ABSTRACT

Microplastic Ingestion by Freshwater and Marine Fish from the Brazos River Basin and Texas Nearshore Marine Waters

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This dissertation serves as one of the first comprehensive system investigations of microplastic ingestion by freshwater and marine fish from the Brazos River basin and Texas nearshore marine waters. In total, 436 freshwater sunfish were sampled and 45% contained ingested anthropogenic materials, consisting of -macro (4%) and -micro (96%) sized contaminants. Microplastic ingestion was greater within fish collected from urban areas, in comparison to upstream and downstream sites, suggesting that human development (i.e. paved roadways) and local urbanization are two possible factors impacting microplastic ingestion by sunfish. The marine portion of this research (Chapter Four) examined a total of 1,381 fish, inclusive of six species of a shared ecological guild, and 42.2% of stomachs contained microplastic fibers (86.4%), beads (12.9%) and fragments (<1%). Despite a substantial overlap in diet, ordination of ingested prey items clustered samples into distinctive species groupings, reflective of the foraging gradient among species. Grunt displayed the lowest overall frequency of microplastic ingestion and the most distinctive ordination grouping, indicating their selective invertebrate foraging preferences. While all six species had ingested microplastic, the results suggest

that grunt, as selective invertebrate foragers, are less likely to ingest microplastic than species which exhibit generalist foraging preferences and methods of prey capture. When comparing microplastic ingestion between freshwater sunfish and marine pinfish, species which serve as ecological analogs between the systems, there was no significant difference in the overall frequency or mean number of microplastics ingested. However, the pinfish stomach content contained microplastic fiber, bead, and fragment morphologies, while the sunfish stomach content only contained microplastic fibers. Pyr-GC/MS analysis classified forty-three of the marine microplastic samples as polyvinyl chloride (34.8%), polyethylene terephthalate (9.3%), nylon (9.3%), silicone (2.3%), and epoxy resin (2.3%). Approximately 42% of samples could not be classified into a specific polymer class, due to a limited formation of pyrolytic products, low product abundance, or a lack of comparative standards. Overall, this dissertation demonstrates that microplastic ingestion is ubiquitous throughout Texas aquatic environments, influenced by species diet and foraging methods, and reflective of local land use patterns and major sources of pollution.

Microplastic Ingestion by Freshwater and Marine Fish from the Brazos River Basin and Texas Nearshore Marine Waters

by

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A Dissertation

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CHAPTER ONE

Introduction

Plastic debris is ubiquitous throughout global aquatic environments, contaminating freshwater rivers and lakes, and coastal, deep-sea, near-shore, and open ocean systems (do Sul and Costa, 2014; Dreidger et al., 2015; Eerkes-Medrano et al., 2015; Free et al., 2014; Mathalon and Hill, 2014). Following its commercialization in the 1960's, plastic production has grown exponentially, increasing 560-fold over the past sixty years. (Jambeck et al., 2015). Currently, worldwide plastic production exceeds 300 million tons per year, an estimated 10% of which ultimately ends up in marine systems (Gourmelon, 2015).

Plastic enters the environment via intentional (e.g. illegal discards) and unintentional (e.g. surface runoff) pathways. Major sources of plastic pollution include coastal tourism, the fishing industry, inadequately lined landfills, and natural disasters (Jambeck, 2015; Tibbetts, 2015). Although the total proportion of improperly discarded plastic is unknown, estimates predict that 80% of marine debris originates from terrestrial sources (Jambeck et al., 2015; Thompson, 2006). Following its release into aquatic systems, plastic disperses via wind and currents, resulting in the long-range transport and widespread distribution of this contaminant throughout the entirety of the water column. Computational modeling has aided in the estimation of the global distribution, residence time, and convergence zones of plastics throughout aquatic systems, however, external forces, such as the mixing of the sea-surface boundary and re-suspension from sediments, makes it difficult to determine the exact route of plastic dispersal throughout aquatic

environments. Additionally, the varying physiochemical properties of plastic, inhibits the identification of transport and settling pathways (Eerkes-Medrano et al., 2015).

Chemical and photolytic degradation processes are also main factors which influence the fate of plastics in the environment, contributing to the degradation of macroplastics and resulting in the formation of microplastic. Plastic can be broadly categorized into –macro (i.e. greater than 5mm in diameter) and -micro (i.e. less than 5 mm in diameter) size classifications, however, there is no internationally recognized lower boundary for the category of –micro sized plastics. Oftentimes the lower size boundary classification is determined by the methodologies utilized for research, for instance the use of 333 μm as the lower boundary for microplastic classification in neuston trawl studies. This inconsistency in size classification is problematic as it limits the ability to compare results between studies, subsequently inhibiting the progression of microplastic research.

Microplastics are created through both primary and secondary methods. Primary microplastics are plastics which are produced at a microscale and include items such as microbeads and as vectors for drugs, whereas secondary microplastics are formed via the breakdown of macroplastics (Browne et al., 2011; Dubaish and Liebezeit, 2013; Fendall and Sewell, 2009; Mathalon and Hill, 2014; Wagner et al., 2014;). Studies examining microplastic pollution have confirmed this contaminant within all types of aquatic systems, including freshwater, marine, and deep ocean environments (do Sul and Costa., 2014; Driedger et al., 2015; Dris et al., 2015; Eriksen et al., 2013; Free et al., 2014; Moore et al., 2011).

As microplastic pollution research has increased, a growing subset of literature has investigated consequences resulting from the interaction of wildlife and microplastic, such as microplastic ingestion. To date, microplastic ingestion has been discovered within a large variety of species, ranging from filter-feeding and deposit-feeding invertebrates, to lobsters, copepods, and fish (Cole et al., 2013; Desforges et al., 2015; Graham and Thompson, 2009; Murray and Cowie, 2011). Studies examining the ingestion of microplastic by fish report a wide range of deleterious effects, including reduced physical health, a reduction in predatory performance, a histological change to internal organs, a physical blockage of the digestive organs, and an interference with feeding (Jovanović, 2017). Common goby exposed to microplastics were found to confuse microplastics with prey items, resulting in a reduction of predatory performance and efficiency (de Sá et al., 2015). The uptake and accumulation of microplastic has also been shown to induce histological change and oxidative stress in the liver of zebrafish and result in a pathological alteration to the distal portion of the intestine of European sea bass (Lu et al., 2016; Pedà et al., 2016). Despite the growing body of literature examining microplastic and fauna interactions, research examining microplastic ingestion and subsequent toxicological effects is still in its infancy.

Limitations of the current body of available research examining microplastic ingestion by fish includes the wide variety of species and locations examined, the lack of a standard microplastic size classification, and the variation in methodologies utilized for microplastic identification (Foekema et al., 2013; Lusher et al., 2013; Nadal et al., 2016; Ramos et al., 2012; Romeo et al., 2015). The following research was performed in response to current microplastic literature limitations and provides one of the first

comprehensive investigations of microplastic ingestion by fish from North America via the following goals and aims:

1. Goal One: Examine the occurrence and frequency of microplastic ingestion by a freshwater sentinel taxon (i.e. the sunfish, Centrarchidae)
    a. Aim: Evaluate the impact of local urbanization on the frequency and type of microplastic ingested
    b. Knowledge Gap: This research will add to the growing body of literature examining microplastic ingestion by freshwater fish as the majority of available scientific literature focuses extensively on marine systems. This research will also offer insight into the impact of local urbanization on microplastic ingestion, specifically investigating whether various forms of urbanization serve as vectors for microplastic pollution.
2. Goal Two: Examine the occurrence and frequency of microplastic ingestion by six marine fish species from the Texas Gulf Coast
    a. Aim: Evaluate the influence of varying methods of prey capture and foraging preferences on microplastic ingestion by six species of a shared ecological guild
    b. Knowledge Gap: This research is the first to investigate microplastic ingestion from an ecological standpoint, seeking to determine if and why species of a shared ecological guild ingest varying levels of microplastic.

3. Goal Three: Compare microplastic ingestion (i.e. overall frequency of ingestion and types of particles ingested) between freshwater sunfish and a marine ecological analog (i.e. pinfish)
    a. Aim: Investigate shifts per the frequency and type of microplastic ingested between the freshwater and marine systems examined
    b. Knowledge Gap: The freshwater and marine studies are unique in that they occupy the same watershed (Brazos River), because of this, we are able to directly compare frequencies and types of microplastic ingested. Pinfish were specifically chosen for this purpose as they serve as ecological analogs to freshwater sunfish. Both species are suction feeders and occupy a similar niche within their environment.
4. Goal Four: Investigate the applicability of pyr-GC/MS for the polymer identification of microplastic extracted within fish stomach content
    a. Aim: To identify microplastic polymers extracted from the stomach content of the marine fish samples.
    b. Knowledge Gap: The primary techniques utilized for microplastic polymer identification (i.e. FTIR and Raman spectroscopy) are inhibited by microplastic size and complexity, and by time constraints, thus current polymer identification results are skewed towards larger particles. As pyr-GC/MS does not exhibit these limitations, it can serve as an effective and efficient alternative method for microplastic polymer identification.

CHAPTER TWO

Literature Review

Plastic Distribution

Plastic pollution is ranked by The United Nations Environmental Program as one of the top global environmental issues (Mason et al., 2016). Since its commercialization during the Second World War, plastic production has increased exponentially, growing from 150 million tons produced annually during the 1930's-1950's, to over 300 million tons today. In the United States, plastic production accounts for the third largest manufacturing industry, producing 32.5 million tons of plastic annually, approximately 90% of which is discarded (U.S. EPA, 2015).

Estimates predict that 10% of annual plastic waste ends up in marine environments, 80% of which is attributed to terrestrial based sources (Jambeck et al., 2015; Thompson, 2006). Due to the complex and dynamic nature of environmental transport pathways, it is difficult to trace plastic back to it originating source, however, intentional (e.g. illegal discards) and unintentional (e.g. surface runoff) discards, coastal tourism, the fishing industry, inadequately lined landfills, and sewage treatment facilities are major contributors of plastic waste into aquatic environments (Galgani et al., 2017). While computational modeling has successfully estimated global marine distribution, residence time, and convergence zones of plastic pollution, external forces, such as mixing of the water-surface boundary and re-suspension from sediments, makes it difficult to determine exactly where and how plastics disperse and settle throughout aquatic systems.

Physiochemical properties of plastic, such as specific density, may offer limited insight as to where and how plastic will disperse once it reaches aquatic systems. For instance, plastic with a density less than that of marine water (approximately 1.03 g/cm^{-3}) is positively buoyant and likely to float, while plastic with a density greater than 1.03 g/cm^{-3} is likely to sink (Andrady, 2011). However, this assumption only holds true without the inclusion of external factors, such as vertical mixing, underlying currents, degradation, or biofouling, thus it is not truly reflective of plastic transport within the environment (Engler, 2012; Legarde et al., 2016). Examples of this include the long-range transport of high-density particles and the settling of low-density particles in benthic environments (Frere et al., 2017; Ryan, 2015).

Plastic Size Categorization

Plastic size categorization is inclusive of: 1. Macroplastic: plastic ≥ 5mm in diameter; and 2. Microplastics: plastic < 5 mm in diameter. To date, there is no standardized lower bound for the categorization of microplastic, it is instead determined via the research focus and subsequent methodologies utilized, such as 333μm set as the lower bound for microplastic collections via neuston net, or 53μm for fish stomach content analysis (Arthur et al., 2009; Peters and Bratton, 2016). While the division of plastic into -macro and –micro size categories is becoming more standardized throughout the scientific literature, there are categorization schemes which expand upon or conflict with these divisions. For instance, GESAMP (2015) utilizes the following plastic size categorization scheme: Megaplastic: plastic ≥ 1m in diameter; Macroplastic: 2.5 cm ≤ plastic ≤ 1m; Mesoplastic: 1mm ≤ plastic ≤2.5 cm; Microplastic: 1μm ≤ plastic ≤1mm; and Nanoplastic: plastic ≤1μm in diameter.

In addition to size, microplastics may be categorized via their production source as either primary or secondary. Primary sourced microplastics are plastics manufactured at a micro-scale (e.g. vectors for drugs, exfoliates within body scrubs) while secondary sourced microplastics are plastics derived from the mechanical, photolytic, or chemical degradation of larger plastics, resulting in plastics with micro-sized dimensions (Dubaish and Liebezeit, 2013; Fendall and Sewell, 2009; Mathalon and Hill, 2014; Wagner et al., 2014).

Microplastic Identification

Hidalgo-Ruz et al. (2012) published the first review article examining the methods utilized for the identification and quantification of microplastics within marine matrix samples. Of the 68 studies included in this review, density separation, filtration, sieving, and visual analysis were the primary methods utilized for microplastic identification. Of these methods, visual analysis was the most common and research inclusive of this method identified microplastic via the criteria of shape, type, degradation, and color (Hidalgo-Ruz et al., 2012). Hidalgo-Ruz et al. (2012) subsequently utilized this information to develop a set of standards to be met when utilizing visual analysis for microplastic identification.

The Hidalgo-Ruz et al. (2012) Standards:

1) The particles' largest dimension is less than 5mm

2) The particle must have no visible structures of organic origin

3) Particles in the shape of a fiber should be equally wide throughout the entirety of its length

4) Particles must be homogeneously colored

5) If particle composition is in question, its characteristics must be further examined under a compound microscope

These standards were then utilized by the Marine and Environmental Research Institute (Guide to Microplastic Identification, 2012) as a basis for their guide on the identification of microplastics, with the following alterations:

1) Biofouling may alter the appearance of the particle by adhering to the surface, thus if organic structures are noted on only a section of the particle, further analyze the particle under a compound microscope

2) Particle splitting or fraying may occur due to degradations, thus equal thickness is not always standard

3) Particles may exhibit patterns or color alterations, thus, homogenous color is not always standard

The combination of the Hidalgo-Ruz et al. (2012) standards and the additional alterations by the Marine and Environmental Research Institute are now widely utilized as standard guidelines for the visual identification of microplastics within environmental samples and were applied as standards for microplastic identification within this research.

Microplastic Characterization

Morphology and color are the most common dimensions utilized for microplastic characterization. Microplastic morphology is inclusive of: 1. Fiber: a strand or filament plastic; 2. Film: a thin sheet or membrane-like plastic; 3. Sphere (microbeads): plastic that is round or ball-like in shape; and 4. Fragments: irregular or angular shaped plastic (Figure 2.1). Microplastic color is typically categorized into main color blocks (e.g. red, blue, green, black, white), however, this method is all inclusive and may be subjective.

Chapter 4 addresses this limitation by utilizing a color categorization based off of the standardized Munsell Color System (Munsell Color Company, 2012). While previously utilized in the field of soil science, the Munsell Color System is widely applicable for the identification of microplastic color per the dimensions of hue, value, and chroma. Hue is the dimension which distinguishes between color families, value is the dimension measures the lightness or darkness of color, and chroma is the dimension which measures the intensity of color (Munsell Color Company, 2012). This method, while more precise than previous color classifications, is only applicable for particles which exhibit homogenous color.

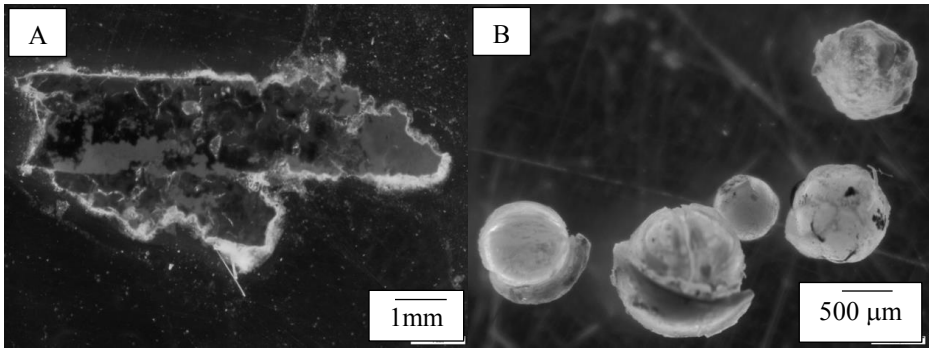

Figure 2.1: Photographs of macroplastic fragments (A) and microplastic spheres (i.e. microbeads) (B)

Microplastic Pollution

Marine Environments

Microplastic pollution first appeared within the scientific literature in the early 1970's with the discovery of microplastic fibers within water samples from the North Sea (Buchanan,1971). Following this, polystyrene pellets were discovered within surface waters and plankton samples from the Atlantic Ocean (Carpenter et al., 1972; Colton et

al., 1974). Since this early research, microplastics have been discovered within every type of aquatic system (e.g. rivers, oceans, and lakes) and are ubiquitous contaminants worldwide (Baldwin et al., 2016; Desforges et al., 2014; Horton et al., 2017).

The most widely investigated marine systems for microplastic pollution are the Atlantic and Pacific Oceans. Microplastics have been confirmed within the waters of the Atlantic Ocean, ranging from 0.26 particles/m^{-3} in waters from the South Atlantic to 2.46 particles/m^{-3} in the Northeastern Atlantic (Lima et al., 2014; Lusher et al., 2014). Studies of microplastic concentrations within the Pacific Ocean are greater than that of the Atlantic, ranging from 8-9180 microplastics/m^3 within the surface waters of the North-East Pacific and approximately 16,000 microplastics/m^3 in surface waters off of the coast of Geoje Island, South Korea (Desforges et al., 2014; Song et al., 2014). The variation in microplastic concentration per location are likely the result of numerous mechanisms, including winds, currents, coastline geography, and human factors such as local land use, and levels of anthropogenic disturbance (Barnes et al., 2009).

The combination of environmental and human mechanisms on microplastic transport pathways also result in microplastic "hotspot" areas (i.e. areas of high plastic abundance) (Sharma and Chatterjee, 2017). Confirmed hotspot areas include coastal waters nearby heavy industrialization, such as harbor waters in Sweden contaminated with 100,000 microplastics/m^{-3} (Norén and Naustvoll, 2010) and accumulation zones far removed from waste sources (i.e. gyres). Gyres, also referred to as "garbage patches" are known areas of debris accumulation due to their unique position amongst zones of oceanic currents. Gyres have been confirmed within every major marine system and may contain anywhere from 17-184 trillion microplastic particles. A survey of five sub-

tropical gyres (North Pacific, North Atlantic, South Pacific, South Atlantic, Indian Ocean) estimates that these areas contain upwards of 462 trillion pieces of microplastic, in addition to macroplastics and additional waste materials that contribute to these major zones of accumulation (Eriksen et al., 2014).

Freshwater Environments

Despite the growing body of literature examining microplastic pollution of marine systems, research pertaining to freshwater systems is limited. However, similar to marine systems, microplastics have been confirmed as ubiquitous contaminants throughout freshwater environments, impacted by similar ecological and anthropogenic transport pathways (Fischer et al., 2016; Horton et al., 2017; Lechner et al., 2014). For instance, urbanization has been identified has a major factor impacting the prevalence of microplastic within freshwater systems. A study of the Laurentain Great Lakes (USA) found that areas downstream of highly populated metropolitan areas (i.e. Detroit and Cleveland) contained elevated microplastic concentrations (280,947-466,305 particles/km^{-2}) in comparison to samples collected from less urbanized areas of Lake Huron (456-541 particles/km^{-2}) (Eriksen et al., 2013). Baseline investigations of microplastic pollution within the neuston of Lakes Huron, Superior, and Erie, found an average of approximately 43,000 microplastic items per km^{-2}, ranging from 450-466,000 particles/km^2, while nearby tributaries contained an average of 0.05-32 microplastic per m^{-3} (Baldwin et al., 2016; Eriksen et al., 2013). Despite the comparatively low concentration of microplastic within Laurentian lakes, it is clear that tributaries are contributing sources of microplastic into the lakes.

Originating sources of microplastic may include commercial and industrial activity, road runoff, littering, atmospheric deposition, and wastewater effluent (Horton et al., 2017). A study of nine rivers near Chicago (USA) found that microplastic concentrations were greater downriver of wastewater effluent (5.7 particles/m^{-3}) in comparison to upstream sites (2.4 particles/m^{-3}) (McCormick et al., 2014). Microplastics typically associated with wastewater effluent include microplastic beads, a by-product of facial scrubs, and microplastic fibers, a result of the breakdown of synthetic or semi-synthetic materials such as clothing. Browne et al. (2011) examined the breakdown of manufactured clothing and found that a single article may release more than 1,900 microplastic fibers per washing cycle. Following clothing breakdown, fibers are transported within washing machine wastewater and sent to wastewater treatment plants, where they are either filtered out during the filtration process or released into the environment via wastewater effluent.

Microplastic Sinks

Benthic and shoreline soils and sediments serve as major areas of microplastic deposition and accumulation, however, the mechanisms controlling microplastic sinks are not fully understood. Reports from shallow water (i.e. coastal zone) sediments of the Southern Baltic Sea showed higher microplastic concentrations (15-27 particles/kg^{-1} d.w.) than deep-water sediments (0-3 particles/kg^{-1} d.w.) (Bozena et al., 2017). This trend was mirrored within Belgian sediments, which contained significantly higher microplastic concentrations within coastal harbor sediments (166.7±92.1 particles/ kg d.w.) than continental shelf and beach sediments (97.2±18.6 particles/kg dry and 92.8±37.2 particles/kg dry, respectively) (Claessens et al., 2011). When comparing

between urban and rural sites, German beach sediments from urban sites contained greater microplastic concentrations (5000-7000 particles/m^3) than rural sites (150-700 particles/m^3) (Ballent et al., 2012). It is likely that this is a direct result of local urbanization and anthropogenic disturbance, however, factors such as polymer density and water column density stratification may also play an important role in microplastic deposition and accumulation.

Microplastic Ingestion by Fish

The small size of microplastic and its prevalence throughout aquatic systems significantly increases the likelihood of microplastic ingestion by fish species. Many studies have investigated this interaction and report that a wide range of species, from varying locations and trophic guilds, have ingested microplastic (Bellas et al., 2016; Lusher et al., 2016; Murphy et al., 2017). Overall, there are two hypotheses as to why fish ingest microplastic, 1. Fish intentionally ingest microplastics, mistaking them for prey due to a similar size or color; or 2: Fish accidentally ingest microplastic, either simultaneously during normal foraging behavior or during the ingestion of prey in which microplastics are located on or within the item (Jovanović, 2017). In addition to this, factors such as microplastic availability and fish foraging behavior influence microplastic ingestion, adding to the complex nature of this interaction.

Several studies examining microplastic ingestion by fish have found nylon blue fragments and fibers to be the primary type of microplastic recovered. This was fist noted in one of the earliest reports of microplastic ingestion by fish, which discovered that 18-33% of marine catfish sampled, inclusive of three species, had ingested microplastic (Possatto et al., 2011). Separate examinations of microplastic ingestion by Gerreidae and

estuarine drums from the Goiana Estuary also found that nylon blue fragments were the only type of microplastic ingested (Dantas et al., 2012; Ramos et al., 2012). All three of these studies attribute high levels of local and artisanal fishing as sources of nylon into the systems, suggesting that microplastic ingestion was directly a result of nearby anthropogenic disturbance.

In addition to availability, particle color may be a factor influencing rates of microplastic ingestion. A study examining fish from the North Pacific Gyre reported that 35% of mesopelagic fish samples had ingested microplastic and that blue, white, and clear were the most common microplastic colors recovered (Boerger et al., 2010). These colors are similar to plankton, a common prey item of the mesopelagic species examined, suggesting that the microplastics may have been mistaken for prey items. While this method of microplastic ingestion is active, it appears that a majority of available literature suggests that microplastic ingestion more commonly results via passive methods of ingestion (e.g. simultaneously ingesting microplastic during normal foraging behavior). For instance, Tanaka and Takada (2016) cite incidental ingestion during filter feeding as the cause for 77% of Japanese anchovy from Tokyo Bay containing ingested microplastics. Comparatively, a study of three demersal fish species from the Spanish, Atlantic and Mediterranean Coasts found that only 17.5% of individuals contained ingested microplastic. Of the three species examined (Lesser spotted dogfish, European hake, and Red mullet), Red Mullet displayed the highest frequency of microplastic ingestion, which may be reflective of their foraging behavior which results in the ingestion of sediment along with prey items (Bellas et al., 2016).

Numerous studies have also examined the role the fish habitat (i.e. oceanic zone) may have on microplastic ingestion. A comparison of 26 benthic and pelagic fish species from the Portuguese coast found that 19.8% of total fish had ingested microplastic (Neves et al., 2015). When comparing between fish species of the benthic and pelagic zones, there were no significant differences in overall frequencies of microplastic ingestion. This result was also shown by Lusher et al. (2013), which found that there was no significant difference in microplastic ingestion (36.5% overall) between five species of pelagic and demersal fish examined. Davison and Asch (2011) conducted a similar study but instead investigated the influence of vertical migratory foraging on microplastic ingestion. Overall, 9.2% of mesopelagic fish from the North Pacific Subtropical Gyre had ingested microplastic, however, non-vertically migrating fish displayed lower overall frequencies of ingestion (4.8%) in comparison to 11.6% of vertically migrating fish which had ingested microplastic. These results indicate that microplastics are ubiquitous contaminants of marine systems, and available to all species regardless of habitat, trophic role, or methods of prey capture.

Freshwater Species

While reports of microplastic ingestion by marine fish species are becoming more frequent within scientific literature, studies examining microplastic ingestion by freshwater fish are limited. A study of fish collected from freshwater streams in France reported that 11-26% of fish had ingested microplastic and that the highest rates of microplastic ingestion were associated with levels of local anthropogenic pressure (Sanchez et al., 2014). Freshwater fish from South America were also found to ingest high numbers of microplastic (83% had ingested anthropogenic debris, microplastic

constituted 88.6% of ingested anthropogenic material) and fish from urbanized sites contained a greater number of ingested microplastics than those from rural sites (Silva-Cavalcanti et al., 2017). In North America, studies examining microplastic ingestion by fish are limited to that of Phillips and Bonner (2015), which reported that 8% of freshwater fish contained ingested microplastic and that fish from urban sites displayed higher frequencies of microplastic ingestion in comparison to fish from rural sites.

Adverse Effects

Microplastic ingestion by fish may cause a wide range of adverse effects, the extent and severity of which are a result of multiple factors, such as the species of examination and the size and type of plastic ingested. Known adverse effects include a physical blockage of digestive organs, interference with feeding, and a reduction of predatory performance and efficiency (de Sá et al., 2015; Jovanović, 2017; Pedà et al., 2016). Microplastic ingestion has also been observed to induce histopathological changes within Sea bass and Zebrafish, resulting in an alteration of function within the distal portion of the intestine and oxidative stress within the liver (Lu et al., 2016; Pedà et al., 2016). Furthermore, hard plastics with sharp edges may penetrate internal organs, causing mechanical injuries or ulceration (Jovanović, 2017).

In addition to adverse effects via an alteration of biological processes, ingested microplastics may serve as vectors for chemical exposure via the leaching of additives, such as plasticizers, colorants, and stabilizers, or the adsorption/desorption of chemical contaminants from the environmental matrix. A study of Japanese medaka found that fish exposed to polyethylene deployed within the marine environment contained greater concentrations of polybrominated diphenyl ethers (PBDEs) and polycyclic aromatic

hydrocarbons (PAHs) than those exposed to virgin polyethylene and control treatments (Rochman et al., 2013). Studies have also found that the ingestion of microplastics contaminated with sorbed chemical pollutants may result in hepatic stress and endocrine disruption within fish, thus the impacts of microplastic ingestion may include both biological and toxicological effects (Rochman et al., 2013; Rochman et al., 2014).

Despite these preliminary results, the exact nature of chemical transfer from plastic to fish is relatively unknown (Koelmans et al., 2016). Simulation and computational modeling results indicate that ingested microplastics containing hydrophobic organic chemicals (HOCs) are not likely routes of chemical exposure based on the chemical transfer rate from microplastic to organism, simulated desorption rate in artificial gut fluid, and bioaccumulation potential (Bakir et al., 2016; Koelmans et al., 2016). This is supported by Ziccardi et al. (2016), which found that although HOCs can partition from microplastic to organisms, there is not enough evidence to support ecologically significant adverse effects on aquatic life due to HOC exposure via microplastic ingestion. This contradiction between model and laboratory results is indicative of the large knowledge gap pertaining to the role of microplastics as vectors for chemical exposure. Thus, further research is needed which examines the complex interactions involved in microplastic ingestion, including the desorption and adsorption of chemical pollutants and subsequent potential adverse effects (Crawford and Quinn, 2017).

Literature Limitations

Microplastic pollution research is still fairly new within the scientific literature, thus there are major limitations that exist. Overall, methods of reporting vary widely per

the environmental matrix examined and the methodologies utilized. Major limitations of research examining microplastic pollution of aquatic environments include variations in methods of collection, such as the size of net mesh utilized, the location of water samples (e.g. surface vs. mi-water column), the length of trawl time, and the methods of reporting results (e.g. particles/L, particles/ m^{-3}). These methods of reporting do not translate well for comparisons with investigations of microplastic contamination of soils and sediment, which also vary widely per the methods of reporting (e.g. particles/ m^{-3}, particles/kg dry weight).

Literature examining microplastic ingestion by fish is also limited by the variation in the methodologies utilized for analysis and methods of reporting. Major variations in methodologies include discrepancies in the organ of examination (e.g. stomach or entire GIT) and the methods utilized for microplastic identification (e.g. visual analysis, density separation, digestion). A lack of a standardized lower-bound for the definition of microplastic size complicates comparisons between results and can lead to an under estimation of microplastic concentrations. Additionally, there needs to be a standardized method for polymer identification, without which, sources of microplastic contamination can only be hypothesized. These discrepancies collectively limit the enhancement of current microplastic research and need to be addressed by the scientific community before universal progress can be made.

CHAPTER THREE

Urbanization is a Major Influence on Microplastic Ingestion by Sunfish in the Brazos River Basin, Central Texas

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Abstract

Microplastics, degraded and weathered polymer-based particles, and manufactured products ranging between 50 and 5000 mm in size, are found within marine, freshwater, and estuarine environments. While numerous peer-reviewed papers have quantified the ingestion of microplastics by marine vertebrates, relatively few studies have focused on microplastic ingestion by freshwater organisms. This study documents microplastic and manufactured fiber ingestion by bluegill (*Lepomis macrochirus*) and longear (*Lepomis megalotis*) sunfish (Centrarchidae) from the Brazos River Basin, between Lake Whitney and Marlin, Texas, USA. Fourteen sample sites were studied and categorized into urban, downstream, and upstream areas. A total of 436 sunfish were collected, and 196 (45%) stomachs contained microplastics. Four percent (4%) of items sampled were debris on the macro size scale (i.e. >5 mm) and consisted of masses of plastic, metal, Styrofoam, or fishing material, while 96% of items sampled were in the form of microplastic threads. Fish length was statistically correlated to the number of microplastics detected ($p = 0.019$). Fish collected from urban sites displayed the highest mean number of microplastics ingested, followed by downstream and upstream sites. Microplastics were associated with the ingestion of other debris items

(e.g. sand and wood) and correlated to the ingestion of fish eggs, earthworms, and mollusks, suggesting that sunfish incidentally ingest microplastics during their normal feeding methods. The high frequency of microplastic ingestion suggest that further research is needed to determine the residence time of microplastics within the stomach and gut, potential for food web transfer, and adverse effects on wildlife and ecosystemic health.

Introduction

The United States discards plastic waste at a rate of 29.6 million tons per year (U.S. EPA, 2015). Plastic is a versatile, lightweight, and strong material composed of various elements, such as carbon, hydrogen, oxygen, nitrogen, chlorine, and sulfur and is ideal for a variety of applications in many industries (Andrady, 2011). Early reports of plastic waste in marine systems occurred in the 1960's (Harper and Fowler, 1987; Kenyon and Kridler, 1969) and plastic has now been reported in both freshwater and deep ocean environments (Dris et al., 2015a; Galgani, 2015). A rough estimate predicts that 70-80% of plastic-based marine litter originates from inland sources and is transported by rivers to oceans (Wagner et al., 2014). Potential sources include wastewater treatment plants, cargo shipping, human litter from beaches, and fisheries (Wagner et al., 2014). While most marine studies assign inland waters as the most realistic sources, the proportional contribution of various point and non-point sources have not been established at either the regional level for the Gulf of Mexico or for marine systems as a whole (Thiel et al., 2013).

Primary microplastics are plastics manufactured at a microscopic scale (i.e. <5 mm) and used in products such as facial cleansers, boat cleaners, and drug vectors.

Secondary microplastics form from the prolonged mechanical, photolytic, or chemical degradation of primary macroplastics and often result in fragmented pieces or fibers (Mathalon and Hill, 2014). To date, sources of microplastics in freshwater systems have not been fully characterized. Due to the variation in physicochemical properties for the different types of plastics (e.g. specific gravity, molecular weight, functional groups), the difficulties of developing accurate detection and quantification methods, and the variation in transport pathways; the relative availability of microplastics in freshwater is largely unknown (Eerkes-Medrano et al., 2015).

Studies of plastic contamination have reported microplastics within freshwater rivers (Dris et al., 2015b; Klein et al., 2015; Moore et al., 2011), lakes (Eriksen et al., 2013; Free et al., 2014), and shoreline sediments (Zbyszewski and Corcoran, 2011). Field studies on microplastic interaction with fish are mainly the result of marine system studies and indicate an occurrence of ingestion ranging from 2.6% to 36.5% (Foekema et al., 2013; Lusher et al., 2013; Ramos et al., 2012; Romeo et al., 2015). Freshwater system studies inclusive of microplastic ingestion by fish are limited. One study by Sanchez et al. (2014) reports a 12% occurrence of microplastic ingestion within wild gudgeons (*Gobio gobio*) from French rivers.

This study defines microplastics as plastics, artificial polymers (e.g. polyester or Nylon), and manufactured products, that range in size from 50 to 5000 mm (Masura et al., 2015). The aim of this work is two-fold; first, to examine a sentinel taxon (i.e. the sunfish Centrarchidae) for microplastic ingestion; and second, to evaluate the influence of urbanization on microplastic ingestion. This research compared the frequency of microplastic ingestion by two species of sunfish, bluegill (*Lepomis macrochirus*) and

longear (*Lepomis megalotis*), collected from 14 geographic sites representing upstream, downstream, and urban areas within the Central Brazos River Basin, Texas.

Methods

The Study Region and Selection of Sampling Sites

The Brazos River watershed originates in the Texas panhandle and reaches the Gulf of Mexico at Freeport, Texas, southwest of Houston (Fig. 3.1). Three major tributaries: the Salt Fork, the Double Mountain Fork, and the Clear Fork of the Brazos River, converge west of Dallas-Fort Worth to form the Brazos River Basin. The basin has a contributing drainage area of approximately 109,000 km^2 (Brazos River Basin and Bay Expert Science Team BBEST, 2012).

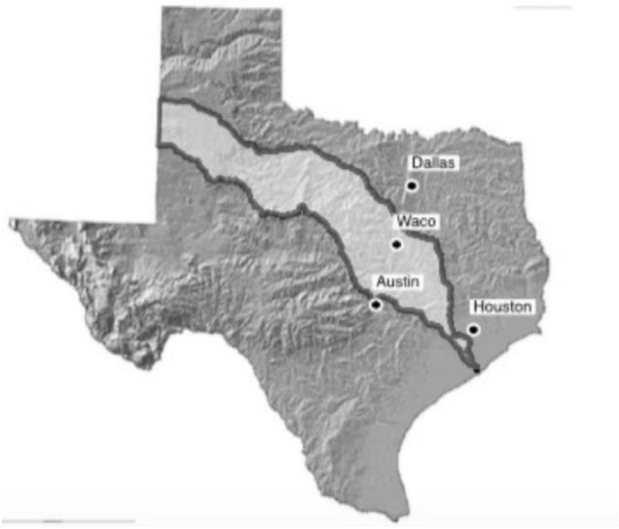


Figure 3.1: Map of the Brazos River Basin. Major cities are noted within the figure. Map shows the topography across the state of Texas. Dark line represents the boundary of the Brazos River Basin.

In order to provide an array of conditions relative to the position of urban areas and the structure of reservoirs, sampling sites incorporated a variety of areas along the Brazos River. The immediate land use around the sample areas includes natural forested river- banks and wetlands, mowed lawns, docks with boat ramps, marinas, paved roads, and parking areas (Table 3.1). True color remote sensed imagery was utilized from Google Earth to create 40,000 m^2 land plots (200 × 200 m) and a set of three 1000 m transect lines associated with each sample location. Land plots centered on the sampling site and the nearest point on the river and extended directly landward. Transects were placed 100 m apart, centered on the sampling site, and extended directly landward. Land use, associated with sample site, was categorized into the following divisions: park, road, development, dock, pasture, plow field, forest/wetland, and manmade structure.

Species Examined in the Study

The Brazos River Basin sustains a variety of sunfish species, such as bluegill (*L. macrochirus*), longear (*L. megalotis*), green (*L. cynellus*), and redear (*L. microlophus*) (Armstrong, 1998). Bluegill and longear sunfish served as the study specimens and are found throughout Central Texas freshwater systems and reside within streams, ponds, and reservoirs (Texas Parks and Wildlife, 2015). Both study species forage throughout the entirety of the water column and utilize methods, such as suction feeding, to capture prey (Mecozzi, 2008; Rider and Margraf, 1998). Bluegill and longear sunfish were chosen as the study specimens because of their abundance throughout the study area, accessibility for collection, and position within the food chain. Sunfish, as a sentinel species, can be used as an indication of ecosystem health and offer insight into the potential impacts of microplastic ingestion on other organisms.

Table 3.1: Sample site land use characteristics

Site name	Same collection location	Geography/Distribution	Development
1. Lake Waco South	Dock	Upstream (Bosque River)	Park, parking area, dock
2.Lake Waco North	Riverbank	Upstream (Bosque River)	Park, parking area
3.Lake Waco Marina	Riverbank	Upstream (Bosque River)	Marina, multiple docks & houseboats
4.Thornton Farm Pond	Riverbank	Upstream (Navasota Watershed)	Pasture, livestock grazing
5.Lake Whitney North	Riverbank	Upstream (Brazos River)	Private resort, parking, multiple docks
6.Lake Whitney Marina	Dock	Upstream (Lake Whitney)	Marina, multiple docks, parking area
7.South of Lake Whitney Dam	Riverbank	Upstream (Brazos River)	Farmland, forest
8.Bosque River Suburban	Riverbank	Urban (Bosque River)	Boat ramp, parking area, athletic fields
9.Bosquee-Brazos Confluence	Riverbank	Urban (Brazos Bosque Confluence)	Park, parking area, picnic shelters, dock
10.Suburban	Dock	Urban (Brazos River)	Park, parking area
11.Waco center	Dock	Urban (Brazos River)	Park, parking area, stone dock
12.Waco (Baylor Marina)	Dock	Urban (Brazos River)	Marina, multiple docks, parking area, athletic fields
13.Below Low Water Dam	Riverbank	Downstream (Brazos River)	Highway overpass
14.Falls of the Brazos	Dock	Downstream (Brazos River)	Park, parking area, dam

Sample Collection and Laboratory Analysis

Between 21 March 2014 and 25 July 2014, 318 bluegill and 118 longear sunfish were collected from 14 sample locations using hook-and-line and cast nets (RS-750 Series, Fitec, Memphis, TN). In the field, samples were taken directly from a riverbank or dock (Table 3.1), thus samples were collected in shallow water generally between 50 cm and 5 m in depth. Upon capture, fish were immediately euthanized via pithing and cutting through the spinal column. Animal use was in accordance with the American Veterinary Medical Association guidelines on euthanasia and was approved by the Baylor University Institutional Animal Care and Use Committee. Sunfish were placed into sealed freezer

bags, labeled with location, temperature, date, time, and capture method, and transferred in to a -4 °C freezer for storage. In the laboratory, each fish sample was defrosted, weighed, measured, and grouped into a length class based on three major size categories: ≤10 cm (n = 91), 10.1-13.9 cm (n = 203), and ≥14.0 cm (n = 142). Stomach contents were also removed, weighed, and stored in glass vials containing 70% ethanol. Stomach contents were washed with distilled deionized water through four filters, 1000 mm, 243 mm, 118 mm, and 53 mm (Wildco Supply Company, Yulee, FL). This process resulted in the separation of individual ingested items into unique size populations. During this process, each filter was visually inspected for laboratory dust or filter particle contamination under a dissecting microscope. Resultant stomach contents were examined using a stereomicroscope with 10X oculars (Motic, DMW 143, VWR). Contents were separated and categorized as organic (i.e. biological) or inorganic (i.e. manufactured). Items determined to be inorganic and on the micro scale consisted of a variety of materials. These materials were collectively classified as microplastic and manufactured materials because of the variation in functional groups and physiochemical properties and included items such as woven or dyed natural to reconstituted materials, manufactured materials coated in plastic, materials developed from mixtures of manufactured products, and chemically treated materials. Items classified as microplastic were then characterized using the following criteria: the number of microplastic items residing within each stomach, form (e.g. thread, sphere, or block) (Fig. 3.2A and 3.2B), and color (e.g. red, blue, gray, or black). Specific to the thread form, the microplastic was found to be either intact or frayed (Fig. 3.2C). Microplastic fray was characterized as the wear or breakdown of the thread.

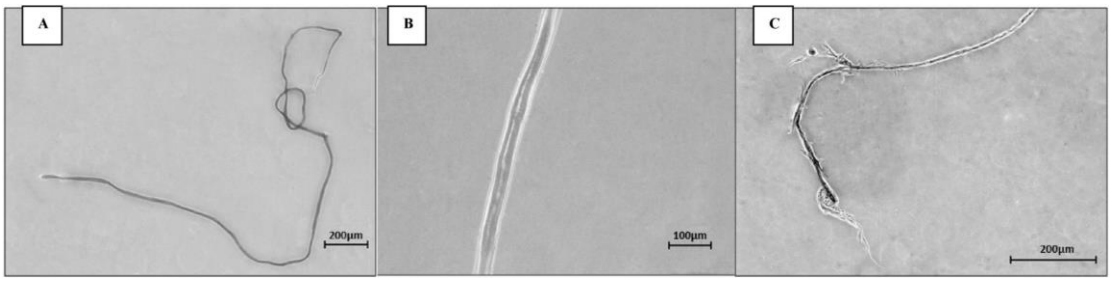


Figure 3.2: Photographs of microplastic threads collected from the stomach contents of the sunfish.

Statistical Analysis

Statistical analysis utilized IBM SPSS Statistics for Windows, Version 22.0, at a significance level of 0.05 to examine differences between microplastic frequency within the fish population related to the sample site. Linear correlations were calculated via the Pearson method and Kruskal-Wallis was used for non-parametric tests. To identify major categories of ingested items, Pisces software (Community Analysis III Package) was used for multivariate analysis. This analysis included Principal Components Analysis and Agglomerative Clustering via Wards method, utilizing Whittakers Index as the distance measure. Specific to microplastic color classification, microplastics were separated into six major color categories; red, blue, gray, black, tan, and green. All additional colors were consolidated into a category classified as "other".

Results

Size and Weight of Fish

Four hundred and thirty-six (436) sunfish were analyzed, inclusive of 318 bluegill and 118 longear (Table 3.2). One hundred and ninety-six (196) fish stomachs (45% of the total 436 fish) contained ingested microplastics (the term microplastic is inclusive of all

manufactured materials). Correlation coefficients (cc) based on the combined sample indicated that the length of the fish was significantly correlated to the number of microplastics found (cc = 0.112, p = 0.019), while the weight of the fish was not (cc=0.068, p = 0.154), despite the expected strong positive correlation be- tween length and weight (cc = 0.928, p = 0.000). Stomach weight was also significantly correlated to the number of microplastics ingested (cc = 0.125, p = 0.011). The capture method and sunfish species influenced the mean size of the fish. Cast nets captured smaller fish (mean = 10.24 cm) and hook-and-line captured larger fish (mean = 13.87 cm, p = 0.000). Longear sunfish were most often captured with cast nets (55.9% of the total), while bluegill sunfish were more often caught with hook-and-line (68.9% of the total). Overall, cast nets were responsible for 96% of fish captured from downstream locales, 44% of fish from upstream locales, and 10% of fish from urban locales.

Table 3.2: Mean values and range of fish length and weight for each species (*Lepomis macrochirus; Lepomis megalotis*). The number of stomachs containing microplastics is also reported.

Species	L. macrochirus	L. megalotis
Number of stomachs examined	318	118
Mean fish length ± SD (cm)	13.3 ± 2.7	10.5 ± 2.2
Length range (cm)	7.6 ± 20.7	7 ± 16.8
Mean fish weight ± SD (g)	54.2 ± 32.8	28.1 ± 18.0
Weight range (g)	7.8 ± 174.6	7.3 ± 100
Number of stomachs containing microplastics	144	52
Mean stomach weight ± SD (g)	0.93 ± 0.92	0.38 ± 0.31

Microplastic Ingestion Relative to the Urban Areas

Frequency of microplastic ingestion (f_{mp}) differed significantly among sample sites (p = 0.000). Waco Center had the highest frequency of microplastic ingestion (f_{mp} = 75%) and mean number of particles per fish (1.63), while Lake Waco South had

the lowest frequency of ingestion (f_{mp} = 19%) and mean number of particles per fish (0.19) (Fig. 3.3).

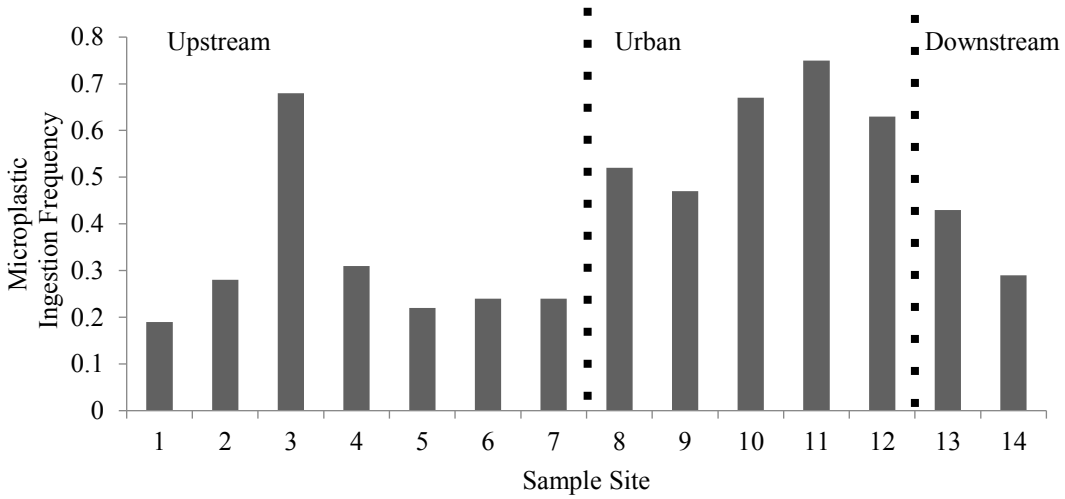


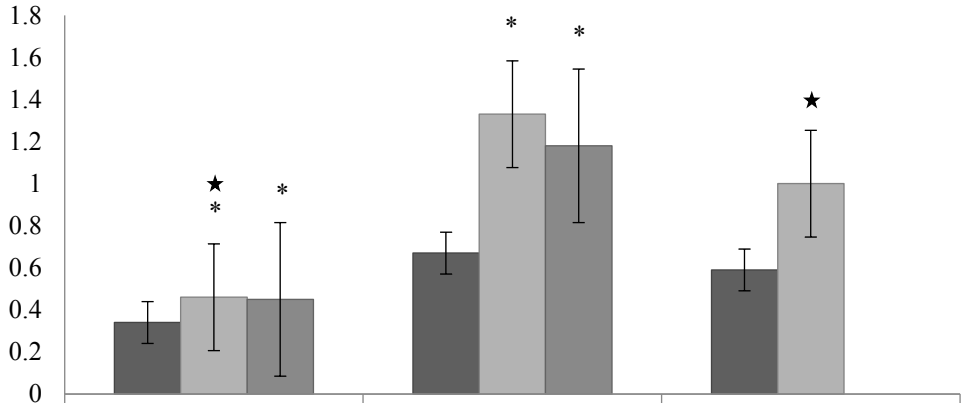
Figure 3.3: Frequency of ingested microplastics at each sample site. The frequency of ingestion ranged 19%-75% in the samples used in the study. See Table 3.1 for additional information on each sample site.

The mean number of microplastics per fish differed significantly between upstream and urban areas for the 10.1-13.9 cm sunfish size class (p = 0.000) and the ≥14 cm sunfish size class (p = 0.000), while there was no significant difference in the ≤10 cm sunfish size class (p = 0.078) (Table 3.3 and Fig. 3.4). There was also no significant difference in the mean number of microplastics between downstream and urban areas for either comparable size class ≤10 cm (p = 0.170) and 10.1-13.9 cm (p = 0.706), while there was no comparison for the ≥14 cm size class. Mean microplastic ingestion differed significantly between the upstream and downstream areas for the 10.1-13.9 cm size class (p=0.037), while there was no significate difference in the ≤10cm size class (p=0.745) and no comparison in the ≥14 cm size class (Table 3.3 and Figure 3.4). Overall, the greatest difference in the mean number of microplastics ingested occurred within the

10.1-13.9 cm size class between the upstream (0.46), urban (1.33), and downstream (1.00) areas (Figure 3.4).

Table 3.3: Mann-Whitney U values comparing samples upstream and downstream of the urban area of Waco by fish size class.

Size Class	Upstream-Urban	Downstream-Urban	Upstream-Downstream
<= 10 cm	z=-1.76, p =0.078	z= -1.37, p =0.170	z=-0.326, p=0.745
10.1 cm to 13.9	z=-4.69, p=0.000	z=-.378, p= 0.706	z=-2.08, p=0.037
=>14 cm	z=-3.95, p=0.000	No comparison	No comparison



	Upstream	Urban	Downstream
<=10	0.34	0.67	0.59
10.1-13.9	0.46	1.33	1
>=14	0.45	1.18	No Comparison

Figure 3.4: Mean number of microplastics per sunfish, by sample site and size class distribution.

Correlation of Microplastics and Land Use

The mean number of microplastics ingested positively correlated with the area of major roadways located within 40,000 m plots ($p = 0.029$, $r^2 = 0.338$) and the area of major roadways located along 1000 m transects ($p = 0.010$, $r^2 = 0.440$) (Fig. 3.5). The mean number of microplastics ingested was not correlated with any other land use category in either sample plots or transects. Frequency of microplastic ingestion was

positively correlated with the area of lawn located within 40,000 m sample plots (cc = 0.650, p = 0.012), but was not correlated with any other land use category in either sample plots or transects.

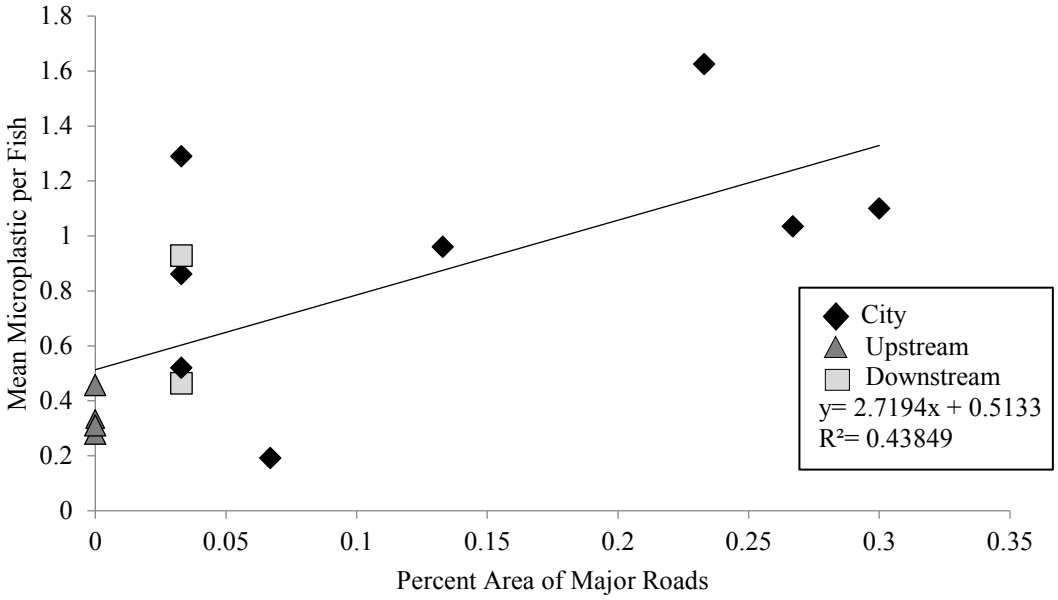


Figure 3.5: Linear regression of mean microplastics per sunfish versus the percent area of major roads located within 40,000 m² plots adjacent to each sample site. The regression line includes all data points in the graph and closely fits with the urban data points.

Microplastic Characteristics

Three hundred and forty-nine items were identified. Four percent (4%) of items sampled were debris on the macro size scale (i.e. >5 mm) and consisted of masses of plastic, Styrofoam, or fishing material, while 96% of items sampled were in the form of threads. Gray and blue were the most common microplastic colors collected, comprising 79.1% of the total sample (Fig. 3.6). Microplastic color was associated with the distribution of sample sites ($X^2 = 23.93$, $p = 0.021$). Microplastics from urban sites had the largest distribution of color (7), followed by upstream (6), and downstream (5) sites.

Microplastic color was not significantly associated with fish size or length, stomach weight, microplastic body fray, or microplastic end fray.

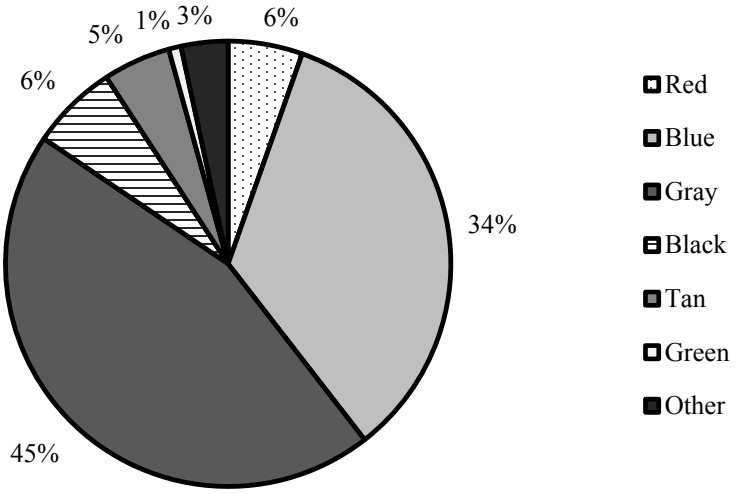


Figure 3.6: Distribution of microplastic color compared to the total of all samples analyzed. Gray and blue were the most common colors found, while green was the least common color found in the samples.

Thirty-seven percent (37%) of microplastics displayed body fray. Microplastic body fray was associated with urban sites ($p= 0.044$) but was not associated with upstream or downstream areas. Overall, microplastics from samples collected from urban sites had the lowest percentage of body fray (33%) in comparison to upstream (41%) and downstream (51%) sites. Microplastic end fray was not associated with the distribution of sample sites; but urban and upstream sites had a lower percentage of end fray (61% and 60%, respectively) when compared to downstream sites (70%).

Correlation of Microplastics and Organic Food Items

Microplastic ingestion classified with the ingestion of other debris, such as sand and wood, but did not classify with the ingestion of high-nutrient organic food items (Fig. 3.7). Of the twenty-six organic items sampled, the ingestion of microplastics only significantly correlated with the ingestion of fish eggs (cc = 0.195, p = 0.000), vegetation (cc = 0.108, p = 0.025), earthworms (cc = 0.147, p = 0.000), and mollusks (cc = 0.103, p = 0.030). In addition, microplastic ingestion did not correlate with the distribution of organic food items grouped by probable location within the water column (Table 3.4).

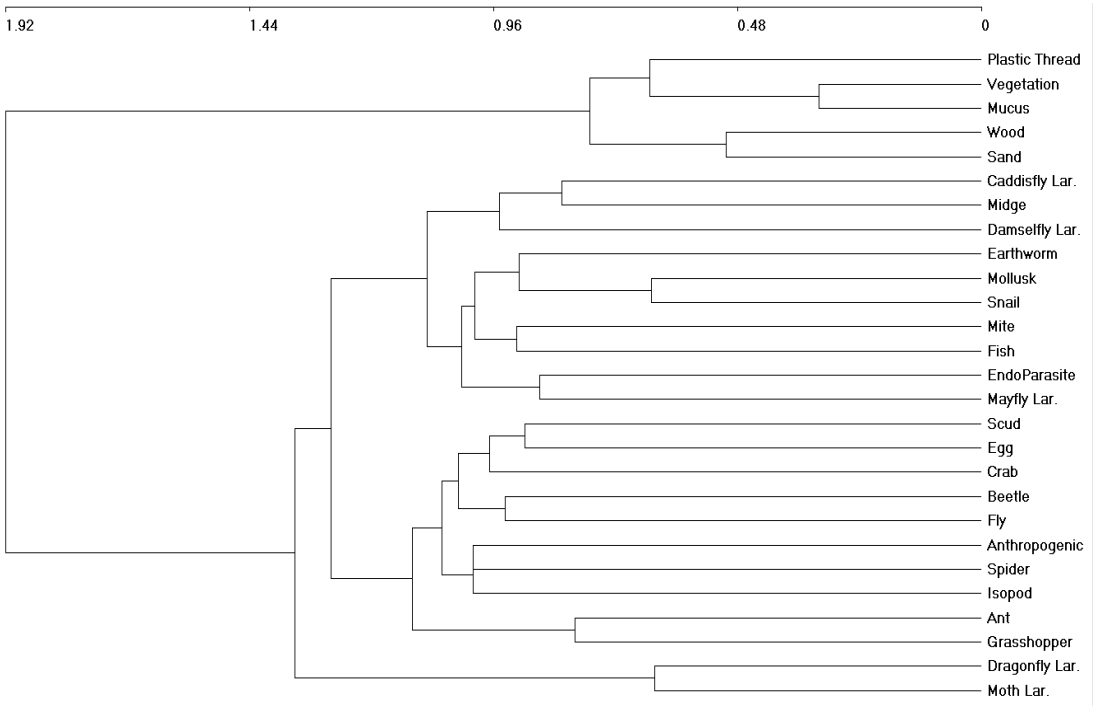


Figure 3.7: Cluster analysis of the sunfish ingested items. Data was clustered based on group average of ranked Whittakers Indices. *Note that the term "anthropogenic" refers to the macroplastic items discovered within the fish stomach content.

Table 3.4: Pearson analyses using the Decorana Axes to correlate microplastic ingestion frequency versus location of sunfish ingested items within the water column.

		Surface to Benthos	Land to Water
Number Thread	Pearson Correlation	-0.081	-0.014
	Sig	0.095	0.775
	N	425	425

Discussion

Forty-five percent of the sunfish sampled had ingested microplastics. This result is significantly higher than similar freshwater (Sanchez et al., 2014), estuarine (Dantas et al., 2012; Ramos et al., 2012), and marine studies (Foekema et al., 2013; Lusher et al., 2013; Possatto et al., 2011; Romeo et al., 2015). When separated into size classes relative to the distribution of sample sites, fish collected from urban areas averaged the highest mean occurrence of microplastic ingestion. A hypothesis by Browne et al. (2011), suggests that washing machine effluent, via sewage sludge, is a major contributor of microplastics to marine systems. However, as all five of the urban sites are located upstream of the Waco sewage effluent, the elevated levels of ingestion suggest that non-point source pollution in the form of illegal trash disposal, urban runoff, and aerial transport, may be major contributors to the availability of microplastics within this fluvial ecosystem.

One potential source of non-point source pollution is the area of major roadways adjacent to each sample site. Roadways act as vectors for non-point source pollution by allowing the accumulation of debris due to illegal trash disposal and wear-and-tear of vehicles. Debris is then released into the environment, via surface runoff, during storm events. In 2013, the Texas Department of Transportation conducted two surveys documenting the most prevalent types of waste on Texas roadways. Plastic waste ranked

in the top three most common types of litter sampled and comprised 17% of total litter sampled (Environmental Resources Planning, 2013). In addition, waste was classified as visible litter (greater than 12.9 cm^2) and micro-litter (less than 12.9 cm^2) with micro-litter comprising 71% of total litter sampled. The volume of illegally dumped waste, entering the fluvial system around the City of Waco, Texas, has become such a serious esthetic and water quality concern, that the City of Waco has purchased a workboat specifically to clean-up trash and woody debris accumulating along the riverbanks of the Brazos, following major precipitation events.

Surface runoff, via roadways, could also be a contributing factor to the reported microplastic characteristic results. Gray and blue were the most common microplastic colors identified. While changes in microplastic color are not fully understood, the high percentage of gray and blue and consistent form (thread) of microplastics sampled, suggests that ingested microplastics originate from a similar source. Microplastics from urban sites displayed the greatest color diversity and the lowest percentage of body fray, in comparison to upstream and downstream sites. Microplastics from urban sites also displayed a lower percentage of end fray in comparison to downstream sites. These findings suggest that urban areas contribute a greater variety of relatively un-weathered anthropogenic materials into the watershed.

This study reported similar microplastic characteristic findings as previous studies on marine (Foekema et al., 2013; Lusher et al., 2013; Possatto et al., 2011; Romeo et al., 2015), estuarine (Dantas et al., 2012; Ramos et al., 2012) and freshwater (Sanchez et al., 2014) fish. While these studies are all similar, there were differences in the species sampled and methodologies utilized for laboratory analysis. Dantas et al. (2012), Possatto

et al. (2011), Ramos et al. (2012), and Romeo et al. (2015), analyzed all stomach contents and collectively reported an occurrence of ingestion ranging from 7.9 to 33%. Lusher et al. (2013) analyzed the content of the entire gastrointestinal tract and reported a 36.5% occurrence of microplastic ingestion, which is more similar to the results in this analysis. Foekema et al. (2013) examined the esophagus, stomach, and intestines, and reported the presence of microplastics in 2.6% of the fish examined. Sanchez et al. (2014) examined the digestive tract and reported 12% of specimens examined were contaminated by microplastics. Aside from a difference in the amount of gut content analyzed, the greatest difference in methods was due to this study's use of a gut content 4-staged filtration method. By separating gut content into four distinct size classes, we found an improvement in both microplastic detection and analysis efficiency. Possible factors contributing to the variation in results also include the difference in species, feeding methods, sample site locations, and levels of anthropogenic disturbance within the systems.

Sunfish, as suction feeders and omnivores, opportunistically ingest prey and other items throughout the entirety of the water column. While stomach content analysis revealed over 25 commonly found organic items, microplastic ingestion only classified with the ingestion of debris items that are not essential to fish survival. Debris items (e.g. wood, vegetation, and sand) are most likely ingested simultaneously with high-nutrient organic items (e.g. insect larvae). In addition, microplastic ingestion correlated with the ingestion of eggs, earthworms, and mollusks all of which are high-nutrient organic food items. We suggest that items which are sticky or structurally intricate entrap microplastics, causing them to adhere to or entangle

within the items. Sunfish then incidentally ingest microplastics during their normal feeding methods.

Field observations of anthropogenic debris within the river, taken during sample collection, indicated that a high percentage of macroplastic debris was polystyrene. Small fragments of polystyrene cups and containers were also common along the riverbanks and in sediments. Despite this, only one fish had ingested an obvious block of polystyrene and macro-debris was rare in sunfish stomachs. Further research is needed to determine the specific composition of the manufactured fibers examined. These fibers include materials such as woven or dyed natural or reconstituted materials, natural fibers coated in plastic, materials developed from blends of agricultural and petroleum products, and chemically treated materials. Manufactured blends may include cotton- polyester, cotton-elastane (Spandex), or cotton blended with viscose rayon, which is semi-synthetic and formed of reconstituted cellulose from bamboo, sugar cane, or other agricultural sources. In addition, some fibers are from a single synthetic source, such as monofilament fishing line. Sunfish do not appear to be ingesting plastics, artificial polymers, and manufactured items relative to their proportional weight or volume in the Brazos River, but instead relative to the shape and size of the anthropogenic debris. It is possible that sunfish are able to reject macro-debris which are unpalatable, while fibers, which adhere to or are entangled throughout organic food items, are not recognized.

Conclusions

The present study examines and confirms the ingestion of microplastics by bluegill and longear sunfish within the Brazos River Basin, Central Texas. Microplastic ingestion occurs at significant levels and is greater within fish from urban areas in

comparison to upstream and downstream sites. Human development (in the form of roadways) and local urbanization are two possible factors influencing the occurrence of microplastic ingestion by sunfish. Sunfish averaged greater levels of microplastic ingestion in comparison to similar freshwater, estuarine, and marine studies (Dantas et al., 2012; Foekema et al., 2013; Lusher et al., 2013; Possatto et al., 2011; Ramos et al., 2012; Romeo et al., 2015; Sanchez et al., 2014). Microplastic ingestion was associated with the ingestion of debris items and correlated to the ingestion of fish eggs, earthworms, and mollusks, suggesting that sunfish accidentally ingest microplastics during their normal feeding methods. The incidental ingestion of microplastics has also been reported in pelagic fish (Ramos et al., 2012), marine catfish (Possatto et al., 2011), and estuarine drums (Dantas et al., 2012). It is probable that the occurrence of microplastic ingestion is influenced by species, feeding methods, location, level of anthropogenic disturbance, presence of point-source and non-point source pollution, and local urbanization. The overall high frequency of microplastics suggests that further research is needed to determine the residence time of microplastics within the stomach and gut, potential for food web transfer, and adverse effects on wildlife and ecosystemic health.

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CHAPTER FOUR

Foraging Preferences Influence Microplastic Ingestion by Six Marine Fish Species from the Texas Gulf Coast

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Abstract

This study evaluated the influence of foraging preferences on microplastic ingestion by six marine fish species from the Texas Gulf Coast. A total of 1,381 fish were analyzed and 42.4% contained ingested microplastic, inclusive of fiber (86.4%), microbead (12.9% %), and fragment (<1.0%) forms. Despite a substantial overlap in diet, ordination of ingested prey items clustered samples into distinctive species groupings, reflective of the foraging gradient among species. *Orthopristis chrysoptera* displayed the lowest overall frequency of microplastic ingestion and the most distinctive ordination grouping, indicating their selective invertebrate foraging preferences. Cluster analysis of *O. chrysoptera* most closely classified microplastic with the ingestion of benthic invertebrates, whereas the ingestion of microplastic by all other species most closely classified with the ingestion of vegetation and shrimp. *O. chrysoptera*, as selective invertebrate foragers, are less likely to ingest microplastics than species exhibiting generalist foraging preferences and methods of prey capture.

Introduction

Plastics are synthetic materials constructed of organic polymers and composed of a variety of elements, such as carbon, oxygen, nitrogen, and sulfur (American Chemistry Council, 2005). The composition and structure of plastic causes it to be lightweight, durable, and cheap, properties that promote its utilization by all sectors of industry. Since its commercial development in the 1930's and 1940's, plastic has become dominant throughout the consumer marketplace, and as its use has continued to increase, so has its presence and impact on the environment (Jambeck et al., 2015).

The improper disposal of plastic traces back to the early 1900's, however, the incidence of plastic pollution does not appear within the scientific literature until the 1960's (Kenyon and Kridler, 1969). Currently, worldwide plastic production exceeds 299 million tons per year, an estimated 22-43% of which ends up in landfills (Gourmelon, 2015). The United States produces approximately 32.5 million tons of plastic annually and discards approximately 29.6 million tons (U.S. EPA, 2015). While the proportion of plastic waste that ultimately ends up in aquatic systems is unknown, estimates predict that 10% of all plastic waste enters the sea each year, 80% of which is attributed to terrestrial based sources (Thompson, 2006; Jambeck et al., 2015).

Once released into aquatic systems, plastic undergoes mechanical, chemical, and photolytic degradation processes, resulting in the formation of secondary microplastics. Microplastics may also be released directly into the environment (i.e. primary microplastics) and include materials such as plastic abrasives utilized for boat cleaners. While the proportion of literature examining microplastic pollution has increased, the variation in the physiochemical properties of different types of plastic has limited the

available knowledge pertaining to the relative availability, transport, and settling of microplastics throughout aquatic systems (Eerkes-Medrano et al., 2015). Despite this, microplastic pollution has been reported in coastal waters (Ng and Obbard, 2006), surface waters (Eriksen et al., 2013), rivers (Moore et al., 2011), estuaries (Sadri and Thompson, 2014), and suspended throughout the water column (Lattin et al., 2004).

In addition to microplastic pollution, studies have also examined the interaction between wildlife and microplastic, resulting in microplastic ingestion. Current studies investigating fish ingestion of microplastic report frequencies of ingestion ranging from 2.6% to 68%, however, these reports vary widely per the species and locations examined and the methodologies utilized for analysis (Lusher et al., 2013; Romeo et al., 2015; Nadal et al., 2016; Possatto et al., 2011; Sanchez et al., 2014; Vendel et al., 2017). Major variations in methodologies include: the organ of examination (i.e. stomach or gastrointestinal tract), the size of filter utilized for the lower bound of -micro categorization, and the inclusion of microplastic fibers reported within the results. Furthermore, there is a lack of research regarding microplastic ingestion by fish species from North America, and very few studies have examined microplastic ingestion by fish from the Gulf of Mexico. Available research includes a study by Peters and Bratton (2016) which reports microplastic ingestion by 45% of freshwater sunfish within the Brazos River basin (a drainage basin into the Gulf of Mexico), and a study by Phillips and Bonner (2015) which reports microplastic ingestion by 8% of freshwater fish from Gulf of Mexico drainage systems and 10% of marine fish from Laguna Madre, a bay system of the Gulf of Mexico.

This study was conducted in response to the limitation of research examining microplastic ingestion by fish from North America and defines microplastics as plastics, artificial polymers (e.g. polyester or nylon), and manufactured products (i.e. manufactured natural and non-natural material), that range in size from 50 to 5000 μm (Masura et al., 2015; Peters and Bratton, 2016). The aims of this study were to examine the occurrence and frequency of microplastic ingestion by six marine fish species from the Texas Gulf Coast, and to evaluate whether ecological factors (i.e. foraging preferences and methods of prey capture) influence microplastic ingestion by species of a shared ecological guild.

Methods

The Study Region and Species

The present study was conducted along the Texas (TX) Gulf Coast, spanning from the Galveston Bay (29.4720° N, 94.7692° W) to Freeport (28.9541° N, 95.3597° W), TX. Local land use includes a variety of natural systems (e.g. barrier island interior wetlands and tidal fringe wetlands), protected state and city parks, and industrialized and urban areas. Water bodies include estuarine and marine environments, which support a variety of commercial and sport fish species. Six fish species were examined for the purposes of this study: Southern kingfish (*Menticirrhus americanus*), Atlantic croaker (*Micropogonias undulates*), Atlantic spadefish (*Chaetodipterus faber*), Sand trout (*Cynoscion arenarius*), pinfish (*Lagodon rhomboids*), and grunt (*Orthopristis chrysoptera*). These species occupy a benthivore ecological guild, whereas they are mainly demersal foragers, and a large proportion of their diet is inclusive of benthic

invertebrates. Variations among species, per foraging preferences and methods of prey capture, include Southern kingfish and Sand trout, which include piscivory within their diet; Atlantic croaker and pinfish which include vegetation within their diet and utilize suction feeding to capture prey; Atlantic spadefish which forage for both benthic and water column invertebrates around manmade structure, and grunt which are selective benthic invertebrate foragers. These fish species were selected for investigation due to their abundance throughout the study area, accessibility for collection, shared ecological guild, and varying foraging preferences and methods of prey capture.

Sample Collection and Laboratory Analysis

Between September 2014 and September 2015, 1,381 fish, inclusive of six species, were collected from seven sample locations. Sample locations were organized from the gulf side of barrier islands, to inlets and passes, and bays behind offshore barrier islands. The collection locations included: 1) the Galveston Beach Front on the Gulf of Mexico; 2) the Surfside Jetty facing the Gulf of Mexico; 3) San Luis Pass, connecting the Gulf of Mexico to west Galveston and Christmas Bays; 4) Pelican Island, facing the Galveston/Houston ship channel connecting the Gulf of Mexico to Galveston and Trinity Bays; 5) the Brazos River estuary, a channelized section of the Brazos river located within Freeport, TX; 6) North Galveston Bay near LaPorte, TX; and 7) Bastrop Bayou, a river fed area surrounded by extensive tidal wetlands, on the landward side of Bastrop Bay. The sample sites located within Galveston, Freeport, and upper Galveston Bay are the most heavily urbanized, while Bastrop Bayou is the most isolated, located just outside of Brazoria National Wildlife Refuge. Specimens were collected via hook and line, from a pier, dock, or shoreline, and were immediately euthanized via pithing and cutting

through the spinal column. Animal use followed the American Veterinary Medical Association guidelines on euthanasia and was approved by the Baylor University Institutional Animal Care and Use Committee. Following euthanasia, fish were placed into sealed freezer bags, labeled with the location, date, and time, and transferred to the laboratory for storage in a -4^{o} C freezer.

All laboratory analysis, including dissection and stomach content separation (minimum bound of filter mesh size: 53µm), replicated the protocol of Peters and Bratton (2016). Following dissection and stomach content separation, all resultant material was categorized as natural or anthropogenic. Natural items were classified into one of eleven general taxonomic and functional prey groups: vegetation, wood, fish, sand, shrimp, crab, mollusk, squid, annelid, midge, and egg. Items determined to be anthropogenic were further characterized, via morphology, into size (i.e. macro and micro), form, and color categories. Microplastic form was comprised of fibers (Figure 4.1 A.), particles slender or elongated in appearance; spheres (microbeads) (Figure 4.1 B.), particles round or ball-like in shape; and fragments, particles angular in appearance (Hidalgo-Ruz et al., 2012). Microplastic color was classified utilizing the Munsell Color System, a universally standardized system categorizing color per hue, value, and chroma. Hue is the dimension which distinguishes between color families, value is the dimension measures the lightness or darkness of color, and chroma is the dimension which measures the intensity of color. Microplastic fibers were classified via hue, value and chroma due to the overall evenness of color throughout the entirety of the particle, while microbeads and fragments were only classified via hue, as color often varied throughout the particle.

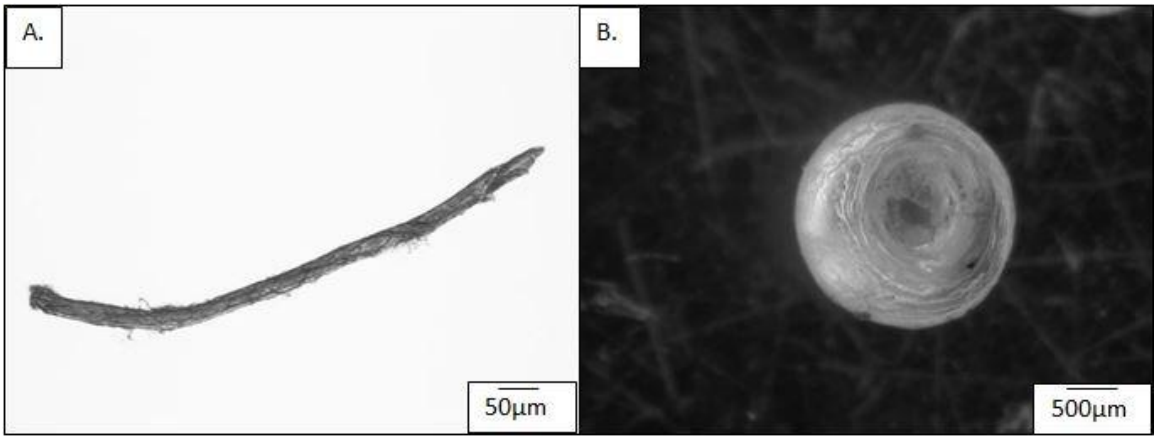

Figure 4.1: Photographs of microplastic fibers (A) and microbeads (B) collected from the stomach content of marine fish

Contamination Control

Measures to avoid contamination were adopted while handling and processing samples. In order to minimize the risk of contamination, stomach content was transferred directly from the sample specimen into glass vials and stored with 70% ethanol. Before analysis, all lab surfaces, tools, and equipment were disinfected, and all filters and instruments were cleaned and examined for contamination under the stereoscope. During analysis, samples were covered at all times except for during analysis under the microscope, at which point samples were exposed to the air. At the start of the study, sample blanks were placed at various points throughout the laboratory in order to determine the risk of possible airborne pollution. Sample blanks revealed negligible levels of contamination, thus airborne contamination was not a risk in accordance with the control measures taken.

Statistical Analysis

Statistical analysis utilized IBM SPSS Statistics for Windows, Version 24.0, at a significance level of 0.05. Chi-square test of homogeneity was utilized to determine if

there was a significant difference in the frequency of microplastic ingestion among species, and pairwise comparisons were performed using a z-test of two proportions with a Bonferroni correction in order to determine where significant differences occurred. Correlations were calculated via Fisher's exact test. Pisces software (Community Analysis V Package) was used for Agglomerative Clustering via Wards method, utilizing Whittakers Index as the distance measure and for DECORANA ordination.

Results

A total of 1,381 fish were analyzed**,** 150 Southern kingfish, 383 Atlantic croaker, 103 Atlantic spadefish, 139 Sand trout, 449 pinfish, and 157 grunt (Table 4.1). Approximately 42% (585 individuals) contained ingested microplastics, averaging 1.93 particles per individual. The average number of particles ingested by the total number of fish sampled (n=1,381) was 0.82 particles per individual. When separated via species, the frequency of microplastic ingestion (f_{mp}) ranged from 26.8% to 46.6% (grunt and Atlantic spadefish, respectively), and there was a significant difference in f_{mp} between grunt (26.8%) and Atlantic croaker (f_{mp}=45.2%, p=0.001), Atlantic spadefish (f_{mp}=46.6%, p=0.023), and pinfish (f_{mp}=46.5%, p=0.000) (Table 4.2).

Table 4.1: Mean values and range of fish length, weight, and stomach weight for all species

Common Name	Number of Stomachs examined	Mean Length (cm) ± SD	Length range (cm)	Mean Weight (g)± SD	Weight range (g)	Mean Stomach Weight (g)± SD
Southern kingfish	150	19.8±3.1	15.0-31.6	96.6±55.3	31.6-400.0	2.1±1.8
Atlantic croaker	383	17.7±2.4	6.8-26.0	75.9±35.0	11.9-270.0	1.3±1.4
Atlantic spadefish	103	13.1±1.9	8.8-20.9	99.4±37.4	33.0-280.0	2.9±1.9
Sand trout	139	20.5±4.4	13.5-31.6	105.2±54.9	38.9-370.0	1.9±1.8
Pinfish	449	14.4±1.9	9.4-20.3	57.3±20.1	15.2-140.8	1.2±1.1
Grunt	157	17.1±2.1	12.0-22.0	76.9±27.6	8.7-168.4	1.1±0.9

Table 4.2: Frequency of microplastic ingestion and average number of particles per species

Common Name	Number of stomachs with plastic	Percentage with plastic	Average plastic per fish ± SD	Average plastic per fish with plastic ± SD
Southern kingfish	53	35.3%	0.57±1.22	1.62±1.58
Atlantic croaker	173	45.2%	0.87±1.47	1.93±1.67
Atlantic spadefish	48	46.6%	1.38±3.84	2.96±5.21
Sand trout	60	43.2%	0.79±1.27	1.83±1.34
Pinfish	209	46.5%	0.96±1.75	2.07±2.07
Grunt	42	26.8%	0.54±2.23	2.00±4.00

Species frequency of microplastic ingestion (f_{mp}) varied by sample site. Of the six sample species, grunt displayed significantly lower frequencies of microplastic ingestion at all sample sites where comparisons were available, except for those collected from Pelican Island and Surfside Jetty (Figure 4.2). Grunt f_{mp} (61.1%) was significantly higher than pinfish (f_{mp}=30.8%) at Surfside Jetty ($p=0.029$) and there was no difference in f_{mp} between grunt, Atlantic croaker, and pinfish (f_{mp}=30.4%, 43.3%, 34.0%, respectively) from Pelican Island ($p=0.373$) (Figure 4.2). The highest mean frequencies of microplastic ingestion for a single sample were 83.3% within Atlantic croaker from San Luis Pass

(followed by 80.9% within Atlantic croaker from Bastrop Bayou) and 76.0% within pinfish from Galveston (followed by 75.0% within pinfish from the Brazos River estuary).

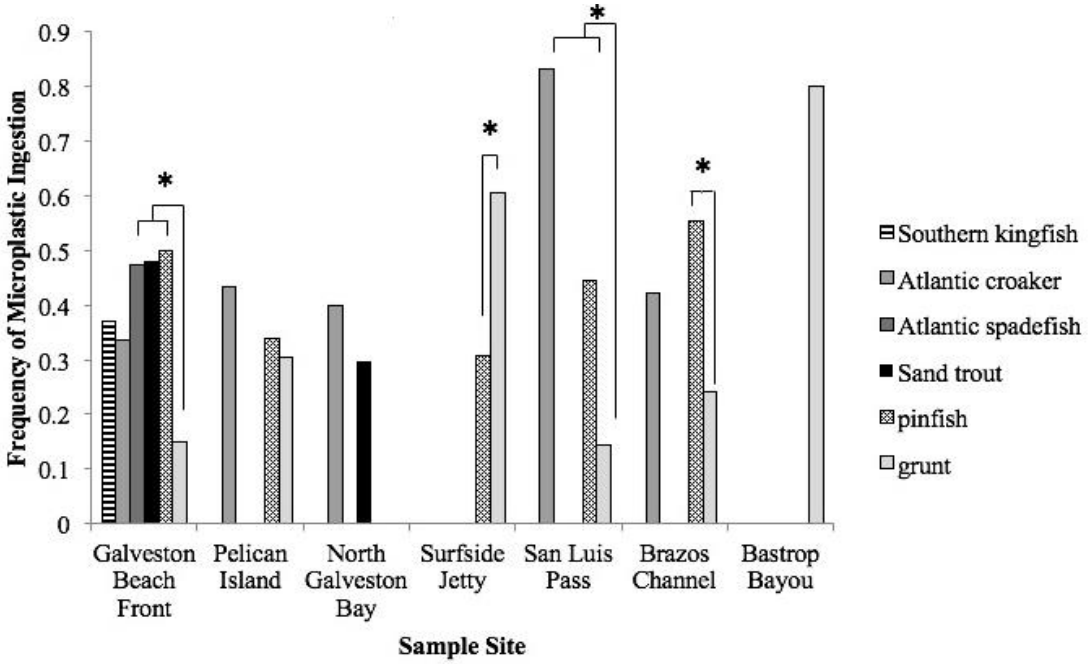

Figure 4.2: Frequency of ingested microplastic per species and sample site (* indicates a significance level of $p<0.05$)

Association of Ingested Microplastics and Prey Groups

In addition to microplastic, fish stomachs contained eleven taxonomic and functional prey groups, which were common for all study species. When separated via species, Southern kingfish, Atlantic spadefish, and grunt displayed no association between ingested microplastics and any other prey group. While ingested microplastics were associated with the ingestion of vegetation ($p=0.036$) by Atlantic croaker; crab ($p=0.014$) by Sand trout; and wood ($p=0.028$) and fish ($p=0.008$) by pinfish. Furthermore, ingested microplastics most closely classified with the ingestion of vegetation and shrimp

for all species, except for grunt, whose ingestion of microplastic most closely classified with the ingestion of mollusk, sand, and crab (Figure 4.3).

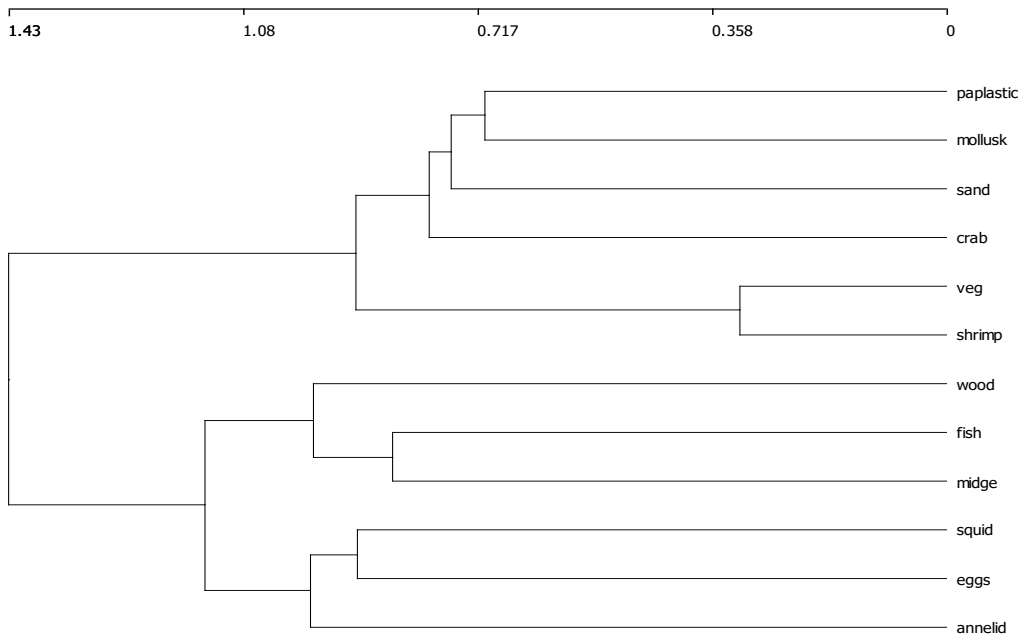

Figure 4.3: Cluster analysis of the combined grunt stomach content, in which the presence of ingested microplastic (paplastic) most closely classified with mollusk, sand, and crab.

Ordination of sample collections (per species, date of collection, and sample site), via the means of ingested food items, clustered collections into distinctive species groups, as opposed to clustering via the date of collection or sample site. Grunt collections clustered into the most distinctive species group, displaying only minimal overlap with pinfish, whereas all other species groups displayed significant overlap with one or more species. Additionally, both axis represent gradients within the data which are reflected by the species groupings. Ordination results suggest that axis one represents the foraging gradient among species, from invertebrate foragers to carnivorous foragers and axis two

represents the microhabitat distribution, from structure and rocky shoreline to the water column and open channel. The overall trend displayed by the species groupings reflects these gradients among species and the variation between species foraging preferences (Figure 4.4).

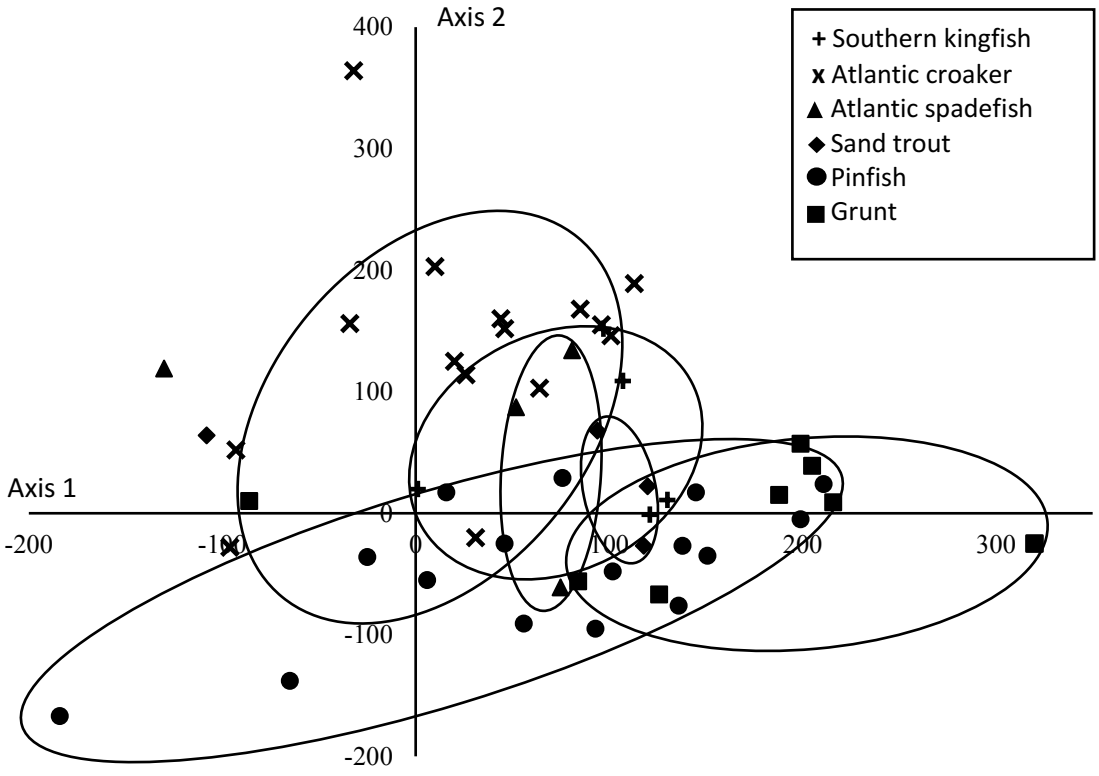

Figure 4.4: Ordination of species samples per the means of ingested food items.

Microplastic Characteristics

A total of 1,141 anthropogenic items were identified. One percent of items were on the macro-size scale (i.e. >5mm) and consisted of fishing materials, while 99% of items were on the micro scale (i.e. <5mm) and included fiber (976), microbead (146), and fragment (8) forms. Fiber form microplastics constituted over eighty percent of ingested particle types for Southern kingfish (97.1%), Atlantic croaker (83.5%), Sand trout

(92.6%) and pinfish (97.1%), and approximately 60% of ingested particle types for Atlantic spadefish (61.8%) and grunt (59.1%). Of the 585 individuals that had ingested microplastics, 41 stomachs contained a total of 146 microbeads. A significant portion of ingested microbeads (33%) were found within the stomach content of two spadefish, while 29.5% of microbeads were found within the stomach content of twenty-two Atlantic croaker, and 25.3% of microbeads within the stomach content of four grunt. Microplastic ingestion ranged from 0-32 particles per fish and was evident in all species. The greatest number of ingested microplastics per individual (32 particles) was observed in Atlantic spadefish, consisting of four microplastic fibers and twenty-eight microbeads. Microplastic fiber color was inclusive of a total of ten hue dimensions, eight value dimensions and eight chroma dimensions, however, the majority of microplastic fibers were categorized as hue purple/blue (35.5%) and purple (23.0%), value 3 (19.5%) and 4 (19.2%), and chroma 2 (27.4%) and 4 (23.9%) (Table 4.3).

Table 4.3: The distribution of microplastic fiber hue (A), value (B), and chroma (C) dimensions.

A. Hue	Percent	B. Value	Percent	C. Chroma	Percent
Red	8.3	2	0.2	2	27.4
Blue	10.9	2.5	14.3	4	23.9
Purple/Blue	35.5	3	19.5	6	15.1
Purple	23	4	19.2	8	19.1
Green	2.7	5	14.1	10	12
Yellow	2.3	6	10.3	12	1.7
Red/Purple	5	7	10.6	14	0.4
Green/Yellow	3.9	8	11.8	16	0.5
Yellow/Red	7.3				
Blue/Green	1.3				

All microbeads (100%) were categorized as hue yellow/red and microplastic fragments were categorized as hue blue (25%), yellow/red (37.5%), green (25.0%), and red/purple (12.5%).

Discussion

Microplastic Ingestion by Fish

The present study confirms the ingestion of microplastic by six marine fish species from the Texas Gulf Coast and examines the influence that species foraging preferences and methods of prey capture have on microplastic ingestion. Forty-two percent of the sample specimens had ingested microplastic, averaging 1.93 particles per fish. Results of similar marine studies have reported frequencies of ingestion ranging from 2.6-68% and particles per individual ranging from 0-3.75+-0.25, thus the results of this study are within the high-end range of multi-species studies reported within the literature (Boerger et al., 2010; Neves et al., 2015; Romeo et al., 2015; Bellas et al., 2016; Nadal et al., 2016). When comparing between species, the frequency of microplastic ingestion ranged from 26.8-46.6% and 0.54-1.38 particles per specimen, which is similar to the results of individual species studies reported within the literature (Choy and Drazen, 2013; Lusher et al., 2013).

The closest comparative study is that by Phillips and Bonner (2015), which reported that 10% of marine fish from Laguna Madre, an estuary of the Gulf of Mexico, had ingested microplastic. The overall frequency of microplastic ingestion reported within the present study is approximately four times greater than the results of Phillips and Bonner (2015), which may be due to variations in methodology or due to Laguna

Madre's unique system characteristic as a negative estuary in terms of salinity gradient. However, this variation in results is most likely due to the greater degree of urbanization and industrialization located around Houston, Galveston, and Freeport, and our examination of fish which occupy a benthivore ecological guild. Furthermore, this study reported maximum frequencies of microplastic ingestion, per species sample, ranging between 75.0% and 83.3%**,** suggesting that Phillips and Bonner's (2015) data may be an underestimate of microplastic ingestion by fish within the most developed areas of the Gulf of Mexico.

Overall, grunt displayed a significantly lower frequency of microplastic ingestion in comparison to pinfish, Atlantic croaker, and Atlantic spadefish. This pattern was further reflected when examining frequencies of microplastic ingestion per sample site, except for grunt collected from Surfside Jetty, which displayed a significantly higher frequency of microplastic ingestion in comparison to pinfish. Overall, the most urbanized sites produced the highest frequencies of microplastic ingestion per sample collection (e.g. pinfish from Galveston beach front, f_{mp}=76.0%), however, the most remote site (Bastrop Bayou) also resulted in one of the highest reports of microplastic ingestion (Atlantic croaker, f_{mp}=80.9%). As the species examined in this study are non-territorial and the retention time of microplastic within the gut is unknown, levels of ingested microplastic cannot be attributed to the site of collection. This was apparent as species collections were not always readily available at each sample site throughout the extent of the study. In order to assign frequencies of ingested microplastic to the sample site, further research is needed to determine the retention time of microplastic within the gut and the average distances traveled for non-territorial species. Additionally, research

examining microplastic loads throughout the system (i.e. water and sediment) would provide a better understanding of the influence that microplastic availability has on the occurrence and frequency of ingestion.

Species Diet

Despite a substantial overlap in diet, sample collections ordinated into distinctive species groups, which was reflective of the foraging gradient among species. Of the six study species, grunt are the most selective foragers, feeding almost exclusively on benthic invertebrates, and subsequently displayed the most distinctive ordination group. In comparison, the other five study species display generalist foraging preferences and methods of prey capture, such as the inclusion of piscivory within species diet (e.g. Southern kingfish and Sand trout), the utilization of suction feeding to capture prey (e.g. pinfish and Atlantic croaker) or foraging throughout benthic and water column habitats around manmade structure (e.g. Atlantic spadefish). The results of the cluster analysis further support the selective foraging patterns displayed by grunt, as grunt ingestion of microplastic most closely classified with the ingestion of crab, sand, and mollusk, while microplastic ingestion by all other species most closely classified with the ingestion of vegetation and shrimp. The results of both the ordination and cluster analysis suggest that grunt, as selective invertebrate foragers, are less susceptible to microplastic ingestion than species which display generalist foraging preferences and methods of prey capture.

Several hypotheses exist as to why fish ingest microplastic. A hypothesis by Romeo et al. (2015) suggests that secondary ingestion, via bioaccumulation, may be a main form of microplastic ingestion by fish. While to the best of our knowledge, there are no studies confirming secondary microplastic ingestion, there are numerous reports

confirming the ingestion of microplastic by common prey species (e.g. invertebrates, *Bogue boops boops*) of larger predatory fish (Nadal et al., 2016; Graham and Thompson, 2009). In contrast, Güven et al. (2017) found that trophic level had no influence on microplastic ingestion by fish, suggesting that bioaccumulation and biomagnification are not main routes of ingestion. Other hypotheses suggest that direct ingestion, via the targeting of microplastic as food or by mistaking it as prey, as a primary route of exposure (Lusher et al., 2013; Boerger et al., 2010; Foekema et al., 2013). However, the purple/blue color categorization of a majority of microplastic fibers suggests that they were not distinctively visible to fish and the frequency and occurrence of microplastic ingestion by all species supports the hypothesis by Peters and Bratton (2016), which suggests that microplastic ingestion is predominantly incidental. This hypothesis explains why, despite the overlap in commonly ingested prey items, grunt displayed a significantly lower frequency of microplastic ingestion in comparison to Atlantic croaker, pinfish, and Atlantic spadefish.

Characterization of Microplastic

Of the 1,141 microplastics recovered, fibers were the most prominent form, followed by microbeads and fragments. The most common microplastic fiber hues were purple/blue and purple, value 3 and 4, and chroma 2 and 4, thus the majority of fibers were darker in color and displayed a less vivid color intensity. The dominance of blue/purple fibers agrees with the findings from planktivorous fish from the North Pacific Central Gyre (Boerger et al., 2010), estuarine drums from the Goiana Estuary (Dantas et al., 2012), fish from the Mediterranean Sea (Romeo et al., 2015; Güven et al., 2017), and sunfish from the Brazos River basin (Peters and Bratton, 2016), however, these studies

classified microplastics into broad color categories and did not further classify color via value and chroma. Possible sources of fiber form microplastics include wastewater treatment plant discharge, sewage sludge, and macroplastic degradation (Lusher et al., 2013; Neves et al., 2015; Ramos et al., 2012; Peters and Bratton, 2016). The overall consistency of microplastic form and color, both of this study and that reported within the literature, suggests that these materials may be originating from a similar manufacturing process, such as bulk fabricated materials, which are produced in the form of sheets, films, or fibers (Bart, 2006). However, further research is needed to determine major sources of microplastic pollution and the main polymer types of microplastics recovered.

Microbeads were the second most prominently ingested microplastic form and were found within every study species. However, a limited number of grunt and Atlantic spadefish were responsible for a large proportion of ingested microbeads, suggesting that there may have been instances of intentional microplastic ingestion, possibly due to the mistaking of microbeads for a similar prey item (e.g. fish eggs). While microbeads are less commonly cited within the microplastic ingestion literature, they have been confirmed as residue of personal care products and it is suggested that they may enter aquatic systems via wastewater discharge (Nalbone, 2015). Microplastic fragments were the least commonly ingested form of microplastic and are likely the result of macroplastic degradation. It is possible that fish are able to identify and expel larger plastic materials, whereas micro-sized particles are not easily identifiable and subsequently incidentally ingested.

Conclusions

This study is one of the first to examine the influence that foraging preferences and methods of prey capture have on microplastic ingestion by species of a shared ecological guild. Results of the study report high frequencies of microplastic ingestion by six marine fish species from the Texas Gulf Coast and suggests that grunt, as selective benthic invertebrate foragers, are less susceptible to microplastic ingestion than species which exhibit generalist foraging preferences and methods of prey capture. The occurrence and high frequency of ingested microplastic reported by this study highlights the ubiquitous nature of this pollutant throughout the Texas Gulf Coast and the importance of targeting microplastics in future pollution control efforts.

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CHAPTER FIVE

A Comparison of Microplastic Ingestion between Freshwater Sunfish and Marine Pinfish from the Brazos River Basin and Nearshore Marine Waters

Introduction

Fish ingestion of microplastic has been confirmed within approximately 150 marine, estuarine, and freshwater species, ranging in frequency from a few percent to over two thirds of the specimens sampled (Jabeen et al., 2017; Nadal et al., 2016; Davison and Asch, 2011). However, a majority of the available research reports on a single species or a limited number of individuals from a wide variety of species, thus statistical comparisons between studies are difficult. Additionally, a large proportion of reports are quantitative, while few studies further investigate ecological or environmental factors which may have an important role in this type of interaction.

Numerous studies have investigated variations in microplastic ingestion by species of differing aquatic zones (e.g. pelagic vs. benthic), such as Lusher et al. (2013), which found no significant difference in microplastic ingestion between demersal and pelagic fish from the English Channel (35% and 38%, respectively). Research has also examined variations in microplastic ingestion per fish distance to shore, confirming higher microplastic concentrations within coastal fish species (45.2-51.1%) in comparison to offshore species (0-10%) (Murphy et al., 2017), and per vertical foraging pattern, reporting lower frequencies of ingestion (4.8%) in non-vertically migrating foragers in comparison to vertically migrating foragers (11.6%) (Davison and Asch, 2011). Despite a recent increase in microplastic ingestion research, there is still a lack of

comprehensive system investigations, such as those examining variations in microplastic ingestion between freshwater and marine species of a shared watershed.

The goal of this research was to compare the microplastic ingestion results of sunfish and pinfish, which serve as ecological analogs between Texas freshwater and marine systems (Peters and Bratton, 2016; Peters et al., 2017). These similarities offer a unique opportunity to investigate differences in the type and frequency of microplastic ingested between freshwater and marine fish from the Brazos River Basin and Texas nearshore marine waters.

Methods

The Study Region and Species

The Brazos River Basin originates in the Texas panhandle and reaches the Gulf of Mexico at Freeport, Texas, southwest of Houston. It supports bluegill (*Lepomis macrochirus*) and longear (*Lepomis megalotis*) sunfish, species which reside within freshwater streams, ponds, and reservoirs (Texas Parks and Wildlife, 2015). Both species are omnivores and forage throughout the entirety of the water column, utilizing methods, such as suction feeding, to capture prey (Mecozzi, 2008; Rider and Margraf, 1998; Armstrong, 1998; Brazos River Basin and Bay Expert Science Team BBEST, 2012). Pinfish (*Lagodon rhomboides*) are a common inshore marine species belonging to the Sparidae family. The diet of juvenile and subadult pinfish primarily consists of shrimp, mysids, amphipods, fish eggs, insect larvae, and bivalves, while adults exhibit an ontogenetic shift and include plant material within their diet (Masterson, 2008).

Sample Collection and Analyses

Bluegill and longear sunfish were collected from March to July 2014, from 14 freshwater sample locations, spanning from Lake Whitney to Falls of the Brazos, TX (Peters and Bratton, 2016). Pinfish were collected from September 2014 to September 2015, from five marine sample locations, spanning from Galveston Bay to Freeport, TX (Peters et al., 2017). Laboratory analysis of the freshwater and marine samples, including dissection and stomach content separation, followed the protocol of Peters and Bratton (2016). Statistical analysis utilized IBM SPSS Statistics for Windows, Version 24.0, at a significance level of 0.05. Pearson chi-squared was utilized to determine if there was a significant difference in the frequency and number of microplastics ingested between the freshwater and marine species.

Results

A total of 885 fish were sampled, 436 bluegill and longear sunfish (318 and 118, respectively) and 449 pinfish. Approximately 46% of fish contained ingested microplastic (45.0% of sunfish and 46.5% of pinfish), which did not significantly differ between species ($p=0.634$). Sunfish were on average smaller than pinfish (i.e. fish length and weight) but displayed a greater range in length and weight (Table 5.1). The combined sunfish sample averaged 0.80 microplastics per individual, which did not significantly differ from pinfish (0.96 microplastics per individual; $p=0.744$). Of the fish that had ingested microplastic, sunfish averaged 1.80 microplastics per individual and pinfish averaged 2.07 microplastics per individual ($p=0.686$). Additionally, pinfish contained a greater maximum number of microplastics per fish (17 microplastics per individual) than sunfish (11 microplastics per individual). Overall, sunfish frequency of microplastic

ingestion ranged from 19-75% and significantly differed between sample site (p=0.000) (See Figure 3.3). Pinfish frequency of microplastic ingestion also significantly differed between sample site (p=0.004), ranging from 30.8%-55.5%. When separated via the month and location of collection, pinfish frequency of microplastic ingestion was closer to that of sunfish, ranging from 18.8%-76.0%, (p=0.000) (Figure 5.1).

Table 5.1: Mean values and ranges for sunfish and pinfish length, weight, and stomach weight

Common Name	Sunfish	Pinfish
Mean Length (cm) ± SD	12.6±2.8	14.4 ± 1.9
Length Range (cm)	7.0-20.7	9.4-20.3
Mean Weight (g) ± SD	47.2±31.7	57.3±20.1
Weight Range (g)	7.3-174.5	15.2-140.8
Stomach Weight (g)	.80±.8	1.2±1.1
Stomach Weight Range	0.1-8.81	0.2-6.0

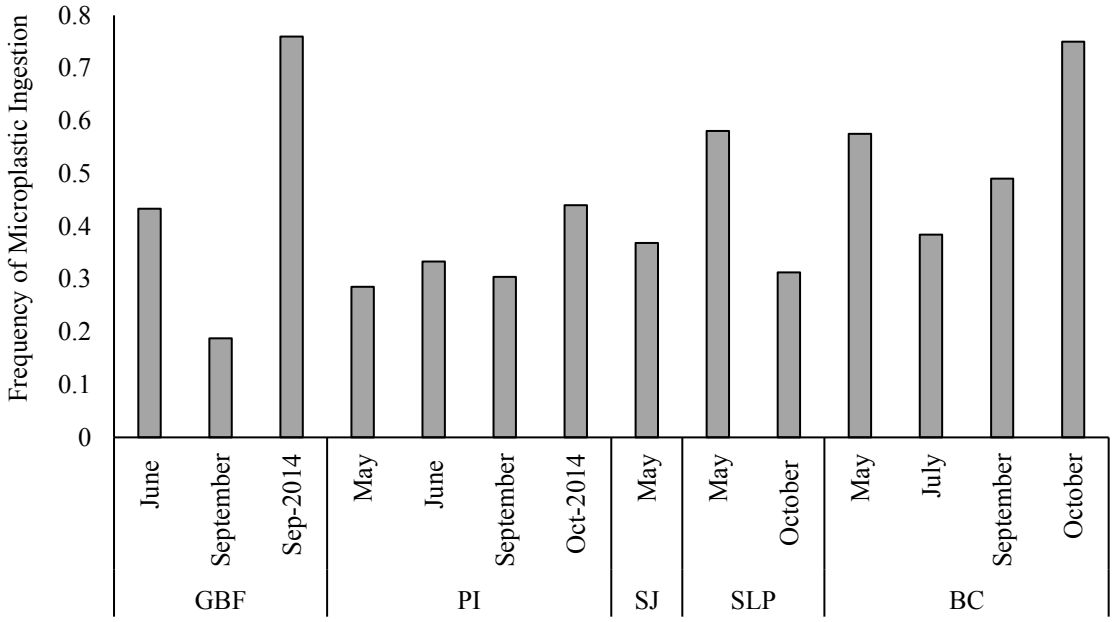

Figure 5.1: The frequency of microplastic ingestion per sample site (i.e. Galveston Beach Front (GBF); Pelican Island (PI); Surfside Jetty (SJ); San Luis Pass (SLP); and Brazos Channel (BC) and month of collection. All samples were collected in 2015 unless labeled as 2014.

A total of 764 anthropogenic items were discovered within the fish stomach content. Sunfish stomach content contained 349 items, 4% of items were on the macro scale (i.e. >5mm in diameter) and consisted of masses of plastic, Styrofoam, or fishing material, and 96% of items were on the micro scale (i.e. <5mm in diameter) and consisted of particles with a fiber morphology. In addition to anthropogenic material, sunfish stomach content contained twenty-six prey groups, of which, microplastics were associated with eggs ($p=0.020$) vegetation ($p=0.025$), earthworms ($p=0.000$) and mollusks ($p=0.030$) (See Figure 3.7). Pinfish stomach content contained a total of 415 items, 100% of which were on the micro scale and consisted of fiber (97.1%), bead (2.0%), and fragment particle morphologies (0.90%). In addition to microplastic, pinfish stomach content contained a total of eleven prey groups, of which, ingested microplastics were associated with wood ($p=0.028$) and fish ($p=0.008$) (See Figure 4.3).

Discussion

Despite the variations between freshwater and marine habitats, there was no significant difference in overall microplastic ingestion between sunfish and pinfish. There was also no significant difference in the mean number of microplastics per fish, despite pinfish containing one individual with approximately 33% more particles than the highest recorded number of microplastics within any sunfish sample. While sunfish were on average smaller than pinfish (i.e. length and weight), they also contained the smallest and largest individuals of the combined species sample.

When separated via sample site, sunfish displayed a greater range in microplastic ingestion frequency than pinfish. Of the combined 19 freshwater and marine sites sampled, the four sites from which samples displayed the highest frequencies of microplastic ingestion were located within the freshwater sample area, 75% of which were located within urban city district of Waco (See Figure 3.3). When further separated via sample site and the month of collection, pinfish displayed a similar range in microplastic ingestion frequency (18.8-76%) to that of sunfish. However, the samples with the highest and lowest frequencies of microplastic ingestion were from the same location, collected one year apart, suggesting that external factors may play an important role in microplastic availability. Brief observations into local climatic events, surrounding the time of sample collection, suggest that weather events (e.g. events resulting in heavy wrack), may increase microplastic availability, likely as a result of increased surface runoff or the resuspension of particles from the sediment.

A similar originating source, such as surface runoff, may contribute to the overall uniform morphology (i.e. fiber) of microplastics collected (97% of the total). All

microplastics within the sunfish stomach content displayed a fiber morphology, while pinfish stomach content contained ingested microplastic fiber, bead, and fragment morphologies. The inclusion of beads and fragments indicates a greater diversity of microplastics within marine waters, likely a result of high levels of local industrialization, waste water effluent, and tourism, all of which may serve as vectors of microplastic pollution into the system.

In addition to the ingestion of microplastic, stomach content analysis revealed twenty-six commonly ingested prey groups within the sunfish samples (See Figure 3.7) and eleven commonly ingested prey groups within the pinfish samples (See Figure 4.3). Despite the sunfish diet encompassing over twice the variety of prey than that of the pinfish diet, both species displayed limited associations between ingested microplastic and other prey items, thus ingestion is likely incidental and occurs during normal foraging behavior. The use of suction feeding may be one factor which enhances the likelihood of microplastic ingestion, especially in areas containing high levels of microplastic pollution.

Conclusions

The present study compares microplastic ingestion between freshwater sunfish and marine pinfish. Both species are similar in body shape and size and occupy a similar ecological guild and niche, thus enabling comparisons of microplastic ingestion between the freshwater and marine habitats. Overall, there was no significant difference in the frequency of microplastic ingestion or the average number of microplastics per fish between the freshwater and marine species sampled. Microplastic fibers were the most common particle morphology ingested by both species, however, pinfish stomach content

also contained beads and fragments. The results indicate that there is a greater variety of microplastics within Texas coastal waters, likely resulting from local land use patterns. Furthermore, despite sunfish stomach content encompassing a greater number of prey groups than pinfish, microplastic ingestion by both species shared limited associations with additional prey items, suggesting incidental microplastic ingestion during normal foraging behavior.

CHAPTER SIX

Pyr-GC/MS Analysis of Microplastics Extracted from the Stomach Content of Benthivore Fish from the Texas Gulf Coast

Introduction

Microplastics are major global contaminants, ubiquitous throughout freshwater and marine systems (Eriksen et al., 2013; Lattin et al., 2004; Moore et al., 2011; Ng and Obbard, 2006; Sadri and Thompson, 2014). Due to their small size (i.e. less than 5mm), it is difficult to predict particle transport following release into aquatic systems, however, microplastics have been discovered within waters from the near shore to open ocean (Eriksen et al., 2014; Kang et al., 2015), from the surface to benthos (Song et al., 2014; Woodall et al., 2014), and from subtropical to polar seas (Law et al., 2010; Obbard et al. 2014). While the environmental impact of these contaminants is not fully understood, microplastic ingestion has been identified within taxa spanning from invertebrates to large marine mammals (Hurley et al., 2017; Taylor et al., 2016).

Fish ingestion of microplastic has been confirmed within freshwater, marine, and estuarine species, ranging from a few percent to more than two-thirds of all fish examined (Lusher et al., 2013; Nadal et al., 2016; Peters and Bratton, 2016; Peters et al., 2017; Possatto et al., 2011; Romeo et al., 2015; Sanchez et al., 2014; Vendel et al., 2017). It is likely that variations in microplastic ingestion are the result of several factors, such as the species of examination, location of collection, methodologies employed for microplastic extraction, and the analytical analyses utilized for polymer identification. Due to the complex nature of these micro-contaminants, it is now becoming standard to

employ two or more identification techniques for the confirmation of plastic. Initial identification routinely involves a physical characterization of the particle (e.g. size, morphology, and color), aided by microscopy, followed by a secondary identification via chemical characterization (e.g. spectroscopy) to identify the specific type of plastic polymer (Shim et al., 2017).

Polymer characterization often employs Fourier transform infrared spectroscopy (FTIR) or Raman spectroscopy (Lenz et al., 2015; Shim et al., 2017). Both techniques use electromagnetic radiation to profile samples, however, FTIR is a measure of the particle's covalent chemical bonds using the absorbance of their vibrational modes while Raman is a measure of the particle molecular structure using light scattering from key vibrational modes after excitation with a visible light source (Käppler et al., 2016; Löder and Gerdts, 2015). While both techniques explore molecular vibrations, the differences in their approach mean that different types of bonds are highlighted by each technique (e.g., discussion in Rubinson and Rubinson, 2000). FTIR spectra highlight polar covalent bonds, while Raman spectra highlight more purely covalent bonds such as carbon to carbon (C-C) or sulfur to sulfur (S-S). FTIR is coupled with varying modes of measure (e.g. micro-FTIR, transmission, reflectance, and attenuated total reflectance), some of which can minimize method limitations such as the requirement of an extensive sample pretreatment and inhibited analysis of plastics which contain irregular surfaces, (Ng and Obbard, 2006; Song et al., 2014). While FTIR can analyze particles as small as 10 μm (the size of the IR beam aperture), particles of this size often require multiple analysis runs or produce unclear results, thus FTIR is most applicable for particles that are greater than 50μm (Shim et al., 2017). Comparatively, the laser aperture utilized in Raman

spectroscopy is smaller than that of FTIR, thus it can identify particles as small as a few μm in size. Raman has been found to be sensitive to additive and pigment chemicals, which are often incorporated during the production phase of plastics, resulting in the interference in polymer identification (Lenz et al., 2015). FTIR may also be challenged by additives and plastic co-polymers; work by Hendrickson et al., 2018 indicates that ATR-FTIR may mask chemical constituents within heterogeneous particles that appear when the particle undergoes pyr-GC/MS analysis.

A third method of polymer identification is the coupling of thermal desorption or pyrolysis with gas chromatography-mass spectrometry or GC/MS (Dümichen et al., 2015; Frias et al., 2013). Pyrolysis-GC/MS (pyr-GC/MS) uses heat in an inert environment (i.e., no oxygen) to decompose polymeric material in a predictable fashion. The pieces of polymer generated can then be separated by gas chromatography on the basis of their size and polarity and analyzed by mass spectrometric detector at the outlet of the gas chromatography column. (Frias et al., 2013). This method yields a total chromatogram (abundance vs time) for the separated pyrolysis products and provides mass spectrometry data throughout the chromatogram as well. These can be compared against a known reference library to determine the specific class of polymer being analyzed. This method is beneficial over FTIR and Raman spectroscopy as it can characterize particles less than 10μg when measured in splitless mode, and the utilization of a thermal analysis combined with GC/MS, enables the separation and analysis of chemical additives as well as the polymer material (Hendrickson et al., 2018). However, pyr-GC/MS is destructive, resulting in the total loss of the particle and subsequently eliminating further particle analysis.

Despite recent advancements in these analytical methods as applied to microplastics, the applicability and feasibility of each is somewhat incomplete due to the wide range of microplastic polymers, including weathered polymers, and additives found throughout the environment. This research serves as one of the first applications of pyr-GC/MS for microplastic polymer identification within a fish ingestion study, and specifically investigates the polymer distribution of microplastic recovered from the stomach content of six marine fish species from the Texas Gulf Coast. The pyr-GC/MS method utilized within this study has previously been applied to the identification of microplastic recovered from the waters of Western Lake Superior (Hendrickson et al., 2018). The use of this method here enabled a comparison of polymer results between freshwater and marine systems and an investigation of method applicability across sample matrixes.

Methods

Microplastics were collected from the stomach content of 1,381 marine fish, inclusive of six species (i.e. southern kingfish (*Menticirrhus americanus*), Atlantic croaker (*Micropogonias undulates*), Atlantic spadefish (*Chaetodipterus faber*), sand trout (*Cynoscion arenarius*), pinfish (*Lagodon rhomboids*), and grunt (*Orthopristis chrysoptera*) from the Texas Gulf Coast (Peters et al., 2017). Fish collection took place from September 2014 to September 2015, and stomach content analysis followed the protocol of Peters and Bratton (2016). Following identification, microplastics were characterized via particle size, morphology, and color (Peters et al., 2017).

Approximately five percent of recovered microplastics were selected and transferred from Baylor to the University of Minnesota Duluth for pyr-GC/MS analysis.

Particle mass was measured via a Mettler Toledo XP2U microbalance and particles <10 μg were measured in splitless introduction into the gas chromatograph, while particles >10 μg were introduced using a 1:100 split (Hendrickson et al., 2018). Samples were analyzed using an Agilent 7890B Gas Chromatograph with Agilent 5977A mass-selective detector (MSD) Mass Spectrometer and Gerstel Pyrolysis/Thermal Desorption Unit (Gerstel GmbH & Co. KG, Germany). All pyrolyzer and GC unit parameters adhered to the protocol of Hendrickson et al. (2018). The MSD utilized electron impact (EI^+, 70eV) for the ionization source and scanned for ions from m/z 10-550 (Hendrickson et al., 2018).

Following analysis, ion chromatograms were assessed with the National Institute of Standards and Technology (NIST) mass spectra library (Version 2.0, 12/4/12, available through the mass spectrometer's software package) and the following standards: medium-density polyethylene (MDPE, catalog #: EV306010), polystyrene (PS, catalog #: ST316051), polyvinyl chloride (PVC, catalog #: CV316010), and polyethylene terephthalate (PET, catalog #: ES306030), all in powder form (250-350 μm) (Goodfellow, Inc.). Samples which yielded a low number of pyrolytic products (<4 total) or low pyrolytic product abundances were evaluated via Mass Hunter qualitative analysis software which was utilized to integrate total ion chromatogram peak areas and calculate a 3:1 signal-to-noise ratio (Hendrickson et al., 2018).

Results

A total of 43 microplastic samples were analyzed, inclusive of 30 fibers, 3 fragments, and 10 spheres (microbeads). Particles were identified into the following five polymer classes: PVC (Figure 6.1; Table 6.1) and PET, constituting approximately 44.1%

of the total sample, silicone (2.3%), epoxy resin (2.3%), and nylon (9.3%) (Figure 6.2). Half of the nylon particles were further classified as Nylon 6 due to the high abundance of caprolactam within the pyrogram results (Lehrle et al., 2000). In addition to the five polymer classes, approximately 42% of particles were classified as sample unknowns, 21% of which displayed a similar chromatogram result inclusive of seven common pyrolytic products (i.e. Unknown Subsample A) (Figure 6.3; Table 6.3).

PVC polymers were inclusive of microplastic fibers (73.3%), fragments (20.0%), and spheres (6.7%), while all PET, epoxy resin, and nylon polymers were in the form of microplastic fibers and the single particle identified as silicone was in the form of a microbead. Particles classified as "Unknown" contained fiber (55.6%) and sphere (44.4%) morphologies (Figure 6.4) and particles further categorized as "Unknown Subsample A" contained sphere (55.6%) and fiber (45.4%) morphologies. Diethyl phthalate was found in 16.3% of all particles analyzed, including PVC (14.3%), Silicone (14.3%), Nylon (14.3%), Unknown (28.6%), and Unknown Subsample A (28.6%).

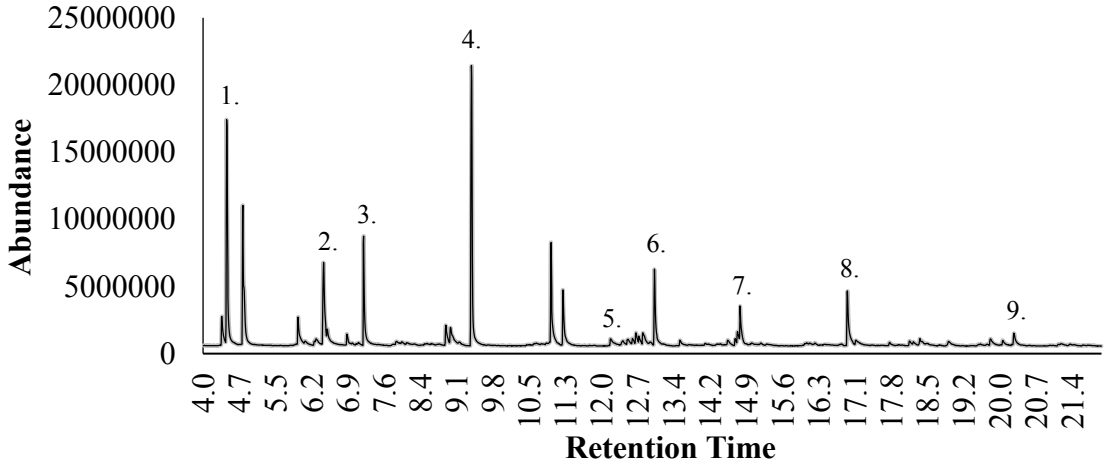

Figure 6.1: Total chromatogram of PVC particle with pyrolytic products numerically labeled.

Table 6.1: PVC pyrolytic products with retention times and parent m/z's.

Sample Peak	Pyrolytic Product	Retention Time (±0.5 minutes)	Parent m/z
1	Styrene	4.4	104
2	Mesitylene	6.5	120
3	Indene	7.2	116
4	Naphthalene	9.5	128
5	Biphenyl	12.1	154
6	Acenaphthylene	13.1	154
7	Diethyl phthalate	14.8	222
8	Anthracene	16.9	178
9	Pyrene	20.2	202

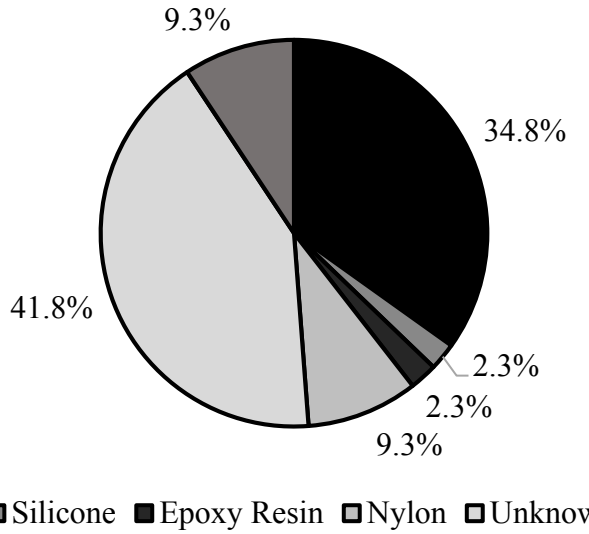

Figure 6.2: Distribution of polymer classes identified via pyr-GC/MS.

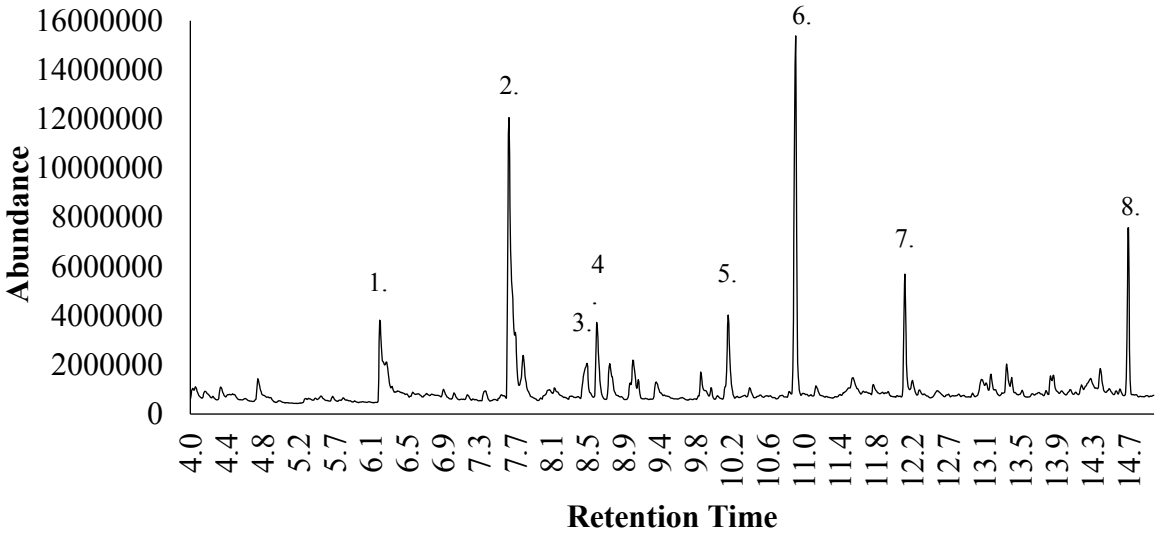

Figure 6.3: Total chromatogram of Unknown Subsample A, a fiber particle with pyrolytic products numerically labeled.

Table 6.2: Unknown Subsample A pyrolytic products with retention times and parent m/z's. Pyrolytic products of peaks 1-7 are indicative of the particles within this classification.

Peak number	Retention Time (±0.5 minutes)	Pyrolytic Product	Parent m/z
1	6.2	Phenol	94
2	7.7	4-Methylphenol	108
3	8.5	2,5-Pyrrolidinedione	99
4	8.7	Benzonitrile	103
5	10.1	Benzenepropanenitrile	131
6	10.9	Indole	117
7	12.2	3-Methylindole	131
8	14.7	Diethyl Phthalate	222

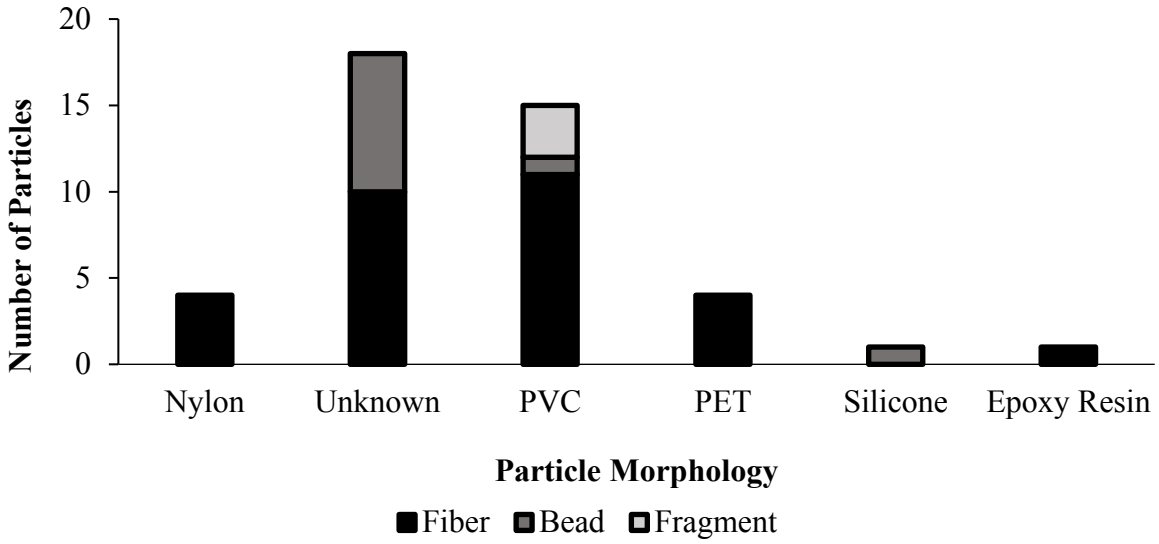

Figure 6.4: Distribution of particle polymer classes and associated morphologies.

Discussion

Of the original marine microplastic data set (i.e. Peters et al., 2017), the most common particle morphologies were fibers (86.4%), followed by spheres (12.9%), and fragments (<1.0%), thus, samples chosen for pyr-GC/MS analysis proportionally favored sphere and fragment morphologies, which were on average larger than microplastic fibers (Peters et al., 2017). Overall, the total number of samples analyzed was limited by time and monetary constraints, while the particles chosen for analysis were also limited by a minimum size constraint. These three factors contributed to the distribution of particle morphologies chosen for analysis and the comparatively low proportion of microplastic fibers sampled. Previously, microplastic form has been utilized to hypothesize originating sources of pollution (e.g. fibers resulting from wastewater effluent), however, due to varying environmental conditions and a limited knowledge of microplastic transport pathways, it is difficult to assign microplastics to a single source. While pyr-GC/MS cannot distinguish originating sources of pollution, it can identify specific polymers and

subsequently a measure of polymer distribution throughout the sample matrix. Polymer identification is also a first step toward predicting ultimate fate in an aquatic environment; for example, different plastic polymers have different susceptibilities to various biologically or photochemically mediated degradation processes (Gewert et al., 2015).

Of the five polymer classes identified, PVC and PET were the most common, while all other polymer classes collectively constituted less than 15% of the total sample. PVC is one of the leading polymers worldwide, accounting for 20% of all plastics manufacturing and prominent within applications for building, transport, and packaging, and as a strengthening coating for fabrics (British Plastics Federation, 2018). PET is the most common thermoplastic polymer resin of the polyester family and is widely utilized for packaging and bottle applications and for the production of synthetic fibers (i.e. polyester). Both PVC and PET are high density polymers (approximately 1.38g/cm^3), thus they are likely to sink within freshwater and marine habitats. As the fish species originally examined are benthivore foragers, the high proportion of PVC and PET within the sample is not unexpected. However, a study of microplastic pollution within Western Lake Superior identified PVC as the most common microplastic polymer collected within surface water samples and PET as the 4th most commonly identified polymer, suggesting that polymer density does not ultimately determine particle fate and transport (Hendrickson et al., 2018).

In total, 42% of all particles analyzed could not be classified into a specific polymer class, due to either a lack of pyrolytic products formed, low pyrolytic product abundance, or the lack of a clear polymer match, and were subsequently categorized as

"Unknown". Fifty percent of unknown particles displayed a similar pyrogram result, suggesting a shared compositional origin, and were subsequently separately classified (i.e. Unknown Subsample A) despite the lack of polymer match. The particles classified as Unknown Subsample A were not cohesive in morphology, containing both spheres and fibers, which indicates a variation in origin. Additionally, half of the fibers within Unknown Subsample A exhibited diethyl phthalate, a known plasticizer, as a pyrolytic product. One possible hypothesis is that the particles of Unknown subsample A are associated with the petroleum industry (e.g. manufacturing waste or pitch byproducts or perhaps even products from plastics manufacturing, whose feedstock is usually from petroleum). The petroleum industry is highly concentrated along the Texas Gulf Coast and reflective of local land use patterns, such as high levels of local petroleum and natural gas industrialization and major petroleum transport zones (i.e. Port of Galveston). It is possible that particles with a sphere morphology are a direct result of the petroleum industry, while the fiber particles were in fact known plastic polymers which had been coated in or had absorbed waste products, subsequently inhibiting polymer identification.

In addition to a high concentration of petroleum industrialization, major pollution events, such as the British Petroleum Deepwater Horizon oil spill, have impacted the Texas Gulf Coast (Nelson et al., 2015; Singleton et al., 2016). Studies investigating the impact of events, such as oil spills or petroleum waste contamination, cite inhalation, aspiration, ingestion via contaminated sediment, water, or prey, and absorption through the skin, as routes of exposure to aquatic organisms (NOAA, 2017). Laboratory studies investigating potential adverse effects, via the exposure of fish to oil contaminated sediment, indicate that fish experienced reduced growth (i.e. length and weight) and

fecundity (Raimondo et al., 2016). Fish exposure to oil via contaminated water has also been linked to decreased swimming speeds, maximum metabolic rate, and aerobic scope (Stieglitz et al., 2016). While Peters et al. (2017) did not investigate adverse effects, the hypothesis of this study suggests that Texas Gulf Coast fish may experience adverse effects resulting from the ingestion of petroleum contaminated particles.

A secondary hypothesis is that the particles of Unknown Subsample A are associated with coal tar, which is a liquid oil byproduct of coal pyrolysis and is utilized in its raw form for industries such as synthetic fiber, dyestuff, and coatings (Fardhyanti and Damayanti, 2015; Jiang et al., 2007). Major coal tar compound groups include: oxygen containing compounds (e.g. phenols), nitrogen containing compounds (e.g. amines), aromatic hydrocarbons (e.g. benzene hydrocarbons), and inorganic constituents (Fisher, 1938). Of the seven pyrolytic products indicative of Unknown Subsample A, phenol and 4-methylphenol (i.e. phenol group), and indole, 3-methylindole, and benzonitrile (i.e. amines) have been identified as constituents of coal tar. Additionally, diethyl phthalate was identified as a product of coal tar pyrolysis, suggesting that its presence does not always indicate a parent plastic polymer (Jiang et al., 2007).

One primary use of coal tar is for the production of coal tar creosote (i.e. creosote), formed via the fractional distillation of crude coal tar (World Health Organization, 2004). Creosote is an oily liquid, consisting of aromatic hydrocarbons, anthracene, naphthalene, and phenanthrene derivatives and comprised of approximately 85% PAH's and 2-17% phenolics (U.S. Department of Health and Human Services, 2002). Similar to both coal tar and petroleum waste, creosote is a multicomponent mixture that varies per the source and preparation parameters utilized for production.

Because of this, creosote components are rarely consistent per collective type and relative concentrations, however, display similar compositional chemical groups as that of coal tar. Creosote is utilized along the Texas Gulf coast as a wood preservative and water-proofing agent for marine pilings and as a preventative agent for plant and animal growth along concrete marine pilings (U.S. Department of Health and Human Services, 2002). Because of this, and similar to previous petroleum and coal tar hypotheses, creosote may serve as a vector for chemical pollution throughout Texas Gulf Coast waters and subsequently affect microplastic polymer identification.

Although the hypotheses associating Unknown Subsample A with petroleum waste, coal tar, and creosote are preliminary, extensive research was conducted to investigate probable alternatives and none were readily identified. In addition to a literature search, the sample chromatogram results were compared against a NIST polymer library, and a reference text polymer library, inclusive of 163 standard polymer samples (Shin et al., 2011). Major groups represented within the library include: polyolefins, vinyl polymers with ethylene units, vinyl polymers with styrene units, vinyl polymers with styrene derivatives, acrylate-type polymers, chlorine-containing vinyl polymers, fluorine-containing vinyl polymers, diene-type elastomers, polyamides, polyacetals and polyether polymers, thermosetting polymers, polyimides, and polyimide-type engineering plastics, polyesters, silicone polymers, polyurethanes, and natural and cellulose-type polymers, none of which shared more than two common pyrolytic products with Unknown Subsample A (Shin et al., 2011). Additional research is needed in this area of study, particularly that which investigates the potential for varying polymers to adsorb chemical and environmental pollutants.

To the best of the authors' knowledge, pyr-GC/MS has not been previously utilized for microplastic polymer identification within a fish ingestion study. However, the polymer classes identified within the current study are similar to those in Neves et al. (2015), which utilized μ-FTIR to identify polypropylene, polyethylene, rayon, polyester, and nylon 6 within the stomach contents of fish off of the Portuguese coast, Lusher et al. (2013) which utilized FTIR to identify rayon and polyamide polymers within fish from the English Channel, and Possatto et al. (2011) which visually identified blue nylon fragments within marine catfish. Additionally, the results of this study are similar to that of Hendrickson et al. (2018), which identified PVC, PP, PE, and PET within microplastic particles in the surface waters of Lake Superior. However, Hendrickson et al. (2018) did not detect compounds similar to those of Unknown Subsample A, suggesting that this chemical configuration may be more prevalent within the Gulf of Mexico than in the Great Lakes and similar freshwater systems. Further research of petroleum or chemical processing byproducts, resulting in the formation of particles or potential absorption, would help to clarify this discrepancy and direct future microplastic polymer research in areas with high levels of local industrialization.

Overall, the results of this study indicate that pyr-GC/MS is an applicable tool for microplastic polymer identification within a fish ingestion study. However, this method is limited by the following factors: 1. Size Constraint: Analysis success significantly decreases when particles are less than ten micrograms, thus the smallest microplastic size classes are underrepresented within the literature; 2. Time Constraint: Pyr-GC/MS is time intensive and requires a minimum of 45 minutes per particle for sample prep and analysis; 3. Monetary Constraint: It is difficult to obtain a representative pyr-GC/MS

sample for studies that have large number of microplastic particles; and 4. Standard Constraint: There is a lack of comparative standard data available, especially that which represents pyrolytic products associated with polymers which have undergone environmental transformations. Future research is needed which addresses these limitations and focuses on the development of a more efficient and effective method for microplastic polymer identification.

Conclusions

The quantification of microplastic polymer ingestion by fish is an important first step towards understanding the breadth of microplastic pollution throughout aquatic systems and potential adverse effects resulting from microplastic exposure via ingestion. This study confirms pyr-GC/MS as an applicable analytical tool for microplastic polymer identification and is one of the first to utilize pyr-GC/MS for polymer identification within a fish ingestion study. Overall, the polymer classes identified within this study are similar to that Hendrickson et al. (2018), however, unique to this study was the identification of particles hypothesized to be related to petroleum industrialization. Future research is needed which focuses on the development of polymer identification methods that maintain sample integrity, are not limited by particle size or type, and can be quickly and efficiently utilized for polymer identification.

Acknowledgments

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CHAPTER SEVEN

Conclusions

Despite the growing body of literature examining microplastic pollution, there is a large knowledge gap pertaining to microplastic ingestion by fish, including a lack of investigation into the occurrence and frequency of microplastic ingestion per species, the potential for trophic level transfer, originating microplastic sources, and potential adverse effects. This dissertation serves as a baseline assessment of microplastic ingestion within freshwater and marine fish species from the Brazos River Basin and the Texas nearshore marine waters and is one of the first comprehensive North American investigations of microplastic ingestion by fish.

The first goal of this dissertation was to examine the occurrence and frequency of microplastic ingestion within freshwater fish from the Brazos River basin and to investigate the role of local urbanization as a source of microplastic pollution into the system. Chapter Three addresses this goal by confirming the ingestion of microplastic by 45% of 436 bluegill and longear examined. Frequency of microplastic ingestion differed significantly between sample sites, ranging from 19% at Lake Waco South (an upstream sample locale) to 75% at Waco Center (an urban sample locale). When separated into size classes relative to the distribution of sample sites (i.e. urban, upstream, downstream), fish collected from urban areas averaged the highest mean number of microplastics per fish (0.67-1.33 microplastics). Additionally, the mean number of ingested microplastics positively correlated with the area of major roadways located within sample plots and

along sample transects, suggesting that developed roadways and urban areas may serve as vectors of non-point source pollution.

The second goal of this dissertation was to investigate the occurrence and frequency of microplastic ingestion by six marine fish species from the Texas Gulf coast. Chapter Four addresses this goal by examining microplastic ingestion by 1,381 fish, inclusive of six species, collected from Texas nearshore marine waters spanning from Galveston Bay to Freeport, TX. A secondary aim of Chapter Four was to examine the influence that foraging preferences (e.g. methods of prey capture) had on microplastic ingestion by species of a shared ecological guild, some of the first research to examine microplastic ingestion from an ecological standpoint as opposed to a baseline or quantitative report.

Chapter Four confirmed the ingestion of microplastic by 42.4% of all Southern kingfish (*Menticirrhus americanus*), Atlantic croaker (*Micropogonias undulates*), Atlantic spadefish (*Chaetodipterus faber*), Sand trout (*Cynoscion arenarius*), pinfish (*Lagodon rhomboids*), and grunt (*Orthopristis chrysoptera*) sampled. These species occupy a benthivore ecological guild, whereas they are mainly demersal foragers and benthic invertebrates constitute a significant proportion of their diet. Despite the significant overlap in diet, ordination of ingested prey items clustered samples into distinctive species groupings, reflective of a foraging gradient among species. Variations in foraging include Southern kingfish and Sand trout, which include piscivory within their diet; Atlantic croaker and pinfish which include vegetation within their diet and utilize suction feeding to capture prey; Atlantic spadefish which forage for both benthic

and water column invertebrates and preferentially forage around manmade structure, and grunt, which are selective benthic invertebrate foragers.

Of the six species examined, grunt displayed the lowest overall frequencies of microplastic ingestion and the most distinctive ordination grouping, indicating their selective foraging preferences. Additionally, cluster analysis results most closely classified microplastic ingestion with the ingestion of vegetation and shrimp for all species, except for grunt, which most closely classified the ingestion of microplastic with benthic invertebrates. Thus, the results indicate that grunt, as selective invertebrate foragers, are less likely to ingest microplastics than fish which utilize generalist foraging preferences and methods of prey capture.

Chapter Four also introduced a novel method for microplastic color classification, via the Munsell Color System. Previous attempts at classifying microplastic color utilized broad color schemes, consisting of mainly primary colors (e.g. red, blue, green, white). This form of classification is generalized and prone to user bias, especially in cases where a single project utilized multiple personnel for microplastic color identification. Because of this, it was difficult to compare microplastic color results between studies and the lack of available color detail inhibited investigations into the association between microplastic color and polymer type or originating source.

The Munsell Color System is an internationally recognized color system and has previously been widely utilized for soil investigations. To the best of our knowledge, this research is the first to apply the Munsell System for microplastic color classification, which enables microplastics to be assigned to a wider range of colors and also minimizes user error as all colors are set standards, against which the particle can be directly

compared. With the use of this system, the microplastics recovered within the Chapter Four study were successfully classified into ten hue dimensions, eight value dimensions, and eight chroma dimensions.

The third goal of this dissertation was to compare the frequency and type of microplastic ingested between the freshwater and marine species examined. The research of Chapters Three and Four are unique in that both took place within the Brazos River Basin, enabling a comparative systems investigation. Additionally, pinfish were specifically chosen for inclusion in the Chapter Four study as they are ecological analogs to sunfish (i.e. fulfill similar trophic roles, share similar feeding methods, and are opportunistic foragers).

In total of 885 fish, inclusive of 436 sunfish and 449 pinfish were examined. Overall, approximately 45.0% of sunfish and 46.5% of pinfish had ingested microplastic. Sunfish averaged 0.80 particles per fish, which did not significantly differ from pinfish ($p=0.744$), which averaged 0.96 particles per fish. In total, sunfish had ingested 349 anthropogenic items, 4% on the macro-scale and 96% on the micro-scale and pinfish had ingested a total of 415 anthropogenic items, all of which were on the micro-scale and consisted of fibers, fragments, and spheres.

Despite variations between the freshwater and marine habitats, both sunfish and pinfish displayed similar frequencies of microplastic ingestion, number of particles per fish, and particle morphologies. While the results indicate that microplastics are ubiquitous contaminants throughout the entirety of the Brazos River watershed, freshwater fish are more likely to ingest macroplastics, which may be due to levels of local urbanization and runoff. Furthermore, as microbeads were only found in marine

fish, it is likely that these contaminants originate downstream of the sunfish study boundary or are a result of local petroleum pollution. This knowledge is an important first step in understanding point and non-point sources of microplastic pollution into Texas aquatic systems and can be utilized for the development of source reduction strategies and targeted, cost-effective remediation efforts.

The fourth goal of this dissertation was to examine the applicability of pyr-GC/MS for microplastic polymer identification. This method was applied for the analysis of microplastics recovered from the Chapter Four (marine system) study and was conducted in collaboration with researchers at the University of Minnesota, Duluth, whom developed this method for the successful analysis of microplastics recovered within the water of Western Lake Superior.

Chapter Six presents one of the first successful utilizations of pyr-GC/MS for microplastic polymer identification within a fish ingestion study. A total of five polymer classes were identified, including PVC, PET, epoxy resin, Nylon, and silicone. Fifty percent of the Nylon particles were further classified as Nylon 6 due to the presence of caprolactam as a main pyrolytic product. In addition to polymer classifications, 42% of particles were classified as sample unknowns, approximately 21% of which displayed similar chromatogram results (i.e. Unknown Subsample A). Possible hypotheses for Unknown Subsample A include association with the petroleum industry, coal tar, or coal tar creosote, which may serve as source of pollution into the system and are reflective of local land use patterns (i.e. marine pilings).

Overall, the results of the pyr-GC/MS analysis were similar to that of Neves et al. (2015) and Luster et al. (2013), which identified PP, PE, rayon, polyester, and Nylon 6

within the stomach content of fish from the Portuguese Coast and the English Channel. The best comparative aquatic systems investigation was conducted by Hendrickson et al. (2018), which originally developed and utilized this method of pyr-GC/MS for the analysis of microplastics collected from Western Lake Superior. Hendrickson et al. (2018) also identified PVC as a primary microplastic polymer class, however, they did not identify any particles associated with petroleum waste byproducts. These results indicate that although microplastic pollution is ubiquitous, microplastic polymer distribution is reflective of local land use patterns and major sources of microplastic pollution into the system.

The successful utilization of pyr-GC/MS within a fish ingestion study offers an efficient and effective alternative to FTIR and Raman spectroscopy, methods which are limited by the inclusion of particle additives or chemicals, particle size, and time constraints. While pyr-GC/MS is not limited by these factors, analysis success decreases when sample particles weigh less than 10μg in weight, thus this method has limited applicability for the analysis of smaller microplastic fibers. The greatest limitation of all available polymer identification techniques is the lack of ability to successfully analyze the smallest microplastic size classes, a consequence which may result in the under representation or lack of identification of major polymer groups.

Future Research Needs

This research is the first to confirm microplastic ingestion within freshwater and marine fish from a shared watershed in North America. While the results of this dissertation fill critical knowledge gaps within the literature, there are still many

unknowns pertaining to fish ingestion of microplastic. The following are suggestions for the focus of future research:

1. Standardization of the lower bound of the –micro size categorization
    a. Currently, the lower bound utilized for microplastic research is determined via the methodologies utilized (e.g. 333μm for trawl studies and 53μm utilized for fish ingestion studies). This variation in size inhibits comparisons between studies, especially between studies of varying environmental matrixes (e.g. fish ingestion, water, and sediment). The development of a set lower bound standard would not only enhance comparative research between studies, but also, enable researchers to compare overall microplastic loads which would offer better insight into the extent of microplastic pollution throughout aquatic systems.
2. Standardization of microplastic collection techniques per matrix
    a. As microplastic research is fairly new within the scientific literature, many of the collection techniques are developmental, thus they vary per study. Research examining microplastic ingestion by fish utilizes methods of identification including: visual identification, digestion, and density separation, all of which yield varying results. Of these methods, visual identification may reflect the largest measure of human error, dependent upon the skill of the observer. However, both digestion and density separation can lead to an underestimation of recovered microplastic, via the digestion of particles along with organic content or a loss of particles during density separation. Due to the variation in methodologies, reported

results may be over estimations or under estimations of the actual total. While a standardization of methods would reduce the margin of error when comparing between studies, it would not reduce the margin of error within each method.

3. Improvement in microplastic analytical analysis
    a. Although analytical techniques are becoming a standard for microplastic polymer identification, there is still a lack of methods which are able to identify small polymers or particles with complex surfaces and additives (i.e. FTIR and Raman spectroscopy), thus the use of these techniques is biased towards large, uniform particles. While pyr-GC/MS does not exhibit these limitations, it is destructive and prevents further particle analysis. Because of these limitations, it is important to develop a technique which can analyze small, complex particles, while simultaneously maintaining particle integrity.
4. Potential adverse effects
    a. As microplastic ingestion has been confirmed within numerous taxa, across all trophic levels, it is important to investigate potential adverse effects resulting from this type of interaction. Preliminary research has confirmed the potential for microplastic bioaccumulation/biomagnification and the transfer of chemicals from microplastic to fauna, however, the full extent of adverse effects associated with these factors is relatively unknown.

APPENDIX

APPENDIX A

Microplastic Pyrolytic Analyses

The following figures and tables (A.1-A.43) pertain to the 43 marine microplastic samples analyzed via pyr-GC/MS (Chapter Six). Each figure contains x,y parameters specific to the sample, thus the x-axis displays the retention time pertinent to the pyrolytic products formed and the y-axis displays the product abundance corrected for background noise. The inclusion of an "*" following the figure name indicates that the sample pyrolytic products were limited, however, the available products were deemed sufficient for polymer characterization.

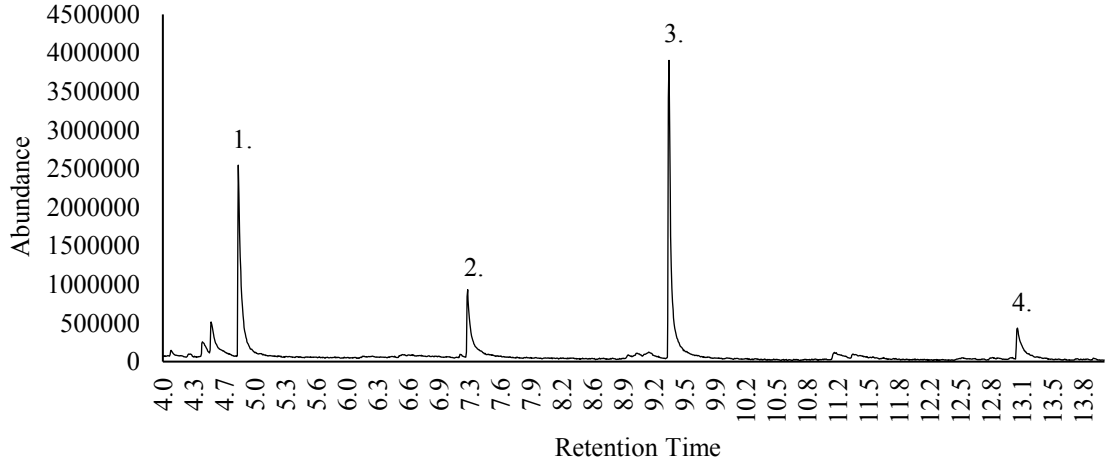

Figure A.1: Total chromatogram of PVC identified fiber particle with pyrolytic products numerically labeled.

Table A.1: Pyrolytic products of Figure A.1 with retention times and parent m/z's.

Sample Peak	Pyrolytic Product	Retention Time (±0.5 minutes)	Parent m/z
1	Styrene	4.9	104
2	Indene	7.3	116
3	Naphthalene	9.4	128
4	Acenaphthylene	13.2	154

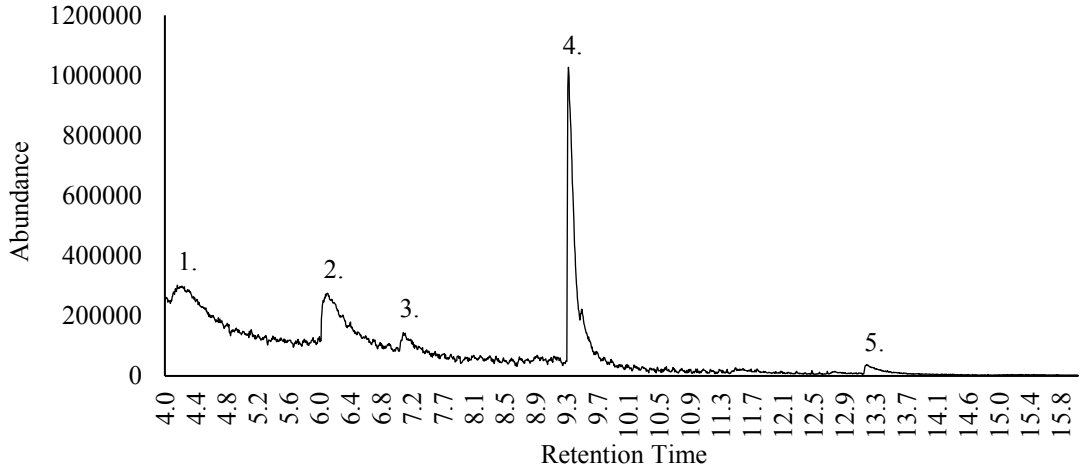

Figure A.2: Total chromatogram of PVC identified fiber particle with pyrolytic products numerically labeled.

Table A.2: Pyrolytic products of Figure A.2 with retention times and parent m/z's.

Sample Peak	Pyrolytic Product	Retention Time (±0.5 minutes)	Parent m/z
1	Styrene	4.3	104
2	Benzonitrile	6.1	103
3	Indene	7.2	116
4	Naphthalene	9.4	128
5	Acenaphthylene	13.4	152

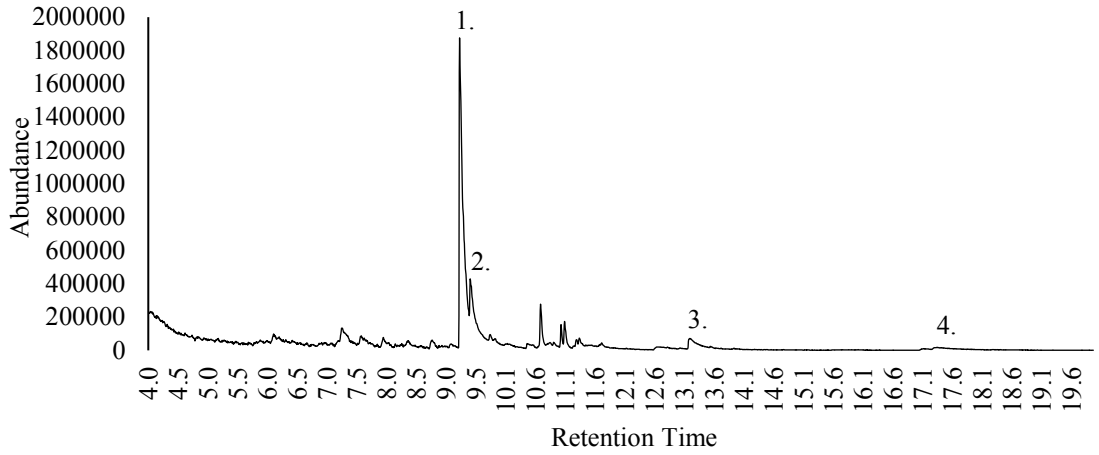

Figure A.3: Total chromatogram of PVC identified fiber particle with pyrolytic products numerically labeled.

Table A.3: Pyrolytic products of Figure A.3 with retention times and parent m/z's.

Sample Peak	Pyrolytic Product	Retention Time (±0.5 minutes)	Parent m/z
1	Naphthalene	9.3	128
2	Benzothiophene	9.4	134
3	Biphenyl	12.7	154
4	Acenaphthylene	13.2	154

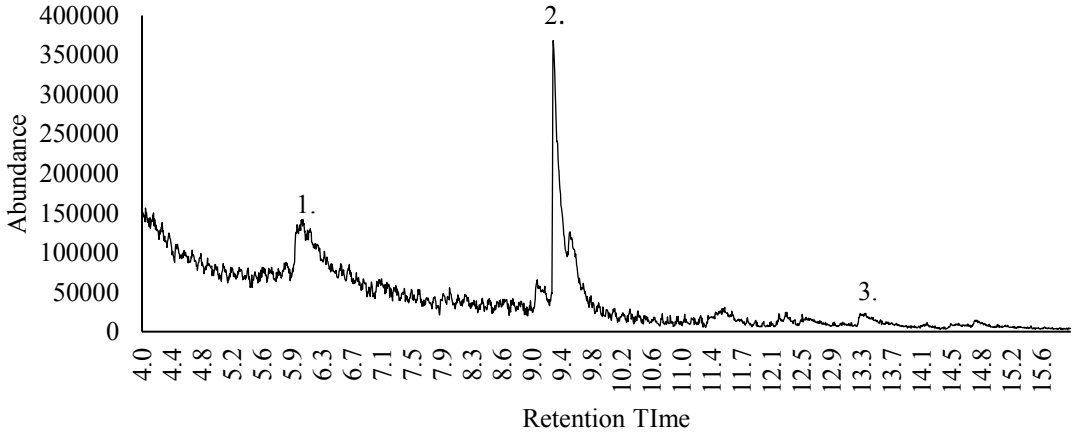

Figure A.4: Total chromatogram of PVC identified fiber particle with pyrolytic products numerically labeled.

Table A.4: Pyrolytic products of Figure A.4 with retention times and parent m/z's.

Sample Peak	Pyrolytic Product	Retention Time (±0.5 minutes)	Parent m/z
1	Benzonitrile	6.0	103
2	Naphthalene	9.4	128
3	Acenaphthylene	13.4	154

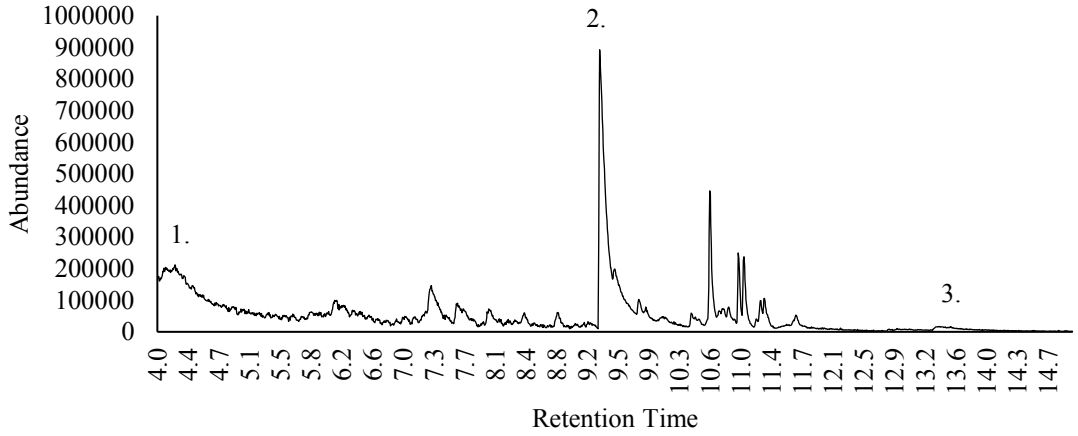

Figure A.5: Total chromatogram of PVC identified fiber particle with pyrolytic products numerically labeled.

Table A.5: Pyrolytic products of Figure A.5 with retention times and parent m/z's.

Sample Peak	Pyrolytic Product	Retention Time (±0.5 minutes)	Parent m/z
1	Styrene	4.2	104
2	Naphthalene	9.3	128
3	Fluorene	13.6	166

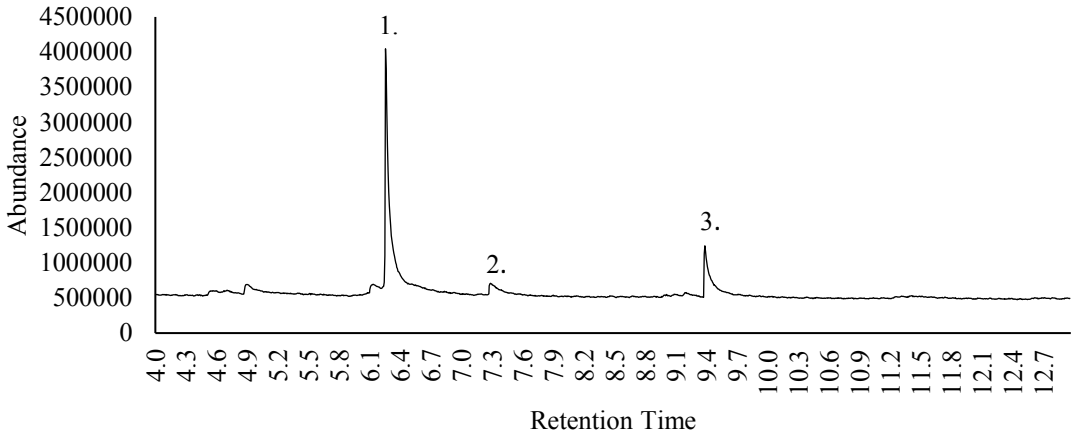

Figure A.6: Total chromatogram of PVC identified fragment particle with pyrolytic products numerically labeled.

Table A.6: Pyrolytic products of Figure A.6 with retention times and parent m/z's.

Sample Peak	Pyrolytic Product	Retention Time (±0.5 minutes)	Parent m/z
1	Benzonitrile	6.3	103
2	Indene	7.3	116
3	Naphthalene	9.5	128

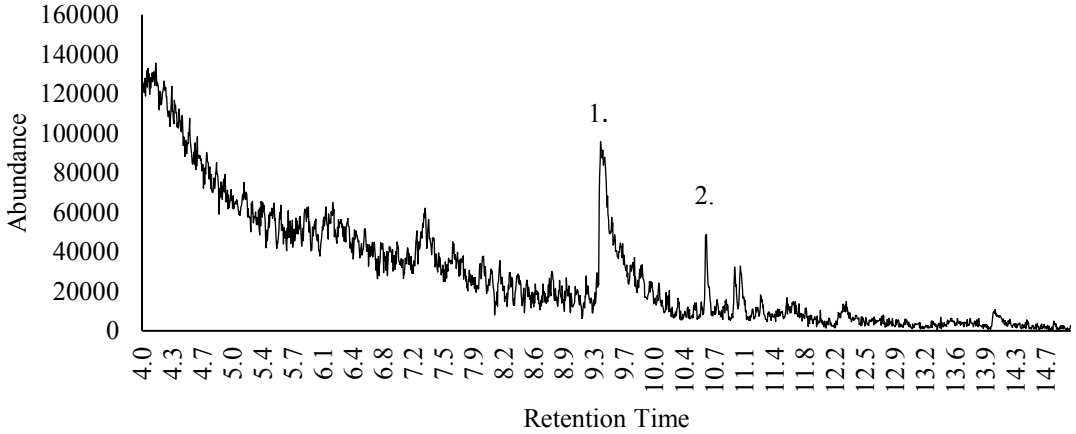

Figure A.7*: Total chromatogram of PVC identified fiber particle with pyrolytic products numerically labeled.

Table A.7: Pyrolytic products of Figure A.7 with retention times and parent m/z's.

Sample Peak	Pyrolytic Product	Retention Time (±0.5 minutes)	Parent m/z
1	Naphthalene	9.6	128
2	Biphenyl	10.8	154

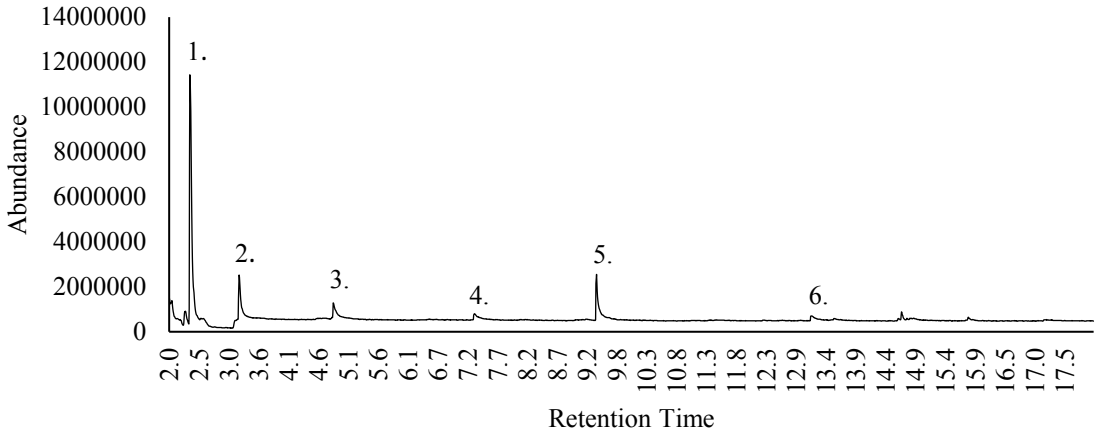

Figure A.8: Total chromatogram of PVC identified fragment particle with pyrolytic products numerically labeled.

Table A.8: Pyrolytic products of Figure A.8 with retention times and parent m/z's.

Sample Peak	Pyrolytic Product	Retention Time (±0.5 minutes)	Parent m/z
1	Benzene	2.5	78
2	Toluene	3.3	92
3	Styrene	4.8	104
4	Indene	7.3	116
5	Naphthalene	9.4	128
6	Acenaphthylene	13.1	154

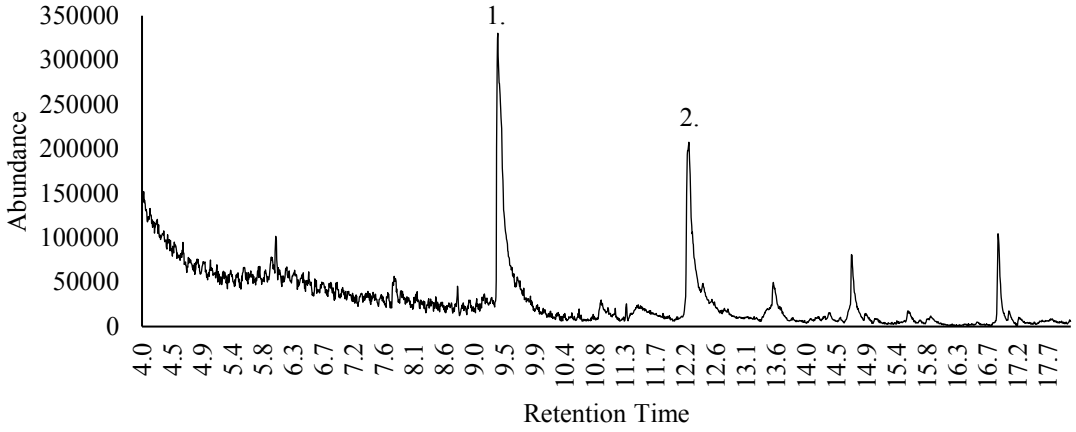

Figure A.9*: Total chromatogram of PVC identified fiber particle with pyrolytic products numerically labeled.

Table A.9: Pyrolytic products of Figure A.9 with retention times and parent m/z's.

Sample Peak	Pyrolytic Product	Retention Time (±0.5 minutes)	Parent m/z
1	Naphthalene	9.4	128
2	Biphenyl	12.2	154

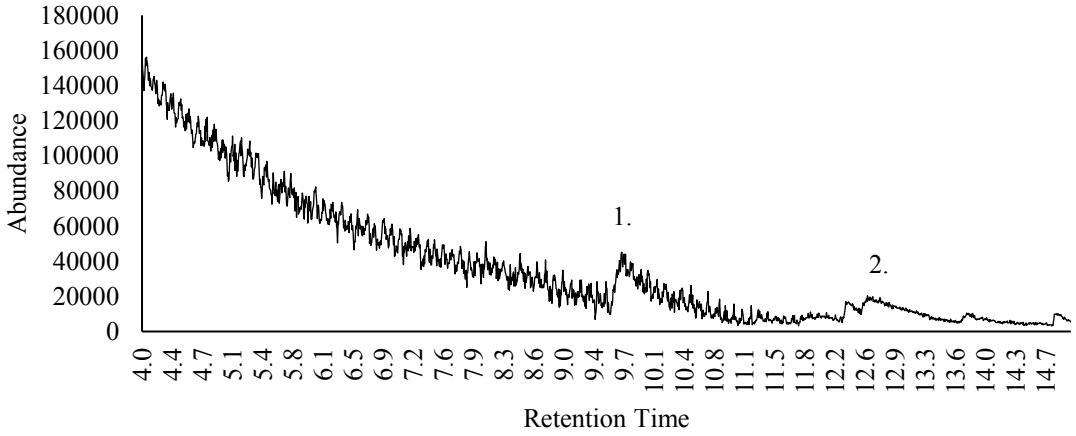

Figure A.10*: Total chromatogram of PVC identified fiber particle with pyrolytic products numerically labeled.

Table A.10: Pyrolytic products of Figure A.10 with retention times and parent m/z's.

Sample Peak	Pyrolytic Product	Retention Time (±0.5 minutes)	Parent m/z
1	Naphthalene	9.8	128
2	Biphenyl	12.6	154

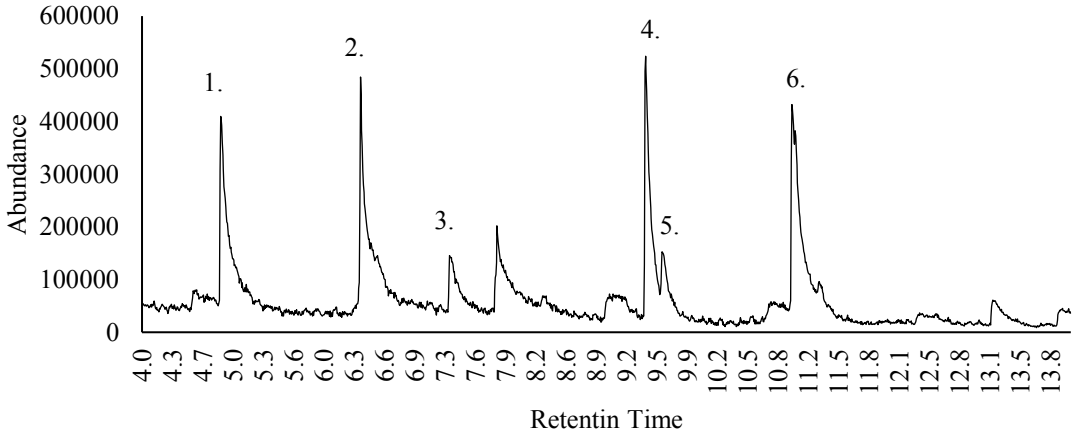

Figure A.11: Total chromatogram of PVC identified bead particle with pyrolytic products numerically labeled.

Table A.11: Pyrolytic products of Figure A.11 with retention times and parent m/z's.

Sample Peak	Pyrolytic Product	Retention Time (±0.5 minutes)	Parent m/z
1	Styrene	5.0	104
2	Benzonitrile	6.4	103
3	Indene	7.4	116
4	Naphthalene	9.4	128
5	Benzothiophene	9.6	134
6	Indole	11.0	117

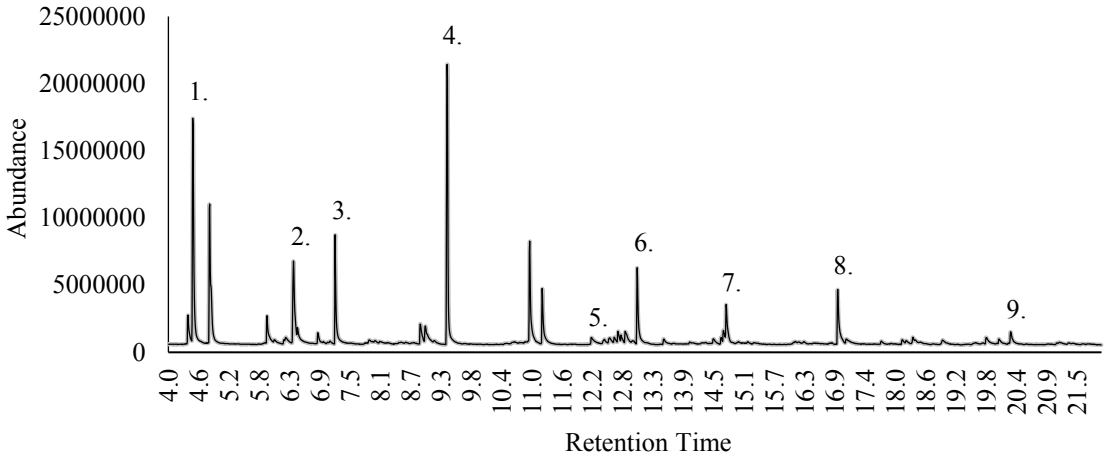

Figure A.12: Total chromatogram of PVC identified fragment particle with pyrolytic products numerically labeled.

Table A.12: Pyrolytic products of Figure A.12 with retention times and parent m/z's.

Sample Peak	Pyrolytic Product	Retention Time (±0.5 minutes)	Parent m/z
1	Styrene	4.4	104
2	Mesitylene	6.5	120
3	Indene	7.2	116
4	Naphthalene	9.5	128
5	Biphenyl	12.1	154
6	Acenaphthylene	13.1	154
7	Diethyl phthalate	14.8	222
8	Anthracene	16.9	178
9	Pyrene	20.2	202

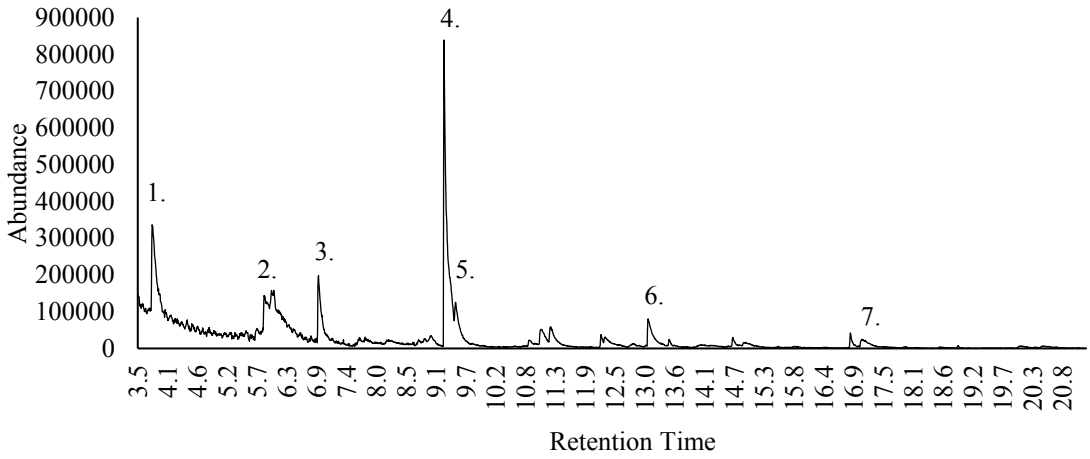

Figure A.13: Total chromatogram of PVC identified fiber particle with pyrolytic products numerically labeled.

Table A.13: Pyrolytic products of Figure A.13 with retention times and parent m/z's.

Sample Peak	Pyrolytic Product	Retention Time (±0.5 minutes)	Parent m/z
1	Styrene	3.7	104
2	Benzonitrile	6.1	103
3	Indene	7.0	116
4	Naphthalene	9.3	128
5	Benzothiophene	9.5	134
6	Acenaphthylene	13.1	152
7	Phenanthrene	17.2	178

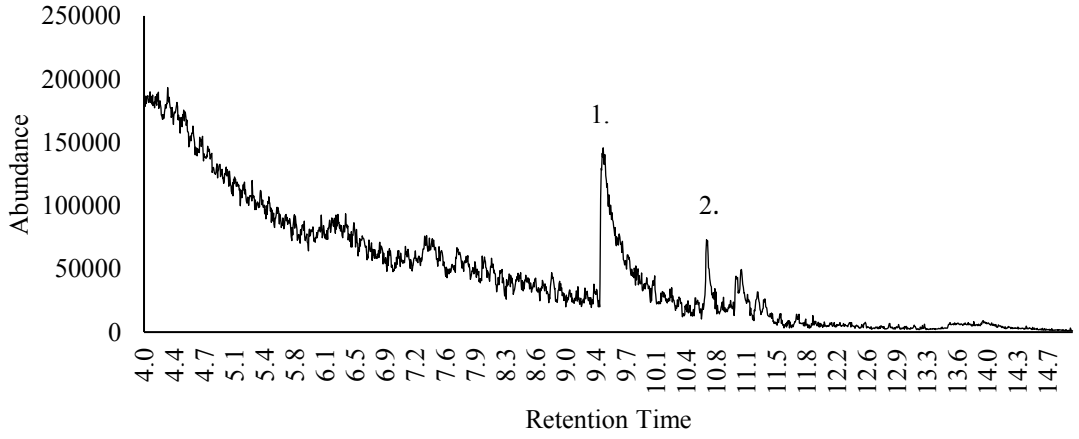

Figure A.14*: Total chromatogram of PVC identified fiber particle with pyrolytic products numerically labeled.

Table A.14: Pyrolytic products of Figure A.14 with retention times and parent m/z's.

Sample Peak	Pyrolytic Product	Retention Time (±0.5 minutes)	Parent m/z
1	Naphthalene	9.5	128
2	Biphenyl	10.6	154

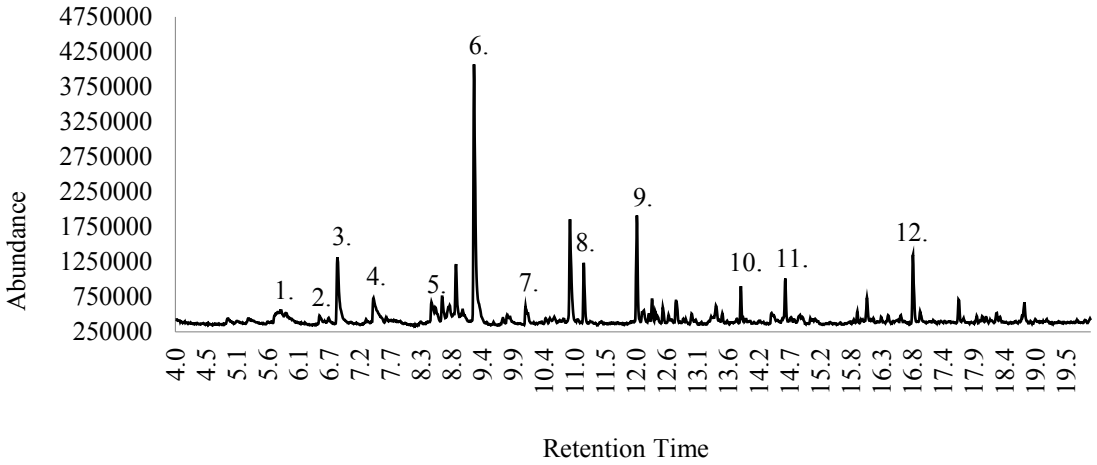

Figure A.15: Total chromatogram of PVC identified fiber particle with pyrolytic products numerically labeled.

Table A. 15: Pyrolytic products of Figure A.15 with retention times and parent m/z's.

Peak number	Pyrolytic Product	Retention Time (±0.5 minutes)	Parent m/z
1	Phenol	5.9	94
2	Indane	6.4	118
3	Indene	6.8	116
4	4-Methylphenol	7.5	108
5	1H-Indene, 2-methyl	8.5	130
6	Naphthalene	9.2	128
7	Benzenepropanenitrile	10.2	131
8	1-Methylnapthalene	11.1	142
9	Biphenyl	12	154
10	Bibenzyl	13.9	182
11	Fluorene	14.6	166
12	Phenanthrene	16.9	178

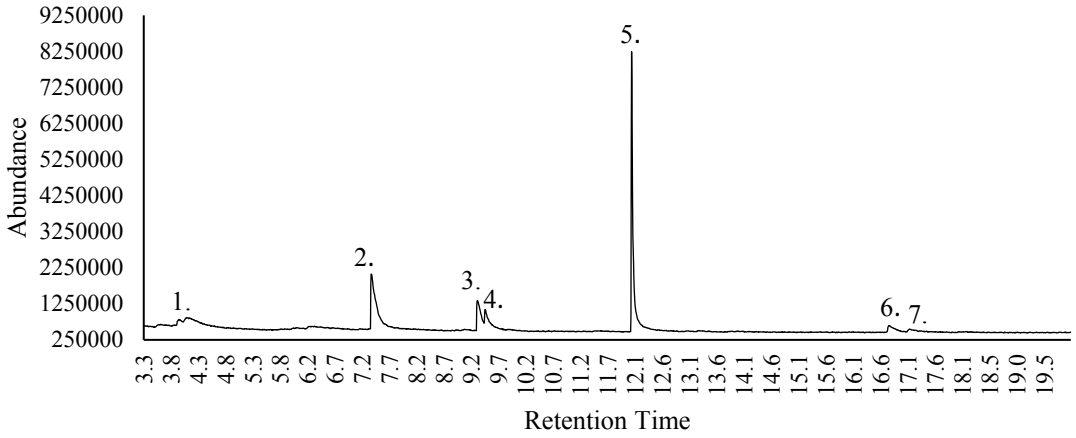

Figure A.16: Total chromatogram of PET identified fiber particle with pyrolytic products numerically labeled.

Table A.16: Pyrolytic products of Figure A.16 with retention times and parent m/z's.

Sample Peak	Pyrolytic Product	Retention Time (±0.5 minutes)	Parent m/z
1	Styrene	4.2	104
2	Acetophenone	7.5	120
3	Naphthalene	9.4	128
4	Benzothiophene	9.5	134
5	Biphenyl	12.1	154
6	Dibenzothiophene	16.8	184
7	Phenanthrene	17.2	178

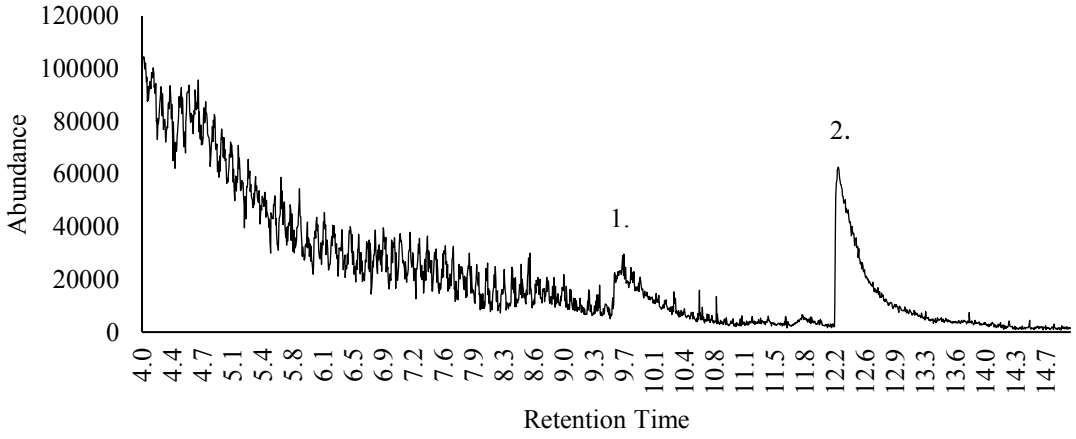

Figure A.17*: Total chromatogram of PET identified fiber particle with pyrolytic products numerically labeled.

Table A.17: Pyrolytic products of Figure A.17 with retention times and parent m/z's.

Sample Peak	Pyrolytic Product	Retention Time (±0.5 minutes)	Parent m/z
1	Naphthalene	9.8	128
2	Biphenyl	12.4	154

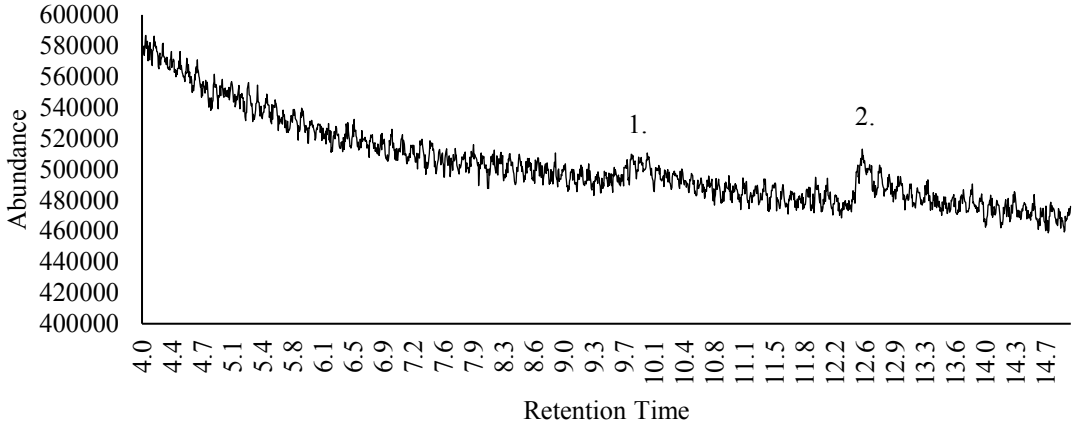

Figure A.18*: Total chromatogram of PET identified fiber particle with pyrolytic products numerically labeled.

Table A.18: Pyrolytic products of Figure A.18 with retention times and parent m/z's.

Sample Peak	Pyrolytic Product	Retention Time (±0.5 minutes)	Parent m/z
1	Naphthalene	9.8	128
2	Biphenyl	12.4	154

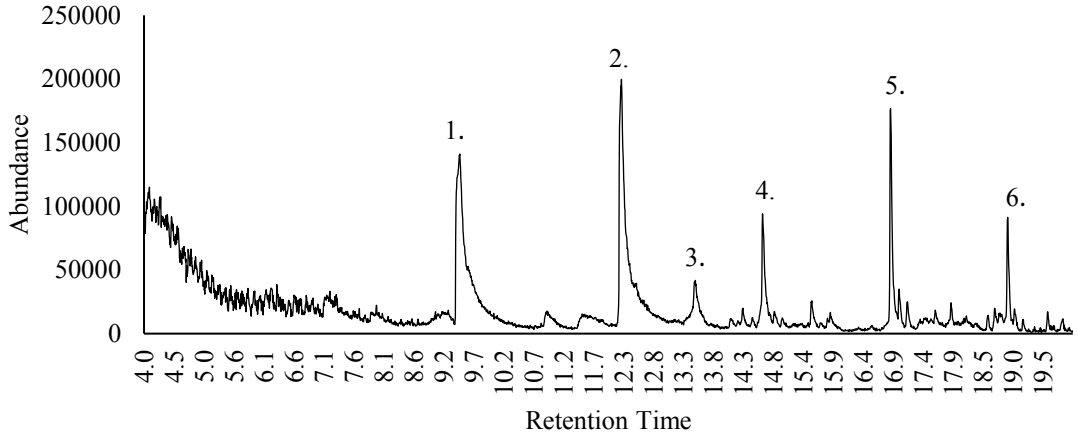

Figure A.19: Total chromatogram of PET identified fiber particle with pyrolytic products numerically labeled.

Table A.19: Pyrolytic products of Figure A.19 with retention times and parent m/z's.

Sample Peak	Pyrolytic Product	Retention Time (±0.5 minutes)	Parent m/z
1	Naphthalene	9.4	128
2	Biphenyl	12.4	154
3	Cetene	13.5	224
4	1-Hexadecanol	14.7	242
5	Octadecene	16.9	252
6	1-Eicosene	19.0	280

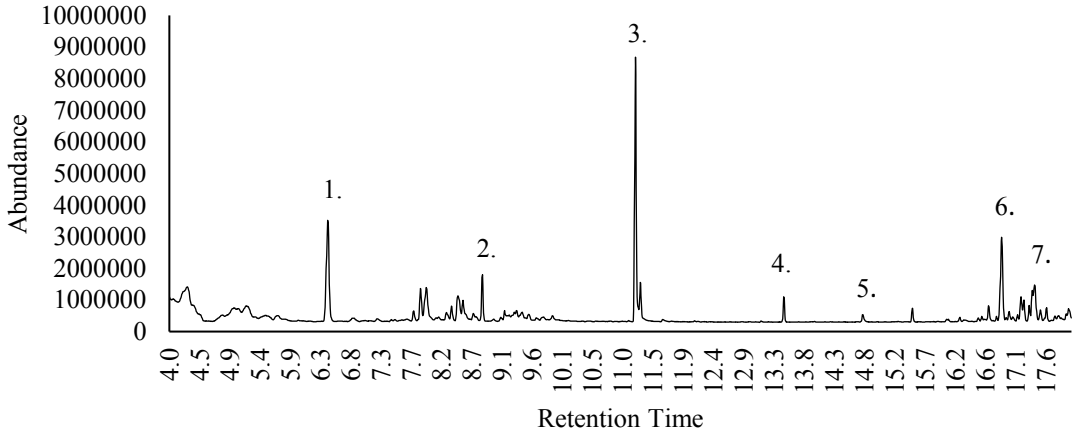

Figure A.20: Total chromatogram of Silicone identified bead particle with pyrolytic products numerically labeled.

Table A.20: Pyrolytic products of Figure A.20 with retention times and parent m/z's.

Sample Peak	Pyrolytic Product	Retention Time (±0.5 minutes)	Parent m/z
1	Cyclotetrasiloxane, octamethyl	6.5	296
2	Cyclopentasiloxane, decamethyl	8.8	370
3	1,2-Benzenedicarboxylic acid	11.1	166
4	Cycloheptasiloxane, tetradecamethyl	13.6	519
5	Diethyl phthalate	14.8	222
6	Benzoic acid, heptyl ester	16.9	220
7	Benzoic acid, octyl ester	17.4	234

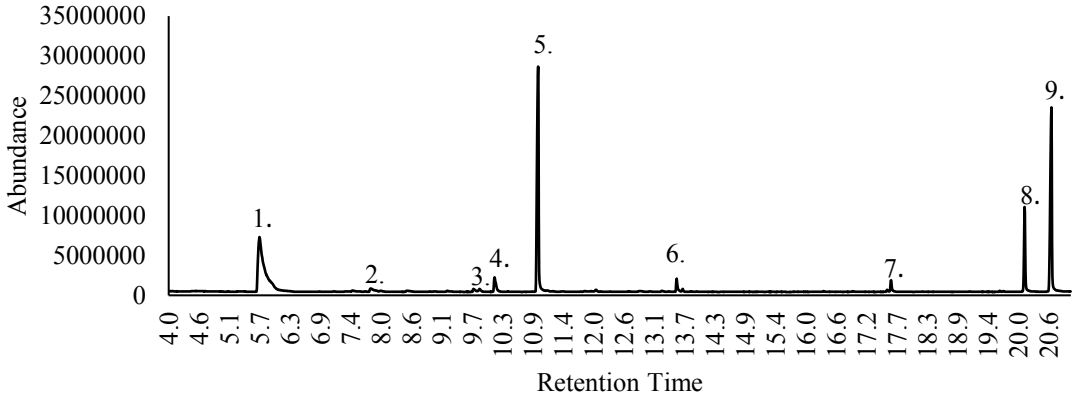

Figure A.21: Total chromatogram of Epoxy Resin identified fiber particle with pyrolytic products numerically labeled.

Table A.21: Pyrolytic products of Figure A.21 with retention times and parent m/z's.

Sample Peak	Pyrolytic Product	Retention Time (±0.5 minutes)	Parent m/z
1	Phenol	5.8	94
2	Benzoic acid, methyl ester	7.9	136
3	Benzofuran, 2,3-dihydro-	9.7	120
4	Benzene, 1-methoxy,2	10.3	122
5	P-Isopropylphenol	10.9	136
6	1-4-Benzenedicarboxylic acid, dimethyl ester	13.7	194
7	Phenol	17.6	94
8	2-2-Propane	20.1	132
9	Bisphenol A	20.6	228

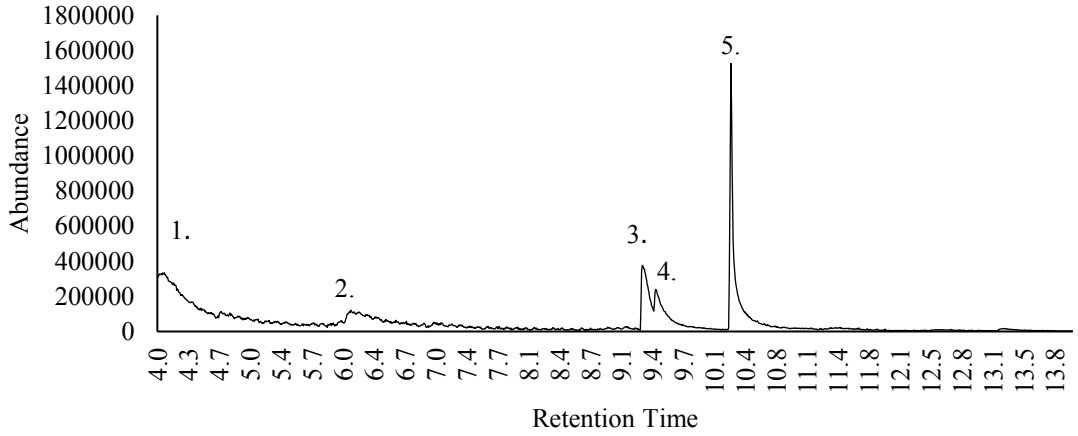

Figure A.22: Total chromatogram of Nylon 6 identified fiber particle with pyrolytic products numerically labeled.

Table A.22: Pyrolytic products of Figure A.22 with retention times and parent m/z's.

Sample Peak	Pyrolytic Product	Retention Time (±0.5 minutes)	Parent m/z
1	Styrene	4.1	104
2	Benzonitrile	6.2	103
3	Naphthalene	9.3	128
4	Benzothiophene	9.4	134
5	Caprolactam	10.3	113

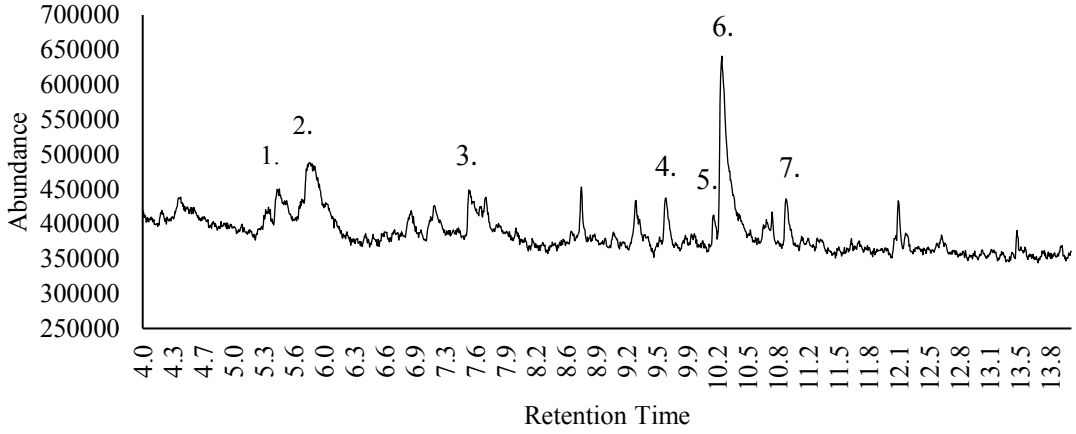

Figure A.23: Total chromatogram of Nylon identified fiber particle with pyrolytic products numerically labeled.

Table A.23: Pyrolytic products of Figure A.23 with retention times and parent m/z's.

Sample Peak	Pyrolytic Product	Retention Time (±0.5 minutes)	Parent m/z
1	Phenol	5.5	94
2	4-Methylphenol	5.9	108
3	Benzyl nitrile	7.6	117
4	Benzofuran, 2,3-dihydro-	9.7	120
5	Benzenepropanenitrile	10.2	131
6	Indole	10.3	117
7	Debrisoquine	11.0	175

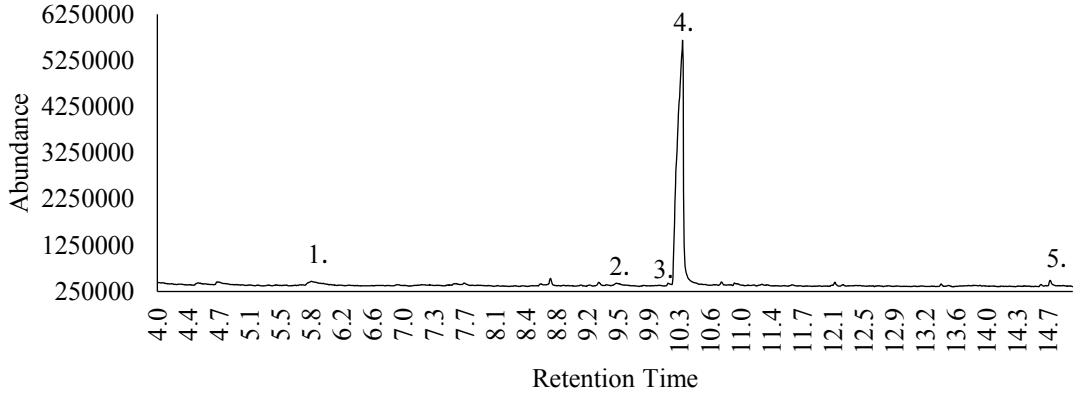

Figure A.24: Total chromatogram of Nylon 6 identified fiber particle with pyrolytic products numerically labeled.

Table A.24: Pyrolytic products of Figure A.24 with retention times and parent m/z's.

Sample Peak	Pyrolytic Product	Retention Time (±0.5 minutes)	Parent m/z
1	Phenol	5.9	94
2	2-Pyrrolidinone, 1-ethenyl	9.3	111
3	Benzenepropanenitrile	10.0	131
4	Caprolactam	10.3	113
5	Diethyl phthalate	14.7	222

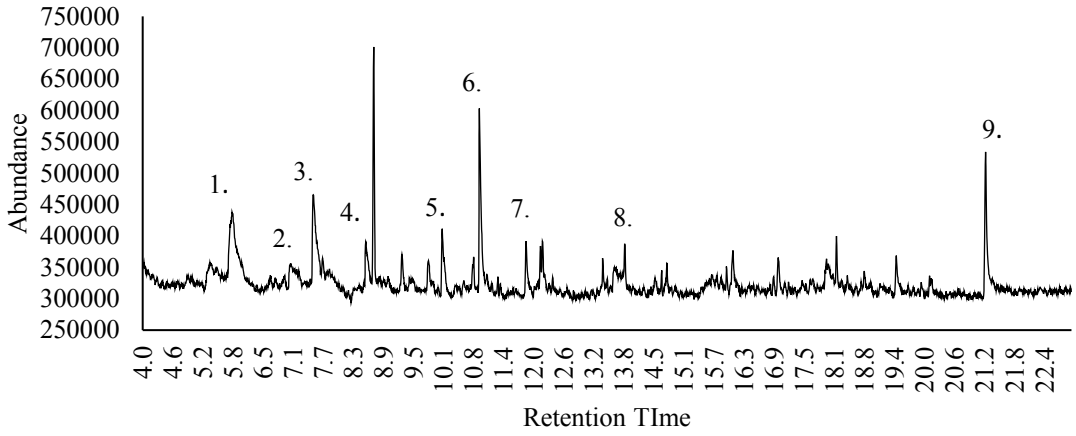

Figure A.25: Total chromatogram of Nylon identified fiber particle with pyrolytic products numerically labeled.

Table A.25: Pyrolytic products of Figure A.25 with retention times and parent m/z's.

Sample Peak	Pyrolytic Product	Retention Time (±0.5 minutes)	Parent m/z
1	Phenol	6.0	94
2	2-Pyrrolidinone, 1-methyl	7.1	99
3	3-Methyl phenol	7.5	108
4	Benzyl nitrile	8.6	117
5	Benzenepropanenitrile	10.2	131
6	Indole	10.9	117
7	Hexane	11.9	86
8	Bibenzyl	14.0	182
9	1,8-Diazacycloterradecone-2,7-dione	21.3	226

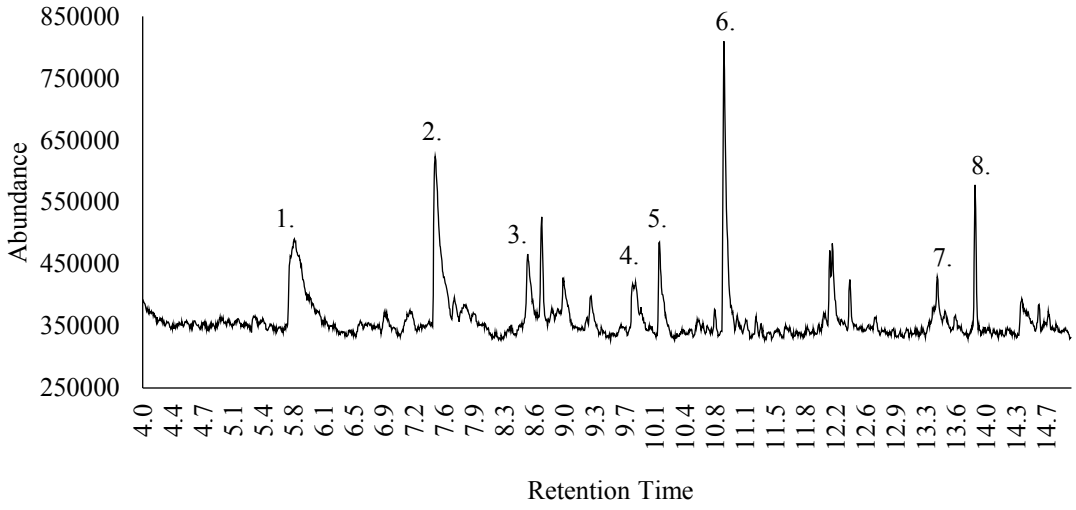

Figure A.26: Total chromatogram of Unknown Subsample A identified bead particle with pyrolytic products numerically labeled.

Table A.26: Pyrolytic products of Figure A.26 with retention times and parent m/z's.

Sample Peak	Pyrolytic Product	Retention Time (±0.5 minutes)	Parent m/z
1	Phenol	5.8	94
2	4-Methylphenol	7.5	108
3	Benzyl nitrile	8.6	117
4	Benzofuran, 2,3-dihydro-	9.9	120
5	Benzenepropanenitrile	10.2	131
6	Indole	10.9	117
7	Debrisoquine	13.4	175
8	Bibenzyl	13.8	182

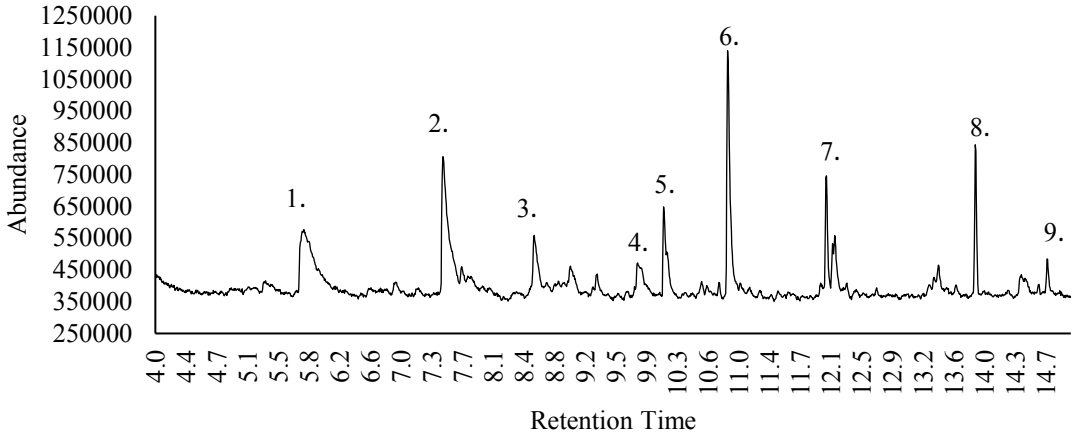

Figure A.27: Total chromatogram of Unknown Subsample A identified fiber particle with pyrolytic products numerically labeled.

Table A.27: Pyrolytic products of Figure A.27 with retention times and parent m/z's.

Sample Peak	Pyrolytic Product	Retention Time (±0.5 minutes)	Parent m/z
1	Phenol	5.8	94
2	4-Methyl phenol	7.5	108
3	Benzyl nitrile	8.6	103
4	Benzofuran, 2,3-dihydro	9.9	120
5	Benzenepropanenitrile	10.1	131
6	Indole	11.0	117
7	1,3,5-Triazine-2,4,6-trione	12.0	
8	Bibenzyl	13.9	182
9	Diethyl phthalate	14.9	222

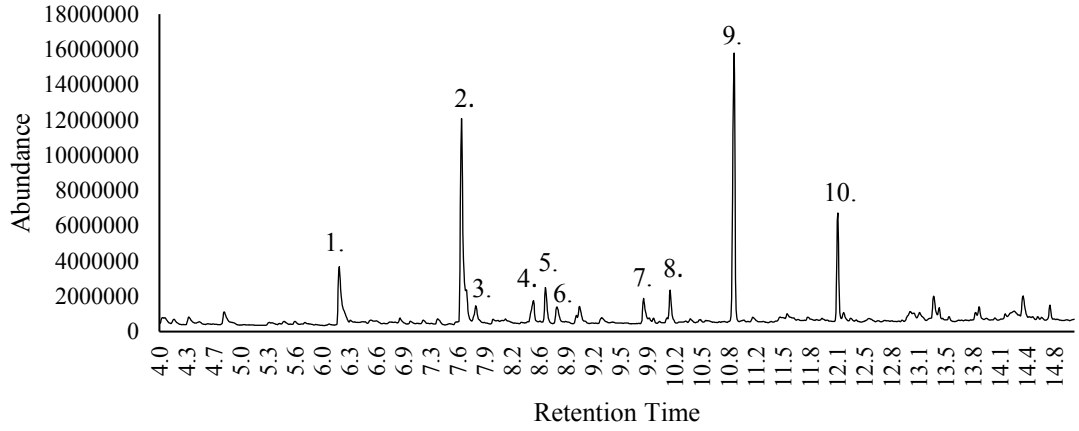

Figure A.28: Total chromatogram of Unknown Subsample A identified bead particle with pyrolytic products numerically labeled.

Table A.28: Pyrolytic products of Figure A.28 with retention times and parent m/z's.

Sample Peak	Pyrolytic Product	Retention Time (±0.5 minutes)	Parent m/z
1	Phenol	6.3	94
2	4-Methyl phenol	7.6	108
3	Butanenitrile	7.7	69
4	2,5-Pyrrolidinedione	8.5	99
5	Benzyl nitrile	8.6	117
6	Phenol, 2,4-dimethyl	8.8	122
7	Benzofuran, 2-3-dihydro	9.8	120
8	Benzenepropanenitrile	10.1	131
9	Indole	10.9	117
10	1-H, Indole, 3,methyl	12.2	131

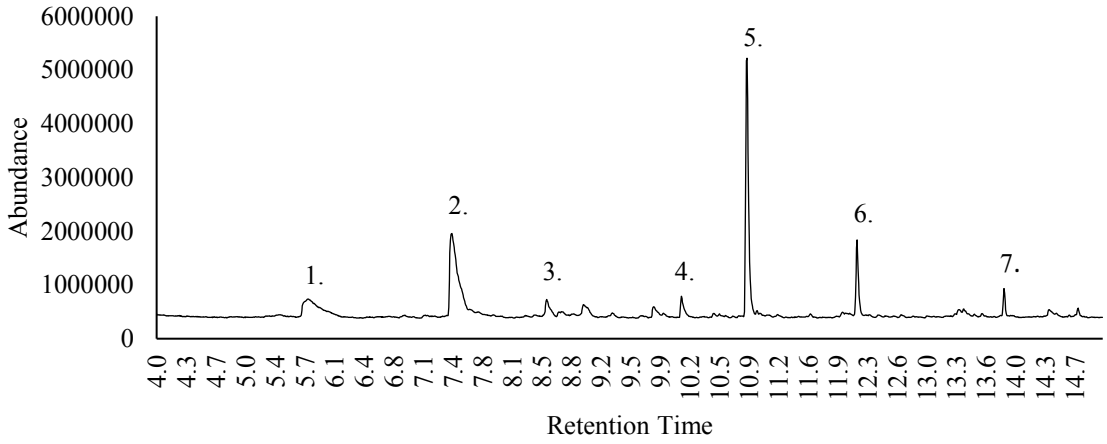

Figure A.29: Total chromatogram of Unknown Subsample A identified fiber particle with pyrolytic products numerically labeled.

Table A.29: Pyrolytic products of Figure A.29 with retention times and parent m/z's.

Sample Peak	Pyrolytic Product	Retention Time (±0.5 minutes)	Parent m/z
1	Phenol	5.8	94
2	4-Methyl phenol	7.5	108
3	Benzyl nitrile	8.6	117
4	Benzenepropanenitrile	10.2	131
5	Indole	10.8	117
6	1H-Indole, 3-methyl	12.1	131
7	Bibenzyl	13.8	182

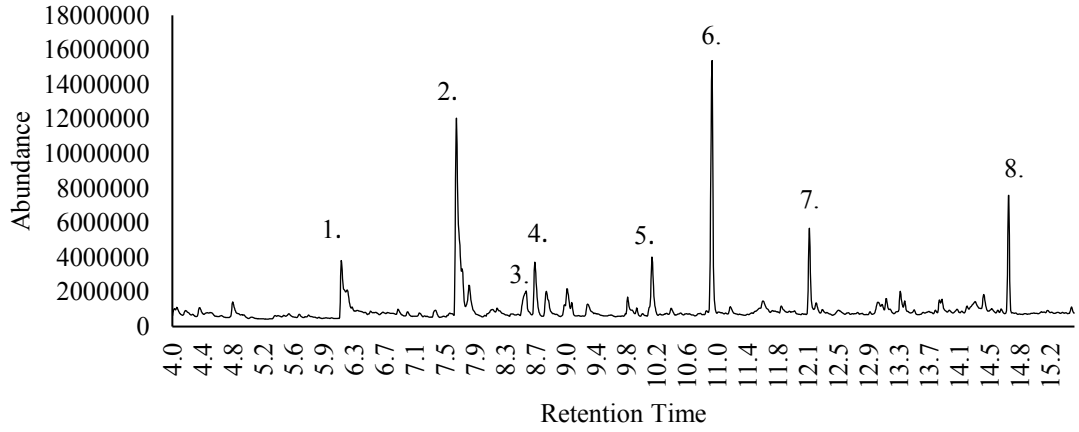

Figure A.30: Total chromatogram of Unknown Subsample A identified fiber particle with pyrolytic products numerically labeled.

Table A.30: Pyrolytic products of Figure A.30 with retention times and parent m/z's.

Sample Peak	Pyrolytic Product	Retention Time (±0.5 minutes)	Parent m/z
1	Phenol	6.2	94
2	4-Methyl phenol	7.6	108
3	2,5-Pyrrolidinedione	8.6	99
4	Benzyl nitrile	8.7	117
5	Benzenepropanenitrile	10.1	131
6	Indole	10.9	117
7	1H-Indole, 3-methyl	12.2	131
8	Diethyl phthalate	14.8	222

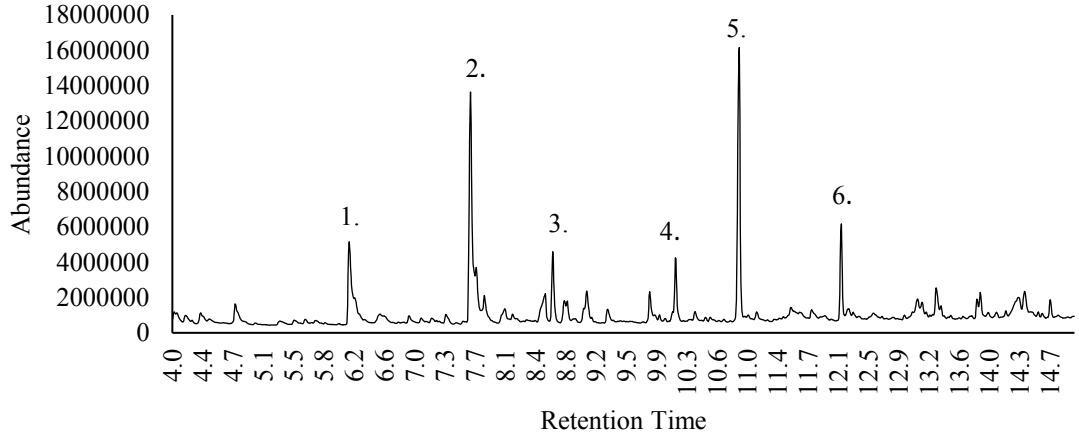

Figure A.31: Total chromatogram of Unknown Subsample A identified bead particle with pyrolytic products numerically labeled.

Table A.31: Pyrolytic products of Figure A.31 with retention times and parent m/z's.

Sample Peak	Pyrolytic Product	Retention Time (±0.5 minutes)	Parent m/z
1	Phenol	6.2	94
2	4-Methylphenol	7.7	108
3	Benzyl nitrile	8.7	117
4	Benzenepropanenitrile	10.1	131
5	Indole	10.9	117
6	1-H-Indole, 2,2-methyl	12.1	131

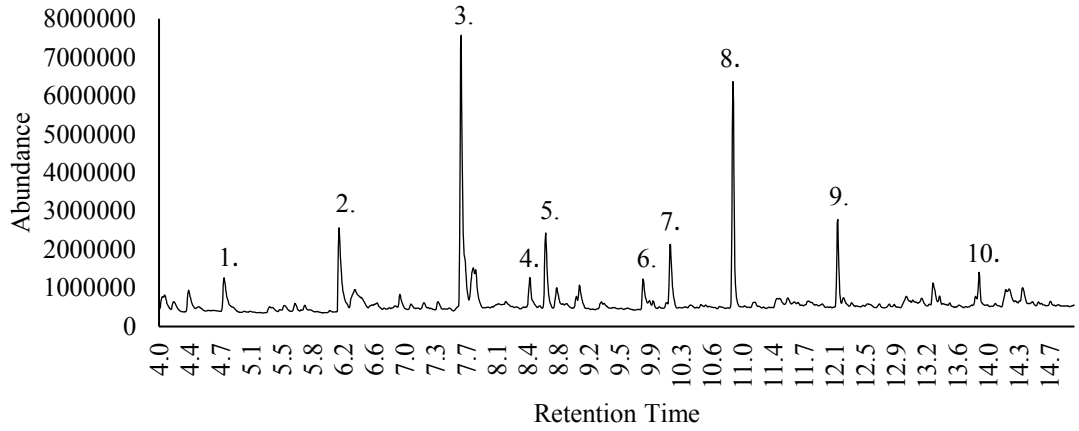

Figure A.32: Total chromatogram of Unknown Subsample A identified bead particle with pyrolytic products numerically labeled.

Table A. 32: Pyrolytic products of Figure A.32 with retention times and parent m/z's.

Sample Peak	Pyrolytic Product	Retention Time (±0.5 minutes)	Parent m/z
1	Styrene	4.8	104
2	Phenol	6.2	94
3	4-Methylphenol	7.7	108
4	2,5-Pyrrolidinedione	8.5	99
5	Benzyl nitrile	8.7	117
6	Benzofuran-2,3-dihydro	9.9	120
7	Benzenepropanenitrile	10.1	131
8	Indole	10.9	117
9	1H-Indole, 4-methyl	12.2	131
10	Bibenzyl	13.9	182

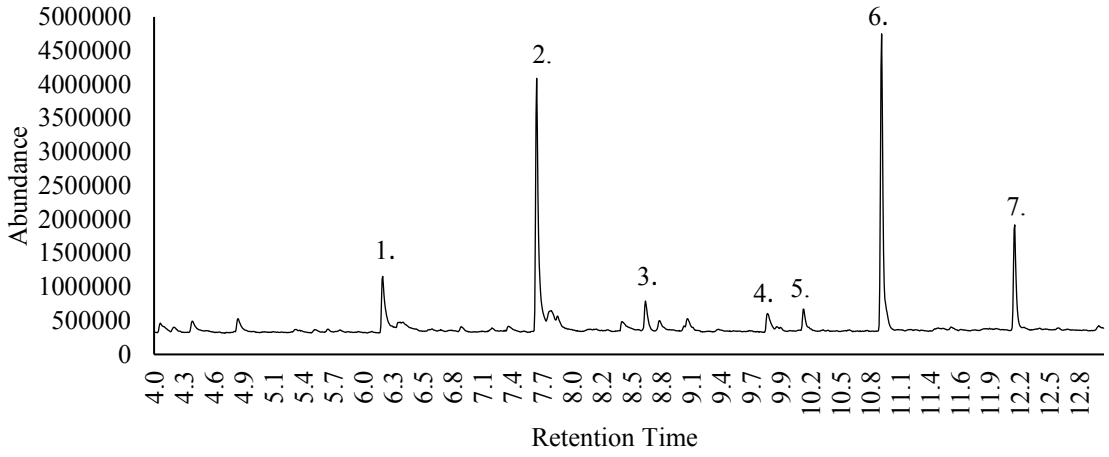

Figure A.33: Total chromatogram of Unknown Subsample A identified bead particle with pyrolytic products numerically labeled.

Table A.33: Pyrolytic products of Figure A.33 with retention times and parent m/z's.

Sample Peak	Pyrolytic Product	Retention Time (±0.5 minutes)	Parent m/z
1	Phenol	6.2	94
2	4-Methylphenol	7.6	108
3	Benzyl nitrile	8.7	117
4	Benzofuran-2,3-dihydro	9.9	120
5	Benzenepropanenitrile	10.2	131
6	Indole	11.0	117
7	1H-Indole, 4-methyl	12.2	131

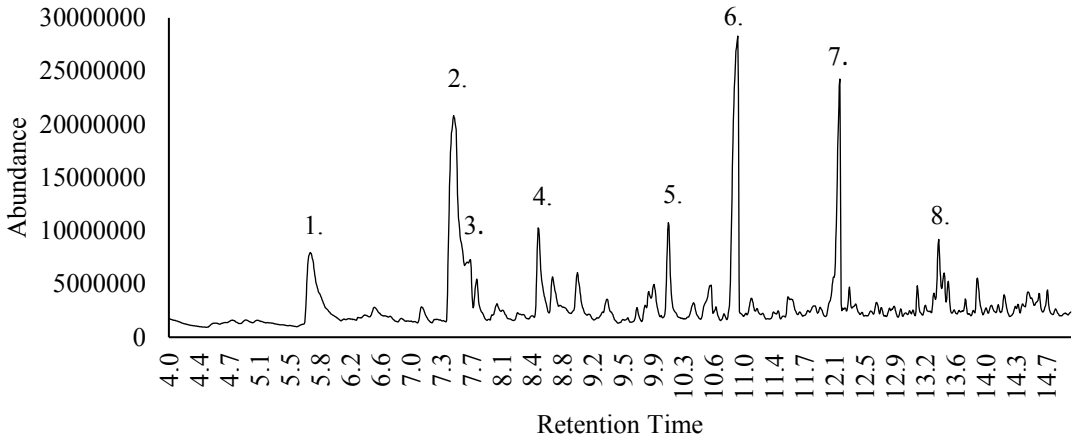

Figure A.34: Total chromatogram of Unknown Subsample A identified bead particle with pyrolytic products numerically labeled.

Table A.34: Pyrolytic products of Figure A.34 with retention times and parent m/z's.

Sample Peak	Pyrolytic Product	Retention Time (±0.5 minutes)	Parent m/z
1	Phenol	5.8	94
2	4-Methyl phenol	7.5	108
3	2,5-Pyrrolidinedione, 1-methyl	7.8	113
4	Benzyl nitrile	8.5	117
5	Benzenepropanenitrile	10.1	131
6	Indole	10.9	117
7	1-H-Indole, 3-methyl	12.1	131
8	Ethaneperoxoic acid, 1-cyano-1-ethyl ester	13.4	219

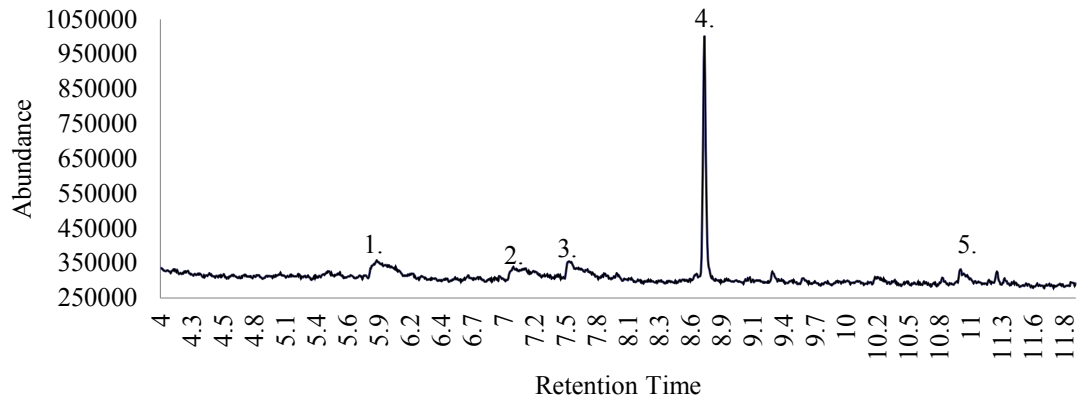

Figure A.35: Total chromatogram of Unknown fiber particle with pyrolytic products numerically labeled.

Table A.35: Pyrolytic products of Figure A.35 with retention times and parent m/z's.

Sample Peak	Pyrolytic Product	Retention Time (±0.5 minutes)	Parent m/z
1	Phenol	5.9	94
2	2-Pyrrolidinone, 1-methyl	7.1	99
3	4-Methyl phenol	7.6	108
4	Cyclopentasiloxane, decamethyl	8.7	370
5	Indole	11.1	117

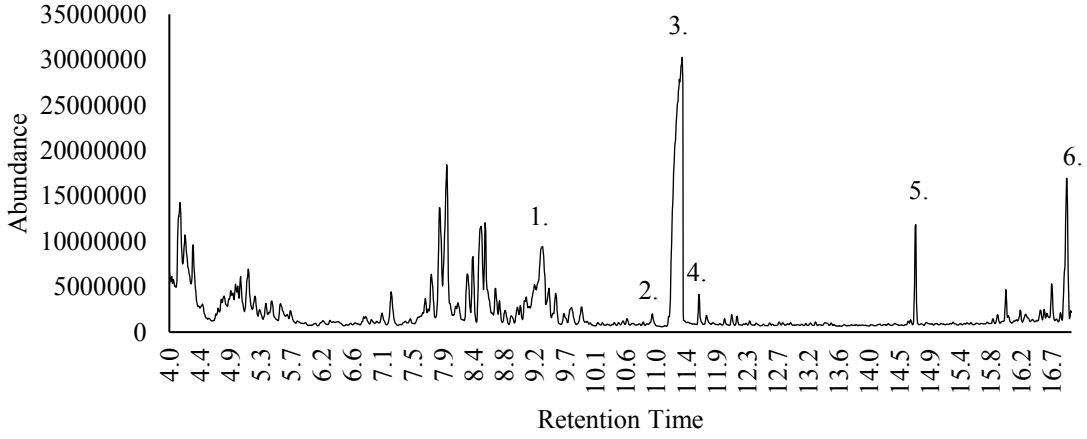

Figure A.36: Total chromatogram of Unknown fiber particle with pyrolytic products numerically labeled.

Table A.36: Pyrolytic products of Figure A.36 with retention times and parent m/z's.

Sample Peak	Pyrolytic Product	Retention Time (±0.5 minutes)	Parent m/z
1	p-Chlorophenyl carbamate	9.2	
2	Naphthalene	11.0	128
3	1,2-Benzenedicarboxylic acid	11.3	166
4	Triacetin	11.6	218
5	Diethyl phthalate	14.8	222
6	Benzoic acid, heptyl ester	16.8	220

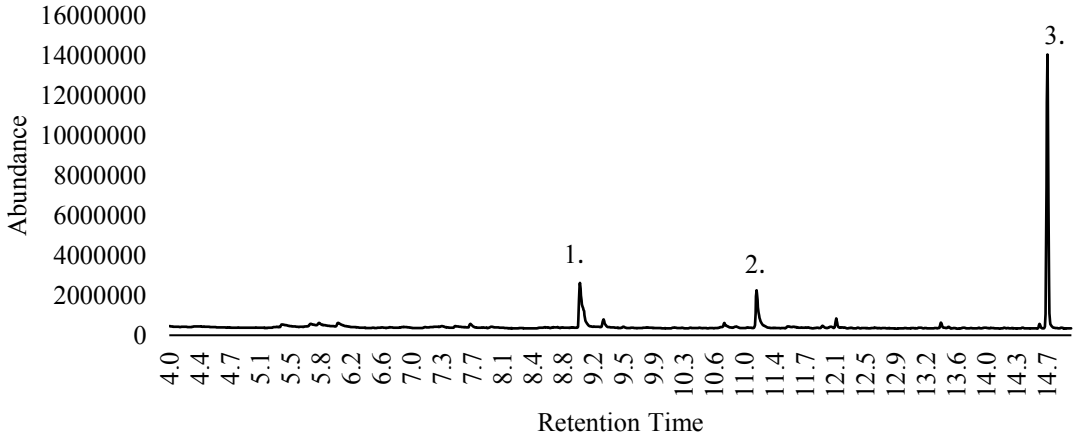

Figure A.37: Total chromatogram of Unknown fiber particle with pyrolytic products numerically labeled.

Table A.37: Pyrolytic products of Figure A.37 with retention times and parent m/z's.

Sample Peak	Pyrolytic Product	Retention Time (±0.5 minutes)	Parent m/z
1	Ethyl benzoate	9.0	150
2	1,2-Benzenecarboxylic acid	11.2	166
3	Diethyl phthalate	14.7	222

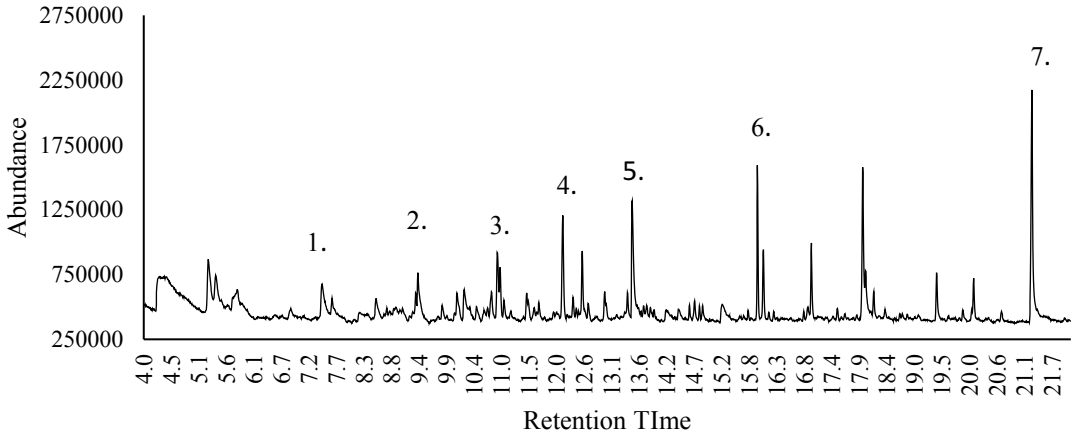

Figure A.38: Total chromatogram of Unknown fiber particle with pyrolytic products numerically labeled.

Table A.38: Pyrolytic products of Figure A.38 with retention times and parent m/z's.

Sample Peak	Pyrolytic Product	Retention Time (±0.5 minutes)	Parent m/z
1	4-Methyl phenol	7.5	108
2	Hexanedinitrile	9.3	108
3	Indole	10.7	117
4	Cyclopentanone	12.0	84
5	2,3,7-Trimethylindole	13.5	159
6	1,2,3,7-Tetramethylindole	16.0	173
7	1,8-Diazacycloetetradecane	21.3	198

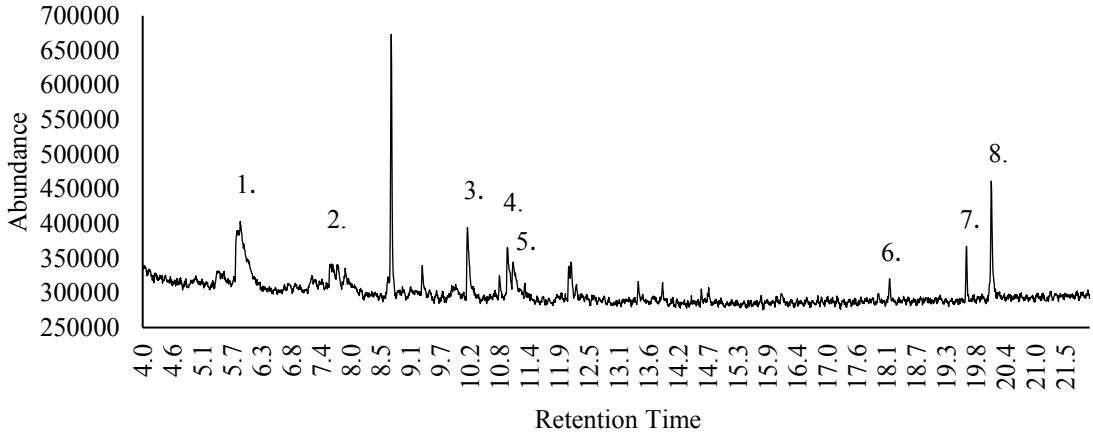

Figure A.39: Total chromatogram of Unknown fiber particle with pyrolytic products numerically labeled.

Table A.39: Pyrolytic products of Figure A.39 with retention times and parent m/z's.

Sample Peak	Pyrolytic Product	Retention Time (±0.5 minutes)	Parent m/z
1	Phenol	6.0	94
2	4-Methylphenol	7.5	108
3	1,3-Cyclopentadiene	10.2	66
4	Indole	10.9	117
5	Benzofuran, 2,3-dihydro-2-methyl	11.0	134
6	Hexadecanoic acid, methyl ester	18.0	270
7	Benzene	19.7	78
8	2-2-Propane	20.2	132

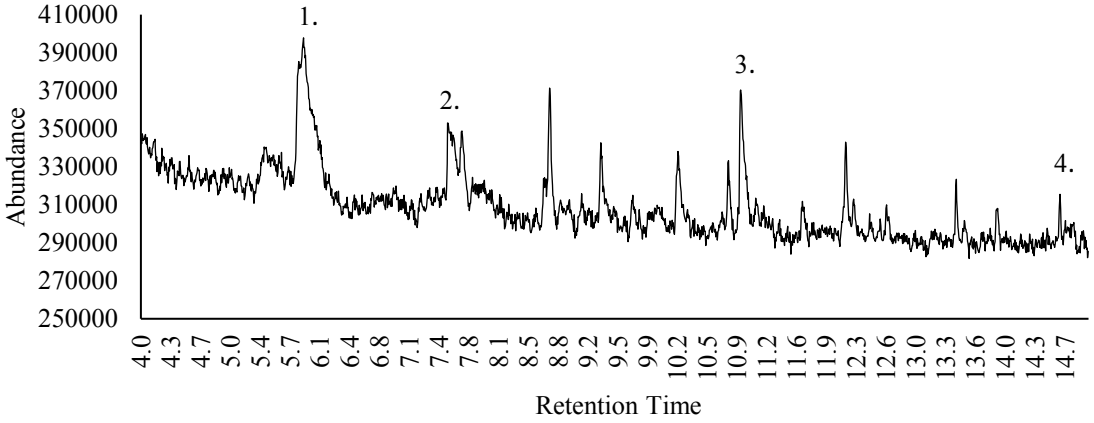

Figure A.40: Total chromatogram of Unknown fiber particle with pyrolytic products numerically labeled.

Table A.40: Pyrolytic products of Figure A.40 with retention times and parent m/z's.

Sample Peak	Pyrolytic Product	Retention Time (±0.5 minutes)	Parent m/z
1	Phenol	6.0	94
2	4-Methylphenol	7.5	108
3	Indole	11.0	117
4	Bibenzyl	14.6	182

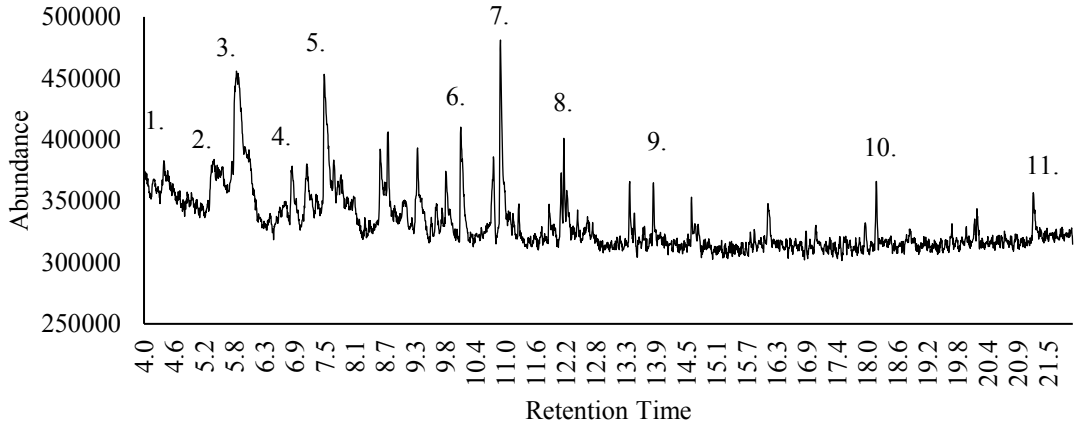

Figure A.41: Total chromatogram of Unknown fiber particle with pyrolytic products numerically labeled.

Table A.41: Pyrolytic products of Figure A.14 with retention times and parent m/z's.

Sample Peak	Pyrolytic Product	Retention Time (±0.5 minutes)	Parent m/z
1	Styrene	4.4	104
2	Benzaldehyde	5.2	106
3	Phenol	5.8	94
4	Indene	6.7	116
5	4-Methylphenol	7.5	108
6	Benzenepropanenitrile	10.1	131
7	Indole	10.9	117
8	1,3,5-Triazine-2,4,6-trione	12.2	249
9	Bibenzyl	13.9	182
10	Hexadecanoic acid, methyl ester	18.1	270
11	1,8-Diazacyclotetradecane, 2,7-dione	12.2	226

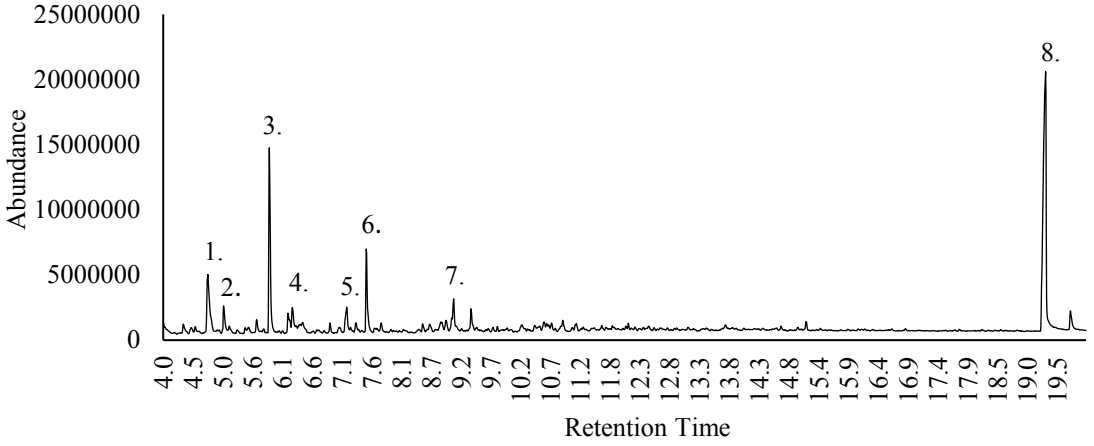

Figure A.42: Total chromatogram of Unknown bead particle with pyrolytic products numerically labeled.

Table A. 42: Pyrolytic products of Figure A.42 with retention times and parent m/z's.

Sample Peak	Pyrolytic Product	Retention Time (±0.5 minutes)	Parent m/z
1	Styrene	4.7	104
2	2,4-Hexadienal	5.0	96
3	Benzaldehyde	5.8	106
4	Benzonitrile	6.3	103
5	Indene	7.1	116
6	Acetophenone	7.5	120
7	Naphthalene	9.0	128
8	1,3,5-Triazine-2-4-diamine, 6 phenyl	19.3	187

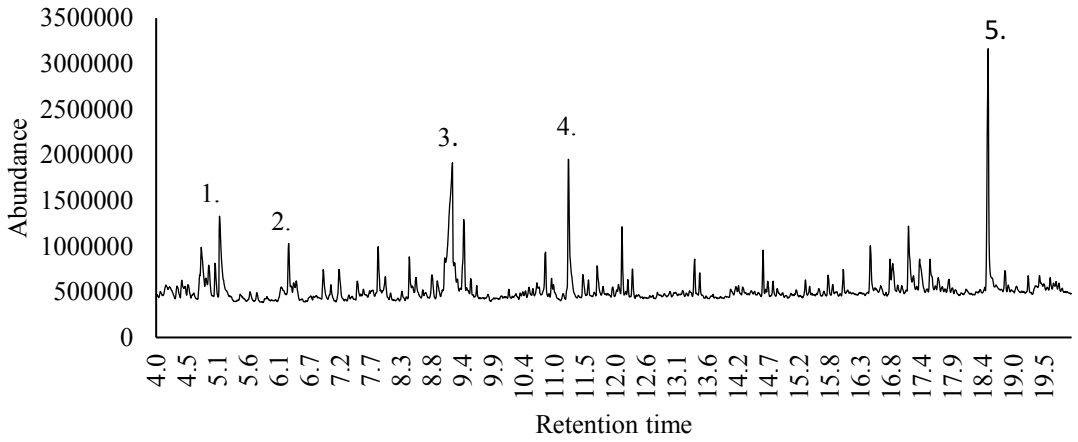

Figure A.43: Total chromatogram of Unknown bead particle with pyrolytic products numerically labeled.

Table A.43: Pyrolytic products of Figure A.43 with retention times and parent m/z's.

Sample Peak	Pyrolytic Product	Retention Time (±0.5 minutes)	Parent m/z
1	Butanoic acid, 4-hydroxy	5.2	104
2	1-Decene	6.3	140
3	Benzoic acid	9.1	122
4	1,2-Benzenedicarboxylic acid	11.2	166
5	n-Hexadecanoic acid	18.6	256

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