Microplastic accumulation in New Zealand green-lipped mussels *Perna canaliculus* and the role of microplastics in the uptake of triclosan

A thesis submitted in partial fulfilment for the degree of a Master of Science in Environmental Science

University of Canterbury

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2017

UNIVERSITY OF CANTERBURY
Te Whare Wānanga o Waitaha
CHRISTCHURCH NEW ZEALAND

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Acknowledgements

I would firstly like to thank my senior supervisor Associate Professor Sally Gaw. You have been the best supervisor I could ask for and also a great friend. Your hands on approach and belief in my abilities has made this all possible. Also, Professor Islay Marsden, my co-supervisor, thank you for your valuable input and assistance in the biological aspects of this project.

I gratefully acknowledge the Brian Mason Scientific and Technical Trust for funding this research.

I would never have completed had it not been for the technical staff at the University of Canterbury including Rennie Bishop, Jan McKenzie, Matthew Polson, Jonathan Hill, Alistair Duff, Rob Stainthorpe, Reijel Gardiner and Nathan Alexander. Thank you all for your time and patience with me and letting me continuously borrow equipment.

A particular thank you must go to all who have assisted me with sampling. Rennie Bishop, Chris Sampson, Natalie Pilcher and Ben Fraser, thank you for accompanying me on my sampling trips even in some less than ideal weather. Also, a huge thank you goes to the people who kindly collected samples for me from all around New Zealand. Dr Megan Oliver (Greater Wellington Regional Council), Anna Madarasz-Smith (Hawkes Bay Regional Council), Emily Roberts (Taranaki Regional Council), Dudley Bell and Rex Fairweather (University of Waikato), Brian Roughan (Ministry of Primary Industries), Ricky Eyre (Northland Regional Council), Brian Stewart (Ryder Consulting) and James Lambie (Horizons Regional Council). Thank you to Marcus Cameron (Auckland Council) for assistance and advice.

To the Environmental Chemistry and Marine Physiology research groups – thank you for letting me bounce ideas off you all and for your presentation help. Particularly Chris and Natalie, thanks for your positive words when I needed encouragement and getting me out of the lab when I needed a break. Phil Clunies-Ross, thank you both for your help and expert guidance throughout the project. Also, thank you Nicole McRae for your assistance in biochemical biomarker analysis.

To my family – Mum, Dad, Michelle and Andy – thank you for listening to me stress, giving me encouragement and trying to sound interested when I complain about broken instruments.

Finally, thank you to my partner Matt. Thank you for having dinner ready for me when I crawl through the door after a hard day, for encouraging me through the road blocks and for your complete faith in me from beginning to end.

Abstract

Small plastic particles, known as microplastics, are of increasing concern for marine and freshwater species. Microplastics have been reported globally in a wide range of marine species including mussels, crabs, fish and seabirds. This was the first study to investigate microplastic accumulation in the New Zealand green-lipped mussel, *Perna canaliculus*. This thesis investigated microplastic accumulation in the New Zealand green-lipped mussel and the effects of microplastics, both singly and in combination with triclosan (a hydrophobic, anti-microbial compound), on the green-lipped mussel.

Microplastic accumulation in green-lipped mussels around New Zealand was investigated using a field survey whereby mussels were collected from eight mussel beds around New Zealand with a more in depth survey conducted in Canterbury. Mussels were acid digested and the resulting digests observed under fluorescence coupled microscope to identify potential microplastic particles. Across both National and Canterbury mussel surveys, 35% of mussel samples analysed contained microplastics, the majority (78%) of the plastics isolated were fragments, with fibres (13%) and beads (9%) also located.

Microplastics can sorb hydrophobic contaminants from the water, potentially providing an additional pathway of exposure of marine species to contaminants. An acute 48 h laboratory study was conducted to investigate the effects of microplastics and triclosan, both individually and combined, on green-lipped mussels. The range of biomarkers assessed included clearance rate, oxygen respiration rate, byssus production, superoxide dismutase (SOD) activity, glutathione-S-transferase (GST) activity and lipid peroxidation. Microplastics had adverse effects on mussel physiology including decreased oxygen respiration rate and byssus production when present alone. These physiological impacts were not observed when the microplastics were sorbed with triclosan. Triclosan, both alone and with microplastics, adversely affected mussel oxidative stress markers including SOD activity and lipid peroxidation. A potential synergistic effect was observed on the SOD enzyme activity when mussels were exposed to triclosan sorbed to microplastics. No effect on the biochemical biomarkers was observed for mussels exposed to microplastic only. Microplastics enhanced the uptake of triclosan in the mussel tissue when triclosan was sorbed to microplastics compared with triclosan in the absence of microplastics. This indicates that microplastics potentially provide an additional pathway of exposure of hydrophobic contaminants to mussels in the marine environment.

This research illustrates that green-lipped mussels accumulate microplastics in the New Zealand marine environment. If concentrations of microplastics in the marine environment continue to increase, adverse effects on mussel physiology and uptake of sorbed contaminants may occur.

Abbreviations

ACN	Acetonitrile
ANOVA	Analysis of variance
BPC	2,2-bis(4-hydroxy-3-methylphenyl) propane
BSA	Bovine serum albumin
DAD	Diode array detector
DCM	Dichloromethane
EDTA	Ethylenediaminetetraacetic acid
GC-MS	Gas chromatography – mass spectroscopy
GST	Glutathione-S-transferase
HNO_3	Nitric acid
HPLC	High performance liquid chromatography
MDA	Malondialdehyde
MeOH	Methanol
MSTFA	N-methyl-N-(trimethylsilyl) trifluoroacetamide
PAHs	Polyaromatic hydrocarbons
PBDE	Polybrominated diphenyl ether
PE	Polyethylene
POPs	Persistent organic pollutants
PP	Polypropylene
PTFE	Polytetrafluoroethylene
PVC	Polyvinyl chloride
SOD	Superoxide dismutase
SPE	Solid phase extraction
UV	Ultraviolet

1. Introduction

The marine environment is under threat from a wide range of pressures. Human settlement in coastal regions has resulted in loss of coastal habitats, overfishing, nutrient loading and introduction of invasive species. Provisioning services such as aquaculture, construction and bioprospecting (exploration of marine biodiversity for resources) have added to these pressures. Most services that are derived from marine ecosystems are being used unsustainably causing an increased rate of degradation (UNEP, 2006).

Anthropogenic marine debris including metal, glass and plastic are commonly reported throughout the oceans. Plastics are among the most abundant marine debris due to high consumption, inappropriate methods of disposal and persistence in the environment. The threat plastic debris poses to marine life is of particular concern due to the impacts of ingestion and entanglement (Gall and Thompson, 2015).

A plethora of other contaminants are found in the marine environment resulting from human activities including: human and animal pharmaceuticals, personal care products, brominated flame retardants and household chemicals. Many of these are lipophilic compounds so bioaccumulate in living aquatic organisms (Llorca et al., 2016). They are present in the marine environment due to inefficient wastewater treatment systems and have the potential to sorb to microplastic particles (Bakir et al., 2012). These contaminants are of increasing concern to researchers investigating the pressures on the marine environment due to their potential toxicological effects on biota (Llorca et al., 2016).

1.1. Plastics

The invention of plastics is considered one of the most important technological advancements in recent history. Plastics have infiltrated all aspects of society and are now used daily by almost every human in the world. Mass production of plastics begun in the 1950's with approximately 1.7 million tonnes produced per year, rising to 311 million tonnes by 2014 (Statista, 2016). Plastics are used in a wide range of applications including packaging, storage and personal care products (Van Cauwenberghe et al., 2015). The key benefits of modern day plastics lie in their versatility and low cost. The most commonly used plastics include high and low density polyethylene, polyvinyl chloride, polypropylene and polystyrene (Table 1.1, Halden, 2010). They provide important applications in medicine and food packaging as they can be manufactured cost-effectively and hygienically for single-use. During the production of plastics, additives such

as plasticizers, antimicrobial compounds and flame retardants can be added to influence the properties. Many of these additives such as Bisphenol A and di-(2-ethylhexyl) phthalate (DEHP) have human health risks in the form of endocrine disrupting properties (Halden, 2010). As a result the environmental concerns regarding plastics arise from both the chemical additives and the physical properties.

Table 1.1: Common plastic types and their uses (Halden, 2010).

Plastic type	**Use**
High density polyethylene	Bottles and packaging
Low density polyethylene	Plastic grocery bags and shrink wrap
Polyvinyl chloride	Food wrap and medical devices
Polypropylene	Packaging and bottle tops
Polystyrene	Building materials, disposable utensils and toys.

The appropriate disposal of plastics is of significant environmental concern due to the volume of plastics produced and the extremely long time that it can take for them to degrade completely. The properties that are desirable for the applications of plastic materials, result in significant environmental concerns. An example of this is the long residence times of decades to millennia (Kukulka et al., 2012).

Disposal of plastics is difficult when the most effective way of completely disposing of non-biodegradable wastes, waste incineration, is known to produce carcinogenic polychlorinated dibenzo-p-dioxins/furans (PCDD/Fs) and organohalogens which are toxic and persistent (Halden, 2010). Recycling of plastics is encouraged in many locations around the world. However, effective “closed-loop” recycling (processing to manufacture a product with equivalent properties) is only possible under a range of conditions. For example, the waste stream for processing must consist of a narrow range of polymer grades and types and the end result must have the properties required for the designated use (e.g. extrusion into bottles; Hopewell et al., 2009).

1.1.1. Microplastics

Microplastics can be separated into two main categories, defined by the method of production. The first category, primary microplastics, includes the plastic particles manufactured to be of microscopic size. These are used as cosmetic abrasives in personal care products or for air

blasting (Cole et al., 2011). When used as abrasives in personal care products, microplastic beads are rinsed directly down the drain to the wastewater treatment plant. Primary wastewater treatment processes are able to remove a majority of microplastic particles from the influent; however, some particles are released into the environment via the effluent (Carr et al., 2016). Secondary microplastics are formed when macroplastics (larger plastic particles) are subjected to ultraviolet (UV) radiation and friction due to wave action in the marine environment, breaking larger pieces into smaller particles, eventually resulting in microplastics (Cole et al., 2011; Van Cauwenberghe et al., 2015). Secondary microplastics are thought to be the main contributor to microplastic pollution (Carr et al., 2016). Sources of macroplastics include fishing nets, food packaging, bottles and cigarette filters (Sheavly and Register, 2007).

It is estimated that approximately 10% of plastics produced make their way into the oceans (Cole et al., 2011) and that 80% of the world's marine pollution comes from land based sources (GESAMP., 1991). Sources of marine debris are often classified under two main categories: land-based and ocean/waterway-based depending on where the debris enters the water. Land-based debris is released on land and is washed, blown or discharged into the waterways. This can include illegal dumping of wastes, ineffective wastewater treatment, public littering and discharges from manufacturing operations. Ocean/waterway-based debris is generated by human activities at sea such as commercial and recreational fishing, offshore petroleum platforms and military vessels (Sheavly and Register, 2007).

Researchers have adopted different size limits and exclusion criteria for microplastic analysis with some stating that microplastics are less than 1 mm in diameter (Mathalon and Hill, 2014; Van Cauwenberghe et al., 2015) and some using 5 mm (De Witte et al., 2014; Devriese et al., 2015; Santana et al., 2016). For the purposes of this investigation, microplastic particles are classified as less than 1 mm in diameter.

1.1.2. Microplastic accumulation in marine organisms

Microplastics have the potential to be ingested by a range of marine organisms due to their small size and high persistence (Cole et al., 2011). Trophic level transfer of microplastics has also been reported where higher level organisms predate other species that have already ingested microplastics, raising the possibility for biomagnification (Farrell and Nelson, 2013). The extent of microplastic pollution in oceans is vast with over 600 marine species including invertebrates, crustaceans, fish, seabirds and mammals, adversely affected by microplastics in the marine environment (Claessens et al., 2013; Table 1.2).

Table 1.2: Microplastic contamination in marine organisms.

Location	Organism	Microplastics	Reference
Bivalves			
French-Belgian-Dutch coastline	*Mytilus edulis*	0.2 ± 0.3 microplastics g^{-1}	Van Cauwenberghe et al. (2015)
Santos Estuary, Sao Paulo, Brazil	*Perna perna*	75% of mussels had ingested microplastics	Santana et al. (2016)
Belgian Coastline and Netherlands	*Mytilus edulis, Mytilus galloprovincialis, Mytilus edulis/galloprovincialis* hybrids	2.6 – 5.1 fibres 10 g^{-1}	De Witte et al. (2014)
Commercial mussel farm - Germany	*Mytilus edulis*	0.36 ± 0.07 particles g^{-1} (ww)	Van Cauwenberghe and Janssen (2014)
Commercial mussel farm - Germany	*Crassostrea gigas*	0.47 ± 0.16 particles g^{-1} (ww)	Van Cauwenberghe and Janssen (2014)
Nova Scotia – wild mussels McCormack's Beach Rainbow Haven Beach	*Mytilus edulis*	 126 particles $mussel^{-1}$ 106 particles $mussel^{-1}$	Mathalon and Hill (2014)
Nova Scotia – farmed mussels	*Mytilus edulis*	178 particles $mussel^{-1}$	Mathalon and Hill (2014)
China	*Mytilus edulis*	0.9 to 4.6 items g^{-1}	Li et al. (2016)
Other invertebrates			

British Columbia, Canada	*Venerupis philippinarum*	0.07 – 5.47 particles g^{-1}	Davidson and Dudas (2016)
Germany	*Crassostrea gigas*	0.47 particles g^{-1} (ww)	Van Cauwenberghe and Janssen (2014)
China	*Scapharca subcrenata*	10.5 items g^{-1}	Li et al. (2015)
Crustacea			
North Clyde Sea	*Nephrops norvegicus*	83% of specimens had ingested microplastics	Murray and Cowie (2011)
Channel and southern part of North Sea	*Crangon crangon*	0.68 ± 0.55 microplastics g^{-1} (ww)	Devriese et al. (2015)
Polychaeta			
French-Belgian-Dutch coastline	*Arenicola marina*	1.2 ± 2.8 particles g^{-1}	Van Cauwenberghe et al. (2015)
Fish			
Mediterranean Sea	*Trachinotus*	24.3% occurrence	Battaglia et al. (2016)
Goiana Estuary, Brazil	*Cathorops spixii*, *Cathorops agassizii* and *Sciades herzbergii*	18 - 33% of fish had plastic	Possatto et al. (2011)
North Pacific Central Gyre	Planktivorous fish	2.1 pieces of plastic per fish	Boerger et al. (2010)
English Channel	Pelagic and demersal fish species	1.90 ± 0.10 microplastics $fish^{-1}$	Lusher et al. (2013)
Seabirds			
Canadian Arctic	*Fulmarus glacialis* (petrel)	0.094 g plastics $fulmer^{-1}$	Provencher et al. (2009)

Southern Brazil	Procellariiformes	38.3% of bird samples had plastic objects	Colabuono et al. (2009)
North Pacific Ocean	*Phoebastria immutabilis* and *P. nigripes*	51.7 – 83.3% of birds had ingested plastics	Gray et al. (2012)
Marine mammals			
The Netherlands	*Phoca vitulina* (Harbour Seals)	11% of seal stomachs contained plastics	Bravo Rebolledo et al. (2013)

Potential impacts of microplastics on marine organisms include blockages or abrasions of the digestive system, obstruction of enzyme production, decreased feeding stimulus, dilution of nutrients, reduced growth rates, reduced steroid hormone levels, reproductive difficulties, starvation and toxin adsorption (Wright et al., 2013). Microplastics (> 9.6 µm) have even been observed to translocate from the gut to the circulatory system in mussels. These particles were present in the hemolymph of *M. edulis* for over 48 days, suggesting they are a further risk of microplastic accumulation in predators such as birds, crabs and starfish through trophic level transfer (Browne et al., 2008). Microplastic particles decreased clearance rate, respiration rate and byssus production in mussels (*Perna viridis*) in response to microplastics following a 7 day exposure (Rist et al., 2016)

1.1.3. Sorption of organic contaminants to microplastics

Plastic materials may contain organic compounds, either incorporated during the manufacturing process (discussed in Section 1.1), or through sorption of contaminants from the water. It has been proposed that they can concentrate sorbed contaminants from water by up to six orders of magnitude (Farrell and Nelson, 2013). This has led to considerable interest from researchers regarding how plastics contribute to the transport of organic contaminants and whether ingestion of contaminated plastics by marine species contributes to bioaccumulation of the contaminant in the organisms (Koelmans et al., 2016).

The sorption of pollutants by microplastics is facilitated by the high surface area to volume ratio of microplastics and can include pollutants such as aqueous metals (Brennecke et al., 2016; Khan et al., 2015; Teuten et al., 2009), endocrine disrupting compounds (Cole et al., 2011) and persistent organic pollutants (POPs; Frias et al., 2010). Organic chemical pollutants including polychlorinated biphenyls, polyaromatic hydrocarbons and organochlorine pesticides have been isolated on microplastics in the environment at concentrations in the ng g^{-1} to µg g^{-1} range (Cole et al., 2011). Polyethylene has been shown to accumulate higher concentrations of organic contaminants than polypropylene and polyvinyl chloride based on both model calculations and experimental observations. Sorption of contaminants to plastics may also inhibit biodegradation of the contaminants, furthering their persistence (Teuten et al., 2009).

Ingestion of microplastics may provide an additional pathway of exposure of contaminants to organisms (Bakir et al., 2014a; Teuten et al., 2009). Desorption of contaminants from plastics is enhanced by the presence of surfactants and organic matter, indicating an increased rate of desorption will occur in gut conditions (Teuten et al., 2009). For example, Bakir et al. (2014a)

reported that desorption of POPs from microplastics under gut conditions could be up to 30 times greater than in seawater alone.

1.1.4. Regulating microplastic pollution

The International Convention for the Prevention of Pollution from Ships (MARPOL) was signed in 1973 to curb the increasing rate of marine plastic pollution from ocean based sources. However, the disposal of plastics at sea was not banned until 1988. It is suggested that this policy has done little to combat plastic accumulation with concentrations of plastics in the North Pacific increasing by two orders of magnitude since the policy was signed (Rochman et al., 2013).

Internationally governments are introducing regulations to reduce plastic use. For example, several countries have placed taxes on plastic carrier bags which has resulted an up to 90% reduction in their use in the European Union (EU) (Steensgaard et al., 2017). The New Zealand government recently produced a consultation document regarding the use of plastic microbeads in personal care products. This aims to bring New Zealand in line with other countries including the United States and the United Kingdom in banning plastic microbeads in personal care products (Ministry for the Environment, 2017).

Recycling of plastics is encouraged by governments particularly due to the increasing costs of disposal and the related environmental concerns (Bryce et al., 1997). Countries including Switzerland, Germany, Austria and Belgium have reported up to 99% of plastics are recycled or energy recovered in these countries. However, other countries including Malta, Greece and Cyprus, have no recycling schemes with > 75% of plastics disposed of in landfills (Steensgaard et al., 2017). New Zealand has a kerbside recycling collection scheme which includes recycling of plastics bottles, containers and shopping bags but excludes plastic wraps or films and lids (Christchurch City Council, 2016). However, New Zealand supermarkets have recently introduced a soft plastic recycling scheme which facilitates recycling of plastic films and wrappers (Hewett and Mayes, 2016).

1.2. Triclosan

A range of chemicals including flame retardants, non-polar pesticides, pharmaceuticals and personal care products are considered contaminants of emerging concern (CECs) in the aquatic environment. Many CECs are persistent, widely distributed, have the potential to bioaccumulate and are toxic to marine and freshwater species (Llorca et al., 2016). They enter the environment via a range of pathways including: municipal and industrial wastewater effluent, agricultural

runoff, seepage of landfills and storm water discharges. Pharmaceuticals and personal care products in particular can cause adverse effects in aquatic organisms including effects on growth, reproduction and development (Bolong et al., 2009).

Triclosan or 5-chloro-2-(2,4-dichlorophenoxy)phenol is a common broad-spectrum anti-microbial agent (Figure 1.1). It has a relatively high octanol-water partitioning coefficient (log K_{ow}) of 4.8 at pH = 7 meaning it has a high bioaccumulation potential (Fang et al., 2010; Ricart et al., 2010). Triclosan was first introduced to the healthcare industry in the 1970's as a synthetic, lipid-soluble antimicrobial agent. It is used in a wide range of personal care products, household items and medical items including toothpastes, hand soaps, deodorants and cleaning products. It is also incorporated into textiles such as carpets, sportswear and shoes (Fang et al., 2010). However, the necessity and effectiveness of triclosan in non-healthcare settings is the subject of ongoing debate (Yueh and Tukey, 2016).

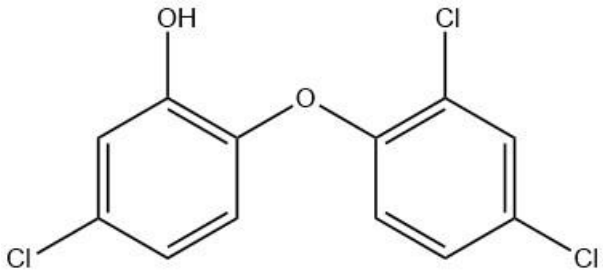

Figure 1.1: Chemical structure of triclosan (5-chloro-2-(2,4-dichlorophenoxy)phenol)

Current wastewater treatment plant (WWTP) systems are often not designed for the removal of organic compounds such as those present in pharmaceuticals and personal care products. Subsequently, triclosan has been reported in WWTP effluents in concentrations as high as 2.7 µg L^{-1} (Ricart et al., 2010) and in estuarine sediment at up to 800 µg kg^{-1} (Dhillon et al., 2015). Due to the widespread use and disposal into sewage systems, triclosan has been reported in finished drinking water, surface water, wastewater and sediments (Fang et al., 2010; Yueh and Tukey, 2016). Additionally, if chlorine is used in the disinfection process, chlorinated triclosan derivatives may result which can be more toxic than triclosan itself with the toxicity increasing with increasing chlorine substitutions (Yueh and Tukey, 2016). In seawater, triclosan has a half-life of approximately four days and can photodegrade to form 2,8-dichlorodibenzo-*p*-dioxine (DCDD) (Aranami and Readman, 2007). The wide spread use of triclosan and ineffective removal mechanisms from wastewater results in pseudo-persistence. This is where the compound is

continually released into the environment and is therefore, continually present, regardless of transformation or degradation mechanisms (Mackay et al., 2014).

High concentrations of triclosan in surface water, suggest it may sorb to microplastics in the environment (Yueh and Tukey, 2016). It is also manufactured into plastics to cause inherent anti-microbial properties (Fang et al., 2010). Triclosan has also been observed to bioaccumulate in snails, algae, fish and marine mammals (Yueh and Tukey, 2016). There is, therefore, an increased need for research into triclosan and microplastics and the impact on marine organisms.

1.2.1. Impact of triclosan on organisms

Triclosan's primary mechanism of action is that it can enter the cell membrane of bacteria and disrupt membrane activities (Fang et al., 2010). It attaches via the hydroxyl group to the upper region of the phospholipid membrane with the remainder of the molecule lying perpendicular to the phospholipid chain (Guillén et al., 2004). Triclosan has also been shown to interfere with bacterial lipid biosynthesis by inhibiting the enoyl-reductase of type II fatty acid synthase and it also has anti-viral, anti-fungal and anti-malarial activity (Fang et al., 2010).

Triclosan is not considered genotoxic or mutagenic in most animal models; however, it induced DNA damage in zebra mussels at concentrations as low as 1 nM. It has been suggested that aquatic organisms are more susceptible to its genotoxic and mutagenic effects (Fang et al., 2010). Triclosan has also been shown to induce oxidative stress in bivalves (Binelli et al., 2009; Binelli et al., 2011; Matozzo et al., 2012). This toxicity mechanism is discussed further in Section 4.1. Antimicrobial resistance, whereby bacteria are able to survive in concentrations of antibiotics that typically inhibit the growth of other bacteria, is thought to be influenced by triclosan. Resistance to triclosan and multidrug resistance was reported to increase in microbial communities exposed to triclosan (Carey and McNamara, 2015).

1.2.2. Regulation of triclosan

Triclosan has been a significant topic of discussion in the international environmental science community in the last two years. In 2016, the United States Federal Drug Administration (USFDA) determined that there was insufficient evidence to justify the use of triclosan in over-the-counter consumer antiseptic washes. The European Union (EU) has ruled that triclosan is prohibited from goods that come into contact with food and is not approved for use in human hygiene products as a disinfectant. In New Zealand, limits for triclosan in toothpastes, cosmetics and mouthwashes are 0.3% under the Hazardous Substances and New Organisms (HSNO) Act (Environmental Protection Agency, 2016).

1.3. Mussels as bioindicators

Molluscs, in particular mussels, are considered the bioindicators of choice due to their wide distribution, accessibility and experimental suitability (Viarengo et al., 2007). Mussels are sessile, filter-feeding animals that are widely distributed along most coastlines around the world, making them an important species for monitoring water quality and for biomonitoring studies (Krieger et al., 1981). They feed on plankton and other microscopic marine species by drawing water through the incurrent siphon and transporting the water via the gills to the branchial chamber where the food is separated from the wastewater and funnelled to the mouth for digestion. Mussels have high bioaccumulation and low biotransformation potential for organic and inorganic contaminants (Stankovic and Jovic, 2013). They satisfy many of the criteria required for an effective monitoring species including ease of access to relatively large populations, sedentary adulthood and large size, tolerance to a range of environmental conditions and filtration of (and therefore, exposure to) large volumes of water for respiration and nutrition (Krieger et al., 1981).

1.3.1. The green-lipped mussel, Perna canaliculus

The New Zealand green-lipped mussel (*Perna canaliculus*), also known as kuku or kutai, is an endemic bivalve species from the molluscan family *Mytilidae*. It is distinguished from other mussel species by a green region around the posterior ventral margin of the shell and due to its relatively large size compared with the blue mussel *Mytilus edulis* reaching a maximum length of around 200 mm compared with 130 mm for the blue mussel (Figure 1.2; Murphy et al., 2002). Wild green-lipped mussels can also be harvested from low tidal regions around the coast of New Zealand and are generally located in slightly deeper waters than blue mussels (*M. edulis*).

Figure 1.2: New Zealand green-lipped mussel, Perna canaliculus.

New Zealand green-lipped mussels are farmed commercially in regions including Marlborough, Coromandel, Canterbury, Southland and Tasman. In 2011, exports of green-lipped mussels, under the trade name New Zealand Greenshell™ mussels, produced a revenue of NZ$218,100,000 contributing to 85% of aquaculture exports by volume from New Zealand (Aquaculture NZ, 2012). Green-lipped mussels are a source of essential vitamins, minerals, iron, lipids and protein (Sivakumaran et al., 2014).

1.4. Biomarkers

Biomarkers are used in biomonitoring programmes to examine the effects of contaminants on the physiology or biochemistry of a species and can be defined as a measured response that can indicate that an organism has been exposed to a stress inducing factor (Shugart et al., 1992). When selecting analyses for biomonitoring experiments, the suite of biomarkers should include a range stress sensitivities from a whole organism level down to a cellular level (Viarengo et al., 2007).

1.4.1. Whole body physiological biomarkers

Physiological biomarkers provide an indication of the physiological health status of the mussel (Chandurvelan et al., 2012). These biomarkers are relevant at community and population levels so are considered ecologically significant (Widdows and Donkin, 1992). Clearance rate, oxygen respiration rate and byssus production are three important physiological biomarkers for mussel stress response (Bayne et al., 1979; Chandurvelan et al., 2012; Lurman et al., 2013; Marsden and Shumway, 1992; Widdows and Staff, 2006). These assays provide an integrated assessment of an organism's well-being (Lam, 2009). Physiological biomarkers including clearance rate, oxygen respiration and byssus production have been previously used to assess the effects of microplastics on the Asian green mussel, *Perna viridis* (Rist et al., 2016).

1.4.2. Cellular biochemical biomarkers

In response to toxic chemicals, adverse cellular and biochemical changes can occur in aquatic organisms. A range of assays have been developed to assess these responses for biomonitoring processes (Walker, 1995). Oxidative stress is one such response, caused by a higher level of reactive oxygen species than antioxidants potentially leading to cell damage (Narra et al., 2017). Two key enzymes in the reactive oxygen species defence system include superoxide dismutase (SOD) and glutathione-S-transferase (GST; Viarengo et al., 2007). GST catalyses a wide range of conjugation reactions of glutathione with xenobiotic compounds and SOD facilitates the scavenging of superoxide anion radicals (Regoli and Principato, 1995). The amount of oxidative

damage occurring can be determined as lipid peroxidation measured via the formation of malondialdehyde (MDA). This occurs when phospholipids are oxidised by reactive oxygen species producing MDA (Valavanidis et al., 2006).

1.5. Thesis objectives and layout

The main objectives of this study were to (a) investigate microplastic accumulation in New Zealand green-lipped mussels, *Perna canaliculus*, including the size of microplastics the mussels ingest; (b) determine if there is a relationship between microplastic concentrations in seawater and in green-lipped mussels; (c) investigate the effects of microplastics and triclosan both individually and combined on the green-lipped mussel using a range of physiological and biochemical biomarkers; and (d) determine whether microplastics enhance the uptake of triclosan by green-lipped mussels. The cultural and economic importance of green-lipped mussels and their proven significance as an indicator species contributed to the selection of this species for use in this project.

A detailed summary of the methods and analyses performed in this study is presented in Chapter 2. Chapter 3 presents the results from the field survey where mussels were collected and analysed from around New Zealand with a more detailed sub-study performed in the Canterbury region. In Chapter 4, the effects of microplastics and triclosan on green-lipped mussels are reported. Whole body physiological and cellular biochemical biomarkers were used in this investigation to compare the effects of microplastics and triclosan on the green-lipped mussels with the effects of the two combined. A summary of the thesis is included in Chapter 5 including recommendations for future work and implications of the findings on microplastic research and policy for marine pollution in New Zealand.

2. Materials and Methods

2.1. Chemicals and Materials

All solvents used in this study were HPLC grade and purchased from Fisher Scientific. These included methanol (MeOH), dichloromethane (DCM), acetonitrile (ACN), hexane, iso-octane and acetone. Ultra-pure water (< 18 MΩ) was sourced from an in-house water filtration system (Santorius, USA). Nitric acid (HNO_3, analytical grade 69%) was purchased from Univar.

Whatman GF/C filter paper (1.2 µm pore size, 47 mm diameter) and Whatman Grade 1 filter paper (11 µm pore size, 55 mm diameter) were purchased from Sigma Aldrich. Biochemical assay kits were also purchased from Sigma Aldrich included Lipid Peroxidation (MDA), Glutathione-S-transferase (GST) and Superoxide dismutase (SOD). Fluorescent orange polyethylene microspheres (diameter 38 - 45 µm, density 1.004 g cc^{-1}) were purchased from Cospheric LLC, USA. Seachem Reef Phytoplankton was purchased from Hollywood Fish Farm (Auckland, NZ).

Trizma Base (≥ 99.9% purity) was purchased from Sigma Life Sciences. Magnesium chloride ($MgCl_2.6H_2O$, ≥ 99.0% purity) was purchased from Biolab Australia Ltd. Ethylenediaminetetraacetic acid disodium salt (EDTA, 99.4% purity) was purchased from J T Baker. Bovine Serum Albumin (BSA) was purchased from GIBCO, Invitrogen Coorporation.

Solid standards of triclosan (5-chloro-2-(2,4-dichlorophenoxy)phenol, 99.5% purity) were purchased from Dr. Ehrenstorfer GmbH. Solid standards of methyl triclosan (2,4,4'-trichloro-2'-methoxydiphenyl ether, 99.1% purity), 2,2-bis(4-hydroxy-3-methylphenyl) propane (BPC, 97% purity) and ammonium iodide (NH_4I, ≥ 99% purity) were purchased from Sigma-Aldrich. 2-mercaptoethanol (2-sulfanylethan-1-ol, ≥ 99.0% purity) and *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA, 98.5% purity) were also purchased from Sigma-Aldrich. Carbon-13 labelled triclosan (^{13}C-triclosan, 99% purity) was purchased from Cambridge Isotope Laboratory Inc.

Solid phase extraction (SPE) cartridges (Phenomenex®, Strata Fluorisil, 1000 mg/6 mL) were purchased from Thermo Fisher Scientific. Silica gel 60 (230-400 mesh) was purchased from Merck. Sulfuric acid (98.5% purity) was purchased from IXOM. Helium gas (instrument grade) was purchased from Southern Gas.

2.2. Cleaning

All glassware used for field analysis were rinsed three times with ultra-pure water and once with acetone and allowed to dry before use (Oliveira et al., 2013). Aquaria and glassware used for exposures were acid washed in 10% HNO_3 overnight and rinsed with ultra-pure water before being solvent washed using three rinses each of DCM, ACN and MeOH and allowed to dry. All other glassware used for laboratory analysis was solvent washed as described above and dried before use.

All cleaning, preparation and analysis was performed in a foil lined fume cupboard to minimise plastic contamination.

2.3. Field study of microplastic accumulation

2.3.1. Sample site selection and collection

National mussel sampling

Regional Council and Ministry for Primary Industries employees from around New Zealand were contacted to gain local knowledge about where in their respective regions, green-lipped mussels could be sampled. In most cases, they replied with knowledge and offers of collecting the mussels as part of their routine sampling regime. Eight sampling sites were selected in order to provide a wide range of environments and spatial locations around New Zealand (Table 2.1). Auckland was not included as a sampling location as Auckland Council reported that there were no suitable mussel sampling locations available.

Plastic chilly bins (10 L Esky, purchased from Bunnings Warehouse NZ) were lined with aluminium foil and couriered to Regional Council, Ministry of Primary Industries or University staff. An aluminium foil lined freezer pack was included in the chilly bin. Samplers were given instructions regarding sampling including to sample 12 *Perna canaliculus* with a minimum of 50 mm in length and from a similar tidal level. They were asked to avoid exposure to plastics during sampling and handling and to provide GPS coordinates of the sample location. The chilly bins containing harvested mussels were then couriered to the University of Canterbury.

Table 2.1: Location and date of collection of samples from national sampling sites.

Site Name	Region	Date of sampling	GPS coordinates (NZTM)
Bay of Islands	Northland	31/05/2016	6064463 Northing, 1733575 Easting
Mount Maunganui	Bay of Plenty	01/05/2016	5830650 Northing, 1880332 Easting
Ahuriri Estuary, Napier	Hawkes Bay	07/04/2016	5622444 Northing, 1934521 Easting
New Plymouth	Taranaki	06/04/2016	5678892 Northing, 1695940 Easting
Wellington Harbour	Wellington	30/05/2016	5427156 Northing, 1753574 Easting
Port Underwood	Marlborough	26/03/2016	5427786 Northing, 1694692 Easting
Westport	West Coast	21/04/2016	5376849 Northing, 1472792 Easting
Lawyers Head, Dunedin	Otago	04/08/2016	4913059 Northing, 1408600 Easting

Canterbury sampling

Sampling sites in the Canterbury region were chosen pairwise to represent areas expected to exhibit high and low levels of microplastic contamination (Table 2.2, discussed further in Section 3.1.1). Surface water samples were also collected at Canterbury sampling sites. This was involved collecting approximately 3 L of water using 4 L amber glass bottles in triplicate.

Table 2.2: Canterbury mussel and water sampling sites and date of sampling. Paired samples are indicated by bold grey lines.

Sampling Location	Anticipated level of contamination	Date of Sampling
Avon-Heathcote Estuary	High	26/02/2016
Taylors Mistake	Low	06/05/2016
Pigeon Bay	High	25/05/2016
Little Akaloa	Low	25/05/2016
Lyttelton Port	High	20/06/2016
Lyttelton Harbour	Low	20/06/2016
Akaroa Harbour	High	22/06/2016
Damon's Bay	Low	22/06/2016

Processing of collected mussel samples

Upon arrival at the laboratory, the mussel species was visually confirmed by identification of green or brown areas around the lip of the shell. Samples were weighed and the length and width of the shell was measured. They were then shucked using a metal knife on an aluminium foil lined wooden board. The tissues were removed from the shells and parcelled individually in two layers of aluminium foil. The specimens were frozen at -80°C.

2.3.2. Microplastic extraction from mussel tissue

Method development

A modification of the digestion method proposed by Claessens et al. (2013) was trialled. In their investigation, *Mytilus edulis* was the field organism, 40 – 45 mm in length. They added 20 mL of HNO_3 (22.5 M) to three mussels, left to deconstruct overnight, followed by boiling for 2 h, diluting to 200 mL using deionised water and filtering (Claessens et al., 2013). Due to the larger size of the *P. canaliculus* compared to *M. edulis*, this method was initially altered to include just two mussels per replicate and 60 mL HNO_3. Observation during and following this trial showed significant deconstruction of the tissue after 17 h and subsequent 2 h of boiling caused complete dissolution of tissues. It was concluded that 60 mL HNO_3 was too much acid and 40 mL would be sufficient to digest the mussel tissue.

Filtration of the trial solutions resulted in a thick layer of debris on the filter paper. The thickness of this layer allowed for the possibility of masking some of the plastic particles. Subsequently, each replicate of two mussels was filtered across four filter papers to minimise the masking effects of the debris. This was done by filtering approximately 50 mL of the solution through a filter paper and rinsing the paper with ultra-pure water, quickly replacing the filter paper and repeating three times. Following filtration of the final 50 mL of solution, filtered through the fourth filter paper, approximately 20 mL warm (~80°C) ultra-pure water was used to rinse the flask and this was poured through the filter paper. This rinse step was repeated two more times. This method proved to be effective in minimising masking due to debris and ensuring all of the contents of the flask were rinsed onto the filter paper.

Optimised mussel tissue digestion

Six specimens from each location were removed from the freezer one hour prior to digestion and thawed. Samples were weighed individually using aluminium weigh boats and transferred to three, 250 mL conical flasks, two mussels per flask. Concentrated HNO_3 (40 mL) was transferred to the flasks using a clean glass measuring cylinder including an empty 250 mL conical flask, to be used as a negative control. The flasks were covered using watch glasses and left overnight at room temperature. The following day, the solutions were boiled for two hours on a hot plate. Warm, ultra-pure water (~80°C, 160 mL) was added to the flasks and the solution was swirled and immediately vacuum filtered through Whatman GF/C filters (1.2 µm pore size, 47 mm diameter) using a glass filtration system. Four filter papers were used per sample to minimise masking of the plastic particles by other debris. Additional 80°C water (approx. 20 mL in triplicate) was used to ensure all of the contents was rinsed from the flasks. Filter papers were placed in cleaned petri-dishes and oven dried. The petri-dishes were then removed from the oven, wrapped in aluminium foil and stored for analysis.

2.3.3. Water filtration

Due to the small volume of water to be analysed (2 L) and the relative cleanliness of the water, it was decided that density separation and digestion of organic matter would not be required. A filter pore size of 11 µm was determined to be sufficient due to the size limit of detection of particles possible with the microscope setup and to allow rapid filtration.

Two litres of seawater from each sample site was vacuum filtered through Whatman Grade 1 filter paper (11 µm pore size, 55 mm diameter) in triplicate. Ultra-pure water (approx. 50 mL in triplicate) was used to rinse any debris from the measuring cylinder and to dissolve salt crystals that had accumulated on the filter paper. The filter papers were placed in clean petri-dishes and

oven dried. The samples were then removed from the oven, wrapped in aluminium foil and stored for analysis.

2.3.4. Microplastic Detection

Filter papers were observed under Leica MZ10f fluorescence coupled microscope at 15 – 120x magnification. Filter papers were scanned in non-fluorescent mode at 15x magnification to locate possible microplastic particles. When located, potential particles were observed under UV, Green and GFP-Plus fluorescence modes and images of each particle were collected at the optimal magnification. Details for each particle were recorded including dimensions, shape and fluorescence wavelength. Particles were characterised as fragment, fiber or bead.

Identification criteria

Strict criteria were adhered too for identification of microplastic particles in water and mussel samples. The criteria included:

- Objects present on blank filters of the same colour/shape/size were not included;
- Fibers were to be uniform thickness throughout the entire length;
- Particles were to have homogenous colours. If they were white or clear they were further examined using fluorescence (Hidalgo-Ruz et al., 2012);
- Fibres that fluoresced blue under UV light were not included as these were a common contaminant.

The estimated size limit for detection of microplastic particles was 40 µm in diameter for all fluorescent and non-fluorescent modes.

2.4. Impact of microplastic and triclosan on green-lipped mussels

2.4.1. Sample collection

Green-lipped mussels (*Perna canaliculus*) were collected from Taylors Mistake beach (5174281.563 Northing, 1582162.917 Easting). Samples were collected during low tide, using scissors to cut byssus threads and transported to the laboratory in an aluminium foil lined chilly bin. Mussels were transported emersed (in air) at approximately 12°C (Chandurvelan et al., 2013b). Upon arrival, the shells were cleaned by gently scraping to remove epibionts and transferred to a 50 L tank with aerated seawater. Mussels were left for two days in a temperature control room at 15°C to acclimatise prior to exposures.

2.4.2. Sorption of triclosan to microplastics

The method used for the sorption of triclosan to the polyethylene microspheres was adapted from Browne et al. (2013). A triclosan spiking solution (1000 mg L^{-1}) was prepared by dissolving 25 mg of triclosan in 25 mL MeOH. MeOH (20 mL) was added to five 100 mL beakers which were then spiked with 2 mL of the 1000 mg L^{-1} triclosan spiking solution. The beakers were wrapped in aluminium foil and left on an orbital mixer for 20 min in a dark fume cupboard. Fluorescent orange polyethylene microspheres (2 g) were added to each beaker. After returning the beakers to the orbital mixer, they were left uncovered in a dark fume cupboard at room temperature to allow evaporation of the MeOH to dryness. The microplastics were then removed from the beakers, combined in a 40 mL amber vial and mixed thoroughly by rolling the cylindrical vial back and forth along the bench for 20 min. Three aliquots (0.1 g) of the microplastics were removed from the vial to determine the concentration of triclosan sorbed to the plastics.

Determining the concentration of triclosan sorbed to microplastics

The sorbed concentration of triclosan was measured by adding 2 mL DCM to each 0.1 g aliquot (n = 3), vortex mixing and allowing to settle. The DCM was transferred to a 15 mL amber vial using a fine tipped glass Pasteur pipette. This process was repeated two further times to ensure complete desorption of the triclosan from the plastics. The 6 mL DCM solution was filtered using pre-cleaned 0.45 µm PTFE (polytetrafluoroethylene) syringe filters and glass syringe barrels and then dried at 40°C under nitrogen gas before being quantitatively transferred to amber HPLC vials using 500 µL MeOH followed by 2 x 250 µL MeOH.

The method for quantification of triclosan by HPLC (high performance liquid chromatography) was adapted from Ricart et al. (2010). Separation was achieved using a Dionex UltiMate® 3000, UHPLC+ focussed, HPLC system with UltiMate® 3000 pump, auto-sampler and diode array detector (DAD). Chromeleon 7 Chromatography Data System software (version 7.2 SR4) was used to control the HPLC system and integrate peaks. Samples were injected via auto-sampler through a Phenomenex Gemini® (5 µm, C18, 110 Å, 150 x 2 mm) HPLC column with an isocratic mobile phase of 90:10 MeOH:water at 0.2 mL min^{-1}. The triclosan peak was detected at 280 nm.

The limit of detection of triclosan by this method was 0.3 mg L^{-1} in the 1 mL extract. The concentration of triclosan sorbed to the microplastics was 0.73 ± 0.03 mg g^{-1}. This concentration was used to calculate the required concentration of triclosan for the triclosan only treatment (0.36 mg L^{-1}).

2.4.3. Exposures

The exposure and acclimatisation was performed in a dark, climate controlled room due to potential photodegradation of triclosan. Following acclimatisation, mussel byssus threads were carefully cut with scissors and the mussels were placed randomly into five acid washed and solvent rinsed 4 L glass vessels, six mussels per vessel. Mussels were left for 48 h in 3 L filtered seawater (1 µm pore size) with the following conditions: control (C1), acetone control (C2), microplastics only (MP), triclosan only (TCS) and triclosan sorbed to microplastics (MP_{TCS}; Figure 2.1). At the beginning of the exposures, 2 mL Seachem Reef Phytoplankton (a concentrated algal solution (von Moos et al., 2012)) was added to each vessel and vessels were aerated and covered with aluminium foil (Figure 2.2). There were six replicates for each treatment. The acetone control was included as the triclosan spike solution was dissolved in acetone due to the hydrophobic nature of triclosan (Oliveira et al., 2013). The solvent concentration in the C2 and TCS treatment vessels was < 0.01% v/v.

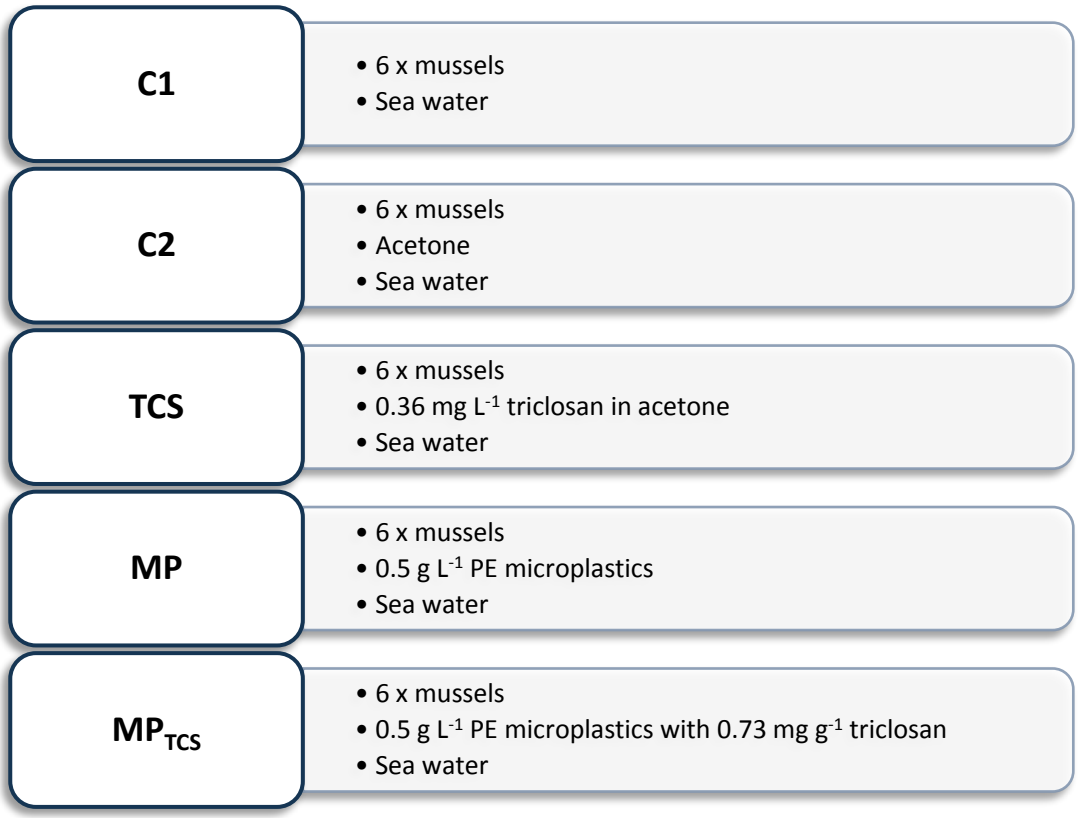

Figure 2.1: Setup for exposure treatments. Entire setup was repeated six times. C1 = control 1; C2 = control 2; MP = microplastics only; TCS = triclosan only; MP_{TCS} = triclosan sorbed to microplastics. PE = polyethylene.

Figure 2.2: Exposure setup. Each vessel contained six mussels. Entire setup was completed six times.

A 10 mL water sample was collected from each vessel (plus a duplicate for each sampling period) at the beginning and after 48 h for analysis of triclosan concentration. The water samples were filtered through glass wool in glass syringes to remove microplastics and 2 mL DCM was added to each vial and stored at 4°C. After the 48 h exposure, mussels were removed and prepared for analysis or analysed immediately for physiological biomarkers (Figure 2.3).

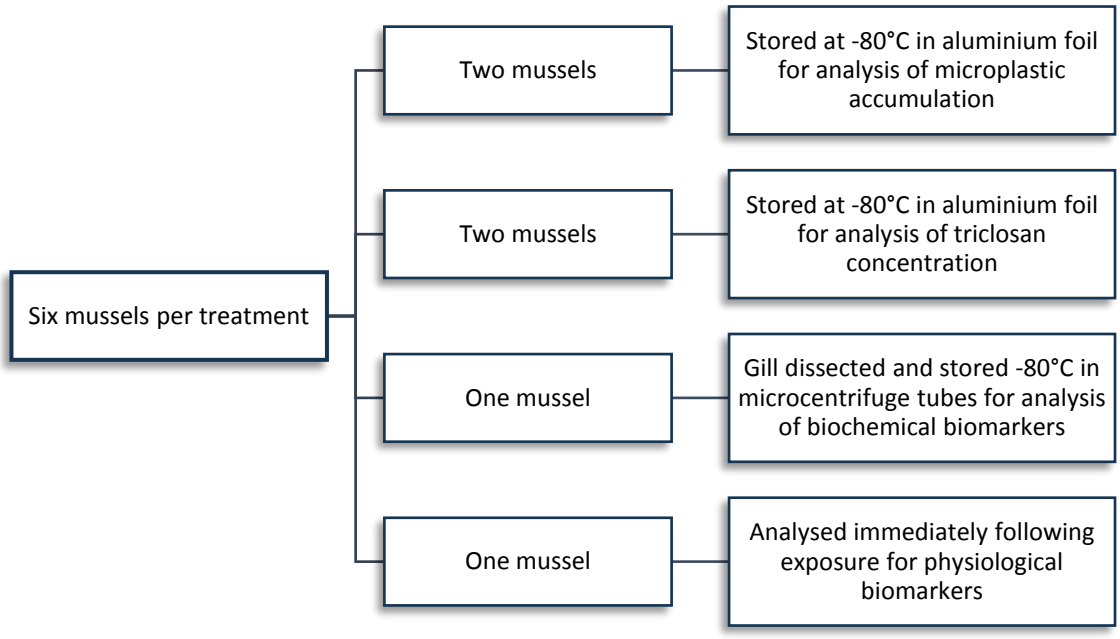

Figure 2.3: Flow chart illustration of sample analysis for each treatment (n=6).

2.4.4. Physiological biomarkers

Clearance rate

The clearance rate can be defined as the volume of water cleared of particles per hour (Widdows and Staff, 2006). The method for measuring the clearance rate by the mussel was adapted from Widdows and Staff (2006). Mussels were placed individually into 350 mL containers with 200 mL filtered seawater. After 1 h, the seawater was replaced with 200 mL filtered seawater containing 490,000 cells mL^{-1} of Seachem Reef Phytoplankton (cell size 1-20 µm). This concentration was chosen to prevent production of psuedofaeces. A 1 mL water sample was collected immediately after addition of the feed solution and mussels were left for 1 h. A 1 mL water sample was then taken from each container and 10 µL Lugol's was added. Cell concentrations were calculated by counting cells using a Marienfeld-Superior Haemocytometer under a Nikon compound microscope.

Oxygen respiration rate

The rate of oxygen respiration was measured using a closed respirometer held in a water bath at 15°C. Mussels were sealed in 400 mL containers filled with oxygen saturated water (Figure 2.4). The rate of decline in oxygen partial pressure was measured by taking 5 mL water samples every 30 min and measuring partial pressure using a Strathkelvin oxygen meter (Model 781). This was performed for 90 min and the partial pressure of oxygen was converted to oxygen concentration using equation 2.1:

$$C_{ti}\left(\mu mol\ L^{-1}\right) = \frac{Exptl\ PO_2 \times 259.6\ \mu mol\ L^{-1}}{PO_2\ max} \qquad \textit{Equation 2.1}$$

Where Exptl PO_2 = the partial pressure (mm Hg) measured by the electrode; and PO_2 max = the oxygen partial pressure at maximum saturation (mm Hg). The respiration rate was calculated using equation 2.2 (modified from Widdows and Staff (2006)):

$$Rate\ of O_2 uptake\ (\mu mol\ O_2\ g^{-1}h^{-1}) = \frac{(C_{t0} - C_{ti}) \times V_r \times 60}{W(t_0 - t_i)} \qquad \textit{Equation 2.2}$$

Where C_{t0}, C_{ti} = concentration of oxygen (µmol O_2 L^{-1}) at the start (t_0) and finish (t_i) of the measurement period; V_r = volume of the respirometer minus the animal; W = mussel dry weight (g) (Chandurvelan et al., 2012). Oxygen respiration was expressed as µmol O_2 g $(dw)^{-1}$ h^{-1}.

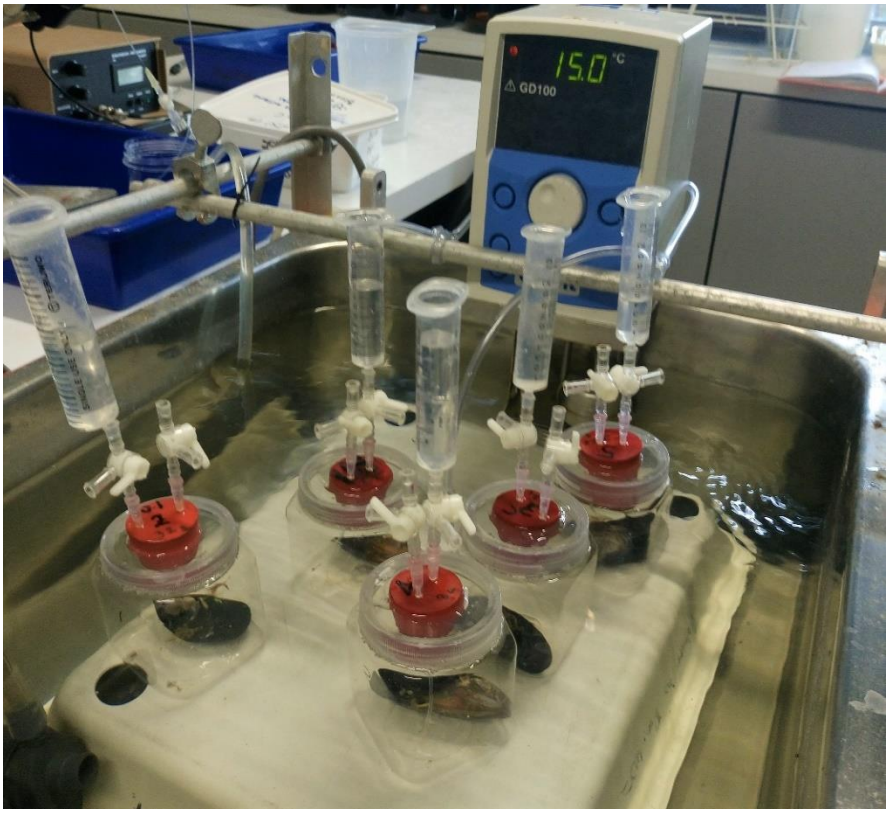

Figure 2.4: Setup for measurement of oxygen respiration.

Byssus thread production

The number of byssus threads produced in the 48 h exposure were carefully counted and the number recorded (Marsden and Shumway, 1992). Results are expressed as number of threads g $(dw)^{-1}$ (dry weight).

Mussel dry weight

To determine mussel dry weight (dw), mussels were shucked, weighed and dried at 60°C for 48 h. Dried samples were then removed from the oven and dry weight calculated (Chandurvelan et al., 2016).

2.4.5. Biochemical Biomarkers

All manipulation and analysis were performed on ice.

Gill tissue homogenisation for biochemical assays

Dissected gill tissue was homogenised in 1.5 mL microcentrifuge tubes using ice cold tris-buffer. The tris-buffer was prepared by dissolving Trizma® base (3.03 g), magnesium chloride (0.146 g) and EDTA (0.254 g) in ultra-pure water (250 mL). The pH was adjusted to pH 7 using dilute hydrochloric acid.

The buffer (300 µL) was added to the microcentrifuge tube containing gill tissue and the contents homogenised using a plastic pallet homogeniser for 2 mins, a further 700 µL buffer was added and tubes were centrifuged at 18,000 g for 20 min. Aliquots of supernatant were transferred to clean microcentrifuge tubes for subsequent analyses. Supernatants were stored at -80°C and used for analysis of protein content, lipid peroxidation, superoxide dismutase and glutathione-S-transferase (Chandurvelan et al., 2013a).

Bradford assay

The method described by Bradford (1976) was used to determine the protein content in the gill tissue. Supernatants were diluted x 40 and 10 µL of diluted supernatant was transferred in triplicate to 96 well plates. Biorad Protein Assay Reagent Concentrate was diluted five times using ultra-pure water and 200 µL added to each well. Absorbance was measured using a Molecular Devices SpectraMax M5 plate reader at 595 nm and readings were calibrated against standard concentrations of Bovine Serum Albumin. Results were reported in mg μL^{-1} (Bradford, 1976). All replicates had a relative standard deviation of < 10%. Calibration curve showed a strong linear relationship (R^2 = 0.990).

Lipid peroxidation assay

A Lipid Peroxidation (MDA) Assay Kit was used to quantify the lipid peroxidation of the gill tissue. Malondialdehyde (MDA) is a product of the attack of reactive oxygen species on polyunsaturated lipids. The method involves the reaction of MDA with thiobarbituric acid (TBA) to form a colorimetric species with absorbs at 532 nm, directly proportional to the amount of MDA and therefore, degree of lipid peroxidation (Sigma-Aldrich, Technical bulletin – Lipid peroxidation (MDA) assay kit). MDA lysis buffer (300 µL) and butylated hydroxytoluene (3 µL) was added to 200 µL of tissue supernatant and the mixture was centrifuged at 13,000 g for 10 min. The resulting supernatant (200 µL) was added to a microcentrifuge tube containing 600 µL TBA solution. The solution was incubated for 60 min at 95°C, then cooled to room temperature. Once cooled, 200 µL of the solution was transferred in triplicate to 96-well plates and absorbance measured at 532 nm using a Molecular Devices SpectraMax M5 plate reader. Lipid peroxidation was expressed as µmol MDA mg $protein^{-1}$ (McRae et al., 2016). All replicates had a relative standard deviation of < 13%. The calibration curve showed a strong linear relationship (R^2 = 0.998).

Superoxide dismutase (SOD) assay

Superoxide dismutase (SOD) activity was measured using a SOD Determination Kit from Sigma-Aldrich. This assay used Dojindo's tetrazolium salt, WST-1 (2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) which produced a formazan dye upon reduction with a superoxide anion. The reduction with O_2 is inhibited by the SOD enzyme. The formazan dye absorbs at 440 nm (Sigma-Aldrich, Product information – SOD determination kit). The sample supernatant (20 µL) was added in triplicate to 96-well plates, 200 µL of a WST working solution and 20 µL of an enzyme working solution were added. The plate was incubated at 37°C for 20 mins and then absorbance read at 440 nm using a FLUOstar Optima plate reader (BMG Labtech). SOD activity is reported as U mg protein^{-1}. The standards fit a hyperbola, single rectangular, two parameter curve (R^2 = 0.989). Blanks were included with each sample and replicates had a relative standard deviation of < 30%.

Glutathione-S-transferase (GST) assay

Total activity of the GST enzymes was quantified using a Glutathione-S-Transferase (GST) Assay Kit. GST was quantified by measuring the reaction product of the GST catalysed conjugation of L-glutathione to 1-chloro-2,4-dinitobenzene (CDNB). The reaction product absorbs light at 340 nm which is directly proportional to the GST activity (Sigma-Aldrich, Technical bulletin – Glutathione-S-transferase (GST) assay kit). The substrate solution was prepared by combining Dulbecco's Phosphate Buffered Saline (9.8 mL), L-glutathione reduced (200 mM, 0.1 mL) and CDNB (100 mM, 0.1 mL). Sample supernatant (20 µL) was added in triplicate to 96-well plates. Substrate solution (180 µL) was added to each well and the absorbance read using a FLUOstar Optima plate reader (BMG Labtech), at 340 nm immediately following the addition. Readings were then taken every minute for 6 min. The change in absorbance per minute was used to calculate GST activity, expressed in µmol min^{-1} mg protein^{-1}. All replicates had a relative standard deviation of < 20%.

2.4.6. Triclosan measurement in water samples

Solvent extraction

Water samples (10 mL) were stored in 15 mL amber glass vials with 2 mL HPLC grade DCM (as described in Section 2.4.3). The DCM was removed using a fine tipped glass Pasteur pipette and transferred to a clean glass vial. A further 2 mL DCM was added, the solution mixed using a vortex mixer and allowed to settle. The DCM layer was removed as above and the process repeated for a third time. The DCM extracts were then evaporated to dryness in the presence

of nitrogen gas at 40°C. The dried sample was then quantitatively transferred to weighed amber glass HPLC vials using 500 µL followed by 2 x 250 µL of MeOH and the HPLC vials were reweighed to determine final volume. Extracted samples were stored at 4°C and analysed by HPLC within one week.

QA/QC

Spiked sea water samples at two concentrations (0.1 mg L^{-1} and 0.4 mg L^{-1}) were included with each batch of samples as well as a blank control. The spikes were prepared by adding 100 or 400 µL of 10 mg L^{-1} triclosan standard to 10 mL filtered sea water. Comparative standards were prepared by dispensing the respective volume of spike directly into a HPLC vial for analysis. Spike recoveries for 0.1 and 0.4 mg L^{-1} spikes were 98 ± 3% and 91 ± 6% respectively relative to the comparative standards. A randomly chosen duplicate sample was included in each set of water samples which had < 1% relative standard deviation. The limit of quantification (LOQ) was 0.29 mg L^{-1} and the limit of detection (LOD) was 0.1 mg L^{-1} in the 1 mL concentrated extract (0.029 and 0.01 mg L^{-1} respectively in the 10 mL water sample). All blanks and control samples were below the LOQ and concentrations were not recovery corrected.

HPLC analysis

Separation and quantification of triclosan was achieved as described in Section 2.4.2. Methyl-triclosan was also monitored for, however, it was below detection limits in all water samples. A six-point triclosan calibration curve (0, 0.3, 0.5, 1, 3 and 5 mg L^{-1}) was prepared using a 10 mg L^{-1} working stock solution. All standards were prepared in MeOH. The 0, 0.3 and 5 mg L^{-1} standards were analysed every 10 - 15 samples to confirm stability of the calibration. The calibration curve was linear (R^2 = 0.9999) over the concentration range. The 0.3 and 5 mg L^{-1} standards were reproducible with relative standard deviations of 3% and 1% respectively.

2.4.7. Measurement of triclosan accumulation in mussel tissue

Triclosan extraction

The method of extraction of triclosan from tissue was adapted from a method by Kookana et al. (2013). The two mussels collected from each replicate (n = 6) for each treatment (Section 2.4.3) were shucked and weighed and tissues were placed in 50 mL centrifuge tubes and freeze dried in a Labconco FreeZone® 2.5 Benchtop Freeze Dry System. Freeze dried tissue was weighed, homogenised and 0.5 g transferred to glass 50 mL centrifuge tubes. A surrogate standard, ^{13}C-triclosan (100 µL of 1 mg L^{-1} ^{13}C-triclosan standard), was added to each sample, spike and blank prior to extraction.

Preliminary investigations showed that using hexane as the extraction solvent (as described by Kookana et. al. (2013)) resulted in insufficient recovery of analytes. Therefore, a mixture of 95:5 DCM:MeOH was used which has a higher polarity, providing enhanced extraction of the polar triclosan from the tissues.

Mussel tissue and solvent blanks and spikes (0.1 mg L^{-1}) were included with each batch (Table 2.4). The DCM:MeOH solvent (95:5) was added (10 mL) to the centrifuge tubes and samples sonicated using an Elma S30 Elmasonic sonic bath (Total Lab Systems Ltd) for 10 mins with sweeping. The samples were then placed on an orbital mixer for 30 mins followed by centrifugation at 2400 g using a Hettich Rotina 420 centrifuge for 12 min. The supernatant was carefully transferred using fine-tipped glass Pasteur pipettes to 40 mL amber vials. Addition of solvent and sonication was repeated two further times. The 30 mL extracts were dried to approximately 5 mL at 40°C under nitrogen gas. Extracts were passed through Strata Fluorisil solid phase extraction (SPE) cartridges (1000 mg/6 mL) with a 2 cm bed of sulfuric acid activated silica gel on the top (Figure 2.5; Canosa et al., 2008). The silica gel was prepared by adding 2.2 mL sulfuric acid to 40 g silica and homogenising on an end-over-end mixer for 3 h. The columns were preconditioned with 2 x 5 mL 95:5 DCM:MeOH, samples were then added and vials were rinsed into the columns using 2 x 3 mL solvent to ensure all contents were rinsed from the vials. A further 5 mL DCM:MeOH was added directly to the columns to ensure all analytes were eluted. Eluents were collected in 40 mL amber vials which were evaporated to dryness at 40°C under a constant stream of nitrogen gas.

Figure 2.5: Solid phase extraction setup for removal of lipids and unwanted material from tissue extracts.

Derivatisation of tissue extracts

A six or seven point calibration curve of ^{13}C-triclosan, triclosan and methyl-triclosan was included with each analysis batch (0, 10, 25, 50, 100 and 250 µg L^{-1}, 500 µg L^{-1} was also included for triclosan and methyl-triclosan). Tissue extracts and standards were derivatised using MSTFA (N-methyl-N-(trimethylsilyl) trifluoroacetamide) for analysis. The derivatisation mixture was prepared by combining ammonium iodide (NH_4I, 11.4 mg), 2-mercaptoethanol (17 µL) and MSTFA (285 µL). The solution was vortexed and incubated at 65°C until NH_4I was fully dissolved. The vial was cooled to room temperature and an additional 2715 µL MSTFA added. The mixture was purged with nitrogen gas and stored at 4°C for a maximum of ten days.

Dried extracts were transferred to 2 mL amber vials using 500 µL followed by 2 x 250 µL ACN. Aliquots of extracts (100 µL) were then transferred to 5 mL reacti-vials. Internal standard (BPC (2,2-bis(4-hydroxy-3-methylphenyl) propane), 100 µL of 1 mg L^{-1}) was added to each sample and evaporated to dryness at 40°C under nitrogen gas. Derivatisation mixture (30 µL) was added to each react-vial, then vials were vortexed and incubated at 65°C for 45 min. The reacti-vials were removed from the heating block and cooled to room temperature before addition of 970 µL iso-octane. Solutions were vortexed and transferred to labelled GC vials and stored at 4°C before analysis and analysed within five days of derivatisation.

Gas Chromatography – Mass Spectroscopy (GC-MS) Analysis

The derivatised samples and standards were analysed by GC-MS using a Shimadzu GC-2010 Gas Chromatograph with a Shimadzu AOC-20i Auto Injector and a Shimadzu GCMSQP2010Plus detector. A Shimadzu SH-Rxi-5Sil MS Crossbond® column (5% diphenyl/95% dimethyl polysiloxane, 30 m length, 0.25 mm ID, 0.25 µm df) was used to separate the analytes. Shimadzu GCMS Solution software (Version 2.70) was used to control the instrument and process data.

A Shimadzu 10 µL syringe was used to inject 1 µL of derivatised sample or standards into the injection port in splitless mode at a temperature of 280°C (Emnet et al., 2015). The initial oven temperature was held at 100°C for 5 min, then increased at a rate of 10°C min^{-1} to 300°C where it was held for 5 min, for a total run time of 30 min per sample. Helium was used as the carrier gas at a flow rate of 5.5 mL min^{-1}. The ion source was held at 200°C and the GC-MS interface at 250°C. The analytes were quantified using Shimadzu GCMS Solution software (Table 2.4). The detection limits were determined using EPA Method 8280A (1984).

Table 2.3: Detection limits, retention times and SIM (selective ion monitoring) mode detection parameters for analytes.

Analyte	Detection limit ($\mu g\ kg^{-1}$)	Retention time (min)	Quantifier ion (*m/z*)	Qualifier ions (*m/z*)
^{13}C-triclosan	5	19.83	206	357, 359
Methyl-triclosan	5	19.82	252	302, 254, 232
Triclosan	5	19.83	360	362, 310
BPC	-	21.15	385	386, 400

QA/QC

The relationship between peak area ratio and concentration was linear for all analytes (R^2 > 0.993). Spikes, control samples and duplicates were included in each extraction batch (Table 2.4). The tissue used in mussel tissue blanks and spikes was prepared by freeze drying and milling six *Perna canaliculus* (purchased from New World Ilam, Canterbury) and combining to make a bulk mussel tissue. Prior analysis of this bulk tissue confirmed that no triclosan was present.

Table 2.4: Mussel tissue triclosan extraction example batch.

Sample or Spike	Volume 1 mg L^{-1} triclosan + methyl-triclosan (µL)	Volume 1 mg L^{-1} ^{13}C-triclosan (µL)
Solvent Blank	0	100
Solvent Spike	100	100
Mussel Blank	0	100
Mussel Spike	100	100
Comparative Standard*	100	100
C1	0	100
C2	0	100
MP	0	100
TCS	0	100
TCS duplicate	0	100
MP_{TCS}	0	100

** Comparative standard involved spiking of standards directly into an HPLC vial.*

Recovery of ^{13}C-triclosan was used to determine the extraction efficiency for each sample and this was used to calculate the concentration of triclosan. A duplicate extraction of the TCS sample for each batch was included and a duplicate injection of the TCS extraction was included in the GC-MS run. All duplicate extractions and injections were within 5% relative standard deviation.

Lipid content analysis

The total lipid content of the mussel tissue was analysed according to a method by Wick et al. (2016). Freeze dried tissue samples (0.5 g) were weighed into 10 mL centrifuge tubes. Propan-2-ol (1.6 mL), cyclohexane (2 mL) and ultra-pure water (2 mL) were added to the tubes. After sonicating for 5 mins, samples were centrifuged for 10 mins at 1400 g. The upper organic layer was transferred to pre-weighed glass vials. This procedure was repeated with 2 mL of cyclohexane/propanol (87/13 v/v) and the upper layer combined with the first extracts. Extracts were dried at 103°C until constant weight. The lipid content was calculated in mg g^{-1} (dw). A duplicate sample was included in the analysis (relative standard deviation < 1.3%) as well as blanks.

2.4.8. Measurement of microplastic accumulation in mussel tissue

Preliminary investigations showed that accumulation of microplastics was high and counting plastic beads was unfeasible. Consequently, microplastic accumulation was quantified by weight. Two mussels per replicate, per treatment, were weighed, measured and shucked and the tissues transferred to clean 250 mL glass conical flasks. Digestion was then completed using HNO_3 (as described in Section 2.3.2). Following heating, solutions were filtered through pre-weighed filter papers (Whatman GF/C, 47 mm), one filter per replicate. The conical flasks were rinsed twice with ultra-pure water and dried in an oven at 50°C overnight until completely dry. Filter papers were then weighed and stored in a desiccator.

The weight of residual tissue per gram wet tissue weight (ww) of control samples was calculated and used to control for left over debris on the filter paper in the microplastic treated mussels. Microplastic accumulation was expressed as mg microplastics g $tissue^{-1}$ (ww). The number of spheres was calculated according to data from Cospheric where for 38-45 µm spheres there are 2.67×10^7 spheres g^{-1} (Cospheric LLC., Spheres per gram calculation table).

2.5. Statistics

Statistics were analysed using RStudio. Grubbs tests were performed to identify and exclude outliers. Normality of data was tested and data were normalised by log transformation where appropriate. Analysis of variance (ANOVA) were performed and where $p \leq 0.05$, post-hoc Tukey tests were performed to identify significant differences between treatments. Welch two sample t-tests assuming unequal variances were performed. Confidence intervals of 95% were assumed for all tests. All values are expressed as mean ± standard error.

3. Field Study of Microplastic Accumulation

3.1. Introduction

While the presence of microplastics in the marine environment was first highlighted in the 1970's, the scientific community has become increasingly aware of and concerned about microplastic pollution in the last decade. Of particular concern is their potential to harm biota and the threat to organisms throughout the food-web (Cole et al., 2011).

Microplastics can be defined as plastic particles less than 1 mm diameter (Mathalon and Hill, 2014; Van Cauwenberghe et al., 2015). Microplastics have been reported in concentrations of up to 102 000 particles m^{-3} in coastal waters in Sweden (Noren and Naustvoll, 2010) and 621 particles 10 g^{-1} sediment on a beach in Germany (Liebezeit and Dubaish, 2012). While gyres and industrial coastal areas have been identified as 'hotspots' for microplastic accumulation, they are found in oceans around the globe. For example, one study reported microplastics on the shores of six continents indicating the ubiquitous nature of this pollutant (Browne et al., 2011). Microplastics have been identified in the New Zealand marine environment with recent study reporting eight out of 10 sediment sampling locations in Canterbury contain microplastics (Clunies-Ross et al., 2016).

A wide range of marine species have been reported to ingest microplastics in the environment due to the abundance, small size and persistence of microplastic particles (Cole et al., 2011; refer to Table 1.2). The bioavailability of microplastics to organisms is influenced by a range of key factors including size, density and colour. The size is determined by wave friction and UV radiation. These natural processes cause larger plastic particles to break down to a size where they can be mistaken for food and ingested by lower trophic organisms (Cole et al., 2011; Van Cauwenberghe et al., 2015; Wright et al., 2013). The density determines the location of microplastics in the water column. Lower density particles inhabit the upper water column making them available to filter and suspension feeding organisms and higher density particles accumulate on the sea floor, available to deposit feeding organisms. The colour of the microplastic particles can induce prey item resemblance for visual predators, increasing ingestion (Wright et al., 2013).

Setälä et al. (2016) reported that bivalves ingested significantly more 10 µm microplastic beads than free swimming crustaceans and benthic deposit feeding animals indicating that bivalves may be more exposed to microplastics than other species. The New Zealand green-lipped mussel (*Perna canaliculus*) is a sessile, filter feeding organism. It is an important bioindicator species

and commercially and culturally valuable (Chandurvelan et al., 2013b). Mussels have been demonstrated to accumulate microplastics in the marine environment in many locations around the world (De Witte et al., 2014; Mathalon and Hill, 2014; Santana et al., 2015; Van Cauwenberghe and Janssen, 2014; Van Cauwenberghe et al., 2015). However, the accumulation of microplastics by New Zealand green-lipped mussels has not been investigated to date. Mussels exhibit a high degree of selectivity towards particle selection which is variable between species (Ward and Shumway, 2004). Due to its larger size (green-lipped mussels can grow up to 200 mm in length, Murphy et al., 2002) it is hypothesised that the green-lipped mussel may have the capacity to ingest larger microplastic particles than other previously studied species including *Mytilus edulis, Mytilus galloprovincialis,* and *Perna perna*.

This study is the first to investigate microplastic accumulation in aquatic organisms in New Zealand. It was designed to be a preliminary investigation to determine if further research into microplastic pollution in New Zealand is warranted. Mussels were collected from eight locations around New Zealand incorporating both the North and South Islands with a more in depth study undertaken in the Canterbury region where mussel and surface water samples were collected from a further eight locations on the Canterbury coastline. Mussel tissue was acid digested and observed under fluorescence coupled microscope to isolate microplastic particles. Surface water samples were filtered and also observed under microscope (see Section 2.3 for further details).

3.1.1. Sampling locations

National Survey

To investigate the microplastic contamination in green-lipped mussels, mussels were collected from a wide range of locations around the New Zealand coastline. Local information regarding mussel bed locations was sourced from local authorities and universities. A range of locations were selected including urban and rural sites and harbour/estuary and coastal locations on the North and South Islands of New Zealand (Section 2.3.1, Table 2.1). This diverse range of locations was used to provide an indication of the overall levels of microplastic contamination in New Zealand green-lipped mussels and to determine if further investigation is warranted.

Canterbury Survey

A more in-depth sub-study was performed in the Canterbury region to determine the regional variation in microplastic pollution and the relationship between water and mussel microplastic concentrations. Sampling sites in the Canterbury region were chosen pairwise to represent areas

expected to exhibit relatively high and low levels of microplastic contamination (Section 2.3.1. Table 2.2).

The Avon-Heathcote Estuary and Taylors Mistake beach were chosen as a pair of areas of expected high and low microplastic contamination respectively. The Avon-Heathcote Estuary was expected to show higher levels of plastic particles as the two contributing rivers, the Avon River and the Heathcote River, both travel through residential areas, with the Avon River also travelling through commercial areas and the Heathcote through industrial areas. Until March 2010, the estuary was also the site of discharge of treated domestic wastewater and industrial effluent (Chandurvelan et al., 2016). Taylors Mistake beach is in close proximity to the Avon-Heathcote Estuary but is relatively sheltered and more remote.

Mussels collected from a jetty within the Lyttelton Port were expected to exhibit higher levels of plastic contamination when compared to samples collected from a shellfish harvesting location within Lyttelton Harbour. This is due to the daily operational activities performed at the Port which have the potential to release microplastic particles into the water and the proximity to wastewater and stormwater discharges in Lyttelton.

Another pair of samples included the Akaroa Main Wharf and Damon's Bay. The environment surrounding the Akaroa Main Wharf was anticipated to have a higher level of microplastic contamination than the isolated waters of Damon's Bay. This is due to the recreational and commercial activities associated with the wharf and surrounding area. In comparison, little human activity was noted at Damon's Bay which is only accessible by boat and has no residential or commercial activities.

The final pair of sites included Pigeon Bay and Little Akaloa on the northern side of the Banks Peninsula. Chandurvelan et al. (2016) measured unexpectedly high trace metal concentrations in green-lipped mussels collected at Pigeon Bay. Therefore, it was decided to include this location as a site of potential high contamination with Little Akaloa, a neighbouring Bay included as the site of anticipated lower microplastic contamination.

3.1.2. Objectives

The objectives of this study were to determine:

- Whether microplastics accumulate in New Zealand green-lipped mussels;
- The size of microplastics green-lipped mussels accumulate; and
- Whether there is a relationship between microplastics isolated in surface water and those found to have accumulated in green-lipped mussels in the Canterbury region.

3.2. Results

3.2.1. Blank extractions

Blanks were included with each batch of mussel extraction and water filtration (one blank for every three samples). Any microplastic particles located on the blank filters were imaged and recorded. Microplastic particles resembling anything located on the blanks based on colour, type and size were excluded from the sample results. Fibres that fluoresced blue under UV light were present on all blanks so all such fibres were excluded from the results (refer to Section 2.3.4).

3.2.2. Microplastics in National mussel samples

There was no significant difference in the concentration of microplastics isolated from mussels in each location of the National mussel survey (statistics summarised in Appendix 4). Microplastic concentrations from the Avon-Heathcote Estuary are included in the National Survey. Microplastic particles were isolated in mussel samples collected from around New Zealand at frequencies of 0 to 2 particles per mussel. Concentrations of microplastics in mussel tissue were 0.04 ± 0.01 particles g^{-1} (ww). Lawyers Head, Dunedin was the only location where microplastic particles were isolated in all mussel samples (Table 3.1). A potential microbead was isolated in a mussel sample from New Plymouth (Figures 3.1 and 3.3A).

Fifty six percent (56%) of particles isolated in the National survey mussel samples were greater than 200 µm in length (Figure 3.1). The largest fiber isolated was approximately 2000 µm in length (Figure 3.3B) and the largest fragment was approximately 1000 µm (Appendix 1). Clear was the dominant colour of particle observed (44%, Figure 3.2). A large tangle of fibers was observed in mussels collected from Napier (Figure 3.3B). This was a combination of two fibers of different colours (blue and black) and different fluorescent signatures.

Table 3.1: Microplastics identified in green-lipped mussel samples collected from locations around New Zealand. An average of the Canterbury mussel sample results is included for comparison (includes Avon-Heathcote data). Two mussels per replicate.

Location	Replicate	Fibres	Fragments	Beads	Total particles per replicate	Average particles per mussel
Bay of Islands	A	0	0	0	0	
	B	0	1	0	1	
	C	0	0	0	0	0.17
Mount Maunganui	A	0	0	0	0	
	B	0	1	0	1	
	C	0	0	0	0	0.17
Napier	A	1	0	0	1	
	B	0	0	0	0	
	C	0	0	0	0	0.17
New Plymouth	A	0	1	1	2	
	B	0	0	0	0	
	C	0	0	0	0	0.33
Wellington Harbour	A	0	0	0	0	
	B	0	2	0	2	
	C	0	0	0	0	0.33
Port Underwood	A	0	1	0	1	
	B	0	0	0	0	
	C	0	0	0	0	0.17
Westport	A	0	0	0	0	
	B	1	0	0	1	
	C	0	0	0	0	0.17
Dunedin	A	0	2	0	2	
	B	0	1	0	1	
	C	0	2	0	2	0.83
Avon-Heathcote	A	0	0	0	0	
	B	0	0	0	0	
	C	0	2	0	2	0.32
Canterbury (Average)		0.04 ± 0.04	0.3 ± 0.1	0.04 ± 0.04	0.7 ± 0.4	0.18 ± 0.08

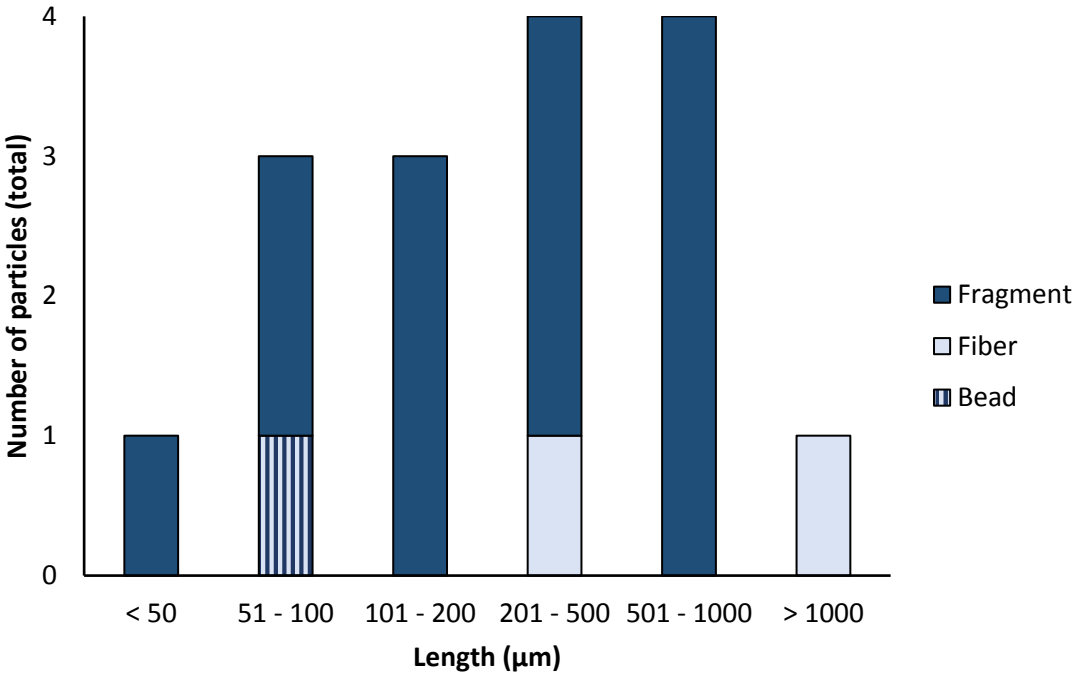

Figure 3.1: Microplastics extracted from National survey mussel samples. Categorised by length and particle type.

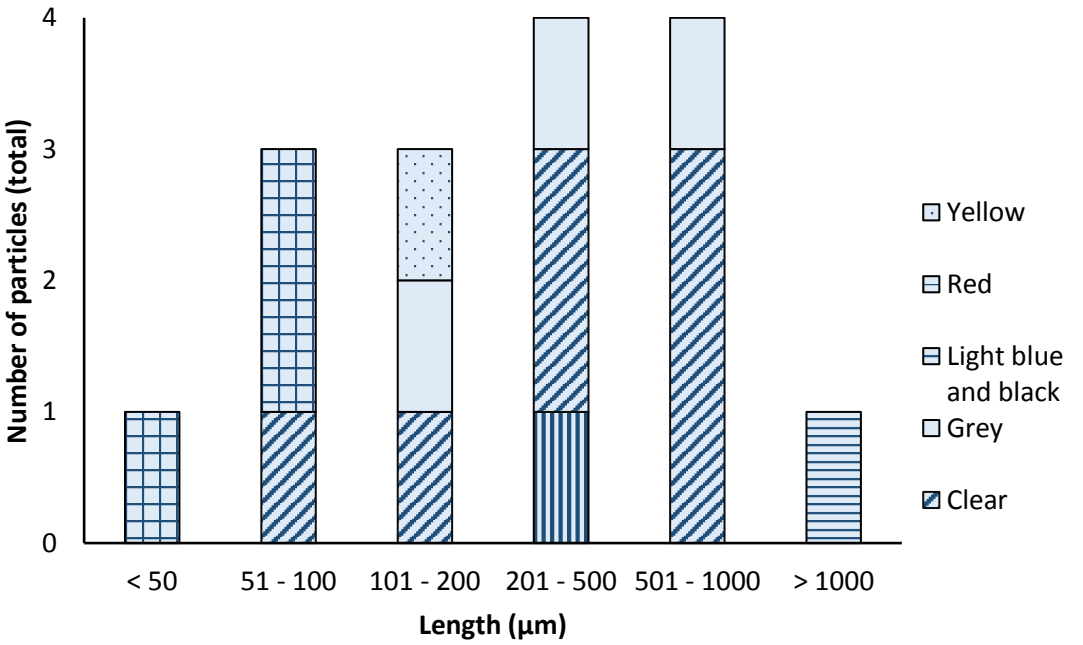

Figure 3.2: Microplastics extracted from mussels collected in the National survey. Categorised by length and colour.

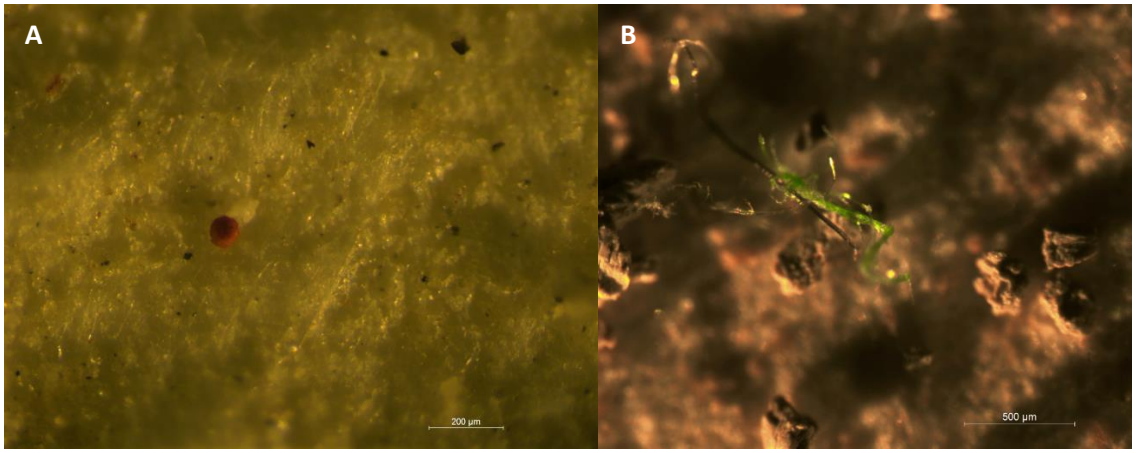

Figure 3.3: Microplastics isolated from mussels from around New Zealand. (A) Possible microbead from New Plymouth viewed under normal light. Scale bar is 200 µm; (B) tangle of fibres from Napier viewed under normal light. Scale bar is 500 µm. Images of all potential plastics isolated are in Appendix 1.

For statistical analysis of National mussel samples, the data for the mussels collected from the Avon-Heathcote Estuary were included in the data set. There was no difference in the number of microplastics per mussel between the North Island and South Island of New Zealand ($t = 0.870$, $df = 3.40$, $p \geq 0.1$). There was also no significant difference in the number of microplastics isolated from mussels from each location. The number of microplastic particles per mussel was compared for urban (Mount Maunganui, Napier, Wellington Harbour, Dunedin and Avon-Heathcote Estuary) and rural (Bay of Islands, Port Underwood, New Plymouth and Westport) sampling sites. Similarly, there was no statistical difference between these categories ($t = 1.223$, $df = 4.89$, $p \geq 0.1$). Harbour/estuary (Port Underwood, Napier, Wellington, Avon-Heathcote Estuary) and coastal (New Plymouth, Westport, Bay of Islands, Dunedin and Mount Maunganui) sampling sites were also compared; however, no statistical difference was measured ($t = -0.605$, $df = 5.06$, $p \geq 0.1$).

3.2.3. Microplastics in Canterbury

A detailed sub-study was performed in the Canterbury region to assess the relationship between microplastic contamination in green-lipped mussels and surface water (statistics summarised in Appendix 4).

3.2.3.1. Microplastics in Canterbury surface water

Potential microplastic particles were isolated from water collected in the Canterbury region (Table 3.2). Microplastics were isolated in 33% of water samples analysed and 60% of those were fragments (Figure 3.4).

Table 3.2: Microplastics identified in water samples collected from the Canterbury region.

Location	Replicate	Fibres	Fragments	Beads	Total particles per replicate	Average concentration (particles L^{-1})
Avon-Heathcote	A	0	0	0	0	
	B	0	0	0	0	
	C	1	0	0	1	0.17
Taylors Mistake	A	0	0	0	0	
	B	0	0	0	0	
	C	0	0	0	0	0
Pigeon Bay	A	0	0	1	1	
	B	0	0	0	0	
	C	0	0	0	0	0.17
Little Akaloa	A	0	0	0	0	
	B	0	0	0	0	
	C	0	0	0	0	0
Lyttelton Port	A	0	1	0	1	
	B	0	0	0	0	
	C	0	0	0	0	0.17
Lyttelton Harbour	A	0	0	0	0	
	B	0	1	0	1	
	C	0	0	0	0	0.17
Akaroa Harbour	A	0	0	0	0	
	B	0	0	0	0	
	C	0	0	0	0	0
Damon's Bay	A	0	1	0	1	
	B	0	0	0	0	
	C	0	0	0	0	0.17

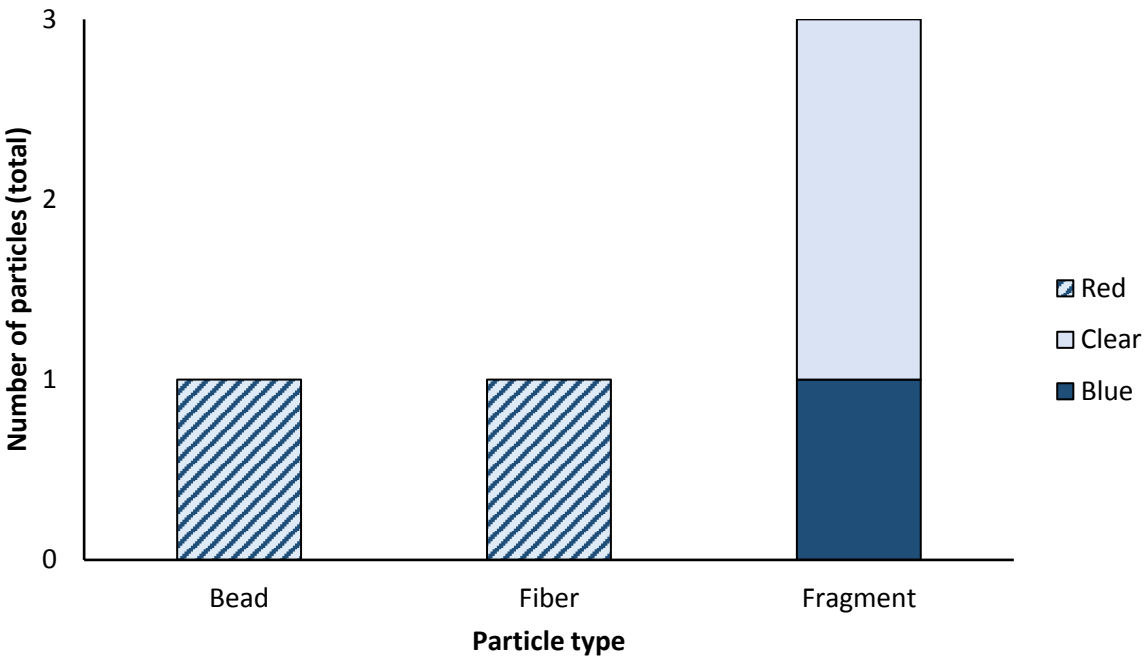

Figure 3.4: Microplastic particles isolated in water samples from the Canterbury region categorised by colour and type.

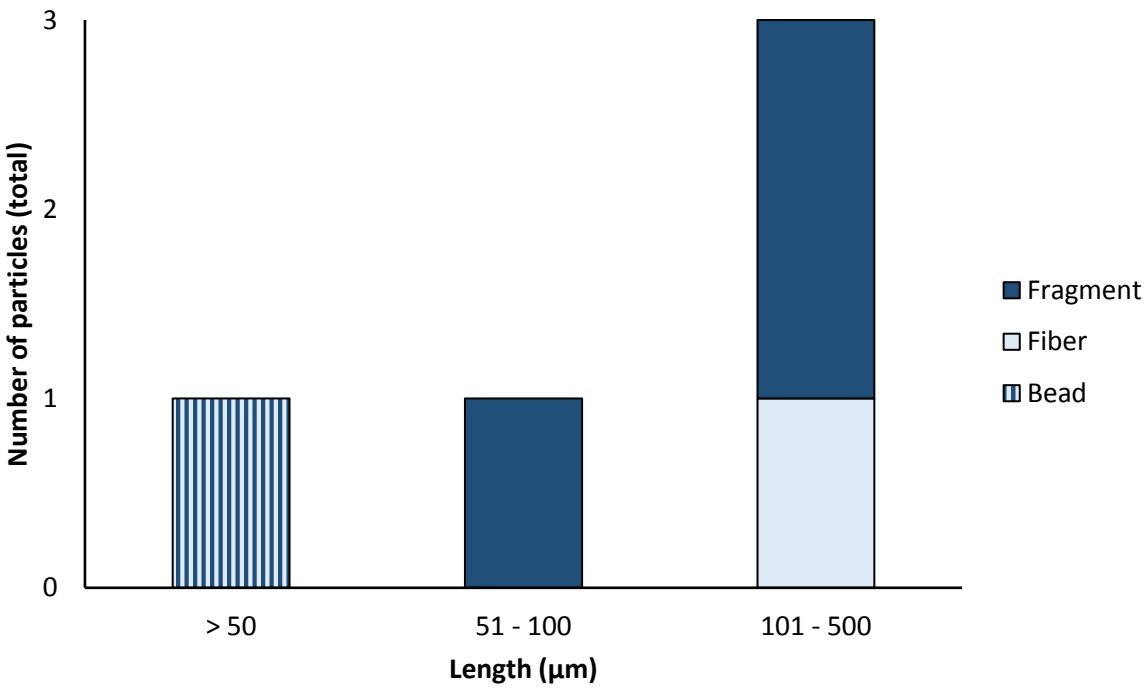

Figure 3.5: Microplastics particles isolated in water samples from the Canterbury region categorised by length and type.

An opaque, blue coloured fragment was isolated from a water sample collected from Lyttelton Harbour. This was not fluorescent but was of a similar size (approx. 100 µm) to the particles isolated in the mussel tissue samples so could be available for ingestion by mussels (Figures 3.5 and 3.6A). A suspected microplastic bead was located in a water sample from Pigeon Bay. The bead was dark red in colour and had a strong fluorescent signature, fluorescing bright red under GFP-Plus fluorescent light (Figure 3.4 and 3.6B).

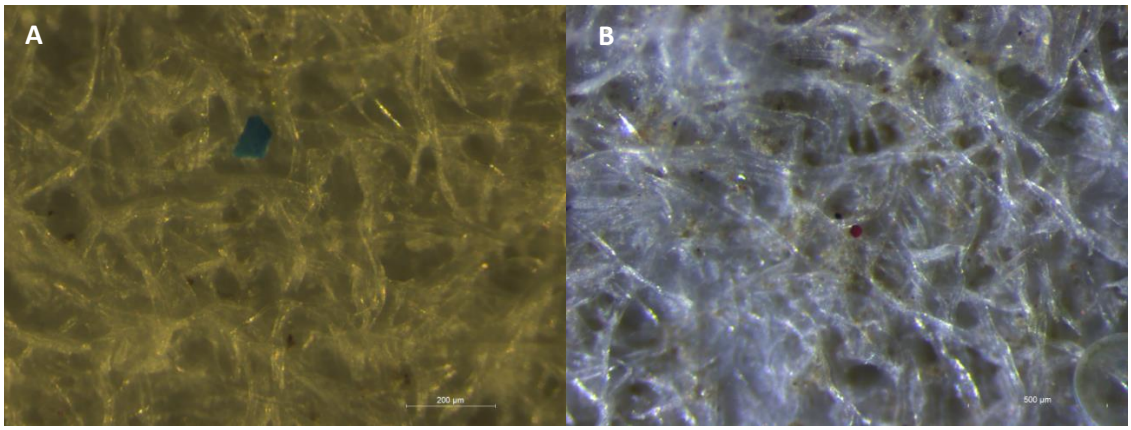

Figure 3.6: Microplastic fragments isolated from water samples. (A) Particle from Lyttelton Harbour viewed under light. Scale bar is 200 µm; (B) Suspected microbead from Pigeon Bay viewed under light. Scale bar is 500 µm. Images of all potential plastics isolated in Appendix 2.

3.2.3.2. Microplastics in Canterbury mussels

Potential microplastic particles were isolated in green-lipped mussels collected from the Canterbury region (Table 3.3). Microplastics were isolated in 29% of mussel samples analysed. The mussels accumulated 0.03 ± 0.01 particles g^{-1} (ww). The largest particle identified in Canterbury mussel samples was 250 µm in length (Figure 3.9A).

Table 3.3: Microplastics identified in green-lipped mussel samples collected from the Canterbury region. Two mussels per replicate.

Location	Replicate	Fibres	Fragments	Beads	Total particles per replicate	Average particles per mussel
Avon-Heathcote	A	0	0	0	0	
	B	0	0	0	0	
	C	0	2	0	2	0.33
Taylors Mistake	A	0	0	0	0	
	B	0	0	0	0	
	C	0	0	0	0	0
Pigeon Bay	A	0	0	0	0	
	B	0	0	0	0	
	C	0	0	0	0	0
Little Akaloa	A	0	1	0	1	
	B	0	0	0	0	
	C	0	0	0	0	0.17
Lyttelton Port	A	0	0	0	0	
	B	0	0	0	0	
	C	0	0	0	0	0
Lyttelton Harbour	A	0	0	1	1	
	B	0	0	0	0	
	C	0	0	0	0	0.17
Akaroa Harbour	A	0	2	0	2	
	B	0	1	0	1	
	C	1	0	0	1	0.67
Damon's Bay	A	0	1	0	1	
	B	0	0	0	0	
	C	0	0	0	0	0.17

Five of the nine (55%) microplastic particles isolated from the mussels collected in the Canterbury region were 51 – 100 µm in length. Seventy seven percent (7%) of the particles were fragments and 77% were between 51 and 200 µm in size (Figure 3.7). The most common colours of particles were clear and red (each 33%, Figure 3.8 and example in Figure 3.9A). A suspected microplastic orange bead was isolated in a mussel from Lyttelton Harbour which was 100 µm in diameter (Figures 3.7 and 3.9B).

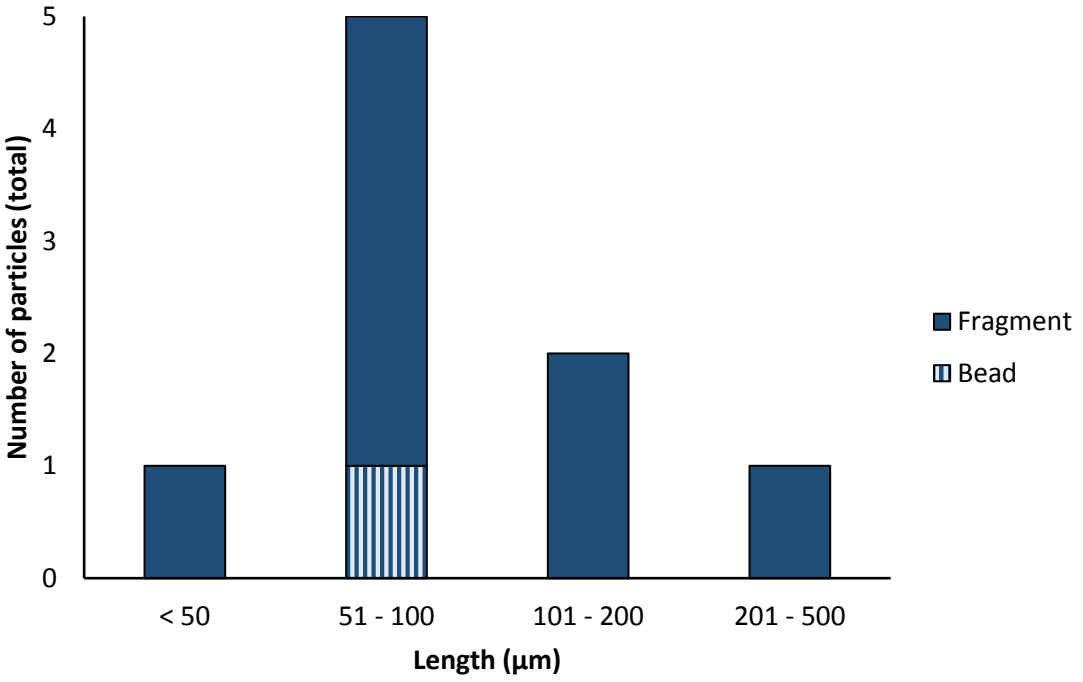

Figure 3.7: Microplastics extracted from mussels collected in Canterbury. Categorised by length and particle type.

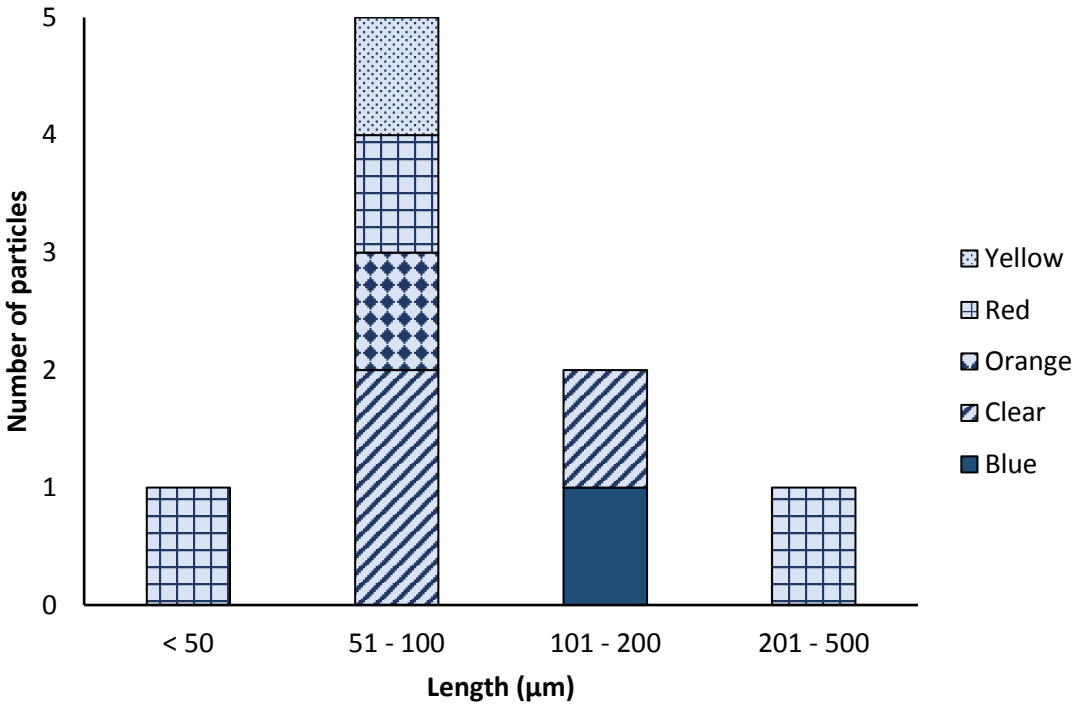

Figure 3.8: Microplastics extracted from mussels collected in Canterbury. Categorised by length and colour.

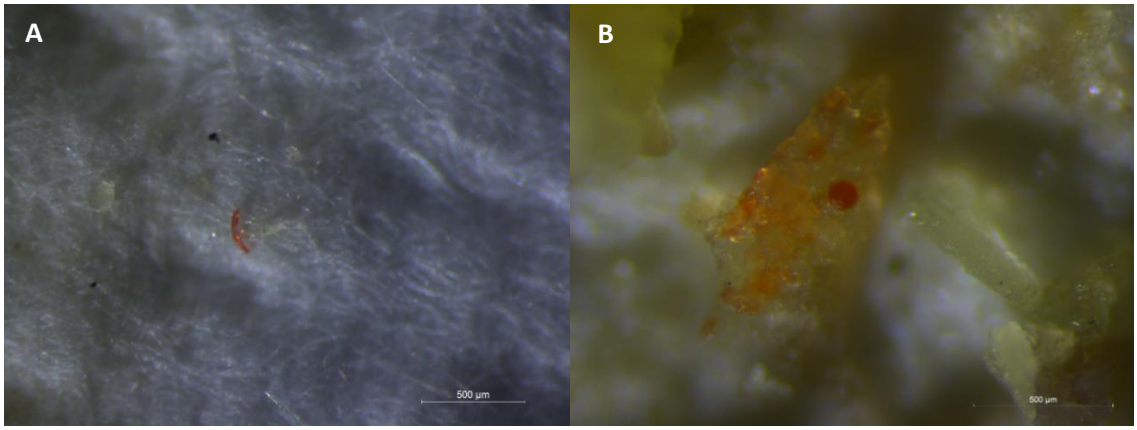

Figure 3.9: Microplastic fragments isolated from mussels collected in Canterbury. (A) Fragment from an Akaroa Harbour mussel viewed under light. Scale bar is 500 µm; (B) suspected microbead form a Lyttelton Harbour mussel viewed under normal light. Scale bar is 500 µm. Images of all potential plastics isolated in Appendix 3.

3.3. Discussion

The objective of this study was to provide a preliminary indication of the extent of microplastic contamination in New Zealand using a small sample size (n = 6) from a limited number of sampling sites. This assessment indicates that microplastic particles are of concern for New Zealand's marine wildlife. Other studies from various locations around the world have reported that invertebrates accumulate microplastic particles (De Witte et al., 2014; Mathalon and Hill, 2014; Santana et al., 2015; Van Cauwenberghe and Janssen, 2015; Van Cauwenberghe et al., 2015). However, this is the first such assessment in New Zealand and the first to assess microplastic accumulation in New Zealand green-lipped mussels, *Perna canaliculus*.

Microplastic fragments, fibres and beads had accumulated in the tissues of green-lipped mussels in New Zealand. Thirty five percent (35%) of samples analysed (two mussels per sample) contained suspected microplastics. Eighteen (18) of the 23 (78%) microplastic particles isolated from mussels were classified as fragments with three fibres (13%) and two (9%) suspected microbeads.

3.3.1. Identification of microplastics

Water and mussel samples were visually assessed to confirm the presence of microplastics. Due to limitations of time and equipment it was not possible to spectroscopically confirm that particles identified were plastic. Isolated particles were treated with caution in confirming that they were indeed plastic and strict criteria were adhered to (Section 2.2). Accordingly, if there was doubt over whether a particle was plastic, it was not included in the results.

3.3.2. Microplastics in New Zealand green-lipped mussels

Microplastics were isolated in at least one mussel from all of the sites analysed in the National survey (Table 3.1). Forty one percent (41%) of the mussels analysed from around New Zealand contained microplastics at an average of 0.29 ± 0.08 particles mussel^{-1} or 0.3 ± 0.1 particles g^{-1} tissue (ww). These results are similar to concentrations of microplastics in mussels reported in some recent studies (Van Cauwenberghe and Janssen, 2014; Van Cauwenberghe et al., 2015) but significantly lower than others (Li et al., 2016; Mathalon and Hill, 2014; Santana et al., 2016). However, the studies by Li et al. (2016) and Mathalon and Hill (2014) included fibers in their assessment. In this study, fibers were treated with extreme caution due to the potential for airborne fiber contamination. For example, Mathalon and Hill (2014) reported an average contamination of 100 fibers per filter.

Van Cauwenberghe and Janssen (2014) and Van Cauwenberghe et al. (2015) reported very similar concentrations of microplastics in *Mytilus edulis* of 0.36 ± 0.07 particles g^{-1} (ww) and 0.2 ± 0.3 particles g^{-1} (ww) respectively. The particles reported in the study by Van Cauwenberghe and Janssen (2014) were significantly smaller than those isolated in the current study. The estimated limit of detection for the current study was 40 µm. This is larger than the largest of the size categories in the Van Cauwenberghe and Janssen (2014) study of > 25 µm. Therefore, it is possible that more microplastic particles may have been measured in the current study if the detection limit was lower. Van Cauwenberghe et al. (2015) did not report the type, size or colour of the microplastics isolated but did state that fibers were excluded.

A majority of the particles isolated in the National mussel samples were clear fragments, > 200 µm in length. This was significantly larger than expected and confirms the hypothesis that as New Zealand green-lipped mussels are larger than the other mussel species investigated to date, they may be able to ingest larger sized microplastics. James et al. (2001) reported that *P. canaliculus* are non-selective for phytoplankton 5 – 100 µm in size and large dinoflagellates and diatoms (around 200 µm) were cleared by green-lipped mussels greater than 60 mm in length. It is unknown whether the potential microplastics found in mussel tissue in the current study were ingested recently and were going to be released or had been retained in the tissue. The particle selection and retention is also likely to vary when food levels are low (James et al., 2001).

Some personal care products such as facial cleansers and hand soaps contain microplastic particles that act as exfoliators (Fendall and Sewell, 2009). In the National survey, one potential microbead was observed in a mussel sample from New Plymouth. This was classified as a bead based on its spherical shape and texture. Particles that were less uniform in shape and

containing sharper edges were classified as fragments. However, a study by Fendall and Sewell (2009) investigated microbeads in facial cleansers from New Zealand reported that not all microplastics in these products are spherical but are in fact a range of irregular shapes and sizes. Three potential microbeads were isolated in total in this study (including all mussel and water samples), one each from New Plymouth, Pigeon Bay and Lyttelton Harbour (Figures 3.3A, 3.6B and 3.9B respectively). Clunies-Ross et al. (2016) also reported two suspected microbeads in sediment from the Canterbury coastline. Results from the current study may underestimate the exposure of mussels to plastic microbeads in personal care products as some of the fragments seen may also have derived from these products.

3.3.3. Microplastics in Canterbury

Microplastics were identified in water and mussel samples in the Canterbury region. Taylors Mistake was the only location investigated where no microplastics were identified in mussel nor water samples. Microplastics were isolated in both mussel and water samples collected from Lyttelton Harbour, Avon-Heathcote Estuary and Damon's Bay (Figure 3.10).

Water samples were included to compare microplastic particles in surface water with those isolated in the mussel tissue as mussels are filter feeders. There was no correlation between the microplastic particles in each sample type or in the presence or absence of microplastics. This is possibly due to the small volume of water sampled and larger water sample volumes may be needed to fully understand the relationship. Also, water sampling provides an indication of microplastic pollution at a specific point of time while mussels may accumulate microplastics over a long period of time depending on retention. This must be taken into account when considering mussels as passive samplers for indications of microplastic pollution in surface water.

The sample sites were chosen on a pairwise basis to identify expected high and low contaminated areas (Section 2.3.1). The areas with expected high contamination levels were selected due to their proximity to industrial or commercial activities or wastewater discharges. There was no correlation between the anticipated pollution levels and the microplastic particles isolated in the water samples or mussel tissue. This indicates that more research is required into the transport and behaviour of microplastics in the New Zealand coastline.

In the current study, fragments made up 60% and 78% of microplastics isolated in water and mussels respectively. This is consistent with previous research into microplastic contamination in Canterbury sediment. Clunies-Ross et al. (2016) investigated microplastic pollution in Canterbury sediments and reported microplastics at eight out of ten sampling sites. The

microplastics reported in the Clunies-Ross et al. (2016) study were polystyrene, polyethylene and polypropylene and 86% were fragments.

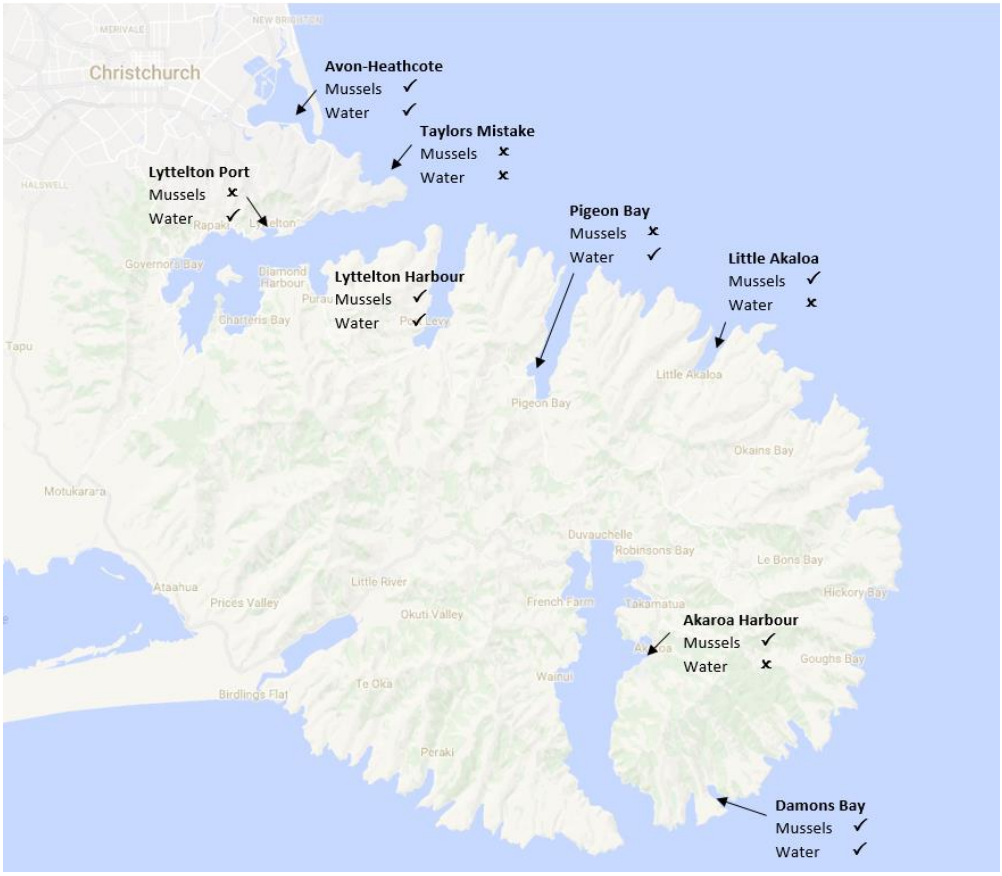

Figure 3.10: Map illustrating where microplastics were identified in green-lipped mussel and water samples in Canterbury. Map adapted from Google My Maps.

Canterbury mussels had comparable levels of contamination and frequency of detection to those collected from elsewhere in New Zealand. Microplastic particles were isolated in 29% of the mussels analysed from Canterbury with an average of 0.19 ± 0.08 particles mussel^{-1} or 0.03 ± 0.01 particles g tissue^{-1} (ww). As microplastics cause adverse effects on mussels (Browne et al., 2008; De Witte et al., 2014; Van Cauwenberghe and Janssen, 2014; Section 4.1.4 of the current study), increasing concentrations of microplastics are likely to result in observable impacts on mussel physiology.

3.3.4. Environmental implications

The results from this study indicate that microplastics are of significant concern for New Zealand marine life. The New Zealand green-lipped mussels ingest microplastics which have the potential

to cause adverse physiological effects (Section 4.2). Mussels are an important food source for many marine animals including snails and crabs (Alfaro, 2006; Farrell and Nelson, 2013). This means there is a potential for bioconcentration of microplastics to higher organisms through trophic level transfer (Farrell and Nelson, 2013).

This study has indicated the need for further research into microplastic contamination in New Zealand's marine environment. As microplastics also have the ability to impact a wide range of organisms, further research is required to investigate whether other New Zealand freshwater and marine species accumulate microplastics. Shellfish exports, including green-lipped mussels, are extremely important to the New Zealand economy so potential impacts should be thoroughly investigated.

3.3.5. Recommendations

This study was a preliminary investigation into microplastic contamination in New Zealand. In future investigations, researchers should perform extractions and digestions in a glove box to minimise contamination by airborne fibers. Also, density separation could be included in the extraction process to remove any undigested tissue from the filter papers to further minimise any masking of microplastic particles. Microplastic detection via microscope could be improved by using gridded filter paper, a higher magnification microscope and by utilising spectroscopic analysis of plastic type wherever possible.

A more in depth assessment of microplastic accumulation in New Zealand marine species is recommended. This should involve collection of a larger number of samples (> 12) per sampling site and multiple locations in the same sampling area (For example, mussel bed). Other marine and freshwater species should also be investigated from microplastic accumulation. Water sampling should also be included for each sample site. In order to give an accurate representation of water microplastic concentrations, a vacuum pump with filter attachment is recommended for extraction on site so that larger volumes of water can be analysed (e.g. 20 L).

3.4. Conclusions

This study has illustrated that the New Zealand green-lipped mussel from a wide range of locations around the New Zealand coastline have accumulated microplastic particles. A majority of the particles isolated were fragments but fibres and beads were also observed. There was no relationship between surface water and mussel microplastics, but further investigation should be performed with larger water volumes. Concentrations of microplastics in green-lipped

mussels were comparable with studies of other mussels internationally. However, the sizes of microplastics measured were larger than those observed from other mussel species indicating that due to the larger size of green-lipped mussels, they are able to ingest larger particles making them potentially at risk of harm from a wider range of particles.

4. Impact of Microplastics and Triclosan on Green-Lipped Mussels

4.1. Introduction

Plastics are a significant concern for marine pollution with the concentration of plastic in the marine environment still increasing (Besseling et al., 2013). Microplastic particles are of particular concern as they can accumulate in marine organisms, potentially causing adverse effects on physiology (Wright et al., 2013). Further research is required into how interactions with other contaminants in the environment alter the impact of microplastics on marine organisms.

Microplastics are thought to act as scavengers for organic pollutants in the aquatic environment. The main types of microplastics observed in the marine environment are polypropylene, polystyrene and polyethylene, all of which have been demonstrated to accumulate hydrophobic organic chemicals in seawater (Lee et al., 2014). For example, Frias et al. (2010) reported a wide range of organic pollutants in microplastics collected from beaches in Portugal including polycyclic aromatic hydrocarbons (e.g. pyrene, phenanthrene and fluoranthene), polychlorinated biphenyls (15 congeners) and DDTs (dichlorodiphenyltrichloroethane and derivatives). Aquatic species ingest these contaminated plastics, potentially providing an additional mechanism for the transport of organic pollutants to organisms (Bakir et al., 2014b).

Microplastics and triclosan have been individually shown to induce adverse physiological and biochemical responses in bivalves. Microplastics induced physiological changes including altering clearance rate, oxygen respiration and byssus production (Oliviera et al., 2013; Paul-Pont et al., 2016; Rist et al., 2016; Sussarellu et al., 2016; Wegner et al., 2012). Triclosan caused oxidative stress in bivalves including oxidative damage and increases in anti-oxidative enzyme activity (Binelli et al., 2009 and 2011; Canesi et al., 2007; Cortez et al., 2012; Goodchild et al., 2016; Kookana et al., 2013; Matozzo et al., 2012). These effects are described further in Sections 4.3.3 and 4.3.4 respectively. However, the impact of triclosan and microplastics combined has not been investigated to date.

A range of physiological and biochemical analyses were selected as part of this study to assess the impacts of microplastics and triclosan both singly and in combination on green-lipped mussels. The physiological tests included clearance rate, oxygen respiration and byssus production. The clearance rate is the volume of water that a mussel can filter to remove particles over a period of time (Bayne et al., 1979; Chandurvelan et al., 2012). This is the main mechanism

of acquiring energy for a mussel so is an important measure of physiological status (Gardner, 2002). The oxygen respiration rate is another important aspect of energy acquisition and is defined as the rate of oxygen consumption by an individual mussel over a period of time (Widdows and Staff, 2006). A mussel's byssus are the extracellular, collagenous threads excreted by the foot to secure the mussel to a surface (Bell and Gosline, 1996). Byssus production in mussels is affected in response to stresses including microplastics (Rist et al., 2016), hypoxia (Clarke and McMahon, 1996) and toxic algal blooms (Marsden et al., 2016). These physiological biomarkers were selected to give an overall status of the physiological health of the mussel.

Triclosan has been reported to induce oxidative stress in mussels (Binelli et al., 2019; Binelli et al., 2013). Accordingly, the biochemical biomarkers selected for this study included two measures of antioxidant enzyme activity and one measure of oxidative damage. These biomarkers were chosen to determine the mussel's response to microplastics and whether this response was sufficient to prevent oxidative damage. The two antioxidant enzymes selected included the superoxide dismutase (SOD) enzyme and the glutathione-S-transferase (GST) enzyme. The role of GST is to conjugate a wide range of compounds containing electrophilic atoms to glutathione, an important step in detoxification (Singhal et al., 2015). This produces free radical intermediates which can be later transformed into oxygen and hydrogen peroxide by the SOD enzyme to prevent oxidative damage (Binelli et al., 2011). Lipid peroxidation is a measure of oxidative damage and is considered a good biomarker of general stress levels in intertidal mussels (Chandurvelan et al., 2016).

In the current study, green-lipped mussels (*Perna canaliculus*) were exposed to microplastics (MP), triclosan (TCS) and microplastics with triclosan sorbed to the surface (MP_{TCS}). This investigation was an acute study using high concentrations of microplastics and triclosan to assess the potential impacts of these contaminants on green-lipped mussels. The objectives of this study were to determine:

- The physiological and biochemical impacts of microplastics, triclosan and triclosan sorbed to microplastics on green-lipped mussels following an acute 48 h study; and
- Whether sorption of triclosan to microplastics enhanced the accumulation of triclosan in the mussel tissue.

The mussels were exposed to five treatments in seawater for 48 h (refer to Section 2.4.3 for further details):

- Control 1 – mussels were fed an instant algae feed;

- Control 2 – mussels were exposed to acetone (< 0.01% v/v) and fed instant algae;
- MP – mussels were exposed to polyethylene microplastics (0.5 g L^{-1}) and fed instant algae;
- TCS – mussels were exposed to triclosan (0.36 mg L^{-1}) spiked using acetone as the carrier solvent (< 0.01% v/v) and fed instant algae; and
- MP_{TCS} – mussels were exposed to microplastics (0.5 g L^{-1}) with triclosan pre-sorbed to the surface of the plastics (0.73 mg g^{-1} triclosan on microplastics. Equivalent to 0.36 mg L^{-1} triclosan in the 3 L exposure).

4.2. Results

No mortality was observed throughout the exposures for any of the treatments.

4.2.1. Control treatments

Two control treatments were included in each batch of exposures, Control 1 and Control 2 (acetone control; described in Section 2.4.3). For all analysis, there were no significant difference in the physiological and biochemical biomarkers between the two controls ($p \geq 0.1$, Appendix 5) so Control 2 was used for comparison with the treatments containing microplastics and triclosan.

4.2.2. Triclosan concentration in water

In the water from the TCS treatment, the triclosan had decreased to below detection limits in the water following the 48 h exposure. This was less pronounced in the water containing microplastics and triclosan with 50% of water samples taken after 48 h containing triclosan above detection limits (Table 4.1, all data shown in Appendix 6). No methyl-triclosan was detected in any water samples.

Table 4.1: Concentration of triclosan in water after 0 and 48 h. Values are expressed as mean ± standard error.

	Concentration (mg L^{-1})	
Treatment	**0 h**	**48 h**
Control 1	< LOD	< LOD
Control 2	< LOD	< LOD
Microplastics only	< LOD	< LOD
Triclosan only	0.20 ± 0.02	< LOD
Microplastics and triclosan	0.10 ± 0.03	0.02 ± 0.01

LOD = limit of detection

4.2.3. Triclosan in mussel tissues

Triclosan and methyl-triclosan were extracted from all mussel samples analysed from the TCS and MP_{TCS} treatments (Table 4.2, all data shown in Appendix 7). Significantly higher concentrations of both analytes were isolated in the MP_{TCS} exposed mussels than the TCS exposed mussels ($p \leq 0.05$, Welch t-test, Figure 4.1). Concentrations of triclosan and methyl-triclosan in control and MP exposed mussels were below detection limits for all replicates.

Table 4.2: Average tissue triclosan and methyl-triclosan concentration in µg g^{-1} (dw) and µg mg $lipid^{-1}$. Values are expressed as mean ± standard error.

	Concentration (µg g^{-1} (dw))		Concentration (µg mg $lipid^{-1}$)	
Treatment	**Triclosan**	**Methyl-triclosan**	**Triclosan**	**Methyl-triclosan**
TCS	1030 ± 50	93 ± 9	21 ± 2	1.9 ± 0.2
MP_{TCS}	2100 ± 400	230 ± 70	34 ± 6	4 ± 1

dw = dry weight

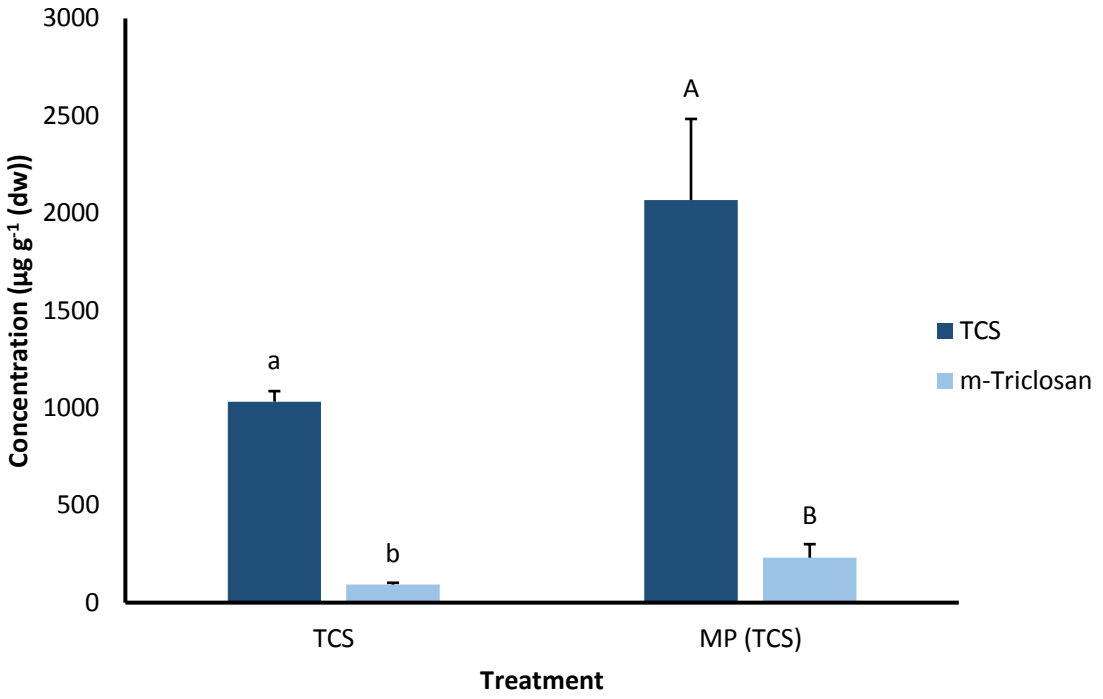

Figure 4.1: Concentration of triclosan and methyl-triclosan in mussel tissue of mussels exposed to TCS and MP_{TCS} treatments. Detection limits were 0.005 µg g^{-1} for both analytes. Capitalised data labels indicate significant difference from lower case ($p \leq 0.05$). Data are expressed as mean ± standard error.

4.2.4. Microplastics in mussel tissues

All mussels in the MP and MP_{TCS} treatments ingested microplastics during the 48 h exposure period. After opening the shells and removing the tissue, the orange microbeads were present in the digestive tract (Figure 4.2). There was no significant difference in the microplastic concentration in tissues of mussels exposed to the MP and MP_{TCS} treatments ($p \geq 0.05$, Table 4.3, data and statistics shown in Appendix 8).

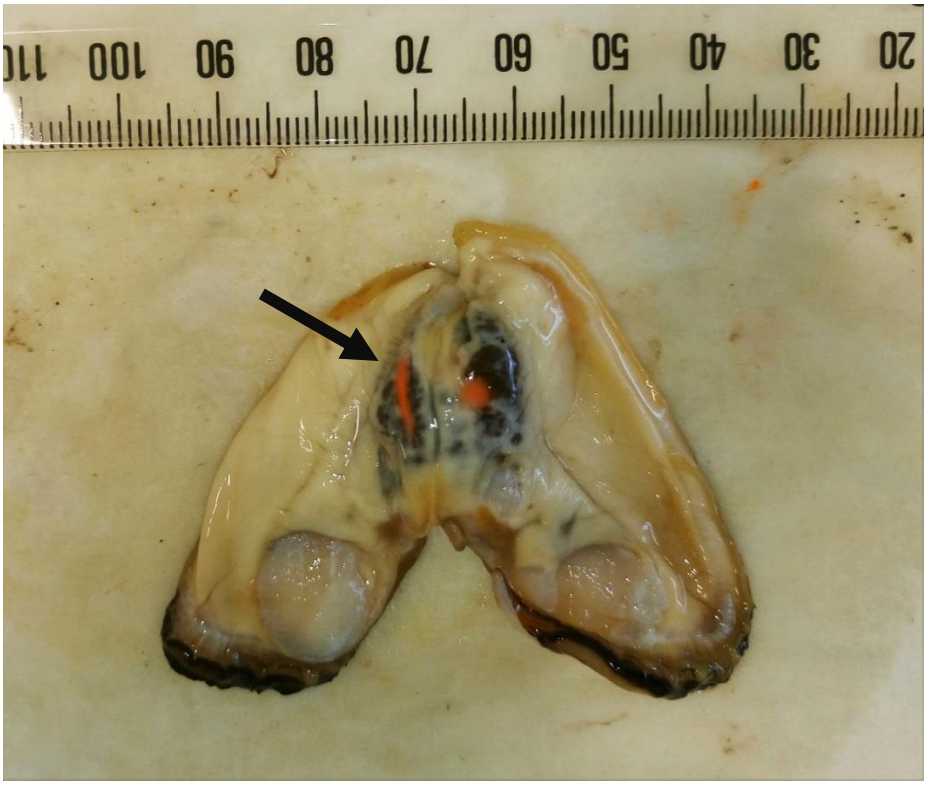

Figure 4.2: Tissue of green-lipped mussel exposed to microplastics. Orange microbeads can be seen in the tissue as indicated by the black arrow.

Table 4.3: Microplastic concentration in green-lipped mussels following acute exposure (48 h). Values expressed as mean ± standard error.

Treatment	Microbeads per gram tissue (mg g^{-1} (ww))	Number of microbeads (1000) per mussel	Number of microbeads (1000) per gram tissue (g^{-1} (ww))
MP	6 ± 1	2000 ± 500	150 ± 30
MP_{TCS}	3 ± 1	1300 ± 300	90 ± 20

ww = wet weight

The filtered digests were observed under microscope to confirm the presence of the microbeads (Figures 4.3 and 4.4). The fluorescent signal had decreased due to the acid digestion but the particles still appeared smooth and regular in shape. There was no observable difference between the number and appearance of microbeads between the two treatments. A small number of particles (< 50 per replicate or < 0.01% of the microplastic treatments) were observed on the blank filter papers and on the filters from the control mussels. This can be attributed to the microbeads becoming airborne during sample processing and becoming caught on the filter paper during filtration.

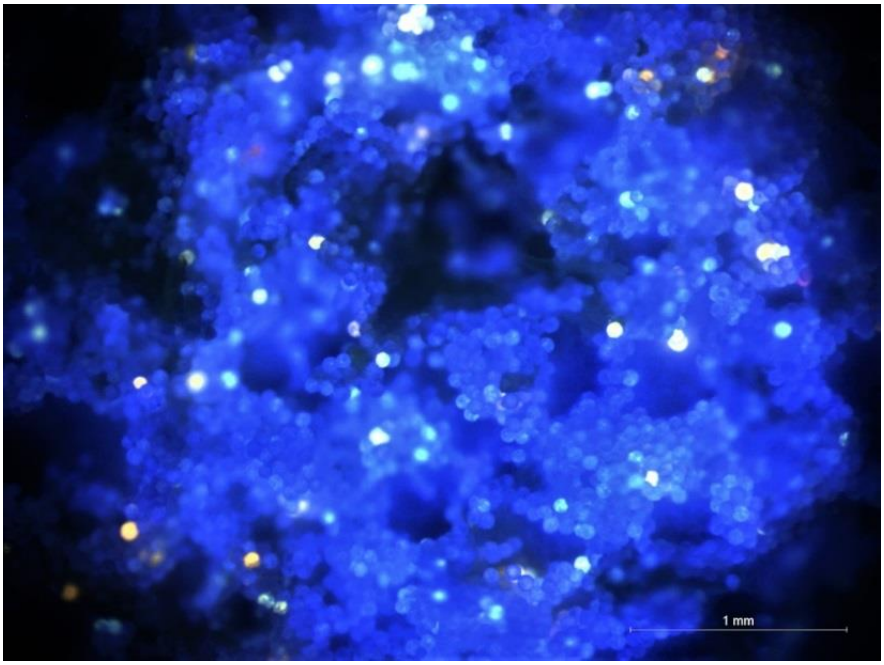

Figure 4.3: Filtered digested tissue of mussels exposed to MP_{TCS}. Scale bar is 1 mm. Observed using a Leica MZf10 fluorescent coupled microscope under UV fluorescent light.

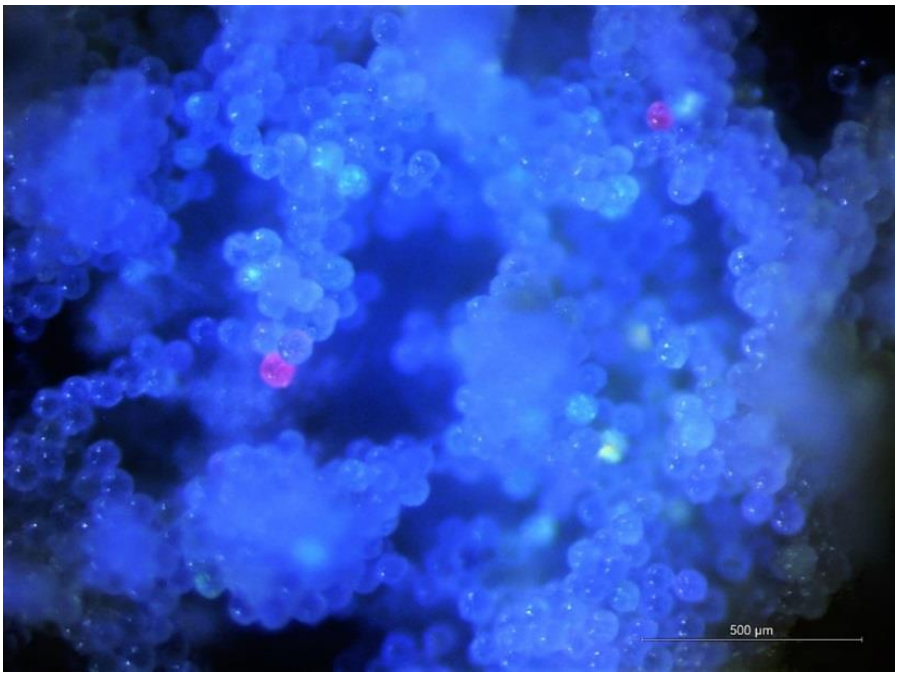

Figure 4.4: Filtered digested tissue of mussels exposed to MP. Scale bar is 500 µm. Observed using a Leica MZf10 fluorescent coupled microscope under UV fluorescent light.

4.2.5. Physiological biomarkers

Clearance rate

There was no significant difference in the clearance rate between the controls and the other treatments ($F = 1.305$, $df = 4$, $p \geq 0.05$, Figure 4.5). The average clearance rate for MP treated mussels was 0.14 ± 0.04 L g^{-1} h^{-1}, slightly lower than that of the controls 0.24 ± 0.03 L g^{-1} h^{-1} (Appendix 9 and 11).

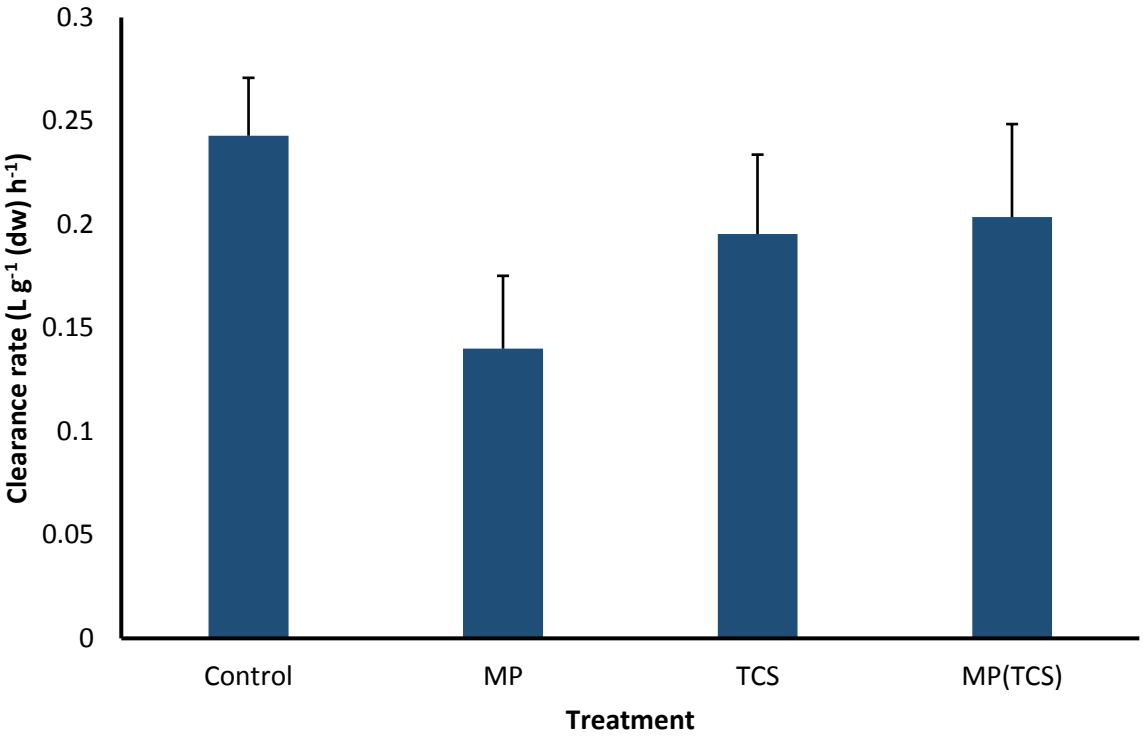

Figure 4.5: Clearance rates in green-lipped mussels after acute microplastic (MP), triclosan (TCS) and microplastic and triclosan (MP_{TCS}) exposures (n = 4). Values are expressed as mean ± standard error. Rates are not significantly different (p ≥ 0.05).

Oxygen respiration rate

Microplastics on their own caused a decrease in the oxygen respiration rate of green-lipped mussels compared with the controls (12 ± 3 µmol O_2 g^{-1} (dw) h^{-1} and 21 ± 2 µmol O_2 g^{-1} (dw) h^{-1} respectively; two tailed t-test assuming unequal variance; t = 2.5156, df = 5.97, $p \leq 0.05$; Appendix 9 and 11). There was no difference in the clearance rate between mussels exposed to treatments containing triclosan and control mussels (Figure 4.6).

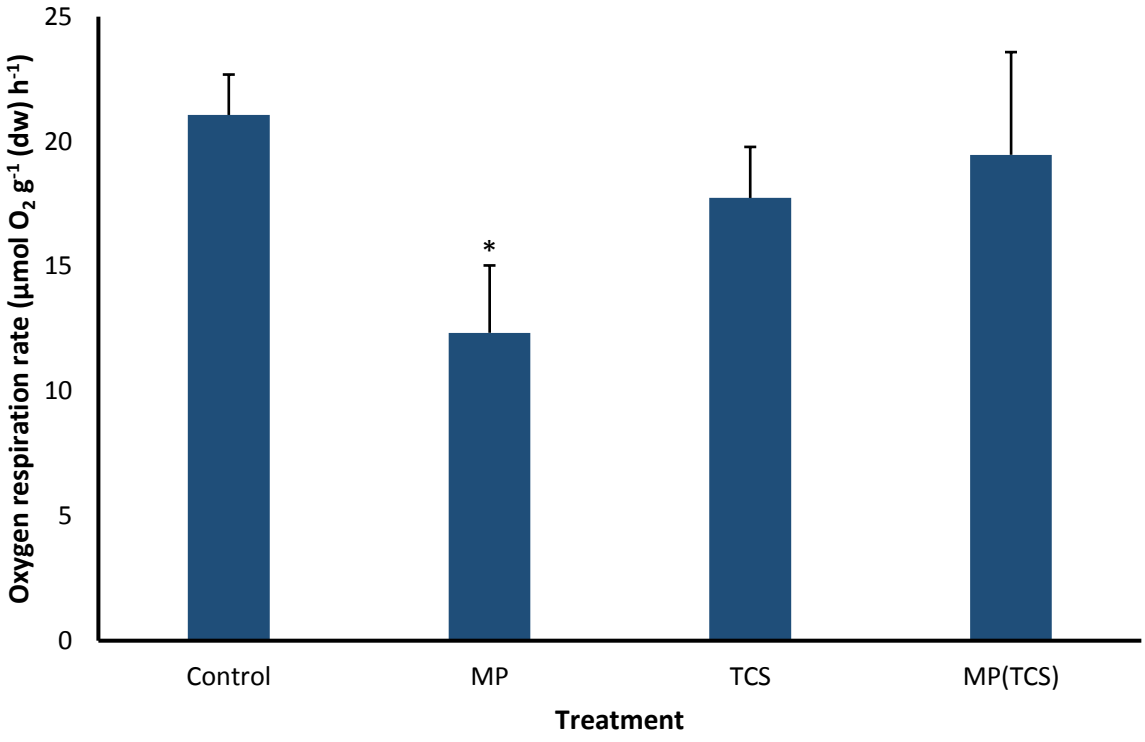

*Figure 4.6: Oxygen respiration rates in green-lipped mussels after acute exposure to microplastics (MP), triclosan (TCS) and combined microplastics and triclosan (MP_{TCS}, n = 6). Values are expressed as mean ± standard error. * indicates significant difference ($p \leq 0.05$) from control.*

Byssus thread production

Mussels exposed to the MP treatment produced 3.3 ± 0.2 threads g^{-1} (dw); significantly fewer byssus threads than the control mussels which produced 10 ± 1 threads g^{-1} (dw) ($F = 5.75$, $p \leq 0.05$, Figure 4.7). There was no significant difference in byssus thread production between the TCS and MP_{TCS} treatments when compared to controls ($p \geq 0.05$; 6 ± 1 and 6 ± 2 threads g^{-1} (dw) respectively, Appendix 9 and 11).

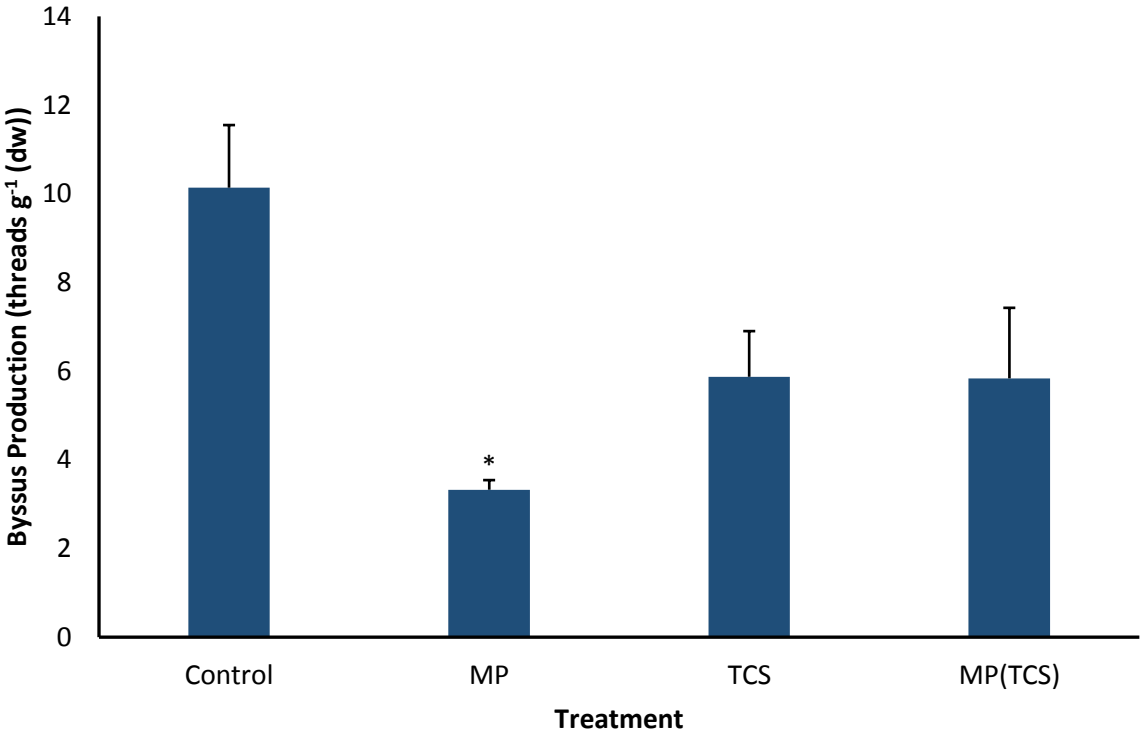

*Figure 4.7: Number of byssus threads produced by green-lipped mussels over the 48 h exposure period with acute exposure to microplastics (MP), triclosan (TCS) and combined microplastics and triclosan (MP_{TCS}, n = 6). Values are expressed as mean ± standard error. * indicates significant difference ($p \leq 0.05$) from control.*

4.2.6. Biochemical biomarkers

Lipid peroxidation

TCS and MP_{TCS} treatments caused significant increases in the amount of lipid peroxidation in gill tissues compared with the controls ($F = 6.382$, $p \leq 0.05$, Figure 4.8). Both TCS and MP_{TCS} treated mussels had 0.015 ± 0.002 µmol MDA mg protein^{-1} whereas the control mussels had 0.009 ± 0.001 µmol MDA mg protein^{-1} (Appendix 10 and 11).

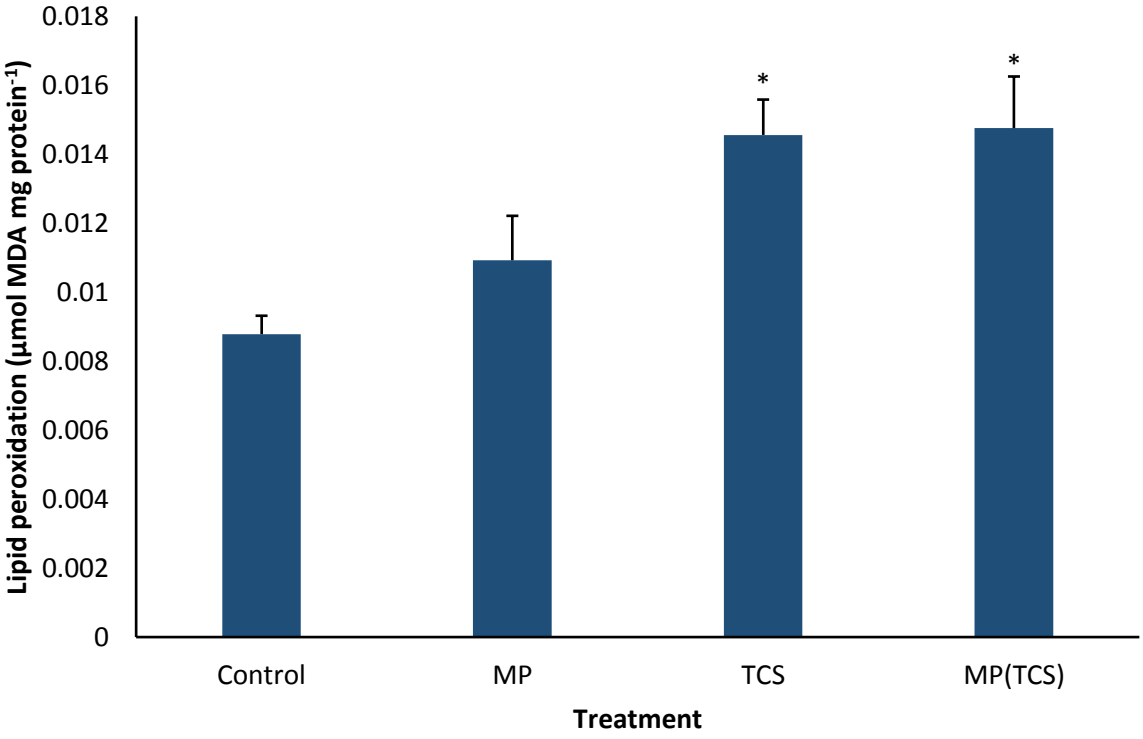

*Figure 4.8: Lipid peroxidation in green-lipped mussel gill tissue following acute exposure to microplastics (MP), triclosan (TCS) and combined microplastics and triclosan (MP_{TCS}, n = 5-6). Values are expressed as mean ± standard error. * indicates significant difference ($p \leq 0.05$) from control samples.*

Superoxide dismutase (SOD) activity

SOD activity in the gill tissue of mussels exposed to MP_{TCS} (2.9 ± 0.7 U mg protein^{-1}) was significantly higher than in the control mussels (1.0 ± 0.2 U mg protein^{-1}; F = 3.831, p ≤ 0.05, Appendix 10 and 11). There was no difference in SOD activity between mussels exposed to the other treatments and control samples (Figure 4.9).

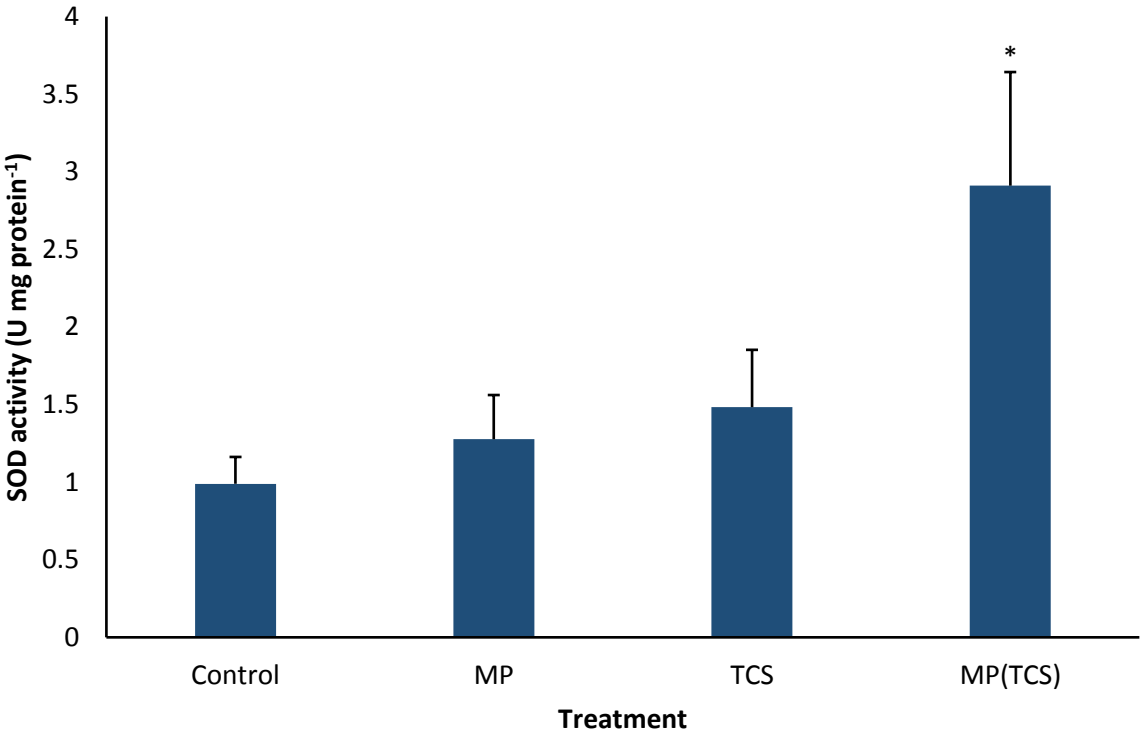

*Figure 4.9: SOD activity in green-lipped mussel gill tissue following acute exposure to microplastics (MP), triclosan (TCS) and combined microplastics and triclosan (MP_{TCS}, n = 4-6). Values are expressed as mean ± standard error. * indicates significant difference (p ≤ 0.05) from control samples.*

Glutathione-S-transferase (GST) activity

There was no significant change in the GST activity between the different treatments ($F = 1.86$, $p \geq 0.05$, Figure 4.10). The GST activity for MP_{TCS} exposed mussels was 0.7 ± 0.2 µmol min^{-1} mg $protein^{-1}$, slightly lower than that of the control mussels (1.6 ± 0.4 µmol min^{-1} mg $protein^{-1}$, Appendix 10 and 11).

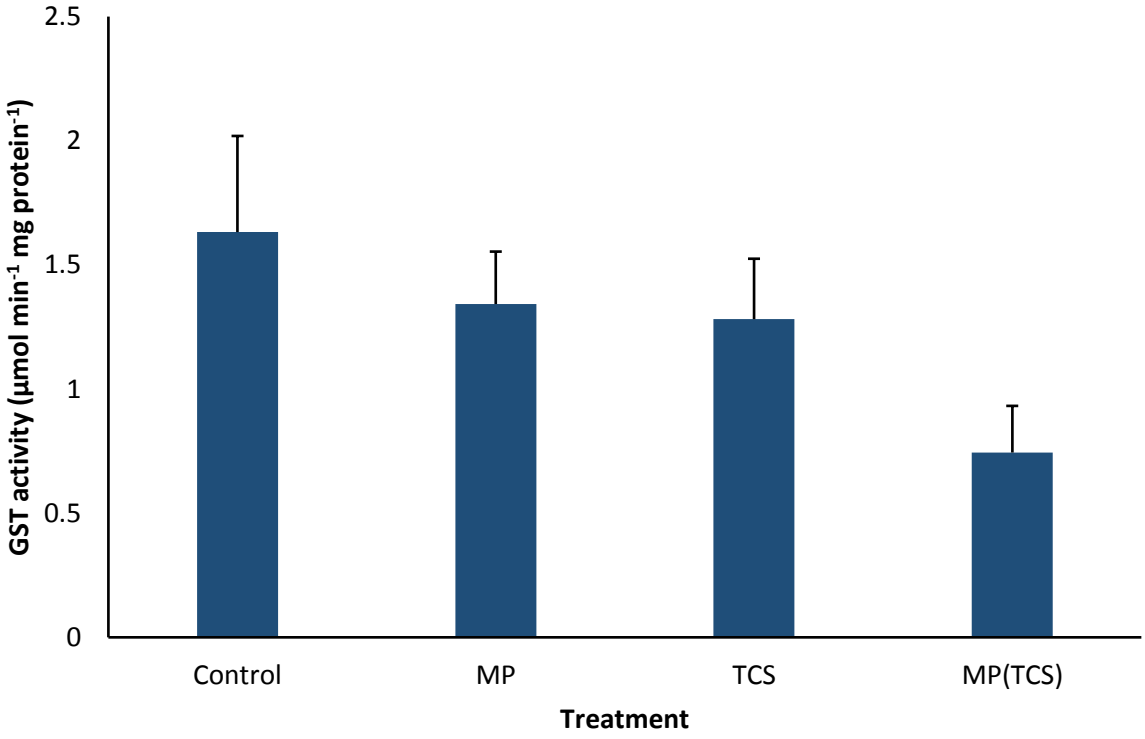

Figure 4.10: GST activity in green-lipped mussel gill tissue following acute exposure to microplastics (MP), triclosan (TCS) and combined microplastics and triclosan (MP_{TCS}, n = 6). Values are expressed as mean ± standard error.

4.3. Discussion

This investigation assessed the impact of microplastics and triclosan individually on green-lipped mussels (*Perna canaliculus*) and how this differs when microplastics are used as an exposure pathway of the mussels to triclosan. Microplastics and triclosan, both individually and combined, had adverse effects on green-lipped mussels. When triclosan was sorbed to microplastics, mussels that ingest these microplastics have increased exposure to triclosan. Triclosan and microplastics acted in a synergistic manner on the activity of the SOD enzyme involved in minimising oxidative damage.

The two control samples (blank control – Control 1, and acetone control – Control 2) were not significantly different from each other. The volume of acetone was low (< 0.01% v/v) and the low density and high volatility of acetone meant that it is likely to have evaporated soon after addition (Sigma-Aldrich, 2016). Therefore, the contribution of acetone in the triclosan only treatment was negligible.

4.3.1. Microplastics in tissues

All the mussels exposed to microplastics ingested significant quantities of microplastic beads (1,300,000 – 2,000,000 microbeads per mussel). This could be seen distinctly through the tissue in the digestive tract (Figure 4.2). While there was no difference in microplastic concentration in tissues between the MP and MP_{TCS} treatments, there was a high amount of variation between replicates (Appendix 8).

The microplastic beads floated near the top of the vessel after they were added to the sea water due to the lower density (1.004 g cc^{-1}). The bubbling meant that they were constantly mixed and swirling but this appeared to be limited to the top half of the 3 L vessel. Over the 48 h period, the microplastics began to fully disperse in the water as they attached to faeces, pseudo-faeces and other debris. After 48 h they were circulating evenly in the water. Therefore, it is suggested that biofouling of the plastics may have contributed to microplastic accumulation and the high concentrations of microplastics measured in the tissue.

During the physiological assessments, the mussels that had been exposed to microplastics continued to produce pseudo-faeces containing the microplastics. This was observed for the entire period of analysis – approximately 6 h, following removal from the treatment conditions. This may have had an impact on the clearance rates (Section 4.1.4).

Although the microplastic particles were significantly larger than that of the instant algae feed (38 – 45 µm microplastic particles and 1 – 20 µm algae), the mussels still ingested the microplastics. The size of microbeads used this study was selected to be comparable with other microplastic exposure studies (Avio et al., 2015; Rist et al., 2016; von Moos et al., 2012). They were required to be small enough to be consumed by the mussels while large enough to be assessed under microscope. A recent study reported green-lipped mussels ingest zooplankton, greater than 60 µm in size (Zeldis et al., 2004). Therefore, using a slightly larger microplastic than previous studies was considered acceptable (38 – 45 µm in the current study compared with 0.03 – 16 µm in previous studies; Browne et al., 2008; Paul-Pont et al., 2016; Wegner et al., 2012). The microplastics used in the current laboratory study were smaller than a majority of those isolated in green-lipped mussels in the environment (Section 3.2)

Other microplastic exposure studies used a wide range of microplastic concentrations in water ranging from 32 µg L^{-1} to 20 g L^{-1} (Avio, et al. 2015; Paul-Pont, et al. 2016; von Moos, et al. 2012). However, they did not assess the concentration of microplastics ingested or accumulated in the tissues. This analysis was included in the current study to confirm that the mussels had ingested the plastics and also to compare with the concentration of triclosan in tissue for the microplastic and triclosan treated mussels. While the concentrations of microplastics measured in mussel tissues this investigation were considerably higher than those observed in green-lipped mussels in the environment (refer to Section 3.2), if microplastics concentrations in the oceans increase, concentrations in mussels are also likely to increase.

4.3.2. Triclosan

Seawater

The triclosan concentration in the sea water decreased significantly over the 48 h exposure period. For most batches, the triclosan concentration in the water at the end of the exposure period was below the detection limit for both the TCS and MP_{TCS} treatments. These results are inconsistent with previous studies. Kookana et al. (2013) investigated triclosan bioaccumulation in *M. galloprovincialis* and reported only a 20% reduction in triclosan concentration in the seawater after 72 h. This differs significantly from what was observed in the current study. Later studies have used the results from Kookana et al. (2013) to justify not measuring the triclosan concentration in water analytically (e.g. Goodchild et al., 2016). While species specific differences are expected, the present study suggests that analytical analysis of water samples for all exposures is recommended.

High concentrations of triclosan were measured in the mussel tissue but were not sufficient to account for the complete loss of triclosan from the water. The loss of triclosan from the water may have occurred via a range of mechanisms including bacterial or photolytic transformation, and loss via sorption to container walls (Yueh and Tukey, 2016). The main bacterial degradation byproduct for triclosan is methyl-triclosan (Dhillon et al., 2015). This transformation product was monitored for in the water and all results for methyl-triclosan in water were below detection limits. Methyl-triclosan was however, measured in all tissue samples analysed from the triclosan containing treatments. Triclosan is present in anionic form in seawater when phototransformation is a more important transformation pathway than if triclosan were present in neutral form (Tixier et al., 2002). A potential phototransformation product of triclosan in sea water is 2,8-dichlorodibenzo-p-dioxin (DDCD), which was not monitored for in the current study (Aranami and Readman, 2007). However, the exposures were performed in dark laboratories and in amber vessels covered with aluminium foil so photolytic degradation is unlikely. Losses

due to sorption were minimised in the exposures by using glass aquaria and metal tubes for the bubblers. Future studies should monitor a wider range of degradation products and measure triclosan accumulation on the mussel shells to determine where the losses occur.

Mussel tissue

Mussels exposed to the TCS and MP_{TCS} treatments accumulated very high concentrations of both triclosan and methyl-triclosan. The concentration of triclosan and methyl-triclosan in mussel tissue was significantly higher in the mussels exposed to the MP_{TCS} treatment than the TCS treated mussels. Depuration was not performed so mussel tissues analysed from the triclosan and microplastic treatment still contained microplastics, potentially with triclosan sorbed. Therefore, the concentrations measured should be considered an indication of the total amount of triclosan the mussels were exposed to, rather than the amount accumulated in the tissues for this treatment.

Both TCS and MP_{TCS} treatments had similar proportions of methyl-triclosan to triclosan (8 ± 1% and 9 ± 2% methyl-triclosan respectively of concentrations of triclosan and methyl-triclosan combined; Welch t-test, $t = -1.128$, $df = 8.6821$, $p \geq 0.1$). Kookana et al. (2013) exposed mussels in a laboratory study to 100 ng L^{-1} triclosan and reported after 30 days approximately 20% of the combined concentration was methyl-triclosan. The relatively higher levels of methyl-triclosan compared with triclosan over time is suggested to be due to the higher persistence and accumulation potential of methyl-triclosan as well as the fact that triclosan can undergo internal methylation in fish to form methyl-triclosan (Rüdel et al., 2013). Consequently, methyl-triclosan has been measured at higher concentrations in tissues than triclosan in environmental samples. For example, Rüdel et al. (2013) measured methyl-triclosan:triclosan ratios in the range of 3-300 in fish from German rivers between 1994 and 2008.

The concentration of triclosan and methyl-triclosan in the mussel tissues were at least four orders of magnitude higher than those reported in environmental samples (Table 4.4). Kookana et al. (2013) deployed mussels (*Mytilus galloprovincialis*) in cages for 70 days near wastewater treatment discharges in the Gulf of St Vincent, South Australia. Triclosan and methyl-triclosan had accumulated at similar concentrations to each other in the mussel tissue, however, methyl-triclosan concentrations were 1000 times lower, and triclosan concentrations were 100,000 times lower than measured in the current study.

Table 4.4: Studies investigating field bioaccumulation of triclosan and methyl-triclosan by bivalves.

Species	Location	Conc. triclosan	Conc. methyl-triclosan	Reference
Mytilus galloprovincialis	Gulf St Vincent, South Australia	9.87 (±1.34) µg kg^{-1} (dw)	6.99 (±2.44) µg kg^{-1} (dw)	Kookana et al. (2013)
Coregonus sp. and *Rutilus rutilis*	Switzerland		365 ng g $lipid^{-1}$	Balmer et al. (2004)
Modiola barbatus L., Mytilus galloprovincialis and *Venus gallina*	Thermaikos Gulf and Lesvos Island (Greece)	146.1 µg kg^{-1} (dw)		Gatidou et al. (2010)

Higher concentrations of triclosan were measured in the mussel tissue of mussels exposed to MP_{TCS} than those exposed to TCS supporting the hypothesis that microplastics provide another pathway for the transport of triclosan to mussel tissues (Table 4.2). This pathway has also been reported for other chemical pollutants. For example, previous studies have illustrated that polyaromatic hydrocarbons (PAHs) can be transported the tissues of marine species via ingestion of the microplastics and later desorption of the contaminant (Besseling et al., 2013; Browne et al., 2013; Chua et al., 2014; Wardrop et al., 2016). Transport and desorption is dependent on the partition coefficient of the contaminants and the type of plastic studied (Wardrop et al., 2016). Wardrop et al. (2016) exposed Rainbow fish (*Melanotaenia fluviatilis*) to microplastics with sorbed polybrominated diphenyl ethers (PBDEs) and reported significantly higher concentrations of ∑PBDEs than the controls. However, they did not include a PBDE only control and are therefore unable to say what influence the microplastics had on the accumulation. Similarly, Avio et al. (2015) investigated pyrene bioaccumulation in *M. galloprovinvialis* using polyethylene and polystyrene to transport the pyrene. They reported significant concentrations of pyrene in the mussel tissues for both types of plastic. However, they also did not include pyrene in the absence of plastics as a control. In a similar study, no difference was reported in fluoranthene concentration in gill and digestive gland tissue in *Mytilus* spp. exposed to fluoranthene both alone and with microplastics (Paul-Pont et al., 2016).

4.3.3. Whole animal physiological biomarkers

Microplastics on their own caused an adverse effect on the physiology of the mussels. This was particularly pronounced for the production of byssus threads. Microplastics alone caused a significant decrease in the production of byssus threads compared to control mussels. However, this was not observed for mussels exposed to microplastics with triclosan sorbed to the surface. The method used to sorb triclosan to the surface of the microplastics may have changed the surface properties of the plastic beads, potentially removing the adverse effects resulting from the uncoated plastic beads. This may account for the higher level of byssus attachment compared with the microplastic only treatment. Rist et al. (2016) also reported a decrease in byssus production in response to microplastic exposure.

A significant decrease in the oxygen respiration rate was observed for the mussels exposed to microplastics only compared with the control mussels. These results are consistent with a study by Rist et al. (2016) who reported a decline in *Perna viridis* oxygen respiration rate and clearance rate in response to microplastics following a 7 day exposure. Similarly, Watts et al. (2016) reported a significant decrease in oxygen consumption, 1 h following addition of microplastics but no change after 24 h in shore crabs, *Carinus maenas*. In contrast, exposure to microplastics caused increased clearance rates in Pacific oysters when exposed to polystyrene microplastics for two months at a concentration of 23 µg L^{-1} compared to controls (Sussarellu et al., 2016) and Green (2016) reported no change in clearance rate and oxygen respiration in European flat oysters (*Ostres adulis*) when exposed to 80 µg L^{-1} high-density polyethylene for 60 days. These two studies investigating organisms with similar feeding mechanisms reported very different results to the current study indicating that species react differently to microplastic exposure. Also, the exposure period was considerably longer in these studies which may affect the responses.

No adverse impacts on the physiological biomarkers were observed when triclosan was present with the microplastics even though microplastic concentrations were the same as for the MP treatment. Rist et al. (2016) hypothesised that adverse effects of microplastics on physiological biomarkers could be due to prolonged periods of valve closure in response to the plastics. Wegner et al. (2012) also noted a decrease in the filtering activity of blue mussels (*M. edulis)* exposed to nano-polystyrene. Future studies should include an assessment of the valve opening times (Riisgård et al., 2006). This could give an indication of if the mussels attempt to limit their exposure to the microplastics. Comparing the opening times for the mussels exposed to MP and those exposed to MP_{TCS} may help ascertain as to why no adverse effects on the physiological biomarkers were seen in the mussels exposed to MP_{TCS}.

In the present study, no adverse effects on mussel physiology were observed for mussels exposed to waterborne triclosan on its own. No other studies could be located that have investigated the effect of triclosan on bivalve clearance rate, respiration rate or byssus production. However, the behaviour of aquatic species has been reported to be effected by triclosan. Goodchild et al. (2016) reported that 900 ng L^{-1} triclosan exposure impaired burrowing and movements behaviours in freshwater mussels (*Elliptio complanata*). Triclosan caused a reduction in swimming speed in Japanese medaka fish when exposed to triclosan for eight days at 0.17 mg L^{-1} (Nassef et al., 2010). Behavioural assessments should be included in future studies investigating the effects of triclosan on green-lipped mussels.

4.3.4. Cellular biochemical biomarkers

There were no differences in the biochemical biomarkers between the controls and the mussels exposed to microplastics only. This is consistent with results from a study by Avio et al. (2015) where *Mytilus galloprovincialis* were exposed to microplastics for 7 days at 1.5 g L^{-1} (< 100 µm) and no effects on lipid peroxidation or GST activity were observed in digestive gland tissue. Oliveira et al. (2013) also reported no change in lipid peroxidation or GST activity in *Pomatoschistus microps* exposed to 1 – 5 µm polyethylene spheres at up to 184 µg L^{-1} for 96 h.

Triclosan had a significant impact on the biochemical biomarkers investigated in the present study. Triclosan, both alone and in conjunction with microplastics, caused an adverse effect on the ability of the mussels to minimise oxidative damage. An increase in the activity of the SOD enzyme was observed in mussels exposed to MP_{TCS}. This enzyme is used in the reduction of oxyradicals to hydrogen peroxide, preventing oxidative damage (Binelli et al., 2011). This increase in activity was not sufficient to prevent oxidative damage as an increase in lipid peroxidation was also observed. There is limited data available for comparison and other studies in the literature to date have investigated the impact of lower concentrations of triclosan on bivalves (Table 4.5). Binelli et al. (2011) reported significant activation of the GST enzyme after 48 h with triclosan concentrations down to 1 nM (290 ng L^{-1}). They also measured a significant increase in SOD activity at 3 nM triclosan (869 ng L^{-1}). These concentrations are much lower triclosan concentrations in this study; however, there was no change in the GST activity in the current study. Binelli et al. (2011) used whole organism samples for measuring the enzyme activity so it is possible that the activation in the GST enzyme could be occurring in an area other than the gill tissue. In a longer period exposure, Matozzo et al. (2012) exposed clams (*Ruditaes philippinarum*) to triclosan at up to 900 ng L^{-1} for seven days and reported a significant increase in SOD activity in the gills but no changes in lipid peroxidation. In the current study the opposite effect was observed with an increase in lipid peroxidation but no significant change in SOD

activity in response to triclosan only. This could be due to the lower triclosan concentration in the study by Matozzo et al. (2011) in which enzymes other than the SOD enzyme were able to minimise the impact of on oxidative damage.

The impact on the SOD activity of the mussels exposed to MP_{TCS} in the present study was significantly higher than the changes in activity in MP and TCS exposed mussels combined. This indicates a potential synergistic effect on the activity of the SOD enzyme when these two contaminants are present together. To my knowledge, this is the first study to report a synergistic effect of microplastics and sorbed contaminants on antioxidant enzymes.

There was the same level of lipid peroxidation for the TCS and MP_{TCS} treatments but higher SOD activity for the MP_{TCS} exposed mussels as discussed. This indicates that the combination of triclosan and microplastics has the potential to cause more oxidative damage than triclosan alone, but the SOD enzyme is capable of increasing activity to minimise this additional damage. However, there was a significant increase in lipid peroxidation for these treatments compared with the controls, indicating that the SOD enzyme and other antioxidant enzymes involved in this process are insufficient to minimise the level of oxidative damage caused by the triclosan.

Table 4.5: Exposure studies investigating effects of triclosan on bivalves.

Species	**Exposure details**	**Conc. triclosan**	**Endpoint**	**Reference**
Mytilus galloprovincialis	Acute 30 min study on hemocytes	1 µM	Decrease in lysosomal membrane stability	Canesi et al. (2007)
Mytilus galloprovincialis	Acute 24 h exposure	2.9 ng g^{-1}	Altered hemocyte and digestive gland function	Canesi et al. (2007)
Dreisena polymorpha	Acute 96 h exposure	1 - 3 nM	Genotoxic and cytotoxic endpoints	Binelli et al. (2008)
Dreisena polymorpha	Acute 96 h exposure	1 - 3 nM	Antioxidant enzyme activation	Binelli et al. (2011)
Elliptio complanata	28 day exposure	300 - 900 ng L^{-1}	Reduction in burrowing and movement behaviours, increase in total AMPK protein abundance and AMPK activity	Goodchild et al. (2016)
Ruditapes philippinarum	7 day exposure	300 - 900 ng L^{-1}	Increase in SOD activity, decrease in AChE activity in gills	Matozzo et al. (2012)
Unio tumidus	14 day exposure	500 ng L^{-1}	DNA fragmentation	Falfushynska et al. (2014)

AMPK = AMP-activated protein kinase.
AChE = acetylcholinesterase.

4.3.5. Study limitations

This was an acute study investigating the effects of high concentrations of microplastics and triclosan on mussels. These impacts may not be seen at environmentally relevant concentrations but should be viewed as a worst-case scenario. In addition, the microplastics used in this study were smooth beads, effects may be different if fragments with sharp edges or odd shapes were used which is more representative of microplastics observed in seawater and green-lipped mussels in New Zealand (refer to Section 3.2). Other studies have reported high concentrations of fibres in the environment (Mathalon and Hill, 2014; Li et al., 2016). Fibres may also cause different changes in mussel physiology.

Depuration was not included after the 48 h exposure so it is not clear whether the mussels would expel the microplastics if allowed. However, the mussels used for physiological biomarker tests were held in fresh, filtered seawater for approximately 6 h after the exposure time due to the nature of the tests. Following the physiological tests, the mussels were shucked and dried to determine dry weight. When shucked, the orange microplastic particles could still be observed in the digestive tract, so the mussels were still likely to be exposed to microplastics after a six hour depuration period. A depuration period was not performed to give an indication of the impact of the microplastics at the time of exposure, rather than following the exposure, wherever possible.

4.4. Conclusions

Acute exposure to microplastics and triclosan had adverse effects on green-lipped mussels. Microplastics adversely affected mussel physiology while triclosan affected mussel biochemistry. When triclosan was sorbed to microplastics, a potentially synergistic impact was observed on the activity of the SOD enzyme. Mussels ingested significant concentrations of microplastics both when exposed to microplastics only and microplastics sorbed with triclosan. There were no significant differences in the ingested quantity between the two treatments. Microplastics may increase the exposure of hydrophobic contaminants to mussels in the marine environment as mussels exposed to triclosan sorbed to microplastics had higher tissue concentrations of triclosan than mussels exposed to triclosan only.

5. Conclusions

5.1. Summary of findings

The objectives of this thesis were to (a) investigate microplastic accumulation in New Zealand green-lipped mussels, *Perna canaliculus*, including the size of microplastics the mussels ingest; (b) determine if there is a relationship between microplastic concentrations in seawater and green-lipped mussels; (c) investigate the effects of microplastics and triclosan both individually and combined on the green-lipped mussel using a range of physiological and biochemical biomarkers; and (d) determine whether microplastics enhance the uptake of triclosan by green-lipped mussels. This was the first study to assess microplastic accumulation in an aquatic species in New Zealand and the first study to assess the effects of microplastics and triclosan both individually and combined on the New Zealand green-lipped mussel *Perna canaliculus*.

The uptake of microplastics by green-lipped mussels around New Zealand was assessed using a field collection survey where mussels were collected from a range of locations around New Zealand with a more in depth survey conducted in Christchurch (Chapter 3). Mussels were digested and observed under fluorescence coupled microscope to identify potential microplastic particles. Green-lipped mussels had ingested microplastic particles including fragments, beads and fibers. Thirty five percent (35%) of samples analysed (two mussels per sample) contained microplastics. A majority (78%) of the particles isolated in mussel tissue were fragments. At least one mussel from each of the National survey sampling locations contained a microplastic particle. There was no relationship between microplastics isolated in seawater and mussel samples.

The physiological and biochemical impacts of microplastics and triclosan, both individually and combined was assessed in an acute 48 h exposure (Chapter 4). The objectives were to determine (a) the physiological and biochemical impacts of microplastics (MP), triclosan (TCS) and triclosan sorbed to microplastics (MP_{TCS}) on green-lipped mussels following an acute 48 h study; (b) whether the sorption of triclosan to the microplastics enhanced the accumulation of triclosan in the mussel tissue and (c) whether the sorption of triclosan to the microplastics effects the accumulation of microplastics in the mussel tissue. Microplastics caused adverse effects on mussel physiology including decreased oxygen respiration rates and byssus production when present alone. This was not observed when the microplastics were present with triclosan. This may be due to changes in the surface properties of the plastic beads when triclosan was sorbed to the microplastics which removed the potential adverse effects resulting from the original plastic beads. Triclosan, both alone and in combination with microplastics, adversely affected

the mussel oxidative stress markers including SOD activity and lipid peroxidation. SOD activity of the mussels exposed to MP_{TCS} was significantly higher than the activity in MP and TCS exposed mussels combined. This indicates a potential synergistic effect on the activity of the SOD enzyme when these two contaminants were present together. This is believed to be the first study to report a synergistic effect of microplastics and sorbed contaminants on antioxidant enzymes in bivalves.

Green-lipped mussels exposed to MP_{TCS} had higher concentrations of triclosan in the tissue than mussels exposed to triclosan only. This means that microplastics potentially increase the exposure of hydrophobic contaminants to mussels in the marine environment providing an additional pathway of exposure. Mussels ingested high concentrations of microplastics in the laboratory exposure experiment. There was no difference in the microplastic accumulation in the two microplastic treatments, MP and MP_{TCS}. Biofouling influenced the behaviour of the microplastics in seawater, causing them to disperse more, making them more available to be ingested by the mussels.

5.2. Recommendations and implications

This preliminary study illustrates that microplastics are ingested by green-lipped mussels in New Zealand. This investigation involved collection of a relatively small number of mussel samples from a limited number of locations. Further research is required to fully understand the level of microplastic contamination in the New Zealand coastal environment and in particular the ingestion by bivalves. A more targeted sampling study is recommended where mussels are collected from mussel beds that are near wastewater outfalls or high levels of commercial marine activity (for example, ports or aquaculture farms). Microplastics have also been reported in sediments in New Zealand (Clunies-Ross et al., 2016). Consequently, research is required to assess the impact of sediment dwelling organisms such as deposit feeding bivalves and worms in New Zealand in both environmental field surveys and laboratory exposures.

The results of the current study indicate that regular, nationwide monitoring of microplastics in wild, marine mussels may be unnecessary. Mussels may not be an appropriate passive sampling method for microplastic contamination in coastal surface water as there was no relationship between microplastics in mussels and in surface water samples. However, there is a potential for higher microplastic contamination in mussels from aquaculture facilities due to expanded polystyrene buoys or polypropylene and polyamide (nylon) fishing equipment (Mathalon and Hill, 2014; Jang et al., 2016). Jang et al. (2016) recently reported that farmed marine mussels

accumulated expanded polystyrene particles thought to originate from polystyrene buoys used on the mussel farm. Also, Mathalon and Hill (2014) recently reported that farmed mussels ingested higher concentrations of microplastics than wild mussels in Nova Scotia. The aquaculture industry is extremely important to the New Zealand economy. In 2013, fisheries and aquaculture contributed $896 million to gross domestic product (GDP), totalling 0.4% of the national economy (Statistics New Zealand, 2016). In 2011, green-lipped mussels accounted for 85% of aquaculture exports by volume from New Zealand (Aquaculture NZ, 2012). Therefore, it is recommended that green-lipped mussels harvested from aquaculture facilities be routinely monitored for microplastic accumulation.

The microbeads used in the laboratory exposure study (Chapter 4) were similar in size to some of the particles observed in the field study (Chapter 3). However, most the particles isolated in the mussels analysed in the field study were fragments with sharp edges. It is unknown what impact the variations in shape may have on the physiological and biochemical biomarkers monitored. It is suggested that they may have a more significant impact on the physiological biomarkers in particular. Future work should perform a similar exposure experiment using milled polyethylene fragments as this may better represent the microplastics observed in the marine environment which have been eroded and broken down by friction and UV radiation. Larger particles were also isolated from mussels in the field survey than those used in the laboratory exposures. An investigation should be performed to determine how the larger size particles effect mussel physiology.

When performing future laboratory exposures to assess the effect of microplastics on mussels, additional biomarkers should be included. Future studies investigating the effects of organic emerging contaminants sorbed to microplastics on mussels should include behavioural assessments including valve opening and movement behaviours. This may indicate why in the current study, no effect on the physiological biomarkers was observed when the microplastics were present with triclosan. The biochemical impacts of triclosan on mussels should be further investigated using a wider range of enzyme activity and oxidative damage assessments. These biochemical assays should also incorporate digestive gland tissue to determine if there is a difference in the biochemical impacts in this organ compared with gills.

To understand the impact of organic contaminants sorbed to microplastics on marine organisms, further research is required to determine which hydrophobic contaminants accumulate in microplastics in the New Zealand marine environment and at what concentration. This may involve collecting plastics from the ocean or estuaries or placing plastics in cages to measure

accumulation of the compounds over time. For example, Frias et al. (2010) collected plastics off the Portuguese coast and reported polyethylene and polypropylene microplastics with polyaromatic hydrocarbons including pyrene, phenanthrene and fluoranthene and a range of polychlorinated biphenyls sorbed to the particles. It would be valuable to have some targeted New Zealand results to allow for more environmentally relevant exposures.

This research illustrates that green-lipped mussels interact with microplastics in the New Zealand marine environment and that further research is required to gain better understanding of microplastic pollution in New Zealand. Microplastics have the potential to cause adverse effects on mussel physiology and provide transport pathways from hydrophobic contaminants so a more in depth understanding of microplastic contamination is required.

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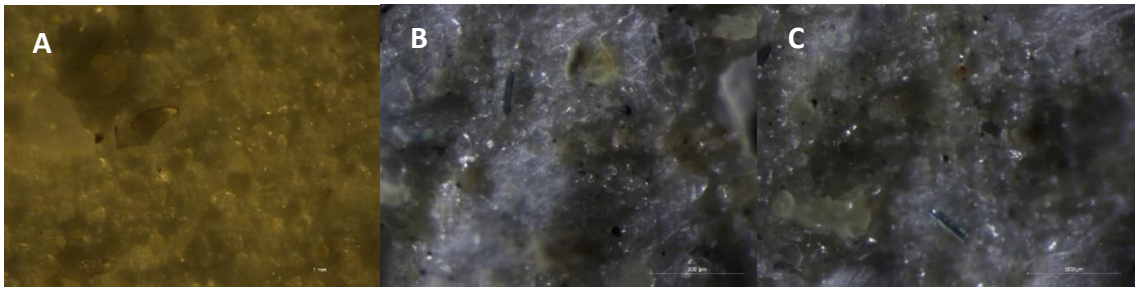
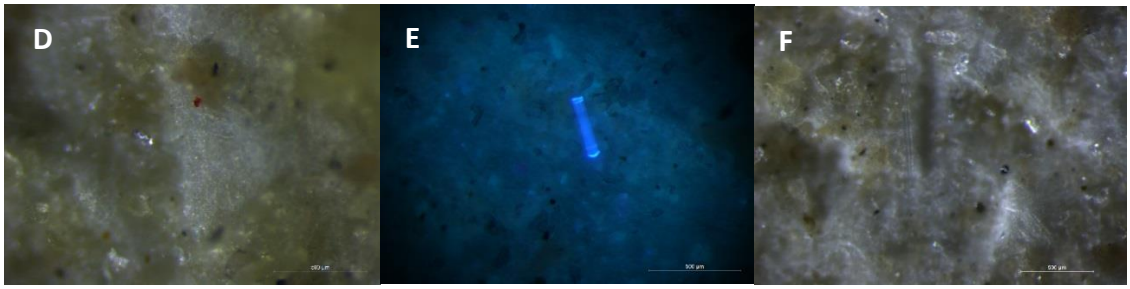
7. Appendices

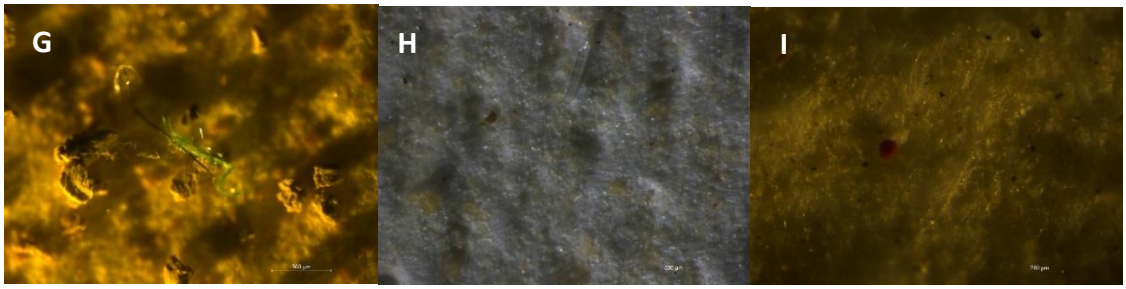
1. Microplastics isolated in the National mussel sample survey



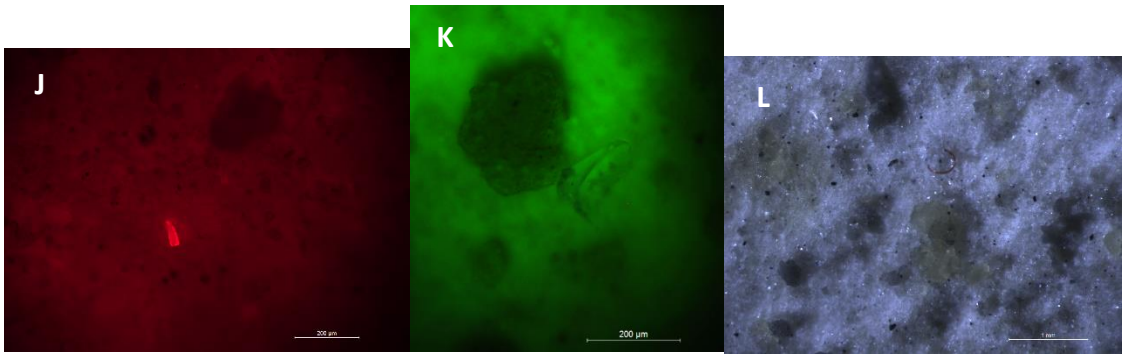
A = Mount Maunganui; B and C = Dunedin



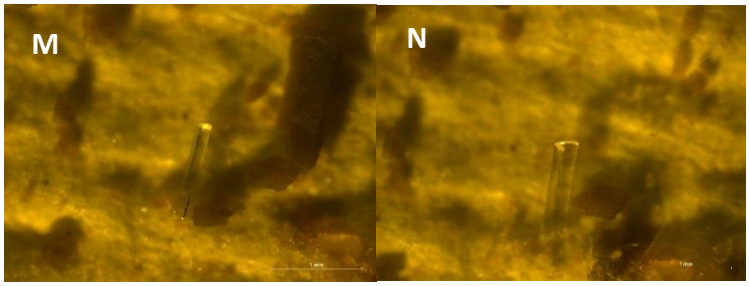
D, E and F = Dunedin



G = Napier; H = Bay of Islands; I = New Plymouth

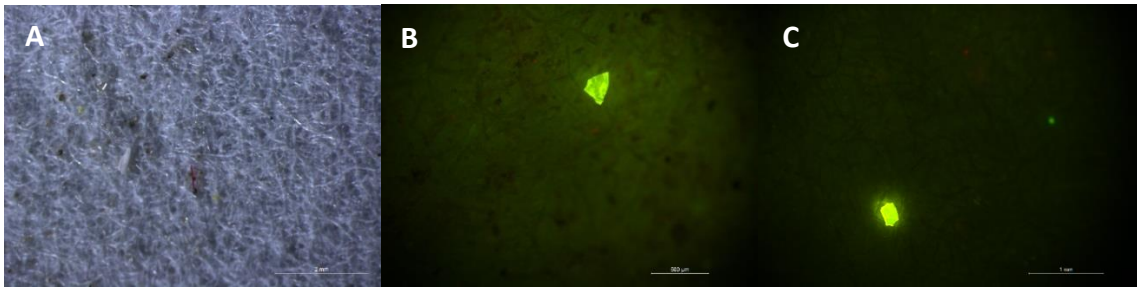


J = New Plymouth; K = Port Underwood; L = Westport

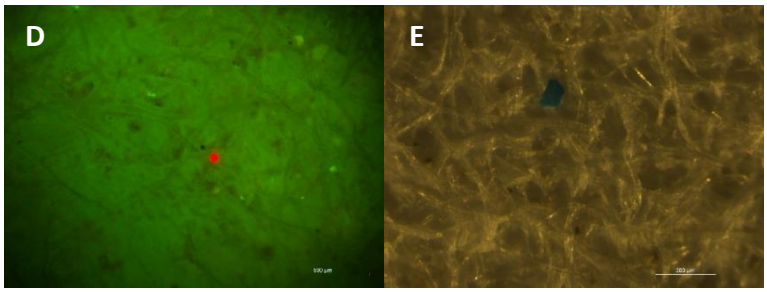


M and N = Wellington Harbour

2. Microplastics isolated in the Canterbury surface water survey

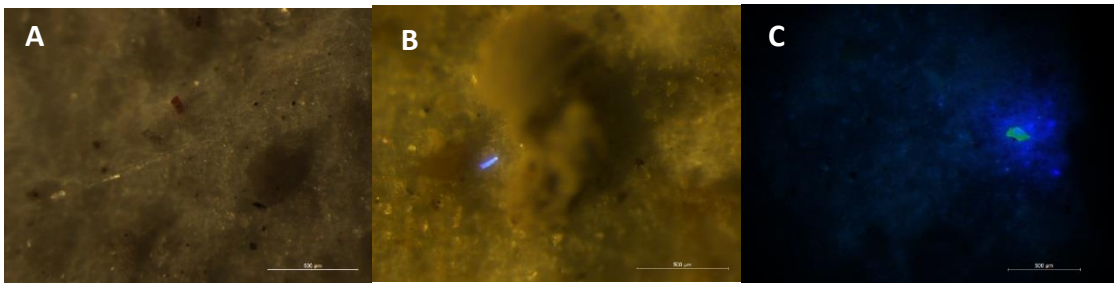


A = Avon-Heathcote Estuary; B = Damon's Bay; C = Lyttelton Port

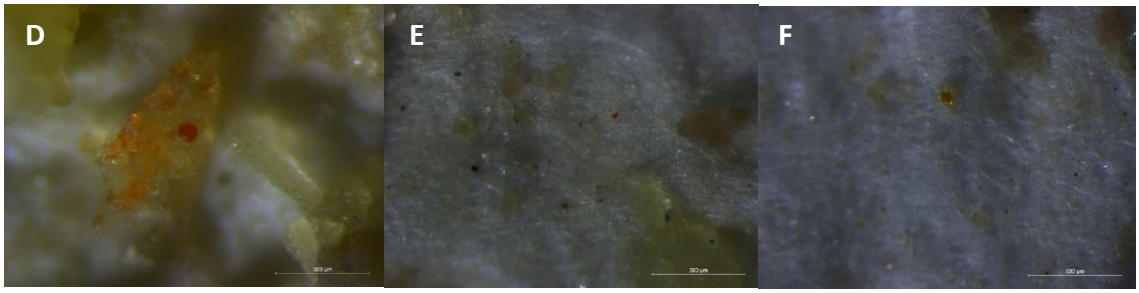


D = Pigeon Bay; E = Lyttelton Harbour

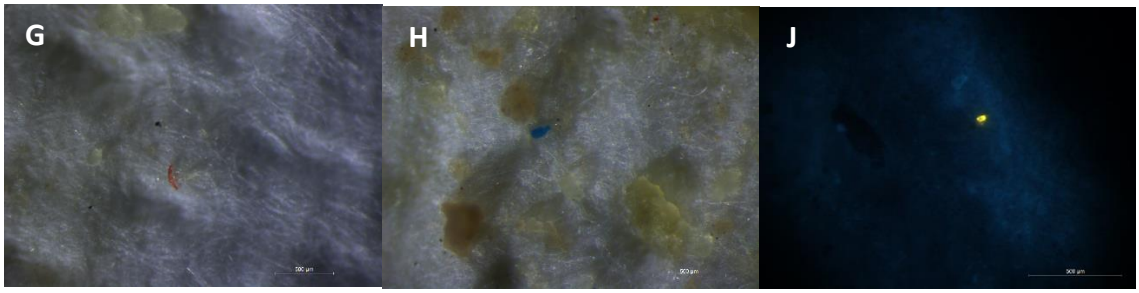
3. Mussels isolated in the Canterbury mussel survey



A and B = Avon Heathcote Estuary; C = Little Akaloa



D = Lyttelton Harbour; E and F = Akaroa Harbour



G and H = Akaroa Harbour; H = Damon's Bay

4. Summary of statistics for mussel and water field survey

ANOVA comparing microplastic per mussel concentrations for each location.

ANOVA			
	F	**df**	**p-value**
National mussel survey	1.102	7	0.4076
Canterbury mussel survey	0.4286	7	0.8702
Canterbury water survey	1.8393	7	0.1481

National mussel survey data analysis

Welch t-test			
Categories	**t**	**df**	**p-value**
South Island vs North Island	-0.87053	3.4014	0.4411
Urban vs Rural	1.2232	4.8903	0.2769
Harbour/Estuary vs Coastal	-0.60486	5.0582	0.5714

5. Summary of statistics comparing control 1 and control 2.

Analysis	t	df	p-value
Byssus thread production	-1.6459	6.7317	0.1455
Clearance rate	-0.26147	5.9955	0.8025
Oxygen respiration	-1.6492	7.0356	0.1429
Glutathione-S-transferase activity	0.49173	6.3489	0.6395
Lipid peroxidation	-0.33663	7.053	0.7462
Superoxide dismutase activity	1.4843	8.1081	0.1755

6. Concentration of triclosan in water samples

All other treatments were below detection limits.

	Concentration (mg L^{-1})			
Treatment	**Triclosan**		**Triclosan and microplastics**	
Batch	**0h**	**48h**	**0h**	**48h**
1	0.306	< LOD	0.223	0.032
2	0.164	< LOD	0.098	< LOD
3	0.181	< LOD	0.047	< LOD
4	0.162	< LOD	0.105	< LOD
5	0.153	< LOD	0.079	0.034
6	0.23	< LOD	0.04	0.035
Average	0.199	< LOD	0.099	0.017

Spike recoveries from sea water samples.

	Recoveries (%)	
Batch	**Low spike**	**High spike**
1	99.0	96.4
2	100.8	86.2
3	98.3	94.0
4	103.6	87.3
5	94.3	83.0
6	92.8	102.5
Average	98.1	91.6

7. Triclosan concentration in mussel tissue

All other treatments (C1, C2 and MP) were below detection limits.

		Concentration (µg g^{-1} (dw))		Concentration (µg mg^{-1} lipid)	
Treatment	**Replicate**	**Triclosan**	**Methyl-triclosan**	**Triclosan**	**Methyl-triclosan**
TCS	1	960.9	67.3	24.7	1.73
	2	1182	101.6	25.6	2.62
	3	1123	121.4	16.7	1.44
	4	1012	84.7	18.0	1.94
	5	882.8	89.6	21.6	1.81
	Average	1032	92.9	20.8	1.90
MP_{TCS}	1	953.3	86.3	20.1	1.82
	2	1738	153.4	22.3	1.97
	3	3717	531.0	57.1	7.30
	4	2787	333.7	51.0	6.11
	5	1911	155.4	27.6	2.25
	6	1299	124.7	26.5	2.54
	Average	2067	230.7	34.1	3.66

	Recovery of ^{13}C-triclosan surrogate, using comparative standard (%)					
			Replicate			
Sample	**1**	**2**	**3**	**4**	**5**	**6**
C1	74.4	121.4	-	73.2	68.2	93.4
C2	92.1	91.2	94.6	96.3	67.0	78.8
MP	117.0	99.7	106.0	101.0	92.6	68.4
TCS	80.4	103.4	76.3	97.4	96.9	95.5
TCS-B	83.3	121.3	110.6	92.9	72.5	114.9
MP_{TCS}	104.3	93.2	114.8	100.7	110.9	87.3

\- *= not recovered*

Mussel tissue spike recoveries

	Recovery of ^{13}C-triclosan, using comparative standard (%)		
	Analysis batch		
	1	**2**	**3**
Solvent blank	122.7	84.3	113.5
Solvent spike	101.9	110.7	110.6
Mussel blank	87.9	94.0	83.9
Mussel spike	90.1	86.5	84.0

	Recovery of triclosan, using comparative standard (%)		
	Analysis batch		
	1	**2**	**3**
Mussel spike	82.7	87.0	55.5

Mussel tissue duplicate extractions and injections

	Repeat extractions	Repeat injections
Analysis Batch	**RSD (%)**	**RSD (%)**
1	0.4	4.6
2	5.0	3.0
3	0.7	0.6
4	5.1	1.0

RSD = Relative standard deviation

8. Microplastic concentration in mussel tissue following acute exposure

Control 1 mussel digests

Replicate	Undigested tissue per g tissue (g g^{-1} (ww))
1	0.0065
2	0.0101
3	0.0079
4	0.0087
5	0.0095
6	0.0108
Average	0.0089

Microplastic concentration in mussel tissue

		Microplastic concentration in mussel tissue		
Treatment	**Rep**	**Mass microplastics per gram tissue (g^{-1} tissue(ww))**	**Mass microplastics per mussel (g $mussel^{-1}$)**	**Beads per mussel ($mussel^{-1}$)**
MP	1	0.0012	0.0176	4.7E+05
	2	0.0038	0.0602	1.6E+06
	3	0.0049	0.0503	1.3E+06
	4	0.0063	0.0929	2.5E+06
	5	0.0088	0.1367	3.7E+06
	6	0.0088	0.0980	2.6E+06
	Average	0.0056	0.0759	2.0E+06
MP_{TCS}	1	0.0033	0.0505	1.3E+06
	2	0.0037	0.0546	1.5E+06
	3	0.0036	0.0419	1.1E+06
	4	0.0060	0.0969	2.6E+06
	5	0.0030	0.0312	8.3E+05
	6	0.0006	0.0070	1.9E+05
	Average	0.0033	0.0470	1.3E+06

Summary of statistics for microplastic concentration in mussel tissue

Welch t-test - two sided assuming unequal variance			
	t	df	p
Microbeads per gram tissue (mg g^{-1} (ww))	1.617	8.034	0.1444
Number of microbeads (1000) per mussel	1.3788	9.0262	0.2012
Number of microbeads (1000) per gram tissue (g^{-1} (ww))	1.3632	8.9753	0.206

9. Results from physiological biomarker analysis

Outliers are excluded from the dataset as determined by Grubbs test.

	Clearance rate (L g^{-1} (dw) h^{-1})			
Replicate	**Control**	**MP**	**TCS**	**MP_{TCS}**
1	0.294	0.080	0.083	0.186
2	0.288	0.079	0.211	0.085
3	0.204	0.191	0.238	0.291
4	0.185	0.210	0.249	0.251
Average	0.243	0.140	0.195	0.204

	Respiration rate (µmol O_2 g^{-1} (dw) h^{-1})			
Replicate	**Control**	**MP**	**TCS**	**MP_{TCS}**
1	23.68	3.975	17.40	22.62
2	26.84	9.771	23.92	17.65
3	21.20	7.821	9.246	11.67
4	18.43	12.98	19.18	8.226
5	20.75	22.15	15.87	37.01
6	15.46	17.32	20.80	19.61
Average	21.06	12.34	17.74	19.46

	Byssus production (threads g^{-1} (dw))			
Replicate	**Control**	**MP**	**TCS**	**MP_{TCS}**
1	5.848	3.528	4.739	3.138
2	14.67	4.076	4.447	9.640
3	13.94	2.896	2.276	4.257
4	8.154	3.741	8.911	2.200
5	8.675	2.907	8.292	4.019
6	9.535	2.801	6.567	11.76
Average	10.137	3.325	5.872	5.837

10. Results from biochemical biomarker analysis

Outliers are excluded from the dataset as determined by Grubbs test.

	Lipid peroxidation (µmol MDA mg protein^{-1})			
Replicate	**Control**	**MP**	**TCS**	**MP_{TCS}**
1	0.00562	0.01379	0.01121	0.01145
2	0.01414	0.01124	0.01563	0.01754
3	0.00757	0.00823	0.01241	0.01062
4	0.01041	0.00890	0.01923	0.01997
5	0.00885	0.01402	0.01821	0.01422
6	0.00611	0.00939	0.01067	
Average	0.00878	0.01092	0.01456	0.01476

	SOD activity (U mg protein^{-1})			
Replicate	**Control**	**MP**	**TCS**	**MP_{TCS}**
1	0.700	0.546	0.840	4.607
2	0.397	1.546	1.489	3.217
3	1.106	1.145	0.734	4.196
4	0.882	1.869	0.780	0.679
5	1.592		2.055	1.853
6	1.260		3.000	
Average	0.989	1.277	1.483	2.910

	GST activity (µmol min^{-1} mg protein^{-1})			
Replicate	**Control**	**MP**	**TCS**	**MP_{TCS}**
1	0.410	2.000	1.802	0.308
2	0.458	0.869	1.431	1.251
3	2.496	1.349	1.030	1.329
4	2.393	1.786	0.584	0.430
5	1.986	0.648	2.091	0.336
6	2.050	1.402	0.751	0.811
Average	1.632	1.342	1.281	0.744

11. Summary of statistics for biomarker analysis

ANOVA			
	F	**df**	**p-value**
Byssus production	5.75	3	0.00527*
Clearance rate	1.305	4	0.313
Lipid peroxidation	6.3823	4	0.001315*
SOD	3.831	3	0.02902*
GST	1.86	4	0.15

** = significant result ($p \leq 0.05$)*
Outliers were removed according to Grubbs test

Welch t-test - two sided assuming unequal variance			
	t	**df**	**p-value**
Oxygen respiration t test (C2 and MP)	2.5156	5.9701	0.04575*
GST (C2 and MP_{TCS})	2.0631	7.2321	0.07672

** = significant result ($p \leq 0.05$)*
Outliers were removed according to Grubbs test