The Sources of Microplastics in San Francisco Bay, California, USA

by

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Abstract
Understanding the sources of microplastics to an ecosystem informs mitigation. Here, I determined the importance of specific sources of microplastics to San Francisco Bay – a heavily populated bay in California, USA. I quantified and characterized microplastics in surface waters of the bay and adjacent National Marine Sanctuaries (NMS). I also quantified and characterized microplastics in source waters: wastewater effluent and stormwater runoff. I compared the diversity of microplastics in source waters to surface water. My results suggest that stormwater runoff influenced microplastic concentrations in Central Bay and South Bay, while wastewater effluent influenced microplastics in North Bay and Lower South Bay. Our findings show that effective mitigation strategies may vary by location. Throughout this project, we came across difficulties identifying fibers using spectroscopic techniques and developed a method to circumvent barriers. The success of the method was confirmed and applied to samples from The Bay to demonstrate its applicability.
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Chapter 1

1 The Sources of Microplastics in San Francisco Bay, California, USA

1.1 Introduction

Each year, approximately 4 to 12 million metric tonnes (MMT) of plastics enter the marine environment from diverse sources. By count, most of the plastics in the environment are microplastics, which are plastic pieces less than 5 mm in their longest dimension. Some of these microplastics are small upon production – also referred to as primary microplastics - and others are a product of breaking up into smaller pieces – also referred to as secondary microplastics. When they are produced to be small in size, they come from primary sources, such as industrial pellet spills from plastic manufacturing plants or microbeads from personal care products. Secondary sources are generated from the chemical, biological and/or physical breakdown of larger pieces of plastic. Secondary microplastics include microfibers from textiles, tire wear particles and plastic fragments from single-use products like straws, bottles and containers. Not only are microplastics contaminating the marine environment, but we now know they are ubiquitous in freshwater and terrestrial ecosystems as well. As the rate of plastic production increases each year, it is expected that more plastic pollution will enter the environment.

As a result of this growing and widespread contamination, more than 220 species are documented to have ingested microplastics. Microplastic exposure may pose a variety of risks to living organisms. Microplastics may physically harm organisms through perforation and blockage of the gastrointestinal tract or lead to starvation. The smaller the plastic particles, especially for the smallest fraction of micro- and nano-sized plastics, the greater their potential to accumulate and biomagnify up food chains because of their potential to translocate outside the gut to other tissues. In addition to physical harm, microplastic pollution may chemically harm organisms by transferring toxins (i.e., additive chemicals or those sorbed from ambient water) to living organisms upon exposure. It is important that more research is conducted to fully characterize the exposure and
potential risk that microplastics pose to living organisms. In parallel, and to help mitigate microplastic pollution, it is important that we work to understand the sources and pathways by which microplastics are entering the environment.

Plastic products and microplastics in aquatic environments originate from a variety of sources – which over time are predicted to generate more and more microplastic particles via fragmentation. Sources include fishing activities, litter from land, personal care products, and shedding of synthetic fibers from textiles\(^1\,^2\). Microplastics also enter the environment via different pathways, including industrial spills, wastewater effluent, and stormwater runoff. Since characteristic polymers are used to make characteristic products, identifying the polymer types of microplastics in the environment is an important step to source-apportioning microplastic pollution\(^2\). The shapes or categories of plastic, in combination with their colour, are also important in determining sources\(^2\). For instance, pink polyester fibers may have originated from washing clothes, while blue PP fibers may have originated from fishing rope. Once microplastic pollution is source-apportioned, its input can be more effectively mitigated\(^2\).

San Francisco Bay (herein after, The Bay) is an urban bay located in California, USA and was previously found to have relatively high densities of microplastic pollution\(^3\). Given the large population that surrounds the Bay Area, the high number of wastewater treatment plants that drain into it, the diversity of recreational activities in The Bay, as well as the many surrounding and diverse industries (e.g., agriculture, shipping), there are likely multiple sources of microplastic pollution to The Bay. This, in combination with the importance of this estuarine ecosystem, makes The Bay an ideal location for a case study to look closer at the contamination of microplastics and to use the assessment to inform sources and fate of microplastic pollution.
Here, baseline concentrations of microplastic contamination across The Bay were measured in surface water and the relationship between microplastics and their pathways to the environment were explored. The two pathways for microplastic pollution to the environment that we investigated are stormwater runoff and wastewater treatment plant (WWTP) effluent. We measured and characterized microplastics in wastewater and stormwater as well as in surface water across the Bay and adjacent marine sanctuaries. We used this data to gain a better understanding of the relative importance of WWTP effluent and stormwater runoff as pathways for microplastic pollution to reach SF Bay and the adjacent marine sanctuaries. Our overall aim is to analyze the distribution of microplastics in The Bay and to better understand the sources of microplastics entering The Bay. We predict that concentrations of microplastic particles in The Bay will be different from concentrations in the marine sanctuaries. We also predict that concentrations within The Bay will vary among sites due to different current patterns and inputs from land. Finally, to inform sources, we explore whether quantities and characterization of microplastics in WWTP effluent and stormwater runoff correlate with those in surface water.
1.2 Materials and Methods

**Sampling Locations**

The Bay is an estuarine system fed by freshwater from the Sacramento and San Joaquin rivers in the north and connected to the Pacific Ocean in the west via the Golden Gate strait (Figure 1). The Bay is the largest estuary on the west coast of North America, and drains roughly 40% of the waters of California. The Bay Area is home to more than 7 million people who rely on The Bay for agricultural, sanitary, food, and recreational purposes. Wastewater treatment plants release effluent into The Bay from over 30 discharge points. The Bay consists of four main regions: North Bay (NB; incorporating San Pablo Bay and Suisun Bay), Central Bay (CB), South Bay (SB), and Lower South Bay (LSB; Figure 1). In this study, we sampled from each region. On the west side of the Golden Gate strait adjacent to The Bay are the National Marine Sanctuaries (NMS; Figure 1). NMS contain nationally important coastal and marine areas including whale migration corridors, estuarine habitats, kelp forests, and rocky coasts among others, which are managed and protected by The National Oceanic and Atmospheric Administration’s (NOAA) Office of NMS. In this study, three NMS were sampled: Cordell Bank, Monterey Bay, and the Greater Farallones Sanctuaries.

**Sample Collection**

To measure the contamination and assess the fate of microplastics in the Bay, we sampled surface water. To better understand sources and pathways, we sampled stormwater and wastewater. Samples of all matrices were taken across the Bay. Forty-eight samples were also taken in the NMS. Surface water samples, across all sites, were taken during two different seasons (wet and dry). In California, the dry season occurs between June and September and the wet season extends from November to April. Table 1 shows the final number of surface water (bulk-water and Manta net), stormwater, and
wastewater samples collected, quantified, and characterized in this study for baseline monitoring of concentrations across the bay and NMS. The methods for collection of each sample type are as follows.

**Surface Water**

Two types of surface water samples were collected for microplastics. Larger sized microplastic particles were sampled using a surface Manta Trawl with a 333 µm mesh. Smaller-sized microplastics were sampled via one-liter grab samples. The Manta Trawl consisted of a modified Neuston net with a rectangular opening 16 cm high by 60 cm wide. The net was towed off to the side of the vessel for 30 min at less than 3 knots. A flow meter with a standard rotor (General Oceanics, Miami FL) was attached to the Manta net. Manta net contents were rinsed with deionized (DeI) water onto a 333 µm sieve, and sieve contents were rinsed into a glass jar using tweezers and a spoon. For storage, rubbing alcohol was added to the jar to prevent algal growth. A total of 65 Manta samples were collected and included in this study. One-liter grab samples were sampled to quantify and characterize smaller-sized particles, including microfibers, more accurately than Manta samples. At each site, a one-liter amber glass bottle was lowered into the water, rinsed with sampled water three times, and then filled to the top with surface water. A total of 52 one-liter grab samples were collected and analyzed in this study. Grab samples were taken less frequently than Manta samples, but when they were sampled they were sampled alongside a Manta sample for comparison. Surface water samples were taken from Lower South Bay, South Bay, Central Bay, and North Bay (including Suisun Bay and San Pablo Bay) within The Bay, as well as from the Greater Farallones, Cordell Bank, and Monterey Bay in the National Marine Sanctuaries. For a description of how many samples were taken at each location see Table 1. In addition, field blanks and field duplicates were taken at certain sites. Field blanks were sampled to account for procedural contamination and field duplicates were collected to measure within-site
variability. For Manta field blanks, the number collected at each region were: 2 from North Bay, 1 from Central Bay, 2 from South Bay, 1 from Lower South Bay, and 1 from each of Cordell Bank, Greater Farallones, and Monterey Bay. For Manta field duplicates, the number collected at each region were: 4 from Central Bay, 1 from South Bay, 1 from Lower South Bay, 2 from Greater Farallones, and 1 from Monterey Bay. For one-liter grab field blanks, 2 were collected in South Bay and 1 was collected in Greater Farallones. For one-liter grab field duplicates, the number collected at each region were: 1 from Central Bay, 1 from Greater Farallones, and 1 from Monterey Bay. All samples were collected in the summer of 2017 (dry season) and winter of 2017-2018 (wet season).

**Table 1.** Summary of samples collected in The Bay and in NMS for Manta, one-liter grab, stormwater, and wastewater that were used in this study (not including field blanks and lab blanks).

<table>
<thead>
<tr>
<th>Location</th>
<th># of One-Liter Grab (&gt; 100 µm) Samples</th>
<th># of Manta (&gt; 355 µm) Samples</th>
<th># of Stormwater (&gt; 125 µm) Samples</th>
<th># of Treated Wastewater Effluent (&gt; 125 µm) Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>North Bay</td>
<td>6</td>
<td>6</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Central Bay</td>
<td>13</td>
<td>17</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>South Bay</td>
<td>7</td>
<td>9</td>
<td>3</td>
<td>N/A</td>
</tr>
<tr>
<td>Lower South Bay</td>
<td>4</td>
<td>7</td>
<td>1</td>
<td>7</td>
</tr>
<tr>
<td>National Marine Sanctuaries</td>
<td>22</td>
<td>26</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Tomales Bay</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Total</td>
<td>52</td>
<td>65</td>
<td>13</td>
<td>18</td>
</tr>
</tbody>
</table>
Wastewater Effluent

Final effluent from wastewater treatment plants were collected from eight different facilities across The Bay: Central Contra Costa Sanitation District in North Bay, San Francisco Public Utilities Commission-Southeast, East Bay Municipal Utility District and East Bay Dischargers Authority in Central Bay, Fairfield Suisun in South Bay, and Sunnyvale, Palo Alto and San Jose Santa Clara in Lower South Bay. Final effluent was sampled directly from the final effluent pipe at the plant through 355 µm and 125 µm metal sieves stacked together for a period of 24 hours. The contents on the sieves were then rinsed into respective clean glass sampling containers with DI water. Rubbing alcohol was added to each sample to prevent algal growth. Sampling occurred in the fall of 2017. One field blank was taken from San Francisco Public Utilities Commission-Southeast. One field duplicate was taken from Palo Alto.

Stormwater Runoff

At each location, an ISCO sampler was used to pump stormwater through 125 µm and 355 µm sieves stacked together over the course of several storm events. The amount of stormwater sieved at each sampling location differs depending on how much rainfall was collected; total liters of stormwater collected ranged from 25 L to 295 L in a given sample. Sieve contents were rinsed into a clean glass jar using reverse osmosis (RO) water. Rubbing alcohol was added to each sample to prevent algal growth. The number of samples collected by region were: 2 from North Bay, 6 from Central Bay, 3 from South Bay, and 1 from Lower South Bay. Sampling took place from winter of 2017 through spring of 2018 during various storm events. One field blank was collected in South Bay and one field duplicate was collected in Central Bay.
Sample Extraction and Quantification

Surface Water

We used methods commonly cited in the literature for the extraction of microplastics from Manta Trawl samples\textsuperscript{6-8}. Ten Manta samples were digested in three times the volume of 20\% KOH solution for seven days to remove dense organic matter. The Manta samples were emptied from the clean glass jar onto a stack of 125 \( \mu \text{m} \), 355 \( \mu \text{m} \), 500 \( \mu \text{m} \), and 1 mm stainless steel mesh sieves. Large non-plastic objects in the sample jars were first removed with tweezers, rinsed three times with reverse osmosis (RO) water back onto the corresponding sieve, and then discarded. Large plastic items were picked up with tweezers, rinsed three times with RO water, and saved for later identification. Contents remaining on each of the sieves were rinsed into new clean sample jars labeled with each size fraction. The 125 - 355 \( \mu \text{m} \), 355 - 500 \( \mu \text{m} \), 500 - 1000 \( \mu \text{m} \), and greater than 1 mm fractions were each manually sorted under a dissection microscope (20x-40x magnification) and the first ten anthropogenic pieces of each colour-category combination (e.g. blue fragments, white spheres) were removed from the sample and placed onto double-sided tape in a Petri dish (see examples of categories and colours in Rochman et al., 2019). The remaining microplastic particles in each colour-category combination were tallied but not removed from the sample. For one-liter grab samples, the entire sample was vacuum filtered onto one or more 20 \( \mu \text{m} \) PC filters (Sterlitech). The volume of the sample was measured with a graduated cylinder. Subsequently, and like above for manta samples, microplastic particles were picked off the filters under a stereomicroscope (Olympus SZ61 Stereo Microscope, zoom range: 0.67-4.5x) and placed onto double-sided sticky tape in a Petri dish.
Wastewater

To remove the organic matter from this matrix, water was removed by sieving (125 µm samples were sieved through the 125 µm sieve and 355 µm samples were sieved through the 355 µm sieve) and three times the volume of 20% KOH (in RO water) was added to each size fraction in a clean polypropylene jar (modified from Foekema et al.)⁹. Samples were left to soak in the KOH solution for seven days at room temperature. Samples were then vacuum-filtered and rinsed with RO water. Subsequently, microplastic particles were extracted from each sample under a stereomicroscope (Olympus SZ61 Stereo Microscope, zoom range: 0.67-4.5x) and microplastic particles were picked out as described above for surface water samples.

Stormwater

The two size fractions of stormwater samples were mixed together for extraction. Each sample was first sieved using 106 µm (to capture all size fractions) and 500 µm stainless steel mesh sieves. The >500 µm fraction was rinsed back into the original sample jar and stored for microplastic quantification. The >500µm fraction were sorted without further treatment because particles were large enough to visually sort. Cleaner samples with little particulate matter were only sieved through the 106 µm sieve and also did not undergo further treatment. The 106 µm fraction for the remaining samples were extracted using density separation in a separatory funnel for 1.5 to 2.5 hours using 1.4 g/cm³ filtered CaCl₂ (aq). The bottom denser material was released onto a 106 µm sieve and transferred with RO water to a glass jar for storage. The supernatant was then sieved through a clean 106 µm sieve and stored in a separate jar for microplastic quantification. The 106 µm and 500 µm sieve fractions were combined and re-sieved through a stack of 125 µm, 355 µm, 500 µm and 1 mm sieves to separate the sample into four size fractions. Using as little RO water as possible, the contents of each sieve were washed into a clean glass jar. Each size fraction was examined using a
stereomicroscope (Olympus SZ61 Stereo Microscope, zoom range: 0.67-4.5x) and microplastic particles were picked out as described above for surface water samples.

**Microplastic Characterization**

**Pictures and Measurements**

All particles were characterized by color and shape. All particles that were mounted on double-sided tape were imaged and measured. These particles were a representative subsample of the color, shape and size of particles in each sample. OMAX ToupView software was used to take pictures of particles and measure their lengths and widths.

**Raman and FTIR spectroscopy**

Due to the large number of particles found across our samples, we used a representative subsampling strategy that allowed us to cut down on the total number of particles chemically analyzed. If there were 10 or less particles of a particular category, all particles were analyzed. If there were between 10 and 100 of a category, e.g. fibers, we randomly selected and analyzed 10. If there were between 100 and 200 particles within a category, we randomly selected and analyzed 10%. Finally, if there were more than 200 within a category, we randomly selected and analyzed 20. Chemical analysis was run for 23% of all particles from manta sampling, 39% from bulk water, 40% from wastewater and 6% from stormwater.

Raman spectroscopy was used to identify particles that were too small to be picked up and manipulated using tweezers. An XploRA PLUS Raman micro-spectrometer from HORIBA (New Jersey) was used. Particles were focused under 100x magnification using an Olympus objective (NA 0.8) and the 532 nm and/or 785 nm lasers were used to take spectra. The XploRA PLUS CCD
Microscope camera was used to manually focus each particle and various parameters including laser intensity, acquisition time, accumulation, grating, and hole size were adjusted to produce a spectrum with a signal to noise ratio of at least 10. This resulting spectrum was matched to a Bio-Rad reference library and our own reference library (Munno et al. in prep) that we produced from several polymer types to determine the chemical identity of each particle.

A Bruker ALPHA II Attenuated Total Reflectance Fourier transform-infrared (ATR-FTIR) spectrometer with a DTGS detector operating in the 500 to 4000 cm\(^{-1}\) wavenumber range with OPUS software was used for polymer identification of larger microplastic particles that could be picked up using tweezers (roughly > 1 mm in diameter). Each individual microplastic particle is placed on a transparent diamond of refractive index 2.4 and secured in position using a lever. A total of 24 scans are taken of the background and sample. The resulting spectrum is then matched with reference spectra from Primpke et al.\(^\text{10}\) and from the Bruker built-in library.

**QA/QC**

Spike and recovery tests were used to develop the standard operating procedures (SOPs) used for extraction of microplastics from all sample matrices to ensure appropriate recovery (>80%) of microplastics from the samples (see A.1.). Lab blanks were run with every batch of 10 samples to quantify procedural contamination. Field blanks were collected to detect sources of procedural contamination during sampling. Field duplicates were collected to measure variability between samples at a single site. A natural sponge was used for cleaning to reduce introduction of contaminant fibers into the samples, and cotton lab coats were worn at all times in the lab. A HEPA filter was placed in the lab to reduce airborne fiber contamination, and every morning, the lab countertops were cleaned using water and Kimwipes. Glassware and tools such as tweezers and spoons are washed with soap, rinsed three times with tap water, and rinsed three times with RO water before and after use.
The area around the dissection microscope was wiped down before each use. We work to reduce the amount of time each sample is exposed to possible airborne contamination by working in a clean cabinet and covering samples with a glass Petri dish, parafilm, or aluminum foil as much as possible to prevent contamination. The number of particles within each color/category in our lab blanks and field blanks from each matrix were averaged. The sum of the averages between the lab and field blanks were then subtracted from each relevant individual sample for one-liter grab, Manta, WWTP and stormwater samples.

**Statistical Analysis**

To visualize and present concentrations of microplastics found across samples for baseline monitoring, ArcGIS Pro was used to plot maps of concentrations for all four sample types. To visualize the trends in particles sizes, RStudio version 1.1.453 was used to plot histograms of particle length for all four sample types.

We ran analyses to assess source-apportionment using surface water, wastewater and stormwater data. For surface water, this includes samples taken via Manta (where fibers were excluded) and one-liter grabs (where fibers were included). To assess sources, we use manta and one-liter together to compare microplastics in surface water to stormwater and wastewater. For the purpose of these analyses, because one-liter grab samples are expected to have a greater concentration of microplastics because they are sampling a larger size range⁵, we corrected for this where both manta and one-liter samples were collected. The conversion described below for manta samples was only used to make stacked bar charts showing the percentages of each shape category, stacked bar chart for polymer types, RDAs, and PCAs. The conversion allows us to use manta and one-liter samples together in the same analysis. For the conversion, we calculated how many particles or polymers of each type would
be in the Manta if it was sampled using a 20 µm mesh which corresponds to the one-liter grab filter size using the relationship (Equation 1)

\[ C_{\text{grab}} = C_{\text{manta}} \times \frac{e^{8.421 - 0.009m_{\text{grab}}}}{e^{2.573 - 0.009m_{\text{low}}}} \] (Equation 1)

from Covernton et al.\(^5\), where \( C_{\text{manta}} \) is the manta concentration in particles/L, \( m_{\text{grab}} \) is 20 µm, and \( m_{\text{low}} \) is 333 µm. RStudio version 1.1.453 was used to create bar-plots of category abundance and polymer abundance across all sites of The Bay and NMS for surface water, wastewater, and stormwater. Individual redundancy analyses (RDA) were performed for both microplastic category and colour-category to investigate the relationship between surface water samples in terms of site and the environmental variables of sampling depth, current velocity, and seasonality. Two extreme outliers were removed from RDA analyses to better observe microplastic community trends within the rest of the dataset. We used Principal Component Analysis (PCA) as a descriptive tool to analyze microplastic community patterns between surface water, stormwater and WWTP samples and thereby help source apportion microplastics in surface water. By using PCA, an ordination plot was created that contained surface water, wastewater, and stormwater sample data on the same axes and the way they grouped together was assessed. Similar to RDA, two extreme outliers were removed from PCA analyses to better observe microplastic community trends within the rest of the dataset.
1.3 Results and Discussion

Baseline concentrations of microplastics across The Bay and NMS

Surface water

Sampling locations for Manta and one-liter grab samples are shown in Figure 1a. Microplastics were found in 64 out of 65 manta samples and 42 out of 52 one-liter grab samples after blank subtraction. Microplastics in Manta samples ranged from 0.1 to 195 mm (25th to 75th percentile: 0.6 to 2.2 mm, median 1.0 mm) in length, while microplastics in one-liter grab samples ranged from 0.04 to 8.08 mm (25th to 75th percentile: 0.3 to 1.3 mm, median 0.7 mm) in length (Figure 2). Overall, the ranges and medians show that Manta samples generally contain longer pieces of microplastics compared to one-liter grab samples. Microplastic concentrations in Manta samples ranged from 0 to 0.056 particles/L (mean ± s.e.: 0.00015 ± 0.00086 particles/L) and concentrations in one-liter grab samples ranged from 0 to 35.7 particles/L. Within The Bay, the average concentration of microplastics in Manta samples was 0.0024 ± 0.0014 particles/L, while the average concentration in manta samples from the NMS was 0.00012 ± 0.00002 particles/L. For one-liter grab samples, the average concentration within The Bay was 3.5 ± 0.7 particles/L compared to 4.7 ± 1.8 particles/L for NMS. If we exclude one grab sample from Monterey Bay with an exceptionally high microplastic concentration, the average concentration for NMS becomes 3.2 ± 1.1 particles/L. Thus, in general, the trend is that microplastic concentrations are higher within The Bay than in the NMS. For Manta specifically, microplastic concentrations are roughly an order of magnitude greater in The Bay than outside The Bay. This trend may be more muted for one-liter samples where fibers are dominant. Other studies have shown unexpectedly large concentrations of fibers in remote regions\textsuperscript{11,12}. In general, this trend supports our first prediction that concentrations of microplastics within The Bay are greater than those outside of The Bay.
Microplastic concentrations in Manta samples for each of the regions within The Bay ranged from 0.000059 to 0.0014 particles/L (mean ± s.e.: 0.0004 ± 0.0002 particles/L) for North Bay, 0.000023 to 0.056 particles/L (mean ± s.e.: 0.0046 ± 0.0032 particles/L) for Central Bay, 0.0001 to 0.0029 particles/L (mean ± s.e.: 0.0010 ± 0.0004 particles/L) for South Bay, and 0.00015 to 0.00098 particles/L (mean ± s.e.: 0.00048 ± 0.00012 particles/L) for Lower South Bay. Central Bay Manta samples had the highest average microplastics concentration out of all the regions. In terms of one-liter grab samples, concentrations for each region within The Bay ranged from 1 to 5.24 particles/L (mean ± s.e.: 3.3 ± 0.8 particles/L) for North Bay, 0 to 18.7 particles/L (mean ± s.e.: 4.0 ± 1.4 particles/L) for Central Bay, 0 to 9.9 particles/L (mean ± s.e.: 2.9 ± 1.5 particles/L) for South Bay, and 1 to 7.6 particles/L (mean ± s.e.: 3.1 ± 1.5 particles/L) for Lower South Bay. Like Manta samples, Central Bay one-liter grab samples had the highest microplastic concentrations. This observation rejects our prediction that microplastic concentrations within The Bay are greatest in Lower South Bay; instead, concentrations seem to be highest in Central Bay. We thought that microplastic particles would accumulate in Lower South Bay because it is an area with limited flushing. However, it seems that perhaps the quantity of input of microplastics to Lower South Bay is not as great as inputs to Central Bay. Central Bay may have the highest microplastics concentration because of population size and land use in the area.

Microplastic concentrations in Manta samples collected during the dry season ranged from 0 to 0.036 particles/L (mean ± s.e.: 0.0016 ± 0.0001 particles/L), whereas those collected during the wet season had concentrations between 0.0075 and 0.18 particles/L (mean ± s.e.: 0.10 ± 0.03 particles/L). Wet season concentrations are greater than dry season concentrations by two orders of magnitude.

Microplastic concentrations in one-liter grab samples collected during the dry season ranged from 0 to 8.7 particles/L (mean ± s.e.: 2.6 ± 0.5 particles/L), while wet season concentrations ranged from 0 to
35.7 particles/L (mean ± s.e.: 5.3 ± 1.6 particles/L). Again, similar to Manta samples, the wet season concentrations are greater than the dry season concentrations by roughly two-fold.
Figure 1. a) Map of Manta and one-liter grab sample collection locations. One-liter grab samples are shown as pins and Manta samples are shown as circles. Manta symbols show start coordinates of Manta trawl. b) Map of stormwater runoff and wastewater effluent sample collection locations. Wastewater samples are shown as triangles and stormwater samples are shown as squares. Colour of symbols represent concentration split into five quantiles.

Figure 2. Histogram of particle sizes in one-liter grab, Manta, wastewater, and stormwater. Inset corner graphs show histograms zoomed into the less than 5 mm length range.
Wastewater

Sampling locations for wastewater are shown in Figure 1b. Microplastics were found in all 18 wastewater samples. Microplastics in wastewater ranged from 0.03 to 30.31 mm (25th to 75th percentiles: 0.4 to 1.6 mm, median 0.8 mm) in length. Concentrations of microplastics in wastewater ranged from 0.003 to 0.181 particles/L (mean ± s.e.: 0.055 ± 0.015 particles/L) (Figure 2).

Microplastic concentrations in wastewater samples for each region of The Bay ranged from 0.0037 to 0.0778 particles/L (mean ± s.e.: 0.039 ± 0.020 particles/L) for North Bay treatment plants (CCCSD and FSSD), 0.011 to 0.182 particles/L (mean ± s.e.: 0.106 ± 0.026 particles/L) for Central Bay treatment plants (EBDA, EBMUD, and SFPUC), and 0.0030 to 0.0233 particles/L (mean ± s.e.: 0.013 ± 0.003 particles/L) for Lower South Bay treatment plants (PA, SJ, and SUNN). No wastewater treatment plants are located in South Bay. Central Bay wastewater effluent contained the highest microplastic concentrations on average. This observation may partly explain the high concentration of microplastic particles in Central Bay surface waters.

Wastewater samples collected during the dry season had concentrations ranging from 0.003 to 0.178 particles/L (mean ± s.e.: 0.036 ± 0.014 particles/L) while samples collected during the wet season had concentrations ranging from 0.007 to 0.182 particles/L (mean ± s.e.: 0.105 ± 0.031 particles/L). The mean wet season wastewater effluent contained roughly three times the average amount of microplastics sampled during the dry season, although the range of concentrations were similar. This could be a result of stormwater infiltrating wastewater treatment plants during periods of intense rainfall and thus contributing more microplastic particles to the wastewater effluent. For instance, SFPUC has a combined sewer system.
Stormwater

Sampling locations for stormwater are shown in Figure 1b. Microplastics were found in all 13 stormwater samples, and ranged from 0.04 to 73.50 mm ($25^{th}$ to $75^{th}$ percentile: 0.4 to 1.6 mm, median 0.8 mm) in length. Concentrations in stormwater ranged from 1.1 to 33.2 particles/L (mean ± s.e.: 10.1 ± 2.7 particles/L) (Figure 2). In terms of concentrations by region of The Bay, the ranges were: 1.63 to 1.65 particles/L (mean ± s.e.: 1.64 ± 0.01 particles/L) for North Bay, 4.9 to 33.2 particles/L (mean ± s.e.: 15.9 ± 3.8 particles/L) for Central Bay, 1.1 to 7.2 particles/L (mean ± s.e.: 4.6 ± 1.8 particles/L) for South Bay, and 2.3 particles/L for the one sample collected in Lower South Bay. Coinciding with Manta, one-liter grab, and wastewater effluent, Central Bay stormwater also contained the highest average microplastic concentrations. All stormwater samples were collected during the wet season.

On average, microplastic particles found in wastewater and stormwater samples do not differ greatly in length. Stormwater samples occasionally contained some large particles. The average microplastic concentration in stormwater runoff is almost 200 times the average microplastic concentration in wastewater effluent. This observation shows that in terms of quantity of microplastics per volume, stormwater is a much larger source of input of microplastics to The Bay. However, the frequency of input of these sources are different in that wastewater effluent is continuous year round while stormwater runoff only occurs during periods of rainfall.

Characterization of microplastics in various matrices

Surface water

Relative abundances of categories found in surface water at each location of The Bay and NMS are shown in Figure 3 side-by-side with wastewater and stormwater. For this analysis, Manta and one-
liter grab sample data were combined to form the surface water category. This is why the calculated conversion was necessary. A diversity of microplastic category types (Figure 3) and polymer types (Figure 4) were found in San Francisco Bay and NMS waters (see A.2., A.3. for images of example particles in surface water). Black fragments were included as their own category to account for the large amount of black rubber-like particles found in stormwater.

In surface water across all locations, fragments – including black fragments - were most abundant (57%) followed by fibers (23%) and foams (11%). For each region of The Bay, the most common shapes were: fibers (55%), fragments (34%), and films (9%) for North Bay; fragments (69%), foams (15%), and fibers (7%) for Central Bay; fragments (50%), fibers (30%), and films (12%) for South Bay; fibers (46%), fragments (43 %), and films (9%) for Lower South Bay; and fibers (62%), fragments (29%), and films (8%) for NMS. Thus, there is an overwhelming presence of fragments in surface water, especially for Central Bay and South Bay. Fibers were highly abundant in North Bay, Lower South Bay, and NMS. An interesting trend is that the quantity of fragments is correlated with overall concentration of microplastics, while in regions of The Bay with relatively lower concentrations such as North Bay and Lower South Bay as well as NMS, fibers are the dominant microplastic category.

Relative abundances of polymers found in surface water at each location of The Bay and NMS are shown in Figure 4 side-by-side with wastewater and stormwater. The most abundant polymers in surface water were, apart from unidentifiable particles (15%) and natural material (7%): non-synthetic textiles (12%), PE (10%), and PP (6%) (for examples of spectra see SI Figure 3). Specifically for each region, the most common polymers aside from unidentifiable particles and natural material were: non-synthetic textiles (31%), polyester (14%), and PE (10%) for North Bay; PE (20%), non-synthetic textiles (13%), and PS (11%) for Central Bay; non-synthetic textiles (25%), PE
(15%), and PS (8%) for South Bay; PE (23%), polyester (17%), and PP (14%) for Lower South Bay; and non-synthetic textiles (9%), other anthropogenic particles such as asphalt, rubber, paint and wax (7%), and cellulose acetate (7%) in NMS surface water. The diversity of polymer types found across The Bay and the NMS support our prediction that there is a multitude of sources contributing microplastics to the water.
Figure 3. Abundance of different categories of microplastic by location within The Bay and in the NMS.
Figure 4. Bar-plots showing abundances of polymers found in surface water, wastewater, and stormwater for each region of The Bay and the NMS.

**Wastewater characterization**

There was also a diversity of microplastic categories and polymer types found in wastewater effluent from The Bay Area (Figure 3, 4; see A.4. for images of example particles found in wastewater).

Overall, the most common shapes in wastewater effluent were fibers (50%), fragments (26%), and foams (16%). By region, the most common shapes included: fibers (57%), fragments – including black fragments (22%), and foams (9%) for North Bay; fibers (47%), fragments (27%), and foams (18%) for Central Bay; and fibers (58%), fragments (25%), and spheres (7%) in Lower South Bay. Overall across all regions, fibers were the predominant category type found in wastewater effluent. This may be a result of fibers being released from products such as toilet paper, wet wipes, and clothing which eventually ended up in effluent water.

The most abundant polymers in wastewater were, apart from unidentifiable particles (10%) and anthropogenic particles with unknown base (33%): PE (17%), anthropogenic synthetic particles such as resins and lubricants (14%), and non-synthetic textiles (11%) (see A.5. for example spectra). Apart from unidentifiable particles and anthropogenic particles with unknown base, by region the most common polymers were: PE (14%), anthropogenic synthetic particles (9%), and non-synthetic textiles (7%) for North Bay; PE (22%), anthropogenic synthetic particles (20%), and non-synthetic textiles (12%) for Central Bay; and non-synthetic textiles (15%), PE (11%), and anthropogenic synthetic particles (4%) for Lower South Bay wastewater effluent. A large portion of synthetic particles found in wastewater were lubricants. PE particles in wastewater included microbeads, fragments, and films. The majority of non-synthetic textiles consisted of cellulose which may have originated from toilet paper, wet wipes, or cotton clothing.
Stormwater characterization

Stormwater runoff also contained various microplastic categories and polymer types (Figure 3, 4; see A.6. for images of example particles found in stormwater). Overall, the most common shapes in stormwater runoff were fragments (67%), fibers (31%), and film (1%). Interestingly, 85% of the fragments found in stormwater were black fragments – many of which were rubber-like. By region:

North Bay contained fibers (82%), fragments (14%, 64% of which were black), and films (3%);
Central Bay contained fragments (70%, 86% of which were black), fibers (28%), and films (1%);
South Bay contained fragments (54%, 81% of which were black), fibers (44%), and films (2%); and
Lower South Bay contained fragments (59%, 37% of which were black), fibers (36%), and foams and fiber bundles (both 2%). There is a substantial amount of black fragments in stormwater, most of which feels and looks like rubber. This may be tire wear particles or pieces of road.

Apart from the unidentifiable particles (29%) and anthropogenic particles with unknown base (17%), the most abundant polymers in stormwater were PE (9%), cellulose acetate (6%), and non-synthetic textiles (6%) (see SI Figure 3 for example spectra). Apart from the unidentifiable particles and anthropogenic particles with unknown base, for each region the common polymers found were: North Bay contained cellulose acetate (19%), non-synthetic textiles (11%), and PE (8%); Central Bay contained PE (9%), PP (6%), and non-synthetic textiles (6%); South Bay contained PE (12%), anthropogenic synthetic particles (6%), and cellulose acetate (6%); and Lower South Bay contained PET (13%), PE (12%), and PVC (12%). Noticeably, stormwater contained a large amount of cellulose acetate which may indicate the breakdown of cigarette filters.
Unidentified particles and anthropogenic particles with an unknown base

Anthropogenic (synthetic) particles are particles of human origin which do not fall under a traditional polymer category; examples include resins and lubricants. Some difficulties with Raman spectroscopy were encountered which led to poor identification of particles. When this happened, if the particle is dyed, it was labeled ‘anthropogenic (unknown base)’. If the Raman did not return a dye or the particle is not evidently dyed, it was labeled ‘No signal, unknown, poor spectra, unmatchable’. Across Bay surface water samples, the sum of unidentified particles and non-synthetic textiles usually adds up to around half or more than half of all polymers. Across stormwater samples, the sum of unidentified particles alone usually equates to more than half of all polymers. A large portion of these unidentified particles are black fragments and feel like rubber – so even though stormwater contained an overwhelming amount of rubber, they ended up being categorized as ‘unknown’ during our spectroscopic identification. Compared to Raman spectroscopy, ATR-FTIR worked better for dark particles and the spectra had better quality library matches. Still, in our lab, FTIR could only be used for larger particles. The large quantity of potentially non-plastic particles emphasizes that better methods are needed to isolate and identify microplastic particles from environmental samples. This may include the development of application-based libraries, spectral libraries, and other methods specific to microplastics.

Source apportionment of microplastics in The Bay

RDA of surface water

RDA derives a specific number of synthetic variables that summarizes the original data set. The original data set consisted of a matrix of microplastic categories as columns and all the surface water samples collected as rows. For our purposes, RDA analysis was used to determine how similar the
microplastic communities were between different sampling locations based on the proximity of the sites to one another on the ordination plot, plotted in Euclidean space. RDA was also used to determine how environmental variables such as seasonality, sampling depth, and current velocity influenced microplastic concentrations. Sites or individual samples are represented by points and environmental variables are shown as vectors. Species, which are the microplastic category types, were plotted onto the RDA as tri-plot arrows with text, to determine which samples contained high concentrations of certain category types.

Results of RDA analysis by microplastic category are shown in the tri-plot ordination diagram in Figure 5 (see SI Figure 6 for the colour-category RDA ordination plot). The first two axes of the RDA analysis accounted for 97.7% of the variance in the data, thus other axes were insignificant and not included in the ordination plot. The first axis (horizontal) most noticeably drives the separation between Central Bay microplastic categories and those of North Bay, Lower South Bay, and NMS. The environmental variables most responsible for this separation are current velocity and sampling depth. The second axis (vertical) drives the separation between Central, South Bay microplastics and microplastics communities from North Bay, Lower South Bay, and NMS and this separation is largely driven by season. Since the first axis accounts for the largest amount of variability in the data, sampling depth is the most important factor distinguishing microplastic category communities between sites. This observation is interesting because it means that perhaps depth influences the fate of the various densities and shapes of microplastics within the estuary. Our analysis also suggests that, since there is clustering of samples by region, multiple microplastic sources are contributing to The Bay and that these sources vary by location.
Figure 5. RDA ordination plot of surface water samples analyzed according to category. Individual samples are shown as points while environmental variables are shown as vectors.
Categories and source apportionment – Barplot analysis

The common presence of fibers and fragments in Bay surface waters suggests that important processes of microplastic input to The Bay may include the shedding of fibers as well as the breakdown of hard plastics from larger objects. Specifically, examples of such sources may include the shedding of fibers in laundry effluent, ropes and lines discarded from fishing activity, and fibers from other materials such as textiles, as well as hard plastic objects that have broken down into smaller pieces over time including water bottles, plastic bags, tires and other litter. North Bay, Lower South Bay, and NMS have fibers as the most common category in surface water, while Central Bay and South Bay surface water have relatively more fragments. Since wastewater has more fibers for all regions (except South Bay since there are no treatment plants in South Bay), and stormwater more fragments for all regions except for North Bay, our data suggests that wastewater is more important as a source of microplastics to North and Lower South Bay while stormwater is a more important source to Central Bay and South Bay. This is supported by our RDA results because we see a clear distinction between North and Lower South Bay surface water microplastic communities from Central and South Bay microplastic communities, which may be a result of greater wastewater influence on North and Lower South Bays versus greater stormwater influence on Central and South Bay surface water. Another observation is that pellets were only found in surface water, and not a single pellet was found in stormwater or wastewater. Its absence in wastewater and stormwater suggests that pellets may not have originated from these two sources, or perhaps that our wastewater and stormwater sampling methods did not capture pellets well. In the case of the former, other activities such as ship spills and industry spills may be responsible for the presence of pellets in surface water and should be further investigated in future studies. Furthermore, both surface water
and stormwater contained spheres, yet spheres were rarely found in stormwater. Its high presence in wastewater may indicate that wastewater is an important source of spheres to The Bay.

Noticeably, foams are found more often in wastewater and films were found in relatively higher abundances in stormwater. This highlights another difference between the two source waters.

Moreover, rubber, included within the ‘black fragment’ category, was present in large quantities in stormwater runoff compared to wastewater effluent. This observation points to the prevalence of tire dust in our stormwater samples which is entering The Bay. Overall, all of these observations support our third prediction that microplastics within The Bay can be source apportioned by investigating the similarities between sources and sinks.

We found diverse communities of microplastic polymers throughout all of our source water and surface water samples. However, they were not as informative as microplastic categories when it came to source apportionment.

**PCA of surface water and source waters**

PCA analysis summarizes the variability of a data matrix of microplastic categories and sample sites onto an ordination plot of new variables or components, which are the axes. The total variability of these new components should be 75% or greater\(^{13}\). Individual samples are plotted as points onto the ordination plot and confidence ellipses are added to aid in identifying clustering of points. PCA is a descriptive tool that allows relationships between sites to be explored because the distances between points are correlated with inherent differences between the sites themselves, and thus can be effective for source apportioning contaminants. A matrix with plastic categories as columns and all the surface water and source water samples as rows was used in the PCA analyses. In this plot (Figure 6), surface
water sites are plotted along with source water sites to help source apportion microplastic communities found in The Bay (see SI Figure 7 for the colour-category PCA ordination plot). Sample type is indicated by shape and cos2, or quality of representation by the PCA components, is indicated by point size. Species, which are the microplastic category types, were plotted onto the PCA as trip-plot arrows, to analyze relationships between category concentrations and sample type.

Two components comprising a total of 84.4% of the variance were distinguished for the microplastic category data (Figure 6). The first component accounted for 48.8% of the variance and is characterized by high concentrations of fragments in surface water samples. The second component accounted for 35.6% of the variance in the data and is characterized by high concentrations of black fragments in stormwater samples. Noticeably, the fragment arrow is driven by Central Bay and South Bay samples, which agrees with category trends in NMS samples. The surface water ellipse overlaps the stormwater ellipse, showing that stormwater samples exhibit similar microplastic community composition to those surface water samples within the overlap region. Wastewater samples cluster within the region where the surface water and stormwater ellipses overlap, indicating that wastewater samples contain similar community composition to certain surface water samples and stormwater samples. Clustering of wastewater effluent samples is noticeable while surface water and stormwater sites are more spread apart, which indicates that the microplastic composition of wastewater is more similar within the sample set itself. Analyses show that Central Bay and South Bay samples are located near stormwater samples while NMS samples are in closer proximity to wastewater samples. This implies that stormwater input has a greater influence on microplastic communities in Central Bay and South Bay and this is further supported by our category analysis and RDA results.
Figure 6. PCA tri-plot of surface water, wastewater, and stormwater samples analyzed according to category. Sampling sites are shown as points and the species or microplastic categories are shown as arrows. Size of points indicate cos2 or the quality of the representation by the PCA components. Ninety-five percent confidence ellipses were added for each sample type.
1.4 Conclusions and Next Steps

Implications for Management

We have found noticeable microplastic contamination in the surface waters of San Francisco Bay as well as in source waters to The Bay. The most common particle lengths were less than 1 mm which include a combination of primary microplastic as well as secondary plastic breaking down into smaller pieces. Microplastic concentrations in surface water are higher in The Bay compared to outside The Bay, and within The Bay the region with the highest concentration of microplastics on average was Central Bay. This result shows that Central Bay is a priority region for the reduction of microplastic input. We have also determined that stormwater runoff and wastewater effluent are important pathways for microplastics to The Bay. Most noticeably, it seems that wastewater is a more important source in the North and Lower South Bay while stormwater is more important for the Central and South Bay. The differences in the importance of the sources shows that, depending on the location within The Bay, mitigation strategies may more effectively target one source more than the other. Moreover, the polymer types of microplastic particles in surface and source waters were characterized, and we found that large quantities of PE and non-synthetic textiles are found in high abundance across all regions of The Bay and NMS. The large amount of PE contamination suggests that sources of PE including fragmentation from littering, films and packaging waste, and water-related sources such as pellet spills need to be tackled. Contamination of The Bay by other anthropogenic particles such as natural fibers and glass beads is an interesting avenue for research in the future. We have discovered that cellulose acetate as well as large quantities of rubber are being inputted into The Bay from stormwater runoff. The presence of these polymers suggests that sources of microplastics to The Bay may include cigarette butts and tire dust, and that these sources need to be effectively reduced to prevent contamination of The Bay.
Based on our results, we suggest that mitigation strategies to reduce microplastic input to The Bay include strategies to filter microplastic from stormwater runoff and wastewater effluent before it reaches The Bay. An example of such mitigation strategies would be the installation of raingardens around the San Francisco Bay Area and household washing machine filters to collect fibers during laundry. For plastic already in The Bay, remediation strategies may include local clean-ups. The contamination of San Francisco Bay with microplastics shows how an entire ecosystem can be affected by a single contaminant. Using our knowledge of sources and fate, however, there can be opportunities to reduce further input of microplastics and target mitigation strategies at those parts of The Bay that are most heavily burdened.
Chapter 2

2 Identifying Microfibers using Multiple Lines of Evidence

2.1 Introduction

Plastic pollution is ubiquitous in the environment and its input into the environment is increasing every year\(^1\). As a consequence, plastic debris has become a common contaminant in the environment and can be found across the open oceans\(^5,26,33,34\), estuaries\(^24\), freshwater systems\(^6,8\), and in organisms\(^35–37\). This widespread contaminant has consequently been found in our resources, including sea salt\(^38\), seafood\(^17,36\), and drinking water\(^39\).

A large proportion of the plastics in the environment are in the form of fibers\(^40–42\), which can be produced from the shedding of clothing, towels, rope, and other products made from textiles\(^22\). When fibers are less than 5 mm in length, they are referred to as microfibers\(^43\). Today, it is understood that microfibers are pervasive, and unless measures are taken, increasing amounts will enter the environment\(^1,40\).

Still, we do not yet understand how this ubiquitous contaminant affects living organisms both physically and chemically. For instance, Jemec et al.\(^44\) found no correlation between gut microfiber content and mortality of *Daphnia magna*, whereas Au et al.\(^45\) found that a chronic exposure of polypropylene (PP) microfibers resulted in significantly less growth in amphipods. Overall, the literature on microfiber risk is not comprehensive. To better understand risk, further research is needed to study mechanism of effect, and exposure concentrations in the environment.

Quantifying microfibers in the environment and identifying the polymer type are critical to understanding exposure, in addition to determining the origin of the contamination. For example, PP is commonly used in monofilament fishing line\(^46\). Nylon, due to its toughness, is often used in musical strings, carpets, and rope\(^47\). Cotton is used in yarn for denim\(^48\). Wool, silk, acrylic and polyester are
commonly used in clothing. Finally Rayon and cellulose acetate, semi-synthetic fibers made from cellulose, are used in the production of clothing and cigarette butts, respectively. The most common methods for identifying microfibers are spectroscopic analyses using FTIR and Raman spectroscopy. However, the small size of microfibers as well as the presence of dyes that interfere with the spectrum present challenges when identifying fiber polymer types. For example, conventional Total Attenuated Resonance-Fourier Transform Infrared (ATR-FTIR) spectroscopy is a form of contact analysis, and thus requires significant contact surface area to generate sufficient signal to identify the material. Thus, if the microfiber is too short in length or too thin, the signal is too weak to be able to identify the polymer type. Although Raman spectroscopy and micro-FTIR do not require contact with the microfiber, they require that the fiber be fixed in place for the entire duration of the analysis. It is difficult to keep the fiber in the exact same location under the microscope due to its light weight and small diameter. While fixing the microfiber on tape can be a solution, interference from dyes in the fiber can pose additional problems during identification and this is especially true for Raman spectroscopy. Dyes complicate polymer identification because dye absorbance of the laser can result in reduced intensity of polymer bands and therefore less successful matching of the spectrum to a polymer in the Raman spectral database. Furthermore, dye bands may obscure polymer bands leading to a reduced match rate (this is also referred to as band overlay). Finally, it is also common to get a dye signal instead of a polymer signal on the Raman when the laser excites the dye instead of the polymer. Most microplastic studies do not deconvolute fluorescence and dye signals from polymer signals due to a lack of spectroscopic training or equipment. As a result, polymer identification is hampered, and in some cases biased, due to the signal from the dyes such as indigo. Thus current methods enable the determination that a microfiber is anthropogenic, but cannot necessarily determine whether it is microplastic (e.g., polyester, acrylic), cotton or wool for example.
To help mitigate these issues, we developed a user-friendly method for analyzing microfibers to material type. The proposed method, consisting of four lines of evidence (related to textile chemistry, density, and surface morphology), circumvents the Raman dye interference issue and provides a convenient and inexpensive way to identify microfibers when chemical structural analysis equipment is not available.
2.2 Materials and Methods

To develop this method, we first gathered information from industry and other resources (e.g., textbooks) regarding which dyes are commonly used with different material types. These dye-polymer associations help with material identification of microfibers when the resulting spectrum matches only dyes in the spectral library. The second step uses density tests that provide a further line of evidence regarding material type based on densities of different textile materials. The third step uses surface morphology to provide evidence relevant to fiber material type. The final step uses a staining technique that is specific to certain material types. All four steps, or lines of evidence, help circumvent issues in determining material type when a spectrum only matches with dyes in the library and not the actual material, as discussed in many previous reports. A second workflow which exploits the physical and chemical features of fibers was also developed when Raman or FTIR is unavailable.

Below, we break the methods section into two parts. First, we describe how we developed methods relevant to each line of evidence. Second, we describe a test used to assess the accuracy of the method with microfibers from new textiles. Finally, we conclude with applying the method to real environmental samples.

Part One – Method Development

Matching Dyes to Textile Material Types

A compilation of polymer-dye links was created by consulting textbooks on dye chemistry and polymer staining in the textile industry. These polymer-dye associations were then confirmed by discussing with experts in the textile industry (see acknowledgements). This information allowed us to link common dyes to specific material types (Figure 7).
Fibers for Proof-of-Concept Tests

To prepare natural and synthetic microfibers, textiles were obtained from a variety of brands and materials purchased from Amazon, King Textiles, and ULINE Canada. Polymer type was confirmed using FTIR before use in experiments (A.7.). These specific materials were used: packets of loose wool fibers (“Felting Wool Roving 36 Assorted Colors Soledi Wool Fiber Roving 0.1 ounce/color for Needle Felting” from Amazon); gauze pad, fabric, and thread for cotton fiber (Honeywell North Bulk First Aid Kit” from Amazon, white cotton fabric from King Textiles, and “Singer Mercerized Cotton Hand Thread, Assorted Colors, 12 Spools” from Amazon); towel and fabric for nylon fibers (“Kokubo Extra Long Rougher Textured Nylon Washcloth” from Amazon, white nylon fabric from King Textiles); yarn for acrylic (“LIHAO Crochet Yarn Acrylic Set Sewing Thread Coloured” from Amazon); thread and rope for polyester (“Sodial – 24 Assorted Colors Polyester Sewing Thread” from Amazon, white “Polyester Rope” from ULINE); rope for PP (yellow and white “Polypropylene Rope” from ULINE), and thread and fabric for silk (“YLI 20210-WHT 100wt T-12 Silk Thread, 200m, White” from Amazon, white silk fabric from King Textiles). Fibers were cut into 1-3 mm long pieces using scissors, and then placed onto double-sided tape on glass slides for subsequent tests. Each material type had a unique color for easy identification during proof-of-concept tests.

Density Tests to Confirm Materials

Our density tests were inspired by studies that exploit the difference in densities between plastic and sediment to isolate plastic in environmental samples, a common technique used during sample cleanup\textsuperscript{64–66}, and the fact that polymers have characteristic densities\textsuperscript{64,65}. To develop the density tests, individual fibers were placed in deionized (DI) water, 1.2 g/mL CaCl\textsubscript{2} (aq) (anhydrous, Thermo Fisher Scientific), and 1.3 g/mL CaCl\textsubscript{2} (aq) in 50 mL PP tubes. CaCl\textsubscript{2} solutions were prepared by adding CaCl\textsubscript{2} pellets to reverse osmosis water until the hydrometer read 1.2 g/mL. A similar procedure is followed to make 1.3 g/mL CaCl\textsubscript{2} solution. Based on the densities of each material, we knew whether it was expected to float or
sink in each solution (Table 2). For each material, we tested whether fibers sink or float to confirm they behaved as expected. Individual fibers were rubbed across the meniscus of the solution for several seconds using tweezers to thoroughly soak the fibers in the solution and remove any electrostatic charge or air bubbles before they were brought to the middle of the solution and released. PP tubes were then centrifuged at 4695.6 x g for 10 minutes. Centrifugation effectively amplified the float/sink signal, and avoided the otherwise long wait time for some fibers to settle. After centrifugation the fiber was observed and its position in the solution was recorded, either at the top or the bottom. The observed behaviour of each material in each of the solutions is summarized in Table 2.

<table>
<thead>
<tr>
<th>Material</th>
<th>DI Water (1.0 g/mL)</th>
<th>1.2 g/mL CaCl\textsubscript{2}</th>
<th>1.3 g/mL CaCl\textsubscript{2}</th>
</tr>
</thead>
<tbody>
<tr>
<td>PP (0.84-0.91 g/mL\textsuperscript{67})</td>
<td>Float</td>
<td>Float</td>
<td>Float</td>
</tr>
<tr>
<td>Acrylic (1.11-1.18 g/mL\textsuperscript{67})</td>
<td>Sink</td>
<td>Float</td>
<td>Float</td>
</tr>
<tr>
<td>Cotton (1.48-1.63 g/mL\textsuperscript{67})</td>
<td>Sink</td>
<td>Sink</td>
<td>Sink</td>
</tr>
<tr>
<td>Silk (1.36 g/mL\textsuperscript{67})</td>
<td>Sink</td>
<td>Sink</td>
<td>Sink</td>
</tr>
<tr>
<td>Nylon (1.06-1.16 g/mL\textsuperscript{67})</td>
<td>Sink</td>
<td>Float</td>
<td>Float</td>
</tr>
<tr>
<td>Wool (1.36 g/mL\textsuperscript{67})</td>
<td>Sink</td>
<td>Sink</td>
<td>Sink</td>
</tr>
<tr>
<td>Polyester (1.3-1.46 g/mL\textsuperscript{67})</td>
<td>Sink</td>
<td>Sink</td>
<td>Sink</td>
</tr>
</tbody>
</table>

Densities of the fibers at 20°C are in parentheses in the column on the left.
Morphology as a Measure of Material Type

Images of surface features of all fibers were taken under 100x, 500x, and 1000x total magnification (Numerical Aperture (NA) 0.25, 0.5, and 0.8 respectively) using an XploRA™ PLUS Raman spectroscopy instrument equipped with an Olympus microscope from HORIBA Scientific. At least two different fiber products were examined for each polymer and trends in surface morphologies were noted. These characteristic identifying surface features were recorded for each fiber type to aid in identification (Table 3). The unique surface morphologies of the fibers is a direct result of how they were created; for instance, polyester is generally thinned by drawing it out to several times its length. Normal polyester is long and smooth (Krystle Moody, Sustainable Materials Development Consultant, personal communication, July 26, 2018). Crimping or compression into folds can be applied to the polyester but its smoothness is retained. On the other hand, animal hair like wool grow in segments like overlapping scales. These fiber surface morphology trends were also confirmed on fibers sampled from storm water, wastewater, and surface water from San Francisco Bay, CA, USA (A.8.).

Table 3. Surface morphological features of each material type.

<table>
<thead>
<tr>
<th>Fiber Polymer (Total magnification)</th>
<th>Natural or Synthetic</th>
<th>Unique Morphological Feature</th>
<th>Image of each material type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polyester (1000x total)</td>
<td>Synthetic</td>
<td>Tubular with colored dots or holes.</td>
<td><img src="image" alt="Image of Polyester" /></td>
</tr>
<tr>
<td>Material</td>
<td>Type</td>
<td>Description</td>
<td></td>
</tr>
<tr>
<td>------------</td>
<td>--------</td>
<td>------------------------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>Nylon</td>
<td>Synthetic</td>
<td>Tubular with colored dots or holes.</td>
<td></td>
</tr>
<tr>
<td>PP</td>
<td>Synthetic</td>
<td>Streaks on surface like tree bark.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Streaks run parallel to fiber length.</td>
<td></td>
</tr>
<tr>
<td>Acrylic</td>
<td>Synthetic</td>
<td>Streaks on surface like tree bark.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Streaks run parallel to fiber length.</td>
<td></td>
</tr>
<tr>
<td>Cellulosic</td>
<td>Natural</td>
<td>Tends to be bumpy and spirals. Has fat lips at the edges of the fiber and concaves inward like a deflated red blood cell.</td>
<td></td>
</tr>
<tr>
<td>Wool</td>
<td>Natural</td>
<td>Segmented. Segments run perpendicular to fiber length.</td>
<td></td>
</tr>
<tr>
<td>Silk</td>
<td>Natural</td>
<td>Similar to cellulosic fibers: bumpy and spirals. May be a bit tubular.</td>
<td></td>
</tr>
</tbody>
</table>
The brightness and contrast of images were enhanced for better effect. See SI for San Francisco Bay fiber morphology images and images of fibers from more fiber products (A.8.).

**Staining Techniques to Identify Material Types**

Microfibers (1-3 mm in length) that were previously placed on double-sided tape were used for staining. Dye concentrations were optimized for the most selective staining possible. A final concentration of 1 mg/mL was chosen for Direct Red 23 (Dye content 30%, Sigma-Aldrich) and 1 µg/mL for Sulfo-Cyanine5 Free Acid (Lumiprobe). Microfibers were immersed in Phosphate Buffer Saline (PBS, pH 7.4, Gibco) and their fluorescence intensity was recorded under 40x total magnification (NA 0.13) using a fluorescence microscope (EVOS FL Auto Imaging System) in both RFP and CY5 channels before staining as a control (Table 3 shows excitation and emission bands for both dyes). The RFP channel has excitation maximum at 555 nm and emission maximum at 584 nm. The CY5 channel has excitation maximum at 649 nm and emission maximum at 666 nm. Next, a 1 mg/mL solution of Direct Red 23 was added to the glass slide with the taped fibers (see dye reaction mechanism in A.9.). Fibers were left submerged in the dye solution for 20 minutes with frequent shaking by hand. It is expected that cellulosic microfibers would stain red in the RFP channel from Direct Red 23 (Table 4). The dye was rinsed off with PBS and fluorescent images of the fibers were taken using the same imaging system with consistent parameters in both RFP and CY5 channels. Finally, a 1 µg/mL Sulfo-Cyanine5 Free Acid solution with citric acid and a pH of 1 was added to the fibers (see dye mechanism in A.9.). The fibers were left to soak for 20 minutes with frequent shaking. It is expected that protein (wool, silk) microfibers and nylon would dye blue in the CY5 channel from Sulfo-Cyanine5 Free Acid (Table 4). The dye was rinsed off with PBS and microscope images of the fibers were taken using the same fluorescence microscope in both RFP and CY5 channels. Light, exposure, and gain parameters were chosen while taking photos of the controls and kept consistent after dyeing: most notably, if fibers were too dark before staining, parameters were increased to allow for better detection of fluorescence changes and if fibers were too bright before
staining, parameters were decreased to allow for better detection of change. No filters or automatic modes were used. The fluorescence of the fibers after staining was controlled by subtracting the original fluorescence values measured before staining in ImageJ. Fibers may already have been fluorescent due to dyes pre-added during manufacture. The fibers were considered to be successfully stained if the fluorescence intensity was increased by ten-fold compared to the intensity prior to staining. This 10x cut-off was established in proof-of-concept tests with natively fluorescent fibers.

Table 4. Fluorescent dyes used in the experiment to selectively stain microfibers (please refer to A.9. for more information regarding staining mechanisms).

<table>
<thead>
<tr>
<th>Fluorescent Dye</th>
<th>Molecular Structure</th>
<th>Expectation</th>
<th>Fluorescence Images after Staining</th>
</tr>
</thead>
<tbody>
<tr>
<td>Direct Red 23 or Pontamine Fast Scarlet 4B (1 mg/mL)</td>
<td><img src="image" alt="Direct Red 23 or Pontamine Fast Scarlet 4B" /></td>
<td>Dyes cellulose fibers Excitation: 507 nm; Emission: 588 nm Fluoresces in RFP Channel</td>
<td><img src="image" alt="Fluorescence Image" /></td>
</tr>
<tr>
<td>Sulfo-Cyanine5 Free Acid (1 µg/mL)</td>
<td><img src="image" alt="Sulfo-Cyanine5 Free Acid" /></td>
<td>Dyes protein and nylon fibers Excitation: 646 nm; Emission: 662 nm Fluoresces in CY5 Channel</td>
<td><img src="image" alt="Fluorescence Image" /></td>
</tr>
</tbody>
</table>

Fluorescence images (top: cotton, bottom: wool) have contrast enhanced and color added to make them easier to see (40x total magnification, NA 0.13, scale bar 200 µm in length). More images showing positive and negative results after fluorescence staining can be found in Supporting Information (A.10.).
Part Two – Proof of Concept Tests

Three volunteers were tasked with testing our method. Before testing the new method, all three testers were asked to identify fibers using only microscope images of the fibers with total magnification of 40x. This is the typical magnification at which researchers pick out microplastics from samples and is the procedure by which researchers would identify microfibers without any fiber identification method. The overall objective of this was to determine whether the method we developed has a higher success rate than using only traditional microplastic counting methods with no chemical identification.

Tests were carried out with three subjects (n = 3) to determine the success rate of the new fiber identification method using the workflow without Raman spectroscopy (Figure 8). All three testers were assigned the same ten fibers and instructed to perform the density tests, assess surface morphology, and conduct the fluorescence staining tests. There is at least one fiber from each of the seven fiber polymer categories in the set of test fibers and thus three polymer repeats. With all lines of evidence in hand, they were asked to determine the material type. Their average success rate was calculated.

Experimenters were guided through the tests with detailed instructions but did not know which fibers matched which polymer type (see A.11. for detailed instructions). The microfibers used in the tests originated from: a black nylon towel, yellow PP rope, white cotton thread, blue polyester thread, purple wool stuffing, gray PP rope, brown polyester thread, brown wool stuffing, white silk fabric, and green acrylic yarn. Identities of the materials were confirmed using ATR-FTIR (A.7.).

Experimenters were asked to conduct density tests for each type of fiber, pre-cut to 1-3 mm in length, as described above. They were given images of fibers taken under 100x, 500x, and 1000x total magnification and asked to describe surface morphology of the fibers. Finally, experimenters were asked to stain fibers first in Direct Red 23 solution and record fluorescence changes, then in Sulfo-Cyanine5 Free Acid solution and record fluorescence changes. Dye and density test solutions were prepared in
advance for the experimenters. At the end of all the experiments, the data was compiled in a spreadsheet and experimenters were asked what they thought the fiber identities were for each fiber from the list of polyester, nylon, silk, wool, cotton (cellulose), acrylic, and PP. They were asked to decide on their reasoning starting with the results of the density tests. As soon as they see a fiber float in all three solutions, the experimenters are asked to identify that fiber as PP. For the remaining fibers, their surface morphology is described using the high magnification microscope images. For any fiber that is segmented, the experimenters are asked to conclude the fiber(s) as wool. Using only density test results and surface morphology, the experimenters are asked to determine the fiber identities to the best of their ability. For any remaining unidentified fibers, fluorescence staining evidence was used. For the full Standard Operating Procedure (SOP), see A.11.

Part Three: Validation with Environmental Samples

To validate this fiber identification method, we applied the second workflow to microfibers we had extracted from San Francisco Bay surface water samples. These samples were collected and analyzed as part of a San Francisco Estuary Institute microplastics analysis project, and we were able to use some of these samples to develop this method. Two testers used the method to blindly identify 18 randomly chosen microfibers each, that have already been identified using ATR-FTIR that fell under one of the microfiber categories pertaining to this method. Firstly, images of the microfibers were taken under 100x magnification using the Raman microscope (NA 0.8). Then, fibers were fluorescently stained and their fluorescence intensity changes determined. Finally, density tests were conducted on the microfibers. Density tests were performed last since fibers may become lost from being manipulated and centrifuged, especially clear fibers, and we wanted to gather as much information from them as possible for this test.
2.3 Results and Discussion

*Part 1: Introducing the Method*

Overall, we created two different workflows. One workflow includes Raman spectroscopy (Figure 7) and the other can be used without spectroscopy (Figure 8). Both workflows use surface morphology and density tests. The workflow with no Raman spectroscopy includes fluorescent staining using chemical dyes. As confirmation, surface morphology should be applied to both as an extra line of evidence but is only critical in the second workflow that does not include spectroscopy. Overall, these fiber identification methods help to solve a pre-existing dye interference issue with Raman spectroscopy and proposes a more affordable alternative to spectroscopic identification of microfibers.

For the first workflow, with Raman spectroscopy, a list of major polymer dyes and dyeing methods were extracted from literature sources along with all of the possible polymers they could dye. Dyes are conjugated aromatic organic molecules derived from petroleum. These coloring agents can be either water-soluble or water-insoluble based on their structure. Besides dyes, other colorants include pigments and fluorescent brightening agents. They contain alternating single and double bonds capable of absorbing certain wavelengths of light thereby taking on the color of the wavelengths they do not absorb. Dyes can be added to polymers in a variety of ways and some of these ways are described below.

Dye classes of major commercial importance were selected, especially those often detected using Raman spectroscopy. The major dye classes of commercial importance include: disperse, basic, indigo (a special type of vat dye used exclusively to dye denim), reactive, vat, phthalocyanine, mineral, direct, and acidic dyes (Figure 7). The possible polymer types associated with each dye class were then narrowed down based on current industry practices. Although dyes can be used to stain several polymers, some polymers
stain better with one dye over another, and thus the textile and apparel industries tend to use specific dyes for specific polymers.

Disperse dyes are water-insoluble dyes that have an affinity for hydrophobic synthetic plastic polymers, namely polyester and polyolefin fibers (i.e. PP and polyethylene (PE) fibers) usually applied as a fine aqueous dispersion\(^6\). Basic dyes primarily stain acrylic due to the strong ionic attraction between the dye and carboxylic acidic dye sites in acrylic fibers\(^6\). Indigo is combined almost invariably with cotton to produce denim fabric\(^\footnote{7}\) and this was confirmed to be the case by industry contacts (Stephanie Karba and Elissa Foster of Patagonia, Environmental Researcher and Senior Manager of Product Responsibility respectively, personal communication, February 6, 2018). Reactive dyes stain primarily cellulosic fibers such as cotton, viscose, modal, lyocell, and linen as well as the protein fibers wool and silk; however, the textile industry pointed out that reactive dyes in practice currently most likely indicate cellulosic fiber\(^6\) (S Karba and E Foster of Patagonia, pers. comm., February 6, 2018). Practices may have been different in the past. Reactive dyes form covalent bonds with cellulosic fibers\(^6\). Phthalocyanine, mineral, direct, and vat all stain cellulosic fibers as well\(^6\). Vat dyes are added to cotton denim and other cellulosic fibers as a liquid dispersion\(^6\). Phthalocyanine dyes are typically used to dye cellulose a bright blue or turquoise-blue color\(^6\). Mineral dyes are water-soluble metal salts which transform into insoluble metal oxides upon contact with cellulosic fibers\(^6\). Direct dyes are water-soluble and are applied to cellulosic fibers in an aqueous bath containing an electrolyte. Finally, acidic dyes are anionic dyes applied predominantly to nylon and protein fibers in an acid or neutral dyebath\(^6\) (S Karba and E Foster of Patagonia, pers. comm., February 6, 2018).

Dyes have Color Index (CI) Generic Names such as ‘CI Vat Green 1’. On the Raman, dyes tend to be named a common name. These names do not have the class of dye as part of the name. However, with a quick Internet search it is possible to find the CI Generic Name of the dye and then figure out what type
of dye it is. For instance, CI Reactive Blue 103 is also known as Levafix Blue EGRN. Pigment Blue 15:3 is also known as Heliogen Turquoise, a phthalocyanine dye\textsuperscript{63}.

Finally, it is important to note that although these dye classes are predominantly used to dye certain specific polymers, it is possible to get a dye to stain a broader range of fibers by manipulating the conditions of the staining process such as pre-treatment of fibers and pH of solution\textsuperscript{62,63}. These stains on non-typical fibers can still be performed, but not necessarily with the best results\textsuperscript{62,63}, and thus it is expected that these deviations are uncommon.

The above information alone leads some microfibers to be identified immediately (Figure 7). Some dyes, however, are capable of staining multiple polymer types. In these cases, density and surface morphology tests can be conducted to identify microfibers to polymer type. For instance, acidic dyes are capable of staining nylon, wool, and silk fibers. Assuming the fiber is large enough to be picked up by tweezers, it can undergo the density test using 1.2 g/mL CaCl\textsubscript{2} (aq) as described above. If the fiber floats, then the fiber is nylon. If the fiber sinks, then it can be either wool or silk. Finally using knowledge of surface morphology, these fibers are easy to tell apart: wool is segmented while silk is not. However, if the fiber is too small to pick up, surface morphology can still be used to give the experimenter hints to the polymer type.
Figure 7. This flowchart shows how microfibers can be identified to polymer type using a combination of Raman spectroscopy, knowledge of dye-polymer associations, and density tests, in addition to surface morphology as an extra easy step (which is not shown in the flowchart).

When Raman is unavailable, a second workflow can be used that only utilizes the density tests, surface morphology and an additional step with fluorescent staining (Figure 8). These pieces of evidence exploit unique features of different textile materials to distinguish them from one another.

The density tests, using widely available and low-toxicity solutions, proved that it is possible to group microfibers based on their behaviour in solutions of different densities. Three solutions are used (water, 1.2 CaCl₂ (aq) and 1.3 g/mL CaCl₂ (aq)) because several fibers have densities hovering around 1.3 g/mL. If a microfiber is neutrally buoyant in 1.3 g/mL CaCl₂ (aq), we can assess whether they sink in 1.2 g/mL CaCl₂ (aq). If it sinks, wool, silk, cellulose, or polyester are possible identities. If the fiber floats, then nylon or acrylic are possible identities.
Density tests coupled with the unique surface morphologies of different materials help narrow down possible material types. Surface morphology of a fiber refers to its shape and texture. For instance, cellulosic fibers tend to be shaped like a deflated red blood cell and look bumpy while polyester fibers tend to be tubular-shaped and look smooth.

Fluorescence staining patterns of the fibers can narrow down identification even further taking advantage of the differences in hydrophobicity of materials. Due to the chemical structure of the fiber polymers, they stain differently when exposed to Direct Red 23 and Sulfo-Cyanine5 Free Acid dyes. Direct Red 23 belongs to the direct dye class which preferentially stains cellulosic fibers through stacking and hydrogen bonding forces\textsuperscript{62}. Sulfo-Cyanine5 Free Acid belongs to the acid dye class which preferentially stains nylon and protein fibers through nucleophilic attack of their electrophilic amine groups by the carboxylic acid group on the dye\textsuperscript{62} (A.9.).

Thus, using differences in density, surface morphology, and their chemistries, the fibers can ultimately be identified to polymer type without the use of expensive equipment. This method – without Raman spectroscopy - works only for fibers that can be picked up with tweezers because the density tests involve dropping the fibers in solutions. However, even if the fiber is too small to pick up, surface morphology and/or staining can still be used to give the experimenter hints to the polymer type of the fiber.
Figure 8. This flow chart shows how microfibers can be identified to polymer type without Raman spectroscopy, using a combination of density tests, surface morphology and fluorescent staining.

Overall, for both methods, we included only the most common fibers used for clothing, fishing, carpet, and upholstery. Other less common fibers exist, such as polyurethane foam fibers and polyvinyl chloride
fibers, which were not included in this study. We did not test them using this method. Polyethylene (PE) fibers are never or seldom reported to be found in environmental samples including mussels, coastlines, marine sediments, and atmospheric fallout which speaks for its lack of abundance and thus were not included in this method\textsuperscript{72–75}. Other fibers can be distinguished from each other using properties such as density and surface morphology. Fibers such as Rayon, Modal, cellulose acetate, and other cellulosic derivatives – just like cotton – can also be identified as ‘Cellulose’ using this method. Segmentation is a characteristic of all animal hair, and wool is just one type of animal hair commonly used in clothing. Clothing that is a blend of cotton and polyester means that individual cotton fibers and individual polyester fibers are woven together in a specific ratio to form the clothing. This means that, in the case of these seven polymers, individual fibers exist uniquely as one polymer type and will be identified using this method. Therefore our method works for fiber blends. Finally, it is assumed that this fiber identification step takes place after sample cleanup using appropriate protocols such as density separation, chemical digestion, and sieving to remove non-anthropogenic materials such as organic matter, glass, and chitin (see Hidalgo-Ruz et al.\textsuperscript{64} for particle isolation techniques). One caveat is that sometimes it is not possible to have a pristine fiber depending on the effectiveness of the cleanup procedure and the extent that it has been biofouled or degraded in the environment.

\textit{Part 2: Results of Proof of Concept Tests – without Raman spectroscopy (using Flowchart 2)}

With instruction, all three experimenters identified all ten of their fibers correctly to polymer type (Table 5). This success rate is much greater than the rate of success for Experimenters 1 and 2 when observing fibers under the dissection microscope alone (Table 5). Detailed results for each tester can be found in Supplementary Information (A.12., A.13., A.14.) and are described below.
Table 5. Summary of overall blind test results using all three lines of evidence: density tests, surface morphology, and fluorescence staining. Experimenter 3 did not identify fibers using a 40x magnification dissection microscope first, which is why the result for that section is ‘N/A’.

<table>
<thead>
<tr>
<th>Blind Test Results</th>
<th># of Fibers Correctly Identified using a microscope with 40x magnification</th>
<th># of Fibers Correctly Identified using the Fiber Identification Method</th>
<th>Success Rate of the Fiber Identification Method (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experimenter 1</td>
<td>2 out of 10</td>
<td>10 out of 10</td>
<td>100</td>
</tr>
<tr>
<td>Experimenter 2</td>
<td>1 out of 10</td>
<td>10 out of 10</td>
<td>100</td>
</tr>
<tr>
<td>Experimenter 3</td>
<td>N/A</td>
<td>10 out of 10</td>
<td>100</td>
</tr>
</tbody>
</table>

Experimenter 1 correctly assigned surface morphological features to all ten fibers (A.12). For the density separation tests, two of the ten microfibers (both wool) incorrectly floated in 1.3 g/mL CaCl₂ (aq). One reason for this could be that the wool fiber was not left soaking in solution for long enough, leaving air bubbles to facilitate floating despite centrifugation. Experimenter 1 still obtained a perfect score due to the order in which the evidence was assessed: specifically, the rules indicate that if the fiber is segmented, regardless of other evidence the fiber is automatically wool (Supplementary Information). For fluorescence tests, Experimenter 1 did not assess staining data for two of the microfibers, because their identities were already determined from density tests and surface morphology alone. Experimenter 1 correctly assigned fluorescence results to five of the remaining eight microfibers. Even so, due to the difficulty in staining colored fibers, fluorescence staining evidence does not need to be assessed when the identity of the fiber has already been deduced.

Experimenter 2 correctly assigned surface morphological features to all ten microfibers (A.13.). For the density tests, all microfibers behaved as expected and were assigned correctly. Fluorescence staining
results were correct for five of ten microfibers. Still, with the strength of the multiple lines of evidence, the errors in the fluorescence analysis did not result in errors in the final assessment. Specifically, based on density test results and surface morphology evidence, microfibers were ultimately assigned to material types correctly.

Experimenter 3 correctly assigned surface morphological features to all ten microfibers (A.14.). For the density tests, the nylon microfiber did not float in 1.2 g/mL CaCl\(_2\) (aq) as expected, and thus nine out of ten microfibers were assigned correctly. The nylon may not have floated due to a contaminated test tube with water which diluted the CaCl\(_2\) solution. The experimenter was still able to obtain a perfect score when considering all the evidence together in the specific order (Supplementary Information). This is because even though the nylon microfiber was assigned incorrect density results, these density results (float in DI, sink in 1.2 g/mL and 1.3 g/mL CaCl\(_2\) (aq)) are not possible for any of the seven fiber polymers. So by using the smooth tubular surface morphology of the fiber to decide between nylon and acrylic, Experimenter 3 concluded that the fiber is nylon. Fluorescence staining results were correct for one of two microfibers assessed. Eight of the microfibers simply were not assessed because the material type had already been deduced from the other two lines of evidence.

It is worth noting that we performed two rounds of tests and used lessons learned from the first round to improve the second round. The first round of tests did not use a centrifuge and resulted in several ambiguous density test results. Moreover, the fluorescent dye concentrations were not optimized and resulted in poor results. Lastly, the surface morphology images were not taken in full focus and the experimenters were not given enough surface morphology identification practice, resulting in poor interpretations of the fibers’ surface features. Overall the quality of the physical and chemical fiber characteristics data was inadequate, and indicated areas where the protocol could be improved. We learned that more detailed instruction with examples was necessary to guide the experimenters through the method.
Examples of how experimenters were guided through the test include: experimenters were given hands-on training on fluorescence microscopy and ImageJ processing, and shown examples of what sunken fibers looked like in solution versus floating fibers. When the experimenter missed a fiber that had floated or sunk or in the case where an incorrect surface morphology was assigned, the experimenter would be asked to reconsider the evidence. This reminder did not reveal the correct answer to the experimenters, but instead prompted them to study their examples more carefully and assess the evidence again. This points to the importance of experience and a second pair of eyes to the success of the fiber identification method. This method does not guarantee perfect success rate but works better the more experienced the tester is with identifying the physical characteristics of fibers.

Moreover, we learned that the order of the assessments of evidence are important for more accuracy. Density tests should be taken into consideration first followed by surface morphology and then fluorescence staining, except for the two important rules that trump all the other evidence: that segmentation ultimately indicates wool and floating in water automatically indicates PP. The order of assessment is such because surface features are open to some degree of interpretation, and fluorescence staining does not work well on dark-colored fibers.

One of the major limitations of this method is that dark-colored fibers or fibers already pre-dyed during manufacture are harder to stain again. Therefore, the fluorescence staining results for these fibers are not as robust as the results from the other two lines of evidence. Therefore, fluorescence staining evidence is assessed with the least priority. Another limitation of this method is that depending on the fiber length, not all the lines of evidence can be used. For instance, only fibers that are physically able to be picked up by tweezers can undergo density tests. If the fiber is too small, surface morphology tests and fluorescence staining can still be performed on the fibers but as a result not every fiber can be identified to polymer type. Finally, it is important to note that since the fibers obtained their unique surface morphologies from the way they were produced, if the fibers have been drastically altered through chemical degradation or
physical manipulation such as flattening or tearing, then it can be difficult to assess their surface morphology correctly. Similarly, if fibers are biofouled in the environment, their density may also be altered. For these reasons, we tested our method with environmental samples and our results are outlined below.

While the four lines of evidence together help the experimenter identify the material type, each line of evidence alone can identify certain types of microfibers. This is useful in cases where researchers have access to limited equipment. For instance, surface morphology only requires a high magnification (100x-1000x) microscope. It alone is capable of identifying wool and can tell you whether the fiber is synthetic vs natural. When CaCl₂ (aq) or other dense solutions and a centrifuge are available to conduct density tests, these tests alone can unequivocally identify polyolefin (PP and PE) fibers, which are the only fiber types that float in water. These tests can be performed in the field. Fluorescence staining tests alone can unequivocally identify cellulosic fibers which are selectively stained by Direct Red 23. Fluorescence staining tests require a fluorescence microscope, fluorescent dyes, and an image processing software such as ImageJ.

Part 3: Method Validation with Environmental Samples

Results of environmental microfiber identification are shown in Table 6.

Table 6. Summary of method validation results on environmental samples using three lines of evidence: density tests, surface morphology, and fluorescence staining.

<table>
<thead>
<tr>
<th>Results of Method Validation on Environmental Samples</th>
<th># of Environmental Microfibers identified correctly</th>
<th>Success Rate of microfiber identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tester 1</td>
<td>13 of 18</td>
<td>72%</td>
</tr>
<tr>
<td>Tester 2</td>
<td>11 of 18</td>
<td>61%</td>
</tr>
</tbody>
</table>
Overall, Tester 1 correctly identified 13 out of 18 fibers (Table 6). Tester 1 was able to correctly match 12 out of 18 fiber surface morphologies (A.15.). For three fibers, the tester noticed more than one type of surface morphology was present on the fiber, which made identification difficult. In the other three cases, the fibers were cracked and flattening, making it hard to determine its surface morphology. For fluorescence staining, nine out of 18 fibers matched the expected staining behaviour. Three fibers were lost during the staining itself, because the mixing of the dye solution with the fibers dislodged them from the double-sided tape that secured them to the glass slide. Not including the lost fibers, the tester successfully matched the fluorescent staining behaviour of nine out of 15 fibers. Specifically, for the wool and nylon cases: clear fibers stained well with Sulfo-Cyanine5 Free Acid while black or dark blue fibers did not fluoresce noticeably. For density tests, 10 of the 18 outcomes matched the expectations of the fibers’ behaviour. However, three fibers were previously lost during fluorescence staining and three more were lost during the density tests because of their clear color or small size which made them difficult to see. Not including the lost fibers, 10 of 12 outcomes matched the expected behaviours. It is possible that the densities of the fibers may have been altered in the environment from degradation or biofouling.

Overall, Tester 2 was able to correctly match 11 out of 18 fibers (Table 6). Tester 2 correctly matched 10 out of 18 surface morphologies (A.16.). For five of the fibers with misidentified surface morphologies, the tester identified multiple morphologies on its surface. For the other three misidentified morphologies, the morphologies were incorrectly assigned. For fluorescence staining, eight out of 18 fibers were correctly assigned. The success rate is different from the other tester perhaps because of slight differences in how the fiber fluorescence was analyzed in ImageJ. Finally, the results for density tests are again that 10 out of 12 fibers have outcomes matching expectations, after accounting for lost fibers.

As per our protocol, we evaluated density and surface morphology results first then took into consideration fluorescence staining results where necessary. Although we obtained the incorrect fiber identity on a few occasions, it was from guessing after narrowing down the options using the existing
evidence. For instance, for one polyester fiber we narrowed down the options to polyester or cellulose after examining its surface morphology and density results. It did not fluoresce at all in the RFP or CY5 channel, but because it was a dark fiber, cellulose could not be ruled out. So we guessed incorrectly that it was cellulose. There were several cases where guesses were taken after narrowing down the options for fiber polymers. Thus, as recommended above, not all steps are necessary if the fiber type can be discerned with less lines of evidence.

Overall our method had a 67% success rate when identifying microfibers from environmental samples. It is evident that getting poor quality spectra and dyes from Raman is a major obstacle when identifying microplastics, as is the case for Karami et al., where the presence of dyes led them to have a 57% microplastic polymer identification rate\textsuperscript{76}. Thus, although our success rate is not 100%, it can be better than conventional Raman spectroscopy for microplastics identification because it tackles the dye problem. It can also be useful when scientists do not have access to spectroscopy. Studies that use micro-FTIR, where an FTIR is coupled to a microscope, have achieved high polymer identification rates\textsuperscript{77,78}. The chemical and physical principles behind the method still hold; however, the major challenge is that fibers in the environment have become degraded over time and thus the intrinsic properties they possess can become altered. This is most noticeable in density changes in the fibers as well as cracking and flattening of the surfaces of the fibers which makes surface morphology identification difficult. Moreover, we realized that it is easy to lose fibers while handling them during the tests; this is especially true for clear fibers. Although this fiber identification method may be hampered by environmental degradation and biofouling of fibers, these same processes have the same effect on other fiber identification technologies – biofouled fibers burn more easily under Raman and both Raman and FTIR may detect impurities in the fibers which may hinder the identification process. In other words, fibers from the environment are inherently more difficult to identify compared to pristine fibers. Ultimately this method, like others,
works best on clean and larger fibers, so being able to isolate the fibers effectively from environmental matrices is an important precursor to the use of this fiber identification method.
2.4 Conclusions and Next Steps

Overall, we aim for this method to be used as a tool to help determine the material type of microfibers in a sample. It offers a convenient and cost-effective method to improve Raman identification of microfibers and identify microfibers without the use of spectroscopic instrumentation. Here, we utilize surface morphology, density, selective staining, and spectroscopic properties of microfibers. We hope this method can be helpful in the field for identifying material-type. Using new and environmental samples, we have demonstrated that this method complements existing technologies for microfiber identification.

When fibers can be identified to polymer type, it is more possible to do source-apportionment. Understanding the source of the contamination is useful information for mitigation to prevent further inputs into the environment. For instance, finding an abundance of nylon and PP may indicate that fishing activity is contributing to plastic contamination in the area. Alternatively, finding an abundance of polyester and acrylic fibers may indicate that synthetic textiles from clothing are a possible source.

Although this method does not differentiate between artificially-modified cellulose and natural cellulose due to the similarity of their densities and chemical structure, future research can look into ways of further differentiating these two types of cellulose. Thus, improved or accessible methods to identify plastic pollution to material type is beneficial to help inform policies that can mitigate contamination.
Chapter 3

3 Conclusions and Future Directions

3.1 Conclusions

Over the course of my Master of Science, we have extracted, quantified, and characterized microplastic particles from hundreds of samples from San Francisco Bay, an urban bay surrounded by numerous industries and over seven million people, in order to better understand the sources and fate of microplastics within this ecosystem. We found microplastics in almost all surface water samples within The Bay, and in every single wastewater and stormwater sample. The highest concentration of microplastics was in Central Bay and their most common size was < 1 mm. Great quantities of PE and non-synthetic textiles were found in surface water samples across all sites including the NMS. Noticeably, wastewater contained a great deal of fibers while stormwater carried large amounts of rubber fragments to The Bay. Sources of microplastics to The Bay varied depending on region and seasonality, and sampling was affected by sampling depth as well as current velocity. Overall, our results show that effectively mitigating microplastics may be specific to location and sources that are most prominent in that region. In this case study, a variety of inputs including tires, littering, and washing clothes are likely sources. Our findings are applicable to other urban bays around the world and help answer major questions in the field pertaining to the sources and fate of plastic pollution in the environment. While undertaking this project, we encountered difficulties identifying dyed microfibers from The Bay using Raman spectroscopy and developed a fiber identification method that can help circumvent the dye interference problem and which can be used without Raman or FTIR spectroscopy. This method can allow more microfibers to be identified and can also act as an alternative to Raman or FTIR spectroscopy when such technology is not available.
3.2 Future Directions

There are many interesting future avenues of research that can stem from this project. Firstly, fish and sediment samples can be analyzed to better inform sources and to understand fate of microplastics to The Bay. Understanding the fate of microplastics helps pinpoint areas with a high burden of microplastics which can be targeted first by mitigation strategies. Furthermore, research into the effectiveness of certain mitigation strategies can be performed. Rain gardens have been shown to reduce microplastics input into The Bay already\textsuperscript{31}. Other strategies that can be tested include the installation of microplastic filter mechanisms in wastewater treatment plants or in washing machines\textsuperscript{32}. Finally, this study investigated two major transport mechanisms of microplastic input into The Bay. However, there are specific pinpoint sources of microplastics that can be better quantified including littering, pellet spills, fishing activity, tire abrasion, and more. Better understanding these sources will further help to control the amount of microplastics entering The Bay.

Overall, there are important research topics that remain to be explored in the field of plastic pollution. Some of these important research areas include: methods, mitigation, fate, and effects. For instance, especially since the field of plastic pollution is relatively new, we are always in need of better techniques for the extraction, quantification, and identification of microplastics from environmental samples. Firstly, it is difficult to extract microplastics well from sample matrices because inevitably some will be missed and false positives may be picked out\textsuperscript{53}. Recently, some new techniques have been developed such as an extraction method using magnetism\textsuperscript{79}. More techniques like these which exploit the unique chemical and physical properties of plastic need to be developed to improve their extraction success rate. Furthermore, efforts should be made to quantify microplastics automatically such as by using computer software. Our novel fiber identification method helps circumvent dye interference issues with Raman spectroscopy, but there remain other barriers to overcome such as size limitations. Pyrolysis GC-MS is a technique that is gaining prominence\textsuperscript{80}. It involves burning plastics
and using their unique monomer signatures to identify them, but needs further optimization and testing\cite{80}. Aside from microplastics, another group of plastic particles that are becoming more and more researched are nanoplastics due to their potential risks to living organisms\cite{81}. A lot more knowledge is needed about this recently discovered group of plastic particles, including their abundance, transport, fate, and health effects\cite{81}.

Furthermore, a lot of research is focused on mitigation strategies and how to reduce input of microplastics into the environment. Research includes modelling how certain policy changes would impact microplastic input quantities over time, source apportionment studies like our San Francisco Bay study, and behavioural studies focusing on how to change people’s behaviour of littering and use of single-use plastic products. Alternatives to plastics are another significant research topic which can help to reduce plastic input into the environment; examples of biodegradable alternatives to plastic being developed at the moment include starch films\cite{82}, sheets made of banana stem extract\cite{83}, as well as bioplastics made of corn and rice starch\cite{84}.

Moreover, a big question in the field of microplastics is: what is the fate of plastic pollution? To date, there is very little information on where plastic ends up in the environment, including its quantities, distribution, and transport mechanisms. In fact, the only well characterized reservoir of plastic pollution is the ocean surface\cite{26,34}, but most of the plastic by mass does not reside there. I am hoping to study this question in detail during my PhD by quantifying the amount of plastic in other major reservoirs including: coastlines, ocean column, animals, shallow benthic sediments, and deep ocean sediments.

Finally, the potential risk that microplastics pose on living organisms is one of the most important research topics in this field. Laboratory ecotoxicology tests are a major area of research\cite{85}, as researchers try to determine the toxicity of plastic particles on living organisms. For my PhD
specifically in relation to risk, I plan on compiling data from around the world on physical and chemical risks of plastic pollution to living organisms and synthesizing them to determine a ‘risk factor’ for different regions of the world where plastic pollution may affect living organisms. I plan on linking this ‘risk factor’ to potential sources and highlighting which sources of plastics may pose the most risk.
Literature Cited


14. Wilcox, C., Van Sebille, E. & Hardesty, B. D. Threat of plastic pollution to seabirds is global, pervasive, and increasing. doi:10.1073/pnas.1502108112


38. Karami, A. et al. The presence of microplastics in commercial salts from different countries. doi:10.1038/srep46173


76. Karami, A. et al. The presence of microplastics in commercial salts from different countries. doi:10.1038/srep46173


84. Marichelvam, M. K. et al. Corn and Rice Starch-Based Bio-Plastics as Alternative Packaging
Appendices

A. 1. Spike and recovery: results from spiking Bay sediment with microplastics and recovering them using sediment extraction methods involving sieving and density separation, which were eventually used to extract real sediment samples.

<table>
<thead>
<tr>
<th></th>
<th>Trial 1 45-500µm</th>
<th>Trial 1 &gt;500µm</th>
<th>Trial 1 Total</th>
<th>Trial 2 45-500µm</th>
<th>Trial 2 &gt;500µm</th>
<th>Trial 2 Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>PE</td>
<td>10</td>
<td>0</td>
<td>10 (100%)</td>
<td>9</td>
<td>0</td>
<td>90 (100%)</td>
</tr>
<tr>
<td>PET</td>
<td>4</td>
<td>4</td>
<td>8 (80%)</td>
<td>6</td>
<td>2</td>
<td>8 (80%)</td>
</tr>
<tr>
<td>PS</td>
<td>0</td>
<td>9</td>
<td>9 (90%)</td>
<td>0</td>
<td>10</td>
<td>10 (100%)</td>
</tr>
<tr>
<td>CA</td>
<td>0</td>
<td>3</td>
<td>3 (100%)</td>
<td>0</td>
<td>3</td>
<td>3 (100%)</td>
</tr>
<tr>
<td>Fiber</td>
<td>0</td>
<td>9</td>
<td>9 (90%)</td>
<td>2</td>
<td>8</td>
<td>10 (100%)</td>
</tr>
</tbody>
</table>

A. 2. Example particles found in one-liter grab samples from San Francisco Bay. Scale bar indicates 1 mm.
A. 3. Example particles found in Manta samples from San Francisco Bay. Scale bar indicates 1 mm.
A. 4. Raman and ATR-FTIR sample and reference spectra with corresponding particle photographs of common polymers found in surface water, stormwater, and wastewater samples from SF Bay. a) pink fragment from a Manta sample identified as PE using ATR-FTIR, b) light blue fragment from a Manta sample identified as PP using ATR-FTIR, c) clear fiber from a wastewater sample identified as
cellulose using Raman spectroscopy, and d) clear fiber bundle from a stormwater sample identified as cellulose acetate using Raman spectroscopy.

A. 5. Example particles found in wastewater treatment plant effluent to San Francisco Bay. Scale bar indicates 1 mm.

A. 6. Example particles found in stormwater runoff to San Francisco Bay. Scale bar indicates 1 mm.
A. 7. SOP for Proof of Concept Tests on the Microfiber Identification Method using Multiple Lines of Evidence

**SOP for Microfiber Identification to Polymer Type using Fluorescent Staining, Raman Spectroscopy, Density Tests, and Surface Morphology**

This SOP uses density tests, surface morphology, and fluorescent dye stains to identify fibers in environmental samples. These methods are based upon the physical characteristics of fibers and dye chemistry theory. In this SOP, the methods will be validated for their robustness.

**MATERIALS**

- Pontamine Fast Scarlet (Direct Red 23, Sigma-Aldrich)
- Sulfo-Cyanine5 Free Acid (Lumiprobe)
- Phosphate Saline Buffer (pH = 7.4, Gibco)
- Calcium chloride (CaCl₂) solid pellets (anhydrous, Thermo Fisher Scientific)
- Tweezers
- 10 mL Falcon tubes
- Deionized water
- Nylon, Polyester, Acrylic, Wool, Silk, PP, and Cotton Fibers
- Scissors
- Fluorescence Microscope (EVOS FL Auto Imaging System)
- Hydrometer
- 500 mL beakers
- Glass slides
- Double-sided tape
- Citric Acid powder
- Squirt Bottle
- Magnifying Glass

**PROCEDURE**

1. Preparation of Dyes (10 minutes)
1.1 Take dye solutions out of freezer and bring them to room temperature. Make a 1 mg/mL solution of Direct Red 23 by diluting stock solution with PBS.
1.2 Cover the falcon tube with foil to prevent photobleaching of dye.
1.3 Store remaining Direct Red 23 stock in the freezer.
1.4 Make a 1 ug/mL solution of Sulfo-Cyanine5 Free Acid using phosphate saline buffer (pH = 7.4) and place in a Falcon tube. Add enough citric acid to make a solution with pH of 1 to activate the dye.
1.7 Cover with foil when not in use.

2. Preparation of Known Dyed Fibers by Experimenters (Do Not Show to Blind Tester; 20 minutes)
2.1 Soak fibers in PBS buffer to rid of any proteins which may be deposited on the fibers due to contaminants in the air or from human contact. Use scissors to cut fibers of length 1-3 mm of a variety of colours for ten fibers of your choice. They may include any of: polyester, silk, cotton, wool, acrylic, nylon, and PP. Make sure fibers are the correct length by observing them under a light microscope. Make sure bundles of fibers are separated into single strands. Rinse scissors and tweezers and blow dry with a stream of air in-between each polymer type. Stick the fibers onto glass slides covered with double-sided tape.

3. Experiments Part 1: Surface Morphology (10 minutes)
3.1 Take pictures of fibers under 50x AND 100x (NA 0.5 and 0.8 respectively) total magnification using bright field microscopy. Make sure that the surface of the fiber is in focus before taking the picture. Well-focused images are necessary in order to see the surface features clearly, so make sure that the images are clear and high contrast.
3.2 In ImageJ, make the fiber pictures black and white and increase contrast so you can see the texture of the fiber clearly.

4. Experiments Part 2: Density Tests (30 minutes)
4.1 Add 10 mL of DeI water to a 15 mL Falcon tube. Using tweezers, rub the first fiber across the meniscus of the solution against the wall of the Falcon tube for several seconds. Then, dip the fiber into solution and hold for several seconds before letting go. Let go of fiber. Repeat for the other nine unknown fibers. Place all ten Falcon tubes into a centrifuge and balance accordingly. Set speed to 4695.6 x g and time to 10 min.
4.2 Turn centrifuge on. Let centrifuge decelerate to a stop.

4.3 Record observations: sink or float. Look carefully for fibers at the bottom of the Falcon tube. When labelling that a fiber ‘floated’ in a solution, make sure that not a single fiber sank to the bottom. Even if 99% of the fibers are floating on the solution, one fiber at the bottom is enough to turn this observation around. This is because water tension often makes fibers float which aren’t supposed to float. So observing the fibers in each of the centrifuged solutions carefully is very important to obtain trustworthy evidence from the density tests.

4.4 Repeat these density tests for the other two solutions, 1.2 g/mL CaCl$_2$ (aq) and 1.3 g/mL CaCl$_2$ (aq).

5. Experiments Part 3: Fluorescence (1 hour)

5.1 Place glass slides with fibers on them in a Petri dish. Collect control images of fibers soaked in PBS without dye and use light of 6, exp of 45, and gain of 0 at magnification 4x. For other models of fluorescence microscopes, make sure to record starting parameters and use them consistently throughout the experiment. Pour Direct Red 23 (1 mg/mL) solution on slides.

5.2 Let fibers sit for 20 minutes in dye mixture with frequent agitation to ensure thorough coating of fibers with dye. Use Pasteur pipette to frequently squirt dye onto fibers. Cover Petri Dish with aluminum foil immediately.

5.3 Rinse dye off of fibers with squirt bottle of PBS. Repeat three times or until all of dye is gone.

5.4 Place glass slide with fibers on fluorescence microscope stage and record fluorescence values from TRANS (visual light), RFP, and CY5 channels. Use same parameters as control images. Specific detailed instructions for using the fluorescence microscope can be found below:

5.5 Place well plate onto fluorescence microscope stage. Cover with dark container to prevent light from saturating detector.

5.6 Turn power ON.

5.7 Move dot on diagram around until you see the fiber on the computer screen and focus.

5.8 Select ‘Capture All’ to obtain picture of fiber through in TRANS, RFP, and CY5 channels.

5.9 Repeat steps 3.1-3.8 but with Sulfo-Cyanine5 Free Acid dye (5 ug/mL).
5.10 Save files onto USB to analyze them in ImageJ.

5.11 Open each image in ImageJ, select 3-5 areas where fibers are using rectangular selection tool, and record average intensity by clicking ‘Measure’. Correct for intensity from control images (see ‘Instructions for determining polymer type’). Record final fluorescence intensity changes in Excel.

6. Experiments Part 4: Control blind study (15 min)

6.1 Using only the TRANS images recorded using the fluorescence microscope, attempt to identify the polymer type of the fibers. Dissection microscope images of the fibers (40x) can also be used for this step. These results will serve as the reference to which the fiber identification success rates are compared.

Once all of these steps are completed, assess evidence in this order: density tests, surface morphology, and fluorescence staining and determine the polymer type of each of the ten fibers.

Instructions for determining polymer type:

2. Determine fiber polymer type using dissection microscope (40x) images or TRANS images alone.
4. Next, move onto the ‘Surface Morphology’ spreadsheet. Describe each of the fiber surface morphologies using visual descriptors such as: spirals, straight, bumpy, smooth, dots and holes on surface, streaky, segmented, concave towards middle, and/or deflated red blood cell. Usually at this stage, using only density tests and surface morphology, some of the fibers can already be uniquely identified. Only when absolutely necessary use fluorescence staining evidence.
5. If the fiber is segmented, it is automatically wool regardless of other evidence.
6. If the fiber floats in DeI water, it is automatically PP regardless of other evidence.
7. For fibers that need extra information and are light-coloured (white, yellow, gray) fibers, prepare a final spreadsheet called ‘Fluorescence Staining’. Add four columns: RFP background, RFP, CY5 background, CY5. Fibers are listed in the rows.

8. In ImageJ, open each of the fluorescence images and measure the average fluorescence of the fibers (‘grayscale intensity’ in ImageJ). Pick 3-4 locations on each fiber and average the grayscale intensity value. Copy it into Excel.

9. In Excel: divide the fluorescence intensity after Direct Red 23 and Sulfo-Cyanine5 staining respectively by the background fluorescence of the fiber soaked in PBS solution before staining.

10. If this number is 10 or greater than 10, then the fiber is considered to fluoresce after being stained by that specific dye. Cellulosic fibers fluoresce in RFP channel after staining by Direct Red 23. Nylon and protein fibers fluoresce in CY5 channel after staining by Sulfo-Cyanine5 Free Acid.

11. Using all the evidence together, determine the fiber polymer type.

REFERENCES

A. 8. FTIR spectra of fibers used to develop fiber identification method and in proof-of-concept tests.

<table>
<thead>
<tr>
<th>Material</th>
<th>FTIR Spectrum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nylon Towel</td>
<td><img src="image" alt="FTIR Spectrum" /></td>
</tr>
<tr>
<td>PP Rope</td>
<td><img src="image" alt="FTIR Spectrum" /></td>
</tr>
</tbody>
</table>
### Cotton Thread

![Graph of Cotton Thread](image1)

<table>
<thead>
<tr>
<th>Color</th>
<th>Hit Quality</th>
<th>Compound name</th>
<th>CAS Number</th>
<th>Molecular formula</th>
<th>Molecular weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>788</td>
<td></td>
<td>CELLULOSE 3WAB</td>
<td>9004-34-6</td>
<td>C6H10O5n</td>
<td></td>
</tr>
</tbody>
</table>

![Chemical Structure of Cellulose](image2)

### Polyester Thread

![Graph of Polyester Thread](image3)

<table>
<thead>
<tr>
<th>Color</th>
<th>Hit Quality</th>
<th>Compound name</th>
<th>CAS Number</th>
<th>Molecular formula</th>
<th>Molecular weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>434</td>
<td></td>
<td>POLY(ETHYLENE TERIPHthalate)</td>
<td>25038-89-5</td>
<td>(C10H12O4)n</td>
<td></td>
</tr>
<tr>
<td>426</td>
<td></td>
<td>POLYESTER FIBER</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

![Chemical Structure of Polyester](image4)

![Library Information](image5)
A. 9. Images of fibers from San Francisco Bay samples and multiple images of fibrous products showing similar trends in surface morphology for the same polymer.

<table>
<thead>
<tr>
<th>Fiber Origin (total magnification)</th>
<th>Raman Microscope Image (50x objective lens has NA 0.5, 100x objective lens has NA 0.8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Purple Polyester from San Francisco Bay (1000x total)</td>
<td><img src="image" alt="Image of purple polyester fiber" /></td>
</tr>
<tr>
<td>• tubular and smooth, with dots</td>
<td></td>
</tr>
<tr>
<td>Material</td>
<td>Description</td>
</tr>
<tr>
<td>----------------------------------------------</td>
<td>------------------------------</td>
</tr>
<tr>
<td>Pink Polyester from San Francisco Bay (1000x total)</td>
<td>• tubular and smooth with dots</td>
</tr>
<tr>
<td>Red Polyester Thread (500x total)</td>
<td>tubular and smooth with dots</td>
</tr>
<tr>
<td>Yellow PP Rope (500x total)</td>
<td>• streaky</td>
</tr>
<tr>
<td>Material</td>
<td>Description</td>
</tr>
<tr>
<td>--------------------------</td>
<td>-----------------------------------------</td>
</tr>
<tr>
<td>Gray PP Rope (500x total)</td>
<td>• streaky</td>
</tr>
<tr>
<td>White Nylon Fabric (500x total)</td>
<td>• tubular and smooth, dotted</td>
</tr>
<tr>
<td>Black Nylon Towel (500x total)</td>
<td>• tubular and smooth, with holes</td>
</tr>
</tbody>
</table>
White Cotton Thread (500x total)
- bumpy, concave with fat lips

White Cotton Gauze Pad (500x total)
- bumpy, spirals

Cellulosic Fiber from San Francisco Bay (1000x total)
- bumpy, spirals, concave towards middle like deflated red blood cell

The brightness and contrast of images were enhanced for better effect.
**A. 10. Reaction mechanisms of fluorescent dyes with polymers.**

<table>
<thead>
<tr>
<th>Dye and Polymer</th>
<th>Mechanism</th>
</tr>
</thead>
</table>
| Direct Red 23 and Cellulose              | - Hydrophobic stacking, van der Walls forces  
                                         | - Hydrogen bonding<sup>62</sup>                                                                                                          |
| Sulfo-Cyanine5 Free Acid and Nylon       | - Citric acid deprotonates carboxylic acid group on Sulfo-Cy5  
                                         | - Citric acid protonates amine group of nylon  
                                         | - Carboxylic acid attacks electrophilic amine group<sup>62</sup>                                                                      |
A. 11. Fluorescence microscope images showing positive and negative results of staining (40x total magnification, NA 0.13). Silk (left) fluoresces and acrylic (right) does not fluoresce after staining with Sulfo-Cyanine5 Free Acid (scale bar indicates 200 µm).

<table>
<thead>
<tr>
<th>Fiber Polymer</th>
<th>TRANS Channel Image</th>
<th>CY5 Channel Image</th>
</tr>
</thead>
<tbody>
<tr>
<td>Silk (Fluorescent)</td>
<td><img src="image1.png" alt="Image" /></td>
<td><img src="image2.png" alt="Image" /></td>
</tr>
<tr>
<td>Acrylic (Non-fluorescent)</td>
<td><img src="image3.png" alt="Image" /></td>
<td><img src="image4.png" alt="Image" /></td>
</tr>
</tbody>
</table>

Images have colour added for better effect.
### A. 12. Summary of expectations versus outcomes for each line of evidence as part of proof-of-concept tests for Experimenter 1.

<table>
<thead>
<tr>
<th>Fiber Material</th>
<th>Fluorescence Material</th>
<th>Fluorescence Expectation</th>
<th>Density Expectation (float): 1.2, 1.3, or Del</th>
<th>Density Outcome</th>
<th>Surface Morphology Expectation</th>
<th>Surface Morphology Outcome</th>
<th>Deduced Polymer Identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Black Nylon Towel</td>
<td>CY5</td>
<td>Neither</td>
<td>1.2, 1.3</td>
<td>1.2, 1.3</td>
<td>Tubular and smooth with dots or holes</td>
<td>Smooth, tubular, with dots/holes</td>
<td>Nylon</td>
</tr>
<tr>
<td>Yellow PP Rope</td>
<td>Neither</td>
<td>N/A</td>
<td>Del, 1.2, 1.3</td>
<td>Del, 1.2, 1.3</td>
<td>Streaky surface</td>
<td>streaks</td>
<td>PP</td>
</tr>
<tr>
<td>White Cotton Thread</td>
<td>RFP</td>
<td>RFP</td>
<td>None</td>
<td>None</td>
<td>Bumpy and spirals; fat lips and concave center</td>
<td>Bumpy, spiral</td>
<td>Cotton</td>
</tr>
<tr>
<td>Blue Polyester Thread</td>
<td>Neither</td>
<td>Neither</td>
<td>None</td>
<td>None</td>
<td>Tubular and smooth with dots or holes</td>
<td>smooth, tubular, with black dots</td>
<td>Polyester</td>
</tr>
<tr>
<td>Purple Wool Fibers</td>
<td>CY5</td>
<td>Neither</td>
<td>None</td>
<td>1.3</td>
<td>Segmented</td>
<td>Not smooth, Segmented</td>
<td>Wool</td>
</tr>
<tr>
<td>Gray PP Rope</td>
<td>Neither</td>
<td>N/A</td>
<td>Del, 1.2, 1.3</td>
<td>Del, 1.2, 1.3</td>
<td>Streaky surface</td>
<td>Smooth, tubular, with streaks</td>
<td>PP</td>
</tr>
<tr>
<td>Material Type</td>
<td>Fluorescence Expectation</td>
<td>Fluorescence Outcome</td>
<td>Density Expectation (float): 1.2, 1.3, or DeI</td>
<td>Density Outcome</td>
<td>Surface Morphology Expectation</td>
<td>Surface Morphology Outcome</td>
<td>Deduced Polymer Identity</td>
</tr>
<tr>
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<td>----------------------------</td>
<td>--------------------------</td>
</tr>
<tr>
<td>Black Nylon Towel</td>
<td>CY5</td>
<td>Neither</td>
<td>1.2, 1.3</td>
<td>1.2, 1.3</td>
<td>Tubular and smooth with dots or holes</td>
<td>Tubular with coloured dots or holes along fiber.</td>
<td>Nylon</td>
</tr>
<tr>
<td>Material</td>
<td>Color</td>
<td>Type</td>
<td>Location</td>
<td>Texture</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Yellow PP Rope</td>
<td>None</td>
<td>Neither</td>
<td>DeI, 1, 2, 1.3</td>
<td>Streaky surface</td>
<td>Streaks on surface like tree bark</td>
<td></td>
<td></td>
</tr>
<tr>
<td>White Cotton Thread</td>
<td>RFP</td>
<td>Neither</td>
<td>None</td>
<td>Bumpy and spirals; fat lips and concave center</td>
<td>Spirals Cotton</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blue Polyester Thread</td>
<td>Neither</td>
<td>RFP</td>
<td>None</td>
<td>Tubular and smooth with dots or holes</td>
<td>Tubular with polyester</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Purple Wool Fibers</td>
<td>CY5</td>
<td>Neither</td>
<td>None</td>
<td>Segmented</td>
<td>Segmented Wool</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gray PP Rope</td>
<td>Neither</td>
<td>CY5</td>
<td>DeI, 1, 2, 1.3</td>
<td>Streaky surface</td>
<td>streaky and tubular PP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brown Polyester Thread</td>
<td>Neither</td>
<td>Neither</td>
<td>None</td>
<td>Tubular and smooth with dots or holes</td>
<td>Tubular and polyester dots</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brown Wool Fibers</td>
<td>CY5</td>
<td>Neither</td>
<td>None</td>
<td>Segmented</td>
<td>Segmented Wool</td>
<td></td>
<td></td>
</tr>
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<td>White Silk Fabric</td>
<td>CY5</td>
<td>CY5</td>
<td>None</td>
<td>Spiral and bumpy</td>
<td>Spirals Silk</td>
<td></td>
<td></td>
</tr>
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<td>Plastic Type</td>
<td>Fluorescence Expectation</td>
<td>Fluorescence Outcome</td>
<td>Density Expectation (float): 1.2, 1.3, or DeI</td>
<td>Density Outcome</td>
<td>Surface Morphology Expectation</td>
<td>Surface Morphology Outcome</td>
<td>Deduced Polymer Identity</td>
</tr>
<tr>
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<td>-----------------------------------------------</td>
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<td>-------------------------------</td>
<td>----------------------------</td>
<td>--------------------------</td>
</tr>
<tr>
<td>Black Nylon Towel</td>
<td>CY5</td>
<td>N/A</td>
<td>1.2, 1.3</td>
<td>1.3</td>
<td>Tubular and smooth with dots or holes</td>
<td>Synthetic, dots</td>
<td>Nylon</td>
</tr>
<tr>
<td>Yellow PP Rope</td>
<td>Neither</td>
<td>N/A</td>
<td>DeI, 1.2, 1.3</td>
<td>DeI, 1.2, 1.3</td>
<td>Streaky surface</td>
<td>streaky</td>
<td>PP</td>
</tr>
<tr>
<td>White Cotton Thread</td>
<td>RFP</td>
<td>RFP and CY5</td>
<td>None</td>
<td>None</td>
<td>Bumpy and spirals; fat lips and concave center</td>
<td>not synthetic, bumpy</td>
<td>Cotton</td>
</tr>
<tr>
<td>Blue Polyester Thread</td>
<td>Neither</td>
<td>N/A</td>
<td>None</td>
<td>None</td>
<td>Tubular and smooth with dots or holes</td>
<td>synthetic smooth, streak, dots</td>
<td>Polyester</td>
</tr>
</tbody>
</table>

‘N/A’ means evidence not considered or necessary to determine final polymer type.

**A. 14.** Summary of expectations versus outcomes for each line of evidence as part of proof-of-concept tests for Experimenter 3.
<table>
<thead>
<tr>
<th>Purple Wool Fibers</th>
<th>CY5</th>
<th>N/A</th>
<th>None</th>
<th>None</th>
<th>Segmented (pink)</th>
<th>Wool</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gray PP Rope</td>
<td>Neither</td>
<td>N/A</td>
<td>DeI, 1.2, 1.3</td>
<td>DeI, 1.2, 1.3</td>
<td>Streaky surface</td>
<td>PP</td>
</tr>
<tr>
<td>Brown Polyester Thread</td>
<td>Neither</td>
<td>N/A</td>
<td>None</td>
<td>None</td>
<td>Tubular and smooth with dots or holes</td>
<td>Polyester</td>
</tr>
<tr>
<td>Brown Wool Fibers</td>
<td>CY5</td>
<td>N/A</td>
<td>None</td>
<td>None</td>
<td>Segmented very segmented</td>
<td>Wool</td>
</tr>
<tr>
<td>White Silk Fabric</td>
<td>CY5</td>
<td>CY5</td>
<td>None</td>
<td>None</td>
<td>Spiral and bumpy twisty</td>
<td>Silk</td>
</tr>
<tr>
<td>Green Acrylic Yarn</td>
<td>Neither</td>
<td>N/A</td>
<td>1.2, 1.3</td>
<td>1.2, 1.3</td>
<td>Streaky surface</td>
<td>Acrylic</td>
</tr>
</tbody>
</table>

‘N/A’ means evidence not considered or necessary to determine final polymer type.
**A. 15.** Summary of expectations versus outcomes of fiber identities as part of method validation on environmental samples for Tester 1.

<table>
<thead>
<tr>
<th>Plastic Type</th>
<th>Fluorescence Expectation</th>
<th>Fluorescence Outcome</th>
<th>Density Expectation (float): 1.2, 1.3, or DeI</th>
<th>Density Outcome</th>
<th>Surface Morphology Expectation</th>
<th>Surface Morphology Outcome</th>
<th>Deduced Polymer Identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red polyester</td>
<td>Neither</td>
<td>Neither</td>
<td>None</td>
<td>None</td>
<td>Tubular with coloured dots or holes.</td>
<td>Holes and dots, smooth surface</td>
<td>Polyester</td>
</tr>
<tr>
<td>Green PP</td>
<td>Neither</td>
<td>Neither</td>
<td>None</td>
<td>None</td>
<td>Streaks on surface like tree bark</td>
<td>Streaky, but also bumpy</td>
<td>Cellulosic</td>
</tr>
<tr>
<td>Dark blue cellulose</td>
<td>RFP</td>
<td>Lost during staining</td>
<td>None</td>
<td>Lost during staining</td>
<td>Bumpy, concave center, spirals</td>
<td>Fat lips and concave center</td>
<td>Cellulosic</td>
</tr>
<tr>
<td>Dark blue cellulose</td>
<td>RFP</td>
<td>Lost during staining</td>
<td>None</td>
<td>Lost during staining</td>
<td>Bumpy, concave center, spirals</td>
<td>Fat lips and concave center</td>
<td>Cellulosic</td>
</tr>
<tr>
<td>Black nylon</td>
<td>CY5</td>
<td>Lost during staining</td>
<td>1.2, 1.3</td>
<td>Lost during staining</td>
<td>Tubular with coloured dots or holes.</td>
<td>Smooth with coloured dots</td>
<td>Nylon</td>
</tr>
<tr>
<td>Black PP</td>
<td>Neither</td>
<td>Neither</td>
<td>DeI, 1.2, 1.3</td>
<td>DeI, 1.2, 1.3</td>
<td>Streaks on surface like tree bark</td>
<td>Streaky but also bumpy</td>
<td>PP</td>
</tr>
<tr>
<td></td>
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<td>--------------------------</td>
<td>--------------------------</td>
</tr>
<tr>
<td>Black wool</td>
<td>CY5</td>
<td>CY5</td>
<td>None</td>
<td>None</td>
<td>Segmented</td>
<td>Segmented</td>
<td>Wool</td>
</tr>
<tr>
<td>Black polyester</td>
<td>Neither</td>
<td>Neither</td>
<td>None</td>
<td>None</td>
<td>Tubular with coloured dots or holes.</td>
<td>Smooth, fat lips, some dots</td>
<td>Cellulosic</td>
</tr>
<tr>
<td>Red polyester</td>
<td>Neither</td>
<td>CY5</td>
<td>None</td>
<td>None</td>
<td>Tubular with coloured dots or holes.</td>
<td>Cracked and flattened – hard to tell; there are dots on the surface</td>
<td>Polyester</td>
</tr>
<tr>
<td>Black wool</td>
<td>CY5</td>
<td>Neither</td>
<td>None</td>
<td>None</td>
<td>Segmented</td>
<td>Segmented</td>
<td>Wool</td>
</tr>
<tr>
<td>Clear PP</td>
<td>Neither</td>
<td>CY5</td>
<td>DeI, 1.2, 1.3</td>
<td>Lost during density tests</td>
<td>Streaks on surface like tree bark</td>
<td>Holes and dots on surface</td>
<td>Polyester</td>
</tr>
<tr>
<td>Dark blue cotton</td>
<td>RFP</td>
<td>Neither</td>
<td>None</td>
<td>None</td>
<td>Bumpy, concave center, spirals</td>
<td>Spirals with fat lips</td>
<td>Cellulosic</td>
</tr>
<tr>
<td>Black acrylic</td>
<td>Neither</td>
<td>Neither</td>
<td>1.2, 1.3</td>
<td>1.3</td>
<td>Streaks on surface like tree bark</td>
<td>Streaky</td>
<td>Acrylic</td>
</tr>
<tr>
<td>Blue nylon</td>
<td>CY5</td>
<td>Neither</td>
<td>1.2, 1.3</td>
<td>1.2, 1.3</td>
<td>Tubular with coloured dots or holes.</td>
<td>Bumpy; cracked and flattened; hard to tell</td>
<td>Nylon</td>
</tr>
<tr>
<td>Plastic Type</td>
<td>Fluorescence Expectation</td>
<td>Fluorescence Outcome</td>
<td>Density Expectation (float): 1.2, 1.3, or DeI</td>
<td>Density Outcome</td>
<td>Surface Morphology Expectation</td>
<td>Surface Morphology Outcome</td>
<td>Deduced Polymer Identity</td>
</tr>
<tr>
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<td>--------------------------</td>
</tr>
<tr>
<td>Red polyester</td>
<td>Neither</td>
<td>Neither</td>
<td>None</td>
<td>Lost during density tests</td>
<td>Tubular with coloured dots or holes.</td>
<td>Bumpy; cracked and flattened; hard to tell</td>
<td>Acrylic</td>
</tr>
<tr>
<td>Black wool CY5</td>
<td>Neither</td>
<td>None</td>
<td>None</td>
<td>None</td>
<td>Segmented</td>
<td>None</td>
<td>Polyester</td>
</tr>
<tr>
<td>Black PP CY5</td>
<td>Neither</td>
<td>Neither</td>
<td>DeI, 1.2., 1.3</td>
<td>DeI, 1.2., 1.3</td>
<td>Streaks on surface like tree bark</td>
<td>Holes and dots on smooth surface, streaks</td>
<td>PP</td>
</tr>
<tr>
<td>Clear wool CY5</td>
<td>CY5</td>
<td>CY5</td>
<td>None</td>
<td>Lost during density tests</td>
<td>Segmented</td>
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<td>Wool</td>
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</table>

A. 16. Summary of expectations versus outcomes of fiber identities as part of method validation on environmental samples for Tester 2.
<p>| | | | | | | | |</p>
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</tr>
</thead>
<tbody>
<tr>
<td>Green PP</td>
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<td>None</td>
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<td>Streaky</td>
<td>Polyester</td>
</tr>
<tr>
<td>Dark blue cellulose</td>
<td>RFP</td>
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<td>None</td>
<td>Lost during staining</td>
<td>Bumpy, concave center, spirals</td>
<td>Red blood cell, concave, bumpy</td>
<td>Cellulosic</td>
</tr>
<tr>
<td>Dark blue cellulose</td>
<td>RFP</td>
<td>Lost during staining</td>
<td>None</td>
<td>Lost during staining</td>
<td>Bumpy, concave center, spirals</td>
<td>Bumpy? segmented?</td>
<td>Wool</td>
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<td>CY5</td>
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<td>Tubular with coloured dots or holes.</td>
<td>Smooth with dots</td>
<td>Nylon</td>
</tr>
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<td>DeI, 1.2, 1.3</td>
<td>Streaks on surface like tree bark</td>
<td>Streaky</td>
<td>PP</td>
</tr>
<tr>
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<td>CY5</td>
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<td>None</td>
<td>None</td>
<td>Segmented</td>
<td>Scales</td>
<td>Wool</td>
</tr>
<tr>
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<td>Neither</td>
<td>None</td>
<td>None</td>
<td>Tubular with coloured dots or holes.</td>
<td>Smooth with dots</td>
<td>Polyester</td>
</tr>
<tr>
<td>Red polyester</td>
<td>Neither</td>
<td>CY5</td>
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<td>None</td>
<td>Tubular with coloured</td>
<td>Streaky?</td>
<td>Wool</td>
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<td>None</td>
<td>Segmented</td>
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<td>Wool</td>
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<tr>
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<td>Lost during density tests</td>
<td>Streaks on surface like tree bark</td>
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<td></td>
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<td></td>
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<td></td>
<td>Nylon</td>
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</tr>
<tr>
<td>Dark blue cotton</td>
<td>RFP</td>
<td>Neither</td>
<td>None</td>
<td>None</td>
<td>Bumpy, concave center, spirals</td>
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<td>Smooth no dots, twisty</td>
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<td>Cellulosic</td>
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<td>1.2, 1.3</td>
<td>1.3</td>
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<td>Acrylic</td>
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</tr>
<tr>
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<td>1.2, 1.3</td>
<td>Tubular with coloured dots or holes.</td>
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<td></td>
<td></td>
<td></td>
<td>Acrylic</td>
<td></td>
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</tr>
<tr>
<td>Red polyester</td>
<td>Neither</td>
<td>Neither</td>
<td>None</td>
<td>Lost during density tests</td>
<td>Tubular with coloured dots or holes.</td>
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<td>Ragged/bumpy PP</td>
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<td>CY5</td>
<td>Neither</td>
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<td>None</td>
<td>Segmented</td>
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<td>More streaks than dots</td>
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<td>Polyester</td>
<td></td>
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<tr>
<td>Black cotton</td>
<td>Neither</td>
<td>Neither</td>
<td>Del, 1.2, 1.3</td>
<td>Del, 1.2, 1.3</td>
<td>Streaks on surface like tree bark</td>
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<td></td>
<td></td>
<td>Smooth with dots</td>
<td></td>
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<td></td>
<td></td>
<td>PP</td>
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<td>CY5</td>
<td>CY5</td>
<td>None</td>
<td>Lost during density tests</td>
<td>Segmented</td>
<td>Scales</td>
<td>Wool</td>
</tr>
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