AN ECOTOXICOLOGICAL ASSESSMENT OF THE IMPACT OF MICROPLASTICS ON *DAPHNIA MAGNA* USING ACUTE AND CHRONIC TOXICITY ENDPOINTS WITH A FOCUS ON STRESS BEHAVIOUR

by

Rachel Kelly Elizabeth McNamee

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Abstract

An Ecotoxicological Assessment of the Impact of Microplastics on *Daphnia magna* using Acute and Chronic Toxicity Endpoints with a Focus on Stress Behaviour

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Rachel McNamee
Environmental Applied Science and Management Ryerson University

Microplastics are ubiquitous in freshwater ecosystems and pose a physical and/or chemical threat to biota. While past research has focused on source, transport, and fate of this contaminant, impact assessment studies are limited. Thus, this research used *Daphnia magna*, an environmentally-relevant, model freshwater zooplankton to assess toxicity. Existing protocols were comprehensively reviewed, tested, modified, and subsequently implemented, to produce healthy, age-synchronized organisms ready for rigorous experimentation. Bioassays included microplastics (microbeads), alongside an organic contaminant, triclocarban. The thesis objectives assess whether microplastics posed a chemical and/or physical impact, either on their own, or in conjunction with an additional contaminant. Acute and chronic toxicity endpoints included mortality, reproductive, and behavioural measurements and microscopy was utilized to visualize microbeads within, and surrounding, the daphnids. Preliminary studies suggest that *D. magna* were not sensitive to environmentally-relevant concentrations of polyethylene microbeads (20-27 μm) alone. However, in conjunction with triclocarban, microbeads seemed to impact *D. magna*.

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1 Introduction

1.1 Overview

Plastic is the most prevalent type of debris found in the oceans and Great Lakes (NOAA, 2018). The appearance of plastic in water comes from various sources, but researchers agree that the vast majority of plastic waste, and thus microplastic waste, originates from land, although ocean vessels can be a significant source (GESAMP, 2015). Microplastics are small pieces of plastics; their exact definition has not yet been coherently and consistently outlined in literature, nor by government organizations. The definitions that have been presented simply specify that microplastics are small pieces of plastic (less than 5 mm, (GESAMP, 2015; *Microbeads-Free Water Act*, 2015; *Microbeads in Toiletries Regulation*, 2017; Bucci *et al.*, 2020)). Research on microplastic began as an offshoot to macro-plastic or, more simply, plastic research. Plastic is a synthetic material composed of repeated monomers. Plastics, and by consequence, microplastics can range in their size, shape, density, and colours. Since plastics are synthetic entities, they are resilient to most biodegradation. Additionally, because of their variation in sizes, shapes, and densities, plastics, and microplastics, sequester in different compartments of the ecosystem, including the water column. A more comprehensive definition of microplastics follows in section 1.3.1.1.

Plastic contamination throughout the environment has negative impacts both abiotically and biotically (Bucci *et al*., 2020). Plastic threats are more initially obvious as physical harm through blockage, entanglement, and choking. Another type of physical threat posed by plastics is a false sense of satiation, deterring the animal from seeking out nutritious food and ultimately leading to starvation. There are also two chemical circumstances in which plastics may be toxic. Firstly, plastics can adsorb toxins and pollutants on their surface (some plastics are pre-treated with toxins, while others can adsorb them once in the environment). Additionally, some plastics release monomers that are carcinogenic or estrogenic (Teuten *et al*., 2009). Although Teuten *et al*. (2009) were initially positing these threats from macro-plastics, the same major threats are posed by microplastics to small organisms. The abundance, prevalence, sources, and sinks of

macro-plastics in oceanic waters have been well-documented (Rochman, 2018). Concentrations in freshwaters are comparable to those noted in oceanic environments, and both are rising.

From an ecotoxicology standpoint, impact assessments are foundational in assessing the toxicity of a contaminant. However, presence, even in high abundance of a contaminant, does not necessitate impact to the environment. Giesy & Hoke (1989) argued for the relevancy of bioassays over chemical residue analysis because "bioassays are a direct measure of functional responses." Furthermore, McCarthy (1994) reiterated stating that toxicity "can only be truly assessed with living systems." Baun & Nyholm (1996) affirmed bioassay's importance as it concludes the "overall toxic properties." In the McCarthy lab, subsequent research has focused on this principle (Spearin, 2003; Marshall, 2009; Puddephatt, 2013; Raby, 2013) Therefore, it is not enough simple to collect and measure the abundance and distribution of microplastics in an aquatic system in order to properly understand impact to the environment.

Daphnia magna have long been used in ecotoxicology because of their environmental relevancy, sensitivity to a range of contaminants, and relative ease of culturing (USEPA, 2002). *D. magna* are important macroinvertebrates in the aquatic food web, as well as their near ubiquity in freshwater environments (USEPA, 2002). Moreover, they are considered good bio-indicator organisms for assessing the toxicity of freshwater environments (Environment Canada, 2016).

1.2 Objectives

As with research on macro-plastic, research on microplastic in oceans and freshwaters has largely focused on the abundance, sources, transportation, and fate of microplastics. However, the presence of a contaminant does not necessarily equate impairment to the environment. A recent meta-analysis reviewing impact assessments for a variety of organisms from plastic and microplastic, found there is not currently enough research to indicate a negative impact from environmentally-relevant concentrations of microplastics (Bucci *et al*. 2020); they called for more research assessing impact from microplastics. Additionally, there has been recent concern in ecotoxicology for upholding the rigorous

experimental standards (Harris *et al*. 2014), suggesting a great need for sound experimental designs.

This thesis project will therefore have the main research goal to address this research gap, by assessing the impacts of microplastics on a freshwater zooplankton (the model organism for this thesis is *Daphnia magna*). To accomplish this goal, and to address the recent lack of rigorous standards in ecotoxicology a few sub-objectives have been set up that are intended to successively move this project forward:

1. To implement and build on previous knowledge pertaining to procedures for culturing bio-indicator organisms (*Daphnia magna*) that produces and maintains a healthy stock of age-synchronized organisms ready for toxicity assays. This will be accomplished by documenting reference conditions and refining behavioural and reproductive endpoints in short- and long-term studies. (This will be detailed in section 1.4.)
2. To refine sub-lethal endpoints, with a specific emphasis on behavioural toxicity endpoints in *Daphnia magna*. Short- and long-term toxicity assessments were carried out, with daily observations of behaviour and reproductive outputs.
3. To perform a pilot study exposing *Daphnia magna* to microplastics, and gathering preliminary observations.
4. To assess the impact of microplastics as either a chemical or physical contaminant using behavioural, reproductive, and lifecycle endpoints for *Daphnia magna.*

Microplastics for this project, were intentionally manufactured of this size and would also constitute "microbeads". These microbeads are fluorescent green polyethylene of size 20 – 27 μm and density of 1.025 g/cm^3. The surface of the microbead is pure polyethylene and smooth. To assess toxicity due to potentially adsorbed chemicals, triclocarban will be used.

1.3 Literature Review of Plastic and Microplastics Pollution

Plastic research (both macro-plastic and microplastic) has largely focused on oceanic pollution prior to examining the ubiquity of plastics, including microplastics, in

all environments. Therefore, the following Literature Review will first examine research on macro-plastic distributions, followed by microplastic studies on abundance. Subsequently, there will be a compilation of microplastic impact assessment research for marine animals, to be followed by studies in the same order for freshwater ecosystems, with an emphasis on *Daphnia* spp. research.

1.3.1 Overview of Research on Plastic Pollution in Marine Environments

Concern regarding plastic pollution first started to emerge in scientific research in the 1970s, with research focused on documenting the amount plastic pollution occurring along shorelines. There were three notable studies: Carpenter & Smith (1972), Scott (1972), Cundell (1973). The source of plastic waste from these collections was debated amongst scientists as either trash from beachgoers (Carpenter & Smith, 1972; Cundell, 1973) or as deposition from the waves as Scott (1972) believed. Pruter (1987) reported that major sources of oceanic plastics were from litter off ships, and litter carried by the river and wastewater treatment plants effluents. This was generally accepted as the source of plastic in the marine environment, as a combination between litter off ships and wastewater treatment plants.

Thompson *et al*. (2004) discovered plastic fragments in the waterways, in Plymouth, United Kingdom, and named these small pieces "microplastics." Their report also noted the ability for macro-plastic to photolyze, whereby a larger piece of plastic will break apart in sunlight. It was thus hypothesized that this process was the source of microplastic. Subsequently, microplastics have been much more heavily researched, and globally, information about their abundance, sources, fates, and effects has increased. Most of this subsequent research has focused on marine plastics, as this was thought to be the ultimate sink (Rochman, 2018).

Obbard *et al*. (2014) found microplastics trapped in ice cores of the Arctic sea ice. This was followed by Woodall *et al*. (2014) documenting microplastic pieces in the deep seas of the Atlantic Ocean, Mediterranean Sea, and Indian Ocean. This research indicates contamination throughout the water column, and in nearly every ecosystem, including those that remain largely untouched by recreational human activity. The debate regarding

sources of plastics continued; was it from waste disposal from the fishing sector or from urban run-off? Researchers Li *et al*. (2016) settled the debate by quantifying pollution, finding nearly 80% of oceanic marine debris originated on land, and that 20% is from commercial fishing.

1.3.1.1 Microplastics: Definitions, Sources, and Abundance

1.3.1.1.1 Definition of Microplastics

As previously discussed, microplastics have yet to be comprehensively and consistently defined in the literature or by government institutions. A couple of attempts at defining them have been made and are listed below:

1.3.1.1.1.1 The European Definition of Microplastics

The Organisation for Economic Co-operation and Development (OECD), in a report published by the Joint Group of Experts on the Scientific Aspects of Marine Environmental Protection (GESAMP) (GESAMP, 2015), defined microplastic with a size range of 1 nm to ≤ 5 mm. Having an upper and lower limit separates the terms microplastics from larger pieces known as macro-plastics, as well as smaller pieces known as nanoplastics.

1.3.1.1.1.2 The American Definition of Microplastics

The United States Environmental Protection Agency (USEPA), in their *Microbeads Free Water Act* (2015) characterized microplastics as an umbrella-term including all small pieces of plastics, from both primary and secondary sources. The specific size range is stipulated as smaller than 5 mm in size, with no specified lower limit. Without a lower limit, this could imply that nanoplastics are subsection of microplastics.

1.3.1.1.1.3 The Canadian Definition of Microplastics

Meanwhile, the Canadian definition for microplastics is similar to the OECD stating an upper and lower limit: smaller than 5 mm in diameter, but the plastic must be larger than 0.1 μm (*Microbeads in Toiletries Regulation*, 2017 under the Canadian Environmental Protection Act). Again, an upper and lower limit segregates microplastics

from either macro- or nano-plastics. The *Microbeads in Toiletries Regulation* also confirms the USEPA's definition of microplastics as an umbrella-term; including specifications of microbeads and microfibers.

These definitions were lifted from Acts and Regulations seeking to ban primary produced microplastics for personal care products (*Microbeads Free Water Act*, 2015; *Microbeads in Toiletries Regulation*, 2017). Unfortunately, the ban on primary produced microplastics may not be a cure-all solution, Law & Thompson (2014) suggested that the abundance of microplastics in the environment is likely to increase despite bans placed on microbeads. Their opinion is based on the idea of primary- and secondary- sourced microplastics. The USEPA denotes microplastics can be created in two general manners: through primary production, which is intentionally manufactured as a microplastic; or through secondary production, which is the fragmentation of macro-plastics into smaller-sized pieces. Thus, because microplastics can be formed as a result of macro-plastic breakage (through weathering, animal chewing, photolysis, and abrasion from wind, waves, and rocks) the overall concentration of microplastics in the environment is likely to increase (Law & Thompson, 2014).

Moreover, since government organizations have yet to coherently define a size range for microplastics, this could pose problems in consistency for policymaking, leading to potentially faulty regulations or unintentionally promoting a macro-or nano-plastic. In academic literature, researchers working on this topic will usually define their specific size range, polymer type, density, and other pertinent characteristics of microplastics. However, this can lead to a lack of consistency making experiments challenging to replicate and results difficult to extrapolate.

1.3.1.1.2 Sources and Abundance of Microplastics

Microplastics have two main sources in the marine environment known as primary and secondary production of microplastics. Primary production is from intentionally manufacturing plastics of a small size, usually in the form of pellets. These spherical plastics are commonly referred to as microbeads (*Microbeads in Toiletries Regulation*, 2017). Primary microplastics are then used in: (i) industry as raw plastic

material, (ii) pharmaceuticals and personal care products, and (iii) abrasives component for air-blasting (to clean industrial machinery). These primary microplastics are transferred through the wastewater treatment system or as leachate from landfilling, which eventually lead into marine environments (*Microbead-Free Waters Act* of 2015; Li *et al*., 2016). Fendell & Sewell (2009) note that most wastewater treatment centers are not designed to capture microplastics, and thus, they are released with wastewater effluent into the surrounding environment.

Meanwhile, secondary microplastic production is from the breakage of macro-plastics into smaller pieces. Synthetic fibers shed from clothing made of polyesters and other textiles are known as microfibers. In a similar manner to microbeads, microfibers from clothing (through washing machine effluent) will drain to wastewater treatment centers, and then to the environment (Zhu *et al*., 2019). Macro-plastics littering the environment can also break apart due to photolysis as suggested by Thompson *et al* (2004), or by weatherings, or by the abrasion of macro-plastics, which causes breakage into fragments (Microbead Free Waters Act of 2015).

Plastic does not distribute evenly within the water column, nor throughout the ocean. Many plastics have a lower or similar density than that of water, and should be buoyant; however, due to weathering and water currents, they will slowly sink (Shaw & Ignell, 1990). NOAA published the work of Shaw & Ignell (1990), who studied the distribution, abundance, and characteristics of plastics, and they noted that wind currents and wind are important factors for plastic distribution. However, this report gained little attention until the oceanographer Moore, in 1997, noticed a large patch of debris, which was then dubbed the “Eastern Garbage patch” and is one part of the “Great Pacific Garbage Patch”. While the name “patch” is misleading, most of the pieces of plastic are buoyant but do not appear as a singular mass, as the pieces float in a “soup-like” mixture (Moore *et al*., 2001).

Moore later noted that due to storms and turbulence, microplastics could resuspend and redistribute throughout the water column (Moore *et al*. 2002).

Additionally, microplastics from just one "parental source" will weather and foul in varying ways and these differences will affect the plastic density and consequently its' distribution in the water column (Anderson *et al*., 2016). Secondary microplastics originating from the same macro-plastic could weather and foul differently, consequently sequestering at different levels of the water column.

Apart from observed plastic and microplastic, Jambeck *et al*. (2015) estimated the prevalence of plastic pollution by linking plastic production and waste disposal of 192 coastal countries using 2010 statistics. They estimated that 275 million metric tons of plastic were generated annually. This figure was expected to rise by an order of magnitude by 2025. Researchers were also able to calculate 4.8 to 12.7 million metric tons of plastic are entering the oceans annually (Jambeck *et al*., 2015).

1.3.1.1.3 Microplastics Pollution in Canadian Marine Environments

Canadian researchers have also reinforced observations of the ubiquity of microplastics in marine environment. Huntington *et al*. (2020) published a first assessment of microplastics in the Canadian North, which included investigating marine water, sediment and zooplankton samples collected in the summer of 2017. Researchers highlighted that much of the area samples is untouched territories. In surface waters, they found that 89% of their samples (19 of the 21) included anthopogenic particles. Microplastics were also found in zooplankton at a similarly high rate, with 90% of samples (18 out of 20) contained particles. Anthopogenic particles in zooplankton ranged from 0-16 g/zooplankton. Some of their samples were near local hamlets and townships; however, using population statistics, they devised no correlation between nearby populations and microplastic contamination.

1.3.1.1.4 Review of Marine Impact Assessments (Table 1)

Microplastics impact assessments have yielded conflicting results, as evident from Table 1. This may be due to large discrepancy in microplastic polymer types, sizes, organisms, and overall procedures. As is evident by the dates of these publications, research on microplastic impact is still relatively novel. The units for exposure concentrations has not yet been standardized. Some of the more insightful impact

assessments will be summarized in this section, and can be overviewed in Table 1 (this chart summarized the plastic polymer, size, exposure concentration, species of bioassay, bioassay design and endpoint)

Table 1 Browne *et al.* (2013) were among the first to try and differentiate if microplastics were causing an effect and if this was due to adsorbed pollutants. Their bioassay examined the effects of polyvinylchloride with and pollutants (nonylphenol and phenanthene) and additives (triclosan and PBDE-47) on *Arenicola marina* (lugworms). They exposed the plastics in the overlaying water at a concentration rate 5% plastic-sediment (43.9 particles/mL), and found that microplastics can transfer these pollutants into lugworms. However, they noted no significant effects from plastics alone. These finding suggest that microplastics may not be harmful themselves but collect harmful pollutants. Thus, the need for more research is crucial and microplastics must be viewed as a complex contaminant, which could interact with other pollutants and additives.

Hämer *et al*. (2014) (Table 1) investigated the effects of microbeads and microfibers by feeding fluorescent polystyrene microbeads, polystyrene fragments, and polyacrylic fibers (1-2500 μm) to isopods (*Idotea emarginata*). The plastic was combined with seaweed-powder agar based food at concentrations that varied between 12-350 microplastics/mg, and fed weekly for 6 weeks (two moult cycles). The experiment examined food choice, localization of microplastics inside the digestive system, growth, and inter-moult duration. The short-term study found isopod ate microplastic indiscriminately (ingesting roughly equal amounts of food with and without microplastics). This was confirmed by viewing microplastics in the stomachs of these animals. Additionally, it appeared microplastics were passing straight through the guts and not bio accumulating, as feces had roughly equivalent microplastic concentration as the food. Overall, this study demonstrated no significant effect on mortality, growth, or inter-moult duration.

Table 1 Microplastic Impact Assessments in Marine Environment

Study/Author	Species	Plastic Polymer	Size/Density	Concentration	Effect
Browne *et al*., 2013	*Arenicola marina* (Lugworms)	Polyvinlylchloride (PVC) with pollutants (nonylphenol and phen- anthene) and additives (Triclosan and PBDE-47)	0.23 mm	42.9 particles/mL	No effect
Hämer *et al*., 2014	*Idotea emarginata* (Isopods)	All fluorescent: Polystyrene microbeads and fragments; polyacrylic fibers	Polystyrene (1-100 µm) and Polyacrylic fibers (20-2500 µm)	12-350 microplastics/mg	No effect
Avio *et al. (*January) 2015	*Mytilus galloprovincialis* (Mediterranean mussels)	Polyethylene and polystyrene	1000-100 µm for pyrene adsorption test, and >100 µm for bioassay	Adsorption test: 20 g/L; plastic bioassay 1.5g/L	Yes
Luís *et al. (*July) 2015	*Pomatoschistus microps* (Common goby)	polyethylene (fluorescent)	1-5 µm, and density 1.2g/cc	Between 0.012-12 mg/L	Yes
Cole & Galloway, (November) 2015	*Crassostrea gigas* (Pacific oysters)	Standard and fluorescent polystyrene	70 nm - 120 µm	1, 1, 10, 100, or 1000 microplastics/mL	No effect
Cole *et al. (*November) 2015	*Calanus helgolandicus* (Copepods)	Polystyrene microplastic beads	20 µm	75 microbeads/mL (represents 10% of food)	Yes
Watts *et al. (*January) 2016	*Carcinus maenas* (European green crabs)	Polystyrene microspheres	8 µm	10^6 and 10^7 particles/L	No effect
Paul-Pont *et al. (*April) 2016	*Mytilus spp.* (Blue mussels)	Polystyrene microbeads	2 and 6 µm	final concentration 2000 microbeads/mL*day (32 µg/L*day)	Yes
Martinez-Gómez *et al. (*June) 2017	*Paracentrotus lividus* (Sea urchin)	polystyrene microspheres (red) and high density polyethylene fluff	Polystyrene: 6 µm. And polyethylene irregular shaped 0-80 µm	4 concentrations: 0, 10^3, 10^4, 10^5 microplastic/mL	Yes
Alomar *et al. (*November) 2017	*Mullus surmuletus* (Striped red mullets)	Field study found: Polyethylene terephthalate (PET) Cellophane Polyacrylate Polyacrylonitrile (PAN) Alkyd Polystyrene acrilonitrile methyl methacrylate	0.1 - 1 mm plastic sizes	Field study (no intentional exposure)	No

Avio *et al*. (2015) (Table 1 research focused on the ability of different polymers to adsorb pollutants. They examined polyethylene and polystyrene (>100 μm), and found several effects at the transcriptional and cellular level of Mediterranean mussels (*Mytilus galloprovincialis).* These bioassays had two objectives; first, Avio *et al*. (2015) measured the adsorption of pyrene (a common PAH) and were able to decipher that both microplastics would adsorb pyrene in a dose dependent relationship. Second, they exposed pristine- and pyrene- adsorbed microplastics in a bioassay and both pristine- and polluted- microplastics caused effects. This further solidified evidence that microplastics should be considered complex as they may adsorb pollutants and could be dangerous as a "sorbent".

Luís *et al.* (2015) (see Table 1 examined the effects of microplastics with the addition of added pollutants. Using common goby (*Pomatoschistus microps)* for a 96-h bioassay, researchers exposed the fish to fluorescent polyethylene (1-5 μm), with and without the heavy metal chomium (VI). To properly understand the effects of this added pollutant to microplastics, they first conducted a Cr(VI) experiment and found for 96-h toxicity dose of LC_{50} 14.4-30.5 mg/L. Again, microplastics that had been pretreated with a pollutant, and in this case, Cr(VI) caused adverse health effects on animals. Lastly, Luís *et al*. (2015) reported decreased predation performance and performance of acetylcholinesterase activity (enzyme found in postsynaptic neural muscular junctions, primarily responsible for breaking down acetylcholine).

Cole & Galloway (2015) examined feeding and growth of larvae Pacific oysters (*Crassostrea gigas)* when exposed to in varying concentrations of microplastics (1-1000 microplastics/mL) (see Table 1). They chose fluorescent polystyrene beads (70 nm - 120 μm), and suspended the beads in artificial seawater prior to exposing them to the animals. They found that organisms readily took up nanoplastics and microplastics, with a strong significant correlation between particle amount and consumption (with increased microplastics concentrations correlated to increased consumption). Exposure to

microplastics had no significant effect on feeding and growth at less than 100 micoplastics/mL.

Cole then followed this research with colleagues (Cole *et al*., 2015) by examining copepods, *Calanus helgolandicus* to polystyrene microplastic beads (20 μm) (Table 1). They directly added the microbeads to the media (mostly composed of filtered sea water and algal food source). A short- and long-term assays were undertaking examining ingestion-egestion rates (24 h) a egg production (9 day exposure) of microplastics, at a concentration of 75 microbeads/mL (representing 10% of their food). Ingestion of microplastics caused significant impact on feeding strategies (consuming 11% less algae cells, and 40% less carbon mass as they were mostly consuming smaller algae cells). Furthermore, prolonged exposure significantly reduced egg size. Cole *et al*. (2015) assumed any effect caused by the polystyrene was due to physical complications, rather than a chemical threat as styrene is considered non-toxic.

Subsequently, Watts *et al*. (2016) exposed crabs (*Carcinus maenas)* to polystyrene microspheres (8 μm) of varying coating (neutral, carboxylated, or aminated) see Table 1. Coatings were added to compare the influence of surface composition. They found that microplastics with different coating would distribute differently across crab gills, but regardless of coating microplastics did not have adverse effects on gill function.

Next, Paul-Pont *et al. (*2016) exposed mussels (*Mytilus spp)* to polystyrene microbeads (of two differing sizes, 2 and 6 μm) (Table 1). During this experiment, mussels were exposed to a concentration of 2000 microbeads/mL daily (or 32 μg/L). Additionally, this experiment attempted to shed light on the adsorption and bioaccumulation of the pollutant fluoranthene by comparing treatments with fluoranthene alone, microbead alone, fluoranthene and microbeads, and a reference control (microbeads had been combined with 0.001%v/v Tween-20). Fluoranthene has a high affinity for microplastic and mussels exposed to both microbeads and fluoranthene had an increased concentration of fluoranthene. Furthermore, microplastics alone under these lab conditions was toxic to the tissue, cellular, and molecular levels

Martínez-Gómez *et al*. (2017) (Table 1) also used polystyrene microspheres (red, sized 6 μm) and high-density polyethylene "fluff" (0-80 μm) in an experiment exposing these microplastics to sea urchins (*Paracentrotus lividus*). Researchers followed procedures developed by previous experts in the field (Saco-Álvarez *et al*. (2010) and Beiras *et al*. (2012)) in order to expose microplastics (at concentrations of 0, 10^3, 10^4, 10^5 microplastics/mL) to embryos for 10 minutes, incubated, and then examined. This resulted in toxic effects of fertilization, and abnormalities in embryo development. The authors noted that it important for research to examine the impact of plastic that has been weathered or aged as opposed to just pristine microplastics.

Lastly, Alomar *et al*. (2017) (Table 1) completed a field experiment examining the effects of microplastics with striped red mullet (*Mullus surmuletus)* by taking samples of the organisms from 5 different ports of Mallorca Island. This field work sampled 417 fish, finding that just over a quarter (27.3%) had plastic in their stomachs. The average number of plastic particles was 0.42 microplastics/individual. In the livers of fish that had ingested plastic there were no signs of oxidative stress. This would suggest that in the wild animals are ingesting microplastics without much observable effect.

1.3.2 Research on Plastic Pollution in Freshwater Environments

Rochman (2018) notes that freshwater systems are highly diverse and highly dependent on the terrestrial environment surrounding them, making most freshwater particularly vulnerable to contaminant inputs from the land. Furthermore, plastic and microplastic pollution contamination in freshwater was assumed to be similar to that of oceanic plastic problems (Rochman, 2018). Thus, it wasn't until recently that research began to focus on freshwater systems.

As the Great Lakes and their surrounding watersheds make up the largest bodies of freshwater on Earth, detecting then reducing contaminants is paramount for clean, safe freshwater. Erikson *et al*. (2013) focused on examining the Laurentian Great Lakes for the presence of plastics. During expeditions in the Great Lakes, researchers used a fine

mesh-net (mesh size: 333 μm) to collect plastic, and then categorized these plastics by size. The average abundance of plastics was calculated at 43,157 plastic particles/km^2 (± 115,519), and microplastics (sized 0.355-0.99 mm) made up 81% of the fragments. Additionally, Corcoran *et al*. (2015) found that polyethylene accounted for the majority (74%) of the microplastic polymer found in sediment cores.

1.3.2.1 Microplastics Pollution in Canadian Freshwater:

Implications for Canadian freshwater ecosystems have been summarized by Anderson *et al*. (2016), emphasizing that only a few studies have looked at Canadian freshwater microplastic assessments. The OMOECC responded to this concerning research by partnering with Western University to examine plastic abundance in water samples from February 2014 to September 2016 (Ballent *et al*., 2016). A total of 66 watersheds were analyzed and microplastics were identified in every sediment sample. The average concentration was 760 microplastic particulates/kg of sediment. Polyethylene was the most common type of plastic polymer (Ballent *et al*., 2016). This research confirmed high concentration of microplastics in the Great Lakes. Furthermore, it also highlighted that similar to oceanic plastic and microplastic, freshwaters are also contaminated by analogous pathways – through urban run-off into streams and tributary rivers leading to the lakes. Furthermore, microplastic ultimate sources are still through primary or secondary production. Finally, research in freshwater systems have consistently focused on the fate and abundance of plastics in freshwater while few studies have assessed the impact of these plastics in this aquatic environment.

1.3.2.2 Review of Freshwater Impact Assessments (Table 2)

Various studies have attempted to assess impact of plastics to wildlife and the environment. Similar to marine ecosystems, researchers also provided inconsistent results across microplastic type, organism, and procedure. Likewise, units for the exposure concentrations have yet to be standardized. The following section is a summary of some of the most notable freshwater impact assessments, Table 2 provides an overview of this information (including the plastic polymer, size, exposure concentration, species of bioassay, bioassay design and endpoint).

Bhattacharya *et al*. (2010) examined the impact of microplastics on two genus of freshwater algae, *Chlorella* and *Scenedesmus* (Table 2). Their findings suggested that algae could be negatively impacted by exposure to microplastics, possibly through the physical blocking of light or airflow. Polystyrene beads (20 nm) were exposed to these algal species separately, and both species showed reduced photosynthesis and increased divergence of oxygen instead of carbon dioxide in photosynthetic pathways.

Rochman *et al*. (2014) Table 2 explored the impact of pristine and weathered microplastics by conducting bioassays on Japanese medaka (*Oryzias latipes*) using pristine microplastics and microplastics that had been deployed in the San Diego Bay (California, USA) for three months prior. To simulate environmentally-relevant concentrations they used exposure concentrations of 8 ng/mL. Their results suggested that plastic debris may alter endocrine system function since altered gene expression in female fish was noted for both pristine and pre-exposed plastic. Gene function in males was only observed in pre-exposed plastics. This research suggests that weathered microplastic might present more impact as an endocrine disruptor, and that pristine microplastic can also pose at least a chemical threat.

Table 2 Impact Assessments in Freshwater Ecosystems

Study/Author	Organism	Plastic Polymer	Size/Density	Concentration	Effect
Bhattacharya *et al*., 2010	*Chlorella* and *Scenedesmus* (Green algae)	Positively and negatively charged polystyrene (Amidine Latex and Carboxyl Latex)	20 nm	0.08 -0.8 mg/mL	Yes
Rochman *et al*., 2014	*Japanese medaka* (Japanese rice fish)	Polyethylene pre-production pellets. When deployed in the bay they were 3 mm, but then prior to exposure all plastics were ground to <0.5 mm	0.5 mm	8 ng/mL	Yes
Au *et al*., 2015	*Hyalella azteca* *(*Amphipods)	fluorescent blue polyethylene microplastic particles and black polypropylene microplastic fibers (marine rope)	Polyethylene 10 µm to 27 µm in diameter (density of 1.13 g/cc). Polypropylene from marine rope 20 µm - 75 mm in length	Polyethylene: acute (0–100000 microplastics/mL) and chronic (0–20 000 microplastics/mL). Polypropylene: 0–90 microplastics/mL	Yes
Imhof & Laforsch, (November) 2016	*Potamopyrgus antipodarum* (New Zealand mud snails)	Polyamide, polyethylene terephthalate, polycarbonate, polystyrene, polyvinylchloride	Derived from raw pellets but were now irregular shaped 118 µm (min 4.64 max: 602 µm)	particles dose of 0%, 30% and 70%.	No effect
Hu *et al*., (December) 2016	*Xenopus tropicalis* (Western clawed frogs)	Uncoated polystyrene pellets (labeled fluorescent green)	1 and 10 µm (density 1.05 g/cm3)	Accumulation experiment 1 µm had three concentrations: 0.1 - 10^5	No effect
Blarer & Burkhardt-Holm, (September) 2016	*Gammarus fossarum* (Amphipods)	Polyamide (PA) fibres (500 × 20 µm) and fluorescent Polystyrene (PS) beads (diameter of 1.6 µm, labelled with fluorescent dye Nile Red 0.01 %)	1) PA fibres - 500 × 20 µm (would sink in water) and 2) PS beads diameter of 1.6 µm (stayed in water column)	3 set ups: i) PA fibers at 4 concentrations: 100, 540, 2680 and 13 380 fibers/cm2 (for 24 hs) ii) four time periods (concentration 2680) at 0.5, 2, 8, 32 hs. iii)PS beads for 24 h concentration: 500, 2500, 12500, 60000 beads/mL. - four post-exposure time for PS beads (depurinated and checked at 1, 2, 4, 16 hs later)	No effect
Grigorakis *et al. (*February) 2017	*Carassius auratus* (Goldfish)	Microfibers from a commercial scarf. Microbeads from a facial cleanser (polyethylene)	Fibers sieved sized 50-500 µm. And beads 250 - 63 µm	50 MP per 1 pellet	No effect
Straub *et al. (*July) 2017	*Gammarus fossarum* (Amphipods)	biodegradable MP polyhydroxybutyrate (PHB) and a petroleum-based MP polymethylmethacrylate (PMMA)	Fragments of varying sizes (32-250 um). PHB white, opaque, density 1.24 g/cm2. PMMA transparent, density: 1.19 g/cm2	0-100 000 MP/individual	Yes
Rochman *et al. (*November) 2017	*Corbicula fluminea* and *Acipenser transmontanus* (Asian clams and White sturgeon)	polyethylene terephthalate (PET), polyethylene, polyvinylchloride (PVC), and polystyrene	Each microplastic micronized for irregular shapes. 12-704 µm	0.0003% microplastic by volume of water (2.8 - 4.2 mg/L)	No Effect

To date, there is one notable study solely focusing on the freshwater macroinvertebrate *Hyalella azteca* (Table 2). Au *et al*. (2015) conducted research exposing *Hyalella azteca* to microplastics (of varying material, density, and size). In accordance with USEPA protocols two studies were undergone, 10-day lethal concentration bioassay and 42-day chronic bioassays. Au *et al*. (2015) used two types of microplastic for the acute bioassay, polyethylene particles (sized 10-27 μm) and polypropylene fibers from a marine rope (cut to 20-75 μm). The 10-day exposure served as a basis of comparison, as the authors noted that polypropylene fibers had LC_{50} of 1.43 microplastics/mL; whereas polyethylene had an LC_{50} of 4.64×10^4 microplastics/mL. This led them to conclude polypropylene fibers were far more toxic than polyethylene beads. However, this conclusion is not fully supported by their work, since they lacked controls in their different types of plastic (different material, sizes, densities, as well as chemical adsorptions). Following the acute exposures, a chronic 42-day lifecycle bioassay was then conducted with the polyethylene beads. Chronic exposure to the polyethylene beads demonstrated a significant decrease in growth and reproduction at low and intermediate exposure concentrations (Au *et al*., 2015).

Imhof & Laforsch (2016) (Table 2) sought to shed light on the dynamic of exposure to multiple types of plastic to benthic grazers. This experiment fed mud snails (*Potamopyrgus antipodarum*) four types of irregularly shaped microplastics (4- 602 μm). Adults were supplied their typical food with the plastic entities (polyamide, polyethylene terephthalate, polycarbonate, polystyrene and, polyvinylchloride) "smeared" atop at two different concentrations (30% and 70%) and a control (0%). The researchers followed OECD guidelines for culturing and testing the snails and examined sublethal highly sensitive endpoints such as: shell size, number of embryos, proportion of embryos with shells, and number of juveniles produced. Their results showed no morphological changes, no effects on embryogenesis, and juveniles development-until-maturity unaffected.

In 2016, Hu *et al*. started experimenting with uncoated polystyrene pellets (sized 1 and 10 μm; 1.05 g/cm^3) and tadpoles (*Xenopus tropicalis*). They performed two types

of experiments: (i) bioaccumulation, which was conducted as a short-term at high concentrations; and (ii) elimination experiments, which followed tadpoles for 1-6 days of depuration. Microplastics were identified in the gills and digestive tract within 1 h of exposure, and egestion followed 6 hours later. Accumulation of microplastics inside a tadpole was concentration, not time dependent. Lastly, tadpoles that were unfed prior to the beginning of the experiment accumulated significantly more microplastics than those pre-fed. This experiment did not comment on the effects on their ability to ingest and egest microplastics.

Blarer & Burkhardt-Holm (2016) (Table 2) examined ingestion and egestion endpoints in amphipods (*Gammarus fossarum*), with polyamide fibers (500 x 20 μm) and fluorescent polystyrene beads (1.6 μm). The results again showed a positive correlation between microplastic concentration and ingestion rate. Microplastics were only found in the gut lumen, implying that epithelial cells did not absorb the microbeads, and suggesting that these plastics would pass through organisms. Again, this bioassay does not comment on effects.

Grigorakis *et al*. (2017) (Table 2) fed microplastics from commercial products to goldfish (*Carassius auratus*) and observed ingestion and egestion of microplastics as compared to their normal food. First, two types of microplastics were extracted: (i) microfibers from a scarf (50 – 500 μm), and (ii) polyethylene from microbeads from a facial cleanser (60 – 250 μm). Second, these microplastics were added to individual fish food pellets at 50 microplastic particles/pellets. Each goldfish was given one pellet either containing the microfiber, microbead, or an untouched pellet (control). Once it was consumed the goldfish were allowed to eat until satiated on normal pellets. All goldfish consumed their initial pellet. Microbeads and microfibers had similar retention times and neither appeared to accumulate in the gut. This is one of the few studies were organisms were actually fed a microplastic, as opposed to exposed to microplastics in their experimental environment.

Given the growing use of biodegradable plastics, Straub *et al*. (2017) (Table 2) conducted a bioassay to assess toxicity differences between petroleum and biodegradable

plastics. Two types of plastics were used, biodegradable polyhydroxybutyrate (PHB) and petroleum-based polymethylmethacrylate (PMMA) (both of sizes 31 – 250 μm). The amphipod (*Gammarus fossarum*) ingested both types of microplastics after 24 h and almost completely egested after 64 h. Over 4 weeks experiments, both microplastics led to a significantly lower weight gain. Petroleum microplastics treatments showed significant decreased assimilation efficiency compared to the biodegradable microplastics. This suggests that pristine microplastics would be more harmful to the health of amphipods than the bio-plastic alternatives.

While many bioassays focus on single organism effects to microplastic ingestion, Rochman *et al*. (2017) was among the first researhers to explore the effects of plastic on a population dynamic between predator and prey (Table 2). This experiment exposed a prey to microplastics and then allowed a natural predator to feed. They chose Asian clams (*Corbicula fluminea*) as a filter feeder and their predator white sturgeon (*Acipenser transmontanus*). This research included 10 treatments, including two controls (one completely referenced, and one with PCB), 4 pristine plastic treatments (polyethylene terephthalate (PET), polyethylene, polyvinylchloride (PVC), and polystyrene), and 4 treatments each of the plastic with pre-sorbed PCB. The pristine plastic treatments showed subtle effects for both prey and predator. However, upon chemical analysis PCB was not detected in prey or predator, suggesting that either PCB did not sorb to the plastic or did not desorbed into the organisms. Though modeling, they determined that polyethylene would sorb the most PCB.

1.3.2.3 Impact Assessments for Daphnia spp. (Table 3)

Research on the impacts of microplastics on *Daphnia* spp. is still novel, through some researchers are working to fill this gap. The following section will provide a synopsis of the bioassays published using *Daphnia* spp. Table 3 provides an overview of these studies summarizing the plastic polymer, size, exposure concentration, species of bioassay, bioassay design and endpoint for some of the more significant impact assessments. There are two studies included in this chart pertaining to zooplankton, but due to species similarity are included with the *Daphnia* spp. The following section includes a synopsis of these studies in chronological order, along with a brief comment on how this research was important for following discoveries.

One of the first assessments on the impacts of microplastics using *Daphnia* spp. was conducted by Besseling *et al*. (2014), as seen in Table 3. Their study used polystyrene beads (70 nm), and suggested that microplastics could be toxic. Following OECD guidelines, they conducted 21-day bioassay, with malformation visible at 30 mg/L. This left much to still be discovered, including acute toxicity. Following this study a series of *Daphnia* spp. bioassays reported conflicting results.

Rehse *et al*. (2016) conducted a study using *Daphnia magna* to attempt to assess the impact of microplastics (Table 3). The researchers used two polyethylene fibers; the first was sized 1-4 μm, the second 90-106 μm. They chose two widely different sizes to begin identifying the specific sizes of microplastics that could particularly affect *Daphnia* spp. The experiment was designed to expose microplastics [12.5 – 400 mg/L] to *Daphnia magna*. Under the OECD guidelines for acute immobilization (OECD guidelines 202 for *Daphnia* spp. Immobilization, (OECD, 2004)) researchers concluded that neonates can be immobilized (not able to swim after agitation). Immobilization only occurred with exposure to microplastics sized 1-4 μm (Rehse *et al*., 2016). The microfibers that were size 90-106 μm did not yield any notable results, likely due to neonates being unable to ingest this size. This was an acute (96-h) exposure study, which, while demonstrating impact to *Daphnia* spp., was unable to decipher how the microplastics caused impact (through a physical or chemical threat.) Additionally, while this study noted

immobilization it did not specify if the microplastics were impairing swimming by weighing or attaching to the swimming appendages.

Jemec *et al*. (2016) (Table 3) found similar results, with increased mortality to *Daphnia magna* when acutely exposed to high concentrations of microplastics (48-h mortality and growth (EN ISO 6341:2012)). Their study used red coloured polyethylene terephthalate (PET) fibers of varying sizes (length 62 - 100 μm; Width: 31- 528 μm; Thick: 1-21.5 μm) at concentrations that varied from 12.5 – 100 mg of microplastic/L. Jemec *et al*. (2016) added the microplastics directly into the vessels with the *Daphnia magna.* The experiment was designed to test whether *Daphnia magna* that were exposed solely to microplastics without another food source would experience detrimental impacts. Thus, Jemec *et al*. (2016) set up two treatments, one where neonates were pre-fed algae, and a second treatment where neonates were not pre-fed. *Daphnia magna* that were not pre-fed had increased mortality, and a 24-h incubation period post exposure did not aid recovery (Jamec *et al*., 2016). However, this bioassay did not yield statistically significant results, which while notable in impact, would need re-assessment (Jemec *et al*., 2016). Furthermore, the study did not consider how the microplastics could be impacting the organism (i.e.: impact caused through physical or chemical impact), nor examine any chronic or reproductive endpoints.

Table 3 Impact Assessments of Microplastics for *Daphnia magna*

Study/Author	Species	Plastic Polymer	Size/Density	Concentration	Effect
Besseling *et al*., 2014	*Daphnia magna*	Polystyrene beads with carboxylic acid side chains	70 nm	0.22−150 mg nano-PS/L.	Yes
Rehse *et al. (*June) 2016	*Daphnia magna*	Polyethylene fibers	1 µm and 100 µm	12.5 - 400 mg of microplastic/L (20 Daphnia per concentration)	Yes
Jemec *et al. (*December) 2016	*Daphnia magna*	Polyethylene Terephthalate (PET); red colour	Length: 62 - 100 µm; Width: 31- 528 µm; Thick: 1-21.5 µm	12.5 - 100 mg of microplastic/L (20 Daphnia per concentration)	No Effect
Cui *et al. (*September) 2017	*Daphnia galeata**	Polystyrene	52 nm	5 mg/L	Yes
Rist *et al. (*September) 2017	*Daphnia magna*	2 µm and 100 nm fluorescent polystyrene beads	spherical Polystyrene beads	Experiments 1 and 2: 1 mg/L	No Effect
Kim *et al. (*October) 2017	*Daphnia magna*	Polystyrene plastic with carboxyl group, and without	Pristine: 201.5 nm, with carboxyl group 191.3 nm. Density: 1.05−1.06 g/mL	1, 5, 10, 20, and 30 mg/L	Yes
Ziajahomi *et al. (*October) 2017	*Ceriodaphnia dubia**	Polyester fibers and polyethylene beads	Polyester fibers from: orange fluorescent clothing (100% polyester, density 1.38 g/ cm^3). Polyethylene beads (1−4 µm, density of 0.987 g/cm^3) - spheres treated with Tween (0.1% v/v)	Acute: 0.5−16 mg/L of PE beads and 0.125−4 mg/L of polyester fibers, which corresponds to 1.7 × 104−5.4 × 105 particles/L for PE beads and 1.1 × 103−3.4 × 104 particles/L for polyester fibers.	Yes
Imhof *et al. (*November) 2017	*Daphnia magna*	2 plastic mixes from raw pellets: A: polyamide, polycarbonate, polyethylene terephalate, polyvinyl chloride; B: Acrylonitrile-burtdiene-styrene terpolymer, plasticized polyvinyl chloride, polyoxymethylene, homopolymer, styerene-acrylonitrile copolymer	irregular shaped particles with average size 40 µm	1% of food particle = microplastic (290 particles/ml)	No Effect
Frydkjær *et al. (*December) 2017	*Daphnia magna*	Polyethylene's, and phenanthene	pristine white microbeads (10-106 µm) and irregular shaped microbeads from recycled polyethylene (10 - 75 µm)	6 concentrations between 0.001 - 10 g/L	Yes
Aljaibachi & Callaghan, (April) 2018	*Daphnia magna*	polystyrene	2 µm carboxylate-modified polystyrene, fluorescent yellow-green (density 1.050g/cm3)	Microplastic uptake: 1.46x10^-2 mg/L. Chronic exposure between 0 - $1.1x10^{-2}$	Yes
Canniff & Hoang, (August) 2018	*Daphnia magna*	Fluorescent green polyethylene beads	63-75 µm (density 0.99 - 1.01 g/cm3)	0, 25, 50, 100 mg/L	No effect

Cui *et al. (*2017) used *Daphnia galeata* in an exposure study with polystyrene nanoplastics (52 nm) (Table 3). They added the plastic to moderately hard water with algae as a food source, and examined survival, reproduction, and lipid storage in adults, additionally, survival, development and hatching rates of embryos were observed. The concentration was relatively low in comparison to other *Daphnia spp.* studies at just 5 mg/L. Additionally, they used Tween to disperse the microbeads at a concentration of 0.1% (they also conducted adjacent Tween toxicant tests). The researchers were able to visually confirm transfer of microplastics from the media into the internal organs (including intestine, thoracic appendages, ovaries, brood chamber, and to the developing embryos). Additionally, embryos showed abnormal development and lowered hatching rates. While intriguing these findings still leave much to uncovered, including if these microplastics on internal organs can cause an impact and if so how (including if they can be a transfer contaminant to these internal organs, and they can in turn cause an impact).

Rist *et al*. (2017) (Table 3) conducted *Daphnia magna* bioassays to quantify ingestion and egestion rates of microplastics. These experiments examined short-term (24 h) ingestion and egestion rates as well as examined 21-day reproductive endpoints. In this case researchers used 2 μm and 100 nm polystyrene beads, with 7-day old *Daphnia magna*. Both sizes were ingested; however, the 2 μm particles were ingested at a rate five times higher than the 100 nm particles. This difference in rate of ingestion as a function of particle size is not entirely surprising as Daphnia prefer particles between 1-25 μm. However, it does lead to interesting questions if the fate of microplastics is to fragment into these smaller sizes. During the 21-day exposure, no effect was observed regarding reproductive impairment for either microplastic sizes.

Kim *et al*. (2017) conducted research with the intent of addressing microplastics and their ability to sorb pollutants by using nickel as a heavy metal additive with different microplastics (as seen in Table 3). Their study used polystyrene (191.5 -201.5 nm, with a density of 1.05-1.06 g/mL), that was either pristine or coated with a carboxyl group. Neonates were exposed for a short term and mortality was used as the end point. The

toxicity of nickel in the presence of different microplastics differed from the toxicity of nickel alone. The assay implemented several concentrations varying from 1-30 mg/L of microplastics. *Daphnia magna* exposed to microplastics that had carboxyl functional groups and nickel had higher immobilization rates then the untreated microplastics with nickel. These results suggested that the coating of microplastics will effect it's sorbing capacity to nickel and consequently the toxicity. The results would suggest that microplastics are harmful from a chemical stance, however further tests would be important to understand if this is just with nickle or other contaminants as well.

Ziajahomi *et al*. (2017) exposed *Ceriodaphnia dubia* to polyester fibers and polyethylene beads microplastics (Table 3). Two types of microplastics were chosen, polyester fibers from an orange fluorescent scarf (simulating fibers that might come out from a washing machine effluent), and the polyethylene beads (as a pristine sources). For an acute (48 h) study, they were able to determine LC_{50} with polyethylene beads of 2.2 mg/L (7.4×10^4 particles/L), and the polyester fibers of 1.5 mg/L (1.3×10^4 particles/L). Chronic exposures did not significantly impact mortality; however, they were able to find EC_{50} for both microplastics: polyester fibers 429 µg/L, and 958 µg/L. The fibers showed more effects than the beads. Other studies would be important is deciphering sub-lethal endpoints for microplastics at environmentally relevant concentrations.

Imhof *et al*. (2017) rejected the previous work (see Table 3), finding no significant changes in mortality, growth, or reproduction when exposing *Daphnia magna* to microplastics. Researchers used two different mixtures of raw pellets: treatment A (polyamide, polycarbonate, polyethylene terephalate, polyvinyl chloride) and treatment B (acrylonitrile-burtdiene-styrene terpolymer, plasticized polyvinyl chloride); the average size was 40 µm. They suspended the microplastics at a concentration of 1% of food particles in a *Daphnia* medium which consisted of purified water and algae. Adult *Daphnia magna* were found to have an average of 30 microplastic particles in their gut; however, researchers concluded no significant impact (Imhof *et al*., 2017). The set up for experimenting with *Daphnia magna* and only using exposure rates of 1% was environmentally-relevant and rigorous. However, by exposing the *Daphnia magna* to a

medley of microplastics at once, if there was impact it would be impossible to tell which microplastic caused the most impact and how. Additionally, Imhof et al. (2017) used adult *D. magna*, however the use of neonates is important as research often demonstrates neonates are more sensitive.

Another study published in 2017 found microplastics had an impact if they were irregular-shaped rather than regular-shaped (Frydkjær *et al*., 2017) (Table 3). This study used 2 types of plastics: polyethylene microbeads, which were pristine-spherical white microbeads, sized 10-106 μm; and phenanthene, which were irregular-shaped recycled and sized 10-75 μm. Frydkjær *et al*. (2017) exposed plankton to microplastic at concentrations between 0.001 – 10 g/L for 24 hs, then, fed the microplastic and plankton to *Daphnia magna*. *D. magna* ingested both the regular and irregular shapes of plastics. However, they egested regular shapes much more quickly. An EC_{50} of 0.065 g/L was found for the irregular shapes and it was concluded that irregular shapes of microplastics cause more of an impact. While these finding are intriguing, the study lacked many necessary controls; for example, the two types of plastics (different materials, shapes, sizes, and sources), and the *Daphnia magna* age was not listed (which could mean they did not age-synchronize). Moreover, Frydkjær *et al*. (2017) were not able to distinguish if impact of plastic was chemical or physical, and only examined acute toxicity.

Aljaibachi and Callaghan (2018) found that daphnids might be able to selectively feed, and subsequently avoided microplastics (Table 3). They conducted short- and long-term experiments with adult daphnids (18-day olds) using 2 μm carboxylate-modified polystyrene (density 1.050g/cm3). The experimental set up examined ingestion of microplastics at 30, 60, 120, and 240 minutes, in treatments with and without algae at increasing microplastic concentrations. In treatments of microplastics and algae as a food source, *Daphnia magna* ingested significantly fewer microplastics. This trend continued even as microplastic concentrations increased. This imbalance in ingestion could suggest that *Daphnia magna* may have been avoiding the microplastics. In their chronic study, the researchers observed microplastic treatments showed an increase mortality after 7 days. This avoidance behaviour leaves much still to be researched, including

understanding if the daphnids would ingest microplastics that covered in a food source, or if the polymer is to blame for their selectivity. Additionally, this project examines microplastics solely from the lens of ingestion but microplastics could be causing impact in other physical and chemical ways (on the carapace, swimming appendages, or with the thoracis legs).

Cannif and Hoang (2018) more recently completed a study on *Daphnia magna* ingestion of microplastic (Table 3). The study used fluorescent green microbeads (sized 63–75 µm with a particle density of 0.99–1.01 g/cm^3), at three concentrations (25, 50 and 100 mg/L). The study first combined microplastics with algae, and then exposed *Daphnia magna* to the liquid medium. The micro-algae selected as a food source was *Raphidocelis subcapitata,* and grew more quickly in the presence of microplastics. Researchers suspected the micro-algae were using the microbeads as a surface on which to grow. The assay used age synchronized 7-day old *Daphnia magna* exposing them to algae-microplastic medium for 21 days. When testing for both acute and chronic impact on *Daphnia magna*, they found that microplastics did not significantly affect survival and reproduction. This could have been due to the algae growing on the microplastics, allowing the *Daphnia magna* to obtain the necessary nutrients despite ingesting microplastics (Cannif & Hoang, 2018).

To sum, research exposing microplastics to daphnids has already begun. However, the research is not clear, with some studies finding effects (and the severity of these effects vary) while other studies concluded no noticeable effects. Additionally, the designs of these studies are not designed to decipher if microplastics pose a chemical or physical threat.

1.3.3 The Bioassay Approach

Testing the toxicity of a contaminant through exposure to a sensitive test organism has long been implemented as an effective tool for measuring toxicity. Thus bioassays are critical for impact assessment. The bioassays employed will test for both acute and chronic impact on the organisms. Dr. McCarthy's laboratory has worked extensively with

D. magna using established protocols and measuring both behavioral and lethal endpoints (McCarthy 1994, Fleet, 2010; Doobay, 2011; Raby 2013). The procedures in this thesis and endpoints will be guided by previous work. McCarthy's 1994 work studied partial lifetime exposure by assessing impact of contaminants until the first clutch. Fleet (2010) also worked with *D. magna,* measuring reproductive and behavioral endpoints. Doobay (2011) examined reproductive endpoints. While Raby (2013) worked with both behavioral and partial lifecycle end points. She notes that bioassays with behavioral endpoints are ecologically-relevant and reflect the cumulative stress on the whole organism. Behaviour can be observed with relatively inexpensive tools. As such, much of the information on *D. magna*, culturing, and bioassays will follow their close instructions.

As microplastics are a physical entities *D. magna* will be examined under a microscope throughout the bioassays. It is thus crucial to understanding the morphology, behaviour, reproduction and life cycle of both test organisms.

1.3.3.1 Daphnia magna

Daphnia magna is a crustacean commonly found in freshwaters (Dodson & Hanazato, 1995), and the genus normally comprise an important component as zooplankton to the foodweb.

1.3.3.1.1 History of *Daphnia* spp. in Bioassay Use

Daphnia spp. are routinely used as a bioassay organism. They are highly sensitive to toxic substances, are ecologically-relevant, and are relatively easy to culture (Nebeker *et al*., 1986; Giesy, & Hoke, 1989). The history of *Daphnia* spp. as a bioassay organism began with Warren (1900) testing concentrations of sodium chloride. Since then a plethora of bioassays using *Daphnia* spp. have been performed. *Daphnia* spp. are planktonic crustaceans, the genus contains more than 100 species (Ebert, 2004).

1.3.3.1.2 Environmental Relevancy

Dodson & Hanazato (1995) referred to this zooplankton as "trophodynamic" in order to express the important role *Daphnia* spp. play by being a primary consumer and key prey for high trophic levels. This dynamic makes them essential in the ecosystem for energy and nutrient transport from autotrophs such as algae to the larger predators. This dual importance was also emphasized by Luecke *et al*. (1992) when regarding macroinvertebrates for controlling clarity (by algae consumption) and prey for fish populations. Environment Canada (2016) and the USEPA (2002) in their protocols for bioassays also noted the importance of *Daphnia* spp. in the aquatic food webs. Figure 1 demonstrates an ideal food chain in a freshwater aquatic environment, and *Daphnia* spp. are influential (Dodson & Hanazato, 1995).

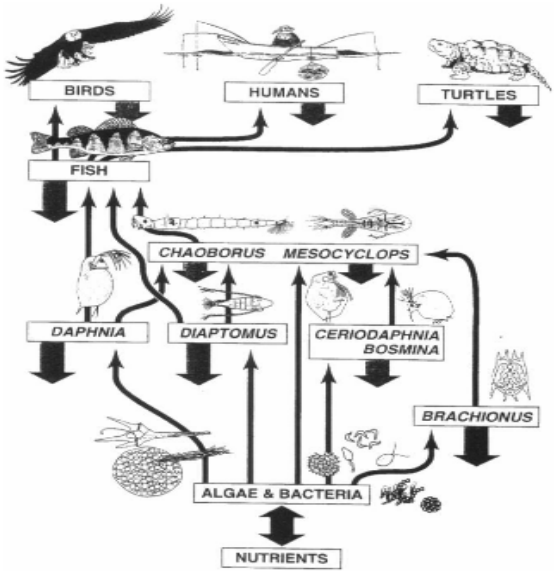

Figure 1 An Idealized Freshwater Food Web (Dodson & Hanazato 1995)

Daphnia magna are non-native to the Great Lakes, and instead, another species *Daphnia plexus*, dominates this ecosystem watershed. However, bioassay protocols (USEPA, 2002) assert that *Daphnia magna* can be used as analogous to *Daphnia plexus*. This is due to their shared sensitivity to toxins and the ease of culturing *Daphnia magna* over *Daphnia plexus*.

1.3.3.1.3 Morphology and Lifecycle

A deep understanding of morphology and lifecycle is crucial for properly assessing impact of microplastics to *Daphnia* spp. *D. magna* grow continuously throughout their lifetime. The species *D. magna* are relatively large in comparison to their genus, ranging between 0.5- and 5-mm. Figure 2, depicts an image of their morphology.

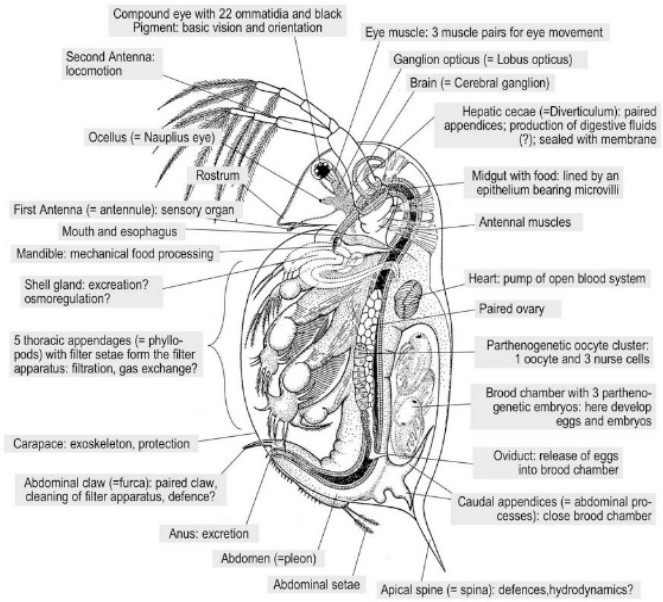

Figure 2 Functional Anatomy of a *Daphnia magna* (Ebert 2004)

As members of the order Cladocera, they are leaf-shaped bodies, cloaked in a carapace. Their carapace covers their cephalothorax and body (Figure 2), and is composed of chitin and fortified with calcium. This carapace is hydrophobic, it is unwetted. Evolutionarily, this prevents algae and protozoa from growing on their carapace. Additionally, because of the hydrophobic properties, if in contact with the surface tension of still water, they could easily become stuck (Fryer, 1991). They shed their carapace every few days during a moult, allowing them to grow (Ebert, 2005).

As seen in Figure 2, above their head reaches out a pair of secondary antennae. These secondary antennae are swimming appendages, used to propel *Daphnia* spp. through the water column in search of food and away from predators. *D. magna* are in near constant motion, swimming. Their swimming pattern has been well documented as a saltatory style (Dodson & Hanazato, 1995). The jump-like motion occurs from strokes of their antennae propelling them through the water.

Daphnia spp. filter-feed on organic matter, using their thoracic legs to collect food (Figure 2). Fryer (1991) details the process, briefly, their biramous legs move, creating a current of water that passes over their setae. The setae collect algae electrostatically. Dodson specifies their setae act as filters (not sieves) (As seen in Covich & Thorp, 2009). Once collected algae is mixed with mucus creating a bolus. *Daphnia* spp. can accept or reject the bolus, but have little choice on the exact make-up of a bolus. *D. magna* can ingest bolus' up to 100 µm, but the average size is between 1-25 µm (Dodson, as seen in Covich & Thorp, 2009). Their gut is composed of three main sections: a foregut, midgut, and hind gut. The fore-and hind-gut are lined with epithelial cells, while the midgut is lined with microvilli and is the location of absorption (Fryer, 1991) *Daphnia magna'*s coloration is related to their diet, well fed animals have a stronger colour; if their diet is predominantly algae, they'll appear clear with a tint of yellow or green.

Under normal conditions, *Daphnia* spp. population will fluctuate cyclically throughout the year with low numbers in the winter, a steep increase in the spring with a population peak, and in the summer months stagnate population sizes, with a smaller second peak in population size in the autumn (USEPA, 2002). During optimal conditions *Daphnia* spp. reproduce parthenogenically (Dodson & Hanazato, 1995) this is depicted in Figure 3 They have four life stages: egg, juvenile, adolescence and adult. Adult females produce a clutch of eggs after every moult; the eggs are held in the brood chamber. Under optimal conditions these eggs hatch after 1 day, but remain in the brood chamber for development. After about 3 days, *D. magna* moult, releasing the neonates. The neonates then undergo 3-5 instars in which they grow rapidly through the juvenile stage. Within the first 5-10 days, (at 20°C) *Daphnia magna* typically produce their first eggs, repeating

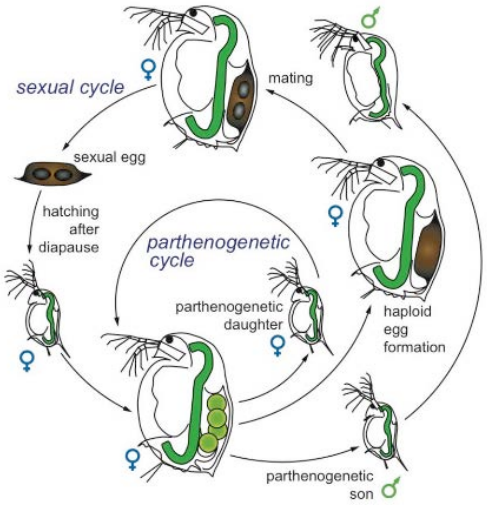

Figure 3 Lifecycle of a Cyclic Parthenogenetic *Daphnia* spp. (Ebert 2005)

the process approximately every 3-4 days until death (Pennak, 1989; Dodson & Hanazato, 1995; Ebert 2005).

1.4 Assessing Impact: The Importance of Sublethal Endpoints

The objectives of this thesis specify that *Daphnia magna* culturing protocols will be reviewed and applied, that sub-lethal endpoints will be refined, and toxicity impact will be assessed. The following section will outline the importance of behaviour as an endpoint.

From an ecotoxicology standpoint, toxicity assays should be conducted for both short-(acute) and long- (chronic) term assessments to properly understand the impact a contaminant is having on the organism. Traditionally, lethality has been used as the endpoint for environmental assessments. However, sublethal endpoints have been lauded as early-warning signs of distress (Hellou, 2011). One sub-lethal endpoint is behaviour. Observing behavioural disturbances in zooplankton could provide useful early informations for preventing more serious problems and maintaining the health of an ecosystem (Døving, 1995 [as seen in Dell'Omo, 2002]). Behaviour is defined by Dell'Omo (2002) as the cumulative interactions of biotic and abiotic factors that represent an animal's response to factors (both internal and external) and relates one organism to another. The study of behaviour is the junction of three separate disciplines: ethology (the study of behaviour), ecology (the study of relationships between animals and their environment), and toxicology (the study of toxic agents) (Dell'Omo, 2002).

1.4.1 Refining Sub-Lethal Bioassay Endpoints

Sublethal endpoints are important as they act as early warning signs for the health of a population and the overall ecosystem (Hellou, 2011). In ecotoxicology, these endpoints can vary from species to species, but generally include development through life stages, reproductive impairment, growth, and behaviour. Kane *et al.* (2005) notes behaviour represents an integrated whole-organism approach. Additionally, behaviour links the physiological and ecological factors of an organism and its environment, which is unique as an endpoint (Kane *et al*., 2005). Observing behavioural disturbances in

zooplankton could provide useful information for preventing more serious problems and maintaining the health of an ecosystem (Døving, 1995 [as seen in Dell'Omo, 2002]).

1.4.1.1 Sub-Lethal Endpoint: Behaviour

Daphnia magna behaviour can be examined through swimming patterns. This thesis would be impossible without building on previous knowledge. Research from Dodson *et al. (*1995), Dodson & Hanazato (1995), Fryer (1991), Schmidt *et al. (*2005) and Szulkin *et al*. (2006) submitted detailed work on *Daphnia* spp. behaviour for use in assessments. In the McCarthy lab, this was further detailed by Marshall (2009) and again discerned by Raby (2013). In line with their work, the three behavioural endpoints have been refined and were applied to bioassays. They are (I) Mobility, (II) Movement through the water column, and (III) swimming style.

1.4.1.1.1 Mobility

Daphnia spp. are typically mobile; and under stressed conditions they have been observed to be immobile either on the bottom of the vessel or caught in the surface tension at the water level. Mobility was considered the first sign of distressed behaviour as it is paramount to the other two patterns (if *Daphnia* spp. are immobile there should be no movement throughout the water column, nor swimming style). Mobility is so crucial to *Daphnia* spp. the OECD has an entire protocol developed with immobility as the toxicity endpoint (OECD, 2004). To measure mobility, vessels were checked daily for 30 seconds, and free swimming daphnids were noted.

1.4.1.1.2 Movement Though the Water Column: Boundary Crossing

As filter feeders, *Daphnia* spp. typically move throughout the waters in search of food particles. Their main source of food is microalgae, and thus, daphnids will spend time in the upper quadrant of the water column as light penetrates the water allowing for high levels of photosynthesis (this is where microalgae predominate). With increased light also comes increased visibility; therefore, to avoid predation, *Daphnia* spp. have been known to move back down through the water column (Dodson & Hanazato, 1995). The result of these two behaviours is a swimming pattern of moving throughout the water column. Schmidt *et al*. (2005) also examined swimming depth of *Daphnia* spp. through the water column (test vessels were 60 mm in depth), but their work did not conclude specifics on normal displacement (mm)/time of daphnids, merely that some degree of

displacement was normal. Dodson *et al*. (1995) noted that different clonal *Daphnia* spp. will have different swimming speeds. *Daphnia* spp. used in these experiments are from the same clonal grandmother, and movement through the water column will be compare with reference conditions. Test vessels had been prepared by marking three equal sized ranges onto the test vessel (at 100 mL filled the water column was 4 cm in height, each range had a depth of 1.33 cm); daphnids' movement through the water column was checked daily for 30 seconds, during which time the displacement of daphnids from one boundary to another was summed.

1.4.1.1.3 Swimming style: Body Score/Orientation

Daphnia magna swimming style is usually a saltatory motion. CO'Keefe *et al.* *(*1998) noticed that under normal laboratory conditions, two distinct patterns and dubbed them "hop-and-sink" or "zooming". The "hop-and-sink" motion consists of a power stroke from their secondary antennae propelling them upwards; this is then followed by a very brief stationary moment where, due to gravity they sink slightly. Fryer's (1991) depiction of these two motions are depicted in Figure 4, with the "hop" or "power-stroke" described as a the working oars, and the "sink" also described as resting. The second swimming style "zooming" is describes as rapid flicks of these antennae with no resting moment in between for quick movements. These periods of zooming are often brief. The combined action of these swimming styles would amount to a swimming pace of between 10 – 15 mm/s (CO'Keefe *et al*., 1998). Movement through the water column should be compared to a healthy reference condition.

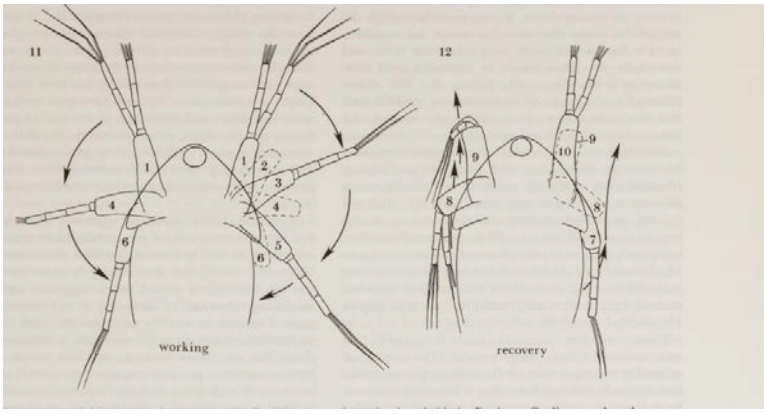

Figure 4 Daphnia spp. Swimming Anatomy (As seen in Fryer 1991)

To assess swimming style, animals were evaluated in the vessel for 30 seconds and each *daphnid* was scored. The scores for each animal was then averaged and an overall swimming style was recorded for the vessel. To account for sensitivity of *D. magna*, the average score was rounded down. This system was heavily based on Raby (2013):

- Score 0: The animal's swimming style was no different than reference conditions. The *D. magna* were swimming, in upright positions, and movements were combinations of "hop-and-sink" and "zooming". Feeding off the bottom was not indicative of stress; thus, animals exhibiting this behaviour were also given a score of 0.

- Score 1: The animal's swimming pattern is "slightly erratic". *D. magna* were swimming, but body orientation was no longer upright; instead, animals were horizontal or upside down. This orientation did not appear to be for the purposes of feeding at the bottom of the vessel, but the result of erratic movements. A score of 1 was also assigned if the animals were continuously zooming for the 30 seconds (as this is unsustainable).

- Score 2: The animals are showing "highly erratic" swimming patterns. If the *D. magna* were swimming, this motion appeared uncontrolled, with twirling, somersaulting, or twitching. These turbulent types of movements in brief moments were described as a normal response to escape predators, but prolonged twirling preceded death from exhaustion (Dodson *et al*., 1995) Immobility was also considered high stress behaviour.

1.4.1.2 Sub-Lethal Endpoint: Reproduction

Another sub-lethal endpoint often applied to *Daphnia* spp. is measuring reproductive impairment. The OECD have published guidelines for a 21-day reproductive impairment bioassay with *Daphnia* spp. (OECD, 2008). This thesis follows these protocols with modifications. Time of first brood, number of broods, and average number

of viable neonates have been used in previous experiments as a sub-lethal bioassay endpoint (See introduction, 1.3.3.1). During a long-term experiment (12-days), daphnids were checked daily for neonates, if any were produced, they were recorded and removed. *D. magna* under normal conditions will reproduce parthenogenically, with most of their time through asexual reproduction. *D. magna cultured* here produced their first brood at about 10 days, with about 6 neonates in the first brood, and then ever 3 days afterwards producing 8-30 neonates.

1.5 The Use of an Organic Contaminant, Triclocarban

This thesis focuses on microplastic impact. However, to determine if this stressor is a chemical or physical threat, an organic contaminant was added as a positive toxicant. Rochman (2015) emphasizes that plastic debris can adsorb organic contaminants during manufacturing and from their environment, additionally Teuten *et al.* (2009) suggested plastics may be chemically toxic themselves. As a physical contaminant it is also possible to cause physical harm such as a blockage or a false sense of satiation (Teuten *et al.*, 2009).

For this study, triclocarban was used as the positive toxicant. triclocarban (3, 4, 4'trichlorocarbanilide; *N*-(4-chlorophenyl)-*N'*-(3,4-dichlorophenyl)-urea, abbreviated TCC, was once a very popular antimicrobial used in many personal care products, including soaps, shampoos, and some creams (Halden & Paull, 2005). In 2005, nearly 80% of antimicrobial bar soaps sold in United States contained triclocarban (Orsi *et al*., 2011). The Food and Drug Administration in 2016 issued a ban on triclocarban, finding it no more effective than soap and water, and manufacturers were given one year to remove it from their products entirely. TCC is insoluble in water, and has a moderate K_{ow} and thus persists through wastewater treatment plants, and persists in the environment (Snyder *et al*., 2011). Previous research found *Daphnia magna* reproductive impairment occurred at 10 µg/L (Raby, 2013)

2 Methods

2.1 Overview

One of the major objectives of this thesis is to review, experiment, implement and ultimately improve upon existing protocols for culturing and assessing sub-lethal endpoints for *D. magna.* It is thus crucial to have properly tested reference conditions. Additionally, when performing the microbead assay properly understood reference conditions ensures any extraneous factors can be excluded. This research followed protocols that have been outlined by Environment Canada for culturing and toxicity testing using *Daphnia magna* with minor modifications. Environment Canada bases their toxicology protocol heavily on the OECD, the USEPA, which, in turn, are guided by scientific research. These protocols provide specific details in order to mitigate the chance of erroneous results due to too many irrelevant factors. This should increase data repeatability, reproducibility and ultimately knowledge generation. Dr. McCarthy's laboratory has been following and revising these protocols since her time at Ryerson, with great success (Spearin, 2003; Fleet, 2010; Gebert, 2010; Doobay, 2011; Tiley, 2012; Raby, 2013; Puddephatt, 2013, Fernandes, 2015).

To distinguish if microplastics cause impact, and whether this impact is due to a chemical or physical threat (or both), 4 treatments were set up for the bioassays using *D. magna.* These treatments were: (I) a treatment with pristine microplastics; (II) a treatment with microplastics that have been exposed to an organic contaminant (triclocarban); and then two controls, (III) a treatment with the organic contaminant; (IV) a reference treatment with no added microbeads and no organic contaminant. Solvent controls were also run as triclocarban was first dissolved in dimethyl sulfoxide (DMSO); and the microbeads were dissolved in a 0.1% Tween-20-20 solution.

2.2 Microscopy

To ensure bioavailability of the contaminant, organisms were examined with a compound light microscope (Leica DM500), and micrographs were taken with Leica image software. Organisms were removed from their experimental vessel with a pipette

and gently placed on a flat microscopic slide. Enough water was transferred onto the slide for the organism's comfort, but not enough for the organism to move freely.

The microscope has two lenses, the ocular lens magnifies 10x, and the objective lens magnifies at 4x, 10x, 100x. As the lenses work in combination, the total magnification is the product of the magnification of the objective and ocular lenses. Before viewing an organism, two slides were prepared for reference: one containing only microbeads, and one containing only a water droplet. To prepare the microbead-alone slide, 1 µL of microbead solution was pipetted onto a slide, and a coverslip was applied to spread the microbeads evenly out on the slide. The slide was placed on the stage, clipped into place, centered, and the coarse adjustment moved all the way up, with the 4x ocular lens in place. Images of the microbeads were taken using a 4x objective and a 10x ocular lens, for a total magnification of 40x. With young *D. magna,* as they are small, the 10x objective lens was used (100x total magnification), this strategy required only fine adjustments between specimens. However, as they daphnids matured and grew, it was necessary to switch to the to the 4x objective (total 40x) to get detailed images. Magnification in the results is always listed as total magnification.

2.3 General Bioassay Preparations

Before setting up any bioassays, *D. magna* needed to be cultured and age synchronized. Several abiotic and biotic factors needed to be addressed. As a guide the flowchart (Figure 5) was created documenting the process accomplished in Ryerson-Lab. The boxes correspond to several different procedures under over-arching headings and should correspond with subheadings and details in the following sections: "Abiotic Bioassay Conditions" is detailed in 2.4, and "Biotic Bioassay Conditions" is detailed in section 2.5 and 2.6; "Ryerson-Lab Pilot Study" is further detailed in section 3.4.

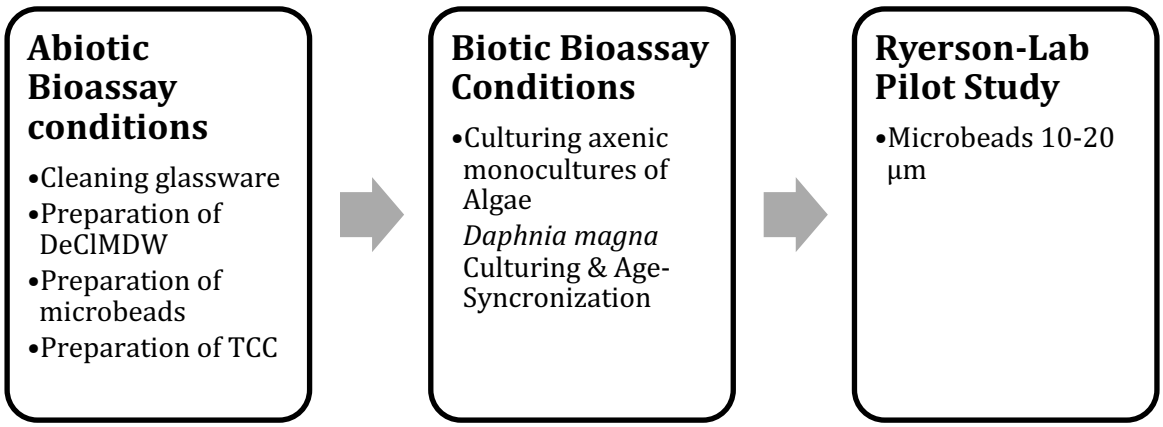

Figure 5 Procedure of Methods for Ryerson-Lab Experiments

2.4 Abiotic Bioassay Conditions

Several abiotic factors were measurencend and maintained including temperature, light intensity, dissolved oxygen, and appropriate pH. To maintain consistent population size, it is important to keep several abiotic conditions constant, because these organisms have population cycling (see Introduction, section 1.3.3.1). The temperature was maintained at 20 ± 2°C, as prescribed in Environment Canada protocol (2016). Temperature was measured using a thermometer and recorded twice daily during the experiments (once in the morning and once in the late afternoon) in order to monitor possible variation throughout the day. Vessels containing organisms were placed on the tabletop, with two nearby windows. A photometer (Field Scout Light Meter) was used to measure light intensity once during the day. Cultures were placed under cool white fluorescent lights at an intensity of 8-12 umols/m^2/s (approximately 500-800 lux). Lights automatically turn on for 16 hours (representing days) and turn off for 8 hours (representing nights). Dissolved oxygen was kept between 60-100%; this was accomplished by pumping oxygen into the vessels using a bubbler, tubing, and a glass Pasteur pipette (for small vessels) or a 1 mL pipette (for larger vessels). Dissolved

oxygen was measured with a dissolved oxygen meter, and concentrations were recorded once weekly (assuming the bubblers were continuously working.)

Several preliminary steps were taken prior to setting up live organism cultures, including cleaning glassware, and preparing dechlorinated water; these preliminary procedures were important as they eliminated potential contamination from other sources. (The outline of these procedures are also listed in Figure 5)

2.4.1 Cleaning Glassware

To eliminate contamination and thus assure that experimental results are conclusive, glassware was cleaned using a procedure based on Puddephatt, (2013), and modified by Fernandes (2015), both based on Environment Canada (1990). First, a non-phosphate detergent (Extran, in powder form) was mixed with tap water to a concentration of 2% w/v, in a large container. Then, glassware was completely submerged into the soapy solution for a minimum of 30 minutes. Subsequently, glassware was scrubbed and rinsed with tap water. Next, the glassware was rinsed with hydrochloric acid (HCl) 10% v/v three times; this step ensures removal of trace or heavy metals, and calcium build-up (from the tap water rinsing). Then, the glassware was rinsed with deionized water three times to ensure that HCl is properly rinsed out. Lastly, the glassware was left in an inverted position to air dry.

2.4.2 Preparation of Dechlorinated Municipal Drinking Water (DeClMDW)

Municipal drinking water in Ontario is treated for human consumption using chlorine. The water pipes at Ryerson campus potentially release copper into the water. The combined water is unsuitable for bioassay organisms, and needs to be filtered. Thus, a filtration system was used to ensure the water is sufficiently safe for the aquatic organisms (Personal Communications Dr. Jorge Loyo, 2013).

A filter system (after Raby, 2013) was set up using two 20 L Nalgene carboys. Both carboys were covered in a thick dark tarp to block any sunlight from entering the containers and prevent unwanted algal proliferation and contamination. The first carboy contained municipal drinking water, fitted with a 10 mL pipette attached to tubing

leading into a filter atop the second carboy. Water moved from the first carboy through the pipette and tubing through capillary action into the filter. The filter was made up of a few layers for different functions. The first layer was granular activated carbon (approximately 125 cm^3), which adsorbed heavy metals. The second and third layer were sand and cotton gauze and these layers were to catch any additional particulates. Water would then pass through a Brita filter. Once the water was filtered, it was then oxygenated with a pipette and bubbled for at least 24 hours. Bubbling ensures the chlorine will volatize off and that the water will have high dissolved oxygen concentrations.

The filtration system was cleaned every 6 months, or when water clarity was affected. To clean the system both carboys were emptied, and filled with soapy water (Extran, 2% w/v), and shaken vigorously. The carboys were then rinsed with dechlorinated water, then acetone (acetone disrupts biofilms), and finally with deionized water. Tubing was examined and replaced, if needed. The filter was also be replaced.

Additionally, in order to conduct these four treatments, pristine plastic microbeads were purchased, and then treated with a Tween solution. Triclocarban stock solution was prepared in laboratory in a stock solution with dimethyl sulfoxide (DMSO).

2.4.3 Preparation of Microbeads

The microbeads used during this experiment were fluorescent green microbeads purchased from Cospheric (USA, California). They were spherical in shape (two sizes were used over the course of this project: (i) 10-20 µm, with a density of 1.026 g/cm^3; and (ii) 20-27 µm, with a density of 1.025 g/cm^3). The fluorosphore of the microbeads is surrounded in pure polyethylene, and the surface is smooth, and pristine (Cospheric correspondence, 2019). The microbeads are hydrophobic and thus needed to be coated with a surfactant in order to prepare a stock solution. Microbeads should be prepared in a 0.1% Tween-20-20 solution at a concentration of 5:1 v/v. (The 0.1% Tween-20-20, is abbreviated in the results as simple Tween solution.) To create the 0.1% Tween-20-20, 1 mL of Tween-20 was combined in 1 L of deionized water, and vigorously shaken until

completely mixed. This 0.1% Tween-20 was then used as a solvent to create a stock solution of microbeads, at a volumetric ratio of 5:1 Tween to microbeads.

2.4.4 Preparation of Triclocarban

Triclocarban (3, 4, 4'trichlorocarbanilide; *N*-(4-chlorophenyl)-*N'*-(3,4-dichlorophenyl)-urea (TCC) solution was prepared as follows: 0.1033 g of TCC was added to 101 mL of DMSO, resulting in a stock solution concentration of 1.023 mg/mL (Raby, 2013), and stored at room temperature. A "dilution 1" was prepared by combining 1 mL of the TCC/DMSO stock solution with 1 L dechlorinated municipal drinking water (the concentration of dilution 1: was 1.023 mg/L). Then, this dilution 1 was used to create final concentrations of 5 and 10 ppb (5 and 10 µg/L) for respective toxicity assays.

2.5 Biotic Bioassay Conditions

The outline of biotic conditions is depicted in Figure 10. Prior to setting up *D. magna* cultures, algae were first purchased and cultured as a food source. Two cultures of algae were prepared in individual batch cultures, as recommended by Environment Canada (2000; 2016). The algae selected were *Raphidocelis subcaptitata* (formally known as *Pseudokirschneriella subcapitata,* and previously, *Selenastrum capricornutum)* and *Chlorella fusca*. *Raphidocelis* is a crescent-shaped microalgae 8-14 µm in length. *Chlorella* is also a microalgae, spherical in shape, and between 2-10 µm. Both cultures were ordered online from Boreal Science (formally Ward's Science),and arrived in test tube Bristol agar slants. Upon receiving the slants, the instructions were to keep the tubes upright, unseal the cap (not remove), and it was recommended to use them immediately. The slants were kept in sealed containers in a refrigerator (between 2–4°C) for up to 1 year. These slants were used as the initial source for culturing.

To culture algae, a nutrient rich broth was prepared. Bold-Basal medium (Appendix, 6.1) is based on Bristol-Roach's (1928) recipe, which included macro- and micro-nutrients in dechlorinated water. Bold (1942) removed the additional glucose and grew algae under high light conditions; glucose can be removed as algae are photosynthetic and will produce their own sugars in the presence of light. This recipe is

high in nutrients, simulating eutrophication, which allows the algae to grow quickly, thus providing a large enough algal food source. More information on the modified Bold-Basal medium can be found in the Appendix (section 6.1).

Batches of algae were grown in 1 L monocultures, in 2 L glass vessels. They were placed on a shaker, under high fluorescent lights. The first batch of algae grew in typical lag-log growth format; *Raphidocelis* was at a sufficiently high concentration and dark green in color by 20 days; *Chlorella* took about 22 days and the medium also appeared dark green. The vessels were then sealed firmly, removed from the shaker and placed in a refrigerator for at least 24 hours, or until all the algae had sunk to the bottom, and the broth overlay. Next, the vessels were decanted using aseptic technique, removing most of the overlaying broth (50-60% of the contents). The remaining contents were swirled and decanted into an amber colour glass bottle. (Storage bottles were previously autoclaved, and if there were no amber ones available, the bottle was covered in aluminum foil). Stock algae could be stored or used immediately. Stored stock algae could be stored for up to 2 months, otherwise a fresh stock solution was again set up from the initial slants. Subsequent batches of algae were cultured by inoculating the Bold-Basal medium with 10 mL from the previous batch instead of from the slants as this was found to be more efficient (approximately 10 and 12 days for *Raphidocelis* and *Chlorella*, respectively).

To ensure each bioassay received the same amount of algae, the stock algae concentration was checked three times using a hemocytometer with a light microscope. As this step examines the algae under a microscope, stock algae were also checked to ensure sterility. If an algae stock solution was found to be contaminated, the solution was discarded and a new broth was immediately prepared. The hemocytometer was first washed with ethanol and wiped with Kimwipes, a fresh coverlip was added to the hemocytometer, and 1 mL of algae from the stock solution was carefully loaded into hemocytometer. Average cells counts were taken for 5 squares, counting the top and left borders. Concentrated algae were then added to *D. magna* with their water in "*Daphnia* growth medium" as listed, based on Raby (2013). *Daphnia* growth medium consisted of 50 mL of algae (equal parts of the two monocultures, each at a concentration of 10^6

cells/mL), 1 mL of B12, 1 mL of Selenium, topped up to 1 L with dechlorinated municipal drinking water. Additional notes on supplemental vitamins and Bold-Basal recipe can be found in the appendix (sections 6.2)

2.6 *Daphnia magna* Culturing and Age-Synchronization

Three types of zooplankton cultures were established and maintained: (i) a mass mixed culture, (ii) a brood stock, and (iii) age-synchronized *D. magna* for the experiment, each of these will be described in detail below. Raby (2013) depicted the various cultures using a flowchart (Figure 6), this depiction was very useful for understanding the role of each culture. This has been recreated in Figure 6 (types of cultures have been bolded). The source of *D. magna* for each culture and demonstrating the flow of setting up cultures. The cultures were maintained in glass vessels.

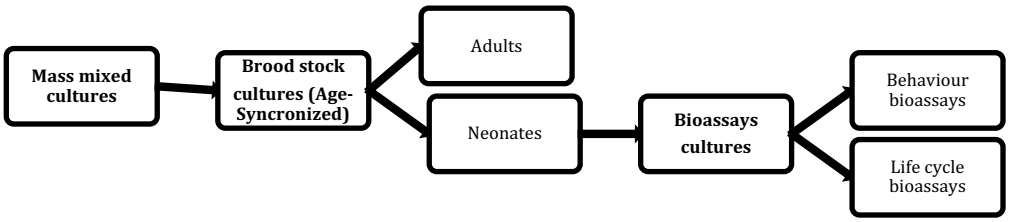

Figure 6 *Daphnia magna* Culturing Flowchart (adapted from Raby (2013)).

2.6.1 Developing a New Experimentally Suitable Population from an Undefined, Pre-Existing Population

Originally in the laboratory, there were two mass mixed cultures in 38-L tanks. These aquaria contained dark green waters (coloration was a combination of different algae growing), had a thick layer of sediment, and unknown but presumed low number of animals. The tanks were not being maintained, and in order not to disrupt their population dynamics, it was decided to set up two additional mass cultures of the same size. These two new mass cultures contained *Daphnia magna* and the amphipod *Hyalella azteca* of mixed-age dimensions.

2.6.1.1 Mass Mixed-Cultures

Two mass mixed cultures of *Daphnia magna* and *Hyalella azteca* of mixed-age organisms were set up in in 38 L tanks (aquaria) by preparing the tanks prior to adding any animals. Each tank contained 3 cotton gauze squares (5 x 5 cm, 3-layers, presoaked to ensure they would sink), 30 L of dechlorinated municipal drinking water, 4 L of water from the original tanks, 25 mL of *Raphidocelis* (at 10^6 cells/mL), 25 mL of *Chlorella* (at 10^6 cells/mL) and 1 g of finely ground Tetramin. If the cotton gauze floated to the surface it was pushed down using a pair of tongs, until it sunk to the bottom. Each aquarium was fitted with a Plexiglas lid, and bubbler, connected with tubing and a 1 mL pipette. These tanks were left for 24 hours. Then, 20 *D. magna* and 40 *H. azteca* were added to the mass cultures from the original aquaria.

Mass cultures were tended weekly by adding 1 g of ground Tetramin, until the algae population was observed to be in high enough concentration, upon visual inspection, so as to maintain the diet of the zooplankton. On the third week, the cotton gauze was replaced; this was done by removing the gauze, rinsing with dechlorinated water, and shaking off all the animals, and a fresh cotton gauze was then placed in the mass culture tanks. Animals that were shaken off the old cotton gauze were returned to their tanks. After 6 weeks, the *Hyalella* population was flourishing; thus, the cotton gauze was removed and not replaced.

After the initial 2-4 weeks, maintaining the cultures on a weekly basis consisted of monitoring for controlling population size. If the zooplankton population size was not controlled, the population would grow exponentially, peak, and then crash. This transition was notable not only in *D. magna* numbers, but also in water clarity; when the *D. magna* peaked, their feeding cleared the water, upon a population crash, the algae would immediately build up. To thin a population a mesh screen was dipped into the water, and run along the surface in several smooth motions, collecting animals on the screen. The screen was immediately dipped into another aquarium, and animals could swim off, effectively transferring into a secondary aquarium.

Mass culture aquaria were cleaned once-monthly by siphoning off one quarter of the water, wiping down the walls of the aquaria using a brown paper towel, and restocking with fresh dechlorinated municipal drinking water. Similar procedures have been suggested in Environment Canada (2000), Fleet (2010) and Raby (2013); however, these methods suggest a shorter cleaning interval, but this was found to be unnecessary and the populations flourished with less handling. During this cleaning, a 2 L pitcher was used to scoop water from the aquaria and gently poured over dual-layered mesh screen. The mesh screens were held on top of a large water-collecting beaker. This water would be checked for any animals, before being discarded down the drain. The mesh screens were identical to those used in Raby (2013), they separated adult and juvenile organisms based on size. This screen method was effective when handling or maintaining the mass culture, but a plastic finger pipette (2 mL, cut with a 5 mm opening) was found to be less invasive and easier when handling organisms for initiating and handling the brood stock.

2.6.1.2 Brood Stock Cultures and Age Synchronization

Several brood stock cultures would be set up prior to beginning any experiment (this depended on the number of neonates needed). Brood stock culture consisted of one adult daphnid, and her offspring. Brood stocks were held in 1 L glass vessels, containing 1 L of *Daphnia* growth medium, fitted with a bubbler from a glass Pasteur pipette.

To initiate a brood stock, one large *D. magna,* containing embryos in her brood chamber, was placed in the glass vessel. This vessel was then checked every 24 hours for neonates. Once the neonates were born, they were removed using a plastic pipette, and placed in individual identical culturing vessel marked with the date. These known-aged neonates, in their 1-L vessels, made up the brood stocks. The mother daphnid initially acquired from the mass culture was replaced back into the mass culture. Her neonates, now the age-synchonized brood stock, would grow and develop. Neonates from the brood stock were used in experiments, while the adults remained in their culture vessels, producing more neonates every few days. In this way, neonates used in the experiments all shared the same grandmother, and were from the same clonal line. To prevent buildup of waste material, half the contents of the vessels were replaced with fresh medium weekly.

A brood stock was considered healthy if they passed three criteria outlined by Environment Canada (2016). According to Environment Canada *Daphnia magna* protocol, there are 4 criteria: (I) no ephippia would be present in the brood stock; (II) neonates must be born within the first 12 days of starting a brood stock; (III) females 2-5 weeks old must produce at least 15 neonates per average brood; and (IV) no more than 25% of the brood stock could die. If one of these criteria were not met, then the animals pertaining to this brood stock would have been retired and a new culture would be set up to replace it. This ensured a vigorous, healthy clonal population, ready for use in rigorous, reproducible bioassay experiments.

2.6.1.3 Bioassay Cultures

Daphnia magna used in the bioassays were age-synchonized, neonates from the brood stock cultures. Vessels for the bioassay were set up in 250 mL beakers containing 100 mL of *Daphnia* growth medium, and fitted with a bubbler (glass Pasteur pipette) and lid, prior to the neonates being added. Vessels were marked with water height, and divided into three equal ranges. Care was taken to transfer as little as possible carry-over water, and the pipette tip was inserted under the water level to ensure neonates were in the water column and not trapped on the surface. Testing conditions were the same as general culturing, and specific measurements of light intensity, temperature, pH, and dissolved oxygen were taken at the beginning and end of each experiment.

2.7 Bioassay Endpoints

Environment Canada (2016) suggests using mortality as an endpoint, as defined by the cessation of movement, including the heartbeat as checked under a microscope. In the current study, this was used as one endpoint. However, sub-lethal endpoints are more sensitive, and with training can be performed efficiently. Thus, behaviour and reproductive endpoints were used in combination with mortality in short- (acute) and long- (chronic) term assessments.

2.8 Bioassay Design

Two types of studies were conducted: (1) a pilot, to check preliminary interactions of the microbeads with *D. magna* (2) a short- and long-term toxicity assay with low microbead doses, and concentrations of triclocarban.

2.8.1 Short-term Toxicity

A single concentration, 48-h acute toxicity test was set up according to Environment Canada (2016) protocols. This assay allowed for two end points: acute behaviour and acute mortality. Behaviour and mortality were checked at 10 minute, and 1, 6, 24, 48 hour intervals. *D. magna* were checked under a microscope at each of these time points except the 1-hour time point. After being examined with a microscope this daphnid would be retired, into a retirement aquarium. Environment Canada (2016) outlines that a minimum of 10 *D. magna* divided among three replicates are a minimum.

2.8.2 Long-term Toxicity

Daphnia magna lifecycle toxicity tests were based on a modified OECD (2008) protocol. This assay will allow for chronic behaviour assessment, chronic mortality, and reproductive endpoints. Toxicity bioassays were conducted for 21 days, measuring lethality, behaviour and reproductive end points. Reproduction was monitored daily, by checking vessels for neonates. Mortality and behaviour were the same end points as in the short-term assays; they were also assessed at the same time intervals (10 minutes, and 1, 6, 24, 48 hours) and every day thereafter. Here too, 1 daphnid from each treatment was sacrificed, for examination under the microscope at 10 minute, 6 hour, 24 h, and 48 hs, and on day 5, 7, 9,11, 13, 15, 17, and 19.

Vessels were set up and left largely untouched (except during short assessment moments) for the first 6 days. After their assessment on the 6th day and every second day thereafter, the contents were refreshed via a modified static renewal procedure. Half of the contents would be slowly pipetted out (~50 mL), and then 50 mL of fresh media would be slowly pipetted into the vessel. A slow displacement of the media and renewal ensured *D. magna* did not die due to shock as a result to osmotic concnetration change, nor to waste build up in the vessel.

3 Necessary Amendments to the Methods:

During the unfolding of this project, a global pandemic ensued known as COVID-19. As this was a novel virus, many countries were unprepared and issued quarantines, lockdowns, and other public health measures to slow the spread of the virus. Canada was no exception to these public health measure and Ryerson University officially cancelled classes on March 12th. Shortly thereafter, for the health and safety of students and staff, laboratory access was switched to "essential needs" only.

3.1 Relocating the Lab from Ryerson-Lab to Home-Lab

In order to continue work for this project, and in adhering to objective 1 an at Home-Lab was established in my family home's guest bedroom. On June 4th lead by Dr. McCarthy, a team (including Dr. Hausner, Dr. McCarthy's daughter Julianna, and myself) collected necessary materials from the Ryerson lab to set up this new lab space nicknamed "The Home-Lab" . The Home-lab was located in a guest bedroom, which had been reorganized to accommodate a large desk, on which the aquarium and other test vessels would be placed, Figure 8 is an image of the room before, and after moving into this new lab setting. After this initial trip, biweekly trips into the Ryerson-Lab were also needed for necessary restocking and cleaning. Attempts were made to keep experiments as scientifically-rigorous as possible, however some alterations were necessary, and they are highlighted here.

(A)

(B)

Figure 7 Arrangement of the Home-Lab (Redecorated guest-bedroom)

3.2 General Home-Lab Set up

Once in the Home-Lab procedures leading up to toxicity assays needed to be restarted. Figure 8 has been provided as a guide of the workload undertaken. The boxes group together similar sets of tasks; “Relocating the lab” has further details in section 3.1, “General Home-Lab Set up” is detailed in section 3.2, “Home Pilot Study” is specified in 3.5, and the respective toxicity assays are detailed in sections 3.6 (Toxicity Assay: Microbeads and TCC) and 3.6.1 (Toxicity Assay Microbeads and lower TCC dose)

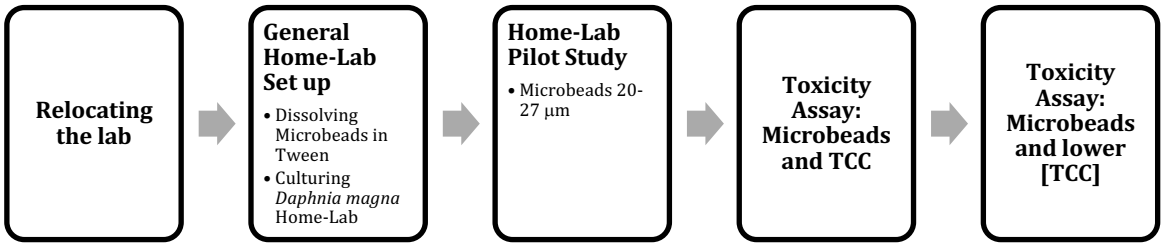

Figure 8 Procedure for Methods for Home-Lab Experiments

In order to conduct the four treatments, the chemicals for each treatment were considered for their safety; MSDS sheets, and internet safety procedure were assessed and the chemicals were deemed "safe for use". Tween was considered safe for handling and storage in a home setting. The 0.1% Tween-20 stock solution was prepared in lab and 1 L was brought to the Home-Lab. Likewise, the stock solution for triclocarban in DMSO was also deemed safe and brought home setting. A small vial of DMSO was also brought to the Home-Lab.

3.2.1 Dissolving the Microbeads in Tween

The microbeads (in powder form) were hand delivered to the Home-Lab by Dr. Hausner and combined in 0.1% Tween-20 solution in this Home-Lab. Personal protective equipment (a mask, gloves, and protective eyewear) was used when combining the microbeads with the Tween solution in order to eliminate the possibility of inhalation. Initially, 3 mL of Tween was combined with 0.5 g of microbeads and shaken vigorously, as per manufacturer's instructions (Section 2.4.3). However, after 4 days the beads separated out of the solution. The manufacturer recommended that due to the high high

surface area: volume ratio, these microbeads would need a higher volume of Tween to dissolve completely. The stock solution was then topped up with 0.1% Tween-20 to 20 mL, and shaken gently until all contents were completely combined. The manufacturing website suggested centrifuging for 5 minutes to combine, but this was considered ineffective since centrifuged solutions separate based on density. A vortex proved to be too powerful, and it was more effective to gently shake the vial until homogenous. Once the microbeads were combined, particles/mL were checked using a hemocytometer, and exposure concentrations were based on manufacturing details and particles/mL.

In terms of abiotic conditions, only temperature and light could be measured. As this was a family home, daily temperature remained consistent throughout the day. The room contained two windows, and the overhead light was left off; thus, cultures were illuminated with indirect natural light, vessels were also randomized on the desk-space. Temperature was measured once daily with a thermometer in the mass culture; light intensity was also measured at this time with the photometer. All four corners of the desk received similar amounts of sunlight. Glassware was brought to the Home-Lab clean, and would be brought back into the lab for cleaning. Dechlorinated municipal drinking water water was brought to the Home-Lab in a single 20-L carboy (filtered in lab) and would bubble for at least 24 hours before being used.

Monocultures of algae had been cultured, harvested, and counted in the Ryerson-Lab so that Home-Lab could refrigerate the bottles, and when needed consisted of opening the algae bottles around a gas stove top (for sterility) and pouring the necessary amounts into 1 L *Daphnia* growth media vessels.

3.3 Culturing *Daphnia magna*: Home-Lab

Similarly, to the Ryerson-Lab conditions, three types of cultures were set up (a mass mix culture, a brood stock, and the experimental culture). This was accomplished by draining a mass mixed culture from the Ryerson-Lab, and carrying the contents in ten lidded 1-L mason jars to the Home-Lab. Upon arriving at Home-Lab, seven of the mason jars were carefully poured into a 10 L aquarium, this was topped with a Plexiglas lid and fitted with a bubbler, thus setting up the mass mix culture. The other three 1-L mason jars

were also fitted with bubblers, but they were kept separate from the mass culture for a few days as a precaution against losing the population due to the stress from changing environments. Maintenance of mass mixed culture was the same procedure as at the Ryerson-Lab. Brood stock initiation and experimental cultures were the same procedures and maintenance as Ryerson-Lab practices. Figure 9 depicts the desk during an experiment, the mass culture can be seen on the left end of the desk, brood stocks are in 1-L mason jars, fitted with bubblers at the far end of the Table, and the experimental vessels take up the majority of the tabletop space.

The length of long-term was shortened (to better accommodate spacing and limited bubblers) from the original 21-day to a time till first brood: 12-day study. Water was still refreshed on day 6 and every second day thereafter.

Figure 9 Desk Configuration During Experiments

3.4 Specific Set Up for Ryerson-Lab Pilot Study (Microbeads 10 - 20 μm)

Three treatments were set up, each with three replicates (a total of 9 vessels); the first treatment contained neonate daphnids (aged <24 hs); the second treatment contained 2-day old daphnids; and the third treatment contained 7-day old daphnids. Each vessel contained microbeads at a concentration of 12.5 mg/L (fluorescent green polyethylene 10-20 μm, 1.026 g/cm^3, Cospheric (UVPMS-BG-1.026 10-20um - 0.1g), USA). *D. magna* were examined with a light microscope at four time intervals: 10-minutes, 1-hour, 24-hours and 48-hours after their initial exposure.

3.5 Specific Set up for Home-Lab Pilot Study (Microbeads 20 - 27 μm)

The microbeads from the 1st pilot study precipitated out of the Tween solution. Thus, a new batch of microbeads were purchased, but due to merchandising this product was no longer in stock, thus slightly different sizes (fluorescent green polyethylene 20-27 μm, 1.025 g/cm^3, Cospheric (UVPMS-BG-1.025 20-27um - 0.5g), USA). A second pilot study was deemed necessary to assess the same objectives but with these new microbeads (i.e. how will the microbeads interact with *D. magna*, and in *Daphnia* growth medium).

Two treatments were set up, a Reference (control) and a microbead exposure. Each treatment had three replicates, with 4 neonates in each vessel. The experiment was stopped after 48 hours, at which point micrographs of daphnids were captured. Vessels were checked for mortality and behaviour at 10 minutes; 1, 6, 24, and 48 hours.

3.5.1 Repetition of at Home-Lab Pilot Study (Microbeads 20 - 27 μm)

Three treatments (control, vehicle control, and Microbead treatment) had 5 replicates (with 4 *D. magna* per vessel). The experiment was stopped after 7 days. Micrographs were only taken during the experiment if neonates were born during the experiment and of deceased daphnids. This was done because once the daphnids was removed from the vessel, they retired, and no longer participated in the experiment.

3.6 Specifications for Toxicity Assay: Microbeads and TCC

For the Toxicity assays, 6 treatments were set up: (I) reference control, (II) vehicle control for 0.1% Tween-20 Solution, (III) vehicle control for DMSO, (IV) triclocarban at 10 ppb, (V) microbeads at 0.025 mg/L and (VI) triclocarban and microbeads. Each treatment had 5 replicates (with 4 *D. magna* per vessel). One daphnid was sacrificed from each treatment at various time intervals (10 minutes; 4, 24, and 48 hours); this sacrificed daphnid was removed from the test vessel and checked with a microscope and subsequently retired. The experiment was stopped after 6 days.

3.6.1 Toxicity Assay Microbeads and Lower TCC Dose

This experiment was carried out with the same treatments and experimental procedures as the previous toxicity assay. The only difference was a 5 ppb triclocarban test vessel. One daphnid was sacrificed from each treatment at various time intervals (10 minutes; 6, 24,48, 120, 144, and 264 hours); this sacrificed daphnid was removed from the test vessel and checked with a microscope and subsequently retired. The experiment was carried out for 12 days to allow for the production of the average first brood +2 days.

3.7 Statistical Analysis

The raw data was discrete, and therefore non-parametric in nature. First the data for each treatment was averaged in terms of median (considering the max and min values for each treatment) and mean (considering the standard deviation). The medians for each treatment were then charted in bar graphs to compare each treatment showing the endpoint for most replicates along with the max and min for each treatment. The Friedman test was employed to compare statistical differences between control and treatment results. Each time interval was a block interval, and the mean value for each treatment were ranked against (A. Laursen, Ryerson University Pers Comm, 2020).

4 Results and Discussion

The Results and Discussion section are divided into the 4 experiments that were conducted, and further divided by the type of data being presented. Each experiment had specific objectives, and their results paved the way for the next set of experiments. Figure 10 depicts the four experiment titles.

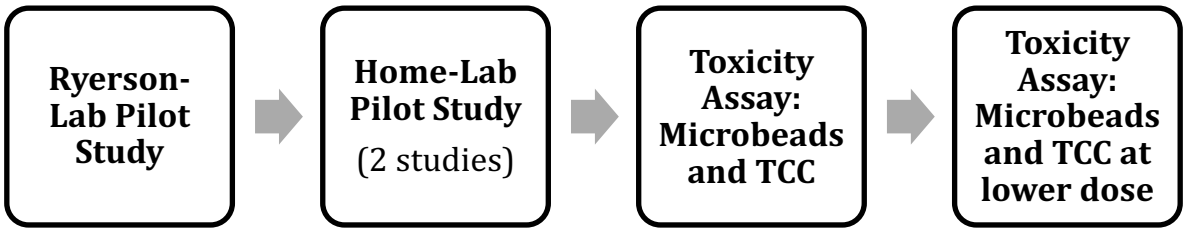

Figure 10 Flowchart of the Experimental Results

For these experiments various treatments were used, and for consistency they are listed here:

-Reference (a control for reference conditions)

-0.1% Tween-20, listed as Tween (a vehicle control)

-Dimethyl sulfoxide, listed as DMSO (a vehicle control)

-Microbeads

-Triclocarban, listed as Triclocarban and in figures as TCC

-Triclocarban & Microbeads, listed in as TCC & Microbeads

4.1 Ryerson-Lab Pilot Study (Microbeads 10-20 μm)

As the main objective during their initial experimentation was to examine microbead interaction in solution with *Daphnia magna,* only preliminary observations were made. The microbeads appeared in the water column and to the naked eye indistinguishable from any algae build-up. As there were no Reference in this Ryerson-Lab Pilot Study, no meaningful endpoint assessments could be drawn. However, these endpoints will be address in a Home-Lab Pilot Study to follow.

Microscopic images (Figure 11 and Figure 12) of neonates, 2-day old, and 7-day old daphnids show microbeads in and on daphnids; additionally, microbeads appeared in the gut, at every time point. The microbeads appearing inside the main body of daphnids are likely underneath the carapace and not inside the body cavity. This can be seen in Figure 11 image (A) where the microbeads are under the carapace near the gut; image (B) shows a separate neonate's gut is full of spherical masses, which were believed to be microbeads. The "lumps" in the gut in B could not at this time be ruled out as masses of spherical algae. Figure 12 depicts a neonate born during the experiment (from one of the 7-old daphnid after 24 hours of exposure to microbeads). The microbeads appeared to stick to daphnids' carapaces and could be seen underneath the carapace surrounding the body. Neonates appeared to "collect" the most, while the 7-day old appeared less encumbered by the microbeads.

4.1.1 Visual Observations of Microbeads and *D. magna*:

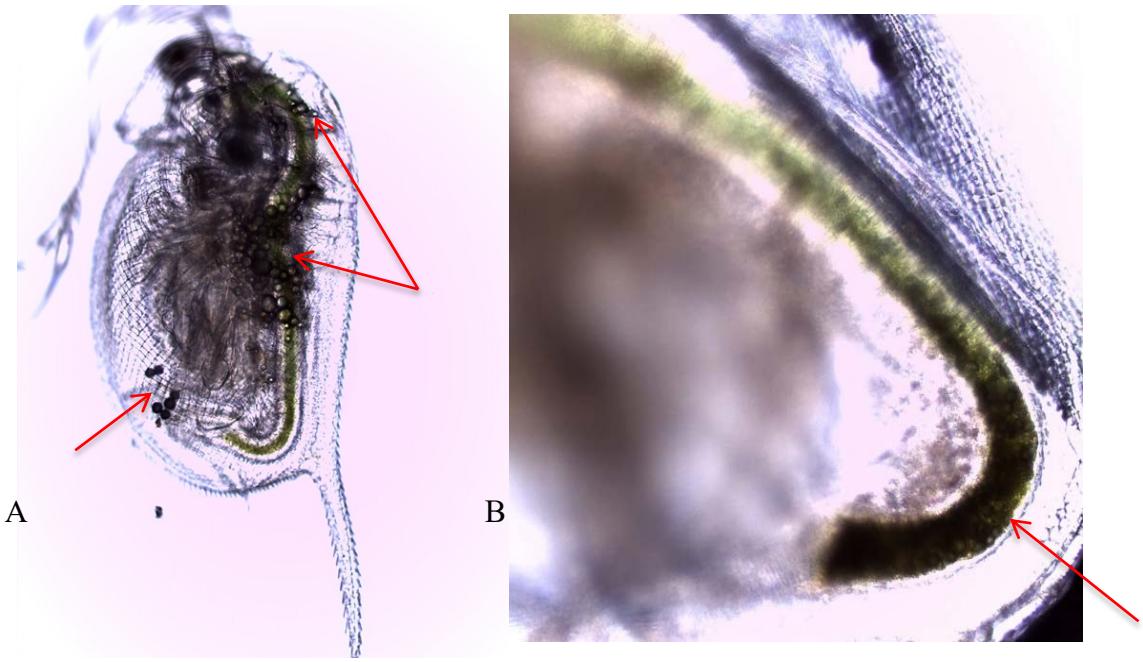

Figure 11 Ryerson-Lab Pilot Study (Microbeads 10 – 20 µm): *Daphnia magna* with microbeads after 10-minute exposure. (A) 40x Magnification;(B) 100x Magnification. Arrows point to microbeads inside under the carapace (A) and to round masses in the gut (B).

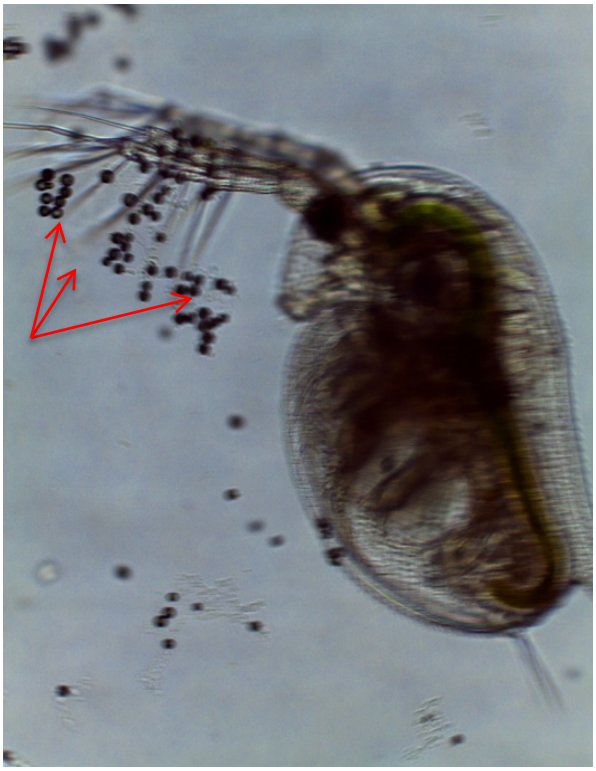

Figure 12 Ryerson-Lab Pilot Study (Microbeads 10 – 20 µm): Neonate born after 24 hours of Microbead Exposure (40x Magnification). Arrows point to microbeads caught on the secondary antennae.

4.1.2 Conclusions from Ryerson-Lab Pilot Study (Microbeads 10-20 µm)

The microbeads were in the water column, and indistinguishable with the naked eye from algae. Microbeads were most visible in and on the neonates. Younger *D. magna* appeared to have greater concentrations, suggesting that neonates should be used in on-going bioassays.

4.2 Home-Lab Pilot Study (Microbeads 20 - 27 µm)

As the microbeads had been changed from the 1st pilot study, a new pilot study was deemed necessary to address the same research question needed to be addressed with these specific microbeads. (Namely, how will the microbeads interact in the *Daphnia* growth medium, and how will *D. magna* interact with the microbeads?) The results for this pilot study are presented as bioassay endpoints and micrographs. The bioassay endpoints are split into mortality and behaviour. For the bioassay endpoints, the medians for each treatment are charted with their respective max and min as error bars. As the

data was discrete, the statistical analysis was Friedman testing and was performed on the means.

4.2.1 Microplastics Effect on Mortality

Effects on mortality can been seen by examining survivorship in Figure 13 depicting the median with the max and min as error bars. During the first three time checks (10 minutes, 1 and 6 hours) there were no deaths in either treatment. After 24 hours, 1 daphnid had died in the Reference and 3 in the Microbead treatment. After 48 hours, the Reference had a mortality rate of 33%, while the Microbead treatment had a mortality of 50%. These values are not significantly different. Environment Canada *Daphnia magna* culturing protocol requires a higher survivorship in the control treatment (Environment Canada, 2016), and thus, it was deemed necessary to repeat this pilot study.

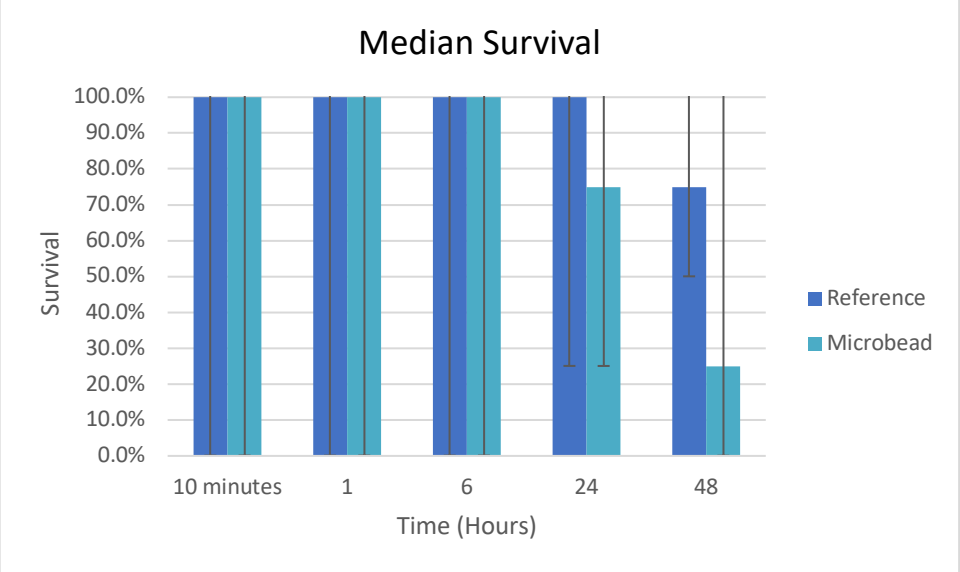

Figure 13 Home-Lab Pilot Study (Microbeads 20 - 27 µm): Survivorship

4.2.2 Microplastics Effect on Behaviour

Behaviour was examined through mobility, boundary crossing, and body score (based on body orientation). Figure 14 depicts all three behavioural assessments in 3 separate charts. In each case, the reference and Microbead treatments were not

significantly different. As a very preliminary conclusion, this would suggest microbeads do not have an impact on behaviour in *Daphnia magna*.

Mobility followed the same trend as mortality, with increasing immobility as time passed. After 48 hours, 50% of *D. magna* were mobile under Reference conditions, while 42% of *D. magna* exposed to microbeads were mobile. Boundary crossings in both treatments varied greatly between vessels for the first 3 time checks. After 48 hours both Reference and Microbead treatments had low number of boundary crossings. Both Reference and the Microbead treatments had increasingly stressed swimming styles as time continued and this is noticeable in the increased body score.

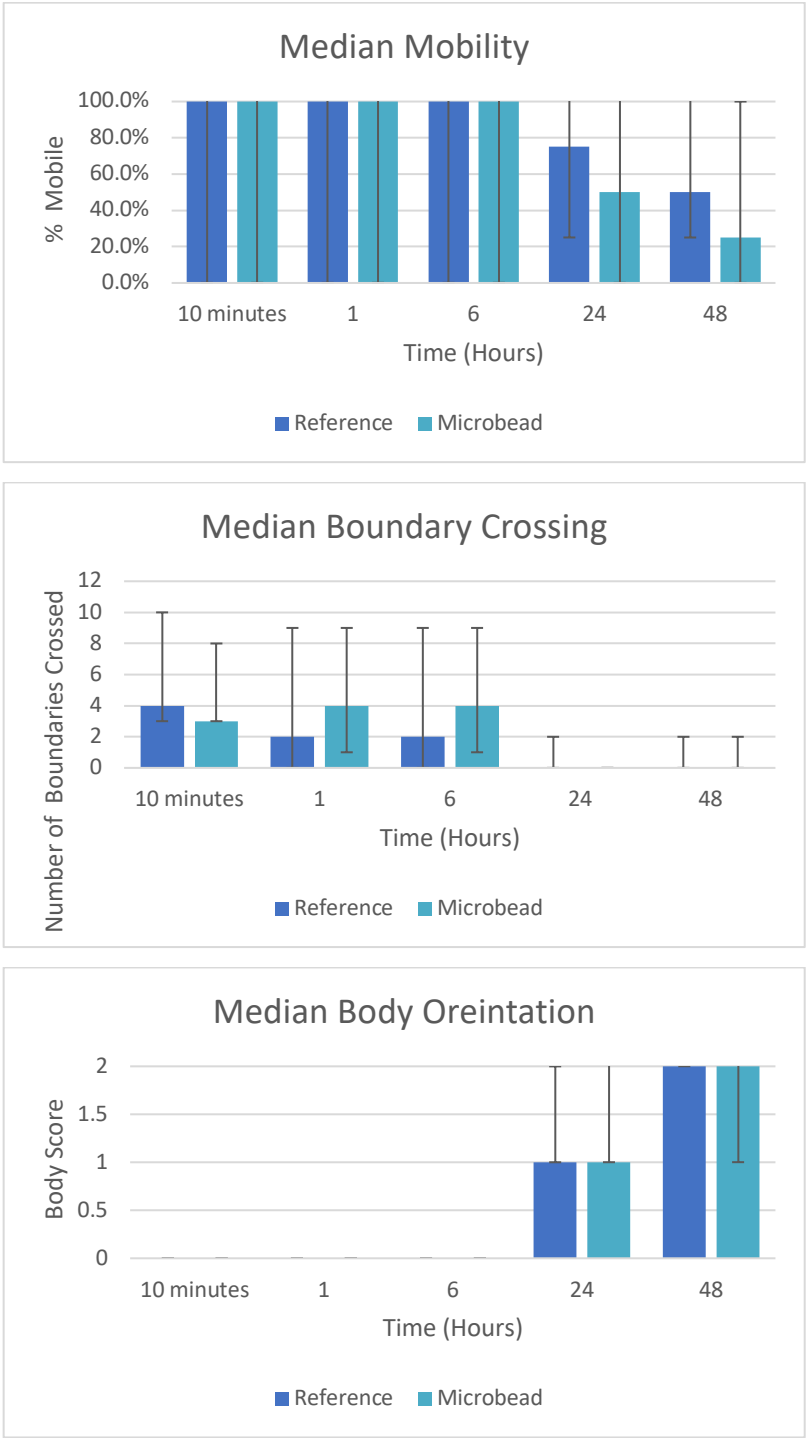

Figure 14 Home-Lab Pilot Study (Microbeads 20 - 27 µm): Median Behaviour

4.2.3 Visual Observations of Microplastics and *Daphnia magna*

Assessments on mortality and behaviour examine the medians, however, there were some striking singular observations from this experiment. In one of the microbead vessels, after 48 hours, three daphnids were immobile, while one appeared to swim with reference 'normal' behaviour. Figure 15 depicts one of the immobile daphnid, found at the bottom of the vessel. The image is 40x magnification. Microbeads appear to have aggregated and can be seen on her carapace, spiny tail, and secondary antennae. Figure 16 compares the daphnid with "normal" swimming behaviour from the Microbead treatment (B, and B') with a reference daphnid that also had "normal" swimming behaviour (A and A'). From this comparison, it is clear from both the whole-body comparison between the reference and daphnid exposed to microbead (at 40x Magnification), and the comparison between their respective intestines (at 100x Magnification) that this swimming daphnid has ingested microbeads. The gut contents of the daphnid exposed to microbeads (B') is full of spherical masses and appears bumpy (see red arrow); in contrast, the reference daphnid (A')'s gut appears full uniform slur which is smooth (see blue arrow). Additionally, the "normal" swimming daphnid from Figure 16 (B and B') does not appear to have any microbeads on her carapace as compared to her vessel-mate from Figure 15.

Figure 15 Home-Lab Pilot Study (Microbeads 20 - 27 µm): Immobilized *D. magna* with Microbeads on Carapace (40x Magnification). Arrows point to aggregates of microbeads on secondary antennae, carapace, and spiny tail.

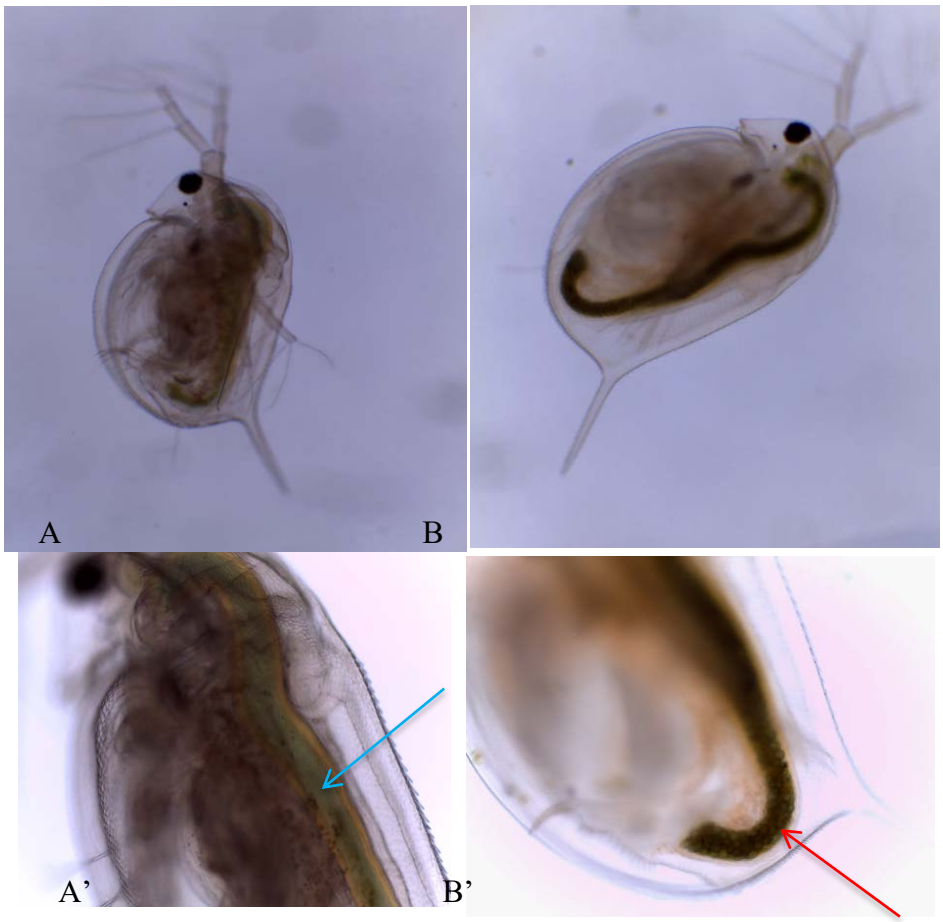

Figure 16 Home-Lab Pilot Study (Microbeads 20 - 27 µm): Healthy Behaving daphnids from Reference Condition (A) and Microbead Exposure(B) at 40x Magnification; Gut contents for respective daphnids' (A' and B') at 100x Magnification. Arrows point to gut contents, the blue arrow emphasizes smooth reference conditions (A') the red arrow identifies bumpy microbead exposure (B')

4.2.4 Conclusions from Home-Lab Pilot Study (Microbeads 20 - 27 µm)

The microbeads were in the water column, but after 48 hours, some of the microbeads appeared to aggregate on the bottom of the vessel. Both the reference and Microbead treatments experienced high mortality rates, that were not significantly different from one another. Additionally, there was no detectable effect on behaviour. This high mortality could be due to *D. magna* acclimatizing to Home-lab conditions.

Aggregates of microbeads were not identifiable on or in *D. magna* with the naked eye; however, micrographs of daphnids revealed microbeads on the carapace of

immobilized daphnids. Additionally, microbeads were visible in the gut of a "normal" swimming daphnids (this was confirmed by comparing to a reference *D. magna*). These micrograph observations would lead to the very preliminary conclusion that *Daphnia* spp. ingesting microbeads may not cause impact, but that microbeads sticking to the carapace could be immobilizing (either physically or chemically).

4.3 Home-Lab Pilot Study Repetition (Microbeads 20 – 27 μm)

Because of high mortality in both the reference and Microbead treatment, this preliminary study was repeated. An additional treatment, Tween was added as a vehicle control. The results section is separated into bioassay endpoints and micrograph photos. The bioassay endpoints section is further divided into mortality and behavioural endpoints. For the bioassay endpoints, the medians for each treatment are charted with their respective max and min as error bars. As the data was discrete, the statistical analysis was Friedman testing and was performed on the means.

4.3.1 Microplastic Effect on Mortality

Survivorship is depicted in Figure 17 as the median for each treatment with error bars are max and min. Mortality in the reference conditions remained relatively low, with 1 daphnid dead on day 4, and an additional two on day 7; overall the mortality rate in reference condition was 15%. The Tween treatment (a vehicle control, to ensure 0.1% Tween-20 is not toxic to daphnids) had a mortality rate of 30%, which was unexpectantly high given that this treatment is a vehicle control. The Microbead treatment also had a mortality rate of 15%. These values were not statistically different, given the small sample size.

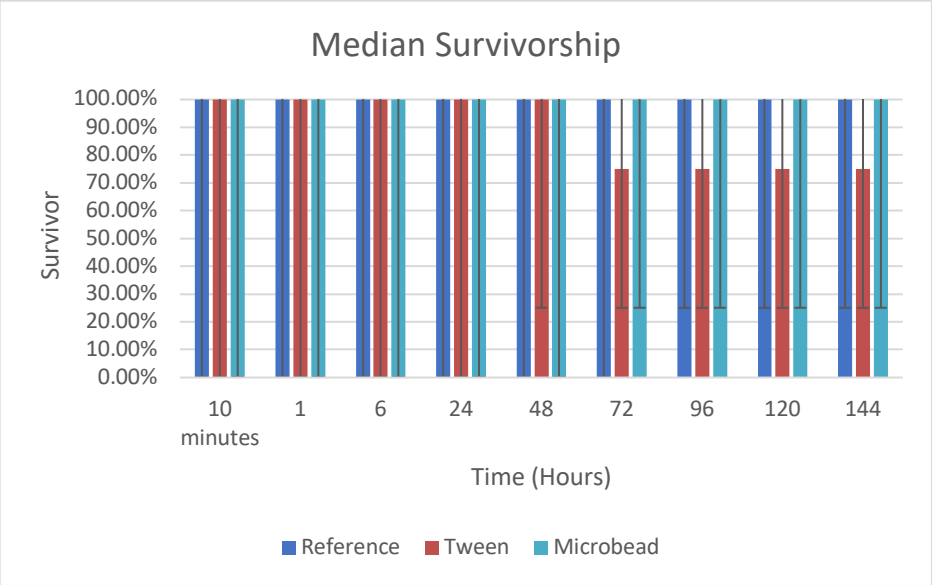

Figure 17 Home-Lab Pilot Study Repetition: Survivorship

4.3.2 Microplastic Effect on Behaviour

Behavioural endpoints for mobility, movement throughout the water column, and swimming style were all assessed and the median are shown in Figure 18. Throughout the entire study, there was no statistical difference in median mobility, number of boundary crossings, and swimming style.

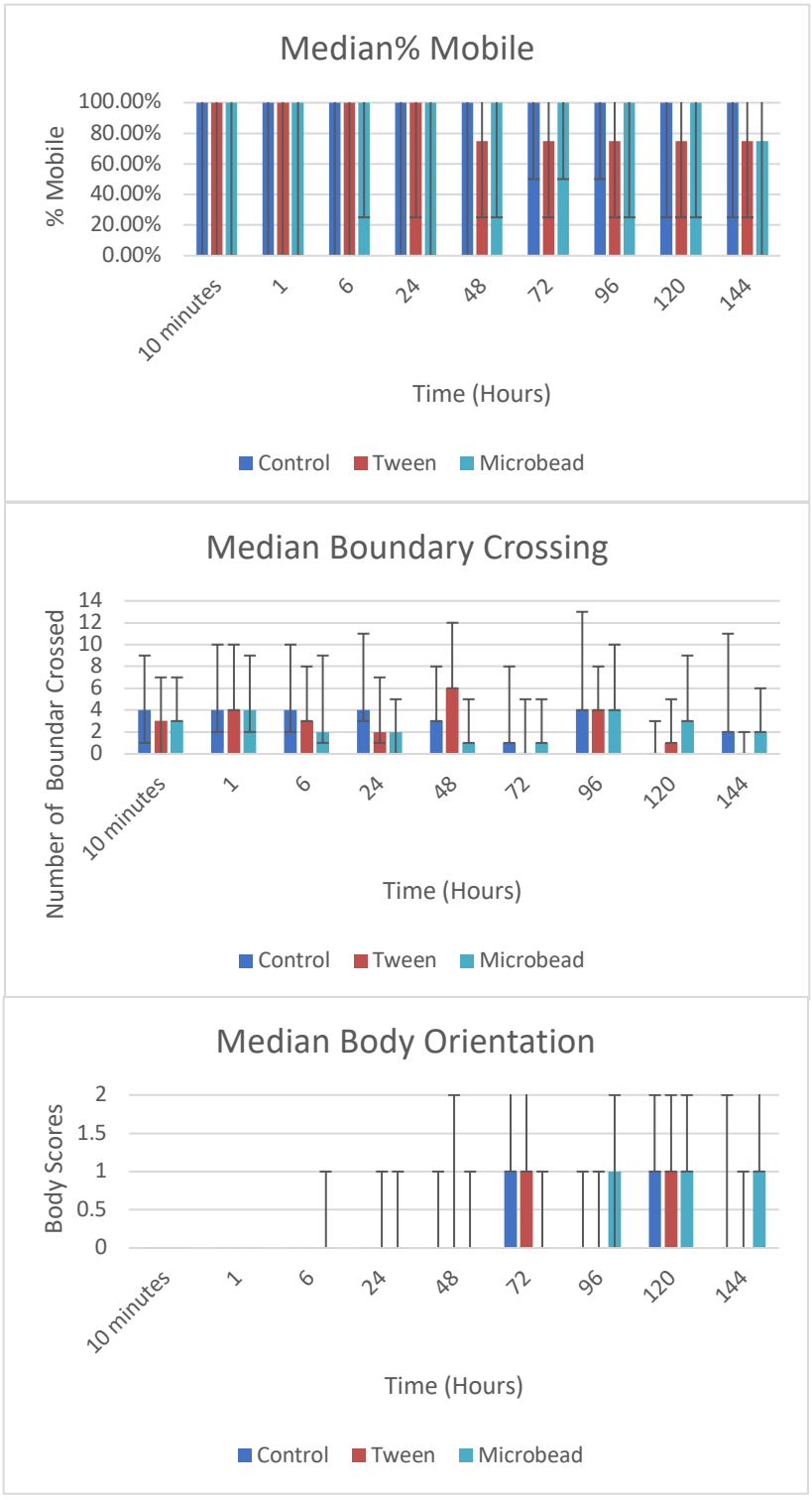

Figure 18 Home-Lab Pilot Study Repetition: Median Behaviour

4.3.3 Visual Observation of Microplastics and *Daphnia magna*

As the preceding pilot studies captured images of microbeads ingested and adhering to *D. magna*, no specific time-dependent photos were taken. Instead, neonates and deceased daphnids were collected and checked under the microscope. Unfortunately, the microbeads came out of solution on day 6, prior to refreshing the water. Half the water was exchanged and no new microbeads were added. In this case, the experiment was allowed to go on for 2 more days, as some of the daphnids already had eggs and seemed likely to yield very preliminary insights on *D. magna* reproduction with microbead exposures.

Figure 19 depicts two neonate daphnids (100x Magnification), born on day 7, one from the Reference treatment (A), and the other from the Microbead treatment (B). A microbead can be seen in the hindgut of the daphnid exposed to microbeads (see the red arrow). The concentration of microbeads in the vessels was unknown but theoretically should be half the initial amount (0.85 mg/L or 1.25 x 10^5 beads/L).

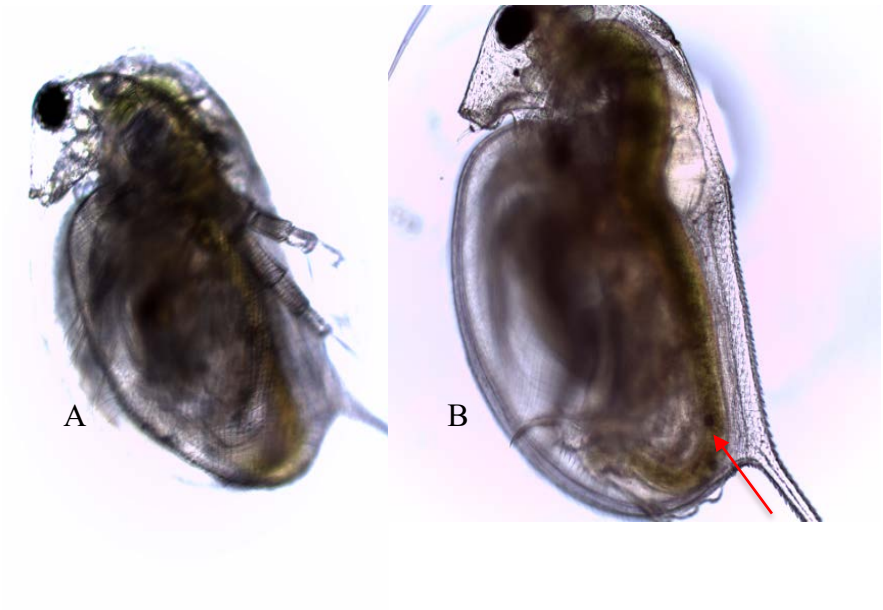

Figure 19 Home-Lab Pilot Study Repetition: Neonates born during the Experiment (100x Magnification). Arrow points to microbead in the gut contents.

A representative of the deceased daphnid from the pilot study can be seen in Figure 20. (A) depicts a daphnid from the reference conditions, and (B) depicts a daphnid

from the Microbead treatment (both are 40x Magnification). Microbeads are visible around the daphnid and on her carapace. For a better view, images (B') and (B'') are 100x Magnification of her secondary antennae and spiny tail. As a preliminary observation it appears, as if deceased *D. magna* from the Microbead treatment have more algae build up and decay.

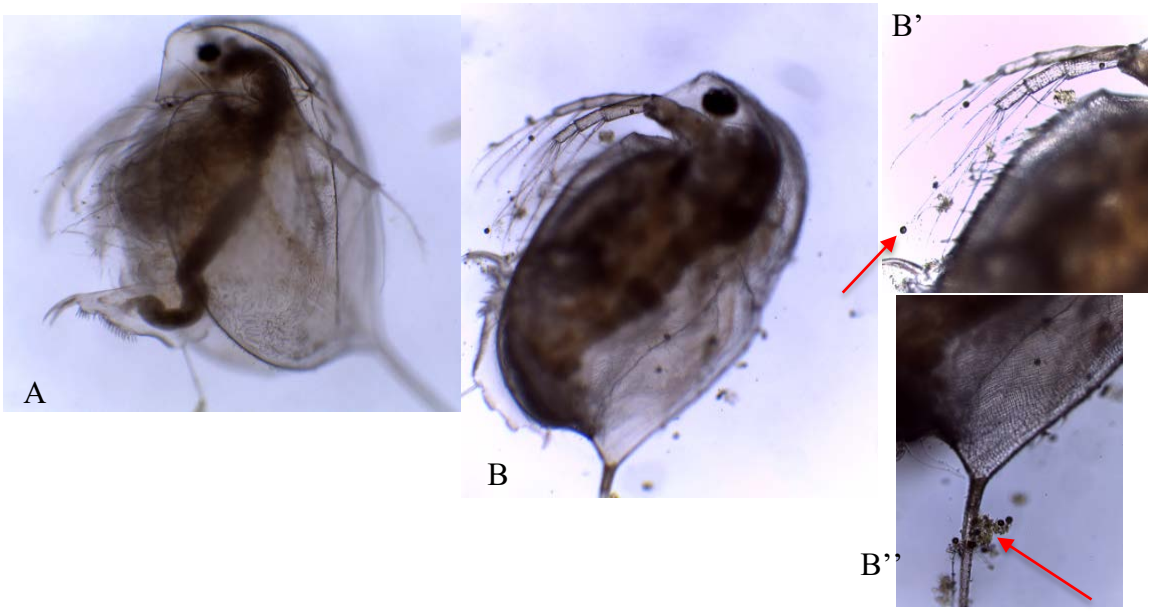

Figure 20 Home-Lab Pilot Study Repetition: Deceased *D. magna*, Reference (A) & Microbead Exposure (B) 40x Magnification; B' and B'' 100x Magnification. Arrows point to microbeads on secondary antennae and spiny tail.

4.3.4 Conclusion from Home-Lab Pilot Study Repetition (Microbeads 20 – 27 µm)

This pilot study repetition demonstrated higher survivorship in reference and microbead conditions, suggesting proper *D. magna* culturing conditions. As this was a pilot study, no decisive conclusions can be drawn. As a preliminary remark, microbeads do not appear to be impacting *D. magna* as measured by mortality or behaviour. Microbeads should have been added during the refreshing of the water on day 6; however, because of aggregation issues were not.

Visually, microbeads could still be seen in neonates and deceased *D. magna* after refreshing, despite theoretically lower concentration. Previous research from Cui *et al.* (2017) suggested neonates born in microbead treatments were often malformed, but this preliminary pilot study did not find developmental issues with neonates. As a preliminary

observation between the deceased *D. magna*, the microbead-exposed daphnids appeared to be more deteriorated than the reference.

4.4 Toxicity Assay: Microbeads and TCC

This experiment fulfills the thesis overall objective by using *Daphnia magna* that had been cultured for toxicity bioassays, with mortality, reproductive and behavioural endpoints to assess the impacts of microbeads. The results section is separated into bioassay endpoints and micrograph photos. The bioassay endpoints section is further divided into mortality and behavioural assessments. For the bioassay endpoints, the medians for each treatment are charted with their respective max and min as error bars. As the data was discrete, the statistical analysis was Friedman testing and was performed on the means.

4.4.1 Toxicity Effects on Mortality

Survivorship is depicted in Figure 21, with the bars representing median survival with a range of the max and min to represent all replicates for each treatment. During the 6-day experiment, reference conditions had a low mortality (6.25%). The two vehicle controls (Tween and DMSO) also had very low mortality, with 0 deaths (0% mortality). This indicates good reference conditions, and denotes that mortality was not impacted by the laboratory set-up. Furthermore, it also rules out the vehicle controls as having an effect on *D. magna* mortality.

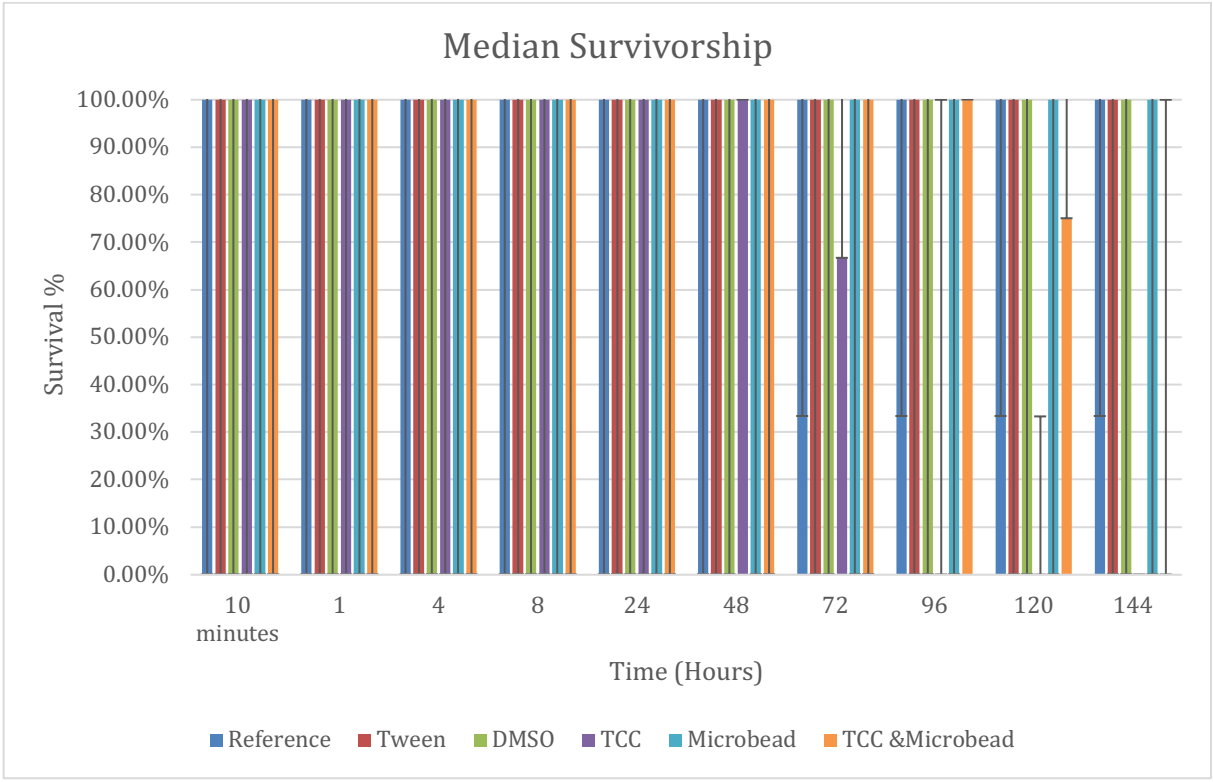

Figure 21 Toxicity Assay: Microbeads and TCC: Survivorship

In an overall assessment, there is no statistical difference between each treatment. Ecotoxicology must take into account the acute (<24 hours) and chronic (>24 hours) effects of a contaminant; thus 4 assessments were made during the first 24 hours, and 1 assessment everyday thereafter. To better assess chronic impact, the values between 24 – 144 hours were considered. Again, the vehicle controls and the Microbead treatment are not statistically different. Conversely, the TCC treatment is statistically different from the controls ($X^2 = 9.9$, $p = 0.05$). The TCC treatment is different from the TCC & Microbead treatment ($X^2 = 4.16$, $p = 0.05$). Together these findings indicate impact on *D. magna* from microplastics is likely due to a chemical (TCC) rather than physical threat from the microbeads. Moreover, there appears to be a delayed effect seen in TCC & Microbead treatment as compared to TCC, this would suggest that microbeads may have an antagonistic effect on TCC, potentially by sorbing TCC and partially removing this contaminant from bioavailability.

4.4.2 Toxicity Effects on Behaviour

Behavioural assessment for % mobility, vertical movement through the water column (boundary crossing), and swimming style (through body orientation based on body scoring) was assessed and depicted in the following three figures (Figure 22, Figure 23, and Figure 24 respectively).

4.4.2.1 Mobility

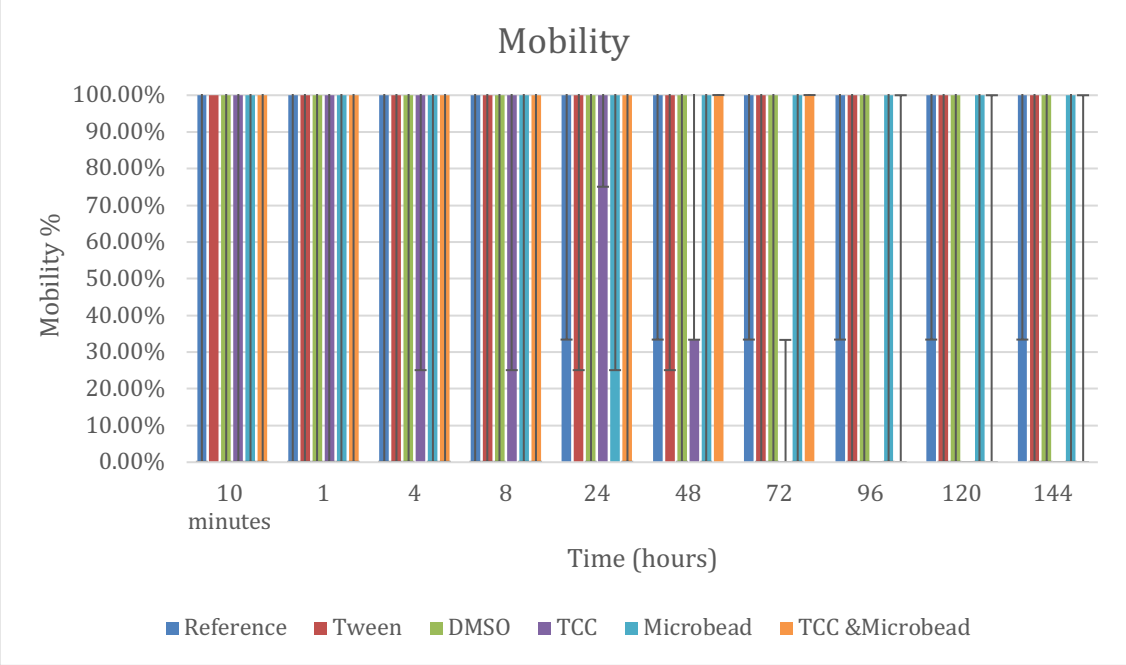

Figure 22 Toxicity Assay: Microbeads and TCC: Mobility

Mobility follows a similar trend to mortality (Figure 22), and median and ranges of max-min give an accurate portrait of the range in each vessel for their respective treatments and time-intervals. The reference and two vehicle controls had low variance, which indicate *D. magna* had normal mobility and rules out the vehicle controls as the reason for any impact. The microplastic alone treatment also maintained a high % mobility, signifying no effect from the microplastics on mobility.

Both the triclocarban (TCC and TCC & Microbead) treatments showed decreased mobility as compared to the controls ($X^2 = 16.9$, $p = 0.01$). In the TCC treatment, all *D.*

magna cease to be mobile after the 3rd day, whereas the TCC & Microbead treatment continue to have some mobile daphnids for the entirety of the experiment. Statistically this delay is also noted as TCC and TCC & Microbeads are different from one another ($X^2 = 6.4$, $p = 0.01$). Mobility is influenced by TCC not microbeads, and it appears as if the TCC & Microbead may be antagonistic in effect, whereby the stress due to TCC is delayed by the microbeads.

4.4.2.2 Movement through the Water Column: Boundary Crossing

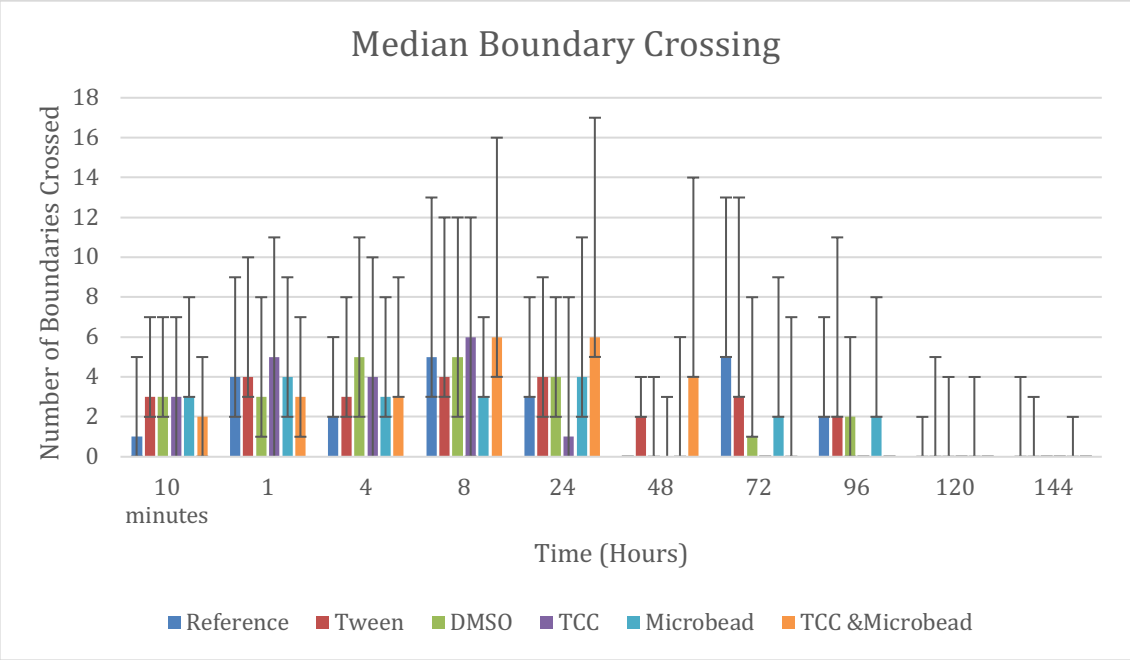

Figure 23 Toxicity Assay: Microbeads and TCC: Boundary Crossing

The second behaviour assessed was movement through the water column as measured by boundary crossing to denote movement from one depth to another. Figure 23 depicts the median and range of max and min for each treatment throughout the experiment. Little has been published on specific movements through a vertical water column, merely that it is typical to see *Daphnia* spp. forage for food and to avoid predation. This lack of comparative literature may be due to different clones having different swimming speeds, which would in turn result in different average boundary crossings (Dodson *et al*., 1995). All daphnids used in this experiment originate from the same clonal grandmother, and thus it is useful to compare results with the Reference condition.

There was no statistical difference among treatments, however some preliminary trends can be drawn by examining means. In every treatment, there is a subtle increase from initial assessments, followed by a downward trend crossing the boundary less frequently. The subtle increase could be the result of daphnids acclimatizing to their environment. The trend towards less boundaries crossed was accompanied by increased feeding off the bottom of the vessel by many of the daphnids. In association with mobility trends (see Figure 23), it is important to note that the increased immobility would also result in fewer boundaries crossed.

4.4.2.3 Swimming Style: Body Score/Orientation

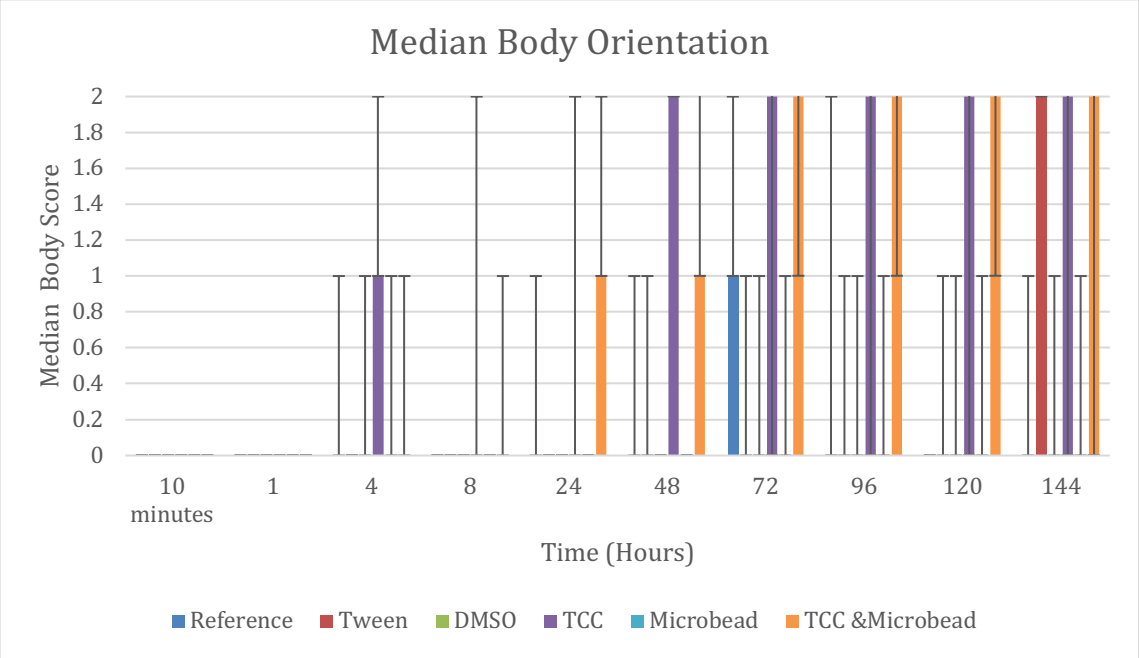

Figure 24 Toxicity Assay: Microbeads and TCC: Swimming Style

The third behavioural assessment was a scored measure of swimming style (Figure 24). The reference showed nearly consistent low scores, representing normal swimming styles. The two vehicle controls also showed normal swimming styles, with low variability between these three controls. The Microbead treatment also had low body scores. Meanwhile the combined triclocarban treatments demonstrated differences from the controls ($X^2 = 16.5$, $p = 0.01$). This was due to the TCC. As TCC showed an impact

($X^2 = 11.73$, $p = 0.01$), while the TCC & Microbead was not different from the controls. Further TCC and TCC & Microbeads were statistically different from one another ($X^2 = 6$; $p= 0.05$). Collectively, these results suggest microbeads are not causing harm on their own, rather chemical sorption could be a potential threat.

4.4.3 Visual Observations of Microplastics and *Daphnia magna*

Visual observations of the microbeads (through micrographs) in and on *D. magna* confirm the bioavailability of this contaminant. Daphnids were sacrificed throughout this experiment by removing them from their test vessel, and observing them under the microscope, after which these daphnids were retired. Observations were made at various time intervals: 10 minutes; 4, 24, 48, and 144 hours.

Figure 25 compares a reference condition neonate (A) with a TCC & Microplastic neonate (B) after 10 minutes (Both photos are 100x Magnification). A single microbead is visible on the TCC & Microbead treatment *Daphnid,* near her spiny tail. The presence of the microbead picked up with a *Daphnid* suggests that the microplastic is present and bioavailable in the water column. Additionally, it is worth noting these photos depict non-uniform gut contents, unlike the images from the pilot studies. This is likely algae ingested from their brood stock chambers prior to beginning this experiment. Previous *D. magna* microplastic research pre-starved the *D. magna,* which may have biased the bioassay endpoints resulting in daphnids eating large quantities of microplastics (Rehse *et al*., 2016; Jamec *et al*., 2016; Aljaibachi & Callaghan, 2018).

The exposure concentration during this experiment was aspiring to be similar to environmentally relevant concentrations. The minimum amount that could be added was 0.1 mL of the stock microbead solution as a result, the micrograph photos of daphnids from microbead treatments did not always contain microbeads. Throughout the whole experiment, microbeads were visible under the microscope, and thus, it seems likely they were in the water column (bioavailable for the daphnids), but due to their low concentration (as compared to the pilot studies) were not always present at the time of inspection for photographs. Figure 26 demonstrates this predicament, as images A, B, and

C depicting a *D. magna* from the reference, microbead, and TCC & Microbead exposures, respectively, are not noticeably different from one another.

Figure 25 Toxicity Assay: Microbeads and TCC: 10-minute Exposure Reference (A) and TCC & Microbead (B) (at 100x Magnification). Arrow points to microbead on the spiny tail.

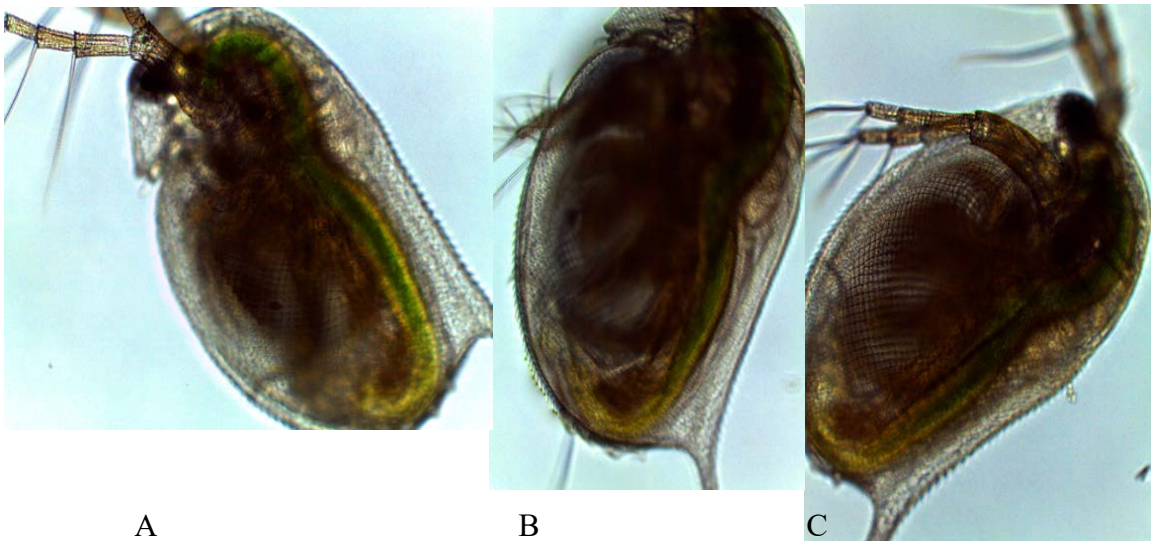

Figure 26 Toxicity Assay: Microbeads and TCC: 24-hour exposure: Reference (A), Microbeads (B), and TCC & Microbeads (C) (100x Magnification)

Figure 27 compares reference conditions (images on the left) and microplastic treatment (images on the right) of *D. magna* after 6 days of exposure. Both images of the reference *D. magna* (A) and the microbead *D. magna* (B) are full body images at 40x Magnification. These adult daphnids carry a young neonate in their brood chamber (in the reference conditions there is an egg in her brood chamber, while in the microplastic treatment (B) there is an embryo). Had this experiment continued, it seems highly likely that both the daphnids in Reference and Microbead treatments would have survived and reproduced. The gut contents for these daphnids are displayed below at 100 Magnification. The reference daphnid (A')'s gut content is a smooth homogeneous mixture, meanwhile the microbead exposed *D. magna* has 5 visible microbeads in her gut.

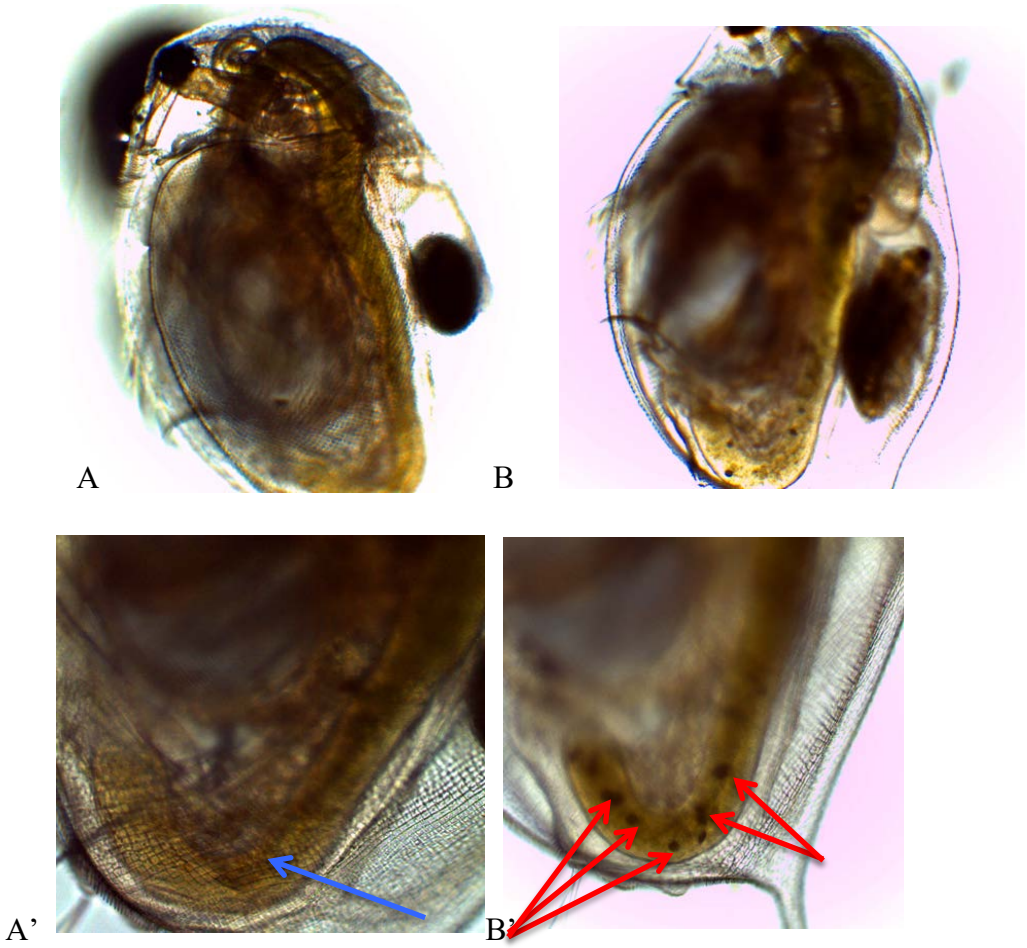

Figure 27 Toxicity Assay: Microbeads and TCC: Reference (A) and Microbeads (B) Exposed (40x Magnification); Gut content: Reference (A') and Microbead (B') at 100x Magnification. Arrows emphasis the gut contents, the blue arrow shows the smooth reference condition, the red arrows point to microbeads inside the gut contents.

Figure 28 depicts two deceased daphnids. Unfortunately, the image of the reference deceased daphnid from this experiment was lost, so as a comparison a representative of a dead TCC daphnid and one from the TCC & Microbead treatment are shown here (labelled as such). The image of the daphnid from TCC & Microbead has microbeads visible on her secondary antennae, and in her gut (see red arrows). Similarly, as previously seen in the pilot study repetition, it appears also that the daphnid from the TCC & Microbead treatment looks more deteriorated than a reference organism.

Figure 28 Toxicity Assay: Microbeads and TCC: Deceased daphnids from TCC and TCC & Microbead exposures (40x Magnification). Arrows point to microbeads on secondary antennae, and inside gut contents.

4.4.4 Summary of Conclusions from Toxicity Assay: Microbeads and TCC:

These findings confirm that reference conditions are suitable for culturing healthy *D. magna.* This is evident from the Reference conditions yielding low mortality, high mobility, and scoring low for swimming style. The two vehicle controls (Tween and DMSO) showed no impact to *D. magna*. Furthermore, *D. magna* are sensitive to triclocarban at 10 ppb. Raby (2013) had previously found reproductive delays from this concentration; however, the mortality rates here suggest *D. magna* are far more sensitive.

As mentioned in the introduction, researchers are divided on their conclusions regarding whether microplastics are causing a negative impact; there is evidence for both viewpoints. A 2020 meta-analysis on plastic and microplastic toxicity noted this inconsistency. Additionally, they saw a trend of reported effect with "extreme, environmentally-unrealistic concentrations" (Bucci *et al*., 2020). The work of this thesis answer's their call for environmentally-relevant doses of around 10 particles/mL (Bucci *et al*., 2020) with ~3.7 microbead/mL; at this level, microbeads alone produced no impact to *D. magna*.

Lastly, it would appear that triclocarban and microbeads fit an antagonism model, as the TCC & Microbead treatment follow the trend set by TCC in a delayed onset (see Figure 21, Figure 22, and Figure 24). Few other researchers have explored the phenomena of combined stressors from a contaminant and microplastic. One study, found nickel toxicity on *D. magna* differs in the presence of microplastic (Kim *et al*., 2017). Together, these preliminary findings and the work done by Kim *et al*. (2017) suggest that there is still much left to be researched regarding the complicated interactions of microplastics with stressors and their combined effect.

Additionally, microbeads seen in the gut confirm the previous pilot studies that *D. magna* can ingest the microbeads. This is consistent with most previous studies of *Daphnia* spp. microbead impact assessments. The early comparison of these results with work published by Aljaibachi & Callaghan (2018) in particularly noteworthy. In Aljaibachi & Callaghan, (2018) found that in the presence of algae as a food source, *D. magna* would selectively reject microplastics. But the micrographs here would suggest *D. magna* are not discriminating entirely.

4.5 Toxicity Assay: Microbeads and TCC at Lower Dose

During the first experiment (section 4.4) mortality in the triclocarban treatment prevented measuring behaviour and reproductive endpoints. Thus, a second study with a lower dose of triclocarban from 10 ppb to 5 ppb was set up. The results section is separated into bioassay endpoints, and micrograph photos. The bioassay endpoints section is further divided into mortality, reproduction, and behavioural assessments. For the bioassay endpoints, the medians for each treatment are charted with their respective max and min as error bars. As the data was discrete, the statistical analysis was Friedman testing and was performed on the means.

4.5.1 Toxicity Effects on Mortality

Survivorship is depicted in Figure 29, as medians with max and min range. Reference conditions show high survival rates throughout the experiment, with a mortality of 8.33% by the end of this experiment. The two vehicle controls are

statistically different from the Reference. This suggests that reference conditions were good and that effects in the test treatments are not impacted by the 0.1% Tween-20 solution or DMSO.

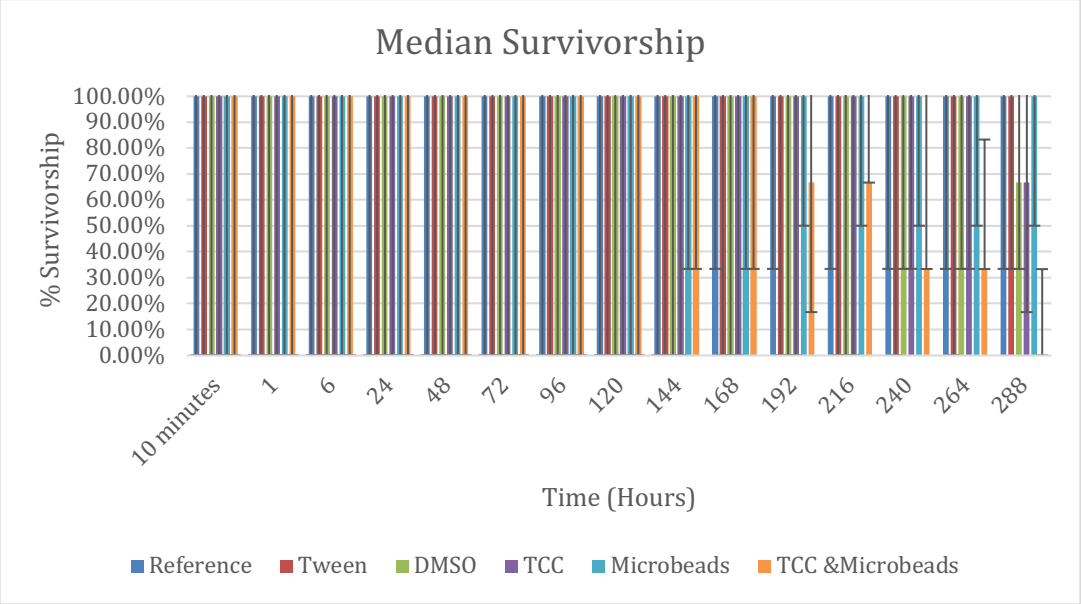

Figure 29 Toxicity Assay: Microbeads and Lower TCC: Survivorship

The overall assessment gives extra emphasis to the first 24 hours, by having 3 time-intervals, whereas following days only have one assessment each. Moreover, mortality for all treatments are the same for the first 5 days. Thus, to examine chronic effect, the assessment ranged from 24 – 288 hs. When examining chronic mortality, it appears the TCC & Microbeads treatment has an effect, as it is significantly different from the control ($X^2 = 8.775$, $p = 0.05$). Based on the pilot studies, and the previous experiment, it was expected that TCC would also show a chronic effect on mortality, and that this effect would be no different from the TCC & Microbead treatment. This was not the case. The triclocarban treatments were significantly different from one another ($X^2 = 4.083$, $p = 0.05$), and the TCC treatment was not different from the controls. The TCC & Microbeads were also independent of the Microbead treatment ($X^2 = 4.083$, $p = 0.05$). Collectively, this presents the very preliminary conclusion that microbeads might be able to magnify the effects of low TCC concentrations (this experiment used 5 ppb).

There are few studies to date that consider the effects of microplastics with an additive on *D. magna*. Cui *et al. (*2017) suggested microbead coating will influence the impact of microplastics. However, there still is much more research to be conducted regarding the interactions between microbeads and pollutants.

4.5.2 Toxicity Effects on Reproduction

This toxicity assay was the first to continue the life-cycle timeframe of the bioassay allowing reproductive endpoints. Not every vessel produces neonates, and because of the on-going sacrificing of *D. magna,* no statistical analysis has been done for reproductive output. As a preliminary result, Table 4 summarizes the treatment vessels that did produce eggs. By examining the number of vessels from each treatment where reproduction occurred; the DMSO treatment and TCC & Microbeads have the least number of vessels that produced neonates, while the TCC treatment and Microbead treatment had more vessels produce neonates. There is a large variance in the time until first brood, between 6 and 12 days. By examining number of neonates produced from each treatment the TCC & Microbead treatment had the least total number of neonates: meanwhile, the Microbead treatment had the most neonates.

Canniff & Hoang (2018), when exposing *D. magna* to polyethylene microbeads, observed no reproductive impairment from the microbeads. This toxicity repetition supports their conclusions with what appears to be no impact from microbeads for initial reproductive endpoints.

Table 4: Toxicity Assay: Microbeads and Lower TCC: Reproduction

Treatment and Vessel number	Time till 1st Brood (Day)	Number of Neonates in 1st Brood	Number of Neonates in 2nd Brood
Reference (1)	7	3	1
Reference (2)	7	2	--
Reference (5)	12	1	--
Tween (1)	7	2	--
Tween (2)	7	1	2
Tween (3)	7	2	--
DMSO (1)	12	2	--
DMSO (2)	7	2	5
TCC (1)	6	1	1
TCC (2)	7	2	--
TCC (4)	12	3	--
TCC (5)	10	1	--

Microbeads (2)	12	7	--
Microbead (3)	7	1	1
Microbead (4)	12	2	--
Microbead (5)	12	2	--
TCC & Microbeads (2)	6	3	--
TCC & Microbeads (5)	12	2	--

4.5.3 Toxicity Effects on Behaviour

Behavioural assessment for % mobility, vertical movement through the water column (boundary crossing) and swimming style (through body orientation based on body scoring) was assessed and depicted in the following three figures (Figure 30, Figure 31, and Figure 32 respectively).

Mobility

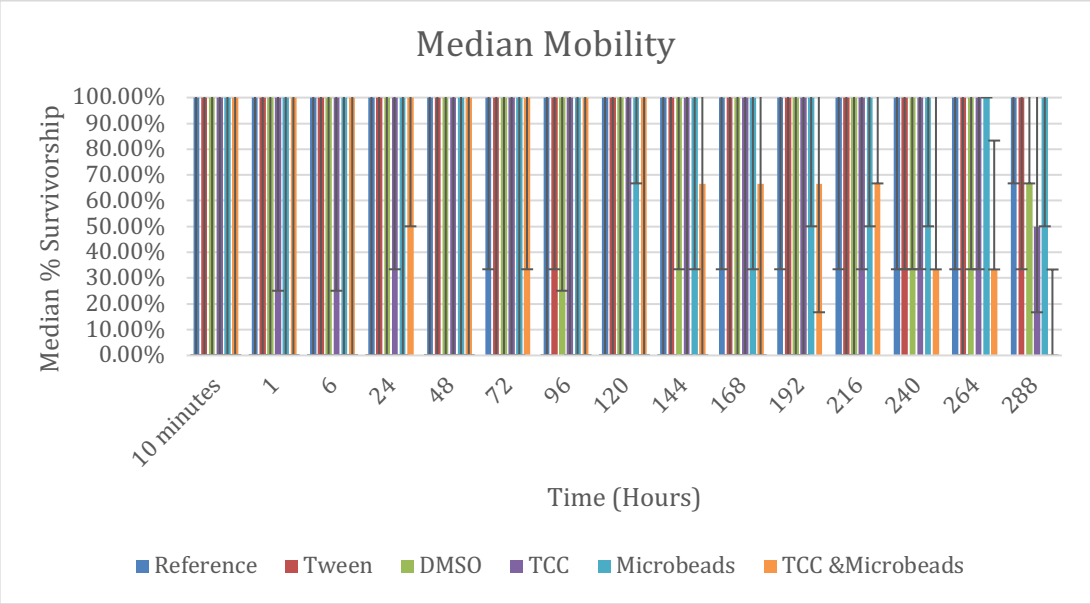

Figure 30 Toxicity Assay: Microbeads and Lower TCC: Mobility

Median and the range of mobility for each treatment is depicted in Figure 30. When comparing the overall change in mobility, the treatments are not statistically different. Unlike survivorship, mobility measurements are more fluid, as a daphnid could be stuck in the surface tension, or at the bottom of the vessel during one assessment but then regain vigour and be actively swimming for the next assessment. These results may explain the large Max-Min error bars within the first 5 days (120 hours). However, as the experiment continues beyond these first 5 days, the trend towards regaining seen in swimming activity happens less frequently, and when cross referenced with the survivorship Figure 29, corresponds – if a daphnid has died it is also no longer mobile.

To better understand the data from a chronic toxicity point of view, the range of assessments was shifted to examine variance between treatments from the 24 – 288 hours (days 1-12). During this time period, the reference treatments have low variance, signifying appropriate bioassay conditions and that Tween and DMSO do not impact daphnid's mobility. *D. magna* were not significantly impacted by the TCC treatment. Moreover, the mobility of *D. magna* in the Microbead treatment is not statistically significant. The lack of sensitivity of *D. magna* to triclocarban at 5 ppb was expected as

Raby (2013) noted no behavioural differences at this concentration. As previously discussed, *D. magna*'s overall sensitivity to microplastic is still inconclusive. *D. magna* sensitivity to microplastics for mobility was previously examined by Rehse *et al. (*2016), and they concluded immobility from polyethylene microplastic fibers. This discrepancy could be due to high concentrations used by Rehse *et al.* (2016) (12.5-400 mg of microplastic/L) as compared with the concentrations of this study (0.025 mg of microplastic/L).

From a chronic toxicity perspective (24 – 288 h, or 1-12 days), the TCC & Microbead treatment shows reduced mobility as compared to the controls ($X^2 = 8.775$, $p = 0.05$); and as compared to the Microbead (alone) counterpart (the Microbead treatment, $X^2 = 4.083$, $p = 0.05$); and as compared to the TCC (alone) counterpart (the TCC treatment, $X^2 = 4.083$, $p = 0.05$). This nonconformity in impact from both its counterparts suggests that the combinations of low concentrations of triclocarban and microplastics could form a synergetic effect, and significantly impact *D. magna*.

Movement through the Water Column: Boundary Crossing

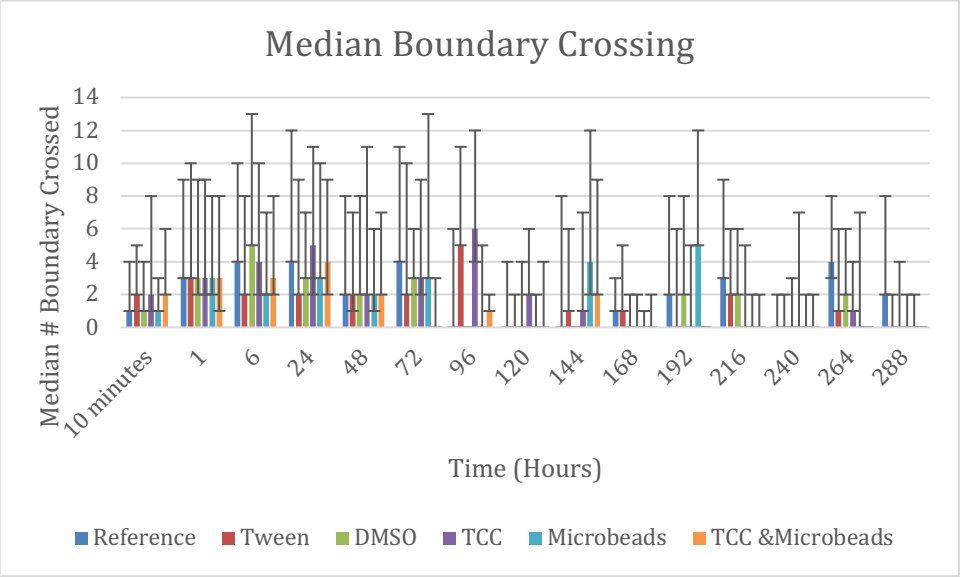

Figure 31 Toxicity Assay: Microbeads and Lower TCC: Boundary Crossing

Boundary Crossing throughout the experiment for each treatment is displayed in Figure 31, noting the median number of movements and the max and min movements within the respective 5 replicates. Again, the expectation was that *D. magna* would move through the water column, and results would need to be compared against the reference conditions. No singular treatment was statistically different from the control treatments.

As a point of comment, the different experimental treatments demonstrate statistical differences from one another. Both microbead treatments (Microbeads and TCC & Microbeads) are not statistically different from one another. Additionally, the two triclocarban treatments were statistically different from one another ($X^2 = 6.67$, $p = 0.01$). What is more the TCC and Microbead treatment were also different from another ($X^2 = 5.4$, $p = 0.01$). As stand-alone information, the differences in these experimental treatments could suggest no behavioural impact from microbeads and a sensitivity to triclocarban.

Swimming Style: Body Score/Orientation

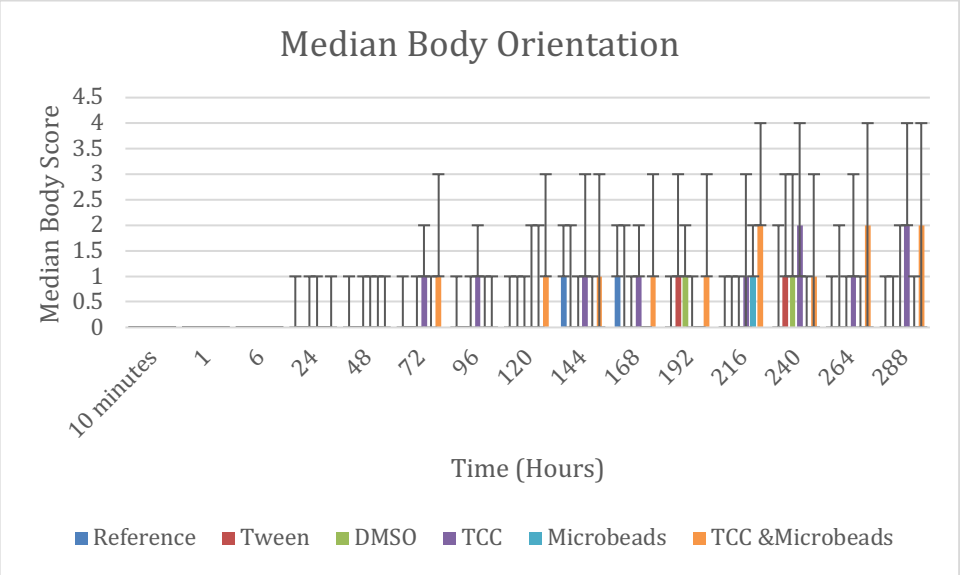

Figure 32 Toxicity Assay: Microbeads and Lower TCC: Swimming Style

The third behavioural assessment, swimming style, is depicted in Figure 32. Overall, the controls (Reference, Tween, and DMSO) received low behavioural scores with little variance; this is indicative that *D. magna* exposed to control (Reference) or vehicle control (Tween and DMSO) behaved with normal swimming styles. This was also true of the Microbead treatment, i.e. *D. magna*'s swimming pattern did not vary when exposed to the microbeads. Conversely, when exposed to the triclocarban treatments, *D. magna* displayed stressed swimming styles ($X^2 = 18.63$, $p = 0.01$). This was also true when comparing each triclocarban treatment independently against the controls (TCC and the controls was significantly different ($X^2 = 11.06$, $p = 0.025$); and TCC & Microbeads was significantly different from the controls ($X^2 = 11.9$, $p = 0.01$)). Lastly, the triclocarban treatments were not different from one another. These joint findings suggest *D. magna* do not show signs of distressed swimming styles when exposed to microbeads, but will show signs of stress when exposed to low concentrations of triclocarban.

4.5.4 Visual Observations of Microplastics and *Daphnia magna*

Again, visual observations of the *D. magna* with microbeads (through micrographs) confirm the bioavailability of this contaminant. *D. magna* were sacrificed

throughout this experiment by removing them from their test vessel, and observing them under the microscope, after which these *Daphnia magna* would be retired. Observations were made at various time intervals: 10 minutes; 4, 24, 48, 120, 144 and 264 hours. As seen in to the previous experiments there were time points (particularly the initial time points) where images of the microbead exposed *D. magna* did not yield micrographs with microbeads. Again, this was likely the result of the concentration of microbeads. Because an image of this was already portrayed in the previous section's visual results (see section 4.4.3, Figure 26), results will described now focus on images that contain microbeads.

Figure 33 depicts two daphnids, one from the reference (A and A') and one from the microbead exposure (B and B'), after 24 hours. The images (A) and (B) show the full body of these daphnids and microbeads do not appear on the carapace or secondary antennae. However, there does appear to be masses in the gut. Images (A') and (B') more clearly depict these masses as the spheres of microbeads in her gut, in contrast the reference gut is clear of debris.

A few days later, prior to refreshing the water (day 5), daphnids were again sacrificed and examined. These daphnids are represented in Figure 34. At 40x Magnifications the gut contents of the Reference (A) and the Microbead (B) do not appear very different. Visually, there is a difference between these two images and the TCC & Microbead daphnids (C), where her gut appears studded with dark spheres. The magnified image of their respective gut contents depicts debris in the reference daphnid's gut (A'). This is likely a result of build-up in the vessel from the first 5 days. The gut contents of the daphnid exposed to microbead alone has some microbeads, but the majority of her gut appeared to contain algae. In contrast, the TCC & Microbead daphnid has many microbeads visible in her gut. This observation was made again at the end of the 12 days in Figure 35. As a comparison through time, it would appear that the longer the *D. magna* are exposed to microbeads, the more microbeads they will ingest (as seen while comparing images in Figure 33 and Figure 35).

As with the last set of photos from the Toxicity Assays (section 4.4.3) daphnids were photographed with eggs or embryos in the brood chambers. Again had this experiment run longer seems likely they would have reproduced. Canniff & Hoang (2018) captured stunning photos of *D. magna* with intestines full of microbeads, yet reported no effect on survivorship or reproduction. The images in Figure 35 (along with bioassay endpoints) confirm that at least with microbeads alone, there is no impact to survivorship. Canniff and Hoang (2018) concluded a proportional relationship between increased microplastic concentration and ingestion rates of *D. magna*. The images in this current study would suggest another piece of the puzzle: with added stressors, *D. magna* may increase be increasing microplastic ingestion, despite fixed concentrations of food and microbeads.

Figure 33 Toxicity Assay: Microbeads and Lower TCC: 24 h Exposure : Reference (A) and Microbead (B) Exposed daphnids (40x Magnification); Gut content: Reference (A') and Microbead (B') at 100x Magnification

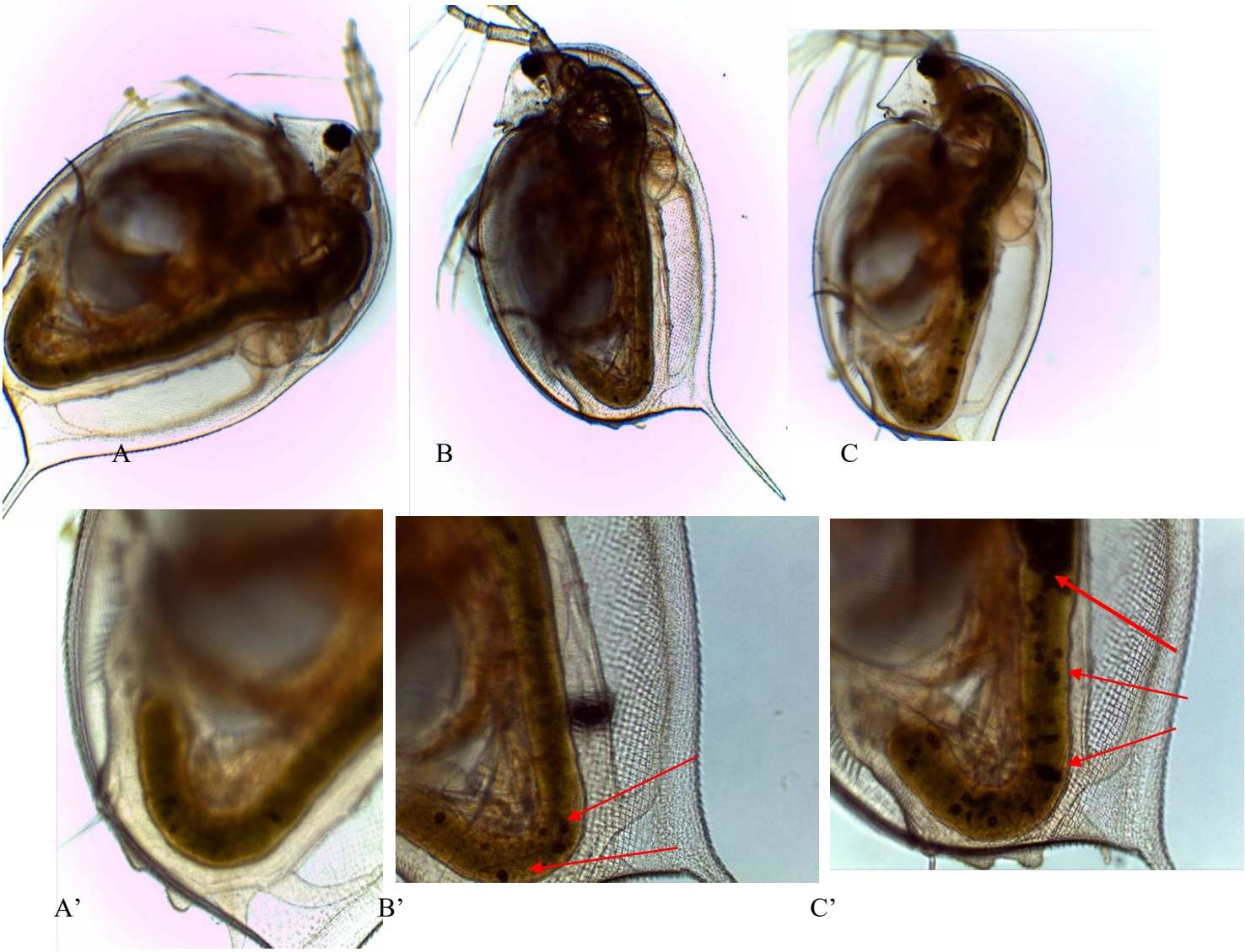

Figure 34 Toxicity Assay: Microbeads and Lower TCC: Exposure Day 5. Above: Reference (A), Microbead (B), TCC & Microbead (C) (40x Magnification). Below: Gut content of Reference (A'), Microbead (B'), and TCC & Microbead (C') (100x Magnification)

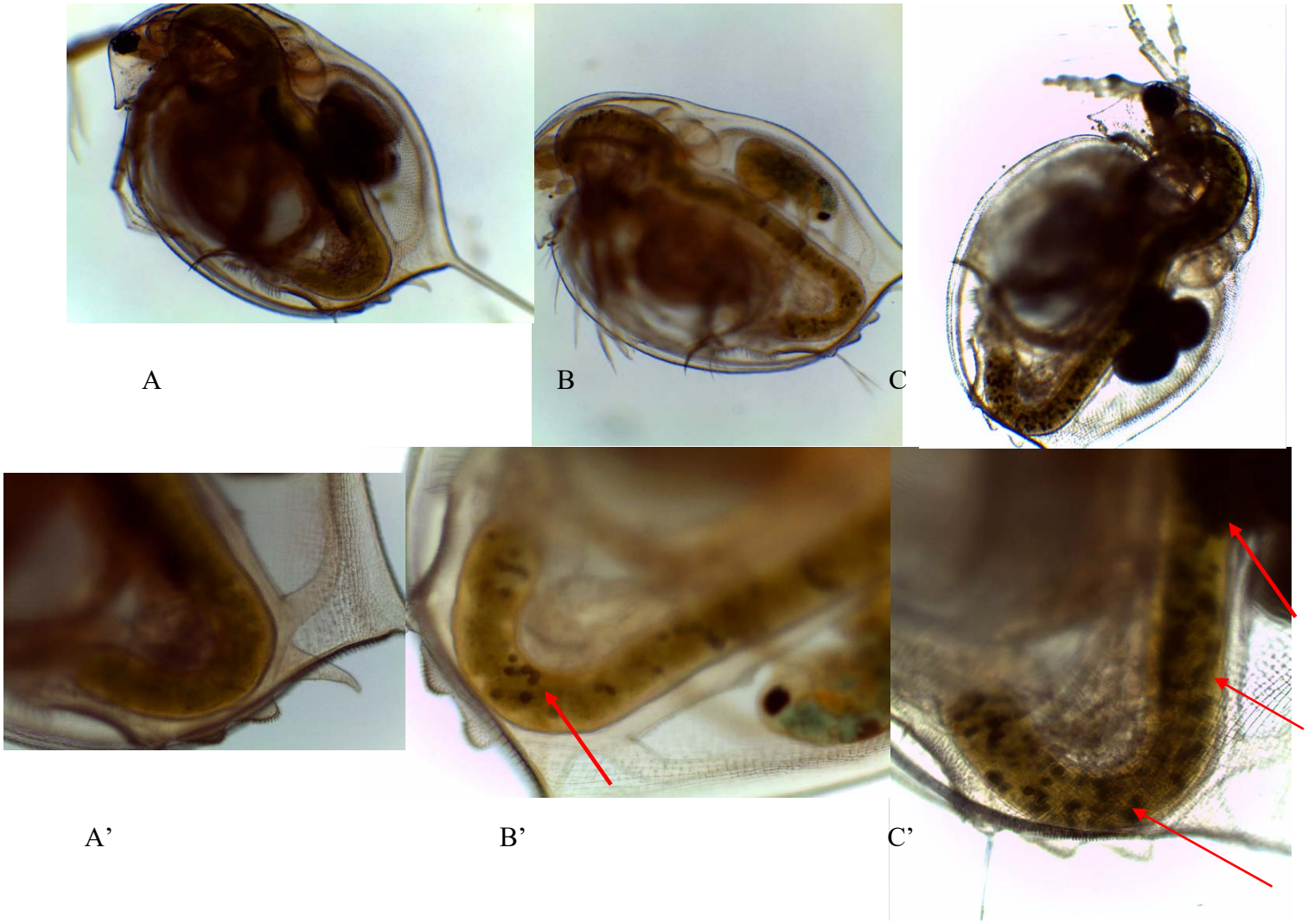

Figure 35 Toxicity Assay: Microbeads and Lower TCC: Exposure Day 12: Above: Reference (A), Microbead (B), TCC & Microbead (C) (40x Magnification). Below: Reference (A'), Microbead (B'), and TCC & Microbead (C') (100x Magnification)

Neonates born during the experiment were collected and examined daily. A representative of the reference (A), microbead (B), and TCC & Microbead (A) are depicted in Figure 36. Neonates did not appear to have microbeads in their gut, but microbeads were observed under the carapace and around the gut area (see the red arrows). Cui *et al. (*2017) found reproductive impairment (embryonic abnormal development and low hatching rates.) with *D. magna*. The size of this study did not permit any assessment of embryonic development; however, as a proxy to this, fully-formed neonates do not appear to have abnormal development.

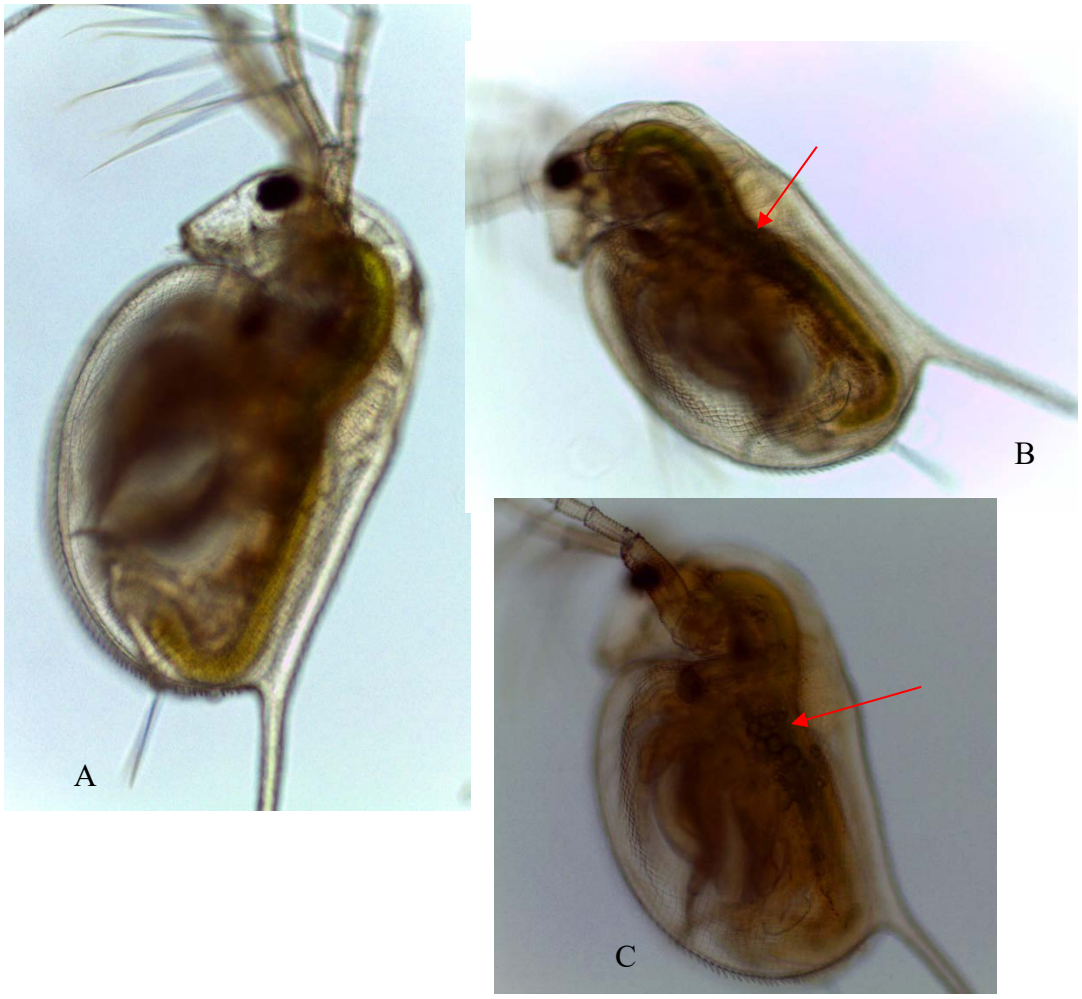

Figure 36 Toxicity Assay: Microbeads and Lower TCC: Neonates born during the experiment. Reference (A), Microbead Exposure (B), TCC & Microbead (C) (100x Magnification)

Deceased *D. magna* were examined before being discarded and Figure 37 depicts some of these daphnids. The single Reference daphnid (A) to die during the experiment did not photograph well, and there appears to be a shadow overlaying the image. For comparison, a deceased daphnid from the TCC treatment (B) was included here. Visually, the daphnids in the microbead treatments (C and D) appear more deteriorated. The daphnid from the Microbead treatment (C) appears to have two eggs on her back, suggesting she was persisting through different developmental stages. Microbeads are visible on her carapace (see red arrow). Image (D) is a daphnid from the TCC & Microbead treatment, and it is believed that, in death, the epidermis pulled away from the carapace, concentrating body fluid, which appears as a brownish colouration behind her gut.

Deceased *D. magna,* like all other deceased animals, and organic matter in the ecosystem, will decompose, feeding detritivores. Carapaces follow a similar route of feeding detritivores. Figure 38 depicts 3 carapaces picked up from 3 vessels: the Reference (A), Microbead (B) and TCC & Microbead (C). The microbeads adhering or inside of daphnids, (e.g. Figure 37, image C) or on the carapace could remain in the food web via these routes.

Figure 37 Toxicity Assay: Microbeads and Lower TCC: Deceased from Reference (A), TCC (B), Microbead (C) and TCC & Microbead treatments (40x Magnification)

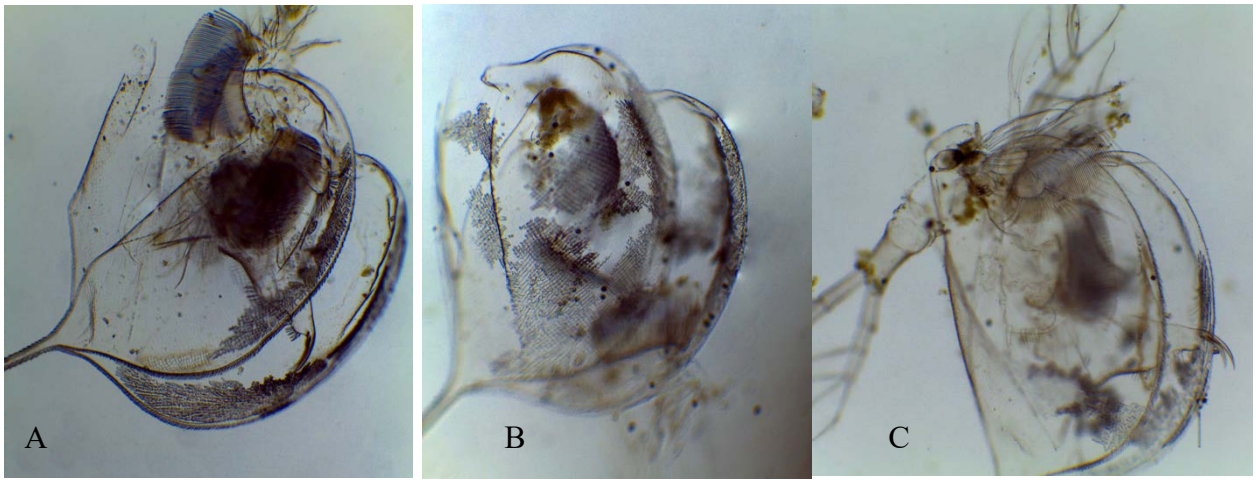

Figure 38 Toxicity Assay: Microbeads and Lower TCC: Carapaces found in Reference (A), Microbead (B) and TCC & Microbead vessels (40 x Magnification)

4.5.5 Conclusions from Toxicity Assay: Microbeads and TCC at Lower Dose

The Microbead and TCC toxicity repetition had strong controls, signifying conditions were tolerable for *D. magna* survival, reproduction, and for normal behaviour to be assessed. Additionally, the vehicle controls did not vary markedly from the Reference treatment, indicating that 0.1% Tween-20 and DMSO had no effect on bioassay endpoints. The Microbead treatment consistently yielded results similar to these controls. The TCC treatment did not affect mortality, mobility, or ability to swim through the water column. However, both triclocarban treatments did show stressed swimming styles (such as twirling and spinning). From a chronic perspective (considering the time interval 24 – 288 hours), the TCC & Microbead treatment increased mortality and decreased mobility.

Consequently, it was concluded that microbeads unaccompanied by additives or pollutants at environmentally-relevant concentrations do not impact *D. magna;* again, there is research both supporting and contesting the effects of microplastics. Furthermore, sensitivity to TCC at 5 ppb for effect on swimming style opposes Raby (2013)'s previous conclusions that swimming style begin showing an effect at 10 ppb. Furthermore, because of increased mortality in the TCC & Microbead treatment, it seems likely triclocarban and microbeads are acting synergistically at low concentrations of triclocarban. While neither TCC or the Microbead treatments independently impacted mortality, the effects of TCC & Microbeads increased mortality. Few studies have examined the effects of contaminants and microplastics.

Visually microbeads were again identified in the intestines and on the carapaces of *D. magna*. The images from this experiment are similar in concept to the images previously captured in the pilot studies and first trial of this toxicity assay. Neonates born throughout the experiment were also seen with microplastics. Furthermore, microbeads also appeared on deceased *D. magna*, and the empty carapaces. Again, these images contrast the conclusions drawn by Aljaibachi & Callaghan (2018), as microbeads were found in the guts of *D. magna.* Images of daphnids from the TCC & Microbead treatment appeared to have ingested more microbeads than the daphnids from the Microbead

treatment. These preliminary photos may be hinting at a separate unknown: *D. magna* in the presence of a contaminant (triclocarban) may not be discriminating against microbeads. Lastly, neonates born during these experiments did not appear to have abnormalities or developmental issues as stated in Cui *et al*. (2017).

5 Overall Summary and Future Directions

This thesis fulfilled its three complementary objectives. First, healthy age-synchronized *Daphnia magna* were cultured for use in toxicity assays. This occurred in the Ryerson-Lab and in the Home-Lab condition. Subsequently, healthy age-synchronized *D. magna* were used to further refine sub-lethal bioassay endpoints, specifically emphasizing behaviour and reproduction. Subsequently, these sub-lethal bioassay endpoints were used alongside mortality to assess the toxicity of microbeads with the added contaminant, triclocarban. The Home-Lab had suitable conditions for culturing of healthy age-synchronized stocks of *Daphnia magna*, and their behaviour could be well documented for the use in bioassays as an endpoint.

From an ecotoxicology standpoint, bioassays are crucial for determining impact to the organism. Lethality is traditionally used as a bioassay endpoint, however, sublethal endpoints are important as early-warning signals. Behaviour offers a unique perspective, as it presents a whole-organism approach, representing the cumulative environmental stress on an organism. Abundance and distribution of a contaminant does not necessitate a negative impact to a particular animal. The literature is quite clear that microplastics are ubiquitous; however, research surrounding impact is still novel. Currently, microplastic impact is under debate by many researchers. This is also true when considering the weight of evidence for impacts of microplastics on *Daphnia* spp.. Part of this confusion stems from microplastics being treated as a single contaminant (rather than as a class of contaminants), and from a lack of environmental relevancy in bioassays.

This toxicity assay exposed *Daphnia magna* to microbeads of polyethylene at environmentally-relevant concentrations (0.025 mg/L = 3.2 microbeads/mL) during short- and long-term assessment periods. Alone, microbeads were ingested by *D. magna*, and did not result in any observable impact to mortality, behaviour or reproduction endpoints. Additionally, *D. magna* were also exposed to triclocarban at 10 ppb and 5 ppb. *D. magna* were sensitive to triclocarban at 10 ppb, but did not show signs of stress at 5 ppb.

The TCC & microbead treatments had varying effects depending on the concentration of TCC. At higher concentrations (10 ppb of TCC) the microbeads appeared to have an antagonistic effect, delaying the onset of the TCC. At lower concentrations (5 ppb of TCC), the microbeads appeared to have a synergetic effect, magnifying their combined effects. Microbeads were confirmed on and in *D. magna* in the pilot and toxicity assays by identifying them with a compound light microscope. *D. magna* did not appear to discriminate against microbeads during feeding processes.

5.1 Future Directions and Limitations

The experiments that unfolded here were very preliminary and need to be part of on-going research involving microplastics. To confirm these preliminary findings, a larger summation experiment needs to be set up including both concentrations of triclocarban with and without the microbeads. Additionally, there needs to be an analysis of the interactions between the microbeads and triclocarban. A further investigation needs to examine the effects of tween and the hydrophobic carapace. No chemical analysis was completed. Likewise, this research lacks the experimental design to include the chemistry of inclusion channels with triclocarban and microbeads on and in *D. magna*.

Research in this area needs to dovetail with environmentally-relevant concentrations of microplastics and logically-sound experimentation to provide evidence for the effects of microplastic pollution. It is also crucially important that we examine early-warning signs as concentrations of microplastics are expected to rise. Additionally, rigorous bioassay designs are needed for conclusive consistent results. Further bioassays should be set up to examine the physical and chemical characteristics of microplastics (as a physical and chemical contaminant); and experiments should be designed such that they can be repeated with different polymers, shapes, and sizes.

6 Appendix

6.1 Bold-Basal Medium For Algae Cultures

To prepare the Bold-Basal medium, concentrations of stock nutrients were prepared in tables 1, 2, and 3 below. This recipe is based on Raby (2013) "Modified Bristol Medium." Each nutrient was in solid form, weighed using an analytical scale and combined with MilliQ water in individual volumetric flask. Macronutrients were combined with 500 mL, minor nutrients were combined in 250 mL, and micronutrient were all added 1 L volume. Tables 1, 2, and 3 list the concentration in g/L. The stock solutions were then filter-sterilized using a vacuum, this is suggested in Environment Canada 2007 instead of autoclaving the solutions. The sterilized stock solutions were then stored in glass bottles.

Table 1: Macronutrient for Bold-Basal Medium

Stock Solution	Concentrations g/L
1. Sodium Nitrate, NaNO3	25
2. Calcium chloride, CaCl2	1.89
3. Magnesium sulfate heptahydrate, MgSO4 7H2O	7.5
4. Dipotassium hydrogen phosphate, K2HPO4	7.5
5. Monopotassium phosphate KH2PO4	17.5
6. Sodium chlorine, NaCl	2.5

Table 2: Minor Nutrient for Bold-Basal Medium

Stock Solution	Concentrations g/L
7. Disodium ethylene diamine tetracetic acid, Na2EDTA	50
8. Potassium hydroxide, KOH	6 mol/L
9. Ferric chloride hexahydrate, FeCl3 6H2O	4.84
10. Boric acid, H3BO3	11.4

Table 3: Micronutrient for Bold-Basal Medium

Stock Solution	Concentrations g/L
1. Manganese chloride, MnCl2 4H2O	0.385
2. Zinc sulfate heptahydrate, ZnSO4 7H2O	2.205
3. Sodium molybdate dihydrate, Na2MoO4 2H2O	0.30
4. Copper sulfate pentahydrate, CuSO4 5H2O	0.395
5. Cobalt dinitrate, Co(NO3)2	0.079

Stock solutions were then combined in the concentrations listed in Table 4 into a large 2 L glass vessel, and topped up to 1 L with MilliQ water and inoculated with algae. Vessels were covered with a lid, but not sealed and placed on a shaker. This allowed for airflow, but maintained sterility. The algae were allowed to grow until vessels were dark green, at which point they were removed from the stir plate, sealed and stored in a refrigerator for 24-h. After such time, the algae were concentrated by decanting around a Bunsen burner (for sterility), this was done by gently removing the top half of the growth medium. Once concentrated, the monocultures were stored in amber glass bottles for up to two months.

Table 4: 1 L Batch of Bold-Basal Medium

Stock Solution	mL
1. Sodium Nitrate, NaNO3	5
2. Calcium chloride, CaCl2	5
3. Magnesium sulfate heptahydrate, MgSO4 7H2O	5
4. Dipotassium hydrogen phosphate, K2HPO4	5
5. Monopotassium phosphate KH2PO4	5
6. Sodium chlorine, NaCl	5

7. Disodium ethylene diamine tetracetic acid, Na2EDTA	0.5
8. Potassium hydroxide, KOH	0.5
9. Ferric chloride hexahydrate, FeCl3 6H2O	0.5
10. Boric acid, H3BO3	0.5
11. Micronutrients	2

6.2 *Daphnia* growth medium

*D. magna w*ere cultured in semi-defined growth medium, which consisted dechlorinated water, algae, and vitamins. Growth medium was prepared in 1-L bottles and then poured or pipetted into vessels as needed. Preparing the media in 1-L batches ensured consistency in each batch.

1 L of *Daphnia* growth medium consisted of:

25 mL of *Raphidocelis* (at a concentration of 10^6 cells/mL)

25 mL of *Chlorella* (at a concentration of 10^6 cells/mL)

1 mL of B12

1 mL of Selenium

Topped up to 1 L with dechlorinated municipal drinking water.

6.2.1 Stock Solution of Vitamin B12

Vitamin B12 was prepared according the Raby (2013), who in turn based her information of MOE (2012) standards. The stock solution for vitamin B12 was 10 mg/L. This was prepared my weighing out 0.001 g (1 mg) of vitamin B12 and dissolving it in 100 mL of deionized water, in a volumetric flask. With this stock solution, "*Daphnia* growth medium" would contain 1 μg of B12 / L of solution. Vitamin B12 was stored in an amber bottle, in the refrigerator and could be kept for 4 weeks.

6.2.2 Stock Solutions of Selenium

Stock solutions of Selenium were also prepared according to Raby (2013) (again she based her stock solution from MOE 2012). The Selenium stock solution was 3 mg/L. This was prepared by weighing out 1.4 mg of sodium selenite decahydrate ((Na_2SeO_4

$10H_2O$) and dissolving it in 100 mL of deionized water in a volumetric flask. With this stock solution, "*Daphnia* growth medium" would contain 3 μg of Selenium / L of solution. Selenium was stored in a media vessel for up to 1 year, in the refrigerator.

6.4 Microbead Calculations: Ryerson-Lab Pilot Study

Given:
0.1 g microbeads dissolved in 8 mL of Tween solution
Sized: 10-20 μm diameter,
Density: 1.026 g/cm^3
Spherical in shape

1) Using Concentrations

0.1 g microbeads/ 8 mL = 0.0125 g/mL
= 12.5 g/L
= 12 500 mg/L

$C_1V_1 = C_2V_2$
(12 500 mg/L)V_1 = (12.5 mg/L) (100 mL)
V_1 = 0.1 mL

0.1 mL of stock solution added to each vessel.

2) Using the g amount and density the volume of the total amount can be calculated:

0.1 g ÷ 1.026 g/cm^3 = 0.0975 cm^3

Assuming a normal distribution of microbeads between 10-20 μm, the average diameter size should be 15 μm.
Volume can be calculated by $\frac{4}{3}\pi r^3$
The volume of an individual bead should be 1 767.15 μm^3 = 1.7 x 10^{-9} cm^3

Total volume ÷ Volume of 1 bead = Number of beads
0.0975 cm^3 ÷ 1.7 x 10^{-9} cm^3 = 55 173 713 beads in 0.1 g
→ 55 173 713 beads in 8 mL = 6 896 714 beads/mL
→ 6 896 714 beads in 0.1 mL (exposed to *Daphnia)*

6.6 Microbead Calculations: Home-Lab Pilot Study

Given:
0.5 g Microbeads
Sized: 20-27 μm diameter,
Density: 1.025 g/cm^3
Spherical in shape
1.47 x 10^8 Beads/g (Manufacturing detail)

Total volume of microbeads can be calculated based on amount and density
0.5 g ÷ 1.025 g/cm^3 = 0.487 cm^3
= 0.487 mL

Advised to dissolve in 0.1% Tween-20 solution 5:1 v/v (Manufacturing detail)
→ Theoretically 2.44 mL of 0.1% Tween-20 solution
→Used 3 mL

June Pilot Study concentration:
0.5 g microbeads/ 3 mL = 0.167 g/mL
= 166.67 g/L
= 166 666. 67 mg/L

$C_1V_1 = C_2V_2$
(166 666. 67 mg/L)*(0.001 mL) = C_2 (100 mL)
C_2 = 1.67 mg/L

0.001 mL of stock solution added to each vessel, final concentration of 1.67 mg/L.

Assuming an even distribution of microbeads in the solution:
Using the amount of microbeads and the manufacturing beads/g we can calculate number of microbeads in total, beads/L, and beads exposed to *Daphnia*
1.47 x 10^8 Beads/g * 0.5 g = 73 500 000 Beads
→ 73 500 000 Beads in 3 mL = 24 500 000 beads/mL
= 24 500 000 000 beads/L
→24 500 beads in 0.001 mL (exposed to *Daphnia)*

6.8 Microbead Calculations: Toxicity Assay: Microbeads and TCC

Given:
0.5 g Microbeads
Sized: 20-27 μm diameter,
Density: 1.025 g/cm^3
Spherical in shape
1.47 x 10^8 Beads/g (Manufacturing detail)

Total volume of microbeads can be calculated based on amount and density
0.5 g ÷ 1.025 g/cm^3 = 0.487 cm^3
= 0.487 mL

Advised to dissolve microbeads in ~ 20 mL 0.1% Tween-20 solution (Correspdence with Cospheric July 2020)
→ Already in 3 mL, topped up to 20 mL
Checked with hemocytometer for density of beads in stock solution: 3.2 x 10^6 microbeads/mL

July Toxicity concentration:
0.5 g microbeads/20 mL = 0.025 g/mL
= 25 g/L
= 25 000 mg/L

$C_1V_1 = C_2V_2$
(25 000 mg/L)*(0.0001 mL) = C_2 (100 mL)
C_2 = 0.0025 mg/L

0.0001 mL (0.1 μL) of stock solution added to each vessel (100 mL), final concentration of 0.0025 mg/L.

Assuming an even distribution of microbeads in the solution:
Using the density of microbeads in the stock solution (measured my hemocytometer), and the volume of stock solution added to vessels, we can calculate concentration of microbeads exposed to *Daphnia*
3.2 x 10^6 microbeads/mL * 0.0001 mL = 3.2 x 10^3 microbeads
3.2 x 10^3 microbeads in 0.1 μL stock solution (exposed to *Daphnia)*

→ $C_1V_1 = C_2V_2$
(3.2 x 10^6 microbeads/mL) * (0.0001 mL) = C_2 (100 mL)
C_2 = 3.2 microbeads/mL exposed to *Daphnia*

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