Microplastics for lunch? Understanding the effects of ingesting a range of microplastics on New Zealand triplefin fish health

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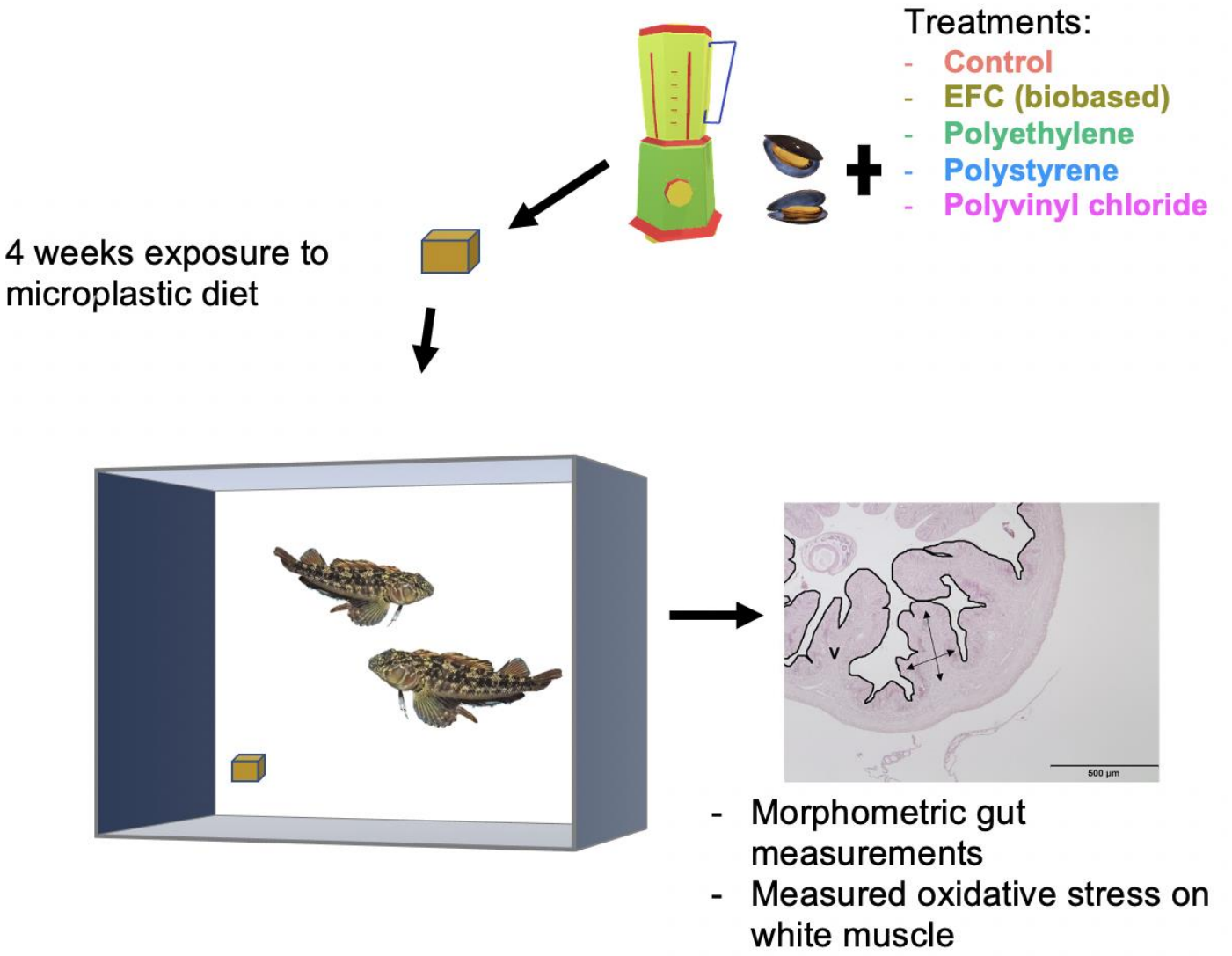
Abstract

Mismanaged plastic waste has resulted in the global accumulation of plastic pollution in the marine environment and consequently, accumulation of microplastics (MP). Some of the most prominent MP types in the marine environment are polyethylene (PE), polystyrene (PS), polyvinyl chloride (PVC). Attempts to mitigate this anthropogenic pollutant have resulted in the increased production of biodegradable plastics (biopolymers). However, there is limited knowledge of the effects these can have once ingested. Multiple studies have looked at the effects of ingesting one or two MP types, yet variation in physical and chemical properties of these different plastic types can cause a variety of adverse effects. Therefore, the primary aim of this study was to identify how a range of MP types affect New Zealand triplefin fish health and condition, specifically looking at changes in gut-morphology and oxidative stress. To address this aim, a 28-day feeding experiment exposing triplefins (*Forsterygion capito*) to either a biodegradable edible film coating (EFC), PE, PS or PVC MP was undertaken. Changes in gut-morphology were examined by measuring villi characteristics such as height, width, and surface area. Further, goblet cell coverage was quantified to understand effects on mucus layer coverage in the gut. Triplefins from all MP diets showed signs of mechanical damage with decreased villus height and width, decreased surface area, and reduced goblet cell abundance however, the magnitude of damage varied dependent on MP type. Ingestion of PVC MP incurred the most mechanical damage as evidenced by a significant reduction in villi surface area. Changes in oxidative stress was addressed by measuring antioxidant enzyme activity and oxidative damage biomarkers in the white muscle. A clear physiological cost of MP ingestion was demonstrated by an increased antioxidant response and subsequent oxidative damage for EFC, PS and PVC treated fish. This was most apparent in PVC treated fish where oxidative damage biomarkers increased more than 7-fold compared to control fish. Triplefins retained relatively good liver condition suggesting resource allocation due to stress. Although the oxidative effects were not reflected in the gut-morphology for EFC, PE and PS MPs, the significant change in oxidative damage highlights the importance of investigating a range of responses. Due to the continual increase in marine plastic pollution, understanding how organisms are

directly affected by these pollutants can providing insight into which plastics may be more problematic and influence future management.



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Chapter 1: *General introduction*

1.1 Plastics

Plastic is one of the most widely consumed resources. In 2019, global plastic production was estimated at 368 million tonnes (PlasticsEurope 2020). Plastic is a synthetic polymer and is comprised of large molecules with numerous subunits (Geyer et al., 2017). The first true synthetic polymer was created in 1903 called Bakelite which was commonly used for old telephones. (Amirkhanian, 2020; Andrady, 2004; Ashby, 2013). The first modern plastic developed was polyvinyl chloride (PVC). Friedrich Klatte polymerised vinyl-chloride to make PVC by heating it with sunlight in 1912. This, however, was not deemed a useable material due to a rapid degradation rate. As technology improved, so did the ability to produce higher quality synthetic polymers (Mulder & Knot, 2001). In 1930, the first PVC products entered the market for items including cable insulation and textiles such as raincoats. Today, PVC is the second most widely used plastic across the globe and has replaced the use of many natural fibres. Common uses of this material include packaging, medical devices (e.g., blood transfusion bags and tubing), plumbing and construction (Andrady, 2004).

Modern PVC is plasticised with di(2- ethylhexyl) phtalate (DEHP), a chemical additive used to improve durability and stability of the plastic. DEHP is not chemically bound to PVC and can leach into the environment (Andrady, 2004). For example, DEHP has been found to leach into aquatic environments (Li et al., 2015), or become airborne, for example in construction sites or PVC plants (Fong et al., 2014; Shinohara & Uchino, 2020). DEHP is a known carcinogen and exposure can lead to endocrine disruption (Adeogun et al., 2018; Manikkam et al., 2013), reduced growth (Zanotelli et al., 2010), and increased risk of cancer (Crobeddu et al., 2019). In rodents, DEHP exposure can accelerate puberty, reduce fertility (Rattan et al., 2018) and cause ovarian and testicular disease (Manikkam et al., 2013). DEHP exposure has been documented to lead to reduced growth in guppy fish (*Poecilia reticulata)* (Zanotelli et al., 2010) and alterations to male gonads in African sharptooth catfish (*Clarias garipinus*). Such alterations included distortion of seminiferous tubes and the production of ovotestis (Adeogun et al., 2018). Further, studies have demonstrated increased cancer risk in

humans (Crobeddu et al., 2019), especially following long-term indoor exposure (Miao et al., 2017).

Following PVC, polyethylene (PE) was developed by the Imperial Chemical Industries in 1937 and is now the most used plastic globally. PE was accidentally discovered by using ethylene to test an apparatus. Here, there was a leak in the apparatus where oxygen entered and left behind a white waxy solid. This product was investigated further and proved to have good chemical resistance and insulation properties (Ronca, 2017). This material is today's low-density polyethylene (LDPE). Other types of PE are high-density polyethylene (HDPE) and linear low-density polyethylene (LLDPE). Generally, most milk bottles and food containers are made of HDPE. Some common LDPE items are disposable gloves, general purpose packaging and pharmaceutical bottles. Items like heavy duty bags used for rubbish, cling wrap and many food packaging's are made from LLDPE. Most PE is created using ethylene produced by the petrochemical industry. This is via "cracking," a process which breaks down high molecular weight petroleum into gasoline. The waste product of this is ethylene which is then polymerised through a gas-phase process (Andrady, 2004). The early 1930's also saw the development of polystyrene (PS) (Andrady, 2004). PS is common in two forms that are both widely used by consumers. Clear polystyrene is often very hard and used for items such as pens, rulers and hard DVD cases to name a few. It is easily formed and cheap to produce (Ashby, 2013). Expanded foam PS is largely seen in packaging and the use of disposable foam cups (Khaksar & Ghazi-Khansari, 2009). These fossil-based plastics make up the majority of plastics and none are biodegradable (Geyer et al. 2017).

1.2 Plastics in the environment

Owing to the large production volume of plastic and the difficulties in disposing of it, plastic pollution is a growing environmental concern as the global amount of mismanaged plastic waste increases. Meijer et al., (2021) estimated 67.5 million metric tonnes of mismanaged plastic waste (MMPW) is generated annually. However, the rate of MMPW generation depends on waste management practises in a given location. For example, in developing countries, waste management can be more challenging. In Africa, nearly 57% of plastic waste is not collected by waste

management and instead is littered or burned (Gourmelon 2015). However, even in countries with waste management schemes such as USA, plastic pollution is an issue. For example, in 2012, it is reported that the US recycled only 9% of its plastic waste (Geyer et al. 2017) with the remainder ending up in landfills or in the environment

Around 91% of mismanaged plastic waste is transported by rivers to the ocean (Lebreton & Andrady 2019; Li et al. 2020). Van Emmerik et al. (2019) estimated a total of 2100 tonnes of plastic waste is transported from land to sea annually from all Jakarta rivers and canals. In Vietnam, it is estimated that between 7,500 – 13,700 tons of plastic enter the South China sea from the Saigon River annually (Lebreton et al., 2017). Of the top 122 polluting rivers globally, 103 are in Asia, eight in South and Central America, eight in Africa and one in Europe (Fig. 2) (Lebreton et al., 2017). In 2019, the greatest recorded amount of plastic waste entering the marine environment originated from Malaysia, Thailand, Vietnam, Philippines and Indonesia. Interestingly, these countries also receive 58% of New Zealand's plastic waste (PW) exports (Farrelly & Green, 2020). From 2018, China banned many PW imports including household PW (Kojima, 2020). With China previously being NZ's main PW export market and, not having the domestic capacity to recycle the amounted plastic in NZ, exporting has been diverted largely to Southeast Asia. Due to fast economic growth, the demand for plastic in these countries often exceeds their waste management ability (Beattie, 2019).

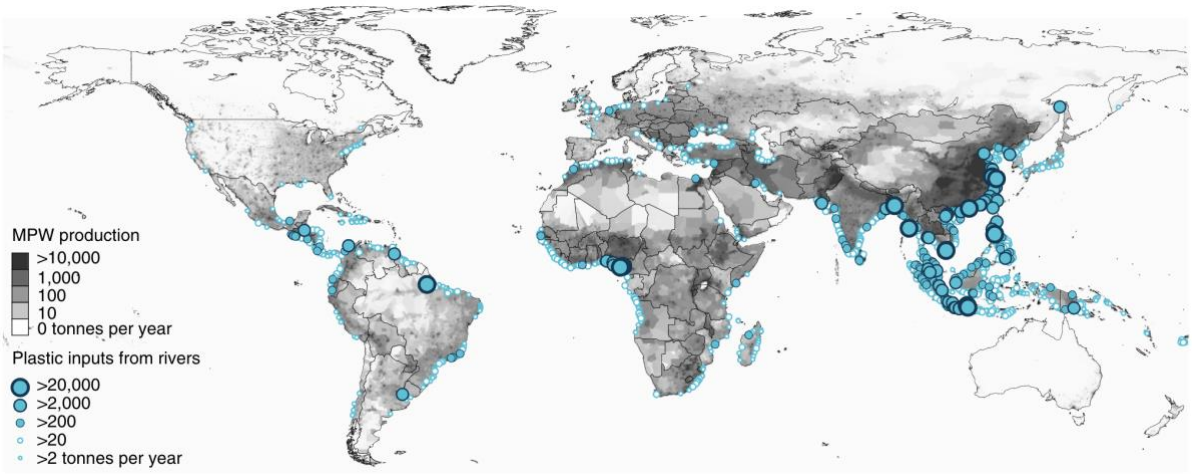


Figure 2: Taken from Lebreton et al., (2017). Shows the global mass of river plastic flowing into oceans in tonnes per year. River contribution data taken from individual watershed characteristics, mismanaged plastic waste (MPW) production per country and monthly averaged runoff.

Given the prevalence of plastic pollution, it is unsurprising that plastic is now found throughout all marine environments, including previously thought of pristine environments such as the polar regions and the deep sea (Bergmann et al. 2017). Lower-density plastics (e.g., PE) are prominent in the surface waters and higher-density plastics (e.g., PVC) often sink and are common in ocean and beach sediments (Schwarz et al., 2019). Surface currents transport plastic particles where they can accumulate in subtropical gyres. The South Pacific gyre has accumulated an average mass of 26,898 particles per km^{-2} (Eriksen et al., 2013) and globally, there is an estimated 250 million tonnes of plastic in the ocean at any one point in time (Eriksson et al. 2014).

1.3 Plastic alternatives – biodegradable biopolymers

Given the threat that increased plastic pollution poses to marine environments (sections 1.4, 1.5), there have been efforts into finding new plastic alternatives, such as biopolymers. Biopolymers are created using material from living organisms e.g., polysaccharides, proteins, or derivatives from renewable bio-based monomers such as polylactic acid (PLA). The most common plant source used is starch, a naturally occurring polysaccharide (Kuddus & Roohi, 2021). Many manufacturers market their products as degradable. This is a loose term which can mean only partially degradable as they are often a blend of starch and LDPE (Dorairaj, 1988). Producing these biopolymers can put pressure on other resources with their high water and land use (Fig. 3). There has been research into plastic alternatives such as edible film coatings (EFC). This bio-based food packaging could be an important way to reduce consumer plastic waste. Sustainable, accessible and 100% biodegradable plastics could change our current “plastic world” situation.

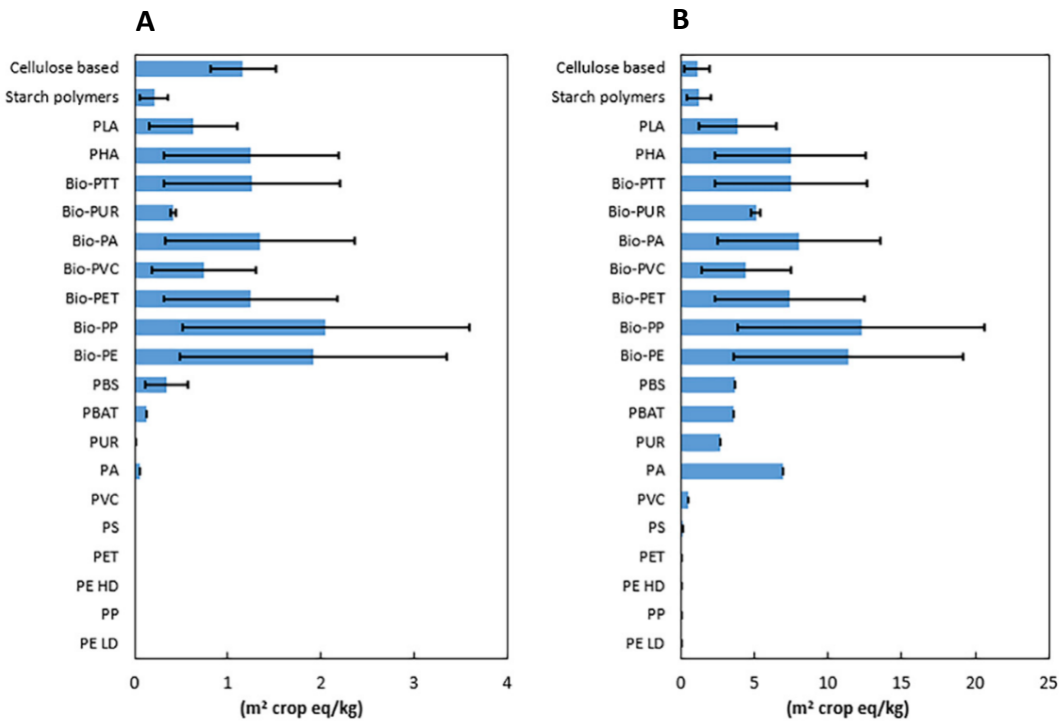


Figure 3: Depicts land use (A) and water use (B) for plastic production. Figure highlights the greater resource use for biobased plastics over fossil based. Full bars show means, and error bars show maximum and minimum levels. Taken from Brigzon et al. (2020).

1.4 Plastic degradation

The accumulation of plastic is driven by its persistence in the environment as degradation rates are slow. This is due to plastics stability and durability, which is what makes it a valuable material (Li et al. 2017). In the marine environment, there are four main mechanisms by which plastic degrades. Seawater is constantly enacting hydraulic degradation while simultaneously, photodegradation occurs from solar radiation. This weakens the structure of the plastic, allowing oxygen atoms to enter the plastic via thermo-oxidative degradation. The combined effects of these mechanisms make the plastic brittle and fragment. The polymer chains of the fragmented pieces have a lowered molecular weight which then allows microorganisms to slowly metabolise them via biodegradation (Fig. 4). This process can take a minimum of 50 years (Webb et al., 2012).

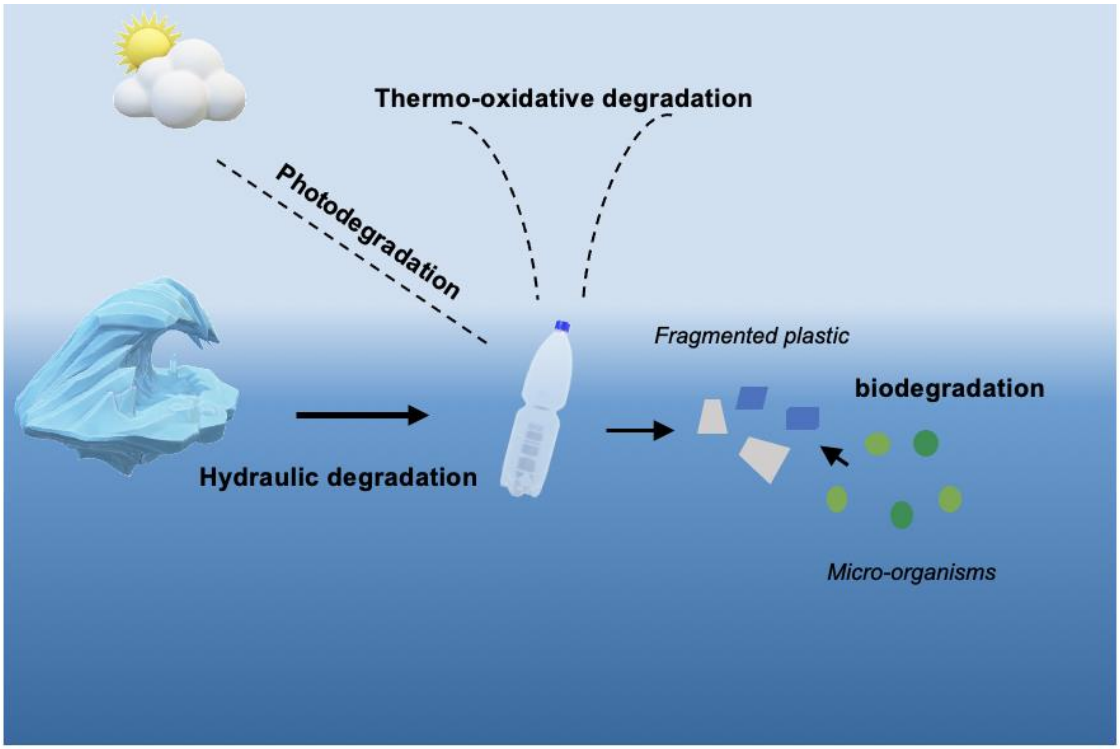


Figure 4: Diagram depicting plastic degradation process in a marine environment.

1.5 Macroplastics

Plastic particles greater than 5mm are termed macroplastics. Common oceanic macroplastics include packaging, bottles, discarded/broken fishing gear and plastic bags. For wildlife, these pollutants are threatening as a variety of taxa frequently become entangled such as cetaceans (Fossi et al., 2018), pinnipeds (Jepsen & de Bruyn, 2019), and reptiles (Barreiros & Raykov, 2014). Globally, 265 bird species have been recorded entangled in plastic, representing 36% of all bird species. Of these species, the majority (83%) were entangled in discarded fishing gear (Ryan, 2018). Entanglement can cause injury and impair mobility, which impacts foraging ability and survival (Gregory, 2009). In New Zealand, entanglement, mostly in trawl netting, is the second-highest call out for the department of conservation (DOC) in the Kaikoura region (Boren et al., 2006). This is especially problematic for NZ fur seals, and can result in serious injury or mortality, influencing population dynamics. For the South American fur seal, it is estimated that even the lowest levels of entanglement can have significant effects on population growth rates (Perez-Venegas et al., 2021). Plastics can also be harmful for marine organisms through ingestion which has been frequently

documented (Hall et al. 2015; Steer et al. 2017; Vendel et al. 2017; for review see Markic et al. 2020)

1.6 Microplastics

Of particular concern is the presence of microplastics (MP). MPs are defined as any plastic particles 5mm or less in size and are termed primary or secondary depending on the origin. Primary MP are direct inputs of small-sized plastics (<5mm) often found in cleansers and toothpastes in the form of microbeads. Secondary MP originate from larger plastic debris that due to biological and mechanical forces (e.g., microbial breakdown or wave action), have fragmented into smaller particles. They also fragment from clothing and textiles during cleaning (Do Sul & Thompson 2014; Law & Thompson 2014). Some of the most prevalent MP in the marine environment are polyvinyl chloride (PVC), polyethelene (PE), and polystyrene (PS) (Doyle et al. 2011; Ramos et al. 2012; Lusher et al. 2013; Do Sul & Thompson 2014; Law & Thomspon 2014: La Daana et al.2017; Bridson et al. 2020). Similar to macroplastics, where they end up is dependent on their density and ocean currents. In New Zealand, a survey of 41 beaches across the country found that plastic debris are the most prevalent of all beached anthropogenic debris, this was followed by metal and wood (van Gool et al., 2021). In Auckland, MP particle concentrations can range from 0-2615 particles per m^2 in beach sediments (Bridson et al., 2020). The first study that recognised MP pollution was Carpenter & Smith (1972) who observed small spherical plastics in plankton tows. Later that year, another study was published by Carpenter et al. (1972) that closely observed these plastics. Here they suggested that perhaps ingestion of the plastics may lead to intestinal blockage in small fish. This is the first time the idea that plastic ingestion could affect fish gut morphology was seen in the literature. This was further supported by the observation that 8 out of 14 sampled species had ingested small plastics (Carpenter et al., 1972). This has since been a growing area of research and in 2004, the term “microplastic” was introduced to describe these particles (Thompson et al. 2004).

Marine organisms frequently interact with plastic debris. Sessile organisms such as barnacles (*Elminius modestus*) have been documented rafting on plastic debris (Barnes & Milner 2005). This can be problematic as a vector for invasive species to

disperse. Polychaetes have been known to use polystyrene debris as habitat as well ingesting plastics due to burrowing (Jang et al., 2018). For example, a single polychaete worm can create hundreds of MP particles whilst burrowing. Owing to their small size, MP are easily ingested by marine organisms through filter feeding, or inadvertently as they can be hard to distinguish from similar-sized organisms. MP ingestion has been observed across multiple taxa (see Table 1), including fish. Lower trophic level fish often directly come into contact with these particles ingesting them either passively or, mistaking them for their natural diet (i.e. phytoplankton and zooplankton). When predators consume these lower-level organisms, they can indirectly consume MPs via trophic transfer, for example, mysids (*Neomysis spp*.) have shown to ingest MP, which are then transferred to a benthic fish (*Myoxocephalus brandti*) that consume them (Hasegawa & Nakaoka, 2021). Further, trophic transfer has been observed in megafauna such as grey seals that have consumed plastic-contaminated mackerel (Nelms et al., 2018). The ability for MPs to transfer through trophic levels has led to increasing concern for MP ingestion in humans as many commercial bivalve species may contain MP and are consumed whole such as NZ green lipped mussels or clams (Webb et al., 2019).

Table 1: Evidence of microplastic ingestion in multiple marine metazoan taxa

Reference	Phylum	Species	Study	Results
Modica et al. 2020	Porifera	79 species of sponges (Arboreal, encrusting and massive)	Examined presence of MP in museum specimens to date MP ingestion in sponges.	Plastic fibres were encountered in 57 out of 79 species sampled. Some from over 20 years ago.
Devereux et al. 2021	Cnidaria Ctenophore	*Cyanea capillata (scyphozoa),* *C. lamarckii (scyphozoa),* *Aurelia aurita (scyphozoa),* *Cosmetira pilosella (hydrozoa),* *Beroe curcumin,* *Pleurobrachia bachei*	Evaluated MP concentrations ingested in 4 species of cnidarian and 2 ctenophore from the North Sea.	All Species had ingested MP. Highest MP concentrations recorded in *B. cucumis* (0.956 ml^{-1}).
Rozaimi et al. 2021	Mollusca, Gastropda	Nerita articulata *Nerita polita* *Chicoreus capucinus*	Evaluated the number of MP ingested in 3 species of tropical estuarine gastropod, Malaysia.	MP detected in all species. Fibres = most prominent. Mean abundance 0.92 particles/g of MP detected in gastropod sample.
Van cauwenberghe & Janssen 2014	Mollusca, Bivalve	*Mytilus edulis* *Crassostrea gigas*	Investigated the presence of MP in bivalves cultured for human consumption in Germany.	M. edulis contained average 0.36 ± 0.07 particles g−1. C. gigas contained average of 0.47 ± 0.16 particles g−1. Annual dietary exposure for European shellfish consumers can amount to 11,000 MP per year.
Oliveira et al. 2020	Mollusca, cephlapoda	*Sepia officinali*	Investigated MP ingestion in wild-caught and cultured cuttlefish.	MP identified in all fish sampled. Fibres were the most common type (≈90% of total count) but were ≈2× higher in relation to body weight in wild vs. cultured animals.
Ianilli et al. 2018	Arthropoda	*Talitrus saltator*	Investigated MP presence in gut contents of sandhoppers sampled from Italy.	Detected PE and PP in all samples. Presumably ingested the particles with natural detritus.
Mohsen et al 2018	Echinodermata	*Apostichopus japonicus*	Examined MP ingestion in farmed sea cucumbers from china.	MP ranged from 0 to 30 particles per intestine. Shows MP widely existed in sea cucumber farms.
Duncan et al. 2019	Chordata	*Chelonia mydas* *Caretta caretta* *Dermochelys coriacea* *Lepidochelys kempii* *Natator depressus* *Eretmochelys imbricata* *Lepidochelys olivacea*	Sampled all 7 species of Marine turtles and from 3 different ocean basins to assess MP ingestion.	Found MP present in all 120 sampled individuals.
Vengas et al. 2020	Chordata	*Arctocephalus philippii* *Otaria byronia* *Arctocephalus australis* *Arctocephalus australis*	Monitored scats of four pinniped (seal & sealion) species for MP ingestion at in Peru and Chile.	Found MP fibres in all studied rookeries). Suggests trophic transfer of MP.
Desforges et al. 2015	Chordata	*Neocalanus cristatus* *Euphausia pacifia*	Examine MP ingestion of the calenoid copepod and a euphasiid from the North Pacific.	Ingesting 1 particle/every 34 copepods and 1/every 17 euphausiids. Evidence of zooplankton mistaking MP for food.
Vendel et al. 2017	Chordata	69 tropical estuary species of fish from 5 feeding guilds	Analysed MP ingestion in 2233 fish from 69 species collected from 2 tropical estuaries in Brazil.	24 out of 69 species had MP in gut contents.

1.7 Effects of microplastic ingestion

It is clear that multiple marine taxa consume MP and it has been well documented that exposure/ingestion can be harmful. Numerous studies have investigated the potential effects of fish ingesting one or two MP types (Table 2, Table 3). For example, Yin et al., (2018) found exposure to PS MP reduced nutrient quality, energy reserve and growth of the Korean rockfish (*Sebastes schlegelii*). Tissues can be affected by PS ingestion as demonstrated by Abarghouei et al., (2021). Here, they found lesions in the intestine, liver and gills of goldfish that had consumed these particles. Other studies have looked at effects of MP ingestion on endocrine disruption which can have follow on effects on reproductive output. Rochman et al., (2014) found that ingesting PE caused a down-regulation of important genes involved in endocrine function like estrogenic receptors and vitellogenin. Further, some studies have investigated biochemical effects such oxidative stress. Using catfish as a model organism, Iheanacho & Odo, (2020) found PVC ingestion induced oxidative stress via decreased antioxidant enzyme activity (superoxide dismutase, catalase and glutathione peroxidase). Consequently, increased lipid peroxides indicated oxidative damage. This leaves an organism more susceptible to pathogens and disease. The range of effects incurred by MP ingestion reduce the condition and thus, fitness of the organism which can affect population dynamics.

There are multiple ways of documenting changes in condition. The liver provides important detoxification and acts as an energy store. If the energy reserve is low, it can be an indicator of stress (Hismayasari et al. 2015). Hepatosomatic index (HSI) is a crude way of measuring the energy reserves within the liver by measuring the ratio of liver to body weight. An organism's reproductive health is another important sign of condition. Gonadosomatic index measures the gonad weight to body weight ratio and indicates the proportion of body tissue that is allocated to gamete production. This is an indicator of reproductive health and stage as generally, gamete size increases with the maturation of the fish, which is correlated with increased fecundity (Islam et al. 2012). Another way to look at direct effects on condition is by using histology. This takes a cross sectional view of tissues and organs which can provide insight into damage and alterations induced by MP exposure (Bernet et al. 1999). Alterations could include lesions and structural cellular changes (Table 2). In addition

to physical changes, chemical changes can also signify damage. An important indicator of toxicity in fishes is oxidative stress. Oxidative stress occurs when the production of reactive oxygen species (ROS) exceeds an organism's ability to remove them from cells and tissues using the antioxidant system (Lushchak, 2016). Biochemical changes associated with oxidative stress such as antioxidant enzyme activity is a useful parameter to document this. Catalase and superoxide dismutase enzymes are the first line of defence against free radicals to avoid oxidative damage. Glutathione peroxidase exists in high concentrations within muscle tissue. It plays an important role by targeting the superoxide anion ($O2^-$) radical and breaking down lipid peroxides (Ighodaro & Akinloye, 2018). Measuring enzymatic activity and oxidative damage markers can provide information on condition at a functional level (Table 3). Organisms respond to changes in condition in a variety of ways and these responses are often linked to each other, which highlights the importance of investigating a range in responses.

Table 2: Histological effects of microplastics ingestion on marine fish.

Reference	Species	Study	Plastic details	Results
Espinosa et al. 2019	European sea bass (*Dicentrarchus labrax* L.)	Seabass were fed different concentrations of PE & PVC for 3 weeks to asses histological changes in liver & intestine.	PE & PVC diet concentrations were Control (0), 100 or 500 mg kg^{-1}.	Fish fed PVC 100mg showed similar morphology to control. PVC 500 mg fish showed <villus thickness, & <goblet cells. PE fish showed >villus height & >goblet cells.
Usman et al. 2021	Javanese medaka (*Oryzias javanicus*)	Exposed to PS for 21 days. Investigated histological alterations in liver, intestine, kidney & brain.	PS beads of 3 different concentrations including 100 µg/L, 500 µg/L & 100 µg/L.	PS exposed groups showed Villi blunting, destruction and inflammation. Liver tissue also showed inflammation. interstitial inflammation, and oedema.
Ahrendt et al. 2020	*Girella laevifrons*	Exposed juvenile intertidal fish PS MP for 45 days to assess alterations in the intestine using histology.	Pellets containing either 0, 0.01 g, or 0.1 g PS plastic per 0.5 g of food.	PS treated fish has PS present in the intestine. All PS fish showed intestinal lesions. PS fish had villi cell loss and crypt cell loss.
Qiao et al. 2019	Zebrafish *(Danio rerio)*	Exposed zebrafish to PS and PP MP for 21 days. Histology was used to see changes in gut morphology.	Exposed to either PS beads, PS fragments or PP fibres.	Fibres caused more intestinal damage than fragments and beads. MP fibres also cause a decline in goblet cells.
Varó et al. 2021	Gilthead sea bream *(Sparus aurata)*	Fish fed 1 of 3 LDPE diets for 30 days. Histology was used to identify intestinal lesions, inflammatory reaction, and epithelial vacuolisation.	Diet 1 = control (0 MP) Diet 2 = 200-500 µm LDPE Diet 3 = 500-100 µm LDPE	Fish fed diets 2 & 3 showed severe intestinal lesions. Inflammatory reaction was observed.

Table 3: Oxidative effects of microplastic ingestion on marine fish

Reference	Species	Study	Plastic details	Results
Wang et al. 2019	Marine medaka (*Oryzias melastigma*)	Exposed fish to PS MP for 60 days. Enzymatic activity of SOD and CAT was determined in intestines, livers, gills, and testis.	Treatments were 0, 2, 20 and 200 µg/L PS MP. Particles were suspended in the water when feeding with brine shrimp.	CAT enzyme in testis and liver were significantly decreased in all PS treated fish. CAT was inhibited in the liver after exposed to 200µg. This also caused an increase of SOD in the gill and intestine.
Solomando et al 2020	Gilthead sea bream *(Sparus aurata)*	9 fish samoled at 0, 30, 60, 90 and 120 days for biochemical analysis. Fish were fed a diet of either MP or no plastic (control) for 90 days + 30 days of no plastic for both.	MP feed was 90% fishmeal and 10% LDPE MP.	CAT, SOD & GPOX enzymes progressively increase in MP group through time until they reach a max at day 90. CAT was significantly higher for MP group at day 90 than the other days.
Espinosa et al. 2019	European sea bass (*Dicentrarchus labrax* L.)	Seabass were fed different concentrations of PE & PVC for 3 weeks to asses changes in antioxidant enzyme activity.	PE & PVC diet concentrations were Control (0), 100 or 500 mg kg^{-1}.	CAT & SOD enzymes significantly decreased in liver of all PE treatments. No significant effect on antioxidant enzymes for PVC treated fish.
Xia et al. 2020	Common carp (*Cyprinus carpio*)	Exposed larvae to 0%, 10%, 20% & 30% PVC MP concentration for 30 & 60 days. SOD, CAT & GPOX activities were analysed in liver, intestine & gills.	Pure PVC particles. The average diameters of the MP were from ~100–200 µm.	After 30 days exposure SOD decreased in liver for all MP groups. After 60 days SOD weakened in all exposure groups for intestine & gills. CAT inhibited in intestines & gills after only 30 days.
Qiao et al. 2019	Zebrafish *(Danio rerio)*	exposed to MP (5 µm beads; 50 µg/L and 500 µg/L) for 21 days and monitored for changes in enzymatic biomarkers.	Pristine PS (5-µm diameter).	CAT & SOD activity increased in both MP groups when compared to control.

1.8 Triplefins - a model animal to evaluate effects of MP in coastal fish

When investigating the effects of MP ingestion, it is important to look at a range of responses. *Forsterygion capito* (New Zealand mottled triplefin) was chosen as a model animal for the purpose of this MSc thesis. *Forsterygion capito* is a small, abundant rocky reef fish found throughout New Zealand's coastline (Feary & Clements, 2006). New Zealand triplefins have an important role in the trophic system. They act as dominant predators on intertidal invertebrates as well as important prey items for larger fish such as sea perch (*Helicolenus percoide*) and blue cod (*Parapercis colias)* (Feary et al., 2009; Wellenreuther et al., 2010). During the breeding season, males typically

remain in the intertidal zone guarding their nests from predators while females are found predominantly in the subtidal zone (Northcott & James, 1996). Triplefins belong to the family *Tripterygiidae.* Species of this same family can be found in the Atlantic, Pacific and Indian oceans (Jawad, 2007; Lin & Hastings, 2013) which may enable us to apply these finding to the broader field. Further, triplefins are an ideal model organism as they have been used extensively in laboratory studies due to their rapid adjustment to aquaria (Feary et al., 2009).

1.9.1 Thesis aims

In Aotearoa New Zealand, the average household consumes 941 plastic bottles and containers per annum. This extrapolates to 1.76 billion items across all NZ (WasteMINZ TAO Forum 2020). Given marine plastic pollution is increasing, it is important to understand the effects of consumption on fish morphology and physiology on fish who consume these particles. It is critical to investigate a range of MP types due to the different chemical and physical properties of differing plastic types. Therefore, the overall aim of this MSc was to identify how a range of MP types affect triplefin overall health. To do this I ran a 28-day feeding experiment where triplefins were exposed to either PE, PS, PVC or a biopolymer (Chapter 2, Chapter 3).

The overarching aim of Chapter 2 was to assess whether exposure to a range of MPs would affect gut morphology. This was undertaken by measuring morphological changes to the villi, the site of nutrient absorption within the gut. Further, I quantified goblet cell coverage to understand effects on mucus layer coverage in the gut. Mechanical damage can occur when abrasive MP, through ingestion, come in contact with the gut structures. I hypothesised that MP ingestion will have a negative effect on triplefin gut morphology due to mechanical damage incurred by plastic ingestion. Further, I expect an increase in the number of goblet cells as an adaptive response to ingesting MP.

The overarching aim of Chapter 3 was to assess whether exposure to a range of MPs induce oxidative stress. This was achieved by measuring antioxidant enzyme activity and oxidative damage biomarkers. ROS species are produced in greater quantities

during periods of stress. An organism's ability to combat this elevation in ROS is dependent on the physiological state of the organism. I hypothesised that MP exposure will cause a negative effect on triplefins antioxidant capacity therefore, resulting in oxidative damage.

Chapter 4 provides an overview of the studies presented in this MSc. Then, the effects of MP ingestion on gut-morphology are discussed, followed by a discussion on the effects of MP ingestion on oxidative biomarkers. Next, Chapter 4 goes into detail of the variation of effects caused by a variety of MP types. The results of the study are then related back to the ecological paradigm of sublethal effects followed by a section on future work. Then finally, an overall conclusion is presented.

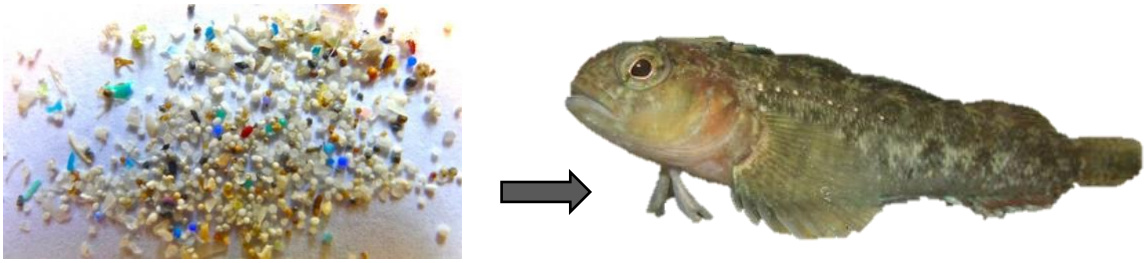
There is to be some repetition expected in the two data chapters as they are intended as two, separate publications.

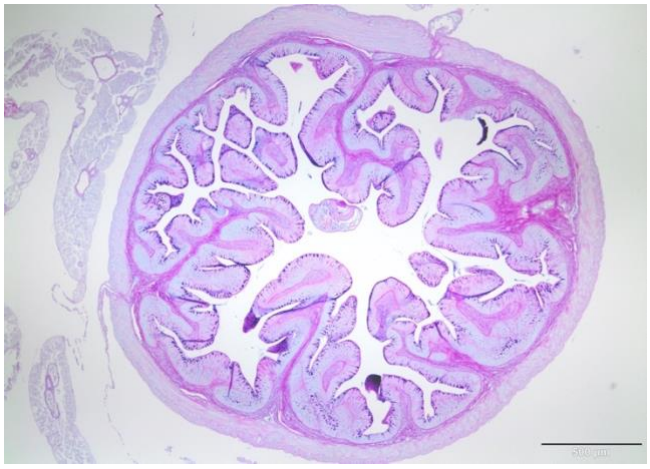
1.9.2 Research roles and responsibilities

This research was supervised by Dr Bridie Allan, Associate Professor Mark Lokman and Associate Professor David Burritt. Formulation of overarching research goals, investigation and formal analyses were carried out by Paige Olmstead. Methodology was adapted in collaboration with Dr Bridie Allan, Associate Professor Mark Lokman, Associate Professor David Burritt and Matthew Downes. Provision of reagents, materials and instrumentation by the Department of Marine Science, Department of Zoology and Department of Botany at the University of Otago. Analysis tools included ImageJ (Fiji extension) (version 2.1.0/1.53C), Microsoft Excel version 16.54 (Excel 2021), R (version 3.6.3).

Chapter 2

Microplastic ingestion affects gut-morphology in New Zealand triplefin fish





2.1 Introduction

A major anthropogenic issue, in the marine environment, is the accumulation of mismanaged plastic waste (MMPW). Weathering and fragmentation of plastic waste has resulted in a global distribution of microplastics (MP) (Gove et al. 2019). Coastal ecosystems are particularly vulnerable due to the high levels of mismanaged plastic waste (Lamb et al. 2018, Kako et al. 2014), which can directly compromise the health of marine ecosystems, with flow-on effects to fisheries and tourism (Lamb et al. 2018). Without improvement of waste management systems, plastic discharged into the marine environment is predicted to increase by an order of magnitude within the decade (Jambeck et al. 2015). To date, most studies investigating the effect of plastic exposure and ingestion have focused on a single plastic type (e.g., polystyrene (PS) or polyethylene (PE)). However, the ingestion of different plastic (e.g., PE, PVC and PS) can affect a number of different pathways and tissues (see Table 2, Chapter 1) as they have different physical and chemical properties (for full breakdown see Chapter 1, section 1.1).

The gastrointestinal tract (GIT) is where multiple processes occur for nutrient absorption in fish. Nutrients are delivered to the intestine (Bakke et al. 2010) which is lined with membranous structures called villi. These structures serve to increase the surface area over which nutrient absorption can occur (approximately 40-fold). Damage or alterations to these villi such as direct rupturing or erosion can reduce nutrient intake and reduce overall body condition and fitness (Jabeen et al. 2018; Lei et al. 2018). Mechanical damage can occur when abrasive MP, through ingestion, come in contact with the gut structures. This can lead to a significant decrease in villus height and width. For example, ingestion of poly vinyl-chloride (PVC) and polyethylene (PE) MP (particles less than 5mm in diameter) by European seabass (*Dicentrarchus labrax*) induced immune system dysfunction in the GIT as well as changes in gut morphology suggestive of gut injury (e.g., decreased villus height and a decrease in goblet cells) (Espinosa et al. 2019). Similarly, Pedà et al. (2016) found structural changes in the intestine of European seabass fed both virgin and polluted PVC MP (polluted PVC were MP deployed in a known polluted harbour for 3 months to represent natural accumulation of marine pollutants in MP). As exposure time increased, so did intestinal damage. After 90 days exposure, 50% of the fish showed

severe mechanical damage with significant loss of the regular intestinal structure. At this level of change, cell functions can be greatly reduced, leading to overall physiological costs.

The epithelial layer of villi is lined with enterocyte cells that absorb nutrients (Fig. 5). MP ingestion has been found to cause vacuolization and necrosis of these cells which is usually incurred by pathogen exposure (Pedà et al. 2016). This can eventually lead to necrosis (cell death) (Henics & Wheatley 1999). An important defence barrier within the GIT is the mucus layer. Goblet cells secrete glycoproteins called mucins which generate the protective mucus layer. This protects the villi epithelium from pathogens, toxins and dietary components that may threaten the intestinal mucosa (innermost layer). When MPs are ingested, it can stimulate a gut adaptive response by increasing the number of goblet cells (Pedà et al. 2016). In other cases, the fish can be so stressed that it is not able to perform normal gut function and therefore have a decrease in goblet cell abundance. Qiao et al. (2019) using zebrafish, found that after ingesting PS MP fibres, goblet cell coverage was significantly reduced. This was thought to be associated with the rough surface of the PS fibres, causing significant epithelial cell damage.

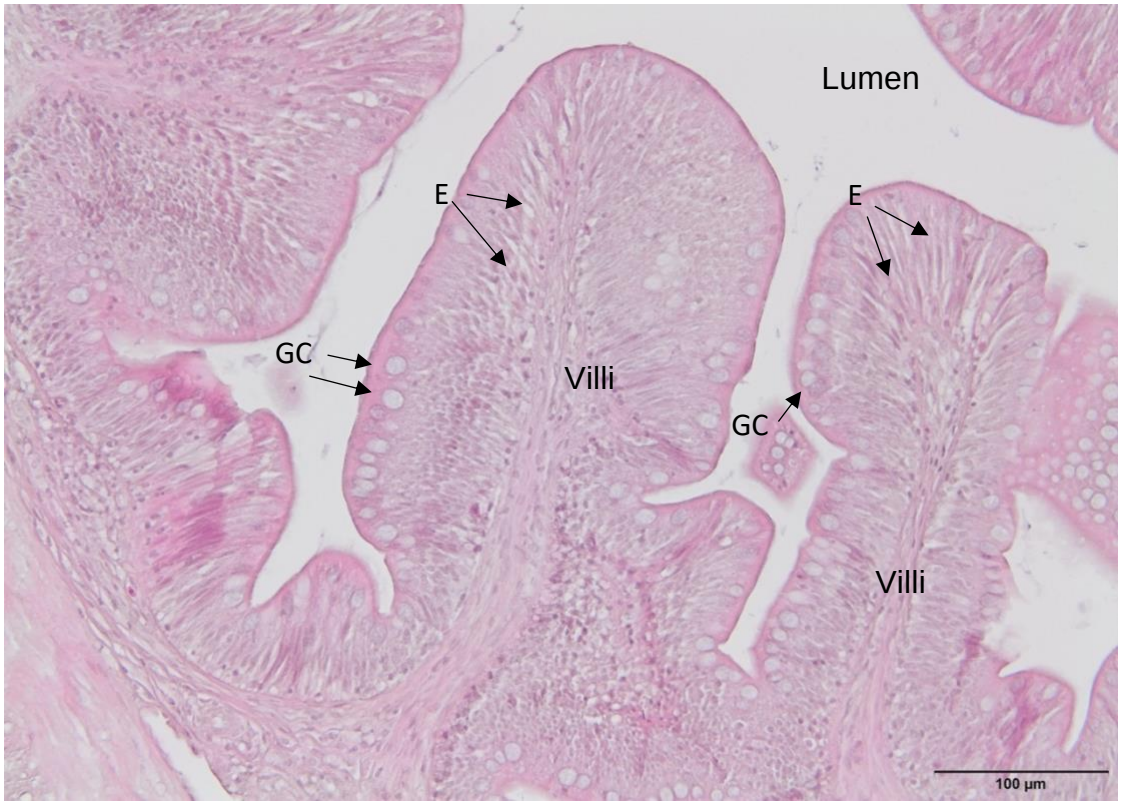


Figure 5: Histological example of triplefin villi structure. Stained with hematoxylin and eosin. “E” arrows indicate enterocytes, “GC” arrows indicate goblet cells.

Similar to humans, gut health is essential for absorption of nutrients required for survival. The human GIT is responsible for digestion/nutrient absorption, defence against diseases and brain signalling. Intestinal damage leads to poor digestion and nutrient deficiency which results in less energy allocated to growth and reproduction. Fish must reach a certain nutritional status to meet their basic metabolic demands before they can put energy into reproduction (Caceres et al., 1994). Villus damage (e.g., erosion, necrosis) observed in MP-treated fish is analogous to the reaction of a human with coeliac disease to gluten ingestion (Tack et al. 2010). Similar to fish, humans have a mucus layer secreted by goblet cells to protect the GIT from pathogens. Figure 6 shows the similarity in villi structure between humans and fish.

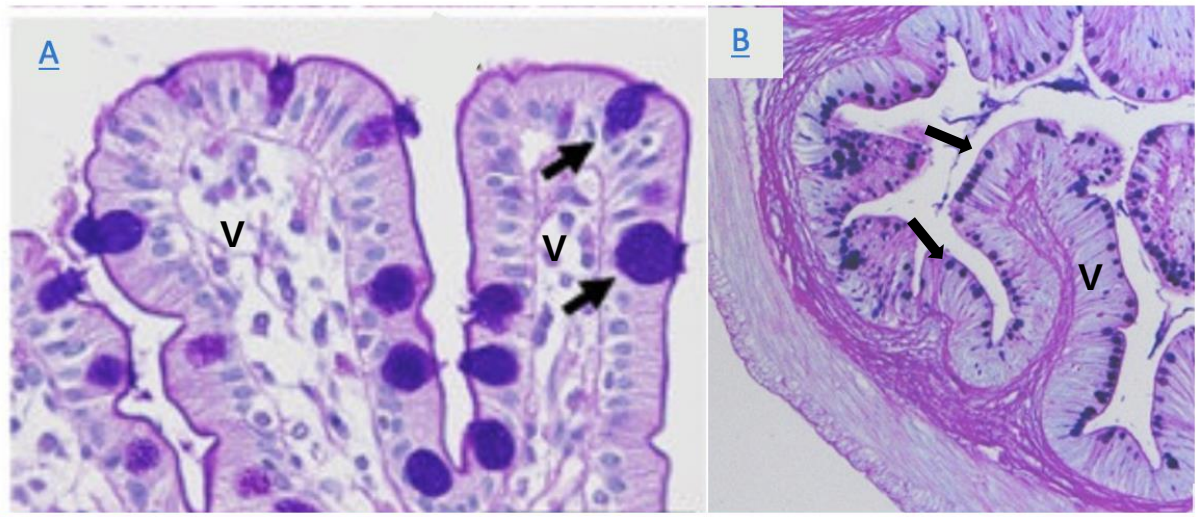


Figure 6: Histology images stained with PAS + Alcian Blue showing villi structure for both human (A) and triplefin fish (Forsterygion capito). Arrows indicate goblet cells, V is villi. Image A taken from Matsubara et al. (2015).

There are a number of different end points that can be used to determine the effect of MP exposure to stressors. For example, histology is a rapid way to see alterations to tissues and organs (Bernet et al., 1999.). This method allows for a cross-sectional view into different tissue types (i.e., GIT) to determine the effects of irritants (i.e., MPs) (Bernet et al. 1999). Measures of condition include gonadosomatic index (GSI) (the proportion of body tissue devoted to gamete production) and hepatosomatic index (HSI) which is a proxy for fish condition (Loret & Planes 2003) and is used as an indicator of energy reserves in the liver (Hismayasari et al. 2015). The liver serves important function for overall health and can be a bioindicator of contaminant exposure due to the liver's detoxification processes. (Critchell & Hoogenboom 2018, Arias et al. 2019).

Given marine plastic pollution is increasing (Farrelly & Green, 2020), it is important to understand the effects consumption may have on the fish that consume these particles. If we are able to understand which polymer types are potentially more harmful than others, we may be able to limit our input of that pollutant. Therefore, the aim of this study was to assess the effects of MP exposure on gut morphology and whether some plastic types are more harmful than others. It is anticipated that MP ingestion will have a negative effect on triplefin gut morphology due to mechanical

damage incurred by plastic ingestion. Further, I expect an increase in the number of goblet cells as an adaptive response to ingesting MP.

2.2 Methods

2.2.1 *Ethics statement*

All work in this study was completed in accordance with the University of Otago Animal Ethics guidelines (AUP 19-70).

2.2.2 *Study Location and Study Species*

To address the above aims, the mottled triplefin (*Forsterygion capito)* was used as a model organism (Chapter 1, section 1.8). Triplefins (Tripterygiidae) are small rocky reef fish that are abundant throughout New Zealand where they have diversified more than anywhere else (Feary & Clements 2006). They are cryptobenthic, opportunistic feeders (Feary et al. 2009) found predominantly in shallower areas under cobbles (Feary & Clements 2006). Small cryptobenthic fish are important prey items in rocky reef ecosystems (Goatley & Brandl 2017). Due to their overwhelming larval abundance, they can account for up to 60% of all consumed reef fish biomass (Brandl et al. 2019). This research was conducted in Portobello on the eastern side of the Otago Peninsula, Dunedin, New Zealand (-45.828172, 170.640949). This area is characterised by a rocky intertidal zone (Fig. 7).

2.2.3 *Animal Collection*

A total of sixty adult, male mottled triplefins were collected using hand nets from the intertidal zone at Back beach, Port Chalmers, New Zealand (Fig. 7) (July 2020). All fish were transferred in insulated bins filled with fresh, aerated sea water to the Portobello Marine Lab (PML) (travel time via boat approximately 5 minutes). Individuals were weighed and placed briefly (10 seconds) into fresh water to remove any parasites (Hadfield & Clayton, 2011).

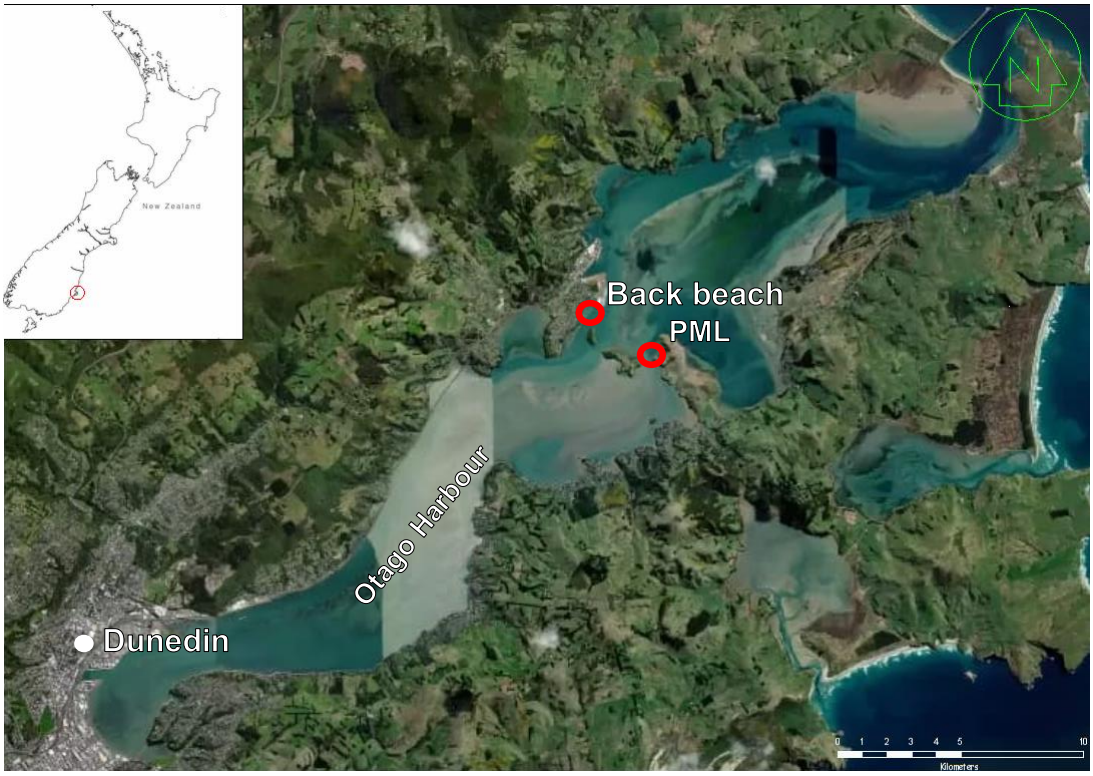


Figure 7: Map depicting research lab and sample collection site in Dunedin, New Zealand.

2.2.4 *Experimental protocol*

A total of 5 treatments including 4 plastic treatments and a control (0% plastic) were used. Plastic treatments were polyvinyl chloride (PVC), polystyrene (PS), polyethylene (PE) and a biodegradable film (biopolymer) or edible food coating (EFC). The plastics treatments were selected based on their prevalence in the marine environment. PVC, PS and PE are the most widely used plastics in the consumer market (Wang et al. 2020). The biodegradable film was produced by the University of Otago food science department. Similarly sized fish were randomly allocated to each treatment tank with two fish per tank. In total, there were 12 replicate fish per treatment housed in 6 x 20L glass tanks per treatment (i.e., 2 fish per tank). These tanks had constant aeration provided by an air stone and the temperature maintained at ambient conditions by a flow through system (Portobello sea temperature, average July range 8.4°C – 10.1°C). The flow rate of tanks was 780ml per minute which provided a complete water change every 30 minutes. All tanks were kept in a temperature control room set to 7am-7pm daylight. Water temperature, flow rate and aeration were monitored daily and recorded. Each tank was blacked out around the sides to prevent fish from other

treatments interacting with each other. Each tank contained 2 terracotta shelters (Fig. 8). During the experimental period two fish jumped out of their tanks and were unable to be replaced therefore, N=58.

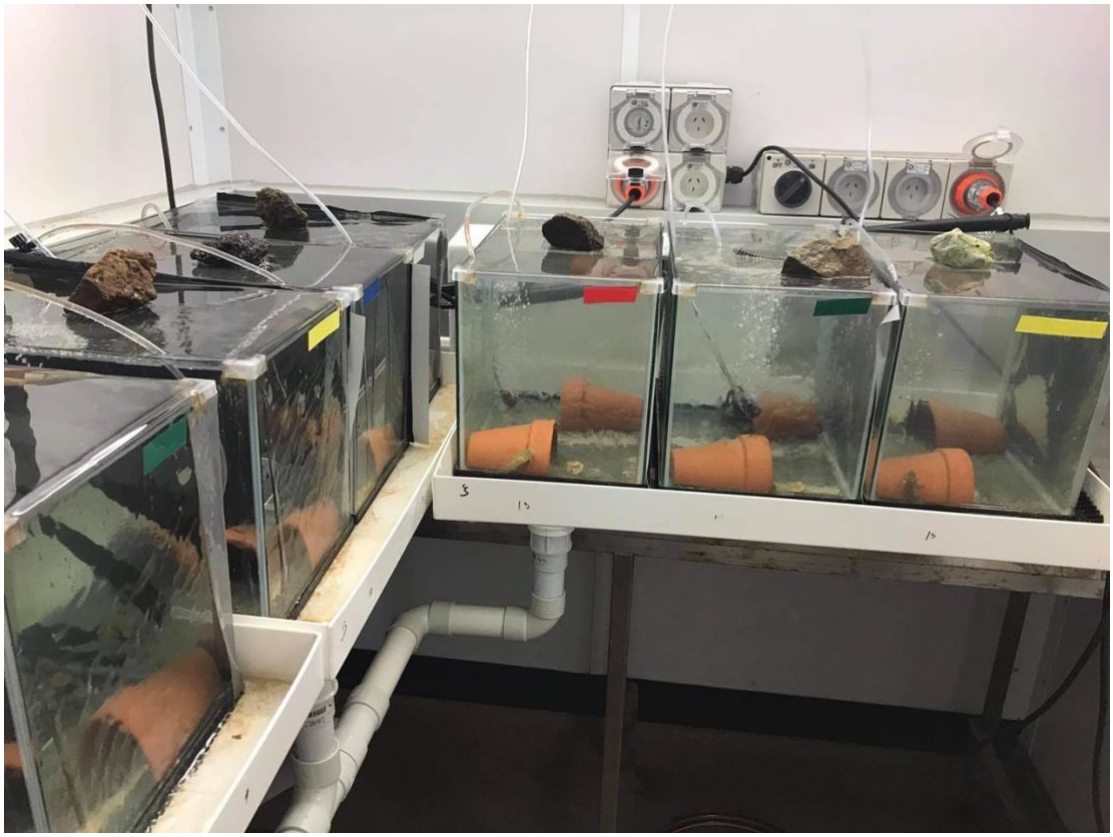


Figure 8: Tank set up for treatments. 2 fish were housed in each tank with an air stone and 2 terracotta pots for shelter.

2.2.5 *Plastic preparation*

The film-forming solution was prepared by mixing stock solutions of corn zein, chitosan, polyvinyl alcohol, and polyethylene glycol to obtain a weight fraction (% w/w) of 0.40, 0.30, 0.10, and 0.20, respectively. This was then stirred for 20 min at 400 rpm (22 °C) and homogenized at 8000 rpm for 2 min (IKA® T 50 ULTRA-TURRAX®, Krackeler Scientific, NY, USA). Following that, using an ultrasonic machine (37 kHz) (Elmasonic S 30 H, Elma Schmidbauer GmbH, Singen, Germany), degassing was carried out for 5 min at 25 ± 3ºC. Next, the solutions were cast on 10 cm × 10 cm polystyrene Petri dishes using a constant rate (~6.8 mg total solids /cm^2) to standardise the film thickness. The drying conditions were kept constant at 20 ± 2ºC

using forced draft (airflow rate v = 0.5 m/s). Dried films were conditioned at 40°C for 12 hours. They were then stored for a minimum of seven days at 22 ± 2°C in a vacuum desiccator at a relative humidity of 53 ± 3 % using magnesium nitrate hexahydrate ($Mg(NO_3)_2 \cdot 6H_2O$). The EFC films were cut into ~ 0.5 cm x 0.5 cm pieces and ground in a mortar and pestle using liquid nitrogen to make the film brittle. This was then sieved to obtain particles of sizes between 600 – 300 µm.

The PS particles were prepared at James Cook University, Australia, using the following method. First, 305 grams of polystyrene was dissolved in one litre of dichloromethane and left to stir overnight using a magnetic stirrer at room temperature in a 2-litre bottle. This solution was then drop cast on watch glasses (50 mm in diameter) and allowed to evaporate in a fumehood for 60 hours to form a thick white membrane. The membranes were dried further in vacuo overnight to form brittle plates of polystyrene. The PS was ground into MP particles using a food processor (NutriBullet, 900 Series) and sieved in the range of 600 – 300µm (Geo-Con).

PE was sourced from PE plastic bags and cut into 0.5 cm x 0.5 cm pieces. The pieces were added into a Warring blender with liquid nitrogen to make the plastic brittle. Next the particles were blended for 2 minutes at the highest speed, followed by sieving to obtain particles of between 600 – 300µm. After each sieving, the grinding process was repeated with particles > 600µm until the desired size was obtained.

The PVC was taken from a PVC pipe. Sawing the pipe produced shavings which were added to a Warring blender. Liquid nitrogen was added and allowed to rest for around 30 seconds. Next the particles were blended for 2 minutes at the highest speed, followed by sieving to obtain particles of between 600 – 300µm. After each sieving, the grinding process was repeated with particles > 600µm until the right size was obtained.

2.2.6 *Food treatments*

The different types of plastic particles were individually added to pulverized fresh green-lipped mussels at a ratio of 2.5% plastic to mussel weight and frozen in 2g feeding blocks using Teflon moulds and stored at -20°C. Green lipped mussels were used as they are easily available, co-exist in Portobello and are consumed by NZ triplefins (Feary et al. 2009, Clements et al., 2010). Mussels have also been documented to accumulate MPs through filter feeding (Webb et al. 2017). Because triplefins are benthic feeders, the food blocks were negatively weighted with small, food grade, stainless-steel nuts. Fish were fed control food (i.e., no plastics) for two weeks to ensure habituation to the food and life in aquaria before being subjected to any plastic treatments. To simulate the inherent patchiness associated with plastic exposure in the marine environment (Clunies-Ross et al. 2016), fish were exposed to plastic treatments every second day. The concentration of MP particles in the Otago region is currently unknown. Therefore, we based MP concentrations for the present study on existing literature from Tanhua et al., (2020). They found an average of 34 particles m^{-3} (~3%), sampled from locations around the South Pacific, including sites around Auckland New Zealand. MP concentrations used in the present study were a lower level at 2% to allow for an underestimation and to reduce any stress that may not be relevant to the natural environment. Prior to feeding, tanks were syphoned to remove any excess food. This was to ensure each fish was receiving the same amount of food each day. Triplefins were exposed to the treatments for 4 weeks before being euthanised under the Otago Animal Ethics guidelines (AUP 19-10) using Aqui-S in fresh seawater (1ml/L). Each fish was dissected for the gastrointestinal tract (GIT), white muscle, gonad, and liver.

Table 4: Showing treatment groups, treatment type, number of replicates and the exposure time.

Group	Treatment	Number of replicate fish	Exposure time
Control	0% Plastic: mussel	12	28-days
PVC	2.5% PVC: mussel	12	28-days
Polyethylene	2.5% nylon: mussel	12	28-days
Polystyrene	2.5% polystyrene: mussel	12	28-days
EFC	2.5% EFC: mussel	12	28-days

2.2.7 *Condition*

To measure fish condition, we looked at changes in weight, hepatosomatic index (HSI) and gonadosomatic index (GSI). Due to fish being of similar size and morphology, I was unable to distinguish between the individuals in each tank for tracking their weight, therefore, Fulton's condition factor was not appropriate for this study. Instead, individual fish were weighed, and a tank average was calculated pre and post experimental period to identify an average weight change among treatments. The two tanks with a fish missing were excluded from this calculation. User error during data recording meant that two other tank values are missing (weight results N = 26). Average HSI was calculate by dividing the liver weight (Lw) by the final body weight (Bw) post-treatment (N=58). GSI was calculated by dividing the gonad weight (Gw) by the sum of gonad weight and body weight. Due to dissection error, some gonad weight values are missing (N = 55).

$$\boldsymbol{HSI} = (\frac{Lw}{Bw})\ 100$$

$$\boldsymbol{GSI} = \frac{Gw}{(Gw + Bw)}$$

2.2.8 *Histology*

The GIT portions were placed in labelled cassettes and dehydrated in graded ethanol and xylene. The purpose of fixing samples in this way is to prevent alterations to the tissues through natural degradation. Samples were dehydrated in progressive strengths of ethanol to avoid degrading the gut tissue. To remove the ethanol from the samples, they were twice soaked in xylene (Pajak 2002). The cassettes were lightly agitated using a benchmark orbi-blotter horizontal shaker during each solvent step. After dehydration, the samples were infiltrated with paraffin wax heated to 60°C. The embedding was repeated 3 times to remove all of the xylene and the contaminated

wax was discarded after each embedment (Appendix, Table 1). Using a microtome (Leica RM2125 RTS), 5µm sections were taken from each GIT portion and placed onto warm water to stretch and then a glass slide. The glass slides were then put on to a hot plate to dry. The dry slides were subjected to a series of solvents prior to staining (Appendix, Table 2). The xylene removes the wax from the slide and the ethanol removes any of the xylene remaining. The slides were then stained in haematoxylin which stains nuclei blue and then eosin which stains cytoplasm, collagen, and any other cells pink. The slides were soaked again in ethanol and xylene to remove excess stain and then cover-slipped using Thermo Fisher's Immu-Mount mounting medium.

Morphometric measurements

Completed slides were photographed using a light microscope (Olympus BX51) and camera (Olympus SC100). The images were analysed both visually and using Image J (Fiji extension) (version 2.1.0/1.53C) to look for signs of histopathological effects. For this purpose, the morphometric parameters measured were villus height, villus width, enterocyte length and absorptive surface area. Absorptive surface area consisted of measuring the entire perimeter of all the villus structures within each gut sample. Villus height and width were an averaged result of the 3 longest villi to standardise the data. (Fig. 9). Due to the nature of this assay, some samples did not result in a high quality image for analysis. Only full gut images were selected (i.e. no edges missing) for morphometric measurements (N = 47).

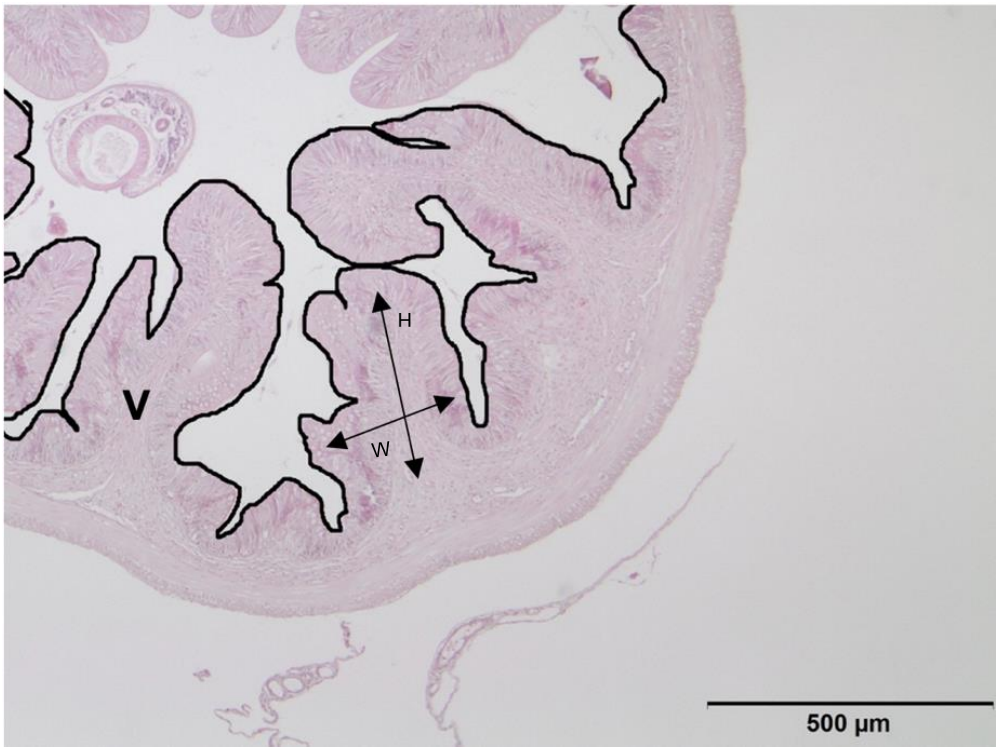


Figure 9: Diagram indicating morphometric measurements made. Black line indicates absorptive surface area perimeter. Arrows indicate example of villus height (H) and width (W) measurements. V = villus.

Goblet cells

To quantify the average number of goblet cells in each gut cross section, a PAS stain was used on 5 µm sections of gut. These were randomly taken from the distal intestine of each fish. The sections were first deparaffinized and then rehydrated using a series of solvents. This was followed by a 5-minute submersion in periodic acid. The sections were then rinsed in distilled water and submerged in Schiff reagent for 30 minutes. This was followed by a counterstain with hematoxylin for 3 minutes and then subsequently were rehydrated (Appendix, Table 3). Goblet cells were quantified using a transect method. Pictures of each gut were taken at 10 x magnification using a light microscope (Olympus BX51) and camera (Olympus SC100). A grid overlaid with nine 4x4 inch squares were placed over each gut image, over the computer screen. The grid was always placed in the same position and gut-images were consistent in size (actual gut sizes do vary and samples that were too big for the grid were not used) (N = 30). Squares were represented by a number between one and nine, three numbers between one and nine were randomly

generated and the respective square was used for counting. Using Image J (Fiji extension) (version 2.1.0/1.53C), the perimeter of each villus present was measured and the goblet cells within these villi were counted. Goblet cells are propagated at the base of the crypt and move towards the apical side as they mature. Eventually the goblet cells shed and are replaced. (Specian & Oliver 1991). Goblet cells were only counted if they were regularly shaped and on the apical side of the villi as these are the mature cells (Fig. 10, Fig. 11).

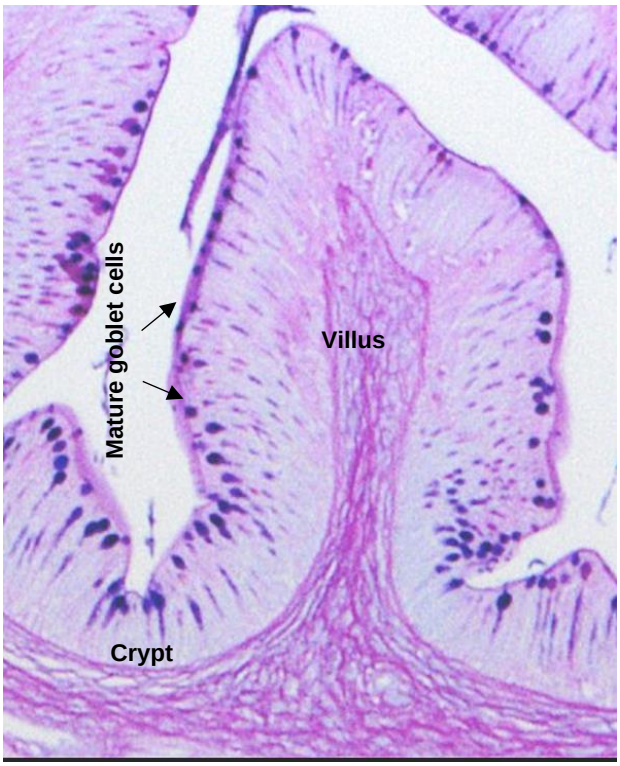


Figure 10: Histological example image of triplefin gut stained with PAS + Alcian Blue. Labels indicate villus structure, the crypt and where the mature goblet cells reside.

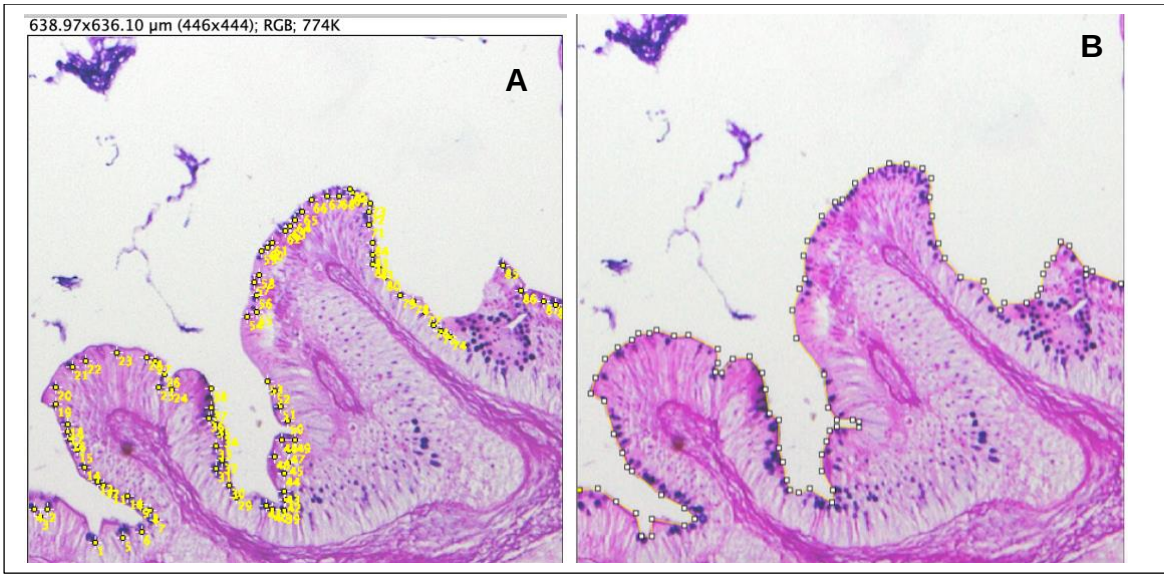


Figure 11: Example of goblet cell quantification. “A” shows how each regular shaped cell at apical region of villi were counted using the multi point tool on ImageJ. “B” shows how the villi perimeter was measured using the segmented line tool on ImageJ.

2.2.9 *Lectin histochemistry*

Goblet cells were also quantified using a variety of lectin stains. Stock solutions of fluorescein-labeled lectins and Carbo-Free Blocking Solution were obtained from Vector Laboratories (Catalog #: FLK-2100) and stored in the dark at 4 °C until use. The kit contained seven fluorescein labeled lectins. This included Con A, SBA, DBA, UEA I, RCA_{120}, PNA, and WGA (Table 5). These lectins bind to a variety of sugar specificities including mannose (Con A), galactose (RCA_{120}, PNA), fucose (UEA I), N-acetylgalactosamine (SBA, DBA), sialic acid (WGA), and N-acetylglucosamine (WGA). The lectin-conjugated fluorescent tags become excited at a wavelength of 495nm allowing us to see if the lectins have bound to any goblet cells. The sections were first deparaffinized and rehydrated using a series of graded alcohols (Appendix, Table 4). The sections were then incubated in diluted Carbofree blocking solution for 30 minutes at room temperature to remove any non-specific binding and block out interference. The blocking solution was cleared and the sections incubated in the diluted lectin solution for 30 minutes in the dark. The slides were then washed twice using HBS buffer containing 2% Tween-20 and cover-slipped using Thermo Fisher’s Immu-Mount mounting medium. This method was tested as a potential way of

quantifying the goblet cells. This method proved useful but did not identify goblet cells as clearly as the PAS+AB stain.

Table 5: Showing origin and binding specificity of each lectin tested.

Lectin	Origin	Binding Specificity
Concanavalin A (Con A)	*Canavalia ensiformis*	Mannose, Glucose
***Dolichos biflorus* agglutinin (DBA)**	*Dolichos biflorus*	N-acetylgalactosamine
Peanut agglutinin (PNA)	*Arachis hypogaea*	Galactose
***Ricinus communis* agglutinin (RCA120)**	*Ricinus communis*	Fucose, Arabinose
Soybean agglutinin (SBA)	*Glycine max*	Galactose, N-acetylgalactosamine
***Ulex europaeus* agglutinin I (UEA I)**	*Ulex europaeus*	Fucose, Arabinose
Wheat germ agglutinin (WGA)	*Triticum*	N-acetylglucosamine, Sialic acid

2.3 Data analysis

Datum was entered into Microsoft Excel version 16.54 (Excel 2021) and analysed in R (version 3.6.3). Graphs were made in R (version 3.6.3) using packages ‘ggplot2’ (version 3.3.0) and ‘plyr’ (version 1.8.6).

2.3.1 *Condition*

Due to the inability to track individual weights, a tank average weight was taken for the 2 fish in each tank before and after the treatment period. This average was calculated in Excel 2021 and imported in to R for analysis on averaged weight difference post-

treatment. Residual analysis indicated that weight difference data met the assumptions of normality and homogeneity of variances as simulated residuals did not deviate from observed residuals (R software package 'DHARMa' version 0.4.1). Therefore, a linear model was applied to the averaged weight gain after MP exposure. This was followed by a least-squared means test, adjusted to the Tukey method (R software package 'lsmeans' version 2.30-0). For HSI and GSI, residual analysis indicated that not all of the data met the assumptions of normality and homogeneity of variances. Therefore, a non-parametric Kruskal Wallace test was performed.

2.3.2 *Morphology*

Residual analysis indicated that absorptive surface area data did not meet the assumptions of normality and homogeneity of variances therefore, the data was log transformed. A one-way ANOVA was conducted on the log-transformed data. This was followed by a least squared means test, adjusted to the Tukey method (R software package 'lsmeans' version 2.30-0). For villus height, log and square-root transformations did not improve the fit of the residuals, and therefore, a non-parametric Kruskal Wallis test was performed. The raw villus width data was log transformed to meet the assumptions of homogeneity of variances and normality. A one-way ANOVA was performed followed by a least squared means test, adjusted to the Tukey method (R software package 'lsmeans' version 2.30-0). To analyse goblet cell counts data, a non-parametric Dunn (1964) Kruskal-Wallis multiple comparison with p-values adjusted using the Holm method was chosen. This was performed using R software package 'FSA' (version 0.9.3).

2.4 Results

2.4.1 *Condition*

There was no observed mortality during the experimental period for each treatment. Each pair of fish gained weight during the treatments (see fig 12) and there was no statistically significant difference between treatment groups ($F_{4,21}$ = 0.8116, p-value = 0.2114) although there was high variability. The EFC group gained the least weight on average during the experiment. Further, results showed no significant difference in fish

condition after the treatment period for HSI (Chi-squared$_4$ = 2.9454, p = 0.567) and GSI (Chi-squared$_4$ = 5.4681, p = 0.2425).

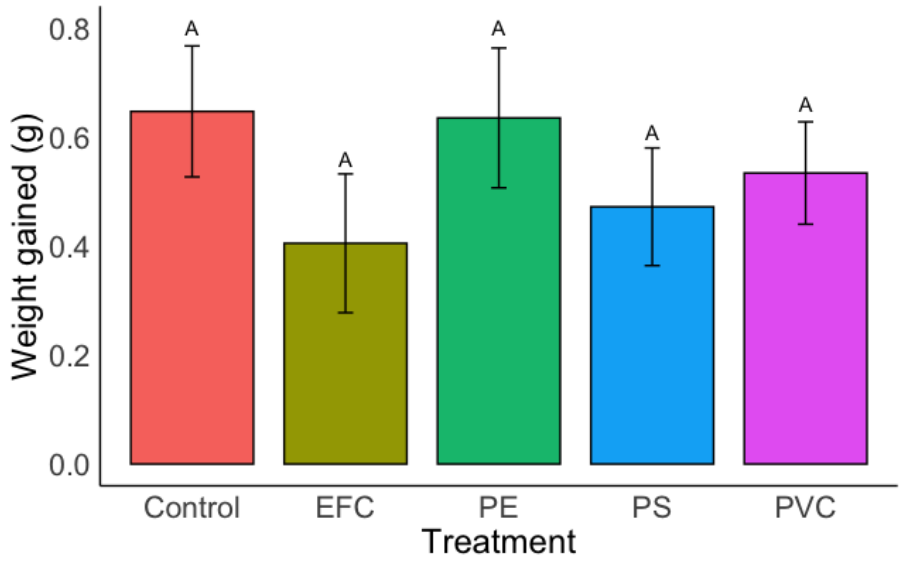


Figure 12: Bar graph showing average body weight gain (g) of triplefins exposed to different microplastic types during the treatment period (28 days). Treatments are Control N=8, EFC, N=11, PE N =11, PS N = 11 and PVC N=10. Error bars represent ± SE.

2.4.2 *Histopathology*

Morphology

Histological analysis of the GIT in triplefins after 28 days exposure to the treatments indicated signs of intestinal damage as a result of MP ingestion. On average, the control group had the largest villi and surface area for nutrient absorption in comparison to the plastic treated groups. This was most distinct in the PVC group where villi were smallest and the lumen was generally larger. Interestingly, there appeared to be PVC MPs retained in the lumen of some individuals (Fig. 13), this was not apparent in the other MP treatment groups. A one-way ANOVA and a Tukey's HSD test revealed that the PVC group had a significantly smaller absorptive surface area ($F_{4,47}$ = 2.999, p-value = 0.0276) than the control group (Fig. 14). Individuals from the EFC and PE groups also showed evidence of intestinal injury with less absorptive surface area than the control group. However, this was not significant. Morphometric

differences were apparent between treatment groups whereby a one-way ANOVA and a Tukey's HSD test found a significant decrease in villi width in the PVC group compared to the control and the EFC group. ($F_{4,47}$ = 4.094, p-value = 0.00629) (fig. 15). There was no significant difference in average villus height (Chi-squared$_4$ = 5.8404, p = 0.2114). Visual observation indicated signs of putative necrosis in some MP treated individuals (Fig. 16).

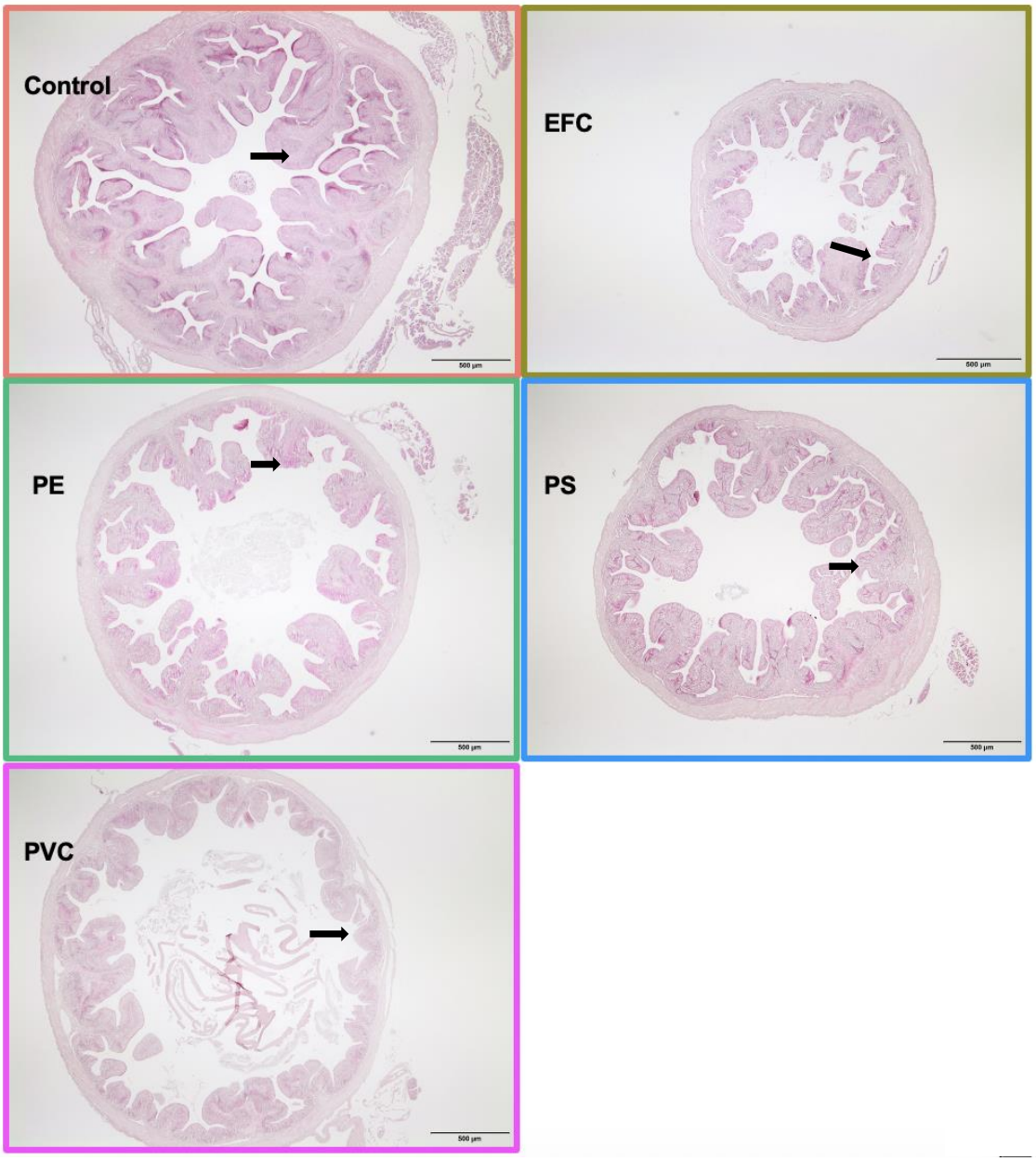


Figure 13: Histological section of triplefin gastro-intestinal tract exposed to different microplastic treatments for 28 days. This is the most extreme example per treatment Hematoxylin and eosin stained. Treatments are Control N = 11, EFC N = 10, PE N = 7, PS N = 10 and PVC N = 9.

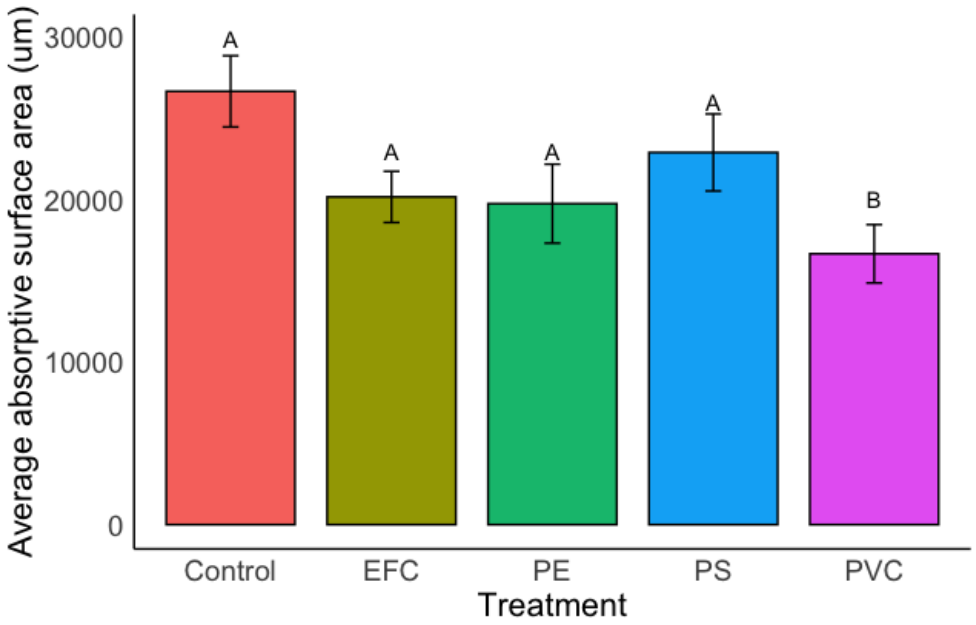


Figure 14: Bar graph showing the average absorptive surface area in the gastro-intestinal tract of triplefins exposed for 28 days to 5 different treatments. Treatments are Control N = 11, EFC N = 10, PE N = 7, PS N = 10 and PVC N = 9. Absorptive surface area is the total villi perimeter in each gut. Error bars represent ± SE.

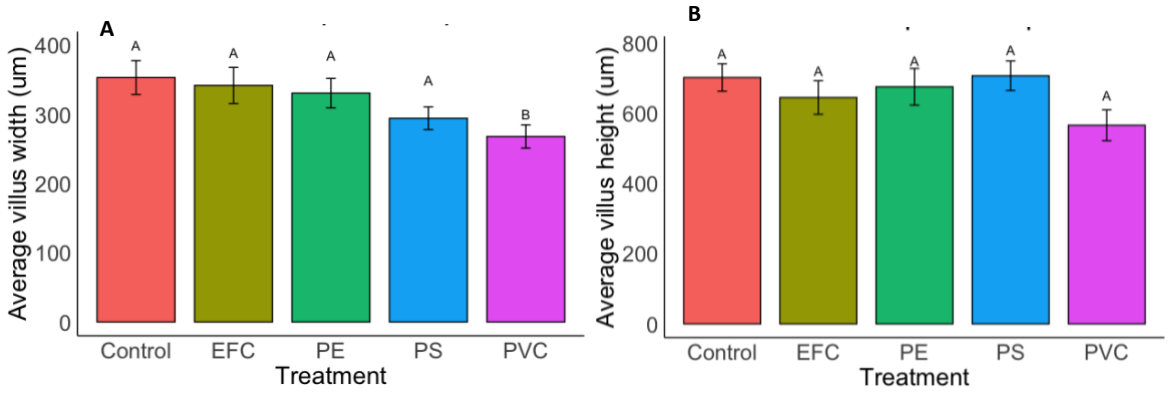


Figure 15: Bar graphs showing the average villus width (**A**) and average villus height (**B**) in the GIT of triplefins under 5 different treatments exposed for 28 days. Treatments are Control N = 11, EFC N = 10, PE N = 7, PS N = 10 and PVC N = 9. Error bars represent ± SE.

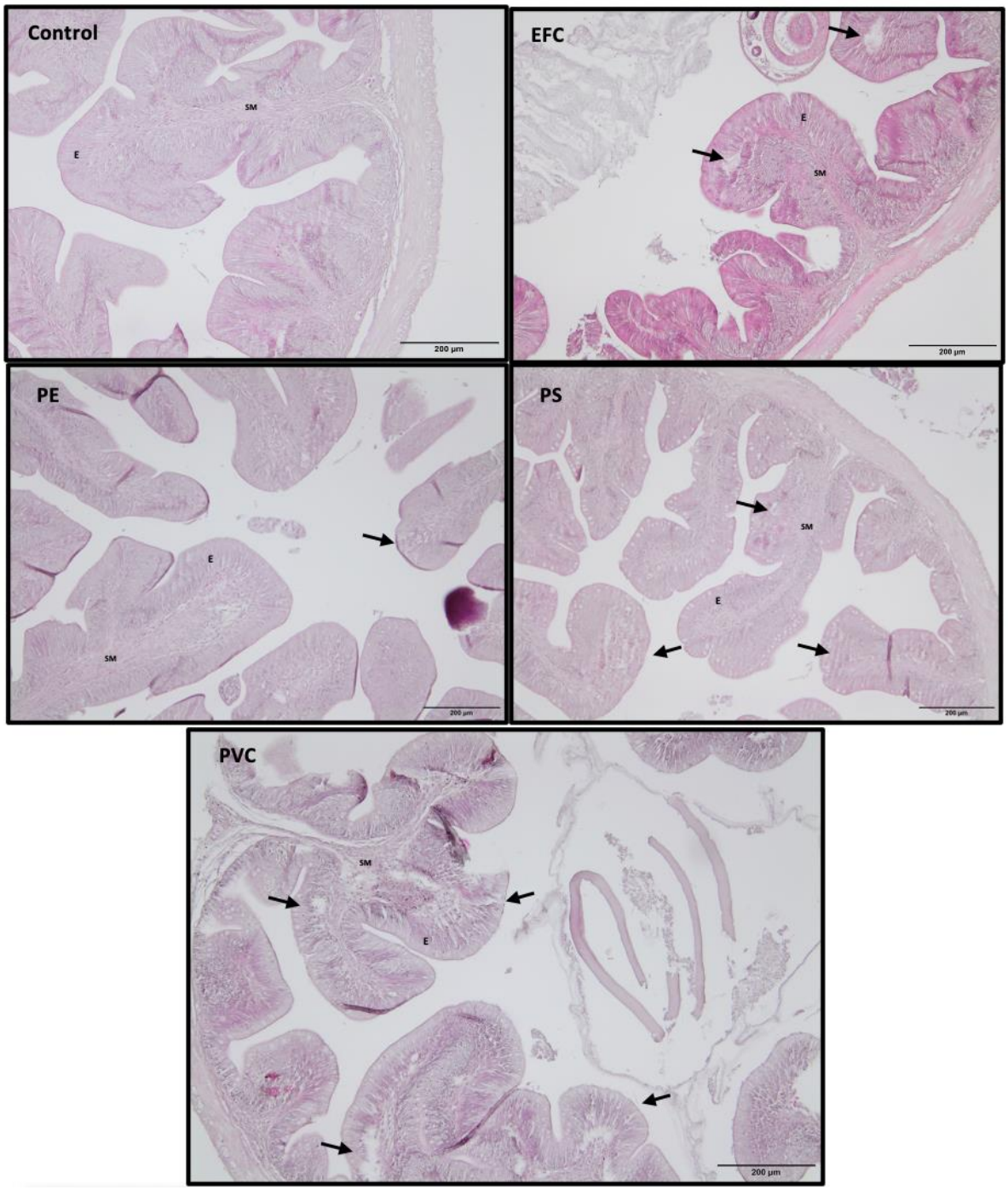


Figure 16: Histological analysis of the triplefin GIT villi fed 5 different treatments for 28 days. Treatments are Control (0% plastic) N = 11, EFC N = 10, PE N = 7, PS N = 10 and PVC N = 9. Arrows indicate possible necrosis; SM is submucosa and E is enterocytes.

Goblet cells

A PAS + Alcian Blue stain resulted in a positive stain. Upon visual analysis, most goblet cells were stained a deep blue. Very few were stained a bright magenta. Alcian Blue stains acidic mucins deep blue and PAS stains neutral mucins a bright magenta colour, suggesting most are acidic. After quantification, Residual analysis indicated goblet cell data was normally distributed. Stimulated residuals did not deviate from observed residuals (R software package 'DHARMa' version 0.4.1), therefore a linear model was conducted on the number of goblet cells per um of surface area. This was followed by a least squared means test, adjusted to the Tukey method (R software package 'lsmeans' version 2.30-0). There were significantly fewer goblet cells per um of surface area in PE ($F_{4,25}$ = 5.289, p-value = 0.0090), PS ($F_{4,25}$ = 5.289, p-value = 0.0182) and PVC ($F_{4,25}$ = 5.289, p-value = 0.034) compared to the control. There was no significant difference between EFC treated fish and the control however, there is a trend showing a decrease in goblet cell abundance ($F_{4,25}$ = 5.289, p-value = 0.076).

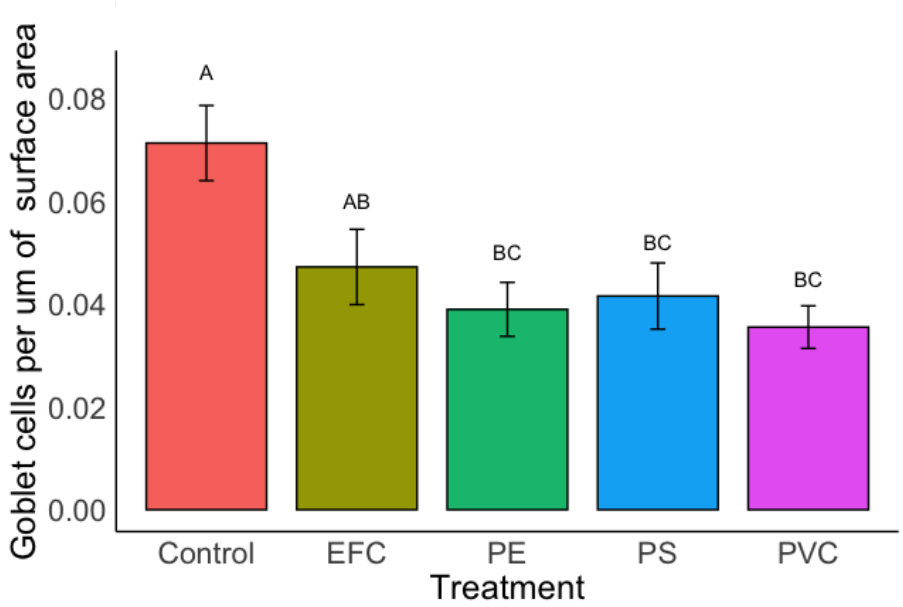


Figure 17: Bar graph showing the average absorptive surface area in the gastro-intestinal tract of triplefins exposed for 28 days to 5 different treatments. These treatments are Control N = 6, EFC N = 6, PE, N =6, PS N = 6 and PVC N = 6. Error bars represent ± SE.

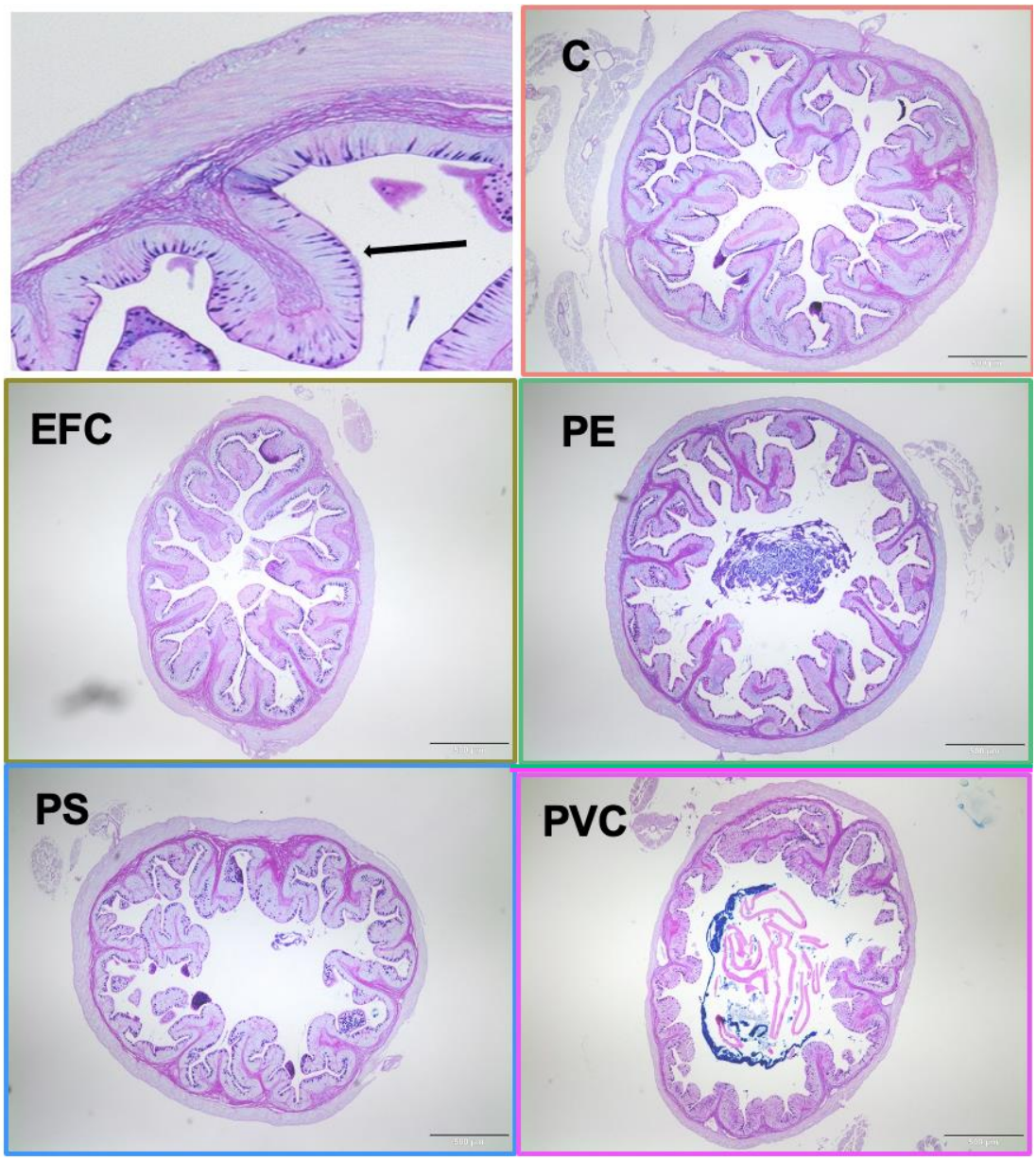


Figure 18: Histological analysis of the triplefin GIT villi fed 5 different treatments for 28 days. These treatments are Control N = 6, EFC N = 6, PE, N =6, PS N = 6 and PVC N = 6.

Lectin histochemistry

Of the 7 lectin stains tested, only wheat germ – agglutinin (WGA) had a strong affinity for goblet cell mucins. This lectin has a binding specificity to N-acetylglucosamine and sialic acid. There was a very weak result from *Dolichos biflorus* agglutinin (DBA) lectin, which too, binds to N-acetylglucosamine

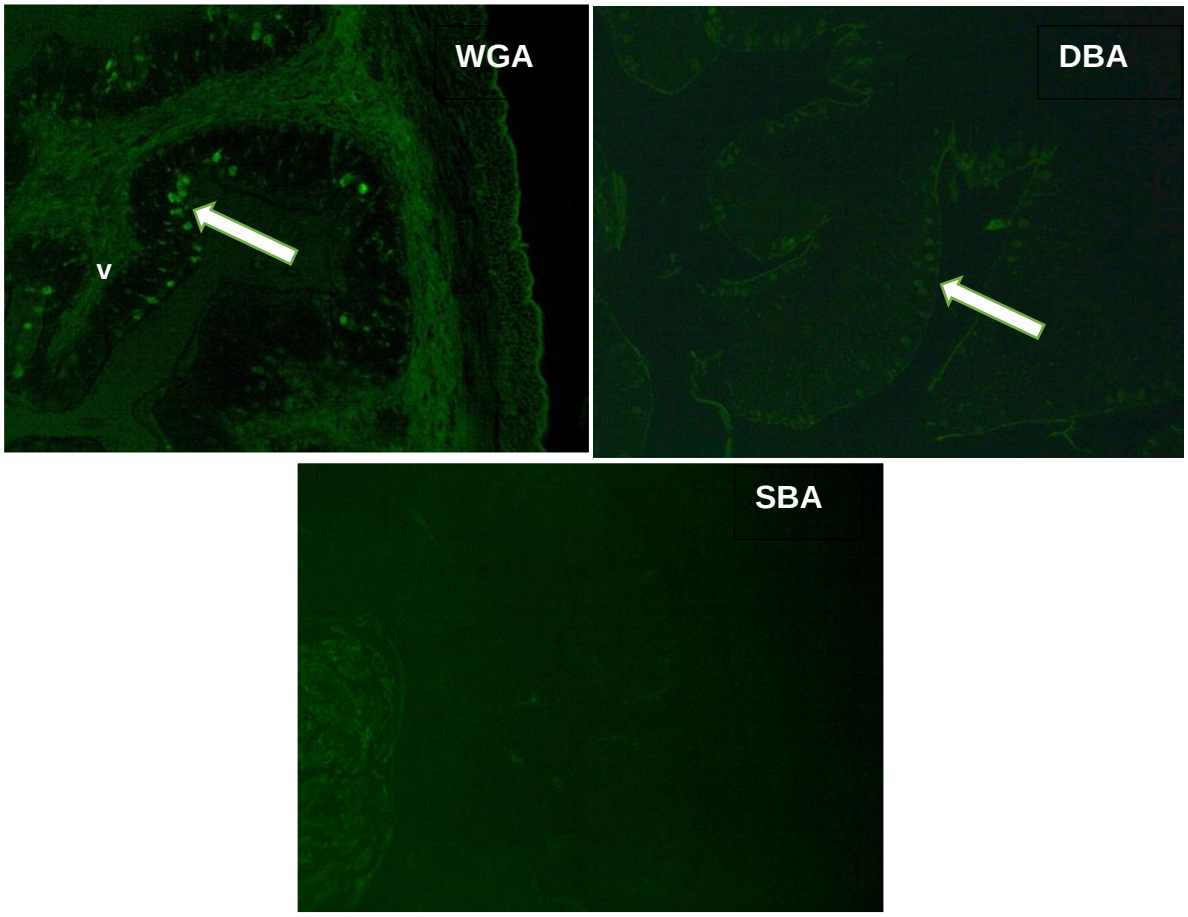


Figure 19: Sample of lectin-stained gut under a fluorescent microscope. WGA is wheat germ agglutinin and SBA is soybean agglutinin lectins. Arrow indicating goblet cells. V is villi. WGA highlights strong signal from this lectin. DBA highlights weak signal from this lectin. SBA shows example of no signal.

2.5 Discussion

The magnitude and extent of plastic pollution in marine environments highlights the need to investigate the effects of MP exposure on marine fishes. To date, multiple studies have investigated the effects of fish ingesting one or two MP types (Pedà et al 2016; Espinosa et al. 2019; Ahrendt et al. 2020), yet little is known of the effects when comparing multiple different types of plastic. To the best of my knowledge, this is the first study to compare direct effects of ingesting multiple MP types. Histological examination of the GIT indicated that on average, fish fed MPs had a smaller surface area for nutrient absorption. Specifically, consumption of PVC MPs significantly reduced the surface area for nutrient absorption in triplefin GIT.

Similar results were found by Pedà et al. (2016) after exposing European sea bass to both virgin and polluted PVC MPs. After 30 days exposure, they saw moderate structural alterations such as shortening and swelling of the villi, the most important site for nutrient absorption. However, this was a qualitative assessment, whereas the present study is quantitative. For example, I observed a significant decrease in villus width in the PVC group compared to the control group. It is thought that chemical plasticisers can leach out of plastics such as the DEHP and BPA found in many PVC items (Erythropel et al., 2014). These can be harmful when ingested and affect gut morphology such as findings from Feng et al., (2020). In mice, they found that ingesting BPA damaged microvilli and the epithelial cells within the GIT. It is possible the plasticizers in PVC may have contributed to the greater negative effect in our PVC treated fish. Morphometric measurements (i.e., villus height and width) are useful to predict nutrient absorption in fish GIT (Magouz et al. *2020)*. Surprisingly we did not find a significant difference in villus height across treatments. Espinosa et al. (2019) found contrasting results after exposing European sea bass to PE where they observed a significant decrease in villus height. Although this study was undertaken for a similar period (21 days) to the present study, European sea bass were exposed to MPs every day. Here, triplefins were administered MP every second day (total of 14 days) to account for environmental variability. It is feasible to predict that a longer exposure time to MPs would have yielded a similar result.

Villus height for the PS group was the most morphologically similar to the control group. This could be related to nature of this plastic. Hard MP types such as PVC often have sharp edges and are irregularly shaped which are more likely to penetrate intestinal structures than soft plastics. Lei et al. (2018) also found similar morphology in PS exposed zebrafish when compared to control fish. Similar to Jabeen et al. (2018) and Lei et al. (2018), cracking and erosion to villi in some PVC treated individuals was observed. On average, fish who consumed PVC had lower villus height. This implies there is less surface area for absorption of available nutrients in these fish. Nutrient absorption influences metabolism, which converts food into energy. Energy is required for all functions including growth, reproduction, and immune responses. Insufficient nutrients can lead to reduced fitness and leave the organism more susceptible to disease (Jobling 2016).

Damage to villi as seen in PVC fish, can be described as villus atrophy, a common indicator in humans for coeliac disease. These changes in intestinal structure are an immune response to dietary gluten found in grains such a wheat and barley. This can lead to malabsorption which with continued ingestion, can lead to serious health complications. Fish in this study have shown a similar response to ingested MPs. Enterocytes line the villi and absorb solubilised nutrients that are released during the digestion process, which then passes to the circulatory system (Bakke et al. 2010). Visual observation saw signs of putative necrosis in the enterocytes from individuals of all MP treatments. This was most distinct in the PVC group. This observation is supported by findings from Varó et al., (2021). After 30 days of MP exposure gilthead seabream showed severe mechanical damage in the intestine including necrosis to the enterocyte cells of the villi. The observed necrosis in the enterocytes is important as it highlights an immune response was likely induced by the foreign MP particles. Severe enterocyte damage may reduce the amount of nutrients absorbed and can lead to colitis thus, reduce fitness (Brugman et al. 2000).

HSI and GSI results suggested there was no significant change in fish condition. This highlights that any morphological changes are likely due to treatments rather than the stress of an experimental environment. A similar response has been demonstrated in female marine medaka (*Oryzias melastigma*) that showed no difference in HSI when exposed to PS MPs. However, they did observe tissue damage and oxidative stress

(Wang et al., 2019). Food availability and water temperature can influence HSI and GSI where unfavourable conditions correlate with a lower HIS and GSI index (Jafor Bapary et al., 2012). This highlights that the morphological changes observed are likely treatment effects. It is interesting that some individuals in the PVC group appeared to retain MP in the gut. It is possible that this could lead to intestinal blockage however, Jovanović et al. (2018) found potential long-term retention of MP in the gut to be low. Similar to humans, the GIT serves an important function for survival as the source of nutrient absorption and impairment can increase susceptibility to infections and inflammatory diseases (Bischoff 2011). The damage to villi, enterocytes and reduction of absorptive surface area seen in these results is analogous to the reactions of a coeliac who consumes gluten. This is characterised by a decrease in villi size and intestinal malabsorption (Tack et al. 2010; Adriaanse et al. 2013). Excessive enterocyte damage is thought to be the responsible mechanism for villi loss in the intestine of those with coeliac disease, leading to villus atrophy (Adriaanse et al. 2013).

treated groups than the control group. It is possible that alterations to the villi incurred by mechanical damage, limited the ability of goblet cell function. This has been seen in mice that have ingested polystyrene particles. Lu *et al.* (2018) found significantly less mucins in PS treated mice as their barrier had been disturbed by the PS particles. We know that the mucus layer provided by these goblet cells acts as an important chemical barrier against pathogens (Specian & Oliver 1991, Scillitani *et al.* 2012, Salinas & Parra 2015). Disruption of this caused by MP ingestion may leave fish vulnerable and more susceptible to disease.

Surprisingly, I found the number of goblet cells to be significantly less abundant in the PE, PS and PVC treated groups. I expect this to be associated with the trend in less villi surface area. It is possible that alterations to the villi incurred by mechanical damage, limited the ability of goblet cell function. Lu et al. (2020) found similar results for PS treated MP treated juvenile yellow perch (*Perca flavescens*), as their barrier had been disturbed by the PS particles. The significant reduction in surface area and villi damage seen in the PVC group likely explain the lower abundance of goblet cells. There was no significant difference in goblet cell abundance between EFC treated fish and control. This was only marginally insignificant and perhaps with a larger sample size we may have seen a different result. The mucus layer provided by these goblet

cells acts as an important chemical barrier against pathogens (Specian & Oliver 1991, Scillitani et al. 2012, Salinas & Parra 2015) and disruption of this may leave fish vulnerable and more susceptible to disease. Interestingly, WGA showed a strong affinity for goblet cell mucins across all treatments. This lectin has a binding specificity to N-acetylglucosamine and sialic acid. There was a very weak result from *Dolichos biflorus* agglutinin (DBA) lectin, which too, binds to N-acetylglucosamine. It is likely that there is more sialic acid residue on the mucins than N- acetylglucosamine due to the weaker DBA result. This was supported by finding majority of goblet cells from the PAS + Alcian Blue stained deep blue, indicating acidity. Blue stained mucins can imply that they are producing sialic acid (Forder et al., 2007). Producing sialic acid can be beneficial as it can disturb a virus's receptor detection in the GIT (Khojasteh, 2012). This provides extra protection from harmful pathogens.

Triplefins are important prey items for many species as cryptobenthic fish larvae can account for the majority of consumed rocky reef biomass (Feary & Clements 2006; Brandl et al. 2019). Mature fish are predated on by large carnivorous fish like the commercially important blue cod (*Parapercis colias*) and sea perch *(Helicolenus percoides)* (Wellenreuther et al. 2010). Triplefins are also important predators on rocky reef habitats where they predate on small intertidal invertebrates. Impairment to their GIT may reduce fitness which could result in population declines. As important prey items, this has potential to cause negative effects on ecosystem dynamics. It is important to note that although we have found these results, which highlight the negative effect of MP ingestion on triplefin gut morphology, there are limitations to this methodology. Using histology only takes a singular 2D cross sectional plane of a 3D object. This could result in missing signs of damage within the same GIT. It could be useful to in future compare multiple sections from the same GIT.

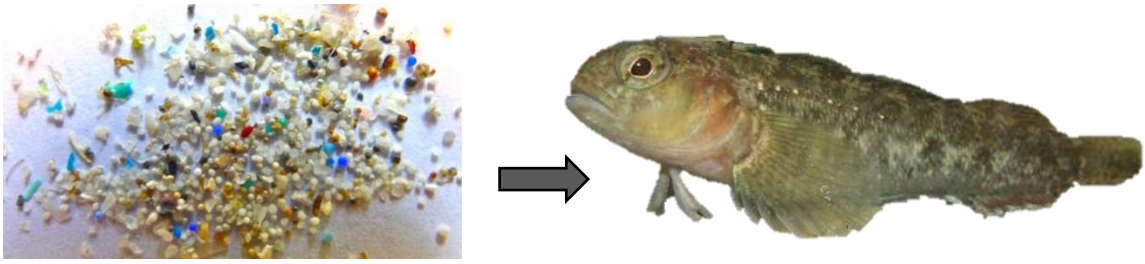
2.6 Conclusion

The results from this study clearly demonstrate a negative effect of MP ingestion on gut morphology in triplefins. Our results suggest that PVC MPs cause a greater reduction in absorptive surface area for nutrients. Further, PE, PS and PVC MP can reduce the number of goblet cells. This can lead to a decrease in fitness and increased risk of infection. There is an increasing amount of plastic pollution entering the marine

environment due to our heavy consumer demand and poor plastic waste management. Understanding how organisms are directly affected by these pollutants can promote awareness and influence future management.

Chapter 3

Microplastic ingestion affects oxidative stress biomarkers in New Zealand triplefin fish



3.1 Introduction

With the global rise in mismanaged plastic waste, microplastics (MP) have become more common in the marine environment (Chassignet et al., 2021; L. Lebreton & Andrady, 2019). An estimated 10 million metric tonnes of mismanaged plastic waste enters the marine environment annually via rivers and streams where it weathers and fragments over time (Meijer et al., 2021). A direct, MP source into coastal environments are wastewater treatment plants (WWTP) as filters are not equipped to trap MP. Ruffell et al., (2021) estimates that WWTPs in New Zealand discharge around 2.4 x 10^5 MP each day into coastal environments. Consequently, MP abundance estimates in beach sediments from the North and South Island found between 0 (none detected) – 2615 and 177-1933 particles.m^{-2} respectively. Some of the most prevalent MP types in the marine environment are polyethylene (PE), polystyrene (PS) and polyvinyl chloride (PVC) as these are the most widely used plastic types in the consumer market (Andrady, 2003, 2004; L. Lebreton & Andrady, 2019). Efforts to reduce plastic production and subsequent plastic pollution has seen a rise in the development and use of biobased plastics over the mostly fossil derived plastics (for review see Kuddus & Roohi, 2021). Yet despite the increasing use of these products, there is limited knowledge on the biological effects of these plastics should they be encountered in the marine environment by marine taxa.

Owing to their small size, MPs are easily ingested by marine taxa including fish (Chapter 1, Table 1.). MP ingestion can cause adverse effects such as altering behaviour (McCormick et al., 2020), damage to internal organs, such as the gastrointestinal tract (GIT) (Pedà et al., 2016) (Chapter 2), endocrine disruption (Rochman et al., 2014) and toxicity (Mak et al., 2019). An important indicator of toxicity in fishes is oxidative stress. Oxidative stress occurs when the production of reactive oxygen species (ROS) exceeds an organism's ability to remove them from cells and tissues using the antioxidant system (Lushchak, 2016). Molecular oxygen is not very reactive however, if it is converted to free radicals, it becomes highly reactive due to an unpaired electron in the outer orbit. Three common ROS are the superoxide anion (O_{2-}), the hydroxyl radical (HO) and hydrogen peroxide (H_2O_2). The superoxide anion (O_{2-}) and hydroxyl radical (HO) are free radicals as they have an unpaired electron, while hydrogen peroxide (H_2O_2) is not a free radical as it has no unpaired electron yet,

they are all called ROS as they are highly reactive. In most aerobic organisms, the reduction of molecular oxygen is a source of energy that gets stored in ATP molecules which provides energy for normal cellular processes (Almroth, 2008). ROS can escape from the mitochondrial electron transport chain where they can react with molecular oxygen to become superoxide anion (O_{2^-}). ROS are constantly formed as a by-product of the metabolism of oxygen however, excess amounts of these can be damaging. The number of electrons escaping, and the ability to combat an imbalance, depends on the physiological state of the fish (Lushchak, 2016). Further, ROS are generated in greater amounts in pathological situations and during environmental stressors (Repetto et al., 2012). Oxidative stress is widely studied in humans where it is linked to multiple diseases such as Alzheimer's disease, diabetes, Parkinson's disease and cancer (Almroth, 2008; Stadtman & Levine, 2011.).

Both humans and fish share the same enzymatic defence against excess dangerous oxidants to reduce cellular damage. Crucial antioxidant enzymes involved in combatting ROS are superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPOX). First recognised in 1969, SOD is the first line of defence and most powerful antioxidant in the cell. It combats superoxide anion by converting it into hydrogen peroxide (Ighodaro & Akinloye, 2018; McCord & Fridovich, 1969). Catalase is the second antioxidant involved where it destroys hydrogen peroxide by converting it to oxygen (O2) and water (H2O) (Lushchak, 2016). Similar to catalase, glutathione peroxidase (GPOX) plays a key role in targeting hydrogen peroxide by converting it to water. GPOX specialises in reducing lipid peroxyradicals (Fig. 20).

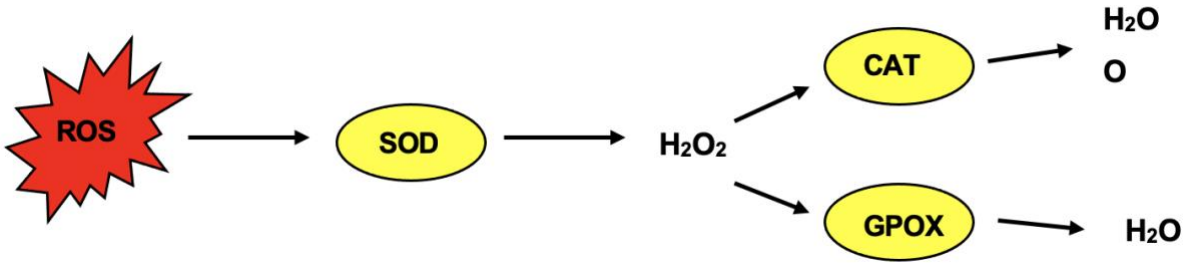


Figure 20: Diagram depicting enzymatic defence process against reactive oxygen species (ROS). SOD is superoxide dismutase, CAT is catalase, GPOX is glutathione peroxidase.

Oxidative stress targets three main macromolecules. These are lipids, proteins, and DNA. For the purpose of this thesis, I will be covering lipids and proteins, but work has been done on DNA for future publication (Appendix 5.2). When an organism is under increasing stress, the enzymatic defence cannot cope with the number of ROS and incurs oxidative damage to the cells. An important biomarker of oxidative damage is lipid peroxidation (LPOX), a toxic consequence of oxidative stress. ROS attack lipid membranes by removing a hydrogen atom from an unsaturated fatty acid which produces a lipid peroxyradical. These lipid peroxyradicals form a chain sequence by removing a hydrogen atom from an adjacent lipid until this is intercepted by GPOX. This means that one initiation event can lead to hundreds of conversions to lipid peroxides. Lipid peroxidation causes adverse effects by targeting polyunsaturated fatty acids in cellular membranes. This decreases the barrier function and membrane permeability which induces cellular functional loss (Halliwell & Chirico, 1993; Moore & Roberts, 1998; Niki, 2009). Fish are particularly susceptible to this as they are generally high in polyunsaturated fatty acids. Not only do lipid peroxides degrade cells, but they also produce toxic products such as hydroperoxides. These can alter chemotactic signals and inhibit protein synthesis (Lushchak, 2016; Repetto et al., 2012). Protein molecules are targeted and oxidised in a similar way to lipids by ROS, which can cause irreversible damage. Oxidative damage to proteins causes loss of function and cell death (Quiney et al., 2011). The level of protein oxidation can be quantified by looking at the number of carbonyl groups (ie. aldehydes and ketones) which are produced when proteins are oxidised by ROS (Dalle-Donne et al., 2003; Madhusudhanan et al., 2004; Parvez & Raisuddin, 2005). ROS can directly attack amino acids in proteins or, secondarily from products of lipid peroxidation (e.g., hydroxyl radicles). This results in the formation of protein carbonyls (PC), an important bioindicator of oxidative damage (Hematyar et al., 2019).

MP ingestion is known to induce oxidative stress in fish, however, most studies have examined this using only one or two plastic types (such as PVC, PE, PS) (Espinosa et al., 2019; Kim et al., 2021; Usman et al., 2021; X. Xia et al., 2020). For example, Solomando et al, (2020) exposed gilthead seabream to polyethylene (LDPE) for 90 days and observed a significant increase in intestinal SOD, CAT and GPOX antioxidant enzyme activity after just 30 days exposure, this gradually increased with

exposure time. Elevated antioxidant enzyme activity suggests that the organisms is under oxidative stress as it requires more antioxidants to combat elevated ROS. Consequently, these fish also showed higher levels of lipid peroxidation. Differing plastic types can cause different effects due to the variation in their chemical composition. For example, PVC is commonly plasticised with di(2-ethylhexl)phthalate (DEHP) which is a known carcinogenic and can cause reproductive damage (Chang et al., 2017; Hokanson et al., 2006; Rank, 2005; Xia et al., 2018). In contrast, many bioplolymers are made using chitosan, a polymer made from chiton taken from the outer skeleton of crustaceans (Kasmuri & Zait, 2018). This substance is known to be effective at absorbing heavy metals from water (WeiBpflog et al., 2020). High concentrations of heavy metals can be harmful to fish, by causing behavioural changes and cellular damage (Mishra & Mohanty, 2008; van Dyk et al., 2007).

To date, the majority of marine fish research investigating the effects of MP exposure on oxidative stress have focused only on antioxidant enzyme activity, with few examining both enzymes and oxidative damage markers (PC and LPOX). Given that marine MPs are continuing to increase in ubiquity, it is important to understand the biochemical effects this can have on fish that consume them. Multiple types of plastic account for the global accumulation of oceanic MP pollution. Among the most prominent are PE, PS and PVC. Due to the variation in physical and chemical properties of different plastic types, its critical to test the effects of a range of MP types. Therefore, the aim of this study is to understand the effects of ingesting a range of MP types on oxidative stress in triplefins. Specifically, looking at antioxidant enzyme activity and oxidative damage markers. If we can understand the effects of multiple plastic types on both antioxidant enzymes and damage markers, and further, which plastic type is more harmful, we may be able to limit the use of more problematic pollutants. With the increased production of biopolymer, and the limited knowledge on their bio-implications, it is important to include them in this field of study.

3.2 Methods

3.2.1 *Ethics statement*

All work in this study was completed in accordance with the University of Otago Animal Ethics guidelines (AUP 19-70).

3.2.2 *Study species*

To address the above aims, the mottled triplefin *Forsterygion capito* (Fig. 21) was used as a model organism. Triplefins (Tripterygiidae) are small rocky reef fish that are abundant throughout New Zealand and have been used extensively in laboratory studies as they readily adjust to aquaria (Feary et al., 2009). A total of sixty adult, male mottled triplefins were collected using hand nets from the intertidal zone at Back Beach, Port Chalmers, New Zealand in June 2020 (Fig. 22).

Figure 21: Image depicting Forsterygion capito. Credit NZMSC, taken from https://www.mm2.net.nz/news/triplefin-fish.

3.2.3 *Experimental protocol*

The same experimental fish from Chapter 2 were used for this study. In brief, a total of 5 treatments including 4 plastic treatments and a control (0% plastic) were used. Plastic treatments were polyvinyl chloride (PVC), polystyrene (PS), polyethylene (PE) and a biodegradable film (biopolymer) or edible food coating (EFC). The plastics treatments were chosen based on their prevalence in the marine environment. PVC,

PS and PE are the most widely used plastics in the consumer market (Wang et al. 2020). In total, there were 12 replicate fish per treatment housed in 6 x 20l glass aquaria per treatment (i.e. 2 fish per tank). Fish were exposed to MP for 4 weeks. MP were administered on every second day. MP diet consisted of 2g frozen food blocks containing pulverized green lipped mussel and MP ratio of 2.5% plastic to mussel weight. After the exposure period, triplefins were euthanised under Otago animal ethics guidelines (AUP 20-01) using Aqui-S in fresh seawater (1ml/L).
For full experimental protocol see Chapter 2: 2.3 – 2.7.

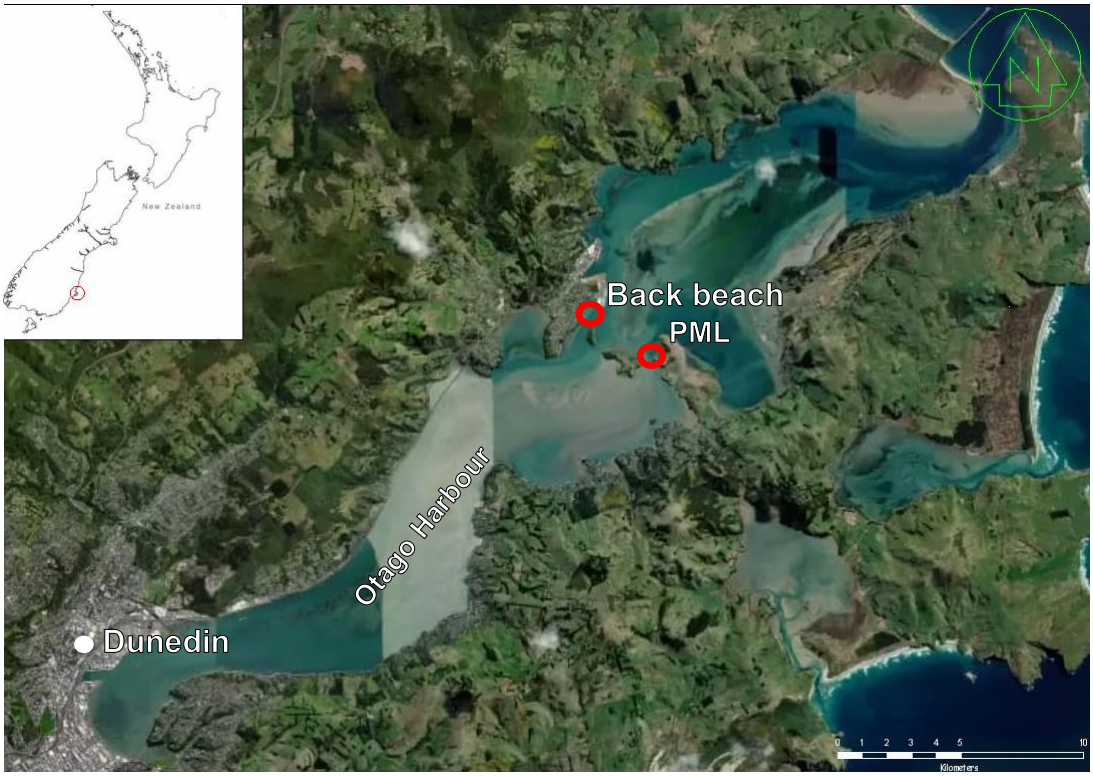


Figure 22: Map depicting research lab and sample collection site in Dunedin, New Zealand.

3.2.4 *Enzyme activity*

White muscle preparation

Antioxidant enzyme activity was measured in frozen white muscle tissue (~100 µg of tissue per fish). Samples were prepared by extracting total protein from the tissue using methods adapted from Lister et al., (2015). This was done by adding 750 µl of extraction buffer (pH 7.0) (containing 0.2 mM Na_2 EDTA, 1% PVP-44, 1mM PMSF,

and 0.5% v/v Triton X-100) and six zirconium beads (Biospec mini™) to the tissue sample tube. Tubes were bead beaten for 10 seconds, five times in a bead beater (Biospec mini™) set to speed 46. Between each 15 second bout, samples were returned to ice to keep cool. Samples were then centrifuged (Eppendorff™ 5424R) for fifteen minutes at 4°C and 14000 rpm. Ultrafiltration was used to remove any small molecules that could interfere with subsequent assay. This was done by adding 500 µl of the supernatant into an ultrafilter (Amicon™, 10 kD MWCO) and spinning down for 15 minutes at 4°C and 10000 RPM. The protein extract was then washed by adding 500µl of phosphate buffer (pH 7.0) and spun again at 4°C and 10000 RPM for 20 minutes in the centrifuge. The remaining sample was reconstituted within the ultrafilter by adding 500 µl of phosphate buffer. The sample was separated in to three aliquot tubes of 100 µl which was then frozen at -80°C until biochemical analysis.

Liver preparation

Antioxidant enzyme activity also was measured in liver tissue (~50 µg of tissue per fish). Samples were prepared by extracting total protein from the tissue using methods adapted from Lister et al., (2015). Here, 950 µl of extraction buffer and six zirconium beads were added to the tissue sample tube. Each tube was bead beaten for 10 seconds in a bead beater (Biospec mini™) set to speed 46, followed by centrifugation (Eppendorff™ 5424R) for fifteen minutes at 4°C and 15000 RPM. Next 1ml of phosphate buffer was added to the tube and further centrifuged for two minutes at the same RPM. From this 900 µl of the supernatant was removed and placed into a new tube with a further 1ml of phosphate buffer (pH 7.0) added to the tube for further dilution. Samples were centrifuged for 30 minutes at 15000 RPM. In a new tube, 500 µl of the liquid was put into an ultrafilter (Amicon™, 10 kD MWCO) and centrifuged for 15 minutes at 10000 RPM. Next the rest of the liquid was added to the ultrafilter and the centrifuge step was repeated. The ultrafilter was then washed by adding an additional 500 µl of phosphate buffer and discarding the filtered-out liquid in the bottom of the tube. This was then centrifuged for 30 minutes at 10000 RPM. In a new tube, the supernatant was reconstituted within the ultrafilter by adding 500 µl of phosphate

buffer. The sample was separated in to 3 aliquot tubes of 100 µl which was then frozen at -80°C until biochemical analysis.

Biochemical analysis

Assays were conducted using methods outlined by Lister et al., (2015) to determine enzyme activity in triplefin white muscle and liver. To determine the soluble protein contents, a Lowry protein assay using a bovine serum albumin (BSA) standard was conducted in line with methods described by Fryer et al., (1986). Using a Sigma-Aldrich 9160 SOD Determination Kit, an assay based upon WST-1 [2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)- 2H- tetrazolium, monosodium salt] reduction was used to determine superoxide dismutase (SOD) activity, where WST-1 produces a water-soluble formazan dye when reduced by superoxide anions. Catalase (CAT) activity was determined using methodology described by Maral et al. (1997) and adapted for 96-well microplates by Janssens et al. (2000). Spectrophotometric methods were used to determine glutathione peroxidase (GPOX) activity as outlined by Paglia and Valentine (1967) with minor modifications.

3.2.6 *Lipid peroxidation*

White muscle

The remaining tissue pellet from the protein extraction was prepared to extract total lipids. The tissue (~100mg) was homogenised in 600µl of methanol: chloroform (2:1 v/v). After 1 minute 0.4 mL of chloroform was added and centrifuged for 30 seconds at ambient temperature. Then 400µl of deionised water was added and centrifuged again for 30 seconds. After a few seconds, the phases separated and 600 µl of liquid from the bottom phases was transferred in a new tube and stored at -80˚C until lipid peroxide analysis. A method described by Mihaljevic et al. 1996 involving ferric thiocyanate, adapted for measurement in a microtitre plate reader was used to determing Lipid hydroperoxides (LPOX) levels in samples. This was determined by the absorbance at 500 nm. The LPOX content was calculated as nmol of lipid hydroperoxide per g of fresh weight using a calibration curve with t-butyl hydroperoxide.

Liver

Lipid peroxides were measured using the remaining liver tissue (~50 µg of tissue per fish). The tissue was homogenized in 0.75 mL of methanol: chloroform (2:1 v/v). After 1 minute 0.5 mL of chloroform was added and mixed by inverting 3 times. 0.5 mL of deionised water was then added to the tube and mixed again using the same technique. The tube was then centrifuged twice for 30 seconds and 550 µl was extracted from the lower phase. 0.05 mL was transferred to a well plate.

3.2.7 *Lipid content*

Liver

To quantify the liver lipid content, the remaining 500µl of supernatant from lipid peroxide test was used for gravimetric analysis. Tubes of 500µl with a known weight were evaporated overnight and then weighed again. The final weight (Fw) was subtracted from the initial weight (iw) and then adjusted for tube weight. the adjusted for liver weight (Lw) to identify lipid %.

3.2.8 *Wild Fish*

Liver data (both enzyme activity and lipid peroxides) for experimental fish was not deemed useable as these livers appeared too fatty for clean extractions (Figure 2.), which raised an interesting question "*Was the overly fatty liver an artefact of the experimental conditions and the readily available, high-quality food (pulverised green lip mussels) or is it a natural feature of triplefins?"* Laboratory experimentation is essential as it provides a controlled environment for reliable data collection (Henshel, 1980). Controlled environments allow you to test treatment effects by keeping all other possible impacting variables constant across treatments (e.g. food availability). Further, laboratory settings can provide insight for future predictions as it allows you to observe phenomena not possible in the field. However, it is also possible that

laboratory environments can cause stress or create unnatural situations. Food is often a limiting factor in the natural environment and so it is entirely possible the fish in the present study were overfed, thereby creating an unnatural physiological state. Therefore, a secondary aim was to identify whether there is a difference in general health between laboratory kept triplefins and wild triplefins. To the best of our knowledge, *F. capito* lipid content has not been quantified which, could provide insight to how applicable our findings are to the natural environment.

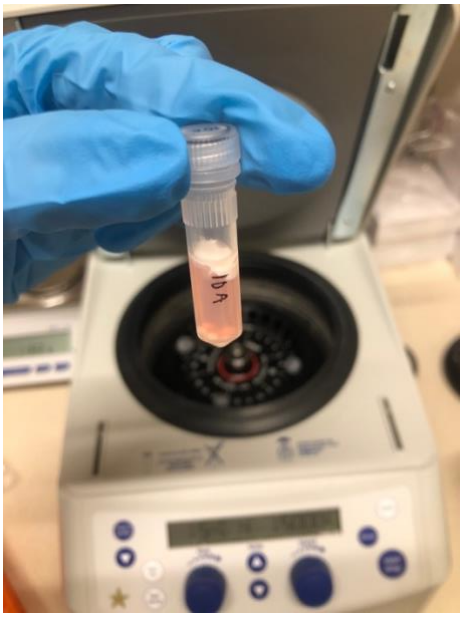


Figure 23: Example of a protein extraction tube. Image depicts a clear white layer of fat at the top of the supernatant. This fat layer makes it difficult to abstract clean proteins from the supernatant.

Secondary data collection was conducted for 10 wild, untreated triplefins. These were collected in September 2021 using hand nets from the intertidal zone North Portobello Beach, Portobello, New Zealand. These were transported in buckets approximately 100m to the Portobello Marine Lab where they were euthanised under the Otago animal ethics guidelines (AUP 19-70) using using Aqui-S in fresh seawater (1ml/L). White muscle and liver were removed from each fish and snap frozen in liquid nitrogen with the same protocols described above for lipid content and lipid peroxide undertaken.

3.3 Data Analysis

Datum was entered into Microsoft Excel version 16.54 (Excel 2021) and analysed in R (version 3.6.3).

3.3.1 *Antioxidant enzyme activity*

Residual analysis indicated SOD, CAT and GPOX data was normally distributed. Simulated residuals did not deviate from observed residuals (R software package 'DHARMa' version 0.4.1), therefore, a linear model was conducted on antioxidant enzyme activity. This was followed by a least squared means test, adjusted to the Tukey method (R software package 'lsmeans' version 2.30-0).

3.3.2 *Oxidative damage markers*

Residual analysis showed that square root transformation of PC data and log transformation of LPOX data met the assumption of normal distribution. Stimulated residuals did not deviate from observed residuals, therefore a linear model was conducted on these oxidative damage markers. This was followed by a least squared means test, adjusted to the Tukey method (R software package 'lsmeans' version 2.30-0).

3.3.3 *Liver lipid content*

Residual analysis indicated SOD, CAT and GPOX data was normally distributed. Stimulated residuals did not deviate from observed residuals (R software package 'DHARMa' version 0.4.1), therefore a linear model was conducted on liver lipid content. This was followed by a least squared means test, adjusted to the Tukey method (R software package 'lsmeans' version 2.30-0).

3.4 Results

3.4.1 *Antioxidant enzyme activity*

All antioxidant enzyme activity was elevated following 28-day exposure to dietary MPs. A linear model revealed that activity of SOD ($F_{4, 44} = 72.25$), CAT ($F_{4, 44} = 84.14$) and GPOX ($F_{4, 44} = 58.3$) enzymes was significantly higher for fish fed EFC, PS and PVC MP diets compared to the control (0% plastic) (p-value ≤ 0.002), indicating oxidative stress. Treatment effect was most distinct in EFC and PVC fed fish as they had the highest levels of enzymatic activity, increasing more than two-fold. For PE treated fish, although appearing to increase, was not deemed significantly greater (Fig. 24).

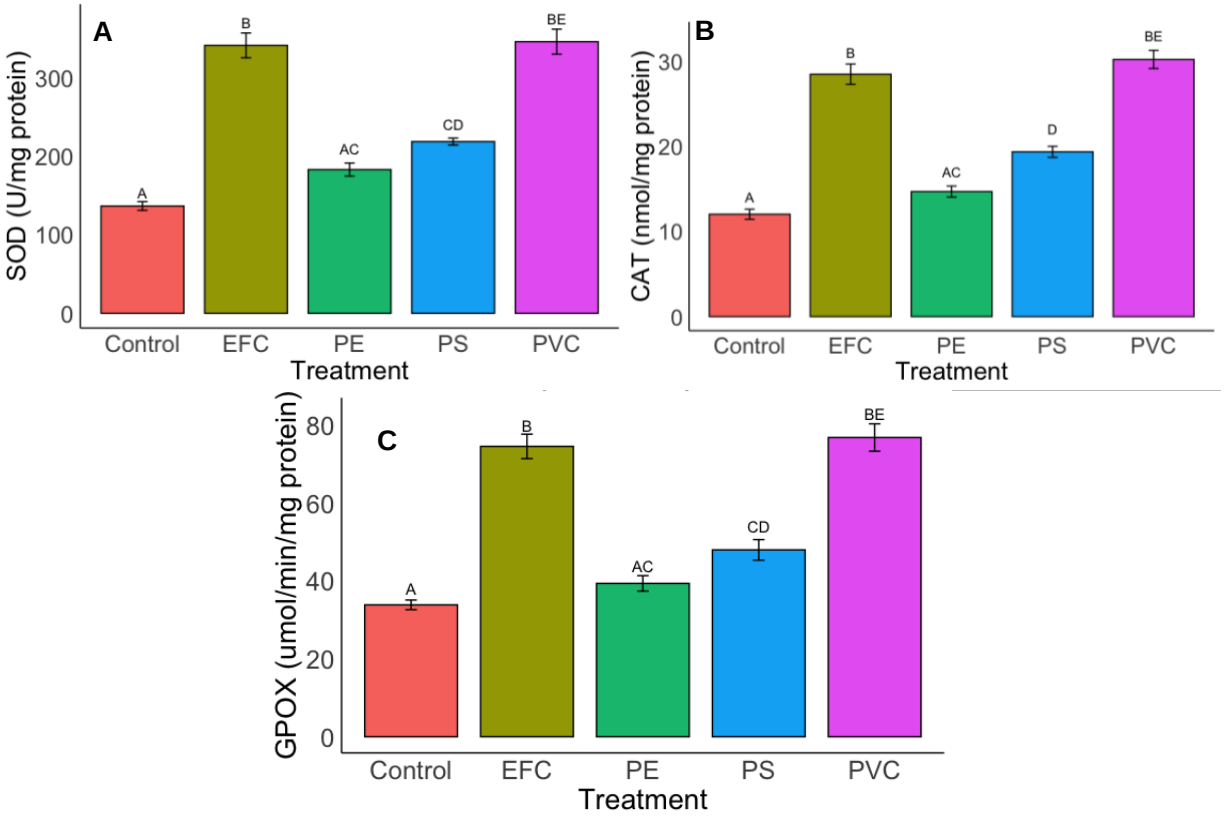


Figure 24: Figures show effects of an edible film coating (EFC) (N=11), polyethylene (PE) (N=8), polystyrene (PS) (N=10) and polyvinyl chloride (PVC) (N=9) microplastic ingestion on antioxidant enzyme activity. Enzymes being, superoxide dismutase (SOD) (**A**), catalase (CAT)(**B**) and glutathione peroxidase (GPOX) (**C**) compared to a control (N=11).

3.4.2 *Oxidative damage*

A similar trend was observed in the oxidative damage biomarkers after 28 days exposure. A linear model indicated a significant increase in PC for fish exposed to EFC, PS and PVC MP diets ($F_{4, 44}$ =423.7, p-value = <0.0001). Similar to enzyme activity, PE did not show sign of a significant increase in PC ($F_{4, 44}$ =423.7, p-value = >0.05). Lipid peroxidation significantly increased in fish fed all MP diets ($F_{4, 44}$ =315.8, p-value = <0.0001). On average, the greatest increase difference was seen in PVC (148.46 nmol/g FW). This was more than a 7-fold increase from the control average (19.21 nmol/g FW). These results are indicative of significant oxidative damage to both proteins and lipids (Fig. 25).

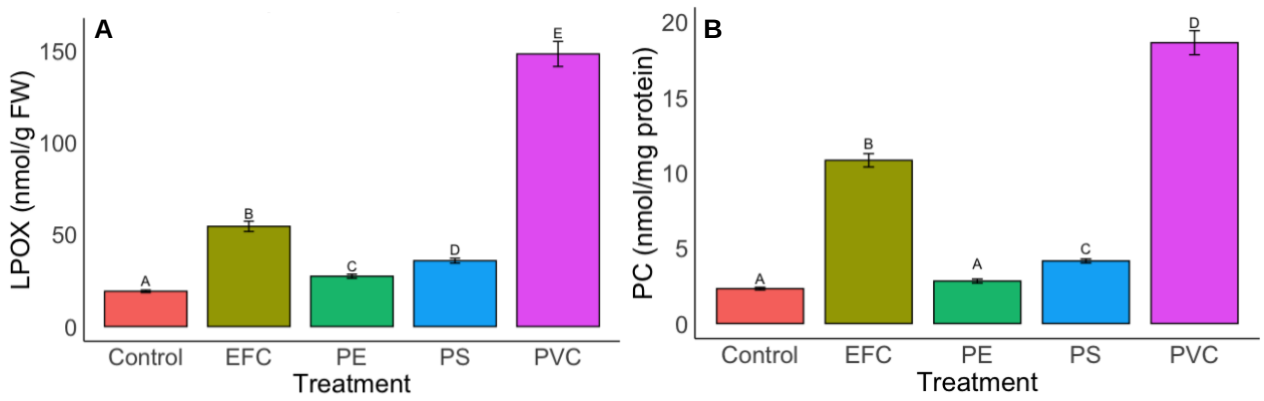


Figure 25: Figures show effects of an edible film coating (EFC) (N=11), polyethylene (PE) (N=8), polystyrene (PS) (N=10) and polyvinyl chloride (PVC) (N=9) microplastic ingestion on oxidative damage biomarkers. Biomarkers being lipid peroxides (LPOX) (**A**) and protein carbonyls (PC) (**B**)compared to a control (N=11).

3.4.3 *Lipid content*

Using a linear model, we identified a clear difference in the percentage of lipids for livers of captive lab fish, to those we found in the wild ($F_{5, 26}$ =6.735, p-value = 0.0002). On average, our control fish had more than double the lipid percentage (37.3%) compared to the wild fish (15.5%). There was no significant difference among lab fish where, plastic treated fish had a similar lipid percentage as the control fish (p-value = >0.05) (Fig. 26).

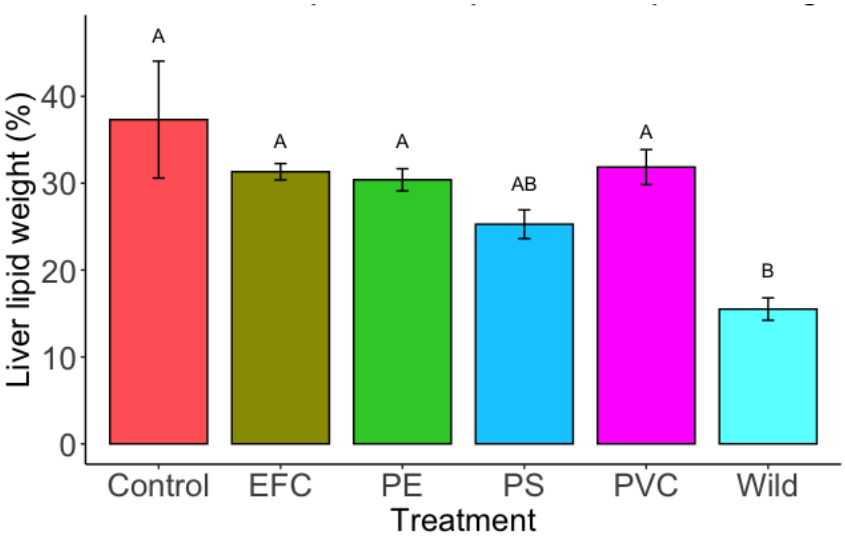


Figure 26: Effect of an edible film coating (EFC) (N=11), polyethylene (PE) (N=8), polystyrene (PS) (N=10) and polyvinyl chloride (PVC) (N=9) microplastic ingestion on the percentage of lipids in triplefin livers. Here we also compare a captive lab fish (control) with fish taken straight from the wild.

3.4.4 *Wild fish comparison*

A one-way ANOVA revealed that the wild fish had significantly lower PC levels than control fish ($F_{1, 19}$ =90.24, p-value = 1.2e-08) (Fig. 27). This suggests there is some oxidative damage to proteins in our control fish however, the magnitude of which is considerably lower than the other PC data (Fig. 25). Although there is some elevation, it is significantly lower than fish fed dietary MP (more than 7-fold for PVC). This is supported by finding no difference in SOD and CAT activity between control and wild fish suggesting no imbalance in ROS. However, we did find an upregulation of glutathione peroxidase (GPOX) ($F_{1, 19}$ =42.25 p-value = 3.16e-06) in control fish (33.907) compared to the wild fish (24.048) (Table 9).

Table 6: Displaying mean values for superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPOX) in untreated captive lab fish (control) (N=11) compared to wild untreated fish (N=10). Significant difference indicated with *

	SOD (U/mg protein)	**CAT** (umol/min/mg protein)	**GPOX*** (umol/min/mg protein)
Control	136.647	12.038	33.907
Wild	148.738	12.566	24.048

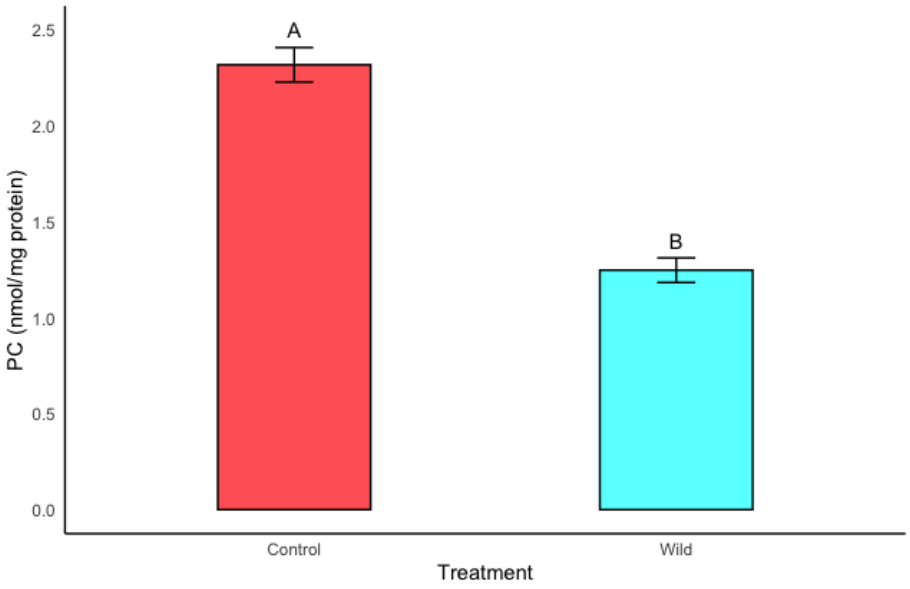


Figure 27: Figure showing a comparison in protein carbonyl (PC) levels in white muscle between non-microplastic treated lab triplefins (Control) (N = 11) and tripelfins analysed immediately from the wild with no treatments (Wild) (N = 10).

3.5 Discussion

The effects of MP exposure on oxidative stress in marine taxa has been an expanding area of research. Most research has focused on the effects of ingesting one or two MP types (Espinosa et al., 2019; Kim et al., 2021; Richardson et al., 2021; Usman et al., 2021; X. Xia et al., 2020). However, given it is likely that wild animals will encounter more than one plastic type, it is important to understand which of these plastic types could be the most damaging. In the present study, we found clear evidence of variation in effects between MP types. Fish from all MP diets showed signs of oxidative stress due to an upregulation of antioxidant enzymes and oxidative damage biomarkers. However, ingestion of PVC incurred the greatest physiological costs.

ROS are generated in greater amounts in pathological situations and during exposure to environmental stressors (Repetto et al., 2012). Further, it has been documented that ingestion of MP can induce an increase in the number of ROS in marine taxa (Table 1). When there is an increase in ROS, antioxidant enzymes become elevated to counteract this. We saw a significant upregulation of SOD, CAT and GPOX enzyme activities for triplefins exposed to dietary EFC, PS and PVC MP, indicating oxidative stress was occurring, suggesting that SOD enzymes were converting more superoxide to H_2O_2 compared to controls. Further, both CAT and GPOX enzymes were actively scavenging the H_2O_2 to convert into water. We found exposure to PVC and EFC MPs caused the greatest level of oxidative stress, due to the major upregulation of SOD, CAT and GPOX enzymes. Romano et al., (2020) also found exposure to PVC induce oxidative stress using goldfish as a model organism. Romano et al., (2020) suggested that oxidative stress can lead to to an increase in cortisol, a stress related hormone, which diverts energy from growth to other stress responses. Although this was not tested in the present study, it does offer an interesting extension to be considered for future research. Interestingly, dietary exposure to PE MPs showed little change in enzyme activity. However, on average, we did see a trend in slight elevation compared to controls. This is in contrast to findings from Qiao et al., (2019) where exposure to dietary PS for 21 days led to significant upregulation of SOD and CAT enzymes in gut tissue of zebrafish compared to controls. It is possible that in general, PE does not induce the same high level of oxidative stress as other MP tested in this study. Alternatively, it is possible that PE treated fish saw an earlier spike in their level of

enzyme activity, which had then decreased again by the end of the experimental period.

With the high levels of enzymatic activity in PVC and EFC treated fish, it is unsurprising that these fish also experienced the most oxidative damage. This was most striking in fish exposed to PVC, as both LPOX and PC levels were more than 7 times higher than the control. This indicated that although the antioxidant enzymes were functioning, this was not enough to prevent oxidative damage to proteins and lipids. ROS attack lipid and protein membranes by removing a hydrogen atom causing the formation of lipid peroxides and protein oxidation. This can be harmful as protein oxidation results in cell death. Further, lipid peroxides are destructive, producing toxic by-products including lipid hydroperoxides which act as mutagens, carcinogens and can produce more free radicals (Helbock et al., 1993; Lushchak, 2016; Quiney et al., 2011; Repetto et al., 2012). Previous studies have demonstrated oxidative damage incurred by PVC ingestion such as findings from Iheanacho & Odo, (2020) who exposed juvenile African sharptooth catfish (*Clarias gariepinus)* to dietary PVC. They sampled three times over a 45-day period and found PVC ingestion elevated lipid peroxide levels which continued to increase over time. Although the present study only sampled post-treatment, we saw a significant elevation after 28-days exposure, indicating oxidative damage to lipids. It would be interesting to consider sampling kinetically for future work to understand how this might change overtime. Other studies have looked PVCs effects on protein oxidation such as Solomando et al., (2020). They found PVC caused a significant increase in protein carbonyls in gilt-head seabream *(Sparus aurata*) after 45 days. A 30-day depuration period allowed for the antioxidant enzymes to combat existing ROS and normalise PC levels in *S. aurata*. We saw significant protein oxidation after 28-days exposure to PVC indicating oxidative damage. it would be interesting to consider a depuration period for future work to understand recovery potential.

It is possible that the observed variation in effects from different plastic types can be related to the different physical and chemical properties of individual plastic types. For example, most PVC is plasticized with dis(2-ethylhexyl)phthalate (DEHP) a known carcinogenic and endocrine disrupting chemical (Adeogun et al., 2018; Manikkam et al., 2013). Recent research has demonstrated that DEHP can affect a suite of

behaviours and physiological functions in marine fish. For example, Yang et al., (2018) exposed marine medaka (*Oryzias melastigma*) to waterborne DEHP for 21 days and found reductions in body weight and length. Further, these juveniles showed signs of altered swimming behaviour. DEHP exposure can be harmful to humans such as its ability to potentially alter genes that are critical to foetal development (Hokanson et al., 2006). For example, workers from a PVC factory in Taiwan have shown long-term effects from DEHP exposure, including increased sperm denaturation and decreased sperm motility, both of which can reduce fertility (Huang et al., 2011). This was attributed to exposure to airborne MP that contained DEHP. In addition, PVC can be abrasive, which depends on its morphology. The MP used in this study had rough edges as they came from shavings of a PVC pipe. Despite being sieved to be in the same size range as the other plastics used, it is possible that they were more abrasive than the other plastic types. Therefore, the observed trend of increased oxidative damage in fish exposed to PVC could, in part, be due to increased mechanical damage after ingestion disrupting the fish physiological processes. Indeed, there were clear differences in the gut morphology in fish exposed to PVC compared to the other plastic types suggesting that this may be the case. It is likely that the observed trends in the present study were a combination of toxic stress as well as stress incurred from mechanical damage (Chapter 2, section 2.4.2).

Many biobased plastics, including EFC used in this study, are produced using chitosan. This biopolymer is sourced from shells of molluscs and crustaceans (Fernandez et al., 2018.; Hasan et al., 2018; Kasmuri et al., 2018). Something of interest is chitosan's applicability in removing heavy metals from aqueous solutions (Cervera et al., 2003). It is possible that these properties are transferable to the EFC used in this study, which may have exposed the fish via ingestion. Heavy metal analysis of the EFC showed accumulation of Cu (0.22 ± 0.137 ppm mg/kg), Ni (1.27 ± 0.134 ppm mg/kg), Cr (4.23 ± 0.196 ppm mg/kg) and Cd (0.003 ± 0.002 ppm mg/kg) however, these are all deemed food safe levels. Exposure to heavy metals such as Fe, Cu and Pb has been shown to cause an upregulation of SOD and CAT enzymes in *Leuciscus cephalus* which could lead to oxidative damage (Hermenean et al., 2015). In humans, oxidative damage is linked to numerous diseases such as cancer, Alzheimer's and diabetes (Almroth, 2008; Stadtman & Levine, 2011.). Oxidative

damage is equally detrimental in fish leading to disease, reducing growth and reproductive output (Kim et al., 2021; Romano et al., 2020).

In fish, the liver is an important storage site for lipids as energy reserves. These are reserved for periods of reproduction and unfavourable conditions such as environmental stressors (Martin et al., 2017; Stallings et al., 2010). Our lack of success in extracting clean proteins from our triplefin livers, lead us to investigate the lipid content in our experimental fish, compared to those in the wild. We found that our experimental control fish had more than double the liver lipid content compared to those that we found in the wild. Although we collected the experimental fish and wild fish at different times of the year (April versus September), it is unlikely that reproductive state was the cause of this variation as both groups were sampled within their breeding season (Montenegro et al., 2022; Northcott & James, 1996). However, it is possible that other environmental factors may have contributed to this finding (i.e., food availability and the temperature of the water). For many temperate fish species, lipid storage is crucial to cope with the colder temperatures lower food availability associated with winter (Fernandes & McMeans, 2019). This is reflected in their lipid storage cycles, for example in cod, their liver lipids reach a max in autumn. These then drop over winter and remain at a low level until spring (Jangaard et al., 1967).

Excess lipid accumulation in the liver can also be linked to disease and induce oxidative stress (Lu et al., 2014; Lu et al., 2017). Using blunt snout bream as a model organism, Dong et al., (2022) tested a low fat (5%) and high fat (15%) diet. Blunt snout sea bream fed high fat diets experienced a significant increase in ROS. We questioned whether our results could be linked to a high fat diet however, NZ greenlipped mussels contain only around 2% fat (Dernekbasi, 2015; Taylor & Savage, 2006). Studies looking at the effects of high-fat diets on fish species tend to identify high-fat diets as 15-22% fat (Du et al., 2006; Lu et al., 2014; Lu et al., 2017), therefore it is unlikely our fish are experiencing stress related to a high fat diet. To address whether there was a difference in fish general health, we looked at the oxidative markers in the white muscle of the wild fish. We found no difference in SOD and CAT antioxidant enzyme activity when comparing our control to wild fish. This suggests that there is probably not a huge imbalance in ROS. We did see an elevation in GPOX for our control but, it is possible that this is linked to the higher lipid content. The slightly higher level of PC

in the control fish could reflect stress from being kept in aquaria. However, this is not at levels that would indicate damage occurring as it is not to the same magnitude as MP treatments. Producing some low levels of PC is part of normal cellular processes (Augustyniak et al., 2015). Therefore, we assume the oxidative damage observed in fish exposed to dietary MP is due to treatment effects.

3.6 Conclusion

The effects of MP ingestion of oxidative stress in fish is a developing area of research where knowledge of testing multiple plastic types is limited. We found clear evidence of MP induced oxidative damage. Here, we identified PVC ingestion to be more harmful than PE and PS MPs. EFC showed a degree of oxidative damage which highlights the need to be mindful of biodegradable plastic composition when looking for plastic alternatives. Our results were not impacted by general fish health during the experimental period and has opened questions on seasonal variation in triplefin lipid content. As marine MP pollution amplifies, understanding how these pollutants effect organisms is important for implementing mitigation strategies.

Chapter 4

General discussion

4.1 Overview

Plastics durability, stability and low production cost have made it an indispensable resource in our daily lives. Items such as packaging, insulation, and medical equipment all contribute to the estimated 367 million tonnes of plastic produced globally in 2020 (PlasticsEurope, 2021). Plastics have many societal benefits, but, at what cost? Mismanaged plastic waste has resulted in the global accumulation of plastic pollution in the marine environment and consequently, accumulation of microplastics (MP). The ubiquity of MP in the marine environment is concerning as multiple taxa are known to ingest these particles (Chapter 1, table 2). The variation in physical and chemical properties of differing plastic types cause a variety of adverse effects (chapter 1, table 2, table, 3). Although MP ingestion is widely studied in fish, most published research tends to focus on the effects ingesting only one or two MP types. Therefore, the primary aim of this study was to identify how a range of MP types affect triplefin health and condition. To address this aim, a 28-day feeding experiment exposing triplefins to either polyethylene (PE), polystyrene (PS), polyvinyl-chloride (PVC) or a biodegradable edible film coating (EFC) was undertaken. In Chapter 2, I explored whether MP ingestion affected triplefin gut-morphology. To achieve this, morphometric measurements including total surface area for nutrient absorption, villus height and villus width were quantified in addition to goblet cell abundance. In chapter three, I investigated whether ingestion of MP would result in an increase in antioxidant enzymes and whether oxidative damage occurred. Overall, the findings in this MSc thesis contribute to the understanding of how ingesting different types of MPs affect fish health. Given marine plastic pollution is increasing, it is important to understand the effects ingestion may have on fish morphology and physiology who consume these particles. If we are able to understand which polymer types are potentially more harmful than others, we may be able to limit our input of that pollutant.

4.2 Effects of microplastic ingestion on gut-morphology

Histological examination of triplefin gastrointestinal tracts (GIT) revealed that MP ingestion can directly affect an organism's gut-morphology (Chapter 2). Overall, fish from all MP diets showed signs of mechanical damage with decreased villus height and width, decreased surface area, and reduced goblet cell abundance. However, the magnitude of damage varied dependent on MP type. Interestingly, ingestion of PVC MP incurred the most mechanical damage as evidenced by a significant reduction in villi surface area, attributed to the significantly reduced villi width (Chapter 2, Fig. 14, Fig 15). In addition, PVC treated fish showed a significant reduction in the number of mucus producing goblet cells. The GIT is an important interface between a fish's internal and external environment, critical for hormone secretion, immune protection, digestion, and nutrient absorption. Digestion and the associated nutrient absorption provide energy for growth, reproduction, and cellular repair (Bakke et al., 2010). Therefore, fish must optimise their nutrient absorption by maximising their absorptive surface area. Here, I demonstrated that when triplefins consume PVC, the absorptive surface is significantly reduced. Given the importance of nutrient absorption across the gut epithelia it is possible that affected fish may not have received the same nutritional input as control fish. A similar response to PVC has been observed in other species such as European seabass (*Dicentrarchus labrax*) (Espinosa et al., 2019; Pedà et al., 2016) and common carp (*Cyprinus carpio*) (Xia et al., 2020) where ingestion resulted in severe villus damage. In terrestrial organisms, exposure to other MP types have shown similar mechanical damage such as findings from Song et al., (2019) who exposed terrestrial snails to PE MP. They found PE exposure incurred lesions to the GIT and caused shortening and breakage to intestinal villi. Lesions have also been observed in mice exposed to PE MP (Djouina et al., 2022). At these sites of intestinal injury, nutrient absorption is impaired and insufficient nutrients can lead to reduced fitness and leave the organism more susceptible to disease.

Goblet cells represent an important defence by secreting a chemical barrier from pathogens and providing lubrication for undigested material. Other species have demonstrated an increase in goblet cell abundance such zebrafish (*Danio rerio)* exposed to PE and PS MP (Limonta et al., 2019). The authors suggested this response was adapted to provide extra barrier protection from mechanical damage. The

decrease in goblet cell coverage in the present study is likely attributed to the reduction in villi surface area in our PVC treated group. These fish are not able to produce as much mucus as those in the control treatment which leaves them vulnerable to microbes. Such consequences have been observed in mice where those deficient in specific mucin coverage resulted in spontaneous initiation of ulcerative colitis (van der Sluis et al., 2006). Although PVC was found to incur the greatest negative effect, observations of other species with a similar response to different MP types highlights the importance of this stressor and to continue including a range of MPs in future work.

There are structural similarities in gut-morphology between humans and most teleost fishes (Chapter 2, Fig. 6). The responses observed in triplefins are analogous to the response of a human with coeliac disease who ingests gluten. Coeliac disease is an autoimmune disorder triggered by the ingestion of gluten. Upon consumption, histological alterations can be seen in the villi. These include inflammation and shortening and swelling of villi until continued exposure eventually results in villus atrophy. Consequently, nutrient absorption is affected, and untreated coeliac patients often suffer from symptoms of malabsorption such as weight loss, and anaemia. Humans with ulcerative colitis and Crohn's disease can be characterised by GIT tissue damage and goblet cell depletion, similar to what was observed in the present study (Theodossi et al., 2006). Gut health often goes hand in hand with oxidative damage as ROS are increasingly produced under periods of stress. Patients with untreated coeliac disease have demonstrated increased oxidative stress. In the present study, the disrupted GIT might have disturbed how nutrients are allocated within the tissue and impaired dietary antioxidant absorption thus, minimising the antioxidant capacity.

4.3 Effects of microplastic ingestion on oxidative biomarkers

I found a clear physiological cost of MP ingestion demonstrated by an increased antioxidant response and subsequent oxidative damage. ROS are constantly being produced as part of normal metabolism. However, when triplefins ingested EFC, PS or PVC MP these were produced in greater amounts due to the stress of ingesting these particles. As a response, antioxidant defences were initiated. However, despite the increase in antioxidant enzymes, it was not enough to restore balance in the number of reactive oxygen species (ROS) being produced, and consequently, damage

occurred (evidenced by elevated lipid peroxides (LPOX) and protein carbonyls (PC)), this was especially apparent in PVC treated fish. Mounting an antioxidant response is an energetically costly process, energy which is often redirected from growth or reproduction. This can be related to the trade-offs associated with life history theory which is based on the assumption that an increase in resource allocation into one function results in resource diversion from other functions (Stearns, 2000) (e.g., increased investment into growth at the cost of reproduction). For example, in Atlantic cod (*Gadus morhua*), females who skip spawning grow significantly larger than females spawning for the second consecutive year. Given the correlation with increased size and greater fecundity in fish, this could be an adaptive life-history for the future potential gain in fecundity (Folkvord et al., 2014). Similarly, when an organism is under oxidative stress, it must invest its energy into removing these oxidants, resulting in reduced growth and fecundity. A study on the alpine swift (*Tachymarptis melba*) found that females with a low resistance to oxidative stress laid poorer quality eggs that were less likely to hatch (Bize et al., 2008).

Although I did see a clear effect of MP ingestion on oxidative stress, I did not see a change in growth or liver condition. It is possible there is a trade-off with resource allocation, "*is liver health prioritised? Or are muscles kept working in optimum condition?".* I saw significant oxidative damage to the triplefins white muscle tissue but the liver remained in relatively good condition. The liver is a highly important organ for survival in periods of unfavourable conditions due it is energy reserve and detoxification abilities (Martin et al., 2017; Stallings et al., 2010). It could be beneficial for an organism to invest more energy into this during periods of oxidative stress. As a result, when their muscles are working, generating energy and subsequently, producing ROS, there is the observed elevation in oxidative damage markers. Although we did not see a strong effect of ingesting EFC, PS and PE MP types on gut morphology, the significant changes in oxidative stress highlight the importance of studying whole organism response.

4.4 Variation of effects caused by different plastic types

Plastics have replaced many historically used resources (e.g., ivory once used for items such as piano keys, combs and billiard balls and Hawksbill turtles shells used for combs and jewellery (Freinkel, 2011)). The expansion of plastic as a resource has taken pressure off other organisms but at a new cost. In the assays used in the present thesis, PVC was the most harmful plastic to ingest. This has been related to the physical and chemical properties of the polymer. In this study, PVC MP were hard with rough edges and likely, abrasive to the GIT. In addition, PVC is often plasticised with carcinogenic chemicals like di(2- ethylhexyl) phthalate (DEHP) which are known to leach out of the plastic. While DEHP can be used in other plastics, it is mainly used in PVC. The New Zealand Ministry for Primary Industries (MPI) released findings on DEHP migration into food products. Here, it was reported that an average of 4.4mg /kg of DEHP was found in corn chips packaged in plastic/foil bags and 0.39mg /kg in frozen meat patties when packaged in a plastic tray with plastic clingwrap. However, the risk of human consumption via contaminated food is within a safe limit (MPI, 2017). There is currently no regulation in the NZ/AUS food standard code (FSC) for plasticiser migration into commercial food. Interestingly, I did not see significant mechanical damage when triplefins ingested PS MP however, I did see signs of oxidative damage to muscle tissue.

In mammalian test subjects, such as mice, PS exposure has demonstrated significant oxidative stress in the testes. This resulted in a decrease in the abundance of sperm and an increase in sperm malformation (Xie et al., 2020). Although not as abrasive, PS MP are chemically harmful, leading to oxidative stress and reproductive toxicity. I did not see an increase in oxidative defence for fish that consumed PE. However, I did see a slight elevation in lipid peroxides but, this could be attributed to fat in the liver. The MP in the present study were originally sourced from plastic bags that had not been exposed to the aquatic environment pre-experimental period. PE has effective absorption properties and can actively accumulate persistent organic pollutants (POP) present in contaminated marine environments. For example, phenatherene, Dichlorodiphenyltrichloroethane (DDT), Perfluorooctanoic acid (PFOA) and DEHP have all demonstrated absorption from PE MP (Bakir et al., 2014). This results in

bioavailability of these pollutants through MP ingestion. This has been observed in blue mussels (*Mytilus edulis*) that accumulated pyrene; a carcinogenic chemical absorbed by the PE MP (Avio et al., 2015). This was physiologically costly for the mussels, resulting in significant oxidative stress. A similar response has also been documented in peppery furrow shell clam (*Scrobicularia plana*) after ingestion of polluted low-density polyethylene (LDPE) particle, resulting in oxidative damage (O'Donovan et al., 2018). Perhaps if the PE MP used in the present study were periodically in the natural marine environment, they may have absorbed POP which would induce greater negative effects on triplefin health. Both blue mussels and peppery furrow clam are important commercial species that are consumed whole which may expose humans to POP.

Similar to PE, there was little evidence of mechanical damage incurred from ingestion of EFC. This is likely attributed to the structure and role of the EFC in that it is an edible film that should break down following ingestion (Giteru et al., 2019). However, the potential for heavy metal absorption highlights the need for careful consideration of biopolymer composition. The results showed a strong antioxidant response suggesting these triplefins were under a level of oxidative stress. Although heavy metal analysis revealed levels of Cu, Ni, Cr and Cd (Appendix, section 5.3) were at food safe levels, this is not to say they won't absorb any POP in the natural marine environment. In the present study, it is likely that the chitosan that was produced from mussel shells was likely contaminated due to the mussels themselves bioaccumulating materials. This highlights the need for consideration of the components used to create biopolymers. While it is important that the use of petroleum-based thermoplastics (i.e. PE, PS and PVC) is reduced due to the serious biological threats they impose, it is also important that the development and use of "sustainable" biopolymers is regulated. To date, there is very little research that investigates whether biopolymers cause negative effects to the taxa that ingest them. However, the findings from the present thesis suggest that there may be "hidden costs" associated with these "ecologically" important products. These "costs" may potentially translate into more community wide effects, such as changes to trophic dynamics.

4.5 Microplastic induced sublethal effects can influence food webs

An important ecological paradigm that fits to this research is “the influence of sublethal effects on food webs.” This research highlights that sublethal effects can depress an organism’s fitness, which has the potential to affect population dynamics. Changes in population dynamics have secondary effects on food webs. Early ecological thinking focused on ideas such as changes in growth, birth, death and its impact on abundances with less importance on their indirect effects. Initially these were tests looking at chemical exposure. Lemke & Mount (1963) looked at the sublethal effects of alkyl benzene sulfonate on bluegill (*Lepomis macrochirus*). The authors focused on growth rate as a response and concluded their findings of continued impairment induced by sublethal effects could result in depleted populations. Later, studies began looking at environmentally relevant sublethal toxins such as metals but, studies still applied their findings to direct effects such as survival and reproduction (McKim et al. 1970). Rand (1995) discussed that histology is a useful method for testing sublethal effects as you can identify alterations in the function of tissues and organs that are not necessarily externally observed. The idea that this could cause “ecological death” was introduced by Scott & Sloman (2004). Although the animal is not overly harmed, they are unable to function in their ecological context as their normal behaviour has been altered. This idea has expanded into looking at the secondary outcomes of sublethal effects. Werner & Moran (2008) suggested that a decreased population size incurred by sublethal effects, has the ability to alter the structure of biological communities. I have demonstrated that MP ingestion has the potential to cause sublethal effects on triplefins. I found structural damage through mechanical abrasion and chemical toxicity reflected in oxidative biomarkers after consumption. Although this did not cause mortality, it negatively affected the most important area for nutrient absorption which can lead to decreased fitness. Sublethal toxicity also reduces fitness by decreasing the energy budget, growth performance and developmental success as more energy is allocated to detoxification (Werner & Moran 2008). This was apparent in triplefins as they demonstrated resource partitioning by diverting energy away from the muscles to keep the liver in good condition. As a result, the muscles were more susceptible to oxidative damage. This has potential negative effects on the wider ecosystem as these fish have decreased fitness which can lead to a population decline and in turn, impact food webs. Food webs with low biodiversity and few interactions are generally the

worst affected due to the positive relationship between species richness and community stability (Kondoh 2003).

4.6 Future work

In future research, it would be informative to sample for oxidative stress biomarkers kinetically. This would allow us to get a better understanding on the entire antioxidant response during exposure. Further, it would be interesting to investigate whether a depuration period would be beneficial in recovery such as findings from Capó et al., (2021) or if the response remains (Mbugani et al., 2022). Although I did take samples and extract DNA from the gonads of triplefins used in this study, COVID restrictions that led to reduced lab availability meant I was unable to complete this on time. However, this data will be included in publications that arise from this thesis (See Appendix section 5.3). Future work is planned to take livers from wild triplefins in July (the same month as initial collection). This would allow us to account for temporal variation in liver lipid stores to get a better understanding of energy budgets in triplefins.

4.7 Conclusion

As marine MP pollution continues to increase, understanding how these pollutants affect organisms is important for implementing mitigation strategies. Results from the present studies highlight the need for expansion in research investigating the range in responses of ingesting multiple MP types. Here, the results identified PVC ingestion resulted in the greatest physiological cost. Specifically, they show PVC ingestion to negatively affect gut-morphology (Chapter 2) and generate the greatest oxidative damage response (Chapter 3). Although the effects of ingesting EFC, PS and PE MP types were not reflected in gut morphology, the significant changes in oxidative stress highlight the importance of studying whole organism response. Understanding how organisms are directly affected by these pollutants can promote awareness and influence future management.

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Appendix

5.1 Supplementary text and figures to accompany chapter 2 methods section 2.2

Figure 28: Example of food preparation. 2 g feeding blocks consisting of mussels 87.5% mussel and 2.5% MP frozen in chocolate moulds. The food blocks were negatively weighted with a stainless steel nut

Table 7: Wax embedding process for 5um sections of gut. Repeated solvents require a fresh change between soaking durations

Solvent	Duration
70% Ethanol	1 hour
95% Ethanol	45 minutes
100% Ethanol	30 minutes
100% Ethanol	30 minutes
Xylene	20 minutes
Xylene	20 minutes
Paraffin Wax	40 minutes
Paraffin Wax	40 minutes
Paraffin wax	40 minutes

Table 8: Staining procedure for hematoxylin and eosin. Repeated solvents require a fresh change between soaking durations

Solvent	Duration
Xylene	2 minutes
Xylene	2 minutes
Xylene	2 minutes
100% Ethanol	2 minutes
100% Ethanol	2 minutes
70% Ethanol	2 minutes
Distilled water	2 minutes
Hematoxylin	4 minutes
Tap water	4 minutes
Scotts water	2 minutes
Tap water	3 minutes
EOSIN	4 minutes
70% Ethanol	4 minutes
100% Ethanol	2 minutes
100% Ethanol	2 minutes
100% Ethanol	2 minutes
Xylene	2 minutes
Xylene	2 minutes
Xylene	2 minutes

Table 3: Staining procedure for Periodic acid-shiffs (PAS) + Alcian blue for goblet cell quantification. Repeated solvents require a fresh change between soaking durations

Solvent	Duration
Xylene	2 minutes
Xylene	2 minutes
Xylene	2 minutes
100% Ethanol	2 minutes
100% Ethanol	2 minutes
70% Ethanol	2 minutes
Distilled H_2O	2 minutes
Alcian Blue	30 minutes
Tap water rinse	10 minutes
0.6% Periodic Acid	5 minutes
Distilled H_2O	2 minutes
Schiff reagent	30 minutes
Hematoxylin	4 minutes
Tap water rinse	5 minutes
Scotts water	2 minutes
Tap water rinse	3 minutes
70% Ethanol	2 minutes
100% Ethanol	2 minutes
100% Ethanol	2 minutes
Xylene	2 minutes
Xylene	2 minutes
Xylene	2 minutes

Table 4: Deparaffinisation steps. Repeated solvents require a fresh change between soakings

Solvent	Duration
Xylene	2 minutes
Xylene	2 minutes
Xylene	2 minutes
100% Ethanol	2 minutes
100% Ethanol	2 minutes
70% Ethanol	2 minutes
Distilled water	2 minutes

5.2 Supplementary text for chapter 3 methods section 3.2

DNA extractions

To extract DNA from the gonads ~25 µg of tissue per fish was used with an ISOLATE II genomic DNA kit. First for pre-lysis, 180µl of lysis buffer and 25µl of proteinase solution was added to the tube and vortexed. The tubes were then incubated for 3 hours at 56°C with a few intermittent vortexing. Sample tubes were then lysed by briefly vortexing before adding 200µl of the lysis buffer. The tubes were then vortexed vigorously and incubated for 10 minutes at 70°C. Following this, the DNA binding conditions must be adjusted by vortexing, adding 210µl of 100% ethanol and then vortexing again. To bind the DNA the samples were centrifuged for 1 minute in a ISOLATE II genomic DNA spin column nested in a 2ml collection tube. The flow-though was discarded and the liquid in the collection tube was kept. The samples were then washed by adding 500µl of wash buffer to the tube and using repeating the centrifuge step. The flow through was discarded and the collection tube again reused. To dry the sample , it was centrifuged for one minute to remove any residual ethanol. To elute the DNA, 100µl of preheated elution buffer (70°C) was placed on to the center of the silica membrane and incubated for one minute at room temperature

5.3 Heavy metal analysis for EFC

Elemental analysis of heavy metals in the ingredients and formulated fish feeds. Results in ppm (mg/kg)

Material	Cr	Ni	Cu	As	Cd	Pb
Polyethylene glycol 400	<0.001	<0.001	<0.0025	0.02 ± 0.005	<0.00025	<0.0001
Zein	2.54 ± 0.42	2.49 ± 0.414	0.8 ± 2.769	0.08 ± 0.036	0.05 ± 0.027	<0.0001
Chitosan	12.11 ± 1.195	3.39 ± 0.335	0.41 ± 0.041	<0.0025	0.01 ± 0.001	0.06 ± 0.006
Polyvinyl alcohol	0.12 ± 0.016	0.02 ± 0.002	<0.0025	<0.0025	0.01 ± 0.001	<0.0001
Edible film (control)	4.23 ± 0.196	1.27 ± 0.134	0.22 ± 0.137	<0.0025	0.003 ± 0.002	<0.0001
Polyethylene (control)	0.18 ± 0.03	0.14 ± 0.018	1.52 ± 0.372	<0.0025	<0.00025	<0.0001
Meat pellet control	0.05 ± 0.031 (0.187–0.369)	0.09 ± 0.023 (1.2–3.44)	1.7 ± 0.32 (0.6–1.5)	2.19 ± 0.121 (0.009–0.011)	0.26 ± 0.02 (0.002–0.028)	0.19 ± 0.036 (0.14–0.62)
Meat pellet (+ PE)	0.08 ± 0.062	0.16 ± 0.073	2.38 ± 0.504	2.11 ± 0.095	0.3 ± 0.01	0.27 ± 0.065
Meat pellet (+ EFC)	0.07 ± 0.13	0.18 ± 0.127	2.21 ± 0.493	1.99 ± 0.107	0.3 ± 0.023	0.25 ± 0.072

Note. Values are +/- standard deviation (n = 3). Values from the nitric acid blank have been deducted from the displayed results

Values in bracket indicate the range of reported values (Pereira et al., 2018).