ANALYSIS OF POLYSTYRENE MICROSPHERE UPTAKE IN PASSIVELY FED DANIO RERIO LARVAE VIA FT-IR SPECTROSCOPY AND FLUORESCENCE-BASED DETECTION

By

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Abstract

Microplastic pollution of marine environments is a pressing problem for the health of aquatic ecosystems and for global food supply. Many studies have been done on detecting and quantifying microplastics in the environment and their impact on marine life. However, most studies exclude nanoscale particles from consideration, focusing instead on larger pieces amenable to visual inspection and common filtration techniques. Studies on microplastic accumulation *in vivo* tend to focus on large fish and sea mammals, and experimental models often use less-than-realistic methods of microplastic exposure (e.g., injection, spike-in, etc.). The focus of this project is to validate Fourier-Transform Infrared (FTIR) spectroscopy as an efficient, accessible tool for detecting and quantifying microplastics in fish tissue. FTIR was used to detect 0.5 um polystyrene particles in passively fed zebrafish larvae up to 7 days post-fertilization. Fluorescent microscopy and spectroscopy also revealed that the fluorescent polymer beads are taken up by the larvae. This accumulation was found to decrease survivability of the larvae up to 7 dpf.

Introduction

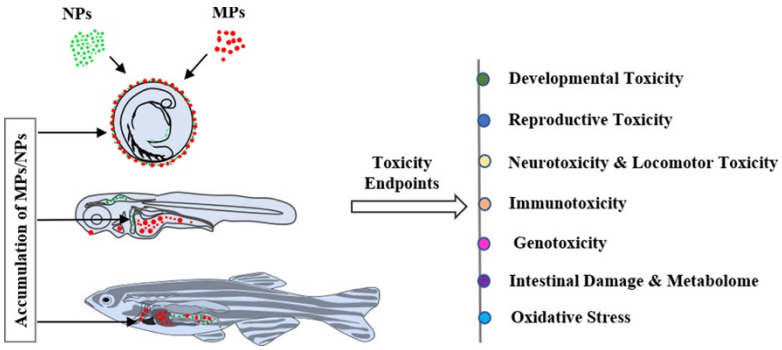
In recent years, much attention has been focused on understanding the sources, distribution, and effects of microplastics in marine environments. The most common plastics found in the ocean include polystyrene, polyethylene, and polypropylene, although nylon, polyester, polyurethane, polyvinyl chloride, and polyethylene terephthalate are also abundant.[1] Microplastics, commonly defined as being 5 mm in diameter or smaller, are of particular concern due to their insolubility in water, durability, and ability to absorb toxic pollutants such as polycyclic aromatic hydrocarbons (PAC).[2] As of 2017, nearly 360 million tons of plastic are mass produced yearly, and roughly 10% of plastics produced end up in the ocean through various means. Most aquatic plastic pollution derives from residential areas, construction, litter, fishing activities, offshore drilling, and sewage outflows.[3] Plastics are often transported to the ocean via rivers, wind dispersal, stormwater runoff, erosion, and illegal dumping. Fishing vessels alone are estimated to contribute anywhere from 500,000 to 1 million tons of plastic pollution to the ocean annually. Plastics derived from these primary sources are problematic due to their ability to entangle marine organisms or be mistaken for food.[4] In fact, a 2016 report recorded over 800 marine species that become entangled or regularly ingest plastic.[5] Plastic shopping bags are often confused with jellyfish by preying sea turtles, and sea mammals can also become contaminated via ingestion of prey species contaminated with plastic.[6] Eriksson and colleagues found microplastics in the excrement of fur seals, which feed primarily on pelagic fish.[6] Once in the ocean, large plastics subjected to mechanical fragmentation, chemical alteration, and ultraviolet photodegradation, which breaks them down into microplastics. Primary sources of microplastics include cosmetics, medicinal products, and personal care items, although several countries have prohibited the use of microplastics for such applications, including the USA and Canada.[4] Other

sources include residential washing machine, the effluent of which contains roughly 1900 synthetic clothing fibers per item per wash cycle.[7] Much of the plastic degradation that occurs is thought to happen on beaches, where micro-collisions with sand and direct exposure to sunlight contribute to a more rapid breakdown. Plastic floating at sea is thought to degrade slower due to lower temperatures and surface films that form; sunken pieces are further shielded from sunlight, contributing to even slower degradation. The anoxic conditions of marine sediment further contribute to the longevity of plastic.[3] Understanding patterns of degradation by identifying surface characteristics can shed light on the type, origin, and history of a particle, as physical forces affect similar plastics in similar ways. This is useful in pinpointing sources and evaluating the trajectory of environmental plastics.[3]

The variable physicochemical properties (size, density, buoyancy, etc.) allow plastics to contaminate nearly every trophic level of aquatic ecosystems, with the lower levels being at the highest risk.[9] The exposure route (e.g., ingestion, contact, etc.) that contributes most to bioaccumulation of microplastics and whether biomagnification from lower to higher trophic levels occurs remain to be determined.[8] Once in the food chain, however, plastics have several remarkable effects at the organismal and ecological level. A study of ascidians along the Israeli coast found high levels of phthalate acid esters (PAE) and microplastics, indicating that sampling invasive filter-feeders could serve as a means of monitoring pollution levels in certain environments.[10] PAEs are widely used as plasticizing agents, and their ubiquity has raised concerns about potential developmental and reproductive toxicity, with quite a bit of evidence suggesting that such effects do occur.[11] Other classes of compounds are used as plastic additives to serve as pigments, surfactants, flame retardants, and UV absorbers.[12] Microplastics are understood to increase the bioavailability of certain compounds in vivo, including bisphenol A

(BPA) and butylated hydroxyanisole (BHA). BPA has been shown to be an endocrine disruptor that contributes to male and female infertility and increase the risk of developing hormone-related tumors (e.g., breast, prostate cancer) and polycystic ovarian syndrome (PCOS).[13] Other reports have shown the neurological and behavior effects of BPA in young children, including increased aggression and hyperactivity.[14] Though BPA usage has been reduced due to increasing health concerns, it is still widely used in the linings of food containers, cans, reusable bottles, carbon paper, and electronics.[14] A 2012 study detected BPA, along with other endocrine disruptors 4-nonylphenol (4-NP) and 4-t-octylphenol (4-t-OP), in wastewater, drinking water, as well as human blood serum and urine.[15]

Of primary significance is the ability of plastics to serve as vectors for the transport of additive compounds throughout the environment and ultimately the human diet. Persistent organic pollutants in the environment, such as polychlorinated biphenyls (PCBs), hexachlorobenzene, and dichlorodiphenyltrichloroethane (DDT), have a higher affinity for polymers than for water, meaning that otherwise very dilute pollutants could concentrate in plastic particles circulating the environment.[5] As large pieces of plastic break down, the surface area-to-volume increases, which increases the rate of leaching of additive compounds into the surroundings.[5] Many experiments involving microplastics use zebrafish (*Danio rerio*) as an animal model. Zebrafish are ideal due to their transparency in the early stages of development, which makes them suitable for studying the localization of fluorescent particles. Given that zebrafish are widely used in developmental and toxicological studies, much is known about how to evaluate relevant endpoints following exposure to a potentially harmful substance.[2]



General scheme of the evaluation of toxicological endpoints in zebrafish following MP exposure (Baghat et al.).

In zebrafish, there is evidence that BPA alters the expression of biomarkers of neurological toxicity (most notably acetylcholinesterase), and that co-occurrence with nanoplastic particles exacerbates this effect by increasing the bioavailability of BPA in vivo.[16] BPA was detected in various tissues of adult zebrafish following exposure with pretreated nanoplastic particles, with the highest concentrations accumulating in the viscera, gills, head, and muscle, in that order. This trend is likely due to the higher lipid content in the viscera than other tissues. A great concern is that microplastics that accumulate to an appreciable degree in certain trophic levels could magnify the uptake of potentially harmful substances by shellfish and other seafood. This is even true of the lower trophic levels consisting of planktonic larvae.[17] A study of fish larvae in the English Channel found that 2.9% of samples contained ingested microplastics, nearly two-thirds of which were blue fibers.[18] It was further discovered that levels of ingestion decreased at greater distances from the shore, highlighting the spatial relationship between the point-sources of plastic and ingestion by marine organisms.[18] Given that microplastics tend to accumulate in the gut, bivalves, crustaceans, and fish eaten whole are suggested to pose a greater risk for human exposure.[19] One study found that farm-raised mussels contained more microplastic fibers than wild ones.[19] Seafood is not the only route for the introduction of

microplastics from the ocean into the human diet. Ocean water and sea salt are often used in food-making processes; consequently, microplastics have been detected in beer and commercially available sea salt.[5,20]

In terms of analytical methods used to detect microplastics in marine organisms, the prevailing trends involve removing the intestinal tract of (usually adult) fish or sea mammals, digesting the organic material with a chemical agent, and sieving out plastic particles with a filter or via density separation for subsequent visual and physical analysis. Karami and colleagues surveyed the performance of a variety of digesting agents under different conditions, evaluating oxidative agents (NaClO and H_2O_2), acids (HCl and HNO_3), and bases (KOH and NaOH) at temperatures ranging from room temperature to 60^{o}C.[21] They performed digestions on African catfish tissue spiked with fragments of various polymers and determined the recovery rate of polymer following density separation with saturated NaI solution and morphological changes via scanning electron microscopy and Raman spectroscopy. It was found that KOH at 40^{o}C for 48 hours had the best results (highest recovery rate with lowest corrosion of particles).[21] For that reason, this treatment method was chosen for the present study. Current research on microplastics suffer from lack of comparability across studies due to the wide variety of SOPs and type/concentration of polymer studied, a problem that is exacerbated by the lack of consensus on concentrations of synthetic polymer in the ocean.[22,23,24] Moreover, most studies on the subject tend to exclude nanoplastic which are too small for conventional density separation, filtration, and visual inspection. Interesting work is underway on the effects of nanoplastics in vivo. In a study on the physiological impact of 20 nm polystyrene particles in the developing zebrafish brain, Sokmen et al. found that particles injected into the embryo accumulate in the brain and cause oxidative damage to DNA.[25] While this work with injected particles is

informative, it remains to be seen whether passively ingested nanoparticles accumulate to similar levels with similar effects. After all, passive ingestion is likely the most environmentally relevant mode of exposure. To that end, passive feeding was used as the exposure method for experiments described here.

Materials and Methods

Establishing Calibration Curves with Scallop Tissue and Spiked-In Polymer

Scallops were purchased from a local supermarket and lyophilized overnight to remove all moisture. The dried tissue was ground into a powder using a mortar and pestle, which was then combined with various types (polystyrene and poly-lactic acid) and weight percentages (1.5%, 2.5%, 5.0%, 10.0%, and 20.0%) of polymer. The tissue-polymer mixtures were used to make potassium bromide (KBr) optical membranes (2.0 mg mixture with 98.0 mg KBr). Mixtures were added to a manual hydraulic press (Specac™ Cat # GS25011) and pressurized at 10 tons for 15 minutes each. Membranes were then collected and kept in a desiccator when not in use. A PerkinElmer Spectrum Two FT-IR Spectrometer (Perkin Elmer, Cat # L160000A) was used to perform analysis on the membranes, and Spectragryph® spectroscopic analysis software was used to process and visualize data. The scan rate was set at 20 scans/minute and data were collected for one minute per sample.

Once the FT-IR spectra of the membranes were collected, they were baselined and normalized to the highest peak. This allows for the closer evaluation of regions of interest and the contribution to the spectra of the spiked-in polymer. To gain a precise evaluation of the polymer spectra, a scaled subtraction was performed whereby the control tissue spectrum was removed from the overall spectrum, and what remains should in theory be contributed by the polymer. The peak absorbances of the various concentrations of polymer were used to construct a calibration curve. Calibration curves for PLA and polystyrene were constructed and will be used in future studies to quantify unknown levels of these polymers in commercially bought fish and experimental samples, provided that the tissue spectrum is approximately constant and equal amounts of material are used to construct the KBr membranes.

Zebrafish Larvae Studies

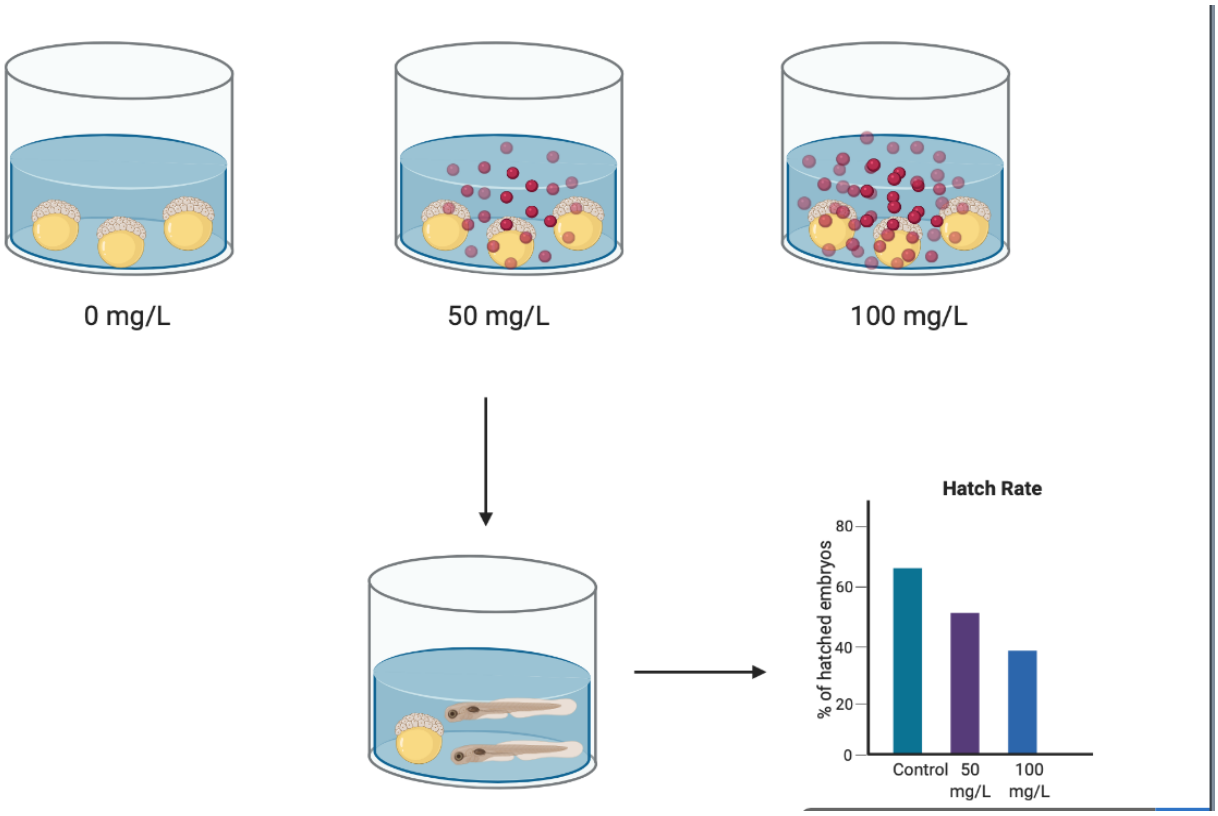
Danio rerio larvae were a generous gift from the Cretton Lab at Brown University. Embryos were collected via natural spawning and reared in egg water medium (Instant Ocean and 0.1% methylene blue). Larvae were kept at 28° C on a 12:12 hour light/dark cycle in a 25-liter incubator with an interior source of white light. Specimens were fed Zeigler® Larval AP100 Diet (Zeigler, Cat # LD100) once daily. For all experiments, two groups of embryos were collected -- an untreated control group and a treatment group – each of which was placed in a separate tank. The larvae were kept in a semi-static environment, meaning that the egg water medium was changed every other day to avoid poor water quality.

Fluoresbrite® Polychromatic Red-dyed 0.5-micron polystyrene microspheres were purchased from Polysciences Inc. (Polysciences Inc., Cat # 19507-5). The beads were kept at 4°C prior to use in experiments. These beads excite at 491 nm and 512 nm, and they emit at a maximum of 565 nm.

Embryo Hatch Rate and Survivability Assessment

Groups of 20 embryos were collected from wild-type adult zebrafish following natural spawning and placed into separate 2.8-liter tanks. Approximately six hours after collection, unfertilized embryos, which appear white and opaque, were removed and excluded from study. Within hours of collection, the groups were exposed to various concentrations of polystyrene microspheres: 0 mg/L, 25 mg/L, 50 mg/L, and 100 mg/L. Each trial was done in duplicate. The exposure was done in a semi-static manner, meaning that the egg water and microspheres were replaced every 24 hours. This was done to prevent contamination and

accumulation of waste products in the tanks. At 3 days post-fertilization, when most embryos are expected to have hatched, a count was taken of the hatched and unhatched embryos. At 7 dpf, a final count of viable larvae was done.



Uptake Studies Using Fluorescence Microscopy

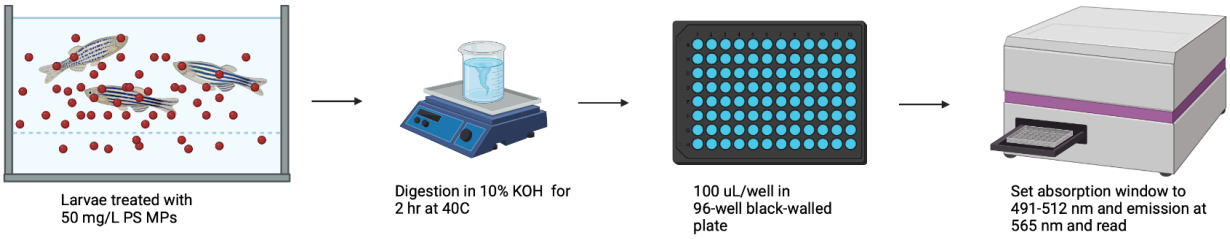
Groups of 20 embryos were collected as in previous experiments and reared in tanks of egg water in a semi-static environment. For a period of seven days post-fertilization, the treatment groups were exposed to 100 mg/L of microspheres. At the conclusion of the treatment period, larvae were euthanized via hypothermic shock. This was done by placing specimens in an ice bath for at least 30 minutes. The sacrificed larvae were then rinsed with egg water to remove any residual microspheres and debris. For samples not being imaged immediately after sacrifice, the specimens were fixed in 4% paraformaldehyde (PFA) in

phosphate-buffered saline (PBS) overnight and treated with a gradient of glycerol concentrations, ranging from 10% to 80% before being placed in 100% glycerol and stored at 4^{o}C. Glycerol was used to assist in clearing pigment from the organisms and to make them easier to position for imaging.

The fixed and cleared specimens were placed onto single-concavity depression slides (BoliOptics, Cat# SL39101004) and imaged with a Zeiss Axiovert 200M fluorescence fitted with an X-Cite LED Illuminator. The microscope is in the Leduc Bioimaging Core at the Brown University Biomedical Center. Brightfield and FITC fluorescence images were captured at 5X objective at 100% LED power each time. Images were processed and fluorescence intensity values were determined with ImageJ.

Uptake Studies Using a Fluorescence Microplate Reader

Approximately 200 embryos were collected and hatched in egg water in a semi-static environment. One group was treated with 100 mg/L of microspheres for up to 7 dpf and another group served as an untreated control (100 larvae per group). At the conclusion of treatment, the larvae were euthanized as previously described, rinsed, and digested in 20% KOH to liberate the fluorescent microbeads. The digested mixtures were then added to a black-walled 96-well plate along with a range of known concentrations of microbeads and analyzed with a multiplate reader, courtesy of the Morgan Lab at Brown. The excitation wavelength range was set to 491-512 nm and the emission was set to 565 nm. These values are included in the technical data sheet from Polysciences Inc.

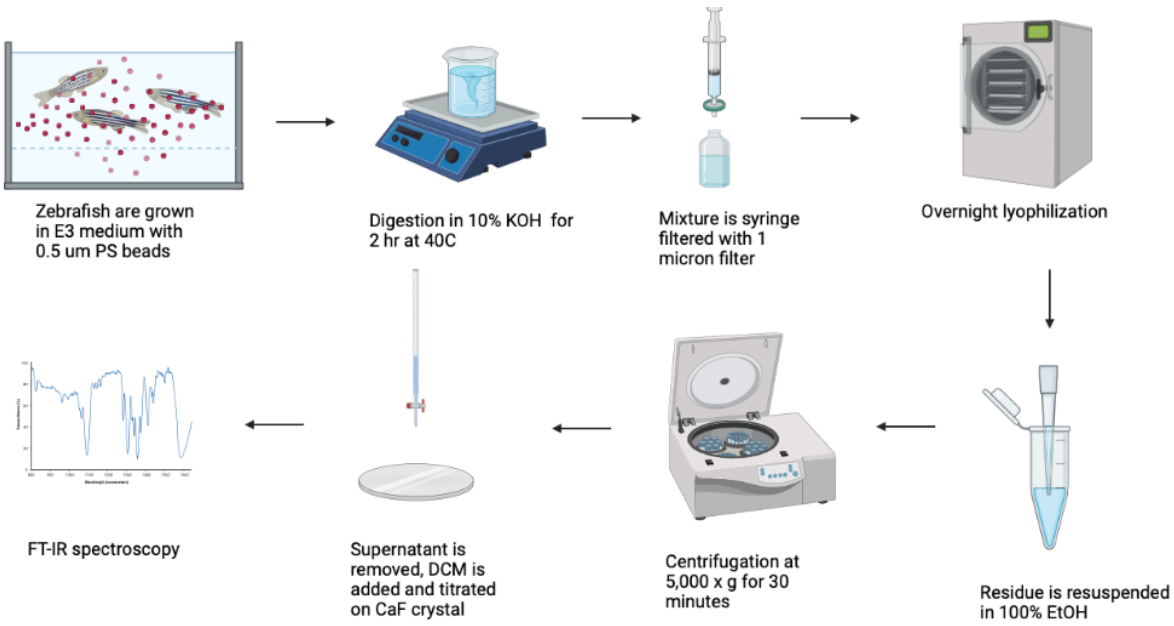


Uptake Studies Using FT-IR

Approximately 400 embryos were collected and hatched in egg water in a semi-static environment. One group served as an untreated control and the other group was exposed to 100 mg/L of 0.5-micron microparticles up to 7 dpf. Following exposure, the larvae were euthanized via hyperthermic shock and lyophilized in glass vial overnight. The samples were then ground into a fine powder with a mortar and pestle, which was used to make KBr membranes as previously described. The membranes were analyzed via FT-IR and the resulting spectra were processed with Spectragryph.

In order to remove tissue and organismal residue from the sample, a crude filtration technique was devised. First, the larvae were hatched and reared as previously described. Following euthanasia, the samples were digested with 20% potassium hydroxide (KOH) and syringe filtered with a 1-micron filter. The filtrate was then lyophilized overnight, creating a saponified, gelatin-like residue, which was then dissolved with 100% ethanol. Samples were spun down at 5000 X g for 30 minutes to pellet the microspheres, and dichloromethane (DCM) was added to dissolve the polymer. 3.0 mL of each solution was then titrated onto a calcium fluoride (CaF_2) optical crystal with a glass pipette. The crystal was analyzed via FT-IR. The crystal was washed several times with 100% ethanol between samples. In addition,

various known amounts (1.0 mg, 2.5 mg, 5.0 mg, 10 mg, etc.) of polystyrene were dissolved in DCM and titrated onto a CaF_2 crystal for analysis in order to construct a calibration curve from which unknown concentrations could be determined.



General scheme of microplastic extraction and analysis via FT-IR spectroscopy.

Results

Scallop Tissue Spiked with Polymer Reveals Unique Peaks for Identification

One of the goals of spiking in known amounts of polymer into tissue is to identify peaks unique to each on the FT-IR spectrum of the mixture. Peaks corresponding to polymer bonds with minimal overlap of tissue peaks would allow for identification of even minute amounts of material in a complex biological matrix. However, there are several polymers whose peaks overlap with or are totally subsumed by peaks belonging to tissue (which makes up most of the sample by weight percentage). PLA and polystyrene were chosen because they exhibit unique and easily identifiable peaks within the tissue matrix, unlike polyvinyl chloride (PVC) which is difficult to differentiate from tissue using FT-IR and was thus excluded from this study. Figure 1 shows the overlaid spectra of un-spiked scallop tissue and pure polystyrene, revealing the distinct peaks for each sample: one at ~697.0 cm^{-1} corresponding to aryl C-H bending in polystyrene and another at ~1650 cm^{-1} belonging to the amide I group of abundant fish collagen. A second fish-specific peak at ~1550 cm^{-1} was also identified and used for analysis, and literature confirms that this peak is derived from the amide II group. Another region of minimal overlap is at 1450-1500 cm^{-1} which corresponds to the C=C bonds in polystyrene.

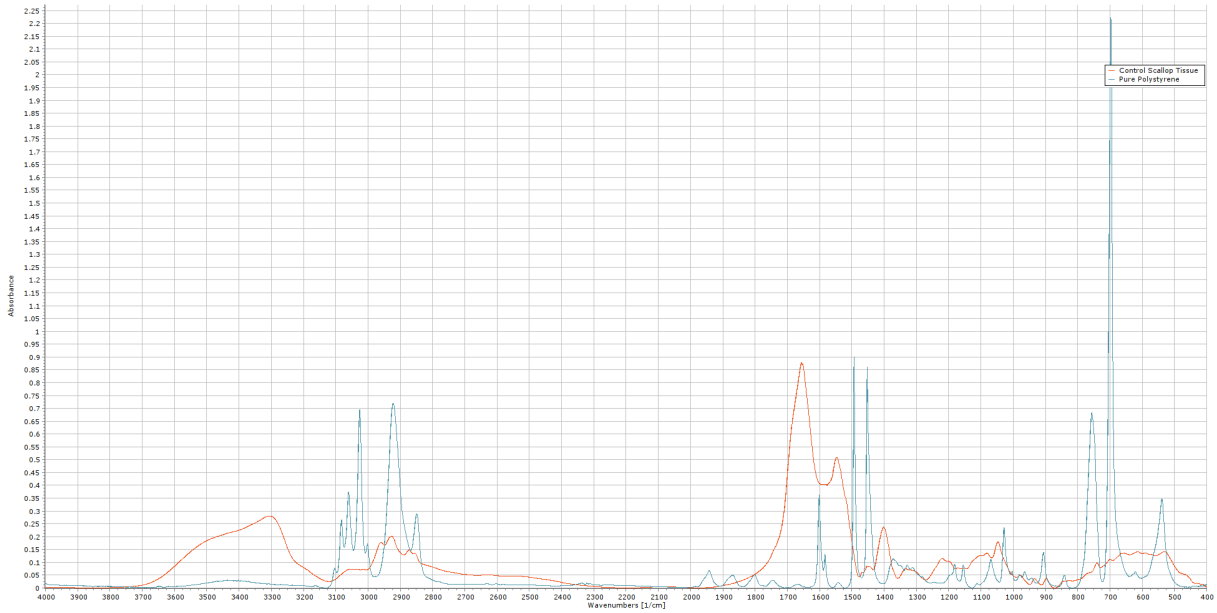


Figure 1. Overlaid FT-IR spectra of untreated scallop tissue and pure polystyrene.

Once obtained, the spectral bands for all spiked-in samples and untreated control were overlaid (Fig. 2, 3), which revealed a clear gradient of peak absorbances at the polystyrene-specific wavelengths, with pure scallop having a basal absorbance of approximately 0.15 AU and the 20% spike-in sample having a peak absorbance of over 0.70 AU. The spectral band of the control tissue was then removed from all samples by performing a 1:1 scaled subtraction in Spectragryph (Fig. 4, 5, 6). Following subtraction, the remaining spectra represent the contribution by the polymer alone, beyond what is hidden by the tissue spectrum. From this, the peak absorbances of the polymer were used to construct calibration curves (Fig. 7). This process was repeated for PLA spike-in samples and control (Figures 8-13).

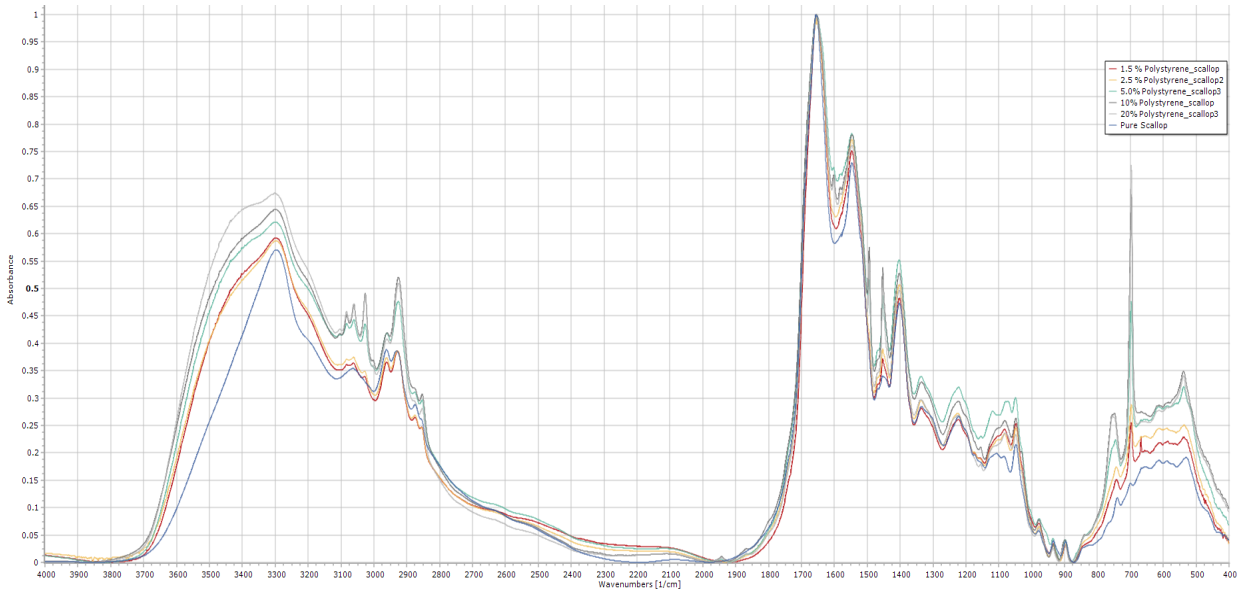


Figure 2. Overlaid FT-IR spectra of scallop tissue with spiked-in weight percentages of polystyrene ranging from 0 to 20%.

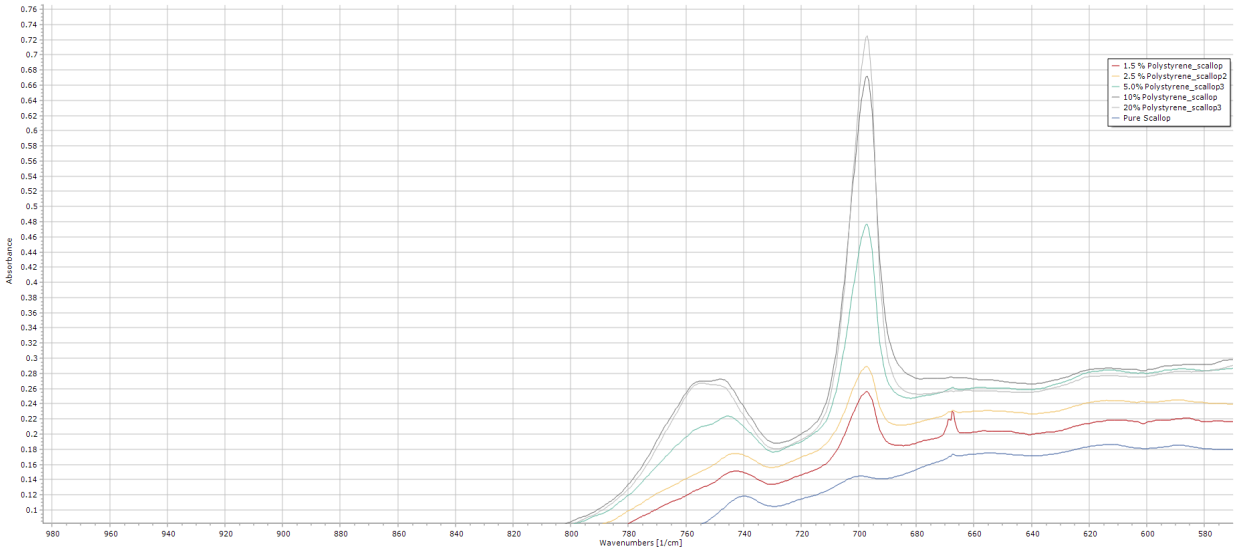


Figure 3. For clarity, a truncated window of the same spectra from 570-1000 1/cm. Note the gradient of peak absorbances at 698 1/cm as the concentration of MP increases.

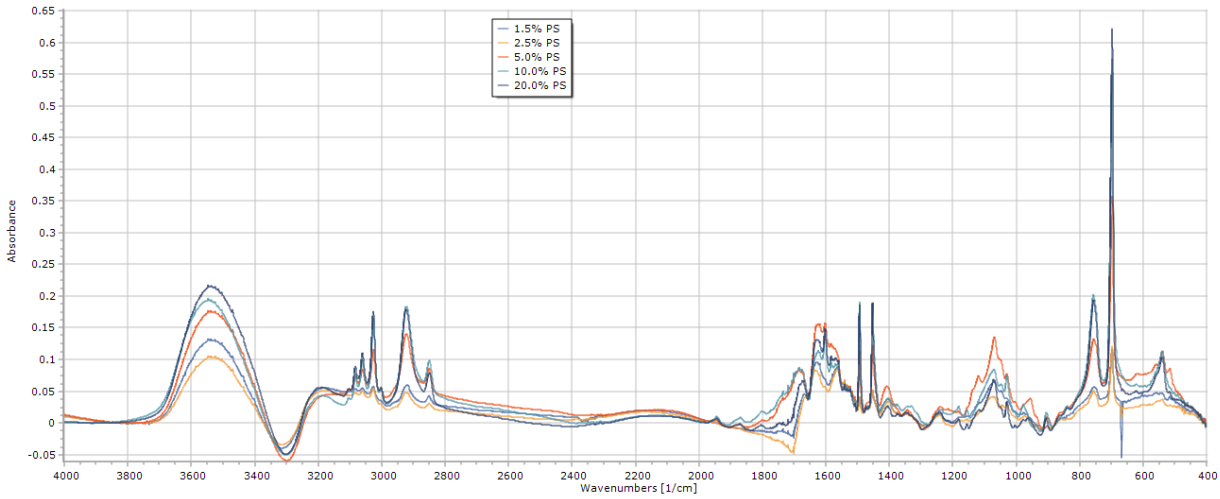


Figure 4. Overlaid spectra of the treated samples following subtraction of control tissue spectrum.

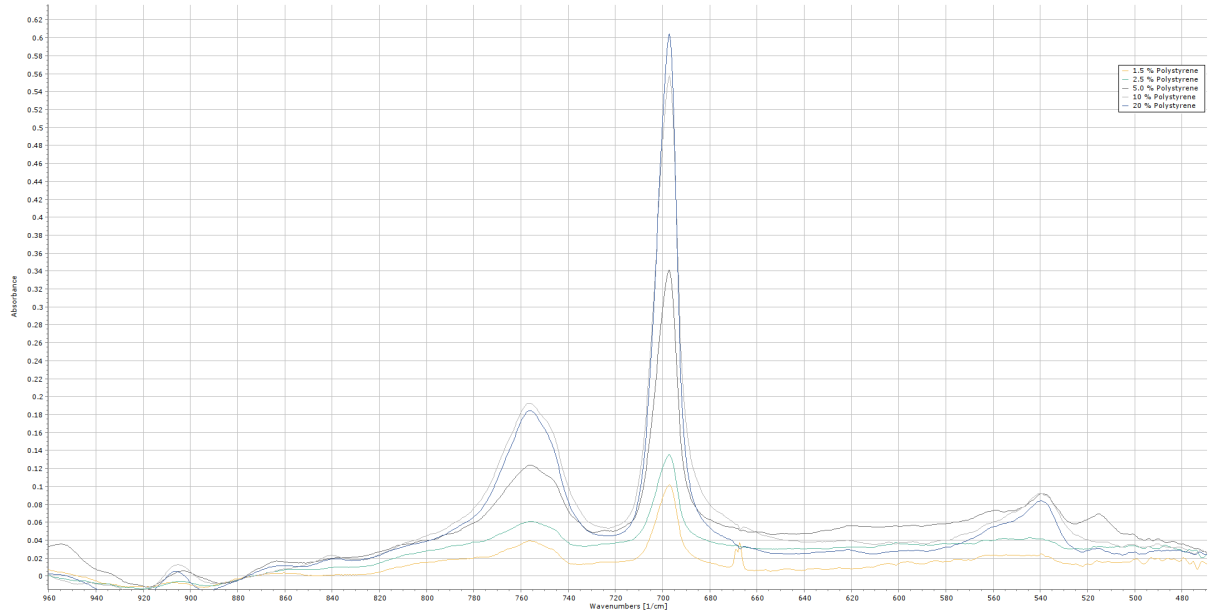


Figure 5. Same spectra as Figure 4 with window set from 500-1000 1/cm.

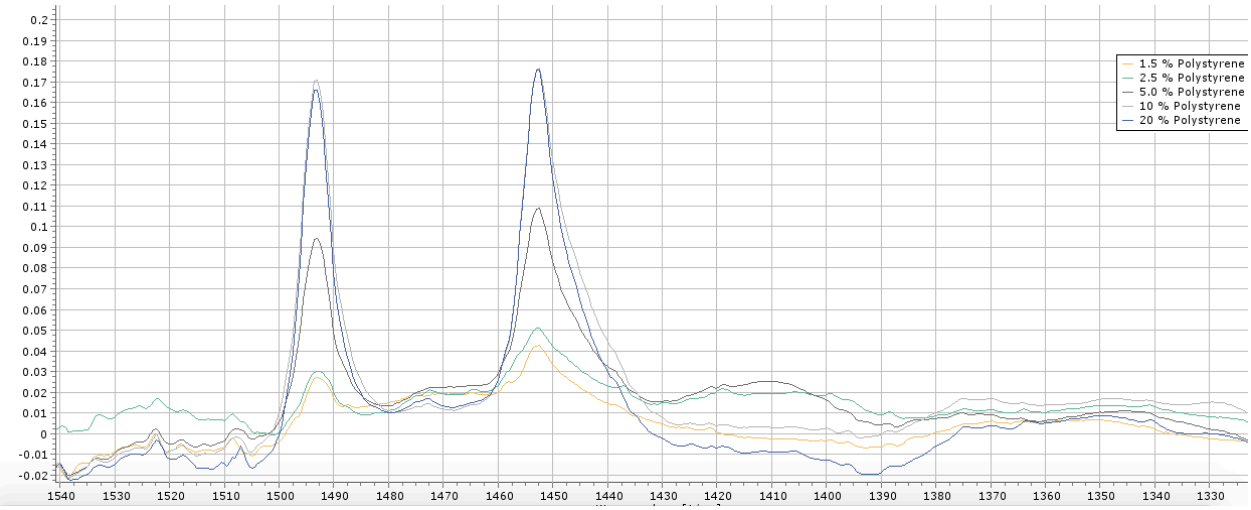
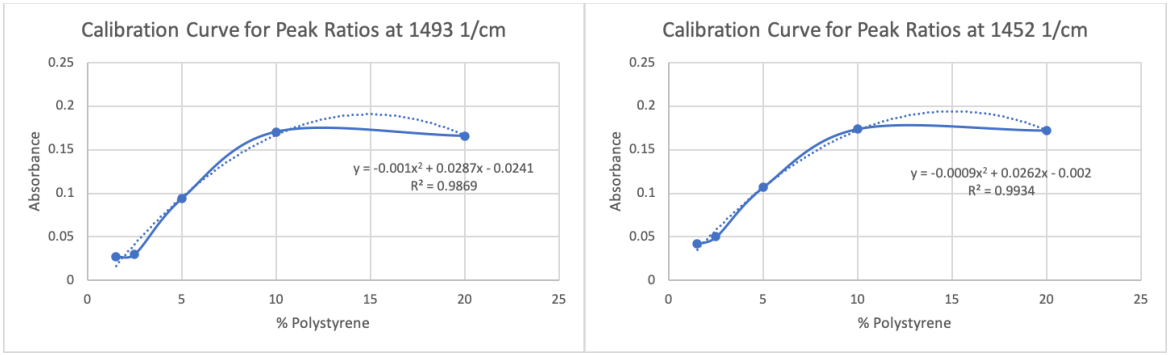


Figure 6. Same spectra from Figure 4 with window set from 1320-1550 1/cm.



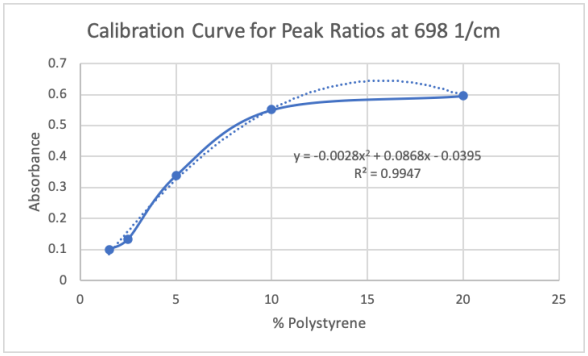


Figure 7. Calibration curves for peak values at 1493 1/cm, 1452 1/cm, and 698 1/cm.

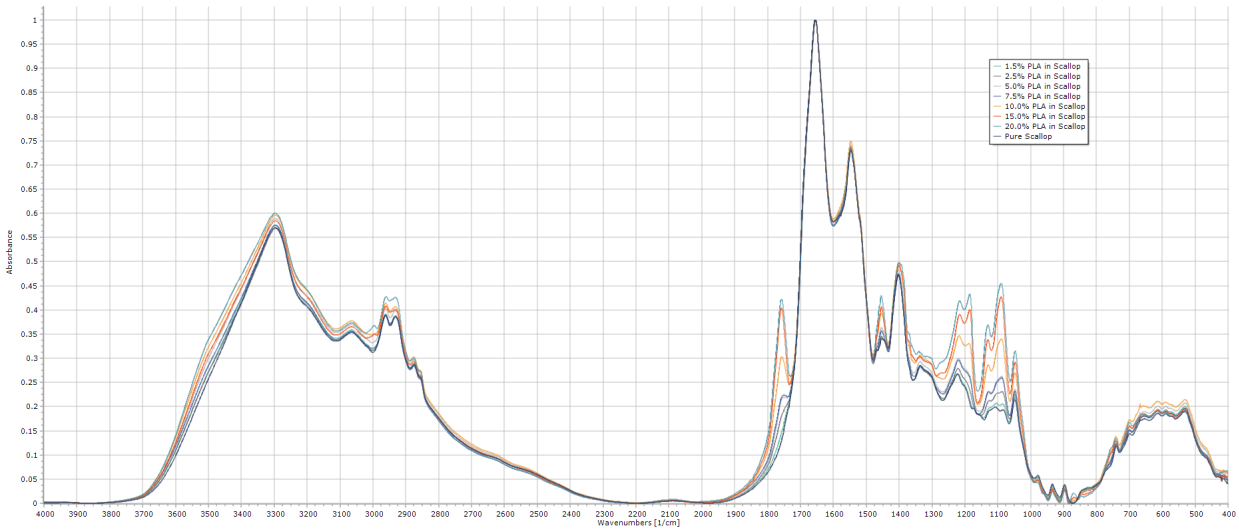


Figure 8. Overlaid spectra of scallop tissue with spiked-in PLA of weight percentages ranging from 0 to 20%.

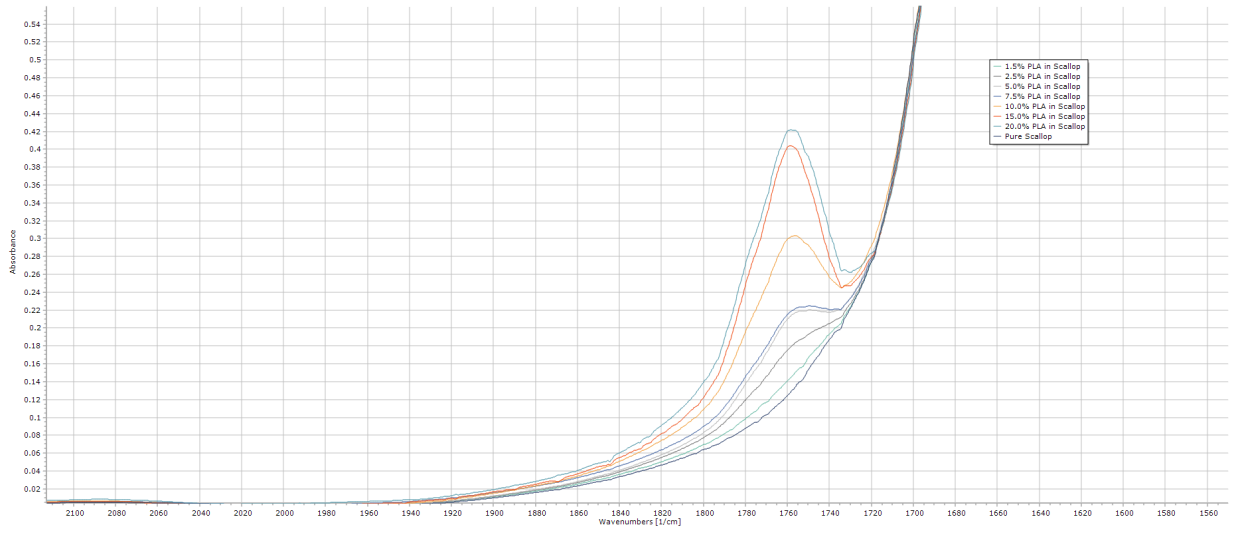


Figure 9. Same spectra as Figure 8 with window set from 1550-2100 1/cm.

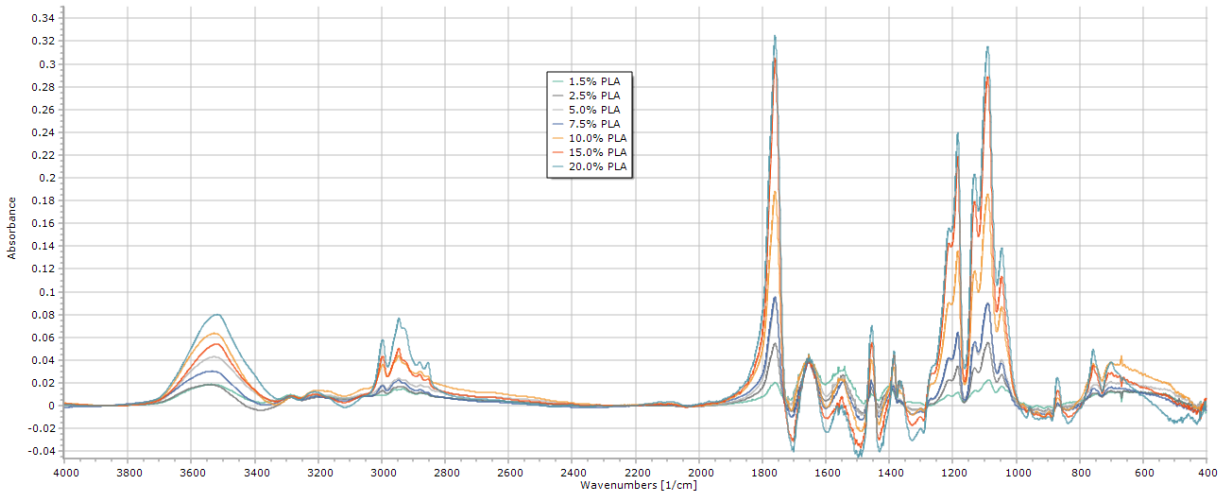


Figure 10. Spectra of scallop tissue with spiked-in PLA post-subtraction of untreated control.

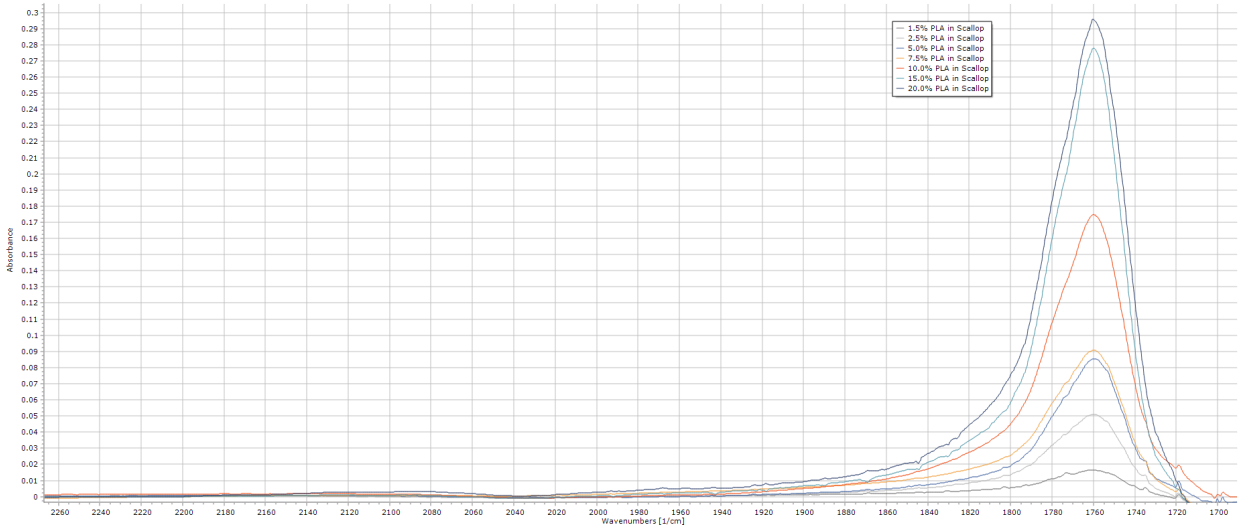


Figure 11. Same spectra as Figure 10 with window set from 1700-2260 1/cm.

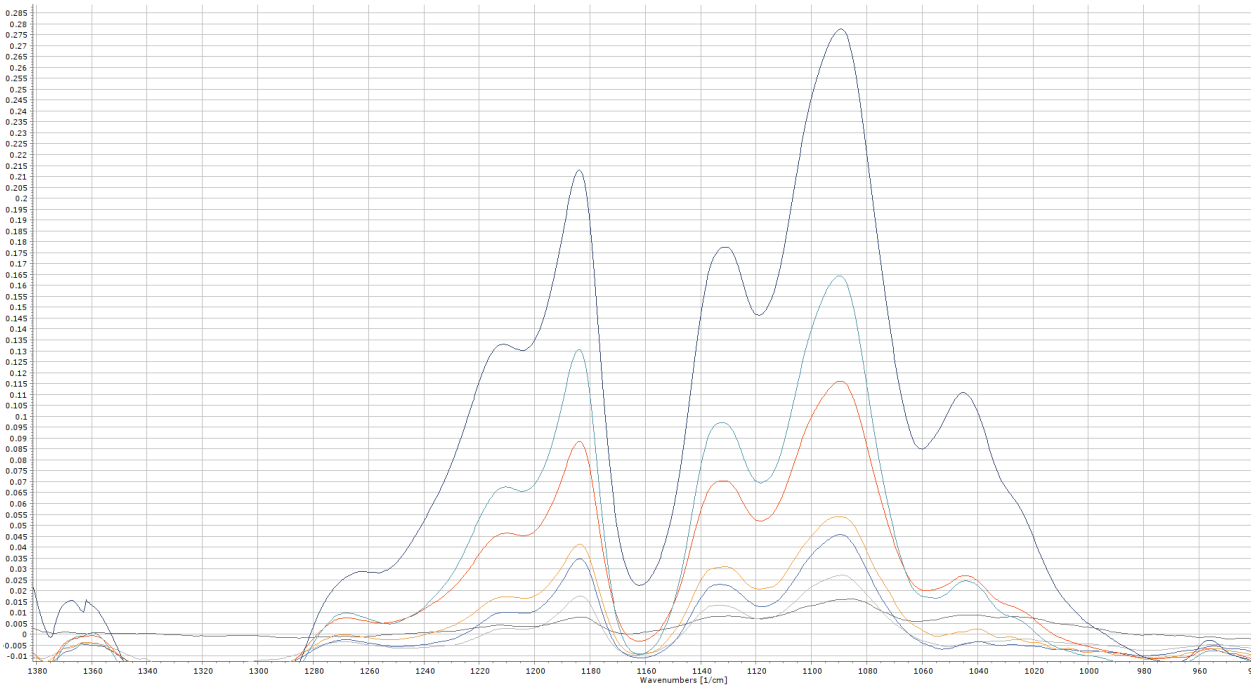
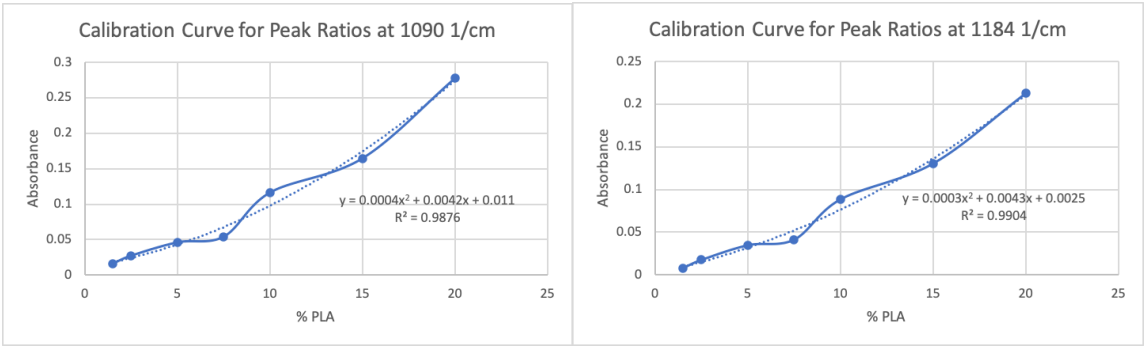


Figure 12. Same spectra as Figure 10 with window set from 960-1380 1/cm.



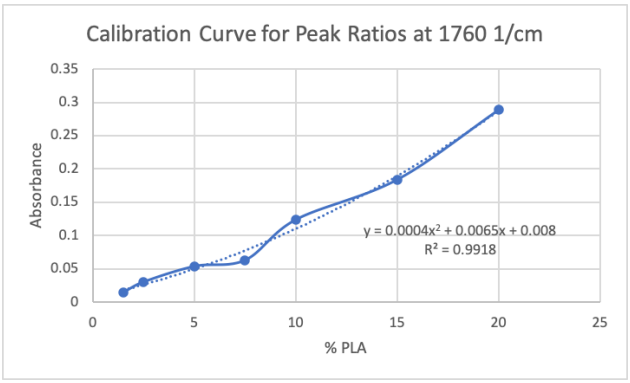


Figure 13. Calibration curves for peak values at 1090 1/cm, 1184 1/cm, and 1760 1/cm.

Nanoscale Polystyrene Beads are Detectable in Danio rerio Larvae via FT-IR Spectroscopy

The distinct peak at 697 cm^{-1} -- characteristic of C-H bending in polystyrene – is present in the passively-fed larval specimens. This can be seen when overlaying the spectra of the treated larvae, untreated control larvae, and pure polystyrene (Fig. 14, 15, 16). The samples include 0 mg/L, 25 mg/L, 50 mg/L, and 100 mg/L, and a larger peak at 697 cm^{-1} is present as dose of polystyrene increases, with the highest absorbance value reaching over 0.70 AU. This indicates that zebrafish larvae take in microplastics from their surrounding environment, although the specific route of uptake is beyond the scope of this study. While the oral route is the most obvious, studies done elsewhere report uptake via the gills and microplastics becoming embedded in the exterior mucosa.[26]

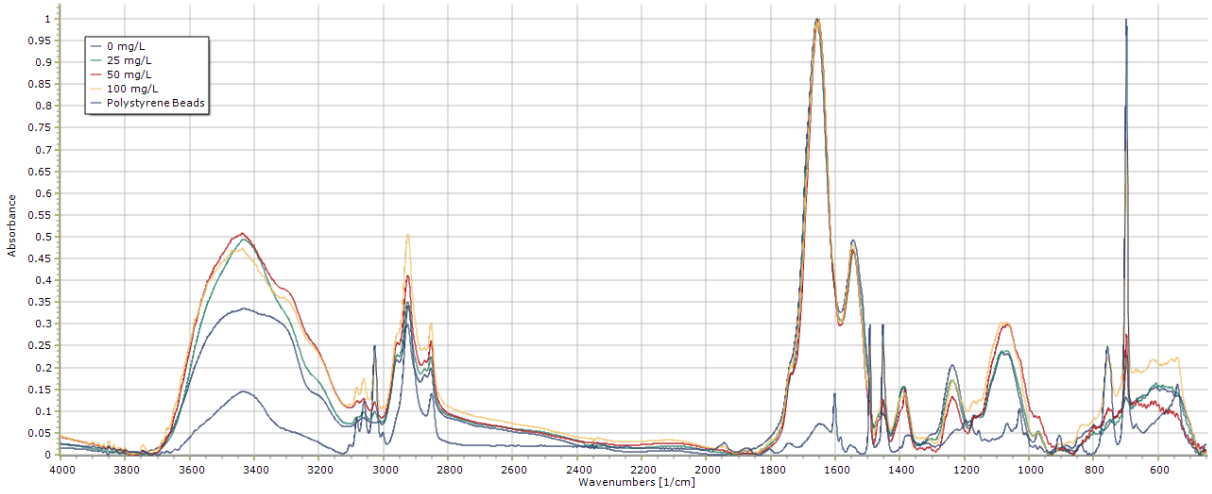


Figure 14. FT-IR spectra of passively fed Danio rerio larvae and pure polystyrene beads.

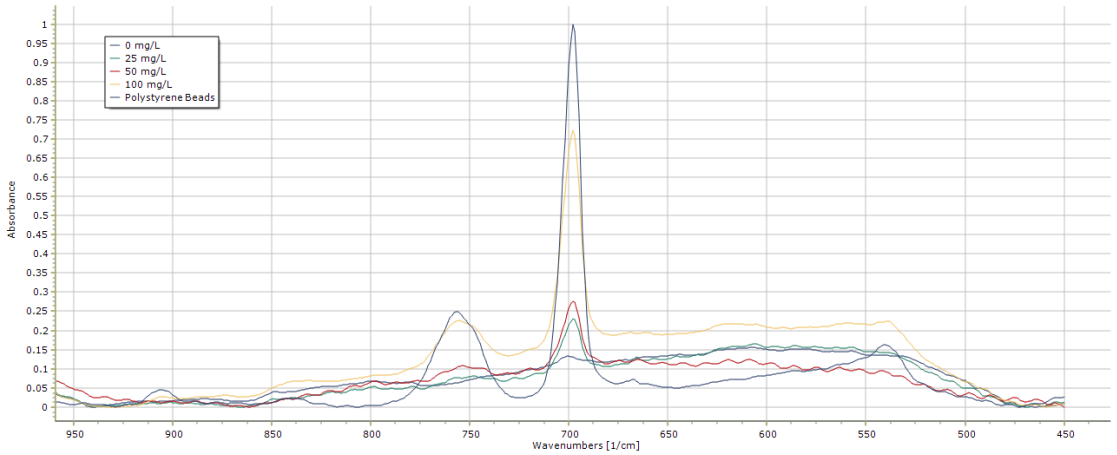


Figure 15. Spectra from Figure 14 with window set from 450-950 1/cm.

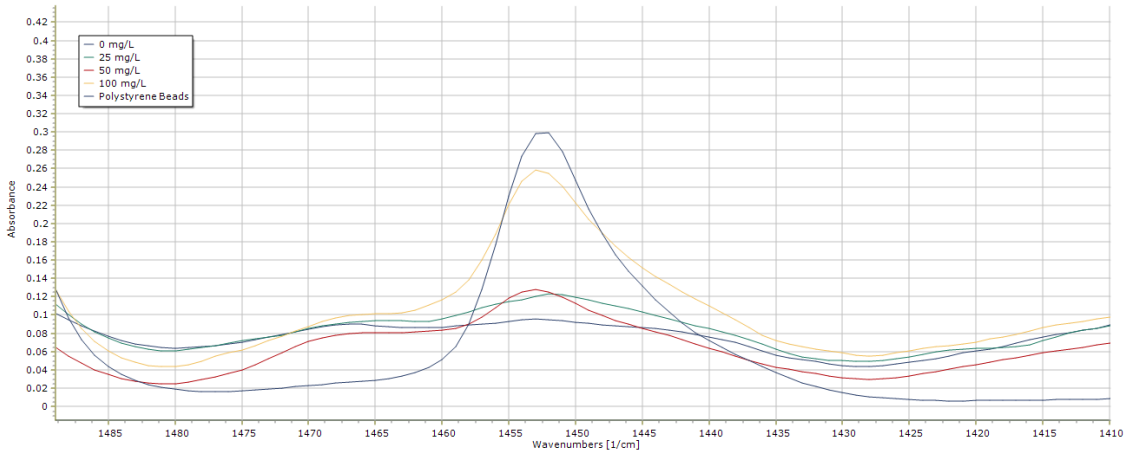


Figure 16. Spectra from Figure 14 with window set from 1410-1470 1/cm.

A Crude Extraction Method Improves Detection of Beads with FT-IR

The previous experiments using FT-IR to detect polymer within a matrix of biological tissue were successful, yet it would conceivably only work for samples which have non-overlapping peaks that can be used to identify the polymer. Even for these samples, there are only a few peaks that serve this need. However, treating the samples by removing as much biological material as possible and separating the polymer could allow the method to be applied to a much broader range of polymer types and would provide cleaner spectral bands

that align more closely with those of the pure polymer material. To that end, a crude extraction method was devised as previously stated, and a simplified workflow for this method is also presented in 'Materials and Methods'. Digestion with KOH at 40^{o}C has been shown in other studies to be an effective means of breaking down organic material from fish with minimal damage to the polymer morphology and yield.[21] Hydrogen peroxide is also a suitable agent for digestion, yet it typically requires higher heat and longer incubation time. As shown in Figure 17, the extraction method allows for visualization of a greater number of polymer-specific peaks, although the maximum peak absorbances for those peaks are lower, perhaps due to differences in the membranes used for FT-IR analysis (i.e., the titrated CaF_2 crystal has a different path length from the KBr membranes). Overlaid spectra of the control larvae, PS-fed larvae, and pure beads reveals significant overlap between the treated sample and the beads, with minimal overlap with the control in polymer-specific wavenumbers. The control larvae do not appear to show any peak similarity with either the treated larvae or the pure beads, indicating that the peaks in the treated sample are likely due to the presence of polymer and that the control sample is free of polymer contamination. The appearance of peaks at 1300 cm^{-1} and 1700 cm^{-1} in the treated sample that are not in the control sample or pure beads could be due to an effect caused by the preceding KOH digestion, although this has not been confirmed or investigated further. The ethanol fraction, which should contain dissolved lipids and no polystyrene, was also analyzed via FT-IR using solvent casting. Figure 19 depicts the resulting spectra, which do not contain any detectable peaks that could be associated with the polystyrene beads. The spectra do, however, contain noisy peaks which are likely due to the complex makeup of the material separated by the extraction. The purpose

of assessing the ethanol fraction is to confirm that no polymer was separated with the ethanol and that it pelleted out.

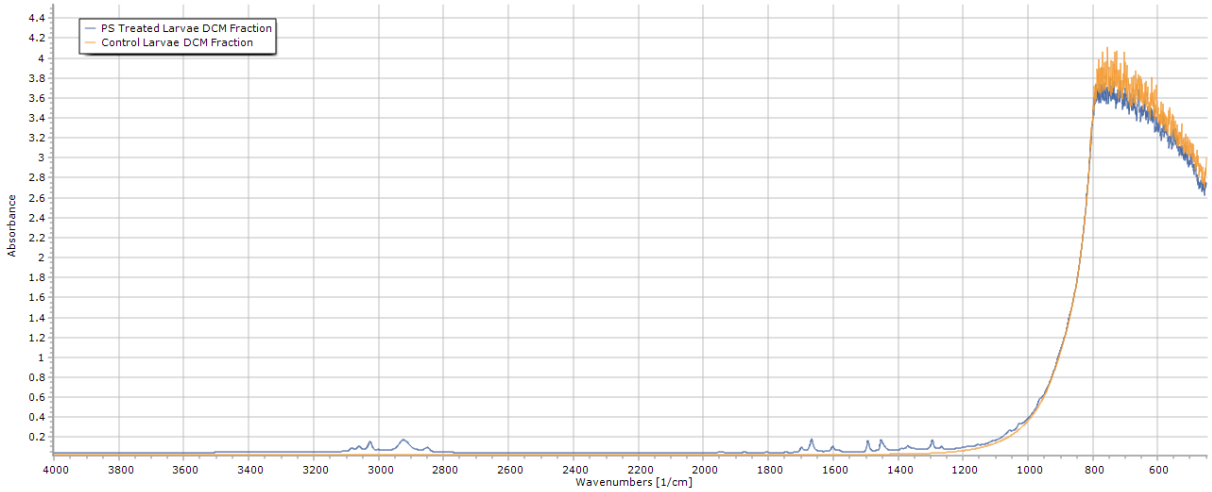


Figure 17. Overlaid spectra of DCM fraction of control and MP-treated larvae.

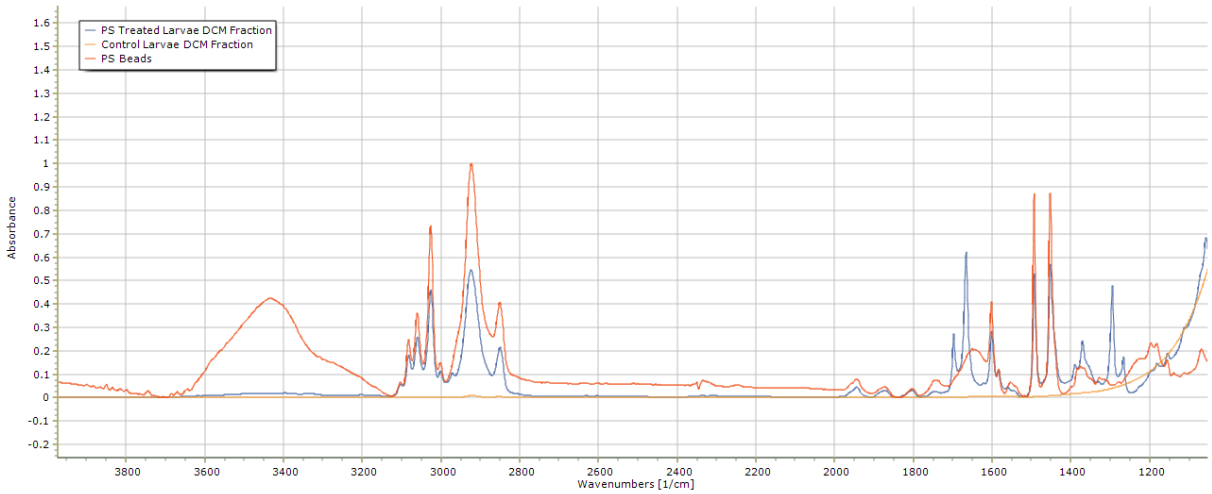


Figure 18. Same spectra from Figure 17 with the window set from 1200-3800 1/cm and the addition of a PS bead control, which contains significant overlap with the treated sample, indicating the presence of polystyrene.

Cross-Validation with Fluorescence-Based Detection

Fluorescent beads were chosen for these experiments because of their amenability to imaging and ease of detection using a fluorometer, which could be used to validate findings from other methods. To that end, a calibration curve was constructed by measuring the fluorescence of known amounts of beads (Figure 19). The curve was then used to calculate the

amount of beads in the larval samples (Figure 20). According to this method, it was determined that the larval digestate (from 200 larvae) had a concentration of roughly 75 mg/L of polystyrene beads, corresponding to a total mass of 0.075 mg. There is a chance that the experimental conditions affect the fluorescence of the beads, which could skew these calculations. For instance, fading might have been caused by prolonged exposure to white light in the larvae incubator or by repeated washing steps.

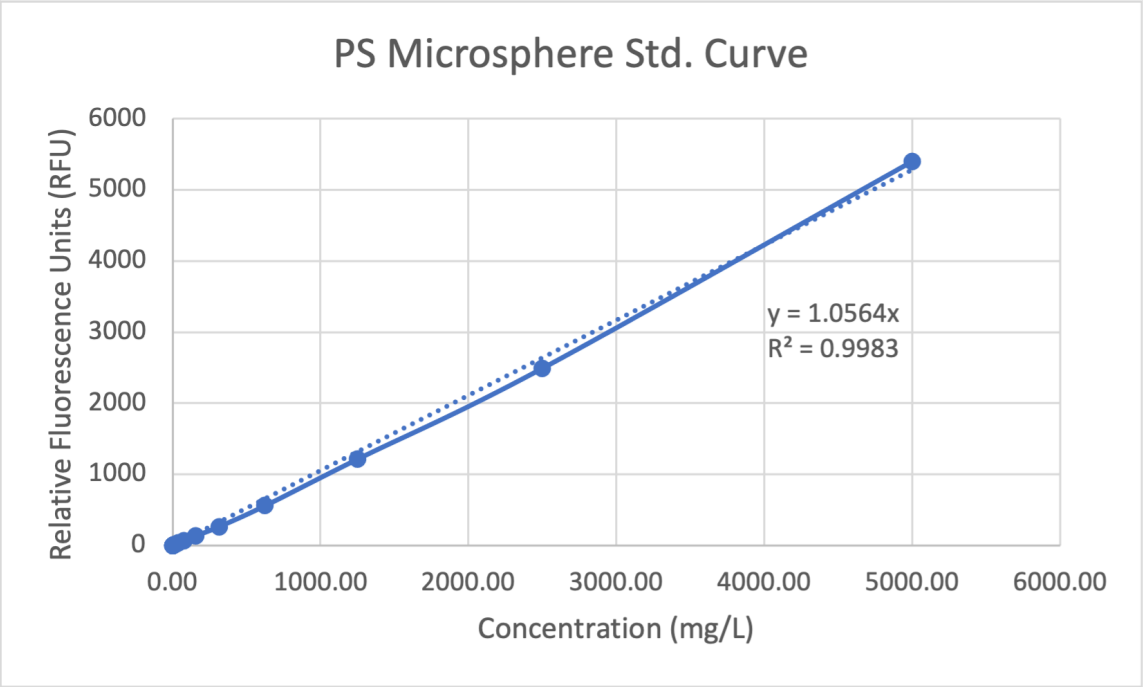


Figure 19. Calibration curve constructed with known concentrations of fluorescent PS beads.

Column1	Control Larvae	50 mg/L Treated Larvae
RFU	0.603	79.14
Concentration (mg/L)	N/A	74.91
Mass (mg)	N/A	0.075
Estimated Mass/Larvae (ug)	N/A	0.75

Figure 20. Quantification of PS beads in control and 50 mg/L treated larvae samples.

Fluorescence Microscopy Reveals Microplastic Uptake

In order to determine whether zebrafish larvae can orally ingest microplastics in a passive environment, as opposed to being injected, fluorescence microscopy was performed following the previously described experimental treatment. It was consistently shown that larvae take in fluorescent microbeads from the surrounding environment whereas untreated larvae do not. Figures 21 and 22 shows a clear fluorescence signature in the larval midsection, indicating the presence of beads. Furthermore, Figure 23 shows the mean fluorescence intensities of both groups as determined by analysis in ImageJ. The mean fluorescence calculation was applied to the entire image taken with a FITC filter. Basal levels of fluorescence appear in some of the images, which is likely due to the persistence of pigment interference. Although larvae are mostly translucent in the early stages of development, melanocytes (melanin producing cells) begin to populate the dorsal, lateral, and ventral sides of the organism during the first week post-fertilization.[27] Despite the pigmentation effect, there is still a pronounced difference in the mean pixel intensity of treated versus control larvae (~550 vs 340 AU, respectively).

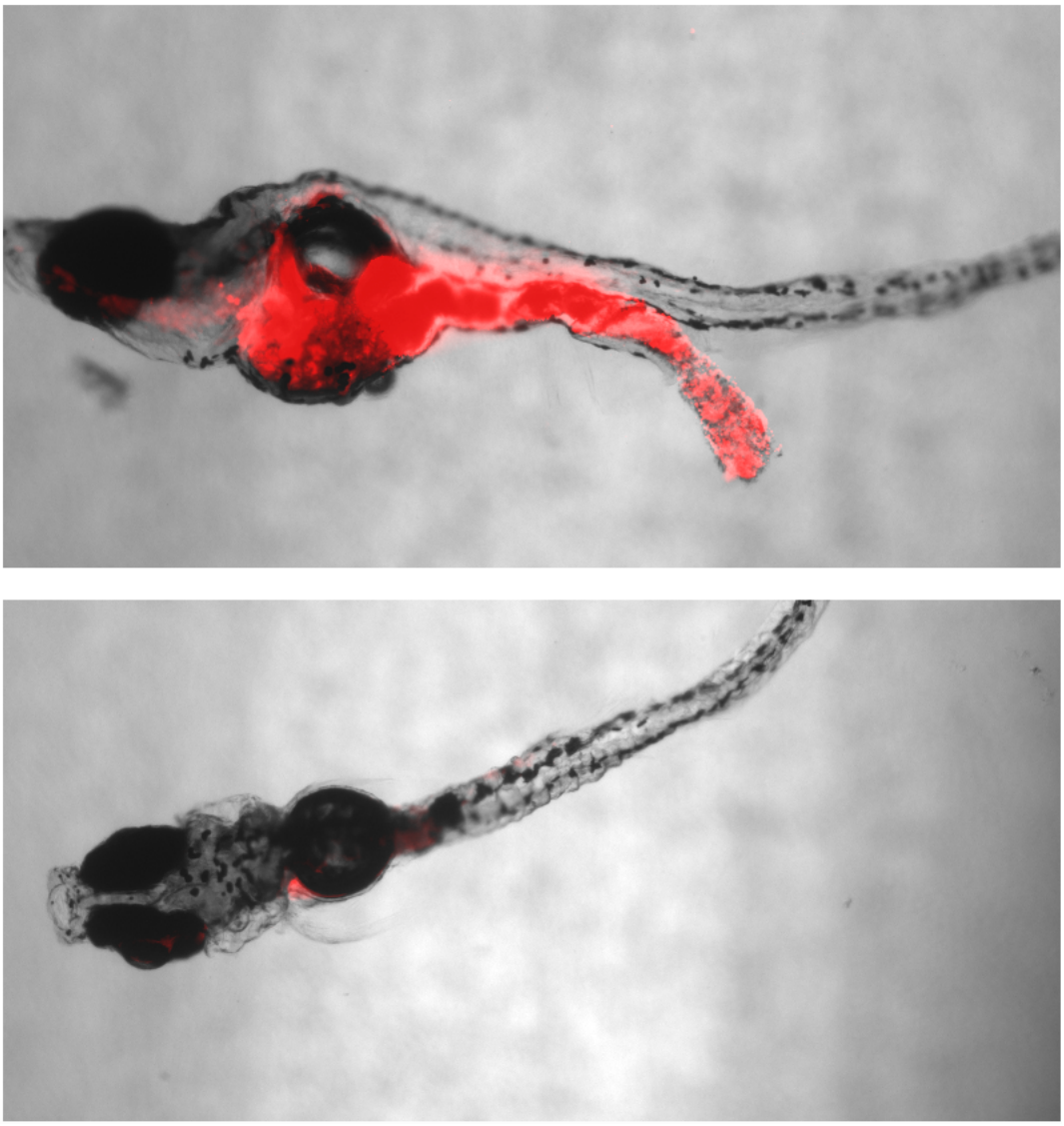


Figure 21. Merged brightfield and FITC images of treated (top) and untreated (bottom) larvae. Basal levels of fluorescence (red) are noted in the control sample, likely indicating pigmentation.

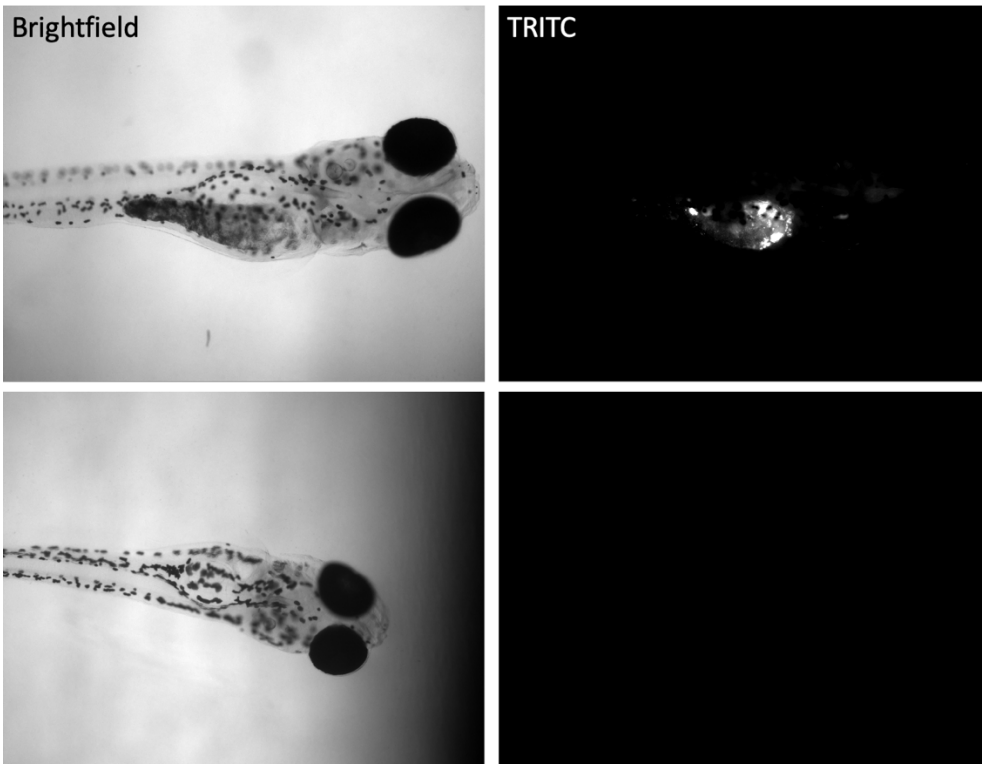


Figure 22. Brightfield and TRITC images of control (bottom) and treated (top) larvae taken at 5X objective. Note the bright signature in the viscera of the treated larvae.

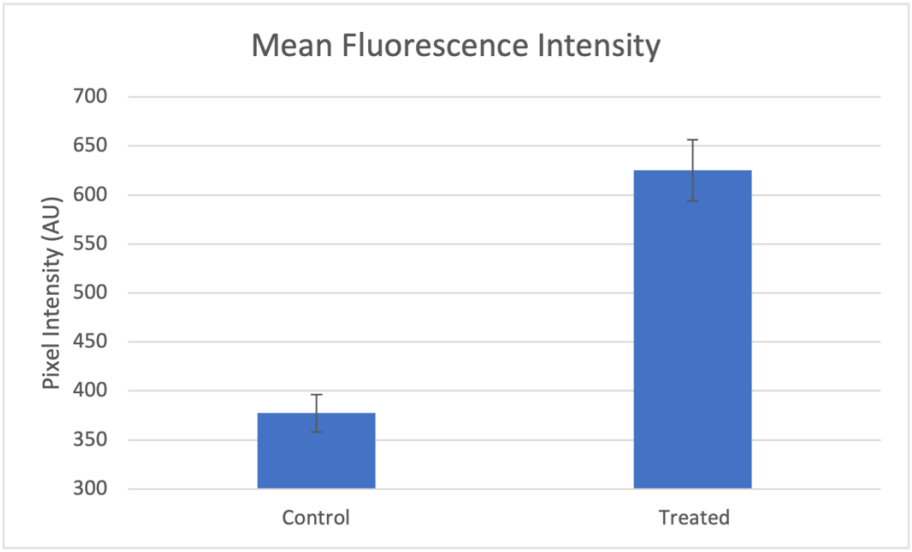


Figure 23. Mean Fluorescence Intensity of FITC images of control and treated specimens.

Exposure to Polystyrene Beads Affects Survivability but Not Hatch Rate

As shown in Figure 24, exposure of zebrafish embryos to microplastics, at least at the tested concentrations, appears to have no effect on hatch rate. However, there is a noticeable trend in survivability that decreases as the concentration of microspheres increases, with the most pronounced effect at 100 mg/L. This is in line with what has been previously shown; Zhao et al. discovered that the five-day survivability of embryos exposed to 500 mg/L was as low as 12%.[28]

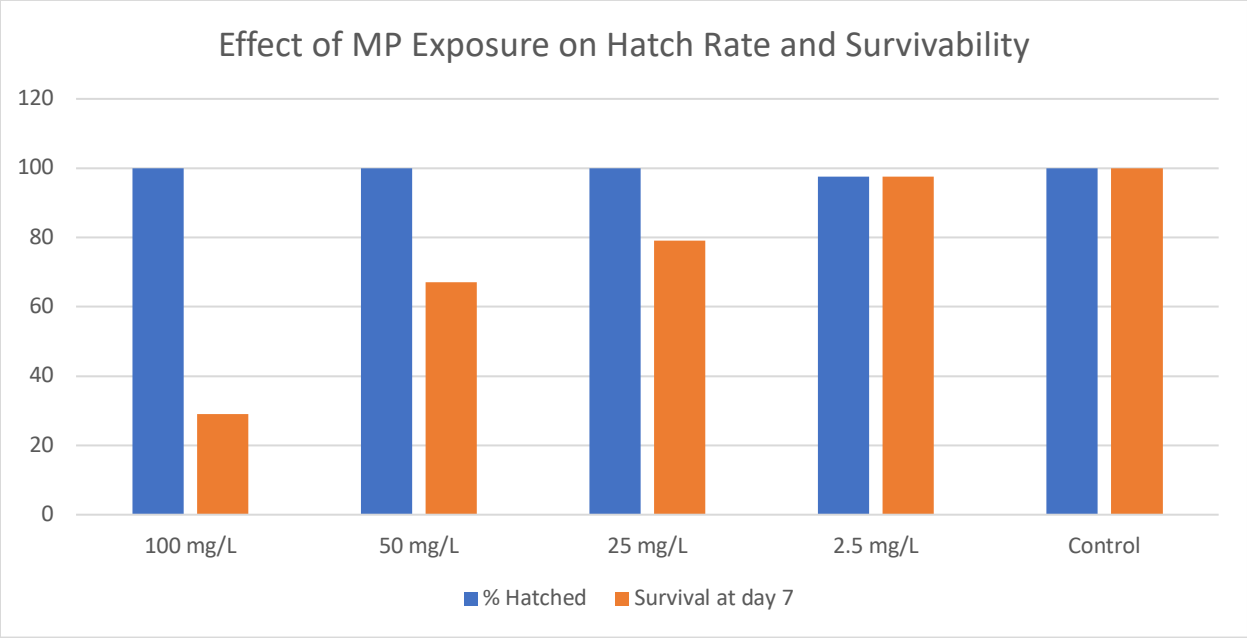
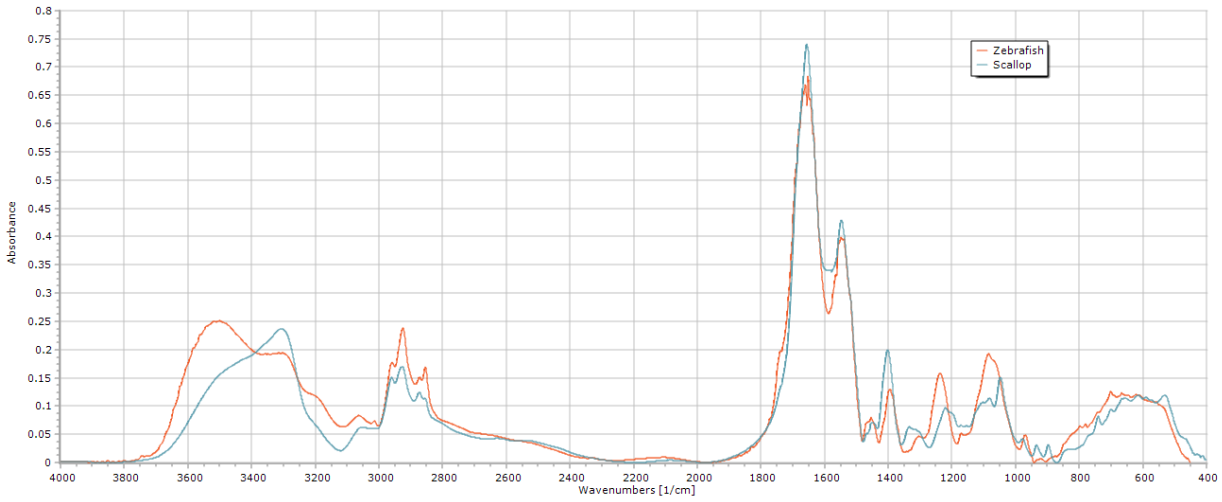


Figure 24. The effect of MP exposure on hatch rate (blue) and survivability at 7 dpf (orange). Although no significant effect on hatch rate is observed, there is a clear decrease in survivability as the concentration of MPs increases.

Discussion

This work represents an exploratory project into the detection and quantification of micro- and nano-plastics in marine organisms. It was discovered that polymers could be clearly identified in complex biological matrix such as zebrafish tissue using FT-IR spectroscopy and that separation of tissue from polymer, although imperfect, can serve to "clean" the spectra to a certain degree. A key takeaway is that standard FT-IR setups are sensitive enough to detect such polymers in biological samples, which provides a cheaper, easier-to-operate alternative to gas chromatography/mass spectrometry. Although GC/MS is the gold standard for polymer identification/quantification, FT-IR may be suitable when detection is the main goal and great precision in quantification is not necessary. Another crucial finding is that zebrafish larvae do, at least in the conditions entailed here, take in microplastics from their environment in a passive manner without the need for injection or spike-in, each of which is suitable for less sensitive analysis.

This work is not without limitations. Importantly, for calibration curves derived from FT-IR spectra to be useful, there must be negligible difference in the tissue composition in each sample, otherwise the spectral subtraction used to construct one set of curves might lead to inaccurate determinations for a different set of samples. However, it seems that for samples using the same kind of tissue (in equal amounts), the minor variations in spectra are inconsequential. There is also a high degree of spectral overlap across species due to the inherent similarity of biological tissue. The two types of tissue used here, zebrafish and scallop, tend to have many regions of overlap and an overall similar shape. However, enough difference exists such that analysis of samples from either species would likely require calibration curves made with that specific tissue.



A key factor in ensuring consistent results is mode of tissue preparation. It is known that IR spectra of desiccated and formalin-fixed tissues differ from those of hydrated tissues, particularly in the amide I region which is indicative of protein secondary structure.[29] For that reason, tissue pretreatment was consistent across experiments here.

Future Directions

The logical next step in the lifecycle of this project is a rigorous investigation of the physiological effects of microplastics and how they relate to polymer type, size, and concentration. One jumping-off point for this would be sectioning of zebrafish larvae to understand the localization and distribution of microplastics, primarily in the gut but also in other organ systems, such as the brain, liver, and gills. It would be informative to stain tissue sections for mucus and immune cell infiltration, which would shed light on the effects of microplastics on the innate immune response. Limonta et al. reported that immunity-related transcriptional changes and alterations to mucosal secretion occur in zebrafish larvae following microplastic exposure.[30] An interesting follow-up to this study would be to see if these changes effect survivability following immune challenge with specific pathogens like *P. neurophilia* and *M.*

chelonae, which often target zebrafish in laboratory environments.[31] If such an effect exists, it would clearly demonstrate that persistent exposure to abundant microplastics leads to increased susceptibility to pathogens – a phenomenon that could manifest in fish species that are critical to the global food supply and the health of aquatic ecosystems. Another avenue of interest would be to interrogate the effects of microplastics on oxidative stress in zebrafish. Qiao et al. reported that catalase (CAT) and superoxide dismutase (SOD) activity was significantly increased in the intestines of adult zebrafish treated with microplastics.[32] It would be informative and inexpensive to replicate these experiments in a larval model. While CAT and SOD are standard enzymatic biomarkers for oxidative stress, there is also a simple protocol for evaluating global oxidative damage to the proteins in an organism. Protein carbonylation is a common alteration caused by excessive oxidation that can be assayed via Western blot with specific antibodies or HPLC. A more accessible procedure is described by Colombo et al., whereby total protein carbonyl content is measured spectrophotometrically following a reaction with 2,4-dinitrophenylhydrazine (DNPH), which yields an adduct that absorbs at around 366 nm.[33] Comparing absorbance values to untreated samples could provide a rough estimate of the global protein carbonylation caused by exposure to microplastics. In addition, it would be instructive to probe whether and to what degree microplastics introduced via passive feeding are retained in larvae before being excreted, and by what means this occurs.

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