

DEVELOPMENT AND USE OF A TISSUE-DESTRUCTION METHOD TO EXTRACT
MICROPLASTICS IN BLUE CRABS (CALLINECTES SAPIDUS)

A Thesis

by

ELIJAH NATHANIEL WADDELL

BS, Oregon State University, 2014

Submitted in Partial Fulfillment of the Requirements for the Degree of

MASTER of SCIENCE

in

ENVIRONMENTAL SCIENCE

Texas A&M University-Corpus Christi
Corpus Christi, Texas

May 2018

© Elijah Nathaniel Waddell

All Rights Reserved

May 2018

DEVELOPMENT AND USE OF A TISSUE-DESTRUCTION METHOD TO EXTRACT
MICROPLASTICS IN BLUE CRABS (CALLINECTES SAPIDUS)

A Thesis

by

ELIJAH NATHANIEL WADDELL

This thesis meets the standards for scope and quality of
Texas A&M University-Corpus Christi and is hereby approved.

“Jeremy L. Conkle, PhD”
Committee Chair

“Hussain Abdulla, PhD”
Committee Member

“Lee Smee, PhD”
Committee Member

May 2018

ABSTRACT

Plastic materials are nearly ubiquitous throughout the marine environment and are often ingested by marine organisms. Assessing this contamination requires extraction and identification of consumed microplastics, which can be challenging due to their small size and the complexity of the tissues in which they accumulate. Existing methods often favor tissue destruction techniques that use corrosive chemicals to break down organismal tissue to assess microplastic contamination. However, extensive validation of these methods is required because the chemical interactions between the digestants and plastic polymers are not fully understood.

For this study, the chemical compatibility between four digestants (hydrochloric acid (HCl), nitric acid (HNO₃), sulfuric acid (H₂SO₄), and hydrogen peroxide (H₂O₂)) and three polymers (low-density polyethylene (LDPE), polystyrene (PS), and nylon) was assessed. All three acids (HCl, HNO₃, H₂SO₄) readily attacked nylon upon contact. Because of this, a microplastic recovery method involving low-temperature tissue destruction with 30% H₂O₂ as a digestant was developed. This method was then validated on blue crabs by spiking known quantities of green nylon fibers, clear nylon fibers, white polyvinyl chloride (PVC) fragments, blue polyethylene (PE) fragments, and black PS fragments into collected stomach tissue before processing them using the developed method. High recovery rates were achieved for PS, PE, and green nylon fibers (>95%) while clear nylon fibers (84%) and white PVC fragments (63%) yielded sub-optimal recoveries. Once validated, the developed method was then used to assess microplastic contamination in 39 blue crabs collected near Corpus Christi Bay, TX. From these blue crabs, 0.44 synthetic fragments/fibers per crab were recovered, with 25.6% of collected blue crabs observed to contain synthetic fragments and fibers within their stomach.

The developed method provides a less destructive alternative for assessing microplastic contamination in marine organisms when compared to published acid-based methods. Additionally, this study provides initial evidence of microplastic contamination in blue crabs, with approximately 25% of sampled blue crabs found to have microplastics or synthetic fibers within their stomachs.

TABLE OF CONTENTS

CONTENTS	PAGE
ABSTRACT.....	v
TABLE OF CONTENTS.....	vii
LIST OF FIGURES	viii
LIST OF TABLES	ix
INTRODUCTION	10
MATERIALS AND METHODS.....	14
Materials for plastic recovery method	14
Chemical selection and plastic compatibility testing.....	15
Testing of tissue destruction and method development.....	16
Microplastic extraction method validation	18
Assessment of microplastic contamination in blue crabs	20
RESULTS	22
Chemical selection and plastic compatibility testing.....	22
Microplastic recovery method	25
Assessment of microplastics in Corpus Christi blue crabs	27
DISCUSSION AND CONCLUSION	28
LIST OF APPENDICES.....	34
LITERATURE CITED	47

LIST OF FIGURES

FIGURES	PAGE
Figure 1: Plastic fibers and fragments used for method validation	19
Figure 2: Blue crab sampling locations	21
Figure 3: Total recovery of suspected fibers and fragments from fifty-four blue crab stomachs ..	26
Figure 4: Spectra for recovered cellulose fibers	43
Figure 5: Spectra for recovered polyester fibers	44
Figure 6: Spectra for recovered acrylic fibers	45
Figure 7: Spectra for recovered fragments and film	46
Figure 8: Observed bleaching of tissue	29

LIST OF TABLES

TABLES	PAGE
Table 1: Summary of microplastic contamination in invertebrates.....	35
Table 2: Summary of utilized tissue destruction methods efficacy.....	37
Table 3: Initial chemical resistance testing.....	23
Table 4: Long term polymer chemical resistance to H ₂ O ₂	24
Table 5: Important Absorbance bands and assignments for ATR-FTIR spectra.....	38
Table 6: Observed suspected microplastics in method validation blue crabs.....	27

1. INTRODUCTION

Plastic debris has been increasingly documented in terrestrial, freshwater and marine systems worldwide over the past 40 years, where it has altered ecosystems and negatively impacted numerous marine species (Colton et al., 1974; Ivar Do Sul et al., 2013). Global plastic production in the 1980's was close to 100 million tons, and it was estimated that 639,000 plastic articles entered marine systems per day from marine sources alone (Horsman, 1982; Barnes, 2005; Ryan et al., 2009). Since then, global plastic production has more than tripled (PlasticsEurope, 2012; PlasticsEurope, 2016), and approximately 10% of all solid municipal waste (by mass) is now composed of plastic materials (Barnes et al., 2009). Jambeck et al. (2015) estimated that 4.8 to 12.7 million tons of plastic material entered marine systems in 2010 due to terrestrial inputs from communities within 50 km of the coast but may have underestimated the quantity of plastics by ignoring inland inputs. How much of this waste ultimately ends up in marine systems is currently unknown, but it is thought that plastics now form the majority (60-80%) of marine debris around the world (Derraik, 2002).

Plastic debris is commonly grouped into two categories: macroplastics, plastics that are greater than 5 mm in size, and microplastics, which are smaller than 5 mm in size (Thompson, 2004; Arthur et al., 2009). Macroplastics in marine environments often consist of discarded plastic products (cups, containers, packaging, fishing gear, etc.) or fragments of larger material (Cole et al., 2011; Wilcox et al., 2016). With prolonged environmental exposure, plastic debris increasingly fragments due to UV irradiation, oxidation, and physical weathering that produces smaller particles (Shah et al, 2008; Andrady, 2011). Microplastics in marine systems include fibers from synthetic textiles, rope, virgin pellets (nurdles), air-blasting media, and microspheres from

personal care products (Fendall and Sewell, 2009; Cole et al., 2011). Common plastic polymers include low and high-density polyethylene (LDPE and HDPE), polystyrene (PS), polyamide (PA, Nylon), polypropylene (PP), Polyethylene terephthalate (PET) and Polyvinyl chloride (PVC), each with their own unique physical characteristics and chemical properties.

In marine systems, plastics can alter ecosystems and negatively impact marine organisms (Laist, 1987; CBD, 2016). Mechanical interactions with plastic, such as entanglement or ingestion, may cause sub-lethal and lethal effects, including strangulation, drowning, choking, pseudo-satiation, disfigurement, maiming, reduced fitness, and the alteration of behavior (Laist, 1987; Laist, 1997; Gregory, 2009; Wright et al, 2013; Ivar do Sul and Costa, 2014). Plastics also frequently contain additives or sorbed chemicals from the environment that can leach into the organism upon uptake (Browne et al., 2013, Teuten et al., 2009) and further transfer between trophic levels (Browne et al., 2008; Batel et al., 2016). Microplastics are also more easily ingested by a wider range of organisms than macroplastics and can take weeks to pass through an organism's digestive tract (Browne et al, 2008; Watts et al, 2014). At least 812 coastal species have been confirmed to be negatively impacted by plastics (which includes ingestion, entanglement, ghost fishing, dispersal and habitat alteration), with those affected by ingestion and entanglement alone totaling 519 species (CBD, 2016). Furthermore, most organisms affected by plastics are disproportionately formed by fish, birds, and marine mammals, with relatively little *in situ* data available on the effects of plastics on marine invertebrates (Laist, 1997; Claessens et al., 2013; CBD, 2016).

Plastic contamination in invertebrates commonly focuses on bivalves or crustaceans as they are often commercially or environmentally important species with indiscriminate feeding strategies that lead to plastic exposure (Andrady, 2011; Ivar Do Sul and Costa, 2014). For example, blue mussels (*Mytilus edilus*) in the North Sea and coastal China contained plastic loads ranging from

0.2 to 4.6 plastic items/g tissue (Table 1). Microplastic contamination was also observed in nine other bivalve species in coastal China (2.1 to 10.5 plastic items/g tissue; Table 1), and concentrations were positively correlated with proximity to human populations (Li et al., 2015). Contamination was observed in the Norway lobster (*Nephrops norvegicus*), with microplastics found in 83% of 120 sampled individuals (Table 1). Most plastics found were synthetic strands, often tangled into balls that were intertwined with fibrous algae (Murray and Cowie, 2011). A similar study investigating Chinese mitten crabs (*Eriocheir sinensis*) off the coasts of Poland and Portugal found that 13% of the 302 specimens sampled had plastics in their stomachs (Table 1). Both studies investigating microplastic contamination in crustaceans (Norway lobster and Chinese mitten crab) found that the majority of recovered plastics consisted of clear balled fibers. A likely source of ingested fibers is the fishing industry, as some of the fiber bundles recovered from these organisms were observed to be like those originating from fisheries (nets, ropes, fishing line) (Wójcik-Fudalewska et al., 2016) or had matching Micro-Raman spectroscopy spectra (Murray and Cowie, 2011). Additionally, laboratory trials have shown that mussels, polychaetes, sea cucumbers, and green crabs are all capable of taking up plastics from their food and the environment, either through ingestion or respiration (Graham and Thompson, 2009; Browne et al., 2013; Claessens et al., 2013; Watts et al., 2014).

Given the diverse feeding strategies of invertebrates and their presence in coastal zones that are often heavily developed and receive anthropogenic debris from upstream, it is likely that these organisms are regularly exposed to plastics. This is of concern for invertebrate fisheries due to economic and human health concerns. The blue crab, *Callinectes sapidus*, is one such species. Blue crabs support a \$219 million dollar fishery in the U.S, with \$74 and \$5.5 million dollar fisheries in the Gulf of Mexico and Texas, respectively (NMFS, 2015). Blue crabs forage in

sediment for plants, animals, detritus, and carcasses (Laughlin, 1982), exposing them to denser plastics, like PVC, and those fouled by biofilms that sink out of the water column, such as PE or PP (Wright et al, 2013). Blue crabs are unlikely to distinguish between their natural foods and plastics and may even preferentially target them, as seen in other organisms (Graham and Thompson, 2009; Murray and Cowie, 2011; Lönnstedt and Eklöv, 2016). Additionally, fibers and other particles may become entangled in their gills as water passes over them during respiration (Watts et al, 2014). Microplastics have been observed to transfer additives to organisms (Browne, 2013), and the accumulation of plastic particles and fibers may lead to similar risks of exposure to plasticizers and other harmful additives in blue crabs. Microplastic accumulation in blue crabs has not been thoroughly investigated and it is unclear to what extent they have been impacted.

While the uptake of microplastics has been increasingly documented, there is currently no consensus or established methodology for investigating contamination in marine organisms. Typically published methods consist of manual dissection or tissue destruction followed by numerous approaches to sorting the material collected (Murray and Cowie, 2011; Wójcik-Fudalewska et al., 2016, Karami et al., 2017). Recently published methods often favor tissue destruction, as it simplifies plastic recovery by dissolving a large portion of non-plastic material using some combination of strong oxidizers, bases, and acids, such as hydrogen peroxide (H_2O_2), potassium hydroxide (KOH), sodium hydroxide (NaOH) and nitric acid (HNO_3) (Table 2). These methods are often enhanced by increasing reaction temperatures ($>50^\circ\text{C}$) and physical disturbance (stirring or sonicating) to facilitate the breakdown of tissue (Table 2). However, many of the methods used for plastic recovery from tissue were either not properly tested across the variety of polymers found in the environment (Table 2) or not validated before use (De Witte et al., 2014). This is problematic because strong acids like HNO_3 , HCl , or mixtures involving these chemicals

dissolve nylon and partially degrade polymers like PVC, PP, and PS (Claessens et al., 2013; Karami et al., 2017). Studies that utilize these methods could underestimate plastic presence by failing to account for unknown interactions with plastics in the samples (Phuong et al., 2016). To help address these shortcomings and provide preliminary data on an important commercial fishery based around the blue crab we tested the chemical interactions of several digestants and polymers and sought to create a less destructive and more inclusive method for recovering plastics that could be used to assess plastic contamination in marine organisms. This created method was then applied to blue crabs collected near Corpus Christi Bay to further assess its viability and provide initial data on possible microplastic contamination.

2. MATERIALS AND METHODS

2.1 Materials for plastic recovery method

Equipment used for this method included the filtering apparatus, cellulose acetate membrane filters (47 mm diameter, Advantec) with 0.8 μm pore size purchased from VWR, and a Meiji Technology EMZ-8TR stereomicroscope. Chemicals used included HPLC grade Acetone and HPLC grade hexane purchased from Fischer Science, and Hydrogen peroxide (30% and 40% by volume Salon Care clear developer) purchased from Sally's Beauty Supply store. All H_2O_2 was pre-filtered at 0.8 μm , stored in a refrigerator at 4 °C in a clean amber glass bottle when not in use, and replaced after 30 days to maintain potency.

Plastics used in this study consisted of Nylon 6, PS, PP, and HDPE plastic nurdles (3 mm diameter) purchased from ResMart (Fort Worth, Texas), and white PVC powder (white-PVC) (250 μm) purchased from Goodfellow (Coraopolis, PA). Blue HDPE fragments (blue-HDPE), black PS fragments (black-PS), green nylon fibers (green-nylon), and clear nylon fibers (clear-nylon) were created from water bottle caps, coffee cup lids, green paracord, and nylon rope, respectively. Nylon fibers from fishing line and blue-LDPE film from plastic bags were used in preliminary testing. Green and clear nylon fibers were cut to lengths between 1.8 and 0.4 mm, while blue-HDPE and black-PS were ground into fragments and sieved to a 200-300 μm size range. Polymer types tested were selected to both represent commonly used plastics and gauge this method's effectiveness on microplastics with differing structures, coloration, chemical resistance, and densities.

Blue crabs used for method development and validation were purchased from a local seafood supplier and were collected from Corpus Christi Bay, TX. Live crabs were euthanized immediately upon returning to the lab. Crabs not immediately processed were frozen at $-20\text{ }^{\circ}\text{C}$ and were thawed out on the day of the experiment.

2.2 Chemical selection and plastic compatibility testing

Initial testing was performed to determine the chemical resistance of three plastic types (nylon, LDPE, and PS) to concentrated hydrochloric acid (HCl , $\sim 12\text{M}$), sulfuric acid (H_2SO_4 , $\sim 16\text{M}$), nitric acid (HNO_3 , $\sim 12\text{M}$), and hydrogen peroxide (H_2O_2 , $\sim 40\%$). Each polymer was tested against each chemical separately to determine its chemical resistance. Nylon fishing line ($\sim 0.02\text{ g}$), blue LDPE plastic bag fragments ($0.10\text{-}0.11\text{ g}$), and black PS coffee lid fragments ($0.18\text{-}0.24\text{ g}$) were exposed to individual chemicals at $20\text{ }^{\circ}\text{C}$ for 48 hours. Percent recovery, calculated

from mass before and after exposure, was used as an indicator of each plastics chemical resistance when there were no obvious visual differences after treatment. The most compatible digestant was then selected for long-term testing wherein PP, PS, HDPE, Nylon, and PVC nurdles and fragments were exposed for 7 days at 20 °C. Hydrogen peroxide was determined to be the most suitable out of the possible digestants as outlined in results section 3.1.

2.3 Testing of tissue destruction and method development

Likely pathways for plastic uptake in blue crabs are through ingestion, as observed in other crustaceans (Table 1), or respiration, when water passes over the gills (Watts et al., 2015). Therefore, blue crab stomachs and gills were selected for initial testing. Blue crabs were purchased, euthanized, and dissected to remove their gills and cardiac stomach, which were collected in pre-cleaned petri dishes and sealed. Samples were then immediately frozen at -20 °C, and thawed the day of processing. Tissue samples were placed individually in 50 ml glass centrifuge tubes before adding 5 ml of 30% H₂O₂ dropwise to the samples, letting them react at 20 °C for 24 hours before repeating. After this initial digestion, 10 ml of 30% H₂O₂ was added to the test tube before heating the sample to 70 °C for 2 hours, followed by subsequent filtration onto a 0.8 µm cellulose acetate filter. However, heating the H₂O₂ to 70 °C sometimes resulted in excessive bubbling that could lead to loss of material, and plastics recovered from the environment can disappear into solution if heated above 50 °C (Personal communication, Chelsea Rochman). Blue crab gills were also found to be highly resistant to treatment by H₂O₂, visibly bleaching but remaining intact. To address this, samples were to be heated at reduced temperatures (20-40 °C) for longer periods (7

days) and the gills were to be excluded from further testing as the method was inadequate. With these improvements, the following digestion method was developed.

Blue crab cardiac stomachs were first dissected and removed from the organism and placed in a clean 50 mL centrifuge tube and dried (with the caps loosened) in a drying oven at 40 °C for 7 days. Once dried, the stomachs were gently crushed with a glass stirring rod to increase the tissue surface area. To ensure no materials remained on the rod, it was rinsed three times into the centrifuge tube with 2 mL of 30% H₂O₂ (dispensed from a glass pipette, for a total of 6 mL). The glass stirring rod was then visually inspected under a microscope to ensure no materials remained attached. Samples then digested overnight at 20 °C before the addition of another 2 mL of 30% H₂O₂ and gentle swirling for 15 seconds. This was repeated two more times, adding 4 mL more over 48 hours, before the centrifuge tubes digested at 20 °C for a final 48 hours. The centrifuge tubes were then heated at 40°C in a hot water bath for 2 hours before vacuum filtration with a 0.8 µm cellulose acetate membrane filter. The filtering apparatus and now-empty centrifuge tube were inspected under a microscope to ensure the complete transfer of material. Filters were then visually inspected under a Meiji Technology EMZ-8TR stereomicroscope for plastic contaminants.

Contamination was prevented by washing all glassware and utensils with detergent before subsequently rinsing them with DI water and hexane and muffling at 500 °C for 4 hours. Prior to use, all utensils were successively rinsed with hexane and acetone before being visually inspected under a microscope. Laboratory contamination of samples was assessed by running a method blank, a pre-cleaned empty vial that was processed alongside the samples using the created method at a rate of one for every three samples processed. Sample contamination from laboratory sources was assessed by establishing limits of detection (LOD) (Mean plastic contamination from sample

blanks + 3*Standard deviation) for each type of plastic (based on coloration and structure) observed in these method blanks (De Witte et al., 2014).

2.4 Microplastic extraction method validation

Fifty-four blue crabs were purchased for the method validation and randomly divided into two groups consisting of twenty-seven individuals. Stomachs were then dissected from the crabs and placed into separate 50 ml centrifuge tubes. Each test tube was then spiked with between 4 and 20 fragments/fibers of each respective plastic depending on the group. Spiked plastics were added to the centrifuge tube directly as their relative location within the sample was intended to be irrelevant (tissue is dried, broken apart, and destroyed). The variation in spiking amounts was done to prevent the researcher from knowing the exact amount of plastics in each sample, thereby reducing bias that could influence recoveries. The first group was spiked with clear-nylon and white-PVC while the second group was spiked with blue-HDPE, black-PS, and green-nylon (figure 1). Samples were then processed according to the finalized methods described in section 2.3, and recovery efficiency for each plastic was recorded and compared. To determine the tissue destruction efficiency, the change in dry weight ($\frac{Initial\ Dry\ Weight - Final\ Dry\ Weight}{Initial\ Dry\ Weight} \times 100$) for twenty samples were compared. Initial dry weights were recorded from tissue dried at 40 °C for 7 days while final dry weights were obtained by drying processed sample filters at 60 °C for 48 hours.

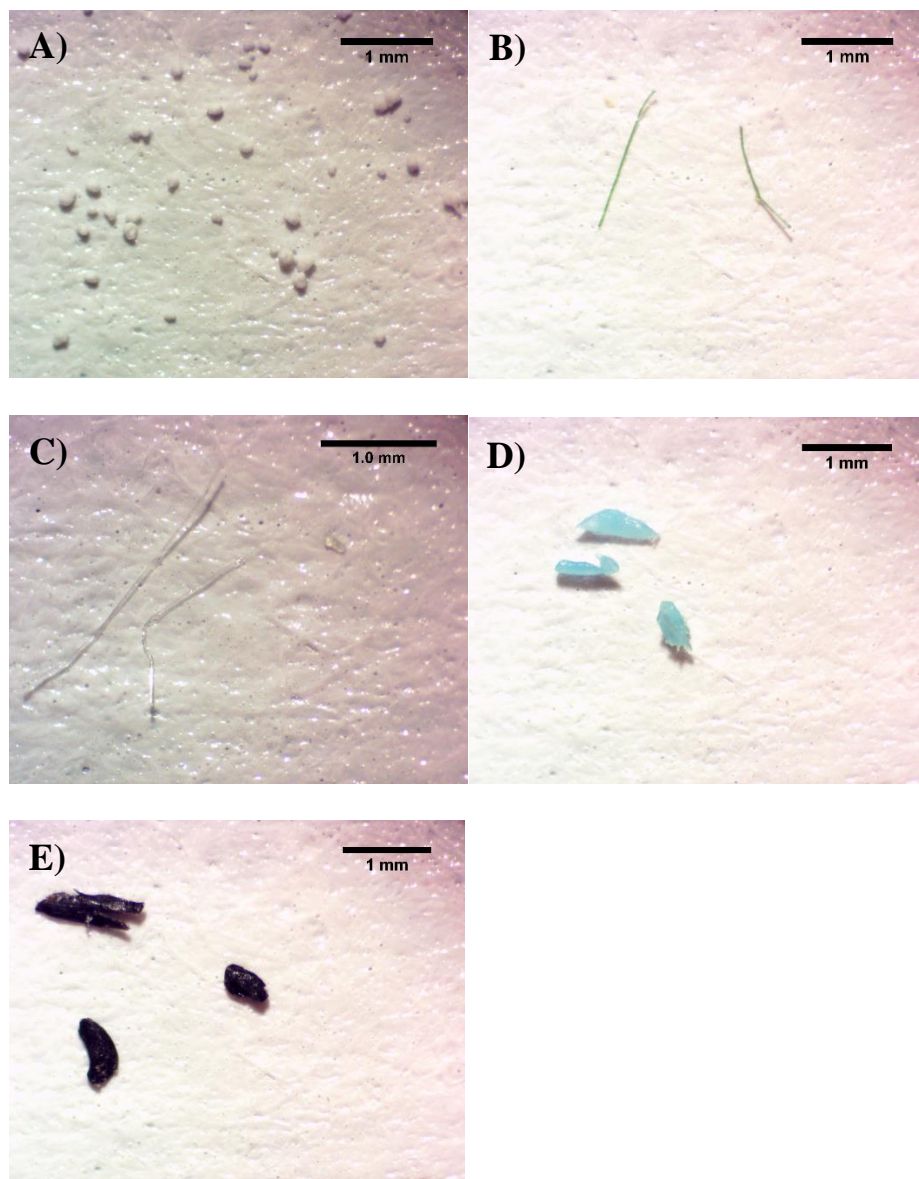


Figure 1. Plastic fibers and fragments used for method validation: A) White PVC powder. B) Green nylon fiber. C) Clear nylon fiber. D) Blue HDPE fragments. E) Black PS fragments.

2.5 Assessment of microplastic contamination in blue crabs

Microplastic contamination in local blue crabs around Corpus Christi Bay was assessed by collecting specimens from three separate sites (Figure 2). Sites were selected for their accessibility, location, and proximity to roads, population centers, and neighboring bodies of water. Blue crabs were collected between July 7th and July 22nd using lines baited with raw chicken. A total of 39 blue crabs were collected: 12 from Site A, 15 from Site B, and 12 from Site C (Figure 2). Upon returning to the lab, the length, mass, and sex of the crabs were recorded, and the crabs were euthanized. Blue crabs were then dissected and their stomachs collected and individually placed into clean 15ml scintillation vials. Stomachs were then processed using the microplastic extraction method outlined in section 2.3.

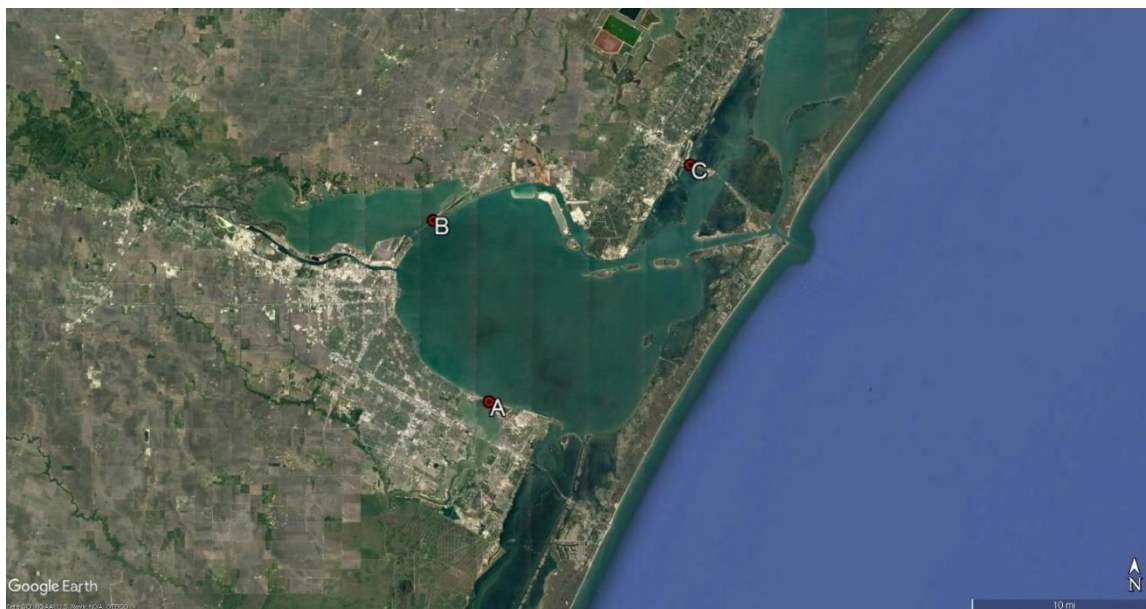


Figure 2. Blue crab sampling locations: Sites around Corpus Christi Bay where blue crabs were collected for assessing microplastic contamination in local blue crabs using the developed tissue destruction method.

Recovered suspected plastics and fibers were then analyzed using FTIR-ATR on a Nicolet iN10 Fourier Transform Infra-Red (FTIR) spectrometer equipped with an iS5 microscope using an ATR-Germanium Crystal and a Mercury Cadmium Telluride IR detector. Sample spectra were collected with 256 scans at a resolution of 8 cm^{-1} over the range of $650 - 4000\text{ cm}^{-1}$. Backgrounds were collected before each sample run and all collected spectra were compared to the “Forensic Comprehensive”, “HR sprouse polymers by ATR”, “Nutraceuticals/herbs”, “ICHEM Nicodom ATR, ATR 100 Spectra Dema Library”, and “Hummel polymer sample library” databases for identification. Samples that positively matched the database ($>65\%$ confidence) were then manually confirmed and considered plausible if they matched with the respective spectra bands outlined in table 5.

3. RESULTS

3.1 Chemical selection and plastic compatibility testing

Nylon was dissolved within the first hour when introduced to any of the strong acids (0% recovery for HNO_3 , HCl , and H_2SO_4), but remained visually unaffected when introduced to H_2O_2 (92.76% recovery) (Table 3). LDPE fragment recoveries ranged from 96.61% to 106.79% and displayed better resistance in all chemicals than nylon (Table 3). However, the LDPE turned brown upon contact with the HNO_3 , indicating a possible chemical interaction. The PS fragments showed no signs of interaction (evolution of gas, bubbling, heating, degradation, or visual discoloration) and had good recovery (100.08-100.77%) in all of the solutions (Table 3). Of the initial chemicals tested, H_2O_2 was the only chemical that did not fully degrade either of the tested plastics in the short-term so a long-term exposure test (7 days) was performed. Triplicates of PVC fragments, nylon, PS, PP, and HDPE nurdles were placed in 400 mL beakers and exposed to 50 ml of pre-filtered 30% H_2O_2 at 20 °C for 7 days. HDPE ($100.00\% \pm 0.01$), PP ($99.47\% \pm 0.42$) and PS ($99.94\% \pm 0.09$) displayed little to no change in mass, while the PVC decreased (98.79 ± 0.13) and the nylon nurdles slightly increased ($102.92\% \pm 1.14$) in mass (Table 4). It is unknown why nylon's mass increased, but it could indicate a chemical interaction with H_2O_2 . With recoveries near 100%, H_2O_2 was used for the remaining method development.

Table 3. Initial chemical resistance testing: Summary of chemical resistance testing for three different polymers exposed to four different chemicals used for tissue digestion over 48 hours.

Polymer	Digestant	Initial mass (g)	Final mass(g)	Percent recovery (%)
PS	HCl	0.2375	0.2379	100.17
PS	H ₂ O ₂	0.2457	0.2459	100.08
PS	HNO ₃	0.182	0.1834	100.77
PS	H ₂ SO ₄	0.1871	0.1882	100.59
LDPE	HCl	0.1095	0.1047	95.62
LDPE	H ₂ O ₂	0.1095	0.1095	100.00
LDPE	HNO ₃	0.1104	0.1179	106.79
LDPE	H ₂ SO ₄	0.1024	0.1019	99.51
Nylon	HCl	0.0236	0	0.00
Nylon	H ₂ O ₂	0.0221	0.0205	92.76
Nylon	HNO ₃	0.0231	0	0.00
Nylon	H ₂ SO ₄	0.0236	0	0.00

Table 4. Long term polymer chemical resistance to H₂O₂: Percent recovery of three different plastics after 7 days of exposure to H₂O₂ at 20 °C.

Polymer	Trial	Initial Plastic Mass (g)	Final plastic mass (g)	Percent recovery (%)
HDPE	1	15.3518	15.3520	100.0013
HDPE	2	14.1229	14.1216	99.9907
HDPE	3	16.8298	16.8289	99.9946
HDPE	Mean			99.9956
PS	1	14.8896	14.8678	99.8536
PS	2	12.9526	12.9510	99.9876
PS	3	13.8288	13.8056	99.8322
PS	Mean			99.8912
Nylon	1	14.7373	15.1762	102.9782
Nylon	2	13.4054	13.9464	104.0357
Nylon	3	16.6506	16.9436	101.7597
Nylon	Mean			102.92
PP	1	5.4375	5.3830	98.9977
PP	2	5.4779	5.4560	99.6002
PP	3	5.1150	5.1051	99.8064
PP	Mean			99.4681
PVC	1	0.9986	0.9853	98.6681
PVC	2	0.9962	0.9840	98.7753
PVC	3	0.9901	0.9795	98.9294
PVC	Mean			98.7909

3.2 Microplastic recovery method

Mean percent recovery (recovery efficiency) for the successful retrieval of all spiked plastics was $89.09 \pm 31.19\%$ while mean tissue destruction efficiency was $65.11 \pm 10.88\%$ ($n=20$). Recovery efficiency varied significantly between spiked plastic type and coloration. White-PVC and clear-nylon fibers had the lowest recovery efficiency at 62.98% and 84.04%, respectively, while green-nylon, blue-HDPE, and black-PS had mean recovery efficiencies of 96.01%, 97.72%, and 99.35%, respectively.

In addition to the plastics spiked in for method validation, non-spiked materials that included suspected synthetic fragments (tested using the hot needle test, coloration, and physical structure), suspected metal fragments (non-ferrous, silver in coloration, and malleable), and fibers (synthetic and non-synthetic) were also found in the samples (Figure 3). Blue fibers, red fibers, and black fibers were found within both the samples and method blanks, which is consistent with contamination observed in other studies (De Witte et al., 2014; Devriese et al., 2015). Limits of detection (described above) were determined to be 3.3, 1.4, and 1.8 fibers/sample for blue, red, and black fibers and were rounded up to the nearest whole number. From the 54 blue crabs sampled, a total of 16 black fibers and 8 red fibers were recovered, but found in quantities below the established LOD's and not included in reported recoveries. A total of 55 blue fibers were recovered, with two individuals containing quantities greater than the determined LOD (5 fibers and 8 fibers respectively). Despite this, no blue fiber recoveries were included in the results as there was no reliable way to tease out laboratory contamination from ingested fibers. After accounting for the LOD, a total of 43 non-spiked fragments and fibers were recovered from the processed blue crabs, including 4 metal fragments, 14 fibers (consisting of fiber types not found

in method blanks), and 25 fragments (Figure 3). Recoveries indicate that there were, on average, 0.80 items per individual, with 42.59% of sampled blue crabs containing objects not introduced during testing (after accounting for contamination) (Table 6). No objective confirmation/identification using micro-FTIR or Raman spectroscopy was possible at the time.

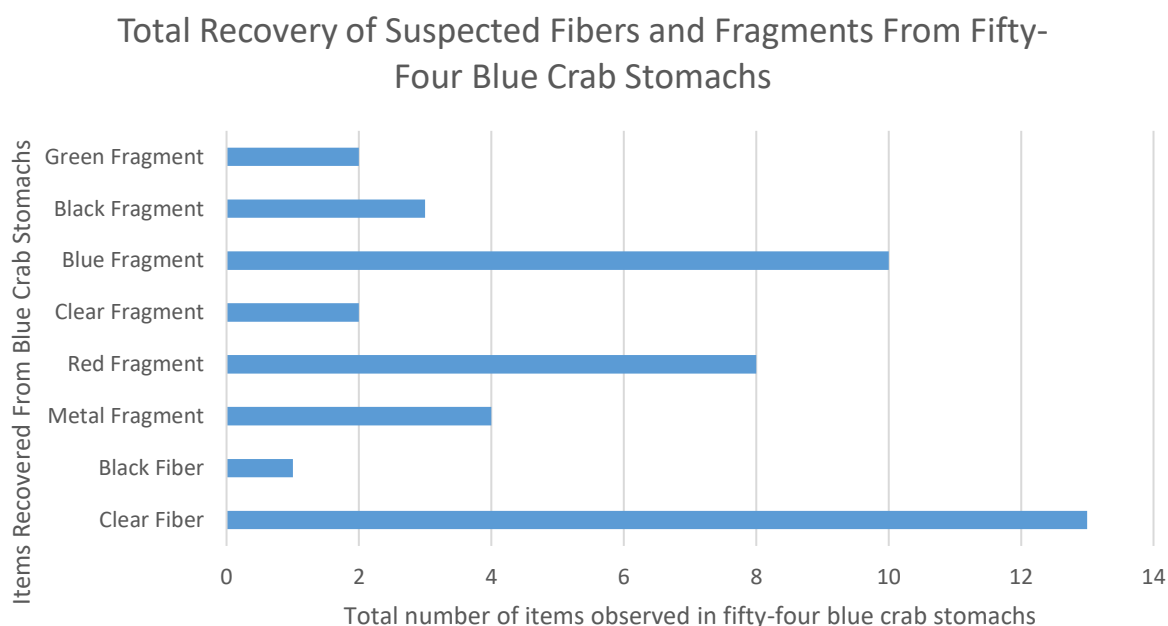


Figure 3. Total recovery of suspected fibers and fragments from fifty-four blue crab stomachs: Total number and types of non-spiked material recovered from the stomachs of 54 blue crabs, after accounting for background contamination. Fibers were not differentiated into synthetic and non-synthetic categories.

Table 6. Observed suspected microplastics in method validation blue crabs: Presence of non-spiked suspected plastics, fibers, and metal fragments observed in the 54 blue crabs processed using the H₂O₂ tissue destruction method.

Type of object recovered	Number of individuals observed	Percent of total sample size observed (%)
Fibers	4	7.41
Fragments	10	18.52
Fibers and Fragments	5	9.26
Metals	1	1.85
Metals and Fibers	1	1.85
Metals and Fragments	1	1.85
Fibers, Fragments, and Metal	1	1.85
None	31	57.41

3.3 Assessment of microplastics in Corpus Christi blue crabs

A total of 157 suspected plastics (126 fibers, 29 fragments, and 2 films) were initially collected from the 39 blue crabs collected from around Corpus Christi Bay. Of this initial recovery, 52 items could be confirmed when analyzed using micro-FTIR. These 52 items consisted of 49 fibers (14 clear/white, 22 blue, 5 red, 7 black, and 1 purple fiber), 1 clear fragment, 1 green fragment, and 1 gray film. Of the recovered fibers, 24 (48.98%) were identified as a cellulose/rayon

blend (Figure 4), 15 (30.61%) as polyester (Figure 5), 9 (18.37%) as acrylic/acrylic blends (Figure 6), and 1 (2.04%) as Polystyrene. The clear fragment, green fragment, and gray film were identified as a PET polymer, polycarbonate, and a phenoxy resin, respectively (Figure 7). Laboratory blanks were then analyzed and 19 clear fibers, 17 clear/white/yellowed fragments, 2 black fragments, 2 blue fibers, and 1 red fiber were recovered. Limits of detection were calculated to be 5.52 for clear fibers, 5.13 for clear/white/yellowed fragments, 0.95 for red fibers, and 1.33 for blue fibers. Clear and blue fibers recovered from method blanks were comprised of either polyester or cellulose while the red fiber was identified as polyester. All of the fragments recovered from the blanks were identified as PS. Following these LOD's, all clear fibers and red polyester fibers were excluded from the results, and only those samples with 2 or more blue fibers were included. After accounting for laboratory contamination 17 synthetic fragments and fibers (not including cellulose/Rayon fibers) were recovered, consisting of 8 polyester fibers, 6 acrylic/acrylic blend fibers, 1 polycarbonate fragment, 1 PET fragment, and 1 phenoxy resin film. Using these final recoveries, 10 crabs (25.6% of sampled blue crabs) were found to have synthetic fragments within their stomach with an average of 0.44 synthetic objects per blue crab.

4. DISCUSSION AND CONCLUSION

For all recovered spiked plastics, no discoloration or obvious signs of chemical interaction or degradation were observed when viewed under a stereo-microscope at 50x magnification. Bleaching of the tissue was observed in samples, with remaining biological matter altered to a pale cream or white color (Figure 8), consistent with past observations (Nuelle et al., 2014). Tissue destruction efficiency of this method (50-80%) was lower when compared to other published tissue

destruction methods (Table 2), and is likely due to the lack of prior depuration of blue crabs (presence of shell hash, grit, and other digestant resistant material), persistence of gastric ossicles, and reduced heating. Some samples had large quantities of grit, shell hash, and other digestion resistant matter that remained after the tissue was dissolved. Blue crab cardiac stomachs contain hard, bonelike-plates that assist in the breakdown of food matter and are largely resistant to digestion by H_2O_2 . Most published methods recommend heating steps that range from 2-96 hours and vary in intensity from 40-80 °C, often with greater intensities for shorter time periods (Table 2). Intensive heating results in greater tissue destruction potential but can destroy or alter plastics within the sample (Vandermeersch et al., 2015; Karami et al., 2017).



Figure 8. Observed bleaching of tissue: Dried blue crab stomach before the digestion method (left), after digestion by H_2O_2 (center), and after filtration (right). Tissue changes from a dark orange to cream/white throughout the process.

Low recovery for clear plastics is likely due to the incomplete destruction and subsequent bleaching of tissue by H_2O_2 (Figure 8). Bleaching changes tissues to colors similar to the clear/white plastics, reducing visual detection and leading to lower recoveries. Recovery of clear

nylon (84.04%) was lower than the recovery of colored nylon (96.01%). White PVC (62.98%) had lower recoveries than all the other polymer types but had no colored analog to compare. Difficulty in identifying clear/white objects was likely responsible for the low recoveries of white PVC, but no observations within this study can rule out the degradation of plastics by H₂O₂. Karami et al. (2017) found reduced recovery of nylon (84-87%) and PVC (81.6%) when introduced to H₂O₂ at 50 °C for 96 hours (Table 2). While our method uses significantly less heating (20-40 °C) to try and mitigate such an interaction, it is still possible that the digestant attacked the PVC or nylon.

Colored plastics (green-nylon, blue-HDPE, and black-PS) had high recovery (96.01%, 97.72%, and 99.35%, respectively) using this method. Visual identification was easily performed as all colored polymers contrasted starkly against the bleached sample background. Plastics were observed on the walls of the filtering apparatus and were all carefully rinsed into the filter. However, the apparatus needed to be dismantled before examination under a microscope and minor losses of spiked plastics could have occurred during this processor when the filtered sample was moved from the apparatus to the microscope.

Blue crabs initially processed during method validation were observed to have several suspected synthetic fragments, fibers, and metal pieces within their stomachs in addition to those deliberately spiked in. Out of the 54 blue crabs processed in method validation, 23 (42.59%) had non-spiked items within them. The most common type of item observed in the blue crabs used for method validation were what we believe to be synthetic fragments (31.48% of individuals), followed by fibers (20.37% of individuals), and metal fragments (7.41% of individuals). Suspected synthetic fragments were observed in a variety of colors, including blue, red, black, green, and clear (Figure 3). None of these fragments were observed in any of the method blanks, so it is unlikely that they were introduced into the sample during processing. Blue, red, and black fibers

were observed in both the samples and the method blanks, while clear fibers were only observed in samples. After accounting for contamination, total number of items per individual ranged from 0 to 4 and may indicate that the number of polymers spiked into the samples (4-20) were unrealistically high.

When this method was field tested on blue crabs collected from Corpus Christi Bay, the final recovery consisted of 17 synthetic plastic fragments/films, and fibers. The most common type of synthetic item recovered were fibers (82.4%), which were composed of either polyester or acrylic material. These fibers were observed in a variety of colors, including blue, red, black, and purple, which is consistent with those found during the method validation. Fourteen clear fibers, identified with FTIR as polyester and cellulose/rayon, were observed in blue crab samples but ultimately excluded from the results due to their presence on method blanks. Fragments and films comprised a far smaller proportion of the recovered synthetic material (17.6%). Of the initial 29 suspected fragments collected, only 3 were identified as plastics or resin. The remaining suspected fragments were either identified as chalcedony/quartz (9 black and 8 red fragments), chitin and undestroyed organic material (1 black and 1 yellow fragment), or could not be determined (5 blue and 2 clear fragments). Collected synthetic fragments/film included 1 green fragment, 1 clear fragment, and 1 gray film, which were identified as polycarbonate, PETE, and phenoxy resin, respectively.

Despite FTIR instrumentation being unavailable during method validation, it is worth comparing the initial recovery of suspected plastics from the method validation and field samples. Suspected fibers and fragments were recovered from both sets of samples, and while the relative abundance of each type of suspected plastic differed (quantity of fibers vs fragments), similar physical characteristics were observed between each. Most suspected fragments and fibers

recovered from both sets of samples were either clear, blue, black, or red, with blue and clear being the most common fiber coloration. However, out of the 157 suspected fragments, films, and fibers initially recovered from field samples, only 52 (33.12%) were correctly identified. Fibers were successfully identified as synthetic only 38.88% of the time while fragments and films were only identified 12.90% of the time. Given the low success rate for identifying plastics without instrumentation, it is possible that the initial suspected recoveries for method validation samples were overestimated.

Based on results from this and other studies, H_2O_2 appears to be an acceptable digestant for plastic recovery methods (Table 2). Minimal interaction between the digestant and PS, nylon, and PE polymers was observed when they were exposed at 20 °C for 7 days ($99.94\% \pm 0.09$, $102.92\% \pm 1.14$, $100.00\% \pm 0.01$ recovery), and no obvious deterioration was observed in any of the samples processed in this study. Hydrogen peroxide has been observed to degrade nylon and PVC when heated to 50 °C for extended periods of time and may alter Raman spectral response when trying to identify the polymer (Karami et al., 2017). However, successful identification of PS, nylon, and PE using FTIR has been performed after treatment with 30% H_2O_2 at 65 °C for 24 hours followed by 24-48 hour digestion period at room temperature (Li et al., 2014). Temperatures were purposefully kept low to prevent the degradation of polymers, but the developed method may have been too conservative given the low tissue destruction efficiency (50-80%). Undigested material, once bleached, can make the visual identification of clear/white objects more difficult. Pairing this method with a density separation step using NaI solution (Claessens et al., 2013; Avio et al., 2015), or a method to better visualize the plastic with a dye, such as Nile red (Shim et al., 2016), would likely improve its recovery efficiency for clear plastics. Further testing of this method is required as only four polymer types, PS, HDPE, nylon, and PVC were tested, and no instrument data was

examined in tandem with the method. As FTIR and Raman spectroscopy are two of the most frequently used identification techniques for plastics, making sure this method is compatible will be important in the future (Harrison et al., 2012, Li et al., 2014).

As shown by the preliminary tests (Table 3) in this study and in observations from Karami et al. (2017), strong acids can rapidly destroy nylon and may attack other polymer types. Nylon is commonly used in fishing gear, nets, and ropes, and has been found in coastal ecosystems and organisms (Gregory, 2009; Murray and Cowie, 2011). Without proper testing and validation, the use of strong acid destruction methods may result in underestimates of plastic pollution and contamination. While strong acids are efficient at destroying tissue and convenient to use, they may be too harsh for recovering plastics.

This developed method described herein successfully uses a low-temperature, tissue destruction approach with 30% H_2O_2 as a digestant for plastic recovery from blue crabs. High recovery rates were achieved for PS, PE, and nylon (>95%) while clear/white polymers (63-84%), and more specifically PVC (63%), yielded sub-optimal recoveries. This study has also provided evidence of microplastic contamination in blue crabs from Corpus Christi Bay, TX, with an average of 0.44 synthetic fragments/fibers per sampled blue crab, with 25.6% of collected blue crabs observed to contain synthetic fragments and fibers within their stomach.

LIST OF APPENDICES

APPENDICES	PAGE
Appendix 1: Tables and Figures	35

APPENDIX 1: TABLES AND FIGURES

Table 1. Summary of microplastic contamination in invertebrates: experimental evidence of microplastic contamination, detailing the organisms, location, quantity of plastics, percent of individuals contaminated, and the method used to extract the plastics.

Organism	Location	Microplastic Contamination	Individuals with Plastics (%)	Method Used	Reference
Crustacea					
<i>Crangon crangon</i>	Southern North Sea (UK, France, Belgium, and The Netherlands)	0.68 ± 0.55^a	63	HNO ₃ :HClO ₄	Devriese et al. 2015
<i>Nephrops norvegicus</i>	Clyde Sea	n.r	83	manual dissection	Murray and Cowie, 2011
<i>Eriocheir sinensis</i>	Baltic Sea (Poland), Coast of Portugal	n.r	13	manual dissection	Wójcik-Fudalewska et al., 2016
<i>Lepas</i> spp.	Northeast Pacific Ocean	1-30 ^b	33.5	manual dissection	Goldstein and Goodwin, 2013
<i>Euphausia pacifica</i>	Northeast Pacific Ocean, Canada	0.059 ^b	n.r	HNO ₃	Desforges et al., 2015
<i>Neocalanus cristatus</i>	Northeast Pacific Ocean, Canada	0.029 ^b	n.r	HNO ₃	Desforges et al., 2015
Polychaeta					
<i>Arenicola marina</i>	North Sea Coast (France, Belgium, Netherlands)	1.2 ± 2.8^a	100	HNO ₃	Van Cauwenberghe et al., 2015
Bivalvia					
<i>mytilus edulis</i>	China	0.9-4.6 ^a	n.r	30% H ₂ O ₂	Li et al., 2016

<i>Scaphara subcrenata</i>	China	2.1-10.5 ^a	n.r	30% H ₂ O ₂	Li et al., 2015
<i>Tegillarca granosa</i>	China	2.1-10.5 ^a	n.r	30% H ₂ O ₂	Li et al., 2015
<i>Mytilus galloprovincialis</i>	China	2.1-10.5 ^a	n.r	30% H ₂ O ₂	Li et al., 2015
<i>Patinopecten yessoensis</i>	China	2.1-10.5 ^a	n.r	30% H ₂ O ₂	Li et al., 2015
<i>Alectryonella plicatula</i>	China	2.1-10.5 ^a	n.r	30% H ₂ O ₂	Li et al., 2015
<i>Sinonovacula constricta</i>	China	2.1-10.5 ^a	n.r	30% H ₂ O ₂	Li et al., 2015
<i>Ruditapes philippinarum</i>	China	2.1-10.5 ^a	n.r	30% H ₂ O ₂	Li et al., 2015
<i>Meretrix lusoria</i>	China	2.1-10.5 ^a	n.r	30% H ₂ O ₂	Li et al., 2015
<i>Cyclina sinensis</i>	China	2.1-10.5 ^a	n.r	30% H ₂ O ₂	Li et al., 2015
<i>Perna perna</i>	Brazil	n.r	75	HNO ₃	Santana et al., 2015
<i>Mytilus edulis</i>	Germany (Mussel Farm)	0.36 ± 0.07 ^a	n.r	HNO ₃	Van Cauwenberghe and Janson, 2014
<i>Crassostrea gigas</i>	Atlantic Ocean (France)	0.47 ± 0.16 ^a	n.r	HNO ₃	Van Cauwenberghe and Janson, 2014
<i>Mytilus edulis</i>	North Sea Coast (France, Belgium, Netherlands)	0.2 ± 0.3 ^a	100	HNO ₃	Van Cauwenberghe et al., 2015

. n.r = Not reported; ^a = # of plastic items/individual; ^b = # of plastic items/gram of tissue

7 Table 2. Summary of utilized tissue destruction methods efficacy: method validation results of various tissue destruction methods for
8 recovering plastics, Identifying the digestant used, heating period and temperature, percent recovery for various types of plastic, and
9 the tissue destruction efficiency of the method.

Digestant	Temperature (°C)	Duration	PVC (%)	PS (%)	PP (%)	PE (%)	PET (%)	Nylon (%)	Destruction efficiency (%)	Reference
density separation (1.2g/ml NaCl solution)	NA	NA	n.t	73	n.t	73	n.t	n.t	n.t	Avio et al., 2015
30% H₂O₂ digestion	55	7 days	n.t	70	n.t	70	n.t	n.t	n.t	Avio et al., 2015
22.5 M HNO₃	100	10 minutes	n.t	4	n.t	4	n.t	n.t	n.t	Avio et al., 2015
Density Separation (1.2g/ml NaCl solution), 15% H₂O₂	NA	NA	n.t	95	n.t	95	n.t	n.t	n.t	Avio et al., 2015
KOH (10%)	40	4 days	93.3	104.2	104.2	103 - 104.4	96.7	99.5 - 104.1	98.6	Karami et al., 2017
HNO₃ (69%)	25	4 days	68.9	87.9	86.3	91.9 - 93.9	93.3	0	99.9	Karami et al., 2017
HCl (37%)	25	4 days	78.5	100.7	102.6	106.3 - 108.4	89.6	3.9 - 5	100	Karami et al., 2017
H₂O₂ (35%)	50	4 days	81.6	105.4	102.5	97.3 - 99.5	99.5	84 - 87.1	98.3	Karami et al., 2017
HNO₃ (69%)	25, 100	1 day, 2 hours	n.t	93.6 - 97.9	n.t	n.t	n.t	0 - 98.3	99.85	Claessens et al., 2015

10 NA = not applicable; n.t = not tested

11 Table 5. Important Absorbance bands and assignments for ATR-FTIR spectra: polycarbonate,
 12 acrylic, poly(ethylene terephthalate), polystyrene, polyester, and natural and man-made cellulose
 13 fiber ATR-FTIR spectra.

14

Material	Absorption Bands (cm ⁻¹)	Assignment	References
Polycarbonate	2966	CH stretch	Jung et al., 2018
	1768	CO stretch	
	1503	Aromatic ring stretch	
	1409	Aromatic ring stretch	
	1364	CH ₃ bend	
	1185	CO stretch	
	1158	CO stretch	
	1013	Aromatic CH in-plane bend	
	828	Aromatic CH out-of-plane bend	
acrylic	2240	CN stretch	Causin et al., 2005
	1720	CO stretch	
	1370	CH stretch	
Poly(ethylene terephthalate)	713	CO stretch	Jung et al., 2018

	1241	CO stretch	
	1094	CO stretch	
	720	Aromatic CH out-of-plane bend	
Polystyrene	3024	Aromatic CH stretch	Jung et al., 2018
	2847	CH stretch	
	1601	Aromatic ring stretch	
	1492	Aromatic ring stretch	
	1451	CH ₂ bend	
	1027	Aromatic CH bend	
	694	Aromatic CH out-of-plane bend	
	537	Aromatic ring out-of-plane bend	
Polyester	2969, 2907	CH stretch	Peets et al., 2017
	1711	CO stretch	
	1504	Aromatic ring CC stretch	
	1472, 1405, 1340	CH bend	

	1241	Ester's CO Stretch	
	1093	CO stretch	
	871	Aromatic ring CH bend	
	718	CH bend, CH rock in CCH ₂ fragment	
Manmade cellulose fibers	3487	OH stretching, intra and inter H bonds	Comnea-Stancu et al., 2017
	3445	OH stretching, intra and inter H bonds	
	2892	CH stretch	
	1640	HOH bend of absorbed water	
	1420	HCH, OCH bend	
	1364	symmetric CH ₃ deformation	
	1335	OH bend	
	1313	CH wagging	
	1263	CH bend	

	1230	COH bend	
	1199	OH bend	
	1156	COC antisymmetric stretch	
	1057	COC symmetric stretch	
	1023	CO stretch	
Natural cellulose fibers	3330	OH stretching, intra and inter H bonds	Comnea-Stancu et al., 2017
	3289	OH stretching, intra and inter H bonds	
	2897	CH stretch	
	1735	CO stretch	
	1640	HOH bend of absorbed water	
	1458	OH bend	
	1425	HCH, OCH bend	
	1366	symmetric CH ₃ deformation	
	1335	OH bend	
	1317	CH wagging	

	1279	CH bend	
	1247	CC, CO, CO stretch	
	1203	OH bend	
	1160	COC antisymmetric stretch	
	1105	COC antisymmetric stretch	
	1030	CO stretch	

15

16

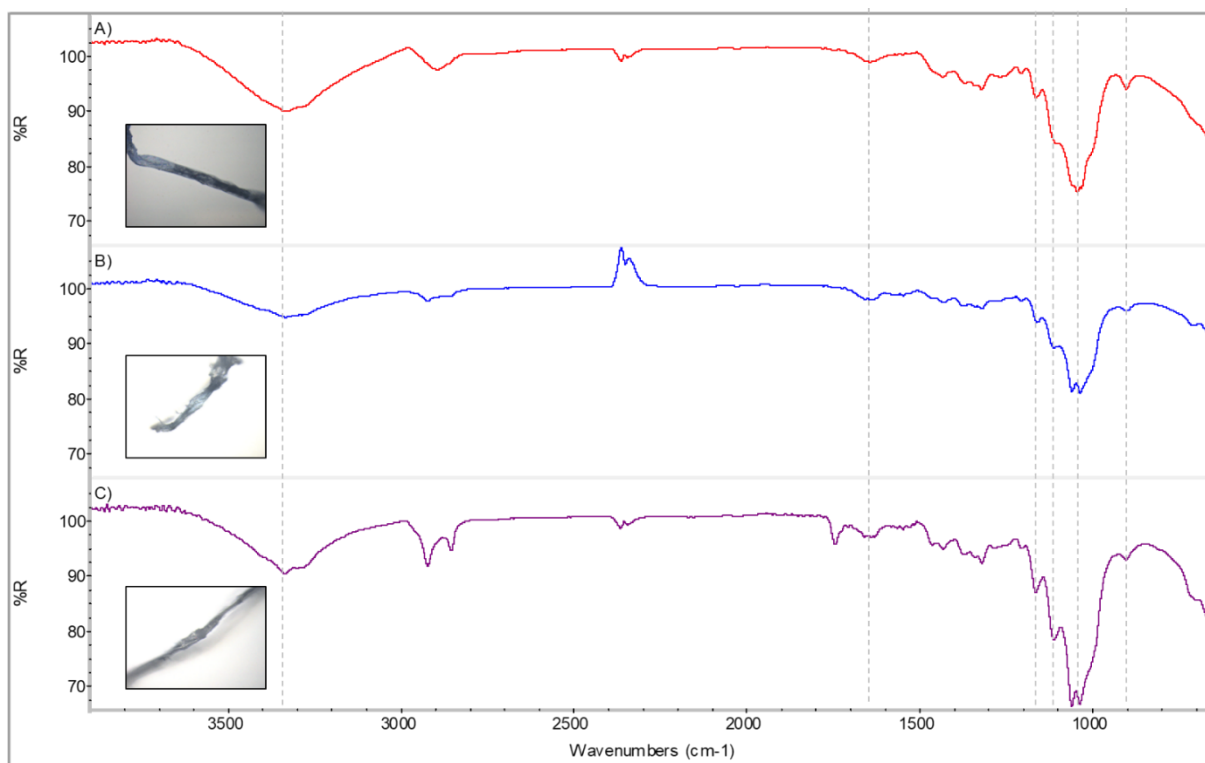


Figure 4. Spectra for recovered cellulose fibers: FTIR-ATR Spectra for three representative cellulose fibers recovered from blue crabs sampled from Corpus Christi Bay.

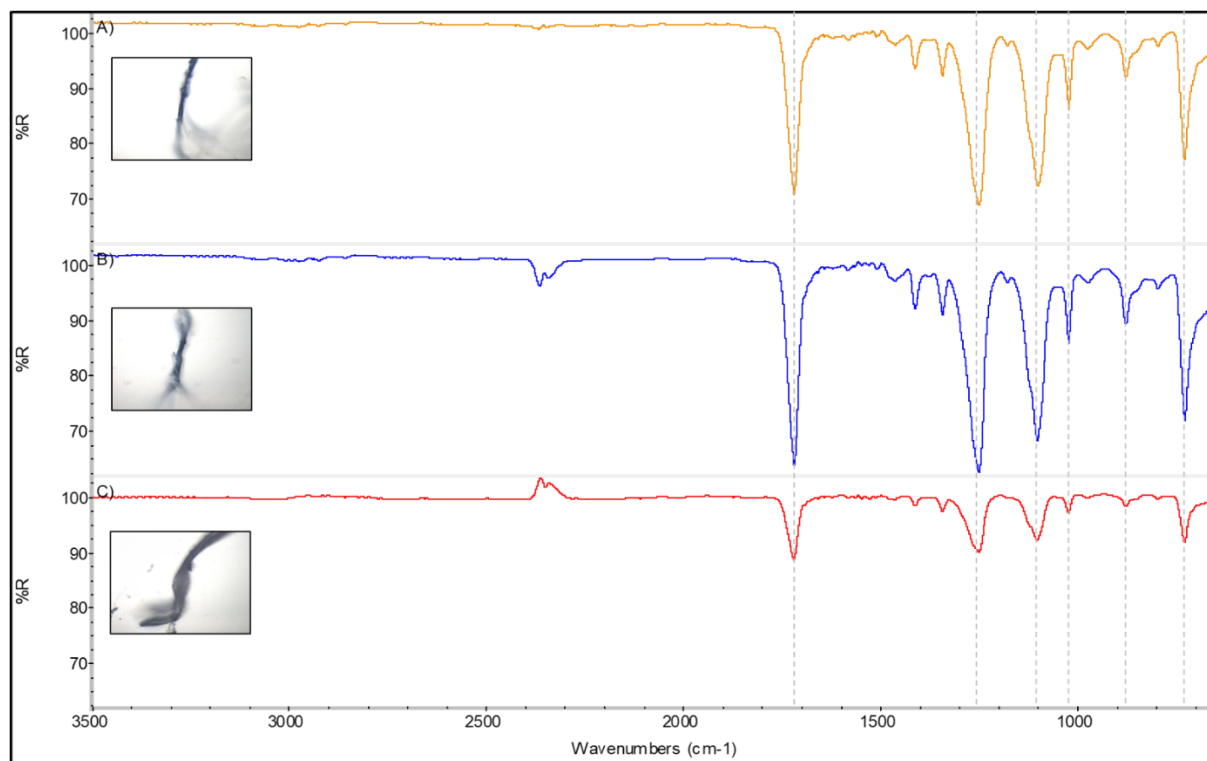


Figure 5. Spectra for recovered polyester fibers: FTIR-ATR Spectra for three polyester fibers recovered from blue crabs sampled from Corpus Christi Bay.

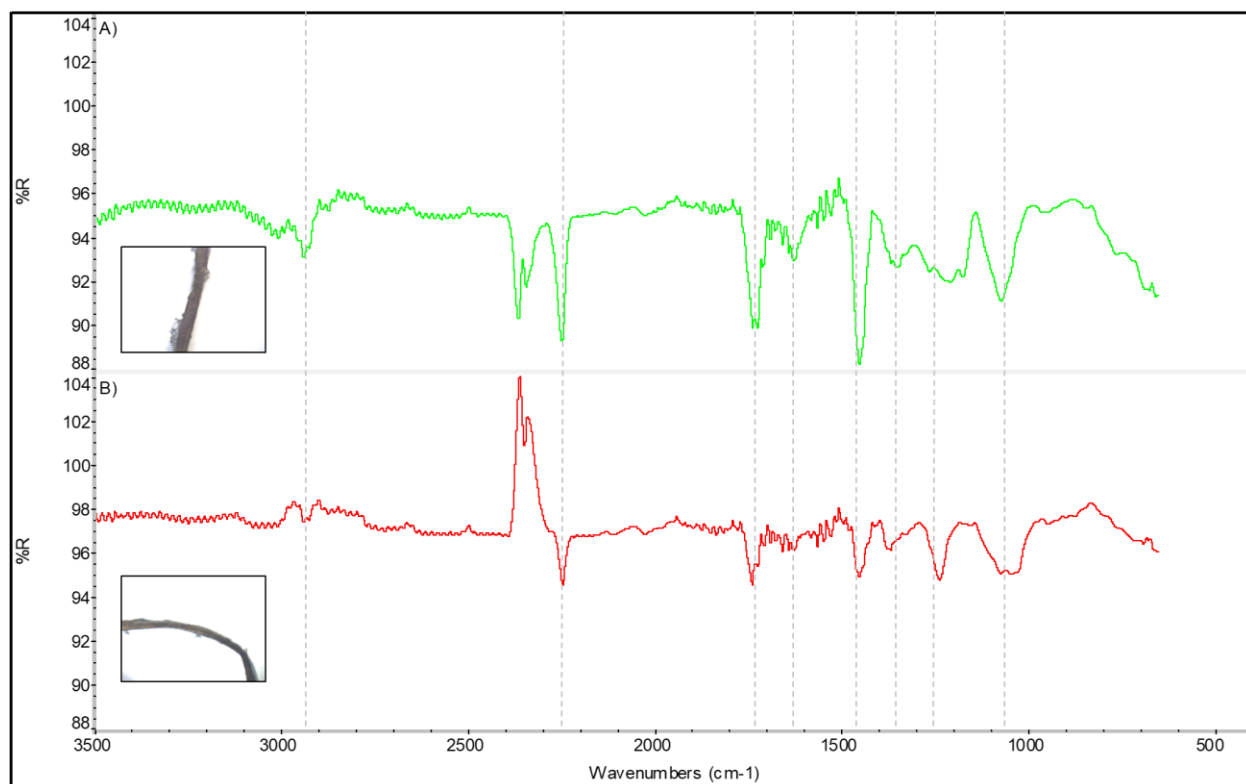


Figure 6. Spectra for recovered acrylic fibers: FTIR-ATR Spectra for two suspected acrylic/acrylic blend fibers recovered from blue crabs sampled from Corpus Christi Bay.

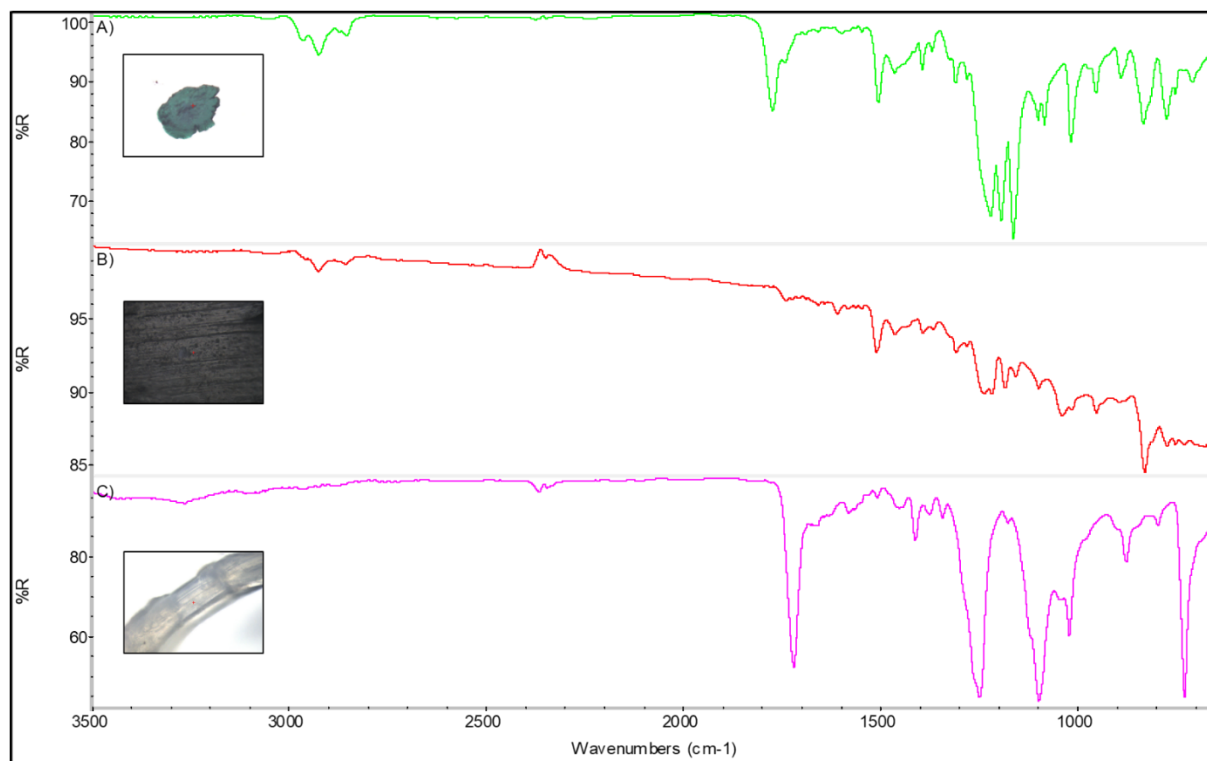


Figure 7. Spectra for recovered fragments and film: FTIR-ATR Spectra for A) a polycarbonate fragment B) phenoxy resin film C) PETE fragment recovered from blue crabs sampled from Corpus Christi Bay.

LITERATURE CITED

- Andrady, A.L., 2011. Microplastics in the marine environment. *Marine Pollution Bulletin* 62, 1596–1605. doi:10.1016/j.marpolbul.2011.05.030
- Avio, C.G., Gorbi, S., Regoli, F., 2015. Experimental development of a new protocol for extraction and characterization of microplastics in fish tissues: First observations in commercial species from Adriatic Sea. *Marine Environmental Research, Particles in the Oceans: Implication for a safe marine environment* 111, 18–26. doi:10.1016/j.marenvres.2015.06.014
- Arthur, C., J. Baker and H. Bamford (eds). 2009. *Proceedings of the International Research Workshop on the Occurrence, Effects and Fate of Microplastic Marine Debris*. Sept 9-11, 2008. NOAA Technical Memorandum NOS-OR&R-30.
- Barnes, D.K.A., 2005. Remote Islands Reveal Rapid Rise of Southern Hemisphere Sea Debris. *The Scientific World Journal* 5, 915–921. doi:10.1100/tsw.2005.120
- Barnes, D.K.A., Galgani, F., Thompson, R.C., Barlaz, M., 2009. Accumulation and fragmentation of plastic debris in global environments. *Philos Trans R Soc Lond B Biol Sci* 364, 1985–1998. doi:10.1098/rstb.2008.0205
- Batel, A., Linti, F., Scherer, M., Erdinger, L., Braunbeck, T., 2016. Transfer of benzo[a]pyrene from microplastics to *Artemia nauplii* and further to zebrafish via a trophic food web experiment: CYP1A induction and visual tracking of persistent organic pollutants. *Environ. Toxicol. Chem.* 35, 1656–1666. doi:10.1002/etc.3361
- Baztan, J., Carrasco, A., Chouinard, O., Cleaud, M., Gabaldon, J.E., Huck, T., Jaffrès, L., Jorgensen, B., Miguelez, A., Paillard, C., Vanderlinden, J.-P., 2014. Protected areas in the

- Atlantic facing the hazards of micro-plastic pollution: First diagnosis of three islands in the Canary Current. *Marine Pollution Bulletin* 80, 302–311.
doi:10.1016/j.marpolbul.2013.12.052
- Browne, M.A., Dissanayake, A., Galloway, T.S., Lowe, D.M., Thompson, R.C., 2008. Ingested microscopic plastic translocates to the circulatory system of the mussel, *Mytilus edulis* (L). *Environ. Sci. Technol.* 42, 5026–5031.
- Browne, M.A., Niven, S.J., Galloway, T.S., Rowland, S.J., Thompson, R.C., 2013. Microplastic Moves Pollutants and Additives to Worms, Reducing Functions Linked to Health and Biodiversity. *Current Biology* 23, 2388–2392. doi:10.1016/j.cub.2013.10.012
- Causin, V., Marega, C., Schiavone, S., Marigo, A., 2005. A quantitative differentiation method for acrylic fibers by infrared spectroscopy. *Forensic Science International* 151, 125–131.
<https://doi.org/10.1016/j.forsciint.2005.02.004>
- Claessens, M., Van Cauwenberghe, L., Vandegehuchte, M.B., Janssen, C.R., 2013. New techniques for the detection of microplastics in sediments and field collected organisms. *Marine Pollution Bulletin* 70, 227–233. doi:10.1016/j.marpolbul.2013.03.009
- Cole, M., Lindeque, P., Halsband, C., Galloway, T.S., 2011. Microplastics as contaminants in the marine environment: A review. *Marine Pollution Bulletin* 62, 2588–2597.
doi:10.1016/j.marpolbul.2011.09.025
- Colton, J.B., Burns, B.R., Frederick D. Knapp, 1974. Plastic Particles in Surface Waters of the Northwestern Atlantic. *Science* 185, 491–497. doi:10.1126/science.185.4150.491
- Comnea-Stancu, I.R., Wieland, K., Ramer, G., Schwaighofer, A., Lendl, B., 2017. On the Identification of Rayon/Viscose as a Major Fraction of Microplastics in the Marine Environment: Discrimination between Natural and Manmade Cellulosic Fibers Using

79 Fourier Transform Infrared Spectroscopy. *Appl Spectrosc* 71, 939–950.
80 <https://doi.org/10.1177/0003702816660725>

81 De Witte, B., Devriese, L., Bekaert, K., Hoffman, S., Vandermeersch, G., Cooreman, K.,
82 Robbens, J., 2014. Quality assessment of the blue mussel (*Mytilus edulis*): Comparison
83 between commercial and wild types. *Marine Pollution Bulletin* 85, 146–155.
84 doi:10.1016/j.marpolbul.2014.06.006

85 Derraik, J.G.B., 2002. The pollution of the marine environment by plastic debris: a review.
86 *Marine Pollution Bulletin* 44, 842–852. doi:10.1016/S0025-326X(02)00220-5

87 Desforges, J.-P.W., Galbraith, M., Ross, P.S., 2015. Ingestion of Microplastics by Zooplankton
88 in the Northeast Pacific Ocean. *Arch Environ Contam Toxicol* 69, 320–330.
89 doi:10.1007/s00244-015-0172-5

90 Devriese, L.I., van der Meulen, M.D., Maes, T., Bekaert, K., Paul-Pont, I., Frère, L., Robbens, J.,
91 Vethaak, A.D., 2015. Microplastic contamination in brown shrimp (*Crangon crangon*,
92 Linnaeus 1758) from coastal waters of the Southern North Sea and Channel area. *Marine*
93 *Pollution Bulletin* 98, 179–187. doi:10.1016/j.marpolbul.2015.06.051

94 Esiukova, E., 2017. Plastic pollution on the Baltic beaches of Kaliningrad region, Russia. *Marine*
95 *Pollution Bulletin* 114, 1072–1080. doi:10.1016/j.marpolbul.2016.10.001

96 Farrell, P., Nelson, K., 2013. Trophic level transfer of microplastic: *Mytilus edulis* (L.) to
97 *Carcinus maenas* (L.). *Environmental Pollution* 177, 1–3.
98 doi:10.1016/j.envpol.2013.01.046

99 Fendall, L.S., Sewell, M.A., 2009. Contributing to marine pollution by washing your face:
100 Microplastics in facial cleansers. *Marine Pollution Bulletin* 58, 1225–1228.
101 doi:10.1016/j.marpolbul.2009.04.025

- Gasperi, J., Dris, R., Bonin, T., Rocher, V., Tassin, B., 2014. Assessment of floating plastic debris in surface water along the Seine River. *Environmental Pollution* 195, 163–166. doi:10.1016/j.envpol.2014.09.001
- Graham, E.R., Thompson, J.T., 2009. Deposit- and suspension-feeding sea cucumbers (Echinodermata) ingest plastic fragments. *Journal of Experimental Marine Biology and Ecology* 368, 22–29. doi:10.1016/j.jembe.2008.09.007
- Gregory, M.R., 2009. Environmental implications of plastic debris in marine settings—entanglement, ingestion, smothering, hangers-on, hitch-hiking and alien invasions. *Philosophical Transactions of the Royal Society B: Biological Sciences* 364, 2013–2025. doi:10.1098/rstb.2008.0265
- Hanke, G., Werner, S., Galgani, F., Veiga, J.M., Ferreira, M., 2013. Guidance on Monitoring of Marine Litter in European Seas. Joint Research Centre Scientific and Policy Reports. Scientific and Technical Research Series. doi:10.2788/99475.
- Harrison, J.P., Ojeda, J.J., Romero-González, M.E., 2012. The applicability of reflectance micro-Fourier-transform infrared spectroscopy for the detection of synthetic microplastics in marine sediments. *Science of The Total Environment* 416, 455–463. doi:10.1016/j.scitotenv.2011.11.078
- Horsman, P.V., 1982. The amount of garbage pollution from merchant ships. *Marine Pollution Bulletin* 13, 167–169. doi:10.1016/0025-326X(82)90088-1
- Ivar do Sul, J.A., Costa, M.F., 2014. The present and future of microplastic pollution in the marine environment. *Environmental Pollution* 185, 352–364. doi:10.1016/j.envpol.2013.10.036

124 Ivar do Sul, J.A., Costa, M.F., Barletta, M., Cysneiros, F.J.A., 2013. Pelagic microplastics
 125 around an archipelago of the Equatorial Atlantic. *Marine Pollution Bulletin* 75, 305–309.
 126 doi:10.1016/j.marpolbul.2013.07.040

127 Jambeck, J.R., Geyer, R., Wilcox, C., Siegler, T.R., Perryman, M., Andrady, A., Narayan, R.,
 128 Law, K.L., 2015. Plastic waste inputs from land into the ocean. *Science* 347, 768–771.
 129 doi:10.1126/science.1260352

130 Jung, M.R., Horgen, F.D., Orski, S.V., Rodriguez C., V., Beers, K.L., Balazs, G.H., Jones, T.T.,
 131 Work, T.M., Brignac, K.C., Royer, S.-J., Hyrenbach, K.D., Jensen, B.A., Lynch, J.M.,
 132 2018. Validation of ATR FT-IR to identify polymers of plastic marine debris, including
 133 those ingested by marine organisms. *Marine Pollution Bulletin* 127, 704–716.
 134 <https://doi.org/10.1016/j.marpolbul.2017.12.061>

135 Karami, A., Golieskardi, A., Choo, C.K., Romano, N., Ho, Y.B., Salamatina, B., 2017. A high-
 136 performance protocol for extraction of microplastics in fish. *Science of The Total*
 137 *Environment* 578, 485–494. doi:10.1016/j.scitotenv.2016.10.213

138 Laist, D.W., 1997. Impacts of Marine Debris: Entanglement of Marine Life in Marine Debris
 139 Including a Comprehensive List of Species with Entanglement and Ingestion Records, in:
 140 Coe, J.M., Rogers, D.B. (Eds.), *Marine Debris*, Springer Series on Environmental
 141 Management. Springer New York, pp. 99–139. doi:10.1007/978-1-4613-8486-1_10

142 Laist, D.W., 1987. Overview of the biological effects of lost and discarded plastic debris in the
 143 marine environment. *Marine Pollution Bulletin* 18, 319–326. doi:10.1016/S0025-
 144 326X(87)80019-X

145 Laughlin, R.A., 1982. Feeding Habits of the Blue Crab, *Callinectes Sapidus* Rathbun, in the
 146 Apalachicola Estuary, Florida. *Bulletin of Marine Science* 32, 807–822.

147 Lechner, A., Keckeis, H., Lumesberger-Loisl, F., Zens, B., Krusch, R., Tritthart, M., Glas, M.,
148 Schludermann, E., 2014. The Danube so colourful: A potpourri of plastic litter
149 outnumbers fish larvae in Europe's second largest river. *Environmental Pollution* 188,
150 177–181. doi:10.1016/j.envpol.2014.02.006

151 Li, J., Qu, X., Su, L., Zhang, W., Yang, D., Kolandhasamy, P., Li, D., Shi, H., 2016.
152 Microplastics in mussels along the coastal waters of China. *Environmental Pollution* 214,
153 177–184. doi:10.1016/j.envpol.2016.04.012

154 Li, J., Yang, D., Li, L., Jabeen, K., Shi, H., 2015. Microplastics in commercial bivalves from
155 China. *Environmental Pollution* 207, 190–195. doi:10.1016/j.envpol.2015.09.018

156 Lönnstedt, O.M., Eklöv, P., 2016. Environmentally relevant concentrations of microplastic
157 particles influence larval fish ecology. *Science* 352, 1213–1216.
158 doi:10.1126/science.aad8828

159 Morritt, D., Stefanoudis, P.V., Pearce, D., Crimmen, O.A., Clark, P.F., 2014. Plastic in the
160 Thames: A river runs through it. *Marine Pollution Bulletin* 78, 196–200.
161 doi:10.1016/j.marpolbul.2013.10.035

162 Murray, F., Cowie, P.R., 2011. Plastic contamination in the decapod crustacean *Nephrops*
163 *norvegicus* (Linnaeus, 1758). *Mar. Pollut. Bull.* 62, 1207–1217.
164 doi:10.1016/j.marpolbul.2011.03.032

165 National Marine Fisheries Service (2016) Fisheries of the United States, 2015. U.S. Department
166 of Commerce, NOAA Current Fishery Statistics No.2015. Available at:
167 <https://www.st.nmfs.noaa.gov/commercial-fisheries/fus/fus15/index>

168 Nuelle, M.-T., Dekiff, J.H., Remy, D., Fries, E., 2014. A new analytical approach for monitoring
 169 microplastics in marine sediments. *Environmental Pollution* 184, 161–169.
 170 doi:10.1016/j.envpol.2013.07.027

171 Peets, P., Leito, I., Pelt, J., Vahur, S., 2017. Identification and classification of textile fibres using
 172 ATR-FT-IR spectroscopy with chemometric methods. *Spectrochimica Acta Part A:*
 173 *Molecular and Biomolecular Spectroscopy* 173, 175–181.
 174 <https://doi.org/10.1016/j.saa.2016.09.007>

175 Phuong, N.N., Zalouk-Vergnoux, A., Poirier, L., Kamari, A., Châtel, A., Mouneyrac, C.,
 176 Lagarde, F., 2016. Is there any consistency between the microplastics found in the field
 177 and those used in laboratory experiments? *Environmental Pollution* 211, 111–123.
 178 doi:10.1016/j.envpol.2015.12.035

179 PlasticsEurope, 2012. *Plastics – the Facts 2012*. Retrieved from
 180 [http://www.plasticseurope.org/Document/plastics-the-facts-](http://www.plasticseurope.org/Document/plastics-the-facts-2012.aspx?Page=DOCUMENT&FolID=2)
 181 [2012.aspx?Page=DOCUMENT&FolID=2](http://www.plasticseurope.org/Document/plastics-the-facts-2012.aspx?Page=DOCUMENT&FolID=2).

182 PlasticsEurope, 2016. *Plastics – the Facts 2016* An analysis of European plastics production,
 183 demand and waste data. Retrieved from
 184 <http://www.plasticseurope.org/Document/plastics-the-facts-20142015.aspx?FolID=2>

185 Ryan, P.G., 1988. The characteristics and distribution of plastic particles at the sea-surface off
 186 the southwestern Cape Province, South Africa. *Marine Environmental Research* 25, 249–
 187 273. doi:10.1016/0141-1136(88)90015-3

188 Ryan, P.G., Moore, C.J., Franeker, J.A. van, Moloney, C.L., 2009. Monitoring the abundance of
 189 plastic debris in the marine environment. *Philosophical Transactions of the Royal Society*
 190 *B: Biological Sciences* 364, 1999–2012. doi:10.1098/rstb.2008.0207

191 Santana, M.F.M., Ascer, L.G., Custódio, M.R., Moreira, F.T., Turra, A., 2016. Microplastic
 192 contamination in natural mussel beds from a Brazilian urbanized coastal region: Rapid
 193 evaluation through bioassessment. *Marine Pollution Bulletin* 106, 183–189.
 194 doi:10.1016/j.marpolbul.2016.02.074

195 Secretariat of the Convention on Biological Diversity (CBD), Montreal. 2016. Marine Debris:
 196 Understanding, Preventing and Mitigating the Significant Adverse Impacts on Marine
 197 and Coastal Biodiversity. Technical Series No.83., 78 pages.

198 Shah, A.A., Hasan, F., Hameed, A., Ahmed, S., 2008. Biological degradation of plastics: A
 199 comprehensive review. *Biotechnology Advances* 26, 246–265.
 200 doi:10.1016/j.biotechadv.2007.12.005

201 Shiber, J.G., 1979. Plastic pellets on the coast of Lebanon. *Marine Pollution Bulletin* 10, 28–30.
 202 doi:10.1016/0025-326X(79)90321-7

203 Teuten, E.L., Saquing, J.M., Knappe, D.R.U., Barlaz, M.A., Jonsson, S., Björn, A., Rowland,
 204 S.J., Thompson, R.C., Galloway, T.S., Yamashita, R., Ochi, D., Watanuki, Y., Moore, C.,
 205 Viet, P.H., Tana, T.S., Prudente, M., Boonyatumanond, R., Zakaria, M.P., Akkhavong,
 206 K., Ogata, Y., Hirai, H., Iwasa, S., Mizukawa, K., Hagino, Y., Imamura, A., Saha, M.,
 207 Takada, H., 2009. Transport and release of chemicals from plastics to the environment
 208 and to wildlife. *Philosophical Transactions of the Royal Society B: Biological Sciences*
 209 364, 2027–2045. doi:10.1098/rstb.2008.0284

210 Thompson, R.C., Olsen, Y., Mitchell, R.P., Davis, A., Rowland, S.J., John, A.W.G., McGonigle,
 211 D., Russell, A.E., 2004. Lost at Sea: Where Is All the Plastic? *Science* 304, 838–838.
 212 doi:10.1126/science.1094559

213 Tsang, Y.Y., Mak, C.W., Liebich, C., Lam, S.W., Sze, E.T.-P., Chan, K.M., 2017. Microplastic
 214 pollution in the marine waters and sediments of Hong Kong. *Marine Pollution Bulletin*
 215 115, 20–28. doi:10.1016/j.marpolbul.2016.11.003

216 Van Cauwenberghe, L., Claessens, M., Vandegehuchte, M.B., Janssen, C.R., 2015. Microplastics
 217 are taken up by mussels (*Mytilus edulis*) and lugworms (*Arenicola marina*) living in
 218 natural habitats. *Environmental Pollution* 199, 10–17. doi:10.1016/j.envpol.2015.01.008

219 Van Cauwenberghe, L., Janssen, C.R., 2014. Microplastics in bivalves cultured for human
 220 consumption. *Environmental Pollution* 193, 65–70. doi:10.1016/j.envpol.2014.06.010

221 Vandermeersch, G., Van Cauwenberghe, L., Janssen, C.R., Marques, A., Granby, K., Fait, G.,
 222 Kotterman, M.J.J., Diogène, J., Bekaert, K., Robbens, J., Devriese, L., 2015. A critical
 223 view on microplastic quantification in aquatic organisms. *Environmental Research, Non-*
 224 *regulated environmental contaminants in seafood: contributions of the ECsafeSEAFOOD*
 225 *EU project 143, Part B*, 46–55. doi:10.1016/j.envres.2015.07.016

226 Watts, A.J.R., Lewis, C., Goodhead, R.M., Beckett, S.J., Moger, J., Tyler, C.R., Galloway, T.S.,
 227 2014. Uptake and Retention of Microplastics by the Shore Crab *Carcinus maenas*.
 228 *Environ. Sci. Technol.* 48, 8823–8830. doi:10.1021/es501090e

229 Wilcox, C., Mallos, N.J., Leonard, G.H., Rodriguez, A., Hardesty, B.D., 2016. Using expert
 230 elicitation to estimate the impacts of plastic pollution on marine wildlife. *Marine Policy*
 231 65, 107–114. doi:10.1016/j.marpol.2015.10.014

232 Wójcik-Fudalewska, D., Normant-Saremba, M., Anastácio, P., 2016. Occurrence of plastic
 233 debris in the stomach of the invasive crab *Eriocheir sinensis*. *Marine Pollution Bulletin*
 234 113, 306–311. doi:10.1016/j.marpolbul.2016.09.059

235 Wright, S.L., Thompson, R.C., Galloway, T.S., 2013. The physical impacts of microplastics on
236 marine organisms: A review. *Environmental Pollution* 178, 483–492.
237 doi:10.1016/j.envpol.2013.02.031
238