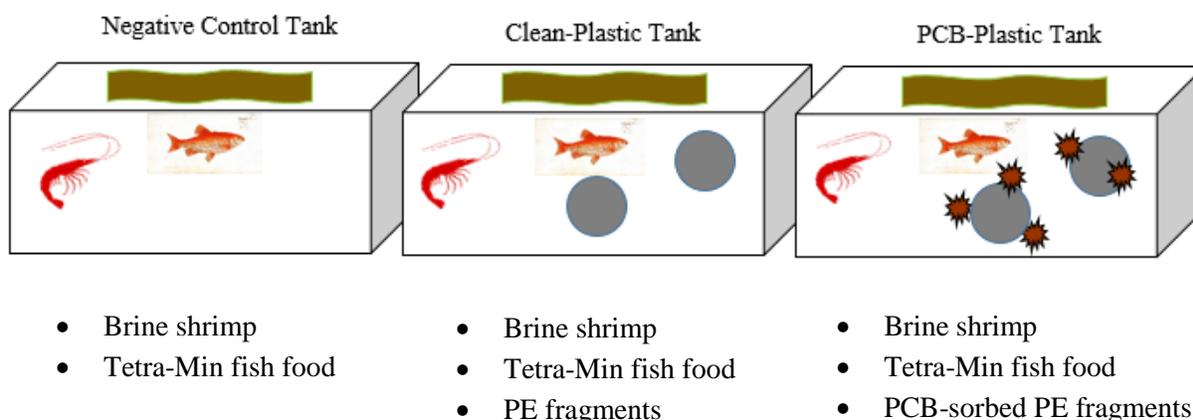
4.2. FISH TREATMENT AND DIETARY EXPOSURE

4.2.1. Fish Housing and Care

Twenty-one adult Japanese medaka fish were kindly provided by Dr. Tomoko Inagaki, University of Puget Sound (Tacoma, WA). Fish were distributed randomly (with roughly equal gender ratios) among three 5-gallon tanks filled with prepared tank water; seawater was made in accordance with University storeroom policy (0.187 g/L Instant Ocean, 0.0086 g/L $CoSO_4$, 0.0126 g/L $NaHCO_3$ in 1 L of DI water). Three ramshorn snails (*Planorbarius* spp.) also were deposited in each tank for cleaning purposes. Fish were allowed to acclimate to vertebrate cold room for one week prior to plastic exposure (set on an 11-hour light-cycle at 22°C). 2 liters of water was replaced every 2 days throughout the acclimation and trial period.

4.2.2. Dietary Exposure

For the first week, fish were fed a diet of brine shrimp nauplia (6 mL, or 2 squirts per tank) and supplemental Tetra-Min fish food (a pinch) twice a day in the morning and night (2% of body weight per day). Brine shrimp eggs were purchased from Brine Shrimp Direct. Brine shrimp nauplia were hatched in accordance with Siddharth Ramakrishnan and Tomoko Inagaki's lab protocol (0.5 g Instant Ocean and 0.3 g brine shrimp eggs in 100 mL DI water bubbled under a heat lamp for 24 hours prior to feeding, or until mixture is dark orange and shrimp are visible). Tetra-Min fish food and all fish equipment was purchased from Petco.

During the plastic exposure trial, one control tank received the normal brine shrimp nauplia and Tetra-Min diet. In accordance with Rochman's study, fish in the second tank (treated with "clean-plastic") were fed 1 g of blended PE plastic fragments mixed into Tetra-Min fish food over the course of 1 month. Fish in the third tank (treated with "PCB-plastic") were fed 1 g of blended PCB-exposed PE plastic fragments mixed in Tetra-Min fish food over the course of 1 month (10% plastic). The purpose of the "clean-plastic" treatment was to distinguish any PCB contaminant that may have leached from the PE fragments as a primary plastic constituent from PCB that was environmentally adsorbed. After gas chromatography with mass spectrometry detection of PCB, we may then subtract the amount of PCB identified in fish exposed to "clean-plastic" from that found in fish exposed to "PCB-plastic" conditions to determine the amount of PCB transferred from marine matrix to fragment to biotic tissue. For a visual schematic of dietary exposure, see Figure 5.

Negative Control Tank	Clean-Plastic Tank	PCB-Plastic Tank
• Brine shrimp • Tetra-Min fish food	• Brine shrimp • Tetra-Min fish food • PE fragments	• Brine shrimp • Tetra-Min fish food • PCB-sorbed PE fragments

Figure 5. Dietary treatments for Japanese medaka fish.

4.3. IN-STUDY OBSERVATIONS

Fish were observed throughout the study for changes in survival, behavior, and morphology.

4.3.1. Fish Survival

To assess fish survival, the number of living fish were counted each day. When an individual died, it was removed from the tank and placed in 10% formalin solution; time and date were noted.

4.3.2. Behavior and Morphological Characterization

While highly qualitative, changes in behavior were observed as marked increases or decreases in activity level in response to food stimuli. Changes in morphology were observed as changes in fish

coloration and visible increases or decreases in egg production; however, these were informal observations to supplement dissection findings and, as such, were not subjected to statistical analysis.

4.4. EUTHANASIA AND SAMPLE DISSECTION

4.4.1. Fish Euthanasia and Preservation

After the one-month plastic exposure trial, all fish were euthanized by 500 mg/L of tricaine mesylate (MS-222; obtained from Western Chemical (Ferndale, WA)) for 30 minutes in accordance with the American Veterinary Medical Association Panel on Euthanasia, weighed, and placed in separate vials of 10% neutral buffered formalin solution (Sigma-Aldrich, catalogue number HT501128).

4.4.2. Gut Extraction

Fish were dissected under a Leica dissecting microscope in accordance with gut extraction protocols developed in the Hodum Lab. All dissecting equipment (Petri dish, scalpel, tweezers) were cleaned with ethanol prior to each dissection. The gut (digestive tract and stomach) of each fish was extracted in a Petri dish and pieces of plastic were identified, quantified, and removed from the gut to assess of plastic consumption and accumulation after one month of exposure (Figure 6). Removing the plastic was also important for future analysis of gas chromatography and mass spectrometry (GC/MS) detection of PCB contaminant; because the purpose of GC/MS analysis would be to identify the amount of PCB that has leached off of the PE fragments into biotic tissue, PCB still adsorbed on the fragments themselves must first be eliminated. The fish tissue and gut were then dried in an drying oven, placed in vials, and frozen at -80ºC.
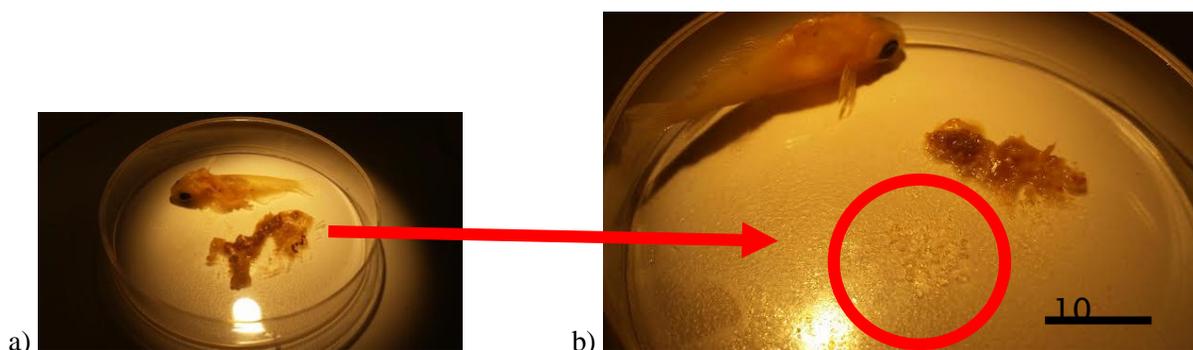
Figure 6. Gut extraction of Japanese medaka fish exposed to plastic dietary treatment. a) Process of gut extraction. b) Plastic PE fragments detected in stomach (encircled).

4.4.3. Plastic Quantification Using UV Fluorescence

In an attempt to ascertain plastic identification from fish tissue, ultraviolet (UV) fluorescence was used to identify and quantify plastic fragments found in the stomach. This is a technique developed by

University of Puget Sound student Nick Lyon in 2014 for quantifying plastic particles in mussel tissue (*Mytilus* spp.) and is now becoming a standard method of measuring plastic concentration in biotic tissue in the Hodum Lab. A Phileex 395 nanometer wavelength 51 UV Ultralight was held over the dissected sample to determine pieces of plastic; however, as collagen fibers and scale tissue also auto-fluoresce under UV light, distinguishing plastic relied purely on rudimentary observation of its physical properties. These include a flat and rounded fragmental shape and blue auto-fluorescence under UV light, distinct from the greener auto-fluorescence of biotic tissue. While this provided a rough estimation of plastic contamination in each fish, further refinement of this methodology is likely needed to ascertain identity of plastic particles.

4.4.4. Assessing Morphological Characterization and Egg Production

In addition to quantifying microplastic fragments in the fish stomachs, the gastrointestinal tract was characterized based on structural integrity to assess influence of microplastic ingestion. The gut was morphologically described based on qualitative observation of gut integrity (i.e., evidence of rupture, abnormalities), using control fish anatomy as a baseline for structural integrity.

In light of Rochman *et al*.'s (2013) findings suggesting inhibited reproductive capacity as a possible physiological consequence of plastic exposure, reproductive health of the fish was also assessed. Reproductive health was measured based on egg production (number of eggs in each roe), with higher egg counts indicating more robust reproductive capacity.

4.5. STATISTICAL ANALYSES

Statistical analyses were conducted to assess significance of differences in fish weight, plastic contamination, and egg production across the three treatments groups. All statistical analyses were conducted using the R Commander statistical program.

4.5.1. Fish Weight

A 1-way ANOVA was employed to test for differences in mean weight of fish between dietary treatment groups.

4.5.2. Plastic Quantification

To provide a more holistic account of variable interactions, plastic data were treated as both count data and continuous. A chi-square test of independence and 1-way ANOVA were used to assess dependence effects of microplastic contamination on dietary treatment.

4.5.3. Egg Production

A 1-way ANOVA was conducted to assess significant differences in egg production across dietary treatment groups. To assess whether microplastic quantity had an effect on egg production, a correlation and simple linear regression were both used.

4.6. PREPARATION FOR CHEMICAL ANALYSIS

This report concerns itself with the first component of a larger study on microplastic contamination. The second component will be chemical analysis of fish samples to test for contamination of PCB using a soxhlet extraction and gas chromatography with mass spectrometry detection of PCB congeners (see Scheme 1). The extraction, clean-up, and detection procedures involved in this component will be similar to University of Puget Sound student Brad Heusinkveld's report on detection of phthalate in Northern fulmar brains (Heusinkveld 2015; unpublished). Remaining fish tissues will be homogenized, and extracted lipid content will be determined gravimetrically using 10% of sample extract prior to clean-up. PCB standards will be used as internal standards, in accordance with the EPA's PCB action plan (U.S. EPA 2007). Sample extracts for PCBs will be analyzed using the UPS Chemistry Department's series gas chromatograph and mass spectrometer with ultrapure grade helium as a carrier gas. Selected ion monitoring will be used to detect PCB congeners and standards (1.0 mL sample injected at 300°C in splitless mode: 90°C for 1 min, 150°C at 5°C/min, 260°C at 3°C/min, 320°C at 20°C/min for 5 min.). Blanked levels of PCB congeners measured in procedural blanks will be subtracted from reported concentrations of total PCB extracted from tissue samples.
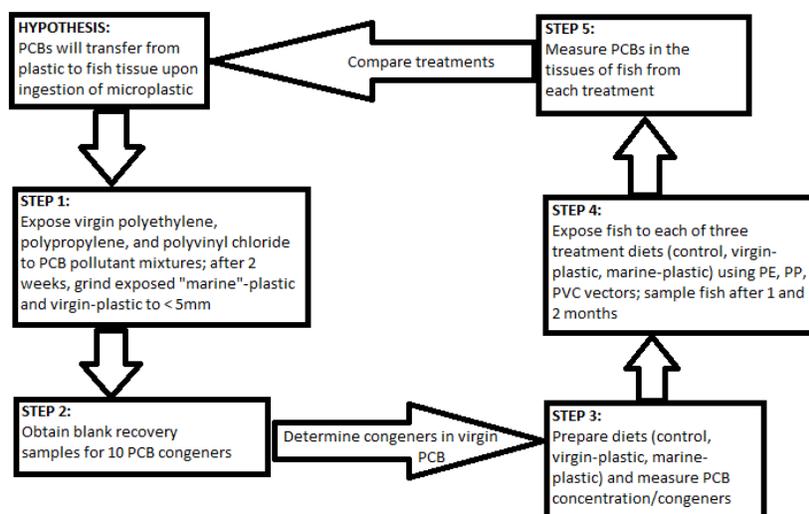
Scheme 1. Schematic diagram of experimental design for chemical detection of PCBs in medaka fish tissue.

5. RESULTS AND DISCUSSION

5.1. IN-STUDY OBSERVATIONS

        While in-study observations were purely qualitative and not subjected to statistical analysis, they provided a rudimentary, first-glimpse understanding of how microplastic and PCB exposure affects Japanese medaka health. Therefore, observations of fish survival and changes in behavior and morphology potentially opened new avenues of study for future research focused specifically on *in-vivo* studies of microplastic exposure. Indeed, some of the observations made during the exposure trials were previously unacknowledged in past studies, contributing new knowledge to the existing wealth of microplastic literature.

5.1.1. Fish Mortality

        Fish survival success varied across dietary treatment group (Figure 7). While control fish survived for the entire 31-day period, fish exposed to clean-plastic conditions suffered 1 premature death (10 days before the end of the trial period) while those exposed to PCB-plastic conditions suffered 3 premature deaths (at 13, 8, and 4 days before the end of the trial period). As none of the control fish suffered premature mortality, they were used as a baseline measure of health with which to compare fish mortality in contaminated environments. The increasing death count across contamination conditions (clean-plastic and PCB-sorbed plastic) indicates a possible correlation between fish survival and exposure to contaminant; however, death counts were too few to conduct statistical analysis. While the singular premature death of the clean-plastic fish (14% of fish) may be due to several other factors (i.e. preexisting health problems, poor nutrition intake, etc.), the presence of microplastic as a potential cause or contributor of death should not be ignored. On the other hand, the higher survival of fish in clean-plastic conditions than PCB-plastic conditions possibly indicates an increased capacity for medaka fish to cope with microplastic in the environment when environmental toxins are not present.

        Perhaps more telling is the higher and earlier death count of fish exposed to PCB-sorbed plastic. The first of these deaths occurred earlier on in the trial (13 days before the end), suggesting a possible effect of early contamination and subsequent lethal physiological response. Additionally, the higher death count (42% of fish) strongly implicates an inability to cope with a changing variable in the surrounding environment (i.e., long-term exposure to PCB-plastic). Furthermore, the combined effect of PCB leaching from the plastic to biotic tissue and possible accumulation of microplastic within the stomach may have induced greater physiological stress upon fish exposed to PCB-sorbed plastic. These results also are consistent with mortality rates documented by Rochman *et al*. (2013), with fish in PBT-treated plastic conditions exhibiting a higher death rate (6%) than those in clean-plastic conditions (4%) over a two-month period. However, Rochman *et al*. (2013) notes these differences were not significant and therefore

should not be used as an indication of contamination effect on medaka fish. Similarly, the small sample size of this study renders mortality a significant event in the course of the trial, with a single fish constituting 14% of the treatment population. Therefore, what may be merely random mortalities caused by disparate factors have a disproportionate effect on total fish survival; a significantly larger sample size is needed to more accurately assess the effect of plastic exposure on medaka fish. Nonetheless, the higher mortality of the PCB-plastic population may be evidence of microplastic partitioning of PCB and subsequent transference to biotic tissue via microplastic ingestion and consequently should not be ignored in this study.

As a side note, all three cleaning snails in the PCB-plastic tank died towards the end of the trial; whether this also is a consequence of PCB exposure or if it is the result of another contaminant or nutritional deprivation is unknown. However, increased mortality among fish and snails in the tank treated with PCB-plastic may indicate possible absorption of PCB via microplastic exposure/ingestion.
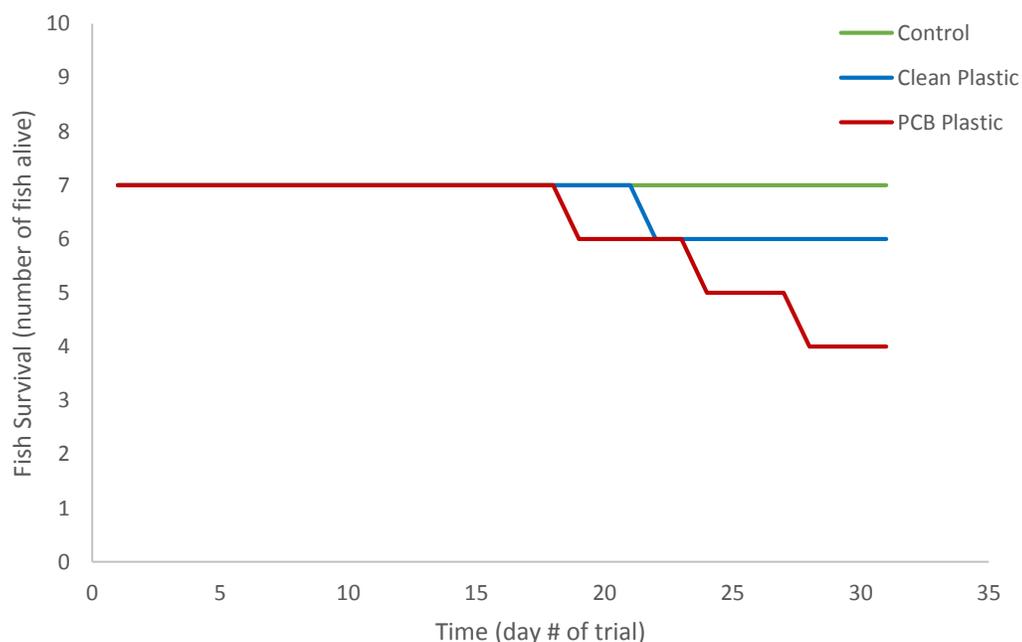
Figure 7. Survival success of fish exposed to different dietary treatments over one-month exposure. Lines indicate number of surviving fish, with the green line indicating control fish, blue indicating clean-plastic, and red indicating PCB-plastic. Control fish exhibited 0% morality (100% survival), while clean-plastic and PCB-plastic exhibited 14% and 42% mortality, respectively.

5.1.2. Behavior and Morphological Characterization

Behavioral and morphological characterizations were neither quantified nor time-stamped; however, they may offer supplementary evidence in assessing the effect of microplastic exposure on medaka fish. Upon feeding events throughout the trial periods, fish held in control and clean-plastic conditions exhibited consistently high levels of activity, as marked by immediate response to feeding

stimuli (rapid pursuit of brine shrimp nauplia or Tetra-Min diet). Individuals exposed to PCB-plastic conditions, however, were observed to be notably more lethargic about halfway through the trial period, as marked by a slower response to feeding stimuli (delayed recognition and pursuit of food source). These observations are interesting when overlaid with observations of mortality, as PCB-plastic fish exhibited both a higher death rate and lower energy levels than those exposed to control and clean-plastic conditions, implicating PCB as the possible factor affecting activity level. Therefore, lack of energy to pursue a food source may be the indirect cause of death among those fish fatally affected by PCB exposure.

Additionally, changes in physical morphology were informally observed between treatment groups throughout the trial period. Fish in control and clean-plastic conditions maintained a bright orange coloration throughout the 31-day exposure period, indicating robust physiological health. In contrast, fish exposed to PCB-plastic treatment were observed to fade in coloration to a light- or gray-orange over the course of the trial period, indicating physiological stress (fewer energy reserves allocated to maintain orange coloration). This suggests that fish exposed to PCB-plastic conditions were being forced to allocate energy usually reserved for maintenance of orange pigmentation to other physiological systems. While the fading of this coloration was neither graded nor time-stamped and may be due to other factors (such as existing pigment mutations within the fish), it may also be a symptomatic response to physiological stress brought about by exposure to PCB. Additionally, nutritional deprivation from decreased energy levels may also have resulted in faded coloration due to decreased consumption of orange-colored brine shrimp nauplia. This is the first documented observation of pigment change in a fish species exposed to PCB and microplastic conditions.

Finally, egg production was informally observed throughout the trial period. While fish in control conditions exhibited a consistent and robust fertility (near-daily evidence of egg production), those exposed to clean-plastic and PCB-plastic conditions exhibited slightly reduced rates of egg production (fewer eggs present with longer periods of time between reproductive events). These observations suggest that fish fertility may have been adversely inhibited by the presence of microplastic within the water matrix and are in line with previous observations of reduced fertility in fish and oysters exposed to microplastics. In both studies, exposure to microplastic was shown to inhibit gene expression of estrogen-receptors and depress overall energy levels and reproduction in organisms exposed to moderate to high levels of polyethylene and polystyrene (Rochman *et al.* 2014; Sassarulu *et al.* 2016). Therefore, observations of low egg production in both treatment groups exposed to microplastics may implicate them as a fertility-inhibitor. Table 1 offers a summary of all in-study observations.

TABLE 1: In-Study Observations of Fish Response across Dietary Treatment Group

Observation	Control	Clean-Plastic	PCB-Plastic
Morality (# fish)	0	1	3
Egg Production	Normal	↓	↓
Activity Level	Normal	Normal	↓
Coloration	Orange (healthy)	Orange (healthy)	Light orange/faded
Other			Snail deaths

5.2. POST-TRIAL WEIGHT

Dietary treatment had a negligible effect on postmortem fish weight (1-way ANOVA, F = 2.02, df = 2, 18, p = 0.162; Figure 8). Mean weight of control fish (284.89 ± 16.99 mg) was similar to that of clean-plastic fish (258.01 ± 11.25 mg; p = 0.348) and PCB-plastic fish (248.44 ± 10.78 mg; p = 0.157). Mean weight of clean-plastic fish was statistically similar to mean weight of PCB-plastic fish (p = 0.868). Subsequently, microplastic exposure did not appear to have a significant effect on fish weight. However, control fish appeared to exhibit a slightly higher mean weight; this is interesting given that accumulation of microplastic within the stomach would likely increase the weight of the fish overall. On the other hand, increased weight may be possible evidence of a healthier physiology (i.e., preserved internal integrity or greater egg load). Raw statistical analyses and R-output may be found in the Appendix.
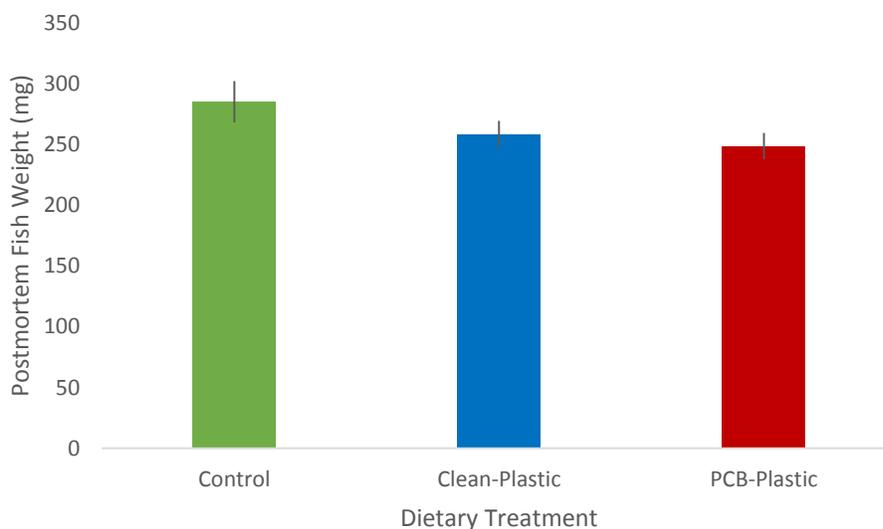
Figure 8. Postmortem fish weight as a function of dietary treatment. Bars represent the mean weight of fish (in mg) exposed to control (green), clean-plastic (blue) and PCB-plastic (red) conditions. Differences across treatment groups were insignificant (p = 0.162).

5.3. GUT ANALYSIS

While qualitative in-study observations were useful in assessing how microplastic exposure affects live-fish physiological and function, quantification of microplastic contamination via fish dissection was necessary to validate these observations and measure levels of plastic transference. Raw statistical analyses and R-output may be found in the Appendix.

*5.3.1. Plastic Quantification*

To this end, the guts of each fish were extracted and dissected and the microplastic fragments within them quantified. PE microplastic appeared as clear, flattened fragments between 1-3 mm in diameter within the stomach of the gut, which then auto-fluoresced blue under UV light. Fragments were identified in fish exposed to clean-plastic and PCB-plastic, verifying the consumption by and accumulation of plastic within fish over time. However, a few fish in control conditions also exhibited some degree of microplastic contamination, evidence of cross-contamination or pre-existing presence of plastic within the tank.

When microplastic quantity was analyzed as count data, microplastic contamination depended on the dietary treatment of the fish (chi-square test of independence, $X2 = 75.93$, df = 12, $p < 0.001$; Figure 9). Fish exposed to clean-plastic conditions were 25% more likely to exhibit microplastic contamination than control fish while those exposed to PCB-plastic were 50% more likely to exhibit contamination. Fish exposed to PCB-plastic conditions were 20% more likely to exhibit plastic contamination than those exposed to clean-plastic conditions.
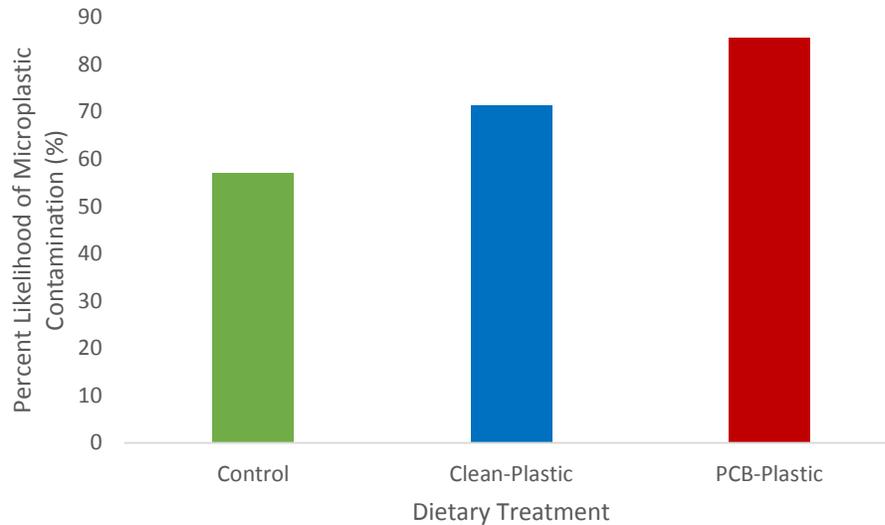
Figure 9. Percent likelihood of containing microplastic in medaka fish as a function of dietary treatment. Bars represent the percent likelihood of finding any degree of microplastic contamination in fish exposed to control (green), clean-plastic (blue) and PCB-plastic (red) conditions (n = 21). Differences across treatment groups were significant (p < 0.001), with clean-plastic and PCB-plastic groups exhibiting a significantly higher chance of containing plastic than the control group.

When microplastic quantity was analyzed as a continuous variable, dietary treatment had a statistically negligible effect on the amount of microplastic contamination in fish (1-way ANOVA, F = 3.28, df = 2, 18, p = 0.061; Figure 10). The mean number of microplastic fragments found in control fish (1.00 ± 0.44 fragments) was statistically similar to that found in clean-plastic fish (7.29 ± 2.91 fragments; p = 0.158) and PCB-plastic fish (8.86 ± 2.68 fragments; p = 0.065). Additionally, microplastic contamination was similar between clean-plastic and PCB-plastic fish (p = 0.880). However, microplastic contamination in clean-plastic and PCB-plastic fish was still notably higher (629% and 786% greater, respectively) than in control fish. Furthermore, the maximum number of microplastic fragments was higher in clean-plastic fish (17 fragments) and PCB-plastic (18 fragments) than in control fish (3 fragments), indicating a higher level of contamination in fish exposed to plastic conditions.
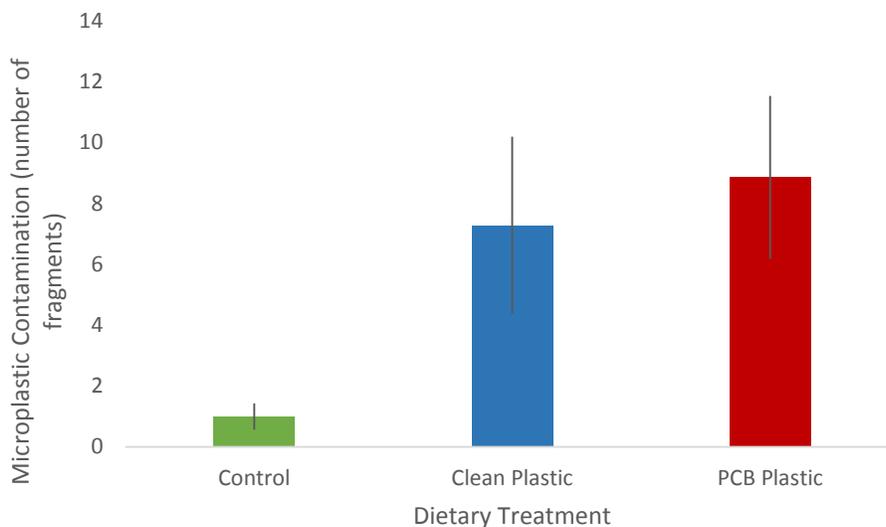
Figure 10. Microplastic contamination (number of fragments) of Japanese medaka fish as a function of dietary treatment (control, clean-plastic, and PCB-plastic). Bars represent the mean number of PE fragments found in fish exposed to control (green), clean-plastic (blue) and PCB-plastic (red) conditions; error bars represent standard error of each mean. Due to the low sample size (n=21), differences across mean groups were insignificant (p = 0.061).

These results verified critical assumptions inherent within this methodology. The presence of PE microplastic in fish confirms their consumption and accumulation within medaka fish. However, whether this plastic was directly or indirectly consumed is uncertain. Because plastic particles were shaped to emulate those found in ocean samples (fragments suspended on or just below the surface), it is possible their consumption was inadvertent. However, their shape also strongly resembled the flakes of Tetra-Min diet being simultaneously administered (in accordance with Rochman et al. 2013), indicating that their consumption may have been a result of direct pursuit. In this way, their food-like shape may also have facilitated their consumption; however, because this shape is among the most common forms of microplastic, the conditions may have been more-or-less comparable to true oceanic conditions. Nonetheless, observation of consumed microplastic both adheres to previous examination of microplastic in the guts of marine vertebrate predators and is a necessary step towards the characterization of possible physiological stress induced by internal microplastic bioaccumulation. Therefore, the very presence of microplastic within the gut is a positive step towards refining a methodology for observing the physiological effects and PCB-transference capacity of microplastics in vertebrate species.

While differences in microplastic quantity were largely insignificant when treated as a continuous variable, their treatment as count data verified their dependence on the dietary treatment of the fish. In other words, those fish exposed to clean-plastic and PCB-plastic contained higher concentrations of plastic than those in control conditions. This also provides a critical verification of the selected methodology because fish exposed to similar amounts of plastic consumed statistically similar amounts of plastic. Future

studies could therefore use the amount of plastic fed to the fish and the amount quantified within the gut to observe rates of consumption under different environmental conditions. Differences in microplastic contamination between clean-plastic and PCB-plastic fish were insignificant; however, fish in PCB-plastic were slightly more likely to consume plastic than those in clean-plastic conditions, a variation possibly due to slight differences in the amount of diet fed to the fish daily. Future refinements of this feeding methodology should likely include reducing exposure of the control treatment to sources of plastic contamination and ensuring standardized feeding quantities and equal fish food-to-plastic ratios. Additionally, a more robust sample size would provide a stronger and more reliable statistical analysis of data.
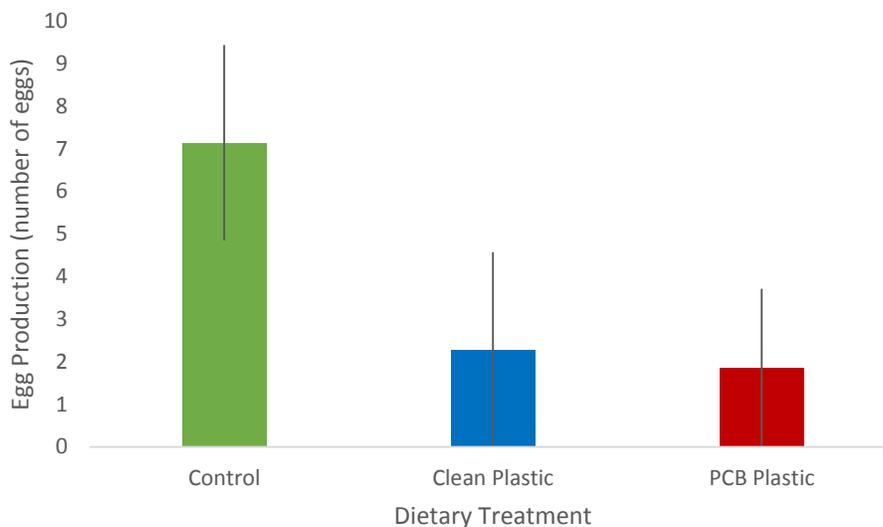
5.3.2. Egg Production

Egg production has been previously established as a strong indicator of fish physiological health by numerous studies (Wagner *et al.* 2002; Scott and Sloman 2004; Sassarulu *et al.* 2016). Furthermore, previous evidence of adverse reproductive effects due to microplastic exposure identify egg production as a possible point of physiological comparison across treatment groups. Therefore, this study quantified and compared egg production of medaka fish across treatment groups to assess potential toxic effects (Rochman *et al.* 2013; Sassarulu *et al.* 2016). While egg production was technically measured as count data (similar to microplastic data), lack of observations prevented its analysis as count data; therefore, egg counts were treated as continuous data to provide a rudimentary understanding of the interactions between different environmental contaminants. Eggs (roe) appeared as clear spheres in a gelatinous cluster near the tail-end of the fish. Eggs were identified in fish exposed to all treatment groups, suggesting that exposure to clean-plastic or PCB-plastic did not completely disable reproductive function. However, slight differences in egg production between treatment groups indicate potential influence of contaminants on reproductive capacity.

Due to the small number of observations, a chi-square test of independence could not be conducted to determine the level of dependence between dietary treatment and egg production. However, fish exposed to control conditions were 80% more likely to exhibit any level of egg production when compared to fish exposed to both clean- and PCB-plastic conditions (Figure 11).
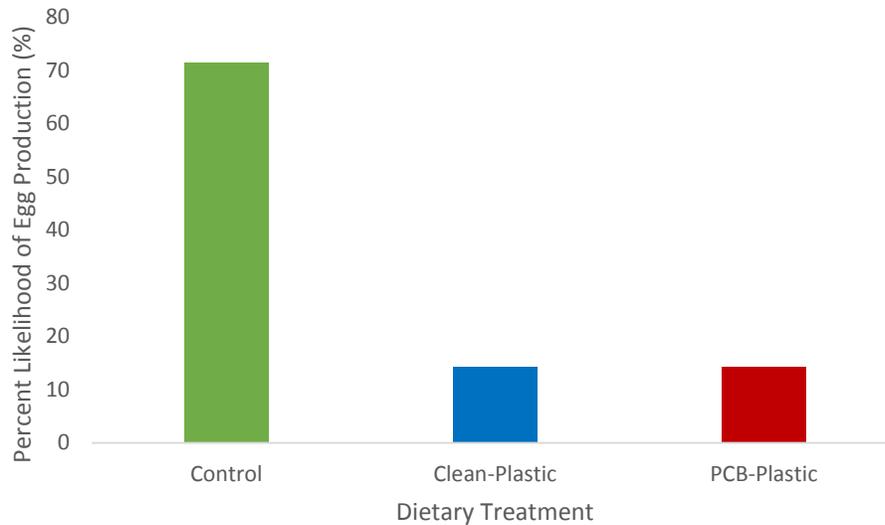
Figure 11. Percent likelihood of egg production by medaka fish as a function of dietary treatment. Bars represent the percent likelihood of finding any eggs in fish exposed to control (green), clean-plastic (blue) and PCB-plastic (red) conditions. Differences across treatment groups were null due to particularly low number of egg observations in clean-plastic and PCB-plastic groups.

When eggs were treated as a continuous variable, dietary treatment had a statistically negligible effect on fish egg production (1-way ANOVA, $F = 1.86$, df = 2, 18, $p = 0.185$; Figure 12). The mean number of eggs found in control fish (7.14 ± 2.29 eggs) was statistically similar to that found in clean-plastic fish (2.29 ± 2.29 eggs; $p = 0.274$) and PCB-plastic fish (1.86 ± 1.86 eggs; $p = 0.220$). Additionally, egg production was similar between clean-plastic and PCB-plastic fish ($p = 0.989$). However, egg production in control fish was still notably higher (212% and 285% greater, respectively) than in clean-plastic and PCB-plastic fish. Furthermore, the maximum number of eggs produced was the same in both control fish and clean-plastic fish (16 eggs in one roe) and somewhat higher than in PCB-plastic fish (13 eggs in one roe).

Figure 12. Egg production (number of eggs/roe) by Japanese medaka fish as a function of dietary treatment (control, clean-plastic, and PCB-plastic). Bars represent the mean number of eggs found in fish exposed to control (green), clean-plastic (blue) and PCB-plastic (red) conditions; error bars represent standard error of each mean. Due to the small sample size (n=21) and low number of observations, differences across mean groups were insignificant (p = 0.185).

Due to the small sample size and small number of observations, these results offer little statistical weight to the overall analysis of microplastic effects on physiology. However, a clear trend is visible in mean egg production across treatment groups, with more control fish exhibiting a notably larger mean egg production than those in plastic-treated conditions. Additionally, the reproductive capacity of fish exposed to PCB-plastic may be slightly disproportionately affected by the combined effects of microplastic and leaching from the environmental toxin. These results are in line with the qualitative in-study observations of egg production depicting lower production in clean- and PCB-plastic treated conditions and therefore may provide rudimentary evidence for the inhibitory effect of microplastic exposure on medaka fish reproductive capacity. In the study by Rochman *et al*. (2013), researchers found evidence of altered gene expression in female fish exposed to clean- and PBT-exposed plastic, in particular the down-regulation of choriogenins (Chg H), precursors of mature egg envelope subunit proteins, and vitellogenin, a precursor protein of egg yolk (Vtg I) (Sugiyama *et al*. 1999; Rochman *et al*. 2014). Both Chg H and Vtg I are used as biomarkers for exposure to environmental estrogens; therefore, there is evidence that the primary constituents and environmentally-sorbed chemicals on microplastics may be estrogen repressors (Rochman *et al*. 2013). Furthermore, gene expression for the estrogen receptor itself (ERα) was found to be significantly down-regulated in female fish exposed to both clean-plastic and PBT-plastic conditions (Rochman *et al.* 2013; Rochman *et al.* 2014). Yet another study recently assessed the reproductive effects of microplastic exposure on oysters, finding that polystyrene microplastic causes reproductive disruption

and inhibits offspring health and viability (Sussarellu *et al.* 2016). In particular, oysters exposed to microplastic conditions exhibited significant decreases in oocyte production and size, sperm velocity, and offspring survival due to loss of energy uptake and energy reallocation from reproduction to structural growth (Sussarellu *et al.* 2016). Therefore, there is existing evidence implicating microplastic as the cause of inhibited reproductive capacity in fish, both as structural inhibitor of necessary physiological processes and as carrier for deleterious environmental toxins. As the results from this study align with those found in Rochman *et al.*'s (2013) and Sussarellu *et al.*'s (2016), our findings suggest that microplastic exposure may be repressing egg production in this study.

An obvious limitation of assessments of egg production is differences in gender ratios between treatment populations of fish. If the control group had a disproportionate number of reproductive females, this would easily explain observed differences in egg production across treatment groups. However, in randomly distributing fish, an approximately equal ratio of supposed males and females were distributed into each tank, which should have evenly distributed reproductive capacity in terms of gender. Nonetheless, male and female medaka fish are difficult to differentiate and often relies on the presence of an egg sac to discern females from males. Additionally, the small sample size would have drastically affected any differences in gender ratios, as would have any number of non-reproductive females. Therefore, alternative explanations for differences may reside in an uneven distribution of males and reproductive females between treatment groups. Future studies should have a more effective means of distributing equal gender ratios among treatment groups to avoid disproportionate reproductive capacity in certain populations.

5.3.3. Microplastic and Egg Production - Correlation and Regression

In order to assess the relationship between microplastic quantity and egg production, correlation and simple linear regression tests were both conducted. While microplastic quantity and egg production did not have a significant relationship, they did exhibit a weak negative association (correlation, r = -0.40, df = 19, p = 0.069; Figure 13).

Among these fish, variation in microplastic quantity explained 16% of the variation in egg production. As microplastic quantity increased by 1 fragment, egg production decreased by 0.36 eggs (regression, F = 3.7, df = 1, 19, p = 0.069; Figure 13).
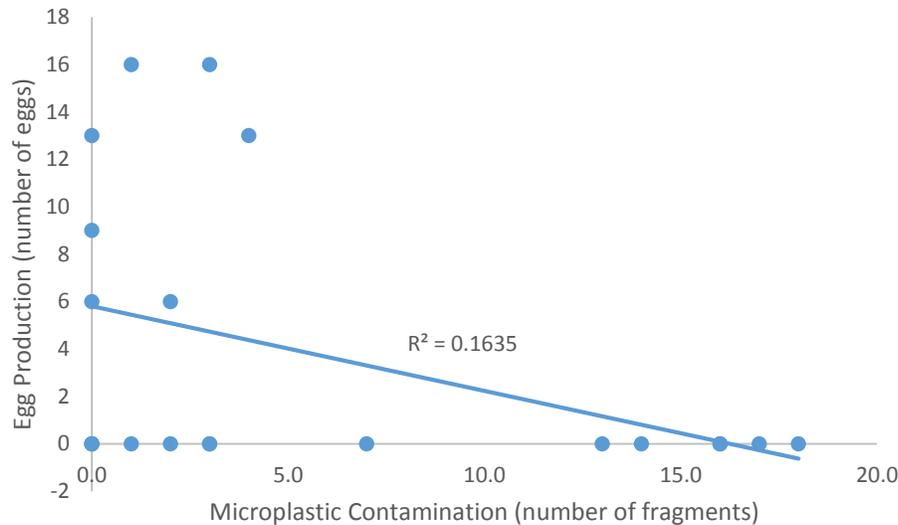
Figure 13. Correlation between microplastic contamination (number of fragments) and egg production (number of eggs) of medaka fish. Blue dots indicate disparate data points; trendline represents the relationship between the two variables. The downward slope of the trendline indicates a slightly negative correlation between microplastic contamination and egg production (r = -0.40); however, this relationship is statistically insignificant (p = 0.069).

While these results offer little in terms of statistical significance (possibly a function of small sample size), they nonetheless contribute to our understanding of how microplastic contamination and egg production interact more directly. Results indicate a slight negative relationship between degree of microplastic contamination and egg production; therefore, those fish exposed to higher concentrations of microplastic may be adversely affected in terms of reduced reproductive capacity, as perceived in Rochman *et al.*'s (2013) and Sussarellu *et al.*'s (2016) studies.

5.3.4. Other Physiological Effects

In addition to quantification of microplastic and egg production, observations were made on the structural integrity and apparent health of the gut; variations were found across treatment groups with regards to pre-ruptured digestive tracts and internal abnormalities. While gastro-intestinal (GI) integrity was more or less preserved in control fish, there were a few instances of pre-rupture in fish exposed to clean-plastic and PCB-plastic (2 and 3, respectively). GI pre-rupture appeared as small ruptures in the intestinal tract and/or stomach, causing the contents to swim freely through the body matrix without manual puncture of the digestive tract. This condition was observed solely in fish exposed to plastic conditions, implicating the consumption of microplastic as a possible cause of internal organ rupture. However, this condition was relatively infrequent and may have arose posthumously in formalin or as a result of manual movement and manipulation of the fish. Nonetheless, it is an interesting piece of evidence

supporting the organ-disrupting impacts of microplastic ingestion and introduces a new avenue for potential future research.

Yet another internal morphological discrepancy observed between treatment groups was the presence of an unidentified abnormality near the liver of fish exposed to PCB-plastic conditions. This abnormality appeared within two different fish as a white circular mass and was distinct from the digestive, reproductive, and hepatic systems. As such masses are previously undescribed by past microplastic studies, it is unknown whether this was a consequence of microplastic exposure, PCB-leaching, or another biological or environmental factor altogether. However, cellular abnormalities have been previously identified in the internal anatomies of fish exposed to PBT-saturated microplastic conditions. In Rochman *et al.*'s (2013) study, eosinophilic foci of cellular alteration (precursors of tumors) and eosinophilic hepatocytes (tumor cells) were identified within the livers of male medaka fish exposed to clean-plastic and PBT-sorbed microplastic, respectively (Rochman *et al.* 2013). These findings suggest that long-term exposure to microplastic may have adverse effects on fish liver growth and development, effects which may be exacerbated or hastened by internal chemical leaching of PBT-sorbed microplastics. While this study did not include liver histopathology, the presence of a white globular mass near the liver may be evidence of potential hepatic damage brought about by exposure to PCB-sorbed microplastic. Tissue samples of these abnormalities were not preserved due to a need for chemical analysis of PCB presence; however, their observation opens yet another avenue of interesting potential research involving physiological characterization of internal anatomy after exposure to contaminated microplastic. However, it is important to note that this mass was unidentified and has not been characterized by any other morphological study of microplastic contamination in fish; indeed, the abnormality could have been a pre-existing condition with the fish or a simple morphological disconformity. Therefore, to draw any grand assumptions about its connection to liver function or microplastic contamination would be overstepping the bounds of this study.

6. LIMITATIONS AND BENEFITS OF STUDY
6.1. ASSESSING METHOD PERFORMANCE

The overall methodology of this study was more or less successful in its confirmation of polyethylene microplastic transference to Japanese medaka fish, quantification of microplastic within internal systems, characterization of morphological discrepancies across treatment groups, and methodological refinement of a more complicated and expensive study. Nonetheless, there were several limitations that likely inhibited methodological accuracy. These limitations involved both exposure techniques and analytical methods.

6.1.1. Microplastic Preparation

　　This methodology attempted to emulate oceanic conditions by recreating weathering processes for the creation of microplastic (blender) and current action for the partitioning of PCB by polyethylene fragments (spinning rod and magnetic plate). While the weathering process worked better than anticipated, more or less accurately recreating the fragmental plastic pieces previously observed in environmental surveys (Wirth 2014; Lyon 2014; Mitchell 2015), the successful partitioning of PCB by plastic was less certain. Determining an accurate concentration of PCB mixture was particularly difficult due to the lack of closed-system studies assessing microplastic as a transport vector for environmental chemicals (many studies merely exposed plastics to environmental conditions). Therefore, the concentration of PCB used may have been significantly higher than would be truly found in the environment, resulting in unrealistic leaching effects on exposed fish, or lower than usually found in the environment, resulting in an underassessment of polyethylene's partitioning capacity. However, in-study and post-dissection observations of lower egg production and morphological abnormalities in fish exposed to PCB-plastic tend to suggest that at least some amount of leaching likely occurred. Future studies should work towards a more definitive method of determining environmentally-relevant concentrations of PBT and saturating microplastics in a way that more naturally facilitates partitioning. Additionally, future studies should expose microplastics to PBTs in artificial seawater rather than DI water to more accurately recreate the chemical conditions surrounding PCB adsorption onto microplastics, as lower pH may play a small role in partitioning chemistry (Andrady 2011).

6.1.2. Fish Exposure to Plastic

　　Methodological techniques for dosing fish with microplastic treatment likely contained the greatest amount of uncertainty in the study. Equal and consistent dosages of microplastic relied on equal amounts of plastic being distributed with each feeding; however, as the Tetra-Min fish food and microplastic particulates were homogenized to create a single mixture, obtaining consistently equal ratios of plastic each time was difficult. Therefore, some tanks may have received more plastic than others during a single feeding in a given day, possibly disproportionately affecting contamination of fish. Additionally, plastic tended to accumulate along the glass walls of the tank - partially due to evaporation of water and partially due to their hydrophobic chemical properties - requiring daily rinsing to re-suspend them within the "neustonic zone". Therefore, periodic absence or reduction of plastic within the water matrix may have reduced the likelihood of consumption by and contamination of fish.

　　Yet another limitation arose in the gradual accumulation of plastic over time within the tanks. While this was more or less controlled for by removing bottom debris with each feeding and changing the artificial seawater every two days, it is likely that plastic particulates suspended in the neustonic zone

remained and accumulated over the month-long exposure period, resulting in an ever-increasing concentration of microplastic. Indeed, this may explain the increasing frequency of mortality near the end of the trial period in the population exposed to PCB-plastic conditions (accumulating concentrations of both PCB and plastic).

Finally, there was no definitive means of preventing environmental leaching of PCBs pre-ingestion within the treatment tanks; therefore, PCB particles may have dissociated themselves from the plastic suspended in the water and entered the fish via a different transport mechanism than the plastic (i.e., the water matrix). Indeed, a recent study by Koelmans *et al*. (2016) questions the strength of adsorption interactions between hydrophobic organic chemicals and microplastics, finding that microplastic partitioning of toxins is much more dynamic and equilibrium-driven than previously assumed (toxins may spend equal amounts of time adsorbed and free-floating in the matrix) (Koelmans *et al*. 2016). My study has subsequently made evident its heavy reliance on the hydrophobic properties of both PCBs and polyethylene in assessing the vector capacity of microplastics. One way to account for this uncertainty would involve taking water samples from each treatment tank and analyzing the amount of microplastic and PCB found to determine the degree of pre-ingestion leakage. Future studies would therefore attempt to more accurately recreate environmental conditions in exposing fish to microplastic treatments, minimize avenues of cross-contamination between treatment tanks, and develop a more consistent method for equal dosage of fish populations.

6.1.3. Gut Analysis and Plastic Quantification

While the procedures for gut extraction and dissection were fairly straightforward and established, limitations such as fish suspension within formalin solution and misidentification of plastic particulate may have affected accuracy of data. Fish were preserved in a formalin solution prior to dissection; however, all fish per treatment were housed together in the same vial, possibly facilitating some degree of cross-contamination between individuals within the same treatment group. Additionally, fixing the fish in formalin post-sacrifice may affect future analysis of PCB concentration within the samples due to the sensitive nature of gas chromatography/mass spectrometry instruments; future studies pursuing chemical analysis of PCB should freeze rather than fix fish samples in formalin.

Identification of microplastic particulate in animal dissections has been significantly improved with the use of ultraviolet light as a sorting mechanism, allowing for rudimentary discernment of microplastic from biotic tissue (Lyon 2014; Mitchell 2015). However, as collagen, bones, and other organic tissue also auto-fluoresce, identification of microplastic may at times be difficult. While polyethylene autofluoresced at a slightly different coloration than did the fish tissue in this study (blue vs.

light green), future microplastic studies may explore the use of spectroscopy to identify microplastic by their specific density via measurement of emitted wavelengths.

6.1.4. Sample Size

The small sample size (n = 21) affected nearly every statistical test involved in this study. It is therefore important to note that any observations and conclusions made therein are singular to this study, subject to high levels of uncertainty, and likely inflated due to the small sample sizes. In contrast, Rochman's study involved over 200 sample organisms from which to make observations and draw conclusions. This limitation is a difficult one to overcome, particularly at the undergraduate level where resources and access to resources (such as study organisms) are often limited, and will likely continue to inhibit the accuracy and depth of student projects involving animal subjects. However, even slightly increasing the sample size by 5-10 fish would very likely increase the robustness of statistical analysis and allow for greater exploration of physiological effects induced by microplastic exposure.

6.2. VERSATILITY AND BENEFITS OF METHODOLOGY

In spite of its several limitations, this study provides a working template for future closed-system microplastic studies. Closed-system studies are important in microplastic toxicology research for assessing how much environmental contaminant is partitioned from the environment to the plastic vector and then to the biotic tissue of exposed organisms. In spite of this logic, the methodology proposed herein is among the first to provide a completely closed-system study assessing microplastic partitioning of PBTs from the environment, contributing valuable knowledge to current understanding of chemical transference across contaminant vectors.

Furthermore, this methodology (developed and refined from Rochman *et al.*'s (2013)) allows for a simplified and more cost-efficient means of testing microplastic transference and accumulation within aquatic vertebrate tissue at the undergraduate level. This simplicity also makes it highly-versatile; a similar methodology may subsequently be applied to test transference of a variety of different environmental contaminants across a wide range of microplastic vectors (polystyrene, polypropylene, etc.).

Lastly, this study attempted to characterize physiological effects of microplastic exposure at the behavioral, morphological, reproductive, and bioaccumulative levels. While limited by sample size, these observations supported old lines of evidence regarding the physiological effects of microplastic ingestion (patterns of bioaccumulation, increased mortality, reproductive inhibition) as well as provided new avenues of potential research (behavioral changes, threatened integrity of internal anatomy, structural abnormalities). Therefore, these observations – while limited in scope – may contribute to current and

future understanding of microplastic as a transport vector for environmental contaminants and subsequent effects on the physiology of exposed organisms.

7. AVENUES OF FUTURE RESEARCH

7.1. CHEMICAL ANALYSIS

This study opens up the door for several potential avenues of further research. While this paper concerns itself with the quantification of microplastic contamination within medaka fish and the qualitative observation of any physiological effects produced therein, the second component of this project involves the detection and quantification of PCB transferred to fish tissue by the PE vector. Therefore, next steps for this project involve refining an extraction methodology and clean-up for medaka fish tissue and detection of sorbed-PCB via gas chromatography/mass spectrometry. Additionally, it will be interesting to compare contamination level across the six PCB congeners used for microplastic exposure to identify possible differences in partitioning affinity.

7.2. VARIATIONS IN EXPOSURE METHODOLOGY

The versatility of this methodology allows for easy replacement of plastic type and PBT contaminant. Few studies have compared PBT transference across different microplastic vectors; because polypropylene and polystyrene are the two most common plastics being produced and discarded after polyethylene, they may be good candidates for alternate plastic vectors. Furthermore, exposing microplastics to different environmental contaminants may enhance current understanding of how different PBTs interact with microplastic in marine conditions. Exposure time may also be extended to two or even three months to more accurately reflect marine conditions.

7.3. HISTOPATHOLOGICAL STUDY

Given previous observations of inhibited liver function in fish exposed to PCB-plastic conditions (Rochman *et al.* 2013; Oliveira *et al.* 2013), a histopathological analysis may provide a more robust characterization of fish physiology and allow for more refined observation of cellular abnormalities.

7.4. COLLABORATION WITH FIELD SURVEYS

Microplastic studies are a highly diverse and constantly evolving field, requiring interdisciplinary exchange and communication among conservationists, marine biologists, toxicologists, chemists, and policymakers. The findings from this study may therefore provide valuable physiological and quantitative contributions to the environmental field data currently being collected at the University of Puget Sound, creating opportunities for collaboration and cross-disciplinary dialogue. With increasing interest in

microplastic research, the development of a simplified and versatile methodology also may be used to shed new light on patterns of microplastic contamination in marine environments, further extending the scope of this and current studies.

8. CONCLUSIONS

This study offered a simplified methodology to assess microplastic transference of PBTs to aquatic vertebrate organisms. Plastic presence within the guts of Japanese medaka confirmed the accumulation of microplastic via ingestion, rendering this study valuable in its dosing methodology. Additionally, the physiological consequences observed in PCB-plastic fish implicated the possible transference of PCB via microplastic; however, whether this occurred indirectly via microplastic consumption or directly through premature leakage in the water matrix remains uncertain. Therefore, the first two hypotheses were largely supported; microplastic appeared to accumulate within fish gut via ingestion (Hypothesis 1) and measures of health evidenced physiological stress and inhibited reproductive capacity in fish exposed to clean-plastic and PCB-plastic environments (Hypothesis 2). This study thus contributes to a growing wealth of research examining the physiological effects of microplastic contamination on marine vertebrates.

In light of rapid and increasing accumulation of plastic debris and chemical pollutants in marine environments, the transfer of toxic chemicals to biota via microplastic ingestion is of significant concern (Cole *et al.* 2011; Rochman and Browne 2013). This research is especially important in benthic organisms and forage fish, which pass on bioaccumulated pollutants to higher trophic level predators and subsequently infiltrate larger ecological systems (Ivar do Sul and Costa 2014). While preliminary research strongly implicates microplastic fragments as vectors of PBT transfer, fundamental questions about how pollutants are transferred to biotic tissue remain unresolved. Inconsistent and complex sampling methodologies diminish the accuracy and comparative value of quantitative field studies to determine distribution. Enhanced laboratory studies are thus needed to identify mechanisms of pollutant transfer and characterize consequences of microplastic bioaccumulation to inform more effective field sampling strategies. This study aimed to refine and simplify existing methodologies for quantification of microplastic accumulation at the underground level. By using it as a template for future studies, we may improve our understanding of pollutant transfer and prioritize critical microplastics for reclassification and replacement (Rochman and Browne 2013).

9. ACKNOWLEDGMENTS

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11. APPENDIX – R OUTPUT

<u>Fish Weight</u>

1-way ANOVA

> summary(AnovaModel.1)

   Df Sum Sq Mean Sq F value Pr(>F)

Treatment 2 5000 2500 **2.017 0.162**

Residuals 18 22311 1240

> with(weight, numSummary(Weight, groups=Treatment, statistics=c("mean",

+ "sd")))

    mean sd data:n

cleanplastic 258.0129 29.75603 7

control 284.8929 44.94113 7

PCBplastic 248.4429 28.52051 7

> local({

+ .Pairs <- glht(AnovaModel.1, linfct = mcp(Treatment = "Tukey"))

+ print(summary(.Pairs)) # pairwise tests

+ print(confint(.Pairs)) # confidence intervals

+ print(cld(.Pairs)) # compact letter display

+ old.oma <- par(oma=c(0,5,0,0))

+ plot(confint(.Pairs))

+ par(old.oma)

+ })

   Simultaneous Tests for General Linear Hypotheses

Multiple Comparisons of Means: Tukey Contrasts

Fit: aov(formula = Weight ~ Treatment, data = weight)

Linear Hypotheses:

      Estimate Std. Error t value Pr(>|t|)

control - cleanplastic == 0 26.88 18.82 1.428 **0.348**

PCBplastic - cleanplastic == 0   -9.57   18.82 -0.509   **0.868**

PCBplastic - control == 0   -36.45   18.82 -1.937   **0.157**

(Adjusted p values reported -- single-step method)

             Simultaneous Confidence Intervals

Multiple Comparisons of Means: Tukey Contrasts

Fit: aov(formula = Weight ~ Treatment, data = weight)

Quantile = 2.5529

95% family-wise confidence level

Linear Hypotheses:

                    Estimate lwr   upr

control - cleanplastic == 0   26.8800 -21.1630 74.9230

PCBplastic - cleanplastic == 0 -9.5700 -57.6130 38.4730

PCBplastic - control == 0   -36.4500 -84.4930 11.5930

cleanplastic   control   PCBplastic

     "a"   "a"   "a"

Microplastic Quantification

Chi-Square Test of Independence

data: .Table

X-squared = 75.926, df = 12, p-value = 2.455e-11

> .Test$expected # Expected Counts

   control cleanPlastic PCBPlastic

1 1.0500000   7.650 9.300000

2 0.2916667   2.125 2.583333

3 0.8750000   6.375 7.750000

4 1.0500000   7.650 9.300000

5 1.8083333   13.175 16.016667

6 1.1666667   8.500 10.333333

7 0.7583333   5.525   6.716667

1-way ANOVA

> summary(AnovaModel.1)

Df Sum Sq Mean Sq F value Pr(>F)

Treatment   2 242.0   121.0   3.279 0.0611 .

Residuals   18 664.3   36.9

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

> with(plastic2, numSummary(Plastic, groups=Treatment, statistics=c("mean",

+   "sd")))

          mean   sd data:n

cleanPlastic **7.285714** 7.696629   7

control   **1.000000** 1.154701   7

PCBPlastic   **8.857143** 7.081162   7

> local({

+   .Pairs <- glht(AnovaModel.1, linfct = mcp(Treatment = "Tukey"))

+   print(summary(.Pairs)) # pairwise tests

+   print(confint(.Pairs)) # confidence intervals

+   print(cld(.Pairs)) # compact letter display

+   old.oma <- par(oma=c(0,5,0,0))

+   plot(confint(.Pairs))

+   par(old.oma)

+ })

        Simultaneous Tests for General Linear Hypotheses

Multiple Comparisons of Means: Tukey Contrasts

Fit: aov(formula = Plastic ~ Treatment, data = plastic2)

Linear Hypotheses:

Estimate Std. Error t value Pr(>|t|)

control - cleanPlastic == 0   -6.286   3.247 -1.936 **0.1575**

PCBPlastic - cleanPlastic == 0   1.571   3.247   0.484 **0.8798**

PCBPlastic - control == 0   7.857   3.247   2.420 **0.0651 .**

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

(Adjusted p values reported -- single-step method)

Simultaneous Confidence Intervals

Multiple Comparisons of Means: Tukey Contrasts

Fit: aov(formula = Plastic ~ Treatment, data = plastic2)

Quantile = 2.5517

95% family-wise confidence level

Linear Hypotheses:

Estimate lwr   upr

control - cleanPlastic == 0   -6.2857 -14.5717   2.0002

PCBPlastic - cleanPlastic == 0   1.5714 -6.7145   9.8574

PCBPlastic - control == 0   7.8571 -0.4288 16.1431

cleanPlastic   control   PCBPlastic

    "a"   "a"   "a"

Egg Production

Chi-Square Test of Independence

data: .Table

X-squared = NaN, df = 12, p-value = NA

> .Test$expected # Expected Counts

  control cleanPlastic PCBPlastic

1 8.227848   2.632911 2.1392405

2 3.797468   1.215190 0.9873418

3 10.126582   3.240506 2.6329114

4 0.000000   0.000000 0.0000000

5 5.696203   1.822785 1.4810127

6 12.025316   3.848101 3.1265823

7 10.126582   3.240506 2.6329114

1-way ANOVA

> summary(AnovaModel.2)

        Df Sum Sq Mean Sq F value Pr(>F)

Treatment   **2** 120.7   60.33   **1.856 0.185**

Residuals   **18** 585.1   32.51

> with(eggs2, numSummary(Eggs, groups=Treatment, statistics=c("mean",

+   "sd")))

            mean   sd data:n

cleanPlastic 2.285714 6.047432   7

control   7.142857 6.067085   7

PCBPlastic   1.857143 4.913538   7

> local({

+   .Pairs <- glht(AnovaModel.2, linfct = mcp(Treatment = "Tukey"))

+   print(summary(.Pairs)) # pairwise tests

+   print(confint(.Pairs)) # confidence intervals

+   print(cld(.Pairs)) # compact letter display

+   old.oma <- par(oma=c(0,5,0,0))

+   plot(confint(.Pairs))

+   par(old.oma)

+ })

        Simultaneous Tests for General Linear Hypotheses

Multiple Comparisons of Means: Tukey Contrasts

Fit: aov(formula = Eggs ~ Treatment, data = eggs2)

Linear Hypotheses:

Estimate Std. Error t value Pr(>|t|)

control - cleanPlastic == 0   4.8571   3.0476   1.594   **0.274**

PCBPlastic - cleanPlastic == 0 -0.4286   3.0476 -0.141 **0.989**

PCBPlastic - control == 0   -5.2857   3.0476 -1.734 **0.220**

(Adjusted p values reported -- single-step method)

Simultaneous Confidence Intervals

Multiple Comparisons of Means: Tukey Contrasts

Fit: aov(formula = Eggs ~ Treatment, data = eggs2)

Quantile = 2.5511

95% family-wise confidence level

Linear Hypotheses:

Estimate lwr   upr

control - cleanPlastic == 0   4.8571 -2.9177 12.6319

PCBPlastic - cleanPlastic == 0 -0.4286 -8.2034   7.3462

PCBPlastic - control == 0   -5.2857 -13.0605   2.4891

cleanPlastic   control   PCBPlastic

    "a"   "a"   "a"

Relationship between Microplastic and Egg Production

Correlation

data: Eggs and Plastic

t = -1.9273, **df = 19, p-value = 0.06903**

alternative hypothesis: true correlation is not equal to 0

95 percent confidence interval:

 -0.71181622 0.03306793

sample estimates:

cor

-0.4043915

Simple Linear Regression

Call:

lm(formula = Eggs ~ Plastic, data = plasticeggs)

Residuals:

   Min   1Q Median   3Q   Max

-5.8012 -4.7306 -0.0912 0.9126 11.2694

Coefficients:

        Estimate Std. Error t value Pr(>|t|)

(Intercept)   5.8012   1.6122   3.598 0.00192 **

Plastic   -0.3569   0.1852 -1.927 0.06903 .

Signif. codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 5.574 on 19 degrees of freedom

Multiple R-squared: **0.1635**,   Adjusted R-squared: 0.1195

F-statistic: 3.715 on 1 and 19 DF, p-value: 0.06903