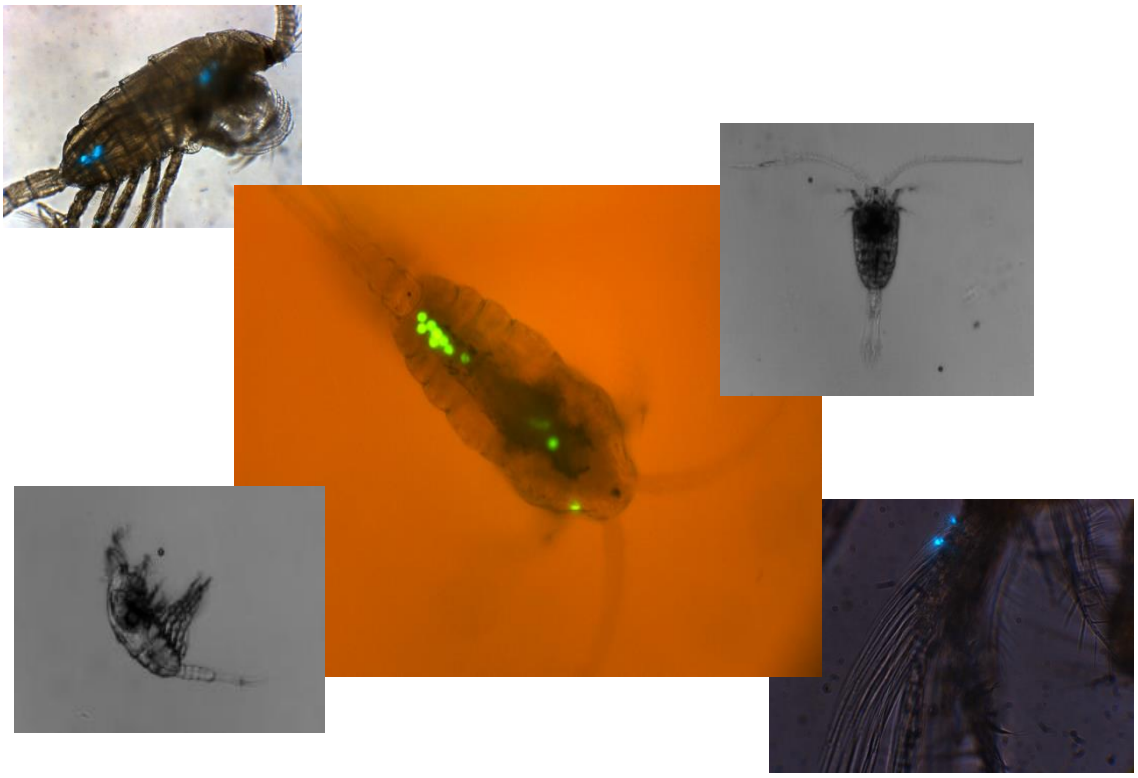
Investigating microplastic ingestion by zooplankton



Submitted by *Craig John Dedman*, to the University of Exeter as a thesis for the degree of *Masters by Research* in *Biosciences*, *November 2014*.

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Abstract

Microplastic pollution is a ubiquitous threat in the marine environment. The ingestion of microscopic debris (<5 mm) by marine zooplankton is a phenomenon of high ecological concern. This thesis presents new information regarding the ingestion of microplastics by marine zooplankton. The calanoid copepod, *Centropages typicus,* abundant in North Atlantic and Mediterranean coastal waters was found to ingest Polyamide-6 Nylon powder (μ = 30 μm), Polyethylene microbeads (μ = 20 μm) and artificial rope fibres (μ = 14.76 μm) that had been labelled with RADGLO fluorescent powder (475 nm) in the presence and absence of natural prey. Feeding experiments were conducted upon four zooplankton species; *Acartia tonsa*, *Porcellanid* larvae, *Calanus helgolandicus* and *Oithona similis*. Exposure to microplastic particles resulted in an energy deficit in all species with the exception of *O. similis* when feeding upon a natural assemblage of algae for 24 hours, though this was only statistically significant for *A. tonsa* exposed to a mixture of 10 μm and 20 μm Polystyrene spheres (100 particles mL^{-1}). Zooplankton displayed altered feeding behaviour. High-speed video analysis allowed for the mechanisms of microplastic detection, capture and subsequent rejection or ingestion to be observed. It was found that long-range chemodetection is unlikely to occur; rather cells are detected upon contact with setae. Individuals appear to reject microplastic particles in response to the physical properties of microplastic particles. These studies provide fundamental information on the ingestion and biological effects of microplastic debris upon zooplankton, knowledge of which is important given the key role that zooplankton play in the transfer of energy to higher trophic levels and, thus, ecosystem function. These findings provide pathways for further research and highlight the influence that feeding strategy and prey selectivity may have in determining the negative effects associated with microplastic uptake.

Acknowledgements

Particular thanks are given to Professor Tamara Galloway and Dr Matthew Cole for their continued support and guidance throughout the entirety of this research project. Gratitude is also shown to Elaine Fileman and Dr Pennie Lindeque for their support during research carried out at Plymouth Marine Laboratory. I would like to give thanks to Dr Thomas Kiørboe for his hospitality and opportunity to use facilities at Denmark Technical University, and to Dr Rodrigo Gonçalves for his expertise and assistance with high-speed filming. The use of facilities and materials at all centres of research carried out during this project is greatly appreciated, as well as, the advice and support of research technician staff, especially Darren Rowe of the University of Exeter.

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Chapter 1 – Introduction

What are microplastics? Their effects and occurrence in the marine environment

1.1 *Microplastics and their occurrence in the marine environment*

The release of anthropogenic waste into the marine environment is becoming an increasingly prominent concern, highlighted by the EU Marine Strategy Framework Directive (EC, 2008) as a key area of research in the fields of ecotoxicology and conservation. Although a relatively recent concern, past research carried out during the 1992-93 'British Steel Round the World Yacht Race' estimated that 6 billion kilograms of waste was dumped into the sea each year (Tait & Dipper, 1998). Plastics are recognised as the most common type of marine debris, constituting 60-80% of all marine waste and 90% of all floating particles (Gordon, 2006). There is a considerable global demand for plastic products, the annual production of plastics in 2012 stood at an estimated 288 million tonnes, representing a 2.8% increase upon the previous year (PlasticsEurope, 2013). It is believed that 10% of plastics manufactured are likely to end up in the marine environment (Thompson, 2006).

Of particular concern is the occurrence of small particles of plastic, termed "microplastics", in the ocean. Microplastics are small plastic fragments, varying in shape and size, less than 5 mm in diameter (Arthur, et al., 2009), which enter the marine environment in one of two ways. Microplastics that are manufactured to be of microscopic size, such as those used in air blasting or "microbeads" or "microexfoliates" in cosmetic products (Fendall & Sewell, 2009), are referred to as primary microplastics (Cole, et al., 2011). Secondary microplastics refer to microplastics that are produced by the degradation and breakdown of larger plastic debris (Cole, et al., 2011) by photo-degradation, oxidation and chemical abrasion (Andrady, 2003; Browne, et al., 2007). Such microplastics can enter the environment directly via run-off, or indirectly as a result of activities including fishing and shipping (Andrady, 2011). Degradation of plastics is defined as a chemical change that considerably decreases the average molecular weight of the polymer (Andrady, 2011), leading to the eventual break up of plastic material as it becomes brittle enough to fall apart. Typically there are four types of degradation that can occur within the marine environment, each categorised by the agency causing the degradation (Andrady, 2011);

- Biodegradation, the action of living organisms, primarily microbes;
- Photodegradation, the action of light;

- Thermooxidative degradation, the action of slow oxidative breakdown at moderate temperatures;
- Hydrolysis, the breakdown of polymers following reactions with water.

The primary mechanism of degradation occurring within the oceans is photodegradation, with the UV-B radiation in sunlight initiating the breakdown of polymers such as low-density polyethylene, high-density polyethylene, polypropylene and polyamides (Andrady, 2011). This initial breakdown then allows the action of thermooxidative degradation to occur, when oxygen is available, further degrading plastic material (Andrady, 2011). It is thought that if biodegradation does occur, however, it is several orders of magnitude slower than photodegradation (Andrady, 2011). Lobelle and Cunliffe (2011), however, found that over a 3 weeks investigation into the effects of microbial action upon microplastics no plastic-degrading microorganisms were present, suggesting that biodegradation may not be widespread. It was identified, though, that biofilms rapidly formed upon microscopic debris (Lobelle & Cunliffe, 2011). As a result the physiochemical properties of the plastic are altered, making particles more neutrally buoyant and changing their position within the water column (Lobelle & Cunliffe, 2011). Hydrolysis in seawater, like biodegradation, is not considered a significant mechanism of degradation of plastic (Andrady, 2011).

Microplastics have been accumulating in the world's oceans for over four decades (Thompson, et al., 2004; 2005) and are likely to continue to be of concern for future generations as certain polymers can take over 500 years to decompose (Gorman, 1993; UNESCO, 1994). Studies have attempted to gain an insight into the distribution of plastic debris across the global ocean. Cózar et al. (2014) provide a summary of findings on the abundance and distribution of plastic debris upon the sea surface (Figure 1). In this study, data from regional surveys, published reports and collected via the Malaspina 2010 circumnavigation was synthesised and used to produce a world map of floating plastic debris distribution (Cózar, et al., 2014).

Figure 1 Adapted from Cózar et al. (2014): Concentration of plastic debris in oceanic surface waters across the globe. The legend (top right) represents mass concentrations of plastics at each sample point. Average concentrations of 442 survey sites are provided (taken from 1127 net surface trawls). Zones where microplastics are predicted to accumulate are represented by grey shading, with dark grey indicating inner zones of accumulation, and light grey indicating outer zones of accumulation. White areas are predicted as areas unlikely to experience accumulation of plastic debris (Cózar, et al., 2014).

The highest densities of plastic litter occur in the convergence zones of the five subtropical gyres (see Figure 1), the most notable of which is the North Pacific central gyre, referred to as the “Great Pacific Garbage Patch”, first observed by the oceanographer Charles Moore. Plastics tend to collect in oceanic gyres due to the fact they are readily carried by ocean currents, and where these currents converge, plastics are deposited and collect in high densities (Maximenko, et al., 2012). The plastic abundance of coastal areas was lower than that of the open ocean. Other studies also indicate the lower abundance of plastic debris in coastal areas; studies upon the microplastic concentration of the North Western Mediterranean Sea found the highest concentrations of >0.36 particles/m^2 with particles ranging in the size of 0.3 mm-5 mm in shelf areas (Collignon, et al., 2012). Such concentrations are relatively low compared to those collected from the open ocean as displayed in Figure 1. Data collected during in this study was carried out relatively close to the coast, 90% of the 40 stations examined had plastics present (Collignon, et al., 2012). However, Cózar et al (2014) concluded that the occurrence of plastic pollution in the ocean was less than

expected and there appears to be an issue of missing plastic particularly in the size range below 200 μm. This issue may result from the fact that sampling particles <100 μm presents many technical difficulties (Hidalgo-Ruz, et al., 2012; Cole, 2014). Resolving the fate of missing plastic is of great importance and it is put forward by Cózar et al. (2014) that there are four possible sinks for floating plastic pollution; shore deposition, thought unlikely in the open ocean; nano-fragmentation by photodegradation and ocean forces; biofouling, increasing the density of plastic particles causing them to sink; and ingestion by zooplankton and other marine biota (Cózar, et al., 2014). In examining the plastic content of the stomachs of mesopelagic fish, Davidson and Asch (2011) reported that plastic occurrence was on the same order of magnitude as those found on the ocean surface (Cózar, et al., 2014). Therefore, although uncertainties still remain in the abundance and distribution of microplastics in the global ocean exist. The evidence available at this time suggests that microscopic plastic debris is a widespread and prolific contaminant, which is likely to increase in the future given the rate at which plastics are produced, their resistance to degradation and disposable nature (Thompson, et al., 2004).

1.2 *The effects of microplastics upon marine biota*

The effect of larger items of plastic debris upon marine biota has been widely studied (Laist, 1987; Derraik, 2002), with 267 species recorded to be affected by plastic pollution worldwide (Moore, 2008). For example, there is clear evidence for the obvious physical harm of plastics on marine organisms through direct contact. Due to the rigidity and complex structures of plastics many marine species can become entangled in plastic materials. The 2008 ICC Report, Ocean Conservancy, found that 443 animals and birds were found entangled in plastic debris within their study area. This is a conservational concern as it is often protected species such as turtles and dolphins that are affected. Damage to coral reef has also been recorded, as plastic due to its rigid structure comes into contact with coral, causing physical damage. A study carried out upon the Florida Keys reef shows significant damage by fishing equipment on the reef (Chiappone, et al., 2005). Damage to coral reefs is an environmental concern, due to the large biodiversity that relies on coral systems.

Less studied is the effect of smaller microplastic particles, discussed above, upon marine biota. Studies have found interactions between such particles and a range of marine organisms, the significance of which is highlighted by the fact that in such investigations microplastics were present in 94% of sampled seabirds (Lozano & Mouat, 2009) and 35% of sampled plankton-feeding fish (Boerger, et al., 2010), suggesting that interactions between the contaminant and animals is likely.

Due to their small size, microplastics are considered bioavailable to a large range of marine organisms (Cole, et al., 2011) and ingestion of microplastic particles has been recorded in a number of species, including; zooplankton (Cole et al, 2013), fish (Boerger, et al., 2010; Davison & Asch, 2011), seabirds (van Franeker, et al., 2011), decapod crustaceans (Murray & Cowie, 2011), mussels (Browne, et al., 2008) and amphipods, lugworms and barnacles (Thompson, et al., 2004). However, it is considered that it is those species at lower trophic levels that are most susceptible to microplastic ingestion (Wright, et al., 2013). Many of these species display limited selectivity of food particles and feed upon any particles that are of an appropriate size (Moore, 2008). The ingestion of microplastics has the potential to cause a number of adverse effects upon biota, as observed in laboratory studies. Setälä et al. (2014) studied zooplankton and found that ingested particles could either pass through the gut, or block and accumulate in the digestive tract of organisms, thus mechanically disturbing feeding and digestion. Differing gut retention times of microplastics have been found during laboratory studies. Experiments carried out by Cole et al. (2013) upon ingestion of microplastic by zooplankton saw gut retention times for polystyrene particles (7.3-30.6 μm in diameter) of up to 7 days, although the majority of particles were passed through the gut in a matter of hours (Cole, et al., 2013). Watts et al. (2014), examined gut retention of polystyrene particles by the shore crab, *Carcinus maenus*. Here, particles of 10 μm were retained in the gut for up to 14 days. In other cases microplastic particles were translocated from the gut to other internal organs of the organism. For example, Browne et al. (2008), found that microplastics could transfer from the gut of blue mussels (*Mytilus edulis*) into the circulatory system, where they remained for up to 48 days, although not having a significant biological effect upon the individual (Browne, et al., 2008). Increased gut

retention times, and persistence of microplastics within animal tissues, is a concern due to the fact that it may give a false sense of satiation and hence reduce feeding rates in organisms (Gregory, 2009), and increase the likelihood of chemically induced problems occurring (Setälä, et al., 2014), due to the leaching of additives such as plasticisers into organisms, many of which are considered to be toxic.

Following ingestion and retention of microplastic particles, the potential for trophic level transfer of microplastics occurs as a result of predation. This process has been demonstrated in several laboratory studies (Farrell & Nelson, 2013; Setälä, et al., 2014; Watts, et al., 2014). Farrell and Nelson (2013), exposed the mussel species, *Mytilus edulis*, to 0.5 µm fluorescent polystyrene spheres, and subsequently fed them to crabs. Tissue samples of the crabs was then analysed over 21 days, where spheres were detected in the stomach, hepatopancreas, ovary and gills (Farrell & Nelson, 2013). Evidence for translocation to the haemolymph was also present (Farrell & Nelson, 2013), however, spheres of a very small size were used (<3 µm), and it is not known whether translocation would occur with larger particles. The number of spheres was highest in the haemolymph after 24 hours. After 21 days of analysis the haemolymph was almost clear of spheres (Farrell & Nelson, 2013). This study clearly showed the trophic transfer of very small microplastic particles between two benthic species. Trophic transfer of microplastics has also been demonstrated in planktonic species; Setälä et al. (2014) exposed a range of planktonic species including mysid shrimp, copepods and polychaete larvae to 10 µm fluorescent polystyrene spheres. Ingestion was recorded in all taxa examined. Zooplankton that had ingested the spheres were then offered as prey to mysid shrimp and analysis via microscopy of the mysid intestine showed the presence of zooplankton prey and polystyrene spheres after 3 hours of feeding (Setälä, et al., 2014).

Other than ingestion, other means by which microplastic can interact with, and cause effects upon marine biota have been identified. Watts et al. (2014) examined the routes of entry of 8-10 µm fluorescent polystyrene spheres into the shore crab, *Carcinus maenus*. It was shown in this study that microplastics could be taken up by the crab via inspiration across the gills, as well as through ingesting previously exposed food, in this case the mussel, *Mytilus edulis*

(Watts, et al., 2014). The spheres showed retention times of up to 14 days in the gut following ingestion, and up to 21 days following inspiration (Watts, et al., 2014), once again displaying the issue of microplastic retention in organisms. The findings showed that benthic species such as crabs could be at risk of plastic exposure via inspiration, and also displays another example of trophic level transfer of microplastic particles, a phenomenon which is likely common in the marine environment. Additional research is required to confirm this in higher trophic levels.

The transfer of adhered pollutants to wildlife via microplastic particles is a process of great interest, and ecological concern. Microplastics readily adsorb waterborne pollutants such as persistent organic pollutants (POPs). Due to the large surface area-to-volume ratio of microplastics, it is thought that if ingested, marine biota may be exposed to leached additives, which could interfere with important biological processes, including; reproduction, development and carcinogenesis (Barnes, et al., 2009). Studies have suggested that small plastic particles exposed to organic pollutants have the potential to pass on these substances to organisms, potentially resulting in a toxic effect. Seabird chicks were fed a diet of fish or fish and resin pellets both containing polychlorinated biphenyls (PCBs), which are highly persistent and hydrophobic contaminants widely found in the marine environment. After 42 days, preen gland oil was extracted from the birds and analysed. Results showed that in both groups, PCB concentrations increased (Teuten, et al., 2009). However, to determine the uptake of PCBs from the resin pellets alone, a different form of PCB was added in a higher concentration to the resin pellet. Following a repeat experiment, chicks eating the fish alone showed no change in PCB uptake, whereas the chicks being fed the resin pellets had significantly increased levels of the PCB present (Teuten, et al., 2009). The significance of this study was to prove that by ingesting microplastics it was very likely that marine organisms would be subjected to the effects of the chemicals present in the plastic, highlighting the potential for such chemicals to bioaccumulate through the food chain.

1.3 *Microplastics and zooplankton*

The zooplankton consists of a group of free-floating heterotrophic animals which inhabit the world's aquatic environments. By living in this manner zooplankton, particularly the holoplankton which spend their entire life cycle as plankton, are

exposed to any contaminant present within the water column. The density of plastic debris in the sea appears high in the sea surface (Cózar, et al., 2014), where zooplankton are also potentially in their highest abundances. In over 60% of 6136 surface plankton net tows, carried out upon the surface of the western North Atlantic Ocean and Caribbean Sea from 1986 to 2008, plastic particles of microscopic size were identified (Law, et al., 2010). It can be assumed then that it is feasible that interactions between zooplankton and marine debris such as microplastics are likely to occur. The effects of such interactions are of great interest and high importance to examine, given that zooplankton provide a key transfer of energy to higher trophic levels, and if contaminated may pass accumulative pollutants to higher trophic levels. There are, therefore, great economic and human health interests in the ingestion of microplastic particles by zooplankton, as it has the potential to affect the quality and safety of commercial fishery products, and the health of the natural ecosystem.

1.4 *Aims*

This thesis aims to explore the ingestion of microplastics of different types by a range of zooplankton species.

In Chapter 2, we aim to investigate whether different microplastic types, for example polystyrene spheres and nylon fibres both common in the marine environment, will be ingested by zooplankton at different rates.

It is hypothesised, here, that the feeding strategy demonstrated by a particular zooplankton species, will influence the extent to which ingestion of microplastics affects feeding behaviour. This hypothesis will be investigated in Chapter 3. This is examined using different zooplankton species exposed to a mixture of natural prey and microplastic particles.

In Chapter 4, the current research suggesting that zooplankton can accept or reject microplastic particles prior to their ingestion, will be tested. This hypothesis is investigated by utilising high-speed video recording. Such experimentation also allows for a more detailed analysis of how zooplankton handle microplastic particles.

It is anticipated that this comprehensive study will provide fundamental scientific knowledge to allow for further advancements in the field of microplastic

ingestion by zooplankton, a phenomenon which has crucial implications in terms of energy transfer for the marine ecosystem as a whole.

Chapter 2

Microplastic ingestion by zooplankton

The aims of this thesis, outlined in Chapter 1, focus upon the effects that microscopic plastic debris present in the marine environment may have upon zooplankton. However, ingestion of plastic particles by zooplankton, in particular copepods, has been recorded in laboratory studies since the early 1970's. This earlier work mainly aimed to observe feeding mechanisms and prey selection in study species, where polystyrene particles were often used to represent prey of differing size or nutritional value. Examples of such work can be found in papers by Frost et al., and Donaghay and Small, (Frost, 1972, 1977; Donaghay & Small, 1979).

In recent years, following increased awareness of microscopic debris occurring within the marine environment, studies have been carried out to assess the potential for microplastic particles to enter and pass through the food chain. Subsequently, effects of such ingestion upon the individual had been assessed.

In experiments carried out upon a range of zooplankton by Cole et al. (2013), ingestion of microplastics (2-30 μm diameter) was found in 14 out of 16 taxa. In Cole's study it was demonstrated that exposure to 7.3 μm polystyrene beads significantly reduced the feeding rate of algae in the copepod *Centropages typicus*. Ingestion of microplastics was not seen in species that display raptoral predation (Kiørboe, 2011), which feed actively by grasping mobile prey and did not proceed to capture the immobile microplastic particles (Cole, et al., 2013). Microplastics were found in Cole's study to adhere to external appendages with the potential to reduce the fitness of the organism by impacting upon prey detection, feeding, mating and predator avoidance (Cole, et al., 2013).

Microplastic ingestion by zooplankton has also been recorded by Setälä et al. (2014) in a range of taxa including; mysid shrimp, copepods, polychaete larvae and rotifers. In this study 10 μm fluorescent polystyrene microspheres were actively ingested by animals, a similar size range as studied by Cole et al. (2013). Ingestion of smaller particles (0.05-6 μm) has also been recorded in the copepod species *Tigriopes japonicas* (Lee, et al., 2013) suggesting that microplastic particles in a wide size range (~0.05-30 μm) are bioavailable to zooplankton species.

2.1 *Bioavailability of microplastics*

As described in Chapter 1, microplastics commonly occur in the upper water column (Law, et al., 2010), where zooplankton are also in their highest abundances. Indeed plastic debris has been recorded in a number of surface plankton samples across the globe, for example 59% of 203 samples carried out in waters between the Japan and Bering Seas (Day, et al., 1989) and 62% of 247 samples from Cape Cod to the Caribbean (Colton, et al., 1974) contained plastic material. Since microplastics are predicted to be carried along ocean currents in a similar manner to zooplankton, interactions are likely.

The small size of microplastics means that they are biologically available to a wide range of marine organisms (Cole, et al., 2011), particularly those occupying lower trophic levels (Wright, et al., 2013) and under experimental conditions this has been proven. It has been shown that as plastic fragments into smaller particles, the chances of ingestion by marine biota may increase. For example particles of 3 μm were more readily ingested by bivalve molluscs than 9 μm particles of polystyrene (Browne, et al., 2007). In addition to size, several other factors have been identified as influential in determining the bioavailabilty of microplastic particles within marine environments. Wright et al. (2013) addresses each of these factors individually, and here I will summarise the key findings from this work.

Size is the first and most obvious factor that will determine a particle's bioavailability, as a small size of an item increases its availability to a number of organisms across a larger range of trophic levels (Wright, et al., 2013). It is thought that many lower trophic level species display limited selectivity between potential food particles and subsequently capture anything within an appropriate size range (Moore, 2008). As a result if a particle falls within a set size boundary it will be ingested. The small size of microplastics may also cause passive ingestion through normal feeding by higher trophic level organisms (Wright, et al., 2013). Such passive ingestion is thought to occur in a number of cetacean species, for example the fin whale (Fossi, et al., 2012).

The second factor identified as playing a part in determining a microplastic particle's bioavailability is density. Density of particles will determine the position of a particle within the water column (Wright, et al., 2013) and therefore

determine which species are likely to encounter such items. Due to the similarity between the density of microplastic particles and algae, microplastics have the potential to be prey alternatives for planktivores and as a result may be captured and ingested in a similar manner (Brillant & MacDonald, 2000).

Abundance of a particular microplastic type will also play a role in the determination of bioavailability of a microplastic particle. With increased abundance of particles the likelihood of encounters between biota and microplastic will be increased, therefore increasing the potential for ingestion (Wright, et al., 2013).

The final characteristic of microplastic particles addressed by Wright et al. (2013) is colour. It is thought that colour may increase the likelihood of ingestion if the microplastic resembles natural prey (Wright, et al., 2013).This would rely on the organism's ability to detect and recognise colour. Some commercially important fish and their larvae displaying visual predation may ingest microplastics in this manner, as they resemble their natural zooplankton prey (Wright, et al., 2013). Studies have found that the most common particles reported in monitoring studies are transparent, making up 49% of sampled particles, followed by white (25.5%), blue (16.9%) and black/grey (5.2%) (Shaw & Day, 1994).

Although it is likely that the four factors described above are the main determinants of a microplastic particle's bioavailability in the marine environment, there are other factors that must be considered including; shape and surface characteristics, surface charge and degree of degradation or biofouling.

2.2 *The effects of microplastic ingestion by zooplankton*

Previous studies have concluded that exposure to microplastics can significantly impact upon the health and fitness of copepods (Cole, 2014).

Following ingestion of microplastic the initial effects upon the organism are likely to occur in the digestive tract, or gut, of the animal. In ingestion studies examining the uptake of polystyrene spheres by copepods, particles had the potential to be retained in the gut for up to 7 days (Cole, et al., 2013). However, in the same study generally microplastics were egested in a number of hours, at a rate similar to that of natural prey (Cole, et al., 2013). Thus egestion may

provide a potential source of secondary uptake via coprophagy of faecal pellets by other zooplankton or marine species. The retention of microplastics has the potential to cause physical harm to the individual. Clumping or knotting with other plastic particles or algal filaments was reported in the decapod crustacean, *Nephrops norvegicus* (Murray & Cowie, 2011). Such gut retention and blockages may negatively affect the manner in which zooplankton species ingest and subsequently digest food, and potentially may increase the likelihood of chemical effects being endured by the individual.

Exposures using 7.3 μm polystyrene spheres carried out by Cole et al. (2013) revealed that the presence of microplastics could reduce the ingestion of algal cells. This was a dose-response relationship and a concentration of 4000 microplastics (MP) mL^{-1} significantly reduced algal ingestion (Cole, et al., 2013). Such a reduction in feeding of natural prey has the potential to have negative consequences for the individual by limiting energy uptake (Cole, et al., 2013). This problem may be exacerbated in species that have low lipid reserves. Whose limited energy uptake is likely to lead to increased mortality, and decreased fecundity and growth (Ayukai, 1987). Additional studies investigating the effects of microplastic upon copepod feeding were carried out upon the well-studied calanoid copepod, *Calanus helgolandicus* (Cole, 2014), a common species found throughout Europe and the North East Atlantic (Bonnet, et al., 2005). Here, using 20 μm polystyrene spheres 75 MP mL^{-1}, the ingestion rate of the copepod was significantly reduced; with a decreased carbon uptake and a shift in ingested algal cell size (Cole, 2014).

However, such negative effects of microplastic exposure were not identified during a study carried out upon the marine larvae of the sea urchin, *Tripneustes gratilla* (Kaposi, et al., 2014). Here, the larvae were found to ingest polyethylene microspheres in a dose-related relationship, however, no significant effect upon survival was identified (Kaposi, et al., 2014). Kaposi et al. (2014) argued that environmentally relevant concentrations of microplastics appeared to have little effect upon the planktonic larvae stage of this species. Ingestion rates were decreased following biofouling of the microplastic particles (Kaposi, et al., 2014). Such biofouling increased the size of particles and caused aggregates to form, thus, reducing the attractiveness of particles to larvae. This may account for the reduction in uptake displayed in this study (Kaposi, et al., 2014).

Lee et al. (2013) examined the effects of exposure on the copepod *Tigriopes japonicas.* Here, animals were exposed to a range of polystyrene spheres, sized 0.05 μm, 0.5 μm and 6 μm (Lee, et al., 2013). All sizes of spheres were ingested and no mortality was suffered by adult copepods (Lee, et al., 2013). However, nauplii of the species suffered increased mortality when exposed to 0.05 μm and 6 μm particles in the F_0 generation, and 0.5 μm spheres resulted in significant reductions in survival in the F_1 generation (Lee, et al., 2013). Such findings suggest that negative impacts upon juvenile copepod stages may result following exposure to microplastics such as polystyrene beads (Lee, et al., 2013). The lack of negative effects occurring to adult copepods in this study may result from the fact that study plastics were <6 μm in size and may not have been detected significantly, or if ingested may have been egested at a higher rate than larger particle sizes.

Imaging of individuals following exposure to microplastics reveals the occurrence of adherence of particles to the feeding appendages and swimming legs of copepods (Cole, et al., 2013). The adherence of particles is likely to adversely affect an individual due to the role external appendages play in swimming and feeding behaviour (Cole, et al., 2013).

2.3 Investigating the ingestion of different plastic types by *Centropages typicus*

It is clear that microplastic ingestion under laboratory conditions is widespread across a number of species (Cole, et al., 2013; Lee, et al., 2013; Kaposi, et al., 2014; Setälä, et al., 2014). However, to date, it is mainly commercially produced polystyrene spheres, uniform in size and shape that have been offered to study organisms in ingestion studies. Such particles are very useful in experimentation due to their ease of use and effectiveness in quantifying uptake through measurements carried out by multisizer equipment. Polystyrene particles, therefore, can act as an effective way of quantifying uptake of microplastics under different scenarios, and present a relatively labour-free process to examine ingestion by a particular species. However, particles used commonly in research do not effectively match those microplastic particles that wild zooplankton are likely to come into contact with within the marine environment.

A large range of plastic types are regularly deposited into the marine environment, the most common of which include; polyethylene, polystyrene and polypropylene. Studies carried out by Sadri and Thompson (2014) upon the Tamar Estuary, UK, found the listed plastics to constitute 40%, 25% and 19%, respectively, of the total particles identified (Sadri & Thompson, 2014). Such plastics degrade differently under marine conditions, and therefore will produce microplastics on different scales, and in differing forms, such as; fibres, spherical and non-uniform shapes. Such microplastics, as described in Chapter 1 enter the marine environment via two routes; primary microplastics are produced to be of small size for their initial use and enter the ocean via run-off (Andrady, 2011), whereas secondary plastics are larger pieces of debris that have degraded to a microscopic size following degradation within the ocean (Andrady, 2011). It is likely, therefore, that zooplankton species will react differently to the various microplastic types that they are likely to come into contact with, hence, making some particles more likely to be ingested than others.

Investigating the ingestion of various microplastic types in a zooplankton species, which can effectively represent the likely behaviour of wild zooplankton, therefore is a high priority. *Centropages typicus* is a copepod species which is well-studied, displaying behaviour that is shared with other copepod species. The study outlined below aimed to investigate whether *C. typicus* ingested a range of microplastic types, representing the key plastics likely to be bioavailable to organisms within the marine environment; primary microplastics, secondary microplastics and fibrous microplastics.

2.3.1 Methods

2.3.1.1 *Preparing plastics for exposure*

In order to examine the ingestion of different microplastic types by zooplankton, a number of test plastics had to be identified. In order to attain a comprehensive overview of microplastic ingestion, it was decided to study a primary microplastic, secondary microplastic and microfibre, all thought of as common in the natural environment. The plastics selected for investigation are outlined in Table 2.1.

Table 2.1 Microplastics used for investigation.

Microplastic Type	Polymer	Source	Form	Size
Positive Control	Polystyrene	Yellow Fluorescent Polystyrene Spheres (Spherotech)	Microsphere	20 µm
Primary Microplastic	Polyethylene	Exfoliating Face Wash (Clearasil)	Microbead	Mean – 30 µm Range - 8.77-127.34 µm
Secondary Microplastic	Polyamide-6 Nylon	Polyamide-6 Nylon Powder	Powder	Mean – 20 µm Range - 8.83-123.42 µm
Fibrous Microplastic	Nylon	Artificial rope	Fibres	Mean – 14.76 µm Range - 8.58-134.56 µm

All test plastics were fluorescently labelled using RADGLO powder, as described below, to allow for imaging of the potential ingestion of microplastics. In order to dye the microplastics, it was essential to ensure the particles were of a sufficient small size to be used for experimentation. The Polyamide-6 Nylon Powder was kindly provided by Dr Bert van Bavel, University of Orebro, Sweden. It had an average size of 15-20 µm, range 5-50 µm. Polyamide-6 Nylon was used to represent secondary microplastic in this study by giving an example of a material that was present in a powder form, such as a larger piece of debris that had been degraded within the marine environment. Polyethylene (PE) microbeads were extracted from a common household face wash produced by Clearasil™. The face wash was passed through a 30 µm mesh and all soap removed to leave only the microbeads, which were subsequently rinsed with 0.2 µm filtered seawater (FSW). Artificial rope was provided by the University of Exeter and was cut into small pieces using laboratory scissors. The exact size of extracted microbeads and microfibres was unknown, therefore, to ensure beads and powder were of a sufficiently small size each was added to a mortar and using snap-freezing by liquid nitrogen, particles

were ground to decrease their size. The subsequent particles were then weighed and using the protocol set-out by (Lindegarth & Jonsson, 1991) particles were dyed with RADGLO Radiant Color™ powder. RADGLO was added to the ground particles in the ratio of 20:1 (dry weight) and mixed in air thoroughly. The RADGLO powder and particle mixture was then transferred to a fume hood where sufficient acetone (approximately 50 mL) was added to cover the entire mixture and left to evaporate overnight to complete the dying process. The resultant mass was then re-homogenised using a pestle and mortar and passed through a 65 µm mesh to remove any larger particles. The labelled particles were then suspended in 0.2 µm FSW and retained in a foil covered bottle to prevent any degradation to fluorescence from exposure to light. In order to ensure microfibre particles remained well mixed and to prevent aggregation, 50 µL Tween 20 (0.001% v/v), known not to have a toxic effect (Lindegarth & Jonsson, 1991) was added to the microfibre suspension.

Stock cultures were then diluted using 0.2 µm FSW and passed through a 10 µm mesh to size fraction particles and remove any unattached RADGLO powder that may alter results. To ensure stock cultures were well mixed, suspensions were sonicated for 2 minutes. The subsequent stock was passed though FlowCAM to measure the concentration of plastics present within the stock as well as the average size and size range (see Table 2.1), in order to prepare microplastics for study. The volumes of microplastic stocks required to be added to seawater was calculated using the equation of $V_1 \times C_1 = V_2 \times V_2$, to produce a test concentration of 100 MP mL^{-1}.

2.3.1.2 *Copepod sampling*

Copepods were collected from Station L4 (50° 15.00' N, 4° 13.02' W) (see Figure 2.1), 12 km South of Plymouth, by the Plymouth Quest Research Vessel, operated by Plymouth Marine Laboratory (PML). A 200 µm net was used to sample zooplankton via vertical tows and samples were transferred to bottles

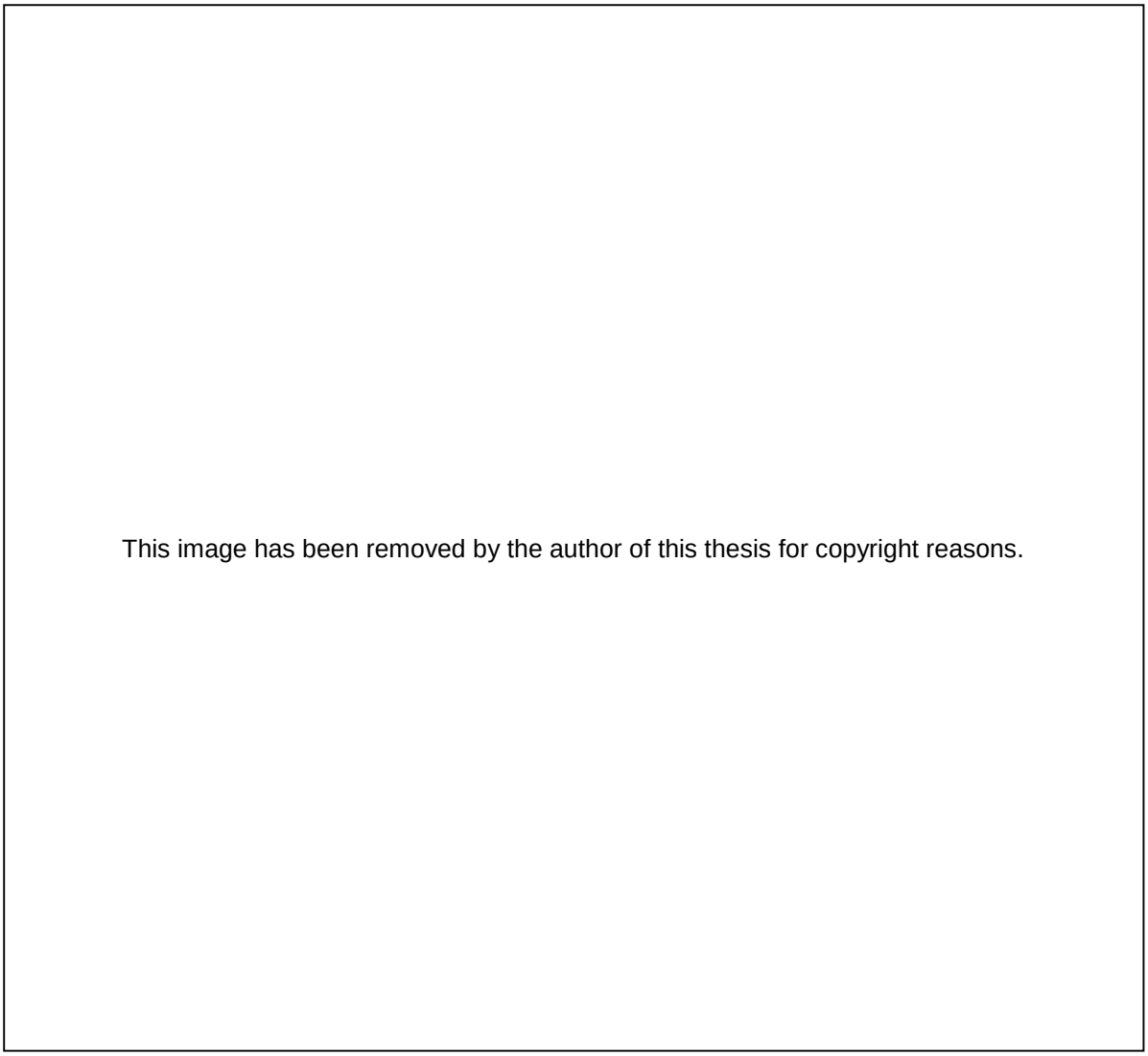

Figure 2.1. Station L4 (50° 15.00' N, 4° 13.02' W), indicated by the red point. Located 12 km South of Plymouth. Image adapted from Google Maps™.

and transported within insulated boxes to PML. Once returned to the laboratory zooplankton were assessed in terms of abundance using a WILD M5-48084 optical microscope, and the calanoid copepod *C. typicus* was chosen as the subject of this study due to its abundance and owing to the fact that *C. typicus* has been recorded in previous studies as ingesting microplastic (Cole, et al., 2013). Adult female *C. typicus* were picked out and transferred to an aerated 5L beaker filled with 0.2 µm FSW and conditioned without food overnight at ambient sea temperature (~17°C).

2.3.1.3 *Natural seawater*

In order to replicate natural conditions, natural seawater containing a natural assemblage of phytoplankton was used to examine uptake of microplastics in the presence of natural prey. Seawater was collected alongside zooplankton samples at Station L4 by the Plymouth Quest Research Vessel at a depth of 10m. Samples were then returned to PML and stored at ambient sea temperature (~17°C). Seawater was passed through a 100 µm mesh before experimental set-up to ensure removal of any microzooplankton which may have altered findings of the investigation.

2.3.1.4 *Experimental set-up*

Individual females of *C. typicus* were added to 35 mL glass bottles using stork-billed forceps. Bottles had been treated with either 0.2 µm FSW or natural seawater, spiked with the corresponding microplastic type to produce a test concentration of 100 MP mL^{-1}. A negative control was also studied, where no microplastic was added to seawater samples. Five replicates of all treatments; negative control, positive control, primary microplastic, secondary microplastic and fibrous microplastic; were set-up and fixed to a plankton wheel, to ensure suspensions remained well mixed, rotating at <5 rpm. Therefore, for each plastic type, five individual females of *C. typicus* were exposed to microplastic particles in the presence and absence of algal prey. Exposures were carried out for 24 hours in darkness at ambient sea temperature (~17°C) in order to best replicate natural conditions and prevent growth of algal cells. Replicates were carried out to ensure reliability of data and provide grounds for statistical analysis.

2.3.1.5 *Assessing the ingestion of microplastic types by C. typicus*

Following exposure samples were passed into a 50 µm mesh and washed into a sample tube using 0.2 µm filtered FSW. Formalin (4%) was then added to the sample tube to preserve specimens for imaging.

Individuals were placed on slides and imaged using a TBM1000 microscope with Prior V31LD4 fluorescent emission attachment and QIClick™ camera. A fluorescence wavelength of 475 nm was used and camera settings set to optimise the view of fluorescence, such that fluorescence appeared as blue on

images. All individuals were imaged, and all examples of ingestion and adherence of microplastic particles were recorded.

2.3.2 Results

2.3.2.1 *Ingestion of microplastics*

Figure 2.2 displays the percentage of individuals that were recorded to ingest microplastic particles during this experiment. As can be seen, microplastic particles were present in all treated copepod samples with all test microplastic types, both in the presence and absence of natural prey. Ingestion was recorded in the positive control, as expected. In the absence of natural prey, 80% individuals were recorded to have ingested PE microbeads, representing Primary Microplastic, an example of which is displayed in Image A, Figure 2.3. When offered microbeads alongside natural prey 100% individuals exposed to the plastic recorded ingestion, see Image A, Figure 2.4 for an example. Microbeads were visible in the upper and lower digestive tract and appear to have aggregated. Ingestion of Polyamide-6 Nylon powder particles were recorded in all individuals exposed to particles (see Image B, Figure 2.3 and 2.4), both in the presence and the absence of natural prey. However, the extent of ingestion does not appear as pronounced as with microbeads upon examining the intensity of fluorescence. As with microbeads, powder particles are visible in the upper and lower digestive tract, however they do not appear to have aggregated in the same manner as microbead particles. In the absence of natural prey, 40% individuals were recorded to have ingested microfibre particles, whereas, when algal cells were available, 100% individuals ingested particles. Upon studying images where phytoplankton was present in comparison to when no prey was available (see Image C, Figures 2.3 and 2.4) it appears that the ingestion of microfibres is considerably higher when natural prey in present. As with the other plastic types, particles were visible in the upper and lower digestive tract, and similarly to microbeads appear to have aggregated.

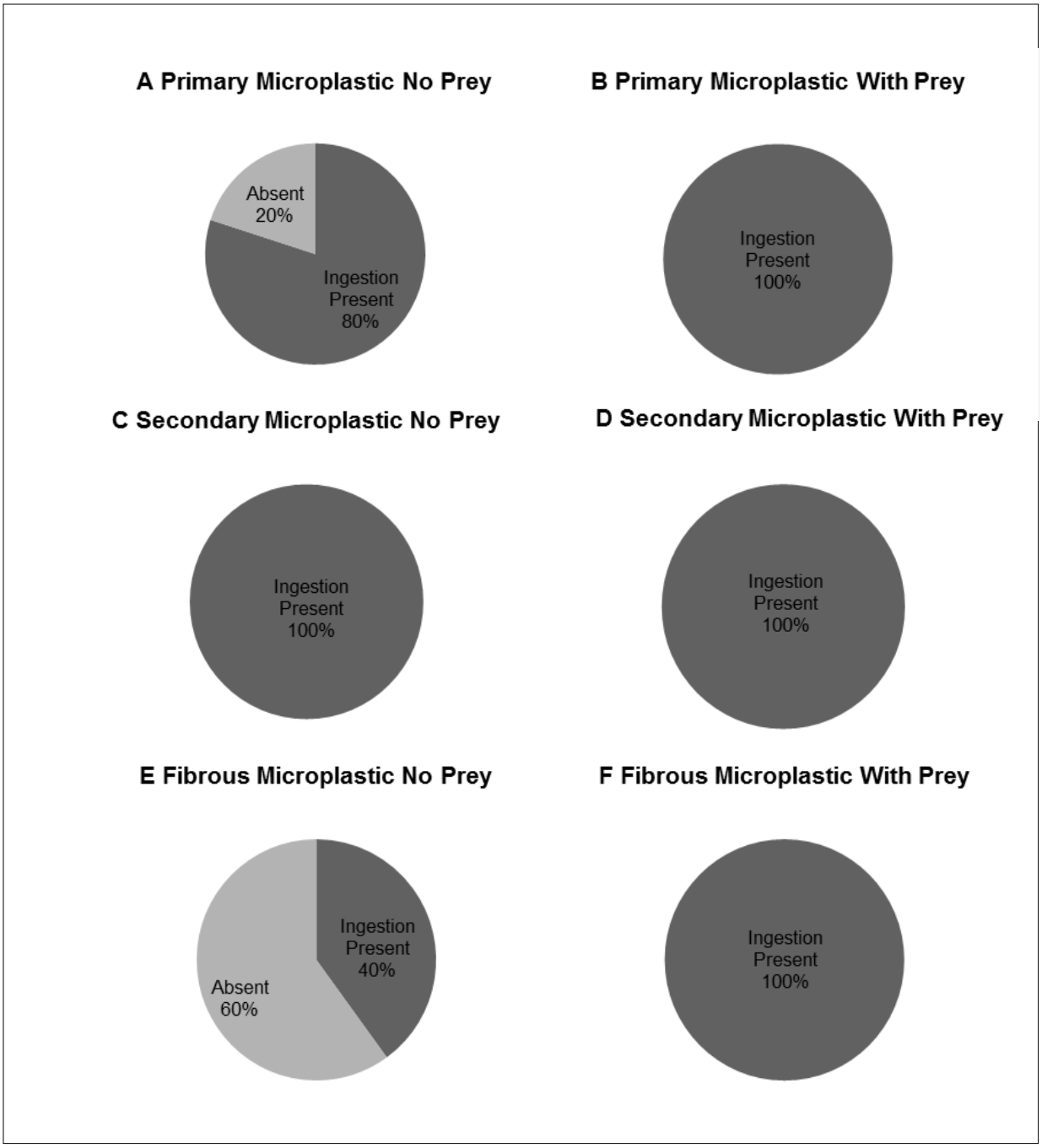

Figure 2.1 Ingestion of microplastics by *C. typicus*. Pie charts displaying the percentage of individuals recorded as ingesting microplastic particles when exposed to different plastic types in the concentration of 100 MP mL^{-1}. N = 5 for all treatments, except for D - Secondary Microplastic with prey (n = 4). 100% ingestion indicates microplastic particles present in all 5 individuals exposed to each treatment.

2.3.2.2 *Investigating the ingestion of different microplastic types in the absence of natural prey*

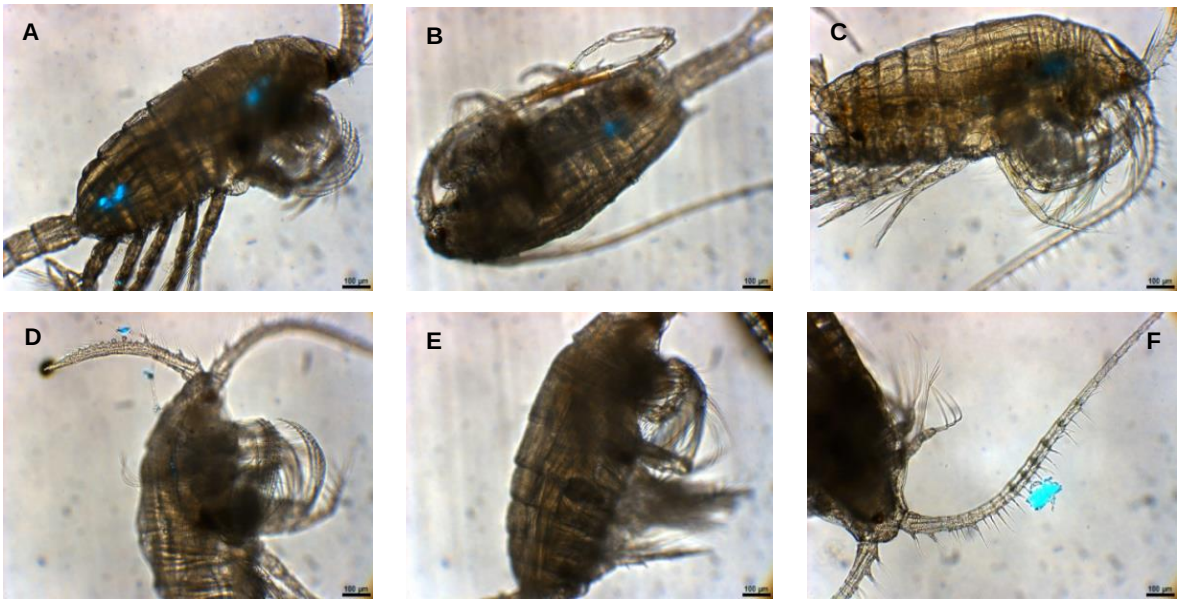

Figure 2.3 Example images of ingestion and adherence of RADGLO labelled microplastic particles in the absence of natural prey by *C. typicus*. A – Ingestion of PE microbeads. B – Ingestion of Polyamide-6 Nylon powder, visible in the lower digestive tract. C – Ingestion of microfibres, present in the upper digestive tract. D – Ingestion of PE microbeads in the upper digestive tract and adherence of particles to the antennae. E – Adherence of Polyamide-6 Nylon powder to the feeding appendages of *C. typicus*. F – Adherence of a microfibre to the antennae.

2.3.2.3 *Investigating the ingestion of different microplastic types in the presence of natural prey*

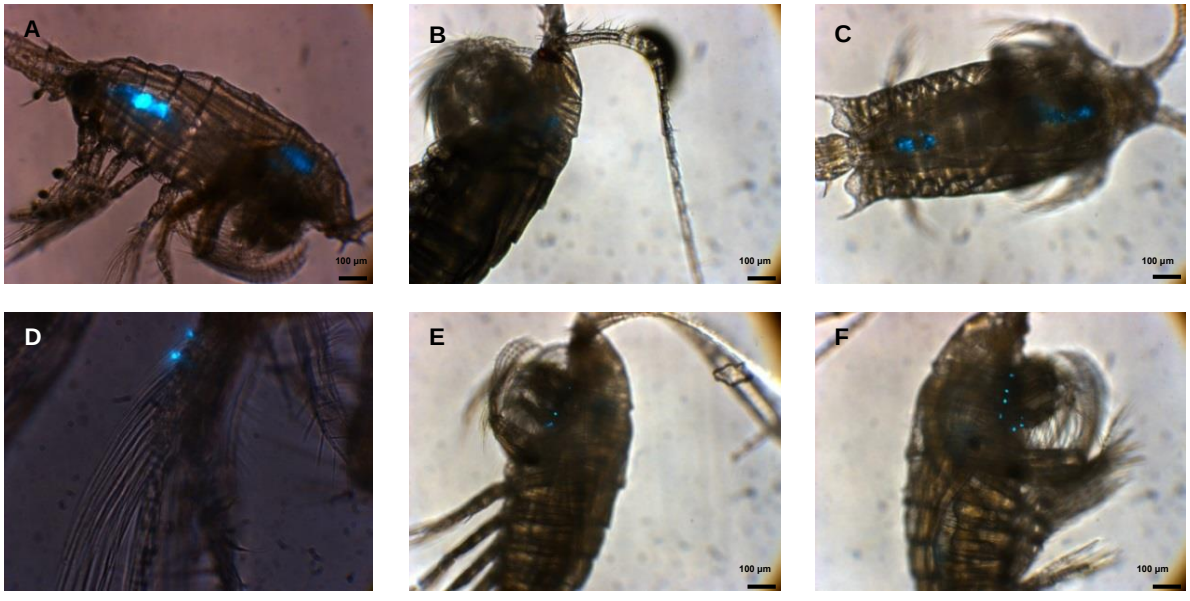

Figure 2.4 Ingestion and adherence of RADGLO labelled microplastic particles in the presence of natural prey by *C. typicus*. A – Ingestion of PE microbeads, present in the upper and lower digestive tract. B – Ingestion of Polyamide-6 Nylon powder, visible in the upper digestive tract. C – Ingestion of microfibres, as seen in the upper and lower digestive tract. D – Adherence of PE microbeads to the feeding appendages, image taken at 400x magnification. E – Adherence of Polyamide-6 Nylon powder to the feeding appendages. F – Adherence of microfibres to the feeding appendages.

2.3.2.4 *Adherence of microplastics*

Figure 2.5 shows that in the presence and absence of natural prey, adherence of microbead particles was visible on individuals exposed to all test plastic types. However, adherence was not observed in the positive control in the absence of prey. Adherence of microbeads, was present on all individuals exposed to the beads, which adhered primarily to the feeding appendages, see Image D, Figure 2.4, but also to the swimming legs, urosome and antennae (Image D, Figure 2.3) . Eighty per cent of the individuals exposed to powder particles had particles adhered to them in the presence of phytoplankton, whereas 100% individuals showed evidence of adherence in the absence of algal prey. Powder particles adhered to multiple body parts, primarily the feeding appendages (see Image E, Figures 2.3 and 2.4) and swimming legs, as well as, the urosome and head of the copepod. In both the absence and presence of natural prey 60% individuals showed evidence of adherence of microfibre particles. Microfibre particles were visible adhered to the feeding appendages (Image F, Figure 2.4), swimming legs, urosome and antennae (Image F, Figure 2.3). The full set of images used for analysis along with a comprehensive summary of each image is provided in Appendix 1. The adherence of microfibre particles appears less common that that of microbeads and powder particles. Appendix 1 provides all images used to assess ingestion and adherence of particles. Tables 7.1.1 and 7.1.2 summarise the data collected from each image.

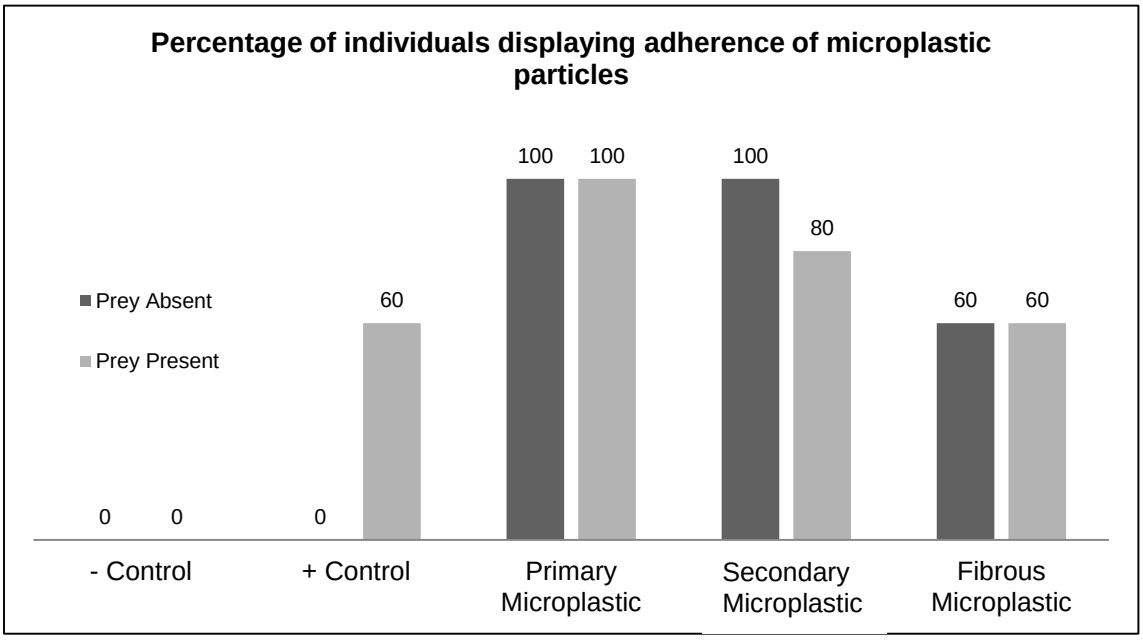

Figure 2.5 Percentage of individuals displaying adherence of microplastic particles. *C. typicus* exposed to different plastic types in the concentration of 100 MP mL^{-1}. N = 5 for all treatments, except for Secondary Microplastic prey present (n = 4). 100% ingestion indicates evidence of microplastic particles adhered to all 5 individuals exposed to each treatment.

2.3.3 Discussion

The results presented here, provide the first evidence that a common marine copepod can readily ingest a range of microplastic types, including Polyethylene microbeads, Polyamide-6 Nylon powder and artificial rope microfibres. Such information has the potential to gain further insight into the entry of microplastic into the food chain, and provides methods which may be utilised in future research.

In previous laboratory studies the ingestion of microplastic particles by zooplankton has been recorded (Cole, et al., 2013; Kaposi, et al., 2014; Lee, et al., 2013), although in most cases it is polystyrene or polyethylene microspheres, uniform in shape and size that have been used in this research. In order to produce environmentally relevant results it is important to consider that microplastic particles present within the marine environment are unlikely to be of uniform shape or size. With this in mind, this study was designed to examine the uptake of the different forms of microplastic likely to be found in the marine environment. Although in order to confirm microplastic ingestion as seen in previous studies, fluorescently labelled polystyrene microspheres were utilised as a positive control. It is important to examine microplastic ingestion in the presence of natural prey sources, which would be available to organisms alongside any microscopic marine debris in the environment. To address this the study was replicated using seawater collected from the same site as study organisms, and in the absence of prey.

The RADGLO dyeing methodology presented here is considered effective in microplastic research. RADGLO was implemented by researchers initially as a manner to examine hydrodynamics and settling behaviour of bivalve larvae (Lindegarth & Jonsson, 1991). In these studies larvae were encouraged to ingest labelled polyvinyl chloride (PVC) particles to allow for visualisation of individuals (Lindegarth & Jonsson, 1991). RADGLO adhered to PVC due to electrostatic forces (Lindegarth & Jonsson, 1991) and it was predicted that the dye would be likely to bind to a number of plastic types. The protocol outlined by Lindegarth and Jonsson (1991) was utilised to dye plastics and altered to meet the demands of the study presented here. All plastics were dyed effectively, and as can be seen in Figures 2.3 and 2.4, particles were clearly visible under fluorescence. The novel method of using RADGLO to dye plastic particles,

therefore, presents a wide range of opportunities in microplastic research. It remains that potentially results may be skewed due to the ingestion of small particles of the RADGLO powder, rather than microplastic particles. This issue was addressed by passing stock plastic suspensions through a 10 μm mesh to remove any remaining powder, though, the possibility that some remained in samples exists and must be considered.

Primary microplastics refer to those particles which enter the marine environment directly (Andrady, 2011) and are manufactured to be of microscopic size, such as particles used in the pharmaceutical or manufacturing industry. Recently much focus has been put upon the use of facial cleansers with “microbeads” or “microscrubbers” made from plastics, commonly polyethylene (PE). *C. typicus* was found to ingest Polyethylene (PE) microbeads extracted from a commercial fash wash in the presence and absence of natural prey. Particles were visible in the digestive tract of all but one of the individuals exposed to the microplastic. Upon examining the presence of microbeads within the digestive tract of exposed individuals it appears that the particles seem to aggregate. This is particularly visible in Image A (Figure 2.3) and Image A (Figure 2.4). The results display a number of cases of adherence of PE microbeads to the feeding appendages and swimming legs. Image D (Figure 2.4) shows clearly how microbead particles have adhered to the setae of the feeding appendages. Evidence of adherence of particles to the attenae of individuals is also apparent, see Image D (Figure 2.3).

The second microplastic type investigated in this study was Polyamide-6 Nylon powder, representing an example of secondary microplastic. Secondary microplastic particles are said to enter the marine environment indirectly, following, the breakdown of larger plastic debris (Cole, et al., 2011). Polyamide-6 Nylon powder, was chosen to represent this microplastic type as it was provided in a powder form, so could be considered as plastic that had been continuously degraded, in a size range appropriate for ingestion study. As with PE microbeads, the results presented above display ingestion of Polyamide-6 Nylon powder by *C. typicus* in the presence and absence of natural prey sources. Fluorescence of particles indicated the presence of powder particles in the upper (Image B, Figure 2.4) and lower (Image B, Figure 2.3) digestive tract.

However, it appears that powder particles do not aggregate in the same manner as was seen with microbeads. Therefore, it can be predicted that powder particles are more likely than microbeads to progress through the digestive tract in the same manner as natural prey and be egested effectively. This may result from the fact that the average size of of powder particles was 20 μm, compared to the 30 μm microbeads, meaning that they are less likely to aggregate and block the digestive tract. It is possible, therefore, that powder particles were ingested and subsequently egested, a process that can occur in a number of hours (Cole, et al., 2013). Considering the images displayed, it can be argued that the ingestion of powder particles was significantly lower than ingestion of microbeads, in terms of the intensity of the fluorescent signal given off by particles in the digestive tract. Such information may hint towards a higher likelihood of ingestion of primary microplastics in the marine environment compared to seconday microplastics. However, due to the nature of imaging studies, in this case it is impossible to test whether this difference in ingestion is statistically significant, and thus, requires further investigation. It remains that lower ingestion may result from the fact that Polyamide-6 powder has a density of 1.13 g cm^{-3} (Goodfellow, 2014), suggesting that particles may sink, so reducing the likelihood of interacting with individuals. Adherence of powder particles to feeding appendages and swimming legs was common when natural prey was present and absent.

The final microplastic type studied in this investigation was fibrous microplastic. It is believed that fibres make up a large proportion of microscopic debris, for example in coastal surveys off the coast of Belgium, plastic fibres made up 59% of plastic debris sampled (Claessens, et al., 2011). Therefore, it is important to study their potential uptake by marine organisms to which they are bioavailable. Following exposure it was found that all individuals offered microfibres in the presence of natural prey ingested particles, and 40% individuals exposed to microfibres in the absence of algal cells ingested the fibres. As with the two other plastic types particles were observed in the upper and lower digestive tract (see Image C Figure 2.3 and 2.4). The aggregation of microfibre particles is expected, as recorded in previous studies (Murray & Cowie, 2011), and examples of such aggregation is visible in the images provided. Adherence of

microfibres to body parts of individuals occurred in a similar manner as recorded with microbeads and powder particles.

The ingestion of microplastic particles, such as that recorded in this study has the potential to adversely affect individuals. The aggregation of particles in the digestive tract may cause a blockage of the tract by clumping or knotting (Murray & Cowie, 2011). It is also possible given that gut retention times of microplatics have been recorded as lasting up to 7 days (Cole, et al., 2013), that the individual may feel a false sense of satiation (Gregory, 2009; Wright, et al., 2013). As a result feeding behaviour may be altered, reducing energy uptake, negatively affecting the individual and its potential progeny, if egg quality and hatchling success is reduced (Cole, 2014). The fact that Polyamide-6 Nylon powder did not appear to aggregate in the same manner as the other two test plastic types, suggests that the powder is more likely to pass through the digestive tract in a similar manner to natural prey and so may not produce a negative effect.

Another clear observation from the images dispalyed above (see Images D, E and F Figure 2.3 and Figure 2.4), is the occurrence of adherence of microplastic particles to zooplankton, as described in previous studies (Cole, et al., 2013). Adherence to feeding appendages (highlighted in Image D, Figure 2.4) and swimming legs, appeared common across all test plastics, as well as, cases of adherence to the antennae, carapace and urosome. Such adherence, as described by Cole et al. (2013) has the potential to affect feeding, predator avoidance and mating. Adherence to these body parts might alter the individual's buoyancy and limited swimming ability may increase the chances of predation due to altered movement causing an increased disturbance in the water, so enabling predators to detect the copepod more easily, or reducing the effectiveness of avoidance strategies such as the escape jump displayed by a range of nauplii and zooplankton prey (Jakobsen, 2001; Titelman & Kiørboe, 2003). It has also been studied that many copepods possess multiple chemoreceptors upon their feeding appendages and setae (Jiang, et al., 2002); therefore, adherence of microplastics may cause disruption to these receptors and alter the detection of prey or mates, as males appear to detect females via chemically signalling alongside hydromechanical cues (Katona, 1973; Griffiths & Frost, 1976; Lazaretto, et al., 1994; Kiørboe & Bagøien, 2005). The feeding

appendages and antennae also utilise mechanoreceptors, which similarly may be disrupted by adherence of foreign particles. Given, the importance of the antennae in predator and prey detection (Strickler & Bal, 1973; Fleminger, 1975; Strickler, 1975; Viitasalo, et al., 1998) and positioning within the water column it can be predicted that adherence of particles to the antennae is likely to interefere with these processes, and as such reduce the fitness of the organism. It would be highly interesting to examine the length of time that particles remain attached to individuals, or investigate the effect upon swimming and feeding behaviour.

Although the results appear to provide clear evidence that ingestion of a number of microplastic types occurs in the copepod species, *C. typicus*, a number of limitations to this study exist and must be addressed before presenting conclusions of this investigation. Firstly, due to the difficult nature of sampling microplastic debris (Hidalgo-Ruz, et al., 2012; Cole, et al., 2014) the concentration of microplastics sized <100 µm within the natural environment is currently unknown, and requires addressing. With that in mind it can be argued that the concentrations used in this experiment do not represent those that are found in the natural environment. However, as this experiment was proposed initially as an exploration into the ingestion of various plastic types it can be considered that the concentration used is not of high importance. It can be argued, though, that differences observed between the uptake of plastics in the presence and absence of natural prey may be due to the fact that the relatively lower number of potential prey particles per mililitre of seawater present in samples where no prey was offered to copepods meant that feeding rate was reduced to conserve energy (Lam & Frost, 1976), as a result less microplastic was ingested. Similarly patterns of adherence between the two treatments may also have been affected in this way.

Another factor that may have affected results was the aggregation of particles within suspensions. Any aggregation producing too large particles to be ingested by *C. typicus* would affect results, therefore, there is a possibilty that ingestion was reduced in some cases due to aggregation. However, it can be argued that similar aggregation may occur in the natural environment. Observed in a few cases was the occurrence of large fibres which were unlabelled and adhered to individuals (see Image F1b, Figure 7.1.1; Image NP4a, Figure 7.1.2,

Appendix 1). The presence of such fibres may have altered results or led to the adherence of other particles, not caused by the behaviour of the copepod.

Further research is required to address the limited evidence that exists on the relative abundances of different microplastic types within the natural environment. Such data would be highly beneficial and allow research to be directed at polymers which are abundant, but new methods must be formulated to overcome the difficulties arising from sampling debris <100 μm (Hidalgo-Ruz, et al., 2012; Cole, et al., 2014).

Regardless of the limitations outlined above, the results provided here display evidence of ingestion of a range of plastic types by a common copepod species. The recorded effects of microplastic ingestion examined by previous research were addressed earlier in this chapter. Although a number of different negative effects are presented, all centre around the problem of reduced energy reserves resulting from an increased sense of satiation as described by Gregory (2009), decreased feeding or altered feeding behaviour (Cole, 2014). The decrease in energy reserves caused by microplastic ingestion is a ecological concern considering the importance of zooplankton in transfering energy to higher trophic levels. A reduction in energy available to species that feed upon zooplankton, including a number of commercially important fish and their larvae, may negatively affect the stability of populations within environments where microplastic polltuion is present. Such an occurrence would be a concern for those associated with the fisheries industry and conservationists alike. By utilising methods such as those presented in this study we may be able to identify species most at risk from ingesting commonly found microplastic particles, and assess the potential of such particles being passed through the food chain to higher trophic levels.

Chapter 3

The effects of microplastic exposure upon marine copepods with varying feeding strategies

3.1 *Feeding strategies in the zooplankton*

The zooplankton represent a large and taxonomically diverse group of species, which display a range of physical forms and life strategies in order to live successfully within the world's ocean currents and water systems. Such areas represent a "viscous and nutritionally dilute world" and as a result in order to survive, a volume of water 10^6 times their own body size must be covered daily by zooplankton (Kjellerup & Kiørboe, 2012).Therefore, it is of great importance that the feeding strategy adopted by a species is efficient. The ingestion of microplastics further reduces the nutritional value of feeding and increases the requirement for effective prey capture, which may also be inhibited by the presence of microplastic within the environment. Generally zooplankton display one of three major feeding strategies (Kjellerup & Kiørboe, 2012); they generate a feeding current and capture prey within this current; they are ambush feeders and capture prey that pass within a capture radius; or they cruise through water and capture encountered prey (Kjellerup & Kiørboe, 2012). All of these feeding types have different methods by which they detect and subsequently capture prey, therefore, it is likely that those species displaying different feeding strategies to one another are likely to interact with and be affected by microplastic exposure in different manners.

3.2 *Prey detection by Feeding-current and Ambush feeding zooplankton*

It could be hypothesised that those copepod species which feed by generating a feeding current are more likely to ingest microplastics as they draw particles towards feeding appendages and may ingest particles in a passive manner whilst filtering. Ambush predators on the other hand, which tend to feed on motile prey (Saiz & Kiørboe, 1995), theoretically, should not detect immobile microplastic particles and as a result significant ingestion is not predicted in these species. However, experimental studies have shown microplastic ingestion in both feeding-current strategists and ambush feeders (Cole, et al., 2013). The manners in which ambush feeders and feeding-current strategists detect prey will be discussed below.

It has been studied and appears widely accepted that ambush feeders detect prey using hydromechanical cues (Yen, et al., 1992). Prey is detected due to changes in the hydrodynamics of ambient water caused by a particle, which as

a result causes bending of the setae and allows the individual to identify the location of the prey particle (Yen, et al., 1992). Such is the case in the copepod *Oithona similis* where it is proposed that motile prey is perceived remotely by hydrodynamic disturbances generated in ambient water by a prey particle (Kiørboe & Visser, 1999) detected by numerous long mechanoreceptory setae on the antennules (Svensen & Kiørboe, 2000). The presence of mechanosensory setae upon antenna appears common in copepods which are blind and rely on such appendages to perceive motile prey (Strickler, 1975). This mechanism of prey detection, however, limits ambush-feeding zooplankton to preying upon motile prey as non-motile cells would fail to produce a disturbance in ambient water and would as a result not be detected. Ambush feeders have been found to feed upon faecal pellets falling from above; hence, producing a hydromechanical signal, such behaviour is displayed by *Oithona similis* (Gonzáles & Smetacek, 1984).

In laboratory studies upon the mechanisms by which prey is captured, the movement of feeding appendages is described as similar between feeding-current generating and ambush feeding zooplankton (Koehl & Strickler, 1981). It has been studied using high-speed filming techniques that feeding-current strategists propel water past themselves by flapping their appendages and then actively capture small parcels of water that contain food particles by opening and closing their second maxillae (Koehl & Strickler, 1981). The ambush feeding copepod *O. similis* captures prey by detecting the location of the cell by detection of hydromechanical cues by the antennules or telson and propels itself towards the cell placing its mouthparts at the location where the prey cell was discovered (Svensen & Kiørboe, 2000). In this case the prey remains almost stationary and unaffected by the motion of the copepod until the feeding basket is opened and prey directed towards the mouth (Kiørboe, et al., 2009). Such evidence counters previous ideas that attack jumps, which are used by some ambush feeders, should be theoretically ineffective as prey cells should be pushed away by the forward jumping attacker due to the thick viscous boundary surrounding the attacking zooplankton (Bruno, et al., 2012). Hence it has more recently been revealed that like feeding-current generating individuals, ambush feeders direct prey towards the mouth by motions of the

feeding appendages, rather than approaching prey cells by direct attack jumps (Bruno, et al., 2012).

Feeding-current strategists are believed to detect prey using biochemical cues and have the potential to select prey based upon their biochemical composition (Tiselius, et al., 2013). Tiselius et al. (2013) provide a model of detection which can generally be applied to feeding-current producing zooplankton; it is proposed that the sphere of chemicals surrounding a potential prey cell elongates when the prey cell is situated within the feeding current. As a result the leading edge of this chemical signal reaches the individual before the prey cell itself, giving the opportunity for the zooplankton to accept or reject the prey cell based upon its chemical signal and alter its feeding-current away or toward the capture area (Tiselius, et al., 2013). This mechanism of chemical reception is also represented by Kjellerup and Kiørboe (2012), where it is suggested that the chemical signal arrives at the zooplankton approximately 0.5 seconds before the prey cell itself allowing a feeding-current strategist time to accept or reject prey. Ambush feeders, on the other hand, do not have this ability and as a result must feed upon particles purely on the extent to which they disturb the hydrodynamics of ambient water. Two processes of chemoreception occur in feeding-current feeding zooplankton; first long-range chemoreception governs the generation of the feeding-current and particle capture, subsequently short-range chemoreception at the mouth induces particle ingestion (Koehl & Strickler, 1981).

It can be argued, therefore, that the key difference between prey detection in ambush and feeding-current strategists, is that ambush feeders cannot detect prey through biochemical means, so limiting selectivity. Feeding-current strategists, on the other hand, have the potential to select particles based on a biochemical signal. Secondly, due to the mechanistic manner in which ambush feeders detect prey, it is only motile prey which are likely to be detected, whereas, feeding-current strategists that possess both mechanistic and biochemical detection are able to detect motile and non-motile prey, so increasing the number of prey sources available to them.

3.3 *The effects of microplastic exposure upon feeding in the zooplankton*

Studies carried out by Cole et al. (2013) and Setälä et al. (2014), alongside results presented in this thesis (Chapter 2), have provided evidence that ingestion of microplastic particles is widespread among a range of species and polymer types under laboratory conditions. One major concern of this is that microplastic ingestion may disrupt normal feeding behaviour, and thus, negatively affect the fitness of the individual.

Further work carried out by Cole (2014) focussed upon the effects of microplastics upon the fecundity and feeding of the calanoid copepod, *Calanus helgolandicus*. Here it was found that exposure to <75 polystyrene MP mL^{-1} negatively affected the feeding rate and carbon uptake in the study species, an 11% reduction in algal uptake and 40% reduction in carbon biomass ingested was observed (Cole, 2014).

In the work carried out by Cole (2014) it was also discovered that the common calanoid species *C. helgolandicus* displayed a switching behaviour in terms of prey selection when exposed to microplastics. It was observed that individuals feeding upon *T. weissflogii* alone ingested all size ranges of the species (11.6–17.0 µm), and those exposed to 20 µm polystyrene beads only ingested algal cells in the size range of 11.6–14.8 µm (Cole, 2014).

However, it is not only copepods which have been found to show altered feeding activity when exposed to small plastic particles. The blue mussel, *Mytilus edulis,* showed a decrease in filtering activity when exposed to polystyrene nanoplastics (30 nm); however, the concentration of plastic still decreased in treatments (Wegner, et al., 2012). Despite the smaller size of the nanoplastics, it could be suggested that exposure to polystyrene microplastics may also produce an adverse effect. Other stress effects were also seen such as the production of pseudofaeces, a mechanism known to be a cleaning mechanism, preventing the gills being blocked by particulate matter (Jørgensen, 1981), and as a rejection mechanism for inedible particles (Wegner, et al., 2012). Similarly polystyrene microplastic spheres have been recorded to adhere to feeding appendages of copepods (Cole, et al., 2013), as mentioned in Chapter 2. Such adherence may adversely affect feeding and swimming

behaviour (Cole, et al., 2013) and thus, reduce the effectiveness of natural feeding, potentially creating an energy deficit for affected individuals.

Reduced feeding activity as a result of microplastic exposure was also recorded in the marine worm, *Arenicola marina* (Wright, et al., 2013). This reduction in feeding was attributed to suppressed feeding activity or adverse effects caused by the unplasticised polyvinylchloride (UVPC) microplastics used in the study. The lack of a protein coat on the particles was thought to decrease the adherence of particles to the worm's feeding appendages, so reducing feeding efficiency (Wright, et al., 2013). Once again the reduction in feeding shown produced a decrease in energy levels recorded in the worms, therefore, reducing their fitness. Due to the fact that *Arenicola* is a keystone species within its natural environment (Wright, et al., 2013), this energy deficit is likely to have knock-on effects throughout the ecosystem.

It appears then that microplastic exposure causes alterations in normal feeding behaviour, reducing the energy uptake of individuals, and thus, creating an energy deficit in the trophic level. Due to the fact that that the species that tend to feed on prey in the size range of microplastics, generally occupy lower levels in the food web, it is particularly important to examine the effects that microplastics are having upon feeding to best inform decision makers of the negative effects of microplastic debris within the marine environment.

3.4 Investigating the effects of microplastic exposure upon feeding in the zooplankton

To further examine the effects that microplastic particles are having upon the feeding behaviour of zooplankton, it was decided to carry out two grazing experiments, examining two zooplankton species, each displaying a different feeding strategy and role within the ecosystem.

The first study was designed to examine the effects of microplastic exposure upon a holoplankton species, *Acartia* tonsa, and a meroplankton species, *Porcellanid* larvae. A*. tonsa* is a copepod species common throughout coastal areas and neritic temperate seas (Saiz & Kiørboe, 1995) which displays two

feeding strategies (Jonsson & Tiselius, 1990). When feeding upon non-motile phytoplankton prey, *A. tonsa* generates a feeding-current, and feeds upon cells captured within this current (Saiz & Kiørboe, 1995). On the other hand, when feeding upon motile prey such as ciliates, the species adopts ambush feeding (Jonsson & Tiselius, 1990). Prey is perceived via mechanical detection through disturbances in the water column caused by the moving prey, and subsequently using an attack jump motion the cell is captured (Saiz & Kiørboe, 1995). *A. tonsa* can therefore, be described as representative of a number of copepod species, feeding in two of the three common feeding modes displayed by copepods. As a result, the species is a highly interesting subject of research into effects of contaminants upon feeding behaviour. Generally it is believed that *A. tonsa* adopts the feeding strategy that is most likely to increase energy intake, so altering feeding behaviour due the relative abundance of motile and non-motile prey in the water column (Kiorboe, et al., 1996). It could be predicted then that in a closed system, such as those used in experimental studies, the addition of microplastic particles, representing non-motile prey, may lead to the adoption of the feeding-current strategy by *A. tonsa* due to the increase in non-motile cells available. For this reason, along with the evidence displayed by Cole et al. (2013) displaying ingestion of 7.3-30.6 μm polystyrene spheres by another member of the *Acartia* genus, *Acartia clausii*, as well as the potential for adherence of microplastics to feeding appendages, which appears common across a range of taxa (see Chapter 2). It can be assumed likely that microplastic exposure will affect feeding behaviour and associated carbon uptake in *A. tonsa*.

To date, microplastic research has focussed upon copepod species spending their entire life history in the plankton and less research into potential effects upon meroplankton species exists. It is therefore, of great interest to examine the effects of microplastic exposure upon larvae whose development is likely to be affected by changes in energy uptake and nutrient availability. *Porcellanid* larvae feed upon algal prey, detecting prey as it comes into contact with the individual (Gonor & Gonor, 1973), therefore relying on chance for encounter (Mooler, 1978; Meyers & Hagood, 1984; Mootz & Epifanio, 1974; Kurmaly, et al., 1989, 1990; Stickney & Perkins, 1981; Barros & Valenti, 2003). However, in order to avoid predation if a cell comes into contact with the individual's

posterior or the top or sides of the carapace the larva reacts quickly and moves away from the cell immediately (Gonor & Gonor, 1973). On the other hand, cells that come into contact with the setae of the larva are captured and passed to the mouthparts (Gonor & Gonor, 1973). Individuals are thought to be sensitive to disturbances within the water column, however, if contact is not made with the potential prey item attempts to capture the cell typically fail (Gonor & Gonor, 1973). By feeding in this manner *Porcellanid* larvae are unable to distinguish between natural prey and microplastic particles until cells are passed to the mouth. It can be predicted then that individuals are likely to interact with microplastic particles if they are present within the marine environment. Studies carried out by Cole et al. (2013) provide evidence for the ingestion of 30.6 µm polystyrene microspheres by *Porcellanid* larvae. Therefore, it can be suggested based on the effects recorded in copepods (Cole, 2014) and other invertebrates such as marine worms (Wright, et al., 2013) that effects upon energy uptake are likely to occur in decapod larvae when exposed to microplastic particles.

The second study was designed to investigate whether the effects upon feeding were influenced by feeding type. It was hypothesised that the effects upon feeding should be less pronounced in an ambush feeding species, *Oithona similis*, compared to a feeding-current strategist, *Calanus helgolandicus,* owing to the fact that ambush feeding species are not expected to detect non-mobile prey such as microplastic particles. Feeding-current strategists on the other hand filter particles into a capture radius, thus, are likely to capture microplastic particles where concentration of microplastics are high. With this in mind, it is hypothesised that feeding behaviour and subsequent carbon ingestion will be unaffected when the ambush predator, *O. similis,* is exposed to microplastics. However, *C. helgolandicus*, a species that feeds using a feeding-current is expected to suffer an energy deficit following exposure to microplastic particles.

3.4.1 Methods

3.4.1.1 *Zooplankton for experimentation*

Zooplankton samples for experimentation were collected from Station L4 (see Figure 2.1, Page 33) by the Plymouth Quest Research Vessel operated by PML via a medium (200 µm) and fine (63 µm) surface trawl of plankton nets. Samples were then transferred to the laboratory at PML for species

identification and sorting. Using the WILD M5-48084 optical microscope species chosen for study were picked out and transferred to 5 L beakers containing 0.2 µm filtered seawater. Subsequently zooplankton samples were transferred to a temperature controlled room, set at ambient sea temperature (~17°C), where they were conditioned overnight without feeding.

3.4.1.2 *Natural Algae*

In order to represent natural conditions as closely as possible, microplastic suspensions were made in natural seawater (NSW) collected from Station L4 by the Plymouth Quest Research Vessel at a depth of 10 m. Following sampling, seawater was transported back to PML, and stored at ambient sea temperature (~17°C). In order to remove any microzooplankton that may alter ingestion rates in samples, seawater was passed through a 100 µm mesh prior to experimentation. During experimentation the phytoplankton community inhabiting the waters of Station L4 varied. NSW collected for use in studies upon *C. helgolandicus* and *O. similis* was dominated by a *Phaeocystis* bloom.

3.4.1.3 *Microplastic Suspensions*

Microplastic suspensions were created by spiking natural seawater with polystyrene microspheres, see Table 3.1. Spheres were first added to 0.2 µm FSW to create a stock solution, the concentration of which was calculated using a Beckman Multisizer Coulter Counter. Suspensions were then produced by spiking 4 L of NSW with microplastic stock solutions of known concentrations, using the formula $C_1 \times V_1 = C_2 \times V_2$, to calculate the required volumes of stock solutions required.

3.4.1.4 *General experimental set-up*

Table 3.1 outlines the size of vessel, number of individuals, microplastic size, concentration, and number of replicates used for each species studied. All studies were carried out in glass bottles which were then fixed to a plankton wheel rotating at <5 rpm.

Prior to experimentation T_0 samples of NSW were fixed using 5ml Lugol's iodine solution (2% final concentration) and stored in brown glass bottles. T_{24} samples, where no animals were added, were also set-up for studies upon *Acartia tonsa* and *Porcellanid* larvae, to examine natural growth of algae. Exposures were

carried out over 24 hours in darkness at ambient sea temperature (~17°C) in order to best replicate natural conditions and prevent growth of algal cells.

Following exposures, samples were fixed using Lugol's iodine solution (2% final concentration), and stored in brown glass bottles to be analysed using FlowCAM.

Table 3.1 Summary of Experimental Set-Up

Treatment	n	Vessel Size (mL)	Microplastic Size (µm)	Concentration (Microplastic particles (MP) mL^{-1})	Number of Reps.
A. tonsa Control	3	325	N/A	N/A	5
A. tonsa Treated	3	325	10 µm Polystyrene Spheres (Fluka) 20 µm Polystyrene Spheres (Fluka)	50 50 = 100	5
Porcellanid Control	2	325	N/A	N/A	5
Porcellanid Treated	2	325	10 µm Polystyrene Spheres (Fluka) 20 µm Polystyrene Spheres (Fluka)	50 50 = 100	5
C. helgolandicus Control	3	74	N/A	N/A	3
C. helgolandicus Treated	3	74	20 µm Yellow Fluorescent Polystyrene Spheres (Spherotech)	75	6
O. similis Control	5	74	N/A	N/A	3
O. similis Treated	5	74	20 µm Yellow Fluorescent Polystyrene Spheres (Spherotech)	75	6

3.4.1.5 *FlowCAM analysis*

FlowCAM enables the user to sort phytoplankton samples into specific categories of interest; for this study we examined the uptake of diatoms, dinoflagellates, pennates, ciliates, unidentified <10 µm cells and unidentified 10-20 µm cells. Samples were passed through FlowCAM in autoimage mode with a particles used per image (PPI) of approximately 1.10. All samples were viewed using a 10x Flow Cell. Following sampling through FlowCAM and classification into prey categories, summary information including cells mL^{-1}, average size (µm) and biovolume ($µm^3$) were exported for further analysis.

3.4.1.6 *Calculation of Ingestion rates and Carbon uptake*

Using the equation of Frost (1972) the ingestion rate and subsequent carbon uptake of each algal type and total algae was calculated for each species.

3.4.1.7 *Statistical Analysis*

Using Microsoft Excel, Student's T-tests were used to assess whether any significant differences were apparent between the ingestion rates and carbon uptake of each prey type, and in total, between the control and treated groups. Results were classed as significantly different to one another when the p-value was <0.05. Any cases of negative ingestion rate or carbon uptake were omitted from statistical analysis.

3.4.1.8 *The effects of a mixture of 10/20 µm polystyrene microspheres on the feeding behaviour of the copepod, Acartia tonsa, and Porcellanid larvae*

Following identification, *Porcellanid* larvae of a similar size, and adult female *A. tonsa* were transferred to 5 L beakers containing 0.2 µm FSW, and conditioned overnight at ambient sea temperature (~17°C).

Based on previous research it was decided to use a concentration of 100 MP mL^{-1} for the study. This was made up of 50 10 µm polystyrene microspheres (Fluka) mL^{-1} and 50 20 µm polystyrene microspheres (Fluka) mL^{-1}, in order to represent natural conditions where microplastics are unlikely to all be of the same size

Microplastic solutions were added to 325 mL bottles, with 5 replicates for control (no microplastic) and treated (with microplastic) groups for each species. Four

T_0 samples of NSW were fixed using 5 mL Lugol's iodine solution (2% final concentration). Four 325 mL samples of NSW were also bottled in order to record algal growth without grazing. Two *Porcellanid* larvae and 3 *A. tonsa* individuals were added to their respective bottles and fixed to a plankton wheel set at <5 rpm. Exposures were carried out for 24 hours at ambient sea temperature (~17°C) in darkness. Following exposure samples were filtered through a 100 µm mesh to remove zooplankton and fixed in Lugol's iodine solution (2% final concentration).

T_0 and T_{24} samples without grazing were run through the FlowCAM at a rate of 1 mL min^{-1} with a total sample volume of 45 mL, recording all cells in the size range of 8-100 µm. T_{24} samples with grazing, both control and treated groups, were concentrated from 90 mL to 30 mL and run through the FlowCAM at 0.625 mL min^{-1}. A total sample volume of 30 mL was analysed with a concentration factor of 3, recording cells in the size range of 8-100 µm.

3.4.1.9 *The effects of microplastic exposure upon the feeding rate of a feeding-current strategist and an ambush feeder under Phaeocystis bloom conditions.*

Adult female *C. helgolandicus* and *Oithona similis* were picked out and conditioned overnight at ambient sea temperature (17°C). The following day, 3 individuals of *C. helgolandicus* and 5 individuals of *O. similis* per replicate were added to 74 mL glass bottles treated with NSW. The community of phytoplankton inhabiting NSW collected at the time of experimentation was dominated by a *Phaeocystis* bloom, a species argued to have multiple effects upon zooplankton feeding ecology (Turner, et al., 2002), therefore, of interest in studies upon feeding behaviour. Control treatments had no microplastics added, other bottles were spiked with 20 µm Yellow Fluorescent Polystyrene spheres (Spherotech) at a concentration of 75 MP mL^{-1}. This concentration of microplastics was chosen following work carried out by Cole (2014) who found effects upon feeding following exposure to 20 µm beads displayed by *C. helgolandicus* at this concentration. Controls were repeated 3 times, and treatment groups were repeated 6 times. Exposures were run for 24 hours fixed to a plankton wheel rotating at <5 rpm at ambient sea temperature (17°C).

Due to a technical error T_{24} samples with no animals added were not recorded, therefore, algal growth was assumed to be 0, due to the fact that experiments were carried out in darkness.

Following exposure, copepod specimens were filtered out of samples using a 100 µm mesh and preserved in 5% ethanol solution and transported to the imaging unit at PML. Individuals were then placed on slides and imaged using the Olympus IMT-2 microscope with Canon camera attachment. A fluorescence wavelength of 475 nm was used to excite microplastics and camera settings set to optimise the view of fluorescence. All individuals were imaged, and all examples of ingestion and adherence of microplastic particles were recorded.

Samples were fixed using Lugol's iodine solution (2% final concentration) and passed through FlowCAM as described above.

3.4.2 Results

3.4.2.1 *A. tonsa exposure to 100 MP mL^{-1} 10/20 µm mixed suspension with natural assemblage seawater.*

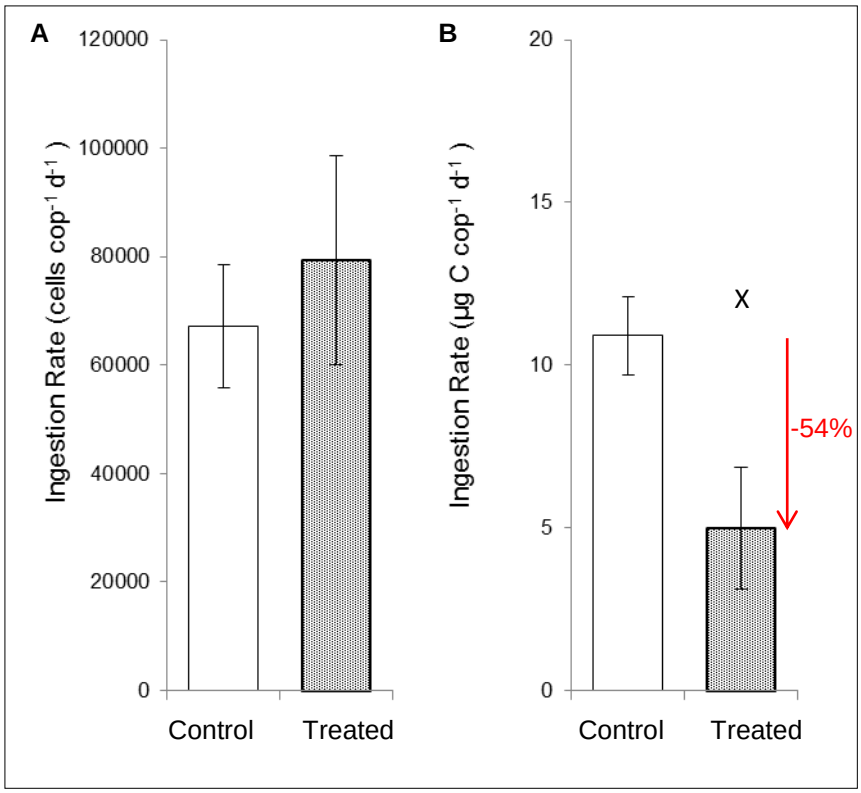

Figure 3.1 Ingestion rate of algal prey by *A. tonsa* and associated carbon uptake when exposed to 10/20 µm Polystyrene spheres 100 MP mL^{-1}. A – Ingestion rate of total algal prey per individual. B – Carbon uptake per individual. X indicates a significant difference $p<0.05$. Data are presented as the mean ± SE for replicate samples.

Figure 3.1 displays the overall ingestion rate and subsequent carbon uptake for *A. tonsa* when feeding on a natural assemblage of algae in the presence of 10 µm and 20 µm polystyrene microspheres. No significant difference appears in the ingestion rate of algal cells per copepod per day (p-value 0.290). However, there appears to be an increase in the average number of cells consumed per day in the treated group, though a large amount of variation exists, reducing the significance of this difference. The carbon uptake has been negatively affected by microplastic exposure with a significantly lower amount of carbon ingested in the treated group when compared to the control, with a p-value of 0.014. The decrease in carbon ingested in the treated group represents an approximate 54% decrease in carbon uptake. The decrease in carbon uptake evident in the

treated group appears surprising as results display an increase in ingestion rate when compared to the control. The reduction in carbon biomass ingested by the treated group, therefore, may be attributed to an alteration of prey type ingested, possibly reducing food quality.

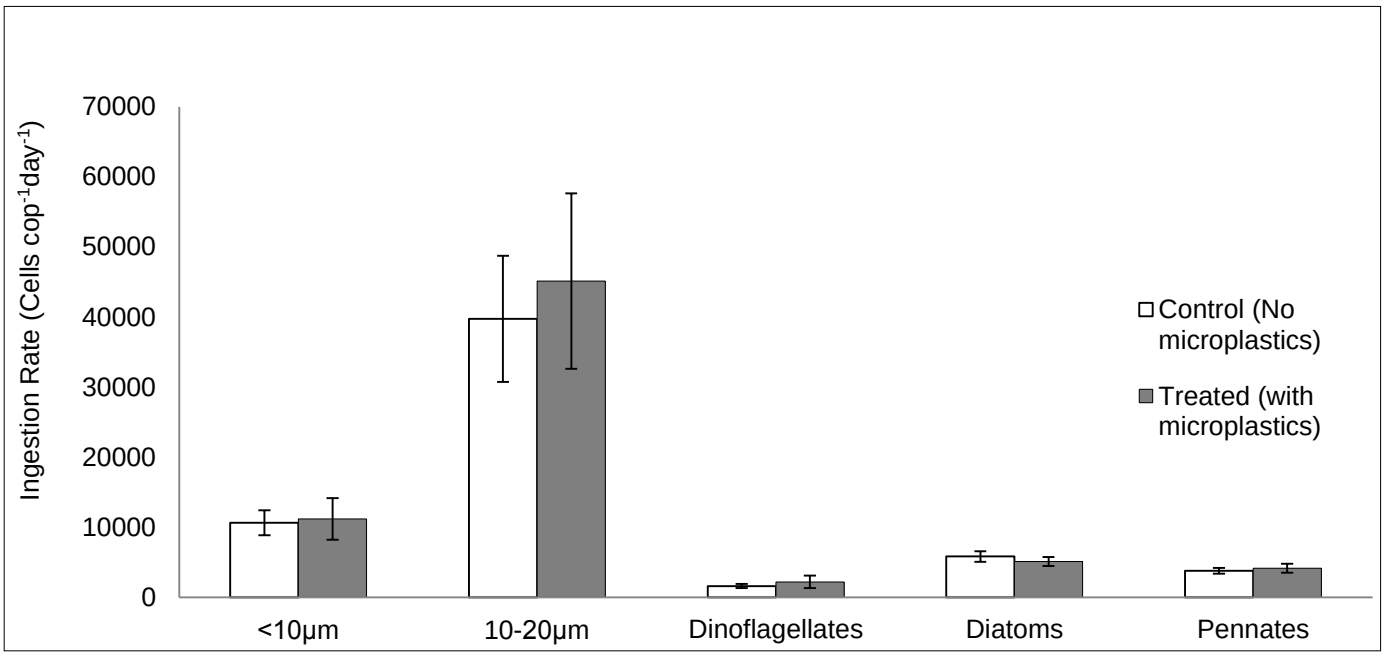

Figure 3.2 Ingestion rate of individual prey types by *A. tonsa* when exposed to 10/20 µm Polystyrene spheres 100 MP mL^{-1}. Data are presented as the mean ± SE for replicate samples.

Figure 3.2 displays the ingestion rate data of each prey type. Statistically there are no significant differences between the control and treated groups for any prey type, although a large amount of variation occurs between values for some groups. The highest ingestion rate can be seen for cells in the 10-20 µm size range; here an increase in the average number of cells of this prey type consumed can be seen in the treated group. A slight decrease can be seen in the ingestion of diatoms when looking at the treated group in comparison to the control. For cells below 10 µm, dinoflagellates and pennates a small increase in the average number of cells ingested can be seen in the treated group, but as mentioned above this difference was not significant.

3.4.2.2 *Porcellanid larvae exposure to 100 MP mL^{-1} 10/20 µm mixed suspension with natural assemblage seawater.*

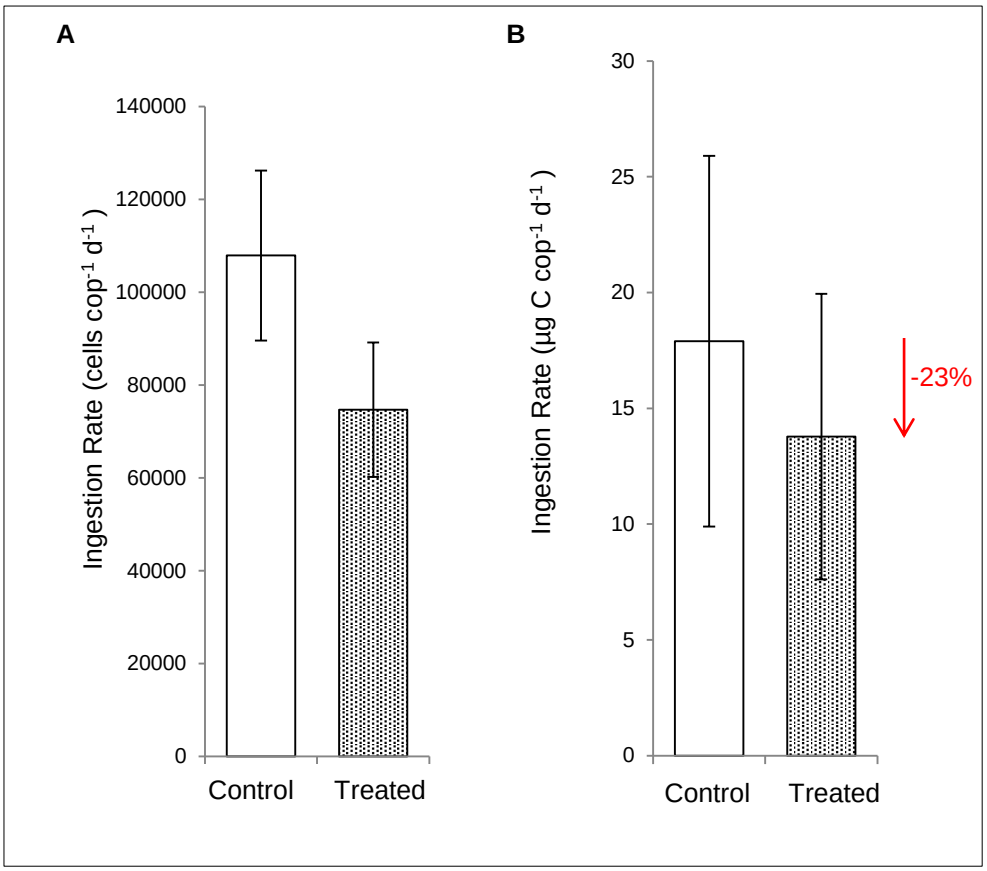

Figure 3.3 Ingestion rate of algal prey by *Porcellanid* larvae and associated carbon uptake when exposed to 10/20 µm Polystyrene spheres 100 MP mL^{-1}. A – Ingestion rate of total algal prey per individual. B – Carbon uptake per individual. Data are presented as the mean ± SE for replicate samples.

Figure 3.3 displays the ingestion rate of total algal cells and subsequent carbon uptake for *Porcellanid* larvae when feeding upon a natural assemblage of algae. The ingestion rate of number of cells consumed per individual per day was on average lower in the treated group, with an average of 74731 cells consumed per day per individual, compared to 107921 cells consumed per individual per day in the control group. However, due to a large amount of variation this difference was not statistically significant with a p-value of 0.096. The carbon uptake similarly to the ingestion rate was lower in the treated group compared to the control, representing an approximate 23% decrease in carbon ingested on average. However, once again due to variation in data this difference was not statistically significant, with a p-value of 0.210.

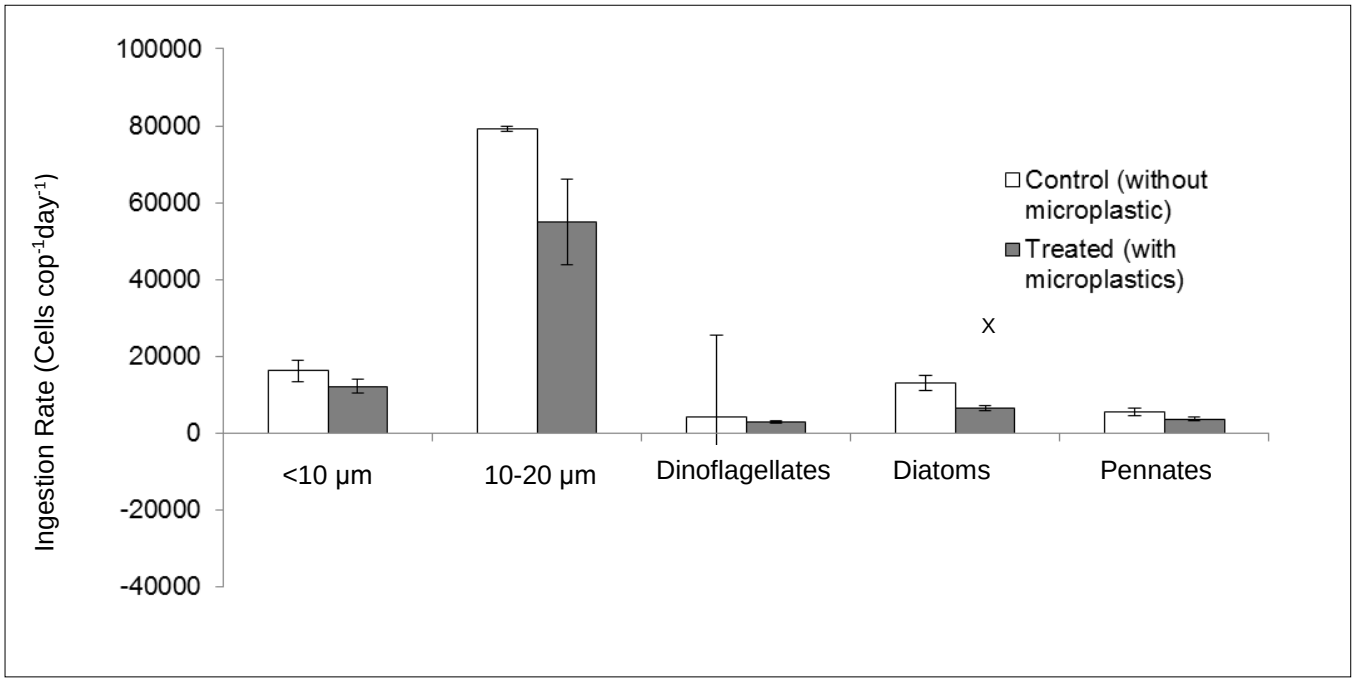

Figure 3.4 Ingestion rate of individual prey types by *Porcellanid* larvae when exposed to 10/20 µm Polystyrene spheres 100 MP mL^{-1}. X indicates a significant difference $p<0.05$. Data are presented as the mean ± SE for replicate samples.

Figure 3.4 represents the ingestion rate of each individual prey type. As can be seen across all prey types the average ingestion rate is lower in the treated group when compared to the control. However, with the exception of diatoms none of these differences are statistically significant. The ingestion of diatom cells was significantly lower in the treated group when compared to the control with a p-value of 0.007. The ingestion rate of dinoflagellates was highly variable in the control group, no significant difference was examined between the ingestion rate of dinoflagellates cells in the control and treated group.

3.4.2.3 *Ingestion of 20 µm Polystyrene spheres by C. helgolandicus and O. similis*

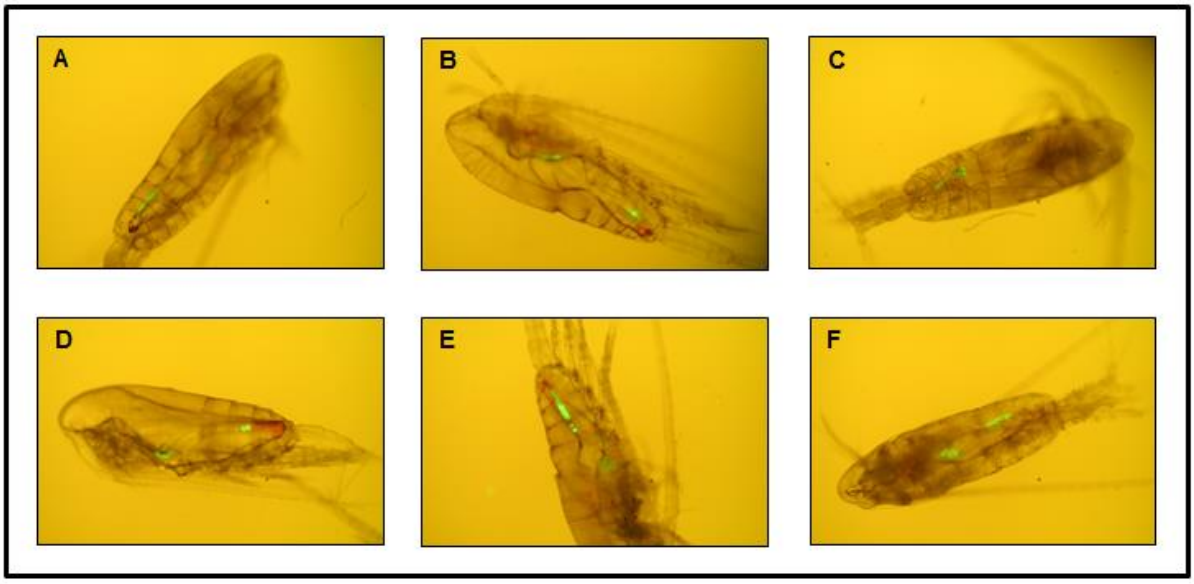

Figure 3.5 Examples of microplastic ingestion by *C. helgolandicus* when exposed to 20 µm Yellow Fluorescent Polystyrene spheres. In all images spheres are visible in the lower digestive tract, where they appear to aggregate.

Figure 3.5 displays examples of ingestion of 20 µm Yellow Fluorescent Polystyrene spheres. In all images presented particles appear to have collected in the digestive tract. Polystyrene spheres were present in 16 of the 18 individuals of *C. helgolandicus*. As expected, all controls were clear of microplastic. The extent of ingestion varied between samples greatly, however, due to the qualitative nature of imaging, it is impossible to test this variation statistically. 5 individuals of *C. helgolandicus* showed evidence of adherence of microplastics to body parts. Particles tended to adhere to the feeding appendages, however, in 2 cases adherence to the carapace also occurred. Upon studying images of *O. similis*, no evidence of microplastic ingestion was present. In 3 cases, adherence of particles to individuals occurred, adhering to the carapace and urosome. Control samples for *O. similis* did not display any evidence of microplastic ingestion or adherence.

3.4.2.4 *The effects of microplastic exposure upon feeding behaviour in the presence of a Phaeocystis bloom.*

a. C. helgolandicus

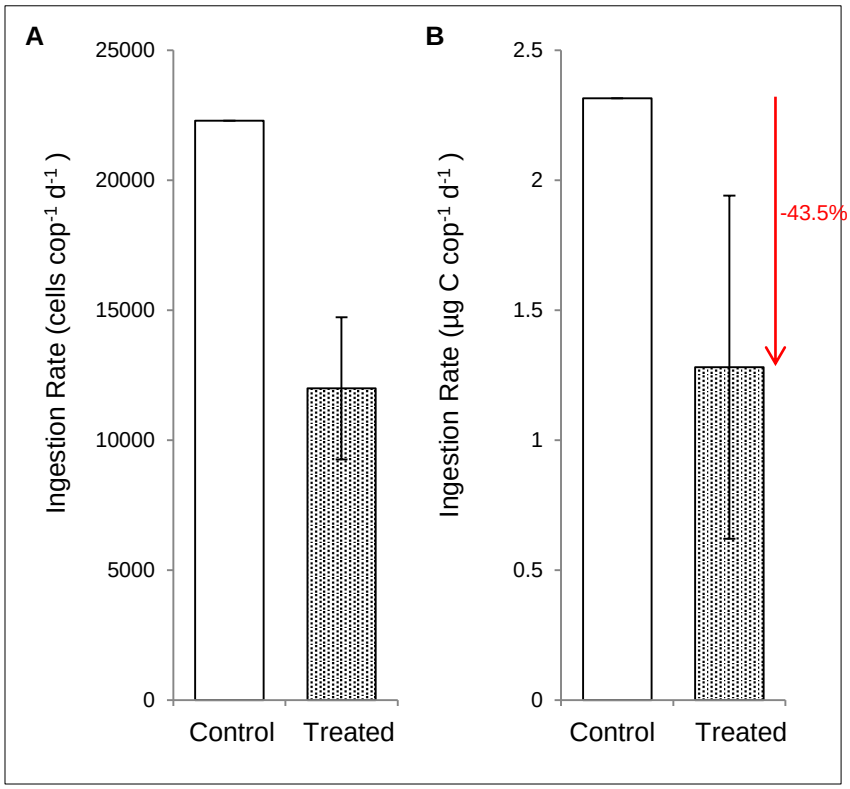

Figure 3.6 Ingestion rate of algal prey by *C. helgolandicus* and associated carbon uptake when exposed to 20 µm Polystyrene spheres 75 MP mL^{-1}. A – Ingestion rate of total algal prey per individual. B – Carbon uptake per individual. Data are presented as the mean ± SE for replicate samples.

The findings displayed by Figure 3.6 show that the average ingestion rate of algal cells and associated uptake of carbon biomass by *C. helgolandicus* was lower in the presence of microplastic particles compared to the control. However, due to the omission of negative ingestion rates only one rep for the control group was available for analysis, meaning that it was not possible to test the statistical significance of the pattern shown. The average ingestion rate of the control group was 22301 cells cop^{-1} day^{-1}, whereas, in the treated group this value was 12002 cells cop^{-1} day^{-1}, representing an approximate 46% reduction in the number of cells ingested. Likewise the carbon biomass consumed by *C. helgolandicus* was approximately 43.5% lower in the treated group compared to the control group, with ingestion of 1.3 µg C cop^{-1} day^{-1} and 2.3 µg C cop^{-1} day^{-1} respectively.

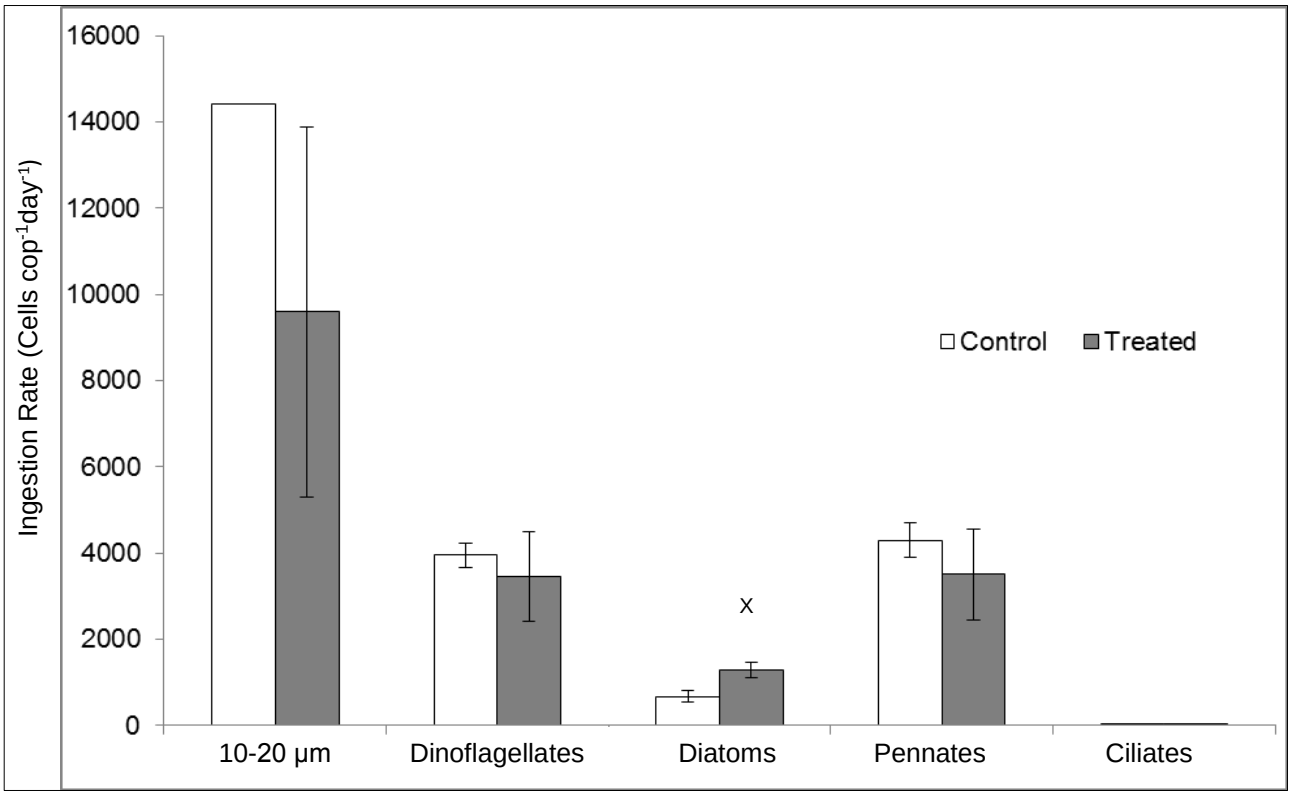

Figure 3.7 Ingestion rate of individual prey types by *C. helgolandicus* when exposed to 20 µm Polystyrene spheres 75 MP mL^{-1}. X indicates a significant difference $p<0.05$. Data are presented as the mean ± SE for replicate samples.

Figure 3.7 displays the average ingestion rate of classified prey types by *C. helgolandicus*. For ingestion of cells <10 µm only negative ingestion was recorded, hence, this prey classification was omitted from analysis. For 3 of the 5 prey types, average ingestion was lower in the treated group compared to the control; 10-20 µm, dinoflagellates and pennates. However, due to the large amount of variation that occurred between samples none of these differences were statistically significant. The average number of diatom and ciliate cells was higher in the treated group compared to the control, this trend was significant for diatoms with a p-value of 0.046, but was insignificant for ciliates.

b. O. similis

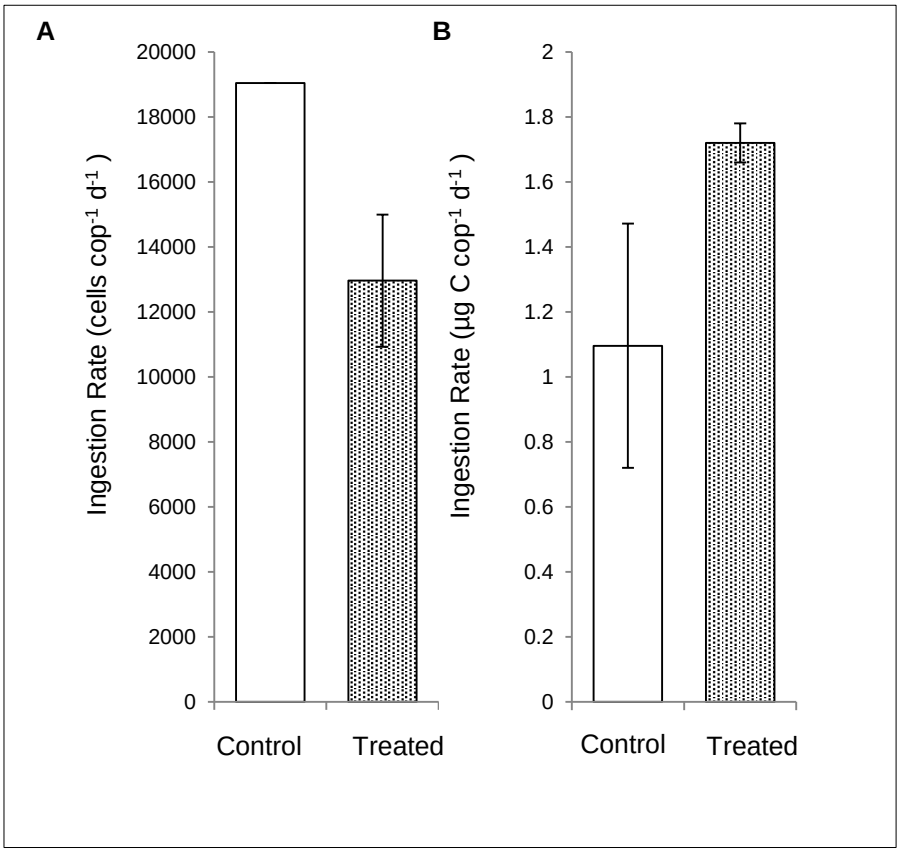

Figure 3.8 Ingestion rate of algal prey by *O. similis* and associated carbon uptake when exposed to 20 µm Polystyrene spheres 75 MP mL^{-1}. A – Ingestion rate of total algal prey per individual. B – Carbon uptake per individual. Data are presented as the mean ± SE for replicate samples.

The results displayed above, Figure 3.8, show the ingestion rate of total algae and the associated carbon biomass ingested by *O. similis* when exposed to 20 µm Yellow Fluorescent Polystyrene spheres. The average ingestion rate appears lower in the treated group compared to the control, with an average of 12962 cells cop^{-1} day^{-1}, compared to 19045 cells cop^{-1} day^{-1} recorded in the control, representing an approximate 32% decline. However, due to the omission of negative results for ingestion rate, it was not possible to test this difference statistically. The carbon biomass ingested by individuals, on the other hand, was higher in the treated group compared to the control, although this difference was not significantly different, with a p-value of 0.091. The lack of significance is thought to be due to the large variation between samples in the control group. The ingestion of carbon is approximately 55% higher in the treated group.

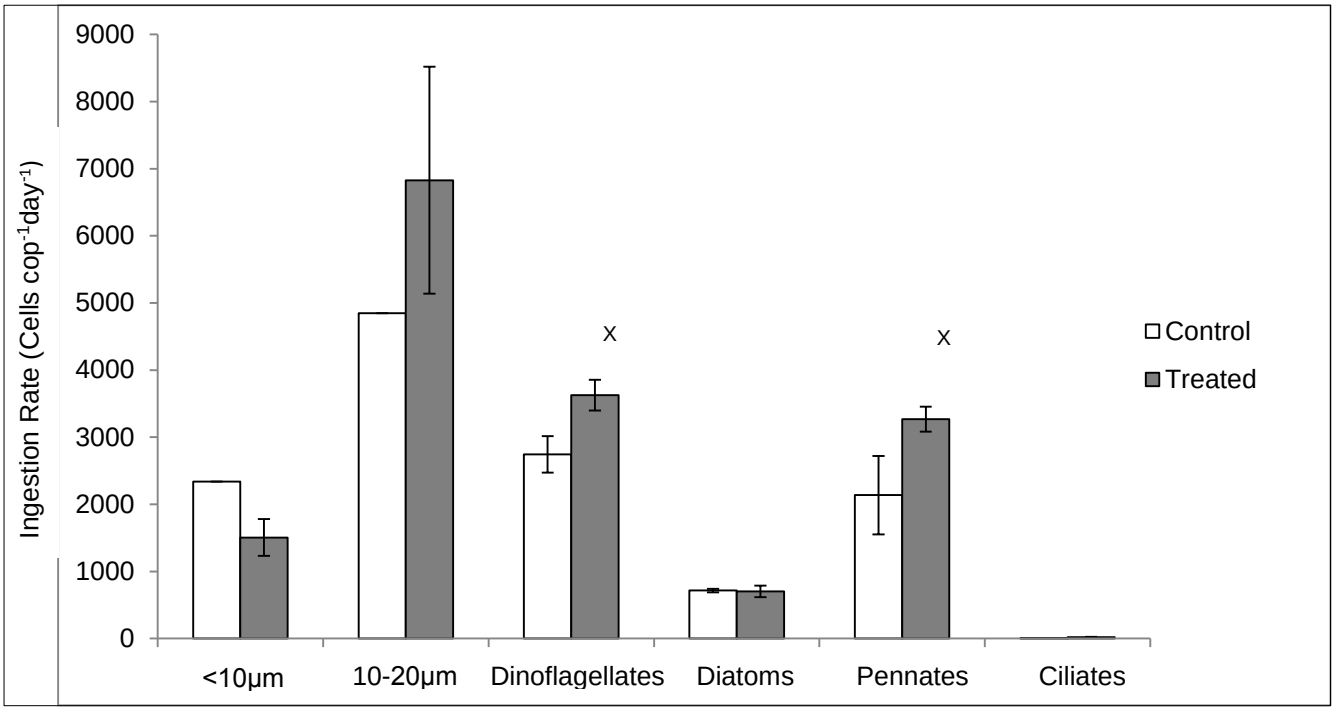

Figure 3.9 Ingestion rate of individual prey types by *O. similis* when exposed to 20 µm Polystyrene spheres 75 MP mL^{-1}. X indicates a significant difference $p<0.05$. Data are presented as the mean ± SE for replicate samples.

Figure 3.9 displays the average ingestion rates of different prey types by *O. similis* during this study. The ingestion of cells <10 µm appears lower in the treated groups compared to the control, however, due to the omission of negative ingestion rates it was not possible to test this change statistically. Similarly, the omission of results meant that it was not possible to test for statistical differences between the ingestion rate of cells sized 10-20 µm in the control and treated groups. However, on average the number of cells in this category ingested was higher in the treated group compared to the control. The average ingestion of dinoflagellates and pennates followed the same pattern, with higher ingestion rates displayed by individuals in the treated group compared to those in the control. This difference was found to be statistically significant with p-values of 0.034 and 0.042, for dinoflagellates and pennates respectively. There was no significant difference between the ingestion rate of diatoms or ciliates shown by the two groups.

3.4.3 Discussion

The results presented here provide further support for the suggestion that exposure to microplastic particles alters the feeding behaviour and associated carbon uptake of zooplankton species, as proposed by Cole et al. (2014). The findings display a reduction in the average uptake of carbon biomass in three out of the four species investigated. Although only significant for the copepod *A. tonsa*, these findings suggest a negative impact of microplastics upon the health of zooplankton as found in previous studies (Cole, et al., 2013; Cole, 2014). By examining the uptake of various prey types we present a new insight into the mechanisms by which zooplankton alter feeding behaviour in response to microplastic exposure.

3.4.3.1 *The effects of microplastic exposure upon the feeding behaviour of zooplankton*

Figure 3.1 displays the ingestion rate of total algae and related carbon biomass uptake by *A. tonsa* when exposed to a mixture of 10 µm and 20 µm polystyrene spheres. Two size ranges were used in this study to attempt and best replicate natural conditions where microplastic particles are unlikely to be of the same size. Although, it is accepted that spheres uniform in size are unlikely to be present in the environment, such particles are practical for laboratory studies. The two sizes are chosen to represent the range of prey naturally consumed by *A. tonsa*, the optimum size believed to be 15-25 µm (Støttrup & Jensen, 1990). Following 24 hour exposure no significant difference was apparent between the total ingestion rate of the control and treated groups (Graph A, Figure 3.1). In fact a slight increase in the number of algal cells ingested was seen in the treated group, an unexpected finding. Upon studying the carbon biomass ingested (Graph B, Figure 3.1), however, it becomes clear that the microplastic particles appear to have had a significant negative effect upon the energy consumed by the copepod. An average of 10.9 µg C cop^{-1} d^{-1} was ingested by the control group, compared to 5 µg C cop^{-1} d^{-1} in the treated group, representing an approximate 54% reduction in carbon biomass consumed, significant to a 98% level. Such a reduction in carbon ingested is similar to findings presented by Cole (2014), where carbon uptake was reduced by 40% in *C. helgolandicus* when exposed to 20 µm polystyrene spheres in the concentration of 75 MP mL^{-1}. However, the reduction in carbon biomass

ingested by *A. tonsa* appears to conflict with the ingestion rate recorded by the treated group during the study, which increased in comparison with the control. It is therefore important to consider the feeding behaviour in terms of prey types ingested by *A. tonsa* to examine whether although ingestion may have increased, the quality of particles ingested may have altered.

The results presented in Figure 3.2, show the ingestion rates of the different prey types available to *A. tonsa* during this experiment. The natural assemblage of algae used during the experiment contained a relatively high abundance of non-motile prey, compared to motile prey. For example, diatoms had an average abundance of 77 particles mL^{-1}, whereas, ciliates were present in the concentration 1 particle mL^{-1}. Therefore it can be suggested that individuals were likely to adopt the feeding-current strategy, as this would achieve the higher energy uptake with non-motile prey being the most abundant prey type (Kiørboe et al, 1996). The adoption of feeding-current feeding behaviour is likely to have exacerbated the effects of microplastic exposure, as if ambush feeding was displayed by *A. tonsa,* microplastic ingestion is unlikely as non-motile cells are not detected by this strategy (Kiørboe, et al., 2009). Figure 3.2 shows no significant differences in the ingestion rate of the different prey types resulted between the control and treated groups following microplastic exposure. However, this may be due to the large amount of variation between replicates. There appears to be a reduction in the number of diatoms ingested in the presence of microplastics, and an increase in the consumption of cells sized 10-20 μm. Due to the relative increase in carbon biomass ingested through feeding upon larger diatom cells (Irigoien, et al., 2000), even a slight decrease in the number of cells ingested may considerably decrease the amount of carbon ingested by an individual. It is proposed, that this sequence of events is the cause of the reduction in carbon biomass ingested by *A. tonsa* displayed here. It is possible though that although such a finding suggests the presence of a switching behaviour in prey type fed upon, reduced feeding upon diatoms may result from the fact that the average length of diatom cells in the study was approximately 39 μm, outside the optimal size range for prey for *A. tonsa* (Støttrup & Jensen, 1990), and may account for the variation in results recorded.

The effects of microplastics upon meroplankton species such as *Porcellanid* larvae remains little understood. The study conducted here presents an initial insight into these effects. Figure 3.3 displays the overall ingestion rate of algal cells and the associated uptake of carbon biomass exhibited by *Porcellanid* larvae when exposed to 10 µm and 20 µm Polystyrene spheres. It is clear in the figure that the average ingestion rate and carbon uptake is lower in the treated group compared to the control, however, due to the large variation between replicates these differences were not statistically different. The average ingestion rate of the treated group was approximately 30% lower than that of the control group, and the associated average carbon uptake was reduced by approximately 23%, from 17.9 µg C cop^{-1} d^{-1} in the control group to 13.8 µg C cop^{-1} d^{-1} in the treated group. Decapod larvae have been recorded to increase ingestion rate as the concentration of food items increases (Barros & Valenti, 2003). The reduction in ingestion rate, therefore, is unexpected as the larvae feed through chance encounter and the increased number of cells caused by the addition of microplastic particles would have increased encounter rates of potential prey cells and so the number of cells ingested. As seen in Figure 3.4 the average ingestion rates of all prey types appears to be reduced in the treated group compared to the control, however, only the ingestion rate of diatoms is significantly lower than the control. These changes in feeding behaviour as a result of microplastic exposure give rise to the overall decrease in average ingestion rate displayed by the treated group seen in Figure 3.3. Unlike *A. tonsa* a switching behaviour in prey type ingested in response to the presence of microplastics does not appear to have been displayed by *Porcellanid* larvae. Rather, individuals appear to reduce feeding behaviour overall, in particular ingestion of high value diatom cells (see Figure 3.4). Clearly, a reduction in the number of cells ingested is likely to reduce the carbon biomass ingested by an individual. The significant reduction in feeding upon diatoms, representing a high value food source will act to exacerbate the energy lost through reduced feeding. Such losses in energy are likely to have adverse effects upon the individual, and potentially the health of the ecosystem as a whole.

The calanoid copepod, *C. helgolandicus*, represents a well-studied species and as described above, exposures to microplastics by Cole (2014), have caused a

reduction in carbon uptake. Following exposure to 20 µm polystyrene microspheres in the study presented here, *C. helgolandicus*, suffered a loss in the average carbon biomass ingested, caused by a reduction in average ingestion rate of total algae cells (see Figure 3.6). The average ingestion of unidentified cells sized 10-20 µm, dinoflagellates and pennates was lower in the treated group compared to the control (see Figure 3.7), however, due to the large amount of variation that occurred between samples none of these differences were statistically significant. However, the average number of diatom and ciliate cells ingested was higher in the treated group compared to the control, this trend was significant for diatoms with a p-value of 0.046, but was insignificant for ciliates. These changes suggest an alteration in feeding behaviour in the presence of microplastics.

In work carried out by Cole (2014) it was recorded that *C. helgolandicus* showed a switching behaviour. Individuals feeding upon *Thalassiosira weissflogii* alone ingested all size ranges of the species (11.6–17.0 µm), and those exposed to 20 µm polystyrene spheres only ingested cells in the size range of 11.6–14.8 µm. Such a shift in feeding behaviour may be the cause of a reduction in carbon uptake recorded in Cole's (2014) study as feeding upon smaller algal cells represents feeding upon a less valuable food source, as described by Irigoien (2000). Although it was thought by Cole (2014) that this switch in prey size is unlikely to have a major ecological effects due to the vast range of phytoplankton available to species in the wild. However, it could be that in times of low nutrient quality the adverse effects are seen at their greatest, further worsening conditions for species when low food availability exists. The results presented here, however, show an increase in feeding upon larger diatom cells, which is an unexpected finding. The increase in feeding upon diatom cells may potentially represent another switching behaviour displayed by *C. helgolandicus*, preferentially feeding upon non-spherical cells so as to avoid the low quality plastics. Previous research has identified that calanoid copepods, such as *C. helgolandicus,* have the ability to select for particles according to quality (Huntley, et al., 1983; Cowles, et al., 1988), size (Mullin, 1963; Richman & Rogers, 1969; Frost, 1972) and shape (Price, 1988). Therefore, it can be suggested that *C. helgolandicus* may be able to assess that the polystyrene microspheres are of low nutritional quality, of spherical shape

and in a particular size range and alter its feeding behaviour so as to only accept non-spherical or smaller prey.

The ambush feeder, *O. similis*, unlike the three other species examined here, did not appear to suffer from the negative effects that resulted from microplastic exposure as recorded in previous studies (Cole, 2014). Upon studying Figure 3.8 it is clear that no significant difference occurred between the ingestion rate of total cells and associated carbon biomass uptake of control and treated groups. The results support the prediction that little or no effect will be suffered by an ambush feeder following exposure to microplastic. By feeding in such a manner, non-motile particles such as microplastics are unlikely to be detected since they do not create a disturbance within the water column (Kiørboe, et al., 2009). This being said, Figure 3.9, displaying the ingestion rates of specified prey types suggests that alterations in feeding behaviour have been caused by the presence of microplastic particles. The ingestion of non-spherical prey, diatoms and pennates, was significantly higher in the treated group compared to the control. Such a finding is unexpected as *O. similis* feeds as an ambush predator and by feeding in this manner should not detect non-motile prey such as diatom and pennate cells. The findings presented in this study, therefore, suggest *O. similis* may possess the ability to switch feeding strategies in a similar manner to *A. tonsa*.

3.4.3.2 *Drivers of altered feeding in the presence of microplastics*

There are a number of factors which may play a role in the alteration of feeding activity of zooplankton, as a result of microplastic exposure. The first of which may be due to a false sense of satiation which may act to reduce feeding rates in organisms. Such processes could be attributed to the recorded extended gut retention times seen in microplastic exposure studies, including those carried out upon zooplankton (Cole, et al., 2013), and other organisms such as mussels (Browne, et al., 2008). Increased gut retention times also increases the length of time for the chemical constituents of plastics to leach into organisms and cause an adverse effect (Setälä, et al., 2014). The decreases in fitness that arise from such stresses may result in reduced feeding activity or an alteration in feeding activity, as the organism may not be able to feed efficiently. Another negative effect of the extended retention times of microplastics is the possibility

of particles to clump and knot (Murray & Cowie, 2011), a process seen in decapod crustaceans, which again is likely to alter feeding in some way.

The ability of copepods to select particles in terms of their food quality has been examined and will be discussed in detail in Chapter 4. It is argued that the copepod species studied possess the ability to actively accept or reject particles based on their quality (Donaghay & Small, 1979). However, in essence quality selection is in some way size selection (Irigoien, et al., 2000), as with increased size, particles offer a larger amount of biovolume to be ingested so increasing nutritional value. Therefore, if we assume that a copepod species is able to detect that a microplastic particle of a certain size is of low nutritional value and they are in high density so that the encounter rate of such particles is high, the copepod may actively reject particles in the size range of the microplastic particles. This may be the mechanism behind the switching behaviour seen in this study and in studies such as those carried out by Cole (2014) where an exposure to uniformly sized polystyrene spheres was used. However, it is unlikely that such behaviour would be observed in the wild as it is not likely that microplastics are of a uniform size, shape and structure.

The lack of a switching behaviour shown by *Porcellanid* larvae suggest that, unlike copepods, decapod larvae do not possess the same selective behaviour as displayed by copepods during feeding. As a result it can be assumed that different mechanisms are driving the reduction in feeding by *Porcellanid* larvae when microplastic particles are present. Cole et al. (2013) recorded that *Porcellanid* larvae are capable of ingesting microplastic particles (Cole, et al., 2013). Therefore, reduced feeding is more likely caused by individuals feeling a false sense of satiation, as described by (Gregory, 2009; Wright, et al., 2013), (Murray & Cowie, 2011), (Cole, et al., 2013).

Similarly to copepods, *Porcellanid* larvae possess appendages used in feeding and swimming; For example the endopodites are used during feeding and require frequent cleaning (Gonor & Gonor, 1973) by the individual to ensure feeding efficiency. Microplastics have been recorded to adhere to feeding appendages (Cole, et al., 2013) as described in Chapter 2. Therefore, if particles adhere to the feeding appendages of *Porcellanid* larvae as has been studied with copepods, it may disrupt normal functioning of the endopodites,

thus, reducing feeding efficiency and the number of cells ingested. The generation of a feeding-current by *C. helgolandicus* and *A. tonsa* during feeding is likely to increase the likelihood of adherence of microplastic particles to feeding appendages, due to the continual movement of appendages in order to generate the current. Such adherence is likely to negatively affect feeding by reducing the ability to catch prey cells.

3.4.3.3 *The effects of Phaeocystis upon grazing studies*

During the preparation of studies involving *C. helgolandicus* and *O. similis* a *Phaeocystis* bloom occurred in the Western Channel. As a result, the natural assemblage of algae used during the investigation was dominated by *Phaeocystis*, a species argued to have multiple effects upon zooplankton feeding ecology (Turner, et al., 2002). *Phaeocystis sp.* is common in phytoplankton communities and its range spreads across worldwide waters in coastal and open ocean systems (Kashkin, 1963). The species is problematic as it possesses a rapid growth rate allowing it to become dominant over a few days (Bautista, et al., 1992). The life cycle of *Phaeocystis* alternates between a solitary stage of motile flagellated cells sized 3.5 µm which group to form non-motile colonies of up to 10 mm in diameter (Gieskes & Kraay, 1975; Verity, et al., 1988). The alga is believed to have negative effects upon the fitness of zooplankton due to the fact that it is a member of the *Prymnesiophyceae* family, recognised as producing toxic compounds (Bautista, et al., 1992). As well as this, the concentration of polyunsaturated acids and vitamin C is low in *Phaeocystis* (Claustre, et al., 1990), meaning that it may not represent a nutritionally valuable food source for zooplankton (Bautista, et al., 1992). However, research conducted by other groups suggests that *Phaeocystis* may not be as problematic as first predicted. *C. helgolandicus* fed *Phaeocystis* received adequate energy for egg production and survivorship and hatchling success was high, suggesting that *Phaeocystis* does not have a toxic effect upon *C. helgolandicus* (Turner, et al., 2002). The presence of *Phaeocystis* then may not have altered results presented in this study for *C. helgolandicus*. Although it was conceded by Turner et al. (2002) that the Mediterranean strain of *Phaeocystis* used in their study may not induce as toxic effects as those that inhabit the North Sea and Polar regions known to produce toxicants such as acrylic acid (Turner, et al., 2002). Therefore, it can be argued that the results of

this study may have been significantly affected by the presence of *Phaeocystis* in samples reducing the fitness of individuals and altering feeding behaviour. Hence, its presence may be a factor in the occurrence of negative ingestion rates which were omitted from analysis. As a result it would be highly beneficial to repeat the experiment at a time where *Phaeocystis* is absent from the phytoplankton community to remove any possibility that its presence altered results.

3.4.3.4 *Limitations of research*

As well as the possible negative effects of *Phaeocystis*, a number of other limitations may have altered the results studies presented here. The first and most apparent limitation is the extent of omitted data following the production of negative ingestion rates in a number of samples. Secondly, the use of 3 *C. helgolandicus* and 5 *O. similis* per 74 mL may have overstocked bottles and resulted in stress on copepods and insufficient prey availability, thus, altering behaviour. Fluorescent microspheres were not used in studies upon *A. tonsa* and *Porcellanid* larvae, hence imaging to assess ingestion of particles was not possible. By imaging individuals further insights into the drivers of altered feeding behaviour could have been made. Overall it is felt that grazing studies such as those carried out here can be effective in analysing the effects of microplastics and other contaminants upon the ingestion rates and associated carbon biomass uptake by zooplankton species. The use of FlowCAM to categorise algae into specific prey types provides an interesting and insightful manner in which to examine results, and allows for additional consideration of the mechanisms that may be driving any patterns observed in altered uptake of cells and carbon.

3.4.3.5 *Conclusions*

Although limitations of this study are apparent and not all results are supported by statistical significance, the discussion above allows for additional consideration of the role of plankton type and feeding type in determining the effect that the presence of microplastics has upon the feeding behaviour of zooplankton. It is felt that by repeating studies in the absence of *Phaeocystis*, and using a larger vessel volume for studies upon *C. helgolandicus* and *O. similis* that results will be more reliable allowing for statistical testing and

confirmation of findings. Results suggest that species displaying ambush feeding strategies feeding upon motile prey are less affected by the negative effects associated with microplastic exposure compared to those species that feed by generation of a feeding current. Meroplankton species appear negatively affected by microplastic exposure in a similar manner to holoplankton, however, additional research is required to confirm findings. Microplastics seem to alter the feeding behaviour of zooplankton in terms of size and type of prey ingested, such changes have the potential to have ecosystem effects where microplastic pollution is high.

Overall the results presented in this study provide additional support for the growing amount of evidence that exposure to microplastic particles causes an energy deficit to marine organisms (Wright, et al., 2013; Cole, 2014). Such a response is highly concerning due to the vital role played by zooplankton in transferring energy to higher trophic levels in the ecosystem.

Chapter 4

Assessing the detection and subsequent acceptance/rejection of microplastic particles by marine copepods

The ability of copepods to accept or reject potential food particles has been a subject of investigation for a number of years. The opportunity now exists to record subjects in high detail using high-speed video recording equipment, allowing the researcher to clearly examine the physical characteristics of interactions between prey and individuals. During this chapter the ability of copepods to select particular prey will be reviewed, before introducing an exploratory study investigating microplastic ingestion by *Temora longicornis.* A study carried out in order to examine the species' ability to reject microplastic particles.

4.1 *Prey selection in copepods*

Zooplankton represents the first trophic level in marine food webs, playing a fundamental role in making primary production available to higher trophic levels (Tiselius, et al., 2013). It is, therefore, of great importance to the ecosystem as a whole that zooplankton species such as copepods are able to detect and feed upon prey effectively, and select prey of high nutritional value. The manner by which zooplankton typically detect and capture prey is described in Chapter 2, however, it has been found that a number of zooplankton species possess the ability to discriminate between particles of different quality (Donaghay & Small, 1979). Copepods, particularly calanoid species of the genus *Calanus*, have been studied intensively, analysing the feeding behaviour they display in terms of selecting prey. For example it has been noted that *Calanus* species possess the ability to reject or actively select prey cells that they consider suitable for ingestion (Leising, et al., 2005). The abilities of studied copepod species to select prey and distinguish one prey species from another will be evaluated, in order to identify whether such behaviours are likely to affect the ingestion of microplastics in copepod species.

Many studies address the issue of size of prey particles, examining whether there is any preference displayed by groups of individuals when offered prey of differing sizes. Frost (1972) found that there was a greater uptake of larger cells over smaller particle as a higher feeding efficiency is apparent when feeding upon larger cells. In Frost's experiments *Calanus pacificus* fed on both large and small particles, but handled the larger particles with much greater effectiveness (Frost, 1972). This study mirrored views by Mullin (1963) and Richman and Rogers (1969) who also noted that copepods seemed to prefer

larger cells over smaller ones. Other studies also note that small particles are not effectively captured; the lack of feeding on pelagophytes and cyanobacteria by *Calanus helgolandicus* in feeding experiments carried out by Irigoien (2000) was believed to be due to size selection and the reduced effectiveness of capturing smaller prey items (Paffenhöfer, 1988; Kleppel, et al., 1998)

The ability of calanoid copepods to discriminate between particles of different food quality has also been addressed in a number of studies (Huntley, et al., 1983; Cowles, et al., 1988). However, it could be argued that size selection described above is in essence quality selection (Irigoien, et al., 2000). The reason for this is that a larger particle represents an important increase in the volume of cytoplasm ingested (Irigoien, et al., 2000), therefore increasing the nutritional value of the cell. As a result a zooplankton species may be at a greater advantage ingesting fewer large cells retaining energy, rather than actively searching and feeding upon a large number of small cells. Though, it has been found that copepods are better able to handle large cells compared to small cells when feeding and tend to be inefficient at feeding on particles smaller than 5-10 µm (Nival & Nival, 1976; Berggreen, et al., 1988).

This suggests that rather than selecting for prey because of its size or nutritional value, copepods simply feed on prey cells most efficiently captured. This being said, a number of zooplankton species have been found to discriminate between particles of different quality (Donaghay & Small, 1979). Some calanoid copepods have been studied to distinguish between valuable (phytoplankton cells) and non-valuable particles (polystyrene beads) either by rejecting un-natural prey (Donaghay & Small, 1979) or by only ingesting them in low quantities (Frost, 1972; Fernandez, 1979; Huntley, et al., 1983). However, other calanoids such as *C. helgolandicus* ingest large quantities of low value particles when natural prey is also offered (Paffenhöfer, 1972; Paffenhöfer & Sant, 1985). However, this behaviour may be deliberate action by the species to diversify the diet, a behaviour seen in other studies where there is higher selectivity for other prey types, when one prey species is dominant (Irigoien, et al., 2000). This behaviour may ensure that the species obtains all the essential elements and nutrients it requires for full fitness.

The action of actively selecting for variability in the diet appears common place across many studied calanoid species. For example, in grazing experiments

conducted by Irigoien et al. (2000) upon *C. helgolandicus* it was found that the species displayed great variability in prey selection. It was suggested that *C. helgolandicus* may vary its diet by actively selecting prey items other than the most abundant available (Irigoien, et al., 2000). It appears clear, then, that significant differences exists in the abilities of distinct species in selecting for prey.

Prey selection due to shape and taste has also been recorded in some species (Price, 1988; Atkinson, 1995). The taste of particular toxic dinoflagellates, for example, caused rejection by copepods (Huntley, et al., 1986). More recent studies have also displayed an ability of some zooplankton to actively reject cells that could cause toxic damage to them (Leising, et al., 2005). It was suggested by Huntley (1986) that copepods may use chemical signals in order to identify and actively reject unsuitable or toxic prey items. This theory was echoed by studies carried out by Leising et al. (2005) upon the calanoid copepod, *Calanus pacificus,* where it was noted that this species may have "considerable abilities" to reject or actively select particles considered most suitable for ingestion. Toxic particles may be avoided due to the selection pressure that exists if the prey item has the potential to adversely affect reproduction (Leising, et al., 2005).

The selection mechanisms used by zooplankton have a vital role in the dynamics of the marine ecosystem as a whole. The reasons for this is that by feeding upon prey items of different nutritional quality, or toxic value, the resulting growth and development of the copepod may be altered (Kleppel, 1993; Kleppel & Burkart, 1995; Ban, et al., 1997; Hygum, et al., 2000; Irigoien, et al., 2002; Paffenhöfer, 2002; Ianora, et al., 2004; Leising, et al., 2005), affecting both the individual and its future progeny. Therefore, the selectivity of prey items made by a particular species has the potential to affect the energy transfer to higher trophic levels. This could be a concern as many zooplankton species, such as *C. helgolandicus*, are key prey species for many species of commercially important fish larvae (Cole, 2014). Microplastics therefore, if selected for due to size parameters or lack of chemical cues, may negatively affect energy reserves and growth in zooplankton as they represent little or no nutritional value. As recorded above (Chapter 3) and in previous studies (Cole, 2014), the ingestion of microplastic particles appears to affect zooplankton

health and potentially the health of commercial fish larvae stocks in the local environment.

4.2 *The utilisation of high-speed filming in microplastic research*

High-speed filming techniques offer a great opportunity to study an interaction for which the exact mechanisms remain unknown and hard to examine. Such equipment has been used effectively by research groups studying the mechanisms by which zooplankton feed and move through the water column, particularly by the Kiørboe group at the Denmark Technical University (see Kiørboe, 2011). Through grazing studies and implementing fluorescent microscopy it has been shown that a range of zooplankton species do ingest microplastics (Cole, et al., 2013) and the effects of such ingestion include reductions in fitness and fecundity (Cole, 2014), as well as, changes in feeding behaviour, as described in Chapter 3. However, due to the small size of microplastics and copepods themselves, the exact mechanisms behind the interactions between individuals and particles and subsequent behaviours are little understood.

High-speed filming allows the researcher to study the way that a microplastic particle is handled by an individual and allows for the investigation into whether such copepod species are able to actively reject microplastic particles. As well as this, by introducing natural prey, there is the opportunity to examine size or food quality selectivity behaviour in more detail. Such research sheds light on the abilities of copepods to cope with microplastics within their environment, once more allowing for environmental effects to be inferred.

With this in mind a study was designed to best display the range of behaviours of copepod species when exposed to microplastics in detail. Subsequent Imaging of individuals exposed to fluorescent microplastic particles would then provide clear evidence for ingestion of microplastic particles, which may not be caught on film, as well as any evidence of adherence of particles to individuals. The research would then allow us to further understand copepod’s abilities to accept/reject prey particles based upon their nutritional value, chemical composition, shape and structure.

4.3 Using high-speed filming to assess the ability of *Temora longicornis* to accept/reject microplastic particles

4.3.1 Methods

4.3.1.1 *High-speed video analysis of microplastic ingestion by T. longicornis*

4.3.1.1.1 *Experimental set-up*

Over 20 individuals of *T. longicornis* cultivated under laboratory conditions at Denmark Technical University (DTU) of varying life stages were added to a 50 mL filming tank and filming took place for approximately one hour per treatment. A new filming tank and set of copepods were utilised for each treatment.

4.3.1.1.2 *Microplastic suspensions*

In order to examine the effects of a range of microplastic treatments on the feeding behaviour of *T. longicornis*, six microplastic suspensions were set-up to be added to filming tanks for filming; 200 µL 10 µm Yellow Fluorescent Polystyrene spheres (Spherotech) were added directly to the 50 mL filming tank; 200 µL 20 µm Yellow Fluorescent Polystyrene spheres (Spherotech) were added directly to the 50 mL filming tank; 25 µL 30 µm polystyrene beads (Fluka) added directly to the 50 mL filming tank; 25 µL 30 µm polystyrene beads (Fluka) added directly to the 50 mL filming tank alongside *Rhodomonas salina* prey; 25 µL 30 µm polystyrene beads (Fluka) stored in 1 mL effluent from *R. salina* culture overnight to attempt to give particles a biological signal and added directly to the 50 mL filming tank; And finally, 25 µL 30 µm polystyrene beads (Fluka) were suspended in 1 mL seawater taken from copepod culture vessels overnight, to encourage biofilm formation, and added directly to the 50 mL filming tank.

4.3.1.1.3 *High-speed filming*

Interactions between individuals and microplastics were filmed using a Phantom v210 high speed camera, at 1000 frames per second. After an interaction was viewed on screen, video was recorded for 5 seconds previous to the interaction in order to capture the entirety of the interaction. Infra-red lighting was used during filming so that behaviour was not altered by the presence of natural light.

4.3.1.1.4 *Analysis of High-speed videos*

Following recording, videos were placed into three classifications; capture and ingestion; capture and rejection; and miss. The life stage of the individual was recorded, along with the number of spheres interacted with and a summary of actions observed in the recording. Freeze-frame images were produced from recordings to summarise the key actions of the interaction between copepod and microplastic particle.

4.3.1.2 *Ingestion of microplastic particles by the marine copepod, T. longicornis*

4.3.1.2.1 *Experimental set-up*

Over 20 individuals of *T. longicornis* cultivated under laboratory conditions at DTU of varying life stages were added to a 50 mL culture bottle and exposed to high densities of 10 µm and 20 µm Yellow Fluorescent Polystyrene spheres (Spherotech) for 2-3 hours.

4.3.1.2.2 *Microplastic suspensions*

Microplastic suspensions of 10 µm and 20 µm Yellow Fluorescent Polystyrene spheres were produced as described above (see Section 4.3.1.1.2).

4.3.1.2.3 *Imaging of microplastic ingestion*

Following exposure, specimens were preserved in 5% ethanol solution and transported back to the imaging unit at Exeter University's Aquatic Resource Centre. Individuals were then placed on slides and imaged using the TBM1000 microscope with Prior V31LD4 fluorescent emission attachment and QIClick™ camera. A fluorescence wavelength of 475 nm was used and camera settings set to optimise the view of fluorescence. All individuals were imaged, and all examples of ingestion and adherence of microplastic particles were recorded.

4.3.2 Results

4.3.2.1 *High-speed filming of copepod feeding behaviour when exposed to microplastic particles*

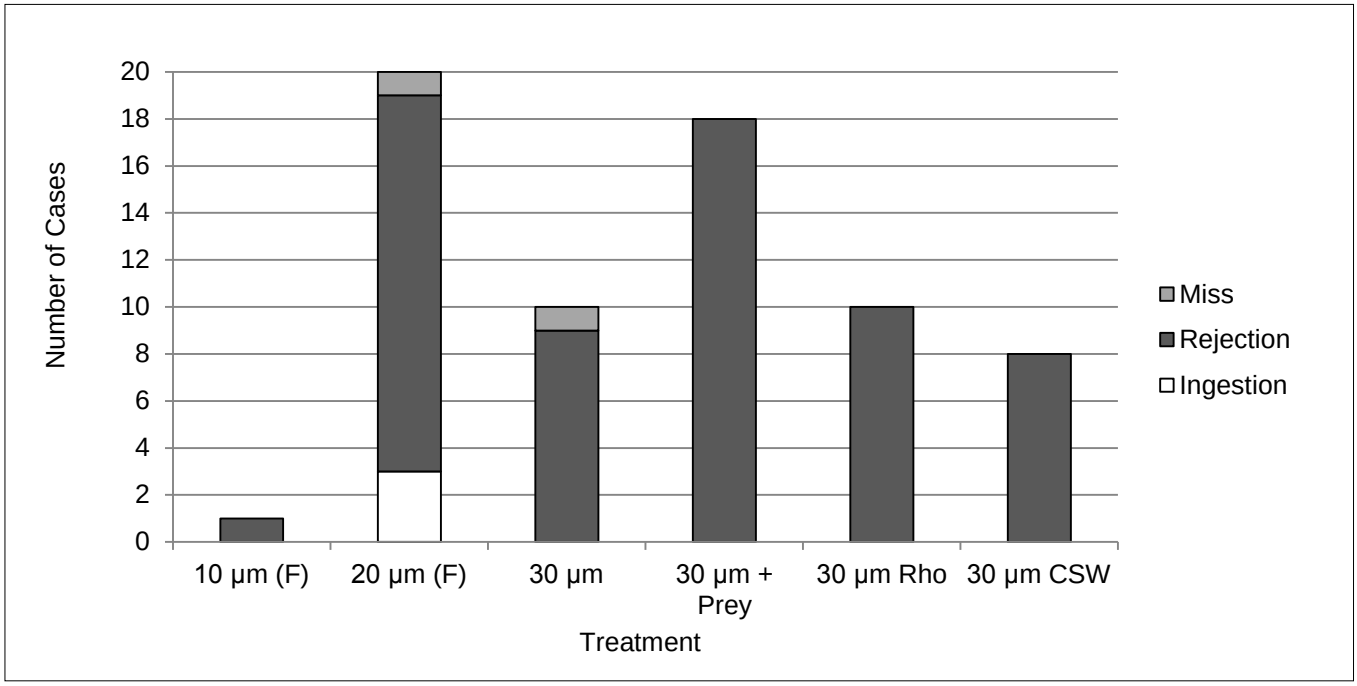

Figure 4.1 – Bar chart summarising video analysis of acceptance/rejection behaviour of *T. longicornis* when exposed to a range of microplastics particles. F indicates fluorescently labelled polystyrene spheres; + Prey indicates filming where *R. salina* was provided as prey alongside microplastic particles; Rho indicates beads treated with effluent water from *Rhodomonas* algal cultures; CSW indicates spheres treated with unfiltered seawater taken from copepod culture vessels.

The results above (Figure 4.1) summarise the data obtained by studying the 68 videos recorded when exposing copepods to a varying range of microplastic suspensions. 3 out of the 68 recordings displayed a capture and ingestion behaviour by *T. longicornis*, 1 video did not clearly show acceptance/rejection behaviour and in the remainder of recordings the microplastic particle was rejected or missed. Acceptance behaviour was only recorded with 20 µm fluorescent polystyrene spheres. All other treatments and microplastic sizes displayed only capture and rejection behaviour in video analysis apart from two cases where the particle was missed. The three cases of ingestion were seen in interactions involving adult *T. longicornis*, no nauplii were recorded to accept the microplastic spheres. A table is provided in Appendix 3, displaying summary data for each of the videos used for analysis.

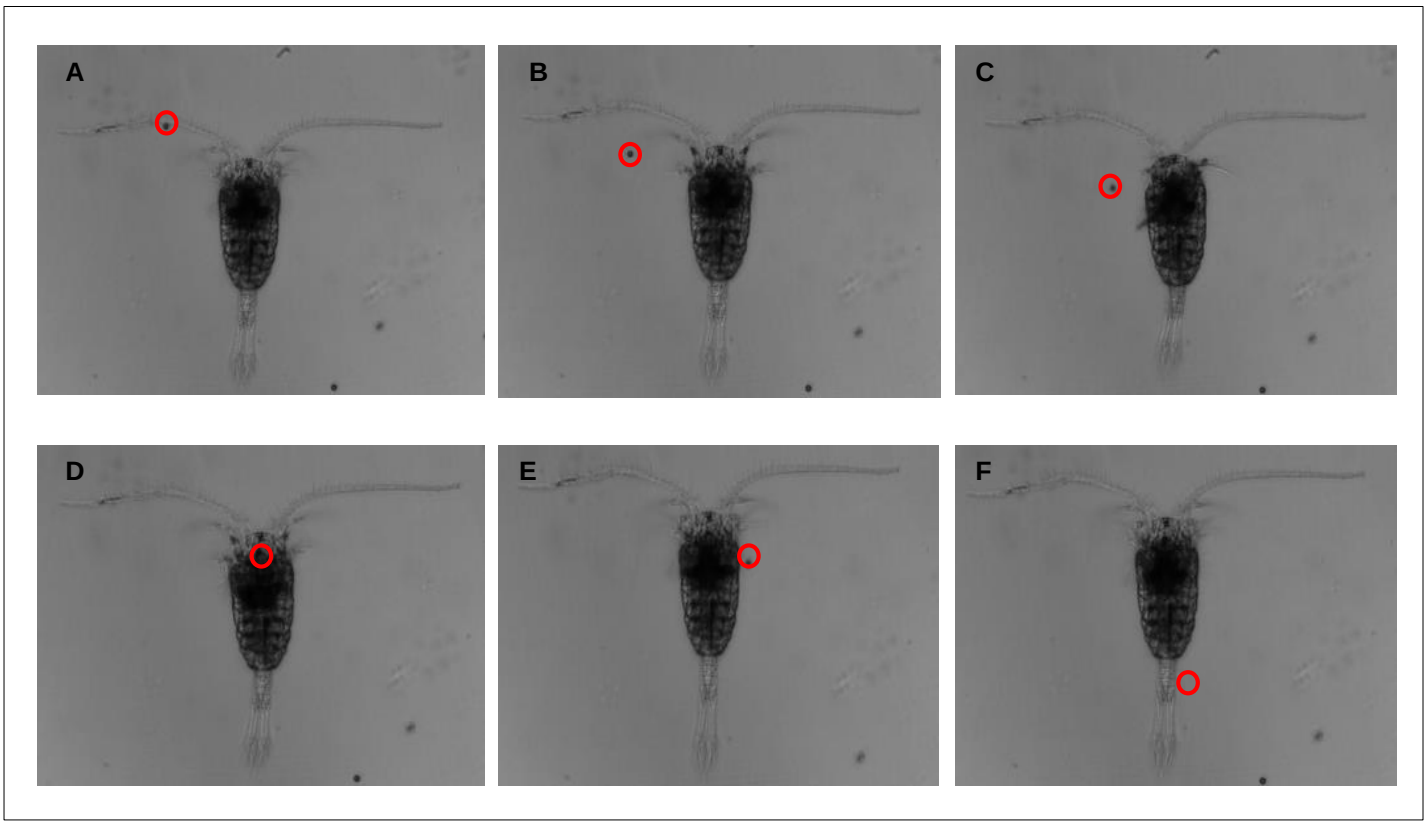

Figure 4.2 Freeze-frame images taken from high-speed video recording of microplastic particle (30 µm) rejection by an adult *T. longicornis* (vertical view). The red circle indicates the position of the microplastic particle.

Figure 4.2 displays the sequence of events that take place in the rejection of a microplastic sphere (30 µm) by a *T. longicornis* adult, filmed in a vertical view. Image A shows the microplastic sphere, circled, being drawn towards the copepod due to the generation of a feeding current. In Image B the sphere is detected by coming into contact with the setae of the copepod. The individual then redirects its feeding-current to transport the microplastic to its mouthparts (Image C). Once at the mouth the copepod appears to try and “bite” the particle as seen in Image D. Image E then shows the copepod rejecting the particle having failed to ingest or actively rejecting the microplastic, which then re-enters the water column (Image F).

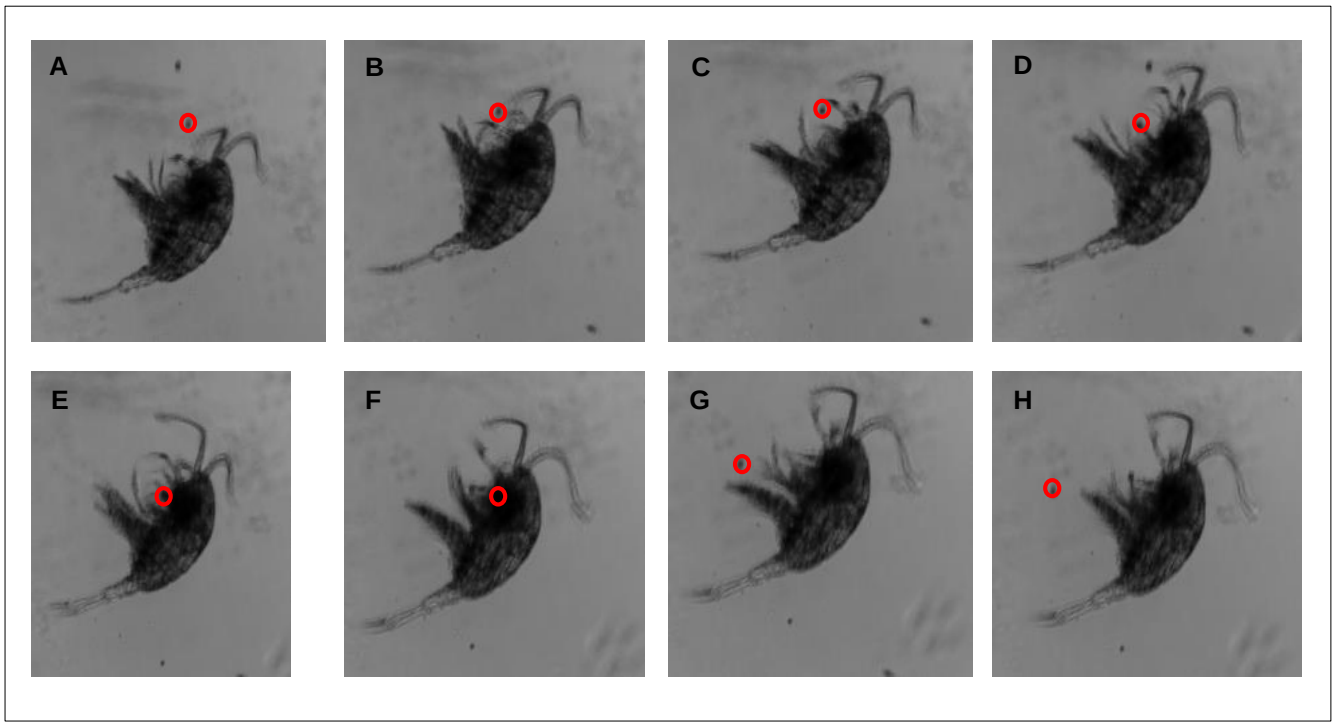

Figure 4.3 Freeze-frame images taken from high-speed video recording of microplastic particle (30 µm) rejection by an adult *T. longicornis* (lateral view). The red circle indicates the position of the microplastic particle.

The images above (Figure 4.3) display the rejection of a microplastic sphere (30 µm) by an adult *T. longicornis*, from a lateral view. Image A shows the microplastic particle being directed towards the copepod as a result of the feeding-current being generated by the individual. The particle is then detected by the copepod once it has made contact with the setae, or feeding appendages (Image B). The individual then proceeds to use its feeding appendages to re-direct its feeding-current (Image C), and in doing so directs the particle towards its mouthparts (Images D and E). Once at the mouth the copepod attempts to ingest the particle by using a “bite-like” behaviour (Image F). However, the particle is then rejected by the upward motion of the swimming legs producing a small current to take away the particle (Images G and H).

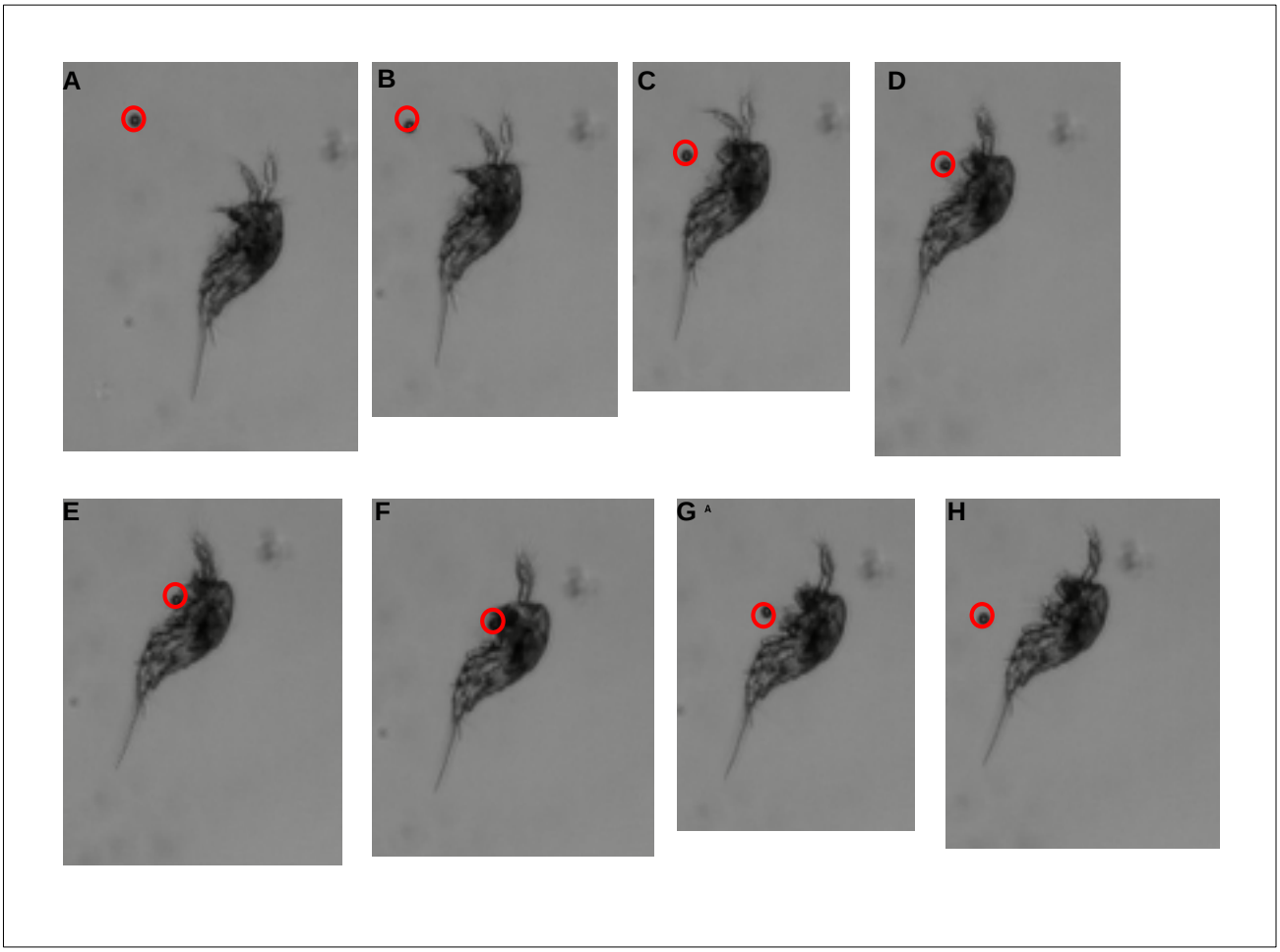

Figure 4.4 Freeze-frame images taken from high-speed video recording of microplastic particle (30 µm) rejection by a *T. longicornis* nauplius (lateral view). The red circle indicates the position of the microplastic particle.

Figure 4.4 shows the rejection of a 30 µm microplastic particle by a *T. longicornis* nauplius filmed in a lateral view. Images A and B show the particle being pulled towards the individual due to the generation of a feeding-current. The particle is then detected when coming into contact with the feeding appendages of the nauplius, which subsequently uses its feeding appendages to re-direct its feeding-current to direct the particle towards its mouth (Images C and D). Once at the mouthparts the nauplius attempts to “bite” the particle (Images E and F). However, it seems that the particle is either too big or robust to be ingested by the nauplius, and is subsequently rejected or dropped by the individual and sent back into the water column by the generation of a small current by the upward motion of the swimming legs (Images G and H).

4.3.2.2 *Imaging of T. longicornis exposed to Yellow Fluorescent Polystyrene Spheres*

Figure 4.5. Ingestion of Yellow Fluorescent Polystyrene Spheres by *T. longicornis*. A – Ingestion of 20 µm polystyrene spheres, spheres visible in the upper and lower digestive tract. B – Ingestion of 20 µm polystyrene spheres, spheres visible in the upper and lower digestive tract, adherence of spheres to the feeding appendages also evident. C – Ingestion of 10 µm polystyrene spheres, spheres visible in the upper and lower digestive tract

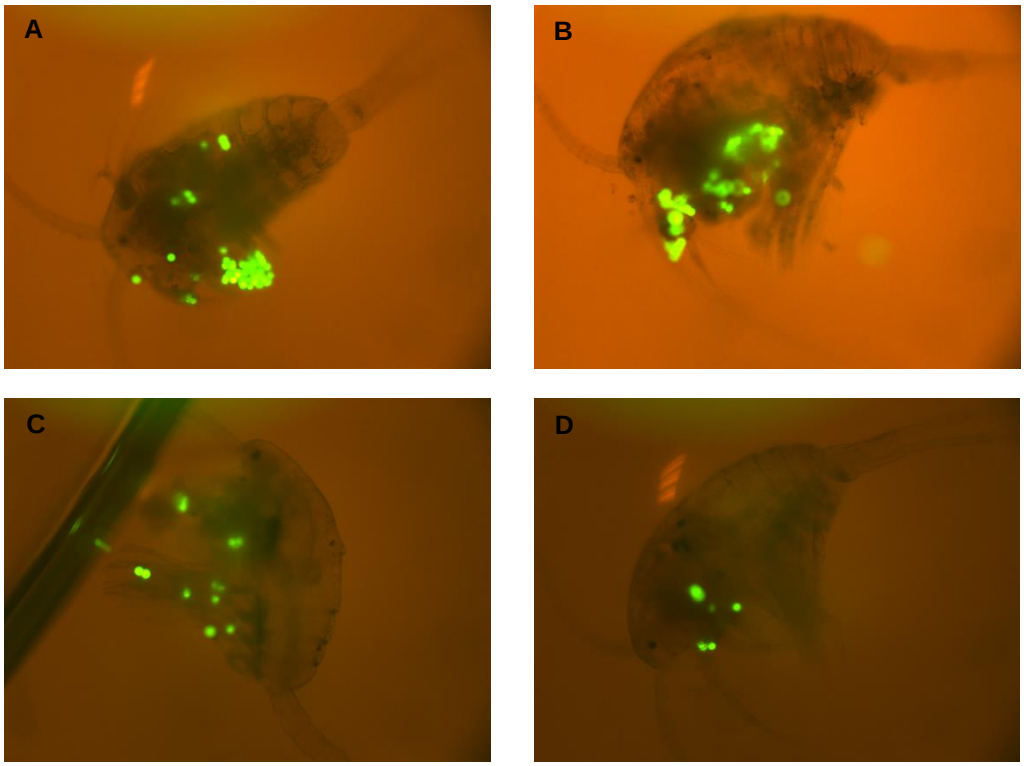

Figure 4.6 Adherence of 20 µm Yellow Fluorescent Polystyrene Spheres to *T. longicornis*.
A – Adherence of spheres to the feeding appendages, swimming legs and carapace. Spheres have appeared to aggregate. B – Spheres have adhered to the feeding appendages, and swimming legs. Aggregation of spheres has occurred. C – Adherence of spheres to the feeding appendages and swimming legs. D – Adherence of spheres to the feeding appendages.

Figure 4.5, presented above displays evidence for the ingestion of polystyrene microspheres by *T. longicornis*. Images A and B display ingestion of 20 μm, spheres, whereas, Image C presented ingestion of 10 μm spheres. In all images spheres are visible in the upper and lower digestive tract, where particles appear to aggregate. Images confirm the ingestion of microplastic particles by *T. longicornis* under laboratory conditions. The adherence of 20 μm polystyrene microspheres to *T. longicornis* is displayed in Figure 4.6. As can be seen a large range in the scale of adherence exists, with considerable adherence in Image A, compared to reduced adherence in Image D. Particles appear to adhere primarily to the feeding appendages and swimming legs. Once adhered, aggregation of microspheres can be observed, see Images A and B.

Table 7.2.2 provided in Appendix 2 summarises the results of fluorescent microscopy of 30 individuals of *T. longicornis* when exposed to 10 μm yellow fluorescent polystyrene spheres. Spheres were visible in 12 samples, of which 8 displayed adherence of particles to individual copepods, 1 displayed possible adherence and 4 individuals displayed ingestion of spheres, with another 4 displaying possible ingestion. The adherence of particles appeared to occur upon the feeding appendages and swimming legs of individuals. A range of scale of spheres adhered to individuals was observed. Those individuals which appeared to ingest the spheres had the particles visible in their digestive tract, or upper digestive tract. Of the samples where ingestion was confirmed, >5 spheres were visible. Adherence and ingestion was observed in both adult copepods and nauplii. A full set of images used for analysis is provided in Figure 7.2.1, Appendix 2.

Table 7.2.3, provided in Appendix 2 displays the summary data of the analysis of images taken using fluorescent microscopy of *T. longicornis* when exposed to 20 μm yellow fluorescent spheres. Of the 50 images analysed, 37 had spheres present, 31 displayed adherence of the spheres to the individual, a further 3 displayed possible adherence, 2 displayed clear ingestion of spheres (see Images 33 and 42 Figure 7.2.2, Appendix 2), and 8 images displayed possible ingestion. Similarly to results obtained from the analysis of images taken proceeding exposure to 10 μm spheres, the majority of adherence of spheres occurred on the feeding appendages of individuals, occurring in 30 of the 31 confirmed adherence cases. Adherence also occurred on the legs of

individuals, head and in one case the urosome (see image 20, Figure 7.2.2, Appendix 2). A vast range of the scale of spheres that adhered to individuals was seen once again, for example a large scale of adherence seen in Image 2 (Figure 7.2.2, Appendix 2), and a low range of adherence as seen in Image 46, (Figure 7.2.2, Appendix 2). Ingested spheres appeared to be present in the digestive tract or upper digestive tract. Like exposures to 10 µm spheres, adherence and ingestion was seen in both adult copepods and nauplii alike. A full set of images used for analysis is provided in Figure 7.2.2, Appendix 2.

4.3.3 Discussion

Active selection is described as “a behavioural response with active dietary choice”, where an individual rejects or accepts a prey cell based upon its physical or chemical properties (Kiorboe, et al., 1996). Passive selection, on the other hand, implies a more simple interaction where differences in encounter rates and handling or capture efficiencies, determine the ingestion of particular prey (Schultz & Kiørboe, 2009). It would appear by studying high-speed video evidence, that the copepod *Temora longicornis* and its nauplii in general possess the ability to reject microplastic particles in the size range of 10-30 µm once captured. However, the mechanism behind this rejection is one which is difficult to analyse. Much discussion is required to assess whether this rejection is an action carried out by the individual in response to the microplastic particle’s physical and chemical properties, or whether the rejection is more of a passive mechanism due to the mechanics of microplastic ingestion.

Fluorescent imaging showed that for 10 µm and 20 µm microplastic exposures both adult *T. longicornis* and nauplii displayed evidence for ingestion of microplastic spheres in accordance with previous studies (Cole et al, 2013). Therefore, contradictions occur between the findings of video analysis and imaging, raising questions over the mechanisms behind interactions between copepods and microplastics, and the effectiveness of high-speed filming for analysing such behaviour, all of which will be explored below.

T. longicornis can be described as a feeding-current strategist (Jakobsen, et al., 2005). Feeding by the generation of a feeding-current which draws particles out of the water column towards the individual, where they are subsequently detected and directed to the mouthparts and ingested (Kjellerup & Kiørboe,

2012). It is believed that feeding-current strategists are capable of both chemical and mechanical detection, detecting prey due to chemical cues (Tiselius et al, 2013) and by disturbances within the water column. The theory of chemical detection is based on the belief that a prey cell is surrounded by a chemical signal which elongates when under the influence of the feeding-current produced by a copepod, therefore, sending a chemical signal to the individual before the prey cell arrives itself (Tiselius et al, 2013). This allows the individual the opportunity to alter its feeding current to either direct the prey cell towards its mouthparts or back into the water column.

However, there is a growing argument among those studying the mechanisms behind prey detection by zooplankton, arguing that such long-range chemical detection is unlikely to occur and instead only mechanical detection is used to detect prey cells (Gonçalves, personal communication). In this case a prey cell is directed by the feeding-current of a copepod, which is produced at all times during feeding behaviour. Eventually the prey cell comes into contact with the setae, or feeding appendages, of the individual which then uses its feeding appendages to re-direct the cell towards the mouth and subsequently ingests the particle. Short-range chemical detection, or assessment, of potential prey cells once caught is thought likely to occur (Koehl & Strickler, 1981), with research suggesting that copepods possess the ability to taste (Price, 1988) and use chemoreceptors located on the setae (Friedman & Strickler, 1975) to assess the biochemical composition of the captured particle. Copepods are also believed to be able to display size and quality selection of prey particles (Frost, 1972; Donaghay & Small, 1979)

The videos recorded during this study support the views above, that long-range chemoreception is not likely to occur and it seems that detection of potential prey occurs after the contact of cell to the feeding appendages of the individual. This was observed in all cases where interactions occurred between copepods and particles. As well as this, microplastic particles produced for research are unlikely to have a significant biofilm on their surface that could produce a biochemical signal, and therefore if long-range chemoreception was apparent they should not be detected. However, as evident in recordings, feeding-currents are still produced in their presence. In all of the 68 videos analysed, detection of the particle occurred after coming into contact with the feeding

appendages of the individual (see Figure 4.2, Image B), after which the feeding appendages were used to direct the microplastic sphere towards the mouth (Figure 4.2, Image C). The individual then appeared to spend time handling the particle, before using a “biting” action (Figure 4.2, Image D) to attempt to ingest the particle. This handling time may be a use of chemoreceptors at the mouth and setae (Friedman & Strickler, 1975) to assess the biochemical composition of the particle just captured, suggesting the evidence of short range chemodetection.

However, it may also be that the microplastic particle is very hard to handle, or could be described as “sticky” due to surface charge. Therefore microplastic particles may be difficult to pass from the feeding appendages to the mouth due to adherence, a process evident from fluorescent imaging following exposures see Figure 4.6. The “biting” motion observed in the majority of videos may display mechanodetection, where the copepod is assessing whether the cell captured is appropriate prey. However, it appears that the copepod is attempting to ingest the microplastic, yet the particle is too hard to be ingested. Although similar in size to a natural algal prey cell, a microplastic particle is much firmer, and where an algal cell would be broken down and subsequently ingested, the microplastic cannot be broken by the copepod. It can be suggested that either the copepod rejects the particle actively as it comes to the conclusion that it is not normal food, or the microplastic is rejected passively simply because it cannot be physically ingested by the copepod. The rejection of the particle appears active because the copepod uses the action of lifting its swimming legs to produce a small current which serves to carry the particle back into the water column. Passive rejection would suggest that the copepod simply drops the particle and continues normal feeding behaviour.

During the investigation, microplastics were also offered in the presence of an algal prey source, *R. salina*. However, of the 18 interactions recorded, all were observed as rejection of microplastic particles, suggesting that individuals are able to detect the particles are not a natural prey source. It was also attempted to give the microplastic particles a chemical signal, to assess long-range chemodetection and examine whether short-range chemodetection at the mouth might lead to ingestion of the particle if given properties that might liken it to a biological cell. By suspending 30 µm polystyrene spheres overnight in algal

culture effluent and seawater taken from copepod culture vessels, it was believed that biofilm formation would occur (Zettler, et al., 2013). However, these particles were treated as described above and rejected in the same manner. Suggesting that chemoreception is unlikely to be the determining factor in rejection of microplastic particles. Rather, it appears mechanistic factors are driving the behaviour observed in videos. However, it must be considered that for the majority of treatments 30 μm spheres were used for this study, and it may be that this size was too large for the copepod to ingest, especially for nauplii. Though ingestion of 30.6 μm spheres by *T. longicornis* has been recorded in previous studies (Cole, et al., 2013). Therefore it would be of interest to observe the same study with smaller particles, and any findings presented here must be treated with caution and in an exploratory nature. It seems, then, from analysing video data alone that the copepod species, *T. longicornis*, possesses the ability to reject microplastic particles when offered as prey, and given a chemical signal. However, by utilising fluorescent polystyrene spheres to represent microplastic particles, the opportunity arises to examine whether any ingestion of particles which was not recorded on film exists. By doing this, the issue that only rejections are recorded due to the relative ease at which rejections are seen on screen when recorded, compared to ingestion, is negated. Imaging resulting from fluorescent microscopy allows for additional information regarding the interaction of copepods and microplastic particles to be recorded.

When we study the images collected from fluorescent microscopy for 10 μm (Figure 7.2.1, Appendix 2) and 20 μm (Figure 7.2.2, Appendix 2) polystyrene spheres it is clear that there is a large number of copepods encountering microplastics within a closed system. The majority of images where spheres are present display adherence of microplastics to the copepod, particularly to the feeding appendages (see Figure 4.6), such adherence has also been recorded by Cole et al. (2013). The adherence of microplastics to the feeding appendages of individuals, has been discussed in detail in Chapter 2, and has the potential to significantly reduce the fitness of the organism as feeding appendages play vital roles in feeding, swimming and mating behaviour (Cole et al, 2013). Adherence may act to reduce motility of feeding appendages, affecting the production of the feeding-current, or may act to disrupt the

detection of particles and subsequent capture. Although the majority of images display adherence of particles, a number of cases of ingestion or possible ingestion of microplastic spheres were apparent. Providing evidence that ingestion of microplastics by *T. longicornis* does occur, as found by (Cole, et al., 2013), recording ingestion of particles sized 7.3-30.6 μm. The ingestion of microplastics is potentially problematic, as discussed in previous chapters. However, the number of cases of ingestion versus rejection raises the question; why were only 3 out of 68 video recordings examples of ingestion? And, are individuals of *T. longicornis* rejecting more microplastic particles than they are accepting? Such questions could only be answered by combining a grazing experiment with high-speed filming. This study would be difficult to set-up due to the labour intensive nature of high-speed filming and the length of grazing experiments, typically run for 24 hours. However, despite the difficulties in set-up, such a study would be greatly beneficial in microplastic ingestion research, and thus, should be considered in the future.

High-speed filming represents a novel and highly interesting manner of studying the interaction between individuals and particles within a laboratory environment. However, there are uncertainties in results where interactions recorded on film, such as those presented in this study, may repeatedly show the same individual interacting with particles rather than a set number of recordings of each individual placed into the filming chamber. This may result in findings that are not representative of the entire group, rather are individual responses. For example, of the 68 recordings used for analysis in the study presented here, it is not possible to state how many videos were of each individual copepod. Such a limitation could be addressed by only filming one individual at a time, however, such a set-up is not likely to produce many recorded interactions and is likely to be very time consuming. If a technique could be developed to identify individual members of the group so that recordings could be analysed in terms of each individual's response it would be highly beneficial. However at this time such a method does not exist, and it is felt that the probability of the same individual being filmed repeatedly is unlikely. As such in analysing recordings we assume that interactions are representative of the group, however, we treat the evidence with caution regarding the

limitations discussed and experimental nature of research, thus, it is used alongside consideration of findings from other studies.

Through consideration of the findings outlined in this study, and in reference to the mechanisms by which prey is detected and subsequently captured by different feeding strategies it is proposed here, that, feeding strategy is likely to influence microplastic uptake by zooplankton. As described in Chapter 3, feeding-current producing zooplankton perceive prey chemically and such possess the ability to select prey based upon the biochemical composition of the particle (Tiselius et al, 2013). This mechanism in theory should reduce the likeliness of a feeding-current feeding individual ingesting a microplastic particle which does not carry a biological signature. However, it has been studied that microplastics in the marine environment can develop biofilms, hence giving the particle an altered biochemical composition which might be perceived by the predating zooplankton. This pattern has been observed under laboratory conditions where clean particles used in experiments were less likely to be ingested than older particles that have developed biofilms (Lobelle et al, 2011). However, results of this study suggest that mechanistic rejection is the main driver behind rejection of microplastic particles by *T. longicornis*, and that long-range chemodetection appears unlikely to occur. However, short-range chemodetection may be apparent in zooplankton displaying both ambush feeding and feeding-current feeding, allowing for rejection of a non-prey item following capture.

High-speed filming has been identified as a novel and highly interesting method by which to examine interactions between microplastic particles and zooplankton, and the opportunities for future research are endless. Filming allows multiple endpoints to be collected, such as; real handling time of particles, ingestion rate, the effects of adherence, length of adherence of particles and effects upon locomotion. It is the belief of the researcher here that focus should be put upon the differences in interactions between copepods displaying the two major feeding strategies; ambush feeding and feeding-current strategists; in order to assess the differences in microplastic uptake and detection as proposed above. Assessing any differences in uptake of aged particles more representative of those found in the natural environment, compared to “clean” particles typically used in the laboratory would be highly

beneficial. By combining high-speed filming and the use of fluorescent particles and microscopy it is thought that a comprehensive study into the ingestion of microplastic particles and the specific mechanisms behind such ingestion can be achieved.

Chapter 5

Microplastics; small problem? Or major issue?

The work presented in this thesis provides further evidence for the ingestion of microplastics by a range of zooplankton taxa under laboratory studies as recorded by Cole et al. (2013) and Setälä et al. (2014). In addition, a new insight into the effects that microplastic exposure has upon the feeding behaviour of zooplankton is presented. Through the utilisation of a novel technique in the field of microplastic research, high-speed filming, the ability of copepod's to reject microplastic particles is assessed, and a new way in which interactions between microscopic plastic debris and zooplankton can be investigated is introduced.

To date, it is primarily only polystyrene microspheres that have been utilised in order to assess microplastic ingestion by zooplankton. Such particles have high effectiveness in laboratory study, however, being of uniform shape and size do not accurately represent those particles likely to be present in the environment. The research presented in Chapter 2 aimed to investigate the ingestion of three plastic types, considered abundant in the marine environment; primary microplastics, secondary microplastics and fibrous microplastics. As a result of this study ingestion of all plastic types was recorded in the calanoid copepod, *Centropages typicus*, suggesting that ingestion of microplastic particles within the environment is likely. As well as supporting previous findings regarding microplastic ingestion, the study outlined in Chapter 2 presents a novel and effective way in which to fluorescently label plastics for use in ingestion studies (see Section 2.3.1.1, Chapter 2). The RADGLO labelling protocol opens a wide range of future research opportunities, allowing for the study of ingestion of common plastic contaminants or those collected from environmental sampling, so as to further increase our knowledge in this area. Although in study used to examine individual effects, such particles labelled with RADGLO could be used to examine tropic level transfer of microplastic particles collected from the environment. Studies such as those carried out by Setälä et al. (2014) could be adapted so as to assess whether particles are passed onto a higher trophic following feeding of previously exposed zooplankton to their predators. A study carried out in this manner would allow the consideration of whether microplastics found in the environment, varying in shape and form are likely to be passed through the food chain.

The second major research topic investigated within this thesis, is the effects that microplastic exposure has upon feeding behaviour of zooplankton. It has been recorded that exposure to microplastic particles has caused reduced feeding activity in marine worms (Wright, et al., 2013) and the copepod *Calanus helgolandicus* (Cole, 2014), resulting in an energy deficit. Due to the importance of such species, which occupy lower trophic levels in the marine environment, in terms of energy transfer, such reductions in energy uptake are likely to have knock-on effects for the entire ecosystem. By examining a range of copepod species, displaying a range of feeding behaviours, as well as one holoplankton species, *Porcellanid* larvae, this thesis provides a new insight into the effects that microplastic exposure is likely to have upon different zooplankton species. Carbon ingestion was reduced in three of the four species studies, although only one reduction was statistically significant. Such findings support previous findings by Cole (2014). It was only those copepod species displaying feeding-current feeding behaviour, *C. helgolandicus* and *Acartia tonsa*, that suffered a reduction in carbon uptake following microplastic exposure. *Oithona similis,* an ambush predator did not appear to suffer any effect on carbon uptake in the presence of microplastic particles, suggesting that feeding-current strategists are more at risk from the negative effects of microplastic exposure. A decrease in carbon uptake was also evident in *Porcellanid* larvae.

During this study FlowCAM was utilised to examine the uptake of various prey types by each of the four zooplankton species, in order to investigate the mechanism of reductions in carbon uptake, or any changes in ingestion of particular prey. Such switching behaviour was recorded by Cole (2014) where *C. helgolandicus* showed a shift in prey size ingested, away from cells in the size range of the microplastics added to suspensions used in investigation. Similarly the findings presented in Chapter 3 of this thesis display a range of switching behaviours displayed by all of the copepod species studied. As well as altering ingestion of prey in distinct size ranges, *C. helgolandicus* and *O. similis* appeared to increase ingestion of non-spherical prey. This suggests that copepods may be able to detect spherical microplastics and decrease ingestion on spherical items. However, *O. similis* being an ambush feeder, theoretically should not be able to detect non-motile prey (Kiørboe, et al., 2009), therefore, this study also suggests that *O. similis*, like *A. tonsa*, may be able to display

feeding-current and ambush feeding strategies depending on the relative concentrations of motile, non-motile or high-quality prey. *Porcellanid* larvae were not recorded to display switching behaviour, instead reduced feeding of all prey types. This suggests that reduced feeding may be the result of satiation (Gregory, 2009), blocking of the digestive tract (Browne, et al., 2008), or the presence of a selective behaviour to reduce feeding rate due to the presence of non-valuable microplastic particles.

The energy deficit suffered by individuals as recorded in studies presented in this thesis and previous research (Wright, et al., 2013; Cole, 2014) is a concern. As discussed in Chapter 3, a reduction in energy ingested by an individual not only is likely to cause a reduction in the fitness of the individual but has the potential to affect its progeny and the ecosystem as a whole. As studied by Cole (2014) an energy deficit can lower egg quality and hatchling success, therefore potenitally reducing the population of a species within an ecosytem over a long timescale. As well as this, due to trophic transfer, the reduction of energy at a low level in the food chain is likely to considerably lower the energy available to higher trophic levels. The decreased carbon ingested by larval stages, such as *Porcellanid* larvae, is likely to reduce survivorship and development into adults. All effects are a cause of concern for fisheries and conservationists alike, as the reductions of energy available to organisms within the ecosystem are likely to reduce the health and quality of populations and commercial stocks. As a result it would be of high interest to examine the altered energy uptake by higher trophic level organisms after feeding on zooplankton prey that had been offered a diet of natural algal prey and algae mixed with microplastic particles, such as those suspensions used in the study presented in Chapter 3. Such research would clearly demonstrate the potential ecosystem level effects of microplastic exposure, and potentially allow us to model any alterations in energy transfer through trophic levels. In terms of meroplankton, such as the *Porcellanid* larvae examined in this thesis, long term studies examining the development of larvae into adults when exposed to microplastic particles would enable us to understand whether microplastics are affecting larval development in any manner.

Chapter 4 of this thesis aimed to investigate the ability of copepod species to reject microplastic particles. By utlising high-speed filming techniques, typically

used to study locomotive mechanisms in zooplankton, it was possible to observe the manner in which a copepod detected, captured and handled a microplastic particle. It was found that *Temora longicornis* appeared to be able to reject microplastic particles, however, this rejection was observed to be likely due to mechanistic properties where the microplastic particle was too large or rigid to be ingested by the individual. Through this research it was also possible to assess the presence of chemodetection by the species. It appeared that long-range chemodetection, believed common in feeding-current feeding copepods (Koehl & Strickler, 1981) did not exist, rather particles were detected after coming into contact with feeding appendages. The use of high-speed filming introduces a novel technique to be implemented in microplastic research providing many opportunities for further research, including the examination of various plastic types, as well as examing the effects of adherence of particles in producing feeding-currents. However, one limitation of high-speed filming is that it is possible to miss ingestion of particles, therefore, by utilising fluorescent particles it is possible to assess ingestion following exposure.

Overall it appears that microplastic ingestion is likely to occur in zooplankton species where concentrations of microplastics are high in the marine environment. The effects of microplastic exposure seem to be negative, especially in species that feed by generation of a feeding current. Zooplankton appear to alter feeding behaviour to avoid microplastic particles, such switching behaviour in turn affects the carbon ingested by the individual. It is of high importance that research efforts are placed on discovering the concentration of microplastic debris present in the environment sized <100 μm, this will allow for more environmentally relevant studies to be produced and more ecosystem related effects to be considered.

In terms of this thesis a range of further research opportunities exist. Firstly the RADGLO fluorescent labelling protocol presented in Chapter 2 allows a wide range of plastic types to be examined in laboratory investigations, reducing the limitation that polystyrene microspheres do not accurately represent particles in the environment. Secondly, the effects of reduced carbon uptake require further investigation. It would be highly benficial to repeat studies outlined in Chapter 3 to gain more insights into alterations in feeding behaviour. The examination into effects on other holoplankton species would also be interesting, as well as

examining the development of meroplankton larvae under long-term exposures to microplastic particles. Investigating the loss of energy to higher trophic levels would also be a key area of research, feeding species occupying higher trophic levels with zooplankton exposed to microplastics, in order to assess the net loss of energy transferred. Finally, high-speed filming techniques provide an opportunity to gain a clearer understanding of how individuals interact with microplastic particles.

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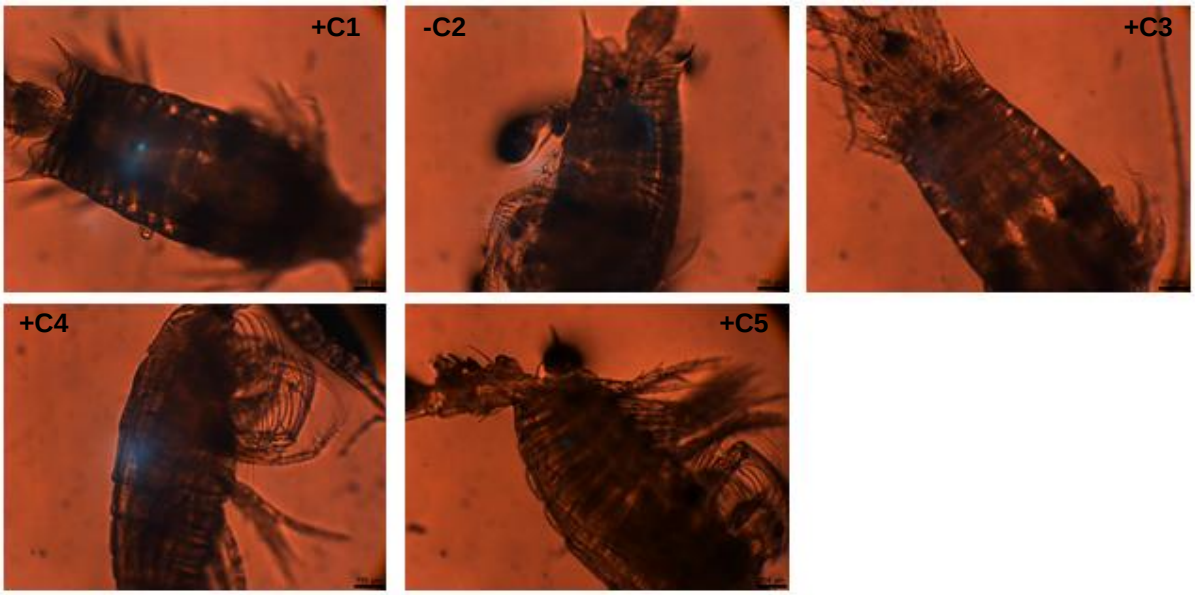
7. Appendices

7.1 Appendix 1

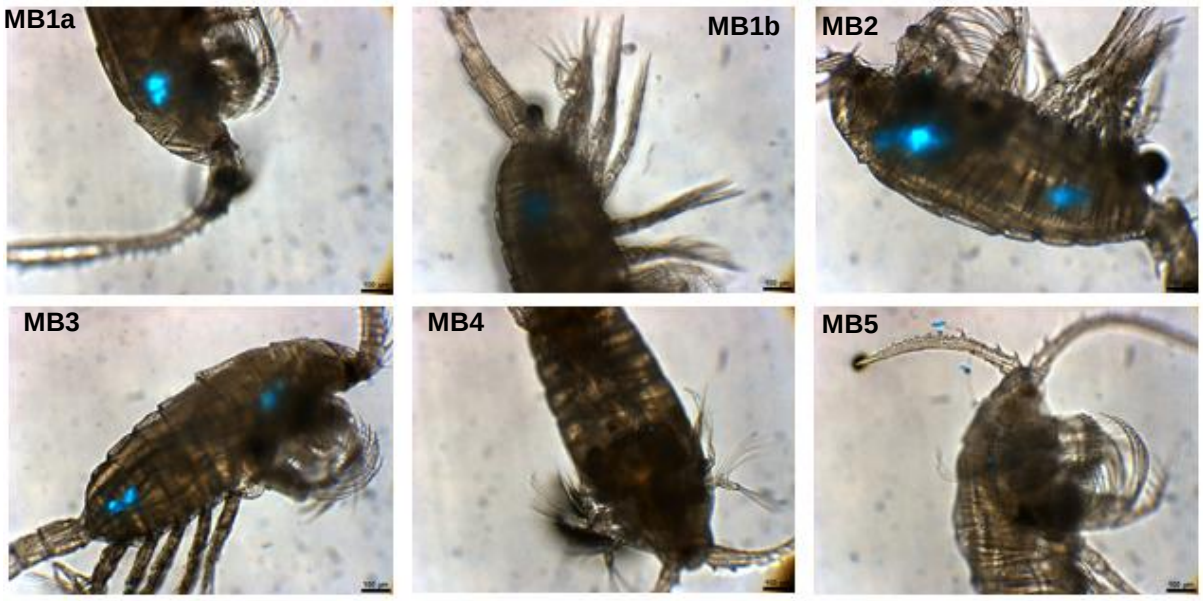
Figure 7.1.1 Investigating the ingestion of various microplastic types in the absence of natural prey

Negative Control (No microplastics added)

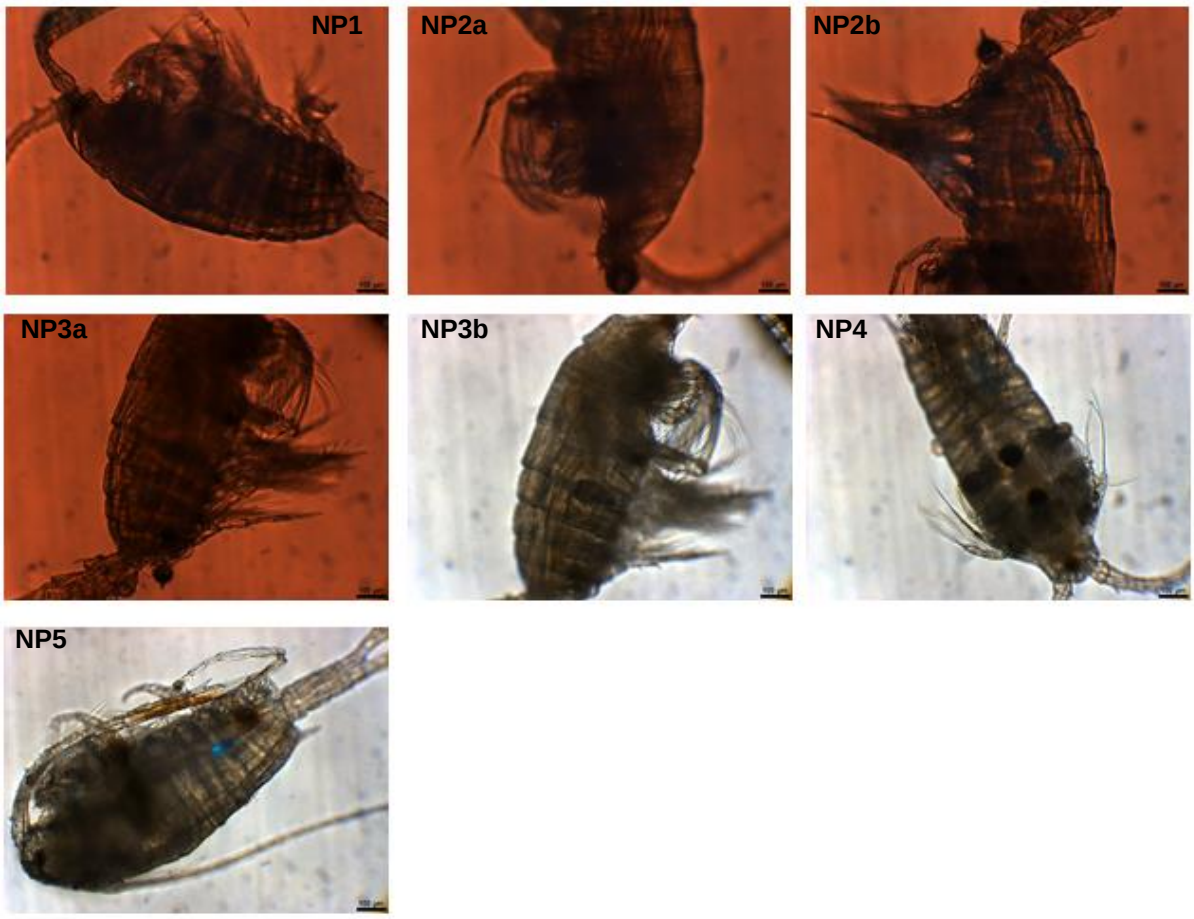
Positive Control - 20µm Yellow Fluorescent Polystyrene Spheres



Primary Microplastic – RADGLO labelled Polyethylene Microbeads



Secondary Microplastic – RADGLO labelled Polyamide-6 Nylon Powder



Fibrous Microplastic – RADGLO labelled Nylon rope microfibers

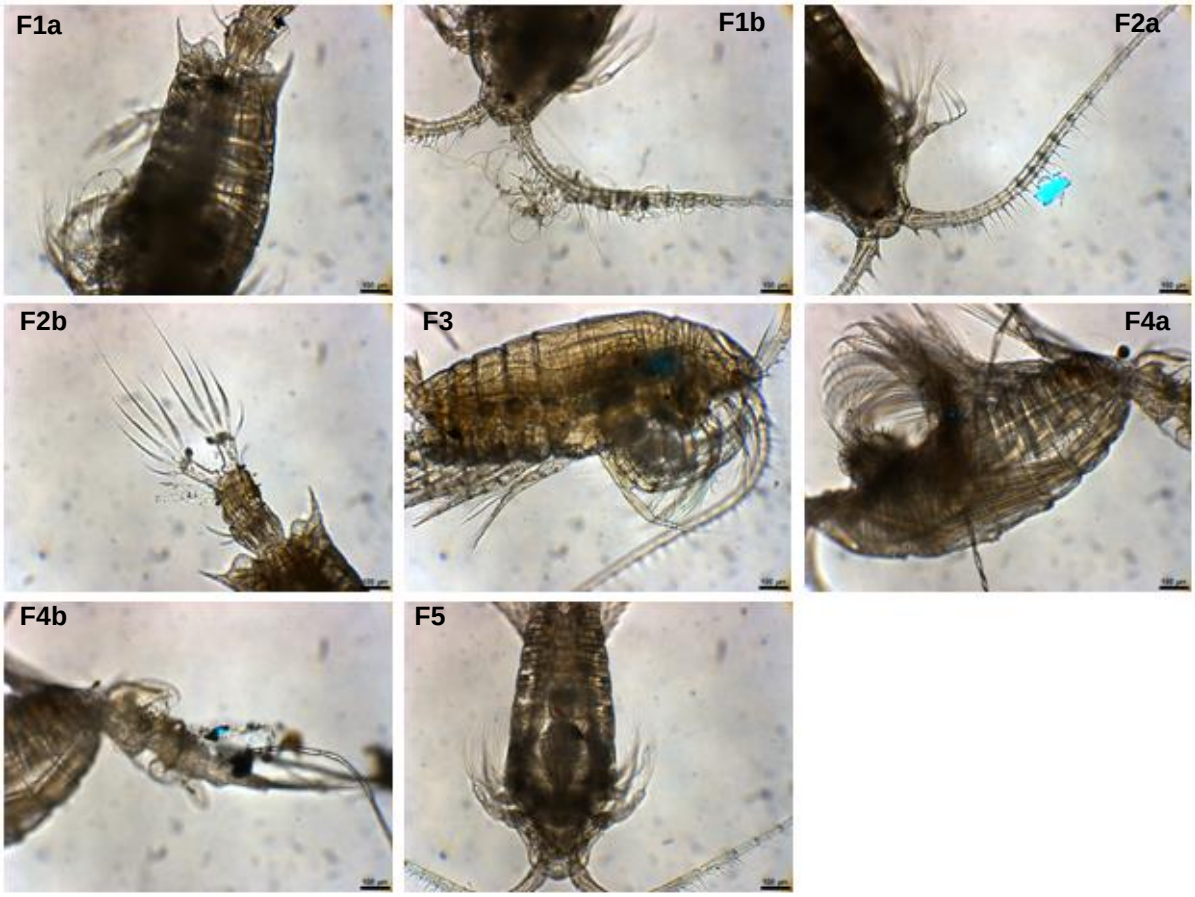


Table 7.1.1 Summary of image data for the ingestion of various microplastic types in the absence of natural prey.

Sample ID	Treatment	Microplastic Present?	Ingestion	Adherence	Notes
-C1	Negative Control (No plastic)	No	N/A	N/A	Blue haze in image –C1 is the result of interference by natural light in imaging.
-C2	Negative Control (No plastic)	No	N/A	N/A	Blue haze in image –C2 is the result of interference by natural light in imaging.
-C3	Negative Control (No plastic)	No	N/A	N/A	Blue haze in image –C3 is the result of interference by natural light in imaging.
+C1	Positive Control (Polystyrene Spheres)	Yes	Yes	No	Sphere appears to be ingested, and is present within the digestive tract.
+C2	Positive Control (Polystyrene Spheres)	Yes	Yes	No	A group of spheres is visible in the lower digestive tract of the individual.
+C3	Positive Control (Polystyrene Spheres)	Yes	Possible	Possible	Fluorescence indicating the presence of particles can be seen on the left of the image, although, it is difficult to identify whether the particle has

					adhered to swimming legs or has been ingested by the individual.
+C4	Positive Control (Polystyrene Spheres)	Yes	Yes	No	Microplastic can be clearly seen in the digestive tract.
+C5	Positive Control (Polystyrene Spheres)	Yes	Yes	No	Microplastic can be clearly seen in the digestive tract.
MB1a	Primary Microplastic (PE Microbeads)	Yes	Yes	Yes	Microbeads clearly visible in the upper digestive tract and smaller fractions adhered to feeding appendages.
MB1b	Primary Microplastic (PE Microbeads)	Yes	Yes	No	Fluorescence in the digestive tract indicates ingestion of microbeads.
MB2	Primary Microplastic (PE Microbeads)	Yes	Yes	Yes	Microbeads are present in the upper and lower digestive tract, aggregation appears to have occurred. Adherence to feeding appendages and swimming legs is also visible.
MB3	Primary Microplastic (PE Microbeads)	Yes	Yes	Yes	Microbeads are present in the upper and lower digestive tract, aggregation appears to have occurred. Adherence to feeding appendages and swimming legs is also visible.
MB4	Primary Microplastic (PE Microbeads)	Yes	No	Yes	Adherence to feeding appendages can be seen, however, no evidence for ingestion is visible.
MB5	Primary Microplastic (PE Microbeads)	Yes	Yes	Yes	Microbeads can be seen in the upper digestive tract. A relatively large particle has adhered to the antennae of the individual.
NP1	Secondary Microplastic (Polyamide-6 Nylon Powder)	Yes	Possible	Yes	Some evidence of fluorescence in the lower digestive tract may indicate ingestion of particles. Adherence to feeding appendages is clear.
NP2a	Secondary Microplastic (Polyamide-6 Nylon Powder)	Yes	No	Yes	Adherence to feeding appendages and carapace visible.
NP2b	Secondary Microplastic (Polyamide-6 Nylon Powder)	Yes	Yes	No	Particles clearly visible in the lower digestive tract, indicating ingestion.
NP3a	Secondary Microplastic (Polyamide-6 Nylon Powder)	Yes	Yes	Yes	Presence of particles is evident in the lower digestive tract. Adherence to the urosome is also visible.
NP3b	Secondary Microplastic	Yes	No	Yes	Evidence of adherence to the feeding appendages.

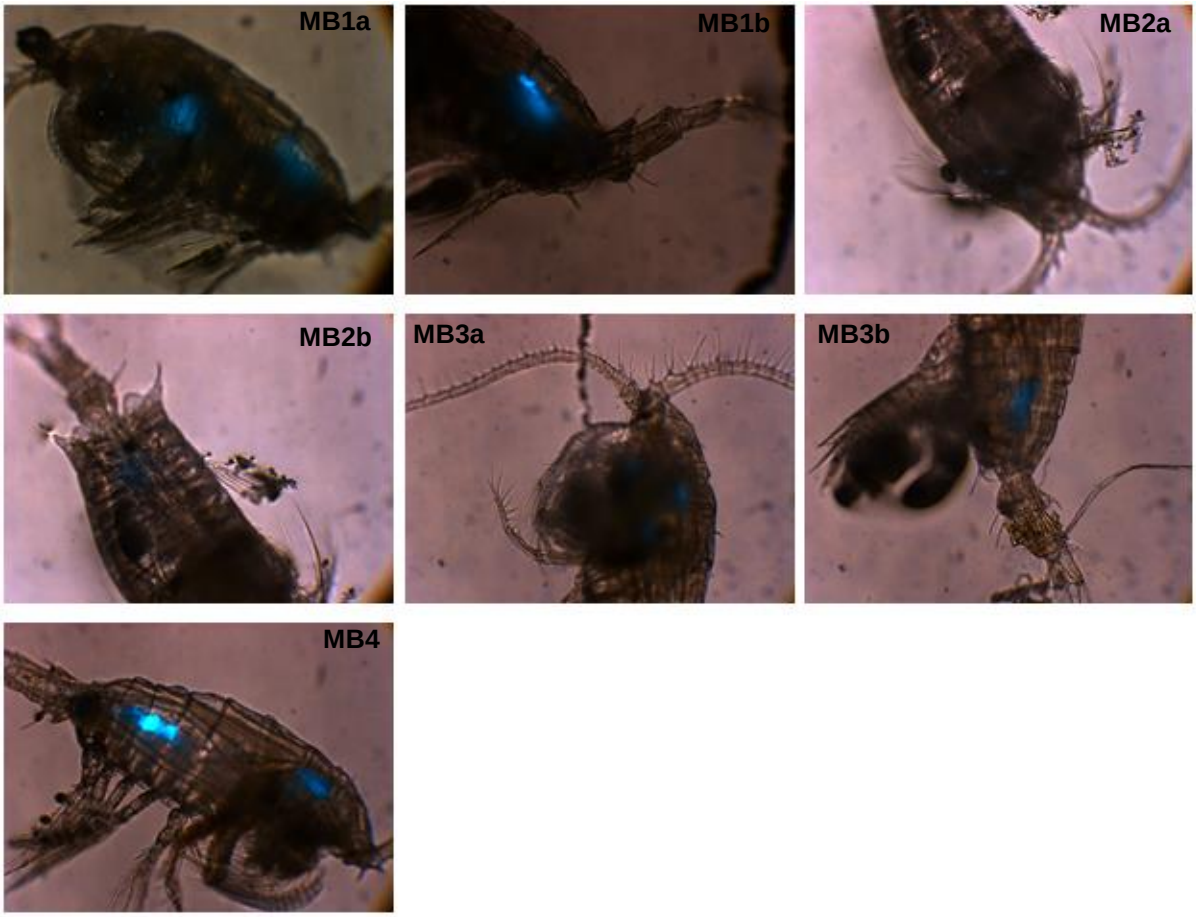
	(Polyamide-6 Nylon Powder)				
NP4	Secondary Microplastic (Polyamide-6 Nylon Powder)	Yes	Yes	Yes	Particles appear to have been ingested and are visible in the upper and lower digestive tract. Adherence to the swimming legs also appears to have occurred.
NP5	Secondary Microplastic (Polyamide-6 Nylon Powder)	Yes	Yes	Yes	Adherence is visible on the feeding appendages. Ingestion is evident from the presence of particles within the lower digestive tract where particles appear to have aggregated.
F1a	Fibrous Microplastic (Nylon rope Microfibres)	Yes	Yes	Possible	Microfibres are visible in the lower digestive tract, indicating ingestion. Evidence for adherence to feeding appendages appears visible, although is out of focus so this cannot be confirmed.
F1b	Fibrous Microplastic (Nylon rope Microfibres)	No	No	No	This image of the attenules of individual F1 does not show further presence of microplastics on this copepod, but shows the adherence of a foreign fibrous particle to the attenules.
F2a	Fibrous Microplastic (Nylon rope Microfibres)	Yes	No	Yes	Clear evidence of adherence of microplastic to the attenule of this individual.
F2b	Fibrous Microplastic (Nylon rope Microfibres)	Yes	No	Yes	Adherence to the urosome is clear in this image.
F3	Fibrous Microplastic (Nylon rope Microfibres)	Yes	Yes	Yes	Microfibre particles can be clearly seen in the upper digestive tract, where aggregation appears to have occurred. Adherence also appears visible on the feeding appendages.
F4a	Fibrous Microplastic (Nylon rope Microfibres)	Yes	No	Yes	Adherence of particles to the feeding appendages is clear. A large unlabelled fibre appears to have also adhered to the individual.
F4b	Fibrous Microplastic (Nylon rope Microfibres)	Yes	No	Yes	Adherence of labelled particles to the urosome is present in image F4b, as well as adherence of a relatively large unlabelled fibre.
F5	Fibrous Microplastic (Nylon rope Microfibres)	No	No	No	Individual F5 appears clear from microplastics both in terms of adherence and ingestion.

Figure 7.1.2 Investigating the ingestion of various microplastic types in the presence of natural prey

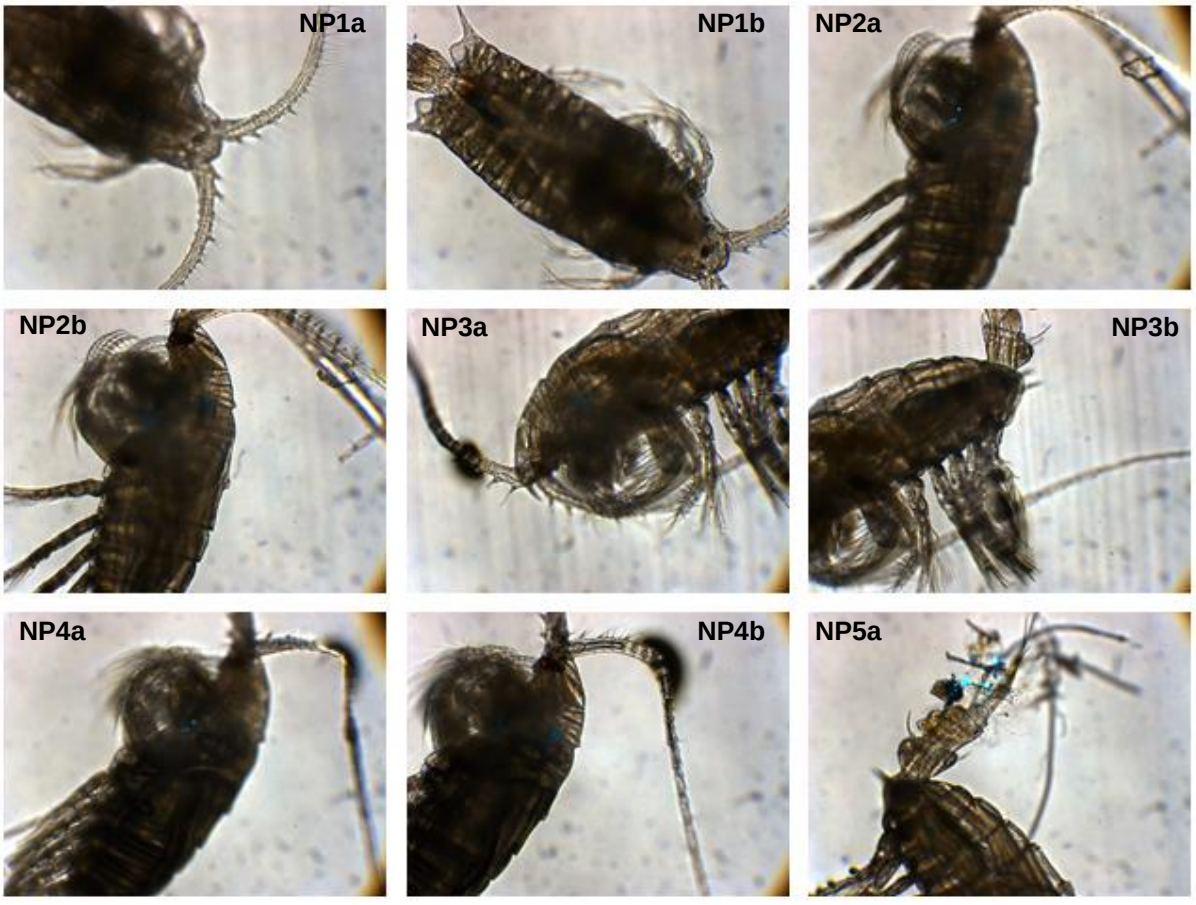
Negative Control (No microplastics added)

Positive Control - 20µm Yellow Fluorescent Polystyrene Spheres

Primary Microplastic – RADGLO labelled Polyethylene Microbeads



Secondary Microplastic – RADGLO labelled Polyamide-6 Nylon Powder



Fibrous Microplastic – RADGLO labelled Nylon rope microfibers

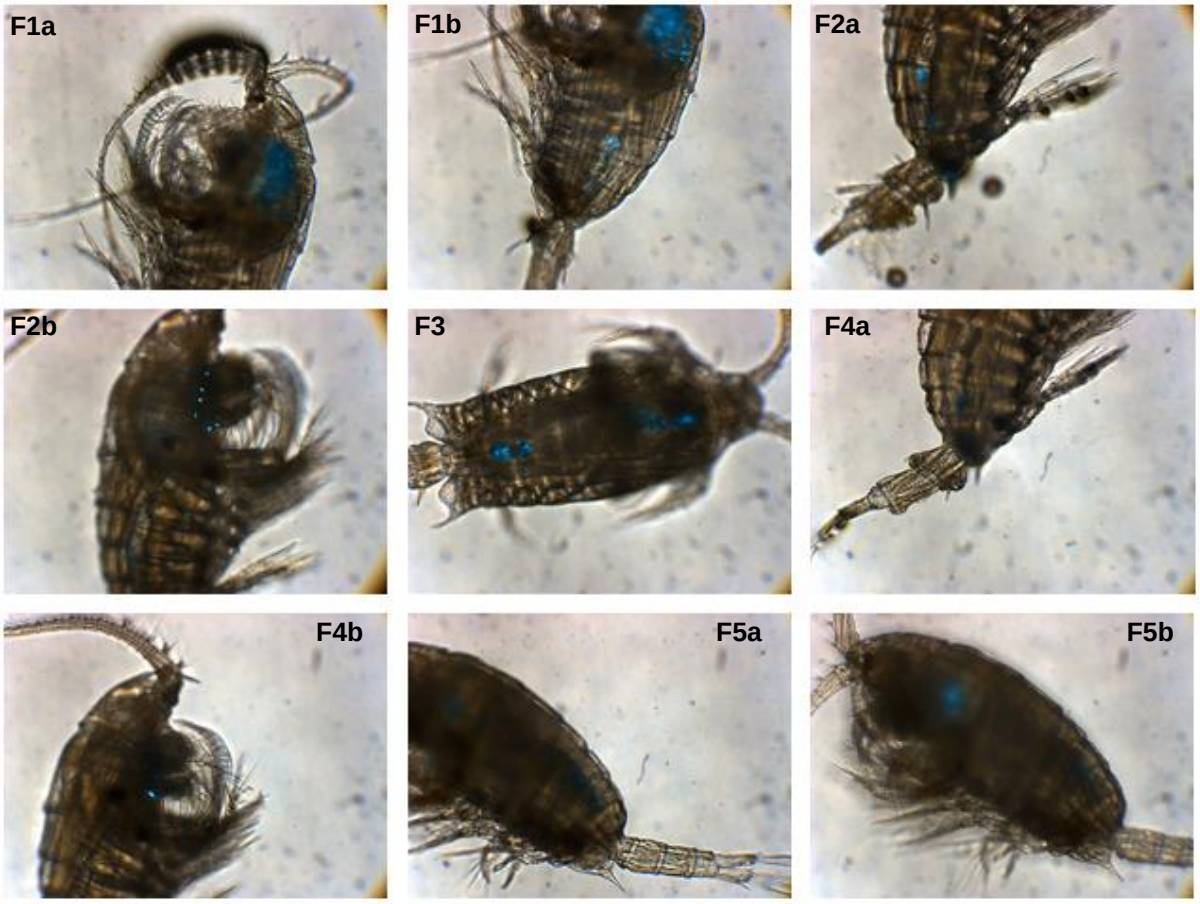


Table 7.1.2 Summary of image data for the ingestion of various microplastic types in the presence of natural prey.

Sample ID	Treatment	Microplastic Present?	Ingestion	Adherence	Notes
-C1	Negative Control (No plastic)	No	N/A	N/A	
-C2	Negative Control (No plastic)	No	N/A	N/A	
-C3	Negative Control (No plastic)	No	N/A	N/A	

+C1	Positive Control (Polystyrene Spheres)	Yes	Yes	Possible	Presence of spheres is evident in the lower digestive tract. Particles are also visible around the upper digestive tract, however, it is possible these particles are adhered to feeding appendages on the front of the organism.
+C2	Positive Control (Polystyrene Spheres)	Yes	No	Yes	Adherence to the urosome is clear in this image.
+C3	Positive Control (Polystyrene Spheres)	Yes	Yes	Possible	Presence of spheres is evident in the lower digestive tract. Particles are also visible around the upper digestive tract, however, it is possible these particles are adhered to feeding appendages on the front of the organism.
+C4	Positive Control (Polystyrene Spheres)	Yes	Yes	No	Polystyrene spheres are clearly visible in the upper digestive tract, indicating ingestion. Evidence of spheres in the lower digestive tract is also present.
+C5	Positive Control (Polystyrene Spheres)	Yes	Yes	No	Ingestion is indicated by the presence of spheres in the lower digestive tract.
MB1a	Primary Microplastic (PE Microbeads)	Yes	Yes	Yes	Evidence for adherence to feeding appendages is clear, as well as, substantial evidence of ingestion in the upper and lower digestive tract.
MB1b	Primary Microplastic (PE Microbeads)	Yes	Yes	No	Image MB1b focusses upon the lower digestive tract of individual MB1, where microbeads appear to have aggregated following ingestion of a substantial volume of particles.
MB2a	Primary Microplastic (PE Microbeads)	Yes	No	Yes	Adherence of microbeads to the feeding appendages is clear in this image.
MB2b	Primary Microplastic (PE Microbeads)	Yes	Yes	No	Microbeads are present in the lower digestive tract, where considerable aggregation appears to have occurred, following ingestion.
MB3a	Primary Microplastic (PE Microbeads)	Yes	Yes	Possible	Particles are visible within the upper digestive tract. It is possible that particles are adhered to feeding appendages on the front of the organism.
MB3b	Primary Microplastic (PE Microbeads)	Yes	Yes	No	Microbeads are present in the lower digestive tract, where considerable aggregation appears to have occurred, following ingestion.

MB4	Primary Microplastic (PE Microbeads)	Yes	Yes	Yes	Substantial evidence of ingestion in the upper and lower digestive tract. Particles appear to have aggregated. Adherence to the feeding appendages and swimming legs is also present.
NP1a	Secondary Microplastic (Polyamide-6 Nylon Powder)	Yes	Yes	No	Particles appear present in the upper digestive tract, indicating ingestion. Particles appear not to aggregate.
NP1b	Secondary Microplastic (Polyamide-6 Nylon Powder)	Yes	Yes	No	Ingestion is indicated by the evidence of particles in the lower digestive tract. Particles appear not to aggregate.
NP2a	Secondary Microplastic (Polyamide-6 Nylon Powder)	Yes	No	Yes	Adherence to feeding appendages is clear in this image.
NP2b	Secondary Microplastic (Polyamide-6 Nylon Powder)	Yes	Yes	Yes	Adherence is indicated as with NP2a, but is out of focus. Ingestion can be seen by the presence of particles in the upper digestive tract.
NP3a	Secondary Microplastic (Polyamide-6 Nylon Powder)	Yes	Yes	Yes	Particles have adhered to the head of the copepod in this image. Microplastic is also visible in the upper digestive tract, indicating ingestion.
NP3b	Secondary Microplastic (Polyamide-6 Nylon Powder)	Yes	Possible	No	Evidence for ingestion appears present in the lower digestive tract, however, it is not possible to confirm this due to the focus of the image.
NP4a	Secondary Microplastic (Polyamide-6 Nylon Powder)	Yes	Yes	Yes	Adherence to the feeding appendages is clear in this image. Out of focus fluorescence also indicates the presence of particles in the upper digestive tract.
NP4b	Secondary Microplastic (Polyamide-6 Nylon Powder)	Yes	Yes	Yes	This image confirms ingestion of particles due to their presence in the upper digestive tract.
NP5a	Secondary Microplastic (Polyamide-6 Nylon Powder)	Yes	Yes	Yes	Fluorescence is visible in the lower digestive tract. Particles have adhered to the urosome entangled in algal matter.
NP5b	Secondary Microplastic (Polyamide-6 Nylon Powder)	Yes	Yes	Yes	Ingestion is indicated by the presence of particles within the digestive tract. Evidence for adherence to the swimming legs also exists in this image.
NP5c	Secondary Microplastic	Yes	Yes	Yes	Adherence to the feeding appendages is present in this image.

	(Polyamide-6 Nylon Powder)				
F1a	Fibrous Microplastic (Nylon rope Microfibres)	Yes	Yes	Yes	Considerable ingestion of microfibers is present in the upper digestive tract. Aggregation of particles appears to have occurred. Adherence of smaller particles to the feeding appendages also is evident.
F1b	Fibrous Microplastic (Nylon rope Microfibres)	Yes	Yes	Yes	Ingestion is indicated by a considerable amount of microfibers present in the lower digestive tract. Adherence to the carapace is present.
F2a	Fibrous Microplastic (Nylon rope Microfibres)	Yes	Yes	Yes	Ingestion is indicated by a considerable amount of microfibers present in the lower digestive tract. Adherence to the swimming legs and urosome is present.
F2b	Fibrous Microplastic (Nylon rope Microfibres)	Yes	No	Yes	Adherence to the feeding appendages is clear in this image.
F3	Fibrous Microplastic (Nylon rope Microfibres)	Yes	Yes	No	Microfibres are present in the upper and lower digestive tract. Considerable ingestion appears to have occurred and particles appear to have aggregated.
F4a	Fibrous Microplastic (Nylon rope Microfibres)	Yes	Yes	Yes	Ingestion is indicated by a considerable amount of microfibers present in the lower digestive tract. Adherence to the swimming legs is present.
F4b	Fibrous Microplastic (Nylon rope Microfibres)	Yes	No	Yes	Adherence to the feeding appendages is clear in this image.
F5a	Fibrous Microplastic (Nylon rope Microfibres)	Yes	Yes	No	Microfibres are present in the upper and lower digestive tract, indicating ingestion.
F5b	Fibrous Microplastic (Nylon rope Microfibres)	Yes	Yes	No	Focussing on the upper digestive tract reveals the extent of ingestion, which appears considerable.

7.2 Appendix 2

Table 7.2.1 Summary data of High-speed video recordings of *Temora longicornis* exposed to a range of microplastic particles.

Video No.	Life Stage	Bead Size (µm)	Treatment	No. beads	Ingestion/Rejection/Miss
1	Adult	10 (F)	None	4	Unsure
2	Nauplius	10 (F)	None	1	Rejection
3	Adult	20 (F)	None	4	Miss
4	Nauplius	20 (F)	None	2	Rejection
5	Adult	20 (F)	None	1	Ingestion
6	Adult	20 (F)	None	1	Rejection
7	Adult	20 (F)	None	3	Ingestion
8	Adult	20 (F)	None	3	Ingestion
9	Nauplius	20 (F)	None	4	Rejection
10	Nauplius	20 (F)	None	1	Rejection
11	Nauplius	20 (F)	None	2	Rejection
12	Nauplius	20 (F)	None	4	Rejection
13	Adult	20 (F)	None	3	Rejection
14	Adult	20 (F)	None	3	Rejection
15	Adult	20 (F)	None	2	Rejection
16	Nauplius	20 (F)	None	1	Rejection
17	Nauplius	20 (F)	None	2	Rejection
18	Adult	20 (F)	None	>5	Rejection
19	Adult	20 (F)	None	1	Rejection
20	Nauplius	20 (F)	None	2	Rejection
21	Nauplius	20 (F)	None	4	Rejection
22	Adult	20 (F)	None	4	Rejection
23	Nauplius	30	None	1	Rejection
24	Adult	30	None	1	Rejection
25	Nauplius	30	None	1	Rejection
26	Adult	30	None	1	Rejection

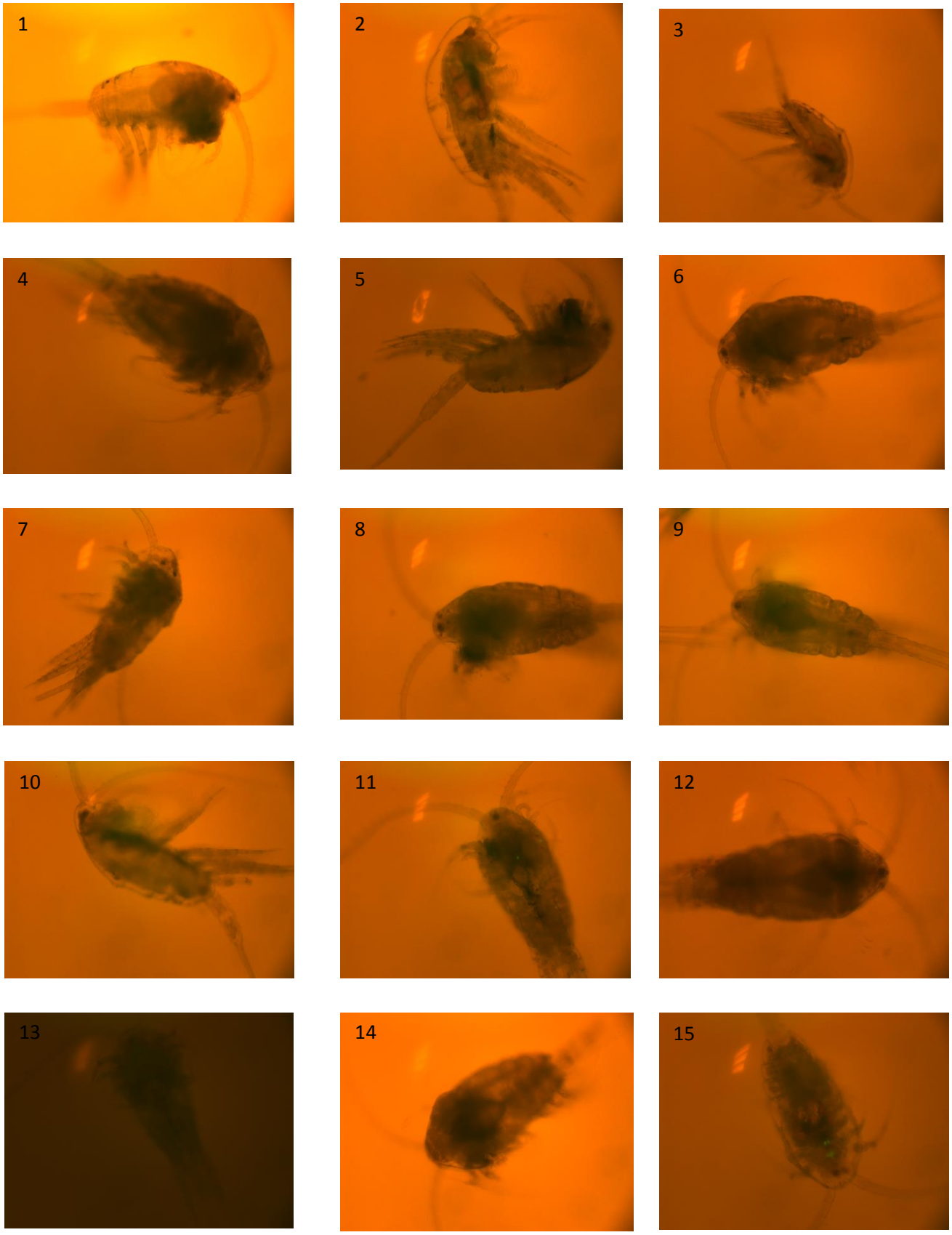
27	Nauplius	30	None	1	Rejection
28	Adult	30	None	1	Rejection
29	Adult	30	None	1	Rejection
30	Adult	30	None	1	Rejection
31	Adult	30	None	1	Rejection
32	Adult	30	None	1	Miss
33	Adult	30	+ Prey	1	Rejection
34	Adult	30	+ Prey	2	Rejection
35	Adult	30	+ Prey	1	Rejection
36	Adult	30	+ Prey	1	Rejection
37	Adult	30	+ Prey	2	Rejection
38	Adult	30	+ Prey	1	Rejection
39	Adult	30	+ Prey	1	Rejection
40	Adult	30	+ Prey	1	Rejection
41	Adult	30	+ Prey	1	Rejection
42	Adult	30	+ Prey	1	Rejection
43	Adult	30	+ Prey	2	Rejection
44	Adult	30	+ Prey	2	Rejection
45	Nauplius	30	+ Prey	1	Rejection
46	Adult	30	+ Prey	2	Rejection
47	Adult	30	+ Prey	2	Rejection
48	Adult	30	+ Prey	2	Rejection
49	Adult	30	+ Prey	1	Rejection
50	Nauplius	30	+ Prey	2	Rejection
51	Adult	30	Rho	1	Rejection
52	Nauplius	30	Rho	>5	Rejection
53	Adult	30	Rho	1	Rejection
54	Adult	30	Rho	1	Rejection
55	Adult	30	Rho	1	Rejection
56	Adult	30	Rho	1	Rejection
57	Adult	30	Rho	1	Rejection

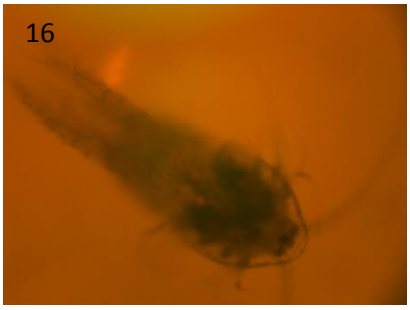
58a	Adult	30	Rho	1	Rejection
58b	Adult	30	Rho	1	Rejection
59	Adult	30	Rho	1	Rejection
60	Adult	30	CSW	1	Rejection
61	Adult	30	CSW	1	Rejection
62	Nauplius	30	CSW	1	Rejection
63a	Adult	30	CSW	1	Rejection
63b	Adult	30	CSW	1	Rejection
64	Adult	30	CSW	1	Rejection
65	Nauplius	30	CSW	1	Rejection
66	Nauplius	30	CSW	1	Rejection

Table 7.2.1 Summary data of high-speed video recordings of *T. longicornis* exposed to a range of microplastic particles. F indicates fluorescently labelled polystyrene spheres; + Prey indicates filming where *R. salina* was provided as prey alongside microplastic particles; Rho indicates beads treated with effluent water from *Rhodomonas* algal cultures; CSW indicates spheres treated with unfiltered seawater taken from copepod culture vessels.

Ingestion of microplastic spheres by T. longicornis when exposed to high densities over a short time period.

Figure 7.2.1 Exposure to 10 µm Yellow Fluorescent Polystyrene spheres

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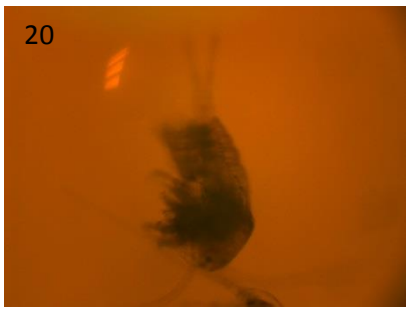
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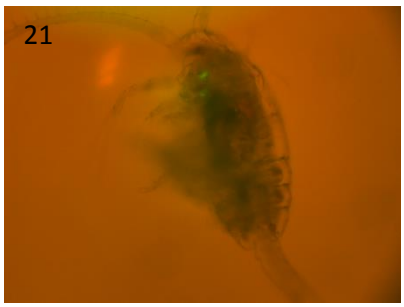
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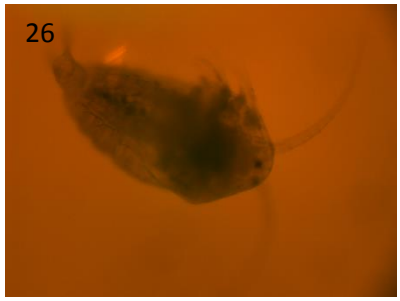
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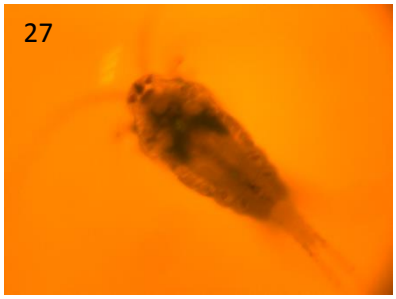
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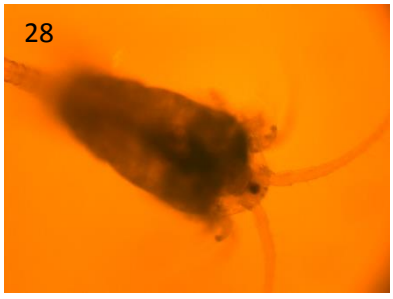
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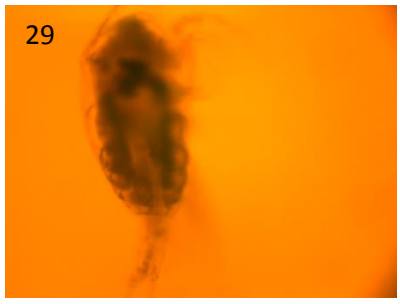
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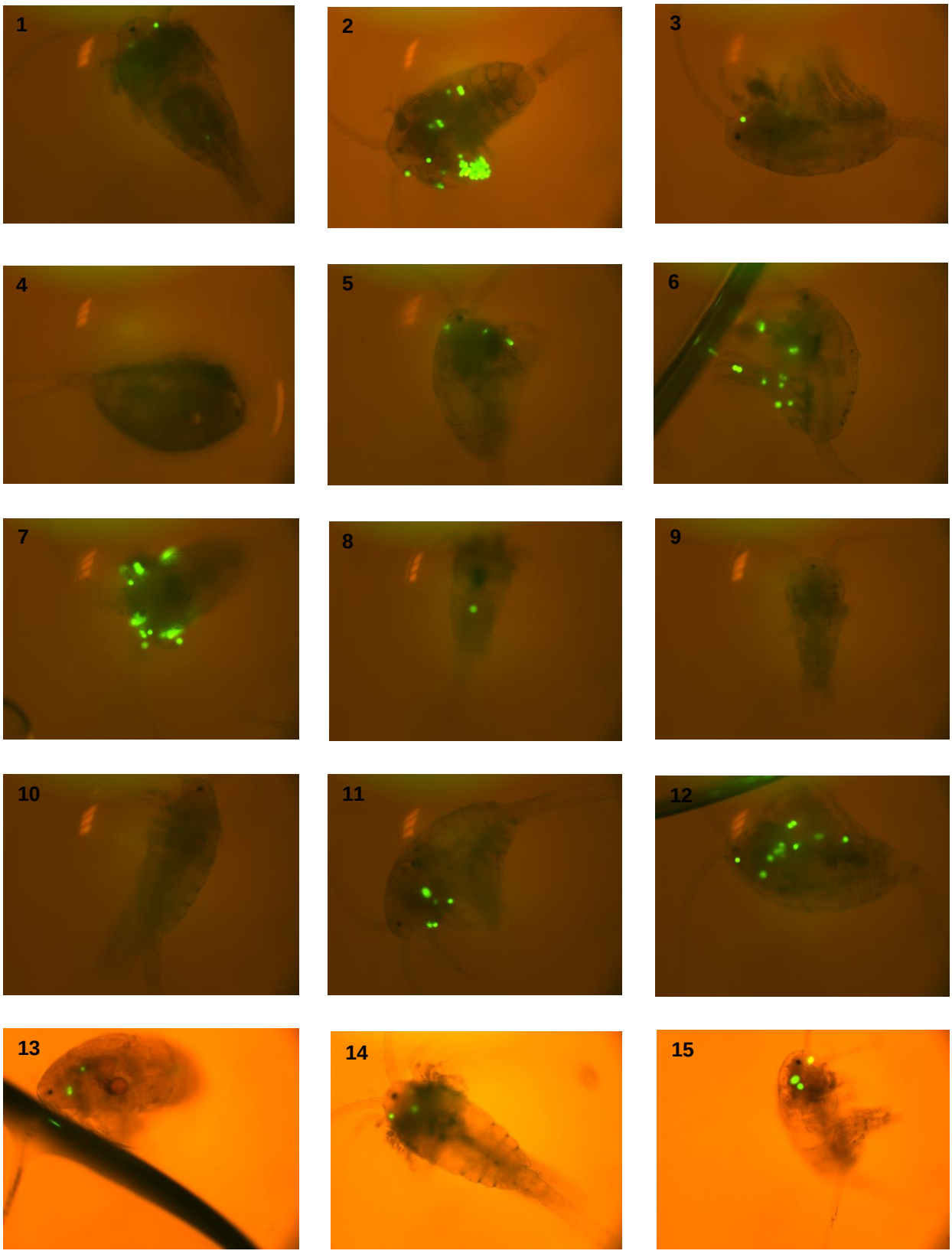
Table 7.2.2 Summary data of imaging of *T. longicornis* exposed to 10 µm Yellow Fluorescent Polystyrene spheres

Individual	Life Stage	Sphere Size (µm)	Spheres Present	Adherence	Adhered to	Scale of Adherence (1-5)	Ingestion	Sphere Location	Approx. number of spheres ingested
1	Adult	10	no	no			no		
2	Adult	10	no	no			no		
3	Nauplius	10	no	no			no		
4	Adult	10	no	no			no		
5	Adult	10	no	no			no		
6	Adult	10	no	no			no		
7	Adult	10	no	no			no		
8	Adult	10	no	no			no		
9	Adult	10	no	no			no		
10	Adult	10	no	no			no		
11	Adult	10	yes	yes	Feeding Appendages	1	possible	Upper Digestive Tract	1
12	Adult	10	no	no			no		
13	Adult	10	yes	yes	Feeding Appendages	2	no		
14	Adult	10	no	no			no		
15	Adult	10	yes	yes	Feeding Appendages	2	no		

Individual	Life Stage	Sphere Size (µm)	Spheres Present	Adherence	Adhered to	Scale of Adherence (1-5)	Ingestion	Sphere Location	Approx. number of spheres ingested
16	Adult	10	yes	yes	Feeding Appendages	2	no		
17	Adult	10	yes	yes	Feeding Appendages	1	yes	Digestive Tract	>5
18	Nauplius	10	no	no			no		
19	Adult	10	no	no			no		
20	Nauplius	10	no	no			no		
21	Adult	10	yes	yes	Feeding Appendages	2	no		
22	Nauplius	10	yes	no			yes	Upper digestive tract/Digestive tract	>10
23	Adult	10	no	no			no		
24	Adult	10	yes	yes	Feeding Appendages/ Legs	2	possible	Upper digestive tract/Digestive tract	
25	Adult	10	yes	no			yes	Digestive Tract	>5
26	Adult	10	no	no			no		
27	Adult	10	yes	possible	Feeding Appendages	1	yes	Upper digestive tract/Digestive	>5
28	Adult	10	no	no			no		
29	Adult	10	yes	no			possible	Digestive Tract	1
30	Adult	10	yes	yes	Feeding Appendages	5	possible	Digestive Tract	>5

Table 7.2.2 Summary data of imaging of *T. longicornis* exposed to 10 µm Yellow Fluorescent Polystyrene spheres. “yes” indicates the presence of spheres and occurrence of adherence or ingestion. “Possible” refers to cases where ingestion or adherence could not be confirmed. “No” indicates the absence of ingestion or adherence. Scale of adherence (1-5) indicates the number of spheres adhered to the individual, with 1 being a low number of spheres attached and 5 representing a large number of spheres adhered to the copepod.

Figure 7.2.2 Exposure to 20 µm Yellow Fluorescent Polystyrene spheres

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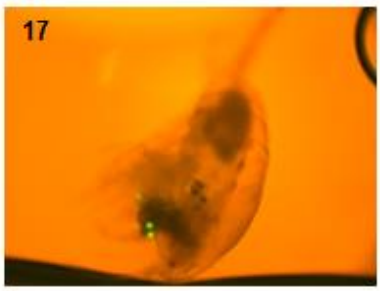
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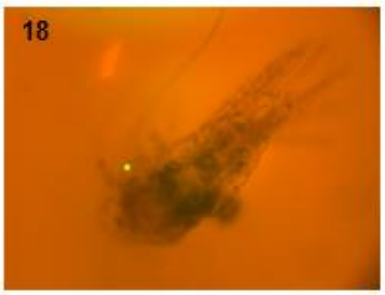
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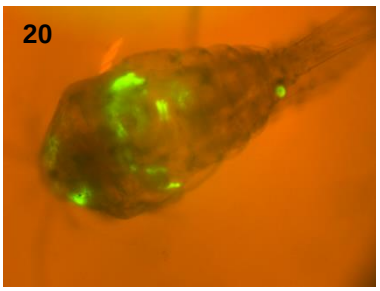
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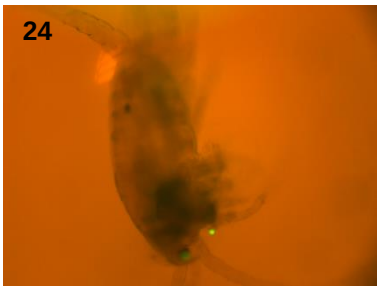
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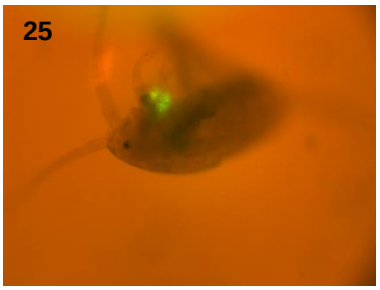
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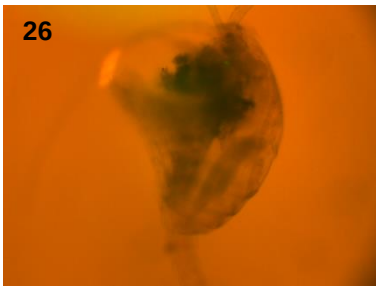
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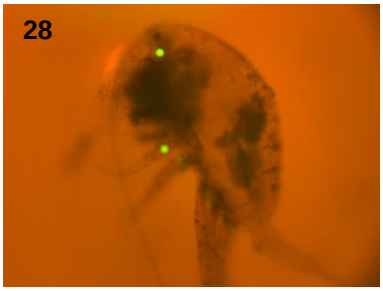
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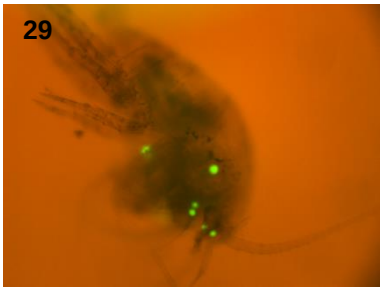
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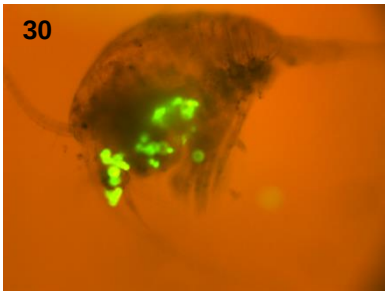
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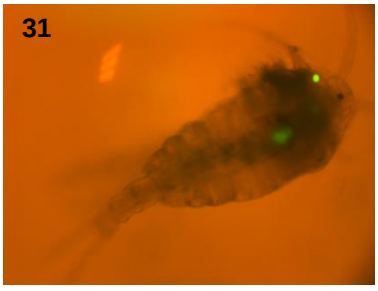
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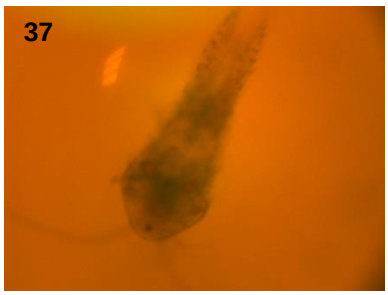
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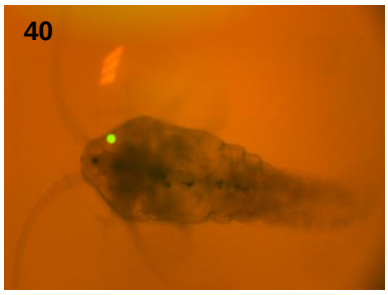
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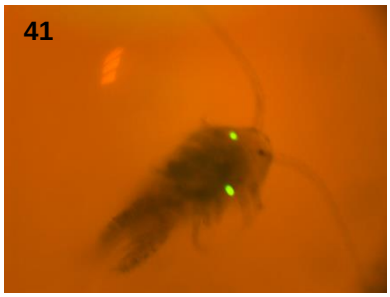
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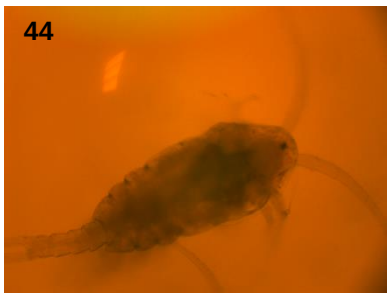
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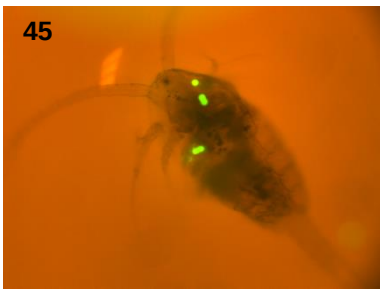
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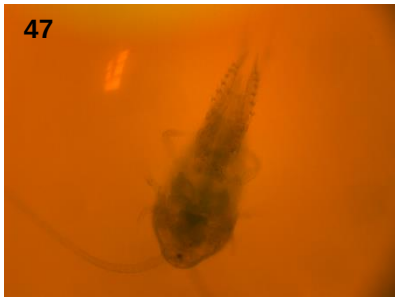
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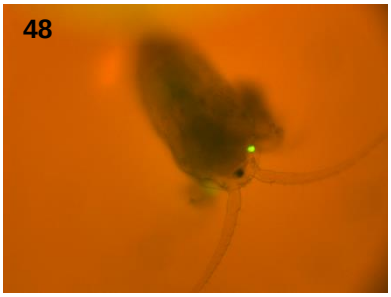
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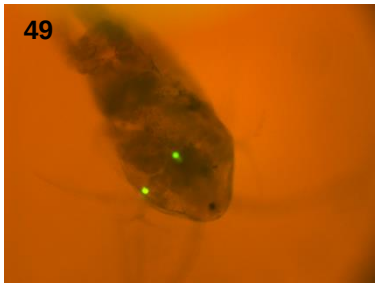
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Table 7.2.3 Summary data of imaging of *T. longicornis* exposed to 20 µm Yellow Fluorescent Polystyrene spheres

Individual	Life Stage	Sphere Size (µm)	Spheres Present	Adherence	Adhered to	Scale of Adherence (1-5)	Ingestion	Sphere Location	Approx. number of spheres ingested
1	Adult	20	yes	yes	Head/Feeding Appendages	3	possible	Digestive Tract	1
2	Adult	20	yes	yes	Head/Feeding Appendages/ Legs	5	no		
3	Adult	20	yes	yes	Feeding Appendages	1	no		
4	Adult	20	no	no			no		
5	Adult	20	no	yes	Head/Feeding Appendages	2	no		
6	Adult	20	yes	yes	Feeding Appendages	3	no		
7	Adult	20	yes	yes	Head/Feeding Appendages/ Legs	5	no		
8	Nauplius	20	yes	possible	Head/Feeding Appendages/ Legs	1	yes	Digestive Tract	1
9	Nauplius	20	no	no	Legs		no		
10	Adult	20	no	no			no		
11	Adult	20	yes	yes	Head/Feeding Appendages	3	no		
12	Adult	20	yes	yes	Head/Feeding Appendages/ Legs	4	no		
13	Adult	20	no	possible	Head/Feeding Appendages	1	no	Upper Digestive Tract	3
14	Adult	20	yes	possible	Head/Feeding Appendages	1	possible	Upper Digestive Tract	1
15	Nauplius	20	yes	yes	Head/Feeding Appendages	1	possible	Upper Digestive Tract	3

Individual	Life Stage	Sphere Size (µm)	Spheres Present	Adherence	Adhered to	Scale of Adherence (1-5)	Ingestion	Sphere Location	Approx. number of spheres ingested
16	Nauplius	20	yes	yes	Feeding Appendages	1	no		
17	Adult	20	yes	yes	Feeding Appendages	1	no		
18	Adult	20	yes	yes	Feeding Appendages	1	no		
19	Adult	20	yes	yes	Feeding Appendages	2	no		
20	Adult	20	yes	yes	Head/Feeding appendages/Legs/ Urosome	5	no		
21	Adult	20	no	no			no		
22	Adult	20	yes	yes	Head	1	no		
23	Adult	20	yes	yes	Head/Feeding Appendages	2	possible	Upper digestive tract	5
24	Adult	20	yes	yes	Head/Feeding Appendages	1	no		
25	Adult	20	yes	yes	Feeding Appendages	4	no		
26	Adult	20	yes	yes	Feeding Appendages	1	no		
27	Nauplius	20	no	no			no		
28	Adult	20	yes	yes	Feeding Appendages	1	no		
29	Adult	20	yes	yes	Head/Feeding Appendages	2	no		
30	Adult	20	yes	yes	Feeding Appendages/Legs	5	no		

Individual	Life Stage	Sphere Size (µm)	Spheres Present	Adherence	Adhered to	Scale of Adherence (1-5)	Ingestion	Sphere Location	Approx. number of spheres ingested
31	Adult	20	yes	yes	Feeding Appendages	2	possible	Upper Digestive Tract	3
32	Adult	20	no	no			no		
33	Nauplius	20	yes	no			yes	Digestive Tract/ Upper Digestive Tract	>10
34	Nauplius	20	no	no			no		
35	Adult	20	yes	yes	Feeding Appendages		no		
36	Adult	20	yes	yes	Feeding Appendages	2	no		
37	Adult	20	no	no			no		
38	Adult	20	no	no			no		
39	Nauplius	20	no	no			no		
40	Adult	20	yes	yes	Feeding Appendages		no		
41	Nauplius	20	yes	yes	Feeding Appendages		no		
42	Adult	20	yes	yes	Feeding Appendages	1	yes	Digestive Tract	4
43	Adult	20	yes	yes	Feeding Appendages		no		
44	Adult	20	no	no			no		
45	Adult	20	yes	yes	Feeding Appendages	5	no		

Individual	Life Stage	Sphere Size (µm)	Spheres Present	Adherence	Adhered to	Scale of Adherence (1-5)	Ingestion	Sphere Location	Approx. number of spheres ingested
46	Adult	20	yes	yes	Feeding Appendages	2	no		
47	Nauplius	20	no	no			no		
48	Nauplius	20	yes	yes	Feeding Appendages		no		
49	Adult	20	yes	yes	Feeding Appendages		possible	Upper Digestive Tract	2
50	Adult	20	no	no			no		

Table 7.2.3 Summary data of imaging of *T. longicornis* exposed to 20 µm Yellow Fluorescent Polystyrene spheres. “yes” indicates the presence of spheres and occurrence of adherence or ingestion. “Possible” refers to cases where ingestion or adherence could not be confirmed. “No” indicates the absence of ingestion or adherence. Scale of adherence (1-5) indicates the number of spheres adhered to the individual, with 1 being a low number of spheres attached and 5 representing a large number of spheres adhered to the copepod.