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Determination of Distribution of Microplastics in Rappahannock River, VA, and Toxicity of Polyethylene Microplastics with Interaction of Methoxychlor on Daphnia magna

Thanh-Binh Duong

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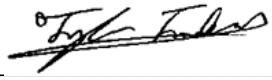

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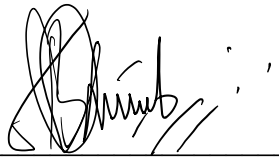

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Determination of Distribution of Microplastics in the Rappahannock, VA, and Toxicity of Polyethylene Microplastics with Interaction of Methoxychlor on *Daphnia magna*

By

Thanh-Binh Duong

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ABSTRACT

Microplastics have become an emerging contaminant of concern in freshwater systems as a component of wastewater treatment plant (WWTP) effluent, household discharge, and industrial outflows. While microplastics have been detected in aquatic environments throughout the world, our knowledge regarding microplastics in the Chesapeake Bay Basin and their impacts on aquatic invertebrates combined with chemical pollutants is limited. This study consisted of two parts: a field-based assessment examining the spatial distribution of microplastics in the Rappahannock River, VA; and a lab-based analysis examining the effects of polyethylene microplastics and the pesticide methoxychlor on the viability and mobility of *Daphnia magna*. Microplastics were more abundant in sediment downstream of a major WWTP outfall site and equally abundant in surface waters at both sites. We suggest that residence time plays a major role in microplastic deposition and that downstream zones are at increased risk. Methoxychlor was found to decrease mobility after 48 hours, whereas microspheres alone caused no significant debilitation. Combinations of the two toxicants resulted in increased mortality rates at moderate mixture treatments and decreased mortality at the highest mixture treatment. Thus, our results suggest that level of microplastic contamination influences the degree of contact organic pollutants may have on vulnerable species.

DEDICATION

Dedicated to my family

Ngan H., Thanh-Diep C., and Quoc H. Duong

ACKNOWLEDGEMENTS

I would like to first thank the University of Mary Washington's College of Arts and Sciences Undergraduate Research Fund, Dr. and Mrs. Ericson, and Mr. and Mrs. Doumas for their generous financial support for my projects throughout the past two years. I also want to thank Dr. Jack Frankel and Dr. Clarence Lee of the Department of Biology at Howard University for providing laboratory space for portions of this study.

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CHAPTER 1 - INTRODUCTION

BACKGROUND ON MICROPLASTICS

Due to its durability and low manufacturing costs, plastic has become an indispensable factor in everyday life in households and industries beginning in the early 20th century (Andrady & Neal., 2009). Several types of polymers are commonly manufactured for various uses (Table 1.1). One of the most-common plastics is polyethylene, which can be found in a high-density and low-density variations and originates from containers for food/beverage and household cleaning products, netting, and wire cables. By comparison, polypropylene is typically used in the manufacturing of bottlecaps, folders, and car bumpers, and polystyrene is most commonly found in disposable dishware and food packaging (Wang et al., 2016). Although they are designed to withstand physical impacts and chemical exposure, fragmentation of plastics can occur over time due to physical and chemical processes resulting in the formation of smaller and smaller particles. Additionally, small plastic fragments are often purposefully manufactured to serve as mechanical exfoliation agents in personal care products (Gregory, 1996; Fendall & Sewell, 2009; Kalicíkova et al., 2017). Plastic fragments that are >5mm in size are known as microplastics which may occur in the form of fibers, randomly shaped fragments, and spheres or beads (Eerkes-Medrano et al., 2015). Due to their wide range of sources, microplastics have become ubiquitous in the natural environment and are an emerging contaminant of concern.

Table 1.1: Different types of plastics, categorized by density and use (Wang et al., 2016)

Polymer	**Abbreviation**	**Density (g/cm^3)**	**Applications**
High Density Polyethylene	HDPE	0.94	Food and beverage containers, detergent and cleaner bottles
Low Density Polyethylene	LDPE	0.91-0.93	Plastic bags, straws, six-pack rings
Polypropylene	PP	0.85-0.93	Bottle caps, folders, car bumpers, netting
Polystyrene	PS	1.05	Disposable plates and cups, food trays
Polyvinyl chloride	PVC	1.38	Detergent and cleaner bottles, piping, medical equipment

As plastics are often manufactured with chemical additives such as plasticizing agents and flame retardants (Chua et al., 2014), these additional components have garnered increased concern by the scientific community due to fact that such compounds can leach from the polymer into the environment and exert their own toxicological effects (Teuten et al., 2009). Concentrations of PBDEs, which are commonly utilized flame retardants, have been detected between 0.002 - 0.082 ng/L in Hong Kong (Wurl et al., 2006) and between 4.2 - 19 ng/L in the Mediterranean Sea along Spain (Sanchez-Avila et al., 2012). Phthalates, a class of plasticizing agents, have been detected at concentrations up to 23.42 μg/L throughout the ocean (Hermabessiere et al., 2017). It has been hypothesized that such contamination originates from plastic pollution in aquatic environments due to their use as additives in the formation process.

Plastics have also been reported to be able to adsorb exogenous organic pollutants, enabling the transport of such compounds across large geographical distances (Wang, et al., 2016). A study by Bakir et al. (2014) examined the transport of DDT and phenanthrene through binding onto plastic. Results showed that DDT was able to bind to polyvinyl chloride as well as polyethylene, indicating plastic's ability to transport organic contaminants. PBDE has been shown to be able to bind to virgin polyethylene particles (not previously containing PBDEs) and upon microplastic ingestion by *Allorchestes compressa,* deposit into amphipod bodies (Chua et al., 2014).

DETECTION OF MICROPLASTICS IN THE ENVIRONMENT

Due to their size, microplastics can easily navigate into aquatic systems through a multitude of pathways. Studies have identified microplastics in samples collected at various stages of treatment at wastewater treatment plants (WWTP), both post-treatment

effluent (Carr et al., 2016; Mason et al., 2016) and treatment plant sludge (Mahon et al. 2016). Particles have been found in household sewage discharge (Peng et al., 2017) and industrial discharges (Anderson et al., 2016), as well. Due to plastic's varying density based on polymer type, microplastics can remain suspended in the water column and disperse throughout bodies of water. Over time, these particles may also deposit into beds of sediment as a result of biofouling. In this instance, biofilm forms on the particulates overtime, enabling the attachment of bacteria, larvae, and spores (Dobretsov, 2010). This induces the overall density of the debris to increase, causing the material to sink below the surface and deposit into the stream bed (Lobelle & Cunliffe, 2011; Wang et al., 2016). Yonkos et al. (2014) found microplastics in surface water samples collected in four rivers in the northern Chesapeake Bay, USA with abundance up to ~250,000 particles or ~245 g per square kilometers. A study by Mathalon and Hill (2014) which examined the presence of microplastic fibers in Nova Scotia, Canada, detected an average of ~20 to ~80 microplastics per 10 g of sieved sediment collected throughout the intertidal system surrounding Halifax Harbor. In the Changjiang Estuary of China, 2 to 34 microplastics were found per 100 g of dry sediment extracted from 53 sites throughout the system (Peng et al., 2017). Microplastics have also been found in deep-sea sediment extracted from the Atlantic Ocean, detected up to approximately 1 fragment per 25 cm^2 (van Cauwenberghe et al., 2013).

IMPACT OF MICROPLASTICS ON BIOTA

Microplastics have been found in various aquatic biota, alluding to its ability to be ingested by organisms in the environment. Mussels from beaches around the Halifax Harbor area of Nova Scotia, Canada were found to have an average of ~106 to ~126

microplastic fibers per specimen (Mathalon & Hill, 2014). In the western English Channel, larvae of *Merlangius merlangus, Microchirus variegatus, Trisopterus minutus, Callionymus lyra,* and *Anguilla* were found to have ingested 1 to 2 particles (Steer et al., 2017). Microplastics have also been found in the intestinal tracts of sunfish collected in the Brazos River Basin in Texas, USA. An average of 1.63 particles per fish was determined out of 436 fish analyzed (Peters & Bratton, 2016). In addition to ingestion patterns found in the environment, an experimental study found that zooplankton were capable of ingesting polystyrene beads ranging from 1.4 to 20.6 μm in diameter (Cole et al., 2013).

The impact of microplastic ingestion has been primarily assessed in aquatic invertebrate species. A study by Wright et al. placed *Arenicola marina* in sediment containing plasticized polyvinylchloride particles, resulting in the depletion of energy reserves of the specimens by up to 50% as indicated by a decrease in lipid reserves. Such decreases may result in an inhibition of maintenance, growth, and reproduction. In addition, microplastic ingestion caused an increase in phagocytic activity of the marine worm's immune cells, which is metabolically demanding. This same study suggested that ingestion caused feeding activity reduction induced by increased residence time of ingested material (Wright et al., 2013). A 48-hour toxicity exposure of *Daphnia magna* to microplastic fibers resulted in average mortality ranging from ~15% to ~40% (Jemec et al., 2016). A 24 h exposure of *D. magna* to nano (100 nm) and microplastics (2 μm) found that ingestion was not size-dependent, and that feeding rate can decrease significantly by 20% following ingestion (Rist et al., 2017). A study on the European green crab (*Carcinus maenas*) found a significant decrease in feeding frequency in the crabs which consumed mussels embedded with polypropylene fibers over a 4 week period, and significant reduced

scope in growth was also observed in crabs with microfibers added to their food (Watts et al., 2015). A study by Green (2016) analyzed the effects of microplastics on the oyster species *Ostrea edulis* and its benthic community through a mesocosm experiment. Polylactic acid (a biodegradable polymer) and high-density polyethylene were inserted into the simulated environments and species richness and abundance was observed for 60 days along with additional indicating factors of oyster health. Species richness was shown to be lower in the mesocosm containing high density polyethylene in comparison to the control and polylactic acid containing vessels, and individual count was lower in both treatment mesocosms in comparison to the control setup, indicating the presence of microplastics can induce a multi-trophic impact rather than a population-exclusive effect.

BACKGROUND ON ORGANOCHLORINE PESTICIDES

Organochlorine pesticides (OCPs) are a class of organic pesticides that are primarily characterized by a chlorinated hydrocarbon structure. OCPs pose a major concern due to their neurotoxicity as well as endocrine disrupting abilities to biota. They may stimulate the central nervous system involuntarily and bind to receptors associated with vital hormones (Jayaraj *et al*., 2016). These compounds are volatile agents that can become suspended in the atmosphere, enabling a more widespread distribution of its contamination in natural environments (Simonich & Hites, 1995). A study which analyzed the exchange of organochlorine pesticides between Australia and Antarctica by means of air-seawater exchange found concentrations of OCPs up 28 pg/L in seawater samples and 35 pg/m^3 in atmospheric samples collected along the cruise track which moved throughout the oceanic region between Australia and Antarctica (Bigot *et al.*, 2016). Along with atmospheric deposition, precipitation may drive the entry of organochlorine compounds into aquatic

environments through runoff (Lefrancq *et al.*, 2017). Fourteen organochlorine pesticide residues have been detected in sediment and water samples from the Densu river basin in Ghana with concentrations up to 14.21 μg/kg and 0.16 μg/, respectively (Kuranchie-Mennsah *et al.*, 2012).

BACKGROUND ON METHOXYCHLOR

Methoxychlor (MXC) is a chlorinated hydrocarbon introduced following the ban of DDT in 1972 (Cupp *et al.*, 2003) as a substitute to previously utilized pesticides (USEPA, 2004). Though MXC is now banned in the United States and Europe since 2003 and 2002, respectively, studies regarding its toxicity remain relevant due to its function as a model endocrine disrupting chemical (EDC) (Akgul et al., 2011). MXC has been shown to perturbate the endocrine system, induce detrimental changes in growth and development, alter reproduction, and affect behavior (Monneret, 2017). As an EDC, MXC signals estrogen receptors in the body (Haschek, 2013) and can cause disruptions in male reproductive development (Aly & Azhar, 2013; Du, et al., 2014). Additionally, its metabolites may also exhibit estrogenic and antiandrogenic properties (Cupp et al., 2003). MXC also remains relevant due to its implications as a transgenerational toxicant (Aoyama et al., 2012; Haque et al., 2014).

In the previously mentioned study assessing the presence of OCPs in the Densu river basin, concentrations of MXC were reported to be below detectable levels in water samples and up to 0.18 μg/kg in soil. It is hypothesized that due to pesticides' low solubility in water, the compound may have instead adsorbed to sediment particles, thus resulting in it not being detected in the water (Kuranchie-Mensah et al., 2012). In addition, MXC was detected by Bigot et al. (2016) at concentrations up to 3.1 pg/L in seawater samples and

1.9 pg/m^3 in atmospheric samples that were collected throughout the Southern Ocean. MXC is also is noted to have a shorter half-life than OCPs such as DDT (Chen et al., 2014). Therefore, while it may not be present in its original parent form in the environment its metabolites may persist in nature. MXC has been additionally found in the fat tissue of *Crocodylus niloticus* from iSimangaliso Wetland Park, South Africa. Concentrations ranged from 79 to 300 ng/g wet weight (Buah-Kwofie et al., 2018). This corresponds to OCPs high lipophilicity, in which they have a greater affinity for lipids. This suggests a greater concern that MXC may accumulate in fat-rich foods, making humans more susceptible to contamination (Jayaraj et al., 2016).

IMPACT OF METHOXCHLOR ON BIOTA

Research on the effects of MXC has primarily focused on mammalian species. A study by Aoyama et al. (2012) examined the developmental and reproductive effects of MXC ingestion on two generations of SPF Sprague Dawley rats by mixing the toxicant with basal feed at varying concentrations. Concentrations of 500 and 1500 ppm were shown to have caused significant suppression in weight gain and food consumption in both sexes of the parent generation. Fertility index in F1 females administered 1500 ppm of MXC significantly decreased, in which eight of the 13 F1 females were unable to become pregnant even when mating with untreated males. Sperm count in both generations of males administered 1500 ppm significantly decreased, as well. An additional study on Sprague Dawley rats instead administered MXC via injection at 1.0 and 2.0 mg. Results found an increase in the days of which female rats were in estrus, indicating a significant impact on reproductive development. Also observed was a decrease in lordosis amplitude, suggesting that the nervous system is vulnerable to the effects of MXC during development

(Bertolasio et al., 2011). Additionally, a multi-generational study on C57BL/6 mice found that the male offspring of female rats administered MXC had lower production in spermatocytes and spermatids than the vehicle offspring (Du et al., 2014). A study assessed the impacts of the hormone estradiol and MXC on body and testis weight of adult male rats previously exposed to ethane dimethanesulfonate. This toxicant is identified as cytotoxic to Leydig cells, which produce testosterone. This study aimed to assess the efficacy of Leydig cell regeneration, assumingly impacted by estradiol or MXC. Results found a greater decrease in body weight 58 days post injection of ethane dimethanesulfonate in the rats treated with MXC than those treated with estradiol as well as the control groups. Additionally, serum testoterone levels of MXC treated rats were significantly lower 58 days post injection than that of groups treated with estradiol or the control specimens (Chen et al., 2014).

RESEARCH OBJECTIVES

Field-Based Assessment

While microplastics have been widely detected in nature in various parts of the world, there is limited knowledge about the distribution of microplastics in the Chesapeake Bay Watershed, with exception to one study by Yonkos et al. (2014). The goal of the first phase of this study is to assess the degree of microplastic pollution in the Rappahannock River, located in the lower Chesapeake Bay Basin. Using the locations of WWTPs in Virginia obtained from the U.S. EPA Facility Registry Service, collection sites were be selected to assess the presence of microplastics in locations upstream from, at, and downstream from WWTP outfall points.

Laboratory Exposures

While the toxicity of microplastics has been investigated in many studies overall, its impact on the behavior has not been investigated. It is less known whether sublethal levels of microplastics can induce a change in behavior, which influences how organisms interact with their environment. Additionally, while the impacts of microplastic ingestion in aquatic biota have been investigated in a variety of species, the knowledge of the sorption behavior of microplastics with neighboring contaminants in the aquatic environment and how it affects biological systems is rather limited in its span of toxicants studied. There have been studies examining the transport and uptake of organic pesticides and insecticides (Bakir et al., 2014; Hüffer & Hofmann 2016; Horton et al., 2018), but the interaction of MXC with plastics and whether it elicits a synergistic or antagonistic effect on aquatic systems has yet to be determined.

The purpose of this study is to analyze the interaction of the organochlorine pesticide MXC and microplastics. Both agents are noted as anthropogenic contaminants in the natural environment. It is yet to be determined whether the presence of microplastics in suspension with MXC can exacerbate or reduce the adverse impacts of the pesticide toxicant. Due to that MXC is hydrophobic and requires an organic solvent as a vehicle, it is hypothesized that it may bind to plastic fragments in the environment due to also being hydrophobic.

CHAPTER 2 - METHODS

FIELD-BASED ASSESSMENT

Study Area

The Chesapeake Bay is an estuary of approximately 4,480 square miles in the Mid-Atlantic region of the United States that stretches to the states of Virginia, Maryland, and Delaware. A multitude of major rivers including the Potomac, Rappahannock, and James, empty into the bay. Its watershed is 64,000 square miles and incorporates not only those states but also Pennsylvania, New York, and West Virginia, thus establishing a large network which connects the residing population in its watershed and aquatic habitats which encompass the area. The size of the population which the watershed supports is approximately 18.1 million people (Chesapeake Bay Program). In the past 20 years, the Mid-Atlantic Basin has received an average annual rainfall of 44.66 inches (NOAA). A large watershed and great population to support results in growth in urbanization and thus an increase in impermeable surfaces including parking lots, driveways, and streets. As a result, a greater portion of the received rainfall becomes runoff, thus driving the microplastics resulting from everyday use or landfill residence to become into nearby rivers and tributaries and, ultimately, the bay itself. As a result, the lower basin of the bay ultimately receives the discharge which is emitted from all ends of the watershed.

Along the eastern boundary of Virginia is the Rappahannock River, which flows into the Chesapeake Bay. The aim of this study is to assess the concentration of microplastics found in samples extracted from varying locations throughout this river to determine the extent of microplastic pollution in the lower Chesapeake Bay basin. Through

extracting microplastics from both water and sediment samples, spatial distribution of microplastics were assessed.

Sample Collection

Water and sediment samples were collected at each sampling site. Collection sites were determined by proximity to WWTP locations, identified by the U.S. EPA Facility Registry Service. Sampling was conducted at areas adjacent to WWTP outfall points and downstream from the WWTP location (Figure 2.1). Sediment samples were obtained using a Van Veen Grab Sampler, and water samples were collected by dip sampling and contained in 19 L buckets. Sediment samples were stored frozen at -16°C until extraction.

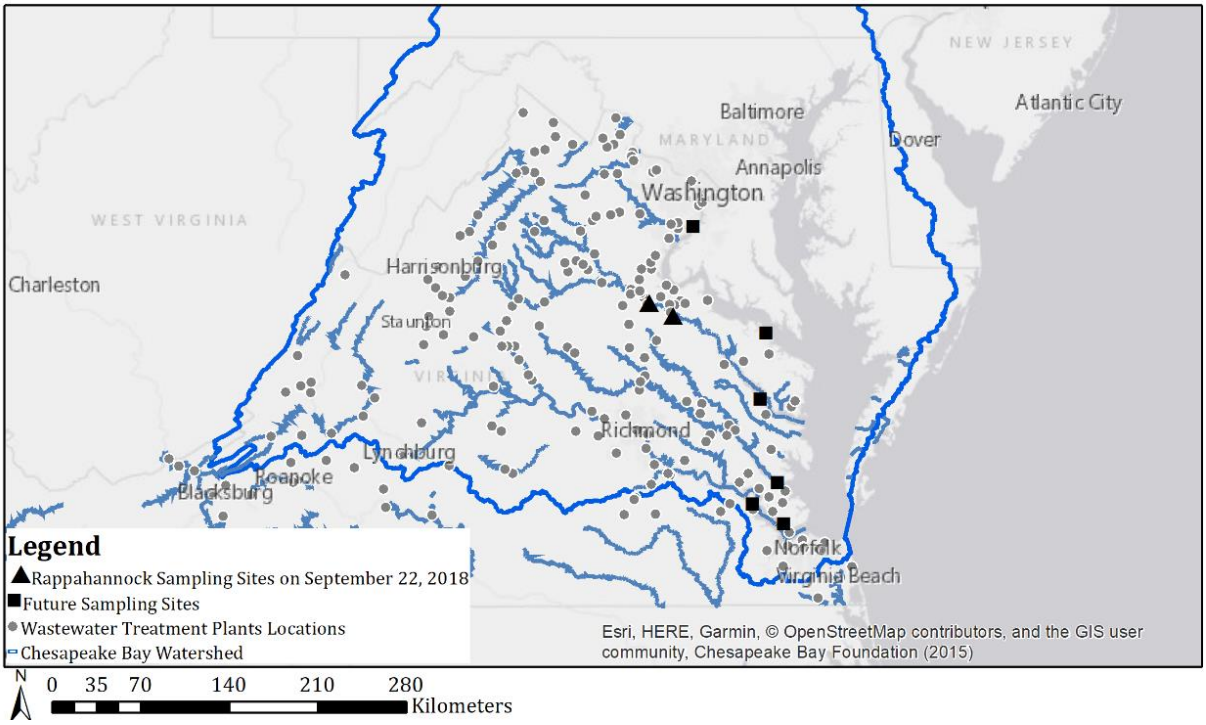

Figure 2.1: Locations of WWTP's and sampling sites in Virginia, USA. Created in ArcGIS Online and ArcMap 10.7 (Esri).

Microplastic Extraction

Sediment samples were dried in a drying oven at 90 ^{o}C overnight. Mass was measured following drying period and prior to extraction protocol. To remove organic material from the samples, 0.05 M Fe(II) solution (Ferrous Sulfate Heptahydrate (CAS: 7782-63-0; Fisher Scientific)) in water and concentrated sulfuric acid (CAS: 7664-93-9; Fisher Scientific)), then 30% H_2O_2 (CAS: 7722-84-1; Fisher Scientific) was added to the sample. The sample was then heated at 75 °C on a hotplate. Following wet peroxide oxidation, to increase density of solution in order to separate microplastics from remaining particulates, 6 g of NaCl (per 20 mL sample) was added. Solution was transferred to a glass separating funnel. After allowing solids to settle overnight, the supernatant was extracted and filtered by means of vacuum filtration using a Vacuubrand ME-1 Vacuum Pump over a 55 mm Whatman filter paper (8μm particle retention). Filter papers were covered with aluminum foil to reduce airborne contamination and air dried overnight (Erni-Cassola et al., 2017; Hidalgo-Ruz et al., 2012; Shim et al., 2015; Masura et al., 2015).

Microplastic Quantification

Filter papers containing the extracted fragments were examined through an Olympus SZ-CTV Stereo Microscope and an OMAX Binocular Stereo Microscope. Fragments were counted individually and characterized by shape and color and categorized by sample type, location which sample was collected from, fragment shape, and color. To quantify the distribution of particles, total fragments accounted for per site were converted to a frequency per dry mass or volume count to compare between locations in order to assess the degree of which microplastics are present in the target study sites.

LABORATORY EXPOSURE

Within the laboratory exposures stage of this study, three main experiments were conducted; 1) a rangefinder assay to determine the impacts of virgin polyethylene beads on *D. magna* mortality, 2) a rangefinder assay to determine the impacts of MXC on *D. magna* mortality, and 3) a microplastic-MXC mixture assay to determine the ability of polyethylene beads to modify the toxic effects of MXC. All stages were conducted as 48 hour acute toxicity tests standardized by the United States Environmental Protection Agency (EPA).

Per treatment level, there were 10 adult organisms evaluated for immobilization and mortality following the 48-h exposure period. Test tubes with a volume capacity of 55 mL were utilized as test vessels for all experiments. Per test tube, there were 20 mL of treatment medium and 1 daphnid. A 12 h dark, 12 h light cycling was maintained through a light timer. Test vessels were kept on lab bench in ambient conditions (20°C ± 1°C)

Test organisms

In this study, *Daphnia magna* was utilized as the test species. *D. magna* is a planktonic crustacean species found abundant in freshwater environments. It is reported to be a keystone species, in which the ecosystem which it inhabits largely depends on the species for continuous stability throughout trophic levels. *D. magna*'s feeding of phytoplankton drive the primary connection between primary producers and many fish species of aquatic environment. If removed, *D. magna* can cause a major collapse of its ecosystem (Martin-Creuzburg et al., 2005). *D. magna* were maintained in a 37.8 L glass aquarium with synthetic daphnia water (Table 2.1) based on standard methods issued by

the U.S. EPA for toxicity testing with media change occurring weekly. Test organisms were fed every 2-3 days with spirulina algae mix. *D. magna* have a considerably short lifespan of 7 to 8 weeks and can reach sexual maturity 6 to 8 days of leaving the brood chamber. They can reproduce both sexually as well as through parthenogenesis, allowing for a reduction in genetic variability amongst test subjects if bred from the same female parent (Dang et al., 2012). In effect, results from toxicity tests can be evaluated primarily based on the toxicant rather than the possibility of genetics inducing significant change. To reduce the variability of genetics and age of test specimens, the test specimens were spawned from the same female parent at approximately the same time (within 2-3 days of each other).

Table 2.1: Chemicals used to compose synthetic *Daphnia* water

Compound	**Concentration**
$NaHCO_3$	0.192 g/L
$CaSO_4 \cdot 2H_2O$	0.120 g/L
$MgSO_4$	0.120 g/L
KCl	0.006 g/L

Polyethylene Microspheres

The first pilot study determined the degree of mortality which *D. magna* exhibit when exposed to vary concentrations of virgin microplastic beads in water. Fluorescent green polyethylene microspheres were purchased from Cospheric, Inc. (Santa Barbara, CA, USA) at 10-20 μm in size with a density of 1.00 g/cm^3 (Item # UVPMS-BG). This density enabled the suspension of the particles rather than settling or flotation. Concentrations of microplastics were determined by weight per volume (mg/L). Treatment levels consisted of 0, 12.5, 25, 50, 100 mg/L (Jemec et al., 2016). Particles were added to the test medium and mechanically mixed through manual agitation for 15 minutes to induce dispersal and suspension of particles. 20 mL of microplastic treatment solution was then transferred to test vessels, a daphnid introduced to treatments, and vessels kept in the testing room which contained temperature and light control for the 48 h exposure period. Immobilization or death of specimens was recorded after 24 hours and after 48 hours to assess impact resulting from toxicant exposure, and mobility was analyzed after 24 hours of exposure. To examine mobility, individual daphnids were transferred to a 50 mL beaker containing 20 mL of synthetic water, which was then placed into a light-controlled recording chamber and acclimated for 3 minutes. Following the acclimation period, each organism was recorded from an aerial view using a Logitech C930 HD Pro webcam for 3 minutes. Following recording, test organisms were transferred back into testing mediums to continue the exposure period. Footage was analyzed using the software ToxTrac, which enables automated tracking of organisms (Figure 2.2) to render quantitative results for the following endpoints: speed, mobile speed, acceleration, total distance travelled, and frozen events (Table 2.2). Statistical analysis of mobility data was performed using GraphPad

Prism8© analytical software. Treatment groups were compared using Welch and Brown-Forsythe ANOVA Test with Dunnett's T3 test with significance set at $p < 0.05$.

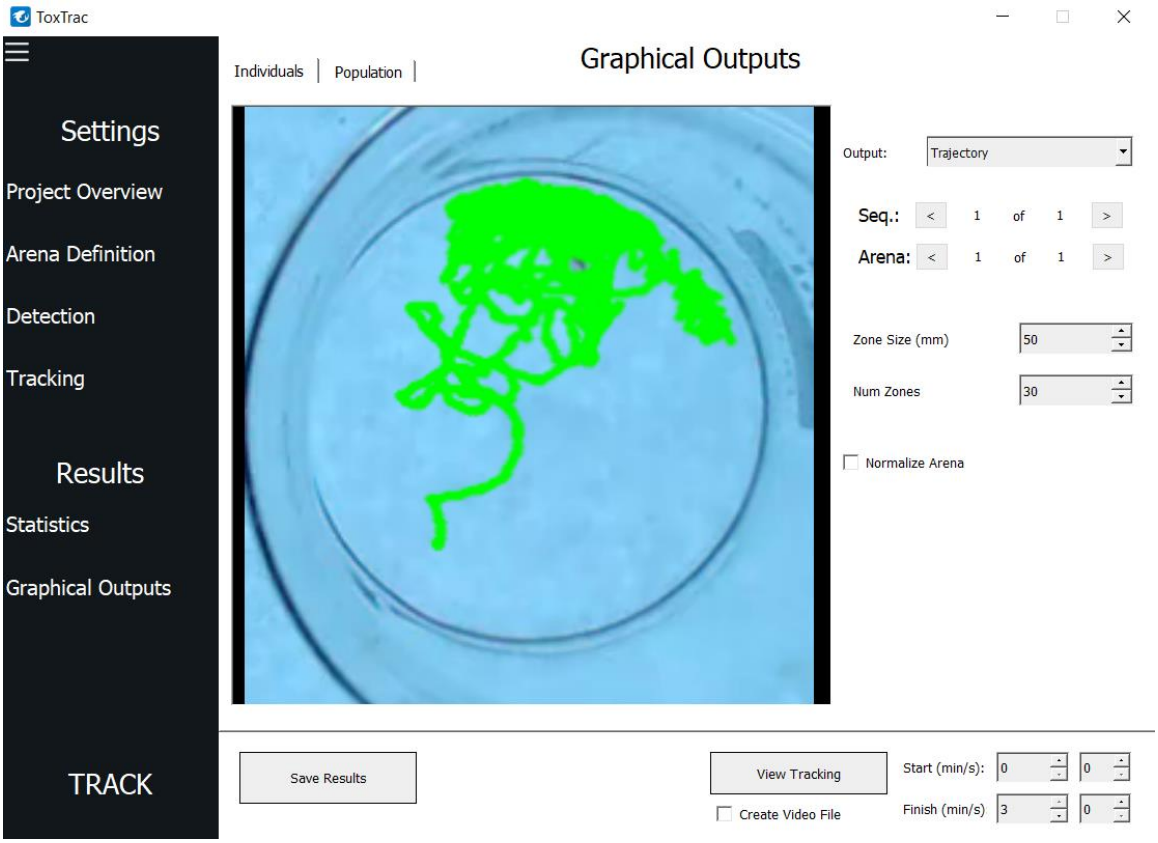

Figure 2.2: Tracking output of ToxTrac. Green linework indicates path of travel by detected organism.

Table 2.2: Target mobility parameters to be studied in behavior assessment

Mobility Endpoint	**Description**
Speed (mm/s)	Rate of movement of the organism for the entire 3-minute recording period
Mobile Speed (mm/s)	Rate of the movement of the organism, calculating with exclusion to periods of lack of movement during the recording period
Acceleration (mm/s^2)	Rate of which the organism increases its speed
Total Distance Travelled (mm)	Total amount of movement by the organism in the 3-minute assessment period
Frozen Events (# of events)	Frequency of which the test organism ceases movement for a brief period of time during the set period of recording

Methoxychlor

The second experiment examined sublethal concentrations of MXC on *D. magna*. MXC was purchased from Millipore-Sigma (CAS: 72-43-5) (Burlington, Massachusetts, USA). As MXC must be dissolved in 200 proof ethanol (CAS: 64-17-5; Fisher Scientific) in order to be suspended in water, a vehicle control was included. In addition to the vehicle control, the levels of treatment were 1.0, 2.5, 5.0, and 10 μg/L based upon concentrations used in previous studies (Baer & Owens, 1999; Horton et al., 2018). Superstock solutions were created per each treatment level to ensure equal amounts of ethanol present in each exposure medium. 40 mL of MXC treatment solution were transferred to test vessels, daphnids were added to each vial, and vessels kept at ambient conditions. Immobilization or death of specimens were recorded after 24 hours and after 48 hours to assess impact resulting from toxicant exposure. Mobility was examined after 24 hours of exposure using the same previously listed methods for *Experiment 1*. Statistical analysis of mobility data was performed were GraphPad Prism8© analytical software. Treatment groups were compared using Welch and Brown-Forsythe ANOVA Test with Dunnett's T3 test with significance set at $p < 0.05$.

Polyethylene Microspheres and Methoxychlor Mixtures

The third study examined the interaction of MXC and polyethylene spheres through its effect on its predetermined toxicity on *D. magna*. Treatment levels were determined through combining high, medium, and control concentrations of each toxicant. Test mediua were created using equal portions of MXC solutions from superstocks and polyethylene microbeads solutions, each at varying concentrations. The treatment levels for this portion of the study were (formatted in [polyethylene microspheres] + [MXC]) was 0 mg/L + 0

µg/L, 0 mg/L + 5.0 µg/L, 0 mg/L + 10.0 µg/L, 0 mg/L + 50 µg/L, 50 mg/L + 5.0 µg/L, 50 mg/L + 10.0 µg/L, 100 mg/L + 0 µg/L, 100 mg/L + 5.0 µg/L, and 100 mg/L + 10.0 µg/L. Mortality checks were conducted following 24 and 48 hours of exposure and mobility assays were performed after 24 hours of exposure, following the same protocol implemented in the other phases of the laboratory exposure study.

CHAPTER 3 - RESULTS

FIELD-BASED ASSESSMENT

Little Falls WWTP

Total dry mass of sediment analyzed from Little Falls WWTP outfall site was 567.8 g. Total microplastic count from sediment was 458, in which 142 particles were filaments and 315 particles were beads (Table 3.1). Fragments and shavings were not detected in sediment samples. Colors of the particles found included black, blue, white, and red (Figure 3.1). The relative abundance of microplastics was approximately 40 microplastics per 50 g dry weight (Table 3.2). Total volume of surface water analyzed was 3800 mL. Total microplastic count in water samples was 110, in which 105 particles were filaments and 5 particles were fragments (Table 3.1). Colors found included blue, red, black, white, brown, green, white, and light blue. The relative abundance of microplastics in surface waters was 2.9 microplastics per 100 mL (Table 3.2).

Table 3.1: Total number of microplastics found in sediment and surface water samples collected from Little Falls WWTP. Total counts and colors of each shape are listed. Total dry mass of sediment sampled and total volume of surface water sampled was 567.8 g and 3800 mL, respectively.

Sample	**Shape**	**Color(s)**	**Count**
Sediment	Filament	Blue, Black, White, Red	142
Sediment	Sphere	Black	315
		Total	**458**
Water	Filament	Blue, Red, Black, White, Brown	105
Water	Fragment	Green, Light Blue, Blue, White	5
		Total	**110**

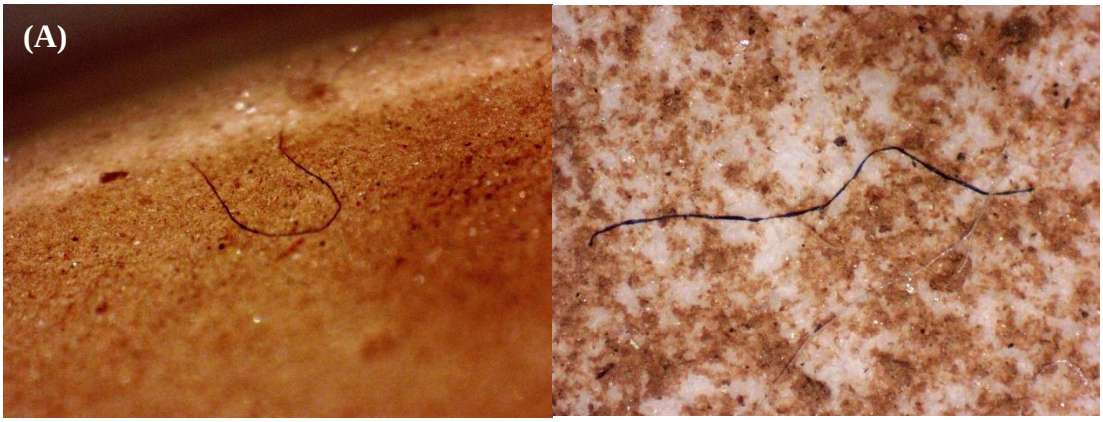

Figure 3.1: Microfibers extracted from sediment (A) and water (B) samples collected from Little Falls WWTP Outfall.

Table 3.2: Abundance of microplastics per 50 g of dry sample in sediment or per 100 mL of surface water collected from Little Falls WWTP.

Sample Type	Concentration
Sediment	40 per 50 g dry weight
Surface Water	2.9 per 100 mL

Hick's Boat Landing

Total dry mass of sediment analyzed from Hick's Boat Landing was 358.6 g. Total microplastic count from sediment was 608, in which 79 particles were filaments and 539 particles were beads (Table 3.3). Fragments and shavings were not detected in sediment samples. The relative abundance of microplastics in sediment was 86 microplastics per 50 g dry weight (Table 3.4). Colors found included black, white, brown, and blue (Figure 3.2). Total volume of surface water analyzed was 5000 mL. Total microplastic count in water samples was 140, in which there were 132 filaments, 5 fragments, 1 bead, and 2 shavings detected (Table 3.3). Colors found included black, blue, grey, pink, white, and red. The relative abundance of microplastics in surface waters at this site was 2.8 microplastics per 100 mL (Table 3.4).

Table 3.3: Total number of microplastics found in sediment and surface water samples collected from Hick's Boat Landing. Total counts and colors of each shape are listed. Total dry mass of sediment sampled and volume of surface water sampled were 358.6g and 5000 mL, respectively.

Sample	**Shape**	**Color(s)**	**Count**
Sediment	Filament	Black, white, Brown, Blue	79
Sediment	Bead	Black	539
		Total	**608**
Water	Filament	Black, Blue, Grey, Pink, White, Red	132
Water	Fragment	Blue, Black, Grey	5
Water	Bead	Black	1
Water	Shavings	White	2
		Total	**140**

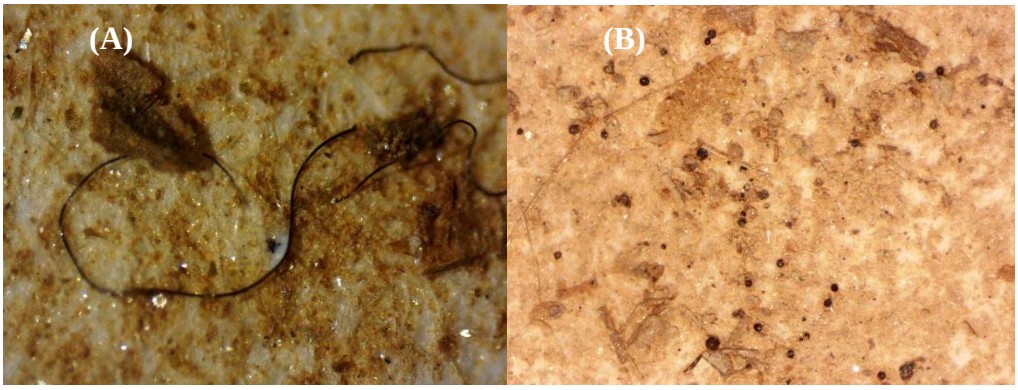

Figure 3.2: Microfilaments (A) and microbeads (B) extracted from sediment collected from Hick's Boat Landing sample site.

Table 3.4: Abundance of microplastics per 50 g of dry sample in sediment or per 100 mL of surface water collected from Little Falls WWTP.

Sample Type	Concentration
Sediment	86 per 50 g dry weight
Surface Water	2.8 per 100 mL

LABORATORY EXPOSURES

Polyethylene Microbeads

All *D. magna* survived after 24 hours of exposure at the varying concentrations of the microspheres. Mortality rates at 48 hours of exposure were 0% at 0 mg/L, 11.8% at 12.5 mg/L, 5.9% at 25 mg/L, 23.5% at 50 mg/L, and 11.8% at 100 mg/L (Table 3.5). Effects of the polyethylene microbeads on speed, acceleration, mobile speed, total distance traveled, and the total count of frozen events were analyzed after 24 hours exposure (Figure 3.3). No significant differences were observed between each of the treatment levels, indicating lack of effect of polyethylene microspheres on the mobility of *D. magna*.

Table 3.5: Percentage of mortality of *Daphnia magna* observed 48-h exposure to water control, 12.5, 25, 50, and 100 mg/L polyethylene microspheres (n = 17).

	% Mortality		
Treatment	**0 h**	**24 h**	**48 h**
0 mg/L	0	0	0
12.5 mg/L	0	0	11.8
25 mg/L	0	0	5.9
50 mg/L	0	0	23.5
100 mg/L	0	0	11.8

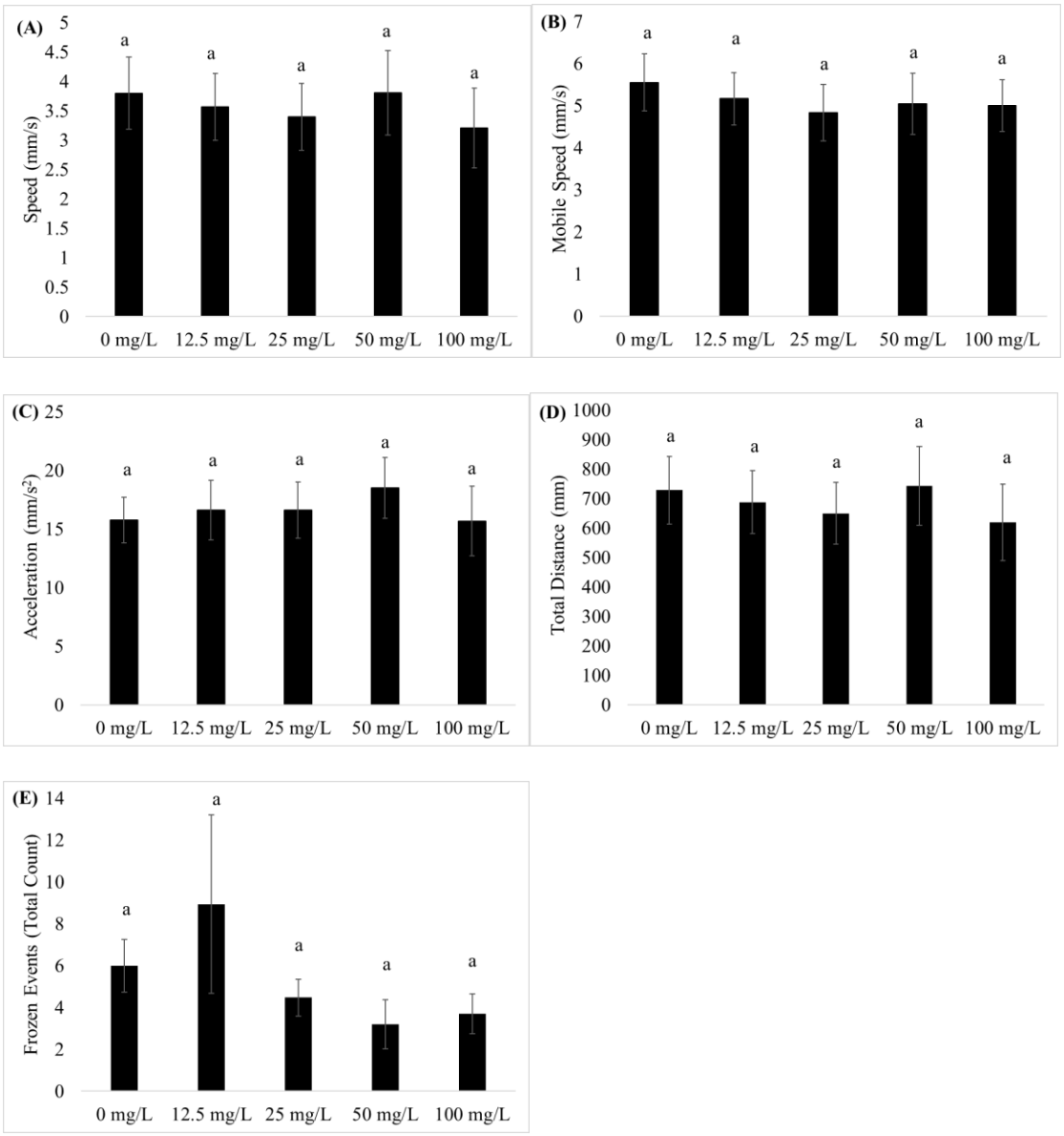

Figure 3.3: (A) Average speed (mm/s) ± standard error of the mean (SEM), (B) average mobile speed (mm/s) ± SEM, (C) average acceleration (mm/s2) ± SEM, (D) average total distance traveled (mm) ± SEM, and (E) average number of frozen events ± SEM exhibited by individual *Daphnia magna* exposed to water control, 12.5, 25, 50, and 100 mg/L polyethylene microspheres for 24 hours (n=17). Letters indicate significant differences ($p < 0.05$).

Methoxychlor

After 24 hours of exposure, *D. magna* exhibited 0% mortality at all levels of treatment with exception to the 10 µg/L treatment (13% mortality). At 48 hours of exposure, the D. magna of the ethanol control level exhibited 0% mortality. Mortality rates of the experimental treatment levels after 48 hours were 7.1% at 0 mg/L, 0% for 1.0 µg/L, 14.3% for 2.5 µg/L, 21.4% for 5.0 µg/L, and 33% for 10.0 µg/L (Table 3.6). Speed, acceleration, mobile speed, total distance traveled, and the total count of frozen events were analyzed after 24 hours of exposure to MXC (Figure 3.4). Significant decreases in speed were found at the 5.0 and 10.0 µg/L treatments. MXC was also found to have caused significant decreases in total distance traveled at 10.0 µg/L. Frozen event count was significantly lower at 1 µg/L and higher at 5.0 µg/L.

Table 3.6: Percentage of mortality of *Daphnia magna* observed during 48-h exposure to ethanol control, 1.0, 2.5, 5.0, and 10.0 µg/L MXC (n = 14).

		% Mortality	
Treatment	**0 h**	**24 h**	**48 h**
Ethanol Control	0	0	7.1
1.0 µg/L	0	0	0
2.5 µg/L	0	0	14.3
5.0 µg/L	0	0	21.4
10.0 µg/L	0	13	33

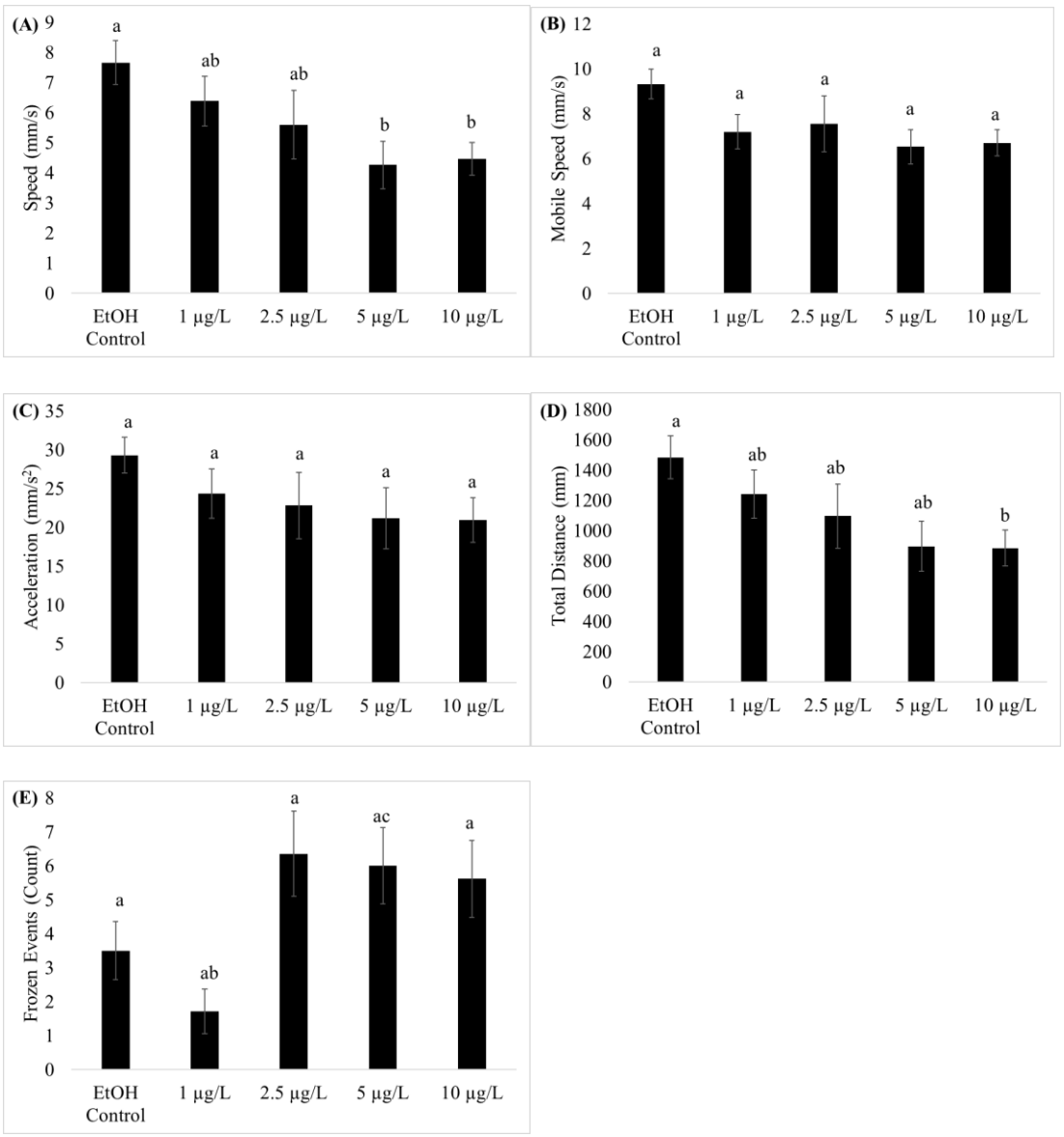

Figure 3.4: (A) Average speed (mm/s) ± standard error of the mean (SEM), (B) average mobile speed (mm/s) ± SEM, (C) average acceleration (mm/s2) ± SEM, (D) average total distance traveled (mm) ± SEM, and (E) average number of frozen events ± SEM exhibited by individual *Daphnia magna* exposed to ethanol control, 1.0, 2.5, 5.0, and 10.0 µg/L MXC for 24 hours (n=14, 14, 14, 14, 13 respectively). Letters indicate significant differences ($p < 0.05$).

Polyethylene Microspheres and Methoxychlor Mixtures

Exposure to the control levels (0 mg/L) of microspheres and either ethanol vehicle only or 5µg/L MXC resulted in partial mortality at 24 hours of exposure and increased mortality at 48 hours. The 0 mg/L microspheres and 10 µg/L MXC resulted in no mortality after 24 hours of exposure and partial mortality after 48 hours of exposure. Treatment levels of 50 or 100 mg/L of microspheres and 0, 5, or 10 µg/L MXC, with exception to the 100 mg/L microspheres and 10 µg/L MXC level, resulted in mortality after 24 hours of exposure. All treatment levels resulted in partial to complete mortality at 48 hours of exposure (Table 3.7).

Mobility parameters were assessed based on differing levels of polyethylene microspheres in test mediums (Figure 3.5). Mixture treatment levels containing 0 µg/L MXC exhibited decreases in average speed, mobile speed, acceleration, and total distance traveled at the highest concentration of microspheres, 100 mg/L. Average frozen events count was highest at the 0 mg/L microspheres treatment. Speed and acceleration were higher in the 50 mg/L microspheres treatment than the 0 and 100 mg/L treatments. In the 5 µg/L MXC treatments, average speed, mobile speed, acceleration, and total distance were highest in the 50 mg/L microspheres treatment level. Average frozen events count was lowest at the 50 mg/L level. In the 10 µg/L MXC treatment, the 100 mg/L microspheres treatment exhibited increases in average speed, mobile speed, acceleration, and total distance. Frozen events were not highly variable throughout varying microspheres levels.

Table 3.7: Percentage of mortality of *Daphnia magna* observed during 48-h to varying mixtures of polyethylene microspheres and methoxychlor (n=6).

		% Mortality	
Treatment	**0h**	**24h**	**48h**
PE0-MXC0	0	16.7	50
PE50-MXC0	0	16.7	16.7
PE100-MXC0	0	16.7	33.3
PE0-MXC5	0	16.7	33.3
PE50-MXC5	0	60	100
PE100-MXC5	0	16.7	50
PE0-MXC10	0	0	80
PE50-MXC10	0	16.7	66.7
PE100-MXC10	0	0	40

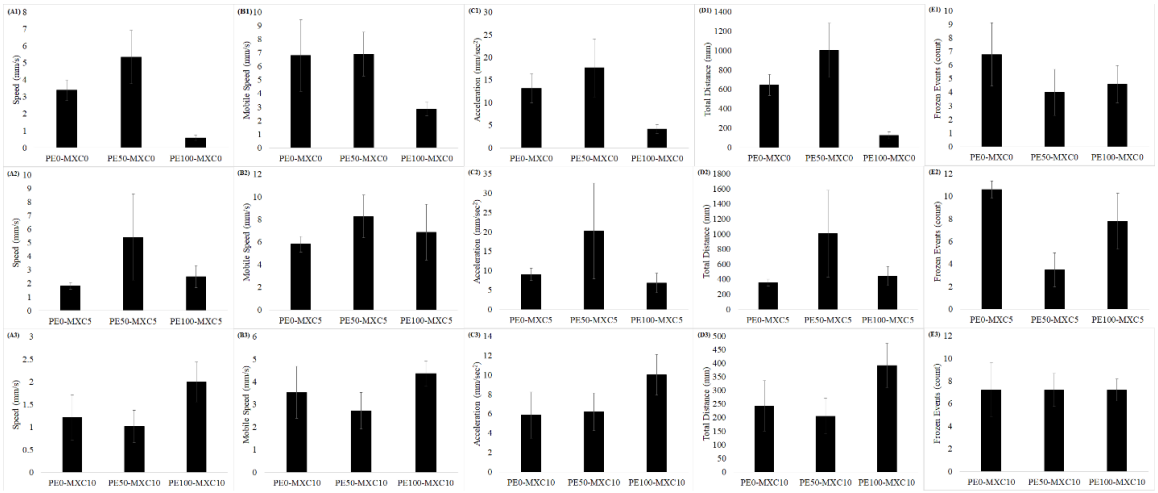

Figure 3.5: (A) Average speed (mm/s) ± standard error of the mean (SEM), (B) average mobile speed (mm/s) ± SEM, (C) average acceleration (mm/s^2) ± SEM, (D) average total distance traveled (mm) ± SEM, and (E) average number of frozen events ± SEM exhibited by individual Daphnia magna exposed to mixtures of varying concentrations of polyethylene microplastics (0, 50, or 100 mg/L) and 0 (A1-E1), 5 (A2-E2) or 10 (A3-E3) µg/L methoxychlor (ethanol control) for 24 hours (n = 2 for PE50-MXC5, n = 5 for all other treatments).

CHAPTER 4- DISCUSSION

FIELD-BASED ASSESSMENT

Microplastics were present in both sites in both types of samples in the Rappahannock River. Concentration in sediment was higher in the downstream site, Hick's Boat Landing, and microplastic concentration in surface waters was higher in the WWTP outfall site, Little Falls. Higher abundance of microplastics in sediment collected from the downstream location compared to the outfall site indicates greater degrees of deposition of plastic particles into the alluvial beds in freshwater environments. In order to accumulate biofilm and undergo sedimentation, microplastics typically have overall longer residence time in the environment (Thompson et al., 2009; Wang et al., 2016). As microplastics may enter the fluvial system through an upstream source point, the particles have longer residence compared to particles entering at the downstream site. Longer residence time overall enables greater buildup of biofilm, which enables aggregation of plastic particles, ultimately resulting in deposition into the sediment bed at the downstream location (Michels et al. 2018). Relatively similar abundances of microplastics in surface water samples from both study sites do not necessarily suggest that WWTP effluent is a main contributor of microplastics in this local river system. The data, however, confirms the occurrence of microplastic contamination in the Rappahannock River System. Ziajahromi et al. assessed wastewater treatment effluent as a route for microplastics to enter the environment. Effluent at the primary, secondary, and tertiary levels of treatment contained microplastics at concentrations of 1.54, 0.48, and 0.28 microplastics/L, respectively (Ziajahromi et al., 2017). While these concentrations may be low, such results from Ziajahromi's study nonetheless validate WWTP's role as a contributor of microplastic

pollution. As such, additional data from varying locations along the river system should be collected in order to support whether or not WWTPs serve as a main pathway of entry for microplastics into aquatic environments.

The two major shapes of microplastics found throughout both sites were filaments and beads. Microplastic fibers or filaments are frequently associated with the breakdown of textile fibers and fishing nets (Watts et al., 2015; Peng et al., 2017). At both sites sampled, the majority of the microplastics detected in sediment were microbeads. Microbeads primarily originate from personal care products such as facial washes as vectors of mechanical exfoliants. Using the program ImageJ by Fiji, the size of the microbeads was determined to range between 9 to 66 µm. A study by Kalcíkova et al. assessed the presence of polyethylene microbeads in cosmetic products to assess their pathway into freshwater systems through wastewater treatment plant effluent. The microbeads found in the products tested were less than 100 µm in size and were not entirely able to be removed through as simulated tertiary treatment system (Kalcikova et al. 2017). In effect, it is suggested that the microbeads found in samples collected originate from personal care products and enter freshwater environments through wastewater treatment plant effluent. Microbeads are commonly detected in other aquatic systems, such as in the southern coastal region of Hong Kong, where they were present in surface waters up to 380,129 pieces per km^2 (So et al. 2018). In the Rhine River, which runs through West Germany, microbeads were present up to 9.2 particles/m^3 (Mani et al. 2018). The relative lack of fragments and shavings suggest that while physical breakdown occurs to plastic debris in the environment overtime, the resulting material does not readily enter aquatic

environments and likely remains in terrestrial environments, accumulating in soil and sediment instead (Hurley & Nizzetto, 2018).

Furthermore, the presence of microplastics in both surface waters and the fluvial bed indicate that microplastics may be dispersed throughout the water column, and, thus, a variety of aquatic biota with various feeding patterns are at risk of exposure to microplastics. Previous studies have supported this hypothesis through the discovery of microplastic contamination in multiple species of varying food acquisition behavior. In the Musa Estuary of the Persian Gulf, a variety of fish and crustacean species were found contaminated with microplastics. Demersal and pelagic fish were found with microplastics throughout the gastrointestinal tracts, gills, and liver, and prawn species were found with microplastics throughout the exoskeleton and muscle. The results of this study found approximately 8 microplastics per prawn and 20 per demersal, or bottom-feeding, fish (Abbasi et al. 2018). A study by Jabeen et al. collected from the Yangtze estuary, East China Sea, South China Sea, and Taihu Lake and assessed various species of different distributions within aquatic environments for microplastic pollution. In 26 species, microplastics were present throughout the gastrointestinal system. The fish assessed were characterized by either pelagic, benthopelagic, demersal, and benthic distribution, indicating that species of varying feeding behavior are capable of exposure and uptake to microplastics (Jabeen et al. 2017). As a result, it is expected that the microplastics distributed throughout the water column in the Rappahannock River can exhibit a similar pathway, contaminating various aquatic species.

LABORATORY EXPOSURE

Polyethylene Microspheres

Results presented in this study indicate that exposure to polyethylene microspheres alone do not induce severe decreases in viability or behavior of *D. magna* after 24 hours. After 48 hours of exposure, partial mortality was observed at all treatments containing the microspheres. The observed lack of change in mobility and low mortality rates may indicate that while the ingestion of the microspheres is apparent in exposed organisms, the size of particles and duration of exposure as well as the shape of microplastics can influence the degree of impact or accumulation.

For instance, Ma et al.'s study also observed no mortality or immobilization in *D. magna* that were exposed to 5, 10, or 15 µm polystyrene microspheres at concentrations up to 100 mg/L for 48 hours, whereas exposure to 50 nm particles at 1 mg/L induced severe immobilization. In this study, particles of the nano scale were able to accumulate throughout the thoracic appendages, structures are essential to swimming and filter feeding. Microparticles, however, were solely ingested and were not found sticking on the body (Ma et al. 2016). Due to their size and lipophilicity, plastic nanoparticles can also have the capacity to be absorbed by the body on the cellular level. Brun et al.'s study assessed the uptake of polystyrene nanoparticles (25 nm) by *D. magna* and noted that particles were able to accumulate in and on cells. It was suggested that nanoplastics are capable of being taken up into the cell through an endocytotic mechanism (2017). As such, the chosen size of microspheres is less likely to interact with exposed *Daphnia* beyond modes of ingestion and egestion. In Caniff and Hoang's work, *D. magna* exposed to 63-75 µm polyethylene microspheres exhibited greater degrees of accumulation after 21 days, compared to 5 days

(2018). Rist et al.'s study in 2017, which exposed *D. magna* to 2 µm and 100 µm polystyrene beads, reported similar findings, in which the number of particles accumulated in the digestive tract increased with exposure time. In effect, longer periods of exposure with continued deprivation from algae feeding could potentially result in greater stress overall and, therefore, higher mortality rates and significant mobility changes.

Exposure studies of microplastics have frequently utilized microspheres as the standard morphology used. However, previous works have found that fibers can elicit a greater adverse effect on exposed organisms. Jemec et al.'s study exposed *D. magna* to 62-1400 µm polyethylene terephthalate microfibers at 12.5, 25, 50, 100 mg/L for 48 hours. Exposure at all treatment levels for 48 hours induced significant mortality compared to the control (Jemec et al. 2016). Compared to Ma et al.'s study which found no significant effects of 5-15 µm polyethylene microspheres on *D. magna* after 48 hours (Ma et al. 2016), Jemec et al. demonstrates that the shape of microplastics plays a significant role in the extent of damage the particles can have on susceptible organisms.

Observing the ingestion of microplastics by *D. magna* suggests that the accumulation of plastic particles in lower trophic species, such as this study's model species, can enable bioconcentration of microplastics in upper level predators. Elizalde-Velazques et al. simulated trophic transfer of polystyrene microplastics from *D. magna* and to *Pimephales promelas* and indicated that particles ingested by *D. magna* can accumulate into their predators, including small fish (2020). This pattern of biomagnification can continue into higher trophic levels and can ultimately result in contamination in fish which are commonly caught for human consumption. In South Korea, carp, bluegill, bass, catfish, and snakehead caught in the Han River were reported to contain microplastics at

abundances up to 48 particles in the intestine and 16 particles in the gills (Park et al. 2020). Additionally, larger filter feeding organisms, such as bivalves commonly harvested and farmed for commercial reasons, are capable of taking up and retaining microplastics. Marine bivalves sold at a large fishery market in Shanghai, China were reported to be contaminated with microplastics at levels up to 70 particles per individual (Li et al. 2015). In effect, the pathway for human exposure to microplastics is highly driven by the accumulation and trophic transfer of plastics in aquatic systems.

Methoxychlor

MXC alone was shown to induce significant decreases in the speed and total distance and significant increases in frozen events frequency after 24 hours of exposure. After 48 hours of exposure, partial mortality was observed. Results of the MXC portion of this study contributes to the body of work surrounding the impacts of organochlorine pesticides on aquatic invertebrates as well as further introduces *D. magna* as a model species which can be used in MXC exposure work. While MXC has been assessed for its impacts on mammals and fish, there are fewer studies which assess sublethal effects on aquatic invertebrates, particularly on *D. magna*.

Estrogens and testosterone have been detected in *D. magna*, but their biological pathway is less understood (LeBlanc 1998). As MXC's endocrine disrupting capabilities is identified by estrogen mimicry in mammals, it is suggested that in *D. magna*, MXC's impact is similar to how other estrogenic compounds interact (Haschek et al., 2013). However, in *D. magna*, synthetic estrogenic compounds have been shown not to induce any changes in sex determination, development, or reproduction as they would in mammals (Zou & Fingerman, 1997; Kashian & Dodson, 2003). It is therefore suggested that

estrogenic compounds and endocrine disruptors of the same nature may illicit an alternate physiological response. This is further supported through stunted mobility found in the *D. magna* after 24 hours of exposure to MXC. The results of this study introduce additional adverse impacts of MXC. MXC's intended mode of action for target pest insects is through the central nervous system to induce hyperactivity and convulsions, ultimately resulting in death (Schuh et al. 2005). The observed stunted mobility in the *D. magna* is likely a result of the neurotoxic function of MXC and serves as a symptom of the whole-body tremors that occur from exposure to organochlorine pesticides (Bloomquist, 1996). DDT and related pesticides, including MXC, target the voltage-gated sodium channels. Exposure to these compounds induces involuntary depolarization of the sodium channels, leading to increased neurotransmitter release. Ultimately, this mode of action results in paralysis then death (Bloomquist, 1996; Davies et al., 2007) Previous studies have assessed the impacts of MXC on mobility and found supporting results. *Ambystoma macrodactylum* that were exposed to 0.3 µM or higher MXC during embryonic development later exhibited shorter travel distances upon startle stimulus (Eroschenko et al. 2002). Adult *Plarnobella duryi* exposed to MXC at 12.5 µg/L or higher exhibited significant decreases in speed and increases in frozen events after 48 hours (Frankel et al. 2019). In effect, debilitated mobility and subsequent mortalities observed after 48 hours of exposure further support this mechanism, in which malfunction of the central nervous system precedes complete paralysis or death in exposed individuals. Drastic changes to the mobility of *D. magna* in acute exposure time indicates that within short periods of time, individuals can potentially exhibit significant difficulty in food acquisition, predator evasion, as well as escaping contamination. Zooplankton, including *Daphnia*¸ are known for predator avoidance

behavior such as diel vertical migration, in which vulnerable organisms ascend and descend throughout the water column to reduce immediate risk of predation (Brzezinski & von Elert, 2015). Various microcrustaceans have been additionally shown to exhibit escape behavior upon exposure to chromium and copper, in which treated organisms displayed hyperactivity and elevated alertness (Gutierrez et al., 2011). While toxicants such as trace metals can induce a stimulatory effect and allow for greater escape abilities, contaminants such as MXC may inhibit affected individuals' ability to remove themselves from adverse conditions through the pesticide's mode of action. In spite of its ban in the United States and Europe (USEPA 2004), MXC continues to be detected in the environment throughout the world due to continued legalization as well as illegal usage. Throughout the Southern Ocean, MXC has been detected at concentrations up to 3.1 pg/L in seawater and 1.9 pg/m^3 in the atmosphere (Bigot et al. 2016). In the Densu River Basin in Ghana, MXC residues were found in sediment up to 0.18 µg/kg (Kuranchie-Mensah et al. 2012). The continuous persistence of MXC throughout aquatic systems, furthermore, indicates the potential for disruptions in the behavior and responses of vulnerable biota, such as *D. magna*, which can lead to long-term impacts beyond a single species but throughout trophic levels in a system.

Polyethylene Microspheres and Methoxychlor Mixtures

Treatment mixtures overall resulted in mortalities at 24 hours with exception to the mixture of 100 mg/L polyethylene microspheres and 10 µg/L MXC. The observed delay and overall lower rate of mortality in the PE100-MXC10 treatment, which is the highest mixture level, compared to the sequence and rate of mortality of the PE50-MXC5, PE50-MXC10, and PE100-MXC5 treatments suggest the potential for an antagonistic dynamic between the two toxicants at high concentrations. The lack of debilitated mobility in the

PE100-MXC10 treated *D. magna* further propose this interaction. The high concentrations of polyethylene microspheres may have enabled maximum adsorption of the pesticide onto the surface of the particles, reducing the levels of MXC in solution and, thus, minimizing contact of the compound directly onto the body of the exposed *D. magna.*

As an organochlorine compound, MXC has the capacity to act as both a stomach and contact pesticide, in which both the dermal exposure as well as ingestion of the chemical can trigger the mode of action to debilitate the exposed individual (Genuis et al. 2016). Hirai et al.'s study DDT and its metabolites were found adsorbed onto the surfaces of microplastic debris throughout the open ocean and beaches, indicating that organochlorine compounds are capable of being taken up and transported by means of plastic particles (Hirai et al. 2011). In effect, MXC is suspected to have been adsorbed by the polyethylene microspheres, reducing the amount of chemical present in the aqueous solution. This ultimately alters the proportion of MXC which enters *D. magna* by means of bodily contact to MXC which enters through ingestion. At the PE50-MXC5 concentration, such a proportion may allow MXC to make contact directly onto the body as well as potentially through the digestive tract by means of the microbeads. In the case of the PE100-MXC10 treatment, rather than magnifying the impact of MXC on *D. magna*, it is suggested that the microspheres inhibited the contaminant's mode of action of incapacitating the exposed individual.

Previous studies have found similar means of interaction between microplastics and organic pollutants. A study by Zhang et al. analyzed the antagonistic interaction between polystyrene microplastics and the antibiotic roxithromycin on *Oreochromis niloticus*. This study exposed fish to these toxicants either individually or in mixture for 14 days. Results

had found that microplastics alleviated the neurotoxic potential of the pharmaceutical on *O. niloticus* (Zhang et al., 2018). Horton et al.'s investigated the interaction of dimethoate and deltamethrin with polystyrene microplastics on the mobility and survival of *D. magna*. This study concluded with no significant increased toxicity when these pesticides were each combined with the microplastic particles, suggesting that microplastics do not exacerbate uptake of pesticides into tissue (Horton et al. 2018). As a result, microplastics may not interact with pesticides in such a way that they serve as a vector of accumulation. While it is possible for microplastics to adsorb organic pollutants, plastic's affinity for organic compounds may prevent the deposit of these contaminants into the body. The chemical partitioning model for polyethylene suggests that the partition coefficient for PCB is high, indicating that the polymer material can readily uptake these class of chemicals (Choi et al. 2013). As MXC is similar in composition and chemical properties (McGovern, 2006), it is, thus, suggested that the polyethylene microspheres acted as a strong vehicle for MXC but a poor vector of transfer in these exposure studies. In order to draw clearer conclusions regarding this potential antagonistic interaction between the toxicants, additional series of assays should be conducted to increase the sample size overall.

Behavioral Toxicology

Behavioral studies for toxicology testing have emerged as an important basis of assessing the impact of contaminants on biological systems in the environment. In the past, research in toxicology has focused on lethal levels of hazardous chemicals. At times, lethal and sublethal concentrations of toxicants are not found in the environment and, thus, corresponding studies may not reflect current environmental conditions. As such, approaching toxicology through nonlethal endpoints such as mobility and reproductive

behavior can elucidate the fate of toxic substances in the environment beyond population declines. A study by Frankel et al. investigated the impacts of progestins on the reproductive behavior of *Gambusia holbrooki* and observed that females exposed to 100 ng/L levonorgestrel exhibited male-like courtship behaviors with control males (Frankel et al. 2016). Bertolasio et al.'s study on MXC found that neonatal injections of 2.0 mg MXC in female Sprague Dawley rats induced decreases in proceptive behavior and increases in rejection behavior (Bertolasio et al. 2011). While mortality was not a key endpoint in these studies, the results of behavioral analysis indicated that nonlethal impacts can affect populations drastically, such as through reproductive success.

Behavioral toxicology has previously been approached through time-intensive, manual efforts or through high cost software for analysis. In contrast, ToxTrac is a free software that provides capabilities to track multiple organisms and render quantitative data on mobility. The software does not require any potentially subjective assessment by the user and allows for unbiased analysis that can be repeated. ToxTrac has been utilized in previous studies for behavioral analysis of other aquatic invertebrates, including *Planorbella duryi* (Frankel et al. 2019), *Artemia franciscana* (Henry et al. 2019), and *Ciona intestinalis* (Rudolf et al. 2019). In effect, ToxTrac is an option in behavioral toxicology work that is expanding in its application across a multitude of species. This study provides additional examples on the use of tracking software to conduct nonintrusive and objective analyses on nonlethal effects of toxicants beyond those engineered to target mobility, which can explicate the impacts of contaminants that are generally considered less harmful to biological systems.

CHAPTER 5 – FUTURE STUDIES

FIELD-BASED ASSESSMENT

Additional Study Sites

This project assessed microplastics pollution in two sites in the Rappahannock River: a wastewater treatment plant outfall site and a relative downstream site. To expand this study, additional sites throughout the Rappahannock River can be sampled in order to further elucidate the dispersal of microplastics throughout the river. By collecting samples from locations that are further downstream as well as locations which are upstream from WWTP sites, it can be further determined how heavily WWTPs play a role in microplastic contamination.

Temporal Analysis of Microplastic Pollution

To expand the scale of the field-based portion of this study, the temporal distribution of microplastics throughout the same study area. While microplastics are readily detected in sediment and water throughout a multitude of aquatic systems throughout the world, there is less known regarding how long microplastics have been prevalent in the environment as well as the types and abundances of microplastics present throughout different periods of times. Temporal analysis of microplastics would be conducted through collecting a sediment core at these various sites and cutting each into individual segments. Through isotopic analysis, each segment represents a specific time period. By analyzing the abundance and types of microplastics in each segment, a timeline of microplastic pollution over the span of multiple decades can be constructed.

LABORATORY EXPOSURES

Growth Assays

In this study, the neurotoxic potential of MXC was assessed through observing changes in viability and mortality of *D. magna*. While these results find that MXC does induce a similar mode of action that is intended for target insects, there is still the potential for MXC to cause additional effects, such as impaired juvenile development. As an endocrine disruptor, MXC has potential to interact with impacted individuals through alternative pathways beyond its capabilities as a neurotoxin. Through conducting assays which investigate changes in the growth of *D. magna* as a result of exposure to MXC, the fate and toxicity of this contaminant in aquatic environments can be further expanded.

Chemical Analysis of Methoxychlor

To further assess whether sorption occurred between MXC and polyethylene spheres, test media created for exposure assays can be analyzed for MXC concentrations. Mixtures would be filtered through vacuum filtration to remove microspheres and then transferred through a solid phase extraction cartridge to collect the target analyte into the filter. The compound would be eluted using dichloromethane, concentrated with nitrogen gas, then reconstituted with hexane for GC-MS analysis. Chemical analysis can further support the model for polyethylene's sorption capacity, and, thus, confirm the change in MXC concentration in aqueous solution following the addition of microplastics.

CHAPTER 6 – CONCLUSIONS

This study consisted of two key objectives: i) assess the presence and distribution of microplastics in a major river system in Virginia and ii) examine the interaction between microplastics and a legacy contaminant to determine the impacts on viability and behavior of a dominant low-trophic level freshwater species. These objectives were selected to expand on the current knowledge of microplastic pollution in the Chesapeake Bay Basin as well as how microplastics may interact with concurrent contaminants present in these environments.

In the field-based assessment, microplastics were present in sediment and surface waters collected from both sample sites. Microplastics loads were especially heavy in the fluvial bed downstream of the effluent source, suggesting receiving areas of outfall sites are prone to greater degrees of microplastic pollution. While there were limitations in the time available to complete this work due to the 2020 COVID-19 pandemic, there is a strong indication that microplastic contamination is occurring in the local environment and suggests the potential for impacts to local wildlife.

Polyethylene microspheres alone were not found to be a severe toxicant to *D. magna* overall. In contrast, acute exposure to MXC induced significant changes to the locomotive capacity of *D. magna*, further expanding our knowledge on organochlorine pesticides and how they may impact non-target organisms. Exposure to binary mixtures of microplastics and MXC, however, resulted in a unique response, in which mobility was not hindered and mortality was delayed. The observed effect suggests that microplastics may act as a protective vector for organochlorine pollutants, reducing the impact susceptible species may encounter as a result of exposure.

Although the scope and intensity of these projects were hindered by the COVID-19 pandemic, our results have been able to provide a foundation of knowledge surrounding microplastics in a region previously overlooked and continued to expand the magnitude of exposure work that focuses on microplastics and their impacts on aquatic species. By continuing these two avenues of research, a greater understanding of microplastic pollution and its extent and impact in freshwater systems can be obtained in order to thoroughly understand how the advancement of humanity has shifted the natural world.

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