Uptake and Biological Impacts of Microplastics and Nanoplastics in Sea Squirts

Submitted by *Victoria Helen Dewar-Fowler*, to the University of Exeter as a thesis for the degree of *Masters by Research in Biosciences, September 2017*.

I certify that all material in this thesis which is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree by this or any other University.

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Abstract

Microplastics have been detected in all marine regions and habitats, from the poles to the deep seas. A number of marine species are known to ingest microplastics, resulting in detrimental impacts. Preliminary work carried out on sea squirts suggested that microplastics may have a negative impact upon their health. Here, the solitary sea squirt, *Ciona intestinalis,* is used as a model species to observe the impacts of microplastics on sea squirts. This species has a transparent body form and is widely available in coastal waters. Laboratory exposures were carried out using a range of different microplastics; fluorescently labelled polystyrene (PS) and polyamide (PA) microbeads, polyhydroxybutyrate (PHB), low density polyethylene (LPE), polypropylene (PP), nylon fibres (NF), nylon particles (NMP) and polystyrene nanoparticles (PNP), cryo-ground polypropelyne rope fibres, rubber loom bands, high density polyethelyne (HDPE) and polyvinyl chloride (PVC). *C. intestinalis* ingested all but the cryo-ground rope fibres. Buoyancy is likely to have been an important factor for ingestion by *C. intestinalis*. Polystyrene and polyamide microbeads were ingested at all concentrations tested (100 and 500 PS beads mL^{-1}, 500 and 1000 PA beads ml^{-1}). However, there were significantly more PS beads in *C. intestinalis* at 24 hours than at 72 hours. Microplastic egestion was also observed, following ingestion plastics were found to be present in faecal matter. There was no distinct pattern of bead content within the sea squirts or surrounding water with time after removal from plastic contaminated water. *C. intestinalis* readily ingested microplastics of the tested size ranges, but were able to rapidly eject them without obvious

detrimental effects. Wild specimens of *C. intestinalis* were also analysed for the presence of microplastics. A number of plastic-like particles were found to be present within these organisms, suggesting that ascidians may be susceptible to microplastic ingestion in the marine environment.

Acknowledgements

Particular thanks are given to Dr Ceri Lewis for her support and guidance throughout the entirety of this research project. Thanks are also given to Dr Matt Cole, Corin Liddle, Adam Porter and Dr Andy Watts for their guidance and support. The use of facilities and materials at the University of Exeter during this project was greatly appreciated, as well as, the advice and support of research technician staff. Gratitude is also shown to Lucy Porter for her assistance in field collections.

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List of Abbreviations

Dw- dry weight

HDPE- high density polyethylene

LDPE- low density polyethylene

NF- nylon Fibres

NMP- nylon Microparticles

PA- polyamide particles

PHB-polyhydroxybutyrate

PNP – polystyrene nanoplastic

PP- polypropylene

PS MP – polystyrene microplastic

PVC-polyvinyl chloride

Chapter 1:

A Review of Microplastics in the Marine Environment

1.1 Introduction

Plastic production has increased dramatically in the last four decades due to its durability, flexibility and low production costs. As a result of this increase in plastic production, plastic debris is now being found at ever-increasing quantities in all marine environments. The annual global plastic production is now estimated to be 300 million tonnes (Plastics Europe, 2016) and it is thought that around 10% of this plastic enters the oceans (Thompson et al., 2004). Plastics may enter the ocean through accidental release, sewage discharge, run-off from land, blowing from landfill sites and purposeful discard on land or at sea (Barnes et al., 2009). Recent estimates have calculated that there were between 15 to 51 trillion microplastic particles in the oceans in 2014 (van Sebille et al., 2015). Once plastic enters the ocean it breaks down to form microscopic and nanoscopic fragments that are able to persist for decades (Andrady, 2015); there is increasing and widespread concern that these microplastics might represent a major threat to marine ecosystems (Shim and Thompson, 2015; Clark et al., 2016; Galloway and Lewis, 2016).

Microplastics are often defined as plastic particles with a diameter of less than 5 mm (Cole, 2011). They can be purposefully manufactured to be of microscopic size, known as primary microplastics, which include virgin plastic pellets and beads those contained in cosmetics (Thompson et al., 2009; Cole et al., 2011).

Microplastics can also be formed as a result of the breakdown of larger plastics through a variety of processes, termed secondary microplastics. These processes may include biodegradation (microbial fragmentation of plastics), photo-degradation (a process by which light weakens the plastic structure), thermo-oxidative degradation (breakdown through slow oxidative processes at moderate temperatures), hydrolysis (water action causing plastic fragmentation) (Andrady, 2011). However, due to the haline environment and cooling effect of the sea, plastic degradation is slowed and microplastic fragments accumulate (Barnes et al., 2009).

This fragmentation along with the ever-increasing production is leading to microplastics having a greater abundance in the marine environment. It is thought that plastics within the ocean have been decreasing in size over the last three decades, with samples from the west North Atlantic showing a decrease from 10.66 mm in the 1990s to 5.05 mm in the 2000s (Wright et al., 2013a). This decrease in size is likely to be as a result of an increase in primary microplastics as well as fragmentation of older plastics into greater number of fragments. Studies are now using a mass-conserving fragmentation process to predict this size decrease, using this process it is thought that particles will increase by the inverse of the particle radius to the power of 3 (Lenz et al., 2016). However, it is commonly reported that the increase is slightly lower with an increase of particles being inversely related to the particle diameter to the power of 2.96 (Lenz et al., 2016). This difference in particle fragmentation may be due to the variety in particle shapes found in the environment; each may have a different fragmentation process. However, it is likely that this decrease in particle size and

increase in particle number will make microplastics available to a greater number of species.

1.2 Marine microplastic abundances and distribution

Microplastics have now been detected in all marine regions and habitats, from the poles to the deep seas (do Sul et al., 2013; Goldstien and Goodwin, 2013; Van Cauwenberghe et al., 2013; Thompson, 2015; Lusher, 2015; Van Sebille et al., 2015). However, microplastic accumulation is not uniform across all habitats; with gyres being of particular interest due to the high concentrations of debris found within them (Van Sebille et al., 2015; Clarke et al., 2016). Microplastic concentrations within the North Pacific Subtropical gyre have been recorded at densities of 32.76 particles m^3 (Goldstein et al., 2012). This high concentration indicates a high bioavailability within the oceanic gyres; however, Clarke et al. (2016) demonstrated that these areas of high microplastic concentration coincided with areas of low sea surface chlorophyll. This may be of significance, as areas containing low surface chlorophyll are thought to be areas with the least biological productivity and low biomass. If these high densities of microplastics are occurring in areas of least productivity then it is likely that the impacts on marine fauna arising from microplastic interactions will be of relatively low concern. However, high plastic concentrations are now being recorded in areas of greater productivity, such as coastal areas (Clark et al., 2016).

Coastal areas are often densely populated areas, which leads to an increase in the volume of microplastic entering the water due to direct discharge (Clarke et al., 2016). This direct discharge causes an accumulation of plastic debris in

productive, shallow coastal areas. Numerous studies report microplastics within coastal zones and sediments (e.g. Browne et al., 2011; Woodall et al., 2014; Von Cauwenberghe et al., 2015; Phuong et al., 2016), with concentrations ranging from 2.3 items kg^{-1} in Nordernay beach, Germany (Thompson et al., 2004) up to 4137.3 particles/m^3 in China (Zhao et al., 2014). Generally, these studies note that, in line with Clarke et al. (2016), areas of high microplastic abundance appear to coincide with industrial areas, thereby, implying that marine animals living in industrial coastal areas are likely to be at greatest risk of microplastic ingestion. The coastal human population is predicted to rise from 625 million in 2000 to 880 million in 2030 and to reach 1 billion before 2060 (Neaumann et al., 2015). With this increase in coastal population the volume of plastic entering the oceans is likely to increase, potentially leading to greater concentrations of microplastics in these areas. It has been reported that 15-21 % of oceanic primary production occurs in shallow coastal areas (Jahnke, 2010). Due to this increase in potential interactions with microplastics in an area of greater primary productivity, it is of paramount importance that we gain a greater understanding of how organisms in these areas will respond to this threat.

1.3 Biological impacts of marine microplastics

A review carried out by the Secretariat of the Convention on Biological Diversity and Scientific and Technical Advisory Panel (GEF) in 2012, found that plastic was present in over 80% of cases where animals were either using marine debris for dispersal or habitat or had become entangled or ingested marine debris. Microplastics were thought to be present in 11 % of these cases. The impacts of plastics can include entanglement, concentration and transfer of toxins and

pollutants, lacerations, and impediments resulting from adherence (Baulch and Perry, 2014; Cole et al., 2015; Gall and Thompson, 2015; Wright et al., 2013b). Previously studies investigating the impacts of plastics in the marine environment focused upon ingestion and entanglement of vertebrate species, due to the impacts being highly emotive and often attract media interest (Wright et al., 2013a). Microplastic research to date has also been biased towards vertebrates, as highlighted by do Sul and Costa (2014) who report that out of 101 publications reviewed 26 focused upon microplastic ingestion by vertebrates, whilst only 11 reported impacts upon invertebrate species. However, recently there has been a vast increase in literature focusing on the impacts microplastics may be having upon invertebrates. This literature covers a broad range of organisms from sediment dwelling worms (Wright et al., 2013) to commercially farmed mussels (Mathalon and Hill, 2014) to pelagic zooplankton (Cole et al., 2013).

Over 600 species have now been reported to have encountered microplastics, with over 300 of these species being found to have ingested plastics (Galloway et al., 2017). Ingestion is the most likely way in which organisms interact with marine microplastics (Lusher, 2015). Microplastics occupy the same size range as many plankton prey species and are often ingested either through indiscriminate filter feeding or through selective feeding in which they are mistaken for natural prey. A number of marine organisms have been found to ingest plastic in the field, these include 100 % of marine turtle species, 59 % of whale species, 92 fish species (Kühn et al., 2015) and 43 invertebrate species, across 11 phylum, were found to ingest microplastics in laboratory studies (Lusher, 2015) and 13 out of 15 zooplankton species readily ingested microplastic beads in laboratory settings (Cole et al., 2013). A range of detrimental impacts of

microplastic ingestion, such as limiting feeding uptake, blocking feeding appendages or translocation of plastic particles into the circulatory system have now been found to occur in marine invertebrates (Browne et al., 2008; Watts et al., 2015; Wright et al., 2013; Cole et al., 2013). For example, Cole et al. (2013) found that microplastic beads were indiscriminately ingested by 13 zooplankton species, including four species of copepods, which led to a significant decrease in feeding rates. These beads were later egested in faecal pellets; however gut retention times were up to 7 days in the *Calanus helgolandicus*. They also reported that microplastics were able to adhere to the organisms with beads being lodged between appendages used for prey capture, predator detection and movement. This adherence may have repercussions for feeding, mating, locomotion and predator avoidance (Cole et al., 2103; Galloway et al., 2017).

Adherence of plastics may also lead to an increased energy demand for locomotion and combined with increased gut retention times, microplastics were likely to increase energy expenditure as well as decrease energy intake, leading to negative impacts upon fecundity and growth. Detrimental effects arising from microplastic ingestion have also been reported in the polychaete worm, *Arenicola marina* (Wright et al., 2013b). Here, it was demonstrated that a low level (0-5 % sediment mass) of polystyrene bead (130 µm diameter) ingestion caused a reduction in feeding activity leading to a 50 % reduction in lipid energy reserves, potentially causing negative impacts upon the physiology. Watts et al. (2015) found that shore crabs, *Carcinus maenas*, also reduced their food consumption from 0.33 to 0.03 g d^{-1} and the energy available for growth decreased by 90 KJ individual d^{-1} when fed a diet containing 1 % (by weight) polypropylene microfibers for 4 weeks. Browne et al. (2008) found that polystyrene particles

were translocated to the circulatory system within 3 days, potentially disrupting physiological processes. While other studies have found that when exposed to microplastics mussels had an inflammatory response (Von Moos et al., 2012), oxidative damage, altered gene expression and increased haemocyte mortality (Paul-Pont et al., 2016).

In a key study, Sussarellu et al. (2016) looked at the impact of polystyrene ingestion upon Oysters. Oysters exposed to polystyrene microspheres at concentrations of 2,062 ± 170 and 118 ± 15 beads ml^{-1} for 2- and 6-µm particles, respectively for 2 months during their reproductive cycles were found to have significantly decreased numbers of oocytes and lower sperm velocities. The impacts of microplastic exposure were also seen in the offspring of those exposed, with the offspring having a lower larval yield and development when compared to those with no parental microplastic exposure. This study also found that oysters exposed to microplastics increased their microalgae consumption and absorption efficiency, possibly as a compensatory mechanism to increase energy intake as a response to digestive disruption caused by microplastic ingestion. However, this study used a concentration of beads that is several orders of magnitude above the concentration reported in the environment (Lenz et al., 2016). Therefore, while a number of detrimental effects were reported in the oysters it is not clear whether the current environmental levels of microplastics would elicit the same responses in real-world populations. Nonetheless, these studies show that microplastics are able to have a significant impact upon resource and energy allocation. The ingestion of microplastics has the potential to disrupt energy intake, meaning that less energy may be available for reproduction and survival. However, only a few groups of organisms have been

studied in this detail and while ingestion may be occurring it is not clear if microplastic ingestion always leads to detrimental impacts on energy allocation.

Further to these detrimental impacts, there is widespread concern that plastics are capable of transferring toxins into organisms. Additives, often referred to as “plasticisers”, are often added to plastics during their production, these are used to improve its functionality, i.e. provide heat resistance, protect against oxidative damage and microbial degradation or make them more malleable (phthalates) (Browne et al., 2007; Talsness et al., 2009; Thompson et al., 2009). The level of additives varies with the plastic type, for example polyvinyl chloride, phtalates can make up 50 % of its weight (Oehlmann et al., 2009) and polycarbonates are often found to cantain Bisphenol A. These additives have the potential to leach into the environment as the plastic breaks down, both Bisphenol A and phtalates have been found in elevated concentrations in aquatic environments, particularly around landfills (vom Saal and Myers, 2008). The leaching of these additives may provide a mechanism for them to be taken up my organisms either through their presence in the environment or through leaching post-ingestion (Barnes et al., 2009; Lithner et al., 2011; Talsness et al., 2009).

Microplastics have a large surface area to volume ratio, increasing the risk of leaching chemicals once ingested (Cole et al., 2011). It is thought that these additives may lead to disruptions of biological mechanisms such as reproduction, movement and growth (Barnes et al., 2009; Lithner et al., 2011). Bisphenol A has previously been shown to be an endocrine disrupting chemical that mimics, competes with or disrupts synthesis of hormones (Talsness et al., 2009),

potentially leading to disruptions in development and reproduction (Cole et al., 2011). While phthalates have been shown to have a range of effects on aquatic organisms, these include genetic damage, locomotive disruption and intersex in fish (Oehlmann et al., 2009). However, while Oehmann et al. (2009) found that these plasticisers can induce negative effects at low concentrations in a number of organisms, there has been little research into whether plastics are transferring these chemicals into organismsin the marine environment. Evidence for this often comes from studies mechanically leaching plastics in a laborotory setting (e.g. Bejgarn et al., 2015) and leachates collected ready for exposure to organisms. This methodology is unlikely to represent the chemical composition and complexity resulting from plastic degradation found in natural environments.

As plastics fragment they increase their surface area and their hydrophobic properties mean that many hydrophibic organic contaminants (HOCs) and bacteria adhere to their surface (Mato et al., 2001). These toxins may then disassociate from the plastic once ingested (Mato et al., 2001). Plastics are composed of monomers that break down in the marine environment (Browne et al., 2007). A number of plastics (e.g polyvinyl chloride, polystyrene and polycarbonate) are known to release toxic monomers that have been associated with cancer and reproductive abmormalities in invertebrates (Browne et al., 2007). Laboratory exposures have shown that a low dosage of 0.074 % of plastics in the sediment led to PCB bioaccumulation in lugworms increasing by a factor of 1.1 relative to the control group (Besseling et al., 2012).

1.4 Does microplastic shape matter?

Microplastics found within the water come in a variety of shapes and sizes, with fibres, film and pellets amongst others being reported. However, fibres are more common within the environment due to the fragmentation processes (Andrady, 2011; Hidalgo-Ruz et al., 2012; Erikson et al., 2014; Woodall et al., 2014). Despite microfibres being the most numerous in environmental samples, laboratory studies to date have mainly focused on the impacts of microplastic beads. Therefore, there would appear to be a mismatch between laboratory studies to date and the environment. Fragments have a greater potential to impact upon organisms due to their shape, making them less able to pass smoothly through an intestinal system. Fibres have been found to be present in the gut contents of decapods (Murray and Cowie, 2011), copepods and euphausiids (Desforges et al., 2015). Laboratory studies have found that ingesting microplastic fibres can have consequences, such as reduced growth rates. Shore crabs were found to have reduced energy available for growth and reproduction along with a reduced food consumption rate when fed microfibres (Watts et al., 2015). Au et al. (2015) found that toxicity of microfibers was greater than that of particles in the freshwater amphipod, *Hyalella azteca*. This toxicity manifested itself in in greater gut residence times and lower growth rates in amphipods exposed to polypropylene fibres relative to those seen in amphipods exposed to polyethylene particles (Au et al., 2015). Due to the increase in the toxicity of impacts being reported in these organisms, it is important to expand the current knowledge of how fibres will interact with marine animals.

Microplastics are widely available to a range of organisms; however, feeding strategies may affect upon the likelihood of an organism to ingest microplastic (Setala et al., 2015). In a small-scale mesocosm containing, filter feeding bivalves (*Macoma balthica* and *Mytilus trossulus)*, free-swimming crustaceans (*Gammarus* spp. and *Mysid shrimps)* and deposit feeding worms (*Monoporeia affinis* and *Marenzelleria* spp.), it was found the filter-feeders ingested significantly more plastics (Setala et al., 2015). This is likely to be due to their non-selective feeding strategy. Filter feeders are often unable to reject microplastics thereby making them vulnerable to ingestion and the associated impacts. For example, Rosa et al. (2015) examined particulate uptake in *M. edulis*. These authors found that mussels fed polystyrene particles that ranged in size from 2-45 µm had a capture rate of almost a 100 % for particles over 4 µm, whilst particles under this size had a lower capture efficiency. This suggests that size of particles plays a role in mussel prey capture and the mussels were not able to reject the plastic particles before ingestion.

A filter feeder that is yet to receive any attention in relation to microplastics is the sea squirt. Sea squirts are prolific filter feeders (Ruppert et al., 2004) that are often found in areas of high anthropogenic activity (Kourakis and Smith, 2015). These traits make them a potentially vulnerable organism to ingestion and associated impacts of environmental microplastics. Filter feeders, such as sea squirts, play a role in ecosystems by filtering particles out of the water column and either absorb and store them, re-suspend them or alter their properties, possibly causing them to have a greater density. This alteration of their properties may make them important in bentho-pelagic coupling, a process in which nutrients are transported from the water column to the sediment below. Filter-

feeders ingest plankton and particulate organic matter, which is then egested in the form of faeces and psuedofaeces. This egested matter often has a higher density than the plankton and particulate organic matter causing it to sink to greater depths, delivering nutrients to deeper water.

It is possible that microplastic is also being transported to ocean sediments in this way. Cole et al. (2013) found that after ingestion microplastics were packaged into the faecal pellets of copepods. This caused the faecal pellets to have a greater buoyancy, slowing their sinking rates, potentially leading to the plastic laden faecal pellets being available to pelagic organisms for longer. Conversely, by being packaged into faecal pellets microplastics were contained in aggregates denser than the microplastics separately, leading to a greater volume of microplastics sinking. This would lead to a greater volume of microplastics being available to benthic organisms. In line with Cole et al. (2013), it is likely that many organisms ingesting plastics are also producing microplastic-laden faeces. Sea squirts occupy coastal areas of high bioproductivity and by processing microplastics into waste products may enhance microplastic availability to other organisms.

1.5 Sea Squirt Ecology

Ascidians, commonly known as sea squirts, play many roles within intertidal communities (Ruppert et al., 2004). Often found on the underside of rocks they increase the surface area available for settlement of other species allowing the formation of diverse cryptic communities (Lambert, 2005). Ascidians are also

predated upon by specialised species including gastropod, echinoderms, fish and flatworms, making them an important link within local food webs (Lambert, 2005). This potentially enables them to play a role in the bioaccumulation of microplastics within higher trophic levels. Sea squirts are efficient filter feeders capable of filtering the equivalent of their body volume every second (Ruppert et al., 2004). This efficiency in water filtration enables them to purify water by extracting and storing toxins, such as vanadium (Ruppert et al., 2004).

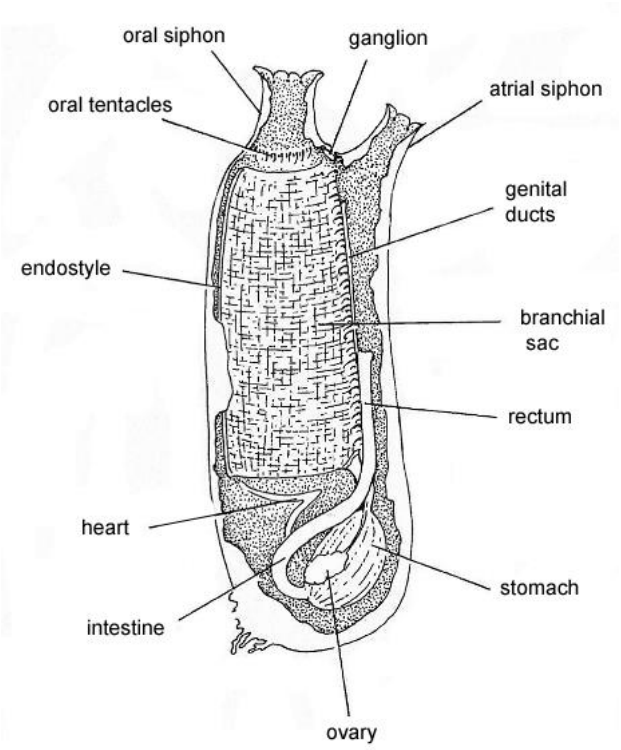

Figure 1.1: diagram of *Ciona intestinalis*, showing locations of key organs. Adapted from Millar (1970)

It is clearly important, therefore, that a greater understanding of how microplastic pollution will impact upon sea squirts and affect their ecological roles within ecosystems is gained. The indiscriminate filter feeding of ascidians lends them to microplastic studies. Ascidians remove particulates from the water current pumped through the pharyngeal basket. The current is produced by lateral cilia

on the margins of pharyngeal basket gill slits (Ruppert et al., 2004). The endostyle excretes a mucus net to cover the pharyngeal basket lining, trapping suspended particles (Ruppert et al., 2004). These particles are then transported by cilia to the oesophagus (Ruppert et al., 2004). Once through the pharynx, the digestive tract is a U-shape (Ruppert et al., 2004). The oesophagus leads in a dorsal direction to the stomach, at the base of the U. The stomach is the site of extra-cellular digestion and is lined with secretory cells (Ruppert et al., 2004). The intestine then ascends from the stomach, terminating with a rectum and an anus. The intestine forms faeces and is likely to be the site of absorption (Ruppert et al., 2004). The anus discharges faeces into the atrium for expulsion through the exhalant siphon. This simple gut structure will influence the ways in which microplastic ingestion will affect sea squirts. It is possible that small plastics can be transported from the intestine via absorption along with other particles.

Ciona intestinalis is a sea squirt that has a global distribution. *C. intestinalis* is native to the UK but has been transported via ballast water to North America where it is now a prolific invasive species (Carmen et al., 2010). Within the UK, sea squirts including *C. intestinalis* are regularly found in rock pools, harbours and marinas. *C. intestinalis* has an opaque tunic allowing some of the internal organs to be viewed without invasive methods. This species was chosen as the test species for this study due to is commonality and opaqueness, as can be seen in figure 1.2.

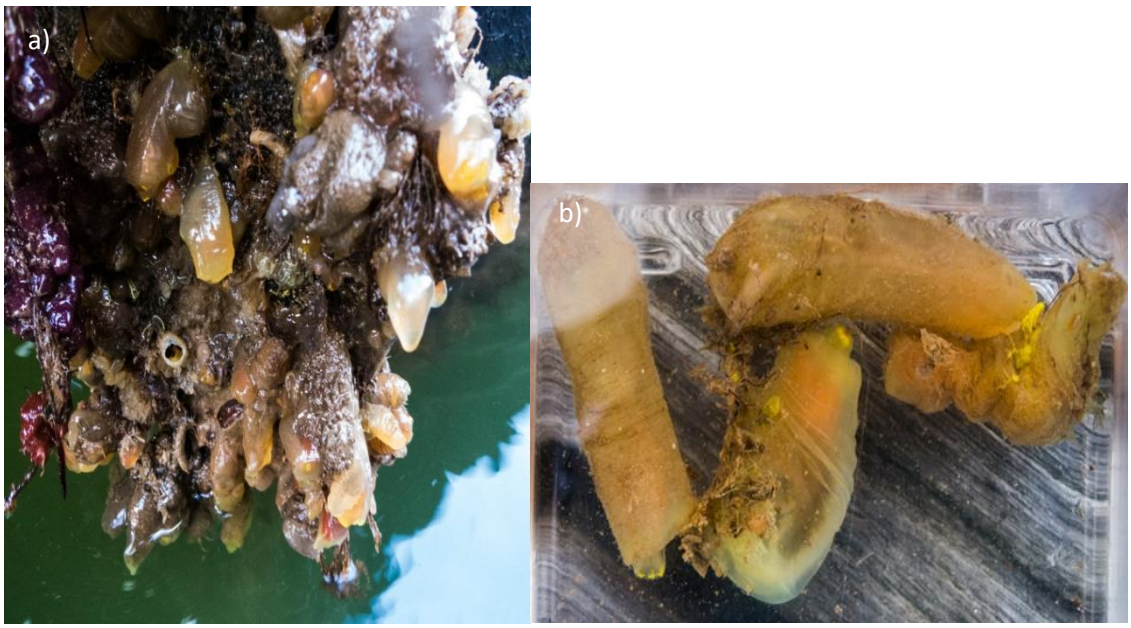

Figure 1.2: *Ciona intestinalis* collected from Millbay Marina. A) *C. intestinalis* found living on structures in Millbay marina, forming a cryptic community. B) *C. intestinalis* after removal from structures.

1.6 Thesis Aims and Hypotheses

This body of work aims to establish whether the sea squirt *Ciona intestinalis* will ingest microplastics and if so whether there are any associated negative effects. The following hypotheses are tested:

(H_1) Sea squirts will readily ingest a variety of microplastics in laboratory studies;

(H_2) Once ingested the microplastics will negatively affect sea squirt feeding rates;

(H_3) Sea squirts are ingesting microplastics in the natural marine environment.

Chapter 2:

The Uptake of Different Microplastics in *Ciona intestinalis*

2.1 Introduction

2.1.1 Microplastics and Marine Organisms

The quantity of marine plastics in our oceans is increasing, with their global production being estimated at 300 million tonnes per year (Plastics Europe, 2016). Plastics are lightweight and durable, meaning that once in the ocean they persist and accumulate. Once in the oceans these plastics break down through a variety of processes, including biodegradation, photo-degradation, thermo-oxidative degradation and hydrolysis (Andrady, 2011). These processes cause plastics to fragment into microplastics, generally defined as plastics with a diameter of less than 5 mm (Cole, 2011). It is now thought that there are more than 50 trillion microplastic particles within the oceans (van Sebille et al., 2015). Recently, Galloway and Lewis (2016) produced a tentative adverse outcomes pathway (AOP) to show the effects microplastics may have upon aquatic organisms. This pathway, based on the work of Sussarellu et al. (2016), shows the mechanisms of microplastic uptake and associated impacts. This pathway, combined with other studies, show that ingestion of microplastics can lead to range of negative effects. These include gut blockages, decreased food intake, reduced energy reserves for growth and reproduction (Cole et al., 2013; Wright et al., 2013b; Sussarellu et al., 2016; Galloway and Lewis, 2016), oxidative damage, alterations in gene expression (Jeong et al., 2016; Paul-Pont et al., 2016) and decreases in survival (Ogonowski et al., 2016; Oliveira et al., 2013; Rist et al., 2016).

2.1.2 Microplastic Properties affecting Ingestion

Microplastics vary in size, shape, density and abundance and are transported within the oceans based on these characteristics. These factors will also play a role in ingestion by marine organisms. The ubiquity and high abundance of microplastics in marine habitats means that all organisms are likely to come into contact with a range of microplastics. Microplastics can have a range of impacts on organisms from sub-cellular alterations (Jeong et al., 2016; Rochman et al., 2014) through to impacts at organismal level such as ingestion and absorption (Cole et al., 2013; Van Cauwenberghe et al., 2015; Wright et al., 2013a). However, it is thought that ingestion is the greatest threat posed by microplastics (Lusher, 2015). Microplastics occupy the same size range as plankton and sand grains, making them bioavailable for a wide range of marine organisms (Wright et al., 2013a). Often organisms are unable to differentiate between natural prey items and microplastics, leading to unintentional ingestion (Cole et al., 2013). Ingestion of plastics has now been reported in over 300 marine species (Galloway et al., 2017). It has been suggested that many lower trophic organisms are not able to select for nutritious food particles, but capture all particles within a size range (Moore, 2008). This is reason for concern in our environment as plastics are often occupy the same size range as prey species and are therefore likely to be ingested in relatively large quantities by low trophic level organisms.

Microplastics vary in type and density meaning that they are likely to be in all habitats. The density may play a role in the availability for different organisms (Wright et al., 2013a). Buoyant polymer types, such as polyethylene (PE), accumulate on the surface (Wright et al., 2013a; Clark et al., 2016) and tend to

be transported by winds and surface currents (Cózar et al., 2014; Lusher et al., 2015) to oceanic gyres (Law et al., 2010; Maximenko et al., 2012). Therefore, these plastics are likely to be encountered by organisms inhabiting the upper water column, such as plankton (Wright et al., 2013). Negatively buoyant polymers, such as polyvinyl chloride (PVC), will sink in the water column where they may be encountered by benthic suspension and filter-feeders, deposit feeders and detritivores (Wright et al., 2013). Negatively buoyant plastics within the water column may be transported through oceanic currents (Van Cauwenberghe et al., 2013). These transport mechanisms allow microplastics the reach a wide range of marine regions and habitats, including polar waters (Lusher et al., 2015; Waller et al., 2017), mid-ocean gyres (Goldstein and Goodwin, 2013; Lusher, 2015), coastal regions (Clark et al., 2016), the deep-sea (Van Cauwenberghe et al. 2013) and sediments (Doyle et al., 2011; Thompson et al., 2004). This density related transport increases the availability of plastics to many organisms.

Microplastics have a variety of shapes, with fibres being the most common in the marine environments (Thompson et al., 2004; Claessens et al., 2011; Erikson et al., 2014; Mathalon and Hill, 2014). The shape plays a role in the uptake and impact the plastic may have upon organisms. It is thought that fibres are more likely to cause blockages within intestinal tracts and toxicity may be greater. Au et al. (2015) compared the impacts of fibres and beads on freshwater amphipods. This study found that fibres had greater gut residence times than beads and led to decreased growth rates. Fibres have also been found to be present in a number of organisms in the environment including mussels (Li et al., 2016; De Witte et al., 2014; Van Cauwenberghe and Jansenn, 2014; Van Cauwenberghe

et al., 2015), oysters (Van Cauwenberghe and Jansenn, 2014) and lobsters (Murray and Cowie, 2011), but very few studies have been performed in the laboratory using microfibers to look at their uptake rates under controlled conditions. The majority of laboratory studies use beads to assess the impacts of microplastics on marine organisms. This is possibly due to the ease of using ready-made beads that can be easily quantified and diluted. In order to subject organisms to fibres, the fibres often have to be manually prepared from larger fragments and often form aggregates once in the water (Gutow et al., 2015; Welden and Cowie, 2016). However, organisms are likely to encounter fibres more often than beads within the environment and therefore it is important that the impacts microfibers have upon organisms are investigated.

The abundance of plastic polymer types may also affect their availability to organisms. An increased abundance is likely to lead to increased ingestion of these particle types. The most commonly found plastics in the marine environment are polypropylene (PP), polyethylene (PE) and polyvinylchloride (PVC) (Andrady, 2011). Therefore, it would be expected that these plastics would be the most common polymers ingested by organisms. However, due to the difficulties in accurately assessing polymer types few studies report the numbers of each polymer found to be ingested. Often studies analyse a subsample of the plastics they find within organisms in order to confirm the presence of plastics (e.g. Neves et al., 2015). One study that has reported polymer types is Lusher et al. (2013). This study analysed particles found in the digestive tracts of fish caught in the English Channel. This study reported rayon to be the most common polymer (57.8 %) followed by polyamide (35.6 %), polyester (5.1 %) polystyrene

(0.9 %) and polyethylene (0.3 %). These findings suggest that abundance may be an important factor in ingestion by marine organisms; however, other factors may be more important in making polymers available to organisms. As a variety of plastics increase in abundance within marine environments, it is important to understand whether the polymer type affects ingestion in a range of organisms, and if ingestion occurs, the potential impacts this may have. Here, I look at ingestion of a variety of plastic types and shapes in the common sea squirt, C*iona intestinalis*.

Feeding strategies may also influence the impact microplastics have upon organisms (Setala et al., 2015), with benthic suspension feeders possibly being more susceptible to sinking microplastics (Wright et al., 2013a) than buoyant polymers. Setala et al. (2015) found that when exposed to microplastics in a mesocosm study, filter-feeders ingested the greatest volume of plastic. Filter-feeders capture particles using specialized structures, often cilia that create a current leading to an inhalant siphon, where ingestion occurs. This feeding strategy is non-selective and microplastics are likely to be ingested due to the inability to reject particles before ingestion. The impacts of microplastics have been studied in very few filter feeders. However, one that has received a great deal of attention is the common mussel, *Mytilus edulis*. *M. edulis* has been shown to ingest microplastics ranging from 2 to 90 µm (Browne et al., 2008; Ward and Kach, 2009; Ward et al., 2003, Von Moos et al., 2012; Van Cauwenberghe et al., 2015; Paul-Pont et al., 2016) and are therefore susceptible to ingestion of a wide range of microplastics in the environment.

2.1.3 Ascidians and Microplastics

Ascidians are efficient filter-feeders and are therefore likely to be susceptible to the impacts of microplastics. *C. intestinalis* feed by filtering sea water through their inhalant siphon which in the large specimens measured in Du Clos et al., (2017) was found to have an opening diameter of 7 to 10 mm (Figure 2.1). This large diameter is likely to enable microplastics to enter into the sea squirt digestive system with relative ease. Sea squirts typically grow in coastal areas, such as marinas and rock pools (Lambert, 2005). They provide refuge and are preyed upon by a variety of organisms (Lambert, 2005). However, they have been overlooked when assessing the impacts of microplastics. In line with the AOP produced by Galloway and Lewis (2016), the first step to establishing whether microplastics have an impact upon organisms is to investigate whether ingestion occurs. Following the establishment of ingestion, gut residence times and quantification of ingestion is required to ascertain whether there are likely to be any further biological effects.

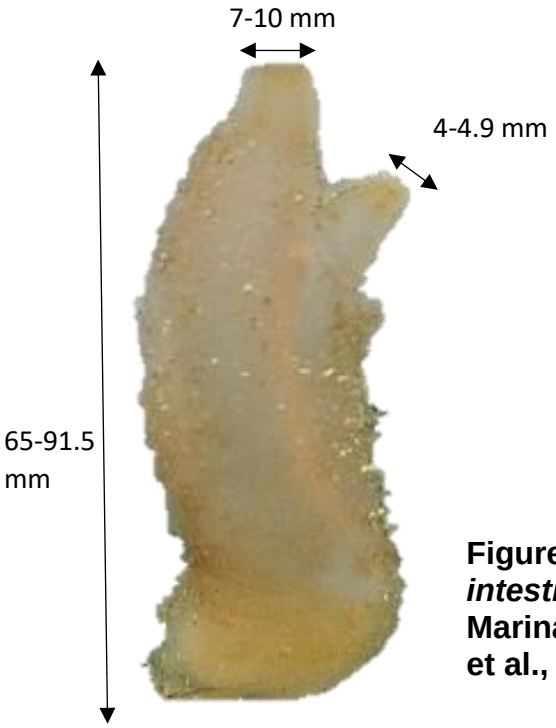

Figure 2.1: Photograph of a *Ciona intestinalis*, collected from Millbay Marina. Dimensions found in Du Clos et al., (2017) are shown.

2.1.4 Chapter 2 Aims and Hypotheses

In this study, the ingestion of a variety of microplastics types in *C. intestinalis* is quantified under laboratory conditions in order to test the hypothesis that all plastics will be ingested due to the indiscriminate filter feeding mechanism employed by sea squirts. It is also hypothesised, that in line with other studies, algal ingestion will decrease when microplastic ingestion occurs. I also aim to establish whether ingestion of microplastics is affected by the microplastic concentration in the water or by the exposure length. The hypothesis is that an increased concentration and increased exposure time would lead to an increase in plastic ingestion by *C. intestinalis.* Depuration was also looked at in order to establish gut residence times and the ability to egest plastics by *C. intestinalis*. Depuration was hypothesized to occur after removal from plastic contaminated water, with the greatest number of plastics being seen in sea squirts immediately after removal from a plastic exposure.

2.2 Methods

2.2.1 Animal collection and Maintenance

Specimens of *Ciona intestinalis* were collected by hand from Millbay Marina, Plymouth on a number of occasions. Collections were carried out on clear, calm days. Adult specimens, ranging in size from 0.4 to 8 g were collected individually and transported in aerated seawater back to the laboratory. The animals were then maintained at 32ppt at 15°C for a minimum of 48 hours prior to any experimentation.

2.2.2 Microplastic Ingestion Exposures:

To first establish whether sea squirts ingest microplastics, individual sea squirts (75 in total, 25 per plastic concentration) were exposed to 10 µm fluorescently labelled polystyrene (PS) spheres (Sphero^{TM} Flourescent Particles, Yellow, 10.0-14.0 µm). Sea squirts were exposed to one of three treatments; a control (no microplastics added), a microplastic concentration of 100 PS bead ml^{-1}, or a concentration of 500 PS beads ml^{-1}. These concentrations were chosen to ensure ingestion was likely to occur and to be comparable to those used in previous studies (Lee et al., 2013; Ogonowski et al., 2015; Van Cauwenberge et al., 2015). Stock concentrations were established using a Beckman Coulter Counter to quantify the concentration of beads per ml.

Initially, 10 µm polystyrene beads were used to investigate microplastic ingestion. Each individual sea squirt was placed in a 400 ml glass beaker with 300 ml of 0.2 µm filtered artificial seawater. Sea squirts were then divided between six treatments: 1) 24-hour exposure to no plastic; 2) 24-hour exposure to a nominal plastic exposure of 100 beads ml $^{-1}$; 3) 24-hour exposure to a nominal plastic exposure of 500 beads ml^{-1}; 4) 72-hour exposure to no plastic; 5) 72-hour to a nominal plastic exposure of 100 beads ml $^{-1}$; 6) 72-hour exposure to a nominal plastic exposure of 500 beads ml^{-1}. Water samples were taken at regular intervals throughout the exposure and analysed via a Beckman Coulter Counter to give a bead count per ml in the exposure water.

A second experiment was then carried out in order to further explain the initial results. This exposure was carried out using 2 L glass beakers containing 1 L of

0.2 µm filtered artificial seawater. Each beaker contained a magnetic stir bar and a specifically designed mesh on which the sea squirt was placed (Figure 2.2). The beakers were placed on magnetic stir plates set at 130 rpm to ensure there would be no plastic settlement (Figure 2.2). Air was provided by airlines and Pasteur pipettes. Three exposure times were selected: 24, 48 and 72 hours. Six sea squirts were exposed to a nominal concentration of 500 10 µm polystyrene beads per ml for each of the exposure times. Water samples were taken from the top, middle and bottom of the beaker throughout the exposure and analysed using the Beckman Coulter Counter. This was done to establish where the plastic was within the water column over time and help explain the previous results.

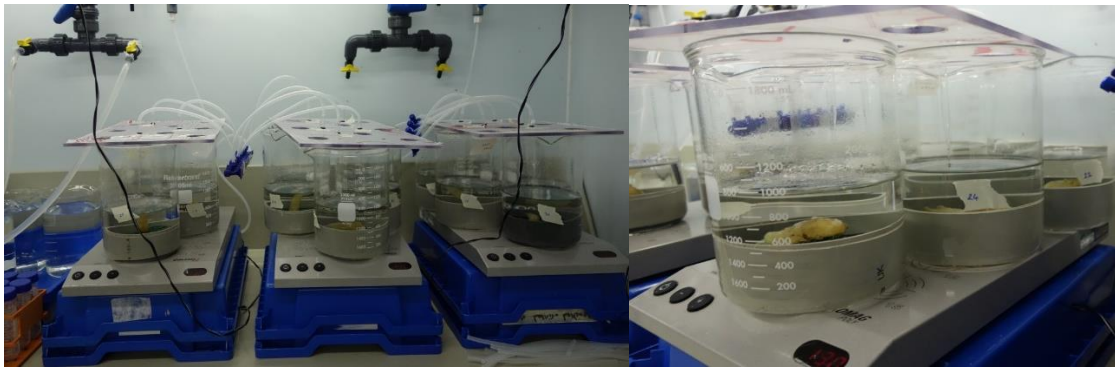

Figure 2.2 Photographs demonstrating microplastic exposure. *C. intestinalis* can be seen resting on mesh suspended above magnetic stir bars set to rotate at 130 rpm, air was supplies through glass pipettes.

Subsequent exposures were carried out using fluorescently labelled polyamide (PA) microbeads (Spherotech Ltd). This exposure was carried out in the same manner as the PS exposure using stirrers. Sea Squirts were exposed to a nominal concentration of 500 beads ml^{-1} for 24 or 72 hours, in order to allow direct comparison to PS ingestion. Water samples were taken throughout for coulter counter analysis.

2.2.3 Creation of Microplastics Used

Once polystyrene and polyamide ingestion had been established, exposures were carried out using a variety of plastic types. These plastics included cryo-ground rope fibres, loom bands and high density polyethylene (HDPE) milk bottle tops of approximately 500 µm, as well as fluorescently labelled polyvinyl chloride (PVC), polyhydroxybutyrate (PHB), low density polyethylene (LDPE) and polypropylene (PP) (purchased from Spherotech Ltd.). The cryo-ground plastics were made using the methodology found in Watts et al. (2015). In brief these plastics were cut into sections if required, i.e. blue rope, before being snap frozen in liquid nitrogen. Once frozen the plastics were ground using a commercial coffee grinder in 3 x 10s bursts. This grinding was repeated until plastics were uniform in shape. Once ground these microplastics were manually counted using an inverted microscope (Leica DMI 4000B) to achieve an approximate concentration per gram, sea squirts were placed in individual beakers containing 1 L of ASW and a stir bar set to 130 rpm. These exposures were carried out for 24 hours. As this experiment was to see whether *C. intestinalis* would ingest these plastics exposure concentrations were higher at 1000 particles ml^{-1}.

2.2.4 Depuration experiment

A similar experimental design was used to investigate microplastic depuration in *C. intestinalis* in order to establish whether sea squirts were egesting plastics and if so how quickly. Sea squirts (N=36) were exposed to either fluorescently labelled PS or PA beads as described for the ingestion experiments for two hours. Concentrations of 1000 beads ml^{-1} for PS and 500 beads ml^{-1} for PA were used to ensure ingestion occurred within the short exposure period. Following the

exposure, sea squirts were removed from the contaminated water, rinsed with clean ASW and placed, separately, in a 400 ml glass beaker containing 200 ml of ASW. Sea squirts were left in these beakers for the duration of their depuration time. Three sea squirts were weighed and snap frozen at a number of time points after plastic exposure to establish the rate of depuration. These were 0, 1, 2, 4, 8 and 16 hours after removal from polystyrene exposure and 0, 15, 30, 45 and 60 minutes after the end of the exposure to polyamide. Water samples, 5 ml from each beaker, up until the point of sea squirt removal, were also taken at these time points of those exposed to polystyrene.

2.2.5 Quantification of microplastic uptake:

Upon completion of exposures sea squirts were rinsed to remove any plastics that may have adhered to the outer surface. The sea squirts were then either preserved in 5 % formaldehyde or snap frozen in liquid nitrogen and stored at -80°C, until analysis could be carried out. Sea squirts were thawed and dissected, using sterile, stainless steel scissors and scalpels, to separate out the tunics, intestinal tract and heart. The internal tissues were then weighed and homogenized in 15 ml of milliQ water. Tunics were discarded immediately to avoid contamination. After homogenization 20 µl of homogenate was placed into a well plate. The well plate was then observed under a fluorescent light using an inverted microscope (Leica DMI 4000B). The microplastic beads were then counted in each well. This was repeated six times for each sea squirt in order to give an average bead count for 20 µl of homogenate. The average bead was then used along with the wet weight of the internal organs to calculate the bead count per gram of internal tissue.

Sea squirts preserved in formaldehyde were dissected in order to locate and look for the presence of plastics with the hearts. However, this proved to be unsuccessful due to the hearts not being visible in the preserved sea squirts. In order to ascertain whether plastics were in the hearts the exposures were repeated and hearts removed from the sea squirts immediately after the end of the exposure. Hearts were dissected out in the following manner: The tunic was removed following a lateral cut with a clean blade. This was then discarded in order to avoid contamination. A longitudinal incision was made into the mantle to expose the visceral cavity. The heart was then either removed from this cavity by cutting through blood vessels and removing the digestive system or viewed under a fluorescent microscope whilst still attached. Dissected hearts were then placed under a microscope (Leica DMI 4000B) for visual identification of any plastics.

2.2.6 Quantification of microplastic depuration

Once removed from the water, sea squirts were immediately snap frozen in liquid nitrogen and stored at -80 °C. The sea squirts were then defrosted and their tunics removed, as described above, and discarded. The internal organs were weighed before being homogenized in 15 ml of milliQ water. After homogenization six lots of 20 µl of homogenate from each sea squirt (total of 36 individuals) was pipetted into a well plate. The beads within the homogenate were then counted using an inverted microscope (Leica DMI 4000B) with a fluorescent light. Microplastics in the water samples were quantified by using a Beckman Coulter Counter.

2.2.7 Statistical Analysis

All statistical analysis were carried out using R (version 3.3.2). Analysis of variance (ANOVA) tests followed by Tukey post-hoc tests were carried out to establish any significant differences between microplastic ingestion rates across microplastic concentration exposures, microplastic types and exposure times.

2.3 Results

2.3.1 Microplastic Ingestion

The majority of sea squirts (18 out of 21) were found to have ingested the microplastics they had been exposed to. Ingestion was recorded as a presence or absence of microplastics within the digestive system due to difficulties in quantifying the ingestion of these plastics. However, the ingestion of microplastic was not uniform across all plastic types. Rope fibres did not appear to be ingested by any of the exposed sea squirts (Figure 2.3). All samples taken from sea squirts exposed to PVC, PHB, LPE or PP contained plastics. While HDPE and loom bands were found in 56 and 22%, respectively, of the samples taken from exposed sea squirts.

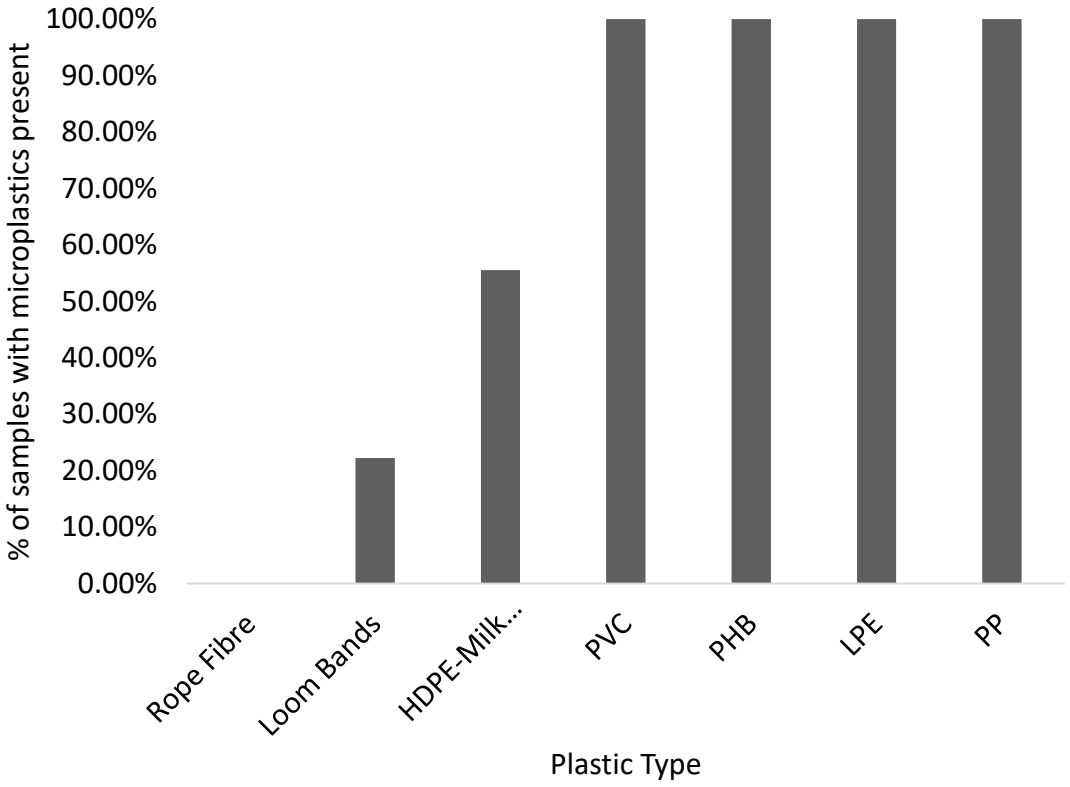

Figure 2.3: Exposure of a variety of microplastic to *C. intestinalis* (data as percentage of samples found to contain plastics in each treatment, 6 sub-samples per sea squirt, N=3 for each treatment).

2.3.2 Polystyrene Ingestion

Sea squirts were found to ingest 10 µm PS beads when no stir bars were present. Polystyrene beads were found in the internal tissues of all sea squirts exposed to both 100 and 500 beads ml^{-1} at both 24 hours and 72 hours. A greater number of beads were found in the internal organs of sea squirts exposed to 500 PS beads ml^{-1} (25536.7 beads sea $squirt^{-1}$) than 100 PS beads ml^{-1} (7678.4 beads sea $squirt^{-1}$) at 24 hours (Figure 2.4). However, after 72 hours there fewer PS beads in the internal organs of sea squirts exposed to both concentrations, with the lowest concentration being seen in those exposed to 500 PS bead ml^{-1} for 72

hours (994.2 beads sea squirt^{-1}) (Figure 2.4). No beads were recorded in sea squirts in either control treatment. Ingestion was significantly different between the nominal concentrations of PS beads (Two-way ANOVA, $F_{2,24}$=4.555, P=0.021) as well as over time (Two-way ANOVA, $F=_{1,24}$=7.998, P=0.009). An interaction effect between time and bead concentration was also seen (Two-way ANOVA, $F_{2,24}$=4.045, P=0.031).

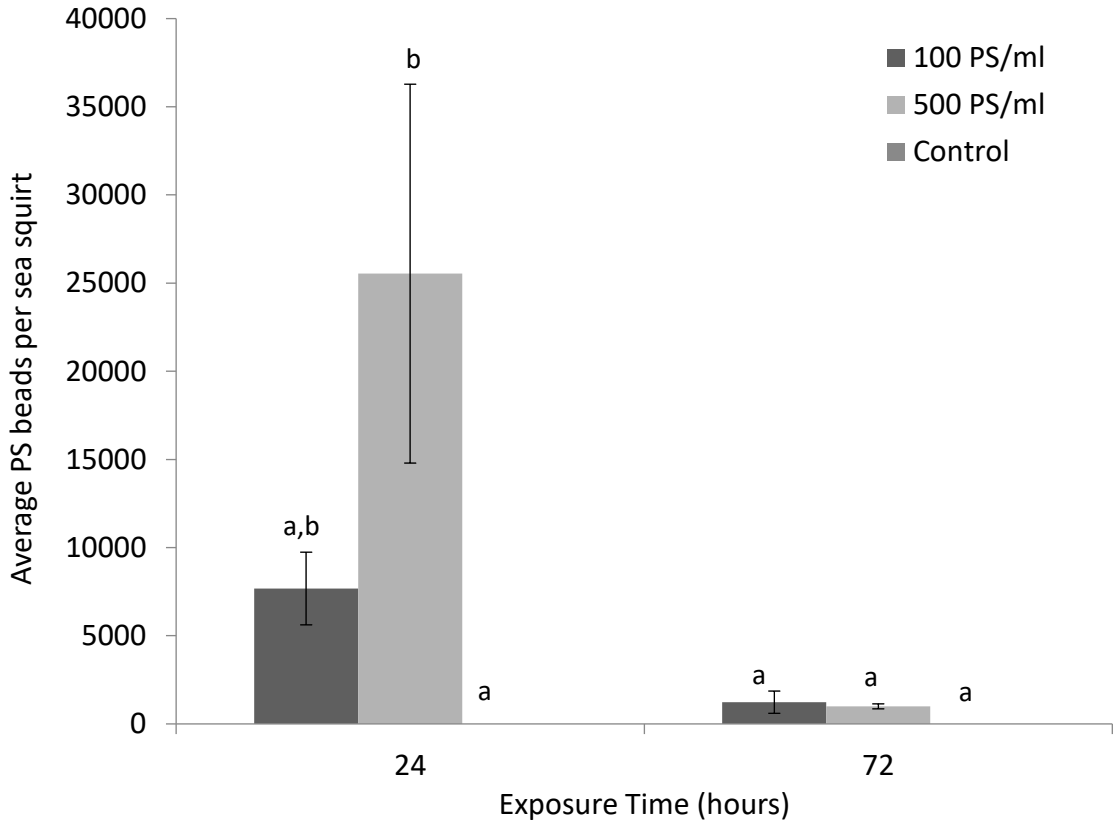

Figure 2.4: Bead content of *Ciona intestinalis* exposed to polystyrene beads (data as average +/- standard error, letters denote significance as found by a Tukey HSD test, N=5).

The bead counts found within the internal organs of the sea squirts were converted to beads per gram of tissue to account for sea squirt size variations in bead uptake. A significant difference in ingested bead concentration was found

between nominal bead concentration (Two-way ANOVA, $F_{2,24}$=9.576, P<0.01) and exposure time (Two-way ANOVA, $F_{1,24}$=16.985, P<0.001). Sea squirts exposed to a nominal concentration of 500 beads ml^{-1} for 24 hours were found to have significantly higher concentration of beads in their internal organs (47354.4 beads g^{-1}) when compared to all other sea squirts (Figure 2.5). No significant differences were found between any other treatments. The lowest concentration of beads was found in sea squirts exposed to 100 beads ml^{-1} for 72 hours (3450.7 beads g^{-1}), exclusive of sea squirts in the control group. No beads were found in the control sea squirts. This is in contrast to concentrations found in whole sea squirts, where sea squirts exposed to 500 beads ml^{-1} had a lower bead content at 72 hours than those exposed to 100 beads ml^{-1} (Figure 2.4).

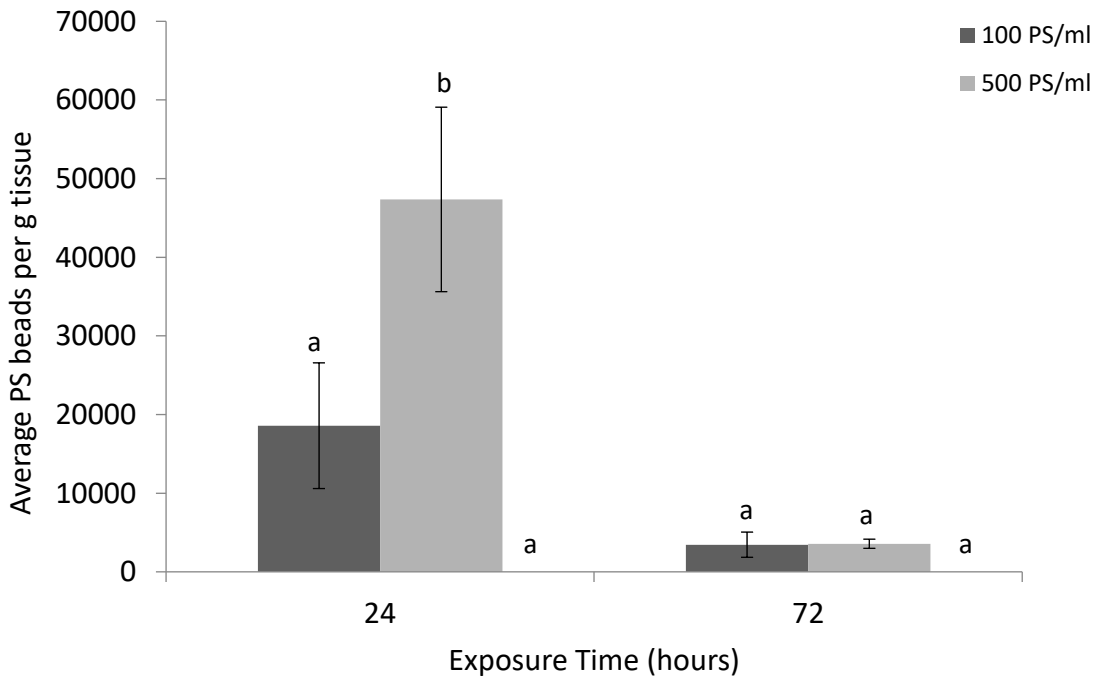

Figure 2.5: Average bead content of *Ciona intestinalis* exposed to polystyrene beads per gram of tissue (data as average +/- standard error, letters denote significance as found by a Tukey HSD test, N=5).

When exposed to a concentration of 500 PS beads ml^{-1} suspended in the water column by a magnetic stirrer, the number of beads in the internal organs of sea squirts decreased significantly (One-way ANOVA, $F_{3,20}=21.22$, $P<0.001$) from 156706.9 beads g^{-1} after 24 hours to 17231.6 beads g^{-1} at 72 hours (Figure 2.6). There was a significant decrease in bead numbers from 24 hours to 48 hours (Tukey HSD, $P<0.001$), however the drop in bead concentration between 48 hours and 72 hours was not found to be significant. Stir bars appear to increase the number of beads ingested by sea squirts. After a 24 hour exposure sea squirts were found to contain approximately 3 times more plastic when a stir bar was present (156706.8 beads g^{-1}) compared to those exposed to plastics without a stir bar (47354.4 beads g^{-1}). This difference appears to increase with exposure time, after a 72-hour exposure sea squirts exposed to plastics suspended by a stir bar contained approximately 5 times more plastic than those exposed to plastics with no stir bar present (17231.6 beads g^{-1} and 3569.9 beads g^{-1}, respectively).

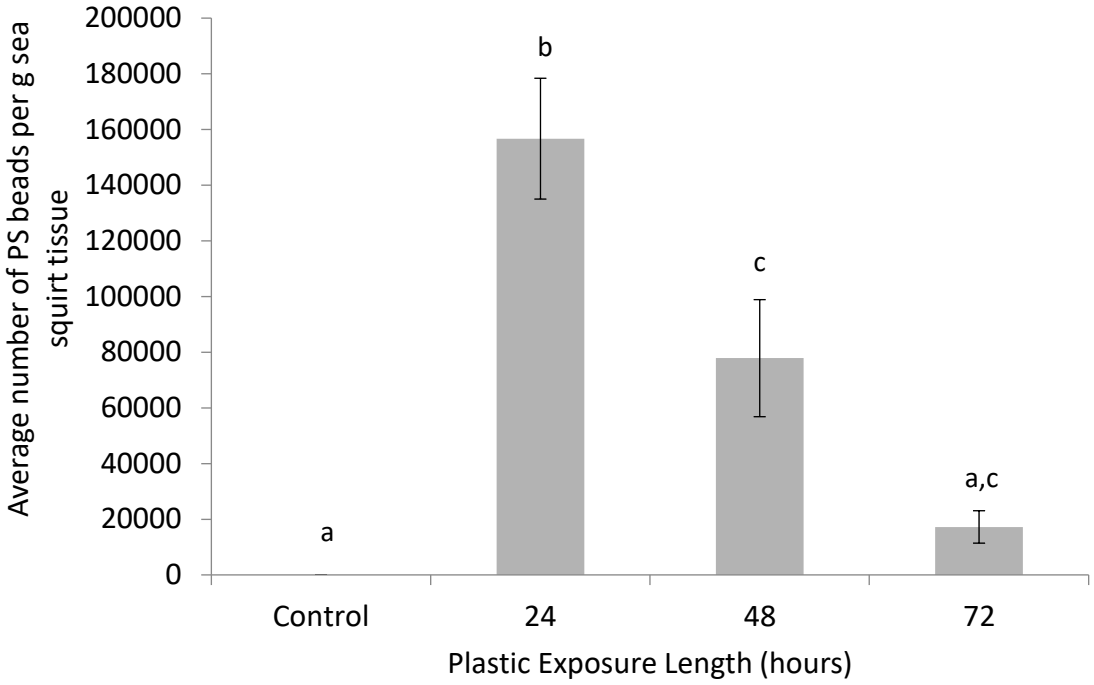

Figure 2.6: Average bead content per gram of *Ciona intestinalis* at different time points exposed to 500 polystyrene beads ml^{-1} (data as average +/- standard error, letters denote significance as found by a Tukey HSD test, N=6).

A coulter counter was used to read the number of particles of approximately 10 µm within water samples. These readings appear to show a smaller number of particles in water samples from controls with no plastic exposure across all time points and water column locations (Figure 2.7a). The greatest concentrations of particles were found in water from plastic controls without sea squirts at the top of the water column at 0 hours (212.4 particles ml^{-1}) and in the middle of the water column at 24 hours (196 particle ml^{-1}) (Figure 2.7b). The number of particles throughout the water column appears to decrease with time in both the plastic control (Figure 2.7b) and the sea squirt exposures (Figure 2.7c).

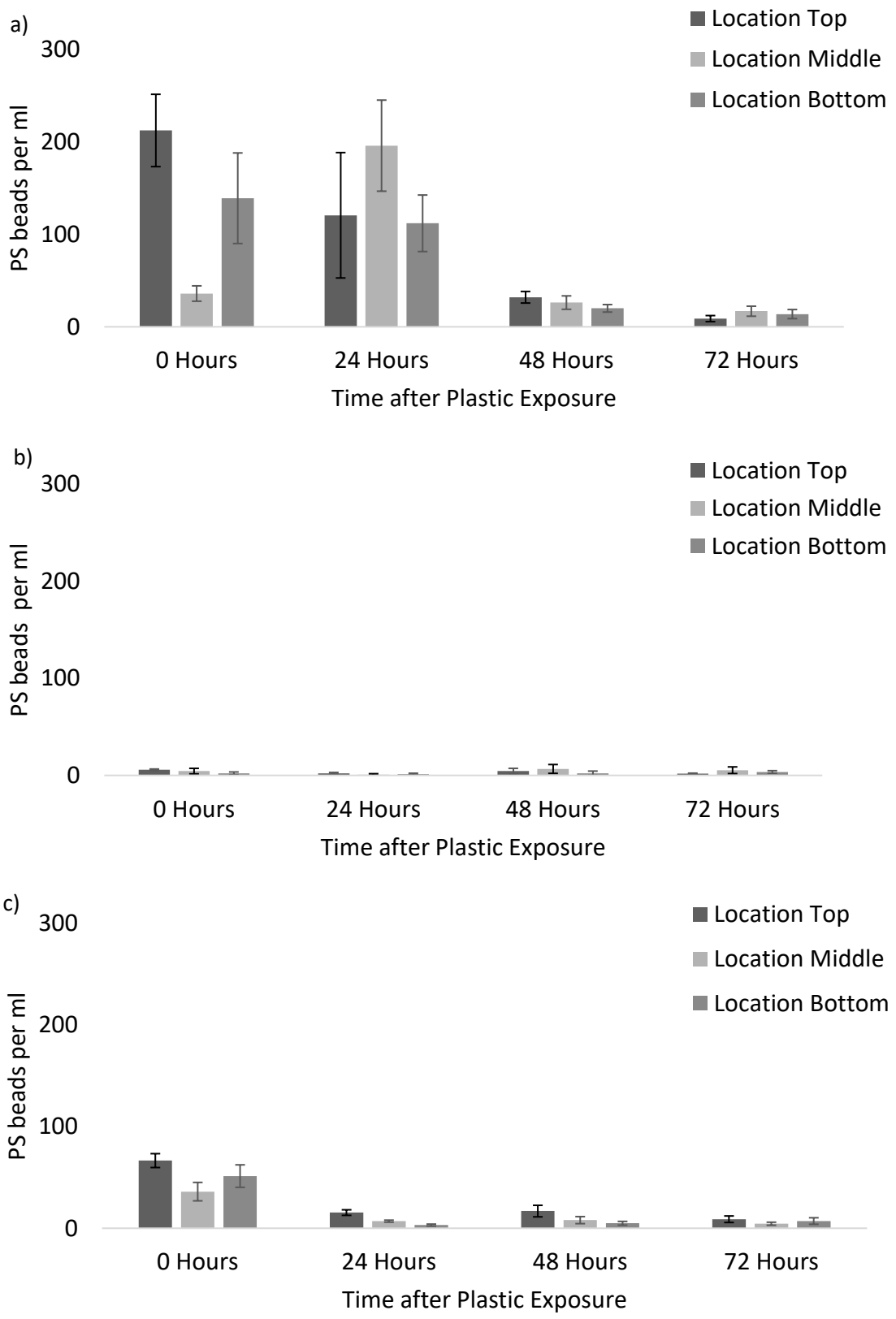

Figure 2.7: Bead content of water samples taken from water in exposure beakers (data as average +/- standard error). a) water samples taken from control vessels containing *C. intestinalis* in clean water. b) water samples taken from beakers with a concentration of 500 PS bead ml^{-1}, but no *C. intestinalis*. c) water samples taken from beakers containing *C. intestinalis* exposed to a concentration of 500 beads ml^{-1}.

2.3.3 Polyamide Exposures

An initial 24-hour exposure to polyamide beads at a nominal concentration of 500 beads ml^{-1} resulted in an average of 213565.67 (± 75409.4) beads g^{-1} within the internal tissues.

A similar pattern was seen in PA ingestion to that seen in PS ingestion. A greater number of beads were seen in the internal organs of sea squirts exposed to a concentration of 500 PA beads ml^{-1} at 24 hours (394876 beads g^{-1}) than at 72 hours (119265.9 beads g^{-1}) (Figure 2.8). This was not found to be significant (One-way ANOVA, $F_{1,8}$=2.153, P=0.18). However, this pattern was not consistent across all exposures carried out. A second experiment looking at sea squirts ingestion of PA beads found a greater number of beads in the internal organs of sea squirts exposed to a concentration of 500 PA beads ml^{-1} at 72 hours (56585.9 beads g^{-1}) than at 24 hours (41452.6 beads g^{-1}) (Figure 2.9). This was also not found to be significant (One-way ANOVA, $F_{1,10}$=0.183, P=0.678).

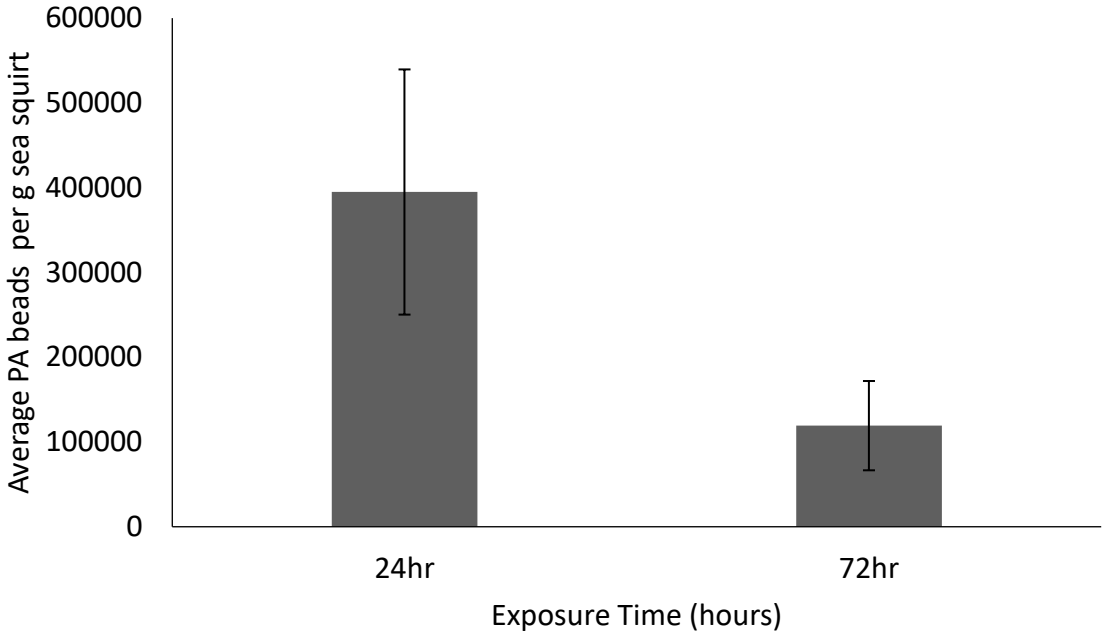

Figure 2.8: Bead content of *Ciona intestinalis* exposed to polyamide beads per gram of tissue (data as average number +/- standard error, N=6 (24 hours), N=4 (72 hours)).

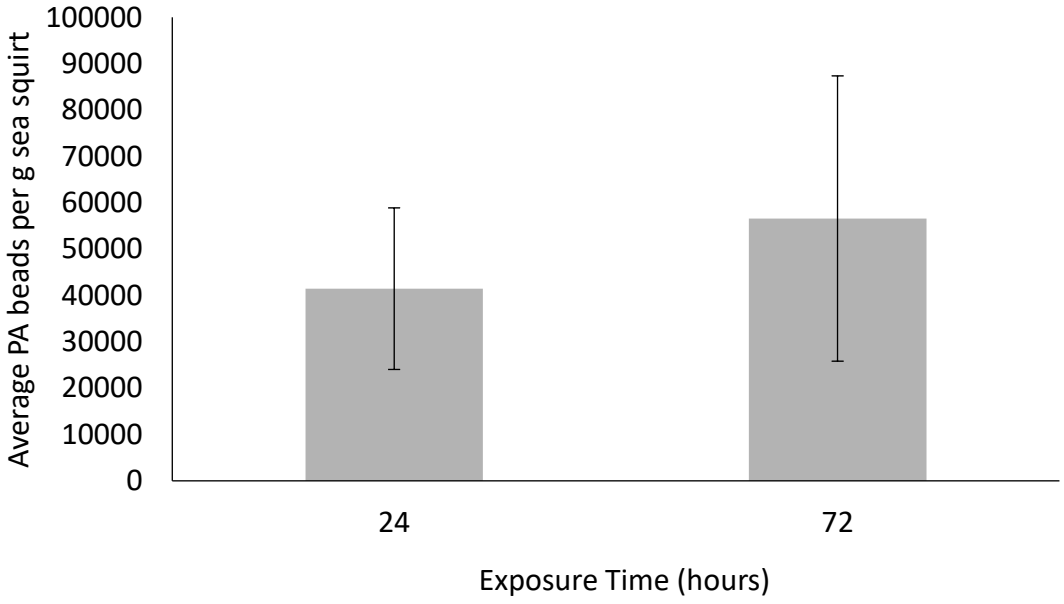

Figure 2.9: Bead content of *Ciona intestinalis* exposed to polyamide beads per gram of tissue in a repeat exposure (data as average number +/- standard error, N=6).

2.3.4 Plastic Translocation

Hearts were not visible in the preserved sea squirts and therefore attempts to dissect them out were unsuccessful. No plastic was observed in the hearts when viewed under a fluorescent microscope (Figure 2.10), suggesting that no translocation of either PS or PA beads into the circulation took place in *C. intestinalis*.

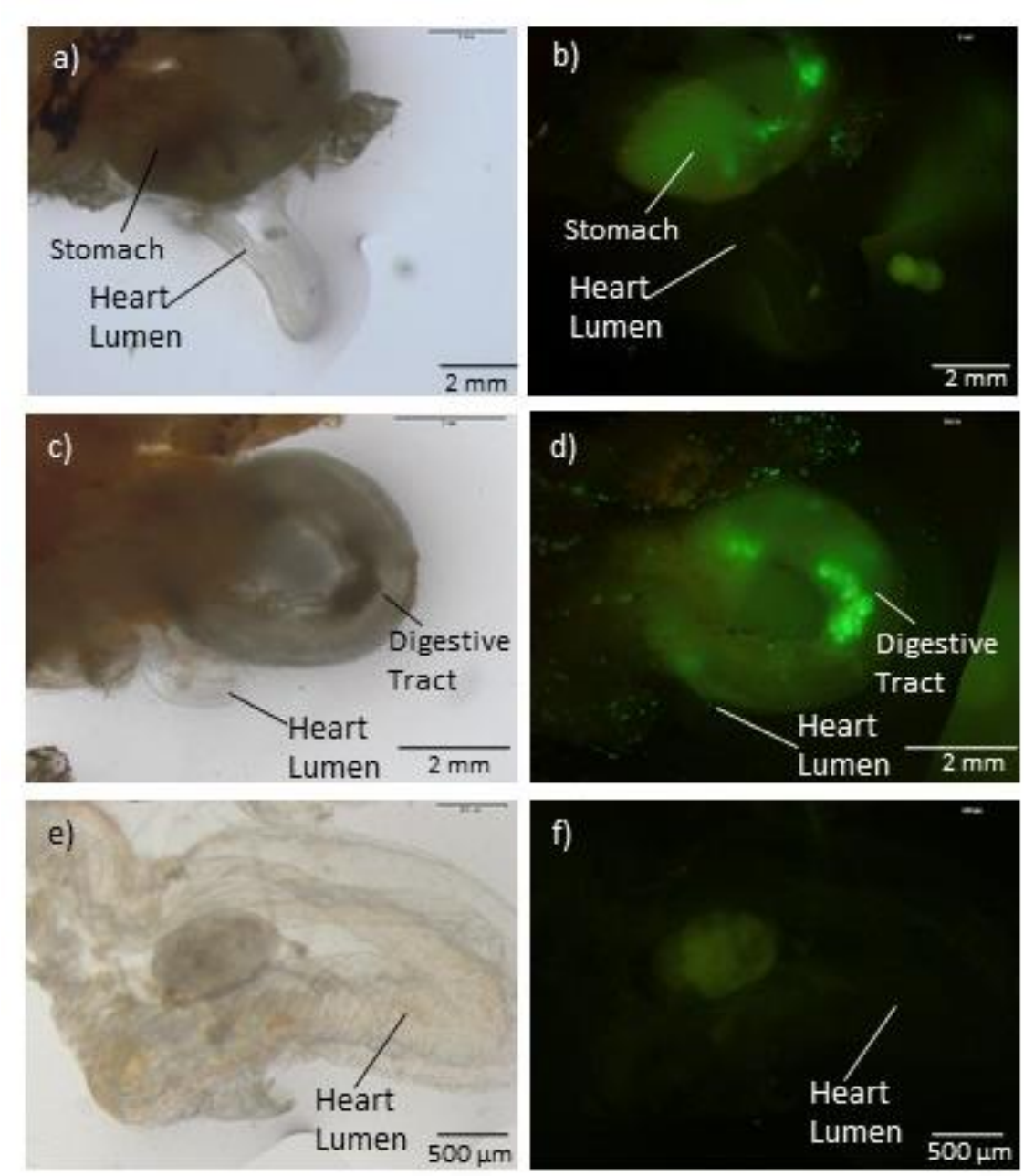

Figure 2.10: Examples of hearts dissected from *Ciona intestinalis* exposed to fluorescent plastics. Photos showing *C. intestinalis* hearts after microplastic exposures. Left hand photos show dissected hearts under white light, right hand pictures show the same hearts as those seen in the left hand photos under green fluorescent light, observed using an inverted microscope (Leica DMI 4000B). Plastics can be seen as fluorescent green particles in the intestines and stomachs in 8b and 8d.

2.3.5 Polystyrene Depuration

Following a two-hour exposure to a concentration of 1000 PS beads ml^{-1} sea squirts were left to depurate. The number of beads found within the internal organs of sea squirts left to depurate did not differ significantly with time after removal from plastic (One-way ANOVA, $F_{5,12}$=2.523, P=0.0877). There was no apparent pattern or decrease in beads within the sea squirts with time (Figure 2.11). An initial increase in plastic beads was seen before the concentration dropped to an average of 211 beads g^{-1} after four hours of depuration time (Figure 2.11). An increase was then observed with the greatest number of beads being found in the internal organs of sea squirts 8 hours (19734 beads g^{-1}) after the end of PS exposure (Figure 2.11). A decrease is then seen to 6998.2 beads g^{-1} in sea squirts given 16 hours to depurate.

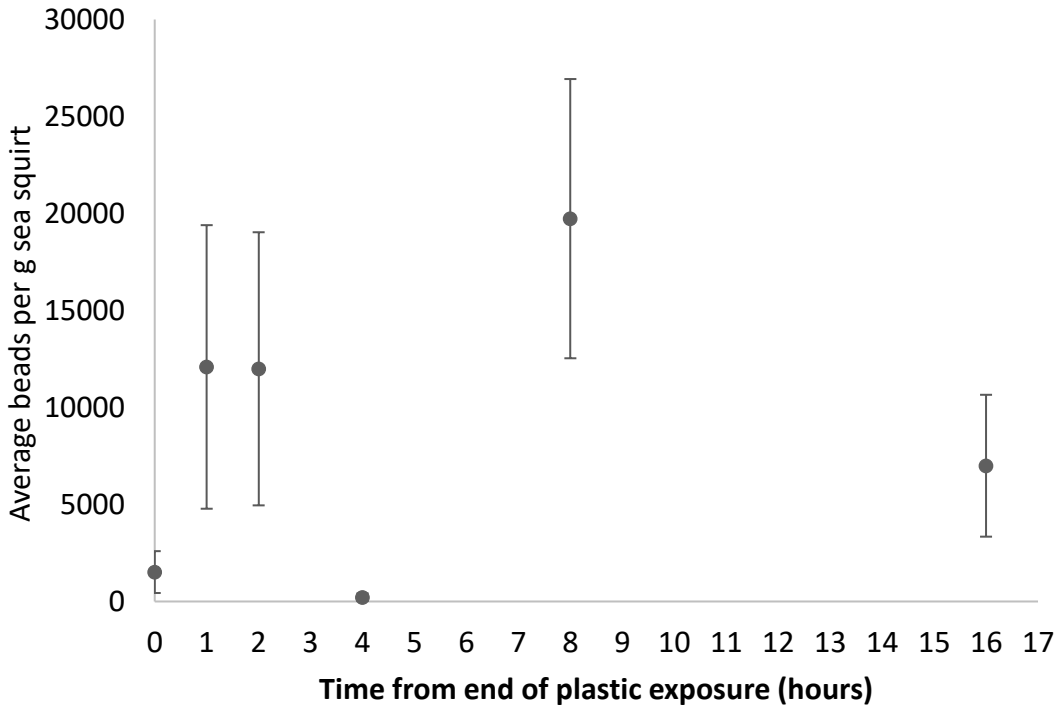

Figure 2.11: Depuration of polystyrene plastics from *Ciona intestinalis* following a 2 hour exposure to a concentration of 1000 beads ml^{-1} (data as average +/- standard error, N=3).

Water samples were taken from the beakers of all sea squirts placed in clean water to depurate after a two-hour exposure to PS beads. Polystyrene beads were found in all water samples. Significant differences in particle concentrations were found with time from removal from the plastic exposure (One-way ANOVA, $F_{5,52}$=4.388, P<0.01) (Figure 2.12). An initial increase in PS beads in the water is seen from 12.8 particles ml^{-1} at 0 hours to 34.7 particles ml^{-1} at 2 hours after removal from the plastic exposure (Figure 2.12). Following this increase a decrease was seen with the lowest concentration of particles (11.2 particles ml^{-1}) being recorded at 8 hours after removal from the plastic exposure. However, the greatest number of beads was found in water taken 16 hours after the sea squirts had been removed from the plastic exposure (Figure 2.12). This pattern of rising

and falling over time appears to compliment, to an extent, the number of beads found within the internal organs of the sea squirts (Figure 2.12). At 8 hours after removal the water contains the least number of particles (Figure 2.12), while the sea squirts contain the greatest number of particles (Figure 2.11). However, after 16 hours this is reversed with the sea squirts containing relatively low number of beads (Figure 2.11), whilst water samples have the greatest number of particles within them (Figure 2.12), indicating a relationship between the sea squirts and the water.

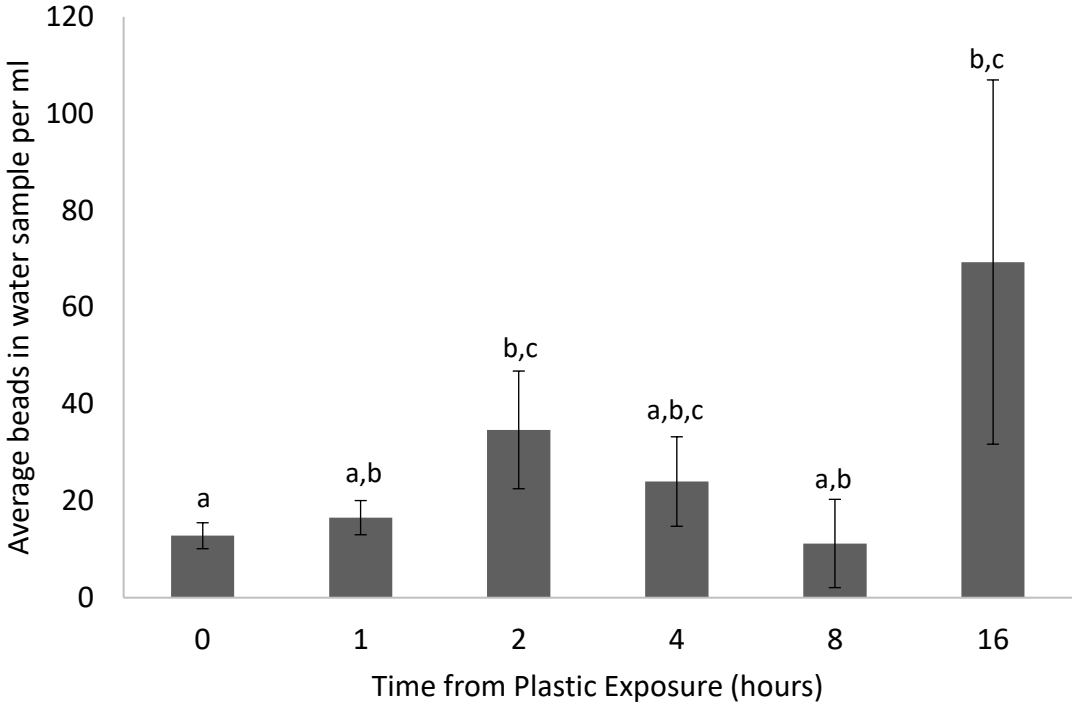

Figure 2.12: Polystyrene content of water from depuration experiments following a 2 hour exposure (data as average +/- standard error, letters denote significance as found by a Tukey HSD test, N=3).

2.3.6 Polyamide Depuration

A depuration exposure was also carried out using polyamide beads. Similarly, to the PS depuration an initial decrease in the number of beads found in the internal

organs was seen before a rise to peak at 20081.7 beads g^{-1} after 30 minutes (Figure 2.13). This was then followed by a decrease to 6128 bead g^{-1} after 45 minutes. However, no significance was found between internal organ plastic content with time (One-way ANOVA, $F_{4,25}$=1.943, P=0.134). Water samples taken from this depuration exposure were not analysed.

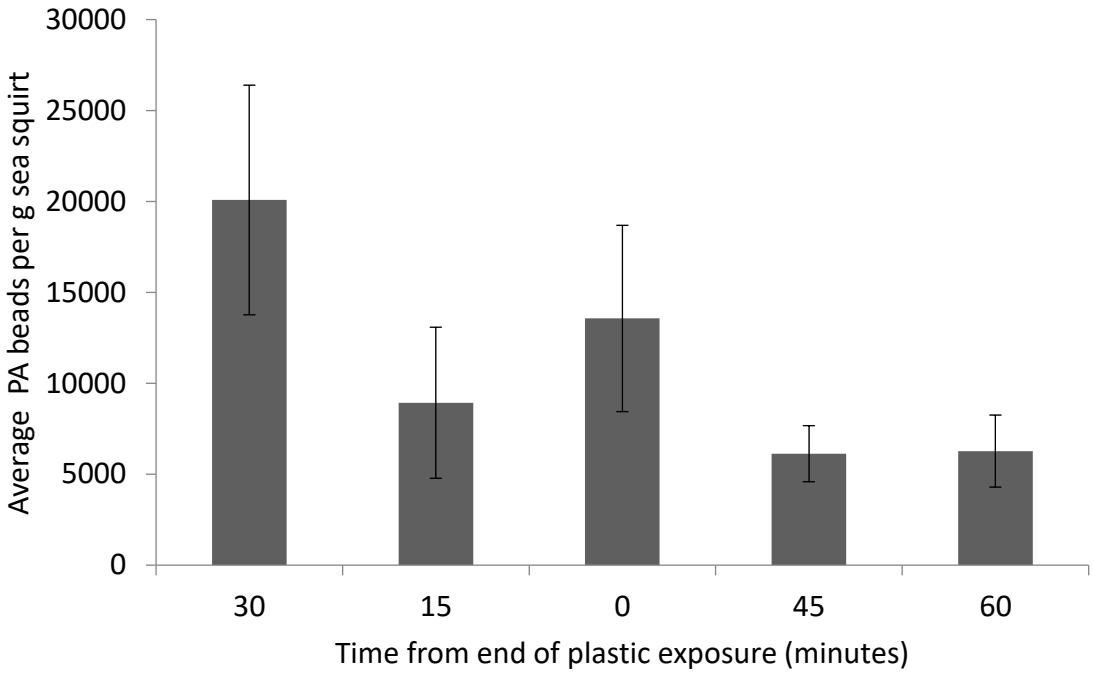

Figure 2.13: Depuration of polyamide plastics from *Ciona intestinalis* following a 2 hour exposure to a concentration of 500 beads ml^{-1} (data as average +/- standard error, N=6).

2.4 Discussion

This study has clearly shown, for the first time, that a common sea squirt, *C. intestinalis* readily ingests a variety of microplastics, adding to the growing number of organisms found to have the potential for microplastic ingestion. *C. intestinalis* were exposed to nine different plastics, ranging in shape, polymer type and density.

The results presented here are in line with many studies looking into microplastic ingestion by filter feeders (e.g. Browne et al., 2008; Von Moos et al., 2012; Cole et al., 2013, 2105; Wright et al., 2013; Van Cauwenberghe et al., 2015; Green et al., 2016; Paul-pont et al., 2016; Sussarellu et al., 2016). Mussels have previously been shown to ingest polystyrene microspheres (Browne et al., 2008; Van Cauwenberghe et al., 2015; Paul-Pont et al., 2016) and HDPE (von Moos et al., 2012). While oysters have been reported to ingest polystyrene (Sussarellu et al., 2016) and HDPE (Green et al., 2016). These studies report that following ingestion of polystyrene, mussels displayed a range of signs from no effects when ingesting 110 particles ml^{-1} (Van Cauwenberghe et al., 2015) through to oxidative damage, increased haemocyte mortality and altered gene expression when exposed to a very high concentration of 2000 particles ml^{-1} (Paul-Pont et al., 2016). Browne et al. (2008) also showed that ingestion of high concentrations of polystyrene beads by mussels led to translocation of the plastics into the circulatory system, possibly leading to extra stresses on the mussels. Ingestion of HDPE has also been shown to lead to lysosomal membrane damage and inflammatory responses in mussels (von Moos et al., 2012).

Oysters have also been shown to have adverse responses from microplastic ingestion. Reductions in feeding rates and offspring survival have been reported in relation to polystyrene ingestion (Sussarellu et al., 2016), while HDPE ingestion appeared to lead to slight decreases in feeding and growth rates (Green et al., 2016). The end points reported in these studies were not looked at in the work presented here, however, it is possible that ingestion of microplastics in sea squirts would lead to one or more of these impacts.

Out of the nine microplastics used in this experiment, eight were found to be present in the internal organs of exposed sea squirts. A key factor determining uptake appears to be the buoyancy on the plastic polymer. The one plastic found to not be ingested was cryo-ground polypropylene rope fibres. These fibres were relatively large (3-5 mm long) and were seen to form aggregates during which remained at the surface for much of the exposure. Polypropylene has a density of 0.91 g cm^{-1}, making it very buoyant. Buoyant polymers are likely to be encountered by organisms inhabiting the upper water column, such as zooplankton (Wright et al., 2013b) and pelagic fish (Neves et al., 2015). However, sea squirts are benthic organisms attaching themselves to surfaces and are less likely to encounter these buoyant particles. It is likely that within this study the rope fibres remained out of reach of the sea squirts.

It was also found that cryo-ground loom bands, made of synthetic rubber, and HDPE had a lower presence in the sea squirts than the other plastic types investigated, excluding the rope fibres. All sea squirts exposed to these plastics were found to contain plastic particles within at least one sample taken from their

internal organs. These plastics are also positively buoyant and formed aggregates during the exposure. Further to this, these plastics also adhered to the glass beakers and plastics within the exposure. This adherence along with positive buoyancy was likely to decrease the occurrence of ingestion by *C. intestinalis*.

Nonetheless, these buoyant polymers were seen in a number of tissue samples taken from the sea squirts. This would indicate that the plastics are being transported downwards in the exposure beaker to reach the sea squirt. The beakers used in this exposure were 2 L; therefore a relatively small water column was formed above the sea squirts. Sea squirts feed by using cilia to produce water currents, this water current then passes through the pharyngeal basket, where particulates are caught (Ruppert et al., 2004). This current has been recorded to reach velocities of 7.4 L h^{-1}, capturing particles 30 mm from the inhalant siphon after a pumping duration of 20 seconds (Du Clos et al., 2017). It is likely that a longer pumping duration will lead to a greater capture region and the feeding current will impact upon water beyond this region. *C. intestinalis* were exposed to plastics for 24 hours, making it likely that their capture region was far greater than 30 mm. Due to the relatively small water column above *C. intestinalis* in the exposures it is possible that the inhalant current would reach the surface, actively drawing plastics downwards towards the inhalant siphon.

High density polymers such as PHB (density: 1.25 g cm^{-3}) and PVC (density 1.4 g cm^{-3}) were kept in suspension by bubbling air into the beaker. All samples from sea squirts taken from these exposures were found to contain plastic particles.

The ingestion of PVC is in line with other benthic filter feeders. Graham and Thompson (2009) found that filter-feeding sea cucumbers ingested both nylon and PVC when it was present in the sediment they were feeding on. Wright et al. (2013b) also found that the lugworm, *Arenicola marina*, ingested PVC when it was present in the sediment. Ingestion of PVC in lugworms led to a decrease in lipid energy reserves, possibly as a result of gut blockages caused by the plastic. The only study, to date, looking at ingestion of PHB in aquatic organisms found that a biodegradable PHB polymer led to decrease in weight gain in *Gammurus fossarum* (Straub et al., 2017).

Low density plastics such as polystyrene (density: 1.03 g cm^{-3}), polyamide (density: 1.14 g cm^{-3}), LPE (density: 0.94 g cm^{-3}) and PP (density: 0.925 g cm^{-3}) would also be expected to remain at the top of the water column making them unavailable to the sea squirts. However, all samples taken from sea squirts exposed to these plastics were found to contain plastic particles. This would suggest that buoyancy was not the only factor determining uptake in sea squirts. Size may also play an important role. Sea squirts are unable to select particles prior to ingestion and will filter all particles in a given size range out of the water column. *C. intestinalis* has been found to have a 100 % filtration efficiency when exposed to inorganic particles ranging from 2 to 50 µm (Randlov and Riisgard, 1979; Robbins, 1984). Rope fibres, loom bands and the HDPE used in this exposure were cryo-ground to be approximately 500 µm in length. This is a magnitude larger than inorganic particles reported to be ingested by *C. intestinalis*. However, some ingestion was seen in those exposed to loom bands and HDPE, suggesting that these particles may not have been uniform in size. The polystyrene, polyamide, LPE and PP used in this study occupied the size

range of 2-50 µm and therefore any particles remaining in the water column had a greater bio-availability to the sea squirts.

Initial ingestion of PS appeared to be concentration dependent with a greater volume of beads being seen in sea squirts exposed to 500 PS beads ml^{-1} for 24 hour when compared to those exposed to 100 PS beads ml^{-1} for the same time. This finding is in line with other studies such as Kaposi et al. (2014). This study found that when sea urchin larvae were exposed to polyethylene microbeads at concentrations of 1, 10, 100 and 300 beads ml^{-1}, a greater volume of beads was present in the stomachs of larvae exposed to 100 and 300 beads ml^{-1}. They also found that larvae exposed to a concentration of 300 beads ml-1 had smaller body widths and a decreased survival rate, suggesting an energy imbalance resulting from PE ingestion. Besseling et al. (2013) also report that the polystyrene gut content was highest in lugworms exposed to a concentration of 100 beads L^{-1}, compared to worms exposed to polystyrene concentrations of 1 and 10 beads L^{-1}. Feeding activity appeared to have the greatest decrease, while weight gain was the least, in the worms exposed to the greatest concentration of plastics. This suggested that higher ingestion of plastics had a greater impact upon the worms activity and weight gain.

To date, few studies have looked at concentration dependent uptake of microplastics in marine animals. However, from these studies it would appear that a greater uptake is seen with greater exposure concentrations. If microplastics continue to increase in abundance in the environment (Claessens et al., 2011), ingestion by marine organisms may also increase. This greater

uptake also appears to lead to greater toxicity of microplastic effects. As concentration appears to play a role in uptake and toxicity, it would be useful for future laboratory exposures to determine concentrations at which toxic effect are seen in organisms.

No microplastics were visible in the hearts of the sea squirts exposed to plastics. It was thought that the microplastics used in this study were able to be translocated due to earlier work carried out on the sea squirt, *Ecteinascidia diaphanis* (author's unpublished work, Gbri.org.au, 2017). This work showed a translocation of microplastic beads into the hearts of the sea squirts (unpublished data). Microplastic translocation has been shown in before in mussels (Browne et al., 2008) and fiddler crabs (Brenneke et al, 2015). Mussels were exposed to a total of 15, 000 polystyrene particles for 3 hours, before being transferred to clean sea water. Mussels were found to contain microplastics in their circulatory fluid after 3 days, showing that polystyrene microplastics have the ability to be translocated within the body of mussels. Fiddler crabs were exposed to either 108 mg or 1000 mg of weathered polystyrene plastic pellets, broken down to be 180- 250 µm. Following exposure, it was found that the crabs had a greater number of plastic fragments in their hepatopancreas than in their stomachs, suggesting translocation from the digestive system post-ingestion. Translocation of microplastics has not been studied in many organisms and the toxicological affects remain unknown. However, the previous findings of Browne et al. (20008) and Brenneke et al. (2015) show that translocation is possible in some organisms and should be investigated further.

Sea squirts were found to have a decreased microplastic content after 72 hours when compared to those sampled at 24 hours in most exposures. This decrease in microplastic content during exposures has also been seen in sea urchin larvae (Kaposi et al., 2014). The urchin larvae were found to egest microplastics and this decrease in microplastic content was thought to be due to egestion rather than reduced feeding. Depuration experiments showed that *C. intestinalis* were able to egest the different plastics investigated. Egestion typically occurred within a matter of hours, although a small number of plastics were still present in sea squirts after a depuration of 16 hours.

Microplastic egestion has also been previously shown in copepods. Cole et al. (2013, 2016) found that ingested polystyrene beads were egested from copepods in faecal pellets. Similarly, to the study presented here, plastics ingested by the copepods were found to have a similar gut retention time to natural prey items, but a number of individual were found to retain plastics for longer periods (Cole et al., 2013). Plastic laden faecal pellets egested by copepods were found to sink to the base of exposure beakers (Cole et al., 2016), suggesting that these faecal pellets remained denser than seawater. However, the density and sinking rates of faecal pellets were altered by the incorporation of microplastics. This incorporation reduced the faecal pellet density leading to a 2.25 fold reduction in sinking rates. Coprophagous organisms often consume faecal pellets and a reduction in the sinking rates due to microplastics may lead to an increase in ingestion of microplastics by these organisms (Cole et al., 2016).

To understand whether ingested plastics were being re-suspended after egestion, water samples were taken from beakers during exposures. These water samples showed a decrease in plastics within the water column over the exposure time. Wegner et al. (2012) also noted a decrease in the volume of plastic seen in the water surrounding mussels during a polystyrene exposure. This decrease in particles within the water was attributed to mussel ingestion rather than the plastic settling. They found that the mussels were removing 4.7 mg of plastics from the water every hour when exposed to a concentration of 0.3 mg L^{-1}. This decrease in water plastic concentration in the study reported hear and Wegner et al. (2012) lend support to the theory that ingested plastic is being packaged into faecal matter and as reported by Cole et al. (2016), sinking to the base of exposure vessels.

Microplastic depuration has also been investigated in mussels and oysters collected from the environment (Van Cauwenberghe and Jannsen, 2014). These animals were left to depurate in clean water for 3 days after collection. Water was changed daily to prevent re-ingestion of any plastics. It was found that after 3 days plastics remained in the tissues of both mussels and oysters suggesting that the organisms were unable to egest all plastics. Brilliant and MacDonald (2000) found that smaller particles had a shorter gut retention time than larger particles in the sea scallop. Therefore, it was thought that the plastics remaining in the mussels and oysters were small and had probably been translocated to other tissues beyond the digestive system (Van Cauwenberghe and Jannsen, 2014). This study found that the smaller polyamide particles were egested from sea squirts quicker than larger polystyrene particles, supporting the finding of smaller

particles having shorter gut residence times, supporting the findings of Brilliant and MacDonald (2000).

This study has shown that *C. intestinalis* are able to ingest a wide range of microplastic polymers making it an organism of concern. Large volumes of plastics were ingested with some plastics remaining within internal tissues for 16 hours after removal from contaminated waters. However, sea squirts were found to have decreased concentrations of plastics within them after longer exposures, suggesting short gut retention times and efficient egestion. The next chapter will further investigate the fate of microplastics in these exposure and whether the properties of microplastics affect ingestion by *C. intestinalis.* An alternative and less time-consuming method for microplastic quantification will also be used and compared to the methodology used in this study.

Chapter 3:

The impact of shape and size of micro- and nano-plastic particle on uptake and biological effect in *Ciona intestinalis*.

3.1 Introduction

3.1.1 Microfibres

Microplastics have now been detected in all marine regions and habitats, from the poles to the deep seas (do Sul et al., 2013; Goldstien and Goodwin, 2013; Van Cauwenberghe et al., 2013; Thompson, 2015; Lusher 2015; Van Sebille et al., 2015). Recent estimates have predicted that there may be as many as 51 trillion pieces of microplastic in ocean waters across the globe (van Sebille et al., 2015). Microfibres microscopic filaments of plastics, have been reported to be the most common plastic type to be found in many marine waters and sediments. Lusher et al. (2014) found that 95 % of all plastic collected from net tows in the Norths Atlantic Ocean were found to be fibres, while concentrations of 213 fibres kg^{-1} were found to be present in sediments from Cornish estuaries (Thompson et al., 2004) and 200-800 fibres kg^{-1} have been found in sediment from Nova Scotia, Canada (Mathalon and Hill et al., 2014). These high concentrations of microfibres make them likely to be the most bioavailable plastic to marine organisms, and fibres have been found in the stomachs of marine organisms collected from the environment (e.g. Li et al., 2016; De Witte et al., 2014; Van Cauwenberghe and Jansenn, 2014; Van Cauwenberghe et al., 2015).

To date, very few laboratory studies have used microplastic fibres within exposures, due to the difficulty in producing fibres for experiments, with the majority of studies still focusing on microbeads such as those found in cosmetics. This has led to a mismatch in the organism responses reported from bead exposures and what may be occurring in the natural environment. However, the ingestion of plastic microfibres has been found to cause reductions in energy availability for growth and reproduction and decreases in feeding rates in shore crabs (Watts et al., 2015).

It has also been suggested that microfibres have the potential to exert a greater toxicity to organisms than particles (Au et al., 2015), due to their shape and potential to cause gut blockages. Au et al. (2015) have provided some evidence in support of this where they tested the impacts of ingestion of polypropylene microfibres and polyethylene microplastic particles in the freshwater amphipod, *Hyalella azteca.* They found that fibres, once ingested, had a six-fold increase in gut residence times compared to the particles which then resulted in lower growth rates after 42 days. This increase in toxicity along with the high encounter rates of microfibres in the marine environment demands a greater effort to understand the impacts ingestion of fibres may be having upon our marine organisms.

3.1.2 The decreasing size of microplastics

Once in the marine environment plastics undergo fragmentation through processes such as biodegradation, photo-degradation, thermo-oxidative degradation and hydrolysis (Andrady, 2011). It is therefore likely that the average size of plastic particles in the oceans is decreasing. Moret-Fergerson et al. (2010) were able to show that the average plastic particle size had reduced from 10.66

mm in the 1990s to 5.05 mm in the 2000s. These particles were captured using a nueston net, with a 335 µm mesh, and identified by eye and density, therefore, it is likely that many particles across the size range as well as those smaller than the mesh used were likely to have been missed. There also appears to be a mismatch in the size distribution of plastic reported in the oceans compared to the expected size spectra, with a lack of plastics of less than 1 mm being recorded (Cozar et al., 2014; Lenz et al., 2015). Most studies report plastics at the millimetre scale (do Sul et al., 2009; Browne et al., 2010; Claessens et al., 2011; Van Cauwenberghe et al., 2015).

There are a number of reasons as to why smaller plastics are not being recorded: smaller plastics may be being diluted within the ocean to a greater extent than larger particles (Koelmanns et al., 2015), technical difficulties are present when measuring plastics smaller than this scale (Cozar et al., 2014; Fillela, 2015), the majority of sampling efforts use a mesh of 335 µm which enables any plastic particles smaller than tis to pass through. Cozar et al. (2014) proposed the idea that once plastics reach this size fragmentation may be accelerated resulting in a number of far smaller and difficult to detect smaller plastics. This idea was taken further by a recent study using a solar reactor to prove fragmentation of microplastics through photo degradation (Gigault et al., 2016). These smaller plastics have been termed nanoplastics, whilst there is currently no definition of the maximum size of nanoplastics is often referred to as 100 nm (Klaine et al., 2012; Koelmanns et al., 2015).

3.1.3 The impacts of nanoplastics

I have previously found that *Ciona intestinalis* readily ingests polystyrene microbeads with no excessive gut residence times being seen and no apparent translocation of plastics to the heart (Chapter 2). However, nanoplastics may have greater implications for marine organisms than microplastics, due to their smaller size. A smaller size may allow it to move through biological barriers and be translocated into tissues and cells (Galloway et al., 2017). Nanoplastics are also known to behave differently to their larger counterparts; they have a greater tendency to form aggregates (Koelmans et al., 2015), which will increase their density and potentially speed up sinking of these particles (Galloway et al., 2017). The increased sinking of plastics will increase the availability of plastic to benthic organisms. While, microplastics ranging from 0.8 µm to 5 mm have been shown to be ingested by a number of organisms in the marine environment (e.g. Mathalon and Hill, 2014; Van Cauwenberghe and Janssen, 2014; Wesch et al., 2016), there are no reports of marine organisms interacting with nanoplastics in the field to date. This lack of data is most probably due to the technical difficulties currently faced in the detection of these particles in the environment. However, a limited number of studies have investigated ingestion of nanoplastics within laboratory settings.

Nanoparticles have been found to have negative impact upon a number of organisms. Mussels exposed to nanoparticles had been found to have increased oxidative stress responses and tissue cell injuries when compared to control mussels (Koehler et al., 2008), suggesting that the ingestion on nanoplastics by mussels results in cellular toxicity. Ward and Kach (2009) also investigated the uptake of fluorescently 100 nm polystyrene beads in both mussels, *Mytilus edulis*,

and oysters, *Crassostrea virginica*. Both species were found to ingest the nanopolystyrene, particularly when the particles were aggregated. These nanoplastics had a greater gut retention time than 10 µm polystyrene microplastics, demonstrated by egestion of nanoparticles increasing over 72 hours, whilst egestion of 10 µm was highest at 6 hours and decreased over 72 hours. This suggested that the nanoplastics may have a greater toxicity than microplastics when ingested by these organisms.

Wegner et al. (2012) have also looked at the impacts nanoplastics may have upon mussels. The uptake of 30 nm polystyrene particles was measured indirectly, through measuring the nanoplastic content of the water and the mussel valve opening time. It was found that the nanoplastic content of the water decreased suggesting ingestion by the mussels. This was measured using valve opening as a proxy for feeding activity. Control mussels had a valve opening of 4 mm throughout the exposure. Following exposure to nanoplastics mussels were seen to close their valves within 20 minutes, before reopening to approximately 1 mm within an hour. Valves remained at 1 mm for the remainder of the 4-hour duration of the exposure. This reduction in valve opening was assumed to correspond to a reduction in feeding activity, suggesting a preference to stop ingesting nanoparticles. These studies appear to support the idea that nanoplastics may pose a greater threat to marine life than microplastics and therefore far more research into this emerging area is required.

Nanoplastics have also been shown to have population effects upon other organisms, for example, Besseling et al. (2014) show that *Daphnia manga* exposed to nanopolystyrene had a reduced body size and reproductive success.

This was seen at the population level with population growth reducing due to 68 % of the neonates produced from exposed Daphnia having malformations. Nanoplastics have also been seen to have an impact on copepods at the population level. Female and nauplii *Tigriopus japonicas* were found to ingest three different sizes of polystyrene particles, 0.06, 0.5 and 6 µm, for 96 hours, with no mortality occurring (Lee et al., 2013). It is not clear if the nanoplastics had other impacts during this exposure. A chronic exposure was also carried out looking at the impacts of microplastics over two generations, found that when exposed to 0.05 µm beads at a concentration of 1.25 mg L^{-1} mortalities in the next generation occurred. Exposure to larger particles also caused a reduction in fecundity and reproduction but no mortalities were observed. These studies suggest that ingestion of nanoplastics has the potential to impact upon marine organisms at the population level as well as the cellular and individual level.

3.1.4 Laboratory techniques for quantifying plastics

A common technique in laboratory experiments investigating micro- and nanoplastic ingestion is to use fluorescently labelled plastics, these tend to be manufactured to fluoresce at set wave lengths or can be dyed with Nile Red (e.g. Ward and Kach, 2009; Lee et al., 2013; Besseling et al., 2014; Cole et al., 2014; Setala et al., 2014). Fluorescently labelled plastics are able to be viewed under a microscope containing a fluorescent bulb, meaning that the passage of plastics can be tracked through organisms, easily identified in water columns and reduces the contamination potential. Fluorescently labelled plastics can be purchased or plastics can be fluorescently dyed within the laboratory (Cole, 2016). A well-established method in quantifying the uptake of fluorescent plastics is to count the plastic particles in a subsample of the target tissue. This method has the

advantage that it provides a relatively accurate assessment of the volume of plastics ingested, if enough subsamples are counted. However, it is a time consuming methodology and can be liable to operator error (Hussain, 2001). This technique is also unsuitable for quantifying the uptake of nanoparticles due to their sizes preventing them being visible for optical counting. Another advantage of using fluorescently labelled plastics for laboratory studies is that they can be excited at certain wavelengths. This excitation is then able to give a signal that can be utilised by alternative fluorometric techniques.

These alternative techniques are not commonly employed when studying microplastics but are far more common in nanoparticle studies, for example, Ward and Kach (2009) analysed the nanoplastic content in the digestive glands of mussels after exposure using a spectrophotometric technique. This technique was also employed by Wegner et al. (2012) to quantify the nanoplastic concentrations within the water column following nanoplastic exposure to mussels. These techniques rely on a calibration curve being created in which known concentrations of the fluorescent particles can be matched to emission readings. If the these techniques can be applied to microplastic studies as well as nanoplastic studies it will enable better comparisons of ingestion rates, reduce operator error and save the time required to accurately count a number of sub-samples.

3.1.5 Chapter 3 Aims and Hypotheses

This chapter investigates whether the size, shape and type of plastic effects microplastic ingestion in the sea squirt, *C. intestinalis* using polystyrene and nylon

microbeads, nylon fibres and polystyrene nanoplastics. The following hypothesis are tested:

H_1) All microplastics will be ingested by *C. intestinalis.* Microplastics will be found in tissues of *C. intestinalis* following a microplastic exposure;

H_2) A greater volume of nanoparticles will be ingested compared to microplastics as a result of their decreased size;

H_3) Fibres will have the greatest reduction in feeding rates within the microplastic treatments, due to their potential to cause gut blockages is also tested;

H_4) It is predicted that in line with previous studies nanoplastics will have a greater negative impact upon *C. intestinalis*. Feeding rates are used as a measure of biological effect to see if nanoplastics have an effect;

H_5) Sea squirts in nanoplastic treatments will have a decreased feeding rate when compared to those in microplastic treatments;

H_6) Finally, the two techniques, optical counting and a fluorometric technique are compared. It is hoped that by comparing these methods of ingested microplastic within the sea squirt tissues, each method can be validated with the aim of developing a less time consuming practices for micro- and nanoplastic uptake studies.

3.2 Methods

3.2.1 Animal collection and maintenance

Specimens of the transparent sea squirt (*Ciona intestinalis*) were collected by hand from Millbay Marina, Plymouth, Devon (UK). Adult specimens were transported in aerated seawater within a cool box back to the laboratory within 2 hours of collection. Animals were maintained in 32 ppt 0.2 µm filtered artificial sea water (FSW) at 15°C for a minimum of 48 hours prior to experimentation, to allow for depuration of any gut contents. A stock concentration of algal solution (Shellfish diet 1800^{TM}) was created by diluting 20 ml of algal solution in 2 L of artificial seawater (TropicMarine).

3.2.2 Microplastics

Here, the uptake and effects of four different types of plastic: polystyrene microbeads are compared: nylon microplastic particles, nylon fibres, polystyrene microplastic beds and polystyrene nanoplastic beads. (1) Yellow fluorescently labelled 20 µm polystyrene beads (PS MP) were purchased from Spherotech (FP-20052-5) and rinsed several times in order to remove any sodium azide (a biocide) on them. (2) Nylon microplastic fragments (NMP) of 23-30 µm were purchased from Goodfellow (AM376010). (3) Nylon fibres (NF) of approximately 30 µm were created using the cryogenic microtome preparation method described by Cole (2016). In brief: microfibres were created by wrapping a reel of Nylon (10 µm diameter, AM325705, Goodfellow) around a spool and coating them with a water-soluble freezing agent (Neg 50TM, Richard-Allen) before freezing at -80°C for 10 minutes. The embedded nylon was cut into lengths of ~10 mm and bound together, in parallel, with more freezing agent. Embedded

fibres were mounted and cut to 30 µm lengths with a cryogenic microtome. Fibres were placed in a Pyrex beaker containing ultrapure water and heated to 60°C, and then filtered through an 8 µm polycarbonate. Both the NMP and NF were fluorescently dyed using Nile Red prior to use in the exposures. This was done by dissolving 100 mg of Nile Red (technical grade, N3013, Sigma Aldrich) into 200 ml of acetone to produce a stock solution. The prepared nylon and 2.5 ml of stock solution were placed in a 5 ml Eppendorf, vortexed and left for 10 minutes to allow the Nile red to coat the nylon. The nylon was then vacuum filtered onto 8 µm polycarbonate filters, rinsed with acetone and washed with ultrapure water to remove any excess dye. This process was then repeated to ensure all particles were adequately dyed. (4) Polystyrene nanoplastics (PNP) were obtained from Magspere Inc.

3.2.3 Experimental setup

Exposures were carried out in 2 L glass beakers containing FSW aerated via a glass Pasteur pipette. A total of 30 sea squirts were used in this exposure, 6 individuals per treatment for each of the four experimental treatments plus 6 individuals in the control group containing no plastics. Each sea squirt was placed in a separate beaker. Sea squirts were placed on a plastic mesh, suspended approximately 2 cm above the base of the beaker. The mesh allowed any detritus (e.g. faeces) to sink to the base of the beaker for later collection. Animals were depurated for 24 h prior to experimentation. Microplastics (PS MP, NF, NMP) were added to experimental vessels at a concentration of 100 particles per ml, and PNP added at a dose of 500 µg/l. In all cases, plastics were added to the beakers part way through the water change to optimise mixing.

One hundred ml of algal stock solution was added immediately after the water change and mixed thoroughly. Water samples (10 ml) were collected from the beaker of each sea squirt via pipette at 0, 6 and 24 hours and split into two; one sample was used for chlorophyll analysis to calculate the algal concentration within the water column in order to ascertain ingestion rates. The remainder of the water sample was used to quantify the concentration of plastic in the water. All sea squirts were exposed to a treatment for 24 hours before exposures were terminated and sea squirts were snap frozen in liquid nitrogen and stored at -80 °C. Water remaining in the beaker was immediately filtered through a 30 µm mesh to collect faeces; faecal matters was transferred into a Falcon tube prior to further analysis.

3.2.4 Tissue analysis

Sea squirts were thawed and dissected, using sterile, stainless steel scissors and scalpels, to separate out the tunics, intestinal tract and heart. Tunics were placed in pre-weighed falcon tubes, and the internal organs placed in pre-weighed Eppendorf tubes. Each sample was sonicated, using a probe sonicator (Autotune series high intensity ultrasonic processor) set to a maximum output power of 100 watts, in ultrapure water (tunics, 20 mL water; organs, 4 mL water) to homogenise the tissues. Sonication was carried out until the tissues were homogenised. Latterly, the remainder of each sample was dried in a 60°C oven, and weighed to ascertain dry weight (dw). After sonication, representative subsamples of homogenised tissues were taken for either manual plastic counts, fluorescence readings or both. Plastics were quantified in tissues by microscopy or fluorescence analysis. For sea squirts exposed to control, PS MP, NF and NMP treatments, six subsamples (20 µl) of sonicated and homogenised tissue were

placed on a slide, and viewed under an inverted fluorescent microscope (Leica DMI 4000B). The number of microplastics in each subsample was quantified, and the total volume of homogenised tissue used to calculate the amount of plastic in each tissue type. The yellow fluorescence of the manufactured particles was used to quantify plastics in the tissues of sea squirts exposed to control, PS MP and PNP treatments. Here, a representative 2 ml subsample of homogenised tissue was plated out, and analysed per the protocol previously described.

3.2.5 Faecal Samples

Faeces produced by exposed sea squirts were centrifuged at 5000 rpm for 20 minutes, and then the supernatant removed and discarded. Faecal matter was transferred into pre-weighed Eppendorf's, placed in a 60°C oven overnight before being weighed. Subsequently, 4 ml of ultrapure water was added to each sample and faeces homogenised. Microplastics were quantified using microscopy or fluorescence, as per the protocols previously described.

3.2.6 Waterborne plastic concentration

Microplastic concentrations were measured in water samples indirectly by measuring the fluorescence of the plastic particles as a proxy as well as by manual counts. Fluorescent analysis, as described in Hussain (2001), was carried out on the control, PS MP and PNP treatments. This method was chosen due to a high extraction efficiency being reported in previous studies (Hussain, 2001). Water samples for fluorescent analysis were placed in a -80°C freezer in order to prevent any biological changes within them. These samples were defrosted at room temperature. A volume of 2 ml was taken from each sample

and added to an Eppendorf containing 2 ml of solvent, 2-(2-ethoxyethoxy) ethyl acetate. Eppendorf's were placed on a shaking plate overnight, before being centrifuged at 5000 rpm for 10 minutes. The supernatant was then transferred into clean Eppendorf's and vortexed. Subsamples of 200 µl were taken from the vortexed supernatant and pipetted into well plates. Three subsamples were taken from each sample to be read.

The fluorescence within the water samples from the control, PS MP and PNP was measured using a plate reader (Spectramax m5). The reader was set to excite the particles at a wavelength of 470 nm, corresponding to the fluorescence of the particles, and read the corresponding emissions at the wavelength 525 nm. Serial dilutions of known concentrations of PS MP and PNP were created and each dilution measured in triplicate by the plate reader to gain an average fluorescence reading for each concentration. A standard curve was then created from these readings for both the PS MP (Appendix 1, Figure 6.2) and PNP (Appendix 1, Figure 6.3). These standard curves were used to infer the concentration of plastics in each sample from the florescent readings. Manual counts and the counts inferred from fluorescence readings were compared. Pearson's product-moment calculations were carried out using R studio in order to test for significant relationships between fluorescent readings and sample masses.

3.2.7 Algal Ingestion rates

Ingestion rates of algae were calculated from chlorophyll A absorbance measurements in water samples compared to a standard curve taken at two time points. Water was vacuum filtered through a 1.2 µm GF/C Whatman filter. Filter

papers were placed in an Eppendorf containing 10 ml of 90% acetone and stored at -4°C overnight to allow cellular disruption to occur. Once removed from the freezer, filter papers were centrifuged at 5000 rpm for 5 minutes to remove any chlorophyll from the filter paper into suspension in the acetone.

Following centrifugation triplicate 200 µl aliquots of supernatant was placed in a 96 well plate and read spectrophotometrically 440 nm excitation and 670 nm emission to ascertain chlorophyll *a* fluorescence. Each plate was read at 25°C three times to gain an average reading for each well. In order to calculate the chlorophyll a concentrations, it was necessary to compare fluorescence data with the absorbance readings of a chlorophyll A standard (Sigma-Aldrich). Serial dilutions of the Chlorophyll A standard were prepared to pre-determined concentrations (0-100 % Chlorophyll A concentration), and then analysed in triplicate on the plate reader using the aforementioned settings. A reference graph was created from these readings (Appendix 1, Figure 6.1) and linear regression used to establish the relationship between Chlorophyll a concentrations and fluorescence.

Ingestion rates were then calculated by using the starting chlorophyll concentration (C_0) and the concentration calculated at 6 or 24 hours (C_t). The following equations, adapted from Frost (1972) were used for calculating ingestion rate (I):

(1) $g' = (1/t) * \log (C_t/C_0) * -1$

(2) $[C] = C_0 * (1 – EXP((g' * -1) * t))/ (t * g')$

(3) $F= (C_0-C_t) * V/ ([C] * t)$

(4) $I = F * [C]$

Where g' is the instantaneous change in algae concentration coefficient, t is the incubation time, [C] is the average chlorophyll concentration, F is the clearance rate of algae, and V is the volume of water in the exposure.

The nanoplastic (PNP) treatment was separated from the microplastic treatments for analysis due to the different particle concentration used during the exposure, meaning that the results are not directly comparable. A Pearson's product-moment calculation was carried out using R studio to check for significance between ingestion rates and sea squirt mass. ANOVAs and Tukey post-hoc tests were carried out in R studio to test for significant differences in ingestion rates between treatments.

3.2.8 Statistical Analysis

All statistical analysis were carried out using R (version 3.3.2). Pearson's product moment correlation tests were undertaken to test whether Chlorophyll a ingestion varied with dry weight. Analysis of variance tests followed by Tukey post-hoc tests were carried out to establish any significant differences between chlorophyll ingestion rates and the plastic treatments. To check whether there were any significant relationships between organ dry weight and fluorescence readings Pearson's product-moment correlation tests were conducted.

3.3 Results

3.3.1 Where are microplastics found?

Following 24 hours of exposure to polystyrene microplastics, all tissues of sea squirts, their faeces and the water was observed for plastics. No plastics were found in the water samples. It would appear that a large proportion of the plastic added to the water column is ingested through the inhalant siphon. Once ingested the plastic travels through the intestinal system and is egested in faecal matter (Figure 3.1). However, after 24 hours of being exposed to PS MP, 1.5 % of the nominal concentration added to the water was found to be in the internal tissues of sea squirts (Figure 3.2). Adherence of PS MP to the tunics was also seen, with 1.8 % of the nominal concentration being found on the outside of the exposed sea squirts. However, it would appear that sea squirts egest most of the plastic they ingested with 90 % of the nominal concentration being seen in the faeces.

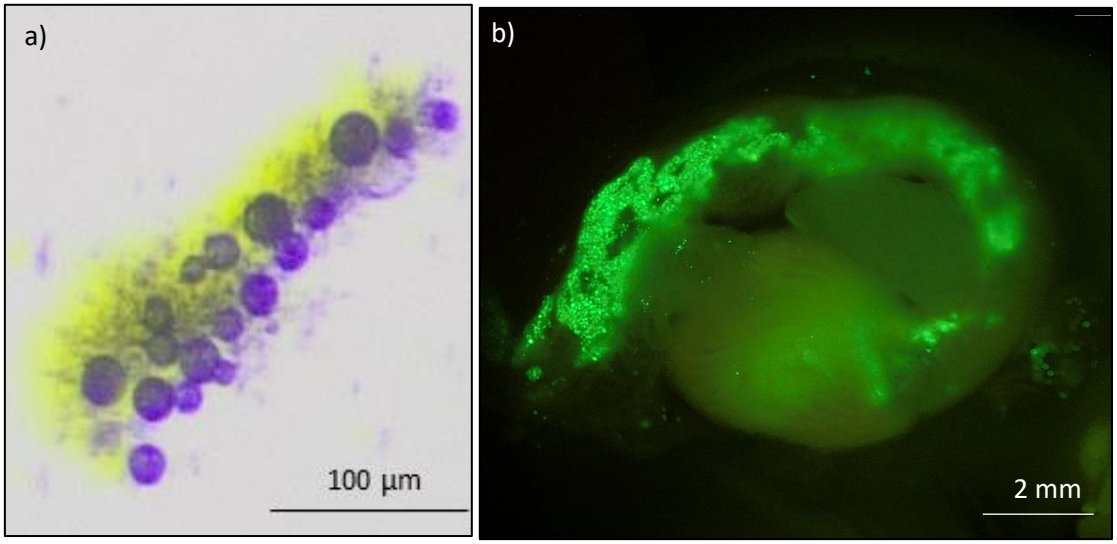

Figure 3.1: Photographs showing 20 µm polystyrene microplastic spheres in *C. intestinalis* faeces and digestive tract. a) An enlarged photograph of a faecal pellet egested by a sea squirt exposed to 20 µm polystyrene microbeads, the beads can be seen in purple held together by the green algae. b) An enlarged photograph of a *C. intestinalis* digestive tract, taken using a fluorescent microscope (Leica DMI 4000B). Fluorescent green beads can be seen within the stomach and oesophagus.

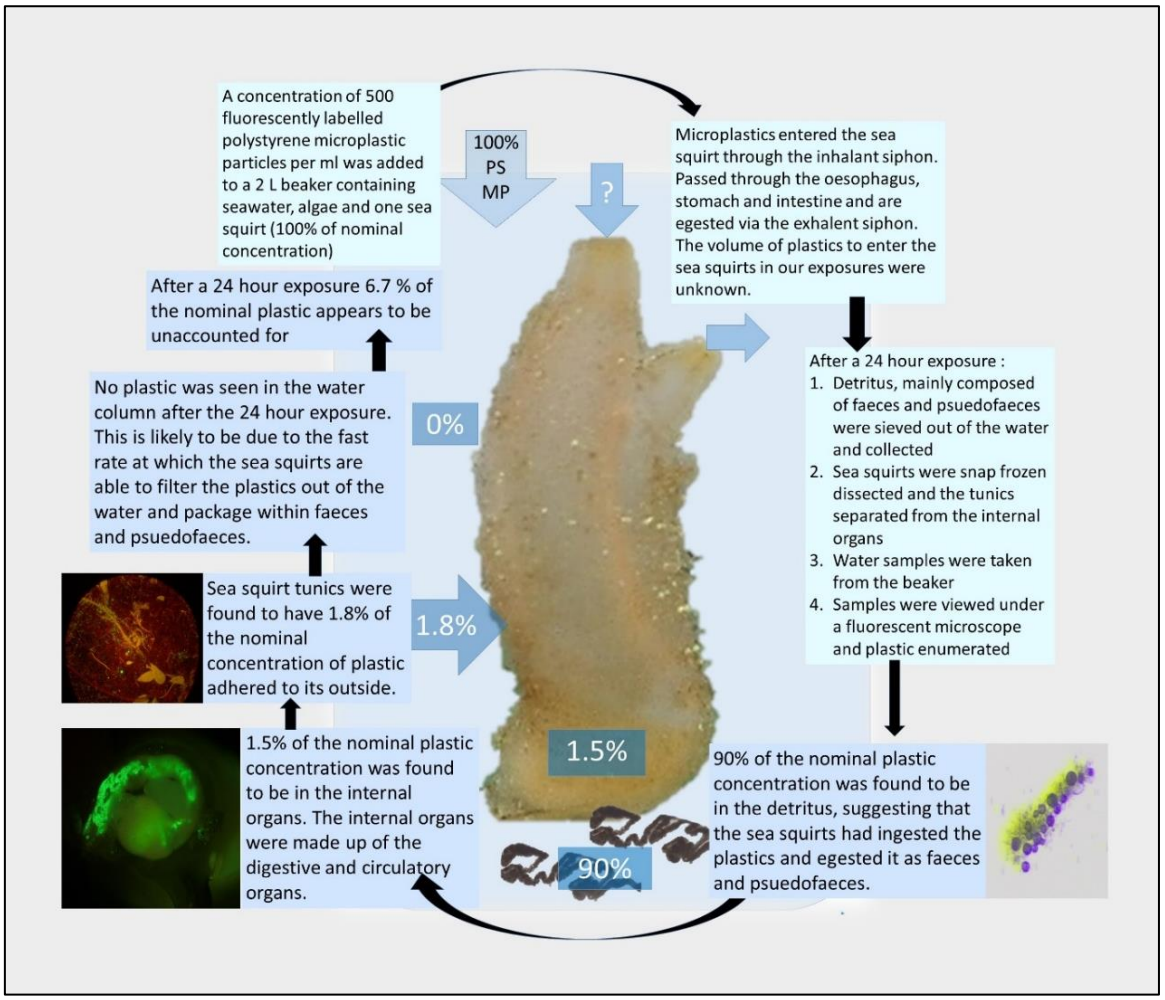

Figure 3.2: Schematic diagram showing the path and locations of 20 µm polystyrene microplastic spheres (PS MP) through *C. intestinalis*, during a 24 hour exposure.

3.3.2 Tissue analysis for presence of plastics

When the tissues of sea squirts exposed to polystyrene microplastic spheres and nylon fibres were viewed under a microscope with a fluorescent bulb, numerous plastic particles could be seen in many of the tissues. Manual counts of polystyrene beads and nylon fibres show that a significantly higher number of polystyrene beads were found in exposures (Two-way ANOVA, $F_{1,40}$=8.24, P<0.005) (Figure 3.3). These counts showed a large volume of plastics in the faecal matter of exposed sea squirts. *C. intestinalis* with those exposed to

polystyrene beads having significantly more plastic in their faecal matter (1807.4 ± 500.6 plastics mg^{-1}) than those exposed to nylon fibres (333.9 ± 165.7 mg^{-1}) (One-way ANOVA, $F_{1,10}=7.74$, $P<0.05$). Similarly, to the faecal matter, sea squirts exposed to polystyrene had significantly more plastics present in their internal organs (29.2 ± 7.4 plastics mg^{-1}) than those exposed to nylon fibres (10.5 ± 2.4 plastics mg^{-1}) (One-way ANOVA, F1,10=5.7, $P<0.05$). This was also seen in the tunics of *C. intestinalis*, with tunics from those exposed to polystyrene having a greater plastic load (35.2 ± 4.9 plastics mg^{-1}) than those in the nylon fibre treatment (7.2 ± 2.1 plastics mg^{-1}) (One-way ANOVA $F_{1,10}=26.86$, $P<0.05$. The number of plastics found in water samples taken at 24 hours was also counted in the same way. Water from polystyrene exposures was found to contain 4.2 ± 1.9 plastics ml^{-1}, while water from nylon fibre exposures contained 1.4 ± 1 plastics ml^{-1}, this was not found to be significantly different (One-way ANOVA, $F_{1,10}=1.43$, $P=0.26$). Sea squirts exposed to nylon microplastics were unable to be included in these results due to the size and fluorescence of the particles being similar to natural particles found within sea squirts. Nanoplastic particles occupy a size range too small to be seen with the microscope in use and so could not be quantified in this way.

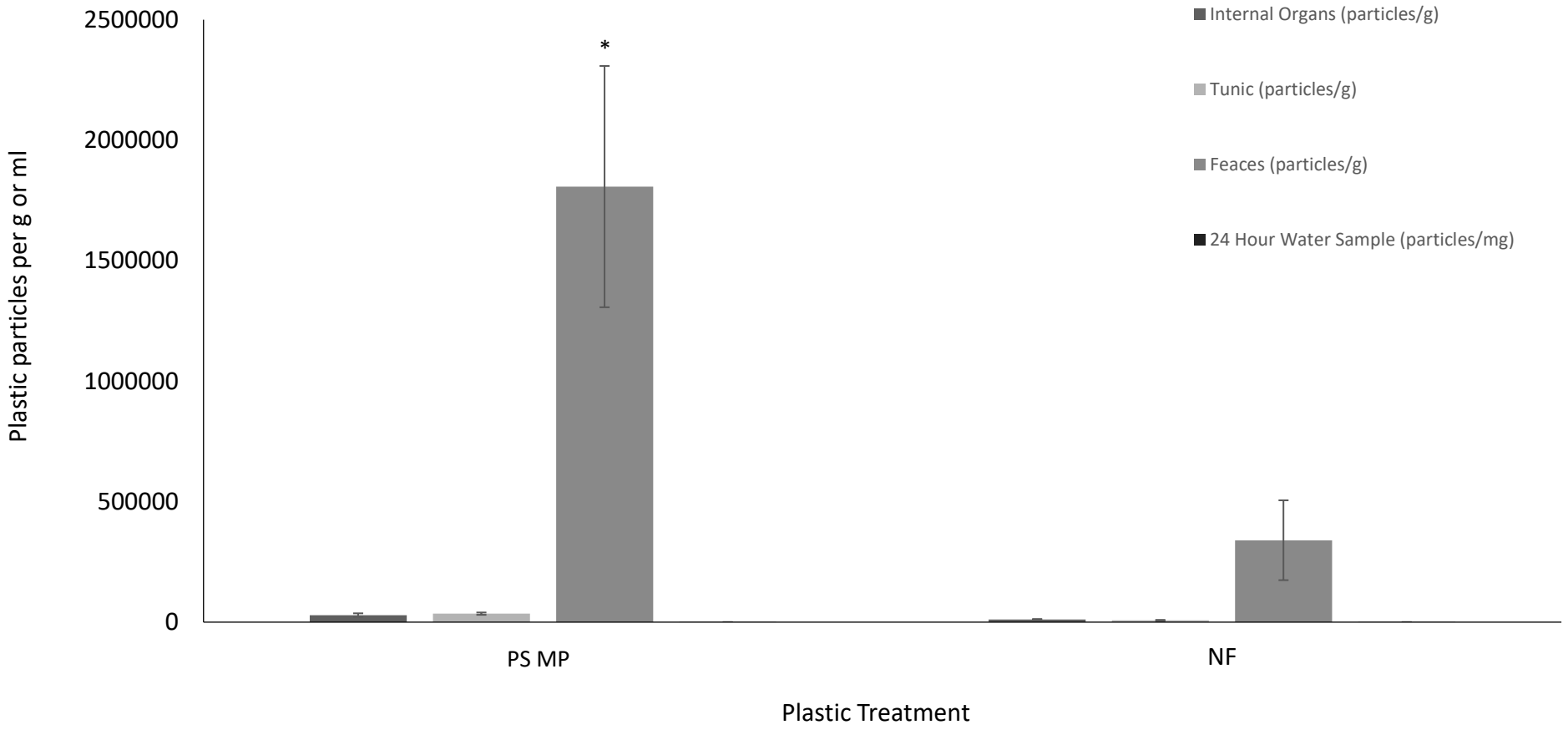

Figure 3.3: Microplastic locations in *Ciona intestinalis* as shown by manual counts (data as average +/- standard error, * denotes significance, N=6). PS MP refers to 10 µm polystyrene spheres, NF represents pre-prepared nylon fibres.

Fluorescent readings appear to show far fewer plastics within the tissues of sea squirts (Figure 3.4) than manual counts (Figure 3.3). The fluorescent readings show a greater volume of plastics in all tissues of sea squirts exposed to polystyrene nanoplastics (PNP) than those exposed to polystyrene microplastics (PS MP) (Figure 3.4). The internal organs of sea squirts exposed to polystyrene nanoplastics contained an average of 318 ± 85 plastic g^{-1}, while those exposed to polystyrene microplastics contained 230 ± 29 plastic g^{-1} on average. This is in contrast to the results seen in manual counts, where feaces contained the greatest number of plastics (Figure 3.3), fluorescent readings appear to show a negligible number of plastic in the faeces in both the polystyrene microplastic exposure (0 ± 0.3 plastics g^{-1}) and the polystyrene nanoplastics exposure (31 ± 7 plastic g^{-1}). The tunics of sea squirts exposed to PNP also contained on average 166 ± 28 plastics g^{-1}, while sea squirts exposed to PS MP contained 94 ± 8 plastics g^{-1} in their tunic, on average. The water samples taken at 24 hours from the PS MP and PNP exposures had similar levels of plastics within them; these were 4.7 ± 0.4 plastics ml^{-1} in the PS MP exposure and 4.1 ± 0.4 plastics ml^{-1} in the PNP exposure.

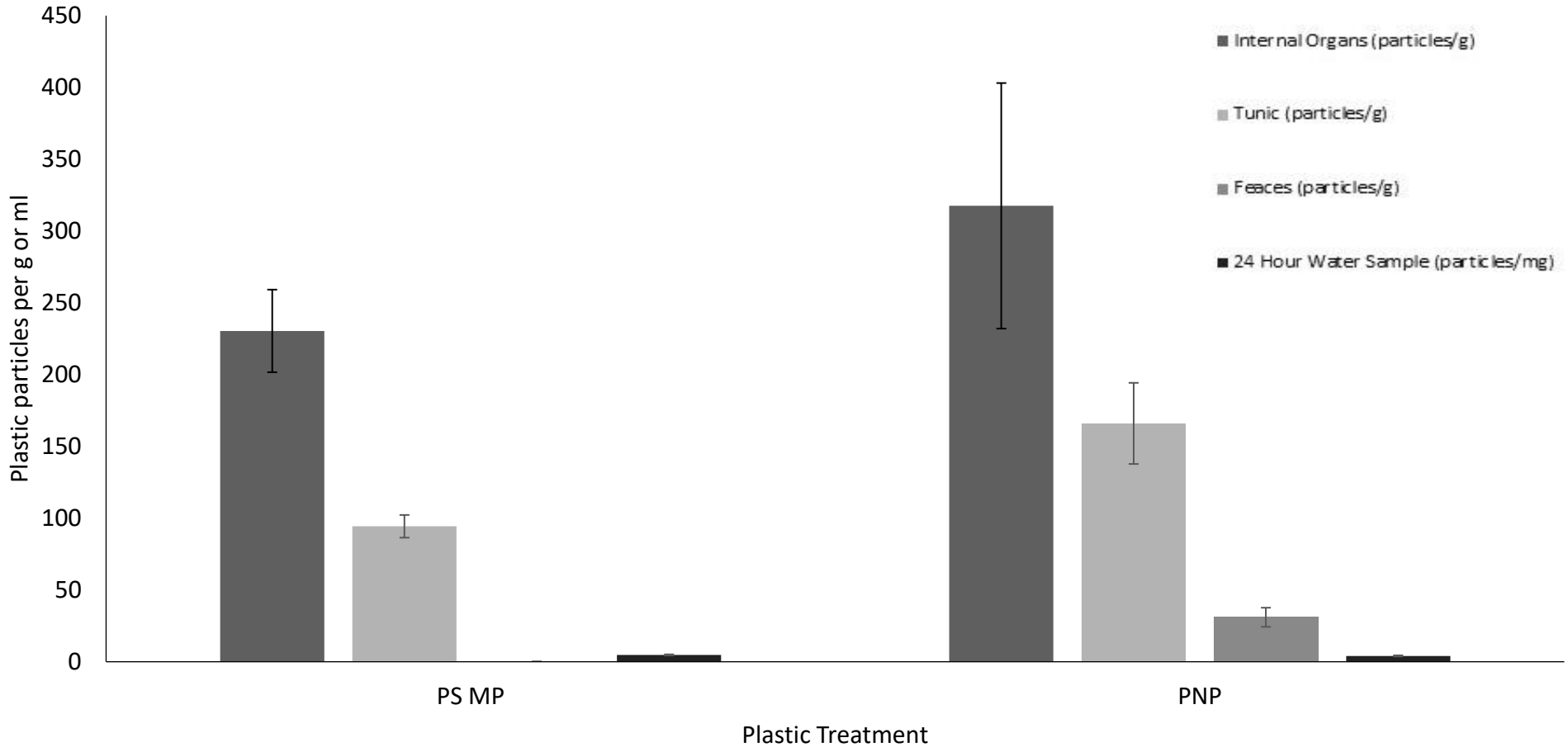

Figure 3.4: Micro- and Nanoplastic locations in *Ciona intestinalis* as shown by fluorescent readings (data as average +/- standard error, N=6). PS MP refers to 10 µm polystyrene spheres, PNP represents polystyrene nanoplastics.

3.3.3 Egestion of Plastics

Fluorescence readings appeared to have a positive relationship with sample weight with the exception of the faeces of those exposed to PNP (Figure 3.5). However, a number of these relationships were not found to be significant. A significant positive relationship was found in the Internal organs of those exposed to PS MP (Pearson's product-moment correlation, R_{10}=0.708, P=0.010) (Figure 3.5a) and in the tunic samples of both the PS MP (Pearson's product-moment correlation, R_4=0.626, P=0.030) (Figure 3.5c) and PNP (Pearson's product-moment correlation, R_{10}=0.876, P=0.023) (Figure 3.5d). A weak positive relationship was found between the internal organ mass and faeces mass in both PS MP (Figure 3.5g) and PNP (Figure 3.5h).

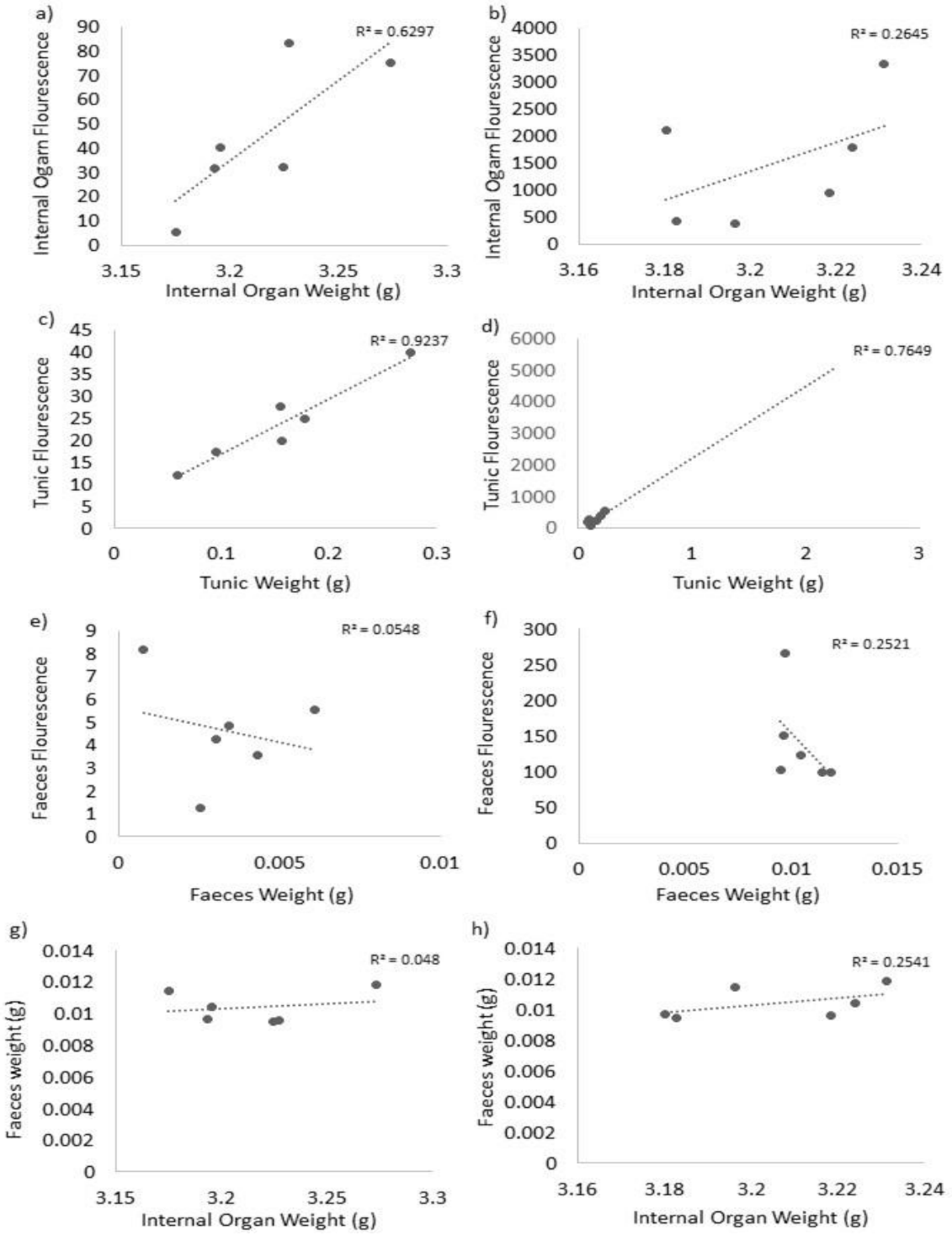

Figure 3.5: Relationships between fluorescent readings and sample weights. a) and b) Relationship between Internal organ fluorescence and weight in the 10 µm polystyrene microplastic spheres (PS MP) treatment (a) and the polystyrene nanoplastic (PNP) treatment (b). c and d) Relationship between Tunic fluorescence and weight in the PS MP treatment (c) and the PNP treatment (d). e) and f) Relationship between Faeces fluorescence and weight in the PS MP treatment (e) and the PNP treatment (f). g) and h) Relationship between Internal organ and Faeces weight in the PS MP treatment (g) and the PNP treatment (h).

3.3.4 Normal algal ingestion rates of *Ciona intestinalis*

Sea squirt body mass did not seem to influence algal ingestion rates after 6 hours (Figure 3.6) (Linear Regression, $F_{1,27}$=0.078, P=0.782) or 24 hours (data not shown). Due to body mass having no influence on algal feeding rate in any treatment group, algal ingestion rates were not normalized but reported per individual in further results.

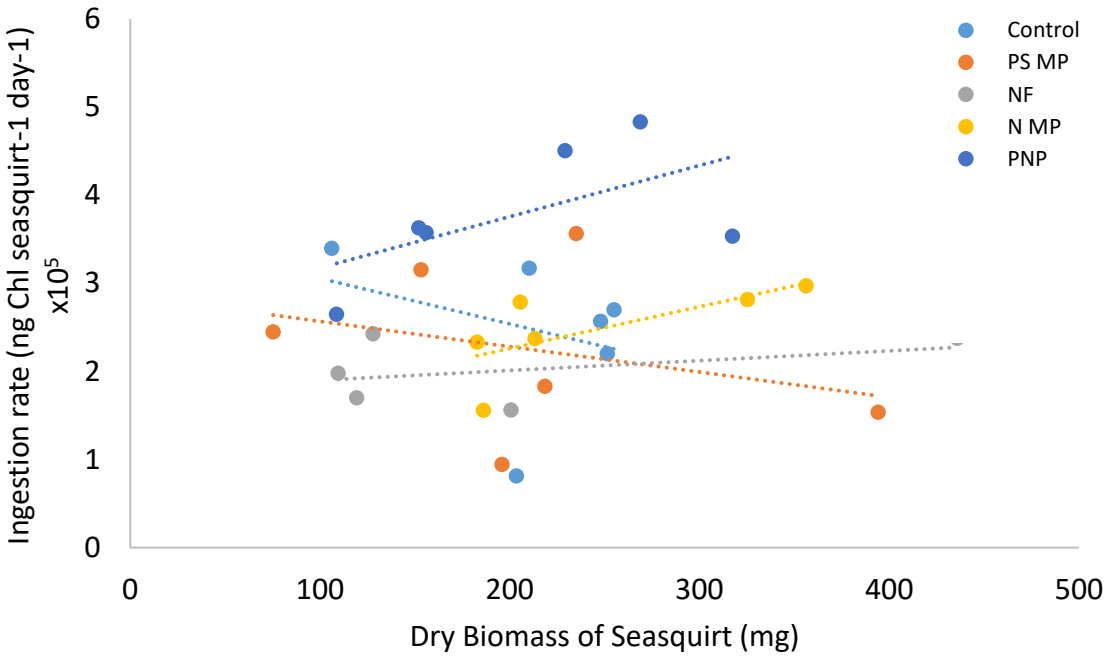

Figure 3.6: Algal ingestion rates of *Ciona intestinalis* when exposed to micro- and nano-plastics (data per individual +/- standard error, N=6). PS MP- 10 µm polystyrene microplastic spheres, NF- nylon fibres, NMP- nylpn microplastics, PNP- polystyrene nanoplastics.

3.3.5 Are there impacts of plastics on feeding rate?

Exposure to microplastics for 6 hours did not appear to alter sea squirt ingestion rates (One-way ANOVA, $F_{3,19}$ =0.454 , P= 0.717) (Figure 3.7). After 6 hours, sea squirts in the control group ingested 0.25 ± 0.04 mg chlorophyll sea squirt day^{-1}

on average, whereas those exposed to microplastics ingested 225504 ± 16590 ng chlorophyll sea squirt day^{-1}. However, a slight increase to 247115 ± 21034 ng chlorophyll sea squirt day^{-1} was seen in sea squirts exposed to Nylon microplastics, while exposure to polystyrene microplastics and nylon fibres appeared to reduce ingestion rates to 224478 ± 40691 and 226693 ± 29534 ng chlorophyll sea squirt day^{-1}, respectively.

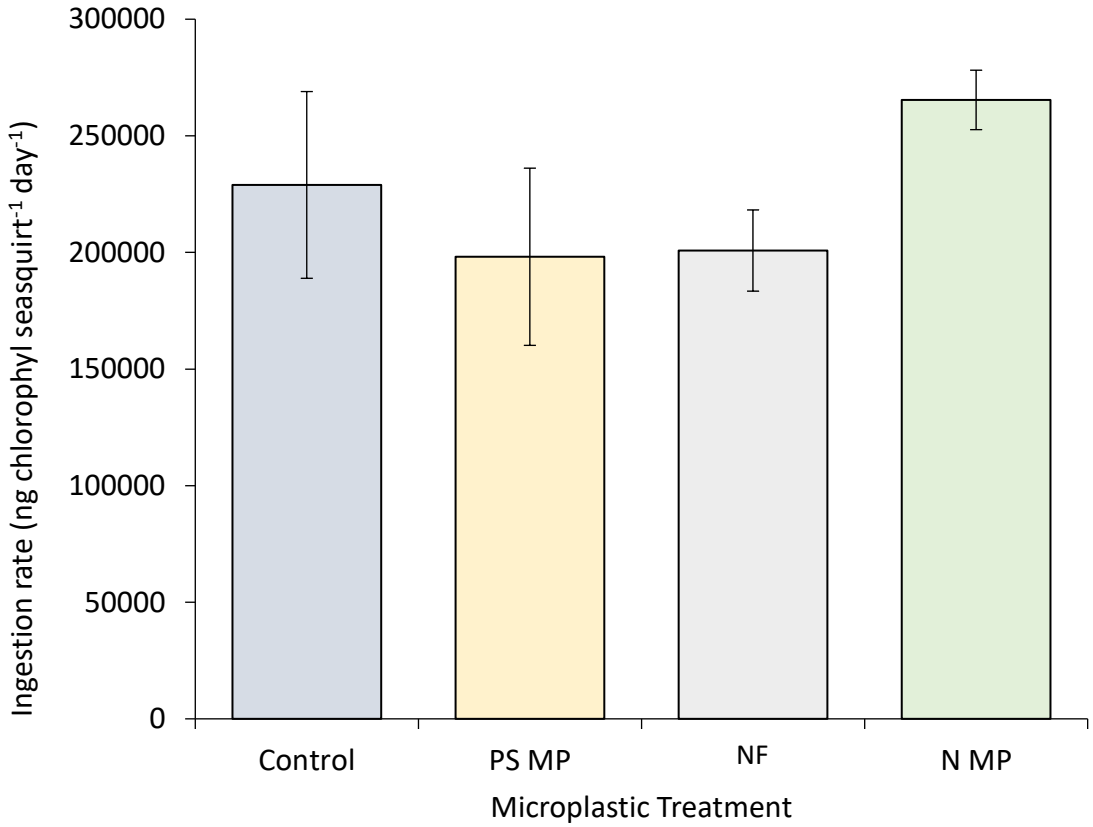

Figure 3.7: Algal ingestion rates of *Ciona intestinalis* exposed to microplastics for 6 hours (data as average +/- standard error, N=6). PS MP- 10 µm polystyrene spheres, NF- Nylon fibres, N MP- nylon microplastic.

After 24 hours the ingestion rates of all the sea squirts appears to show a slight decrease. However, there are no significant differences between the ingestion rates of the control group and any of the microplastic treatments (One-way ANOVA, $F_{3,15} = 1.805$, $P = 0.189$) (Figure 3.8). Contrary to the pattern seen after

6 hours at 24 hours the control group has a lower ingestion rate of 158 ± 17 ng chlorophyll sea squirt day^{-1}, than the microplastics treatments that have an average ingestion rate of 191 ± 15 ng chlorophyll sea squirt day^{-1}. Polystyrene microplastics maintain the lowest ingestion rates within the microplastic treatments with an average of 168 ± 12 ng chlorophyll sea squirt day^{-1}, while those sea squirts exposed to nylon fibres have the highest ingestion rates after 24 hours with an average of 209 ± 14 ng chlorophyll sea squirt day^{-1}.

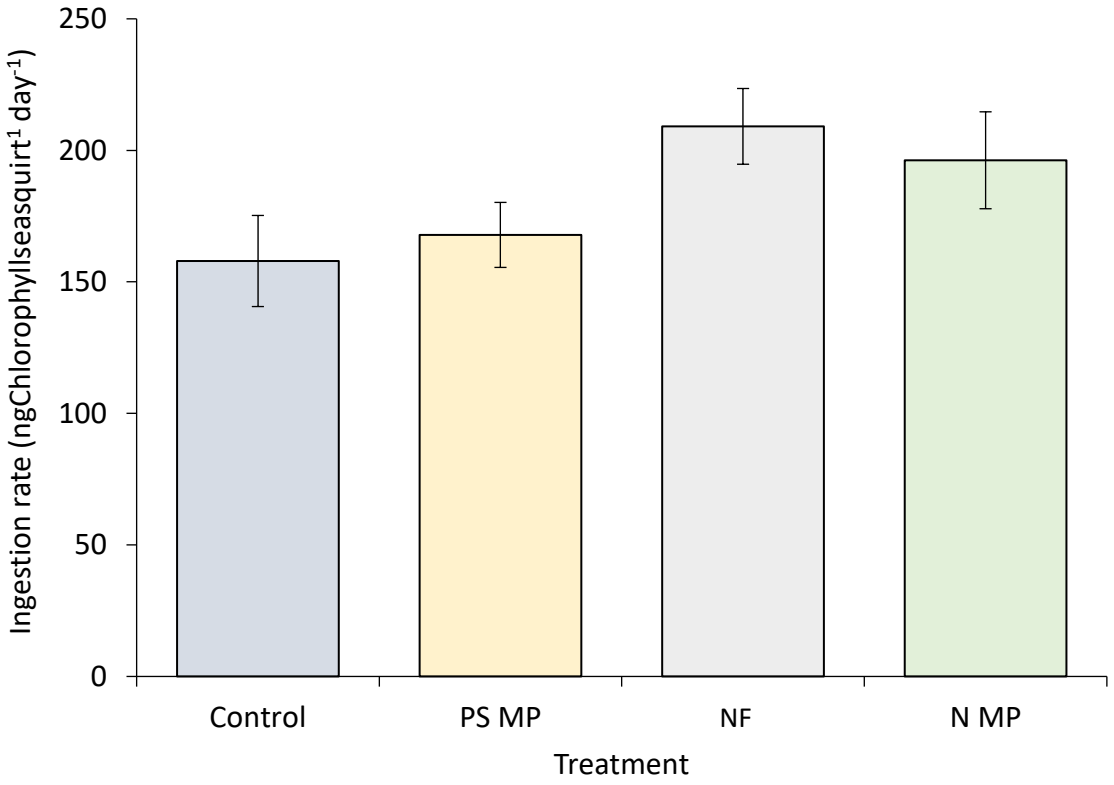

Figure 3.8: Algal ingestion rates of *Ciona intestinalis* exposed to microplastics for 24 hours (data as average +/- standard error, N=6)**.** PS MP- 10 µm polystyrene spheres, NF- Nylon fibres, N MP- nylon microplastic.

In contrast to the microplastic exposures, nanoplastic exposure appeared to have a significant impact in the ingestion rates of sea squirts (One-way ANOVA $F_{1,10}$=7.105, P= 0.0237) (Figure 3.9). After being exposed to nanoplastics for 6 hours sea squirts had an ingestion rate of 364192 ± 34806 ng chlorophyll sea

squirt day^{-1}, on average, compared to an average of 247373 ± 37526 ng chlorophyll sea squirt day^{-1} for sea squirts in the control group.

After 24 hours of exposure, the ingestion rate of the sea squirts exposed to nanoplastics remained slightly higher than those in the control group (Figure 3.10) but had fallen to 220 ± 27 ng chlorophyll sea squirt day^{-1}. This rate is no longer significantly different from the control group and microplastic treatments. (One-way ANOVA, $F_{1,7}$=3.085, P=0.122).

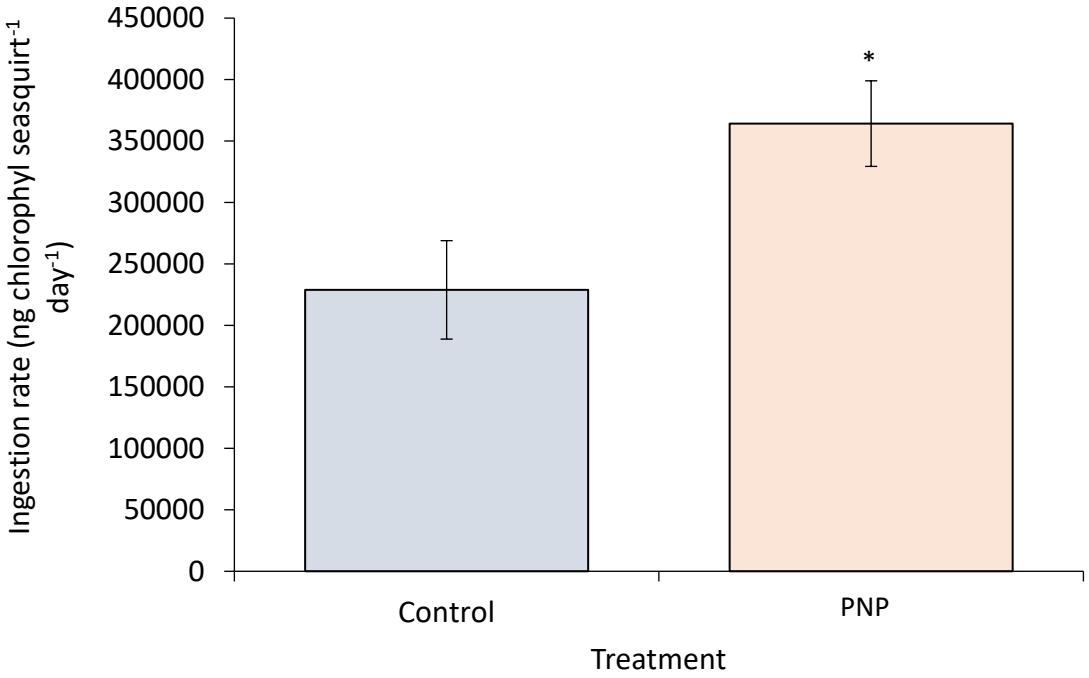

Figure 3.9: Effect of the presence of polystyrene nanoplastics (PNP) on algal ingestion rates of *Ciona intestinalis* after 6 hours (data as average +/- standard error, N=6).

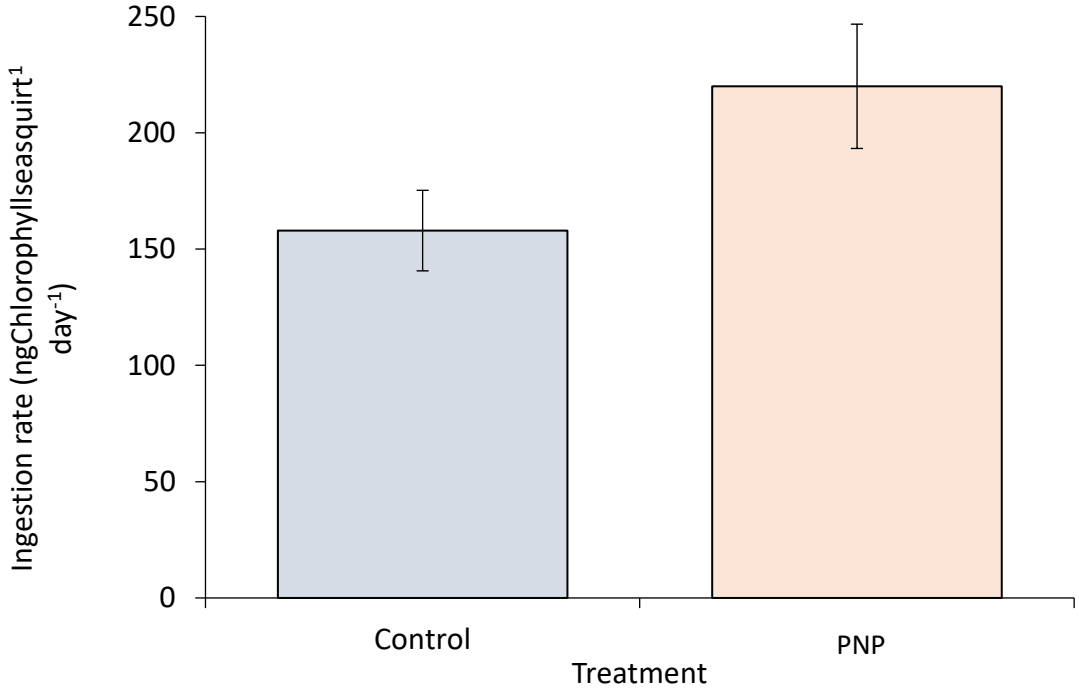

Figure 3.10*:* Effect of the presence of polystyrene nanoplastics (PNP) on algal ingestion rates of *Ciona intestinalis* after 24 hours (data as average +/- standard error, N=6).

3.4 Discussion

This study investigated the uptake and egestion of a variety of microplastics in the sea squirt, *C. intestinalis*. It was hypothesised that *C. intestinalis* would ingest all plastics, however nanoplastics would be ingested in greater volumes and will cause a reduction in feeding rate. It was also hypothesised that ingestion of fibres would lead to the greatest decrease in feeding rates. In line with my previous findings, all sea squirts exposed to plastics were found to readily ingest plastic, regardless of type, shape or quantity, proving the hypothesis that *C. intestinalis* would ingest all plastics. Plastic was found to be egested in faecal matter within 72 hours of ingestion. Microplastics were seen in the digestive system, faecal matter and adhered to external surfaces. This result is of significance as it suggests that wild sea squirts are likely to be ingesting a wide variety of microplastic types within the environment.

Here sea squirts were found to readily ingest polystyrene microbeads, nylon particles, nylon fibres and polystyrene nanoparticles. All of these plastic types have been reported in environmental samples collected by both sea surface tow (Moret-Ferguson, 2010) and benthic sampling (Claessens et al., 2011) suggesting they are present throughout marine ecosystems, and are therefore available for ingestion by marine organisms. Ingestion of microplastics in laboratory conditions have been reported in a number of organisms, including copepods (Cole et al., 2013, 2015, 2016), lugworms (Wright et al., 2013b), mussels (Browne et al., 2008; Van Cauwenberghe et al., 2015; Paul-Pont et al., 2016) and oysters (Sussarellu et al., 2016). Ingestion of polystyrene resulting from exposures to high concentrations (2000 beads ml^{-1}) has been shown to

result in oxidative damage, increased haemocyte mortality, altered gene expression (Paul-Pont et al., 2016) and translocation of these plastics from the digestive tract to the haemolymph were observed in mussels exposed to 15000 beads (Browne et al., 2008). Few studies have looked at ingestion of nylon under laboratory conditions. However, nylon has been shown to be ingested by holothurians under laboratory conditions (Graham and Thompson, 2009). This study does not go onto explore any impacts associated with this ingestion. It is likely that ingestion of nylon particles will illicit the same toxicological responses as polystyrene. Here, the impacts of polystyrene and nylon ingestion in sea squirts are explored.

Plastics were found to be present in the internal organs and faeces and adhered to the external surface of the tunics of all sea squirts exposed to plastics. Microplastics have been shown to cause gut blockages in a number of species, including the decapod *Nephrops* (Murray and Cowie, 2011) and planktivorous fish (Boerger et al., 2010). However, faecal matter was found to contain up to 90% of the nominal plastic concentration after only 24 hours, suggesting that plastics were able to pass through the intestinal tracts of *C. intestinalis* with very little negative impact. Faecal matter is important in the vertical flux of material such as organic carbon, nutrients and energy (Turner, 2002). The presence of microplastics in faecal matter is likely to alter this vertical flux. Cole et al. (2016) found that ingested polystyrene beads were egested from copepods in faecal pellets. These faecal pellets were found to have a greater buoyancy than faecal pellets containing no plastics, increasing the time they spent in the water column. This decrease in sinking rates may decrease the vertical transfer of material to benthic habitats. Sea squirt faecal density was not measured in this study;

however, it is likely that in line with the findings of Cole et al. (2016), the incorporation of microplastics will increase faecal matter buoyancy. Sea squirts are important in bentho-pelagic coupling, a process in which nutrients from the water column are transferred to the benthos. This is done, in sea squirts, by removing particulates from the water column by filter feeding and egesting them in the form of faeces or psuedofaeces. Alterations to the faeces is likely to alter the nutrient transfer carried out by sea squirts. Conversely, this mechanism may also aid in transporting buoyant polymers to benthic habitats. Ingested buoyant polymers may be packaged into faecal matter with a greater density than the polymers increasing their sinking rates. This mechanism may increase the bioavailability of plastics to benthic organisms.

The methodology used in this study did not allow visualization of plastics within the intestinal tract, therefore it is uncertain whether coalescence of plastics within specific regions of the digestive tract, similar to that seen in copepods (Cole et al., 2103), was occurring. However, only 1.5 % of the nominal concentration of polystyrene microplastics were found to be in the internal organs. The internal tissues were comprised of all digestive and circulatory organs, lending further support to the suggestion that microplastics were egested with few negative impacts. Plastic was also found to be adhered to the external surfaces of the sea squirts. This has been previously reported in copepods (Cole et al., 2013, 2015). It was suggested that this adherence would alter the energy budgets of the organisms due to possible limitations in activities such as prey detection, predator evasion and locomotion. Adult sea squirts are sessile animals and do not require locomotion to evade predators or catch prey, thereby meaning that adherence of plastics is less likely to alter a sea squirts energy budget.

Sea squirts are prolific filter feeders (Ruppert et al., 2004), filtering a vast volume of water every hour, enhancing their chances of ingesting plastics in the water column. Previous studies have shown that the ingestion rates of *C. intestinalis* increase in a linear way with body size (Peterson and Riisgard, 1992; Peterson and Svane, 2002). This is in contrast to the results presented in this study, which show no relationship between chlorophyll ingestion rates and the dry biomass of the sea squirts. It is not clear why this is the case as it is expected that larger animals would ingest a greater volume of algae, however this may simply be an experimental artefact of our exposure systems. One suggestion may be that the chlorophyll concentration in the water was low and combined with the high filtration rates of the sea squirts almost all sea squirts were able to remove the vast majority of the algae within a short time period. Peterson et al. (1999) found that *C. intestinalis* decreased their cilia beats with increasing algal cell concentrations, suggesting that ingestion rates decrease with increased food availability, possibly as a result of gut fullness. It is possible that this was seen in the exposures. Ingestion rates were found to be lower after 24 hours than after 6 hours. Following a period of depuration prior to the exposure sea squirts may have had an initial increase in ingestion rate until they reached satiation. After this point, the frequency of cilia beats may have decreased leading to a decrease in ingestion rate.

The presence of microplastics of any size, shape or polymer type of plastic did not significantly influence algal ingestion rates in *C. intestinalis*. This finding is in contrast to the vast majority of studies looking at the influence microplastic ingestion has upon algal ingestion rates. Previous studies using copepods (Cole et al., 2013, 2015) lugworms (Wright et al., 2013b), crabs (Watts et al., 2015) and

lobsters; (Welden and Cowie, 2016) all found the presence of microplastics led to a decreased intake of natural food when exposed together. It is likely that feeding strategy plays a role in these results, with certain feeding modes being more susceptible to the presence of microplastics than others. Unlike the organisms mentioned, sea squirts are indiscriminate filter feeders and a therefore likely to ingest higher quantities of plastics.

Copepods employ two feeding mechanisms, ambush and filter-feeding (Keorboe, 2011) depending on species, with some species employing both mechanisms whilst other species use just one or the other. Firstly, filter-feeding copepods feed by creating a water current by vibrating feeding appendages (Keorboe, 2011). Prey within this current are then detected by chemical cues and strained from the water current via setae on the maxillae (Keorboe, 2011). Secondly, ambush feeding relies on copepods sensing their prey moving in the water through hydromechanical signals (Visser, 2001). This signal is picked up by mechanoreceptory setae on the antennules, and the copepod can then jump onto the prey (Keorboe, 2011). The presence of microplastics is likely to decrease algal ingestion in these feeding strategies. Ambush feeders are likely to respond to plastics, decreasing the time available for algal prey capture (Cole et al., 2013). Filter-feeding copepods may not be able to reject plastic particles or rejection prior to ingestion will decrease feeding time (Cole et al., 2013). Sea squirts are sessile filter feeders and are not able to reject particles prior to ingestion. This indiscriminate filtering mechanism will not lead to a decrease in time spent feeding as a result of microplastic ingestion as seen in copepods.

Wright et al. (2013b) found that the filter-feeding lugworm reduced its feeding activity when exposed to 5 % (by weight) plastic in the sediment. Lugworms feed by ingesting sediments and filtering out particles post ingestion (Rijken, 1979). Ingestion of microplastics led to a 1.5 % increase in gut retention times, possibly leading to increased levels of satiation and decreased feeding. Crabs and lobsters were also found to decrease their feeding rates when being fed contaminated food (Watts et al, 2015; Weldon and Cowie, 2016). These animals are scavengers and carnivores and are likely to ingest plastics contained within prey species. Similarly to microplastic ingestion in the lugworms, plastic ingestion in these organisms leads to greater gut retention times and increased satiation, leading to reductions in feeding (Weldon and Cowie, 2016). These organisms have complex gut structures when compared to sea squirts. Sea squirts have a simple U-shaped digestive tract, consisting of an oesophagus, a stomach and an intestine leading to the anus (Ruppert et al., 2004). This simple system is likely to lead to quicker egestion of unwanted particles than in organisms with more complicated structures. This egestion will decrease microplastic-initiated satiation, allowing sea squirts to continue feeding.

However, in line with this study, Sussurellu et al. (2016) found that exposure to microplastics led to an increase in microalgae consumption in oysters. It was suggested that this increase was a compensatory mechanism to increase the energy intake after a disruption in the energy balance arising a result of microplastic ingestion. It is possible that a similar compensatory mechanism will be present in sea squirts, explaining the increased ingestion rates reported in this study.

The idea that feeding mode is a key determinant of whether microplastics will have a negative impact of natural food ingestion has been presented previously. Setala et al. (2016) looked at deposit feeding polychaetes and crustaceans, free swimming crustaceans and bivalves in a mesocosm experiment. They suggested that feeding strategy plays a role in the ingestion of microplastics. They found that filter-feeding bivalves ingested ten times more plastics than deposit feeding worms and free-swimming crustaceans. Sea squirts are indiscriminate filter feeders, feeding on any particles that pass through their cilia, suggesting that they may have high microplastic ingestion rates.

This study used two different methodologies to assess the fate of plastics within exposures of *C. intestinalis*. Both methodologies used fluorescently labelled plastics in order to ascertain plastic concentrations. The first methodology used was to manually count microplastics in subsamples using a microscope with a fluorescent bulb. This method is commonly used in the field of microplastics (e.g. Ward and Kach, 2009; Lee et al., 2013; Besseling et al., 2014; Cole et al., 2014; Setala et al., 2014) as it allows microplastics to be easily distinguished from other particles. If enough subsamples are counted, it is thought that this methodology gives an accurate count of microplastics within the sample being looked at. These counts are then scaled up to give a concentration of microplastics in the whole sample. The second methodology used here, a fluorometric technique, is to read the intensity of the fluorescence within set wavelengths, when excited. The higher the intensity, the greater the plastic concentration. This methodology is less time consuming and can be used on nanoplastics, which are too small to be counted using the first methodology (Hussain, 2001). Fluorometric techniques are not

commonly used in microplastic studies and this is the first study to compare these two methodologies.

In this study, the two methodologies show contrasting results. Due to the limitations of both methodologies only sea squirts exposed to polystyrene microplastics could be analysed using both methods. Manual counts show that there is a far greater proportion of polystyrene microplastics in the faecal pellets of exposed sea squirts. This is not reflected in the fluorescent readings, which appear to show a negligible amount of plastics in the faecal matter. Manual counts appear to show concentrations of polystyrene of several magnitudes higher than fluorescent readings. The reasons for these discrepancies between the results are unclear. However, it is possible that the process of ingestion may alter the properties of the plastics. *C. intestinalis* use mucous to trap particles for ingestion as well as for egestion (Ruppert et al., 2004). If the particles are covered in this mucous when being analysed via the fluorometric technique it is possible that they may not be excited at the same wavelength or may not give the same level of response. This may lead to an underestimation of plastic particles present and explain the differences seen in this study. Unfortunately this study was not able to determine whether ingestion altered plastic properties or coated particles in mucous, therefore the reasons for the discrepancies in the methodologies remain unclear. It would appear from these results that fluorescent techniques are not yet able to match manually counting plastics within samples. However, as plastics fragment into smaller pieces and research into nanoplastics increases reliable alternative techniques are going to be required.

This chapter and the previous chapter, have shown that *C. intestinalis* ingests plastics of varying shapes and sizes. It would appear that these sea squirts are able to egest these plastics with few negative impacts. However, plastics in the environment have a greater range of sizes, shapes and type to those used within these studies. These studies have also used clean plastics, in the environment it is thought that plastics have the potential to transfer toxins through leaching post-ingestion, leading to greater impacts (Barnes et al., 2009; Lithner et al., 2011). Therefore, plastic ingestion by organisms in the environment needs to be studied. The next chapter looks at whether *C. intestinalis* inhabiting a marina in Plymouth, UK are ingesting microplastics.

Chapter 4:

Plastic ingestion by *Ciona intestinalis* in the field

4.1 Introduction

4.1.2 Microplastic Ingestion in the Environment

It is now very well established that a wide variety of marine organisms can ingest microplastics in the environment (do Sula and Costa, 2014; Rochman et al., 2014) these include 100 % of marine turtle species, 59 % of whale species, 92 fish species (Kühn et al., 2015). Many more species have been found to ingest microplastics in laboratory exposures including 43 invertebrate species, across 11 phylum (Lusher, 2015) and 13 out of 15 zooplankton species (Cole et al., 2013). However, the majority of studies aiming to establish the impacts microplastics are having upon organisms have been carried out in laboratory settings. The impacts of microplastic ingestion within natural environments is less well known, with only three studies reporting environmental microplastic ingestion prior to 2013 (Goldstein and Goodwin, 2013). However, as the awareness of microplastic and their potential to cause harm is rising, so too is the research effort in this area and an increasing number of species are now found to have ingested microplastics in the natural environment. Microplastics have now been found to be ingested by a number of organisms across our oceans. Lusher et al. (2013) samples the gut contents of 184 fish, spanning 10 species, caught in the English Channel. These authors found that 36.5 % of the fish had ingested plastic and at least one fish of each species contained plastics. Shrimp caught in the North Sea were found to contain an average of 1.23 plastic particles (Devriese et al., 2015). Murray and Cowie (2011) found that 83 % of 120 lobsters

caught in the Clyde Sea contained plastics in their stomachs. Mussels containing plastics have been found along the coast of Belguim (De Witte et al., 2014), the Chinese coast (Li et al., 2016), the French-Dutch-Belgian coast (Van Cauwenberghe and Janssen, 2014) and in Nova Scotia (Mathalon and Hill, 2014). However, these are just a few of the organisms inhabiting our oceans, in order to gain a greater understanding into the impacts of microplastics more organisms need to be looked at. Invertebrates are often at the base of marine food webs, therefore, establishing whether invertebrates are ingesting microplastics is important step as ingestion of plastics by invertebrates has the potential for plastics to enter specialist feeders as well as to bio-accumulate throughout the food web.

One invertebrate species that has received a lot of attention is the common mussel, *Mytilus edulis*, due to its commercial importance and ease of sampling. Mussels have been documented to have ingested microplastics in Nova Scotia (Mathalon and Hill, 2014), the German North Sea (Van Cauwenberghe and Janssen, 2014) and along the coast of China (Li et al., 2016). These studies show a variable amount of plastics in mussels from different regions. Along the coast of China wild mussels appeared to have greater plastic content (2.7 items g^{-1}) than farmed specimens from the same coastline (1.6 items g^{-1}). In contrast to this, farmed mussels in Nova Scotia were found to contain 75 microfibres per mussel on average, while wild mussels contained significantly less (34 microfibers per mussel). However, this study sampled 20 wild mussels and 10 farmed mussels and pooled these mussels for analysis, possibly explaining the very high microplastic content reported. Further to this, the farmed mussels were purchased from markets; therefore, plastic contamination during the

transportation process cannot be eliminated. Mussels in the German North Sea were found to contain less plastic than those in China or Nova Scotia with approximately 0.36 particles per gram of soft tissue being reported. In spite of this, the mussels reported to contain the highest levels of microplastics across these studies are farmed mussels in Nova Scotia, possibly due to the high human population inhabiting the bay in which they were grown (Mathalon and Hill, 2014).

While the mussel appears to have received the greatest coverage in terms of microplastic ingestion there are a small number of other marine invertebrates that have been found to contain microplastics. Van Cauwenberghe and Janssen (2014) also looked at oysters in the same region and found that oysters contained a higher concentration of around 0.45 particles per gram of soft tissue. Murray and Cowie (2011) reported that 83 % of the 120 decapods collected from the Clyde Sea contained plastic fragments, with 62 % of the plastics being balls of tangled strands. Goldstien and Goodwin found 33.5% of the 385 gooseneck barnacles they sampled contained between one and 30 pieces of microplastic. Desforges et al. (2015) report finding one plastic particle for every 34 copepods samples and 1 particle for every 17 euphausids sampled in the Northeast Pacific Ocean, respectively. It is clear from these studies that microplastics are being ingested by a range of organisms across all trophic levels.

4.1.2 Microplastic Polymers in the Environment

The previous chapter in this thesis show that under laboratory conditions *Ciona intestinalis* readily ingests microplastics. However, the oceans are a far more complex system than can be replicated in the laboratory. The majority of plastic

is buoyant and transported through currents making them available to a wide range of organisms across different habitats. This buoyancy might be predicted to reduce the potential for interactions between microplastics and sea squirts which are shallow water, benthic organisms. However, it is becoming clearer that microplastics are being found in high concentrations in all areas of our oceans including deep sea sediments and organisms. Deep sea benthic organisms such as corals have been found to contain microplastics, including those from buoyant polymer types, including rayon and polyester (Woodall et al., 2014). Rayon, while not a true plastic is a synthetic polymer and was found to make up 56.9% of the fibres seen. Polyester was the most common plastic making up 23% of the fibres found. These concentrations are thought to be up to four times those found in the corresponding surface waters (Woodall et al., 2014), demonstrating that the transportation of plastics out of the surface layers to deeper regions must occur. The transport mechanisms behind this movement of buoyant particles to deeper waters is not yet clear.

An area that has recently received attention for the accumulation of plastic are those in regions of high anthropogenic activity. The majority of plastic entering the oceans occurs in coastal areas with high human populations (Clark et al., 2016). While these areas constitute a small volume of the global ocean they have high rates of productivity, shallow seas over continental shelves are responsible for 15-21% of global oceanic primary production (Jahnke, 2010). Due to this high productivity and proximity to plastic sources coastal areas have a high potential for the overlap of plastic debris and species interactions (Clark et al., 2016). Polychaete worms living in tidal sediments in areas of high anthropogenic activity have been shown to ingest microplastics (Van Cauwenberghe and Janssen,

2014), demonstrating that microplastics are available to organisms inhabiting sediments.

4.1.3 *Ciona intestinalis*

C. intestinalis inhabit coastal areas and thrive in polluted waters and marinas (Kourakis and Smith, 2015) placing them in this area of high plastic content, making them a likely to target for plastic ingestion. Sea Squirts play a vital role in coastal ecosystems, they are primary consumers within the marine food web, are prey for a number of specialized species such as nudibranchs, echinoderms and fish. These organisms are prolific filter feeders capable of filtering the equivalent of their body volume every second (Ruppert et al., 2004). This filtering process is important for local ecosystems due to their ability to purify the filtered water by removing and sequestering toxins (Ruppert et al., 2004). The high filtration rate means that sea squirts are highly likely to ingest any microplastics in the water column. However, there have been no reported incidents of sea squirts ingesting microplastics outside of controlled laboratory conditions to date.

4.1.4 Chapter 4 Aims and Hypotheses

Here we aim to establish whether *C. intestinalis* are ingesting plastic *in situ* and to ascertain the type of plastic ingested by sea squirts in their natural environment. We hypothesize that sea squirts collected from a marina will have microplastics present within their intestinal systems and that these plastics will primarily be fibres.

4.2 Methods

4.2.1 Settlement Plate Placement

Settlement plates were constructed from clean Perspex acrylic plastic sheets. The sheets were cut to a size of 90 x 91 cm. Each rectangular plate had two holes drilled into the top corners in order for attachment to the marina by a heavy strength three strand hemp rope, to avid plastic contamination (Figure 4.1). Six settlement plates were then suspended at a depth of 1m from the board walks at Millbay Marina, Plymouth (50.363479, -4.151538), Devon, U.K. This marina was selected due to its previously known population of *C. intestinalis*. Plates were deployed on the 27th of April 2016 in order to recruit *C. intestinalis* larvae throughout the Spring and Summer. Plates were removed from the marina on the 8th September after a period of just over 3 months. Adult *C. intestinalis* were removed from the plates immediately and placed in 50 ml falcon tubes for transportation to the laboratory. To reduce contamination whilst collecting the organisms cotton clothing was worn and the time the organisms spent in the air was limited to a little as possible.

Figure 4.1: Settlement plate before being placed in Milbay Marina (Photograph provided by A. Porter).

4.2.2 Digestion of *Ciona intestinalis*

Once in the laboratory the sea squirts were classified by size, with small sea squirts being those less than 1.5 cm in length and those between 1.5 and 5 cm. All sea squirts were rinsed in 0.2 µm filtered artificial sea water to remove any microplastics that may have adhered to the outsides. Small sea squirts were placed, whole, into a 50 ml falcon tube containing 10 ml of 3% NaCl. Tunics were removed from sea squirts over 1.5 cm and the internal organs and tunics placed in separate falcon tubes containing 10 ml of 3% NaCl. A maximum of 15 sea squirts was placed in each falcon tube. Samples were pooled into five samples, 15 sea squirts per analysis, due to their size and small abundance of microplastics expected to be found in each one. The falcon tubes containing

bleach and the sea squirts were then left on a shaking plate overnight. The sea squirts were removed from the NaCl and filtered through a 30 µm mesh. Any matter caught on the mesh was rinsed before being placed in 10% KOH for two weeks, to allow further breakdown of organic matter. Clean lab coats were worn throughout to minimize contamination from clothing.

4.2.3 Microplastic Analysis

After 2 weeks the 10 % KOH, containing small sea squirts and the internal organs of large sea squirts, were filtered through a GF/C Whatman filter paper using a vacuum pump. The pump was enclosed to avoid drawing any plastics from the atmosphere onto the filter papers. The falcon tubes were rinsed thoroughly with MilliQ water which was also filtered. Each falcon tube of 10% KOH was filtered through a separate filter paper. Filtration was carried out under controlled conditions and all samples were covered immediately after being transferred from one vessel to the next to avoid airborne contamination. Filter papers were viewed using an inverted fluorescent microscope (Leica DMI 4000B) to check for any plastics. Plastic particles were analysed using an FTIR (Agilent Cary 630) to ascertain the type of plastic.

4.3 Results

4.3.1 *Ciona intestinalis* recruitment and settlement

C. intestinalis was found to be present on all six settlement plates retrieved from Millbay marina (Figure 4.2). A total of 58 individuals were collected from the settlement plates for analysis.

Figure 4.4: Settlement plate after retrieval from Milbay Marina, Plymouth. *C. intestinalis* can be seen in the right hand corners, highlighted by the red circles.

4.3.2 Ingested Particles

A total of four filter papers were analysed for microplastics, one filter paper was discarded prior to analysis. This was due to the digestion process being unsuccessful in breaking down the tunics. Filtration of the tunics onto a filter paper

was then unsuccessful. All analysed filter papers had plastic –like particles on them. However, due to the pooling of the sea squirts for analysis it is not clear that all animals contained plastics. A total of 48 plastic-like particles were seen on the filter papers (Table 4.1). This suggests an average of 0.8 particles per sea squirt. Fibres were the most common particle seen with these accounting for 34 (71 %) of the particles seen. A variety of particles were seen on the filter papers, these included: relatively long fibres (Figure 4.3A, B and E), small dark fibres (Figure 4.3I, H and J), flecks of small coloured particles (Figure 4.3C) and variously coloured thicker fibres (Figure 4.3D and G). All plastics found were too small to be analysed by the FTIR (Agilent Cary 630, lower size limit 50 µm).

Table 4.1: Summary table of plastic-like fragments found on filter papers following digestion of *C. intestinalis* collected from Millbay Marina, Plymouth.

Particle Description	**Filter Paper 2**	**Filter Paper 3**	**Filter Paper 4**	**Filter Paper 5**
Dark Fibre	2	2	3	12
Clear Fibre	1			2
Blue Fibre	1	2	4	2
Red Fibre	1	1		1
Dark Particle	8	1	1	2
Red Particle		1		
Green Particle				1
Total Fragments	**13**	**7**	**8**	**20**

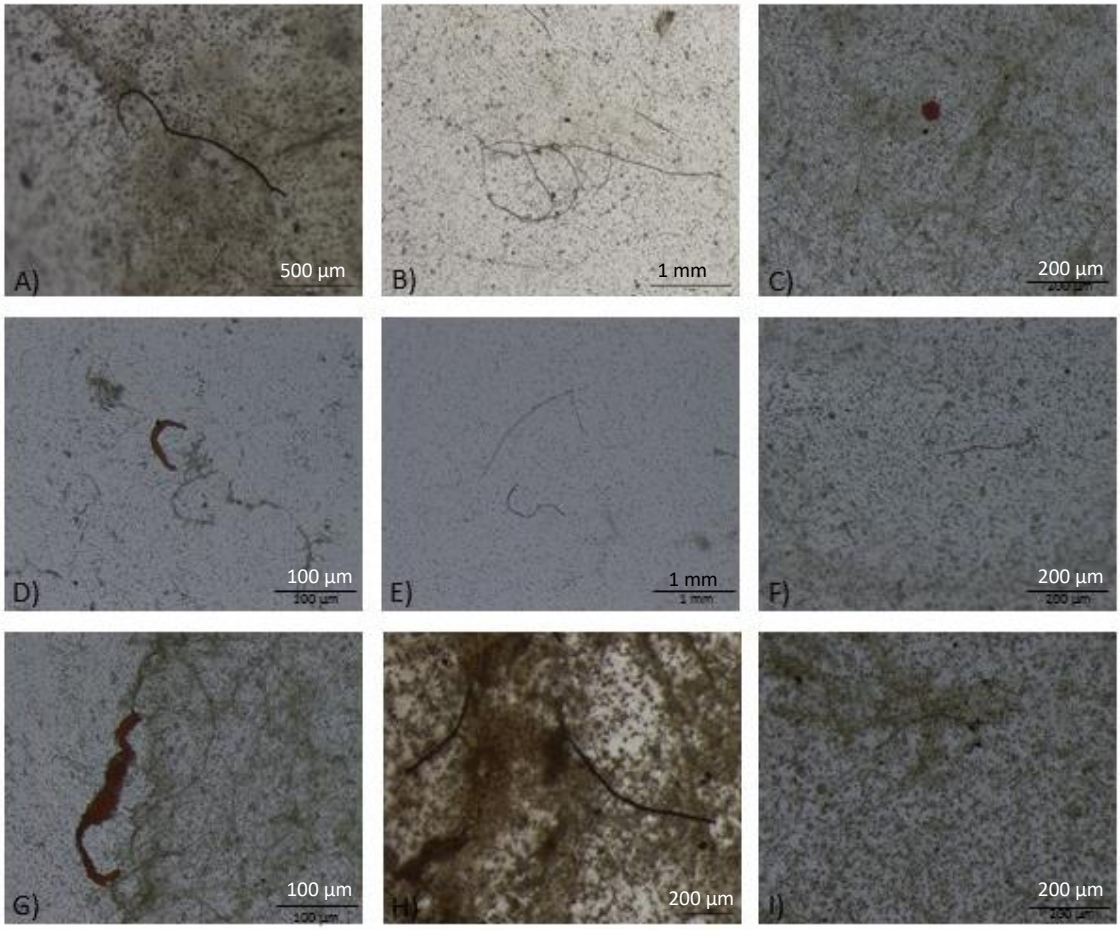

Figure 4.5: A selection of photos of particles found on meshes through which previously digested sea squirts were filtered. Photos A, B, E, F, H and I show plastic-like fibres. Photo C shows a small coloured particle. Photos D and G show larger red coloured fragments found in sea squirts.

4.4 Discussion

In this study *C. intestinalis* from one marina in Plymouth were investigated to see whether microplastic ingestion was occurring in the natural environment. Settlement plates placed in Millbay Marina were colonised by *C. intestinalis* during the summer of 2015. A total of 58 sea squirts were removed from the plates giving an average recruitment of 9.7 sea squirts per settlement plate over the four month placement. Plastic-like particles were observed on all filter papers, suggesting that Sea squirts are ingesting microplastics in the environment. This finding adds to a growing number of organisms reported to have ingested microplastics within the environment.

The average abundance of 0.8 plastic-like particles per individual is in line with other studies that have shown filter feeders such as mussels from German farms contained 1.19 particles mussel^{-1} (Van Cauwenberghe and Janssen, 2014). However, the number of particles found in this study is much lower than those reported in mussels in other parts of the world, Mathalon and Hill (2014) reported very high levels of an average of 34-178 particles per mussel collected from Canada. These mussels were transported 800 km after being cultured to markets therefore airborne contamination whilst in transport may have occurred leading to the high concentrations seen. It is not clear why the concentration of particles seen in *C. intestinalis* is towards the lower range of plastics being reported in other animals. However, as seen in previous chapters *C. intestinalis* appeared to be able to egest plastics efficiently. *C. intestinalis* in marine habitats may have shorter plastic retention times than other species, resulting in a lower volume of plastics inside them at any one time. Alternatively, the sea squirts collected in this

study may have been inhabiting an area with relatively low plastic concentrations. If this is the case the opportunity for *C. intestinalis* to ingest plastics would have been lower.

Mathalon and Hill (2014) found mussels from farms contained a greater number of microplastics than wild mussels in the wild. It was thought that this might be due to the technique of growing mussels on polypropylene ropes providing an increased localised source of plastic that may be ingested. If this is the case, it is also likely that the sea squirts in this study were subjected to high levels of plastic emanating from the rope used to attach the settlement plates. However, Le et al., (2016) dispute this, they found a greater number of plastics in wild mussels found around China than those farmed. Further analysis of these mussels found no polypropylene within the farmed mussels suggesting that there was no greater plastic contamination. The explanation given for the greater number of plastics in the wild mussels was due to them being collected in heavily polluted industrial area, while farmed mussels are grown in high quality waters. These mussels grown in high quality waters had an average of 2.4 particles individual^{-1}, which is also higher than the sea squirts analysed in this study. It is unlikely that the sea squirts analysed are from an area of greater cleanliness, therefore, this suggests that a low plastic abundance is not reflective of the plastic content of the water.

There a number of other factors that have yet to be explored in sea squirts to establish why the plastic abundance within them is low. It may be that all sea squirts analysed were a maximum of 4 months old. This may not have been long enough to ingest a vast number of particles. Most studies looking at microplastic

ingestion have used mature animals that have been inhabiting an area for longer than 4 months. This may allow accumulation of plastics within them that is not seen within the sea squirts analysed here.

Sea squirts are a group of organisms that have not been studied in terms of microplastic ingestion before. This study shows that sea squirts are very likely to be ingesting plastic in the environment, however it is not clear what the impacts of this plastic ingestion are or if sea squirts are able to egest unwanted particles. Sea squirts feed through filtration using cilia and mucous to trap particles to guide particle to the inhalant siphon. However, once ingested it is possible for particles to be egested through the inhalant siphon during the “squirting” motion. This mechanism is usually triggered as a predator response, but may also be utilised for particle egestion. No studies to date have looked at sea squirts using this mechanism to egest unwanted particles.

Fibres were the most common particle type to be seen in this study, they made up 71 % of the total particles recorded. This is in line with the majority of other studies looking into microplastic ingestion. Fibres are now thought to be the most common plastic in the ocean (Erikson et al., 2014), and are being found in a range of marine organisms. Similarly, Lusher et al. (2016) found that 68.3 % of the plastics found within fish collected from the North Sea were fibres. While Desforges et al. (2015) found that 50 % of plastics found in copepods collected from the Pacific were fibres, yet fibres made up 68 % of the plastics found in euphausiids from the same area. While these results are slightly lower than those seen in the present study, it would appear that the *C. intestinalis* in this study are

filtering the same plastic compositions as reported in other organisms. Sea squirt feeding mechanisms are similar to those utilised by copepods and euphausiids, therefore, this similarity is to be expected.

A combination of digestion protocols were used in this study, due to the difficulties in digesting sea squirts. Currently there is no recommended protocol for the digestion of animals with respect to viewing plastics contained within them. Initially H_20_2 was chosen to digest the sea squirts due to its use in previous microplastic digestion studies (Mathalon and Hill 2014; Li et al., 2015), however, very little digestion occurred. KOH was then selected to digest the sea squirts further, this method was chosen due to it having the least impact on microplastics when compared to other commonly used digestion methods (Dehaut et al., 2015). Despite this Dehaut et al. (2015) found that KOH had a slight impact on cellulose acetate. This polymer is not common but if present would not be represented in the samples following digestion, possibly leading to an underestimate in the number of particles ingested by the sea squirts. Different digestion protocols have been found to have varying success rates in plastic recovery due to digestion of the plastics or plastic adhering to equipment used (Claessens et al., 2013). This may impact the number of plastics recorded in organisms and makes comparing ingestion within different organisms and from different locations very difficult.

The sea squirts collected from Millbay Marina were growing at 1 m below the sea surface. Animals at this depth are likely to come into contact with buoyant particles that are transported by wind and surface currents. These plastics are likely to build up in areas such as marinas which are protected somewhat. This would mean that organisms inhabiting these areas are more likely to be subject

to buoyant plastics than those in the open ocean. However, this study was unable to determine the nature of the particles present within the sea squirts, due to the particles being too small to be detected by FTIR. FTIR machines analyse the spectral composition of a plastic fragment and match them to a fingerprint of a known polymer, making them a powerful tool in plastic identification. Unfortunately, there are also a number of issues associated with using an FTIR machine and it is not always possible to do so (e.g. De Witte et al., 2014; Mathalon and Hill, 2014; Devriese et al., 2015). FTIR analysis relies on the plastic being placed above a narrow infrared beam (Song et al., 2015). As plastic fragments decrease in size they become harder to see with a microscope and may not be noticed and analysed by FTIR. If plastics are successfully placed onto the FTIR they may still be too small to be detected, FTIRs have a lower detection limit of 50 µm (Agilent Technologies 2011). Small plastics also adhere to larger plastics due to their hydrophobic properties, this may mean that the FTIR machine is unable to distinguish and the small plastic will not be analysed.

These issues are likely to lead to an under representation of the small microplastics found within samples. However, plastics of this size range are likely to be bio-available to the greatest number of organisms and are similar in size to many phytoplankton and algal species. Another constraint of using an FTIR combined with identification of particles using a microscope is that particles that are the same colour as the background object will not be visually identified and placed on the FTIR. This may also lead to an under representation of particles of certain colours, typically white and clear (Song et al., 2015). The use of an FTIR also carries a time cost and many studies analyse a portion of the particles seen (e.g. Cooper & Corcoran, 2010; Li et al., 2016), possibly leading to an

underrepresentation of plastics (Song et al., 2015). The use of an FTIR machine is advantageous when identifying microplastics, however development of new techniques are required to look into small microplastics.

Thus far, sea squirts have been overlooked when looking at the impacts microplastics are having upon our oceans. However, they are an important group of organisms. They are prolific filter feeders, playing a role in water purification. If this is affected by ingestion of plastics, localised water clarity may decrease having further consequences for the local ecosystems. On the other hand, sea squirts may be able to assist in removing microplastics from the environment and store them away from other organisms. Sea Squirts are able to filter and store toxins found in the water column, such as vanadium, this process might be able to help remove plastics from the water trigger the same process.

These results show that *C. intestinalis* are likely to be ingesting microplastics, but associated impacts are currently unknown. Understanding whether sea squirts are ingesting microplastics in the environment is also important to understand the impacts this may be having at the ecosystem level. Sea Squirts play a role in providing habitats and as prey for specialised predators. If plastic ingestion has a detrimental impact upon sea squirts, it is possible that the impacts could reduce the populations of sea squirts could decrease, providing less shelter for organisms dependent upon their shelter.

This study has shown that *C. intestinalis* collected from the field have ingested plastic like particles. Unfortunately, the particles were not able to be analysed to

determine their nature, therefore, it is still unclear as to whether sea squirts are ingesting plastics in the wider environment. Further research is required to ascertain the nature of the particles found to confirm whether these sea squirts have ingested plastics. This study has only looked at a small number of sea squirts from one location within the UK to gain a greater understanding into the impacts of microplastic ingestion by sea squirts a range of species need to be analysed from a wide range of areas.

Chapter 5:

General Discussion

This study aimed to establish whether *C. intestinalis* were able to ingest plastics in laboratory conditions, and whether this microplastics ingestion had any impacts on their feeding physiology. Further to this, *C. intestinalis* collected from a natural marine environment were analysed for plastic ingestion. Ascidians have received very little attention when looking at the impacts of marine plastic, here I show, for the first time that ascidians are able to ingest a range of different plastics shapes and sizes, but that microplastics ingestion had no impact on normal feeding rates. These laboratory exposures were complimented by the presence of microplastic-like particles in *C. intestinalis* collected from the environment, suggesting that plastic ingestion is occurring in this species in their natural habitats.

These new findings add to the growing body of literature that microplastics are readily ingested by a wide range of species. Here, I have shown that plastic ingestion by ascidians varied with polymer type. To date, many studies looking at microplastic ingestion under laboratory conditions have only looked at ingestion of one type of plastic, and most commonly in the form of beads (e.g. Browne et al., 2008; Wright et al., 2013; Cole et al., 2013, 2016; Sussarellu et al., 2016). However, a wide variety of plastics are being found in marine environments, with the most common being polypropylene, polyethylene and polyvinylchloride (Andrady et al., 2011). It would be expected that organisms inhabiting a range of marine environments would come into contact with a variety of these plastics.

Therefore, establishment of ingestion of a range of plastics is important to aid the assessment of the impacts plastics may have upon the marine environment. This is one of the first studies to look at ingestion of a number of different types of plastics in an organism to look for differences in ingestion or impact according to shape or polymer type. The properties of the plastic particles is likely to affect their behaviour within the marine environment. One factor that may influence behaviour and bioavailability is buoyancy. The buoyancy of a polymer influences its position in the water column, with buoyant polymers such as polyethylene inhabiting the surface layers and denser polymers such as polyvinyl chloride being found deeper within water columns (Wright et al., 2013a; Clark et al., 2016). Plastics inhabiting the surface layers would be expected to be bioavailable to pelagic organisms, inhabiting these layers, such as plankton (Wright et al., 2013a).

Benthic organisms, such as sea squirts and mussels, are more likely to come into contact with negatively buoyant polymers (Wright et al., 2013a). However, buoyant polymers have recently been recorded in the guts and tissues of benthic organisms. For example, low density polyethylene, high density polyethylene and polystyrene were found in the guts of mussels (Van Cauwenberghe et al., 2015) from the French- Belgian-Dutch coast, and rayon, polyester, polyamides, acetate and acrylic have been recorded in deep-sea corals and sediments from depths of up to 2000 m (Woodall et al., 2014), indicating that they are available in all habitats despite their buoyant properties as virgin plastics. The presence of buoyant plastics within these animals suggests that plastics within the marine environment are being transformed in some way, causing their buoyancy and behaviour to be altered. A number of ways in which plastics are being

transformed have been hypothesised. These may include, ingestion and egestion of plastics may alter their properties (Cole et al., 2016) or by biofouling (Lobelle and Cunliffe, 2011). Egestion of microplastics may lead to plastics being incorporated into faecal matter of a different density to the plastics prior to ingestion. Biofouling is a process in which pollutants, sediments and organic matter adhere to the surface of the plastics, and this adherence of materials with differing densities is likely to alter the density of the plastic.

Despite buoyancy playing a role in the bioavailability of plastics, studies have not looked at this in laboratory exposures. Here, I used a range of plastics with different densities, which were therefore likely to have different availabilities to these benthic, filter feeding sea squirts. These plastics were polystyrene beads, polyamide beads, cryoground polypropylene rope fibre, cryoground rubber loom bands, high density polyethylene particles, polyhydroxybutyrate particles, low density polyethylene particles, polypropylene particles, nylon particles, nylon fibres and polystyrene nanoplastics. All but one of the plastics used were found to be readily ingested, suggesting ascidians may have a high capacity for ingestion of plastics in the wider environment. However, ingestion did not occur at the same rate for all of the plastics tested. Polypropylene rope fibres did not appear to be ingested by any of the exposed sea squirts, while HDPE and loom bands were only found to be present in some of the exposed *C. intestinalis* samples. All other plastics were abundant in all samples taken from exposed *C. intestinalis*, suggesting that uptake of these plastics occurred at a greater rate than polypropylene, HDPE and loom bands. These plastics found to be ingested at a decreased rate have high buoyancies and were observed to be floating on

the surface of the water within exposure beakers, potentially decreasing their availability to *C. intestinalis* within the exposure beakers.

Further to this, subsequent exposures to microplastics found that a greater number of beads were ingested by *C. intestinalis* than fibres when exposed at the same concentrations. This provides further evidence that different plastics have different bioavailabilities to organisms. Prior to this study, very few studies have looked at the uptake of differently shaped microplastics. One study that has looked at the ingestion of both beads and fibres is Au et al. (2015). This study exposed the freshwater amphipod, *Hyalella azteca,* to polyethylene particles and polypropylene fibres. Ingestion of both plastics occurred, however, the rate of ingestion could not be directly compared due to differences in exposure concentrations.

The beakers used in these exposures were 2L, therefore had a relatively small water column above the sea squirt. Sea squirts feed by creating a current in the surrounding water, from which particulates are filtered and ingested (Ruppert et al., 2004). A small water column is likely to result in the sea squirts being able to incorporate surface waters into this current. In spite of this, differences in the ingestion of plastic types occurred. The columns of water overlying benthic organisms in natural environments are likely to be much greater than those used in this experiment. The surface waters of these larger columns are unlikely to be reached by the feeding currents produced by sea squirts. Therefore, a larger water column is likely to decrease the bioavailability of buoyant plastics to benthic filter feeders further unless the behaviour and buoyancy of plastics are altered

through environmental processes, such as biofouling and transformation through ingestion (Lobelle and Cunliffe, 2011; Cole et al., 2016; Clark et al., 2016). However, buoyant fibres are being recorded in benthic organisms within the natural environment (Van Cauwenberghe et al., 2015; Woodall et al., 2014). This study also found plastic-like particles to be present in sea squirts collected from the natural environment. Whilst this study was unable to determine the polymer type observed in these sea squirts, it is likely that a number are buoyant plastics. The presence of these polymers in benthic organisms suggests that the polymers are present in benthic habitats. The marine environment is a complex environment, with many interactions and processes taking place. Microplastics are long-lasting and hydrophobic, making them ideal surfaces for the adherence of waterborne pollutants (Teuten et al., 2009). Accumulation of a variety of pollutants, sediments and organic material may lead to the formation of an ecocorona on the surface of the plastic (Galloway et al., 2017). This ecocorona may influence the behaviour and fate of the plastic in a variety of ways. One of these is thought to be by increasing the density of the plastic causing it to sink within the water column, increasing the bioavailability of plastics to benthic organisms (Galloway et al., 2017).

Despite sea squirts readily ingesting large numbers of a variety of plastics, no effect on their natural algal feeding rates were seen in this study. This is in contrast to a number of studies looking at the impacts of microplastics on organisms, including a 30 % reduction in copepod feeding rates following exposure to polystyrene (Cole et al., 2013), a 40 % reduction in feeding activity in lugworms fed polystyrene (Besseling et al., 2013). Significant reductions in food consumption resulting from microplastic ingestion have also been reported

in crabs (Watts et al., 2015) and lobsters (Weldon and Cowie, 2016). A reduction in food consumption is likely to lead to an energy decrease (Wright et al., 2013a). Crabs de 1% microplastics (by weight) were found to have an energy reduction of 90 KJ individual day^{-1} (Watts et al., 2015), while lugworms exposed to low concentrations of polystyrene were found to have a 50 % decrease in lipid energy reserves when compared to worms in plastic free conditions (Wright et al., 2013b). The findings presented in this study on *C. intestinalis* may indicate that the ingestion of plastics may not be detrimental to *C. intestinalis* at the individual level.

However, microplastics may have ecosystem impacts (Wright et al., 2013a), through a variety of processes. Firstly, ingestion of plastics may also lead to trophic transfer of plastics (and associated pollutants). Microplastics ingested by low-trophic organisms, such as ascidians, may lead to a transfer and bioaccumulation of plastics higher up the food chain. Transfer of plastics has been previously reported in crabs fed contaminated mussels (Farrell and Nelson, 2013), mysid shrimps feeding on contaminated zooplankton (Setala et al., 2014) and lobsters fed contaminated fish (Murray and Cowie, 2011). It would appear that trophic transfer of microplastics is possible in laboratory studies, and is therefore likely to be occurring in the environment. Murray and Cowie (2011) believe that lobsters collected from the Clyde Sea may have ingested plastics through feeding upon contaminated prey, due to the omnivorous feeding strategy of the lobsters. The role an organism will play in the trophic transfer of plastics to other organisms will depend upon all organisms involved. Short microplastic retention times in the prey animals is likely to decrease the volume of plastic transferred to predators. Trophic levels and chain length may also be important,

greater accumulation through the food chain is likely to occur in the higher levels if the lowest trophic levels are ingesting plastics and the chain is relatively long. Sea squirts are preyed upon by a number of specialised species including gastropods, echinoderms and fish, making them a potential vector organism for plastics to get into the food chain. Bioaccumulation of plastics in higher trophic organisms is likely to lead to adverse impacts arising from microplastic toxicity.

Secondly, microplastics may be transformed through ingestion. Sea squirts are important organisms for bentho-pelagic coupling, a process in which nutrients and other matter is transferred from the water column to the benthos. Sea squirts do this by filtering particles from the water column and egesting faeces and psuedofaeces, which then sinks, transporting matter to the benthos. Microplastics are likely to be transported from the water column to the benthos in similar ways. Plastics were seen in the faeces of *C. intestinalis* within these exposures, suggesting that sea squirts could play a role in altering the behaviour of plastics. Previous studies looking at alterations in faecal pellet behaviour as a result of microplastics have found that plastics increase faecal pellet buoyancy (Cole et al., 2016). It was found that the presence of microplastics in faecal pellets, increased the sinking rates by a factor of 2.25. Extrapolations carried out in this study estimated that faecal pellets would take 53 days longer to sink to the seabed, assuming an average ocean depth of 3682 m. This increase in sinking time would lead to an increase in the bioavailability of plastics to coprophagous organisms (Cole et al., 2016). Further to this, increasing the time faecal pellets remain within the water column would also make them more susceptible to fragmentation and degradation, resulting in carbon and nutrients being released back into the upper layers of the water column, reducing the flux of these

materials to deeper waters (Turner et al.,2015; Buesseler et al., 2007; Giering et al., 2014). Conversely, the incorporation of microplastics into faecal matter may also increase the availability of plastics to benthic organisms. Cole et al. (2016) found that all faecal pellets sank to the base of exposure beakers, showing that the incorporation of plastics did not cause faecal pellets to become negatively buoyant. While these faecal pellets may take longer to reach the benthos, they are a mechanism in which microplastics may be transferred from the water column to benthic habitats.

Microplastic in the marine environment are thought to be decreasing in size due to fragmentation processes (Moet-Ferguson et al., 2010). This decrease in size is leading to an increase in the abundance of nanoplastics. It has been suggested that nanoplastics have a greater toxicity than microplastics (Galloway et al., 2017). This greater toxicity combined with increasing abundance makes nanoplastics an important area of research. Previous studies have found that mussels and oysters ingest nanoplastics (Ward and Kach, 2009). Nanoplastics were found to have a greater gut retention time than microplastics in these organisms. Exposure to nanoparticles has also been shown to lead to oxidative stress responses and tissue cell injuries in mussels (Koehler et al., 2008). *C. intestinalis* readily ingested polystyrene nanoparticles. The fluorometric methodology used in this study appeared to show 6 % of the nominal nanoplastic and 0% of the microplastic concentration being present in faecal matter following a 24 hour exposure. This would suggest that, in contrast with Ward and Kach (2009), nanoplastics had a shorter gut retention time in *C. intestinalis*. Conversely, the manual technique found that 96 % of the total microplastics accounted for after a 24 hour exposure were in the faecal matter. This

discrepancy between the two methods indicates that no conclusions regarding the impacts of ingesting nanoplastics on *C. intestinalis* can be drawn.

Laboratory studies investigating the effects of microplastic ingestion have shown that microplastics can produce toxicological effects, including reduced feeding rates (e.g. Murray and Cowie, 2011; Besseling et al., 2013; Cole et al., 2013, 2015; Wright et al., 2013b; Watts et al., 2015), reduced fecundity (e.g. Wright et al., 2013; Cole et al., 2016; Oganowski et al., 2016; Sussarellu et al., 2016) and increased mortality (e.g. Lee et al., 2013; Kaposi et al., 2014; Cole et al., 2015; Oganowski et al., 2016). However, all of these studies have used microplastic concentrations above those reported in the environment (Lenz et al., 2016). This study used microplastic concentrations ranging from 100 to 1000 particles ml^{-1}. These concentrations are also several orders of magnitude higher than those currently reported in the environment. These high concentrations were selected in order to be comparable to other studies and to be high enough for ingestion in the sea squirts to be seen. It was hoped that with high concentrations, the process of ingestion and any translocation as well as any toxicity could be easily identified. In order, to see if *C. intestinalis* are ingesting plastics at environmentally relevant concentrations, sea squirts were collected from the environment and analysed for plastic ingestion.

C. intestinalis were collected from settlement plates, placed in Milbay Marina Plymouth over the summer of 2016. An average of 0.8 plastic like particles per individual were recorded in these sea squirts. This is the first time ingestion of

plastic-like particles has been recorded in ascidians collected from the marine environment. Unfortunately, the particles seen were not able to be identified as plastics due to the limitations of the FTIR available. However, in line with other studies assessing microplastic ingestion in marine organisms fibres were the most common particle. In this study, fibres made up 71 % of the total particles seen, this is similar to the findings of fibres making up 68 % of plastics seen in euphuasiids (Desforges et al., 2015) and 68.3 % in fish collected from the North Sea (Lusher et al., 2016). This finding is not surprising as fibres are possibly the most common plastic shape in the marine environment (Erikson et al., 2014). This result of fibres being the most common particle in *C. intestinalis* collected from the marine environment provides further evidence that plastic ingestion by ascidians requires further research. Previous studies have concluded that fibres may have greater toxicity than microbeads (Au et al., 2015) and have the potential to cause more gut blockages due possible entanglement in intestinal tracts (Murray and Cowie, 2011; Cole et al., 2013). This study was unable to ascertain any toxicity arising from microfiber ingestion; therefore, this should be an area of future research.

Plastics are increasingly abundant in marine habitats, with recent estimates ranging from 15 to 51 trillion pieces with a buoyant load of plastics less than 200 mm amounting to between 93, 300 and 236, 000 million tonnes (van Sebille et al., 2015). However, there appears to be a missing fraction in the oceans. The global annual production of plastics is now estimated to be 300 million tonnes (Plastics Europe, 2016), of which 10% is predicted to enter the oceans (Thompson et al., 2004). Estimates based on net samples and trawls appear to reach only 1-10% of the buoyant plastics predicted to enter the oceans (Clark et

al., 2016). This is likely to be due to a number of reasons. Firstly, sampling tends to be carried out on surface waters and shallow sediments due to technical difficulties in sampling deep water and deep sediments. Secondly, smaller plastics may be missed using current sampling techniques (Cozar et al., 2014). Thirdly, smaller plastics fragment at a faster rate leading to an increase in the amount of plastic currently unable to be detected (Andrady, 2015). Finally, biological interactions, such as biofouling or entanglement with planktonic aggregates and ingestion may lead to plastics being undetected or transported from the surface layers (Clark et al., 2016). This missing fraction may be posing a greater threat to wildlife than is currently recognised and understanding any biological interactions leading to transformations is important to assess the impacts plastics may have upon a range of habitats. Ingestion of microplastics has now been reported in over 300 species (Galloway et al., 2017). However, few studies have looked at the impacts of ingestion upon the plastics.

The body of work presented here adds to the growing evidence that marine plastics are being transported within the marine environment via ingestion and subsequent egestion processes. Currently sampling efforts are only reporting 1-10 % of the total buoyant plastics thought to be entering the oceans (Clark et al., 2016). This may be as a result of biological processes, such as those seen in this study, removing plastics from surface waters. Present estimates of the plastic load within oceans tends to focus on surface plastics and transportation through oceanic processes. A greater understanding of the ways in which biological processes are transporting plastics throughout the marine environment may lead to increases in estimates of plastic concentrations in marine habitats.

The transportation of plastics to benthic habitats may also pose a risk to economically important species. A number of economically important fishery species, such as mussels and oysters, are benthic feeders. An increase in plastics in these habitats may lead to increased ingestion by these species, which could then be transferred to humans. Mussels collected from a mussel farm in Germany and oysters taken from supermarkets in France have been found to contain 0.36 and 0.47 plastics g^{-1}, respectively (Van Cauwenberghe and Jansssen, 2014), suggesting consumption of marine plastics by humans is already occurring. The toxicity of these plastics to humans is currently unknown; however, it is likely that bioaccumulation of associated pollutants, such as bisphenol A may have implications for human health. Bisphenol A has been shown to have adverse effects such as the onset of obesity and cardiovascular disease in humans (Melzer et al., 2012; Cipelli et al., 2013, Galloway, 2015). It is likely that other associated pollutants will also have adverse impacts, suggesting that consumption of contaminated seafood may have implications for human health. In light of the findings presented in this work and those discussed, further studies investigating marine plastics should now focus upon biological processes associated with marine plastics. Studies should be carried out with the intention of gaining a greater understanding of how and where plastic related biological processes are occurring along with the implications for marine wildlife and human populations.

Appendix

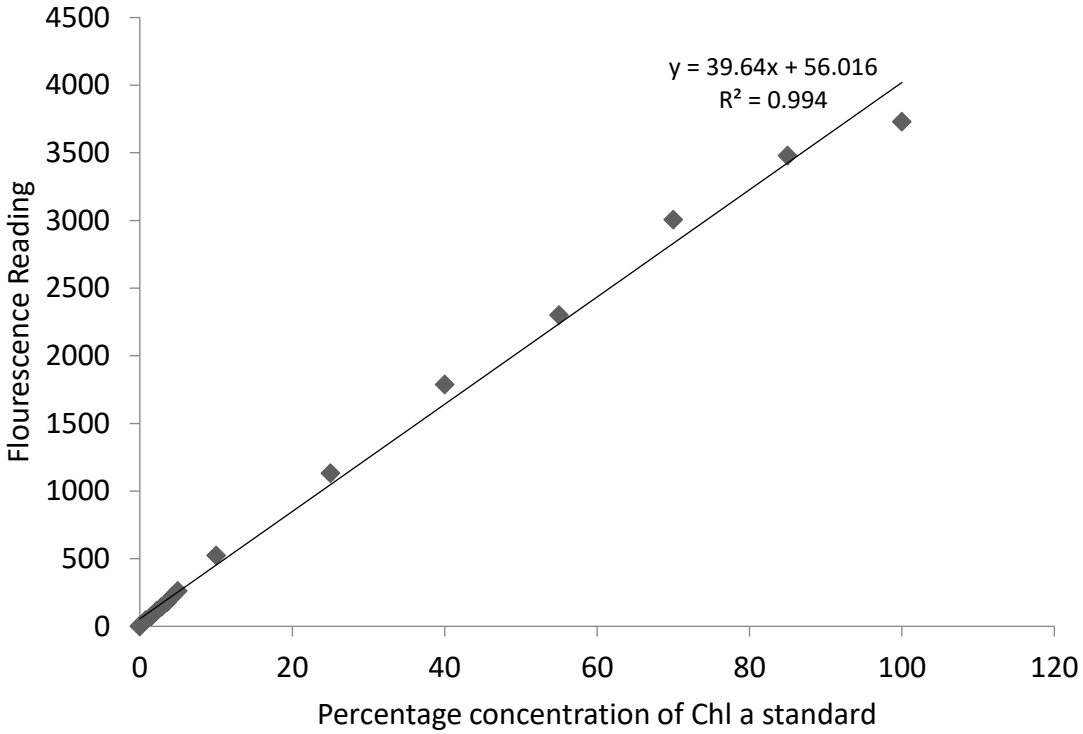

Figure 6.1: Reference graph for Chlorophyll a. A dilution series of standard chlorophyll a with corresponding fluorescent reading

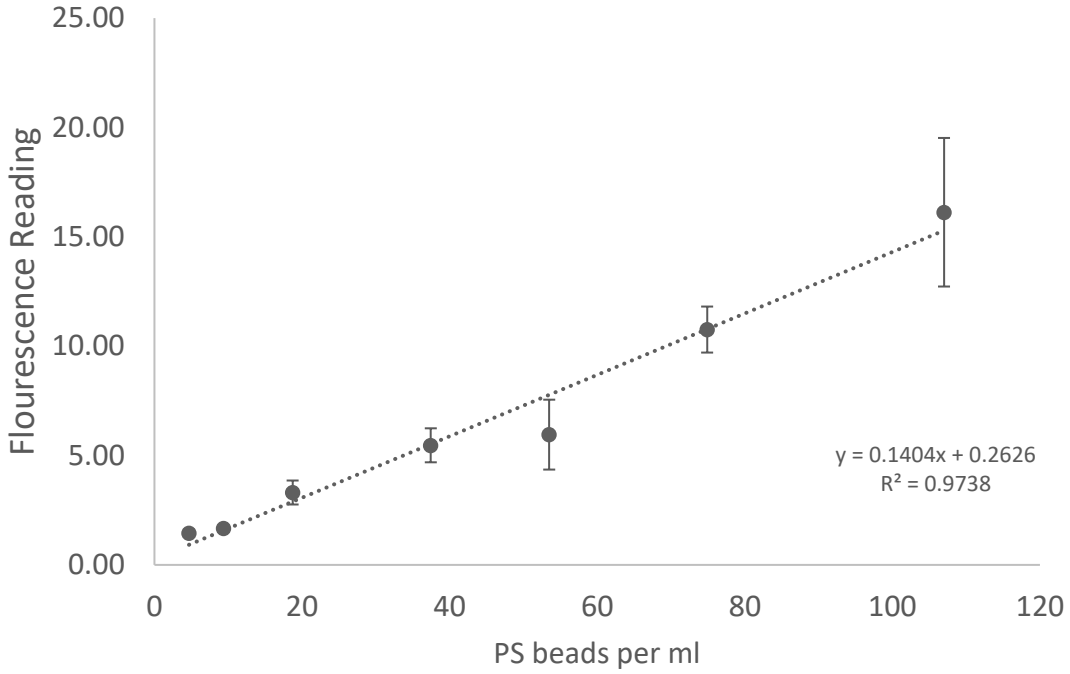

Figure 6.2: Reference graph for Polystyrene microplastic (PS MP). PS MP bead concentration within water against the corresponding fluorescent reading

.

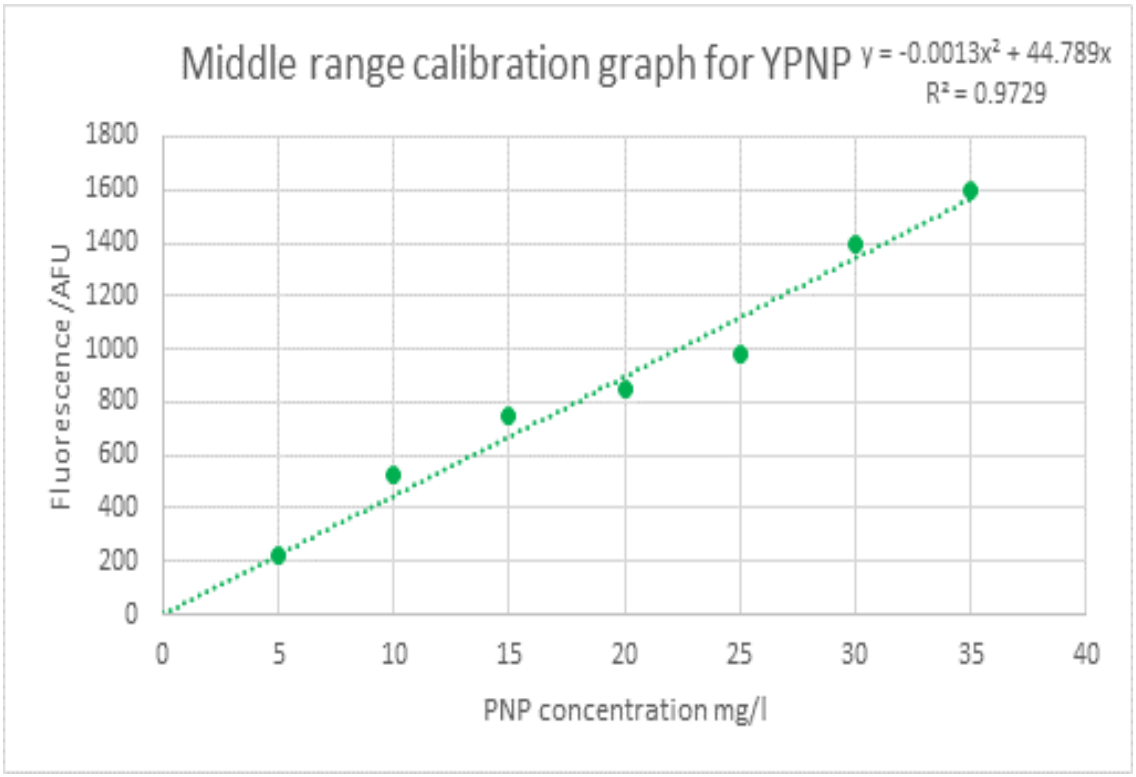

Figure 6.3: Reference graph for Polystyrene nanoplastic (PNP). PNP bead concentration within water against the corresponding fluorescent reading (Figure provided by C. Liddle).

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