THE DISTRIBUTION AND EFFECTS OF MICROBEADS ON HOST-PARASITE INTERACTIONS OF ONTARIO WETLAND FAUNA: TADPOLES, SNAILS AND TREMATODES

By

Mary Katherine Claudia Balsdon

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ABSTRACT

The distribution and effects of microbeads on host-parasite interactions of Ontario wetland fauna: tadpoles, snails and trematodes

Mary Katherine Claudia Balsdon

Master of Applied Science, 2018

Program of Environmental Applied Science and Management

Ryerson University

Plastic microbeads pose an environmental problem as they easily enter into waterbodies, take a long time to break down, and their ingestion can have negative effects on aquatic organisms. I found that microbead consumption had a significant negative effect on the growth of northern leopard frog (*Lithobates pipiens*) tadpoles, as well as their susceptibility to trematode parasite (*Echinostoma trivolvis*) infection, but minimal effects on leukocyte profiles and infection tolerance. Freshwater snails (*Stagnicola elodes*) given microbead diets exhibited a non-monotonic response in their production of trematode (*Haematolechus parviplexus*) infectious stages, with those in the highest microbead treatment tending to exhibit greater growth and shorter longevity. I also found that algae (*Chlorella pyrenoidosa*) and microbeads created density-dependent aggregations that could be a potential ingestion pathway for herbivorous fauna. Lastly, I found many particles in Ontario wetlands and ponds that may be used for controlled pesticide release, thus potentially posing a threat.

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Chapter 1 General introduction

Introduction

Format of Thesis

This thesis is presented in a manuscript-based format. Chapter One is a literature review. The following chapters (Chapter Two, Three, Four, and Five) are stand-alone papers. Each of these chapters include its own abstract, introduction, methods, and discussion. Since Chapter One is a literature review, there will be some repetition in the proceeding chapters. Chapter Two and Three will be submitted for publication. Chapter Six concludes the thesis with discussion, conclusions, and future work.

Contributions of Authors

Manuscript in Chapter Two, Three, Four, and Five

Mary Balsdon and Dr. Janet Koprivnikar both contributed to the conceptual design, data analysis and writing, while Mary Balsdon performed the experiments. For Chapter Five, Dr. Chelsea Rochman gave feedback, advice and the facility for the analysis of field samples.

Overview

Worldwide, waterbodies are contaminated by many compounds, including various plastics. Small plastic particles are called microplastics. Microbeads are a type of microplastic with the diameter ranging from 0.1 μm to 5 mm (Government of Canada, 2017). Microbeads are commercially available in sizes 10 μm to 1 mm and are used in various industries including: automobile, industrial, medical, agricultural and cosmetic (Pettipas *et al*., 2016). In the cosmetic industry, microbeads are used as abrasives in personal care products such as facial cleansers and toothpaste. The problem with using microbeads in these products is that once used, they get washed down the drain and can contaminate aquatic ecosystems because wastewater treatment plants are not designed to handle them. Notably, treatment plant filters are not fine enough to catch microbeads and so they end up in waterbodies through various pathways (Rochman *et al.*, 2015). The final effluent containing microbeads can be discharged directly into waterbodies or enters by runoff from biosolids applied to some agricultural land (United States Environmental Protection Agency, 2007; Saruhan *et al*., 2010). In the agricultural industry, microplastics are used in applications including the controlled release of pesticides (Roy *et al*., 2014; Campos *et*

al., 2015). These microplastics can enter waterbodies as well by runoff from some agricultural fields, so not necessarily entering in all inland waterbodies. By entering into waterbodies, microbeads can have negative effects on animals in aquatic ecosystems. Freshwater fauna are the most imperiled of all in North America (Ricciardi & Rasussen, 2009), so it is important to understand how habitat contamination by microbeads may affect such organisms.

Although there has been much recent attention paid to the problems associated with microplastic contamination of aquatic ecosystems, most studies have been limited to marine environments. Those studies that have been conducted on the distribution and abundance of microplastics in freshwater, have focused on the Great Lakes (Zbyszewski & Corcoran 2011; Eriksen *et al*., 2013 Castañeda *et al*., 2014; Zbyszewski *et al*., 2014; Corcoran *et al*., 2015; Ballent *et al*., 2016). Consequently, the occurence of microplastics in other smaller freshwater bodies, like wetlands and ponds is unknown, or their possible consequences for aquatic organisms using those habitats. In wetlands and ponds, herbivorous organisms like *Stagnicola elodes* (freshwater snails) and *Lithobates pipiens* tadpoles (northern leopard frog) could potentially ingest microplastics through the aggregation of algae upon which they feed, such as *Chlorella pyrenoidosa* and microplastics (Lagard *et al*., 2016). Beyond direct effects on these free-living animals, *S. elodes* and *L. pipiens* are potential hosts for parasites as well, and thus could also suffer indirect effects of microbead ingestion through alterations of host-parasite dynamics. For instance, the trematode parasites, *Echinostoma trivolvis* and *Haematolechus parviplexus* have free-living aquatic stages which are susceptible to environmental contaminants, and their production and transmission success could be affected by microbeads if these influence their snail and amphibian hosts (Pietrock & Marcogliese, 2003) . It is therefore important to understand the occurrence of microplastics in various freshwater ecosystems, as well as their potential direct and indirect effects on aquatic organisms if ingested, to know the extent to which microplastic pollution can cause harm.

Consequently, the mandate of this thesis was to attempt to build upon previous microplastic literature, (see below) that largely pertains to marine systems and to the Great Lakes to carry out specific aims: 1) determine the distribution and abundance of microplastics in ponds and wetlands by creating an approach for sampling; 2) explore a possible pathway of microbead ingestion by aquatic herbivores through their aggregation with a common freshwater algae

(*Chlorella pyrenoidosa*); and, 3) determine the effects of microbead ingestion on the common herbivorous freshwater organisms (*S. elodes* and *L. pipiens*) both directly, and also with the added stressor of parasites (*E. trivolvis* and *H. parviplexus*).

Microbead properties and occurrence in freshwater ecosystems

Properties of microbeads

Microbeads are made of various types of plastic, which allows variation in shape, size and purpose. The types of plastic used for microbeads include: polyethylene, polyethylene terephthalate, polypropylene, polyamide, polyester, polystyrene and polyvinyl chloride. Depending on the type of plastic used, this affects its density, and ultimately determines the fate of microbeads made from that material in aquatic ecosystems. Polyethylene and polypropylene are typically low-density plastics, and are therefore buoyant, initially ending up in the surface water. Polyamide, polyester, polystyrene and polyvinyl chloride are high-density plastics which sink to the sediment (Cole *et al*., 2011). The water turbulence also affects the fate of microbeads in aquatic environments.

Sampling methods of microplastics in freshwater

Microplastics have been found not just in marine environments, but also in freshwater bodies. Previous studies have detected microplastics in various locations across the world from, Asia, Europe and North America (**Table 1.1**).

Table 1.1: Freshwater microplastic studies

Study/Authors	Water Body/ Location	Size Classes/ Mesh Size	Sampling and Analysis Method	Surface Water or Sediment	Max Abundance
Anderson *et al*., 2017	Lake Winnipeg, Canada	Size Classes: >333 µm, <5 mm Mesh Size: 333 µm	Manta trawl, scanning electron microscopy with energy dispersive x ray spectroscopy	Surface water	748 027 microplastics/km^2
Ballent *et al*., 2016	Lake Ontario, Canada	Size classes: 2 mm, 5.6 mm	Sediment trap, core and grab sampling techniques, Raman spectroscopy, x ray fluorescence spectrometry	Sediment	~ 28 000 particles per kg dry sediment
Castañeda *et al*., 2014	St. Lawrence River, Canada/USA	Mesh Size: 500 µm	Ponar grab, Peterson grab, dissecting microscope, scanning calorimetry	Sediment	~136 926 items/m^2
Corcoran *et al*., 2015	Lake Ontario, Humber Bay, Canada	Size Classes: <0.5 mm, 0.5-0.1 mm, 0.71-0.85 mm, 0.85-1 mm, >1 mm	CCGS Limnos using a mini boss borer, Fourier transform infrared spectroscopy	Sediment	Humber Bay: 1634 2118 pellets Lake Ontario: 35 microplastic particles in box cores
Eriksen *et al*., 2013	Lake Superior, Lake Huron, Lake Erie, Canada/USA	Size Classes: 0.355-0.999 mm, 1-4.74 mm, >4.75 mm Mesh Size: 333 µm	Manta trawl, scanning electron microscopy	Surface Water	463 423 items/km^2
Faure *et al*., 2012	Lake Geneva, Europe	Size Classes: >5 mm, <5 mm Mesh Size: 300 µm	Manta trawl, sieve	Surface Water	48 146 items/km^2 water
Free *et al*., 2014	Lake Hovsgol, Mongolia, Asia	Size Classes: 0.355-0.999 mm, 1-4.749 mm, >4.75 mm Mesh Size: 333 µm	Transect line, manta trawl	Surface Water	44 435 items/km^2
Lechner *et al*., 2014	Danube River, Australia, Europe	Size Classes: <2 mm, 2-20 mm	Stationary conical driftnets	Surface Water	141 647.7 items/ 1000 m^3
Sadri & Thomposn 2014	Tamar estuary, United Kingdom, Europe	Size Classes: <1 mm, 1-3 mm, 3-5 mm, >5 mm	Manta net, Fourier transform infrared spectroscopy + Bruker Hyperion 100 microscope	Surface Water	204 pieces of plastic
Vaughan *et al*., 2017	Edgbaston Pool, Birmingham, United Kingdom	Size Classes: >1 mm, 500 µm-1 mm	Transect lines, boat using HTH gravity corer	Sediment	25-30 particles/ 100g dried sediment
Zbyszewski & Corcoran 2011	Lake Huron, Canada/USA	Size Classes: <5 mm, >5 mm	Stainless steel trowel, transect line, Fourier transform infrared spectroscopy and scanning electron microscope	Sediment	3209 pieces of plastic
Zbyszewski *et al*., 2014	Lake Erie, Lake St. Clair, Canada/USA	Size Classes: <2 cm	Steel trowel, transect line Fourier transform infrared spectroscopy and scanning electron microscope	Sediment	Lake Erie: 1576 pieces of plastic Lake St. Clair: 817 pieces of plastic

These studies have used different techniques and identification methods to determine the presence of microplastics in freshwater bodies. These techniques also vary depending upon whether sampling was from the sediment or surface water. For example, Zbyszewski & Corcoran (2011) sampled the sediment of Lake Huron using a stainless steel trowel across a transect line, and identified the microplastics found by Fourier Transform Infrared Spectroscopy (FTIR) and scanning electron microscopy. Some other methods for sampling sediment include: sediment traps, core and grab sampling techniques (Ballent *et al*., 2016), ponar grab, Peterson grab (Castañeda *et al*., 2014), using a mini boss borer from a Canadian Coast Guard Ship (CCGS) (Corcoran *et al*., 2015), using a HTH gravity corer with transect lines and a boat (Vaughan *et a*l., 2017), and using a steel trowel (Zbyszewski *et al*., 2014).

There are also various methods of identifying micoplastics including: FTIR (Zbyszewski *et al*., 2014; Corcoran *et al*., 2015), scanning calorimetry (Castañeda *et al*., 2014), x-ray fluorescence spectroscopy (XRF), and Raman spectroscopy (Ballent *et al*., 2016). FTIR functions by identifying particles via the shining of an infrared light and an instrument that measures which wavelengths are absorbed (Hollas, 2004). The computer takes the data and conducts a math process (Fourier Transform) to generate the readable absorbance spectrum. The resulting spectrum is then compared to a database to find a match and identify the material (Hollas, 2004). XRF measures the energy of electrons moving to different orbitals to identify a specific plastic. Scanning calorimetry identifies a plastic by determining the amount of heat required to increase the temperature of a given sample. Raman spectroscopy is based on the scattering of monochromatic lights from a laser source (Hollas, 2004). The photons of the laser are absorbed by the sample and reemited. The frequency of the reemited photons is shifted up or down in comparison with the original monochromatic frequency- this is called the Raman effect (Hollas, 2004). Various approaches are thus used to collect microplastics and identify the plastic polymers.

For instance, Eriksen *et al*., (2013) collected microplastics in the surface waters of Lake Superior and Lake Huron using a manta trawl and identified the particles caught with scanning electron microscopy. A manta trawl with a net, or in combination with a sieve seems to be the most common method of microplastic collection in surface water (Faure *et al*., 2012; Eriksen *et al*., 2013; Lechner *et al*., 2014; Anderson *et al*., 2017). Another method for surface water

sampling of microplastics is to use stationary driftnets (Sadri & Thompson, 2014). For sampling the surface waters of small ponds and wetlands, it is not feasible to use a manta trawl to collect microplastics. Ideally, there should be a method to sample the surface water in such a way that it can be sieved to collect microplastics within specific size ranges. However, identification methods for microplastics are similar regardless of sampling either the sediment or surface water. For surface water, microplastics are often identified using scanning electron microscopy with energy dispersive x-ray spectrometry (Anderson et al., 2017) or FTIR (Sadri & Thompson, 2014).

There are no standardized approaches for sampling and identifying microplastics in aquatic ecosystems because it is not practical to do so considering the variation in environments and plastics, and there are always factors like location, accuracy and feasibility to consider (Rochman *et al*., 2017; Shim *et al*., 2017). It is also difficult to have standardized approaches for sampling microplastics because of the very nature of these tiny plastics in that they vary in size, shape, colour, and polymer type, making it a challenge to compare various microplastic data (Shim *et al*., 2017). However, some aspects of the methodologies for sampling and identifying microplastics have the same general procedures. For sampling microplastics in the sediment or surface water, there is always a size range of the specific microplastics of interest, methods for the extraction of microplastics from collected samples, and a way to identify and confirm a sample as a microplastic (Rochman *et al*., 2017). By having these same basic steps in various microplastic studies, it allows the same general procedure to be applied in different waterbodies for comparative purposes. Importantly, it is vital to understand how widely microplastics are distributed across various freshwater bodies to know the extent of the problem that they might pose to aquatic fauna.

Microplastics in the Great Lakes

The Great Lakes in Canada and the United States contain 18% of the world's fresh surface water, and are a source of drinking water for 10 million Canadians (Environment and Climate Change Canada, 2017). Microplastics contribute to the other contaminants and toxic substances which enter the Great Lakes. The known studies of microplastics in the Great Lakes with given latitude and longitude coordinates were mapped to illustrate their distribution (**Figure 1.1**).

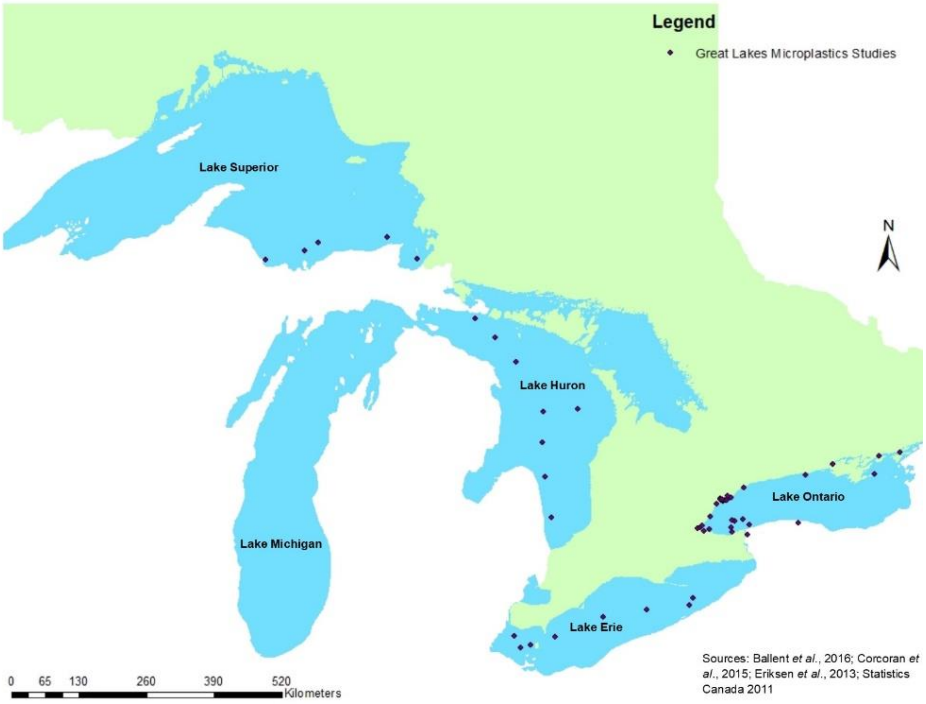

Figure 1.1: Microplastics studies in the Great Lakes based on published literature with given latitude and longitidue coordinates.

To date, studies have shown that thousands of microplastics are distributed in each of the Great Lakes (Zbyszewski & Corcoran 2011; Eriksen *et al*., 2013; Castañeda *et al*., 2014; Zbyszewski *et al*., 2014; Corcoran *et al*., 2015; Ballent *et al*., 2016). However, it is unknown whether microplastics from the Great Lakes can be re-distributed into other freshwater bodies, such as coastal wetlands, or the extent to which they occur in spatially-separated habitats such as inland ponds and wetlands. While the Great Lakes are a critical habitat for freshwater organisms, and a vital resource for humans, microbeads may affect other smaller aquatic ecosystems that also serve key ecologial functions.

Wetlands

Wetlands are defined as land that is saturated by surface or ground water regularly for a duration that is suffificient to support vegation adapted for saturated soil conditions (Davis, 1994). Canada is home to 6% of the world's wetlands (Ontario Ministry of Natural Resources and Forestry, 2017). Ontario contains 25% of Canada's wetlands, covering over 330 000 square kilometers (Ontario Ministry of Natural Resources and Forestry, 2017). The level of water saturation determines how the soil develops and the types of plants and animals that live in and around a wetland. There are four types of wetlands, which include bogs, fens, swamps and marshes. Bogs are old wetlands, representing areas of peat depressions which get filled with water by precipitation or surface runoff and are strongly acidic and are nutrient-poor (Ontario Ministry of Natural Resources and Forestry, 2017). Bogs contain Sphagnum mosses, ericaceous shrubs and black spruce trees (National Wetlands Working Group, 1997). Fens are similar to bogs but only receive water from melted glaciers through the ground and are thus less acidic and more nutrient-rich than bogs (Ontario Ministry of Natural Resources and Forestry, 2017). The vegetation in fens includes black spruce, tamarack, sedges, grasses and various mosses (National Wetlands Working Group, 1997). Swamps are considered to be woodeded or forested wetlands with coniferous or deciduous trees (National Wetlands Working Group, 1997). Marshes are defined as either coastal (being primarily along marine coastlines or that of large water bodies) or inland if they are along the borders of streams and rivers (Ontario Ministry of Natural Resources and Forestry, 2017). The vegetation in marshes typically consists of reeds, rushes, sedges, cattails, pickerel weed, arrowheads, pond lilies and coontails (National Wetlands Working Group, 1997).

In Ontario, wetlands along the Great Lakes are known as the Great Lakes Coastal wetlands. These wetlands are very valuable as they improve the water quality of the Great Lakes by filtering pollutants, protecting against flooding and erosion damage, cycling nutrients and organic matter, and providing habitat for various animals (McGarry, 2014). Ponds are small bodies of water which have a maximum depth of 8 m and light reaching the bottom, resulting in temperatures that are relatively uniform (Oertli *et al*., 2005). For my thesis, ponds and wetlands were chosen for the sampling of microplastics based upon having a location adjacent to the Great Lakes and/or those known to have snails and tadpoles present to determine if these freshwater fauna are realistically exposed to microbeads. It is important to understand the distribution and abundance of microplastics in ponds and wetlands because these could have negative impacts on various species within them, and ultimately on these ecosystems.

Potential for effects of microplastics in aquatic ecosystems

Microplastic effects on freshwater animals

Although there have been various studies looking at the effects of microplastics on freshwater organisms from all around the world (**Table 1.2**), many potential influences are still unknown (Pettipas *et al*., 2016).

Table 1.2: Studies looking at the effects of microplastics on freshwater organisms

Study/Authors	Microplastic Type	Microplastic Size and Concentration	Taxa	Location	Length of Exposure	Effects of Exposure
Blarer & Burkhardt-Holm 2016	Polystyrene beads	Size:1.6 µm Concentration: 500, 2500, 12 500, 60 000 beads/mL water	*Gammarus fossarus* (amphiopod)	Europe	28 days	Reduced assimilation efficiency
Grigorakis *et al*., 2017	Polyethylene microbeads	Size: 63 µm Pellet with 50 particles	*Carassius auratus* (gold fish)	East Asia	1.5 hours to 6 days	Microbeads did not accumulate in the gut
Hu *et al*., 2016	Polystyrene microspheres	Size: 1 µm and 10 µm Concentration: 1 µm- 10, 10^3, 10^5 particles/mL; 10 µm- 0.1, 10, 10^3 particles/mL	*Xenopus tropicalis* tadpoles (tropical clawed frog)	Gambia, Ghana	1-48 hours	Tadpoles ingest and egest microplastics fairly quickly
Imhof & Laforsch 2016	Mixture of polyamide, polyethylene terephthalate, polycarbonate, polystyrene, polyvinylchloride	Size: 4.64-602 µm Concentration: 0%, 30% and 70% on top of diet food	*Potampoyrgus antipodarum* (mud snail)	New Zealand	8 weeks	No effects on morphology and shell thickness
Karami *et al*., 2016	Polyethylene with phenanthrene	Polyethylene 50 or 500 µg/L and treatments loaded with phenanthrene 10 or 100 µg/L in water	*Clarias gariepinus* (juvenile African catfish)	Africa and Middle East	92 hours	Phenathrene treatments increased degree of tissue change in the liver and decreased transcription levels
Lönnstedt & Eklöv 2016	Polystyrene microplastics	Size: 90 µm Concentration: 0, 10 000, 80 000 particles/m^3 in water	*Perca fluviatilis* (European perch)	Europe	24 hours	Inhibits hatching, decrease growth rates, alters feeding preference, innate behaviours
Lu *et al*., 2016	Polystyrene microplastics	Size: 5 µm, 70 nm Concentration: 20, 200, 2000 µg/L	*Danio rerio* (zebrafish)	Himalayan region	3 weeks	Microplastic accumulation in tissues, inflammation and lipid accumulation in the liver
Oliveira *et al*., 2013	Polyethylene microspheres and pyrene	Size: 1-5 µm and pyrene 0. 18.4, 184 µg/ L	*Pomataschistus microps* (common goby)	Europe	96 hours	Microplastics modulate bioavailability or biotransformation of pyrene. Microplastics inhibit acetylcholinerase activity
Rist *et al*., 2017	Polystyrene beads	Size: 2 µm and 100 nm Concentration: 1 mg/L	*Daphnia magna* (water flea)	Northern hemisphere, South Africa	24 hours	100nm lower egestion and feeding rates
Rochman *et al*., 2013	Polyethylene	Size: <500 µm Concentration: 300 ng/mL	*Oryzias latipes* (Japanese medaka, Japanese rice fish)	East, southeast Asia	2 months	Hepatic stress fish sorbed pollutants on microplastics
Setälä *et al*., 2014	Fluorescent polystyrene spheres	Size: 10 µm Concentration: 1000, 2000, 10 000 particles/mL	*Eurytemora affins* (copepod) *Neomysis integer* (mysid shrimp)	Baltic Sea	3-12 hours	Potential microplastic transfer from mesozooplankton to macrozooplankton

The fauna from the studies listed in **Table 1.2** range in their exposure time to microplastics, from short term exposures of 1 hour to the longest exposure of 8 weeks (Hu *et al*., 2016; Imhof & Laforsch, 2016). The various exposure times allow for a range of effects on the organism studied. Lu *et al*., (2016) found that zebrafish (*Danio rerio*) accumulate microplastics in tissues, and have inflammation in the liver, but Grigorakis *et al*., (2017) found that microbeads did not accumulate in the guts of the goldfish (*Carassius auratus*). Blarer & Burkhardt-Holm (2016) found that *Gammarus fossarus* exposed to polystyrene beads had reduced food assimilation efficiency. The effects of microplastics are dependent on the species, exposure time, plastic type, size and concentration. For instance, Oliveira *et al.*, (2013) used polyethylene microspheres between 1-5 μm that were fed to the common goby (*Potataschistus microps*), while Lönnstedt & Eklöv (2016) used 90 μm polystyrene particles to expose European perch (*Perca fluviatilis*). There were also different concentrations of microplastics used across studies, and variation in whether the exposure was through contact in the water or via ingestion (i.e. in the food source). For example, Grigorakis *et al*., (2017) used 50 polyethylene particles per pellet of food, while Blarer & Burkhardt-Holm (2016) had polystyrene beads in concentrations of 500, 2500, 12 500, and 60 000 per mL of water. In my thesis, I decided to use polyethylene microbeads sized 106-125 μm in my controlled experiments because this size range reflects those commercially available given that they are found in personal care products (Pettipas *et al*., 2016).

While there are various modes of effect for microplastics, they are largely considered as a problem because of potential ingestion by various organisms owing to their similarity to prey in size and smell. Notably, microplastics are comparable in size to phyto-and zooplankton, therefore being attractive to consumers such as fish (Sigler, 2014). For example, Savoca *et al*., (2017) found that the northern anchovy (*Engraulis mordax*), a foraging fish, ingests plastic because it gives off a food odour. This finding indicates the potential for the chemical signature of plastic debris to make certain species prone to mistaking it as food. Findings by Setälä *et al*., (2014) also indicate the potential for microplastic transfer from mesozooplankton to macrozooplankton. They found that there was a food web transfer of microspheres after the copepod, *Eurytemora affins* ingested microspheres was in turn eaten by mysid shrimps, *Neomysis integer*. There is thus the potential to have community and ecosystem level consequences of microplastic ingestion. Beyond ingestion owing to the mistaken identification as food items, microplastics can adhere to actual food sources as well, likely increasing the

probability of their consumption. One potential pathway is through adhesion to phytoplankton such as algae.

Algae are defined as a group of organisms which can be either prokaryotic or eukaryotic, multicellular or unicellular, that are capable of producing oxygen through photosynthesis (Raven & Giordano, 2014). Algae lack true stems, roots and leaves, and are commonly found in freshwater and saltwater bodies (Raven & Giordano, 2014). Microplastics adhere to algae based upon hydrophobic interactions (Long *et al*., 2015; Gustov *et al*., 2016). For example, *Chlorella* populations at their exponential growth phase have a highly negative surface charge and cells remain dispersed because they are difficult to neutralise (Vandamme *et al*., 2012; Safri *et al*., 2014). Once *Chlorella* reaches the declining growth phase, the cells decrease their negative charge, and causing them to aggregate together (Vandamme *et al*., 2012; Safri *et al*., 2014). Plastic is typically positively-charged and is attracted to the cellulose of algae due to electrostatic forces (Kalčíkova *et al*., 2017). The adsorption of plastic to algae is enhanced by the roughness of cellulose surfaces, providing numerous binding sites for plastic (Kalčíkova *et al*., 2017). The mixture of algae and microplastics can create hetero-aggregations that are denser than clusters of either alone, causing these to sink towards the sediment (Lagard *et al*., 2016). Such aggregation is dependent on the type of microplastic and algae, as well as their concentrations. Lagard *et al*., (2016) reported that experimental hetero-aggregates were comprised of approximately 50% polypropylene microplastic and 50% microalgae, thereby serving as a potential pathway for microplastics to be travel vertically from surface water to the sediment.

As such, hetero-aggregates of algae and microplastics may represent a mechanism for ingestion by various suspension-feeding and grazing organisms. Common organisms in Ontario wetlands, which eat algae, and thus have the potential to be affected by aggregations with microplastics include freshwater snails such as *Stagnicola elodes*, and larval amphibians such as those of the northern leopard frog, *Lithobates pipiens* (Seale & Beckvar 1980; Colbourn *et al*., 2007). For instance, Seales & Wassersung (1979) found that *Lithobates sp.* tadpoles have similar suspension feeding rates for two types of freshwater algae, *Anabaena sphaerica* and *Chlorella pyrenoidosa*, therefore either are favourable algae species for studying adhesion to microbeads. *Chlorella* is a genus of unicellular green algae which is an eukaryotic organism, while *Anabaena* is a genus of multicellular cyanobacteria which is a prokaryotic. Yokota *et al*., (2017) found that

Anabaena sphaerica (also known as *Dolichospermum flos-aquae*) adhered to irregularly shaped microplastics. Therefore, to contribute to a broader understanding of aggregations between algae and microbeads that potentially create an ingestion pathway for various organisms like freshwater snails and tadpoles, *Chlorella pyrenoidosa* will be the focus in my thesis. Ingestion of microplastics can lead to various harmful effects on organisms, but these can include mechanisms that interact with other aquatic contaminants, such as persistent organic pollutants.

Persistent Organic Pollutants (POPs)

Microplastics cannot only harm animals through direct means such as physical obstruction of the digestive system, they are also problematic because plastics can accumulate persistent organic pollutants (POPs) from water by diffusion, potentially causing even greater harm to organisms which ingest them, including negative effects on growth, reproduction and survival (Rochman *et al.*, 2013; Syberg *et al*., 2015). These pollutants can play a role owing to the structure of plastic, which is composed of polymer chains that contain crystalline and amorphous regions. Amorphous or low crystalline polymer chains are loosely packed together, allowing the adsorption of POPs to take place (Teuten *et al*., 2009; Syberg et al., 2015; Hartmann *et al*., 2017). These amorphous regions are categorized as either glassy or rubbery depending on the polymer glass transition temperature (Teuten *et al*., 2009; Hartmann *et al*., 2017). Glassy polymers have chains that are more condensed and cross-linked, therefore having strong adsorption sites, but creating a slow POP release rate (Teuten *et al*., 2009; Hartmann *et al.*, 2017). Polyvinyl chloride, polyamide and polystyrene are glassy polymers (Teuten *et al*., 2009; Syberg *et al*., 2015; Hartmann *et al.*, 2017). By having a high crystallinity, this decreases the rate of adsorption of the contaminant into the plastic matrix (Mato *et al* 2001; Karapanagioti and Klontza 2008; Teuten *et al*., 2009; Hartmann *et al*., 2017). The hydrophobicity of POPs also pushes these out of the water and into a sorbing matrix (Hartmann *et al*., 2017). Weak van-der-Waals forces then help keep the POP molecules dissolved in the plastic matrix (Hartmann *et al*., 2017). Rubbery polymers include polyethylene and polypropylene (Teuten *et al*., 2009; Syberg *et al*., 2015; Hartmann *et al*., 2017). In comparison to glassy polymers where adsorption is the dominant force for POPs, rubbery polymers have absorption as the dominant force (Hüffer & Hofmann, 2016). Examples of known POPs include polychlorinated biphenyls (PCBs) and phenanthrene, but others are possible and should be considered.

The rate of POP diffusion from microplastics into living organisms is dependent on the structure of the plastics, the physiological conditions of the organism, and external factors such as salinity, pH, and temperature (Syberg *et al*., 2015). For examples, higher salinity causes an increase in the partitioning of POP into the plastic polymer (Bakir *et al*., 2014; Velzeboer *et al*., 2014), and POP desorption rates with gut surfactant were faster in warm blooded organisms compared to seawater (Bakier *et al*., 2014). Low pH and temperatures also increase desorption (Bakir *et al*., 2014). The ingestion of microplastics that contain POPs has been shown to be harmful. For example, Rochman *et al*., (2013) exposed Japanese medaka (*Oryzias latipes)* to polyethylene beads soaked with PCBs that caused hepatic stress. Karami *et al*., (2016) exposed *Clarias gariepinus* (African sharptooth catfish) to polyethylene microplastics soaked in phenanthrene, causing an increased degree of tissue change in the liver and decreased transcription levels. Just as various contaminants, microbeads can have synergistic effects on aquatic organisms, contaminants can also interact with biotic stressors to cause further harm. One way is which this can occur is by contaminant alterations of host-parasite interactions.

Host-parasite interactions with contaminants

A parasite is an organism which lives in (or on) its host and benefits by deriving nutrients at the host's expense (Centers for Disease Control and Prevention, 2016). Parasites are a ubiquitous threat to most organisms and can form a significant portion of food web biomass (Kuris *et al*., 2008). In addition, many parasites have complex, multi-host life cycles with free-living infectious stages that are susceptible to environmental contaminants (Pietrock & Marcogliese, 2003). There are many documented effects of contaminants on various host-parasite systems (select examples for amphibians and freshwater snails given in **Table 1.3**).

Table 1.3: Studies on the effects of contaminants/stressors on amphibian and freshwater snail hosts and their trematode parasites.

Study/Authors	Contaminant/Condition	Taxa	Parasite	Findings
Belden & Kiesecker 2005	Exogenous corticosterone	*Hyla versicolor* tadpoles (Gray tree frog)	*Alaria sp*.	Tadpoles developed higher parasite loads and lower numbers of circulating eosinophilic granulocytes
Bennet & Johnson 1973	2% NaCl 0.04 I.U. of bovine Adrenocorticotropic hormone (ACTH)	*Notophthalmus viridescens* (Eastern newt)	_____	Increase in neutrophils and decrease in lymphocytes
Cary *et al*., 2014	Pentabromodiphenyl ether mixture (DE-71) (flame retardant) 0, 1.1, 6.1, 71.4 and 634ng/g wet duet weight	*Lithobates pipiens* (Northern leopard frog)	_____	Significant decrease in neutrophil counts among treatments
Koprivnikar *et al*., 2006	20 *and* 200 μg/L *atrazine*	*Rana clamitans* tadpoles (Green frog)	*Alaria sp.* *Echinostoma trivolvus*	*Echinostoma trivolvis* mortality increased in 200 *μg/L* atrazine. *Alaria sp*. decreased activity and increased mortality. *Echinostoma* to infect the second intermediate host the intensity of infection in tadpoles was reduced at both 20 and 200*μg/L*
Koprivnikar *et al*., 2007	0, 3, 30 μg/*L atrazine*	*Rana sylvaticus* tadpoles (Wood frog)	*Echinostoma trivolvis*	Tadpoles exposed to 30*μg/L atrazine has* higher intensity of parasitism than 1 or 3μg/L of atrazine
Koprivnikar & Walker 2011	0.33 μg/L *atrazine metabolite*	*Stagnicola elodes* (freshwater snail)	*Gymnocephalus* cercariae *Echinoparyphium sp*.	Increased mortality of snails infected with *gymnocephalus* cercariae but not that of uninfected snails with the dormant infection *Echonoparyphium sp*.
Krist *et al*., 2004	Level of food fed vs starved	*Potamopyrgus antipodarum* (New Zealand mud snail)	*Microphallus sp*.	Host condition did not affect susceptibility to infection. Poor condition hosts have higher parasite induced mortality than hosts in good condition.
LaFonte & Johnson 2013	Chronic corticosterone exposure	*Hyla versicolor* (Gray tree frog)	*Ribeiroia ondatrae* *Echinostoma trivolvis* *Alaria sp.*	Reduced resistance increase infection of all three trematodes- particularly strong increase of *Ribeiroia ondatrae*. 62% decrease in circulating eosinophils
Milotic *et al*., 2017	Road salt up to 1140 mg/L	*Lithobates sylvaticus* (Wood frog) *Lithobates pipiens* (Northern leopard frog)	*Helistoma trivolvis*	Wood frogs exposed to road salt had higher parasite loads infection intensity. Northern leopard frogs the lymphocytes elevated at the highest concentration.
Rohr *et al*., 2010	Age and Size	*Bufo americanus* (Bull frog) *Rana clamitans* (Green frog) tadpoles	*Ribeiroia ondatrae* *Echinostoma trivolvis* *Plagiorchid* trematode cercariae	Younger tadpoles had a greater parasite induced mortality than older tadpoles. Younger tadpoles had lower resistance compared to higher resistance in older tadpoles. Host size was not predictive of resistance but was a positive predictor of the cost of resistance and tolerance.
Schotthoefer *et al*., 2017	Developmental stage	*Rana pipiens* tadpoles (Northern leopard frog)	*Echinostoma*	Stage 25-26 tadpoles successfully encysted compared to tadpoles in stage 37-38.

Abiotic and biotic stressors can have important effects on the susceptibility (i.e. resistance) and/or tolerance of hosts to parasite infections. Resistance is a measure of a host's ability to reduce successful parasite establishment, while tolerance is the host's ability to deal with a given parasite load (Råberg *et al*., 2007). For example, Kris *et al*., (2004) determined that host condition (fed or starved) of *Potamopyrgus antipodarum* (New Zealand mud snail) did not affect susceptibility to infection by the trematode (flatworm) *Microphallus sp*. However, if a host is in poor condition (i.e. stressed) then it can have higher parasite-induced mortality than a host in good conditions, but the parasites found within individuals can also be affected. Koprivnikar & Walker (2011) reported that exposure to a contaminant, the pesticide atrazine (0.33 μg/L), decreased the production of trematode infectious stage (cercariae) within *Stagnicola elodes* (freshwater snails), while snails infected with *Echinoparyphium* sp. had increased mortality rates under pesticide exposure compared to those that were uninfected. This suggests that snails and their parasites fared worse with combined stressors. Similarly, Koprivnikar *et al*., (2007) determined that *Lithobates sylvaticus* tadpoles (wood frog) exposed to relatively high levels of atrazine had a higher intensity of parasitism than individuals subject to a lower or zero concentration. Other factors to consider for the susceptibility of hosts to parasites and contaminants are their age, size, and developmental stage. Rohr *et al*., (2010) found that younger tadpoles had greater trematode-induced mortality than older tadpoles because less developed individuals had a lower resistance. Schotthoefer *et al*., (2003) found similar results as tadpoles in earlier developmental stages had many trematodes that were successfully encysted compared to tadpoles in later developmental stages that were exposed to cercariae.

One major way in which contaminants can affect the susceptibility and/or tolerance of organisms to parasites is by reducing their immune response. For examples, Milotic *et al*., (2017) exposed larval *L. sylvaticus* (wood frogs) and *L.pipiens* (northern leopard frogs) to 1140 mg/L of road salt. Compared to those kept at lower concentrations, wood frog tadpoles exposed to this high-level road salt level had higher parasite loads, and northern leopard frogs had elevated lymphocytes numbers. Belden & Kiesecker (2005) reported that *Hyla versicolor* tadpoles had higher parasite loads and lower numbers of circulating eosinophilic granulocytes, when exposed to exogenous corticosterone- a stress hormone. LaFonte & Johnson (2013) also exposed tadpoles to corticosterone and various parasites (*Ribeiroia ondatrae*, *Echinostoma trivolvis* and *Alaria sp*.), finding a 62% decrease in circulating eosinophils. Bennet & Johnson (1972) found that

Notophthalmus viridescens (Eastern newt) exposed to adrenocorticotropic hormone (ACTH) had increased neutrophils but decreased lymphocytes. Lastly, Cary *et al*., (2014) found that there was a decrease in neutrophil counts for larval *L. pipiens* exposed to a flame retardant. These studies show that various contaminants can affect the number of leukocytes (e.g., lymphocytes, eosinophils or neutrophils). Despite the importance of parasites for many animals, we still know little about how this stressor interacts with others in the environment. For microbeads, there have been no studies to date of how ingestion of these may affect host-parasite interactions, such as those between common pond and wetland hosts and parasites including that of the snail *S. elodes* and the trematode parasite *Haematoloechus parviplexus,* or *L. pipiens* tadpoles and the trematode *Echinostoma trivolvis*. Thus, host-parasite interactions and microbead presence in wetlands must be studied to understand the potential for synergistic effects considering the complex life cycle of parasites such as trematode, and their capacity to affect hosts.

Trematodes (flukes) are a common parasitic flatworm found in many aquatic habitats. These have complex life cycles that include free-living infectious stages and various hosts. Examples of common trematodes found in ponds and wetlands include *Echinostoma trivolvis* and *Haematoloechus parviplexus*. The life cycle of this trematode begins with adult worms found in definitive/final hosts such as birds and mammals or adult amphibians (**Figure 1.2**).

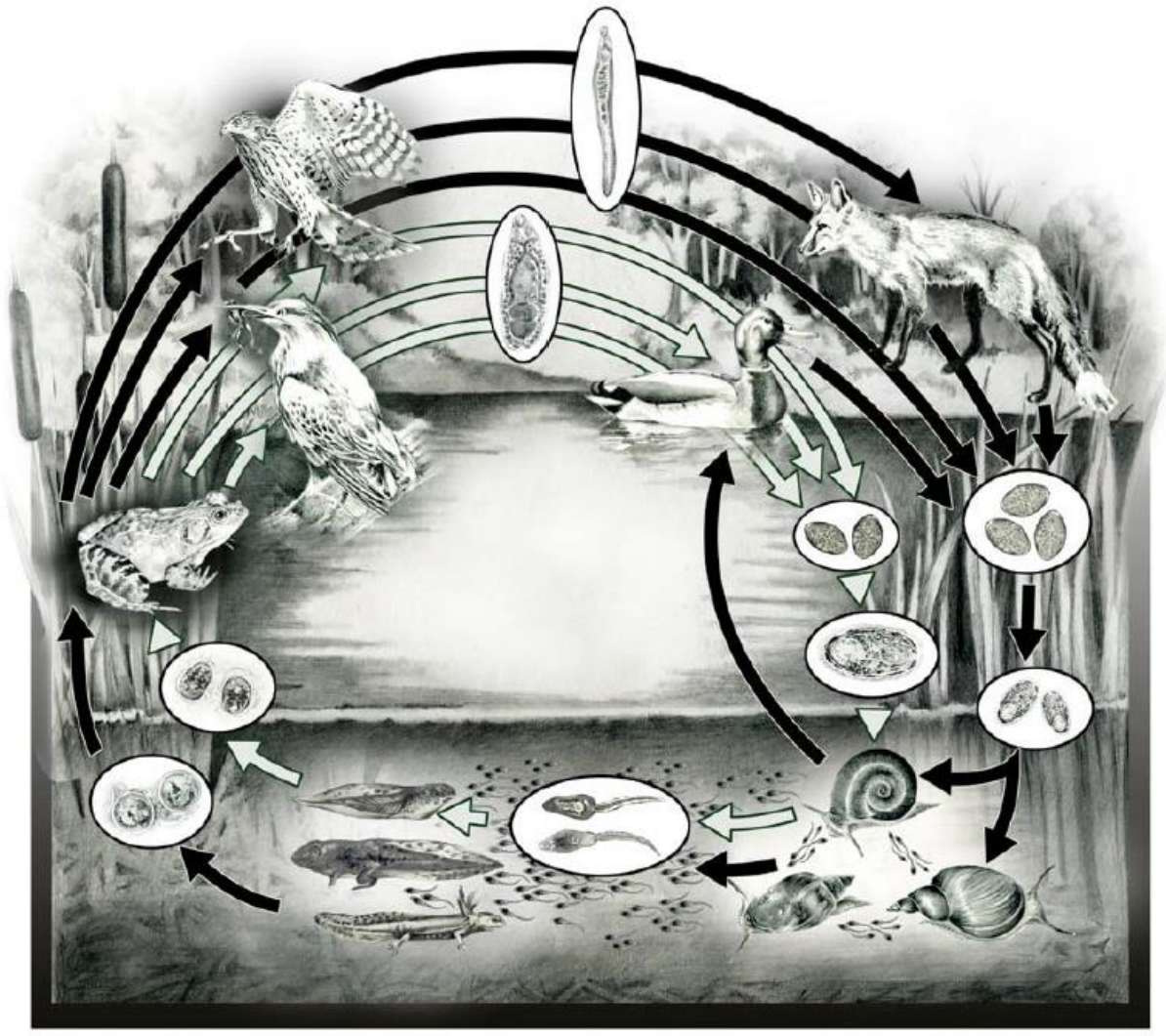

Figure 1.2: The life cycle of freshwater trematodes *Echinostoma trivolvis* (black arrows) and *Ribeiroia ondatrae* (grey arrows).

The adult worms sexually reproduce and pass eggs within the host feces. These eggs hatch into a ciliated, free swimming larval form known as a miracidium that seeks out the first intermediate host, typically an aquatic snail (Sukhdeo, 2012). Within the snail, the miracidia transform into an asexually-reproducing form known as a redia or sporocyst, depending on the species. The redia or sporocyst consumes the snail host tissue to feed and produce the next infective larval stage, the free-living cercariae (Sukhdeo, 2012). The cercariae emerge from the snail and seek out a second intermediate host to encyst within or on (e.g. dragon fly larvae, tadpoles, or fish). The life cycle is completed when the appropriate final host ingests an infected second intermediate host containing viable cysts (Olsen, 1986). Trematodes are important because a significant amount of energy moves through these parasites in ponds and wetlands making up a large portion of biomass in these waterbodies (Preston *et al*., 2013). Also, trematodes can be an added stressor for hosts (Koprivnikar, 2010). *Echinostoma* trematodes can be quite harmful to larval amphibians (Koprivnikar *et al*., 2012), and thus negative effects of microbead ingestion on cercarial production within snails are critical to consider because these could result in decreased transmission to tadpoles or other hosts. Consequently, there are subtle effects of microbeads to consider beyond those on growth and survival of herbivores such as snails. By conducting detailed studies on the occurrence of microbeads in smaller freshwater bodies such as ponds and wetlands, as well as their potential for direct and indirect effects on aquatic fauna, this may aid in shaping policies surrounding microplastic use and management.

Microbeads policies

Due to the risk of mortality and other toxic effects of microbeads on organisms, there are proposals to phase out microbeads in many countries, and these have created different plans to deal with the problem of microbeads. Australia plans on a voluntarily removal of microbeads from personal care products by July 2018, while France, the United Kingdom and Italy have proceeded with risk management-based actions by banning microbeads on January 1, 2018 (Government of Canada, 2017). In the United States of America, microbeads have been eliminated from personal care products since July 2, 2017 under the Microbeads Water Act (2015) (U.S. Government Publishing Office, 2015). Microbeads were classified as toxic in Canada under the Environmental Protection Act enacted on June 26, 2016 (Government of Canada, 2017). Canada's ban on selling personal care products containing microbeads took place on July 1, 2018 under the Microbeads in Toiletries Regulation (Microbeads in Toiletries

Regulation, 2017). However, even when microbeads are banned, there is still the potential for accumulation in aquatic ecosystems, and harm to fauna, owing to their persistence (Wright *et al*., 2013).

Thesis objectives and hypotheses

The primary goal of this thesis is to add to the microbead literature by focusing on specific gaps in our current knowledge. Microbeads are found in large numbers in the Great Lakes, but their abundance in smaller waterbodies like wetlands and ponds is unknown. In wetlands, microbeads have the potential to adhere to algae, creating an ingestion pathway for herbivorous organisms. By investigating this potential pathway of microbead ingestion, the likelihood of effects for select freshwater fauna can be assessed, and extent of direct and indirect harm caused by microplastic pollution if ingested. Based on this framework, microbeads have various pathways by which they may enter into the freshwater environment and have effects on aquatic fauna. This thesis will explore some of these pathways and possible implications for common pond and wetland inhabitants through both direct and indirect effects, including multiple stressors and synergistic interactions (**Figure 1.3**).

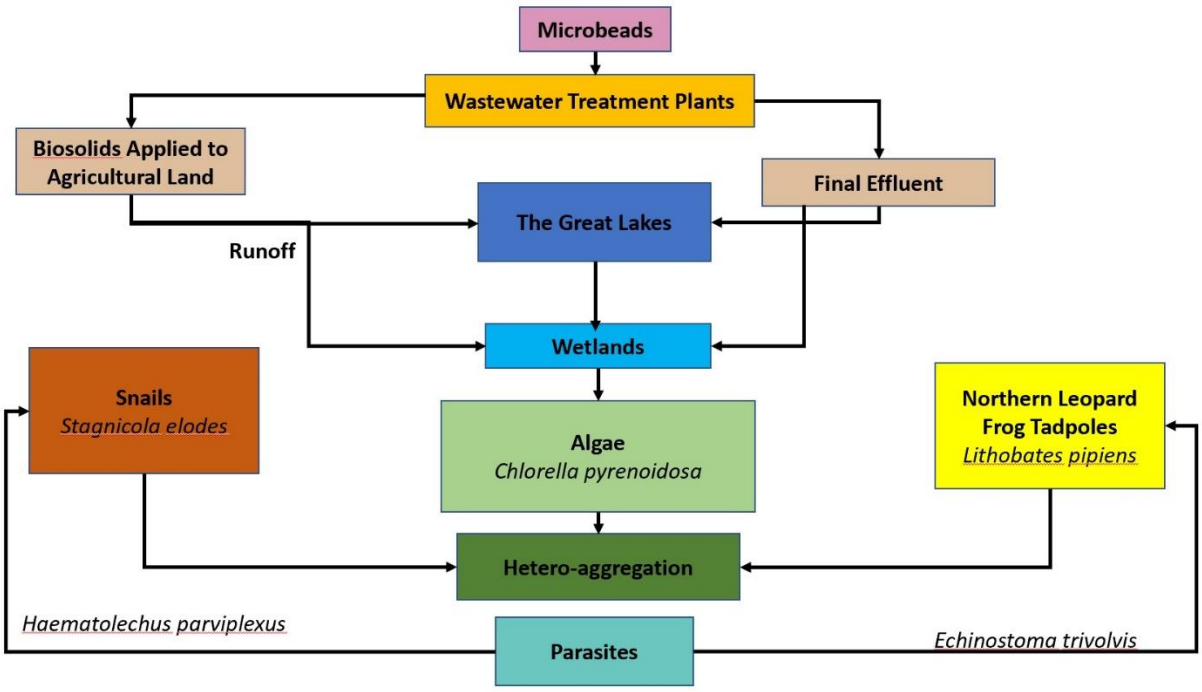

Figure 1.3: Pathways for microbeads to enter freshwater environments and affect select fauna.

Microbeads get washed down drains and slip through the filters at wastewater treatment plants, thus having two major pathways for entering into fresh waterbodies such as ponds and wetlands. They likely either enter through runoff from biosolids left over from treatment plants which are applied to agricultural land, or by the final effluent that is released into large water bodies. These waterbodies include the Great Lakes, which in turn connect to the coastal Great Lakes wetlands. While it is known that there are microbeads in the Great Lakes, the abundance of microbeads in the coastal wetlands has not yet been reported. Similarly, the occurrence of microbeads in small inland aquatic habitats, such as ponds, is not yet understood.

Like most animals, tadpoles and snails are hosts to parasites, and both are susceptible to environmental contaminants. Possible effects of microbead ingestion on these animals that are common inhabitants of freshwater ponds and wetlands are explored in the next two chapters. In Chapter 2, I aimed to determine the effects of microbeads and trematode infection on the immune system and growth of *Lithobates pipiens* tadpoles. I hypothesized that there would be a decrease in the growth and immune response of tadpole in treatments where they were exposed to *Echinostoma trivolvis* and/or microbeads. Because other contaminants can affect the immune response in tadpoles by changing the number of leukocytes, microbeads may act similarly. Tadpoles that are exposed to multiple stressors may shift in their energy allocation towards survival over growth, thus I expected that tadpoles would be smaller in size if exposed to microbeads and/or trematode infection.

In Chapter 3, I aimed to determine the separate effects of microbeads on snail mortality and production of trematode cercariae. I hypothesized that there would be a difference in the mortality and production of cercariae of snails infected with *Haematolechus parviplexus* that were exposed to different levels of microbead treatments. Multiple stressors can cause an increase in host mortality, but also reduce the production of cercariae within snails if host resources are limited.

Microbeads have hydrophobic interactions with, and the capacity to adhere to algae in freshwater habitats, with important implications for potential harm to herbivorous aquatic fauna. In Chapter 4, this interaction is explored through the potential for algae and microbeads to create hetero-aggregations. I hypothesized that there would be a difference in the size of aggregates formed by microbeads or the algae species *Chlorella pyrenoidosa* alone or in combination.

Based on the hydrophobic interactions of microbeads and algae, the creation of hetero-aggregates should cause a change in the aggregate size. If microbeads adhere to algae, this represents a possible pathway for their ingestion by various animals, such as tadpoles and snails, as well as subsequent transfer to higher trophic levels.

In Chapter 5, my aim was to create and conduct a field sampling methodology to determine microbead abundance in ponds and wetlands. I hypothesized that microbeads would be present in coastal wetlands due to their known occurrence in the Great Lakes, and their hydrological connectance. I also hypothesized that microbeads would be found in ponds that were connected to watersheds receiving wastewater effluent, or those located next to agricultural fields because of the runoff of biosolids applied to agricultural lands.

Overall, the goal and expected significance of my research was to have a better understanding of the distribution of microbeads in small freshwater bodies, such as wetlands and ponds, as well as contribute to the understanding of the potential for algae-microbead aggregations to act as a pathway for ingestion by herbivorous organisms. This will furthermore, help us to understand the effects of microbead ingestion on select freshwater organisms, including their interaction with other stressors like by parasites. It is important to be aware of that our actions, such as release of compounds into the environment, can have long term effects and manage these appropriately.

Chapter 2 The effects of microbeads and trematode infection on the growth and leukocyte profiles of *Lithobates pipiens* tadpoles

Abstract

Plastic takes a long time to break down in the environment and can have negative effects on animals. The same is true for small plastics, microbeads which are found in personal care products and toiletries. Tadpoles (*Lithobates pipiens*) were fed agarose algae cubes containing, 0, 10, or 100 microbeads (sized 106-125 µm), assigned to treatments exposed to the trematode, *Echinostoma trivolvis* or no trematode infection for 2 or 4 weeks. Tadpoles on microbead diets had a significant influence on the growth of tadpoles, as well as their susceptibility to infection by the trematode parasite infection, but there were minimal effects on tadpole white blood cell (WBC) measures. Uninfected tadpoles that were kept on microbead diets for 4 weeks had a non-monotonic growth response, while infected tadpoles grew similarly in all the diets. It is not clear why this happened, but it may be due to phenotypic plasticity of the intestines owing to the increased metabolic demands brought on by parasite infection.

Introduction

Global species extinctions are caused by many factors, including habitat loss, climate change, and pollution (Sala *et al*., 2000). Disease is one of the major contributors to the causes of extinction (Smith *et al*., 2009), but is also important to consider how stressors might interact with parasitic diseases to have harmful synergistic effects (Daszak *et al*., 2000). Infectious diseases are a major consideration for amphibian conservation, which makes looking at interactions with any other stressors even more critical (Daszak *et al*., 2003; Blaustein *et al*., 2012). While the chytrid fungus *Batrachochytrium dendrobatidis* and ranaviruses have received the most attention, other infectious agents such as trematode (flatworm) parasites can also have harmful effects. Trematodes (primarily *Ribeiroia ondatrae* and echinostomatids) have been demonstrated to have harmful effects on larval amphibians (Hayes *et al*., 2006; Johnson & McKenzie, 2009; Koprivnikar *et al*., 2012). For instance, *Echinostoma trivolvis* can cause a loss of renal function in the kidneys of tadpoles (Schotthoefer *et al*., 2013).

It is important to consider the interaction of other stressors with parasitism for disease dynamics as these may affect host susceptibility and/or tolerance to infections (Smith *et al*., 2009). Resistance relates to the successful establishment of parasites (a.k.a. host susceptibility), while host tolerance more commonly pertains to how a host deals with its parasite load in terms

of the cost of infection (Rohr *et al*., 2008). There are other stressors faced by amphibians, like aquatic contaminants, but pesticides have been the best studied (Hayes *et al*., 2006; Haas *et al*., 2018). Various studies have illustrated that exposure to pesticides can affect either the susceptibility or tolerance of larval amphibians to trematode parasites (Kiesecker, 2002; Koprivnikar, 2010).

Exposure to environmental stressors, such as contaminants, can cause physiological stress (Beyers *et al*., 1999) that can alter host resistance or tolerance to infections. For instance, high amounts of stress may amphibians to have a lower immunocompetence, leading to greater parasitism (Bennet & Johnson, 1973). This may occur in various ways. Stress causes an increase in glucocorticoid hormones (primarily corticosterone in amphibians), which accelerates energy utilization and metabolism, but causes a decrease in tadpole growth (Glennemier & Denver, 2002). This illustrates how energy allocations can be shifted in stressful conditions to be used for critical functions related to survival instead of energy for growth and reproduction (Moore & Jessop, 2003). Such energy-related trade-offs can also play a role in infectious disease dynamics. For example, host size was not a predictor, but a good proxy for measuring the cost of resistance and tolerance to trematode infection in tadpoles (Rohr *et al*., 2010). Beyond effects on energy allocation, glucocorticoids can also affect various immune parameters (Sapolsky *et al*., 2000). These include influencing cytokine synthesis (Maule & Vanderkooi, 1999), reducing antibody production, and dampening inflammatory responses (Mastorakos *et al*., 1999).

In amphibians, elevated levels of corticosterone have been shown to affect leukocyte (white blood cell) profiles, and these may in turn reduce host susceptibility or tolerance to trematode infections. For example, *Hyla versicolor* tadpoles (gray tree frog) that were exposed to exogenous corticosterone and trematodes of *Alaria sp*. had lower numbers of circulating eosinophilic granulocytes, as well as higher parasite loads (Belden & Kiesecker, 2005). LaFonte & Johnson (2013) also exposed *H. versicolor* to chronic corticosterone, which strongly reduced their ability to clear cysts after infection by the highly pathogenic trematode *R. ondatrae*. Although not challenged with parasites, *Notophthalmus viridescens* (eastern newt) similarly had significantly decreased neutrophil counts after treatments of adrenocorticotropic hormone (ACTH) (Bennet & Johnson, 1973). Importantly, contaminants can alter leukocyte profiles in a manner that could then influence host infections. For instance, *Lithobates pipiens* tadpoles

(northern leopard frogs) exposed to a flame retardant showed a decrease in neutrophil counts (Cary *et al*., 2014). As noted above, atrazine-exposed tadpoles had reduced numbers of eosinophils, increasing their susceptibility to trematode infections (Kiesecker, 2002). Other aquatic contaminants commonly experienced by larval amphibians have been shown to reduce their resistance to trematode infection as well. It was recently reported that road salt caused larval wood frogs (*L. sylvatica*) to have higher parasite intensity, as well as elevated lymphocyte counts in larval northern leopard frogs (Milotic *et al*., 2017).

There is a lack of information regarding the effects of microplastics on freshwater fauna, especially those in small waterbodies such as ponds (refer to **Chapter 1**). Although microplastics are probably a pervasive problem in these habitats, little is known regarding the direct effects of their ingestion, and even less about indirect effects, such as those influencing host susceptibility or tolerance to parasitism. Currently, the effects of microbeads on larvae of common North American amphibians, such as northern leopard frogs, are unknown, and studies with amphibians in general have been extremely limited. Hu *et al*., (2016) studied the effects of exposure to polystyrene microspheres on larval *Xenopus tropicalis* (tropical clawed frog) and found that the microplastics were ingested and egested at fairly quick rates, and the higher the concentration of microspheres, the greater the chances were of accumulation. However, tropical clawed frog tadpoles are not a good representative of how larvae of most frogs may be affected by microbead ingestion because they consume food in different ways and would therefore likely be exposed to microbeads by different pathways, which could affect the possible harm incurred. Tropical clawed frog tadpoles are strictly filter feeders and would therefore ingest microbeads suspended in the water; however, northern leopard frog tadpoles also scrape algae from surfaces, which may be more likely ingested via algae-microbead aggregations (Duellman & Trueb, 1994; see **Chapter 4**). In addition to examining direct effects (i.e. growth and mortality) of microbead ingestion on amphibians native to North America, it is also important to consider their possible indirect effects and interactions with other stressors.

Given the common infection of larval amphibians by trematode parasites, and their known detrimental effects (see above), here I aimed to determine the effects of microbead ingestion on the immune system and growth of *Lithobates pipiens* tadpoles. I hypothesized that there would be a decrease in the growth and immune response for tadpoles exposed to both

Echinostoma trivolvis trematodes and microbeads. I also hypothesized that tadpoles infected with trematodes would have a decreased growth and immune response compared to non-infected tadpoles. Given that other contaminants have been shown to affect the immune response in tadpoles by changing the number of leukocytes, microbeads could act similarly. Tadpoles exposed to multiple stressors can also shift their energy allocation towards survival over growth (Moore & Jessop, 2003).

Materials and Methods

Tadpole and parasite sources

Northern leopard frog tadpoles (*Lithobates pipiens*) were donated by a collaborator at Environment Canada in Ottawa, ON, who followed an established breeding protocol for field-collected adults of this field (see Trudeau *et al*., 2013). Tadpoles were reared in 4 aerated plastic tubs containing 10 L of dechlorinated water that housed approximately 80 tadpoles each until their experimental use once they reached their desired developmental stage (see below). The tadpoles were kept on a 14:10 light/dark cycle at 21°C and were fed commercial flaked fish food (Tetramin) and boiled organic spinach 3 times a week. Note that the Tetramin food and organic spinach were not analyzed for contaminants. Water changes for the group housing tubs were performed every 2 days. After reaching Gosner development stage 25 (Gosner, 1960), 135 tadpoles were haphazardly selected from the 4 communal tubs for inclusion in the experiment. This developmental stage was necessary because the mortality of less- developed tadpoles exposed to *E. trivolvis* is very high (Schotthoefer *et al*., 2003), and I wanted to ensure that a sufficient number survived the tolerance-related portion of the study. Tadpoles were randomly selected for each of the 3 microbead diet treatments, as well as for exposure to *E. trivolvis* or not. This resulted in 6 unique combinations (see experimental design below). The weight of each tadpole was recorded prior to commencing the microbead diet treatments. As aquatic snails typically serve as the first intermediate hosts of trematodes (see **Chapter 1**), I used naturally-infected snails collected from the field as a source of the infectious stage (cercariae) with which to expose tadpoles. *Stagnicola elodes* snails were collected in various wetlands (see **Chapter 3**). Each snail was screened for trematode infection in individual wells of tissue culture plates containing 10 mL of dechlorinated water (see **Chapter 3** for details) and those with emerged *E. trivolvis* cercariae were separated for later use.

Experimental design

The combination of tadpole diet and parasite exposure resulted in 6 unique treatments (**Figure 2.1**). Firstly, 45 tadpoles were randomly assigned to each of 3 experimental diets: no microbeads, 10 microbeads/week (low), and 100 microbeads/week (high). The concentrations for the low and high treatments are based on a combination of those used in previous studies with freshwater animals, as well as levels reported from freshwater environments. However, microbead data for North America are available only for surface waters of the Great Lakes (mean of 43 000 particles/sq.km) and sediment in the St. Lawrence River (median 52 particles/sq. m and a high of 103 microbeads/L) and are thus not easily translated into that which larval amphibians may be exposed (Eriksen *et al*., 2013; Castañeda *et al*., 2014). Consequently a concentration of 10 microbeads/week for the low and 100 microbeads for the high level were chosen based on the study with tropical amphibians (the only one to date with any larval amphibians) and sediment levels of the St. Lawrence River (Castañeda *et al*., 2014; Hu *et al*., 2016).

All tadpoles were fed their experimental diet for 2 weeks, after which 90 were exposed to *E. trivolvis* cercariae and the remaining 45 individuals received a sham exposure that consisted of the addition of 2 mL of water to each of their individual tubs. Of the 90 parasite-exposed tadpoles, 45 were kept for only an additional 48 h- this comprised the resistance aspect of the study. This period of time is sufficient for *E. trivolvis* cysts to fully form (Schotthoefer *et al*., 2003). The remaining 45 parasite-exposed tadpoles, as well as the 45 sham exposed individuals, were then maintained on their experimental diets for another 2 weeks, representing the tolerance component of the study.

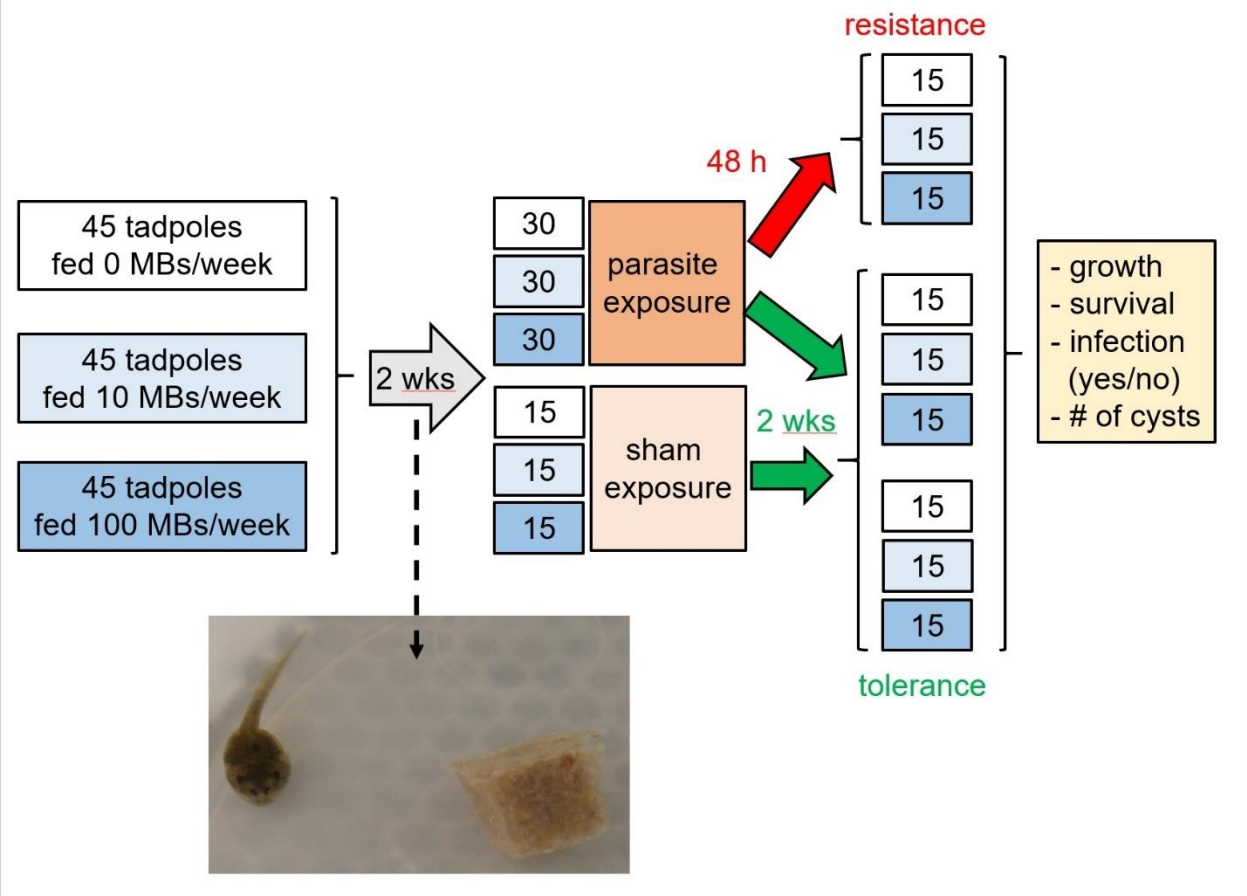

Figure 2.1: Experimental design of the tadpole treatments.

Experimental procedure- microbead diet and parasite exposure

Each of the 135 randomly-selected tadpoles was placed into a 1.5 L plastic container filled with 1 L of dechlorinated water before commencing the diet treatments. The placement of each of each container was randomly distributed among shelves with a random sequence generator. Each tadpole was fed a $1cm^3$ cube consisting of a mixture of a ground Tetramin mix (fish food), water, and agar (similar to Johnson & Hartson, 2009; Imhof *et al*., 2016) with the designated amount of microbeads as per its treatment assignment (0, 10, or 100 microbeads). The microbeads that I used were fluorescent green polyethylene microbeads (sized 106-125 μm) from Cospheric Innovations in Microtechnology (Item # UVPMS-BG-1.00). I chose microbeads of this size and composition because they are commercially available in sizes 10 μm to 1 mm (Pettipas *et al*., 2016). Tadpoles were fed a new cube once a week for their designated time of exposure during water changes for each individual container.

On day 14 (i.e. after 2 weeks) of the designated treatment diets, the 90 tadpoles assigned to parasite exposure had 10 live *E. trivolvis* cercariae (similar to Koprivnikar *et al*., 2007) added to each of their individual containers. For *L. pipiens* tadpoles in early developmental stages (<29), infection by more than 20 *E. trivolvis* can be highly lethal (Schotthoefer *et al*., 2003). I obtained cercariae through the same procedure as used for screening snails for trematode infections but removed the cercariae in each cell-wall and pooled these into Petri dishes. From there, 10 cercariae were pipetted into micro centrifuge tubes filled with dechlorinated water. Cercariae were no more than 3 hours old at the time of their addition, well in line with their period of maximum infectivity (Pechenik & Fried, 1995). The cercariae were added by pouring the contents of a single tube into each container. Of the 90 parasite-exposed tadpoles, 45 were euthanized in a buffered solution of 1% MS-222 after 48 hours because this was a sufficient time for the cysts to form in the kidneys and pronephroi (Martin & Conn, 1990; Fried *et al*., 1997). Each of the euthanized tadpoles also weighed at this time before blood smears were made (see below). Following this procedure, they were individually frozen for subsequent necropsy and evaluation of infection and microbead presence. The remaining 90 individuals were then maintained on their experimental diets for an additional 2 weeks. After a total of 4 weeks passed, these tadpoles were also euthanized, weighed, and frozen after blood extraction. All animal care use was performed under the guidelines of the Canadian Council on Animal Care and was approved by the St. Michael's Hospital Animal Care Committee (protocol #ACC-752).

Experimental procedure- leukocyte and parasite measures

Blood smears were created for each tadpole by using a standard plastic pipette to collect blood and transfer it onto a glass slide following standard protocols (following Davis *et al*., 2008; Milotic *et al*., 2017). Each slide was air dried and fixed by immersion into methanol for 1 minute, followed by Giemsa stain for 30 seconds, dipping into a jar of distilled water for 10 seconds then into another jar of distilled water for 10 seconds, followed by air drying (Milotic *et al*., 2017). The blood smears were examined using standardized methods as well. I examined each slide with a compound microscope using the 100x objective until 150 fields of view or 100 leukocytes were counted (Davis *et al*., 2008; Milotic *et al*., 2017). The number of red blood cells were counted, as well as the total number of white blood cells, including 3 specific types- eosinophils, neutrophils, and lymphocytes (Hadjji-Azimi *et al*., 1987). From these numbers, the ratio of white blood cells (WBC) to red blood cells (RBC), and of each specific WBC type, was calculated (Hadjj-Azimi *et al*., 1987; Milotic *et al*., 2017). Prior to dissection, tadpoles were thawed. The number of cysts within each tadpole was counted under a dissecting microscope and by dissecting the tadpole until the kidneys and pronephroi were visible to locate the cysts. The Gosner developmental stage was also determined for each tadpole. Visible microbeads were counted by thoroughly examining the dissected intestines of each tadpole.

Statistical Analysis

Mass gain was calculated by subtracting the initial mass of each tadpole from its final mass and then dividing the total by the initial mass. This total was multiplied by 100 to make the mass gain a percentage. The data was checked for a normal distribution and then was $\log_{10}$ transformed. To examine the overall effect of microbead diet on mass gain, I only used data from the tadpoles sacrificed after 2 weeks and the sham-exposed individuals that were kept for 4 weeks; for both groups, only those individuals who survived to be weighed (total N=98). Otherwise the length of diet treatment could reflect an influence of infection, which is separately examined below. It is unlikely that those sacrificed after 2 weeks as part of the resistance component of the study had their growth influenced by parasite exposure only 48 hours earlier. I then used a univariate ANOVA to examine mass gain (i.e. growth) based on the fixed effects of microbead diet (categorical), the length of diet exposure (categorical as either 2 or 4 weeks), and their interaction. I did not include developmental stage as a covariate or random effect because this would be confounded with length of diet exposure- tadpoles kept for 4 weeks would be

expected to gain more mass. Tukey LSD post-hoc tests were conducted for the fixed effect of microbead diet.

To determine if there was a relationship between the numbers of microbeads and cysts within individual tadpoles, I considered only infected tadpoles (based on dissections) from both the 2 week and 4 week long sub-experiments. Here, I ran a GLMM using a Poisson distribution with log link function for cyst intensity but used the number of microbeads as the predictor. Length of diet treatment was included as a random effect but tadpole developmental stage was not included for the same reason given above. All other analyses were then separately performed for the tadpoles that were kept for either 2 or 4 weeks and are described below.

The tadpoles sacrificed after 2 weeks formed the susceptibility component of the study. I first examined cyst intensity (number of cysts/tadpole) using a Generalized Linear Mixed Model (GLMM) with a Poisson distribution and log link function. Microbead diet was entered as a categorical fixed effect, and tadpole development stage was included as an ordinal random effect. With a similar approach, I also examined how microbead diet affected susceptibility to infection in terms of individual infection status (either yes or no) as an outcome using a GLMM with a binomial distribution and log link function. As I had hypothesized that microbead diet might affect tadpole leukocyte profiles, I used a series of GLMMs with a normal distribution and identity link function to examine each of the following as a dependent measure after a $\log_{10}$ transformation: total WBC/RBC, lymphocytes/RBC, neutrophils/RBC, and eosinophils/RBC. For these, I included the fixed effects of microbead diet and individual, infection status, as well as their interaction, once again entering tadpole developmental stage as a random effect. Lastly, a GLMM with a Poisson distribution and log link function was used to determine if there was relationship between the number of cysts and total WBC/RBC (predictor).

For the tadpoles kept for 4 weeks, analyses generally similar to those described above for resistance aspect (i.e. maintained for 2 weeks), were conducted to examine tadpole growth and immunocompetence. However, here I used an additional GLMM to investigate host tolerance by examining how the fixed effects of individual infection status and diet, as well as their interaction, influenced mass gain. This could not be done for the tadpoles kept only 2 weeks as they were euthanized 48 h after parasite exposure and would not be expected to gain mass during that time. Individual GLMMs for the same leukocyte measures as above were also conducted. A

final GLMM with a Poisson distribution and log link function was used to determine if there was a relationship between the number of cysts and total WBC/RBC (predictor) for the tadpoles kept for 4 weeks, excluding those that had received the sham parasite exposure. This is best analyzed separately for the tadpoles kept 2 or 4 weeks, respectively, as larval amphibian immune systems change massively during their development (Rollins-Smith, 1998). All analyses were performed in SPSS 24, with non-significant interactions and fixed effects dropped in sequential interactions to obtain the final models.

Results

All tadpoles (2 and 4 week microbead diet treatments)

There was a highly significant effect of both microbead treatment ($F_{2, 92}$ =33.911, P<0.001) and length of treatment exposure ($F_{1,92}$ =28.886, P<0.001) on tadpole mass gain (growth), but no significant interaction of these predictors ($F_{2,92}$ =1.074, P=0.346). Tukey LSD post-hoc tests indicated that there was a significant difference between the none (no microbeads) and low (P<0.001), none and high (P<0.001), and low and high (P=0.003) microbead treatments (**Figure 2.2**). The largest mass gain was seen from the tadpoles in the none microbead diet, followed by the high microbead diet, and the lowest mass gain was seen in tadpoles from the low microbead diet regardless of the length of their diet exposure, but tadpoles gained more mass in 4 weeks of exposure over 2 weeks. For the tadpoles kept for 2 weeks, microbeads were found in the intestines of 0/15 tadpoles in the none diet, 10/15 tadpoles in the 10 microbead (low) diet, and 12/15 tadpoles in the 100 microbead (high) diet. In the tadpoles kept for 4 weeks, microbeads were found in the intestines of 0/30 tadpoles in the none diet, 10/30 tadpoles in the low diet, and 24/30 tadpoles in the high microbead diet. For the tadpoles kept for 2 weeks, the mean (± SE) number of microbeads found in the intestines of those in the low diet was 1.8 ± 0.60 (range 0-7 microbeads) and the mean (± SE) in the high microbead diet was 3.67 ± 0.88 (range 0-12 microbeads). In the low diet tadpoles kept for 4 weeks, the mean (± SE) number of beads found was 0.87 ± 0.26 (range 0-6 microbeads), and the mean (± SE) was 3.8 1± 0.81 (range 0-18 microbeads). There was no relationship between the number of microbeads and parasite cysts within individual tadpoles ($F_{1,48}$ =1.698, P=0.199; **Figure 2.3**).

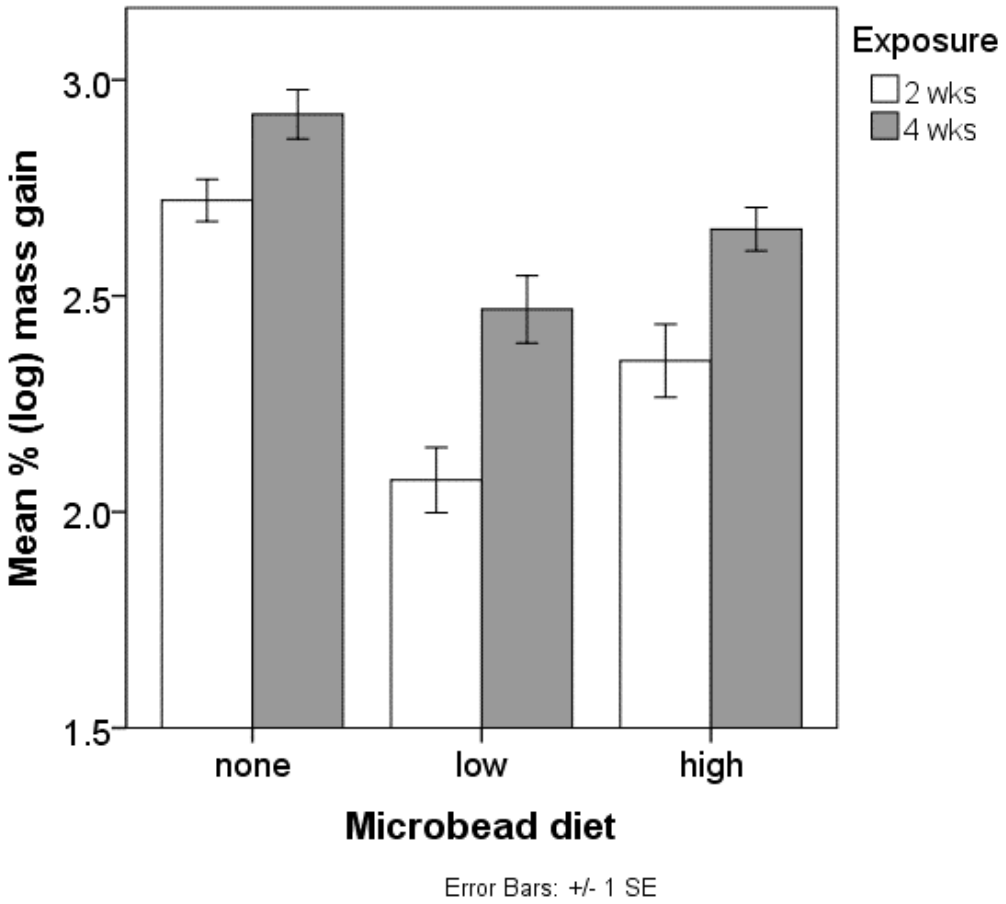

Figure 2.2: Mean (%) (±S.E.) (log_{10}) mass gain by tadpoles given one of three microbead diets (none=0 microbeads, low=10 microbeads, high=100 microbeads) for either 2 or 4 weeks.

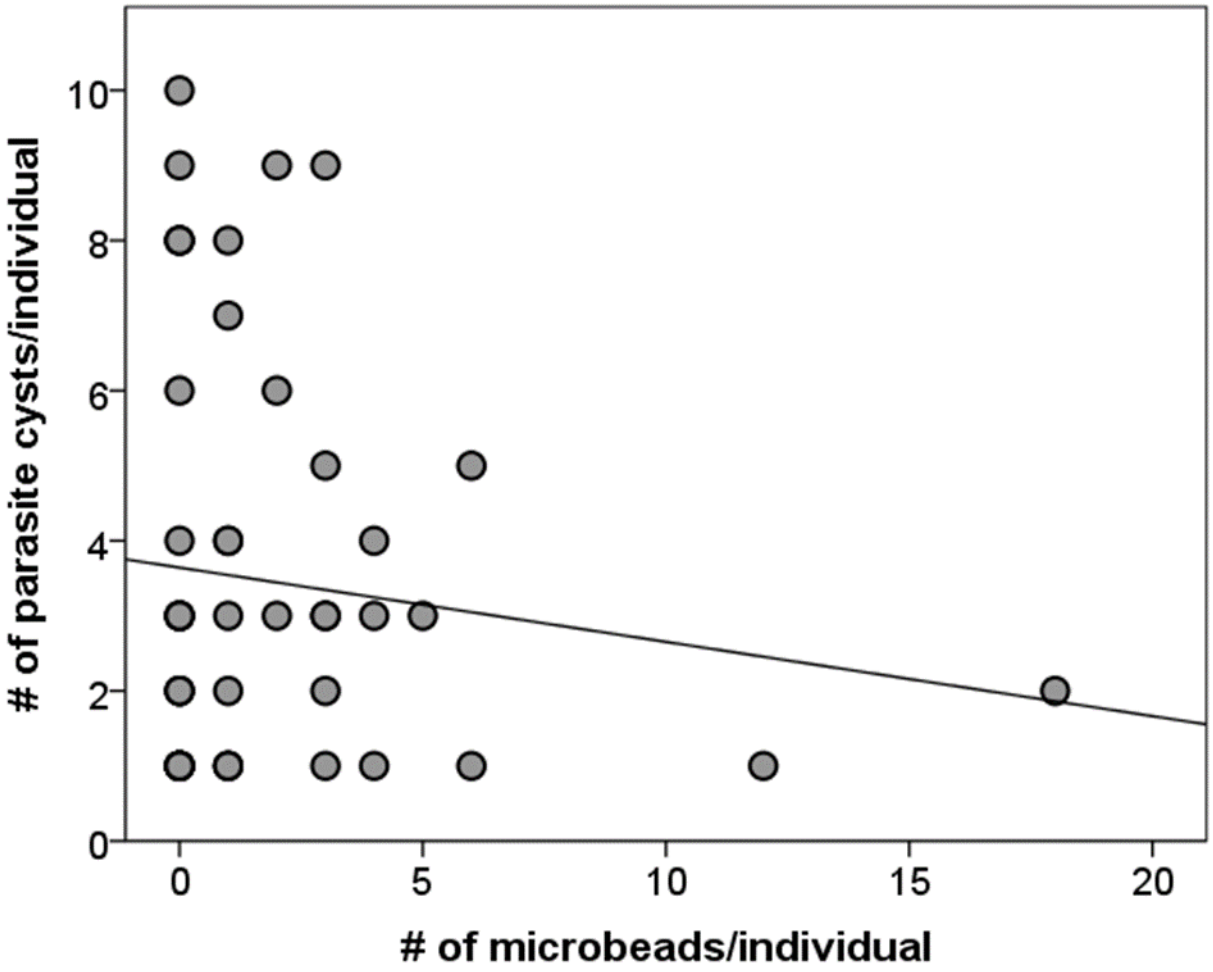

Figure 2.3: There is no relationship between the number of *Echinostoma trivolvis* cysts/individual tadpole and the number of microbeads/individual. Note the line of best fit is for illustrative purposes only.

Two weeks microbead diet treatment

There was a significant effect of microbead diet on susceptibility to infection as measured by cyst intensity ($F_{2,42}$ = 3.531, P=0.038). The posthoc pairwise comparisons indicated a significant difference in cyst intensity between the none and low microbead diets (P=0.035), a marginally insignificant difference between the none and high microbead diets (P=0.052), but no difference between low and high microbead diets (P=0.842) (**Figure 2.4**). However, there was no significant effect of microbead diets on the infection status (yes or no) of individual tadpoles ($F_{2,42}$ =1.148, P=0.327).

There was so significant effect of infection status, microbead diet, nor an interaction between infection status and microbead diet, on the ratio of WBC to RBC (all P>0.805; **Figure 2.5-A**). Results were similar for the ratio of lymphocytes to RBC (all P>0.675; **Figure 2.5-B**), and for the ratio of eosinophils to RBC (all P>0.224; **Figure 2.5-C**). There was a marginally insignificant effect of microbead diet on the ratio of neutrophils to RBC ($F_{2,38}$ = 3.531, P=0.096). The pairwise posthoc comparisons indicated a significant difference between the low and high microbead diets (P=0.043), with a trend for a difference between the none and low microbead diets (P=0.080) as those fed the low microbead diet seemed to have the lowest number of neutrophils overall (**Figure 2.5-D**). There was no significant relationship between the number of cysts found in each tadpole and its WBC/RBC ($F_{1,39}$=1.529 P=0.224; **Figure 2.6**).

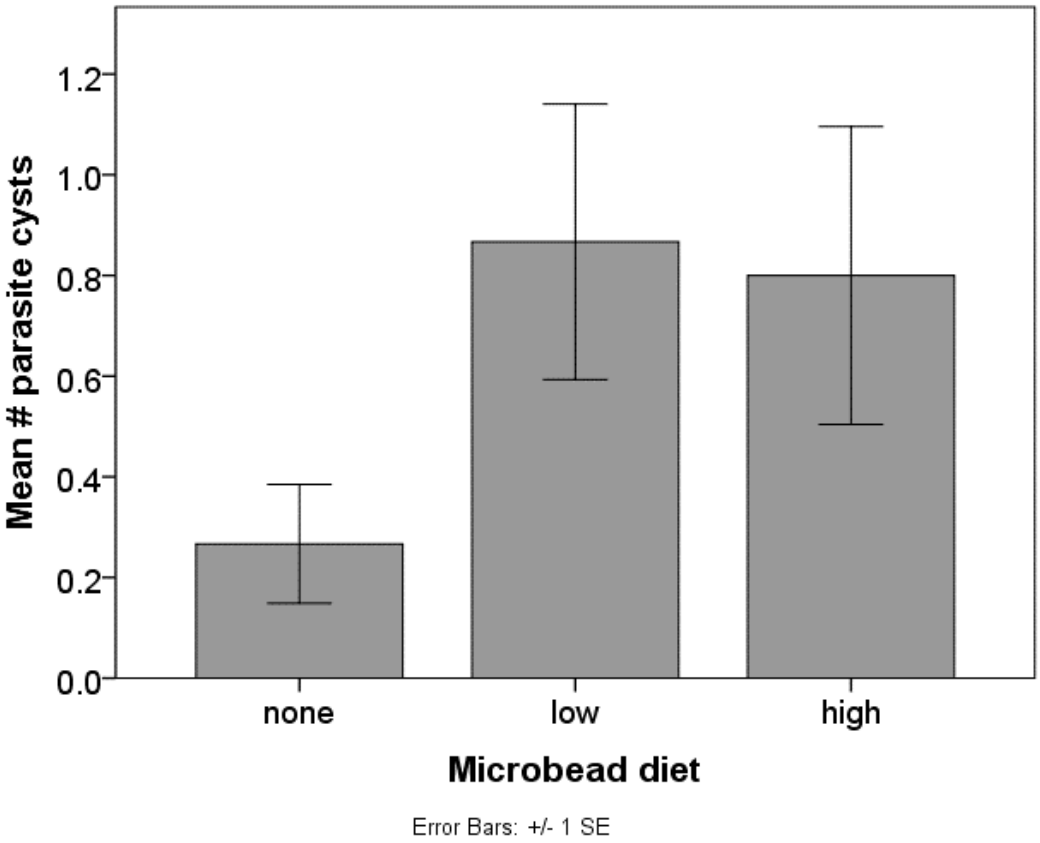

Figure 2.4: Mean (S.E. ±) number of *Echinostoma trivolvis* cysts in *Lithobates pipiens* tadpoles based on their microbead diet for 2 weeks (none=0 microbeads, low=10 microbeads, high=100 microbeads).

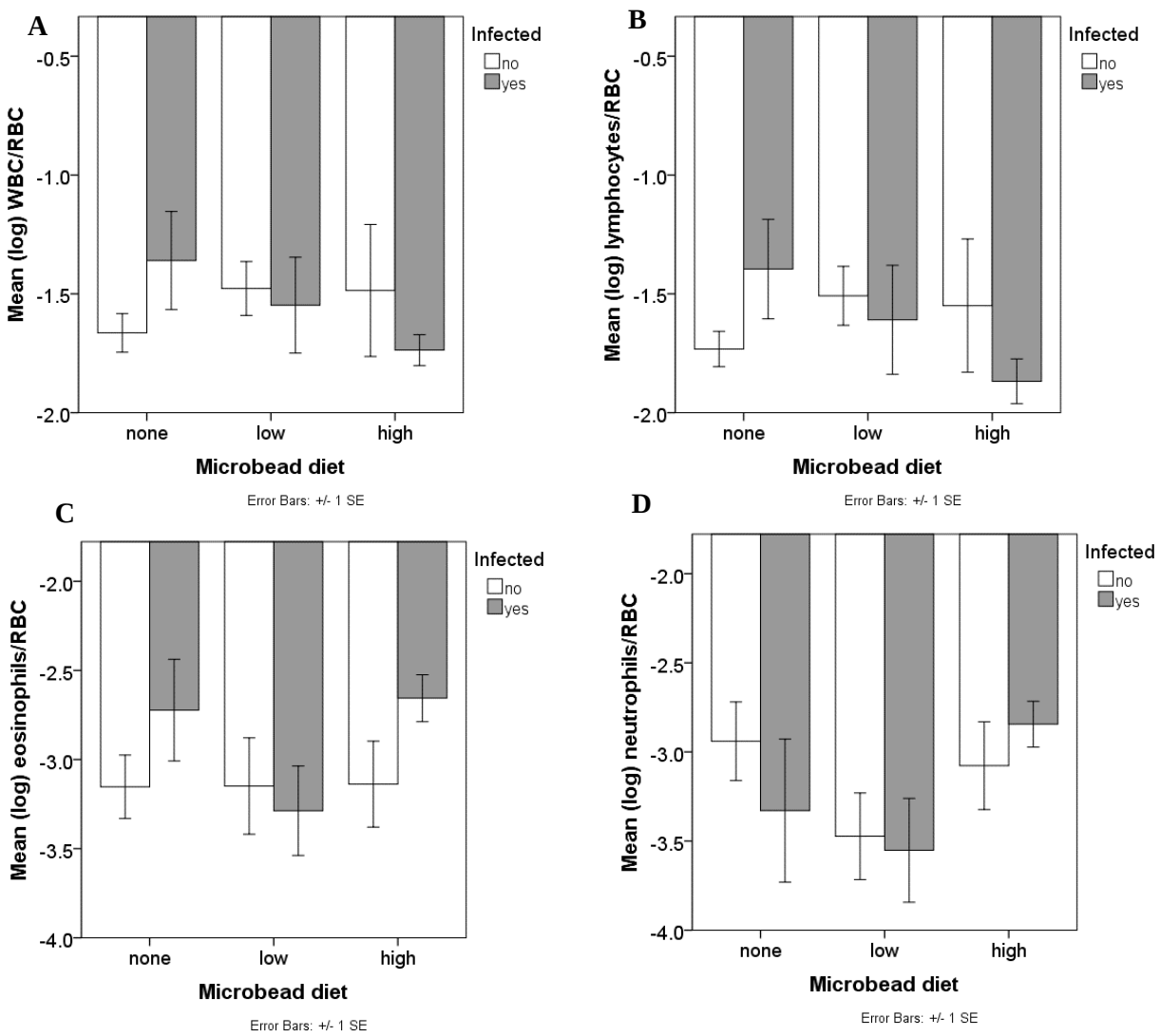

Figure 2.5: Mean (±S.E.) (log_{10}) measures for: **A)** ratio of white blood cells to red blood cells (WBC to RBC); **B)** ratio of lymphocytes to red blood cells; **C)** ratio of eosinophils to red blood cells; **D)** ratio of neutrophils to red blood cells by microbead diet (none=0 microbeads, low=10 microbeads, high=100 microbeads for 2 weeks) with tadpoles either infected or not by *Echinostoma trivolvis*.

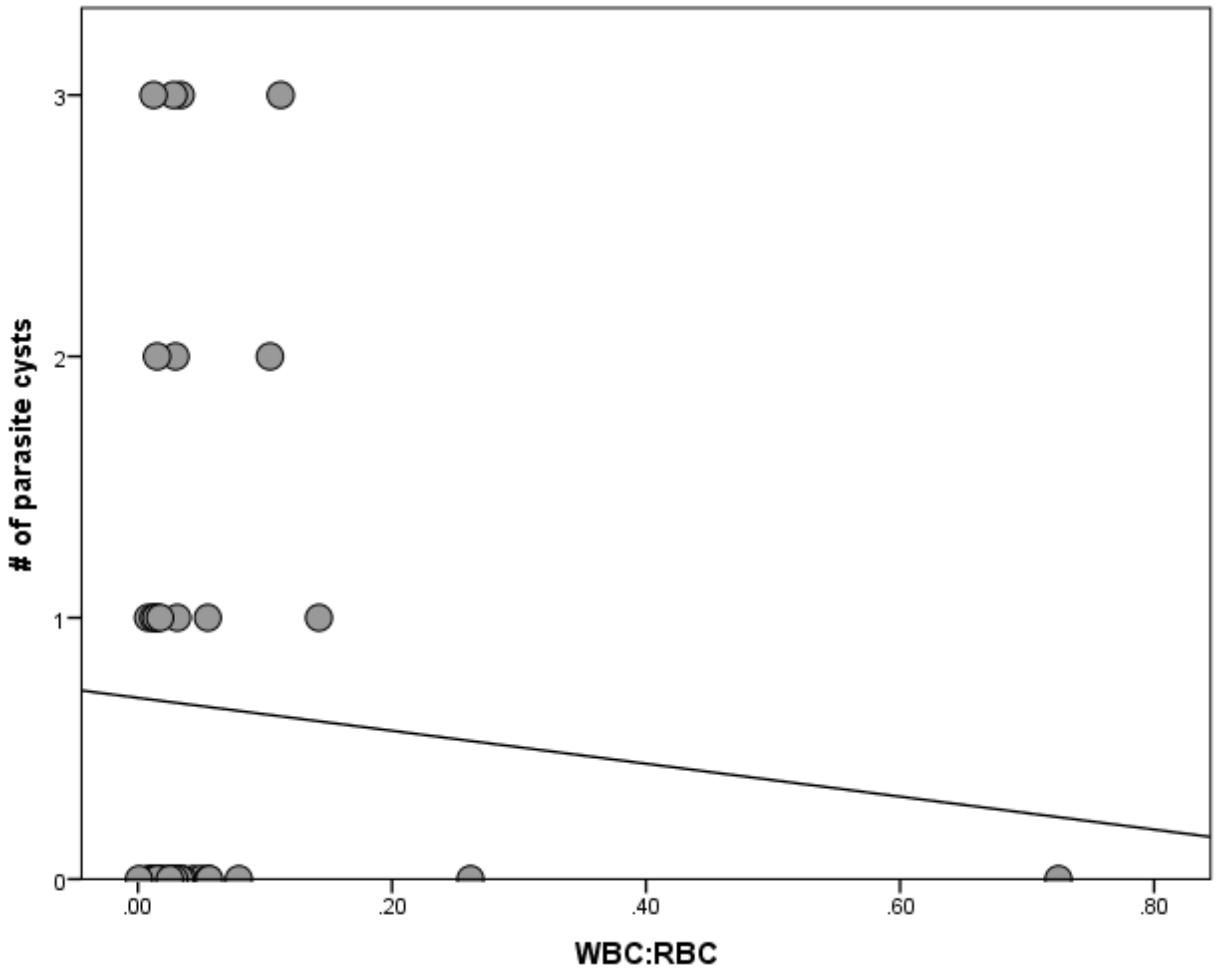

Figure 2.6: There was no relationship between the number of *Echinostoma trivolvis* cysts/individual tadpole and the ratio of white blood cells to red blood cells (WBC to RBC) after 2 weeks of experimental microbead diets.

Four week microbead diet treatment

There were significant effects of infection status ($F_{1,78}$= 8.736, P=0.004) and microbead diet treatment ($F_{2,78}$ =3.846, P=0.026) on mass gain (growth) by tadpoles kept for 4 weeks, as well as a significant interaction of these fixed effects ($F_{2,76}$ =4.554, P=0.013). The tadpoles in the none microbead diet gained the greatest mass followed by those given the high microbead diets, while tadpoles in the low microbead diet gained the least mass (**Figure 2.7**). Neither microbead diet treatment or infection status had a significant effect on the ratio of WBC to RBC, and there was no interaction between these predictors (all P>0.203; **Figure 2.8-A**). Similar results were seen for the ratio of lymphocytes to RBC (all P>0.281; **Figure 2.8-B**), as well as the ratio of eosinophils to RBC (all P>0.197; **Figure 2.8-C**). However, there was a marginally insignificant effect of microbead diet treatment on the ratio of neutrophils to RBC ($F_{2,76}$ =2.942, P=0.059). There is an overall strong trend of microbead treatment on the ratio of neutrophils to RBC (P=0.059) that was driven by a significantly lower ratio (P=0.020) in tadpoles given the high versus low microbead diets (**Figure 2. 8-D)**. There was no relationship between infection intensity and the ratio of WBC to RBC ($F_{1,39}$=0.058, P=0.812) (**Figure 2.9**). See Appendix A for all data set.

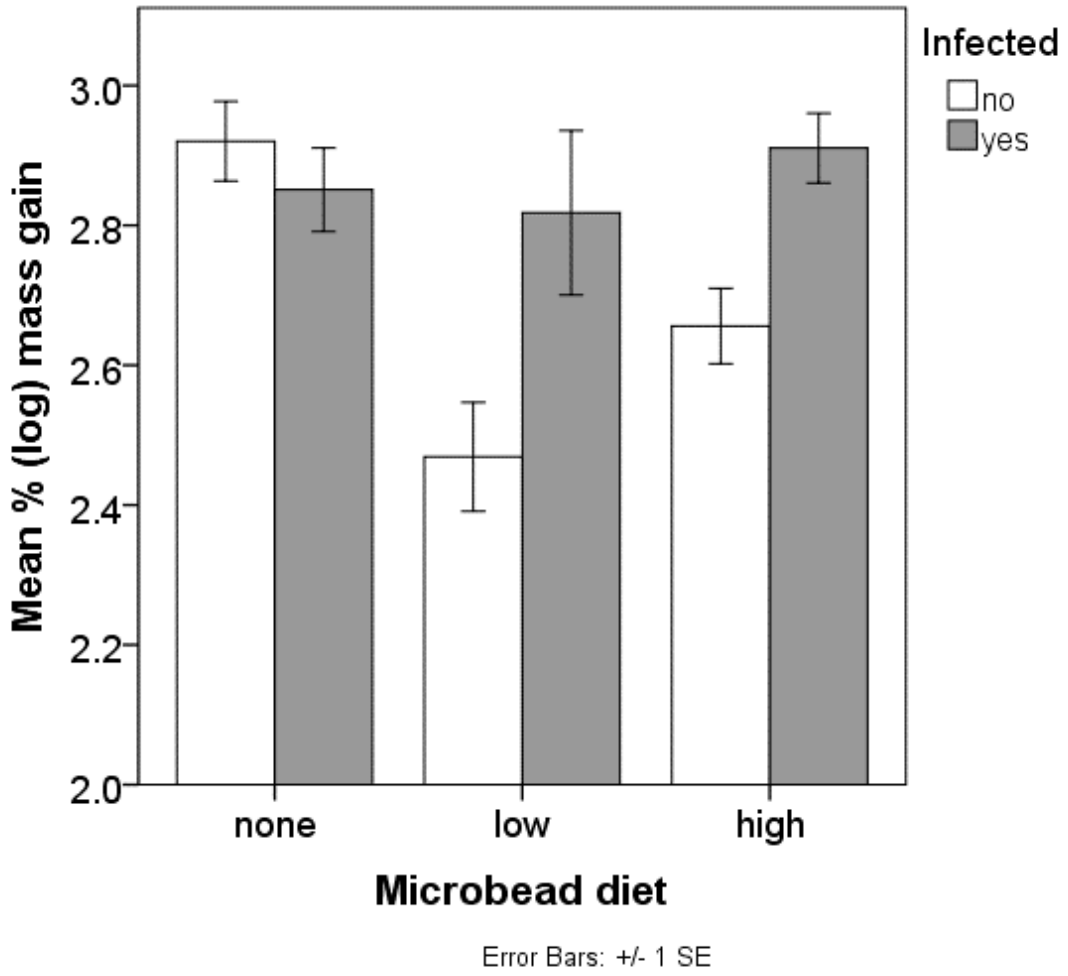

Figure 2.7: Mean (%) (±S.E.) (log_{10}) mass gain by tadpoles fed one of three microbead diets (none=0 microbeads, low=10 microbeads, high=100 microbeads) for 4 weeks, and either infected or not by *Echinostoma trivolvis*.

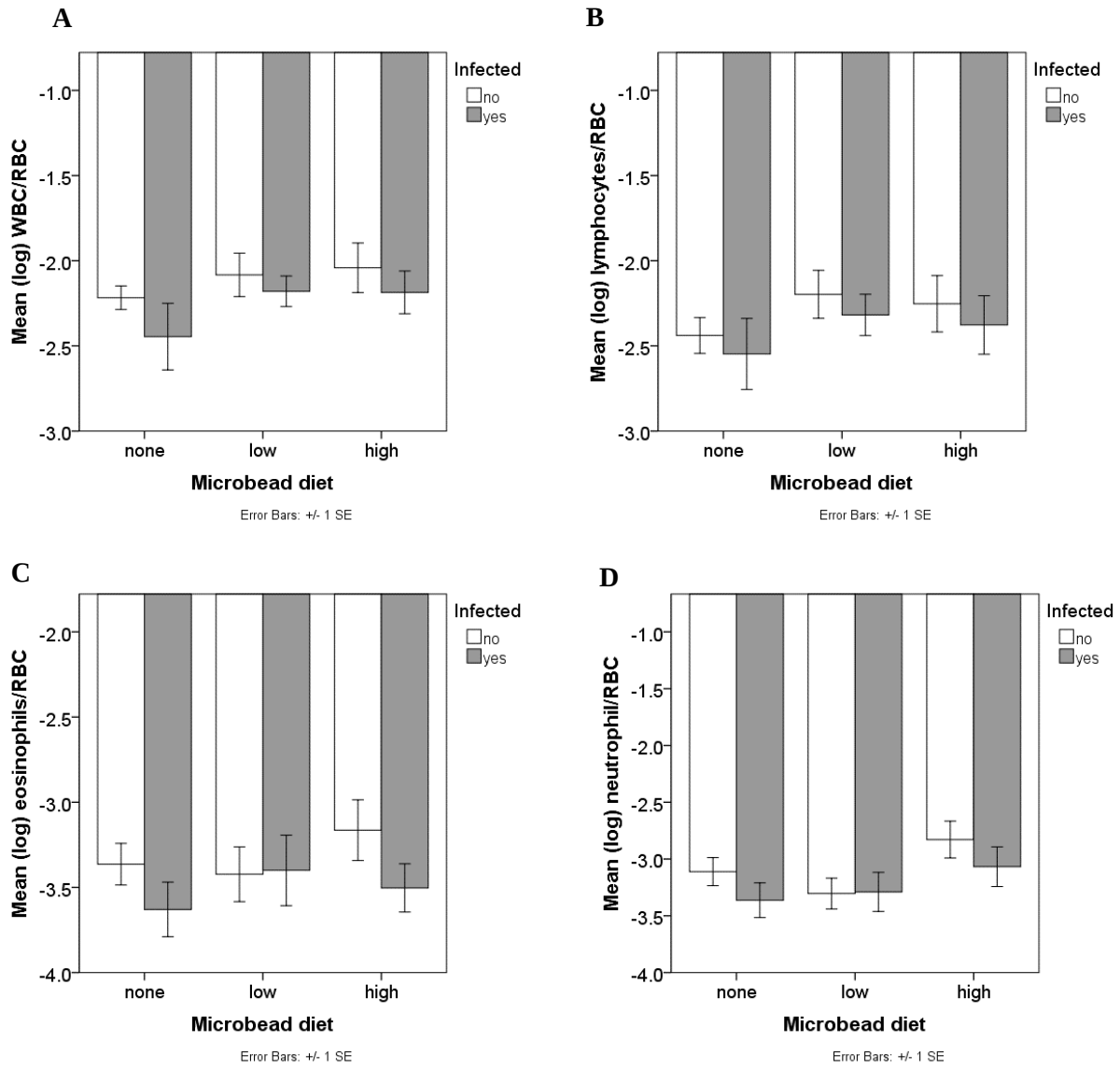

Figure 2.8: Mean (±S.E.) (log_{10}) measures for: **A)** ratio of white blood cells to red blood cells (WBC to RBC) **B)** ratio of lymphocytes to red blood cells **C)** ratio of eosinophils to red blood cells **D)** ratio of neutrophils to red blood cells by microbead diet (none=0 microbeads, low=10 microbeads, high=100 microbeads for 4 weeks) with tadpoles either infected or not by *Echinostoma trivolvis*.

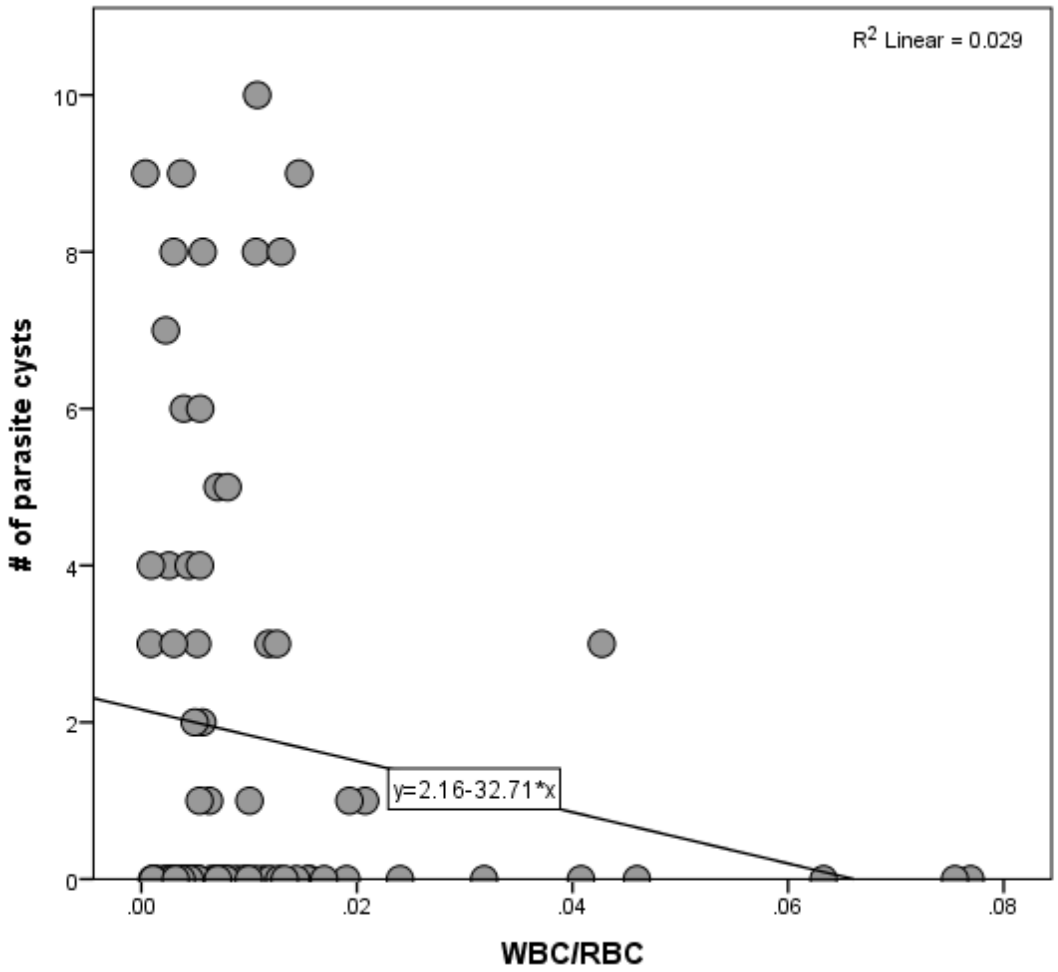

Figure 2.9: There was no relationship between the number of *Echinostoma trivolvis* cysts/individual tadpole and the ratio of white blood cells to red blood cells (WBC to RBC). Note that line of best fit is for illustrative purposes only.

Discussion

I found that experimental diets containing microbeads which correspond to those commonly found in personal care products had a significant influence on the growth of larval northern leopard frogs (*L. pipiens*), as well as their susceptibility to infection by the trematode parasite *E. trivolvis*, but there were minimal effects on tadpole white blood cell (WBC) measures. Tadpoles clearly ingested the microbeads embedded in their experimental food, indicating the possibility for this to also occur in nature under the right circumstances. Tadpoles ate their designated microbead diets and the microbeads travelled through the gut and got excreted. Smaller particles, such as nano-particles could have the potential to cross and enter the bloodstream. Even though microbeads got passed through the gut and excreted, there were still effects on tadpoles. Overall, there was a significant effect of microbead diet on tadpole mass gain (growth), but this was not affected by length of diet treatment (2 or 4 weeks) as both groups exhibited a non-monotonic response. Tadpoles given the none microbead diet grew the most, followed by those in the high microbead diet (100 microbeads/week), with the least mass gained by tadpoles given the low microbead diet (10 microbeads/week). This non-monotonic pattern could result in sufficient feeding and energy in the control diet, not enough food in the low microbead diet and compensatory feeding in the high microbead diet to help these tadpoles to reach their energetic requirements.

Tadpoles could get stressed from ingesting microbeads resulting from a physical obstruction or lack of sufficient energy but could also increase levels of stress hormones such as glucocorticoids. Stress hormones, particularly glucocorticoids like corticosterone in amphibians, go up in stressful environments (Sapolsky *et al*., 2000). If ingesting microbeads causes corticosterone to go up because it is stressful in some way elevated levels could have redirected energy away from growth to help the tadpoles deal with the stress. Changes in glucocorticoids can affect tadpole growth and metamorphosis, as well as immunocompetence. Future studies should measure corticosterone levels along with microbead exposure. Similar to a host investing resources into resistance and tolerance to parasites at the expense of growth (Rohr *et al*., 2008), tadpoles that consume microbeads may face a trade-off as well when attempting to tolerate this type of stressor.

Experimental microbead diets not only had direct effects on tadpole growth as described above, but also indirect effects that related to resistance and tolerance to trematode parasite

infection. Resistance is the ability of potential hosts to reduce successful parasite establishment, whereas tolerance is the ability to deal with a given parasite load so as to minimize its costs (Råberg *et al*., 2007). Tadpoles infected by *E. trivolvis* can suffer from edema, intensity-dependent mortality, inhibited growth, and inflammatory responses (Martin & Conn, 1990; Fried *et al*., 1997). Here, tadpoles given diets containing microbeads for 2 weeks were more susceptible to infection as demonstrated by the significantly higher cyst intensity in tadpoles from the low microbead diet compared to the none, with a strong trend for more cysts in the high microbead diets as well, although cyst intensity did not differ between the low and high microbead diets. This was consistent with literature of tadpoles exposed to stressors like atrazine or road salt that caused an increase in susceptibility to trematode infection (Kiesecker, 2002; Milotic *et al*., 2017). While there was not a significant overall relationship between the number of microbeads and *E. trivolvis* cysts for parasite-exposed tadpoles, it was not possible to know how many microbeads an individual had actually consumed either 2 or 4 weeks based on those found during dissections. Tadpoles in the high microbead diet were fed 10x the number of microbeads as those in the low microbead diet but did not have 10x the number of microbeads in their guts. It is possible that the tadpoles in the high microbead diet learned to selectively eat around the microbeads, whereas in the low microbead diets would have little value to this at the risk of forgoing food. In comparison, Hu *et al*., (2016) also found that microplastics were ingested and egested at fairly quick rates in *Xenopus tropicalis* as found in the digestive tract after 1 hour of exposure and in the feces as quick as 6 hours.

For the tadpoles kept for four weeks, the combination of microbead diet and trematode infection significantly interacted in an unexpected way. I found that infected tadpoles fed either microbead diet gained significantly more mass compared to uninfected tadpoles. In other words, uninfected individuals showed the overall non-monotonic growth response as described above, but parasitized tadpoles grew similarly in all diet treatments. Tolerance to trematode infection can be reduced if tadpoles are exposed to multiple stressors (e.g. Koprivnikar, 2010), but it is unclear why this did not occur here. At low intensity of infection, parasitism is unlikely to affect the tadpoles in the none microbead diet. However, there was significant interactive effect of microbead diet and trematode infection. Parasitism could alter tadpole intestines to become more efficient only in the presence of another stressor to energy, like with the ingestion of microbeads. The higher metabolic demands associated with harboring parasites have been known to cause an

increase in the size (length and width) of host intestines, therefore increasing the rate and efficiency of digestion (Kristan & Hammond, 2003; Schwanz, 2006). The significant increase of mass gain in infected tadpoles irrespective of microbead ingestion could thus have caused by a similar compensation for increased metabolic demands. In contrast, larval pickerel frogs (*Lithobates palustris*) exposed to moderate *E. trivolvis* infection had no significant effects on growth or survival (Orlofske *et al*., 2009). Future studies should therefore look at the size of larval amphibian intestines when they are exposed to *E. trivolvis* in combination with other stressors. It is also possible that tadpoles in the high microbead diet may have engaged in compensatory feeding, which occurs widely when animals are faced with poor-quality diets (Werner & Anholt, 1993).

Although exposure to contaminants can weaken the immune system and make potential hosts more susceptible to parasites (Shutler & Marcogliese, 2011), this is seemingly not the explanation for the reduced resistance of tadpoles fed microbeads here. The leukocytes (white blood cells) of the northern leopard frog are dominated by neutrophils, lymphocytes and eosinophils (Shutler & Marcogliese, 2011). The number of neutrophils is associated with investment in innate immunity, while the number of lymphocytes is associated with adaptive immunity (Shutler & Marcogliese, 2011). Eosinophils are especially important to consider in the context of parasitism because these are cytotoxic cells that stimulate other white blood cells to release histamines to protect themselves from parasites (Edwards, 1994). In spite of this, there was no significant effect of microbead diet or parasite infection on the ratio of WBC to RBC for tadpoles kept either two or four weeks, with similar results for the ratio of lymphocytes to RBC and eosinophils to RBC, although a different pattern was seen for neutrophils. Regardless of whether tadpoles were kept for either two or four weeks, those given the high microbead diet, had the lowest number of neutrophils, followed by the low microbead and none diets, also exhibiting a nonmonotonic response as seen for mass gain.

Other studies have found that circulating neutrophils in amphibians can be affected by exposure to contaminants or elevated levels of stress hormones. For example, *L. pipiens* tadpoles exposed to a pentabromodiphenyl ether mixture displayed a significant decrease in neutrophil counts (Cary *et al*., 2014), as was also seen in *Notophthalamus viridescens* (eastern newt) exposed to adrenocorticotropic hormone (ACTH) (Bennet & Johnson, 1973). If ingestion of

microbeads here potentially caused stress that affected hormones such as glucocorticoids, the outcome with respect to WBC profiles is different than that for grey tree frog tadpoles exposed to exogenous corticosterone who had lower numbers of eosinophils (Belden & Kiesecker, 2005), or the elevated number of lymphocytes seen in northern leopard frog tadpoles exposed to road salt (Milotic *et al*., 2017). In this case, the microbead exposure may be only affecting investment into innate immunity (as represented by the ratio of neutrophils to RBC). However, this will require further study, as well as investigation into other aspects of host defences against parasite infection that could be affected, given the limited literature on the immune system of tadpoles (see Maniero & Carey, 1997; Shutler *et al*., 2009; Davis, 2009).

It is important to understand both the potential direct and indirect effects of microbead ingestion on larval amphibians. Reduced mass gain in response to microbead ingestion may have various implications in natural settings. For instance, a smaller size at metamorphosis can make juvenile frogs more susceptible to predators (Wilbur & Collins, 1973; Skelly, 1994). For example, *L. pipiens* increase in size and decrease foraging in response to the risk of predation (Bennett *et al*., 2013). It is also critical to examine the effects of microbead ingestion in combination with another stressor such as parasite exposure because these can have harmful effects on tadpoles not seen in isolation of one another (Hayes *et al*., 2006; Johnson & McKenzie, 2009; Koprivnikar *et al*., 2012). As pathology owing to trematode infection is strongly intensity-dependent (Schotthoefer *et al*., 2003), that microbead ingestion caused higher parasite loads here not only has implications for tadpole survival and condition, but parasite transmission. Tadpoles are an important intermediate host for many trematode species (Olsen, 1986). By investigating the effects of contaminants and parasite infection on tadpoles, as well as their interaction, the likelihood of parasites reaching final hosts such as birds and mammals can be better understood. It is important to understand how various contaminants can affect larval amphibians considering their worldwide declines (Daszak *et al*., 2003), so this can help better assess their risk. This is the first examination of how microbead ingestion may affect any North American amphibian. My findings will therefore help us to better understand how microbeads could affect fauna in small freshwater bodies, which are understudied at the moment.

Chapter 3 The effects of microbeads on the aquatic snail *Stagnicola elodes* and its production of trematode parasites

Abstract

Microbeads are small plastics that persist in the environment for long periods of time, with important consequences for aquatic fauna in particular; however, little is known regarding potential affects on host-parasite dynamics. This study examined the effects of microbead ingestion on: 1) longevity of freshwater host snails (*Stagnicola elodes*) infected by the trematode *Haematolechus* sp.; and, 2) the emergence of *Haematolechus* sp. cercariae. Through this 3-week lab study, 90 individual host snails were fed food cubes containing either 0, 10 or 100 polyethylene microbeads sized 106-125 μm. 45 snails on these microbead diets, the number of cercariae emerging were counted weekly for the same period. Snails exhibited a significant non-monotonic response to microbead diet in relation their production of cercariae, and snails fed the highest amount of microbeads had the shortest longevity. These results indicate complex effects of microbead diet on potential transmission of cercariae to the next host in the life cycle.

Introduction

Molluscs are important environmental bioindicators because they accumulate pollutants (Dhara *et al*., 2017), but aquatic snails are also a key host for parasites that can affect humans, domestic animals, and wildlife, such as trematodes (flatworms) that cause the harmful disease known as schistosomiasis (Gryseels *et al*., 2006). The trematode life cycle is very complex with multiple intermediate hosts (Sukhdeo, 2012). Molluscs play a key role as the first intermediate host in the complex life cycle of trematode parasites because asexual reproduction within them results in free-swimming cercariae that in turn infect the next required host, including various invertebrates and vertebrates (Olsen, 1986; Sukhdeo, 2012) (refer to **Chapter 1**). Factors that may affect the longevity of infected snails, or the production of cercariae, are therefore important to consider. For example, trematode- infected snails had increased mortality compared to uninfected snails that were also exposed to metabolites of the herbicide atrazine (Koprivnikar & Walker, 2011). For these reasons, it is important to consider possible effects of pollutants not just on molluscs in themselves, but also on their parasites, or how infection may alter host reactions to exposure. By considering other stressors, including infection by pathogens and parasites, it is also possible to look for synergistic effects with contaminant exposure.

Pollutants can affect hosts directly or indirectly by altering their susceptibility or tolerance to parasites, as well as infection intensity, but can also affect parasite transmission stages, especially those that are free-living (Poulin, 1992; Morley *et al*., 2003). This is particularly important for aquatic and motile infectious stages such as trematode cercariae that have to find their next host quickly because they are infectious for about 8 hours after emerging from their snail intermediate host and have a lifespan of about one day (McCarthy, 1999). Various studies have now shown that both infected hosts and their parasites are affected by their aquatic environment. For instance, as salinity increases, this causes harm to both freshwater and marine parasites (Shostak, 1993; Pietrock & Marcogliese, 2003; Studer & Poulin, 2012). There are also various contaminants and pollutants which can cause harm to both hosts and parasites in unpredictable ways. The freshwater snail *Stagnicola elodes* had increased mortality after exposure to a metabolite of the pesticide atrazine, especially if infected with gymnocephalus-type cercariae (Koprivnikar & Walker, 2011), but cercariae production was not affected even though this pesticide can also directly harm them after emergence (Koprivnikar *et al*., 2006). While this has relevance for parasite transmission and pathology, there are still unknown effects for the majority of contaminants, including that of microplastics on molluscs and their parasites (Pettipas *et al*., 2016).

There have been very few studies conducted on the effects of microplastics on snails, let alone the effects with parasites (Imhof & Laforsch, 2016). Imhof & Laforsch (2016) fed *Potampoyrgus antipodarum* (mud snail) food containing a mixture of particles made from various plastic polymers (polyamide, polyethylene, terephthalate, polycarbonate, polystyrene, and polyvinylchloride) sized 4.64-602 μm. The plastic had no effects on the morphology and shell thickness of the snails. Because trematodes can cause a lot of harm to humans and other animals, it is important to know if the development and emergence of cercariae is negatively affected by the ingestion of microplastics in snails serving as hosts. For these reasons, the effects of microplastics on snails and parasites must be studied, as well as the possible effects of pollutants in combination with microplastics.

Not only is the ingestion of microplastics potentially problematic because of issues such as gut blockage (Wright *et al*., 2013), but persistent organic pollutants (POPs) that diffuse from water into microplastics can also cause harm (Rochman *et al*., 2013; Syberg *et al*., 2015). Factors

that effect the sorption of POPs into microplastics include salinity, pH, and temperature (Syberg *et al*., 2015). Higher salinities, low pH and temperatures increase desorption rates of POPs from microplastics (Bakir *et al*., 2014; Velzeboer *et al*., 2014). Plastics with low crystallinity or amphorous regions are packed loosely together which allow POPs to sorb into polymers (Teuten *et al* 2009; Syberg *et al*., 2015; Hartmann *et al*., 2017). Polyethylene is a rubbery polymer which causes adsorption of POPs (Teuten *et al*., 2009; Syberg *et al*., 2015; Hüffer & Hofmann, 2016; Hartmann *et al*., 2017). Hydrophobicity pushes POPs out of the water and then weak van-der-Waals forces keep contaminant molecules in the polymer matrix (Hartmann *et al*., 2017). Common POPs include polychlorinated biphenyls (PCBs) and phenanthrene (Rochman *et al*., 2013; Karami *et al*., 2016). Both PCBs and phenanthrene sorbed to microplastics caused tissue changes and hepatic stress in the Japanese medaka and the north African catfish (Rochman *et al*., 2013; Karami *et al.*, 2016).

There are many potential effects of microbeads that are unknown. Some gaps in knowledge include the following questions: How are molluscs effected by microbead ingestion? Are parasites developing inside hosts such as snails also harmed by microbeads?

The aim of this study was to determine the effects of microbead ingestion on snail mortality, growth and production of cercariae. I hypothesized that there would be an increase in the growth and mortality of snails infected with *H. parviplexus* that were given microbead diets. Because trematode infected snails are often larger in size than non-infected hosts (Sandland & Minchella, 2003; Browne *et al*., 2013), the freshwater snail *Stagnicola elodes* might allocate resources towards growth over reproduction if parasitized (Seppälä *et al*., 2013). However, if infected snails are not receiving enough energy from their specific diet, then this could lead to greater mortality because their basic needs are not being met. Considering that multiple stressors should add more stress to snails. Lastly, I hypothesized that cercariae production would decrease in smails given higher microbead diets. High microbead diets have less energy, therefore giving the parasite to less energy to reproduce if its snail host provides fewer resources. As the rediae and sporocysts in which cercariae develop are located in the digestive gland-gonad (DGG) complex of snails (Probst & Kube, 1999), they may also suffer physical damage from ingested microbeads passing through.

Materials and Methods

Experimental design and snail diet

Stagnicola elodes were locally collected at sites 1 (42°94`77.66 “N; 81°22`68.67 “W), 4 (43°28`34.74 “N; 80°33`19.13 “W) and 5 (43°79`09.46 “N; 79°12`20.74 “W) (**Chapter 5**) and brought back to the laboratory at Ryerson University. Snails were reared in 6 plastic tubs housing approximately 50 snails/ tub, containing approximately 5 L of dechlorinated water. The snails were housed on a 14:10 light/dark cycle and fed store-bought organic spinach to minimize their exposure to other contaminants. Note that the organic spinach was not analyzed for contaminants. The group-housed snails received water changes every two days. Each snail was screened for trematode infection in individual wells of tissue culture plates containing 10 mL of dechlorinated water. To do so, each plate was placed under incandescent bulbs for 1 hour (Koprivnikar *et al*., 2007; Koprivnikar & Walker, 2011) and then each well was examined for emerging cercariae using a dissecting microscope for identification with a standard key (Schell, 1985). The snails infected with *Haematolechus parviplexus* were housed in separate tubs. This trematode was chosen because it was the most common infection found in these snails. Non-infected snails were not chosen because the prevalence of trematode infected snails was so high that it was not possible to achieve equal sample sizes with uninfected snails. The screened snails were then randomly selected from the tubs for the designated microbead diets (0, 10, 100 microbeads; **Figure 3.1**).

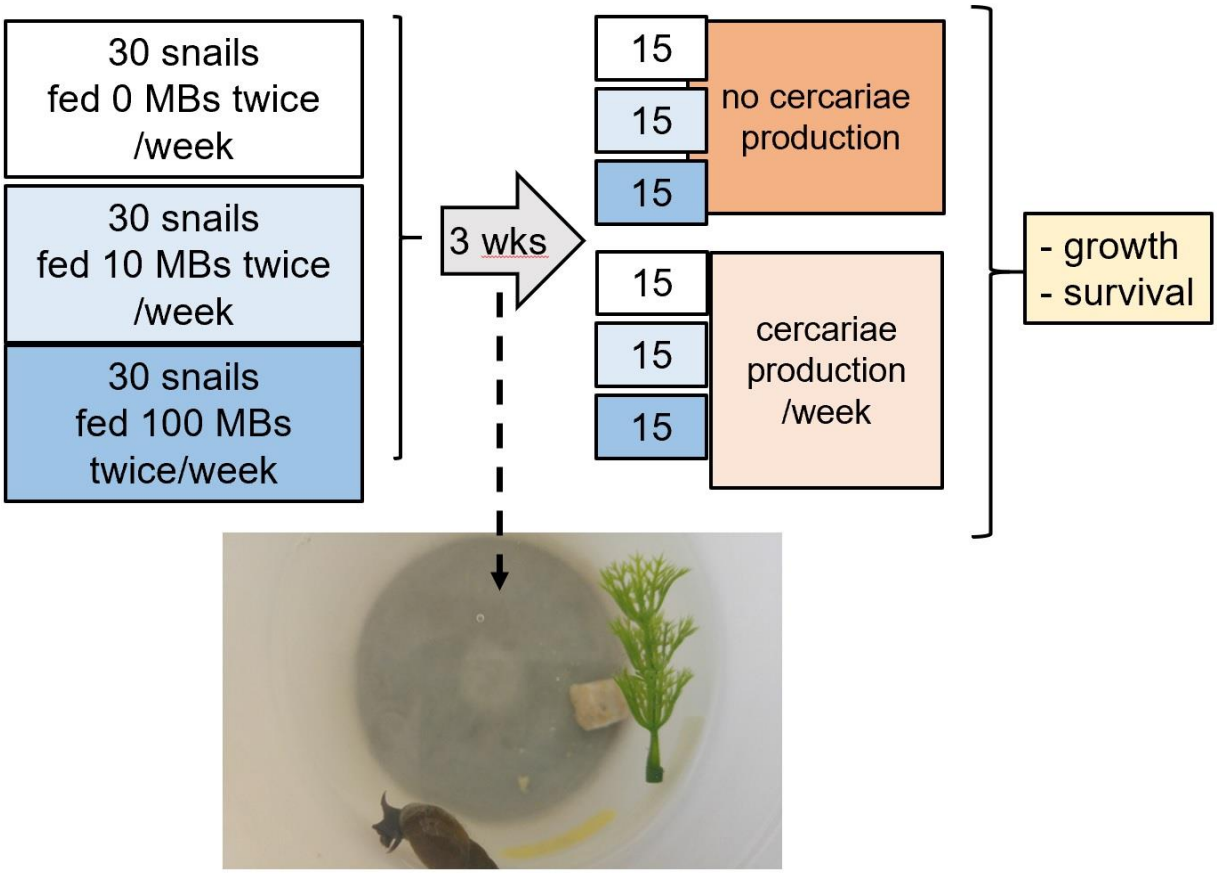

Figure 3.1: Experimental design for snail experiment with exposure to microbead diets.

Based on this experimental design, 90 snails were each individually weighed and their shell length was measured before placing them individually into a 700 mL plastic container filled with 500 mL of dechlorinated water. The placement of each container was randomly distributed among shelves with a random sequence generator. Each snail was fed 1 cm^3 cube of a mixture of a ground Tetramin mix (fish food), water, calcium and agar (similar to Johnson & Hartson, 2009; Imhof & Laforsch, 2016). Note the Tetramin mix was not analyzed for contaminants. Microbeads are commercially available in sizes 10 µm to 1 mm (Pettipas *et al*., 2016). The food cubes contained a designated amount of fluorescent green polyethylene microbeads (sized 106-125 µm from Cospheric Innovations in Microtechnology Item # UVPMS-BG-1.00) that reflected one of the three diets: 0, 10, or 100 microbeads. These concentrations were based on a previous field study if microbeads found in the sediment of the St. Lawrence River, and another looking at the effects of microbeads on African clawed frogs (Castañeda *et al*., 2014; Hu *et al*., 2016), as well as for consistency with an examination of effects on larval amphibians elsewhere in this thesis (**Chapter 2**).

On the feeding days, snails also had water changes and were checked for mortality. The number of *H. parviplexus* cercariae emerging were counted weekly for 45 of the 90 snails (15 per microbead diet) for 3 weeks. The number of cercariae emerging were counted using the screening process described above but instead of simply noting cercariae presence, aliquots of 2 mL were taken from each well and counted for the number of cercariae emerged from each snail. Each snail at the end of the 3 weeks was measured, weighed and frozen for later dissection to confirm infection status and look for microbeads.

Data analysis

Snails were dissected using a dissecting microscope to confirm infection with *Haematolechus* sp. based on established methods (e.g., Koprivnikar & Walker, 2011) and to determine the number of microbeads in the tissues. To examine the cercariae production ($\log_{10}$ transformation of x+1) of each snail over the 4 count time points, I used a General Linear Model (GLM) to conduct a repeated-measures ANOVA (analysis of variance), with microbead diet as a categorical fixed effect, time as the within-subjects effect, and snail length as the covariate because larger individuals tend to produce more cercariae (Koprivnikar & Walker, 2011). Only 22 of the 45 snails with weekly cercariae counts survived until the end of the experiment for inclusion in this analysis. Consequently, I also used a Generalized Linear Model (GLZM) with a

Poisson distribution and log link function for the mean production of cercariae, and for the total production of cercariae by each snail, respectively, over the experimental period so as to use data from all individuals. The mean production of cercariae/snail was calculated as the total number produced divided by the number of count time points (i.e. corrected for length of snail survival) whereas the total production was not corrected for survival. Diet was a categorical fixed effect with initial snail length as a covariate. For the snails that had weekly emerging cercariae counts, another GLZM was used to determine if diet effected longevity of these snails using the week lived until as an ordinal dependent variable with a multinomial distribution and cumulative logit link function. The categorical fixed effect was diet and the length of each snail was again entered as a covariate.

For the dataset with the snails that did not involve weekly counts of cercariae, I also examined the effects of microbead diet on the growth and longevity of each snail. I first calculated the percentage change in the length and mass of each snail (final measure - initial measure/ initial measure). I then used a separate General Linear Model (GLM) univariate ANOVA (analysis of variance) to examine each of the following dependent variables: change of snail length and mass change. The fixed effects for each analysis were diet (categorical). For the longevity of the snails a GLZM with a Poisson distribution and log link function was conducted. Tukey LSD post-hoc tests were conducted for the fixed effect of microbead diet. All analysis was performed in SPSS 24.

Results

Cercariae production results

The repeated measures ANOVA indicated that time did not have a significant effect on cercariae production ($F_{3,54}$= 1.071, P=0.369), and was not affected snail length ($F_{3,54}$=0.935, P=0.430), but there was a significant interaction between time and diet ($F_{6,54}$= 2.658, P=0.025). This is because the number of cercariae that emerged from snails fed the none or low microbead diet peaked at the second time point and then decreased each subsequent week; however, cercariae production remained relatively high for snails fed the highest microbead diet (**Figure 3.2**). The between-subjects test showed that there was no significant effect of diet alone ($F_{2, 18}$= 0.529, P=0.598) on cercariae production for the 18 snails that survived the 3-week experiment.

The GLZM for the total production of cercariae over the experiment (not corrected for individual snail longevity, therefore n=45) indicated that there was a significant effect of diet (Wald X^2=946.358, df=2, P<0.001), as well as the covariate of snail length (Wald X^2=684.375, df=1, P<0.001). The pair-wise comparisons found that there was a highly significant difference among the 1, 10, and 100 microbead diets (each contrast with P<0.001, but lowest total number was from snails in the medium microbead diet (**Figure 3.3**).

The results of the GLZM for mean cercariae production (corrected for individual snail longevity, but n=45) also indicated that diet had a significant effect (Wald X^2 =771.395, df=2, P<=0.001). The follow-up pair-wise comparisons showed that there was a highly significant difference among the 0, 10 and 100 microbead treatments (each contrast with P<0.001) (**Figure 3.4**), with the lowest number again emerging from the snails in the medium microbead diet.

The GLZM for the longevity of the 45 snails undergoing weekly emerging cercariae counts indicates that there was no significant effect of treatment (Wald X^2 = 0.995, df=2, P=0.608) or snail length (Wald X^2 = 0.134, df=1, P=0.714) on their survival (**Figure 3.5**).

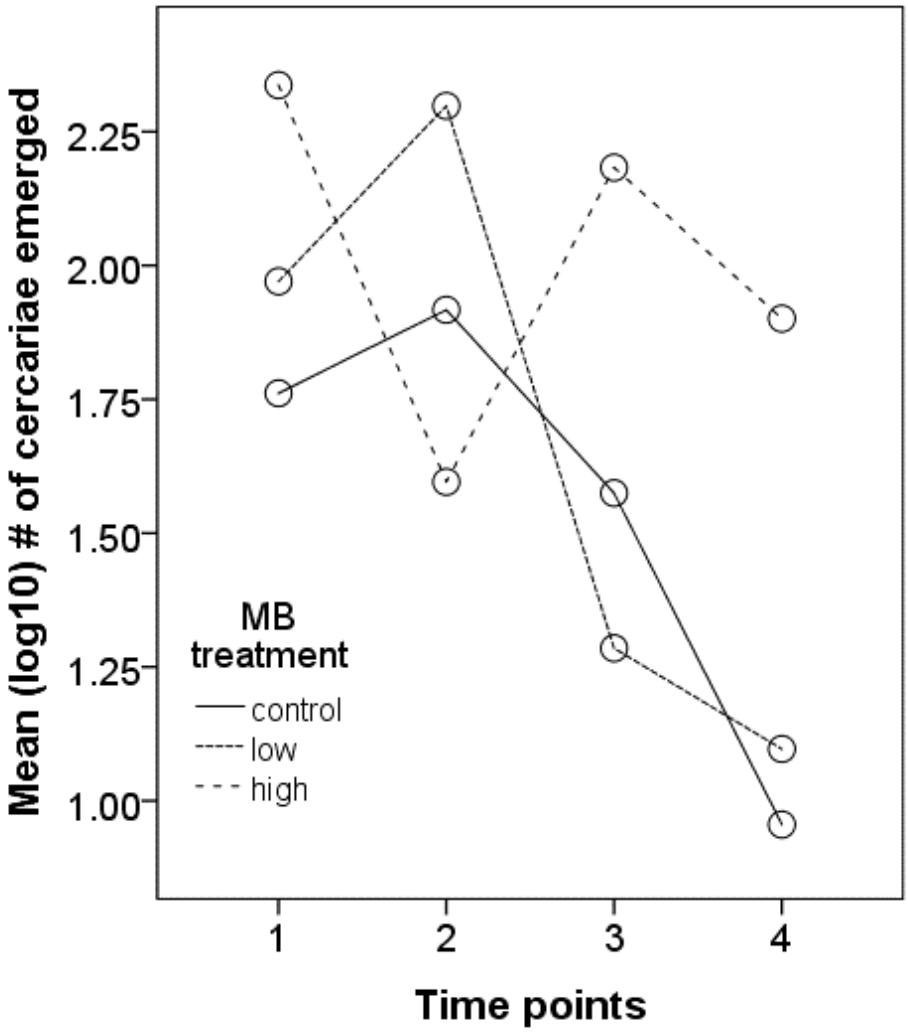

Figure 3.2: The mean (±S.E.) (log_{10}) number of cercariae emerged from snails given one of three microbead diets (none=0, low= 20, and high= 200 microbeads/week) at four time points (1= initial, 2=1 week, 3= 2 weeks, and 4= 3 weeks of diet).

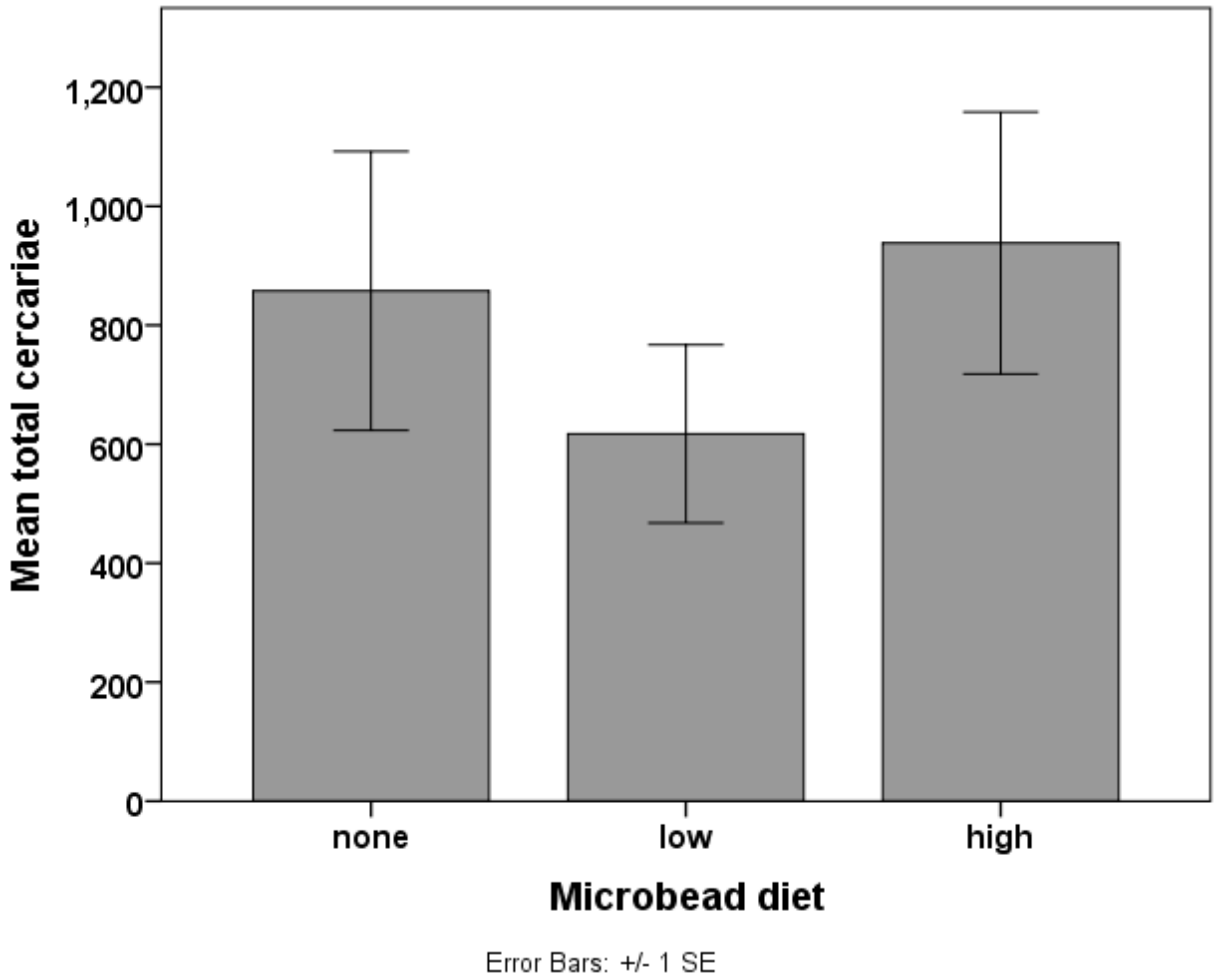

Figure 3.3: Mean (±S.E.) total number of emerged cercariae of *Haematolechus parveplexus* over three weeks from *Stagnicola elodes* fed experimental microbead diets (none=0, low= 20, and high= 200 microbeads/week).

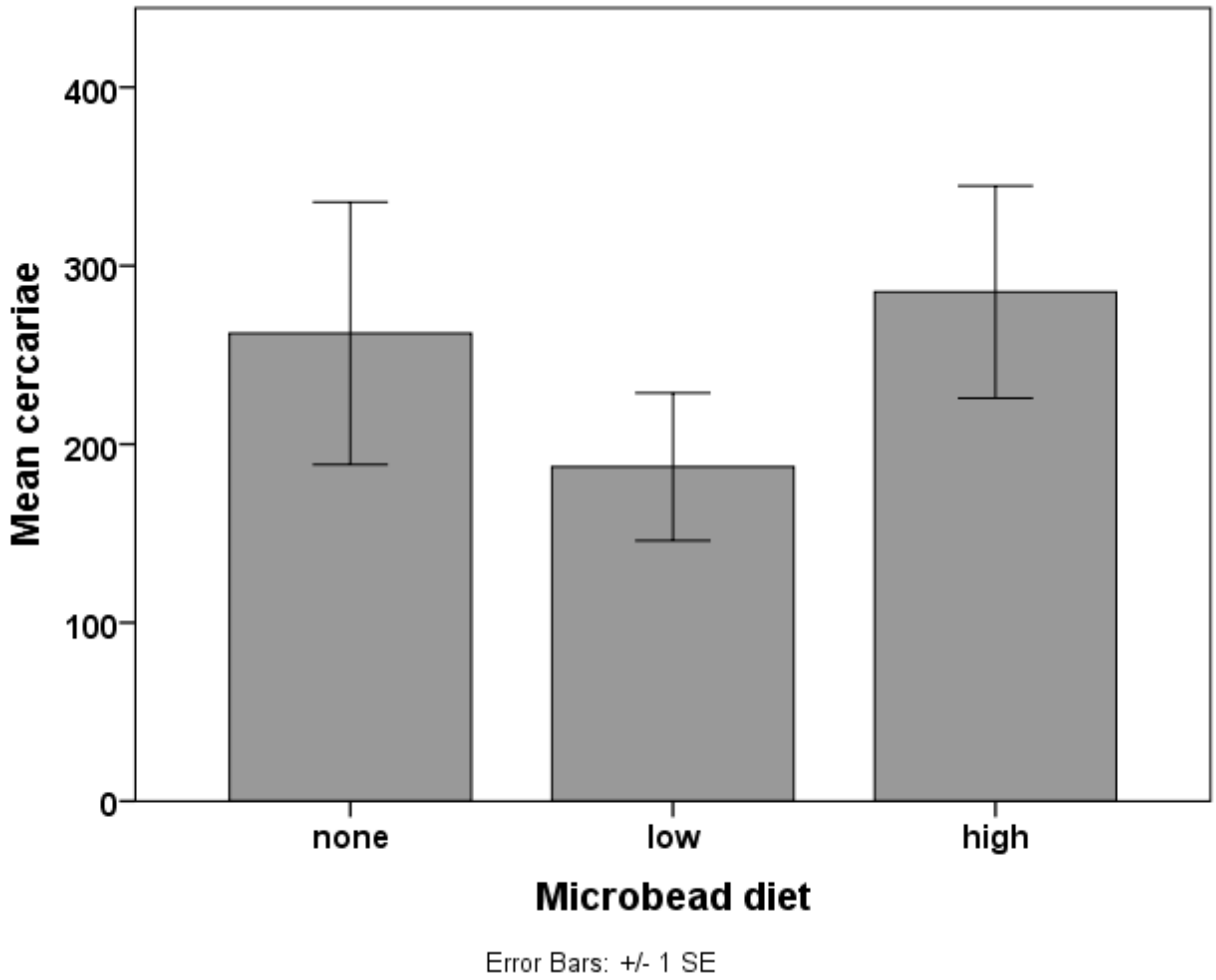

Figure 3.4: Mean (±S.E.) number of emerged cercariae of *Haematolechus parveplexus* from *Stagnicola elodes* fed experimental microbead diets (none= 0, low= 20, and high= 200 microbeads/week), corrected for snail longevity (weeks lived).

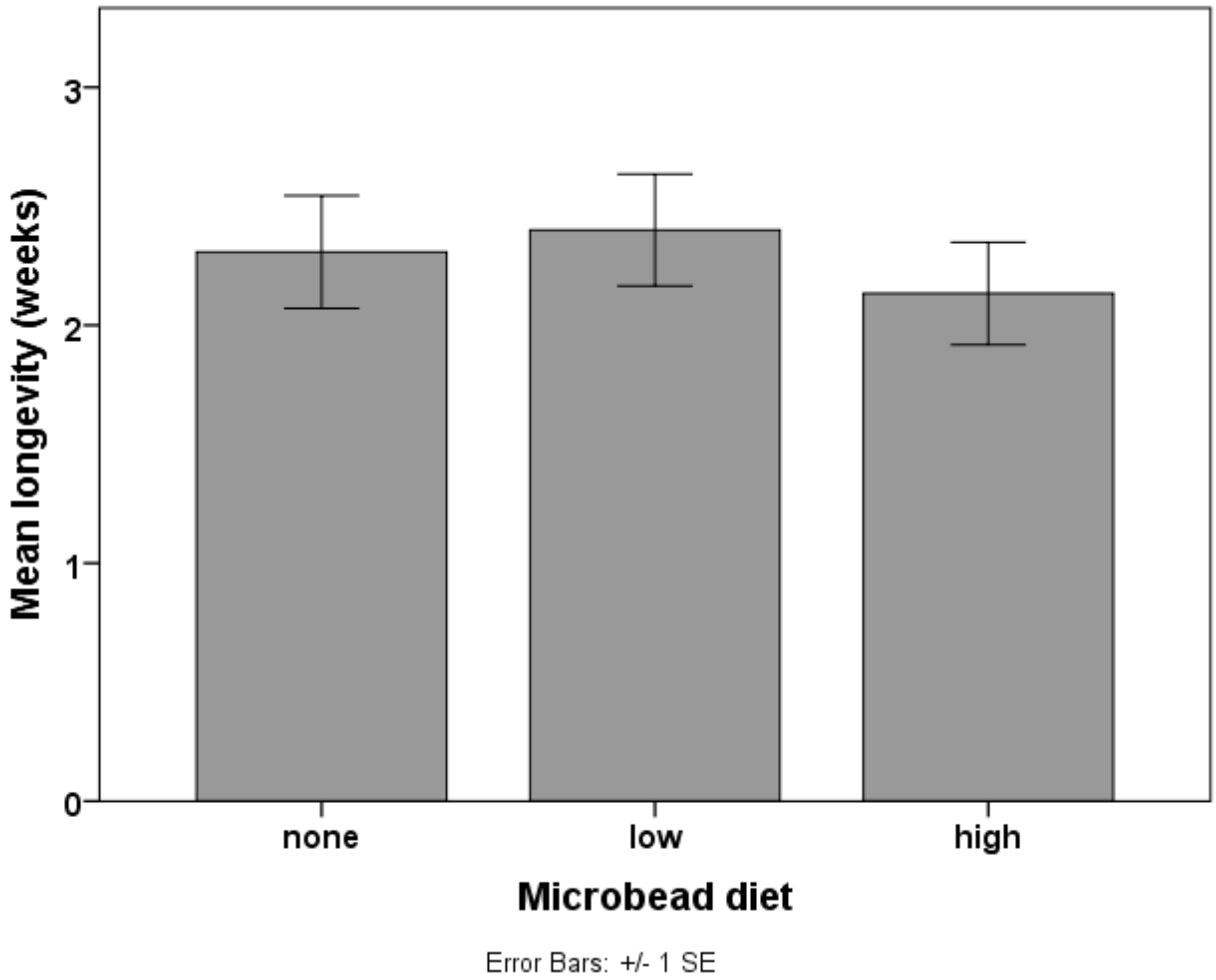

Figure 3.5: Mean (±S.E.) longevity in weeks of *Stagnicola elodes* given experimental microbead diets (none= 0, low= 20, and high= 200 microbeads/week) for which weekly cercariae counts were performed.

Snail growth and mortality- no cercariae production

A General Linear Model (GLM) used to conduct a univariate ANOVA (analysis of variance) for the percentage length change of snails over the course of the experiment indicated that there was no significant effect of diet ($F_{2,33}$ =0.745, P=0.483) (**Figure 3.6**).

There was also no significant effect of diet ($F_{2,33}$ =0.437, P=0.650) on the percentage mass change over the experiment (**Figure 3.7**).

The GLZM indicated that there was a significant effect of diet (Wald X^2 =6.774, df=2, P=0.034) on the longevity of the snails (**Figure 3.8**). Snails lived longest in the none and low microbead diets, and the shortest in the high microbead diet. The pair wise comparisons indicate a significant difference amongst the none and high microbead diets (P=0.019), low and high microbead diets (P=0.026), but no significant difference in the none and low microbead diets (P=0.901). Snails were dissected for the presence of microbeads and I found that 3/90 snails contained visible microbeads. See Appendix B for full data set.

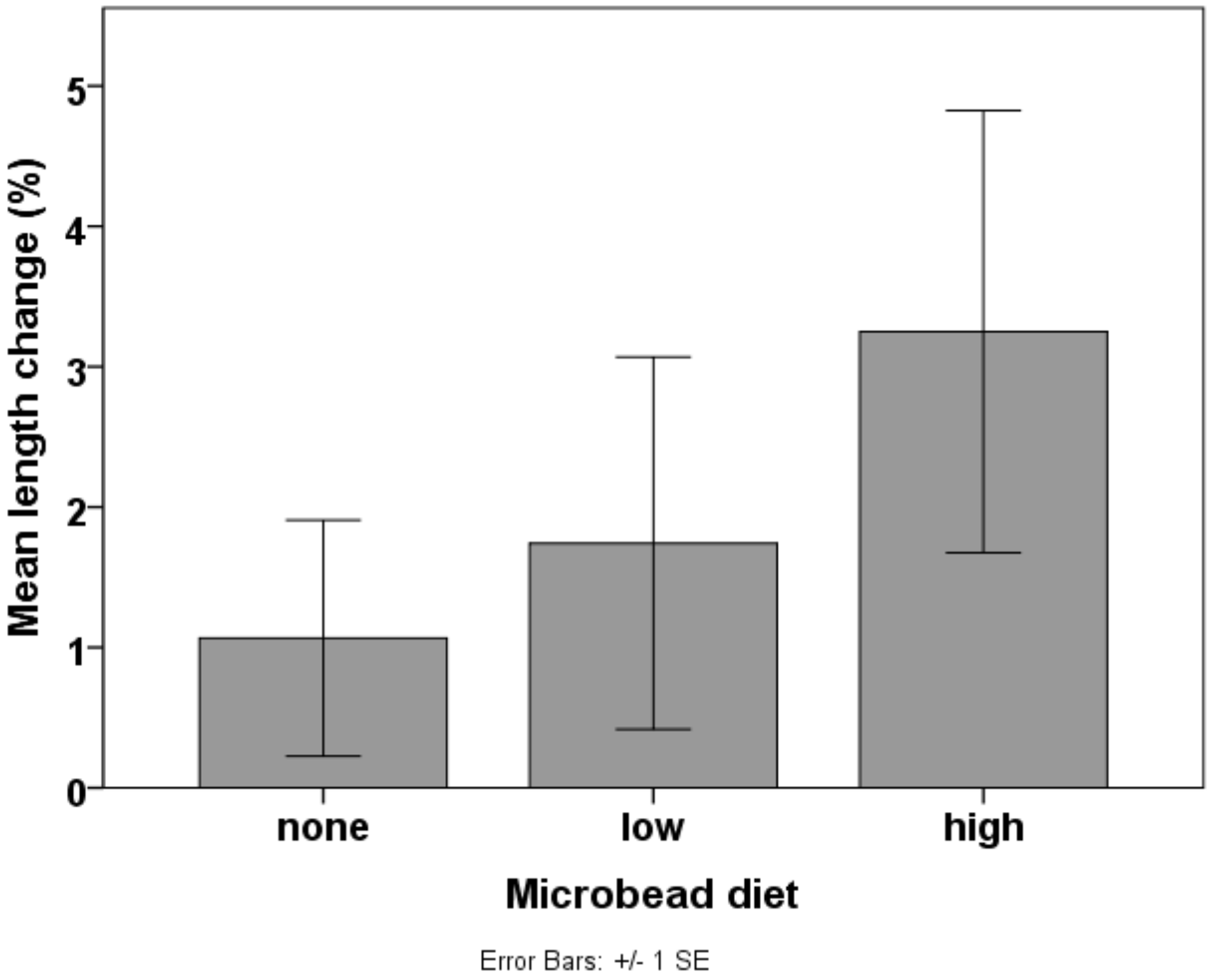

Figure 3.6: Mean (±S.E.) shell length change (%) of *Stagnicola elodes* by microbead diet (none= 0, low= 20, and high= 200 microbeads/week).

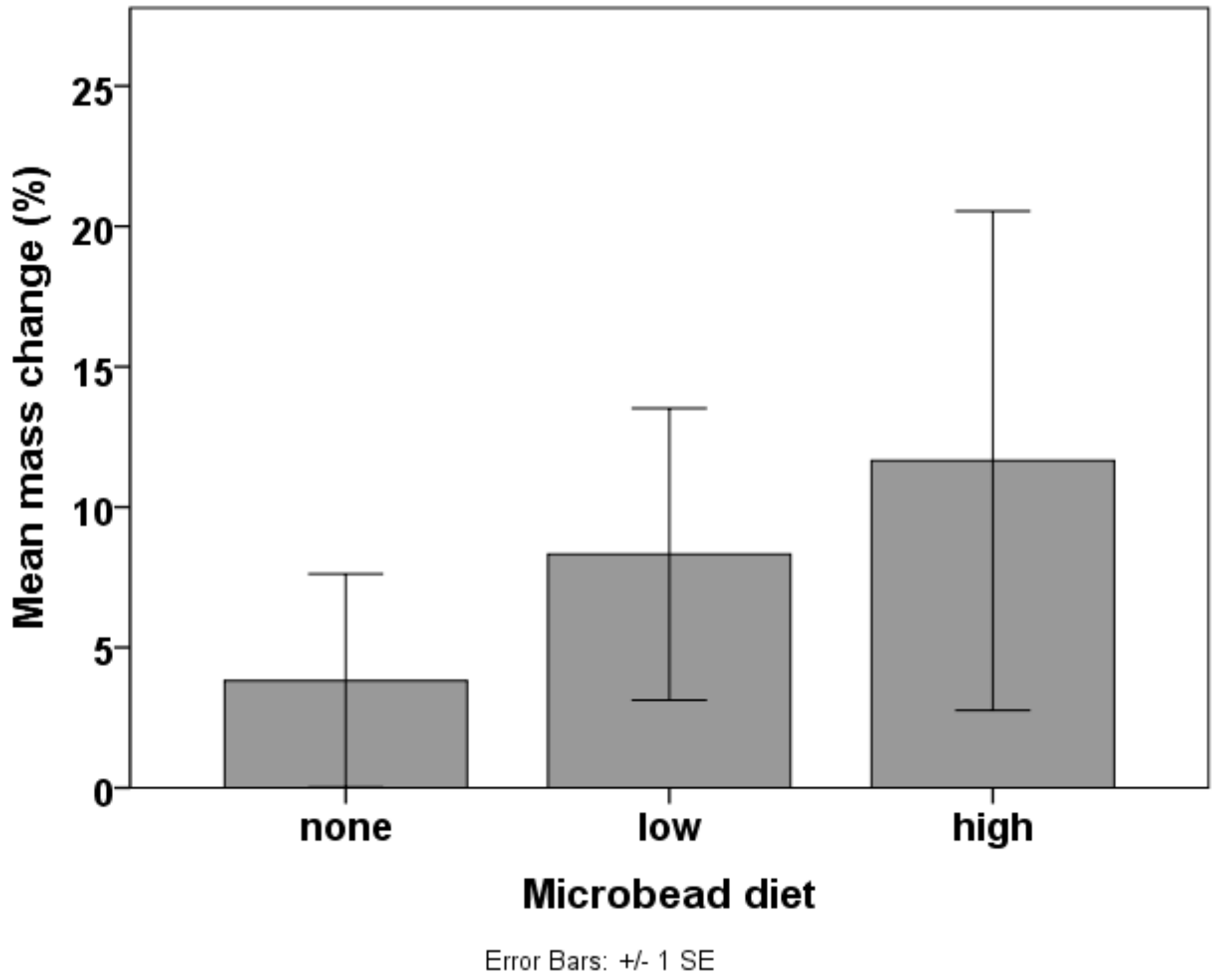

Figure 3.7: Mean (±S.E.) mass change (%) of *Stagnicola elodes* by microbead diet (none= 0, low= 20, and high= 200 microbeads/week).

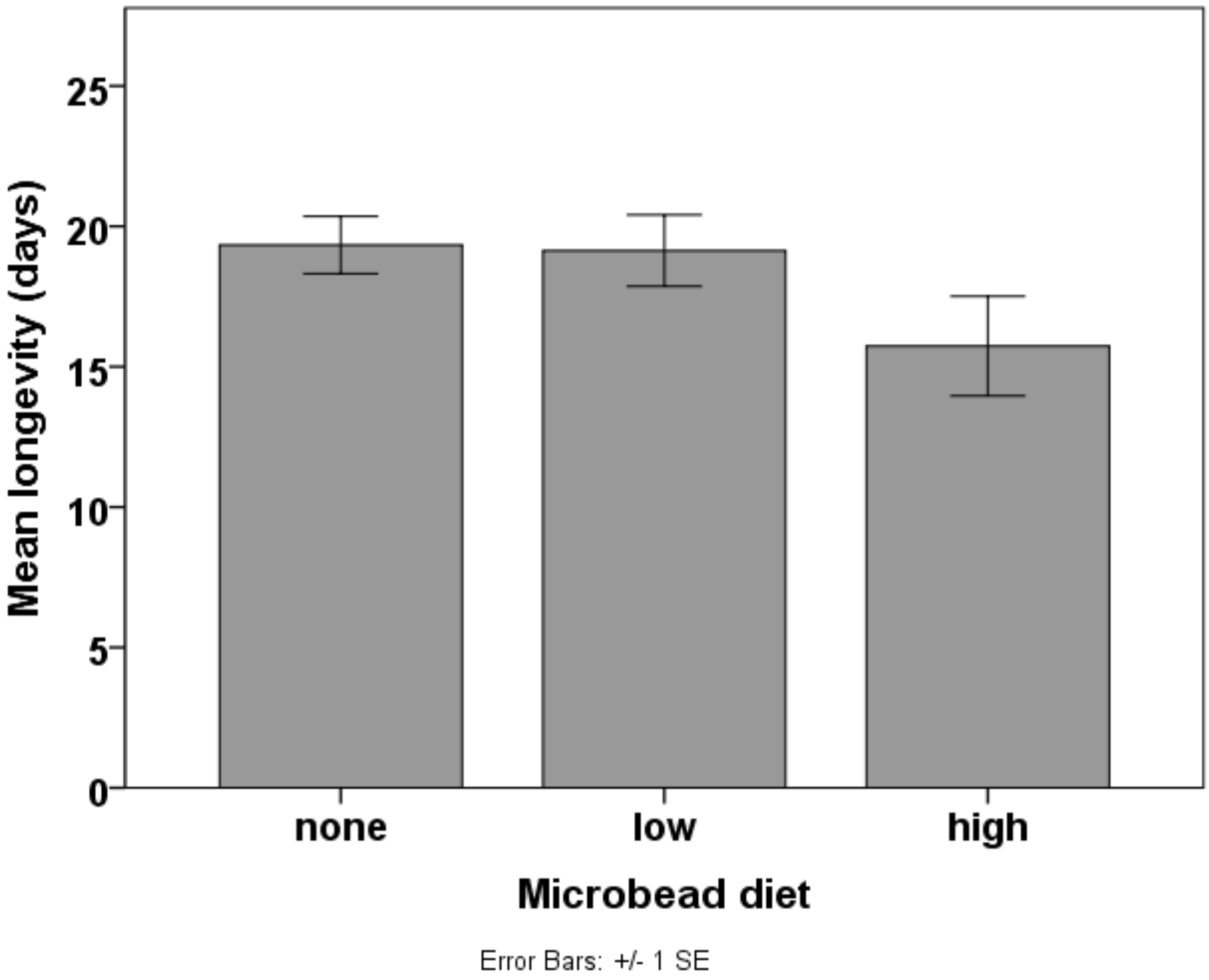

Figure 3.8: Mean (±S.E.) longevity in days of *Stagnicola elodes* by microbead diet (none= 0, low= 20, and high= 200 microbeads/week).

Discussion

The exposure of trematode-infected snails to microbead diets had a highly significant effect on the mean production of cercariae (*Haematolechus parviplexus*) as determined by weekly counts of emerged cercariae. There was a non-monotonic response of mean cercariae emergence (corrected for individual snail longevity) amongst microbead diets, which was different than I had hypothesized. Rather than a dose-dependent negative effect, the production of cercariae was highest in the high microbead diet, and second-highest in the none microbead diet, followed by the low microbead diet. It is not apparent why this occurred, but future studies can examine the mechanisms(s) underlying the non-monotonic responses observed for emergence of cercariae.

While cercariae production increased in snails given the high microbead diets, microbeads were apparently egested at fairly high rates because there were very few microbeads found in the dissected snails. Also, this indicates that microbeads are not likely to accumulate in the gut of the snail. There is the potential though for smaller nano-particles could cross and enter the bloodstream. High egestion rates of microbeads have been seen in *X. tropicalis* tadpoles (Hu *et al*., 2016). This high egestion rates have also been demonstrated for *Carassius auratus* (goldfish), as 0-3 microbeads were found in their gut (Grigorakis *et al*., 2017). Even though snails on microbead diets excreted microbeads at quick rates, there are still effects on each snail. While it is not clear why snails given the diets with none or high ($100/cm^3$) microbeads had more cercariae emerge than those in the low concentration ($10/cm^3$), it should be noted that snail allocation to resources affects the total production of cercariae. This is consistent with literature showing a positive relationship between snail size and trematode parasitism. For examples, *Lymnaea* (*Stagnicola*) *elodes* infected with *Echinostoma revolutum* were larger in size than uninfected snails (Sandland & Minchella, 2003). Trematodes exploit their hosts in various ways and can induce gigantism by causing snails to invest energetic resources into growth instead of reproduction (Seppälä *et al*., 2013). This was reported by Seppälä *et al*., (2013) as growth increased for infected *Lymnaea stagnalis*, while reproduction decreased compared to un-parasitized snails. Greater host size results in more resources for their parasites, especially the rediae that consume host tissue to produce more rediae and cercariae. Also, greater snail size allows the snail to chose riskier behaviour, like more foraging when resources are more abundant (Wojdak, 2009). Considering that snails in the highest microbead diets maintained their growth,

albeit it seemingly at the expense of their survival, this may have allowed cercariae production to continue at a high rate.

As such, shifts in resource allocation under conditions of energetic stress must be considered. For instance, Rollo & Hawryluk (1988) found that *Stagnicola elodes* and *Physella gyrina* allocated resources in different ways when fed artificial diets diluted to various degrees with cellulose. With 75% dietary dilution, *P. gyrina* allocated resources to maintaining reproduction, while *S. elodes* allocated its energy to maintaining growth (Rollo & Hawryluk, 1988). This allocation shift of resources happened before a complete shortage took place (Rollo & Hawryluk, 1988). Most importantly, the ability of these snails to maintain critical functions such as reproduction or growth occurred because of compensatory feeding, i.e. increased ingestion. Both snail species exhibited compensatory feeding in response to diets of diluted food, but *S. elodes* did so to a far greater extent (Rollo & Hawryluk, 1988). It is important to consider how this pertains to ingestion of microbeads by snails infected with trematode infections because their production of cercariae affects downstream hosts like dragonfly larvae, fish, tadpoles, and even humans in the case of schistosomiasis (Olsen, 1986; Sukhedo, 2012). Snails infected by trematodes are typically manipulated to divert their resources away from reproduction and towards growth and maintenance because snails in poor condition constrain parasite replication (Seppäla *et al*., 2013). Compensatory feeding may allow cercariae production to remain high in snails on poor-quality diets, but it is unknown whether this can contribute indefinitely.

For the snail data set (90 snails) where cercariae emergence was not quantified, microbead diet had no significant effect on growth, either by length or mass change. While microbead diet did not have an overall significant effect on the mean length change of snails, the results suggest a possible trend for greater growth by those given the highest concentration of microbeads that requires further exploration, especially if compensatory feeding may have occurred.

There was a significant effect of microbead diets on longevity as snails in 100 microbead diets had shorter longevity compared to those in the 0 and 10 microbead diets. When energy allocation is diverted towards growth, as might be the case here considering that all snails harbored trematode infections, that energy is not necessarily available for vital maintenance, which could have reduced survival. Alternatively, if the snails in the highest microbead diet had

less energy available to them, this may have caused higher mortality. Importantly, all of the snails examined here were infected by the trematode *H. parviplexus*, so this may have represented a situation of multiple stressors. Gustafson *et al*., (2015), found that uninfected snails exposed to atrazine did not show negative effects on growth, reproduction or survival; however, Koprivnikar & Walker (2011), showed that *S. elodes* infected with gymnocephalus-type cercariae had increased in mortality if exposed to atrazine metabolites. It is thus possible contaminants in combination with other stressors like parasites have diverse effects on resource allocation which can differ amongst various types of snails and situations.

Further studies are needed to examine how multiple stressors effect energy allocation and longevity of hosts and parasites directly and indirectly. It is also important to consider how additives could potentially affect the aggregation dynamics of microbeads and food sources for snails and other freshwater herbivores, such as algae, to evaluate the possibility of microbead ingestion in natural systems. Studying the effects of various contaminants, including microbeads, on snails is valuable because these molluscs are good environmental bioindicators by their known accumulation if other pollutants, and are the first intermediate host of trematode parasites that can cause harmful infections in humans and wildlife.

Chapter 4 Aggregation of *Chlorella pyrenoidosa* and microbeads

Abstract

A potential pathway for plastic microbeads to get ingested is through the hetero-aggregation of microbeads and algae. *Chlorella pyrenoidosa* was cultured and pipetted into test tubes with polyethylene microbeads (sized 106-125 μm). To quantitively determine the aggregation of algae and microbeads, samples were taken from the top and bottom of the test tubes to account for the movement of aggregations from the surface water to the sediment. Counts of algal cells were also taken at the top and bottom of the test tubes to account for density-dependent aggregation formation. *Chlorella pyrenoidosa* and microbeads form aggregations in the size range for the latter that are commonly found in personal care products. The interactions of the algae and microbeads were dependent on cell density. Some transport of microbead-algae aggregations from the surface water to the sediment through sinking, may cause more organisms to be affected by microbead pollution if both benthic and surface water inhabitants ingest them.

Introduction

Microplastics can enter waterbodies by the final effluent from wastewater treatment plants, or through runoff of biosolids applied to agricultural lands (United States Environmental Protection Agency, 2007; Saruhan *et al*., 2010). Microplastic contamination is a likely a wide-spread problem in aquatic ecosystems (**Chapter 1**), thus understanding the potential pathways of ingestion by aquatic fauna is important. Microbeads have been examined wihin the paradigm of direct ingestion by aquatic animals (Rochman *et al*., 2013; Hu *et al*., 2016; Imhof & LaForsch, 2016; Lu *et al*., 2016; Grigorakis *et al*., 2017). However, their formation of aggregations with primary producers such as algae also merits consideration as a route for ingestion, especially for herbivores in under-studied systems such as small freshwater bodies.

There are many herbivorous organisms that occur in freshwater wetlands and ponds in North America which could be exposed to microbeads. These can include the aquatic snails *Helisoma trivolvis* and *Stagnicola elodes,* and larvae of amphibians *Lithobates pipiens* and *Lithobates sylvatica* (Chase, 2003; Relyea, 2005). The selection of algae by herbivores is based on the particular distribution of various algal species, largely depending on their location in surface water or sediment (Poore, 2004; Yin *et al*., 2010). Common types of algae found in wetlands and ponds include: *Stigeoclonium, Oedogonium, Cladophora,* and *Chlorella* (Wehr &

Sheath, 2003). The tendency to form aggregations determines if an algal assemblage will remain in the surface layer or sink (Alldredge & Silver, 1988), which in turn affects its likelihood of ingestion by herbivores depending on their microhabitat preferences (Campeau *et al*., 1994).

Aggregations may form through interactions among algae and other materials. For instance, Guenther & Bozelli (2004), studied how suspended clay particles enhance algal sinking through the aggregation of algae and clay. This was conducted by examining how algal populations densities fluctuated over time with different clay concentrations. The population densities of the algae species studied (*Phormidium amoenum*, *Mougeotia* sp., *Staurodesmus convergens*, and *Chlorella* sp.) decreased as the number of algae-clay aggregations increased (Guenther & Bozelli, 2004). This trend was dependent on the morphological characteristics of the algae and the concentration of clay particles (Guenther & Bozelli, 2004). The adhesion of particles and algal cells thus increases the sinking rates of the latter (Avnimelech *et al*., 1982; Alldredge & Silver, 1988; Soballe & Threlkeld, 1988; Cuker *et al*., 1990; Jackson, 1990), and this could occur with various types of inorganic particles, including microplastics. Importantly, hydrophobic interactions can cause microplastics to adhere to algae (Long *et al*., 2015; Gustov *et al*., 2016).

If so, this may shift the distribution of otherwise buoyant microbeads from surface waters and allow their ingestion by herbivores that consume benthic algae. For example, Lagard *et al*., (2016) demonstrated that the formation of aggregations between polyethylene squares ($1cm^2$) and *Chlamydomas reinhardtii* caused these microplastics normally found in the surface water to be transported to the sediment.

The tendency to form hetero-aggregations depends on the characteristics of the materials involved. Positively-charged particles have a high affinity to negatively-charged particles. Algae, bacteria and detritus have negatively charged components, therefore attracting positively-charged particles (Neihof & Loeb, 1972). The size and density of the particles is also important. For instance, the probability of contact between algae and clay particles increases when there is a higher concentration of clay particles or at larger algae cell sizes (Alldredge & Silver, 1988). Another factor influencing the ionic interaction of algae and clay particles is the nature of the algae cell surface. The cell surface has properties that may play a role in the aggregation formation, such as the secretion of polysaccharides like mucilage (Kilorboe *et al*., 1990).

Positively-charged, nanosized plastic beads more readily adsorb to *Chlorella* and *Scenedesmus* than negatively-charged ones due to the electrostatic attraction between the beads and cellulose constituents (Bhattacharya *et al*., 2010). In another study, polystyrene particles (0.05, 0.05, 6 μm in size) that were either negatively- or neutrally-charged were exposed to marine algae (*Thalassiosira pseudonana* and *Dunaliella tertiolecta*) for 72 hours (Sjollema *et al*., 2016). The authors found that microalgal growth was reduced up to 45% by the uncharged polystyrene particles only at high concentrations (250 mg/L), with an increased negative effect at smaller particle sizes.

Chlorella is unicellular, eukaryotic green algae found in freshwater habitats. The herbivores *Stagnicola elodes* and *Lithobates pipiens* are known to consume *Chlorella* (Seale & Beckvar 1980; Colbourn *et al*., 2007). The formation of hetero-aggregations with microbeads is therefore important to see if this could be a route of ingestion in nature. While I have established that there are effects on microbead ingestion on tadpoles and snails through an experimental diet involving microbeads embedded in a solid food mixture (**Chapter 2 & 3**), a more realistic route should be examined. As described above, the sinking of this algae has been shown to be dependent on the amount of clay particles adhered to its cells (Alldredge & Silver, 1988). Because *Chlorella* has a high surface area to volume ratio owing to its relatively small size, cells need a large amount of clay particles adhered in order to sink (Alldredge & Silver, 1988). This small size of individual cells also means that there is a lower probability of *Chlorella* encountering clay particles compared to other larger-sized algae (Alldredge & Silver, 1988). *Chlorella* and clay particles aggregations were formed at the highest concentration of suspended clay particles tested (50 mg/L) by Alldredge & Silver (1988), and the sinking of these aggregations may have been enabled by the mucilage cover of *Chlorella* (Alldredge & Silver, 1988). Hydrophobic interactions cause microplastics to adhere to algae (Long *et al*., 2015; Gustov *et al*., 2016). Owning to these properties, as well as its surface charge, *Chlorella* can perhaps also readily adhere to other inorganic particles.

During the exponential population growth phase, *Chlorella* cells have a highly negative surface charge and remain dispersed because they are difficult to neutralise (Vandamme *et al*., 2012; Safri *et al*., 2014). Once the *Chlorella* population reaches the declining phase, the negative charge of the cells decreases, causing them to aggregate together (Vandamme *et al*.,

2012; Safri *et al*., 2014). Given that plastics are typically positively charged, this material is attracted to the cellulose found algae due to electrostatic forces (Kalčíkova *et al*., 2017). The adsorption of plastic to algae is also enhanced by the roughness of cellulose surfaces providing numerous binding sites (Kalčíkova *et al*., 2017). Previous studies have worked with large fragments, or nano-beads, leaving a gap in knowledge of algae interactions with microbeads in the size of personal care products.

For this study, I aimed to determine if polyethylene microbeads similar in nature to those typically found in personal care products (10 μm to 1mm; Pettipas *et al*., 2016) and *Chlorella* can form aggregations. Due to the positively charged nature of this plastic, and subsequent attraction to the cellulose of algae, I hypothesized that microbeads and *Chlorella* should create aggregations because of electrostatic forces (Kalčíkova *et al*., 2017). Here I set out to: i) determine if *Chlorella* forms aggregations with microbeads representative to those found in personal care products; ii) quantify the relationship between aggregations of *Chlorella* and microbeads and the algal cell density to see if their formation is density-dependent; iii) explore the range in size exhibited by aggregations; and iv) determine if the number and size of aggregates differed between the top and bottom of the experimental containers to test possible implications for surface water and sediment differences in the field. Through this study, I sought to help achieve a better understanding of how the formation of algae-microbead aggregates affects the transport of microplastics and influences the likelihood of their selection as food by common freshwater herbivores such as the snails and larval amphibians examined in earlier chapters of this thesis.

Materials and Methods

Algae culturing

Chlorella pyrenoidosa (Item #470176-674) and Algae Gro ® Concentrate (15-3751) (akin to Bold's media, see James, 2012) were obtained from Boreal Scientific and the Carolina Biological Supply Company, respectively. In a 500 mL flask, I mixed 10 mL of *C. pyrenoidosa* with 100 mL of the Bold's media. The algae was cultured for 2 weeks in this flask on a continuous shaker for 100 rpm under a 16:8 light-dark cycle until it reached exponential growth phase (Environmental Canada, 1992). The light intensity at which the culture was kept was measured using a Solar Electric Quantum Meter (Item #3415FSE) from Spectrum Technologies,

Inc. The light intensity was found to be 4000 ± 400 lux, which along with a temperature of 24 ± 2°C was in keeping with established culturing methods (Environmental Canada, 1992).

Experimental design and procedure

To quantitatively demonstrate the aggregation of microbeads and algae, I used an approach similar to those by Guenther & Bozelli (2004) and Grossart *et al*., (2006) in measuring the aggregation size and looking at the settling of aggregates. After my *C. pyrenoidosa* culture reached exponential growth phase, 10 mL of the culture and 2 mL of Bold's media were pipetted into each of the 10 test tubes (diameter=16 mm, length =125 mm, total volume=20 mL). The test tubes were not on a continuous shaker, unlike a similar experiment (Yokota *et al*., 2017), so as to create realistic environmental conditions representative of wetlands that are relatively stagnant with little water movement. I used a 16:8 light-dark cycle, with a light intensity of 4000 ± 400 lux, and a temperature of 24 ± 2°C (Environmental Canada, 1992). Next, 100 blue polyethylene microbeads (sized 106-125 μm in diameter) from Cospheric Innovations in Microtechnology (Item # BLPMS-1.08) were added to each test tube. These individual cultures were then maintained for 2 weeks under the conditions described above.

Subsamples were taken from each test tube to determine the cell density (cells/mL) and number of aggregations randomly across the 2 weeks. Sub-samples were taken from the top and bottom of the test tubes (following Guenther & Bozelli, 2004) to evaluate the vertical distribution of aggregates. A total of 4 subsamples, each consisting each of 10 μL was taken from each tube using a disposable plastic pipet. I thus took 20 μL from the top (within 1 cm) and 20 μL from the bottom (within 1 cm) of the test tube. From each 20 μL aliquot from the top and bottom of the test tube, respectively, 10 μL was pipetted to a hemocytometer and a light microscope was used to determine the cell density. The other 10 μL was pipetted onto a slide and analysed using a light microscope at 100x magnification. I used the digital image package Nikon 4.3 NIS Elements to capture 10 fields of view (FOV) from each slide. In each FOV, the number of aggregations (anything more than one cell of algae) was counted, and the area of each aggregation was determined by circling the circumference of the latter for calculation by the software. Each aggregation was placed into one of four aggregate size classes that I established (size class 1=25-1000 μm^2, size class 2=1001-5000 μm^2, size class 3= 5000-10 000 μm^2, size class 4=> 10 000 μm^2) (**Figure 4.1**).

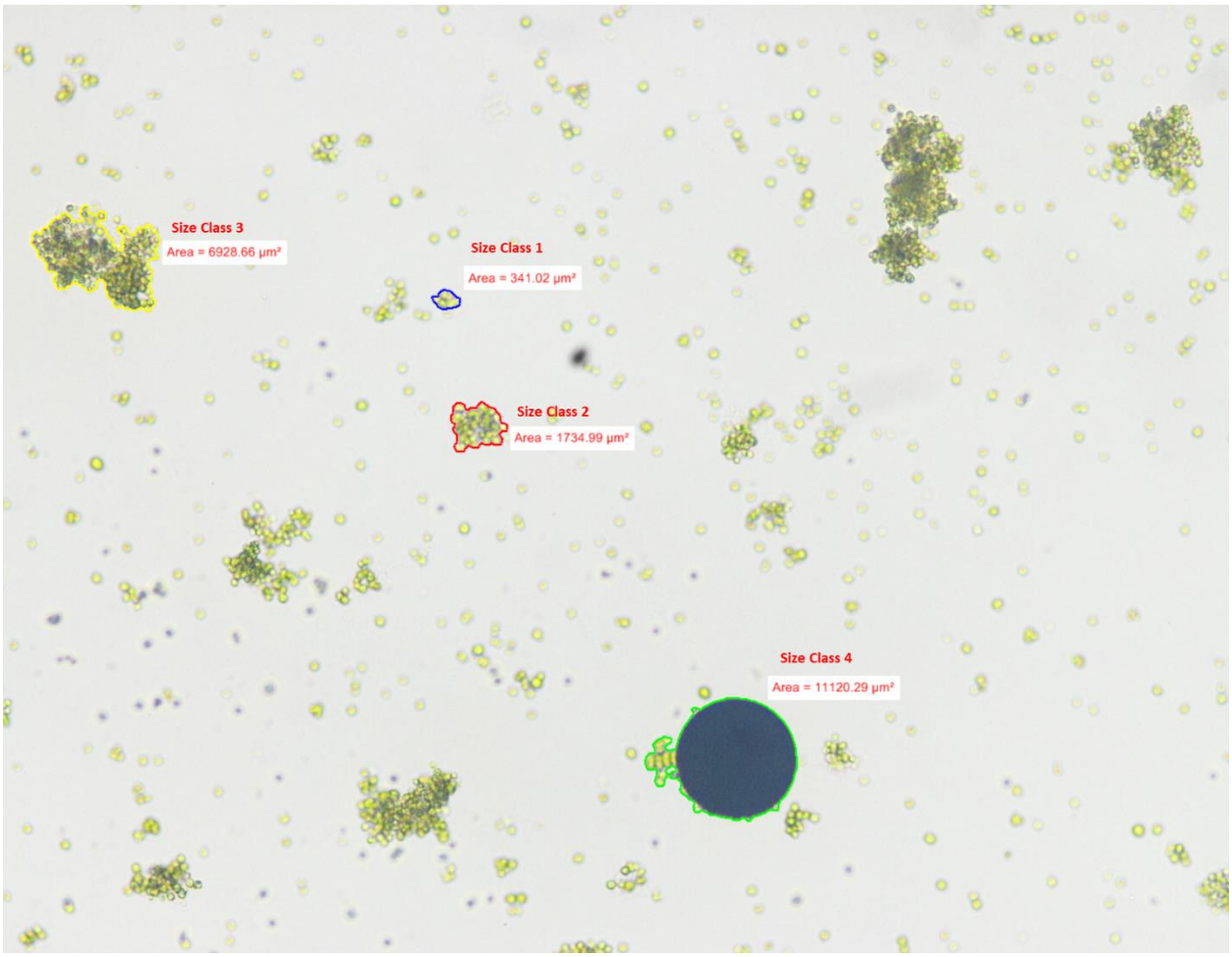

Figure 4.1: A field of view (at 100x magnification) showing live *Chlorella pyrenoidosa* with a polyethylene microbead demonstrating the various aggregation size classes (size class 1=25-1000 µm^2, size class 2=1001-5000 µm^2, size class 3= 5000-10 000 µm^2, size class 4=> 10 000 µm^2).

Data analysis

The total number of aggregations was $\log_{10}$ transformed to help meet the assumption of a normal data distribution. A univariate ANOVA (analysis of variance) was performed using a General Linear Model (GLM) procedure to determine whether the total number of aggregations differed based on the location in the test tube (top or bottom). Each 10 µL sub-sample for which a slide was created for examination was considered as an individual replicate. The total number of aggregations was the dependent variable, location in the test tube was a categorical predictor and the algal cell density of each individual tube was included as a covariate. I then calculated the proportion of total aggregations in each size class for each individual tube and arcsine square-root transformed these values. For each of the aggregation size classes (size class 1, 2, 3, and 4), a series of separate univariate ANOVAs were conducted using a GLM procedure to evaluate if their relative proportions also differed by location in the test tube. The dependent variable was the proportion of aggregations in the size class of interest, the location in the test tube (top or bottom) was the categorical factor, and the algal cell density for that tube was included as a covariate. A separate univariate ANOVA was used with the GLM procedure to determine if the number of aggregations differed by size class. The number of aggregations ($\log_{10}$ transformed after adding a value of 0.1 to account for zeroes) was the dependent variable, the categorical fixed factor was size class (4 total) and the algal cell density for each tube was again included as a covariate.

To determine if the presence or absence of microbeads was affected by the location in the test tube, a Generalized Linear Model (GLZM) was used. To evaluate this, I considered if there were any aggregations in size class 4 for each sample because only this category contained a size range (> 10 000 μm^2) large enough to include a microbead sized 106-125 µm in diameter (8820-12 266 μm^2) with algae adhered. The presence or absence of microbeads was the dependent variable (binomial distribution with a logit function), the location in the test tube was a factor and algal cell density was a covariate. A Generalized Linear Mixed Model (GLMM) was conducted to determine if there was a relationship between the algal cell density (fixed effect) and the number of aggregations in size class 4, with location in the test tube.

Lastly, a series of paired t-tests were conducted to compared the respective areas for the following contrasts: algae + microbead aggregation vs. microbead only; microbead only vs. single algae; microbead only area vs. algae only aggregation; single algae + microbead vs. single

algae; algae only aggregation vs. single algae; and algae + microbead aggregation vs. algae-only aggregation. This was based on the 10 fields of view for each sub-sample.

Results

Location in the test tube (top or bottom) had a significant effect ($F_{1,37}$= 25.802, P<0.001) on the total number of aggregations. The top test tube location had fewer aggregations (mean value of 54.35 ± 22.32 S.D.) compared to the bottom (mean value of 114.35 ± 39.99 S.D.) (**Figure 4.2**). There was no significant difference based on location in the test tube for the proportion of total aggregations comprised by 3 of the 4 size classes, respectively (size class 1: $F_{1,37}$= 0.012, P=0.740; size class 2: $F_{1,37}$= 0.985, P=0.327; and size class 3: $F_{1,37}$= 0.783, P=0.382; **Figures 4.3A-C**). However, test tube location had a significant effect on the proportion of aggregations in size class 4 that made up the total in each tube ($F_{1,37}$= 6.693, P=0.014). Aggregates in size class 4 made up of a greater proportion of the total found in the top of the test tubes (mean value of 2.45 ± 2.65 S.D.) compared to the bottom (mean value of 2.40 ± 2.89 S.D.) (**Figure 4.3D**). The number of aggregations in each size class significantly differed ($F_{3,275}$ =245.488, P<0.001). The post-hoc pairwise comparisons indicated that the number of aggregates in each size class differed from one another (all contrasts with P<0.001), but the greatest number were found in size class 1 and then decreased in each successive size class (**Figure 4.4**).

When considering the occurrence of aggregations specifically in size class 4 as a proxy for the presence or absence of microbeads, location in the test tube had no significant effect (Wald Chi-Square=0.871, P=0.351), indicating that large aggregations were equally likely to be found in the top or bottom (**Figure 4.5**). However, there was a significant relationship between the number of aggregations in size class 4 and the algal cell density in each test tube ($F_{1,158}$ =61.580, P<0.001). The greater the algal cell density, the greater the chances were of finding aggregations of algae and microbeads (**Figure 4.6**).

There was a significant difference between the average algae + microbead area and microbead only area (t_{10} =4.562, P=0.001). On average, the algae + microbead area was 1308.6 μm^2 larger than that of a microbead alone (95% confidence interval (CI) = (669.4-1947.7). There was also a significant difference between the average area of a microbead only compared to a single algae cell (t_{10} =14.987, P<0.001). On average, the area of a microbead was 8315.3 μm^2 larger than a single alga (95% CI = [7079.1-9551.6]). The area of an average microbead was a

significantly different from that of an algae only aggregation ($t_{10} = 4.941$, p=0.001), with the former 5282 μm^2 larger than the latter (95% CI = 2899.9-7664.1). There was a significant difference between the area of algae + microbead aggregation and a single algae cell (t_{10} =15.989, $p \leq 0.001$). On average, an algae + microbead aggregation was 9624 μm^2 bigger than the area of an algae (95% CI = 8282.8-10 965). The area of an average algae only aggregation significantly differed from that of a single algae cell as well ($t_{10} = 3.311$, P=0.008). Average algae clump area was 3033.3309 μm^2 larger than an alga (95% CI = 992-5074.6). An average aggregation formed by algae + microbead was significantly larger compared to that of one algae only ($t_{10} = 5.673$, P<0.001). The area of an average algae + microbead aggregation was 6590.6 μm^2 larger than an algae only cluster (95% CI = 4002.2-9178.9). See Appendix C for full data set. All analysis was performed in SPSS 24.

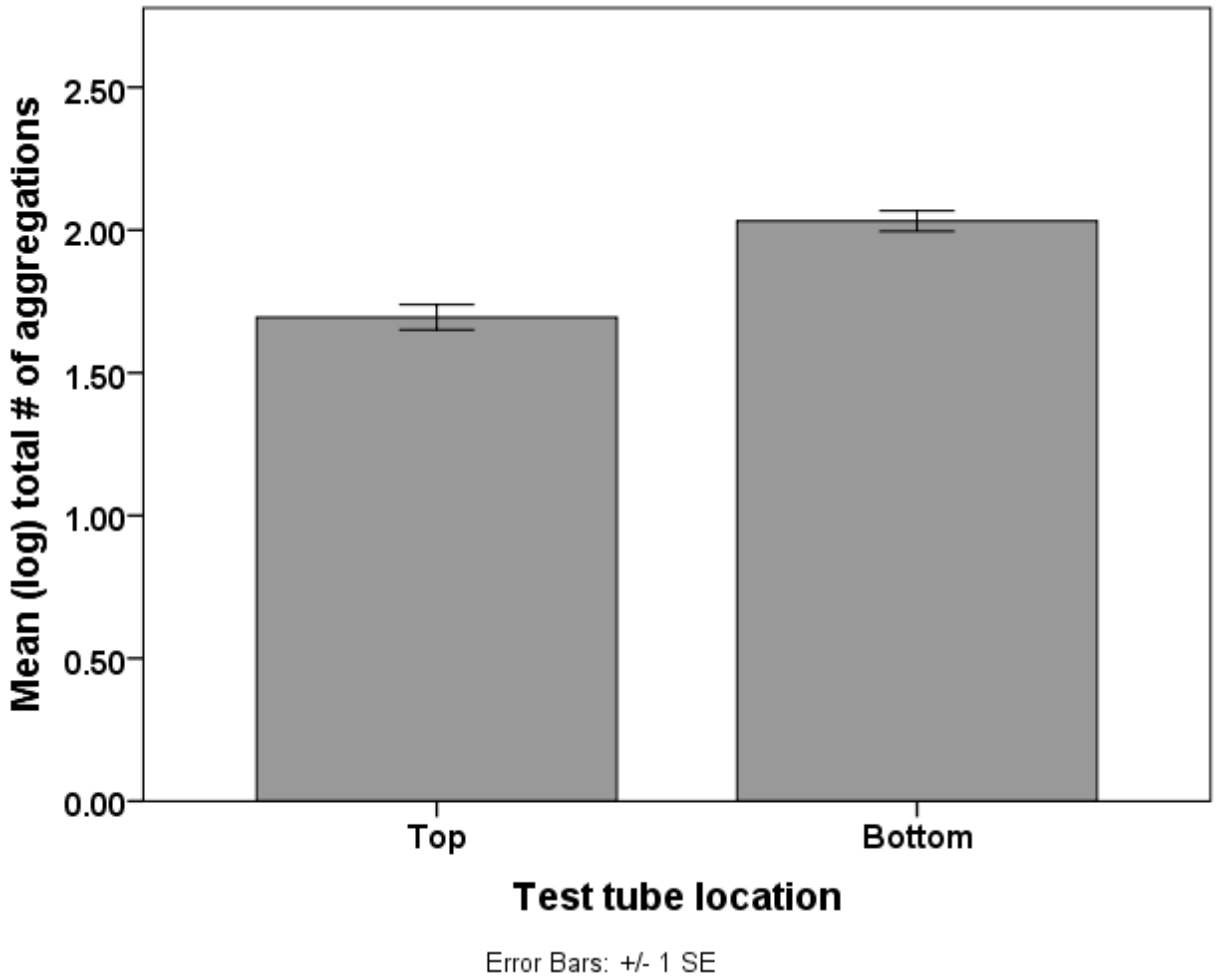

Figure 4.2: Mean (±S.E.) log total number of aggregations in the top and bottom of test tubes containing polyethylene microbeads and live *Chlorella pyrenoidosa*.

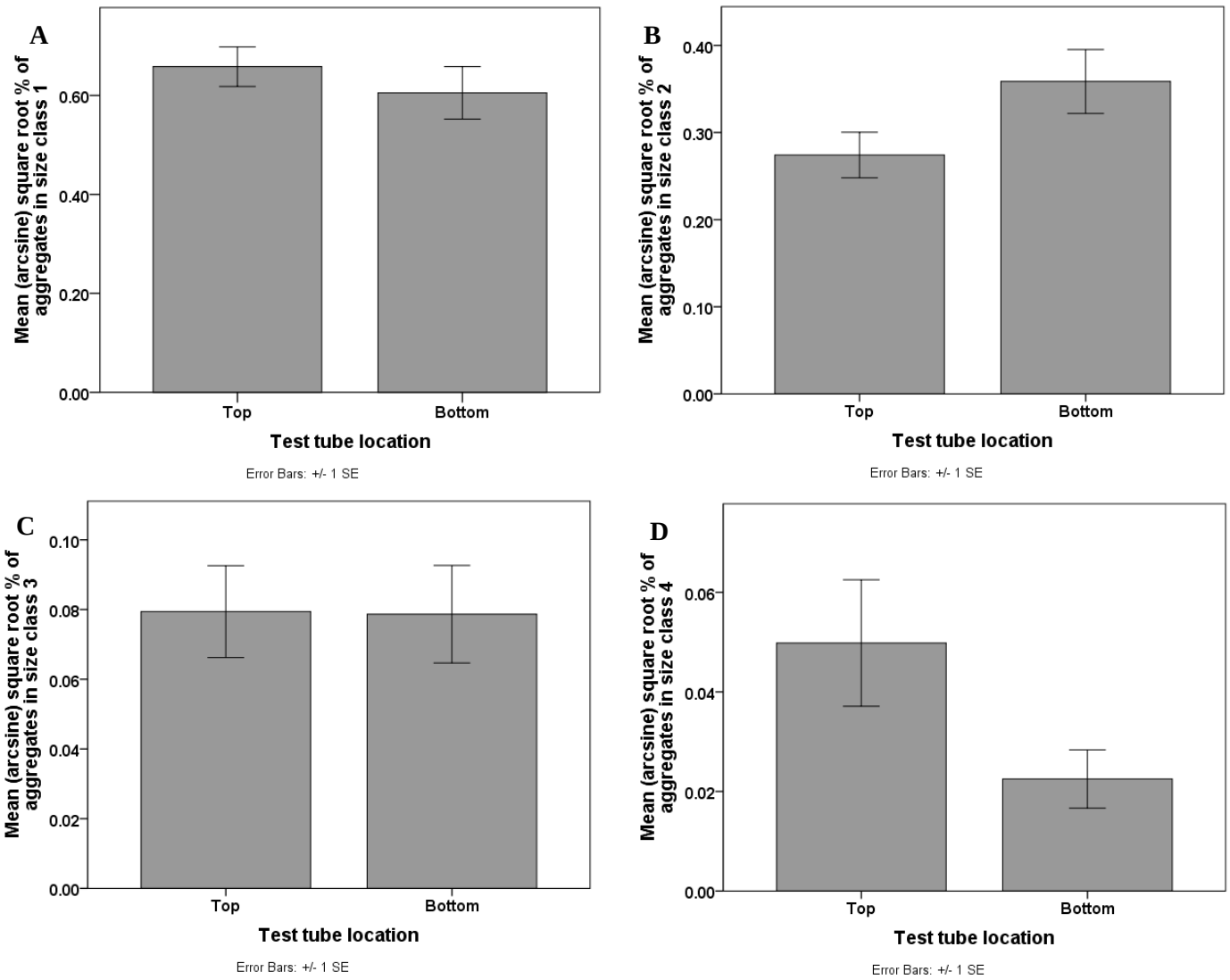

Figure 4.3: Mean (±S.E.) arcsine square root of the percentage of aggregations in: **A)** size class 1 (25-1000 μm^2); **B)** size class 2 (1001-5000 μm^2); **C)** size class 3 (5000-10 000 μm^2); **D)** size class 4 (>10 000 μm^2) in the top and bottom of test tubes containing polyethylene microbeads and live *Chlorella pyrenoidosa*. There was a significant effect of within-tube location for size class 4.

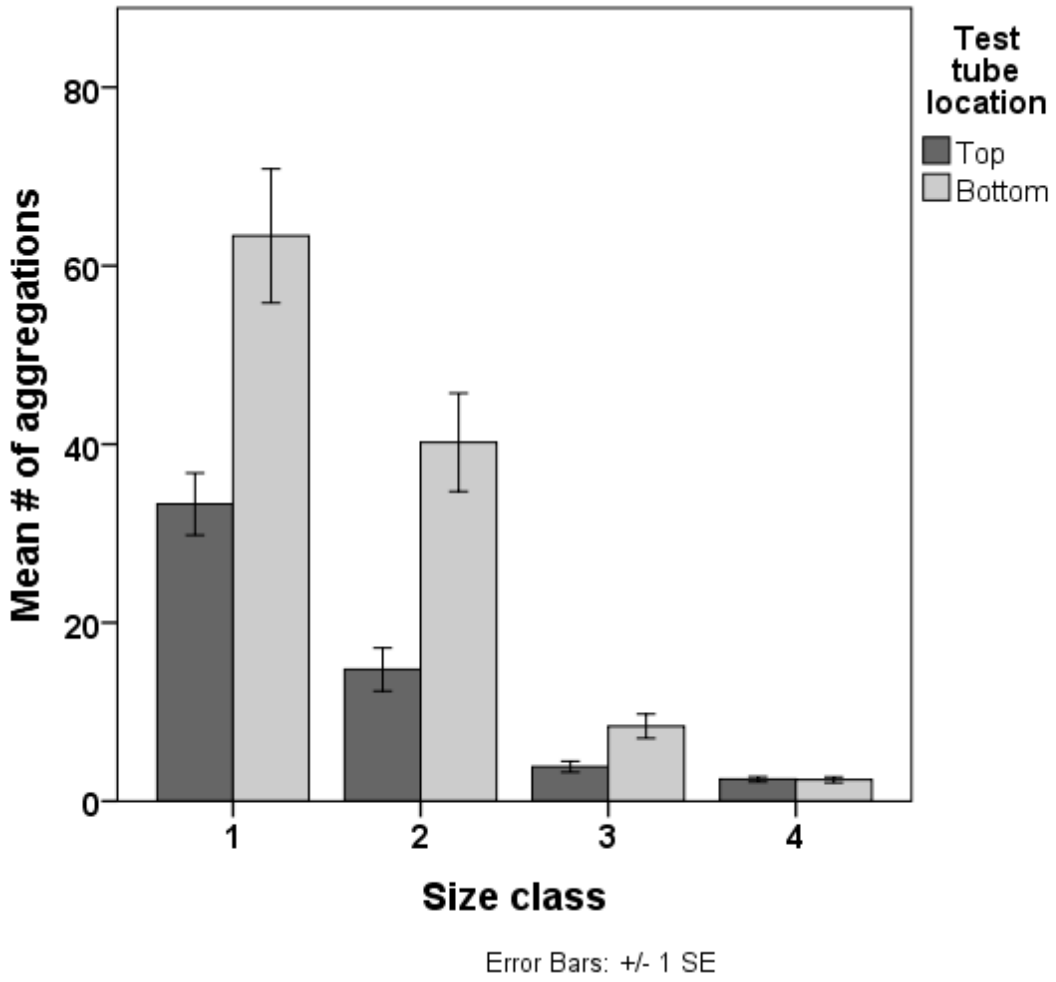

Figure 4.4: Mean (±S.E.) number of aggregations in each size class (size class 1=25-1000 μm^2, size class 2=1001-5000 μm^2, size class 3= 5000-10 000 μm^2, size class 4=> 10 000 μm^2) in the top and bottom of test tubes containing polyethylene microbeads and live *Chlorella pyrenoidosa*.

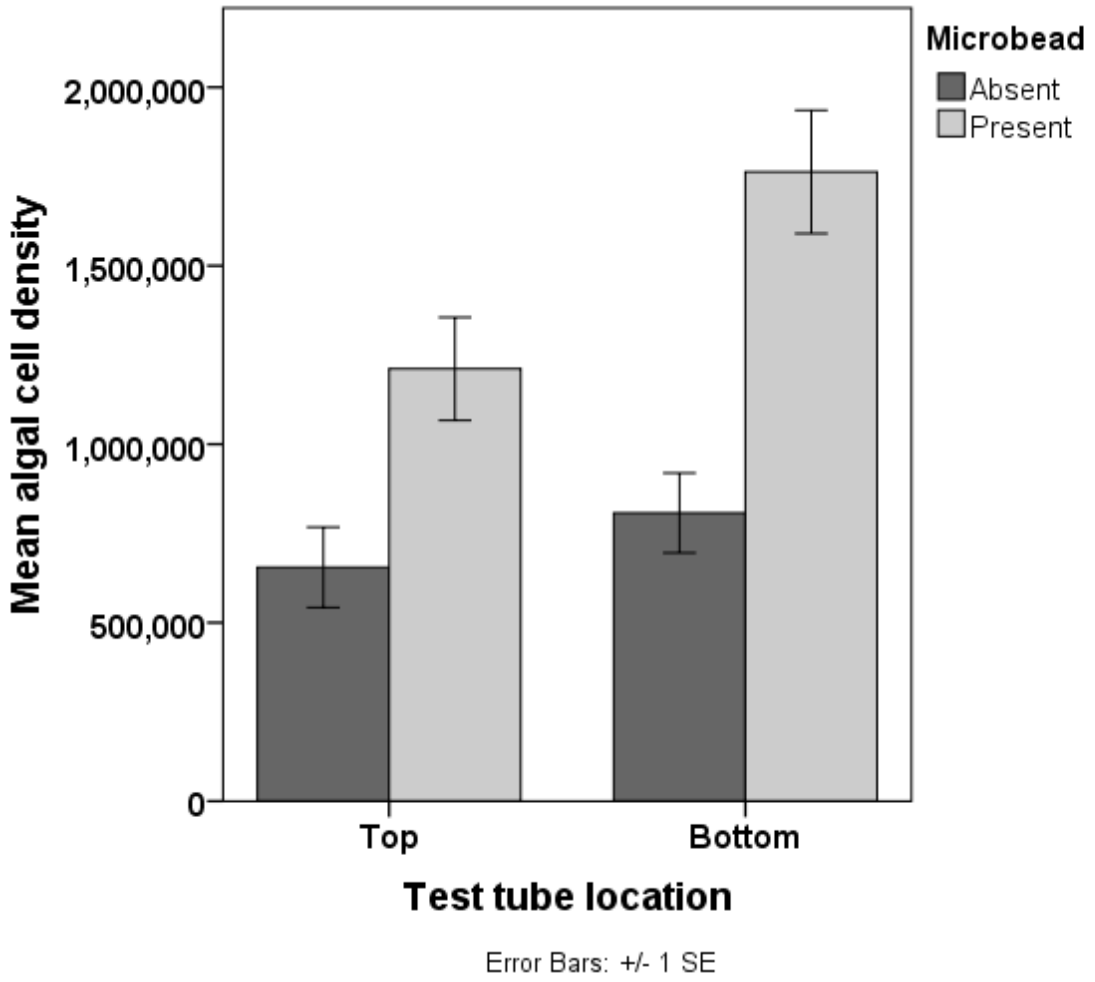

Figure 4.5: Mean (±S.E.) algal cell density for the occurrence of aggregations in size class 4 (>10 000 µm $^{2)}$, indicating when a microbead is absent or present, in the top and bottom of test tubes containing polyethylene microbeads and live *Chlorella pyrenoidosa*.

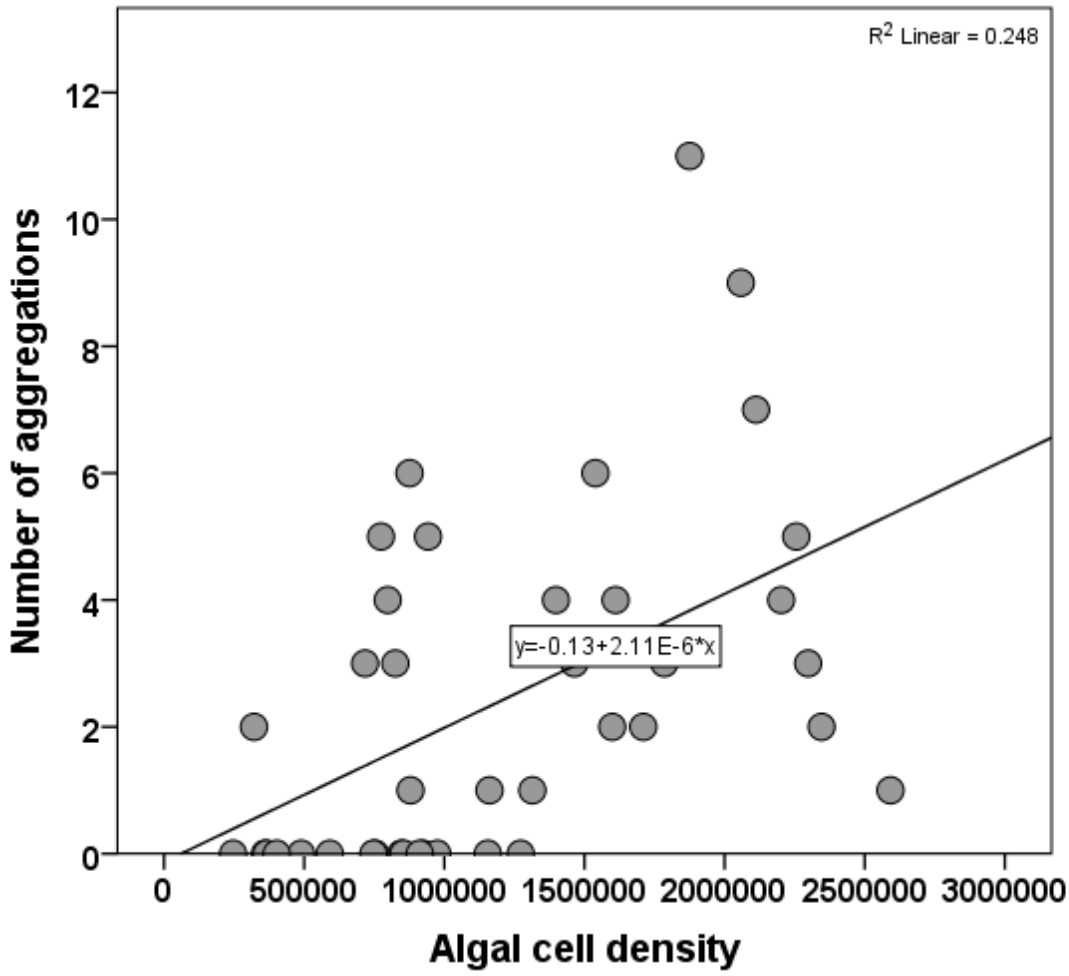

Figure 4.6: The relationship between the number of aggregations in size class 4 (> 10 000 µm$^{2)}$ with algal cell density in test tubes containing polyethylene microbeads and live *Chlorella pyrenoidosa*.

Discussion

Algae is an important source of food for various herbivorous organisms in small freshwater systems such as ponds and wetlands (Chase, 2003; Relyea, 2005), but their formation of aggregations with various substances can affect their location within water bodies, and thus their availability. For instance, clay particles can create aggregations with *Chlorella* and influence the rate of their transfer to the benthos (Alldredge & Silver, 1988; Guenther & Bozelli, 2004). Here, I found that the common freshwater algae *Chlorella pyrenoidosa* can also create aggregations with polyethylene microbeads of the size range commonly found in personal care products (Pettipas *et al*., 2016), and this affected the distribution of aggregates. *Chlorella* cells almost formed a coating around the much larger microbeads, rather than microbeads forming a layer around a central clump of algae. The outer coating of algae was anywhere from one cell to three cells thick layer of algae around the microbeads, and I also observed that there were sometimes a few short chains of algae hanging from the microbeads as well.

Previous studies have shown that algae and microplastics can create aggregates (Lagard *et al*., 2016; Long *et al*., 2015; Yokota *et al*., 2017). Long *et al*., (2015), produced aggregates by growing *Chaetoceros* and *Rhodomonas* (*Crypta phyta*) in rolling tanks (as monocultures and a mixed culture) and found that added microplastics (2 µm polystyrene beads) were incorporated and concentrated into algae aggregates, leading to the accelerated sinking of microplastics. Lagard *et al*., (2016) demonstrated that aggregations formed between polyethylene squares ($1cm^2$) and *Chlamydomas reinhardtii*. Algae has negatively-charged components which are attracted to the positive charges of plastics (Neihof & Loeb, 1972). In its exponential growth phase, *Chlorella* has a highly negative surface charge which is difficult to neutralise (Vandamme *et al*., 2012; Safri *et al*., 2014). Adsorption of plastic to algae is also enhanced by the roughness of the cellulose surfaces, creating numerous binding sites (Kalčíkova *et al*., 2017). This has been demonstrated in the literature as *Anabaena sphaerica* strongly adhered to irregularly shaped microplastics (Yokota *et al*., 2017). It could explain why I found that *C. pyrenoidosa* cell density was significantly positively related to the number of aggregations found in each size class. Importantly, there was a significant positive relationship between algal cell density and the number of aggregations in size class 4 (> 10 000 μm^2). The higher the algal cell density, the greater the chances of their interaction with microbeads to form aggregations in this largest size class.

The formation of aggregations between algae and inorganic particles has implications for their transport from surface water to the sediment, as has been demonstrated for *Chlorella* and clay particles (Alldredge & Silver, 1988; Guenther & Bozelli, 2004), as well as other algae and microplastics (Lagard et *al.*, 2016; Long *et al.*, 2015). I found that there was a significant difference in the total number of aggregations between the top and bottom of the test tubes, with a greater number (all size classes combined) found at the bottom. However, the location in the test tube had no effect on the proportion of total aggregations making up size classes, with size class 4 as the exception. In other words, those in the largest size class (> 10 000 μm^2) tended to dominate the composition of the microbead communities at the tops of the tubes. That being said, microbeads were no more likely to be found at the bottom or tops of the test tubes. Differences in experimental methodology may play a role in explaining this. For example, Yokota *et al.*, (2017), had their algae (*A. sphaerica*) cultured on a continuous shaker, as well as when the algae was exposed to their designated microplastic treatments. This is not representative of environmental conditions that would be typical of inland wetlands and small ponds where one might expect the water to be relatively stagnant with little movement. By having the experimental treatments on a continuous shaker, this may force interactions of algae and microplastics beyond what would otherwise occur. To be environmentally relevant, I cultured *C. pyrenoidosa* on a continuous shaker to make sure the algae received all of the nutrients needed to reach the exponential growth phase but kept the algae stationary once in the designated treatments with microbeads. While I took samples from the top and the bottom of the test tube to account for the movement of aggregates from the surface water to the sediment (similar to Guenther & Bozelli, 2004 who used test tubes with 50 mL of volume), it is also possible that greater distances are needed to detect effects on sedimentation.

The entities with the largest area were algae + microbead aggregates, followed by microbeads only, algae only aggregations, and single algae cells representing the smallest mean area. Given that the mean algae + microbead aggregation area was larger than that those of algae only, this opens up the possibility that the aggregate with greater area may be more attractive to herbivores, and more likely to be ingested than an algae clump on its own. According to optimal foraging theory, animals search for and capture food in a manner that requires the least amount of time or effort for the greatest net energy gain (MacArthur & Pianka, 1966). In other words, animals want to gain the most amount of energy for the lowest cost so as to maximize their

fitness (MacArthur & Pianka, 1966). Aggregations with the largest area should thus appear to contain the most energy if they seemingly represent large clusters of algae looking like a big appetizing ball of food. A cloak of algae may make it more likely that a microbead would get eaten compared to one without algae, otherwise one could argue that microbeads on their own may be more attractive as food compared to algae clumps. That the largest entities consisted of microbead-algae aggregations may also affect their likelihood of ingestion by herbivores in other ways. For instance, tadpoles that engage in suspension feeding can more readily retain large particles compared to small particles (Seale & Beckvar, 1980).

By investigating the formation of aggregates with algae, the movement of microbeads within water bodies, especially transfer from surface water to the sediment, can also be better understood. This is important to determine which herbivorous organisms may be most likely to ingest microbeads, as well as their possible movement through food webs. Some transport of microbead-algae aggregations from the surface water to the sediment may cause more organisms to be affected by microbead pollution if both benthic and surface water inhabitants could ingest them. Here, microbeads were equally likely to occur in the top and bottom of the test tubes, while size class 4 aggregations are more likely at the top. Tadpoles are probably more likely to ingest microbeads if these aggregates tend to float in the short term.

Given that larval amphibians are preyed upon by a variety of animals (Brodie Jr & Formanowicz Jr, 1973), their ingestion and retention (**Chapter 2**) of microbeads could have consequences for other trophic levels. For example, Chae *et al*., (2018), demonstrated this with the transfer of nano-sized polystyrene nanoplastics through various freshwater trophic levels. Nano-sized polystyrene was transferred from the algae *Chlamydomonas reinhardtii* to the primary consumer *Daphnia magna* (water flea), followed by transfer to the secondary consumer *Oryzias sinensis* (fish), and finally to the end consumer *Zacco temminckii* (piscivorous fish) (Chae *et al*., 2018). Future studies should thus test if herbivorous organisms prefer ingesting microbead and algae aggregates over algae-only clumps. By understanding the feeding behaviour of herbivorous organisms, along with the interaction of microplastics with their natural food sources, one can better determine the likelihood of these animals ingesting microbeads and predict possible impacts on aquatic ecosystems.

Chapter 5 Abundance of microbeads in wetlands

Abstract

There have been thousands and thousands of small plastics found in the Great Lakes. It is unknown the number of these small plastics in smaller bodies of water like wetlands and ponds. I developed an approach for the sampling these particles in wetlands and ponds using buckets of water, sieves, a water pump, and filter bags to get particles between 100-500 µm. The collected particles were analyzed using Fourier Transform Spectroscopy (FTIR) and Raman spectroscopy. I found numerous particles in the sampled wetlands and ponds but these appear to be from the agricultural industry and not from personal care products. From sampling of only a few wetlands and ponds, it can be seen that that there are hundreds of particles of various polymers that are likely used for pesticide release which are present. Future studies should therefore examine effects of ingesting polymer beads/particles used in the controlled release of agricultural compounds by organisms in wetlands and ponds.

Introduction

Microbeads are used in various industries, including by the cosmetic industry in personal care products and toiletries, and by the agricultural industry to aid in the controlled release of fertilizers and pesticides from coated beads (Pettipas *et al*., 2016). As personal care products get used by consumers, these small plastics travel down drains and end up at wastewater treatment plants. Microbeads found in personal care products are so small (10 µm to 1 mm; Pettipas *et al*., 2016), that they slip through the filters at wastewater treatment plants and end up in natural water bodies such as lakes and rivers directly through the final effluent, or indirectly by the runoff of biosolids applied to agricultural land (United States Environmental Protection Agency, 2007; Saruhan *et al*., 2010; Rochman *et al*., 2015). In the agricultural industry, microplastics and other polymers are used in applications including the controlled release (CR) of fertilizers and pesticides applied to some crop fields (Roy *et al*., 2014; Campos *et al*., 2015). Polymers used in the agricultural industry vary in diameter from 3 µm to 800 µm (Roy *et al*., 2014). These microplastics can then enter water bodies as well via runoff from agricultural land. Once in the natural water bodies, pollution by microplastics becomes problematic because they take a long time to break down and can have effects on various organisms and the environment. Microplastic ingestion has been documented for various aquatic animals and is known to be harmful (**Chapter**

1). Understanding the distribution and occurrence of microplastics in natural water bodies is therefore important.

The Great Lakes reside in North America and are an important resource as they supply 18% of the world's freshwater (Environment and Climate Change, 2017). However, the Great Lakes face many challenges with various contaminants, including those posed by microplastics. Microplastics have been found in great abundance in the Great Lakes (Zbyszewski & Corcoran, 2011; Eriksen *et al*., 2013; Castañeda *et al*., 2014; Zbyszewski *et al*., 2014; Corcoran *et al*., 2015; Ballent *et al*., 2016). For example, Ballent *et al*., (2016) found approximately 28 000 plastic particles per kg of dry sediment from Lake Ontario. However, the problem of microplastic pollution is not just limited to the sediments and pelagic zones of the Great Lakes. For instance, many of the Great Lakes are associated with coastal wetlands (the Great Lakes Wetlands) that support high levels of biodiversity and primary productivity (Brazner *et al*., 2000), but the presence of microplastics in these key habitats has not been investigated extensively. While there are various studies on the abundance of microplastics in the Great Lakes, smaller freshwater bodies such as inland ponds and wetlands have also not been examined (Zbyszewski & Corcoran, 2011; Eriksen *et al*., 2013; Castañeda *et al*., 2014; Zbyszewski *et al*., 2014; Corcoran *et al*., 2015; Ballent *et al*., 2016). This represents a gap in our knowledge regarding microplastic pollution in certain freshwater habitats that are also critical. For example, wetlands and ponds both support high diversity, as well as performing important ecosystem services (Oertli *et al*., 2005; Gustavson & Kennedy, 2010), and 25% of Canada's wetlands are in Ontario (Ontario Ministry of Natural Resources and Forestry, 2017). Wetlands are land which is saturated with water sufficient enough to support vegetation suited to the saturated soil (Davis, 1994). Ponds are water bodies which stay at a relatively uniform temperature as light reaches the bottom at a maximum defined depth of 8 m (Oertli *et al*., 2005). Wetlands alone provide many benefits, which include providing habitat to animals, flood protection, and improving water quality by filtering pollutants and cycling nutrients (McGarry, 2014).

Microplastics are comprised of different shapes, sizes, colours and types of polymers, all of which make it difficult to have standardized approaches for their detection and quantification (Shim *et al*., 2017). It is important that a sampling methodology thus consider a specific size

range of microplastic, appropriate extraction methods, and a way to identify and confirm the microplastic polymer type (Rochman *et al*., 2017). Sampling methods also differ according to whether one wishes to sample the surface water or sediment. For sampling surface waters, there are various methods that have been employed which include using a manta trawl pulled by a vessel along, transect lines, sieves, and stationary conical driftnets (Faure *et al*., 2012; Eriksen *et al*., 2013; Free *et al*., 2014; Lechner *et al*., 2014; Sadri & Thompson, 2014; Anderson *et al*., 2017). However, most of these sampling methods are not feasible for a smaller freshwater body. For example, using a manta trawl for a wetland would not work due to the much smaller size and amount of water in a wetland, along with the presence of high amounts of emergent and submerged vegetation possible. Instead, it is possible, and more feasible, to use sieves of specific mesh sizes for sampling microplastics in wetlands. There is no specific protocol for microbead sampling in small water bodies, and even though these exist for microbiota, very large volumes are likely needed so there are no false negatives. For this study, I only collected microbeads between 100 μm and 500 μm because these correspond to the size range commonly used in personal care products (Pettipas *et al*., 2016), which I hypothesized may be most prevalent.

Various methods are used to identify the polymer type of collected microplastics. These include the use of scanning electron microscopy (SEM), Raman spectroscopy, or Fourier transform spectroscopy (FTIR) (Faure *et al*., 2012; Eriksen *et al*., 2013; Free *et al*., 2014; Lechner *et al*., 2014; Sadri & Thompson, 2014; Anderson *et al*., 2017). These methods are detailed elsewhere (**Chapter 1**) but are briefly described here. Scanning electron microscopy emits electrons which interfere with the atoms of the sample and produce signals regarding its composition (Hollas, 2004). FTIR functions by identifying particles through infrared light and an instrument that measures which wavelengths are absorbed, while Raman spectroscopy identifies particles by the absorption of monochromatic light from a laser (Hollas, 2004; Shim *et al*., 2017).

For this study, I examined the abundance and types of microbeads present in a few select Ontario wetlands and ponds. These were chosen because the site represented a Great Lakes coastal wetland or was known to contain natural populations of tadpoles or snails (these animals are explored in **Chapter 2 & 3**). To carry out these field collections, I chose a sampling methodology using approaches similar to those used for sampling microplastics in the Great

Lakes, including the use of Raman spectroscopy and FTIR to identify the polymer of each distinct microbead type.

I hypothesized that microbeads would be present in coastal wetlands because the Great Lakes contain thousands of microbeads and these could be transported into their adjacent wetlands. Microbeads could particularly make their way into ponds adjacent to crop fields if biosolids are applied to them, and as well as their use for the controlled release of pesticides or fertilizers. It is important to understand the abundance of microbeads in coastal and inland wetlands, as well as ponds, to know the extent of microplastic pollution in freshwater habitats.

Materials and Methods

Sampling locations

Water samples were taken at 6 wetland or pond locations in Ontario (**Figure 5.1)**. Tadpoles and snails were known to be present in all 6 locations based on previous studies (Koprivnikar & Redfern, 2012) or personal observations. Locations with ID# 1, 2, 3 & 4 are ponds and #5 & 6 are marshes (a type of wetland) based on the definitions above. Site #5 is an inland marsh, and was located along a river. Site#4 consisted of a man-made storm water retention and drainage pond. Site #3 was a pond located beside agricultural crop- land, and site #6 was a coastal marsh located along Lake Ontario near a wastewater treatment plant. Sampling took place in August and September 2017, with one collection visit per site.

Figure 5.1: Wetland sampling locations for field study indicated by black circles, with numbers corresponding to site ID described in the main text.

Figure 5.2: ID#1 - considered a pond.

Figure 5.3: ID#2 – considered a pond.

Figure 5.4: ID#3 – considered a pond, note crop field in background.

Figure 5.5: ID#4 – considered a pond (note: used for storm water retention).

Figure 5.6:ID#5 – considered an inland wetland (along a river)- specifically, a marsh.

Figure 5.7: ID#6 – considered a coastal wetland (along Lake Ontario)-specifically, a marsh.

Sampling methodology and analysis

At each site, I first filled a 18.9 L plastic bucket with water while standing in the water body at a depth between 0.6 and 1.2 m. The spot chosen to collect the samples was conducted randomly based on where it was feasible to stand considering the presence of vegetation and other obstacles and accessibility. The water was collected by skimming surface water using a smaller plastic container (2 L). While collecting the surface water, it is possible that sediment was included owing to movement while I entered the water body. Next, I poured the water from this first 18.9 L bucket through two stacked metal sieves (1.18 mm and 500 μm pore sizes, respectively) into another 18.9 L bucket so as to retain particles smaller than 500 μm. Then the sieved water was pumped from the second bucket using a water pump into a 100 μm nylon monofilament mesh liquid filter bag (7.06" x 16.5") from The Cary Company. I was thus able to retain particles 100-500 μm in size by this sequential filtering procedure. This process was repeated 5 times for a single filter bag, totalling 94.5 L of water per bag. Because 3 mesh liquid filter bags were used at each location, I was able to collect samples totalling 283.5 L of filtered water per site (**Figure 5.8**). Each filter bag was placed into a labelled zip lock bag for safe storage. This process was repeated at each of the 6 sampling locations.

At the lab, the contents of each of the filter bags were analyzed using a dissecting microscope. To do so, I first scraped the contents into Petri dishes and then the particles were counted, recorded and placed in separate micro-centrifuge tubes labeled by their site and type based on physical appearance (size, shape, and colour). I then took 15 pictures of each type of particle to determine its size (diameter) by placing them on a glass slide and using a compound microscope with the 10x objective. To determine the polymer composition for each of the particle types, I prepared 10 particles of each type. I cut a circle (10 cm in diameter) out of transparency film and stuck the particles onto the circle with double sided transparent tape. These particles were analyzed using Fourier Transform Spectroscopy (FTIR; Bruker Alpha II Platinum-ATR). I also prepared one particle of each type for Raman spectroscopy in the same way as for FTIR analysis, with the only difference of cutting the particles in half. A control particle (polyethylene microbead) used in lab studies (**Chapter 2 & 3**) was also prepared for analysis with both FTIR and Raman spectroscopy. The FTIR and Raman spectroscopy was performed at the University of Toronto in the lab of Dr. Chelsea Rochman.

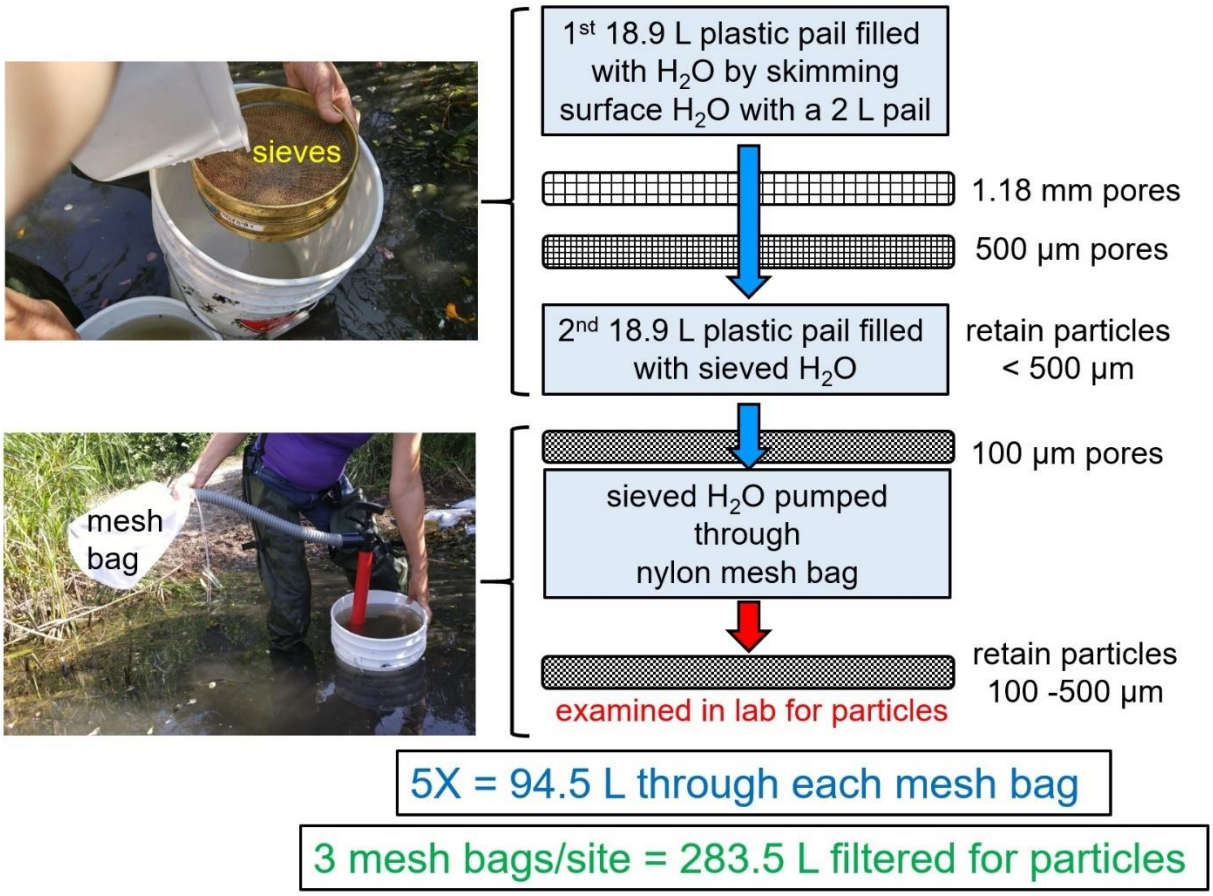

Figure 5.8: Sampling methodology for collection of plastic particles in small water bodies.

Results

I found two distinct types of particles in the water bodies, green fragments and black spheres, that were possibly some type of microplastic based on physical appearance. These varied in their occurrence and abundance as sometimes only one was found at a site, and sometimes they co-occurred (**Table 5.1**). When cut open, the black spheres contained white in their center with black colouration occurring on the outside (**Figure 5.9**). The mean (± standard error SE) diameter of the green fragments was 333.24 (± 24.48) µm (range 189.77 to 513.18µm, **Figure 5.10**). The mean (± SE) diameter of the black spheres was 451.26 (± 14.28) µm (range 374.2 to 593.37 µm, **Figure 5.11**). The FTIR analysis identified the spectra of the green fragments as corresponding to those known for: artistic water colour opaque white paint, rayon fiber, kapok natural fiber, or artists water colour/tempera light brown ocre. For the black spheres, the spectra from FTIR were identified as: modified starch, acid treated starch, g-cyclodextrin, tinting color burned sienna nr. 1510 dried, interior primer paint from 1960, hammerite paint for metal grey, praktik primer paint 100, fabric vison + polyester + lycra (5:4:1), or artistic water colour opaque white. For the manufactured polyethylene microbeads that I used as a reference, FTIR identified these as polyethylene plastic (**Table 5.2**). Raman spectroscopy identified the green fragments as hostasol green dye, and the black spheres as 1,10-Decanediol. For the manufactured polyethylene microbeads, Raman spectroscopy also correctly identified these as polyethylene (**Table 5.3**).

Table 5.1: Total number of particles found at each field sampling location (see map in Fig. 2.1).

Location ID	Site Type	Category	Colour	Total # of Particles
1	Pond	Fragment	Green	643
2	Pond	Sphere	Black	7
2	Pond	Fragment	Green	5
3	Pond	Fragment	Green	392
3	Pond	Sphere	Black	147
4	Pond	n/a	n/a	0
5	Marsh	Sphere	Black	678
6	Marsh	Sphere	Black	190

Figure 5.9: Green fragments (left) and black spheres (right) collected from field sites.

Table 5.2: Size (diameter) of black spheres and green fragments from each field site (location ID)

Sample #	Location ID	Category	Colour	Size (µm)
1	5	Sphere	Black	462.4
3	5	Sphere	Black	418.3
4	5	Sphere	Black	410.28
7	2	Sphere	Black	437.01
9	3	Sphere	Black	374.2
13	3	Sphere	Black	465.07
16	1	Sphere	Black	379.54
28	6	Sphere	Black	523.87
29	6	Sphere	Black	466.41
30	6	Sphere	Black	593.37
186	6	Sphere	Black	419.63
205	3	Sphere	Black	420.97
211	3	Sphere	Black	455.72
239	5	Sphere	Black	473.09
244	5	Sphere	Black	469.08
5	1	Fragment	Green	248.57
8	3	Fragment	Green	383.96
14	2	Fragment	Green	189.77
21	1	Fragment	Green	372.86
22	1	Fragment	Green	513.18
23	1	Fragment	Green	390.23
24	1	Fragment	Green	408.94
25	1	Fragment	Green	251.25
26	1	Fragment	Green	219.17
27	1	Fragment	Green	295.35
93	1	Fragment	Green	374.19
107	1	Fragment	Green	478.43
110	1	Fragment	Green	323.41
112	1	Fragment	Green	303.37
113	1	Fragment	Green	245.9

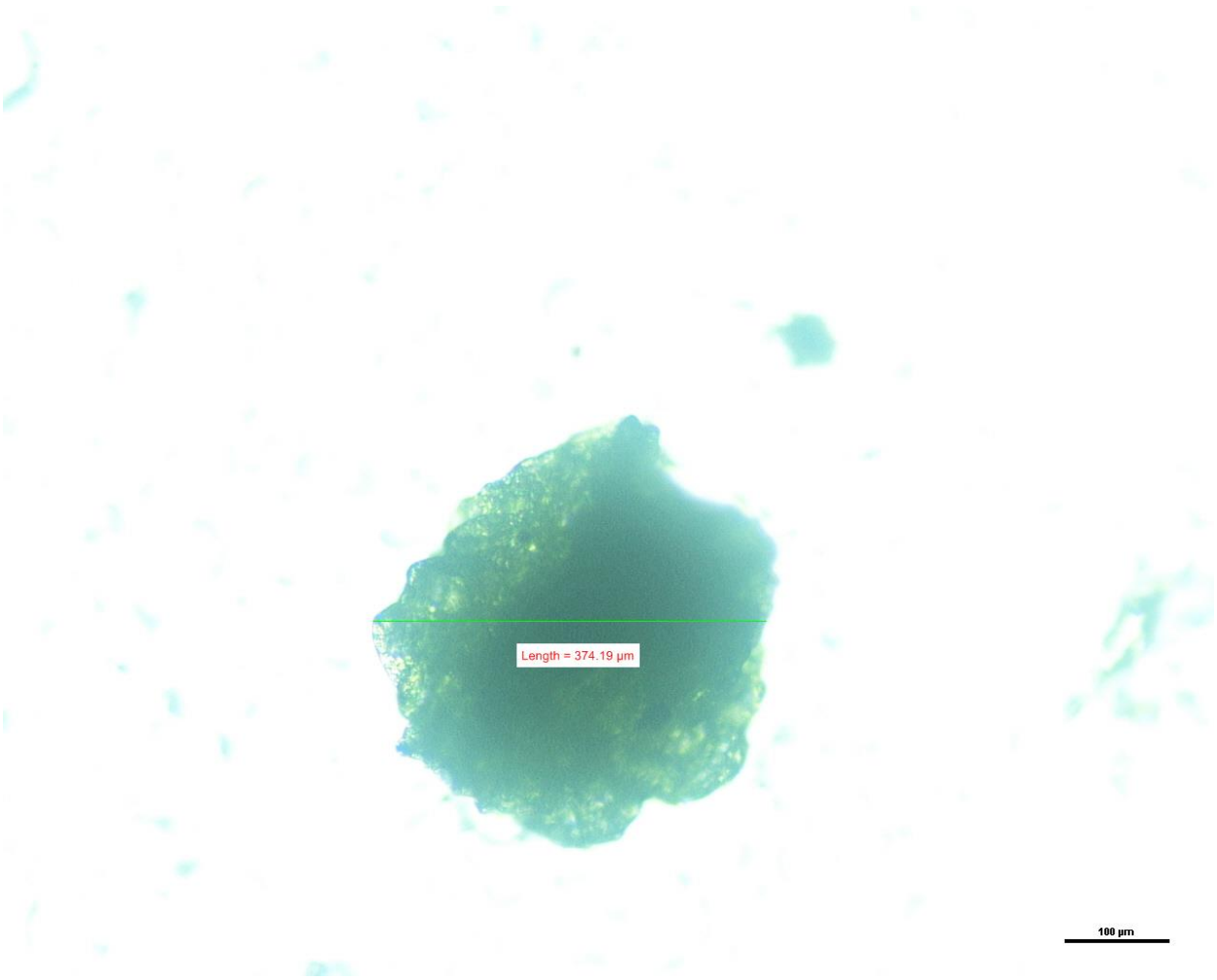

Figure 5.10: Image of field collected particles under a compound microscope- green fragment (374.19 µm).

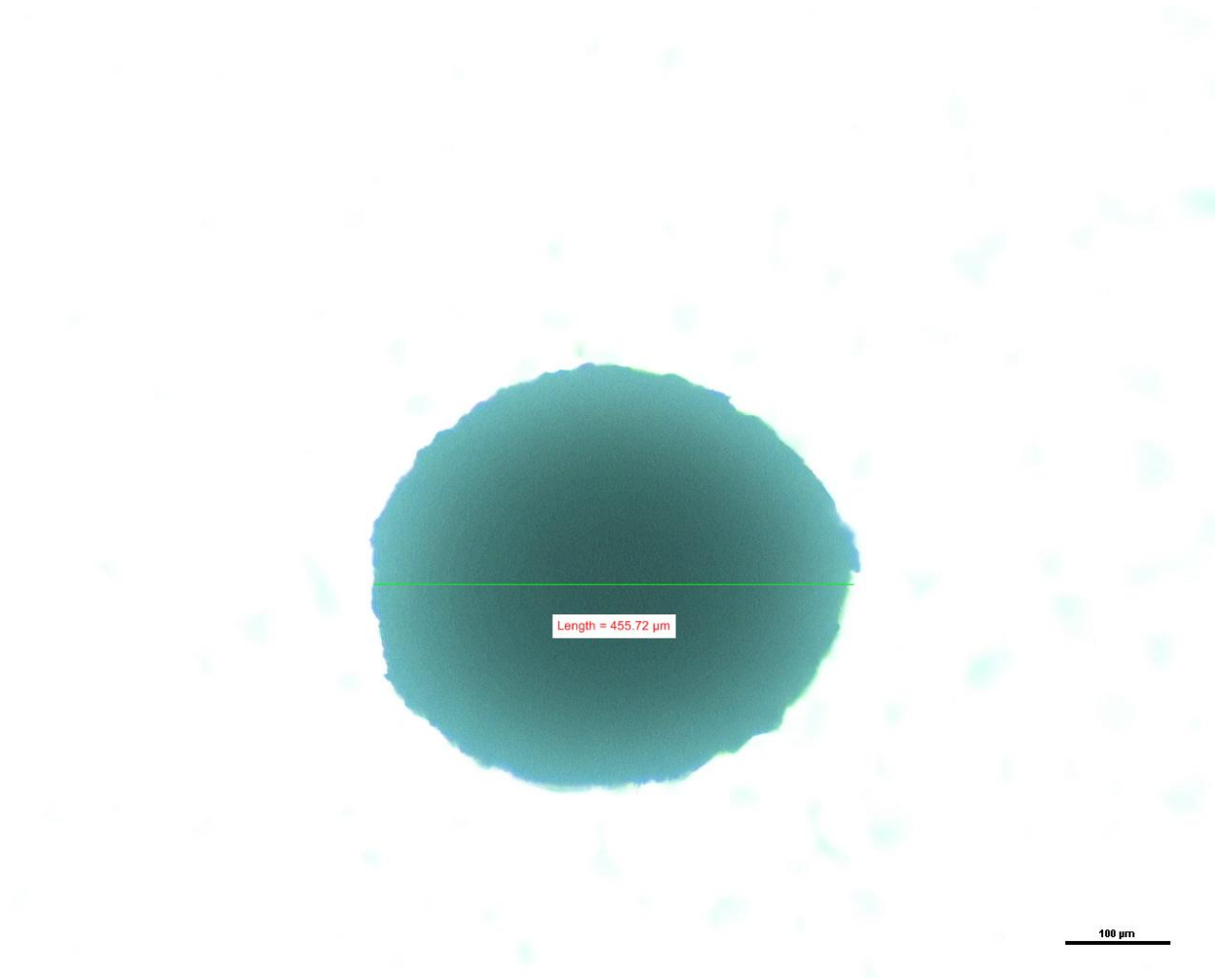

Figure 5.11: Image of field collected particles under a compound microscope- black sphere (455.72 µm).

Table 5.3: FTIR analysis of particles collected from field sites (location ID).

Sample ID	Location ID	Major Component	Category	Colour	FTIR ID
128	1	water colour paint	paint	green	artistic water colour opaque white
174	1	water colour paint	paint	green	artistic water colour opaque white
164	1	water colour paint	paint	green	artistic water colour opaque white
115	1	water colour paint	paint	green	artistic water colour opaque white
113	1	water colour paint	paint	green	artistic water colour opaque white
156	1	water colour paint	paint	green	artistic water colour opaque white
147	1	inorganic	fragment	green	zinc sulfate heptahydrate
149	1	anthropogenic fiber	fiber	green	kapok natural fiber
127	1	tempera paint	paint	green	artists water colour/tempera light brown ocre
109	1	synthetic polymer	paint	green	Artists water colour/tempera light brown ocre
7	2	carbohydrate	starch	black	modified starch, acid treated starch
31	6	carbohydrate	starch	black	g-cyclodextrin
39	6	tinting color	paint	black	tinting color burned sienna nr. 1510 dried
41	6	paint	paint	black	interior primer paint from 1960
177	3	paint	paint	black	hammerite paint for metal grey
188	3	paint	paint	black	praktik primer paint 100
179	3	paint	paint	black	interior primer paint from 1960
178	3	anthropogenic fiber	fabric	black	fabric vison + polyester + lycra (5:4:1)
74	6	synthetic polymer	sphere	black	polyvinyl alcohol fully hydrolyzed
59	6	water colour paint	paint	black	artistic water colour opaque white
manufactured microbead		polyethylene	microbead	green	polyethylene, lupolen 6021 D

Table 5.4: Raman analysis of particles from field sites (location ID).

Sample ID	Location ID	Plastic Type	Category	Colour	Raman ID
98	1	dye	dye	green	hostasol green g-k
214	3	organic	alkanediols	black	potassium antimony (III) tarate hydrate
manufactured microbead	n/a	microbead	microbead	green	polyethylene

Discussion

I found a number of particles in five of the six ponds and wetlands than I sampled; however, based on my analysis of their chemical and physical characteristics, these seem likely to result from activities related to the agricultural, not the cosmetic industry. Agrochemicals are used in the agricultural industry for a number of purposes, including as pesticides, chemical fertilizers, and other growth agents (Milani et *al*., 2017), but agrochemicals from nearby fields can enter into wetlands and ponds by runoff during rainfall events (Vymazal & Březinová, 2015). Based on my findings, it would appear that particles composed of polymers may also enter into water bodies in this way if they are applied to crop fields. In the agricultural industry, various polymers are used in applications like the controlled release (CR) of pesticides (Roy *et al*., 2014; Campos *et al*., 2015). CR give the ability to predict the delivery of pesticides by controlling the rate and timing of release (Roy *et al*., 2014). Polymers used for CR can be natural or synthetic, as well as either biodegradable or non-biodegradable. Natural polymers include starch, chitosan, gelatin, albumin, polysaccharides, cellulose, agarose and dextrin, and alginates (Roy *et al*., 2014). Synthetic polymers include polystyrene, polyacrylamide, polymethyl acrylate, polyesters, and polyamides (Roy *et al*., 2014). The use of natural polymers is preferred because the synthetic polymers may have toxic effects, and take a long time to degrade, negatively affecting the environment (Roy *et al*., 2014).

There are many mechanisms for CR of pesticides, but encapsulation within polymeric matrixes helps to deliver the chemicals in a predictable way. Controlled release particles come in various forms including microcapsules and microspheres (Roy *et al*., 2014). Micro-capsulation involves coating particles which are around 3 µm to 800 µm in size with the desired compound, while microspheres have the CR product dissolved or dispersed in a polymer matrix that is typically 20 nm to 2000 µm in size (Roy *et al*., 2014). The particles that I found are within the size range of both micro-capsulation and microspheres for controlled release of pesticides as the green fragment were 333.24 (± 24.48) µm in diameter, and that of the black spheres was 451.26 (± 14.28) µm. Micro-capsulation seems to be the mechanism of CR for the black spheres. As indicated by Raman spectroscopy, these spheres appeared to have a white center made of potassium antimony (III) tarate hydrate and were coated with a black layer. The black outer layer could be a synthetic polymer like polyvinyl alcohol fully hydrolyzed, a natural polymer like g-cyclodextrin, or a combination of natural and synthetic polymers. The pesticide NeemAzal was

encapsulated with polyvinyl alcohol/alginate with a diameter ranging from 2.2-2.82 mm (Rashidzadeh *et al*., 2014). Unfortunately, the dye of the green fragment interfered or overpowered the resulting spectra, thus it was not possible to determine their composition with this method. However, the use of the FTIR indicated that the green fragments could be made of zinc sulfate heptahydrate. Zinc sulfate is known as an herbicide that is used to control the growth of moss by having the zinc bind to the proteins or organic acids in the plant (Boone *et al*., 2012). If this zinc reaches freshwater, it is highly toxic to fish and invertebrates (Boone *et al*., 2012).

The results of the FTIR indicated that the particles which I found in wetlands and ponds may consist of natural and/or synthetic polymers. In the agricultural industry, there is a demand for natural biodegradable polymers, but they do not fit all the needs for specific applications (Russo *et al*., 2004). Ideal natural polymers with these properties include agar, starches, and cellulose derivatives, while the synthetic biodegradable polymers include polycaprolactones (PCL), polyvinyl alcohol (PVA), and polylactide (PLA) (Russo *et al*., 2004). Notably, polyvinyl alcohol and starches were both possibilities of for the chemical identification of the particles in my field study.

The controlled release of pesticides has various advantages and disadvantages. The advantages to using CR are ensuring a constant level of pesticides or nutrients over a period of time, the use of smaller doses that result in a decrease in environmental pollution, a reduction of evaporation losses, and ease of handling (Roy *et al*., 2014; Milani *et al*., 2017). The disadvantages to using CR are that nutrients or pesticides are known to release prematurely, or do not release, thereby damaging crops, but there is a lack of standardized methods to validate the predicted release of chemicals in the field based on lab conditions (Milani *et al*., 2017). Importantly, there are factors differing from the lab to the field which have not been accounted for. Once the CR particles are placed in the soil, microorganisms degrade the polymeric material, exposing the underlying layers, but the rate of this depends on environmental conditions (temperature, soil and water content) that could disrupt the controlled release of pesticides and nutrients (Milani *et al*., 2017).

While the leaching of chemicals from CR particles can pose a risk to the environment, it is quite possible that these particles in themselves may represent a hazard that needs investigation considering the known harmful effects of microplastic ingestion by aquatic fauna

(see **Chapter 1**). Future studies should thus study the effects of polymers used in the controlled release of pesticides on freshwater bodies and their inhabitants. This is important as I found these polymers used for pesticide release in the hundreds, even in small water bodies such as ponds. In addition, because the conditions used for testing the effects of CR pesticides in the lab are different than in the field, the effects of these polymer particles may be underestimated if they do not consider their possible ingestion. The ingestion of plastic polymers has focused on microbeads of specific composition and size, but clearly there is the need to study whether aquatic fauna will eat CR particles, and effects of this ingestion. There is thus a need to move beyond studies focused on microbeads found in personal care products, even though there are negative effects of ingesting those. Based on my field collections, it seems more realistic that snails and tadpoles would encounter CR particles, so they should be considered for further study.

Chapter 6: General discussion, conclusions, and future work

The primary objectives of my thesis were to: i) study the effects of microbead ingestion on tadpoles and snails in combination with other stressors like trematode infection; ii) explore the aggregation of algae and microbeads as a potential ingestion pathway by these herbivores; and iii) to investigate the occurrence and abundance of microbeads in select Ontario wetlands and ponds.

In Chapter 2, I examined the effects of microbead ingestion on tadpole growth, as well as susceptibility and tolerance to trematode parasite infection. I hypothesized that tadpoles infected with trematodes would have decreased growth and immune response, the latter measured by altered numbers of leukocytes. Tadpoles were exposed to three different microbead diets (0= none microbeads/week, 10= low, or 100 microbeads=high) in the form of agarose-algae mixture cubes for either 2 or 4 weeks. There was a significant effect of microbead diet on the growth of larval *Lithobates pipiens* (northern leopard frogs), as well on their susceptibility and tolerance to infection by the trematode parasite *Echinostoma trivolvis*, but there were minimal effects on leukocyte profiles. Microbead diet had a negative effect on growth, but it was a different response than I had hypothesized because it exhibited a non-monotonic pattern for both the 2 and 4-week diet exposure periods. The tadpoles in the low microbead diet exhibited the least growth (measured as percentage mass gain), followed by the high and no microbead diets. It is also possible that tadpoles in the high microbead diet ate more food to compensate for the likelihood of not getting enough energy.

Tadpoles exposed to trematode infectious stages after 2 weeks on the high microbead diet- exhibited a significantly higher intensity of parasite cysts compared to tadpoles in the low or no microbead diets, indicating greater susceptibility to infection. Uninfected tadpoles that were kept on microbead diets for 4 weeks had the same non-monotonic growth response described above, while infected tadpoles grew similarly in all the diets. It is not clear why this happened, but it may be due to phenotypic plasticity of the intestines owing to the increased metabolic demands brought on by parasite infection (Kristan & Hammond, 2003; Schwanz, 2006), or perhaps a greater rate of food ingestion. With respect to the leukocyte profiles, tadpoles in the high microbead diet had lower numbers of neutrophils for both the 2 and 4-week microbead exposure groups, which may represent a decreased investment in innate immunity in

favour of maintaining their growth under these conditions. Future studies should thus look at the size of larval amphibian intestines when they are exposed to *E. trivolvis* in combination with other stressors, as well as foraging behaviour. To better understand whether microbead ingestion generally represents a physiological stressor, studies should also measure levels of stress hormone corticosterone in relation to microbead exposure in amphibians. By understanding the potential stressors to amphibians, it can be valuable knowledge given the worldwide decline of amphibians (Daszak *et al*., 2003).

In Chapter 3, I examined the effects of microbeads on snails (*Stagnicola elodes*) that were infected by a trematode (*Haematolechus parviplexus*), specifically their growth, longevity, and production of infectious cercariae. I had hypothesized that there would be a decrease in growth and survival for snails given the microbead diets. I also expected that snails given high microbead diets would show a decrease in cercariae production if the within-snail stages creating these (rediae or sporocysts) had increased mortality or reduced availability of nutrients. Snails (n=90) were exposed to diets with microbeads at 3 different concentrations (0=none microbeads/week, 10=low, or 100 microbeads=high) in the form of agarose-algae mixture cubes fed twice weekly for 3 weeks. For the 45 snails that were exposed to diets of microbeads, I conducted weekly counts of their cercariae production (as measured by cercariae emergence).

The mean production of cercariae exhibited a different negative response to host microbead diet than hypothesized because it also had a significant non-monotonic pattern, as seen with tadpole growth above. The lowest cercariae emergence was seen from snails in the low microbead diet. However, snails in the control and low microbead diets grew more (achieved longer length) than the snails in the high microbead diets for which weekly cercariae counts were done. As trematodes exploit their snail hosts by redirecting their energetic resources into growth over reproduction (Seppälä *et al*., 2013), it is possible that this occurred even more so for snails in the high microbead diet. In other words, the greatest production of cercariae by snails given the high microbead diet was only possible because they grew the least. Snails also exhibit compensatory eating once their diet gets diluted with innutritious matter (Rollo & Hawryluk, 1988), so this may have been enough to sustain production of cercariae under food challenge, but not host growth.

There was also no significant effect of microbead diet on growth of snails that did not undergo cercariae production. However, the snails in the high microbead diet had significantly shorter longevity, thus putting their energy into maintaining growth could have reduced their survival. Alternatively, the snails in the high microbead diets had less energy available to them, leading to greater mortality. Future studies are needed to determine how multiple stressors affect energy allocation and longevity of hosts and parasites directly and indirectly. For instance, the mechanisms(s) underlying the non-monotonic responses observed for emergence of cercariae here are unknown.

Overall, ingestion of microbeads has various effects on animals. Tadpoles ingesting microbeads impacted growth and susceptibility to trematode infection, while for snails cercariae production and longevity were affected. Microbeads were excreted at faster rates in snails compared to tadpoles. The variation of excretion rates could be due to the gut of each of these organisms. Snail guts may be wide and short, while tadpole intestines long and thin. Further analysis of the gut of snails and tadpoles can be analyzed once faced with various stressors. Also, future studies can explore the effects of persistent organic pollutants (POPs) accumulated on plastics. Polyethylene is a stable plastic, and it has not been shown to leach compounds which could be endocrine disruptors. Future experiments could use glass beads as a treatment to compare to plastic in order to clarify the role of physical blockage vs. any role of the plastic itself.

In Chapter 4, a pathway of microbead ingestion by herbivores such as snails and larval amphibians was explored by determining if aggregations form between *Chlorella pyrenoidosa* and microbeads. I hypothesized that microbeads should create aggregations with *Chlorella* due to the electrostatic forces between them (Kalčíkova *et al*., 2017). *Chlorella* was cultured and pipetted into test tubes with polyethylene microbeads (sized 106-125 μm in diameter). To quantitively determine the aggregation of algae and microbeads, samples were taken from the top and bottom of the test tubes to account for the movement of aggregations from the surface water to the sediment. Counts of algal cells were also taken at the top and bottom of the test tubes to account for density-dependent aggregation formation. I additionally categorized aggregations into different size ranges to determine if these differed based on their location in the test tubes.

My results show that *Chlorella pyrenoidosa* and microbeads form aggregations in the size range for the latter that are commonly found in personal care products (10 μm to 1 mm; Pettipas *et al*., 2016). Based on my observations, the algae almost forms a coating around the microbead due to the electrostatic forces (Vandamme *et al*., 2012; Safri *et al*., 2014). The interactions of the algae and microbeads were dependent on cell density, with a significant positive relationship between *Chlorella* density and the number of aggregations in the largest size class containing microbeads. There were a greater number of aggregations (all size classes) at the bottom than the top of the test tubes, and the location in the test tubes had no effect on the proportion of aggregations in each size class. The exception was size class 4 (> 10 000 μm^2), with a higher number of these largest aggregations at the top of the test tubes, but microbeads themselves were equally likely to be found at either the top of the bottom of the test tubes.

The entities with the largest area were the algae + microbead aggregates, followed by microbeads only, and algae-only aggregations, and single algae cells representing the smallest mean area. Aggregations with the largest area should thus appear to contain the most energy for herbivores if they seemingly represent large clusters of algae that look like a big appetizing ball of food. Depending on the size of food that herbivores typically eat, this also contributes to their likelihood of microbead exposure. For example, many tadpoles engage in suspension feeding and can more readily retain large particles compared to small particles (Seale & Beckvar, 1980). Some transport of microbead-algae aggregations from the surface water to the sediment through sinking, may cause more organisms to be affected by microbead pollution if both benthic and surface water inhabitants may ingest them. Future studies should thus test if herbivorous organisms prefer ingesting microbead and algae aggregates over algae-only clumps.

In Chapter 5, I sampled a total of 6 Ontario wetlands and ponds to explore the abundance and occurrence of microbeads in these small water bodies. I hypothesized that microbeads would be present in Great Lakes coastal wetlands, as these lakes have been shown to contain thousands of microbeads. I also predicted that microbeads would be found within inland ponds and wetlands if these enter as runoff from biosolid application to agricultural fields. I developed an approach for the sampling of microbeads in wetlands and ponds using buckets of water, sieves, a water pump, and filter bags to get particles between 100-500 μm. The collected particles were analyzed using Fourier Transform Spectroscopy (FTIR) and Raman spectroscopy. I found

numerous man-made particles in the sampled wetlands and ponds but these appear to be from the agricultural industry and not from personal care products.

In the agricultural industry, beads made from various polymers are used for the controlled release of pesticides and fertilizers (Roy *et al*., 2014; Campos *et al*., 2015). Various polymers are used including those that are synthetic, natural, biodegradable and non-biodegradable. There is an increasing desire to use natural polymers, but not all these fit the specific needs of the applications, thus a blend of synthetic and natural polymers are often used (Russo *et al*., 2004). I found two primary types of particle in the samples that I collected in the wetlands and ponds. The first type had microcapsulation of a white center coated with a black outer layer. The white center was identified as potassium antimony (III) tarate hydrate, and the black outer layer could be either polyvinyl alcohol fully hydrolyzed or g--cyclodextrin. The second major type, consisting of green fragments, were identified as zinc sulfate heptahydrate. Both bead/particle types thus contain compounds known to be used for agricultural applications. From my sampling of only a few wetlands and ponds, it can be seen that that there are hundreds of particles of various polymers that are likely used for pesticide release which are present. Future studies should therefore examine effects of ingesting polymer beads/particles used in the controlled release of agricultural compounds by organisms in wetlands and ponds, as well as assess their distribution in freshwater bodies. The effects of these polymers for pesticide release may be underestimated in the field, perhaps posing more of a hazard than microbeads originating from personal care products.

My thesis will contribute to the current scientific knowledge regarding microbeads having focused on particular gaps in information. Notably, there has been limited research on the effects of microbeads on animals dwelling in freshwater. This is the first study to examine the impact of microbead ingestion on the larvae on any North American amphibian (*Lithobates pipiens* tadpoles here), as well as on the common aquatic snail *Stagnicola elodes*. The aggregation of microbeads and *Chlorella pyrenoidosa* is one of only a handful of studies examining this potential pathway of ingestion by herbivores (Bhattacharya *et al*., 2010; Long *et al*., 2015; Lagard *et al*., 2016; Sjollema *et al*., 2016; Yokota *et al*., 2017), confirming that it is important for this particular algae species too. To date, small freshwater bodies such as wetlands and ponds have not been sampled for microbeads, or these data have not been published. My

study is a starting point for examining microbead effects on taxa in small freshwater bodies, which must be expanded upon so as to inform conservation and management polices. My field collections indicate that beads of various polymers likely used for the controlled release of pesticides are present in small water bodies, and this may be a larger problem than expected if they are ingested based on my lab exposures with microbeads used in personal care products. The knowledge gained from these studies will help to understand the extent of pollution by microbeads and other plastic particles, the potential pathways of microbead ingestion by organisms in small freshwater bodies, and some of the direct and indirect effects of doing so. From my understanding there are no discussion of bans of microbeads in other industries besides in the cosmetic industry. This knowledge can be the starting point for conservation efforts for our small freshwater bodies and the organisms that live within these environments, as well as inform policies governing the use of plastic particles that may enter these habitats.

Appendices

Appendix A: Tadpole data. Legend given below table.

Tadpole #	RBC	WBC	Neutro	Lympho	Eosino	Treatment #	Length	Stage	Mass Initial (g)	Mass Final (g)	Mass Change %	# MBs	# Cysts	Infected	Cercariae
1	6280	100	3	88	0	0	0	26	0.1835	0.8108	341.8529	0	0	0	1
2	3904	100	5	94	1	0	0	25	0.1567	0.8829	463.4333	0	0	0	1
3	3231	100	0	93	7	0	0	26	0.2132	1.6859	690.7598	0	1	1	1
4	702	100	0	96	4	0	0	26	0.3402	2.2353	557.0547	0	1	1	1
5	3763	100	21	74	5	0	0	25	0.1436	1.0411	625	0	0	0	1
6	3855	31	0	31	0	0	0	25	0.2662	1.2002	350.864	0	0	0	1
7	2292	100	12	83	5	0	0	25	0.2593	4.1486	1499.923	0	0	0	1
8	2017	100	3	95	2	0	0	26	0.2191	1.8998	767.0927	0	0	0	1
9	1818	100	7	87	6	0	0	26	0.2093	1.5895	659.4362	0	1	1	1
10	2814	100	30	56	14	0	0	25	0.2555	0.9753	281.7221	0	0	0	1
11	5333	80	6	73	1	0	0	25	0.2099	1.1805	462.4107	0	1	1	1
12	6312	68	0	60	8	0	0	25	0.1338	1.0517	686.0239	0	0	0	1
13	4164	80	7	72	1	0	0	25	0.3274	1.2998	297.0067	0	0	0	1
14	4511	74	2	68	4	0	0	26	0.3091	1.5801	411.1938	0	0	0	1
15						0	0	25	0.1243	0.861	592.679	0	0	0	1
16	962	100	0	99	1	1	0	25	0.3041	0.8698	186.0243	0	2	1	1
17						1	0	25	0.1976	0.4822	144.0283	0	1	1	1
18	3269	100	4	96	0	1	0	26	0.382	0.801	109.6859	7	0	0	1
19	1809	100	0	100	0	1	0	25	0.2521	0.5226	107.2987	2	0	0	1
20	3067	78	5	68	5	1	0	25	0.2653	0.5019	89.18206	1	0	0	1
21						1	0	25	0.2313	0.4045	74.88111	1	0	0	1
22	2970	100	0	99	1	1	0	25	0.296	0.3814	28.85135	3	3	1	1
23	2566	35	0	35	0	1	0	26	0.2443	0.9975	308.3095	1	1	1	1
24	2417	77	0	73	4	1	0	26	0.3367	0.6139	82.32848	0	0	0	1
25	1259	100	0	97	3	1	0	25	0.2242	0.3978	77.43087	0	0	0	1
26						1	0	26	0.4015	0.9059	125.6289	1	1	1	1

27	2118	17	0	17	0	1	0	25	0.2147	0.588	173.8705	3	1	1	1
28	3744	47	2	39	6	1	0	25	0.1501	0.7926	428.048	7	0	0	1
29	890	100	1	98	1	1	0	26	0.4225	0.6636	57.06509	1	3	1	1
30	3327	40	13	17	10	1	0	25	0.1911	0.5718	199.2151	0	1	1	1
31	7053	100	11	85	4	2	0	25	0.2937	0.9478	222.7102	1	1	1	1
32	3380	100	1	95	4	2	0	25	0.1019	0.494	384.789	3	2	1	1
33	1781	100	10	87	3	2	0	25	0.262	1.0107	285.7634	9	0	0	1
34	2623	87	12	61	14	2	0	25	0.111	0.5529	398.1081	0	0	0	1
35	138	100	0	100	0	2	0	25	0.2895	0.8158	181.7962	4	0	0	1
36	382	100	0	100	0	2	0	25	0.1373	0.8885	547.1231	2	0	0	1
37	2320	32	9	18	5	2	0	25	0.3865	0.5927	53.35058	4	0	0	1
38	3893	68	13	42	13	2	0	25	0.2263	0.6746	198.0999	12	1	1	1
39	4155	63	5	57	1	2	0	25	0.3251	1.0809	232.4823	7	0	0	1
40	3100	89	3	76	10	2	0	26	0.2851	0.9218	223.3251	3	0	0	1
41	3319	50	6	28	16	2	0	26	0.266	0.9127	243.1203	0	2	1	1
42	3148	79	4	68	7	2	0	25	0.21	0.4944	135.4286	0	0	0	1
43	2506	2	0	2	0	2	0	25	0.1871	0.6015	221.4858	4	0	0	1
44	3575	100	6	86	8	2	0	25	0.1833	1.8346	900.8729	2	3	1	1
45	3900	48	4	31	13	2	0	25	0.1144	0.1724	50.6993	4	3	1	1
46	4214	24	7	16	1	0	1	26	0.215	1.6761	679.5814	0	8	1	1
47	4554	4	2	2	0	0	1	25	0.1682	1.2939	669.2628	0	3	1	1
48	3482	37	0	37	0	0	1	28	0.2609	2.4037	821.3108	0	8	1	1
49						0	1	28	0.164	1.3989	752.9878	0	2	1	1
50	6882	27	4	22	1	0	1	28	0.289	2.1583	646.8166	0	6	1	1
51	6020	13	6	6	1	0	1	28	0.2647	2.0555	676.5395	0	0	0	1
52	6963	45	3	42	0	0	1	25	0.437	3.0594	600.0915	0	0	0	1
53						0	1	25							
54	5299	2	0	2	0	0	1	27	0.1287	0.4866	278.0886	0	9	1	1
55	6693	72	3	63	6	0	1	28	0.1675	2.3241	1287.522	0	10	1	1
56	4298	23	15	4	4	0	1	25	0.1192	1.0698	797.4832	0	0	0	1
57	4309	11	3	8	0	0	1	28	0.2763	2.9342	961.9616	0	4	1	1

58	5107	25	8	15	2	0	1	28	0.1979	2.4452	1135.574	0	0	0	1
59	4013	4	3	1	0	0	1	27	0.1816	1.7073	840.1432	0	0	0	0
60						0	1	25							1
61	2188	3	2	1	0	1	1	26	0.2314	1.6744	623.5955	0	0	0	1
62	2473	14	1	13	10	1	1	25	0.2599	1.3915	435.3982	1	2	1	1
63						1	1	25	0.1716	1.4886	767.4825	0	1	1	1
64	3334	21	10	6	5	1	1	28	0.1323	1.366	932.5019	0	1	1	1
65	4817	100	5	89	6	1	1	28	0.2075	1.8729	802.6024	0	1	1	1
66	4347	18	7	11	0	1	1	28	0.1741	3.459	1886.789	0	0	0	1
67	3651	16	3	9	4	1	1	25	0.1817	1.8618	924.656	1	4	1	1
68	4858	11	4	7	0	1	1	26	0.1871	2.1329	1039.979	1	7	1	1
69	4731	14	4	10	0	1	1	28	0.1792	1.6236	806.0268	1	0	0	1
70	5836	41	4	37	0	1	1	28	0.4516	2.0811	360.8282	0	0	0	1
71	2583	3	0	3	0	1	1	25	0.2329	1.848	693.4736	0	0	0	0
72	4632	60	0	60	0	1	1	25	0.1985	1.1514	480.0504	0	8	1	1
73	6518	77	0	77	0	1	1	28	0.2061	1.6347	693.1587	0	3	1	1
74	5402	20	2	18	0	1	1	26	0.1181	2.2087	1770.195	3	9	1	1
75						1	1	25							0
76	5204	27	0	27	0	2	1	28	0.3279	2.747	737.7554	0	3	1	1
77	3673	26	0	26	0	1	1	27	0.2081	2.7465	1219.798	6	5	1	1
78	5346	16	4	11	1	2	1	28	0.1682	2.3173	1277.705	1	8	1	1
79	5450	44	2	42	0	2	1	28	0.2799	1.9607	600.5002	2	0	0	1
80	2256	33	0	33	0	2	1	26	0.1369	2.3193	1594.156	2	9	1	1
81						2	1	25							1
82	4851	55	22	17	16	2	1	27	0.2891	1.7417	502.4559	4	0	0	1
83	8904	44	0	44	0	2	1	28	0.1642	2.2088	1245.189	18	2	1	1
84	5568	5	3	1	1	2	1	28	0.2296	1.844	703.1359	1	4	1	1
85	6646	20	11	9	0	2	1	28	0.2258	2.004	787.5111	5	3	1	1
86	9722	38	10	26	2	2	1	25	0.3614	1.257	247.8141	3	0	0	0
87	8087	44	13	31	0	2	1	28	0.1981	1.9232	870.8228	4	4	1	1
88	4720	18	5	13	0	2	1	28	0.223	1.7902	702.7803	1	0	0	1

89	7966	43	14	19	10	2	1	26	0.22	2.2286	913	4	1	1	1
90	9633	77	27	44	6	2	1	27	0.1847	1.8425	897.5636	3	5	1	1
91	8621	41	1	38	2	0	1	26	0.2808	2.1249	656.7308	0	0	0	0
92	8732	77	0	76	1	0	1	28	0.2452	3.1469	1183.401	0	0	0	0
93	5880	74	2	66	6	0	1	27	0.2531	2.0959	728.0917	0	3	1	1
94	8092	79	4	74	1	0	1	28	0.2129	3.5449	1565.054	0	0	0	0
95	9189	70	31	24	15	0	1	25	0.0831	1.645	1879.543	0	0	0	0
96	6337	67	0	66	1	0	1	28	0.2455	2.6078	962.2403	0	0	0	0
97	8277	99	10	88	1	0	1	28	0.2278	2.9921	1213.477	0	0	0	0
98	6313	44	12	28	4	0	1	28	0.2009	1.9743	882.7277	0	0	0	0
99	3516	55	2	43	10	0	1	28	0.1184	1.5814	1235.642	0	0	0	0
100	4060	21	2	19	0	0	1	28	0.2236	3.4467	1441.458	0	0	0	0
101	9286	49	10	38	1	0	1	28	0.2083	3.2051	1438.694	0	0	0	0
102	6911	29	8	9	12	0	1	28	0.2277	0.7298	220.5094	0	0	0	0
103	1885	29	9	10	10	0	1	26	0.1894	0.7031	271.2249	0	0	0	0
104						0	1	26	0.1433	0.8383	484.9965	0	0	0	0
105	4888	22	0	21	1	0	1	28	0.1326	0.8815	564.7813	0	0	0	0
106	2133	87	0	84	3	1	1	25	0.1291	0.5952	361.038	2	0	0	0
107	2625	63	3	57	3	1	1	25	0.168	0.4286	155.119	0	0	0	0
108	1422	90	0	90	0	1	1	26	0.2365	0.4535	91.75476	0	0	0	0
109	3842	11	2	8	1	1	1	28	0.2498	0.9306	272.538	1	0	0	0
110	6494	83	19	39	25	1	1	26	0.2203	0.9856	347.3899	0	0	0	0
111	4025	15	2	12	1	1	1	28	0.2323	0.9368	303.2716	0	0	0	0
112	2517	6	0	6	0	1	1	28	0.1873	0.6033	222.1036	0	0	0	0
113								26	0.2091	0.5963	185.1746	2	0	0	0
114	5502	79	5	72	2	1	1	26	0.2397	1.0191	325.1564	4	0	0	0
115	2509	24	0	24	0	1	1	27	0.2736	0.9434	244.8099	2	0	0	0
116								25	0.2647	0.4659	76.01058	0	0	0	0
117	1761	56	3	47	6	1	1	28	0.265	1.0569	298.8302	0	0	0	0
118	2049	39	4	19	16	1	1	25	0.281	0.563	100.3559	0	0	0	0
119	4482	76	0	74	2	1	1	28	0.3055	1.2541	310.5074	0	0	0	0

120	2385	13	4	7	2	1	1	25	0.2228	0.3511	57.58528	2	6	1	1
121	3189	25	0	25	0	2	1	28	0.2268	1.9032	739.1534	1	0	0	0
122	4404	85	6	76	3	2	1	28	0.2651	1.7717	568.3138	0	1	1	1
123	4085	41	11	24	6	2	1	28	0.1896	1.0804	469.8312	6	1	1	1
124	7018	20	7	6	7	2	1	28	0.2045	1.64	701.956	14	0	0	0
125	2175	100	12	64	24	2	1	28	0.3406	2.8659	741.4269	2	0	0	0
126						2	1	25							0
127	2763	3	0	3	0	2	1	26	0.2855	0.9407	229.4921	2	0	0	0
128	4793	34	10	20	4	2	1	26	0.21	0.9128	334.6667	0	0	0	0
129						2	1	25							0
130	4417	44	22	16	6	2	1	26	0.1807	0.6747	273.3813	3	0	0	0
131	1300	100	3	93	4	2	1	25	0.1295	1.0047	675.8301	3	0	0	0
132	2341	100	10	88	2	2	1	28	0.2201	1.1613	427.6238	3	3	1	1
133	3688	49	10	37	2	2	1	28	0.2289	1.5227	565.225	6	0	0	0
134	5976	19	10	6	3	2	1	28	0.2718	0.9012	231.5673	8	0	0	0
135	1325	100	14	83	3	2	1	28	0.2167	1.0989	407.1066	3	0	0	0

Tadpole #- ID of tadpole

RBC- number of red blood cells

WBC- number of white blood cells

Neutro- number of neutrophils

Lympho- number of lymphocytes

Eosinophils- number of eosinophils

Treatment- 0=0 microbeads, 1=10 microbead, 2= 100 microbeads

Length- 0=2 weeks, 1=4 weeks

Stage- Gosner developmental stage

Mass Initial- initial mass of tadpole

Mass Final- final mass of tadpole

Mass Change Percentage- (final mass of tadpole- initial mass of tadpole)/ initial mass of tadpole *100

MBs- number of microbeads found in tadpole

cysts- number of parasite cysts found in tadpole

Infected- 0=not infected with parasite, 1= infected with parasite

Cercariae- 0= no cercariae given, 1=cercariae given

Appendix B-1: Data for snails undergoing cercariae emerging counts. Legend given below table.

Snail #	Treatment	Initial	Week 1	Week 2	Week 3	Total #Cercariae	Average #Cercariae/week	Initial Length (mm)
1	0	897	1605	0		2502	834	29.3
2	0	463	420	595	370	1848	462	31.5
3	0	362	135	45		542	180.67	24.7
4	0	472	5			477	238.5	28
5	0	6	850	0		856	285.33	22.7
6	0	121	665	1270		2056	685.33	20.9
7	0	6	10	25	5	46	11.5	25.3
8	0	5	0			5	2.5	25.4
9	0	611	145	85	0	841	210.25	20.5
12	0	384	445	440	135	1404	351	16
13	0	9	550	0	0	559	139.75	26.7
14	0	12	0			12	6	31.1
15	0	3	0	0	0	3	0.75	27.1
16	1	3	0			3	1.5	25.3
17	1	70	60	0	0	130	65	26.3
18	1	3	380	0	0	383	95.75	25
19	1	6	600	0	0	606	151.5	27.4
20	1	162	140	170	125	597	149.25	26.2
21	1	59	85	5	5	154	38.5	26.1
22	1	483	415	90	40	1028	257	24
23	1	293	15			308	154	23.2
24	1	95	10	0	0	105	26.25	27.1
25	1	102	25	20		147	49	22.3
26	1	283	150	165	15	613	153.25	20.9
27	1	704	135			839	419.5	18.9
28	1	703	675	115	400	1893	473.25	24.1
29	1	208	605	740	250	1803	450.75	14.5
30	1	621	30			651	325.5	16.9

31	2	13	35	140	0	188	47	19.4
32	2	354	1345	15		1714	571.33	18.3
33	2	543	10			553	276.5	17.4
34	2	616	15	655	640	1926	481.5	24.9
35	2	13	10			23	11.5	23.8
36	2	147	15	0		162	54	24.9
37	2	376	10	0		386	128.67	15.9
38	2	608	1480	405	670	3163	790.75	20.9
39	2	334	60	210	190	794	198.5	20.5
40	2	617	45	395	250	1307	326.75	23.9
41	2	227	720			947	473.5	24.4
42	2	485	5	100	40	630	157.5	19.3
43	2	3	10			13	6.5	19.7
44	2	429	795	5		1229	409.67	21.3
45	2	447	580	10		1037	345.67	20.1

Snail #- ID of each snail

Treatment- experimental food: 0=0 microbeads, 1=10 microbead, 2= 100 microbeads

Initial- Initial amount of emerging cercariae

Week 1- Number of cercariae emerging after 1week exposure to treatments

Week 2- Number of cercariae emerging after 2 weeks exposure to treatments

Week 3- Number of cercariae emerging after 3 weeks exposure to treatments

Total #Cercariae- The total number of cercariae emerging amongst exposure to treatments

Average #Cercariae- The average number of cercariae emerging amongst exposure to treatments based on snail longevity

Initial Length- Initial shell length of snail

Appendix B-2: Data for no cercariae production snails. Legend given below table.

Snail #	Location#	Initial Length (mm)	Final Length (mm)	Length Change (%)	Mass Initial (g)	Mass Final (g)	Mass Change (%)	Time (days)	Treatment	MBs
91	1	23.8	23.8	0	1.1302	1.1593	2.574766	21	0	0
92	2	18.6	18.6	0	0.5149	0.5305	3.029715	21	0	0
93	1	25.7	26.3	2.33463	1.0259	0.9526	-7.14495	21	0	0
94	1	23.8	23.9	0.420168	0.7966	0.9517	19.47025	21	0	0
95	2	19.8	20.1	1.515152	0.6211	0.6541	5.313154	21	0	0
96	1	26.1	26.2	0.383142	1.2493	1.0641	-14.8243	21	0	0
97	1	20.7			0.6014			14	0	0
98	2	17.6			0.4262			7	0	0
99	1	22.3	23.4	4.932735	0.9791	0.947	-3.27852	21	0	0
100	3	18.5	18.8	1.621622	0.534	0.6673	24.96255	21	0	0
101	1	24.7	24.7	0	0.9035	0.8444	-6.54123	21	0	0
102	1	23.9	23	-3.76569	1.1515	1.0929	-5.08901	21	0	0
103	1	20.1	19.7	-1.99005	0.5793	0.5867	1.277404	21	0	0
104	3	21.8	23.4	7.33945	0.6719	0.8471	26.07531	21	0	0
105	1	24.4			0.8696			17	0	0
106	2	19.9	19.5	-2.01005	0.5386	0.6378	18.41812	21	1	0
107	1	19.3	19.4	0.518135	0.5633	0.7124	26.46902	21	1	0
108	1	24.8	25.1	1.209677	1.0258	0.9132	-10.9768	21	1	0
109	1	26	26.4	1.538462	1.1883	1.1079	-6.76597	21	1	0
110	1	23.4			0.9835			7	1	0
111	1	23.9			0.9278			7	1	0
112	1	22.4	23.2	3.571429	0.8975	0.649	-27.688	21	1	0
113	1	18.9	20.5	8.465608	0.3717	0.5106	37.36885	21	1	0
114	1	21.9	24.1	10.04566	0.9525	0.9615	0.944882	21	1	0
115	2	19.1	18.4	-3.66492	0.54445	0.5866	7.741758	21	1	0
116	3	23.7			0.9657			21	1	0
117	2	20.8	19.7	-5.28846	0.5361	0.5971	11.37847	21	1	0

118	2	17.7	18.7	5.649718	0.3797	0.4702	23.83461	21	1	0
119	1	28	27.9	-0.35714	1.3961	1.6227	16.23093	21	1	0
120	1	24.3	24.6	1.234568	0.8837	0.9093	2.896911	21	1	0
121	2	19.4			0.5403			10	2	0
122	3	20.4			0.6787			21	2	0
123	1	23.2			0.9886			10	2	0
124	1	25.8	25.8	0	0.9354	0.9205	-1.5929	21	2	0
125	1	21.8			0.5983			10	2	0
126	1	24.7	25	1.214575	1.1176	1.0246	-8.3214	21	2	0
127	1	23.3			0.809			3	2	0
128	3	20.7	21	1.449275	0.6711	0.7449	10.99687	21	2	2
129	2	19	19.8	4.210526	0.5116	0.6066	18.56919	21	2	0
130	1	24.5	25.6	4.489796	0.9329	0.8795	-5.72409	21	2	0
131	1	25.9			0.7947			7	2	0
132	1	26.3	25.6	-2.6616	0.9526	1.0098	6.004619	21	2	0
133	1	20.6	23.5	14.07767	0.5165	0.9258	79.24492	7	2	0
134	2	20.2	20.5	1.485149	0.5648	0.5666	0.318697	21	2	0
135	1	22.1	23.2	4.977376	0.7633	0.8042	5.358313	21	2	0

Snail #- ID of snail

Location- 1=ID #4, 2=ID#1, 3=ID#5

Initial Length- Initial shell length of snail

Final Length- Final shell length of snail

Length Change Percentage= (Final shell length – initial shell length) / initial shell length * 100

Initial Mass- Initial mass of snail

Final Mass- Final mass of snail

Mass Change Percentage= (Final mass- initial mass)/ initial mass *100

Time- Number of days lived (longevity)

Treatment- 0=0 microbeads, 1=10 microbead, 2= 100 microbeads

MBs- number of microbeads present in snail

Appendix C: Algae data. Legend given below table.

Cell Density (cells/mL)	Size Class (1)	Size Class (2)	Size Class (3)	Size Class (4)	Total Aggreg	Microbead Present	Location (0 or 1)
321000	16	10	2	2	30	1	0
246000	33	8	0	0	41	0	0
360000	41	10	0	0	51	0	0
753000	49	11	2	0	62	0	0
366000	30	11	2	0	43	0	0
924000	11	10	2	0	23	0	0
591000	38	10	3	0	51	0	0
1464000	60	14	3	3	80	1	0
1161000	43	48	9	1	101	1	0
825000	11	8	2	3	24	1	0
798000	36	7	5	4	52	1	0
774000	18	4	5	5	32	1	0
1398000	34	12	9	4	59	1	0
876000	53	18	5	6	82	1	0
1611000	42	28	7	4	81	1	0
1539000	15	7	7	6	35	1	0
846000	10	11	5	0	26	0	0
1710000	27	21	5	2	55	1	0
1155000	47	35	4	0	86	0	0
2058000	52	12	0	9	73	1	0
747000	24	15	3	0	42	0	1
489000	50	17	0	0	67	0	1
879000	137	50	0	1	188	1	1
975000	83	24	14	0	121	0	1
402000	75	22	2	0	99	0	1
717000	48	19	4	3	74	1	1
854000	139	10	2	0	151	0	1

912000	77	15	2	0	94	0	1
1599000	120	72	9	2	203	1	1
942000	42	49	17	5	113	1	1
1875000	60	36	20	11	127	1	1
2202000	28	19	18	4	69	1	1
2112000	62	36	12	7	117	1	1
1785000	58	32	10	3	103	1	1
2592000	51	52	11	1	115	1	1
2256000	43	42	6	5	96	1	1
1314000	45	95	13	1	154	1	1
2346000	42	47	7	2	98	1	1
1272000	54	88	9	0	151	0	1
2298000	29	64	9	3	105	1	1

Cell Density (cells/mL)- number of algae cells per mL

Size Class (1)- number of aggregations in size class 1 (25-1000 μm^2)

Size Class (2)- number of aggregations in size class 2 (1001-5000 μm^2)

Size Class (3)- number of aggregations in size class 3 (5000-10 000 μm^2)

Size Class (4)- number of aggregations in size class 4 (> 10 000 μm^2)

Total Number of Aggregations- total number of aggregations from all size classes

Microbead Present- 0=absent, 1=present

Location-location in test tube- 0=top, 1=bottom

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