LOW INCIDENCE OF MICROPLASTIC CONTAMINANTS IN PACIFIC OYSTERS FROM THE SALISH SEA, USA

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15 **HIGHLIGHTS**

16

17 - Microplastics in oysters were found in 5 of 10 sites in the Salish Sea, Washington.

18 - Only ~2% of the microparticles were identified as microplastics by RMS and FTIR.

19 - Sorbitan derivatives, polyamide resins, cellulose, and minerals were also present.

20 - Microfibers were observed but were not confirmed as plastic or polymeric with RMS.

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ABSTRACT

Plastic pollution is a threat to marine life with long term impacts to ecosystems and organisms in the sea. In this study, we quantified the presence of microparticles in wild populations of Pacific oysters (*Crassostrea gigas*) from the Salish Sea, Washington State. Examination under a dissecting microscope revealed 63% of oysters contained microparticles (~1.75 microparticles per oyster) and microfibers were the dominant type of particles. Using Raman microspectroscopy (RMS) and Fourier Transform Infrared microspectroscopy (µ-FTIR) we found that only ~2 % of these microparticles were synthetic and included polymers such as polystyrene, polyethylene, polypropylene, poly(bisphenol A carbonate), rayon, and polyacrylate. It is important to note that of the 447 microparticles analyzed with RMS, 41% showed fluorescence interference, impeding the determination of their identification. The remaining microparticles were cellulose derivatives, shell fragments, biological or proteinaceous material, salts, minerals, and gypsum. Fourier Transform Infrared spectroscopy equipped with a diamond Attenuated Total Reflectance accessory (ATR-FTIR) showed the presence of sorbitan derivatives in all samples examined (n = 213). These findings provide the first baseline for microplastic and other particles in oysters from the west coast of the United States integrating results from ATR-FTIR, µ-FTIR, and RMS, in addition to visual sorting. These results suggest there is low retention of plastic particles in Pacific oysters from the Salish Sea, but further research is needed to determine the composition of microparticles with fluorescence interference.

[229 words]

INTRODUCTION

Plastic pollution is a threat to marine life around the globe with long term impacts to coastal ecosystems and the industries that depend on them. Small plastic particles or microplastics are synthetic solid particles or polymeric matrices, that have regular or irregular shape and range in size from 1μm to 5mm (Arthur et al. 2009, Frias & Nash 2019). Microplastics are insoluble in water and can have either primary or secondary manufacturing origin. Microplastics are considered to be primary in origin when they are manufactured to be small, or secondary when they are a result of a slower, long-term breakdown from larger plastics. Primary and secondary microplastics can reach the ocean from wastewater treatment plant runoff, or form when plastic debris is fragmented by UV radiation and wave abrasion (e.g. Weinstein et al. 2016). Currently, microplastics have been reported along shorelines around the world on all continents (Moore 2008, Browne et al. 2011, Cole et al. 2011, Hirai et al. 2011, Waller et al. 2017) and have been found in sediments, throughout the water column, and in the digestive systems, respiratory structures, and tissues of marine organisms worldwide (Andrady 2011, Browne et al. 2011, Cole et al. 2011, Depledge et al. 2013, Ling et al. 2017, Munari et al. 2017). Because of their small size, microplastics can be easily ingested by marine organisms, particularly by species that have limited ability to select particles during feeding (Ward et al. 1994, Ward & Shumway 2004). The accumulation of microplastics in organisms may have detrimental effects on feeding rates, energy storage, reproduction and overall fitness (Paul-Pont et al. 2016, Sussarellu et al. 2016, Welden & Cowie 2016, Harris and Carrington 2019). Furthermore, microplastics do not degrade quickly and may be transferred through trophic interactions (Farrell & Nelson 2013, Au et al. 2017), creating a pathway to ingestion of microplastics by animals at higher trophic levels, including fish, predatory mammals, and humans (Wang et al. 2019).

Microplastics are usually transported in the water, either by marine currents or in freshwater systems by rivers (see review by Cole et al. 2011 for marine environments, Siegfried et al. 2017, Horton & Dixon 2018). In regions with high population density, it is likely that more microplastics will be released to the waterways (e.g. Miller et al. 2017). Similarly, waters that are stagnant, or have longer residence times (e.g, in a bay, inlet, or fjord), may accumulate more microparticles at depth and in sediments (Wessel et al. 2016). Therefore, marine organisms living under different oceanographic conditions could be differentially exposed to microplastic contaminants. Previous research suggests that microfibers from clothing are the most common type of secondary microplastic worldwide (Carr 2017, Cesa et al. 2017). When clothes are washed, these small fibers are released into the water, and their small size allows them to pass through filters, into grey water and eventually local waterways. Other secondary microplastic sources include the breakdown of plastic bags and containers that are transported into waterways, and the breakdown of fishing or aquaculture gear. Primary sources can be microbeads used in cleaning and beauty products that are also transported by waterways. Distinguishing primary vs. secondary plastics or synthetic vs. natural particles in the marine environment is a difficult task. In our study we only use the term microplastic (regardless of origin) for particles that were identified as synthetic polymers by Raman microspectroscopy (RMS), Fourier Transform Infrared Spectroscopy equipped with a diamond Attenuated Total Reflectance accessory (ATR-FTIR), or FTIR microspectroscopy (μ-FTIR). Other particles that were similar in size and visual characteristics to microplastics but with unknown composition were referred to as microparticles, sometimes also referred to as “suspected microplastics”.

Microplastics have been found in ecologically and commercially important bivalves from

97 Europe, Asia, Brazil, Canada, and the United States (Van Cauwenberghe & Janssen 2014,
98 Mathalon & Hill 2014, Rochman et al. 2015, Li et al. 2016), however, there is a lack of
99 research in the eastern North Pacific, one of the most productive regions in the world and
100 with high potential for marine aquaculture (Baechler et al. 2019, Granek et al. 2020). On the
101 west coast of the United States, two studies have assessed and quantified the presence of
102 microplastics in commercially important bivalves (Rochman et al. 2015, Baechler et al.
103 2019). One study found that 33% of the Pacific oysters sampled in California markets (n =
104 12) contained 0-2 microparticles per individual (Rochman et al. 2015). These particles were
105 identified as microfibers using visual sorting but no further analyses were performed to assess
106 their chemical identity. The other study carried out in the Oregon coast found that
107 microplastics were present in Pacific oysters and razor clams, but only 26 of the 2428
108 microfibers were analyzed with FTIR. In Canada, visual sorting also revealed the presence of
109 microparticles in wild and cultured Manila clams (8-11 particles/clam; Davidson & Dudas
110 2016, Murphy 2018), oysters, and mussels (5.6-6.57 per gram; Mathalon & Hill 2014,
111 Murphy 2018). Murphy (2018) was the only other study in addition to Baechler et al. (2019)
112 that examined the chemical identity of microparticles. Using FTIR analysis, this study
113 showed that only half of the microparticles extracted were synthetic polymers (including
114 plastic). In this context, it is fundamental that microplastic determinations are based on
115 chemically identified polymers, otherwise overestimation of microplastics and
116 misinterpretation of results may occur (Shumway et al. 2018).
117
118 In this study, we examine the presence and distribution of microplastic pollution in the
119 Pacific oyster, *Crassostrea gigas*, an ecologically and economically important shellfish
120 species in Washington State, USA (Fig. 1). As ecosystem engineers, their presence creates a
121 hard-bottom substrate that provides habitat and protection for other organisms. Furthermore,

oysters are important food sources for aquatic animals and can filter high volumes of water removing organic and inorganic particles from the water column, resulting in cleaner water (Coen et al. 2007). In the Salish Sea and Puget Sound in particular, there are multiple self-sustaining *C. gigas* populations in bays and inlets where the oysters grow either individually or in dense mats on rocks and soft-bottom substrates. Economically, the hardiness and rapid growth (10-15 cm in 2 years) of *C. gigas* are major advantages for the aquaculture industry. Pacific oysters currently lead the production output of the aquaculture industry in Washington State, the top producer of shellfish in the US with around 200 million dollars in sales in 2014 (Washington Sea Grant 2015). Understanding the abundance and diversity of microplastics in *C. gigas* will help determine if the species is vulnerable to these contaminants and to what degree. Specifically, our goals are to: (i) determine the abundance and type of microplastics in naturally occurring populations of *C. gigas* in Washington State, (ii) identify the material of these microplastics, and (iii) identify potential areas that are 'hotspots' for microplastic accumulation in Washington State. We hypothesize that microfibers will be the dominant type of microplastics in oysters, and that regions with longer water residence times will have oysters with higher concentration of microplastics due to longer exposure to these contaminants over time. Considering that marine plastic waste is expected to increase (Andrady 2011, Cózar et al. 2014, Lebreton et al. 2018), this study will contribute to establishing a baseline for the eastern North Pacific, and provide evidence to inform and direct mitigation strategies aimed at aiding in the detection of microplastic sources and the production of healthy shellfish.

METHODS

Location and species

146 The study was performed in Puget Sound in Washington State, the leading producer of
147 farmed bivalves in the United States (Fig. 1). Puget Sound is located in the southern end of
148 the Salish Sea, is the second largest estuary in the country, and is a complex glacial system
149 with seven sub-basins that have distinct characteristics due to the degree of water mixing,
150 freshwater influence, and wind fetch. The sampling sites are located throughout Puget Sound
151 to capture the variability in environmental conditions between basins along the estuary (Fig.
152 1, Table 1). Sites in Northern Puget Sound often experience shorter water residence times (2
153 months) than the average for Puget Sound due to their proximity to the Pacific Ocean
154 (Encyclopedia of Puget Sound). In contrast, South Puget Sound has longer water residence
155 times (2-4 months) due to limited water circulation and mixing (Finlayson 2006). Puget
156 Sound estuary is within close proximity to some of the largest cities in Washington State
157 (Seattle, Tacoma and Olympia, Fig. 1) with a population of approximately 3,867,000 people,
158 making it the 14th largest metropolitan area in the United States (United States Census Bureau
159 2017). Previous studies in the Puget Sound region have found that microplastics are present
160 in sediment and water samples (Masura et al. 2015, Eshom-Arzadon 2017). However, there
161 are no published studies examining accumulation of microplastics in shellfish that naturally
162 grow in the area.
163
164 **Sample collection and processing**
165 We collected 30 *C. gigas* individuals during the low tide at ten sites in Puget Sound estuary
166 between January and April 2018 (Table 1, Fig. 1). All collection sites are State Parks or
167 public access beaches with self-sustaining Pacific oyster populations. Within 48 hs of
168 collection, we dissected oysters and measured the right and left valves to the nearest mm with
169 a digital caliper. We then removed the soft tissue from the shells, weighed it to the nearest mg

with an XS304 Mettler Toledo digital scale, and stored it at -20 °C until the microplastic extraction protocol was performed.

Tissue digestion and microplastics extraction

We used a standardized extraction protocol developed by Li et al. (2015) to extract potential microplastics from oysters. We placed each oyster tissue in a 1 L glass beaker and digested it with 250 ml of 30% hydrogen peroxide in an oscillator (3500 VWR Advanced) at 65 °C for 24 hs. In some cases, larger oysters took up to 48 hs to be fully digested. During digestion we covered the beakers with aluminum foil to prevent dehydration and airborne contamination of the samples. Each digestion cycle, we processed three samples and a blank extraction control. This number was determined by the maximum number of beakers that fit into the oscillator in the incubator (4 beakers total). We then added 750 ml of 25% saline solution to the digested solution. The water for the saline solution was filtered with a 1.0 µm pore size nuclepore hydrophilic membrane (VWR Whatman). In the saline solution, positively buoyant microparticles floated to the surface while heavier, biotic (undigested tissue), and abiotic (sediment) sank to the bottom. After 24 hs, we filtered the solution with 5 µm pore size Whatman nitrate cellulose membrane using a vacuum pump system under a fume hood. Finally, we placed each filter membrane in a labeled and sealed Petri dish and left it to dry for 48 hs.

Given that airborne microplastic contamination is very common (Foekema et al. 2013, Torre et al. 2016, Granek et al. 2020), we adopted a series of measures to prevent it. In addition to the control blanks throughout the protocol, we rinsed the glass beakers and other lab material with deionized filtered water three times prior to use (Li et al. 2015). To prevent contamination from our own clothing we used a 100% cotton laboratory coat during all

195 experimental procedures and analyses of the filters (Van Cauwenberghe & Janssen 2014).
196
197 **Observation and quantification of microplastics and microparticles**
198 Determination of microplastics was performed using the definition proposed by Frias & Nash
199 (2019). Only synthetic polymers identified by RMS, ATR-FTIR, or µ-FTIR were considered
200 microplastics. Other particles similar in size and visual characteristics to microplastics but
201 with unknown composition are referred as microparticles throughout the manuscript. Once
202 the filters were dry, we visually sorted and enumerated microparticles under a dissecting
203 microscope. Visual sorting is one of the most common ways to quantify and identify potential
204 microplastics, and differentiate morphotypes such as fibers (slender and elongated), spheres
205 (round, similar to a ball in shape), flakes (small and very thin layer of larger plastic debris),
206 and fragments (isolated or incomplete part of larger plastic debris that did not fit any of the
207 previous categories; Hidalgo-Ruz et al. 2012, Li et al. 2016). The dimensions and coloration
208 of the microparticles on the filters were obtained by taking pictures (Nikon Eclipse Ni camera
209 with a 4x objective attached to a dissecting scope) and estimating their length using the open
210 software ImageJ.
211
212 We used a d'Agostino test to test for normality in our data. The null hypothesis for this test is
213 that data are normally distributed (not skewed) and the alternative hypothesis is that data are
214 skewed. In our case the test indicated the data are skewed (skew = 2.38, $z = 9.21$, $p < 2.2e-16$).
215 16). As a result, we used a Kruskal-Wallis rank sum tests were used to determine if there
216 were significant differences in oyster weight per site and microparticle abundance between
217 sites. A Spearman correlation test was also used to determine if there was a significant
218 positive association between number of microparticles and oyster weight per site. All

219 analyses were carried out in R Statistical Computing software, version 3.5.3 (R Core Team
220 2019).
221
222 To account for potential sources of contamination we visually inspected the filters of blank
223 extraction controls, and compared the microparticles found in them with those retrieved in
224 the filters with sample after digestion. If there was an agreement between the type or color of
225 microparticle in the samples, with the ones found in the controls, these microparticles were
226 not considered towards the total count as they were considered to come from contamination.
227
228 **Validation of microplastic identity through polymer chemical analyses**
229 After categorizing and measuring microparticles retained in filters we employed RMS and
230 ATR-FTIR to determine the identity of the polymers that make up those microparticles.
231 These non-destructive techniques record the signals absorbed or produced from individual
232 microparticles, and are commonly used for microplastic identification due to their high
233 accuracy in polymer recognition (e.g. Enders et al. 2015, Loder et al. 2015, Tagg et al. 2015,
234 Imhof et al. 2016). RMS provides information about the structure of a molecule depending on
235 its polarizability, while FTIR identifies the presence of certain functional groups in an
236 organic molecule depending on the molecules' change in dipole moment (Hind et al. 2001).
237
238 In recent studies, FTIR has been more frequently used for marine microplastic identification
239 than RMS (Lenz et al. 2015). However, RMS can provide advantages over FTIR such as
240 lower minimum microparticle size identification (Lenz et al. 2015, Kappler et al. 2016). In
241 addition, RMS measurements can be done on thicker or strongly absorbing microparticles
242 because measurements do not depend on the transmission of excited light through the sample
243 material (Lenz et al. 2015). Both RMS and FTIR have limitations in the identification of

molecules because of the different approaches used to determine the polymer identity. However, when used in combination they become a powerful tool for polymer characterization.

Across ten locations, we randomly selected 69 oyster samples and 23 controls to analyze by RMS. For each oyster (filter sample) we selected representative microparticles (n = 3-10 depending on microparticle density on the filter) of various sizes and shapes and analyzed them using a Renishaw inVia Raman microspectrometer with 785 nm and 514 nm lasers. Two different objectives (5x and 10x) were used to optimize the analytical laser spot for spectral analysis, and the laser power and acquisition times were varied depending on each microparticle's sensitivity to thermal damage. Then, we individually and manually matched each microparticle's Raman spectrum to a known plastic Raman spectrum in the Renishaw Raman Database of Polymers library, containing 267 polymer entries (Renishaw). Each spectrum was subjected to data processing depending on the signal-to-noise ratio and fluorescent interference (which produces a curved, sloped baseline). Baseline correction allows for the removal of a distorted spectrum before comparing to a known reference spectrum. While some spectra received baseline correction (linear or polynomial baseline correction available in the Renishaw Windows-based Raman environment software) not every spectrum required baseline correction before library searching. The reference spectrum from the library database matched when all the reference peaks (in wavenumbers) appeared in the sample spectrum. Additional peaks appear in the sample spectrum because of proteinaceous or biological contaminants or effects from environmental degradation. It is important to note that all the spectra present in the polymer library correspond to virgin polymers which have not been subject to any environmental degradation. Comparing spectra

268 between virgin polymers and non-virgin polymers exposed the marine environment can
269 hinder the ability to detect and identify microplastics.
270
271 In our samples, physical degradation (such as cracks, color change, or embrittlement) of the
272 microplastics was not observed or quantified visually. However, it is possible that
273 fragmentation, due to physical degradation, occurred as the microplastics identified were all
274 less than 50 µm in size. For the positively identified microplastics in our samples, the Raman
275 spectrum and all of the major peaks matched the reference spectrum exactly indicating that
276 degradation did not hinder our identification analysis via RMS (Lenz et al. 2015).
277
278 We also used a Bruker Vertex 70 ATR-FTIR to determine polymer identification of 70 filters
279 with samples and 24 filters used as controls. Since the diamond ATR crystal has an area of 4
280 mm^2 and the filters have an area of ~1662 mm^2, each filter was analyzed in two different
281 random areas to confirm whether or not the samples or controls were uniform. We first
282 analyzed the control filters to obtain a background spectrum and capture background sources
283 such as light or other residual signals from the atmosphere. Next, we obtained the samples'
284 spectrum and subtracted the background spectrum from the sample's spectrum to obtain the
285 FTIR spectrum which we then matched to an FTIR spectral library. Similar to RMS, the
286 FTIR library contained only virgin polymer spectra.
287
288 µ-FTIR was performed on selected individual microparticles from three filters that had
289 confirmed microplastics from RMS to determine if both techniques detected the same
290 contaminants. µ-FTIR was performed on 5 µm - 50 µm microparticles with a Thermo
291 Scientific Nicolet iNTM10 FT-IR microscope with a fixed focal length 15x objective. The

292 microparticles were isolated from the filters and, depending on the sample, were analyzed
293 with either micro-ATR or micro-reflection techniques.
294
295 **RESULTS**
296 **Observation and measurement of microparticles**
297 Using a dissecting scope, we counted and measured microparticles from 213 of the 300
298 oysters collected throughout ten sites in Puget Sound (Table 2). This difference is due to the
299 fact that several filters had plenty of digested organic matter and we could not process them.
300 Of the 213 filters we were able to process (one filter per oyster), 63% (n = 134) had at least
301 one microparticle visible under the dissecting scope, and in 96% of the cases (n = 129) these
302 microparticles were microfibers. The rest of the microparticles (4% of the total) were
303 classified as flakes or fragments. For 37% of the filters (n = 79) we did not observe
304 microparticles resembling plastic under the dissecting scope. Across all sites, mean
305 microfiber length was 621.93 µm (n = 276), median fiber length was 485.14 µm (n = 276),
306 with minimum and maximum values ranging between 102.45 µm and 2885.49 µm
307 respectively (n = 276).
308
309 When grouped per site, the mean number of microparticles per oyster ranged between 0.69
310 and 3 (Table 2, Fig. 2A) and there were significant differences between sites (Kruskal-Wallis
311 rank sum test, $\chi^2 = 32.84$, $p = 0.0001$), where Oakland Bay and Samish Bay had significantly
312 more microparticles than the other sites (Wilcoxon rank sum test, W = 322, $p = 0.0099$, Fig.
313 2A). Mean oyster weight per site ranged between 12.23 gr to 59.85 gr (Table 2, Fig. 2B).
314 There were significant differences in oyster weights between sites (Kruskal-Wallis rank sum
315 test, $\chi^2 = 103.07$, $p < 2.2^{-16}$), where Oakland Bay oysters were significantly smaller
316 (Wilcoxon rank sum test, W = 101.5, $p = 0.0006$, Fig. 2B) and Samish Bay oysters were

317 significantly larger than the other sites (Wilcoxon rank sum test, W = 415, $p = 1.41^{-10}$, Fig.
318 2B). However, overall, the number of microparticles was not associated with the biomass
319 (size) of the oyster (Spearman's correlation, S = 15983, $p = 0.912$, Fig. 2C).
320
321 In terms of coloration, the majority of the microparticles identified under the dissecting scope
322 were dark (either blue or black), light (yellow, white, silver), clear, red, purple and green
323 (Fig. 3A).
324
325 **Validation of microparticles through polymer identification analyses**
326 We analyzed a total of 447 microparticles from oyster and control filters using RMS
327 (Supplementary Table 1). The number of microparticles analyzed per site differed as it
328 depended on the density of microparticles found on each filter. The shapes of the
329 microparticles varied from fiber, shard, irregular, and spheroid with microparticles sizes
330 ranging from 20 µm to 13000 µm. Out of the 447 microparticles examined, only eight (~2%
331 of the total number of microparticles analyzed) were identified as microplastics (Fig. 4, Fig.
332 5, Fig. 6, Table 3). Seven of those microplastics were found in filters with oyster samples and
333 only one microplastic (polystyrene) was found in one control filter. In the oyster samples,
334 two microparticles were identified as polystyrene (PS), three particles as polypropylene (PP),
335 and two as polyethylene (PE). All of these microplastic samples ranged in size from 50 - 150
336 µm and the shapes were irregular, spheroid, or shard. In terms of coloration, the majority of
337 the microparticles identified using RMS were clear, light (mostly opaque white), dark (either
338 blue or black), and pink or purple (Fig. 3B).
339
340 RMS results showed that 10% (n = 46) of the microparticles that were colorless or opaque,
341 with an irregular or fiber-like shape, were identified as "cellulose", or a mixture of cellulose-

342 derived polymers (Fig. 4). These cellulose-derived microfibers were found on both control
343 samples and oyster samples, but more predominantly on the control samples and had the
344 same composition of the filter papers used. RMS also identified 18% (n = 81) of
345 microparticles as either poly(ethylene glycol) monooleate or polyamide resin (Table 3, Fig.
346 4). Differentiation of these particles were not possible because of a combination of high
347 fluorescence interference and because the spectra for the two polymers are similar.
348 RMS results showed that 29% (n = 127) of the microparticles surfaces were biological, salt,
349 or mineral in nature. These microparticles were predominantly < 75 µm and of various
350 shapes and are labeled as 'others' (Fig. 4). The remaining proportion of the microparticles
351 analyzed, 41% (n = 185), contained high fluorescence interference, possibly from a layer of
352 biofilm, and weak Raman signals which prohibited our ability to identify them despite
353 organic matter removal efforts. Based on their RMS spectra, we suspect that some of these
354 microparticles are biological materials (such as protein and carbohydrates), silicates,
355 minerals, or shell fragments, similar to those found by Wagner et al. (2017).
356
357 Examinations using ATR-FTIR revealed that 100% of the filters examined with oyster
358 samples from all sites (n = 71) contained a mixture of sorbitan derivatives such as sorbitan
359 monopalmitate, sorbitan trioleate, sorbitan monolaurate, sorbitan monooleate, or polysorbate
360 (a representative FTIR spectrum of sorbitan monopalmitate is shown in Fig. 7). Sorbitan
361 derivatives were found consistently across the entire filter for all samples. µ-FTIR was
362 performed on selected particles in a small subset of our samples that had microplastics
363 present. µ-FTIR confirmed our RMS results such that the majority of the microparticles were
364 identified as proteinaceous, fatty acid esters, and additionally identified the presence of
365 polyester, rayon, poly(t-butyl acrylate), and poly(bisphenol A carbonate) (Table 3).
366

Due to the small number of microplastics found in this study we were not able to establish any comparisons between sites with different oceanographic conditions. Similarly, the small number of microplastics per site (Table 2) also limited our ability to determine any hotspots for microplastic contamination in the different basins of Puget Sound estuary.

DISCUSSION

The global demand for plastics has consistently increased over recent years (Andrady 2011, Browne et al. 2011), leading to alarming rates of pollution worldwide (Gallo et al. 2018). Marine plastics have been estimated to exceed 5 trillion pieces (Eriksen et al. 2014), posing challenges for the health of marine ecosystems and the industries that depend on them. This first baseline of microplastic presence in Pacific oysters from Puget Sound revealed five major findings: (1) ~2% of the microparticles were identified as microplastics using RMS or µ-FTIR, (2) microplastics in oysters were present in five out of ten sites examined, (3) sorbitan derivatives were present in all oyster samples, (4) 41% of microparticles showed fluorescence interference and could not be identified with RMS, and (5) when observed under a dissecting scope, fibers were the most common type of microparticle found, but most of these fibers did not have a plastic composition as indicated by RMS and µ-FTIR.

Particles identified under the dissecting microscope

Oysters from all ten sites had microparticles that were identified mostly as microfibers (96% of microparticles) under a dissecting microscope. Relative abundances of these microparticles varied between sites but mean values ranged between 1 and 4 microparticles per oyster per site (Fig. 3). These findings agree with what has been reported for bivalves from Europe (Van Cauwenberghe & Janssen 2014, Li et al. 2018), China (Li et al. 2015, Li et al. 2016, Qu et al.

2018), Brazil (Santana et al. 2016), Canada (Davidson & Dudas 2016), and the United States (Rochman et al. 2015), among many others. Because visual sorting has limitations for accurate polymer identification, we complemented our observations with three different techniques that allow for polymer identification: RMS, ATR-FTIR, and µ-FTIR.

Plastic particles identified using RMS, ATR-FTIR, and µ-FTIR

The majority of microparticles in our study were analyzed using RMS, and this technique was complemented with ATR-FTIR and µ-FTIR for a smaller set of samples. Out of 447 microparticles examined, only eight particles were identified as microplastics by RMS and their spectra matched with polypropylene (PP), polyethylene (PE) or polystyrene (PS) (Fig. 6). These three polymers have been identified in surface waters and sediments in the Atlantic (Moret-Ferguson et al. 2010, Enders et al. 2015, Woodall et al. 2014, Courtene-Jones et al. 2017) and North Pacific Oceans (Rios et al. 2007), and also in bivalves from China and Europe (Li et al. 2016, Li et al. 2018, Li et al. 2018). PE along with PP are the most commonly used hydrocarbon polymers (Da Costa et al. 2018), and there are many different sources and a wide range of domestic and industrial applications for them (Andrady 2011, Zhao et al. 2018). PP for example, is used for rope, bottle caps and nettings (Smith et al. 2018). High and low density PE are used for milk and juice jugs, plastic bags, six pack rings, netting and drinking straws (Smith et al. 2018), and PS is used for plastic utensils and food containers (Smith et al. 2018).

RMS showed that 81 microparticles were either poly(ethylene glycol) monooleate and/or polyamide resin. The similar Raman spectra of these two compounds made differentiation of

414 the exact chemical identification challenging even with samples that have not been exposed
415 to environmental factors. This challenge coupled with high fluorescence interference and
416 weak Raman signal rendered differentiation impossible for the microparticles analyzed.
417 However, poly(ethylene glycol) monooleate is water soluble and it is likely that this
418 compound was washed away during the sample processing. If this was the case, then the
419 compound in the oyster samples is most likely a polyamide resin. Given that polyamide
420 resins are not ubiquitous or widely used, it is surprising they are found in high abundance in
421 the oyster. If these resins are synthetic in origin they should be considered microplastics
422 (Frias & Nash 2019), but the resins could also have a natural origin if they are the result of
423 the oxidation of oyster protein by hydrogen peroxide. To test these hypotheses we ran a
424 simple test to determine if the polyamide resins could be natural in origin by doing the same
425 digestion protocol we used for the oyster samples on an egg. Eggs are also high in protein
426 and are a closed system that should be naturally free of microplastics. The filter resulting
427 from the egg processing was scanned with RMS and the Raman spectra matched the one
428 observed for all the polyamide resin particles found in our samples (Supplementary Image 1).
429 While this test is not conclusive, it provides strong support to the idea that these polyamide
430 resins present in every filter can come from oysters instead of synthetic sources. Further,
431 investigations using ATR-FTIR revealed that 100% of the filters with oyster samples from all
432 sites contained sorbitan derivatives. While ATR-FTIR has a penetration depth of ~2 µm,
433 RMS has a penetration depth of 12 µm at 785 nm. Thus, it is possible the microparticles are
434 poly(ethylene glycol) monooleate or polyamide resins coated with sorbitan derivatives.
435
436 Both sorbitan derivatives and poly(ethylene glycol) monooleate are most often used as

emulsifiers and surfactants (Bobin et al. 1999). Sorbitan derivatives are not always polymeric (polysorbate being a polymeric sorbitan derivative) and are considered synthetic waxes derived from the dehydration of sorbitol. Sorbitan derivatives such as sorbitan monolaureate, monooleate, and monostearate are FDA-approved for oral administration up to 25 mg per kg body weight as food additives (Nielloud and Marti-Mestres 2000). In 2018, the European Food Safety Authority re-evaluated the use of sorbitan derivatives as food additives and found that the acute toxicity of these compounds is low, however, more data is needed to decrease uncertainty in exposure assessment (European Food Safety Authority 2015).

Other ubiquitous polymers present in the samples were cellulose-derived. It is likely that these polymers originated during the extraction protocol. We used filters that are made up of nitrate-cellulose and it is possible that some degradation occurred during the peroxide treatment that led to cellulose particles in the filters. Future studies could benefit from using other types of filters such as glass filters to solve this. Other cellulose-derived polymers that were identified by RMS included cellulose acetate which are used in cigarette filters, hygiene products, and clothing (Woodall et al. 2014, Andrady 2015). This contaminant is usually introduced in marine environments via sewage discharge (Browne et al. 2011).

Lastly, four additional microparticles were identified as plastics/synthetic polymers with µ-FTIR. These microparticles came from three of the same filters also used for RMS and were identified as polyester, rayon, poly(*t*-butyl acrylate), and poly(bisphenol A carbonate). Polyester is a synthetic polymer that is petroleum based and mostly used as fibers used to manufacture fabrics that make up blankets, fleece and other clothing items. Rayon in contrast

460 is considered a semi-synthetic fiber derived from wood pulp. Rayon is mostly used for
461 clothing, and when it is bleached with dioxins to make white clothes it can become toxic.
462 Poly(*t*-butyl acrylate) is a polymer mostly used in paints, coatings, adhesives, fuel and
463 textiles but there is not enough information available about its toxicity to marine animals.
464 Poly(bisphenol A carbonate) or polycarbonate (PC) is a polymer formed by monomers of
465 bisphenol A carbonate (BPA) and is one of the most widely used thermoplastics (Quaranta et
466 al. 2017). PC is used in a wide range of industrial applications, such as automotive and
467 transportation, building and construction, packaging, medical, data storage, and interactive
468 software media (Siddiqui et al. 2018). Traditional methods of PC disposal (such as landfilling
469 and incineration) are related to the leaching of BPA and PBA products (Siddiqui et al. 2018).
470 Previous research has also shown that BPA can be released when polycarbonates are
471 biodegraded by marine microorganisms (Artham & Doble 2012). PC found in one of the
472 oyster filters warrants some concern as degradation of this polymer to BPA may induce
473 differential effects in the gonads of male and female oysters (Luo et al. 2017). BPA has
474 received attention because it is mildly estrogenic (Im & Loffler 2016) and can be an
475 endocrine disruptor toxic to marine organisms (Artham & Doble 2012).
476
477 **Methods to identify plastic polymers and associated caveats**
478 Microplastics research is a field that is growing at an exponential pace but there are still
479 several challenges associated with the chemical identification of microparticles. The
480 identification of polymers is challenging in part due to the large number of mixtures of
481 polymers that are produced by manufacturers today. Therefore, no single analytical approach
482 is perfect to determine the composition of microparticles (Elert et al. 2017), and studies

aiming to chemically identify microparticles can benefit from using different methods to analyze microplastic composition. This is hard to achieve however, as techniques such as RMS, FTIR, and µ-FTIR require training and expertise to use the equipment appropriately (Granek et al. 2020). These methods are also costly and time consuming, particularly in the case of RMS.

Of the three, ATR-FTIR is the least costly and is most suitable to analyze particles that are larger than 100 µm. The caveat of ATR-FTIR is that it can detect materials that are coated on the surface of a sample, but may not identify the underlying material as it only has a penetration depth of ~2 µm. Another disadvantage with ATR-FTIR is that brittle samples can fracture upon contact with the diamond ATR (Wagner et al. 2017). ATR-FTIR and µ-FTIR use mid-IR light while RMS uses sub-micron light (usually a laser source) to probe microparticle identities. Therefore, while IR can only identify particles larger than 10-20 µm, RMS has the ability to resolve samples larger than 1 µm (Silva et al. 2018). Both µ-FTIR and RMS require low amounts of sample, can identify particles with minimal sample preparation, and distinguish between plastics and natural particles from marine organisms or soil (Silva et al. 2018). Compared to Raman, FTIR has been around longer and the technology has evolved with the polymer industry. There is a greater abundance of historical IR spectroscopic data for polymer analysis and thus more available reference spectra for microplastic identification (Araujo et al. 2018).

There are also different caveats that need to be taken into account for each technique, such as the issue of fluorescence interference with RMS (Araujo et al. 2018). In our study, almost 41% of the microparticles analyzed showed high fluorescence and this limited our ability to

detect the RMS signal and identify the composition of the particles. Thus, it is likely we may have underestimated the amount of microplastics present in the samples. The sources of fluorescence in microparticles can be many. Coloring agents, biological material, or degradation products from the environment can attach/adsorb to the plastic particles and influence their spectra. Many colorants such as pigments and dyes strongly fluoresce in visible light and can also hinder the acquisition of spectra, while inorganic pigments are more likely to change the pattern of peaks that are displayed (Frederiks 2012). Bacteria present in biofilm (Araya et al. 2003, Rummel et al. 2017), and algal phaeopigments (Carlson & Shapiro 1981, Mitchell & Kiefer 1988) can also be major contributors to fluorescence. To reduce fluorescence interference in some of our samples, it was necessary to bleach or illuminate them with the laser before spectral acquisition. This treatment, however, occasionally led to microparticles degradation.

Another caveat are additive compounds included to the basic polymer matrix when commercial plastics are manufactured (Lenz et al. 2015). These added compounds can alter the reading of the plastic polymers by RMS. Similarly, plastic microparticles obtained from the marine environment can have altered chemical fingerprints as a result of weathering (Lenz et al. 2015). These alterations can sometimes lead to a falsified polymer type signal (Lenz et al. 2015) and misidentification. This issue has been mentioned as one of the main drawbacks of matching spectra to a library because reference libraries cannot cover the whole variety of particles present in marine samples, both in terms of additives and/or degradation (Lenz et al. 2015). As this challenge begins to be recognized, several authors have recommended that spectra from degraded polymers are also included in polymer libraries in

order to increase the possibilities of identification of microparticles that have been exposed to environmental conditions. Lenz et al. (2015) showed for example, that PVC can be degraded to a point where successful identification is not possible if using a virgin PVC reference. Similarly, when exposed to artificial seawater, PE pellets undergo considerable structural modifications as a result of the formation of new functional groups in the polymer that ultimately yield polymers with a distinct composition (Da Costa et al. 2018).

Conclusions and implications of this research

The findings presented here indicate that visual sorting should not be assumed to be a reliable method for microplastic identification. Over 60% of the oysters analyzed had a microparticles that looked like a microplastics however only 2% of the microparticles observed were confirmed to have a plastic composition. Our research further shows that Pacific oysters from Puget Sound in Washington State, USA, are not accumulating large amounts of microplastics, on the contrary, we have no evidence to support the idea that there is more than one microplastic per oyster. As pointed out previously, 41% of the samples were covered with fluorescent material and the methods have caveats to consider, but based on the 60% of the samples that were identifiable, the presence of microplastics is very low. This does not undermine in any way the seriousness and emergency of plastic contamination in marine environments and its effects on marine biota. This research instead provides the first baseline value for microplastics in Pacific oysters from Washington State based on three different techniques - in addition to visual sorting. Information from this first baseline study will give ecosystem managers, the aquaculture industry, and the general public an important indicator of bivalve health. This pioneer contribution can be considerably improved by analyzing more of the oyster samples with μ-FTIR, by managing fluorescence interference

554 under RMS, and using additional chemical identification techniques. Having a more
555 comprehensive dataset of results as well polymer libraries with spectra of polymers that were
556 exposed to the environmental degradation, will allow scientists to identify with more
557 accuracy the types of microplastics, resins, additives, and other contaminants present in
558 marine organisms.
559
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574

575 **FIGURE CAPTIONS**

576 **Figure 1**. Location of Puget Sound and its basins in Washington State, USA. The five basins
577 indicated are Juan de Fuca (JF) Strait in purple, Hood Canal in green, South Puget Sound
578 (PS) in yellow, Central Puget Sound in red and Whidbey Basin in blue. The image on the
579 right shows the study species, the Pacific oyster, *Crassostrea gigas*.

580

581 **Figure 2**. Panel showing (A) boxplots with the number of microparticles per oyster that were
582 identified using visual sorting under the dissecting microscope. Site names are organized
583 alphabetically. Thick horizontal lines represent median values per site, boxes enclose the
584 25^{th}-75^{th} percentiles and whiskers indicate the minimum and maximum values, (B) boxplots
585 showing mean oyster weight per site, (C) scatterplot of the relationship between mean oyster
586 weight and number of microparticles per oyster for all pooled sites. R-squared value for
587 regression: 0.003.

588

589 **Figure 3**. Pie charts showing (A) the percentage of different colors observed in
590 microparticles identified as microfibers under the dissecting scope (size ranges: 102 to 2885
591 μm) and (B) the percentage of different colors observed in microparticles under RMS (size
592 ranges between 20 to 50 μm). In a clockwise direction, 'clear' indicates transparent particles,
593 'red' indicated red and pink particles, 'purple' indicates violet and purple particles, light
594 indicates white, yellow, silver and amber particles, 'green' indicates greenish particles and
595 'dark' indicates all particles that were blue, black or dark in coloration.

596

597 **Figure 4**. Map showing the location of sampling sites (1-10) in Puget Sound, Washington.
598 Site names are organized N-S as follows: (1) Samish Bay, (2) Sequim Bay, (3) Mystery Bay,
599 (4) Jacoby State Park , (5) Heritage County Park , (6) Illahee State Park, (7) North Bay, (8)

Kopachuck State Park, (9) Penrose Point, (10) Oakland Bay. The barplot to the right shows the proportion of different microparticles identified by RMS per site. It is important to note that we did not process 100% of the particles per filter therefore the graph does not represent all the particles that were present, only the ones that were randomly sampled. It is also of note that the number of particles sampled varied per site as it depended on the density of particles per filter (number of microparticles analyzed per site is shown in Table 2). 'Cellulose' are mainly cellulose fibers, 'fluorescence' are unidentified particles due to high fluorescence interference, 'others' are shell particles, grains of sand, and gypsum, 'resins' are polymers such as sorbitan monopalmitate, and 'plastics' are particles that were identified as polyethylene (PE), polypropylene (PP) and polystyrene (PS).

Figure 5. Size distribution and identification of microparticles from oyster samples using RMS. Most microparticles ranged between 20 to 50 µm in size. The identity of the particle is color coded according to the key in the top right-hand corner of the image. The darker blue color is an overlap between the blue and pink colors. Polystyrene (PS), polypropylene (PP), and polyethylene (PE) microparticles (in red) were all less than 150 µm. One 13000-µm microfiber particle and particles categorized as others (such as gypsum, salt, minerals, or shells) were omitted from the histogram for clarity.

Figure 6. Spectra of microplastic particles identified using RMS. Red spectra are the individual plastic RMS spectra and the blue spectra are the reference library spectra. (a) North Bay Oyster 3a is a 75-µm polystyrene shard, (b) North Bay Oyster 3e is a 100-µm polypropylene irregular-shaped particle, (c) North Bay Oyster 3h is 50-µm polystyrene shard, (d) North Bay Oyster 13d is a 150-µm polypropylene spheroid, (e) Oakland Oyster 4d is a 100-µm polyethylene shard, (f) Samish Control 4-6a is a 100-µm polystyrene irregular-

625 shaped particle, (g) Sequim Oyster 25d is a 150-µm polypropylene shard, (h) Jacoby Oyster
626 31b is a 125-µm polyethylene irregular-shaped particle.
627
628 **Figure 7**. FTIR spectrum of North Bay oyster 1 (in red) and FTIR reference spectrum of
629 sorbitan monopalmitate (in blue). This spectrum is representative of the large majority of the
630 spectra observed with FTIR.
631
632 **Figure 8**. Microplastic particles identified from three filter samples using µ−FTIR. Red
633 spectra are the individual microplastic µ−FTIR spectra and the purple or black spectra are the
634 library reference spectra. (a) Oakland Oyster 4a is a polyester fiber, (b) Oakland Oyster 4b is
635 a rayon fiber, (c) Oakland Oyster 4c is a poly(t-butyl acrylate) irregular-fragment, (d) Jacoby
636 Oyster 31a is a poly(bisphenol A carbonate) particle.
637
638

TABLE CAPTIONS

Table 1. Sampling sites organized N-S according to the Puget Sound basin where they are located (see Fig. 1). Site coordinates and characteristics of the basins are also included. 'SP' indicates State Park and 'CP' indicates County Park.

Table 2. Number of oysters processed under the dissecting scope and RMS per site. For microparticles identified under dissecting scope we present the mean value of the ones that were visually sorted as microplastics, and the mean value per gram of oyster. For microparticles identified under RMS we present the number of particles analyzed per site and the percentage of those that were confirmed as plastic.

Table 3. Number and type of pollutants found per site. The main source of each pollutant is indicated, as well as the method of detection in oyster tissue samples from Washington state.

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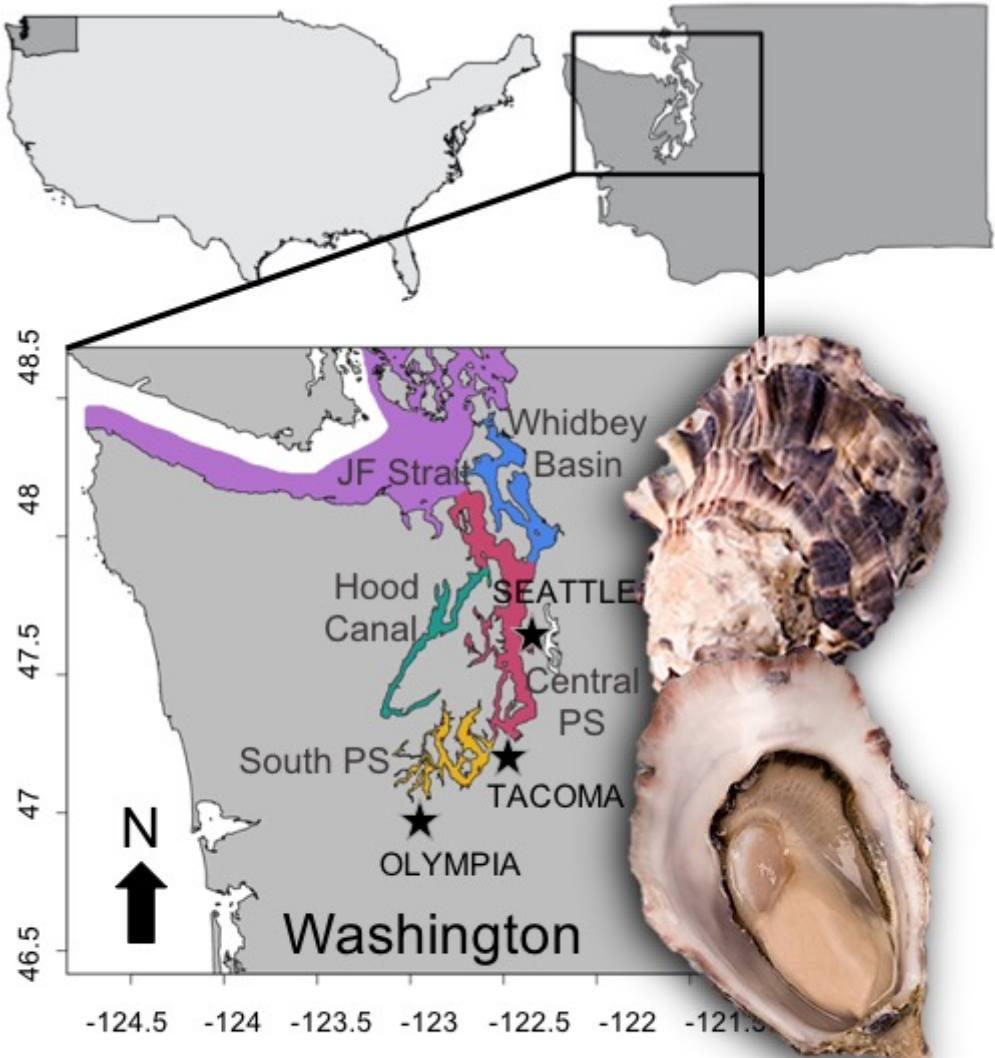
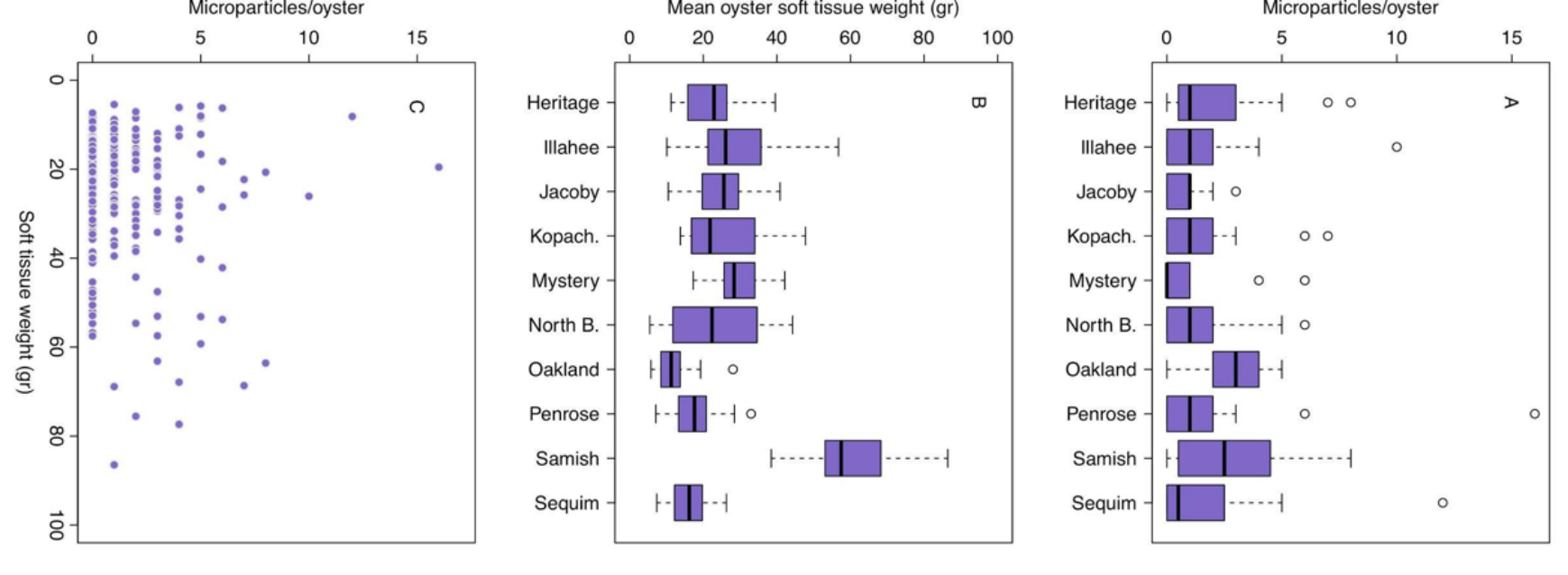
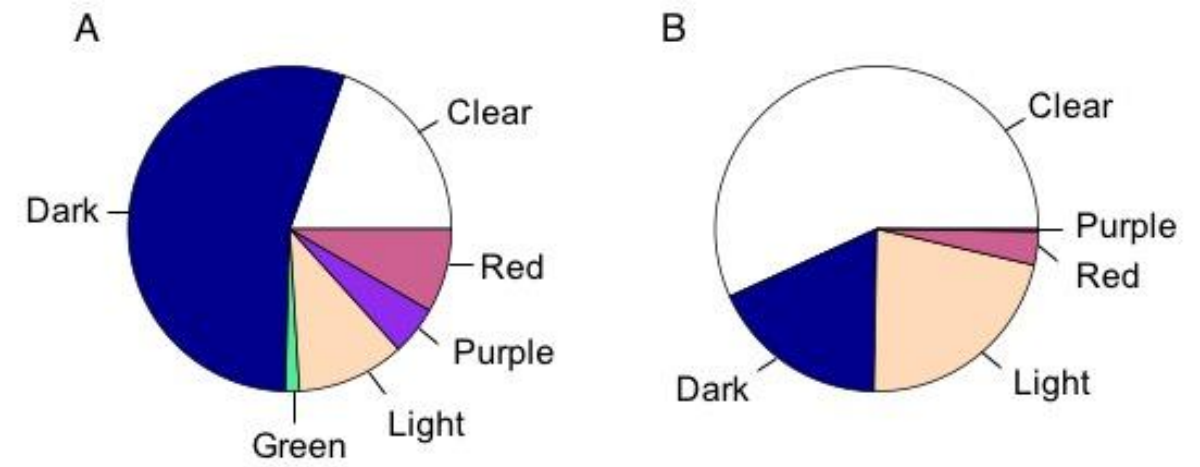
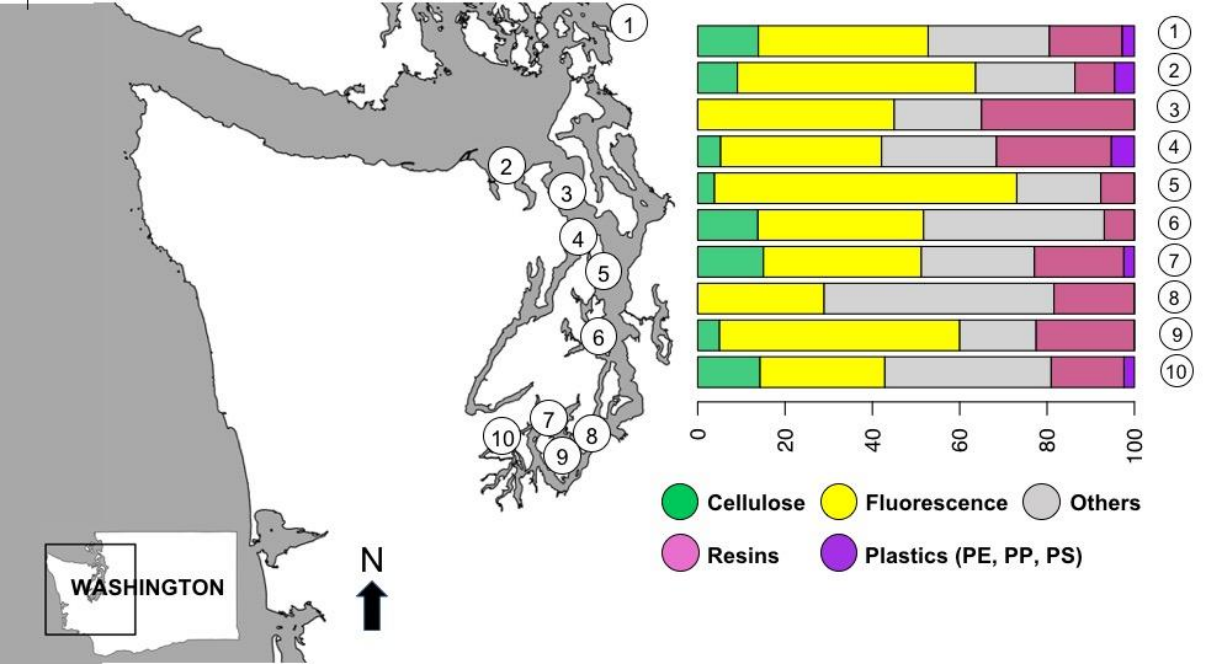
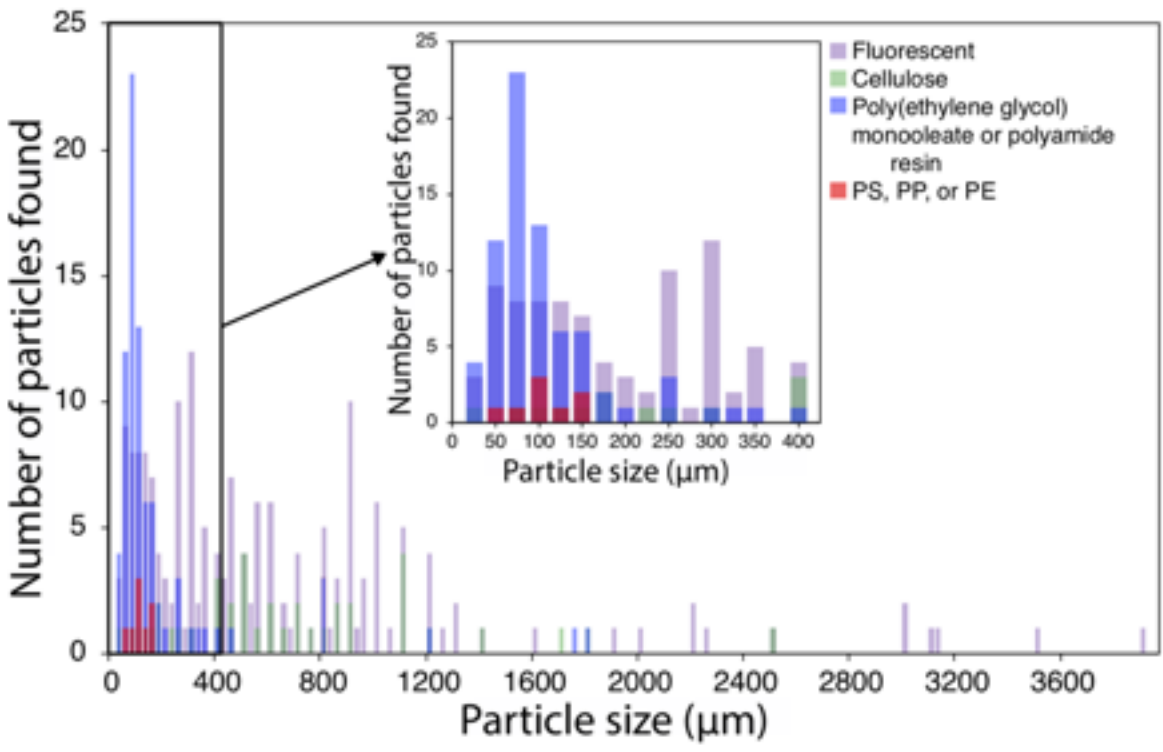
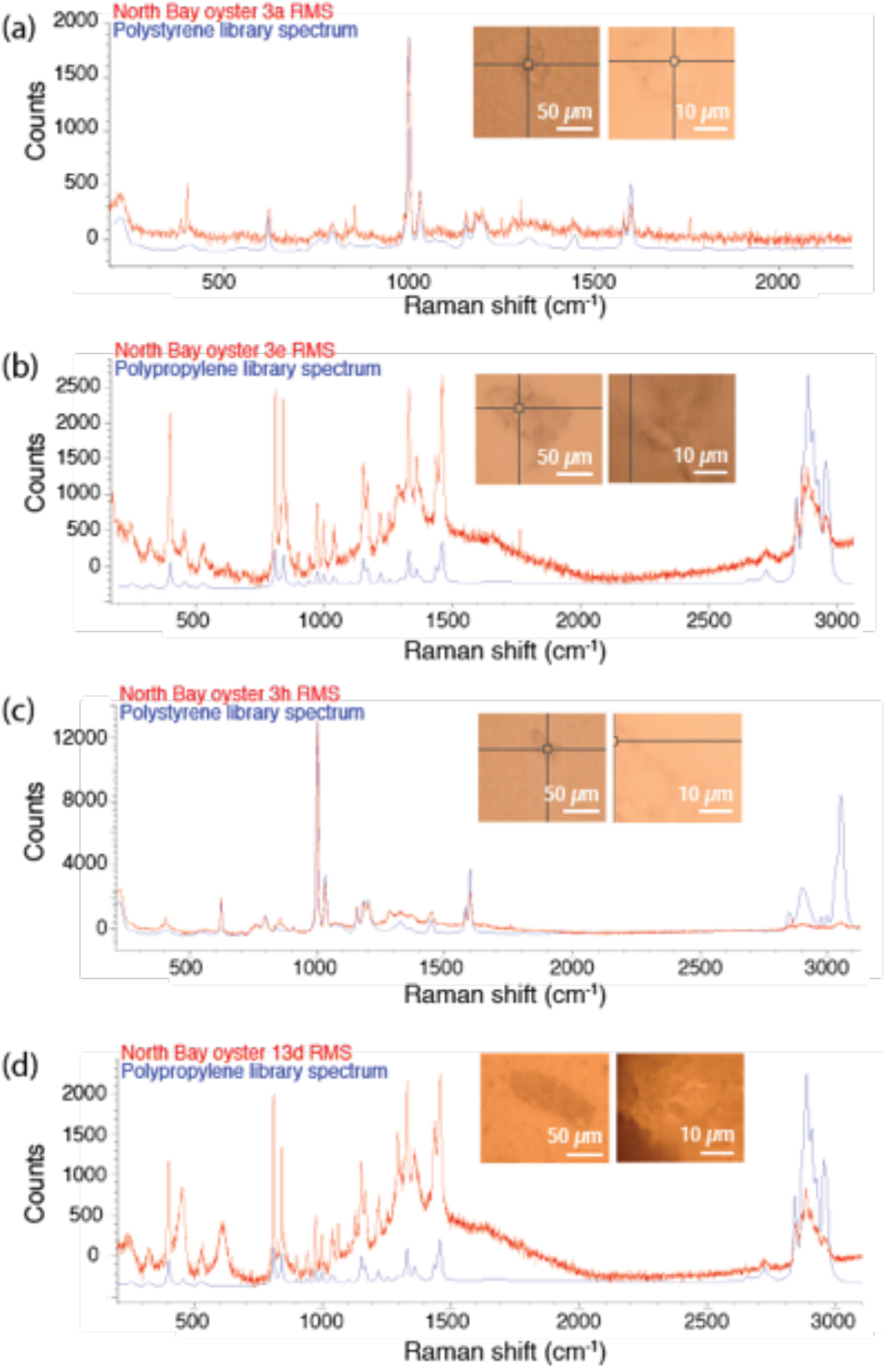

JF Strait
Whidbey Basin
Hood Canal
SEATTLE
Central PS
South PS
TACOMA
OLYMPIA
N
Washington
48.5
48
47.5
47
46.5
-124.5
-124
-123.5
-123
-122.5
-122

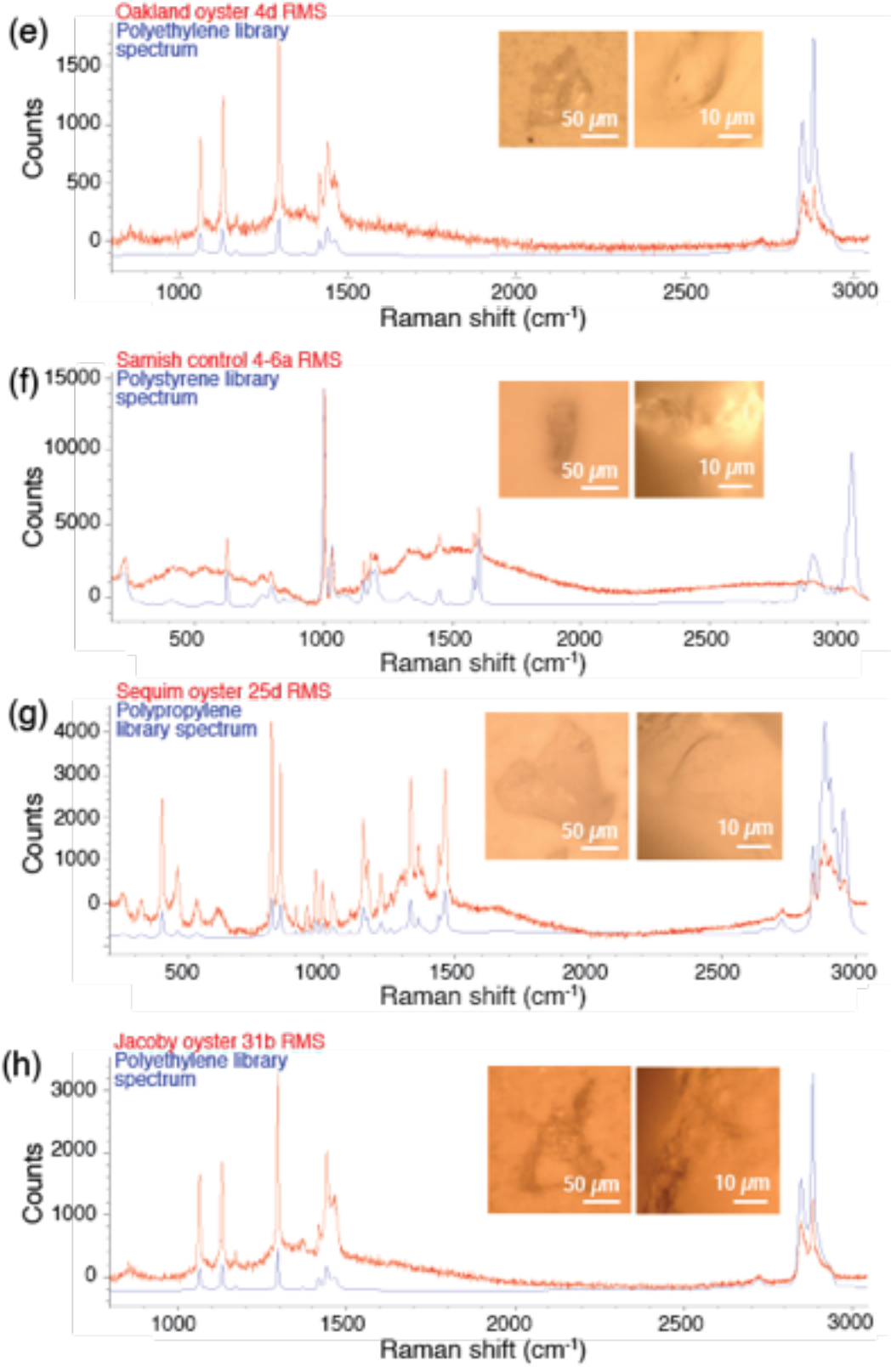

Microparticles/oyster
0
5
10
15
C
Soft tissue weight (gr)
0
20
40
60
80
100
Mean oyster soft tissue weight (gr)
0
20
40
60
80
100
B
Heritage
Illahee
Jacoby
Kopach.
Mystery
North B.
Oakland
Penrose
Samish
Sequim
Microparticles/oyster
0
5
10
15
A
Heritage
Illahee
Jacoby
Kopach.
Mystery
North B.
Oakland
Penrose
Samish
Sequim

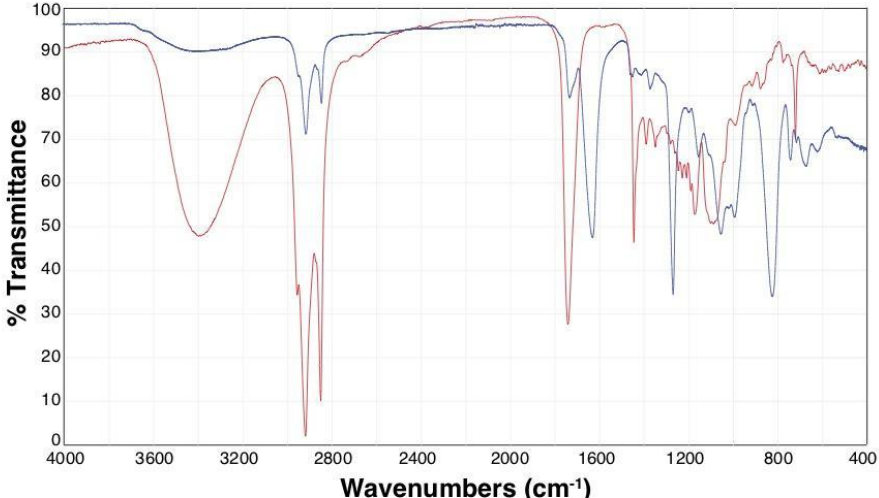

A
Clear
Dark
Red
Purple
Green
Light
B
Clear
Purple
Red
Dark
Light


1
2
3
4
5
6
7
8
9
10
WASHINGTON
N
0
20
40
60
80
100
Cellulose
Fluorescence
Others
Resins
Plastics (PE, PP, PS)


Number of particles found
Particle size (µm)
0
400
800
1200
1600
2000
2400
2800
3200
3600
5
10
15
20
25
Fluorescent
Cellulose
Poly(ethylene glycol) monooleate or polyamide resin
PS, PP, or PE
Number of particles found
Particle size (µm)
0
50
100
150
200
250
300
350
400
5
10
15
20
25


(a)
North Bay oyster 3a RMS
Polystyrene library spectrum
Counts
Raman shift (cm-1)
50 µm
10 µm
(b)
North Bay oyster 3e RMS
Polypropylene library spectrum
Counts
Raman shift (cm-1)
50 µm
10 µm
(c)
North Bay oyster 3h RMS
Polystyrene library spectrum
Counts
Raman shift (cm-1)
50 µm
10 µm
(d)
North Bay oyster 13d RMS
Polypropylene library spectrum
Counts
Raman shift (cm-1)
50 µm
10 µm


(e)
Oakland oyster 4d RMS
Polyethylene library spectrum
Counts
Raman shift (cm-1)
50 μm
10 μm
(f)
Samish control 4-6a RMS
Polystyrene library spectrum
Counts
Raman shift (cm-1)
50 μm
10 μm
(g)
Sequim oyster 25d RMS
Polypropylene library spectrum
Counts
Raman shift (cm-1)
50 μm
10 μm
(h)
Jacoby oyster 31b RMS
Polyethylene library spectrum
Counts
Raman shift (cm-1)
50 μm
10 μm


% Transmittance
100
90
80
70
60
50
40
30
20
10
0
4000
3600
3200
2800
2400
2000
1600
1200
800
400
Wavenumbers (cm-1)

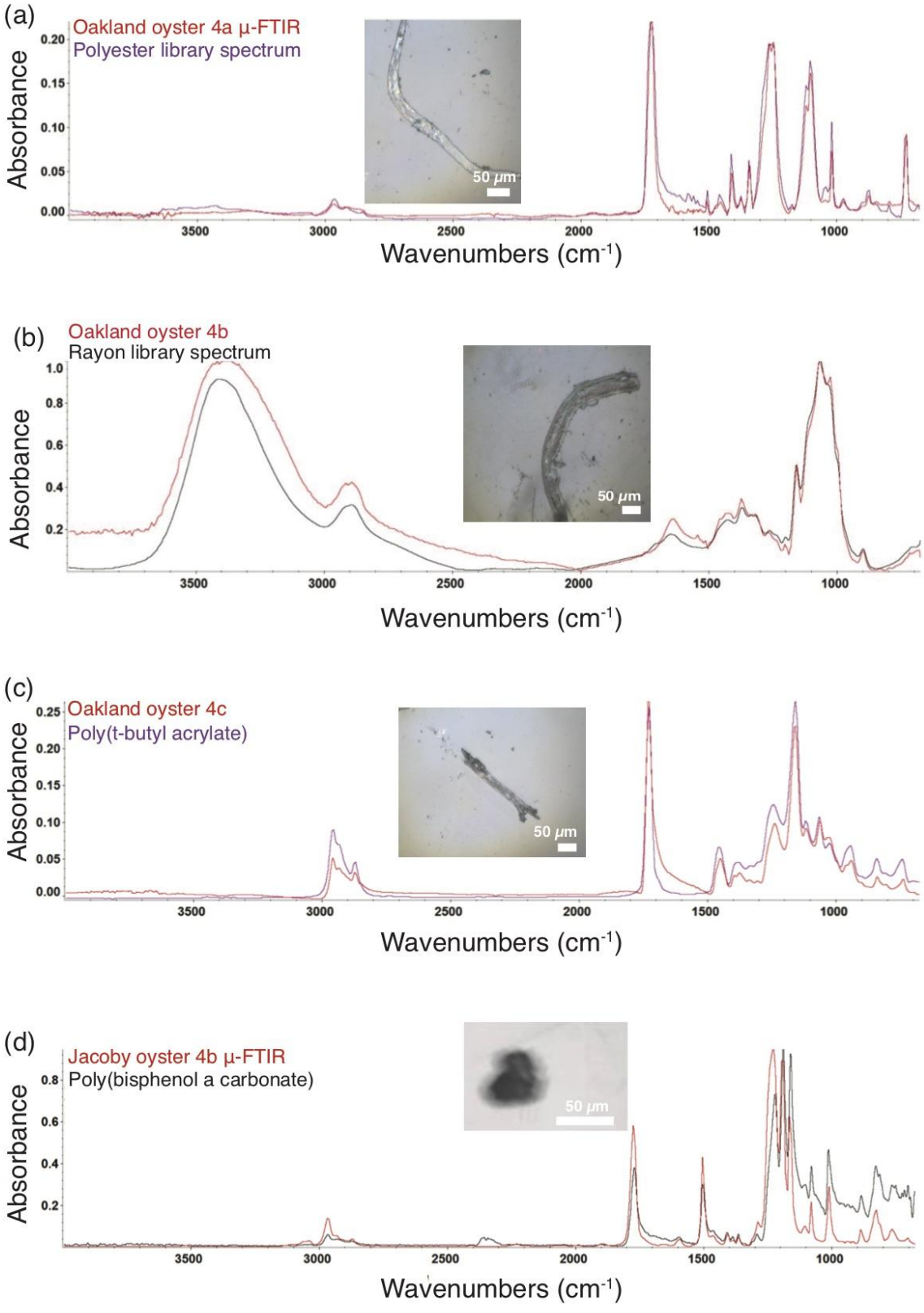

(a)
Oakland oyster 4a µ-FTIR
Polyester library spectrum
Absorbance
0.20
0.15
0.10
0.05
0.00
50 µm
3500
3000
2500
2000
1500
1000
Wavenumbers (cm-1)
(b)
Oakland oyster 4b
Rayon library spectrum
Absorbance
1.0
0.8
0.6
0.4
0.2
50 µm
3500
3000
2500
2000
1500
1000
Wavenumbers (cm-1)
(c)
Oakland oyster 4c
Poly(t-butyl acrylate)
Absorbance
0.25
0.20
0.15
0.10
0.05
0.00
50 µm
3500
3000
2500
2000
1500
1000
Wavenumbers (cm-1)
(d)
Jacoby oyster 4b µ-FTIR
Poly(bisphenol a carbonate)
Absorbance
0.8
0.6
0.4
0.2
50 µm
3500
3000
2500
2000
1500
1000
Wavenumbers (cm-1)

TABLE 1

Basin	Site	Coordinates	Characteristics
Juan de Fuca Strait	Sequim Bay	48° 2' 26" N, 123° 2' 6" W	Open basin connected to the Pacific Ocean shorter water residence times than average for Puget Sound (approximately 1 month). Sequim Bay is however narrow and north-facing, factors that increase water residence times relative to the rest of the basin.
Whidbey Basin	Samish Bay	48° 35' 23" N, 122° 30' 7" W	Close to Juan de Fuca Strait, shorter water residence times than average for Puget Sound (approximately 1 month)
Central Puget Sound	Heritage CP	47° 50' 24" N, 122° 35' 16" W	This basin includes areas draining into small creeks that flow directly into Puget Sound from Seattle and off Vashon Island. Water residence time approximately 2 months.
	Illahee SP	47° 35' 48" N, 122° 35' 57" W	
	Jacoby SP	47° 52' 7" N, 122° 38' 12" W	
	Mystery Bay SP	48° 3' 34" N, 122° 41' 50″ W	
South Puget Sound	Kopachuck SP	47° 18' 31" N, 122° 41' 16" W	Long, narrow bays, islands and small inlets. Water residence time approximately 2 months.
	North Bay	47° 23' 25" N, 122° 48' 51" W	
	Oakland Bay	47° 13' 29" N, 123° 4' 8" W	
	Penrose Point	47° 15' 32" N, 122° 44' 54" W	

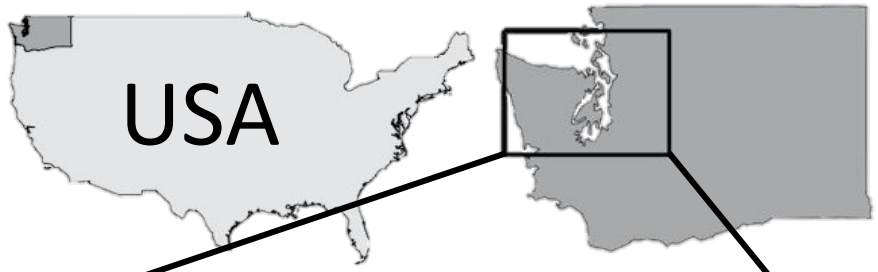
TABLE 2

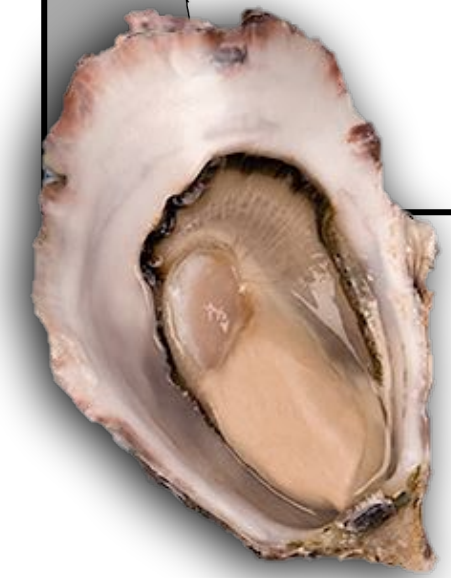
Site	Number of oysters processed	Mean oyster weight	Mean number of particles per oyster (using a dissecting scope)	Mean number of particles per gram (using a dissecting scope)	Particles examined using RMS	Percentage of RMS plastic particles (%)
Heritage CP	20	22.41	2.05	0.09	35	0
Illahee SP	21	28.98	1.71	0.07	28	0
Jacoby SP	23	25.17	0.69	0.03	19	5.3
Kopachuck SP	22	26.66	1.36	0.05	38	0
Mystery Bay SP	21	29.48	0.76	0.02	21	0
North Bay	21	23.48	1.62	0.11	167	2.4
Oakland Bay	21	12.23	3	0.3	42	2.4
Penrose Point SP	24	18.18	2	0.11	40	0
Samish Bay	20	59.85	2.8	0.05	37	2.7
Sequim Bay	20	16.23	1.7	0.14	22	4.5

TABLE 3

Pollutant	Possible Sources	No. particles found	Sites	Method used
Polyethylene (PE)	Milk and juice jugs, plastic bags, six pack rings, drinking straws	2	Jacoby SP, Oakland Bay	RMS
Polystyrene (PS)	Plastic utensils, food containers	3	Samish Bay, North Bay	RMS
Polypropylene (PP)	Rope, bottle caps, nettings	3	Sequim Bay, North Bay	RMS
Polyester	Fabrics, fibers and outerwear	1	Oakland Bay	μ-FTIR
Rayon	Semi-synthetic fiber derived from wood pulp	1	Oakland Bay	μ-FTIR
Poly (t-butyl acrylate)	Paints, coatings, adhesives, textiles	1	Oakland Bay	μ-FTIR
Poly (bisphenol A carbonate)	Automotive and transportation, building and construction, packaging, medical, storage devices, interactive software media	1	Jacoby SP	μ-FTIR
Poly(ethylene glycol) monooleate or polyamide resins	Emulsifiers, surfactants, printing inks	81	All sites	RMS

Sorbitan derivatives	Emulsifiers, surfactants	Non-solid polymers (uncountable)	All sites	ATR-FTIR





300 Pacific oysters

447 microparticles processed