The 'Trojan Horse Effect' of nanoplastics with various aquatic contaminants

by

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Abstract

As of 2016, the global annual production of plastic was 330 million metric tonnes (Plastics Europe, 2017). This staggering number is expected to double over the next 20 years as consumption increases (Plastics Europe, 2017; Lebreton et al., 2019). Less than half of the plastic produced reaches a landfill or recycling depot, leaving the remainder to litter the terrestrial, aquatic and marine environments (Rochman et al., 2013). These plastics break down over time via weathering and degradation to eventually produce micro or nanoplastics (NPs) (Mattsson et al., 2018). The definition of NPs has varied over time, but today is defined as a synthetic organic polymer that has at least one dimension between 1 and 1000 nm (Mattsson et al., 2018). These small dimensions increase the threat posed to aquatic environments because surface area increases exponentially as diameter decreases which leads to strong sorption affinities for contaminants (Velzeboer et al. 2014). The adsorption of contaminants to the NPs is concerning as studies have shown that NPs (and potentially the adsorbed co-contaminant) bioaccumulate and can pass through lipid-membranes (Bergmann et al., 2015).

Co-contaminant uptake is also known as the “trojan horse effect”. This occurs when NPs increase the uptake of the contaminant by adsorption and subsequent transportation through the organism, leading to increased toxicity or accumulation compared to the contaminant presented in the absence of the plastics (Trevisan et al., 2020). Previous research in our lab indicated that NPs can increase the uptake of the hydrophobic organic pollutant phenanthrene in zebrafish embryos, verifying the Trojan Horse effect for that contaminant in the presence of NPs (Zhang et al., 2020).

The overall aim of my research is to contribute to a co-contaminant uptake model that, contributes substantially to the field of NPs toxicology through the provision of a "read across" for risk assessors that will eliminate the need to examine every plastic/contaminant combination.

The first aim of this thesis was to determine the co-contaminant uptake and depuration rate of ^{14}C-Phenanthrene when sorbed to various sized (20 nm and 500 nm) NPs. The model organism *Daphnia magna* was used throughout the uptake and depuration experiments. ^{14}C-Phenanthrene accumulation was lower at multiple time points when sorbed to 500nm NPs when compared to ^{14}C-Phenanthrene alone or ^{14}C-Phenanthrene sorbed to 20 nm NPs. Depuration rates were similar amongst all ^{14}C-Phenanthrene groups.

The second aim of this thesis was to determine the effects of chemical properties such as K_{ow} on the co-contaminant uptake rates when various aquatic contaminants are sorbed to NPs (20 and 500 nm). The model organism *Daphnia magna* was used throughout the ^{14}C-Fluoxetine and ^{14}C-Glyphosate experiments as well. The percentage of ^{14}C-Fluoxetine uptake was only significantly different between groups at one timepoint (2h), where the 500 nm sorbed group had significantly higher uptake compared to both the 20 nm sorbed group and the chemical control. The percentage of ^{14}C-Glyphosate uptake was relatively low amongst all treatment groups, and therefore depuration experiments were not completed. However, at one timepoint (4h) the 500 nm sorbed group had significantly higher uptake than the 20 nm sorbed group and the chemical control. Determination of the uptake-rate using various aquatic contaminants that have differing chemical properties will aid in the development of a model, overall leading to a "read-across" that will provide efficiency in risk assessment.

Overall, this thesis aims to generate empirical data necessary for a co-contaminant uptake-kinetic model to be further developed. This research will impact the field of risk

assessment significantly by allowing prediction of toxicity when plastics and contaminants are co-incidentally present.

Preface

Abigail Robinson is the principal researcher for all experiments and work within the following thesis. All experimental work was conducted at the University of Alberta, in the Department of Biological Sciences solely by Abigail Robinson. Dr. Greg Goss supervised the entirety of this thesis. No component of this thesis has yet been published. The project was completed using funding under the Department of Fisheries and Oceans Canada Increasing Knowledge of Plastic Pollution initiative (IKPP).

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List of Abbreviations

ARO	Aquatic Research Organisms
CCME	Canadian Council of Ministers of the Environment
EC_{50}	effective concentration (50%)
EROD	ethoxyresorufin-O-deethylase
Flx	fluoxetine
Gly	glyphosate
h	hour
ISO	International Organisation for Standardization
L	litre
LC_{50}	lethal concentration (50%)
mg	milligram
$MgSO_4 \cdot 7H_2O$	magnesium sulfate heptahydrate
mm	millimetre
mL	millilitre
mM	millimolar
mg	milligram
n	sample size
$NaHCO_3$	sodium bicarbonate
ng	nanogram

nm	nanometre
nL	nanolitre
NP	nanoplastics
OCD	obsessive compulsive disorders
OECD	Organisation for Economic Co-Operation and Development
p	probability value
PAH	polycyclic aromatic hydrocarbon
Phe	phenanthrene
POP	persistent organic pollutant
PS	polystyrene
PS-NP	polystyrene nanoplastics
µg	microgram
µL	microlitre
YCT	yeast, trout chow and cereal leaf mix
SD	standard deviation
SEM	standard error of the mean
SUP	single use plastic
SSRI	selective serotonin reuptake inhibitor
WHO	World Health Organization

CHAPTER ONE

Introduction

Plastic Production

Commercial plastic production was initiated in 1868 (Anderson & Thompson, 1950) and since then plastic production methods have become more effective, efficient, and widespread (Okoffo et al., 2021). Exponential increases in plastic production have occurred over the past 70 years, with global annual production increasing from 1.7 to 335 million metric tons in 2017 (Plastics Europe, 2017). Common use items such as bags, reusable bottles, and food packaging are typically made of plastic as it is a cheap and durable option. Approximately 10% of household waste is plastics, and approximately 80% of the waste residing on shorelines is plastic (Barnes et al., 2009). The presence of plastics is a large geological footprint left by mankind, adding evidence to the proposed concept of the Anthropocene. This concept, first suggested by Crutzen (2006) claims that the large shift in resource use occurring in the mid-20th century has left a footprint that could become part of the geological time scale (Zalasiewicz et al., 2016). In combination with the many other impacts such as CO_2 emissions, resource extraction, and more, Crutzen, (2006) suggested the generation of a new geological time frame named the 'Anthropocene'. Given that plastics are estimated to remain in the environment for hundreds to thousands of years, they will provide a physical human footprint on a vast range of global environments (Barnes et al., 2009). Developing an understanding of the potential longer-term effects of plastic pollution is critical to ensuring sustainability of the environment as we move into the future.

In 2018, the vast consumption of single use plastics was identified as a global environmental pollution crisis by the United Nations Environment Program (UNEP, 2018). Hopeful future projections anticipate a reduction in annual plastic waste if society can increase recycling efforts and reduce the use of single use plastics (SUPs) (Lebreton, L., & Andrady, A., 2019). These projections allow research scientists to analyze the predicted pollution levels, aiming to reduce potential harmful effects before the effects occur.

The COVID-19 global health crisis has recently added to the growing issue of plastic waste. From 2019 to 2021, plastic production increased at a 5% annual compound rate (Adyel, 2020). Approximately 8.4 million tons of plastic waste were produced during the pandemic between April 2020 and August 2021, and additionally, it is estimated that approximately 25.9 thousand tons of plastic waste were released into the ocean in this time (Peng et al., 2021). This increase in production was due to many factors including but not limited to increase in disposable PPE production, the increase of take-away deliveries from restaurants, and an increase in home delivered products.

As plastics accumulate in natural environments, the strain on species and ecosystems only intensifies (Brandon et al., 2019). A study in the 1960's demonstrated the cumulative effects of plastics in the environment through the examination of seabird gut contents (Kenyon et al., 1969). The results were startling and demonstrated that plastic fragments were present in 74% of the seabirds examined (Kenyon et al., 1969). Since the previously mentioned 1969 study, plastic use has increased and as a result, the abundance of plastic fragments in or near aquatic/marine environments has also increased (Brandon et al., 2019; Wilcox et al., 2019). Wilcox et al. found that the number of plastic fragments in the North Atlantic has increased over time, corresponding with the rates of global plastic production (2019).

The Government of Canada reported that only 9% of plastic waste is recycled, and the remainder ends up in Canadian landfills or natural environments (Canada, 2021). Canada has approximately 25% of global freshwater, therefore it is important that we monitor the effects of modern consumption and waste habits (Canada, 2021). Consequences of co-contaminant nanoplastic uptake on aquatic life are not deeply understood, presenting a large gap in aquatic toxicology research.

Micro/Nanoplastics

Plastics are very durable in the environment and results in a reasonable cause for concern regarding the organisms living in the aquatic environment. Given the longevity of plastics, it is likely that all plastics ever produced and released to the environment still exist in the environment to some extent, except for those that have been incinerated (Thompson et al., 2005). While plastics are durable, they fragment into smaller pieces over time through processes such as photo-oxidative degradation, thermal weathering, biodegradation, and physical/chemical breakdown (Singh et al., 2008). The rate of breakdown and weathering varies depending on the characteristics of the plastics and/or additives in the polymer (Avio et al., 2017). The most common types of plastics include polystyrene (PS), polyvinyl chloride (PVC), polyethylene (PE), and polypropylene (PP) (Avio et al., 2017). Polystyrene is a common plastic (used throughout the following experiments) with an average of 13 million tons produced per year (Lithner et al., 2011). The chemical and physical breakdown of these plastics leads to fragile and brittle particles, which may then break into micro-sized plastics (Avio et al., 2017). Barnes et al. analyzed the number of microscopic plastic fragments in surface waters and found significant increases from the 1960s to the 2000s, corresponding with the increases in annual plastic

production (2009). Given the amount of plastic waste is still increasing annually, the number of microplastics found in the environment will continue to climb, posing a threat to aquatic ecosystems.

Plastics can be broken down into small fragments that cannot be seen with the naked eye. Categorizing these small pieces has proven to be a scientific challenge, with continuous debate about the size ranges that encompass macro, micro and nano-plastics (Hartmann et al., 2019). A recent article has defined NPs as products resulting from the breakdown of industrial plastic objects, with one dimension between 1 and 1000nm (Gigault et al., 2018). For this thesis, NPs will be defined as small plastic fragments with a diameter <1000nm. Plastic fragments with a diameter greater than 1000nm will be defined as microplastics.

It has been argued that a strict definition of nano versus micro plastics may reduce the effectiveness of nanoplastic research as this may suggest that everything within each category has similar effects and characteristics (Hartmann et al., 2019). However, the consensus is that the ecotoxicity of NPs will likely vary with properties other than size and include elements such as porosity, solubility, color, shape, etc. (Hartmann et al., 2019). These other plastic characteristics may affect how the plastic is weathered or the ability of a plastic to sorb contaminants. Dyes or other additives added into the plastics may alter the sorption capacity of a plastic or the toxicity of a plastic, although this has not yet been investigated.

While current knowledge on the presence and abundance of microplastics (plastics with a diameter <5mm) is rapidly growing (Strungaru et al., 2019), the detection and quantification of NPs in natural environments is still limited, mostly by lack of analytical tools. Microplastics will break down to the nanoscale over time and therefore, the presence of microplastic concentrations in any environment will indicate the probable presence of NPs in the system. Microplastics have

been detected in almost every aquatic environment globally, ranging from 0.028 particles/m^3 to 419,000 particles/m^3 (Sadri & Thompson, 2014., Lahens et al., 2018). Research on microplastic concentrations in the freshwater environments of Canada is limited. However, a study of the St. Lawrence River found microplastics along the entire length of the river at an average density of 52 microbeads/m^2 (Castañeda et al., 2014). Microplastic concentrations have been analyzed in the North Saskatchewan River, which runs through Edmonton, AB, Canada (Bujaczek et al., 2021). Microplastics were found at every sampling site throughout the river and ranged from 4.6 to 88.3 plastic particles or fibre per cubic meter of water (Bujaczek et al., 2021).

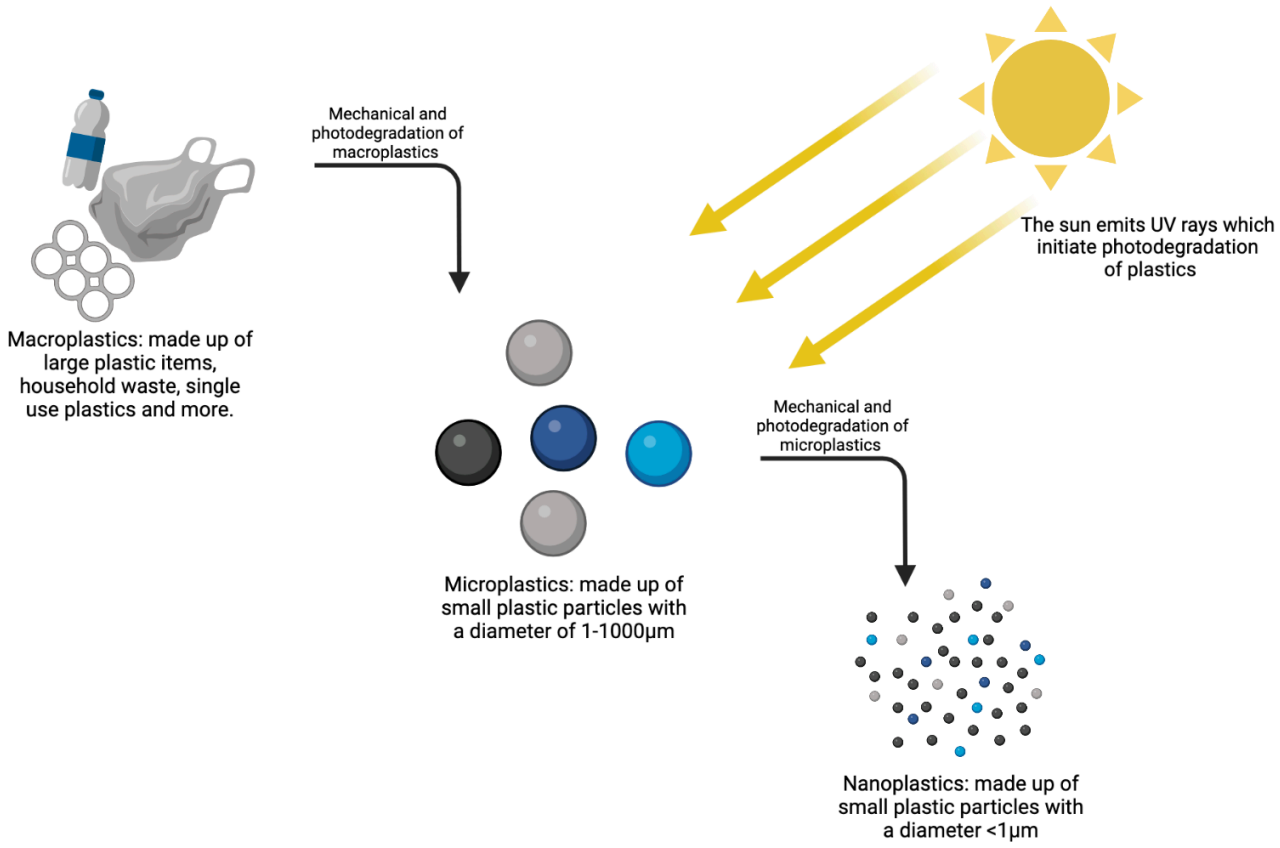

Figure 1.1: Illustration displaying the breakdown process of plastics. Most plastics begin as macroplastics (Single use utensils, plastic bottles, etc.). When these macroplastics are disposed of, they breakdown via UV degradation and mechanical weathering to become microplastics. The process continues and plastics become smaller, eventually reaching the nano scale.

Uptake of Plastics from the Environment

The detection of micro and NPs in aquatic systems has increased scientific curiosity regarding the effects of these plastic particles. Early studies examining the uptake of plastics looked inside the guts of seabirds and counted the plastic particles inside. Surveys of Hawaiian seabird populations found that 90% of the seabirds (including chicks) had plastics in their upper digestive tract (Fry et al., 1987). These plastic particles were macro and micro plastics, physically taking up space in the gut and hindering he ability of the bird to scavenge for food effectively, as the birds were consistently satiated. The sea urchin *Paracentrotus lividus* breaks down macroplastics into smaller fragments via grazing, producing on average 91.7 plastic fragments over a ten-day period (Porter et al., 2019). The uptake and bioerosion completed by the sea urchin decrease the plastic particle size and therefore may increase the bioavailability of the particles to other aquatic/marine species (Porter et al., 2019).

New studies examining the uptake of microplastics from the environment have been alarming and include uptake by humans, fish, birds, and more (Murphy et al., 2017; Prata et al., 2020; Egbeocha et al., 2018). Human studies have demonstrated the presence of microplastics in common food and beverage items, and subsequent findings of microplastics in human tissues (Wright et al., 2017). Estimates of annual microplastic intake for a typical American diet suggests ingestion of 39,000 to 52,000 particles annually (Cox et al., 2019). Recent studies have found microplastics in placenta and newborn babies, suggesting that the avoidance of microplastic ingestion may be incredibly difficult (Ragusa et al., 2021; Zhang et al., 2021).

Detection of NPs in the environment is difficult due to the small size and relatively low environmental concentrations of particles (Lehner et al., 2019). However, new technology such

as surface-enhanced Raman spectroscopy is being examined for its potential to detect 100 nm plastics at concentrations as low as 40 ug/L (Lv et al., 2020). Pyrolysis gas chromatography is also utilized to detect particles > 1 um in size at concentrations of 50 ug/L (Sullivan et al., 2020). Due to the small size of the NPs and relatively low concentrations, the uptake in natural environments is yet to be studied. In laboratory settings, nanoplastic uptake has been demonstrated in many organisms including but not limited to: *Daphnia magna*, *Chorella*, bivalve molluscs, and multiple fish species which suggests similar uptake may be occurring in aquatic environments (Besseling et al., 2014; Hazeem et al., 2020; Al-Sid-Cheikh et al., 2018; Chen et al., 2017; Zhang et al., 2021).

The 'Trojan Horse Effect'

The ubiquitous prevalence of plastics in aquatic environments and the evidence above that the smallest of these particles can cross epithelia is alarming. However, the impact of NPs may also be larger than the physical pollution of plastics themselves. Given that NPs are very small (<1000nm in diameter), they will have an exponentially larger increase in surface area to volume ratio as size decreases (Koelmans et al., 2015) (Figure 1.2). Additionally, given that plastics are made of inert hydrophobic polymers, the plastics in the environment have hydrophobic surfaces, these hydrophobic characteristics provide an optimal surface for partitioning of hydrophobic organic contaminants from the water to the surface of the plastic particle (Velzeboer et al., 2014). The hydrophobic surface of the NPs is known to adsorb a variety of contaminants to their surface, generating an outer layer known as the 'surface corona' (Velzeboer et al., 2014). For example, complex mixtures of metals are known to accumulate on

the surface of NPs in marine environments (Rochman et al., 2014). The increased surface area of NPs in the environment compared to macro and micro plastics means that the sorption of organics is predicted to be much higher in smaller particles than in larger particles for any given mass. Recently, Zhang et al., (2020) did an examination of nanoplastic sorption of ^{14}C-Phenanthrene (a common model polycyclic aromatic hydrocarbon (PAH)) and found that 20 nm polystyrene NPs have higher sorption capacity for ^{14}C-Phenanthrene when compared to 500 nm NPs. The 20 nm plastic particles with sorbed ^{14}C-Phenanthrene also increased ^{14}C-Phenanthrene uptake in zebrafish embryos compared to the ^{14}C-Phenanthrene sorbed to 500 nm plastic particles or ^{14}C-Phenanthrene alone (Zhang et al., 2020). Moreover, exposure of ^{14}C-Phenanthrene sorbed to 20 nm particles displayed significantly higher uptake and ethoxyresorufin-O-deethylase (EROD) activity in rainbow trout than when sorbed to 500 nm particles or ^{14}C-Phenanthrene alone (Zhang et al., 2021). These results clearly indicated that both the size, the surface area to volume ratio, and the hydrophobicity of the NPs and the organic itself will all play a role in the sorption potential and accumulation potential of any given organic for a given size of nanoplastic.

The term 'Trojan Horse Effect' has been applied to the enhancement of organic uptake due to the sorbed carrier function of plastics. Many organisms unintentionally ingest NPs throughout their lifetime, including humans (Cox et al., 2019). Zebrafish embryos exhibited increased EROD activity and increased fluorescence in microscopy samples in embryos exposed to NPs with sorbed PAHs (Trevisan et al., 2020). Another study using mussels indicated increased bioavailability of pyrene after ingestion when sorbed to microplastics, demonstrating the Trojan Horse Effects of plastic fragments (Avio et al., 2015). Research comparing micro to NPs have also demonstrated that NPs with sorbed phenanthrene have significantly higher uptake

compared to microplastics sorbed with phenanthrene or phenanthrene alone (Ma et al., 2016). However, a recent study modelled the potential uptake of various contaminants (including phenanthrene) in marine life when sorbed to microplastics and found that the co-contaminant sorbed microplastics had a negligible impact on biota when compared to other uptake effects of contaminants in the natural environment (Bakir et al., 2016). The modelling study was completed using micro-sized plastics, therefore the surface area to volume ratio (Figure 1.2) was lower than if NPs were used, and potential for crossing the membrane (Figure 1.3) was not considered (Bakir et al., 2016). Overall, the Trojan Horse Effect appear to be a valid concern in that it results in NPs potentiating the uptake of contaminants into organisms.

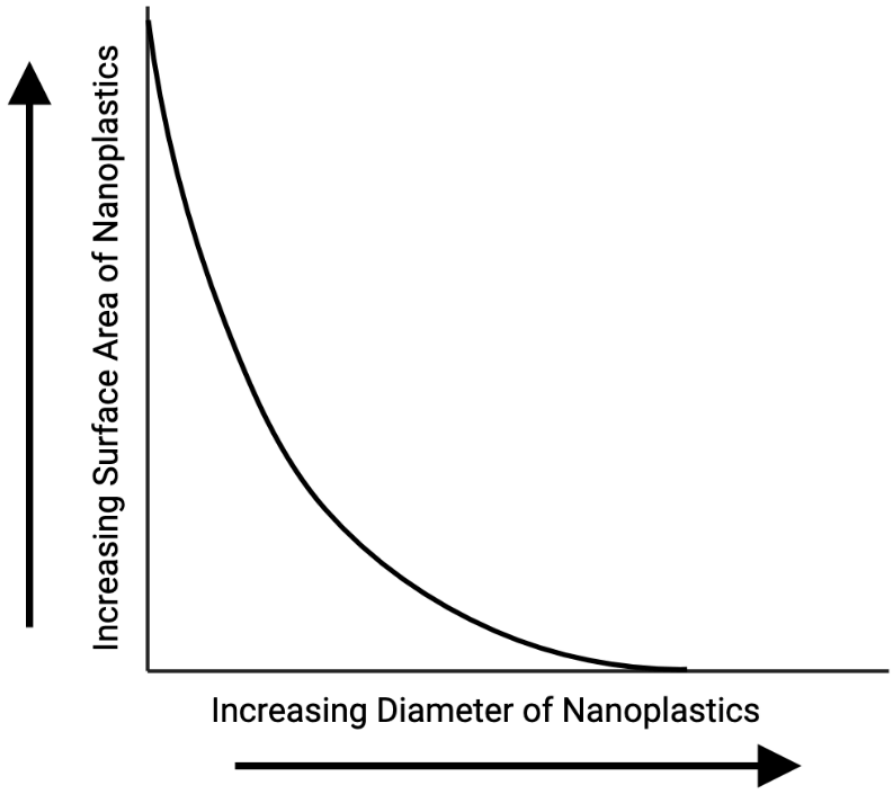

Figure 1.2: Illustration demonstrating the surface area to volume ratio effects. As the diameter of micro/NPs increases, the surface area increases exponentially.

Uptake and Depuration

Previous literature indicates that *Daphnia magna* will ingest micro/NPs (Canniff & Hoang, 2018). Daphnia typically feed on algae approximately 0.019 – 5.0µm in size via a mechanical sieving process (Gophen & Geller, 1984). These dietary habits allow for easy ingestion of micro/NPs, along with any environmental contaminants sorbed to those NPs. It is also possible that smaller NPs enter can the *Daphnia magna* via gill epithelial cells which have a width of 70-130 nm (Kikuchi, 1983). When NPs and sorbed co-contaminants are ingested, there are two possible hypotheses for uptake of the co-contaminant from the gut into the animal. The first hypothesis is that as the nanoplastic nears the intestinal epithelial membrane of the daphnid, the contaminant can offload from the plastic and then be sorbed through the membrane by simply diffusion. The relative hydrophobicity/hydrophilicity of each environmental contaminant can be described by its octanol: water partition coefficient, also known as K_{ow} (Sangster, 1997; Kolpin et al., 2002; Contardo-Jara et al., 2009). It is hypothesized the K_{ow} is one dominant factor affecting the rate at which NPs either adsorb or release the contaminants from various types of nanoplastic particles. This hypothesis is supported with previous literature regarding larger sized microplastics (6µm), whereby the microplastics did not migrate from the gut to surrounding tissue (Elizalde-Velázquez et al., 2020). A second hypothesis is that the NPs themselves can cross the membrane, especially the smaller sized particles < 100 nm, thus "carrying" the organics into the organism. This would be indicated by the presence of both the NPs and the contaminants inside the organism, after the initial crossing of the intestinal epithelium.

After organic contaminants enter the animal, the phase I and phase II biotransformation pathways will be stimulated for elimination of the planar organic compounds (Baldwin &

LeBlanc, 1994). The phase I biotransformation pathways typically induce a loss of pharmacological function or the organic contaminant, which reduces the potential harmful effects of these compounds. Phase II biotransformation pathways form polar compounds that are usually inactive, and this facilitates rapid excretion of the organic. The combination of phase I and phase II biotransformation pathways generally leads to the rapid depuration of organic contaminants of the animal, and therefore a reduction in the overall body burden in the face of continued contaminant exposure.

It is important to examine not only the uptake and effects of micro or NPs, but also the mechanism and rate that the organism can rid of the particles. The depuration/egestion of NPs from *Daphnia magna* via feces has been studied and determined to occur at a higher rate after exposure to larger NPs (Rosenkranz et al., 2009). The presence of 1000 nm NPs (90% depuration over 4 h) resulted in significantly higher depuration rates when compared 20 nm NPs (40% depuration over 4 h) in *Daphnia magna* (Rosenkranz et al., 2009). The mechanism *Daphnia magna* use to excrete NPs is not yet known, and therefore the size dependence noted in previous experiments requires further exploration. Egestion rates of irregularly shaped NPs have also been demonstrated to be slower than the depuration rates of spherical shaped NPs (Frydkjær et al., 2017).

Sources of Organic Contamination in the Environment

Organic contaminants are produced by humans globally, and as a result are found in natural environments globally. There are many different sources of pollution, and these sources can be categorized as point sources or nonpoint sources of pollution (Doust et al., 1994). Point

sources of pollution include direct discharge such as pipes or factory effluent into aquatic systems (Duda, 1993). Non-point sources of pollution include groundwater, agricultural runoff, and atmospheric deposition (Duda, 1993). In aquatic environments, most of the pollution derives from wastewater, runoff, or the burning of fossil fuels (Kolpin et al., 2002; Boivin et al., 2006; Hughes et al., 1997). For this thesis, one contaminant from each of three major group of pollutants was used to effectively compare the co-contaminant effects of various pollutants. Phenanthrene is a common PAH that is emitted from the combustion of fossil fuels (Simpson et al., 1996). Fluoxetine (Flx) (a common antidepressant) is commonly found in wastewater and subsequently in aquatic environments as approximately 60% is excreted into wastewater systems (Metcalfe et al., 2010). Glyphosate (Gly) (also known as Roundup) is present in runoff and therefore is found in aquatic systems, particularly in areas with high amounts of agricultural activity (Battaglin et al., 2005).

Detection of these contaminants in the environment or measurement in experimentation requires specialized methods. These methods have advantages and disadvantages that must be taken into consideration when designing an experiment. Mass spectrometry is a technique used to measure the amount of one or more contaminants in a sample. This technique is beneficial as multiple contaminants can be studied at once. However, mass spectrometry generally requires large volumes of media to be extracted to reach detectable levels, require expensive instrumentations and are generally time consuming. For the following thesis, radioactive labelling methods were employed. Radioactive counting can detect very low and environmentally relevant levels of contaminants. Overall, radioactive labelling is a cost-effective method to detect environmentally relevant levels of contaminant in a sample.

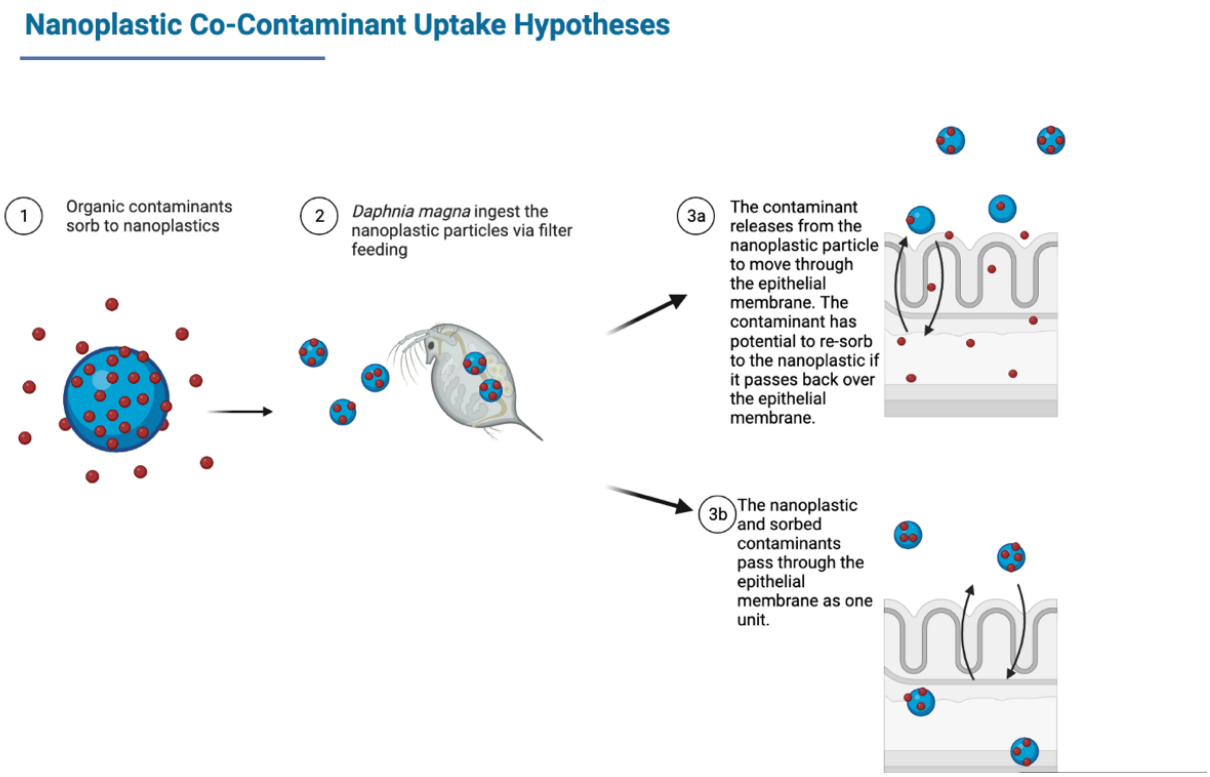

Figure 1.3: Illustration displaying the potential uptake mechanisms of contaminants when sorbed to NPs.

Radiolabeled Chemicals

The experiments conducted for the purpose of this thesis involved the use of radiolabeled chemicals at tracers of uptake and depuration. Radioactive chemicals were used as tracers to allow for sensitive measures of transport even at very small and environmentally relevant exposure concentrations. Furthermore, measurement of organic transported in *Daphnia magna* allows for very small volumes of isotopes to be used in exposures, thereby allowing for resources to be used efficiently. The use of radioactivity in my study allows for experimental exposures with concentrations smaller and more closely related to environmental concentrations than those used in previously published studies.

Phenanthrene

Phenanthrene is a naturally occurring PAH that is produced and released into the environment via combustion and petrogenic processes. Phenanthrene is typically released into the atmosphere through vehicle emissions, steel works, oil shale plants and cigarette smoke (Gad, 2014). Phenanthrene is also considered a Persistent Organic Pollutant (POP) as it is resistant to photochemical, biological, and chemical degradation. Most POPs can bind strongly to lipids, thereby enhancing potential bioaccumulate processes (Geyer et al., 2000). Previous studies using radiolabeled ^{14}C-Phenanthrene indicate that this chemical accumulates in the liver, gallbladder, gill, and gut of *Oncorhynchus mykiss* (Rainbow trout) (Blewett et al., 2021). Canadian Council of Ministers of the Environment (CCME) guidelines indicate that phenanthrene should not exceed 0.4 µg/L in freshwater environments (CCME, 1999). However,

phenanthrene has been found in aquatic environments at concentrations up to 26.1 µg/L (Maskaoui et al., 2002).

The octanol-water partition coefficient (log K_{ow}) is used to measure the extent of hydrophobicity of a compound or substance (Sangster, 1997). Substances with higher K_{ow} coefficients tend to bind to organic matter or organisms in aquatic environments while those with lower K_{ow} coefficients are more water soluble (Djomo et al., 1996). Phenanthrene has a log-K_{ow} of 4.57, these hydrophobic qualities suggest that the phenanthrene will likely bind to the more hydrophobic NP-PS particles in aquatic environments as they are also hydrophobic (Djomo et al., 1996).

This thesis investigates whether the presence of NPs in conjunction with phenanthrene potentiates contaminant transfer into aquatic organisms. It is known that phenanthrene can adsorb to the surface of the NPs and therefore could help carry the toxicant into the organism (Ma et al., 2016) and this may potentiate the noted effects of phenanthrene alone. NPs sorbed with phenanthrene have been shown to display significantly higher toxic effects when compared to larger plastic particles also sorbed with phenanthrene, thereby indicating the potential detrimental effects of nano-sized plastic particles (Ma et al., 2016; Xu et al., 2021) In a recent study by Zhang et al., (2020), simultaneous exposure to NPs and phenanthrene resulted in increased mortality and decreased length in zebrafish embryos when compared to isolated exposure to either phenanthrene or the NPs alone (Xu et al., 2021; Zhang et al., 2020).

Fluoxetine

Fluoxetine (N-methyl-3-phenyl-3-[4-(trifluoromethyl) phenoxy] propan-1-amine) is the leading antidepressant worldwide in terms of total sales (Wong et al., 1995). As a SSRI (Selective serotonin reuptake inhibitor) with limited side effects, it has been used globally to treat depression, OCD (obsessive compulsive disorder) and anorexia (Wong et al., 1995). When fluoxetine is metabolized by cytochrome P450 it becomes norfluoxetine. However, this compound is also a SSRI with a much longer half-life than fluoxetine itself (Kwon & Armburst, 2006). In the body, wastes are filtered and excreted as urine and are commonly discharged into the environment via sewage waste. As a result, fluoxetine is found ubiquitously in aquatic environments (Kolpin et al., 2002). Kolpin et al., found fluoxetine to be present at an average concentration of 0.018 ug/L in surface waters (2002) with effluent fluoxetine concentrations having been recorded at much higher concentrations, reaching 0.540 ug/L levels (Weston et al., 2001). It is important that these substances are analyzed and understood as fluoxetine is prevalent in aquatic systems

As previously noted, the octanol-water coefficient is a common metric used to indicate the hydrophobicity of a substance. Fluoxetine has a K_{ow} of 4.26 (Wan et al., 2007) indicating a potential for bioaccumulation. However, research suggests that the K_{ow} for fluoxetine may not be a reliable indicator of the bioaccumulation factor (BAF) as the substance ionizes in water (Cunningham, 2004). The liposome-water partition coefficient is suggested to estimate BCF of fluoxetine with a value >3, indicating moderate potential for bioaccumulation (Oakes et al., 2010). The positive K_{ow} value indicates that fluoxetine will likely bind to NP-PS particles in aquatic systems. However, to date, research on the impact of NPs in conjunction with fluoxetine is limited.

Glyphosate

Glyphosate is a non-selective herbicide that is commonly known as Roundup®. It is used to control weeds in both terrestrial and aquatic environments (Tsui & Chu, 2003). Many glyphosate-based products are not permitted for aquatic use. However due to widespread terrestrial application, glyphosate has been shown to be almost ubiquitously present in agricultural and domestic run-off. The highest recorded concentrations of glyphosate in aquatic environments reached 105,000 µg/L in Argentina (Sasal et al., 2017). In Canada, the highest recorded concentration of glyphosate in freshwater was 41 µg/L (CCME, 2006). CCME guidelines state that aquatic concentrations should not exceed 65 µg/L glyphosate (CCME, 2006). Therefore, exposure of aquatic organisms to glyphosate is inevitable and the understanding of toxic effects and implications of co-exposure with plastics is extremely important.

Glyphosate is usually applied in conjunction with a surfactant (e.g., methanol) in most practical applications, and this is the formulation know as Roundup®. It is likely that any surfactant use will also exhibit similar toxicity to glyphosate on its own. Therefore, when both glyphosate and NPs are present together in an aquatic media, they display significantly higher toxicity (Tsui & Chu, 2003). Glyphosate toxicity for midge larvae 48 h whereby 50% of the organisms were affected (EC_{50}) was 55 mg/L and for rainbow trout LC_{50} was 140 mg/L, the concentration whereby 50% of the organisms died (Folmar et al., 1979; (World Health Organization (WHO), 1994). Glyphosate has high water solubility and a low log-K_{ow} (-3.2), which indicates that the bioaccumulation threat in aquatic environments is low (Contardo-Jara et al., 2009). Glyphosate has been shown to accumulate in the aquatic organism *Lumbriculus*

variegatus (2009). This accumulation demonstrates a potential for bioaccumulation of glyphosate in *Daphnia magna* when present in conjunction with NPs.

***Daphnia magna* as a model invertebrate organism**

The invertebrate species *Daphnia magna* (also known as the water flea) is found in freshwater environments globally. As a grazer of algae and a primary food source for fish, daphnids are a keystone species in aquatic environments (Carpenter et al., 1963). *Daphnia magna* are filter feeders who filter out bacteria, algae, and other small particles suspended in the water (Tkaczek, 2021). Therefore, the presence of micro and NPs is concerning as these pollutants may be consumed by the invertebrates unintentionally. Daphnids were first used in toxicological testing in 1944 to assess the potential toxicity of industrial wastewater (Anderson, 1944). These invertebrates reproduce and mature rapidly and are easy and inexpensive to care for in the laboratory (Anderson, 1944).

In addition to this, previous research has indicated that *D. magna* ingest both microplastics and NPs, however microplastics are more readily egested (Rist & Hartmann, 2017). Round microplastics are also more readily egested by *D. magna* when compared to rough microplastic particles (Frydkjær et al., 2017). This thesis will examine both the uptake and depuration rate of smooth 20 & 500 nm nanoplastic particles with sorbed aquatic contaminants.

Thesis Aims

With the production, use, and disposal of plastics inevitable in our current society, it is important to understand the impacts and potential effects that these products have on ecosystems and health of organisms. Knowledge of the effects of micro and NPs is limited but increasing rapidly, and this research aims to further contribute to the current understanding of these particles. The aims of this thesis are:

1) To determine whether the presence of NPs (20 nm or 500 nm) increases or decreases the net transport rate of ^{14}C-Phenanthrene, the percentage uptake, and percentage depuration in *Daphnia magna*. I *hypothesize* that uptake and depuration will both be enhanced as the NPs may carry multiple ^{14}C-Phenanthrene compounds at a time.
2) To compare three common aquatic contaminants (^{14}C-Phenanthrene, ^{14}C-Fluoxetine and ^{14}C-Glyphosate) to determine whether the presence of NPs (20 nm or 500 nm) increases or decreases the net transport rate of each of these contaminants. I will also measure the percentage uptake and percentage depuration in *Daphnia magna*. I *hypothesize* that compounds with a higher K_{ow} (phenanthrene and fluoxetine) will have higher net transport rates, uptake rates and possibly depuration rates when comparted to contaminants with a lower K_{ow} (glyphosate). The is primarily hypothesized to be due to higher or more robust sorption of organics with a higher K_{ow} to the nanoplastic surface.
3) I also *hypothesize* that smaller NPs will have an increased net transport rate as they may additionally cross the membranes in *Daphnia magna*.

In the following thesis, the 'Trojan Horse Effect' of NPs present with various environmental contaminants will be analyzed to determine potentiation of toxic effects that may occur with the

presence of NPs. A deeper understanding of co-contaminant uptake effects is essential to regulate and monitor the impact of micro/NPs and chemicals on aquatic life.

CHAPTER TWO

Introduction

Plastic pollution in natural environments is a rapidly growing issue with increases of 1.7 to 335 million metric tons in annual plastic production from 1947 to 2017 (Plastics Europe, 2017). Approximately 80% the waste lying on shorelines is plastic, giving insight into the magnitude of waste in natural environments that is made of plastic (Barnes et al., 2009). When these plastics are released into the environment, they breakdown from macro plastics into smaller sized micro or NPs. This breakdown occurs via UV degradation or mechanical weathering (Singh et al., 2008). Studies have also found that plastic particles are taken up by sea urchin, and bio eroded into smaller sized particles (Porter et al., 2019).

As the particles are weathered and decrease in size the surface area to volume ratio increases exponentially (Koelmans et al., 2015). NPs are defined as fragments with a diameter < 1000 nm in size with a large surface area to volume ratio (Gigault et al., 2018). This increase in surface area to volume ratio allows the hydrophobic micro/nanoplastic particles to sorb metals or other hydrophobic contaminants to their surface (Rochman et al., 2014; Zhang et al., 2020). The nanoplastic particle has potential to carry multiple sorbed contaminants into the organism, this concept is known as 'The Trojan Horse Effect'. This effect has been demonstrated by enhanced toxicological effects when contaminants are sorbed to nanoplastic particles (Trevisan et al., 2020; Zhang et al., 2020). Three common contaminants (phenanthrene, fluoxetine and glyphosate) released into aquatic systems via fossil fuel combustion, wastewater, and agricultural runoff (respectively) were chosen for the co-contaminant uptake study.

Previous literature demonstrated that the invertebrate species *Daphnia magna* will ingest NPs, as they typically feed on algae that is 0.019 – 5.0µm in size (Canniff & Hoang, 2018;

Gophen & Geller, 1984). As the invertebrate species ingests the NPs, they also will ingest contaminants sorbed (Canniff & Hoang, 2018). The first co-contaminant uptake hypothesis is the nanoplastic nears the intestinal epithelial membrane of the *Daphnia magna* and offloads the contaminant which then crosses the membrane by simple diffusion. Variances in octanol: water partition coefficient (K_{ow}) would alter the uptake rates of this hypothesis; the K_{ow} is the relative hydrophobicity/hydrophilicity of each contaminant (Sangster, 1997; Kolpin et al., 2002; Contardo-Jara et al., 2009). The second co-contaminant uptake hypothesis is that the nanoplastic and sorbed co-contaminant both cross the intestinal membrane of the daphnid.

It is important to also examine the rate at which the invertebrate species *Daphnia magna* can excrete or depurate the NPs and/or sorbed co-contaminants. The depuration of NPs with sorbed co-contaminants has not been previously studied. However, the depuration of NPs alone has been examined, and the egestion rate is slow in the absence of food or after exposure to larger NPs (Rosenkranz et al., 2009; Frydkjær et al., 2017; Rist et al., 2017). This study aims to determine the impact of contaminant characteristics on the co-contaminant uptake and depuration effects when sorbed to nanoplastic particles.

Materials and Methods

Animals

Daphnia magna were originally obtained from Aquatic Research Organisms (ARO), Inc (Hampton, New Hampshire, USA). The animals were cultured and maintained in Biological Sciences (University of Alberta) at an 8:16 light: dark photoperiod. The daphnids were stored in 2L glass beakers containing International Organization for Standardization (ISO) test water: magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$) (0.5mM), sodium bicarbonate ($NaHCO_3$) (0.77mM), calcium chloride ($CaCl_2 \cdot 2H_2O$) (2mM), and potassium chloride (KCl) (0.08mM): made into solution using ultrapure water at 20 ± 1°C (OECD, 2004). Water changes were completed every 3-4 days or as needed. Daily feeding included algae (*Raphidocelis subcapitata*), YCT (a yeast, cereal leaf, trout chow mix), and Roti-Rich liquid invertebrate food. Neonates were separated from the reproducing colonies daily and maintained for 10-15 days to be used in the uptake and depuration experiments. Experimental animals were not fed the day of exposure to decrease extraneous variables.

Materials and Chemicals

Fluorescently labeled NPs in sizes 20nm (505 nm excitation, 515 nm emission, Catalog#F8787) and 500 nm (505 nm excitation, 515 nm emission, Catalog#F8813) were obtained from ThermoFisher. The as-purchased plastics were received suspended in water (2% solids) with 2 mM sodium azide added. To remove the azide, the plastics were dialyzed for 7 days in Milli-Q water using Slide-A-Lyzer dialysis Cassettes (2000 molecular weight cut-off, ThermoFisher, Catalog#PI66203). The water was changed once an hour (h) for the initial 12

hours, and then twice daily for the remainder of the seven days. The NPs were kept in a dark area and refrigerated.

Radioisotopes were used to trace movements of specific organic chemicals in the following experiments. ^{14}C-Phenanthrene (55 mCi mmol^{-1};) ^{14}C-Fluoxetine (55 mCi mmol^{-1}) and ^{14}C-Glyphosate (50 mCi mmol^{-1}) were obtained from American Radiochemicals. For detection of radioisotopes, samples were mixed with Ultima-Gold scintillation fluor (PerkinElmer, Catalog #6013309) and a beta counter (HITACHI AccuFLEX LSC-8000) was used to quantify the number of radioactive materials in each of the isotope exposure experiments. Non-radioactive versions of each chemical used for post exposure washes were obtained from Sigma-Aldrich and were used to rinse daphnids after exposure to the radioactive solutions. Phenanthrene was dissolved in Milli-Q water at a stock concentration of 2 mg/L to be used for non-radioactive washes (CAS-No. 85-01-08, 98% purity). Fluoxetine hydrochloride was dissolved in Milli-Q water at a stock concentration of 2mg/L to be used for non-radioactive washes (CAS-No. 56296-78-7, 99% purity). Glyphosate was dissolved in Milli-Q water at a stock concentration of 2 mg/L to be used for on-radioactive washes (CAS-No. 1071-83-06, analytical standard).

Organic Contaminant Uptake and Depuration Test Protocol

^{14}C-Phenanthrene Uptake Protocol

Daphnia magna (10-15 days old) were fasted for 24h prior to experimentation. Test solutions were prepared 1 hour prior to experimentation to allow adequate sorption of the contaminants to NPs. ^{14}C-Phenanthrene was used at a concentration of 6.48µg/L, a hundred times lower than the concentration used by Zhang et al. (2020). ^{14}C-Phenanthrene stock was diluted to desired concentration in OECD water, and subsequently NPs were added to the solution (as required in each experiment).To test if NPs affected the rate of organic uptake, the following treatments were prepared: OECD water only (control), 6.48 µg/L ^{14}C-Phenanthrene, 6.48 µg/L ^{14}C -Phenanthrene + 1 mg/L 20 nm NPs, and 6.48 µg/L ^{14}C -Phenanthrene + 1 mg/L 500 nm NPs. To start each test, 5mL of the test solution was portioned into a glass vial (5 vials per treatment, n=5) and a 500 µL aliquot taken from the treatment to provide a pre-uptake radioactive count of exposure solution. Total counts in the following calculations were obtained by using the cpm measurement of the aliquot and expanding that to the volume of solution (4.5mL). Twenty *Daphnia magna* (10-15 days old) were then added to each vial and at the appropriate exposure time (30 min, 1 h, 2 h, 4 h, 8 h, 12 h, 24 h), the daphnids were removed and were washed thrice with OECD water containing non-radioactive phenanthrene (2 mg/L) and then once with OECD water alone. A post-uptake 500 µL sample was taken from the exposure media. The total counts for the post-uptake solution were obtained by the measurement of the aliquot cpm and multiplying those cpms by the volume of the post-uptake solution (4.5mL). The animals were pat dry, weighed, and incubated at 60°C in 2 N nitric acid for 24 h. 3 mls of Ultima-Gold

scintillation fluor was added to each sample and each sample was counted for 10 minutes in the beta counter.

^{14}C-Phenanthrene Depuration Test Protocol

Adult daphnids (10-15 days old) were fasted for 24 h prior to the beginning the depuration experiments. Testing solutions were prepared as described above in '^{14}C-Phenanthrene Uptake Test Protocol'. 5 mL of the testing solution was pipetted into a glass vial, and 500 µL was removed to quantify radioactivity in the test solution prior to uptake. Total counts in the following calculations were obtained by using the cpm measurement of the aliquot and expanding that to the volume of solution (4.5mL). 20 adult daphnids were then moved into the solution and exposed for 24h. After the 24 h period, the daphnids were removed from the solution and another 500 µL measurement was taken from the exposure solution to quantify total uptake into the daphnids after 24 h. Total uptake was obtained by measurement of the 500 µL aliquot and multiplying the cpms by the total volume of the exposure solution (4.5mL). The daphnids were rinsed 3x with non-radioactive phenanthrene (1 mg/L) and once with OECD water to remove any externally bound ^{14}C-Phenanthrene. The daphnids were then placed into 5 mL clean OECD water for 1, 2, 4, 8 or 24 h to depurate the contaminants. After the depuration time was complete, daphnids were removed from the OECD water, patted dry, weighed, and digested in 2N nitric acid for 24 h at 60°C. All samples had Ultima-Gold and counted for 10 minutes using the HITACHI AccuFLEX LSC-8000.

^{14}C-Fluoxetine Uptake Protocol

^{14}C-Fluoxetine uptake data was obtained using similar steps to those outlined in '^{14}C-Phenanthrene uptake protocol' with radioactive and non-radioactive fluoxetine hydrochloride substituted for the radioactive and non-radioactive phenanthrene solutions. The following treatments were prepared: OECD water only (control), 11.24 µg/L ^{14}C-Fluoxetine, 11.24 µg/L ^{14}C-Fluoxetine + 1 mg/L 20 nm NPs, and 11.24 µg/L ^{14}C-Fluoxetine + 1 mg/L 500 nm NPs. No other alterations to the previously described protocol were made.

^{14}C-Fluoxetine Depuration Protocol

^{14}C-Fluoxetine depuration data was obtained using the steps outlined in '^{14}C-Phenanthrene depuration protocol'. Radioactive and non-radioactive fluoxetine hydrochloride was substituted for the radioactive and non-radioactive phenanthrene solutions. ^{14}C-Fluoxetine was used at a concentration of 11.48 µg/L to achieve significantly enough counts per minute. The following treatments were prepared: OECD water only (control), 11.24 µg/L ^{14}C-Fluoxetine, 11.24 µg/L ^{14}C-Fluoxetine + 1 mg/L 20 nm NPs, and 11.24 µg/L ^{14}C-Fluoxetine + 1 mg/L 500 nm NPs. Daphnids were exposed to radioactive solutions for 24 h prior to the depuration period. No other alterations to the previously described protocol were made.

^{14}C-Glyphosate Uptake Protocol

^{14}C-Glyphosate uptake data was obtained using the steps outlined in '^{14}C-Phenanthrene uptake protocol'. Radioactive and non-radioactive glyphosate was substituted for the radioactive

and non-radioactive phenanthrene solutions. The following treatments were prepared: OECD water only, 6.76µg/L ^{14}C-Glyphosate, 6.76µg/L ^{14}C-Glyphosate + 1mg/L 20nm NPs, and 6.76µg/L ^{14}C-Glyphosate + 1mg/L 500nm NPs. No alterations to the previously described protocol were made.

Calculations

The following calculation was used to determine the percentage of ^{14}C-Phenanthrene taken up by the daphnids:

$$\%\ ^{14}\text{C-Phenanthrene taken up by daphnids} = \left(\frac{\text{Total cpm of daphnid tissues}}{\text{Total cpm of pre-uptake solution}} \right) \bullet 100$$

The following calculation was used to determine the percentage of ^{14}C-Phenanthrene depurated by the daphnids:

$$\%\ ^{14}\text{C-Phenanthrene depurated by daphnids} = \left(\frac{\text{Total cpm of post-depuration OECD water}}{\text{Total cpm of daphnid tissues}} \right) \bullet 100$$

The following calculation was used to determine the net transport rate of ^{14}C-Phenanthrene:

$$\text{Net Rate of Transport (ng g}^{-1}\text{ h}^{-1}) = \frac{\text{(Total cpm of daphnid tissues)}}{\text{[Specific Activity (cpm/ng) • Mass (g) • Time (h)]}}$$

The following calculation was used to determine the percentage of ^{14}C-Fluoxetine taken up by the daphnids:

$$\%\ ^{14}\text{C-Fluoxetine taken up by daphnids} = \left(\frac{\text{Total cpm of daphnid tissues}}{\text{Total cpm of pre-uptake solution}} \right) \bullet 100$$

The following calculation was used to determine the percentage of ^{14}C-Fluoxetine depurated by the daphnids:

$$\%\ ^{14}\text{C-Fluoxetine depurated by daphnids} = \left(\frac{\text{Total cpm of post-depuration OECD water}}{\text{Total cpm of daphnid tissues}} \right) \bullet 100$$

The following calculation was used to determine the net transport rate of ^{14}C-Fluoxetine:

$$\text{Net Rate of Transport (ng g}^{-1}\text{ h}^{-1}) = \frac{\text{(Total cpm of daphnid tissues)}}{\text{[Specific Activity (cpm/ng) • Mass (g) • Time (h)]}}$$

The following calculation was used to determine the percentage of ^{14}C-Glyphosate taken up by the daphnids:

$$\%\ ^{14}\text{C-Glyphosate taken up by daphnids} = \left(\frac{\text{Total cpm of daphnid tissues}}{\text{Total cpm of pre-uptake solution}} \right) \bullet 100$$

The following calculation was used to determine the rate of ^{14}C-Glyphosate Uptake:

$$\text{Net Rate of Transport (ng g}^{-1}\text{ h}^{-1}\text{)} = \frac{\text{(Total cpm of daphnid tissues)}}{\text{[Specific Activity (cpm/ng) • Mass (g) • Time (h)]}}$$

Fluorescence Microscopy

Microscopy was conducted to visualize the uptake of NPs in the aquatic invertebrate species, *Daphnia magna*. Daphnids (8-10 days old) were fasted for 24h and then exposed to 1mg/L fluorescent nanoplastic (20 or 500nm) solutions for 24 or 48h. A group of daphnids were also depurated for 24h after a 24h exposure to the NPs. The organisms were alive throughout the imaging process. Brightfield and fluorescence imaging was conducted using the ZEISS Axio Imager M2 at the Microscopy Facility in the Department of Biological Sciences at the University of Alberta.

Statistical Analysis

Statistical analysis and subsequent graphing were performed using the Prism GraphPad Software (Version 9.3.1 (350), GraphPad Software Inc.). Detection of outliers in the data set was

completed using Grubbs test ($\alpha = 0.05$). The D'Agostino-Pearson test was used to test for normality. Two-way analysis of variance followed by Tukey's multiple comparisons test was used to compare the co-contaminant net transport rate between various sized NPs and aquatic contaminants ($\alpha = 0.05$). All data is presented as the mean ± standard error of the mean.

Results

^{14}C-Phenanthrene Uptake and Depuration

The net transport rate of ^{14}C-Phenanthrene was similar across all timepoints (0.5h-24h) with the exception of the 1h timepoint (Figure 2.3). After 1h of exposure to ^{14}C-Phenanthrene the treatment group with 500nm NPs displayed a significantly slower net transport rate (25 ng mg^{-1} h^{-1}) when compared to the control group (^{14}C-Phenanthrene without NP, 75 ng mg^{-1} h^{-1}). Overall, the net transport rate of ^{14}C-Phenanthrene was high initially and slowed over time to eventually reach levels nearing 0 ng mg^{-1} h^{-1} for all treatment groups. No significant differences were detected between treatment groups for all timepoints with the exception of the 1 h time point.

The percentage of ^{14}C-Phenanthrene detected in *Daphnia magna* without NPs, increased over the 24h exposure period, reaching a plateau of 4% accumulation of the total available ^{14}C-Phenanthrene at the 4h point (Figure 2.1). When ^{14}C-Phenanthrene was present in conjunction with 1 mg/L 20nm NPs, the percentage of ^{14}C-Phenanthrene taken up by the daphnids increased in a similar pattern to the control, plateauing at 4% accumulation at the 4h point (Figure 2.1). However, when ^{14}C-Phenanthrene was present with 1 mg/L 500nm NPs the percentage of ^{14}C-Phenanthrene accumulated appeared to reach equilibrium at the 0.5h mark at 1% ^{14}C-Phenanthrene accumulated. The treatment group with 1 mg/L 500nm NPs and ^{14}C-Phenanthrene

demonstrated a significantly lower percentage of ^{14}C-Phenanthrene taken into the daphnids from 2h to 8h when compared with the ^{14}C-Phenanthrene only and the ^{14}C-Phenanthrene with 20nm NP groups.

The percentage of ^{14}C-Phenanthrene depurated from *Daphnia magna* did not vary between treatment groups (^{14}C-Phenanthrene, ^{14}C-Phenanthrene + 20nm NPs, or ^{14}C-Phenanthrene + 500nm NPs) at any time point (1h, 2h, 4h, 8h or 24h) (Figure 2.2). All groups reached a similar depuration at 1 h with 93% of total accumulated ^{14}C-Phenanthrene remaining. All groups displayed slightly decreased percentages of ^{14}C-Phenanthrene remaining across time, reaching a plateau with 85% of accumulated ^{14}C-Phenanthrene remaining at approximately 8h spent in clean OECD water.

^{14}C-Fluoxetine Uptake and Depuration

The net transport rate of ^{14}C-Fluoxetine was high amongst all groups initially (200-450 ng mg^{-1} h^{-1}) and decreased across time to reach levels nearing 0 ng mg^{-1} h^{-1} (Figure 2.6). ^{14}C-Fluoxetine present in conjunction with 1 mg/L 20nm NPs showed significantly higher (450 ng mg^{-1} h^{-1}) net transport rates compared to the ^{14}C-Fluoxetine control (200 ng mg^{-1} h^{-1}) and ^{14}C-Fluoxetine + 1 mg/L 500nm NP (320 ng mg^{-1} h^{-1}) groups at 0.5 h. At 0.5 h, 1 h, and 2 h of exposure the ^{14}C-Fluoxetine + 1mg/L 20 nm NPs treatment group also showed a significantly higher net rate of transport when compared to the ^{14}C-Fluoxetine only and ^{14}C-Fluoxetine + 1mg/L 500 nm NP groups. At 4h of exposure and thereafter, the control and nanoplastic groups displayed similar net transport rates that decreased over time.

The percentage of ^{14}C-Fluoxetine detected in *Daphnia magna* increased over time, with the highest recorded levels nearing 10% ^{14}C-Fluoxetine accumulation (Figure 2.4). A significant

difference between groups was detected after 2h of exposure, whereby the ^{14}C-Fluoxetine + 1 mg/L 500 nm NPs demonstrated a significantly higher percentage (%10) of ^{14}C-Fluoxetine taken up when compared to the ^{14}C-Fluoxetine only group (6%). All other timepoints (0.5 h, 1 h, 2 h, 8 h, 12 h, and 24 h) displayed similar levels of ^{14}C-Fluoxetine accumulation between groups.

The percentage of ^{14}C-Fluoxetine depurated from *Daphnia magna* did not vary between treatment groups (^{14}C-Fluoxetine, ^{14}C-Fluoxetine + 1mg/L 20 nm NPs, and ^{14}C-Fluoxetine + 1 mg/L 500 nm NPs) (Figure 2.5). All treatment groups displayed a decreasing percentage of ^{14}C-Fluoxetine remaining up to the 8h timepoint, where a plateau was reached at approximately 80% ^{14}C-Fluoxetine remaining.

^{14}C-Glyphosate Uptake and Depuration

The net transport rate of ^{14}C-Glyphosate was 18-22 ng mg^{-1} h^{-1} amongst all groups initially and decreased across time to reach levels nearing 0 ng mg^{-1} h^{-1} (Figure 2.8). No significant differences were found between groups at any time point.

The percentage of ^{14}C-Glyphosate accumulated was low (between 0-1% at any given time point) (Figure 2.7). No significant differences were seen between the groups at 0.5, 1, 2, 8, 12 or 24 h. After 4 h of exposure, the ^{14}C-Glyphosate + 1 mg/L 500 nm NPs treatment group showed significantly higher accumulation of ^{14}C-Glyphosate when compared to the control group or the ^{14}C-Glyphosate + 1 mg/L 20 nm NP group.

Depuration experiments were not completed due to the low uptake rate and low accumulation percentage of ^{14}C-Glyphosate.

Microscopic Imaging of Fluorescent NPs

The microscopic imaging completed for this thesis was analyzed as qualitative data. Figure 2.9 displays the accumulation and depuration of 20 nm NPs at identical concentrations to the radioactive uptake and depuration experiments. Fluorescence was visualized at 24 h, 48 h and after 24 h of depuration for the 20nm NP group. The fluorescence was much more vibrant for uptake and depuration with the 500nm NPs (Figure 2.10). For both the 20 nm and 500 nm groups, the NPs are seen along the intestinal tract (the gut) of the daphnids. However, in frames C and D in Image 1.2 some of the 500 nm NPs appear to have migrated into the body of the daphnid.

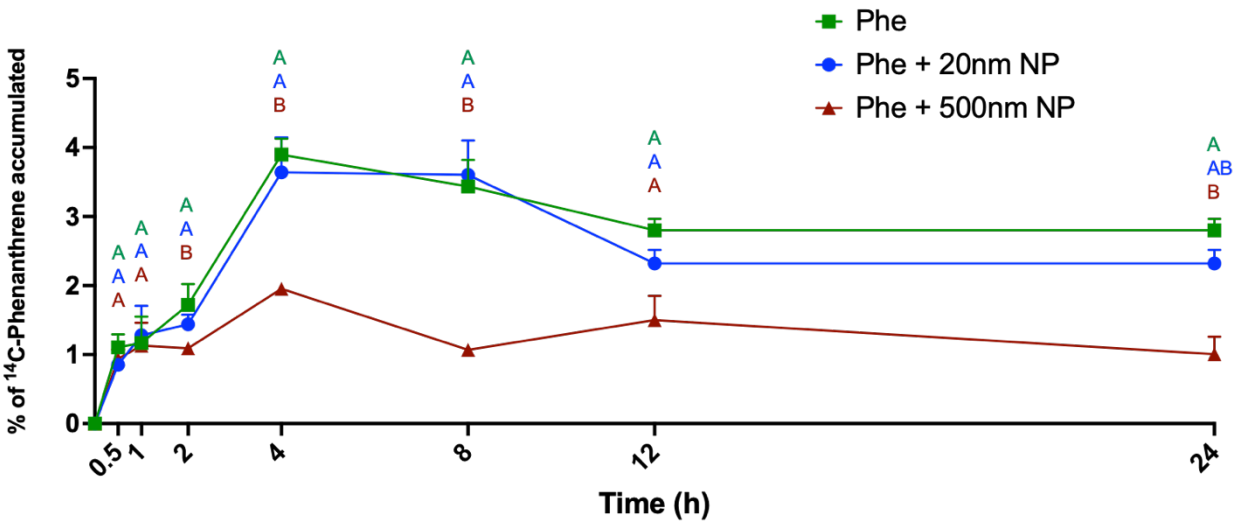

Figure 2.1: Percentage of ^{14}C-Phenanthrene detected in *Daphnia magna* after exposure for various time periods to the chemical control (6.48µg/L ^{14}C-Phenanthrene), the 20nm NP (1g/L 20nm NP + 6.48µg/L ^{14}C-Phenanthrene) and the 500nm NP (1g/L 500nm NP + 6.48µg/L ^{14}C-Phenanthrene) solutions.

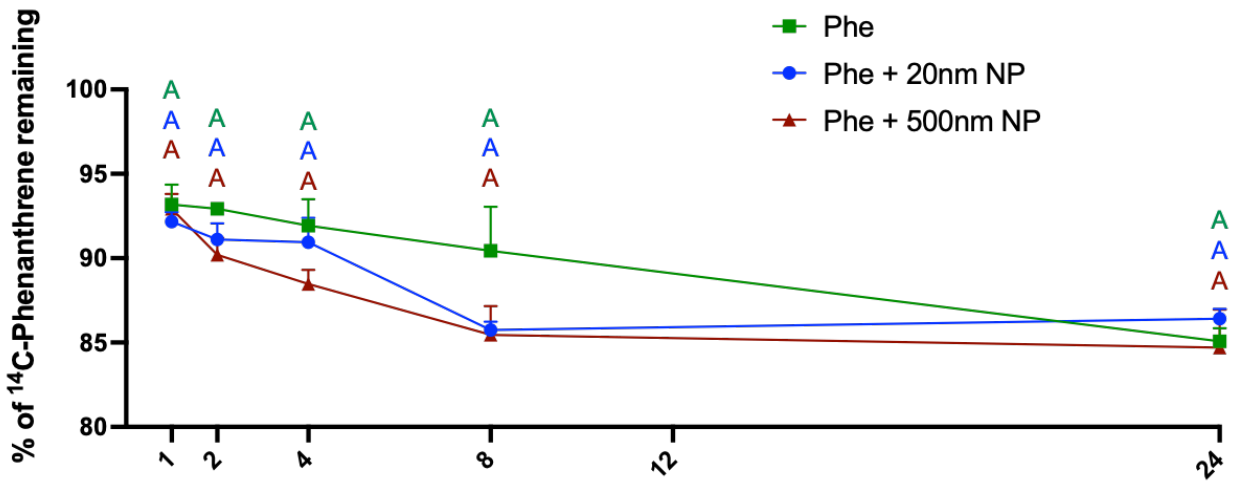

Figure 2.2: Percentage of ^{14}C-Phenanthrene remaining in *Daphnia magna* after accumulation exposure for 24h to the chemical control (6.48µg/L ^{14}C-Phenanthrene), the 20nm NP (1g/L 20nm NP + 6.48µg/L ^{14}C-Phenanthrene) and the 500nm NP (1g/L 500nm NP + 6.48µg/L ^{14}C-Phenanthrene) solutions. Measurements of ^{14}C-Phenanthrene remaining were taken after *Daphnia magna* had been removed from exposure solutions into clean OECD water for various time periods.

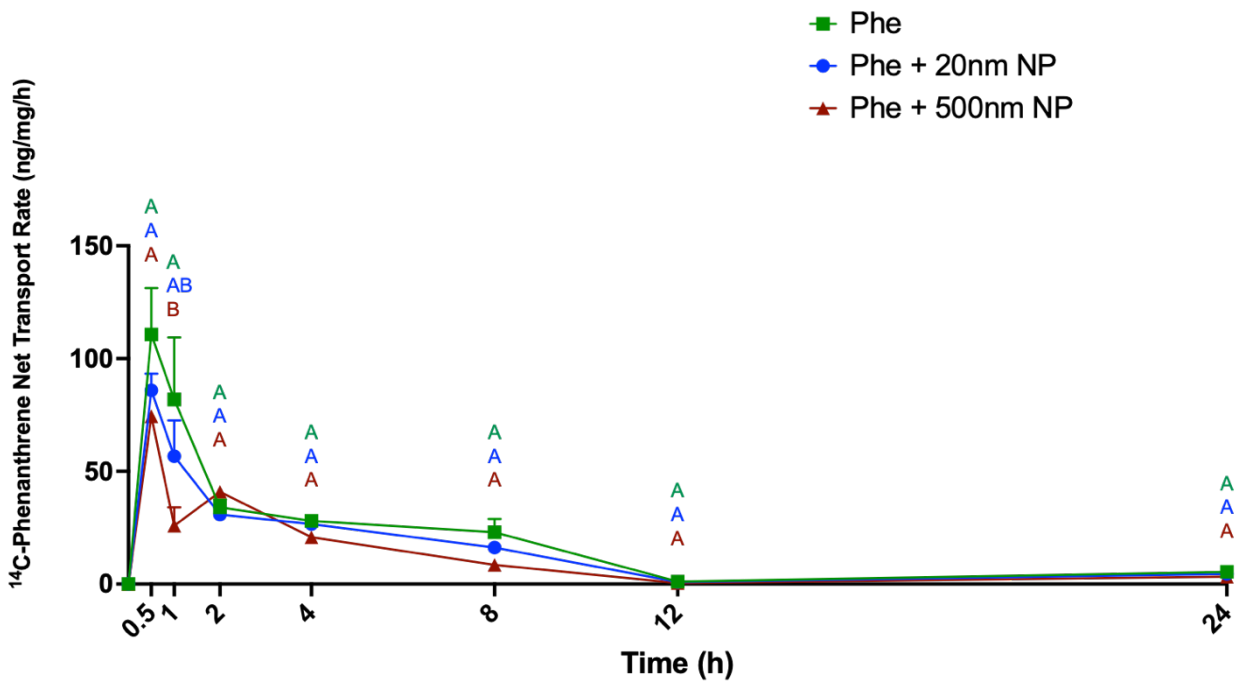

Figure 2.3: Net transport rate of ^{14}C-Phenanthrene in *Daphnia magna* after exposure for various time periods to the chemical control (6.48µg/L ^{14}C-Phenanthrene), the 20nm NP (1g/L 20nm NP + 6.48µg/L ^{14}C-Phenanthrene) and the 500nm NP (1g/L 500nm NP + 6.48µg/L ^{14}C-Phenanthrene) solutions.

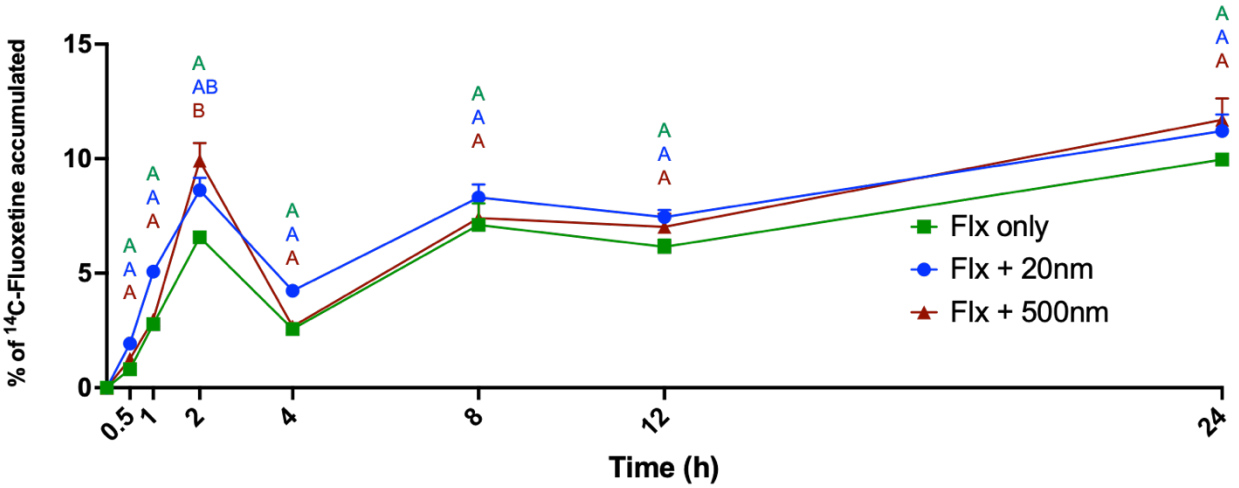

Figure 2.4: Percentage of ^{14}C-Fluoxetine detected in *Daphnia magna* after exposure for various time periods to the chemical control (11.24µg/L ^{14}C-Fluoxetine), the 20nm NP (1g/L 20nm NP + 11.24µg/L ^{14}C-Fluoxetine) and the 500nm NP (1g/L 500nm NP + 11.24µg/L ^{14}C-Fluoxetine) solutions.

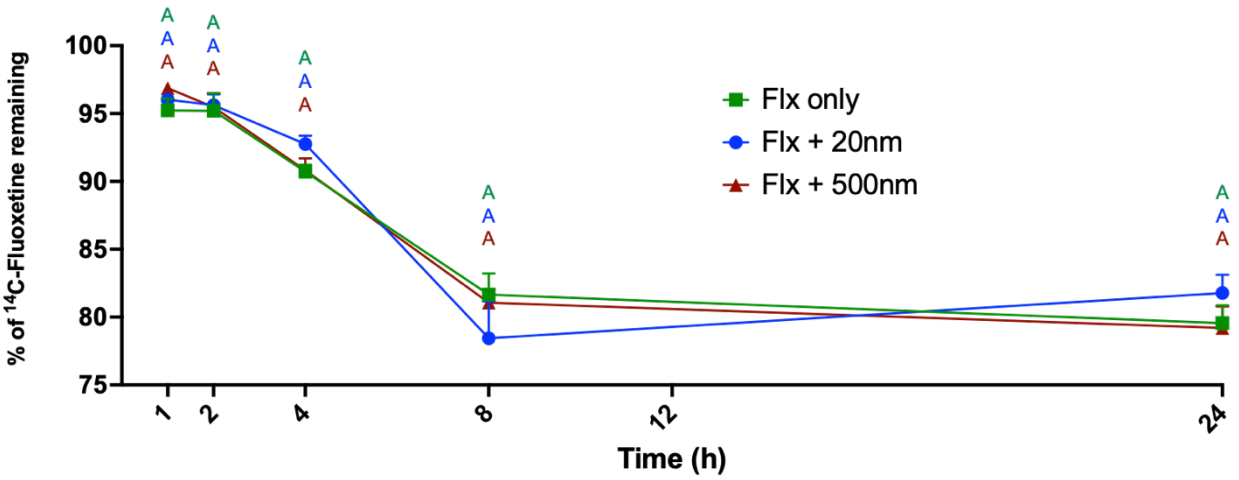

Figure 2.5: Percentage of ^{14}C-Fluoxetine remaining in *Daphnia magna* after accumulation exposure for 24h to the chemical control (11.24µg/L ^{14}C-Fluoxetine), the 20nm NP (1g/L 20nm NP + 11.24µg/L ^{14}C-Fluoxetine) and the 500nm NP (1g/L 500nm NP + 11.24µg/L ^{14}C-Fluoxetine) solutions. Measurements of ^{14}C-Fluoxetine remaining were taken after *Daphnia magna* had been removed from exposure solutions into clean OECD water for various time periods.

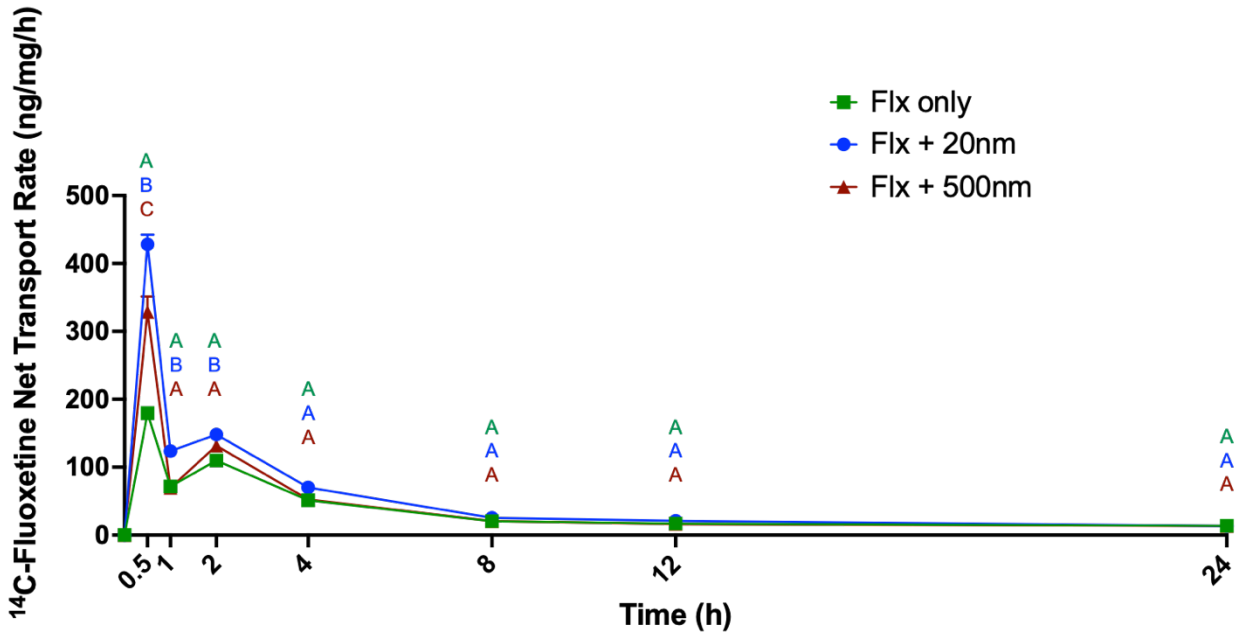

Figure 2.6: Net transport rate of ^{14}C-Fluoxetine in *Daphnia magna* after exposure for various time periods to the chemical control (11.24µg/L ^{14}C-Fluoxetine), the 20nm NP (1g/L 20nm NP + 11.24µg/L ^{14}C-Fluoxetine) and the 500nm NP (1g/L 500nm NP + 11.24µg/L ^{14}C-Fluoxetine) solutions.

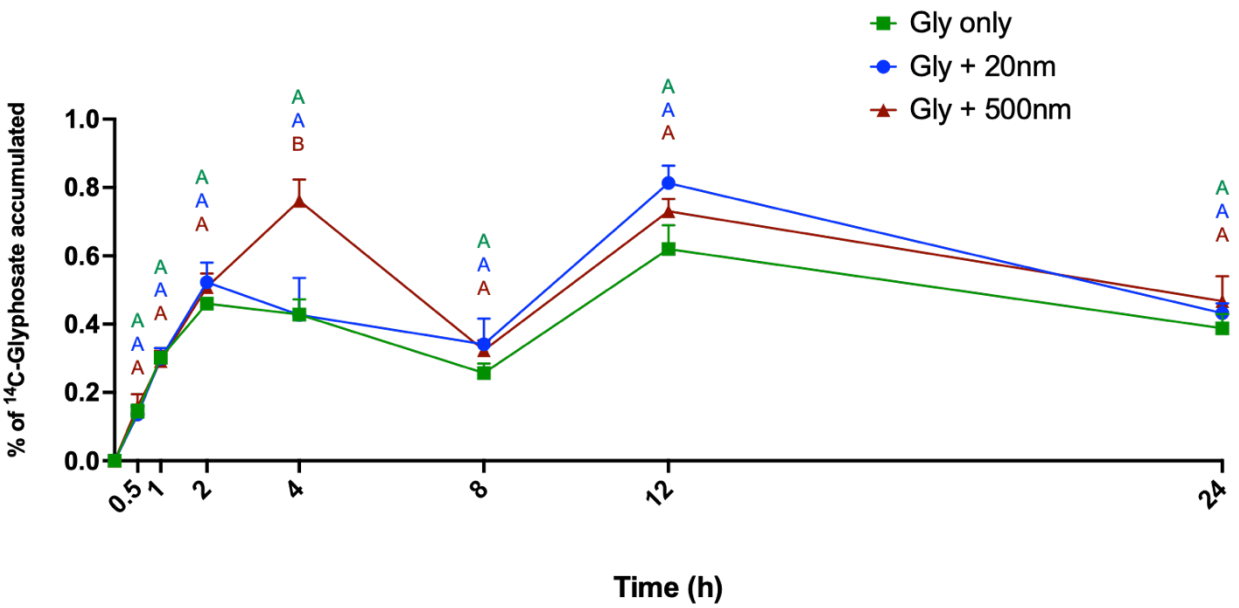

Figure 2.7: Percentage of ^{14}C-Glyphosate detected in *Daphnia magna* after exposure for various time periods to the chemical control (6.76µg/L ^{14}C-Glyphosate), the 20nm NP (1g/L 20nm NP + 6.76µg/L ^{14}C-Glyphosate) and the 500nm NP (1g/L 500nm NP + 6.76µg/L ^{14}C-Glyphosate) solutions.

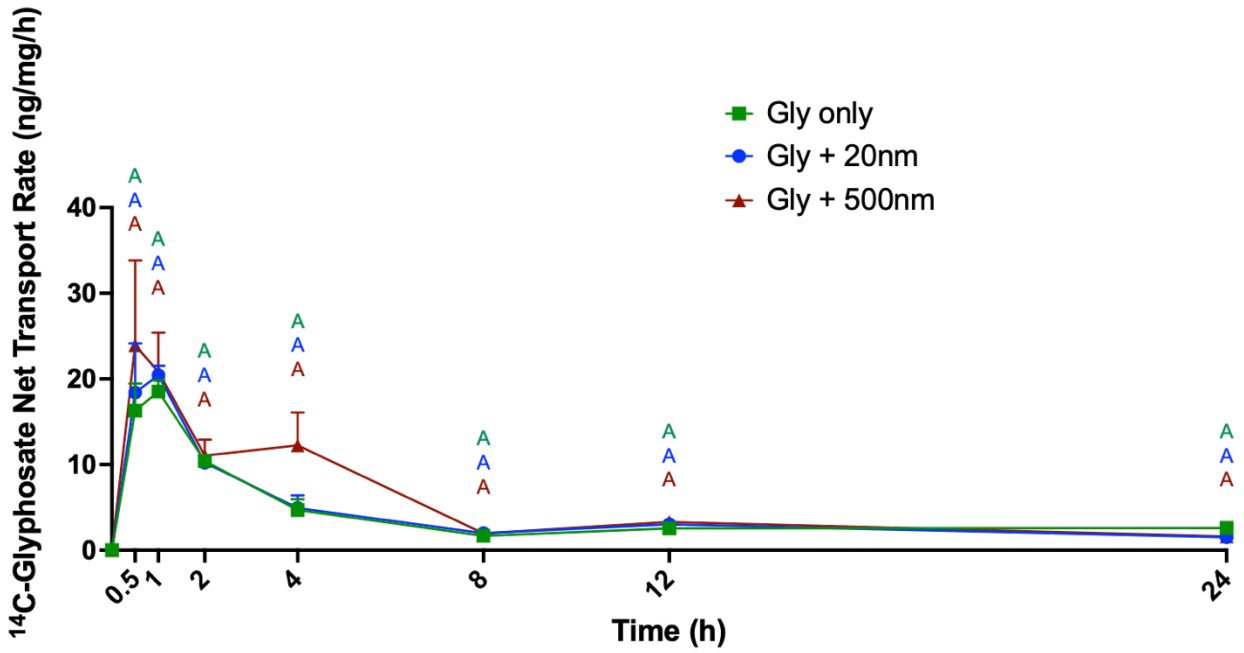

Figure 2.8: Net transport rate of ^{14}C -Glyphosate in *Daphnia magna* after exposure for various time periods to the chemical control (6.76µg/L ^{14}C-Glyphosate), the 20nm NP (1g/L 20nm NP + 6.76µg/L ^{14}C -Glyphosate) and the 500nm NP (1g/L 500nm NP + 6.76µg/L ^{14}C -Glyphosate) solutions.

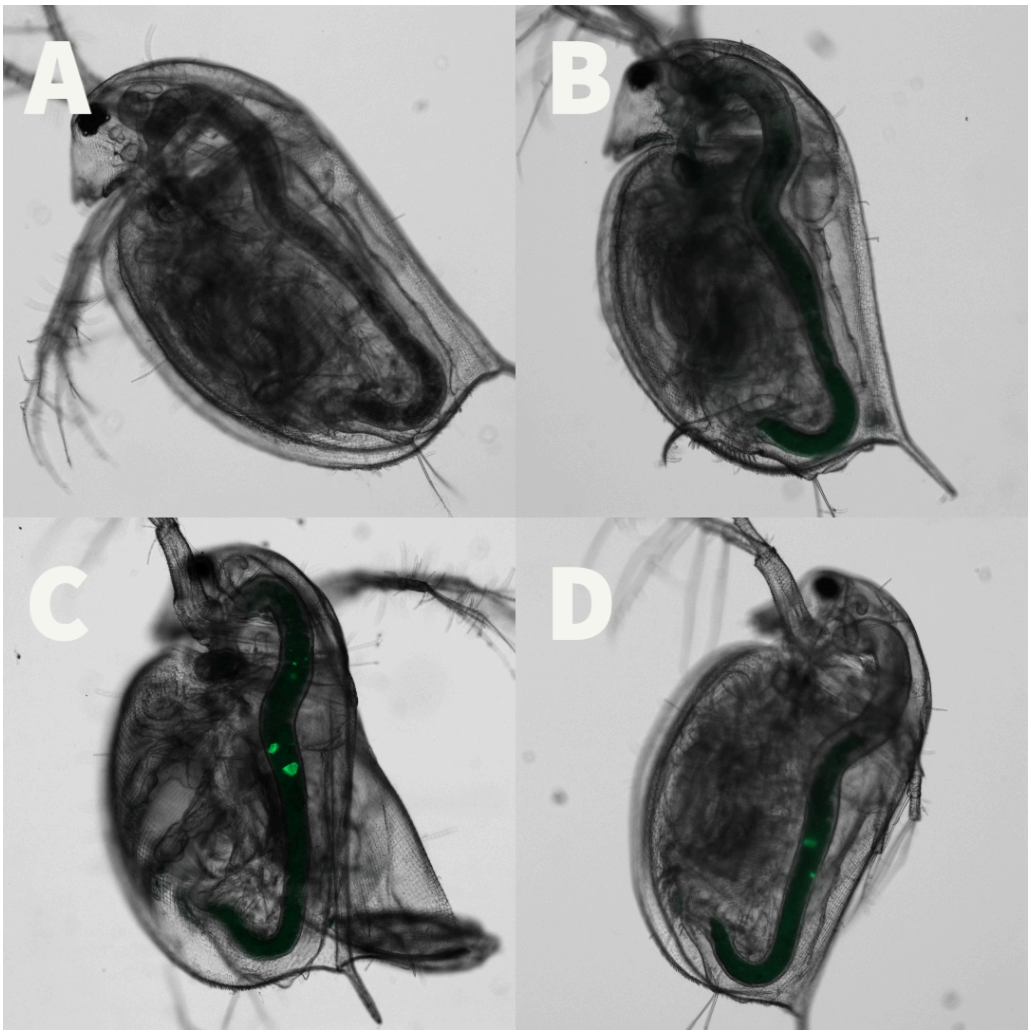

Figure 2.9: Light Microscopic images of living adult (10-15 days old) *Daphnia magna* after exposure to the following conditions. (A) 24h control exposure to OECD water (B) 24h exposure to 1g/L 20nm fluorescent NPs (C) 48h exposure to 1g/L 20nm fluorescent NPs (D) 24h depuration in OECD water after 24h exposure to 1g/L 20nm NPs

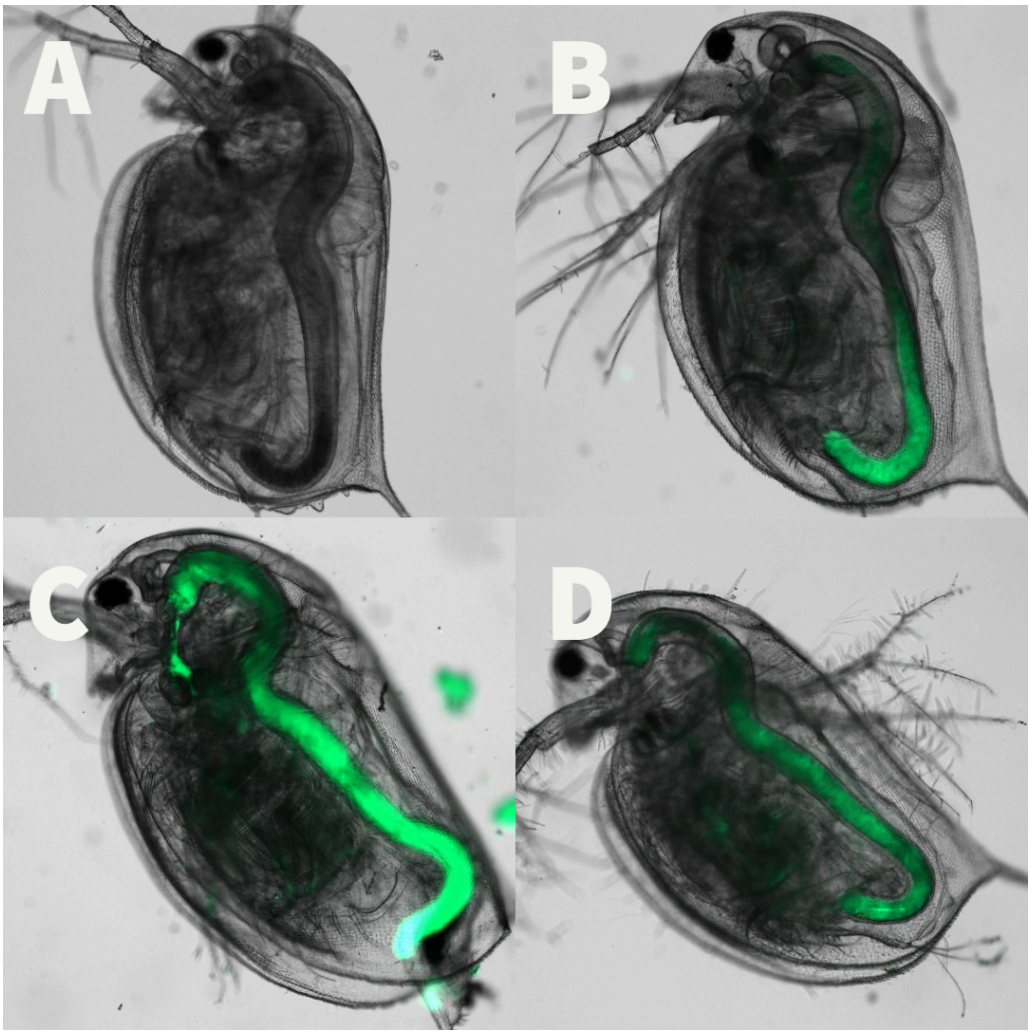

Figure 2.10: Fluorescent Microscopy images of living adult (10-15 days old) *Daphnia magna* after exposure to the following conditions. (A) 24h control exposure to OECD water (B) 24h exposure to 1g/L 500nm fluorescent NPs (C) 48h exposure to 1g/L 500nm fluorescent NPs (D) 24h depuration in OECD water after 24h exposure to 1g/L 500nm NPs

Discussion

This study demonstrated that NPs could act to either increase or decrease the uptake and depuration rates of various aquatic contaminants. Whether the presence of plastics increases or decreases organic contaminant uptake varies based on the characteristics of the contaminant in question. Three contaminants (phenanthrene, fluoxetine, and glyphosate), representing three common classes of aquatic contaminants were investigated. This study demonstrated that when NPs are in the water, they are ingested and can be found in the gut of *Daphnia magna* (Figure 2.9 & Figure 2.10). Furthermore, the particles remain in the gut throughout the 24h period studied, even after the organic contaminants have been depurated. The ingestion of nanoplastic particles by daphnids is due to the filter feeding mechanism by which daphnids can ingesting particles between 0.4-0.7 μm (Gophen & Geller, 1984). These results support previous findings demonstrating that filter feeders such as *Daphnia magna* are able to ingest nanoplastic particles (Rist & Baun, 2017).

NPs visualized in the gut tract of *Daphnia magna*

Microscopy demonstrated that both 20 and 500 nanometer plastics continue to increase in the intestine of *Daphnia* beyond the initial 24-hour period of the experiment, with increases still noted at 48h of exposure (Figure 2.9 & Figure 2.10). These findings suggest that daphnids have either not significantly decreased the uptake of nanoplastic particles or increased the rate of excretion of particles enough to reduce the amount in the gut (i.e., when excretion exceeds ingestion). These results are similar to the results of Rist et al., who examined the ingestion of 2 μm and 100 nm fluorescent plastic particles and found both sizes were readily ingested by

Daphnia magna (2017). Fluorescent microscopy also demonstrated that the NPs primarily reside in the gut of the *Daphnia magna*. These results support the hypothesis in figure 1.3 whereby the NPs enter the gut and subsequently offload contaminants which then pass through the gut tissue and into the animal. The fluorescent microscopy images do not display a large amount of NPs moving from the gut into the body of the organism (figure 2.9 & figure 2.10). However, in figure 2.10c and figure 2.10d, a small number of plastics can indeed be seen outside the gut, having translocated across the gut epithelia to inside the body of the *Daphnia magna* suggesting that a few particles can pass directly through the gut membrane. A study by Brun et al., found that adult *Daphnia magna* ingested nanoplastic particles (as they were visualized in the gut, but were not found in the lipids of the adults (2017). *Daphnia* embryos were also examined and found to sorb NPs to their surface, suggesting that the accumulation does not occur through the maternal body, but through adherence to the embryo itself (Brun et al., 2017). Our results show fluorescence intensity did decrease after 24h (figure 2.9 & figure 2.10) indicating that some of the NPs are indeed excreted, similar to the noted depuration of the sorbed radioactive labelled contaminants in the uptake and depuration experiments. These results are also supported by the findings of Rist et al., who found that egestion occurred over 24h after exposure to 2 μm and 200 nm plastic particles (2017).

A potential confounding factor in the visualization of plastic particles could be the leaching of fluorescein molecules from the plastics (Christoph et al., 2019; Schür et al., 2019). Fluorescein is known to have pK_a values of 2.22, 4.34 and 6.68, while *Daphnia magna* gut has a pH of 5.5-7.2 dependent on the region (Smith & Pretorius, 2002; Ebert, 2005; Davis et al., 2020). Therefore, it is unlikely that leaching of the fluorescein dye is an artifact in the microscopy images. Additionally, if a small amount of leaching does occur at a low gut pH this does not

affect the results as microscopy was used only for qualitative analysis (presence or absence of particles) and not to quantify the amount of particles in the gut.

^{14}C-Phenanthrene uptake reduced when sorbed to 500 nm NPs

Interestingly, ^{14}C-Phenanthrene uptake is significantly lower when compared to the control group in the presence of 500 nanometer plastic particles (Figure 2.1). At each of the two-, four-, and eight-hour time points, the uptake was significantly lower for ^{14}C-Phenanthrene when sorbed to the larger NPs (Figure 2.1). The net transport rate of ^{14}C-Phenanthrene was also significantly lower when sorbed to 500 nanometer plastics at the one-hour time point (Figure 2.3). These results are similar to the findings of Zhang et al., which displayed increased uptake of ^{14}C-Phenanthrene into zebrafish embryos when sorbed to 20 nm plastics compared to 500 nm plastic particles (2020). Additionally, the difference in surface area (SA) between the 20 nm and 500 nm NPs is hypothesized to play a significant role in the uptake of organic contaminants. With the theoretical surface area to volume ratio much larger for the 20 nm plastics compared to the 500 nm NPs, this would allow for more sorbed ^{14}C-Phenanthrene per plastic mg of plastic. However, Zhang et al., conducted sorption experiments and determined that when correcting for surface area the sorption of ^{14}C-Phenanthrene was similar between the 20 nm and 500 nm groups (2020). Additionally, the distribution coefficient (K_d) decreased as particle size decreased, this is caused by a reduced surface area that can be accessed by the phenanthrene molecules (Zhang et al., 2020). Therefore, it is possible that the 20 nm particles readily offload the ^{14}C-Phenanthrene, this explains the similarity between the 20 nm and ^{14}C-Phenanthrene only groups in figure 2.1. As the experiments were completed using a standard of 1mg/L of nanoplastic particles, the 20 nm groups would have a substantially higher number of particles per litre (L) when compared to

the 500nm groups since the 500nm plastic particles have a higher unit weight per particle. Given that the 500nm groups had fewer total nanoplastic particles, the likelihood of the *Daphnia magna* ingesting the particles would be lower, thus potentially lowering the overall uptake percentage of the sorbed ^{14}C-Phenanthrene. Ma et al., examined differences in ^{14}C-Phenanthrene uptake when sorbed to varying (50 nm to 10 um) sized plastic particles, and noted that the small (50 nm) particles displayed significantly higher bioaccumulation of ^{14}C-Phenanthrene when compared to the large (10 um) particles (2016). Overall, these findings display a decreased uptake in ^{14}C-Phenanthrene when sorbed to 500 nm nanoplastic particles.

^{14}C-Fluoxetine uptake is increased when sorbed to 20 nm NPs

The fluoxetine net transport rate was significantly higher when sorbed to the 20 nm plastics at 0.5, 1 and 2-hour time points compared to the 500 nm and control groups (Figure 2.4). Although co-contaminant uptake experiments with fluoxetine and NPs have not been completed previously, the results can be explained via extension of ^{14}C-Phenanthrene results. Phenanthrene (4.57) and fluoxetine (4.26) both have hydrophobic (positive) K_{ow} values, indicating they will sorb to the nanoplastic (Djomo et al., 1996; Wan et al., 2007). Wagstaff et al., reported that fluoxetine demonstrated significant adsorption to microplastics like the ^{14}C-Phenanthrene sorption demonstrated by Zhang et al., (2022; 2020). The rapid rate of uptake in the presence of 20 nm plastics suggests that the 20nm particles are easily ingested by the organism due to their small size (which likely goes undetected) and the higher number of particles in solution. Rist et al., noted that *Daphnia magna* exposed to 100 nm NPs had more than 400 times as many particles in their body compared to the 2 um particles (2017). Gophen and Geller suggested that

particles at the nanoscale may interact with *Daphnia magna* filter feeding structures or be ingested via passive mechanisms (1984). Similar to the ^{14}C-Phenanthrene results, it is likely that the 20 nm NPs have more isotope sorbed/mg plastic and therefore this will act to increase the rate of uptake. The rapid drop in percentage (5%, Figure 2.4) of ^{14}C-Phenanthrene in the *Daphnia magna* at the 4h timepoint can be explained by Figure 2.5 which demonstrates that ~8% of ^{14}C-Phenanthrene is depurated by the 4h timepoint.

^{14}C-Glyphosate sorption kinetics impair experiment quality

When examining ^{14}C-Glyphosate uptake, the 500 nm treatment group was significantly higher after four hours when compared to the 20 nm and control groups (Figure 2.7). Glyphosate is relatively hydrophilic chemical with a K_{ow} of -3.20 (Contardo-Jara et al., 2009), suggesting it may not associate with the hydrophobic surface of the nanoplastic as firmly as other more hydrophobic contaminants. Previous literature indicates that exposure to glyphosate and polystyrene NPs has an additive effect on toxicity to *Daphnia* magna, however this literature does not supply sufficient information to interpret these results as being due to the Trojan Horse Effect (Nogueira et al., 2022). In addition, synergistic effects on the toxicity to aquatic plants was demonstrated after exposure to high concentrations (15 mg/L glyphosate and 25 mg/L microplastics) of the contaminants, these results indicate toxicity but again do not indicate the Trojan Horse Effect is occurring (Yu et al., 2021). The additive effect of polystyrene NPs and glyphosate may be due to the particle and contaminant being taken up separately, and not due to a co-contaminant uptake model. Although there is a significant increase in the uptake percentage of ^{14}C-Glyphosate in the presence of 500 nm NPs at 4h of exposure, this data is not supported by the trend in figure 2.7. ^{14}C-Glyphosate uptake was relatively low (highest uptake = 0.8%, Figure

2.7) when compared to ^{14}C-Phenanthrene (highest uptake = 4%, Figure 2.1) and fluoxetine (highest uptake = 12%, Figure 2.4). As a result, ^{14}C-Glyphosate depuration experiments were not completed due to the low uptake of ^{14}C-Glyphosate in the original experiments (highest uptake = 0.8%, Figure 2.7).

Depuration is not affected by the presence of NPs

Differences in depuration rate were not detected in either the ^{14}C-Fluoxetine or ^{14}C-Phenanthrene treated groups (Figure 2.2 & 2.5). Approximately 85% of the total ^{14}C-Phenanthrene taken up into the Daphnia was still remaining in the daphnids after 24 hours in both the plastic and control groups (Figure 2.2). Similarly, ^{14}C-Fluoxetine remained at approximately 80% of the total taken up after 24 hours of depuration in all groups (Figure 2.5). Rist et al. found similar results after a 24h egestion period where no significant egestion of 100 nm or 2 um plastic particles occurred (2017). The lack of significant egestion/depuration may be due to the fasted nature of the depuration experiments, if food were present, it may require the egestion of feces and subsequently the egestion of nanoplastic particles (Ebert, 2005). ^{14}C-Glyphosate depuration experiments were not completed due to the small amount of uptake after the 24h exposure period (Figure 2.7). The ^{14}C-Phenanthrene and ^{14}C-Fluoxetine findings were supported via fluorescence microscopy which was used to visualize the nanoplastic particles after the depuration period. The particles were found to be in the gut tract of the invertebrates, and many particles remained even after the 24h depuration period. The lack of significant differences between treatment and control groups for either of the two aquatic contaminants

(^{14}C-Phenanthrene or ^{14}C-Fluoxetine) in the radioactive flux experiments suggests that NPs do not alter the way in which the co-contaminant is handled and excreted by the organism (Figure 2.2 & 2.5). In contrast, a study by Ma et al., displayed that the half-life of phenanthrene is significantly increased when sorbed to NPs due to decreased metabolism/breakdown of phenanthrene into metabolites (Ma et al, 2016). These authors argued that phenanthrene molecules were not being broken down due to their sorption to the NPs inside the animals. This suggests that the NPs are being excreted without degradation of the phenanthrene pollutant, which may enhance the environmental accumulation of phenanthrene if the molecule degradation/depuration mechanisms are inhibited. The presence of phenanthrene has been shown to increase EROD activity at low doses in *Daphnia magna,* which is a response needed to further stimulate excretion of the substance (Frutos et al., 2010). The presence of fluoxetine initiates a phase I/II biotransformation pathway due to its hydrophobic nature, inducing CYP enzymes and producing a product with a longer elimination half-life (Neuwoehner et al., 2009). ^{14}C-Glyphosate was not examined for depuration due to the small amount initially taken up by the organism, and the low sorption of glyphosate to the NPs.

Summary of findings

This study is the first of its kind to analyze multiple contaminants and compare the uptake and depuration when associated with NPs (the Trojan Horse Effect). The findings suggest that uptake and depuration of co-contaminants is entirely dependent on the characteristics of the chemical, and the size of the nanoplastic it is sorbed to. The findings indicate that NPs are taken up over a period of one to two days, and that only approximately 20% of the contaminants (either sorbed to NPs or not) are depurated over a 24 h period without food (Figure 2.2 & 2.5).

Comparison of the uptake between contaminants (^{14}C-Phenanthrene, ^{14}C-Fluoxetine, and ^{14}C-Glyphosate) suggests that uptake is enhanced when a contaminant is hydrophobic. Small plastic fragments have potential to increase the uptake of aquatic contaminants, therefore it is essential to study and understand the probability and effects of the increased uptake.

CHAPTER THREE

Summary

To summarize, this study demonstrates the capacity by which NPs can facilitate the uptake of aquatic contaminants into the invertebrate species *Daphnia magna*. The uptake of the NPs and sorbed co-contaminants is enhanced with smaller (20 nm) plastic particles. Contaminants that are slightly hydrophobic, such as fluoxetine show enhanced co-contaminant uptake when sorbed to NPs compared to very hydrophobic (i.e., Phenanthrene) or hydrophilic (i.e., glyphosate) substances. Depuration (the percentage of contaminant remaining) is not altered by the presence of NPs or the characteristics of the sorbed co-contaminant. NPs were found to reside in the gut tract of *Daphnia magna* with a few particles moving from the gut tract to the body of the organism. In addition, nanoplastic depuration was visualized and the presence of particles in the gut tract of the organism decreased after a 24 h depuration period.

This experiment is the first of its kind to integrate radiotracer uptake methods with a comparison of aquatic contaminant uptake/depuration when sorbed to NPs. The experimental design allows for environmentally relevant levels of aquatic contaminants to be used. The field of NPs and co-contaminant uptake has many unanswered questions, and there is much research to be done. This study contributes to the rapidly growing field and provides insight into the impact of contaminant characteristics on the uptake and depuration rates when sorbed to NPs of invertebrates

Future research directions

To expand upon the findings of this thesis, multiple sizes of NPs and types of NPs (polystyrene, polycarbonate, PDBE etc.) should also be investigated. Similarly, the effects of weathering on sorption of organics to NPs should be investigated to mimic the nano and microplastics present in the environment. Previous experiments have examined the uptake and depuration of smooth versus rough NPs, this should be examined in terms of co-contaminant uptake as well (Frydkjær et al., 2017). Rough NPs are more environmentally relevant but also pose significant challenges in research such as the determination of surface area and potentially significant inconsistencies in surface characteristics. Smooth NPs are useful in scientific research because they have a known surface area, which reduces variability within groups of plastics and allows for comparison between groups of plastics (Zhang et al., 2020). A comparison of 20 nm smooth NPs with sorbed ^{14}C-Phenanthrene and 20 nm rough NPs with sorbed ^{14}C-Phenanthrene would provide additional environmental relevance to this body of literature.

If the experimental methods in this thesis were to be repeated, increases of exposure volumes should be completed to ensure *Daphnia magna* do not exhibit overcrowding effects (Lowes et al., 2021). These overcrowding effects may have affected the uptake and depuration previously described.

Future studies should also involve investigation of the biochemical responses to both plastics and organics to determine the effects on the organism itself. For example, extension of this study should include examination of phase one and phase two biotransformation enzymes, or EROD responses, to the net body burden of chemicals sorbed to the NPs. Study of these biochemical responses will also aid in determining whether NPs are excreted using a mechanism similar to the excretion of their sorbed co-contaminant. Phenanthrene has been shown to have a

larger half-life when sorbed to NPs, thus reducing its toxicity but increasing its environmental presence (Ma et al., 2017). Analysis of the biochemical response to NPs will aid in determining their toxicity and the toxicity of sorbed co-contaminants.

It is difficult to find background information regarding the sorption of fluoxetine, or the lack of sorption of glyphosate to NPs. Sorption experiments using common aquatic contaminants and NPs would benefit this area of research. If the contaminant is known to sorb to NPs, the uptake of that nanoplastic and sorbed co-contaminant is likely as multiple studies have displayed the uptake of NPs and co-contaminants (Zhang et al., 2020; Trevisan et al., 2019). Sorption kinetics are also imperative to understanding what may occur once the nanoplastic and sorbed co-contaminant are ingested, whether the contaminant is likely to offload from the nanoplastic or not. To expand upon this point, a comparison of sorption kinetics between similar sized rough and smooth nanoplastic particles would increase the environmental relevance of the sorption experiments.

As suggested by Rist et al., the lack of significant depuration may be due to the absence of food in the experiment (2017). The experiment within this thesis was completed in the absence of food to eliminate extraneous variables, however now that the uptake and depuration has been recorded without food it would be beneficial to examine the effects when food is present. The presence of food in the uptake experiments may alter the daphnid feeding pattern to mimic the natural environment where prey is present (Gophen & Geller, 1984). This may enhance or reduce the uptake of nanoplastic particles and subsequently the sorbed co-contaminants. In addition, depuration may be altered as food entering the gut may increase the feces produced by the daphnid, excreting nanoplastic particles and the sorbed co-contaminants (Ebert, 2005).

All in all, expansion in this field of research is imperative to understanding the impacts of man-made waste on organism and ecosystem health. The production of plastics will be continued for many years to come, and therefore many of the macroplastics will end up in natural environments. It is important that research in this field maintains environmental relevance to ensure regulations can be built upon research to protect the health of natural systems.

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