Increasing the environmental relevance and realism of microplastic exposures changes the degree of toxicity to *Daphnia magna*

By

Katie Marie Reilly

A thesis submitted to the University of Birmingham for the degree of

DOCTOR OF PHILOSOPHY

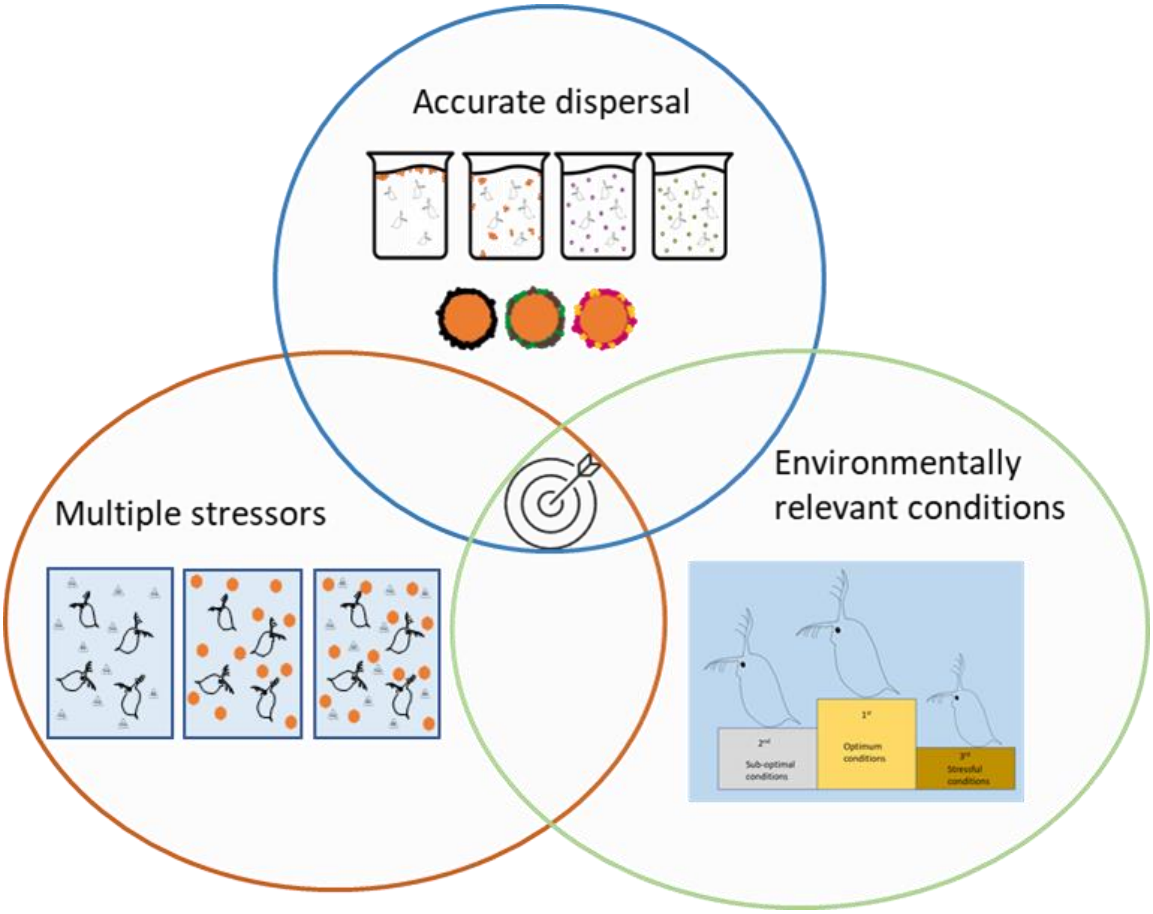
School of Geography, Earth and Environmental Sciences

College of Life and Environmental Sciences

University of Birmingham

March 2021

Abstract



Microplastics (MP) are widely recognised as a contaminant of concern in the environment. Found in all environments sampled, the widespread use of plastic due to the versatile and relatively cheap cost of the material in combination with its long life by design, means that this is likely to be a problem facing the environment for the foreseeable future. Due to this, it is important to understand how microplastics might be impacting their environments, both in terms of physico-chemicals changes and also in terms of potential detrimental impacts to the organisms encountering the plastics. Laboratory based toxicity studies are widely used to explore the issues facing organisms by replicating these encounters. However, unlike chemical

based toxicity tests, microplastics have a range of properties (such as hydrophobic surfaces) that may make this more challenging, and therefore previously established test protocols may need modifications to make them more applicable to this emerging pollutant. Establishing thorough reporting of baseline data and learning from similar fields, such as nanomaterial toxicity, will enable a greater understanding of the mechanistic toxicity and potential threat that MP poses to organisms and subsequently their wider ecosystems.

This thesis explores various elements of toxicity testing encompassing MP dispersal, medium influence on *Daphnia* response and finally mixture toxicity. By exploring the different methods of dispersing hydrophobic polyethylene beads, test designs can be modified to be more environmentally realistic whilst ensuring adequate dispersion (Chapter 3). The significant variation in both control (medium only) and chemical (sodium dodecyl sulphate) exposure response in *Daphnia* highlights the need to consider the testing medium during the experimental design stage for environmentally relevant test endpoints (Chapter 4). Finally, a combination of elements from Chapter 3 and 4 formed the study design for mixture exposure of three chemicals (sodium dodecyl sulphate, triclosan and diclofenac) in various media explored in Chapter 4, to *Daphnia* in chemical only and chemical with MP in combined exposures to ascertain how the effect of the chemical may vary (Chapter 5). Combining the various elements of this thesis explores the variability resulting from the study design within MP toxicity tests and highlights the need for environmental consideration at the design stage to expand the scope of MP laboratory-based toxicity studies to increase the environmental relevance and realism going forwards.

COVID impact statement

The final stages of this thesis were disrupted and delayed due to the COVID19 pandemic. As a result, the work planned for Chapter 5 (chemical and microplastic co-exposures) was not completed as anticipated. The method development for the analysis of the mixture scenarios was underway at the point of the first lockdown and as the laboratory was closed at short notice all culturing and related work was discarded during the university wide closed period (March-September 2020). The COVID-secure working environment for the laboratories made the collaboration with Dr. Daniel Drage for his expertise in the analysis of these samples challenging within the timeframe for the thesis deadline. As a result, this work is expected to be undertaken in the interim between the thesis submission and the viva, and an updated copy of this analysis and interpretation of the results within the context of Chapter 5 will be sent to the examiners at the earliest opportunity.

Acknowledgements

A huge thank you to Professor Iseult Lynch and Professor Jon Sadler for your supervision, advice, and support during my PhD. Thank you for the freedom and guidance to allow me to explore ideas and theories and allowing me to deviate off on tangents of interest throughout the research process. Thank you for all of the opportunities during my PhD to help me build my confidence, gain experience, showcase my work and to develop a wider network of support in the scientific community.

I gratefully acknowledge EU H2020 project RiskGone which provided funding and training opportunities for this project. I would also like to extend my thanks to the College of Life and Environmental Sciences Daphnia facility where parts of the research were undertaken and for technical support throughout the process, I would like to thank Caroline Sewell, for her expertise in all things *Daphnia*, Chris Stark for ICP and elemental analysis, David Tubbs for practicalities of field sampling and Dr Daniel Drage for HPLC-MS expertise, support and guidance throughout Chapter 5 especially.

Thank you to all my friends from the daphnia lab, nano labs and the 425/435 offices for the coffee breaks, lunchtime walks, R coding help and Friday drinks. Thanks especially to Helen, Jenny and Sam, for always being on the other end of the phone during this write up period. I am so grateful for the virtual support network that you have seamlessly transitioned into during 2020/2021, and very much look forwards to a having a Friday drink with you all at the first opportunity.

Laura, Fatima, Julia and Hollie; thank you for listening to me rave and rant about the *Daphnia* in equal measure and for making the basement an enjoyable place to spend hundreds of hours over the past few years. I'm so glad of our friendships that now replaces the basement with brunch.

Finally, a huge thank you to my Mum, Dad, siblings (Paul, Shaun, Abigayle and Georgina) and family. Thank you for the financial, emotional, and logistical support during my PhD and for believing in me when I didn't believe in myself- I appreciate it more than I ever say!

Contents

List of figures

List of tables

List of appendices

Author contributions

Chapter 1: KR research and writing, JS and IL supervision and review.

Chapter 2: KR experiment design, data collection, analysis and writing, LE- assistance in TEM imaging, JS and IL supervision and review.

Chapter 3: KR experiment design, data collection, analysis and writing, JS and IL supervision and review.

Chapter 4: KR experiment design, data collection, analysis, DD and SH HPLC-MS assistance with experiment design and method development for planned chemical analysis, JS and IL supervision and review.

Chapter 5: KR synthesis and writing, JS and IL supervision and review.

Abbreviations

BCA- Bicinchoninic acid assay

BSA- Bovine Serum Albumin

DCS- Disc Centrifuge Sedimentation

DLS- Dynamic Light Scattering

EDC - Endocrine Disrupting Chemical

ENM - Engineered Nanomaterial

FTIR - Fourier-Transform Infrared Spectroscopy

GC-MS - Gas Chromatography Mass Spectrometry

HARN - High Aspect Ratio Nanomaterials

HOC-Hydrophobic Organic Contaminants

HPLC-MS- High Performance Liquid Chromatography Mass Spectrometry

LRTP - Long Range Transport Potential

MARPOL - International Convention of Prevention of Pollution from Shipping

MP - Microplastic

MSFD - Marine Strategy Framework Directive

NOAA - National Oceanic and Atmospheric Administration

NOM- Natural Organic Matter

NP – Nanoparticle

OCS - Operation Clean Sweep

POP - Persistent Organic Pollutant

REACH - Registration, Evaluation, Authorisation and Restriction of Chemicals (EU regulation)

SDS- Sodium Dodecyl Sulphate

SEM - Scanning Electron Microscopy

SI – International System (of units)

SUP - Single Use Plastic

TEM - Transmission Electron Microscopy

TRWP - Tire and Road Wear Particles

WWTW - Water-Water Treatment Works

WFD - Water Framework Directive

A note on terminology: "plastic" is used to describe and discuss the range of polymers of varying sizes and sources throughout this thesis, from macro to nano scale. MP refers specifically to the <5 mm size range and nano plastic applies to particles smaller than 1 µm in line with the most commonly used denominations in the literature at present as there are no formal regulatory definitions as yet.

Chapter 1

1. Introduction and literature review

1.1 Introduction

Microplastics (MP) are a diverse group of pollutants that are frequently identified as a ubiquitous contaminant of concern in all environments studied to date. The field of MP research is both relatively new and rapidly expanding with the intention to establish this baseline knowledge of the scale of the pollution and the severity of the effects this can have on the ecosystem. Due to the complexity of the pollutant, there is a wide range of laboratory-based toxicity assays that have been undertaken to address these concerns, encompassing a variety of both test organisms and microplastic combinations. As a result, several unconscious and/or unintentional assumptions can be made in the study design of toxicity experiments that are currently based on test guidelines designed for chemical exposures. For example, accurate dispersal of hydrophobic particles is a potential issue in toxicity assays which is not accounted for in chemical-based protocols which could therefore lead to inaccurate exposures and reported concentrations. Furthermore, the distribution of MP in the environment is being widely researched to identify potential hotspots and areas of concern. Therefore, it is important to understand how toxicity might vary in media that is more representative of these environments compared to the traditional salt-based culturing medium that if often used in toxicity tests, and how the baseline fitness of the organisms cultured in the varying media can change. In addition, due to the complexity of environmental contaminants, the role of MP with a relatively large surface area to act as a potential 'trojan horse' for chemical contaminants is a current avenue being explored by the microplastic research community.

1.2 What is the microplastic problem?

Plastic is an important resource in the 21st century, and global production has grown in recent years to 359 million tonnes in 2018 (PlasticsEurope, 2019b). Because of this, plastic is increasingly found to be polluting the environment; however, the visible plastic is only part of the problem. The smaller, harder to see plastic pieces, which are often termed microplastics (MP) and are currently classified as plastic particles that are less than 5mm in diameter (Barboza and Gimenez, 2015), are considered to be a greater environmental concern due to their ubiquitous presence, increased biological interactions and difficulties in sampling. Although the size classification of plastic are often discussed within the literature, the most frequently used definition currently of microplastics is the National Oceanic and Atmospheric Administration (NOAA) definition of less than 5mm, but discussions within the research community are underway to re-evaluate this in line with the advancing analytical methods now being developed and implemented (Hartmann *et al.*, 2019). MP are also often reported by morphology in the categories of beads or spheres, fibres and fibre bundles, pellets, film, foam or fragments and are introduced into the environment as either primary or secondary plastic (Rochman *et al.*, 2019).

Primary MP are classified as those that have been manufactured as smaller than 5mm, such as exfoliators in cosmetics (often called microbeads), paint particles and industrial pellets (World Health Organization, 2019). Secondary MP are those that have been introduced into the environment as larger plastics and have subsequently been broken down through chemical, physical and/ or biological processes over time (Rochman *et al.*, 2019). This could include items such as plastic bottles and bags and discarded fishing nets (S. C. Gall and

Thompson, 2015). Although there are some variations in the categorisation of different sources of microplastic pollution, the classification of primary microplastics as being those that are manufactured in the size range of microplastics makes them easier to monitor and manage at a manufacturing level, compared to secondary microplastics which are harder to control. Primary microplastics typically make up a small fraction of the total MP released into the environment, and this fraction is set to further decrease in future years following a targeted response to reduce these inputs by the introduction of bans and phase-out initiatives such as the microbead ban (Mitrano and Wohlleben, 2020).

Although MP are classed as either primary or secondary, both types can be introduced from a range of sources. Potential sources of MP to freshwater environments include industrial outflows, Waste water Treatment Works (WWTW) outflows, washing machine discharge, sewage sludge application to agriculture, urban runoff, tourism and recreational activities, and atmospheric deposition (Wagner *et al.*, 2014). Plastic pollution was previously a very marine focused field of research; however, this has rapidly expanded recently to encompass freshwater, terrestrial, and atmospheric MP for both sources, sinks and potential environments for biological interactions (Allen *et al.*, 2019; Provencher *et al.*, 2019; Triebskorn *et al.*, 2019). MP have been found ubiquitously in samples taken across the globe, including arctic sea ice, coral reefs, mangroves, deep sea trenches, open ocean, coastal zones, estuaries, rivers, lakes and canals (Barnes *et al.*, 2009; Lavender, 2010; Sadri and Thompson, 2014; Wagner *et al.*, 2014; Woodall *et al.*, 2014; Hall *et al.*, 2015; Lusher *et al.*, 2015; Leslie *et al.*, 2017). In addition to environmental samples, MP have also been found in extracted table salts from 2- 367 items per kg depending on source, and in drinking water with 1.2- 118 items per L depending on both location and drinking container/source (Zhang *et al.*, 2020). Due to this

increasing number of studies and data available, models have been developed to estimate the transport of plastics through these different environments, and have approximated that 80% of the plastic in the marine environment originates from freshwater sources inland that have then been transport from the land to the sea via rivers and streams (Hurley, Woodward and Rothwell, 2018). When this is paired with previous knowledge of other pollutants such as oil or heavy metals from industry, and various models looking at the transport and mixing of these in the environment, it can be used to make predictions based on the geography and point sources of pollutants in the vicinity (Mason *et al.*, 2016).

Microplastics are a particularly challenging form of pollutant, and there are several policies that aim to address this. Currently, MP pollution is monitored under the Marine Strategy Framework Directive, which aims for a reduction in marine pollution and has several monitoring efforts to address this (Gago *et al.*, 2016). MP have also been listed as a priority in the UK 25 year environment plan and through the recent passing of the Environment Act which was accepted at the end of 2021 (House of Commons, 2021). The Environment Act outlines several ambitious targets to safeguard the environment and reduce the amount of single use plastics, and plastic packaging in use, and therefore, a good understanding of the current state of the environment will be key to implementing these legislations.

1.2.1 Most common types of plastic

The range of plastics (in terms of polymer, plasticisers and additive combination) is as wide ranging as the number of uses of plastic. However, these six types of polymer categories make up 90% of the plastic demand globally (Bejgarn *et al.*, 2015);

- polyethylene (PE, both LDPE and HDPE)
- polypropylene (PP)
- polyvinylchloride (PVC)*
- polystyrene (PS)
- polyethylene terephthalate (PET)
- polyurethane (PUR)

*From a regulatory point of view, PVC is not classified as a polymer under the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) due to the high content of additives which can make up to 50% of the material's weight. However, within the microplastics research community this has been classified as plastic due to the polymer backbone and the variability of the additive content once it is in the environment (Hartmann *et al.*, 2019).

Plastic properties can vary significantly based on the plasticisers and additives used in combination with the polymer. For example, colour, density, flexibility, melting point, along with properties such as resistance to corrosion and flame retardance, are key factors in why plastics are so useful and widely used, but this also leads to microplastics being a very diverse and multifaceted environmental problem (Rochman *et al.*, 2019). There is also the addition of inorganic monomers such as silicates, that are not technically classified as polymers along with the more recently added group of bioplastics that increase the complexity of this issue (Hartmann *et al.*, 2019).

1.2.2 Breakdown, weathering, and size classification of plastics

The size classification of plastics is under discussion within the microplastics research community, and several frameworks have been suggested in recent years. Currently the classifications do not adhere to the International system (SI) units used within other fields. The most frequently used definition for microplastic size is less than 5mm in the largest dimension, which was largely determined due to the increase in biological interaction potential at that size. Due to advances in analytical and sampling techniques, there is discussion to reclassify this (Hartmann *et al.*, 2019; Rochman *et al.*, 2019) with a compromise between SI units and those most frequently used within the field currently. The size classes proposed by (Hartmann *et al.*, 2019) are;

- Nano plastics - < 1000 nm
- Microplastics (MP) - 1 µm - 1000 µm
- Mesoplastics - 1 mm - 10 mm
- Macroplastics - > 1 cm

Agreeing and harmonising the terminology to be used to report the sizes of plastics in studies will enable clear comparisons between studies going forwards.

Size classes are important in terms of establishing the potential biological interaction and could also be used as an indicator of the potential sources of the plastics to the environment. Plastics have been found to gradually break down in the environment due to a mix of physical, chemical and biological interactions. UV light has a significant effect on the rate of chemical weathering of plastics because the UV light exceed the storage modules energy capacity leading to breaking of the polymer chains (Iñiguez, Conesa and Fullana, 2018). UV light can

also lead to free radicals forming which also affect functional groups within the polymer chains, causing further breakages (Iñiguez, Conesa and Fullana, 2018). Therefore, it is most likely that plastics will gradually transition through the above-mentioned size classes during the particle's lifetime.

The continued degradation of MP into nano plastic particles has been demonstrated using polystyrene in a study that confirmed that the size of the particles in solution decreased over time as the overall number of particles increased under weathering conditions in the laboratory using Nanoparticle Tracking Analysis (NTA) (Lambert and Wagner, 2016a). Using Scanning Electron Microscope (SEM) images, Lambert and Wagner (2016a) theorised that the nano plastic content increased due to nano particles being released from the surface of the MP where there was evidence of bubbling. This indicated that the continual breakdown of plastic in the environment through abiotic and biotic weathering will pose a problem for many years to come. As the surface of the plastic becomes rougher due to the fragmentation, it is also likely that any chemical transfer onto the plastic will also increase due to the increase in surface area (Bakir, Rowland and Thompson, 2014; Napper *et al.*, 2015a), thus enhancing the potential for transfer of co-pollutants.

1.3 Sources of plastic

1.3.1 What are the main inputs and endpoints?

There are a wide range of plastics uses, including in cosmetics, paints, personal care products and clothing in addition to all the plastic resources such as industrial fillers and packaging that we use or benefit from daily. All of these are potential sources of MP to the environment through both direct emissions such as fly tipping or mismanagement of waste, and indirect

emission such as WWTW and agricultural runoff. If a significant point, or diffuse, source can be identified this enables a more focused approach for legislation and policy changes.

1.3.1.1 Personal care products and microbeads

Microbeads is a commonly used term for a subset of primary microplastics that are incorporated into personal care products such as facial scrubs and shower gels. With increasing awareness of this source of MP to the environment, there has been successful campaigns, such as 'Beat the Bead' (Plastic Soup Foundation, 2020), to prevent the sale of products containing microbeads and put legislations in place to ensure this. This included the Microbead Ban in the UK, of which the first phase came into effect in June 2018 (DEFRA, 2018b). Prior to the microbead ban, polyethylene was often used in personal care products, with up to 93% of the cosmetics on the market containing polyethylene (Napper *et al.*, 2015a). (Gouin *et al.*, 2011) calculated that in 2011 the population of the US used 263 tonnes of polyethylene beads in their cosmetics and the European population collectively used 4076 tonnes, most of which is anticipated to not have been filtered out at WWTW and would therefore have made its way into the aquatic environment. A large quantity of the plastic isolated from WWTW effluent samples were blue polyethylene fragments which was identified as a common polymer that was used in toothpastes prior to the microbead ban (Carr, Liu and Tesoro, 2016). Microbeads in personal care products have since been banned in several countries, such as UK, Canada, Germany, US. However, there are sometimes loop-holes in these laws such that this only applied to wash on and wash off products and therefore some sources of microbeads are exempt under this legislation (see section 1.7: People, policy and plastic).

1.3.1.2 Clothing

Clothing has been shown to contribute a significant amount of MP fibres to the environment through washing and pilling. Synthetic fibres are widely utilised due to their beneficial properties such as quick drying, temperature control and flexibility, however during the wash cycle fibres can be released from the fabric at varying rates which end up in the waste-water outflow (Napper and Thompson, 2016). The variability of fibre release is based on the age and type of fabric, in addition to the washing technique used, for example newer items of clothing typically release significantly more fibres compared to older garments. Depending on the wash cycle and material, a 6 kg load can release between 137,951-728,789 fibres per wash (Napper and Thompson, 2016). The type of fabric has a significant impact on the amount of fibres released as fleece shed approximately 1200 fibres per 100 cm^3 compared to polyester and nylon based knit products which produced approximately 10 per 100 cm^3 (Carney Almroth *et al.*, 2018). This highlights the variability of fibre release based on textiles alone, in addition the combination of bio-detergent and fabric conditioning lead to elevated fibre release (Napper and Thompson, 2016). This could be a key source of the microfibres that are frequently found in the environment (Grbić *et al.*, 2020).

1.3.1.3 Tyre and road wear particles (TRWP)

Polymers are often added to tyres to enhance the properties of the rubber such as decreasing the stopping distance when breaking and making them safer and more effective across a range of temperatures (Kole *et al.*, 2017). However, car tyres (and tyre and road wear particles (TRWP) such as fragments worn off from thermoplastic road marking paints) are a potentially significant source of MP to the urban environment due to the abrasion with the road surface over time (tyre balding) with the particles then washing down the drains (Panko *et al.*, 2019).

It has been calculated that the per capita emissions of tyre particles range from 0.23 to 4.7 kg per year with a global average of 0.81 kg per year (Kole *et al.*, 2017). There are further limitations to the analysis of TRWP as they may differ significantly from the original tyre matrix due to the mixing with other anthropogenic pollutants and the abrasion process, such as heat from breaking, which often leads to additives leaching rapidly from the tyre matrix (Kole *et al.*, 2017; Wagner *et al.*, 2018). TRWP have been found in environmental samples, and are theorised to form a large portion of the particles that are found in urban run-off, with an estimate of only 50% removal at WWTW (Wagner *et al.*, 2018).

1.3.1.4 Agricultural plastics

In addition to the use of sewage sludge from WWTW, there are further uses of polymers and plastics in agriculture, often termed “plasticulture”, which can slowly release MP to the environment. This includes polytunnels to aid plant growth and plastic mulch to regulate and insulate ground temperature (Steinmetz *et al.*, 2016). During the use of polytunnels, the coating slowly degrades due to UV exposure which can lead to droplets forming, once the droplets form on the inside of the polytunnel it magnifies the UV rays and causes sun damage to the plants. As a result, polytunnels are often discarded at the end of the growing season as it is cheaper to replace the tunnels than to recoat them or to risk sun damage to crops (Briassoulis *et al.*, 2013). Although the use of plastic in agriculture is variable across different regions, it was calculated that 1.74 million tonnes was used in Europe in 2018, which represents a significant source of plastic to the environment (PlasticsEurope, 2019b). In addition to this, plastic is often used to create microcapsules of pesticides or fertilizers to allow for controlled release during the growing season. This releases primary microplastics directly

into agricultural land in varying concentrations depending on crop type and application, and has been reported of up to 369 mg/kg (Katsumi *et al.*, 2020).

1.3.1.5 Waste-Water Treatment Works (WWTW)

The quantification of MP emissions from WWTW can vary depending on the technology used and therefore this can be significantly different around the world (Rochman, Cook and Koelmans, 2016). To understand the pressures that MP put on WWTW it is important to understand the quantity present in the water column and the amount that is attempting to pass through the WWTW system (Eerkes-Medrano, Thompson and Aldridge, 2015).

WWTW are often hypothesized to be incapable of effectively removing MP from the system due to the size of the filters used. It is estimated that 4 million MP particles per day, per WWTW on average are released in the effluent, due to the tertiary filtration systems being ineffective at removing the smaller and lighter particles (Mason *et al.*, 2016). This study also found a variety of MP in the effluent sampled which was broadly categorised into 59% fibres, 33% fragments, 5% films and 2% foam and 1% pellets, however, all fibres could not be confirmed as plastics due to limitations in spectrometric analysis (Mason *et al.*, 2016).

There are several factors that affect the output of plastic from WWTW including; surrounding population and land use, combined sewer systems, the flow rate through the treatment plant, the filtration methods used and the source of the water (i.e. residential, commercial or industrial) (Mason *et al.*, 2016; Grbić *et al.*, 2020). It has previously been found that beaches in close proximity to sewage disposal sites had 250% more MP fibres compared to beaches further from outflow sites (Browne *et al.*, 2011).

On the other hand, WWTW have been shown to be very effective at removing MP from the system by using a 45-400 µm mesh to sample the WWTW discharge (Carr, Liu and Tesoro, 2016). However, neutrally buoyant plastics are most likely to escape the filtration process due to being missed by the critical removal modes of skimming and settling as the plastic will be mid water column (Carr, Liu and Tesoro, 2016). Although it has been calculated that WWTW have the capability to remove up to 99.9% of MP in the system, due to the volume of MP in the system this might still mean that a large numbers (the 0.1%) could be entering the environment (Carr, Liu and Tesoro, 2016; Horton *et al.*, 2017). The rate of removal is likely to vary depending on the properties of the MP (such as morphology, density etc.) however, WWTW would also increase the contact time between the plastic and other pollutants and therefore has the potential to increase biofouling (Carr, Liu and Tesoro, 2016). In addition, sludge from WWTW is often spread on agricultural soil as fertilizer and therefore any MP that have been contained in the sludge are released back into the environment. The MP from the sludge are then potentially remobilized into the freshwater system within the catchment due to runoff during rainfall (Corradini *et al.*, 2019; Mitrano *et al.*, 2019).

1.3.2 Environmental hotspots

Research is currently underway to quantify and identify MP hotspots in the environment in addition to determining the major sources. MP have been found in all environments studied at varying concentrations from deep ocean trenches (Woodall *et al.*, 2014) to mountain snow (Allen *et al.*, 2019), ocean gyres (Lebreton *et al.*, 2018) to freshwater rivers (Tibbetts *et al.*, 2018), and in both air samples (Wright *et al.*, 2020) and food intended for human consumption (Zhang *et al.*, 2020). The variability in methodology including mesh-size used can make it challenging to compare the results of the studies to identify hotspots of MP deposition.

However, due to this increase in the environmental sampling and model development, estimates have been calculated on the plastic fluxes and potential sinks including how much plastic there is in certain areas, i.e. the ocean gyres. Although only estimates, it enables hotspots to be identified to try and locate potential point, or diffuse, sources that could aide a more targeted approach to preventing the release of MP into the environment (Horton *et al.*, 2017).

The environmental transport of plastics can be heavily influenced by the density of the polymer, for example, the denser the MP the more likely it is to sink, the lighter the more likely it is to be transported further by both aquatic and airborne transport. The density of the particles can change over time depending on the breakdown of the chemical structure of the particle and the release of any additives (such as in PVC) and the addition of particles to the MP surface such as organic matter or biofilm formation (Horton *et al.*, 2017).

The ocean gyres have been identified as a hotspot of MP, due to the convergence of currents that can transport positively and neutrally buoyant plastics. In one model of the Great Pacific Garbage patch it has been estimated that in a 1.6 million km^2 area there are 79,000 tonnes of plastic, made up of 1.8 trillion pieces, with MP making up only 8% of mass but 94% of particle numbers (Lebreton *et al.*, 2018). However, it is worth noting that approximately 60% of the plastic produced are negatively buoyant, and are therefore likely to sink and are expected to eventually settle in the sediment and would not be found in these convergence zones (Lebreton *et al.*, 2018). This has led researchers to question where this 60% might be with a logical hypothesis being that they settle out and accumulate in aquatic sediments. This is widely supported as MP have previously been found in aquatic sediments such as river

catchments in addition to the deep-sea sediments (Woodall *et al.*, 2014; Hurley, Woodward and Rothwell, 2018; Tibbetts *et al.*, 2018). Once settled in the sediment, these MP may also be remobilised during flooding events which could change the expected concentration of MP within an area (Hurley, Woodward and Rothwell, 2018). Aquatic sediments cover a huge area globally, from riverbeds to ocean basins and entails a range of logistical challenges for sampling campaigns which means the spatial (and temporal) resolution of this data is less robust than other environments (such as beach sediments) (Galgani *et al.*, 2013). If the area is logistically hard to sample, it follows logically that any clean-up efforts would be logistically challenging too and therefore current mitigation efforts are focusing on the identified hotspots and sources, such as 'The Ocean Clean-up Project' (see section 1.7) in addition to improving WWTW technology and removing microbeads from cosmetics (Rochman *et al.*, 2019).

1.4 Ecological impacts

1.4.1 Environmental evidence of interaction of MP and biota

Larger items of macroplastics have often been used in environmental campaigns to highlight the harm that this pollution can have on the planet. Campaigns that were widely used were turtles, seals and sea birds choking on beer packs and plastic bottles (S. C. Gall and Thompson, 2015). Entanglement with plastics and physical damage to the digestive tract was widely observed and it was theorised that this could be happening on a smaller scale with MP and had previously been going unnoticed. MP are now acknowledged as being ubiquitous in the environment which consequently leads to MP having a high encounter rate with organisms across the globe. Encounter rates and contamination have been calculated to have risen by

49% in the past 20 years, and a review of 700 animal's encounters with marine debris summarised that 92% of the encounters were plastic debris (S. C. Gall and Thompson, 2015). There has been a range of studies published that have confirmed MP ingestion in organisms sampled in various environments including zooplankton, macroinvertebrates, crabs, fish and birds (Sanchez, Bender and Porcher, 2014; Desforges, Galbraith and Ross, 2015; Reynolds and Ryan, 2018; Giani *et al.*, 2019; Horn *et al.*, 2019; Windsor *et al.*, 2019).

Due to the evidence of MP interaction and ingestion from the environment, it is important to ascertain the effects that this could be having to the individual organisms and the overall consequences for the ecosystem and its services. The amount of plastic found within an organism could be used as a proxy for how polluted their environment is, but this would depend on the residence time of the animal within that environment in addition to other factors which could complicate this proxy measurement.

1.4.2 Laboratory evidence of effects

MP ingestion has been observed in both laboratory and field studies in a range of organisms and conditions. Although the majority of the research to date has focused on the marine environment and the impact on marine organisms, due to the similarity of physiology of some groups of organisms, it can be assumed that similar responses would occur in freshwater organisms (Horton *et al.*, 2017). This has already been demonstrated in some of the toxicology studies undertaken so far, as outlined in Table 1.1 below.

Table 1.1 A summary of a range of organisms (fresh water and marine) and how they are affected by MP based on current scientific literature.

Organism	**Marine/ Fresh water**	**Polymer type and size**	**Observed impacts**	**References**
Marine mussels	Marine	PE & PS 100 µm (with pyrene)	Altered immunological response and induced genotoxicity	(Avio *et al.*, 2015)
		30 nm (with and without algae present)	Decrease in feeding	(Wegner *et al.*, 2012)
			Increase in pseudo faeces production	
Marine lugworm	Marine	PVC (5% of available food)	Pollutants (Triclosan and PBDE) accumulated in the gut (326-3770% higher than the sediment)	(Browne *et al.*, 2013)
			Decreased feeding, anti-oxidative potential, and survival	
Zooplankton (including copepods)	Marine	1.7-30.6 µm Polystyrene	Ingested and excreted up to 7 days later	(Cole *et al.*, 2013)
			Adhered to external appendages limiting movement	
			Reduced successful feeding on algae	
		20 µm PS	Significant decrease in feeding	

			Downward shift in size of algae ingested	(Cole *et al.*, 2015)
			Prolonged exposure leads to decreased reproductive output	
Phytoplankton	Marine	2 µm Polystyrene	Change in permeability and aggregate density	(Long *et al.*, 2017)
Coral	Marine	PP 10 µm-2mm	Accumulated in mesenterial tissue with potential to reduce digestion of prey/food due to blockage	(Hall *et al.*, 2015)
		Mixture of PE, PP, PS, PET, PVC 500-1000 µm	Approx. 8% of plastic was retained for at least 24 hours (most prey is digested in 3-hour average) with potential wasted effort (energy) for digestion	(Allen, Seymour and Rittschof, 2017)
Zebra Fish	FW	125-250 µm	Combination of feed + plastic + contaminant had the greatest detrimental effect on the liver compared to any individually	(Rainieri *et al.*, 2016)
			Indication of plastic acting as a vector for chemicals (BFR, PFC and methyl mercury)	
Algae	FW	PS 0.05. 0.5, 6 µm	Uncharged PS particles reduced algal growth by up to 45%	(Sjollema *et al.*, 2016)

		LDPE PS 70 µm	Changes to the lipid molecules that are key to cellular membrane integrity/function and decreases the food quality for consumers	(Guschina, Hayes and Ormerod, 2020)

In summary the main impacts observed so far (Table 1) are increased mortality, reduced fecundity, decreased growth and changes to lipids. However, there are also studies that report no detrimental effects to the test organisms (Kaposi *et al.*, 2014; Jovanović *et al.*, 2018) and therefore there is a need to understand the underpinning principles that cause these differing results, for example; dose and exposure duration, feeding method, plastic morphology or experiment complexity.

1.4.3 Biomagnification, accumulation, concentration, and trophic transfer

One of the key concerns regarding the potential impacts of MP is how it will affect the food-web through impacts on keystone species and through trophic transfer. Biomagnification is the process effect when contaminants are transferred through the food-web which can lead to bioaccumulation within individual organisms with the top consumers being most at risk (Walker *et al.*, 2012). Bioconcentration is when the concentration of a contaminant in an individual is higher than in the environmental medium which it inhabits (Walker *et al.*, 2012). An infamous example of this is with DDT poisoning which has disastrous, long lasting consequences (Gerber *et al.*, 2016).

The potential for biomagnification of MP throughout food chains has been demonstrated in several laboratory studies. For example, Athey and co-authors (2020) confirmed the potential

for trophic transfer in estuarine systems under lab conditions using ciliates and larval fish (silverside). An initial MP particle per mL concentration of $5x10^5$ was used during the exposure with the ciliates, which although being orders of magnitude higher than currently reported concentrations, is in-line with other toxicity tests (Athey *et al.*, 2020). This led to a rapid uptake of particles in the silverside larvae (with 320 particles per individual on average) retained following ingestion of their ciliate prey (Athey *et al.*, 2020). Although confirmed in laboratory settings it can be more challenging to ascertain the extent of trophic transfer in the environment due to different potential sources, i.e. accidental ingestion (Nelms *et al.*, 2018; Athey *et al.*, 2020). Another study has shown that trophic transfer of MP doesn't have a significant impact on fish behaviour and therefore the response is likely to be dose and organism dependent (as it is with primary ingestion) (Tosetto, Williamson and Brown, 2017). Trophic transfer potential has also been demonstrated in a larger organism by comparing captive grey seal scat to their prey's (Atlantic mackerel) digestive tract which showed similar concentrations, morphology and polymers type in both samples indicating that the mackerel is a likely route of MP ingestion by seals (Nelms *et al.*, 2018).

Bioaccumulation has also been reported in several studies with retention in the guts or stomach most common (Provencher *et al.*, 2019). More recently, MP have been reported to be translocated to other organs including the brain and reproductive tissue in crabs (although this was quantified using fluorescence which is prone to significant experimental artefacts- see section 1.6) (Crooks, Parker and Pernetta, 2019). Studies have also reported the translocation of MP PS into the lipid droplets in *daphnia*, although this could be actually be due to lipophilic dye transfer from fluorescently stained particles into the lipid droplets rather

than the particles themselves, which is a common challenge for bioaccumulation studies (Triebskorn *et al.*, 2019).

There is the potential for MP to have detrimental impacts on primary producers, for example in the fatty acid quality of freshwater algae which can have knock on consequences for the organisms that rely on this for food (Guschina, Hayes and Ormerod, 2020). There is also the potential that predator-prey relationships could be impacted by MP interactions. MP have been shown to cause algae to clump, which could lead to increased algae ingestion as the algae form larger particles and are therefore easier to detect but also ingest, which would also lead to increased inadvertent MP ingestion (Guschina, Hayes and Ormerod, 2020). In addition, MP can also change the behaviour of some organisms which makes them more vulnerable to predation such as decreased swimming abilities or changes to buoyancy (Cole *et al.*, 2015). This effect has also been shown to be intensified if the MP has associated chemical contamination, such as DDT, as the chemical can cause specific, or increased, changes to behaviour or metabolism that would not have been caused by the MP alone and therefore would cause increased threat of predation and trophic transfer of MP (Athey *et al.*, 2020).

1.4.4 Ecosystem consequences and invasive species

In addition to the single organism and trophic transfer effects noted above, there is also the potential for MP to have wide reaching impacts on whole ecosystems. This could be due to disruption to biogeochemical cycles, changes or disruption to microbial communities or via transport of alien species (Krause *et al.*, 2021). Due to the long residence time and variable buoyancy of MP particles in the environment, they are capable of being transported long distances and, in combination with their large surface area, provide an ideal platform to

transport alien/invasive species such as bacteria, viruses and dinoflagellates to different ecosystems (Debroas, Mone and Ter Halle, 2017; Kettner *et al.*, 2019; Bowley *et al.*, 2021).

Due to the sorption potential of MP, the surface of the plastic may have elevated concentrations of organic and inorganic molecules when compared to the surrounding medium which can lead to an increase of the number of plankton subsequently interacting with the particles (Shen *et al.*, 2019). This can then lead to alteration in the olfactory cues presented by the MP due to the presence of the plankton, leading to increased ingestion and trophic transfer of the plastics. Further research needs to be undertaken to evaluate the invasive species transport potential of MP, as the surface conditioning of microplastics has been observed but the subsequent ecosystem consequences and colonisation are still relatively unexplored (Khalid *et al.*, 2021).

In a microcosm study, MP have been shown to have variable effects on salt marsh microbial communities which led to subsequent impacts on the nitrogen cycling ability of the microbes, when compared to controls; PVC was found to inhibit nitrification and denitrification, whereas the polyurethane foam and polylactic acid treatments promoted both processes (Seeley *et al.*, 2020). Studies in the terrestrial environment have also highlighted the impact that MP have on the nutrient cycling processes and the changes to abiotic factors, such as water movement through the soil profile due to the addition of MP which has had a variable impact on plants, as demonstrated by soil spiked with polystyrene MP to make up 2% of the weight leading to a significant increase in root biomass, potentially to compensate for the increase in drainage of the soil (de Souza Machado *et al.*, 2019). The impact of MP on abiotic factors has also been explored in beach sediments which demonstrated the increase in permeability of beach

sediments and consequent delays in temperature increase (insulation in the sediment) due to the addition of plastics, which can make up 30% of the sediment as found in previous environmental samples (Carson *et al.*, 2011). The insulation of the local environment in the sediment could have significant impacts on key species such as sea turtles as their offspring's sex is determined by the temperature of the sediment that the eggs are incubated in (Carson *et al.*, 2011).

1.4.5 Biofilms, eco-corona and olfactory cues

Not only can MP/nano plastics interact in the aquatic environment with other pollutants, MP also interact with the biomolecules secreted by the organisms into the environment. This can include proteins and polysaccharides which are secreted as a standard predator/prey mechanism in freshwater environments. Nasser and Lynch (2016a) investigated the capacity of carboxylic polystyrene NP spheres to adsorb the proteins secreted by *Daphnia magna* and *Chlorella vulgaris* and what effect this has on the subsequent ingestion of the plastics. The authors found that *Daphnia magna* proteins coated the NP which led to an increase in NP agglomeration, uptake and retention once ingested and thus subsequent toxicity. The *Chlorella vulgaris* did not excrete as much protein as the *Daphnia* and therefore it had less of an overall effect. The longer the incubation period the higher the concentration of the proteins, which was detected with mass spectrometry. Nasser and Lynch (2019a) reasoned that the OECD standard test for toxicity is not effective when assessing nanoparticles broadly, which includes nano plastics, without significant modification, and the tests tend to oversimplify the system with no regard for an eco-corona on the particles, which makes a significant difference when working with particulates. This eco-corona is also likely to

influence the MP toxicity tests currently used and therefore it would be beneficial for the MP research community to implement the lessons learned previously in nanotoxicity research.

(Carr, Liu and Tesoro, 2016) found that, in the samples they isolated from WWTW, biofilms were ubiquitous on the plastics, which consequently could affect the interaction of the plastic with organisms, other pollutants and the environment due to changes in the hydrophobic nature and density of the plastic along with the olfactory cues present. Plastic also has the potential to transfer these microbial communities through the freshwater network as it moves through the system which could alter the dynamics and distribution of the biofilms (Hoellein *et al.*, 2014). In addition, microbial communities have been identified as the dominate colony on the surface of MP are not found in abundance in surrounding medium (sea water) which could be an indicator of the changing microbial dynamics that MP can pose to the environment (Rogers *et al.*, 2020).

The change in biomolecule composition in the MP eco-corona and the associated olfactory cue signalling has been demonstrated to increase the grazing rate of copepods by between 72-292% after exposure to MP conditioned with dimethyl sulphide (DMS) as an olfactory stimulant (Procter *et al.*, 2019). This could therefore lead to a significant increase in the amount of plastic ingested in the environment compared to that in laboratory-based studies without this conditioning step. In addition, the presence of polystyrene MP (500 nm and 30 µm) has been shown to inhibit odorant evoked behaviour in goldfish (to L-cysteine and taurocholic acid) after a 28-day MP exposure, again likely due to the binding of these odorous or scented molecules to the MP surface, which could have wide scale implications for the

ecosystem due to changes in fish behaviour as a result of being unable to response to olfactory cues (Shi *et al.*, 2021).

1.5 Chemicals and plastics

1.5.1 The vector potential of MP

Due to the surface chemistry and large surface area to volume ratio, MP also have the potential to concentrate any contaminants already present in the environment. When this is combined with the Long-Range Transport Potential (LRTP) and bioaccumulation facets of this problem it highlights how MP are a key pollution problem globally (Barboza and Gimenez, 2015). Although there have been several studies looking at the potential for chemical transfer of pollutants adsorbed onto plastics into marine organisms it is important to look at the transfer in freshwater as the chemical load and range in freshwater environments is different to that in marine systems, i.e. higher concentrations of pesticides, pharmaceuticals and surfactants due to local inputs (Rochman, 2013a; Wagner *et al.*, 2014).

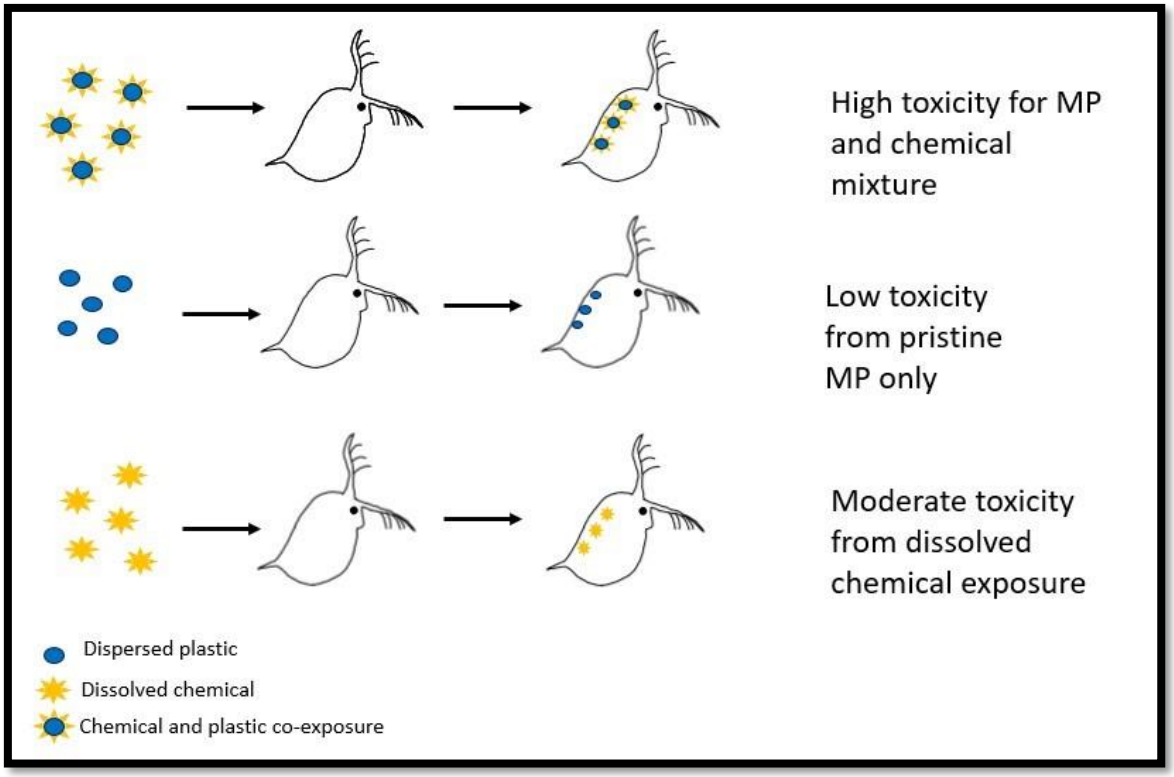


Figure 1.1 Conceptual diagram of the vector potential of MP and the hypothesised potential increase in toxicity due to the changes in exposure scenario/pathway that are currently being investigated in the MP community.

The vector potential of MP is often also referred to as the "Trojan Horse effect" and is the theory that chemicals will be able to accumulate on the surface of the MP and this will lead to an increase in the chemical toxicity within the organism as the chemicals will then be released once the plastic is ingested. This is highlighted in the top row of Figure 1.1 as the concentration of chemicals has increased due to the inadvertent uptake of the chemical via ingestion of MP compared to an aquatic exposure to dissolved chemicals only (bottom row).

The vector hypothesis is commonly referred to in theory, but it needs definitive verification, particularly in the freshwater environment. Vector capacity could have significant ecological implications due to the potential increase in uptake and transfer of MP and any associated chemicals across protective barriers in the organism (Velzeboer, Kwadijk and Koelmans, 2014;

Wagner *et al.*, 2014). It is also possible that binding of co-pollutants to MP can reduce their bioavailability to organisms, through a reduced concentration in solution, if the co-pollutant is strongly bound to the MP and not desorbed easily.

MP in freshwater systems not only pose the opportunity to act as a vector for the transfer of chemicals, but also biological contaminants and pathogens, which could have serious consequences for the water quality and the health of the ecosystem. Plastics could be detained, concentrated and then inadvertently released at WWTW, giving the plastic particles time to accumulate more biological and hazardous contaminants without the ability to remove them from the environment (Murphy *et al.*, 2016).

1.5.2 Key chemicals associated with MP so far

Due to the hydrophobic nature of many plastics they tend to accumulate other hydrophobic (organic) chemicals (HOCs) and pollutants including pharmaceuticals, Endocrine Disrupting Chemicals (EDCs) and other Persistent Organic Pollutants (POPs) present in freshwater systems (Carr, Liu and Tesoro, 2016; Koelmans *et al.*, 2016). There have been a wide range of chemicals-plastic interactions explored, both in terms of plastic additives leaching.

There is a risk of intentionally added chemicals (additives) leaching from the plastics under environmental conditioning. The most common types of additives include:

- Plasticisers to increase pliability: Phthalates*, Bisphenol A*, Nonylphenol*
- Plasticisers used as antimicrobials: Triclosan*
- Plasticisers as flame retardants: Polybrominated biphenyl* (this is now banned), PBDE-47*, Brominated Flame Retardants*

On the other hand, there is also the risk that MP can act as a sink for other environmental chemicals and could potentially concentrate the chemicals within the environmental medium and transport them to new locations or into organisms at a higher concentration (the so-called Trojan horse effect) upon ingestion. The types of co-pollutants for which this is a potential risk include:

- Polycyclic Aromatic Hydrocarbons (PAH) - resulting from the incomplete combustion of organic matter
- Dissolved metals: aluminium**, copper**, zinc**, manganese**
- Detergents and surfactants: Linear Alkyl benzene Sulphonate**, Sodium Lauryl Sulphate**, Tween**
- Pesticides: difenoconazole**, buprofezin**, imidacloprid**
- Pharmaceuticals: 17α-ethinylestradiol and 17β- estradiol- (hormones)**, Diclofenac (Non-Steroidal Anti-Inflammatory) **

Chemicals additives in plastic products have been highlighted as a key factor in toxicity response. Potential transfer of chemical additives in plastics, such as Bisphenol A, in food contact containers has been a long term concern due to the potential of these chemicals to leach into food in high concentrations (Muncke, 2011). Studies have shown that solvent extracted chemicals from clean, cryo-milled shampoo bottles were found to have a significantly detrimental effect on oligochaete health (approximately 50% reduction in the dry weight of worms), and MP that had undergone the solvent extraction step were less toxic compared to MP that had not undergone the solvent extraction treatment which had 100% mortality in the higher dose groups (8.4% of sediment as MP) (Klein *et al.*, 2021).

Chemical adsorption is affected by several factors but polymer type and particle size have significant effects on the capacity for chemical interaction and rate of transfer onto the particle surface (Bakir, Rowland and Thompson, 2014; Eerkes-Medrano, Thompson and Aldridge, 2015). Research on the adsorption and desorption of chemicals onto plastics in the environment is relatively complex as it is logistically challenging to isolate the pollutants and there are many variables that need to be considered (Bakir, Rowland and Thompson, 2012) and it is too simplistic to get a realistic understanding of such a complex problem in the laboratory as hydrophobic POPs are often in complex mixes. Hydrophobicity has a significant effect on the sorption capacity of different chemicals although it is acknowledged that it isn't the only factor (Bakir, Rowland and Thompson, 2012; Koelmans *et al.*, 2016) and it is theorised that due to the nature of the polymers, pore filling plays a large role in the transfer of chemicals onto the plastics. Competitive adsorption could have a range of effects in the environment, as chemicals that are present in lower concentration, but have a higher affinity for adsorption, could outcompete the more abundant chemicals for the binding sites, and as a result of this MP could have a significant effect on the transfer and concentration of chemicals in the environment (Bakir, Rowland and Thompson, 2012).

The majority of chemical binding studies for hydrophobic organic chemicals (HOCs) assume that the plastic surface is unconditioned by other molecules and often don't take into consideration other potential sinks for the chemical that could be present within the medium, such as dissolved organic carbon and organic colloids etc. (Koelmans *et al.*, 2016). However, if the role of the eco-corona, and specifically Natural Organic Matter (NOM) (see section 1.4-Biofilms, eco-corona and olfactory cues) is considered as a factor in these co-pollutant exposures this could change the potential chemical transfer due to the additional binding of

chemicals as a result of the NOM in the eco-corona (Lowry *et al.*, 2012; Petersen *et al.*, 2015; Saavedra, Stoll and Slaveykova, 2019). Nanomaterials have previously been coated with humic acids to optimise the ability of the nanomaterials to remove heavy metals from polluted environments (Tang *et al.*, 2014). This could change the exposure pathway of chemicals of ingested plastics due to the digestion of the surface bound NOM with the associated chemicals.

1.6 Why methods matter: current methodologies

The sampling method can have a significant impact on the amount and types of plastics found in environmental samples. Sampling method differences can arise from differences in density of the salts used in the density separation to the mesh size used to collect the samples. It is therefore imperative that all these details are included in the methodology description (metadata) to allow for a detailed comparison of the methods. A collaborative guidance document has recently been compiled to address the shortfalls in the reporting in the literature rather than in trying to standardise a method (Cowger *et al.*, 2020). This will be beneficial to ensure that sufficient details are included in published work to allow comparisons and continuations of current methods and studies and facilitate dataset integration and modelling.

1.6.1 Collection and separation of MP from environmental compartments

1.6.1.1 Water

Plastic density is key to the distribution of plastic in the water column and the sampling strategy used can have a significant impact on the concentration of plastics collected in the samples (Horton *et al.*, 2017). Although influenced by the hydrodynamic behaviour of each stream, it has been found that on average the concentration of MP in rivers was highest in the mid channel at the surface, compared to near the edges of the bank, therefore care is needed for site selection to prevent accidental bias (Eerkes-Medrano, Thompson and Aldridge, 2015).

MP can be filtered out of the water column at various depths depending on the buoyancy of the plastic. Surface, mid column and deep water or vertical hauls are commonly implemented and the mesh aperture used to filter the water has a significant impact on the amount of plastic collected (Rocha-Santos and Duarte, 2015). For example, using a plankton net (80 µm) compared to a manta net (330 µm) can lead to a 100 fold difference in the number of particles reported within the study (Horton *et al.*, 2017). However, there is often a trade off in studies with the mesh size, as the smaller the aperture the faster the mesh will clog and therefore this can be particularly challenging in areas with high algae or suspended solid contents (Prata *et al.*, 2019).

1.6.1.2 Sand and Sediment

MP are also found in sediments worldwide and samples from beaches and estuaries are commonly taken by a surface scrape or using a box corer, and deep sea sediments are usually taken using a core and a bottom trawl (Rocha-Santos and Duarte, 2015).

Once sediment samples are collected, they are then dried and sieved into the size fractions of interest. Following this the sediment is then mixed with a super saturated salt solution, often sodium chloride (NaCl with a density of 1.2cm^{-3}), sodium iodide (NaI with a density of 1.6cm^{-3}) or zinc chloride ($ZnCl_2$ with a density of 1.8 cm^{-3}). The Marine Strategy Framework Directive (MSFD) and the National Oceanic and Atmospheric Administration (NOAA) recommend the use of NaCl, due to it being inexpensive and widely available (European Commission *et al.*, 2013; Mausra *et al.*, 2015). However, there is the potential when using NaCl that some of the denser polymers are not extracted from the sediment. If NaI or $ZnCl_2$ are used, it creates a denser solution and therefore more of the polymers will be extracted which will lead to more accurate quantities of plastic being reported (Rocha-Santos and Duarte, 2015; Nel *et al.*, 2019). Following the re-suspension of any MP particles, the supernatant is then filtered onto a fine aperture filter paper and visually inspected to quantify MP extracted. This is an area of concern within the methodology, as is often hard to visually determine which particles are plastic and which are biological material (Cole *et al.*, 2013; Prata *et al.*, 2019). There is also the risk of operator selection bias based on the shape of the plastic found, as fibrous and brightly coloured plastics are more visible compared to clear or white particles on a white filter paper (Cole *et al.*, 2014). This can lead to over and under estimation, therefore it is important, to enable accurate reporting, to further analyse suspected MP to confirm they are plastics. Nile red is a widely used fluorescent staining technique used to identify microplastics within sediment samples. However, due to Nile red also staining other biological material (such as wood, chitin etc.) that could be present within the sample it is important to establish baseline levels of pixel brightness from these sources in controls (Nel *et al.*, 2021). Establishing a threshold of pixel brightness to be used for environmental samples during analysis is a key

step in using Nile red effectively to improve the reporting standards of MP in various matrices to allow comparisons between studies and sites (Nel *et al.*, 2021).

1.6.1.3 Biological material

MP samples can be identified in organisms through dissection to extract potential plastics from various tissues; however this would be limited to the larger fraction of the MP due to the physical difficulty of extracting smaller particles using this method (Desforges, Galbraith and Ross, 2015). There are also various digestion methods that can be employed to determine the presence of MP in smaller organisms, for example plankton, and for the digesting of other biological material. This can be acidic, alkaline or enzymatic, with different levels of success for each protocol (Cole *et al.*, 2014).

Acids such as sulphuric acid (H_2SO_4) and nitric acid (HNO_3) are not recommended to be used as they can damage or destroy various polymers including polystyrene and nylon, which will affect the quality and quantity of the plastics reported (Rocha-Santos and Duarte, 2015). Acids can also cause damage to the surface of the plastic which can make further analysis of the particles to determine the cause of breakdown ineffective (Cole *et al.*, 2014). (Desforges, Galbraith and Ross, 2015) employed an acid digestion protocol using HNO_3 and acknowledge the likelihood of underreporting of certain types of plastic as a result of using HNO_3. Alkaline digestion protocols, using sodium hydroxide (NaOH), have also been shown to damage MP including yellowing of uPVC, melding of polyethylene fragments and partial destruction of Nylon fibres, which can also lead to inaccurate classification of MP colours and shapes (Cole *et al.*, 2014; Nuelle *et al.*, 2014).

Cole and co-authors (2014) developed an enzymatic approach to biological digestions, using Proteinase-K, which could digest >97% of the biological material in the sample. Although this method would be more expensive for larger samples, it is much more efficient at removing biological material without damaging any of the plastics present as this protocol specifically targets biological materials leaving the MP intact (Cole *et al.*, 2014). In a comparative study of hydrogen peroxide (H_2O_2), proteinase-K, trypsin and potassium hydroxide (KOH), KOH (with a neutralisation step ahead of filtering) was the recommended treatment due to high recovery rates of the smallest MP fraction (1.2-10 µm) using mussels (Thiele, Hudson and Russell, 2019). This indicates that the material to be digested is an important consideration when deciding which digestion protocol to follow. It is important to remove biological materials from MP as several of the analytical steps can be confounded by biofouling of the particles surface, including particles filtered from sediment or water (Hoellein *et al.*, 2014; Horton *et al.*, 2017).

1.6.2 Characterisation of microplastics - laboratory studies

Due to the physical nature of MP, and the high surface area - volume ratio of the particles, it is important to understand the physio-chemical properties of the MP. Characterisation steps have been well established for nanomaterials and several of these techniques are readily applicable for MP. This can include optical techniques such as Transmission Electron Microscopy (TEM), Scanning Electron Microscopy (SEM), and particle distribution determining techniques such as Dynamic Light Scattering (DLS), UV-VIS spectroscopy and Disc Centrifuge Sedimentation (DCS) in addition to surface charge analysis such as Zeta potential (Nasser and Lynch, 2016a; Reynolds, Giltrap and Chambers, 2019).

SEM has a range of potential applications for MP characterisation and provides a good level of detail of the surface topography and composition of the particles, it works by scanning the surface across an electron beam, allowing detailed mapping of the topographic surface to be captured, for example if there is any evidence of surface pitting of the plastics (Courtene-Jones *et al.*, 2017; Kumar, Pavithra and Naushad, 2019). In addition, TEM images are useful to accurately determine morphology (shape) of the particles and particle size, and gives a clear indication of degree of agglomeration in the sample (Mourdikoudis, Pallares and Thanh, 2018; El Hadri *et al.*, 2020). UV-vis and DLS use similar principles of light interaction with the particles to determine particle size and distribution. UV-vis measures the intensity of the light reflected from the particles when compared to a standard reference material to determine the size, shape and concentration of the particles in solution (Mourdikoudis, Pallares and Thanh, 2018). DLS can be used to measure the hydrodynamic size of the particles in suspension and any potential agglomeration of the particles by measuring the scattering of a light source through a solution as the particles move under Brownian motion. The DLS method is underpinned by the fact that larger particles will move more slowly in solution and therefore the size of the particle can be correlated with the amount of subsequent light reaching the detector. Therefore, if particles are agglomerating in solution, this will lead to slower movement and a change in the detected back scattered light (Kumar, Pavithra and Naushad, 2019; El Hadri *et al.*, 2020).

Disc Centrifuge Sedimentation works by spinning an aliquot of particles in solution through a sucrose disk prior to detection with a light source. The particles move through the beam and are counted which gives an average of the particle size and the relative concentration in the sample. Due to the centrifugation, larger particles will move faster through the sucrose and

will therefore be detected first, finishing with the smallest particles in the sample taking the longest to cross the light source (Mourdikoudis, Pallares and Thanh, 2018).

Zeta potential quantifies the electrophoretic mobility of particles in an ionic solution. Surface charge on the particles leads to formation of a layer surrounding the particle of oppositely charged ions, made up of a strongly bound inner layer and a more loosely bound external layer. The movement between these layers is termed the slipping plane and can be used as a measure for the surface charge and electrostatic stability of the particles (Kumar, Pavithra and Naushad, 2019; El Hadri *et al.*, 2020).

1.6.3 Toxicity studies - *Daphnia magna*

Laboratory studies enable detailed, controlled exposures to investigate MP ingestion and trophic transfer using model organisms. *Daphnia* are an established and popular freshwater model for toxicity testing with both the OECD and REACH, they are a keystone species for regulating the ecosystem and are a very good indicator species (Nasser and Lynch, 2016a). *Daphnia* have a range of toxicity test end points including the OECD 202 (acute toxicity) and OECD 211 (reproductive success) which are some of the most widely used ecotoxicology tests (OECD, 2004a, 2012a). In addition to this, growth over time and changes to lipid deposits and/or tail length can be used as sublethal markers of toxicity with a range of modelling potential for energy uses and reproductive success (Chevalier *et al.*, 2015; Ellis *et al.*, 2020). *Daphnia* have been used in a range of ecotoxicity tests previously outline in Table 1.2 below.

Table 1.2 Summary of *Daphnia* toxicity studies and their test conditions

Study reference	Polymer / particle	Size	Chemicals	End points	Highlights of study
(Rosenkranz *et al.*, 2009)	PS	20 nm and 1000 nm	N/A	Uptake (TEM and confocal)	Comparison of natural particles and PS-NP uptake. Significantly more of the 1000nm plastic was ingested- potentially due to the animals actively selecting the larger particles. Potential misidentification of beads due to use of fluorescence.
(Besseling *et al.*, 2014)	PS	70 nm	N/A	Growth, reproduction	Potential translocation and significant effects on reproduction with reduced neonate sizes. Neonate malformations rose to 68%.
(Ogonowski *et al.*, 2016)	PE	1-5 µm	N/A	Growth, reproduction, feeding	Comparison of natural and anthropogenic particles. Decreased growth. Secondary MP had more of an effect and longer retention.

(Rehse, Kloas and Zarfl, 2016)	PE	1 and 100 µm	N/A	Survival	EC50 (96 hours) for 1 µm was 57.43 mg/L
(Jemec *et al.*, 2016)	PET fibres	62-1400 µm	N/A	Survival, uptake and egestion	Majority of fibres ingested were around 300 µm. Increased mortality in the non-pre-fed group. Daphnids were not able to recover within 24 hours.
(Imhof *et al.*, 2017)	Polymer mixtures	40 µm	N/A	Growth, reproduction, gene expression,	No significant changes to morphological traits in adult *Daphnia* but changes to gene expression. Juveniles showed some subtle changes in morphological traits (body and tail length etc).
(Frydkjær, Iversen and Roslev, 2017)	PE	10-106 µm 10-75 µm	Phenanthrene	Survival, ingestion, egestion	Egestion of irregular shaped MP was slower than spherical
(Horton *et al.*, 2018)	PS	1 µm	Dimethoate and deltamethrin	Mobility and survival	PS exposure alone had no effect but combined effect with chemicals lead to increased

					immobilisation and mortality
(Schür *et al.*, 2020)	PS	63 µm	N/A	Growth, reproduction, and sensitivity to potassium dichromate	MP led to increase mortality and impact life-history end points compared to natural particles under low food when compared to natural particles (kaolin).
(Elizalde-Velázquez *et al.*, 2020)	PS	6 µm	N/A	Uptake and egestion, translocation	Rapid depuration rates and limited retention with no noted translocation
(Kelpsiene *et al.*, 2020)	PS	53 nm	N/A	Reproduction, survival	Decreasing trend of number of offspring per daphnid over life time and a decrease in survival over time.
(Nasser and Lynch, 2016a)	PS	80-100 nm	N/A	Uptake and mortality	*Daphnia* secreted protein eco-corona leads to increase in uptake and toxicity of PS

In addition to already being an established model species for regulatory testing, *Daphnia* are an advantageous model for MP toxicity testing due to their clear body which allows effective visualisation to highlight the extent of uptake and gut retention. Increasingly, machine learning approaches are being applied to assess the toxicity of nanomaterials to daphnids,

which may also be applicable to MP, as they are based on phenotypic changes rather than quantification of the internalised dose (Karatzas *et al.*, 2020).

As outlined in Table 1.2, *Daphnia* have been widely used as a model organism in MP toxicity testing. Prior to this, *Daphnia* have been widely used a model species in chemical testing and ecosystem effects modelling, and more recently in nanomaterial toxicity studies. This makes them an ideal test species for ongoing MP and MP chemical mixture toxicity work, and previously, the modes of toxicity have been explored, particularly with exposures to pristine and environmentally aged particle based pollutants (Ellis, Valsami-Jones and Lynch, 2020). Although *Daphnia* are relatively simple anatomically when compared to higher order aquatic species such as fish, they have an extensive endocrine system which has been demonstrated to be highly responsive to endocrine disrupting chemicals and pharmaceuticals, which makes them a useful species to work with (Peake *et al.*, 2016). In addition, due to the clonal reproduction of *Daphnia* under standard testing conditions, the impacts on the epigenetic profile, transcriptional and phenotypic response can be further explored to look at population level effects (Ellis and Lynch, 2020).

Studies have previously been undertaken using fluorescently labelled industrial beads. This theoretically can aid the identification of the beads to enable confirmation of ingestion, and potential storage within the organism's tissue (Rosenkranz *et al.*, 2009). However only using fluorescence to determine the transfer of MP has been proven to be flawed, as the dye can leach from the beads and be retained in the lipid deposits and other tissues leading to incorrect tracing of MP transfer (Schür *et al.*, 2019). There is also the issue of the change of internal biological conditions which can affect the fluorescent signal from the dye and

therefore can significantly affect the results (Triebskorn *et al.*, 2019). In addition to this, the internal gut pH of test organisms may differ than that of the stock solution or the testing medium, for example, the lower gut in *Daphnia* has been shown to be pH 5.5-6 compared to the culturing medium which was pH 7.8, this could interfere with the dye fluorescence and the dye binding or leaching from the particles (Davis *et al.*, 2020).

1.6.4 Analytical techniques

Two of the most widely used and accepted methods of determining the polymers found in environmental samples are Fourier Transform Infra-Red (FTIR) spectroscopy and Raman spectroscopy, other methods such as thermo-gravimetric analysis (TGA) have been trialled but are currently less widely used (Horton *et al.*, 2017). Raman and FTIR are beneficial as they do not destroy the samples and can be used on particles down to 20 µm and can be used in combination with other methods (Prata *et al.*, 2019). FTIR can be modified to scan the surface of the particle or used in transmission mode for thicker or opaque particles however this is often limited on the window size for a reliable scan (depending on the method this could be around 1mm depending on the particle shape) and can be confounded by additives in the plastic matrix (Horton *et al.*, 2017; Jung *et al.*, 2018). Raman spectroscopy can also be confounded by any additional pollutants on the particle surface such as organic matter or surface coatings such as paint or degradation as this interferes with the Raman scan.

Another method that is increasingly been used is Pyrolysis Gas Chromatography- Mass Spectrometry (pyrolysis GC-MS) which is a destructive method compared to FTIR and Raman, as the sample is used up during the analysis and therefore no subsequent measurements can be taken. However, pyrolysis GC-MS can be used to run bulk samples, and this can speed up

the analysis process (Okoffo *et al.*, 2020). In addition, this can be used to identify additives in the MP particles and can be used to identify chemical degradation, however this method does not give information on particle size or number (Prata *et al.*, 2019). One of the best approaches going forwards would be to combine several complimentary techniques to enable us to build a clear, and more accurate picture of the polymers found, however more techniques used on a sample will decrease the throughput and therefore a balance needs to be established.

1.6.5 Quality control

It is imperative, due to the widespread use of the plastics and the microscopic sizes of the particles being studies, that contamination is prevented and accounted for. This has been done through a range of methods for example using clean metal and glass instruments and vessels for research (Cole *et al.*, 2014). Cotton clothing is commonly worn during research to prevent the transfer of any textile particles whilst the work is being conducted and particle traps or simple agar-filled petri dishes have also been used in some laboratory studies to provide a control for any contamination within the laboratory during the study (Woodall *et al.*, 2015; Catarino *et al.*, 2018). Clean rooms and clean air filters can reduce airborne microfibre contamination of samples by up to 96.5% which could previously have led to an overestimation of the microfibre fraction of environmental samples (Wesch *et al.*, 2017). It has also been highlighted that some of the extraction methods used can lead to contamination of samples with plastic from the nets or sediment separation units that utilise plastic parts, which with some simple modifications could significantly reduce the amount of MP contamination introduced into the samples (Nel *et al.*, 2019). Blanks and controls are also an important part of the QA/QC step in MP studies, and this is often done with fibre traps and

procedural blanks during sample processing to account for and identify potential sources of MP contamination (Prata *et al.*, 2019). The QA/QC steps and any identified contamination identified should be included in the sample reporting (Prata *et al.*, 2019; Cowger *et al.*, 2020).

1.6.6 Realistic concentrations

Often the research undertaken for ecotoxicology studies have very extreme concentrations of contaminants and plastics to entail a response, which are frequently greatly elevated compared to what has been found presently in the natural environment (Bejgarn *et al.*, 2015; Lambert and Wagner, 2016a). It is important to acknowledge the more extreme concentrations as although the results increase our understanding, it is crucial not to directly translate this into potential impacts in the natural environment (Eerkes-Medrano, Thompson and Aldridge, 2015). The distribution of MP reported in the environment is heterogenous and therefore, by identifying environmental hotspots, ecosystems and therefore species at high risk of interaction with MP can also be identified enabling a more holistic understanding of the risk of MP (Nel *et al.*, 2020).

Environmental concentrations can vary based on the sampling method, location and matrix and are often reported in different units making comparisons between regions challenging. Values reported in the literature range from a maximum of 0.32 and minimum 0.00012 particles per litre in river water samples (Great Lake tributaries) and maximum 616.1 and minimum 1.2 particles per kg in lake sediments (Lake Ontario) (Horton *et al.*, 2017). This demonstrates the variability of MP particles that are currently found in the environment due to the range of study approaches in addition to the actual environmental variability. The 100 Plastic Rivers project (University of Birmingham, 2021) aims to collect baseline data of MP

contamination using standardised sampling, extraction, and quantification to allow this wide-scale spatial comparison of 100 rivers globally. This data will help further the understanding of baseline microplastic concentrations and help to identify potential hotspots within this. The current variability in MP concentrations reported highlights the need for more ecotoxicology studies that have more realistic environmental concentrations (spanning the range of concentrations from background to hot-spot concentrations) and conditions to be able to have data sets that can be used to directly feed into policy (Lenz, Enders and Nielsen, 2016). However it has been observed that the methods for sampling environmental MP often uses mesh sizes of 333/330 µm and therefore doesn't accurately represent the plastics used in toxicology studies which are often much smaller than this including down to the nanoscale (Athey *et al.*, 2020).

1.7 Plastic, People & Policy

Following the increasing media attention on the detrimental impact of MP pollution, there has been a growing number of environmental groups and members of the public engaging with this issue. Changes to existing legislation and the addition of new polices are working towards minimising and mitigating the environmental consequences of MP pollution. In addition to this there are technological advances to provide alternatives to plastics and attempt to remove current MP from the environmental system. A combination of these approaches and an international effort is a promising start to addressing this ubiquitous issue.

1.7.1 Environmental legislation: MARPOL, MSFD & WFD

One of the earliest pieces of legislation to protect the marine environment was the International Convention for the Prevention of Pollution from Ships (MARPOL) in 1983, which has since been amended with Annex V in 1988, to take into account the dumping of waste (and plastic specifically) overboard (Rochman, Cook and Koelmans, 2016; IMO, 2020). MP pollution was first explicitly highlighted by the Marine Strategy Framework Directive (MSFD) 2008 which formed a specific marine litter task group which focuses on MP in descriptors 2, 8 and 10 (Galgani *et al.*, 2013; Gago *et al.*, 2016). In addition to the MSFD task force, other organisations have also assigned task forces and focus groups to find ways to address the issue including; NOAA Marine Litter Task Force (USA), US Environmental Protection Agency (USA), Ministry for the Environment (Canada), European Commission (Europe), North-west Pacific action plan (Asia), Department for Environmental Affairs (South Africa) and the UN Environmental Programme (Loizidou, Loizides and Orthodoxou, 2014).

Although there are some policies in place for the monitoring and mitigating of MP pollution in marine systems (such as MARPOL and MSFW), the focus has mainly been on marine litter and there is currently a shortfall in the policy protecting freshwater systems from the same pollutants (Bell, McGillivray and Pedersen, 2013; Eerkes-Medrano, Thompson and Aldridge, 2015). Currently, the Water Framework Directive (WFD) does not cover MP, or engineered nanomaterials (ENM), leaving freshwater environments unprotected (Gago *et al.*, 2016; Lynch, 2016). This is particularly important when considering legislative protection of the environment, it has been calculated that approximately 70% of marine pollution (collectively, not specifically MP) are from freshwater sources and 56% of this pollution is currently regulated at the point of origin (Bell, McGillivray and Pedersen, 2013). This emphasises the

potential effectiveness of enforcing the current regulations and protecting freshwater environments from MP pollution by updating and expanding existing legislation, such as the WFD, to include MP and ENM (Bell, McGillivray and Pedersen, 2013)(Bell, McGillivray and Pedersen, 2013). It is more comprehensive and holistic to manage freshwater and marine ecosystems together and not as isolated entities due to the transboundary nature of the problem, and the legislation needs to reflect this. This transboundary nature of pollution was considered during the implementation of the WFD strategy for managing water by catchment basins as opposed to administrative boundaries (Lynch, 2016).

An example of the complexity of the monitoring issue is the River Danube, Europe's second largest river and the world's most international river, with a drainage basin extending to 19 countries (Lechner *et al.*, 2014). In a study of the transportation of plastic in the Austrian Danube, an estimate of 4.2 tonnes of plastic was reported to be being deposited into the Black Sea each day via the Danube (Lechner *et al.*, 2014). However, it is likely that there is more plastic entering the Black Sea due to (1) the mesh size used for sampling not being efficient at removing the smaller plastics (<500 µm), (2) particles that are negatively or neutrally buoyant are missed during the surface sampling procedure used and (3) not being able to accurately calculate the input from neighbouring countries due to the difference in quality of WWTW and monitoring (Lechner *et al.*, 2014). A subsequent study (Lechner and Ramler, 2015) investigated a point source of industrial microplastics into freshwater systems. They found that the company (Borealis- a plastic production company) emitted approximately 200g of industrial MP per day under standard operating conditions, which is stated as being below the maximum legal limit of MP discharge per day of 30 mg/L. Once scaled up this totals an estimate of 94.5 tonnes per year from a single point source (Lechner and Ramler, 2015) which

highlights how industrial MP are legally emitted into the environment globally, and that the required legal amendments to prevent these emissions are long overdue.

1.7.2 Plastic legislation: Microbead ban, bag charges and latte levy

Although 50% of polymers produced globally are constructed of monomers that the UN Globally Harmonized system considers to be hazardous and are regulated under REACH, this legislation doesn't currently extend to the polymer (Lithner, Larsson and Dave, 2011; Rochman, 2013a). However, evidence based policy change has the potential to be very effective, for example the microbead ban which is currently being implemented or proposed by many countries and states worldwide (including the USA, Canada, UK, Ireland, Australia, the Netherlands, Sweden and Denmark with Taiwan and South Korea currently proposing legislation) (Rochman, Cook and Koelmans, 2016; Schnurr *et al.*, 2018). However, the MP ban in the UK is only addressing the 'wash off' beads in cosmetics and does not cover beads contained in products designed to be left on (DEFRA, 2018b), such as in nail polish and moisturisers. This bead ban also doesn't cover microbeads in other products such as printer toner, cleaning products or industrial abrasives (Schnurr *et al.*, 2018). Therefore, there needs to be more comprehensive legislation or a plan to implement and tighten up on the other sources such as household products and industrial microbeads in the future.

Plastic waste has also been addressed in the 25-year Environment Plan laid out by the UK government in 2018 which is also supported by a call for evidence for the feasibility of using taxation as a method to help address single use plastics (DEFRA, 2018a). The 25-year Environment Plan is supported with statutory targets that have been proposed in the Environment Bill which has been proposed to parliament in March 2020 with targets to reduce

all Single Use Plastics (SUPs) by 2042 (House of Commons, 2020). This Bill encompasses a proposed money back deposit scheme for plastic bottles as well as a ban on straws, cotton buds and stirrers, although this ban has currently been postponed due to the COVID-19 situation (DEFRA, 2020).

Managing the source and effects of plastic pollution forms part of the Environment Bill in terms of implementing a polluter pays principle for certain types of single use plastic waste (such as coffee cups) in the form of a "latte levy", and it is proposed to pass this cost (20-25p) onto the consumer to encourage a change in consumer behaviour (DEFRA, 2020). This will also support the EU strategy for circular economy which is set to reduce the amount of SUPs by 2021 with a targeted approached to reducing the use of the 10 most commonly found items on beaches such as takeaway food containers, balloons and water bottles (accounting for 70% of marine litter found), or substituting them with a less harmful/long lasting material (European Commission, 2018). This is calculated to save €22 billion by 2030 in environmental damage and clean-up costs (European Commission, 2018).

1.7.3 Societal pressure and response

Social science can be used to influence a positive behavioural change (Rochman, Cook, and Koelmans 2016) although an anthropocentric focus is often required to influence large numbers of people. For example, in 2008 it cost the UK €1-2 million in rescue operations to free boats that had been entangled with plastic and €18-19 million in clean-up operations for debris on UK beaches (Rochman, Cook, and Koelmans 2016); this can serve as a good incentive for change.

The Great Nurdle Hunt is an effective social science project organised by Fidra, a charity based in Scotland, UK, but has data from around the world. This has been a useful social science resource to monitor the loss of plastic production pellets, often termed nurdles, into the environment (PlasticsEurope, 2019a). The project encourages individuals and groups to look for nurdles on their local beaches in a set amount of time and report the data. This has allowed them to build the Great Nurdle Hunt map which shows the distribution of nurdles around the world. The social science project is particularly successful because it does not require any equipment and is something that almost anyone can do on a beach visit. Although this project can run all year with data being submitted continuously, there is a Great Nurdle Hunt organised annually for a global effort. This has been successful in previous years with 352 nurdle hunts taking place in 32 countries (spanning all 7 continents) during the 9-day period in 2019 (Fidra, 2019). The results of this campaign can help feed in as evidence for required policy change, and could be used to monitor how effectively initiatives to reduce plastic loss from manufacturing can be (Fidra, 2019) although it would be difficult to pinpoint the exact source of these nurdles without tagging or further analysis of specific compositions.

The plastic bag charge introduced in the UK in October 2015 has had a significant impact on the number of single use plastic bags produced annually. It is estimated that the average person now uses only 10 single use bags per year compared to 140 per year in 2014 before the ban was introduced (DEFRA, 2019). In addition to this, the bag charge has raised £169 million for various charities in this time whilst the use of bags has dropped by 90% (DEFRA, 2019). As a result of this, there has been a 30% decrease in the amount of plastics bags found in a deep sea monitoring programme during this period indicating that the reduction of bags

being used is also having a wider impact on reducing environment pollution (Maes *et al.*, 2018).

There are discussions within the plastic and polymers industry to move away from the single use plastic model and attribute a higher value across the board to plastic as a resource, such as through Operation Clean Sweep (OCS). OCS has 6 main commitments to reduce the spillage of plastic pellets including: improving worksite set ups, providing training in spill prevention and response, auditing performance and suggesting procedures, ensuring adherence to local legislation and encouraging partners to pursue the same goals (Operation Clean Sweep, 2019). OCS is a global initiative and in Europe is headed by Plastics Europe since 2015 and in the 2018 report, there were over 500 signatories - 90% of Plastic Europe's members were taking part in the initiative which accounts for 98% of the market share (PlasticsEurope, 2019a). 100% of signatories completed a questionnaire to enable baseline reporting for progress going forwards, which will form the basis of the 2019 report for OCS. Current analysis shows that 98% of signatories have benefited from the OCS project management in terms of their pellet management. Although there are currently no national or local regulations in Europe for the contamination and loss of pellets, companies are using the OCS as part of their external audit criteria and as evidence towards ISO 14001 and ISO 9001 certification (PlasticsEurope, 2019a) demonstrating the wider implications that this project can have.

1.7.4 Technology changes

The nano research field sets the precedent for a lot of the MP challenges, as similar discussions were held within this field 5-10 years ago. There is discussion to implement a "Benign by Design" approach for nanomaterials, by maintaining the key function of the particles whilst

removing the hazard that can be posed through alternative synthesis stages (Lynch, 2016). Although a lot of MP are not engineered in the same way ENM are, there are lessons to be learned from the ENM examples such as changing the fibre structure and framework in clothing to reduce the amount of fibres that are lost over time (Carney Almroth *et al.*, 2018).

In response to the research on how many microfibres are shed from clothing during wash cycles, filters are being developed to help reduce the number of fibres released from washing machines to wastewater. These filters have variable success rates depending on capture, for example 26% reduction with a Cora Ball (added to the drum with the laundry load) compared to 87% with a lint LUV-R (externally fitted to filter the wash water) when compared to a control (McIlwraith *et al.*, 2019). An additional method for particle capture is the GuppyFriend, which is a polyamide mesh laundry bag that synthetic clothes are placed in before washing, which is claimed to remove about 70% of the microfibres released from washing synthetic clothing. During a study investigating the consumer interest and understanding of the MP fibres issue, when comparing the Cora Ball, GuppyFriend and Filtrol 160 (a similar application to the Lint LUV-R) the most popular choice was the Cora Ball (49%), compared to the least popular GuppyFriend (16%) due to a combination of factors such as cost and perceived effectiveness (Herweyers *et al.*, 2020). This is a large consideration for suggested behaviour changes, as conflicting information, significant costs and difficulty with access or application can be significant barriers to individuals wishing to engage with the issue.

The Ocean Clean-up Project has been proposed as a solution to the vast amounts of plastics that are accumulating in the ocean gyres. Using the movement of the ocean and a floating boom with a submerged skirt, it is intended to capture and concentrate the plastic particles in

sizes from mm to larger macro plastics that are floating in the ocean to allow for easier collection (Ocean Clean-up Project, 2019). This a contentious issue at present due to the uncertainty of the impacts this could have on the biota living in the gyres and the potential for them to be trapped along with the plastic. During the pilot study, observations were conducted from boats to determine if there was any potential for harm to biota, (Ocean Clean-up Project, 2019) but this is limited to larger organisms, such as marine mammals. Due to the lower size range proposed to be collected, there could be a significant impact on the lower trophic levels of the food web or on drifting organisms, such as jelly fish, that would likely be caught up with the plastic (5Gyres, 2020). This could have wider detrimental effects on the ecosystem, despite the positive benefits of the clean-up effort. It also has the potential to appear as an easy fix to the public or policy makers and therefore reduce the concern and efforts of people to reduce the amount of plastic entering the environment (5Gyres, 2020).

1.7.5 What does the future hold for MP?

Due to delays in policy and the future legacy inputs from secondary MP, MP and nano plastics are going to be an environmental pollution issue for many years to come (Eerkes-Medrano, Thompson and Aldridge, 2015). It is anticipated that if a 'business as usual' approach is adopted the plastic debris found in the environment will increase by an order of magnitude by 2025 (Rochman, Cook and Koelmans, 2016). Therefore, further research needs to be undertaken to create evidence-based policy changes which will be effective. The development of a Risk Assessment framework would be a powerful tool for ecosystem managers and policy makers; however, a precautionary approach should be taken until we have a strong enough evidence base to develop this tool (Rochman, Cook and Koelmans, 2016).

There is increasing concern regarding the impact that MP can pose to human health via exposure in the food chain. Although there is very limited evidence and not many studies into the impacts of MP on humans there is evidence that MP are present within the human food chain (including in agricultural crops and plants) (Oliveri Conti *et al.*, 2020) and in organisms that are intended for human consumption (Kosuth, Mason and Wattenberg, 2018; Hernandez *et al.*, 2019). If there is evidence of detrimental impacts to human health this could have disastrous consequences for the aquaculture and fisheries economies (Eerkes-Medrano, Thompson and Aldridge, 2015). There are a wide range of ways that MP can have an impact on humans both directly and indirectly including through drinking water and food, bathing water, water use logistics and ecosystem services (Eerkes-Medrano, Thompson and Aldridge, 2015) and this is likely to be a key concept used to encourage change going forwards.

Due to the complex nature of the chemical transfer and biotic interaction with MP it is important that multifactorial exposures are conducted in the future with a combination of biotic (e.g., organisms and biofilms) and abiotic (temperature, salinity, pH) conditions to further understand this issue (Bakir, Rowland and Thompson, 2012). In the long term, we need to aim policy, technology and societal changes at reducing plastic emissions and leading to a more circular economy and increasing the perceived value of plastics to minimise the MP threat (Kalogerakis *et al.*, 2015; Napper *et al.*, 2015a).

1.8 Objectives and thesis structure

The overarching aim of the thesis was to take an analytical approach to the different stages of study design for assessment of the toxicity of microplastics using the model species *Daphnia*

magna, from dispersion to multi-stressor studies. The three objectives break down the different stages of the study design with the intention of increasing our understanding of how potentially unconscious assumptions in study design may affect the overall toxicity endpoints following microplastic exposure to *D. magna.*

1.8.1 Objective 1

Do dispersal methods affect the subsequent toxicity of polyethene MP exposures?

This chapter explores the following questions in detail: By characterising polyethylene (PE) beads that have been dispersed using a range of methods is it possible to recommend more environmentally realistic dispersion methods compared to the artificial surfactants that are currently recommended by manufacturers and which may themselves be toxic? Do the different methods of dispersion lead to any significant variation in acute toxicity and are there any subsequent impacts on the particle's interactions with daphnia secreted biomolecules (specifically proteins)? (Chapter 3)

1.8.2 Objective 2

How does different culturing medium affect the baseline chronic toxicity response in *D. magna?*

Here, the effect of using different media (standard salt based culturing medium, model river waters of varying ionic strengths and natural organic matter contents, and local borehole water), was explored to understand how life history parameters (total neonates and growth) vary over a 21-day test period for both controls and exposures using a chemical (SDS) or a MP such as PE. From this, the question of whether daphnid fitness in the optimised toxicity testing

medium gives the organisms an unfair advantage, and how this can be factored into extrapolation from lab to field during risk assessment can be explored. (Chapter 4)

1.8.3 Objective 3

Does the addition of MP to chemical exposures change the effect concentration (EC_{50}) of the chemical and if so how?

Standard acute toxicity tests to determine the effect concentration (EC_{50}) of three chemicals were initially undertaken prior to mixture exposures with a combination of chemical and PE MP to determine the effect that microplastic can have on the chemical toxicity endpoints. This was done in various media (explored in chapter 3) to ascertain the variability that multiple parameters can have on well-established acute toxicity endpoints and as a first look into mixture toxicity. Here, the impact of the biomolecule corona was explored, since in real environments co-pollutants must compete with biomolecules to bind to the MP surface which is currently not factored into studies of Trojan-horse type effects. (Chapter 5)

1.8.4 Thesis structure

An initial literature analysis was undertaken to establish the current state of the science in the field of microplastic research in the broader context and to identify potential assumptions or oversights in the design of toxicology studies. The protocols used in the studies performed throughout this thesis are compiled in Chapter 2 to support subsequent research in the field. Following this, three original research chapters are presented in paper-style as self-contained studies. Finally, a synthesis and future directions chapter draws together the key results of the

original research in moving the state of the science for toxicity study designs forwards, with suggestions of avenues for future research to build on this discussion.

Chapter 2

2. Methodology

2.1 *Daphnia* culturing

2.1.1 Introduction and principles

As a model organism, Daphnia are cultured in a controlled environment to establish the baseline health of the organisms in order to effectively test for toxicity response (deviations from the baseline health observations) to a range of different substances and conditions. The *Daphnia* facility at the University of Birmingham has a primary culturing room to maintain the *Daphnia* cultures at 20°C (±1°C) and a 16:8 light: dark cycle. Bham 2 strain *Daphnia magna* were used for all exposures and experimental work.

2.1.2 Resources and reagents

- Aerated medium or borehole water
- Glass jars (1L with metal screw tops)
- *Chlorella vulgaris* algal feed* (refrigerated to 4°C)
- Ethanol spray
- Glass pipette
- Light box

*Algae culturing information can be found in Annex 1.

2.1.3 *Daphnia* medium preparation

Stock solutions of the main salts used for the various *Daphnia* media were prepared and stored in the main prep room in the *Daphnia* facility. Stocks indicated with (*) in the tables below (Table 2.1-2.3) are stored at 4°C in the fridge. Aliquots of these stocks were then added to Milli-Q water as required to make up the working concentration of the various media and left to aerate for a minimum of 2 hours but ideally overnight (12 hours).

Table 2.1 Medium composition and stock salt preparations for Artificial River Water Class 1 based on (Hammes, Gallego-Urrea and Hassellöv, 2013)

ARW1					
pH 7.3-7.7		Stock conc	1L	4L	
		M, g/mol	m, mg	m, mg	(in g)
Powder	Calcium sulphate dihydrate ($CaSO_4$)	172.17	1.722	6.888	0.0068
Powder	Calcium carbonate ($CaCO_3$)	100.09	2.002	8.008	0.008
		C, mol/L	V, µL		V, µL
Aqueous	Calcium nitrate tetrahydrate ($Ca(NO_3)_2$)	1	5		20
Aqueous	Magnesium nitrate hexahydrate ($Mg(NO_3)_2$)	1	48.9		195.6
Aqueous	Sodium bicarbonate ($NaHCO_3$)	1	13.5		108
Aqueous	Calcium chloride ($CaCl_2$)	1	7.5		30
Aqueous	Potassium bicarbonate ($KHCO_3$)	1	11.6		46.4
Aqueous	NOM*	1	1.84		7.36

Table 3.2 Medium composition and stock salt preparations for Artificial River Water Class 5 based on (Hammes, Gallego-Urrea and Hassellöv, 2013)

ARW5					
pH 7.8-8.2		Stock conc	1L	4L	
		M, g/mol	m, mg	m, mg	(in g)
Powder	Calcium sulphate dihydrate ($CaSO_4$)	172.17	155.02	620.08	0.6201
Powder	Calcium carbonate ($CaCO_3$)	100.09	8.007	32.028	0.032
Powder	Magnesium carbonate ($MgCO_3$)	121.41	45.517	182.068	0.182
		C, mol/L	V, µL	V, µL	
Aqueous	Calcium nitrate tetrahydrate ($Ca(NO_3)_2$)	1	165	660	
Aqueous	Sodium bicarbonate ($NaHCO_3$)	1	696.6	5572.8	
Aqueous	Calcium chloride ($CaCl_2$)	1	363.5	1454	
Aqueous	Potassium bicarbonate ($KHCO_3$)	1	85.9	343.6	
Aqueous	NOM*	1	4.6	18.4	

Table 2.4 Medium composition and stock salt preparation for HH COMBO based on (Kilham *et al.*, 1998a)

HH COMBO				
pH 7.6-7.8		Stock conc	1L	4L
		C g/L	V, ml	v, ml
Aqueous	Calcium chloride dihydrate ($CaCl_2$)	110.28	1	4
Aqueous	Magnesium sulphate heptahydrate ($MgSO_4$)	113.5	1	4
Aqueous	Potassium phosphate dibasic (K_2HPO_4)	1.742	1	4
Aqueous	Sodium nitrate ($NaNO_3$)	17	1	4
Aqueous	Sodium metasilicate nonahydrate ($NaSiO_2$)	28.42	1	4
Aqueous	Boric acid (H_3BO_3)	24	1	4
Aqueous	Potassium chloride (KCl)	5.96	1	4
Aqueous	Sodium bicarbonate ($NaCO_3$)	63	1	4
Aqueous	Sodium selinate ($NaSeO_4$)*	40 µg/mL	50 µL	200 µL
Aqueous	VIM*	50 µg/mL	0.5	*2
Aqueous	Animate*	100 mgL	1	*4

2.1.4 Protocol

1) *Daphnia* were cultured in groups within their respective laboratory medium, typically 15 adults were maintained in 900 mL of medium (in 1L jars) with metal screw tops loosely fitted.
2) Medium was prepared following the standard procedures (see below, section 2.1.3 medium preparation) and allowed to aerate for a minimum of 2 hours, but typically overnight.

3) The pH of the medium was checked and altered as required with either 0.1M NaOH or 1M HCl to be within the specified range and any final medium constituent (VIM and animate) were added.
4) Medium was then poured into fresh culturing beakers, and *Daphnia* were carefully transferred using a glass pipette. A lightbox was used to make the *Daphnia* more visible within the jars at this stage if required.
5) Once *Daphnia* were added to the new culturing jars, algal feed was added in the following ratio, 0.5 mg C for days 0-7 and 0.75 mg C for days 7 onwards (based on 15 *Daphnia*/900 mL).
6) Glassware was washed with 70% ethanol spray and hot water to prevent any bacterial growth.
7) To split running cultures, adult *Daphnia* were removed from the medium and neonates were then filtered carefully through a mesh to concentrate them within the medium for exposures, or alternatively a lightbox was used to view the *Daphnia* and neonates were selected and transferred to new culturing jars to start the new generation of running cultures.

   N.B Running cultures were established with neonates from the third brood of running cultures to maintain optimum genetic health of the organisms.

2.2 *Daphnia* exposures- acute

2.2.1 Introduction and principles

The acute exposure protocol follows the guidelines set out by the OECD 202 test for *Daphnia* (OECD, 2004a). The principle of the test is that daphnids are exposed to a series of different concentrations of a toxicant and are exposure for 48 hours. Observations are made at the 24 and 48-hour intervals to assess for "immobilisation" within the *Daphnia* test, which is defined as a daphnid that is not moving/swimming after gently agitating the vessel for 15 seconds (disregarding any movement of their antennae). Immobilisation is often used as it is hard to visually determine *Daphnia* death without the

use of a microscope, and this therefore speeds up the observations. As a result of the immobilisation being used as the end point for the test, results are reported as the effect concentration (EC_{50}) compared to lethal concentrations/dose (LC_{50}).

2.2.2 Resources and reagents

- Aerated medium or borehole water
- Glass pipette
- Light box
- Toxicants/stock solutions
- Test vessels

2.2.3 Protocol

1) Neonates were filtered from the running cultures and pooled from the different culture jars. *Daphnia* exposures were undertaken with broods 3-6 from the healthy running cultures. Cultures were maintained in the same medium that the exposure was conducted in to remove any confounding factors associated with the change in medium.
2) Neonates were then allocated to a test vessel from the pooled stock to ensure that there is no bias associated to the different culture jars that could confound the results.
3) *Daphnia* were grouped, typically 10 individuals per vessel for an acute test unless otherwise stated, with a minimum of 3 vessels per treatment, in fresh medium.
4) The toxicant was then added to the test vessel in the nominal concentration outlined in the study.

   N.B. As stated in the OECD 202 test protocol *Daphnia* were not fed during the duration of the test.
5) The labelled test vessels were then pooled and stored within the CT laboratory for the duration of the test unless otherwise stated.

6) For observation of results, test vessels were randomly selected, and results were recorded to minimise operator bias and fatigue within observations.

2.2.4 Data capture, processing, and presentation

Total immobilisation during the test period was recorded for each of the test vessels. This data was then plotted for a dose response curve with a log transformation of the concentration (x axis) to establish the sigmoid response curve over the 50% effect concentration range. This allows a more accurate Effect Concentration (EC_{50}) to be calculated. EC_{50} values can then be compared to other toxicants to establish a relative ranking of exposure hazard.

2.3 *Daphnia* exposures- chronic

2.3.1 Introduction and principles

Daphnia chronic toxicity response can be established using total reproduction and growth over a 21-day testing duration. The chronic toxicity exposures are based on the OECD 211 *Daphnia* chronic reproduction test (OECD, 2012a). Observations are made over the test durations for time to first brood, time between broods, total neonates per brood (and over the whole period) and growth over time, often measured from the eye to the tail spine. These observations allow for sublethal toxicity to be observed in the *Daphnia* and the impact of the toxicant on the reproductive health of the *Daphnia* to be determined.

2.3.2 Resources and reagents

- Aerated medium or borehole water
- Glass pipette
- Light box
- Test vessels (50mL glass vials) and racks

- *Chlorella vulgaris* algal feed (refrigerated to 4°C)
- Nikon stereomicroscope with camera (or microscope with a camera fitting to enable images to be taken)
- Access to image analysis software (such as Image J- which is open access ImageJ (nih.gov))

2.3.3 Protocol

1) Neonates were filtered from the running cultures and pooled from the different culturing jars. NB. *Daphnia* exposures were done with broods 3-6 from the running cultures. Cultures were maintained in the same medium that the exposure was conducted in to remove any confounding factors associated with the change in medium.
2) Neonates were then allocated to a test vessel from the pooled stock to ensure that there is no bias associated to the different culture jars that could compound the results.
3) *Daphnia* were maintained individually in the 50mL test vessels, with typically 12 replicates per treatment.
4) The toxicant was then added to the test vessel in the nominal concentration outlined in the study.
5) The labelled test vessels were then pooled and stored within the CT laboratory for the duration of the test.
6) For observation of results, test vessels were randomly selected, and results were recorded. Typically, *Daphnia* were imaged on Day 0, 7, 14 and 21 during chronic tests and neonates were counted daily.
7) For imaging, *Daphnia* were removed from the test vessel and placed on a glass slide, excess medium was removed to limit the *Daphnia* movement to enable a clearer image to be taken. The slide was transferred to the microscope stage, focus adjusted, and image taken as quickly as possible to reduce the stress to the *Daphnia*. Once imaged, the *Daphnia* was returned to the test vessel. Light intensity and magnification were recorded on the respective observation

sheets at the time of imaging. A scale bar was included with each photo to enable subsequent growth measurements.

8) Neonates were removed from the test vessel at time of observation. Care was taken to ensure minimal medium was removed.
9) Testing medium was replenished three times per week as outlines in steps 3 and 4 above.
10) Images were measured for total growth (centre of the eye to base of the tail spine) and total length of tail to allow for total growth to be calculated using Image J software.
11) At the end of the 21-day testing period *Daphnia* would be discarded or could be retained for subsequent lipid analysis (see section 5).

2.3.4 Data capture, processing, and presentation

Daphnia observations were recorded in the data sheet outlined in Table 4 below. Daily observations were recorded, and this allows for subsequent analysis for total neonates or potential delays to broods etc.

Table 2. 5 Daphnia observation data capture sheet for chronic toxicity studies

Date	Day of Culture	Culture/Jar						Offspring						Food	Media Change	Observations/Comments
		1	2	3	4	5	6	1	2	3	4	5	6			

In addition, measurements were taken for growth over time using image analysis software, such as Image J. This allowed the growth of the *Daphnia* to be measured by using the captured microscope images from the study. Typically, measurements were taken from the centre of the eye to the base of the tail spine (Figure 2.1) and then from the base to the tip of the tail for tail length. This measurement can then be replicated across all *Daphnia* measurements fairly consistently however, this measurement will use the carapace of the *Daphnia* and therefore will not take into consideration potential variability in mass. Calibration was based on the scale bar for the respective images.

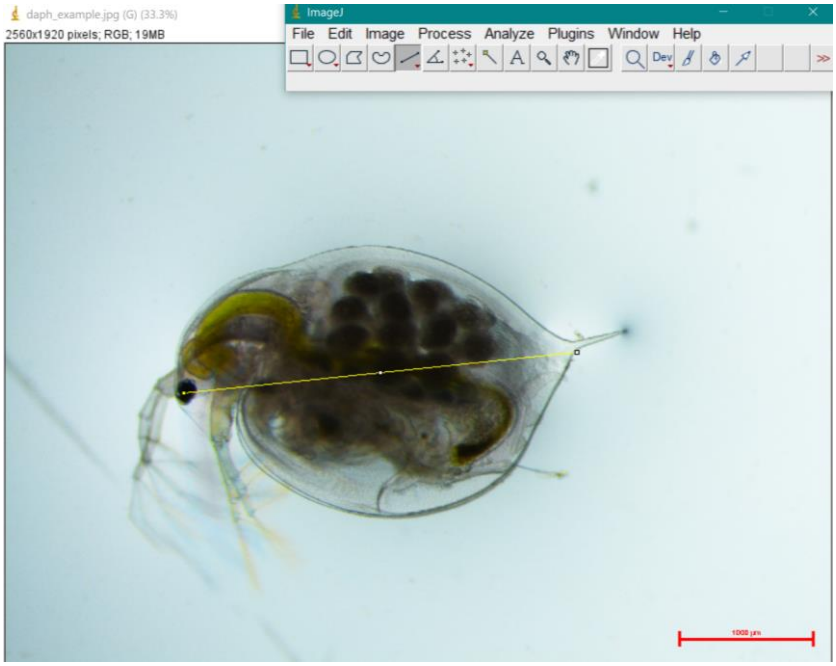


Figure 2.1 Example of image analysis in Image J measuring the growth of the daphnid. Red line depicts the scale bar and yellow line is the length being measured.

The data was then formatted (examples in S4.3 and S4.4) and presented as box plots and the corresponding density distribution plots to visualise the distribution and range across the different populations. The variation in the different populations (exposure and control group's) total neonates or growth over the test duration was analysed using ANOVA with a post-hoc Tukey test to determine significance at the 95% confidence interval.

2.4 Protein analysis- BCA

2.4.1 Introduction and principles

The BCA assay is a commonly used method to determine the total protein concentration of a sample using a bovine serum albumin (BSA) standard curve for absorbance. The principles of the assay are based on the reduction of Cu ions, from Cu^{+2} to Cu^{+1}, by the binding of amino acids within the proteins in the sample binding to the Cu, commonly known as the Biuret reaction. BCA then reacts with the

reduced Cu^{+1} forming a stable violet complex. This colorimetric change can be measured on a plate reader for absorbance at 560 nm.

2.4.2 Resources and reagents

- Peirce BCA Total Protein analysis kit
- CoStar 96 well flat-bottomed plates (clear with lid)
- Plate reader (Tecan Spark)
- Aliquots of conditioned medium
- 1.5mL Eppendorf tubes for sample isolation

2.4.3 Protocol

1) PE beads were exposed to conditioned medium as outlined in the respective study (i.e., exposure orders or chemical combination etc).
2) Beads were then extracted by sequential washing steps with fresh medium to remove i) unbound, ii) loosely bound proteins in solution.
3) Aliquots of the beads in the conditioned medium were pipetted into 1.5mL Eppendorf tubes for centrifugation.
4) Samples were centrifuged at 14,000 rpm for 5 minutes. During this time as visible pellet would form when trailed with green PE beads of the same density and size.
5) The top of the supernatant (25 µL) was removed and added to the plate during the sequential washing steps.
6) The pellet would then be resuspended using fresh unconditioned medium and repeated steps 4-5.
7) Following the washing phase, the pellet (25 µL from the bottom of the Eppendorf tube) was finally carefully pipetted into the 96 well plate.

8) A serial dilution of the 2 mg/L BSA standard was then diluted and 25 µL added to the plate spanning the concentration range of 0-2 mg/L.
9) The BCA reagents were then mixed in a 50:1 ratio of reagent A:B with enough for 100 µL to be added to each of the wells in use in the plate, including the standards and controls.
10) 100 µL aliquots of the reagent were then added to each of the wells in use.
11) The plate was then microwaved with a lid for 20 seconds with a beaker of water as a heat sink.
12) Following this, the plate was then read on the plate reader (without the lid) at 560 nm.

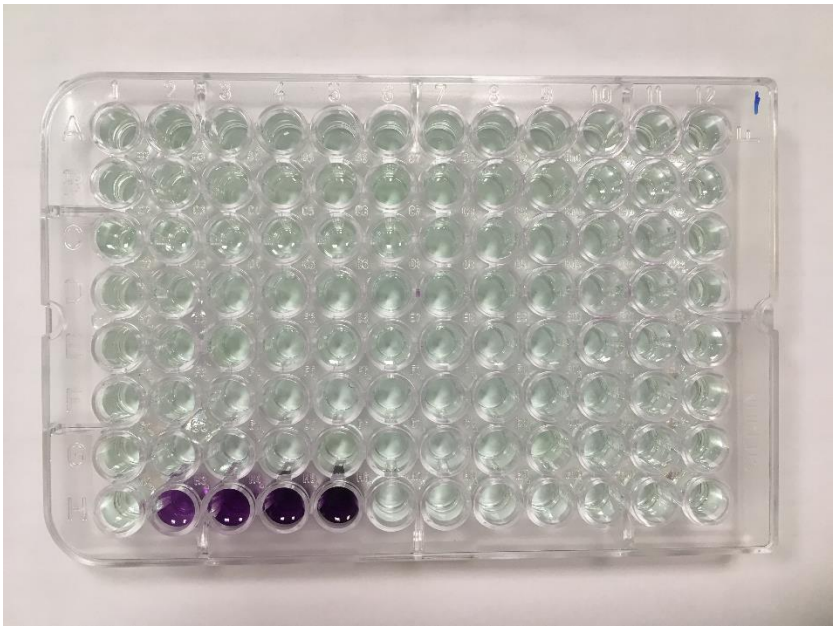


Figure 2.2 Example of the BCA assay it the 96 well plate with the BSA calibration standard in the bottom left.

2.4.4 Data capture, processing, and presentation

Following the measurement of the relative absorbance, the relative absorbance was calculated from the BSA standard, an example is outlined in Figure 2.3 below. By using the equation of the line, the absorbance reading could then be converted into a total protein concentration for the assay.

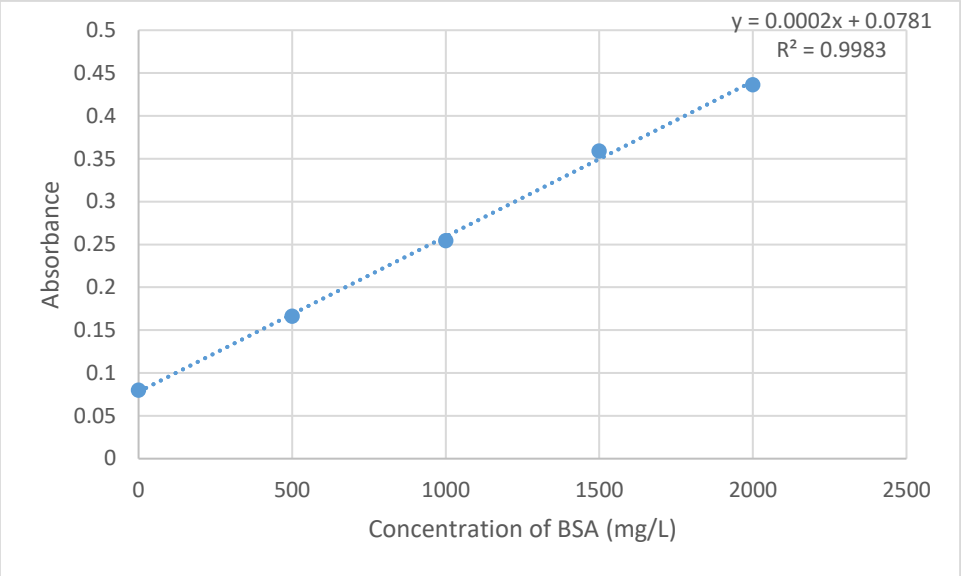


Figure 2.3 Example of a BSA standard for the BCA assay kit to determine total protein concentrations.

2.5 Lipid analysis- Nile red

2.5.1 Introduction and principles

Nile red is a soluble, lipophilic dye that can be exposed to cells, or *Daphnia*, to stain and highlight lipid deposits. Nile red binds to the deposits within the *Daphnia* and then can be identified using a fluorescence filter (FPG) on the microscope due to the excitation and emission potential of the stain (470/525 respectively). In addition, the absorbance of the Nile red can be read on a plate reader, which allows for a relative absorbance intensity to be measured based on the amount of dye that has bound to the lipids in the sample.

2.5.2 Resources and reagents

- Nile red stain 1mg/ml made up in acetone
- Testing vessels for the daphnia
- 96 well plate (costar)
- FPG lighting filter on the microscope camera (U-M49002XL-GFP filter cube)

- Plate reader (Tecan Spark)
- Isopropanol
- Sonification bath

2.5.3 Protocol

1) Removed the daphnid from the testing medium and placed into a test vessel with fresh medium.
2) Added Nile red to the test vessel and allowed to incubate with the *Daphnia* for 2 hours.
3) Removed the *Daphnia* and rinsed with fresh medium twice, transferred to a new vessel with fresh medium.
4) Imaged the *Daphnia* using the FPG filter on the light source, carefully pipetted the *Daphnia* onto a glass slide and removed the excess medium to limit the movement of the *Daphnia* to get a clearer image.
5) Recorded the light intensity and the magnification during the imaging stage.
6) Transferred the *Daphnia* back to the test vessel.
7) Once all *Daphnia* were imaged, removed the excess medium from the Eppendorf tubes and replaced with 300µL isopropanol.
8) Transferred the test vessels into the water bath and sonicated for 10 minutes. Checked the samples to ensure they were a homogeneous liquid, but sonicated for longer if required.
9) Centrifuged the samples for 5 minutes at 12,000 rpm.
10) Removed a 200µL aliquot of supernatant from each sample and added to a 96 well plate.
11) Read the plate at 470 excitation, 525 emission (for Nile red) with 30 scans per sample.

2.5.4 Data capture, processing, and presentation

The imaging of the *Daphnia* allowed observations to be made on the relative intensity and distribution of the lipid deposits of each of the individual *Daphnia*. By homogenising the individuals, it allowed for a quantitative assessment for the changes in the relative lipid concentrations across the *Daphnia* individuals (and populations) within the study. Although there was no standard lipid curve to compare the relative absorbance too, comparisons were made within each of the respective groups.

2.6 TEM of PE beads

2.6.1 Introduction and principles

Transmission Electron Microscopy (TEM) works on the principle of an electron beam interacting with the sample allowing it to be visualised. The TEM is comprised of a cylindrical vacuum to remove the interaction of the electron beam with air, with an electron gun at the top (cathode) and an anode plate at the bottom which accelerates the electrons towards the sample within the cylinder. Throughout the cylinder, the electrons pass through several apertures which focuses the beam on the samples. When the electrons reach the sample, they can be absorbed or scattered by the sample depending on the material properties. The electrons are then captured by a phosphorus screen which allows the image to be generated. This protocol follows the method established in (Ellis, Valsami-Jones and Lynch, 2020).

2.6.2 Resources and reagents

- TEM (JEOL 1400EX 80 kV)
- 300- mesh carbon coated copper TEM grid (Agar Scientific)
- Aliquots of the PE particles in solution

2.6.3 Protocol

1) 25µL drop of each sample was added to a 300-mesh carbon coated TEM grid.
2) Samples were left overnight to dry (under a cover to minimise disruption from air and dust etc).
3) The grid was carefully returned to the grid deck/plate and taken to the TEM for analysis by qualified/experiences operators.

2.7 Particle analysis - DCS

2.7.1 Introduction and principles

Characterisation of particles is important to understand if the particles are suitably dispersed within the medium or if they are agglomerating etc. which can change the way that the particles would react, or their bioavailability to organisms. Disc centrifuge sedimentation (DCS) is a method that measures the amount of time taken for particle to move through a sucrose gradient before it is detected, to determine the average size, and size distribution, of particles in a solution. Assuming all particles have the same density, larger particles will move through the sucrose and be detected quicker than smaller particles. For DCS analysis, samples are injected into the middle of a disc that is rotating at high speeds, and as the particles move through the gradient, they disrupt the light source which is the measured by the detector.

2.7.2 Resources and reagents

- Analytic Disc Centrifuge Sedimentation (DCS) CPS instrument (DCS24000)
- DCS instrument standards and capping agent (dodecane)

- DCS standard (1.6μm PVC)
- Sucrose
- DI water
- DCS sample injection syringes
- Aliquots of dispersed PE solutions for analysis

2.7.3 Protocol

1) Made the sucrose solution following the recommended guidelines for the density of the particles (for 1.3 g/cc PE beads a 24% and 8% sucrose solution is required).
2) Checked that the sucrose gradient wheel cap is securely fitted and close the instrument door.
3) Added the first injection of sucrose (starting with the higher concentration) and waited for the wheel to reach the maximum RPM.
4) Added the 1.6mL sucrose injections to the wheel, in order of decreasing density.
5) Once the gradient was established, topped it with 0.5mL dodecane as the capping agent.
6) Allowed the gradient to establish over 1 hour prior to running any samples.
7) Injected the calibration standard (1.6 μm PS) and a calibration curve was then displayed in real time and the instrument confirmed if the calibration meets the standards required.
8) Once calibration had passed, injected between 0.2-0.5 mL of the sample, when completed the software displayed the particle size distribution.
9) Extracted the data from the software manager as CSV files for each individual measurement.

2.7.4 Data capture, processing, and presentation

The data was stored in the allocated folder in the software directory. Open the files and convert to CSV, export the files to a removable local memory (the computer is not networked).

Import the CSV files to excel and process as required. Particle distribution can be visualised using a scatter plot initial to view distribution and the average particle size is also included as an output in the CSV.

The change in average particle size was recorded for all the PE dispersions across the various timepoints to ascertain if there is significant difference in the average particle size.

2.8 Particle analysis- DLS, Zeta

2.8.1 Introduction and principles

Dynamic Light Scattering (DLS) is a method of determining relative particle size taking into consideration the hydrodynamic layer on the surface of the particle in solution. A laser is shone into a solution and the light is scattered from the particles and the light intensity is then measured by a detector, the difference in particle size will lead to difference in the amount of light scatter which can be determined by the DLS to give an average particle size for the solution.

2.8.2 Resources and reagents

- Malvern Zetasizer 5000 instrument
- Disposable cuvettes
- Disposable zeta sizer capillaries
- Aliquots of the PE dispersals

2.8.3 Protocols

1) 650 µl Aliquots of the PE solution were pipetted into low volume cuvettes.
2) The cuvette was placed within the DLS sample holder, ensuring the clear panels on the cuvette face the laser beam.

3) The respective SOP was selected. This was established based on the Refractive Index and Adsorption values for the PE sample and stored within the SOP files.
4) A new file was generated for each sample, and the file name was assigned for the sample run.
5) Once the file for the sample was selected, the DLS sample could be analysed. The sample would be analysed in triplicate during a sample run with the SOP.
6) Once the sample was finished, an average of the 3 runs was generated.
7) The sample was then removed and a fresh aliquot from the same original stock solution was added. Steps 3-6 were repeated to result in 3 different aliquots of the stock solution being analysed in total for each PE sample and dispersal combination.
8) In parallel, 1mL of sample was also taken and injected into a zeta charge capillary and the caps were replaced. Care was taken to ensure that no bubbles were included within this sample.
9) The capillary was then added to the sample holder and the relevant Zeta SOP was selected. This was based on the same parameters as the DLS SOP.
10) A new measurement file was also selected for the zeta readings.
11) Once the measurement file was selected, the zeta potential of the sample could be measured, this was also done in triplicate (as outlined in steps 6 and 7).

2.8.4 Data capture, processing, and presentation

The data from the DLS and zeta analysis can be exported as CSV files, alternatively the software can be downloaded to enable analysis of the raw files. The particle size distribution can be plotted, and the average particle size can also be determined. The poly dispersity index is also reported in the instrument output for the DLS measurements, and this can be recorded along with the average particle size and reported for the variability in the particle distributions over time. The Z-average score for the zeta potential can be recorded and reported for the particles. This is the average zeta

potential for the sample, which indicated how reactive the surface layer may be with charged ions in the medium.

2.9 ICP-OES analysis

2.9.1 Introduction and principles

Inductively coupled plasma- optical emission spectroscopy (ICP-OES) uses inductively coupled plasma (usually from an argon gas source) to produce excited atoms/ions that are detectable by using the electromagnetic radiation wavelengths. Elements are excited at particular wavelengths. The intensity of these excited ion emissions can be used to calculate a concentration for this based on calibration standards.

2.9.2 Resources and reagents

- ICP-OES (Perkin-Elmer Optima 8000) with argon gas flow
- ICP grade calibration standards of selected elements (B, C, Mg, P, K, Si, Na)
- Aliquots of filtered environmental samples

2.9.3 Protocols

1) Calibration standard of the selected elements were created from the stock solutions in concentrations spanning 0, 0.1, 1, 10 and 100 mg/L and added to the autosampler grid.
2) 15mL of filtered environmental samples were added to the autosampler grid and recorded on the sampling map.
3) Samples were analysed using a radial view plasma with cross flow nebuliser flow of 8L per minute and a sample flow rate of 1mL per minute. Analysis was undertaken in triplicate with blanks every 15 samples and calibration checked against the internal standards on each series.

4) Samples were discarded after analysis.

2.9.4 Data capture, processing, and presentation

Data was stored as a CSV file. Limit of detection (LOD) was calculated and checked against the samples (Table 2.5). Corrected intensities of the elements were used for subsequent analysis and reporting.

Table 2.6 LOD for element analysis for ICP-OES samples

Element	LOD (mg/L)
B	0.535
Si	0.335
P	0.862
Na	0.849
Mg	0.904
Ca	0.386
K	1.3

2.10 Field study sampling

2.10.1 Introduction and principles

Understanding the environmental conditions, the *Daphnia* and microplastics can be found in, is an important consideration for the medium that we culture the daphnids in to ensure that is representative of their natural environment. This field study was undertaken as a collaborative project with 3 distinct strands; *Daphnia* collection, microplastic sampling and *in*

situ water quality analysis in addition to elemental analysis of the water samples. When combined, this project will give a holistic overview of the state of a series of ponds in the Birmingham area (UK), across an urban-rural pollution gradient, as shown in figure 2.4 below.

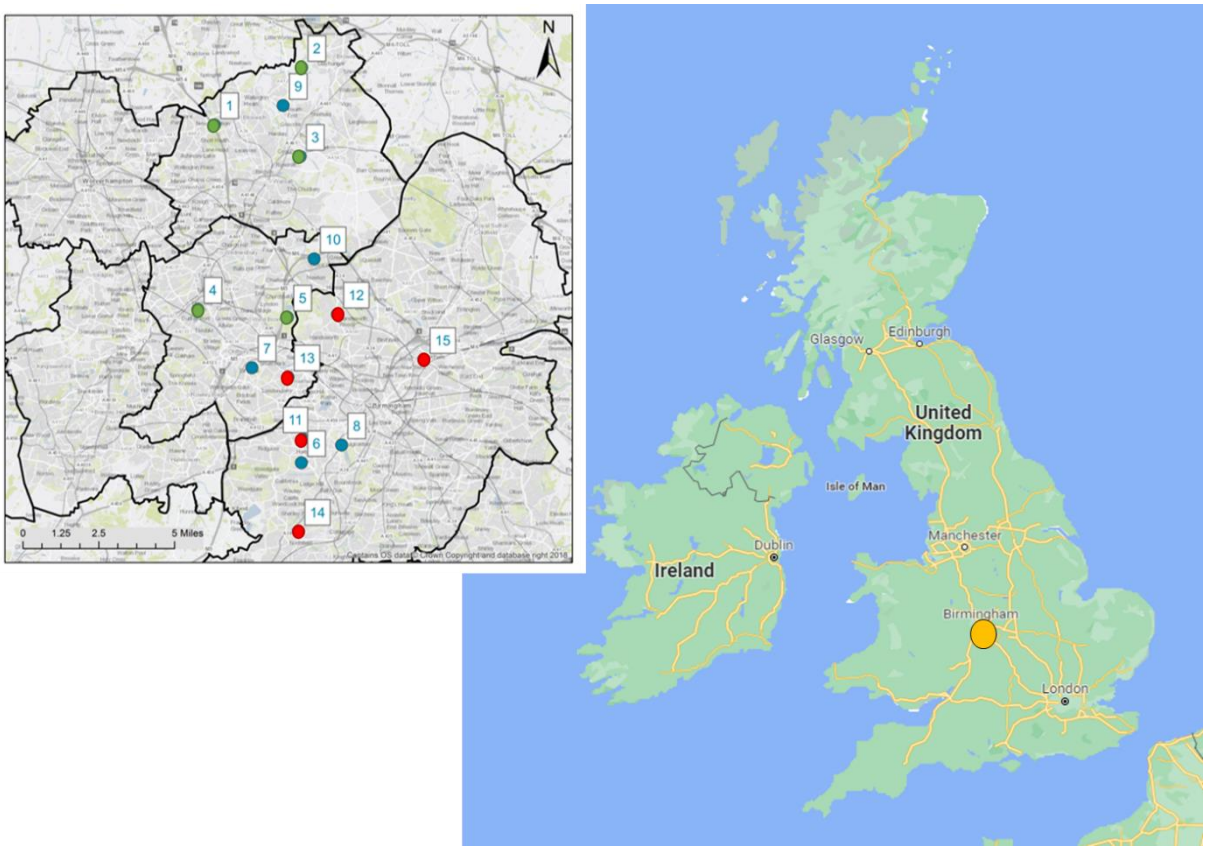


Figure 2.3 Map of the pond sampling sites around the Birmingham region, UK, sites were classified as rural (1-5 (green)), suburban (6-10 (blue)), and urban (11-15 (red)) based on a range of parameters including land-use and population density. (Goole Maps, 2021).

2.10.2 Resources and reagents- water sampling

- Acid washed polypropylene bottles
- Mutliparamter probe (temperature, conductivity, dissolved oxygen,
- Flow meter

- Cool box
- dried 0.45 µm Glass Fibre filter paper

2.10.3 Protocols- water sampling

1) On arrival to site, observations and times were recorded in a field notebook.

2) The *in situ* parameter measurements were taken first to ensure that there was no disruption to the substrate or potential flow as a result of subsequent sampling.

3) Once all parameter measurements had been taken in triplicate and recorded the water samples were collected. Bottle were rinsed thrice in the pond water before a sample was collected. Care was taken to ensure there was no contact to the substrate or vegetation and there was minimal air in the top of the sample bottle. Water samples were also taken in triplicate and stored in a cool box until return to the lab.

5) The samples were then filtered through the dried filter paper to remove suspended matter before being frozen for the subsequent elemental analysis with ICP-OES.

2.10.4 Data capture, processing, and presentation

The *in situ* measurements were taken in triplicate and averaged before being tabulated. The elemental analysis of the water samples was then undertaken (section 2.9) and the results were compiled and presented in a separate report (Appendix 3) to present an overview of the variability of the sites within the study.

Chapter 3

3. The impact of medium composition and dispersal protocols on microplastic surface conditioning and bioavailability in standard laboratory toxicity testing with *Daphnia magna*

Katie Reilly[1], Laura-Jayne. A Ellis[1], Jon Sadler[1] & Iseult Lynch[1]

[1]School of Geography, Earth and Environmental Science, University of Birmingham, UK.

Keywords: *Daphnia magna,* polyethylene, microplastics, medium composition, particle dispersion

3.1 Abstract

Upon entering the aquatic environment microplastics (MP), be they primary microbeads or secondary particles degraded from larger plastics pieces, become 'aged' during their lifetime via chemical, physical and/or biological processes. Ecotoxicology tests, which were designed for soluble chemicals prior to the emergence of MP, often use commercial formulations of spherical beads and simplistic testing medium with high exposure concentrations which are unrepresentative of environmental MP exposures. To demonstrate the consequences of these factors, polyethylene (PE) microbeads were dispersed in a range of culturing media including local borehole water which contains natural organic carbon, and a range of dispersal approaches were compared including ethanol prewetting often used to disperse hydrophobic carbon nanotubes, and the use of TWEEN as a surfactant to confer hydrophilic properties to the MP, and the stability of the PE MP was assessed over 21 days and correlated with the MP acute toxicity to *Daphnia magna.*

Although the stability of the dispersions differed across the range of exposures and media tested, all remained within the nominal exposure size range of 1-5µm. The results show that the difference in the dispersion method can lead to variations in the *Daphnia* secreted proteins associated with the PE particles using a Bicinchoninic acid assay (BCA) assay. For example, PE that was prewetted with ethanol or the dispersion containing high levels of natural organic matter had lower associated concentrations of protein compared to other methods.

Although the use of TWEEN to disperse the PE led to a smaller range in measured size over time, it is also feasible to use alternative methods to avoid any potential confounding effects from the surfactant. This can include using more environmentally relevant dispersion methods such as conditioned medium or natural organic matter, which disperse the particles without significantly impacting the particle stability over time.

3.2 Introduction

Plastics are one of the key materials used in the 21st century, and the plastics industry has continued to grow with 359 million tonnes of virgin plastic produced globally in 2018 (PlasticsEurope, 2019b). Among the main attractions of plastics including their low cost, are the range of accessible properties such as thermal and electrical insulation, durability and flexibility, which make them suitable for a wide range of uses and products, from electrical cable insulation to disposable bottles to car tires (Rochman *et al.*, 2019). Plastic items can end up in the environment due to accidental release or mismanagement of waste, and the larger items can be broken down into MP over time due to environmental degradation (Rochman *et al.*, 2019). In addition, MP can be released directly into the environment as MP through

industrial pellet spillage, abrasive cleaners, or fibre release from clothing (Wagner *et al.*, 2014). MP pollution has been found ubiquitously in all studied environments across the globe, ranging from ocean trenches, coral reefs, freshwater lakes, rivers and arctic ice cores (Hall *et al.*, 2015; Leslie *et al.*, 2017; Peeken *et al.*, 2018; Sighicelli *et al.*, 2018; Jamieson *et al.*, 2019), demonstrating the scale of the issue. Thus, there are increasing concerns about the presence of plastics and their breakdown products as microplastics (MP), in the environment and what consequences this has for both the environment and the organisms at risk of plastic ingestion.

To further the mechanistic understanding of potential toxicity due to ingestion, laboratory-based toxicity assays have been conducted using a wide range of test organisms (from algae to zooplankton through to fishes and birds (McGoran *et al.*, 2018; Reynolds and Ryan, 2018; Windsor *et al.*, 2019; Guschina, Hayes and Ormerod, 2020)), and MP of varying morphology, size, colour, additives and polymer type (Luo, 2020). This range of combinations (of model/test organisms versus MP) has led to various adverse outcomes being reported as a direct result of MP exposure, including decreased feeding, limited movement and mobility, decreased reproductive capability and death (Foley *et al.*, 2018; Kukkola *et al.*, 2021). Such toxicity tests have historically used commercial formulations of spherical beads and simplistic testing medium with high exposure concentrations in order to see an effect. However, with increasing understanding of the scale and complexity of microplastics found in the environment, there is a need for the laboratory testing of MP to be more reflective of the real world conditions (Kukkola *et al.*, 2021), which will require some adaptions to the approaches used for traditional chemical testing (OECD, 2004b), which are based on solubility and equilibrium principles. While there have been calls in the literature to use more environmentally relevant morphologies and concentrations of microplastics in toxicity assays (Kalčíková *et al.*, 2017),

this is often undertaken with commercial MP formulations which contain additional surfactants such as TWEEN to maintain the particles in dispersion due to the hydrophobic nature of many plastics, or preservatives such as sodium azide to prevent microbial growth in the dispersions once opened thus extending their shelf-life (Pikuda *et al.*, 2019). However, the presence of such additives have been shown to have confounding results in toxicity studies (Masakorala, Turner and Brown, 2011; Pikuda *et al.*, 2019), as these additives can themselves be toxic. Nevertheless, it is important to ensure that the MP are adequately dispersed within the testing medium, so that the effect concentration is accurately calculated from the intended exposure and therefore alternative, and more natural dispersion methods were explored. This research aims to study the effect that different dispersal protocols and medium compositions can have on the surface properties of polyethylene (PE) MP spheres, and how this determines their potential agglomeration and thus, bioavailability to aquatic organisms during standard laboratory toxicity testing. To explore this question the OECD standardised tests for acute toxicity with *Daphnia magna* were used (OECD, 2004b), and the impact of dispersion method and medium composition were correlated with the MP toxicity to daphnids. Insights into the mode of action of the differently dispersed PE MP were also explored by evaluating physical versus chemical effects of the PE MP, and the influence of *Daphnia*-secreted biomolecules versus natural organic matter. Polyethylene (PE) beads in the size range of 1-4 µm were used for the study to allow for interaction with, and uptake into the gut of, the daphnids during the toxicity assessment as this is a comparable size range to the algal feed cell size (Schmidt, Rohde and Braumann, 2021).

The toxicity effects associated with MP are typically dose dependent, which highlights the need for environmentally relevant study designs. This has led to discussions on what could be

achieved by standardising the terminology, and methods, used within this field to allow inter-comparison of MP results across the research community (Hartmann *et al.*, 2019). However, points have also been raised to avoid standardising the methodologies before all potential confounding effects are adequately understood, to avoid overlooking a currently unknown aspect of the problem, following on from similar discussions held 5-10 years prior within the engineered nanomaterials community (Hund-Rinke *et al.*, 2016; Nasser and Lynch, 2019a). The standardisation of both the sampling and laboratory testing of MP is fundamental for inter-laboratory testing to validate testing protocols for example, and to ensure sampling consistency between field locations to enable comparisons of disparate data (Kukkola et al 2021). Exploring alternative methods in parallel to currently established and standardised approaches will allow for comparison of the new methods to current standard results in addition to expanding the data available to explore mechanistic responses. However, combining several methods in parallel will significantly increase the time and financial cost of experimental strategies and this needs to be reflected in the design stage.

There is an urgent need to establish an environmentally representative (i.e. not relying on artificial surfactants) method of MP dispersion, that would harmonise the bioavailable particle dose and ensure exposure of the test organisms in a manner that is reminiscent of the MP dispersion in the environment. This is particularly relevant for MP composed of hydrophobic polymers, whose natural tendency is to agglomerate to minimise contact with water (Figure 3.1 A, B), which due to their low density relative to water and consequent buoyancy can lead

to their accumulation at the air-water interface, such that organisms in the water column, such as daphnids, are not actually exposed.

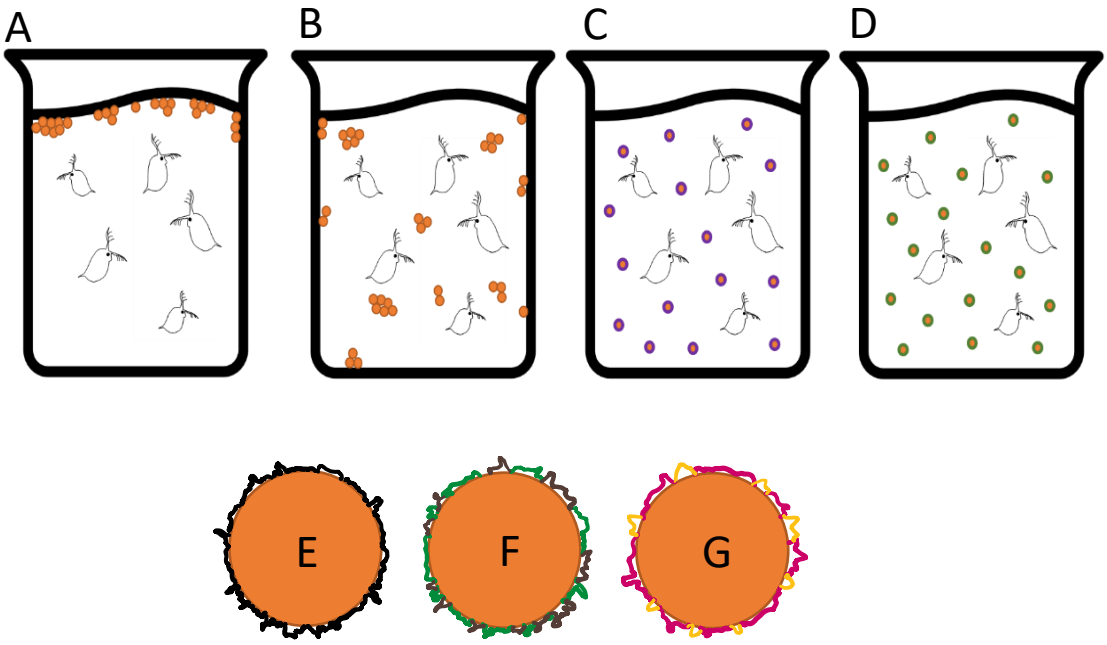


Figure 3.1 The local environment of the particle will have a significant impact on the surface coating and eco-corona formation of the particle, which can have an influence over the dispersal and subsequent bioavailability of the MP to the test organism. A-D represent the theoretical hypothesis that poorly dispersed hydrophobic particles, i.e., in salt only or low NOM-content media, are likely to remain at the surface of the test vessel (A), or agglomerate into size fractions that are not accessible to the test organisms (B), therefore reducing the encounter rate of the organism with the particles. Modifying the dispersal protocol by introducing synthetic surfactants (C) or more natural dispersant methods such as NOM (D) will enhance the MP dispersion as a result of eco-corona formation on the particles. E-G show the potential variation that these dispersion methods could have. E demonstrates the overall conditioning of particles by the local environment, with F having a predominately more biological corona (green) compared to G which is representative of the surface conditioning due to a surfactant-based dispersion (such as in 3.1C).

While this is relevant information in terms of an overall risk assessment (where risk = exposure hazard) it is not helpful for hazard determination, thus requiring the aforementioned dispersion strategies as part of overall hazard study design going forwards. For regulatory purposes, standardised and comparable methods should be used to establish the baseline toxicity, and the adaptations posed for the nanomaterials community to ensure dispersion of hydrophobic particles such as carbon nanotubes and consideration of the role of corona formation through interaction with biomolecules present in the environment are likely also applicable for MP research. These approaches are presented below as part of a revised testing strategy for MP, using the example of PE MP toxicity assessment with daphnids.

Polyethylene (PE) was used within this study as it is one of the most commonly produced polymers (Europe, 2017) making up 36% of total polymers produced between 1950-2015 (Geyer, Jambeck and Law, 2017). It is typically used for food containers, such as milk cartons, and prior to the Microbead ban in 2018 (England Government, 2017), PE was found in a range of personal care products, for example facial scrubs (Napper *et al.*, 2015b). However, despite the phase-out of PE microbeads from inclusion in personal care products, the legacy inputs are anticipated to be an issue for several years to come, as the current products on the market and in people's homes will continue to be a source of PE to the environment. Currently this ban is in effect in the UK with an EU-wide ban currently proposed for 2020 to be phased-in over a number of years (Guerranti *et al.*, 2019). PE MP are commonly reported in environmental studies and are often one of the most common polymers identified within water samples including rivers, lakes and mountain catchments (Tibbetts *et al.*, 2018; Grbić *et al.*, 2020; Allen *et al.*, 2019) and are also frequently used in lab-based toxicity exposures as reported in the literature (Jemec *et al.*, 2016; Imhof *et al.*, 2017; Kalčíková *et al.*, 2017). PE is

a hydrophobic polymer which is important to consider during dispersion in the laboratory, as the particles do not readily disperse in pure water. Therefore, the major salts and potentially Natural Organic Matter (NOM) contained in artificial laboratory media will be key to effective dispersal, in order to ensure reproducible exposure of the organisms and enable correlation of dose and response to determine effective concentrations accurately.

NOM, the decaying plant and animal matter present in natural waters and soils, has been described as containing varying fractions of humic acid, fulvic acids, polymeric substances and a hydrophilic fraction, which have been widely reported to have strong absorption to colloidal materials (Afshinnia, Marrone and Baalousha, 2018; Tayyebi Sabet Khomami *et al.*, 2020). The role of NOM within MP research, particularly in the context of the freshwater environment, has not been greatly studied to date, although there is extensive literature from colloids and nanomaterials (Lowry *et al.*, 2012; Petersen *et al.*, 2015; Markiewicz *et al.*, 2018a). The OECD 211 test for *Daphnia magna* reproduction recommends the avoidance of soil and seaweed extract (due to their heterogeneity and batch-to-batch variability) in the test medium in order to increase the standardisation and ability to replicate studies across laboratories (OECD, 2012b). However, it is worth noting that this assay was developed for dissolved chemicals rather than particles with their enormous surface area and surface energy, and that revisions of the test guidelines to account for the specific features of nanomaterials, such as inclusion of biomolecules, are currently being validated for regulatory testing of nanomaterials (Petersen *et al.*, 2015; Nasser and Lynch, 2019a; Ellis, Valsami-Jones and Lynch, 2020). It is highly likely that such modified OECD tests will also be applicable for MP. The lack of biomolecules in the medium detracts from the environmental realism of the toxicity tests due to the simplification of the medium in the test, and for MP will also result in instability and

poor dispersion of the MP leading to inhomogeneous exposures and likely irreproducible results as shown schematically in Figure 3.1A. It has also been recommended that the total organic carbon (TOC) concentration in the test medium is <2mg/L at the start of the test (OECD, 2012b). This is likely due to the interactions between NOM and chemicals dissolved in solution which can dramatically reduce their bioavailability and thus lead to underestimation of the chemical toxicity. However, this effect is different during MP exposures due to the spheres being dispersed rather than diluted in the medium, and therefore the NOM will interact instead by forming a corona on the particles and thus increase their dispersion and bioavailability (Markiewicz *et al.*, 2018b). Thus, the absence or low concentration of NOM actually acts to decrease the reproducibility of the tests in this case, which should be taken into account when testing particles including microplastics.

The array of exposure scenarios (media conditions and dispersal strategies) used within this study is not exhaustive but encompasses a range of media that are used in MP studies to understand the effects that these dispersal techniques could be having on the plastics surface characteristics and hence stability, agglomeration, and bioavailability. While not a fully comprehensive comparison of all dispersal techniques and methods, this study is a compilation of previous methods and their correlation with impacts of the differently dispersed MP on test organisms to highlight the fact that different techniques have a direct influence on the subsequent toxicity due to particle dispersion properties (Ellis, Valsami-Jones and Lynch, 2020). The ability of the microbeads to disperse within the media is key for how the test organisms will be able to interact with them during the study. Figure 3.1 shows examples of the different dispersion scenarios tested herein leading, namely the ethanol prewetting and use of TWEEN (Figure 3.1C) and the use of media containing NOM (Figure

3.1D) which will result in differently coated MP. By using a more natural method of dispersing plastics we can more accurately design and test MP toxicity, reflecting how realistic environmental exposures would take place. This is an important consideration for toxicity testing going forwards, to make them as accurate and representative as possible to make sure that we are effectively protecting the environment and to increase the reliability and integrity of models and testing scenarios (Jager, Heugens and Kooijman, 2006).

Corona formation on particles plays an important role in changing the surface properties of the material and is representative of the local environment in which the particles are dispersed (Figure 3.1 E-G) including NOM, biomolecules (such as secreted proteins, and other endogenous materials) that form the environmental, or otherwise known as the 'eco-corona' (Nasser and Lynch, 2016b; Ellis and Lynch, 2020). Other environmental constituents that can form a corona around the MP are inorganic compounds (phosphate; sulphates), metabolic resides, and other chemical pollutants (Chetwynd and Lynch, 2020). For example, *Daphnia* will condition their culturing or testing medium with secreted biomolecules such as proteins, polysaccharides and metabolites which are then capable of binding to particles added to the medium (Nasser and Lynch, 2016b). The corona forms due to the instantaneous surface interactions of the MP with other molecules in the water, i.e. by attraction due to the high surface energy of the pristine particles (Ellis, Valsami-Jones and Lynch, 2020). The acquired eco-corona can help to overcome the hydrophobic nature of the 'pristine' PE surface and increase their dispersal and stability. This is an important consideration as the formation of a corona on particles can affect the agglomeration of the spheres in solution and therefore the subsequent size, bioavailability, and toxicity. This is particularly problematic since the eco-corona can 'mask' the MP resulting in them appearing food-like to organisms, leading to

increased uptake and retention, thus leading potentially to toxicity as has been demonstrated for nanomaterials including nanoscale polystyrene beads (Nasser and Lynch, 2016b; Fadare *et al.*, 2019; Ellis and Lynch, 2020).

There is significant discussion within the MP research community regarding the standardising of the terminology, reporting and protocols for MP exposures (Twiss, 2016; Hartmann *et al.*, 2019; Cowger *et al.*, 2020). One aspect is that, if protocols are standardised across the scientific community then it will allow easier comparisons of results and a larger database can be built to establish relationships and patterns in the data faster (Cowger *et al.*, 2020). On the other hand, if the methodologies are standardised too quickly, they run the risks of missing some parameters and pathways which could be having unobserved impacts on the results. This argument follows the ecotoxicology paradox of simplifying the environment to be able to study effects in the laboratory and to allow ranking of toxicity; to be able to explore the relationships the systems are simplified, but it then poses the risk of missing a step that effects the relationship being studied (Jager, Heugens and Kooijman, 2006; Wilson, McHugh and Giltrap, 2014). Therefore, simplifying makes the experiments less environmental realistic which could pose challenges to validating laboratory testing and extrapolating the findings to realistic environmental results. Drawing on previous research from the nanomaterials community, this study aims to ascertain the potential variability in MP studies as a result of the dispersion method used encompassing consideration of potential agglomeration of the MP in the medium, changes to acute toxicity, disparity in protein conditioning of the particle surface, in addition to quantifying any leaching of the particle dye into the stock solution.

3.3 Materials and methodology

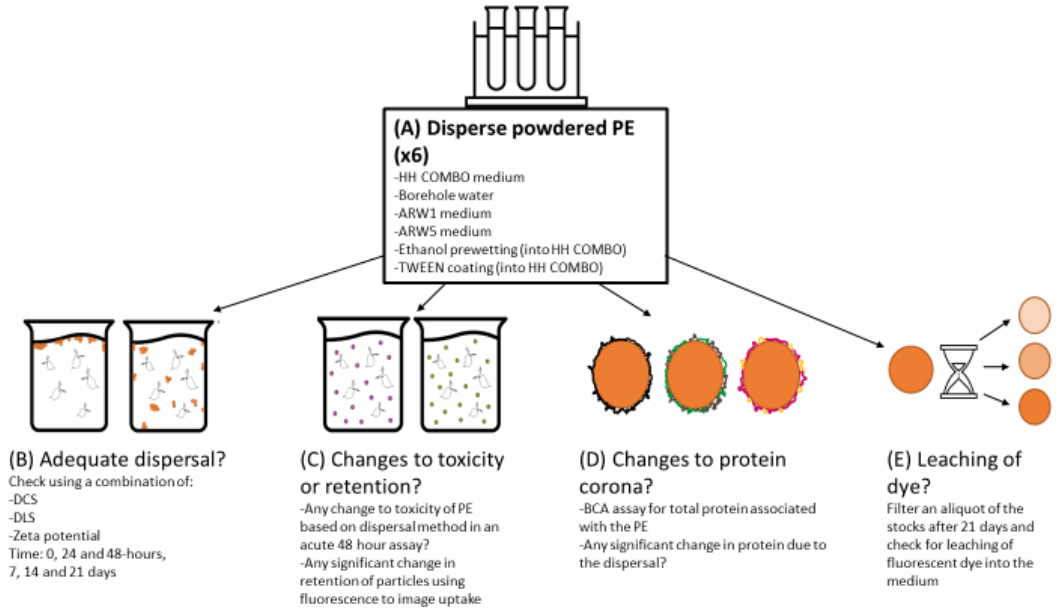


Figure 3.2 An overview of the four elements of the study: dispersion, toxicity, proteins and leaching to determine if the dispersal method has a significant effect on these elements of MP toxicity studies.

3.3.1 Materials (or Particles)

Polyethylene (PE) spheres were sourced from Cospheric, USA. Fluorescently stained green beads were predominantly used, which were 1-5 µm in diameter with a density of 1.3 g/cm^3 and supplied as a dry powder, with no preservatives. In addition, clear beads (also 1-5 µm in diameter with a density of 1.3 g/cm^3 and supplied as a dry powder) were used for the protein analysis steps (3.3.7).

3.3.2 Test Media

Various test media were prepared using laboratory grade chemicals following standardised protocols (Table 3.1) to allow for comparison of the MP dispersal within the medium (Kilham *et al.*, 1998b; Gallego-urrea, Hassello and Hammes, 2013) and during *Daphnia* culturing. The media used for testing included High Hardness Combo medium (HH COMBO) (Kilham *et al.*,

1998b), Artificial River Water Classes I and V (ARW1 and ARW5 respectively), from Hammes, Gallego-Urrea and Hassellöv, (2013) and borehole water taken from the University of Birmingham's on-campus borehole. These media represent a range of waters from natural NOM-containing freshwater (borehole) through to synthetic model waters that are representative of real European waters with different ionic strengths and NOM contents (ARW Class I and 5) and finally, the HH COMBO medium which is salt-only and has been developed as a standard for organism culturing and toxicity testing and is often used with the Bham 2 strain of *D. magna.*

Table 3.1 Artificial medium preparations - key salts, physico-chemical conditions and citations.

Medium	**HH COMBO**	**ARW1**	**ARW 5**
Salt components	$CaCl_2$ $2H_2O$ $MgSO_4$ $7H_2O$ K_2HPO_4 $NaNO_3$ $NaHCO_3$ $NaSiO_3$ 9 H_2O H_3BO_3 KCl	$CaSO_4$*$2H_2O$ $CaCO_3$ $Ca(NO_3)_2$ $Mg(NO_3)_2$ $NaHCO_3$ $CaCl_2$ $KHCO_3$	$CaSO_4$ $CaCO_3$ $4MgCO_3$. $Mg(OH)_2$ $Ca(NO_3)_2$ $NaHCO_3$ $CaCl_2$ $KHCO_3$
Conditions	pH- 7.6-7.8 ionic strength- 11.07 mg/L	pH- 7.3 ionic strength- 10.12 mg/L	pH- 8.1 ionic strength- 15.76 mg/L

	NOM 0 mg/ L	NOM 1.84 mg/ L	NOM 4.6 mg/ L
Key reference	(Kilham *et al.*, 1998b)	(Hammes, Gallego-Urrea and Hassellöv, 2013)	(Hammes, Gallego-Urrea and Hassellöv, 2013)

In terms of particle stability, the 3 media were selected to explore a range of potential combined effects such as ionic strength (which will impact the zeta potential (section 3.3.3.)) with ARW1 having the lowest and ARW5 the highest salt concentration. In addition, the presence of NOM can impact stability, HH COMBO has none added, and ARW5 has the most. In addition, the pH varies between the media, with HH COMBO having the lowest pH and ARW5 the highest pH, which could impact chemical speciation in future work, and therefore is an important aspect to consider at the dispersion stage. In addition to the PE stability measurements, the difference in medium composition can affect the environmental relevance of studies, with HH COMBO being the most controlled as it is a salt only medium, and ARW1 and ARW5 being the most representative of European waters and therefore closer to real-world environmental conditions which is an important consideration in test design (chapter 4).

3.3.3 Particle characterisation and dispersion techniques

The PE spheres (both the clear and fluorescent green) were dispersed in each of the four media. These media have various properties which could affect PE bead dispersal such as differing ionic strengths, absence or presence of biomolecules and the presences or absence

of suspended solids and NOM which are outlined in Table 3.1. Two additional dispersal methods were also reviewed, namely the use of the surfactant TWEEN 20 as recommended by the supplier and an ethanol pre-wetting method (developed for hydrophobic nanomaterials (Jensen *et al.*, 2013)) before subsequently dispersing the prewet beads into HH COMBO medium, totalling six stock solutions of each of the PE beads for analysis. The pre-wetting with ethanol used an 80% ethanol solution to coat the beads ahead of their dispersal in the medium. TWEEN was prepared using the protocol recommended by manufacturer (Cospheric) for their Density Marker Bead dispersal (Desantis, 2014). Briefly, TWEEN 20 was dissolved in hot MilliQ water to form a 0.1% dilution and allowed to cool before adding to the powdered beads at a 1:5 ratio of beads: solution, before further mixing. The particle dispersions were all prepared to be 1 g/L particles from the dry powdered spheres as supplied, and dispersions were monitored for potential agglomeration and changes in average size or variations in the surface charge due to the method used.

Following dispersal, Transmission Electron Microscopy (TEM) was performed using a JEOL 1400EX 80 kV and system. Samples were prepared by depositing a 10 µL drop of the PE suspension onto a 300-mesh carbon-coated copper TEM grid (Agar Scientific, UK) (Ellis, Valsami-Jones and Lynch, 2020). Images were taken of the spheres at time 0 to check the morphology of the particles (See section 3.4.1, Figure 3.3).

Differential Centrifuge Sedimentation (DCS) analysis was conducted using an Analytic CPS DCS24000, with a low-density sucrose solution and a spin rate of 10,000 rpm as per the recommendation based on the density and size of the PE spheres used within the study. DCS relies on the centrifugal force to move the particles through the sucrose gradient, and larger

particles will move faster and therefore be detected first, finishing with the detection of the smaller particles in solution.

Dynamic Light Scattering (DLS) and Zeta potential measurements using a Malvern Nanosizer 5000 instrument were used to analyse potential agglomeration and surface charge, respectively. DLS is used to measure the hydrodynamic diameter/size of the particles in solution by quantifying the intensity of scattered light that is reaching the detector, thus agglomeration of particles will lead to a change in the intensity of light scattering in the sample and therefore the measured size of the particles. Zeta potential is a measure of the electrophoretic mobility of the particles slipping plane in the ionic medium, and is used to measure the movement between the charged ion layers that bind to the particle. This gives and indication of the surface charge and electrostatic stabilisation and particle- stability within the medium, but is sensitive to changes in pH or ionic strength. This allowed assessment of the role of the medium and surfactants / NOM on the stability of the PE in the medium. DCS, DLS, and zeta were used to characterise the MP and to assess dispersal and the surface properties at times 0, 24 and 48 hours in line with the exposure protocol outlined by the OECD for the EC_{50} acute toxicity tests (OECD test 202) (OECD, 2004b). Interim measurements were taken at days 7 and 14 before a final samples were analysed 21 days after initial dispersal to look at any potential longer-term agglomeration in the media and the stability of the particles for the chronic reproductive testing, assuming medium replenishment of particle dispersions during this test was from one original stock (OECD test 211) (OECD, 2012b).

The combined approached of the different characterisation techniques explores different parameters to build up an overview of the particle stability under the various conditions. TEM

is used to measure the core diameter of the particles and provides images to confirm the spherical morphology. DCS and DLS are used to measure the hydrodynamic size of the particle taking into consideration the different dispersal methods and the zeta potential can then be used as a proxy for the surface charge and the (mechanism of) stability of the particles in the medium.

The purpose of this analysis was to ascertain how available the MP particles are during routinely used acute toxicity assays (such as the OECD 202 test). For this purpose, the samples were treated the same as if it they were dispersed in the toxicity assay, using the same temperature (20 °C), lighting (i.e., 16:8 light: dark cycle - see section 3.3.4) and agitation of the medium as used during the *Daphnia* acute and chronic toxicity tests.

3.3.4 *Daphnia* culturing

Bham 2 strain *Daphnia magna Straus* were cultured using pools of the third broods to establish new generations, in each of the 4 medium types prior to the subsequent toxicity tests and maintained at 20 °C in a 16:8 light: dark cycles. Each of the culturing media were refreshed three times per week to ensure healthy maintenance, and cultures were fed *Cholorella vulgaris* algae daily (based on 0.5 mg carbon /day for the first 5 days and 0.75 mg carbon thereafter) and maintained with 15 daphnids per 1L culturing vessel, in 900mL of medium.

3.3.5 Particle- *Daphnia* interaction

To determine if the dispersion method impacted the interaction of the PE MP with *Daphnia*, a preliminary uptake/egestion study was undertaken with the variously dispersed PE samples. *Daphnia* neonates were removed from the respective culturing medium into fresh medium

containing no algae feed and were not fed during the PE acute toxicity assay, as recommended in OECD 202 protocol, nor during the uptake/egestion study to allow clearer imaging of the gut. Exposure concentrations in the initial range finding exposure spanned from 0 mg/L in the control group to 1000 mg/L in the highest exposure, in line with artificially high exposure concentrations reported in the literature (Zimmermann *et al.*, 2020). A concentration of 250 mg/L was used across all groups for the subsequent ingestion-egestion exposures.

Images of the daphnids were taken prior to exposure to PE, 24 hours after exposure and 24 hours post removal into fresh medium to enable an overview of the movement of the PE MP through the *Daphnia* gut.

3.3.6 Dye leachate

At the end of the 21-day incubation period in the respective media, an aliquot of the stock solution for each treatment was taken and filtered through a 0.45 µm PES membrane filter to remove the beads. This solution was then analysed for residual fluorescence in the excitation range of 414 nm on a Tecan Spark plate reader, which corresponds to the dye associated with the beads, to determine if there was any leaching of dye during the 21-day period due to the dispersant method.

3.3.7 Protein corona formation

To ascertain if there are any changes to the associated protein corona on the MP beads due to the dispersion methods, total protein associated with the PE beads were quantified using the Bicinchoninic acid assay (BCA). *Daphnia* were removed from their running cultures into

fresh medium without algae feed and allowed to 'condition' the medium for 24-hours. During this period, the daphnids would be releasing metabolites, polysaccharides and proteins into the medium. After 24-hours the daphnids were then returned to their respective running cultures and the conditioned medium was used for subsequent analysis. An aliquot of each of the six clear PE stock solutions, prepared using the same methods detailed in section 3.3.3, was added to the respective *Daphnia* conditioned medium and allowed to incubate for 1 hour ahead of extraction and analysis following the protocol outlined (Nasser and Lynch, 2016b). Briefly, the particles were isolated using centrifugation at 14,000rpm for 5 minutes, the supernatant was removed, and fresh medium was added before resuspension of the PE pellet and a repeat of the centrifugation steps. This process was initially conducted with the green PE to ensure that a visible pellet formed during the centrifugation step. The total protein was then quantified using BCA with Bovine Albumin Serum (BSA) standard on a Tecan Spark plate reader at 515nm.

3.4 Results and discussion

3.4.1 Dispersion of PE

A fully defined medium is important for comparability across laboratory testing and previous research has highlighted the suitability of modifications of the OECD test medium without EDTA for use in chemical toxicity testing due to the high potential for chelation of metals (OECD, 2012b). However, little consideration is reflected within the literature of plastic toxicity testing. Due to the inert design of the plastic surface, chelation is expected to have limited effects on virgin PE MP, moreover, the high curvature and surface area provides a platform

for chemical interactions, while the hydrophobic nature is important to consider during MP exposures and subsequent dispersal for these toxicity tests. Pikuda and co-authors (Pikuda *et al.*, 2019) have highlighted the confounding effects that surfactants and antimicrobials can have on toxicity testing in commercial formulations of MP dispersions. The use of various dispersion methods or testing medium was undertaken to ascertain the influence that this can have on the variation in average size of the MP particles and their zeta potentials as a potential alternative to the use of artificial surfactants that are currently widely used but which themselves may be toxic to the organisms, for example, by damaging lipid membranes.

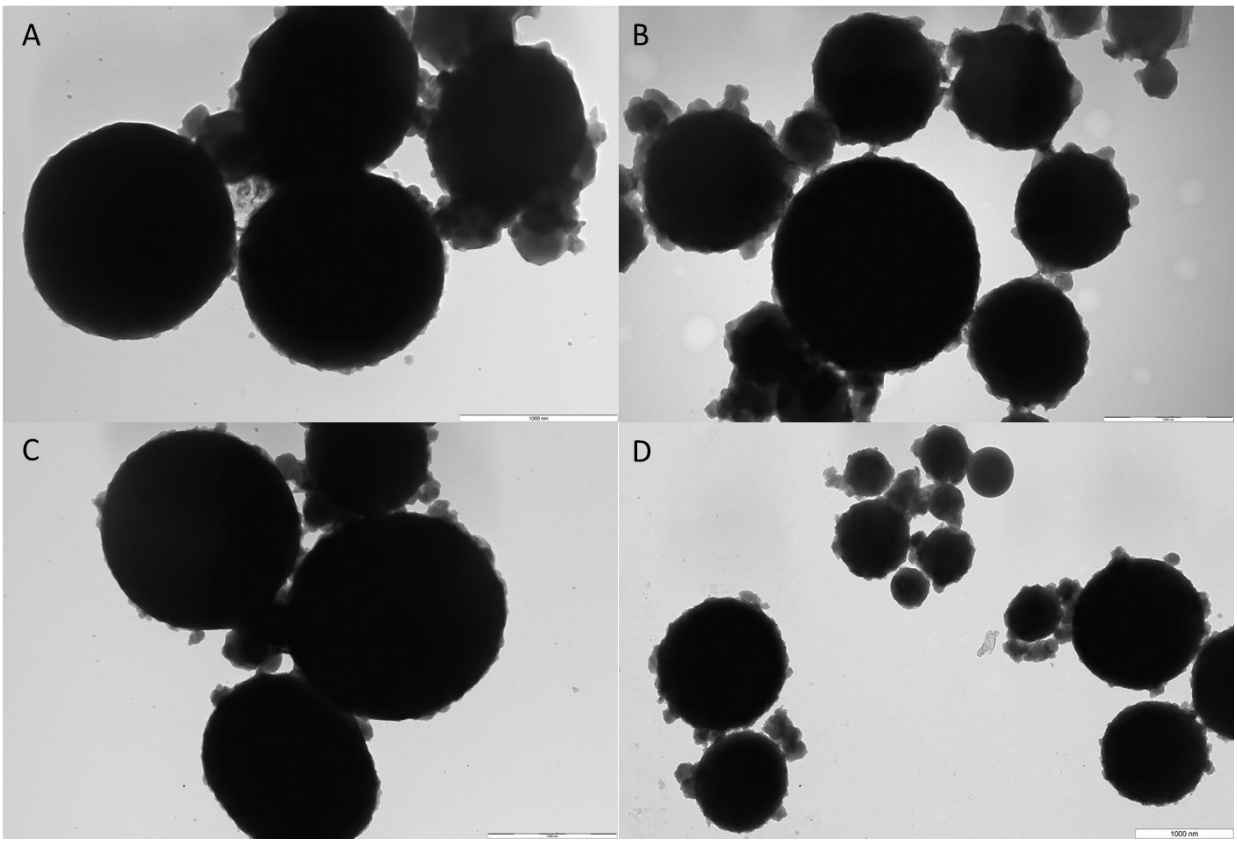


Figure 3.3 Representative TEM images of the PE MP in the different media at different timepoints. A) time 0 in ARW1, B) 24 hours in ARW5, C) 7 days in HH COMBO and D) 21 days in ARW5. The scalebar on all images is 1000 nm.

The particle size was determined with the use of TEM images as part of an ongoing project on particle characterisation and ageing in the synthetic river waters (ARW1 and ARW5) in combination with HH COMBO. The morphology of the particles was confirmed to be spherical and particles of sizes spanning the whole size range stated by the supplier (of 1-5 µm) were observed as shown in Figure 3.3.

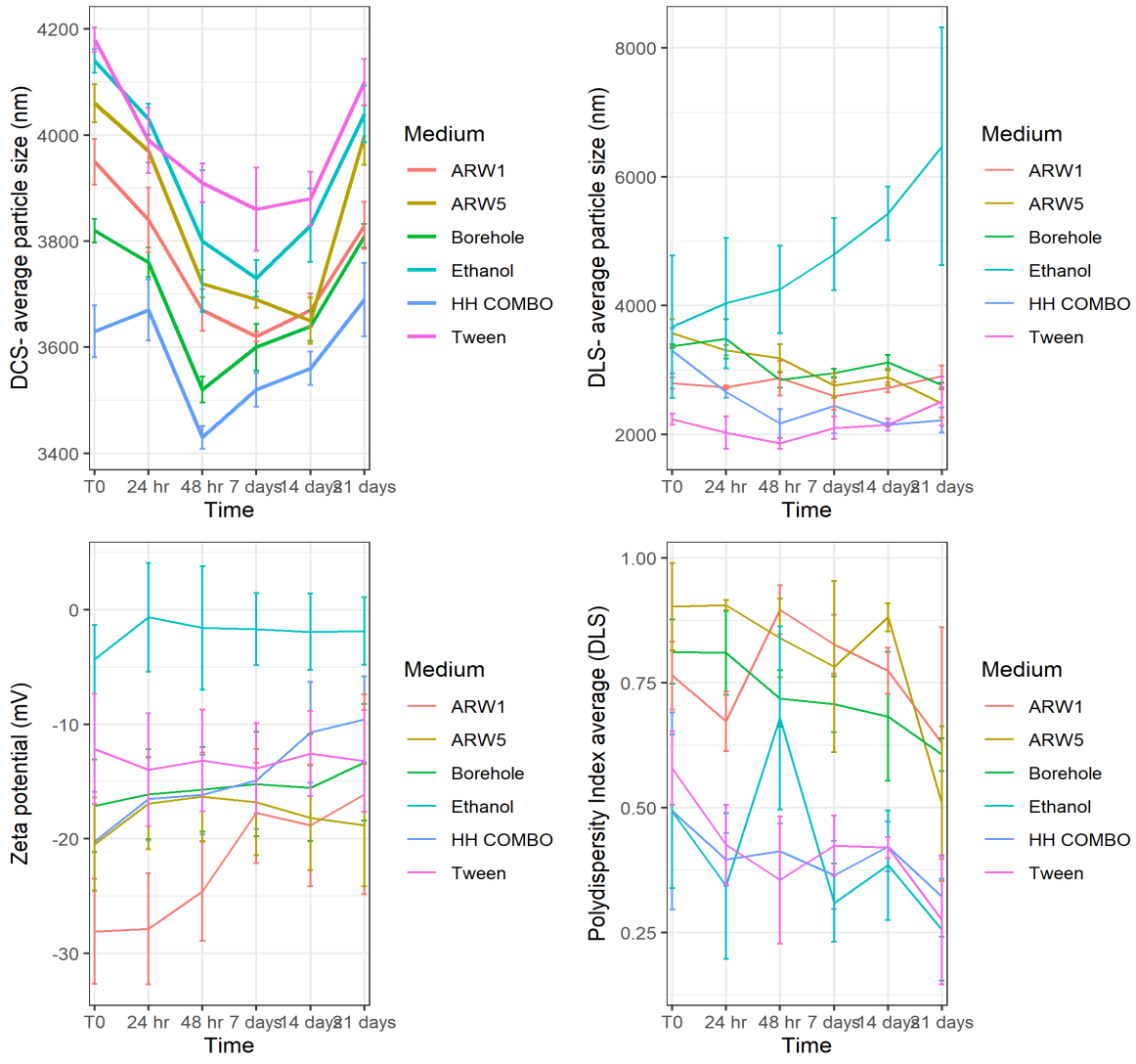


Figure 3.4 The impact of media composition and dispersion method (surfactant, ethanol, NOM) on particle stability over the 21 days as determined by DLS and DCS. A) the average particle size determined using DCS, B) the average particle size determined using DLS, C) zeta potential variation and D) the polydispersity index (DLS).

When measuring the average particle size with DCS (Figure 3.4A), there does not appear to be a significant increase in size, compared to the average size measurements taken with the DLS technique (Figure 3.4B), which could be due to the technique of spinning the particles through a sucrose gradient and this could therefore overcome any potential agglomeration of the PE MP in the test solution. Although both techniques show variability in the average particle size, the solution was not homogenous in the size distribution initially and therefore all the dispersion conditions used had average particle sizes within those expected based on the PE particle size range (1-5 μm). The higher the zeta potential (positive or negative values) the more stable the dispersion is considered to be in the case that it is electrostatically stabilised, and thus the less likely it is that the particles will flocculate/aggregate in the solution. As seen in Figure 3.4C, the zeta potential of the ethanol prewetted particles is considerably lower than that of the other treatments. This is reflected in the DLS measurement of average particle size which indicates an increase in the average particle size over time.

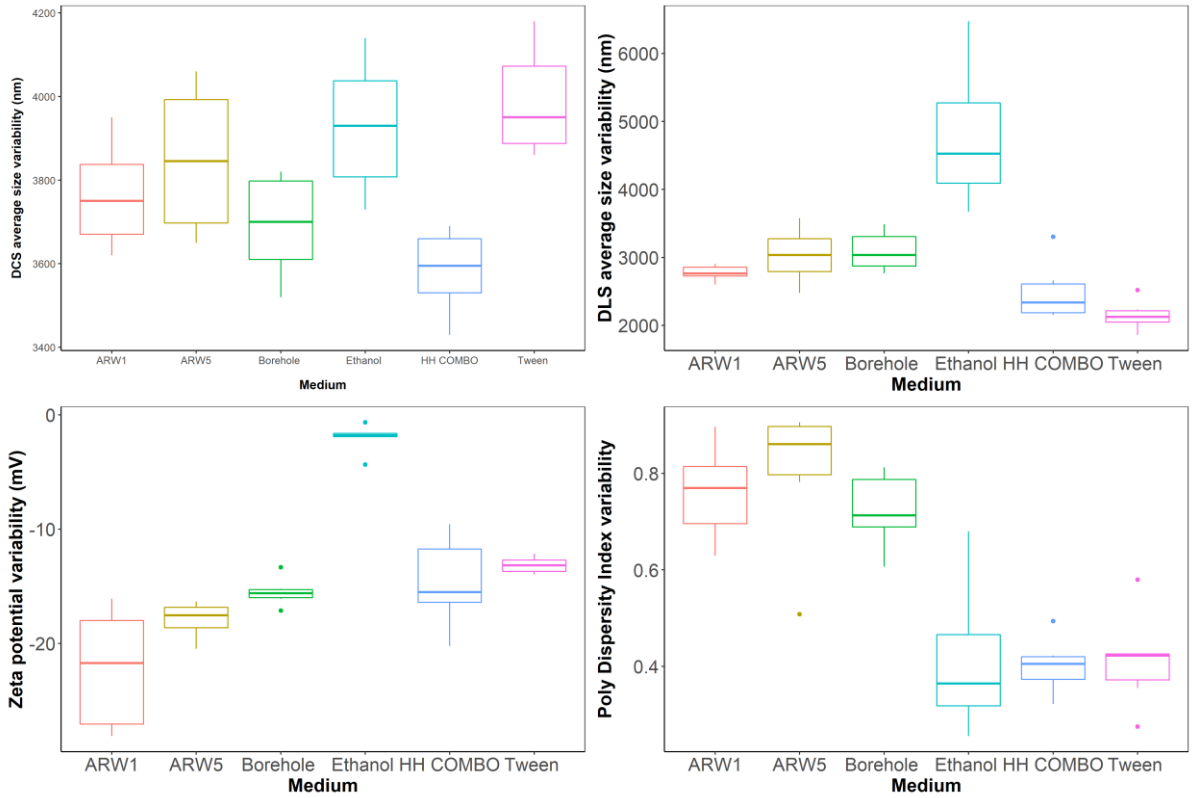


Figure 3.5 Box plots to show the variability in the particle size, zeta potential and PDI for the 21-day period in each of the dispersion methods and media. A) DCS average particle size variation, B) DLS average particles size, C) variability in zeta potential, and D) polydispersity index variation per group. Colours are kept consistent across the plots and are used to identify the different medium/ dispersants used, in order to ease comparison of the groups. Boxplots compare the range within the 9 replicates for the 21 day exposure period for each dispersant method. The box boarders are the upper and lower interquartile ranges (IQR) and the middle line within the box is the median. Whiskers show the extent of the data to 1.5x IQR and any outliers beyond this as presented as points within the plot.

When comparing the total variation in the samples over the 21-day period, the ARW5 and the ethanol samples had the largest variability in MP size (Figure 3.5A, 5B), which could be due to the relatively high concentration of NOM in the ARW5 treatment (Table 3.1) leading to a diffuse absorbed corona and to the low zeta potential of the ethanol prewetted MP reducing the electrostatic repulsion between the MP. Both the TWEEN and ethanol treatments had the lowest variability in the zeta potential (Figure 3.5C) compared to the MP that was dispersed

in medium without the dispersion treatments. Zeta potential was most variable in the HH COMBO and the ARW1 dispersions which could be due to the lack of stabilisers/surface coating properties in these media as HH COMBO is a salt-only medium and ARW1 has a relatively low concentration of NOM compared to the ARW5 (Table 3.1).

Due to the lack of homogeneity in the sample we would expect a higher-than-average PDI for all dispersant methods compared to that of studies using homogenous particles sizes. The zeta potential variability over the 21 days had the smallest range for the MP with a dispersant, the ethanol prewetting and the TWEEN, compared to the MP dispersed in media only (Figure 3.5C). The use of the non-ionic surfactant TWEEN appears to give a more stable characterisation profile across the 21 days when compared to other treatments due to the lower size variability (Figure 3.5). This is consistent with a layer of surfactant coating the MP and providing steric stabilisation preventing the MP from coming too close together. However, the use of various media as dispersant, which have different amounts of NOM (from none to 4.6 mg/L), leads to quite some variability in the apparent particle size over the 21 days, although the mean size never goes much beyond the 5 µm specified by the manufacturer and thus agglomeration is limited, at least of the larger particles which dominate the DLS data. Due to the heterogeneous nature of MP in the environment it is highly unlikely that the variability in the MP exposures from using medium as a dispersant rather than adding the synthetic TWEEN as a dispersant will detract from the environmental accuracy of the exposure, especially when taking into consideration the potential confounding effects of using a surfactant (which is highly unlikely to be factor in toxicity during environmental exposures).

The presence of NOM in natural waters has led to its widespread use for the dispersal of nanomaterials in previous work to improve and refine standard toxicity testing for particles. Increasing the complexity of the testing medium, from standard salt-only culturing medium to NOM containing medium increases the realism of the tests, and improves the reproducibility of the tests by stabilising the particles and facilitating their contact with the test organisms (Lowry *et al.*, 2012; Petersen *et al.*, 2015; Ellis, Valsami-Jones and Lynch, 2020). However, the conditioning of MP for toxicity work, e.g. through dispersion in NOM-containing medium to stabilise them and ensure that their surfaces are closer to how they would be in the environment where interactions with biomolecules occurs instantaneously, is not yet widely considered (Kögel *et al.*, 2020). The role that this pre-conditioning could play within the toxicity exposure could be significant due to the interaction with different molecules in the medium and how this could affect the olfactory cue detection of the plastic by the organisms (Ellis, Valsami-Jones and Lynch, 2020). Although this may vary in sensitivity with each organism, it is an interesting aspect of the ever-growing MP problem, and we would suggest that pre-treatment of MP with artificial surfactants prior to their exposure to the test organisms themselves should be given greater consideration for the variability of confounding effects that surfactants could pose.

3.4.2 Particle retention in the *Daphnia* guts

One of the challenges with assessing MP toxicity is how to effectively quantify the amount of plastic that is potentially ingested. *Daphnia* are a great test organism due to their keystone status in the environment, and their responsiveness in previous testing toxicity testing with chemicals and nanomaterials making them an indicator species (Chapter 4). An additional

benefit of using *Daphnia* in MP research is the transparent body of the *Daphnia* that allow plastics to be visualised in the daphnids guts. This can allow us to observe the uptake and retention of plastics in *Daphnia*.

The uptake of the MP particles by the *Daphnia* was confirmed using both optical and fluorescence microscopy, at varying concentrations during an initial toxicity assessment following the OECD 202 protocol. The initial PE exposures highlight that the PE was ingested in a dose-dependent manner, but that PE beads had negligible toxicity and it appeared that the observed effects from the PE beads were the result of the physical interaction and impedance of movement due to coating of the swimming appendages at extreme concentrations of MP which led to the eventual immobilisation of the *Daphnia* (~1g/L, 72-hours) (Figure 3.6D).

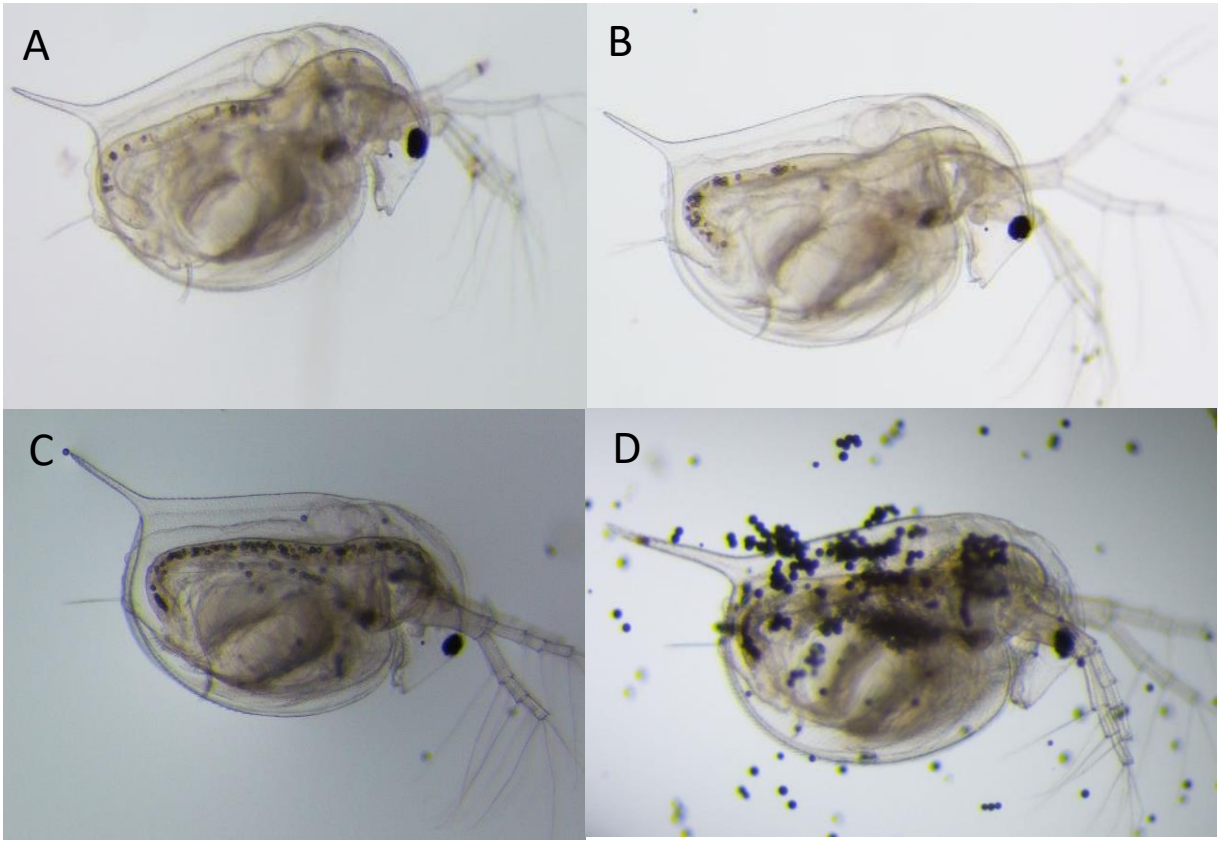


Figure 3.6 An example of the ingestion and interaction with PE MP in *Daphnia* after 24-hours exposure. A- 25 mg/L, B- 50mg/L, C- 200mg/L and D-1000mg/L. Note the limited evidence of MP in daphnid D compared to the external interaction showing the physical toxicity and interreference that occurs during exposures. The PE MP shows evidence of dose-dependent uptake across all exposures, with increasing observed concentrations in the daphnids guts with increase external exposure concentrations.

Although plastics are designed in most cases to have limited toxicity, and indeed limited toxicity was observed in this study for the PE MP tested, we can compare any elevated toxicity arising from the variation in dispersal protocol and/or medium composition. We can also estimate the amount of plastic ingested based on how much plastic is present in the daphnid guts using fluorescence and observe if there is a delay in egestion. The working hypothesis for this part of the study was that medium constituents bound to the surface of the MP, such as the humic acid or secreted biomolecules, might make the MP more attractive for uptake, but that these coatings can be digested in the *Daphnia* gut and therefore reveal the underlying

hydrophobic surface leading to the PE MP becoming stickier and/or interacting more strongly with the gut epithelia and resulting in physical damage and toxicity. Similarly, the TWEEN surfactant could also be displaced or degraded in the gut with its low pH and high enzyme and bacterial content, which could potentially prove toxic in its own right once ingested, leading to elevated toxicity.

For the use of surfactants to disperse the particles, it should be considered that this is a chemical that has been intentionally added to the solution for the purpose of coating the particles and therefore this could have an impact on the overall toxicity of the study. For the toxicity exposure within this study a TWEEN control was conducted, with the same concentration of TWEEN used in the solution to check for any toxicity associated with this. However, this is limited by the fact that TWEEN is used to coat the PE ahead of dispersal rather than being dissolved into solution and therefore the exposure pathways would be different between these two scenarios, however negligible toxicity was observed in the control and the TWEEN PE at the exposure concentrations during the acute toxicity assays.

Images were also taken to assess the potential retention of microplastics in the *Daphnia* gut. Daphnids were exposed to MP for 24 hours before being removed, rinsed, and placed into fresh medium to allow depuration. Daphnids were imaged during the depuration window to assess if there was any delay in egestion (Figure 3.7). Images were taking using both using bright-field and fluorescence light filters during the ingestion-egestion process. Although the use of fluorescence in microplastic ingestion studies have been shown to leave potential artifacts in the data, it is a useful tool in determining uptake and can complement the bright-field images in this study.

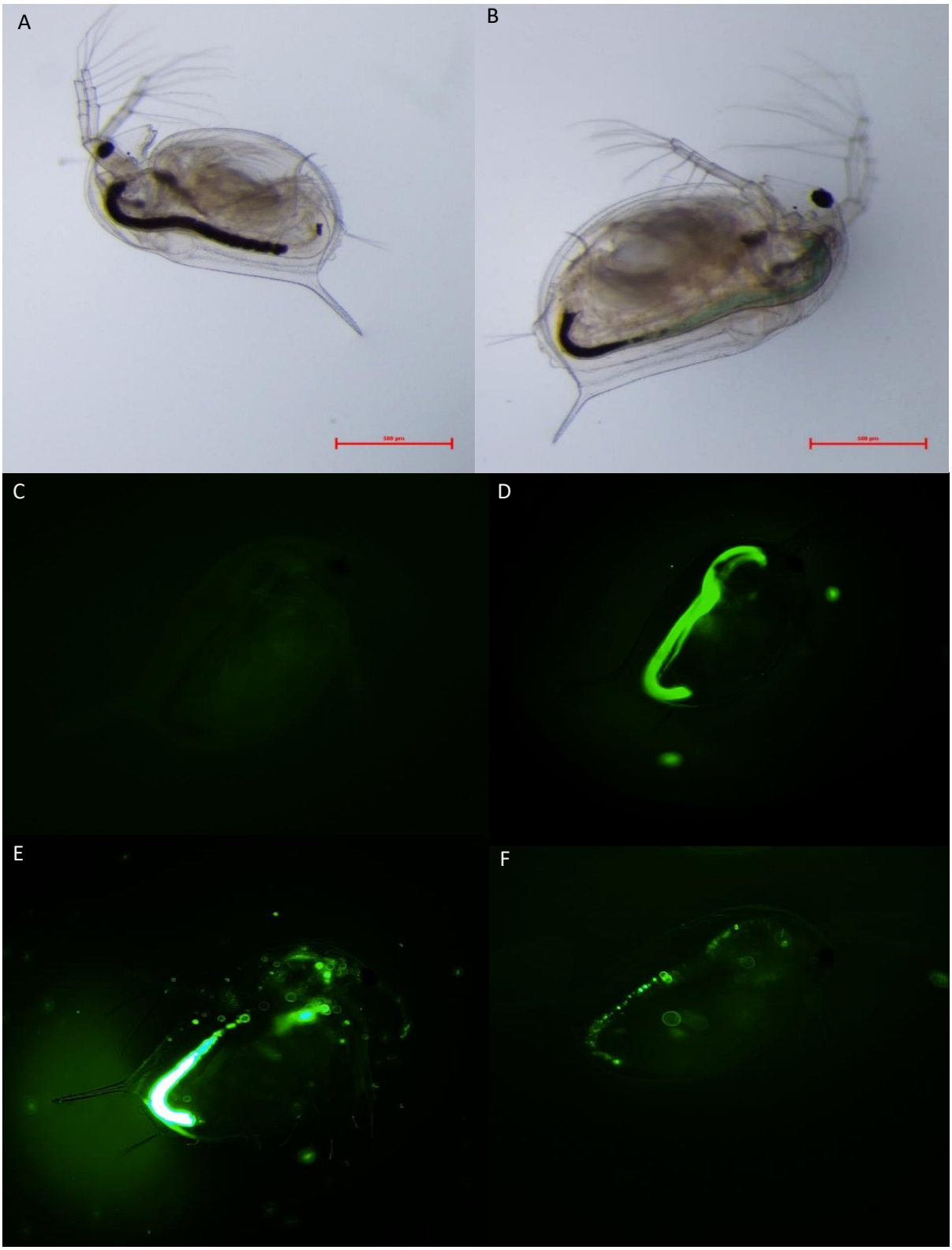


Figure 3.7 Ingestion of PE beads (1-5 μm) by *Daphnia* neonates in the HH COMBO group. Images A and B were taken with a Nikon Optical microscope, C-F were taken using an Olympus optical microscope with a Green Fluorescent Protein (GFP) filter cube and dichroic mirror and a DP74 colour camera and viewed using CellSens software (x70 magnification). A- daphnid after 24-hour exposure to PE with clear evidence of MP throughout the gut. B- daphnid 24-hour after removal from PE

exposure, showing that most of the MP had been depurated but that some remained in the lower gut segments. C- daphnid with fluorescence as control prior to exposure to PE. Note that the slight trace of fluorescence here is autofluorescence. D- daphnid after 24-hour exposure to PE with evidence of MP throughout the gut as shown by the fluorescent green. E- 24-hour after removal from PE exposure, showing that most of the MP had been depurated but that some remained in the lower gut segments as evidenced by the reduced fluorescence. F- daphnid after a 48-hour depuration window showing further reduction in fluorescence in the gut and no visible evidence of dye leaching at these time points.

As the particles can be visually tracked through the gut, we can make an approximation on potential retention or significant increase in residence times of the particles and if this varies based on the dispersion process or medium composition (Figure 3.7). Although the bulk of the particles can be tracked due to the size, there is still the potential that fragments of particles could be tapped in the microvilli in the *Daphnia* gut that could cause longer term inflammation in the gut as a result. There is also the potential that the daphnids would be able to digest the surface coating of the plastics, for example the corona and any associated chemicals or toxicants with this could then also cause a subsequent toxicity response, however this was not observed during the acute exposure with TWEEN during this study. The ability of the daphnids to egest the PE MP once ingested is demonstrated in Figure 3.7, with the gradual decrease in fluorescence (Figure 3.7 C-F) and density of the particles present in the gut in the bright-field images (Figure 3.7 A, B). Daphnids were also imaged under the fluorescence filter prior to exposure to confirm that there was no baseline fluorescence that could confound the particle ingestion images (Figure 3.7C).

3.4.3 Leaching of dye depends on the dispersion method

Following the filtration of an aliquot of the initial MP stock in the various media (21-days after dispersal), there was no discernible leaching of the dye from the beads into the media of the stock. This is an important consideration, as if the dye were leaching from the beads into the stock solution, this would decrease the fluorescent signal of the beads in later exposures using the same stock of MP. For example, during a chronic study during medium renewal and re-exposure, if the dye were leaching from the beads into the stock medium, the beads added to the exposure on day 20 would have less fluorescence than those added to the exposure on day 1, which could subsequently affect the estimation of ingested MP based on the decrease in this fluorescence. Although, as noted in other studies, the pH of *Daphnia* gut can vary from that of the testing medium from pH between 7.3-8.1 (depending on the medium) to 5.6 in the lower area of the *Daphnia* guts (Davis *et al.*, 2020). Therefore, there is still the potential for dye to leach out once ingested due to the change in internal pH and the presence of enzymes which could lead to potential artifacts in the data as a result during internalised or ingestion-based fluorescence studies (Schür *et al.*, 2019). However, leached dye tends to lead to a broad distribution throughout the organism leading to a diffuse background of the dye, which is not evident from Figure 5 (Salvati *et al.*, 2011).

3.4.4 Protein corona formation on the PE surface

The formation of the corona will depend on the local environment of the particle, and can change over the particles lifetime and therefore this should be factored into the experimental design for the assessment of MP toxicity (Ellis, Valsami-Jones and Lynch, 2020). One of the

growing concerns within this field of research is how environmentally relevant the laboratory toxicity testing is. The primary focus of this research was initially the determination of the concentration of the particles actually dispersed in the toxicity tests in order to correlate exposure dose with response. However, there is increasing interest in the range of morphologies arising from MP agglomeration and the importance of testing more complex mixtures with heterogeneous biomolecules to have a better understanding of the MP surface characteristics during the exposure to organisms. Although in this study we used simplistic PE spheres, the approach presented here allows a more accurate estimation of corona coating based on the surface area of the particles. Based on the available literature, there is a clear need to consider the environmental relevance of studies by considering the role that medium and dispersal methods can have within a MP laboratory study.

It has been shown that the formation of a *Daphnia* secreted protein corona on the surface of nano polystyrene spheres leads to an increase in the uptake of the particles during an acute toxicity assay (Nasser and Lynch, 2016b). The surface binding of proteins, polysaccharides or xenobiotics by plastics could also be impacted by the dispersion protocol used, as has been shown for metal and metal oxide nanomaterials (Ellis, Valsami-Jones and Lynch, 2020). For example, a surfactant (sodium dodecyl benzene sulphonate) led to an increase in ionic binding of methylene blue dye to polyvinylchloride (PVC) MP surfaces during exposures (Xia *et al.*, 2020). Even in the absence of proteins or biomolecules in the dispersant medium, overtime the daphnids themselves secreted biomolecules into the medium, and their filter-feeding natures ensures that these secreted biomolecules are released into the surrounding medium and this become available to form a corona on the MP. Proteomics analysis of the corona composition of nanomaterials recovered from exposure medium after 7 days has been used

to revealed insights into how the daphnids responded to the presence of the particles (Ellis and Lynch, 2020).

The variability in total protein associated to the PE MP surface due to the six different dispersion methods was determined by mixing an aliquot of each of the PE dispersal stocks (made of the clear PE beads) with *Daphnia* conditioned medium (24-hours) for a 1-hour incubation before analysis with BCA (section 3.3.7, & Figure 3.1 D).

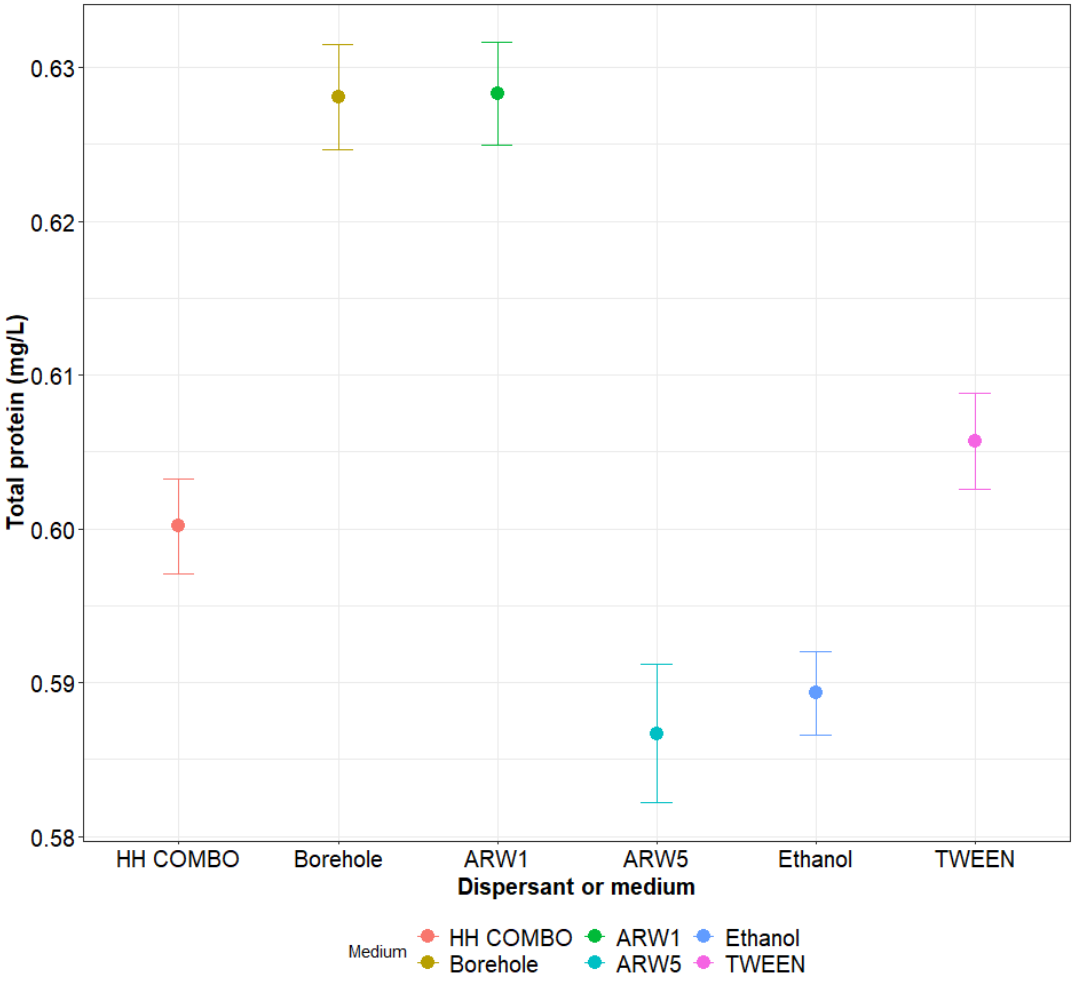


Figure 3.8 The total protein associated with the PE MP that has been dispersed with the various protocols and in the different media (relative to the BSA standard currently)

The amount of protein present in the HH Combo medium is lower than in the ARW1 and borehole as there were no biomolecules present initially and thus all were acquired as a result of daphnid secretions into the water. This was comparable in concentration to the TWEEN treatment, which is most likely to denature/remove the proteins due to the surfactant properties of TWEEN (Maiolo *et al.*, 2014; Jelińska *et al.*, 2017). ARW5 and ethanol had the lowest concentrations of protein, ARW5 may be due to the surface of the MP being conditioned by the high concentration of NOM in the medium already, which could slow the binding of chemicals (and potentially a longer incubation period would allow more transfer to occur). In addition, the low zeta potential (Figure 3.4 C) of the ethanol prewetted MP could impact their subsequent binding to proteins within the medium. The ARW1 and borehole treatments had the highest concentration of protein, potential due to surface coating with NOM that allowed for subsequent interaction with the *Daphnia* excreted proteins within the 1-hour incubation period.

3.5 Conclusion

When using a polydisperse mixture of bead sizes, although the average size of the beads changes over time, it remains within the scope of the study design during the 21-day period. During this timeframe, there was no quantifiable leaching of the fluorescent dye from the PE beads into the respective media, including the ethanol prewetting and TWEEN dispersions. PE microbeads have negligible acute toxicity on *Daphnia magna* within this study, which is increasingly being reported from other MP toxicity studies (Canniff and Hoang, 2018). However, the effect that the dispersion methods can have on the protein corona formation

showed that the prewetting of the MP with ethanol or dispersal in medium with high concentrations of NOM decreased the concentration of associated proteins compared to the other treatments after a 1-hour incubation.

Future work could include a longer-term assessment to ascertain if there is any sublethal toxicity or variability in toxicity end points due to the dispersal processes, and longer incubation periods in test scenarios, for example with the inclusion of algae. In addition to this, there are other factors that could be considered in studies going forwards including the morphology of the particles and any surface modifications such as charge or loosely bound dye due to the change in surface area and hydrophobicity (Saavedra, Stoll and Slaveykova, 2019).

The dispersion process of particles is an important step going forwards, and characterisation steps should be undertaken, such as the steps undertaken within nanotoxicology, to accurately report the dose and study design within the microplastics toxicity field to allow for comparisons between studies.

Chapter 4

4. Effect of culturing media on *Daphnia* fitness and toxicity test performance

Katie Reilly[1], Laura-Jayne Ellis[1], Jon Sadler[1] & Iseult Lynch[1].

[1]School of Geography, Earth and Environmental Science, University of Birmingham, UK.

Key words: *Daphnia magna,* medium, fitness, toxicity testing,

4.1 Abstract & graphical abstract

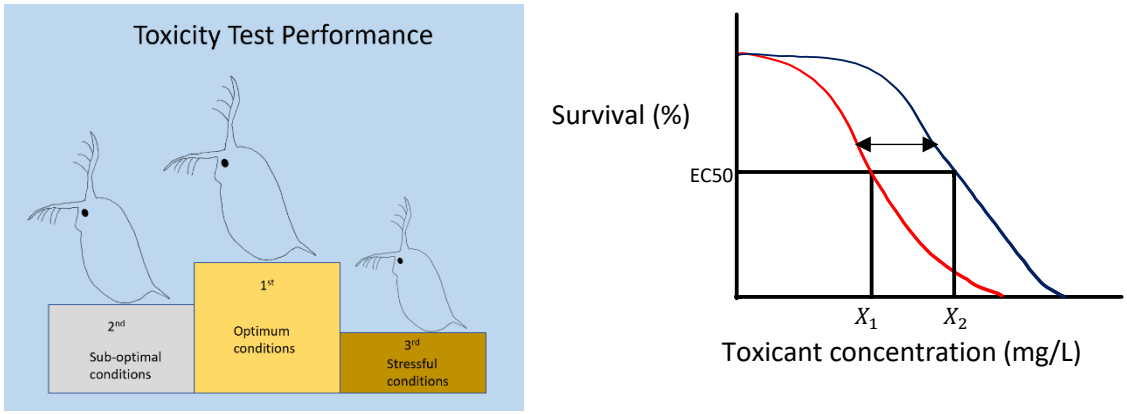


Figure 4.1 Graphical representation of the potential shift in the EC_{50} value as a result of the changing testing conditions impacting test performance.

Differences in the culturing conditions, and therefore testing medium, used in *Daphnia* toxicity assays can lead to considerable variations in the total reproduction and growth of the *Daphnia* in both the control and exposure groups. Given the role of standardised tests in ranking the toxicity of chemicals including nanomaterials and microplastics, as well as the use of data from *Daphnia* toxicity assays for environmental modelling and establishment of threshold levels for pollutants, a deeper understanding of the inherent variability in the test systems is needed.

Similarly, the standardised test media have been developed to optimise the test population health, which does not take into consideration any deficiencies in species health or fitness that occur due to natural environmental variation and adaption to the environment. Utilisation of super-fit daphnids could mean that we underestimate the potential toxicity of chemicals to environmental populations, especially when looking at sublethal toxicity markers such as growth and reproduction. For example, by varying the culturing /exposure medium a significant difference in the growth and total reproduction for *Daphnia,* even with no toxicant present, is demonstrated here. Such life-history trait impacts over multiple generations could have wider implications at a population level due to the multiple stressors present in the natural environment, and warrants further consideration as part of the current efforts to revise standardised test guidelines.

4.2 Introduction

Daphnia magna are a well-established and widely used model species for freshwater toxicity testing due to their keystone status in the environment, their rapid parthenogenic reproductive cycle and their sensitivity to a range of xenobiotics, such as kairomones (Nasser, Constantinou and Lynch, 2020), pharmaceuticals (Peake *et al.*, 2016), nanomaterials (Ellis and Lynch, 2020) and metals (Traudt, Ranville and Meyer, 2017). A suite of behavioural and morphological changes that can be observed in *Daphnia* in response to environmental stimuli have been established and form the foundation of the defined protocols for testing of chemical toxicity to *Daphnia* such as the OECD 202 (Guideline for testing of chemicals- *Daphnia* sp. Acute Immobilisation Test) and OECD 211 (Test Guideline- *Daphnia magna*

reproduction test) and the EPA testing methods (US Environmental Protection Agency, 1996; OECD, 2004a, 2012a). End-points evaluated can range from the more extreme response such as death (often measured as immobilisation), to reproductive changes such as an increase or decrease in the number of neonates or a delay between broods, to delays in growth, or can consider phenotypic changes such as additional spines on the helmet and behavioural changes such as swimming behaviour (Colbourne *et al.*, 2011; Chevalier *et al.*, 2015; Karatzas *et al.*, 2020; Tkaczyk *et al.*, 2021).

Daphnia are commonly found in ponds and slow-moving water bodies and are a keystone species due to their role as the intermediate step in the trophic food web, wherein they are an important food source to many aquatic invertebrates whilst being a key consumer of algae (Ebert, 2005). Ponds are a useful tool for reflecting the physico-chemical impacts of local land use and are representative of the effects of local point and diffuse sources due to their small area and often relatively large catchments. Land use is often reported as having the largest effect on water quality and subsequently the macroinvertebrate community composition (Thornhill, 2013). In addition, *Daphnia* are a well-established indicator species for pollution and are widely used within eco-toxicity testing protocols to access chemical hazards due to their sensitivity (Nasser and Lynch, 2019b). However, the comparison between toxicity end points for laboratory cultured daphnids compared to daphnids in real environments is unclear.

It has also been highlighted that small variations in the testing conditions with *Daphnia* can have a significant impact on the output of the study. This includes variations in temperature, (Rinke and Petzoldt, 2003) and also in the food availability which has been widely studied (Brown and Yan, 2015; Ogonowski *et al.*, 2016). In addition, it is important to take into

consideration the culturing prior to the toxicity testing and therefore to consider the impact of the exposure medium in which the *Daphnia* are maintained and/or acclimatized. The purpose of standardised culturing media and protocols are to ensure the optimum health of the test organisms in order to have a standardised and healthy baseline from which to access toxicity, and as such they have been designed to optimise organism "fitness". It is generally assumed that body size is a strategic trait in daphnia that can be adjusted based on the surrounding conditions (such as food availability and quality) and that daphnids can exploit this by adjusting their growth to provide competitive advantage (Pereira and Gonçalves, 2008). The Size Efficiency Hypothesis (Brooks and Dodson, 1965) provided the theoretical grounds to explain the dominance of large-bodied over the smaller zooplankton species, in lakes where predation pressure is low or absent: the food concentration needed to permit the population maintenance (where population growth equals zero) should decrease as adult body size of coexisting species increases (Brooks and Dodson, 1965). Thus, body size can be roughly correlated with fitness within a single daphnia species but in interspecies competition functional factors must also be considered.

Indeed, a key requirement of standardised toxicity testing is that there can be no effect in the controls to rule out confounding factors; for example, the OECD 202 acute toxicity test is considered invalid if >10% of the control population are immobilised during the 48 hours as this suggests that there is an underlying cause other than the toxicant being evaluated. The OECD 211 test is considered invalid if >20% mortality occurs in the parent populations (of both the exposure and control daphnids) or the mean number of neonates per daphnid is <60 at

the end of the test (OECD, 2012a). If either of these conditions is met, this indicates that the exposure concentration was too high and could be leading to mortality within the test populations when the aim of the test is to assess sublethal toxicity response on the reproductive success of *Daphnia,* therefore invalidating the test. However, the robust health of these animals may give them an advantage in toxicity testing due to the favourable conditions and optimum growth environments of the laboratory compared to the natural environment, which could lead to sub-lethal effects of chemicals being missed.

As the field of microplastics (MP) research has expanded, in parallel with growing public awareness of ubiquity of microplastic pollution globally, potential questions about the validity of the testing methods used for microplastics has arisen. For example, the OECD tests as currently used were specifically designed for dissolved chemicals and not for dispersed particles, however these tests are typically used to enable comparison to previous toxicity studies and data (Nasser and Lynch, 2019b). As we assess the limitations of these testing methods there has also been discussion on their environmental relevance. Particularly within the MP field concerns have been raised both in terms of the concentrations of particles used (i.e., unrealistically high concentrations are needed in order to obtain a response) (Lambert and Wagner, 2016b; Athey *et al.*, 2020) and more recently in terms of the morphology of these particles due to the potential different in retention and mechanistic toxicity, for example, fibres are theorised to be retained longer in organisms due to potential tangling (Kukkola *et al.*, 2021). However, the culturing medium used for the test organisms should also be considered in all fields of toxicity testing, especially when aiming to extrapolate these results to understand environmental problems.

As noted above, optimisation of medium composition to support organisms' health has been an ongoing process. For example, (Kilham *et al.*, 1998b) aimed to address a limitation in the media available for multiple-organism studies such as food chain or tropic transfer, by designing a medium that is suitable to support both algae and zooplankton growth to harmonise conditions for experiments using both test animal and their food organisms. This led to the design of COMBO medium, which was reported to support excellent growth and reproduction of both algae and zooplankton, with algal growth rates similar to those of algae grown in the standard reference medium 'WC' (Guillard, 1975), and *Daphnia* fecundities of *D. pulicaria* were similar to those reared in natural surface waters (Kilham *et al.*, 1998b). COMBO medium resulted from the combination of several media that were commonly used at the time for both algae and *Daphnia* culturing respectively, as outlined in Table 4.1. There is often a narrow concentration range between toxicity and deficiency in minor elements which can be hard to balance when considering a medium for use with more than one organism. COMBO medium has been demonstrated to provide a good level of health to both *Daphnia* and algae and is therefore suitable for long term culturing. However, it was reported that there was a significant difference in the fecundity of the *Daphnia*, and animals fed the higher ratio of algae in the test had lager broods in the filtered Reservoir Water compared to the COMBO medium, which highlights how multifaceted this issue can be.

Table 4.1 Summary table of key media used as the foundation of COMBO to enable optimised culturing of both algae and *Daphnia*. From Kilham et al. 1998.

Algae		***Daphnia***		**Notes**
Guillard's WC	Guillard, 1975	MS	Keating 1985	MS: high glycylglycine content = aseptic
Fraquil	Morel, et al. 1975	M-4	Elendt & Bias 1990	M-4: recommended by OECD toxicity programme in Europe, but does not sustain algae
ASM	Carmicheal & Gorham, 1974			
DYIII	Lehman, 1976			

As many of the studies on the impact of medium composition were performed before the emergence of nanoscale materials and the microplastic pollution problem, a re-look at these issues is timely. Additionally, the comparatively low acute toxicity of pristine spherical microplastics requires consideration of the relevance of testing non-environmentally relevant exposure doses to determine effect concentrations, and evaluation of whether the enhanced "fitness" of organisms in optimised lab conditions is actually reflective of the potential impacts of microplastics in real conditions. To facilitate evaluation of the impact of medium composition on organism fitness and responsiveness during toxicity testing, *Daphnia* fecundity in a panel of representative and increasingly realistic waters was compared. Thus, in addition to HH COMBO, two model synthetic waters that are representative of the

compositions of common waters in Europe (as detailed in (Gallego-urrea, Hassello and Hammes, 2013) in terms of their ionic strengths and natural organic matter content, and a real groundwater taken from the onsite borehole at the University of Birmingham were compared. Although not exhaustive of all testing media utilised in standardised toxicity testing, this set of waters provides a comparison between salt-based laboratory culturing medium (HH COMBO), model environmental waters (artificial river water class 1 and 5- ARW1 and ARW5 respectively) and natural borehole water. This enables a preliminary exploration of the impact of the different types of media on *Daphnia* fitness or fecundity of both controls and exposure populations during chronic studies.

In this study the growth and fecundity of *Daphnia* in the varying media are compared to determine if medium composition has an impact on the organism's overall fitness prior to exposure to toxicants. The second stage of the study assessed whether any inherent differences in organism fitness conferred by the different media compositions impacted the daphnids sensitivity to toxicants, represented here by the widely used surfactant Sodium Dodecyl Sulphate (SDS) assessed according to the OECD 211 test. Nile red quantification of lipids were included as sublethal markers of stress, in addition to evaluation of growth (eye-tail length and tail length) and number of offspring.

4.3 Materials and methods

4.3.1 Laboratory media for *Daphnia* culturing

Three standardised culturing media, outlined in Table 4.2, were made in the laboratory using laboratory grade chemicals following the methods outlined in (Kilham *et al.*, 1998b) and

(Hammes, Gallego-Urrea and Hassellöv, 2013). The media were aerated for a minimum of 8 hours prior to use for culturing and the dissolved oxygen content was measured during each media change. pH was also measured and moderated to within the defined parameters of each medium (see Table 4.2) before being used for the ongoing culturing of Bham 2 strain *Daphnia magna.*

The artificial waters were prepared according to the compositions shown in Table 4.2, and vary in terms of their salt compositions, total ionic strength, and NOM content, and are thus an intermediate between salt-only standard test media and real waters which will also contain suspended matter and a suite of other minerals, biomolecules and pollutants spanning a range of concentrations.

In addition to the artificial laboratory media, borehole water (pH~8-8.2) is often used for the culturing of *Daphnia*, as is the case with Bham 2 *D. magna*. For comparison water from the local borehole (Appendix 3- Table S3.4) was also used within this study and aliquots of the borehole water were routinely taken for further element analysis to monitor any potential confounding factors.

4.3.2 *Daphnia* culturing and acclimatisation

Third brood daphnids were used for all cultures to ensure optimum quality of genetic health of future cultures. The media were refreshed three times per week. All cultures were fed the same daily algal ration of *Chlorella vulgaris* (0.5 mg C days 0-7, 0.75 mg C days 7 onwards) and kept in a 20°C laboratory under a 16:8- hour light: dark cycle. *Daphnia* were acclimatised with a phased media change from the initial HH COMBO culture into the separate cultures (borehole, ARW1 and ARW5) with 25:75, 50:50 and 75:25 ratios of the new medium: HH

COMBO over the sequential water changes of the initial acclimatisation culture. Following this, cultures were maintained in their respective media for 3 generations ahead of the control and exposure observations.

4.3.3 Analysis

The artificial laboratory medium and borehole water were analysed using Inductively Coupled Plasma- Optical Emission Spectroscopy (ICP-OES) (Perkin-Elmer Optima 8000) with a radial view plasma objective using a cross flow nebuliser (argon gas flow of 8 L per minute) and a sample flow rate of 1 mL per minute. The seven elements selected for quantification due to their predominate concentration in HH COMBO medium were boron, calcium, magnesium, phosphate, potassium, silicon and sodium. Calibration standards for ICP-OES were 0, 0.1, 1, 10 and 100 mg/L. The water samples were filtered through a 0.45 µm PES membrane filter ahead of ICP-OES analysis. Samples were analysed in triplicate, with blanks every 15 samples and calibration checked against the internal standards on each series.

4.3.4 Toxicity testing

Daphnia were cultured in the 3 laboratory testing media (outlined in Table 4.2) in addition to the onsite borehole water to ascertain if there is any significant difference in the baseline *Daphnia* health or fitness due to the culturing medium. *Daphnia* were cultured in their respective media for 3 generations ahead of starting the test to allow sufficient acclimatisation and all other testing conditions were kept the same. Borehole water acts as the closest possible water source to the natural water that *Daphnia* would be in, whereas HH COMBO was used as the most controlled water. ARW1 and ARW5 both contain varying concentrations of natural organic matter and nutrient concentration and are model waters based on

European river profiles (Hammes, Gallego-Urrea and Hassellöv, 2013). In parallel, *Daphnia* were exposed to SDS (200 µg/L- approximately EC_5 calculated from the initial range finding exposures) in a chronic exposure to determine if the difference in medium composition significantly impacts the *Daphnia* response in the controls or during the toxicity assay. All other variables were controlled and kept the same across the tests. Culturing controls and SDS exposures were run in parallel, with 12 *Daphnia* per group maintained individually in 50 mL vessels. Media was replaced thrice a week and *Daphnia* were fed daily. Optical microscope images were taken every 7 days to measure growth over the test period (using the increase in length from the eye-tail and tail length measurements as a proxy for total growth over time) and neonates were counted daily. Example measurements and log sheets are included in section 2.3.4.

4.3.5 Nile Red staining of lipids

Following the conditioning of the media, the *Daphnia* were removed into fresh media and exposed to Nile red (0.1 mg/L in the medium) to stain the lipid droplets that have formed over the 21-day period. The *Daphnia* were stained for 1 hour before moving into fresh medium and rinsing any excess stain from the carapace, following the method outlined in (Jordão *et al.*, 2016). *Daphnia* were then imaged on an Olympus MVX-ZB10 microscope with CellSens software and U-M49002XL-GFP filter cube for fluorescence imaging. In addition, images were taken of unstained *Daphnia* as a fluorescence control without the addition of Nile red.

Daphnia were then isolated in individual 1.5 mL centrifuge tubes; excess medium was removed and 300 µL of isopropanol were added before sonication to homogenise the *Daphnia*. The sample was then centrifuged at 12,000 rpm and 200 µL of supernatant from

each sample was then added to a 96 well plate (Costar) and analysed on a Tecan Spark plate reader (470 excitation, 525 emission) with 30 scans per sample. This allowed a relative comparison across the groups for the increase in intensity based on the Nile red staining of the lipids.

Table 4.2 Summary of the various medium compositions used for testing and their key parameters.

Medium	**HH COMBO**	**ARW1**	**ARW 5**
Salt components	$CaCl_2$ $2H_2O$ $MgSO_4$ $7H_2O$ K_2HPO_4 $NaNO_3$ $NaHCO_3$ $NaSiO_3$ 9 H_2O H_3BO_3 KCl	$CaSO_4$*$2H_2O$ $CaCO_3$ $Ca(NO_3)_2$ $Mg(NO_3)_2$ $NaHCO_3$ $CaCl_2$ $KHCO_3$	$CaSO_4$ $CaCO_3$ $4MgCO_3$. $Mg(OH)_2$ $Ca(NO_3)_2$ $NaHCO_3$ $CaCl_2$ $KHCO_3$
Conditions	pH- 7.6-7.8 ionic strength- 11.07 mg/L NOM 0 mg/ L	pH- 7.3 ionic strength- 10.12 mg/L NOM 1.84 mg/ L	pH- 8.1 ionic strength- 15.76 mg/L NOM 4.6 mg/ L
Key reference	(Kilham *et al.*, 1998b)	(Hammes, Gallego-Urrea and Hassellöv, 2013)	(Gallego-urrea, Hassello and Hammes, 2013)

4.4 Results & Discussion

4.4.1 Comparison of the chemical compositions of the various test media

As with many biological elements, there is a narrow concentration range between deficiency and toxicity which needs to be carefully considered. Testing medium needs to be suitable for the test species (i.e., algae and *Daphnia*) and a fully defined medium is preferred for the standardisation of the assessment, as opposed to borehole water. Limitations of key elements, such as calcium for the carapace development, have been shown to significantly impact the growth and development of *Daphnia* (Hessen, 2000; Jeyasingh and Weider, 2005). The medium also has the potential to impact the test species or toxicant in question; for example previous research has highlighted the need to ensure that there are no salts in standardised test medium solutions that will react with potential metal toxicants and thus change the overall metal toxicity, leading to the modification of the OECD medium by removing the EDTA when testing toxicants containing metals, or using an alternative medium that contains no chelating agents (OECD, 2004a). When comparing the laboratory testing media, it become apparent how deficient the ARW1 medium is in the macro elements required by *Daphnia* for healthy growth and maintenance (see Figure 4.2). HH COMBO, ARW5 and borehole were more comparable, although borehole was significantly lower in sodium in comparison to the others.

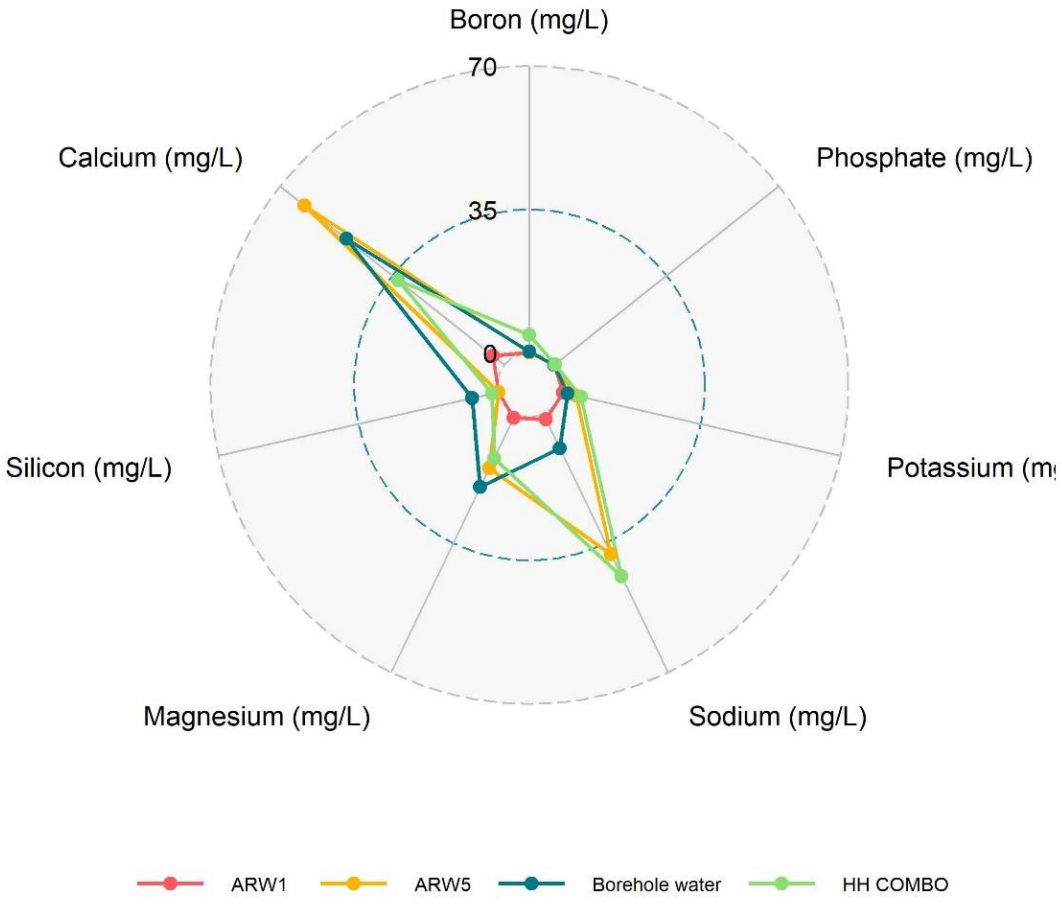


Figure 4.2 Radial diagram comparing the compositions of the 4 test media in terms of their compositions of 7 macronutrients determined to be essential for *Daphnia* health and fecundity.

4.4.2 Variability in medium controls

As noted previously, standardised test media are optimised for organism growth and health, while the artificial river waters were designed to mimic the range of compositions of river waters found throughout Europe with no consideration for their ability to maintain organism health. The medium composition was found to impede the output of the chronic toxicity tests (OECD 211) within the control groups. *Daphnia* in the ARW1 control medium grew less, and had lower fecundity compared to the organisms in HH COMBO, ARW5 and borehole water as shown in Figure 4.3. This is most likely due to the low calcium concentration available within ARW1 medium (Figure 4.2) which can affect the growth and impede moulting of the daphnid's carapace which is an essential step in growth (Jeziorski and Yan, 2006).

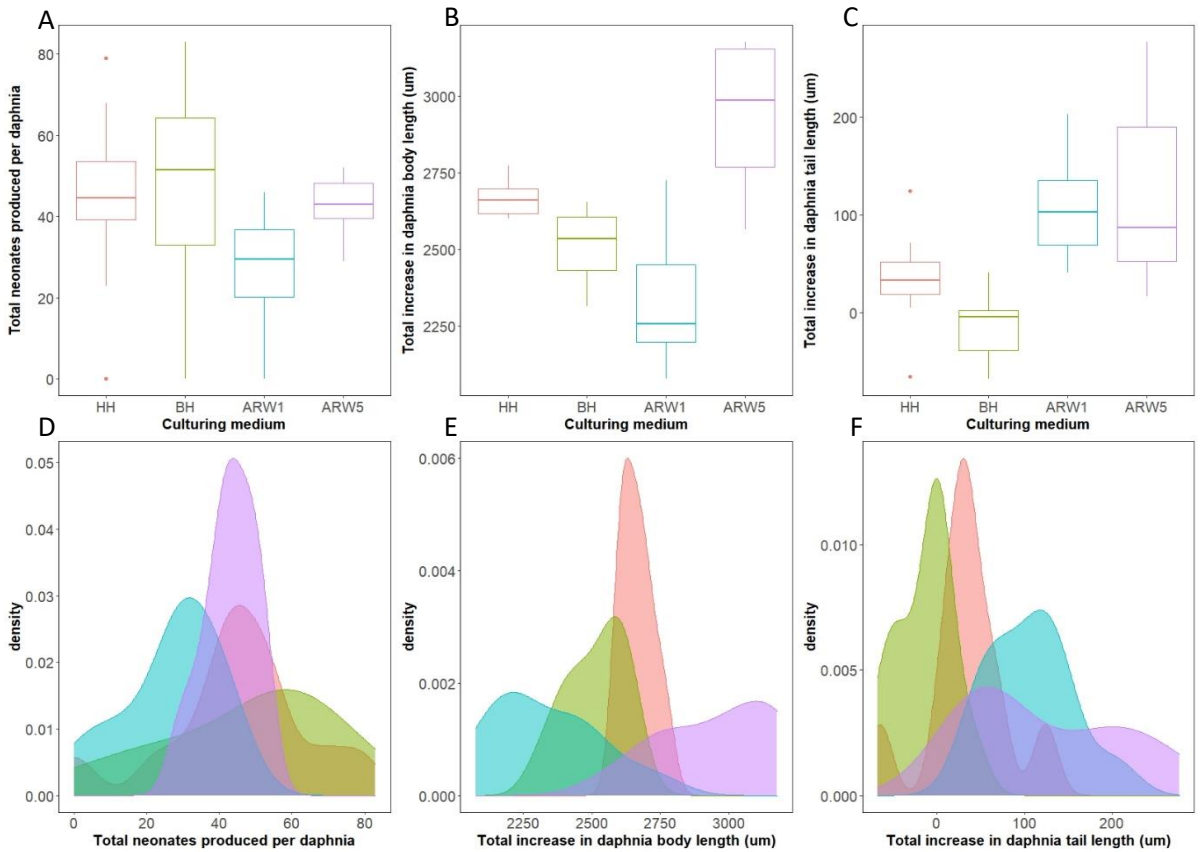


Figure 4.3 Comparison of the growth and reproduction capacity of the control *Daphnia* (medium only) in the four different test media. Top panel shows the boxplots of results and the bottom panel shows the density distribution of the data for each group (colour matched with the boxplots).

For *Daphnia* growth measured using body length, *Daphnia* cultured in ARW5 medium showed the most growth and grew significantly more compared to daphnids from borehole, HH COMBO and ARW1 at the 99.9% confidence interval (p values <0.001). Daphnids cultured in ARW1 were also significantly different to both those grown in borehole ($p < 0.05$) and HH COMBO ($p < 0.001$) as they grew significantly less. Although neonate totals were more variable across all groups, ARW1 has significantly fewer neonates (97% CI) compared to the *Daphnia* cultured in the borehole water ($p < 0.03$).

4.4.3 Variability in chronic toxicity due to medium composition differences

Throughout the duration of the test, the *Daphnia* cultured in ARW1 had much higher mortality compared to the other groups. By the end of the 21-day test period, only 1 individual (from the starting 12) that had been exposed to the SDS survived. Neonates from daphnids cultured in this medium were also immobilised in the testing medium during the interval from release to counting (< 24 hours). This highlights the sensitivity of *Daphnia* cultured in the ARW1 medium to toxicity testing scenarios as all other parameters (i.e., algae rations, temperature etc.) were controlled. As a result of this, the ARW1 exposure group has been removed from further analysis and comparison to the other groups due to lack of observations/data available for statistical analysis of growth and reproduction.

In this study, the addition of a sublethal dose (approximately EC_5 based on average acute toxicity assay results in the various medium- Chapter 5) of SDS had a hormesis effect on the *Daphnia*; the exposed daphnids showed greater growth and increased fecundity over 21 days compared to the controls, as shown in Figure 4.4. Hormesis is an evolutionary low-dose adaptation to environmental stressors that can provide a quantitative estimate of biological

plasticity due to the phenotypic response, on average by 30-60% increase in the stimulatory response range when compared to the controls (i.e. growth, reproduction in this scenario) (Calabrese, 2019). The hormesis response followed the same pattern across the exposure groups as was observed with the control daphnids, whereby those cultured in ARW5 showed the most hormesis (i.e., most growth/highest fecundity), followed by the borehole and HH COMBO daphnids. Interestingly, the exception was the daphnids cultured in ARW1, which although given the same dose that resulted in hormesis in the other groups, led to high mortality (91%) within this group. This highlights the sensitivity of *Daphnia* cultured in the ARW1 medium compared to the other groups.

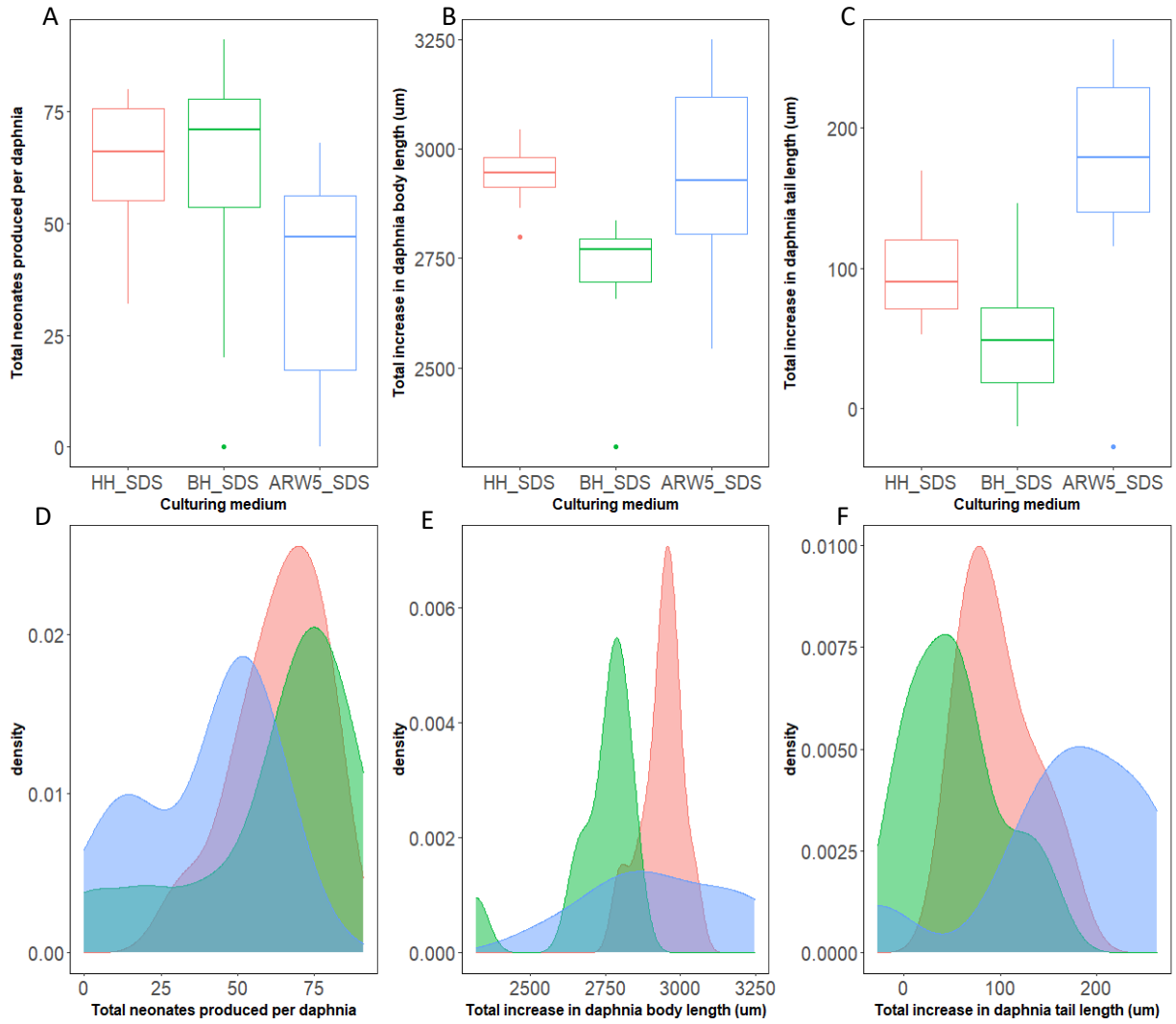


Figure 4.4 Comparison of the growth and reproduction capacity of the exposure daphnids (various medium in combination with SDS) in the three different media, ARW1 data has been omitted due to lack of observations due to the high mortality in this group. Top panel shows the boxplots of results and the bottom panel shows the density distribution of the data for each group (colour matched with the boxplots).

In addition to the removal of the ARW1 group due to high mortality (91%) compared to the hormesis response from the other groups, there was also a significant difference in the total growth of the *Daphnia* during the exposure in the ARW5 group compared to borehole water ($p < 0.02$) and when comparing the HH COMBO and borehole groups ($p < 0.01$). The ARW5

Daphnia were also significantly different in terms of total neonates when compared to borehole *Daphnia* ($p < 0.05$) and HH COMBO ($p < 0.02$) (Figure 4.4A). This could be due to the use of the carbon in the added NOM in this medium as an additional food source. The higher range of observed results in the ARW5 group could be due to contradictory impacts from the presence of the NOM, for example, this could act as a potential carbon (food) source which would be useful for growth, but could also be potentially interacting and absorbing the SDS in the medium which can lead to higher toxicity (see chapter 5).

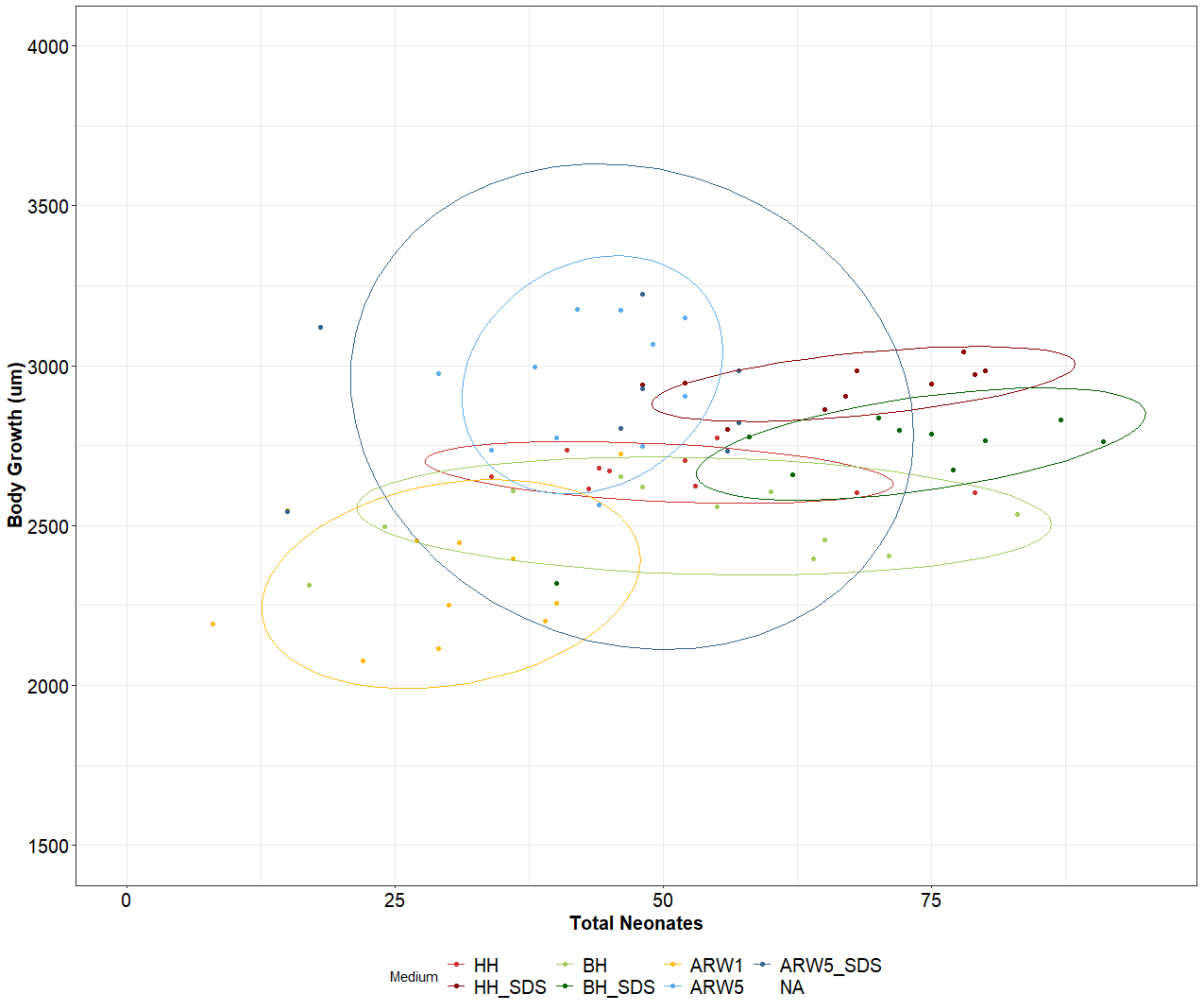


Figure 4.5 PCA plot showing the variability of the different medium and exposure combinations. HH (reds) shows a relatively low range in the results and an optimum level of fitness, borehole (greens) growth and neonates are comparable to HH, and ARW5 (blues) shows the greatest range in results and covers the scope of both HH and borehole. The ARW1 data shows the lowest growth (measured by eye-base of tail length) and neonates for the controls (yellow) when compared to all groups.

Interestingly, the different groups of medium controls and exposures followed similar patterns, and across all groups total number of neonates (offspring) were more variable compared to the change in growth as indicated by the principal components analysis (PCA) shown in Figure 4.5. The HH COMBO, as shown from Figures 4.3 and 4.4, had the smallest range in the data for both control and SDS-exposed daphnids. This could be due to the HH COMBO being the most controlled medium, as this is a salt only medium and therefore there is no heterogenous NOM, or other biological variable that could be impacting the individual daphnids response and growth. This is important to consider, as the results from these exposures are going to have a small range and could therefore over-estimate the environmental populations' ability to respond to stressors. This can further be highlighted by the ARW5 groups (and the ARW1 control) which has the largest range in the data within the groups. This highlights the variability in response that could occur in environmentally relevant model waters. Although the borehole water groups had a similar range to HH COMBO daphnids for both control and exposures, there was less growth / increase in total body length (eye-tail) with this medium and very high variability in the number of neonates, particularly in the controls (Figure 4.4).

Daphnia in testing conditions are fed set algal rations which gives them a steady food supply for growth and development, this would be supplemented in the ARW5 groups by addition of NOM which can be used as another source of carbon by the daphnids. This would differ from environmental samples which would be subject to seasonal variability and potential effects on algal growth from other factors such as light/shading and nutrient availability. This was not a factor in this study as the focus was on *Daphnia* fitness variability due to the culturing medium, but this should be a consideration when extrapolating the results to varying

environments and is summarised succinctly by (Jager, Heugens and Kooijman, 2006) as "The safety factors should reflect our ignorance about the translation from laboratory tests (short-term, high exposure, one species, and controlled environment) to the field (long-term, low exposure, multiple species, variable environment)".

4.4.4 Lipid analysis with Nile red staining

In addition to often being used in MP studies to stain potential polymers, Nile red is a lipophilic dye that is commonly used to stain and subsequently identify lipid droplets using fluorescence in cells and organisms (Jordão *et al.*, 2016; Nel *et al.*, 2021).

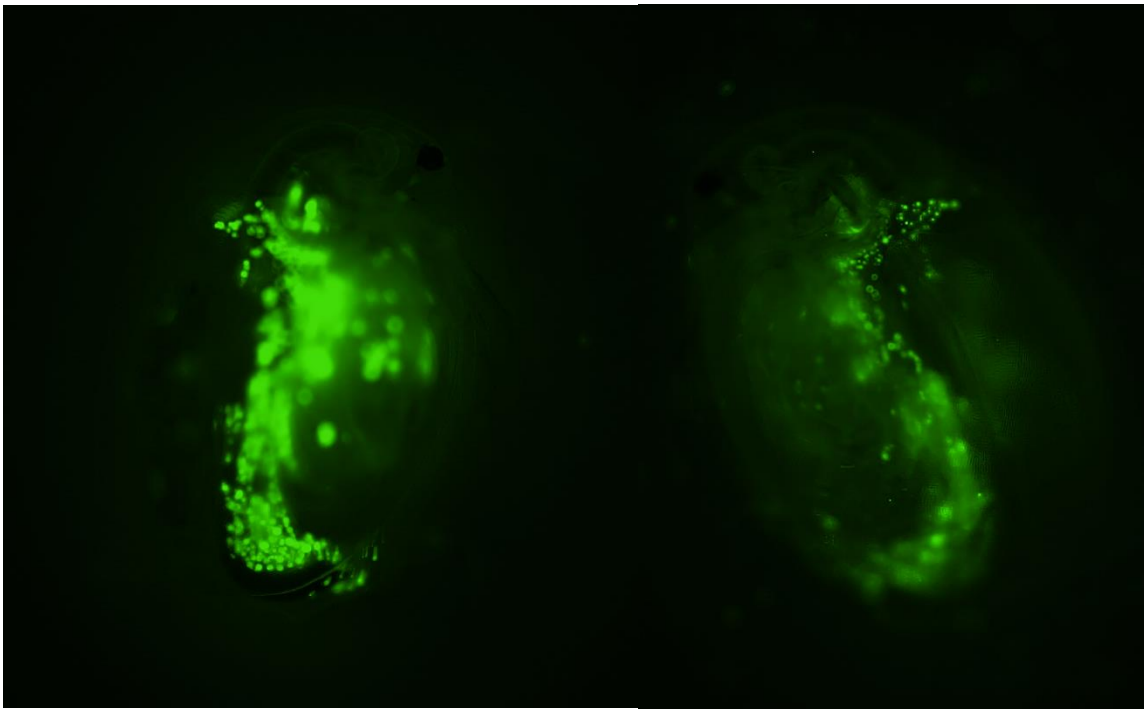


Figure 4.6 Lipid deposit staining in *Daphnia magna* with Nile red (0.1 g/L dose in fresh medium). Images A and B were taken using an Olympus optical microscope with a Green Fluorescent Protein (GFP) filter cube and dichroic mirror and a DP74 colour camera and viewed using CellSens software (x70 magnification). Lipids can be seen following the gut of the *Daphnia,* on the left in a daphnid from the HH COMBO-SDS exposure group and on the right a daphnid from the HH COMBO control group.

Examples of the lipid deposits can be seen in the daphnids in Figure 4.6 differing in intensity, with the majority of the lipid droplets being located along the gut. The use of the stain allows observations of the quantity and distribution of lipid deposits in the daphnids, ahead of further analysis by homogenisation to enable a relative comparison of lipids based on the intensity of the fluorescence (Figure 4.7).

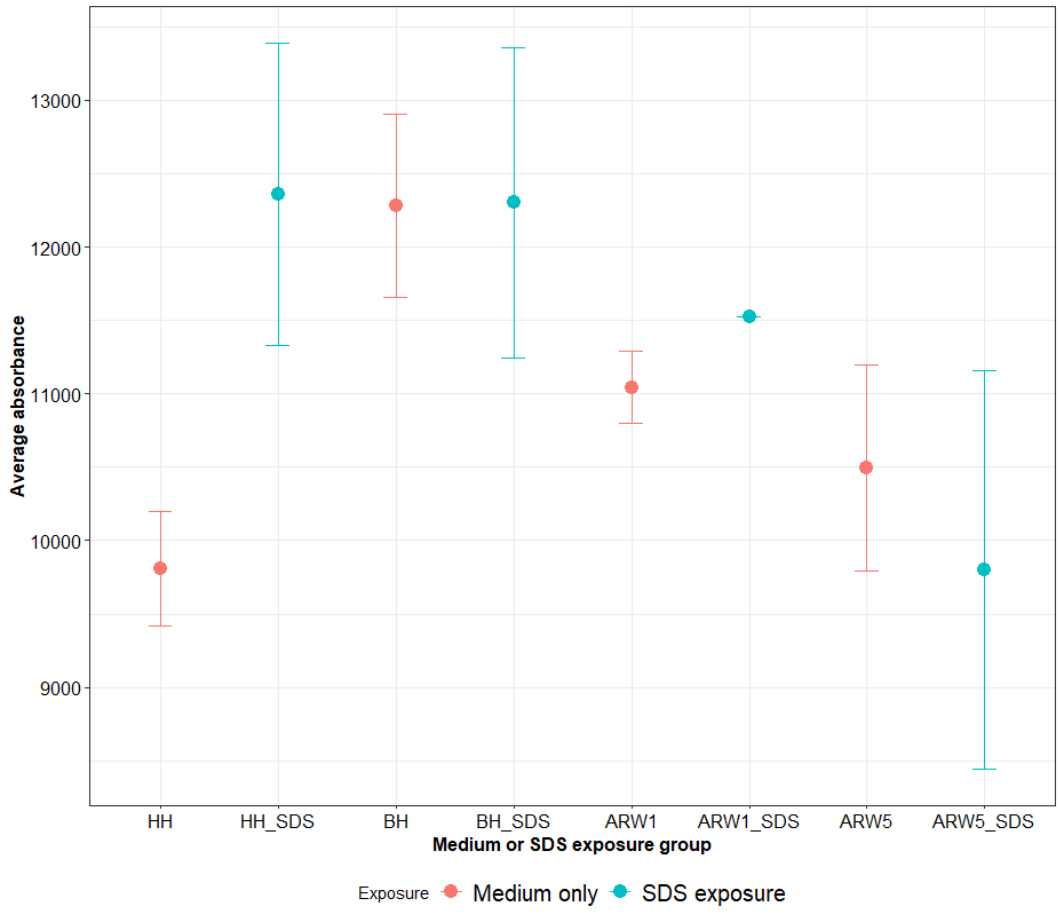


Figure 4.7 Variation in the Nile red fluorescence intensity in the stained lipids in *Daphnia* samples for both the control groups and those that had been exposed to SDS to allow for a relative comparison to the other treatment groups. This method is based on a comparison of exposed *Daphnia* to a control for baseline. The surviving ARW1 daphnid was included for reference to the other groups, although no statistical inferences could be established for the ARW1 lipid changes based on this single surviving replicate.

Although this method currently only allows for a relative comparison between the *Daphnia*, as this method is based on a comparison of exposed daphnids to a control baseline to determine changes in lipid concentrations (Jordão *et al.*, 2016), it shows the variability in the lipid deposits that can arise as a result of the culturing media and exposure combinations which would typically be used as the control populations within exposure studies. There is high variability across all samples, but the populations that had been exposed to the SDS have higher variability in the lipid content detected with the Nile red staining method with the exception of the ARW1 group (as there was only one surviving daphnid). Care was taken at the time of staining to ensure that daphnids did not have any offspring in the brood pouch which could interfere with the relative absorbance reading for the adult daphnid. The ARW5 *Daphnia* had lower lipid concentrations relative to the other groups, which could be due to the presence of the NOM within the media acting as an alternative source of carbon for the daphnids, which therefore would increase the perceived availability of food and decrease the likelihood of the *Daphnia* prioritising lipid storage for potential low-food scenarios as a side effect of the chemical exposure (Wacker and Martin-Creuzburg, 2007).

4.5 Conclusions and future directions

The medium used for culturing *Daphnia* during toxicity studies can have a significant impact on the baseline health of the test organism. This is an important consideration as the medium used should be representative of the target environment that we are aiming to protect, and results for toxicity assays should be moderated to take into consideration a precautionary approach due to the multiple stressors that would be encountered by the organisms living in

the environment. This is reflected in mixture toxicity studies that are being conducted currently and reported (Chapter 5), as well as initial considerations of the combined effect of toxicants and climate change for example (Dr Berta Bonet, personal communication, February 2021- publication pending). As with many elements of ecotoxicity, a balance needs to be found for the different aspects of the study. It is not feasible to use a range of complex culturing medium for all toxicity testing, but it should be considered at the design stage to support the overall aims of the study. If test organisms have an advantage to their environmental counterparts, underestimations of the environmental toxicity and consequences could be an unintentional result of the more simplistic testing methods. This would be more problematic for environmental extrapolation and modelling compared to acute toxicity comparison studies (Jager, Heugens and Kooijman, 2006).

The standard culturing medium, HH COMBO, leads to good performance by the *Daphnia* during the acute toxicity assay, with a similar performance compared to daphnids cultured in borehole water. The ARW1 daphnids had relatively poor fitness compared to the other groups, particularly when exposed to SDS. In comparison, ARW5 had excellent fitness, although slightly more variable when compared to borehole and HH COMBO results. This indicates that the culturing and exposure medium can have a significant influence on the overall performance of *Daphnia* during standard acute toxicity assays. As a result, recommendations of probable effect concentrations and threshold levels for monitoring would benefit from basing these thresholds on toxicity data using medium and conditions most representative of the target environment. Following on from this study, it would be beneficial to determine if *Daphnia* differ in response to a chronic toxicity assay with MP in the varying media to establish

a baseline understanding of any potential variability due to effects of medium composition on particle dispersion in combination with *Daphnia* sensitivity (see Chapter 5).

Supplementary material

Summary statistics for chronic exposures

Table S4.1- Summary statistics for the control populations

Medium	Average total neonates	Standard deviation (total neonates)	Average growth (body µm)	Standard deviation of growth (body µm)
HH COMBO	44.75	20.33	2666.14	58.18
Borehole	47.41	24.26	2513.63	109.57
ARW1	26.91	13.62	2333.13	198.07
ARW5	43.0	7.04	2953.49	208.12

Table S4.2- Summary statistics for the control populations

Medium	Average total neonates	Standard deviation (total neonates)	Average growth (body µm)	Standard deviation of growth (body µm)
HH COMBO + SDS	63.58	14.49	2938.22	68.56
Borehole + SDS	61.0	27.70	2720.57	152.42
ARW5 + SDS	39.0	21.76	2934.11	234.83

Table S4.3 Example *Daphnia* chronic observation data (Growth measurements)

Medium	Daphnia	body_T0	tail _T0	body_T7	tail_T7	body_T14	tail_T14	body_T21	tail_T21	growth_body	growth_tail
HH	1	1127.405	329.444	2404.672	272.925	3577.709	326.826	3901.392	334.617	2773.987	5.173
HH	2	1225.224	432.394	2518.054	443.468	3476.213	499.29	3928.343	465.114	2703.119	32.72
HH	3	1199.74	401.248	2554.264	414.114	3430.752	523.679	3934.298	472.095	2734.558	70.847
HH	4	1205.302	361.166	2520.821	439.288	3466.583	472.274	3808.039	485.336	2602.737	124.17
HH	5	1232.746	417.634	2565.539	461.541	3528.873	421	3835.155	352.647	2602.409	-64.987
HH	6	1198.979	342.56	2609.22	330.878	3432.632	345.237	NA	NA	NA	NA
HH	7	1087.111	375.254	NA	NA	NA	NA	NA	NA	NA	NA
HH	8	1228.394	356.346	2419.107	388.705	3517.265	440.956	3900.433	390.139	2672.039	33.793
HH	9	1236.902	413.031	2537.066	413.065	3622.204	421	3889.093	438.69	2652.191	25.659
HH	10	1225.335	419.923	2349.175	391.175	3503.397	464.959	3849.794	475.977	2624.459	56.054
HH	11	1200.065	381.793	2458.306	437.556	3488.538	472.875	3880.25	398.026	2680.185	16.233
HH	12	1163.156	328.535	2460.401	285.506	3439.898	321.886	3778.868	367.635	2615.712	39.1
HH_SDS	1	1227.828	391.732	2644.736	420.065	3426.687	498.802	4027.43	495.055	2799.602	103.323
HH_SDS	2	1223.525	406.871	2642.802	459.391	3694.16	518.462	4169.472	549.996	2945.947	143.125
HH_SDS	3	1163.378	366.366	2407.575	411.749	3605.276	561.98	4147.671	452.276	2984.293	85.91
HH_SDS	4	1192.43	403.265	2557.024	447.456	3745.932	592.653	4236.086	456.237	3043.656	52.972
HH_SDS	5	1208.512	405.71	2617.513	474.381	3745.087	510.26	4152.253	574.827	2943.741	169.117
HH_SDS	6	1264.43	420.49	2737.887	514.143	3767.492	507.669	4128.712	490.241	2864.282	69.751
HH_SDS	7	1277.561	376.402	2597.733	323.793	3730.196	487.491	NA	NA	NA	NA
HH_SDS	8	1259.668	345.728	2672.164	377.913	3833.809	413.367	4243.272	402.673	2983.604	56.945
HH_SDS	9	1174.605	400.843	2494.497	417.831	3754.953	509.902	4079.203	475.333	2904.598	74.49
HH_SDS	10	1209.183	394.454	2573.077	472.921	3777.187	528.191	4150.347	520.504	2941.164	126.05
HH_SDS	11	1198.441	408.14	2535.121	414.65	3798.927	532.665	4169.81	502.313	2971.369	94.173
HH_SDS	12	1284.054	431.267	2763.669	485.934	NA	NA	NA	NA	NA	NA
BH	1	1319.35	414.012	2065.101	342.639	3226.664	429.774	3633.505	361.748	2314.155	-52.264
BH	2	1210.734	373.372	2396.259	377.339	3396.484	361.482	3816.615	384.544	2605.881	11.172
BH	3	1188.217	381.581	2479.221	349.924	3448.903	429.018	3842.037	385.606	2653.82	4.025
BH	4	1433.615	398.292	2353.489	359.634	3229.234	415.227	3829.259	393.998	2395.644	-4.294

BH	5	1317.354	429.936	2360.171	384.142	3233.101	513.391	3723.339	362.2	2405.985	-67.736
BH	6	1306.686	402.233	2384.253	377.59	3266.605	427.265	3761.769	403.079	2455.083	0.846
BH	7	1139.619	382.072	2359.487	361.777	3224.054	424.885	3747.877	422.836	2608.258	40.764
BH	8	1277.104	421.273	2515.483	374.162	3483.26	443.662	3773.231	375.341	2496.127	-45.932
BH	9	1137.695	346.841	2372.848	357.25	3351.243	397.708	3673.598	340.613	2535.903	-6.228
BH	10	1248.796	382.974	2439.892	377.195	NA	NA	NA	NA	NA	NA
BH	11	1266.45	406.447	2508.377	410	3481.886	453.295	3886.742	406.263	2620.292	-0.184
BH	12	1280.653	389.942	2407.18	356.49	3340.045	390.61	3839.503	358.341	2558.85	-31.601
BH_SDS	1	1246.394	403.265	2460.815	406.778	3567.768	437.446	4007.597	458.472	2761.203	55.207
BH_SDS	2	1194.372	372.611	2462.01	398.355	3662.346	499.939	4029.704	487.018	2835.332	114.407
BH_SDS	3	1129.185	346.446	2398.319	399.41	NA	NA	NA	NA	NA	NA
BH_SDS	4	1198.403	366.075	2506.841	393.422	3668.804	474.673	3996.315	428.79	2797.912	62.715
BH_SDS	5	1171.908	366.172	2476.341	372.673	3482.556	451.142	3936.298	511.949	2764.39	145.777
BH_SDS	6	1175.369	393.901	2439.247	390.62	3490.119	452.4	3951.178	421.188	2775.809	27.287
BH_SDS	7	1145.544	305.151	2560.951	418.901	NA	NA	NA	NA	NA	NA
BH_SDS	8	1247.711	439.813	2098.833	324.964	3202.009	437.77	3567.498	455.429	2319.787	15.616
BH_SDS	9	1236.717	407.655	2419.108	363.834	3622.154	459.385	3894.704	482.336	2657.987	74.681
BH_SDS	10	1244.586	433.518	2464.587	384.495	3517.94	406.289	3919.766	434.898	2675.18	1.38
BH_SDS	11	1175.361	388.926	2464.785	382.798	3604.893	429.727	4007.29	376.157	2831.929	-12.769
BH_SDS	12	1092.467	380.316	2392.339	390.759	3506.609	429.444	3878.727	422.303	2786.26	41.987
ARW1	1	903.167	355.448	1881.824	384.645	3366.998	541.128	3627.831	458.367	2724.664	102.919
ARW1	2	1094.526	358.856	NA	NA	NA	NA	NA	NA	NA	NA
ARW1	3	1023.239	378.351	2214.418	476.122	3083.687	517.287	3571.439	519.863	2548.2	141.512
ARW1	4	1079.584	410.222	2172.901	472.691	2888.334	563.422	3281.763	547.58	2202.179	137.358
ARW1	5	1105.223	452.509	2205.808	690.424	2941.278	578.345	3221.682	519.508	2116.459	66.999
ARW1	6	1096.526	388.339	2062.485	424.976	3078.137	503.94	3289.861	458.905	2193.335	70.566
ARW1	7	994.374	335.726	1966.297	375.573	2933.558	433.112	3245.211	468.124	2250.837	132.398
ARW1	8	1169.27	343.963	2276.16	429.105	3084.003	427.882	3246.23	384.829	2076.96	40.866
ARW1	9	1052.714	416.613	2132.68	438.64	3258.847	541.203	3505.619	538.957	2452.905	122.344
ARW1	10	1082.773	397.974	2300.296	485.124	3208.261	518.11	3528.629	450.363	2445.856	52.389
ARW1	11	1039.884	368.794	2136.594	463.652	3082.898	596.95	3297.047	571.7	2257.163	202.906
ARW1	12	1085.257	410.815	2379.287	448.183	3141.691	504.744	3481.165	512.219	2395.908	101.404
ARW1_SDS	1	1047.798	422.847	NA	NA	NA	NA	NA	NA	NA	NA
ARW1_SDS	2	1075.965	404.546	2156.73	417.864	NA	NA	NA	NA	NA	NA

ARW1_SDS	3	1066.269	410.074	NA	NA	NA	NA	NA	NA	NA	NA
ARW1_SDS	4	1091.106	437.246	2223.788	448.576	3340.246	636.834	3788.462	648.957	2697.356	211.711
ARW1_SDS	5	966.61	328.51	NA	NA	NA	NA	NA	NA	NA	NA
ARW1_SDS	6	1088.691	414.171	NA	NA	NA	NA	NA	NA	NA	NA
ARW1_SDS	7	1064.289	412.908	NA	NA	NA	NA	NA	NA	NA	NA
ARW1_SDS	8	1082.801	401.589	NA	NA	NA	NA	NA	NA	NA	NA
ARW1_SDS	9	1084.36	407.643	NA	NA	NA	NA	NA	NA	NA	NA
ARW1_SDS	10	1129.562	414.282	1888.956	427.205	NA	NA	NA	NA	NA	NA
ARW1_SDS	11	1050.554	407.693	NA	NA	NA	NA	NA	NA	NA	NA
ARW1_SDS	12	1109.308	405.097	NA	NA	NA	NA	NA	NA	NA	NA
ARW5	1	986.833	364.785	2371.859	428.536	3508.348	463.676	3892.499	469.446	2905.666	104.661
ARW5	2	909.361	359.252	2243.063	583.906	3718.546	510.895	4083.276	532.274	3173.915	173.022
ARW5	3	908.446	366.906	2507.874	411.848	3370.556	475.825	3884.59	417.433	2976.144	50.527
ARW5	4	938.328	363.796	2309.851	508.413	3573.355	600.878	4113.978	640.285	3175.65	276.489
ARW5	5	924.18	360.534	2323.726	496.618	3628.717	526.152	3990.053	579.821	3065.873	219.287
ARW5	6	1094.656	391.862	2506.235	422.993	3582.876	509.942	3841.526	444.616	2746.87	52.754
ARW5	7	933.815	387.764	2182.765	405.71	3552.059	449.521	4111.078	452.953	3177.263	65.189
ARW5	8	923	370.098	2594.348	470.044	3488.404	517.64	3919.088	549.996	2996.088	179.898
ARW5	9	1118.282	398.751	2585.492	482.545	3579.211	518.893	3893.092	415.026	2774.81	16.275
ARW5	10	952.377	385.823	2287.394	467.817	3638.832	558.758	4101.667	619.246	3149.29	233.423
ARW5	11	1209.754	403.755	2468.678	447.85	3463.867	423.738	3775.56	440.875	2565.806	37.12
ARW5	12	1107.698	379.235	2551.57	427.49	3555.671	422.971	3842.202	448.234	2734.504	68.999
ARW5_SDS	1	886.843	345.141	2473.684	441.997	3668.107	515.874	4110.174	573.53	3223.331	228.389
ARW5_SDS	2	1159.344	407.904	2561.12	515.013	3602.169	647.824	4087.523	602.7	2928.179	194.796
ARW5_SDS	3	983.993	335.106	2251.325	447.426	3450.067	501.721	NA	NA	NA	NA
ARW5_SDS	4	1096.253	360.435	2555.113	494.099	3702.688	619.101	NA	NA	NA	NA
ARW5_SDS	5	1157.036	378.637	2608.882	430.305	3723.725	484.737	4141.74	493.756	2984.704	115.119
ARW5_SDS	6	1108.444	411.432	2447.291	411.057	3563.125	496.805	3928.734	384.241	2820.29	-27.191
ARW5_SDS	7	1041.025	350.101	2461.476	400.867	3488.538	547.5	3845.646	516.811	2804.621	166.71
ARW5_SDS	8	1143.989	372.625	2514.404	463.652	3460.874	500.304	NA	NA	NA	NA
ARW5_SDS	9	1157.097	352.339	2353.593	398.355	3474.088	504.181	3891.511	614.75	2734.414	262.411
ARW5_SDS	10	909.651	374.62	2157.686	439.474	3755.17	541.84	4028.707	553.662	3119.056	179.042
ARW5_SDS	11	1121.099	347.776	2348.95	411.746	3283.791	523.136	3665.008	604.233	2543.909	256.457
ARW5_SDS	12	1057.904	356.133	2394.447	395.588	3903.631	481.462	436.426	495.957	-621.478	139.824

Table S4.4 Example *Daphnia* chronic observation data (neonates)

Medium	Daphnia	No_1brood	Time_1brood	No_2brood	Time_2brood	No_3brood	Time_3brood	No_4brood	Time_4brood	No_5brood	Time_5brood	Total_neonates
HH	1	8	12	27	16	20	19	NA	NA	NA	NA	55
HH	2	3	11	19	14	15	16	15	19	NA	NA	52
HH	3	10	12	12	16	19	19	NA	NA	NA	NA	41
HH	4	14	9	8	11	26	12	19	16	12	19	79
HH	5	14	14	14	16	19	6	21	NA	NA	NA	68
HH	6	8	9	15	12	NA	NA	NA	NA	NA	NA	23
HH	7	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	0
HH	8	11	12	15	16	19	19	NA	NA	NA	NA	45
HH	9	11	12	17	16	6	19	NA	NA	NA	NA	34
HH	10	5	9	14	12	17	16	17	19	NA	NA	53
HH	11	10	12	21	16	13	19	NA	NA	NA	NA	44
HH	12	9	12	19	16	15	19	NA	NA	NA	NA	43
HH_SDS	1	11	11	25	14	20	16	NA	NA	NA	NA	56
HH_SDS	2	NA	NA	20	14	28	16	4	21	NA	NA	52
HH_SDS	3	21	11	17	14	5	16	15	19	22	21	80
HH_SDS	4	5	9	35	12	30	16	8	19	NA	NA	78
HH_SDS	5	2	11	31	12	32	16	10	19	NA	NA	75
HH_SDS	6	16	11	25	14	21	16	3	21	NA	NA	65
HH_SDS	7	9	9	17	12	28	16	9	19	NA	NA	63
HH_SDS	8	18	12	33	16	17	19	NA	NA	NA	NA	68
HH_SDS	9	23	11	27	16	17	19	NA	NA	NA	NA	67
HH_SDS	10	17	12	25	16	6	19	NA	NA	NA	NA	48
HH_SDS	11	26	11	33	16	20	19	NA	NA	NA	NA	79
HH_SDS	12	22	11	10	14	NA	NA	NA	NA	NA	NA	32

BH	1	9	16	8	19	NA	NA	NA	NA	NA	NA	17
BH	2	2	12	19	14	22	16	17	19	NA	NA	60
BH	3	15	12	14	16	17	19	NA	NA	NA	NA	46
BH	4	5	9	14	12	5	16	19	19	21	21	64
BH	5	11	9	13	12	21	16	26	19	NA	NA	71
BH	6	10	9	13	12	19	16	23	19	NA	NA	65
BH	7	11	12	9	16	16	19	NA	NA	NA	NA	36
BH	8	9	12	15	19	NA	NA	NA	NA	NA	NA	24
BH	9	10	11	14	14	18	16	18	19	23	21	83
BH	10	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	0
BH	11	9	12	20	16	19	19	NA	NA	NA	NA	48
BH	12	21	14	19	16	15	19	NA	NA	NA	NA	55
BH_SDS	1	19	12	36	16	28	19	8	21	NA	NA	91
BH_SDS	2	11	12	33	16	26	19	NA	NA	NA	NA	70
BH_SDS	3	20	12	NA	NA	NA	NA	NA	NA	NA	NA	20
BH_SDS	4	19	14	30	16	23	19	NA	NA	NA	NA	72
BH_SDS	5	7	11	24	12	26	16	23	19	NA	NA	80
BH_SDS	6	10	11	22	14	13	16	13	21	NA	NA	58
BH_SDS	7	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	0
BH_SDS	8	6	11	13	14	21	19	NA	NA	NA	NA	40
BH_SDS	9	9	12	28	16	25	19	NA	NA	NA	NA	62
BH_SDS	10	25	12	26	16	26	19	NA	NA	NA	NA	77
BH_SDS	11	17	12	27	16	32	19	11	21	NA	NA	87
BH_SDS	12	14	12	25	16	25	19	11	21	NA	NA	75
ARW1	1	7	11	10	14	7	19	22	21	NA	NA	46
ARW1	2	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	0
ARW1	3	3	14	12	19	NA	NA	NA	NA	NA	NA	15
ARW1	4	9	9	7	12	9	16	14	19	NA	NA	39

ARW1	5	11	11	4	19	14	21	NA	NA	NA	NA	29
ARW1	6	5	11	3	19	NA	NA	NA	NA	NA	NA	8
ARW1	7	15	12	14	16	1	19	NA	NA	NA	NA	30
ARW1	8	9	11	8	19	5	21	NA	NA	NA	NA	22
ARW1	9	9	12	17	16	1	19	NA	NA	NA	NA	27
ARW1	10	14	12	13	16	4	19	NA	NA	NA	NA	31
ARW1	11	7	9	6	12	16	16	11	19	NA	NA	40
ARW1	12	15	11	13	14	8	19	NA	NA	NA	NA	36
ARW1_SDS	1	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	0
ARW1_SDS	2	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	0
ARW1_SDS	3	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	0
ARW1_SDS	4	13	11	14	14	2	19	5	21	NA	NA	34
ARW1_SDS	5	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	0
ARW1_SDS	6	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	0
ARW1_SDS	7	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	0
ARW1_SDS	8	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	0
ARW1_SDS	9	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	0
ARW1_SDS	10	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	0
ARW1_SDS	11	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	0
ARW1_SDS	12	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	0
ARW5	1	8	12	25	16	19	19	NA	NA	NA	NA	52
ARW5	2	6	11	21	14	12	19	7	21	NA	NA	46
ARW5	3	4	11	9	14	16	19	NA	NA	NA	NA	29
ARW5	4	3	11	15	14	10	19	14	21	NA	NA	42
ARW5	5	15	11	9	14	10	19	15	21	NA	NA	49
ARW5	6	14	12	21	16	13	19	NA	NA	NA	NA	48
ARW5	7	6	11	12	14	7	19	17	21	NA	NA	42
ARW5	8	20	14	14	16	4	21	NA	NA	NA	NA	38

ARW5	9	7	12	17	16	16	19	NA	NA	NA	NA	40
ARW5	10	19	11	18	14	9	19	6	21	NA	NA	52
ARW5	11	11	12	20	16	13	19	NA	NA	NA	NA	44
ARW5	12	10	12	13	16	11	19	NA	NA	NA	NA	34
ARW5_SDS	1	17	12	21	16	10	19	NA	NA	NA	NA	48
ARW5_SDS	2	16	12	20	16	12	19	NA	NA	NA	NA	48
ARW5_SDS	3	4	12	21	16	17	19	NA	NA	NA	NA	42
ARW5_SDS	4	11	12	2	16	NA	NA	NA	NA	NA	NA	13
ARW5_SDS	5	5	11	24	14	23	16	5	21	NA	NA	57
ARW5_SDS	6	12	12	22	16	23	19	NA	NA	NA	NA	57
ARW5_SDS	7	4	11	11	12	21	16	10	19	NA	NA	46
ARW5_SDS	8	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	0
ARW5_SDS	9	8	11	26	14	22	19	NA	NA	NA	NA	56
ARW5_SDS	10	11	14	5	19	2	21	NA	NA	NA	NA	18
ARW5_SDS	11	15	16	NA	NA	NA	NA	NA	NA	NA	NA	15
ARW5_SDS	12	17	11	22	14	16	19	13	21	NA	NA	68

Chapter 5

5. The role of chemical contaminants and plastics; Trojan horse or multi-stressor effects?

Katie Reilly[1], Daniel Drage[1], Laura-Jayne. A Ellis[1], Stuart Harrad[1], Jon Sadler[1] & Iseult Lynch[1]

[1]School of Geography, Earth and Environmental Science, University of Birmingham, UK.

Part of this work has been presented as a virtual poster at SETAC 2020.

The final stages of this work were impacted by the COVID19 University-wide closed period (20th March – 1st September 2020), and the restrictions on access and numbers allowed per laboratory following that. The planned work, that would have been included in this chapter has been outlined in a future work section due to being beyond the timeframe of this thesis.

5.1 Abstract

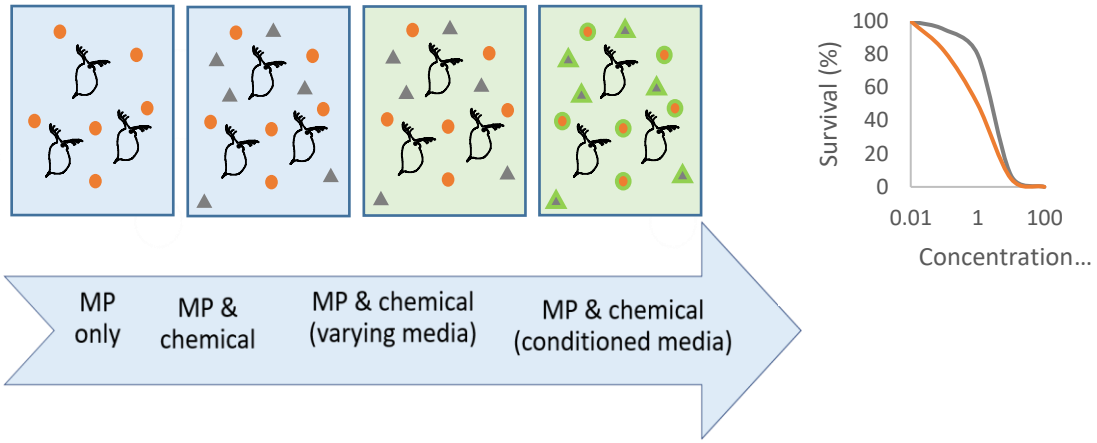


Figure 5.1 Schematic of increasing environmental realism in study designs for MP exposures and correlation with the toxicity of chemicals, MP, and their mixtures.

5.2 Introduction

Microplastics (MP) are becoming a well-established contaminant of concern in the environment with an increase in both scientific and societal interest over recent years. As a

result, environmental sampling and toxicity studies have increased in variety and complexity over recent years, covering a range of test organisms and MP combinations (Gall and Thompson, 2015; de Sá *et al.*, 2018; Foley *et al.*, 2018). The potential of MP to act as vectors for chemicals (co-pollutants) or alien species commonly found in the environment, often termed the Trojan horse effect, is currently being explored due to the large surface area and long transport range potential of MP in the environment (Rochman, 2013b; Ziccardi *et al.*, 2016).

The presence of such multiple stressors, such as chemical pollutants in combinations with MP, poses a complex challenge for ecotoxicology studies to replicate the various pathways of exposures within controlled and simplified testing scenarios in order to understand the drivers of any observed toxicity. Due to their ecological status as a keystone species in freshwater environments, *Daphnia magna* are a well-established test organism for chemical testing, with standardised testing protocols established for this, such as 48-hour acute toxicity and 21-day chronic toxicity assays (OECD, 2004a, 2012a). Due to this, there is a wealth of data for chemical toxicity effects in *Daphnia* against which to establish a baseline response to aquatic pollution stressors. Nanomaterial toxicity, and more recently MP studies can supplement our understanding of how particulate pollution could be impacting the *Daphnia* and the ecosystems more broadly, including potentially altering the bioavailability of other chemicals to organisms. EC_{50} values are used throughout for the *Daphnia* assays, this is because the acute toxicity test (OECD 202) is based on the immobilisation of Daphnids as mortality (Lethal Concentration or LC_{50}) is difficult to determine accurately without using a microscope.

The complexity and range of chemical and plastic combinations that could occur in the environment is incredibly varied, since it is estimated that there are over 100,000 chemicals in the environment (European Environment Agency, 2021)(only a small percentage of which have been extensively tested for their toxicity) and over 40 different types of plastic estimated to be in widespread use in industry alongside numerous blends and copolymers, and over 400 additives used to improve the functional properties of the plastics as determined by ECHA's mapping of the additives in high production volume plastics (European Chemicals Agency, 2021). Thus, it would be impossible to investigate all combinations of plastics and chemicals, and therefore this study aims to quantify the potential of one of the most environmentally abundant plastics, polyethylene (PE), to transfer or exasperate the chemical toxicity to *Daphnia magna* of three representative chemical co-pollutants under various exposure scenarios. This is an important step towards understanding the environmental toxicity of MP in realistic environmental conditions and the potential interactions of MP with other environmental pollutants. The three chemicals selected for this study, Sodium dodecyl sulphate (SDS), Triclosan and Diclofenac, were tested in combination with 1-5 µm polyethylene (PE) beads in order to assess if their toxicity was enhanced (through enhanced uptake through binding to the PE) or reduced (for example by being so strongly bound to the PE that it is no longer bioavailable to organisms). PE was chosen as it is a very commonly found polymer in environmental samples and was also frequently used in microbeads in cosmetics prior to the microbead ban (Napper *et al.*, 2015b) and these chemicals were chosen for a range of reasons including previously established presence in the freshwater environment, combination with plastics as an additive and use in personal care products (and therefore likelihood of legacy interaction effects in combination with microbeads) (Browne *et al.*, 2013;

Wu *et al.*, 2016). Although not exhaustive, it spans across three groups of chemicals that are often found in aquatic environments and WWTW, surfactants (SDS), pharmaceuticals (diclofenac) and antimicrobials (triclosan). Therefore, the selected chemicals pose a realistic risk for co-exposure with MP to daphnids in the environment.

SDS

SDS is a common anionic surfactant used in many household products as a detergent and as a result it is commonly found in aquatic and terrestrial environments (Jardak, Drogui and Daghrir, 2016). Annual consumption of surfactants has been steadily growing, much the same as polymer use. Surfactants are amphipathic molecules that are formed of hydrophilic polar head groups and hydrophobic hydrocarbon tails, which could play a significant role in the relationship and binding capacity of surfactants and hydrophobic polymers such as polyethylene (Wu *et al.*, 2016). SDS has also been used as a dispersant in MP studies, although a study with sea urchins found that it did not have a significant disruption to light absorption when compared to other surfactants (Masakorala, Turner and Brown, 2011). However, SDS has also showed a hormesis effect during a low dose-chronic exposure with *Daphnia* in three different media (Chapter 4). Its amphiphilic nature poses a risk to lipid membranes and is widely used to denature proteins for their detection and quantification suggesting that is highly disruptive to biological organisms (Jelińska *et al.*, 2017).

Diclofenac

Diclofenac is a commonly used Non-Steroid Anti-Inflammatory Drug (NSAID) which is widely found in the environment (Du *et al.*, 2016). Due to the wide spread use of this as a painkiller,

it is often found in the aquatic environment and has been reported at concentrations ranging from 0.1-1 µg/L in waste water treatment work (WWTW) effluent (Loos *et al.*, 2013). Although biological treatment schemes for municipal waste are often classified as very effective for compounds such as ibuprofen (elimination rate ~90%), other pharmaceuticals such as diclofenac are moderately persistent and less likely to be broken down (elimination rate ~ 20-80%) (Loos *et al.*, 2013). Diclofenac was added to the first water framework directive (WFD) Substance of Concern list in 2015, however it has since been removed from the list due to lowered concerns about concentration and effects (Smith, 2016). However, an EQS of 0.04 µg/L was proposed by the European Commission and out of 576 samples in England, 64.6% of these exceeded this EQS and of this, 13.4% exceeded the upper threshold of sensitivity of 0.13 µg/L (Leverett *et al.*, 2021), indicating that it is still a high risk to the environment.

Triclosan

Triclosan is a commonly used antimicrobial agent that is added to a range of products such as toothpaste and some polymers during the production stage (depending on the intended use of the product). Removal of triclosan is typically reported to be fairly good at WWTW, (elimination ~90%), however this is mainly due to degradation and adsorption to sludge (Loos *et al.*, 2013) which could provide an insight into the potential binding of the triclosan with natural organic matter (NOM) in the various media tested and in real environments. In terms of MP research this could pose a significant exposure pathway, as there is increasing research into the potential of sludge to be a source of nano or MP back into the environment, for example via the use of sludge as a soil fertiliser (Gao, Li and Liu, 2020). If we take into

consideration that these MP within the sludge may form part of the adsorption and therefore act as a sink (hotspot for accumulation) for triclosan, there is the potential for elevated concentrations in this phase when we compare this to the WWTW effluent after treatment. In addition, triclosan has been reported in environment water samples of up to 2.3 µg/L in natural streams, which indicates that there is potential for this to remain in the aqueous phase too (Dhillon *et al.*, 2015).

Although *Daphnia* are the model species used within this study, other species have also previously been shown to be detrimentally affected by the three chemicals at varying concentrations, a sample of which is included in Table 5.1.

Table 5.1. Literature values for the key chemicals of interest, SDS, triclosan and diclofenac and the typical EC_{50} values reported for a range of organisms.

Chemical	Species	Concentration	Toxic response	Reference
Triclosan	Lepomis macrochirus (fish)	370 µg/L	96 hr LC_{50}	(Orvos *et al.*, 2002)
Triclosan	Scenedesmus (algae)	1.4 µg/L	96 hr (biomass) EC_{50}	(Orvos *et al.*, 2002)
Triclosan	*Gammarus pulex* (freshwater crustacean)	0.75-1.93 mg/L	48 hr EC_{50}	(Rowett, Hutchinson and Comber, 2016)
Triclosan	*D. rerio* (fish)	0.28-0.21 mg/L	96 hr LC_{50}	(Li *et al.*, 2018)

SDS	*Pseudosida ramose* (Cladocera)	11.1 mg/L	48 hr EC_{50}	(Freitas and Rocha, 2012)
SDS	*Artemia franciscana*	7.49 mg/L	48 hr EC_{50}	(Manfra *et al.*, 2016)
Diclofenac	*D. rerio* (fish)	166.6 mg/L	96 hr LC_{50}	(Praskova *et al.*, 2011)
Diclofenac	*D. subspicatus* (algae)	72-626 mg/L	96 hr (biomass) EC_{50}	(Cleuvers, 2004)
Diclofenac	*Pseudokirchneriella subcapitata*	100 mg/L	96 hr (biomass) EC_{50}	(Grung *et al.*, 2008)

The effects of the three chemicals on *D. magna* were determined alone and in combination with PE, over 21-days to assess chronic and reproductive effects. Building on previous work which has shown that different media compositions influence the inherent fitness of *Daphnia* and thus, their ability to process and mitigate against the toxic impacts of PE and chemicals, the study was performed in three different media (used in Chapter 4). Due to the long-term nature of the exposure, we also included consideration of the impact of the role of protein corona, as even in the absence of a pre-formed corona the natural secretions of the daphnids would result in corona formation during the experiment (Ellis and Lynch, 2020). Additionally, since the goal of the study was to understand the impacts of binding of the chemical co-pollutants to the MP surface it was essential to assess this under realistic exposure conditions, i.e., under competitive conditions whereby the co-pollutants have to compete with proteins

and other biomolecules for the PE surface (Wang *et al.*, 2018). It is well known that initial binding to particle surfaces is based on abundance, but these abundant biomolecules are displaced over time by higher affinity but lower abundance proteins (Cedervall *et al.*, 2007), and it is likely that similar effects apply also to small molecules, and indeed the role of such small molecules in the eco-corona is increasingly being explored and understood (Chetwynd and Lynch, 2020; Chetwynd *et al.*, 2020). Competitive binding of proteins has been demonstrated by many authors (Zhang *et al.*, 2019), and it has recently been shown that the toxicity of cadmium ions were reduced by entrapment into the albumin corona formed around graphene oxide particles (Martinez *et al.*, 2020). Thus, a key aspect of the current study was to assess the impact of the exposure order (e.g., MP exposed to co-pollutant and then conditioned medium, MP exposed to conditioned medium and then co-pollutant, or exposure of the MP to co-pollutant and conditioned medium simultaneously (Figure 5.2) on the amount of the co-pollutant sorbed to the MP and thus taken up into the daphnids during the subsequent toxicity studies. This was complemented by analysis of the total protein content in the PE coronas under the different conditions using a total protein assay (BCA) and assessing the protein in the *Daphnia*-conditioned testing medium and how this varied in the presence or absence of the chemicals.

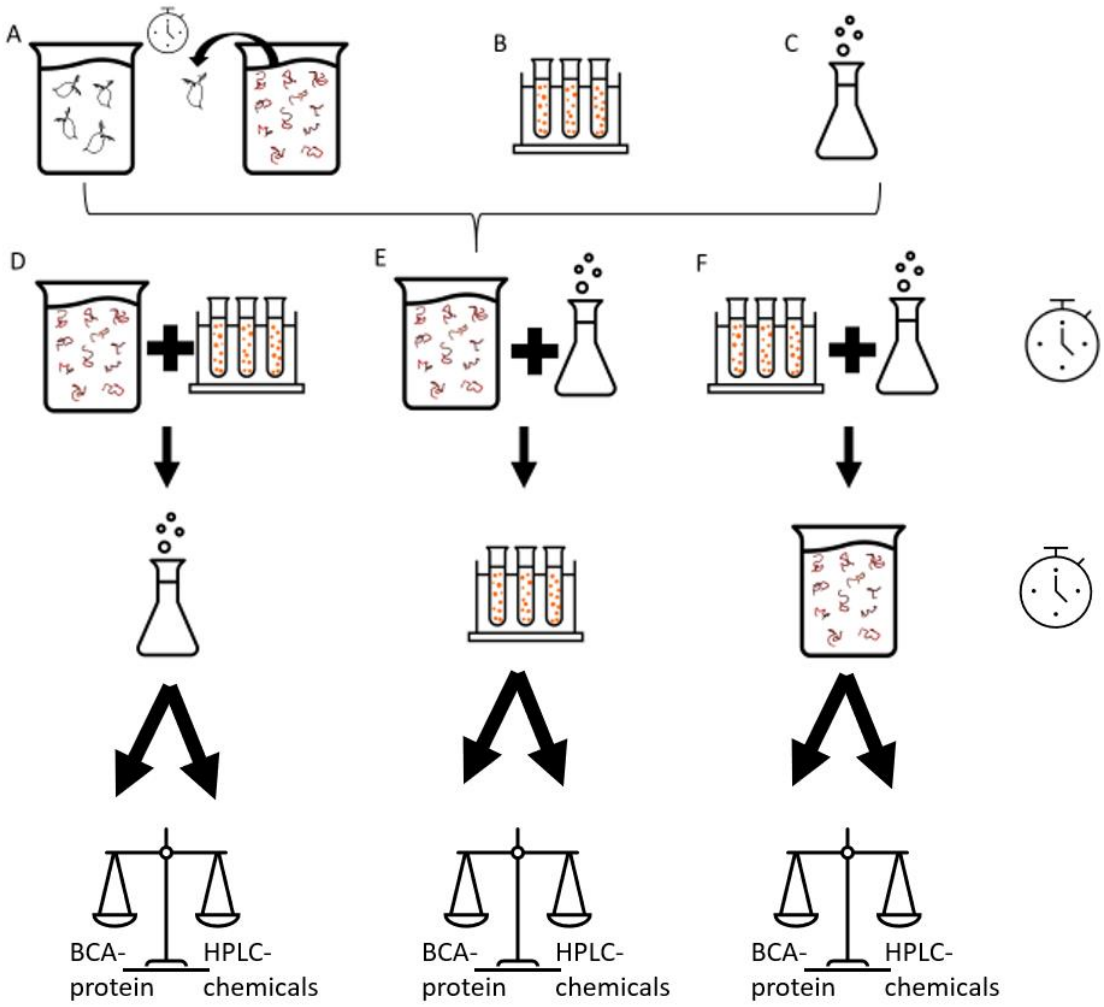


Figure 5.2 Schematic showing the different exposure pathway combinations for the protein and chemical PE interactions: A- *Daphnia* conditioned medium was prepared with a 24-hour conditioning window (see section 5.3.3), B- PE MP dispersions and C- chemical stocks were prepared. Following this preparation step, three different combinations orders were investigated (D-F) to determine if the order has a significant impact on the particle surface conditioning of the MP and compared used total protein assay (BCA) and planned HPLC-MS analysis for chemical concentrations. This involved combining two of the stocks, i.e., conditioned medium and dispersed MP, prior to adding the chemicals (D). The other combination orders were undertaken (E, F) to determine if order of exposure impacts the results.

The pathway of the chemical exposures was quantified during several stages, to assess whether the chemicals remained attached to the PE throughout the exposure duration or whether there was desorption and if so where these occurred, as follows;

1) Variation in *Daphnia* acute toxicity (EC_{50} values) for the three chemicals, as a result of the different media in addition to the combined exposure with PE MP.
2) Change in protein concentration associated with the PE as a result of the combined exposure, and exposure order (Figure 5.2 D-F).
3) Mass balance of the chemicals for the mixture scenarios, determined by measuring the PE load and the chemical load in the medium (Figure 5.2 1D-F)- future work.

5.3 Methodology

5.3.1 Materials

Polyethylene spheres (Copsheric, USA) in the size range 1-5µm were used for the study.

Chemicals (SDS, triclosan and diclofenac) of analytical grade from Sigma Aldrich (physico-chemical properties outline in Table S5.1).

5.3.2 Test organisms

Daphnia magna, Bham 2 strain, were used for the toxicity assessment of the chemical and MP mixtures. *Daphnia* were cultured in 900 mL of medium in 1L jars and fed a daily ration of *Chlorella vulgaris* (0.5 mg C days 0-7, 0.75 mg C days 7 onwards) with a 16:8-hour light: dark cycle in a controlled temperature lab (20 °C). For the exposure, neonates from the running cultures were pooled before being split into test vessels. *Daphnia* were cultured for 3

generations in their respective testing medium ahead of exposures to allow them to acclimatise to the testing medium.

5.3.3 Culturing and conditioning media

Toxicity assessments were undertaken in HH COMBO medium, borehole water from the onsite borehole at the University of Birmingham and in Class 5 artificial river water (ARW5) that contains NOM. This was to establish the variation in observed effect that could happen as a result of the different media compositions, as variation in medium has been shown to have a significant effect on the end point of chronic exposures and their respective controls (Chapter 4). Using a medium that contains NOM in the form of humic acid introduces another potential surface coating which could affect the amount of potential chemical binding through competitive adsorption.

Conditioned media was prepared by adding *Daphnia* from the running cultures to fresh medium without any algae (10 daphnia per 50mL vessel) for 24 hours. During this period, the *Daphnia* were excreting proteins, polysaccharides, and other metabolites into the medium to 'condition' the medium. After 24-hours the daphnids were then returned to the running stocks and the now conditioned medium was kept for the mixture scenario analysis.

5.3.4 Characterisation of the MP in the different media

The stability of the particles in combination with the conditioned medium and the chemicals was also undertaken to ascertain if the chemicals would lead to any variation of the dispersal of the particle that would lead to a change in the toxicity of the MP. Dynamic Light Scattering (DLS) and Zeta potential measurements using a Malvern Nanosizer 5000 instrument were used to analyse potential agglomeration and surface charge, respectively.

5.3.5 Toxicity

Standard 48-hour acute toxicity assays were conducted following the OECD 202 protocol. A suite of range finding exposures was initially undertaken spanning a broad range of values based on reported values in the safety data sheets supplied and reported literature values. Following this, a suitable, narrower range was then selected to undertaken the acute toxicity assays (10 neonates per replicate, 5 replicates per treatment in 5mL of test medium) with the three chemicals to establish an estimated range of toxicity response for the subsequent mixture exposures.

Following this, simultaneous exposures were undertaken with PE from a set stock (0.1 g/L dispersed in their respective medium) in combination with the chemicals over the same concentration range as that used for the chemical only exposures.

Following the acute toxicity tests, an initial comparison of chronic toxicity was undertaken in HH COMBO, comparing the toxicity of diclofenac and SDS over a 21-day period, in chemical only exposures and in combination with PE MP. The EC_5 values of diclofenac and SDS (from the acute tests) were used in the chronic exposure, and for the mixture tests a fixed PE concentration was used also (25 mg/L). Daphnids were kept in individual test vessels of 50mL with 12 *Daphnia* per treatment. The exposure medium was replaced three times per week, and *Daphnia* were fed daily. Images were taken every 7 days to measure growth over the test period (eye-tail and tail length) and neonates were counted daily.

5.3.6 Proteins

Competitive binding with proteins was also assessed by comparing the protein concentration on the surface of the PE alone and when mixed in combination with other chemicals. SDS was hypothesised to have the lowest concentration of protein due to the protein denaturing capabilities of this chemical. PE particles (0.1 g/L stock concentration) were incubated in conditioned medium for 4 hours.

Following the required incubation time, proteins adsorbed to the PE particles were extracted using fresh (unconditioned) medium to wash the particles and centrifuged at 14,000 rpm to pellet the particles allowing removal of the unbound proteins in the supernatant. The particles were washed in four stages and the supernatant was removed at each step and replaced with fresh medium. Following this, each of the supernatants and the pellet were added to individual wells of a 96-well plate (3 wells per supernatant / pellet) included in the plate for analysis. During this study clear PE (1-5 µm) was used to minimise the effect of the particle on the colourimetry reading on the plate. BSA was used as the standard for the protein extraction and the plate was read at 526 nm peak absorbance for colorimetric analysis. The method was followed in detail from (Docter *et al.*, 2014) and adapted by (Nasser and Lynch, 2016b). A control for the pellet formation was conducted with green PE to confirm that the centrifuging steps were adequate to form a PE pellet of the same average size and density within the solution. This was important to allow accurate quantification of the proteins lost at each stage of the wash.

5.3.7 Quantification of the chemicals sorbed to the PE

A method was developed and optimised to quantify the SDS, diclofenac and triclosan from the mixture scenarios using HPLC-MS, based on previous work extracting brominated flame retardants from plastic samples (Abdallah *et al.*, 2017). Samples were taken from the three exposure combinations (Figure 5.2 D-F) for analysis of the associated chemical concentration in the testing medium prior to exposure to the *Daphnia* and on the MP surface on the particles within the medium over the time-course of the exposure. The total chemical concentration was compared to standard calibration of the chemicals diluted in analytical grade methanol. This optimised method will be used for a mass balance study to explore the competitive binding potential- see future work.

5.4 Results

5.4.1 Mixture toxicity - EC_{50} variations

Previous exposures with the PE MP have shown that even high concentrations lead to negligible toxicity in *Daphnia* (Chapter 3), and the toxicity response seemed to be in relation to the physical interaction and impediment of movement rather than a more typical ingestion-based response.

The combination of chemicals and MP led to variable toxicity responses within the *Daphnia* in combination with the different media, as shown in Figures 5.3-5.5 for the three chemicals in the three culturing media.

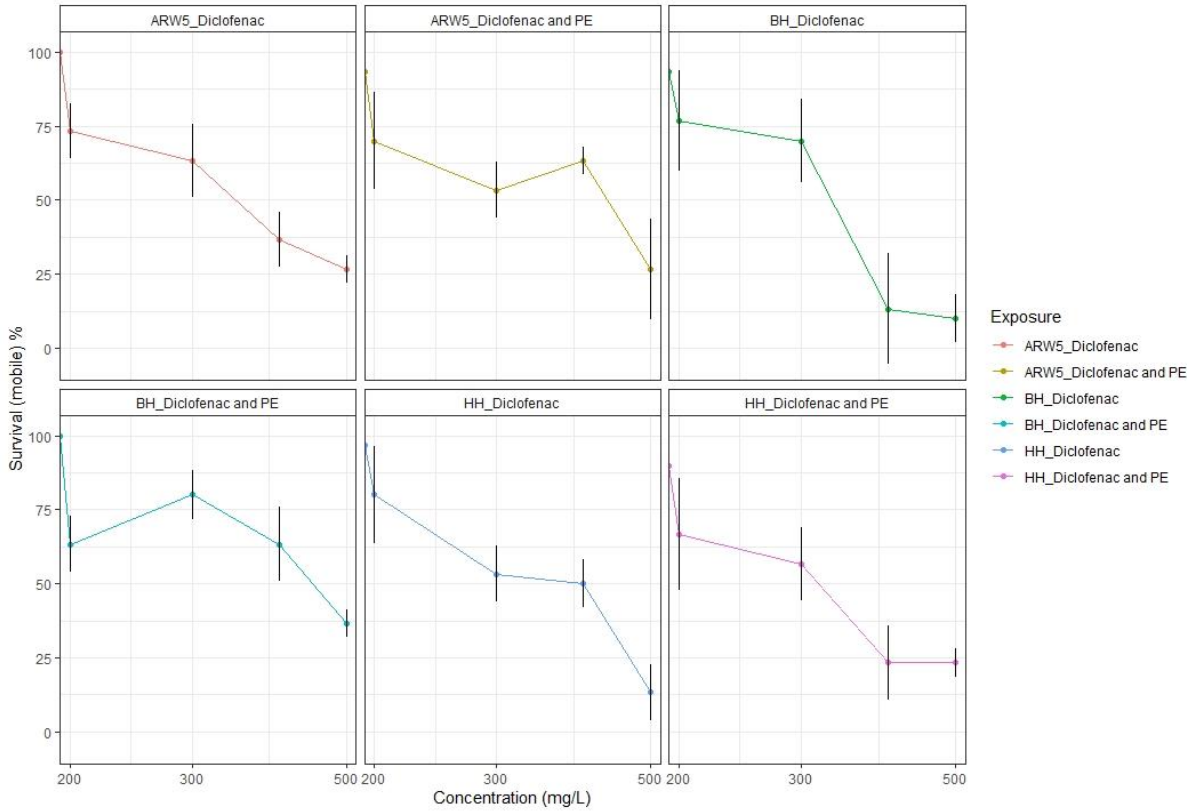


Figure 5.3 Variation in the acute toxicity response of D. magna to diclofenac in HH COMBO, borehole water and ARW5 medium alone or in combination with PE beads, reported as % organism survival.

Daphnia cultured in ARW5 that were exposed to a combination of diclofenac and MP were the most sensitive group, compared to *Daphnia* cultured in ARW5 medium that were exposed to diclofenac only. This could be due to the NOM acting as a sink for the diclofenac, which in the absence of MP led to lower bioavailability of the diclofenac as the NOM itself was not taken up by the daphnids. On the other hand, NOM is known to have a high propensity for binding to particles including MP, and thus binding of the diclofenac-loaded NOM onto the PE MP led to enhanced uptake of the diclofenac by daphnids and thus enhanced bioavailability and toxicity. Variability was also observed in both the HH COMBO and the borehole cultured daphnids, however in both of these groups, the diclofenac only exposure led to a slightly greater toxicity response, potentially due to the lack of NOM to act as this sink for the

chemical. This also suggests that the diclofenac had a relatively low affinity for the PE surface in the absence of NOM, and will be discussed further below.

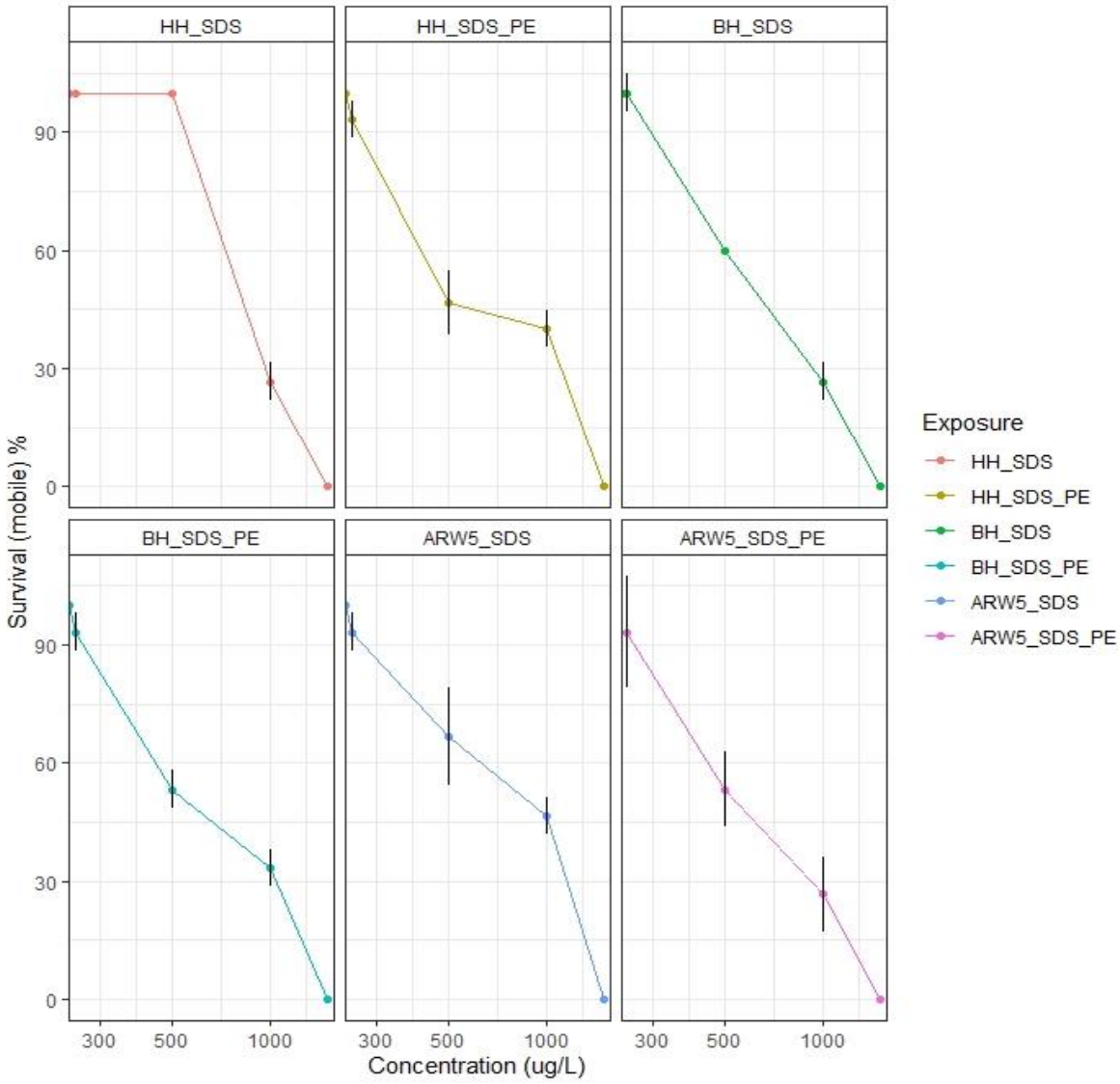


Figure 5.4 Variation in the acute toxicity response of *D. magna* to SDS in HH COMBO, borehole water and ARW5 medium alone or in combination with PE beads.

Within the SDS exposures, the HH COMBO daphnids exposed to a combination of SDS and MP had the lowest threshold for the toxicity response (lowest EC_{50} value) compared to the ARW5 daphnids that have been exposed to SDS only which showed the highest tolerance. This could be due to the fact that SDS readily coats the MP surface due to no NOM in this medium

compared to the other media (borehole and ARW5 which both contain NOM), which might therefore lead to competitive binding on the MP surface between the SDS and NOM.

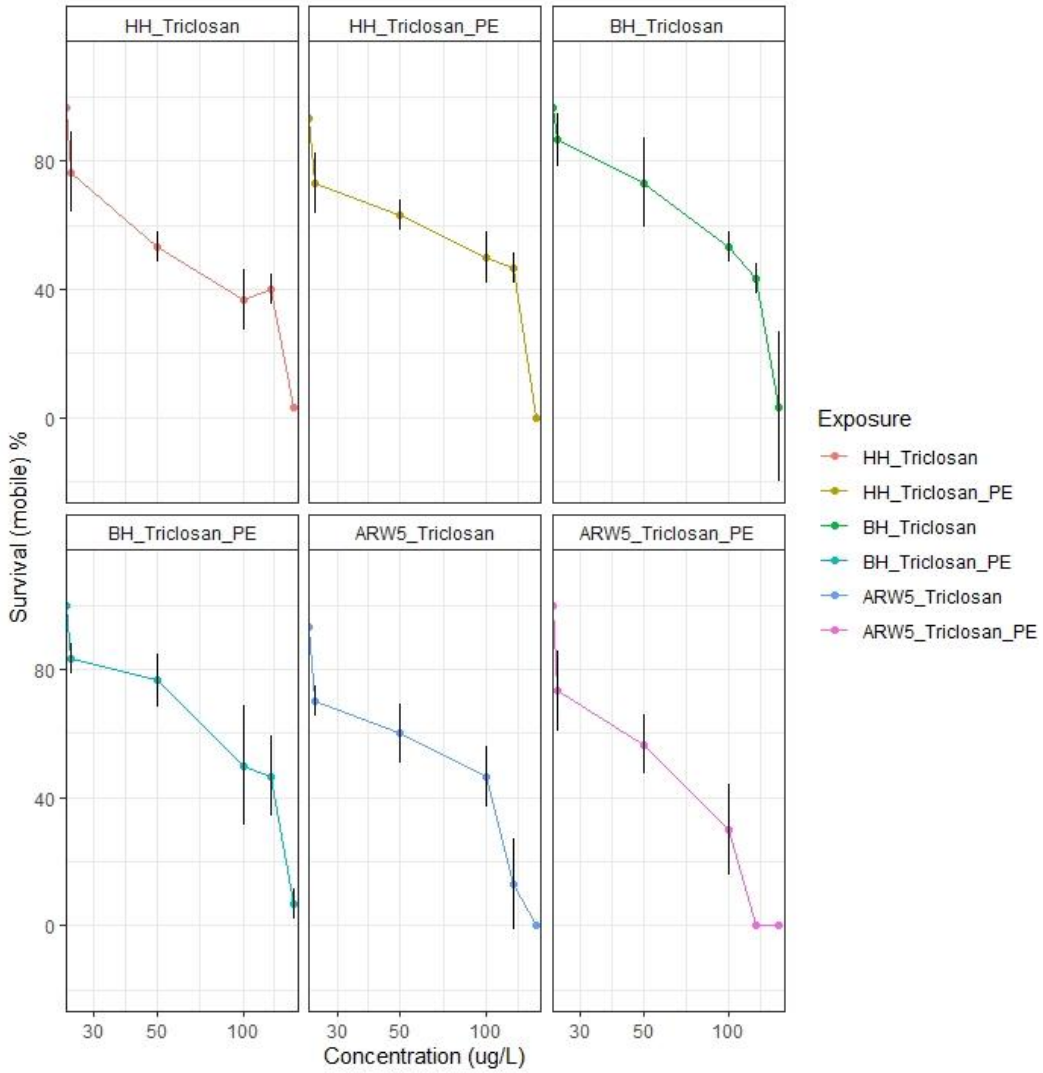


Figure 5.5 Variation in the acute toxicity response of *D. magna* to triclosan in HH COMBO, borehole water and ARW5 medium alone or in combination with PE beads.

Triclosan has already been reported to be readily associated with sewage sludge during WWTW processing, and therefore it is highly likely that it would also associate with the NOM present in relatively high concentrations (4.6 mg/L) within the ARW5 medium. Interestingly, the HH COMBO exposure had the lowest EC_{50} value of 60 µg/L, closely followed by the ARW5

group containing MP with an EC_{50} value of 62.5µg/L (Table 5.2). This could indicate that triclosan and diclofenac have similar mixture toxicity responses and bind to the NOM which then binds to the PE beads, therefore increasing the toxicity of the particles once ingested.

The impact of the media used for the toxicity assessment was significant and varied across all groups, although the two main patterns emerged depending on whether the chemical had a stronger affinity for the PE particle surface or for the NOM. The classical trojan horse effect implies a direct binding of the co-pollutant to the PE surface and being carried into the organism via this route. This was indeed the trend that was observed with SDS which had a lower EC_{50} value (was more toxic) in the presence of the PE than alone. However, for the other two chemicals tested, the impact seemed more complex, in that the strongest interaction was with the NOM and thus in the absence of the PE particles their toxicity was low as the chemicals were not as bioavailable to the daphnids, while the binding of the NOM with the complexed chemical to the PE particles led to an increased uptake and thus increased toxicity, via an indirect binding effect. The observed EC_{50} values are also likely to be influenced by the underlying sensitivity of the test *Daphnia* due to their culturing conditions (Chapter 4) which results in different degrees of "fitness". EC_{50} values for the different chemicals in the different treatment groups can vary significantly, and are shown in Table 5.2.

Table 5.2 Summary of the EC_{50} values in the different exposure combinations of chemical with/without PE in the various unconditioned media showing the significant impact of both medium composition (environmental realism) and the co-exposure toxicity

	HH COMBO	Borehole	ARW5
Triclosan (µg/L)	60	108.3	87.5
Triclosan + PE (µg/L)	100	100	62.5
Diclofenac (mg/L)	291.7	350	350
Diclofenac + PE (mg/L)	329.39	262.46	200
SDS (µg/L)	844.7	649.62	916.6
SDS + PE (µg/L)	482	583.25	562.85

Table 5.2 shows the variability in toxicity as a result of the exposure scenario and toxicant combinations. For example, SDS ranged from 916 µg/L in ARW5 to only 482 µg/L in HH COMBO when combined with PE MP. Interestingly, SDS toxicity increased across all media in combination with MP, most probably due to the surfactant coating the surface of the PE within the mixture leading to enhanced uptake on the PE particles compared to the concentration that could be taken up through diffusion from the water where chemical equilibrium principles would limit the uptake. Thus, the behaviour of the SDS is the only one that seems to follow the classical Trojan horse paradigm of increased uptake / toxicity across all three media (so in the absence or presence of NOM in the medium). This also suggests that the SDS has a higher affinity for the PE surface than NOM does, although we would also expect some interaction between NOM and SDS as it has been shown that SDS adsorbed substantially to native sediment (containing 16% w/w NOM) but not to organic-free sediment or organic-free sediment coated with either a commercial (Aldrich) or natural humic acid (Marshall, House

and White, 2000). The analysis of the chemical loading on the PE particles by HPLC-MS (delayed due to extensive lab closures, limited accessibility to labs and pressure on instrument times) will shed light on this important and interesting question.

NOM has the potential to act as a sink for xenobiotics in the environment, such as metals and other chemicals (Lowry *et al.*, 2012). Due to the likely conditioning of the PE by NOM in the ARW5 group, this then led to adsorption of the chemical by the plastic.

In addition, the concentrations for the EC_{50} values for all three chemicals are higher than those currently reported in the aquatic environment (Dhillon *et al.*, 2015; Leverett *et al.*, 2021). However, due to the potential surface coating of the plastic with the chemicals, or the binding of the chemicals to the organic matter in the solution, it is important to explore this exposure pathway further. This could be undertaken through analysis of environmental samples, such as the suspended organic matter in addition to the water samples to see if this presents an elevated concentration which would be more realistic through this combined exposure pathway with particles compared to dissolved toxicants only.

5.4.2 Chronic mixture toxicity

Following on from the acute exposure, chronic assays were planned (and undertaken in part), using the EC_5 values determined from the acute tests as the sublethal exposure concentrations in order to further explore the variability that the different combinations of chemical, MP and media could have on *Daphnia*. A fixed concentration of PE MP was added for the mixture studies in order to understand how binding to the MP or not affects the chronic toxicity of the co-pollutants, and how this correlates with the protein composition on the PE MP over time.

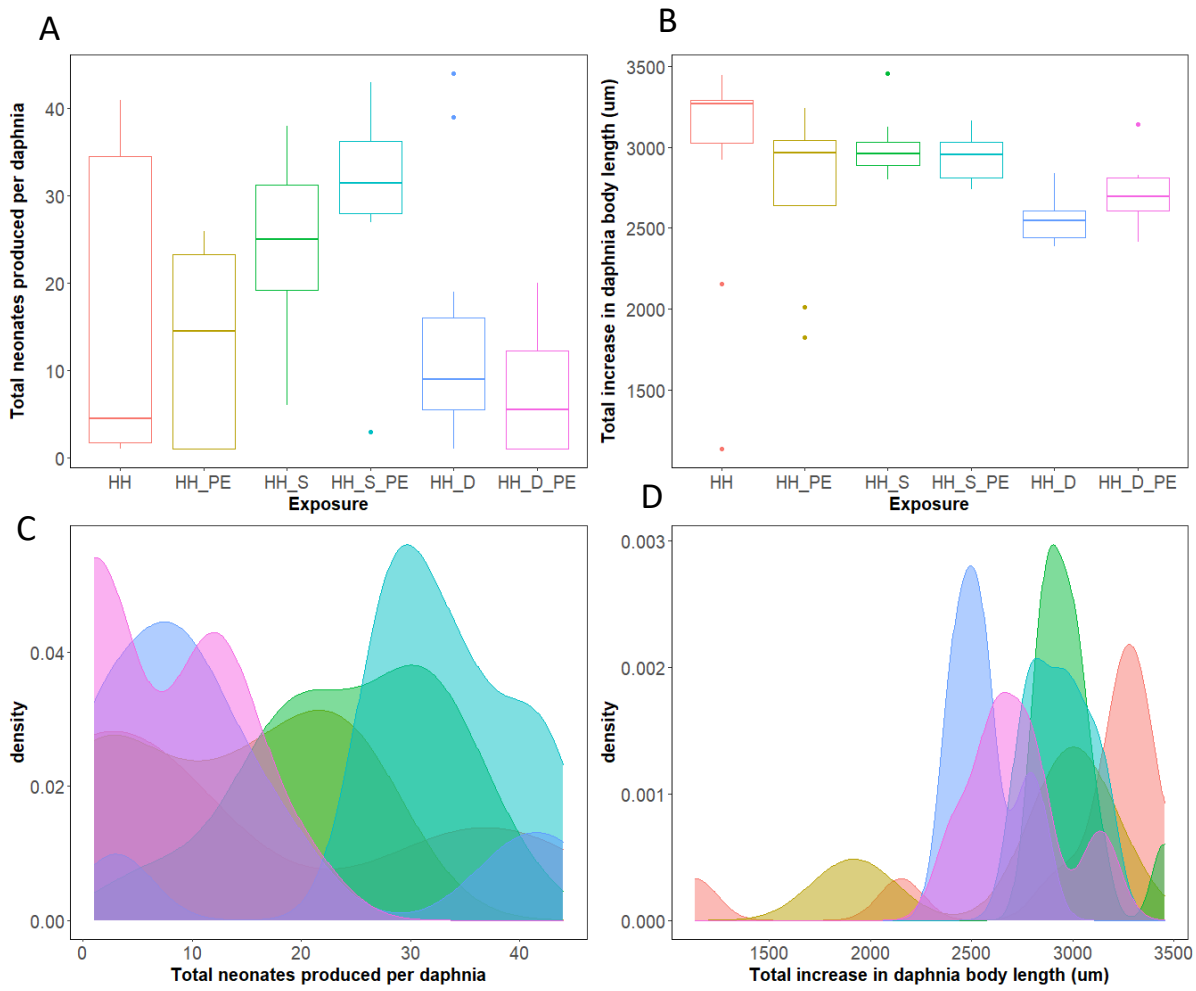


Figure 5.6 Variation in the chronic toxicity response in terms of total numbers of neonates (A,C) and growth (total body length) (B,D) of *D. magna* exposed to an approximate EC_5 concentration of diclofenac and SDS in HH COMBO medium, alone or in combination with PE beads over 21-days. The colour of the density plots matches the boxplots in the first panel. The abbreviations in the top panel correspond to the following exposure combinations: **HH**: HH COMBO medium only (control); **HH_PE**: HH COMBO containing 1-5 µm PE MP at 25mg/L; **HH_S**: SDS at 150µg/L (~EC_5) in HH COMBO medium; **HH_S_PE**: SDS at 150µg/L (~EC_5) plus PE MP at 25mg/L in HH COMBO medium; HH-D: diclofenac at 50 mg/L (EC_5) in HH COMBO medium; **HH_D_PE**: diclofenac at 50mg/L (EC_5) plus PE MP at 25 mg/L in HH COMBO medium. These same abbreviations are used in subsequent figures also.

Although the HH COMBO control and the PE only exposure are both highly variable, comparison of the difference in response to sublethal exposure to SDS versus diclofenac indicates that the diclofenac exposure had a more detrimental impact over 21-days (Figure 5.6, 5.7), both as a single chemical

exposure and in combination with PE, compared to SDS. However, SDS has previously been shown to have a hormesis effect (a positive response to a stressor leading to an increase in growth and/or reproduction). Note however that in some cases the number of surviving daphnids is lower than expected, which as noted below may be evidence of some problems in the running and exposure cultures.

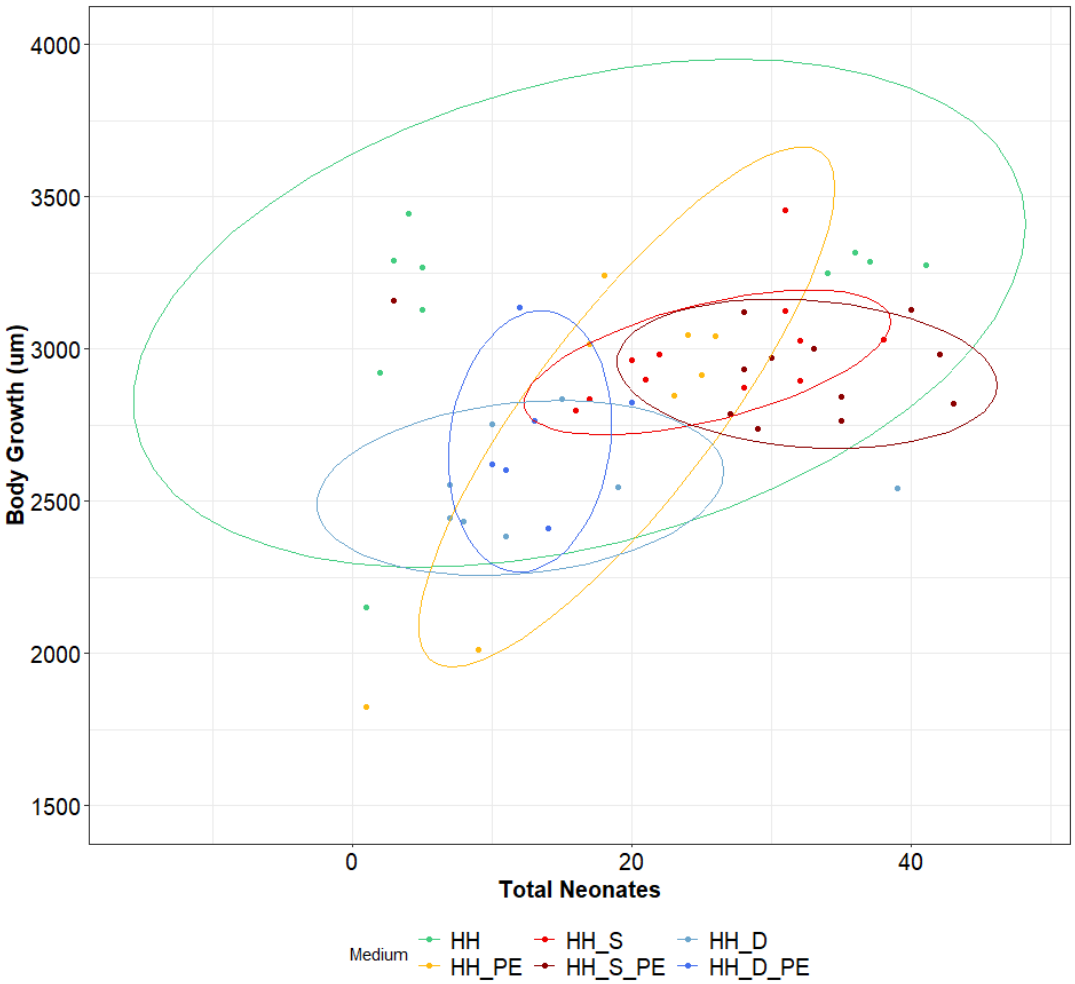


Figure 5.7 PCA analysis of the impacts of SDS (HH_S and HH_S_PE) and diclofenac (HH_D and HH_D_PE) compared to the respective medium controls in HH COMBO with and without PE MP on daphnid growth, determined at day 21.

Although there was relatively high variability in the end points across all exposure populations, the groupings in Figure 5.7 indicate that exposures containing diclofenac generally had fewer

neonates and grew less than daphnids exposed to SDS, irrespective of whether MP was added to the exposure or not. The PE only exposure had higher variability than the four combined exposure treatments, but interestingly less variability than the medium only control. The future planned work aims to explore similar patterns to determine the combinations in the various media and how they might vary in chronic toxicity end points. This is interesting as previous work with the various media and SDS only exposures had the lowest variability in the control group of HH COMBO medium (chapter 4), and therefore further exploration of this change in effect and replicates of these exposures would be helpful to address this difference.

A number of unforeseen challenges were encountered in these longer-term studies, including the Covid-related lab closures noted at the beginning of the thesis which occurred right in the middle of one of the replicates of these exposures. Since the labs have re-opened, we have been working to generate the additional replicates needed but have been facing a number of issues beyond our control that is leading to a general underperformance of all daphnids in our facility, irrespective of which medium or which *Daphnia* strand people are working on. We had previously had a problem with a bacterial infection in the algal food culture and had replaced that, but it seems there is still an issue with the new algal culture as the *Daphnia* are not growing well for the last couple of months. Thus, additional experiments for this chapter / paper are planned to increase the statistical robustness of the data and the conclusions we can draw from it, as well as a deeper analysis of the general repeatability of the standard OECD *Daphnia* acute and chronic toxicity tests (OECD 202 and 211) in our culture facility in different media assessed across the range of fecundity parameters in both the running cultures and during toxicity testing. Such baseline data will be important for early identification of potential issues in the future, and for validation of the assays for use with MP more broadly.

5.4.3 Protein variations

The presence of the chemicals in the different media can affect the chemical signalling and olfactory cues present in the medium for the *Daphnia.* For example, SDS is a well-established surfactant which would significantly reduce the concentration of functional protein signals within the medium (for example by denaturing the proteins and thus preventing them from functioning) in addition to reducing or removing the protein corona associated with the MP. Diclofenac and triclosan are less likely to have a significant impact on the proteins in the medium or at the PE surface in terms of denaturing the secreted proteins, but could impede the signalling in the water due to the increased concentration of the chemicals diluting the olfactory signals for the *Daphnia* (Leduc *et al.*, 2013).

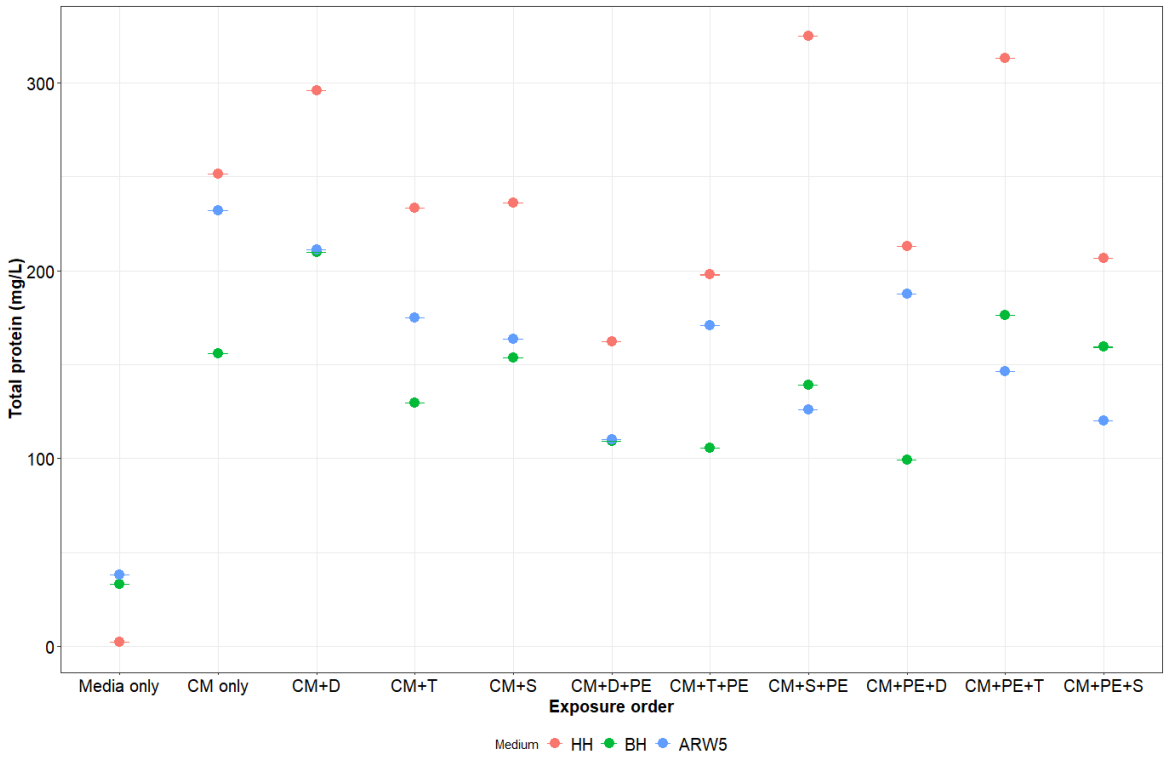


Figure 5.8 The average protein concentration associated with the medium (when no MP added) or bound to the MP surface during the different exposure orders (outlined in Figure 5.2). CM is conditioned media and the labels are representative of the order of mixing, i.e. CM+D+PE was CM with diclofenac added, followed by PE into this mixture.

Figure 5.8 highlights the variability of the amount of protein associated to the MP surface due to the different order of mixing. The conditioning process is where the medium is filtered through the daphnid guts and collects secretions from the daphnids and their gut microbiota (Nasser, Constantinou and Lynch, 2020), which in the environment is part inherited from the mother and part acquired from the surroundings as part of an adaptive response. Thus, the fact that the amounts of proteins in the different CM varies is reasonable and could relate to a number of factors including the overall differences in fitness of the daphnids in the different media (see Chapter 4) and potentially differences in the gut microbiota in the different media, although of course all were cultured in the same facility and fed the same algae so the adaptive components may be similar also – this is a potential topic for future work to undertake proteomic analysis to determine differences in gut microbial secretions. It is clear that borehole CM had the lowest amounts of proteins secreted into it, while the HH COMBO CM has the highest concentrations of proteins. This could be due to the *Daphnia* secreting higher concentration of biomolecules to condition this salt-only medium, when compared to the borehole or ARW5 groups which already contain NOM. The borehole and ARW5 medium appear to have similar variability in concentrations based on the mixture/exposure order.

The order in which the mixture components were added reveals some interesting patterns. The question being explored here was whether the various co-pollutants could effectively compete with proteins to bind to the surface of the PE particles, and/or whether the co-pollutants could even displace proteins that had already bound to the PE surface. Thus, in the

case where the co-pollutants were added to the CM before the PE any reduction in protein content detected on the PE particles relative to the protein concentration in the CM is indicative of the co-pollutant binding to the PE surface and thus reducing the amount of proteins bound. Similarly, where the PE is added to the CM first and the corona allowed to form before the co-pollutant is added, any decrease in the protein content is related to displacement of the proteins by the co-pollutant, indicating a strong competitive interaction. However, as noted in the previous sections, both triclosan and diclofenac bind strongly to NOM in the ARW5 and borehole waters, so in this case it is important also to consider the impact of the NOM-co-pollutant complex competing with the proteins to bind or displace proteins with low affinity for the PE surfaces. Where protein concentrations increased overall relative to the CM, this is indicative of a type of bioconcentration factor wherein the apparent local concentration increases. Additional analysis of the HPLC-MS data to confirm the co-pollutant concentrations and correlate these with the protein concentrations and the total PE surface area will allow additional insights into the competitive interactions.

There was notably more protein associated with the PE that had been added last to the mixture of HH COMBO medium and SDS, compared to the PE that had been added to the conditioned media followed by the SDS, which makes sense as the proteins and SDS would be competing together to bind to the surface in the latter case and indeed it is possible that some of the SDS-denatured proteins might actually have higher affinity for the PE surface than their folder variants might, given that we know SDS plays an important role in denaturing proteins and removing them from particle surfaces for subsequent analysis. Further work is planned to explore the protein conditioning of the particles in more detail in parallel to the HPLC-MS

work, to enable a statistical analysis of the various responses to be undertake in combination with the mass balance results for the chemical concentration.

5.5 Conclusion and future/planned work

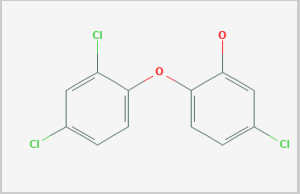
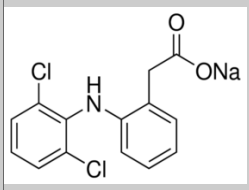
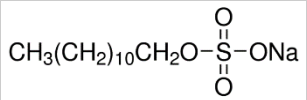
The toxicity associated with chemical and PE MP mixtures is highly variable based on the combination of chemical and testing medium. This highlights how complicated this co-exposure and trojan horse scenario could be in the environment due to the complexity of the different chemical combinations in combination with the additional environmental stressors, such as predator olfactory cues, limited food, variable temperatures etc.

Additional experiments to ascertain the toxicant impacts on the *Chlorella vulgaris* algal feed would be beneficial to further develop the overall understanding of how these various combinations can impact the ecosystem at different levels. This was not undertaken in this study as the assumption was made that due to the regular feeding of the *Daphnia* during the chronic assays, the potential impacts to the *Chlorella vulgaris* would not have a significant impact to the daphnids response. This could be in combination with other species, with different feeding mechanisms, such as sediment dwelling organism, which might be at higher risk from the chemicals that are capable of binding with the NOM in the environment. In addition, although *Daphnia* are an excellent model species for freshwater environments they are relatively simplistic in terms of physiology when compared to higher trophic level organism, i.e., fish. Therefore, a species with a more complex physiology and different mode of action, would add an interesting perspective and further understanding to this topic, and could allow ecosystem level effects to be further explored.

Planned work for this chapter (delayed due to the Covid pandemic), includes the use of HPLC-MS methods to determine if there is a significant change in the chemical concentration associated with the MP that we would hypothesize would be the inverse of the protein concentration. For example, if there is a significant quantity of protein associated with the MP the available surface for binding of chemicals is reduced, however we would expect that particles dispersed in media containing (NOM) might also have lower protein concentrations. In addition, the NOM itself can act as a sink for pollutants such as diclofenac and through binding to the MP, increase its bioavailability and this toxicity relative to its exposure directly with the PE MP. Thus, a complete re-think of how we perform mixture toxicity assessment for particles is needed, to account for direct versus indirect incorporation into the associated corona, and whether the binding is reversible or irreversible under relevant biological conditions and how we relate presence of chemicals in the corona to their bioavailability and uptake from the gut into organisms' tissues. It is hoped that as we build up some initial data to parameterise predictive models, some of these questions can be answered using quantitative-structure activity relationship (QSAR) models and machine learning approaches in the future.

Physico-chemical parameters for test chemicals

Table S5.1 A summary of some key physico-chemical parameters for the test chemicals - triclosan, diclofenac and sodium dodecyl sulphate.

	Triclosan	Diclofenac (sodium salt)	Sodium Dodecyl Sulphate (SDS)
CAS no	3380-34-5	15307-79-6	151-21-3
Formula	$C_{12}H_7Cl_3O_2$	$C_{14}H_{10}Cl_2NNaO_2$	CH 3(CH 2) 11SO 4Na
Molecular weight	289.5 g/mol	318.13 g/mol	288.38 g/mol
Solubility	12 g/L 20°C (OECD 105)	2.37 mg/L at 25 °C	'soluble' and partially soluble in ethanol
Partition coefficient (log Kow)	4.7	8 at pH 8.5	Pow: 0.83 at 22°C
Daphnia magna toxicity (EC50) 48 hour	0.39 mg/L	123.3 mg/L	(*Daphnia dubia*) 5.55 mg/L NOEC: 0.684 mg/L- 7 days (*Daphnia magna*) 1.8mg/L- 48 hours
Dissociation constant	8.14 at 20°C	4.15	1.31 at 20°C
Other			Surface tension 25.2 mN/m at 23°C
Chemical structure			

Chapter 6

6. Key research findings, synthesis, and future directions

By combining the various strands of this research (Chapters 3-5) we can increase our understanding on how to update MP toxicity study designs to reflect more environmentally realistic exposure conditions by considering dispersion protocols and culturing medium used for the study, as well as factoring in the role of the acquired biomolecule corona, whether that comprises natural organic matter, secreted proteins and other biomolecules, co-pollutants present in the aquatic environment or a combination of all of these. It is worth pointing out that even where salt-only media are used, the presence of living organisms results in secretion of biomolecules very rapidly through filter feeding, excretion of waste, shedding of carapace and a host of other natural processes, such that acquisition of an eco-corona by the MP occurs even without conscious design (Nasser, Constantinou and Lynch, 2020).

Chapter 3 discusses the importance of dispersal protocols in MP study design, and the need to report dispersion protocols to allow for metanalysis of toxicity studies going forwards. Although recently, attention of the use of commercial pre-dispersed MP and the variability in toxicity as a results of the surfactants or preservatives has been discussed (Pikuda *et al.*, 2019) few studies report the washing or dispersal process (Ogonowski *et al.*, 2016). This work reviews the stability of a hydrophobic polymer in various dispersion steps (artificial and more natural methods) and the small variations in particle size and zeta potential over time, in addition to surface conditioning with proteins. Although no significant variation in toxicity was found within these acute exposures, potential sublethal stress, or chronic toxicity may arise during longer term or multigenerational studies (Schür *et al.*, 2020). Similarly, there may be

epigenetic changes that result in increased sensitivity or increased tolerance to the MPs that that may only manifest in later generations (Ellis, Kissane and Lynch, 2020).

Following on from dispersion in Chapter 3, Chapter 4 demonstrates the importance of medium consideration for toxicity testing for both chemical and MP exposures with *Daphnia*, particularly with reflection that extrapolating hazard thresholds based on laboratory toxicity endpoints may be an overestimate of the capacity of environmental populations of daphnids to cope with the same level of stressor. This was a particularly interesting aspect of the thesis, as this highlights clearly the variability that can arise during chronic toxicity studies as a result in the variability in the testing medium through the controls in this study alone, which therefore underpins toxicity results reported across the board. For example, there was a 26% increase in the average size of the daphnids in the ARW5 control group compared to the daphnids cultured in the ARW1 medium, and a 76% increase in the total number of neonates produced during the borehole water daphnids during the duration of the study when compared to the ARW1 *Daphnia.*

The underpinning elements of Chapter 3 and 4 formed the basis of the study design for Chapter 5, bring together the elements of MP toxicity and dispersal and the influence of the media on the *Daphnia* fitness and performance in subsequent tests. Chapter 5 highlights the variability in acute toxicity (EC_{50} values) that could arise due to the combination of different SDS, triclosan or diclofenac with polyethylene MP in increasingly realistic environmental medium such as the ARW5 medium, which contains relatively high levels of NOM with the potential to change the interaction with the chemical and plastic mixtures. As NOM has previously been shown to be a sink for other pollutants, such as chemicals and metals in the

environment, the potential binding of NOM to MP could lead to remobilising of these associated pollutants increasing the overall toxicity associated with MP in the environment. For example, when exposure to a combination of SDS and PE MP in ARW5 medium the EC_{50} concentration was 916 µg/L compared to only 482 µg/L in HH COMBO. This study shows that the SDS is most likely coating the particles within the mixture, which would overcome the limitations of a chemical only exposure which would rely on chemical equilibrium principles to determine the uptake concentration, as by binding to the PE surface the SDS can then also be taken up by inadvertent ingestion of the particles. The results of this study indicate that of the three chemicals explored, only SDS exhibited the toxicity profile that follows the classical Trojan horse paradigm (increased uptake leads to increased toxicity) discussed and currently being explored within MP research. However, the Trojan horse effect cannot be confirmed in this study as the HPLC-MS mass balance work planned was not completed, therefore although the SDS and PE MP lead to increase toxicity in *Daphnia* the mechanism of this is still to be determined. The results suggest the SDS may have a higher affinity for the MP surface compared to the NOM present within the ARW5 and borehole waters, and can displace the proteins from the conditioned medium, although intriguingly, some of the potentially denatured proteins may have had a higher affinity for the PE particles potentially due to the exposure of their hydrophobic cores. The planned analysis of the chemical adsorption on the PE particles by HPLC-MS will address this aspect of the competitive interactions and confirm the concentrations of the co-pollutants absorbed to the PE MP in the different waters and the impact of the order of mixing on this.

Although this thesis is focused on one species (*Daphnia magna*) and one type of polymer (polyethylene) the overarching principles explored in the respective chapters can be applied

to the majority of freshwater toxicity testing scenarios, for both dissolved and dispersed toxicants. Polyethylene was originally chosen due to its high occurrence in environmental samples and presence in personal care products, but the principles of dispersion and mixture-based exposures are equally applicable for other types of MP. This is an important aspect of development in the field of MP ecotoxicology to ensure accurate characterisation of the particles and realistic dispersions to understand the mechanistic toxicity potential of plastics. In addition, there is a lot of interest and discussion on the trojan horse potential of MP, and although not confirmed within this study, the planned mass balance work will aim to address this question. However, the linking of different exposure scenarios and chemical mixtures highlighted some interesting results of study design that will be beneficial in moving the state of the science of MP and chemical mixtures forward, by applying these environmentally realistic media and exposure pathways to these topical questions.

The breakdown of the different experimental stages (dispersion, medium, mixtures) and the interesting results and discussion from this could provide useful evidence for updating regulatory testing for particulate based toxicants (Nasser and Lynch, 2019b). For example, the OECD 202 and 211 tests, could be modified to take into consideration more realistic medium compositions and to include NOM that have been shown in this study, and previous work with nanomaterials, to have an effect on the stability and toxicity of particles during the testing window (Ellis, Valsami-Jones and Lynch, 2020).

In conclusion, the different strands of this thesis explore the variability that can arise as a result of study design within MP toxicity tests and highlights the need for clear reporting (dispersal protocols) and consideration (media for testing) during the studies to expand the

scope of MP laboratory-based toxicity studies to increase the environmental relevance and realism going forwards. These data will feed into the overall re-design of standardised toxicity testing for particles and especially for environmentally generated particles such as secondary MP which will inevitably have an associated layer of biomolecules and potentially co-pollutants due to their formation *in situ* in the environment.

Future directions

Following on from this work, the work that was planned for the final months / weeks of thesis preparation and the subsequent papers that were disrupted and delayed due to COVID lab closure will be conducted to further address the chemical variability within the multiple stressor exposure. This will be complemented by the chronic toxicity studies for the chemical and medium variations with MP to explore the sublethal and chronic toxicity variation that could arise as a result (following the hormesis response to SDS in Chapter 3). Future work could build upon several strands of this study, to look at the mass balance and transfer of chemicals in various scenarios and under different environmental conditions. For example, how does the different pH values of the medium effect the MP surface, binding of co-pollutants and consequent mixture toxicity, and how is this set to change under future climate models. In addition, the exposure scenarios and the mixture toxicity could also be assessed with other model species, with different feeding mechanisms, to determine how this might vary the potential exposure to the toxicants. It would also be beneficial to explore the potential chronic toxicity effects over different levels of the trophic food web, for example, algae and fish, to understand how it will impact the ecosystem on different scales.

Building on the theme of increasing the environmental relevance and complexity of study designs, going forwards multigenerational studies should be explored under realistic testing scenarios. Multigenerational work with MP research is expanding with current studies reporting an increase in toxicity in subsequent generations from parents who exhibited no clear signs of a toxic response (Schür *et al.*, 2020). Multigenerational work has also been used with nanomaterials research and highlights the sensitivity to test organisms over this more realistic timeframe (Ellis *et al.*, 2020; Karatzas *et al.*, 2020). Environmental variability could also be factored into this study to explore how potential climate change scenarios could impact the *Daphnia* response, in addition to other variables such as food availability. A key aspect that emerged from the medium conditioning studies in the different media, and which warrants further investigation, is the contribution of the daphnia gut microbiome to the secreted proteins. It is conceivable that the different media support different microbiomes and that this plays a role also in the responses of the daphniids to both the MP and the co-pollutants. The gut microbiota will also be sensitive to climate change and other environmental stressors, making this a rich avenue for exploration. Finally, the corona studies were only conducted on conditioned media – while there is growing evidence that recovery of particles from the medium during chronic exposures can also shed light on the response mechanisms induced in the organisms in response to the pollutants, so recovery of the particles at different timepoints during the chronic exposures (e.g., at days 1, 3, 7, 14 and 21 to explore both acute and chronic responses allowing assessment of recovery pathways also) and proteomic analysis of the coronas might shed important light on how the daphnids respond to the challenges of MP and MP mixtures with co-pollutants.

Exploration of different MP would be an interesting direction to take this work in for the environmental relevance. For example, fibres are one of the most reported groups of MP found in environmental samples. Exploration of the variability in toxicity and stress as a result of the potential increased in residence time of fibres that could be trapped within the gut due to tangling with the microvilli in the daphnids guts could really further our mechanistic understanding of MP toxicity going forwards.

Another avenue to investigate for MP toxicity is the application of adverse outcome pathways that have been identified within nanomaterials research, and assessment of whether pathways triggered by nanomaterials are applicable also to MP or whether there are any modifications required for the MP compared to the nanomaterials. Given the fact that MP are relatively non-toxic themselves, although of course there are potential effects from additives and adsorbed pollutants) the molecular initiating events are potentially different, although this in itself will provide important insights into whether cellular attachment and or uptake of particles, as are currently being considered for nanomaterials, are in fact sufficient to be considered as initiating events for signalling pathways.

Development of predictive models to correlate physicochemical parameters and induction of toxicity is an area of increasing importance. The data generated here will also be utilised by our collaborators within existing EU projects to develop initial models for MP and mixture toxicity, adapting approaches that have been developed for nanomaterials, including image analysis of daphniids following exposure to nanomaterials and implementation of machine learning modelling approaches to predict ecotoxicity (Karatzas *et al.*, 2020). Again, the limitations of the lower toxicity of MP may play provide some limitations here, but on the

other hand the machine learning models had much greater sensitivity than our visual cues, and linked to proteomics data for example on pathways activated could also feed into establishment of baselines for confirmation of zero or low toxicity effects. A final approach that could be explored is that of dynamic energy budget modelling, whereby the trade-offs between growth, reproduction and elimination of toxicants are explored will be applied to the acute and chronic toxicity data alone and in mixtures, opening up another new avenue of research.

7. Bibliography

5Gyres (2020) *Plastic FAQs*, *5Gyres.org*. Available at: https://www.5gyres.org/faq (Accessed: 13 April 2020).

Abdallah, M.A.-E. *et al.* (2017) 'A rapid method for the determination of brominated flame retardant concentrations in plastics and textiles entering the waste stream', *Journal of Separation Science*, 40(19), pp. 3873–3881. doi:10.1002/jssc.201700497.

Afshinnia, K., Marrone, B. and Baalousha, M. (2018) 'Potential impact of natural organic ligands on the colloidal stability of silver nanoparticles', *Science of The Total Environment*, 625, pp. 1518–1526. doi:10.1016/j.scitotenv.2017.12.299.

Allen, A.S., Seymour, A.C. and Rittschof, D. (2017) 'Chemoreception drives plastic consumption in a hard coral', *Marine Pollution Bulletin*, 124(1), pp. 198–205. doi:10.1016/j.marpolbul.2017.07.030.

Allen, S. *et al.* (2019) 'Atmospheric transport and deposition of microplastics in a remote mountain catchment', *Nature Geoscience*, 12(5), pp. 339–344. doi:10.1038/s41561-019-0335-5.

Athey, S.N. *et al.* (2020) 'Trophic transfer of microplastics in an estuarine food chain and the effects of a sorbed legacy pollutant', *Limnology and Oceanography Letters*, 5(1), pp. 154–162. doi:10.1002/lol2.10130.

Avio, C.G. *et al.* (2015) 'Pollutants bioavailability and toxicological risk from microplastics to marine mussels', *Environmental Pollution*, 198, pp. 211–222. doi:10.1016/j.envpol.2014.12.021.

Bakir, A., Rowland, S.J. and Thompson, R.C. (2012) 'Competitive sorption of persistent organic pollutants onto microplastics in the marine environment', *Marine Pollution Bulletin*, 64(12), pp. 2782–2789. doi:10.1016/j.marpolbul.2012.09.010.

Bakir, A., Rowland, S.J. and Thompson, R.C. (2014) 'Enhanced desorption of persistent organic pollutants from microplastics under simulated physiological conditions', *Environmental Pollution*, 185, pp. 16–23. doi:10.1016/j.envpol.2013.10.007.

Barboza, L.G.A. and Gimenez, B.C.G. (2015) 'Microplastics in the marine environment: Current trends and future perspectives', *Marine Pollution Bulletin*, 97(1–2), pp. 5–12. doi:10.1016/j.marpolbul.2015.06.008.

Barnes, D.K.A. *et al.* (2009) 'Accumulation and Fragmentation of Plastic Debris in Global Environments Linked references are available on JSTOR for this article : Accumulation and fragmentation of plastic debris in global environments', *Philosophical Transactions of the Royal Society B: Biological Sciences*, 364(1526), pp. 1985–1998. doi:10.1098/rstb.2008.0205.

Bejgarn, S. *et al.* (2015) 'Toxicity of leachate from weathering plastics: An exploratory screening study with Nitocra spinipes', *Chemosphere*, 132, pp. 114–119. doi:10.1016/j.chemosphere.2015.03.010.

Bell, S., McGillivray, D. and Pedersen, O. (2013) *Environmental Law*. 8th Edition. Oxford: Oxford University Press.

Besseling, E. *et al.* (2014) 'Nanoplastic Affects Growth of *S. obliquus* and Reproduction of *D. magna*', *Environmental Science & Technology*, 48(20), pp. 12336–12343. doi:10.1021/es503001d.

Bouch, J.E. *et al.* (2006) 'Sedimentary and diagenetic environments of the Wildmoor Sandstone Formation (UK): implications for groundwater and contaminant transport, and sand production', *Geological Society, London, Special Publications*, 263(1), p. 129 LP – 153. doi:10.1144/GSL.SP.2006.263.01.07.

Bowley, J. *et al.* (2021) 'Oceanic Hitchhikers – Assessing Pathogen Risks from Marine Microplastic', *Trends in Microbiology*, 29(2), pp. 107–116. doi:10.1016/j.tim.2020.06.011.

Briassoulis, D. *et al.* (2013) 'Review, mapping and analysis of the agricultural plastic waste generation and consolidation in Europe', *Waste Management & Research*, 31(12), pp. 1262–1278. doi:10.1177/0734242X13507968.

Brooks, J.L. and Dodson, S.I. (1965) 'Predation, Body Size, and Composition of Plankton', *Science, New Series*, 150(3692), pp. 28–35.

Brown, A.H. and Yan, N.D. (2015) 'Food Quantity Affects the Sensitivity of *Daphnia* to Road Salt', *Environmental Science & Technology*, 49(7), pp. 4673–4680. doi:10.1021/es5061534.

Browne, M.A. *et al.* (2011) 'Accumulation of microplastic on shorelines woldwide: Sources and sinks', *Environmental Science and Technology*, 45(21), pp. 9175–9179. doi:10.1021/es201811s.

Browne, M.A. *et al.* (2013) 'Microplastic Moves Pollutants and Additives to Worms, Reducing Functions Linked to Health and Biodiversity', *Current Biology*, 23(23), pp. 2388–2392. doi:10.1016/j.cub.2013.10.012.

Calabrese, E.J. (2019) 'The Dose–Response Revolution: How Hormesis Became Significant', in *The Science of Hormesis in Health and Longevity*. Elsevier, pp. 3–24. doi:10.1016/B978-0-12-814253-0.00001-2.

Canniff, P.M. and Hoang, T.C. (2018) 'Microplastic ingestion by Daphnia magna and its enhancement on algal growth', *Science of The Total Environment*, 633, pp. 500–507. doi:10.1016/j.scitotenv.2018.03.176.

Carney Almroth, B.M. *et al.* (2018) 'Quantifying shedding of synthetic fibers from textiles; a source of microplastics released into the environment', *Environmental Science and Pollution Research*, 25(2), pp. 1191–1199. doi:10.1007/s11356-017-0528-7.

Carr, S.A., Liu, J. and Tesoro, A.G. (2016) 'Transport and fate of microplastic particles in wastewater treatment plants.', *Water research*, 91, pp. 174–82. doi:10.1016/j.watres.2016.01.002.

Carson, H.S. *et al.* (2011) 'Small plastic debris changes water movement and heat transfer through beach sediments', *Marine Pollution Bulletin*, 62(8), pp. 1708–1713. doi:10.1016/j.marpolbul.2011.05.032.

Catarino, A.I. *et al.* (2018) 'Low levels of microplastics (MP) in wild mussels indicate that MP ingestion by humans is minimal compared to exposure via household fibres fallout during a meal', *Environmental Pollution*, 237, pp. 675–684. doi:10.1016/j.envpol.2018.02.069.

Cedervall, T. *et al.* (2007) 'Understanding the nanoparticle–protein corona using methods to quantify exchange rates and affinities of proteins for nanoparticles', p. 6.

Chetwynd, A.J. *et al.* (2020) 'The Nanomaterial Metabolite Corona Determined Using a Quantitative Metabolomics Approach: A Pilot Study', *Small*, 16(21), p. 2000295. doi:10.1002/smll.202000295.

Chetwynd, A.J. and Lynch, I. (2020) 'The rise of the nanomaterial metabolite corona, and emergence of the complete corona', *Environmental Science: Nano*, 7(4), pp. 1041–1060. doi:10.1039/C9EN00938H.

Chevalier, J. *et al.* (2015) 'Exploration of *Daphnia* behavioral effect profiles induced by a broad range of toxicants with different modes of action: Effects of different toxicants on *Daphnia* behavior', *Environmental Toxicology and Chemistry*, 34(8), pp. 1760–1769. doi:10.1002/etc.2979.

Cleuvers, M. (2004) 'Mixture toxicity of the anti-inflammatory drugs diclofenac, ibuprofen, naproxen, and acetylsalicylic acid', *Ecotoxicology and Environmental Safety*, 59(3), pp. 309–315. doi:10.1016/S0147-6513(03)00141-6.

Colbourne, J.K. *et al.* (2011) 'The Ecoresponsive Genome of Daphnia pulex', *Science*, 331(6017), pp. 555–561. doi:10.1126/science.1197761.

Cole, M. *et al.* (2013) 'Cole M. Microplastic ingestion by Zooplankton. ES&T 2013', *Environmental Science and Technology*, 47, pp. 6646–6655. doi:10.1016/j.bbrc.2009.05.097.

Cole, M. *et al.* (2014) 'Isolation of microplastics in biota-rich seawater samples and marine organisms', *Scientific Reports*, 4, pp. 1–8. doi:10.1038/srep04528.

Cole, M. *et al.* (2015) 'The impact of polystyrene microplastics on feeding, function and fecundity in the marine copepod Calanus helgolandicus', *Environmental Science and Technology*, 49(2), pp. 1130–1137. doi:10.1021/es504525u.

Corradini, F. *et al.* (2019) 'Evidence of microplastic accumulation in agricultural soils from sewage sludge disposal', *Science of The Total Environment*, 671, pp. 411–420. doi:10.1016/j.scitotenv.2019.03.368.

Courtene-Jones, W. *et al.* (2017) 'Microplastic pollution identified in deep-sea water and ingested by benthic invertebrates in the Rockall Trough, North Atlantic Ocean', *Environmental Pollution*, 231, pp. 271–280. doi:10.1016/j.envpol.2017.08.026.

Cowger, W. *et al.* (2020) 'EXPRESS: Reporting Guidelines to Increase the Reproducibility and Comparability of Research on Microplastics', *Applied Spectroscopy*, p. 000370282093029. doi:10.1177/0003702820930292.

Crooks, N., Parker, H. and Pernetta, A.P. (2019) 'Brain food? Trophic transfer and tissue retention of microplastics by the velvet swimming crab (Necora puber)', *Journal of Experimental Marine Biology and Ecology*, 519, p. 151187. doi:10.1016/j.jembe.2019.151187.

Davis, A. *et al.* (2020) 'Development and application of a ratiometric nanosensor for measuring pH inside the gastrointestinal tract of zooplankton', *Environmental Science: Nano*, 7(6), pp. 1652–1660. doi:10.1039/C9EN01300H.

Debroas, D., Mone, A. and Ter Halle, A. (2017) 'Plastics in the North Atlantic garbage patch: A boat-microbe for hitchhikers and plastic degraders', *Science of The Total Environment*, 599–600, pp. 1222–1232. doi:10.1016/j.scitotenv.2017.05.059.

DEFRA (2018a) *25-year-environment-plan.pdf*. Available at: https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachment_data/file/693158/25-year-environment-plan.pdf (Accessed: 29 April 2020).

DEFRA (2018b) *World leading microbeads ban comes into force - GOV.UK*. Available at: https://www.gov.uk/government/news/world-leading-microbeads-ban-comes-into-force (Accessed: 25 April 2020).

DEFRA (2019) *Plastic bag sales down 90% since introduction of 5p charge*, *GOV.UK*. Available at: https://www.gov.uk/government/news/plastic-bag-sales-down-90-since-introduction-of-5p-charge (Accessed: 6 April 2020).

DEFRA (2020) *Ban on plastic straws, stirrers and cotton buds delayed due to coronavirus outbreak - Defra in the media*. Available at: https://deframedia.blog.gov.uk/2020/04/16/ban-on-plastic-straws-stirrers-and-cotton-buds-delayed-due-to-coronavirus-outbreak/ (Accessed: 29 April 2020).

Desantis, I. (2014) 'Cospheric SOP- Tween dispersal', pp. 3–5.

Desforges, J.P.W., Galbraith, M. and Ross, P.S. (2015) 'Ingestion of Microplastics by Zooplankton in the Northeast Pacific Ocean', *Archives of environmental contamination and toxicology*, 69(3), pp. 320–330. doi:10.1007/s00244-015-0172-5.

Dhillon, G. *et al.* (2015) 'Triclosan: Current Status, Occurrence, Environmental Risks and Bioaccumulation Potential', *International Journal of Environmental Research and Public Health*, 12(5), pp. 5657–5684. doi:10.3390/ijerph120505657.

Docter, D. *et al.* (2014) 'Quantitative profiling of the protein coronas that form around nanoparticles', *Nature Protocols*, 9(9), pp. 2030–2044. doi:10.1038/nprot.2014.139.

Du, J. *et al.* (2016) 'Toxicity Thresholds for Diclofenac, Acetaminophen and Ibuprofen in the Water Flea Daphnia magna', *Bulletin of Environmental Contamination and Toxicology*, 97(1), pp. 84–90. doi:10.1007/s00128-016-1806-7.

Ebert, D. (2005) 'Ecology, Epidemiology, and Evolution of Parasitism in Daphnia', p. 126.

Eerkes-Medrano, D., Thompson, R.C. and Aldridge, D.C. (2015) 'Microplastics in freshwater systems: A review of the emerging threats, identification of knowledge gaps and prioritisation of research needs', *Water Research*, 75, pp. 63–82. doi:10.1016/j.watres.2015.02.012.

El Hadri, H. *et al.* (2020) 'Nanoplastic from mechanically degraded primary and secondary microplastics for environmental assessments', *NanoImpact*, 17, p. 100206. doi:10.1016/j.impact.2019.100206.

Elizalde-Velázquez, A. *et al.* (2020) 'Translocation, trophic transfer, accumulation and depuration of polystyrene microplastics in Daphnia magna and Pimephales promelas', *Environmental Pollution*, 259, p. 113937. doi:10.1016/j.envpol.2020.113937.

Ellis, L.A. *et al.* (2020) 'Multigenerational Exposures of *Daphnia Magna* to Pristine and Aged Silver Nanoparticles: Epigenetic Changes and Phenotypical Ageing Related Effects', *Small*, 16(21), p. 2000301. doi:10.1002/smll.202000301.

Ellis, L.-J.A. and Lynch, I. (2020) 'Mechanistic insights into toxicity pathways induced by nanomaterials in *Daphnia magna* from analysis of the composition of the acquired protein corona', *Environmental Science: Nano*, 7(11), pp. 3343–3359. doi:10.1039/D0EN00625D.

Ellis, L.-J.A., Valsami-Jones, E. and Lynch, I. (2020) 'Exposure medium and particle ageing moderate the toxicological effects of nanomaterials to *Daphnia magna* over multiple generations: a case for standard test review?', *Environmental Science: Nano*, 7(4), pp. 1136–1149. doi:10.1039/D0EN00049C.

Ellis, L.-Jayne.A., Kissane, S. and Lynch, I. (2020) 'Maternal Responses and Adaptive Changes to Environmental Stress via Chronic Nanomaterial Exposure: Differences in Inter and Transgenerational Interclonal Broods of Daphnia magna', *International Journal of Molecular Sciences*, 22(1), p. 15. doi:10.3390/ijms22010015.

England Government (2017) 'The Environmental Protection (Microbeads) (England) Regulations 2017', 2017(1312), p. 2017 No. 1312.

Europe, P. (2017) *Plastics – the Facts 2017*.

European Chemicals Agency (2021) *Mapping exercise – Plastic additives initiative - ECHA*. Available at: https://echa.europa.eu/mapping-exercise-plastic-additives-initiative (Accessed: 1 March 2021).

European Commission *et al.* (2013) *Guidance on monitoring of marine litter in European seas.* Luxembourg: Publications Office. Available at: http://dx.publications.europa.eu/10.2788/99475 (Accessed: 3 June 2020).

European Commission (2018) 'EU strategy for cicular economy fact sheet.pdf'.

European Environment Agency (2021) *Chemicals — European Environment Agency*. Available at: https://www.eea.europa.eu/themes/human/chemicals (Accessed: 1 March 2021).

Fadare, O.O. *et al.* (2019) 'Humic acid alleviates the toxicity of polystyrene nanoplastic particles to *Daphnia magna*', *Environmental Science: Nano*, 6(5), pp. 1466–1477. doi:10.1039/C8EN01457D.

Fidra (2019) *Great Nurdle Hunt 2019*. Available at: https://www.nurdlehunt.org.uk/news-events.html (Accessed: 28 April 2020).

Foley, C.J. *et al.* (2018) 'A meta-analysis of the effects of exposure to microplastics on fish and aquatic invertebrates', *Science of The Total Environment*, 631–632, pp. 550–559. doi:10.1016/j.scitotenv.2018.03.046.

Freitas, E. and Rocha, O. (2012) 'Acute and chronic effects of atrazine and sodium dodecyl sulfate on the tropical freshwater cladoceran Pseudosida ramosa', *Ecotoxicology (London, England)*, 21, pp. 1347–57. doi:10.1007/s10646-012-0888-1.

Frydkjær, C.K., Iversen, N. and Roslev, P. (2017) 'Ingestion and Egestion of Microplastics by the Cladoceran Daphnia magna: Effects of Regular and Irregular Shaped Plastic and Sorbed

Phenanthrene', *Bulletin of Environmental Contamination and Toxicology*, 99(6), pp. 655–661. doi:10.1007/s00128-017-2186-3.

Gago, J. *et al.* (2016) 'Microplastics in Seawater: Recommendations from the Marine Strategy Framework Directive Implementation Process', *Frontiers in Marine Science*, 3. doi:10.3389/fmars.2016.00219.

Galgani, F. *et al.* (2013) 'Marine litter within the European Marine Strategy Framework Directive', *ICES Journal of Marine Science*, 70(6), pp. 1055–1064. doi:10.1093/icesjms/fst122.

Gall, S.C. and Thompson, R.C. (2015) 'The impact of debris on marine life.', *Marine pollution bulletin*, 92(1–2), pp. 170–179. doi:10.1016/j.marpolbul.2014.12.041.

Gall and Thompson, R.C. (2015) 'The impact of debris on marine life', *Marine Pollution Bulletin*, 92(1–2), pp. 170–179. doi:10.1016/j.marpolbul.2014.12.041.

Gallego-urrea, A., Hassello, M. and Hammes, J. (2013) 'Geographically distributed classification of surface water chemical parameters influencing fate and behavior of nanoparticles and colloid facilitated contaminant transport', 7. doi:10.1016/j.watres.2013.06.015.

Gao, D., Li, X. and Liu, H. (2020) 'Source, occurrence, migration and potential environmental risk of microplastics in sewage sludge and during sludge amendment to soil', *Science of The Total Environment*, 742, p. 140355. doi:10.1016/j.scitotenv.2020.140355.

Gerber, R. *et al.* (2016) 'Bioaccumulation and human health risk assessment of DDT and other organochlorine pesticides in an apex aquatic predator from a premier conservation area', *Science of The Total Environment*, 550, pp. 522–533. doi:10.1016/j.scitotenv.2016.01.129.

Geyer, R., Jambeck, J.R. and Law, K.L. (2017) 'Production, use, and fate of all plastics ever made.', *Science advances*, 3(7), p. e1700782. doi:10.1126/sciadv.1700782.

Giani, D. *et al.* (2019) 'Microplastics occurrence in edible fish species (Mullus barbatus and Merluccius merluccius) collected in three different geographical sub-areas of the Mediterranean Sea', *Marine Pollution Bulletin*, 140, pp. 129–137. doi:10.1016/j.marpolbul.2019.01.005.

Goole Maps (2021) *Google map (UK)*, *Google map, UK*. Available at: https://www.google.co.uk/maps/place/Birmingham/@48.523252,-9.2842498,4.96z/data=!4m13!1m7!3m6!1s0x4877497527da4091:0x5680d7e773a3b0bb!2s (Accessed: 03 January 2022).

Gouin, T. *et al.* (2011) 'A thermodynamic approach for assessing the environmental exposure of chemicals absorbed to microplastic', *Environmental Science and Technology*, 45(4), pp. 1466–1472. doi:10.1021/es1032025.

Grbić, J. *et al.* (2020) 'Microplastics entering northwestern Lake Ontario are diverse and linked to urban sources', *Water Research*, 174, p. 115623. doi:10.1016/j.watres.2020.115623.

Grung, M. *et al.* (2008) 'Environmental assessment of Norwegian priority pharmaceuticals based on the EMEA guideline', *Ecotoxicology and Environmental Safety*, 71(2), pp. 328–340. doi:10.1016/j.ecoenv.2007.10.015.

Guerranti, C. *et al.* (2019) 'Microplastics in cosmetics: Environmental issues and needs for global bans', *Environmental Toxicology and Pharmacology*, 68(March), pp. 75–79. doi:10.1016/j.etap.2019.03.007.

Guillard, R.R.L. (1975) 'Culture of Phytoplankton for Feeding Marine Invertebrates', in Smith, W.L. and Chanley, M.H. (eds) *Culture of Marine Invertebrate Animals: Proceedings — 1st Conference on Culture of Marine Invertebrate Animals Greenport*. Boston, MA: Springer US, pp. 29–60. doi:10.1007/978-1-4615-8714-9_3.

Guschina, I.A., Hayes, A.J. and Ormerod, S.J. (2020) 'Polystyrene microplastics decrease accumulation of essential fatty acids in common freshwater algae', *Environmental Pollution*, 263, p. 114425. doi:10.1016/j.envpol.2020.114425.

Hall *et al.* (2015) 'Microplastic ingestion by scleractinian corals', *Marine Biology*, 162(3), pp. 725–732. doi:10.1007/s00227-015-2619-7.

Hammes, J., Gallego-Urrea, J.A. and Hassellöv, M. (2013) 'Geographically distributed classification of surface water chemical parameters influencing fate and behavior of nanoparticles and colloid facilitated contaminant transport', *Water Research*, 47(14), pp. 5350–5361. doi:10.1016/j.watres.2013.06.015.

Hartmann, N.B. *et al.* (2019) 'Are We Speaking the Same Language? Recommendations for a Definition and Categorization Framework for Plastic Debris', *Environmental Science & Technology*, 53(3), pp. 1039–1047. doi:10.1021/acs.est.8b05297.

Hernandez, L.M. *et al.* (2019) 'Plastic Teabags Release Billions of Microparticles and Nanoparticles into Tea', *Environmental Science & Technology*, 53(21), pp. 12300–12310. doi:10.1021/acs.est.9b02540.

Herweyers, L. *et al.* (2020) 'Consumers' Perceptions and Attitudes toward Products Preventing Microfiber Pollution in Aquatic Environments as a Result of the Domestic Washing of Synthetic Clothes', *Sustainability*, 12(6), p. 2244. doi:10.3390/su12062244.

Hessen, D.O. (2000) 'Calcium limitation in Daphnia magna', *Journal of Plankton Research*, 22(3), pp. 553–568. doi:10.1093/plankt/22.3.553.

Hoellein, T. *et al.* (2014) 'Anthropogenic Litter in Urban Freshwater Ecosystems: Distribution and Microbial Interactions', *PLoS ONE*. Edited by S. Bertilsson, 9(6), p. e98485. doi:10.1371/journal.pone.0098485.

Horn, D. *et al.* (2019) 'Microplastics are ubiquitous on California beaches and enter the coastal food web through consumption by Pacific mole crabs', *Marine Pollution Bulletin*, 139, pp. 231–237. doi:10.1016/j.marpolbul.2018.12.039.

Horton, A.A. *et al.* (2017) 'Microplastics in freshwater and terrestrial environments: Evaluating the current understanding to identify the knowledge gaps and future research priorities', *Science of The Total Environment*, 586, pp. 127–141. doi:10.1016/j.scitotenv.2017.01.190.

Horton, A.A. *et al.* (2018) 'Acute toxicity of organic pesticides to Daphnia magna is unchanged by co-exposure to polystyrene microplastics', *Ecotoxicology and Environmental Safety*, 166, pp. 26–34. doi:10.1016/j.ecoenv.2018.09.052.

House of Commons (2020) *Environment Bill*. Available at: https://publications.parliament.uk/pa/bills/cbill/58-01/0009/20009.pdf (Accessed: 29 April 2020).

House of Commons (2021) *Environment Act 2021*. Queen's Printer of Acts of Parliament. Available at: https://www.legislation.gov.uk/ukpga/2021/30/contents/enacted (Accessed: 7 January 2022).

Hund-Rinke, K. *et al.* (2016) 'Regulatory ecotoxicity testing of nanomaterials – proposed modifications of OECD test guidelines based on laboratory experience with silver and titanium dioxide nanoparticles', *Nanotoxicology*, 10(10), pp. 1442–1447. doi:10.1080/17435390.2016.1229517.

Hurley, R., Woodward, J. and Rothwell, J.J. (2018) 'Microplastic contamination of river beds significantly reduced by catchment-wide flooding', *Nature Geoscience*, 11(4), pp. 251–257. doi:10.1038/s41561-018-0080-1.

Imhof, H.K. *et al.* (2017) 'Do microplastic particles affect Daphnia magna at the morphological, life history and molecular level?', *PLOS ONE*. Edited by J.-S. Hwang, 12(11), p. e0187590. doi:10.1371/journal.pone.0187590.

IMO (2020) *International Convention for the Prevention of Pollution from Ships (MARPOL)*. Available at: http://www.imo.org/en/About/Conventions/ListOfConventions/Pages/International-Convention-for-the-Prevention-of-Pollution-from-Ships-(MARPOL).aspx (Accessed: 10 April 2020).

Iñiguez, M.E., Conesa, J.A. and Fullana, A. (2018) 'Recyclability of four types of plastics exposed to UV irradiation in a marine environment', *Waste Management*, 79, pp. 339–345. doi:10.1016/j.wasman.2018.08.006.

Jager, T., Heugens, E.H.W. and Kooijman, S.A.L.M. (2006) 'Making Sense of Ecotoxicological Test Results: Towards Application of Process-based Models', *Ecotoxicology*, 15(3), pp. 305–314. doi:10.1007/s10646-006-0060-x.

Jamieson, A.J. *et al.* (2019) 'Microplastics and synthetic particles ingested by deep-sea amphipods in six of the deepest marine ecosystems on Earth', *Royal Society Open Science*, 6(2). doi:10.1098/rsos.180667.

Jardak, K., Drogui, P. and Daghrir, R. (2016) 'Surfactants in aquatic and terrestrial environment: occurrence, behavior, and treatment processes', *Environmental Science and Pollution Research*, 23(4), pp. 3195–3216. doi:10.1007/s11356-015-5803-x.

Jelińska, A. *et al.* (2017) 'Denaturation of proteins by surfactants studied by the Taylor dispersion analysis', *PLOS ONE*. Edited by E.A. Permyakov, 12(4), p. e0175838. doi:10.1371/journal.pone.0175838.

Jemec, A. *et al.* (2016) 'Uptake and effects of microplastic textile fibers on freshwater crustacean Daphnia magna', *Environmental Pollution*, 219, pp. 201–209. doi:10.1016/j.envpol.2016.10.037.

Jensen, K.A. *et al.* (2013) 'Final protocol for producing suitable manufactured nanomaterial exposure media', *The generic NANOGENOTOX dispersion protocol*, pp. 1–33.

Jeyasingh, P.D. and Weider, L.J. (2005) 'Phosphorus availability mediates plasticity in life-history traits and predator–prey interactions in Daphnia', p. 8.

Jeziorski, A. and Yan, N.D. (2006) 'Species identity and aqueous calcium concentrations as determinants of calcium concentrations of freshwater crustacean zooplankton', 63, p. 7.

Jordão, R. *et al.* (2016) 'Compounds altering fat storage in Daphnia magna', *Science of The Total Environment*, 545–546, pp. 127–136. doi:10.1016/j.scitotenv.2015.12.097.

Jovanović, B. *et al.* (2018) 'Virgin microplastics are not causing imminent harm to fish after dietary exposure', *Marine Pollution Bulletin*, 130, pp. 123–131. doi:10.1016/j.marpolbul.2018.03.016.

Jung, M.R. *et al.* (2018) 'Validation of ATR FT-IR to identify polymers of plastic marine debris, including those ingested by marine organisms', *Marine Pollution Bulletin*, 127, pp. 704–716. doi:10.1016/j.marpolbul.2017.12.061.

Kalčíková, G. *et al.* (2017) 'Wastewater treatment plant effluents as source of cosmetic polyethylene microbeads to freshwater', *Chemosphere*, 188, pp. 25–31. doi:10.1016/j.chemosphere.2017.08.131.

Kalogerakis, N. *et al.* (2015) 'The role of environmental biotechnology in exploring, exploiting, monitoring, preserving, protecting and decontaminating the marine environment', *New Biotechnology*, 32(1), pp. 157–167. doi:10.1016/j.nbt.2014.03.007.

Kaposi, K.L. *et al.* (2014) 'Ingestion of Microplastic Has Limited Impact on a Marine Larva', *Environmental Science & Technology*, 48(3), pp. 1638–1645. doi:10.1021/es404295e.

Karatzas, P. *et al.* (2020) 'Development of Deep Learning Models for Predicting the Effects of Exposure to Engineered Nanomaterials on *Daphnia magna*', *Small*, 16(36), p. 2001080. doi:10.1002/smll.202001080.

Katsumi, N. *et al.* (2020) 'Accumulation of microcapsules derived from coated fertilizer in paddy fields', *Chemosphere*, p. 129185. doi:10.1016/j.chemosphere.2020.129185.

Kelpsiene, E. *et al.* (2020) 'Long-term exposure to nanoplastics reduces life-time in Daphnia magna', *Scientific Reports*, 10(1), p. 5979. doi:10.1038/s41598-020-63028-1.

Kettner, M.T. *et al.* (2019) 'The Eukaryotic Life on Microplastics in Brackish Ecosystems', *Frontiers in Microbiology*, 10, p. 538. doi:10.3389/fmicb.2019.00538.

Khalid, N. *et al.* (2021) 'Linking effects of microplastics to ecological impacts in marine environments', *Chemosphere*, 264, p. 128541. doi:10.1016/j.chemosphere.2020.128541.

Kilham, S.S. *et al.* (1998a) 'COMBO: a defined freshwater culture medium for algae and zooplankton', p. 13.

Kilham, S.S. *et al.* (1998b) 'COMBO : a defined freshwater culture medium for algae and zooplankton', pp. 147–159.

Klein, K. *et al.* (2021) 'Chemicals associated with biodegradable microplastic drive the toxicity to the freshwater oligochaete Lumbriculus variegatus', *Aquatic Toxicology*, 231, p. 105723. doi:10.1016/j.aquatox.2020.105723.

Koelmans, A.A. *et al.* (2016) 'Microplastic as a Vector for Chemicals in the Aquatic Environment: Critical Review and Model-Supported Reinterpretation of Empirical Studies', *Environmental Science & Technology*, 50(7), pp. 3315–3326. doi:10.1021/acs.est.5b06069.

Kögel, T. *et al.* (2020) 'Micro- and nanoplastic toxicity on aquatic life: Determining factors', *Science of The Total Environment*, 709, p. 136050. doi:10.1016/j.scitotenv.2019.136050.

Kole, P.J. *et al.* (2017) 'Wear and Tear of Tyres: A Stealthy Source of Microplastics in the Environment', *International Journal of Environmental Research and Public Health*, 14(10), p. 1265. doi:10.3390/ijerph14101265.

Kosuth, M., Mason, S.A. and Wattenberg, E.V. (2018) 'Anthropogenic contamination of tap water, beer, and sea salt', *PLOS ONE*. Edited by Z. Zhou, 13(4), p. e0194970. doi:10.1371/journal.pone.0194970.

Krause, S. *et al.* (2021) 'Gathering at the top? Environmental controls of microplastic uptake and biomagnification in freshwater food webs', *Environmental Pollution*, 268, p. 115750. doi:10.1016/j.envpol.2020.115750.

Kukkola, A. *et al.* (2021) 'Nano and microplastic interactions with freshwater biota – Current knowledge, challenges and future solutions', *Environment International*, 152, p. 106504. doi:10.1016/j.envint.2021.106504.

Kumar, P.S., Pavithra, K.G. and Naushad, Mu. (2019) 'Chapter 4 - Characterization techniques for nanomaterials', in Thomas, S. et al. (eds) *Nanomaterials for Solar Cell Applications*. Elsevier, pp. 97–124. doi:10.1016/B978-0-12-813337-8.00004-7.

Lambert, S. and Wagner, M. (2016a) 'Characterisation of nanoplastics during the degradation of polystyrene', *Chemosphere*, 145, pp. 265–268. doi:10.1016/j.chemosphere.2015.11.078.

Lambert, S. and Wagner, M. (2016b) 'Characterisation of nanoplastics during the degradation of polystyrene', *Chemosphere*, 145, pp. 265–268. doi:10.1016/j.chemosphere.2015.11.078.

Lavender, K. (2010) 'Plastic Accumulation in the North', *Science*, 329(September), pp. 1185–1188. doi:10.1126/science.1192321.

Lebreton, L. *et al.* (2018) 'Evidence that the Great Pacific Garbage Patch is rapidly accumulating plastic', *Scientific Reports*, 8(1), p. 4666. doi:10.1038/s41598-018-22939-w.

Lechner, A. *et al.* (2014) 'The Danube so colourful: A potpourri of plastic litter outnumbers fish larvae in Europe's second largest river', *Environmental Pollution*, 188, pp. 177–181. doi:10.1016/j.envpol.2014.02.006.

Lechner, A. and Ramler, D. (2015) 'The discharge of certain amounts of industrial microplastic from a production plant into the River Danube is permitted by the Austrian legislation', *Environmental Pollution*, 200, pp. 159–160. doi:10.1016/j.envpol.2015.02.019.

Leduc, A.O.H.C. *et al.* (2013) 'Effects of acidification on olfactory-mediated behaviour in freshwater and marine ecosystems: a synthesis', *Philosophical Transactions of the Royal Society B: Biological Sciences*, 368(1627), p. 20120447. doi:10.1098/rstb.2012.0447.

Lenz, R., Enders, K. and Nielsen, T.G. (2016) 'Microplastic exposure studies should be environmentally realistic', *Proceedings of the National Academy of Sciences of the United States of America*, 113(29), pp. E4121–E4122. doi:10.1073/pnas.1606615113.

Leslie, H.A. *et al.* (2017) 'Microplastics en route: Field measurements in the Dutch river delta and Amsterdam canals, wastewater treatment plants, North Sea sediments and biota', *Environment International*, 101, pp. 133–142. doi:10.1016/j.envint.2017.01.018.

Leverett, D. *et al.* (2021) 'Environmental quality standards for diclofenac derived under the European Water Framework Directive: 1. Aquatic organisms', *Environmental Sciences Europe*, 33(1), p. 133. doi:10.1186/s12302-021-00574-z.

Li, C. *et al.* (2018) 'The pH-dependent toxicity of triclosan to five aquatic organisms (Daphnia magna, Photobacterium phosphoreum, Danio rerio, Limnodrilus hoffmeisteri, and Carassius auratus)', *Environmental Science and Pollution Research*, 25(10), pp. 9636–9646. doi:10.1007/s11356-018-1284-z.

Lithner, D., Larsson, A. and Dave, G. (2011) 'Environmental and health hazard ranking and assessment of plastic polymers based on chemical composition', *Science of the Total Environment*, 409(18), pp. 3309–3324. doi:10.1016/j.scitotenv.2011.04.038.

Loizidou, X.I., Loizides, M.I. and Orthodoxou, D.L. (2014) 'A novel best practices approach: THE MARLISCO case', *Marine Pollution Bulletin*, 88(1–2), pp. 118–128. doi:10.1016/j.marpolbul.2014.09.015.

Long, M. *et al.* (2017) 'Interactions between polystyrene microplastics and marine phytoplankton lead to species-specific hetero-aggregation', *Environmental Pollution*, 228, pp. 454–463. doi:10.1016/j.envpol.2017.05.047.

Loos, R. *et al.* (2013) 'EU-wide monitoring survey on emerging polar organic contaminants in wastewater treatment plant effluents', *Water Research*, 47(17), pp. 6475–6487. doi:10.1016/j.watres.2013.08.024.

Lowry, G.V. *et al.* (2012) 'Transformations of Nanomaterials in the Environment', *Environ. Sci. Technol.*, p. 7.

Luo, H. (2020) 'Aging of microplastics affects their surface properties, thermal decomposition, additives leaching and interactions in simulated fluids', *Science of the Total Environment*, p. 10.

Lusher, A.L. *et al.* (2015) 'Microplastics in Arctic polar waters: the first reported values of particles in surface and sub-surface samples', *Scientific Reports*, 5(1), p. 14947. doi:10.1038/srep14947.

Lynch, I. (2016) 'Water governance challenges presented by nanotechnologies: tracking, identifying and quantifying nanomaterials (the ultimate disparate source) in our waterways', *Hydrology Research*, 47(3), pp. 552–568. doi:10.2166/nh.2016.107.

Maes, T. *et al.* (2018) 'Below the surface: Twenty-five years of seafloor litter monitoring in coastal seas of North West Europe (1992–2017)', *Science of The Total Environment*, 630, pp. 790–798. doi:10.1016/j.scitotenv.2018.02.245.

Maiolo, D. *et al.* (2014) 'Surfactant Titration of Nanoparticle–Protein Corona', *Analytical Chemistry*, 86(24), pp. 12055–12063. doi:10.1021/ac5027176.

Manfra, L. *et al.* (2016) 'Lethal and sublethal endpoints observed for Artemia exposed to two reference toxicants and an ecotoxicological concern organic compound', *Ecotoxicology and Environmental Safety*, 123, pp. 60–64. doi:10.1016/j.ecoenv.2015.08.017.

Markiewicz, M. *et al.* (2018a) 'Changing environments and biomolecule coronas: Consequences and challenges for the design of environmentally acceptable engineered nanoparticles', *Green Chemistry*, 20(18), pp. 4133–4168. doi:10.1039/c8gc01171k.

Markiewicz, M. *et al.* (2018b) 'Changing environments and biomolecule coronas: consequences and challenges for the design of environmentally acceptable engineered nanoparticles', *Green Chemistry*, 20(18), pp. 4133–4168. doi:10.1039/C8GC01171K.

Marshall, S.J., House, W.A. and White, G.F. (2000) 'Role of Natural Organic Matter in Accelerating Bacterial Biodegradation of Sodium Dodecyl Sulfate in Rivers', *Environmental Science & Technology*, 34(11), pp. 2237–2242. doi:10.1021/es990828p.

Martinez, D.S.T. *et al.* (2020) 'Effect of the Albumin Corona on the Toxicity of Combined Graphene Oxide and Cadmium to Daphnia magna and Integration of the Datasets into the NanoCommons Knowledge Base', *Nanomaterials*, 10(10), p. 1936. doi:10.3390/nano10101936.

Masakorala, K., Turner, A. and Brown, M.T. (2011) 'Toxicity of Synthetic Surfactants to the Marine Macroalga, Ulva lactuca', *Water, Air, & Soil Pollution*, 218(1–4), pp. 283–291. doi:10.1007/s11270-010-0641-4.

Mason, S.A. *et al.* (2016) 'Microplastic pollution is widely detected in US municipal wastewater treatment plant effluent', *Environmental Pollution*, 218, pp. 1045–1054. doi:10.1016/j.envpol.2016.08.056.

Mausra, J. *et al.* (2015) *Laboratory Methods for the Analysis of Microplastics in the Marine Environment*. NOS-OR&R-48, p. 39.

McGoran, A.R. *et al.* (2018) 'Ingestion of plastic by fish: A comparison of Thames Estuary and Firth of Clyde populations', *Marine Pollution Bulletin*, 137, pp. 12–23. doi:10.1016/j.marpolbul.2018.09.054.

McIlwraith, H.K. *et al.* (2019) 'Capturing microfibers – marketed technologies reduce microfiber emissions from washing machines', *Marine Pollution Bulletin*, 139, pp. 40–45. doi:10.1016/j.marpolbul.2018.12.012.

Mitrano, D.M. *et al.* (2019) 'Synthesis of metal-doped nanoplastics and their utility to investigate fate and behaviour in complex environmental systems', *Nature Nanotechnology*, 14(4), pp. 362–368. doi:10.1038/s41565-018-0360-3.

Mitrano, D.M. and Wohlleben, W. (2020) 'Microplastic regulation should be more precise to incentivize both innovation and environmental safety', *Nature Communications*, 11(1), p. 5324. doi:10.1038/s41467-020-19069-1.

Mourdikoudis, Pallares and Thanh (2018) 'Characterization techniques for nanoparticles: comparison and complementarity upon studying nanoparticle properties', *Nanoscale*, 10(27), pp. 12871–12934. doi:10.1039/C8NR02278J.

Muncke, J. (2011) 'Endocrine disrupting chemicals and other substances of concern in food contact materials: An updated review of exposure, effect and risk assessment', *The Journal of Steroid Biochemistry and Molecular Biology*, 127(1–2), pp. 118–127. doi:10.1016/j.jsbmb.2010.10.004.

Murphy, F. *et al.* (2016) 'Wastewater Treatment Works (WwTW) as a Source of Microplastics in the Aquatic Environment', *Environmental Science and Technology*, 50(11), pp. 5800–5808. doi:10.1021/acs.est.5b05416.

Napper, I.E. *et al.* (2015a) 'Characterisation, quantity and sorptive properties of microplastics extracted from cosmetics', *Marine Pollution Bulletin*, 99(1–2), pp. 178–185. doi:10.1016/j.marpolbul.2015.07.029.

Napper, I.E. *et al.* (2015b) 'Characterisation, quantity and sorptive properties of microplastics extracted from cosmetics', *Marine Pollution Bulletin*, 99(1–2), pp. 178–185. doi:10.1016/j.marpolbul.2015.07.029.

Napper, I.E. and Thompson, R.C. (2016) 'Release of synthetic microplastic plastic fibres from domestic washing machines: Effects of fabric type and washing conditions', *Marine Pollution Bulletin*, 112(1–2), pp. 39–45. doi:10.1016/j.marpolbul.2016.09.025.

Nasser, F., Constantinou, J. and Lynch, I. (2020) 'Nanomaterials in the Environment Acquire an "Eco-Corona" Impacting their Toxicity to *Daphnia Magna* —a Call for Updating Toxicity Testing Policies', *PROTEOMICS*, 20(9), p. 1800412. doi:10.1002/pmic.201800412.

Nasser, F. and Lynch, I. (2016a) 'Secreted protein eco-corona mediates uptake and impacts of polystyrene nanoparticles on Daphnia magna', *Journal of Proteomics*, 137, pp. 45–51. doi:10.1016/j.jprot.2015.09.005.

Nasser, F. and Lynch, I. (2016b) 'Secreted protein eco-corona mediates uptake and impacts of polystyrene nanoparticles on Daphnia magna', *Journal of Proteomics*, 137, pp. 45–51. doi:10.1016/j.jprot.2015.09.005.

Nasser, F. and Lynch, I. (2019a) 'Updating traditional regulatory tests for use with novel materials : Nanomaterial toxicity testing with Daphnia magna', *Safety Science*, 118(May), pp. 497–504. doi:10.1016/j.ssci.2019.05.045.

Nasser, F. and Lynch, I. (2019b) 'Updating traditional regulatory tests for use with novel materials: Nanomaterial toxicity testing with Daphnia magna', *Safety Science*, 118, pp. 497–504. doi:10.1016/j.ssci.2019.05.045.

Nel, H. *et al.* (2019) 'Simple yet effective modifications to the operation of the Sediment Isolation Microplastic unit to avoid polyvinyl chloride (PVC) contamination', *MethodsX*, 6, pp. 2656–2661. doi:10.1016/j.mex.2019.11.007.

Nel, H.A. *et al.* (2020) 'Citizen science reveals microplastic hotspots within tidal estuaries and the remote Scilly Islands, United Kingdom', *Marine Pollution Bulletin*, 161, p. 111776. doi:10.1016/j.marpolbul.2020.111776.

Nel, H.A. *et al.* (2021) 'Detection limits are central to improve reporting standards when using Nile red for microplastic quantification', *Chemosphere*, 263, p. 127953. doi:10.1016/j.chemosphere.2020.127953.

Nelms, S.E. *et al.* (2018) 'Investigating microplastic trophic transfer in marine top predators', *Environmental Pollution*, 238, pp. 999–1007. doi:10.1016/j.envpol.2018.02.016.

Nuelle, M.T. *et al.* (2014) 'A new analytical approach for monitoring microplastics in marine sediments', *Environmental Pollution*, 184, pp. 161–169. doi:10.1016/j.envpol.2013.07.027.

Ocean Clean-up Project (2019) *The Ocean Clean-up Project- Oceans*, *The Ocean Cleanup*. Available at: https://theoceancleanup.com/oceans/ (Accessed: 13 April 2020).

OECD (2004a) *OECD 202: Daphnia magna Acute Immobilisation Test*. 202.

OECD (2004b) *Test No. 202: Daphnia sp. Acute Immobilisation Test, OECD Guidelines for the Testing of Chemicals, Section 2.* Paris. doi:https://doi.org/10.1787/9789264069947-en.

OECD (2012a) *OECD 211: Daphnia magna Reproduction Test*. OECD Guideline for the Testing of Chemicals 211.

OECD (2012b) *Test No. 211: Daphnia magna Reproduction Test, OECDD Guideline for the Testing of Chemicals, Section 2.* Paris. doi:https://doi.org/10.1787/9789264185203-en.

Ogonowski, M. *et al.* (2016) 'The Effects of Natural and Anthropogenic Microparticles on Individual Fitness in Daphnia magna', *PLOS ONE*. Edited by A. Mukherjee, 11(5), p. e0155063. doi:10.1371/journal.pone.0155063.

Okoffo, E.D. *et al.* (2020) 'Identification and quantification of selected plastics in biosolids by pressurized liquid extraction combined with double-shot pyrolysis gas chromatography–mass spectrometry', *Science of The Total Environment*, 715, p. 136924. doi:10.1016/j.scitotenv.2020.136924.

Oliveri Conti, G. *et al.* (2020) 'Micro- and nano-plastics in edible fruit and vegetables. The first diet risks assessment for the general population', *Environmental Research*, 187, p. 109677. doi:10.1016/j.envres.2020.109677.

Operation Clean Sweep (2019) *The programme to prevent the loss of plastic granules*, *Operation Clean Sweep*. Available at: http://www.opcleansweep.eu/the-program/ (Accessed: 13 April 2020).

Orvos, D.R. *et al.* (2002) 'Aquatic toxicity of triclosan', *Environmental Toxicology and Chemistry*, 21(7), pp. 1338–1349. doi:10.1002/etc.5620210703.

Panko, J. *et al.* (2019) 'Evaluation of Tire Wear Contribution to PM2.5 in Urban Environments', *Atmosphere*, 10(2), p. 99. doi:10.3390/atmos10020099.

Peake, B.M. *et al.* (2016) '5 - Impact of pharmaceuticals on the environment', in Peake, B.M. et al. (eds) *The Life-Cycle of Pharmaceuticals in the Environment*. Woodhead Publishing, pp. 109–152. doi:10.1016/B978-1-907568-25-1.00005-0.

Peeken, I. *et al.* (2018) 'Arctic sea ice is an important temporal sink and means of transport for microplastic', *Nature Communications*, 9(1). doi:10.1038/s41467-018-03825-5.

Pereira, J.L. and Gonçalves, F. (2008) 'Daphnia fitness over a food gradient : is body size the single trait predicting exploitative ability?', *Annales de Limnologie - International Journal of Limnology*, 44(3), pp. 169–179. doi:10.1051/limn:2008001.

Petersen, E.J. *et al.* (2015) 'Adapting OECD Aquatic Toxicity Tests for Use with Manufactured Nanomaterials: Key Issues and Consensus Recommendations', *Environmental Science & Technology*, 49(16), pp. 9532–9547. doi:10.1021/acs.est.5b00997.

Pikuda, O. *et al.* (2019) 'Toxicity Assessments of Micro- and Nanoplastics Can Be Confounded by Preservatives in Commercial Formulations', *Environmental Science & Technology Letters*, 6(1), pp. 21–25. doi:10.1021/acs.estlett.8b00614.

Plastic Soup Foundation (2020) *Beat the Bead*, *Bead the Microbead*. Available at: https://www.beatthemicrobead.org/.

PlasticsEurope (2019a) *Operation Clean Sweep 2018 Report*.

PlasticsEurope (2019b) *Plastic- The Facts 2019*. Brussels, Belgium: PlasticEurope. Available at: https://www.plasticseurope.org/en/resources/publications/1804-plastics-facts-2019 (Accessed: 31 March 2020).

Praskova, E. *et al.* (2011) 'Assessment of diclofenac LC50 reference values in juvenile and embryonic stages of the zebrafish (Danio rerio)', *Polish Journal of Veterinary Sciences*, 14(4), pp. 545–549. doi:10.2478/v10181-011-0081-0.

Prata, J.C. *et al.* (2019) 'Methods for sampling and detection of microplastics in water and sediment: A critical review', *TrAC Trends in Analytical Chemistry*, 110, pp. 150–159. doi:10.1016/j.trac.2018.10.029.

Procter, J. *et al.* (2019) 'Smells good enough to eat: Dimethyl sulfide (DMS) enhances copepod ingestion of microplastics', *Marine Pollution Bulletin*, 138, pp. 1–6. doi:10.1016/j.marpolbul.2018.11.014.

Provencher, J.F. *et al.* (2019) 'Assessing plastic debris in aquatic food webs: what we know and don't know about uptake and trophic transfer', *Environmental Reviews*, 27(3), pp. 304–317. doi:10.1139/er-2018-0079.

Rainieri, S. *et al.* (2016) 'Effects of dietary microplastic exposure on the organ toxicity of a mixture of chemical contaminants in zebrafish (Danio rerio)', *Toxicology Letters*, 258, pp. S285–S286. doi:10.1016/j.toxlet.2016.06.1995.

Rehse, S., Kloas, W. and Zarfl, C. (2016) 'Short-term exposure with high concentrations of pristine microplastic particles leads to immobilisation of Daphnia magna', *Chemosphere*, 153, pp. 91–99. doi:10.1016/j.chemosphere.2016.02.133.

Reynolds, A., Giltrap, M. and Chambers, G. (2019) 'Evaluation of non-invasive toxicological analysis of nano-polystyrene in relative in vivo conditions to D. magna', *Environmental Science: Nano*, 6(9), pp. 2832–2849. doi:10.1039/C9EN00434C.

Reynolds, C. and Ryan, P.G. (2018) 'Micro-plastic ingestion by waterbirds from contaminated wetlands in South Africa', *Marine Pollution Bulletin*, 126, pp. 330–333. doi:10.1016/j.marpolbul.2017.11.021.

Rinke, K. and Petzoldt, T. (2003) 'Modelling the effects of temperature and food on individual growth and reproduction of Oaphnia and their consequences on the population level', p. 12.

Rocha-Santos, T. and Duarte, A.C. (2015) 'A critical overview of the analytical approaches to the occurrence, the fate and the behavior of microplastics in the environment', *TrAC - Trends in Analytical Chemistry*, 65, pp. 47–53. doi:10.1016/j.trac.2014.10.011.

Rochman, C.M. (2013a) 'Plastics and priority pollutants: A multiple stressor in aquatic habitats', *Environmental Science and Technology*, 47(6), pp. 2439–2440. doi:10.1021/es400748b.

Rochman, C.M. (2013b) 'Plastics and Priority Pollutants: A Multiple Stressor in Aquatic Habitats', *Environmental Science & Technology*, 47(6), pp. 2439–2440. doi:10.1021/es400748b.

Rochman, C.M. *et al.* (2019) 'Rethinking microplastics as a diverse contaminant suite', *Environmental Toxicology and Chemistry*, 38(4), pp. 703–711. doi:10.1002/etc.4371.

Rochman, C.M., Cook, A.M. and Koelmans, A.A. (2016) 'Plastic debris and policy: Using current scientific understanding to invoke positive change', *Environmental Toxicology and Chemistry*, 35(7), pp. 1617–1626. doi:10.1002/etc.3408.

Rogers, K.L. *et al.* (2020) 'Micro-by-micro interactions: How microorganisms influence the fate of marine microplastics', *Limnology and Oceanography Letters*, 5(1), pp. 18–36. doi:10.1002/lol2.10136.

Rosenkranz, P. *et al.* (2009) 'A COMPARISON OF NANOPARTICLE AND FINE PARTICLE UPTAKE BY DAPHNIA MAGNA', *Environmental Toxicology and Chemistry*, 28(10), p. 2142. doi:10.1897/08-559.1.

Rowett, C.J., Hutchinson, T.H. and Comber, S.D.W. (2016) 'The impact of natural and anthropogenic Dissolved Organic Carbon (DOC), and pH on the toxicity of triclosan to the crustacean Gammarus pulex (L.)', *Science of The Total Environment*, 565, pp. 222–231. doi:10.1016/j.scitotenv.2016.04.170.

de Sá, L.C. *et al.* (2018) 'Studies of the effects of microplastics on aquatic organisms: What do we know and where should we focus our efforts in the future?', *Science of The Total Environment*, 645, pp. 1029–1039. doi:10.1016/j.scitotenv.2018.07.207.

Saavedra, J., Stoll, S. and Slaveykova, V.I. (2019) 'Influence of nanoplastic surface charge on eco-corona formation, aggregation and toxicity to freshwater zooplankton', *Environmental Pollution*, 252, pp. 715–722. doi:10.1016/j.envpol.2019.05.135.

Sadri, S.S. and Thompson, R.C. (2014) 'On the quantity and composition of floating plastic debris entering and leaving the Tamar Estuary, Southwest England', *Marine Pollution Bulletin*, 81(1), pp. 55–60. doi:10.1016/j.marpolbul.2014.02.020.

Salvati, A. *et al.* (2011) 'Experimental and theoretical comparison of intracellular import of polymeric nanoparticles and small molecules: toward models of uptake kinetics', *Nanomedicine: Nanotechnology, Biology and Medicine*, 7(6), pp. 818–826. doi:10.1016/j.nano.2011.03.005.

Sanchez, W., Bender, C. and Porcher, J.-M. (2014) 'Wild gudgeons (Gobio gobio) from French rivers are contaminated by microplastics: Preliminary study and first evidence', *Environmental Research*, 128, pp. 98–100. doi:10.1016/j.envres.2013.11.004.

Schmidt, M., Rohde, F. and Braumann, U.-D. (2021) 'Chapter 15 - Visualization and co-registration of correlative microscopy data with the ImageJ plug-in Correlia', in Müller-Reichert, T. and Verkade, P. (eds) *Methods in Cell Biology*. Academic Press (Correlative Light and Electron Microscopy IV), pp. 353–388. doi:10.1016/bs.mcb.2020.10.001.

Schnurr, R.E.J. *et al.* (2018) 'Reducing marine pollution from single-use plastics (SUPs): A review', *Marine Pollution Bulletin*, 137, pp. 157–171. doi:10.1016/j.marpolbul.2018.10.001.

Schür, C. *et al.* (2019) 'When Fluorescence Is not a Particle: The Tissue Translocation of Microplastics in *Daphnia magna* Seems an Artifact', *Environmental Toxicology and Chemistry*, 38(7), pp. 1495–1503. doi:10.1002/etc.4436.

Schür, C. *et al.* (2020) 'Microplastics but not natural particles induce multigenerational effects in Daphnia magna', *Environmental Pollution*, 260, p. 113904. doi:10.1016/j.envpol.2019.113904.

Seeley, M.E. *et al.* (2020) 'Microplastics affect sedimentary microbial communities and nitrogen cycling', *Nature Communications*, 11(1), p. 2372. doi:10.1038/s41467-020-16235-3.

Serra, T., Müller, M.F. and Colomer, J. (2019) 'Functional responses of Daphnia magna to zero-mean flow turbulence', *Scientific Reports*, 9(1), p. 3844. doi:10.1038/s41598-019-40777-2.

Shen, M. *et al.* (2019) 'Micro(nano)plastics: Unignorable vectors for organisms', *Marine Pollution Bulletin*, 139, pp. 328–331. doi:10.1016/j.marpolbul.2019.01.004.

Shi, W. *et al.* (2021) 'Microplastics impair olfactory-mediated behaviors of goldfish Carassius auratus', *Journal of Hazardous Materials*, 409, p. 125016. doi:10.1016/j.jhazmat.2020.125016.

Sighicelli, M. *et al.* (2018) 'Microplastic pollution in the surface waters of Italian Subalpine Lakes', *Environmental Pollution*, 236, pp. 645–651. doi:10.1016/j.envpol.2018.02.008.

Sjollema, S.B. *et al.* (2016) 'Do plastic particles affect microalgal photosynthesis and growth?', *Aquatic Toxicology*, 170, pp. 259–261. doi:10.1016/j.aquatox.2015.12.002.

Smith, C. (2016) 'Pollutants from the EU Watch List: a review of their occurrence and water-treatment options', *Water Research*, (473), p. 2.

de Souza Machado, A.A. *et al.* (2019) 'Microplastics Can Change Soil Properties and Affect Plant Performance', *Environmental Science & Technology*, 53(10), pp. 6044–6052. doi:10.1021/acs.est.9b01339.

Steinmetz, Z. *et al.* (2016) 'Plastic mulching in agriculture. Trading short-term agronomic benefits for long-term soil degradation?', *Science of The Total Environment*, 550, pp. 690–705. doi:10.1016/j.scitotenv.2016.01.153.

Tang, W.-W. *et al.* (2014) 'Impact of humic/fulvic acid on the removal of heavy metals from aqueous solutions using nanomaterials: A review', *Science of The Total Environment*, 468–469, pp. 1014–1027. doi:10.1016/j.scitotenv.2013.09.044.

Tayyebi Sabet Khomami, N. *et al.* (2020) 'Validation of a field deployable reactor for *in situ* formation of NOM-engineered nanoparticle corona', *Environmental Science: Nano*, 7(2), pp. 486–500. doi:10.1039/C9EN01090D.

Thiele, C.J., Hudson, M.D. and Russell, A.E. (2019) 'Evaluation of existing methods to extract microplastics from bivalve tissue: Adapted KOH digestion protocol improves filtration at single-digit pore size', *Marine Pollution Bulletin*, 142, pp. 384–393. doi:10.1016/j.marpolbul.2019.03.003.

Thornhill, I.A.G. (2013) 'Water quality, biodiversity and ecosystem functioning in ponds across an urban land-use gradient in Birmingham, U.K.', p. 307.

Tibbetts, J. *et al.* (2018) 'Abundance, Distribution, and Drivers of Microplastic Contamination in Urban River Environments', *Water*, 10(11), p. 1597. doi:10.3390/w10111597.

Tkaczyk, A. *et al.* (2021) 'Daphnia magna model in the toxicity assessment of pharmaceuticals: A review', *Science of The Total Environment*, 763, p. 143038. doi:10.1016/j.scitotenv.2020.143038.

Tosetto, L., Williamson, J.E. and Brown, C. (2017) 'Trophic transfer of microplastics does not affect fish personality', *Animal Behaviour*, 123, pp. 159–167. doi:10.1016/j.anbehav.2016.10.035.

Traudt, E.M., Ranville, J.F. and Meyer, J.S. (2017) 'Age-related differences in sensitivity to metals can matter for *Daphnia magna* neonates', *Integrated Environmental Assessment and Management*, 13(1), pp. 208–210. doi:10.1002/ieam.1858.

Triebskorn, R. *et al.* (2019) 'Relevance of nano- and microplastics for freshwater ecosystems: A critical review', *TrAC Trends in Analytical Chemistry*, 110, pp. 375–392. doi:10.1016/j.trac.2018.11.023.

Twiss, M.R. (2016) 'Standardized methods are required to assess and manage microplastic contamination of the Great Lakes system', *Journal of Great Lakes Research*, 42(5), pp. 921–925. doi:10.1016/j.jglr.2016.07.032.

University of Birmingham (2021) *100 Plastic Rivers*, *100 Plastic Rivers*. Available at: https://www.birmingham.ac.uk/research/water-sciences/projects/plastic-rivers.aspx.

US Environmental Protection Agency (1996) 'EPA daphnia protocol.pdf'.

Velzeboer, I., Kwadijk, C.J.A.F. and Koelmans, A.A. (2014) 'Strong Sorption of PCBs to Nanoplastics, Microplastics, Carbon Nanotubes, and Fullerenes', *Environmental Science & Technology*, 48(9), pp. 4869–4876. doi:10.1021/es405721v.

Wacker, A. and Martin-Creuzburg, D. (2007) 'Allocation of essential lipids in Daphnia magna during exposure to poor food quality', *Functional Ecology*, 21(4), pp. 738–747. doi:10.1111/j.1365-2435.2007.01274.x.

Wagner *et al.* (2014) 'Microplastics in freshwater ecosystems: what we know and what we need to know', *Environmental Sciences Europe*, 26(1), p. 12. doi:10.1186/s12302-014-0012-7.

Wagner, S. *et al.* (2018) 'Tire wear particles in the aquatic environment - A review on generation, analysis, occurrence, fate and effects', *Water Research*, 139, pp. 83–100. doi:10.1016/j.watres.2018.03.051.

Walker, C.H. *et al.* (2012) *Principles of Ecotoxicology*. 4th edn. London: Taylor and Francis Group.

Wang, Fen *et al.* (2018) 'Interaction of toxic chemicals with microplastics: A critical review', *Water Research*, 139, pp. 208–219. doi:10.1016/j.watres.2018.04.003.

Wegner, A. *et al.* (2012) 'Effects of nanopolystyrene on the feeding behavior of the blue mussel (*Mytilus edulis* L.)', *Environmental Toxicology and Chemistry*, 31(11), pp. 2490–2497. doi:10.1002/etc.1984.

Wesch, C. *et al.* (2017) 'Assuring quality in microplastic monitoring: About the value of clean-air devices as essentials for verified data', *Scientific Reports*, 7(1), p. 5424. doi:10.1038/s41598-017-05838-4.

Wilson, J.G., McHugh, B. and Giltrap, M. (2014) 'Biomarkers: Are realism and control mutually exclusive in integrated pollution assessment?', *Marine Environmental Research*, 102, pp. 11–17. doi:10.1016/j.marenvres.2014.07.005.

Windsor, F.M. *et al.* (2019) 'Microplastic ingestion by riverine macroinvertebrates', *Science of The Total Environment*, 646, pp. 68–74. doi:10.1016/j.scitotenv.2018.07.271.

Woodall, L.C. *et al.* (2014) 'The deepsea major sink for microplastic', *Royal Society Open Science*, pp. 1–8.

Woodall, L.C. *et al.* (2015) 'Using a forensic science approach to minimize environmental contamination and to identify microfibres in marine sediments', *Marine Pollution Bulletin*, 95(1), pp. 40–46. doi:10.1016/j.marpolbul.2015.04.044.

World Health Organization (2019) *Microplastics in drinking-water*. Geneva: World Health Organization. Available at: https://apps.who.int/iris/handle/10665/326499 (Accessed: 6 January 2022).

Wright, S.L. *et al.* (2020) 'Atmospheric microplastic deposition in an urban environment and an evaluation of transport', *Environment International*, 136, p. 105411. doi:10.1016/j.envint.2019.105411.

Wu, C. *et al.* (2016) 'Sorption of pharmaceuticals and personal care products to polyethylene debris', *Environmental Science and Pollution Research*, 23(9), pp. 8819–8826. doi:10.1007/s11356-016-6121-7.

Xia, Y. *et al.* (2020) 'Strong influence of surfactants on virgin hydrophobic microplastics adsorbing ionic organic pollutants', *Environmental Pollution*, 265, p. 115061. doi:10.1016/j.envpol.2020.115061.

Zhang, Q. *et al.* (2020) 'A Review of Microplastics in Table Salt, Drinking Water, and Air: Direct Human Exposure', *Environmental Science & Technology*, 54(7), pp. 3740–3751. doi:10.1021/acs.est.9b04535.

Zhang, X. *et al.* (2019) 'The Competitive Dynamic Binding of Some Blood Proteins Adsorbed on Gold Nanoparticles', p. 6.

Ziccardi, L.M. *et al.* (2016) 'Microplastics as vectors for bioaccumulation of hydrophobic organic chemicals in the marine environment: A state-of-the-science review: Role of microplastics in marine

contaminant transfer', *Environmental Toxicology and Chemistry*, 35(7), pp. 1667–1676. doi:10.1002/etc.3461.

Zimmermann, L. *et al.* (2020) 'What are the drivers of microplastic toxicity? Comparing the toxicity of plastic chemicals and particles to Daphnia magna', *Environmental Pollution*, 267, p. 115392. doi:10.1016/j.envpol.2020.115392.

Appendices

Annex 1: Daphnia facility algae culturing SOP.

UNIVERSITY OF BIRMINGHAM

SCHOOL OF BIOSCIENCES, DAPHNIA FACILITY

STANDARD OPERATING PROCEDURE

SOP No.5 Version #2

ALGAL CULTURE

SOP No.5 #2

The purpose of this Standard Operating Procedure (SOP) is to describe the culture of algae in the facility.

Culture of algae:

The algal species cultured will be *Chlorella vulgaris*. Other species may be cultured for specific experimental purposes.

The culture of algae will generally be performed once weekly with preparation and harvesting on Wednesdays. Alternatively, if there is greater demand for algae, then preparation and harvesting can be done on Monday and Thursday mornings. For holiday periods the regime will be altered accordingly. The number of flasks prepared will be dependent on demand in the laboratory.

Harvesting algae:

Make up new cultures by inoculating freshly autoclaved BBM medium in conical flasks from two or more good existing cultures by adding an aliquot of algae to each flask. The aliquot volume added to the fresh medium will normally be 20-25ml for 7 days incubation. Volumes for alternative incubation will be 100-110ml on Monday for a 3-day incubation period, and 75-80ml on Thursday for a 4-day incubation period. Other volumes may be used for other incubation periods. Decant the remaining algal culture into clean beakers and cover with cling-film to prevent aerial contamination.

Aerate the culture flasks vigorously using freshly autoclaved aeration tubes.

After use, place the used conical flasks in a sink and add ≈1 teaspoon of sodium bicarbonate. Fill with hot water then place the used aeration tubes in the flasks. Leave to soak for >1 hour.

Clean conical flasks and aeration tubes and leave to dry.

Centrifugation of algae:

Centrifuge the algae in 500ml centrifuge tubes using a Sorval Centrifuge (located in room S203 off S204) at 3500rpm for 15 minutes using the rotor SLA3000, min temp +4°C and max temp +9°C. The rotor can be found in the corner cupboard in room S204.

After centrifugation, decant off the supernatant. Re-suspend the concentrated algae in the minimum amount of modified standard Combo medium and then decant into a clean beaker. In order to ensure the maximum amount of algae is recovered, rinse the centrifuge tubes with a minimum amount of standard Combo so as not to over-dilute, then add this to the collection beaker.

Measure the optical density (OD) using a spectrophotometer. Measure the absorbance of a 1 in 10 dilution of the algal concentrate at 440nm using 1cm cuvettes. Dilute with standard Combo medium until the absorbance is approximately 0.80.

After adjustment to the correct OD, decant the algae into 1L Duran bottles and store in the fridge at +4°C.

Clean the centrifuge tubes and leave to dry.

Preparation of fresh algal medium:

Prepare 10 litres of BBM medium (SOP No.3) in 10L aspirators or two 5L Duran bottles and dispense (approx. 1650ml) to an appropriate number of conical flasks and autoclave. The volume prepared will vary dependent on the demand for experimental procedures. Prepare and autoclave ready for Wednesday morning.

Approved by: Caroline Sewell **Signature**:

Annex 2- Cospheric Tween dispersion protocol used throughout Chapter 3.

Content taken from: Tween solutions for Suspension of Hydrophobic Particles in Water for Density Marker Beads in Percoll or other gradients or Flow Visualization (cospheric.com) on 28/06/21

Aqueous Solutions of Hydrophobic Particles (Tween)

Technical Information Sheet (MFG-WI-88-rev1)

"Suspension of Hydrophobic Particles in Aqueous Solution"

This document describes the process for preparing suspensions of hydrophobic particles in an aqueous solution by using a surfactant.

Background Information

Many materials are hydrophobic (water-fearing) in nature. Due to their non-polar chemical structure, hydrophobic particles want to minimize contact with polar (water) molecules and, as a result, tend to aggregate on the surface of the water and resist going into suspension. This presents a challenge to scientists and engineers who would like to be able to work with hydrophobic particles suspended in aqueous solution.

Examples of the applications are using fluorescent polyethylene microspheres for flow visualization in aqueous systems, creating density gradients, filtration and contamination control studies.

Fortunately, there is a simple way to overcome the hydrophobic effect. It is called a surfactant, a detergent, or simply “soap.” Surfactant is a magical molecule that has both hydrophobic and hydrophilic properties, which coats the particles and helps them mix into water. The same mechanism applies when we use soap to wash greasy dishes or stained clothes.

Selection of the surfactant depends purely on your process and product requirements. Dishwashing liquid works great, so does Simple Green. For scientists working on biological applications we recommend the use of Tween surfactants. Tween is the commercial name for Polysorbate non-ionic surfactants, which are stable, nontoxic, and often used in pharmacological, cosmetic, and food applications. Non-ionic detergents are considered to be “mild” detergents because they are less likely than ionic detergents to denature proteins. By not separating protein-protein bonds, non-ionic detergents allow the protein to retain its native structure and functionality.

Tween 20 and Tween 80 are frequently used. Both surfactants are yellowish, water-soluble viscous liquids. Primary difference between the two is viscosity. Tween 20 has lower viscosity and is easier to work with.

Suspension Process

There are many ways to suspend the particles (e.g. put a few drops of dish detergent into water and shake with the particles).

The process below is specific for using the minimum amount of Tween for biologically sensitive applications.

Safety:

Gloves and eye protection are to be worn at all times during solution preparation and use.

Care should be taken when handling hot objects/liquids and immersion blender.

Centrifuge should be properly balanced and allowed to come to a full stop before opening.

Recommendations:

We recommend using distilled water to minimize impurities.

We recommend boiling the water to sterilize and to make it easier to disperse a small amount of surfactant uniformly. This also increases shelf-life of prepared solutions and suspensions.

We use an immersion blender to disperse the surfactant in water quickly and effectively.

Process:

Preparing Tween Solution:

Fill a heatproof container with distilled water.

Ensure the water level is high enough to cover the immersion blender.

Heat water to boiling and leave boiling for 5 minutes.

Weigh out 0.1g of Tween per 100ml of water used (creating 0.1% solution).

Slowly add Tween to boiled water while mixing with immersion mixer (~30 seconds).

Some bubbles will form during mixing.

Bubbles will dissipate on cooling and solution will appear clear.

Suspending particles in Tween solution.

Place the desired amount of particles into a container.

Dispense prepared Tween solution on top of particles.

We recommend at least five times greater volume of solution to the volume of particles.

Cover tightly and place containers into a centrifuge.

Centrifuge on highest setting for at least 5 minutes.

If some particles are still floating on the surface of water, more centrifuging may be necessary.

A small quantity of particles may accumulate on the top surface and not enter solution despite additional centrifugation. Typically, these particles will go into suspension over time (hours).

Other Considerations

A greater length of centrifuging or larger volume of Tween solution may be necessary to suspend certain materials and particle sizes.

As a 0.1% Tween solution is sufficient for most applications, concentration levels could be raised to support particles that are more resistant to entering solution.

Once the particles are suspended, solution can may be diluted further to increase the volume.

Particles can be recycled and reused as necessary. The suspension might need to be repeated.

If no centrifuge is available, it is possible to shake the container by hand (up and down, upside down) to achieve the same result.

Here is an example of fluorescent beads being dispersed in a pilot bioreactor:

About Cospheric

Our extensive product line consists of more than two thousand unique spherical microparticle and nanoparticle products, all developed based on customer demand. We work with each individual customer to find a creative solution for their unique needs -- tight particle size ranges, wide selection of colors, densities, properties and formulations. We are the sole global supplier for the majority of our products. We developed a disruptive technology which is redefining the microsphere market and creating a new category of precision spherical particles. Our research department is always excited to tackle new challenging projects. Explore at www.Cospheric.com.

Other Information

The information contained in this document is correct to the best of our knowledge at the date of publication. It should not be viewed as all inclusive, but as a guide only. It does not represent any guarantee of the properties of the product. Cospheric LLC shall not be held liable for any damage resulting from handling of or from contact with the above product. For these reasons, it is important that product users carry out their own tests to satisfy themselves as to the suitability of the safety precautions for their own intended applications.

Annex 3- field campaign report

Water quality report

A collaborative field campaign was undertaken to ascertain the water quality and confirm the presence of microplastics (MP) and *Daphnia* at ponds in the local area (Birmingham, UK). Ponds are very useful tools for reflecting the physio-chemical local land use due to the small area and often relatively large catchments, which is often reported as having the largest effect on water quality and subsequently the macroinvertebrate community composition. This can be affected by point and diffuse sources. Ponds can also be lost due to natural succession, with minimal anthropogenic intervention, vegetation can encroach on pond area leading to a gradual, natural reduction. This coupled with the anthropogenic and climate pressures on pong habitats can have as significant effect on the network both spatially and temporally. Similar to the hedgerows and more recently the marine conservation zones, the important of connectivity crops up as a major issue for these conservation issues and the same is true for urban ponds. To maintain the ecological diversity currently present within the water, the network connections need to be maintained which is often challenging from a land use point of view as ponds span different land users and uses.

The level of protection offered to ponds in terms of conservation stems from the Water Framework Directive (WFD) and the UK Biodiversity Action Plan (UK BAP). Under the WFD ponds may be viewed as artificial depending on the nature of their creation and maintenance which lowers the level of protection, however under the UK BAP they can be

protected as Priority Habitats, but it is important to view this as a network of ponds rather than each water body in isolation.

Within the context of this thesis, this study aims to investigate the water quality across the urban-rural pollution gradient for both site parameters and major ion analysis. This water quality analysis can then be compared to the major elements in the *Daphnia* medium to make links in the future for how realistic the medium is in relation to the local environment.

For the context of the overall collaborative project aims, ponds were an ideal environment to sample as previous work in the group has demonstrated the microplastic concentrations in ponds tends to be higher due to the movement and deposition from areas of high flow, and the potential for urban run off from their local environments (Tibbetts *et al.*, 2018). In addition, *Daphnia* have also been reported to prefer low flow environments and therefore the likelihood of finding daphnids in the pond network was relatively high (Serra, Müller and Colomer, 2019).

Due to the collaborative nature of this field campaign, the results for the water analysis and *in situ* measurements were combined into a separate report (presented at SETAC Europe in 2019) linking the water quality results with the results for the *Daphnia* and microplastic sampling. The *Daphnia* and microplastics work are not included in this report or thesis, as it was undertaken by other PhD students and will be presented in their respective theses.

Environmental sample collection and analysis

Site sampling was undertaken in July and August 2018. This sampling campaign spanned an urban-rural pollution gradient (as shown in Figure A3-1) and encompassed *Daphnia* via a net

sweep sample, microplastics using a surface trawl and water quality using *in situ* parameter measurements and water sampling for further elemental analysis. When collecting water samples contact with the bottom substrate, vegetation and surface debris was carefully avoided. Samples were collected in acid washed polypropylene bottles which were rinsed on site in triplicate. Once collected the samples were kept cool until returned to the fridge in the lab. Site parameters measured *in situ* included temperature (°C), pH, dissolved oxygen (% saturation) and electrical conductivity (µS/cm). Triplicates of all measurements were taken on site and later averaged for data reporting.

Prior to use for the water filtration, glass fibre (GF) filter papers were dried at 100°C to remove any weight from moisture content. The pond water was then filtered through the dried 0.45 µm GF paper to remove suspended material. An example of the filter papers can be seen in Figure A3-2 to highlight the colour difference across the sites, reflective of the different water compositions. The filter papers were then placed in the oven at 50°C to allow the content to be dried slowly. Filter papers were reweighed to determine the mass of the suspended solids within the samples. The mass of the suspended solids was then calculated using the equation:

$$SS\ (mg/l) = \frac{(f2 - f1) * 1000}{V}$$

where *f2* is the weight of the paper post filtration and drying, *f1* is the start weight (both in mg) and *V* is the volume of water filtered (mL).

The results are limited because there was only one sampling season for this field campaign, therefore the water sample is only representative of that specific sampling point and does not take account of any seasonal variability. The chemical flux between the water and surrounding

sediment was not studied within this field campaign due to the lack of comparison between the sites, as at several of the ponds it was not possible to sample for sediment due to the artificial bottoms and lack of substrate.

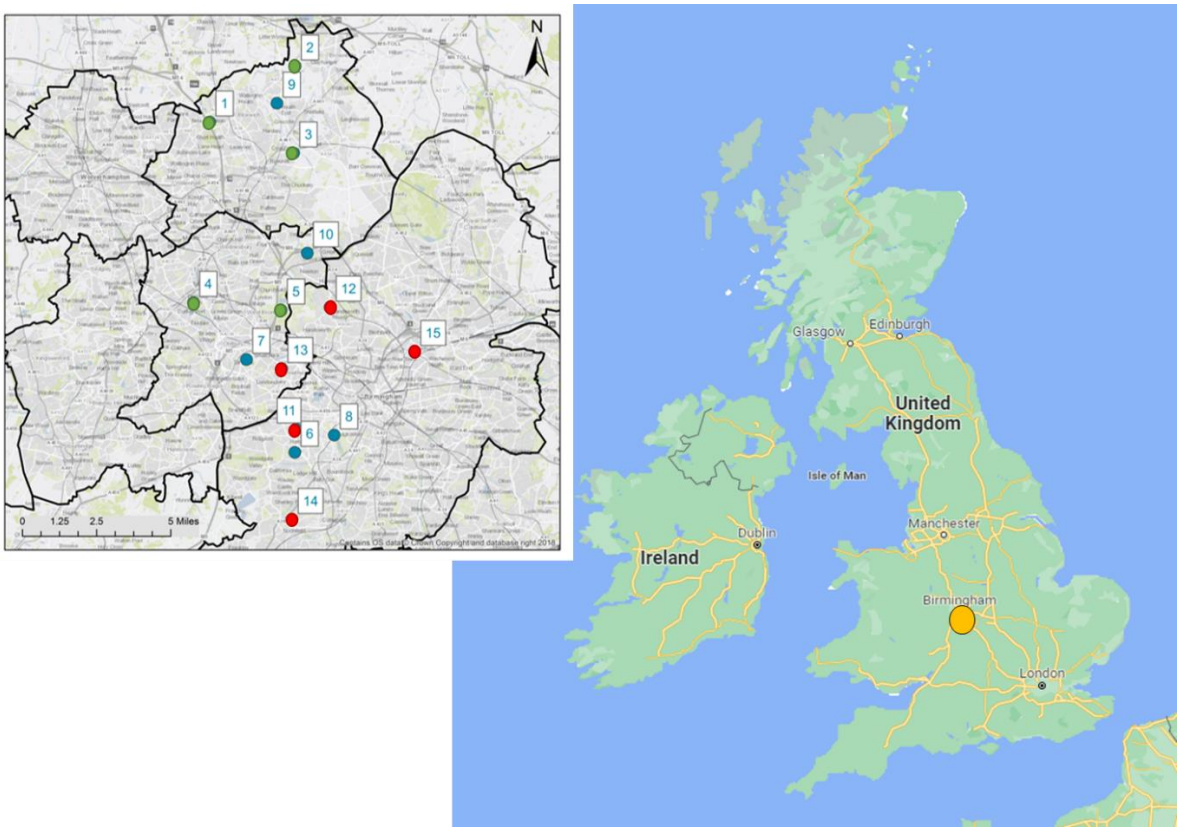


Figure A3-1 Map of the pond sampling sites around the Birmingham region, UK, sites were classified as rural (1-5 (green)), suburban (6-10 (blue)), and urban (11-15 (red)) based on a range of parameters including land-use and population density. (Goole Maps, 2021).

The water samples along with the artificial laboratory medium and borehole water were analysed using Inductively Coupled Plasma- Optical Emission Spectroscopy (ICP-OES) (Perkin-Elmer Optima 8000) with a radial view plasma objective using a cross flow nebuliser (argon gas flow of 8 L per minute) and a sample flow rate of 1 mL per minute. The seven elements selected for quantification due to the predominate concentration in HH COMBO medium were

boron, calcium, magnesium, phosphate, potassium, silicon and sodium. Calibration standards for ICP-OES were 0, 0.1, 1, 10 and 100 mg/L. The water samples were defrosted and filtered through a 0.45 µm PES membrane filter ahead of ICP-OES analysis. Samples were analysed in triplicate, with blanks every 15 samples and calibration checked against the internal standards on each series.

How similar are the natural waters compared to the culturing media.

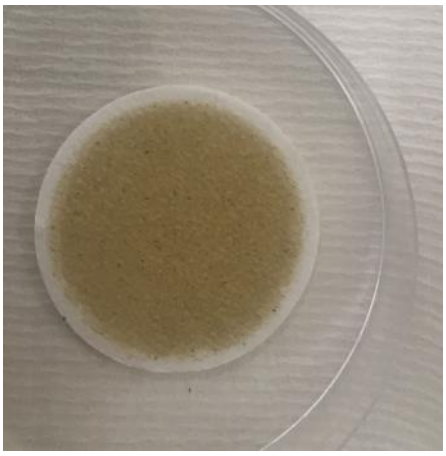
Although it was not feasible to culture the *Daphnia* in the various environmental water samples collected for a prolonged period, we can make comparisons between the macro-element concentrations and how this relates to their bioavailability both in natural waters and laboratory media. High variability in the availability of macro-elements available to all *Daphnia* was found in all the field samples analysed (S3.2 and S3.3)

The 15 sites used for the study were stratified across the land use classifications (urban, sub-urban and rural) and were selected based on accessibility, representation of land use and geographic spread to be able to build a water quality and pollution case study of the Birmingham area. During this sampling campaign *Daphnia* were found at all sites, in addition to the presence of MPs which were also found in the trawl samples at all sites. Coupled with the water chemistry data collected as part of the field campaign, the presence of large numbers of daphniids gives an indication of the overall water quality. In the study of water quality, the urban sites were deemed to have relatively good water quality compared to the sub-urban sites. This could be due to the fact that the urban ponds were well maintained to keep the aesthetic and the investment of the private owners and stakeholders compared to

some of the other sites. Due to the small area of ponds, it should be relatively simple to manage point or diffuse sources of pollution due to the land ownership compared to stream catchments.

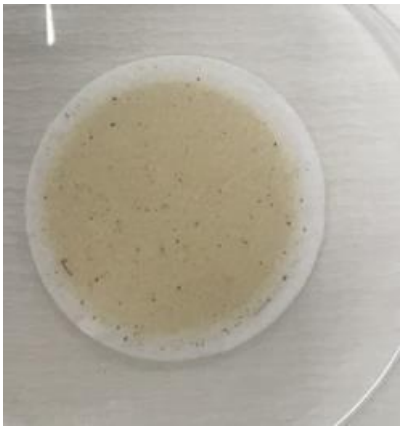
For diffuse sources, the percentage of impermeable surfaces surrounding the pond can have a significant effect on the quantity of pollution that enters the pond from the environment. This indicated that urban areas with more impermeable surface substrates pose a greater risk to ponds due to urban run-off compared to rural sites where this may be delayed due to infiltration. Increased urbanisation and changes to land use also means that the networks are often lost between different ponds, which decreases the species resilience due to loss of connectivity and ecological flow (Thornhill, 2013). This can then exasperate the other issues that impact pond health such as pollution and poor water quality including high nutrient content and low oxygen (typical of eutrophication). The highest variability in the samples was within the rural sites for the major element analysis. This could be due to the ponds ability to act as a representative reflection of local land use which can be highly variable in rural classifications. This is highlighted in Sites 1 and 2 which show the greatest difference across the whole dataset (with the exception of Boron) (A3.2)

Figure A3-2. Example filter papers from each of the 15 field sites.



Site 1

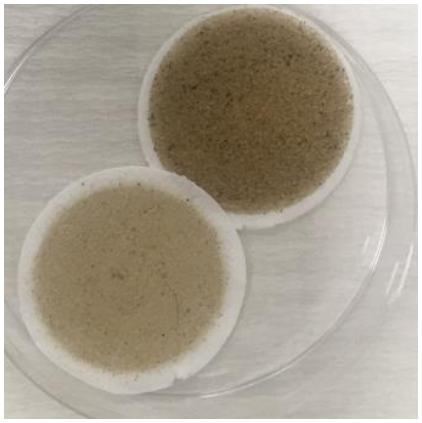
Site 2



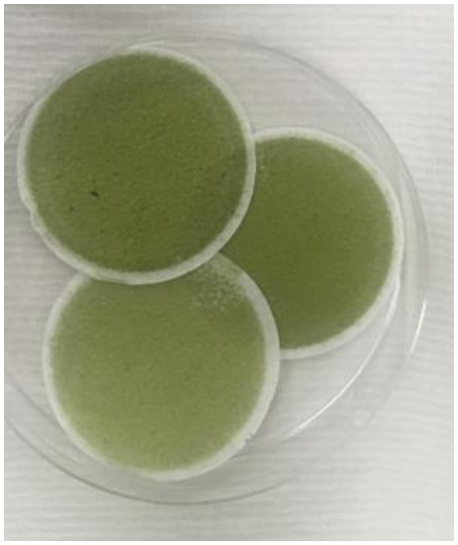
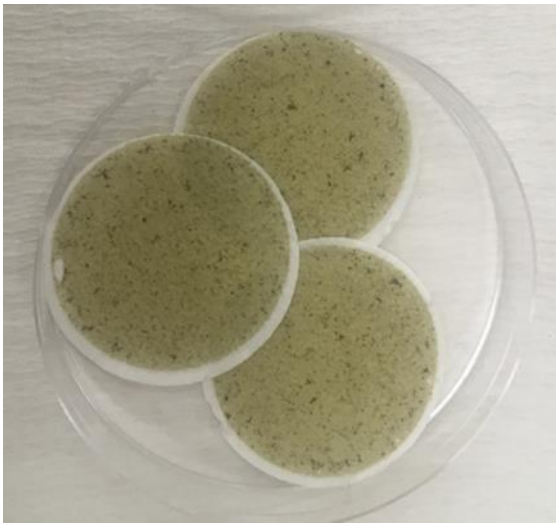
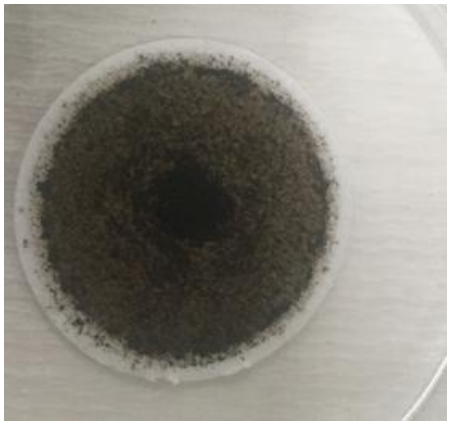
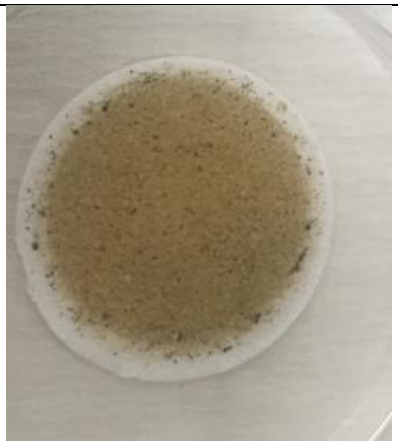
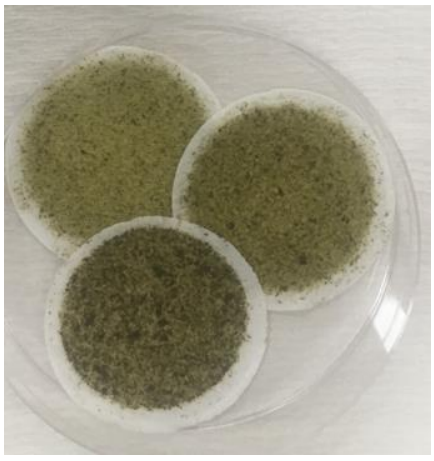
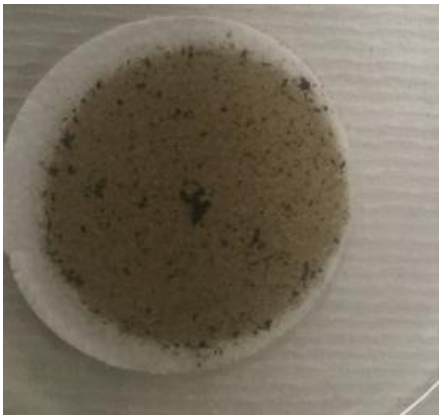
Site 3

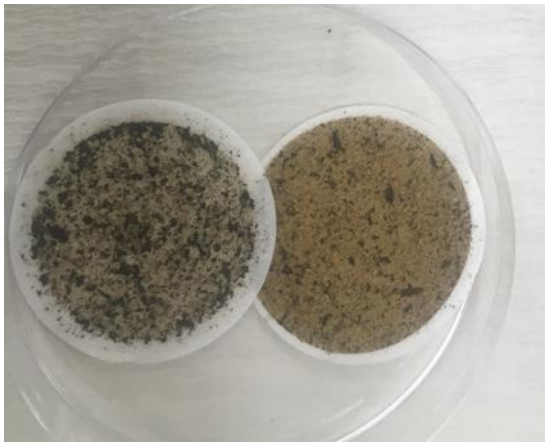
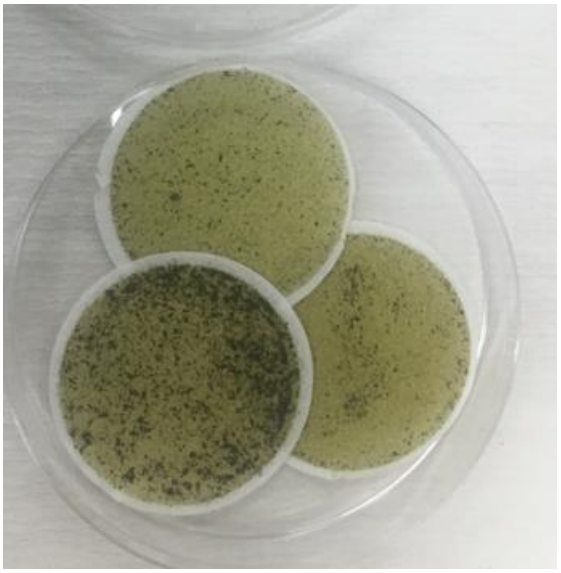
Site 4

Site 5



Site 6

 Site 7	 Site 8
 Site 9	 Site 10
 Site 11	 Site 12

 Site 13	 Site 14
 Site 15	

Environmental site data

Table S3.2. Summary table of the averaged field site parameter measurements

	Site number	Site name	Temperature (°C)	pH	Conductivity (ppm)	Conductivity (µS)	Dissolved oxygen (mg/L)	Dissolved oxygen (%)	Velocity (m/s)	Average SS (mg/L)
Rural	**1**	Oily Goughs	20.07	7.55	365.33	731.33	368.07	488.25	529.22	11.00
	2	Litchfield road	20.70	8.26	NV	NV	8.26	8.26	8.26	114.67
	3	Park Lime Pits	20.30	7.95	271.00	543.00	273.98	362.66	393.21	42.67
	4	Sheepwash Nature Reserve	21.07	8.95	776.00	1552.00	778.98	1035.66	1122.21	42.00
	5	Sandwell Priory	18.80	7.48	192.00	384.67	194.72	257.13	278.84	40.00
Suburban	**6**	Grove Park	21.43	7.44	235.33	470.67	237.81	314.60	341.03	45.33
	7	Victoria park	20.30	7.81	214.00	427.33	216.38	285.90	309.87	31.56
	8	The Vale	21.70	7.52	152.00	304.00	154.51	203.50	220.67	37.67
	9	Pelsall Cricket Club	16.47	7.62	220.00	441.67	223.10	294.92	319.89	48.33
	10	Red House Park	18.57	7.74	209.00	418.33	211.69	279.68	303.23	22.00
Urban	**11**	Moor Pool	20.83	8.78	178.00	356.67	181.15	238.60	258.81	51.33
	12	St Christopher Rd	17.70	7.57	224.67	443.00	225.08	297.58	321.89	25.00
	13	Vic Park Smethwick	21.20	8.38	193.33	386.67	196.13	258.71	280.50	30.67
	14	Bourneville School	19.33	7.43	172.00	343.33	174.26	229.86	249.15	70.67
	15	Edgbaston lake	18.40	7.89	246.00	491.33	248.41	328.58	356.11	41.56

Table S3.3 Summary table of the averaged major elements quantified by ICP-OES analysis of the environmental sites.

	Site number	Site name	Boron (mg/L)	Calcium (mg/L)	Magnesium (mg/L)	Phosphate (mg/L)	Potassium (mg/L)	Silicon (mg/L)	Sodium (mg/L)
Rural	**1**	Oily Goughs	1.162	6.081	3.622	3.622	4.442	3.895	3.986
	2	Litchfield road	0.944	57.805	29.375	29.375	38.852	32.534	33.587
	3	Park Lime Pits	0.728	21.697	11.212	11.212	14.707	12.377	12.766
	4	Sheepwash Nature Reserve	2.448	22.224	12.336	12.336	15.632	13.435	13.801
	5	Sandwell Priory	0.670	20.335	10.503	10.503	13.780	11.595	11.959
Suburban	**6**	Grove Park	0.650	22.992	11.821	11.821	15.544	13.062	13.476
	7	Victoria park	0.691	16.123	8.407	8.407	10.979	9.264	9.550
	8	The Vale	0.803	19.235	10.019	10.019	13.091	11.043	11.384
	9	Pelsall Cricket Club	0.602	24.468	12.535	12.535	16.513	13.861	14.303
	10	Red House Park	0.536	11.268	5.902	5.902	7.691	6.498	6.697
Urban	**11**	Moor Pool	0.700	26.017	13.358	13.358	17.578	14.765	15.234
	12	St Christopher Rd	0.608	12.804	6.706	6.706	8.739	7.384	7.609
	13	Vic Park Smethwick	0.580	15.623	8.102	8.102	10.609	8.938	9.216
	14	Bourneville School	0.516	35.592	18.054	18.054	23.900	20.003	20.652
	15	Edgbaston lake	0.663	21.109	10.886	10.886	14.294	12.022	12.401

Table S3.4- Location and depth information for the Birmingham University Great Hall Borehole array**.** Depths are in metres below ground level (mbgl). Elevation at the site of all three boreholes is approximately 125 m above Ordnance Datum (Bouch et al., 2006)

Borehole name (including BGS borehole identification number)	**Grid reference (BNG)**	**Depth (TD, m)**	**Depth to casing (mbgl)**	**Cored interval (mbgl)**	**Core recovery (m)**	**Optical Televiewer run interval (mbgl)**
Birmingham University 1 (Eastern Borehole; SP08SW 525)	[SP ^{4}04780 ^{2}83397]	50.15	12.35	7.40 – 50.15	40.94	12.35 – 48.40
Birmingham University 2 (Southwestern Borehole; SP08SW 526)	[SP ^{4}04760 ^{2}83397]	50.00	15.65	6.25 – 50.00	39.29	15.65 – 49.80
Birmingham University 3 (Northwestern Borehole; SP08SW 527)	[SP ^{4}04762 ^{2}83408]	50.00	12.30	6.40 – 49.94	39.74	12.30 – 48.90