Drivers and Consequences of Ingesting Plastic Marine Debris for Marine Mammals

by

Gregory Bruce Merrill Jr.

University Program in Ecology
Duke University

Defense Date: March 6th, 2025

Approved:

Douglas P. Nowacek, Supervisor

Daniel Rittschof

Chelsea Rochman

Thomas Schultz

Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the University Program in Ecology in The Graduate School of
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ABSTRACT

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Abstract

Plastic pollution is so ubiquitous in the oceans that it has infiltrated marine ecosystems, with plastic ingestion documented across all trophic levels. This includes marine mammals: the whales, dolphins and porpoises, seals and sealions, walruses, otters, and polar bears. In the case of large, macroplastic debris it is largely assumed that marine mammals mistakenly consume plastic because it visually resembles the physical characteristics of common prey items; for example, a plastic bag or sheet for the gelatinous bodies of squid and jellies. However, some whales dive exceptionally deep to forage, below the photic zone where there is little to no light to see. How then, are deep-diving whales mistaking plastic for food? Smaller microplastics – either intentionally produced or the product of biological, physical, and chemical degradation within the environment are also consumed. Marine mammals are most likely to consume microplastics via contaminated prey in a process called trophic transfer. Whether or not these ingested particles translocate and deposit in various organs outside the gastrointestinal tract in this taxon is unknown. Furthermore, an understanding of the consequences of microplastic exposure to marine mammals is lacking owing to ethical and logistical constraints of studying marine mammals as well as the complicated toxicological nature of microplastics as a suite of contaminants. Therefore, this dissertation aimed to identify drivers and consequences of plastic marine debris in marine mammals.

In Chapter 1, I introduce the issue of plastic pollution in the oceans, exploring sources and fate in the marine environment. I summarize known impacts of plastic on marine organisms with a particular focus on marine mammals. For Chapter 2, I utilized opportunistically obtained tissue samples from twelve different species to investigate whether or not microplastics were translocating within the bodies of marine mammals. Using a complement of Raman spectroscopy and pyrolysis gas chromatography with mass spectrometry, myself and my co-authors demonstrated the occurrence of microplastics, ranging in size, mass concentration, and particle

count concentration from 24.4 – 138 μm, 0.59 – 25.0 μg/g, and 0.04 – 0.39 particles/g, respectively, in four tissues (acoustic fat pad, blubber, lung, and melon). Chapter 3 built upon these findings to explore what some of the consequences of microplastic exposure may be on marine mammals using an *ex vivo* blubber model and an *in vitro* skin cell model. Coupled with glycerol assays, gene expression analyses, protein-protein interaction networks, and functional pathway analyses, I found species-specific, treatment-specific, and tissue-specific responses to microplastic exposure impacting metabolism. Further I identified two key genes – ANGPTL4 and IRS2 – of importance in the lipolytic response to microplastic exposure in common bottlenose dolphins. In Chapter 4, I aimed to identify a sensory mechanism for consumption of plastic in deep-diving cetaceans who forage in the aphotic zone, presumably relying solely on echolocation to locate prey without the assistance of vision. By ensonifying plastic objects and common deep-diver prey items, myself and colleagues demonstrated that the acoustic target strength between the two were similar. This suggests that consumption of plastic by deep-diving odontocetes is driven by a misperception of acoustic signals. I close with Chapter 5, a synthesis of this body of work where I summarize key findings, explore persistent knowledge gaps, highlight limitations of the work, and suggest future research.

Findings from this work extend across multiple disciplines, informing conservation, policy, toxicology and sensory ecology. This dissertation contributes to the field by demonstrating a mechanism of microplastic exposure (translocation), illuminating previously unconsidered sensory mechanisms for consumption (echolocation) and adds to the expansive body of literature teasing apart the complicated impacts of microplastic exposure in a group of species where there exists a paucity of information.

Dedication

First and foremost, to my grandmother Bobbi – whose time ran shy of the completion of this degree – but whose gentle soul, deep love for animals, unwavering support, and dedication to ending plastic pollution continue to inspire me. I miss you dearly.

And to the rest of my family who have tolerated my absence while I have been galivanting around the globe playing with whales:

With all my love, thank you.

Table of Contents

List of Tables

List of Figures

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1 Introduction: Plastic Pollution, an Over-*whale*-ming Problem

Plastic pollution is a global issue due to a combination of factors related to production, usage, waste management, and behavior in the environment. Originally developed in the early 20th century, the first plastic, Bakelite, served to replace ivory-based billiard balls as the game's popularity rose and elephants became more scarce (Geyer 2020a, Walker-Franklin and Jambeck 2023, Olatunji 2024). It is ironic that plastic's origins had positive secondary conservation implications given the burden it now places on societies and ecosystems. Due to their durability, versatility, and cost-effectiveness, following the second World War, plastic became widely produced (Geyer 2020a, Walker-Franklin and Jambeck 2023, Olatunji 2024). Initially, plastics were even perceived as miracle products, marketed as affordable and convenient. *Life* magazine heralded the dawn of "Throwaway Living" with the proliferation of single-use plastic commodities (Rochman 2020, Acaroğlu et al. 2024) . Production of plastic consequently soared from 2 to 380 million tons annually between 1950 and 2015 (Geyer et al. 2017, Geyer 2020b). In recent years, advances in hydraulic fracturing (fracking) and shale gas extraction created a surplus of natural gas, a feedstock of raw plastic manufacturing further fueling (pun intended) plastic generation as cost of production fell (Center for International Environment Law 2017, Sicotte 2020). Additionally, the growth of renewable energy markets has reduced global demand for traditional fossil fuels (Zhang 2024); fossil fuel companies have refocused their attentions to plastic production as a means of diversifying their portfolios to sustain profits as the planet transitions to renewable energy sources, driving a massive expansion of plastic production (Tilsted et al. 2023). Novel material properties of plastics and rapid production throughout the decades has led to inadequate waste management (lack of waste disposal infrastructure, mismanagement, and leakage) subsequently leading to contamination of the environment (Jambeck et al. 2015, Law 2017, Ryberg et al. 2018, Lechthaler et al. 2020, Fayshal 2024).

1.1 Sources in the Marine Environment

Deposition of plastic into the global oceans comes largely from land-based sources, but maritime sources contribute substantially as well (Li et al. 2016, Schwarz et al. 2019, Rochman 2020). From land, mismanaged waste from households, wastewater and industrial activities yield vast quantities of plastic debris (Rochman 2020). A wide array of industries and sectors contribute to the problem including municipal, commercial, industrial, manufacturing, packaging and distribution, construction, textiles, agriculture and energy (Rochman 2020, Koutnik et al. 2021). Via stormwater runoff, wastewater effluent, and industrial runoff, plastic that is littered, blown from landfills/garbage cans, or otherwise mobilized by natural disasters, accidental spills, intentional dumping, or sewage overflow can enter fluvial systems where it may then be transported to sea (Anderson et al. n.d., Ryan et al. 2009, Coe and Rogers 2012, GESAMP 2015, García Rellán et al. 2023a). A landmark estimate of plastic emissions from land into the oceans, found ~8 million metric tonnes entered the marine environment in 2010 (Jambeck et al., 2015). Based on recent estimates, plastic production is expected to increase (MacLeod et al. 2021, Dokl et al. 2024); the Jambeck et al. (2015) figure of plastic waste entering the global oceans from land is therefore now considerably outdated. Although a handful of studies have aimed to refine and update this estimate in recent years (Schmidt et al. 2017, Lebreton et al. 2017, Bai et al. 2018, Borrelle et al. 2020), they focus on more nuanced contexts (Rochman 2020). For example, land-based plastic waste making its way into aquatic ecosystems in general, including freshwater and brackish systems, amounted to roughly 21 million metric tonnes in 2016 (Borrelle et al. 2020). A comprehensive full-scale global estimate of land-derived plastic debris entering the oceans specifically has yet to be reconsidered, likely a result of the complexity of a myriad of sources and sinks that perpetuate a paucity of data. Activities in the maritime sector are identified as major sources of marine plastic debris too (Coe and Rogers 2012, Daniel and Thomas 2023). These include loss of derelict fishing gear, illegal dumping at sea by vessels, accidental container spills from shipping, and wastewater from ships (Coe and Rogers 2012, Rochman 2020, Daniel and Thomas 2023), with fishing and shipping vessels alone contributing up to 14% of global plastic emissions (Richardson et al., 2019).

1.2 Macro-, Micro-, & Nano- Plastics

Plastic debris in the marine environment is primarily classified by size: macroplastics, microplastics, and nanoplastics. The largest plastics, macroplastics, are items exceeding 5 mm (Cyvin et al. 2021). This category spans several orders of magnitude in size and can include anything from a rubber eraser to a kilometers-long nylon fishing net. On the opposite end of the spectrum are micro- and nanoplastics. Microplastics are smaller than 5 mm (Thompson et al. 2004, Andrady 2011) and are either intentionally produced – termed primary microplastics – or secondarily derived from fragmentation of larger objects (Boucher and Friot 2017, Rochman 2020). Examples of this size range include plastic microbeads in exfoliating skin care products and toothpaste, industrial abrasives, nurdles (pre-production plastic pellets), tire wear particles, paint chips, and synthetic fibers derived from clothes laundering including in dryer lint (An et al. 2020). The smallest plastics, termed nanoplastics, are those smaller than 100 nm (Mattsson et al. 2018). Nanoplastics are often produced indirectly through the degradation of larger plastics or the byproduct of industrial processes (Koelmans et al. 2015).

Physical, chemical, and biological degradation processes work in tandem to fragment plastic debris into continuously smaller particles (Albertsson and Karlsso 1988, Stark and Matuana 2004, Brandon et al. 2016, Song et al. 2020). In the marine environment, physical degradation of microplastics involves abiotic forces; fragmentation due to UV radiation, wave action, and mechanical abrasion (Barnes et al. 2009). Oxidation is the primary mechanism of chemical degradation (Andrady and Koongolla 2022). Finally, an increasing number of organisms have been identified that can degrade plastic (Shah et al. 2008, Wei and Zimmermann 2017, Dawson et al. 2018, Danso et al. 2019, Pérez-García et al. 2021). Plastic degrading organisms are largely microorganisms that can produce plastic-degrading enzymes PETase (PET hydrolase) and MHETase (Monohydroxyethyl terephthalate hydrolase (Maity et al. 2021). However, biological degradation efficiency is very limited (He et al. 2024). Combined, these degradation pathways, and plastic's novel and durable nature make plastic a persistent and pervasive pollutant within marine environments. This dissertation focuses on micro- and macroplastics exclusively.

1.3 Fate in the Marine Environment

Both micro- and macroplastics undergo multiple transport, degradation, and accumulation processes once introduced into marine environments that determine their fate. Buoyant macroplastics like shopping bags, some fishing gear, and bottles may drift across large swaths of ocean, entrained in surface currents or propelled by wind (GESAMP 2015). Ultimately, floating plastics may accumulate in ocean gyres, (re)deposit on coastlines, foul and sink to the seafloor, or be ingested by marine organisms (Rochman 2020, Zhang et al. 2020a, Savoca et al. 2021). Physical, chemical, and biological degradation processes work in tandem to fragment macroplastics into secondary microplastics (Browne et al. 2007, Andrady and Neal 2009, Cole et al. 2011a, Hidalgo-Ruz et al. 2012, Song et al. 2020, So et al. 2022). Both primary microplastics and those derived from the fragmentation of macroplastics can be found throughout the water column across the world's oceans. Low density microplastics may remain at or near the surface for some time, but may eventually foul (Fazey and Ryan 2016). Colonization by microorganisms and adherence of organic matter to the microplastic surface increases their weight causing them to begin sinking (Kaiser et al. 2017). These and denser microplastics may finally embed in seafloor sediments in coastal and deep-sea environments (Claessens et al. 2013, Van Cauwenberghe et al. 2013, Zhang et al. 2020a, Carlsson et al. 2021). The bodies of organisms too are reservoirs for plastic debris (Athey et al., 2020; Cole et al., 2011; Coppock et al., 2021; García Rellán et al., 2023). Internalization of marine plastic debris occurs primarily via pulmonary or branchial respiration (Watts et al. 2014, Barboza et al. 2020, Meaza et al. 2021, Sendra et al. 2021, Dziobak et al. 2024) and ingestion (Schuyler et al. 2012, De Stephanis et al. 2013, Goldstein and Goodwin 2013, Allen et al. 2017, Savoca et al. 2017, 2021). Ubiquity of microplastics throughout the marine environment makes organisms with diverse feeding mechanisms susceptible to microplastic ingestion including filter feeders (e.g. baleen whales (Germanov et al. 2018, Zantis et al. 2021a, Kahane-Rapport et al. 2022), bivalves (Zhang et al. 2020b)), suspension feeders (e.g. barnacles (Goldstein and Goodwin 2013)), deposit feeders (e.g. sea cucumbers (Muhammad Husin et al. 2021), polychaetes (Knutsen et al. 2020)), suction feeders (e.g. seahorses (Liu et al. 2022b), groupers (Mardiansyah et al. 2022)), grazers (e.g. manatees (Gowans and Siuda 2023, Zavala-Alarcón et al. 2023), zooplankton), and predation (e.g. seabass (Reinold et al. 2021)). Ingestion of and interactions with both macro- and micro-plastics leads to a myriad of health impacts.

1.4 Effects of Plastic on Marine Organisms & Ecosystems

An increasing understanding of the multidimensional nature of plastic points to a variety of lethal and sub-lethal impacts across all levels of biological organization from cells to ecosystems (Rochman 2015, Bucci et al. 2020). At an individual level, physical pieces of plastic can be harmful via smothering (macro-), entanglement (macro-) or internalization via inhalation (micro-) and ingestion (macro- and micro-). Marine wildlife may misidentify plastic as prey owing to mechanisms related to sensory modalities. For example, gustation (taste) and olfaction (smell), have been identified as drivers of plastic consumption in corals (Allen et al. 2017) and seabirds (Savoca et al. 2016), respectively. Similarities in visual appearance of plastic with typical food items can also drive ingestion (Moore 2008, Mrosovsky et al. 2009, Boerger et al. 2010, Fukuoka et al. 2016, Duncan et al. 2019a, 2019b). Blocked gastrointestinal tracts or a false sense of satiation induced from ingestion of larger pieces of plastic may ultimately lead to malnourishment and death (Simmonds 2012, Poli et al. 2015, Ryan 2016, Alzugaray et al. 2020).

Internal lacerations to the gut or external lacerations resulting from entanglement can cause inflammation or lead to infection (Cassoff et al. 2011, Moore and van der Hoop 2012, Sharp et al. 2019). Organisms may also engage with macroplastics by simple physical contact, resulting in no apparent harm (Tekman et al. 2022). Microplastics too can be physically harmful causing abrasion or perforations prone to infection (Hu et al. 2020a). They may also translocate from the gastrointestinal tract, respiratory systems, or dermis to other organ and body systems (Browne et al. 2008, Brennecke et al. 2015, Løvmo et al. 2017, Messinetti et al. 2019, Zeytin et al. 2020, Elizalde-Velázquez et al. 2020, Mc Ilwraith et al. 2021), or serve as a vector for additives added to plastic polymer blends during manufacturing, pathogens, or environmental pollutants sorbed to the plastic's surface (Browne et al. 2013, Koelmans et al. 2016, Campanale et al. 2020, Gouin 2021, Amelia et al. 2021). Many laboratory-based studies demonstrate methylation and altered gene expression, oxidative stress, inflammation, disrupted feeding, decreased growth and reproductive success, changes in larval development, reduced filtration and respiration rates, altered behaviors, and decreased survival as a result of microplastic exposure (Coe and Rogers 2012, Wright et al. 2013, Bucci et al. 2020, Tekman et al. 2022, Yang et al. 2022). Others reveal endocrine disruption, promotion of cancer cell proliferation, accelerated aging, impaired adipogenesis, impaired lipolysis, obesegenesis, impaired lipid metabolism, and altered cell functions (Bucci et al. 2020, Shiu et al. 2022, Du et al. 2023, Wang et al. 2023, Zhao et al. 2024, Aristizabal et al. 2024, Moon et al. 2024). While laboratory studies demonstrating these impacts are numerous and increasing, where impacts of plastic have been tested, some studies have documented a lack of an effect (Bucci et al. 2020). Ultimately, effects of microplastic exposure vary depending on size, type and concentration of microplastic, exposure duration, and species exposed (Tekman et al. 2022).

Both macro- and microplastics pose risks to marine ecosystems. Ingested microplastics have infiltrated marine food webs and can migrate up ecological positions via trophic transfer, through the consumption of microplastic contaminated food resources (Nelms et al. 2018, Athey et al. 2020, Costa et al. 2020, Elizalde-Velázquez et al. 2020, Hasegawa and Nakaoka 2021). Macroplastic can serve as refuge to smaller organisms and substrates for sessile organisms and pathogens; entrained in currents, plastic can transport these species to new areas potentially resulting in competition, extirpation of resident species, or proliferation of disease (Tekman et al. 2022). Though, the current body of literature suggests these interactions seldom drive critical decreases in populations (Tekman et al. 2022). However, when considering the impacts of plastic pollution on large charismatic species – seabirds, turtles, marine mammals, corals, and sharks and rays – there are potentially serious ramifications for marine ecosystems. Plastic may compound the deleterious impacts of various additional anthropogenic stressors on these oft-dwindling populations (Fossi and Panti 2018). For example, due to smothering and entanglement, research has documented serious damage to coral reefs and mangroves (Frankignoulle and Gattuso 1993, Tekman et al. 2022). Both ecosystems are considered carbon sinks, the destruction of which impacts global carbon cycling, important for both ocean and human health (Tekman et al. 2022).

1.5 Marine Mammals and Plastic

Marine mammals are vulnerable to the effects of plastic marine debris owing to their morphology, anatomy, and behaviors. Susceptibility to plastic is concerning because of the outsized importance of marine mammals to ecosystem structuring and function (Bowen 1997, Kiszka et al. 2015, Lacher et al. 2019). Moreover, marine mammals are commonly identified as sentinels of ecosystem, and increasingly, human health (Wells et al. 2004, Bossart 2011, Reif et al. 2015). Macroplastic debris primarily harms marine mammals via entanglement and ingestion (Lusher et al. 2015, Law 2017, Panti et al. 2019). Adaptations for efficient movement and existence in water – streamlined, torpedo-shaped bodies, short necks and limbs, and baleen plates in mysticetes (baleen whales) – make it difficult to shed fishing gear once entangled (Kruse et al. 2023). Severe entanglements can result in lacerations, severed appendages and death (Cassoff et al. 2011, Kraus 2018). Many examples of stomach content analyses document these animals mistaking plastic for food (Jacobsen et al. 2010, De Stephanis et al. 2013, Zachos 2018, Jerbi et al. 2021, Kruse et al. 2023, Nelms et al. 2023). A stomach full of plastic can result in a false sense of satiation or blocked gastrointestinal tract, preventing nutrient acquisition and ultimately lead to starvation (de Vere et al. 2018, Tuuri and Leterme 2023).

In comparison, the impact of microplastics on marine mammals is relatively understudied (Zantis et al. 2021b, Sanganyado et al. 2021). However, owing to their small size, ubiquity, and persistence, microplastics may be internalized in marine mammals through direct ingestion and/or trophic transfer depending on the feeding strategy a species employs (Zantis et al. 2021b, 2021a, Kahane-Rapport et al. 2022, Werth et al. 2024). Filter feeding mysticetes sieve large volumes of water engulfed during prey capture which can lead to ingestion of microplastic via either pathway: consumption of prey contaminated with microplastics (trophic transfer) or indirect uptake from the water column (Germanov et al. 2018, 2019, Torres et al. 2023). Though, recent estimates identified trophic transfer as a more significant route of exposure to baleen whales (Zantis et al. 2021a, Kahane-Rapport et al. 2022). In contrast, ingestion of microplastics by marine mammals feeding at mid- to high trophic levels – odontocetes (toothed whales), pinnipeds, sea otters, and polar bears – is most likely to occur via trophic transfer (Ivar Do Sul et al. 2014, Nelms et al. 2018).

1.6 Knowledge Gaps & Dissertation Structure

To date, only three literature reviews explore the nexus between plastic and ingestion of it by marine mammals (Simmonds 2012, Baulch and Perry 2014, Zantis et al. 2021b), underscoring a relatively nascent understanding of the impacts of plastic marine debris on this taxon. Owing to ethical, legal, fiscal, and logistical challenges of studying living, free-ranging marine mammals at sea – particularly whales – studies have relied primarily on post-mortem analyses, gut content analyses (Lusher et al. 2015, 2018), fecal analyses (Ryan 2016, Donohue et al. 2019) and biomarkers (Baini et al. 2017) to investigate exposure risk to both macro- and microplastics. Although these efforts have increased in recent years, many questions remain surrounding the drivers and consequences of ingesting plastic by marine mammals. For example, it is not known whether microplastic particles transfer to and embed in the tissues of marine mammals upon ingestion (Perez-Venegas et al. 2018, Zantis et al. 2021b) or whether microplastic exposure subsequently results in any health effects to the animals (Claro et al. 2019, Panti et al. 2019, Zantis et al. 2021b). The former represents my first dissertation chapter and relies on tissues collected opportunistically post-mortem from whale and seal species. Chapter two expands upon the commonly used methodologies listed above by employing *in vitro* cellular assays and *ex vivo* blubber explants to explore metabolic and transcriptomic effects of microplastic exposure to marine mammals. In the case of macro-litter, it is unknown what sensory modalities influence plastic consumption for marine mammals. Visual misidentification is a reasonable hypothesis for species that forage at or near the sea surface. However, some marine mammal species forage below the photic zone (Quick et al. 2020). Because there is no light to see, whether or not acoustic capabilities play a role in plastic consumption in deep-diving, echolocating odontocetes is the focus of my third chapter. Finally, my dissertation concludes with a summary chapter. Chapter five synthesizes key findings from published chapters two and four, contextualizes remaining findings from my dissertation, and recommends pathways for future investigation.

1.7 Collaborative Works

Chapter 2 is published *in Environmental Pollution*, cited as: Merrill, G. B., Hermabessiere, L., Rochman, C. M., & Nowacek, D. P. (2023). Microplastics in marine mammal blubber, melon, & other tissues: Evidence of translocation. *Environmental Pollution*, *335*, 122252. https://doi.org/10.1016/j.envpol.2023.122252. Greg B Merrill was responsible for: conceptualization, investigation, data curation, formal analysis, writing – original draft, writing –

review & editing, visualization, funding acquisition; Ludovic Hermabessiere for: supervision, resources, writing – review & editing, methodology, investigation; Chelsea M Rochman for: supervision, resources, writing – review & editing, methodology; and, Douglas P Nowacek for: supervision, and resources.

Chapter 4 is published in *Marine Pollution Bulletin*, cited as Merrill, G. B., Swaim, Z. T., Benaka, I. G., Bishop, A. L., Kaney, N. A., Kuhlman, S., Matheson, J. C., Menini, E., Goh, S., Lei, S., & Nowacek, D. P. (2024). Acoustic signature of plastic marine debris mimics the prey items of deep-diving cetaceans. *Marine Pollution Bulletin*, *209*, 117069. https://doi.org/10.1016/J.MARPOLBUL.2024.117069. I, Greg B. Merrill, was responsible for: Writing – review & editing, Writing – original draft, Visualization, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation; Z.T. Swaim for: Writing – review & editing, Supervision, Resources, Investigation; I.G. Benaka for: Writing – review & editing, Investigation; A.L. Bishop for: Writing – review & editing, Investigation; N.A. Kaney for: Writing – review & editing, Investigation; S. Kuhlman for: Investigation; J.C. Matheson for: Writing – review & editing, Investigation; E. Menini for: Investigation; S. Goh for: Investigation; S. Lei for: Investigation; and D.P. Nowacek for: Writing – review & editing, Supervision, Software, Resources, and Methodology.

2 Microplastics in marine mammal blubber, melon, & other tissues: evidence of translocation

Chapter 2 is published *in Environmental Pollution*, cited as: Merrill, G. B., Hermabessiere, L., Rochman, C. M., & Nowacek, D. P. (2023). Microplastics in marine mammal blubber, melon, & other tissues: Evidence of translocation. *Environmental Pollution*, *335*, 122252. https://doi.org/10.1016/j.envpol.2023.122252. I, Greg B Merrill, was responsible for: conceptualization, investigation, data curation, formal analysis, writing – original draft, writing – review & editing, visualization, funding acquisition; Ludovic Hermabessiere for: supervision, resources, writing – review & editing, methodology, investigation; Chelsea M Rochman for: supervision, resources, writing – review & editing, methodology; and, Douglas P Nowacek for: supervision, and resources.

2.1 Introduction

Plastic pollution continues to increase in oceanic environments globally at a predicted average rate of 8.75 million metric tonnes per year based on 2010 data (Jambeck et al. 2015, Geyer et al. 2017), impacting ecosystems, societies, and economies (Worm et al. 2017). Incidence of ingestion of plastics is well-documented in 1288 marine species(Santos et al. 2021). In fish alone, nearly 400 species have been documented to consume microplastics (plastic particles 1µm - 5mm) – 54% of which are commercially important (Savoca et al. 2021). Occurrence of plastic ingested by marine fish has more than doubled in the last decade; fish are consuming plastic more frequently over time (Savoca et al. 2021). Marine mammals also ingest microplastics either incidentally from the water column during prey capture or through trophic transfer (Nelms et al. 2018), consumption of lower-trophic level organisms who have in-turn themselves ingested plastic. Baleen whales are estimated to consume between 200,000 and 10 million microplastic particles indirectly through contaminated prey each day, depending on species and foraging strategy (Zantis et al. 2021, Kahane-Rapport et al. 2022). While large anatomical structure of the gastrointestinal tract suggests a majority of microplastics consumed by marine mammals should be excreted through defecation, ultimately the collective fate of ingested microplastic particles is unknown.

Apart from egestion, one possible fate of ingested microplastics is translocation from the gastrointestinal tract to other body organs and tissues. The phenomenon of microplastic translocation has been demonstrated experimentally in various species (Browne et al. 2008, von Moos et al. 2012, Messinetti et al. 2019, De Sales-Ribeiro et al. 2020, Zeytin et al. 2020) with a majority of studies utilizing particles <20µm in size. Field studies of wild-caught species report microplastic particles in various tissues spanning the entire microplastic size classification range, ie. 1µm – 5000µm (Collard et al. 2017, Abbasi et al. 2018, Mc Ilwraith et al. 2021). At present, two mechanisms are predicted to explain microplastic translocation: 1) transcellular uptake through intestinal epithelial cells into circulatory fluid (Fuglem et al. 2010, von Moos et al. 2012, Løvmo et al. 2017) or, 2) paracellelular diffusion between the tight junctions of adjacent cells (Vagner et al. 2022). Together, both mechanisms accommodate microplastic particles < ~130 µm, however those <83 µm are more likely to translocate based on a synthesis of research (Mehinto et al. 2022). The mechanisms by which larger particles >130µm translocate are not understood but may possibly be facilitated by the abrasion and perforation of tissues and/or induction of the inflammatory response (Handy et al. 2008, Mc Ilwraith et al. 2021).

Translocation of microplastics has not been examined in marine mammals. The lack of research on this topic for marine mammals is likely due in part to the logistical challenges and financial constraints of working with large, free-ranging species at sea. However, marine mammals may be at a disproportionately higher risk of accumulating microplastics in their bodies by virtue of their foraging behaviors and anatomy. For example, blubber is a lipid-rich, layered, and vascularized adipose tissue important for energy storage and mobilization (inner layer) (Strandberg et al. 2008a, Koopman 2018) unique to marine mammals. Known lipophilic and hydrophobic properties of microplastics and associated compounds (Teuten et al. 2007), along with their demonstrated ability to translocate via the circulatory system in other species, makes blubber a notable candidate sink destination for microplastics ingested by marine mammals. Similarly, the acoustic organs of toothed whales are composed in large part of fats. While generally considered metabolically inactive, the acoustic tissues are vascularized and provisioned oxygen via the circulatory system, suggesting they too are susceptible to microplastic translocation.

Many questions remain regarding the effects of microplastic exposure, especially for marine mammals. Studies addressing impacts from exposure often utilize environmentally irrelevant plastics and concentrations making it difficult to draw conclusions about effects of mixtures and types of microplastics in nature (Bucci et al. 2020). However, some observed effects include changes in gene expression, decreased growth, inflammation, perforation, abrasion, and denuding of tissues, oxidative stress, and disruption of immune system functioning (Lusher et al. 2015, Bucci et al. 2020, Hu et al. 2020a, Bhuyan 2022, Yuan et al. 2022). Translocation of microplastics in marine mammals could have direct health implications as well as downstream implications for human health. Native subsistence users, for example, depend on marine mammals culturally and for food security. At the time of writing, Iceland, Norway, and Japan engage in commercial whaling and the consumption of whale products, though Iceland has announced the end of its commercial practices by 2024. Presence of microplastics in harvested tissues could pose food safety, food quality, and food availability risks (Mc Ilwraith et al. 2021) to native communities. Furthermore, as sentinel species whose diet overlaps with both commercial and small-scale fisheries, the presence of plastic in marine mammal tissues may serve to warn that consumption of various seafoods may expose consumers to microplastics. As such, this study aimed to identify if microplastics are translocating to various tissues in marine mammals using a combination of Pyrolysis-Gas Chromatography/Mass Spectrometry and Raman Spectroscopy to examine opportunistically obtained samples from stranding events and native subsistence harvests in Alaska, California, and North Carolina, USA.

2.2 Methods

2.2.1 Sample Acquisition

Marine mammal tissue samples from 32 individuals spanning 12 species (Table 1) – including blubber, melon, mandibular acoustic fat pad, and caudal lung – were obtained through partnerships with NOAA Fisheries' Alaska, Southeast and West Coast regional stranding networks under parts authorization letter 1514-08b pursuant to the United States' Marine Mammal Protection Act. Samples were originally procured opportunistically between 2000 and 2021 either from stranded individuals or from subsistence harvests from various locations in Alaska, California, and North Carolina. Samples were not stored with microplastic analyses in mind; tissues were stored frozen at -80°C in a mix of plastic polyethylene Whirl-Pak bags or foil before receipt for this project. For details, see the Quality Assurance & Quality Control section below, but in short to remove any microplastics introduced during sampling and storage, tissues were rinsed with filtered water and the outer edges shaved off and discarded. Tissue was then stored at -80°C in foil until processing.

Table 1. Samples evaluated for microplastics by MAE py-GC/MS or Raman spectroscopy, noting for the former which samples were lost due to system limitations. A. Fat Pad = Acoustic Fat Pad.

Treatment	Animal ID	Species	Year	Strand/ Harvest	Location (US State)	Tissue	Digested Mass (g)	Analysis	Py-GC/MS loss?
072621-T2	Procedural Blank	-	-	-	-	-	-	MAE py-GC/MS	No
072621-T3	JPIER024	Bottlenose Dolphin	2020	Strand	NC	Blubber	15.85	MAE py-GC/MS	No
072621-T4	JPIER025	Bottlenose Dolphin	2020	Strand	NC	Blubber	16.12	MAE py-GC/MS	No
072621-T5	VGT419	Bottlenose Dolphin	2019	Strand	NC	Blubber	15.53	MAE py-GC/MS	No
072621-T6	EB19PH022	Bearded Seal	2019	Harvest	AK	Blubber	15.10	MAE py-GC/MS	No
072621-T7	EB19PH028	Bearded Seal	2019	Harvest	AK	Blubber	19.03	MAE py-GC/MS	Yes
072621-T8	PI19SH009	Spotted Seal	2019	Harvest	AK	Blubber	16.38	MAE py-GC/MS	Yes
080921-T2	Procedural Blank	-	-	-	-	-	-	Raman	NA
080921-T3	OO1010	Minke Whale	2011	Strand	AK	Blubber	23.80	Raman	NA
080921-T4	BP1602_20160706	Fin Whale	2016	Strand	AK	Blubber	19.56	Raman	NA
080921-T5	PH110311_AVPS	Ringed Seal	2011	Strand	AK	Blubber	18.71	Raman	NA
080921-T6	2017221	Beluga Whale	2017	Strand	AK	Blubber	19.61	Raman	NA
080921-T7	2019010	Humpback Whale	2019	Strand	AK	Blubber	23.33	Raman	NA
080921-T8	2019031	Gray Whale	2019	Strand	AK	Blubber	14.74, 16.21	MAE py-GC/MS, Raman	No
080921-T9	EB19PH012	Bearded Seal	2019	Harvest	AK	Blubber	18.36	Raman	NA
080921-T10	EB19PH026	Bearded Seal	2019	Harvest	AK	Blubber	26.76	Raman	NA
0112921-T2	Procedural Blank	-	-	-	-	-	-	Raman	NA
0112921-T3	C-118 UPPER	Gray Whale	2000	Strand	CA	Blubber	21.45	Raman	NA
0112921-T4	C-118 LOWER	Gray Whale	2000	Strand	CA	Blubber	25.80	Raman	NA

0112921-T5	C-671 UPPER	Gray Whale	2021	Strand	CA	Blubber	18.50	Raman	NA
0112921-T6	C-671 LOWER	Gray Whale	2021	Strand	CA	Blubber	18.16	Raman	NA
0112921-T7	C-672 UPPER	Gray Whale	2021	Strand	CA	Blubber	15.65	Raman	NA
0112921-T8	C-672 LOWER	Gray Whale	2021	Strand	CA	Blubber	10.65	Raman	NA
0112921-T9	RT48	Pilot Whale	2005	Strand	NC	Blubber	22.36	Raman	NA
0112921-T10	RT50	Pilot Whale	2005	Strand	NC	Blubber	20.40	Raman	NA
0112921-T11	RT57	Pilot Whale	2005	Strand	NC	Blubber	22.60	Raman	NA
121321-T2	Procedural Blank	-	-	-	-	-	-	MAE py-GC/MS	Yes
121321-T3	CALO2106	Pygmy Sperm Whale	2021	Strand	NC	Blubber	22.89	MAE py-GC/MS	Yes
121321-T4	JPIER033	Pygmy Sperm Whale	2021	Strand	NC	Blubber	25.08	MAE py-GC/MS	Yes
121321-T5	CAVIA517	Bottlenose Dolphin	2021	Strand	NC	A. Fat Pad	24.55	MAE py-GC/MS	Yes
121321-T6	CAVIA517	Bottlenose Dolphin	2021	Strand	NC	Melon	26.83	MAE py-GC/MS	Yes
121321-T7	PL19SH003	Spotted Seal	2019	Harvest	AK	Blubber	27.09	MAE py-GC/MS	Yes
121321-T8	PL19SH019	Spotted Seal	2019	Harvest	AK	Blubber	21.71	MAE py-GC/MS	Yes
121321-T9	PL19SH031	Spotted Seal	2019	Harvest	AK	Blubber	20.69	MAE py-GC/MS	Yes
121321-T10	PH19SH012	Ringed Seal	2019	Harvest	AK	Blubber	16.68	MAE py-GC/MS	Yes
121321-T11	PH19SH047	Ringed Seal	2019	Harvest	AK	Blubber	23.72	MAE py-GC/MS	Yes
060122-T2	Procedural Blank	-	-	-	-	-	-	Raman	NA
060122-T3	CAHA 564	Bottlenose Dolphin	2021	Strand	NC	Melon	17.98	Raman	NA
060122-T4	CAHA 564	Bottlenose Dolphin	2021	Strand	NC	A. Fat Pad	23.07	Raman	NA
060122-T5	2020230	Beluga Whale	2020	Strand	AK	A. Fat Pad	11.75	Raman	NA
060122-T6	2020230	Beluga Whale	2020	Strand	AK	Lung	18.93	Raman	NA
060122-T7	2019384	Beluga Whale	2019	Strand	AK	A. Fat Pad	14.88	Raman	NA
060122-T8	2019384	Beluga Whale	2019	Strand	AK	Lung	11.41	Raman	NA
060122-T9	2019396	Beluga Whale	2019	Strand	AK	A. Fat Pad	14.03	Raman	NA
060122-T10	2019396	Beluga Whale	2019	Strand	AK	Lung	9.92	MAE py-GC/MS	Yes

2.2.2 Tissue Digestion & Filtration

Samples were sectioned, weighed (wet weight ranging between 9.92-27.09g, Table 1), placed in borosilicate glass jam jars (120mL), covered with inverted petri dishes, and baked at 50°C (Econotherm Laboratory Oven, Precision Scientific) for 48h along with a negative control (blank). Blubber tissue from gray whales C-118, C-671, and C-672 were further sectioned in half vertically to evaluate presence of microplastics within the more metabolically active inner layer and the thermoregulatory outer layer and are denoted as lower and upper, respectively. A stock solution of 20% (w/v) potassium hydroxide (KOH; Sigma-Aldrich SKU 221473) was prepared and pre-filtered through a baked (see QA/QC below) 47mm 1µm glass fiber filter (MilliporeSigma SKU APFB04700) prior to use. At 48h, KOH ~3x the volume of the sample was added to each jar and samples were shaken at 50 rpm at 50°C for an additional 48hrs. The combination of KOH with the high-fat content of the chosen marine mammal tissues results in a saponification reaction; after 96h of digestion, the resulting soap is dissolved with the addition of ~30mL 100% ethanol for ~4h (von Friesen et al. 2019). Dissolution of soap was occasionally assisted by stirring with a glass rod. Each dissolved sample was then vacuum filtered through a baked 1µm glass fiber filter using a glass Buchner filtration apparatus. A 20mL aliquot of 30% hydrogen peroxide was added to the Buchner funnel and allowed to oxidize any remaining fatty residues for a minimum of 1 hour before filtering again. Glass fiber filters containing samples were stored in individual, covered glass petri dishes for analysis.

2.2.3 Characterization of Microplastics by Mass using Py-GC/MS

The mass of microplastics in samples was intended to be quantified using microwave-assisted extraction (MAE) coupled with pyrolysis gas chromatography mass spectrometry (Py-GC/MS) following (Hermabessiere and Rochman 2021). Due to system limitations resulting from processing ultra-fatty tissues, only five were successfully processed using MAE and Py-GC/MS with a total of 12 lost (Table 1). The remaining samples were instead characterized using Raman spectrometry, discussed below. Py-GC/MS targets six polymer types: polycarbonate (PC), polystyrene (PS), poly(methyl-methacrylate) (PMMA), polyvinylchloride (PVC), polypropylene (PP) and polyethylene (PE). Briefly, plastic polymers were extracted from samples by microwaving using a Mars6 Microwave Reaction System (CEM Corporation): following digestion and filtration, glass fiber filters (samples), dichloromethane (DCM) blanks, and a reference standard suite composed of PC, PS, PMMA, PVC, PP, PET, PE, were placed in 25mL DCM and microwaved at 180°C for 15min using classic method set-up (Ramp time: 25min; Power: 1800W). After extraction, samples, standards, and blanks were centrifuged (5810R, Eppendorf) for 5min at 2246g at 18°C and then concentrated to a volume of 2mL under a stream of nitrogen (TurboVap, Biotage), loaded into pyrolysis analysis cups, allowed to evaporate completely, and pyrolyzed (decomposed through heating) at 590°C for 12sec (AS-1020E autosampler on an EGA/PY3030-D pyrolyzer, FrontierLab). Pyrolysis products were transferred with a split of 15, on an 8890 GC (Agilent) equipped with a HP-5ms column (30 m; 0.25 mm; 0.25 µm thickness; Agilent). The oven program was set as follows: 35°C for 2 min, then increased to 310°C at 3°C/min, and held for 30 min. Carrier gas used was Helium with a constant flow of 0.8 mL/min. Mass spectra were obtained using an Agilent 5977B MS device. Interface temperature between both instruments was set at 280°C, source temperature was set at 230°C, quad temperature was set at 150°C, ionization voltage was set at 70 eV, and a mass range from 50 to 650 amu was scanned at 2.5 scans/second.

Seven external calibration curves were prepared from the reference standard suite in order to calculate sample polymer mass; reference polymers were weighed on a precision balance (Explorer 125D, Ohaus) and extracted following the MAE method described above. 0.5, 1, 1.5, 2, 3.5, 5, and 10µL of reference standard solution was added to seven separate pyrolysis cups and allowed to evaporate for 30min. After evaporation, 5µL of an internal standard (polystyrene-d5 dissolved in DCM ([0.122mg/mL]) was added to each cup. Concentrations (µg/µL) for each calibration curve were as follows: 1.09 for polyethylene and 1.04 PVC, polypropylene 0.53, polystyrene 0.53, PMMA 0.43, and polycarbonate 0.42. Individual polymer calibration curves were produced using relative response of each polymer by integrating both characteristic peak (see (Hermabessiere and Rochman (2021)) and polystyrene-d5 (m/z=109) peak.

2.2.4 Characterization of Microplastics Particles by Raman Spectroscopy

Under a stereomicroscope, filters were examined visually for microplastic particles following (Munno et al. 2022): suspected plastic particles were categorized according to color and morphology (fragment, fiber, film, foam, sphere) based on visual characteristics and texture. All putatively identified microplastics were removed from the filters with stainless steel forceps and mounted to an acetate sheet using double-sided tape, barring those suspected to be introduced during sample collection or processing discussed below in Quality Assurance & Quality Control. Plastic identity was verified, and polymer type identified, using Raman spectroscopy: a Raman spectrometer (HORIBA Raman Xplora Plus) controlled by LabSpec6 software (version 6.5.1.24) with spectral resolution ranging from 1.325 cm^{-1} per pixel (785 nm excitation laser, 600 grooves/mm) to 3.252 cm^{-1} per pixel (532 nm excitation laser, 1200 grooves/mm) with a 100x long working distance microscope objective and equipped with a charge coupled device detector (–60 °C, 1024 x 256 pixels). Raman spectra were compared with KnowItAll (John Wiley & Sons Inc.), SLoPP, and SloPP-E (Munno et al. 2020) reference libraries of known plastic types. Spectral matches were assigned based on visual confirmation for similar peak alignment and intensity, as well as Hit Quality Index (HQI) score (above 80%) as calculated by the Bio-Rad KnowItAll and ID Expert matching algorithm. Finally, confirmed microplastics were then enumerated by morphology category and color. The size of each particle (longest axis) was measured digitally using a stereomicroscope (Olympus SZX12), camera (Olympus DP71) and ImageJ software (Schneider et al. 2012).

2.2.5 Quality Assurance & Quality Control

To minimize contamination of samples from ubiquitous ambient microplastics, strict QA/QC measures were implemented when processing. Wherever possible, plastic labware and instruments were substituted with glass, metal, and wood. All labware was washed with natural fiber sponges, rinsed three times with deionized water, baked in a muffle furnace (Lindberg/Blue M BF51748C, Thermo Fischer Scientific) at 500°C for 4hrs, and stored covered in aluminum foil in a laminar flow clean cabinet. Samples were collected by the three stranding networks noted above opportunistically without the intention of conducting microplastic analyses; samples were stored in a mix of plastic polyethylene Whirl-Pak bags and foil. As such, upon sample receipt, the outer surfaces of each tissue sample were rinsed three times with pre-filtered (1µm) deionized water, cut off, and discarded to eliminate potential contamination from the environment and original sampling procedures. Tissue was then stored in aluminum foil at 80°C. All procedures were conducted in a laminar flow clean cabinet while wearing a cotton lab coat.

Procedural blanks were implemented to assess potential contamination throughout the tissue digestion and microplastic characterization procedures. If plastic was identified in procedural blank samples, particles identified from samples matching the polymer composition, color, and category (fragment, fiber, film) were presumed introduced procedural contamination and subtracted from the samples (S. Table 1).

2.3 Results

2.3.1 Mass concentration of microplastics using MAE and Py-GCMS

Of 17 non-control samples (16 individual animals) examined using MAE Py-GC/MS, 12 were lost due to a system failure mid-cycle likely caused from residual lipids of the digested tissue (discussed below), leaving five samples (from five individuals) with useable high-quality data. MAE py-GC/MS identified microplastics in the blubber tissue from three of those five samples (60%) which included one bottlenose dolphins, a gray whale, and a bearded seal (Table

2). Dolphin JPIER024 had 25.2µg PVC/g blubber (380.0µg total PVC). 9.5µg PVC/ g blubber (140.0µg total PVC) were found in gray whale 2019031 from Alaska. And finally, a total of 8.87µg PVC was found in the blubber tissue of bearded seal EB19PH022 (0.59µg PVC/g blubber). Representative mass spectrometry data illustrating the presence of PVC are represented in S. Table 1. Microplastics were not detected in dolphins JPIER025 or VGT419, nor on the control filters.

Table 2. Py-GCMS results demonstrating the presence of microplastics in the blubber tissue of four individual marine mammals. Concentrations are expressed as mass of plastic per mass of wet weight blubber tissue. PE = polyethylene, PP = polypropylene, PS = polystyrene, PMMA = poly(methyl methacrylate, PVC = polyvinyl chloride, PC = polycarbonate.

Species	Sample	Year	Strand-Harvest Location	Polymer	Peaks	Peaks Present in Chromatogram?	Indicator Ions (m/z) Present	Styrene Peak area (u.a)	Concentration in Sample (µg/g)	Plastic Mass (µg)	Styrene d5 peak area (u.a.)	Digested Mass (g)
Control	Control	NA	NA	PE	C10, C17	No					21,329,886.53	NA
				PP	2,4-dimethyl-1-heptene	No						
				PS	Styrene trimer	No						
				PMMA	Methyl-methacrylate	No						
				PVC	Indene	No						
				PC	4-isopropenylphenol	No						
Gray Whale	2019031	2019	Alaska	PE	C10, C17	No					20,195,417.25	14.74
				PP	2,4-dimethyl-1-heptene	No						
				PS	Styrene trimer	No						
				PMMA	Methyl-methacrylate	No						
				PVC	Indene	Yes	91 & 115 & 116	6,018,662.94	9.50	140.00		
				PC	4-isopropenylphenol	No						
Bearded Seal	EB19PH022	2019	Alaska	PE	C10, C17	No					30339447.94	15.10
				PP	2,4-dimethyl-1-heptene	No						
				PS	Styrene trimer	No						
				PMMA	Methyl-methacrylate	No						
				PVC	Indene	Yes	91 & 115 & 116	3199839.32	0.59	8.87		
				PC	4-isopropenylphenol	No						
Bottlenose Dolphin	VGT419	2019	North Carolina	PE	C10, C17	No					20,350,459.49	15.53
				PP	2,4-dimethyl-1-heptene	No						
				PS	Styrene trimer	No						
				PMMA	Methyl-methacrylate	No						
				PVC	Indene	No						
				PC	4-isopropenylphenol	No						
Bottlenose Dolphin	JPIER024	2020	North Carolina	PE	C10, C17	No					20,267,768.08	15.08
				PP	2,4-dimethyl-1-heptene	No						
				PS	Styrene trimer	No						
				PMMA	Methyl-methacrylate	No						
				PVC	Indene	Yes	91 & 115 & 116	14,531,915.98	25.20	380.00		
				PC	4-isopropenylphenol	No						
Bottlenose Dolphin	JPIER025	2020	North Carolina	PE	C10, C17	No					23,738,997.06	16.12
				PP	2,4-dimethyl-1-heptene	No						
				PS	Styrene trimer	No						
				PMMA	Methyl-methacrylate	No						
				PVC	Indene	No						
				PC	4-isopropenylphenol	No						

2.3.2 Microplastic particles identified using microscopy and Raman spectroscopy

Small numbers of microplastic contamination were introduced during sample processing; in all but one instance, contaminant particles were identified as blue polyethylene fragments. One additional contaminant particle was characterized as a blue rubber fragment. Supplemental Table 1 outlines samples where blank correction was applied. Additional non-plastic particles were identified in samples including various minerals, as well as various anthropogenic sources (cotton, dyes, cellulose, & glass) which have been excluded from our dataset.

Of 19 individual animals examined using Raman spectroscopy, 13 (~68%) had at least one microplastic particle present in at least one of the four tissues examined: 9 of 14 (64%) blubber samples contained microplastics, 1 of 1 (100%) melon samples, all 3 (100%) acoustic fat pads, and both (100%) caudal lung tissues. A total of 34 particles were observed: 7 in acoustic fat pads, 17 in blubber, 3 in caudal lung, and 7 in melon tissue (Figure 2). See Supplementary Table 2 for detailed breakdown of particles by individual and tissue type including polymer type. Average microplastic particle length ranged from 198μm (min 65.5 – max 436μm, n = 7 particles) in the acoustic fat pads to 537μm (min 31.4 – max 1140μm, n = 3 particles) in lung tissue samples analyzed. Blubber tissue had both the smallest (24.4μm) and largest (1387μm) plastic particles, averaging 383μm (n = 17 particles). The seven plastic particles found in the melon sample ranged between 34.7 and 920μm, averaging 302μm in size. Frequency distribution of particle size by tissue is represented in Figure 1.

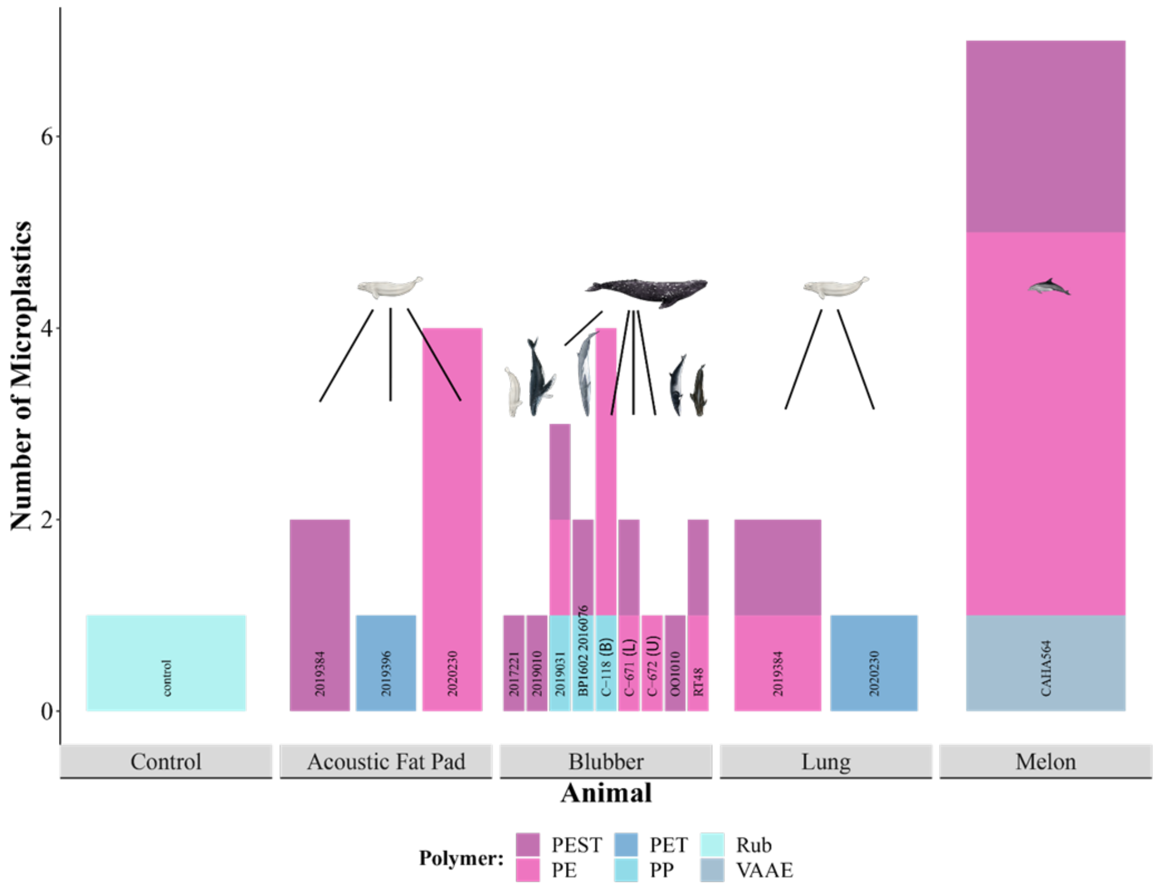

Figure 1: Raman spectrometry results representing number of microplastics per sample grouped by individual and tissue type for samples containing microplastics after blank correction.Vertical alpha-numeric labels represent individual animals (refer to Table 1). (L) = section from the lower, inner blubber layer. (U) = blubber sectioned from the upper blubber layer. (B) = L and U sections processed separately but compiled here. All other blubber samples represent the full blubber depth. PEST: polyester, PE: polyethylene, PET: polyethylene terephthalate, PP: polypropylene, Rub: rubber, VAAE: vinyl acrylic acetate ester.

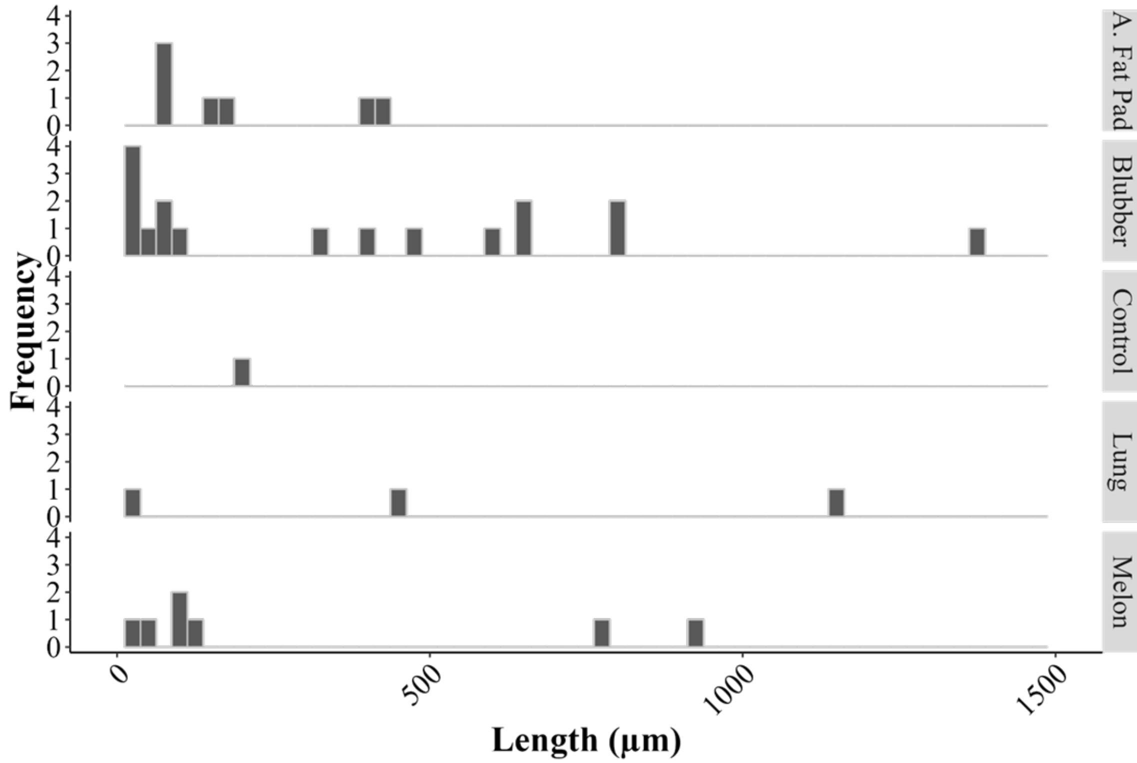

Figure 2: Size (length) histograms of microplastics identified by Raman spectrometry after blank/control subtraction grouped by tissue.

The number of microplastic particles per gram of wet weight tissue ranged between 0.04 and 0.19 in blubber with both extrema found in mysticetes. Mysticetes averaged 0.09 plastic particles per gram of blubber whereas odontocetes averaged 0.07 (range 0.05 – 0.09). Blubber from gray whales C-118, C-671, and C-672 was subsectioned to evaluate propensity for microplastic translocation to the upper or lower layers of blubber tissue but too few samples contained microplastics to make a meaningful comparison, however were present in both layers. The highest concentration across all tissue types was observed in the one melon sample at 0.39 microplastics per g tissue (Figure 3). Acoustic fat pads averaged 0.18 (range 0.07-0.34) while lung tissue averaged 0.11 plastic particles per gram of tissue (range 0.05 to 0.18).

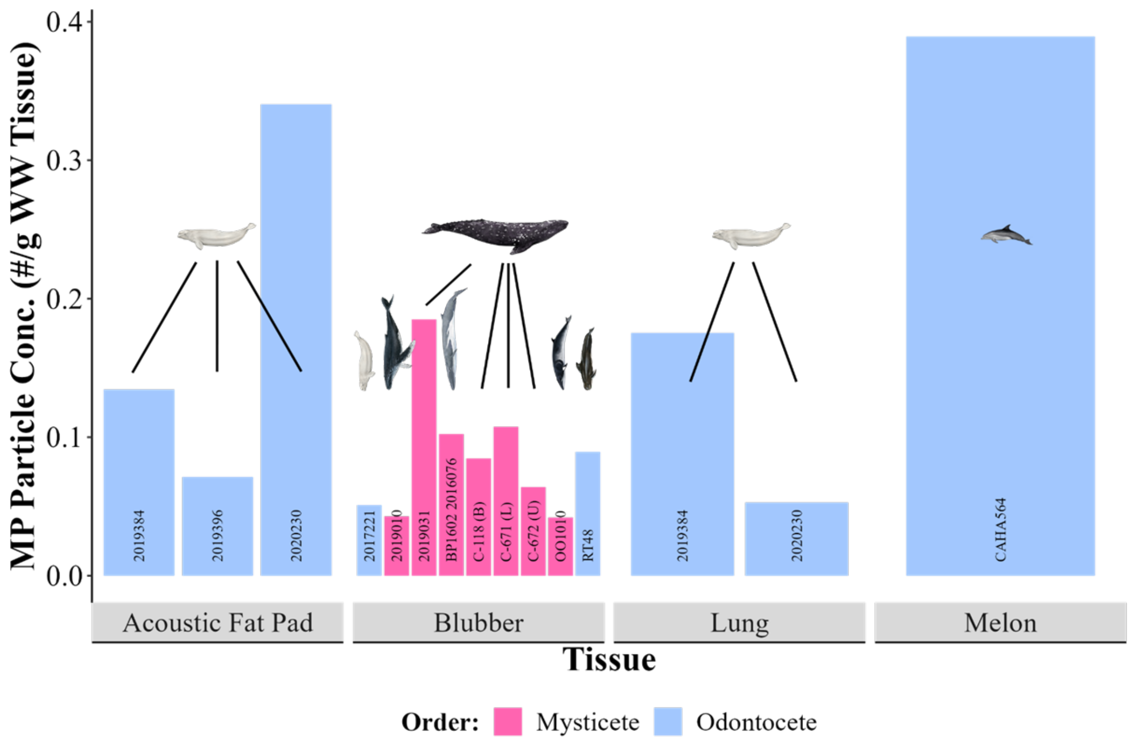

Figure 3: Raman spectrometry results representing number of microplastics (MP) per gram of wet weight (WW) tissue grouped by tissue type and cetacean order for tissues containingmicroplastics after blank correction. Vertical alpha-numeric labels represent individual animals (refer to Table 1). (L) = section from the lower, inner blubber layer. (U) = blubber sectioned from the upper blubber layer. (B) = L and U sections processed separately but compiled here. All other blubber samples represent the full blubber depth.

Raman spectroscopy identified 6 polymers across all tissue types (Supplementary Figure 2). Polyester and polyethylene were the most commonly observed polymer, found in all four tissue types (Figure 4). Polyethylene terephthalate was found in acoustic fat pad and lung tissues. In addition, polypropylene was noted in blubber samples and vinyl acrylic acetate ester was found in melon. Three shape categories were confirmed: fibers and fragments were ubiquitous amongst all tissue types however foams were observed only in the acoustic tissues (Figure 5). No films or spheres were found. With respect to color, eight total were noted (Figure 6). Blue was pervasive across tissue categories. Transparent and purple particles were detected in all tissue types except blubber. Though, blubber demonstrated the greatest diversity of colors, additionally including black, brown, green, and white. Grey particles were noted in the lungs. Controls shown particles composed of blue fragments, mostly polyethylene but one of rubber (Figures 4, 5, 6), and were used to identify and exclude contamination from samples as outlined in Supplemental Table S1.

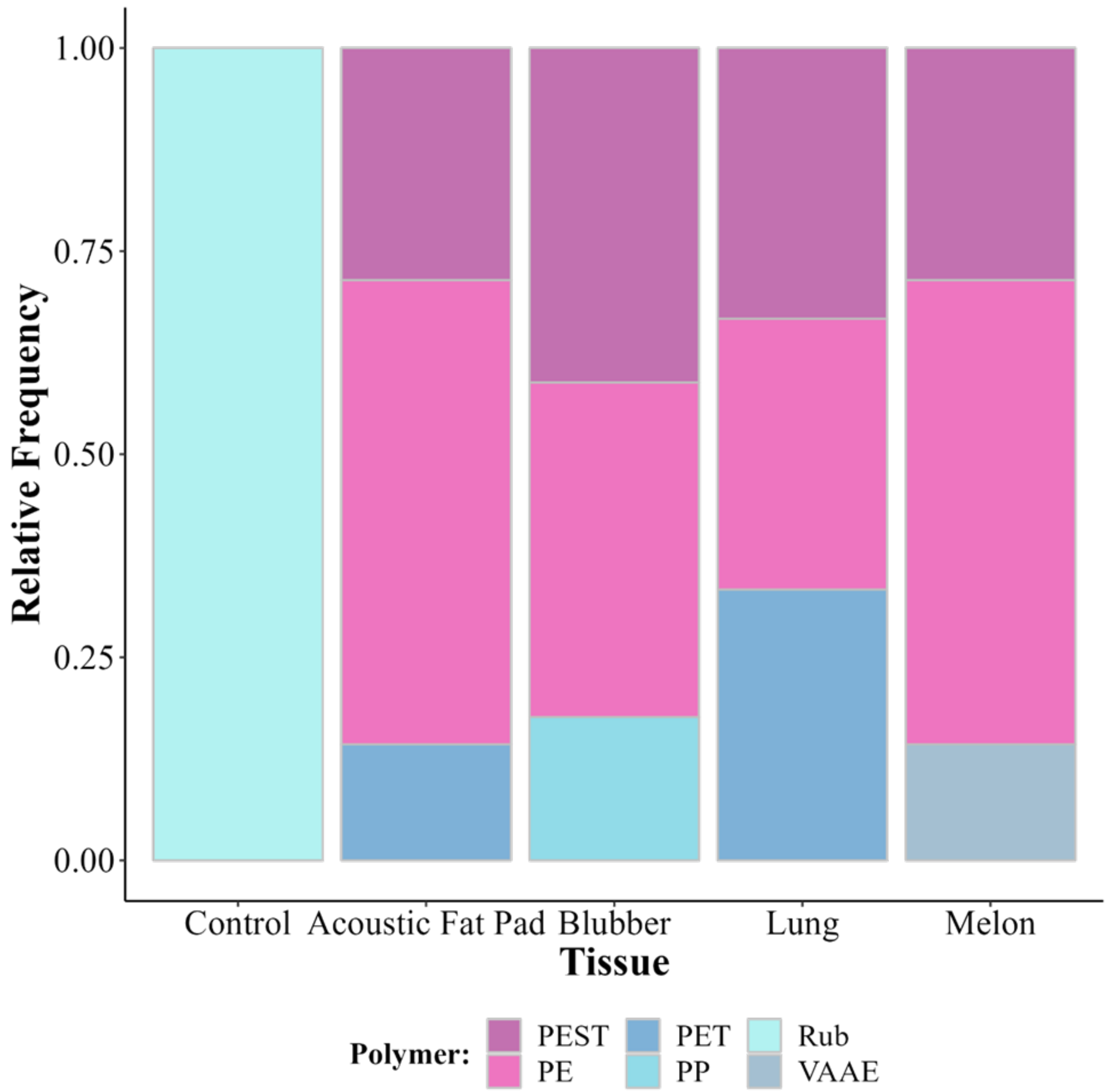

Figure 4: Relative frequency of microplastic polymers by tissue type determined by Raman spectrometry after blank correction.PEST: polyester, PE: polyethylene, PET: polyethylene terephthalate, PP: polypropylene, Rub: rubber, VAAE: vinyl acrylic acetate ester.

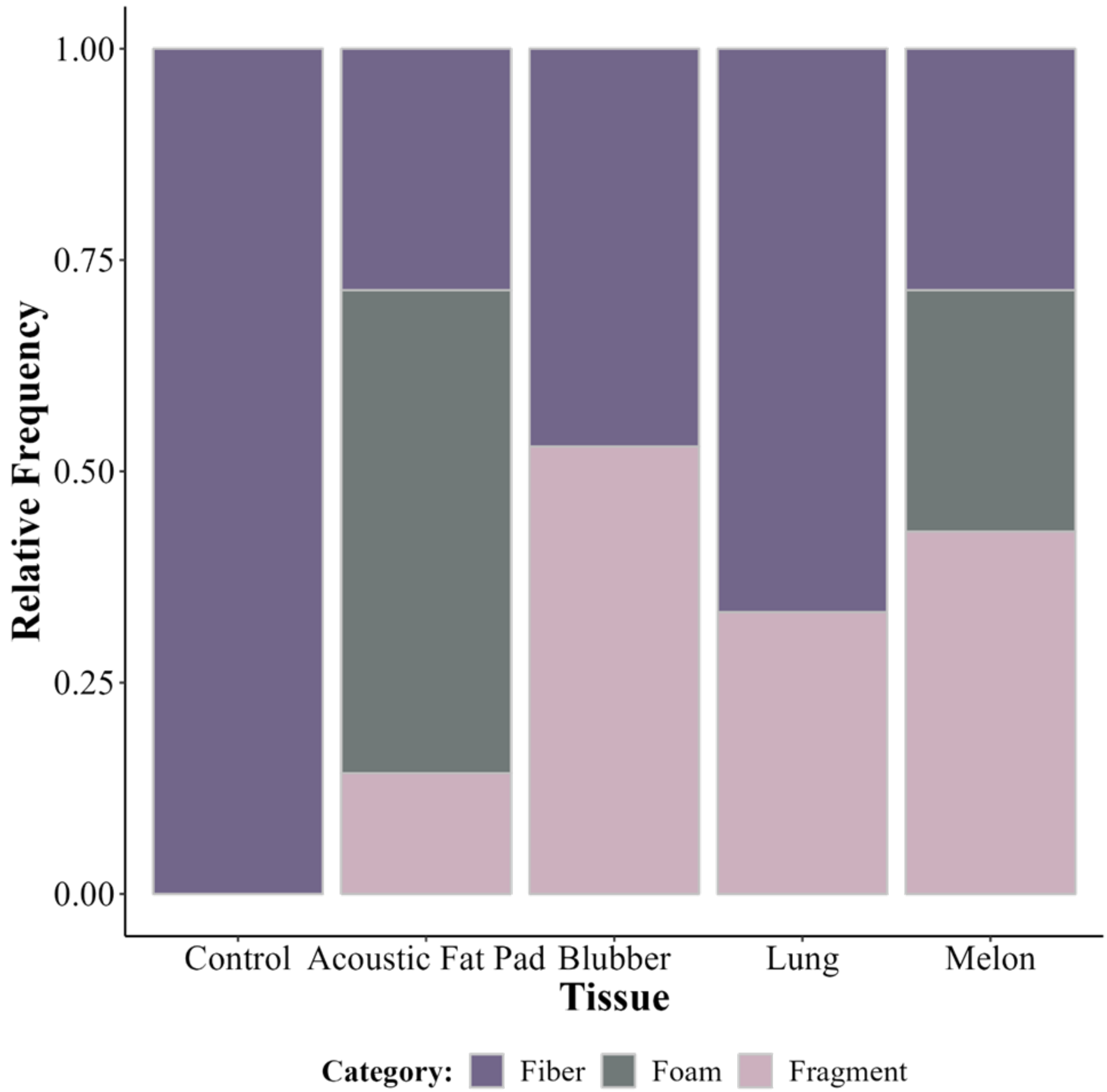

Figure 5: Relative frequency of microplastic shape categories by tissue type determined by Raman spectrometry after blank correction.

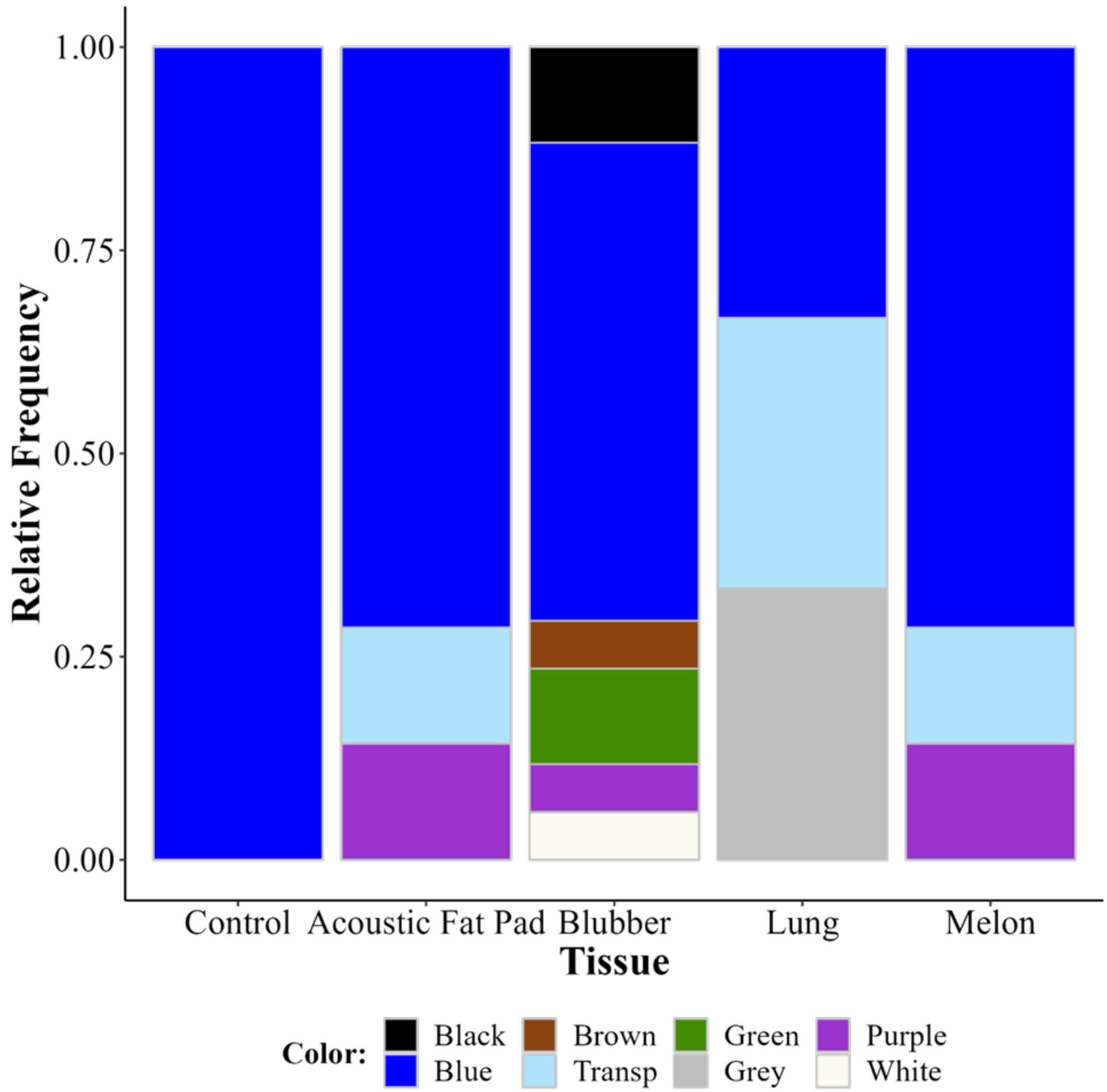

Figure 6: Relative frequency of microplastic colors by tissue type. Transp: transparent determined by Raman spectrometry after blank correction.

2.4 Discussion

The identification of various plastic polymers using both MAE-Py-GC/MS and Raman spectroscopy supports the hypothesis that microplastic particles are translocating to various marine mammal tissues, specifically blubber, melon, acoustic fat pad, and caudal lung, each of which serves vital roles. Here, like for other smaller animals (Collard et al. 2017, Mc Ilwraith et al. 2021), microplastics were found outside the gastro-intestinal tracts of marine mammals. There are three possible exposure routes: dermal contact, inhalation, and ingestion. There is very little research on dermal contact in animals or humans but exposure via the latter two routes is believed to be more significant (Domenech and Marcos 2021). However, the extent to which microplastics are internalized via inhalation in marine mammals is also unknown (Lusher et al. 2018, Nelms et al. 2019). In the case of ingestion, plastic particles observed here would have to have moved from the gastro-intestinal tract to the organs analyzed. The underlying mechanisms of translocation are still unknown, but some can be hypothesized. Translocation following ingestion is thought to occur by transcellular uptake through intestinal epithelial cells into circulatory fluid or paracellular diffusion between the tight junctions of adjacent cells for particles <130μm. Because many of the plastic particles observed here exceed this size threshold, their ability to translocate might otherwise be facilitated by abrasion and perforation of intestinal tissue. Several studies have also observed particles above the theoretical size range for translocation in other species highlighting a research need to understand the mechanisms at work here (Mc Ilwraith et al. 2021). Perhaps shape plays a significant role in translocatability; fibers were the most commonly observed shape in this study and may therefore pass more readily into the circulatory system. However, it is also plausible that exposure to fibers may simply be greater than other observed shapes, driving frequency observations in the tissues. Fragments and then foams were the next most to least commonly encountered shapes, respectively, with relative frequency across the three shapes consistent to observations of relative frequency in the environment (Burns and Boxall 2018, Adamopoulou et al. 2021).

Effects of microplastic exposure are currently understood to be complex; various studies show negative consequences of exposure but just as many demonstrate no effect (Bucci et al. 2020) with effects likely varying dependent on microplastic characteristics (ie. shape, chemical composition, size, color, etc.). Observed effects of microplastic tissue translocation include inflammation, oxidative stress, growth inhibition, and reduced reproductive output and survival

(Ziajahromi et al. 2017, Jaikumar et al. 2018, 2019, Limonta et al. 2019, Xia et al. 2020, Mehinto et al. 2022). Effects specific to marine mammals are unknown but may have an effect on tissues that play vital somatic and ecological functions. For example, acoustic tissues and blubber examined here are highly specialized organs unique to marine mammals affording the ability to echolocate prey as well as thermoregulate and store/mobilize energy, respectively. Negative impacts to these tissues may at worst inhibit their critical functions, or in the least contribute to the cumulative effects of multiple anthropogenic stressors (Pirotta et al. 2022) imposed upon marine mammals in a sublethal, but chronic fashion, that collectively reduce fitness. To date, most studies about the interaction of plastic particles and marine mammals in the environment concern macroplastics (size > 25 mm,(GESAMP 2019)) and how the ingestion of larger pieces of plastic can lead to the death of marine mammals via entanglement and obstructed gastrointestinal tracts (Gall and Thompson 2015, Wilcox et al. 2016) One way to gather more information on the effects of plastic, especially microplastics, on the health of marine mammals would be to implement *ex vivo* assays using organ explants (Panti et al. 2019) or cell cultures.

The presence of microplastics in marine mammal tissues has implications for human health. Because marine mammals are important cultural and subsistence resources for various native communities globally, microplastic-contaminated harvest spoils – as observed here – may directly threaten food security. As sentinel species whose diet overlaps with many commercial and small-scale fisheries globally, the presence of translocated microplastics in marine mammal tissues also indirectly signals that various seafoods may pose a consumption risk to peoples world-wide. Further, samples presented here span over two decades, suggesting microplastics have posed a chronic human (and animal) health exposure risk for some time. The current minimum estimate of plastic entering the oceans annually is based on data from 2010 (Jambeck et al. 2015); research since has modeled increases in plastic in the oceans (Lebreton et al. 2019) with plastic production and waste projected to increase as well (Lebreton and Andrady 2019, Geyer

2020b), further implying the exposure risk to marine mammals and humans alike has and is increasing.

While sample sizes here are too low to conduct additional meaningful statistical analyses for many comparisons of interest (e.g. microplastic concentrations between sexes, age classes, species, geographic location, tissue strata, etc.) it is perhaps worth noting a few interesting observations from the MAE-Py-GC/MS results. First, microplastic mass concentrations found in North Carolinian animals (bottlenose dolphins) were between 2.7 – 42.7x greater than those observed in individuals feeding in Alaskan waters (bearded seal and gray whale; Table 2). With more coastline than the lower 48 states combined, it is inappropriate to generalize all Alaskan waters. However, the pristine quality of the Alaska marine environment – at least relative to the densely-populated continental southeastern United States – may be a contributing factor in this observation. Microplastic inventory of the Chukchi sea, for example, is <1/10th the global average (Ikenoue et al. 2023). Though, foraging behaviors/strategies and life histories may also play a role in exposure risk to translocating microplastic deposition in tissues. For example, gray whales are highly migratory, feeding on highly productive summer feeding grounds for a limited period of time each year before returning to warm-water winter calving grounds. The individual examined here, 2019031, represents the eastern North Pacific gray whale stock with feeding grounds in the Bering and/or Chukchi seas. Microplastic exposure risk in this case is then spatiotemporally dependent on foraging season duration in these locations. Gray whales and bearded seals are benthic and epibenthic foragers, respectively. Such species may therefore be exposed more predominantly to high density microplastics like PVC, PC, PMMA, and PS that presumably sink out of the water column and are subsequently consumed by lower trophic-level benthic organisms that serve as target prey. Indeed, the grey whale and bearded seal examined in this study had high density microplastics in their blubber (Table 2). Dolphins on the other hand are relatively shallow divers, foraging instead on mid-trophic level organisms potentially increasing exposure risk to low density microplastic polymers distributed throughout the upper layers of the water column such as polyethylene as was observed in this study. And lastly, in examination of the Raman spectroscopy data, it is curious that of the colors observed, no warm-colored microplastic particles were identified. This is consistent with at least 20 studies examining microplastics in marine mammal stomachs, gastrointestinal tracts, and feces that identified blue, black, and transparent as most commonly observed (Ugwu et al. 2021). Given trophic transfer is considered the predominant microplastic exposure route in this taxanomic group (Zantis et al. 2021a), perhaps marine mammal prey are selecting against warm-colored particles, or if consumed at depth, are only capable of identifying cool-colored microplastics as particle color, particularly warm colors, is attenuated at depth. Then again, more simply, these colors also correspond to relative occurrence observed in the environment (Martí et al. 2020, Amelia et al. 2021).

Loss of samples due to GC/MS system limitations highlights the need for careful consideration of sample preparation to avoid sample loss and instrument damage. Blubber and acoustic organs are exceptionally fatty tissues. Lipids can affect extraction efficiency, cause matrix effects and introduce baseline disturbance during GC/MS analysis (Chamkasem et al. 2013, Noguera 2021). Here, we successfully analyzed five blubber samples using MAE Py-GC/MS before the GC/MS instrument reached its operational limits. Deliquescence of saponified lipids in ethanol and subsequent treatment of filtered extracts with hydrogen peroxide were intended to limit lipid carryover through to GC/MS analysis, however it is clear from samples lost that additional method development may be required for cleaning up ultra-fatty tissues, such as marine mammal blubber. For example, gel permeation chromatography – an exclusionary methodology based on molecular size – may be necessary to remove lipids for high-throughput of marine mammal samples using MAE-Py-GC/MS, though gel permeation is time consuming and utilizes hazardous chemicals (Jain and Gupta 2006, Noguera 2021). Additional newly-modified methodologies such as quick, easy, cheap, effective, rugged, and safe (QuEChERS) solvent extraction (Anastassiades et al. 2003) with Enhanced Matrix Removal-Lipid (Noguera 2021) represent a promising alternative.

Further, this investigation highlights the utility in the paired complement of Raman with Py-GC/MS methodologies. The former is a non-destructive method that allows for characterization of polymer type, size, shape, color, and category but is limited to particles >~10 µm and does not quantify mass. Py-GC/MS too identifies polymer type with the advantage of being able to detect particles <10µm and quantify mass. However, Py-GC/MS is both destructive and targeted; at the present the method is only capable of detecting PC, PS, PMMA, PVC, PP & PE. Together, the two methods provide a robust complement of microplastic characterization. For example, the MAE-Py-GC/MS data presented here demonstrated the presence of PVC in the blubber sample of grey whale 2019031 whereas no PVC was detected in the sample when analyzed using Raman spectrometry. Raman instead further identified PEST (not quantifiable with the present Py-GC/MS method used), PE, and PP. It is also important to note here that other studies using Py-GC/MS found higher quantities of PVC in comparison with spectroscopic techniques such as Raman (Primpke et al. 2020) even if studies found limited matrix or no effects of natural material on PVC marker such as benzene (not used here; (Okoffo et al. 2020) During this study, PVC was quantified using indene as a marker (Hermabessiere and Rochman 2021) and the presence of other markers such as benzene, toluene, styrene, naphthalene and 3-methylindene were confirmed in order to confirm the presence of PVC. It is possible the PVC particles were too small for Raman analysis based on the technique's limitations and would have otherwise gone undetected without the application of MAE-Py-GC/MS.

While microplastics have been previously documented in marine mammal gastrointestinal tracts, this is the first study to identify translocation and deposition of microplastics into various marine mammal tissues. There is then now a need to explore the consequences of microplastic translocation to the tissues evaluated here (blubber, melon, acoustic mandibular jaw fat, and caudal lung) as well as other organs and tissues with particular focus on eliciting dose-response relationships. Further, identifying the mechanism by which these particles enter the circulatory system (as many here were larger than can be explained by transcellular uptake or paracellular diffusion) can help inform our understanding of microplastic particle retention time in the body: are these particles permanently deposited or are they transitory? Ultimately, the development of a biomarker that can be used to evaluate microplastic contaminant loads in living individual animals, perhaps via biopsy or fecal sample, would allow for the assessment of risk to wild populations, native subsistence users, and marine mammal consumers globally. Whether the concentration of microplastic in the tissues examined here present a health threat to marine mammals remains to be examined, however the presence of microplastics embedded in internal organs underscores the ubiquity of the pervasive plastic pollution problem afflicting the oceans and its inhabitants with rippling implications for humans.

3 Metabolic and transcriptomic responses to microplastics in marine mammals using two ex-vivo models: blubber explants and cell cultures

3.1 Introduction

Plastic pollution continues to befoul oceanic environments with incidence of ingestion well-documented in > 1200 marine species (Kühn and van Franeker 2020, Santos et al. 2021, Savoca et al. 2021, Nanthini devi et al. 2022, Marmara et al. 2023). Marine mammals (whales, seals, sea lions, etc.) consume microplastics (.001 - 5 mm) either incidentally from the water column during prey capture or through trophic transfer (Nelms et al. 2018), consumption of lower-trophic level organisms who have in-turn themselves ingested plastic. Somewhere between 200,000 and 10 million microplastics are ingested by large, filter-feeding baleen whales per day indirectly via trophic transfer, depending on species and foraging strategy (Zantis et al. 2021a, Kahane-Rapport et al. 2022). Merrill et al. (2023) recently discovered that some of those ingested microplastics can migrate from the gastrointestinal tract and embed in various marine mammal organs – such as blubber, melon, acoustic fat pad (mandibular jaw fat), and lungs – in a process called translocation. A complete understanding of the impacts of microplastic particles on the function of these organs, especially organs unique to marine mammals, is lacking.

Many questions remain regarding the effects of microplastic exposure in general, especially for marine mammals which are difficult to study owing to ethical and logistical challenges. Studies addressing impacts from exposure often utilize environmentally irrelevant plastics and concentrations making it difficult to draw conclusions about effects of mixtures and types of microplastics in nature (Bucci et al. 2020). However, some generally observed effects include changes in gene expression, decreased growth, inflammation, perforation, abrasion, and denuding of tissues, oxidative stress, and disruption of immune system functioning (Lusher et al. 2015, Desforges et al. 2016, Limonta et al. 2019, Bucci et al. 2020, Hu et al. 2020b, Bhuyan 2022, Yang et al. 2022, Yuan et al. 2022, Bishop et al. 2024). For marine mammals, skin and blubber are the most tractable tissues to study owing to the procurement methods' relatively low invasive nature and ease of application (Vazquez et al. 2024). Research into the effects of microplastics on skin and adipose tissues in other species reveal endocrine disruption, inhibition of growth, promotion of cancer cell proliferation, accelerated aging, impaired adipogenesis, impaired lipolysis, obesegenesis, and impaired lipid metabolism (Shiu et al. 2022, Du et al. 2023, Wang et al. 2023, Zhao et al. 2024, Aristizabal et al. 2024, Moon et al. 2024). How these findings compare to the highly specialized skin and unique adipose tissues (like blubber) of marine mammals has yet to be examined.

Microplastics have the potential to inhibit specialized functionality of both skin and blubber tissue in marine mammals. Blubber serves three main functions: buoyancy/streamlining, storage of lipid energy reserves and thermoregulation in a highly thermally conductive aqueous environment (Strandberg et al. 2008b, Iverson and Koopman 2018, Koopman 2018) The skin of marine mammals has also evolved thermoregulatory capacity; in cetaceans it is particularly thick and rich in cytoplasmic lipid vacuoles, serving to combat extreme cold conditions outside the protective insulation of the blubber (Menon et al., 2022; Springer et al., 2021). Observed impacts of microplastic exposure on lipid metabolism in other species noted above suggest that the ability to thermoregulate efficiently may be impaired by microplastics in both skin and blubber tissues. Furthermore, exposure to microplastics could impair capacity to store and mobilize (lipolysis) energy reserves in blubber during critical life history events (e.g. migration, reproduction).

Translocation of microplastics to marine mammal tissues could have downstream implications for human health as well. Native subsistence users of the arctic, for example, depend on marine mammals culturally and for food security (Hovelsrud et al. 2008, Huntington et al. 2016, Fauchald et al. 2017). Presence of microplastics in harvested tissues could pose food safety, food quality, and food availability risks to native communities (Mc Ilwraith et al. 2021, Lusher et al. 2022, Miller 2023). Furthermore, as sentinel species whose diet overlaps with both commercial and small-scale fisheries globally (Trites et al. 1997, Gannon and Waples 2004, Jog et al. 2022), the presence of plastic in marine mammal tissues may serve to warn that consumption of various seafoods may expose consumers to microplastics. This study aims to quantify the impacts of microplastic on lipid catabolism using a comparative *in vitro* approach that exposes marine mammal and human tissues to microplastics. Lipolysis assays were used to quantify rates of lipid catabolism by measuring concentrations of glycerol, a byproduct of lipolysis. Bioinformatic analyses (differential gene expression, protein-protein interaction networks, and KEGG functional analyses) were used to explore transcriptomic responses to microplastics.

3.2 Methods

3.2.1 QA/QC

Any and all glassware, aluminum, and stainless steel were baked at 500 °C for 4 hrs in a muffle furnace to remove traces of ubiquitous ambient microplastics.

3.2.2 Biopsy Sampling

This work was conducted under permit no. 22156 issued to D. P. Nowacek by the U.S. National Marine Fisheries Service pursuant to the Marine Mammal Protection Act. Skin and blubber samples were collected from Atlantic common bottlenose dolphins (*Tursiops truncatus*) and a humpback whale (*Megaptera novaeangliae*) using standard biopsy techniques [35]; Using a crossbow, modified bolts equipped with 20 mm stainless steel tips were deployed at distances ranging between 10-30 m targeting the dorsal and lateral body surfaces near the dorsal fin. Upon sampling, bolts were retrieved, and tissue samples were processed according to the protocols below.

3.2.3 Media Preparation

Three types of media were prepared for this experiment: 1) Sampling media (Dulbeco's Modified Eagle Medium + GlutaMAX (4,5 g/L glucose) + PenStrep (1%)), 2) Culture media (sampling media + Fetal Bovine Serum (10%)), and 3) four exposure medias. Exposures consisted of a control (culture media), and three treatments (culture Media + microplastics at 40 µg/mL white 100 µm polyethylene microspheres (Cospheric), 20 µg/mL black 100 µm polyvinyl chloride fragments, 40 µg/mL black 100 µm polyvinyl chloride fragments). Polyvinyl chloride microplastic fragments were produced by synthetically abrading a black PVC plate using a custom instrument following protocols outlined in and provided by Sipe et al. (2022). Microplastics were weighed on baked aluminum weigh pans and transferred to baked Erlenmeyer flasks by carefully rinsing pans repeatedly with culture media using a glass dropper in a sterile tissue culture laminar flow hood.

3.2.4 Biopsy Explant Microplastic Assays

3.2.4.1 Explant Preparation

Aboard the biopsy vessel, blubber tissue retrieved from biopsies was rinsed with Phosphate Buffered Saline (PBS, Gilbeco) sectioned from the skin and placed in 10 mL sampling media in sterile, baked borosilicate scintillation vials with foil-lined lids to avoid contamination from ubiquitous ambient microplastics. Sample vials were then placed in a Ziplock bag and then in the sampler's pocket to keep tissues roughly mammalian body temperature during transport back to the lab.

Upon arrival at the dock, caps of the scintillation vials containing biopsies were loosened and placed in the tissue culture chamber (5% CO_2, 37°C). Working in a laminar flow clean hood, following standard tissue culture procedure, 8 scintillation vials per individual dolphin (n = 3) were filled with 1mL culture media, subsequently weighed and returned to the hood. The biopsies were removed from the culture chamber and sectioned on a bamboo cutting board in the hood into 8 roughly identically sized pieces starting with one cut along the long axis with 3 additional cuts across the two halves along the short axis. These resulting explants were randomly placed into individual scintillation vials containing pre-weighed media, capped, and weighed again to calculate the mass of the explant. Explants were replaced into the culture chamber for three hours to allow the tissue to acclimate and cellular debris from cutting to settle.

3.2.4.2 Explant Exposure Assay

After three hours, explants were gently removed from scintillation vials via forceps, and placed in a 12-well plate, one explant in 1 mL exposure media per well as follows: In duplicate, dolphin explants were exposed to four treatments: Control (culture media), culture media + 40 μg/mL polyethylene, culture media + 20 μg/mL polyvinyl chloride, culture media + 40 μg/mL polyvinyl chloride. The 12-well plate was manually agitated for 5 min before placing in the tissue culture chamber. At this time, $t = 0$ glycerol concentration was measured from the scintillation vials (see Lypolysis Assay below). The explant exposure was maintained for 72 hrs with once-daily media renewals and glycerol concentration quantification.

3.2.4.3 Lipolysis Assay

Lipolysis activity was monitored by colorimetric quantification of glycerol concentrations at four time points ($t = 0$, 24, 48, and 72 hrs). Glycerol (mM) was measured using a glycerol assay kit (MAK117-AKT, Sigma-Aldrich) according to manufacture's protocols and a Spectra Max M2 spectrophotometer/plate reader (Molecular Devices). Measurements were standardized by mass of the explant. Cumulative concentrations per time point were determined by $C\text{cum}(t) = \sum_{i=t}^{t} C(i)$, where $C\text{cum}(t)$ is the cumulative concentration at time *t*, and $C(i)$ is the concentration at time point i.

3.2.5 Skin Fibroblast Microplastic Assays

3.2.5.1 Cell Culture

Skin sectioned from bottlenose dolphin (Ttr) biopsies was used to establish primary cell lines of skin fibroblasts. Skin was washed with PBS, minced and allowed to adhere to a treated tissue culture plate in culture media. Cells attempting to wound heal, expanding on to the plate, became primary cells which were cultured to confluence in a culture chamber at 5% CO_2 and 37°C. Normal human dermal fibroblasts (NHDF) were purchased (MilliporeSigma) and cultured to confluence as well in the same conditions.

3.2.5.2 Fibroblast Assay

Ttr and NHDF were exposed to the same four treatments as the blubber explants: Control (culture media), culture media + 40 µg/mL polyethylene, culture media + 20 µg/mL polyvinyl chloride, culture media + 40 µg/mL polyvinyl chloride. All treatments were plated in duplicate per species at 200,000 cells per well on a 6-well plate in 1 mL exposure media. The assay ran for 120 hours with daily renewals of media. Cumulative glycerol concentrations were measured prior to media renewal for each replicate at timepoints *t* = 0, 24, 48, 72, 96, and 120 hours as outlined in Lipolysis Assay above.

3.2.5.3 RNAseq & Gene Expression Analysis

Following glycerol quantification at test termination, RNA was extracted from each replicate using a Quick-RNA MiniPrep kit in accordance with the manufacturer's instructions (Zymo Research R1054). RNA concentration was estimated using Qubit fluorometer (Life Technologies, USA). Individual libraries were barcoded and pooled. Integrity was evaluated using a 5300 Fragment Analyzer (Agilent Technologies, USA). Mean RIN scores for samples used for RNA sequencing averaged 9.93 ± 0.14 (range 9.5 – 10). cDNA library preparation and

NovaSeq Illumina sequencing were performed by Duke University's School of Medicine Sequencing and Genomic Technologies Core Facility. Quality of reads was evaluated using FastQC v0.11.7. Then reads were trimmed with Trimmomatic v0.39. Trimmed reads were indexed then aligned to the appropriate reference genome (NCBI RefSeq assembly GCF_000001405.40 and GCF_011762595.1) using samtools v1.10 and HISAT2 v2.10, respectively. Number of reads per gene was calculated using FeautreCounts v2.0.3. Finally, differential gene expression analysis was conducted using DESeq2 v1.42.1 with a false discovery rate of 0.05 and a log2 fold change threshold of 1.0.

3.2.5.4 Functional Analyses

To gain functional insight into the biological roles of differentially expressed genes (DEGs), predicted protein-protein interaction (PPI) networks were constructed for each treatment within each species using STRINGdb v2.14.3. These networks provide a systems-level perspective on how DEGs may interact and contribute to cellular processes. Finally, to evaluate the functional biological context of differentially expressed genes, a Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway Enrichment Analysis was performed using ClusterProfiler v4.10.1 and org.Hs.eg.db v3.18.0. This analysis identified enriched pathways associated with DEGs, offering insights into potential molecular mechanisms impacted by microplastic exposure. Pathways were ranked based on adjusted p-values (FDR < 0.05), and results were visualized to highlight key functional disruptions across treatments and species.

3.3 Results

3.3.1 Blubber Biopsy Explant

3.3.1.1 Lipolysis: Microplastic Assay

Glycerol concentrations were measured daily to evaluate the impact of microplastic exposure on fat metabolism in blubber. The rate of lipolysis in blubber explants increased, with glycerol concentrations increasing by 0.48 mM per day over a 72 hr period when exposed to

40µg/mL of PE 100 µm microspheres (Figure 7). This is a 0.13 mM greater increase in glycerol concentrations per day relative to the control (0.35 mM/d) in this treatment ($t = 3.9$, $p = 0.02$). The rate of lipolysis did not differ from the control in any other treatment.

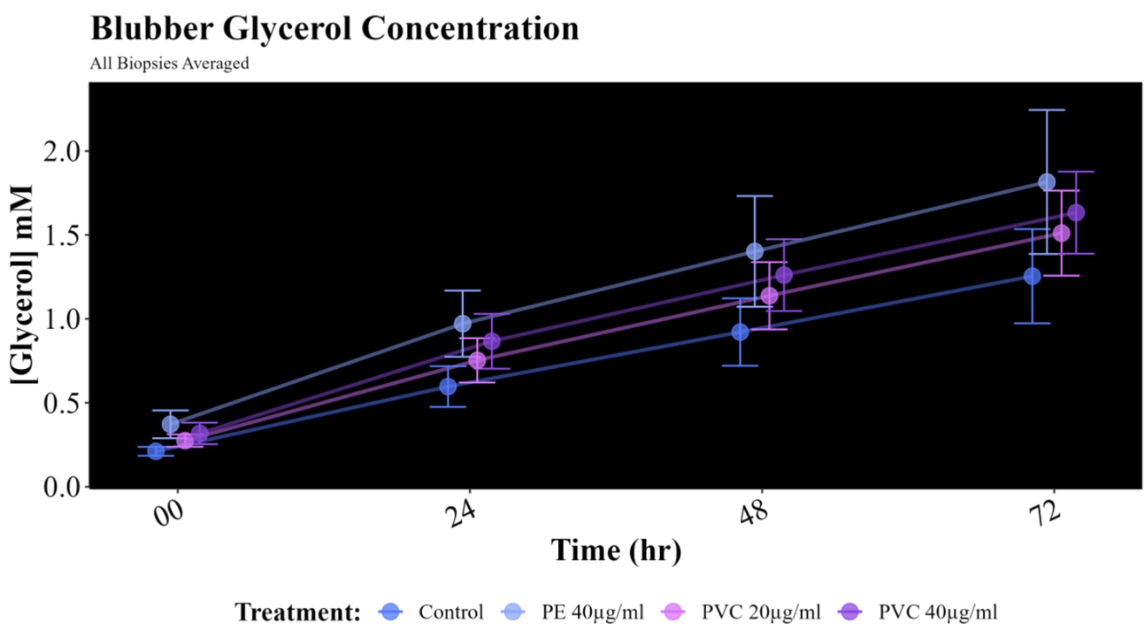

Figure 7: Concentration of glycerol across time produced by dolphin blubber explants exposed to various microplastic treatments.

3.3.2 Skin Fibroblasts

3.3.2.1 Lipolysis: Microplastic Assay

When exposed to 40 µg/mL of PE 100 µm microspheres, dolphin and human skin fibroblasts exhibited species-specific responses with respect to rate of lipolysis as indicated via glycerol concentrations (Figure 8). Dolphin fibroblasts exposed to PE decreased the rate of glycerol production by 0.05 mM/day compared to the control over the 120 hr exposure period ($t = -3.5$, $p = 0.01$). On the other hand, human skin fibroblasts did not significantly alter the rate of lipolysis when exposed to PE (0.011 mM/d, $t = 1.4$, $p = 0.53$). There was no altered response in rate of lipolysis for either species' fibroblasts when exposed to either concentration of polyvinyl chloride microplastics.

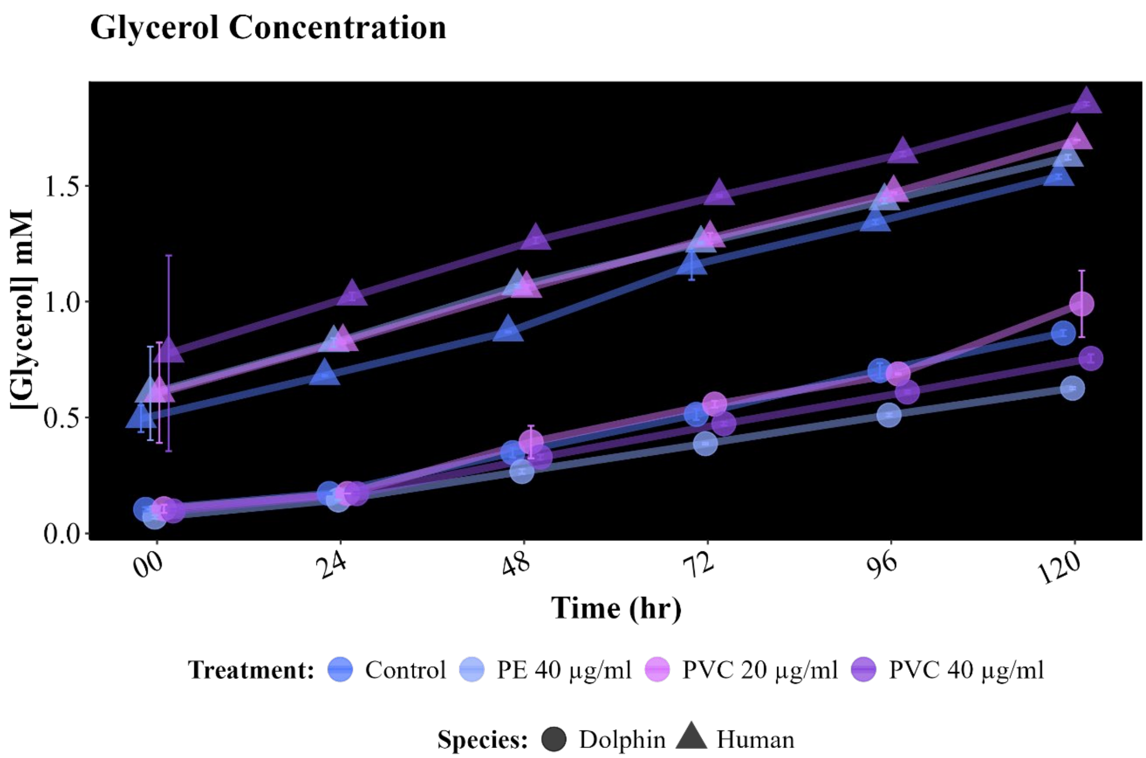

Figure 8: Concentration of glycerol across time produced by dolphin and human skin fibroblasts exposed to various microplastic treatments

3.3.2.2 Differential Gene Expression Analysis

Differential gene expression analysis was conducted at the gene level using DESeq2 comparing each of three plastic treatments (PVC20, PVC40, PE40) to a control within each of the two species (Ttr, NHDF). False discovery rate and log2fold change were set at <0.05, and >1, respectively. Relative to the control, when exposed to PVC20, 2422 Ttr genes were differentially expressed; 1514 upregulate, 908 downregulated (Figure 9). A total of 2306 Ttr genes were differentially expressed when exposed to PVC40 (1502 upregulated, 804 downregulated). In total, 1216 genes were differentially expressed (902 upregulated, 314 downregulated) when exposed to PE40 relative to the Ttr control. Of those 1216 genes, just two – ANGPTL4 and IRS2 – play a role in lipolysis and were upregulated in the PE40 exposure.

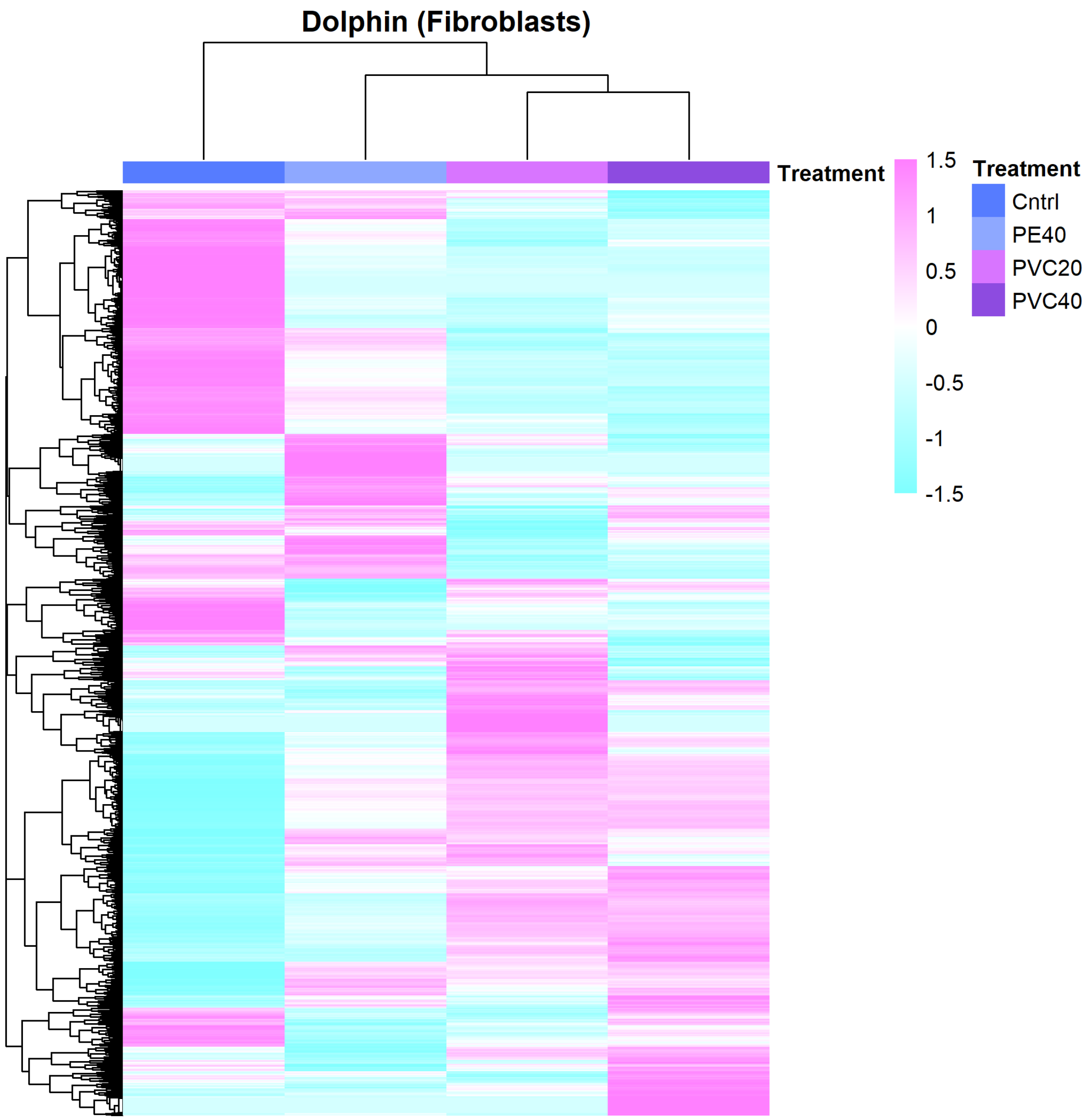

Figure 9: Differential expression results of genes for dolphin skin fibroblasts in response to three microplastic treatments.

Differential expression of NDHF cells to microplastic exposures are represented in Figure 10. In the PVC 20 µg/mL exposure, 188 NHDF genes were significantly differentially expressed (67 upregulated, 121 downregulated). Increase the concentration to 40 µg/mL and the PVC exposure caused differential expression in 202 NHDF genes, 73 upregulated and 129 downregulated. PE40 saw 272 total differentially expressed NHDF genes (94 upregulated, 174 downregulated).

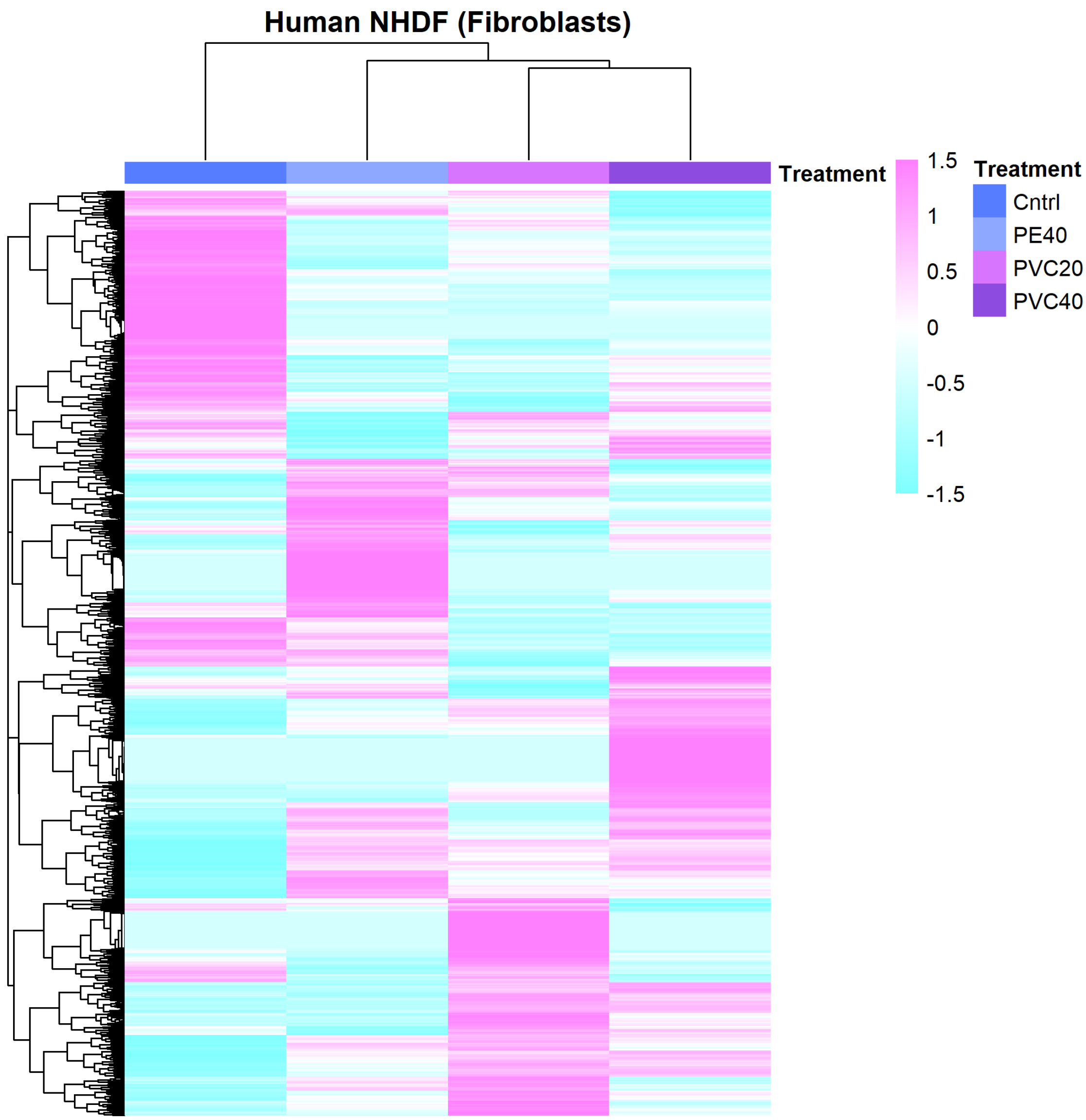

Figure 10: Differential expression results of genes for human skin fibroblasts in response to three microplastic treatments.

3.3.2.3 Protein-Protein Interaction Networks and Analyses

Differentially expressed genes from all treatments were used to predict protein-protein interaction networks utilizing STRINGdb, which imports data from publicly available interaction databases BioGRID, DIP (Database of Interacting Proteins), IntAct, MINT (Molecular

INTeraction database), and PDB (Protein Data Bank). All network statistics are summarized in Table 3.

Table 3. Summary statistics for protein-protein interaction network analyses by species (Ttr = Dolphin, NHDF = Human) and microplastic treatment.

	Ttr			NHDF		
	PVC20	PVC40	PE40	PVC20	PVC40	PE40
Number of Nodes	1841	1764	883	94	112	167
Average Neighbors	30.20	30.03	22.87	16.51	13.32	14.08
Network Density	0.02	0.02	0.03	0.18	0.12	0.08
Network Centralization	0.27	0.28	0.35	0.38	0.33	0.26
Network Heterogeneity	1.37	1.41	1.42	1.16	1.24	1.22
Clustering Coefficient	0.25	0.26	0.30	0.90	0.85	0.78

Dolphin (Ttr) protein-protein interaction networks for skin fibroblasts exposed to PVC20, PVC40, and PE40 are represented in Figures 11, 12, and 13, respectively. Across the three Ttr networks, the number of nodes ranged between 883 (PE40) to 1841 (PVC20). Similarly, the lowest average number of neighbors was the PE40 treatment (22.87) and PVC20 had the highest (30.20). Conversely, PVC20 had the lowest network density (0.016) while PE40 had a density of 0.26. Network centralization was roughly equal for the PVC treatments: 0.267 and 0.276, for PVC20 and PVC40, respectively. PE40 centralization was greatest at 0.346. For both network heterogeneity and clustering coefficient, PVC20 represented the lowest score among all treatments (1.37 and 0.253 respectively). On the other hand, PE40 had the highest heterogeneity and clustering coefficients at 1.42 and 0.295, respectively.

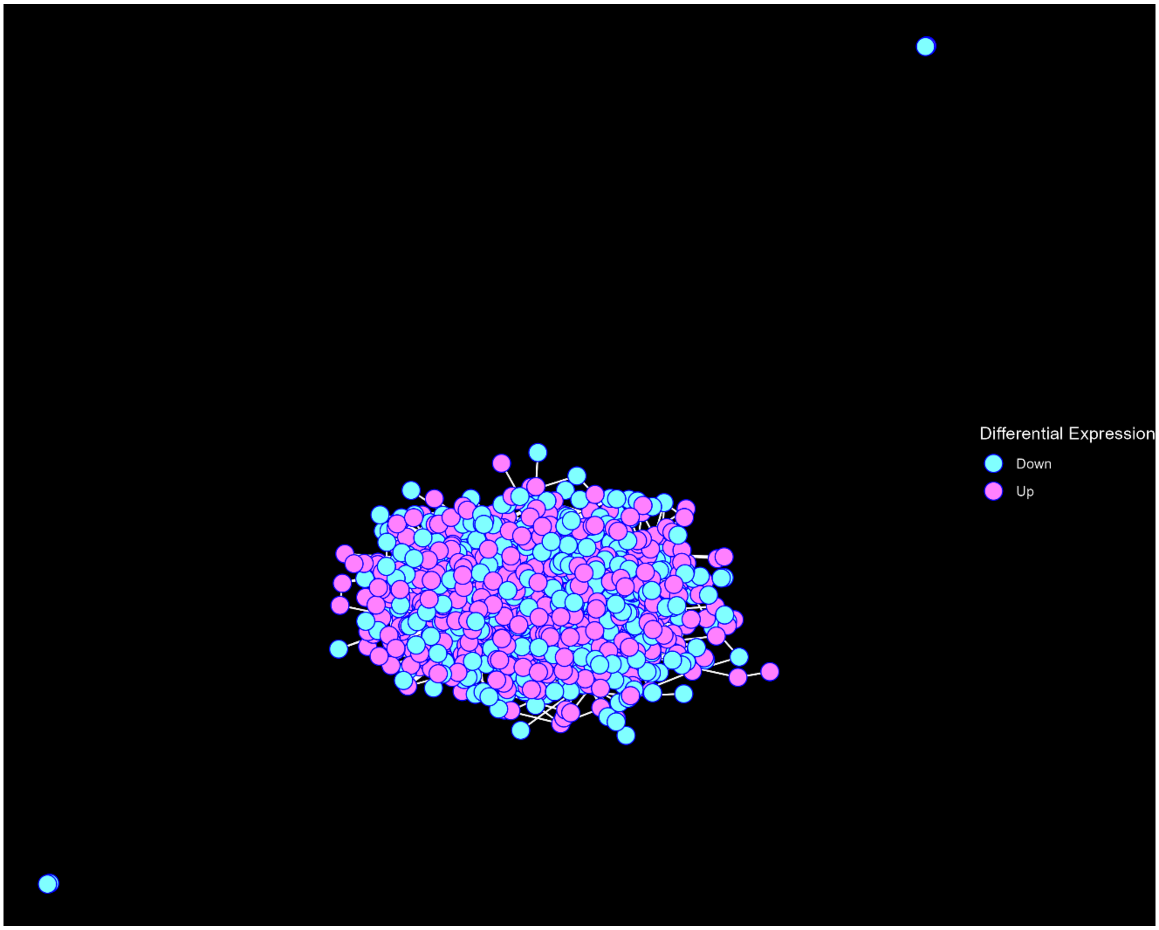

Figure 11: Protein-Protein Interaction Network for Dolphin cells exposed to microplastic treatment PVC20. Node colors indicate differential expression.

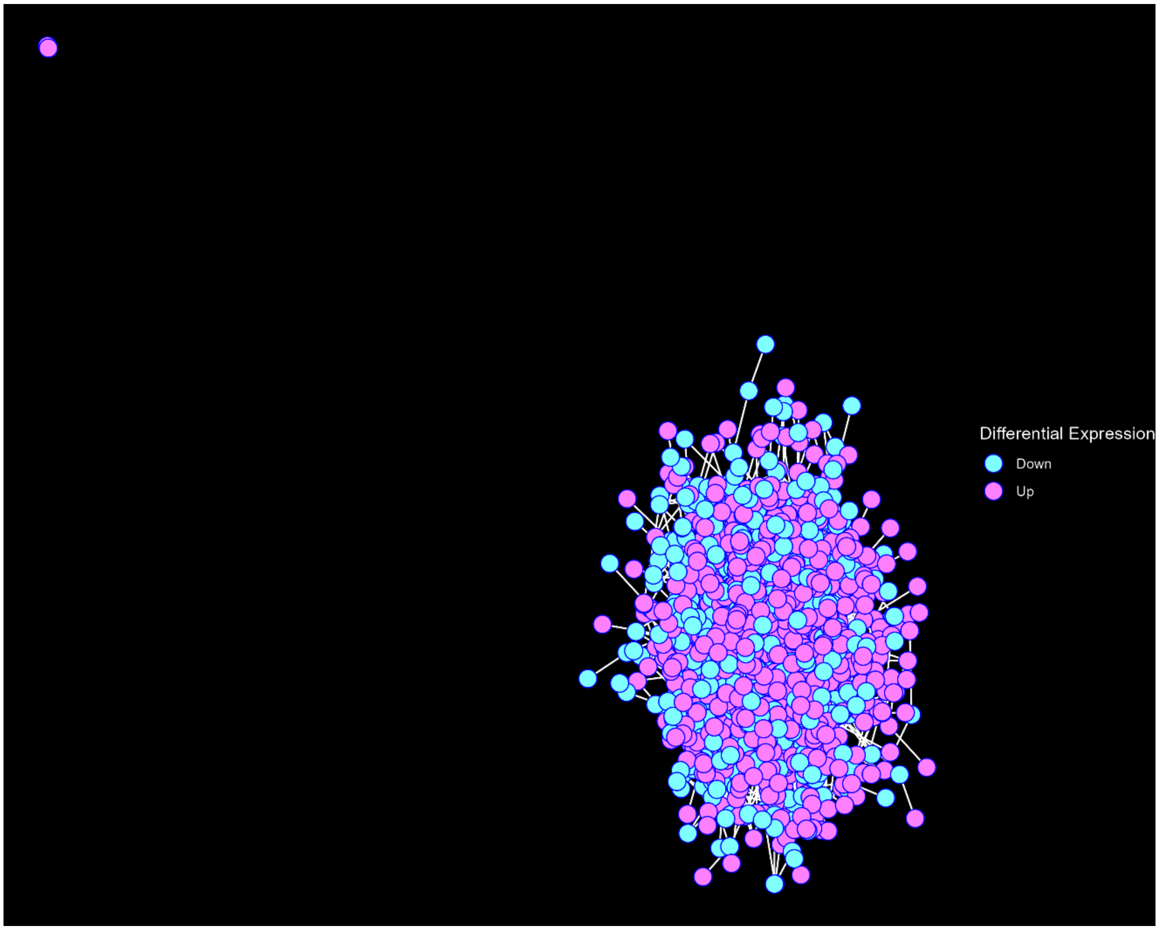

Figure 12: Protein-Protein Interaction Network for Dolphin cells exposed to microplastic treatment PVC40. Node colors indicate differential expression.

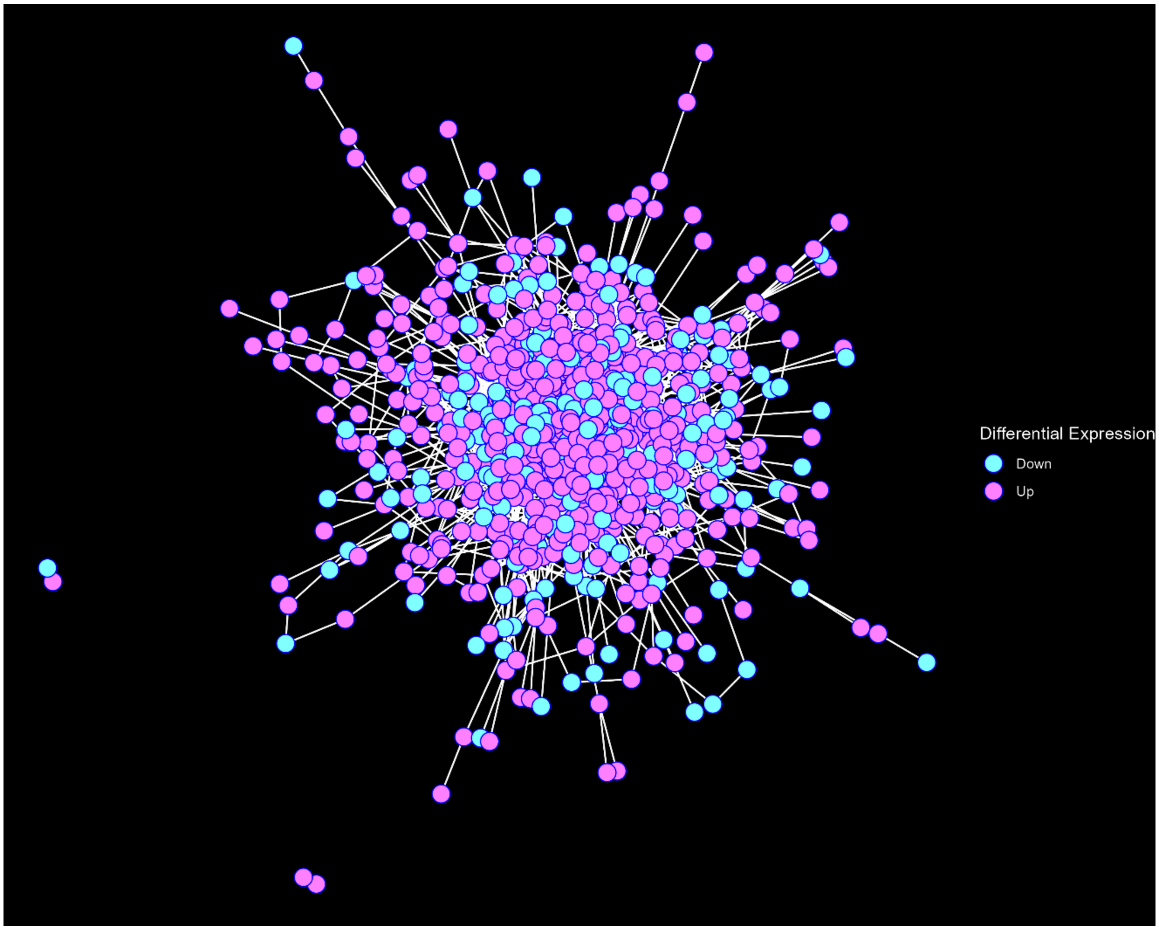

Figure 13: Protein-Protein Interaction Network for Dolphin cells exposed to microplastic treatment PE40. Node colors indicate differential expression.

Human (NHDF) protein-protein interaction networks for skin fibroblasts exposed to PVC20, PVC40, and PE40 are represented in Figures 14, 15, and 16, respectively. NHDF networks ranged in their number of nodes from 94 (PVC20) to 167 (PE40). For this cell type, average number of neighbors was lowest for the PVC40 (13.32) treatment and highest in the PVC20 (16.51). PVC20 had highest network density (0.18) while NHDF cells exposed to PE40 had the lowest (0.08). Network centralization was highest for the PVC treatments (0.33 and 0.38 for PVC20 and PVC40, respectively) and lowest in PE40 (0.26). Heterogeneity ranged between 1.16 (PVC20) and 1.24 (PVC40). Finally, NHDF clustering coefficients ranged between 0.78 (PE40) and 0.90 (PVC20).

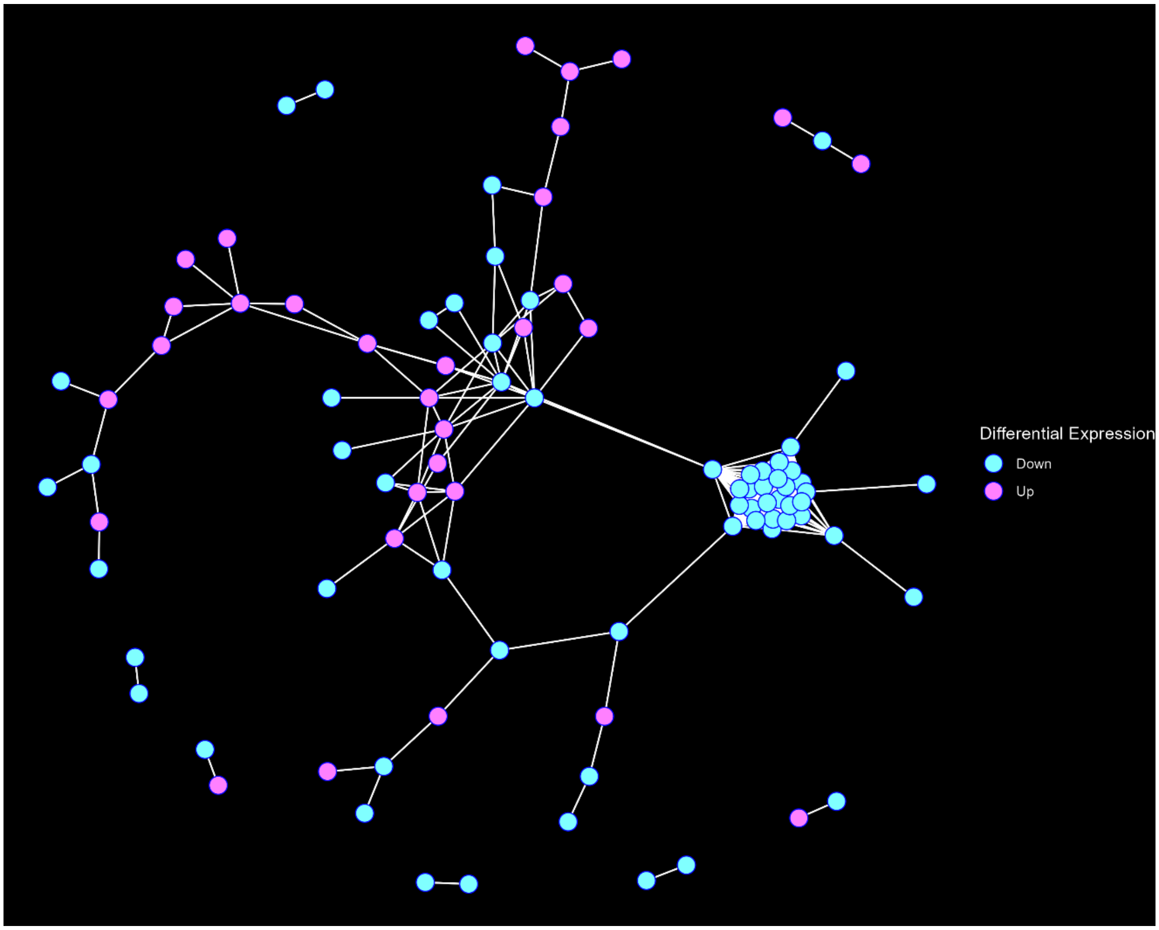

Figure 14: Protein-Protein Interaction Network for Human NHDF cells exposed to microplastic treatment PVC20. Node colors indicate differential expression.

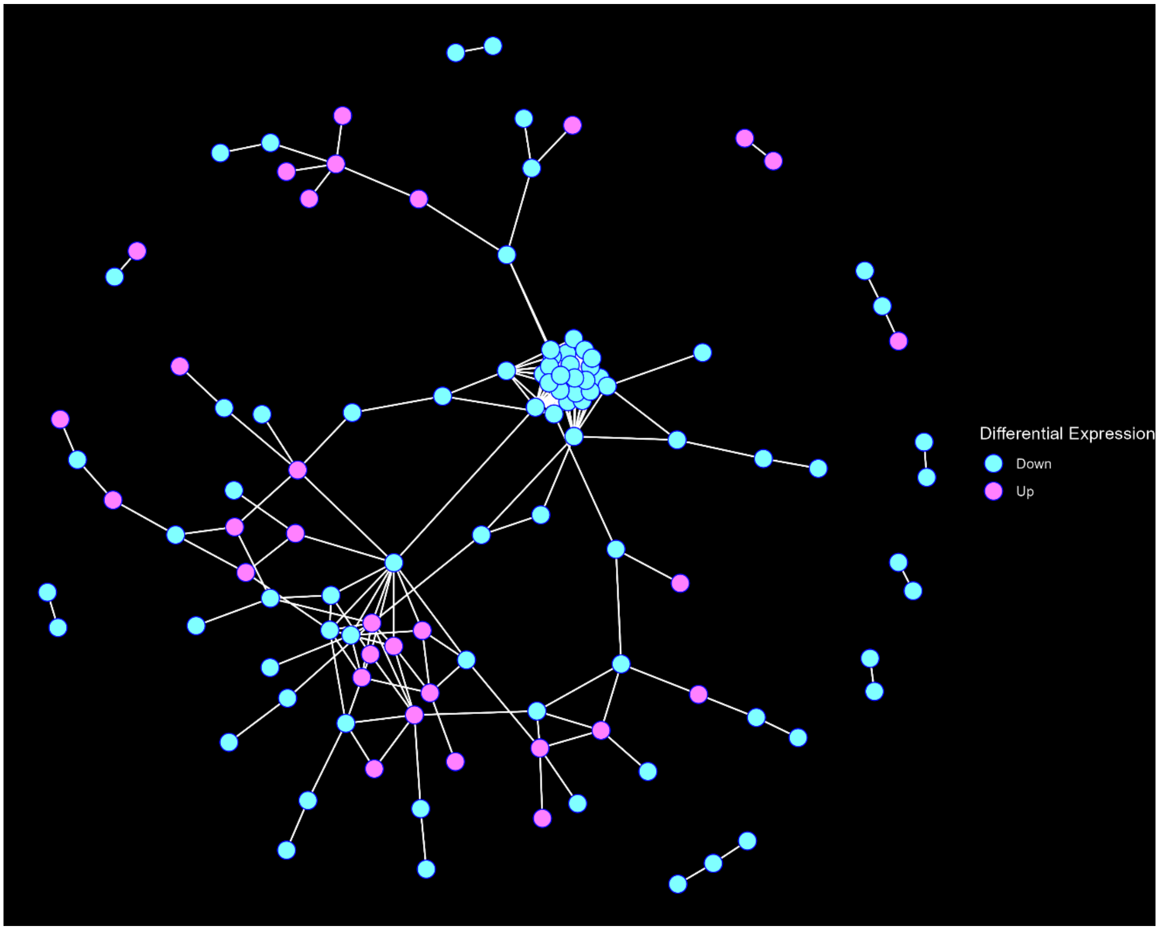

Figure 15: Protein-Protein Interaction Network for Human NHDF cells exposed to microplastic treatment PVC40. Node colors indicate differential expression.

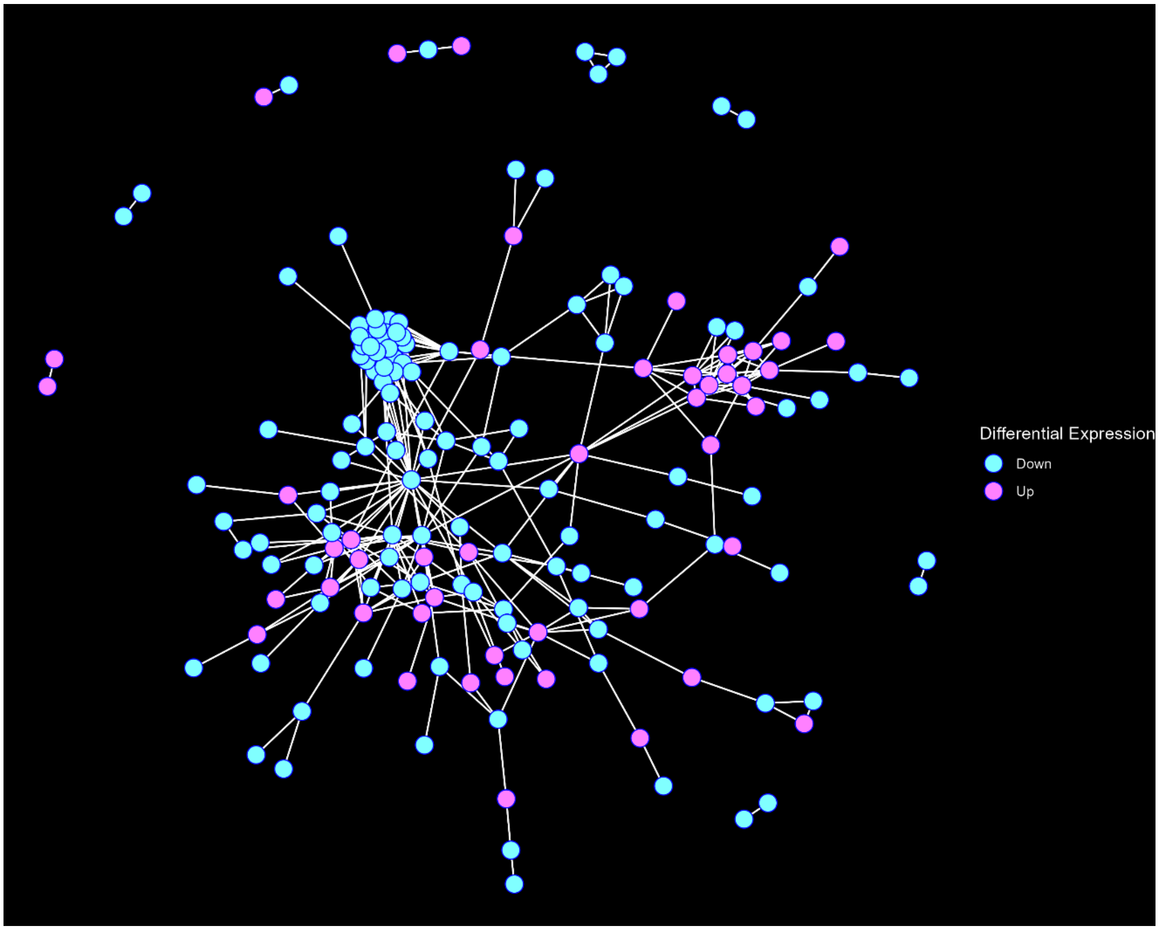

Figure 16: Protein-Protein Interaction Network for Human NHDF cells exposed to microplastic treatment PE40. Node colors indicate differential expression.

3.3.2.4 KEGG Pathway (Functional) Analyses

Functional annotation was used to identify pathways that were enriched in the Ttr and human (NHDF) transcriptomes in response to the three plastic exposures, relative to the human genome.

For Ttr, a total of 57 pathways were enriched with 37 of these pathways (65%) enriched across all three plastic treatments. Eight pathways (14%; hsa00100, 00900, 04020, 04270, 04928, 04931, 05164, 05321) mapped to both PVC treatments but not PE40. Twelve (21%) mapped uniquely to only one of the three treatments. Hsa04022, 04024, 04068, 04936, 05160, 05162, and 05171 uniquely mapped to PVC20. Hsa04014, 04148, and 04380 uniquely mapped to PVC40. Two pathways – hsa04974 and 05140 – mapped uniquely to PE40.

In all three Ttr treatments (PVC20, PVC40, PE40), the top three categories of pathways enriched were Human Diseases (HD; 21, 18, 19 number of pathways per treatment, respectively), Environmental Information Processing (EIP; 16, 14, 12), and Organismal Systems (OS; 8, 11, 9), in that order (Table 4). Other categories enriched included metabolism and cellular processes. Notably, pathways related to drug development or genetic information processing were not enriched. Although there were a greater number of enriched pathways concerned with human diseases (eg. Rheumatoid arthritis, Malaria, amoebiasis, cancer, hypertrophic cardiomyopathy, etc.), the top three most significantly enriched pathways by highest gene count were categorized as either EIP or OS and were the same across all three plastic treatments: hsa04060 Cytokine-cytokine receptor interaction (EIP), hsa04010 MAPK signaling pathway (EIP), and hsa04360 Axon guidance (OS). The top 10 KEGG pathways significantly overrepresented in the Ttr transcriptome for each treatment are shown in Figures 17, 18, 19 and a complete list of enriched pathways per treatment is presented in Table 5.

Table 4. KEGG results demonstrating the functional categories overrepresented in each treatment.

Species	Treatment	Pathway Category	Pathway Count	Average Gene Count	SD Gene Count
Ttr	PE40	Human Diseases	19	18.5	6.28
		Environmental Information Processing	12	24.8	10.3
		Organismal Systems	9	17.3	4.97
		Cellular Processes	2	23	1.41
		Other	1	29	-
	PVC20	Human Diseases	21	26.7	9.24
		Environmental Information Processing	16	36.3	14.5
		Organismal Systems	8	25.6	8.09
		Cellular Processes	2	33	2.83
		Metabolism	2	8	0
		Other	1	43	-
	PVC40	Human Diseases	18	26.3	9.71
		Environmental Information Processing	14	38.2	15.9
		Organismal Systems	11	24.5	6.77
		Cellular Processes	3	30	3.61
		Metabolism	2	8.5	0.707
		Other	1	44	-
NHDF	PVC20	Human Diseases	4	7	0.816
	PVC40	Human Diseases	1	8	-

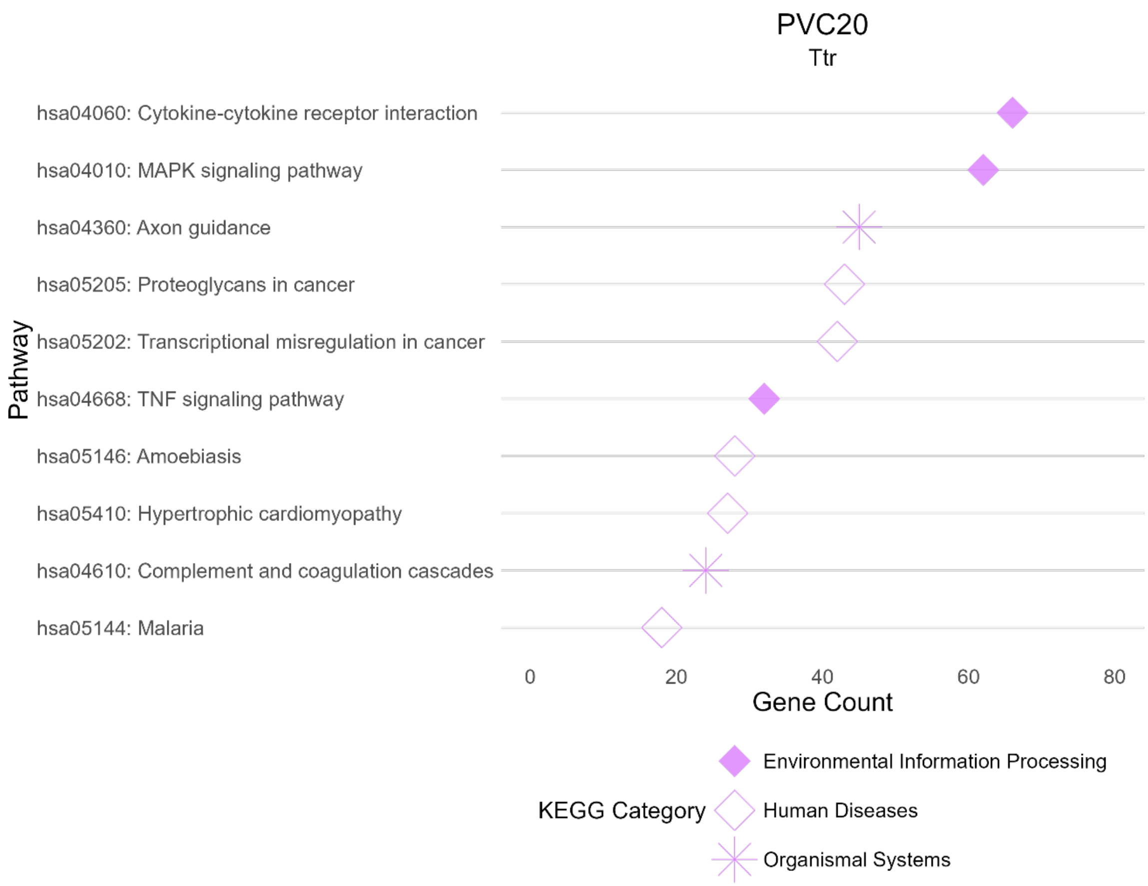

Figure 17: Top 10 KEGG pathways significantly overrepresented in the dolphin skin fibroblast transcriptome when exposed to PVC20 relative to the human proteome (*p* < 0.05) as indicated by the number of transcripts mapping to each overrepresented pathway.

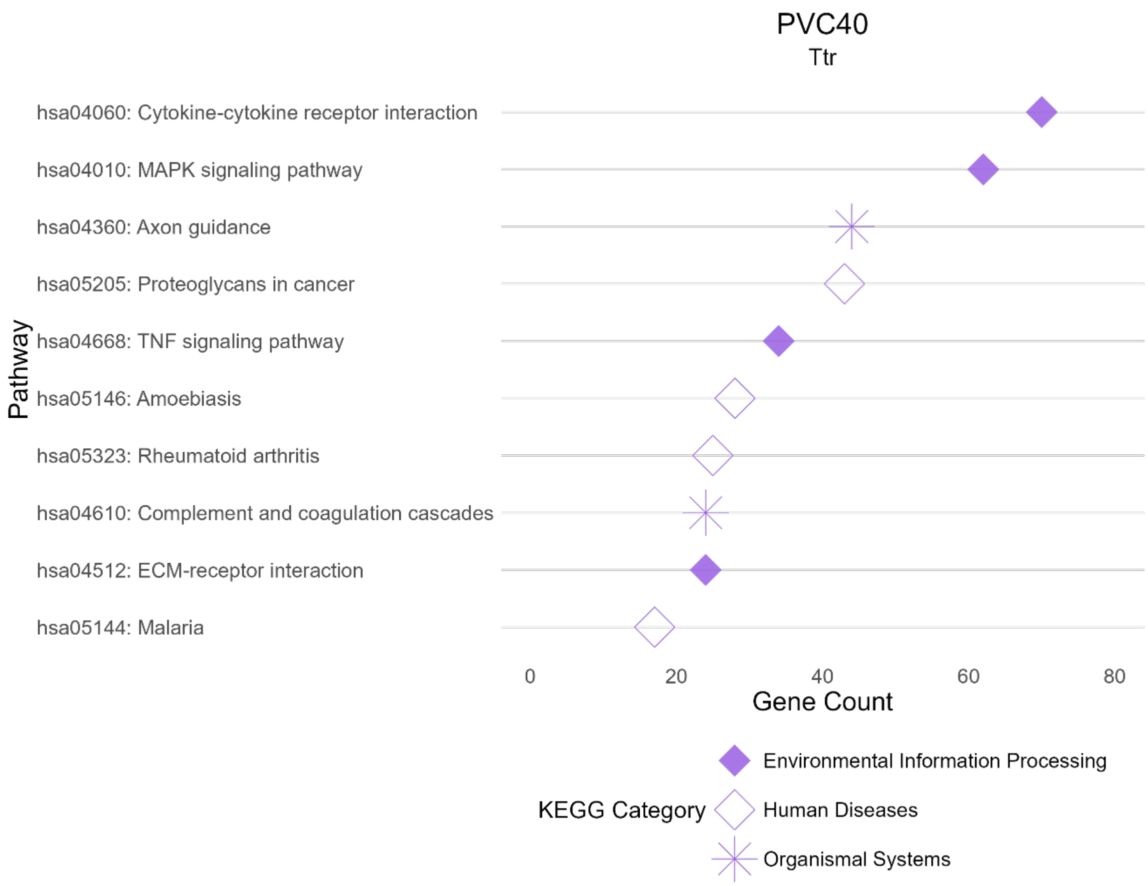

Figure 18: Top 10 KEGG pathways significantly overrepresented in the dolphin skin fibroblast transcriptome when exposed to PVC40 relative to the human proteome ($p < 0.05$) as indicated by the number of transcripts mapping to each overrepresented pathway.

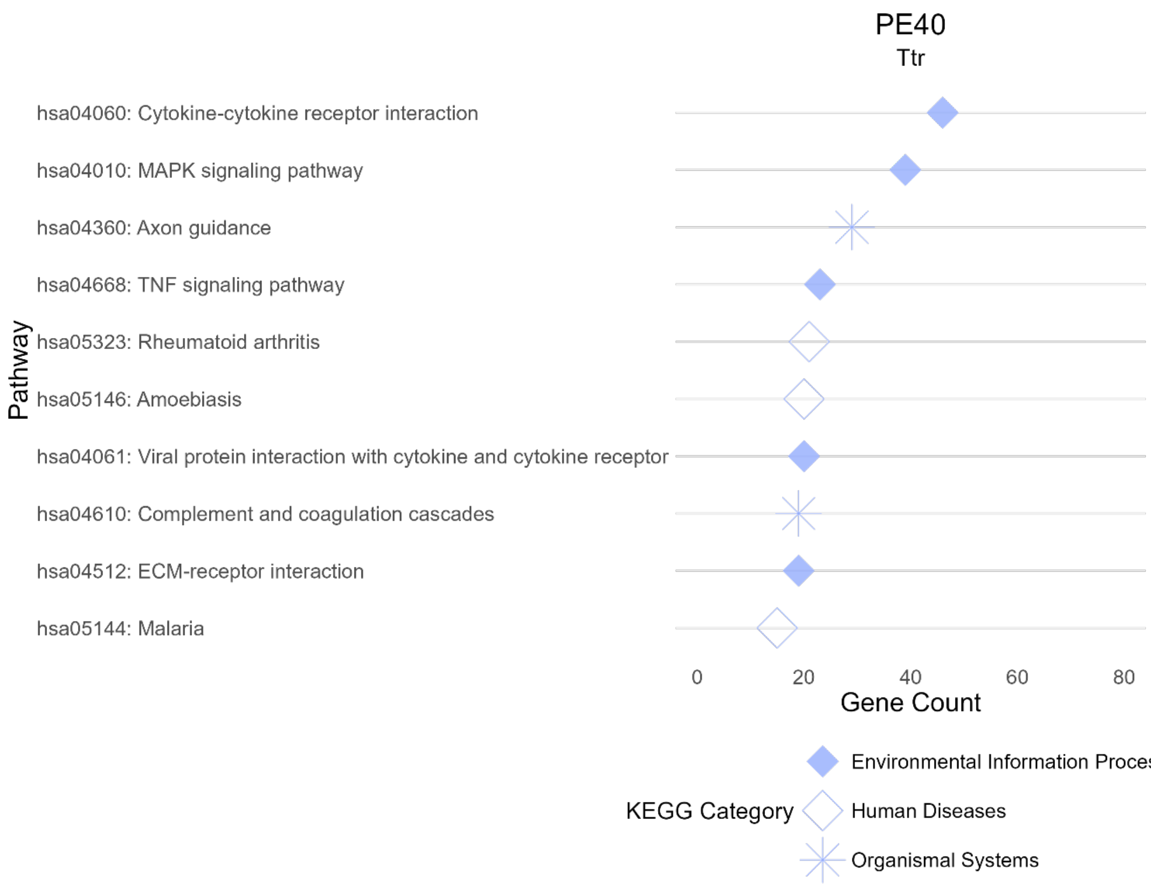

Figure 19: Top 10 KEGG pathways significantly overrepresented in the dolphin skin fibroblast transcriptome when exposed to PE40 relative to the human proteome (*p* < 0.05) as indicated by the number of transcripts mapping to each overrepresented pathway.

Table 5. KEGG analysis results: Enriched Pathways Ttr = dolphin, NHDF = human. EIP = Environmental Information Processing, HD = Human Disease, OS = Organismal Systems, CP = cellular Processes, M = Metabolism.

Species	Treatment	Pathway Category	Pathway ID	Pathway Description	Gene Count
Ttr	PE40	EIP	hsa04060	Cytokine-cytokine receptor interaction	46
Ttr	PE40	EIP	hsa04010	MAPK signaling pathway	39
Ttr	PE40	EIP	hsa04151	PI3K-Akt signaling pathway	38
Ttr	PE40	HD	hsa05165	Human papillomavirus infection	36
Ttr	PE40	Other	hsa04820	Cytoskeleton in muscle cells	29
Ttr	PE40	OS	hsa04360	Axon guidance	29
Ttr	PE40	HD	hsa05202	Transcriptional misregulation in cancer	28
Ttr	PE40	HD	hsa05205	Proteoglycans in cancer	26
Ttr	PE40	CP	hsa04510	Focal adhesion	24
Ttr	PE40	EIP	hsa04015	Rap1 signaling pathway	24
Ttr	PE40	EIP	hsa04668	TNF signaling pathway	23
Ttr	PE40	CP	hsa04550	Signaling pathways regulating pluripotency of stem cells	22
Ttr	PE40	EIP	hsa04390	Hippo signaling pathway	22
Ttr	PE40	HD	hsa05224	Breast cancer	22
Ttr	PE40	HD	hsa05323	Rheumatoid arthritis	21
Ttr	PE40	HD	hsa05418	Fluid shear stress and atherosclerosis	21
Ttr	PE40	EIP	hsa04061	Viral protein interaction with cytokine and cytokine receptor	20
Ttr	PE40	HD	hsa05146	Amoebiasis	20
Ttr	PE40	EIP	hsa04310	Wnt signaling pathway	19
Ttr	PE40	EIP	hsa04512	ECM-receptor interaction	19
Ttr	PE40	OS	hsa04610	Complement and coagulation cascades	19
Ttr	PE40	OS	hsa04657	IL-17 signaling pathway	19

Ttr	PE40	EIP	hsa04514	Cell adhesion molecules	18
Ttr	PE40	HD	hsa05225	Hepatocellular carcinoma	18
Ttr	PE40	HD	hsa05226	Gastric cancer	18
Ttr	PE40	OS	hsa04640	Hematopoietic cell lineage	18
Ttr	PE40	HD	hsa05133	Pertussis	17
Ttr	PE40	HD	hsa05142	Chagas disease	16
Ttr	PE40	HD	hsa05410	Hypertrophic cardiomyopathy	16
Ttr	PE40	OS	hsa04620	Toll-like receptor signaling pathway	16
Ttr	PE40	EIP	hsa04064	NF-kappa B signaling pathway	15
Ttr	PE40	EIP	hsa04350	TGF-beta signaling pathway	15
Ttr	PE40	HD	hsa04933	AGE-RAGE signaling pathway in diabetic complications	15
Ttr	PE40	HD	hsa05144	Malaria	15
Ttr	PE40	OS	hsa04926	Relaxin signaling pathway	15
Ttr	PE40	HD	hsa05414	Dilated cardiomyopathy	14
Ttr	PE40	OS	hsa04974	Protein digestion and absorption	14
Ttr	PE40	HD	hsa05217	Basal cell carcinoma	13
Ttr	PE40	OS	hsa04750	Inflammatory mediator regulation of TRP channels	13
Ttr	PE40	OS	hsa04916	Melanogenesis	13
Ttr	PE40	HD	hsa05134	Legionellosis	12
Ttr	PE40	HD	hsa05222	Small cell lung cancer	12
Ttr	PE40	HD	hsa05140	Leishmaniasis	11
Ttr	PVC20	EIP	hsa04060	Cytokine-cytokine receptor interaction	66
Ttr	PVC20	EIP	hsa04010	MAPK signaling pathway	62
Ttr	PVC20	EIP	hsa04151	PI3K-Akt signaling pathway	59
Ttr	PVC20	HD	hsa05165	Human papillomavirus infection	51
Ttr	PVC20	EIP	hsa04020	Calcium signaling pathway	46
Ttr	PVC20	OS	hsa04360	Axon guidance	45

Ttr	PVC20	HD	hsa05205	Proteoglycans in cancer	43
Ttr	PVC20	Other	hsa04820	Cytoskeleton in muscle cells	43
Ttr	PVC20	HD	hsa05202	Transcriptional misregulation in cancer	42
Ttr	PVC20	EIP	hsa04015	Rap1 signaling pathway	38
Ttr	PVC20	EIP	hsa04024	cAMP signaling pathway	38
Ttr	PVC20	CP	hsa04510	Focal adhesion	35
Ttr	PVC20	EIP	hsa04310	Wnt signaling pathway	32
Ttr	PVC20	EIP	hsa04390	Hippo signaling pathway	32
Ttr	PVC20	EIP	hsa04668	TNF signaling pathway	32
Ttr	PVC20	CP	hsa04550	Signaling pathways regulating pluripotency of stem cells	31
Ttr	PVC20	HD	hsa05224	Breast cancer	31
Ttr	PVC20	HD	hsa05418	Fluid shear stress and atherosclerosis	31
Ttr	PVC20	HD	hsa05225	Hepatocellular carcinoma	30
Ttr	PVC20	EIP	hsa04022	cGMP-PKG signaling pathway	29
Ttr	PVC20	EIP	hsa04514	Cell adhesion molecules	29
Ttr	PVC20	HD	hsa05146	Amoebiasis	28
Ttr	PVC20	HD	hsa05226	Gastric cancer	28
Ttr	PVC20	HD	hsa05410	Hypertrophic cardiomyopathy	27
Ttr	PVC20	HD	hsa04933	AGE-RAGE signaling pathway in diabetic complications	26
Ttr	PVC20	OS	hsa04640	Hematopoietic cell lineage	26
Ttr	PVC20	EIP	hsa04350	TGF-beta signaling pathway	25
Ttr	PVC20	HD	hsa04936	Alcoholic liver disease	25
Ttr	PVC20	HD	hsa05323	Rheumatoid arthritis	25
Ttr	PVC20	OS	hsa04270	Vascular smooth muscle contraction	25
Ttr	PVC20	EIP	hsa04064	NF-kappa B signaling pathway	24
Ttr	PVC20	EIP	hsa04068	FoxO signaling pathway	24
Ttr	PVC20	EIP	hsa04512	ECM-receptor interaction	24

Ttr	PVC20	HD	hsa05414	Dilated cardiomyopathy	24
Ttr	PVC20	OS	hsa04610	Complement and coagulation cascades	24
Ttr	PVC20	HD	hsa04931	Insulin resistance	23
Ttr	PVC20	OS	hsa04620	Toll-like receptor signaling pathway	22
Ttr	PVC20	OS	hsa04657	IL-17 signaling pathway	22
Ttr	PVC20	EIP	hsa04061	Viral protein interaction with cytokine and cytokine receptor	21
Ttr	PVC20	HD	hsa05142	Chagas disease	21
Ttr	PVC20	OS	hsa04928	Parathyroid hormone synthesis, secretion and action	21
Ttr	PVC20	HD	hsa05133	Pertussis	20
Ttr	PVC20	OS	hsa04750	Inflammatory mediator regulation of TRP channels	20
Ttr	PVC20	HD	hsa05222	Small cell lung cancer	19
Ttr	PVC20	HD	hsa05144	Malaria	18
Ttr	PVC20	HD	hsa05217	Basal cell carcinoma	18
Ttr	PVC20	HD	hsa05134	Legionellosis	16
Ttr	PVC20	HD	hsa05321	Inflammatory bowel disease	15
Ttr	PVC20	M	hsa00100	Steroid biosynthesis	8
Ttr	PVC20	M	hsa00900	Terpenoid backbone biosynthesis	8
Ttr	PVC40	EIP	hsa04060	Cytokine-cytokine receptor interaction	70
Ttr	PVC40	EIP	hsa04151	PI3K-Akt signaling pathway	63
Ttr	PVC40	EIP	hsa04010	MAPK signaling pathway	62
Ttr	PVC40	HD	hsa05165	Human papillomavirus infection	50
Ttr	PVC40	Other	hsa04820	Cytoskeleton in muscle cells	44
Ttr	PVC40	OS	hsa04360	Axon guidance	44
Ttr	PVC40	EIP	hsa04015	Rap1 signaling pathway	43
Ttr	PVC40	HD	hsa05205	Proteoglycans in cancer	43
Ttr	PVC40	EIP	hsa04020	Calcium signaling pathway	41

Ttr	PVC40	HD	hsa05202	Transcriptional misregulation in cancer	40
Ttr	PVC40	EIP	hsa04014	Ras signaling pathway	36
Ttr	PVC40	EIP	hsa04668	TNF signaling pathway	34
Ttr	PVC40	CP	hsa04510	Focal adhesion	33
Ttr	PVC40	EIP	hsa04390	Hippo signaling pathway	33
Ttr	PVC40	CP	hsa04550	Signaling pathways regulating pluripotency of stem cells	31
Ttr	PVC40	EIP	hsa04310	Wnt signaling pathway	31
Ttr	PVC40	HD	hsa05224	Breast cancer	30
Ttr	PVC40	HD	hsa05418	Fluid shear stress and atherosclerosis	30
Ttr	PVC40	HD	hsa05146	Amoebiasis	28
Ttr	PVC40	CP	hsa04148	Efferocytosis	26
Ttr	PVC40	EIP	hsa04350	TGF-beta signaling pathway	26
Ttr	PVC40	EIP	hsa04514	Cell adhesion molecules	26
Ttr	PVC40	HD	hsa05226	Gastric cancer	26
Ttr	PVC40	HD	hsa04933	AGE-RAGE signaling pathway in diabetic complications	25
Ttr	PVC40	HD	hsa05323	Rheumatoid arthritis	25
Ttr	PVC40	HD	hsa05410	Hypertrophic cardiomyopathy	25
Ttr	PVC40	OS	hsa04380	Osteoclast differentiation	25
Ttr	PVC40	EIP	hsa04512	ECM-receptor interaction	24
Ttr	PVC40	HD	hsa05414	Dilated cardiomyopathy	24
Ttr	PVC40	OS	hsa04270	Vascular smooth muscle contraction	24
Ttr	PVC40	OS	hsa04610	Complement and coagulation cascades	24
Ttr	PVC40	OS	hsa04640	Hematopoietic cell lineage	24
Ttr	PVC40	EIP	hsa04061	Viral protein interaction with cytokine and cytokine receptor	23
Ttr	PVC40	EIP	hsa04064	NF-kappa B signaling pathway	23
Ttr	PVC40	OS	hsa04657	IL-17 signaling pathway	23

Ttr	PVC40	OS	hsa04926	Relaxin signaling pathway	23
Ttr	PVC40	OS	hsa04928	Parathyroid hormone synthesis, secretion and action	23
Ttr	PVC40	HD	hsa04931	Insulin resistance	22
Ttr	PVC40	HD	hsa05142	Chagas disease	22
Ttr	PVC40	HD	hsa05133	Pertussis	20
Ttr	PVC40	OS	hsa04620	Toll-like receptor signaling pathway	20
Ttr	PVC40	OS	hsa04750	Inflammatory mediator regulation of TRP channels	20
Ttr	PVC40	OS	hsa04916	Melanogenesis	19
Ttr	PVC40	HD	hsa05217	Basal cell carcinoma	18
Ttr	PVC40	HD	hsa05144	Malaria	17
Ttr	PVC40	HD	hsa05134	Legionellosis	14
Ttr	PVC40	HD	hsa05321	Inflammatory bowel disease	14
Ttr	PVC40	M	hsa00900	Terpenoid backbone biosynthesis	9
Ttr	PVC40	M	hsa00100	Steroid biosynthesis	8
NHDF	PVC20	HD	hsa05171	Coronavirus disease - COVID-19	8
NHDF	PVC20	HD	hsa05160	Hepatitis C	7
NHDF	PVC20	HD	hsa05164	Influenza A	7
NHDF	PVC20	HD	hsa05162	Measles	6
NHDF	PVC40	HD	hsa05164	Influenza A	8

Similar to Ttr, in the NHDF exposures to PVC20 and PVC40, the top category of pathways enriched were related to disease; Disease was the only pathway category enriched for these two treatments. PVC20 has four enriched pathways (Figure 20) and PVC40 has two (Figure 21). The gene sets were not of sufficient size to obtain significant functional enrichment in the NHDF PE40 exposure. The hsa05164 Influenza A pathway was shared across both PVC treatments. Other enriched disease pathways in the PVC20 treatment included hsa05171 Coronavirus disease – Covid-19 (8 genes), hsa05160 Hepatitis C (7 genes), and hsa05162 Measles (6 genes).

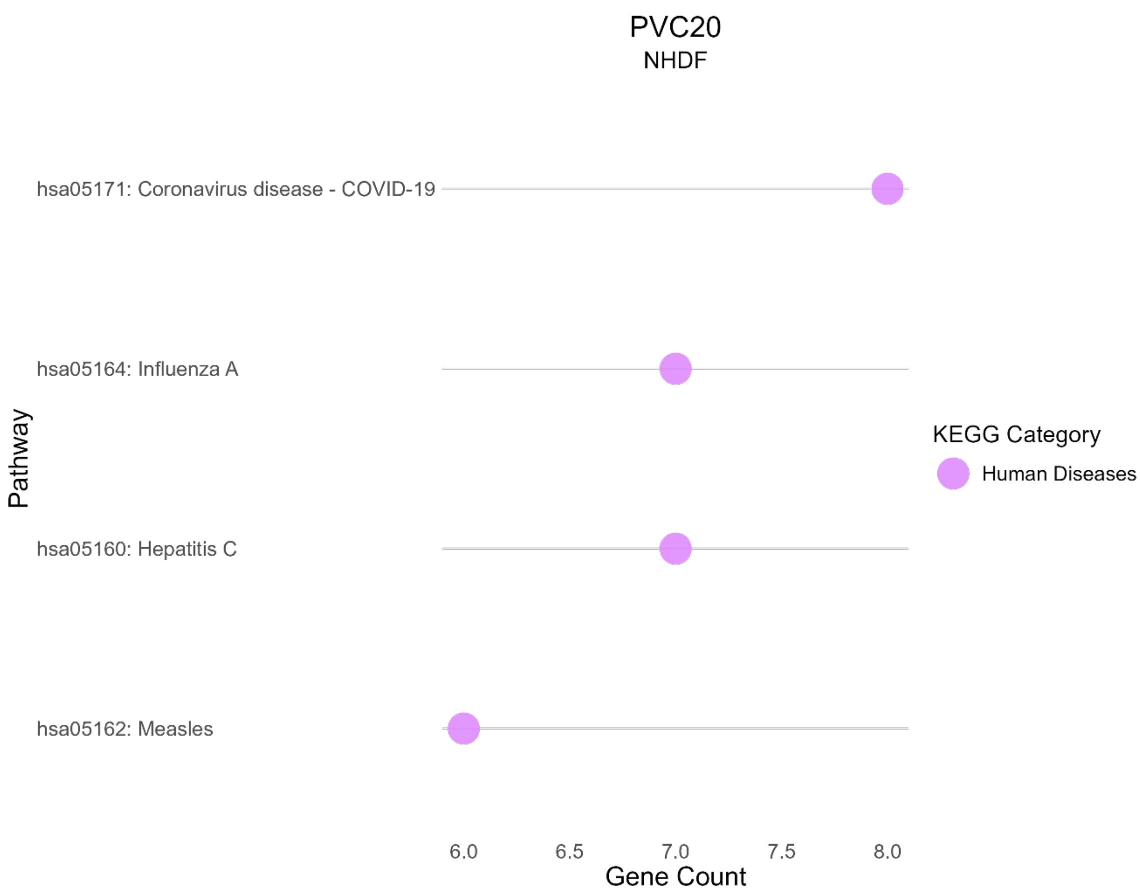

Figure 20: KEGG Functional Analysis showing the number of genes associated with an enriched pathway for NHFD cells exposed to microplastic treatment PVC20.

Figure 21: KEGG Functional Analysis showing the number of genes associated with an enriched pathway for NHFD cells exposed to microplastic treatment PVC40.

3.4 Discussion

In dolphin skin fibroblasts, just two genes with functionality related to lipolysis were differentially expressed in response to exposure to polyethylene microplastics, underscoring their importance in the lipolytic response to microplastics in dolphins. These genes are ANGPTL4 and IRS2. Angiopoietin-like protein 4 (ANGPTL4) plays critical roles in lipid metabolism that are both pro- and anti-lipolytic (Kersten 2021). Upregulation of ANGPTL4 in adipocytes stimulates lipolysis (Kersten 2021), increasing fatty acids and glycerol. Although here I did not conduct a differential gene expression analysis for the blubber explants, this conventional understanding of how upregulation of ANGPTL4 increases lipolysis within adipose tissue is consistent with my finding that the rate of glycerol production increased in blubber exposed to polyethylene. On the other hand, ANGPTL4 also inhibits lipoprotein lipase (LPL; (Fernández-Hernando and Suárez 2020, Xu and Jiang 2024)). LPL controls the breakdown of triglycerides (Wu et al. 2021), the inhibition of which causes a reduction in the uptake of fatty acids to tissues, preserving triglycerides within the bloodstream during stressful or energy-demanding states such as fasting or diving (Yoshida et al. 2002, Ruppert et al. 2020, Sylvers-Davie and Davies 2021). Differential gene expression results for dolphin skin fibroblasts demonstrated upregulation of ANGPTL4 concurrent with reduced rate of glycerol production when exposed to polyethylene microplastics, consistent with the anti-lipolytic effect of Angiopoietin-like protein 4.

Insulin Receptor Substrate 2 (IRS2) also regulates lipolysis though indirectly through its role in insulin signaling (Althaher 2022). During insulin-stimulated conditions, insulin binds to the receptor of IRS2, phosphorylating IRS2 ultimately activating PI3K-Akt (Rametta et al. 2013, Althaher 2022). In turn, PI3K-Akt phosphorylates hormone-sensitive lipase (HSL) causing its inactivation (Althaher 2022). HSL is an important enzyme responsible for the breakdown of triglycerides, thus upregulation of IRS2 indirectly reduces lipolysis (Althaher 2022). Therefore, the upregulation of IRS2 observed in dolphin skin fibroblasts exposed to polyethylene is consistent with IRS2 anti-lipolytic role in metabolism.

Varied response in glycerol production by tissue type highlights tissue-specific impacts to microplastic exposure, in this case polyethylene. *Ex vivo* and *in vitro* models are gaining traction in recent years as models for studying marine mammals such as primary cell lines, induced pluripotent stem cells, explants, and organoids (Godard et al. 2004, Debier et al. 2020, Lam et al. 2020, Vazquez et al. 2024, Allen et al. 2024). For cetaceans, perhaps the most tractable model is skin fibroblasts because they are relatively easy to obtain fresh from live free-ranging species via remote biopsy sampling (Hunt et al., 2013). Ethics, logistics, and policy often preclude sampling of alternative tissue types in whales (Vazquez et al. 2024). However, results from studies employing skin cell models must be careful in extrapolating findings to alternative tissue types and/or the whole organism given the differential responses in glycerol production between blubber explants and skin fibroblasts when exposed to polyethylene observed here.

Protein-protein interaction (PPI) network analyses underscore species-specific and treatment-specific responses to microplastic exposure. In general, dolphin PPI networks are considerably larger, less dense, and exhibit higher heterogeneity and distributed centralizations when exposed to microplastics compared to NHDF cells. On the other hand, human cells exposed to microplastics exhibit smaller, denser, more clustered and more centralized PPI networks. These topologies and centrality measures imply that dolphin skin fibroblasts possess the capacity to mount a broader, more adaptive molecular response via activation of a wide array of proteins to respond to stress of microplastic exposure. Mitochondrial dysfunction and oxidative stress are, for example, repeatedly reported in microplastic studies (Lee et al., 2022; Liu et al., 2022; Tao et al., 2024). PPI topologies reflective of systemic protein responses to these two types of molecular stress are consistent with the topologies of the dolphin networks presented here (Shen et al. 2023). Conversely, human skin fibroblasts appear to activate specific pathways that are more localized, possibly reflecting a less adaptive response.

KEGG functional enrichment analyses demonstrated distinct, yet overlapping transcriptomic responses to microplastic exposures in dolphin and human skin fibroblasts implicative of immune system perturbation. For dolphin cells, a conserved response involving immune modulation, stress signaling, and neurodevelopmental processing was evident by consistent enrichment of pathways related to cytokine signaling, MAPK signaling, and axon guidance across all treatments. This is supported by – and reflects broad systemic impacts – the overrepresentation of pathways categorized as Diseases, Environmental Information Processing, and Organismal Systems. In contrast, human NHDF cells responded to microplastics in a more constrained fashion. Pathways were primarily enriched in the Disease category, with fewer pathways enriched overall. While insufficient gene set size impeded enrichment analysis of PE40 exposure to NHDF cells, overlapping pathways between PVC20 and PVC40, such as Influenza A and Coronavirus disease pathways, reflect immune system interactions similar to the dolphin response. Together, Ttr and NHDF findings align with prior microplastic toxicity research. Microplastic-induced oxidative stress and inflammation are oft-identified mechanisms of toxicity (Von Moos et al. 2012, Chen et al. 2017, Espinosa et al. 2018, Pittura et al. 2018, Tang et al. 2020, Yang et al. 2022, Bishop et al. 2024) Furthermore, they also indicate species-specific differences in pathway enrichment, perhaps unsurprising given the context in which skin functions between the two: a saline marine environment versus terrestrial.

Tissue-specific, species-specific, and treatment-specific (by polymer) responses to microplastics observed here underscore the complexity of microplastics as a pollutant. There is not a straightforward consensus on the toxicity of microplastic exposure because while some studies observe effects of exposure, others report no measurable impact (Bucci et al. 2020). It is for this reason that despite twenty years and exponential growth in publication on the topic, contemporary research still refers to this contaminant as “emerging” (Rubio-Armendáriz et al. 2022, Martín et al. 2022, Podbielska and Szpyrka 2023, Stapleton and Hai 2023, Bhardwaj et al. 2024). Bucci et al., (2020) call for research to consider microplastics as a complex suite of contaminants, stressing the importance of the context in which a system is evaluated. The dosage and duration of exposure, species, polymer type, shape, size, color, charge, and level of biological organization all influence the effects of microplastics and are therefore important factors to be considered holistically when evaluating the complex effects of microplastics. Further research will need to be undertaken to understand if the effects of microplastic exposure presented here rise to a scale of biological relevance to whole organism functioning with respect to marine mammals. To do so, that work will need to carefully employ a holistic approach cognizant of the complexity of microplastic toxicity. New studies should integrate established models of toxicity to provide insights into microplastic exposure: chemical, particulate, endocrine disruption, and comparative models across species. *In vivo* experiments in marine and terrestrial organisms can serve to validate *in vitro* cellular-level findings in the context of whole organism health. Future work must also address dynamics of exposure, prioritizing long-term exposure that evaluate chronic effect of microplastics, better mimicking real-world conditions. Doing so will improve our ability to draw inferences and conclusions regarding the impacts of microplastic exposure in a biologically relevant context.

4 Acoustic signature of plastic marine debris mimics the prey items of deep-diving cetaceans

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4.1 Introduction

Plastic pollution in the oceans is ubiquitous and increasing (Geyer et al. 2017, Geyer 2020b) with more than 1200 marine species known to ingest plastic debris (De Stephanis et al. 2013, Zachos 2018, Santos et al. 2021). In the case of marine mammals, there are hundreds of examples of whales (cetaceans), seals/sea lions (pinnipeds), and manatees (sirenians) consuming plastic, ingestion of which constitutes a major threat to individual health (De Stephanis et al. 2013, Provencher et al. 2017, Fossi et al. 2018b, 2018a, Panti et al. 2019, Eisfeld-Pierantonio et al. 2022, Gowans and Siuda 2023, Kelly et al. 2023, López-Martínez et al. 2023, Nelms et al. 2023, Murphy et al. 2024, Feyrer et al. 2024). Consequences of macroplastic ingestion include abrasion and perforation of tissues, infection, reduced reproduction and growth, suffocation, clogging the baleen filter (Werth et al. 2024), false satiation, occlusion of the gastrointestinal tract (De Stephanis et al. 2013, Zachos 2018), starvation, and ultimately death (De Stephanis et al. 2013, Fossi et al. 2018b, 2018a, Zachos 2018, Panti et al. 2019). However, our understanding of the sensory mechanisms driving this consumption are incomplete and vary between species.

The sensory mechanisms by which plastic is identified by marine animals as potential prey can be broadly categorized as either a sensory bias or prey resemblance, both of which appear to be species-specific. For example, some seabirds consume plastic based on olfactory cues (Savoca et al., 2016). Similarly, northern anchovy (*Engraulis mordax*) use a chemosensory mechanism to detect odors when foraging (Savoca et al. 2017). As time passes, plastic debris can accumulate such chemical signatures from the marine environment driving misidentification of it as prey by the anchovy (Savoca et al., 2017). Additionally, based on models of plastic spectral reflectance, some seabird vision systems can more easily detect plastic reflecting long wavelength light; in other words, warm colored plastics may exploit a sensory bias in this taxon, driving consumption (Patel 2023).

For pinnipeds and toothed whales, it is widely assumed that plastic debris is visually mistaken as common prey resources - either by shape, color, or behavior of the object in the water column - particularly plastic bags and films that physically resemble gelatinous prey species like squid and jellies (Carson 2013, Savoca et al. 2016, 2017). However, deep diving whales such as sperm whales and beaked whales forage at great depths below the photic zone (Watwood et al. 2006, Quick et al. 2020, Sweeney et al. 2022). Instead of sight, these animals rely on echolocation to hunt. That is to say, assuming these animals are ingesting plastic at depth and not at/near the surface, they are consuming plastic without visually identifying it. Deep-diving toothed whales may therefore be misinterpreting acoustic cues when echolocating; presumably plastic's acoustic signature resembles that of primary prey items, driving plastic consumption.

If the acoustic signatures of plastic marine debris resemble that of prey items at relevant frequencies utilized during foraging, deep-diving whales may mistake plastic for food because of a sensory bias. In echolocation, toothed whales generate sound waves by vibrating phonic lips – membranes that function similarly to vocal cords – located below the blow hole. That sound is amplified and directed using a specialized fatty organ called the melon. Sound emission is staged, beginning with "usual clicks" and culminating in relatively high frequency, low amplitude buzzes/creaks. In general, toothed whales use click frequencies ranging from 10 – 160 kHz to echolocate on targets up to several hundred meters away (Tyack and Janik 2013). Some deep-diver examples include: sperm whales (*Physeter macrocephalus*) that click between .01 – 25 kHz and creak between 25 – 30 kHz (Fais 2016 & Andre 2017), goose-beaked whales (*Ziphius cavirostris*, formerly "Cuvier's beaked whales" (Rogers et al., 2024) that click between 28 – 47 kHz (Baumann-Pickering et al. 2013, Southall et al. 2019) and Blainville's beaked whales (*Mesoplodon densirostris*) that signal between 26 – 51 kHz (Jones et al. 2008). Echolocating Pygmy sperm whales (*Kogia breviceps*) utilize bands in the 125 – 130 kHz range (Southall et al. 2019). Emitted sounds bounce off objects in their path and return to the animal as echoes. Finally, the echoes are received by fats in the lower jaw and travel to the inner ear to be interpreted by the brain (Madsen et al. 2013) allowing odontocetes the ability to "see" with sound by reconstructing detailed maps of objects and potential prey in the environment (Madsen et al. 2007). The relatively broadband echolocation signals used (Au and Benoit-Bird 2008, Okamoto et al. 2010) play a role in target discrimination.

The target strength (TS) of an animal or object is a measure of the magnitude of the incident sound that is reflected as an echo, and depends on the material properties of the ensonified object, the frequency at which that object is ensonified, and the angle or orientation of the object at the time of ensonification (Urick 1983, Ladino et al. 2024). For example, the TS of ommastrephid squids examined in a tank ranged from -66.14 to -31.01 dB at 38 kHz but between

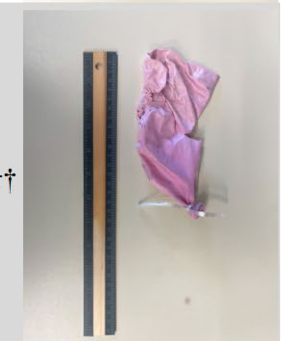
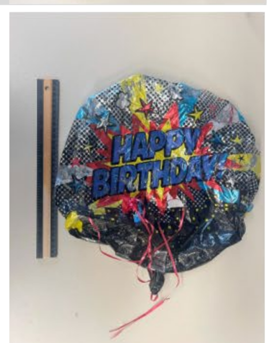
-80.44 and -70.10 dB at 200 kHz (Redaelli et al., 2024). The same study determined target strength of ammoniacal squids to be between -73.96 and -68.4 dB and -82.33 and -75.07 dB at 38 and 200 kHz, respectively (Redaelli et al. 2024). *In-situ* target strength measurements of the jumbo squid (*Dosidicus gigas*) were similarly low: -62.0, -67.4, -67.9, and -67.6 dB at 38, 70, 120, and 200 kHz, respectively (Benoit-Bird et al., 2011). Weak TS, resulting in weak echoes, of cephalopods is generally attributed to their gelatinous body with the mantle scattering the most sound (Arnaya et al., 1989; Madsen et al., 2007; Redaelli et al., 2024), though the beak, eyes, and arms do play a role in acoustic scattering as well (Benoit-Bird et al. 2008); that is, the scattering of sound waves, which changes the direction and intensity of the incident sound, ultimately altering the intensity of the reflected sound, i.e. target strength. Despite their relatively weak overall echoic strength, stomach content analyses of stranded whales indicate cephalopods are an important prey resource of deep diving, echolocating toothed whales (Clarke 1996). For example, world-wide, Cranchiidae, Gonatidae, Histioteuthidae, Octopoteuthidae, Ommastrephidae, Onychoteuthidae, Pholidoteuthidae, and Mastigoteuthidae comprise the majority of goose beaked whales' diet (West et al. 2017). Similarly, squid families Cranchiidae, Gonatidae, Histioteuthidae, Octopoteuthidae, Ommastrephidae, Onychoteuthidae, Pholidoteuthidae, and Mastigoteuthidae are most important for sperm whales; the vast majority of ingested cephalopods were slow-moving, gelatinous and small in size (Clarke, 1996; Foskolos et al., 2020).

To our knowledge, only one prior study has examined the target strength of plastic, and this research was conducted over 50 years ago outside of the context of marine debris, let alone the ingestion of it by marine mammals (Welsby & Goddard, 1973). Additionally, the study examined only a select few polymer types, whereas hundreds can be found in the marine environment today. Here, we aimed to identify whether plastic marine debris' acoustic signature resembled that of prey targeted by deep-diving whales, potentially leading to their misidentification and subsequent ingestion. To do so we implemented an *in-situ* approach, measuring TS at sea of a variety of naturally weathered/fouled plastic marine debris sourced from the environment across the range of frequencies employed by odontocetes; namely at 38, 70, and 120 kHz. Given consumption of plastic debris is a common occurrence in deep-diving whales, we suspect prey items and plastic will have similar target strength measurements.

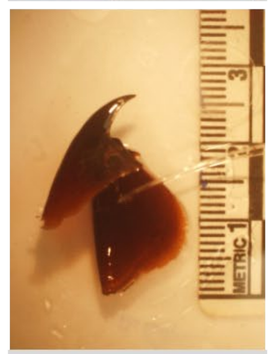
4.2 Methods

The target strength (TS, measured in dB re 1 m^2) of nine plastic marine debris items and representative prey items (Table 6) was measured at three frequencies (38, 70, 120 kHz) using a hull-mounted Simrad® EK80 echosounder with three separate transducers aboard the Duke University Marine Lab's research vessel, *R/V Shearwater*.

Table 6. Sample list of plastic and prey items ensonified

Sample ID	Description	Type	Polymer	Figure	Class
Sample001	Green Rope	Rope/net	PA (Nylon)†		Plastic
Sample002	Black Jug	Fragment	HDPE†		Plastic
Sample005	Pink Balloon	Film-Like	Latex Rubber†		Plastic
Sample008	Black Balloon	Film-Like	PET†		Plastic

Sample010	White Grocery Bag	Film-Like	HDPE		**Plastic**
Sample013	**White Styrofoam**	**Fragment**	**PS**		**Plastic**
Sample014	Gallon Milk Jug	Fragment	HDPE		**Plastic**
Sample015	**Blue Grocery Bag**	**Film-Like**	**HDPE**		**Plastic**
Sample016	Water Bottle	Fragment	PET		**Plastic**

Sample017	**Single *L. brevis***	**Whole**	**N/A**		**Prey**
Sample018	Four *L. brevis*	Whole	N/A		Prey
Sample019	**Single Histioteuthid Beak**	**Beak**	**N/A**		**Prey**
Sample020	Four Histioteuthid Beaks	Beak	N/A		Prey

4.2.1 Sample Selection

4.2.1.1 Plastic

Plastic marine debris was collected opportunistically from the shoreline of Pivers Island, Beaufort NC and Atlantic Beach NC (Table 6). The selected samples were in various stages of fouling at unknown durations of environmental exposure (see images Table 6). Notably, Sample001 (green rope) and 002 (black jug) were fouled with acorn and gooseneck barnacles. The primary types of plastic litter found in stranded cetacean stomachs are categorized as film-like, fragments, and rope/netting (Roman et al., 2021). Representative plastics from these categories were therefore selected for analysis. The polymer types represented amongst these categories were high-density polyethylene (HDPE), polyethylene terephthalate (PET), and polystyrene (PS), identified by labeling on the plastic item. Additionally, some items were suspected to be latex rubber and polyamide (PA) nylon based on visual identification. See sample list in Table 4 for complete list of plastic items ensonified.

4.2.1.2 Prey Items

We utilized *Lolliguncula brevis* and the beaks of an unknown histioteuthid. Whole body *L. brevis*, a small shallow water species, was used as an anatomical proxy of deep-diving whale prey (Clarke 1996, West et al. 2017, Foskolos et al. 2020). Because of the depths many of the common whale prey families inhabit, they are not commercially harvested and therefore not readily accessible. Additionally, beaks from the unknown histioteuthid, a family commonly identified in sperm whale diet (Foskolos et al. 2020), were sourced directly from a stranded sperm whale stomach (stranding ID CALO23-09) and used as an additional prey target. Beaks were identified as histioteuthid using morphological characters outlined by Clarke, (1986). These two types of prey items, whole body *L. brevis* and histioteuthid beak, were each ensonified as individual targets and as groups of four to simulate schooling behavior (Table 6).

4.2.2 Experimental Design

Prior to obtaining sample measurements, the echosounders were calibrated according to manufacturer instructions using a 38.1 mm tungsten steel calibration sphere suspended beneath the vessel on monofilament line using three fishing rods (Foote et al., 1987; Simrad EK80, 2020). As demonstrated in Figure 22, various plastic items commonly found in whale stomachs (eg. balloons, grocery bags, jugs, etc.), and prey items (squid and squid beaks) were mounted one at a time to a rig constructed of 90.7 kg (200 lb) monofilament line (Triple Fish, Valley Twp, PA), 9 kg (20 lb) kettlebell weights, 59 kg (130 lb) three-way swivels, and longline snap clips. The rig was deployed iteratively, once per sample, over the bow with handlers on either side of the vessel and walked aft to the location of the echosounders. Care was taken to ensure all objects were gently submerged and filled entirely with sea water, if applicable, to avoid the effects of air bubbles. The echosounder was configured to a transmitted power of 1500, 750, and 250 W at 38, 70, and 120 kHz, respectively, a pulse duration of 0.256 ms, and a ping rate of 50 ms. Each sample was loaded onto the rig one at a time, held stationary ~ 4 – 5 m beneath the echosounder, and ensonified at 38, 70, and 120 kHz for a minimum of 2 min (~ 2300 pings at each frequency). Resulting echogram data was stored as coupled raw (.raw) and index (.idx) echogram files and processed shoreside with Echoview 13.1 software as described in Data analysis below (echoview, Hobart, Tasmania, Australia). Calibration of the echosounder and sample measurements were performed roughly ~ 35nm off Hatteras Island, North Carolina at 35.395 N, -75.085 W in favorable conditions; sea state < 2, calm with nominal current in a minimum of 20 m depth, 7.7 deg C SST, and 26 ppt salinity.

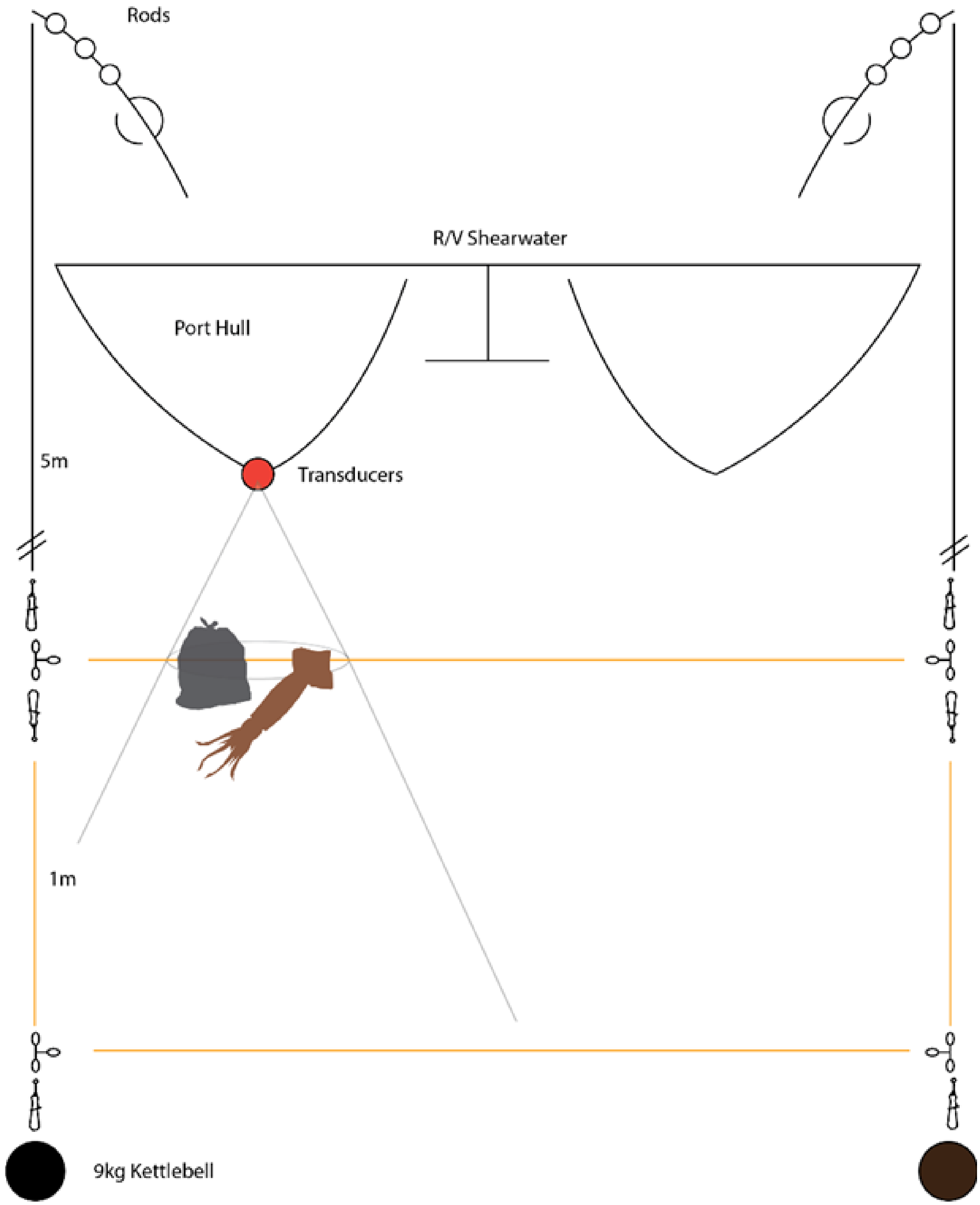


Figure 22: Schematic diagram illustrating rig, constructed of monofilament line, used to deploy plastic and prey items underneath the Research Vessel Shearwater to be ensonified. Sample items ensonified one at a time at 4-5m depth with vessel in a minimum of 20m.

4.2.3 Data Analysis

Raw and index echogram files were imported to Echoview 13.1 for each sample object. Echoview's Background Noise Removal filtering algorithm was applied to pings at all three frequencies for each sample object. Then within each echogram, a region of interest of approximately 100 pings was manually defined synchronously (spatially and temporally) across echograms from all three frequencies around an ensonified sample object. Finally, the Echoview single target detection (single beam – method 2) algorithm was applied at each frequency with a -80 dB threshold to detect individual echoes within each region. Target strength was then extracted and exported to be processed and visualized in R. The following summary statistics were calculated grouped by sample and frequency: median TS, TS range, the interquartile range, and the mean absolute deviation (similar to standard deviation but more resilient to outliers and appropriate for nonparametric data). Additionally, data was further summarized by frequency and type (Film-like, Fragment, Prey, Rope/net) to calculate a minimum and maximum median TS and the percent overlap of that range for each plastic type with the range of median TS values for the prey items. Finally, in order to compare TS of deep-diving cetacean prey items to plastic marine debris, a non-parametric test must be employed; TS is logarithmic by nature and measurements were not normally or homogeneously distributed. Therefore, we utilized a Kruskal-Wallis test to check for significant differences between these two groups. Because all three frequencies reported significant *p*-values, a Dunn's test – a non-parametric pairwise comparison - was subsequently performed. A Bonferroni correction for multiple comparisons was applied to the *p*-value.

4.3 Results

4.3.1 Summary Statistics

Summary statistics for TS, grouped by sample and frequency, are reported in Table 7. For "Film-like" plastics, TS ranged between -55.15 to -33.39 dB at 38 kHz, -68.56 to -49.54 dB at 70 kHz, and -74.31 to -32.97 dB at 120 kHz. Similarly, at 38, 70, and 120 kHz, echoes from plastic "Fragment" items ranged from -74.36 to -52.42, -72.33 to -44.19, and -63.18 to -37.31 dB, respectively. The "Rope/net" type category was represented by one sample, Sample001, and had a median TS of -43.00, -46.54, and -60.68 dB at 38, 70, and 120 kHz, respectively. Prey on the other hand, Samples017 to 020, have TS ranging from -70.90 to -51.21 and -74.06 to -47.39 dB at 38 and 120 kHz, respectively. At 120 kHz, only prey Sample017 was acoustically detectable with a median target strength of -62.59 dB.

Table 7. Summary statistics of target strength per sample by frequency. IQR = interquartile range. MAD = mean absolute deviation. Color indicates Type: Film-like, Fragment, Prey, Rope/net.

Frequency	Sample ID	Type	Median TS	Min TS	Max TS	Range	IQR	MAD
38 kHz	Sample001	Rope/net	-43.00	-61.03	-28.78	32.25	10.18	6.11
	Sample020	Prey	-60.22	-79.96	-53.93	26.03	7.06	5.73
	Sample019	Prey	-70.90	-78.13	-63.23	14.90	3.99	2.90
	Sample018	Prey	-60.23	-79.36	-51.09	28.26	12.23	8.51
	Sample017	Prey	-51.22	-66.10	-43.00	23.10	9.00	5.85
	Sample016	Fragment	-56.41	-79.96	-28.09	51.87	30.98	22.79
	Sample014	Fragment	-74.36	-79.94	-71.73	8.21	3.70	2.65
	Sample013	Fragment	-60.03	-78.21	-43.69	34.52	18.10	13.32
	Sample002	Fragment	-52.42	-67.78	-47.83	19.95	5.91	3.95
	Sample015	Film-like	-33.89	-68.47	-27.94	40.53	8.33	6.20
	Sample010	Film-like	-38.13	-53.16	-30.87	22.30	7.14	5.76
	Sample008	Film-like	-55.15	-77.21	-42.62	34.59	6.62	4.74
	Sample005	Film-like	-46.89	-58.58	-42.87	15.72	7.50	4.98
70 kHz	Sample001	Rope/net	-46.54	-58.31	-25.41	32.90	18.15	14.86
	Sample020	Prey	-53.51	-67.06	-45.52	21.54	8.26	5.92
	Sample019	Prey	-68.67	-78.11	-55.84	22.27	10.91	6.97
	Sample018	Prey	-47.39	-59.06	-38.43	20.63	7.19	5.97
	Sample017	Prey	-74.06	-78.68	-65.74	12.94	3.19	2.58
	Sample016	Fragment	-44.19	-79.25	-31.72	47.53	10.80	7.70
	Sample014	Fragment	-72.33	-79.58	-68.19	11.39	3.79	2.91
	Sample002	Fragment	-65.82	-79.56	-56.43	23.14	8.29	6.16
	Sample015	Film-like	-49.54	-77.37	-36.15	41.22	12.08	9.39
	Sample010	Film-like	-68.56	-79.98	-42.39	37.59	22.93	14.85
	Sample005	Film-like	-61.76	-77.94	-52.31	25.63	15.19	11.21
120 kHz	Sample001	Rope/net	-60.68	-79.67	-44.86	34.81	22.00	16.91
	Sample017	Prey	-62.59	-77.91	-58.50	19.41	9.98	4.04
	Sample016	Fragment	-63.18	-79.93	-34.43	45.51	19.76	15.13
	Sample013	Fragment	-37.31	-53.80	-29.29	24.51	9.88	5.62
	Sample002	Fragment	-58.77	-77.06	-48.51	28.55	7.63	5.57
	Sample015	Film-like	-47.73	-78.34	-38.84	39.50	7.57	5.54
	Sample010	Film-like	-32.97	-79.31	-22.18	57.13	35.21	12.09
	Sample008	Film-like	-74.31	-79.95	-65.16	14.80	8.15	5.85
	Sample005	Film-like	-67.05	-79.93	-60.71	19.22	9.65	6.76

Percent overlap of these median TS ranges was calculated between the plastic and prey types where possible. At 38 kHz, the ranges of median target strengths for film-like plastic and prey overlapped by 18.50%, with the remaining 81.5% of the plastic TS range having a greater echoic strength than the prey. Median fragment TS overlapped with prey by 84.23%. At 70 kHz, range of median film-like TS overlapped with that of prey by 100%. And median fragment TS overlapped with prey by 88.61%.

4.3.2 Kruskal-Wallis Test

Kruskal-Wallis tests conducted for all three frequencies indicated a nonparametric pairwise comparison between plastic and prey TS using a Dunn's Test was appropriate (38 kHz: $X^2 = 502.36$, df = 12, $p = 6.96 \times 10^{-100}$; 70 kHz: $X^2 = 929.53$, df = 10, $p = 2.81 \times 10^{-193}$: 120 kHz: $X^2 = 297.56$, df = 8, $p = 1.36 \times 10^{-59}$).

4.3.3 Dunn's Pairwise Comparisons

Target strength of every plastic object ensonified had at least one non-significant relationship with at least one prey item at at least one of the three frequencies measured. All Dunn's test comparisons, inclusive of Z scores and adjusted *p*-values, are reported in two tables, one for non-significant comparisons (Table 8) and another for significant pair-wise comparisons (Table 9). In 35 of 43 comparisons (81.4%) for which target strength between plastic and prey items differed, the target strength of the plastic item exceeded the strength of the prey item (Table 9). In total, 88.9 % of 72 comparisons between plastic and prey, target strength either matched or exceeded the target strength of prey items across all three frequencies (Table 8 & 9).

Table 8. Non-significant pairwise comparisons (Dunn's Test) of target strength between prey items and plastic marine debris.

Frequency	Sample ID (Prey)	Prey Item	Sample ID (Plastic)	Plastic Object	Z	*P* adj.
38 kHz	Sample017	Single *L. brevis*	Sample016	Water Bottle	-2.06	1.00
			Sample013	White Styrofoam	-3.15	0.06
			Sample008	Black Balloon	-0.61	1.00
			Sample005	Pink Balloon	2.45	0.55
			Sample002	Black Jug	-0.3	1.00
	Sample018	Four *L. brevis*	Sample016	Water Bottle	2.62	0.34
			Sample013	White Styrofoam	0.49	1.00
			Sample008	Black Balloon	2.69	0.28
			Sample002	Black Jug	3.21	0.05
	Sample019	Single Histioteuthid Beak	Sample014	Gallon Milk Jug	-1.55	1.00
	Sample020	Four Histioteuthid Beaks	Sample013	White Styrofoam	0.79	1.00
			Sample008	Black Balloon	3.08	0.08
70 kHz	Sample017	Single *L. brevis*	Sample014	Gallon Milk Jug	0.21	1.00
			Sample010	White Grocery Bag	1.8	1.00
			Sample005	Pink Balloon	1.39	1.00
			Sample002	Black Jug	0.89	1.00
	Sample018	Four *L. brevis*	Sample015	Blue Grocery Bag	-0.34	1.00
			Sample001	Green Rope	2.2	0.77
	Sample019	Single Histioteuthid Beak	Sample014	Gallon Milk Jug	-0.89	1.00
			Sample010	White Grocery Bag	1.51	1.00
			Sample005	Pink Balloon	0.93	1.00
			Sample002	Black Jug	0.14	1.00
	Sample020	Four Histioteuthid Beaks	Sample010	White Grocery Bag	-2.28	0.63
			Sample005	Pink Balloon	-2.24	0.70
120 kHz	Sample017	Single *L. brevis*	Sample016	Water Bottle	1.1	1.00
			Sample008	Black Balloon	-2.48	0.24
			Sample005	Pink Balloon	-1.11	1.00
			Sample002	Black Jug	2.54	0.20
			Sample001	Green Rope	0.47	1.00

Table 9. Significant pairwise comparisons (Dunn's Test) of target strength between prey items and plastic marine debris.

Frequency	Sample ID (Prey)	Prey Item	Sample ID (Plastic)	Plastic Object	Z	*P* adj.
38 kHz	Sample017	Single *L. brevis*	Sample015	Blue Grocery Bag	7.18	2.75E-11
			Sample014	Gallon Milk Jug	-7.19	2.53E-11
			Sample010	White Grocery Bag	7.01	8.97E-11
			Sample001	Green Rope	4.82	5.52E-05
	Sample018	Four L. brevis	Sample015	Blue Grocery Bag	9.96	8.84E-22
			Sample014	Gallon Milk Jug	-4.36	5.13E-04
			Sample010	White Grocery Bag	10.09	2.43E-22
			Sample005	Pink Balloon	5.56	1.04E-06
			Sample001	Green Rope	8.46	1.05E-15
	Sample019	Single Histioteuthid Beak	Sample016	Water Bottle	7.77	3.04E-13
			Sample015	Blue Grocery Bag	13.27	1.33E-38
			Sample013	White Styrofoam	4.32	6.20E-04
			Sample010	White Grocery Bag	13.80	9.41E-42
			Sample008	Black Balloon	6.28	1.34E-08
			Sample005	Pink Balloon	9.07	4.56E-18
			Sample002	Black Jug	7.07	5.87E-11
			Sample001	Green Rope	12.84	3.69E-36
	Sample020	Four Histioteuthid Beaks	Sample016	Water Bottle	3.28	4.09E-02
			Sample015	Blue Grocery Bag	10.59	1.34E-24
			Sample014	Gallon Milk Jug	-4.31	6.48E-04
			Sample010	White Grocery Bag	10.85	7.92E-26
			Sample005	Pink Balloon	6.07	5.02E-08
			Sample002	Black Jug	3.68	9.24E-03
			Sample001	Green Rope	9.36	3.08E-19
70 kHz	Sample017	Single *L. brevis*	Sample016	Water Bottle	9.34	2.57E-19
			Sample015	Blue Grocery Bag	5.82	1.64E-07
			Sample001	Green Rope	7.98	3.88E-14
	Sample018	Four L. brevis	Sample016	Water Bottle	3.15	4.42E-02
			Sample014	Gallon Milk Jug	-9.93	8.25E-22
			Sample010	White Grocery Bag	-6.83	2.35E-10
			Sample005	Pink Balloon	-6.04	4.21E-08
			Sample002	Black Jug	-9.27	5.26E-19
	Sample019	Single Histioteuthid Beak	Sample016	Water Bottle	16.43	3.12E-59
			Sample015	Blue Grocery Bag	6.93	1.14E-10
			Sample001	Green Rope	10.39	7.39E-24
	Sample020	Four Histioteuthid Beaks	Sample016	Water Bottle	10.37	8.87E-24
			Sample015	Blue Grocery Bag	3.63	7.77E-03
			Sample014	Gallon Milk Jug	-5.00	1.60E-05
			Sample002	Black Jug	-4.14	9.63E-04
			Sample001	Green Rope	6.67	7.12E-10
120 kHz	Sample017	Single *L. brevis*	Sample015	Blue Grocery Bag	6.07	2.26E-08
			Sample013	White Styrofoam	9.93	5.43E-22
			Sample010	White Grocery Bag	6.65	5.14E-10

4.3.3.1 At 38 kHz

At 38 kHz, when compared to Sample017 – a single *L. brevis* squid – plastic Samples002 (Black Jug), 005 (Pink Balloon), 008 (Black Balloon), 013 (White Styrofoam), and 016 (Water Bottle) showed no significant difference in target strength (Table 8, See S. Figure 1 for example echograms comparing Samples017 and 008). Likewise, there were no significant differences in target strength between Sample018 – four *L. brevis* squid – when compared to plastic Samples 002, 008, 013, and 016 (Table 8). The comparison of plastic items to Sample019 – a single histioteuthid squid beak – showed only Sample 014 (Gallon Milk Jug) to have similar target strengths (Table 8). And finally, the target strength of four histioteuthid squid beaks – Sample020 – was comparable to that of plastic Samples008 and 013 (Table 8). In all pairwise comparisons of plastic Samples001 (Green Rope), 010 (White Grocery Bag), and 015 (Blue grocery Bag), the plastic items were stronger echoic targets than any of the four prey item samples (Table 9, Figure 23).

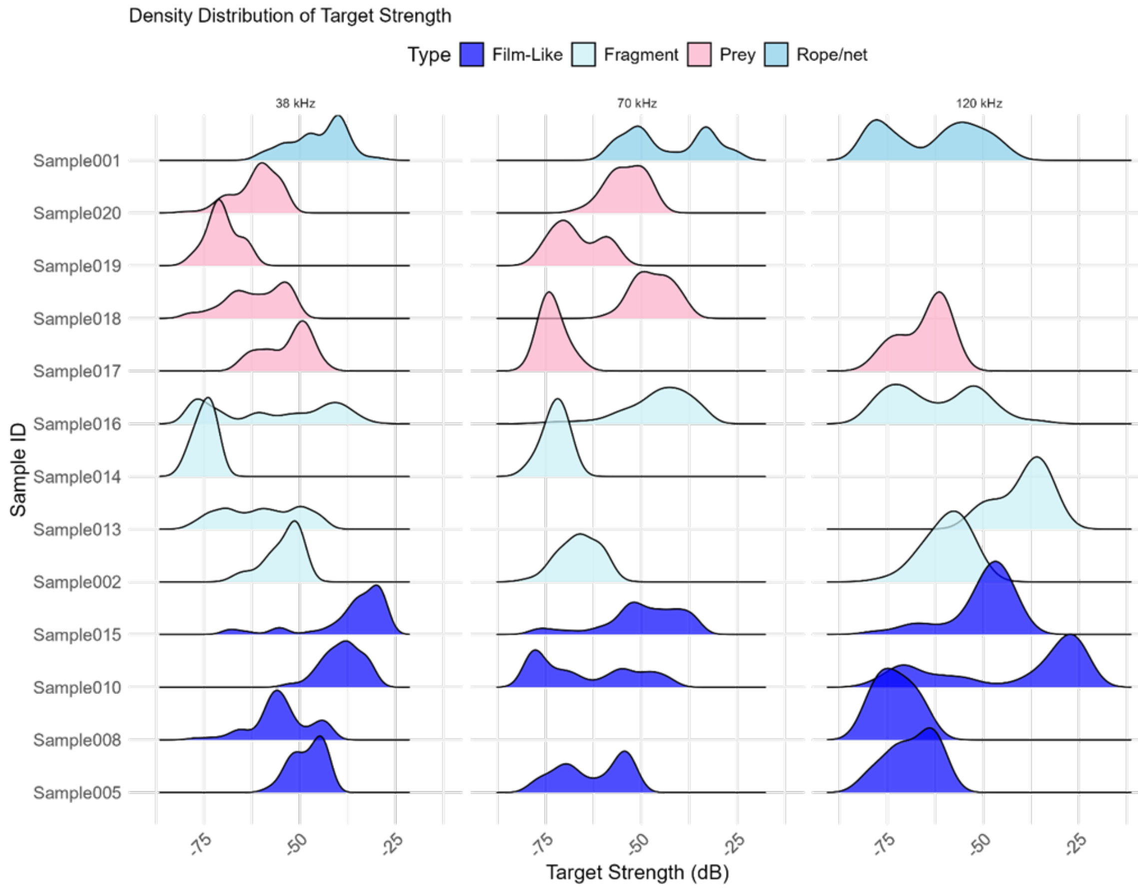


Figure 23: Density distribution of target strength for samples at 38, 70, and 120 kHz, color coded by type of plastic debris or prey item. See Table 4 for sample identification. Samples with no distribution were undetectable at the given frequency.

4.3.3.2 At 70 kHz

At 70 kHz, when compared to Sample017 – a single *L. brevis* squid – plastic Samples002 (Black Jug), 005 (Blue Grocery Bag), 010 (White Grocery Bag), and 014 (Gallon Milk Jug) showed no significant difference in target strength (Table 8). Likewise, there were no significant differences in target strength between Sample018 – four *L. brevis* squid – when compared to plastic Samples001 (Green Rope) and 015 (Blue Grocery Bag, Table 8). The comparison of plastic items to Sample019 – a single histioteuthid squid beak – showed Samples002, 005, 010, and 014 to have similar target strengths (Table 8). And finally, the target strength of four histioteuthid squid beaks – Sample020 – was comparable to that of plastic Samples005 and 010 (Table 8). In all pairwise comparisons with plastic Sample016 (Water Bottle) the plastic item was a stronger echoic target than any of the four prey item samples (Table 9, Figure 23). Plastic Sample008 (Black Balloon) and 013 (White Styrofoam) were undetectable at 70kHz.

4.3.3.3 At 120 kHz

At 120 kHz, when compared to Sample017 – a single *L. brevis* squid – plastic Samples001 (Green Rope), 002 (Black Jug), 005 (Pink Balloon), 008 (Black Balloon) and 016 (Water Bottle) showed no significant difference in target strength (Table 8). Prey Samples018 (four *L. brevis*), 019 (a single histioteuthid squid beak), and 020 (four histioteuthid squid beaks) were undetectable at 120kHz. Plastic Sample014 (Gallon Milk Jug) too was not detected at this frequency. In all pairwise comparisons of plastic Samples010 (White Grocery Bag), 013 (White Styrofoam), and 015 (Blue Grocery Bag) with prey Sample017, the plastic items were stronger echoic targets (Table 9, Figure 23).

4.4 Discussion

This study investigated whether plastic marine debris' target strengths resembled that of prey targeted by deep-diving, echolocating whales, with the aim of determining if the whales' acoustic sensory mechanism may play a role in the misidentification of plastic as prey. Prey items, whole squid and squid beaks, as well as various plastic types routinely found in stomachs of stranded whales, were ensonified at sea to measure and compare target strength. Indeed, these findings support the hypothesis that the consumption of plastic marine debris by echolocating deep-diving odontocete whales is at least partially driven by an acoustic misperception. This is due to similarities in target strength of plastic with that of common whale prey items. At all three frequencies examined, the TS of 88.9% of ensonified plastic marine debris either matched or exceeded the TS of a squid prey item examined (single or multiple *L. brevis* and single or multiple histioteuthid beaks). In no instance was the target strength of a plastic item lower than that of the prey item with the lowest target strength at a given frequency. Because the TS of plastic debris acoustically resembles whale prey, it may therefore be mistaken as a prey target, thus driving the phenomenon of voluntary plastic ingestion in this group of whales. Median fragmented plastic target strength overlapped with median prey target strength by 84.23% and 88.61% at 38 and 120 kHz, respectively. Median TS of film-like plastics overlapped with prey median TS by 18.5% and 100% at 38 and 120 kHz, respectively. The overlap at 38 kHz is low because the median TS of film-like plastics was stronger than that of the prey items in all instances. Considering the TS of film-like and fragmented plastics is similar to or stronger than prey TS, this may explain why these types of plastics in particular are most commonly found in stranded whale stomachs (Roman et al. 2021).

There is a frequency dependence in TS across plastic debris that may translate to species-specific impacts. For example, some film-like samples had lower target strengths at higher frequencies (e.g. Sample005 and Sample010), and in some cases plastic was acoustically undetectable at a given frequency but detectable at others. This might suggest that different plastics therefore have a species-specific impact given that different odontocetes utilize different frequency bands when foraging. For example, goose-beaked whales that forage using 28 – 47 kHz (Baumann-Pickering et al. 2013, Southall et al. 2019), may be more likely to consume something like Sample005 (a pink balloon) than Stejneger's beaked whales (*Mesoplodon stejnegeri*) which produce echoic signals between 46-76 kHz (Baumann-Pickering et al. 2013, Southall et al. 2019); Sample005 had TS roughly between 1-2 orders of magnitude greater when measured at 38kHz than when measured at 70kHz which would make it a stronger target to goose-beaked whales. There are hundreds of types of plastic, and the various material properties including polymer (chemical) composition, additives, shape, size, age/weathering, and degree of fouling likely play a role in the frequency-specific responses observed. For example, target strength increases both with filament radius in plastic netting and area of plastic sheeting (Welsby and Goddard 1973). Thickness and shape (e.g. crumpled) of Melinex and polythene sheets also positively influence target strength (Welsby and Goddard 1973).

At the time of writing this article, Redaelli et al., (2024) published a conference paper showcasing preliminary work also examining target strength between plastic and squids. Their results and conclusions align with those presented here, and methodologies they utilized compliment this work well. Redaelli et al. (2024) conducted their target strength measurements at 38 and 200 kHz *ex-situ* in a tank, after artificially weathering and fouling their plastic samples for a period of one month. They note this design likely does not adequately represent the complex marine acoustic landscape and that it provides limited coverage over the range of frequencies utilized during echolocation by odontocetes. Here we sourced plastic debris directly from the marine environment (naturally fouled and weathered), ensonified our sample objects at sea *in-situ*, across a more inclusive range of frequencies relevant to odontocete echolocation (38, 70, 120 kHz), and with a broader array of plastic items. However, whereas (Redaelli et al. 2024) evaluated the impact of orientation (vertical, horizontal) and configuration ("extended" or "folded") of the ensonified object on target strength, we allowed our objects to move freely beneath the transducer beam of the vessel in calm sea conditions. The concordance in conclusions between the two approaches serves to validate the other and further support the finding that plastic debris acoustically mimics the TSs of deep-diving whale prey.

To more fully understand the role of echolocation in the misidentification of plastic as prey, future work should explore the influence of pressure on target strength of various plastics, evaluate a more comprehensive suite of prey species, and tease apart whether or not whales can discriminate between plastic and prey items with similar target strengths. Here we examined target strength of plastic debris 4-5 m below the water surface. Deep-diving whale prey exists hundreds to thousands of meters below the surface in the aphotic zone and therefore under immense pressure. How different plastics with varying material properties respond to that pressure, to what degree they compress or do not, and how it may impact target strength is unknown. Additional work may investigate target strength of other families of squid important to deep divers unexplored here or by Redaelli et al., (2024) or of fish and crustacean species also consumed by these whales. And while we now know that plastic debris is detectable to deep-divers via echolocation and has similar or stronger target strengths as some prey, whether these animals can discriminate and classify between a given plastic and prey item with similar target strengths is an unanswered question. There is evidence that odontocetes can discriminate between objects of the same dimensions but varying material properties (Au & Turl, 1991). However, what we know about this ability to discriminate is limited almost entirely to captive dolphins. Dolphins would naturally employ a multimodal foraging approach, combining sight with echolocation simultaneously to increase the accuracy of target identification. Presumably, this is not an option for deep-divers foraging in the aphotic zone. Furthermore, owing to the challenges of studying deep divers – which spend a majority of their lives at great depths and are too large for captive studies – research on the ability to discriminate echolocated targets is lacking in this group. That said, a recent cross-modal matching study examining a dolphin's ability to discriminate objects of the same shape but different material properties showed that dolphins had a much more difficult time correctly identifying objects filled with water (Wei et al. 2021). Wei et al., (2021) note that most of the energy in an incident sound striking submerged plastic bags and bottles would transmit through the plastic rather than reflecting back at an echolocating whale, perhaps interfering with the animal's ability to discriminate between these plastic items and prey.

Domestically and globally, we produce staggering amounts of plastic, much of which is superfluous single-use plastic and quickly ends up in the oceans or other undesirable places. With respect to echolocating whales, manufacturers could attempt to produce a "whale-safe" plastic that is acoustically undetectable by altering the material properties of their products. However, such a solution may only instigate another issue as an acoustically undetectable fishing net, for example, may increase the risk of whale entanglement. Ultimately, work-arounds are insufficient solutions compared to the drastic reduction in production of unnecessary plastic. While plastic does serve many important purposes, such as in medical applications or durable long-lasting products, much of it, such as single-use packaging – like the shopping bags and films found in stranded whale stomachs – is gratuitous and pernicious*. This study supports the hypothesis that deep-diving odontocetes are susceptible to plastic ingestion based on the acoustic similarities of plastic with their prey resources. There is therefore an urgent need to further understand the role echolocation plays in plastic ingestion and mitigate plastic pollution to protect vulnerable whale species such as deep-diving odontocetes.

5 Conclusion

Out of sight and mind for those who produce it, plastic debris that infiltrates the oceans poses a multitude of threats to marine organisms, inclusive of marine mammals. Whales, seals and other marine mammals are prone to entanglement in ocean-based plastic fishing gear, active, lost, or otherwise discarded. Consumption of plastic discharges from land-based sources is common evidenced by investigations of stomach contents often revealing plastic bags, balloons and bottles. Meticulous analyses of marine mammal gastrointestinal tracts identified ingestion of microplastics, a practically invisible, yet ubiquitous threat. As air-breathers, inhalation of microplastics by marine mammals was also suspected and recently confirmed in bottlenose dolphins (Dziobak et al. 2024) Across these various plastic forms and exposure routes, animals that encounter or ingest them face severe consequences, including laceration, strangulation, suffocation, malnutrition, starvation, oxidative stress, inflammation, endocrine disruption, and widespread homeostatic imbalance. While our understanding of the extent to which whales and seals become entangled and the resulting repercussions is well-established, the drivers and consequences of ingesting both large and small plastics within this taxon were incomplete. The body of work presented here aimed to address those gaps. Beginning at the small end of spectrum, I addressed consequences of microplastic ingestion and ended by exploring why deep-diving echolocating whales were driven to consume macroplastic.

5.1 Summary of Findings

While the literature to-date cites several incidences of microplastics in the gut contents and feces of marine mammals, it was unclear whether ingested plastic particles were expelled entirely from the animal or if any were retained within the body. Retention within organs would imply translocation from the gastrointestinal tract. Tissue samples from 12 whale/seal species and four organs were collected (blubber, melon, lung, acoustic jaw fat), digested, and evaluated using Raman spectroscopy and pyrolysis gas chromatography mass spectrometry. These methods successfully identified microplastics in 68% of all samples. The most commonly observed polymer and shape were polyethylene and fibers, respectively. Presence of microplastics in these four marine mammal organ tissues provided supporting evidence for translocation. Furthermore, samples digested were collected as early as 2001, underscoring the temporal scale of microplastic pollution and highlighting chronic exposure to this pollutant. The consequences of microplastic exposure became the focus of my third chapter.

Consequences of microplastic exposure in marine mammals are particularly challenging to evaluate and reveal complex responses. This is because known effects vary based on concentration, duration of exposure, and complicated interactions between size, shape, color, chemical composition, and charge of plastic particles. Ethical, legal, logistical and financial considerations further complicate investigations concerning living marine mammals. This is especially true of whales which are primarily accessible at sea unlike their pinniped relatives that haul out on land to breed and rest. However, *in vitro* and *ex vivo* methods have increased the tractability of experimental research to understand effects of anthropogenic pressures on marine mammals. Here, I employed both a cellular and explant model by crossbow biopsying the skin and blubber tissue of living, free-ranging bottlenose dolphins. Skin was used to establish primary cell lines alongside human dermal fibroblasts. Cell lines and blubber were exposed to polyethylene microspheres and polyvinyl chloride fragments, common microplastics, for an acute duration and ultimately transcriptomic and metabolic (lipolytic) responses were evaluated. Rate of lipolysis increased in blubber tissue while decreasing in dolphin skin cells exposed to polyethylene microspheres, highlighting tissue-specific responses to microplastic exposure. But polyvinyl chloride exposures had no impact on rate of lipolysis in either species; a treatment (polymer)-specific response. Differential gene expression and functional analyses implicated ANGPTL4 and IRS2 as the primary genes driving the lipolytic response to polyethylene. Further comparisons across species revealed both overlapping and species-specific responses, which were also treatment-dependent, highlighting the complexity of microplastics as a diverse group of toxicants with non-uniform toxicity.

And finally, switching gears to macroplastics, I identified a plausible and even probable sensory explanation for the consumption of plastic by deep-diving odontocetes who presumably do not use vision to forage. Ingestion of plastic bottles and bags is well documented in the stomachs of sperm whales and other deep-diving whale species like beaked whales. Feeding well below the photic zone, these species cannot rely on visual senses to detect prey resources. But instead, as odontocetes, these species can employ echolocation using specialized fatty organs. Using objects commonly found in whale stomachs, I subjected plastic to sound at frequencies similar to those used in echolocation and found that the resulting echoes (target strength) were similar to those produced when common prey items were ensonified. This suggests that the acoustic properties of plastic result in the accidental consumption of it by deep-diving, echolocating whales.

5.2 Knowledge Gaps & Limitations

Though the methodologies of these chapters bridged several gaps in research, each has its own inherent limitations. In Chapter 2, I used both Raman spectroscopy and pyrolysis gas chromatography mass spectrometry to identify and quantify the number of plastic particles in various marine mammal organs. The former is a non-targeted, qualitative approach capable of identifying plastic particle roughly 10 µm in size, but cannot quantify mass. Py-GC/MS can quantify mass, including that of particles <1 µm, however the method is destructive and targeted, capable of identifying only a small handful of polymers. Here, py-GC/MS identified PVC in some samples, where Raman spectroscopy did not; perhaps PVC particles were generally below the size detection limit of Raman. This underscores the limitations of each approach which may have biased results. Further we learned that py-GC/MS, as currently designed, is not particularly suitable for exceptionally fatty tissues like those from marine mammals resulting in sample loss.

While the complement of the two methods helped alleviate some limitations of each, others remain depending on sample matrix. Chapter 3 aimed to identify metabolic and transcriptomic responses to microplastic exposure using *in vitro* and *ex vivo* models. Tissue-specific responses between skin cell and blubber assays make it challenging to extrapolate findings to potential health implication at the level of the whole organisms or populations. In Chapter 4, while I identified similarities in acoustic target strength of plastic marine debris and deep-diving whale prey, it remains unknown if echolocating animals have the ability to discriminate between the two. Finally, a major limitation to these and other studies relying on marine mammals is the logistic difficulty and expense associated with field research, which limited sample size (Cise et al. 2022). Sample size is further hampered by elusive behaviors of study species, remote habitats, and expertise required for remote sample collection (Cise et al. 2022).

5.3 Broader Impacts

Findings from this work extend across multiple disciplines, informing conservation, policy, toxicology and sensory ecology. It provided empirical evidence of microplastic translocation, species-specific cellular responses to microplastic exposure, and a novel acoustic sensory mechanism explaining the ingestion of macroplastics by deep-diving odontocetes. Demonstration of microplastic accumulation in vital organs from decades past underscores the urgency of regulatory action to mitigate the long-term ecological consequences of marine plastic pollution. Insights into cellular and metabolic responses to microplastics enhance our ability to assess toxicological risks across species, including potential parallels to human health. In identifying echolocation-driven misidentification of plastic as prey, myself and coauthors highlighted an unrecognized risk factor contributing to ingestion. These data may be valuable to conservationists, managers, and policymakers seeking to reduce plastic-related mortality in vulnerable odontocete populations. Overall, this work advances our mechanistic understanding of plastic’s effects on marine life and provides a strong foundation for future interdisciplinary work.

5.4 Future Directions

There are so many exciting questions to pursue that this body of work has only begun to uncover, paving the way for future research on the ecological, physiological, and behavioral impacts of plastic pollution on marine mammals. We now know microplastics are embedded in the bodies of marine mammals. But we do not yet know if those microplastics can be remobilized and eventually expelled, if concentrations are homo- or heterogeneously distributed between or within organs, or how these concentrations vary across species, populations, sexes, or age classes. Some microplastics in this work and other literature exceed a theoretical limit in size for translocation across the epithelial barrier of the intestine; what mechanism(s) are allowing these particles to pass? Right now, diagnosing or quantifying microplastic exposure outside of the gut is limited to opportunistic samples sourced from stranded or harvested animals. I would like to develop a biomarker, potentially using phthalates (a known plastic additive), that could be correlated to microplastic concentrations in blubber tissue. This would allow for an estimation of microplastic exposure in blubber of living individuals via biopsy. Are the lipolytic responses to microplastic exposure observed at the cellular and tissue levels cause for concern? Do they threaten a marine mammal's ability to effectively store and mobilize energy reserves for critical life history events like reproduction and migration? Plastic marine debris can even "sound" like prey to deep-diving echolocators. The capacity for marine mammals to discriminate between the two via echolocation could be explored with living, captive odontocetes. It is my aspiration to tackle some of these outstanding questions as I continue my career in academia.

5.5 Concluding Remarks

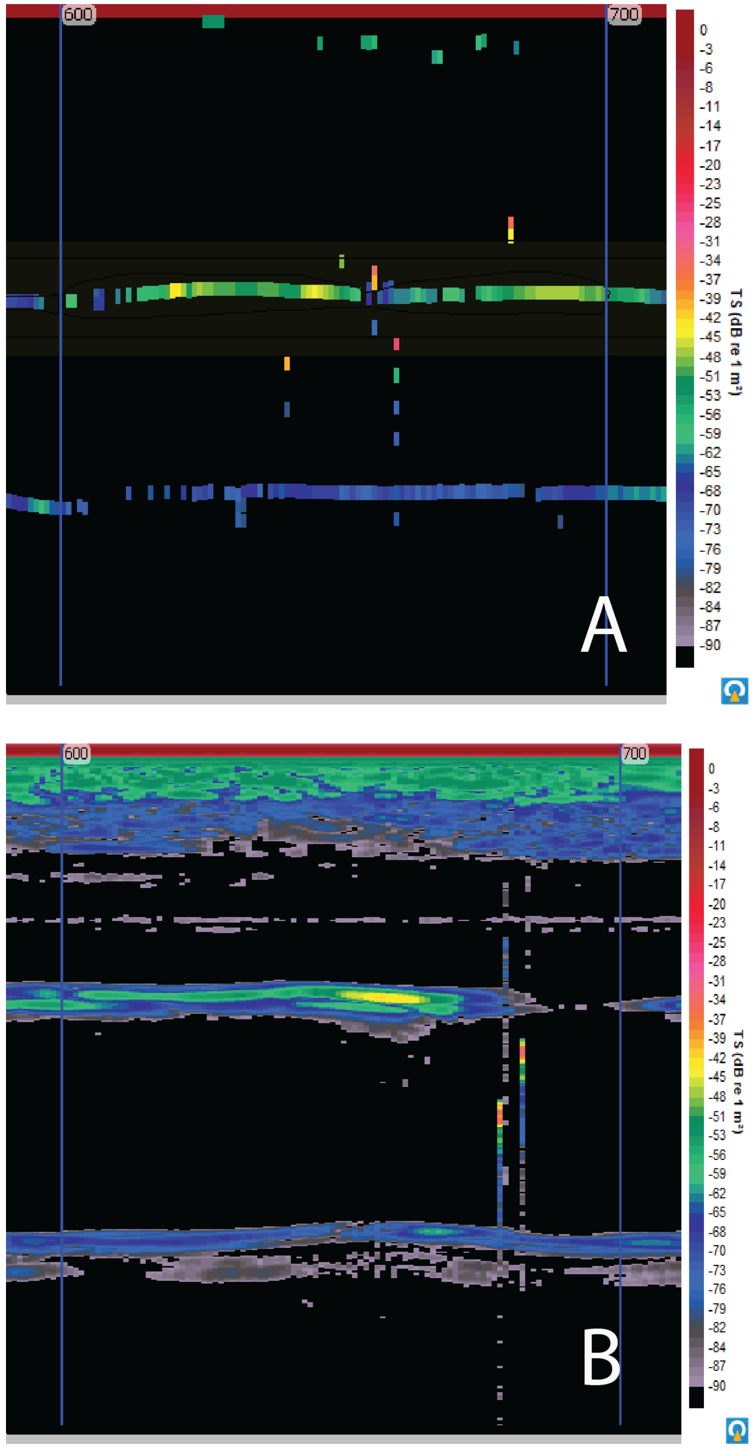
There remains much we do not know about the impacts of plastic pollution. However, it is evident that we have entered a period of consequences. I encourage anyone interested in combatting plastic pollution to take action. Participating in and organizing local cleanups is a great way to spread awareness of the issue. Make changes to your consumer habits by purchasing more sustainable products to reduce your plastic footprint. But most importantly, we need a shift in policy. Advocate for plastic-free initiatives such as single-use bans, encourage your representatives to support limits on plastic production, and vote for legislatures who too envision a world without plastic waste.

Appendix A

S. Table 1.

Sample ID	Animal ID	Polymer	Species	Tissue	Number of Contaminant Particles	Color	Category
060122-T2	control	assumed polyethylene	Ctl	control	1	blue	fragment
060122-T2	control	polyethylene	Ctl	control	7	blue	fragment
060122-T2	control	rubber	Ctl	control	1	blue	fragment
060122-T3	CAHA564	polyethylene	Ttr	melon	3	blue	fragment
060122-T5	2020230	assumed polyethylene	Dle	acoustic fat pad	9	blue	fragment
060122-T7	2019384	assumed polyethylene	Dle	acoustic fat pad	1	blue	fragment
060122-T8	2019384	assumed polyethylene	Dle	lung	9	blue	fragment
080921-T10	EB19PH026	assumed polyethylene	Eba	blubber	1	blue	fragment
080921-T2	control	assumed polyethylene	Ctl	control	4	blue	fragment
080921-T4	BP1602 2016076	polyethylene	Bph	blubber	1	blue	fragment
080921-T5	PH110311AVPS	assumed polyethylene	Phi	blubber	1	blue	fragment
080921-T8	2019031	assumed polyethylene	Ero	blubber	7	blue	fragment

080921-T9	EB19PH012	assumed polyethylene	Eba	blubber	3	blue	fragment
112921-T10	RT50	assumed polyethylene	Gma	blubber	4	blue	fragment
112921-T10	RT50	polyethylene	Gma	blubber	1	blue	fragment
112921-T11	RT57	assumed polyethylene	Gma	blubber	3	blue	fragment
112921-T2	control	assumed polyethylene	Ctl	control	3	blue	fragment
112921-T2	control	polyethylene	Ctl	control	1	blue	fragment
112921-T7	C-672	assumed polyethylene	Ero	blubber	6	blue	fragment
112921-T8	C-672	assumed polyethylene	Ero	blubber	7	blue	fragment
112921-T8	C-672	polyethylene	Ero	blubber	1	blue	fragment
112921-T9	RT48	assumed polyethylene	Gma	blubber	19	blue	fragment
112921-T9	RT48	polyethylene	Gma	blubber	1	blue	fragment



S. Figure 1. Example echograms showing the TS of ensonified objects. Panel A shows prey Sample017 (single L. brevis) at 38 kHz compared to panel B, Film-like plastic Sample008 (black balloon) at 38 kHz. White arrows indicate the given sample while yellow arrows indicate the kettlebell weights used to hold the rig stationary.

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Biography

Gregory (Greg) Bruce Merrill Jr is a first-generation college graduate of the University of California – Davis, where he earned a Bachelor of Science in Biological Sciences in 2014. He was also awarded two minors: 1) Oceanography, and 2) Wildlife, Fish, & Conservation Biology. After graduation Greg worked at the Aquatic Toxicology Laboratory housed within the School of Veterinary Medicine at UC Davis as a Junior Specialist for two years. He also volunteered at The Marine Mammal Center in Sausalito, California rehabilitating sick and injured seals and sea lions. The latter led him to pursue a Master of Science degree in Biological Sciences from the University of Alaska – Anchorage where he studied the foraging ecology of northern fur seals on the Pribilof Islands of Alaska. After successfully earning his Master's degree in the summer of 2019, Greg packed all he could into his car and drove from Anchorage to Durham, NC – an epic 10 day journey across North America – to begin his PhD in Ecology at the Nicholas School of the Environment at Duke University. Across his graduate tenure, Greg was awarded 17 research grants totaling in excess of $262,000, first-authored three publications in high-impact journals, and co-authored an additional four. A complete list of his scholarly work can be accessed here: https://scholar.google.com/citations?user=AnfWSAUAAAAJ&hl=en.