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Research article

Morphometric effects of various weathered and virgin/pure microplastics on sac fry zebrafish (*Danio rerio)*

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Abstract: Microplastics (5 mm to1 nm) and plasticizers are ubiquitous worldwide in waterways, beaches, sediments, and biota. Ingestion of microplastics by various marine species and bioaccumulation of plasticizers continues to be of concern. Additionally, microplastics act as a carrier for the transport of persistent organic pollutants and some harmful microorganisms, increasing the hazard to aquatic species. Microplastics vary in composition based on their monomeric component and the specific plasticizer(s). There is a large data gap in our understanding of the biological toxicity of the different plastic polymers. The results presented here examine gross morphological alterations in sac fry zebrafish as a result of exposure to weathered microplastics and virgin/pure plastic polymers. Embryos were exposed from 3 hours post fertilization (hpf) to 96 hpf with samples of weathered microplastics from estuaries in Newark Bay, NJ, as well as commercially available virgin/pure plastics at concentrations of 1 μg/mL or 10 μg/mL. The Newark Bay microplastics were chemically identified using pyrolysis GC-MS. The three field samples were composed primarily of polyethylene (FPE), polypropylene (FPP) and polyvinyl chloride vinyl acetate mixture (FVA). Significant morphometric changes ($P< 0.05$) were noted between the control zebrafish and the treated groups in the embryonic zebrafish samples for the Newark Bay, weathered samples following statistical analysis of morphometric data. The commercial microplastics tested included: low density polyethylene (LDPE), medium density polyethylene (MDPE), high density polyethylene (HDPE), polypropylene (PP), polystyrene (PS), polyvinyl chloride (PVC), sodium polyacrylate (SPA), polyethylene terephthalate (PET), polyurethane (PUR), poly methyl-methacrylate (PMMA), polyethylene (co-vinyl-acetate) (PEVA), and polystyrene (co-acrylonitrile) (PSAN). Significant changes were seen in total body length in all three Newark Bay field sample microplastics, as well as virgin/pure microplastic treatment

groups PET, PUR, PMMA. The pericardial sac size was found significantly altered in FPP 10 μg/mL sample plastic as well as pure microplastic treatment groups HDPE, SPA, PET, PUR, PMMA, PP, and PEVA. The interocular distance was found to be significantly changed in the pure microplastic treatment groups HDPE and PET. The pericardial sac size was the most sensitive endpoint measured followed by total body length. The least sensitive endpoint was interocular distance. These results highlight the associated toxicity with both weathered and lab standard grade microplastics exposure to treated zebrafish developing embryos. The laboratory induced cardiac and growth alterations following laboratory microplastic exposure could be examined in field populations exposed to high microplastic concentrations.

Keywords: microplastic toxicity; pure plastics; *Danio rerio;* zebrafish; morphometrics

1. Introduction

"Plastic" once meaning to shape or mold, is now an umbrella term for a large class of anthropogenic organic polymers. First synthesized in the mid-19th century and perfected in 1907, Leo Baekeland, a New York chemist, discovered Bakelite, one of the first fully synthetic liquid resins that would retain shape and form under any circumstance [1]. Many variations followed, but not until after World War II did large-scale production begin, dating back to ~1950s [2]. These substances are derived from oil, natural gas, or coal, and sometimes synthesized with other elements such as oxygen, nitrogen, chlorine, or sulfur, to form various hydrocarbon polymers with diverse side chains [3]. When the monomers form a simple polymer chain the plastic is a thermoplastic. Thermoplastics are meltable and constitute approximately 92 percent of all plastics. Plastics in which the carbons form two and three-dimensional networks are known as thermosets, non-meltable plastics. Thermosets, such as epoxy or unsaturated polyester resins (UPRs), are molded after the chemical mixing has occurred but before the plastic cures [3]. Plastics are inexpensive to manufacture, have durable and lightweight properties and as a result, they are used in commercial, industrial, medicinal, and municipal applications. Waste plastics however, have been contaminating the environment due to their mostly single-use intent, low recovery value and resistance to degradation [4]. Plastic production has exponentially grown from approximately two million metric tons (Mt) in 1950, to 380 million Mt in 2015 annually. It is estimated that 8300 million Mt of virgin plastic have been produced to date globally. With approximately 9 percent of plastics recycled, 12 percent incinerated, and 79 percent accumulates in landfills or other natural environments [2], such as oceans, lakes, seas, rivers, coastal areas, and even Polar Regions [5] making plastic a global environmental pollutant. The first reports of plastic debris in the marine environments were reported in the early 1970s but received little notice. In the decades following, data on ecological consequences of plastic debris accumulated and increasing research interest ensued. Of particular concern are the smaller pieces and those not visible to the naked eye [6], since research is lacking into their environmental impacts.

Microplastics are anthropogenic organic polymers, with dimensions of 5 mm to 1 nm according to the National Oceanic and Atmospheric Administration (NOAA) [7–9]. Microplastic particles vary in size, specific density, chemical composition, and shape [5]. Microplastics are either manufactured as small particles known as primary microplastics or are the result of larger plastic pieces degrading known as secondary microplastics. Primary microplastics are found in personal care products marketed

as “micro-beads” for exfoliation, in air-blasting media for removing rust or paint from machinery, engines, or boat hulls, or virgin plastic production pellets to produce larger plastic parts [10]. Secondary microplastics are derived from larger plastic debris that breakdown due to a combination of chemical, physical and even rare biological processes [11]. The breakdown can continue into the nanometer size range.

Microplastics entering freshwater often can be sourced back to wastewater treatment plants (WWTPs). Primary microplastics and secondary microfibers in the effluent of WWTPs are thought to be a major source in freshwater bodies [11]. WWTPs are unlikely to remove microplastics by the existing screening of debris as coarse screens retain particulates >10 mm, and some of the finest screens are only able to retain particulates >1.5 mm [11,12]. The density of microplastic particles, which vary between polymers or vary depending on additional weight from biofouling, contributes to the particle’s fate. Dense particles settle into the sludge, and low-density particles are discharged with the effluent. Effluents enter receiving waters and sewage sludges can be land applied. It is common practice in Europe to process sewage sludge as an agricultural fertilizer. Harmful substances are regulated within sludge applied to land, yet microplastics are not considered harmful or hazardous, and are therefore not regulated. It is estimated that 400,000 tons are inadvertently applied to land annually, which exceeds the mass estimated in ocean surface waters globally [11]. Another major route of microplastics entering the environment is through the degradation of larger plastic debris. A combination of physical, chemical, and biological processes diminishes the structural integrity of macroplastic pieces, which leads to the fragmentation into microplastic particles. Ultraviolet (UV) radiation from the sun causes oxidation of the polymer matrix which leads to bond cleavage and fragmentation of the plastic. Over time the macroplastics turn brittle, crack, yellow and are reduced to smaller and smaller microplastic debris [13].

Microplastic ingestion by aquatic organisms including algae, crustaceans, echinoderms, bivalves, fish, seabirds, as well as higher trophic level organisms has been reported [4,14–17]. Once ingested, the absorption, distribution, metabolism, and excretion (ADME) varies depending on the organism as well as the characteristics of the microplastic [14]. The exposure outcomes for ingestion of macro- and micro-plastics have primarily focused on internal damage due to ingestion, choking hazard and entanglement. Some research in marine invertebrates show links to sub-lethal effects such as reduced reproduction, reduced growth and fitness which result from physical effects of ingestion including lacerations, inflammatory responses, and replacement of digestible food exhibited in lower energy intake and output [11]. Other research focuses on plasticizers, which give plastics their specific physical properties: flexibility, rigidity, UV stability, flame retardation, anti-microbial factors, and coloring [11,18,19]. Toxicity studies examining the produced plastic or specific polymers are generally lacking. Several plasticizers have been identified as toxic or endocrine disrupting including bisphenol-A, di-*n*-butyl phthalate, di-(2-ethylhexyl) phthalate, polybrominated diphenyl ethers (PBDEs) [20,21]. Sorption of persistent organic pollutants (POPs), due to the typical hydrophobicity of the microplastics and their ability to transport these chemicals through the environment [22], as well as the ability for microplastics to attract biofilms and the attachment of harmful microorganisms [14] is of concern.

The aforementioned concerns are valid but frequently overlooked is the inherent toxicity of the microplastics themselves. Plastic is an umbrella term for many chemical compounds with vastly different chemical structures (Figure 1). It has long been thought that plastics are biochemically inert [22], which may be true for some microplastics, but perhaps not all microplastics. Little is known of the toxic effects of microplastics, that can translocate into tissues. Toxic effects of microplastics

may be size dependent, as seen in a study where the marine copepod *Tigriopus japonicas,* treated with 0.05, 0.5 or 6 μm PS microbeads at concentrations of 0, 0.125, 1.25, 12.5 and 25 μg/mL, showed some reproductive effects such as reduced fecundity [11,23]. The chemical backbone and the side chains can undergo metabolism and result in compounds with reactive functional groups or that could result in toxicity. Less is known about the effects of composition specific nano-sized microplastic compounds based on their functional groups and possible interactions in tissues.

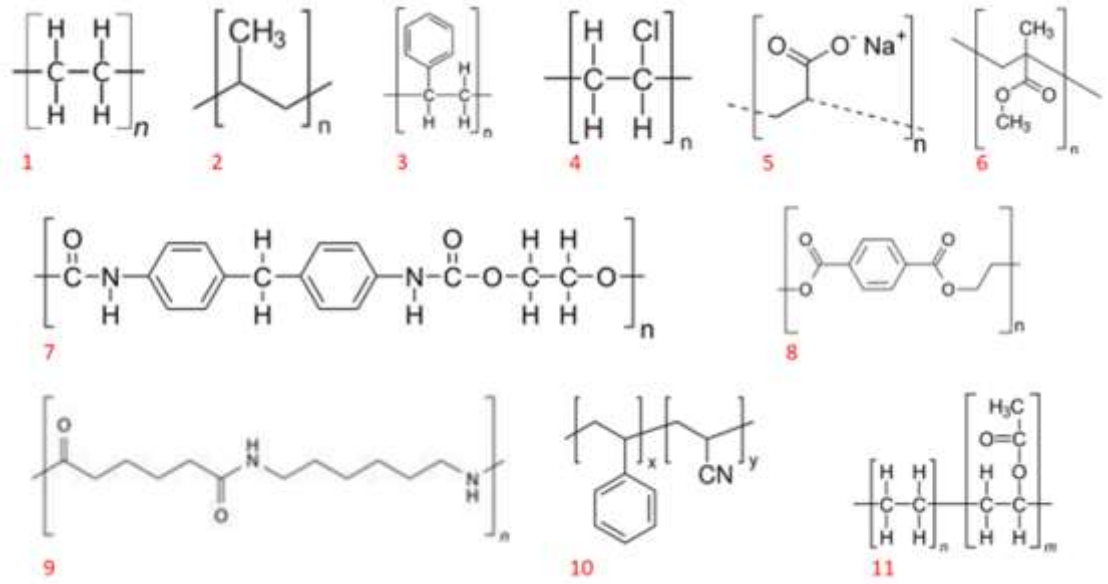

Figure 1. Chemical structures of pure microplastics analyzed. 1) polyethylene (PE), 2) polypropylene (PP), 3) polystyrene (PS), 4) polyvinyl chloride (PVC), 5) sodium polyacrylate (SPA), 6) poly (methyl methacrylate) (PMMA), 7) polyurethane (PUR), 8) polyethylene terephthalate (PET), 9) nylon 6,6 (NYL6/6), 10) poly (styrene-co-acrylonitrile) (PSAN), 11) poly (ethylene-co-vinyl acetate) (PEVA).

This paper presents the results from exposing embryonic zebrafish (*Danio rerio*) to a panel of virgin/pure microplastic compounds (Table 1) with diverse monomeric configurations, as well as weathered field samples from Newark Bay, New Jersey. It was hypothesized that different microplastic compounds would not be biochemically inert and would interfere with development in embryonic zebrafish. Based on morphometric endpoints total body length (TBL), pericardial sac size (PCS), and interocular distance (IOD), structurally different plastics caused significant changes in the examined zebrafish morphometrics.

Table 1. Summary of significant changes in microplastic type with 1 indicating 1 μg/mL and 10 indicating 10 μg/mL, and morphometric endpoints total body length (TBL), Pericardial sac (PCS), and interocular distance (IOD). Arrow indicates significance, downward direction refers to a decrease and upward direction refers to an increase in size. *P-value* ≤0.05.

Microplastic Sample	TBL	PCS	IOD
Field Polypropylene 1			
Field Polypropylene 10	↓	↓	
Field Polyethylene 1	↓		
Field Polyethylene 10	↓		
Field PVC, Vinyl Acetate 1	↓		
Field PVC, Vinyl Acetate 10	↓		
Low Density Polyethylene 1			
Low Density Polyethylene 10			
Medium Density Polyethylene 1			
Medium Density Polyethylene 10			
High Density Polyethylene 1			↑
High Density Polyethylene 10		↑	
Polystyrene 1			
Polystyrene 10			
Sodium Polyacrylate 1		↓	
Sodium Polyacrylate 10			
Polyvinyl Chloride 1			
Polyvinyl Chloride 10			
Polyethylene Terephthalate 1	↓		
Polyethylene Terephthalate 10	↓	↓	
Polyurethane 1	↓	↓	
Polyurethane 10	↓	↓	
Poly (Methyl Methacrylate) 1	↓	↓	
Poly (Methyl Methacrylate) 10	↓	↓	
Polypropylene 1		↑	
Polypropylene 10		↑	
Polyethylene co-Vinyl Acetate 1	↑	↑	
Polyethylene co-Vinyl Acetate 10			
Polystyrene co-Acrylonitrile 1			
Polystyrene co-Acrylonitrile 10			

2. Materials and methods

2.1. Zebrafish husbandry

Wildtype, AB strain Zebrafish (*Danio rerio),* were obtained from Zebrafish International Resource Center (ZIRC) and used for all morphometric experiments in this study. The zebrafish were maintained and bred in a recirculating aquatic habitat system with fish system water, carbon and sand filtered municipal tap water, on a 14-h light: 10-h dark cycle. The system was maintained at 28 ℃, <

0.05 ppm nitrite, < 0.2 ppm ammonia, and pH 7.2–7.7. The zebrafish were fed a diet of hatched Artemia cysts brine shrimp (PentairAES) and a 1:4-part mixture of Aquatox Fish Diet flake food (Zeigler Bros, Inc.) and Tetramin (Tetra) respectively. Husbandry protocol (#08-025) was approved by the Rutgers University Animal Care and Facilities Committee and followed for all experiments.

2.2. Plastic preparation

Table 2. List of virgin/pure microplastics tested in this study. Tested with asterisks indicates compound was tested, without asterisks indicates not yet tested. Abbreviation of microplastic type on table as Abbrev. Grades tested include reference standards (Ref Std), analytical standard (Analyt Std) and laboratory standard (Lab Std) according to Sigma Aldrich.

Tested	#	Compound Name	Abbrev	Index number (lot #)	Grade
*	1	Polyethylene High Density	HDPE	MKBZ2763V	Ref Std.
*	2	Polyethylene Medium Density	MDPE	MKBT4619V	N/A
*	3	Polyethylene Low Density	LDPE	MKCB9440	Ref Std.
*	4	Polystyrene	PS	MKBV4969V	Anylyt. Std.
	5	Polyamide (Nylon) 6,6	PA	L5472375VCSGF96375831	Lab Std.
*	6	Poly (Methyl methacrylate)	PMMA	BCBR6742V	Lab Std.
*	7	Poly (Styrene-co-acrylonitrile)	PSAN	MKBW3169V	Lab Std.
*	8	Sodium Polyacrylate	SPA	MKBT8619V	Lab Std.
*	9	Poly (Vinyl chloride)	PVC	MKBV0958V	Lab Std.
*	10	Polyethylene Terephthalate	PET	LS472375VCSGF27383384	Lab Std.
*	11	Polyurethane	PU	GF2691891	Lab Std.
*	12	Polyethylene (co-Vinyl acetate)	PEVA	MKBH6596	Lab Std.
*	13	Polypropylene	PP	MKCH2207	Lab Std.

Three individual microplastics of unknown composition were chosen from field samples collected from Newark Bay estuarine locations in New Jersey via manta trawl according to Eriksen *et al.* [24], followed by recovery of microplastics after processing with the Fenton reaction at a concentration of 0.05M Iron (Fe(II)) and equal part 30% hydrogen peroxide heated to 75 ℃ for 30 minutes, repeated addition of 30% hydrogen peroxide if necessary, (see *Supporting Information*) protocol. Individual larger plastic particles were pulverized via Dremel with a diamond tip followed by glass homogenization in 95% ethanol and finally the slurry poured through a U.S.A. Standard testing 2 mm sieve number 10 (Fisher Scientific). The filtered effluent was centrifuged at 8000 rpm for 15 min. The supernatant was discarded, and the pellet was completely dried by a stream of air. A small aliquot of the three field samples were sent to the NOAA laboratory, where they were analyzed via Pyrolysis GC-MS (see *Supporting Information*) and identified as polypropylene (PP), polyethylene (PE), and a polyvinyl chloride (PVC)/ vinyl acetate co-polymer (VA). The field samples are referred to as field PP (FPP), field PE (FPE) and field PVC/VA (FVA). Pure microplastic samples were purchased from Sigma-Aldrich and were processed identically to the microplastics of unknown composition. The pure samples, purchased from Sigma Aldrich included and are referred to as: Low density polyethylene (LDPE), medium density polyethylene (MDPE), high density polyethylene (HDPE), polypropylene (PP), polystyrene (PS), polyvinyl chloride (PVC), sodium polyacrylate (SPA), polyethylene terephthalate (PET), polyurethane (PUR), poly methyl-methacrylate (PMMA), polyethylene (co-vinyl-acetate) (PEVA), and polystyrene (co- acrylonitrile) (PSAN), (See Figure 1 for structures and Table 2 for lot # and purity).

2.3. Experimental design

A Fish Embryo Acute Toxicity Test (FET) protocol was used and is described below. Female and male AB strain zebrafish groups were cohabitated for breeding. The following morning the fertilized embryos were collected, cleaned, and rinsed 3 times in egg-water (autoclaved, aerated, 60ppm instant ocean in Millipore water). Randomly sorted embryos at 3-hour post fertilization (hpf) (512-cell stage) [25] were selected and placed in 1-dram borosilicate glass vials with one microplastic type per embryo, with 20 embryos per treatment (Table 1). Each embryo was exposed to a single microplastic type in 1mL of egg-water composed of 0.006% instant ocean in Millipore water, autoclaved and aerated. The microplastics tested were: FPE, FPP, FVA, HDPE, MDPE, LDPE, PP, PS, PVC, PET, PUR, PMMA, SPA, PEVA, or PSAN. The individual embryos were exposed to 1 or 10 μg/mL of a single microplastic particle type. The glass vials were continuously shaken at 26° C for 96 hpf. The shaking maintained the plastics in suspension or in contact with the embryos. At 96-hpf the embryos were sacrificed in 10% buffered formalin. The fixation was uniform across all control and treatment groups, and comparison across all groups were consistent and allow for evaluation of direct chemical effects. The sac fry larvae were then rehydrated with 50% ethanol and stained with alcian blue/alizarin red stain and stored in 100% glycerol. The larvae were photographed with Scion Color Digital Camera, model CFW-1310C on an Olympus SZH-ILLD NO. 005002, Olympus Optical Co., LTD. microscope. Measurements of total body length (TBL), pericardial sac size (PCS), and interocular distance (IOD), were taken using Adobe Photoshop CC 2018, and the data were analyzed with Sigma Plot® 11. The entire experiment was repeated for a total n = 40 per treatment.

2.4. Statistical analysis

SigmaPlot®11 statistical program software was used for all analysis of morphometric endpoints. One-way ANOVA was run to determine significance. Statistical significance was designated at a *P-value* ≤0.05. The software tested for normality and power prior to statistical analysis. The data is presented using box plots that represents area (mm^2) versus microplastic type for pericardial sac size, length (mm) versus microplastic type for total body length as well as interocular distance.

3. Results

3.1. Pericardial sac size

Morphometric measurements on sac fry zebrafish showed significant decrease in pericardial sac size compared to the control in seven of the pure microplastics tested (Figure 2C and 2D). The only significant decrease in pericardial sac size observed from the field collected microplastics was FPP (10 μg/mL) (Figure 2A). Of the pure microplastics tested, significant decrease in pericardial sac size was seen in (panel C) PET (10 μg/mL), PUR (1 μg/mL and 10 μg/mL), and PMMA (1 μg/mL and 10 μg/mL) and in (panel D) SPA (1 μg/mL and 10 μg/mL). Significant increase in pericardial sac size compared to the control was seen in (Figure2B) HDPE (10 μg/mL) as well as in (Figure 3) PP (1 μg/mL and 10 μg/mL), and PEVA (1 μg/mL). All significant results had a *P-value ≤0.05.*

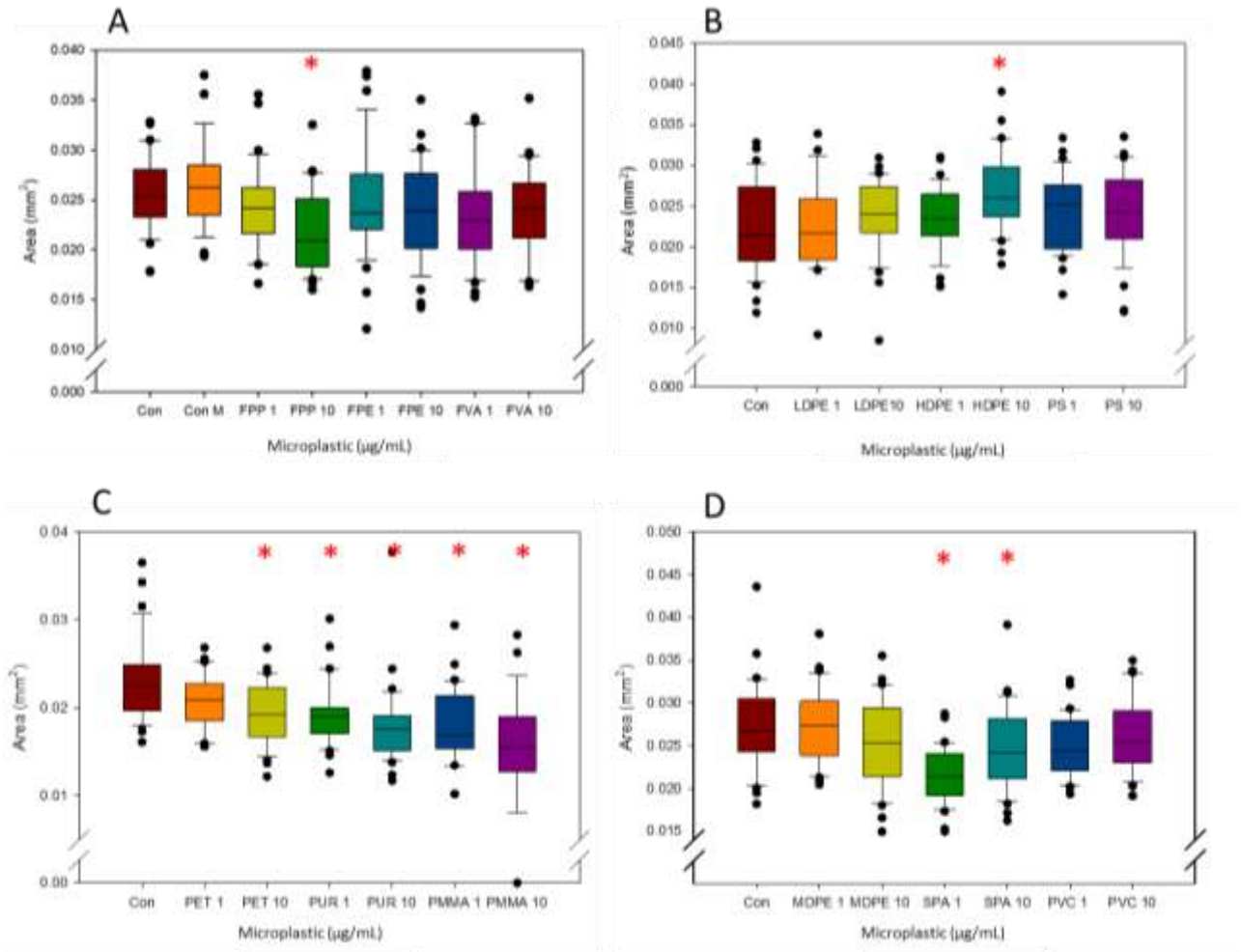

Figure 2. Pericardial Sac Size (in mm^2) versus plastic type, the data are presented as Box Plots. A illustrates on the x-axis Newark Bay Samples Con = control, ConM = control with methylene blue, FPP = Field Polypropylene, FPE = Field Polyethylene, and FVA = Field Vinyl Acetate Copolymer. B illustrates on the x-axis pure microplastics LDPE = low density polyethylene, HDPE = highdensity polyethylene, and PS = polystyrene. C illustrates on the x-axis pure microplastics PET = polyethylene terephthalate, PUR = polyurethane, and PMMA = poly methyl-methacrylate. D illustrates on the x-axis pure microplastics MDPE = medium density polyethylene, SPA = sodium polyacrylate, PVC = polyvimyl chloride. In all box plots along with the plastic type 1 represents 1 µg/mL and 10 represents 10 µg/mL. N = 40. Asterisks indicates significant difference form the control, *P-value* ≤0.05.

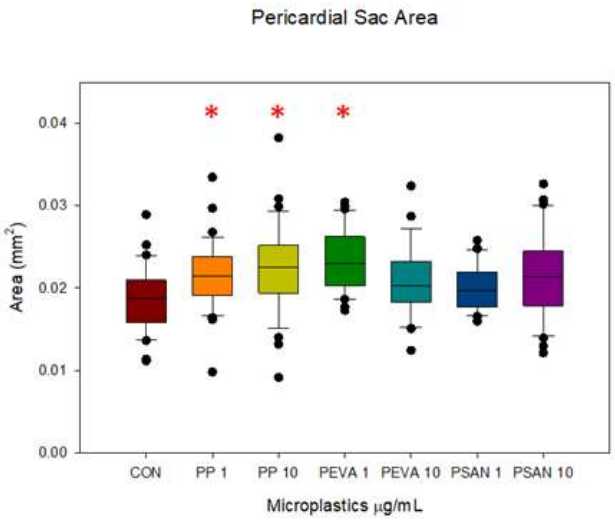

Figure 3. The data are presented as Box Plots Pericardial Sac Size (in mm^2) versus plastic type. In all box plots along with the plastic type 1 represents 1 µg/mL and 10 represents 10 µg/mL. N = 40. Asterisks indicates significant difference from the control *P-value* <0.05.

3.2. Total body length

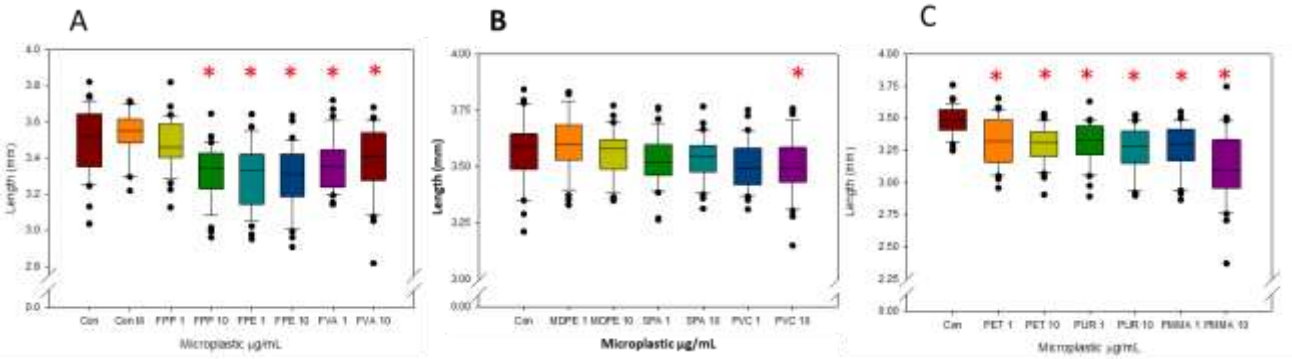

Figure 4. Total Body Length (in mm) versus plastic type, the data are presented as Box Plots.. A illustrates on the x-axis Newark Bay field sample plastics, con = control, con M = control with methylene blue, FPP = Field Polypropylene, FPE = Field Polyethylene, and FVA = Field Vinyl Acetate Copolymer. B illustrates on the x-axis pure microplastics MDPE = medium density polyethylene, SPA = sodium polyacrylate, and PVC = poly vinylchloride. C illustrates on the x-axis pure microplastics PET = polyethylene terephthalate, PUR = polyurethane, and PMMA = poly methyl-methacrylate. In all box plots along with the plastic type 1 represents 1 µg/mL and 10 represents 10 µg/mL. N = 40. Asterisks indicates significant difference from the control *P-value* ≤0.05.

Morphometric measurements on sac fry zebrafish showed significant decrease in total body length compared to the control with the following microplastic samples in (Figure 4.) Figure 4A shows significant decrease in total body length from all field samples tested with the exception of FPP at (1 µg/mL). In Figure 4 B and C PVC (10 µg/mL), PET (1 µg/mL and 10 µg/mL), PUR (1 µg/mL and

10 µg/mL), and PMMA (1 µg/mL and 10 µg/mL) exposure all showed significant decreases in total body length. In Figure 6, PEVA (1 µg/mL) exposure showed a significant increase in total body length. The field collected plastics caused a greater decrease in total body length in comparison to the corresponding pure plastics. Field plastics FPP and FPE resulted in a decrese in total body length that was not observed in the pure PP or PE (See Figure 4A above and Figure 6). All significant results had a *P-value* ≤*0.05*.

3.3. Interocular distance

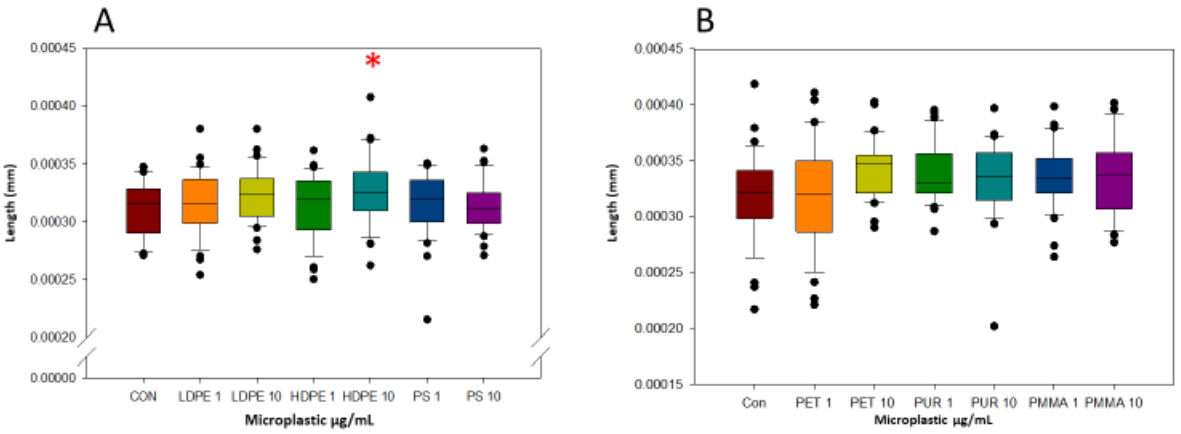

Figure 5. Interocular distance (in mm) versus plastic type, the data are presented as Box Plots. In all box plots along with the plastic type 1 represents 1 µg/mL and 10 represents 10 µg/mL. N = 40. Asterisks indicates significant difference from the control *P-value* ≤0.05.

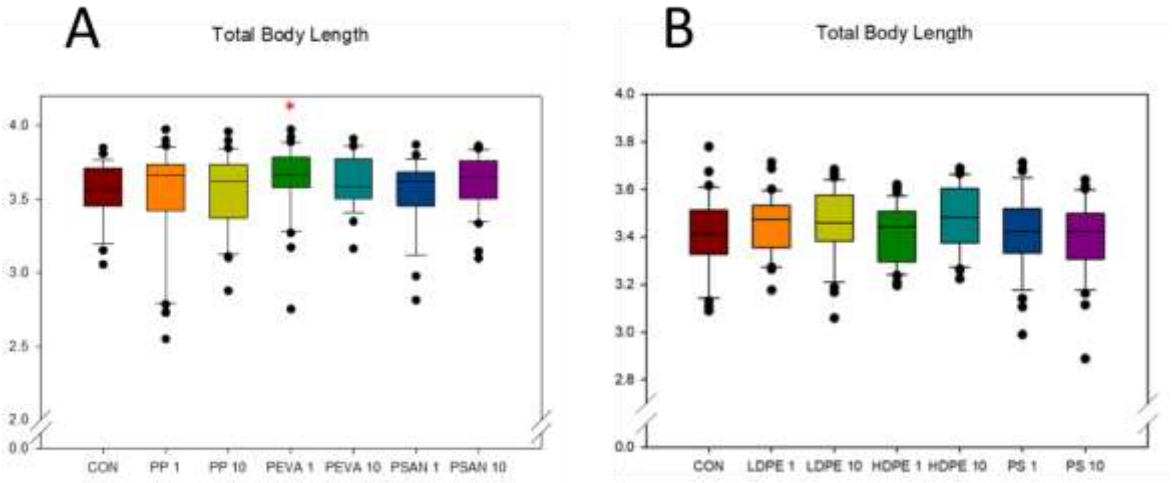

Figure 6. The data are presented as Box Plots Total Body Length (in mm) versus plastic type. All box plots along with the plastic type 1 represents 1 µg/mL and 10 represents 10 µg/mL. N = 40. Asterisks indicates significant difference from the control *P-value* <0.05.

Morphometric measurements on sac fry zebrafish showed significant increase in IOD after microplastic exposure compared to the control with HDPE (10 μg/mL) (Figure 5A***)***. The field collected plastics did not show any significant difference from the control group. IOD showed the least overall effect on the zebrafish development after exposure to field collected and pure plastics. IOD had the least number of significant changes. All significant results had a *P-value ≤0.05.*

4. Discussion

"Plastic," is an umbrella term for hundreds of commercially available polymer materials that all contain structurally unique monomers (Figure 1). There are approximately 20 different groups of pure plastics with differing grades and other diverging characteristics (Thompson et al. 2009b) and close to 5000 grades of plastics mixed with other chemicals, on the market [17,26]. This study focuses on pure microplastics prior to additive modifications. The individual monomers that make up the plastic polymer differ in structure and functional groups. These structural differences influence microplastics' physical and chemical traits and toxicity. Plastics as chemical polymers cannot all be considered the same as their chemical structures give them unique properties for manufacturing and other uses, as well as potential toxicity in living organisms. To date there are few studies looking into potential toxicity of specific pure microplastics polymers, without additives, or sorbed microbiota, or POPs, outside of PE, PS, PP and PVC.

The results in this paper reinforce the hypothesis that structurally different microplastic polymers do result in different observed toxicity in the zebrafish FET assay. It should be noted that buffered formalin fixation followed by morphometric measurements are standard procedures that are accepted for these types of comparisons for toxicant exposure across the toxicological field and can be found in many other studies[27–29]. Borosilicate glass 1-dram vials were used to minimize surface area and the samples were continuously shaken for 96hpf to minimize aggregation, adherence, or floating of microplastic particles. The concentrations of 1 μg/mL and 10 μg/mL were selected to study toxicological responses across plastic polymer types (Figure 1), and likely represent concentrations at the upper end or outside of environmental concentrations. Determining working concentrations of microplastics and nanoplastics for treatments is difficult currently as many studies are not able to collect or quantify particles < 300 μm. The manta trawl collection method collects surface particles [30,31]. In a study by Cohen et al. it was estimated that there are approximately 1 part per billion (ppb), of pieces 300–5000 μm in size, it does not include < 299 μg particles [29]. Besseling et al. points out that the concentration of smaller size fraction microplastics (< 300 μg) are predicted to settle in the water column and accumulate in the sediment [32]. In freshwater system sediments, microplastic concentrations are found to be much higher than in marine environments; in a study from the Netherlands microplastic concentrations were found to be 48–187particles/L [26]. There is a great need to quantify concentrations of nano- and microplastics at all levels of the water column from the air-surface interface, to mid-levels to sediment, due to the varied densities of microplastic types. In fact, the studies of microplastics, especially nanosized, are in their infancy and determining environmentally relevant concentrations are varied and not yet defined.

Total body length (TBL), pericardial sac size (PCS), and interocular distance (IOD) are important developmental endpoints that reflect overall growth, cardiac function, and cranio-facial development. Growth disruption of these early endpoints may lead to change in fitness of larvae. Early life stages of fish are subject to strong selection, due to frequent occurrences of predation [33,34]. In a study by

Duan et al. after treatment of 100nm PS at a concentration of 400 particles/mL in water, at 72hpf particles accumulated in hatched larvae gills and blood, at 96hpf in liver and digestive tract, and after 120hpf in the brain [35]. Other studies have also concluded that nanoplastics impact the zebrafish transcriptome and caused oxidative stress [36,37]. As this study focused on assessing field samples and virgin/pure microplastic types, gross morphometrics were chosen to be quantified to determine if differences in three broad developmental pathways may be affected differently due to the differences in microplastic monomeric structure. At the doses tested (1 and 10 μg/mL) the morphological endpoints did result in statistically significant altered growth parameters. The microplastics did not result in death, delayed hatching, hemorrhage, or a number of other common lesions. Total body length and pericardial sac size were the end points with the largest numbers of statistically significant effects, 12 of 30 microplastic treatments and 11 of 30 microplastic treatments tested, respectively. In 2 of 30 microplastic treatments tested yielded the interocular distance significantly increased (Table 1). These three morphometric parameters combined, highlight the sensitivity of development following exposure to different microplastics at various concentrations.

Regarding the field collected microplastic samples, the total body length was the most sensitive endpoint observed (Figure 4A). The reason for this is not obvious, as the corresponding pure plastic polymers did not result in this effect. FPP and FPE samples showed decreased TBL in comparison to PP and PE pure plastics, which did not produce significant decreases in TBL. Several possibilities may explain these observations: Field collected microplastic samples may have been modified in manufacturing with additives, and/or over time leaching the additives making them more biochemically reactive, or the Fenton procedure may have modified the polymer structure or there may be some other unknown factor. The Fenton treated samples were rinsed in 95% ethanol prior to zebrafish embryo treatment. The ethanol-rinsed Fenton treated field microplastics were not analyzed for iron post rinsing. Non-Fenton treated field plastics were not analyzed for comparison to Fenton treated plastics. In a study by Tagg et al. Fenton reagent had no effect on the surface area of PE, PP, and PVC. Additionally, in their study, the attenuated total reflectance Fourier-transform infrared (ATR-FT-IR) profiles of plastic types tested were found to be insensitive to Fenton's reagent [38]. In contrast, in a study by Hurley et al. the acidity of Fenton reagent was of concern for breakdown of the polymers [39]. Further testing on Fenton treated microplastics prior to embryonic zebrafish treatment would be beneficial. Despite Fenton reagents, structurally (Figure 1), PE and PP would be the least reactive of the plastics tested. Based on the chemical structure of PE and PP, it could be assumed that reactive products upon metabolism would not be formed compared to other microplastics. Based on structure, the FVA would be the most reactive of the three field samples tested in part due to the chlorine and the ester of the vinyl acetate. Although, there may have been an alteration in the field sample that was not solely based on structure. Some effects from field samples not seen in the virgin/pure microplastic samples may have been due to the alterations from ultraviolet (UV) exposure, temperature differences, or additional chemicals to the microplastics while in the field. The breakdown of the mechanical properties of plastics which include impact strength, tensile strength, and elongation of organic polymers is increased when subject to prolonged exposure to UV and temperature differences [40,41].

The PEVA resulted in a statistically significant increase in total body length compared to decreased body length after exposure to FVA (Table 1). The reason for this difference is not obvious, PEVA being the virgin/pure counterpart to FVA, a field sample, have similar monomeric structures but again the FVA most likely was modified with additives. Additional morphometric measurements

of the yolk sac area may be investigated in future studies for further data in the virgin/pure PEVA treated samples compared to the field FVA samples to assess potential metabolic disruption. Pure PET, PUR, and PMMA all caused a decrease in total body length (Figure 4C). No field counterpart was tested for PET, PUR, and PMMA. A general decrease in total body length may suggest an effect on growth parameters related to energy and storage and conversion of energy, since the embryo and sac fry rely on yolk sac for food at this stage. The effect on growth in the developing embryo is a complex interplay between energy source, delivery, and catabolism. In a study done by Lu et al. size-dependent exposure to PS microplastics disrupted lipid metabolism in zebrafish [42,43].

The effect of exposure on the pericardial sac size (PCS) of the developing zebrafish is a unique finding. The field collected samples did not affect pericardial sac size, with the exception of FPP 10 μg/mL, which caused a decrease in PCS. Virgin/pure SPA, PET, PUR, and PMMA all resulted in decreased PCS. In almost all cases of decreased PCS size the organism also had a decrease in TBL. The PCS size may contribute to a decreased cardiac output and decreased nutrient delivery throughout the body potentially resulting in a decrease in TBL. In several cases larval cachexia was observed, which would indicate a disruption in nutrient delivery and uptake. HDPE, PP, and PEVA, caused an increase in PCS. In the cases where PCS size was increased there was no consistent effect on TBL except for PEVA (1 μg/mL). In many cases dealing with POP's and other xenobiotics, the PCS size is normally enlarged due to pericardial edema. In this study it appears that exposure to the pure plastics resulted in a decrease in cardiac size. Further studies of cardiac system components, both physical and mechanical need to be analyzed to determine whether the actual heart is decreased in size and relative to TBL and are the results dependent on micro/nano-plastic type. In a study conducted by Veneman et al. injection of PS particles in the yolk sacs of 2-day old zebrafish embryos, showed that the particles redistributed throughout the bloodstream and accumulated in the heart region [43,44].

IOD did not appear to be a very sensitive endpoint following the exposures except in the case of HDPE (1 μg/mL), where significant increase was observed. This would indicate an alteration in cranio-facial size. To further determine if microplastics are interacting with cranial or neurologic development additional testing to determine individual microplastic particle accumulation regions, as well as behavioral studies for various microplastics. In zebrafish studies, neurobehavioral consequences were seen with treatment of nanoplastics and swimming competence was affected with treatment of nanoplastics [34,37]. In this study the larvae were sacrificed at 96hpf, which may have been premature for accumulation in the brain [35].

The pure microplastics LDPE, MDPE, PVC, PSAN, and PS did not affect the morphometric end points measured. The microplastics that did affect the measured endpoints, PET, PMMA, PUR, and SPA, will be investigated further. LDPE and MDPE, based on chemical structure, seem to be biochemically inert as they have no functional groups. PVC the polymer of vinyl chloride monomers is a known carcinogen and has been linked to angiosarcoma of the liver [45]. Possibly effects of PVC, PS and PSAN are not seen until a later time point or more continuous or chronic treatment

Perhaps as more sophisticated and reactive methods for polymerization of plastics occur, more reactive compounds are created. As microplastics age, the mass/volume of smaller size fractions of micro- and nano-plastics in the environment is increasing. It is often assumed that plastics are biochemically inert, but chemically different microplastics (< 300 nm) need to be thoroughly tested for potential toxicity. Potentially, these compounds in biochemical systems, when small enough to enter tissues, become subject to metabolism and production of reactive intermediates as well as circulating emboli. Future studies will examine specific nanoparticle plastics characterized by

structural characteristics, size distribution, genomic effects within organ systems, and particle distribution within the zebrafish. Based on our current findings, future toxicological studies will concentrate on the mechanism(s) involved in alterations observed in the yolk sac, cardiac development, and growth using biochemical and histological methods.

5. Conclusion

The hypothesis of this study that different microplastic compounds would not be biochemically inert and would interfere with development in embryonic zebrafish, was confirmed. However, between field and virgin/pure microplastics of the relatively same polymer did not always exhibit the same results. The physical and/or chemical differences between the samples may have been enough to elicit different outcomes in morphometric measurements. Some of the microplastic types did not show morphometric differences in the three measurements analyzed, such as LDPE, MDPE, PVC, PSAN, and PS. The microplastics that resulted in organ system effects included PET, PMMA, PUR and SPA and will be examined further.

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Conflict of interest

All authors declare no conflict of interest in this paper.

Supporting Information

Pyrolysis GC-MS

A piece of plastic material <1 mg was placed in a quartz tube that was heat-cleaned three times for 20 seconds at 1200 ℃. The tube then was placed in a platinum coil of CDS-2000 Pyroprobe. The pyroprobe was inserted into CDS-1500 valve GC interface maintained at 320 ℃. The sample in the quartz tube was pyrolyzed by heating the platinum filament to 750 ℃ for 15 seconds, in the presence of hydrogen. The pyrolyzed fragments were then transferred to and separated on a DB-5 fused-silica capillary column. The column oven temperature was initially held at 45 ℃ for 2 minutes and then increased to 320 ℃ at a rate of 20 ℃/ minute. The column oven was held at 320 ℃ for 19 minutes, for a total run time of 34.75 minutes. The hydrogen carrier gas flow rate was 1.2mL/ minute in a constant flow mode.

A two-tier confirmation was used based on unique peak fingerprints and mass spectra of marker peaks providing additional polymer identification.

Ashok Deshpande (NOAA, 2019)

Fenton Reaction (wet peroxide oxidation)

Prepared Iron (Fe (II)) solution at a concentration of 0.05M was made by adding 7.5 g of $FeSO_4$-$7H_2O$ to 500mL of H_2O and 3mL of concentrated Sulfuric acid. Add 20mL of prepared Iron solution (0.05M) to beaker with plastic sample. Add 20mL of 30% hydrogen peroxide to beaker with Iron solution and plastic, let stand at room temperature for 5min. Add stir bar and cover with watchglass, heat to 75 ℃ on hot plate. Use caution as solution can boil violently. Heat for 30 min. If organic material is visible, add 20mL of 30% peroxide, continue to heat. Repeat addition of peroxide until no natural organic material is visible.

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