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Presence and the potential hormonal disrupting capacity of microplastic in a coastal system

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PRESENCE AND THE POTENTIAL HORMONAL DISRUPTING CAPACITY OF MICROPLASTIC IN A COASTAL SYSTEM

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In

MICROBIOLOGY AND ENVIRONMENTAL TOXICOLOGY

By

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ABSTRACT: "Presence and the potential hormonal disrupting capacity of microplastic in a coastal system" Sami Michishita

Microplastic presence and its potential adverse impacts on wildlife are not yet well understood in coastal ecosystems. Using standardized methods such as an alkaline tissue digestion and Raman spectroscopy, we quantified the presence, category (e.g. fiber or fragment), and polymer identification of microplastics (particle size < 5 mm) in three representative sample types of the coastal system: seawater (n=12 17 h trials), northern anchovies (*Engraulis mordax*, n=24), and common murres (*Uria aalge*, COMU, n=19). We assessed the recovered microplastics from COMU for xenoestrogenic activity using an *in-vitro* estrogen receptor activation assay. Particles were recovered from all sample types: 100% prevalence in the 17 h seawater trials, 58% prevalence in anchovies, and 100% prevalence in COMU. Fibers were the most abundant particle (77%), followed by fragments (13%), foam (5.8%), film (2.0%), and bead (0.78%). Raman spectroscopy identified 11 out of 20 particles that were microplastic (synthetic, semi-synthetic, or blends) as polyester. In addition, particles recovered from digestive tracts of three common murres had potential xenoestrogenic activity. To our knowledge, this study is the first to analyze and provide baseline information on the presence and potential hormone-disrupting activity of microplastic in the Monterey Bay (California) coastal system.

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1. INTRODUCTION

Plastic pollution is a global issue that appears to be worsening as plastic production has exponentially increased, from an estimated two million metric tons (Mt) in 1950 to 380 million Mt in 2015[1,2]. With an annually projected eight million metric tons of macroplastic (size >5 mm) debris accumulating in landfills or natural environments such as marine, the finding that over 700 marine species reported with ingested plastic is not surprising[2–4]. Unless prevention measures are implemented, by 2050 the ocean is predicted to have more plastic items than fish and 99% of seabird species will be impacted by plastic ingestion or entanglement[5,6].

Although macroplastic presence in marine species has been widely documented, microplastic (size < 5 mm) presence is also a concern[1,3,4,7]. Microplastics have been reported in multiple types of environmental samples including deep pelagic water columns, arctic ice, and in Western United States atmospheric samples as both a means of transport and as a reservoir[8–10]. Due in part to the environmental permanency of plastic items, which can weather to smaller pieces, marine organisms are exposed to risk of direct ingestion[1–3,11,12].

Plastic can be composed of several compounds such as the structural plastic polymer (e.g. polyester, polystyrene) and additive chemicals (e.g. plasticizers, flame retardants) for malleability and stability. In addition, plastic found in marine systems can adsorb chemicals known as persistent organic pollutants (POPs) [e.g. polychlorinated biphenyls (PCBs) or polybrominated diphenyl ethers (PBDEs)][13,14]. Furthermore, the stomach environment can increase desorption of plastic-associated chemicals and increase risk of tissue absorption[15]. Indeed microplastic ingestion has been correlated with toxicological effects, such as genetic disruption and abnormal cell development[7,16–19].

Many plastic-associated chemicals are endocrine disrupting chemicals (EDCs) that impact several endocrine pathways such as pathways of thyroid, estrogen, androgen, and glucocorticoid hormones[13]. For example, EDCs impacting thyroid hormones in seabirds have impaired the regulation and growth of feathers, which can interfere with waterproofing and migration, both critical to survival[13]. Thus, EDCs have implications for negative effects to reproduction and survival[14,20–24]. Some EDCs are xenoestrogenic, exogenous chemicals that mimic estrogen by binding to nuclear estrogen receptors and disrupting physiological functions[14,25,26]. The estrogen pathway is comprehensive and helps regulate reproductive, metabolic, immune, and cell differentiation systems[25,26]. However, little is known about the hormonal disrupting capacity of ingested microplastic in seabird species[27].

Studies on plastic prevalence in environmental samples typically report visual characteristics such as color, industrial or anthropogenic (or user), particle types (hard plastic, film, fiber, etc.), and size in length, mass, and/or volume[28]. Recently, papers suggest using more quantitative methods for plastic validation[29]. Additional resources such as Fourier-transform infrared (FT-IR) and Raman spectroscopy can identify “molecular fingerprints” of microplastic particles, such as polyester and polystyrene, to improve the identification and understanding of the possible sources and toxicity of microplastics in environmental samples[30]. For example, Choy, *et al.* (2019) used Raman spectroscopy to uncover the source of sampled microplastics from the Monterey Bay to be weathered anthropogenic particles rather than virgin industrial particles.

Here we investigated the presence and potential hormonal disrupting capacity of microplastic in a coastal system. Presence referred to both prevalence (percentage of individuals) and abundance (quantity recovered from positive cases). Specifically, we quantified microplastic presence in three representative sample types of the Monterey Bay coastal system in California: seawater, northern anchovies (*Engraulis modrax*, anchovies), and common murres (*Uria aalge*, COMU). We analyzed the recovered particles with Raman

spectroscopy to validate and identify the polymer compositions. Lastly, we investigated the xenoestrogenic activity of the chemicals extracted from recovered particles with an *in-vitro* estrogen-receptor activation assay. We expected to recover microplastics in all sample types and find fibers as the most common particle type due to its known high presence in the marine environment[30,31]. We also expected to find recovered microplastic to have potentially xenoestrogenic activity. To our knowledge, our study was the first to investigate microplastic presence in Monterey Bay coastal system and to assess the xenoestrogenic activity of ingested microplastic.

2. MATERIALS AND METHODS

2.1 SAMPLE COLLECTION

Samples were collected in the Monterey Bay (Fig. 1), an ecologically and economically valuable region with several marine protected areas supporting a rich kelp forest and biodiversity of marine mammals, seabirds, shorebirds, fishes, and invertebrates. The Monterey Bay is part of the Pacific Flyway for bird migration and the larger Monterey Bay National Marine Sanctuary (MBNMS) located off the coastal central California, USA[32]. The Monterey Bay is also a well-mixed system due to strong upwelling and horizontal circulations of the California Current System[33].

2.1.1 Seawater

Particles were collected from seawater intake systems at the University of California, Santa Cruz Long Marine Laboratory (Santa Cruz, California, USA) and Moss Landing Marine Laboratories (Moss Landing, California, USA) by methods modified from Mason, *et al.* (2016). Access points of the intake systems were located after the main sand filtration, where Long

Marine Laboratory's vertical filter size was 0.7 cm and Moss Landing Marine Laboratories' mesh size was 0.02 cm. As such, quantification of particles in seawater for this study do not represent or approximate the abundance of particles in the Monterey Bay due to the operational restrictions of recoverable particles. At each trial (n=12), seawater gently flowed through two steel sieves, where a 500 µm-mesh sieve was stacked on top a 150 µm -mesh sieve for 17 h overnight. The time duration to fill a one-liter glass beaker was measured in triplicate before and after each 17 h trial and used to estimate the volume of seawater sampled. The average of the six readings (sec/L) was converted to flow rate (L/sec) to calculate the estimated total volume (L). Sieves were covered with a bucket to protect from weather and to reduce atmospheric contamination, as even sea spray can contain microplastic particles[8]. Sieves were retrieved in the morning and covered with aluminum foil until processing.

Particles in Santa Cruz seawater were sampled in August 2020 (4 trials), May 2021 (2 trials), and July 2021 (2 trials). Particles in Moss Landing seawater were sampled in July 2021 (4 trials). August 2020 samples in Santa Cruz were processed as a pilot trial with varying procedures, such as utilizing a 25 µm-pore size paper filter (VWR International LLC, See Appendix Table A1). Thus the pilot trials were not included in the quantification analysis (e.g. mean, particle/L), but were included in the categorization (particle type) and polymer identification analyses.

2.1.2 Northern anchovy

Whole anchovies (n=24) were generously provided by Ocean2Table, a sustainable Community Supported Fishery group, based in Santa Cruz, California, USA. Anchovies were caught within a 100-mile radius from Monterey, California by local fishing partners (Fig. 1) and stored at -20°C until processing.

2.1.3 Common murre

Common murre (COMU) samples (n=19) were provided by California Department of Fish and Wildlife Office and Spill and Prevention Response seabird monitoring program. All individuals were collected between July 2019 to November 2020 (freshly deceased) for the monitoring program of CDFW OSPR or dead-on-arrival to local rehabilitation centers (Fig. 1). Birds from rehabilitation centers did not undergo any treatment prior to death. Birds were examined via systematic necropsy by CDFW personnel, and the full digestive tract was removed following methods described by van Franeker and Meijboom (2002) and van Franeker (2004). Digestive tracts were stored in aluminum foil and frozen at -20°C until processing for particles. Last occurrences of ingestion were unknown. Specific age, size, sex of COMU were not included in this study.

2.2 PARTICLE VISUAL CATEGORIZATION

The lower end in particle size is limited by equipment (i.e. 150 µm-mesh sieves, 10 µm-pore size filters, and forceps). As the access points of the intake systems were located after sand filters, upper ends of recoverable particle sizes were limited by operational boundaries. Nonetheless, particle orientation can still allow passage through filters and permit recoverable particles greater than the operational boundaries. Visual processing of anthropogenic particles followed guidelines by Lusher and Hernandez-Milian (2018) and categorized as: fiber, fragment, foam, film, and bead. Obtaining unanimous agreement by multiple personnel is suggested to reduce bias[34]. However, all visual processing were conducted by one personnel (S.M.) due to COVID-19 pandemic restrictions. Furthermore, visual processing cannot always differentiate a synthetic particle to a natural one, such as

polyester fibers compared to cotton fibers. Until verified as plastic by Raman spectroscopy, we referred to recovered samples as “particles”.

2.3 SAMPLE PROCESSING

2.3.1 Seawater

Using a dissecting microscope (American Optical Company Model 570, total magnification 40X), particles were extracted from sieves with clean forceps and stored in sterile amber vials. Lab filtered Milli-Q water was used to separate sediment, algae, and other natural materials such as micro-invertebrates and shell fragments. A vacuum filtration apparatus with a 10 µm-pore size polycarbonate filter (MilliporeSigma™ Isopore™ Membrane Filters) was used for further processing when necessary.

2.3.2 Northern anchovy and common murre

Digestive tracts were processed following a protocol from Rochman, *et al.* (2015) with minor modifications. Specifically, in a class 100 clean room, digestive tracts were placed in triple-rinsed glass beakers (anchovies) or 500 mL kilned amber jars (COMU), with 20% potassium hydroxide (KOH) at three times the volume of the sample, or minimum 15 mL, and loosely capped for 2-3 weeks at room temperature. Volume of digestive tracts were determined by water displacement. All anchovies were digested with 15 mL of 20% KOH. Twenty percent KOH is not expected to damage the plastic polymer composition[35]. Although heat is often used in the alkaline tissue digestion process, we excluded this step to reduce potential chemical leaching from the particles[29,36]. After the organic materials were digested, samples were filtered through a vacuum filtration apparatus with a 10 µm-pore size polycarbonate filter (MilliporeSigma™ Isopore™ Membrane Filters). Using a dissecting

scope, particles were removed from the filter and stored in sterile amber glass vials using clean forceps.

2.4 PLASTIC VALIDATION AND POLYMER IDENTIFICATION

A subset of particles recovered from seawater (n=29), anchovies (n=20), and COMU (n=6) were analyzed at the University of California, Davis Health Effects of Anthropogenic Litter Lab (Davis, California). Only six particles (one fragment, one foam, one film, and three fibers) from COMU were analyzed due to logistical constraints.

Raman analysis was performed using the LabSpec 6 software suite and a Horiba XploRA™ PLUS Raman confocal microscope, equipped with 785 nm and 532 nm monochromatic lasers, a cooled charge-coupled device detector (1024 x 256 pixels), and an automated stage. Calibration of the unit was performed using zero-order correction of the spectral peak at 520.7cm-1 of a reference silicon wafer for each grating and laser combination.

Point analysis of each target particle was performed by first focusing the microscope on the surface of the particle. Spectra were acquired using 785 nm and/or 532 nm monochromatic lasers with laser power and spectral acquisition times adjusted during repeated spectral acquisitions until an optimized and representative Raman spectra of the particles were obtained. Laser power ranged from 0.025 – 25 mW (for 532 nm laser) and 0.1 mW to 100 mW (for 785 nm laser) and acquisition times ranged from 0.1 sec to 90 sec depending on the individual characteristics of the particle.

After spectral acquisition, the spectrum for each target particle was baseline corrected and then compared to spectral libraries (Wiley KnowItAll, SLoPP, and SLoPP-E) of known chemicals for identification. Based on spectral identification, each particle was classified into one of the following groups: natural, synthetic, semi-synthetic, blends, dye-

prominent, and unknown. Semi-synthetic referred to polymers that are partially synthetic such as cellulose acetate. Blends referred to having a mixture of more than one prominent polymer, such as cotton and polyester. One of the limitations to Raman spectroscopy is that dye can mask the underlying polymer composition; in this case particles were classified as dye-prominent[30,37].

2.5 ESTROGEN RECEPTOR ALPHA (ERα) ACTIVATION ASSAY: COMU (n=19)

2.5.1 Incubation

Particles were transferred to kilned amber glass vials and placed under UV light in a laminar flow hood for 30 minutes to kill bacteria. Incubation 1 (n=15; COMU1-15): Particles were incubated in 1.5 mL of 100% ethanol (EtOH, Fisher Chemical™) for 7 days at 38°C in a temperature controlled shaker (New Brunswick Scientific Co., Inc. Series 25) at 100 rpm to extract any plastic-associated chemicals. Samples were incubated in sets of three along with a particle-free EtOH control (n=5)[14,15]. Vials were tightly sealed with parafilm and Teflon tape. Sample volume and incubator temperature were checked daily during incubation. After the 7-day incubation, particles were separated from the leachates and placed on double-sided tape sealed on a clear film in a clean Petri Dish. Leachates (500 µL) were dried down under forced air in glass tubes to concentrate and resuspended in 50 µL of EtOH. Leachates with 10X concentrations were transfected to cell assay.

Incubation 2 (n=4; COMU16-19): A second incubation was performed to decrease vehicle background effects (See Appendix Section 7.2). Particles were incubated in 1.5 mL 100% EtOH overnight for 16 h in an orbital shaker at room temperature with a 1.5 mL particle-free EtOH control (n=1). Leachates (500 µL) were dried down under forced air in glass tubes to concentrate before resuspending in 50 µL of EtOH (concentration 10X). We performed an additional assay with leachates with higher concentration (20X). We also

combined 45 µL of each leachate (COMU16-19) together (180 µL), similarly dried down and resuspended to 45 µL (concentration 40X).

2.5.2 Cell Assay

Estrogen receptor activation assays were performed as previously described in Felton *et al.* (2015, 2020) with some modifications. Human embryonic kidney cells (HEK293) were co-transfected with the human estrogen receptor 1 gene (hESR1) in a pcDNA3.1+ expression vector (www.cdna.org), pCMX- β-galactosidase (β-gal) and pGL2-3xERE luciferase reporter plasmids (Addgene plasmid 11354)[38]. After 24 h cells were treated, in triplicate, with an endogenous estrogen 17β-estradiol (E_2: 10^{-12}-10^{-7}M; Steraloids, Newport, RI), bisphenol-A (BPA: 10^{-5}M; Sigma, St. Louis, MO), 5µl of a vehicle treatment of 0.1% DMSO or 0.1% EtOH, particle-free EtOH control, or resuspended leachate treatment. After the 24 h final incubation, cells were lysed to measure luciferase and β-gal activity[39,40]. Luciferase activity was standardized to β-gal activity, and the fold activation was measured by normalizing to a vehicle (DMSO or respective particle-free EtOH control). Final results were normalized to maximum E_2 activation as determined by Graph Pad Prism Software (San Diego, CA)[39,40].

2.6 CONTAMINATION CONTROL

Atmospheric contamination is crucial to monitor when studying microplastics[41,42]. Working areas were cleaned prior to use and tools were triple rinsed with lab-filtered Milli-Q water and visually checked for particles before use. Lab coats and gloves were worn and easily-shedding clothes (e.g. fleece, sweaters, etc.) were avoided. Amber glass vials were baked in a muffle furnace at 450°C prior to use. Vial lids were triple rinsed and checked for particles. A triple-rinsed 100 mL-glass beaker filled with Milli-Q water (procedural blank) was

placed at the working station to capture atmospheric contamination. All filtration and chemical digestion processes were completed in laminar hoods and in a class 100 clean room, respectively. Additionally, after initial quantification of particles were recorded, samples were regularly inspected for the addition or loss of particles, with particular caution during transfer of particles (ie: transferring particles leachate vials to Petri dish). Unaccounted for particles were classified as atmospheric contamination.

2.7 STATISTICAL ANALYSIS

Statistical analyses were performed with JMP Pro 16 (Fisher's exact test and Wilcox test)[*]. Significance was determined by *P*-values < 0.05.

3. RESULTS

3.1 PLASTIC PRESENCE

3.1.1 Seawater

Particles were observed in all seawater trials (n=8) and 67 particles were recovered from approximately 39,000 L (See Appendix Table A1), ranging from 5 to 15 particles per trial (mean 8 ± 1 SEM). The mean seawater volume assessed per 17h trial was 4870 L ± 370 L with an estimated 1.89 ± 0.400 particles (mean ± SEM; median=1.56) per 1000 L. While the particle per liter may seem as a small value, the average flow rate of 0.077 L/sec equals approximately 46,300 L of seawater and almost 100 particles per week. Of potential concern, these intake systems deliver seawater to aquatic tanks for research, including maintaining

[*] Statistical revisions to analyze abundance results are in progress.

populations of marine species, at much higher velocity and volume than the flow rate and volume analyzed in this study.

3.1.2 Northern anchovy and common murre

At least one particle was recovered from 58% of anchovy digestive tracts (n=24) with a total of 31 particles. Out of the digestive tracts with particles present, the average abundance was 2.21 ± 0.500 per fish (mean ± SEM, median=1, range 1-7, Fig. 2). Our results were not statistically different than particles found in anchovies in 2014 in Rochman, *et al.* (2015) in both prevalence (p = 0.259, Fisher's exact test) and mean abundance (p = 0.184, Wilcox test). In Rochman, *et al* (2015), 30% of anchovies (n=10) contained one particle.

At least one particle was recovered from 100% of COMU digestive tracts (n=19), with a total of 115 particles. The average abundance was 6.05 ± 0.993 per bird (mean ± SEM, median=5, range 1-17, Fig. 2). Particles recovered from COMU showed significantly higher prevalence (p = 0.001, Fisher's exact test right-tailed) and higher abundance (p < 0.001, Wilcox test) than particles recovered from anchovies (Fig. 2).

3.2 PLASTIC VISUAL CATEGORIZATION

Particle size is described with its longest dimension. Sizes of a subset of the particles from seawater (n=29) and anchovy samples (n=20) ranged from 119 µm to 4545 µm (See Appendix Table A4), with average size of 1383 µm ± 143 µm (mean ± SEM). Fibers were the smallest and longest particles. Regarding particle size relative to operational boundaries, the smallest particle recovered from anchovies (188 nm) was almost 19-fold larger than the 10 µm-pore size polycarbonate filter. The smallest particle recovered from seawater samples

(119 nm) was almost 5-fold larger than the 25 µm-mesh paper filter used in the pilot trials (Long Marine Laboratories, August 2020).

Fibers were the most common particle type recovered from seawater, anchovies, and murres (80%, 71%, and 77%, respectively, Fig. 3). Seawater: 110 particles were extracted from 12 sampling trials representing approximately 45,700 L of seawater (Fiber: 88, fragment: 10, foam: 8, film: 3). Anchovy: 31 particles were extracted from 14 anchovies (Fiber: 22, fragment: 4, foam: 2, bead: 2, film: 1). COMU: 115 particles were extracted from 19 COMU (Fiber: 90, fragment: 20, foam: 5, film: 1). No microbeads were found in seawater samples and COMU digestive tracts.

3.3 PLASTIC VALIDATION AND POLYMER IDENTIFICATION

A subset of particles from seawater (n=29), anchovies (n=20), and COMU (n=6) were analyzed with Raman spectroscopy and classified as natural, synthetic, semi-synthetic, blends, dye-prominent, and unknown polymers (Fig. 4). Dye-prominent referred to particles that contained anthropogenic dye interfering with polymer identification. 33% of recovered particles were dye-prominent, 27% were natural polymers, 25% were synthetic, and 11% were partially synthetic (semi-synthetic and blends; See Appendix Table A4, Section 7.3). At least 57% of particles that had identifiable polymers were microplastic. No animal-based polymers (fur/hair, shells, bones, etc.) were detected from the samples, which confirmed the visual processing guidelines followed to separate out anthropogenic particles (Lusher and Hernandez-Milian 2018).

Dye-prominent (n=18): Dye-prominent was the most common identification category. However, as only six particles were selected from COMU samples, we evaluated particles that were not visibly colored to reduce the likelihood of dye-interference in polymer identification. Particles categorized as dye-prominent were fibers except for one fragment.

Blue dye was the most common detected (67%), followed by viridian (17%) and indigo carmine dye (11%).

Natural polymer (n=15): One film and one fragment were cellulose. Remaining particles were cellulose or cotton fibers.

Synthetic (n=14): Fiber, fragment, foam, and film particles from seawater, anchovies, and COMU were identified as synthetic polymers. All foam particles were identified as polyester or polystyrene. Half of the synthetic particles were identified as polyester, of which six were further identified as polyethylene terephthalate (PET). Other synthetic polymers identified were polypropylene (PP), polyethylene (PE), polyacrylamide-co-acrylic acid, and MMA-co-TMI (acrylic copolymer).

Blends (n=4): Fibers were identified as blends of cotton or cellulose with PET.

Semi-synthetic (n=2): Fibers were identified as cellulose acetate, a non-petroleum based plastic polymer.

Unknown (n=2): Polymers of two fragments were not identified due to poor spectra quality.

3.4 ERα ACTIVATION ASSAY – COMU

Incubation 1 (n=15): From the 7-day incubation at 38°C and 10X concentrated leachates, three COMU samples (COMU03, 04, 09) stimulated 2.8-, 3.3-, 3.2-fold, while COMU05 had 1.4-fold, activation of human ERα (hERα) respective to particle-free EtOH controls (Fig. 5). Vehicle effect was observed in particle-free ethanol controls of COMU10-15 (data not shown, See Appendix). Incubation 2 (n=4): From the overnight incubation at room temperature, no samples stimulated higher activation of hERα, including the more concentrated leachates (20X) or combined COMU leachate (40X), relative to the particle-free

EtOH control (See Appendix Fig. A2). No vehicle effect was observed. Excluding COMU samples exhibiting vehicle effect, 23% of COMU samples exhibited hERα activation higher than hERα activation from EtOH controls.

3.5 CONTAMINATION CONTROL

Atmospheric contamination was monitored during all procedures. Procedural blanks at working stations were particle-free except one fiber was identified when processing a seawater sample (LML04) but did not match (color, type, texture, shape) with any particles found in sample LML04; thus the results of LML04 were not altered. Atmospheric contamination was not observed in COMU leachate samples (See Appendix Table A5). Four fibers from atmospheric contamination were observed during transfer from vial to Petri dish after initial sample processing. One fiber from atmospheric contamination was larger than 5 mm (6042 µm, See Appendix Table A5).

To monitor contamination from PPE, one fiber sample from two different lab coats were analyzed with Raman spectroscopy (See Appendix Table A4). Fibers from lab coats were natural polymers (cellulose), of which showed similar spectra to two natural fibers from atmospheric contamination (See Appendix Table A5, Section 7.3).

4. DISCUSSION

Microplastic presence in the Monterey Bay

Microplastic presence in the Monterey Bay is poorly understood with previous studies primarily focused on seawater[9,43]. We found microplastics in seawater, anchovies, and COMU (Fig. 3, See Appendix Table A2, Table A3), which implies risk of microplastic exposure across multiple levels of the coastal system[44]. Fibers were the most common

particle type (>70%) recovered across three sample types (ie: seawater, northern anchovies, common murre) (Fig.3). Within our size range of a subset of particles (119 µm to 4545 µm), there is no relationship between particle size and particle type, or that particle size did not affect (ingestion) incidentals; fibers were both the smallest and largest particles recovered (See Appendix Table A4).

Our results showed much fewer particles per liter from seawater intake system samples (median 0.00156 particles/L) compared to findings by Choy, *et al.* (2019) reporting median of 2.9 particles/L at 5 m depth water columns nearshore and offshore of Monterey Bay, a similar depth range as the intake to the systems. We suspect this discrepancy is largely due to our upper size limit from operational boundaries of seawater intake systems' sand filters (Long Marine Laboratory: 0.7 cm; Moss Landing Marine Laboratories:0.02 cm) rather than the lower size limit from mesh size as they were similar (150 µm versus 100 µm). As expected, our results (mean 0.00189 particles/L) also showed fewer particles per liter compared to findings by Mason, *et al.* (2016) reporting mean of 0.086 particles/L in wastewater treatment plant effluent to the San Francisco Bay, roughly 30 miles north of Monterey Bay, since wastewater treatment plant effluent is a source of marine microplastic[31]. Difference in findings with other studies also suggests that microplastic presence may not be uniformly distributed even in the same Monterey Bay and is possibly influenced by abiotic conditions such as seasons, surf breaks, or horizontal tidal zones (e.g. intertidal, neritic, pelagic). Nonetheless, assessing presence at several reference points is an important step in understanding microplastic behavior (e.g. accumulation and movement) in MBNMS.

We did not see a significant difference in prevalence nor abundance of particles in northern anchovies from this study (58%, range 0-7, n=24) compared to anchovies collected from the MBNMS in 2014 (30%, range 0-1, n=10)[34]. More studies are required to analyze potential temporal trends or potential threats to the population. Northern anchovies are recreationally and commercially fished in the MBNMS, providing resources to humans and

piscivores such as COMU. Anchovies compose the third highest biomass of the COMU adult diet and the highest biomass of the COMU chick diet[45]. Furthermore, COMU are the most abundant seabird species in the California Current System that serve as a sentinel species when evaluating pollution of local marine system[45]. Similar spectra of PET fibers were found in all sample types (seawater, anchovies, murres) supporting a potential pathway of exposure. Although finding 100% prevalence in COMU (n=19), we cannot determine from this study that microplastics in COMU were due to indirect (or secondary) ingestion (i.e. trophic transfer) as the excretion, bioaccumulation, and biomagnification of microplastics are still unclear[46]. Some studies have shown evidence for trophic transfer of microplastic, as well as of plastic-associated chemicals, to apex predators (i.e. grey seals, *Halichoerus grypus*) and to humans[47–50].

Over 200 seabird species have been reported with macroplastic ingestion; recent studies have documented *micro*plastic ingestion in seabird species as well[4]. Microplastic ingestion has been documented, in portions of the digestive tract as opposed to the full digestive tract done here, in northern fulmar (*Fulmarus glacialis*), flesh-footed shearwater (*Ardenna carneipes*), and thick-billed murres (*Uria lomvia*)[51–53]. Northern fulmars and flesh-footed shearwaters had high prevalence (86%, n=57 and and 91.5%, n=57), while only 17% of thick-billed murres (n=30) had ingested particles[51–53]. We encourage standardizing methods by examining the full digestive tract when studying microplastic ingestion to maximize detectability as Provencher *et al* (2018) suggests seabirds are more likely to retain microplastic than prey fish species due to the unique physiology, specifically their narrow and multi-chambered digestive tract[54].

Microplastic ingestion in COMU in previous studies are unclear due to reporting[55,56]. Most studies performed methods following macroplastic ingestion protocol such as visual inspection and using 1 mm mesh sieves rather than alkaline tissue digestion and smaller filter pore size more standardized for microplastic analysis[55,56]. For example, Acampora, *et al.*

(2016) report mass rather than particle size but the mesh sieve size allows for microplastic collection. Nine studies that report plastic ingestion of COMU show COMU have a low 6% prevalence compared to our findings of 100% prevalence of microplastic ingestion[4]. Our results illustrate that while some seabird species (and other taxa) may be perceived as having low risk for *macro*plastic (>5 mm) ingestion, as observed in this study, those species could have high *micro*plastic (<5 mm) prevalence. As such, our results illustrate that reporting of only macroplastic prevalence could result in a substantially underestimated total plastic exposure risk (macro- and microplastic) in marine species.

Visual categorization and polymer identification

Consistent with other marine microplastic studies, fibers were the most common particle type recovered (seawater: 80%, anchovies: 71%, COMU: 77%, Fig. 4)[30,31,34,52]. Of the 25 fibers with detectable polymers from Raman spectroscopy, 48% were fully or partially synthetic and 32% were identified with polyester (Fig. 4, See Appendix Table A4). The textile industry is a large contributor to plastic fibers in the environment as over 70 million tons of fibers (or 400 billion square meters of fabric) are produced annually, of which 60% are synthetic with polyester being the most common synthetic polymer [57]. A single polyester clothing item can release over 1900 fibers from one wash in a laundry machine and synthetic materials from washing clothes are discharged to marine systems via the wastewater treatment plant effluent [58–60]. A recent study found that laundry machine filter attachments can prevent outflow of fibers to effluent by 87% and underscores how reducing discharge is essential to reducing microplastic in our coastal system [59].

We emphasize the recommendation by other studies to use Raman (or FT-IR) spectroscopy for microplastic research[54]. While there are some limitations to Raman and FT-IR spectroscopy such as cost, detectable particle size, or dye obstruction, visual

categorization can be misleading and may lead to over reporting of microplastic[30,61]. Visually perceived as plastic, we found almost a third of recovered particles (15/55) to be natural polymers with Raman spectroscopy. Therefore, not all anthropogenic particles are microplastic. In addition, identification of specific polymers can provide important information with respect to the abundance and possible sources of anthropogenic or non-natural particles in the environment[9].

Potential hormonal-disrupting capacity of ingested microplastic

To our knowledge, we are the first to investigate the potential hormonal-disrupting capacity of microplastics recovered from seabird digestive tracts. The number and category of particles incubated per COMU leachate sample did not appear to correspond to levels of hERα activation *in vitro* (See Appendix Table A2). For example, three out of 13 COMU samples (COMU 03, 04, 09) exhibiting the highest levels of hERα activation (26%-31% relative to endogenous E_2) did not have the highest observed number of particles (Fig. 5, See Appendix Table A2). COMU01 had the highest number of particles (n=16), but exhibited low activity, while COMU09 had only two particles but exhibited with high activity (Fig. 5, See Appendix Table A2). Of the six COMU particles analyzed with Raman spectroscopy, one foam from COMU03 with potential hormonal-disrupting capacity was identified as polystyrene (Fig. 4C). Studies have found polystyrene to be xenoestrogenic and potentially cytotoxic dependent on size[14,15,62]. Xenoestrogens are also linked to reducing the ability of seabirds to adapt to climate-related environmental stressors[13]. Therefore, detecting xenoestrogenic activity from microplastic is highly concerning as even low doses of EDCs can lead to adverse effects[25].

We speculate the lack of ERα activation in the majority of COMU samples is likely a combination of the following factors: 1) particles were too small in size or quantity to detect

activity (i.e. COMU08 had one fiber), 2) potentially estrogenic chemicals may have leached to tissue as some hydrophobic additives can desorb as quickly as 10 minutes, 3) some plastic polymers that are EDCs are not xenoestrogenic[14,15,63,64]. We acknowledge there could be other contributing factors (See Appendix Section 7.2). Thus, although more studies are needed, our data indicate that 23% of samples had potential hormonal disrupting activity and provide important information about the possible physiological impact of ingested microplastic.

5. CONCLUSION

Our data emphasize the need for further research on microplastic in marine systems with standardized methods, as marine species suspected with low risk of plastic ingestion could have high microplastic ingestion prevalence. Findings of microplastic in seawater, anchovies, and COMU, as well as that some particles were potentially xenoestrogenic, emphasize the concern of microplastic pollution. Recent discussions regarding microplastic management surround the analogy of “turning off the tap”—when the tub is overflowing, it is more efficient to turn off the tap than to mop the floor[65]. We agree that more preventative management practices to reduce or restrict plastic generation and release into the environment (i.e. “turn off the tap”) should be implemented. For example, high prevalence of fibers found in this study supports the need to reduce discharge from sources such as laundry machines and wastewater treatment plants. Nonetheless, continuing to monitor microplastic presence and potential adverse effects are also important to understand the impact on marine systems and inform waste reduction practices.

FIGURES

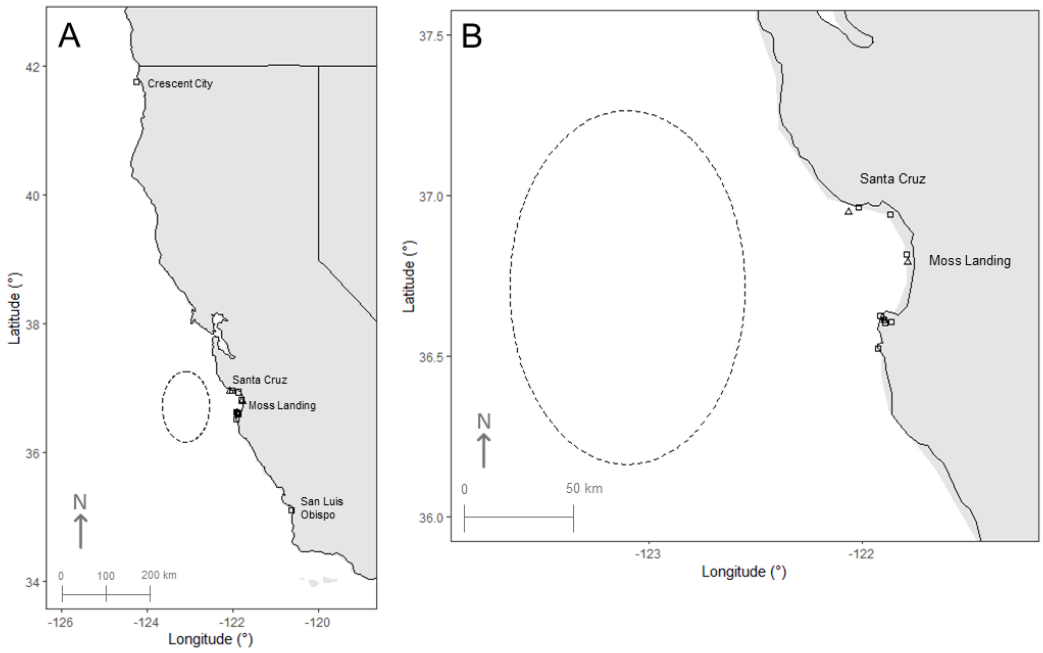


Figure 1: Map of sample collection locations. A) From most northern to most southern sample collection. B) Magnified view of Monterey Bay region. A & B) Open square: common murre collection sites, open triangle: seawater collection sites, open dashed circle: northern anchovies were collected within range of outer boundary lines.

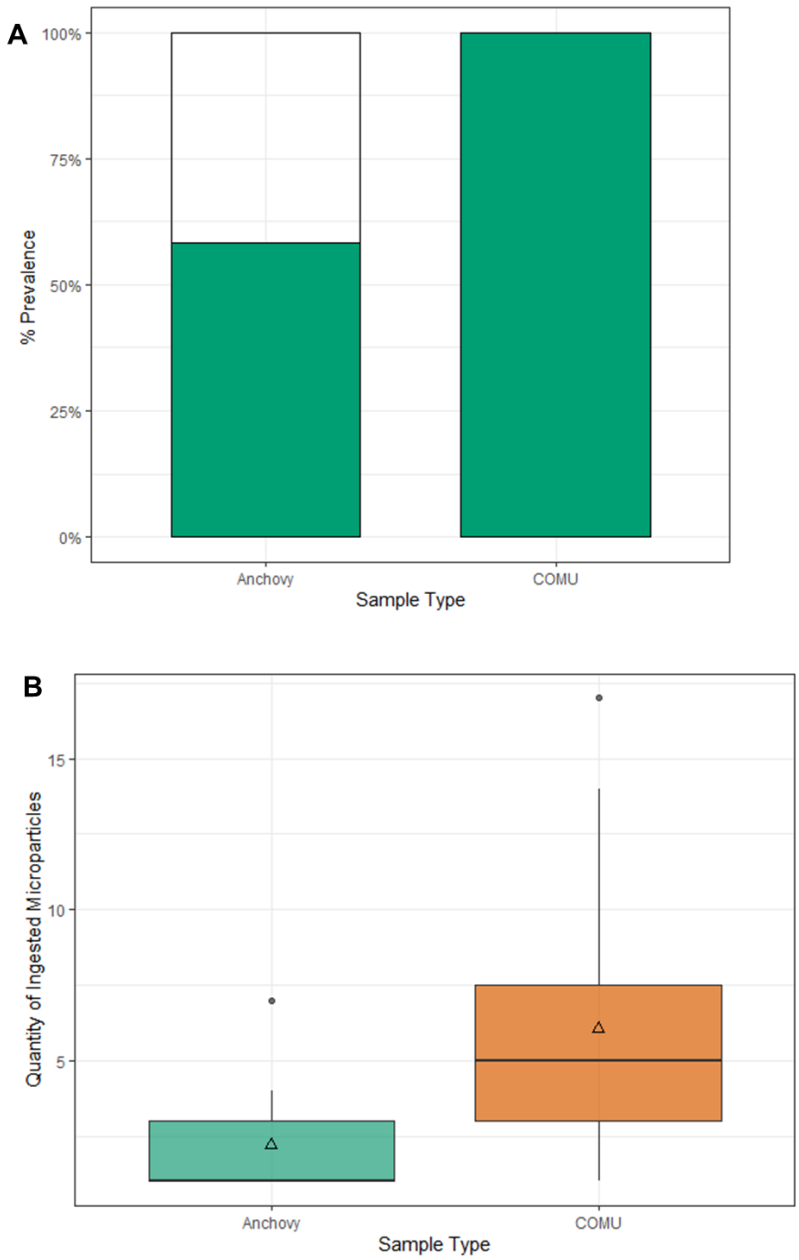


Figure 2: A) Prevalence of particles (size 10µm to 5 mm) in common murres (COMU) was higher compared to northern anchovies (anchovies; p = 0.001, Fisher's exact test). At least one particle was found in 57% of anchovies (n=24) and 100% in COMU (n=19). B) There was a significant difference (p = 0.00012, Wilcox 2-Way Test) between mean abundance of ingested particles by anchovies (n=14) and COMU (n=19). Tukey's boxplot comparing quantity of ingested particles found in anchovies' and COMUs' digestive tracts. Ends of the box represents lower (Q1) and upper (Q3) quartiles, with median line drawn in between. Open triangle represents mean.

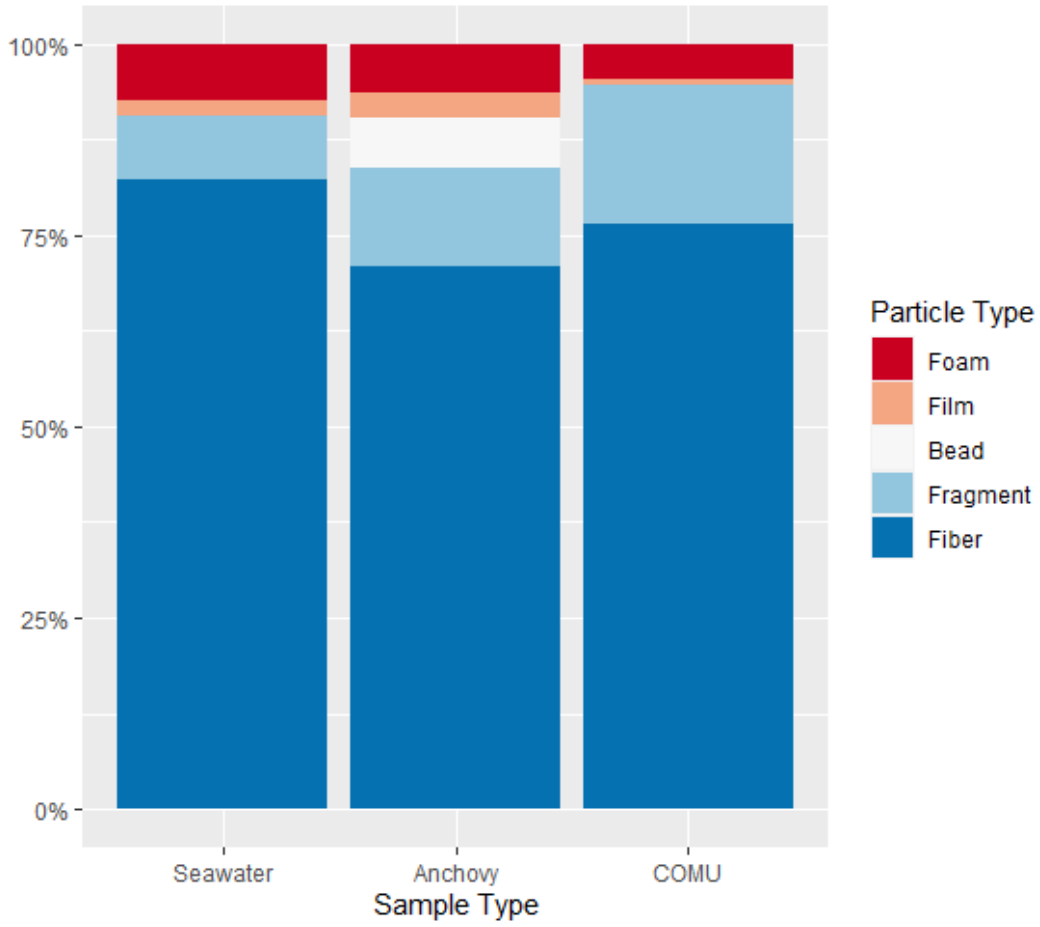


Figure 3: Fibers were the most dominant particle type recovered from all three sample types. A total of 256 particles (size 10µm to 5 mm) were recovered: 110 particles from 12 trials of seawater sampling, 31 particles from 14 northern anchovies (anchovy), and 115 particles from 19 common murres (COMU). Both seawater samples and COMU digestive tracts did not have any microbeads. See Appendix Table A2 and Table A3 for full particle details.

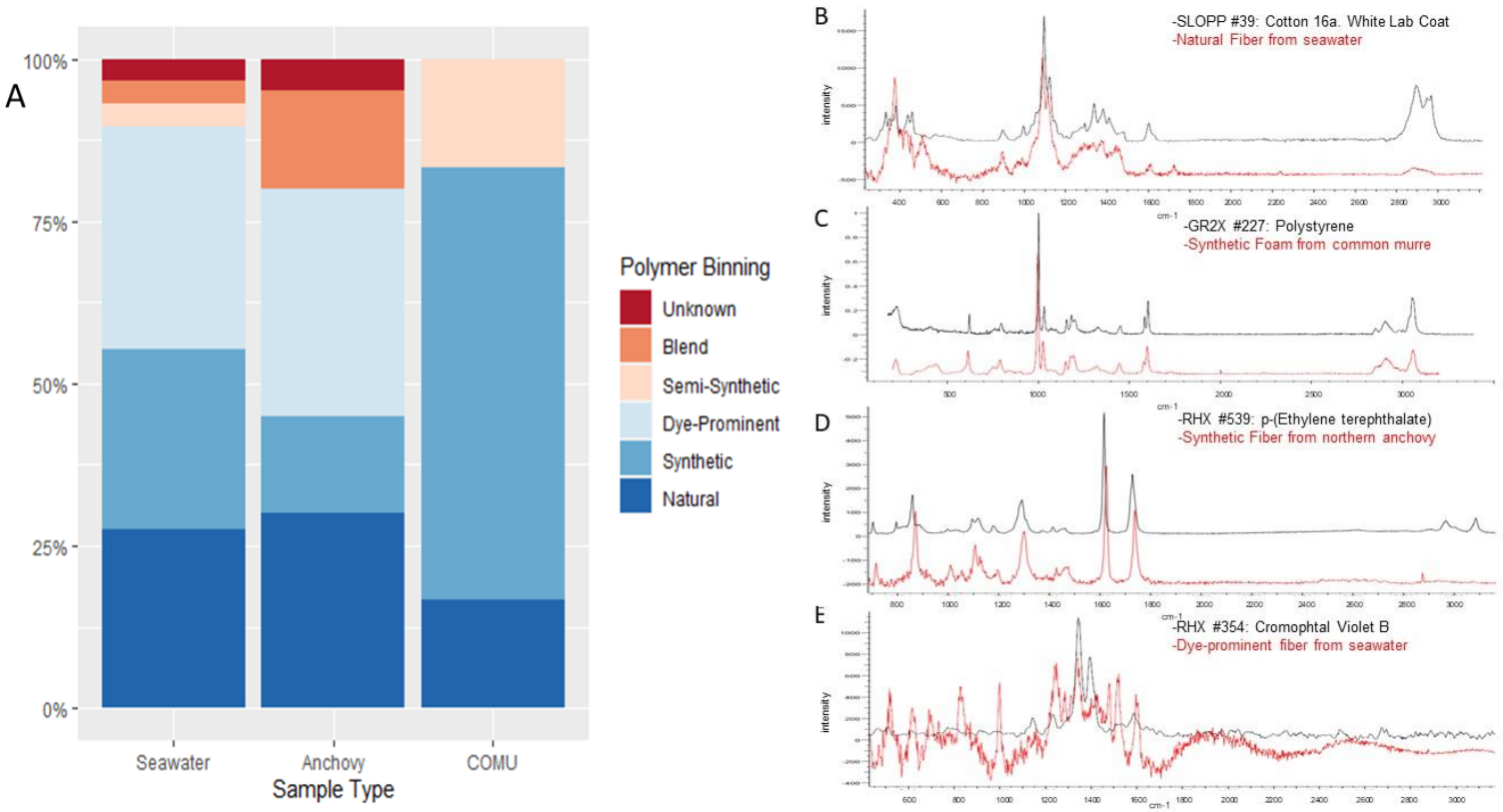


Figure 4: A subset of recovered particles (n=55) were analyzed with Raman spectroscopy to identify polymer compositions: 29 particles from seawater samples, 20 particles from northern anchovies (Anchovy), and 6 selected particles from common murres (COMU). A) Distribution of categorized polymer bins based on Raman identification of particles. B-E) Selected Raman spectra: red line is spectrum of particle; black line is spectrum of matched polymer from spectral libraries (Wiley KnowItAll, SLoPP, and SLoPP-E). Y-axis is intensity and x-axis is Raman effect wavelength (cm^{-1}). B) Natural fiber from seawater identified as cotton (cellulose). C) Synthetic foam from COMU identified as polystyrene. D) Synthetic fiber from anchovy identified as polyethylene terephthalate. E) Dye-prominent fiber from seawater.

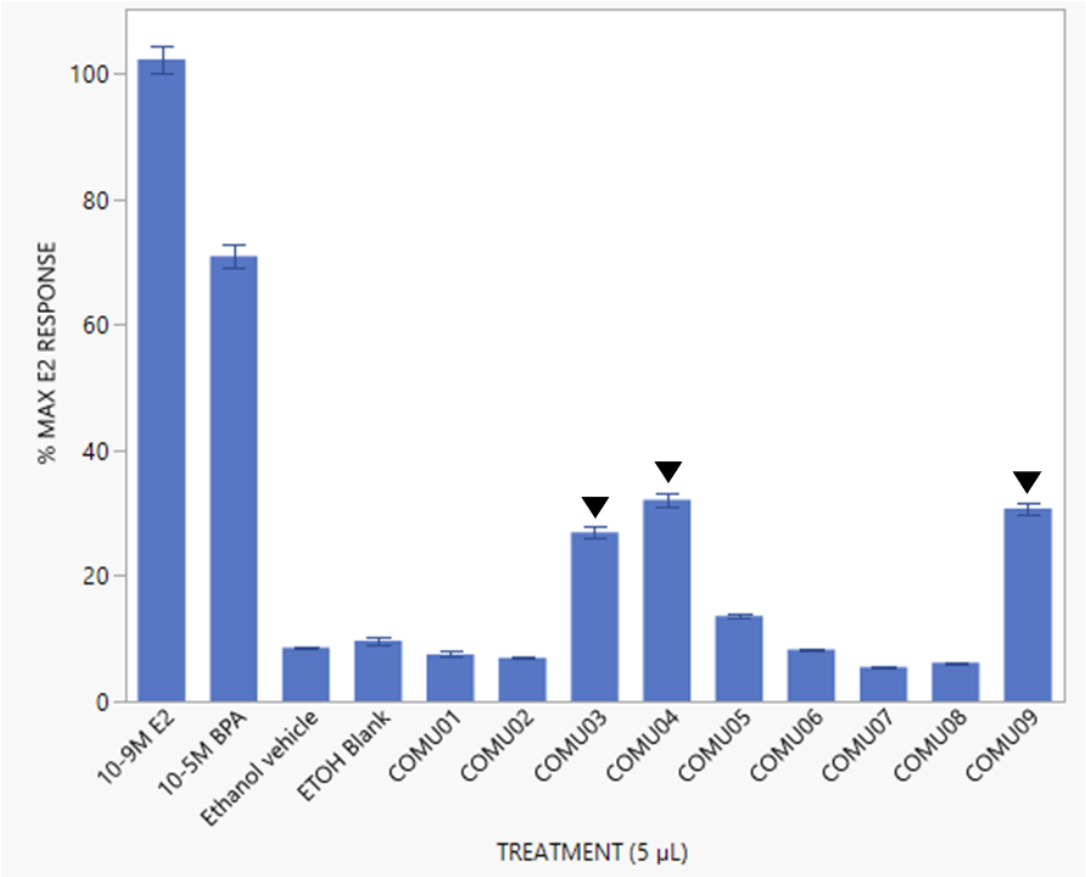


Figure 5: Three common murre (COMU) particle leachates (▼) stimulated 26%-31% activation of human estrogen receptor alpha (hERα) relative to activation from 17β estradiol (E_2). In comparison to the particle-free ethanol blank (ETOH blank), COMU03 stimulated 2.8-fold, COMU04 stimulated 3.3-fold, and COMU09 stimulated 3.2-fold activation of hERα. COMU05 stimulated with less than two-fold activation of hERα than ethanol vehicle (1.6-fold) and ETOH blank (1.4-fold). ETOH blank encompasses three ethanol blanks incubated with each incubation set. Data represent mean ± SEM of fold activation relative to maximal E_2 activation.

7. APPENDIX

This appendix presents information, figures, and tables to provide additional data and clarification.

7.1 Layered Complexity of August 2020 Seawater Samples

We did not include seawater samples from August 2020 ('SEA') in all analyses. Sampling methods varied within all four samples and compared to the LML and MSL samples as it was part of a method testing. SEA01 average flow rate is calculated with the three start time measurements only. SEA02 and SEA03 included paper filters (25 µm, Whatman filter paper Grade 4) on the top sieve and were found overflowing when personnel arrived in the morning, leading to presume particles were lost. SEA04 only had four time measurements due to weather and hazardous constraints: 3 start and 1 end. Santa Cruz, CA, USA experienced climate-related extreme wildfires summer of 2020. During SEA04 sampling, ashes and smoke from the nearby wildlife (CZU Lightning Complex Fire) accumulated to the sampling area. Several ashes were found on the sieves from SEA04. Interesting, SEA04 particle leachates stimulated high activation of human ERα (data not shown), but also had other complications such as full evaporation. We are uncertain whether high activation was due to adsorbed hydrophobic organic contaminants such as polycyclic aromatic hydrocarbons (PAHs) from the wildfire, a vehicle effect from a mechanical issue (such as a pipet or pipet tip contamination), or due to displacement. Multiple ethanol blanks were incubated and analyzed with SEA04: one ethanol blank that also fully evaporated also had high vehicle effect, while another ethanol blank with high vehicle effect did not evaporate and need displacement.

Table A1: Seawater assessments from Long Marine Laboratory (SEA, LML) and Moss Landing Marine Laboratories (MSL). SEA samples were not included in the main quantification analysis as they were collecting during pilot trials and methods varied. Average flow rate was calculated from the six time measurements taken (three at the start, three at the end of assessment).

Sample	Dates conducted	Average Flow (sec/L)	Estimated Total Volume (L)[A]	Number of Particles	Particles per 1000 L[B]
SEA01	10-11 Aug 2020	26	2340	30	12.8
SEA02	11-12 Aug 2020	50	1220	3	2.47
SEA03	12-13 Aug 2020	23	2610	7	2.68
SEA04	18-19 Aug 2020	110	579	3	5.18
LML01	24-25 May 2021	14	4500	10	2.22
LML02	25-26 May 2021	9	6550	7	1.07
LML03	12-13 July 2021	15	4150	10	2.41
LML04	13-14 July 2021	15	4020	6	1.49
MSL01	5-6 July 2021	18	3470	15	4.33
MSL02	6-7 July 2021	11	5620	6	1.07
MSL03	7-8 July 2021	10	5800	5	0.862
MSL04	8-9 July 2021	13	4900	8	1.63
AVERAGE*	N/A	13	4870	8	1.89
ST ERROR*	N/A	1	370	1	0.400

*Excludes SEA01-04
[A]Estimated with average flow (sec/L) and duration of assessment (~17 h)
[B]Calculated with average flow (sec/L) and number of particles observed

Table A2: 115 particles (size 10µm to 5 mm) were recovered from common murres (COMU, n=19). Particles were categorized as fiber, fragment, foam, bead, and film. No beads were found in COMU.

Sample ID	Quantity Found	Quantity Incubated[A]	Fiber	Fragment	Foam	Bead	Film
COMU01	17	16*	16	1	0	0	0
COMU02	11	11	5	3	3	0	0
COMU03	10	10	6	2	2	0	0
COMU04	7	7	3	4	0	0	0
COMU05	6	5*	6	0	0	0	0
COMU06	4	4	3	1	0	0	0
COMU07	4	4	0	4	0	0	0
COMU08	1	1	1	0	0	0	0
COMU09	2	2	1	1	0	0	0
COMU10	3	3	3	0	0	0	0
COMU11	4	4	4	0	0	0	0
COMU12	14	14	14	0	0	0	0
COMU13	2	2	1	1	0	0	0
COMU14	2	2	1	0	0	0	1
COMU15	5	5	3	2	0	0	0
COMU16	7	7	7	0	0	0	0
COMU17	3	2*	3	0	0	0	0
COMU18	8	8	8	0	0	0	0

COMU19	5	5	4	1	0	0	0
--	115	112	89	20	5	0	1

[A]Quantity of particles incubated in ethanol for leachates for ERα activation assay treatment.
*Quantified particles that were not incubated were fibers due to manual limitations.

Table A3: 141 particles (size 10µm to 5 mm) were recovered from anchovies (n=24) and seawater (n=12). Particles were categorized as fiber, fragment, foam, bead, and film. No beads were found in seawater.

Sample Type	Sample ID	Quantity Found	Fiber	Fragment	Foam	Bead	Film
Anchovy	OTNA01	3	2	1	0	0	0
	OTNA02	1	1	0	0	0	0
	OTNA03	0	0	0	0	0	0
	OTNA04	1	1	0	0	0	0
	OTNA05	7	5	2	0	0	0
	OTNA06	4	2	0	0	2	0
	OTNA07	0	0	0	0	0	0
	OTNA08	0	0	0	0	0	0
	OTNA09	3	2	1	0	0	0
	OTNA10	2	2	0	0	0	0
	OTNA11	1	1	0	0	0	0
	OTNA12	0	0	0	0	0	0
	OTNA13	1	1	0	0	0	0
	OTNA14	0	0	0	0	0	0
	OTNA15	0	0	0	0	0	0
	OTNA16	1	1	0	0	0	0
	OTNA17	1	1	0	0	0	0
	OTNA18	0	0	0	0	0	0

	OTNA19	0	0	0	0	0	0
	OTNA20	4	2	0	2	0	0
	OTNA21	1	0	0	0	0	1
	OTNA22	0	0	0	0	0	0
	OTNA23	1	1	0	0	0	0
	OTNA24	0	0	0	0	0	0
Seawater	SEA01	30	27	0	2	0	1
	SEA02	3	1	2	0	0	0
	SEA03	7	4	3	0	0	1
	SEA04	3	2	1	0	0	0
	LML01	10	10	0	0	0	0
	LML02	7	2	3	1	0	1
	LML03	10	9	1	0	0	0
	LML04	6	6	0	0	0	0
	MSL01	15	11	0	3	0	0
	MSL02	6	3	0	2	0	0
	MSL03	5	5	0	0	0	0
	MSL04	8	8	0	0	0	0
Total	--	141	131	110	14	10	2

Table A4: Polymer compositions identified on 57 particles (size 10µm to 5 mm) with Raman spectroscopy: 55 recovered particles and 2 control particles from lab coats worn. The sizes of COMU samples and one seawater sample (LML01) were not measured. Specific sample IDs of anchovy source are unknown.

Sample Type	Sample ID	Particle Type	Size (µm)	Spectra Quality	Polymer Binning	ID 1	ID 2
Common Murre	COMU06	Fiber	N/A	Very good	Natural	Cotton	
	COMU06	Fragment	N/A	Very good	Synthetic	Polypropylene	
	COMU12	Fiber	N/A	Good	Semi-synthetic	Cellulose acetate	
	COMU12	Fiber	N/A	Good	Synthetic	Polyester (Polyethylene terephthalate)	
	COMU14	Film	N/A	Good	Synthetic	Polyethylene	partially obscured by dye
	COMU03	Foam	N/A	Excellent	Synthetic	Polystyrene	
Anchovy*	N/A	Fragment	383 x 218	Excellent	Synthetic	Polypropylene	
	N/A	Fragment	188 x 107	Poor	Unknown	Methyl Vinyl Ether/Maleic	Anhydrase
	N/A	Fiber	2133 x 19	Excellent	Synthetic	Polyester (Polyethylene terephthalate)	
	N/A	Fiber	954 x 48	Good	Blends	Cotton	Polyester (Polyethylene terephthalate)
	N/A	Fiber	468 x 30	Fair	Dye prominent	Blue dye	Yellow dye
	N/A	Fiber	2340 x 37	Good	Natural	Cotton (cellulose)	

	N/A	Fiber	1201 x 54	Fair	Natural	Cotton (cellulose)	blue dye
	N/A	Fiber	3225 x 21	Good	Natural	Cotton (cellulose)	
	N/A	Foam	1802 x 368	Excellent	Synthetic	Polystyrene	
	N/A	Fiber	1146 x 29	Fair	Dye prominent	Blue dye	
	N/A	Fiber	1529 x 30	Fair	Natural	Cotton	Perm bordo (Violet) dye
	N/A	Fiber	1966 x 41	Good	Blends	Cotton	Polyester (Polyethylene terephthalate)
	N/A	Fiber	1362 x 28	Good	Dye prominent	Blue dye	
	N/A	Fiber	754 x 32	Good	Natural	Cellulose	pink dye
	N/A	Fiber	2989 x 30	Fair	Dye prominent	Blue dye	Poly meta phenylene terephthalamide (PMTA)
	N/A	Fiber	844 x 20	Fair	Dye prominent	Blue dye	
	N/A	Fiber	1440 x 49	Fair	Dye prominent	Stains	Viridian
	N/A	Fiber	1643 x 60	Good	Blends	Cellulose	Polyester (Polyethylene terephthalate)
	N/A	Fiber	495 x 46	Fair	Natural	Cotton	black dye
	N/A	Fiber	1628 x 14	Good	Dye prominent	Indigo carmine dye	Viridian
Seawater	LML01	Fiber	N/A	Good	Natural	Cellulose/cotton	
	SEA01	Foam	1487 x 591	Fair	Synthetic	Polyester (Polyethylene terephthalate)	

SEA01	Fragment	453 x 278	Poor	Unknown	Methyl Vinyl Ether/Maleic	Anhydride copolymer
SEA01	Film	933 x 218	Fair	Synthetic	Polyester (Polyethylene terephthalate)	
SEA01	Film	734 x 255	Fair	Synthetic	Polyester	
SEA01	Fiber	1960 x 30	Fair	Dye prominent	Blue dye	
SEA01	Fiber	1174 x 28	Very good	Dye prominent	Blue and Yellow dye	phenolic resin
SEA01	Fiber	1939 x 32	Good	Blends	Cotton	Polyester (Polyethylene terephthalate)
SEA01	Fiber	1418 x 25	Fair	Dye prominent	Dye	
SEA01	Fiber	916 x 28	Very good	Dye prominent	Blue dye	black dye
SEA01	Fiber	1795 x 32	Fair	Synthetic	Polyacrylamide-co-acrylic acid	
SEA01	Fiber	4453 x 18	Very good	Semi-synthetic	cellulose acetate	
SEA01	Fiber	1084 x 29	Fair	Synthetic	MMA co TMI (acrylic copolymer)	
SEA01	Fiber	625 x 33	Very good	Dye prominent	indigo carmine dye	
SEA02	Film	951 x 405	Fair	Natural	Cellulose	Dye
SEA02	Fiber	1598 x 35	Poor	Natural	Cotton	blue dye

SEA02	Fiber	657 x 13	Good	Dye prominent	Blue dye	
SEA02	Fiber	1868 x 24	Very good	Natural	Cotton (cellulose)	
SEA02	Fiber	637 x 42	Very good	Synthetic	Polyester (Polyethylene terephthalate)	Blue Dye
SEA02	Fiber	2572 x 28	Poor	Natural	Cotton	
SEA02	Fiber	746 x 31	Fair	Natural	Cotton	
SEA03	Fragment	1093 x 450	Poor	Dye prominent	Unknown	
SEA03	Fiber	1071 x 26	Good	Dye prominent	Blue dye	
SEA03	Fiber	1891 x 13	Good	Dye prominent	Blue-green dye (Viridian)	
SEA03	Fiber	119 x 38	Excellent	Synthetic	Polyester (Polyethylene terephthalate)	
SEA03	Fiber	436 x 28	Good	Dye prominent	Blue dye	
SEA03	Fragment	250 x 89	Very good	Natural	Cellulose	
SEA04	Fiber	4545 x 47	Good	Natural	Modal (cellulose)	
SEA04	Fiber	2430 x 18	Very good	Dye prominent	blue dye	

Table A5: Quality Control – Polymer compositions identified on four particles from atmospheric contamination and two control lab coats with Raman spectroscopy. Specific anchovy sample ID is unknown. The following particles were not observed in procedural blanks but between procedures after initial processing.

Sample Type	Sample ID	Particle Type	Size (µm)	Spectra Quality	Polymer Binning	ID
Atmospheric contamination	SEA04	Fiber	6042 x 21	Very good	Synthetic	Polyacrylonitrile
	SEA03	Fiber	2035 x 18	Very good	Natural	Cotton (cellulose)
	SEA03	Fiber	2950 x 21	Fair	Natural	Cotton
	Anchovy	Fiber	2645 x 19	Good	Dye prominent	indigo carmine dye
Lab Coat	Clean Lab	Fiber	N/A	Good	Natural	Cellulose
	Cell Culture Room	Fiber	N/A	Good	Natural	Cellulose

7.2 Estrogen receptor activation assay

Vehicle effects observed from incubation 1.

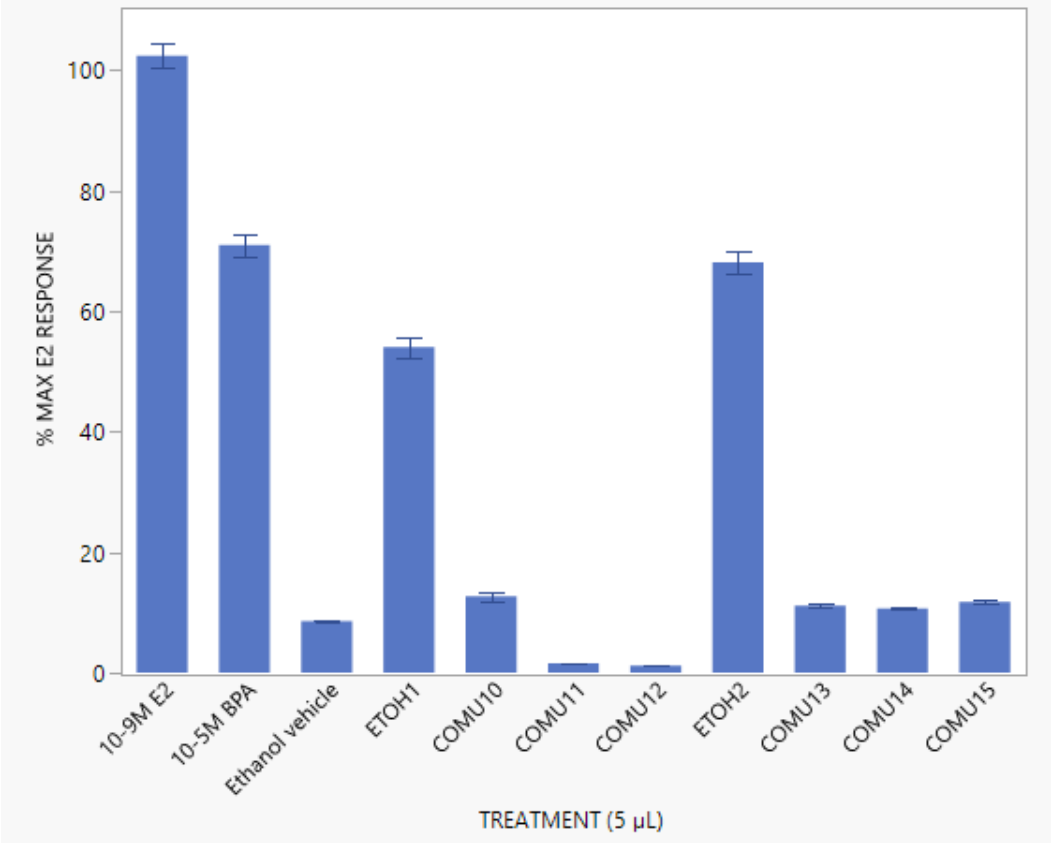


Figure A1: Vehicle effect was observed in two ethanol blanks: ETOH1 and ETOH2; therefore resulting the common murre (COMU) samples to be inconclusive data. ETOH2 stimulated almost as high of activation of human estrogen receptor alpha (hERα) as the positive control (Bisphenol-A, BPA). Data represent mean ± SEM of fold activation relative to maximal E_2 activation.

We observed partial evaporation and displaced the ethanol volume in ETOH1 (600 µL) and COMU10 (800 µL) on day 6 of incubation. We observed partial evaporation in another ethanol blank and COMU but did not observe a vehicle effect (Fig. 5). Therefore, we do not suspect the vehicle effect was due to evaporation and its volume displacement as ETOH2 without any evaporation was observed with the highest activation of all 19 COMU and 5 ethanol blanks analyzed.

No vehicle effect observed in incubation 2.

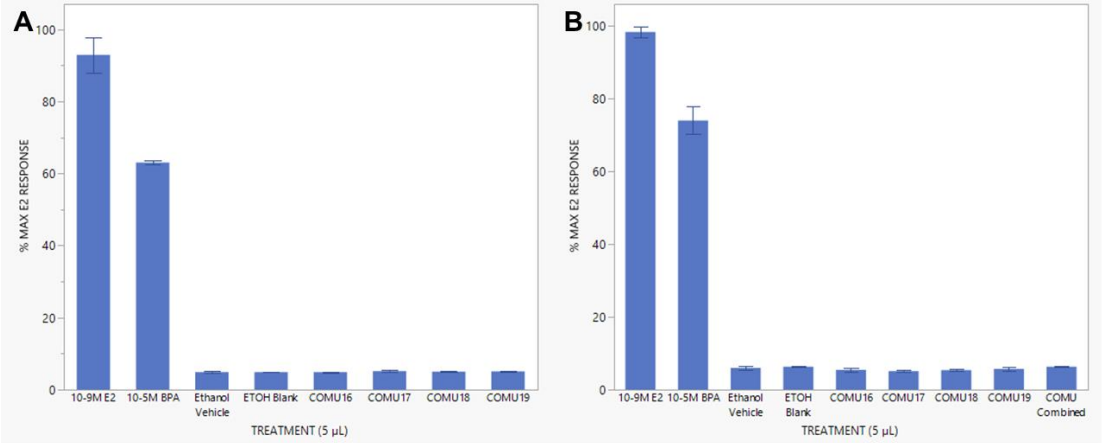


Figure A2: A) Leachates of particles recovered in common murres (COMU) at 10X concentration. B) COMU leachates (COMU16-19) are 20X and pooled leachate (COMU Combined) is 40X in concentration. No estrogenic activity was observed when 5 µL of treatment sample were transfected to human embryonic kidney (HEK293) cells and luciferase gene activation was measured. Leachates were prepared following the second incubation protocol (i.e. 16 h at room temperature compared to 7 days at 38°C). Quantity of particles incubated ranged from two to eight, with all fibers except one fragment in COMU 19—see particle details in Table A2. Data represent mean ± SEM of fold activation relative to maximal E2 activation.

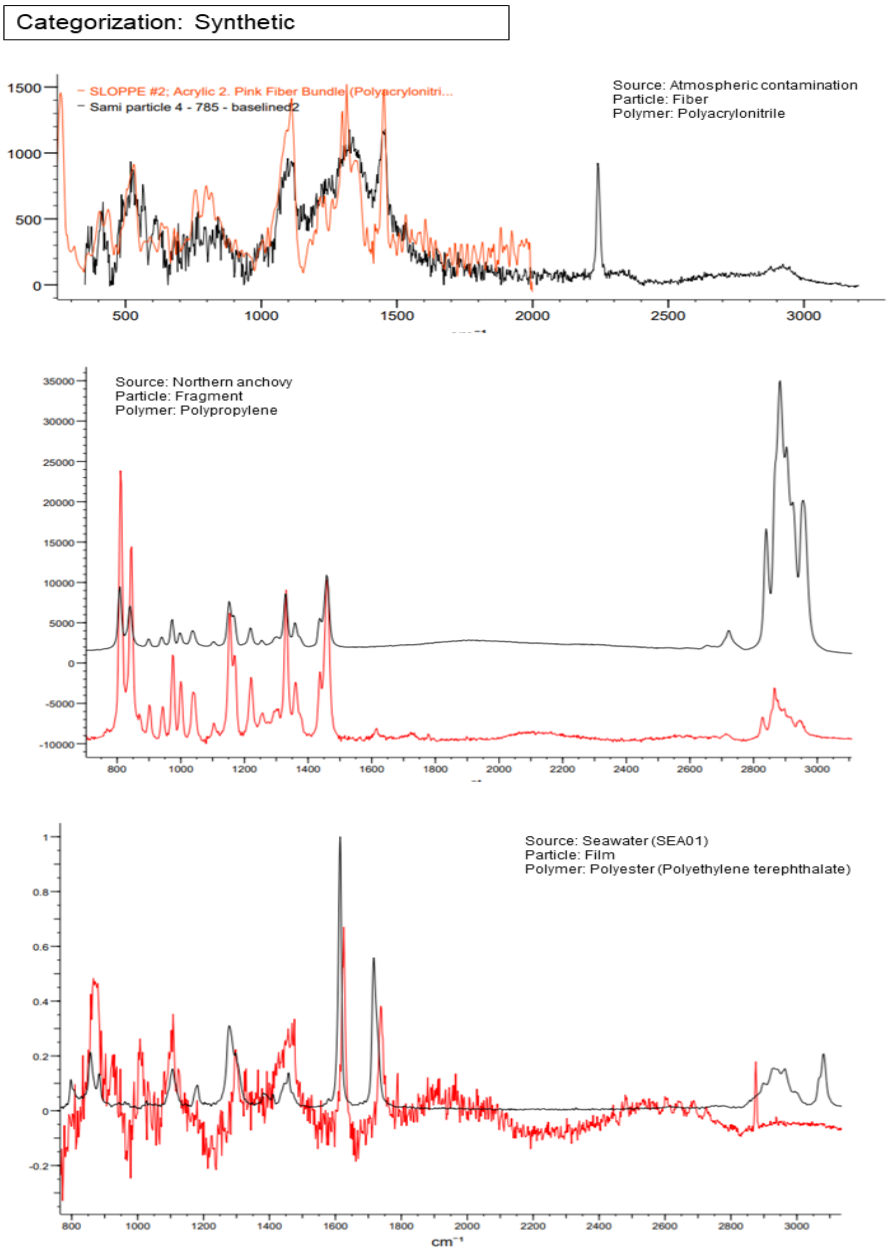
Lessons learned-Vehicle effect: We suspect the vehicle effect was due to any one of these contributing factors: 1) lids of amber vials were plastic and chemicals could have migrated to the leachate or 2) pipet tips or pipets used were partially contaminated; although we acknowledge there could be more. The incubating shaker used regularly fluctuated in temperature; without adjusting the dials the temperature could drop or rise 10°C unexpectedly. High temperatures (above 40°C) and evaporation did not seem to be related; however high temperatures could have caused some vapors to reach the inside plastic layers of the lids or cause chemicals to migrate into the leachate. Based on these observations, we attempted a second protocol of incubation (COMU16-19) by incubating samples at room temperature and only for 16 h. While the second incubation method was successful in reducing vehicle effect and evaporation, we did not observe any activation from the samples;

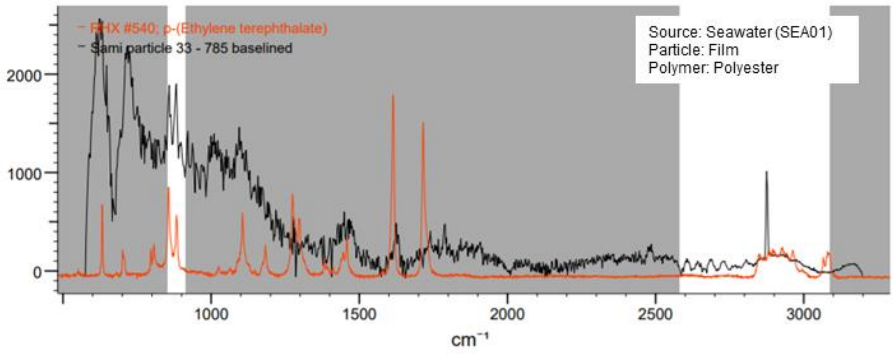
therefore is still difficult to conclude whether the lack of activation in COMU16 to 19 were due to decreased incubation time and/or temperature, or that the particles of those leachates were not xenoestrogenic. In our group's pilot study with ingested macroplastic, we observed substantial stimulation of hERα from leachates when incubated for 16 h at 38°C. Unfortunately, our results are still incomparable as the pilot study was analyzing higher abundance of macroplastic particles such as fishing line, bottle cap, and fragment pieces. Thus, we will need to continue exploring methods to establish best methods to study potential xenoestrogenic activity of ingested microplastics. We hope this study provides preliminary information on successes and failures on measuring xenoestrogenic activity from ingested microplastic for future researchers.

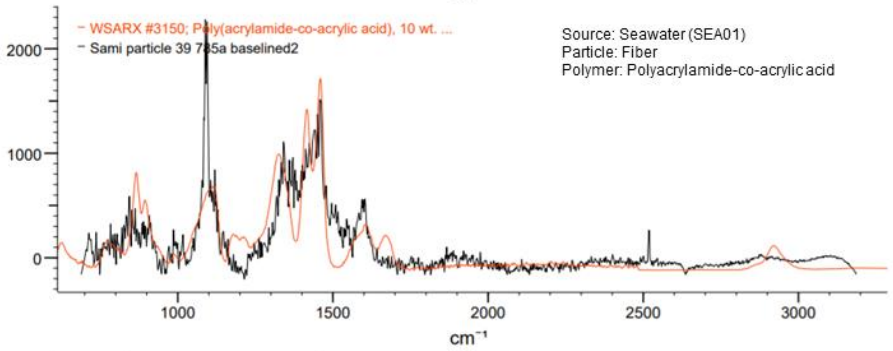
Remaining gaps in knowledge: While many plastic-associated chemicals are EDCs, not all are xenoestrogenic and may target other endocrine pathways. The scope of this study focused on the xenoestrogenic potential of plastic-associated chemicals; therefore did not perform other tests to measure other possible endocrine-disrupting capacity. Another crucial gap in knowledge is there is an influence of KOH digestion on the outcomes of the estrogen receptor activation assay. Potassium hydroxide (20%) has been shown to not alter or damage the plastic polymer, but the impact of KOH on additives and contaminants (if present) as well as the overall influence on the ER assay is unknown[35]. Further tests such as spiking the 20% KOH digestion with a known control such as BPA would help uncover some of these gaps.

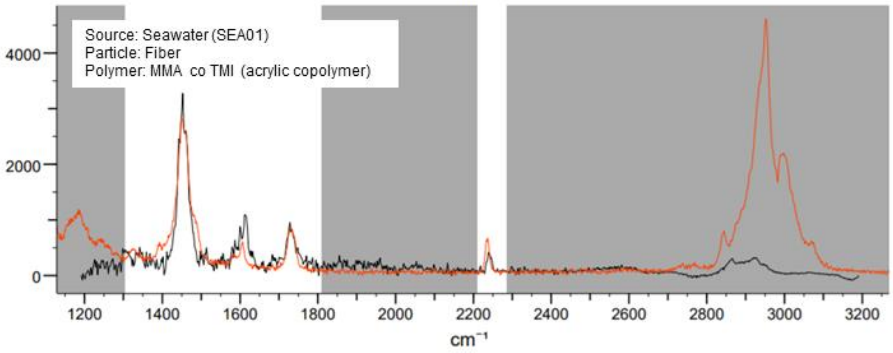
7.3 Polymer identification with Raman spectroscopy

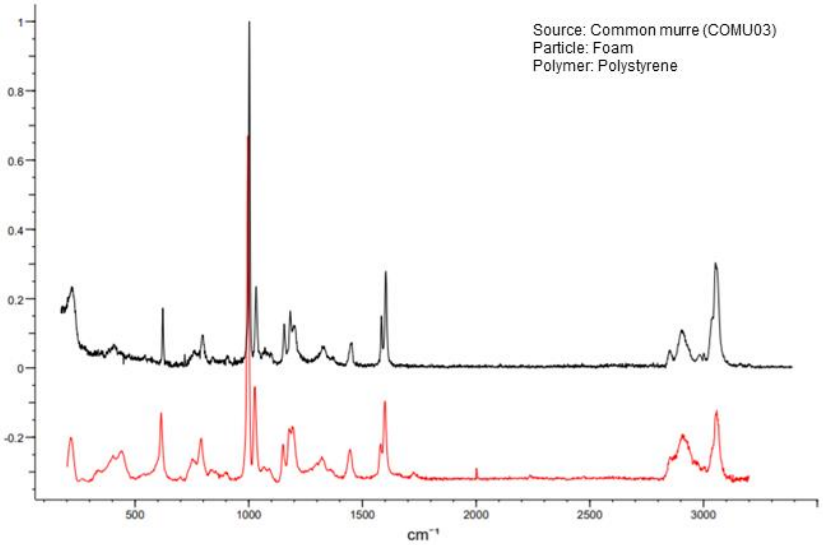
Raman spectra of 57 particles analyzed (excludes four spectra shown in Figure 4): 18 particles recovered from northern anchovies (*Engraulis mordax*), 6 particles recovered from common murres (*Uria aalge*), 27 particles recovered from seawater, 2 control particles from lab coats, and 4 particles from atmospheric contamination. Specific anchovy ID source is unknown. Spectral libraries for polymer matching were Wiley KnowItAll, SLoPP, and SLoPP-E. Red line represents particle spectra. Black line represents matched polymer spectra. For some particles, line representation colors are different and noted with specific spectra.

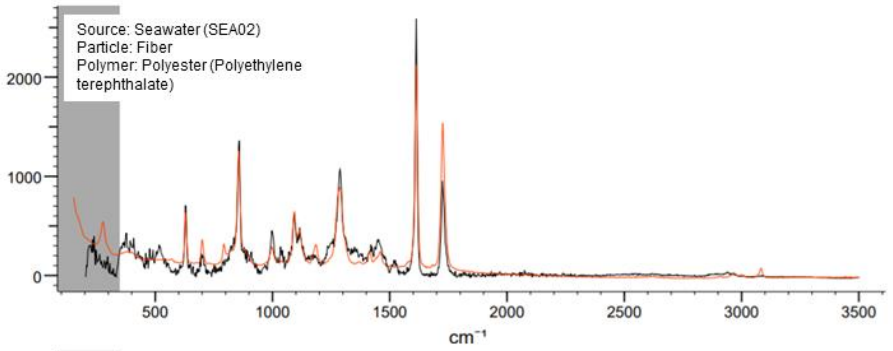


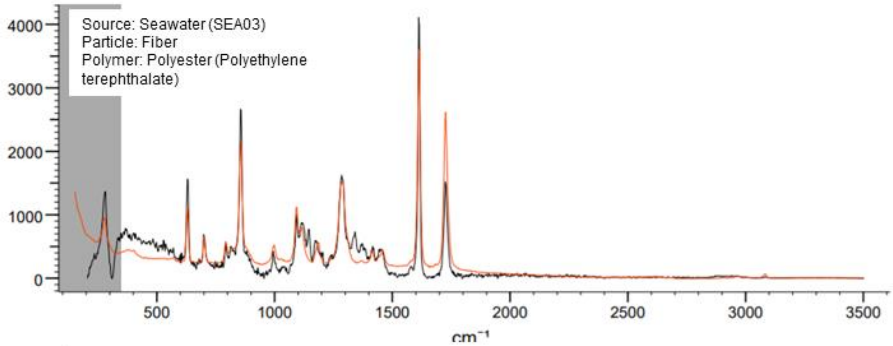

Source: Seawater (SEA01)
Particle: Film
Polymer: Polyester
cm⁻¹

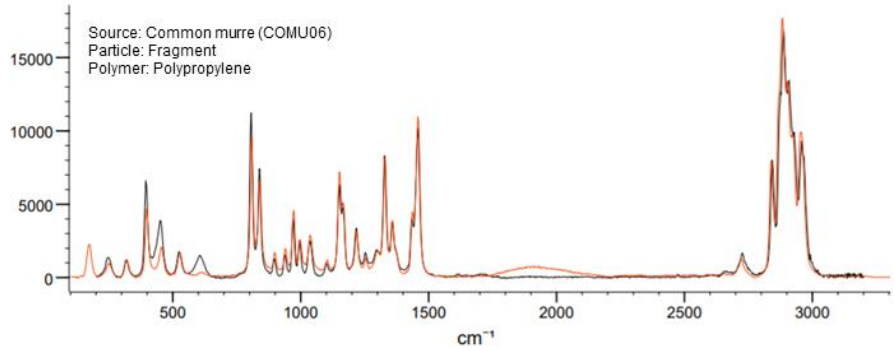

Source: Seawater (SEA01)
Particle: Fiber
Polymer: Polyacrylamide-co-acrylic acid
cm⁻¹

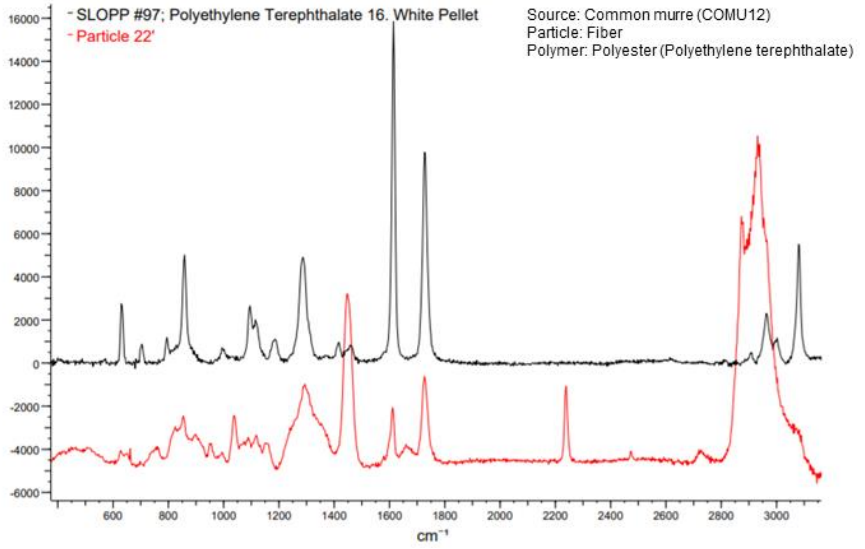

Source: Seawater (SEA01)
Particle: Fiber
Polymer: MMA co TMI (acrylic copolymer)
cm⁻¹

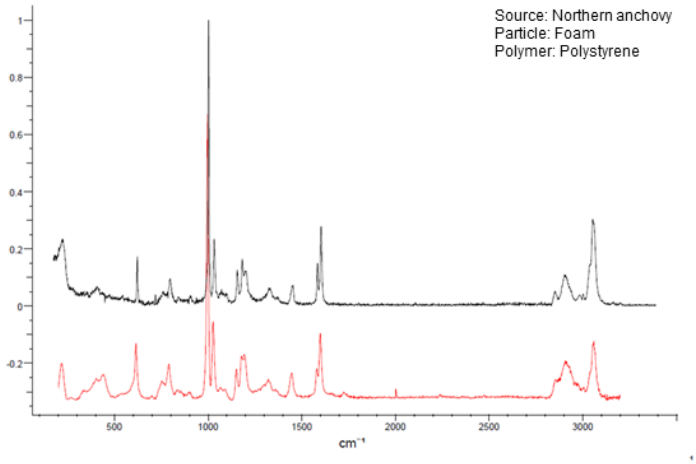

Source: Common murre (COMU03)
Particle: Foam
Polymer: Polystyrene
cm⁻¹


Source: Seawater (SEA02)
Particle: Fiber
Polymer: Polyester (Polyethylene terephthalate)
cm⁻¹

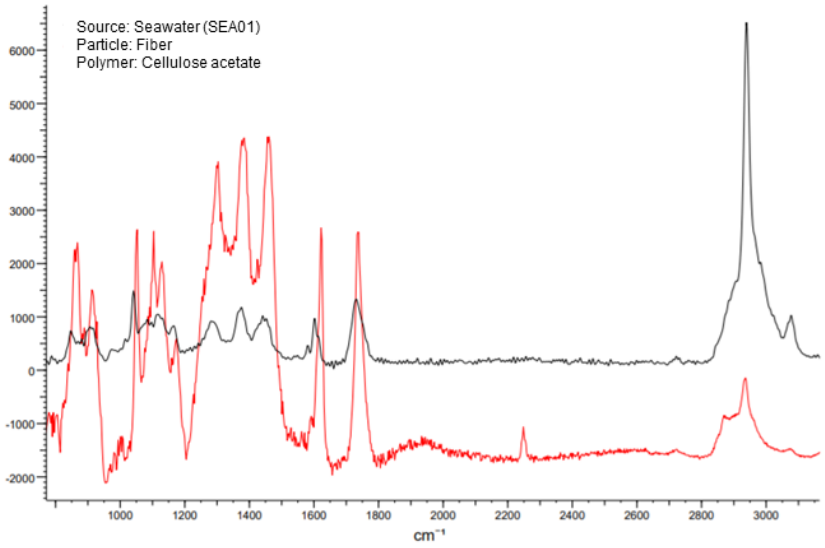

Source: Seawater (SEA03)
Particle: Fiber
Polymer: Polyester (Polyethylene terephthalate)
cm⁻¹

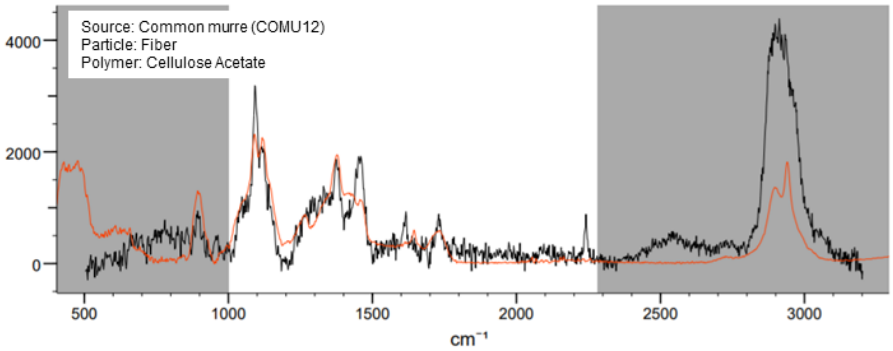

Source: Common murre (COMU06)
Particle: Fragment
Polymer: Polypropylene
cm⁻¹


SLOPP #97; Polyethylene Terephthalate 16. White Pellet
Particle 22'
Source: Common murre (COMU12)
Particle: Fiber
Polymer: Polyester (Polyethylene terephthalate)
cm⁻¹

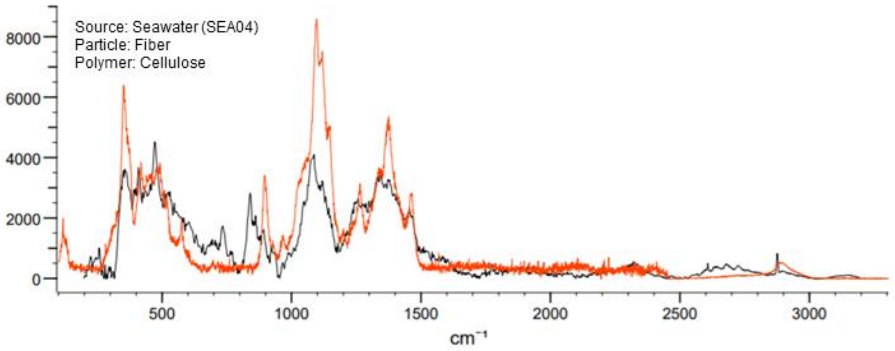


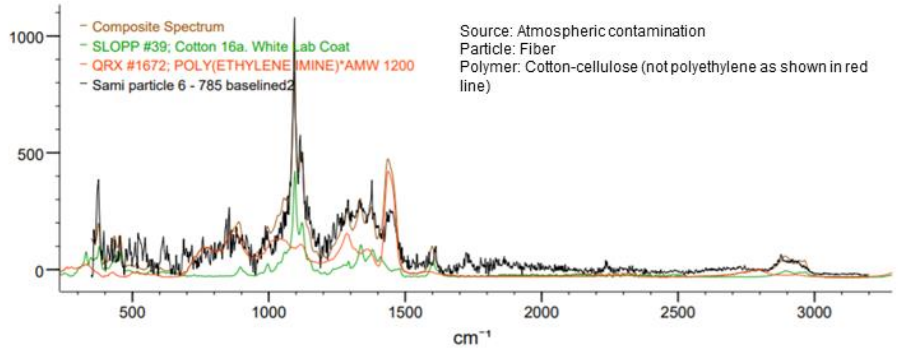
Categorization: Semi-Synthetic

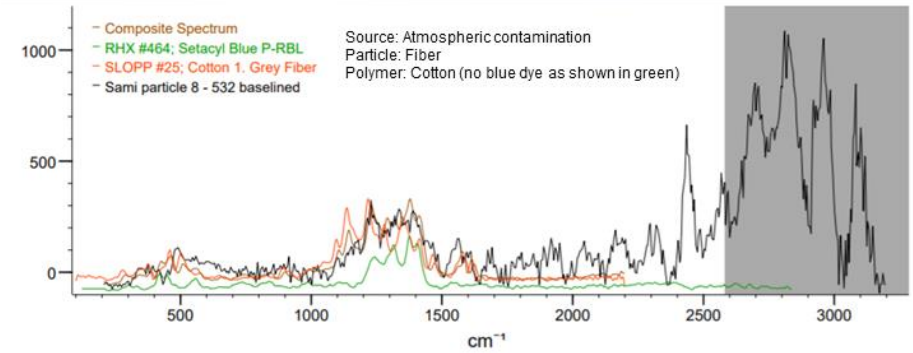


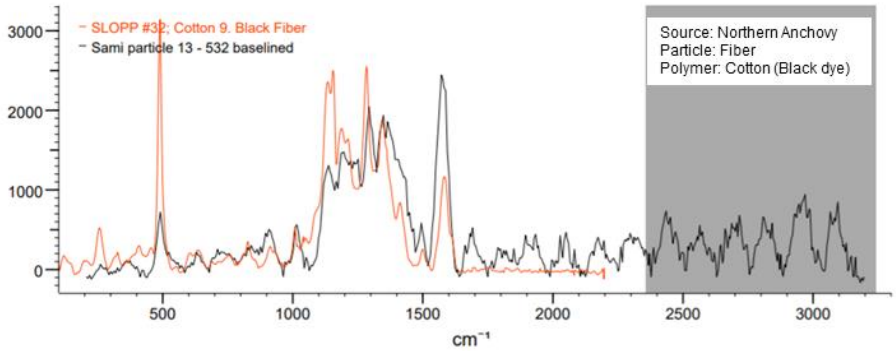


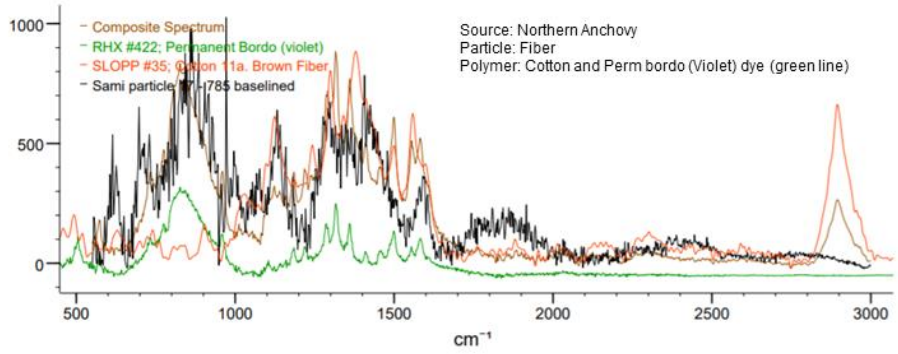
Categorization: Natural

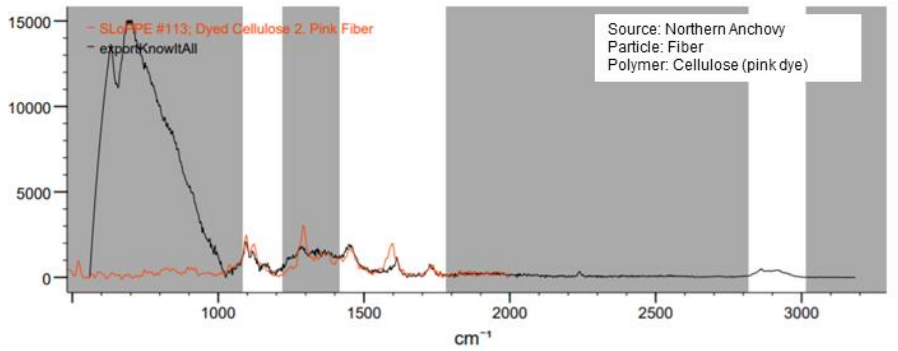


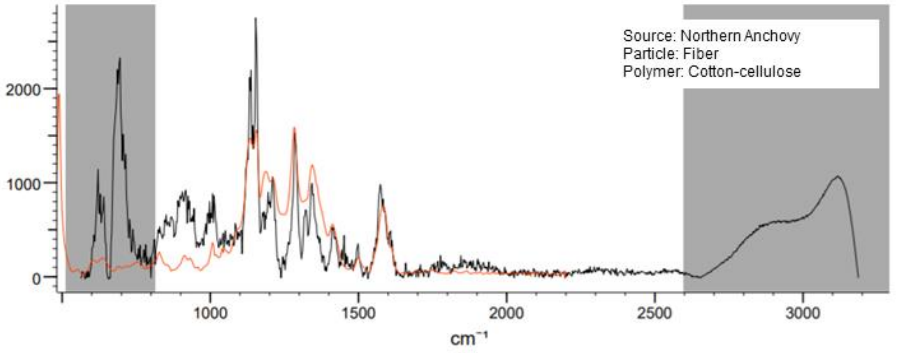


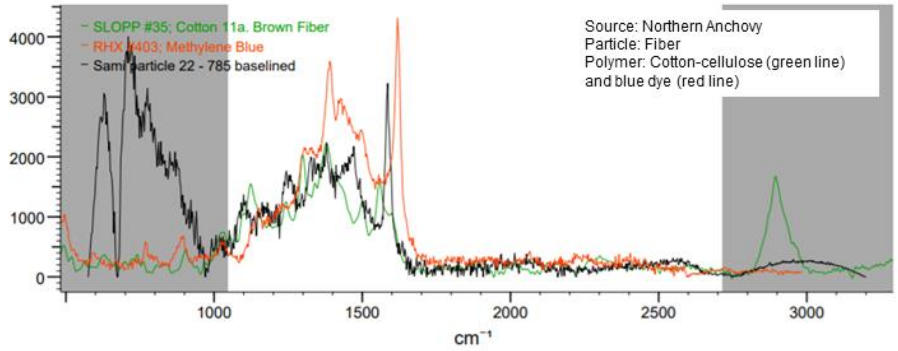


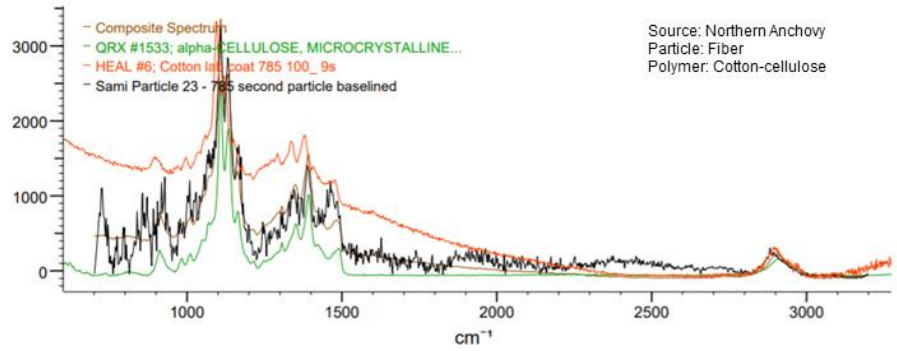


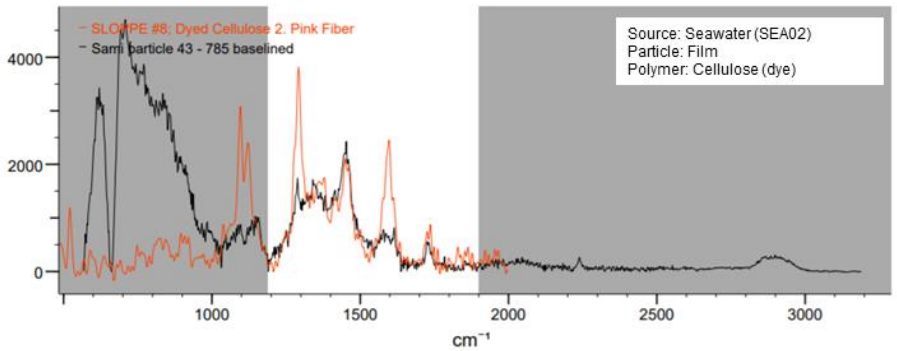

Composite Spectrum
RHX #422; Permanent Bordo (violet)
SLOPP #35; Cotton 11a. Brown Fiber
Sami particle 22 - 785 baselined
Source: Northern Anchovy
Particle: Fiber
Polymer: Cotton and Perm bordo (Violet) dye (green line)
cm⁻¹

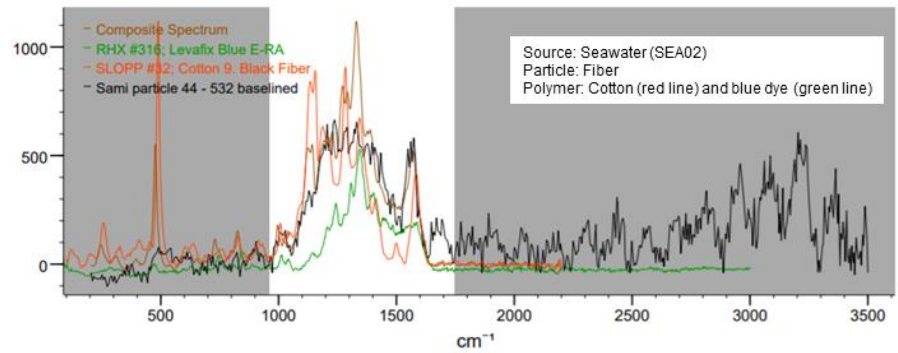

SLOPE #113; Dyed Cellulose 2. Pink Fiber
exportKnowItAll
Source: Northern Anchovy
Particle: Fiber
Polymer: Cellulose (pink dye)
cm⁻¹

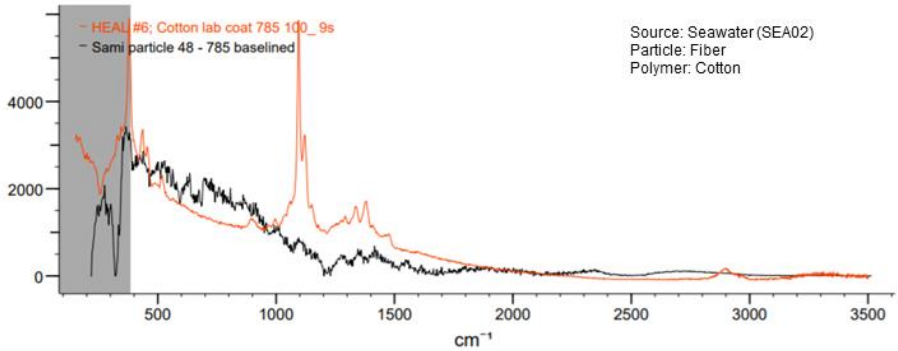

Source: Northern Anchovy
Particle: Fiber
Polymer: Cotton-cellulose
cm⁻¹

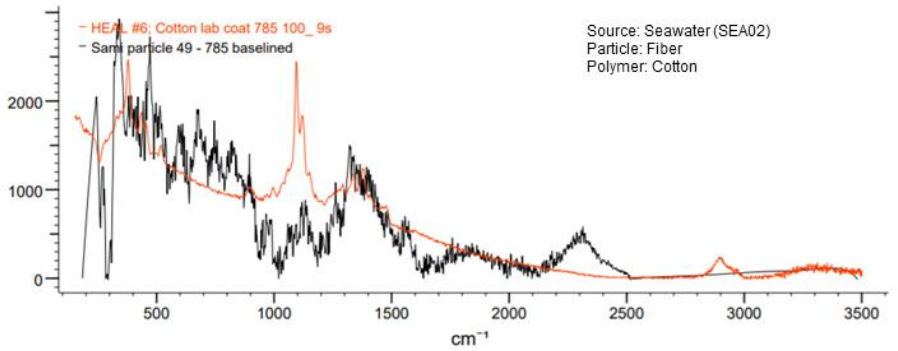

SLOPP #35; Cotton 11a. Brown Fiber
RHX #403; Methylene Blue
Sami particle 22 - 785 baselined
Source: Northern Anchovy
Particle: Fiber
Polymer: Cotton-cellulose (green line)
and blue dye (red line)
cm⁻¹

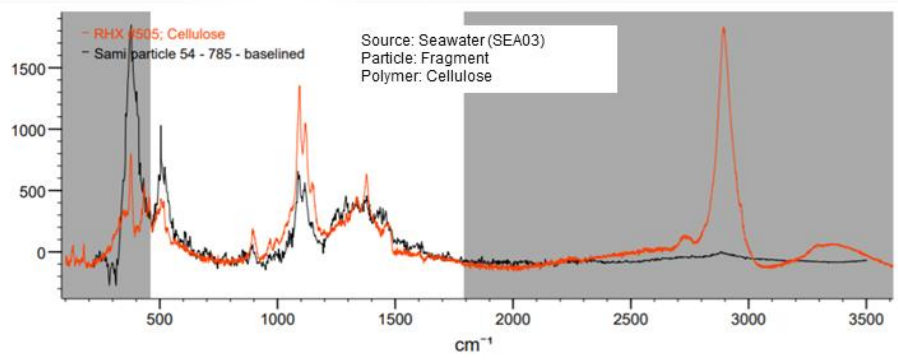

Composite Spectrum
QRX #1533; alpha-CELLULOSE, MICROCRYSTALLINE...
HEAL #6; Cotton lab coat 785 100_ 9s
Sami Particle 23 - 785 second particle baselined
Source: Northern Anchovy
Particle: Fiber
Polymer: Cotton-cellulose
cm⁻¹

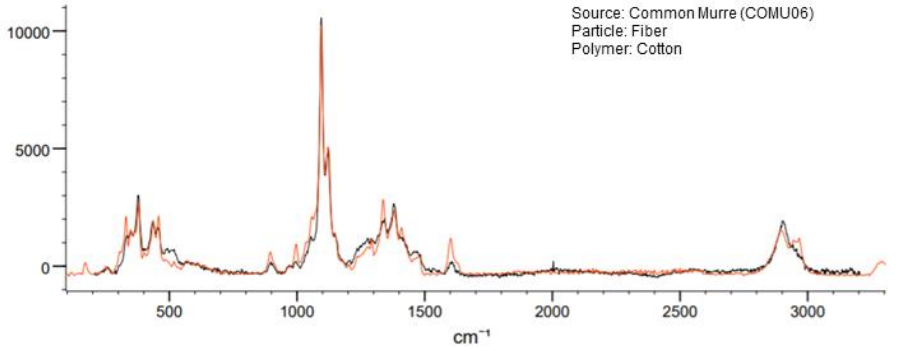

SLOPPE #8; Dyed Cellulose 2. Pink Fiber
Sami particle 43 - 785 baselined
Source: Seawater (SEA02)
Particle: Film
Polymer: Cellulose (dye)
cm⁻¹

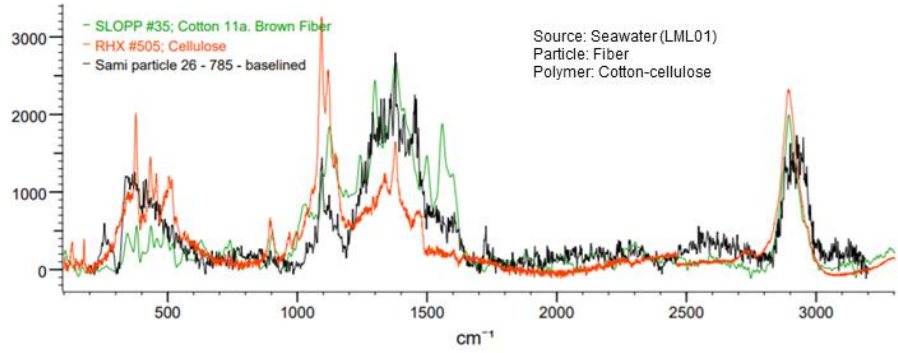

Composite Spectrum
RHX #316; Levafix Blue E-RA
SLOPP #32; Cotton 9. Black Fiber
Sami particle 44 - 532 baselined
Source: Seawater (SEA02)
Particle: Fiber
Polymer: Cotton (red line) and blue dye (green line)
cm⁻¹

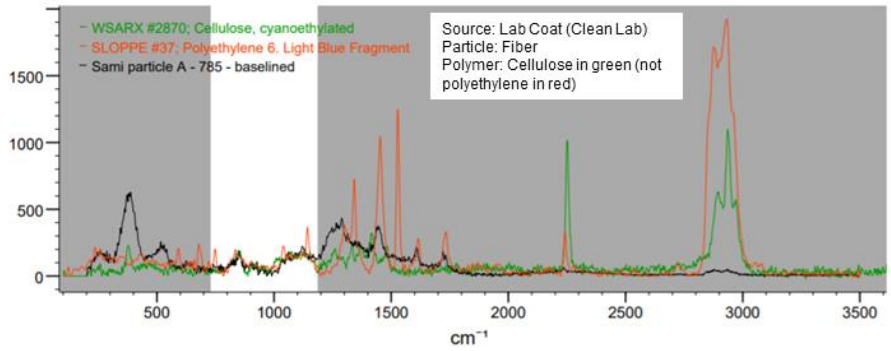

HEAL #6; Cotton lab coat 785 100_ 9s
Sami particle 48 - 785 baselined
Source: Seawater (SEA02)
Particle: Fiber
Polymer: Cotton
cm⁻¹

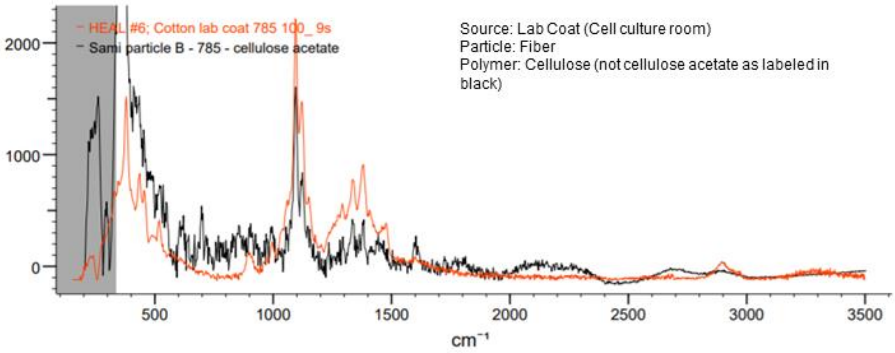

HEAL #6; Cotton lab coat 785 100_ 9s
Sami particle 49 - 785 baselined
Source: Seawater (SEA02)
Particle: Fiber
Polymer: Cotton
2000
1000
0
500
1000
1500
2000
2500
3000
3500
cm⁻¹


RHX #505; Cellulose
Sami particle 54 - 785 - baselined
Source: Seawater (SEA03)
Particle: Fragment
Polymer: Cellulose
1500
1000
500
0
500
1000
1500
2000
2500
3000
3500
cm⁻¹

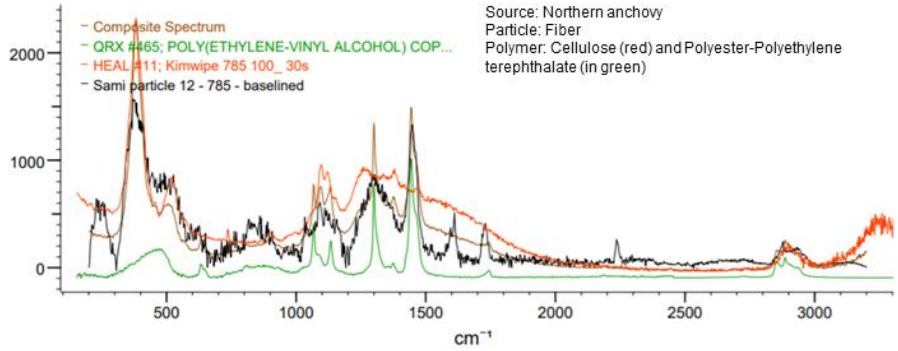

Source: Common Murre (COMU06)
Particle: Fiber
Polymer: Cotton
10000
5000
0
500
1000
1500
2000
2500
3000
cm⁻¹

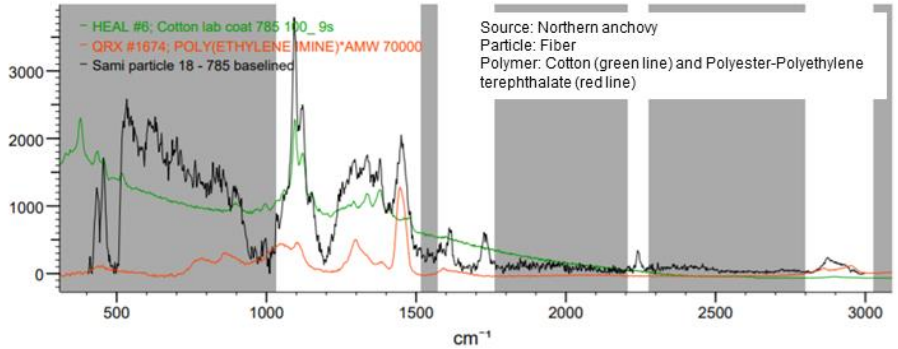

SLOPP #35; Cotton 11a. Brown Fiber
RHX #505; Cellulose
Sami particle 26 - 785 - baselined
Source: Seawater (LML01)
Particle: Fiber
Polymer: Cotton-cellulose
3000
2000
1000
0
500
1000
1500
2000
2500
3000
cm⁻¹

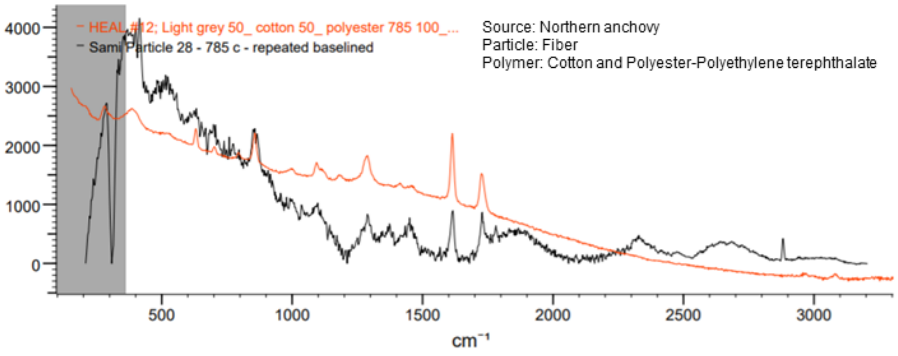


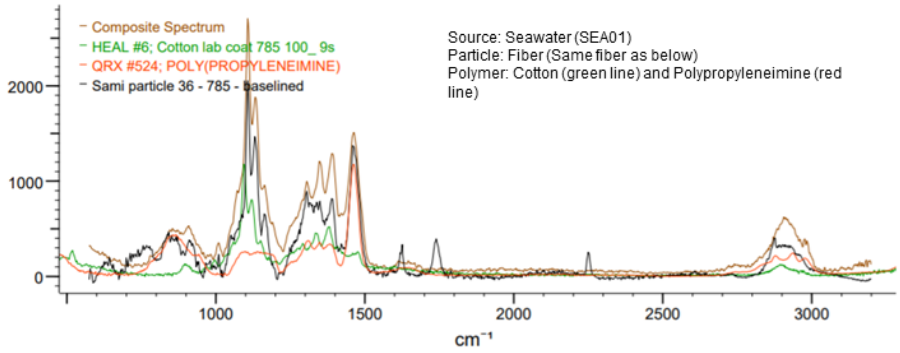


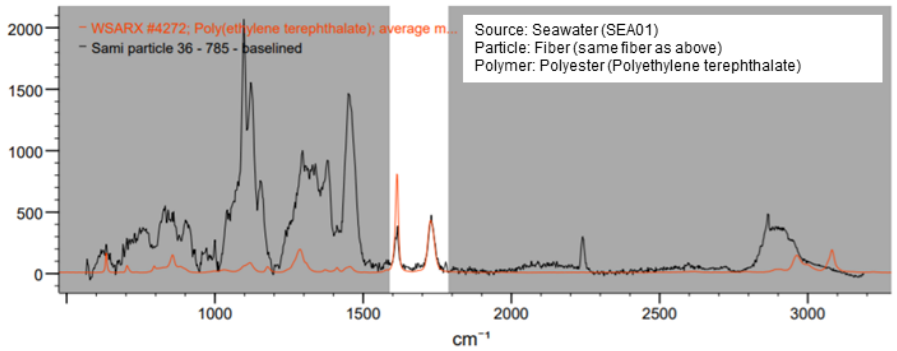
Categorization: Blends



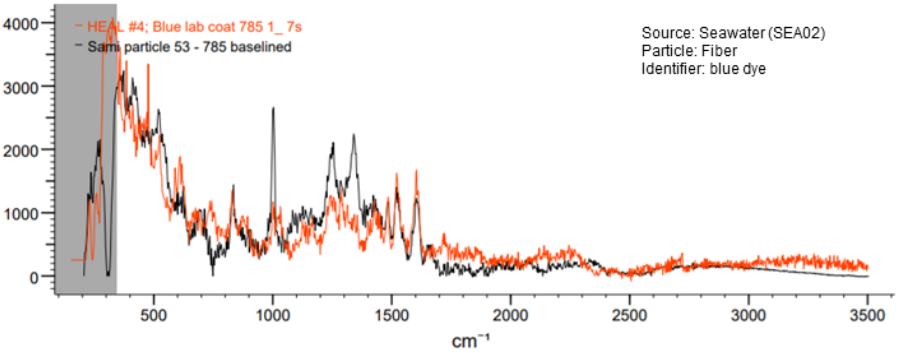


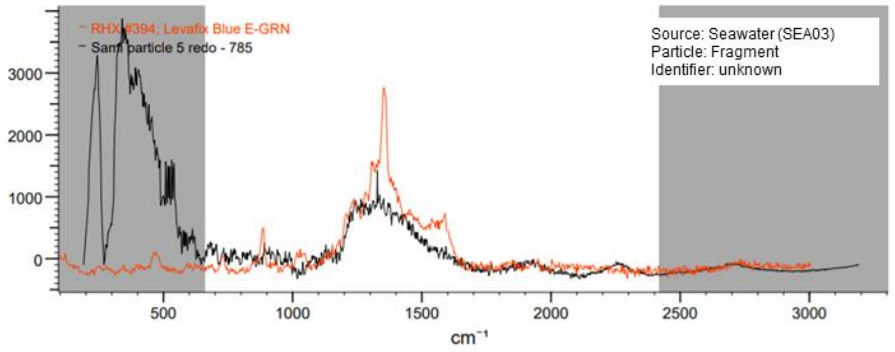


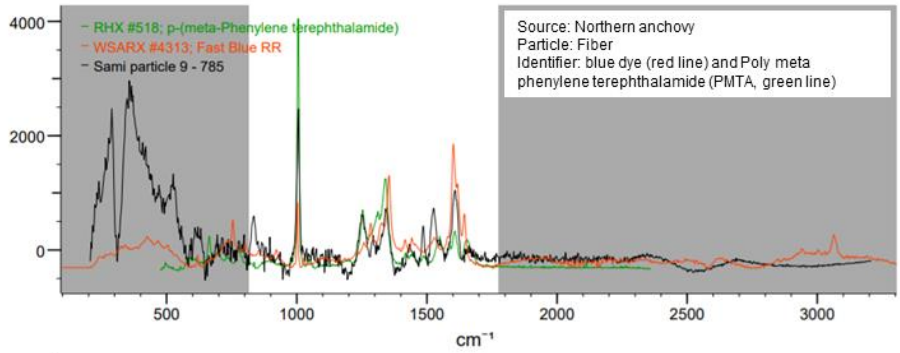


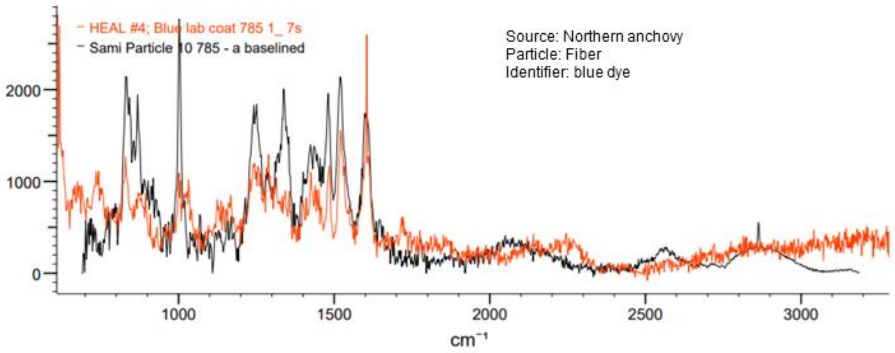


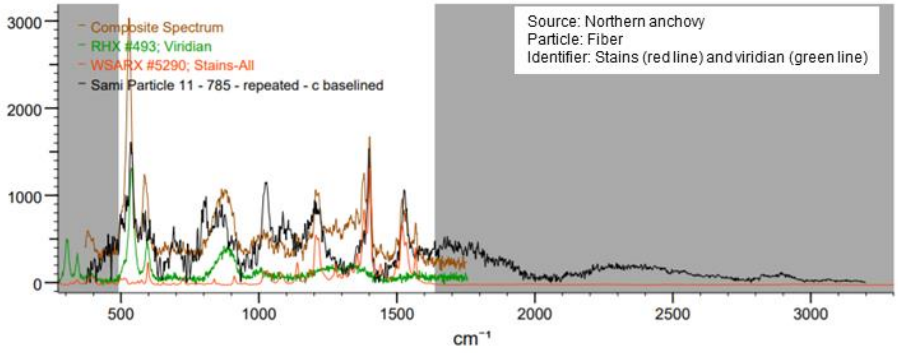
Categorization: Dye-prominent

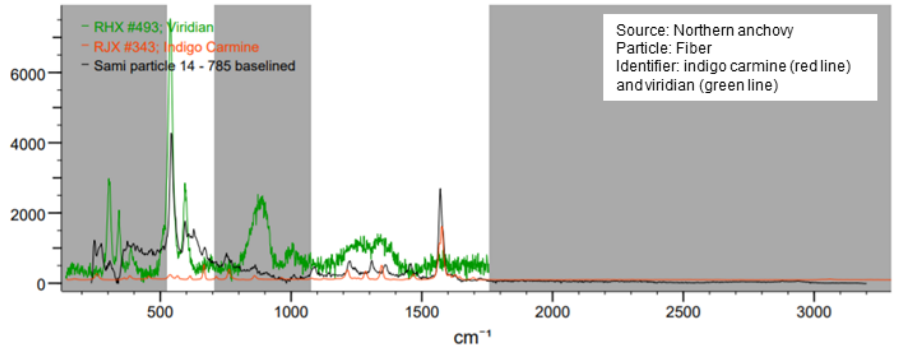


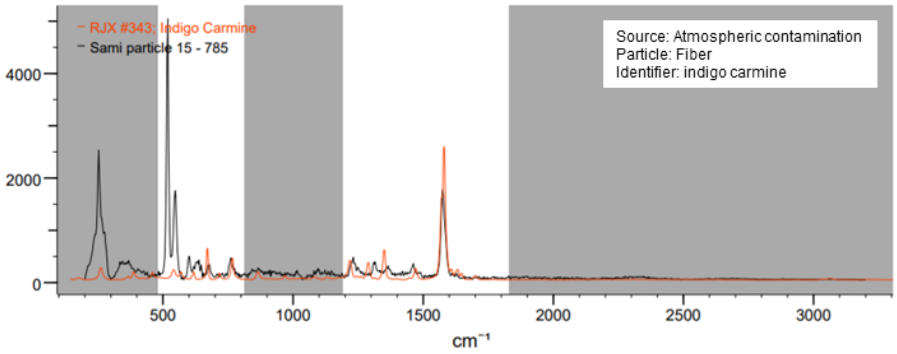

RHX #394; Levafix Blue E-GRN
Sami particle 5 redo - 785
Source: Seawater (SEA03)
Particle: Fragment
Identifier: unknown
3000
2000
1000
0
500
1000
1500
2000
2500
3000
cm⁻¹

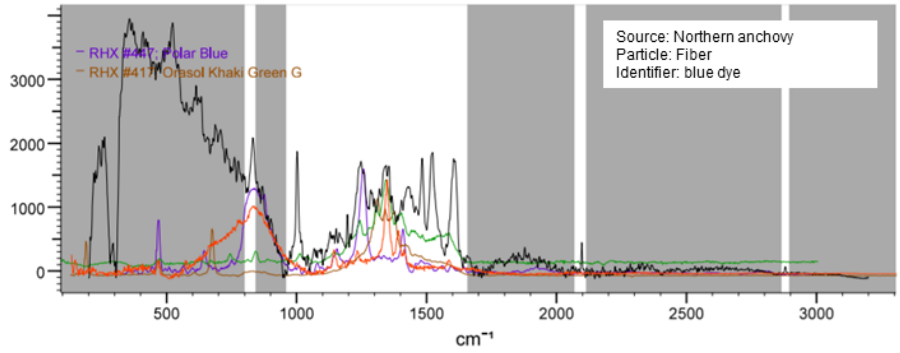

RHX #518; p-(meta-Phenylene terephthalamide)
WSARX #4313; Fast Blue RR
Sami particle 9 - 785
Source: Northern anchovy
Particle: Fiber
Identifier: blue dye (red line) and Poly meta phenylene terephthalamide (PMTA, green line)
4000
2000
0
500
1000
1500
2000
2500
3000
cm⁻¹

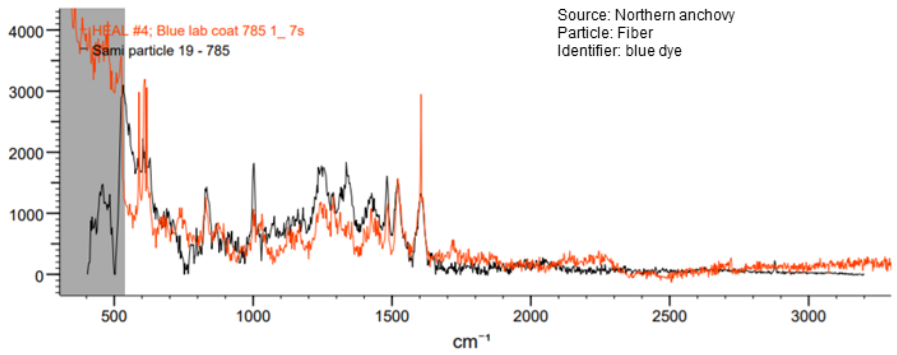

HEAL #4; Blue lab coat 785 1_ 7s
Sami Particle 10 785 - a baselined
Source: Northern anchovy
Particle: Fiber
Identifier: blue dye
2000
1000
0
1000
1500
2000
2500
3000
cm⁻¹

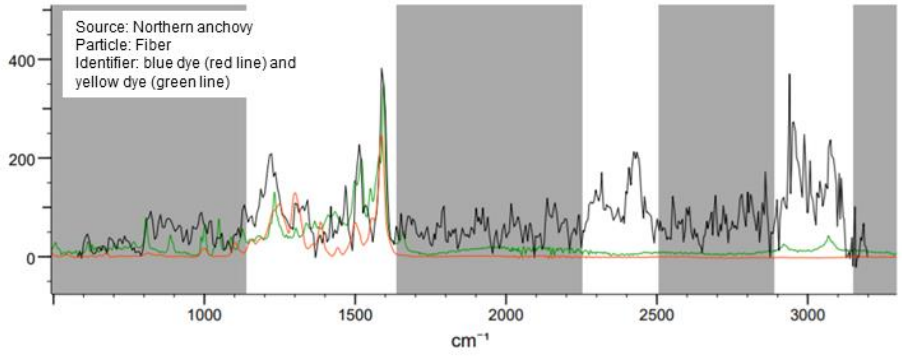

Composite Spectrum
RHX #493; Viridian
WSARX #5290; Stains-All
Sami Particle 11 - 785 - repeated - c baselined
Source: Northern anchovy
Particle: Fiber
Identifier: Stains (red line) and viridian (green line)
3000
2000
1000
0
500
1000
1500
2000
2500
3000
cm⁻¹

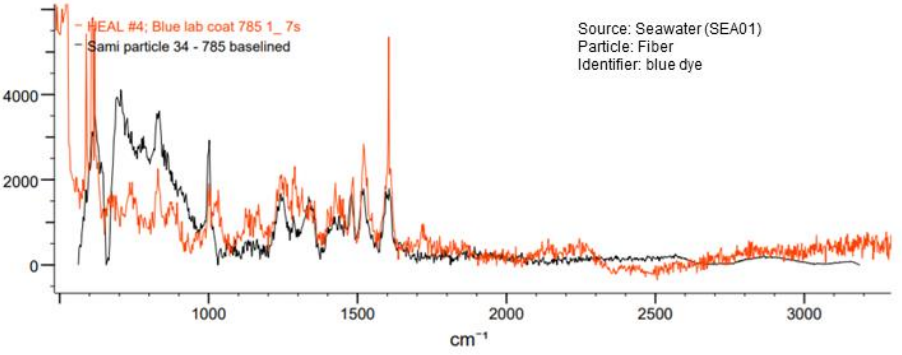

RHX #493; Viridian
RJX #343; Indigo Carmine
Sami particle 14 - 785 baselined
Source: Northern anchovy
Particle: Fiber
Identifier: indigo carmine (red line) and viridian (green line)
6000
4000
2000
0
500
1000
1500
2000
2500
3000
cm⁻¹

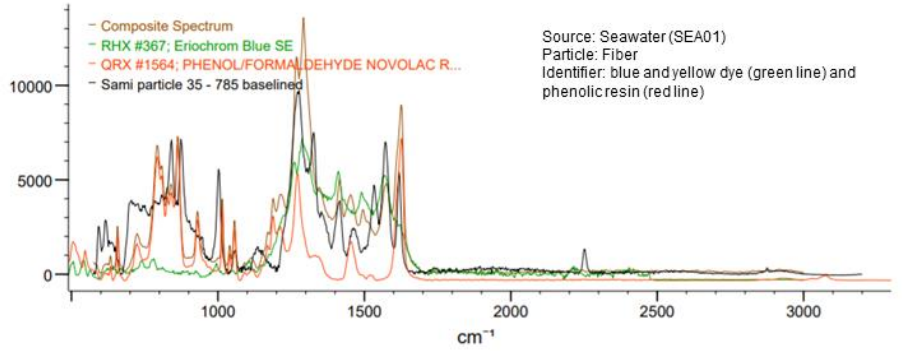

RJX #343; Indigo Carmine
Sami particle 15 - 785
Source: Atmospheric contamination
Particle: Fiber
Identifier: indigo carmine
4000
2000
0
500
1000
1500
2000
2500
3000
cm⁻¹

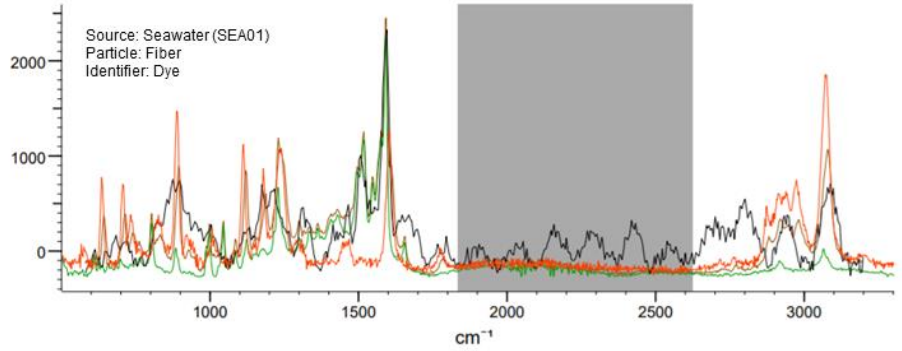

RHX #417; Orasol Khaki Green G
Source: Northern anchovy
Particle: Fiber
Identifier: blue dye
3000
2000
1000
0
500
1000
1500
2000
2500
3000
cm⁻¹

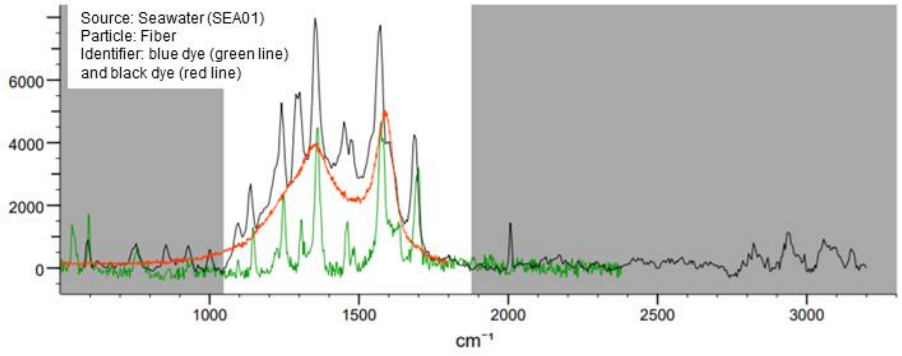

HSAL #4; Blue lab coat 785 1_ 7s
Sami particle 19 - 785
Source: Northern anchovy
Particle: Fiber
Identifier: blue dye
4000
3000
2000
1000
0
500
1000
1500
2000
2500
3000
cm⁻¹

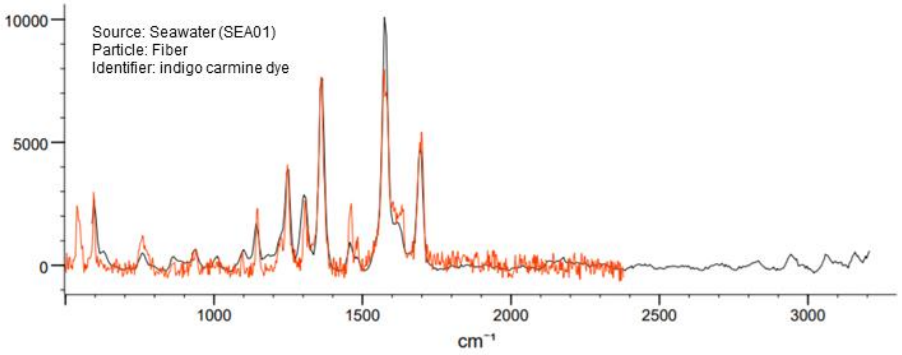

Source: Northern anchovy
Particle: Fiber
Identifier: blue dye (red line) and yellow dye (green line)
400
200
0
1000
1500
2000
2500
3000
cm⁻¹

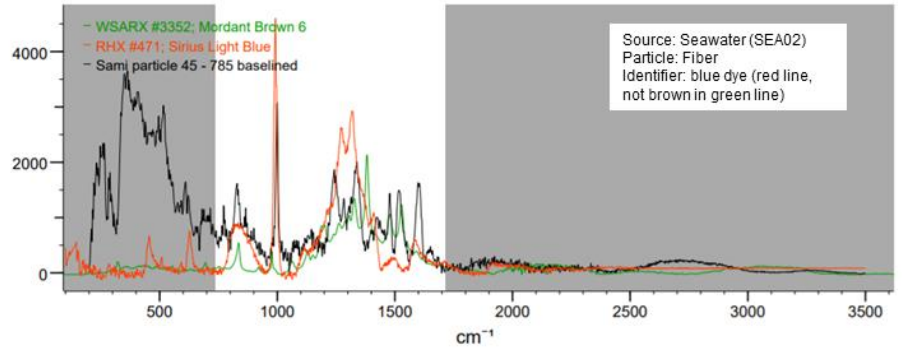

HEAL #4; Blue lab coat 785 1_ 7s
Sami particle 34 - 785 baselined
Source: Seawater (SEA01)
Particle: Fiber
Identifier: blue dye
4000
2000
0
1000
1500
2000
2500
3000
cm⁻¹

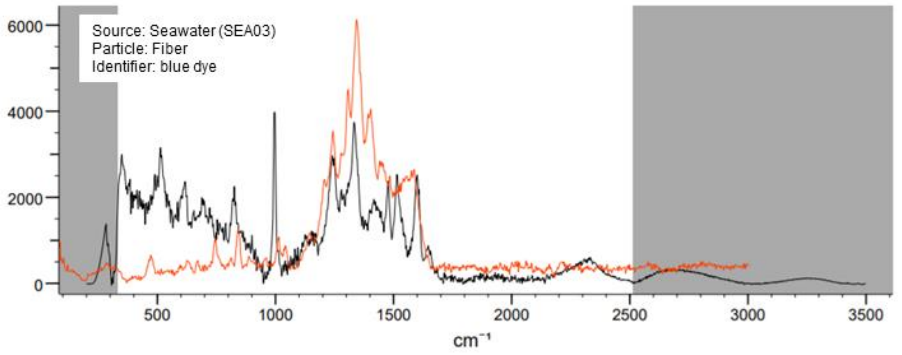

Composite Spectrum
RHX #367; Eriochrom Blue SE
QRX #1564; PHENOL/FORMALDEHYDE NOVOLAC R...
Sami particle 35 - 785 baselined
Source: Seawater (SEA01)
Particle: Fiber
Identifier: blue and yellow dye (green line) and phenolic resin (red line)
10000
5000
0
1000
1500
2000
2500
3000
cm⁻¹

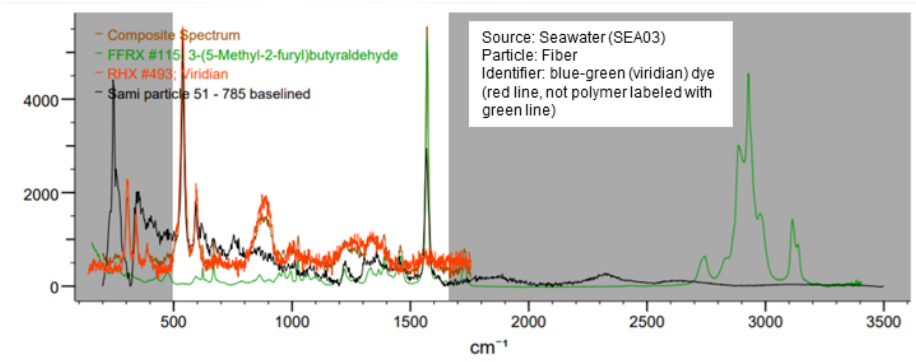

Source: Seawater (SEA01)
Particle: Fiber
Identifier: Dye
2000
1000
0
1000
1500
2000
2500
3000
cm⁻¹


Source: Seawater (SEA01)
Particle: Fiber
Identifier: blue dye (green line)
and black dye (red line)
6000
4000
2000
0
1000
1500
2000
2500
3000
cm⁻¹

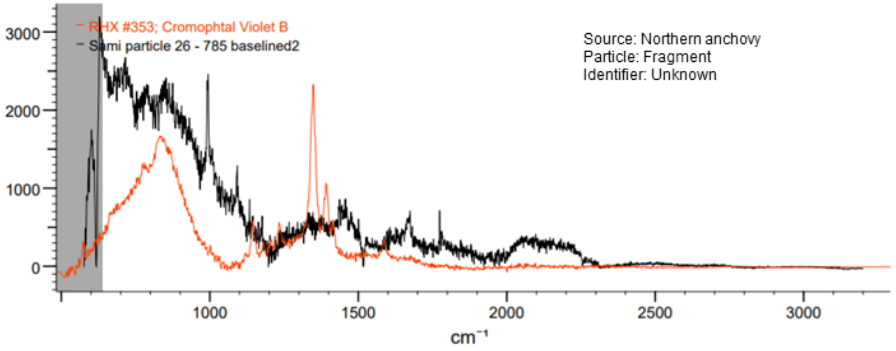

Source: Seawater (SEA01)
Particle: Fiber
Identifier: indigo carmine dye
10000
5000
0
1000
1500
2000
2500
3000
cm⁻¹

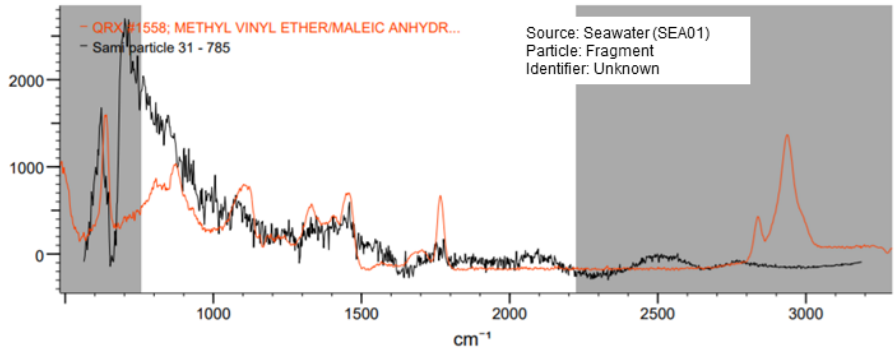

WSARX #3352; Mordant Brown 6
RHX #471; Sirius Light Blue
Sami particle 45 - 785 baselined
Source: Seawater (SEA02)
Particle: Fiber
Identifier: blue dye (red line,
not brown in green line)
4000
2000
0
500
1000
1500
2000
2500
3000
3500
cm⁻¹


Source: Seawater (SEA03)
Particle: Fiber
Identifier: blue dye
6000
4000
2000
0
500
1000
1500
2000
2500
3000
3500
cm⁻¹



Categorization: Unknown





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