POLYSTYRENE PARTICLE UPTAKE AND THEIR POTENTIAL TO ACT AS A TROJAN HORSE FOR PYRENE TO OYSTERS (*CRASSOSTREA VIRGINICA*)

by

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A thesis submitted to the faculty of
The University of North Carolina at Charlotte
in partial fulfillment of the requirements
for the degree of Master of Science in
Biology

Charlotte

2017

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ABSTRACT

TAMARA RENEE GASPAR. Polystyrene particle uptake and their potential to act as a trojan horse for pyrene to oysters (*Crassostrea virginica*). (UNDER THE DIRECTION OF DR. AMY H. RINGWOOD)

The global annual usage of plastics has increased dramatically over the last decade. Polystyrene is the fourth most common plastic material produced annually due to its many versatile applications. Consequently, there has been a coinciding increase in polystyrene wastes, much of which makes its way into waterways and oceanic habitats. While plastic debris has been shown to adversely affect many marine species as a result of ingestion and entanglement, little has been studied regarding the effects of small-scale plastic particles (nano and micro) on cellular responses of marine invertebrates. In this study, we investigated the potential for uptake of polystyrene nano and micron sized beads (50nm and 3um) by the Eastern Oyster, *Crassostrea virginica*, as well as the toxicity of the particles alone and combined with pyrene. Contamination of plastic by PAHs, such as pyrene, which can be toxic to marine life, presents significant issues of concern about impacts on marine species. This research was focused on four key components; 1) the toxicity of nano and micron polystyrene particles, 2) how particle size would affect particle uptake by hepatopancreas (hp) cells *in vitro*, 3) the difference in uptake of micron and nano particles *in vivo* between gill and hp cells, and 4) the cellular response of hp tissue after exposure to polystyrene beads alone, as well as polystyrene beads incubated with pyrene *in vitro*. Generally, this research indicated that oysters accumulate polystyrene beads, with greater intracellular accumulation of nanoparticles in hepatopancreas cells. While polystyrene was generally nontoxic, there was some evidence that nanoparticles could serve as a Trojan horse for pyrene.

ACKNOWLEDGMENTS

I would like express my sincere gratitude to the Department of Biological Sciences and the University of North Carolina at Charlotte for affording me the opportunity to complete my Master's degree. I would like to also thank and acknowledge all of those who helped me along the way throughout my years in graduate school. I would like to thank all of my past and present lab mates, especially, Bushra Khan whose wisdom and passion for biology helped to guide and encourage me. I would like to especially thank my major advisor, Dr. Amy Ringwood, for accepting me into her lab and allowing me the opportunity to pursue higher education despite my status as a non-traditional student, for the education and guidance she provided over the past several years, and for sharing and instilling her love of biology, specifically ecotoxicology, with me. To my committee members, friends, classmates, and colleagues in the department, I would like to thank you for sharing your knowledge and time with me, and for helping me over obstacles along the way. I would also like to acknowledge Sigma Xi Grants in Aid of Research for funding part of my project.

Last, I would like to acknowledge my family for their unending support. To my mom, Janet, I would like to thank you for being there to step in in a pinch, for the countless times you dropped what you were doing to shuttle my girls to their latest activity. To my husband, Kevin, there are not enough words to thank you for your sacrifices to our family and to me during this process. I cannot thank you enough for putting your education on hold while I pursued my dreams, and for always providing stability.

DEDICATION

This thesis is dedicated to two of the most wonderful people I know, my daughters, Kaitlin and Courtney Gaspar. Watching them investigate the world with innocence, joy, and wonder has provided me with immeasurable amounts of inspiration. Watching them dare to be brave and accepting of life's challenges while growing into beautiful, strong, young ladies has provided me with the courage and motivation to pursue my ambitions.

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CHAPTER 1: INTRODUCTION

The global annual use of plastics has increased dramatically over the last decade, with the annual production reaching 311 million tons in 2014, a nearly 30% increase since 2011(Wegner et al. 2012, PlasticsEurope, 2015). Plastic polymers are primarily synthetic and derived from petrochemicals with a long carbon backbone. The side chains vary between plastic polymers resulting in subgroups of plastics, each with a different set of characteristics. Polystyrene is the fourth most common subgroup of plastic produced annually and is commonly used in applications such as packaging, construction, transportation, and food storage. Polystrene has a variety of different formulations, and takes on many forms ranging from hard plastic dash boards to insulating styrofoam and fast food containers, making it a very versatile plastic (EPA 1995).

With increased production and applications, there has been a coinciding increase in plastic wastes, much of which makes its way into waterways and the open ocean (Mato et al. 2001, Cadée, 2002). Roughly 70-80% of all plastic debris in the ocean is a result of the improper handling of land waste (Martins et al. 2011). It is estimated that 4.8 to 12.7 million metric tons of plastic waste entered the ocean in 2010, and at current production growth combined with improper waste handling estimates, 100 to 250 million metric tons will be entering the ocean in the year 2025 (McCarthy et al. 2013). Once in the ocean, currents can carry the waste into gyres resulting in the formation of what has been termed "great ocean garbage dumps". Discovery of these ocean gyres that concentrate plastics

and other garbage have increased concerns about the potential effects of plastics on coastal as well as open ocean ecosystems.

Plastic debris has been shown to adversely affect many marine species as a result of ingestion and entanglement, where much attention has been paid to larger marine vertebrates, such as turtles, manatees and whales. However, less has been studied regarding the effect of ingestion of small scale plastic particles on marine invertebrates (Laist 1987, Derraik 2002). Moreover, animals cannot fully digest plastic marine debris; the debris becomes available to be consumed by higher trophic levels or is released once an animal dies and decomposes, potentially causing harm to other animals and cycling through food webs (Bocchetti et al. 2008, Ward et al. 2009).

Physico-chemical processes and UV radiation serve to break the polymers into smaller and smaller fragments, thereby increasing the surface area, and also pollutant binding capacity (Rios et al. 2007). Microscopic plastic particles have been isolated from beaches and estuaries throughout the world and it is proposed that nano-sized plastic particles are also being generated (Cole et al. 2011). Additionally, the production of nano plastics for applications including drug delivery, cosmetics, electronics, and paints increases the risk of their entry into aquatic ecosystems where they are available for consumption by a variety of organisms (Ward et al. 2009).

It has been reported that an adult oyster can filter up to 200 L of water per day (Foundation). As such, they are an essential organism for the maintenance and stability of estuarine systems, but are also highly susceptible to water-borne pollutants. As oysters feed, food particles are captured and first sorted by the gills, and concentrated into a mucous strand, which carries the material to the labial palps which ejects sediment

particles, debris, etc. Food particles then travel into the mouth, and into the stomach, where preliminary breakdown of the food materials occurs, and then into the digestive gland (aka hepatopancreas) for intracellular digestion and nutrient absorption. Toxic materials may also follow this pathway and may accumulate inside cells and lysosomes, or other organelles, and adversely affect their function. The high filtration capacity mediated by the gills, and the predisposition for particle accumulation by digestive gland tissues make the Eastern oyster, *Crassostrea virginica,* and other bivalves excellent models for evaluating the potential impacts of polystyrene nano and micron particles on estuarine ecosystems. (Browne et al. 2008, Wright et al. 2013) (Ringwood et al. 2002)

Micron and nano sized particles have the potential to mimic food particles to filter feeders, such as oysters. Wegner et al (2012) found that the blue mussel, *Mytilus edilus,* reduced feeding activity and increased pseudofeces production in the presence of freely suspended (non-aggregated) 30nm plastic particles when exposed for eight hours. However, when algae was also present, the animal increased filtering resulting in increased removal of plastic particles from the water. In a study by Ward et al (2009) uptake of 100nm plastic particles has been shown to be facilitated by agglomeration with organic material in the water column. Evidence of freely suspended nanoparticles was not found in hepatopancreas tissue after 72 hours of exposure, whereas it was observed in hepatopancreas tissue when the particles were incorporated in agglomerates. Additionally, Ward found the nanoparticles in fecal pellets for more than 14 days after transfer to clean conditions, as compared to the 10um particles that were fully egested within 6 days after transfer to clean conditions. Ward proposed that the longer gut retention time may be indicative of more extensive intracellular digestion. Likewise, in a

study by Browne et al (2008) non-aggregated 3µm and 9.6µm plastic particles were shown to enter the gut cavity and digestive tubules within 12 hours of exposure, and to translocate into the circulatory system of the mussel, *Mytilus edilus*, where they persisted after transfer to clean water for the 48 days of the study. Additionally, the smaller sized particles (3um) translocated and accumulated in the hemolymph to a higher concentration during the exposure period than the larger sized particles (9.6 um). Therefore, smaller particles may have a greater potential for uptake, increasing the concerns regarding small micro- and nano-particle wastes.

Plastics are hydrophobic substances that provide a highly attractive surface for the binding of lipophilic chemicals (Cole et al. 2011), including polycyclic aromatic hydrocarbons (PAH) and polychlorinated compounds. Plastic pellets recovered from the beaches of Hawaii, Mexico, and California had adsorbed a mixture of polycyclic aromatic hydrocarbons (PAH's) at concentrations ranging from 500 to 1200 ng/g (Rios et al. 2007). In a sample of plastic fragments recovered from a Portugal beach, pyrene, one of the top 17 PAH's listed by the Environmental Protection Agency (EPA) as potentially harmful or toxic (Alberts 2004) was one of the most common PAH's adsorbed to polystyrene at concentrations as high as 319 ng/g (Frias et al. 2010). These concentrations are much higher than those recovered from the surrounding water (Mato et al. 2001), indicating that plastic particles can concentrate organic contaminants, and potentially deliver high doses to animals that consume them.

Contamination of plastic by persistent organic pollutants, such as pyrene, which can be toxic to marine life, presents significant issues of concern about impacts on marine species (Martins et al. 2011). Pyrene is a known carcinogen that is ubiquitous in the

environment with concentrations increasing near urbanized or industrialized areas (Manzetti 2013). Pyrene is formed by the incomplete combustion of fossil fuels, volcanic activity, forest fires, and other industrial activities (Manzetti 2013). It has been documented to have genotoxic, mutagenic, and carcinogenic effects, attributed to its planar conformation which allows it to readily diffuse into the cytoplasm of cells, where it has been shown to increase oxidative stress (Xue et al. 2005, Manzetti 2013).

Organic compounds like PAH's have been shown to induce oxidative stress in aquatic animals (Winston and DiGiulio, 1991; Livingstone, 2001). Because the breakdown and removal of PAH's occurs through the cytochrome P450 system, there are two major pathways that this can occur through. The first involves the formation of a reactive intermediate after phase 1 biotransformation that can directly enter redox cycles, whereas the second involves the conjugation to detoxification enzymes, e.g. glutathione, of that reactive intermediate, leading to a decrease in oxidative defense potential through the excretion of this oxidized form (Lemaire et al 1994). While, several CYP genes have been identified in bivalves, little is known about bivalve mollusk CYP450 systems making this important work in understanding the effects of PAH's on the Eastern oyster (Zanette et al. 2010).

The purposes of this research project were to evaluate the uptake and intracellular accumulation of polystyrene particles, both nano and micron sized, by oysters, *C. virginica*, as well as evaluate the potential for polystyrene particles to act as a Trojan horse to facilitate increased accumulation and toxicity of the organic pollutant pyrene. Experiments were conducted to test the following hypotheses:

H1. Oysters exposed to polystyrene beads, both nano and micro, will experience cellular toxicity.

H2. Nano-sized polystyrene beads will be taken up more readily by hepatopancreas cells than micron sized polystyrene beads.

H3. Gill and hepatopancreas tissues of adult oysters will exhibit differential uptake of polystyrene beads based on particle size and concentration.

H4. Exposure to polystyrene-pyrene particles will be characterized by increased oxidative stress compared to oysters exposed to polystyrene or pyrene alone.

CHAPTER 2: POLYSTYRENE PARTICLE ACCUMULATION AND EFFECTS

2.1 Introduction

The presence of plastic particles in ocean waters and aquatic organisms is an important current topic that is serious and of increasing concern. As global production of plastics increases, and improper handling of plastic wastes continues, an ever increasing quantity of plastic makes its way into aquatic ecosystems, freshwater waterways, the open ocean and estuarine systems (Martins et al. 2011, McCarthy et al. 2013). In 2014, 311 million tons of plastic were produced with disposable packaging being the largest market sector further increasing the concern about plastic waste inputs in the marine environment (Halliwell et al. 1984). An additional source of concern is the use of microplastics from common household products such as toothpaste, facial exfoliators, and hand cleaners (Derraik 2002, Lassen 2015). It has been estimated that if plastic production continues to rise at the same annual rate, by the year 2025 plastic waste inputs into ocean systems will reach between 100 and 250 million metric tons (McCarthy et al. 2013). Furthermore, degradation of those polymers via natural weathering processes has been shown to fragment plastics into sizes smaller than 1um (Tobiszewski et al. 2012), and degradation has been shown to start in as little as 8 weeks (Weinstein et al. 2016). This results in particle fragments that are readily available for consumption by a

multitude of organisms including small marine invertebrates (Council 2003, Martins et al. 2011).

Organisms across trophic levels have been found to ingest plastic marine debris in the natural environment (Cadée 2002, Environment et al. 2008). Estuarine organisms have been shown to have higher levels of plastic waste in their guts as compared to open ocean organisms (Denuncio et al. 2011). Laboratory studies have also confirmed the capacity of estuarine organisms to accumulate plastic (Bocchetti et al. 2008, Ward et al. 2009, Ma et al. 2011, Wegner et al. 2012), and translocation of plastic particles from the gut to the circulatory system has been observed in mussels (Browne et al. 2008).

Oysters are sessile filter feeders common in estuaries and they are known to remove particles in the 4-10um size range with nearly 100% efficiency (Haven et al. 1970, Ringwood et al. 2008) making them an ideal model for particle uptake studies in the micron size range. While there is some debate about the filtration efficiency of bivalves below 1um, studies with oysters have shown that a reduction in waterborne bacteria occurs with oyster feeding indicating that oysters are capable of capturing particles well below 1um (Langdon 1996, Jones et al. 2002).

Filter feeding in oysters and other bivalves is accomplished by the highly-ciliated gills. As the inhalant currents move water through the gills food particles are sorted and captured by the cilia, and incorporated into mucous strings that are then moved towards the labial palps and mouth. Rejected particles are moved away from the labial palps through radial ridges on the mantle and expelled with the exhalent current as pseudofeces. Accepted particles are moved through the labial palps to an oral groove and finally into the mouth and digestive tract. (Ringwood et al. 2002, Gosling 2015)

Post-ingestive particle sorting is continued by cilia on the gut lining. Large particles that are not able to be broken down physically by the rotating abrasion of, or chemically from the released enzymes of the crystalline style have been shown to move through the intestinal groove rather rapidly with a short gut retention time. Smaller particles, and larger particles that have been broken down, are moved into the digestive gland where they are taken up by endocytic pathways, such as pinocytosis (particles <150nm) and phagocytosis (particles >150nm), and shuttled to the lysosomes where the food particles are digested (Alberts 2004, Ward et al. 2009, Gosling 2015). Because these pathways are highly conserved in eukaryotes, they are the most likely routes of PS particle uptake (Moore 2006).

For this research, two types of studies were conducted to assess the accumulation of polystyrene particles in oyster tissues using both micron sized (e.g. 3µm) and nano-sized (e.g. 50nm) particles. *In vivo* studies were conducted to test the hypothesis that gill and hepatopancreas tissues of adult oysters would exhibit differential uptake of polystyrene particles based on particle size. *In vitro* studies were conducted using isolated cells to test the hypotheses that nano-sized particles would be taken up more readily by hepatopancreas cells than micron-sized particles in a concentration-dependent manner. Both *in vivo* and *in vitro* studies were used to assess potential toxicity.

2.2 Materials and Methods

2.2.1 Animal Care and Exposure Conditions

Eastern oysters, *Crassostrea virginica*, collected from Bogue Sound off the North Carolina coast at Emerald Isle were maintained in the lab aquaria with salinity and pH

monitored daily and maintained between 25-30‰ and around 7.8-8.2 pH units, respectively. All seawater (SW) used for laboratory culture and exposures contained a mixture of natural SW, collected off the North Carolina coast, and artificial SW (Instant Ocean©) at a ratio of 2/3 Instant Ocean to 1/3 natural SW. When used for exposures, this mixture was filtered to 0.22µm. Aquaria were kept under constant aeration and carbon filtration was alternated every other day with feeding (carbon filters were removed and the animals were fed cultured algae *Isochyrsis galbana* (CCMP462, Bigelow Laboratory for Ocean Sciences) cultured in autoclaved SW with f/2-Si medium).

The 3um and 50nm fluorescent polystyrene (PS) particles used in this study contained a yellow fluorophore incorporated into the particle during preparation by the manufacturer (Spherotech catalog #'s FP-3052-2 and FP-00552-2 respectively). A stock solution of 100ppm was prepared, based on particle concentration, in DI water and vortexed to obtain a homogeneous mixture. Serial dilutions were made in the filtered SW mixture to produce exposure concentrations of 10ppb, 50ppb and 100ppb.

Dynamic light scattering (DLS) was utilized to assess the effects of concentration and SW on particle size using a Zetasizer Nano ZS (Malvern Instruments). Polystyrene solutions were prepared in the same manner as the exposure solutions using both DI water and the seawater mix at concentrations of 10ppb, 50ppb, and 100ppb. To replicate *in vivo* exposure conditions 50ppb solutions were aerated in glass beakers for 48 hours and then analyzed. To replicate in vitro exposure conditions the 10ppb and 100ppb solutions were kept in tubes and gently mixed every hour for 4 hours and then analyzed. Analysis was completed using a refractive index of 1.590. The average particle sizes reported are based on readings from 2 different exposure days.

2.2.2 *In vitro* Exposures

Primary cell cultures of hepatopancreas cells were generated using standard techniques. Hepatopancreas tissue sections of approximately 5mm^3 were dissected, kept on ice, and manually chopped before being placed into one well of a 24-well plate, shaken in 800µl calcium magnesium free saline (CMFS) for 20 minutes and 800µl CMFS plus 400µl trypsin for an additional 20 minutes. After shaking, tissues were sheared using a glass Pasteur pipette and transferred to 1.7ml microcentrifuge tubes by filtering through 41µm mesh screens. Tubes were spun at 1700 rpm for 3 minutes, washed by removing the supernatant and resuspending in 1ml CMFS. This process was repeated and the isolated cells from each tube were transferred to separate 60mm x 15mm glass exposure dishes containing 8ml of exposure solution of a 1:1 mixture of CMFS and either SW control, 10ppb PS, or 100ppb PS and exposed for 4 hours with gentle shaking throughout. Each treatment was replicated in a total of 6 dishes with pH maintained between 7.4-7.5 to mimic physiological conditions. A 1ml subsample of cells was taken before the addition of PS (control), at 1hr, 2hrs, and 4hrs, and fixed in 1% buffered formalin for review under fluorescent microscopy. Subsamples for lysosomal destabilization analysis were taken at 0hrs and 4hrs.

2.2.3 *In vivo* Exposures

Groups of 3 oysters were placed in 2 L beakers and exposed to 1.2 L of the PS particle solutions of 50ppb in SW for 48 hours. At the end of the exposure period, oysters were removed from the treatment water and placed in clean SW for a 1-hour depuration period to allow for some cleansing of extraneous polystyrene particles. Oysters were then sacrificed and live, freshly dissected hepatopancreas (HP) tissues were used for

lysosomal destabilization assays. Additional samples of gill and HP tissues were processed to isolate cells that were fixed in 1% buffered formalin for fluorescent microscopy. These studies were used to characterize the potential for uptake and toxicity of polystyrene particles in whole adult (*in vivo*) oysters.

2.2.4 Lysosomal Destablization

Lysosomal destabilization assays have been used widely as a valuable cellular damage assay (Regoli 1992, Ringwood et al. 2005, Moore et al. 2014). Briefly, hepatopancreas tissue samples, approximately 5mm^3, were processed into primary cell preparations with CaMg-free-saline (CMFS) and trypsin, and filtered through a 41μm nylon screen. After the cells were rinsed and re-suspended in CMFS (final volume between 80ul-120ul dependent on pellet size), a working solution of neutral red (NR) (0.04mg/mL) was added at a 3:2 ratio of NR to cell preparation volume for a final concentration of 24μg/ml. After a 60-minute incubation period cells were scored as either stable (NR contained within the lysosomes) or destabilized (NR diffusing into the cytoplasm from damaged lysosomes) at 400x magnification, with at least 50 cells scored from each preparation (Figure 1).

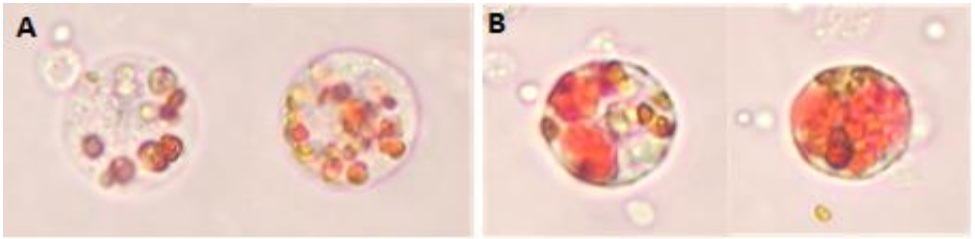

FIGURE 1: Lysosomal destabilization in oyster hepatopancreas cells viewed at 40x. Cells are scored as being stable (A) based on the NR being contained in the lysosomes or unstable (B) based on the NR diffusing in to the cytoplasm. (Ringwood et al. 2005)

2.2.5 Fluorescent Microscopy

Fixed cells were rinsed twice in CMFS to remove freely suspended PS particles (centrifugation at 200g for 5 minutes, removing the supernatant, and resuspending in fresh buffered formalin). After the final rinse, cells were re-suspended in a total volume of 200ul to concentrate the cells. Subsamples of cells were mounted on slides and analyzed using the Zeiss Axio Observer with the Fluo3 filter (ex. 400+495+570; em.460+530+625) at 400x total magnification. One hundred cells per animal (n=6 replicates/treatment) were scored as fluorescing or not fluorescing, and the percent fluorescing cells was determined. Cells were also analyzed using the Delta Vision Elite deconvolution microscope at 600x total magnification which allowed for greater resolution and determination of cellular location of PS. Twenty cells per replicate were cells with particles inside was determined.

2.2.6 Statistics

All data were analyzed using Sigma Stat 2.0 or GraphPad Prism 6. There were no significant beaker effects in the experiments, so data were pooled for statistical analysis. Experiments/treatments performed on separate dates were compared using ANOVA or t-test analyses and data were pooled to increase power when there were no significant differences ($p \geq 0.05$). Significant differences between treatments were determined using either the one-way ANOVA analysis or ANOVA on Ranks. Additionally, pairwise comparisons were performed using the Student-Newman-Keuls method.

2.3 Results

2.3.1 Particle sizing

To evaluate particle characteristics and behavior in seawater, Dynamic Light Scattering (DLS) was conducted on subsamples of the 50nm particle solutions. Results were analyzed to provide mean particle size in exposure solutions as an indicator of agglomeration. Particles did not agglomerate in DI water at any of the tested concentrations and maintained the manufacturer reported size. In seawater, there was no significant increase in particle size across all treatments, although the highest particle size was observed at the highest PS concentration (Table 1). Therefore, based on DLS studies the particles were very stable and did not show high rates of agglomeration even in seawater.

TABLE 1: Summary of polystyrene (PS) particle size studies with DLS (average ± SD) in DI water or SW (25‰). Averages based on sample sizes of 6 replicates from two different exposure days.

Media	[PS NP] (µg/L)	Average Size (nm)
DI	10	59.28 ± 2.76
DI	50	59.41 ± 1.54
DI	100	61.14 ± 5.94
SW 25‰	10	60.26 ± 4.27
SW 25‰	50	59.55 ± 6.32
SW 25‰	100	68.11 ± 10.98

2.3.2 Particle toxicity

Lysosomal destabilization was used as an indicator of cellular toxicity both *in*

vivo and *in vitro*. After both the 4-hour *in vitro* and 48 hour *in vivo* exposures no evidence of toxicity was observed for any of the particle types or concentrations for both time frames (Figure 2).

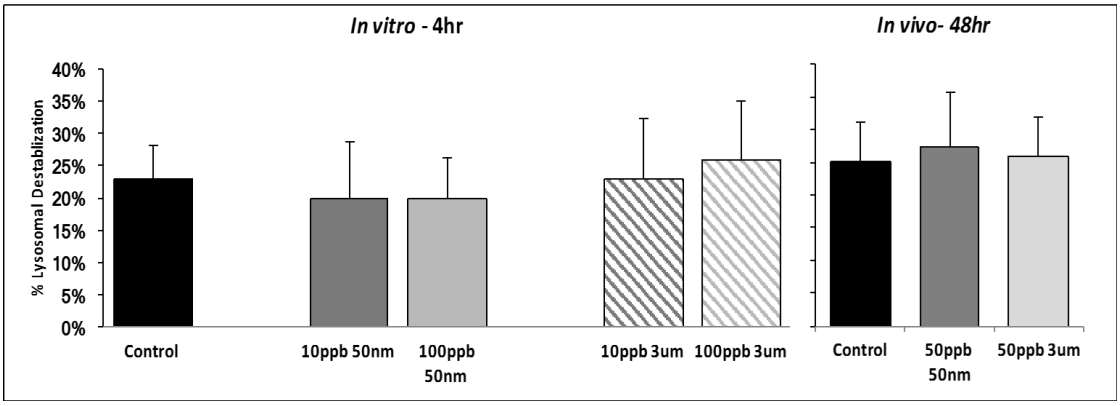

FIGURE 2: Lysosomal destabilization in hepatopancreas cells after exposure to fluorescent polystyrene beads. No significant differences were detected between controls and any of the treatments.

2.3.3 Cellular Accumulation Studies

Epifluorescent microscopy analyses were used to assess the potential for cellular accumulation of PS particles. The 3 um PS particles were observed in solution to confirm visibility (Figure 3). Using epifluorescent microscopy at 400x, both polystyrene nanoparticles and microparticles were found with oyster hepatopancreas cells exposed *in vitro* for 4 hours (Figure 4).

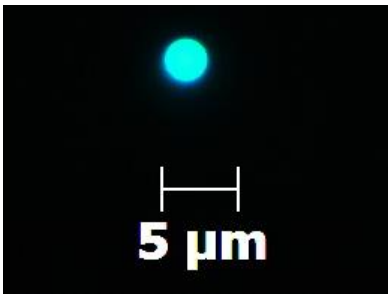

FIGURE 3: Fluorescent microscopy image of 3um bead viewed at 400x using the Zeiss filter set 25(ex. 400+495+570, em. 460+530+625)

FIGURE 4: Hepatopancreas cells as viewed with the Zeiss Axio Observer under light microscopy at 400x magnification (A); Control cells using the Fluo3 filter showing absence of polystyrene particles (B); presence of 50nm polystyrene particles (C); and presence of 3um polystyrene particles (D).

In the case of 50nm particles at both 10 and 100ppb, there was a significant difference in presence across different time points (ANOVA, $p<0.001$), with increasing incidence of fluorescing cells over time (Figure 5). While slightly higher percentages of fluorescent cells were observed for the 100ppb treatment, there were no significant differences between exposure concentrations for the different time points. Regression analysis based on exposure duration indicated a significant increase with time (analysis based on individual replicates, $n=12$ for each time point, $p<0.0001$, $r^2 =0.80$).

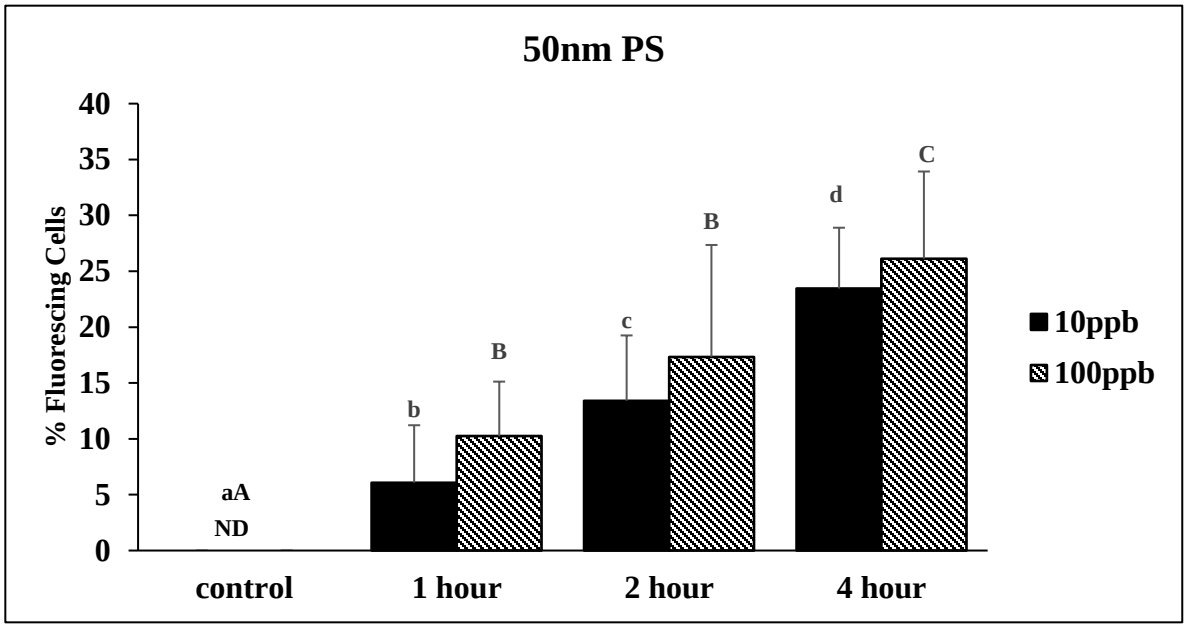

FIGURE 5: Percent fluorescing cells based on epifluorescent analysis after exposure of hepatopancreas cells in vitro to 50nm polystyrene particles at two concentrations for 0,1,2, and 4 hours. Values are means+ standard deviations. Letters indicate significant difference within a concentration. ND= not detected. n=6 replicates/treatment; ≥100 cells/replicate, $p<0.05$.

Accumulation of 3um PS particles was observed after 1 hour of exposure *in vitro*, with significant differences at the later time points ($p=0.005$). (Figure 6 Regression analysis indicated a significant accumulation over time, although the slope and r^2 values were lower for the 3µm than the 50nm PS particles (10ppb - $F(1,22)=13.34$, $p=0.0014$, $r^2=0.38$; 100ppb - $F(1,22)=9.015$, $p=0.0066$, $r^2 = 0.29$). In contrast to the pattern observed with nano-PS, more micron-PS particles were accumulated with cells exposed to the lower PS concentration. To further consider size effects, the data were pooled from both concentrations. The incidence of accumulation was greater with the 50nm particles than with the 3um particles, except in the case of the 2-hour time point (Figure 7).

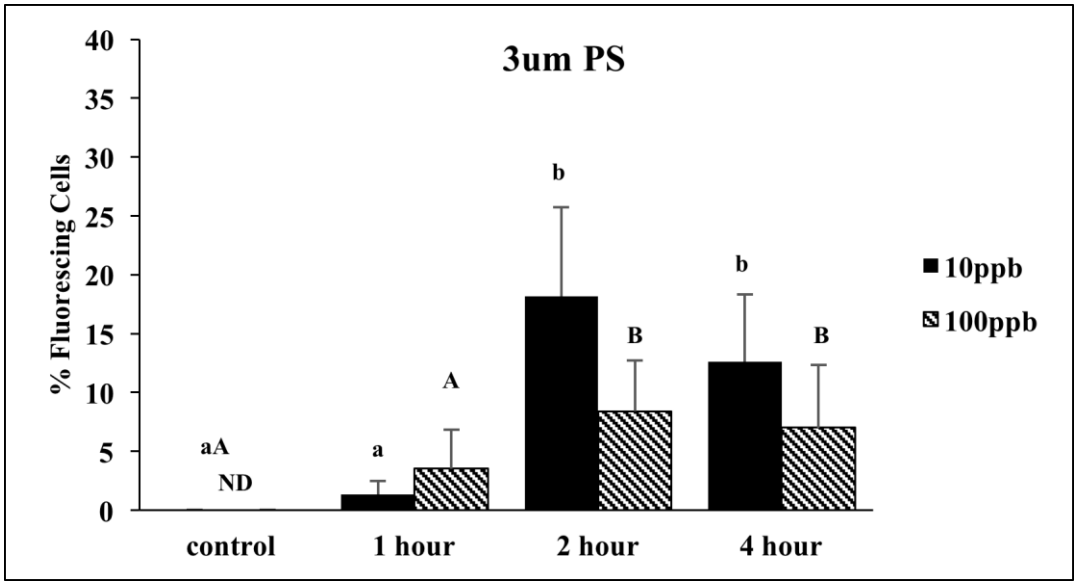

FIGURE 6: Percent fluorescing cells after exposure of hepatopancreas cells *in vitro* to 3um polystyrene particles at two concentrations for 0,1,2, and 4 hours. Values are means+ standard deviations. Letters indicate significant differences within a concentration. An * indicates significant differences within a time point across concentrations ($p<0.05$). ND= not detected. n=6.

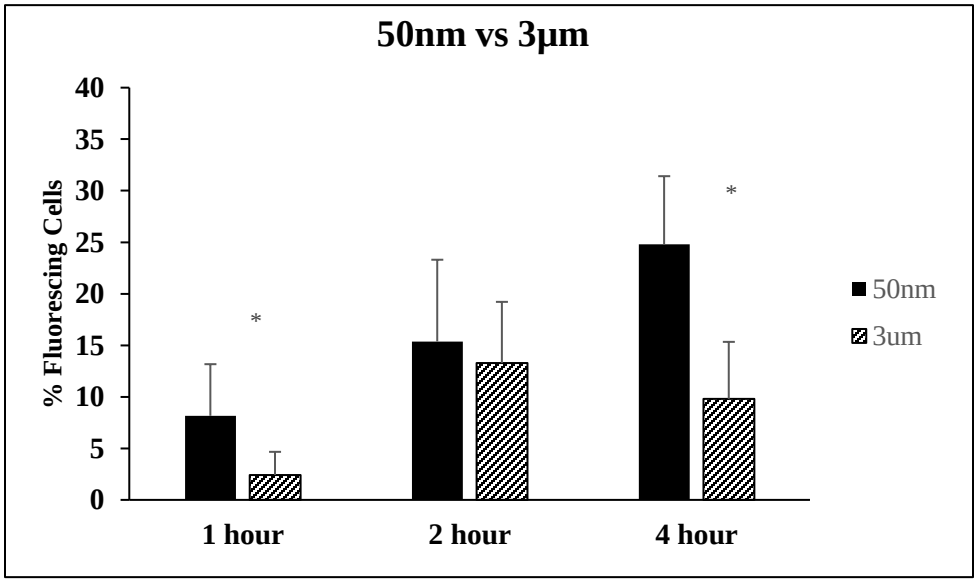

FIGURE 7: Percent fluorescing cells after exposure of hepatopancreas cells *in vitro* to 50nm and 3um polystyrene particles at two pooled concentrations (10 and 100ppb) for 0,1,2, and 4 hours. Values are means+ standard deviations. An * indicates significant differences between particle sizes ($p < 0.05$). ND= not detected. n=12.

Epifluorescent microscopy indicated cellular accumulation that includes both internalized PS particles and PS particles that are bound externally. Deconvolution microscopy was used to confirm internalization of PS particles. After review of the hepatopancreas cells exposed to 50nm particles using 600x magnification, it was determined that only the 50nm particles were internalized by cells (Figures 8 and 9), while 3µm PS particles were observed on the cell margins only. Additionally, the percentage of cells with 50nm particles was not significantly different at each time point from the percent determined using the epifluorescent microscope (compare Figures 5 and 9).

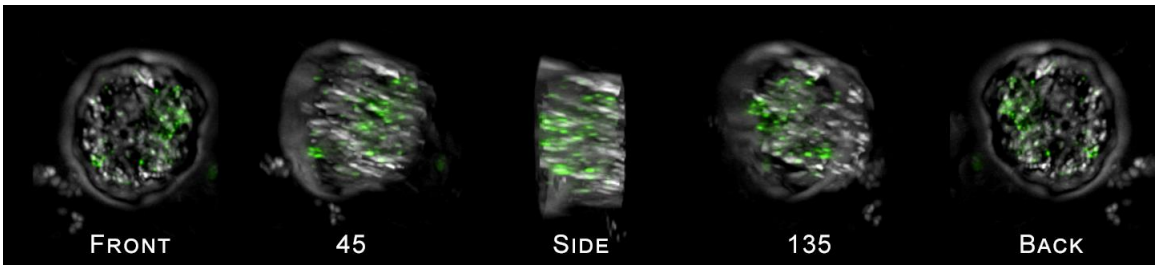

FIGURE 8: Cross section of a single hepatopancreas cell exposed to 50nm PS particles as viewed using the DeltaVision Elite deconvolution microscope at 600x total magnification rotated around the y-axis of the cell by 180°. Images show the presence of 50nm polystyrene beads within the cell.

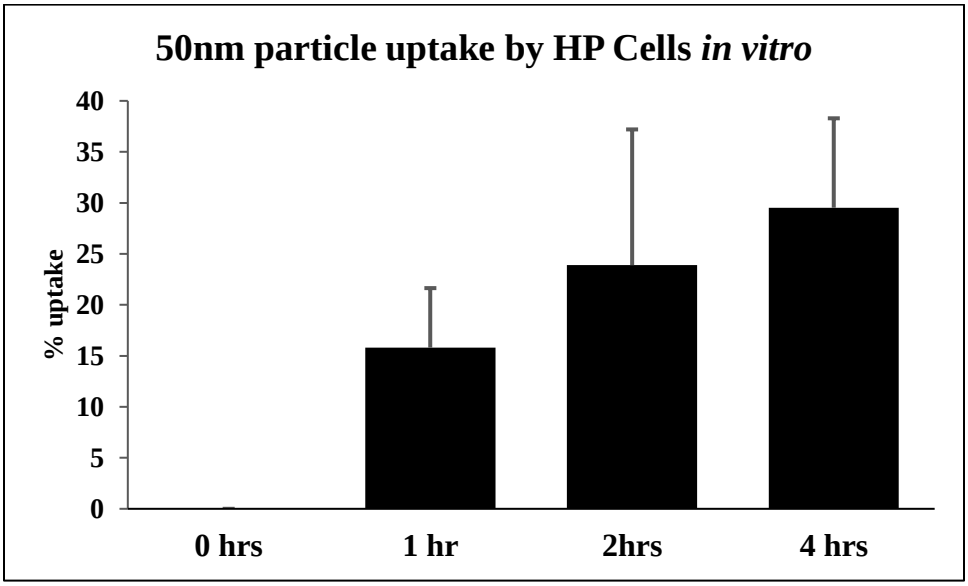

FIGURE 9: Percent of hepatopancreas cells with internalized PS particles after *in vitro* exposure to 100ppb 50nm polystyrene particles for 0,1,2, and 4 hours, determined by fluorescent deconvolution microscopy. Values are means+ standard deviations. ND= not detected. Control N=5; Treatment N=6.

2.3.4 *In vivo* Exposure

To compare if particle uptake occurred in the gills, in the hepatopancreas tissues, or both of intact adult oysters, *in vivo* exposures were conducted using 50ppb of 50nm and 3um particles. Epifluorescent microscopic analyses at 400x magnification indicated that both 50nm and 3um polystyrene particles were found with both hepatopancreas and gill oyster cells when exposed *in vivo* (Figure 10). Comparison between tissue types showed slightly higher incidences of 50nm particles than 3µm but the differences were not significant. However, in gill cells the incidence of fluorescing cells was significantly higher for the 50nm PS particles compared to the 3µm PS particles; and both were lower than those of hepatopancreas cells (Kruskal Wallis One Way ANOVA on Ranks, $p<0.001$). In comparing uptake across tissue types *in vivo*, the hepatopancreas tissues

had a significantly higher accumulation of PS particles than the gills with both 50nm (t-test, p=0.045) and 3um (Mann-Whitney Rank Sum Test p=0.026) particles. Based on this finding, only hepatopancreas cells were further analyzed using deconvolution microscopy which indicated that only 50nm polystyrene particles were internalized by hepatopancreas cells (Figure 11).

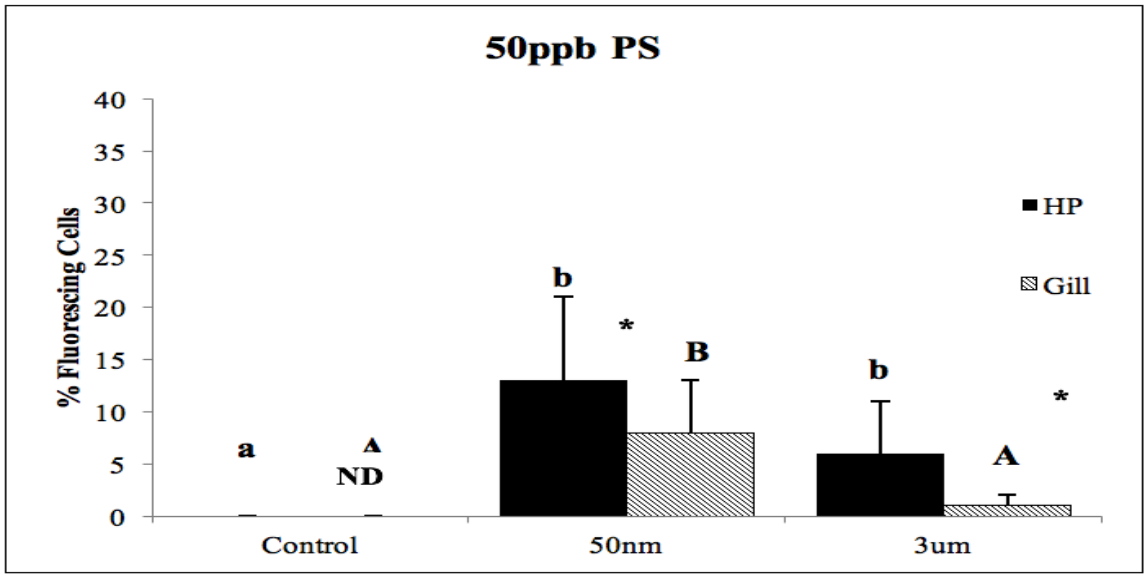

FIGURE 10: Percent fluorescing cells as determined by epifluorescence review at 400x magnification after exposure to PS particles for 48 hours *in vivo*. Values are means+ standard deviations. Different letters indicate a significant difference between particle sizes within each tissue type ($p<0.05$). Asterisks indicate a significant difference between tissue types ($p<0.05$). HP N=18 (control), N=14 (50nm), N=13 (3um). Gill N=18 (control and 3um), N= 15 (50nm). ND = not detected.

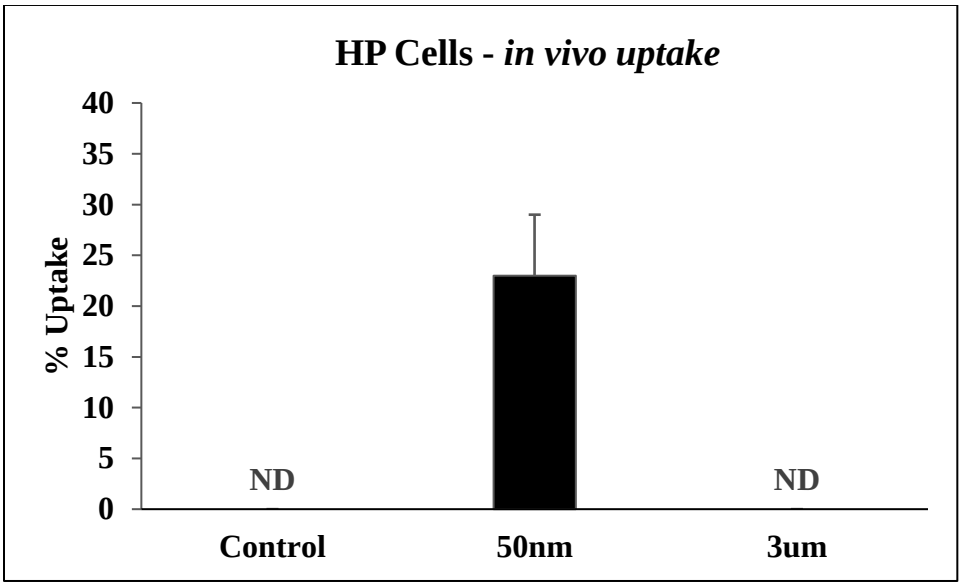

FIGURE 11: Percent fluorescing hepatopancreas cells determined by deconvolution microscopy review at 600x magnification after exposure to polystyrene particles for 48 hours *in vivo*. Values are means+ standard deviations. N=18 (control), N=14 (50nm), N=13 (3um). ND = not detected.

2.4 Discussion

In these studies, PS particle toxicity and uptake at the cellular level were investigated in adult oysters (*in vivo*) and in isolated cells (*in vitro*). The results of these studies confirm that 50nm particles are internalized by oyster cells following both *in vitro* and *in vivo* exposures. However, for the 3µm particle, the fluorescence observed was due to extracellular attachment. Studies with epifluorescent techniques can only confirm association, but closer inspection of the hepatopancreas cells using deconvolution microscopy indicated that only the 50nm particles were internalized, and were clustered in what appeared to be the lysosomes. This finding is consistent with other types of

nanoparticles that have also been observed in the lysosomes of cells after exposure (Ringwood et al. 2003, Ma et al. 2011, Seydoux 2014, Rocha et al. 2016). Due to the hydrophobic nature of PS particles it is like that the PS particles adhered to the outside of hepatopancreas cells by Van der Wahl's forces where the 50nm PS particles were subsequently taken up through pinocytosis, the endocytic pathway used by cells to take up fluid and small particles (<150nm), where they would then be shuttled to the lysosomes (Alberts 2004, Moore et al. 2014).

The percent accumulation for 50nm PS particles determined using the epifluorescent microscope was comparable to the percent uptake calculated from the deconvolution microscope. This important finding indicates that epifluorescent microscopy which is more readily available in teaching and research laboratories can be used to study nanoparticle accumulation. Even in the epifluoresent images in these studies, the nanoparticles appeared more organized and consistent with lysosomal content, whereas the micron particles had a more disorganized arrangement and looked more external.

Using a sensitive cellular toxicity assay, lysosomal destabilization, there was no evidence of toxicity for either the 3µm or 50nm PS particles over the short-term duration of these exposures. Therefore, even though particle accumulation (internal and external) was observed, especially in hepatopancreas cells, the bare PS particles were not overtly toxic. Longer term exposures or chronic accumulation could contribute to cellular or lysosomal destabilization if cells are unable to eliminate PS particles.

Environmental data continue to show increases in the amount of plastic wastes in marine environments, accumulating significantly in estuaries and on beaches (Derraik

2002, Council 2003, Nolde et al. 2006, Claessens et al. 2013). These studies further indicate that breakdown of plastics to less visible nanoparticles can have important implications regarding cellular bioreactivity, and highlight the importance of continued studies regarding potential impacts on aquatic organisms.

CHAPTER 3: THE EFFECTS OF PLASTIC PARTICLES AND PYRENE IN COMBINATION – IN VITRO STUDIES WITH OYSTER HEPATOPANCREAS CELLS

3.1 Introduction

Plastic polystyrene beads have been shown to be ingested by invertebrate animals and to be translocated to other tissues within the organism (Bocchetti et al. 2008, Browne et al. 2008, Environment et al. 2008, Hwang et al. 2014) and ingestion can result in the reduction of food consumption leading to a decrease in fecundity and survival (Cole et al. 2015). An additional area of concern with regards to plastic ingestion is the potential for plastic fragments or pre-production pellets to adsorb pollutants from the surrounding water or sediments and make them more bioavailable to marine organisms, particularly filter feeders and deposit feeders (Mato et al. 2001). Polystyrene, among other plastics, is a hydrophobic polymer capable of binding organic substances such as polycyclic aromatic hydrocarbons, polychlorinated biphenyls, pesticides, etc. (Ziccardi et al. 2016).

Polycyclic aromatic hydrocarbons (PAH) are chemical compounds widely known as coal and oil-related pollutants that are also produced by other combustion processes including cooking, volcanic activity, and forest fires, and as a result are ubiquitous in the environment (Harvey 1997, Council 2003, Environment et al. 2008, Tobiszewski et al. 2012, Shen 2016). Pyrene is one type of polycyclic aromatic hydrocarbon comprised of four benzene rings. Levels of pyrene in the environment have been increasing as oil fueled vehicles become more common in developing nations resulting in increased

extraction and spill (Shen 2016). The EPA lists pyrene among the 15 most toxic polycyclic aromatic hydrocarbons (EPA 1995). Pyrene has been shown to induce cellular damage *in vivo* and *in vitro* in a variety of marine organisms. Studies have shown polycyclic aromatic hydrocarbons to have adverse effects on organism health across trophic levels as metabolization of polycyclic aromatic hydrocarbons can lead to reactive intermediates capable of oxidative damage resulting in oxidative stress and potentially death (Anderson et al. 1986, Grundy et al. 1996, Woo et al. 2014, Bhagat et al. 2016, Dissanayake et al. 2016).

For this research, studies were conducted to determine the potential toxicity of pyrene to oysters when exposed with and without polystyrene beads, both nano sized (e.g. 50nm) and micron sized (e.g 3µm). *In vitro* studies were used to test the hypothesis that oysters, when exposed to polystyrene beads, both nano and micro, in combination with pyrene will experience greater cellular toxicity than to polystyrene or pyrene alone.

3.2 Materials and Methods

3.2.1 Animal Care and Exposure Conditions

Eastern oysters, *Crassostrea virginica,* collected from Bogue Sound off the North Carolina coast at Emerald Isle were maintained in the lab aquaria with salinity and pH monitored daily and maintained between 25-30‰ and around 7.8-8.2 pH units, respectively. All seawater (SW) used for laboratory culture and exposures contained a mixture of natural SW, collected off the North Carolina coast, and artificial SW (Instant Ocean©) at a ratio of 2/3 Instant Ocean to 1/3 natural SW. When used for exposures, this mixture was filtered to .22µm. Aquaria were kept under constant aeration and carbon

filtration was alternated every other day with feeding (carbon filters were removed and the animals were fed cultured algae *Isochyrsis galbana* (CCMP462, Bigelow Laboratory for Ocean Sciences) cultured in autoclaved SW with f/2-Si medium).

The 3um and 50nm fluorescent polystyrene (PS) particles used in this study contained a yellow fluorophore incorporated into the particle during preparation by the manufacturer and were purchased from Spherotech (catalog #'s FP-3052-2 and FP-00552-2 respectively). A stock solution of 100ppm was prepared, based on particle concentration, in DI water and vortexed to obtain a homogeneous mixture. Serial dilutions were made in the filtered SW mixture to produce exposure concentrations of 10ppb and 100ppb.

A 100ppm stock solution of pyrene was prepared using pyrene obtained from Sigma Aldrich (catalog #82648-1G), dissolved in toluene which was then evaporated off before preparation of the exposure solutions of 50ppb and 200ppb in 25psu seawater (1:2 natural to artificial seawater, 0.22 µm filtered).

3.2.2 *In vitro* exposure

Exposure dishes containing 10ppb polystyrene, 100ppb polystyrene, 50ppb pyrene, 200ppb pyrene, or a mixture of polystyrene and pyrene. For the polystyrene and pyrene mixtures, the solutions were prepared from the primary working stocks 4 hours prior to tissue exposures to allow time for the pyrene to adsorb to the polystyrene beads. Hepatopancreas tissues were dissected from 24 animals with each organ separated into three pieces of approximately $5mm^3$. Tissues were kept cool in the SW mixture with 1mg/ml streptomycin and 0.4 mg/ml penicillin during the dissections, 3 tissue pieces were then added to each of the exposure dishes and exposed for 24 hours. At the

conclusion of the 24-hour exposure, one piece of tissue was analyzed for lysosomal destabilization and the remaining pieces were frozen for lipid peroxidation and other analyses.

3.2.3 Lysosomal Destabilization

Lysosomal stability in the hepatopancreas (aka the digestive gland) tissues is a well-established indicator of toxicity (Regoli 1992, Ringwood et al. 2005, Moore et al. 2014). At the conclusion of the 24 hour exposure, hepatopancreas tissue samples approximately 5mm³ were processed into primary cell preparations with CaMg-free-saline (CMFS) and trypsin, and filtered through a 41μm nylon screen. After the cells were rinsed and resuspended in CMFS (final volume between 80ul-120ul dependent on pellet size), a working solution of neutral red (NR) (0.04mg/mL) was added at a 3:2 ratio of NR to cell preparation volume for a final concentration of 24μg/ml. After a 60-minute incubation period cells were scored as either stable (NR contained within the lysosomes) or destabilized (NR diffusing into the cytoplasm from damaged lysosomes) at 400x magnification, with at least 50 cells scored from each preparation (see Figure 1, Chapter 1)

3.2.4 Lipid Peroxidation

Malondialdehyde (MDA) is an abundant reactive aldehyde produced from the breakdown of polyunsaturated fatty acids of membrane phospholipids by oxygen free radicals, and thus is often used a general indicator of oxidative damage (Halliwell et al. 1984). Therefore, a MDA assay was used to measure the levels of oxidative damage in hepatopancreas tissue (Ringwood et al. 1999) Tissues were weighed and homogenized in 4 volumes of 50mM potassium phosphate buffer (pH 7.0), then centrifuged (13000 g at

4°C for 5 minutes); 50µl subsamples of the supernatant were combined with 700µl of 15% trichloroacetic acid containing (0.375%) thiobarbituric acid and 7µl 2% butylated hydroxytoluene. Standards of known MDA concentrations were prepared (800, 400, 200, 100, 50, and 25 µM) from a 10mM malondialdehyde tetraethylacetal stock solution (Acros organics, NJ). Samples and standards were boiled in a water bath for 15 minutes, and then centrifuged (13000 RCF, at room temperature for 5 minutes). The supernatant was then dispensed into a 96-well plate in triplicate (200-µl in each well) and the absorbance of MDA was measured at 532nm using a µQuant spectrophotometer (Biotek Instruments, Inc.). The final data are expressed as nmol/g wet tissue.

3.2.5 Statistics

All data were analyzed using Sigma Stat 2.0 or GraphPad Prism 6. There were no significant beaker effects in the experiments, so data were pooled for statistical analysis based on individual oysters. Experiments/treatments performed on separate dates were compared using ANOVA or t-test analyses and data were pooled to increase power when there were no significant differences ($p \geq 0.05$). Significant differences between treatments were determined using either the one-way ANOVA analysis, ANOVA on Ranks, or two-way ANOVA analysis. Pairwise comparisons were performed using the Student-Newman-Keuls or Bonferroni methods.

3.3 Results

3.3.1 Nano Polystyrene

To determine if nano-sized (50nm) polystyrene (PS) could be acting as a vector for pyrene and causing increased toxicity to oyster tissues, 24 hr *in vitro* exposures were

conducted, and lysosomal destabilization and lipid peroxidation were used as markers of cellular toxicity. After exposure to 50nm PS and pyrene for 24 hours, the results of this study indicated that 50nm PS alone did not increase the rates of lysosomal destabilization compared to controls. The rates of lysosomal destabilization did increase after exposure to pyrene, and pyrene and PS in combination ($p<0.01$). While there were no significant interaction effects based on a two-way ANOVA ($p = 0.264$), the combination of PS and 50 ppb pyrene caused significantly higher destabilization rates than pyrene alone ($p=0.264$) (Figure 12).

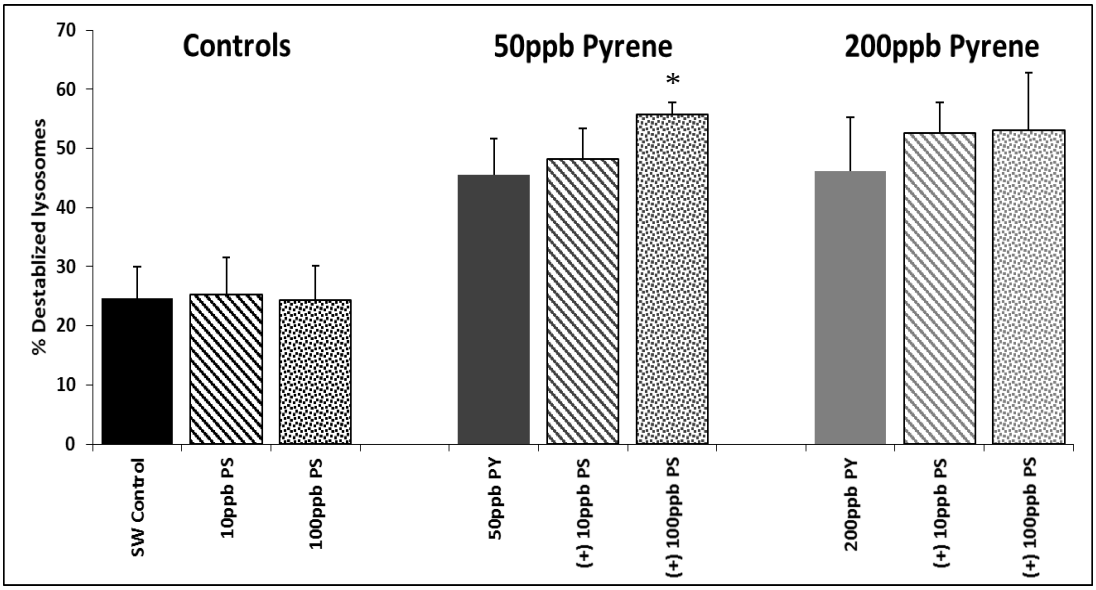

FIGURE 12: The effects of 24-hour *in vitro* exposures of oyster hepatopancreas tissues to 50nm polystyrene (PS) particles and pyrene (PY) on lysosomal destabilization (n=6). All pyrene treatments were significantly different from all comparable controls ($p< 0.0001$). The asterisk (*) indicates a significant difference from 50ppb pyrene treatment only.

Analysis of lipid peroxidation indicated that neither pyrene nor 50nm PS resulted in increased levels of malondealdehyde (MDA) after *in vitro* exposure for 24-hours as

compared to control levels (p=0.35 and p=0.47, respectively). However, there was a slight trend toward decreased lipid peroxidation in the presence of pyrene as the concentration of PS increased (Figure 13).

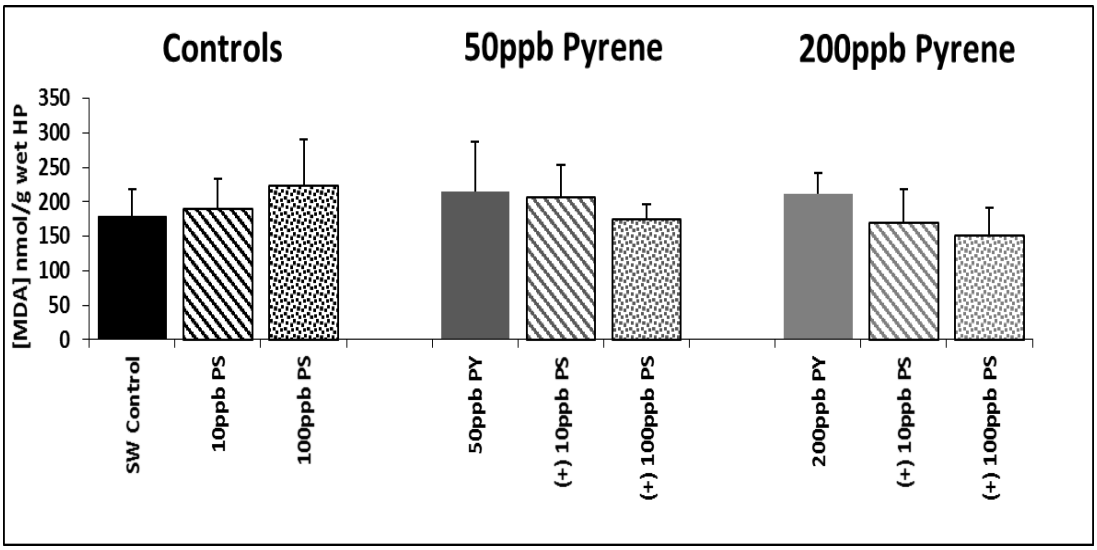

FIGURE 13: The effects of 24-hour *in vitro* exposures of oyster hepatopancreas cells to 50nm polystyrene (PS) particles and pyrene on MDA concentrations. Values are means+ standard deviations (n=6).

3.3.2 Micron-sized Polystyrene

Similar to the nano-PS studies, 24 hour *in vitro* experiments were conducted to determine if micron-sized (3µm) polystyrene (PS) could be acting as a vector for the transport and increased toxicity of pyrene to oyster tissues. Lysosomal destabilization and lipid peroxidation were again used as markers of cellular toxicity. After exposure to 3µm PS and pyrene for 24 hours, PS alone did not increase the rates of lysosomal destabilization compared to controls. The rates of lysosomal destabilization did increase after exposure to pyrene and PS at both concentrations with a significant effect from both

pyrene alone ($p<0.01$), and pyrene and PS in combination ($p<0.01$) compared to controls. There were no significant interaction effects observed in this short-term study although there were overall trends of higher toxicity with the pyrene and PS combinations compared to pyrene alone (p values ranged from 0.034 to 0.132) (Figure 14).

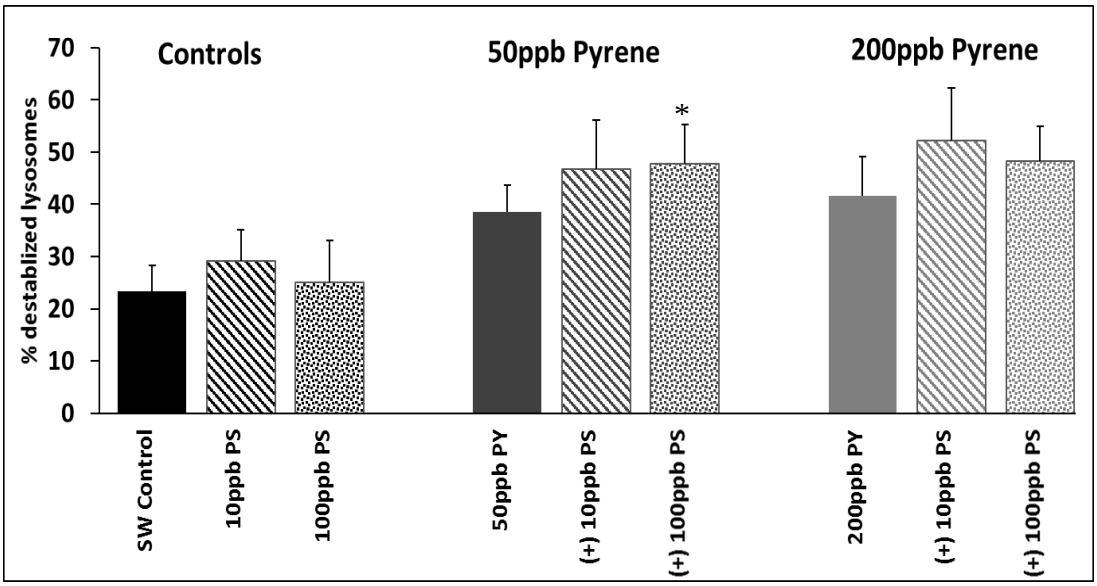

FIGURE 14: The effects of 24-hour *in vitro* exposure of oyster hepatopancreas cells to 3µm polystyrene (PS) particles and pyrene (PY) on lysosomal destabilization (n=6). All pyrene treatments were significantly different from all comparable controls ($p<0.01$). The asterisk (*) indicates a significant difference from the 50ppb pyrene only ($p=0.034$).

Lipid peroxidation levels after 24-hour exposure to 3µm were similar to the 50nm findings in that there were no effects of pyrene or polystyrene alone. There was a significant decrease in lipid peroxidation in both 200ppb pyrene and PS combined treatments as compared to the controls ($p<0.01$) (Figure 15).

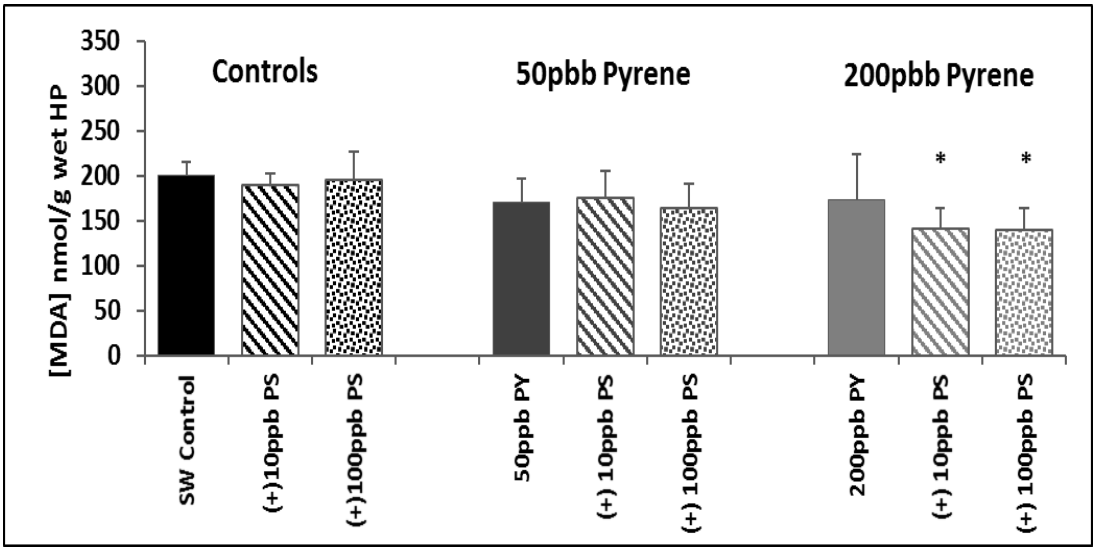

FIGURE 15: The effects of 24-hour *in vitro* exposures of oyster hepatopancreas cells to 3µm polystyrene (PS) particles and pyrene on MDA concentrations. Values are means+ standard deviations (n=6). Asterisks indicate significant difference from controls ($p<0.05$).

3.4 Discussion

Lysosomal destabilization has been demonstrated to be a very sensitive assay for measuring cellular toxicity(Ringwood et al. 2003, Nolde et al. 2006, Hwang et al. 2014, Johnson et al. 2015, Mayrand et al. 2015). While lysosomal assays of PS exposed oyster cells indicated no significant toxicity, high rates of destabilization were observed with pyrene that are associated with impaired oyster health and reproductive success (Ringwood et al, 2004; Edge et al., 2012). While there were overall trends of increased toxicity with pyrene and PS in combination, there were no dramatic differences with either nano- or micro PS.

In these studies, lipid peroxidation levels decreased below control levels with pyrene and PS in combination, especially for the micro-PS particles. This effect could be explained by increased antioxidants as early responses for mitigating increased

cellular perturbation and damage. The possibility that the PS particles served as oxyradical scavengers cannot be ruled out, and based on the lysosomal effects, longer exposure periods could eventually lead to more wide-spread damage and increased lipid peroxidation.

Nano-sized particles should be more readily accumulated inside cells via endocytotic pathways than micro-sized particles, and other studies with these particles did indeed indicate cellular accumulation of nano-PS, but not micron-PS in oyster hepatopancreas cells. In our studies, it is possible that most of the toxicity was related to the release of dissolved and loosely bound pyrene. The ratio of PS particles to pyrene may also be important; the higher toxicity of the 50 ppb pyrene plus 100 ppb nano-PS could reflect accumulation of more pyrene-laden nanoparticles.

Overall these studies indicate that plastic nanoparticles that do move through the digestive track and contact hepatopancreas cells may not cause direct toxicity, but pyrene whether in solution or adsorbed to the particles was very toxic. In these short-term exposures of cells *in vitro*, there were only small increases in toxicity (increased lysosomal destabilization) of pyrene when adsorbed to plastic nano or micron sized particles, but the most significant effects were with the nanoparticles. For the low pyrene concentration (50 ppb) - high nanoparticle concentration (100 ppb), more pyrene may have been transported into the cells as the particles were taken up by endocytosis. It is important to note that lysosomal destabilization rates of pyrene and particles in combination were never lower than just pyrene alone, indicating that the particles did not reduce the bioavailability or toxicity of pyrene. These studies do suggest that adsorbed pollutants can pose significant risks to marine organisms due to increased exposure

potential, but more comprehensive studies including longer term exposures and lower doses of pyrene and other pollutants are needed to address this important issue.

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