Whale, what do we have here? Evidence of microplastics in top predators: analysis of two populations of Resident killer whale fecal samples.

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Abstract

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Environmental microplastics (plastic particles less than 5 mm in size) are a growing ecological issue and are widely documented in marine life. The consequences of microplastic ingestion in top predators are poorly understood but may include physiological and toxicological effects, and the potential for bioaccumulation in apex predators has been suggested. Here, I investigate the presence of microplastics in two populations of North Pacific Resident killer whales and determine if there is a significant difference in the number of microplastics between the populations. This study examined 33 feces samples, 18 from the Southern Resident population, and 15 from the Alaskan Resident population. We implemented multiple contamination-control measures to reduce sample contamination from synthetic clothing and plastic equipment. Microplastics were found in every fecal sample except one, with an average and standard

deviation of 82.5 (±173) per sample. I observed no significant difference in the number of microplastics between the two populations (p-value = 0.799). Preliminary Raman microspectroscopy revealed three plastic polymer types that included polyethylene, nylon, and polyamide. Verified microplastics were found in fecal samples from both populations of resident killer whales, validating the occurrence of microplastic pollution in upper-trophic marine predators. This study is another example of the pervasiveness of microparticles in the marine environment, and the need for a better understanding of the potential effects on apex predators.

1. Introduction

Humans depend on the ocean: (1) nearly 25% of the world's population currently lives within 100 km of the ocean, (2) the ocean produces almost half of global primary production, a large portion of which fuels global fisheries, (3) the ocean hosts substantial biodiversity, and (4) coastal countries benefit economically from the important and continually growing tourism sector (Kühn et al., 2015). However, humans have substantially altered the ocean within the last few centuries, and in recent decades through the input of anthropogenic debris (Bergmann et al., 2015). Anthropogenic debris is "any persistent, manufactured or processed solid material discarded, disposed of or abandoned in the marine and coastal environment" (Bergmann et al., 2015), and is found in almost all oceanic and coastal areas. The majority of anthropogenic debris found on shorelines, the sea surface, and the seafloor is plastic (Galgani et al., 2015). Plastic pollution can affect marine species through entanglement and ingestion (Lusher et al., 2015), and the presence of plastic in the marine environment poses immense threats for marine life (Moore et al., 2020; Kühn et al., 2015). Anthropogenic pollution is thus a tremendous problem in the marine environment.

Plastic debris in the marine environment is composed of a mixture of synthetic polymers and chemical additives, and ranges in size from large macroplastics to microscopic nanoplastic particles (Hahladakis et al., 2018). Plastic polymers are manufactured to be strong, durable, and lightweight; attributes that make them both desirable for consumer products and detrimental as post-consumer waste in the marine environment. Plastics are estimated to take around 500-1,000 years to degrade; thus, almost all plastic created still exists today (Rotjan et al., 2019). The form of the plastic may change as plastics break down into smaller pieces (Rotjan et al., 2019). Of the 230 million tons of plastic produced globally every year, about 10% eventually makes its way

into the marine environment, with an estimated five trillion plastic pieces currently in the ocean (Lusher et al., 2015; Moore et al., 2020). Sources of plastic litter are diverse, including post-consumer items like fishing gear, food packaging, bottles, bags, lids, straws, fibers from synthetic clothing, microbeads found in cosmetics, car tires, city dust, all of which break down into fragments in the ocean (Gallo et al., 2018). Plastics are also transported to the ocean from road run-off, wastewater, winds, and waterways (Rotjan et al., 2019) and can concentrate in particular areas of the ocean, like gyres, upwelling zones, and coastal waters (Lusher et al., 2018).

Microplastics are a form of marine litter, defined as plastic fragments that are less than 5 mm in the largest dimension (Gallo et al., 2018; Jiang, 2018). The various categories of microplastics can be broadly classified as either primary or secondary microplastics. Primary microplastics are pre-consumer plastics such as those used in exfoliating processes like sandblasting or maintenance of plastic products, and microbeads found in cosmetic products. Secondary microplastics are small plastic particles generated from the fragmentation of larger plastic pieces.

Microplastic pollution in the marine environment is widely recognized as a growing environmental problem because of its persistence and rate of accumulation, slow degradation, and the fact that it is difficult, if not impossible to remove microplastic particles from the ocean (Lusher et al., 2015). A 22-year study on plastic pollution in the Western North Atlantic and the Caribbean Sea found that high-density polyethylene (HDPE), low-density polyethylene (LDPE) and polypropylene (PP) float on the surface because they are less dense than seawater; and polyvinyl chloride (PVC), polyethylene terephthalate (PET), and polystyrene solid (PS) sink because they are denser than seawater (Law et al., 2010). Microplastics that float in surface

waters can be transported by currents to areas of low circulation or washed ashore, while denser larger plastics sink and can accumulate in deep-sea sediments (Lusher et al., 2015). The presence of microplastics is thus found throughout the water column, exposing a variety of marine life to this anthropogenic problem.

Organisms can ingest microplastics as food, either by mistaking them as prey when foraging, unintentionally capturing them while filter-feeding, or by eating prey that contains microplastics. Large marine vertebrates and predatory species like marine mammals are likely ingesting microplastics through their prey via trophic transfer or through incidental oral intake during filter feeding (Burkhardt-Holm & N'Guyen, 2019; Gallo et al., 2018). Ingested microplastics may cause chemical harm to higher trophic species through bioaccumulation of contaminants contained in or absorbed by the plastic.

We do not yet know whether microplastics harm organisms, and if a chemical transfer occurs in nature. The hydrophobic properties of synthetic plastic polymers allow persistent organic pollutants (POPs) present in the ocean, like polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), and organochlorine pesticides like dichlorodiphenyltrichloroethane (DDT) (Smith et al., 2018), to adsorb onto the surface and become concentrated on the plastic particles (Nelms et al., 2019). These adsorbed chemicals can desorb and leach into tissues once ingested (Nelms et al., 2019). Low-trophic-level animals can carry toxic chemicals from ingested plastics to all levels of the food web by mistaking microplastics as their food source, which can be similar in size (Zhu et al., 2019). Although, most experimental studies using clean organisms exposed to contaminated microplastics show harm from microplastics, which favor a chemical transfer from the microplastics to the organism's tissues (Ribeiro et al., 2019). There have also been theoretical studies that predict

contaminated microplastics would not favor chemical transfer to tissues because the pollutants would be in equilibrium with their environment (Ribeiro et al., 2019). Recent chemical transfer modeling studies also suggest that when compared with natural pathways like water and sediment, chemical transfer to organisms is potentially low given the low abundance of microplastics (Ribeiro et al., 2019). However, the perfectly spherical (as opposed to fragmented) microplastics used in many laboratory studies are not indicative of what is found in nature, and thus may not truly tell us what is happening under natural circumstances (Cole, 2016).

Endocrine-disrupting chemicals (EDC) are often present in plastics and are a known problem for marine organisms because they mimic hormones naturally present in the organisms. One of these chemical classes, known as plasticizers, are added during the production of plastics. Plasticizers are odorless and colorless esters that are added to plastic to increase the elasticity of the material (Nelms et al., 2019). Plastic polymer components and plastic additives, for example, styrene, phthalates, and other plasticizers (Bang et al., 2012) are also chemicals with endocrine-disrupting properties, and are a significant concern for marine species (Gallo et al., 2018). EDCs can lead to both temporary and permanent changes in the endocrine system (Gallo et al., 2018), either by altering hormone synthesis or by the interaction with hormone receptors (Matthiessen et al., 2018). These EDCs mimic, compete, or disrupt the synthesis of endogenous hormones (Gallo et al., 2018). Effects of EDCs include impaired reproduction, metabolism, thyroid function, and an increased risk of hormone-sensitive cancers (Gallo et al., 2018). Experimental research on mammals, including humans and animals, suggests that embryonic and developmental periods are a critically sensitive time, where EDCs may cause life-long cellular effects (Gallo et al., 2018; Singleton, 2003). In marine mammals, the effects of some lipophilic EDCs are further amplified through vertical transmission to offspring during gestation and

lactation. Marine mammals have a very high milk fat percentage and a long lactation period, which leads to substantial EDC transfer in many marine mammals (cetaceans in particular) (Godfray et al., 2019). As a result, cetaceans could be at special risk from these plastic-borne chemicals.

Long-lived apex predators like marine mammals are extremely susceptible to biomagnification and bioaccumulation of POPs from microplastics (Nelms et al., 2019). Bioaccumulation occurs when primary producers and consumers accumulate POPs from seawater, and it accumulates in their bodies over time (Vinzant, 2017). The highest levels of POPs are found at the top of the food chain due to biomagnification, which occurs when larger organisms feed on lower trophic contaminated organisms, absorbing the POPs of those smaller organisms and, in turn, absorbing the POPs into their tissues at a higher concentration. The more contaminated food an organism eats, the more POPs they will absorb in their body, which is why POPs become more concentrated in top predators (Pierce et al., 2008; Vinzant, 2017). When organisms ingest toxin-loaded microplastics, these microparticles may be another source of POPs in marine food chains, as toxins may leach from the plastics to tissues in organisms (Desai, 2015). Zooplankton are likely ingesting microplastics directly, thereby increasing their POP burden, and threatening the foundation of the marine food web. Through the process of biomagnification, microplastics may play a role in increasing the total POPs in top predators like marine mammals (Desai, 2015).

Marine mammals are often valued as indicators of marine ecosystem health, due to their extreme (in some cases) longevity, migratory behavior, and their role as apex predators, particularly with respect to POPs and other toxins known to bioaccumulate within their tissues (Krahn et al., 2007). Like many other cetaceans, so-called Resident killer whales in the eastern

North Pacific inhabit relatively large home ranges and preferentially feed on the largest of salmonids (Parsons et al., 2009). As such, these piscivorous Resident killer whales may be a valuable indicator of microplastics in the marine food web, to the extent that they accumulate these pollutants from lower trophic levels. As an indicator of marine ecosystem health and microplastics in top predators, killer whales provide an opportunity to examine the negative effects of microplastics on top predators and predict how these effects may translate to human health because their food source is also widely eaten by humans.

In the eastern North Pacific Ocean, there are three contiguous populations of Resident killer whales (fish-eating killer whales) (Parsons et al., 2009). The southernmost population, the Southern Resident killer whales, is currently listed as an endangered distinct population segment (DPS) under the Endangered Species Act (ESA) and continues to decline with a current population of around 72 individuals. Three factors have been identified as significant drivers of the population's decline: vessel noise, lack of prey, and contaminants (Fisheries, 2016). Another population of Resident killer whales is the Southern Alaskan Resident killer whales. This population is increasing by 3% and has for the past 25 years, with a current population around 700 individuals (M. Muto et al., 2018). The population previously faced declines, from interactions with fisheries, and experienced damaging effects from the Exxon Valdez oil spill, from which it has recovered (M. Muto et al., 2018).

These two killer whale populations occupy geographic regions that differ in many ways. The Alaskan Resident killer whales occupy a range that is less densely human-populated, and are found generally in two communities, one which occupies areas in and around Prince William Sound and the other around Kenai Fjords, Cook Inlet, and possibly Anchorage (M. Muto et al., 2018). Southern Resident killer whales are primarily found in the inland waters of the Salish Sea

during the summer months but range from southern British Columbia to California (Fisheries, 2019). The only large city within the range of the Alaskan Residents is Anchorage while the Southern Residents' range includes large coastal cities of British Columbia, Washington, Oregon, and California. The waters and areas around the Salish Sea are also markedly more urbanized, affected by chemical run-off, and contaminants associated with industrial and human activities than the waters in the Gulf of Alaska (Fisheries, 2019).

Here I am investigating the presence of microplastics in marine top predators. I compare the prevalence of microplastics between two North Pacific resident killer whale populations; Southern Alaskan and Southern Resident killer whales. Using fecal samples collected from surface seawater, I isolate microparticles (unverified microplastics) from fecal samples and catalog these microparticles by type (fragment, fiber, or film) and color. Comparing microparticles isolated from both killer whale populations, I examine differences in the prevalence of total microparticles and the different types of microparticles between the two populations. I then verify microparticles and identify polymer types using data from Raman Spectroscopy, and make inference about the origins of identified microplastic particles.

2. Methods

Field Methods

1. Fecal Collection

Fecal samples were collected from both the Southern Resident and the Alaska Resident killer whales (in 2007-2019 and 2016-2018, respectively) from small boats during both dedicated and opportunistic field observations. Sampling regions were determined by the seasonal ranging

patterns of the targeted killer whale populations, focusing predominantly on nearshore waters in the Salish Sea for the Southern Residents and the Gulf of Alaska for the Alaskan Residents (Figures 1a & 1b). Feces were identified as semi-cohesive brownish to greenish material floating in the water column or at the surface and were collected using a long-handled (4 m) fine-mesh net (pool net). Fecal samples were initially stored in clear polyethylene plastic bags or 50mL tubes on ice packs. They were later transferred to long-term storage at -20°C or -80°C before analyses. Samples were collected under the authority of the United States National Marine Fisheries Service (NMFS), Marine Mammal Protection Act research permit # 781-1824, 16163, 21348 issued to the NW Fisheries Science Center.

2. Quality Control

Fecal samples were collected and archived prior to the conception of the current microplastics study. The ubiquity of microplastics in the environment was unknown at the time of collection and explicit controls and efforts to mitigate plastic contamination of samples during field collection were lacking. There may have been potential exogenous contamination of fecal samples during collection, from pieces of the pool nets used for collection and synthetic clothing worn. A potential secondary source of environmental contamination of fecal samples may have occurred during collection. When the fecal samples were collected, water contamination may have occurred from microplastics potentially "hitchhiking" in the fecal samples when they were collected from the surface water. Water samples were collected at the same time as fecal samples in 2019 to attempt to understand this potential contamination. One liter of water was collected with each sample and is representative of the surrounding surface water exposed to the fecal sample before collection.

Laboratory Methods

1. Fecal Selection

Fecal samples were selected from the collection of killer whale fecal samples archived at the Northwest Fisheries Science Center (NOAA Fisheries, Seattle, WA) based on sample volume (>5 ml), with the intention of choosing larger samples to process. All samples were genotyped using previously validated methods (M. J. Ford et al., 2018; Michael J. Ford et al., 2011) to genetically assign an individual whale identification (ID) to each fecal sample based on a reference set of vouchered killer whale nuclear genotypes. For Alaska killer whale fecal samples, individual whale IDs were unknown, but multiple samples originating from the same killer whale could be identified based on genotypic matches. Particularly large (>15ml) fecal samples were processed in two different sets to evaluate intra-sample variability in microparticle composition.

2. Isolation of Microplastics

Methods were adapted from a previously published protocol for isolating microplastics (Masura et al., 2015). One-liter glass flasks were rinsed with filtered deionized (DI) water, dried in a dedicated microplastics incubating oven at 60°C and weighed. Fecal samples were removed from the freezer, thawed, and ~3-4ml were transferred to each sterile, pre-weighed flask. Sample wet weight and visual observations were recorded. Flasks were covered with clean foil and incubated in a drying oven at 60°C until feces visually no longer appeared wet, ~24-48 hours.

After drying, flasks were weighed to obtain the dry weight of fecal material. This was done to normalize samples by dry weight, due to the varying sizes of the fecal samples. Filtered hydrogen peroxide (H_2O_2) solution was added (200 ml for samples and 100 ml for control) to

each flask for digestion following a wet peroxide oxidation protocol using 30% H_2O_2 and heat. Flasks with foil were placed in the oscillation incubator at 60℃ and 80 rpm for 24-48 hours. At 24 hours, flasks were visually inspected, and if mostly digested, the heat was turned off and was left oscillating for 24-36 hours. After fecal samples were fully digested, 800 ml of filtered 0.25 g/ml sodium chloride (NaCl) solution was added, each flask was covered with parafilm, and then inverted three times to mix. The flasks were left to rest in the hood overnight to settle and allow lower density material to rise and higher density material to sink. Once flasks had fully settled, each solution was filtered through a 1μm pore size nitrocellulose-filter-membrane (Whatman, GE Healthcare) in the safety cabinet. If the filter membrane clogged, the remaining solution was filtered through a 5 μm pore size nitrocellulose-filter-membrane (Whatman, GE Healthcare). Filters were placed in sterile, labeled Petrislides (MilliporeSigma™ PetriSlide™), and left to dry in the safety cabinet for 1-2 days.

I processed 11 sets in total. Sets included 4-8 samples processed together, and each set included a procedural control. After set 5, the NaCl density separation step was eliminated to facilitate filtering of the entire digested sample and reduce salt deposition on the dried filters. The NaCl step's purpose was to create a density separation to float the microplastics to the surface and, therefore, not have to filter the entire solution. However, I wanted to filter everything to ensure I was not missing any particles. So, from set 6, after the fecal samples were digested in H_2O_2, the solution was fully filtered. This did not seem to cause any change, which was expected since the microparticles should have been floating at the surface of the density separation. I then rinsed the filter cup and flask with 100-200ml of filtered DI to rinse any leftover H_2O_2. After DI, I filtered 50ml of filtered 95% ethanol to aid in drying and inhibit mold growth on the filters.

3. Visual observation and validation of microplastics

Dried filters were visually inspected with a Nikon SMZ745 stereomicroscope and photographed with a Nikon 5300 camera attached to the scope. All microparticles were cataloged by type (fragment, fiber, film, other) and color. To distinguish microparticles by type and not organic material, I followed guidance from Barrows et al., 2017. To identify microfibers, I looked for fibers that were equally thick throughout their entire length with no cellular or organic structure. For particles, I looked for clear and homogenous color throughout and rough edges. Colors were determined to the best of my ability through the microscope and were recorded as multi if exhibited multiple colors. For analysis purposes, however, I grouped colors into categories (dark, light, multi-colored, reds) and kept white, black, and orange separate. DigiCamControl software was used to control stereoscope images, and ImageJ software was used to add scale bars. Images taken from the camera were saved in Raw format to ensure picture quality. Image format was later converted to .tif as required by ImageJ. Microparticles were measured and scale bars generated for each image in ImageJ.

When the sample was split on multiple filters, the 1-micron filter was inspected like the others. A subsample of 5-micron filters were visually inspected to ensure particles were not missed in the subsequent filters. Controls from each set were also visually inspected in the same way as the sample filters.

A sub-sample (10%) of each type of microparticles (fibers and fragments) from 14 fecal samples and three procedural controls (17 filters) were further analyzed using Raman microspectroscopy (RMS) to validate the microparticles as microplastics, determine the polymer type and determine the size of the microparticle. RMS was chosen to validate my microparticles

because it is a non-destructive approach, commonly used for microplastic identification with high accuracy in polymer recognition (Martinelli et al., 2020). RMS can also identify smaller particle sizes than other polymer identification techniques and can identify thicker or stronger absorbing microparticles because the method does not depend on light transmission through the particle material to identify the polymer type (Martinelli et al., 2020).

Spectroscopy is used to measure the spectra produced when matter interacts with, or emits, electromagnetic radiation (Hurst et al., 2018). RMS works by quantifying the interaction of light with the chemical bonds within a material (Horiba). When light scatters with frequency changes this is called Raman scattering (Kawata et al., 2019). It is possible to analyze the composition of materials by analyzing the spectrum of Raman scattered light and this process is known as Raman spectroscopy. To analyze smaller microscopic particles, like microplastics, Raman spectroscopy is combined with a microscope and is known as RMS (Kawata et al., 2019). Raman scattered light contains information about the molecules in the substance analyzed and using the wavelength of the emitted scattered light you can interpret them. The difference between the wavenumbers and the intensity of the spectra of scattered light is called a Raman spectrum and is how you identify the polymer type of the microplastics (Kawata et al., 2019).

In the Material Science and Engineering Department at the University of Washington, I used a Renishaw inVia Raman microscope equipped with a Leica DMIRBE inverted optical microscope with 514 nm and 785 nm excitation lasers to perform spectral analysis of isolated microparticles. To visually search for microparticles, I applied a range of objectives (10x and 50x) to aid in point to point mapping. Each particle found was also photographed and measured. The laser power and acquisition times varied depending on the sensitivity of the sample to thermal damage to minimize laser-induced damage to the microparticles. A spectral reference

library purchased through Renishaw Inc., of major consumer plastic polymers was used to identify polymer types for each microplastic particle. Spectrogram matching was conducted using an automated software and spectral reference libraries, or hand-matched when needed.

4. Quality Control

To limit the risk of sample contamination with microparticles during laboratory procedures, a 100% cotton lab coat and non-synthetic clothing were worn at all times during sample handling and microplastic isolation. All lab work was conducted inside a Class II, Type A2 biosafety cabinet with air filtration to attempt to reduce any air contamination. Prior to each use, all working surfaces within the biosafety cabinet were wiped with 95% ethanol, and all plastic items were removed, whenever possible. All liquids (DI, 30% hydrogen peroxide (H_2O_2), NaCl, and 95% ethanol) used during microparticle processing, and sample treatments were filtered through a 0.45 μm pore size nitrocellulose-filter-membrane (Whatman, GE Healthcare) using a vacuum filtration in the hood before use. This size filter was chosen because it was a smaller pore size than what was used for filtering fecal samples, and therefore, there would be no introduced contamination from the liquids.

All glassware was thoroughly rinsed with filtered DI water before use, and samples were immediately covered with foil when not in use. Negative procedural controls were run with every set of samples to account for possible procedural contamination. Procedural controls were processed concurrently with fecal samples and subject to all sample processing steps from heating through filtration. After visual inspection of the filter and classification, all microparticles found in procedural controls were subtracted one for one from corresponding samples in the set. Microparticles were removed from samples when both type and color-

matched corresponding particles in control (Moore et al., 2020). I conducted additional air controls to detect any other possible sources of contamination. One air control was conducted in the safety cabinet. I placed a wet filter exposed to the air in the cabinet to determine if there was any possible air contamination while I was isolating the microparticles. The other air controls were laid out while conducting RAMAN micro-spectroscopy to ensure no contamination occurred during spectral analysis.

Statistical Analysis

R software (R Core Team, 2013) was used for all data analyses. A t-test was performed to test for a difference in total microparticles between the two populations, and potential differences between the types of microparticles between the two populations. T-tests and ANOVA were run to determine the statistical significance of the controls. A power analysis was conducted to determine the sample size needed to detect statistical significance, given the observed data.

I determined the human population density of the fecal sample locations using 2010 US census data (*U.S. Census Bureau QuickFacts*, 2010). For Prince William Sound and Kenai Fjords, I used the population density of the Borough, and for the Oregon coast and San Juan Islands, I used the population density at the County level. For the Puget Sound region, I used the population density of eight counties that surround the Puget Sound (Island, Snohomish, King, Pierce, Thurston, Mason, Kitsap, and Jefferson), and averaged these to get the average human population density of the region.

3. Results

Controls

1. Procedural and Laboratory

All controls, procedural and air, contained a varying number of microparticles. All procedural controls contained microfragments; however, procedural controls of two of the eleven sets did not contain any microfiber contamination. The number of microparticles in the controls ranged from 16 to 195 per control, with an average and standard deviation of 51.55 ± 49.34. All controls contained relatively similar levels of contamination, except one outlier (Figure 2). Total number of microparticles in the control in Set 6 was atypically high. However, the samples in set 6 were low, suggesting that the level of contamination found in the procedural control did not affect the samples. The air control placed in the safety cabinet had similar contamination to the procedural controls (Figure 2).

2. Environmental controls

Paired fecal samples and seawater samples were examined to look for evidence of contamination of fecal samples resulting from microparticles present in seawater at the time of collection. In all paired fecal/seawater samples, the water contained a larger number of microparticles than the feces (Figure 3), however, none of these differences were statistically significant (p-value > 0.05). These sample pairs give us the ability to look at the presence of microparticles in one-liter volumes of surface water that potentially could be "hitchhiking" onto the fecal samples when they are collected from the surface water.

Suspected Microplastics

I identified a total of 2,723 microparticles (suspected microplastics) in 33 eastern North Pacific killer whale fecal samples. I found a total of 1,356 microparticles in Alaskan Resident

samples (AK; N = 15), and 1,368 microparticles in Southern Resident samples (SR; N = 18; Figures 4a and 4b). All fecal samples except one SR sample contained microparticles, although there was a large variability of microparticles per sample (mean ± SD, 82.5 ± 173).

To compare the two populations' microparticle burden and control for variance due to fecal volume, I used the total number of microparticles/gram of dry fecal material. In the AK population, I identified an average of 173 (±333) microparticles/gram of feces, and in the SR populations, I identified an average of 165 (±392) microparticles/gram of feces (Figure 5). There was no significant difference observed between the two killer whale populations (t = 0.256, df = 27.568, p-value = 0.7999).

Microparticle types included both fibers and fragments (Figure 6). Microparticle types found in each population were compared per gram of dry fecal material. Fecal samples collected from Southern Residents had more microfragments/gram of feces than the Alaskan Residents (SR = 141 ± 394, AK = 78 ± 143). In contrast, Alaskan Resident fecal samples had more microfibers/gram of feces than the Southern Residents (SR = 19 ± 28, AK = 91 ± 190). However, neither of these differences were statistically significant (microfragments: t = -0.62774, df = 22.151, p-value = 0.5366, microfibers: t = 1.4531, df = 14.492, p-value = 0.1675).

I identified sixteen different colors of microparticles and grouped them into seven color categories. The most common colors were white (63%) and black (17%), which comprised 80% of the isolated microparticles. The remaining colors were light (8%), orange (5%), dark (3%), reds (2%), and multi-colored (1%) (Figure 7).

I found no pre-consumer microparticles (e.g., nurdles, or small plastic pellets used in the manufacturing of plastic (Ellison, 2007)) in any of our fecal samples. All isolated microparticles appeared to be post-consumer in origin. Post-consumer microplastics are made, for example,

from the degradation of larger plastics, fibers from clothing, brake or tire dust. All microparticles found in the killer whale fecal samples, therefore, likely originated from human-created pollution.

Two fecal samples were subsampled and processed in duplicate to investigate how homogeneous the distribution of microparticles is within a single fecal sample. One of the samples processed in duplicate contained relatively similar (11 and 23 microparticles) microparticle totals, while the other had a significant difference in the number of microparticles observed (6 and 322) (Figure 8). Despite the small sample size, these data suggest that the distribution of microparticles throughout fecal samples is not homogenous, or homogeneity may vary depending on the consistency of the sample.

Per sample microparticles, microfragments, and microfibers were compared to the estimated total human population density from the census in 2010 at the fecal sample collection location (Figure 9). All data values used were corrected for fecal size variability (dry fecal weight). For the human population density, locations were grouped into general areas (Kenai Fjords, Prince William Sound (PWS), San Juan Islands (SJI), Puget Sound, and Oregon Coast) to determine if there were any patterns between human population density and the number of microparticles in the fecal samples. The sample with the largest number of microparticles was found in the Puget Sound area, which also has the largest human population density, followed by the Kenai Fjords and Prince William Sound, which had the two lowest human population densities. The Kenai Fjords and Prince William Sound also had the sample with the highest number of microfibers, while Puget Sound had samples with the lowest microfiber totals. Microfragments followed the same pattern as the total microparticles.

I conducted a power analysis to estimate the sample size needed to detect a significant difference (p-value < 0.05) in microparticle burden between the AK and WA/OR fecal samples given the observed variability and 95% power. In my study, to observe a significant difference between the populations microparticle burdens, I would need a sample size for each population of 56,729 (Figure 10). Therefore, these populations' microparticle burden is essentially the same within reasonable bounds.

Verified Microplastics

A subset of 89 microparticles were examined and analyzed using Raman microspectroscopy. Of these particles 34 (38%) were verified synthetic microplastics, 40 (45%) of the microparticles fluoresced, and the remaining 15 microparticles were determined to be filter paper fragments (Figures 11 & 12, Table 1). The 40 microparticles that fluoresced had high fluorescence interference and a weak Raman signal, which hindered our ability to identify these particles. Of the verified microplastics, the identified synthetic polymers include polyethylene (n = 12), nylon (n = 12), and polyamide (n = 10). Synthetic microplastics were found in both Resident Killer whale populations. Due to the small proportion of samples for which spectral analysis was completed, I was unable to compare the prevalence of different polymers between the populations. Measured microplastics ranged in size from 5 - 300 μm with an average size and standard deviation of 91.875 ± 72.986 (Figure 13); therefore, our limit of detection was 5 μm.

4. Discussion

Interpretation and Implication of Findings

Using an archived collection of fecal samples collected from two killer whale populations in the eastern North Pacific, I documented the presence of microplastics in top predators. This study is the first to identify the presence of microplastics in killer whale feces and to compare microparticles in two populations of killer whales. Microparticles were discovered in every fecal sample examined except one, serving as evidence of the pervasiveness of microparticles in the marine environment.

No significant difference was detected in the total number of microparticles isolated from fecal samples from the two populations. This finding was surprising due to the differences in geographic location. The Alaskan Resident's range is bordered by smaller human communities, and the Gulf of Alaska is a very large area of open water. In contrast, the Southern Resident population's location is greatly influenced by the dense human population, found throughout their range. The Salish Sea, commonly occupied by the Southern Residents during the summer/fall sample collection months, is much more protected than the Gulf of Alaska and is disproportionately affected by chemical run-off and contaminants associated with industrial and human activities (Fisheries, 2019). The prevalence of urbanized rivers affected by upstream cities removed from the coast likely contributes to potential microplastic input throughout the range of the Southern Resident killer whale population (Ecology and King County, 2011).

The similarity in microparticle burden between fecal samples from these two populations differs from that of a recent study looking at the relationship between microplastic contamination and coastal area use (Jang et al., 2020). Jang et al (2020) found higher abundance of microplastics in beach sediments collected in urban sites compared to rural sites. However, they also concluded that marine microplastics are generated from both land- and marine-based sources (Jang et al., 2020), so this may explain some of the similarities seen in the microparticle

burden between killer whale populations. A possible marine-based source of the population's fecal sample similarities is that a large proportion of plastic marine debris in the North Pacific originates from Asia (Pan et al., 2019). Through currents and large scale ocean circulation this debris can be distributed throughout the Pacific (Lusher et al., 2015; Moore et al., 2020).

Salmon (a prey species common to both resident killer whale populations) mature in the mid-Pacific and do a large proportion of their feeding during this time (Aydin et al., 2005). Thus, salmon may be ingesting microplastics that originated from Asia. This could potentially mean that microplastics found in the killer whale fecal samples could have originated from Asia and not correlated with the ranges of the populations. Another marine-based source of plastic debris could come from the fishing industry. Both killer whale populations occupy areas that also support commercial fisheries, and Alaska produces 60% of the nation's commercial fisheries (https://www.akrdc.org/fisheries). With a larger proportion of fisheries in Alaska, this may be a basis for the similarity of microparticle burden between populations fecal samples.

Like previously mentioned, the two killer whale populations studied here share common prey species (Fisheries, 2019), which may also explain the similarities in the microparticle burden. The fecal samples were collected in inshore waters, however killer whale prey spends a large proportion of their life out in the Pacific Ocean. So even though these two populations of killer whales may forage on prey from different stocks, it is likely that these stocks overlap in their distribution when out in the mid-Pacific. Microplastics move through ocean currents (Lusher et al., 2015), and a recent study found that the Arctic may be a sink for microplastic pollution due to long-range transport through sea ice and thermohaline circulation (Moore et al., 2020). Plastic pollution, like many other pollutants, has no boundaries. The similarity in the microparticle burden between the two killer whale populations and the presence of

microparticles in every Alaskan fecal sample demonstrates the growing global nature of this pollutant and its transport to more remote regions.

Finding only post-consumer microparticles in the killer whale feces suggests the pervasive amount of anthropogenic litter degrading in the marine environment. In previous microplastic studies on marine mammals, fibers were found to be widespread in the marine environment (Donohue et al., 2019) and the majority of microplastic found (Nelms et al., 2019). This type of microplastic comes mostly from synthetic clothing and often enters the marine environment through washing machine effluent. A study conducted in 2016 found that fibers indeed were released from clothing during both washing and drying, and that fibers were emitted throughout the lifetime of a garment (Pirc et al., 2016). These fibers wash into sewer pipes, pass through treatment plant filters, and empty into the ocean (Sutton et al., 2019). However, in our data as a whole, I found a larger number of fragments than fibers, which is consistent with studies on pinniped species that also feed on fish (Donohue et al., 2019).

When comparing microparticle types between the populations' fecal samples, the Southern Residents had more microfragments than the Alaskan Resident killer whales, and the Alaskan Residents had more microfibers and the Southern Residents. The larger number of fragments found in the Southern Resident population may be due to a more urbanized habitat. Fragments come from the breakdown of larger plastics, and a more urban habitat would have larger plastic input due to more humans occupying the region (Municipal Solid Waste, 2016). A study in San Francisco Bay found that 300 times more microplastics come from storm drains than wastewater, which is the largest source of fragments (Sutton et al., 2019). These drains collect plastic litter from roads, foam food packaging, rubber bits from car tires, and other sources (Sutton et al., 2019). This debris is then delivered to water sources and eventually makes

its way to the ocean (Pirc et al., 2016). The prevalence of microfibers in the Alaskan killer whale fecal samples may be due to a difference in the wastewater treatment facilities in cities around the Salish Sea compared to Southern Alaskan coastal communities (US EPA, 2015). According to the EPA, many small and rural communities struggle with aging and inadequate wastewater treatment facilities (US EPA, 2015). A study of shorelines worldwide found that wastewater treatment facilities receive large amounts of microfibers daily. While processing the water removes most microfibers, some (particularly the smallest microfibers and nanofibers) are released into the local environment (Browne et al., 2011). Inadequate processing may remove less of these fibers and maybe why the Alaskan fecal samples had a larger number of fibers in them.

I found microparticles in a wide range of colors, with black and white microparticles most prevalent. This large quantity of black and white fragments may be due to brake or tire dust and Styrofoam food packaging coming from storm drains (Sutton et al., 2019). Black microparticles were also preferentially captured by fish in an experimental study on microplastic ingestion (Ory et al., 2018). Preferential consumption of black microparticles was inferred to reflect similarities between the black particles and the fish's food type, and other colored microplastics were only co-ingested with black microparticles (Ory et al., 2018). Likely, killer whales are secondarily ingesting microplastics from their prey, and this prey might be ingesting microplastics similar in appearance to their food type. A large number of the white microparticles could have also come from clothing fibers. These may be natural, synthetic, or a combination, and through the isolation of microparticles from the feces potentially lost the color. A large number of orange fibers and other colored microparticles may be from fishing gear or ropes used for buoys.

Broad comparisons of the samples microparticles, microfragments, and microfibers, compared to human population density at general sampling locations suggested potential effects of human population density on microparticles in marine top predators. From the Southern Resident samples, the samples with the most microfragments and all microparticles of any location were both collected in the Puget Sound. Of the three Southern Resident sampling locations, it makes sense that samples with the highest microparticle burden were collected in the Puget Sound Region, as this area has the highest human population density. This region is also protected (Figure 1b), unlike the open ocean, possibly keeping the microplastics in the area rather than letting the currents distribute them elsewhere. However, samples collected from the Puget Sound had low microfiber totals compared to other locations. This may be due to better water treatment facilities in highly populated areas, like previously mentioned.

Similar microparticle burdens were found in the Alaskan Resident samples collected in the Kenai Fjords and Prince William Sound. Both Alaskan regions also had samples with the highest total microfibers than any other locations. The Kenai Fjords and Prince William Sound have the lowest population densities, so likely their plastic input is not from the surrounding land but rather from fishing activities in the area or currents. Considering the foraging habits of odontocete (toothed) cetaceans, such as killer whales, it is most likely that killer whales are acquiring microparticles indirectly from prey. Considering movements of both predator and prey, and killer whale gut transit time, the collection location of feces may not contribute to patterns of recovered synthetic microparticles on a fine geographic scale.

Due to unforeseen challenges, we were not able to fully verify 10% of each type of microparticle for each filter. Raman microspectroscopy was completed on 17 filters, verifying 89 microparticles. Of the 89, 34 were identified as synthetic microplastics based on spectral

analysis, comprising nylon, polyamide, and polyethylene. I found synthetic microplastics in both the Alaskan Resident and Southern Resident populations.

A study on the relationship between microplastic contamination and the prevalence of coastal area use identified polyethylene as one of the most common polymer types at both urban and rural sites (Jang et al., 2020). Polyethylene is one of the most common polymer types found in seawater, marine sediments, and organisms worldwide, is produced in large quantities, and is the most common type of plastic used worldwide (Jang et al., 2020). The low specific density of polyethylene allows it to float on the sea surface and travel through currents, facilitating its wide distribution to even the most remote areas (Jang et al., 2020). Microplastics in rural sites may also originate from local sources from the weathering and fragmentation of polyethylene-based fishing ropes (Jang et al., 2020). These studies findings, and the fact that polyethylene is the most common type of plastic worldwide, support discovering polyethylene particles in fecal samples from both populations of resident killer whales.

Microplastic contamination in coastal areas highlights the prevalence of low-density polymers (like polyethylene) compared to high-density polymers (like nylon) at all sites (Jang et al., 2020). In our study, nylon was one of the commonly identified polymers among microplastic particles isolated from killer whale feces. The third polymer type we identified was polyamide. A review of current microplastics studies found that polyamide was one of the most common microplastic polymer types in biota collected during field sampling (de Sá et al., 2018). All three polymer types identified in this study, were found in similar amounts in the killer whale fecal samples, however polyamide was the least represented among the three. Additional polymer identification from our fecal samples, will reveal more information about the potential synthetic microplastics found in the Alaskan Resident and Southern Resident populations fecal samples.

Raman microspectroscopy has both advantages and drawbacks for microplastic verification. The major advantage is its ability to analyze very small (<20 μm) microparticles compared to other methods like FTIR (Araujo et al., 2018). This is evidenced by identifying microplastics in our samples as small as 5 μm. However, a major drawback to Raman is its vulnerability to fluorescence interference (Araujo et al., 2018). Fluorescence occurs either due to intrinsic properties of the microplastic or due to impurities like coloring agents, biological material, and degradation products (Araujo et al., 2018), which can attach or adsorb to the microparticles and impact their spectra (Martinelli et al., 2020). Bacteria in biofilm and algal phaeopigments could also contribute to fluorescence (Martinelli et al., 2020). The fluorescence can, in the worst cases, completely overshadow the Raman signal (Araujo et al., 2018), which occurred in 45% of my particles. With 45% of my identified microparticles having fluorescence interference, I was unable to detect the Raman signal or identify the composition of these particles. There is the possibility that due to this, I may be underestimating the number of synthetic microparticles present in the samples.

The isolation and identification of microplastics in the killer whale feces provides strong evidence for the prevalence of microplastic particles in the gut of killer whales. Detection and quantification of the relative burden of microplastics in killer whale feces is a critical first step in understanding the potential for microplastics to contribute to physiological or health impacts on wild killer whales. Microplastics research has just scratched the surface of understanding the effects of microplastics on biota, and there is still much debate in the scientific community about the negative effects of microplastics. This study is the beginning of understanding if microplastics are causing harm to a marine top predator. I cannot yet conclude the potential impacts of the presence of microplastics on organisms, but studies have demonstrated the effects

of microplastic ingestion on fish health that includes intestinal blockage, physical damage, histopathological alterations in the intestines, change in behavior, change in lipid metabolism, and transfer to the liver (Jovanović, 2017). Another study on the effects of microplastic ingestion in fish found that the most consistent effect was an overall reduction in the consumption of their natural prey (Foley et al., 2018).

The role of microplastics in the transfer and accumulation of toxicants is currently unknown, but it is possible that these microparticles are playing a role in transferring chemicals from the environment to the organisms. Microplastic particles may act as a vector, carrying and possibly increasing the rate of transfer of POPs to organisms (Nelms et al., 2019). Both populations of killer whales in the current study are known to have relatively high levels of contaminants in their tissues (Buckman et al., 2011; Ross et al., 2000) and understanding whether microplastic particles play a role in the accumulation and transfer of POPs through the food web is currently unknown. The Southern Resident killer whale population is an endangered population confronted by many health and environmental challenges (Gaydos et al., 2004). The presence of microplastics may pose yet another problem for this population, which may compound effects of reduced prey availability and toxicants, especially individuals who already have a lowered immune system or preexisting problems. Comparing microplastics in fecal samples from two killer whale populations exhibiting contrasting population trends is an important first step in addressing whether microplastics are more harmful on already compromised individuals. Developing a better understanding of the toxic effects of microplastics on an organism are critical for a better understanding of the population-level effects.

Microplastics also have the potential to negatively impact the food web at all trophic levels. Zooplankton play a crucial role in marine ecosystems as a primary consumer of the

aquatic food chain (Chatterjee & Sharma, 2019). When exposed to microplastics, they were observed ingesting them, and experienced adverse effects from the microplastics penetrating along the cell wall, resulting in the reduction of chlorophyll absorption (Chatterjee & Sharma, 2019). The effects of microplastics were also studied on fish eggs, larvae, and adult fish. Eggs exposed to high concentrations had slower hatching rates, and larvae were small and slow. Their response to chemical alarms (e.g. olfactory cues to the presence of a predator) was also deficient, resulting in a decreased survival rate (Chatterjee & Sharma, 2019). Fish were also observed ingesting microplastics, presumably mistaking these fragments for prey. Exposure to microplastics caused modifications in the fish intestine and alterations in the typical structure of the fish (Chatterjee & Sharma, 2019). An experimental study conducted at UC Davis in 2013, found that bioaccumulative and toxic substances could sorb from the seawater and transfer from microplastics to fish upon ingestion. This suggests that exposure to microplastics in nature may be a significant route of contaminants in wildlife (Rochman et al., 2013). All of these are examples of potential stressors to the food web.

These microscopic particles may be relatively harmless in small or moderate concentrations, but there are still many unanswered questions about the potential for long-term harm from chronic exposure to microplastics. As marine plastic pollution increased in tandem with increased plastic production, I need to continue to monitor top predators like killer whales, as they can act as essential indicators for microplastic contamination in the marine environment. The presence of microplastics in charismatic megafauna, many of which are also culturally and ecologically important, stresses the need for global action now to better understand the sources, impacts to species, and fate of microplastic pollution.

Challenges and Limitations

The purpose of this pilot study was to determine if microparticles are present in killer whale feces and if there is any difference in total burden between two North Pacific killer whale populations. With a limited sample size, it is difficult to determine what is driving similarities in the population's total microparticles and variations in each fecal sample. This points to the fact that a larger sample size is needed to potentially identify other variables that might be driving the similarities and variations seen in the data.

Due to the nearly ubiquitous distribution of microparticles in the environment, procedural controls are a necessity when isolating microplastics from biological samples. I accounted for contamination in our procedures as previously mentioned; however, despite stringent laboratory practices aimed at limiting potential sources of contamination, microparticles were identified in every control. There was also air contamination in our safety cabinet that could have been from plastic materials in the safety cabinet that I was unable to remove due to safety precautions. Previous microplastics studies had limited contamination in their controls (Donohue et al., 2019; Nelms et al., 2019). Microplastic research greatly benefits from having a designated lab to avoid contamination, as evidenced by our study. Without a designated place for microplastic research, contamination could come from numerous sources like plastic lab equipment, and I cannot control what other people are using in the lab or wearing. During the time I was isolating microplastics from the killer whale feces, construction occurred in the lab. I completed no work during the time of construction, but this may have put more particles into the air than usually is present.

I collected one liter of water to represent the amount of surrounding seawater the fecal samples are exposed to between defecation and collection, to determine if there was any indirect

water contamination of our samples. However, in every pair of samples, there was a larger number of microparticles in the water sample, although this was not significantly different. With the large variability, it is challenging to determine if there was indirect water contamination in our samples. This is a secondary control but still shows the potential that there may be some contamination through this route. This may also tell us with higher microparticles in the water, that there is no biomagnification of microparticles. However, microparticles in the fecal samples are just a snapshot in time. This is not telling us the total microparticles in the gut or how long they were there.

Due to the larger volume of two fecal samples, I ran these samples twice. Both were from the Alaskan Resident population. This potentially would allow us to look at the homogeneity of microparticles distribution throughout the killer whale feces. However, one of our pairs shows homogenous fecal samples while the other heterogenous fecal samples. I would need to have more duplicated samples to be able to determine hetero- or homogeneity of microparticles in the feces. This also allows me to look at our within-sample variance. With only the two samples to compare, we have high within-sample variance and would need to do more within samples to determine the true variance here. I assume with more samples, we would see lower within variance than between sample variance. It may also be effective to attempt to homogenize the fecal samples before isolating the microparticles in the future. This could be done by potentially blending them, lightly enough not to degrade the microplastics but enough to homogenize the sample.

A power analysis was run to determine the sample size needed to detect a difference between the two populations. With our observed within-sample variance, limited between-sample variance and similar mean number of particles per gram of feces for both populations,

our power to detect population level differences in microplastic burden is extremely low (0.05). I estimated that I would need roughly 57,000 samples per population to detect a significant difference. Increasing the number of samples processed in duplicate will allow more accurate estimates of true sample variance. Through processing a larger number of samples, I may discover the true variance is lower than with our current limited sample size due to this being a pilot study. However, there may not be a true difference between the population's microplastic burden, and doing more samples in the future would increase the validity of the study. The power analysis illustrates the need for numerous samples to determine differences in the populations and again validates that plastic is everywhere, and it's unevenly distributed among individual killer whales within a population.

Future Research and Recommendations

This preliminary study to determine the presence of microplastics in top predators using killer whale feces was successful in modifying a previously published methodology to a novel substrate. The data clearly indicate the presence of microparticles in killer whale feces. Still, more research is needed to understand better the potential impacts of these microparticles on the health of individual whales and other top predators. To investigate the health effect of microplastics on killer whales, analysis of fecal samples for xenoestrogenic compounds in subsamples of the fecal samples analyzed for microplastics, is an important next step.

Xenoestrogens, like other EDCs, mimic endogenous hormones and interfere with the endocrine system (Singleton, 2003). Adverse effects of xenoestrogens include a range of potential developmental anomalies in wildlife (Singleton, 2003). The most widely studied mechanism in which xenoestrogens affect the endocrine system is through binding and activation

of estrogen receptors (Singleton, 2003). Some xenoestrogens such as PCBs, DDTs, and alkylphenols are incredibly persistent in the marine environment and accumulate in the food chain (Singleton, 2003). Some plastic additives are also xenoestrogenic compounds, including BPA, PBDEs, phthalates, and PVC ("Xenoestrogens," 2012). Xenoestrogens are an optimal choice to analyze because they should be detectable and quantifiable in the feces, where other EDCs require blood or urine samples (Diamanti-Kandarakis et al., 2009).

Another possible future extension of this research includes investigating the food web of Resident killer whales to determine the relative burden of microplastics on lower trophic organisms. This would provide an indication of modes of ingestion and bioaccumulation of microplastics in top predators like killer whales. I could perform this by isolating microplastics from the gut of salmon species primarily eaten by Resident killer whales. I could further investigate this by determining where the salmon were ingesting the microplastics either through primary ingestion or secondary ingestion. By assessing the number of microplastics in salmon, we could parameterize a model to estimate how many microplastics are directly ingested by killer whales and other top predators.

5. Figures

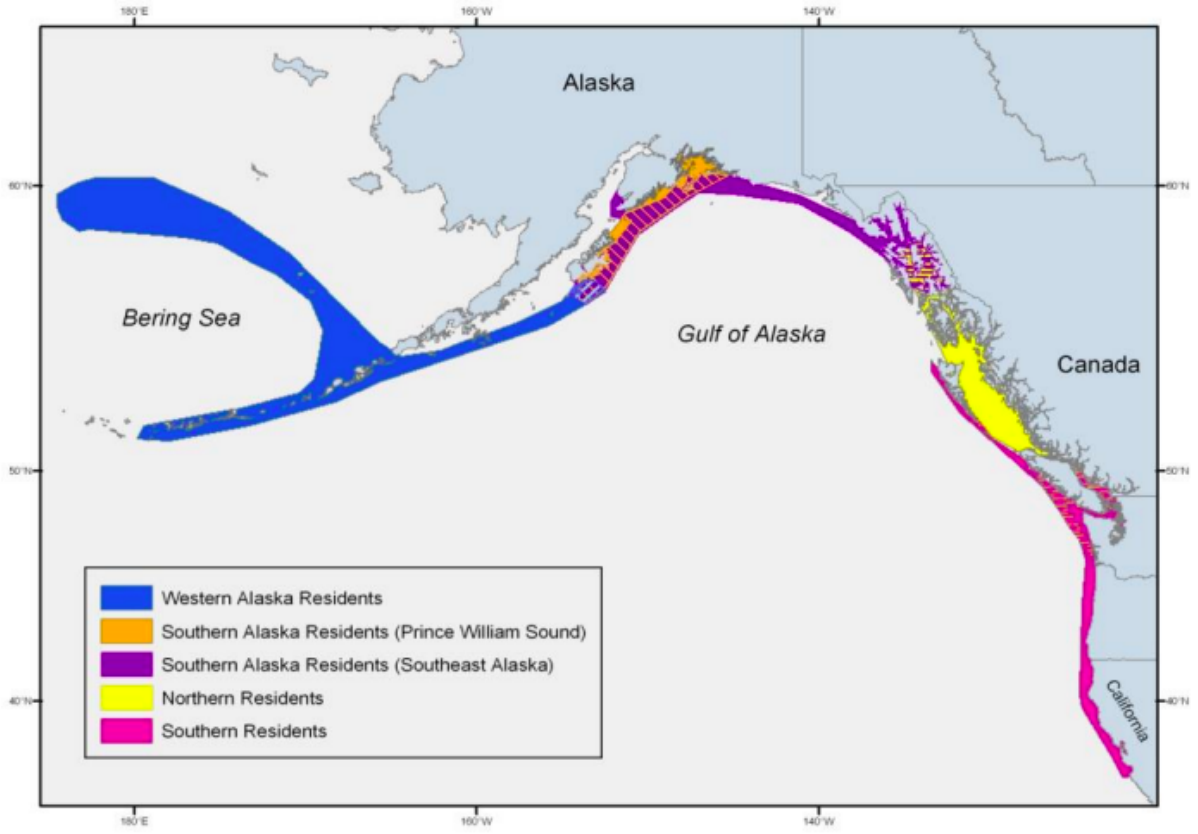

Figure 1a. Map of sampled killer whale populations ranges. The Southern Resident killer whales range is seen in pink, and the Alaskan Resident killer whales range is seen in orange and purple. Map sourced from NOAA Northwest Fisheries Science Center and Washington Department of Fish and Wildlife
https://static1.squarespace.com/static/5626fb1ee4b07be93cf930c1/t/5c3d23392b6a28b6ab9d37a5/1547510609353/Orcas+of+Oregon_MCWC+presentation.pdf

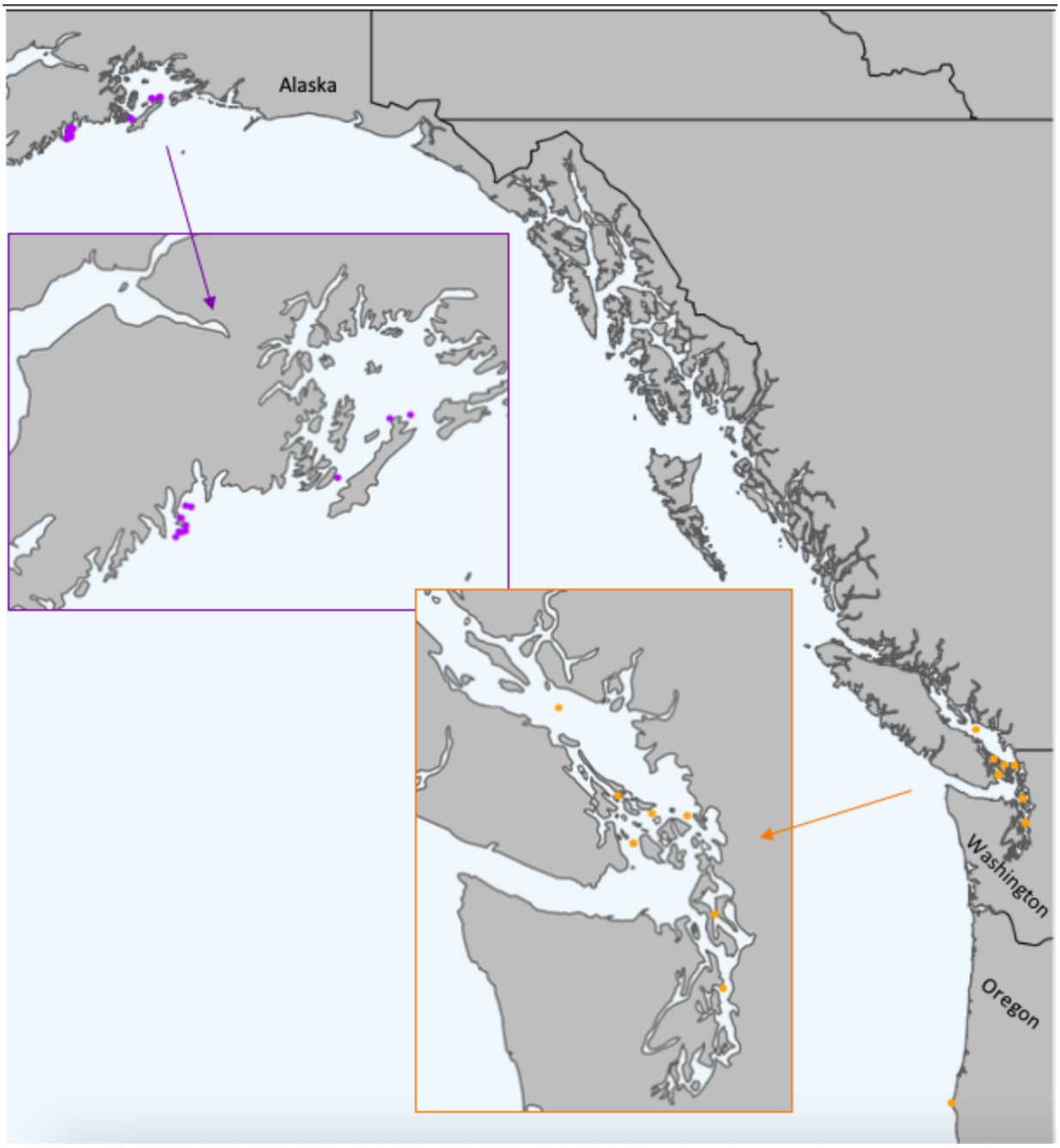

Figure 1b. Map of all sample collection locations. The Alaskan Resident fecal samples can be seen in purple and Southern Resident fecal samples in orange.

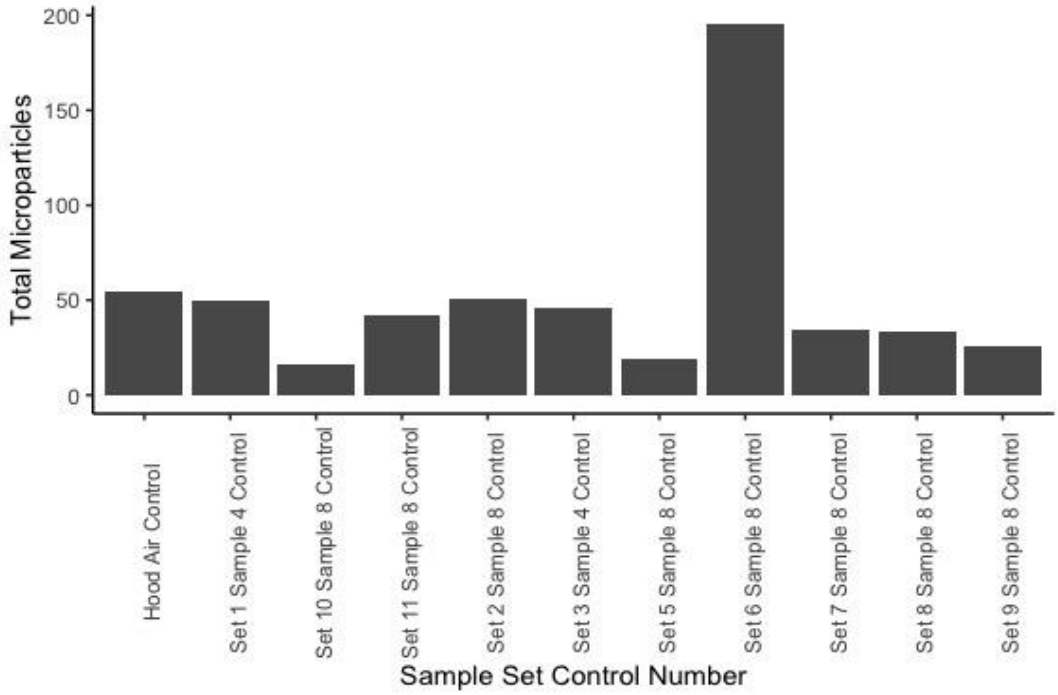

Figure 2. The total number of microparticles identified in each procedural control, (n =10 sample controls; n = 1 one air control).

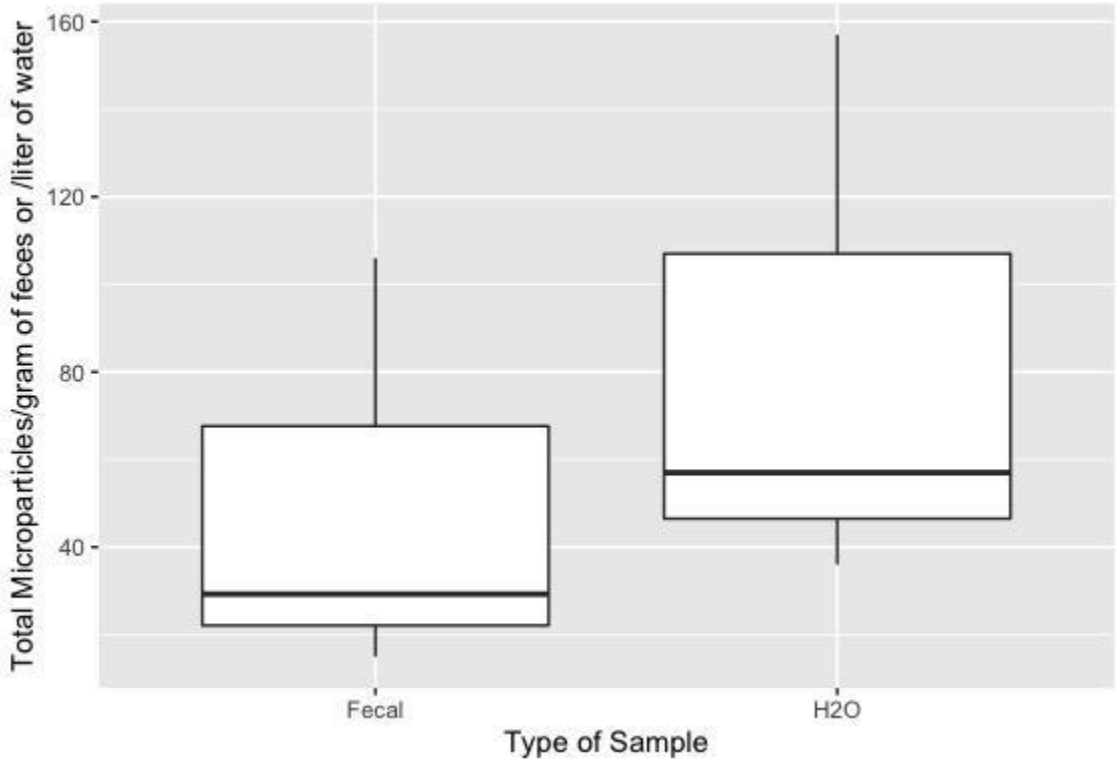

Figure 3. The total number of microparticles per gram of dry fecal material and total number of microparticles per liter of seawater isolated from concurrently collected fecal and water samples.

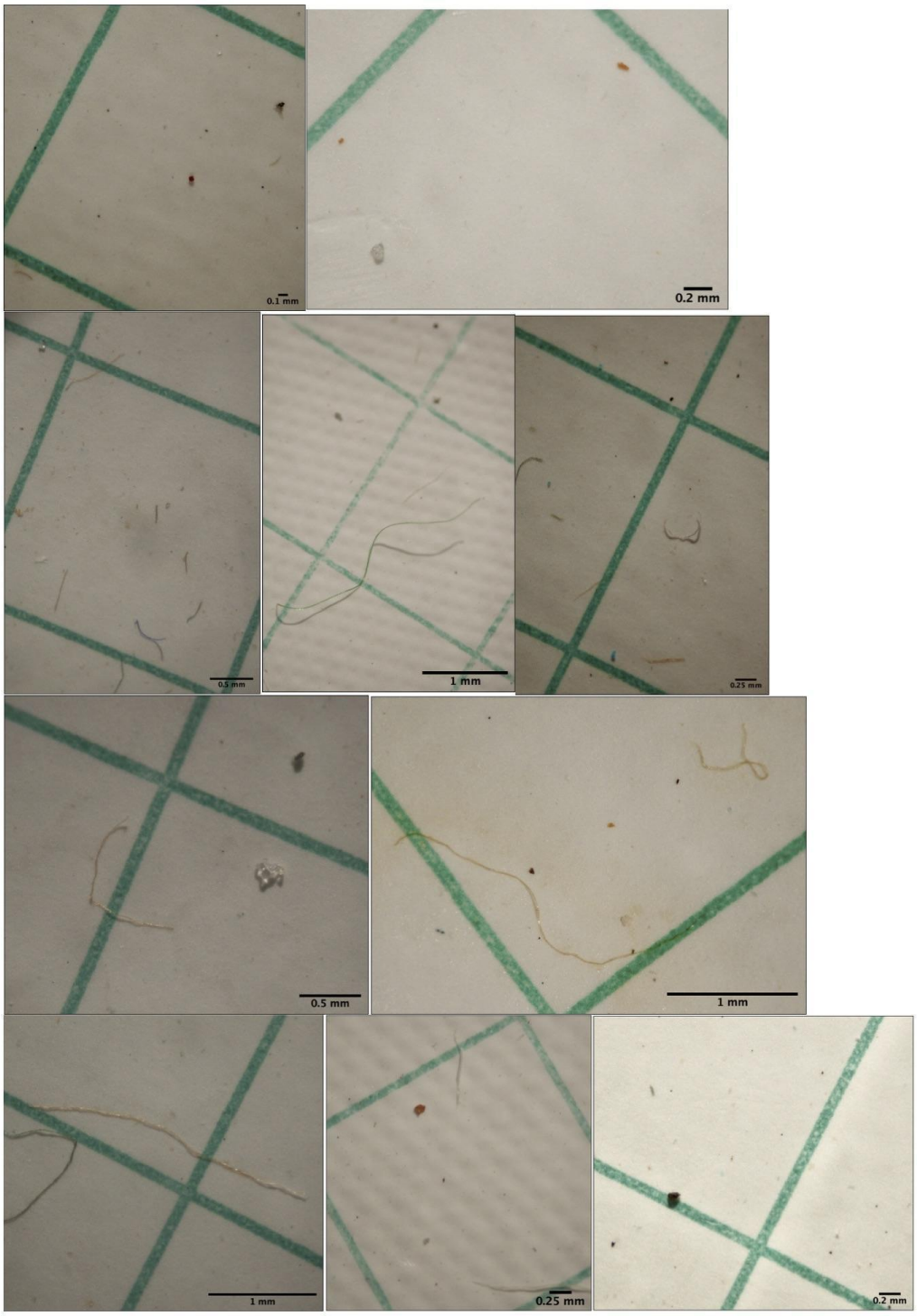

Figure 4a. Examples of imaged microparticles found in the fecal samples of Alaskan Resident killer whales. Images were taken with a Nikon SMZ745 stereomicroscope and photographed with a Nikon 5300 camera attached to the scope at 40X magnification.

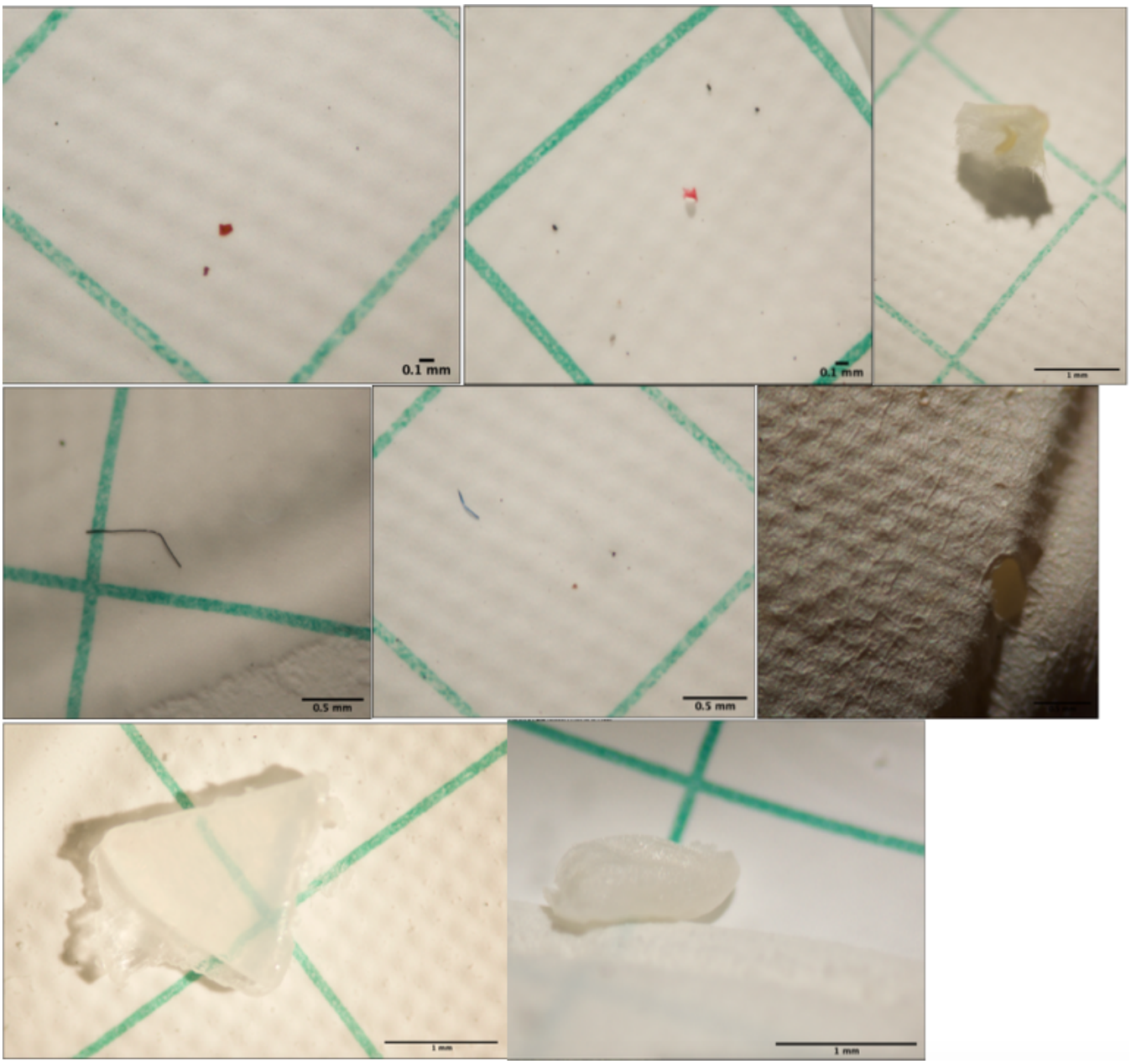

Figure 4b. Examples of imaged microparticles found in the fecal samples of Southern Resident killer whales. Images were taken with a Nikon SMZ745 stereomicroscope and photographed with a Nikon 5300 camera attached to the scope at 40X magnification.

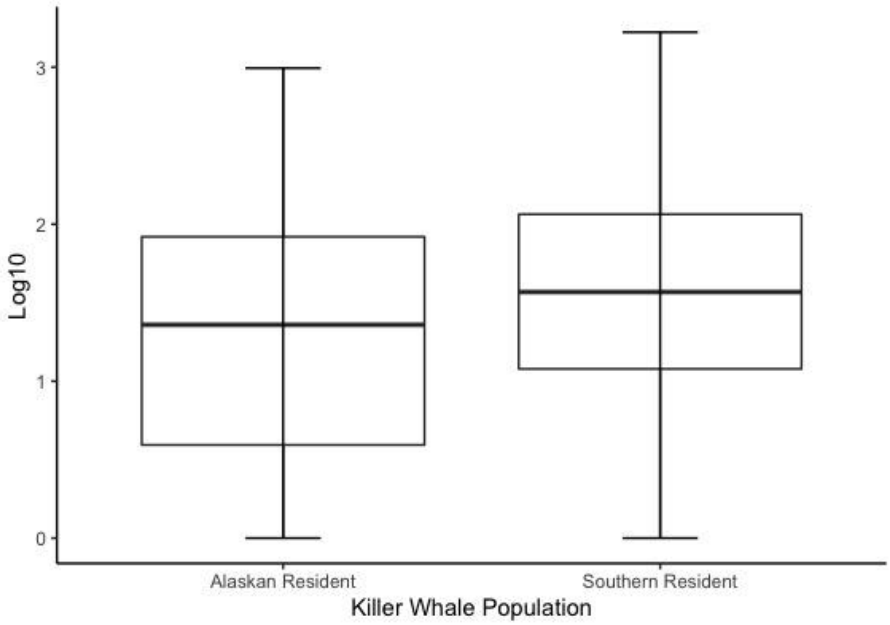

Figure 5. Total number of microparticles per gram of dry fecal matter, per sample in the Alaskan Resident and Southern Resident Killer Whale populations. Thick horizontal lines represent median values per population, boxes enclose the 25th–75th percentiles, and whiskers indicate the minimum and maximum values.

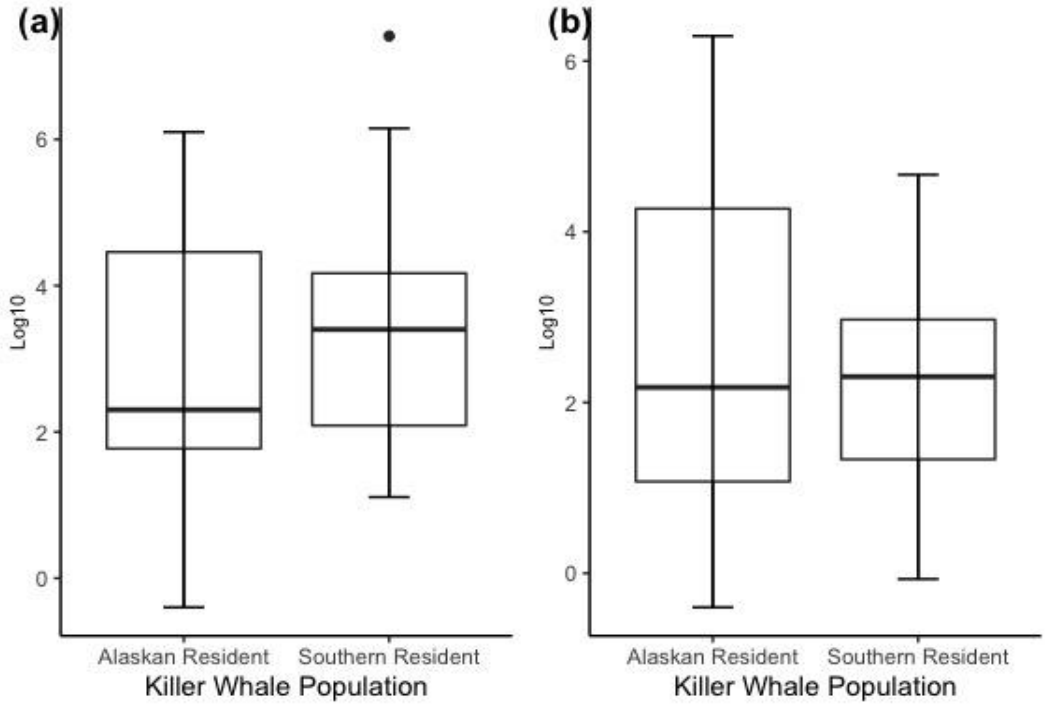

Figure 6. Total number of microparticles per gram of dry fecal matter compared across the two killer whale populations for both (a) microfragments and (b) microfibers. Thick horizontal lines represent median values per population, boxes enclose the 25th–75th percentiles and whiskers indicate the largest variable within 1.5 X above or below the interquartile range and the dots are the samples that fall outside of this and are outliers

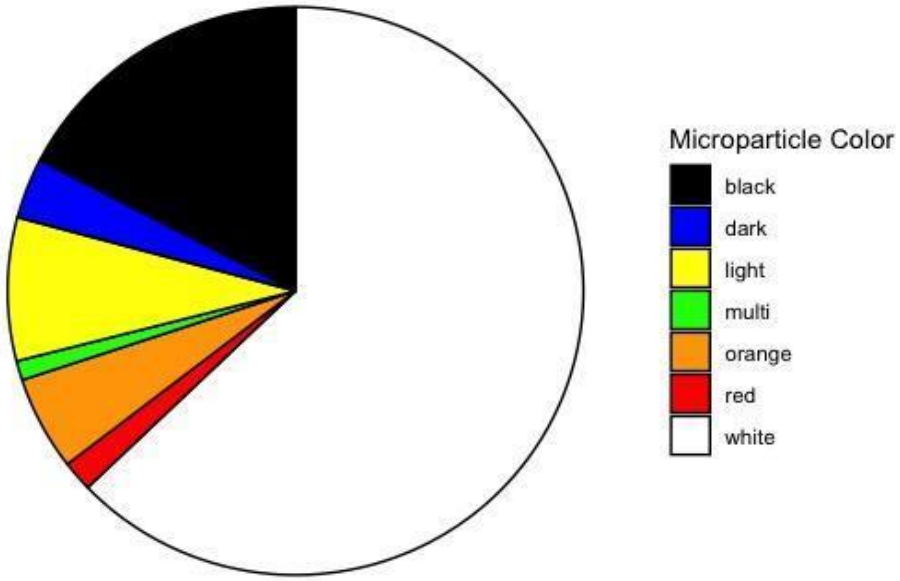

Figure 7. Proportion of different colors of microparticles identified under the stereomicroscope. From top to bottom: ‘black’ includes all black particles; ‘dark’ includes all particles that are blue or dark in coloration except black; ‘light’ includes particles that were transparent, yellow, and other non-white particles; ‘multi’ indicates all particles comprising multiple colors; ‘orange’ indicates all orange particles; ‘red’ includes particles observed as red, pink, purple, and brown; and ‘white’ indicates all the white particles.

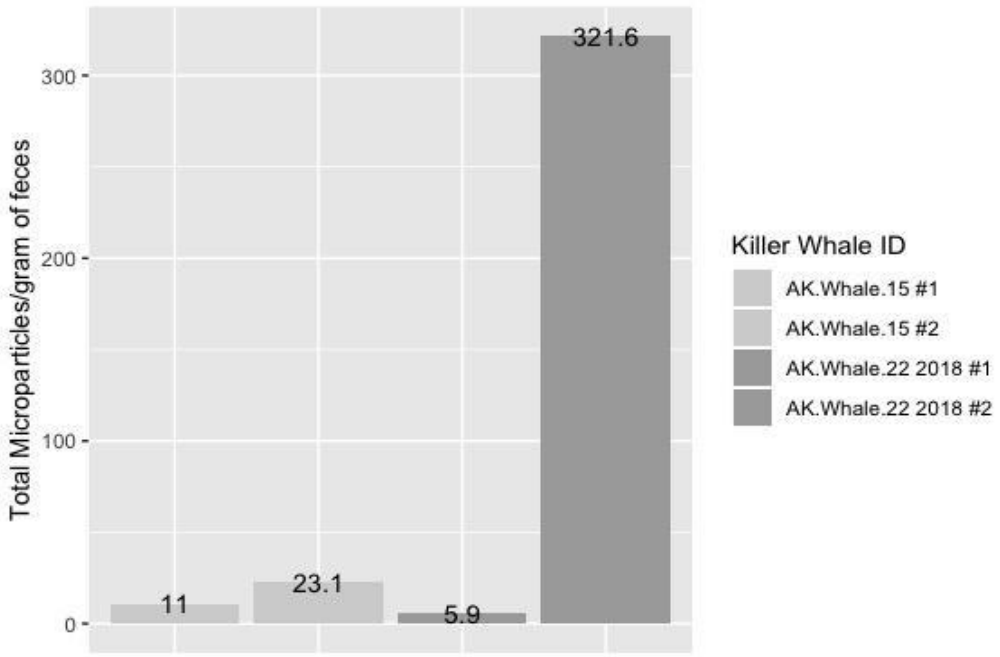

Figure 8. Total microparticles per gram of dry fecal matter from killer whale fecal samples that were processed in duplicate. Duplicate fecal subsamples indicated by color.

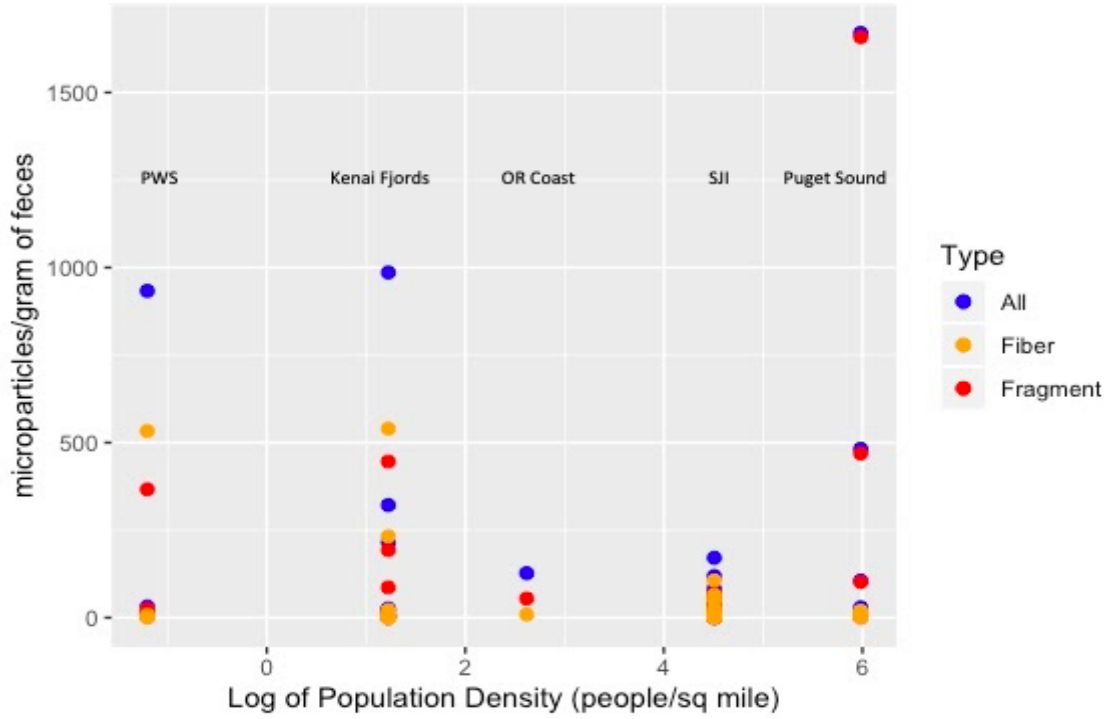

Figure 9. Total microparticles, microfragments, and microfibers per sample corrected for dry fecal weight compared to the human population density in sample collection regions.

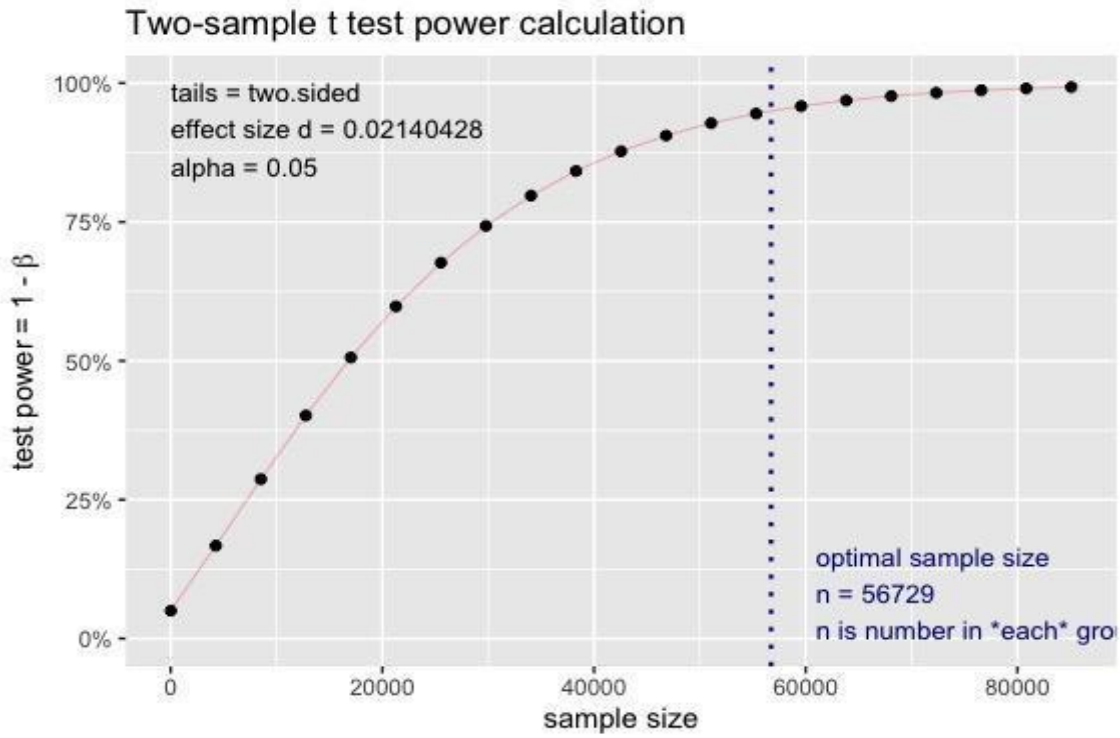

Figure 10. Power analysis using observed variance to determine the sample size needed to detect a significant inter-population difference in microplastic burden. The optimal sample size is when power is 0.95.

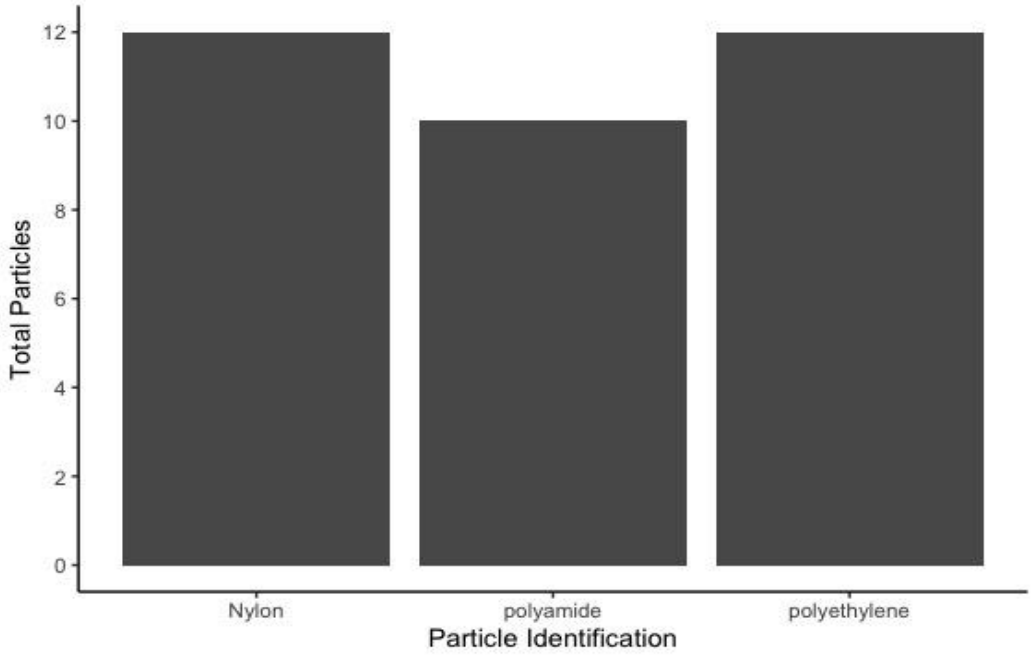

Figure 11. Verification and identification of microparticles polymer type from killer whales' fecal samples using Raman microspectroscopy. Microparticles identified as fluorescence may or

may not be synthetic, these were unable to be identified due to fluorescence interference.

Table 1. Number and type of polymer identified in microparticles isolated from killer whale fecal samples. The main sources of each polymer type are listed (Gent & Stevens, 2016; Martinelli et al., 2020; Vagholkar, 2016), and the locations where the fecal samples containing the microplastics identified were collected.

Polymer Type	**Possible Sources**	**No. particles found**	**Sample Locations**	**KW Population**
Polyethylene (PE)	Milk and juice jugs, plastic bags, six-pack rings, drinking straws (Martinelli et al., 2020)	12	Prince William Sound, Kenai Fjords, and Puget Sound	AK and SR
Nylon	fishing nets, ropes, parachutes and type cords, clothing, carpets, used as plastic for making machine parts, and military applications such as flak vests, and tires for vehicles (Vagholkar, 2016)	12	Kenai Fjord and San Juan Islands	AK and SR

Polyamide	textiles, automotive industry, carpets, kitchen utensils, and sportswear (Gent & Stevens, 2016)	10	Kenai Fjord and San Juan Islands	AK and SR

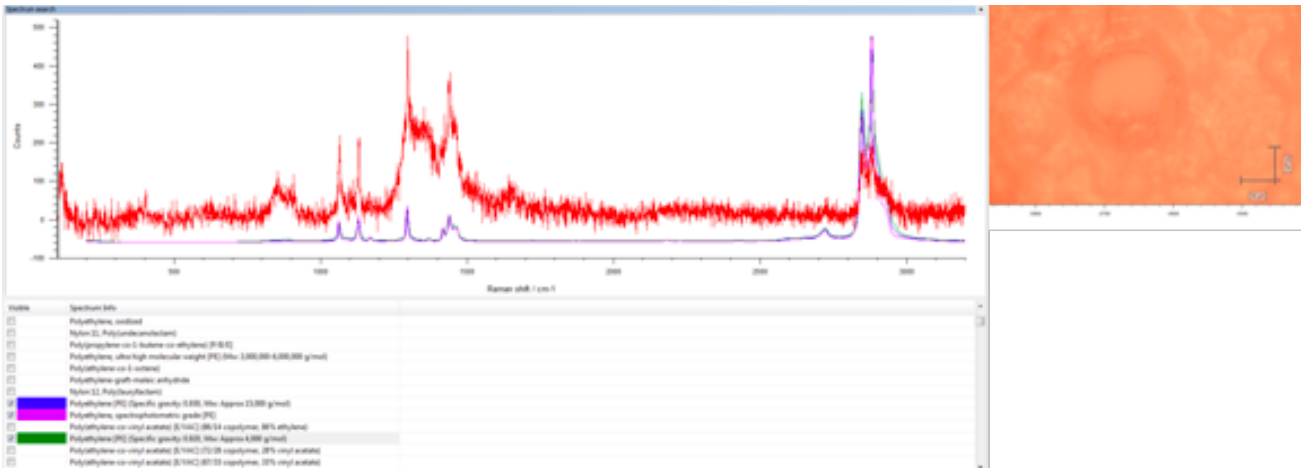

Figure 12. Example spectrum generated from a microplastic particle using Raman microspectroscopy. The red spectrum is the individual microplastic, and the blue spectrum is the reference library spectrum. This microfragment from the killer whale fecal samples was identified as polystyrene.

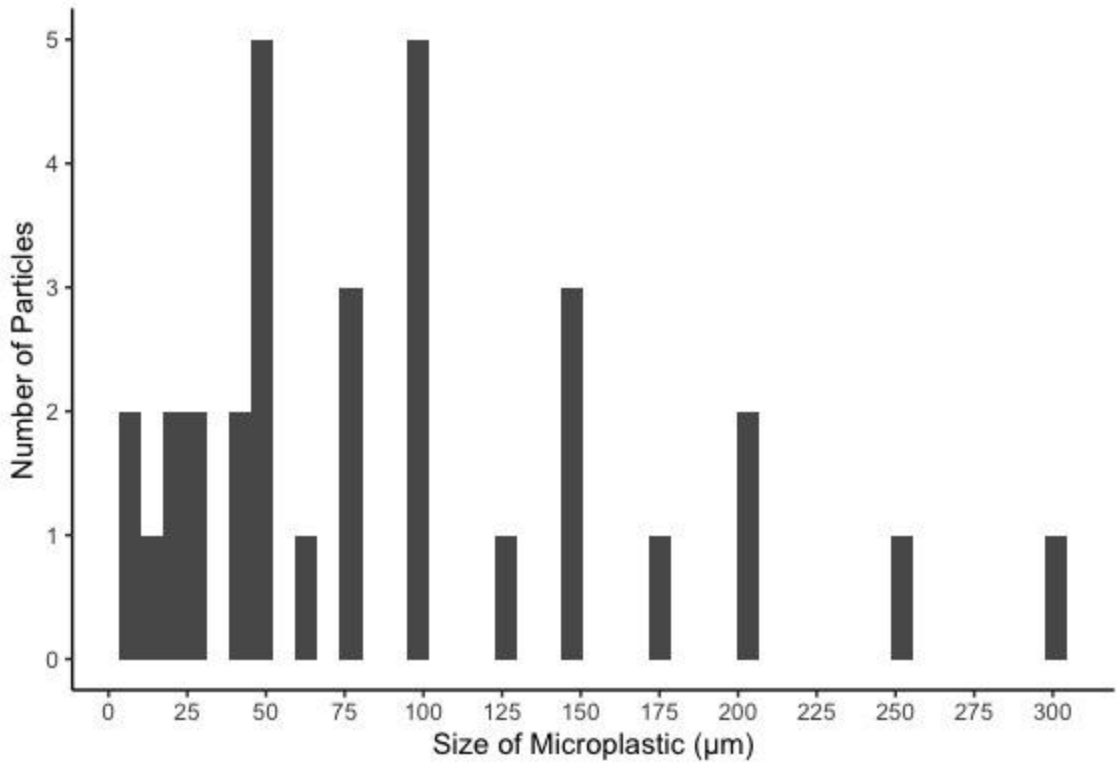

Figure 13. Distribution of the size (µm) of microplastic particles isolated from killer whale fecal samples and analyzed using Raman microspectroscopy.

6. References

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