Assessing the threat of microplastics to commercial oysters, particularly those of the genus *Crassostrea*

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Assessing the threat of microplastics to commercial oysters, particularly those of the genus *Crassostrea*

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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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Signature: ……..

Abstract

Current statistics show that China is the largest producer of plastics in the world, contributing to almost 30% of production worldwide (Plastics Europe, 2017). In particular, mega-cities in China connected with the Pearl River Estuary contribute to much of this production, and locations in the Estuary's proximity are potential sites for plastic pollution. In this thesis, the hypothesis that *Crassostrea hongkongensis* oysters cultured in Deep Bay, Hong Kong would be susceptible to microplastic uptake was tested. Extensive sampling was conducted across the bay through the collection of oysters, water and sediment from five selected sites (rafts). Follow up analysis of samples revealed that an average of 15.1 ± 6.1 microplastics per individual were present within the oysters, and oysters situated in the outer part of the bay took up more microplastics compared to those situated in the inner part of the bay. The numbers and types of microplastics quantified in sediment and water samples across sites did not correspond to the number of microplastics quantified in *Crassostrea hongkongensis* specimens across sites, suggesting that there was an element of selection during the biological uptake of these particles, and this was potentially influenced by particles' properties such as size, shape and polymer type.

Having established that wild *Crassostrea hongkongensis* in Deep Bay were taking up microplastics, an experiment was set up to investigate whether a genetically similar oyster species, *Crassostrea gigas,* would exhibit selective uptake up microplastics according to plastics' size, shape or polymer type, and whether microplastic exposure would induce biological responses within the oysters. Eight microplastic types of various polymers, sizes and shapes were supplied in equal concentrations to *Crassostrea gigas* oysters at a final concentration of 100 microplastics mL^{-1}, where

they were exposed for a 24 hour period. Findings revealed that microplastic polymer type and size did indeed influence uptake (Pr (>Chi)=0.034), and oysters readily took up 0.29% of 8-30 μm polyethylene beads and 0.31% of 115-156 μm polyvinyl chloride fragments supplied when exposed solely to plastics; oysters exposed to a combination of algae and plastics (same microplastic concentrations) took up 0.25% of the former plastic type, and 0.32% of the latter plastic type supplied. A post-hoc Tukey test confirmed that uptake of these two plastic types were significantly higher ($p<0.05$) than with other plastic types.

Crassostrea gigas oysters were then exposed to polyethylene beads (8-30 μm) at a concentration of 100 microplastics mL^{-1} for 14 days and subsequent biological assays were used to identify potential biological impacts upon exposure. Despite the tentative evidence of a small increase in phagocytosis activity and decreases in food assimilation and metabolism, no significant ($p<0.05$) impacts on oxidative stress, DNA damage or changes to oxygen consumption and feeding behaviour were found as a result of microplastic uptake.

The research in this dissertation shows that although the size and polymer type of microplastics affects uptake, exposure to beads at low concentrations does not cause adverse biological impacts or health effects. Since oysters tend to be contaminated with microplastic fibres in the natural environment, and the microplastics used for testing exposure-induced biological impacts consisted solely of microplastic beads, they may be subject to higher levels of oxidative stress and other toxic effects beyond those reported in this study.

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List of Abbreviations

ATP……adenosine triphosphate

BaP……benzo[*a*]pyrene

CFCs……chlorofluorocarbons

CYP1A……cytochrome P450 1A

DDE……dichlorodiphenyldichloroethylene

DDT…… dichlorodiphenyltrichloroethane

DI……deionised water

DMSO……dimethyl sulfoxide

DNA……deoxyribonucleic acid

DW……dry weight

EDTA……ethylenediaminetetraacetic acid

EPS……expanded polystyrene

FTIR spectroscopy……fourier-transform infrared spectroscopy

GLM……general linear model

HDPE……high-density polyethylene

IDH……isocitrate dehydrogenase

LDH……lactate dehydrogenase

LDPE……low-density polyethylene

LPO……lipid peroxidation

MDA……malondialdehyde

NBT…… nitroblue tetrazolium

NR……neutral red

OD……8 oysters cultivated from the bottom of a rope in Deep Bay

ODS-2……octadecyl silane

OS……8 oysters cultivated from the top of a rope in Deep Bay

O_2^-……oxygen free radicals

PA……polyamide

PAHs……polycyclic aromatic hydrocarbons

PBS……phosphate buffered saline

PCBs……polychlorinated biphenyls

PERMANOVA……permutational multivariate analysis of variance

PET……polyethylene terephthalate

PHE…… phenanthrene

POPs……persistent organic pollutants

PP……polypropylene

PS……polystyrene

PVC……polyvinyl chloride

ROS……reactive oxygen species

RPM……revolutions per minute

SD……standard deviation

SE……standard error

SOD……superoxide dismutase

TBARS……thiobarbituric acid reactive substances

WW……wet weight

7D……7 days

14D……14 days

24H……24 hours

Chapter 1: An introduction to microplastics

1.1 Defining ‘plastics’ and ‘microplastics’

The first account of the use of polymers (materials composed of simple recurring units) was in 1600 BC, when ancient Mesoamericans used latex from rubber trees to make rubber (Hosler et al., 1999). Other natural polymers such as waxes, horns and resins were in use up until the 19th century, when the first synthetic polymers were developed, and expanded throughout the following century (Andrady and Neal, 2009). These developments led to the production of the first plastics and the onset of mass production in the 1950s (Barnes et al., 2009; Mattsson et al., 2015).

Plastics are essentially synthetic polymers derived from oil, coal, natural gas and biomass sources (Shah et al., 2008). These polymers soften upon heating and can be moulded (and re-moulded) into specific shapes and sizes as thermoplastics (e.g. films, fillers and floats) or moulded once only as thermosets (e.g. resins and foams) (GESAMP, 2015). Hundreds of types of plastic types are produced worldwide (Geyer et al., 2017), but six main types of plastics – high-density polyethylene (HDPE), low-density polyethylene (LDPE), polyvinyl chloride (PVC), polystyrene (PS), polypropylene (PP) and polyethylene terephthalate (PET) comprise over 90% of global plastic production and are used in a wide range of commercial and industrial applications (Table 1.1) (Andrady and Neal, 2009; Geyer et al., 2017; Rochman et al., 2013b). Due to their desirable properties (e.g. chemical/light resistant, used at a wide range of temperatures, durable, versatile), worldwide demand of plastics has reached an unprecedented level at 335 metric tonnes in 2016 (Plastics Europe, 2017).

Table 1.1: Common uses of plastics. Adapted from (Andrady and Neal, 2009; Geyer et al., 2017).

Type of plastic	Common Applications	2015 Primary Production (million metric tonnes)
Polypropylene (PP)	Film pouches, crates, container caps, bowls, kettles	68
Low-density polyethylene (LDPE)	Carrier bags, plastic wrap	64
High-density polyethylene (HDPE)	Bottles, jugs	52
Polyvinyl Chloride (PVC)	Plastic wrap, buildings, furniture	38
Polyethylene terephthalate (PET)	Bottles, clothing	33
Polystyrene (PS)	Cups, trays, packaging, insulation	25

Plastics can be classified into different size-classes (e.g. mega-, macro-, meso-, micro- and nano-), and there is currently no formal agreement within the scientific community on the upper size limits of the different-size classes of plastics (GESAMP, 2015; Mattsson et al., 2015; Romeo et al., 2015). Most publications and rports, however, have adopted an upper-sized limit of <5 mm to define 'microplastics' (Arthur et al., 2008; Moore, 2008; NOAA Marine Debris Program, 2015; Wright et al., 2013a). The lower size limit of microplastics has also not been defined strictly, however, the International Union of Pure and Applied Chemistry (IUPAC) supports a lower size limit of 100 nm for microparticles (Vert et al., 2012). Even if microplastics at lower observation limits can be magnified sufficiently, these observations are constrained by the ability to make distinctions between synthetic particles from other particles using colour, shape, shininess, texture, and other visual cues; making observations at higher magnifications also require more time to process (Ballent et al., 2016). These constraints primarily apply to field-based studies (chapter 2 of this thesis), however,

they are less evident in lab-based studies when microplastics can be stained with fluorescent dyes (the method used in chapter 3 and 4 of this thesis) and enumerated under a fluorescent microscope. As such, the lowest size category of microplastics enumerated in chapter 2 were prescribed as <100 µm, and the smallest size range of microplastics enumerated in chapters 3 and 4 were 8-12 µm.

Microplastics are produced in one of two ways: through manufacturing as scrubbers, industrial pellets or microbeads (primary microplastics), or through fragmentation and degradation from larger plastics via mechanical, oxidative and/or biological processes (secondary microplastics), (Browne et al., 2011; Hidalgo-Ruz et al., 2012).

1.2 Sources of microplastic debris to the marine environment

Primary plastic pellets of ~5 mm or <0.5 mm sizes are used mostly in the production of larger plastics and they can be lost into the environment through transportation, handling or processing (Derraik, 2002); microbeads (~0.25 mm) are used mostly as industrial abrasives and exfoliants in cosmetic products (Thompson, 2015). The former two products have been widely reported to be susceptible to industrial spillage (Blight and Burger 1997) and much of the latter is reportedly discharged into the sea via wastewater (Thompson, 2015). Industrial abrasives contain, among other materials, polyester and PS microplastics which are used to remove paint on surfaces, or to clean equipment by air-blasting (Gregory, 1996). A recent study showed that the contents of cosmetic products typically contain between 0.05 and 12% PE microplastics and >70% of these plastics are over 450 µm in size (Gouin et al., 2015).

Microplastic debris is a form of marine debris, the latter of which has been defined as “any persistent solid material that is manufactured or processed and directly or indirectly, intentionally or unintentionally, disposed of or abandoned into the marine environment or the Great Lakes” (National Oceanic and Atmospheric Administration US Department of Commerce, 2018). The first reports of microplastic debris were published in the 1970s, and observations were made in subtropical gyres located in the North Atlantic (Carpenter and Smith, 1972; Gregory, 1977). In the same decade, high concentrations of plastic resin pellets were found on beaches in New Zealand close to plastic producing/processing facilities with up to 40,000 pellets/m in some areas (Gregory, 1978). These reports predicted that plastic production would increase in the years to come and that this would likely lead to more plastic debris in the oceans and seas. Current statistics show that the annual production of plastics has increased significantly from 1.5 million tonnes in the 1950s (when mass production of plastics began) to 280 million tonnes in 2011 (Barnes et al., 2009; Depledge et al., 2013; Gourmelon, 2015; Plastics Europe, 2017; Thompson et al., 2004), and total global plastic waste was estimated to be 99.5 million tonnes in 2010, with 4.8 to 12.7 million tonnes entering the ocean that year (Jambeck et al., 2015). However, there are only a handful of studies which have reported temporal trends of microplastic debris, and some studies have described increases in abundance (Goldstein et al., 2012; Thompson et al., 2004), decreases (Morét-Ferguson et al., 2010; van Franeker and Law, 2015), whilst other studies have described “missing/lost” or less-than-expected levels of microplastic accumulation (Cozar et al., 2014; Katsnelson, 2015). In the case of the latter-most descriptions, we now know that this is largely due to the gradual displacement of microplastics from the sea surface through sinking and/or uptake by marine organisms – in fact, a recent study suggested that 99.8% of plastic waste

entering the oceans will eventually make its way to the sea floor (Koelmans et al., 2017). If we consider this figure in the context of the estimated 15 to 51 trillion microplastic particles weighing between 93 and 236 thousand metric tonnes worldwide (Sebille et al., 2015), this equates to a worryingly significant number of microplastics.

Plastic waste is released into the environment through marine-based sources such as equipment or processes involved in fishing, aquaculture and shipping, and land-based sources such as wastewater effluent, run-off and rivers (Figure 1.1) (Desforges et al., 2014; Gouin et al., 2011). It is believed that the majority of waste comes from land-based sources as a result of inappropriate waste management (e.g. not transporting waste properly, or not burying waste properly at landfill sites), littering, illegal dumping, inadequate disposal (Andrady, 2011; Barnes et al., 2009; GESAMP, 2010) and the subsequent distribution of plastics into the ocean by wind, currents, runoff and effluent (Duis and Coors, 2016; Magnusson et al., 2016). Rivers are one of the major transportation pathways of microplastics, with the 10 top-ranked catchments (ranked by mismanaged plastic waste generated per year) transporting 88-95% of the world's global mismanaged plastic waste, and discharging 410,000-4,000,000 tonnes of plastic waste into seas worldwide every year (Schmidt et al., 2017). In the study, the Pearl River ranked 7th of the 10 catchments, and generated 2,515,374 tonnes of mismanaged plastic waste per year in the catchment area.

Synthetic polymers, tetxtiles and fibres constitute a significant proportion of microplastic debris in oceans and shorelines across the globe (Browne et al., 2011). A study which collected 1,393 1-litre grab samples from coastal and open ocean sites revealed that over 91% of plastic particles consisted of fibres (Barrows et al., 2018).

Of the microplastics exported from European rivers into seas, it has been modelled that 42% consists of synthetic polymers, 29% consists of plastic-based textiles derived from laundry, synthetic polymers, and 19% consists of plastic fibres from household dust (et al., 2017). Another study projected that the laundry of synthetic textiles contribute to ~35% of primary microplastics to the world's oceans (Boucher and Friot, 2017). This is likely linked to the plenteous amounts of plastics used in apparel – estimated at a global total of 69.7 million tonnes in 2010, or an average of 11 kg used per person globally (FAO/ICAC, 2013) – and the subsequent shedding and release of fibres from consumers' washing/laundry, whereby over 700,000 fibres can potentially be released from an average 6 kg wash load of acrylic fabric (Napper and Thompson, 2016). it follows that microplastic fibres are amongst the most commonly found microplastics in oceans and shorelines across the globe.

Another common form of microplastic debris are microbeads. In the same study mentioned above, which modelled the export of microplastics from European rivers into seas, 10% consisted of microbeads from personal-care products (Siegfried et al., 2017). A study of six brands of facial scrubs estimated that between 4,594 and 94,500 microplastic beads can be released into wastewater per use (Napper et al., 2015) ; in the USA alone, 200 tonnes of microbeads are used annually and 50% pass through sewage treatment into the ocean (Gouin et al., 2011).

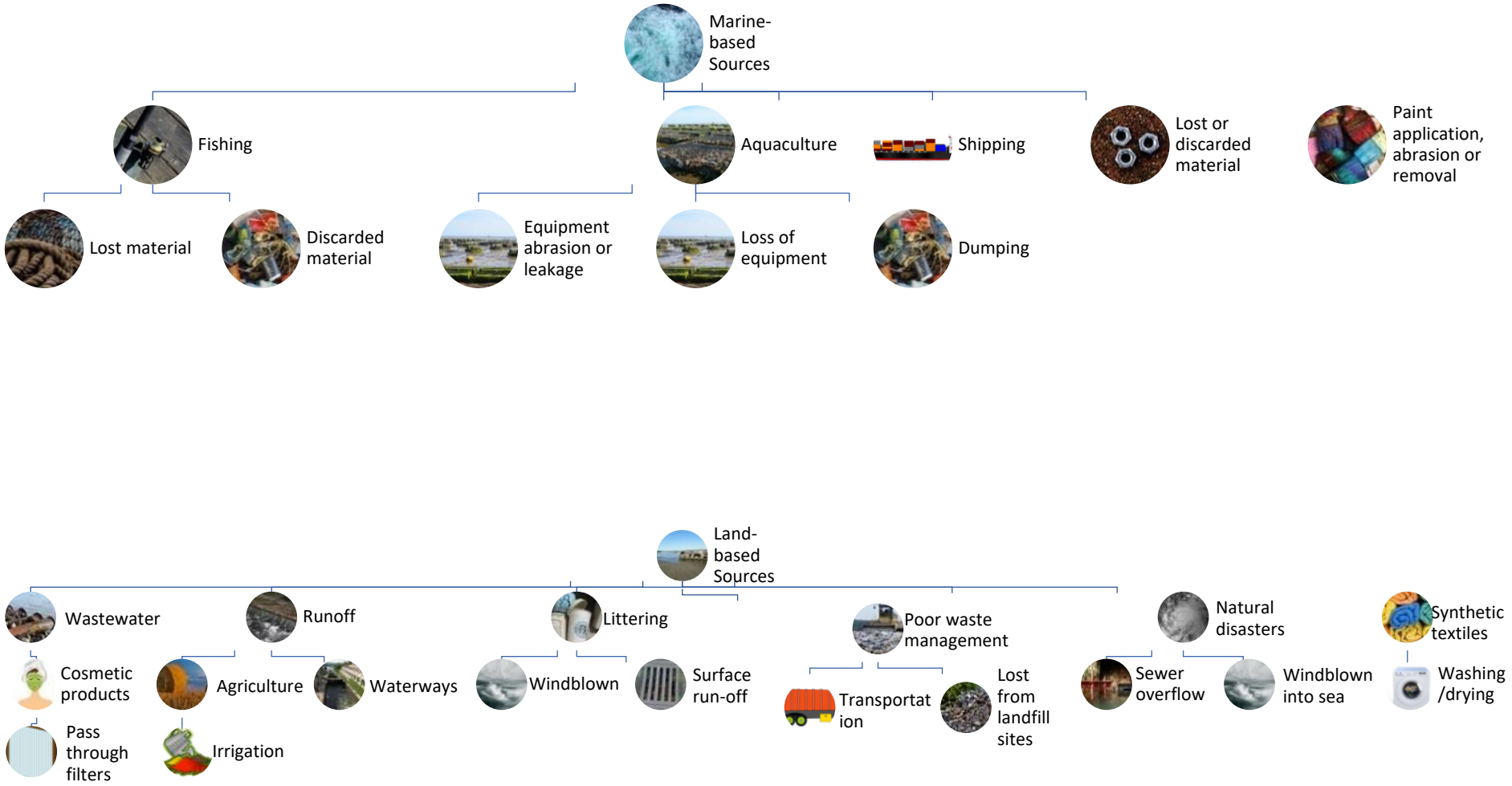

Figure 1.1: Land based vs marine based sources of microplastics (Andrady, 2011).

1.3 Distribution and fate of microplastics

Once in the sea, the distribution of microplastics are dependent upon several factors including the influence of wind (Desforges et al., 2014), currents (including gyres) and wave action (Lusher, 2015), sediments (Uncles and Stephens, 2010), incorporation with marine aggregates (Martin et al., 2017; Porter et al., 2018), weathering processes (such as UV and mechanical fragmentation) (Morishige et al., 2007; Thompson et al., 2004), biofouling (Cozar et al., 2014; Kooi et al., 2017), size of plastic (Kooi et al., 2017) as well as the density and surface area of plastic polymers (Avio et al., 2017). High concentrations of microplastics tend to be transported via wind, waves and currents to distinct accumulation zones which consist mostly of ocean margins and gyres (Eriksen et al., 2013). Gyres are formed from the interplay between surface/geostrophic currents and wind (Maximenko et al., 2012; Moore, 2008), and the oceans' five main gyres - the Indian Ocean gyre, the North and South Pacific gyres, and the North and South Atlantic gyres - make up 40% of the sea's surface (Eriksen et al., 2014); amongst these gyres, the North Pacific gyre acts as the biggest plastic sink due to comparatively high input sources in East Asia (Jambeck et al., 2015). Besides accumulating in gyres, microplastics can also suspend in the water column (Lattin et al., 2004), surface waters (Zhao et al., 2014), and deep sea (Woodall et al., 2014); and accumulate in coastal waters (Costa and Barletta, 2015), estuaries (Yonkos et al., 2014), rivers (Mani et al., 2015), beaches (Laglbauer et al., 2014) and benthic sediments (Castañeda et al., 2014). In order to better predict the distribution of plastics in the oceans, models simulating environmental conditions and parameters can be used, and evidence has shown that in addition to being found in surface waters, many microplastics sink and spread through the water column (Woodall et al., 2014).

Other models have also shown that most microplastics are concentrated at intermediate depths, with smaller and denser particles settling faster without resurfacing, which is due primarily to the progressive formation of biofilm on microplastics (Cozar et al., 2014; Kooi et al., 2017). Tentative evidence from observations made in bottom water and benthic sediments along the Irish continental shelf has shown, however, that over time, microplastic deposition increases as they move through the water column and onto the sea floor, where microplastics' chances of further fragmentation are reduced due to the low energy environment, cold temperatures and lack of UV radiation, leading to longer residence times at deeper depths (Martin et al., 2017). Unlike natural polymers (biopolymers) such as plankton-produced chitin, which easily degrades into CO_2 and H_2O in the ocean in a matter of years (Brandon et al., 2016; Poulicek and Jeuniaux, 1991), plastics are resistant to biodegradation but breakdown through mechanical action over hundreds to thousands of years (likely longer in deep sea and non-surface polar environments) into fragments and microplastic debris (Barnes et al., 2009).

Relative to the specific gravity of seawater (~1.025), the specific gravity of microplastics can range from 0.91 (LDPE) to 1.38 (PVC) (Andrady, 2011). Upon entering marine waters, microplastics with a higher density and a higher specific gravity tend to sink toward benthic substrates and microplastics with a lower density and lower specific gravity tend to float on the water surface (Browne et al., 2011). In waters with turbulence or biofouling, positively buoyant microplastics can become negatively buoyant and sink over a timescale of weeks to months (Lobelle and Cunliffe, 2011); a recent modelling study estimated that 99.8% of microplastics entering the ocean since 1950 had settled below the ocean surface layer by 2016,

with 9.4 million tonnes of additional microplastics settling per year (Koelmans et al., 2017). Another recent model based upon realistic environmental parameters (such as water density, temperature and viscosity) predicted that all microplastics can settle due to biofouling, with most microplastics sinking and concentrating at intermediate depths and a relatively low number of microplastics remaining at the surface (Kooi et al., 2017). Additionally, the model predicted a size selective removal of smaller particles first (<10 μm) and larger particles settling last. This size selective removal process is in line with the observations of (Cozar et al., 2014) - that size-selective sinks involve a combination of nano-fragmentation of microplastics, transfer to the interior of the ocean via food webs and ballasting, and other processes yet to be discovered.

In addition to increased sinking rates due to biofouling, the sinking of microplastics may also be accelerated through their incorporation with organic-rich aggregates (composed of fecal pellets, phytoplankton, larvacean houses, microbes and particulate organic matter), a process which has been evidenced to occur within the top 2 m of the water column (Zhao et al., 2017). A recent laboratory study reported an increase in sinking rates by 818 m day^{-1} and 916 m day^{-1} for polyethylene beads (9-11 μm) and polyamide fibres (10×50 μm) respectively when incorporated with marine snow, and it was suggested that this provided an important pathway by which plastics are rapidly transported downward (Porter et al., 2018). Indeed, this may account for microplastics found in deep sea environments. In one study in deep-sea (1000 m – 3500 m) sediments and coral samples in the Atlantic Ocean, Mediterranean Sea and Indian Ocean, samples were found to contain up to four times as many microplastics as those collected from sea-surface waters (Woodall et al., 2014). The authors reported concentrations of microplastic fibres (most were 2-3 mm rayon and polyester

fibres) found in sediment to range from 1.4 to 40 pieces 50 mL^{-1}, and observed that plastics had also accumulated on the surface of octocorals. Microplastics have also been found in deep sea organisms, which mostly intake food from organic detritus aggregates (i.e. marine snow) from the euphotic zone and can take up microplastics that resemble the size of marine snow in the mid-Atlantic and Indian Ocean (Taylor et al., 2016). Fibrous microplastics consisting of five different materials (including polypropylene, a buoyant polymer) were found in nine organisms from a depth of 334-1,783 m in the former location and 954-1,062 m in the latter location; deposit, predatory and detritivore feeders took up more microplastics than suspension feeders took up less microplastics compared to, implying their greater vulnerability to microplastic pollution (Taylor et al., 2016).

Analysis of surface sediment samples collected from 16 sites within the Lambert Channel and Baynes Sound, British Columbia, Canada, a premier area for *Crassostrea gigas* oyster cultures, revealed densities of up to 25,000 microplastic beads/kg dry sediment (Kazmiruk et al., 2018). An average of 700,000 microplastic particles/km^2 were found in surface water samples collected in San Francisco Bay, an urban estuary in California (Sutton et al., 2016). Surface water samples collected from four estuarine tributaries in Chesapeake Bay, U.S.A contained up to 560 g microplastics/km^2 and correlations between statistically significant ($p<0.05$) positive correlations were found between concentrations of microplastics and population density (Yonkos et al., 2014).

1.4 Impacts of microplastic pollution

Besides distributing through the environment, microplastics can also be taken up by a wide range of marine species (Cole et al., 2013; Van Cauwenberghe and Janssen, 2014; Wright et al., 2013a) – over 280 species (including those with important ecosystem roles such as primary production, filter feeding, detritus feeding) across trophic levels are known to consume microplastics (Galloway et al., 2016) and the number reported has doubled since 1997 (Kühn et al., 2015), likely due to a large increase in research effort; affected organisms reported from field-based studies include microscopic plankton (Beer et al., 2018), mesopelagic fish (Wieczorek et al., 2018), shrimp (Devriese et al., 2015), lobsters (Murray and Cowie, 2011) and whales (Fossi et al., 2016). Amongst bivalves, plastic ingestion has been recorded in specimens collected in Canada (Davidson and Dudas, 2016), China (Li et al., 2015), the UK (Cole and Galloway, 2015) and Germany (Van Cauwenberghe and Janssen, 2014).

Due to the small size of microplastics (<5 mm), they are readily taken up by lower trophic organisms, which exert limited selectivity between microplastics and normal food particles of ingestible size (Moore, 2008). Organisms are particularly susceptible to this uptake when the size range of microplastics overlap with the preferred particle size of animals' feeding habits (Galloway and Lewis, 2016). Through the application of dissection, digestion and density separation methods, scientists are able to enumerate the number/type/size of microplastics within organisms (Lusher et al., 2016). Laboratory-based exposure studies have shown that when exposed to microplastics, specimens are potentially able to discriminate between organic and inorganic particles (e.g. microplastics) through innate selection

mechanisms, and regurgitating them as an acute physiological reaction (Ory et al., 2018; Powell and Berry, 1990). When copepod *Calanus helgolandicus* specimens were exposure to 20 μm PS beads (75 microplastics mL^{-1}) in a lab-controlled setting for 24 hours, they altered their feeding strategy to take up smaller sizes of algal prey (maximum particle size taken up reduced from 17 μm to 14.8 μm), which was cop. Despite using this altered feeding strategy, however, microplastics were still readily taken up, and the authors reported that specimens ingested 11% fewer algal cells and 40% less carbon mass, (Cole et al., 2015). In another feeding study, Copepod *Acartia clausi* specimens pre-conditioned to feed on *Thalassiosira fluviatilis* selectively took up 14 μm *Thalassiosira fluviatilis* algae particles over 20 μm latex spheres and 5 μm *Thalassiosira pseudonana* algae particles, suggesting that pre-conditioned individuals can exhibit complex grazing behaviours which optimises their behaviour to increasing uptake efficiency (Donaghay and Small, 1979). Besides this observation of selective uptake, the authors found that copepods were able to reject captured microplastics, an observation shared by (Powell and Berry, 1990) on their experiments with the copepod *Eurytemora affinis*; this was seen as an effective feeding strategy to reduce copepods' uptake of microplastics. The selective uptake of particles is also evident in oysters - when feeding, oysters capture particles by generating a current by lateral cilia on ctenidial filaments which flow into an inhalant siphon, and these particles are retained by a layer of mucus on filaments in the ctenidium (Ward and Shumway, 2004). Oysters also possess the ability to sort through particles based on their nutritive content, size, mass and other related factors; they can also reject unfavourable particles as pseudo-faeces, but studies have produced mixed results as to the effectiveness of pre-ingestive sorting (Bernard, 1974; Cognie et al., 2003; Garrido et al., 2012; Pastoureaud et al., 1995).

In any case, given that bivalves can retain 3-4 μm particles with 100% efficiency and 1 μm particles with 50% efficiency, and that microplastics in these size ranges occur in coastal environments, it is conceivable that microplastics are captured and retained by bivalves in polluted environments (Gosling, 2007).

A field study supports the idea that lower trophic organisms are particularly vulnerable to the uptake of microplastics – microplastics ranging between 4 to 2,400 μm in size were found in zooplankton samples trawled in the South China Sea, and these consisted mostly of polyester fibres (Sun et al., 2017). The authors opined that carnivorous zooplankton are more susceptible to microplastic uptake given their ability to take up microplastics both directly from seawater (by confusing them with prey) and indirectly via contaminated lower trophic level organisms. Filter feeders, particularly megafana, have also been reported to be particularly vulnerable to microplastic uptake and potential effects thereafter given that they filter copious amounts (up to hundreds of thousands of cubic metres) of water on a daily basis to obtain adequate nutrition (Germanov et al., 2018); they are also long-lived, with toxins associated with microplastics potentially accumulating in their systems over decades and transferred or offloaded from mothers to offspring during their lifetime (Lyons et al., 2013; Rochman et al., 2014). Due to their size, filter-feeding megafauna are capable of ingesting larger plastics - plastics ranging from 1 mm to 17 cm were found in a humpback whale *Megaptera novaeangliae* stranded on a sandbank in the Netherlands, and these consisted of PE, PP, PVC, PET and nylon plastic types (Besseling et al., 2015). Another factor for the vulnerability of filter-feeding megafauna to microplastic ingestion are their habitats – the habitat ranges of the manta ray *Mobula alfredi*, the whale shark *Rhincodon typus* and the fin whale *Balaenoptera physalus* reportedly

overlap regions with high levels of buoyant microplastic pollution including the Mediterranean Sea, the Gulf of Mexico, the Bay of Bengal and the Coral Triangle (Germanov et al., 2018). These levels of microplastic pollution were quantified using predictive mapping from (Sebille et al., 2015), a paper within which the authors defined accumulation zones with microplastic concentrations greater than 106 particles km^{-2}.

Situated at the upper part of the food web, higher-trophic organisms also possess the ability to take up microplastics directly or indirectly via the ingestion of contaminated prey, although this is poorly understood for higher-trophic organisms due to a lack of empirical evidence, unsuitability for monitoring and ethical constraints for laboratory experiments (Bravo Rebolledo et al., 2013; Nelms et al., 2018). Despite this shortfall, tentative evidence has shown that trophic transfer represents an indirect pathway for higher-trophic organisms and species that feed upon whole prey – an average of one to four 1.5 mm (mean length) and 2 mm (mean length) microplastics were found in half of the sampled faeces of the grey seal *Halichoerus grypus* and in one third of sampled Atlantic mackerel *Scomber scombrus* the seals fed upon respectively (Nelms et al., 2018). Amongst the types of microplastics found by the authors, ethylene propylene was the most common in both sample types and this indicated a clear trophic-transfer link between microplastics found in seals and in the fish they preyed upon.

Some laboratory studies have tested for, and demonstrated the transfer of microplastics across trophic levels. Mesoplankton *Marenzelleria* spp. and copepods pre-exposed to 10 µm PS microplastics (<2000 particles mL^{-1}) for 12 hours were fed to *Mysis spp*. shrimp, and microscopy observations revealed that all but one of the

shrimp subsamples had taken up microplastics as a result of feeding on contaminated mesozooplankton (Setälä et al., 2014). The transfer of microplastics has also been evidenced from *Carcinus maenas* crabs' ingestion of *Mytilus edulis* mussels pre-exposed to 0.5 µm PS microplastics (~1.03 million microplastics mL^{-1}) (Farrell and Nelson, 2013). Microplastics in crab haemolymph amounted to <0.04% of the concentrations mussels were exposed to, and 0.28% of the estimated number retained by mussels. Similarly, another laboratory study using the same two model species to test this pathway found that all but one crab sampled had microplastics in their foregut six hours after feeding upon mussels exposed to 10 µm PS microplastics (1,000 microplastics mL^{-1}) (Watts et al., 2015). However, key questions remain as to the implications this has for organisms across food chains, including whether microplastics may aggregate in number through and up food webs and with age of organisms, and whether microplastics may affect the health and mortality of prey species.

One of the first papers to examine organisms' potential health impacts from exposure to microplastics placed *Mytilus edulis* specimens in seawater with PS microplastics (3.0 µm or 9.6 µm; ~42.86 microplastics mL^{-1}) mixed in, and the authors observed that particles were taken up and translocated from the gut to the circulatory system within three days and persisted there for over 48 days; smaller particles were also shown to be more likely to accumulate in the tissues (Browne et al., 2008). Microplastics have been shown to affect feeding rates and energy reserves - significant reductions in algal feeding rates of the copepod *Centropages typicus* (Cole et al., 2013) and <50% reductions in energy reserves of the polychaete *Arenicola marina* (Wright et al., 2013a) were reported upon exposure to PS microplastics (7.3−30.6 µm; <3000 microplastics

mL^{-1}) and unplasticised PVC microplastics (230 µm; 1% and 5% by weight) respectively. There is evidence that this can be dose dependent - when the Asian green mussel *Perna viridis* was exposed to PVC microplastics (1-50 µm; 0-2160 mg L^{-1}) for two hours a day for 44 days, filtration and respiration rates and byssus production were shown to decrease in a dose dependent manner (i.e. higher doses exerting more deleterious effects) (Rist et al., 2016). However, the impact on feeding from microplastic exposure is not always evident in studies. For example, studies on the larvae of the sea urchin *Tripneustes gratilla* (10-45 µm PE microspheres, 1.0 g mL^{-1}) (Kaposi et al., 2014), *Crassostrea gigas* oyster larvae (70nm-20 µm PS beads, 1,000 microplastics mL^{-1}) (Cole and Galloway, 2015) and adult European flat oysters *Ostrea edulis (*0.6-363 µm PLA particles and 0.48-316 µm HDPE particles, 0.8 and 80 µg/L) (Green, 2016) all showed that ingestion was unaffected by microplastic exposure.

The effect on feeding rate has also been shown to have a knock-on effect on reproductive fitness - a 24 hour exposure on the marine copepod *Calanus helgolandicus* to PS microplastics (20 µm; 75 microplastics per mL) resulted in significant reductions in specimens' egg size and hatching success (Cole et al., 2015). Similarly, a study on *Crassostrea gigas* oysters' exposure to PS microplastics reported a significant decrease in oocyte number, oocyte diameter and sperm velocity (Sussarellu et al., 2016); and exposures to 0.05, 0.5 and 6 µm PS microplastics at concentrations of 0.1, 1, 10, and 20 µg mL^{-1} induced reduced growth rates, fecundity, lifespans and increased reproductive times for the rotifer *Brachionus* koreanus in a size dependent manner (i.e. smaller microplastics exerting more deleterious effects) (Jeong et al., 2016).

There is also some evidence that in addition to taking up microplastics, organisms may be susceptible to oxidative stress upon prolonged exposure - *Mytilus edulis* mussels exposed to HDPE microplastics (0-80 µm; 2.5 g HDPE-fluff L^{-1}) for 96 h were found to have accumulated microplastics in their lysosomal system, which resulted in the formation of granulocytomas (indicating a strong inflammatory response) and lysosomal membrane destabilisation (von Moos et al., 2012). Similarly, *Mytilus spp.* Mussels exposed to PS microplastics (2 and 6 µm; 32 µg L^{-1}) for seven days experienced significant increases in haemocyte mortality and decreases in catalase and lipid peroxidation activity; a further seven day depuration period led to increases in messenger RNA (mRNA) levels of superoxide dismutase (antioxidants), lysozyme (involved in immunity), pyruvate kinase (involved in digestion) and decreases in catalase activity (antioxidants) (Paul-Pont et al., 2016). A three day exposure of the European seabass (*Dicentrarchus labrax*) to microplastics (undisclosed polymer type) (1-5 µm; 0.26 and 0.69 mg L^{-1}) caused neurotoxicity through the inhibition of acetylcholinesterase, oxidative damage through increases in lipid peroxidation in the brain and muscles, and impacts on energy levels through effects on the energy-related enzymes lactate dehydrogenase (LDH) and isocitrate dehydrogenase (IDH) (Barboza et al., 2018). However, there have also been studies documenting an absence of oxidative stress in organisms exposed to microplastics. For example, though 27.3% of striped red mullet *Mullus surmuletus* caught in different ports around Mallorca Island were found with an average of 0.42 ± 0.04 microplastics $individual^{-1}$, the wild-caught fish showed no evidence of having experienced oxidative stress or cellular damage in their liver (Alomar et al., 2017). Similarly, lysosomal integrity, lipid peroxidation and deoxyribonucleic acid (DNA) damage in the mussel *Perna viridis* were non-significant following a three month laboratory exposure to PVC microplastics (0.1-1.0 µm; 0.125

g L^{-1}) (Santana et al., 2018); and oxidative stress, cell viability and phagocytic activity in *Mytilus edulis* mussels were non-significant following their <48 day exposure to PS microplastics (3.0 or 9.6 µm; ~42.9 microplastics mL^{-1}) (Browne et al., 2008).

When persistent organic pollutants (POPs) such as polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs) or dichlorodiphenyldichloroethylene (DDE) come into contact with particulate and dissolved organic matter, sediments or microplastics, they can sorb via absorption or adsorption processes largely driven by the hydrophobic properties of the organic compounds and their sorption capacity in seawater (Andrady, 2011; Mato et al., 2001; Thompson et al., 2004). Sorption can depend on several factors including the sorption capacity, length of polymer chains, partition ratios and binding strengths of the particle, organic matter or microplastic (Hartmann et al., 2017). Field measurements documenting the sorption of PCBs and PAHs over a 12-month period in San Diego Bay, California showed that sorption occurred on high-density polyethylene (25 ng g^{-1} PCB, 797 ng g^{-1} PAH), low-density polyethylene (34 ng g^{-1} PCB, 722 ng g^{-1} PAH), polypropylene (27 ng g^{-1} PCB, 122 ng g^{-1} PAH), polyethylene terephthalate (1 ng g^{-1} PCB, 14 ng g^{-1} PAH) and polyvinyl chloride (2 ng g^{-1} PCB, 27 ng g^{-1} PAH) microplastics (Rochman et al., 2013b). The results showed that PCBs and PAHs sorbed to HDPE, LDPE and PP microplastics at concentrations of an order of magnitude greater than PET and PVC due mainly to the latter two plastics' lower sorption capacities and the ability for PCBs and PAHs to reach equilibrium at a comparatively faster rate on these two plastics. Plastics may also play a role in transporting POPs in the marine environment, as was evidenced by high sorption capacity of PE, PP and PS microplastics which was comparable to the hydrophobic organic compound 1-octanol - a frequently used partition coefficient (Lee

et al., 2014); the authors noted that sorption was particularly pronounced for POPs that are persistent or have travelled over long distances. Concentrations of such toxins on microplastics can reach up to a million times higher than concentrations in the sea (Takada, 2013).

In addition to the direct impact of plastic particles, it has previously been suggested that the sorption of POPs into microplastics can potentially lead to toxic effects being bio-magnified up the food chain when they are taken up by sediment, water-column, or surface water-dwelling organisms at the base of the food chain (Moore, 2008; Teuten et al., 2007). In a study simulating a natural food chain from zooplankton to fish, 1 µm to 5 µm microplastics (undisclosed polymer type) and 10 µm to 20 µm PE microplastics (1.2×10^6 particles per 20,000 *Artemia nauplii*) sorbed with benzo[*a*]pyrene (BaP) 60–252 µg were taken up by *Artemia* nauplii following exposure periods of 3-6 hours, and these microplastics were subsequently up by *Danio rerio* zebrafish through the ingestion of the nauplii over a three day period (Batel et al., 2016). Of the fish examined, 38% had microplastic particles retained within intestinal villi and of this 38%, microplastics were clearly taken up by epithelial cells in 7.7% of fish; however, there was no significant induction of cytochrome P450 1A (CYP1A) in the liver (a biomarker of cellular and enzymatic effects), which was attributed to high individual variation and low sensitivity regarding BaP concentration. Given these results, there was limited evidence that POPs in microplastics could potentially desorb in fish intestines and transfer to the epithelium and the liver (Batel et al., 2016).

Besides the study mentioned above, there have been few other studies that have documented the transfer of microplastic-sorbed contaminants across trophic levels

and there are fundamental knowledge gaps how POPs and microplastics are transferred or accumulated across food chains. Even the extent to which microplastics act as vectors to transfer POPs from the environment into organisms is a topic that has divided opinions within the scientific community. One recent paper described that POPs can transfer between microplastics and organisms via water, intra-organismal fluids, direct external contact (such as the skin or exoskeleton) or direct internal contact (such as the gut or gill walls) (Hartmann et al., 2017). The authors argued that other models (e.g. (Bakir et al., 2016; Koelmans et al., 2016)) had not included direct external contact as a potential pathway, and proposed that once microplastics are taken up via water or direct contact, POPs could be desorbed from the sorbent before being taken up by organisms as freely dissolved molecules and transferring to tissues; the governing process for desorption and subsequent uptake of the POPs was stated to be diffusion and partitioning (Hartmann et al., 2017). However, the idea that microplastics play a significant role in transferring POPs to organisms is arguable. Natural exposure pathways, for instance, play a significantly larger role in transferring POPs to organisms. In addition to water, dissolved organic carbon, colloidal carbon, black carbon, natural fibres, bacteria, diatoms and other planktons can dissolve and bind POPs (Dean et al., 1993; Koelmans et al., 2016; Ladewig et al., 2015). A recent modelling/review study on the transfer of POPs to marine animals contested that the overall flux of POPs bioaccumulated from ingesting natural prey outweighed the flux of POPs garnered from taking up microplastics (Koelmans et al., 2016), and another study predicted that POP sorption to polyethylene accounts for less than 0.1% of POPs in the oceans (Gouin et al., 2011). It has also been argued that plastics' role as vectors may only be regarded as significant for transporting plastic additives (flame retardants) that are generated through plastic production and leached into the marine

environment through degradation processes (e.g. UV and oxidation) (Gewert et al., 2015). Additionally, the resulting concentration of plastic additive leaching in water or other environmental media is argued to be extremely low, and only significant in semi-enclosed/enclosed bays with accumulation zones of plastics (Kwon et al., 2017). Despite the questionable extent to which microplastics may acting as vectors to transport POPs, it has been proposed that plastics ought to be treated as pollutants in order to enact policies that may be critical in reducing their environmental impact (Lohmann, 2017).

Due to the mounting evidence that microplastics can be taken up by a wide range of aquatic organisms, there is concern that microplastics have the potential to accumulate up the food chain, ultimately affecting humans in the process (Wright and Kelly, 2017). However, due to the difficulty in studying direct impacts on humans, there is currently no established link between the uptake of food and the subsequent translocation/uptake of microplastics into the human body (Galloway, 2015). It is known that microplastics fragmented to a small enough size (<415 nm in diameter) can diffuse through the mucus lining of the gut into the lymphatic system and enhance gut-related infections, liver stress, endocrine disruption and immune-related problems (Hussain et al., 2001; Rochman et al., 2014). Further research, particularly in localising microplastics (and nanoplastics) inside organisms through procedures such as electron/fluorescent microscopy and mass spectroscopy, measuring processes such as sorption/desorption mechanisms involved in POPs' translocation from microplastics into tissues and identifying the specific impacts of these translocated toxins would advance our insights into present and future risks of microplastic uptake. This is of great importance given that shellfish is a delicacy enjoyed by many, as was illustrated

by an estimate that European top consumers eat as many as 72.1 g shellfish day^{-1} (>4 oysters day^{-1}) and as a result, may consume up to 11,000 microplastics a year. (Van Cauwenberghe and Janssen, 2014).

1.5 Economic impacts and implications for the Hong Kong oyster fishery

Besides inflicting potential biological and environmental impacts, such as those discussed in the previous section, microplastics have also been reported to have potential economic impacts. These impacts can be calculated through a cost-benefit analysis, which considers a variety of direct-use (e.g. tourism and commercial profits from mussel and oyster production) and indirect-use benefits (e.g. ecosystem/biological processes) and consider these against avoidable costs and mitigation costs (e.g. beach cleaning). One study, of which the scope covered the whole of South-England, the South of the Netherands, the whole of Belgium and the Northern part of France, calculated that microplastic pollution would cost between £0.32 million - £503 million a year, avoidable costs amounted to £0.324 million - £514 million a year, and that £250 million could be saved if microplastics were not present in the sea and ocean (Van der Meulen, M.D et al., 2015); calculations were based upon costs to tourism (i.e. bathing water quality, aesthetic disturbance), clean up costs on beaches, damage to motors/fishing gear, shellfish industry imports/exports, losses to the fishing and shipping industry and other factors. Likewise, another study accounted for impacts on UK maritime services, costs of removing marine litter, and potential losses to the shellfish industry due to lower growth rates and higher mortality

rates of shellfish (Lee, 2015); the projected cost of plastic pollution impacts was estimated to be between £1.3 million to £8.04 million per year up to 2100. Across the European Union, the Marine Conservation Society (MCS) has estimated that the costs for cleaning beaches and coasts due to microplastic pollution to be as high as €630 million and costs to the fishing industry amounting to €60 million (Marine Conservation Society, 2016). Missing from these projections and estimations, however, are possible health costs from exposed to pathogens or chemical substances as a result of microplastic uptake. This is due primarily to the paucity of data relating to whether diseases and illnesses are bourne in humans as a result of microplastic exposure, as basic cost-of-illness models require esimates of disease incidences and cost per illness (USDA ERS, 2017).

Though the economic impacts of microplastic pollution have not been projected or modelled for Hong Kong, microplastic pollution may potentially affect the 700-year old Hong Kong oyster fishery, which is located in Deep Bay, a semi-enclosed bay in northwest Hong Kong (Mok, 1973; South China Morning Post, 2016). Two species – *Crassostrea hongkongensis* and *Crassostrea ariakensis* are currently cultivated in the bay and it was estimated that 118 tonnes of oyster meat was produced in 2017, with a valuation of HK$13 million (Agricultural Fisheries and Conservation Department, 2018).

Deep Bay is discharged by four rivers/nullahs – Shenzhen River, Shanpui River, Dasha River, and Tin Shui Wai Nullah, and the mouth of the bay joins the Pearl River Estuary. The Pearl River drains eight provinces, including Guangdong province, a densely populated province and a major plastic producer (>13% of total national

production) in China (National Bureau of Statistics of China, 2016). For this reason, plastic waste could potentially be transported along the Estuary and discharged into surrounding waters, including Deep Bay.

A recent study documenting microplastic distribution on 25 beaches in Hong Kong showed that microplastics are significantly more abundant on beaches located in the western side (e.g. 258,408 items/m^2 on a beach along the southwestern coast of Lantau Island) compared with the eastern side (e.g. 16 items/m^2 on two beaches on the eastern coast of Hong Kong) of the special administrative region; evidence showed that microplastics consisted mostly of expanded polystyrene (EPS) fragments, a plastic type that is commonly found in food containers and buoys used in aquaculture installations (Fok and Cheung, 2015). When accounting for seasonal differences, it has been shown that more microplastics can be found on the west coast during wet seasons (when discharge from the estuary are highest), which indicate that discharges from the estuary may influenced the abundance and distribution of microplastics in Hong Kong (Cheung et al., 2016). This contrasted with the findings from another study in Deep Bay, which revealed that microplastics were significantly more abundant in the dry season compared to the wet season, indicating that microplastics may have come mostly from local land-based sources rather than from the Pearl River Estuary (Tsang et al., 2017). The study reported the mean abundance of microplastics in the seawater and sediment of Deep Bay to range from 51 to 11,222 particles per 100 m^3 of seawater and from 67 to 237 particles per kilogram of sediment.

1.6 Aim of the thesis

Based on the existing evidence of microplastic pollution in the water and sediment of Deep Bay, the evidence of biological impacts that microplastic exposure poses to marine organisms and the importance of the Hong Kong oyster industry, this thesis aimed to address the following research objectives:

1) To assess the extent to which plastic pollution affects the oyster fishery in the bay through the collection and analysis of *Crassostrea hongkongensis* oysters, water and sediment samples in the bay (Chapter 2);
2) To test whether microplastic uptake is affected by size or shape of microplastic via an experiment performed on a genetically similar oyster species – *Crassostrea gigas* (Chapter 3*);*
3) To identify whether prolonged microplastic exposure induced biological effects in *Crassostrea gigas* oysters through a series of biological endpoints (Chapter 4).

Chapter 2: The abundance and environmental partitioning of microplastics in Deep Bay, Hong Kong

2.1 Introduction

Deep Bay is located at the mouth of the Pearl River Estuary in the north-west territory of Hong Kong and the south-west territory of Shenzhen; the length of the bay stretches 13.9 km and covers 112 km^2 (Aiguo Qian, 2003). Oyster rafts occupy approximately 7-8 km of the bay (Fishermen, pers comm). Oyster farming in Deep Bay has a rich historical and traditional background, with the industry dating back 700 years and supplying a delicacy commonly enjoyed at Chinese New Year banquets and local barbeque venues (South China Morning Post, 2016). Additionally, oyster farming is a commercially important industry and annual production was estimated at HK$13 million in 2017 (Agricultural Fisheries and Conservation Department, 2018). Aside from their commercial and cultural value, oysters play vital roles in the ecosystem as they remove particulate matter from the water column and deposit them as pseudo-faeces on the bottom sediment - improving water quality in the process (Ermgassen et al., 2013). Two oyster species are currently farmed in Deep Bay – *Crassostrea hongkongensis* and *Crassostrea ariakensis,* the former species being more commonly farmed and the species of interest in this study (Morton, 1975). The inner part of the bay is fed mostly by local river inputs whilst the outer part of the bay is fed by water flowing from the Pearl River Estuary; highest discharge rates occur over the summer wet season (Aiguo Qian, 2003; Lee et al., 2006). The inner part of the bay has been reported to contain greater numbers of microplastics in the sediment (Tsang et al., 2017), whilst the outer part of the bay has been reported to contain greater numbers

of microplastics in surface waters and on beaches (Fok and Cheung, 2015; Tsang et al., 2017). Up until the 1980s, a bottom-laying method was used to culture oysters in Deep Bay, and this method was criticised as being inefficient due to the inability to produce sufficient spat to ensure sustainable harvests, and being detrimental to oysters' health due to heavier pollution loads on the bottom waters (Morton, 1975). This method has now been replaced by suspended rope-culturing on rafts and this has been adopted across all oyster farms in the bay (Fishermen, pers comm).

In Deep Bay, the site of interest in this study, high levels of metals such as zinc, iron copper and nickel have been found in sampled oysters (Phillips and Yim, 1981; Wong et al., 1981) and water (Liu et al., 2004); PAHs such as phenanthrene, fluoranthene, naphthalene anthracene, fluorine, pyrene and pyrene compounds have been found in sediment samples (Qiu et al., 2009; Zhao et al., 2012); microplastics such as PS, PP and PE have been found in sampled beaches (Fok and Cheung, 2015; Zurcher, 2009), sediment and water samples (Tsang et al., 2017); and high levels of suspended solids, turbidity, organic matter, nutrients and *E. coli* bacteria ("EPD - Marine Water Quality in Hong Kong," n.d.). Amongst sources of anthropogenic pollution are local waste inputs from domestic, industrial and agricultural runoff in drainage systems, sewage treatment waterways and rivers ("EPD - Marine Water Quality in Hong Kong," n.d.). With regard to plastic pollution, over 20.6% of total municipal waste disposed of in Hong Kong in 2016 consisted of plastics, and this number is projected to rise given the lack of recycling facilities in the city and the implemented restriction of solid waste exports to China (Environmental Protection Department (Statistics Unit), 2017). In addition to local waste inputs, it is suspected that the Pearl River Estuary transports waste inputs from surrounding megacities in Guangdong province (a major plastics

processing and manufacturing hub, which contributes to more than 13% of China's plastic production) downstream into other localities including Deep Bay (Fok and Cheung, 2015; Zurcher, 2009).

This study set out to identify whether microplastic pollution poses a potential risk to the oyster shellfishery in Deep Bay, Hong Kong. The environmental partitioning (i.e. the distribution of microplastics within the water column) of microplastics was looked at along a short transect with comparisons drawn between sea surface samples and benthic sediment samples. The abundance, shapes and sizes of identified microplastics in these samples were then compared with cultured *Crassostrea hongkongensis* oysters collected along the transect to see how the environmental types and concentrations of microplastics related to any biological uptake of microplastics into oysters. It was suspected that the inner part of the bay would be influenced more by local river inputs, and that the outer part of the bay would be influenced more by inputs from the Pearl River Estuary (leading to an unequal distribution of microplastics across the bay), and so the transect was designed to span from one side of the bay to the other.

Three hypotheses were tested as part of the study:

1. The biological uptake of microplastics occurs in *Crassostrea hongkongensis* oysters in Deep Bay.
2. The concentration of microplastics increases along a gradient from the inner part of the bay to the outer part of the bay.

3. There is a significant relationship between the type and abundance of microplastics found in environmental samples and in oysters across sites.

2.2 Methodology

a) Study Location

The study was carried out in Deep Bay, (Fig. 2.1) sites during the dry season of 2016/2017 (December to February) during the high tide. Sites 3, 4 and 5 were sampled on December 14, 2016 and sites 1 and 2 were sampled on February 16, 2017. These five sites were situated horizontally across the bay, spanning 6 km in ascending distance from the mouth of the Pearl River Estuary. As the sites were located in the centre of the bay, a boat was used as means of transportation to reach each site.

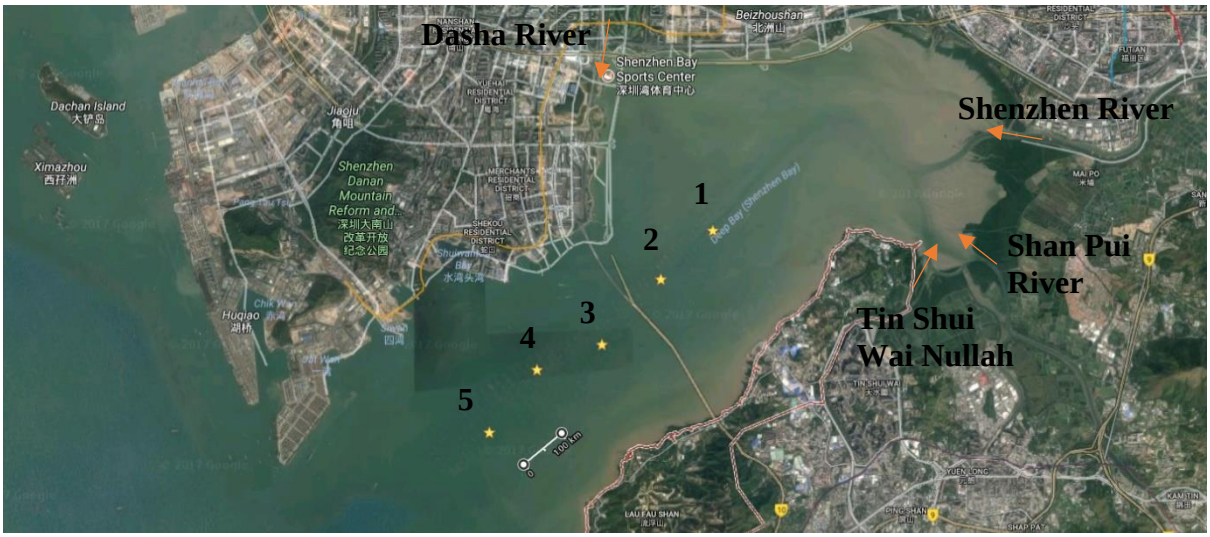

Figure 2.1: Study site in Deep Bay. Numbers 1 to 5 represent sampling locations, and arrows represent rivers/nullahs that flow into the Bay. Scale bar shows 1 km.

b) Seawater samples processing

Four replicate surface seawater samples were collected from each site (Table 2.1), and each replicate was separated by a distance of >2 metres to ensure that samples did not overlap. Samples were collected using a 12V 1000 G/H bilge pump connected to a hose at a water depth of ~1 metre, following a method used by studies such as (Barrows et al., 2018). For each sample, 100 L of water was pumped and filtered through a stacked set-up of a 500 µm and 212 µm sieve. The filtered material was rinsed several times with distilled water and placed in individual nalgene containers; samples were fixed with 4% formalin (final concentration) and stored at room temperature in the laboratory. For analysis, samples were rinsed with Milli-Q® into a 63 µm sieve, and then rinsed back into pre-weighed beakers before being desiccated in an oven at 60 °C for 24 hours. Upon drying, the dry weight of samples was recorded and samples were digested to remove any remaining organic material (40 mL of 1 M KOH per 0.2 g dry weight) in an oven at 60 °C for 24 hours. The digested samples were then vacuum filtered with pre-weighed 0.45 µm Whatman® nitrocellulose filter paper and the filter paper was left to dry in an oven at 40 °C. Filter paper was subsequently observed under a stereoscope for any microplastics.

Table 2.1: GPS locations of sites for oyster, water and sediment sample collection.

Site	GPS Location	Water Depth (m)	Oyster Depth (Shallow/m)	Oyster Depth (Deep/m)
1	22º29'22"N, 113º58'20"E	3.50	0.00 - 0.37	1.00 - 1.37
2	22º28'45"N, 113º57'39"E	3.50	0.00 - 0.45	1.00 - 1.25
3	22º28'1"N, 113º56'58"E	4.50	0.00 - 0.45	1.25 - 1.7
4	22º27'49"N, 113º56'9"E	4.50	0.00 - 0.35	1.00 - 1.15
5	22º27'12"N, 113º55'43"E	5.00	0.00 - 0.35	1.00 - 1.15

c) Sediment samples processing

Four replicate benthic sediment samples were collected from each site (Table 2.1) using a 6 x 6 x 6” Ekman grabber. Samples were placed in sealable plastic bags and stored in a -20 °C freezer. For analysis, samples were de-frosted overnight at room temperature, and four sub-samples of 100 g sediment per replicate were placed into pre-weighed glass beakers and left to dry overnight in a 60 °C oven. When dried, the dry weight of each sub-sample was recorded, and saturated NaCl solution (26% weight/weight) with a density of 1.2 g/cm^3 was added into each of the glass beakers before being mixed vigorously with a glass rod - this density separation step allowed microplastics to float to the surface whilst denser particles sunk to the bottom of the beakers. Samples were left to settle overnight then decanted into 0.45 µm Whatman® nitrocellulose filter paper in a vacuum filter. Filter paper was subsequently observed under a stereoscope for any microplastics.

d) Oyster specimen processing

Within each site, one oyster raft was selected for sampling, and 16 *Crassostrea hongkongensis* oysters of similar sizes were purchased from the raft. As the oysters are grown on varying depths on the ropes in each raft, 8 oysters were collected at shallow (OS) and deep (OD) intervals (Table 2.1); OS was defined as the first 8 oysters from the top of a rope and OD as 8 oysters from the bottom of a rope, with a gap of ~50 cm separating the two intervals. The sampling of oysters at various depths was conducted to test whether the uptake in oysters would be influenced by depth, as microplastics may have been distributed at various depths of the water column and subsequently taken up by oysters at those corresponding depths.

Collected oysters were stored in sealable plastic bags and stored in a -20 °C freezer in the laboratory. For analysis, the bags were taken out and the shell length and wet weight of each oyster was recorded using a caliper. Each oyster was washed in Milli-Q® to remove any externally adhered plastics, after which their tissue was excised (using a scalpel and a pair of scissors) from their shells and placed in a 50 mL falcon tube. They were then placed in a 60 °C oven overnight, and the dry weight of each oyster was recorded the following day. Tissue digestion was conducted using 10% KOH and incubation at 60 °C, 220 rpm on an incubated shaker for at least 24 hours (some larger tissue required longer periods of incubation). Digested tissue was vacuum filtered with pre-weighed Whatman® 5 µm nitrocellulose filter paper. Undigested tissue (if any) in the falcon tube was poured into a petri dish and any microplastics observed under a stereoscope were transferred onto filter paper with a pair of forceps. Filters were left to dry in an oven at 40 °C. After drying, the dry weight of filter paper was recorded and filter paper was subsequently observed under a stereoscope for any microplastics.

e) Quality control

Throughout all sampling procedures, steps were taken to minimise and quantify any contamination of samples with microplastic fibres in the air, including: thoroughly using Milli-Q® to clean all equipment used prior to, and after use; covering equipment with aluminium foil after rinsing; wearing cotton clothing for fieldwork and lab coats at all times during analysis; wearing nitrile gloves in the field and in the lab; and using procedural blanks - additional replicates used in procedures that are absent of biological material or microplastics to assess the level of background contamination.

f) Plastic enumeration criteria

Microplastics were assessed by their size and shape, and the following criteria was used to standardise microplastic selection: no cellular or organic structures visible; fibres equally thick and not tapered at the end; coloured particles homogenously coloured; fibres not segmented or ribbon-like; and particles not shiny in appearance (Cole et al., 2011a; Mohamed Nor and Obbard, 2014). A Leica M205 C stereoscope (with a superimposed scale on the image) was used to observe for, and to enumerate, microplastics. Plastics were categorised by shape (fragment, fibres, film, and pellets), and size (<100 μm, 100-500 μm, 500-1000 μm, and >1000 μm).

Fourier-transform infrared spectroscopy (FTIR) was not available at the time of writing, but samples have been kept for future analysis. As such, where the term 'microplastics' or 'particles' are used in the context of my experimental results/observations, they refer to particles <5 mm that visually looked like plastics and matched the above criteria.

g) Statistical analysis

A permutational multivariate analysis of variance (PERMANOVA) (Anderson, 2001) was conducted to quantify the variability and determine the relative importance of each factor at a 95% confidence interval. For oyster analysis, the factors were site and depth, and for sediment and water data, a single factor (site) was analysed.

2.3 Results

a) Abundance of microplastics

Microplastics were found in all water samples across sites, and an average of 671.3 ± 285.5 microplastics/m^3 of seawater were found in Deep Bay (Fig. 2.2). Whilst there was a significant difference (PERMANOVA; $p<0.001$ pseudo-$F=3.322$) in the abundance of microplastics found between sites, there was no clear trend observed from one end of the bay to the other. The concentration of microplastics was lowest in site 2 (381.25 ± 18 microplastics/m^3) and highest in sites 1 (973.75 ± 36 microplastics/m^3) and 4 (978.75 ± 73 microplastics/m^3). An example of a microplastic fibre found in a water sample is shown in Fig. 2.5.

Microplastics were found in all sediment samples across sites, and an average of 1.22 ± 0.70 microplastics/g (dry weight) of benthic sediment was found in Deep Bay (Fig. 2.3). Whilst there was a significant difference (PERMANOVA; $p<0.001$, pseudo-$F=10.655$) in the abundance of microplastics found across sites, there was no clear trend observed from one end of the bay to the other. Site 3 had the highest concentration of microplastics with 2.23 ± 0.63 microplastics/g (dry weight) and site 5 had the lowest concentration with 0.79 ± 0.28 microplastics/g (dry weight). An example of a microplastic pellet found in a sediment sample is shown in Fig. 2.6.

Ninety five percent of oysters were found to contain microplastics in all sites sampled, taking up an average of 15.1 ± 6.1 microplastics per oyster (Fig. 2.4). The concentration of microplastics was lowest in site 2 (9.4 ± 7.7 microplastics/individual) and highest in site 5 (25.2 ± 11.1 microplastics/individual). An example of a microplastic fibre found in an oyster specimen is shown in Fig. 2.7.

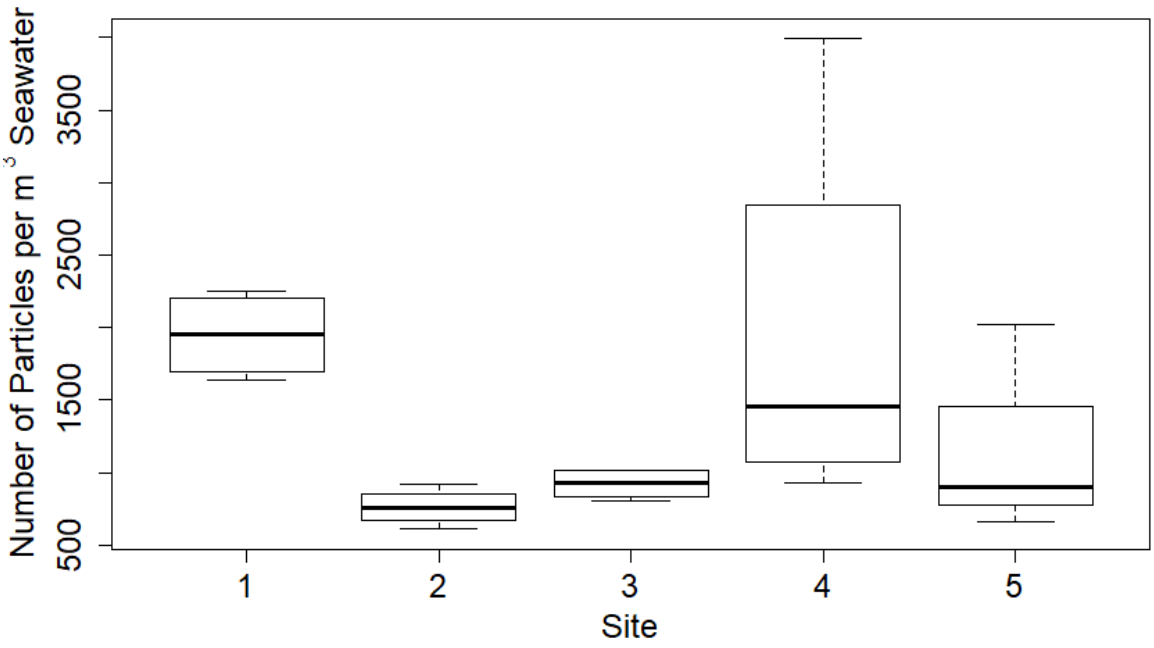

Figure 2.2: Number of microplastics within the water column across sites within Deep Bay, Hong Kong. Data as average ± SD per m^3 seawater.

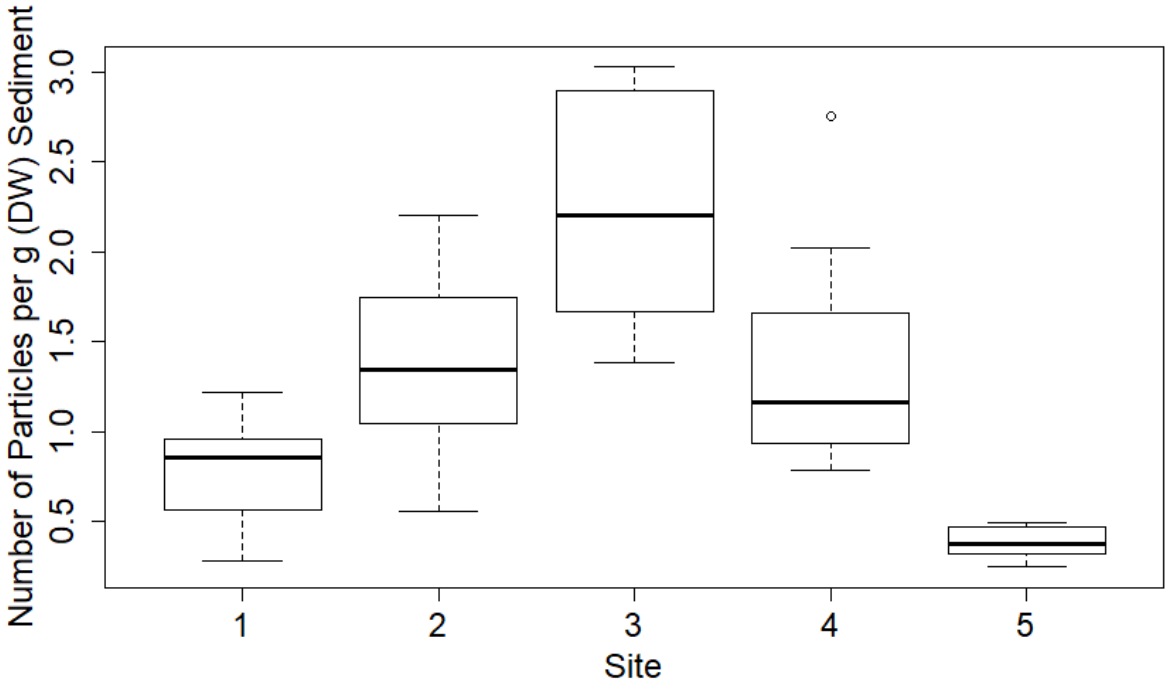

Figure 2.3: Number of microplastics in sediment across sites within Deep Bay, Hong Kong. Data as average ± SD per g (dry weight) of sediment.

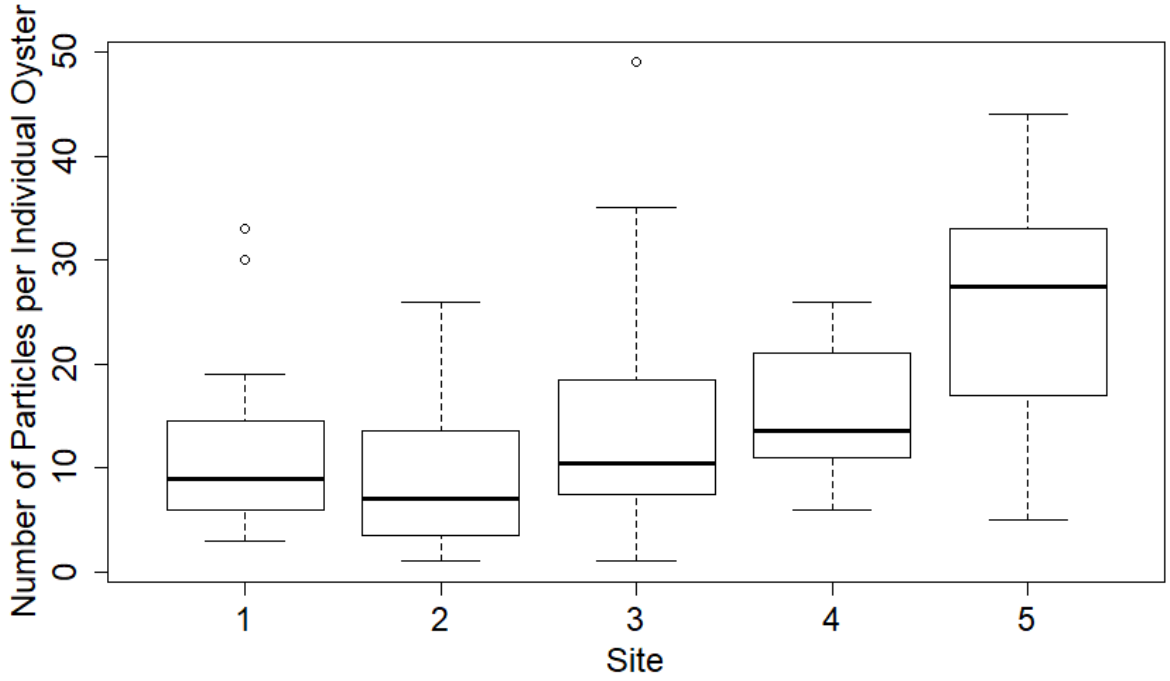

Figure 2.4: Number of microplastics in individual oysters across sites within Deep Bay, Hong Kong. Data as average ± SD per individual.

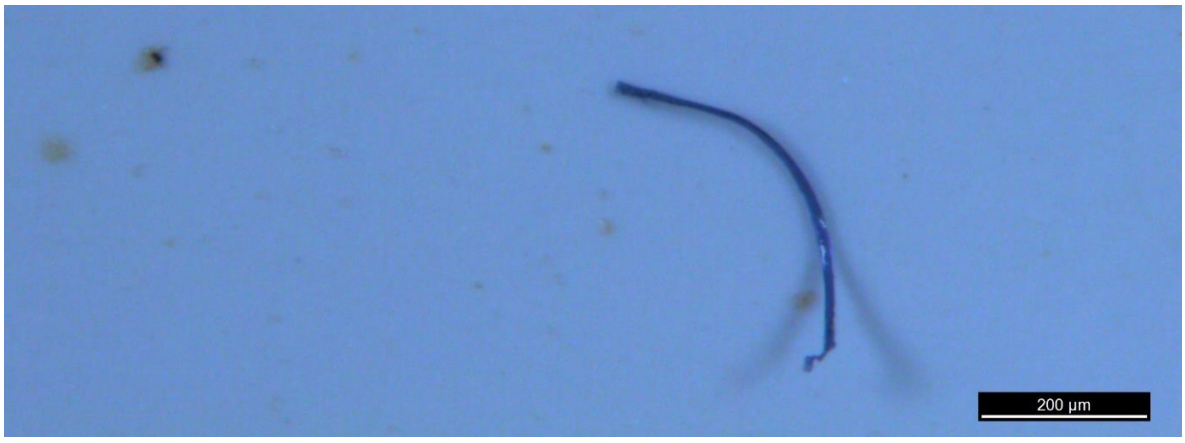

Figure 2.5: Example of a microplastic fibre found in a seawater sample.

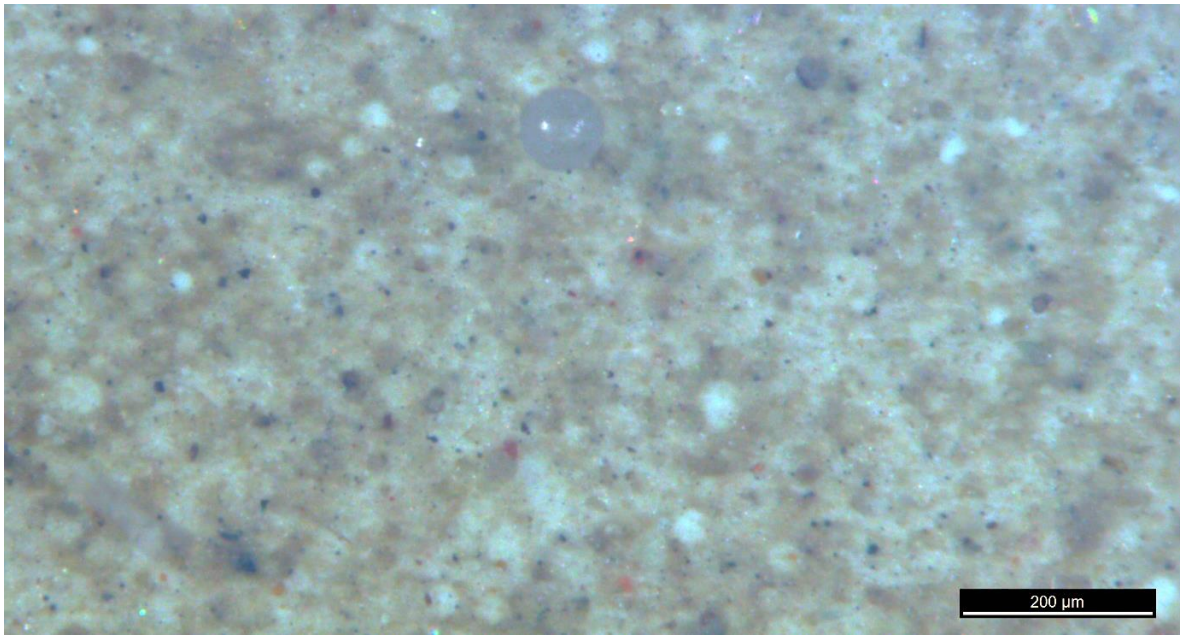

Figure 2.6: Example of a microplastic pellet found in a sediment sample.

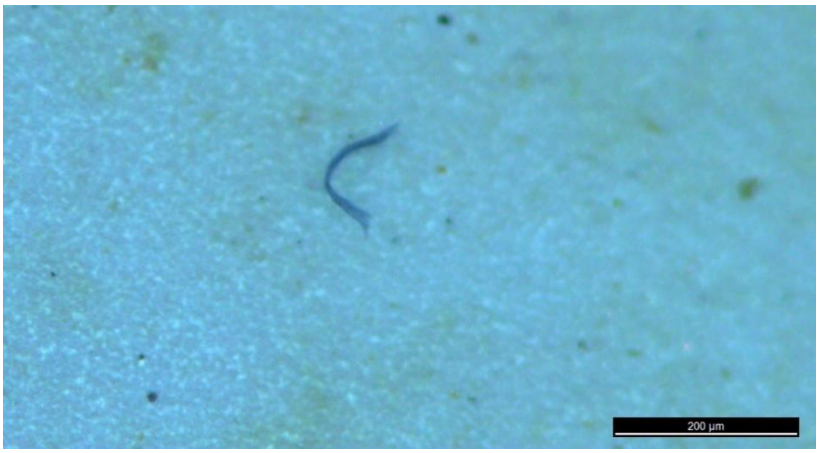

Figure 2.7: Example of a microplastic fibre found in an oyster sample.

b) Comparison between environmental and biological samples

Type, size and number of microplastics were examined in water, sediment and oyster samples across sites. These comparisons were conducted in order to test whether there was a relationship between the number and type of microplastics found in oysters and those found in the surrounding water and sediment across sites. Comparisons between microplastics found in water and oysters across the five sites

did not share a strong relationship and were not statistically significant ($p<0.05$). Pearson's product-moment correlation produced values of P=0.940, t=-0.081 and the R^2 value was 0.002 (Fig. 2.8). The same test was run to make comparisons of microplastics found in sediment and oysters, and as was the case above, the relationship was not strong and statistically non-significant ($p<0.05$). Pearson's product-moment correlation produced values of t=0.900, p=0.440, and the R^2 value was 0.213 (Fig. 2.9).

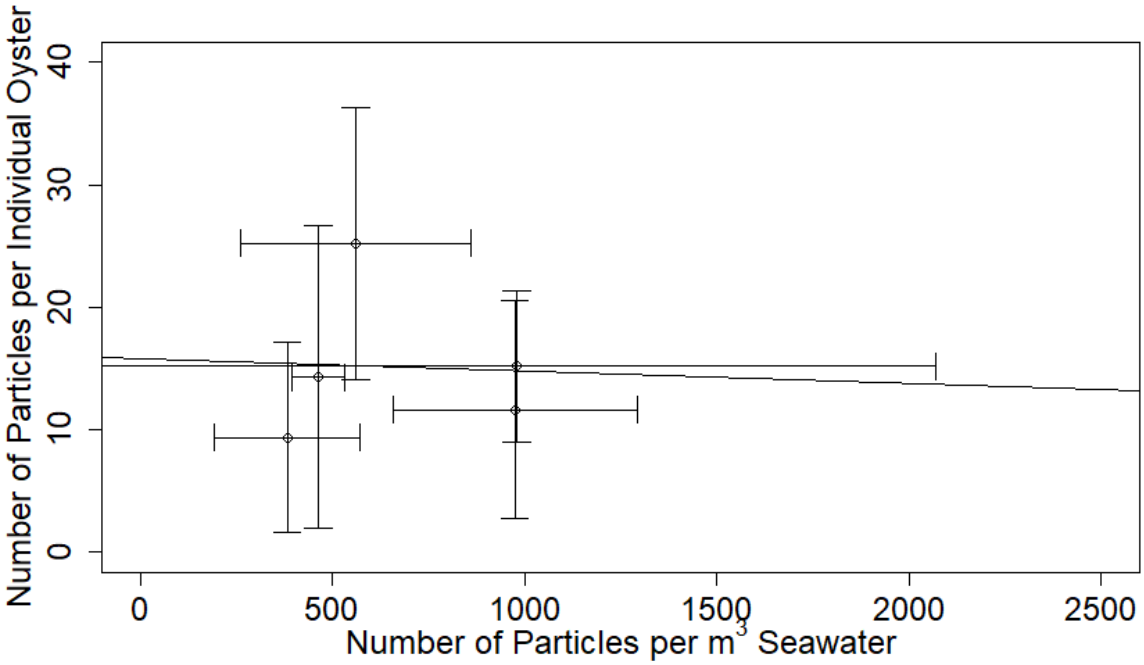

Figure 2.8: Relationship between number of microplastics in seawater (number per m^3 seawater) vs number of microplastics per oyster (individual) ± SD, across sites in Deep Bay; R^2=0.002.

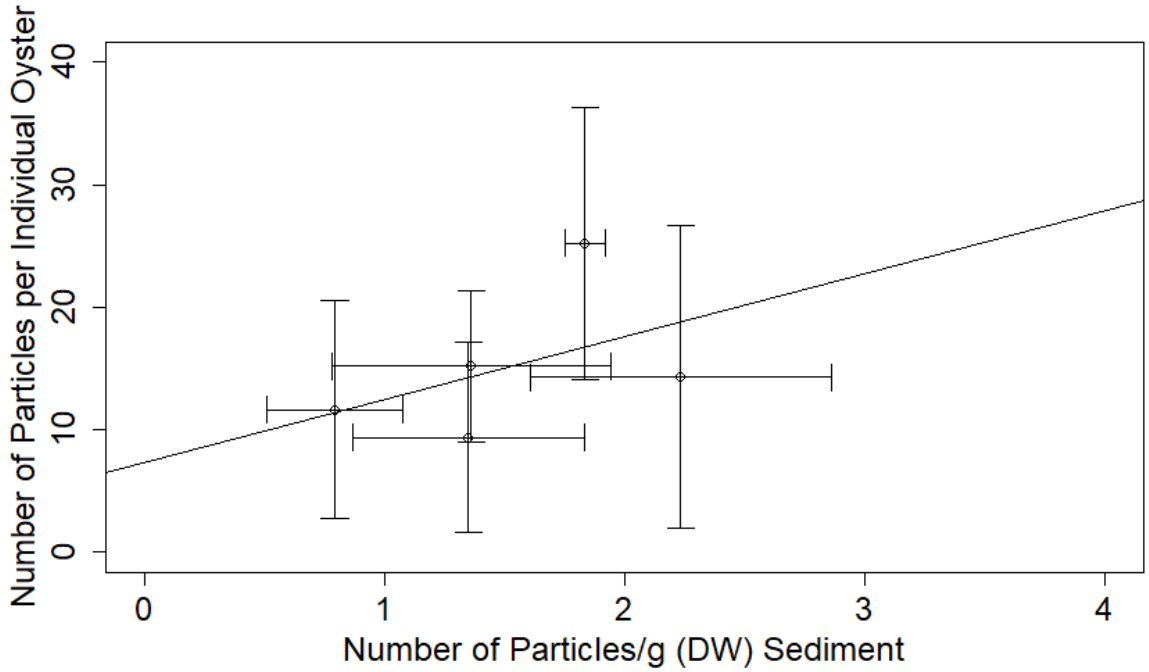

Figure 2.9: Relationship between number of microplastics in sediment (number per g dry weight) vs number of microplastics per oyster (individual) ± SD, across sites in Deep Bay; R^2=0.213.

c) Relationship between water depth and microplastics in oysters

More microplastics were found in oysters at shallow depths in sites 1-3, and at deeper depths in sites 4 and 5 (Fig. 2.10). The concentration of microplastics increased from the mouth of the bay toward the estuary (with the exception of sites 1 and 2). Site (PERMANOVA; p=0.041, pseudo-F=1.556), and the interaction between site and depth (PERMANOVA; p=0.010, pseudo-F=2.326) were confirmed to have an effect on the concentration of microplastics in oysters. Taken as a single factor, depth had no effect on concentration of microplastics (PERMANOVA; p=0.526, pseudo-F=0.651).

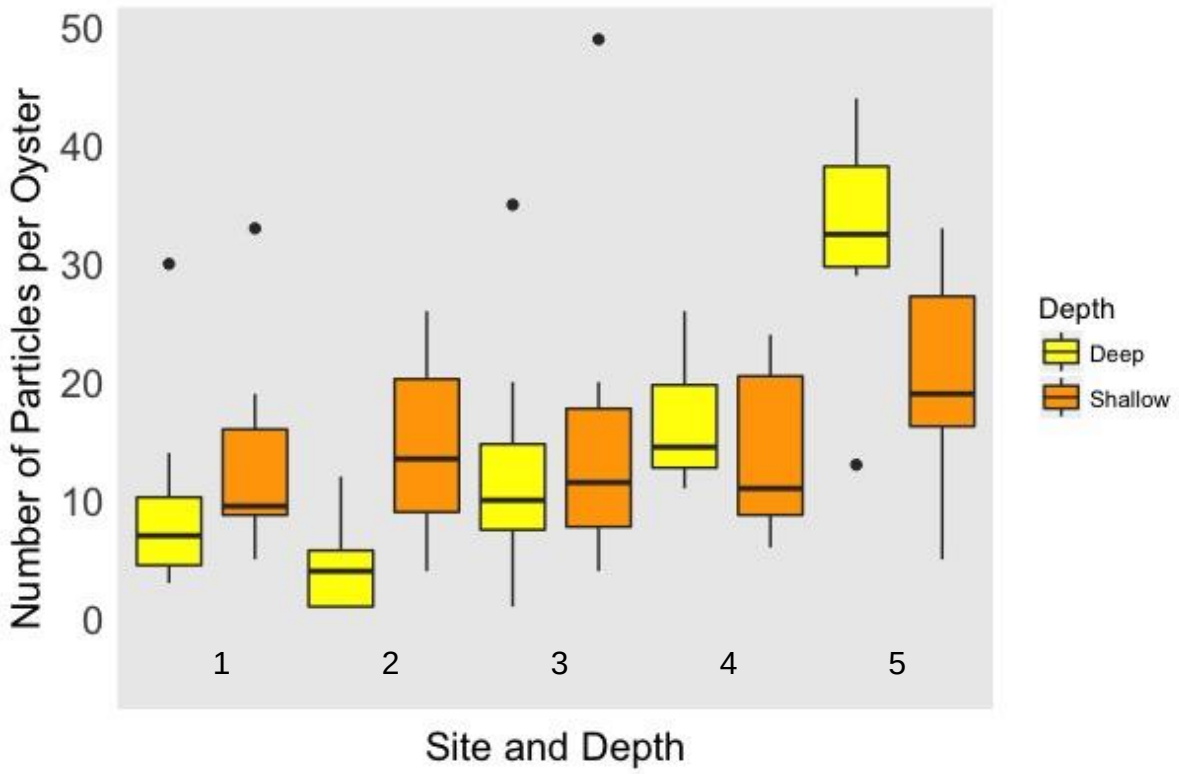

Figure 2.10: Number of microplastics in individual oysters in shallow and deep depths across sites within Deep Bay, Hong Kong. Data as average ± SD per individual.

d) Correlation between oyster size/weight and weight and microplastics recorded

To determine if there was a relationship between the size of oyster specimens and the number of microplastics recorded therein, and whether the relationship was proportional, three size-related parameters - wet weight (g), shell length (mm) and dry weight (g) - were measured from each oyster specimen, and regression analyses of these factors were plotted against the number of microplastics taken up per individual (Figs. 2.11-2.13). Due to the weak relationship (R^2 value) between parameters, none of the above parameters were used for standardisation.

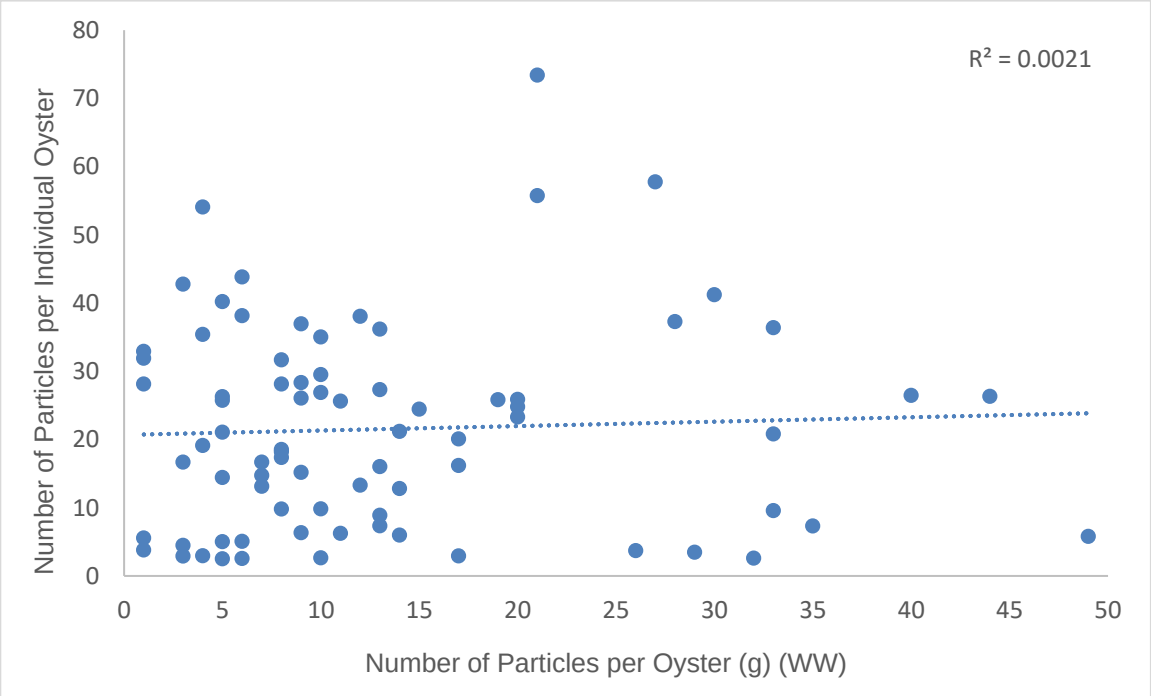

Figure 2.11: Scatterplot showing the relationship between number of microplastics/individual oyster vs number of microplastics/g (wet weight) of oyster.

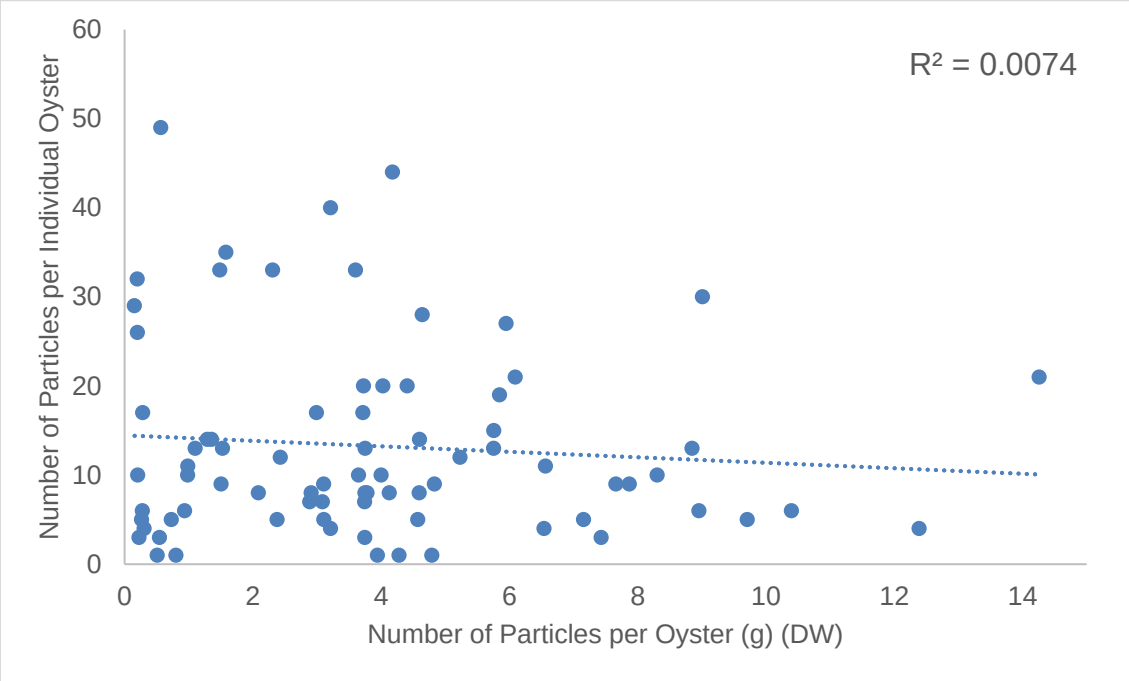

Figure 2.12: Scatterplot showing the relationship between number of microplastics/individual oyster vs number of microplastics/g (dry weight) of oyster.

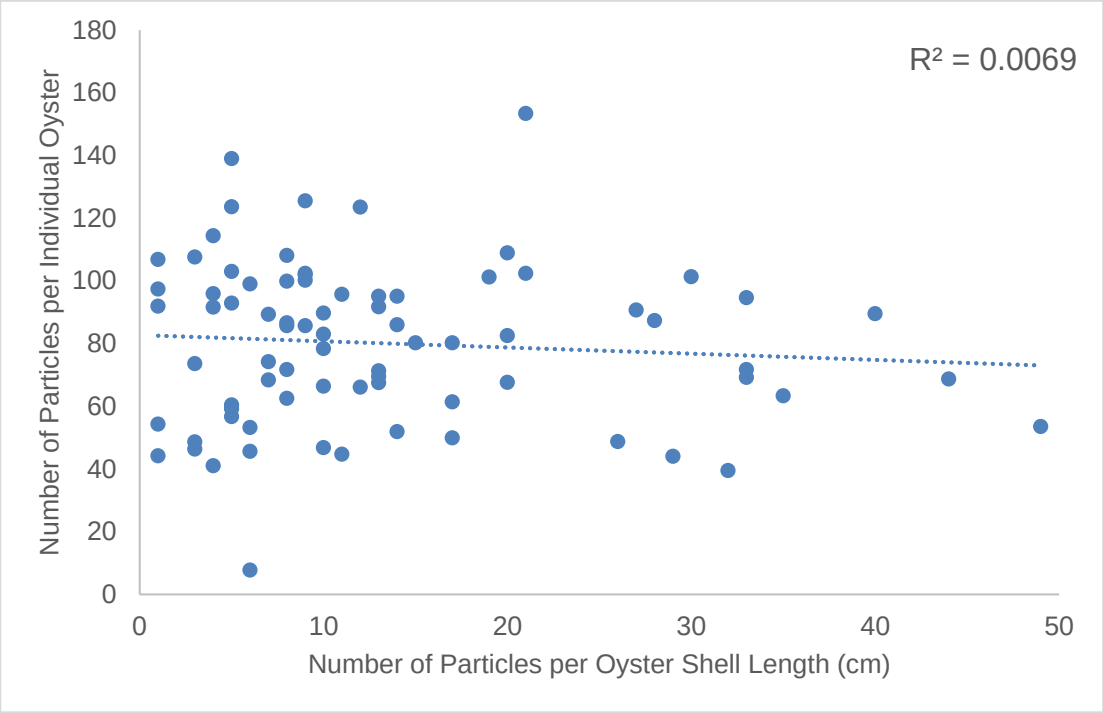

Figure 2.13: Scatterplot showing the relationship between number of microplastics/individual oyster vs number of microplastics/cm (shell length) of oyster.

e) Types of microplastics

In seawater, 77% of microplastics were fibres (Fig. 2.14), which were predominantly white; other particles found were fragments (18%), film (3%) and pellets (2%).

In sediment, most microplastics consisted of pellets (57%) (Fig. 2.15), which were white/pale in colour; other particles found were fibres (21%), fragments (18%) and film (3%).

Of the types of microplastics found in oysters, 84% were fibres (Fig. 2.16), and most particles taken up were white/pale in colour; other microplastics found in oysters included fragments (12%) and pellets (5%).

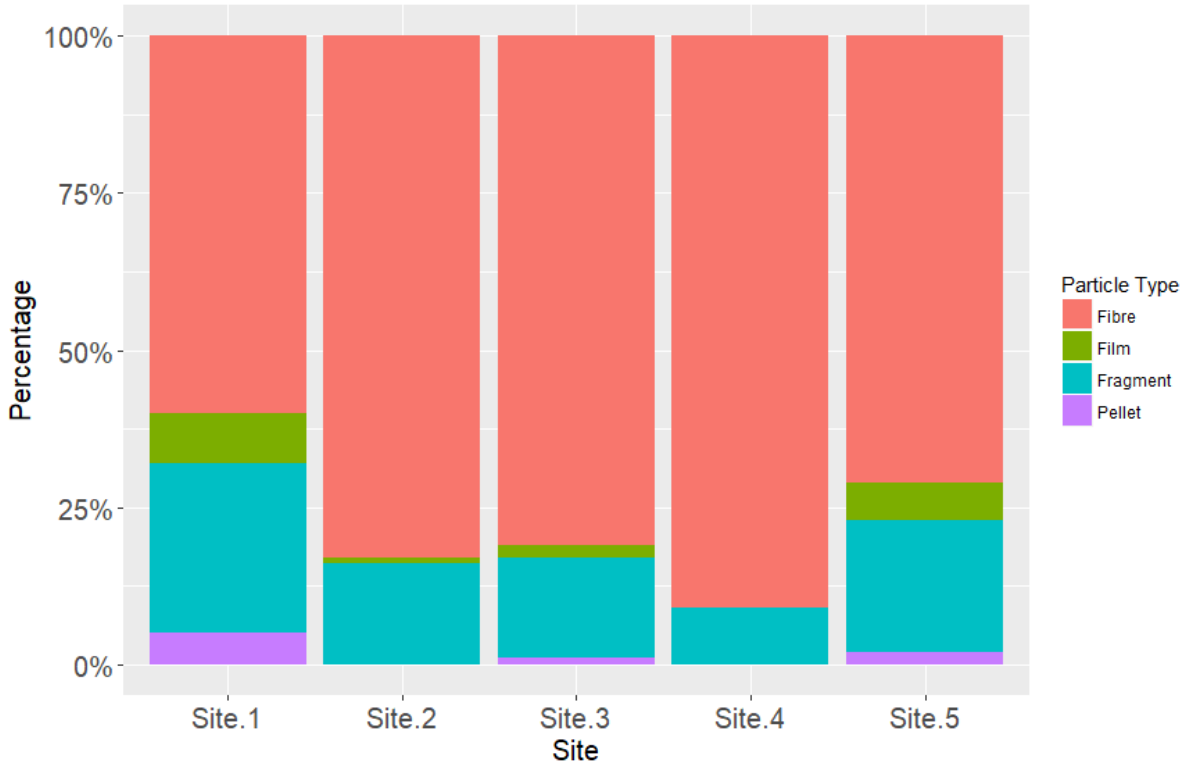

Figure 2.14: Types of microplastics distributed in the water column within Deep Bay, Hong Kong. Data as percentage of fragments, film, fibres and pellets distributed per m^3 of seawater.

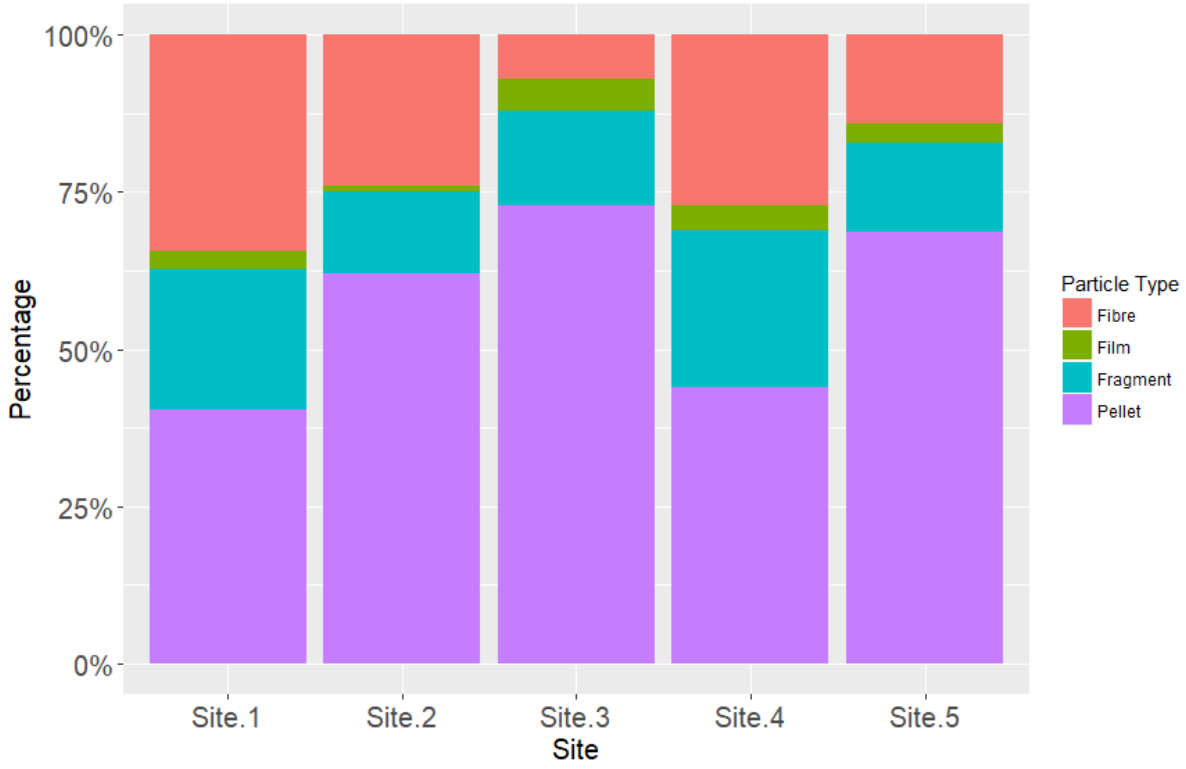

Figure 2.15: Types of microplastics distributed in sediment within Deep Bay, Hong Kong. Data as percentage of fragments, film, fibres and pellets distributed per g (dry weight) of sediment.

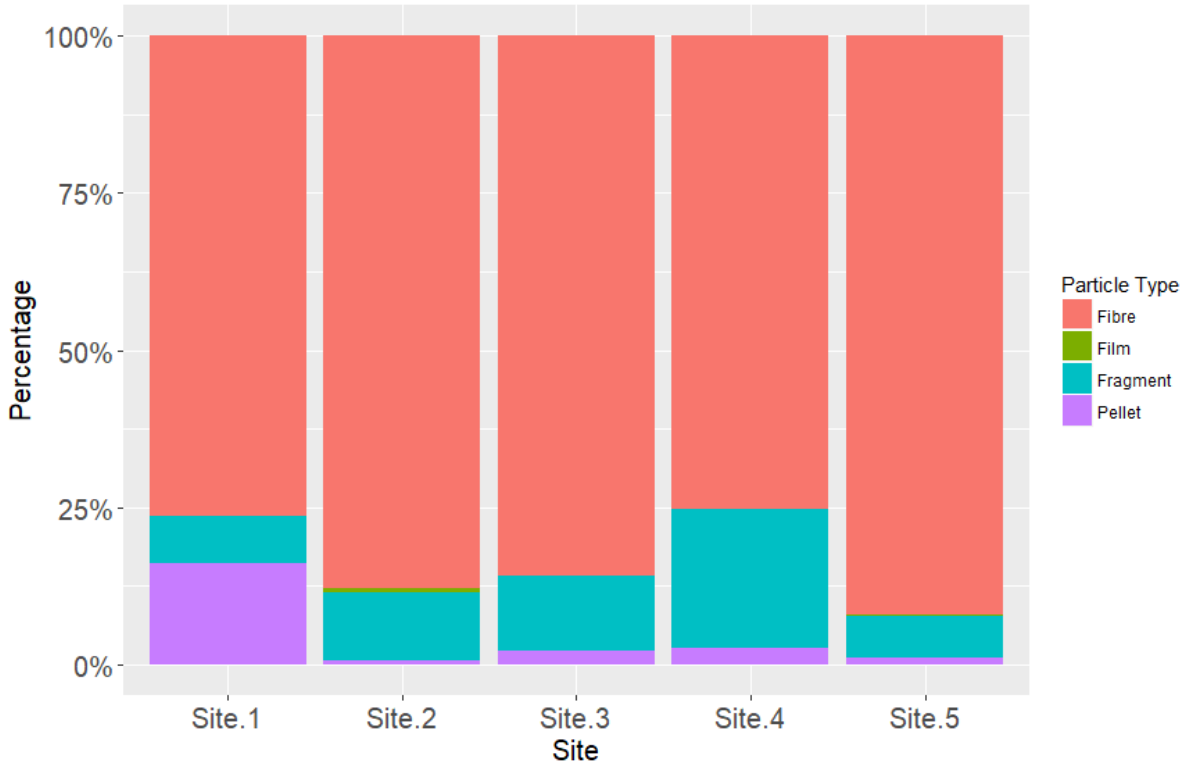

Figure 2.16: Types of microplastics taken up by oysters within Deep Bay, Hong Kong. Data as percentage of fragments, film, fibres and pellets taken up per individual oyster.

f) Size of microplastics

Most microplastics in seawater were in the size range of <100 µm (44%) and 100-500 µm (30%) (Fig. 2.17); other sizes of microplastics included those between 500-1000 µm (17%) and >1000 µm (9%).

Most microplastics in sediment were <100 µm in size (77%) (Fig. 2.18); other sizes of microplastics were 100-500 µm (15%), 500-1000 µm (6%) and >1000 µm (3%).

Most microplastics in oysters belonged to size classes of <100 µm (41%) and between 100 µm-500 µm (30%) (Fig. 2.19); other sizes of microplastics included 500-1000 µm (17%) and >1000 µm (13%).

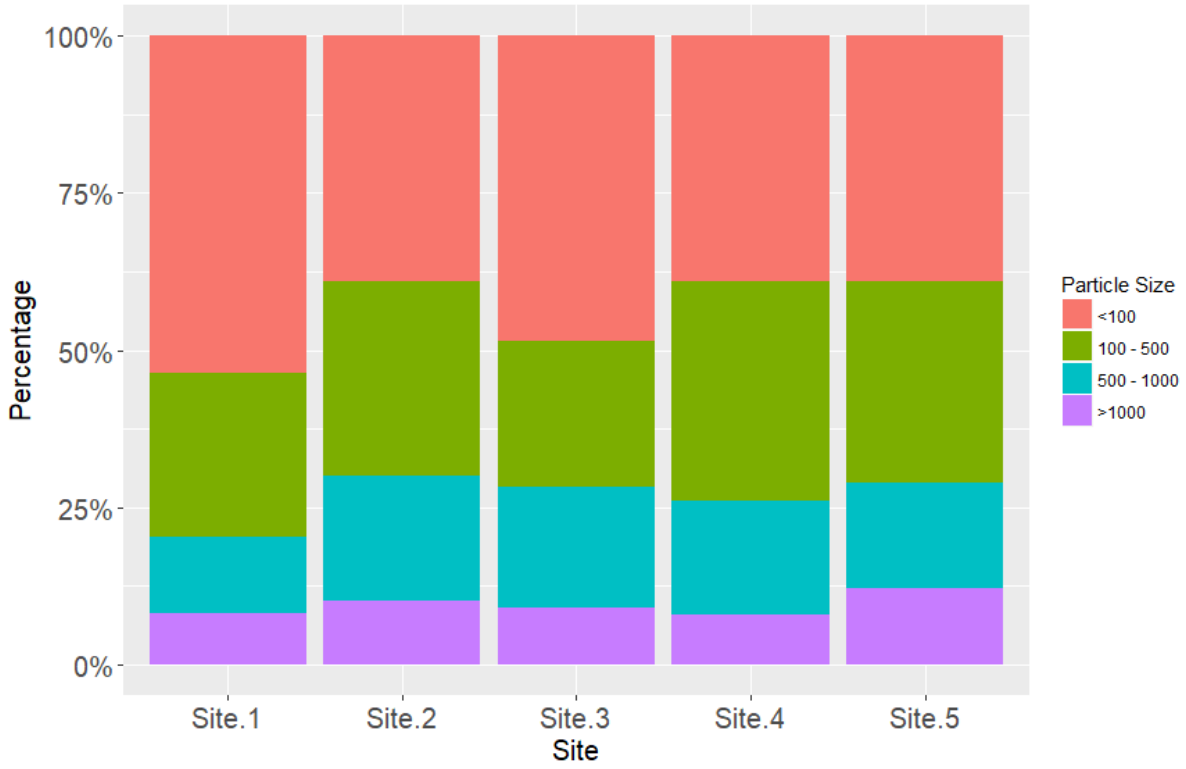

Figure 2.17: Sizes (diameter in μm) of microplastics distributed in the water column within Deep Bay, Hong Kong. Data as percentage of <100 μm, 100-500 μm, 500-1000 μm and >1000 μm microplastics distributed per m^3 of seawater.

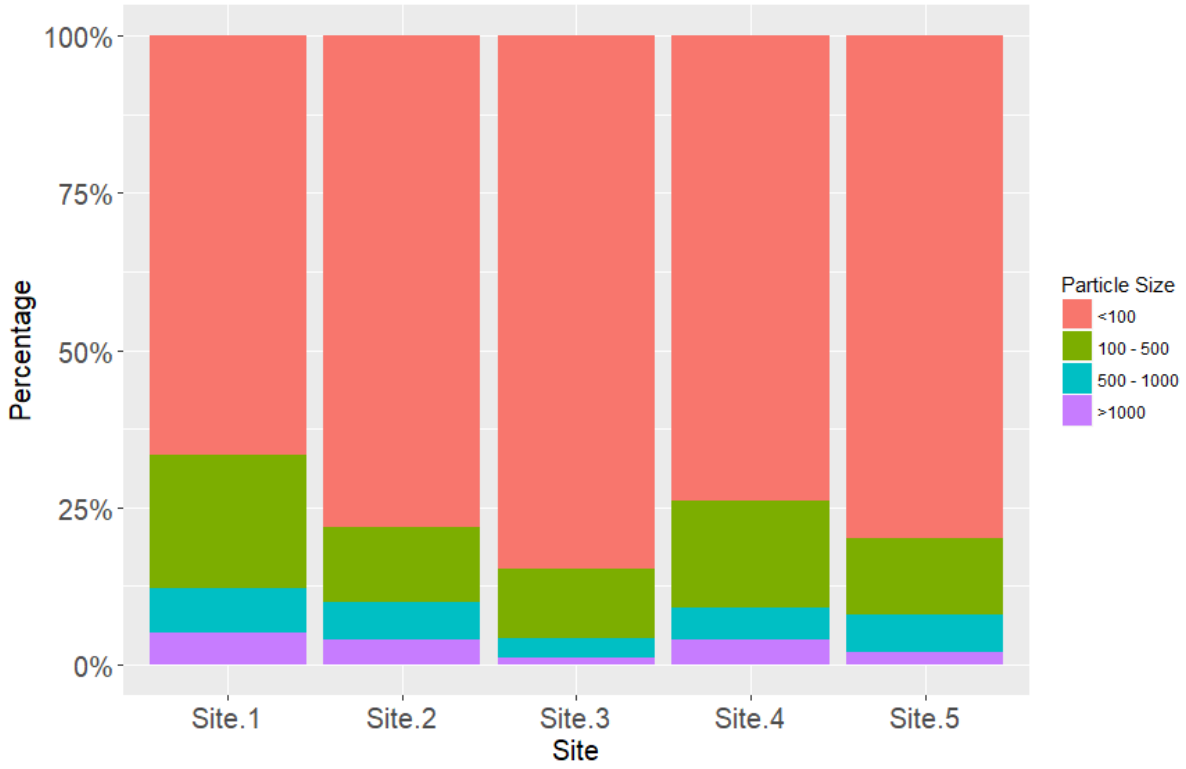

Figure 2.18: Sizes (diameter in μm) of microplastics distributed in sediment within Deep Bay, Hong Kong. Data as percentage of <100 μm, 100-500 μm, 500-1000 μm and >1000 μm microplastics distributed per g (dry weight) of sediment.

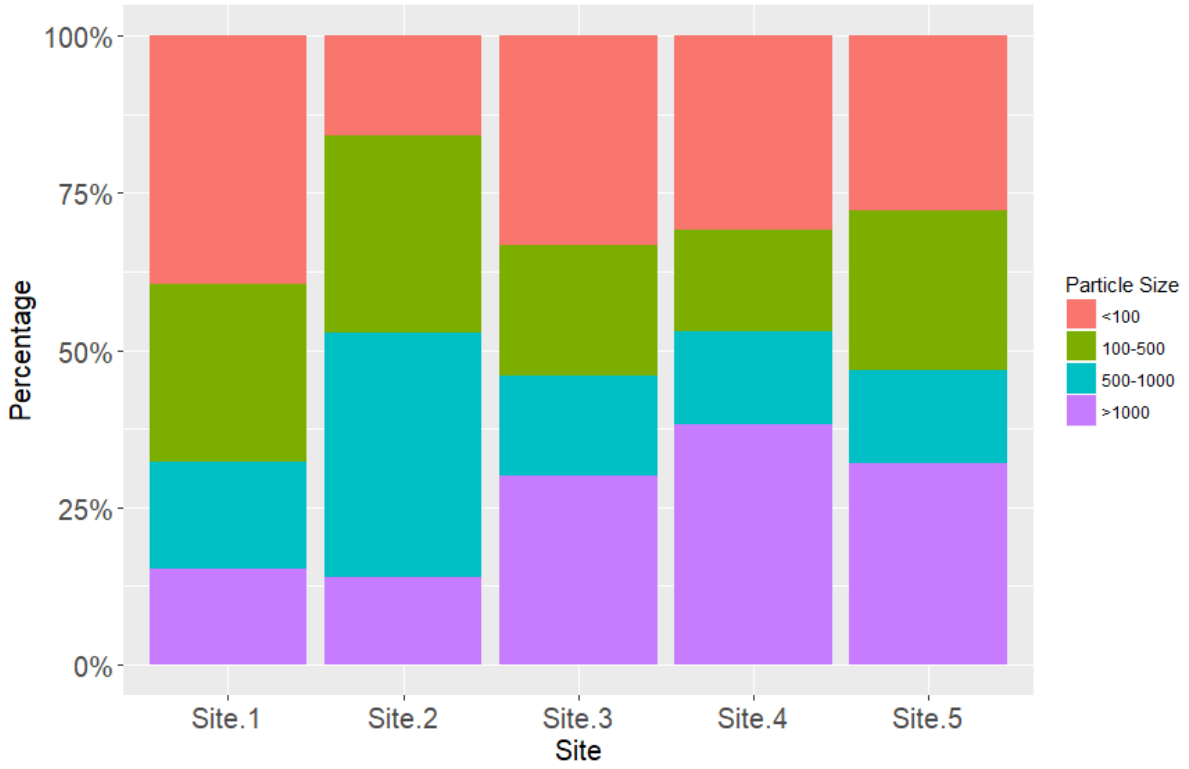

Figure 2.19: Sizes (diameter in μm) of microplastics taken up by oysters within Deep Bay, Hong Kong. Data as percentage of <100 μm, 100-500 μm, 500-1000 μm and >1000 μm microplastics taken up per individual.

g) Procedural blanks

Plastics enumerated in seawater, sediment, and oyster samples were corrected to account for any plastics observed in procedural blanks. Plastics in procedural blanks amounted to an average of 1.83 ± 0.81 plastics/filter paper in seawater processing procedural blanks, 0.30 ± 0.10 plastics/filter paper in the procedural blanks for oyster processing, and 5.3 ± 1.06 plastics/filter paper.

h) Comparisons with other studies

The average number of microplastics taken up by bivalves, water and sediment in this study, and the number of microplastics quantified in biological and environmental samples from other studies are shown in Tables 2.2-2.4.

Table 2.2: Table showing the number of microplastics found in seawater in this study and the number of microplastics found in water in other studies.

Location	**Average No. Microplastics per 100 m^3 of Water**	**Reference**
Deep Bay, Hong Kong	67,125 (microplastics)	This study
Victoria Harbour, Hong Kong	14,008	(Tsang et al., 2017)
Tolo Harbour, Hong Kong	687	(Tsang et al., 2017)
Tsing Yi, Hong Kong	3158	(Tsang et al., 2017)
Deep Bay, Hong Kong	5,637	(Tsang et al., 2017)
NE Pacific Ocean	27,900	(Desforges et al., 2014)
West Coast, Vancouver Island	171,000	(Desforges et al., 2014)
Queen Charlotte Sound, New Zealand	763,000	(Desforges et al., 2014)
Strait of Georgia	321,000	(Desforges et al., 2014)
Yangtze Estuary, China	413,730	(Zhao et al., 2014)
East China Sea, China	17	(Zhao et al., 2014)
Seto Island, Japan	374	(Isobe et al., 2015)
Goiana Estuary, Brazil	26	(Lima et al., 2014)
North Pacific Ocean	220	(Moore et al., 2001)
Taihu Lake, China	14,600	(Su et al., 2016)

Table 2.3: Table showing the number of microplastics found in sediment in this study and the number of microplastics found in sediment in other studies.

Location	**Weight Type (g)**	**Average No. Microplastics per kg of Sediment**	**Reference**
Deep Bay, Hong Kong	DW	1,513 (microplastics)	This study
Victoria Harbour, Hong Kong Tolo Harbour, Hong Kong Tsing Yi, Hong Kong Deep Bay, Hong Kong	Unspecified	170 205.5 123.5 152	(Tsang et al., 2017)
Wanning, China	DW	6,923	(Qiu et al., 2015)
Nievwpoort, Belgium	DW	13	(Van Cauwenberghe et al., 2013)
Nieuwpoort, Belgium Oostende, Belgium Zeebrugge, Belgium	DW	166.7 97.2 92.8	(Claessens et al., 2011)
Venice, Italy	DW	1,455	(Vianello et al., 2013)
Taihu Lake, China	DW	122.8	(Su et al., 2016)
Bijiashan, Bohai Sea Xingcheng, Bohai Sea Dongdaihe, Bohai Sea	DW	102.9 163.3 117.5	(Yu et al., 2016)
Slovenia	DW	177.8 170.4	(Laglbauer et al., 2014)
Kachelotplate, Spiekeroog	DW	671 1.45	(Dekiff et al., 2014)

Table 2.4: Table showing the number of microplastics found in oysters in this study and the number of microplastics found in bivalves in other studies.

Bivalve Species	**Location Collected**	**Weight Type (g)**	**Average Number**	**Controlled for Background Contamination**	**Reference**
Crassostrea hongkongensis	Deep Bay, Hong Kong	WW DW Per individual	1.3 13.2 15.1 (microplastics)	Yes	This study
Crassostrea gigas	Brittany, France (supermarket)	WW	0.47	Yes	(Van Cauwenberghe and Janssen, 2014)
Mytilus edulis	Mussel farm in Germany	WW	0.36	Yes	(Van Cauwenberghe and Janssen, 2014)
Mytilus edulis	Breakwaters in France, Belgium and the Netherlands	WW	0.2	No (excluded all microplastic fibres from analysis)	(Van Cauwenberghe et al., 2015)
Mytilus edulis	Groyne	WW	2.6	Yes	(De Witte et al., 2014)
Mytilus edulis	Quayside	WW	5.1	Yes	(De Witte et al., 2014)
Mytilus edulis	McCormack's Beach	Per individual	34	No (contamination observed)	(Mathalon and Hill, 2014)
Mytilus edulis	Rainbow Haven Beach	Per individual	34	No (contamination observed)	(Mathalon and Hill, 2014)
Mytilus edulis	Newfoundland and Labrador (grocery)	Per individual	75	No (contamination observed)	(Mathalon and Hill, 2014)
Scapharca subcrenata	Fish market in Shanghai	WW	2.1-10.5	Yes	(Li et al., 2015)

Tegillarca granosa	Fish market in Shanghai	WW	2.1-10.5	Yes	(Li et al., 2015)
Mytilus galloprovincialis	Fish market in Shanghai	WW	2.1-10.5	Yes	(Li et al., 2015)
Patinopecten yessoensis	Fish market in Shanghai	WW	2.1-10.5	Yes	(Li et al., 2015)
Alectryonella plicatula	Fish market in Shanghai	WW	2.1-10.5	Yes	(Li et al., 2015)
Sinonovacula constricta	Fish market in Shanghai	WW	2.1-10.5	Yes	(Li et al., 2015)
Ruditapes philippinarum	Fish market in Shanghai	WW	2.1-10.5	Yes	(Li et al., 2015)
Meretrix lusoria	Fish market in Shanghai	WW	2.1-10.5	Yes	(Li et al., 2015)
Cyclina sinensis	Fish market in Shanghai	WW	2.1-10.5	Yes	(Li et al., 2015)
Periwinkle (unspecified species)	Eastern Scheldt (outside)	DW	20	Yes	(Leslie et al., 2017)
Crassostrea gigas	Eastern Scheldt (outside)	DW	87	Yes	(Leslie et al., 2017)
Crassostrea gigas	Rhine Estuary	DW	105	Yes	(Leslie et al., 2017)
Mytilus edulis	Eastern Scheldt (outside)	DW	30	Yes	(Leslie et al., 2017)
Mytilus edulis	Ter Heijde North Sea coast	DW	19	Yes	(Leslie et al., 2017)
Mytilus edulis (wild)	Coastal waters of China	WW	2.7	Yes	(Li et al., 2016)
Mytilus edulis (farmed)	Coastal waters of China	WW	1.6	Yes	(Li et al., 2016)

2.4 Discussion

This study reveals that the seawater, sediment and *Crassostrea hongkongensis* oysters in Deep Bay are contaminated with microplastics, reflecting that microplastic pollution is a local environmental issue. Compared with the abundance of microplastics reported in other locations, the average number of microplastics found in the surface waters of Deep Bay (67,125 microplastics per 100 m^3) fall in between the lower end of reported values (e.g. 17 microplastics per 100 m^3 in the East China Sea, China) and the upper end of reported values (e.g. 763,000 microplastics per 100 m^3 in Queen Charlotte Sound, New Zealand) (Table 2.2). Since reported concentrations vary quite significantly (Table 2.2), it is difficult to establish what the most common concentrations of microplastics in seawater are, and the concentrations found in locations that most closely resemble those found in this study. No clear trend was apparent in microplastics found in sediment samples collected along the transect from the inner part of the bay towards the outer part of the bay (refuting Hypothesis 2).

Similarly, the average number of microplastics found in the benthic sediment in Deep Bay (1,513 microplastics per kg (DW) fall in between the lower end of reported values (e.g. 13 microplastics per kg (DW) in Nievwpoort, Belgium) and the upper end of reported values (e.g. 6,923 microplastics per kg (DW) in Wanning, China) and most closely resembled those found in Venice, Italy (1,455 microplastics per kg (DW)) (Table 2.3). However, reported numbers more commonly range between 100-200 microplastics per kg sediment (DW). Similar to what was observed in seawater samples collected between sites, no trend was apparent in microplastics found in sediment samples collected along the transect (refuting Hypothesis 2).

Of the oysters collected, 95% contained microplastics and an average of 15.1 microplastics were taken up per individual (confirming Hypothesis 1). These concentrations fall within the middle of previously reported data for bivalves – concentrations as low as 0.2 microplastics/g DW were found in *Mytilus edulis* mussels in breakwaters in France, Belgium and the Netherlands, and concentrations as high 105 microplastics/DW were found in *Crassostrea gigas* oysters in the Rhine Estuary (Table 2.4). However, more common are numbers ranging between 2-6 particles per individual. When compared with other datasets available for microplastic uptake in mussels, the number of microplastics taken up by *Crassostrea gigas* oysters in this study most closely resembled those taken up by *Mytilus edulis* mussels farmed in the coastal waters of China (Li et al., 2016). In that particular study, mussels were collected in 10 sites stretching over 12,400 miles of coastline (65% of the total coastline length), and included areas influenced by the flow of the Yangtze River and the Yellow River, which were respectively ranked as catchment areas with the 1st and 3rd highest levels of mismanaged plastic waste generated per year (Schmidt et al., 2017). Unlike the lack of clear trends across sites for water and sediment samples, there was a general trend of increased concentrations of microplastics from the inner part of the bay toward the outer part in oyster specimens (notably, almost three times as many microplastics were taken up in oysters in site 5 compared with oysters in site 2) along the transect that was sampled (confirming Hypothesis 2).

There was a weak correlation and insignificant relationship between the concentrations of microplastics found in environmental (water and sediment) and biological (oyster) samples across sites (refuting Hypothesis 3), suggesting that the distribution of microplastics and their interaction in the environment varied across

depths, and that the biological uptake of microplastics was independent of environmental concentrations. This is supported by the fact that microplastic pellets <100 μm in size were more commonly found in sediment, whereas microplastic fibres <100 μm and 100-500 μm in size were more commonly found in the water column and in oysters. Interestingly, the number, type and size of microplastics taken up by oysters along the transect did not significantly differ between depths, which may be because the various depths that oysters were cultured at were not marked enough to influence microplastic uptake. In this study, oysters were cultured at depths ranging from 0-1.7 m, which closely resembled the 0-2 m reported in *Crassostrea gigas* cultures in Gosung Bay, Korea (Ngo et al., 2006); oysters in that study experienced higher growth rates, gonadal maturity and fecundity than those at 3-5 m depths. Amongst the sites in the present study, a notable difference was observed in site 5, in which oysters at deeper depths (1-1.15 m) took up more than five times the number of microplastics than oysters at shallower depths (0-0.35 m); this is primarily due to the high number of microplastic fibres taken up by two specimens at that depth (40 and 41 fibres respectively). The two oyster specimens in question had the shortest shell length amongst all oyster specimens (33.4 mm and 32.6 mm), and it may be that they had not fully developed adult capabilities for processing and selecting between particles (Cannuel and Beninger, 2007).

The findings from this study also suggested that the size of oyster does not influence the amount of microplastics taken up, and this was indicated by the lack of any significant relationship between the number of microplastics taken up and the corresponding wet weight, dry weight and shell length of the respective oysters. It is possible to infer from this that microplastics do not accumulate in oysters over time

(otherwise microplastic concentrations would increase as oysters grow in size), and that microplastic uptake is not directly proportional to the volume of water filtered (given that larger oysters are expected to filter more water) (National Research Council, 2004). It may be that plastic uptake was more related to chance encounters than the volume of water filtered by oysters.

The trends observed in water and oyster samples suggest that microplastic inputs from the Pearl River Estuary might be higher than those from local rivers/nullahs in the bay (such as Shenzhen River and Dasha River), given that existing evidence has shown that the Pearl River Estuary and local rivers have a stronger influence on the outer and inner parts of the bay respectively (Aiguo Qian, 2003). However, a recent study argued that the high daily discharge volume of local municipal and industrial sewage may offset the Pearl River Estuary's relatively low discharge in transporting microplastics into Deep Bay (Tsang et al., 2017). They attributed this to their observations of more microplastics in surface waters in the outer part of the bay compared with the inner part of the bay, which were in line with my observations, but they observed more microplastics in sediment in the inner part of the bay compared with the outer part of the bay, whilst no clear trends between sites were observed in my samples. The significance of the Pearl River Estuary's role in transporting microplastics into Deep Bay is potentially influenced by its course. The Pearl River Estuary runs through a number of large plastic-producing cities, including Foshan and Dongguan where plastic production exceeded 2.8 million and 1.2 million tonnes in 2015 respectively (佛山市统计信息网, 2016); it was also recently ranked as a catchment area with the 7th highest load of mismanaged plastic waste per year (Schmidt et al., 2017). The potential significance of the Pearl River Estuary's role in

transporting microplastics to Deep Bay is also supported by a study which found a close relationship between economic status and types of industry in areas adjacent to three urban estuaries located in heavily populated and industrialised areas in China – Jiaojiang, Oujiang and Minjiang (Zhao et al., 2015). The authors indicated that the Jiaojiang estuary flows through Taizhou city, where over ten thousand plastic enterprises are situated, and this is suspected to contribute to a significant amount of anthropogenic debris.

Where the Pearl River Estuary meets Deep Bay, stratification and mixing between freshwater (1.00 g cm^{-3}) and saline (1.03 g cm^{-3}) environments would likely determine the movement of plastic debris (Wong et al., 2004). A plume front in the estuary is formed by the interaction between these two environments, and they are known to have low circulation and high sedimentation rates (Zhang, 2017). Organic and inorganic particles within the plume fronts may potentially interact with microplastics' density, size and charge, which would increase the aggregation and deposition of microplastics, and also accelerate the accumulation rates of floating and settling plastics (Besseling et al., 2017). Plume fronts in Deep Bay may have disrupted the movement of microplastics from one end of the bay to the other, a phenomenon which was indicated in a study in the Goiana estuary (Lima et al., 2015). The authors described that in dry seasons, upper and middle parts of the Goiana estuary is a transitionary zone for freshwater and saltwater, generating turbulence and stratification which subsequently hinders the movement from the upper part to the lower part of the estuary, and vice versa.

Plastic pollution of freshwater and marine environments are interlinked, given that a significant proportion of plastic comes from land-based sources, and are transported via rivers and estuaries via processes such as flushing - by a recent estimate, rivers transport 1.15-2.41 million tonnes of plastic waste into the ocean every year (Lebreton et al., 2017). The role of rivers and estuaries as transporters of plastics has been supported by a model demonstrating the transportation of 200-250 μm PE and PVC microplastics from freshwater and estuarine environments into marine environments over a 360 h time frame (Bakir et al., 2014b), and has also been supported by a study which observed the near equal number of microplastics entering and leaving the Tamar River Estuary via rivers (Sadri and Thompson, 2014). However, there is also evidence which suggests that plastic debris can be immobilised in freshwater environments, with documentations of entrapment on river banks (Hoellein et al., 2014; Klein et al., 2015) and river bed sediments (Wagner et al., 2014); this indicates that they may also act as potential sources and sinks of microplastics. With regard to the Deep Bay environment, the specific inflow/outflow of microplastics to and from the Pearl River Estuary and local river catchments are yet to be known, but the data from the environmental samples collected in this study suggests that they might play active roles in transporting microplastics into the bay.

It has been suggested that a large proportion of microplastics found in Hong Kong waters come from microbead-containing personal care products that pass through wastewater treatment facilities (Tsang et al., 2017). In Hong Kong, an estimated 9.4 billion microbeads reach coastal waters every day, primarily due to the ineffectiveness of sewage treatment systems in removing microplastics (Cheung and Fok, 2016). For example, a primary treatment screening in a sewage treatment plant in Wanchai, Hong

Kong utilises a mesh size of >6 mm, which would allow microplastics to pass through at ease (Gulkowska et al., 2008). The data from the present study indicated that spherical-shaped microplastics were ubiquitous in the environment as they were found in all environmental and biological samples. The majority (57%) of microplastics in benthic sediment consisted of plastic pellets, which were 17 times as prevalent as microplastic fibres, and more biofouling may have occurred on microplastic pellets, leading to them to sink and eventually deposit on the benthic sediment. Conversely, in water samples and oyster specimens, the majority of microplastics identified consisted of microplastic fibres, which were respectively 36 times and 17 times more prevalent than microplastic pellets, potentially due to their greater propensity to float within the water column. A potential source of these microplastic fibres may have been from synthetic fibres that had entered the sea through urban runoff and wastewater discharge pathways, due to the ease in which they can pass through wastewater treatment plants (Rochman et al., 2015). Synthetic fibres could likely include those from washing clothes – for example, it has been estimated that 700,000 fibres are released from an average 6 kg wash of acrylic fibre (Napper and Thompson, 2016). They might also have been from fragments from ropes used to culture oysters with, or other components used in oyster rafts.

There was no clear relationship between concentrations of microplastics found in the surrounding water column, benthic sediment and oysters between sites, and this may have been due to a combination of factors such as the influence of differences in polymer type, size and shape on microplastics' distribution in the water column; oysters' feeding efficiency in selecting, retaining and rejecting particles (Long et al., 2015); the shallower depth of water in the inner part of the bay and deeper depth in

water toward the outer part of the bay; and the higher concentrations of algae in the inner part of the bay (Aiguo Qian, 2003).

To build upon the results from this investigation, future studies could account for seasonal variation which would be useful to find out whether hydrographic differences may influence microplastic distribution and abundance in the sediment and water in the bay. As oysters are generally cultured in the bay for a one-year period before being marketed (Fishermen, pers comm), seasonal comparisons on microplastic uptake in *Crassostrea hongkongensis* would provide greater insights on dry/wet season impacts. Due to the potential influence that size and density of plastic polymers may exert on plastics' movement and residence in marine compartments – including sediment on the shore and seafloor, surface water, water column, soft shores and rocky shores (Hidalgo-Ruz et al., 2012), it is recommend that future studies should ensure that multiple marine compartments are sampled, in order to give a more realistic picture on the extent of plastic pollution in a particular area. Setting a standardised methodology for comparison is highly important, as current studies in Hong Kong have reported seasonal variation - one recent study showed there to be considerably more microplastics in Hong Kong during the wet season (Cheung et al., 2016), whereas another study showed the contrary to be the case (Tsang et al., 2017). These differences can partly explained by differences in site selection, sampling methodology and sampling period (Fok and Cheung, 2015; Tsang et al., 2017). Difficulties in making comparisons between studies has been a commonly cited issue due to differences in detecting, isolating and identifying microplastics, and a move towards adopting standardised methodologies has been recommended (Lusher et al., 2016; Twiss, 2016).

Chapter 3: An investigation into whether shape, size, and polymer type influence microplastic uptake by *Crassostrea gigas*

3.1 Introduction

Given the ubiquity of microplastics in the environment – approximately 5.25 trillion particles on the sea surface according to one estimate (Eriksen et al., 2014) - in their many forms, types and sizes, the uptake of these particles by marine organisms and subsequent biological impacts is a growing concern. The major types of plastics used in packaging include PE, PP, PS, PET and PVC, and these are likely to end up in the environment following their disposal (Andrady, 2011). A recent study provided evidence that since 1950, the largest groups of non-fibrous plastic production are PE (36%), PP (21%) and PVC (12%), and the largest groups of fibrous plastic production consist of polyester (77%) (most of which is PET); approximately 6,300 tonnes of plastic waste has been generated from 1950 to 2015, of which 79% have accumulated in landfills or the natural environment (Geyer et al., 2017).

Of the plastics found in the natural environment, a variety have been found in marine specimens in the field, including modified acrylic, polypropylene, viscose, polyester and acrylic taken up by deep sea dwelling Cnidaria, Echinodermata and Arthropoda in the mid-Atlantic and SW Indian Ocean (Taylor et al., 2016); PE, PP, PVC, PET and nylon particles taken up by a humpback whale (*Magaptera novaeangliae*) in the Netherlands (Besseling et al., 2015); PE pellets in larvae and juveniles of the winter flounder *Pseudopleuronectes americanus* in the North Atlantic Ocean (Carpenter et

al., 1972); PE and PP filaments in the decapod crustacean *Nephrops norvegicus;* and cellophane, polyethylene terephthalate and polyester in the mussel *Mytilus edulis* in Chinese coastal waters (Li et al., 2016). Though the polymer types of microplastics found in Deep Bay *Crassostrea hongkongensis* (Chapter 2 of this thesis) have yet to be identified through FTIR analysis, fibres, fragments and pellets were present across collected specimens.

This environmental data confirms that marine microplastics are actually a complex mixture of many shapes, sizes and types of plastic particles, yet little is known of how these factors influence uptake into organisms. This complex mixture is due to the constant change in size, shape, charge, density, surface chemistry and other properties of microplastics as a result of weathering, mechanical action, degradation, oxidation and other similar processes (Andrady, 2011; Gewert et al., 2015). Microplastics' properties (such as the ones mentioned above) as well as factors such as the density of seawater and extent of biofouling in the surrounding environment are likely to influence the types of plastics that are distributed in the water column (Galloway et al., 2017; Turner, 2015). By this reasoning, benthic-dwelling organisms and surface-dwelling organisms for instance, may be exposed to a broad variety of plastic types during their lifetime.

Feeding mode may influence microplastic uptake; for bivalves, a group of suspension filter-feeders which filter large volumes of water, this uptake (and their sorbed contaminants) is potentially very high (Sussarellu et al., 2016; Van Cauwenberghe et al., 2015; Wegner et al., 2012). In order to filter through particles that they come into contact with, oysters have intricate particle selection processes – their feeding is

predominantly controlled within the pallial complex, which consists of a single pump housed in an inhalant chamber that is provided with an inhalant aperture and an exhalent aperture, and both apertures are separated by the ctenidium (Bernard, 1974). As particles enter the mantle cavity through the inhalant aperture, an initial sorting phase occurs where the velocity of the feeding current slows and heavier particles (such as minerals) are settled out by gravity, gathered and carried by mucus on the mantle surface and rejected as pseudo-faeces (Bernard, 1974). A second particle sorting process then occurs in two phases: segregating inorganic particles from nutritious particles and selectivity over seston based on their available nutritious content (Bayne et al., 1977; Bernard, 1974). When particles are rejected, they are coated with mucus and bound with other inorganic particles together before being expelled through oysters' gills and labial pulps as pseudo-faeces; when seston are preferentially selected, they are carried in mucus to the labial palps, and subsequently passed to the mouth for ingestion, digestion and assimilation (Cognie and Barille, 1999).

Particle capture occurs when then particles come into contact with, and are retained by ctenidial filaments; this process is facilitated through the use of latero-frontal cirri- and cilia-generated currents (Riisgård et al., 1996). These capture processes within the cirri and cilia greatly increase particle-retention efficiency and are also known to be aided by active mechanisms and passive processes. Active mechanisms include the redirection of particles to acceptance or rejection tracts based on muscular contractions in the feedings organs, or changes in the activity of cirri and cilia covering these organs due to the latter's stimulation by qualitative aspects of particles such as the presence of epicellular metabolites (Ward and Shumway, 2004). Passive

processes include the selection of particles based on their structures and morphology, and the detection of physiochemical interactions on parameters such as wettability or electrostatic charge between particles and feeding organs (Ward et al., 1991). However, at the level of the ctenidial filaments, particle selectivity is not as effective – video endoscopy showed that natural particles within the size range of seston (4 – 5 µm (Møhlenberg and Riisgård, 1978; Riisgård, 1988)) usually consumed upon by *Crassostrea gigas* would only be qualitatively selected (and rejected) by the oyster if the particle dimension/size allowed the particle to pass through the ctenidial filaments (Cognie et al., 2003). Both large and small particles that were not able to enter oysters' ctenidial filaments were selected upon by the labial palps; larger particles >70 µm (including inedible particles) were able to be transported along the edges of the filaments and grooves at the edges of the gills (Cognie et al., 2003). Given that microplastics above and below this size have been reported in the environment – ranging from <100 µm to >1000 µm in water and sediment samples collected in Deep Bay (Chapter 2 of this thesis), ranging from <100 µm to >500 µm in size in Lagoon sediments in Venice (Vianello et al., 2013), and ranging from <100 µm to >1000 µm in size in sub-surface seawaters of the north-eastern Pacific Ocean and coastal British Columbia (Desforges et al., 2014) - microplastics taken up by oysters in their natural environment may be able to bypass their innate selection and rejection processes, and indeed, this may account for the microplastics found in digested oyster tissue collected in Deep Bay (Chapter 2 of this thesis) as well as microplastics found in other field studies (Leslie et al., 2017; Li et al., 2016; Van Cauwenberghe et al., 2015).

As a general rule, the capture efficiency of particles increases non-linearly with increasing particle size to a maximum, a (Ward and Shumway, 2004). A study on the

retention efficiency of 13 bivalve species through sampling water from the inhalant and exhalant regions of bivalves showed that retention efficiency decreased by size and was species-specific, but all bivalves could capture particles larger than 6 μm at 100% efficiency (Møhlenberg and Riisgård, 1978). Similarly, field experiments using high seston concentrations of 64.4 mg L^{-1} showed that *Crassostrea gigas* were only able to capture particles >12 μm at 100% efficiency (Barillé et al., 1993). This retention efficiency is reportedly partly dependent on the complexity of cirri and cilia of laterofrontal tracts in bivalves (Shumway and Parsons, 2016), which induce lateral flows to reduce particle loss and to increase the probability of encountering particles with the frontal surface of gill filaments (Ward et al., 1998). Selective feeding allows bivalves to maximise their nutritional intake - for example, when offered a variety of algal species to feed on, *Crassostrea gigas* and *Mytilus edulis* samples have been shown to be able to discriminate between algal species in order to maximise their energy and nutritional intake (Bougrier et al., 1997). The authors found that algae were selected independently of their size, volume or carbon content, but selected primarily on the basis of their organic content. Other known parameters affecting the efficiency of feeding on organic matter are the size and concentration of seston made available to oysters (Barille et al., 1992; Zhang et al., 2010), the level of turbulent mixing within the water column (Troost et al., 2009), the inhalant and exhalant current velocities in the organism (Troost et al., 2009), as well as the presence of inorganic particles such as plastics (which potentially dilute available organic matter) (Newell and Jordan, 1983).

There is some tentative evidence of microplastic size influencing the uptake of microplastics into bivalves. In a laboratory study on *Crassostrea gigas* oysters,

specimens were exposed to virgin micro-PS pellets of (2 µm and 6 µm; 0.023 mg $L^{-1)}$ for two months during their reproductive cycle, and it was observed that in addition to taking up a greater proportion of the 6 µm microplastics, exposed oysters consumed significantly more microalgae and experienced significantly higher levels of absorption efficiency (Sussarellu et al., 2016). Similarly, in a two-week exposure study, *Mytilus edulis* mussels were exposed to PS microspheres of (10 µm, 50 particles mL^{-1}; 30 µm, 50 particles mL^{-1} ; and 90 µml,10 particles mL^{-1}); the authors found that of the three particle sizes, 10 µm particles were the most commonly detected in mussel tissues at 2.6 ± 0.4 (SD) particles g^{-1}, whilst there was no evidence of uptake for 30 µm and 90 µm microplastics (Van Cauwenberghe et al., 2015). Based on this information, the authors suggested that the size limit for particle retention was between 10 µm – 30 µm.

With regard to polymer shape, some evidence has shown that it plays a role in influencing microplastic uptake in some species of fish. A field study that investigated the composition of microplastics in the digestive tracts of Japanese anchovy (*Engraulis japonicus*) collected in Tokyo Bay found that most consisted of PE (52.0%) or PP (43.3%), most were fragments (86.0%) (whilst beads were the least common at 7.3%), and 80% were between 150-1000 µm (of which 50% were <500 µm) (Tanaka and Takada, 2016). The plastics found were different from those reported in surface seawaters around Japan, where most microplastics were >1000 µm, dominating over the relatively low numbers (~20%) of small microplastics (<1000 um) found (Isobe et al., 2015). In a laboratory study, Goldfishes' (*Carassius auratus*) exposure to 50 microfibres (50-500 µm) or 50 microbeads (unknown size) for up to 144 hours revealed that the transition to, and retention of both plastic types in the gut were similar, with 1-

3 microfibres and 0-3 microbeads recovered after the 144 hour time point (Grigorakis et al., 2017). With regard to polymer type, there is some evidence that their density influences microplastic uptake in the fish mullet *Mugil cephalus* – exposure to PE or PS particles between 0.1 and 1 mm at a concentration of <2500 particles L^{-1} for a 7 day period revealed that the number of measured items in the gastrointenstinal tract was significantly higher in fish exposed to PS compared with PE microplastics (Avio et al., 2015b). This was suspected to be because the PE (0.92 g/cm^3) supplied was more buoyant (thus, having a higher tendency to re-float) than the PS supplied (1.04 g/cm^3) (which remained more uniformly distributed in the water column), enhancing the probability of the former being ingested by fish.

In the previous chapter, the types and sizes of microplastics taken up by *Crassostrea hongkongensis* oysters in Deep Bay were not directly proportional to those found in the natural environment (i.e. the water column and sediment), which suggests that there may be some influence of size, shape and/ or polymer type on the uptake of microplastics into this species. The aim of this experiment was to test this hypothesis and to investigate whether the properties of plastics – specifically their shape, size and polymer type would influence *Crassostrea gigas (*a genetically similar oyster) oysters' biological uptake of microplastics.

3.2 Methodology

In order to investigate how shape, size and polymer type of plastics influences the biological uptake of microplastic particles in *Crassostrea gigas* oysters, a 24 hour (24H) exposure to a mixture of different microplastics was performed. Oyster specimens were collected from the beach at low tide at Starcross Bay, Dawlish (50°37'07.2"N,

3°26'51.6"W) in April 2017, and upon return to the lab, each shell was carefully scraped using a scraper to remove any attached barnacles. Oysters were kept in a large aquarium tank containing artificial seawater at 15 °C in a temperature-controlled room and fed a daily diet of 5 mL Shellfish Diet 1800®. A 200 L water carboy was used to create artificial seawater with DI and TropicMarine artificial sea salt to a salinity level of 34 PSU and pH_{NBS} of 8.1. In preparation for the experiment, 100 mL of *Isochrysis galbana* (Tahitan strain, T-iso) was cultured for 10-14 days in autoclaved conical flasks in a 15 °C room, and the algae was supplied daily with Guillard's (F/2) marine water enrichment solution (prepared at 20 mL L^{-1} of filtered artificial seawater). Algal concentration and size was determined through the use of a Multisizer™ 3 Coulter Counter®.

To test whether shape, size and polymer type influences the biological uptake of microplastic particles by the oysters, a selection of different microplastics were chosen and combined to create a complex mixture of microplastics, with the aim to reflect the range of commonly recovered plastic types, shapes and sizes from environmental samples (Andrady, 2011; Cole et al., 2011a; Duis and Coors, 2016; Rochman et al., 2013b). The microplastics selected for use were: (1) polystyrene (PS) beads (8-12 μm), (2) polyethylene (PE) beads (8-30 μm), (3) polyamide (PA) fibres (10-50 μm), (4) polyethylene terephthalate (PET) beads (247-390 μm), (5) nylon 6,6 polyamide (PA) fibres (500 μm), (6) nylon 6,6 PA fibres (1000 μm), (7) polyvinyl chloride (PVC) fragments (115-156 μm) and (8) high-density polyethylene (HDPE) fragments (500-1000 μm) (Table 3.1). These were purchased from Cospheric (http://www.cospheric.com) or Goonvean (http://www.goonveanfibres.com). A total

concentration of 100 microplastics mL^{-1} was used with each microplastic type present in equal ratios (i.e. 18,750 microplastics per 187.5 mL seawater for each plastic type). Initially, plastics were coated with 0.01% Tween which was subsequently washed off with Milli-Q® to facilitate mixing of hydrophobic/buoyant microplastics. For staining, Nile Red stock solution was made by preparing 1 mg mL^{-1} in acetone, and microplastics were placed on filter paper and stained using 200 μL of Nile Red working solution (10 μg mL^{-1}) following an approach by (Maes et al., 2017), before being left to incubate for 30 minutes. Stained microplastics were then mixed with Milli-Q® into eight individual falcon tubes. Concentrations were checked by vortexing the falcon tubes, pipetting three replicate 1 mL subsamples from each falcon tube into individual petri dishes, drying the samples in a 60 °C oven and enumerating them under a fluorescent microscope (Olympus SZX16 with Prior Lumen 200) under green light at an excitation of 365 nm and emission wavelength of 470 nm. When the number of microplastics per mL had been established, each of the plastic samples were mixed with Milli-Q® again to formulate a final concentration that would result in 100 microplastics per mL in 1.5 L seawater.

Table 3.1: Table showing the types and sizes of plastics used for the 24 hour exposure.

Plastic Type	**Size (μm)**
PS beads	8-12
PE beads	8-30
Nylon 6,6 PA fibres	10-50
PET fragments	247-390
PVC fragments	115-156
Nylon 6,6 PA fibres	500
Nylon 6,6 PA fibres	1000
HDPE fragments	500-1000

When conducting microplastic exposure experiments, it is important to use environmentally realistic concentrations. It has been pointed out that a number of papers have used concentrations that are orders-of-magnitude higher than those reported in field studies, therefore, conservative amounts (100 microplastics mL^{-1}) were used in this study, and this was based off data retrieved from (Lenz et al., 2016).

A randomised design of “algae only”, “plastic only” and “plastic and algae” treatment groups was used – each specimen across all treatment groups (8 oysters per treatment group) was allocated a random number from 1 to 24 generated from a random number generator. “Algae” was a plastic-free control treatment, “plastic only” was an exposure to plastics only, whilst “plastic and algae” was an exposure to the combined concentrations of “algae” and “plastic” treatments respectively. Both treatments with algae contained 0.75 x 10^6 cells mL^{-1} of cultured *Isochrysis galbana* (Tahitan strain, T-iso), at a size of 3-6 μm, and both treatments with microplastics contained a final concentration of 100 microplastics mL^{-1} (18,750 microplastics per 187.5 mL seawater for each plastic type). The experimental treatments incorporated the use of magnetic stirrers and followers to keep individual beakers well-stirred, which helped prevent clumping at the bottom or the sides of beakers, and to minimise potential effects of particle density. As a precaution, aluminium foil was used to cover the tops of each beaker to prevent splashes and resulting cross-contamination. Specimens were exposed to their respective treatments for 24 hours at 15 ºC.

Following the exposure, oysters were snap frozen and then kept in a -20 °C freezer until later analysis. For analysis, the tissue of the oysters were collected with a pair of

tweezers and scalpel and placed in 50 mL falcon tubes, before being left to digest for 24H in 10% KOH at 60 °C. Digestant was then vacuum filtered through 5 μm gridded nitrocellulose filter paper. To enumerate microplastics, filter paper was placed on a fluorescent microscope (Olympus SZX16 with Prior Lumen 200) under green light at an excitation of 365 nm and emission wavelength of 470 nm, so that any background contamination of microplastics from the air, equipment and other processes could be discounted; for this reason, no procedural blanks were used, and no FTIR analysis was required to identify plastics in digestant.Three 1 x 1 mm grids were sub-sampled for each filter paper and the average number of each microplastic type per grid was photographed and analysed with Image J software (V 1.50) and extrapolated for overall analysis.

A general linear model (GLM) was used to test the effect of polymer type (8 in total) and plastic size (“small” ≤50 μm, “medium” = 51-499 μm, “large” ≥500 μm) on uptake; these two factors were concatenated as part of the analysis. Plastic shape was not included as a factor in the model due to the lack of overlap between the factors of polymer type and size. A quasipoisson distribution was used (to account for over-dispersion) and the presence of microplastics (True/False) and presence of algae (True/False) were nested into the model to account for treatments of “plastic”, “algae” and “plastics and algae”. Chi-square tests ($p<0.05$) were used to test the variables that had a significant effect on the model, and non-significant effects were eliminated from the model. A post-hoc Tukey test ($p<0.05$) was used on the final model to compare significant differences between treatment types.

3.3 Results

An average of 3.88 ± 0.37 (SE) microplastics were found in oysters in the “algae” control group, an average of 118.13 ± 9.29 (SE) microplastics were found in oysters in the “plastic” group and an average of 117.38 ± 8.83 (SE) microplastics were found in oysters in the “plastic and algae” group (Fig. 3.1). In the “algae” control group, small amounts of PE beads (8-30 μm), PVC fragments (115-156 μm) and PA fibres (500 μm) were found in digested oyster tissues, and amongst these three plastic types, PVC fragments were taken up the most (Fig. 3.1).

In the “plastic” group, the dominant plastic types found in oysters were PE beads (8-30 μm) (0.29% of total added microplastics of this type) and PVC fragments (115-156 μm) (0.31% of total added microplastics of this type), and other plastics taken up in oysters included PA fibres (10-50 μm) (0.02% of total added microplastics of this type), PET fragments (247-390 μm) (6.7×10^{-4}% of total added microplastics of this type), PA fibres (500 μm) (9.3×10^{-3}% of total added microplastics of this type), HDPE fragments (500-1000 μm) (1.3×10^{-3}% of total added microplastics of this type) and PA fibres (1000 μm) (2.7×10^{-3}% of total added microplastics of this type) (Fig. 3.1).

In the “plastic and algae” group, the dominant plastic types found in oysters were also PE (8-30 μm) (0.25% of total added microplastics of this type), and PVC (115-156 μm) (0.32% of total added microplastics of this type). Other plastics found in oysters were PA fibres (10-50 μm) (0.039% of total added microplastics of this type), PET fragments (247-390 μm) (2.7×10^{-3}% of total added microplastics of this type), PA fibres (500 μm)

(0.012% of total added microplastics of this type) and PA fibres (1000 μm) (2.0 x 10^{-3}% of total added microplastics of this type) (Fig. 3.1).

There was a significant effect of plastic type on microplastic uptake in oysters. Chi-square tests yielded significant interactions between the presence of microplastics and polymer type/size (Pr (>Chi)=0.034) and between the presence of algae and polymer type/size (Pr (>Chi)=0.002) on the number of microplastics taken up by oysters at a 95% confidence interval. A post-hoc Tukey test on the resulting GLM model showed that small-sized PE beads and medium-sized PVC fragments ("Group B") were significantly ($p<0.05$) taken up more than large-sized PA fibres ("Group A"); small-sized PS beads, medium-sized PE beads, large-sized HDPE fragments and small-sized PA fibres ("Group AB") were not significantly different from the other two groups at a 95% confidence interval (Fig. 3.1 and Table 3.2).

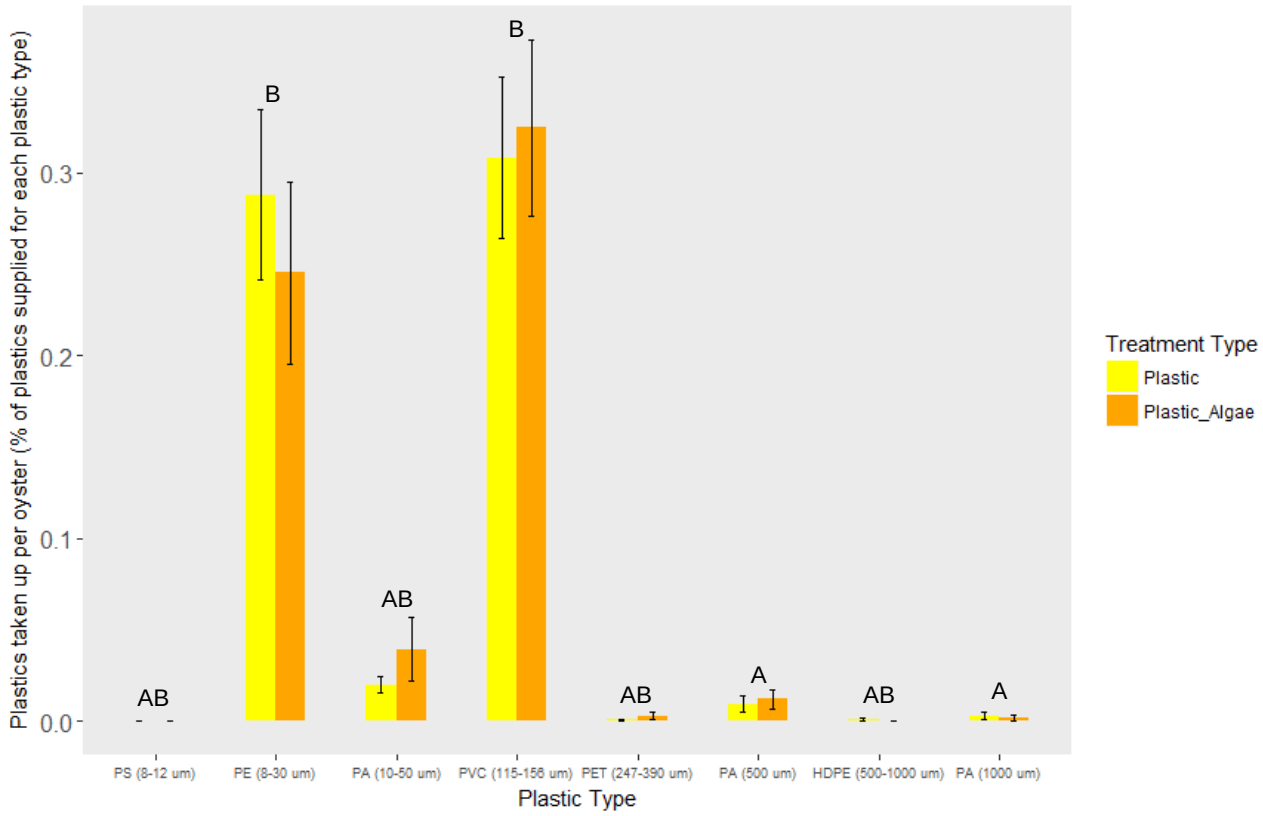

Figure 3.1: Plastics taken up per oyster, expressed as a percentage of the total number supplied of the respective plastic type/size, and categorised according to treatment type. Given that only small amounts of microplastics were found in the "algae" control treatment, data for this treatment is not included. Standard error shown in error bars and labels "A", "B" and "AB" refer to post-hoc Tukey test groups (Table 3.2).

Table 3.2: Table showing the results of the post-hoc Tukey test.

Polymer Type/Size	**Mean**	**Standard Error**	**Group**
PS/small	3.05e-08	2.65e-05	AB
PET/medium	6.18e-05	0.04	AB

HDPE/large	8.75e-05	0.05	AB
PA/small	3.39e-04	0.21	AB
PA/large	0.46	0.14	A
PE/small	6.36	1.31	B
PVC/medium	12.60	1.38	B

3.4 Discussion

The results from the present study indicated that microplastics were readily taken up by *Crassostrea gigas* oysters, and selectivity was based on polymer types - primarily to PE and PVC, plastic sizes – primarily to “small” plastics ≤ 50 µm and “medium” plastics 51-499 µm, and plastic shapes - primarily beads and fragments. With regard to particle size, *Crassostrea gigas* oysters’ selectivity for particle size in this 24H exposure were in agreement with the similar findings from Chapter 2 of this thesis, which showed that 71% of microplastics taken up by *Crassostrea hongkongensis* oysters were <500 µm in size.

The mechanism for capturing, sorting and rejecting particles based on their size has been suggested to be based on a mechanical sieving and redirection mechanism by bivalves’ laterofrontal cirri, which capture particles and redirect flow towards frontal surfaces of the filament located on the ctenidium for retention in a layer of mucus (Tammes and Dral, 1956; Ward et al., 1998). The size of particles that are captured by oysters extend from 4 µm to >250 µm (Cognie et al., 2003), and a greater proportion of larger particles are reportedly rejected compared to smaller particles (Tamburri Mario N. and Zimmer-Faust Richard K., 1996). This study showed that larger plastics were less readily taken up, with microplastics up to 156 µm being readily taken up,

after which the number of microplastics taken up declined, but was still evident for particles up to 1000 µm - above and beyond the typical size range of particles taken up by oysters; this observation is also supported by the fact that relatively low numbers of large PA fibres (500 and 1000 µm) were taken up compared with smaller PA fibres (10-50 µm). On the other end of the size spectrum, the smallest microplastics supplied as part of the experimental exposure were 8-12 µm PS beads, and none of these beads appeared to be taken up by oysters. This was an interesting finding, given that other studies have reported *Crasssotrea gigas* oysters to ingest microplastics between 2 and 6 µm (Sussarellu et al., 2016) and *Mytilus edulis* mussels to ingest particles between 2-16 µm in size (Browne et al., 2008; J. E. Ward et al., 2003; Ward and Kach, 2009; Ward and Targett, 1989). It is likely that the sizes of microplastics more readily taken up by *Crassostrea gigas* oysters in the present study was due to a combination of oysters taking up smaller microplastics and rejecting larger microplastics.

Another possible explanation behind the sizes of microplastics taken up *Crassostrea gigas* oysters is that they may have captured smaller particles with less efficiency and were less able to discriminately select and sort through them (Ward and Shumway, 2004), as supported by studies which report decreased retention efficiencies below 6-10 µm (Heral, 1985) and 6 µm (Møhlenberg and Riisgård, 1978; Riisgård, 1988). The low uptake of microplastics on both ends of the size spectrum of supplied plastics, and the relatively high concentrations of microplastics ranging from 8-156 µm taken up by oysters indicate that feeding occurred across a wide size range, and other factors may have played a stronger role in influencing plastic uptake. The influence of particle size on microplastic particle uptake in bivalves was also evidenced in *Crassostrea*

hongkongensis oyster specimens collected from Deep Bay (Chapter 2 of this thesis), whereby 41% and 30% of particles taken up by oysters were <100 μm and 100-500 μm in size respectively, but a fair proportion (30%) of particles taken up were above 500 μm in size. Feeding across a wide spectra of microplastic size ranges has also been demonstrated in laboratory exposures on organisms such as *Palaemonetes pugio* daggerblade grass shrimp (30–165 μm) (Gray and Weinstein, 2017) and freshwater zooplankton crustacean *Daphnia magna* (62-1400 μm) (Newell et al., 1989).

It has previously been suggested that the density of particles and hence their buoyancy, may play a role in influencing *Crassostrea gigas* oysters' uptake of particles, as they are able to sort between lighter organic particles and heavier inorganic particles before they reach the ctenidia and labial palps (Bernard, 1974). Though density was not included as a factor in our statistical analyses due to the inconclusiveness of specific density values for the plastics supplied, it is unlikely that the density of plastics played a significant role in influencing microplastic uptake. Out of the plastics supplied as part of the experimental exposure, PE (LDPE and HDPE) was of the lowest density (~0.92-0.97 g/cm^3), followed by PS (~1.04-1.1 g/cm^3), PA (~1.13-1.35 g/cm^3), PET (~1.37-1.45 g/cm^3) and PVC (~1.16-1.58 g/cm^3) (Galloway, 2015; "Nylons (Polyamide)," 2018). PE plastics are typically low-density and relatively buoyant compared with other plastics, whereas PVC, PET and PA plastics are typically higher-density plastics that are more likely to sink (Browne et al., 2007); yet, plastic uptake was still considered significant for these plastic types. Furthermore, the experimental treatments incorporated the use of magnetic stirrers and followers to keep individual beakers well-

stirred, which helped prevent clumping at the bottom or the sides of beakers, and to minimise potential effects of particle density. The influence of particle density on the uptake in bivalves has also been played down by findings by (Taghon, 1982). In a field experiment the authors conducted in <2 cm shallow tide pools, the bivalve *Macoma balthica* was exposed to protein-coated glass beads (44-62 μm and 105-124 μm; roughly equal numbers of each bead size) placed directly on the sediment, and results showed that they selectively took up protein-coated glass beads regardless of whether they had lower specific gravity (lower γ_s=2.42) or a higher specific gravity (lower γ_s=2.99) values. In oceanic environments, where water depths can extend to thousands of metres deep, the density of particles may play a stronger role in influencing their distribution in the water column (National Oceanic and Atmospheric Administration, 2017). Buoyant microplastics, for example, were largely found in the top 0-5 m of the North Atlantic subtropical gyre, with concentrations approaching zero at 5 m, indicating that most microplastics were found at or near the surface (Kooi et al., 2016). Conversely, high-density microplastics such as polytetrafluoroethylene (2.10−2.30 g cm^{-3}), polyamide (1.13 g cm^{-3}) and nitrile rubber (1.30 g cm^{-3}) were found in Arctic sediments at depths of 2,340-5,570 m, which are likely to have sunk to the seafloor directly (Bergmann et al., 2017). However, the authors of this study also found low-density microplastics such as PE and chlorinated PE in sediment samples, which indicated that they may have been subject to processes such as biofouling, wind-mixing, incorporated with sinking aggregates (such as marine snow), adsorption of clay minerals or ingestion-egestion (Andrady, 2011; Cole et al., 2011a; Cozar et al., 2014; Lobelle and Cunliffe, 2011; Long et al., 2015). Particles may also be subject to re-suspension or redistribution in the water column through vertical mixing driven by turbulence and storm activity (Cole et al., 2011b; Lattin et al., 2004).

Plastic shape also influences microplastics' buoyancy in the water column, with fibres and fragments with greater surface areas found more commonly in the water column compared with spherical-shaped plastics (Chubarenko et al., 2016). In the present study, beads and fragments were observed to be the two dominant shapes taken up by oysters. Though algal particles vary heterogeneously from spherical-shaped to complex-shaped particles, beads and fragments resemble them more closely compared with fibres (Stephen et al., 2010), likely allowing them to pass through oysters' ctenidial filaments (Cognie et al., 2003). Despite the relative ease in being taken up in a controlled (laboratory) environment, the fact that 84% of microplastics found in *Crassostrea hongkongensis* oysters in Deep Bay (Chapter 2 of this thesis) consisted of fibres, compared with fragments (12%) and beads (5%) is an indication that oysters' contact with plastic beads/fragments in the natural environment were likely to be less than with fibres, and subsequently lowered their chances of taking the former plastic shapes up. The lack of contact is suspected to have been linked with plastic beads' propensity to sink more quickly (due to the relatively high proportion found in benthic sediment) compared with plastic fibres. Particle properties such as density and environmental factors such as vertical mixing may have influenced plastics' distribution in the water column.

Though there is currently only a limited amount of literature on how polymer type specifically influences plastic uptake, evidence has shown that some bivalves selectively take up particles based on surface properties such as wettability or surface charge. For example, *Crassostrea virginica* oysters and *Mytilus edulis* mussels were

shown to ingest PS plastics and reject aluminium oxide particles of the same size, and this was suspected to be due to a combination of particles' respective wettability and charge (Rosa et al., 2013); *Mytilus edulis* mussels were shown to reject ectotrine treated octadecyl silane (ODS-2) particles and to accept ectotrine treated PS particles, and this was suspected to be partly due to lipophilic differences between particle types (Ward and Targett, 1989); and *Mytilus edulis* mussels were found to ingest significantly more bacteria with a reduced negative surface charge (Hernroth et al., 2000). Most plastics are characterised by smooth, hydrophobic surfaces with no net charge, but these properties change in seawater, (Galloway et al., 2017) and this may impact their interaction and ability to form hetero-aggregates with algal particles (Lagarde et al., 2016; Long et al., 2017; Wegner et al., 2012). These interactions may, in turn, affect the bioavailability of plastics and bivalves' selection of food and non-food particles, given that bivalves rely primarily upon chemosensory receptors in the labial pulps for selecting particles, and that chemical cues partially come from ectocrines on the surface of algal cells or boundary layer of surrounding cells (Ward and Targett, 1989). However, in the present study, the interaction between algae and plastic was not strong enough to result in significant differences in microplastic uptake between "plastic" and "plastic and algae" groups. A likely explanation for this is due to the short-time exposure, in contrast with hetero-aggregation formation periods such as <29 days (Long et al., 2017) and 20 days (Lagarde et al., 2016) used in other laboratory studies.

Evidence has shown that retention efficiency of particles by the laterofrontal cirri is not dependent upon size alone, but also on the morphological complexity of the laterofrontal cirri (Ward and Shumway, 2004), the concentration of seston (Barille et al., 1992; Widdows et al., 1979), particle fluorescence (Newell et al., 1989) and surface

charge of particles (Hernroth et al., 2000). Indeed, the microplastics used in our study ranged between 8-1000 μm, which did not overlap with *Isochrysis galbana* algae particles (3-6 μm), and given the lack of differences observed for the presence of algae between treatment groups, it can be inferred that uptake selectivity was not strongly affected by available concentrations of organic matter; this is consistent with observations made from other studies on *Crassostrea gigas* oysters (Barille et al., 1992; Prou, 1995; Zhang et al., 2010). As algal concentration increases, particle retention efficiency has been shown to decrease in proportion (Libini et al., 2017; Wilson, 1983), and this in turn, limits the sizes of particles that are retained at maximum efficiency to larger-sized particles (>12 μm, as reported by (Barille et al., 1992), and 30-35 μm, as reported by (Strohmeier et al., 2012)). Conversely, as the concentration of particulate organic matter decreases and the feeding efficiency becomes negative (as energetic costs of selective feeding exceed the nutrition received from ingested food), bivalves have been reported to reduce their active selection of particles (Velasco and Navarro, 2002). Would this be the case, I would have observed significant differences in particle uptake between “algae” and “plastic and algae” treatment groups.

The uptake of microplastics in oysters within the “algae” treatment suggests that there was an element of contamination in the samples, as they included plastics that were used in “plastic” and “plastic and algae” treatments. In the “algae” treatment, the mean uptake of microplastics taken up by *Crassostrea gigas* oysters amounted to 3.88 ± 0.37 (SE), equivalent to ~3.2% of the mean number of microplastics taken up by *Crassostrea gigas* oysters in the “plastic” treatment; this suggested that oysters were

highly sensitive to microplastic contamination and even able to take up microplastics at low concentrations. A suspected source of this contamination may have been from the bubbling produced from air stones. Though aluminium foil was used to cover beakers as a precaution, there were gaps in the foil which may have resulted in microplastics splashing across beakers, and indeed, splashes were observed around the beakers used in the experimental set up.

Further exposure studies under controlled conditions with varying microplastic types and factors would provide evidence about potential factors that influence selective uptake in *Crasssotrea gigas* oysters. A key limitation from this study was that each polymer type used in this study was restricted to a specific shape – PA microplastics supplied to oysters for instance, were only supplied as fibres, which restricted the model used for analysis. To overcome this limitation, it is recommended that future studies include strong overlaps between factors – including but not limited to concentration, size, shape, polymer type and organic content – in order to make it possible to draw comparisons between factors as well as their relative importance in determining oysters' biological uptake. Better yet, combining empirical evidence from laboratory exposures with field-based studies could provide further insights into what parameters of plastics oysters may be most vulnerable to in the natural environment, as well as how these plastic types influence the capture, sorting and rejection of organic and inorganic particles.

Chapter 4: Biological impacts of microplastic ingestion in the Pacific Oyster *Crassostrea gigas*

4.1 Introduction

To date, studies have shown that the uptake of microplastics can lead to a number of physical and biological impacts (Alomar et al., 2017; Besseling et al., 2015; Sussarellu et al., 2016; Wright et al., 2013c). On a physical level, when organisms' gastrointestinal tract or feeding appendages become blocked or damaged by abrasions and ulcers, this can potentially lead to inflammation, satiation, starvation and debilitation, and ultimately, death (Derraik, 2002; Gregory, 2009; Jin et al., 2018; Wagner et al., 2017). On a biological level, microplastic uptake has been reported to lead to an alteration in feeding behaviour and subsequent energy intake in the crab *Carcinus maenas* (Watts et al., 2015), the lugworm *Arenicola marina* (Wright et al., 2013b), the marine copepod *Calanus helgolandicus* (Cole et al., 2015) and the clam *Atactodea striata* (Xu et al., 2017). Algae have also been reported to experience adverse impacts on photosynthesis upon microplastic exposure – *Chlorella* and *Scenedesmus* exposed to 20 nm PS beads (>1.8 mg L^{-1}) for 24 hours experienced significant decreases in CO_2 depletion (Bhattacharya et al., 2010). Negative impacts on growth and reproduction, and increases in oxidative stress in *Crassostrea gigas* have been documented upon exposure to PS microplastics (2 and 6 µm in diameter; 0.023 mg L^{-1}) for a two month period (Sussarellu et al., 2016). The potential transfer from chemicals associated with plastics to tissues in humans has also been postulated, and these chemicals may disrupt endocrine activities and activating signal transduction pathways relevant to hormone action in the process (Diamanti-Kandarakis et al., 2008).

It has been suggested that the physical and biological impacts of microplastic exposure are likely to be affected by the accumulation capacity of microplastics, as well as the rate at which they fragment into smaller sizes (Wright et al., 2013c). Gut blockage and supressed feeding, for example, are linked with accumulation of microplastics, and evidence has shown that haemocytes' ability to phagocytose microplastics are dependent on the latter being at least below 20 µm in size (Brillant and MacDonald, 2000). Furthermore, as microplastics fragment into smaller sizes it becomes increasingly likely that they can translocated and accumulate within organism' tissues (Wright et al., 2013c). This was demonstrated by the greater abundance (>60%) of 3 µm microplastics in *Mytilus edulis* mussels' that had translocated from the gut cavity to the haemolymph compared with 9.6 µm microplastics (Browne et al., 2008).

Feeding rates can be affected by a range of abiotic (e.g. temperature) and biotic factors (e.g. concentration of food, taste) (Scherer et al., 2017), and evidence has shown that exposure to micro-PS (2 and 6 µm; 0.023 $mg \cdot L^{-1}$) for a two month period is sufficient to induce 3% increases in *Crassostrea gigas* oysters' algal consumption rates, resulting in a compensatory effect on food intake and absorption efficiency, and enhanced mechanical digestion (Sussarellu et al., 2016). The increase in food intake was suspected to be a strategy to adjust energy intake in response to digestive interference in the gut. On the other hand, an 11.2% decrease in *Calanus helgolandicus* copepods' feeding rate of *Thalassiosira weissflogii* was reported upon their exposure to PS microplastics (20 µm; 75 microplastics mL^{-1}) for a 24 hour period (Cole et al., 2015). The authors attributed this to copepods' altered feeding strategy of

shifting the size range of algal prey taken up – microplastic-exposed copepods took up microplastics between 11.6-14.8 µm in size compared with the 11.6-17.0 µm size range taken up by copepods supplied solely with algae. Similarly, clearance rates of *Atactodea striata* clams exposed to PS microplastics (63-250 µm; 1000 microplastics L^{-1}) for five days were reduced by up to approximately 80% (estimated figure from graphs as no actual figures were provided) (Xu et al., 2017). The authors suggested that this may have been an adaptive strategy to reduce the uptake of microplastics.

Microplastic exposure has also been reported to have a potential effect on respiration rate. The energy consumption (respiration) rates of *Mytilus edulis* mussels exposed to PS microplastics (10-90 µm; 110 particles mL^{-1}) for a 14 day period increased by 25% compared to control organisms (Van Cauwenberghe et al., 2015). This was suspected to be due to the increased stress that oysters had in order to retain physiological homeostasis. However, the effect on respiration has been less evident in other studies. For example, respiration rates of *Atactodea striata* clams were shown to be unaffected by exposure to PS microplastics (63 µm-250 µm; 10 or 1000 microplastics L^{-1}) for one, five or ten days (Xu et al., 2017). Similarly, *Calanus helgolandicus* copepods exposed to PS microplastics (20 µm; 75 microplastics mL^{-1}) for a 24 hour period experienced no change in active metabolic rates (Cole et al., 2015). Explanations behind these insignificant differences were not provided, but it has been pointed out that if any reductions in metabolic rate are observed, it may be an adaptive strategy when faced with starvation (Peter, 1992; Thomas, 2014).

Oxidative stress is often measured as an indicator of organismal stress response to environmental pollution. In order to maintain an internal stable state in an ever-

changing external environment, oysters possess a steady state of cellular metabolism and this is maintained by haemocytes (circulating in the haemolymph) which are involved in physiological processes such as digesting, transporting and distributing nutrients, repairing tissue and facilitating cellular defence mechanisms (Bayne, 2017). In this steady state, oxidases reduce oxygen molecules to form water, and any intermediaries produced from incomplete reduction potentially interact with reactive oxygen species (ROS) (Schieber and Chandel, 2014). These interactions can potentially lead to oxidative damage to organisms' tissue, and several biomarkers can be used to measure the level of disturbance brought to this oxidant-antioxidant complex (known as "oxidative stress") and the immunological damage an organism is subjected to. These biomarkers include superoxide dismutase (SOD) activity, lipid peroxidation (LPO), lysosomal stability, phagocytosis activity and levels of deoxyribonucleic acid (DNA) damage (Bartosz, 2009; Pulskamp et al., 2007).

As a means of inhibiting ROS reactions, SOD is produced through metal-protein complexes in oysters (McCord, 1993). A commonly used way to measure the amount of SOD produced is through a SOD assay, which involves the use of xanthine-oxidase to generate oxygen free radicals (O_2^-) and the use of nitroblue tetrazolium (NBT) to quantify this production (Weydert and Cullen, 2010). There is existing evidence that microplastic exposure can induce SOD production in bivalves – for example exposure to PS microbeads (2 and 6 µm, 32 µg L^{-1}) for 7 days resulted in subsequent increases in ROS production in *Mytilus spp. m*ussels' haemocytes; higher amounts of SOD were produced to convert O_2^- into less damaging hydrogen peroxide as a means of preventing cellular oxidative damage (Paul-Pont et al., 2016).

Another reaction induced by ROS is LPO – the peroxidation of polyunsaturated acids that occurs via a chain-reaction with free radicals, and which induces changes to the structure, functionality and permeability of cell membranes (Abuja and Albertini, 2001; Niki, 2008). LPO increases with oxidative stress, and a common way to measure the extent of LPO is with the thiobarbituric acid reactive substances (TBARS) assay, whereby the reaction between thiobarbituric acid (a reagent) and malondialdehyde (MDA) – a by-product of LPO that is produced due to unstable lipid peroxides - is measured; a pink pigment is produced and fluorescence readings are taken to quantify the amount of MDA produced (Moselhy et al., 2013). There is existing evidence that European seabass *Dicentrarchus labrax* exposed to fluorescent red polymer microspheres (1-5 µm, 0.26 and 0.69 mg L^{-1}) for 96 H can experience increases in LPO in the brain and muscle (Barboza et al., 2018).

Lysosomal stability – the stability of the cell membrane is one of the first parameters that is disrupted by organism' exposure to contaminants; it is linked with oxidative stress but also with toxicological, physiological and pathological responses (Pichaud et al., 2008). Lysosomes contain many enzymes, carry out protein degradation and detoxify some foreign compounds, and the lysosomal membrane protects the cell from the leakage of degradative enzymes (Hu et al., 2015). The neutral red retention assay is commonly used as a measurement of lysosomal stability, whereby the neutral red dye enters the cell and accumulates in lysosomes due to ion-trapping of the water soluble dye (Martínez-Gómez et al., 2008); the assay measures the decreased retention time of the dye, with higher values reflecting more stability (Svendsen et al., 2004). It has been suggested that increases in the size, volume and swelling of lysosomes, as well as increases in hydrolase activities serve as evolutionary

responses to environmental and contaminant stress due to increased autophagy (Lowe, 1988). For example, the destabilisation of *Mytilus edulis* specimens' lysosomal membranes were found to significantly heighten with exposure times of up to 96 H with HDPE particles (0-80 µm, 2.5 g particles/L) (von Moos et al., 2012).

Phagocytic activity is often measured as an indicator of oxidative stress and immune function, and there have been tentative suggestions that microplastic exposure can cause irritation and inflammation, which subsequently sets off immune response pathways including recognition, chemotaxis, attachment, ingestion and destruction (Galloway et al., 2017; Pipe and Coles, 1995). The phagocytosis assay is a common method to measure the level of phagocytic activity, whereby a known number of zyomosan particles are mixed with haemolymph and the number of zyomosan particles taken in by cells following a period of incubation give an indication of immune function (Parry and Pipe, 2004). Increases in phagocytic activity from microplastic exposure was shown to be induced in *Arenicola marina* worms exposed to unplasticised PVC microplastics (230 µm; 1% and 5% by weight) for four weeks (Wright et al., 2013a), and increases in the engulfment of microplastics by haemocytes were induced in *Mytilus* spp. exposed to PS microplastics (2 and 6 µm, 32 µg L^{-1}) for 7 days (Paul-Pont et al., 2016).

There is also evidence that contaminants can induce damage to DNA – the carrier of information - in bivalves (Almeida et al., 2011; Gomes et al., 2013; Jha et al., 2005); these toxic contaminants can induce apoptosis and necrosis which introduce strand breaks in DNA (Alberts et al., 2002; Lee and Steinert, 2003). These strand breaks can be measured using a comet assay, a gel electrophoresis-based method which

involves the embedment of haemolymph cells in agarose on a microscopic slide and subsequent lysing to form nucleoids; electrophoresis of these nucleoids results in structures resembling comets and the intensity of the comet tail relative to the head reflects the number of strand breaks (Collins, 2004). Exposure to microplastics has previously been reported to cause DNA strand breaks - *Scrobicularia plana* clams exposed to PS microplastics (20 µm; 1 mg L^{-1}) for 14 days reportedly experienced damage to their DNA (Ribeiro, 2016) and haemocytes of *Mytilus galloprovincialis* mussels exposed to PE and PS microplastic beads at concentrations of 1.5 g L^{-1} for 7 days were shown to have significantly enhanced DNA strand breaks of 30% and 20% respectively (Avio et al., 2015a).

In the previous chapter I demonstrated that oysters readily took up microplastics, particularly high numbers of PE and PVC compared to other plastic types. Here, I assess whether there are biological impacts of PE uptake on the health of *Crassostrea gigas* oysters. A suite of endpoints were used to include both measurements of food assimilation and metabolism (feeding rate and respiration rate) and toxicological indicators of general stress (NR), immune function (phagocytosis), DNA damage (comet) and oxidative stress (SOD and LPO).

4.2 Methodology

a) Animal collection and husbandry

Thirty-two oysters were collected from Starcross Bay, Dawlish (50°37'07.2"N 3°26'51.6"W) at low tide in May 2017 and upon return to the laboratory, each shell was scraped to remove any attached barnacles. Oysters were stored in a large tank with artificial seawater at 15 °C in a wet room, and fed a daily diet of 5 mL Shellfish Diet

1800® until use. A 200 L water carboy was used to create artificial seawater with DI and artificial sea salt (TropicMarine), to a salinity level of 34 PSU and pH_{NBS} of 8.1. In preparation for the experiment, 100 mL of *Isochrysis galbana* (Tahitan strain, T-iso) (www.seahorsebreeder.co.uk) was cultured for 10-14 days in autoclaved conical flasks in a 15 °C room, and the algae was supplied daily with Guillard's (F/2) marine water enrichment solution (prepared at 20 mL L^{-1} of filtered artificial seawater). Algal concentration was determined through the use of a Multisizer™ 3 Coulter Counter®.

b) Experimental set up

To determine any biological impacts of microplastic ingestion on oyster health parameters, oysters were exposed to PE beads (8-30 μm) at a final concentration of 100 microplastics mL^{-1} in two litre beakers for 7 or 14 days; exposed oysters were compared to oysters placed in an algal control treatment with no plastics added. PE beads were selected on the basis that they were shown to be readily taken up by oysters (Chapter 3 of this thesis), and its ubiquity in the environment (Andrady, 2011a; GESAMP, 2015; Gouin et al., 2015). PE beads (8-30 μm) were purchased from Cospheric (http://www.cospheric.com) and coated with 0.01% Tween which was subsequently washed off with DI to facilitate mixing of hydrophobic/buoyant microplastics. Thirty-two oysters were placed in 2 L glass beakers (two oysters per 2 L beaker) which were filled with 1.5 L of 0.33 μm filtered artificial seawater. Oysters were suspended above the bottom of the beaker on a plastic mesh (supported between two PVC cylindrical pieces) and positioned side by side to ensure that one oyster's opening did not overlap another's. Of the 32 oysters, half were supplied with a daily concentration of 100 PE beads (8-30 μm) mL^{-1} seawater and 0.75×10^6 mL^{-1} of *Isochrysis galbana* (Tahitan strain, T-iso); the other half were supplied with the same

concentration of algae but without microplastics (i.e. control conditions). To maintain seawater conditions and microplastic concentrations, the contents of each beaker were drained at the same time each day and resupplied with treatment conditions.

To ensure that the water quality did not deteriorate over the course of the exposure, spot checks on pH, temperature, oxygen, ammonia and salinity were taken daily over the course of the exposure. Ammonia tests were carried out on randomly selected samples throughout the exposure period. For each spot check taken, a blank sample (10 mL DI) was added to a sample cell, which was subsequently placed in a spectrophotometer (LANGE DR 3900) and used to standardise subsequent readings. An ammonia salicyclate powder pillow (PERMACHEM™) (10 mL) was mixed with 10 mL of selected sample in a glass sample cell and left to react for 3 mins. Following this reaction, an ammonia cyanurate powder pillow (PERMACHEM™) (10 mL) was mixed with 10 mL of a selected sample in a glass sample cell and left to react for 15 mins. Samples were read at 655 nm. Oxygen and temperature, pH and salinity levels were measured using a Firesting O_2 consumption kit, pH metre and salinity metre respectively.

In order to keep the algae and/or plastic treatments well circulated, a follower was placed at the bottom of each beaker and a magnetic stirrer was used to rotate the follower at 200 rpm. Air stones were connected via hoses to supply air to each beaker, and the flow of air was adjusted so that all treatments received a similar flow. Air stones were positioned on the upper part of the beaker to minimise disruption to the mixing of particles/feeding of oysters as much as possible. To minimise the effect of splash/spray from bubbles generated from the air stones (and thus potential cross-

contamination), aluminium foil pieces were placed over the top of each beaker. A random design was used to organise the placement of beakers in the room – each sample across treatments (consisting of two oysters) was allocated a random number (generated from a random number generator) running from 1-16 and beakers were placed on the bench in descending order according to the random number.

Oysters were sampled at two time points during the exposure at 7 days (7D) and 14 days (14D). After 7D, one oyster was taken from each beaker (8 from each treatment) and set aside for a feeding assay, before being subjected to the other biological assays (except the oxygen and the phagocytosis assay). The remaining oysters (8 from each treatment) were sampled at 14D for respiration rate and subsequently, all toxicological assays.

Haemolymph extraction was performed using a pair of scissors to pry open the valve from the side opposite the adductor muscle (to avoid damaging the muscle) and a scalpel to cut away any surrounding tissue. A 5 mL syringe and a 23-gauge needle was subsequently used to extract haemolymph from the adductor muscle, which was then placed into an eppendorf tube with 100 µL PBS and kept on ice until use.

c) Feeding assay

Feeding rate assays were performed to assess any impact of microplastic uptake on feeding behaviour. At day 7, one oyster from each beaker was taken out and placed in an individual 2 L beaker with 1.8 L of 0.33 µm filtered artificial seawater. Beakers were then filled with a final concentration of 0.75 x 10^6 per mL *Isochrysis galbana* and three additional beakers (controls) were filled with the same concentration of algae

only (i.e. no oyster added) to account for any naturally occurring change (e.g. die-off) in algal concentration over the 60 minute duration of the assay. The duration of the assay was set at 60 minutes, as findings from preliminary experiments had shown that *Crassostrea gigas* oysters were able to consume >50% of the available algae over this period, which was deemed sufficient to draw potentially meaningful comparisons between experimental and control samples.

From the onset of the assay, and every 20 minutes thereafter up to a 60 minute period, 10 mL samples were obtained from each beaker and 100 μL was analysed per sample with a Multisizer™ 3 Coulter Counter. To prevent contamination, the aperture was rinsed with artificial seawater between readings. Readings on the coulter counter recorded the number of algal particles present this and was based upon the 2.997-6.047 μm range in *Isochrysis galbana* particle size identified in preliminary trials. The aperture size used in the coulter counter was 50 μm. After 60 minutes had passed, all multisizer readings were collated and exported to an excel spreadsheet for further analysis.

d) Oxygen consumption

Oysters were starved for the last three days of the 14D exposure period (i.e. days 11-14), to ensure that their basal metabolic rate was measured rather than digestive processes (as these can increase respiration rates for a few days (Bayne, 2017)). Prior to the start of the assay, preliminary trials were run with oysters placed in various beaker sizes and volumes of water, in order to optimise a suitable combination for the assay. A combination was deemed suitable when the oxygen saturation level did not fall below 80% over the 60 minute period so as to avoid accounting for hypoxic stress.

For the assay, oysters were placed in individual 1 L beakers (with 600 mL of 0.33 μm fully aerated filtered artificial seawater). Three additional beakers with 600 mL of 0.33 μm filtered artificial seawater were used in the experiment to account for any microbial respiration/oxygen diffusion across the water over the course of the assay. A calibrated (100% saturation and 0% saturation) FireSting fibreoptic oxygen meter (PyroScience GmbH) was used to record oxygen concentration levels (mm Hg) in each beaker at the start of the experiment and after 60 minutes had lapsed. Following the measurements, oysters were rinsed in DI to remove salt crystals, placed in a drying oven at 60 °C for 24 hours, and the dry weight (g) of each oyster was obtained for the purposes of normalising oxygen consumption rate against dry weight.

In addition, a single beaker was set aside, and pumped with nitrogen until oxygen values dropped to ~70% of original values. A FireStingfibreoptic probe was left in the beaker, recording oxygen concentration values every second, and was monitored to evaluate the rate of oxygen increase over time. This was done in order to account for the rate of oxygen diffusion from the air into the water.

e) Neutral red assay

The NR assay was conducted with modifications from (Martínez-Gómez et al., 2008) as an indicator of lysosomal stability. Haemolymph (200 μL) was pipetted in triplicate onto poly-lysine treated microplates. Plates were left to incubate on a covered plate shaker at room temperature for 45 minutes to allow cell adhesion to occur. Following incubation, plates were inverted to discard non-adhered cells and 200 μL of neutral red working solution (0.2% of a stock solution consisting of 28.8 mg neutral red in 1

mL DMSO) was added to each well and left to incubate on a covered plate shaker at room temperature for 3 hours. After this, plates were inverted and washed with PBS to discard excess neutral red, and 200 μL of acidified ethanol (1% acetic acid, 50% ethanol) was used to breakdown the cellular membrane in each well to resolubilise the neutral red dye. Absorbance readings were taken at 540 nm with a spectrophotometer (TECAN infinite M200 PRO).

f) Phagocytosis assay

At the end of the 14-day exposure period, a phagocytosis assay was conducted using a modified approach from (Parry and Pipe, 2004). Aliquots of 50 μL samples and 50 μL blanks were pipetted in triplicate into a poly-lysine well plate and incubated for 60 mins at room temperature to allow cell adhesion to occur. At the 50 minute mark, 100 μL of Baker's formol calcium (2% NaCl, 1% $C_4H_6CaO_4$, 4% H_2CO) was added to the blanks only to kill the cells. After the incubation period, the plate was inverted and washed with PBS to remove non-adhered cells, and 50 μL of dyed zymosan (1.1×10^7 particles mL^{-1}) was added to each well and incubated for 30 mins at room temperature. Following the incubation, 100 μL of Baker's formol calcium was added to each well to stop the reaction for 10 mins, and the plate was then centrifuged at 1000 rpm for 5 mins. Supernatant was removed through plate inversion and washed with PBS, and these steps were repeated until the wells of the blanks were clear. Prior to the last centrifugation step, 100 μL of zymosan standards were aliquoted in triplicate to produce a standard curve. To resolubilise the haemocytes, 100 μL of acidified ethanol (1% acetic acid, 50% ethanol) was added to wells and absorbance readings of the plate was taken with a spectrophotometer (TECAN infinite M200 PRO) at 550 nm.

g) SOD assay

SOD activity was conducted using an adaptation of the NBT assay (Parry and Pipe, 2004). Three reagents were prepared, comprising; 1) Reagent A – 2.28 g Na_2CO_3, 1.81 g $NaHCO_3$, 1 L DI; Reagent B – 7.6 mg xanthine, 18.6 mg EDTA, 25 mg bovine serum albumin, 12 mg NBT, 500 mL Reagent A; Reagent C – 40 mg xanthine oxidase, 3.16 g Reagent A. SOD standard solutions were made by preparing a stock solution of 6.126 units mL^{-1} and running a serial dilution of the stock solution with Reagent A and PBS until the concentration reached 0.13 units mL^{-1}. Samples (36 μL) and standards (18 μL) were mixed with 90 μL (for samples) and 108 μL (for standards) of Reagent A and 702 μL of Reagent B. Blanks were prepared by mixing 126 μL of Reagent A with 702 μL of Reagent B. Eppendorf tubes were vortexed and 230 μL of samples, standards and blanks (three replicate aliquots each) were pipetted into a 96-well microplate. Reagent C (10 μL) was then added into each well with a multichannel pipette, and the plate was shaken briefly for 10 s on a plate shaker before absorbance readings were taken with a spectrophotometer at 573 nm. Twenty-five time points were obtained for each aliquot and the inhibition of SOD activity was calculated for each sample.

The optimal units mL^{-1} for the SOD standard was identified through the use of a standard curve. A small amount (< 0.1 mg) of stock SOD from bovine erythrocytes (Sigma-Aldrich), following the procedure shown above, and from the data received, a graph was drawn comparing the dilution level used (100% to 0.003%) against calculated inhibition values. A calibration curve was then run on values leading up to where the graph began to plateau in order to quantify the SOD standard.

h) TBARS assay

Measurement of TBARS was conducted to evaluate ROS-mediated oxidation of polyunsaturated fatty acids. The assay used a microplate method to quantify malondialdehyde (MDA) levels, a by-product produced by lipid peroxidation through its reaction with thiobarbituric acid (Camejo et al., 1998). Preliminary test runs were conducted on oysters prior to the start of the assay to identify whether further dilution of haemolymph samples with PBS were required to produce readings that were within the range of standard curve absorbance readings. Test runs showed 1:9 dilutions (PBS: Haemolymph) provided an optimal absorbance reading.

Diluted oyster haemolymph (100 μl) was added to wells, then 300 μl PBS+EDTA, 150 μl thiobarbituric acid (1.3% in 0.3% NaOH), 100 μl trichloroacetic acid (50% w/v), 20 μl butylated hydroxytoluene were mixed and reacted with standards (a serial dilution of tetraethoxypropane, ethanol and PBS+EDTA) and samples in individual eppendorf tubes, and incubated at 60 °C for 60 mins. After incubation, standards and samples were placed on ice to stop the reaction, centrifuged at 13,500 rpm for 7 mins and pipetted onto a microplate in 200 μl aliquots. Absorbance readings were taken using a spectrophotometer (TECAN infinite M200 PRO) at 530 nm.

i) Comet assay

DNA damage in haemocytes was measured as single strand breaks using a comet assay adapted from (Lewis and Galloway, 2008). For each assay, 100 μL of haemolymph was used from each oyster. Haemolymph was centrifuged at 78 x g (1000 rpm) for 3 mins and the supernatant was removed. The cell concentrate was mixed with 1% low melting point agarose (heated to 37 °C) and pipetted onto slides (pre-coated with 1% high melting point agarose). Slides were protected with coverslips

and allowed to set for 10 mins at 4 °C, and once set, the coverslips were removed. Samples were placed in 270 mL of Lysis solution (2.5 M NaCl, 0.1 M EDTA, 10 mM Tris), 3 mL of Triton X-100 and 30 mL of DMSO at 4 °C for a 1 hour period, followed by 45 mins denaturation in electrophoresis buffer (0.3 M NaOH, 1 mM EDTA at pH 13), electrophoresis for 30 mins at 25 V and 300 mA, and neutralisation with a neutralising buffer (0.4 M Tris at pH 7.5). Cells were stained with 20 µL SYBRSafe solution, and viewed under an ultraviolet fluorescence microscope (Nikon Eclipse 50i and a PRIOR Lumen200 lamp) using a 420-490 nm excitation filter and a 520 nm emission filter. One-hundred cells per preparation were quantified, and the tail migration (%) was measured through the use of Kinetic COMET software.

j) Standardizing assays against protein content

NR, SOD, phagocytosis and TBARS assays were normalised against protein content using a method from (Bradford, 1976). For the standard, 5 µL of samples, blanks (PBS) and 5 µL protein standards (0-1 mg ml^{-1} bovine serum albumin) were pipetted in triplicate onto a microplate and 200 µL Bio-Rad reagent (1:5 in DI) was added to each well. The microplate was placed on a covered plate shaker and allowed to incubate for 20 mins at room temperature. Absorbance readings were taken at 595 nm with a spectrophotometer (TECAN infinite M200 PRO).

4.3 Results

a) Feeding

There was no significant effect ($p<0.05$) of PE microplastic exposure on the algal feeding rate of exposed oysters for the 7D (Wilcoxon test; $w=42$, $p=0.382$) sampling points (Fig. 4.1). A significant difference in the variability of feeding rate between

specimens within the control and plastics treatments was observed, however (f=271,510,000, df =15, p<0.001). The algal feeding rate (units per mL g $(DW)^{-1}h^{-1}$) at the 7D sampling point was 9,967.85 ± 4,087.81 (SE) for plastic exposed oysters and 12,558.1 ± 3,008.39 (SE) for control oysters.

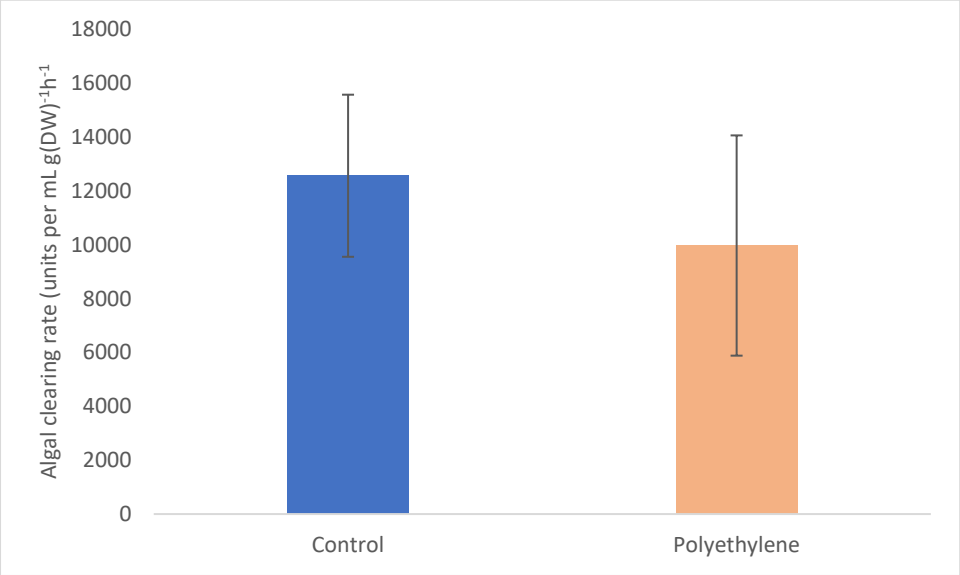

Figure 4.1: Algal clearing rate (units per mL $g(DW)^{-1}h^{-1}$) in control and polyethylene-exposed specimens at the 7 day sampling point. Standard error shown in error bars.

b) Oxygen

There was a slight reduction in oxygen consumption rates ($\mu M(O_2)g(DW)^{-1}h^{-1}$) at the 14 day sampling point 61.075 ± 21.378 (SE) for PE microplastic-exposed oysters compared with the 77.645 ± 45.530 (SE) consumption rate for control oysters, however, PE exposure was shown to have a statistically non-significant effect (p<0.05) on these observed differences (Wilcoxon test; w=27, p=0.645) (Fig. 4.2). A significant difference in the variability of oxygen consumption rate between specimens within the control and plastics treatments was observed, however (f=62,317, df=15, p<0.001).

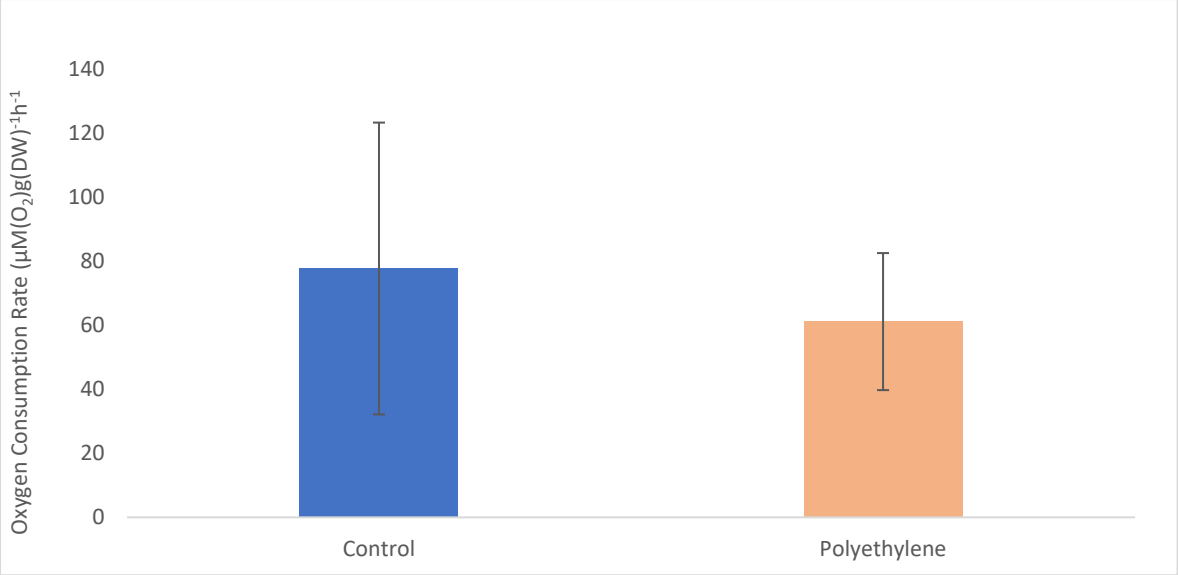

Figure 4.2: Oxygen consumption rate (µM(O_2)g(DW)$^{-1}$h^{-}1) in control and polyethylene-exposed specimens at the 14 day sampling point. Standard error shown in error bars.

c) Neutral red

There was no significant effect ($p<0.05$) of PE microplastic exposure on NR retention in the haemocytes of exposed oysters for either the 7D (two sample t-test; t=0.099, df=12.66, p=-0.923) or the 14D (two sample t-test; t=-0.222, df=11.871, p=0.828) sampling points (Fig. 4.3). A significant difference in the variability of NR retention between specimens within the control and plastics treatments was observed, however, at the 7D (f=0.000148, df=15, $p<0.001$) and the 14D sampling points (f=0.000587, df=15, $p<0.001$ In control specimens, the average NR retention (µg NR mL^{-1}/µg protein) decreased from 0.025 ± 0.002 (SE) to 0.013 ± 0.003 (SE) from the 7D to the 14D sampling points, with a similar decline in PE-exposed oysters over the same period.

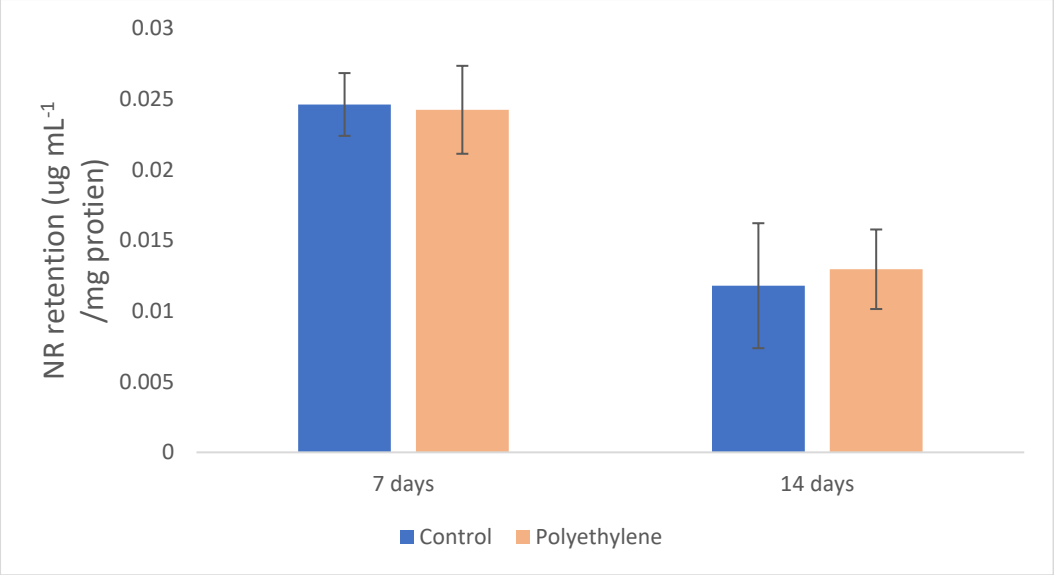

Figure 4.3: Neutral red retention (μg mL^{-1}/mg protein) in control and polyethylene-exposed specimens at 7 day and 14 day sampling points. Standard error shown in error bars.

d) Phagocytosis

Phagocytosis activity (number of zymosan particles phagocytosed/mg protein) was higher in oysters exposed to PE beads, increasing from control levels of 0.00015 ± 5.716E-05 (SE) to 0.00027 ± 5.128E-05 (SE) for plastic exposed oysters at the 14D sampling point; however, this was not a significant increase ($p<0.05$) (two sample t-test; t=-1.601, df=13.838, p=0.132) (Fig. 4.4). A significant difference in the variability of phagocytosis between specimens within the control and plastics treatments was observed, however, at the 14D sampling point (f= 9.803e-08, df=15, $p<0.001$).

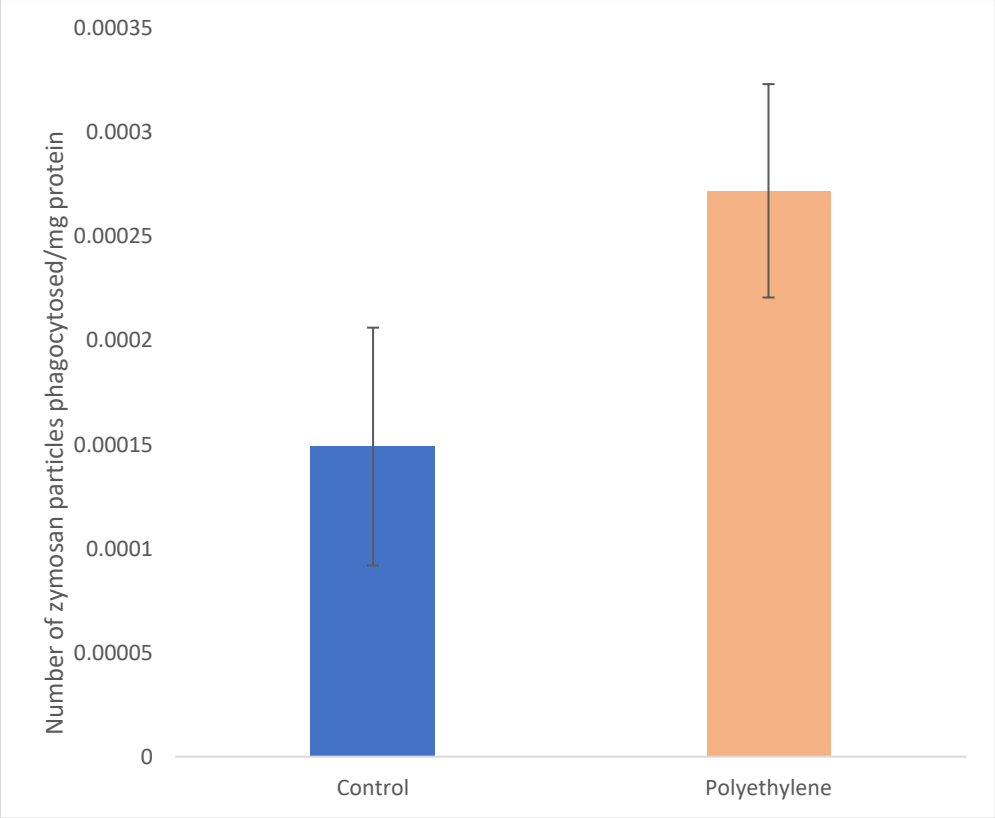

Figure 4.4: Phagocytosis activity of oyster haemocytes (number of zymosan particles phagocytosed/mg protein) in control and polyethylene-exposed specimens at the 14 day sampling point. Standard error shown in error bars.

e) SOD

There was no significant effect ($p<0.05$) of microplastics exposure on SOD activity in the haemocytes of PE microplastic-exposed oysters for either the 7D (two sample t-test; t=-1.203, df=12.205, p=0.252) or the 14D (Wilcoxon test; w=22, p=-0.328) sampling points (Fig. 4.5). There was no significant difference in the variability of SOD activity between specimens within the control and plastics treatments observed at the 7D sampling point (f=0.650, df=15, p=0.580), however, there was a significant difference at the 14D sampling point (f=6.514, df=15, p=0.002). In control specimens, average SOD activity (units/mg protein) decreased from 2.169 $\pm$ 0.147 (SE) to 1.329

± 0.466 (SE) between the 7D and 14D sampling points, and decreased from 2.488 ± 0.220 (SE) to 1.176 ± 0.208 (SE) in the PE-exposed oysters over the same period.

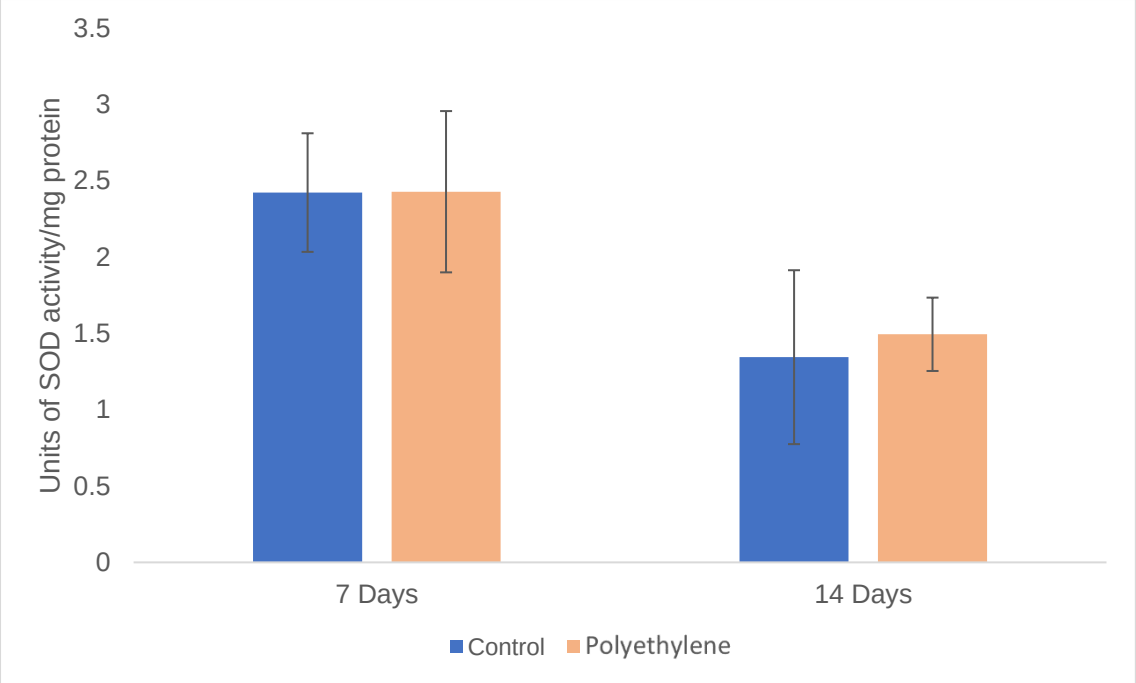

Figure 4.5: Units of SOD activity/mg protein in control and polyethylene-exposed specimens at 7 day and 14 day sampling points. Standard error shown in error bars.

f) TBARS

There was no significant effect ($p<0.05$) of PE microplastic exposure on TBARS activity in the haemocytes of exposed oysters for either the 7D (Wilcoxon test; w=39, p=0.505) or the 14D (two sample t-test; t=0.211, df=10.982, p=0.837) sampling points (Fig 4.6). There was also no significant difference in the variability of TBARS activity between specimens within the control and plastic-exposed specimens observed at the 7D (f=1.644, df=15, p=0.396) and 14D (f=3.307, df=15, p=0.05) sampling points. In control specimens, average TBARS activity (units/mg protein) increased from 0.684 ± 0.234 (SE) to 0.870 ± 0.332 (SE) from the 7D to the 14D sampling points, and

increased from 0.583 ± 0.419 (SE) to 0.729 ± 0.296 (SE) in plastic-exposed oysters over the same period.

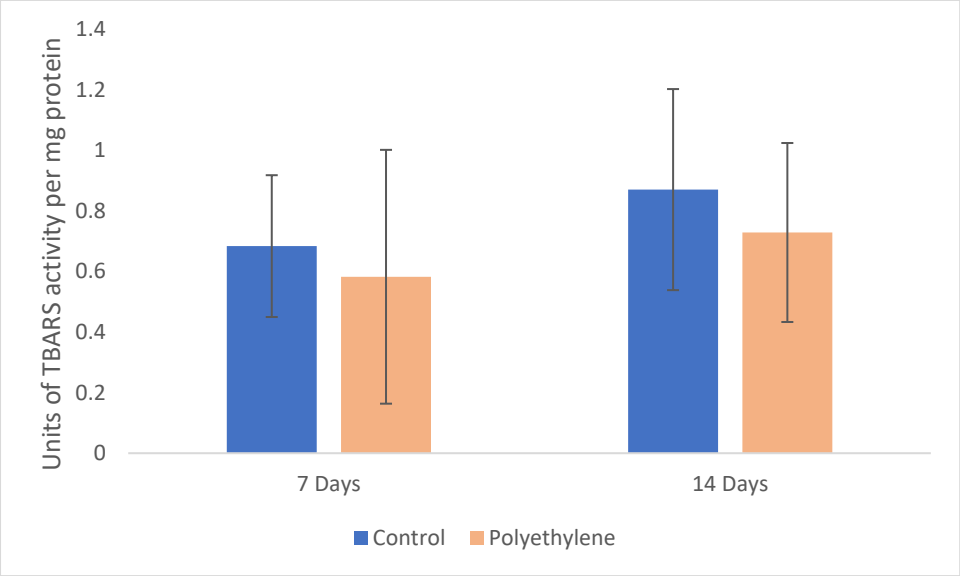

Figure 4.6: Lipid peroxidation of oyster haemocytes, measured as units of TBARS/mg protein in control and polyethylene-exposed specimens at 7 day and 14 day sampling points. Standard error shown in error bars.

g) Comet assay

There was no significant effect ($p<0.05$) of the PE microplastic exposure on tail migration % in the haemocytes of exposed oysters for either the 7D (two sample t-test; $t=0.735$, $df=13.459$, $p=0.475$) or the 14D (two sample t-test; $t=0.409$, $df=13.405$, $p=0.689$) sampling points (Fig 4.7). There was, however, a significant difference in the variability of tail migration % between specimens within the control and plastic-exposed specimens observed at the 7D ($f=77.19$, $df=15$, $p<0.001$), and 14D ($f=112.13$, $df=15$, $p<0.001$) sampling points. In control specimens, average tail migration increased from 21.526% ± 1.604% (SE) to 25.43% ± 1.93% from the 7D to the 14D sampling points, and increased from 23.39% ± 1.97% (SE) to 24.41% ± 1.56% in the plastic-exposed oysters over the same period.

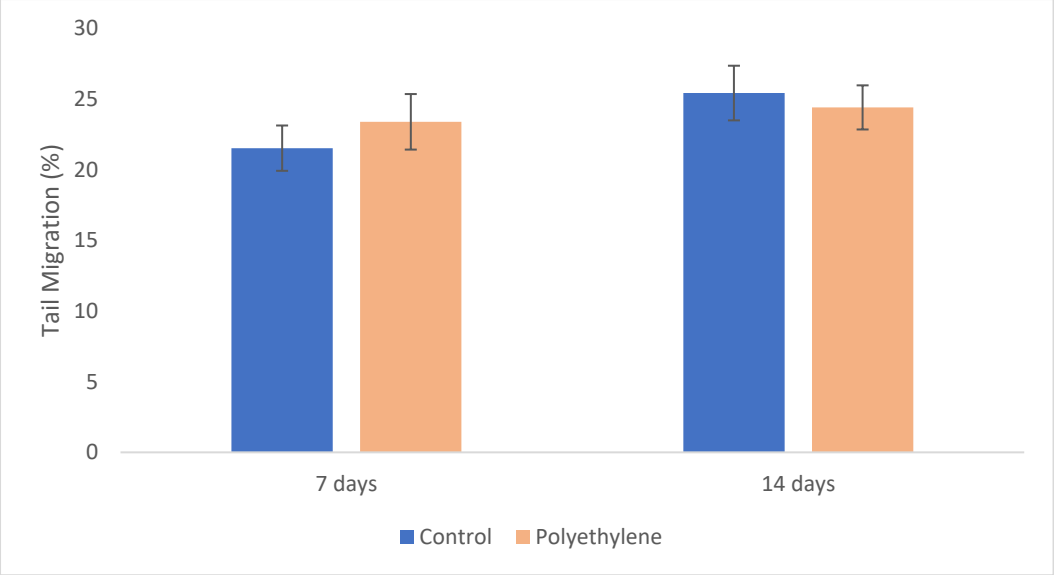

Figure 4.7: Tail migration (%) as a measure of DNA damage in control and polyethylene microplastic-exposed specimens at 7 day and 14 day sampling points. Standard error shown in error bars.

Table 4.1: Sizes, polymers, concentrations and exposure periods of microplastics used in plastic exposure experiments.

Organism	**Size and Polymer Type**	**Concentration**	**Exposure Period**	**Indicated response**	**Reference**
Crassostrea gigas Oysters	8-30 µm PE microbeads	100 microplastics mL^{-1}	7 days and 14 days	Non-significant impacts to feeding, respiration, phagocytosis, SOD, LPO and DNA damage	This study
Danio rerio Zebrafish	5 µm PS microbeads	2.9 x 10^4 mL^{-1}	7 days	Significant increases in SOD and catalase in the liver.	(Lu et al., 2016)

Mytilus edulis Mussels	0-80 µm HDPE fluff	2.5 g L^{-1}	Up to 96 h	Notable histological changes, formed granulocytomas after 6 h and lysosomal membrane destabilisation.	(von Moos et al., 2012)
Mytilus galloprovincialis Mussels	<100 µm PE and PS powder	20 g L^{-1}	7 days	Lysosomal compartment, peroxisomal proliferation, antioxidant system, neurotoxic and genotoxic effects.	(Avio et al., 2015a)
Mytilus galloprovincialis Mussels	1-50 µm PE microbeads	1.5 x 10^7 particles L^{-1}	24 hours	Genes related to oxidative stress (catalase and SOD) and immune response (toll-like receptor, peptidoglycan recognition protein, myticin A and mytilin B) in the mussel were up-regulated in the mantle and digestive gland.	(Détrée and Gallardo-Escárate, 2017)
Mytilus spp. Mussels	2-6 µm PS microbeads	32 µg L^{-1}	7 days	Higher haemocyte mortality and an increase in reactive oxygen species production, as well as an up-regulation of genes involved in glycolysis in the digestive gland.	(Paul-Pont et al., 2016).
Crassostrea gigas Oysters	2 and 6 µm PS microbeads	2 µm particles: 2,062 ± 170 microplastics	2 months	Significant decreases in oocyte number, diameter and sperm velocity, and dynamic energy budget	(Sussarellu et al., 2016)

		mL^{-1}; 6 μm particles: 118 ± 15 microplastics mL^{-1}		modelling suggested that energy allocation shifted from reproduction to structural growth and elevated maintenance costs in oysters.	
Nephrops norvegicus Langoustines	3-5 mm PP microplastic fibres	A total of 360 (5 fibres per feeding)	8 month period	Reduction in body mass, blood protein and stored lipids.	(Welden and Cowie, 2016).
Mytilus edulis Mussels	30-nm PS microplastic beads	0, 0.1, 0.2 and 0.3 g L^{-1}	8 hours	Significant reductions in filtration rate and significant increases in faeces/pseudo-faeces production.	(Wegner et al., 2012)
Arenicola marina Lugworms	125-149 μm unplasticised PVC powder	0.5-5% PVC by sediment weight	28 days	Significantly reduced feeding activity, significantly increased phagocytic activity, significantly reduced energy reserves.	(Wright et al., 2013a)
Dicentrarchus labrax European Seabass	1–5 μm polymer microspheres	0.26 and 0.69 mg L^{-1}	96 hours	Significant reduction of the swimming velocity and resistance time of fish.	(Barboza et al., 2018)
Carcinus maenas Crabs	1–5 mm PP microspheres	0% (0 mg), 0.3% (0.6 mg), 0.6% (1.2 mg), 1% (2.0 mg) added to 2 g of the feed	1 month	Reduced food consumption, reduction in energy available for growth.	(Watts et al., 2015)

4.4 Discussion

The results obtained from the present study revealed that although there was tentative evidence of a small increase in phagocytosis activity (phagocytosis assay) and decreases in food assimilation (feeding assay) and metabolism (oxygen consumption assay) in *Crasssotrea gigas* oysters exposed to PE microplastics, these were statistically non-significant at the level of replication used and at the concentration of microplastics they were exposed to. Nonetheless, they are suggestive of trends observed in other studies which, it is important to emphasise, mostly used higher concentrations of microplastics than what was supplied in the present study. For example, significant increases in phagocytosis activity were observed in *Mytilus galloprovincialis* mussels upon their exposure to LDPE microplastics (20-25 µm; 2.34 x 10^7 particles mL^{-1}) (Pittura et al., 2018) and also in *Arenicola marina* lugworms upon exposure to unplasticised PVC microplastics (230 µm; 1% and 5% by weight) (Browne et al., 2013). Increased phagocytic activity is indicative of an inflammatory response and impairment to the immune system, and phagocytosis by macrophages has been reported to occur for particles >0.5 µm (Yoo et al., 2011).

Decreases in feeding rate were observed in studies on the copepod *Calanus helgolandicus* (20 µm PS microplastics; 75 microplastics mL^{-1}; 11.2% decrease) (Cole et al., 2015); the mussel *Perna viridis* (1–50 µm PVC microplastics; 216 mg L^{-1}; 41% decrease) (Rist et al., 2016); the clam *Atactodea striata* (63-250 µm PS microplastics; 1000 microplastics L^{-1}; ~80% decrease) (Xu et al., 2017); and the mussel *Mytilus edulis* (30 nm PS microplastics; 0.3 g L^{-1}; opening of valves decreased by ~75%) (Wegner et al., 2012). Similarly, decreases in respiration rate were observed in the mussel *Perna viridis* (1–50 µm PVC microplastics; 216 mg L^{-1}; 41% decrease) (Rist et al., 2016). As an adaptive strategy to reduce the uptake of microplastics and inorganic

material or low-quality seston, bivalves may limit the opening of their valves (Rist et al., 2016; Wegner et al., 2012) and increase their level of pseudo-faeces production (Garrido et al., 2012; Shin et al., 2002). This enables them to reduce blockage, injury to epithelia and to prevent the saturation of ingestion and sorting capacity; the increase in pseudo-faeces production reportedly reduces bivalves' uptake of food and gaseous exchange and potentially depletes their energy reserves (Pastoureaud et al., 1995; Riisgård et al., 2011; Wright et al., 2013c). To respond to digestive interference, reduced energy intake and to maintain homeostasis, *Crassostrea gigas* oysters have been reported to increase their feeding rates by 3% when exposed to PS microplastics (2 and 6 µm; 0.023 mg L^{-1}) (Sussarellu et al., 2016) and *Mytilus edulis* mussels reportedly increased their energy consumption (respiration) by 25% when they were exposed to PS microplastics (10-90 µm; 110 particles $mL^{-1)}$ (Van Cauwenberghe et al., 2015).

For the other biological end points measured in this study, there was no indication of impacts on oxidative stress (SOD and TBARS assays), general stress (NR assay) nor DNA damage (comet assay) at the level of replication used. However, evidence for the contrary (i.e. significant effects) have previously been reported for plastic-exposed organisms (Table 4.1), and incidentally, these studies used much higher concentrations of microplastics than what was supplied in the present study. For example, evidence of oxidative stress were found in *Danio rero* zebrafish following their exposure to 5 µm PS microplastics at a concentration of 2.9×10^4 mL^{-1} (Lu et al., 2016), *Mytilus edulis* mussels following their exposure to 0-80 µm HDPE particles at a concentration of 2.5 g L^{-1} (von Moos et al., 2012), *Mytilus galloprovincialis* mussels following their exposure to <100 µm PE and PS microplastics at a concentration of 20

g L^{-1} (Avio et al., 2015a) and *Mytilus galloprovincialis* mussels following their exposure to 1-50 μm PE microplastics at a concentration of 1.5 x 10^7 particles L^{-1} (Détrée and Gallardo-Escárate, 2017). Conversely, other studies that reported non-significant oxidative stress responses in specimens used relatively conservative concentrations of microplastics. There were non-significant impacts on oxidative stress or cellular damage in *Mullus surmuletus* mulletfishes caught in the Balearic Islands; 27.3% of captured individuals ingested an average of 0.32 ± 0.04 MPs $individual^{-1}$ (Alomar et al., 2017). Oxidative stress, cell viability and phagocytic activity in *Mytilus edulis* mussels were non-significant following their <48 day laboratory exposure to PS microplastics (3.0 or 9.6 μm; ~42.9 microplastics mL^{-1}) (Browne et al., 2008); and lysosomal integrity, lipid peroxidation and DNA damage in the mussel *Perna viridis* were non-significant following their three-month exposure to PVC microplastics (0.1-1.0 μm; 0.125 g L^{-1}) (Santana et al., 2018). The successful egestion of plastics (Kaposi et al., 2014), effective neutralisation of ROS production (Paul-Pont et al., 2016) and ability to respond to low concentrations of, and short exposure periods to, microplastics (Santana et al., 2018) have been provided as explanations behind a lack of observed significant biological responses.

Based on this information relating to microplastic concentrations used, and corresponding revelations in organisms' biological responses across studies, it would be premature to make inferences from the present study's results to conclude that *Crassosstrea gigas* oysters do not suffer from adverse health effects when exposed to microplastics. These results simply indicate that oysters were not experiencing direct toxicity from PE particles at the concentration of microplastics they were exposed to. Indeed, studies which used multiple concentrations/dosages in studies

have often reported dose-dependent effects on specimens – the copepod *Paracyclopina nana* experienced developmental delays and reduced fecundity in a dose-dependent manner when exposed to 0.05 µm PS microplastics at concentrations of 0.1, 1, 10 and 20 µg mL^{-1} (Jeong et al., 2016). Similarly, the mice *Mus musculus* experienced significant decreases in adenosine triphosphate (ATP) level and increases in lactate dehydrogenase (LDH) in a dose-dependent manner when exposed to 5 and 20 µm PS microplastics at concentrations of 0.01, 0.1 and 0.5 mg day^{-1}, indicating that energy metabolism in the liver was impaired (Deng et al., 2017).

Studies exposing specimens to high concentrations of microplastics have, in cases, justified these concentrations on data from pollution "hotspots" - where the number of microplastics are concentrated in a polluted area – that are present in the natural environment (Lenz et al., 2016). For instance, the justification for using exposure concentrations of 2,062 $\pm$ 170 microplastics mL^{-1} and 118 $\pm$ 15 microplastics mL^{-1} for 2 and 6 µm PS beads (Sussarellu et al., 2016) was based upon the 81.43 $\pm$ 4.03 mg small plastic concentrations per kg dry weight sediment of a highly polluted area close to a ship-breaking yard in India (Reddy et al., 2006). Other hotspots of plastic debris have been located along the coastlines of southern China and Southeast Asia, the east coasts of Australia, the USA and southern Africa, and oysters residing in these pollution hotspots may well be more exposed to, and susceptible to, comparatively high concentrations of plastics, thus experiencing higher levels of stress (Schuyler et al., 2016). On the other hand, one must also consider areas outside of pollution hotspots, where plastic concentrations may be significantly lower. For example, the number of microplastics per kg dry weight of sediment have been reported to be as low as 1.45 (equivalent to 1.45×10^{-3} microplastics g^{-1}) (Dekiff et al., 2014) and 13 (1.3

x 10^{-2} microplastics g^{-1}) (Van Cauwenberghe et al., 2013). Similarly, the number of microplastics per m^3 of seawater have been reported to be as low as 17 (Zhao et al., 2014) and 26 (Lima et al., 2014). In Chapter 2, I showed that the Deep Bay environment had an average of 1,220 ± 700 microplastics per kg dry weight of sediment (equivalent to 1.22 microplastics g^{-1}) and an average of 671.3 ± 285.5 microplastics/m^3 of seawater (equivalent to 6.7 x 10^{-4} microplastics mL^{-1}), and these figures were in between the low and high ends of reported microplastic concentrations. Given that the concentrations of microplastics found in the Deep Bay environment were considerably lower than those typically used in experimental exposures, it is possible to infer the unlikelihood that *Crassostrea hongkongensis* oysters in Deep Bay environment exhibit biological responses directly due to the presence microplastics.

The size of microplastics used in exposure experiments is likely to influence organisms' biological uptake (as indicated in Chapter 3 of this thesis) and subsequently, their potential biological responses. Many studies reporting significant responses have used smaller-sized particles in their exposure experiments compared to the 8-30 μm sizes particles used in the present study, including sizes of 1-5 μm (Barboza et al., 2018), 5 μm (Lu et al., 2016) and 2-6 μm (Paul-Pont et al., 2016; Sussarellu et al., 2016) (Table 4.1). Evidence from microplastic size-dependent experiments on the monogonont rotifer *Brachionus koreanus* (Jeong et al., 2016) and *Crassostrea gigas* larvae (Cole and Galloway, 2015) have shown that smaller-sized particles are more readily consumed than larger-sized particles, and that the latter is more easily egested. Furthermore, the capture retention efficiency of particles over 6-10 μm by *Crassostrea gigas* is reportedly 100%, with decreasing efficiencies with smaller sizes (Heral, 1985). It follows that smaller inorganic particles that fall outside the preferred range and food

type of oysters are less likely to be egested after capture and subsequent sorting, and potentially more readily taken up by oysters (Ward and Shumway, 2004). Once taken up, the propensity for smaller sized particles to potentially fragment further and to translocate from the gut to the circulatory fluid and phagocytic cells would increase, as their smaller size would enable them to pass through the gut wall and possibly even tissue membranes; as a result, physiological stress responses may be triggered (Galloway et al., 2017; Wright et al., 2013c). To illustrate this point, it has been reported that only particles <150 μm may translocate across the gut epithelium (leading to systemic exposure), with only the smallest size fractions (<1.5 μm) potentially penetrating deeply into organs (EFSA Panel on Contaminants in the Food Chain (CONTAM), 2016). Unfortunately, the lower size limit of analysed microplastics found in Deep Bay (Chapter 2 of this thesis) was limited to <100 μm due to the constraints of the sampling and identification methodology used. This is a common limitation in field sampling, as the sizes of microplastics collected in the field are constrained by the sensitivity of the sampling technique used (mesh size of nets or size of filters), and the identification of samples in the laboratory are similarly dependent on the pore size used for filtration and magnification of stereoscopes/microscopes used to detect plastics (Gago et al., 2016; Hidalgo-Ruz et al., 2012). In particular, colourless particles <100 μm with irregular shapes are difficult to characterise (Song et al., 2015), and more specialist equipment such as fourier-transform infrared (FTIR) spectroscopy, raman spectroscopy or thermal analysis would be required to detect particles below this range (Shim et al., 2016). For the reasons above, the commentary that can be made at this stage on the uptake of smaller particles (<100 μm) in oysters is limited. However, even if microplastics particles did fall outside the preferred feeding size range of *Crasssostrea hongkongensis* oysters to begin with, processes such as

weathering and fragmentation would likely lead to them being at the lower ends of the micrometre scale and possibly even at the nanoscale.

The shape of microplastics may potentially impact observed biological responses. Though many of the studies outlined in Table 4.2 report the use of microplastic beads in exposure experiments, it has been suggested that microplastic fibres indicate may cause greater inflammatory responses, greater difficulties in clearance, phagocytosis, mytosis and chromosome segregation in organisms compared with other plastic shapes, in particular due to their length, composition and large surface area per unit weight (Donaldson and Tran, 2002). Evidence has shown that compared with other plastic shapes, fibres induce further reductions in neonate numbers and adult body size of the waterflea *Ceriodaphnia dubia* (Ziajahromi et al., 2017), higher mortality rates of the daggerblade grass shrimp *Palaemonetes pugio* (Gray and Weinstein, 2017) and higher levels of toxicity and significantly less growth in the freshwater amphipod *Hyalella azteca* (Au et al., 2015). It is suspected that these adverse impacts may be due to the difficulty in egesting fibres compared to other plastic shapes, leading to longer gut residence times and subsequently a higher likelihood for them to entangle within the intestinal tract (Murray and Cowie, 2011; Nimmo et al., 1977). The entanglement of fibres has also been associated with damage of internal structures and gut blockages, leading to heightened inflammatory responses (Gray and Weinstein, 2017; Nimmo et al., 1977). In Chapter 3, it was shown that the shape of plastics did not significantly influence the uptake of microplastics, potentially because the fibres used did not resemble the shape or size of oysters' usual food intake. However, based on the vast majority of microplastics that consisted of fibres in the

water column and, indeed, inside oysters' tissues, it is possible that they would respond more strongly to fibres compared with plastic beads.

Low level biological responses may build up over time. *Crassostrea gigas* samples in the present study were exposed to plastics for a maximum of just 14 days, whereas oysters in the natural environment and farmed oysters might be exposed to plastics over the course of their lifetime, which may cause chronic effects. Some relatively longer term microplastic exposures in laboratory studies have demonstrated this point. *Crassostrea gigas* oysters exposed to PS microplastics (2 and 6 µm; 0.023 mg L^{-1}) for 2 months exhibited significant decreases in oocyte number, diameter and sperm velocity, and dynamic energy budget modelling suggested that energy allocation shifted from reproduction to structural growth and elevated maintenance costs in oysters (Sussarellu et al., 2016). Another study exposed *Nephrops norvegicus* Langoustine specimens to PP fibres (3-5 mm; 5 microplastics per feeding) over an 8 month period, resulting in reductions in body mass, blood protein and stored lipids of exposed specimens (Welden and Cowie, 2016). It has been argued however, that a longer period of exposure to microplastics (Santana et al., 2018) or to stressors such as temperature (Widdows and Bayne, 1971) may increase the ability of organisms to acclimate to stressors in the long term by regulating pseudo-faeces production, clearance rate, growth rate, and other physiological processes. The regulation of *Mytilus edulis* mussels' filtration rates, digestion absorption and rejection of pseudo-faeces upon exposure to microplastics have been documented as responses to maintain a consistent level of nutritive particle uptake (Bayne et al., 1993; Riisgård et al., 2011). More research making use of longer-term exposure periods are undoubtedly required to fill in missing knowledge gaps in these areas. This would also

be useful to supplement the tentative evidence showing that the sorption of organic contaminants into microplastics can increase over time (Rochman et al., 2013b), and the evidence that concentrations can be as high as a hundred-fold and a million-fold of what is found in sediment and water respectively (Teuten et al., 2007).

There is a small, but nonetheless, non-discountable possibility that an increase of ROS may have been present in the exposed oysters in other tissues but not detectable in the haemolymph. Processes within the immune system are energetically costly (Segerstrom, 2007) and although haemocytes and surface epithelia display immune functions through the recognition and response to the presence of foreign and infectious cells (Cheng, 1996; Schmitt et al., 2012), other tissues such as the mantle and digestive glands (which play an important role in transferring energy assimilated from food to other body tissues and glycogen stores) may instead have been sites of concentrated ROS production due to their additional energy requirements to maintain oxidative/antioxidative processes (Patterson et al., 1999). When *Mytilus galloprovincialis* mussels were exposed to HDPE microplastics (1-50 μm;15,000 microplastics mL^{-1}), genes related to oxidative stress and immune response were up-regulated in the mantle and digestive gland, but no significant responses were evident in the gills and haemolymph (Détrée and Gallardo-Escárate, 2017). Similarly, *Mytilus spp.* mussels exposed to microplastics (2 and 6 μm; 2,000 microplastics mL^{-1} day^{-1}) had higher haemocyte mortality, more ROS production and stronger up-regulation of genes involved in glycolysis in the digestive gland (Paul-Pont et al., 2016). However, this is unlikely to have been the main reason behind the observed lack of a biological response to PE exposure, given the consistent non-significant results from the wide array of assays used to measure biological responses. A much more likely reason is

that the concentration of microplastics supplied were simply not high enough to induce a biological response. Incidentally, the studies mentioned above used microplastic concentrations several orders of magnitude above the concentrations used in the present study.

Future research is recommended to involve the use of longer-term experiments and to draw comparisons in order to provide a clearer indication to the conditions oysters would face in the wild. Oysters in Deep Bay were cultured on ropes across wet and dry seasons – which provide varying conditions in current, wave action, salinity and tidal flushing – and a comparison of oysters collected between wet and dry seasons could also provide information on whether environmental conditions would affect microplastic uptake (Aiguo Qian, 2003). In order to better understand the effects of environmental contaminants and the potential interactions they might have with microplastics, it would be useful to conduct a follow up experiment to this study involving the analysis on the adsorption of additives of such chemicals on plastic, which would give a more environmentally realistic picture of contaminants oysters might be exposed to (Browne et al., 2013).

Thesis Discussion

Given the amount of plastic produced in megacities located in the Pearl River Delta region, as well as the growing number of studies documenting microplastic ingestion by marine organisms, this study was timely as it is the first investigation into whether locally cultured *Crassostrea hongkongensis* oysters followed this trend. Earlier studies have reported high levels of metal contaminants (Liu et al., 2004; Phillips and Yim, 1981; Wong et al., 1981), harmful organic contaminants (Qiu et al., 2009; Zhao et al., 2012) and microplastics in the water and sediment in the bay (Fok and Cheung, 2015; Tsang et al., 2017; Zurcher, 2009).

In this body of work I have demonstrated that Deep Bay contains microplastics in water (average of 671.25 particles per m^3) and sediment (average of 1,513 particles per kg dry weight of sediment) and *Crassostrea hongkongensis* oysters were taking up microplastics (average of 15.1 particles per individual). The lack of any clear relationship between microplastics found in water and sediment samples and oyster specimens allude that the biological uptake of particles were potentially influenced by plastic properties such as size, shape and polymer type.

This hypothesis was then confirmed via a set of 24 hour controlled exposure experiments which revealed that biological uptake was significantly influenced by the size and type of plastic supplied. Amongst the microplastics that *Crassostrea gigas* oysters (a genetically similar species to *Crassostrea hongkongensis*) were exposed to, polyvinyl chloride fragments (115-156 μm) and polyethylene beads (8-30 μm) were

the most commonly taken up in these controlled exposures. This is important, as it showed selective uptake was occurring and that the types of plastic taken up in oysters did not simply reflect the types of plastics found in their surroundings, as was evidenced by the weak relationship between biological specimens and environmental samples collected in Deep Bay.

Finally, it was revealed that despite the fact that polyethylene beads were readily taken up by oysters, no clear evidence of biological harm was demonstrated at the concentrations used (100 microplastics mL^{-1}), which were considerably higher than those found in the environment at Deep Bay (e.g. 6.7 x 10^{-4} microplastics mL^{-1} in seawater). Biological assays conducted after a one-week and two-week exposure period showed that oysters exposed to microplastics did not experience significant levels of oxidative stress, cellular damage or alterations to oxygen consumption and feeding rates compared to control treatments.

It can be inferred from this study that *Crassostrea hongkongensis* oysters in Deep Bay are taking up microplastics from the surrounding environment, but are unlikely to suffer any biological responses unless they are exposed to these plastics over a much longer period of time. Time-dependent exposures have shown these responses to exacerbate over time (Sussarellu et al., 2016; von Moos et al., 2012), however few studies have looked at how these responses might change over long periods of time. Considering that the lifespan of unharvested *Crassostrea gigas* oysters can reach up to 40 years (Pauley et al., 1988), and that harvested oysters are marketed between 18-30 months (Helm, 2006), more research is required to better understand the long-term effects of plastic exposure, such as the potential transfer of contaminants or

diseases from plastics to organisms (Breitwieser et al., 2016; Cotou et al., 2013; Van Cauwenberghe and Janssen, 2014; Xie et al., 2017). From a health perspective, oysters' ingestion of microplastics are also cause for concern. It has been estimated that an average of 11,000 microplastics are ingested by European shellfish consumers a year (based on top Belgian per capita consumers of 72.1 g (WW) per day) (Van Cauwenberghe and Janssen, 2014). Assuming the same consumption amount for *Crassostrea hongkonensis* oysters and the number of microplastics found in the present study, that would equate to an average of over 34,000 microplastics per year. The implications for human ingestion are manifold – microplastics <100 μm may pass into the gastro-intestinal tract, where they may accumulate and leach toxic chemicals such as hydrophobic organic contaminants (HOCs) that can subsequently lead to biological responses such as inflammation, genotoxicity, oxidative stress, apoptosis and necrosis (Galloway, 2015; Wright and Kelly, 2017). These impacts are likely to be more severe with higher concentrations and doses of ingested microplastics.

From an economic perspective, sales of *Crassostrea hongkongensis* oysters from Deep Bay have been in decline since the 1980s – considered the golden decade of the industry - due to many consumers' preference for imported oysters (Wong et al., 2014). With 118 tonnes of oyster production and an economy valued at US$13 million in 2017, (Agricultural Fisheries and Conservation Department, 2018), the oyster fishery is commercialy important to Hong Kong, and it is imperative to further research potential impacts of microplastics on oysters and consumers to safeguard fishermens' livelihoods and to instill confidence in consumers. Culturally, oysters are a delicacy often enjoyed at traditional festivals, and to satisfy local demand, have been cultured in Deep Bay for over 700 years (South China Morning Post, 2016). Safeguarding

fishermen's livelihoods and consumers' confidence in local oysters is of upmost importance. In Europe, this necessity has been recognised by the European Food Safety Authority (EFSA), which has recommended the use of quality assurance in seafood products as well as research into local effects in the gastrointestinal tract (EFSA Panel on Contaminants in the Food Chain (CONTAM), 2016).

In China, the government has recognised the damage waste has had on the environment and on the public health of its citizens. The latest government figures show that China imported over 49.6 million tonnes of trash, and over the past few years, it has taken steps to gradually reduced the import of solid waste, including plastics (Agence France-Presse, 2018; Environmental Protection Department (Statistics Unit), 2017). This year, it notified the world trade organization that it would stop importing 24 categories of solid waste, including plastics, and this has had serious repercussions in Hong Kong, the largest exporter of used plastics to China (Lau, 2017). To illustrate this point, the recycling rate of plastics has decreased from 32% in 2012 compared to 14% in 2016, which is likely to generate more plastic waste through alternative disposal methods – the leading cause of microplastic pollution (Andrady, 2011a; Environmental Protection Department (Statistics Unit), 2017). The ban has also had significant impacts on the United States and the United Kingdom – the amount of scrap plastics the former country exported to China dropped from 280 to 41 million tonnes (an 85% decrease) between January and December 2017, and the latter country has experienced a marked build-up of waste at recycling plants, given that it has been reliant on China for exporting two thirds of its used plastics (Resource Recycling, 2018; Taylor, 2018)

In order to limit the amount of plastic waste that enters the natural environment and into the food chain, one viable option is to enforce laws and regulations to restrict plastic pollution. The United States introduced a *Microbead-Free Waters Act* in 2015 to prohibit the manufacturing, packaging and distribution of cosmetic products containing microplastic beads, and the United Kingdom has followed in its footsteps by implementing a similar ban on microplastic beads which has already come into force since the end of 2017 (Vaughan, 2016). Some critics claim that more need to be done to restrict plastic use. (Rochman et al., 2013a) proposed that plastics should be labelled as “hazardous”, analogous to how chlorofluorocarbons (CFCs) were treated before their eventual ban, and plastic manufacturers should be required to declare their products “safe” before distribution, as is the current case for food and pharmaceutical manufacturers. The implementation of an “Ecocyclable” standard has also been suggested as the way forward by setting specific requirements for plastics to be non-toxic, degradable and readily able to assimilate into the carbon cycle (McDevitt et al., 2017).

In order to assist with the designation and prioritisation of areas for action, scientific research plays a crucial role in revealing specific life-cycle processes, ecosystems and biological interactions that are most at risk due to microplastic exposure. This study has highlighted the issue of microplastic pollution in a local bay popularised with oyster farms, and has come to show that what may ordinarily be perceived as a drop in the ocean has much farther-reaching consequences than we might imagine.

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