Assessing the Biological Effects of Exposure to Microplastics in the Three-Spined Stickleback (*Gasterosteus aculeatus*) (Linnaeus 1758)

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Abstract

The global pollution of aquatic environments with microplastics and their interaction with wildlife is of concern. Ingestion of plastic has been reported for a wide range of species but little is known about the potential effects of such ingestion. The aim of this thesis was to assess the biological effects that are induced by the ingestion of microplastic in the three-spined stickleback (*Gasterosteus aculeatus*). Novel data for the ingestion, retention, egestion and induced effects of microplastic in multiple ontogenetic life stages are presented. Microplastics of different types and sizes were found to be ingested from the water and diet. Additionally, trophic transfer of microplastic was found as a further route for ingestion. Whereas ingestion of relatively small plastic was not found to induce blockages or obstructions of the gastrointestinal tract, ingestion of relatively large plastic had the potential to result in prolonging effects on food digestion. However, all used microplastics were found to be egested successfully. Effects on length, weight and condition index K were found but varied between exposure types and data suggests that observed short term effects were induced by plastic associated chemicals. Whereas molecular analysis of cytochrome P450 1A confirmed exposure to xenobiotics, relative expression of vitellogenin indicated no exposure to oestrogenic, plastic derived chemicals. Degraded polymer structures showed to have a higher biological activity due to enhanced leaching of endocrine disrupting, plastic derived chemicals and showed a potential to disturb energy metabolism. In addition, plastic was found to act as vectors for absorbed bisphenol A from the water column via trophic transfer to stickleback larvae where desorption of accumulated chemicals had the potential to induce toxic effects. The data presented in this thesis indicate that microplastics can be ingested and can induce negative effects in multiple ontogenetic life stages of sticklebacks.

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Author´s declaration

I confirm that the chapters presented in this thesis are my own original research undertaken as a PhD student at the Department of Biological Sciences at the Institute of Marine Sciences at the University of Portsmouth (October 2012 – September 2014) and at the Environment Department at the University of York (December 2014 - November 2015). Whilst registered as a candidate for the above degree, I have not been registered for any other research award.

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Chapter 1

Introduction

1.1 Overview

Plastic products are an essential part of modern life with applications ranging from clothes, electronics and furniture to highly specified products like prosthetics and engineered nanoplastics for drug delivery. However, since mass production of plastic products started in the 1950s to 1960s, reports have emerged documenting the contamination of the marine environment with synthetic plastics (Hays and Cormons, 1974, Colton *et al.*, 1974, Carpenter *et al.*, 1972, Carpenter and Smith, 1972, Kartar *et al.*, 1973). The plastic industry has grown to a global production of 299 million tonnes of plastic in 2013 (PlasticsEurope, 14/15) and it is estimated that nowadays plastic accounts for 60 - 80% of all marine debris (Derraik, 2002, Gregory and Ryan, 1997, Barnes *et al.*, 2009). Further there is evidence for the contamination of both marine and fresh water environments with plastic litter on a global scale (Derraik, 2002, Eriksen *et al.*, 2013a, Moore *et al.*, 2011, Galgani *et al.*, 2015, Cózar *et al.*, 2014, Ivar do Sul and Costa, 2014, Lusher, 2015). Deleterious effects following the entanglement in larger plastic items (Sazima *et al.*, 2002, Gudger and Hoffmann, 1931, Schwartz, 1984, Barreiros and Raykov, 2014, Carr, 1987, Laist, 1997, Kühn *et al.*, 2015) have been reported. However, the presence of small plastic fragments, so called microplastics (< 5 mm) and their reported ingestion (consumption of a substance through the mouth into the gastrointestinal tract) by a wide range of aquatic species (Kühn *et al.*, 2015, Setälä *et al.*, 2014, Cole *et al.*, 2013, Browne *et al.*, 2008, Mazurais *et al.*, 2014, Besseling *et al.*, 2013) is of concern. Even though numerous reports are present that document the ingestion of plastic, the knowledge about the effects of such ingestion remain unclear for many species (Zarfl *et al.*, 2011).

The aim of this thesis was to assess the effects of ingested microplastic in fish using the three-spined stickleback (*Gasterosteus aculeatus*) as a model organism. Contributing to a first report that nanoplastics can be transferred through the food chain from zooplankton to fish (Cedervall *et al.*, 2012), Chapter 3 assessed the potential for the trophic transfer of microplastic from zooplankton to fish and determined retention, egestion (discharge of undigested material from the gastrointestinal tract via the anus as faeces) and biological effects following the exposure in the adult fish. First evidence that ingestion of plastic by fish larvae can have negative effects on development (Mazurais *et al.*, 2014) was further investigated in Chapter 4 which assessed the biological effects following the accidental ingestion of microplastic particles from a

contaminated water column by young stickleback larvae. Furthermore, there is a considerable lack of knowledge concerning the effects of ingested, commonly used consumer plastic items. Chapter 5 measured the effects arising from a dietary exposure in adult sticklebacks to fragments of degraded and non-degraded plastic carrier bags. Finally, adding to first evidence that ingested plastic can act as a vector for absorbed chemicals (Teuten *et al.*, 2009) and induce biological effects in fish (Rochman *et al.*, 2013b, Rochman *et al.*, 2014) Chapter 6 assessed the plastic mediated trophic transfer of absorbed chemicals by microplastic and the arising effects in exposed young stickleback larvae.

1.2 Plastic in aquatic environments

The pollution of the oceans and fresh water ecosystems with plastic litter is of concern since the durable characteristics of synthetic polymers in aquatic environment leads to accumulation and therefore higher levels of plastic over time (Barnes *et al.*, 2009). The various pathways through which plastic enter aquatic environments, the global scale of this introduction and the wide distribution of plastic litter from lakes (Eriksen *et al.*, 2013a, Imhof *et al.*, 2013) and rivers (Moore *et al.*, 2011, Lechner *et al.*, 2014) to coastal areas (Sadri and Thompson, 2014, Browne *et al.*, 2010), the open ocean (Cózar *et al.*, 2014, Moore *et al.*, 2001), the sea floor (Schlining *et al.*, 2013, Woodall *et al.*, 2014), remote islands (Hirai *et al.*, 2011, Heskett *et al.*, 2012) and the Arctic (Obbard *et al.*, 2014, Bergmann and Klages, 2012) make accurate predictions about the total plastic burden of aquatic environments difficult. In addition, the presence of unknown levels of microplastics (see section 1.2.3), adds to these uncertainties. However, a growing number of reports document the presence of plastic litter in aquatic environments and highlight the potential extent of the pollution. Based on these more recent investigations plastic has been reported to be the dominant type of debris in the marine environment (Coe and Rogers, 1997).

1.2.1 Plastic introduction and presence in the marine environment

It is estimated that around 80% of marine debris originates from land based sources (GESAMP, 1991). Especially rivers have been shown to be heavily contaminated with plastic litter (Moore *et al.*, 2011, Lechner *et al.*, 2014, Morritt *et al.*, 2014) and have the potential to carry this plastic burden into the oceans. The plastic litter introduction of the

Danube into the Black Sea has been estimated to be 4.2 tons of plastic debris day^{-1} and plastic debris at 12,932 pieces of plastic m^{-3} with a total weight of 30.4 tons of two rivers draining the densely populated Los Angeles Basin was found to be carried towards coastal waters over a 72h sampling (Lechner *et al.*, 2014, Moore *et al.*, 2011). Focussing on the effects of urban areas on the input of plastic in the oceans Jambeck *et al.* (2015) presented a framework to calculate the input of 192 costal countries into the oceans (Figure 1.1). Their results suggest that an estimate of 4.8 to 12.7 million metric tonnes of plastics entered the oceans in 2010 as a result of mismanaged plastic waste. The same study also hypothesised that quantities of plastic waste entering the oceans are expected to rise by one order of magnitude from 2010 to 2025 due to population growth and increased plastic production (Jambeck *et al.*, 2015). The remaining 20% of plastic litter entering the oceans are thought to derive from ocean based sources like cruise ships, commercial fishing vessels and recreational boaters which dump their debris directly in the ocean (Horsman, 1982, UNESCO, 1994).

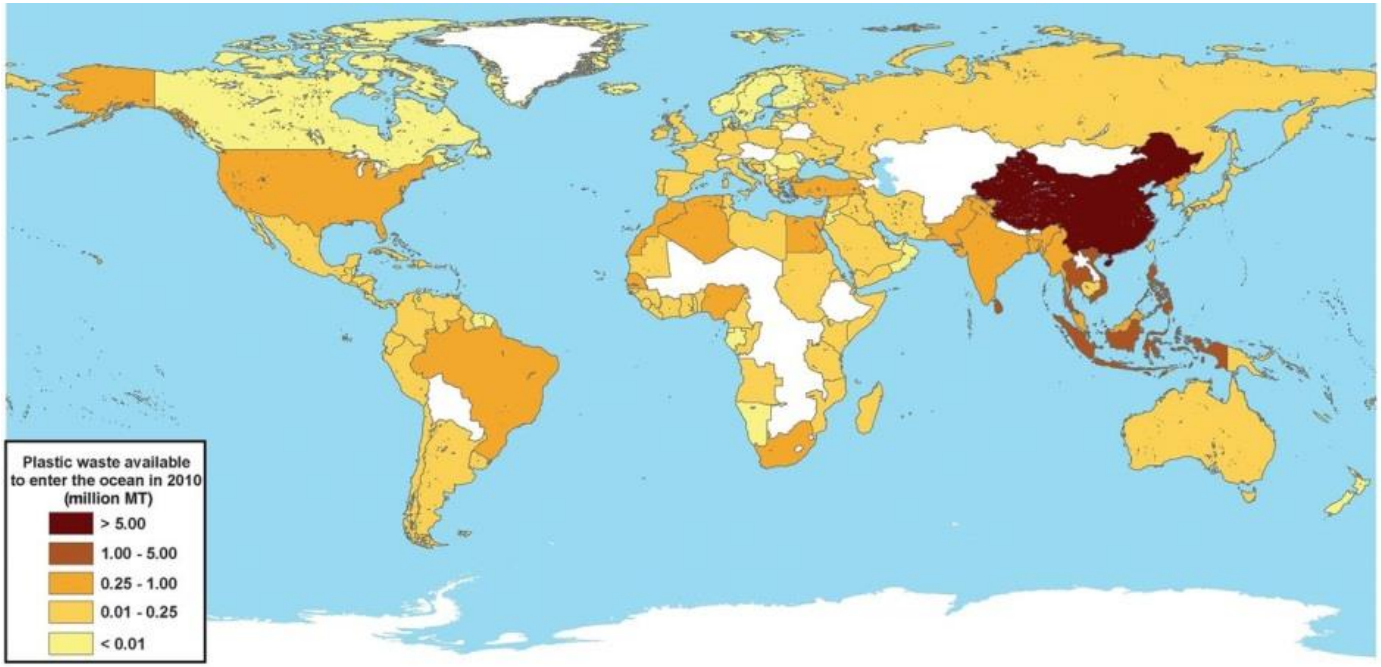

Figure 1.1: Plastic waste input from land into the ocean. Global map with each country shaded according to the estimated mass of mismanaged plastic waste [millions of metric tonnes (MT)] generated in 2010 by populations living within 50 km of the coast. From: Jambeck *et al.* (2015).

Many studies worldwide have investigated the plastic pollution of coastal regions, mainly beaches. In 2002 a review by Derraik (2002) listed 37 studies that evaluated the composition of marine debris for plastic content, 24 of them on beaches. Plastic contamination of beaches has been found to be correlated with their proximity to urban areas (Leite *et al.*, 2014) but shorelines are also a sink for plastic litter carried over the oceans and then deposited in downwind sites as a result of tidal movements (Browne *et al.*, 2010, Eriksson *et al.*, 2013, Williams and Tudor, 2001). It is reported that plastic litter can account for 51 - 90% of shoreline debris (Derraik, 2002) and can accumulate with rates as high as 484 pieces day^{-1} (Cooper and Corcoran, 2010). As a result, beach sediments have been found to be contaminated with plastic fragments at concentrations of up to 80,000 items m^{-2} (Khordagui and Abu- Hilal, 1994) or 300,000 items m^{-3} (Costa *et al.*, 2010).

Estuaries are very special environments forming transition areas from land based fresh water drainage and fully marine conditions and are seen as one of the most biologically productive aquatic environments (Kennish, 2002). However, their often close proximity to urban areas and waste water treatment outflows make them particularly vulnerable for anthropogenic pollutants contaminating estuaries in high concentrations, including plastic (Kennish, 2002, Williams and Simmons, 1997, Browne *et al.*, 2010, Holmes *et al.*, 2014, Bakir *et al.*, 2014b, Smith and Edgar, 2014). Additionally, plastic input from the ocean poses a potential introduction route (Browne *et al.*, 2010). As a consequence, sediment and water samples have been found to be polluted with plastic litter; Thirty sediment samples from the Tamar Estuary, UK were found with a total of 952 plastic items of which 65% were microplastics compared to macroplastic litter items (Browne *et al.*, 2010). Water samples have been found with high plastic densities of up to 4137.3 ± 2461.5 plastic pieces m^{-3} which were found in trawls from the Yangtze Estuary, China (Zhao *et al.*, 2014).

In the open ocean plastic debris are suspected to accumulate in 5 major gyres (IPRC, 2008, Lebreton *et al.*, 2012, Law *et al.*, 2010, Martinez *et al.*, 2009, Barnes *et al.*, 2009, Ryan *et al.*, 2009, Cózar *et al.*, 2014) (Figure 1.2) at high levels (Table 1.1). However, the data that is available to date is produced from the investigation of just small surface areas of these accumulation zones and makes the calculation of accurate numbers for the total plastic burden of the oceans difficult. Estimated quantities are reported with a total of 5.25 trillion plastic particles and a combined weight of 268,940 tons to litter the

oceans globally (Eriksen *et al.*, 2014). However, these numbers are likely to be an underestimation of the real extent of the plastic pollution due to the constant pollution of the marine environment with vast amounts of plastic debris (Jambeck *et al.*, 2015) and sampling techniques that exclude small plastic fragments (Norén, 2007). An extensive list of studies investigating the presence of plastic in the world's oceans can be found in Lusher (2015).

Table 1.1: Levels of plastic accumulation in the 5 major gyres. A list of selected studies to display the high levels of plastic debris within the five accumulation zones.

Gyre	Sampling method	Plastic density	Reference
North Pacific	333µm manta trawls	334,271 pieces km^{-2}	Moore *et al.* 2001
		5,114 g km^{-2}	
	200µm neuston nets	1,150 g km^{-2}	Cózar *et al.* 2014
South Pacific	333µm manta trawls	396,342 pieces km^{-2}	Eriksen *et al.* 2013
	200µm neuston nets	840 g km^{-2}	Cózar *et al.* 2014
North Atlantic	plankton net trawls	580,000 pieces km^{-2}	Law *et al.* 2010
	200µm neuston nets	1,200 g km^{-2}	Cózar *et al.* 2014
South Atlantic	Visual spotting	100 pieces km^{-2}	Ryan *et al.* 2014
	200µm neuston nets	1,200 g km^{-2}	Cózar *et al.* 2014
Indian Ocean	200µm neuston nets	790 g km^{-2}	Cózar *et al.* 2014

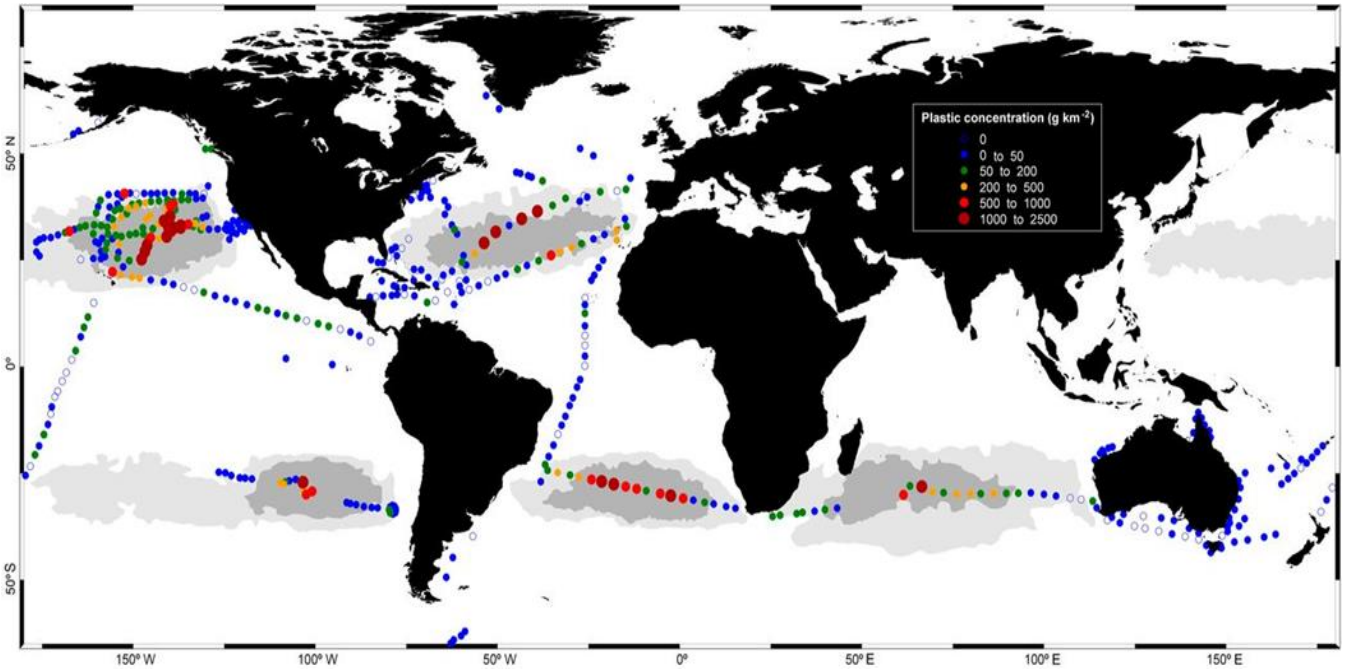

Figure 1.2: Concentrations of plastic debris in surface waters of the global oceans. Coloured circles indicate mass concentrations (legend on top right). The map shows average concentrations in 442 sites (1,127 surface net tows). Grey areas indicate the accumulation zones. Dark and light grey represent inner and outer accumulation zones, respectively; white areas are predicted as nonaccumulation zones. From: Cózar *et al.* (2014).

1.2.2 Plastic introduction and presence in freshwater environments

Contamination from landfill sites or recreational litter has been proposed (Barnes *et al.*, 2009, Jayasiri *et al.*, 2013, Thornton and Jackson, 1998) but also small plastic fragments and fibres released by consumer products and clothes (Browne *et al.*, 2011, Fendall and Sewell, 2009) contribute to the pollution of freshwater environments. Even though recycling schemes are developed further in Europe, many countries still choose to landfill most of their plastic waste over recycling or energy recovery (PlasticsEurope, 14/15). With about 70% of its plastic waste being landfilled, the UK was part of the bottom 7 (out of EU-27+CH/NO) countries in Europe regarding recycling and energy recovery for plastic waste in 2012 (PlasticsEurope, 14/15). With a plastic demand in the UK of approximately 4 million tonnes annually (PlasticsEurope, 14/15), large amounts of landfilled plastic waste have the potential to enter the environment.

It became prominent over the past years that many personal care products like toothpaste, body scrubs, cosmetics and soaps contain small plastic fragments as substitutes for natural ingredients like crushed nut shells, almonds, oatmeal and salts (Hintersteiner *et al.*, 2015, Fendall and Sewell, 2009, Risk, 2012, Zitko and Hanlon, 1991) (Figure 1.3). Added synthetic polymers in personal care products can account for 10.6% of the product in weight, meaning that a 100 ml cream can contain up to 10.6 g of microplastics (Risk, 2012).

Figure 1.3: Microplastic particles can be found in consumer products as substitutes for natural ingredients like crushed nut shells. These microplastics can be as small as 10 µm which is too small to be filtered out by waste water treatment plants. Accordingly theses small plastic particles have the potential to end up in the environment. http://time.com/74956/states-are-cracking-down-on-face-wash/.

These designed microplastics, made of polyethylene (PE), polypropylene (PP), polyethylenterephthalat (PET) and polymethylmethacrylate (PMMA) as well as Nylon are too small to be filtered by waste water treatment plants (typically coarse > 6 mm, and fine screens 1.5 - 6 mm) (Vesilend, 2003) and have the potential to end up in the environment (Gregory, 1996, Habib *et al.*, 1998, Browne *et al.*, 2011). Furthermore, the washing of synthetic clothes might add to the contamination of the environment with plastic; Browne *et al.* (2011) reported a strong resemblance of fibres found in coastal samples compared to ones found in areas that receive sewage discharges and sewage effluents. The study linked the found fibres to washed clothes and reported that a single wash of synthetic clothing can result in the release of over 1900 fibres into the environment (Browne *et al.*, 2011). Additionally, the accidental release of plastic resin pellets or powder used for airblasting or as pre-production materials is suspected to contribute to the pollution of freshwater and marine environments (Gregory, 1978, Gregory, 1996).

These multiple input sources have led to the contamination of fresh water environments with plastic, especially close to urban areas (Moore *et al.*, 2011, Eriksen *et al.*, 2013a). In general the contamination of fresh water environments with plastic waste has received less attention by the scientific community, compared to the marine environment (Thompson *et al.*, 2009, Wagner *et al.*, 2014, Eerkes-Medrano *et al.*, 2015). However, rivers, lakes and affiliated beaches have already been reported to be polluted with plastic debris; Eriksen *et al.* (2013a) found numbers of plastic particles as high as 466,000 particles km^{-2} downstream of urban areas with an average of 43,000 particles km^{-2} in the Laurentian Great Lakes in the USA (Eriksen *et al.*, 2013a). A study conducted by Moore *et al.* (2011) revealed high numbers of plastic particles in two urban rivers and reported a total of 2.3 billion plastic particles with a maximum density of 12,932 items m^{-3} of varying sizes collected during a 72h sampling (Moore *et al.*, 2011). The amount of plastic pollution found in the Austrian part of the Danube river, over a two year study using driftnets reported that the mass of drifting plastic debris (4.8 ± 24.2 g per 1000 m^{-3}) was higher than those of fish larvae (3.2 ± 8.6 g 1000 m^{-3}) (Lechner *et al.*, 2014). Not just water bodies but also sediment samples from shorelines of the Great Lakes in North America were found to be contaminated with plastic and samples were found to contain 1576 pieces of plastic, most of them plastic fragments or pellets (Zbyszewski *et al.*, 2014). In sediment samples taken from a beach affiliated to the subalpine Lake Garda contamination with plastic was found to be as high as 483 ± 236 macroplastic particles m^{-2} and 1,108 ± 983 microplastic particles m^{-2} (Imhof *et al.*, 2013).

1.2.3 Microplastics in aquatic environments

Whereas larger plastic items are easily counted and sampled, smaller fragments have the potential to get overseen and are considered a less well studied component of marine debris (Doyle *et al.*, 2011). These partly microscopic plastic fragments are separately classified to distinguish them from bigger plastic debris like meso (5 - 25 mm) and macro debris (> 25 mm) (Lee *et al.*, 2013b) since their high abundance in the environment makes them a pollutant in their own right (Ryan *et al.*, 2009, Thompson *et al.*, 2004). However, microplastic has not yet been uniformly defined on the basis of its size (Thompson, 2015). An upper size limit of 5 mm has been widely accepted (Arthur *et al.*, 2009, Galgani *et al.*, 2010). A lower size limit for microplastics of 333 µm has been proposed simply due to the lower mesh size limit of neuston nets (Arthur *et al.*,

2009). However, this lower classification limit of microplastic excludes particles in the small micrometre range. Sampling of surface waters with 80 µm mesh sizes found concentrations of collected plastic to be 1000 times higher compared to the proposed 333 µm mesh sizes and 100,000 times higher compared to 450 µm nets, highlighting the amount of microscopic particles that are missed with larger mesh sized sampling techniques (Norén, 2007).

In this thesis the term microplastics will be used to describe plastic fragments of < 5 mm.

This classification which includes plastic particles in the low micrometre range to pieces of 5 mm as microplastic is the to date most widely used classification (Thompson, 2015) and has been applied previously for studies investigating the effects of ingested plastic particles (Browne *et al.*, 2008, von Moos *et al.*, 2012, Cole *et al.*, 2013, Farrell and Nelson, 2013, Setälä *et al.*, 2014, Mazurais *et al.*, 2014).

In general microplastics in aquatic environments can be divided in two categories:

1. Primary microplastics

Primary microplastics are manufactured specifically in the size range of a few micrometres for personal care products like toothpaste (10 µm) (Hintersteiner *et al.*, 2015) up to the maximum size range of the here used definition of microplastics (< 5 mm) for plastic resin pellets (typically 2 - 5 mm). These primary microplastics are used for a wide range of applications including personal care products (Hintersteiner *et al.*, 2015, Fendall and Sewell, 2009, Risk, 2012, Zitko and Hanlon, 1991), airblasting (Gregory, 1996) and as precursor for final plastic products and medical applications like drug delivery (Brem and Gabikian, 2001). The release into the environment as part of their application process or due to accidental release during transport and handling has been reported (Gregory, 1996, Habib *et al.*, 1998, Browne *et al.*, 2011, Hays and Cormons, 1974, Harper and Fowler, 1987).

2. Secondary microplastics

During its lifetime in aquatic environments plastic is exposed to environmental factors causing stress to the material, resulting in an accelerated aging and embrittlement of the material in a process called degradation; Andrady (2011) defined the process of degradation of synthetic polymers as "a chemical change that drastically reduces the average molecular weight of the polymer" and that "the mechanical integrity of plastics invariably depends on their high average molecular-weight, any significant extent of degradation inevitably weakens the material" (Andrady, 2011). Accordingly, degradation of the polymer structure can cause the loss of useful properties and mechanical integrity (Gregory and Andrady, 2003, Andrady *et al.*, 1998). The process of degradation affects large plastic items as well as microscopic ones, resulting in ever smaller fragments, possibly leading to nano-sized particles. The final outcome of the process of degradation of plastic would be the complete mineralisation of the polymer which is the conversion of all the plastics organic carbon into carbon dioxide (Andrady, 2011, Andrady, 1994, Eubeler *et al.*, 2009). However, depending on plastic characteristics and environmental conditions this process may take up to 500 years before a plastic item is completely decomposed (Gorman, 1993, UNESCO, 1994).

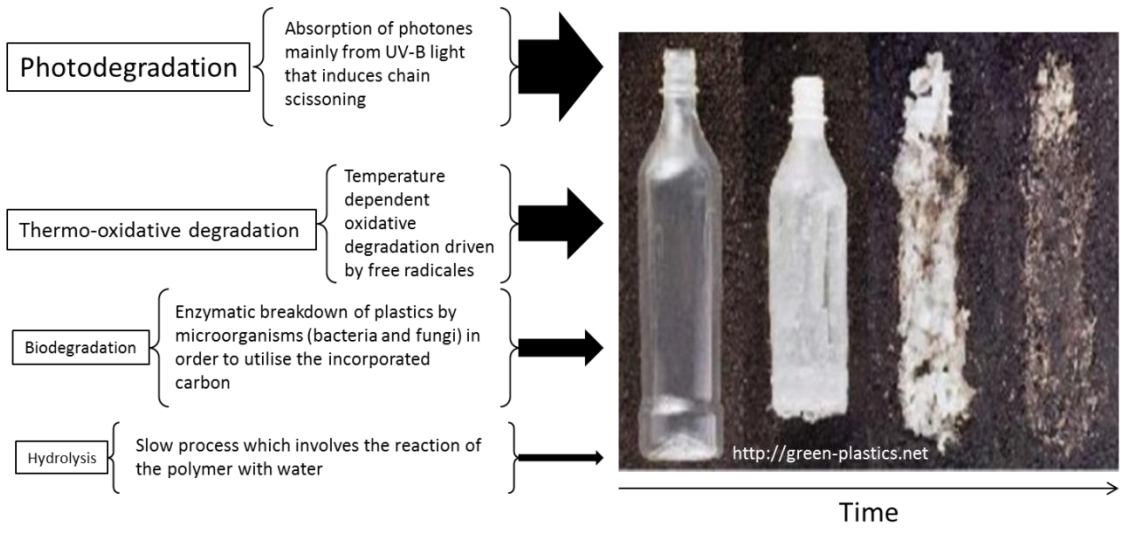

Figure 1.4: Environmental chemical factors of plastic degradation identified by Andrady (2011). Individual contribution of the single factors is to the degradation of a plastic item in the environment, representative for land based degradation. Larger size illustrates a higher contribution of the factor to the degradation process.

Four environmental, chemical degrading factors were identified by Andrady (2011)) as (1) Photodegradation, (2) Thermo-oxidative degradation, (3) Biodegradation and (4) Hydrolysis (Figure 1.4) In addition to these chemical processes mentioned by Andrady (2011) the breakdown of polymer structures due to physical forces like abrasion, wave action, collisions, saltation and traction also occurs (Cooper and Corcoran, 2010). Photodegradation and thermo-oxidative degradation are the primary causes for the accelerated polymer aging in aquatic environments whereas biodegradation and hydrolysis play just a minor role (Andrady, 2011, Gregory and Andrady, 2003). One reason for the higher plastic degradation by photo-thermal-oxidative processes is that nearly all plastics are produced using extrusion, injection moulding or extrusion blowing. These techniques of polymer processing include heat and high shear to form the raw material into end products which causes impurities and reaction products that make them susceptible to UV radiation, heat and oxidative degradation (Andrady *et al.*, 1998). The degradation of plastics via photodegradation and thermo-oxidative degradation is a combination of different chemical processes, strongly influenced by the level of UV exposure and temperature. Therefore, plastic present in different environments break down at different speeds. Due to exposure to higher UV radiation and temperatures of plastic litter on land, like stranded items on beaches, undergo a more accelerated degradation compared to plastics floating in the water body (Pegram and Andrady, 1989). The degradation of the plastic material also strongly depends on the composition of added fillers of the plastic itself (Andrady *et al.*, 2003). Plastic´s resistance against photo-oxidative degradation can be changed to create a more resistant or less resistant product. The use of certain additives like metal oxides as catalysts to enhance photodegradation or oxidation processes accelerates the degradation (Sivan, 2011, Scott, 2000). On the opposite, the use of photostabilisers, achromatic pigments, extenders and thermal stabilizers enhances the resistance to degradation processes driven by UV light, radicals and heat and therefore the plastics lifetime (Saron *et al.*, 2008, Feldman, 2002). As described, plastic items are exposed to multiple environmental factors that lead to an embrittlement of the polymer structure, resulting in the falling apart of the plastic into smaller fragments (Andrady, 2011). Hence larger plastic items can be seen as the origin of a considerable amount of microplastic (Andrady *et al.*, 2003, Andrady, 2011). This process can theoretically continue until these fragments have reached nano sized diameters, however, the smallest plastic fragments found in the environment to date is 1.6 µm (Galgani *et al.*, 2010).

Primary and secondary microplastics have been found to litter aquatic environments globally (Browne *et al.*, 2011, Claessens *et al.*, 2011, Desforges *et al.*, 2014, Eriksen *et al.*, 2013a, Free *et al.*, 2014, Fendall and Sewell, 2009, Barnes *et al.*, 2009, Gregory, 1996, Costa *et al.*, 2010, Doyle *et al.*, 2011, Ashton *et al.*, 2010, Eriksen *et al.*, 2014). Surface water samples from the plastic gyres are not only contaminated with larger (meso and macro) plastic items but also microplastics, which can make up a substantial amount of the total collected plastic; In the North Pacific gyre microplastic accounted for over 92% of the total abundance of 334,270 pieces km^{-2} (Moore *et al.*, 2001). In the South Pacific subtropical gyre microplastic accounted for 55% of the total particle count and 72% of total weight of plastic sampled with a 333 µm mesh (average abundance of 26,898 particles km^{-2} with an average weight of 70.96 g km^{-2} and a maximum concentration of 396,342 particles km^{-2}) (Eriksen *et al.*, 2013b). In the North Atlantic gyre, a study using a 22 year old data set from ship surveys, found microplastic levels to be as high as 20,328 particles km^{-2} of which 88% were sized below 10 mm (Law *et al.*, 2010). Even though this study highlighted that 88% of found plastic pieces were below 10 mm no further classifications were made and therefore no statement for microplastic pollution for the here used classification. However, data from a study collecting neuston net samples (200 - 500 µm mesh size) from the plastic gyres suggested that 80% of present microdebris collected was below 5 mm (measured range was 0.2 - 1000 mm) (Cózar *et al.*, 2014). It is therefore likely that the plastic particles classified as < 10 mm by Law *et al.* (2010) consisted mostly of microplastic (< 5 mm). Similar to investigations of the single subtropical gyres, 680 net tows with a 333 µm mesh size in all five subtropical gyres reported that microplastic accounts for 92.4% of the global particle count (Eriksen *et al.*, 2014). Additionally, water samples from coastal regions found microplastic contaminations to range from 0.022 items m^{-3} near the Californian coast (Gilfillan *et al.*, 2009) to 102,550 items m^{-3} (mainly white spheres (102,400 items m^{-3})) for a heavily polluted industrial harbour with an associated polyethylene production (Norén, 2007). Recent investigations into abundance and distribution of microplastics in the oceans have revealed a constant increase of those small plastic fragments over the past decades (Claessens *et al.*, 2011, Thompson *et al.*, 2004, Goldstein *et al.*, 2012) while the average size of plastic debris has been reported to decrease (Barnes *et al.*, 2009).

Also beaches have been found to be contaminated with micro-sized plastic debris. Out of 22 reviewed studies to report plastic contamination of beach sediments with plastic,

microplastic abundance was found to range from 0.21 items m^{-2} to 77,000 items m^{-2} on a global scale (Hidalgo-Ruz *et al.*, 2012). In terms of volume, microplastic concentration of sediments was found at 185 to 80,000 items m^{-3} (Browne *et al.*, 2010, Martins and Sobral, 2011) and has been observed with a maximum concentration of 300,000 items m^{-3} (Costa *et al.*, 2010). An extensive list of reports to document microplastic abundance in the marine environment can be found in (Lusher, 2015).

1.3 Plastics and incorporated chemicals

Plastic products contain a substantial amount of fillers that are incorporated into the plastic matrix to alter the plastics properties in order to increase its benefits and lifetime (Lithner *et al.*, 2011).

Plasticisers can account for 67 to 80% of the weight of the finished product, depending on the plastic type (Giam *et al.*, 1984, Buchta *et al.*, 2005). Plasticisers are incorporated into the plastics matrix to reduce the chemical affinity between molecules of the polymer chain to enhance the materials flexibility (Oehlmann *et al.*, 2009). There are over 300 different types of plasticisers of which approximately 50 are in commercial use (ECPI, 2015). Around 6 million tonnes of plasticisers are used annually on a global scale and phthalates which account for 85% of this consumption are the most commonly used plasticisers (ECPI, 2015). Plasticisers are especially important in polyvinylchloride (PVC) materials and nearly 90% of the annual production is used for PVC products (Cadogan and Howick, 2000). Phthalate based plasticisers are not considered a persistent pollutant (Stales *et al.*, 1997) but can still be found in the environment on a broad scale which indicates a slow biodegradation (Fatoki and Vernon, 1990, Fromme *et al.*, 2002, Heemken *et al.*, 2001). Phthalate plasticisers as well as bisphenol A (BPA) are known to bioaccumulate in organisms and bioconcentration factors (BCFs) have been reported for fish to be between 42 and 842 but are bound in much higher BCFs in invertebrates (up to 5380 for copepods) (EU, 2003).

A wide range of additives, additional to plasticisers, are commonly incorporated into the plastics matrix for many plastic products (OECD, 2004). The British Plastic Federation (BPF) listed 18 different groups of additives, including numerous chemical compounds. These additives range from fragrances and dyes over various stabilisers and antimicrobials to flame retardants (BPF, 2015). Like plasticisers, these additives can

account for a substantial part of the plastics total weight; Flame retardants for example can account for up to 30% of a plastic product´s weight (EHC162, 1994).

1.3.1 Leaching of incorporated additives

Both, plasticisers and other additives are known to leach from the plastic, resulting in the release of potentially harmful chemicals (Berens, 1997, Loyo-Rosales *et al.*, 2004, Sajiki and Yonekubo, 2003, Koelmans *et al.*, 2014, Guart *et al.*, 2011) (Figure 1.5). Additives used for plastic production can be found in the environment; Flame retardants for example have been found in the environment (air water and soil samples) as well as in aquatic vertebrates, aquatic mammals and birds at concentrations of up to 1700 µg kg^{-1}, 650 µg kg^{-1} and 1800 µg kg^{-1}, respectively (EHC162, 1994) with increasing concentrations (Alaee and Wenning, 2002). Also humans are affected through the ingestion of contaminated fish and shellfish (van Leeuwen and de Boer, 2008). Groups of additives of special concern are alkylphenols and BPA since they have been reported to have endocrine disrupting properties (Anupama-Niar and Sujatha, 2012). Accordingly, concerns have been raised that ingested plastics might leach incorporated chemicals from plastic to aquatic organisms (Teuten *et al.*, 2009, Oehlmann *et al.*, 2009, Browne *et al.*, 2013). Even though the release of additives is a constant process during the plastics lifetime in the environment, the rate at which these chemicals leach from the plastic depends on environmental parameters, the structure of the plastic matrix in combination with the incorporated chemical, the processes used to incorporate the additive into the plastic and properties of the additive itself (Teuten *et al.*, 2009, Bauer *et al.*, 1998, Andrady *et al.*, 2003, Sjödin *et al.*, 2001). Temperature is generally positively correlated to additive leaching (Teuten *et al.*, 2009) and indicates a stronger leaching of additives in homoeothermic animals like marine mammals. Also differences in pH in the gastrointestinal system can promote or hinder the leaching of chemical compounds from the plastic matrix. Acidic media with high ionic strength have been found to have poor extraction effects on incorporated hydrophobic plasticisers and additives, whereas neutral media show higher leaching rates (Teuten *et al.*, 2009). Accordingly, acidic and neutral conditions of gastrointestinal surfactants can be important factors when evaluating the leaching of plastic associated chemicals during the digestion process. An additional factor that can promote the release of plastic incorporated chemicals is the degradation of the synthetic polymer in the environment. Since plastic materials might be present for extended periods of time in the environment

(Derraik, 2002) degradation processes, causing cracks in the polymer structure can increase the surface area and induce the release of additives from the inner part of the matrix (Ejlertsson *et al.*, 2003), resulting in an increased release of potentially harmful chemical compounds.

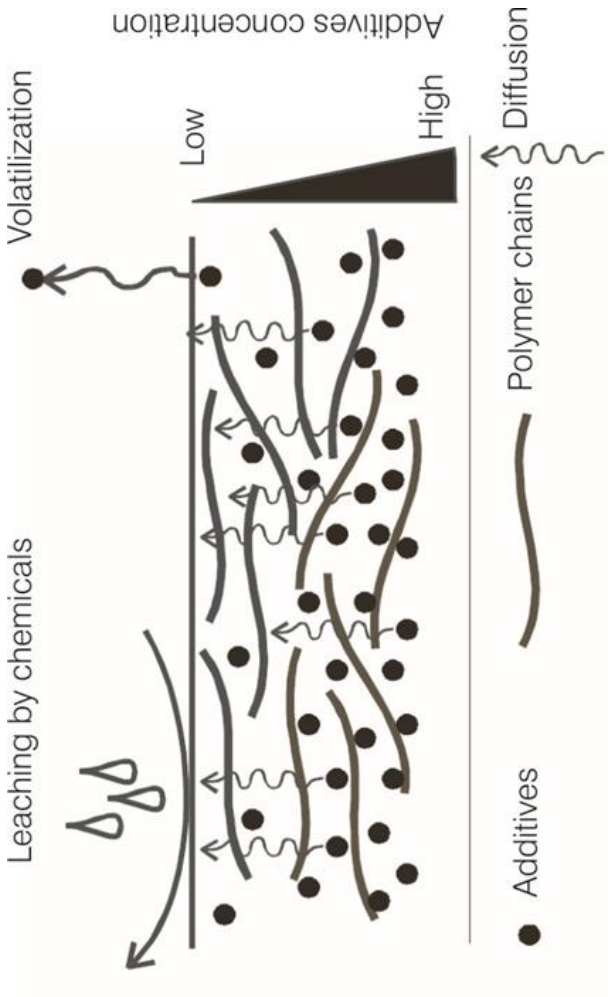

Figure 1.5: Leaching of additives from synthetic polymers. Incorporated additives can leach from the plastic product over the plastic´s surface via volatilization or leaching by chemicals, resulting in an additive concentration gradient in the plastic product. This gradient causes additives from the inner part of the plastic (higher additive concentration) to migrate (via diffusion) to the surface (lower additive concentration). http://www.masterbuilder.co.in/durability-and-long-term-performance-of-high-density-polyethylene-geomembranes/.

1.4 Effects of plastic litter on aquatic life

As a consequence of the high levels of plastic waste accumulating in global environments, marine debris has been identified as a potential factor that may contribute to the loss of biodiversity in the oceans (Gall and Thompson, 2015). Even though no evidence is present to date, plastic may also have the potential to affect human health and activities (Derraik, 2002, Thompson *et al.*, 2009). Additionally, plastic debris has been classified as a top environmental problem (UNEP, 2005). Accordingly, it is important to investigate and understand the interactions and arising effects of wildlife with this anthropogenic debris.

1.4.1 Effects of macro plastic in the aquatic environment

One of the major impacts of macro plastic items in the oceans is the reported entanglement of numerous species (Figure 1.6). The number of species worldwide to be affected by plastic debris (entanglement and/or ingestion) has risen from 267, in 1984 reported by Laist (1997) to 557 in 2014 reported by Kühn *et al.* (2015). Reports for the entanglement in larger plastic items ranges from marine mammals (Laist, 1997, Clapham *et al.*, 1999, Eriksson and Burton, 2003) over birds (Moser and Lee, 1992, Cadée, 2002) to turtles (Bjorndal *et al.*, 1994, Bugoni *et al.*, 2001, Tomás *et al.*, 2002) and many more (Kühn *et al.*, 2015). The entanglement of marine wildlife in lost or discarded fishing gear (Page *et al.*, 2004, Votier *et al.*, 2011, Sazima *et al.*, 2002, Bugoni *et al.*, 2004, NOAA, 2015) in a process called `ghost fishing´ (Bullimore *et al.*, 2001, Carr and Harris, 1997) has been reported, which can lead to deleterious effects (Sazima *et al.*, 2002, Gudger and Hoffmann, 1931, Schwartz, 1984, Barreiros and Raykov, 2014, Carr, 1987). Entanglement in plastic can lead to suffocation (Gregory, 2009) and severe and lethal wounds (Gregory, 1991). Data presented by Wallace (1984) suggested that annually 50,000 - 90,000 marine mammals die as a cause of plastic ingestion or entanglement in the North Pacific Ocean alone (Wallace, 1984). However, not just lost or discarded fishing gear has been found to entangle marine life. Plastic packing loops like detachable lid parts from plastic bottles or six pack holders, monofilament lines and even automobile tires have been recorded to entangle animals, causing severe abrasions and deformations (Sazima *et al.*, 2002, Gudger and Hoffmann, 1931, Schwartz, 1984), lethal lesions, amputation and affect survival (Barreiros and Raykov, 2014, Carr, 1987).

Figure 1.6: Marine organisms entangled in plastic macro debris. A total of 557 marine organisms are reported to be affected by plastic debris. A common threat of macroplastics is the entanglement in discarded fishing gear, packing loops like detachable lid parts from plastic bottles or six pack holders, monofilament lines and even automobile tires which can cause severe abrasions and deformations, lethal lesions, amputation and ultimatively affect survival.

1.4.2 Plastic ingestion

Due to its omnipresence in the marine environment and a wide variety of shapes, colours and sizes plastic debris has been found to be ingested by a wide range of marine biota (Kühn *et al.*, 2015, Laist, 1997, Derraik, 2002) (Figures 1.7, 1.8 and 1.9). Even though the size of a plastic item limits the ingestion to a group of animals that can physically ingest it, colours and shape seem to play an important role as well and can promote selective feeding (Shaw and Day, 1994, Gramentz, 1988, Carpenter *et al.*, 1972). The ingestion of plastic litter ranging from 16 m^2 found in sperm whales (Jacobsen *et al.*, 2010) to just 1.7 µm for zooplankton (Cole *et al.*, 2013) has indicated that the largest as well as the smallest animals in the marine environment may ingest plastics. However, an increasing number of reports adds to a growing list of species with ingestion records and it becomes clear that biota from every trophic level have the potential to ingest plastic debris (Kühn *et al.*, 2015).

First evidence for plastic ingestion by seabirds was given by Harper and Fowler (1987) who reported that ingestion of plastic pellets already occurred in the 1960s (Harper and Fowler, 1987). Since then, multiple field work investigations, have extended the knowledge about plastic ingestion by marine birds and highlighted that ingestion is not limited to an individual but can be found on a species level; It was reported that out of 144 examined seabird species, 82 were found to have small plastic debris in their stomachs with some species showing contaminated stomachs of over 80% of individuals (Ryan, 1990). Additionally, a study by Moser and Lee (1992) found that out of 1033 birds collected off the coast of North Carolina, USA individuals from 55% of the recorded species had plastic in their gastrointestinal tracts (Moser and Lee, 1992). Furthermore, multiple field work studies investigating marine birds have provided evidence that feeding techniques might be of importance for the incidence of plastic ingestion and planktivores species are more likely to ingest plastic particles compared to piscivores (Laist, 1987, Laist, 1997, Moser and Lee, 1992, Azzarello and Van Vleet, 1987).

Figure 1.7: Dead marine bird with ingested plastic litter. Many marine bird species have been reported to ingest plastic which has the potential to accumulate in the stomach, leading to the blockage of the gastrointestinal tract and reduced food ingestion through reduced appetite. As a result the organism is starving with a full stomach which has the potential to lead to the death of the animal. Imaged sourced from http://plasticfreetuesday.com.

An increasing number of reports also documents the ingestion of plastic by marine turtles (Schuyler *et al.*, 2014) and it is estimated that 86% of all turtles species are affected by plastic debris through entanglement and/or ingestion (Laist, 1997). A more recent comparison of ingestion records even reported that 100% of investigated turtles ingested plastic (Kühn *et al.*, 2015). Ingestion records highlighted the importance of the shape of plastic items for the species specific ingestion. Turtles were found to be especially threatened by the ingestion of floating plastic bags since they can be confused for jelly fish (medusoids), their natural food source (Gregory, 2009, Bjorndal *et al.*, 1994, Balazs, 1985, Bugoni *et al.*, 2001).

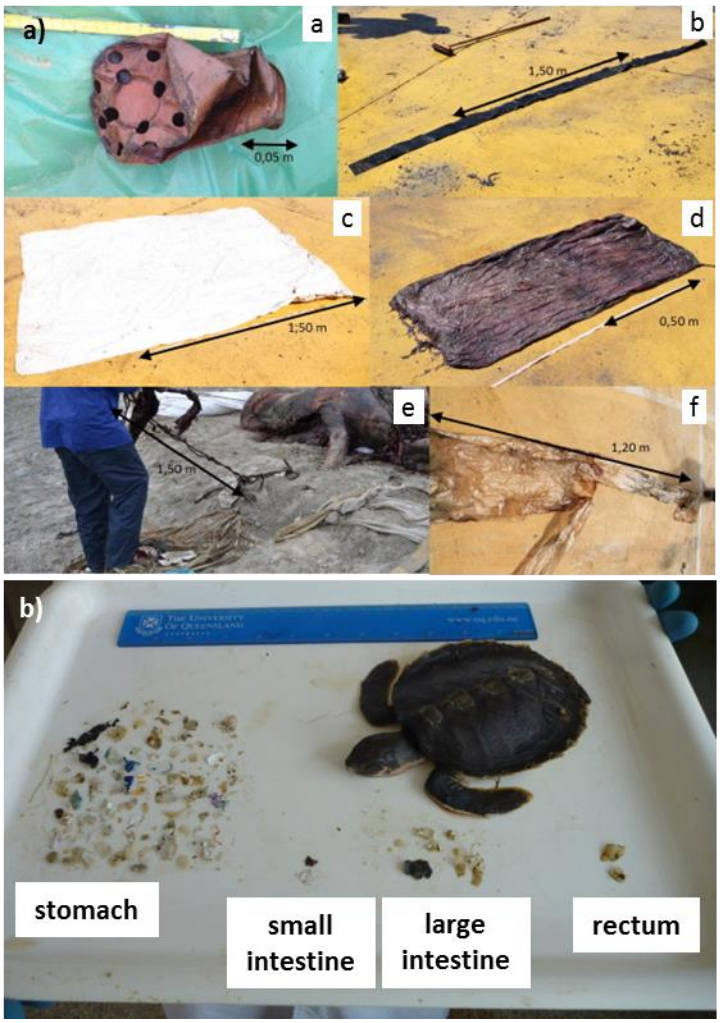

Figure 1.8: Plastic ingestion by a turtle and a whale. a): Plastic debris found in the stomach of a whale (a) flower pot, (b) hosepipe, (c) greenhouse cover material, (d) plastic burlap, (e) rope, and (f) plastic mulch of greenhouse cover material, (d) plastic burlap, (e) rope, and (f) plastic mulch of greenhouse cover material, (d) plastic burlap, (e) rope, and (f) plastic mulch of greenhouse. Image modified from de Stephanis *et al.* (2013). b): Plastic ingestion by a turtle. Recovered plastic fragments from the stomach, small intestine, large intestine and rectum (from left to right). Imaged modified from https://lifescienceexplore.wordpress.com.

Representing some of the oceans largest animals, 50.4% of all investigated marine mammals are reported to be affected by plastic ingestion (Kühn *et al.*, 2015). A total of 26 cetacean species have been documented to ingest plastic litter (Baird and Hooker, 2000, Jacobsen *et al.*, 2010, de Stephanis *et al.*, 2013). Also manatees (Laist, 1987, Beck and Barros, 1991), seals (Bravo Rebolledo *et al.*, 2013, Eriksson and Burton, 2003) and dolphins (Denuncio *et al.*, 2011) have been recorded to have ingested plastic debris.

Unlike most ingestion records of plastic for birds, turtles and marine mammals, data for fish does not solely rely on field studies of dead and decomposing organisms but includes laboratory studies as well. Together, field samples and laboratory experiments have provided evidence for plastic ingestion by many species of teleost fish of varying sizes, development stages and feeding techniques.

First field studies reported that 8 out of 14 collected species of fish larvae ingested plastic particles (Carpenter *et al.*, 1972) and a list of early studies provided evidence for the plastic ingestion by larval and juvenile fish (Table 1.2) (Hoss and Settle, 1990). More recent field work has provided evidence for the ingestion of plastic by fish for many species globally. Foekema *et al.* (2013) reported that out of 1203 sampled fish from seven species in the North Sea a total of 3.2% were found to have ingested plastic with the highest frequency (> 33%) found in the English Channel (Foekema *et al.*, 2013). Another study focussing on the English Channel by Lusher *et al.* (2013) also found high and similar plastic ingestion rate of 36.5% in 504 assessed fish from 10 species (Lusher *et al.*, 2013). Plastic ingestion of 141 mesopelagic fish from 27 species from the North Pacific Gyre was found to be at 9.2% and the authors estimated the ingestion rate of microplastic by mesopelagic fish from the North Pacific Gyre to be as high as 12,000 to 24,000 tonnes year^{-1} (Davison and Asch, 2011). Also investigating the incidence of plastic ingestion by fish from the central North Pacific, Choy and Drazen (2013) showed that among 595 individuals of 7 large predatory fish species, 19 % of individuals had ingested plastic. Species were found with a maximum of 58% of individuals to have ingested plastic (Choy and Drazen, 2013). Focusing on the ingestion of plastic particles by planktivores fish in the North Pacific Gyre, Boerger *et al.* (2010) reported that a total of 35% of caught fish had plastic in their gastrointestinal tracts (Boerger *et al.*, 2010). Lusher *et al.* (2015) presented a review of field studies to report the ingestion of microplastics by fish and found that 55 species of fish from 13 Orders

were reported to ingest plastic. Information for fresh water fish is rare but Sanchez *et al.* (2014) found that 12% of collected fish (gudgeons (*Gobio gobio*)) from a French river had ingested plastic (Sanchez *et al.*, 2014).Early laboratory studies found that larval Atlantic herring (*Clupea harengus*) (Hjelmeland *et al.*, 1988) do ingest polystyrene spheres and Hoss and Settle (1990) found that 5 out of 6 tested fish species ingested plastic spheres (Hoss and Settle, 1990). More recent laboratory based studies confirmed findings from field studies and early laboratory experiments and provided further evidence for the ingestion of plastic particles by fish; Up to 33% of all ontogenetic stages (larvae, juvenile and adults) of 66 investigated marine catfish were found to ingest plastic during a laboratory experiment (Possatto *et al.*, 2011), proposing that all developmental stages of fish can be affected by plastic ingestion. A laboratory study by Oliveira *et al.* 2013 provided evidence for the ingestion of 1 - 5 µm sized polystyrene spheres by juveniles of the common goby (*Pomatoschistus microps*) (Oliveira *et al.*, 2013). The ingestion of 3 mm polyethylene pellets was reported for Japanese medaka (*Oryzias latipes*) (Rochman *et al.*, 2014, Rochman *et al.*, 2013b). Additional evidence for the ingestion of plastic particles by fish has been provided by (Santos and Jobling, 1992, Mazurais *et al.*, 2014). Considering the findings of early and more recent field and laboratory reports, strong evidence is provided that fish from many different species, varying developmental stages and feeding guilds do ingest plastic.

Figure 1.9: Plastic ingestion by fish. Plastic particles and plankton recovered from a fish´s stomach. Similarities in colour and shape might lead to the confusion of plastic with natural prey. Modified from: http://axisoflogic.com.

Table 1.2: List of early reports to document plastic ingestion by fish. Hoss and Settle (1990). ´Ingestion of plastic by teleost fishes´. Proceedings of the Second International Conference on Marine Debris 2-7 April 1989, Honolulu, Hawaii, volume 1. NOAA Technical Memorandum, NMFS-SWFSC(154). US Department of Commerce, NOAA: Panama City XIII, 774 pp..

Clupeidae		
Brevoortia patronus, gulf menhader	7.6	Govoni (pers. commun.)
Clupea harengus, Atlantic herring	42	Carpenter et al. 1972
Gadidae		
Ciliata mustela, five-beard rockling	NA	Kartar et al. 1976
Pollachius virens, pollok	30	Carpenter et al. 1972
Atherinidae		
Menidia menidia, Atlantic silverside	16	Carpenter et al. 1972
Sciaenidae		
Micropongonias undulatus, Atlantik croaker	6.3	Govoni (pers. commun.)
Labridae		
Tautogolabrus adspersus, tautog	91	Carpenter et al. 1972
Gobiidae		
Govius minutus, sand goby	NA	Kartar et al. 1976
Cottidae		
Myoxocephalus aenus, grubby	5.8	Carpenter et al. 1972
Cyclopteridae		
Liparis liparis, striped seasnail	NA	Kartar et al. 1976
Pleuronectidae		
Platichtys flesus, flounder	20-50	Kartar et al. 1973
Pseudopleuronectes americanus, winter flounder	4.6	Carpenter et al. 1972

At the base of the food chain and reliant on small food particles, zooplankton are especially susceptible to the ingestion of small microplastic as many of them are indiscriminate filter feeders with limited ability to differentiate between plastic particles and food (Moore, 2008). Studies by Cole *et al.* (2013 and 2015, Figure 1.10) provided evidence that 13 out of 27 investigated zooplankton taxa ingested 1.7 - 30.6 µm polystyrene beads (Cole *et al.*, 2013) and that the copepod *Calanus helgolandicus* ingested 20 µm polystyrene spheres from the water column (Cole *et al.*, 2015). In addition, the ingestion of 10 µm polystyrene spheres was reported for mysid shrimps, copepods, cladocerans, rotifers, polychaete larvae and ciliates (Setälä *et al.*, 2014). Additional evidence for the ingestion of plastic particles by zooplankton has been provided by (Desforges *et al.*, 2015, Cedervall *et al.*, 2012). The ingestion of plastic particles by zooplankton is of concern since larval, juvenile and adult stages of many marine organisms rely on it as a food source.

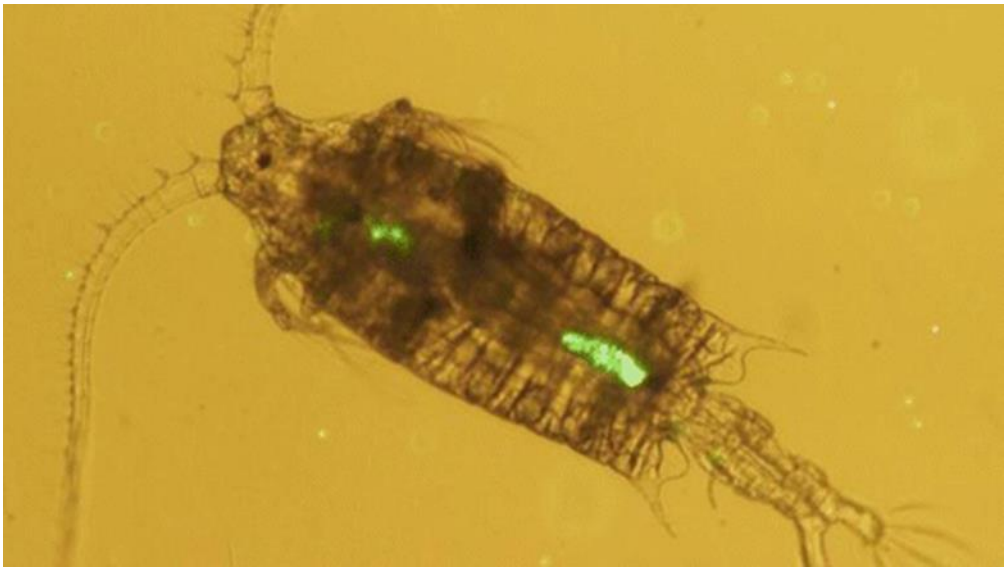

Figure 1.10: Plastic ingestion by zooplankton. Zooplankton that were exposed to a water column contaminated with fluorescent polystyrene plastic spheres (0.4–30.6 µm) were found to actively filter and ingest the plastic from the water. Plastic particles in the gastrointestinal tract of the zooplankton was confirmed using Coherent Anti-Stokes Raman Scattering (CARS) Microscopy. From: Cole *et al.* (2013).

Evidence for plastic ingestion is also available for a wide range of other marine organisms from many trophic levels. Sea urchin embryos *Paracentrotus lividus* (Della Torre *et al.*, 2014) and larva (Kaposi *et al.*, 2013), sea cucumber *Echinodermata, Holothuroidea* (Graham and Thompson, 2009), corals (Hall *et al.*, 2015), mussels (Browne *et al.*, 2008, von Moos *et al.*, 2012, Wegner *et al.*, 2012), crabs (Watts *et al.*, 2014), isopods (Hämer *et al.*, 2014), worms (Browne *et al.*, 2013, Wright *et al.*, 2013), penguins (Brandão *et al.*, 2011) and sharks (Cliff *et al.*, 2002) have all been found to ingest plastic. Additionally, uptake of polystyrene nano particles has been reported for green algae (Cedervall *et al.*, 2012, Bhattacharya *et al.*, 2010, Long *et al.*, 2014). Evidence for the ingestion of plastics by fresh water organisms was reported for a wide range of invertebrates from different habitats and feeding guilds (Imhof *et al.*, 2013, Rosenkranz *et al.*, 2009).

1.4.3 Trophic transfer of plastic particles

The term trophic transfer describes the dietary transfer of compounds along the food chain. During this transfer, biomagnification of consumed dietary components like environmental pollutants is possible (Figure 1.11). Hereby the term biomagnification is defined as “the process in which the chemical concentration in an organism achieves a level that exceeds that in the organism's diet, due to dietary accumulation” (Gobas and Morrison, 2000) and is considered a major mechanism for the accumulation of contaminants in organisms of higher trophic levels (Borgå *et al.*, 2004). Since species of many trophic levels but especially zooplanktonic organisms at the bottom of the food chain have been reported to ingest plastic particles (see section 1.4.2), the accumulation of plastic along the food chain is of concern (Cedervall *et al.*, 2012, Farrell and Nelson, 2013, Setälä *et al.*, 2014, Murray and Cowie, 2011, Watts *et al.*, 2014, Eriksson and Burton, 2003). To date just a very limited amount of data from few species is available to provide evidence for the trophic transfer of ingested plastic. However, some field work and laboratory based studies suggests that indirect ingestion of plastic via trophic transfer occurs.

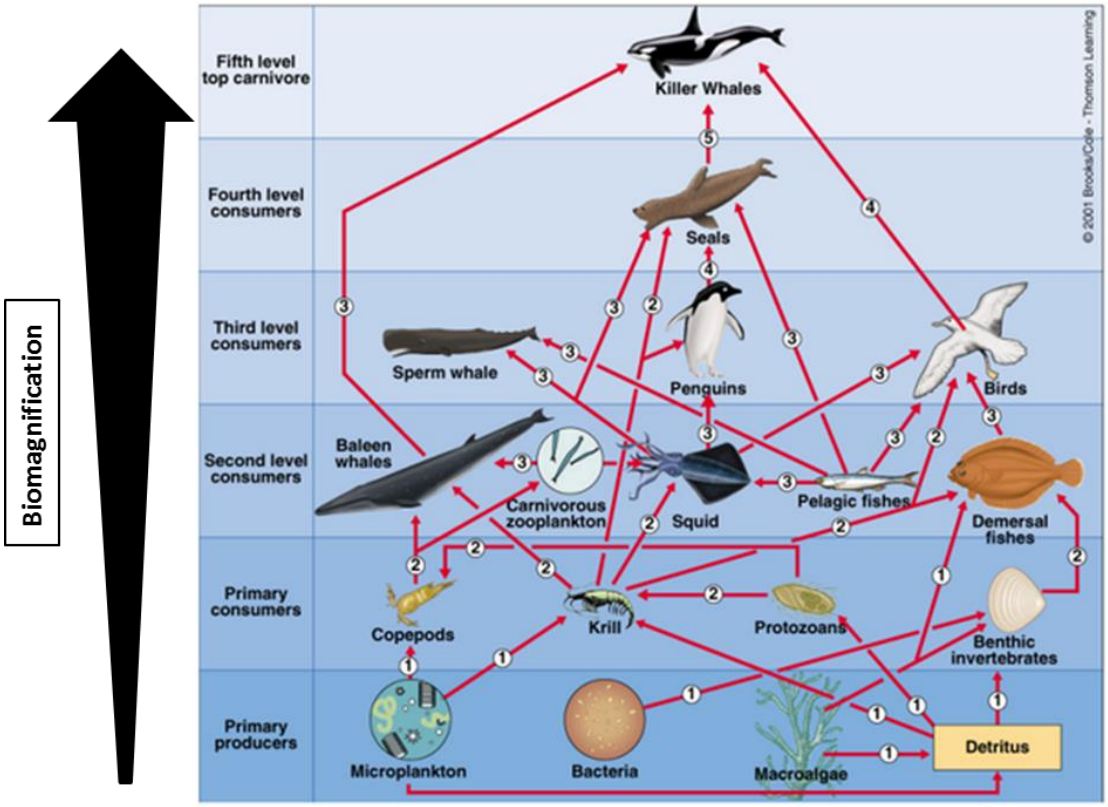

Figure 1.11: Trophic transfer and biomagnification of pollutants in a simplified marine food chain. Through the process of biomagnification low concentrations of environmental pollutants in organisms of a lower trophic level have the potential to reach bioactive concentrations in organisms of a higher trophic level, accumulated via the diet. Modified from: Brooks/Cole, Thomson Learning.

Field work using the Great skuas (*Stercorarius skua*) which are known to predate on small sea bird species examined regurgitated indigestible matter and showed a link with the amount of ingested plastic and their main prey species (Bourne and Imber, 1982, Ryan and Fraser, 1988). Further evidence for the trophic transfer of plastic in birds was reported for goose fish (*Lophius americanus*) which had ingested a little auk (*Alle alle*) which in turn had ingested nylon fishing line (Perry *et al.*, 2013). Evidence for trophic transfer of plastic to marine mammals was presented by Eriksson and Burton (2003) who measured plastic content in faeces samples of fur seals on Macquarie Island and linked the presence of plastic to the ingestion of contaminated myctophid fishes (Eriksson and Burton, 2003).

Laboratory based studies reported the transfer of plastic particles over trophic levels via ingestion of contaminated prey. Using zooplankton as lower trophic level organisms, Setälä *et al.* (2013) reported the transfer of 10 µm polystyrene spheres from copepods (*Eurytemora affinis*) to pelagic mysid shrimps (*Neomysis integer*). Contaminated zooplankton was found in the gastrointestinal tract of shrimps as soon as 3h after start of the exposure (Setälä *et al.*, 2014). Additional evidence for the trophic transfer of 0.5 µm and 10 µm polystyrene spheres was provided from the blue mussel (*Mytilus edulis*) to crabs (*Carcinus maenas*); Two studies exposed *Carcinus maenas* to previously contaminated, cut open *Mytilus edulis* and found plastic particles in stomach, hepatopancreas, ovary and gills (Farrell and Nelson, 2013) and foregut (Watts *et al.*, 2014). Using the crustacean (*Nephrops norvegicus*) as a top consumer, the transfer of plastics from dead fish (*Merlangius merlangus* and *Micromesistius poutassou*) was observed (Murray and Cowie, 2011). However, since already dead fish were hand-contaminated with 5 mm strands of polypropylene rope, the relation to an actual trophic transfer is questionable. Using a more complex approach, a trophic transfer study conducted by Cedervall *et al.* (2012) reported the transfer of 28 nm polystyrene nanoparticles from algae (*Scenedesmus sp.*) over zooplankton (*Daphnia magna*) to fish (*Carassius carassius*) and provided first evidence for the trophic transfer over three trophic levels to be an additional ingestion route to the direct ingestion of plastic in fish (Cedervall *et al.*, 2012).

1.4.4 Effects of ingested plastic

Although there are numerous reports that aquatic organisms ingest plastics, very little is known about the effects that might be induced by ingested plastic and it still has to be established whether the ingestion of virgin plastics has any significant negative health effects on biota (Zarfl *et al.*, 2011). Since the carbon backbone of plastic itself is considered biochemically inert (Teuten *et al.*, 2009, Roy *et al.*, 2011, Lithner *et al.*, 2011), induced effects might be limited to its physical properties like size or shape but also levels of ingested plastic. Characteristics of the plastic could determine how severe the effects will be and on which level of the biological organisation effects might be induced. A list of to date experimental exposures of marine organisms to microplastics and related effects can be found in Lusher *et al.* (2015).

1.4.4.1 The importance of relative size

Plastic debris is present in many different sizes in aquatic environments, from plastics in the micrometre and millimetre range to objects measuring multiple m^2 and ingestion has been reported for all of these size classes. The relative size of the ingested plastic can be of importance since larger plastic objects might lead to the blockage of the gastrointestinal tract (GI) (Gregory, 2009, Balazs, 1985, Tourinho *et al.*, 2010) which can cause further accumulation, leading to acute effects. In marine birds, blockage of the GI has been reported to reduce food ingestion, cause blockage of gastric enzyme secretion, induce internal injuries and reproductive failure and lead to death (Azzarello and Van Vleet, 1987, Rothstein, 1973, Ryan, 1988, Harper and Fowler, 1987, Carpenter *et al.*, 1972). Similar effects have been reported for turtles where the blockage of the oesophagus (Gregory, 2009, Balazs, 1985) affected feeding behaviour and gastrointestinal function (Bjorndal *et al.*, 1994) and hindered survival, especially for young sea turtles (Carr, 1987). Furthermore, ingestion of large plastic items is thought to have fatal consequences on marine mammals; Jacobsen *et al.* (2010) concluded that the death of two sperm whales (*Physeter macrocephalus*) was due to the ingestion of plastic which caused a ruptured stomach for one individual and the emaciated state of the second one (Jacobsen *et al.*, 2010). A ruptured stomach, filled with ingested plastic in a dead, stranded sperm whale was also observed by de Stephanis *et al.* (2013). Similar effects can be seen for other marine mammals like manatees where plastic content in the stomach has been linked to the death of four individuals through

blockages of the gastrointestinal tract (Beck and Barros, 1991). Even though numerous reports are present for fish to ingest plastic, data for the effects of ingested large plastic items are rare (Hoss and Settle, 1990). However, similar to observations from birds and turtles, it was proposed that plastic poses a potential threat to block GIs of fish (Carpenter *et al.*, 1972) and larger plastic particles are retained in the gastrointestinal tractfor prolonged times compared to smaller particles (dos Santos and Jobling, 1991).

Even though smaller fragments have the potential to block the GI if orientated in the wrong way (Bjorndal *et al.*, 1994) they generally pose a lower risk to cause acute effects like internal injuries or blockage of the GI, leading to severe effects in the short term. However, smaller particles might lead to obstructions of the GI which can result in prolonged food processing times (Pierce *et al.*, 2004) and pseudo-satiation resulting in lower food consumption, suppressed appetite and reduced growth (Derraik, 2002, Thompson, 2006, Ryan, 1988). Since plastics do not contain any nutritional value, filling effects and lower food consumption can lead to a starving organism with a full stomach (Dickerman and Goelet, 1987), possibly resulting in its death (Pierce *et al.*, 2004). Hence, small plastic particles may induce long term effects due to the induction of sub lethal effects like minor injuries or slow energy depletion due to poor nutrition.

Invertebrate studies provided evidence for the need to consider more subtle effects when investigating the effects of ingested, smaller plastic fragments; As for birds, also ingestion of plastic particles by zooplankton was linked to the reduced consumption of available food and it was suggested that zooplankton might suffer from energetic depletion due to a decrease of ingested carbon biomass in the long term (Cole *et al.*, 2015). Five day old larvae of the sea urchin *Tripneustes gratilla*, which were exposed for 5 days to 1, 10, 100, and 300 spheres ml^{-1} of 10 - 45 µm polyethylene microspheres, were found to ingest plastic particles in a dose dependent manner and were able to egest all ingested particles within 420 min (Kaposi *et al.*, 2013). The exposure had just limited effects on growth (body width and length) and no effect on survival for larvae after 5 days of exposure. However, even though not statistically different, a trend for lower survival in the 300 spheres ml^{-1} treatment and smaller body widths for all plastic treatments except the 1 spheres ml^{-1} treatment was observed when compared to the control, indicating that a longer exposure might lead to effects on survival and growth. Using the copepod (*Tigriopus japonicas*), Lee *et al.* (2013c) supported findings that microplastics may induce long term effects in zooplankton by exposing copepods to

concentrations of 0, 6, 13, 31, 63, 187, 250 and 313 μg ml^{-1} of 0.05, 0.5 and 6 μm polystyrene beads for 96 hours. Ingestion of all particle sizes was observed but no effect on survival was found for this acute toxicity test. However, exposure of a F_0 and F_1 generation in a chronic exposure to the same bead sizes at lower concentrations (0, 0.125, 1.25, 12.5, and 25 μg ml^{-1}) induced significant effects on survival, development and fecundity compared to the control and highlighted the importance of exposure time and plastic size; The 0.05 μm polystyrene beads decreased survival at concentrations > 12.5 μg ml^{-1} in the F_0 generation and > 1.25 μg ml^{-1} in both the nauplii and copepodites of the F_1 generation. In the 0.5 μm polystyrene bead treatment no effect on the F_0 generation was found but the highest concentration (25 μg ml^{-1}) induced a significant decrease in the survival of the F_1 generation. The 6 μm polystyrene beads did not affect the survival over two generations. The duration of the nauplius phase was significantly longer with 1.25 μg ml^{-1} of 0.05 μm polystyrene beads and in the 0.5 μm polystyrene bead treatment, the concentration of 25 μg ml^{-1} caused a significant developmental delay in the F_1 generation, possibly indicating plastic induced delays. No effects were found for the 6 μm polystyrene beads over two generations. However, 0.5 and 6 μm polystyrene beads caused significant decreases in fecundity at all concentrations measured with the number auf nauplius produced per female (Lee *et al.*, 2013c). A study conducted by Cole *et al.* (2015) using zooplanktonic organisms which were exposed for 9 days to 20 μm polystyrene microplastic found no effect on survival of exposed organisms but fecundity, measured with the reduction in hatching success was negatively affected.

Similar to results of studies using invertebrates, evidence that the ingestion of uncontaminated plastic as part of the diet might cause energy depletion is also given for fish; When exposing Japanese medaka (*Oryzias latipes*) for two months to 8 ng ml^{-1} of virgin low density polyethylene (LDPE) pellets as part of their diet, plastic exposed fish showed glycogen depletion in the liver compared to the control group (Rochman *et al.*, 2013b). Glycogen has been reported to be one of the main energy reserves to be mobilised during periods of starvation (Rios *et al.*, 2006) and thus the observed glycogen depletion in the liver indicates a potential change in energy reserves of plastic exposed fish. Whereas short term utilisation of energy reserves is a normal adaptation to cope with food deprivation (Rios *et al.*, 2006), long-term starvation can lead to the consumption of structural biomass (Kooijman, 2000). In line with this it was reported that the partial blockage of food passage through the GI may cause slow deterioration of

body condition of fish (Hoss and Settle, 1990). Evidence that plastic ingestion can lead to a reduced condition was provided by Foekema *et al.* (2013) who assessed the condition of 1203 wild caught fish (herring, gray gurnard, whiting, horse mackerel, haddock, atlantic mackerel, and cod) from the North Sea related to plastic presence in GI. One out of the five assessed species was found with a decreased condition index. However, the authors argued that the small size of plastic pieces found in the GI of fish did not cause blockages and were unlikely to cause a satiation effect which is why they concluded that the plastic was unlikely to have significant effects on the condition. The authors, however, proposed that the found plastic might have a more serious impact on larval stages of fish (Foekema *et al.*, 2013).

Additional evidence for a plastic related decrease in condition in vertebrates was provided by Ryan *et al.* (1988) who fed chicken (*Gallus domesticus*) with polyethylene pellets and concluded that the observed reduced food ingestion was due to filling effects of the plastic and may result in a limited ability of seabirds to accumulate energy reserves and thus reduce fitness. Evidence for long term effects of ingested plastic for marine birds was presented by Auman *et al.* (1997) reported that plastic induced partial blockage and minor damage of the GI of Laysan albatross (*Phoebastria immutabilis*) chicks did not have a direct effect on mortality, but may contribute to poor nutrition or dehydration (Auman *et al.*, 1997).

Also turtles might be susceptible to energy depletion due to ingested plastic since a reduced volume of the stomach due to ingested plastics caused lower nutrient and energy intake (McCauley and Bjorndal, 1999, Lutz, 1990).

An additional concern is that due to their small size, micro and nano-sized particles could translocate from the gastrointestinal lumen into other tissues like the circulatory system, the liver and the brain of marine organisms (Browne *et al.*, 2008, von Moos *et al.*, 2012, Kashiwada, 2006, Oberdörster, 2004) (Figure 1.12). Before such translocation was investigated for marine biota, rodents and humans provided the first evidence for the translocation of small spheres (Jani *et al.*, 1989, Jani *et al.*, 1992, Volkheimer, 1975, Hussain *et al.*, 2001). These findings are applied in modern medicine and are now used for micro- and nanoparticles to act as systems for drug and vaccine delivery (O'Hagan, 1996). Browne *et al.* (2008) and von Moos *et al.* (2012) provided evidence for the translocation of plastic particles in marine invertebrates using *Mytilus edulis* (Browne *et*

al., 2008, von Moos *et al.*, 2012). Browne *et al.* (2008) reported that a 3h exposure to 3 and 9.6 µm polystyrene microplastics lead to the translocation of particles into the circulatory system (haemolymph) after 3 days. Smaller particles were found to translocate in greater numbers (up to 60% more) compared to larger particles. Translocated particles could still be found in the haemolymph and faeces samples after 48 days. No adverse health effects (measured with cell viability assays for neutral red uptake by haemocytes and phagocytosis capability) were observed to follow the exposure (Browne *et al.*, 2008). In line with findings from Browne *et al.* (2008), von Moos *et al.* (2012) found that high density polyethylene (HDPE) microplastic ranging from 0 - 80 µm could be found in the haemolymph as soon as 3h after initiating the exposure, indicating a rapid translocation after ingestion. Additionally, prolonged exposure was found to increase levels of translocated plastics in the haemolymph which nearly doubled when assessed at 96h (end of exposure) compared to the first measurement after 3h. Particles that translocated to the haemolymph induced a reduced lysosomal membrane stability from 6h of exposure but endpoints lipofuscin accumulation (for oxyradical damage), neutral lipid content (disturbance of lipid metabolism) and condition index were not affected over the duration of the exposure. This difference in observed effects between the two studies of Borwne *et al.* (2008) and von Moos *et al.* (2012) could be explained by the different particle sizes used but also the longer exposure period of 96h by von Moos *et al.* (2012) compared to 3h by Browne *et al.* (2008). Additionally to the translocation of ingested particles, microplastics were also found to be taken up into gill tissue, indicating that translocation of particles can occur over other exposed tissues beside of the GI; Plastic particles in gill tissues caused effects on a cellular level measured with lysosomal membrane stability and formation of granulocytomas as part of an inflammatory response (von Moos *et al.*, 2012). According to this study, translocation of micrometre sized plastic particles can have adverse effects on a tissue level and further investigations are needed to investigate if such evidence can be found for other species. However, even smaller particles in the nanometre range are believed to be able to permeate into the lipid membranes of cells and as a result alter the membrane structure, membrane protein activity, and therefore cellular function (Rossi *et al.*, 2013).

Investigating the effects of translocated plastic nano particles in fish, Cedervall *et al.* (2012) reported a reduced food ingestion and suspected differences in weight loss and lipid metabolism in Crucian carp (*Carassius carassius*) to be caused by the exposure to

a contaminated diet (*Daphnia magna* contaminated with 28 nm polystyrene spheres). During the 39 days starvation study, fish exposed to plastic contaminated diets were found to take twice as long to consume 95% of the presented food. However, whereas the control treatment continuously lost weight over the exposure period, weight loss slowed down for plastic exposed treatments from day 15 of the study. On day 39 plastic exposed fish were even found to have gained weight over the past 12 day period whereas the control treatment continued to lose weight. Differences in triglycerides:cholesterol ratio in blood serum, and the distribution of cholesterol between muscle and liver of sampled fish from the control and exposure groups suggested a disturbance in lipid metabolism (Cedervall *et al.*, 2012). In a separate experiment using fish blood serum from Crucian carp (*Carassius carassius*), Bleak (*Alburnus alburnus*), Rudd (*Scardinius erythrophthalmus*), Tench (*Tinca tinca*), Pike (*Esox esox*), and Atlantic salmon (*Salmo salar*) mixed with 200 nm polystyrene particles the same study found that the used nanoplastics can bind to apolipoprotein A-I in fish serum in vitro and concluded that the observed difference in weight loss could be explained by the translocation of nanoparticles which hindered the utilisation of fat reserves (Cedervall *et al.*, 2012). Also using nanoparticles, a study by Kashiwada (2006) exposed adult Japanese medaka (*Oryzias latipes*) to 10 mg L^{-1} of 39.4 nm polystyrene nanoplastics in the water column. Blood samples were found to be contaminated with plastic at concentrations of 16.5 ± 0.7 ng mg^{-1} blood protein in male and 10.5 ± 2.2 ng mg^{-1} blood protein in female fish. Dissected tissue samples of gills and intestines showed the highest accumulations of plastics but testis, liver and brain were also found to be contaminated with nanoplastics. The author identified the gills and the intestines as the main uptake routes for nanoplastics that are dispersed in the water column. The presence of plastic particles in the brain suggests that nanoplastics might be able to cross the blood-brain barrier (Kashiwada, 2006). Data from these studies highlight the risk for plastic particles to be translocated from the GI to surrounding tissues and highly sensitive organs like the brain, having the potential to cause adverse effects in aquatic organisms. Even though the translocation of plastic particles in the nanometre range, followed by adverse effects has been reported for fish and evidence is given that micrometre sized particles can translocate from the gastrointestinal tract to the haemolymph of mussels, it remains unclear if particles in the micrometre range pose a threat for translocation in fish.

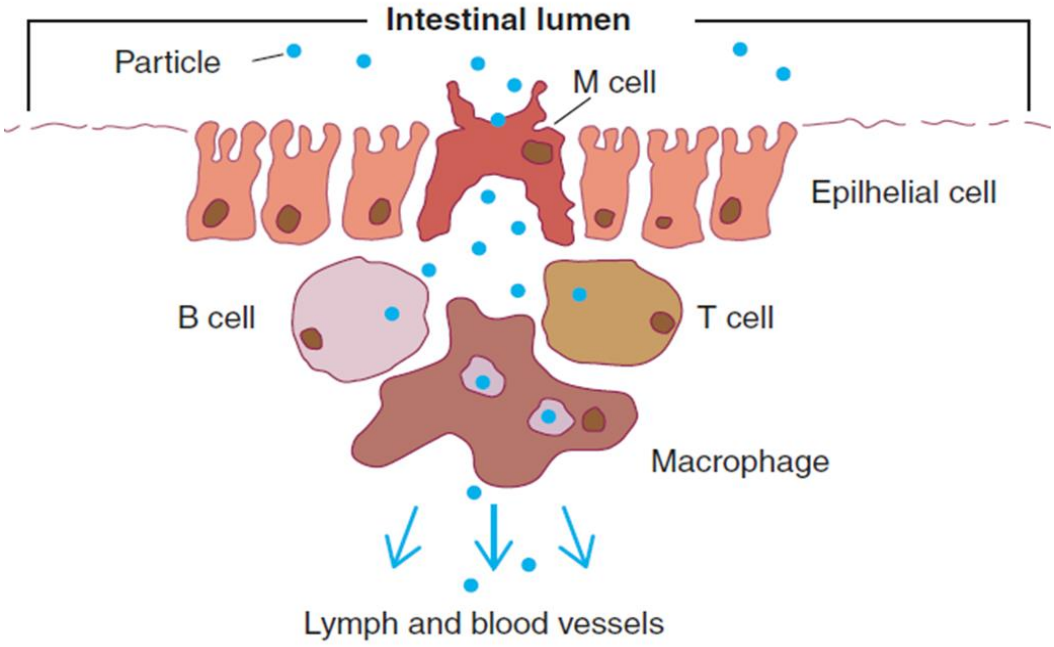

Figure 1.12: Translocation of nanoparticles to the blood from the intestine via M-cells and the lymphatic system from which transport into other tissues can occur. Modified from: Galloway (2015): Micro- and Nano- Particles and Human Health. In: Marine Anthropogenic Litter. Melanie Bergmann, Lars Gutow, Michael Klages (eds.). Springer, 343-366.

1.4.4.2 Concentration related effects

There are few reports concerning concentration related effects of ingested plastic on biota. As previously mentioned, dose dependent effects of a plastic exposure were found for *Tigriopus japonicas* during a chronic exposure by Lee *et al.* (2013c). Copepods showed a dose dependent effect when exposed to 0.05 and 0.5 µm polystyrene spheres; Survival was negatively affected at higher concentrations of the plastic beads for both particles sizes, and the highest concentration (25 µg ml^{-1}) caused a significant developmental delay in the F_1 generation for the 0.5 µm spheres, compared to the control and lower plastic concentrations (Lee *et al.*, 2013c). *Tripneustes gratilla* larvae that were exposed to water columns that had previously been contaminated with polyethylene spheres (1, 10, 100, or 300 spheres ml^{-1}) provided evidence for a dose dependent ingestion of microplastic spheres and a trend for reduced survival and growth for the highest concentration (Kaposi *et al.*, 2013). However, a study by Hämer *et al.* (2014) in which isopods (*Idotea emarginata*) were fed on a diet contaminated with low (12 mg^{-1} of food) and high (120 mg^{-1} of food) plastic concentrations of polystyrene spheres (1 - 100 µm) did not find any differences concerning ingestion, presence in the GI and egestion (Hämer *et al.*, 2014). No dose dependent effects were obtained for fish when Oliveira *et al.* (2013) exposed the common goby (*Pomatoschistus microps*) to 18.4 µg L^{-1} or 184 µg L^{-1} polyethylene spheres (1 - 5 µm) which did not reveal any significant differences for assessed biomarkers (acetylcholinesterase (AChE), isocitrate dehydrogenase (IDH), glutathione *S*-transferase (GST) and lipid peroxidation (LPO)) (Oliveira *et al.*, 2013).

1.4.4.3 Effects of shape

Nearly all of the above mentioned laboratory based studies investigating the effects of ingested plastic used spherule or round plastic pieces. Even though those types of plastics occur in aquatic environments, differently shaped materials are present as well. Accordingly, the shape and type of polymer may be of importance when evaluating the effects of ingested plastics. Hard materials with sharp or pointy edges have the potential to cause internal wounds and bleedings (Gregory, 2009). An examination of dead albatross chicks (Laysan Albatrosses *Diomedea* (*Phoebastria*) *immutabilis*) sampled in 1994 and 1995 from the Midway Atoll in the North Pacific Ocean found that some of the chicks that had ingested plastic, had punctures or tears in the lining of their

proventriculus (Auman *et al.,* 1997). In contrast, plastic fibres and lining, which are less likely to puncture through tissue, may still have the potential to clump together and form larger accumulations that could lead to the blockage of the GI (Murray and Cowie, 2011). Fragments with high surface areas like plastic films can adhere to the stomach or intestine lining and hinder enzyme secretion (Azzarello and Van Vleet, 1987) and could possibly also hinder nutrient absorption (uptake of a substance by the volume of another substance). A study by Hämer *et al.* (2014) investigating the effects of 3 different types of plastics (polystyrene spheres, polyethylene fragments and polyacrylic fibres) did not find any significant differences in ingestion, presence of up taken plastics in the GI or egestion between the plastic shapes (Hämer *et al.,* 2014). However, not only the plastic shape but also the used sizes (spheres and fragments 1 - 100 µm and fibres 20 - 2500 µm) differed, which is why the results cannot be limited just to the plastic´s shape.

According to the findings presented in 1.4.4, acute effects seem to be limited to the ingestion of larger plastic items and more attention should be given to the chronic and sub lethal effects when assessing the ingestion of smaller plastic particles. Sub lethal effects like energy depletion could be of importance since wildlife is continuously exposed to plastic litter. According to findings from birds and invertebrates a continuous ingestion of plastic and the related mobilisation of energy reserves due to the reduced energy assimilation can have limiting effects on growth, especially for fish larvae and a reduction in the condition of the fish as well. However, just little information is available to date. Easy and reliable endpoints to assess such plastic induced starvation can be the measurement of weight and length to evaluate growth and applying a length-weight related condition index to assess utilisation of energy reserves. Additionally, to assess if translocation of ingested micrometre sized plastic particles can occur, histological assessment of sectioned tissue samples and analysis of blood samples can be applied.

1.4.5 Effects of plastic derived chemicals

Due to incorporated chemicals like plasticisers and additives, virgin plastic has the potential to leach bioactive compounds (Berens, 1997, Loyo-Rosales *et al.,* 2004, Sajiki and Yonekubo, 2003, Koelmans *et al.*, 2014, Ahmad and Bajahlan, 2007). As previously described in section 1.3, a substantial part of the finished plastic product can comprise of plasticisers and additives. However, such incorporated chemicals have the

potential to be released during production and use but also after disposal of the item and are suspected to have harmful effects on biota (Oehlmann *et al.*, 2009, Teuten *et al.*, 2009, Lithner *et al.*, 2011, Halden, 2010). Due to the extent of additives used to manufacture plastic products > 50% of produced plastic is currently classified as hazardous due to their composition of monomers, additives and by products (Lithner *et al.*, 2011).

1.4.5.1 Plasticisers and bisphenol A

There is evidence for many phthalate plasticisers but also BPA to affect reproduction in invertebrates (Brennan *et al.*, 2006), fish (Santos *et al.*, 2007) and amphibians (Ohtani *et al.*, 2000), impair development in crustaceans (Wollenberger *et al.*, 2005) and amphibians (Lee *et al.*, 2005) and induce genetic aberrations (Dixon *et al.*, 1999) as well as death in acute toxicity tests (Lee *et al.*, 2005). Additionally, numerous studies are listed in Latini *et al.* (2004) that present evidence for histological damage of livers and kidneys, testicular toxicity, negative effects on fertility and altered gene expression in the liver following an exposure to Di-(2-ethylhexyl)-phthalate (DEHP), the most widely used plasticiser for PVC (Latini *et al.*, 2004).

1.4.5.2 Other additives

Stabilisers such as organotin which are used to prolong the lifetime of the polymer are of concern since endocrine disruption as well as negative effects on the human immune function have been observed (Batt, 2006). Additionally, flame retardants which consist of bromine, phosphorus, nitrogen, chlorine or inorganic compounds are of concern. Brominated flame retardants (BFRs) which are the most widely used (ACC, 2015, Birnbaum and Staskal, 2004) additives from this additive class have been reported to induce negative effects on neuro-behavioural development and in higher concentrations also on thyroid hormone levels in rats and mice as well as foetal toxicity/teratogenicity in rats and rabbits and thyroid, liver and kidney morphology (Darnerud, 2003). These types of additives can become bioavailable for biota upon the ingestion of the plastic; PBDEs, a type of flame retardant incorporated into the plastic matrix, was found to accumulate in lipids of crickets (*Acheta domesticus*) fed with polyurethane foam for 28 days at concentrations of up to 80.6 mg kg^{-1} (Gaylor *et al.*, 2012). Furthermore, there is evidence that additives like UV-stabilizers and nonylphenol induce endocrine disrupting

effects through an estrogenic and/or antiandrogenic mode of action (Harris *et al.*, 1997, Fent *et al.*, 2014). However, just limited effects were observed in Japanese medaka (*Oryzias latipes*) that were exposed to < 0.5 mm virgin polyethylene pellets as part of their diet for two months. Fish did not show any effects for tested biomarkers (choriogenin, vitellogenin (VTG) and oestrogen receptor ERα and gonad phenotype) which indicates that the virgin plastics did not leach substantial amounts of incorporated chemicals (Rochman *et al.*, 2014).

1.4.5.3 Monomer building blocks

Even though the polycarbonate matrix of a plastic product, which is formed out of long chains of monomers, is believed to be biochemically inert due to its molecular size (Teuten *et al.*, 2009, Lithner *et al.*, 2011), unreacted monomers from the polymerisation process or free monomers as a result of degradation processes have been found to have harmful effects (Xu *et al.*, 2004, Halden, 2010, Lithner *et al.*, 2011). Leached monomers like BPA, a building block of polycarbonate, can have endocrine disrupting effects (Iguchi *et al.*, 2006, Vandenberg *et al.*, 2009, Oehlmann *et al.*, 2009, Halden, 2010). Styrene used for the synthesis of polystyrene can be carcinogenic and/or mutagenic (Xu *et al.*, 2004, Lithner *et al.*, 2011). Accordingly, effects of plastic exposure might be linked to polymer type.

1.5 Accumulation of environmental pollutants on plastic, desorption and effects upon ingestion

Plastic items can accumulate a broad range of chemicals from aquatic environments on their surface (Gouin *et al.*, 2011), including metals (Ashton *et al.*, 2010) and persistent bioaccumulative and toxic compounds (PBTs) like polycyclic aromatic hydrocarbons (PAHs) and persistent organic pollutants (POPs) (Mato *et al.*, 2000, Rios *et al.*, 2007, Ogata *et al.*, 2009) (Figure 1.13). POPs are defined by the European Commission as "chemical substances that persist in the environment, bioaccumulate through the food web, and pose a risk of causing adverse effects to human health and the environment" (EU, 2015). This class of pollutants consist of a broad range of chemicals including pesticides, industrial chemicals and unintentional by products of industrial processes (EU, 2015). Typical POPs are polychlorinated biphenyl **(**PCBs), polybrominated

diphenyl ethers (PBDEs), PAHs and different organochlorine pesticides (e.g. DDT and phenols) (Jones and de Voogt, 1999).

POPs are in general lipophilic compounds that tend to accumulate on hydrophobic surfaces which is why they partition strongly to solids and mainly organic matter in sediments but can also accumulate in lipids of organisms (Jones and de Voogt, 1999). Plastics as an additional contaminant in aquatic environments have been found to accumulate POPs on their surface from the surrounding water (Ogata *et al.*, 2009, Endo *et al.*, 2005, Pascall *et al.*, 2005, Lee *et al.*, 2013a, Rios *et al.*, 2007, Heskett *et al.*, 2012, Teuten *et al.*, 2007, Rochman *et al.*, 2013a) at concentrations up to six orders of magnitude greater than ambient sea water (Ogata *et al.*, 2009). Even higher differences can be expected for floating plastic since the sea-surface microlayer has been found with concentrations of organic contaminants up to 500x greater than in the underlying waters (Wurl and Obbard, 2004). Hence, floating plastic debris can accumulate relatively high concentrations of chemical pollutants (Mato *et al.*, 2000). However, concentrations of accumulated chemicals on plastic do not just exceed concentration of pollutants found in the water column but also compared to other solids; Concentrations of chemical contaminants have been reported to accumulate on plastic at concentrations up to two orders of magnitude higher than sediment or other suspended particles (Mato *et al.*, 2000, Teuten *et al.*, 2007).

Different plastic types and their physical and chemical properties like surface area (Teuten *et al.*, 2007), diffusivity (Rusina *et al.*, 2007) and crystallinity (Mato *et al.*, 2000) affect the adsorption (accumulation of a substance on the surface of another substance) of the POPs to the plastic. Hydrophobic compounds are adsorbed more easily than less hydrophobic ones (Smedes *et al.*, 2009, Rochman *et al.*, 2013a). A study by Rochman *et al.* (2013a) exposed five types of plastic pellets (PET, HDPE/LDPE, PVC and PP) for a maximum of 12 months to the environment in the San Diego Bay and found that HDPE, LDPE and PP had the highest concentration of absorbed chemicals with PCB concentration of 25, 34 and 27 ng g^{-1} of pellet respectively (Rochman *et al.*, 2013a). The same study showed that concentrations of PAHs were even greater with 797, 722 and 122 ng g^{-1} of pellet for HDPE, LDPE and PP respectively, suggesting that polyethylene polymers accumulate more POPs than other commonly used polymer types. However, since plastic waste can be present in the environment for decades, concentrations of absorbed POPs on plastics measured on a

global scale have been found to range from 1 to 10,000 ng g^{-1} of pellet (Hirai *et al.*, 2011, Ogata *et al.*, 2009, Rios *et al.*, 2007), highlighting the high adsorption capacities of plastic for POPs (Lee *et al.*, 2013a, Velzeboer *et al.*, 2014) (Figure 1.14).

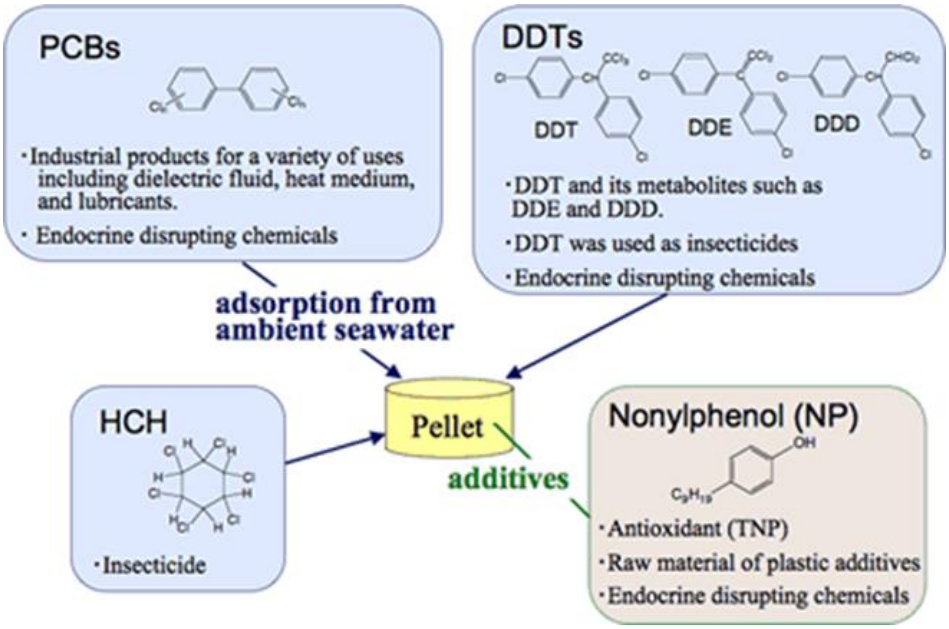

Figure 1.13: Adsorption of Persistent Organic Pollutants (POPs) from the surrounding sea water by plastic. As a result, plastic particles can accumulate substantial amounts of potentially harmful environmental pollutants on their surface. http://www.pelletwatch.org.

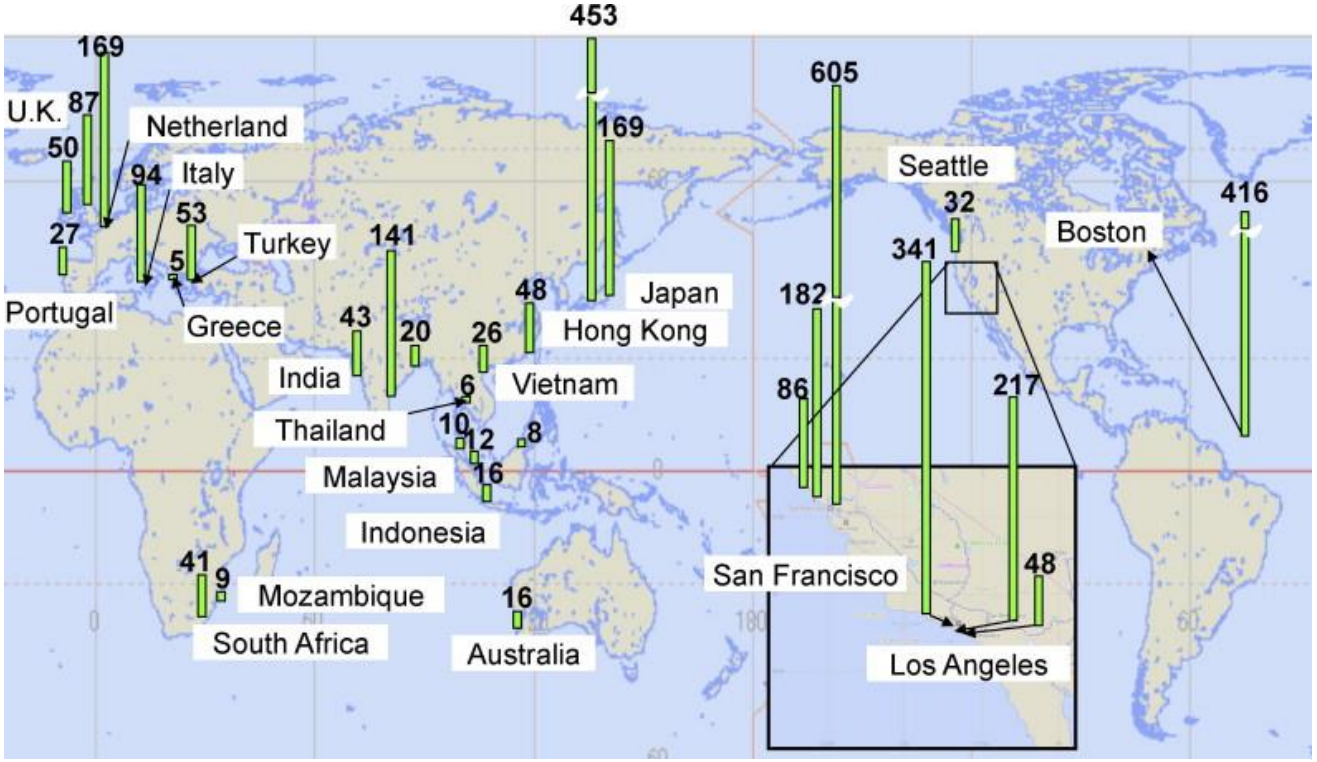

Figure 1.14: PCBs on plastic pellets from beaches around the world. Median concentrations of Σ13 PCBs (ng/g-pellet) in beached plastic pellets. Σ13 PCBs = sum of concentrations of CB# 66, 101, 110, 149, 118, 105, 153, 138, 128, 187, 180, 170, 206. Ogata *et al.* (2009):´International Pellet Watch: Global monitoring of persistent organic pollutants (POPs) in coastal waters. 1. Initial phase data on PCBs, DDTs, and HCHs`, Marine Pollution Bulletin, 58, 10, 1437-1446.

Concerns have been raised since the United States Environmental Protection Agency (USEPA) and the EU list many of the pollutants detected to be absorbed to plastics as priority pollutants because they are persistent, bioacummulative and/or toxic (USEPA, 2013, EuropeanComission, 2014). POPs are known to have endocrine disrupting effects (Daston *et al.*, 1997, Kelce *et al.*, 1995) in humans and wildlife and are also suspected to have carcinogenic effects (Jones and de Voogt, 1999) and might even damage the immune system of top predators (Safe, 1994, Ross *et al.*, 1995). Evidence suggests that plastic absorbed chemicals can be transferred to organisms (Teuten *et al.*, 2009, Besseling *et al.*, 2013, Chua *et al.*, 2014, Mato *et al.*, 2000) (Figure 1.15) and have been found to become bioavailable to a wide variety of species upon ingestion (Fossi *et al.*, 2012, Fossi *et al.*, 2014, Teuten *et al.*, 2009, Tanaka *et al.*, 2013, Lavers *et al.*, 2014, Chua *et al.*, 2014, Gaylor *et al.*, 2012, Besseling *et al.*, 2013, Browne *et al.*, 2013, Rochman *et al.*, 2013b). In line with these findings, desorption rates of accumulated absorbed pollutants from the polymer surface have been reported to be 30 times greater under physiological conditions when compared to conditions in sea water (Bakir *et al.*, 2014a). Therefore, plastics might act as a transport vector for POPs to organisms (Teuten *et al.*, 2009, Besseling *et al.*, 2013, Chua *et al.*, 2014, Mato *et al.*, 2000). Due to their low metabolism rates, environmental chemicals can lead to the accumulation in biota and along food chains (Campfens and Mackay, 1997, Jones and de Voogt, 1999). As a result, POPs can accumulate in organisms of higher trophic levels like marine birds (Helgason *et al.*, 2008), fish (Weber and Goerke, 2003) and marine mammals (Tanabe, 2002).

Indeed, the presence of PCBs in tissues of short-tailed shearwaters (*Puffinus tenuirostris*) was linked to the presence of plastic particles in the stomach by Tanaka *et al.* (2013) who argued that detected PCB congeners were not present in their natural diet and might therefore have derived from the ingested plastic (Tanaka *et al.*, 2013). A study by Yamashita *et al.* (2011) also using sampled short-tailed shearwaters reported that accumulation of PCB concentrations in tissues and found mass of ingested plastics were related (Yamashita *et al.*, 2011). These findings are supported by similar results from Ryan *et al.* (1988) who found that the mass of ingested plastics was positively correlated with PCB levels in great shearwaters (*Puffinus gravis*) (Ryan *et al.*, 1988). Laboratory based studies investigating the effects of the ingestion of contaminated plastic support field based data that plastics can act as carriers for POPs to organisms; Besseling *et al.* (2013) used the lugworm (*Arenicola marina* (L.)) and sediments that

were contaminated with 18 different PCBs. The addition of different concentrations of polystyrene spheres (0, 1, 10, and 100 g PS L^{-1} sediment) was found to enhance the bioaccumulation (accumulation of environmental chemicals in an organism) of PCBs in worms. The lowest plastic concentration was observed to cause the greatest increase in bioaccumulation with a factor of 1.1 - 1.5 compared to non-plastic sediment whereas higher plastic concentrations did not result in higher accumulation factors (Besseling *et al.*, 2013). Also for fish, the transfer of absorbed environmental pollutants on plastic was demonstrated (Rochman *et al.*, 2014, Rochman *et al.*, 2013b).

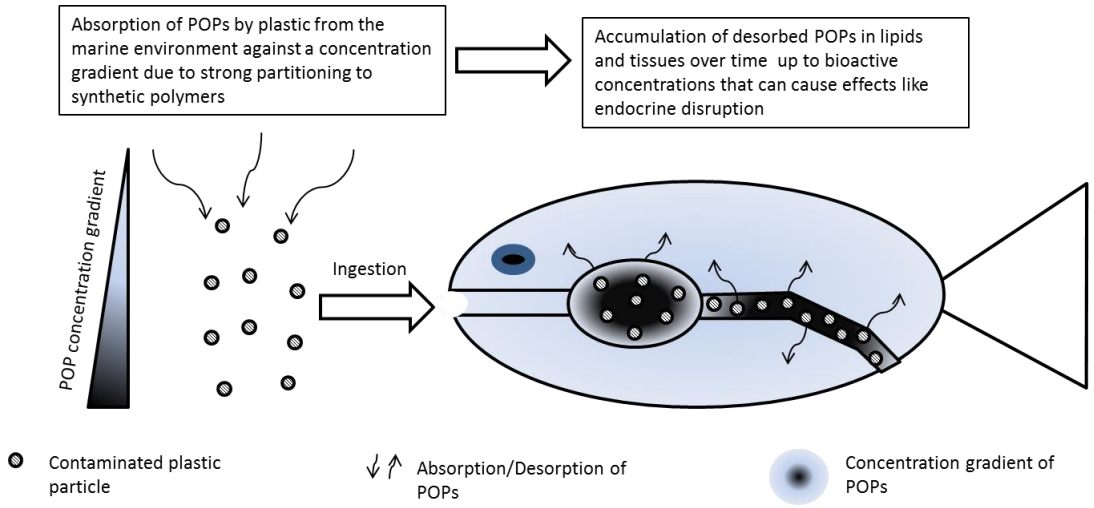

Figure 1.15: Desorption of accumulated POPs upon ingestion. Plastic particles accumulate high burdens of environmental pollutants during their lifetime in the environment at higher concentrations than the ambient sea water. After ingestion these particles can leach these absorbed chemicals at enhanced rates under physiological conditions which then accumulate in lipids and body tissues of the organism up to possibly bioactive concentrations which have the potential to induce endocrine disruption and other negative effects.

Rochman *et al.* (2013) exposed Japanese medaka (*Oryzias latipes*) for 2 months to a diet mixed with virgin or contaminated LDPE plastic pellets (previously exposed to an urban bay for 3 months to absorb POPs from the environment). After 2 months of exposure fish were found with significantly higher PBDE concentration and a trend for higher PAH and PCB accumulation in fish tissues compared to the negative control and virgin plastics (Rochman *et al.*, 2013b). Measured effects on liver toxicity and pathology as tested with glycogen depletion, fatty vacuolation and single cell necrosis was observed for virgin and contaminated plastics whereas fish exposed to contaminated plastics were found with the most severe effects (Rochman *et al.*, 2013b). Another study by Rochman *et al.* (2014b) also using Japanese medaka (*Oryzias latipes*) and the same experimental design as Rochman *et al.* (2013b) provided evidence for endocrine disruption in fish exposed to contaminated plastic diets; Female fish were found with a significant reduction in hepatic VTG and choriogenin expression after one month of exposure whereas after 2 months VTG and oestrogen receptor ERα expression was significantly reduced. Whereas no effects were observed for male fish after 1 month of exposure, expression of choriogenin was significantly reduced in males after 2 months, indicating exposure to anti-oestrogenic compounds (Rochman *et al.*, 2014).

Beside the presented evidence that ingested plastic, contaminated with POPs, can transfer harmful levels of biological active compounds to organisms and induce adverse effects, controvert suggestions from other studies should be mentioned as well. Teuten *et al.* (2007) reported that plastics have the potential to reduce bioaccumulation of POPs; This effect was suggested by the reduced bioavailability of phenanthrene in sediments spiked with polyethylene (Teuten *et al.*, 2007). In this study Teuten *et al.* (2007) argued that the addition of polyethylene to sediment can result in a reduction of phenanthrene tissue concentrations of 13% in *Arenicola marina*. Koelmans *et al.* (2013) who presented a model to predict the transport of POPs to marine organisms by plastics concluded that the ingestion of clean plastics has the potential to lead to a depuration of POPs from the organism and that the extend of bioaccumulation of POPs driven by plastics is not relevant from a risk assessment perspective (Koelmans *et al.*, 2013). In addition, the leaching of nonylphenol (NP) and BPA, both plastic additives, from polyethylene plastics to *Arenicola marina* and North Sea cod (*Gadus morhua*) were found to be negligible for fish and just occasionally relevant for worms (Koelmans *et al.*, 2014). However, for these proposed clearing properties by Teuten *et al.* (2007) and Koelmans *et al.* (2013), virgin plastics or plastics with low contamination levels are

needed. The previously mentioned high partition of chemicals to plastic and the long lifetime of plastics in the environment might therefore limit these findings to virgin and newly introduced plastics.

More research is needed to understand the full extent of the effects of ingested plastics with associated chemicals. Negligible effects found for adult organisms might have adverse effects for larval stages or juveniles of the same species and as it was discussed, plastic types do have a strong influence on the sorption and desorption mechanisms of chemicals in the environment.

1.5.1 Biomagnification of environmental pollutants through trophic transfer of plastic

Trophic transfer of environmental pollutants and their biomagnification over the food chain up to concentrations of 1 million times higher when compared to the surrounding water (Mason *et al.*, 1996), can lead to deleterious effects and even death in top consumers (MIBS, 1997). The trophic transfer and biomagnification of POPs in the marine food web for zooplanktonic species, benthic invertebrates and fish up to sea birds and seals was suggested using stable nitrogen isotopes ($\delta^{15}N$) (Fisk *et al.*, 2001).

As previously mentioned, POPs can be found at concentrations of up to 10,000 ng g^{-1} (Ogata *et al.*, 2009) on plastic pellets. Since plastics are subject to trophic transfer from primary to top consumers (see section 1.4.3) ingestion of contaminated plastic via the diet is possible. With findings of Bakir *et al.* (2014a) who reported enhanced desorption of POPs from plastic under physiological conditions, a plastic mediated dietary accumulation of environmental pollutants is possible.

However, to my knowledge no data is available to document the plastic mediated trophic transfer of environmental pollutants and the possible biomagnification due to desorption processes under physiological conditions. Furthermore, no reports are present to document effects that arise from the ingestion of contaminated plastic via trophic transfer.

1.6 Biomarkers

Fish, like other aquatic biota are exposed to a variety of environmental and anthropogenic contaminants. To determine effects that are induced by the exposure to such contaminants, a vast array of biomarkers on different levels of biological organisation have been established (Viarengo *et al.*, 2007). Ribeiro *et al.* (2013) stated that biomarkers may be derived from morphological, biochemical and physiological alterations in organisms following the exposure to xenobiotics, integrating biological responses to contamination and may thereby indicate sub lethal exposure (Ribeiro *et al.*, 2013). Fish are particularly sensitive to the environmental contamination of water and, therefore, pollutants may significantly interfere with several of their biochemical processes. Additionally, the importance of somatic indices to explain the effects of pollutants on growth, longevity and reproductive ability of organisms has been described (Alberto *et al.*, 2005, Ribeiro *et al.*, 2005, Tejeda-Vera *et al.*, 2007). As a result, morphological endpoints on an organism level like growth in fish is a commonly analysed endpoint (Chovanec *et al.*, 2003).

The ability to maintain normal somatic growth (length and weight) is an important indicator of an organisms fitness which in turn is related to its ability to acquire and assimilate energy, maintain homeostasis and regulate metabolism (Beckman, 2011). The importance of growth in length during larval stages (Bailey and Houde, 1989) of fish shifts towards growth in weight for adults (Nellen, 1986, Mommsen, 2001). The respective growth is of great importance for predator avoidance in larval (Bailey and Houde, 1989) and reproductive success (Munro, 1990) in adult fish. Therefore, effects on length, weight and condition of exposed fish can not only have negative effects on performance and survival for the individual but might also result in effects on the population level (Smolders *et al.*, 2005). Important factors for growth in teleost fish are food availability and nutritional status (Pedroso *et al.*, 2006), water temperature (Handeland *et al.*, 2008) and photoperiod (Barimani *et al.*, 2013). These factors are thought to be related to levels of growth hormone (GH) expression in fish (Björnsson, 1997, Gabillard *et al.*, 2003) which in turn leads to elevated levels of insulin like growth factors (Funkenstein *et al.*, 1989).

The condition index K calculated after Fulton (Ricker, 1975) provides a useful tool to evaluate the well-being of fish and is one of the most widely used measurement for fish

condition (Jones *et al.*, 1999). Fulton´s condition index has been shown to be a good indicator for lipid reserves in fish, including sticklebacks (Herbinger and Friars, 1991, Chellappa *et al.*, 1995). Even though not a direct measurement for fitness, lipid reserves can be seen as fitness indicators of an animal (Pietrobelli *et al.*, 1996); It is commonly assumed that the fitness of an animal is positively correlated with its lipid reserves due to their utilisation during starvation und during time of higher energetic requirements but also help the organism to deal with harsh abiotic factors and may even protect from injury (Wilder *et al.*, 2015). Therefore, a reduced condition index as a measurement for reduced lipid reserves is an important endpoint to assess the changes in the fish´s wellbeing which might result in changes in fitness in the longer term.

Therefore, the choice of growth and condition to test for plastic induced effects in this thesis is in line with multiple studies that have previously applied growth measurements and energy reserves as biological endpoints for plastic exposure (Kaposi *et al.*, 2013, Besseling *et al.*, 2014, Hämer *et al.*, 2014, Besseling *et al.*, 2013, Cedervall *et al.*, 2012, Wright *et al.*, 2013, Donnelly-Greenan *et al.*, 2014, Foekema *et al.*, 2013).

In addition to the endpoints on an organism level, molecular endpoints can be included to test for effects on gene expression. The cytochrome system is a large superfamily of heme proteins comprising of 221 genes divided into 36 subfamilies that are involved in the oxidative metabolism of numerous lipophilic xenobiotics like aromatic hydrocarbons and pesticides which has been demonstrated and validated for field and laboratory studies (Goksoyr, 1995, Boon *et al.*, 2002, Livingstone, 1998, Bucheli and Fent, 1995, Stegeman and Hahn, 1994). Chemicals induce CYP1A through the binding to aryl hydrocarbon receptor (AhR) (Goksoyr, 1995), thereby initiating the induction process leading to increased amounts of CYP1A mRNA, protein and catalytic activity (Goksoyr, 1995). Additionally, cytochrome P450 is involved in the hydroxylation of alkanes (Blasig *et al.*, 1984). As a result, CYP1A has been found to increase in the presence of xenobiotic chemicals (Egaas *et al.*, 1998) and polycyclic aromatic hydrocarbons in flatfish (Myers *et al.*, 2008). Certain subfamilies are particular responsive to xenobiotic compounds, prominent examples being the induction of CYP1A subfamily by planar aromatic hydrocarbons and chlorinated hydrocarbons (Nebert *et al.*, 1989, Stegeman and Hahn, 1994). The induction of CYP1A in fish has been evaluated as a sensitive, convenient, “early warning” signal of organic xenobiotics

in the aquatic environment (Bucheli and Fent, 1995, Goksoyr, 1995, Stegeman and Hahn, 1994).

The yolk precursor VTG is normally produced in parenchymal liver cells of females and is then transported to the ovary, incorporated into the growing oocytes and then processed into the two main yolk proteins, lipovitellin and phosvitin and other components in a process called vitellogenesis (Byrne *et al.*, 1989, Hiramatsu *et al.*, 2002, Wahli *et al.*, 1981). VTG is normally undetectable in the plasma of immature females and male animals because they lack circulating oestrogen, however, VTG expression can be induced by oestrogen exposure (Heppell *et al.*, 1995, Sumpter and Jobling, 1995). Not just the natural oestrogen 17β-estradiol, which is the primary hormone responsible for vitellogenesis (Specker and Sullivan, 1994) but also endocrine disrupting chemicals with oestrogenic mode of action (Sumpter and Jobling, 1995, Thorpe *et al.*, 2001) have been found to induce VTG expression in exposed fish. Accordingly, VTG is an established molecular biomarker to assess the exposure to oestrogenic compounds in fish (Tyler *et al.*, 1996). Since alkylphenols and styrene which can leach from plastic polymers have been found to display oestrogenic properties (Bang *et al.*, 2012), VTG was used to test for oestrogenic plastic derived and absorbed chemicals.

Both, expression of CYP1A and VTG has been previously used to investigate the exposure of desorbed chemicals by plastic to fish (Rochman *et al.*, 2013b, Rochman *et al.*, 2014).

1.7 Study Aims

As described, the contamination of aquatic environments globally with plastic litter is of great concern. The accumulation of this anthropogenic debris over the past decades has led to an omnipresent pollutant that, due to its characteristics, poses a threat to the wild life. Of special concern is the ingestion of small plastic particles. Even though ingestion records are numerous for a broad range of species from different feeding guilds and life stages, just very little is known about the effects that can follow such ingestion. First evidence for various adverse effects has been reported, however, many questions still remain unanswered and the wide range of study organisms used leaves an incomplete picture on a phylum and species level. A range of knowledge gaps concerning microplastic in the marine environment have been identified in a recent review about current trends and future perspectives of microplastics in the marine environment by Barboza and Gimenez (2015) (Figure 1.16).

This thesis addresses some of these identified gaps by contributing to the current lack of knowledge concerning the impact of microplastic ingestion on marine organisms and the food chain.

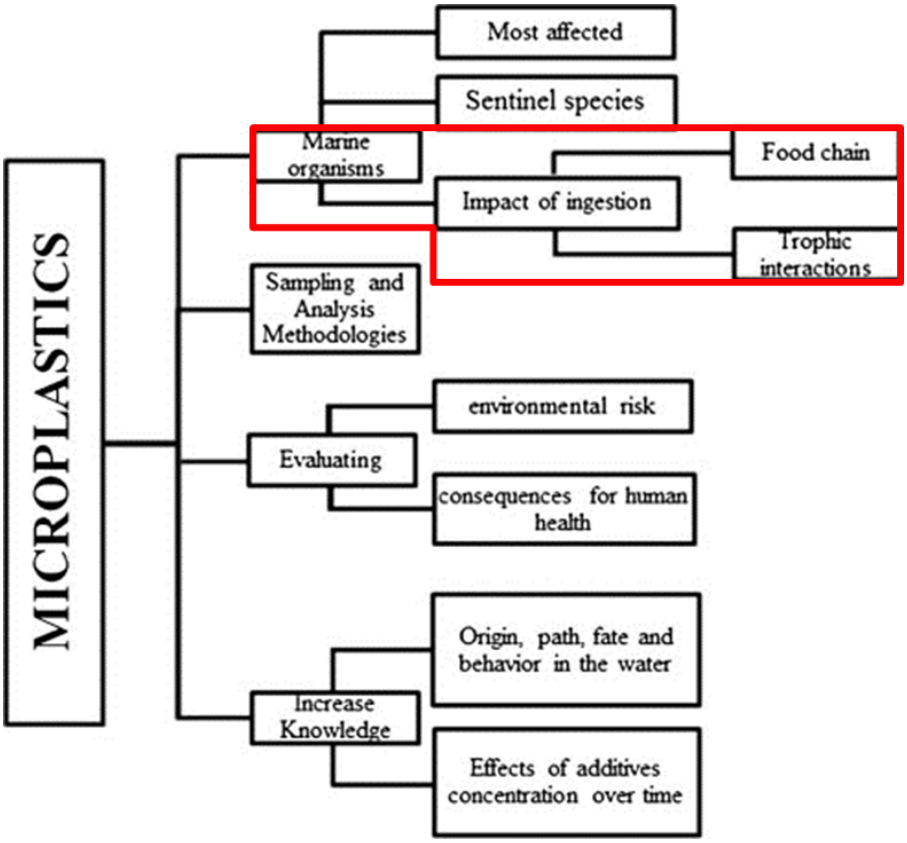

Figure 1.16: Research aspects of interest in the future with regard to marine environment microplastics as identified by Barboza and Gimenez (2015). The chapters presented in this thesis contribute to the knowledge gap in the red framed area. Figure sourced from (Barboza and Gimenez, 2015): Microplastics in the marine environment: Current trends and future perspectives. Marine Pollution Bulletin, In Press, corrected proof.

The overriding aim of this thesis was to assess the biological effects of ingested plastic particles in a small predatory teleost, the three-spined stickleback. An ecotoxicological approach with laboratory based experiments has been chosen to address the following aims:

1. To assess ingestion, accumulation and elimination of microplastic ingested via trophic transfer by a small aquatic vertebrate and to determine biological effects following the exposure. Chapter 3 exposed adult (10 months old) sticklebacks for 7 days on contaminated live diets of *Artemia sp*., followed by a further 14 day depuration period where just non-contaminated *Artemia sp*. were fed. Faeces samples, sampling data (length, weight, condition index K), blood samples and molecular endpoints were assessed to test for biological effects. The hypothesis of this chapter was that exposure to a plastic contaminated live *Artemia sp*. diet will result in the ingestion and egestion of plastic particles and will lead to biological effects on weight and condition index K, will lead to the translocation of microplastic from the gastrointestinal tract to the circulatory system and result in a response in the measured detoxification biomarker CYP1A in adult three spined stickleback.

2. To investigate the effects of ingested microplastic from a plastic contaminated water column on larval three-spined stickleback. Chapter 4 addressed this research question using larval (7 days post hatch (dph)) sticklebacks which were exposed to a water column spiked with ascending concentrations of fluorescent polystyrene plastic spheres over a duration of 7 days. Ingestion and induced effects on growth (length and weight), condition index and molecular endpoints were assessed. The hypothesis for this chapter was that exposure to a water contaminated water column will result in a dose dependent ingestion and egestion of microplastic and induce negative effects on length and condition index K and induce a response in the measured detoxification biomarker CYP1A as well as the oestrogen biomarker VTG B in larval three spined sticklebacks.

3. To assess the effects of exposure to ‘real’ plastics (fragments of conventional plastic bags) on an adult aquatic vertebrate the three-spined stickleback and determine whether degradation of the plastics through weathering influences their potential to induce biological effects. Chapter 5 exposed adult (14 months

old) sticklebacks to a plastic contaminated diet for a total of 28 days. Faeces samples, dissection of GI tracts and sampling data (length, weight, liver weight, condition index K) were used to test for biological effects. Additionally, plastic fragments were analysed for their potential to leach incorporated chemicals under simulated stomach pH conditions via GC-MS. The hypothesis of this chapter was that ingestion of a food source contaminated with microplastic sized plastic bag fragments results in the ingestion of microplastic, effects on food digestion through ingested plastic and effects on weight and condition index K of adult three spined sticklebacks. Aditionally, it is hypothesised that degraded plastic bag fragments have a higher potency to induce biological effects through the leaching of incorporated chemicals.

4. To determine whether a live diet of *Artemia sp*. with ingested plastic particles that had previously been contaminated with bisphenol A have a higher potential to induce biological effects compared to *Artemia sp*. with ingested non-contamianted plastic and *Artemia sp*. that had been exposed to bisphenol A without ingested plastic.. In Chapter 6 larval (10 dph) sticklebacks were exposed to live *Artemia sp*. that had been previously cultured in BPA solutions with or without the addition of polystyrene plastic spheres. Faeces samples, dissections of the GI, sampling data (length, weight and condition index K), mortality and molecular work were used to test for biological effects. The hypothesis of this chapter was that ingestion of *Artemia sp*. that had ingested the bisphenol A contaminated microplastic spheres by three spined stickleback larvae will result in biological effects on length, condition index K and induce a response in the measured detoxification biomarker CYP1A as well as the oestrogen biomarker VTG B in larval three spined sticklebacks.

Chapter 2

Material and Methods

2.1 Study organisms

2.1.1 Three-spined stickleback (*Gasterosteus aculeatus*)

With over 27,700 different species, fish are one of the largest and most diverse groups of animals and are of immense ecological and economic importance (Nelson, 2006). Fish are widely used as sensitive model organisms (Powers, 1989) and bioindicators for the pollution of aquatic environments (van der Oost *et al.*, 2003) and are used for observational as well as experimental assessment of chemical pollution (Slooff and De Zwart, 1983). Since fish are not only widely used as bioindicators for chemicals but are also known to ingest plastic (see Chapter 1, section 1.4.2), they provide a useful tool to assess the effects of an environment polluted with virgin and contaminated plastic.

The three-spined stickleback (*Gasterosteus aculeatus* (Linnaeus 1758)) is a small teleost fish species (Figure 2.1) of the Gasterosteidae family and can be found in habitats in Atlantic, Pacific and Arctic coastal waters as well as in inland waters in Europe, North America and Asia (Östlund-Nilsson *et al.*, 2007). This distribution along habitats of the northern hemisphere with salinities ranging from brackish water to fully freshwater conditions and its good attributes as a lab held organism has led to the stickleback being one of the best studied species of fish (Östlund-Nilsson *et al.*, 2007). Although sticklebacks have largely been used as model organisms for behavioural and evolutionary aspects, they have been proven to be useful model species to study the effects of endocrine disrupting chemical compounds (Katsiadaki *et al.*, 2007, Östlund-Nilsson *et al.*, 2007). Today, the three-spined stickleback is a well-established ecotoxicological model species (Katsiadaki, 2006) and has been used to investigate endocrine disrupting effects (Hahlbeck *et al.*, 2004b, Hahlbeck *et al.*, 2004a, Katsiadaki *et al.*, 2010), metal contamination (Sanchez *et al.*, 2005), host-parasite interactions (Barber and Scharsack, 2009), behaviour (Iersel, 1953) and evolutionary biology (Bell and Foster, 1994). Since the pollution of aquatic environments with plastic waste is of global concern and freshwater as well as brackish and marine habitats are affected, sticklebacks pose an ideal model species to compare observed effects on a global scale. Biomarkers for the exposure to a food source contaminated with POPs (Holm *et al.*, 1993) and water bodies contaminated with oestrogens and androgens (Katsiadaki *et al.*, 2002) have been previously applied. Since many plasticisers and additives that leach from plastics show endocrine disrupting effects and POPs are known to accumulate on

the surface of plastic, sticklebacks are a good candidate to study chemical related effects of plastic exposure. Additionally, sticklebacks possess a well-developed gastrointestinal tract. Following the mouth, the oesophagus leads into a functional stomach which can be separated into three regions. Following the oesophagus a cardiac region exists which is a non-secretory region and mainly used to store food, which is commonly found in fish that feed to satiation like the three-spined stickleback (Gill and Hart, 1998). The other two regions (fundic and pyloric) have secretory properties in which digestive enzymes and acidic stomach pH digest the food. The posterior part of the stomach is separated from the anterior part of the intestine by a pyloric sphincter which allows controlled release of food matter from the stomach to the intestine. The intestines lead to a rectum over which processed food is egested as faeces (Wootton, 1984, Ostrander, 2000, Hale, 1965). This developed gastrointestinal tract allows for the detailed analysis of the presence of ingested plastic and might also lead to higher accumulation of ingested plastic in the stomach of sticklebacks due to satiation feeding and storage of food in the cardiac region of the stomach.

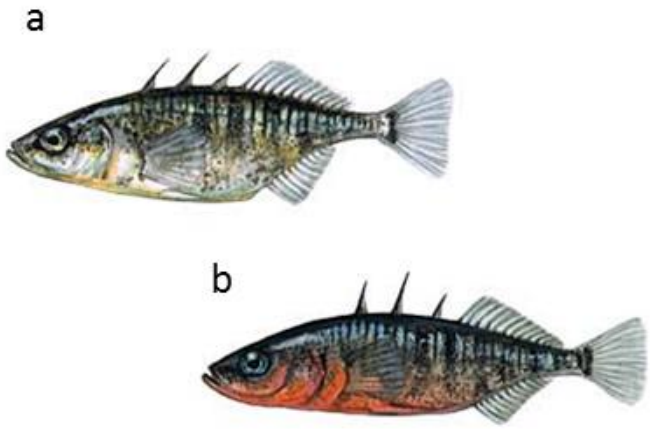

Figure 2.1: Three-spined Stickleback (*Gasterosteus aculeatus*). a: Female fish, b: Male fish with sex specific characteristics during reproductive season. http://www.crsmsodry.cz

2.1.1.1 Production of the F_1 and F_2 generation

As previously described in Chapter 1, section 1.2, plastic particles are present in high levels in aquatic environments. Laboratory hatched F_1 and F_2 generations were used for experiments (Table 2.1) to avoid the possibility of a pollution history of wild caught fish. Additionally, reverse osmosis (RO) water (Select Fusion, Purite) was used for the preparation of all stock and experimental related media (artificial fresh water (AFW) and artificial sea water (ASW)) and test solutions. ASW (3.0 ppt) and AFW (0.5 ppt) used for stock maintenance and exposures were created by the addition of synthetic marine salts (Tropical Marine Centre, UK) to RO water.

To produce these uncontaminated generations of fish a laboratory stock was established. A total of 280 three-spined sticklebacks were caught from brackish conditions (Salinity of 3.0 ppt) at Farlington Marshes (Figure 2.2) and transported to the Institute of Marine Sciences of the University of Portsmouth during March 2012. Farlington Marshes is a 125,000 hectares large coastal grazing marsh and lagoon which has several pools, both freshwater and brackish and is managed by the Hampshire & Isle of Wight Wildlife Trust (HIWWT, 2015).

Figure 2.2: Sampling site for F_0 generation from Farlington Marshes in March 2012 which was used to produce an F_1 generation used for the microplastic exposures. Images sourced from Google Earth.

The fish were acclimatised from environmental (salinity: 3.0 ppt, temperature: 10°C and photoperiod: 13h light and 11h dark) to laboratory holding conditions (salinity: 3.0 ppt, temperature: 18°C and photoperiod: 16h light and 8h dark) over a period of 3 months by

gradually increasing the temperature and photoperiod. Fish were then maintained under these conditions until reproductively mature. During the acclimatisation period, fish were fed defrosted brine shrimp (Tropical Marine Centre; 3x day^{-1} week days and 1x day^{-1} on weekends). Mortality during the 3 months acclimatisation period was recorded to be less than 10%. Over the acclimatisation process fish were held in four 80 L aerated glass tanks (working volume 65 L) with semi static water conditions and attached recirculation units in an air-conditioned room. Two to three water changes were performed per tank per week, depending on the water quality.

Once wild caught fish were acclimatised and mature, salinity was gradually decreased in 0.2 ppt steps every second day to slowly mimic migration into fresh water conditions. This imitated migratory process to fresh water conditions was necessary, since the here used sticklebacks derived from a brackish population that displays anadromous behaviour to breed in fresh water. Once AFW conditions were reached (holding conditions salinity: 0.5 ppt, temperature: 18°C and photoperiod: 16h light and 8h dark), 15 males were chosen to be transferred to spawning tanks which contained 2 cm of sand at the bottom and were supplied with cotton fibres and left to build a nest. Fifteen individual female fish were presented to males with finished nests. After spawning and fertilisation nest were removed from the tanks, any eggs removed and placed in fresh AFW and left to water-harden for 30 min. After water-hardening, eggs were separated under a dissecting microscope using stainless steel, straight teasing needles and tweezers and placed individually in wells of a 96-well plate containing 200 µl AFW using a plastic Pasteur pipette and stored under spawning conditions. Eggs were kept in separated chambers of 96-well plates to avoid cross infection from unhealthy eggs. AFW of the wells was replaced daily with new AFW and the development of larvae assessed daily. Any unhealthy eggs were removed. Upon hatching of the first larvae, unhatched healthy eggs and larvae were transferred to a holding tank containing 5 L AFW under stock conditions. Larvae were fed with suspended frozen rotifers from 3 dph (since yolk reserves are completely depleted at 4 dph in stickleback larvae (Swarup, 1958)) before live, freshly hatched *Artemia sp*. (Ocean Nutrition, Aquatics online, UK) gradually replaced the rotifers. Larvae were kept on a live *Artemia sp*. diet until they were found to accept defrosted blood worms as a food source which replaced the live *Artemia sp*. diet from then on. After 3 weeks, larvae were transferred to mildly aerated 30 L tanks (working volume 20 L) with static water conditions before being transferred

to the final 80 L aerated glass tanks (working volume 65 L) with semi static water conditions and attached recirculation units.

F_2 generation fish were produced using the same techniques as outlined above for the production of the F_1 generation with the exception that just 3 or 4 breeding pairs were used due to the limited number of larvae needed for the experiments for Chapter 4 and 6, respectively. Upon hatching of the first larvae, unhatched healthy eggs and larvae were transferred to 1 L glass beakers containing 200 ml AFW.

Table 2.1: Generation and age of sticklebacks used for the experiments.

Chapter	Generation	Development stage	Age
3	F_1	Adult	10 month
4	F_2	Larvae	7 dph
5	F_1	Adult	14 month
6	F_2	Larvae	10 dph

2.1.1.2 Laboratory stock conditions

The F_1 stock fish used for the production of the F_2 generation and experiments performed in Chapter 3 and 5 were maintained in aerated, semi static holding tanks (90 L, 70 L working volume) with attached recirculation units. Prior to the experiments using fish from the produced F_1 generation, salinity was gradually raised from hatching conditions (Salinity: 0.5 ppt, temperature: 18°C and photoperiod: 16h light and 8h dark) in 0.2 ppt steps every second day to transfer the fish to brackish conditions. Holding conditions at brackish salinity was: Salinity: 3.0 ppt, temperature 18°C and a 16:8h light-dark photoperiod. Experiments were conducted at brackish conditions to test for effects of plastic exposure under normal environmental and not breeding conditions. Fish were acclimatised to these brackish conditions prior to the start of the experiments. Prior to being transferred to the experimental setup, F_1 sticklebacks were fed frozen blood worms (Tropical Marine Centre; 3x day^{-1} week days and 1x day^{-1} on weekends).

Larval F_2 fish were maintained in 1 L beakers containing 200 ml (Chapter 4) or 500 ml (Chapter 6) AFW (Salinity: 0.5 ppt, temperature: 18°C and a 16:8h light-dark photoperiod). Prior to experiments, larvae were kept under these conditions to test for effects of plastic in their natural environment after hatch. Larvae were fed 3 drops of defrosted rotifers (Tropical Marine Centre, UK) two times daily from 2 days post hatch (Chapter 4 and 5). For Chapter 5, freshly hatched *Artemia* nauplii were added from 4 days post hatch. The amount of *Artemia sp*. per feed was then gradually increased from 0.5 to 2 ml per feed as the amount of suspended rotifers decreased from 3 drops to 0 drops per feed.

2.1.1.3 Experimental conditions

All experiments were conducted in an air conditioned aquarium room with an automated light:dark cycle of 16:8. Exposure vessels were placed on elevated shelves (Figure 2.3). For all experiments, the test organisms were randomly distributed to the exposure vessels.

For adult experiments, fish were transferred to 10 L aerated glass aquaria (working volume 6 L) containing ASW (3.0 ppt). A static renewal system was used with 80% of the water in each tank being removed and replaced every second day. Temperature was checked daily with a spirit thermometer (Sigma), whereas pH and dissolved oxygen

(DO) were checked twice weekly using HI-98130 and HI-9146N probes (Hanna Instruments Ltd), respectively. See individual chapters for parameter ranges. Over the experimental procedure sticklebacks were fed according to experimental procedures as detailed in each chapter.

Larval exposures were conducted using 1 L non-aerated glass beakers (working volume 200 ml for Chapter 4 and 500 ml for Chapter 6) containing AFW (0.5 ppt) as exposure vessels. A static renewal system was used with 80% of the water in each beaker being removed and replaced every second day. Temperature was checked daily with a spirit thermometer (Sigma), whereas pH and DO were checked twice weekly using HI-98130 and HI-9146N probes (Hanna Instruments Ltd), respectively. See individual chapters for parameter ranges. Over the experimental procedure stickleback larvae were fed according to experimental procedures as detailed in each chapter.

Figure 2.3: Layout of exposure vessel arrangement. a= 10 L exposure tanks for adult exposures (Chapter 3 and 5). b= 1 L beakers for larval exposures (Chapter 4 and 6).

2.1.1.4 Ethical Review

All procedures involving the three-spined stickleback (*Gasterosteus aculeatus*), were performed in line with UK 2013 Home Office Licence regulations under the Home Office Licence No. PPL70/7467 and the individual experiments were approved by the ethical commission of the University of Portsmouth.

2.1.2 Brine shrimp (*Artemia sp.*)

Artemia sp. are small filter feeding crustaceans that can be found in water bodies in Africa, Australia, New Zealand and North America (Triantaphyllidis *et al.*, 1998). Due to their highly efficient osmoregulation the habitat of *Artemia sp*. are hypersaline aquatic environments with salinities 10 times higher (340 g L^{-1}) than normal marine conditions (Gajardo and Beardmore, 2012). Females are capable of switching between two reproductive modes; During unstable environmental conditions females produce cysts (oviparity) which are able to survive even under critical environmental conditions like severe dehydration, whereas under good environmental conditions free swimming nauplii (ovoviviparity) are produced (Gajardo and Beardmore, 2012). The production of cysts which can be dried and easily shipped have led to the use of *Artemia sp*. on a commercial scale as a live fish food in aquaculture (Sorgeloos *et al.*, 2001, Nash, 1973) and provide not only basic nutritional requirements but also enzymes and other valuable dietary elements as well as forming an attractive prey for predatory fish (Gajardo and Beardmore, 2012). Additionally, *Artemia sp*. are a well-recognised model organism for ecotoxicological studies to test water quality (Persoone and Wells, 1987, Kalčíková *et al.*, 2012). Furthermore, *Artemia sp*. have been previously used as live food in laboratory studies investigating the effects and the biomagnification of POPs on fish (Lyche *et al.*, 2011, Lyche *et al.*, 2013) and plastic ingestion in teleost fishes (Hoss and Settle, 1990). Hence, *Artemia sp*. poses a good model organism for zooplanktonic organisms of the lower trophic levels. In addition, their indiscriminate filter feeding foraging technique to ingest small food particles (algal cells, bacteria and detritus of 1-50 µm) (Dhont and Van Stappen, 2003) indicates their potential to ingest small microplastics. Even though *Artemia sp*. are not part of the diet of the three-spined stickleback in the wild, their size compares to the small crustaceans the can be found in a stickleback´s natural diet (Hynes, 1950, Allen and Wootton, 1984). *Artemia sp*. was

also chosen as a live diet for laboratory held sticklebacks due to the ease of culturing daily batches of freshly hatched nauplii.

Artemia sp. cysts (1 g L^{-1}) were freshly hatched for each experiment in aerated artificial sea water (ASW; 20 ppt) at a temperature of 25°C and a photoperiod of 24h light. After 24h, the freshly hatched *Artemia* nauplii (< 24h old) (Figure 2.4) were harvested by using a light source to separate the live nauplii from the unhatched cysts. Harvested *Artemia sp*. were transferred to a clean culture vessel and used as live food for larval stock fish or matured for a further 24h under culture conditions. Matured *Artemia sp*. were then transferred to the exposure vessels to be contaminated prior to being used as live food for the experiments in Chapter 3 and 6 (Figure 2.5).

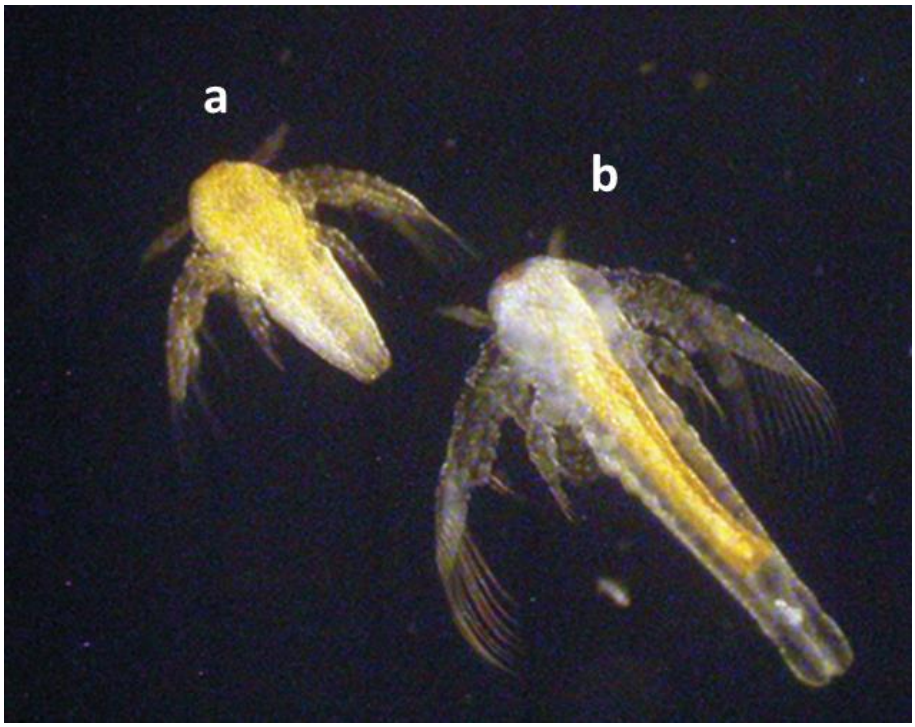

Figure 2.4: *Artemia sp*. development stages. a: freshly hatched nauplii (Instar I). Instar I nauplii were separated from the culture medium and used as live food for larval stock fish or matured for a further 24h under culture conditions to be contaminated according to experimental procedures. b: 72h old nauplii (Instar V). The 72h old nauplii represent the developmental stage of *Artemia sp*. after the contamination for the experiments (24h maturing + 24h exposure to the respective experimental contamination set up. http://www.akvaryumblog.com.

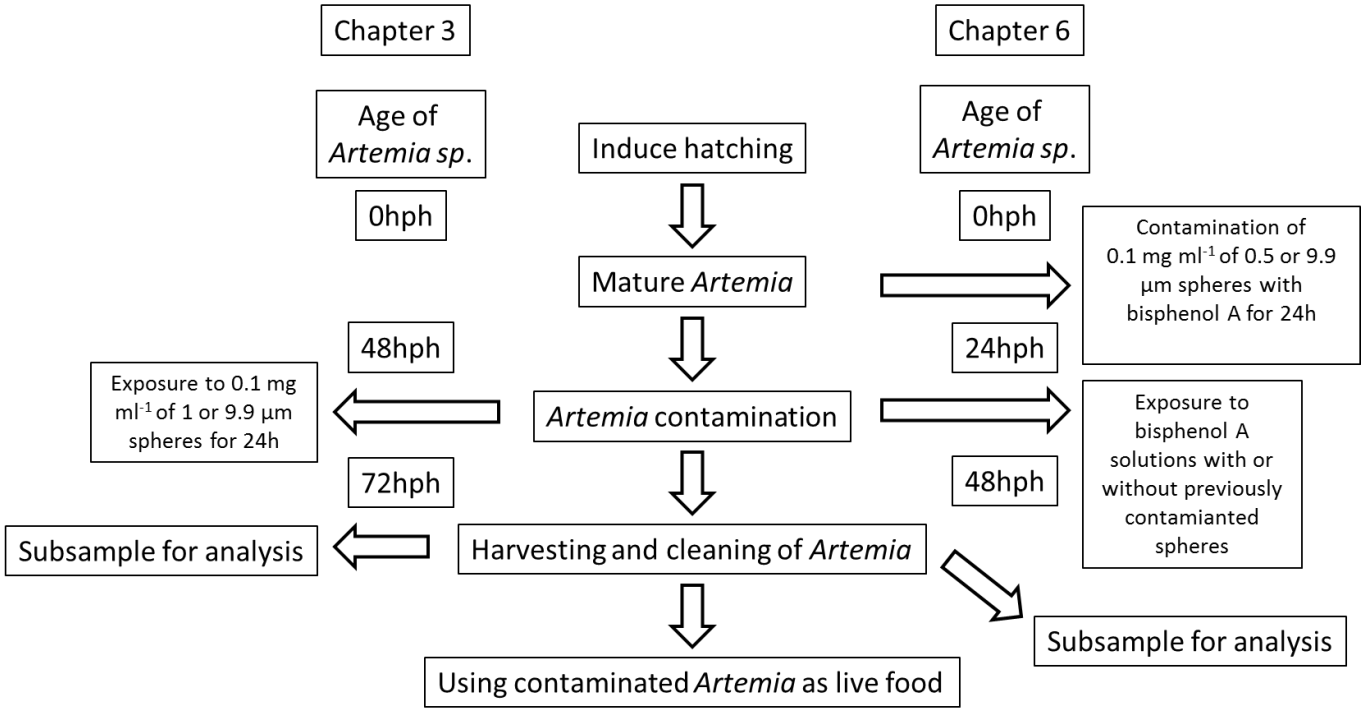

Figure 2.5: Flow diagram of *Artemia sp*. contamination for the different experimental set ups. *Artemia sp*. cysts were used to be incubated (salinity: 20 ppt, temperature: 25°C and photoperiod: 24h light) for 24h to induce hatching and then matured for 24h (Chapter 6) or 48h (Chapter 3) before contamination of 24 hours post hatch (hph) (Chapter 6) or 48hph (Chapter 3) old *Artemia sp*. started.

2.2 Microplastic spheres

Fluro-Max^{TM} green fluorescent polystyrene microspheres (1.0 (Chapter 3 and 4) and 9.9 µm (Chapter 3, 4 and 6); lot no's 40831 and 41359, respectively) were purchased from Thermo Scientific (Fremont, CA, USA). The particle count for the 1 and 9.9 µm spheres per millilitre was $1.81x10^{10}$ and $1.81x10^{7}$, respectively. Firefli^{TM} green fluorescent polystyrene microspheres (0.5 µm (Chapter 6)) were purchased from Duke Scientific Corporation (Palo Alto, CA, USA). The particle count for the 0.5 µm polystyrene spheres per millilitre was $1.4x10^{14}$. All fluorescent microspheres were in aqueous solution (1% solids), had a specific particle density of 1.05 g cm^{-3} and had their maximum refraction at 589 nm. Fluorescent spheres were stored away from light at 7°C in a fridge until needed.

The here used fluorescent plastic spheres were chosen due to the previous successful application in numerous studies; Similar sized fluorescent polystyrene (Browne *et al.*, 2008, Cole *et al.*, 2015, Cole *et al.*, 2013, Besseling *et al.*, 2013, Farrell and Nelson, 2013, Hjelmeland *et al.*, 1988) and polyethylene (Mazurais *et al.*, 2014, von Moos *et al.*, 2012, Kaposi *et al.*, 2013) spheres have been previously shown to be a good model plastic to investigate ingestion and related effects on multiple aquatic species. Polystyrene plastic spheres were used since field data reported their abundance in coastal waters (Carpenter *et al.*, 1972) and reports from the five subtropical gyres reported polystyrene macroplastic as the most abundant plastic type (Eriksen *et al.*, 2014). The here used sphere sizes were chosen due to their suitable size range to be ingested by *Artemia sp.* which naturally feed on small phytoplankton like *Dunaliella sp.* which measure 3 to 13 µm in width (Hosseini Tafreshi and Shariati, 2009) but also algal cells, bacteria and detritus of 1-50 µm (Dhont and Van Stappen, 2003). However, the here chosen sizes of plastic spheres were too small to be directly ingested by the adult and larval fish; The lower size limit for directly ingested food particles by first feeding cod larva has been reported to be 30 - 40 µm which is in accordance with the minimum size of particles that fish larvae theoretically manage to catch by visual feeding (Olsen *et al.*, 2007). Effects of plastic ingestion presented in Chapter 3, 4 and 6 therefore refer to small spherical plastics that are too small to be directly ingested.

2.3 Sampling and analysis

2.3.1 Sampling

Fish were sacrificed in a lethal dose (500 mg L^{-1}) of MS222 (3-aminobenzoic acid ethyl ester, methanesulfonate salt, Sigma, UK) buffered with sodium bicarbonate (Sigma, UK) to pH 7.4. Total lengths were recorded to the nearest 1 mm and wet body weight to the nearest 0.01 mg. Condition index K was calculated after Fulton (Ricker, 1975):

$$K = 100(W/L^3)$$

Where W = wet weight (mg), L = standard length (mm) and 100 as a factor to bring the value of K near unity. Body parts for analysis of relative expression of target genes were fixed in RNAlater (Sigma Aldrich, UK) and stored at -80°C (Table 2.2). Tissue samples for other analysis were fixed in Bouin's solution for 24h or 10% buffered formalin for 2 days before being transferred to 70% ethanol for storage prior to further processing (Table 2.2). See Chapter 2, section 2.3.3 for general histological procedures. Chapter specific methods are described within the related Chapters.

Table 2.2: Target tissues and fixation methods for endpoint analysis. Tissues fixed in RNAlater were used for subsequent analysis for relative expression of target genes. Tissues fixed in Bouin´s solution or 10% buffered formalin were used for histological analysis.

Chapter	Tissue	Fixation medium	Endpoint analysis
3	Liver	RNAlater	Gene expression
	Whole body	Bouin´s solution	Histology
4	Whole body	RNAlater	Gene expression
	Whole body	10% formalin	Microscopy
5	Liver	RNAlater	Gene expression
	Gonads	Bouin´s solution	Histology
	GI tract	10% formalin	Dissection
6	Head region	RNAlater	Gene expression
	Remainder of the body	10% formalin	Microscopy

2.3.2 Fluorescent determinations

Fluorescent determinations were carried out using a Zeiss LUMAR.V12 stereo microscope with an AxioCam MRm camera and AxioVision software (ZEISS, Germany) and a Green Fluorescent Protein (GFP) filter (excitation 485 nm; emission 520 nm) at magnifications detailed in Table 2.3.

Artemia sp.: To test for the presence of ingested fluorescent plastic spheres in the gastrointestinal tract (GI) of *Artemia sp*., sub sampled *Artemia sp*. were immobilised in a final concentration of 50% ethanol in clear, flat bottom 96-well plates to avoid egestion of the ingested spheres (Chapter 3 and 6).

Faeces: Presence of plastic spheres in faecal samples was confirmed using faeces pellets collected from the exposure vessels, which were then stored in clear, flat bottom 96-well plates (Chapter 3 and 6).

For analysis, *Artemia sp*. and faeces samples were kept in the 96-well plates in which they were stored.

Fluorescent determinations specific to exposures are detailed in the respective chapter.

Table 2.3: Fluorescent determinations of samples from data chapters to provide evidence for the presence of fluorescent plastic spheres (Chapter 3, 4 and 6) or nile red stained plastic bag fragments (Chapter 5).

Chapter	Sample	Method	Magnification
3	*Artemia sp*.	Fluorescent microscopy	80x
	Faeces	Fluorescent microscopy	20x
	Faeces	Fluorescent plate reader	NA
	Histol. sections	Fluorescent microscopy	80x
	Blood smears	Fluorescent microscopy	80x
4	Detritus	Fluorescent microscopy	80x
	Larvae	Fluorescent microscopy	20x
	Larvae	Fluorescent plate reader	NA
5	Faeces	Fluorescent microscopy	20x
	Stomach scrapes	Fluorescent microscopy	80x
	Food sub samples	Fluorescent microscopy	20x
6	*Artemia sp*.	Fluorescent microscopy	80x
	Faeces	Fluorescent microscopy	20x
	GI tracts	Fluorescent microscopy	12.5x
	LR white samples	Fluorescent microscopy	80x

2.3.3 Histological analysis

Tissue that had been previously fixed in Bouin´s solution or 10% buffered formalin (see Table 2.2) and then stored in 70% ethanol was used for histological analysis. Whole bodies of adult fish from Chapter 3 were transversely sectioned into 5 mm pieces using a microtome blade whereas gonads (Chapter 5) were used as whole for the following procedure: Tissue was washed by dipping into distilled water, then dehydrated in ascending ethanol concentrations (80, 90 and 100%) and cleared with Histoclear before being infiltrated with molten paraffin wax in two steps using an automated embedding system (Citadel). Each step (dehydration, clearing and infiltration) had a duration of 2h. After paraffin embedding and positioning in a wax block, samples were sectioned (7 µm section thickness) using a manual heavy duty microtome (Leica Jung Biocut 2035) with disposable steel blades (Thermo Scientific MB35 Premier). Produced sections were transferred to a hot water bath (between 35 and 37°C) before being floated onto glass microscope slides and transferred to a hot plate (57°C) for 20min to fix the section onto the slide. For both, whole body and gonads, one slide for each fish was stained with hematoxylin and eosin (H&E) stain (Figure 2.6) and examined with a light microscope to determine the gender of the fish. For Chapter 3, a second slide was left unstained and examined with a fluorescent microscope (Zeiss LUMAR.V12 with an AxioCam MRm camera and AxioVision software using a GFP filter (excitation 485 nm; emission 520 nm)) at x80 magnification, as previous work had shown that this method could be used to identify fluorescent polystyrene plastic spheres in paraffin embedded samples (Sussarellu *et al.*, 2014, Browne *et al.*, 2008). H&E stained sections of gonads from Chapter 5 were assessed using a stereomicroscope (Zoom 2000, Leica) at 30x magnification.

Step	Time/Procedure	Reagent
1	2 x 10 min	xylene
2	2 min	100% ethanol
3	2 min	90% ethanol
4	2 min	70% ethanol
5	2 min	50% ethanol
6	2 min	RO-water
7	10 min	haematoxylin
8	5 min	with running tap water
9	5 sec	acid ethanol
10	10 min	with running tap water
11	5 min	eosin
12	Rinse of stain with running tap water	
13	3 sec	70% ethanol
14	1 min	90% ethanol
15	2 min	100% ethanol
16	2 min	100% ethanol
17	2 x 5 min	xylene

Figure 2.6: H&E staining protocol for histological tissue sections of Chapter 3 and 5.

2.3.4 Molecular analysis

Molecular analysis were performed for Chapter 3, 4 and 6. Methods for the molecular analysis for Chapter 3, 4 and 6 (experiment 1) which were performed by Dr Karen L. Thorpe can be found in Appendix I. Below, the methods for the molecular analysis of Chapter 6 (experiment 2) which were carried out by myself are described.

RNA isolation

Total RNA was isolated from the anterior part of the larvae including head and liver using a Macherey-Nagel NucleoSpin® RNA extraction kit. Tissue samples were transferred from RNAlater to new 2 ml Eppendorf tubes containing 600 µl of buffer solution before tissue disruption and homogenisation with a bead mill (TissueLyser II, Quaigen) (2x 2 min at 20 Hz in a 4°C room) and prepared for RNA isolation (following the supplementary protocol: NucleoSpin® RNA – disruption and homogenization of RNA*later*® stabilized tissue, Macherey-Nagel). The lysate (700 µl) was then further processed following the standard protocol at room temperature. Total RNA concentration was estimated from absorbance at 260 nm (A_{260nm}; Nanodrop 8000; Thermo Scientific) and RNA quality verified by $A_{260\ nm}/A_{280\ nm}$ ratios > 2.0. Samples were kept at -80°C until cDNA synthesis.

cDNA synthesis

The cDNA was synthesised from 0.5 µg total RNA using an equal mix of Oligo$(dT)_{15}$ and random primers following the manufacturers protocol (GoScript™ Reverse Transcription System, Promega). Prior to cDNA synthesis, tests were undertaken to determine the optimal $MgCl_2$ concentration for the used reverse transcriptase (GoScript™ Reverse Transcriptase). No differences in levels of synthesised cDNA were found (verified by PCR and gel electrophoresis), at $MgCl_2$ concentrations of 2, 4 and 6 mM (suppliers recommended range 1.5 to 5 mM). Due to these findings 5 mM of $MgCl_2$ was chosen for the cDNA synthesis since a high $MgCl_2$ concentration for short cDNA products (product size of target gene (CYP1A) 196 bp and housekeeping gene 18S ribosomal RNA (rRNA) 100 bp) is recommended by the supplier. A no reverse transcriptase (-RT) control was included for every sample to test for genomic DNA contamination of synthesised cDNA. Quality of cDNA was tested for each reaction with polymerase chain reaction (PCR) and gel electrophoresis.

Quality control of cDNA

PCR was performed using a GoTaq® G2 Flexi DNA Polymerase kit (M7801, Promega) and the primers for the chosen housekeeping gene (18S rRNA) (Table 2.4). A master mix was prepared and dispersed in aliquots of 22.5 µl before 2.5 µl of cDNA (diluted 1 in 2 in nuclease free water) was added. The PCR was performed using a MyCycler PCR machine (BioRad) with heated lid at 110°C. An initial 2 min denaturation step at 94°C, followed by 35 cycles of: 94°C for 30 sec denaturation, 49°C for 30 sec annealing and 72°C for 25 sec extension was performed. Following the last cycle the reactions underwent a final extension at 72°C for 5 min. Samples were kept at 14°C in the PCR machine until further analysis.

The PCR products were mixed with the supplied loading dye and gel electrophoresis was performed using a 1.2% agarose gel with SYBRE® safe DNA gel stain (Invitrogen) at 110 V for 45 min. Gels were analysed using a U:Genius (Syngene).

Primers

Previously successfully applied primers for molecular analysis performed for Chapter 3, 4 and 6 (see Appendix I) were used for molecular analysis in Chapter 6 (Table 2.4). Desalted primers were synthesised by Invitrogen (Life Technologies, UK) and reconstituted with nuclease free water to a stock concentration of 100 µmole and stored at -20°C. Working solutions of primers were created by diluting the stock solution with nuclease free water to a concentration of 5 µmole for quality control of synthesised cDNA and 7 µmole for real time-quantitative PCR (RT-qPCR) analysis and were stored at 4°C. Primers were tested under PCR and RT-qPCR conditions for linear amplification and were found to be suitable for analysis. Additionally, melt curves during the RT-qPCR showed specific amplification of used primers (Figure 2.7).

RT-qPCR

Real time-quantitative PCR was performed using a Fast SYBRE® Green Master Mix (Life Technologies) and a StepOnePlus™ Real Time PCR system (Applied Biosystems) using the StepOne software v.2.2.2. Two microliters of individual, undiluted cDNA samples were analysed in triplicates for the reference gene (18S rRNA) and the target gene (Cytochrome P450A) with an initial incubation at 95°C for 20 sec, followed by 40

cycles of 95°C for 3 sec and 60°C for 40 sec. Following the final cycle the reactions underwent a 15 sec 95°C denaturation step followed by a 1 min, 60°C hybridisation step before PCR product melt curves were determined during a last temperature increase to 95°C for 15 sec. The relative expression of the CYP1A gene was determined by averaging the triplicate data for 18S rRNA and CYP1A genes which were then used to calculate the δCt for each sample before the mean δCt of each treatment group was computed. Mean δCt values for treatments were then used to calculate the δδCt relative to the reference samples. Finally, the fold change was calculated using the formula $2^{-\delta\delta Ct}$.

Table 2.4: Nucleotide sequences for real time PCR primers.

Transcript (gene)	Forward primer (5'-3')	Reverse primer (5'-3')
18S rRNA (ENSGACG00000021687)	CGGCTACCACATCCAAGGAA	TCCTGTATTGTTATTTTTCGTCACTACCT
CYP1A (ENSGACT00000019429)	ACGTGCAGATGTCAGACGAG	TTGGGTTTGTCGGAGAGAAG

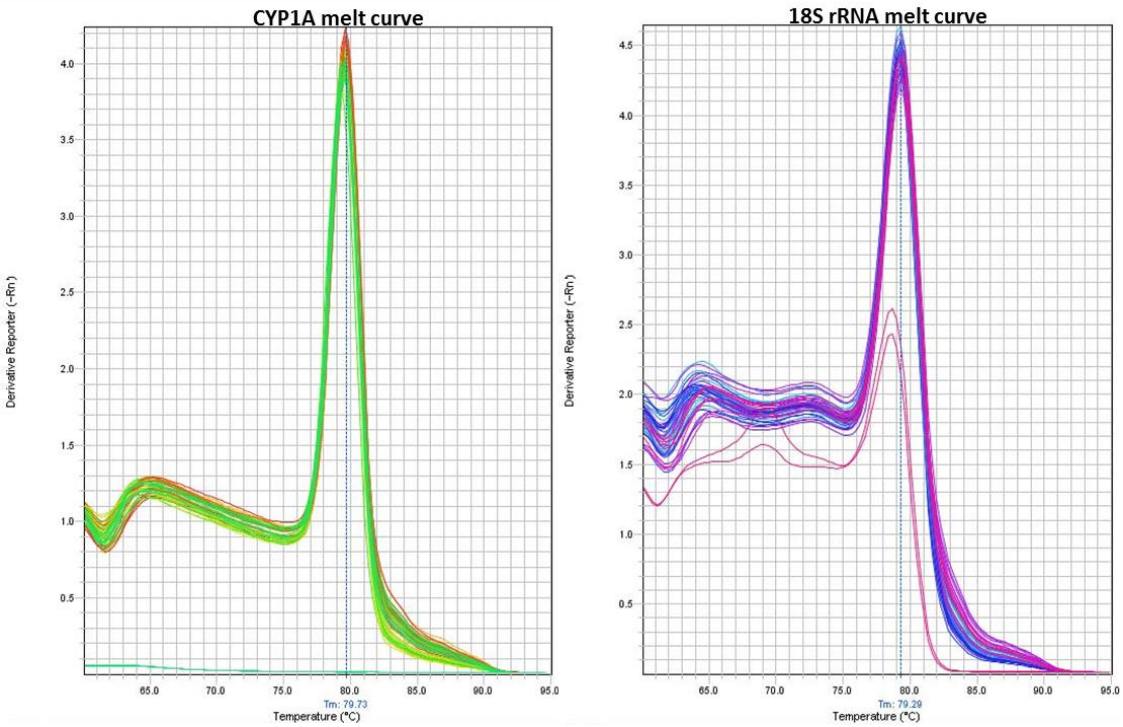

Figure 2.7: Melt curves of amplification products following a 40 cycle qPCR to test for specific binding of used primers.

2.3.5 Statistical analysis

All data were analysed using the SPSS© Statistic package version 22 (IBM©). All data were tested to meet the assumptions for parametric tests, normality (Kolmogorov-Smirnov test) and equal variances (Levene´s test). Data that did not meet the assumptions were transformed using either log or Johnson´s transformation. Data were then tested using One-Way ANOVAs with Tukey HSD and 2-Sided Dunnett´s Post Hoc tests to test for differences between treatment groups and relative to the control, respectively. Data that did not meet the assumptions for parametric tests after transformation were tested with a non-parametric Kruskal-Wallis test. Pearson´s correlations were performed to test for correlations between measured endpoints. Outlier testing was performed using the outlier labelling method (Hoaglin *et al.*, 1986) with g = 2.2 as proposed by (Hoaglin and Iglewicz, 1987). For all statistical analysis statistical significance was set to $p \leq 0.05$ to allow for 95% confidence limit. See data chapters for specific statistical test methods of data. All data are expressed as the mean ± standard error of the mean (SEM).

Chapter 3

Trophic transfer of microplastic spheres from zooplankton (*Artemia sp.*) to adult three-spined stickleback (*Gasterosteus aculeatus*)

The molecular work presented in this chapter was performed by Dr Karen L. Thorpe.

3.1 Abstract

The trophic transfer of plastic particles along the food chain is of concern since ingestion of contaminated diets could lead to an accumulation of plastic in organisms of higher trophic levels. Even though some reports are present to document this trophic transfer between two or three trophic levels, there is just very limited knowledge about the effects of the ingestion of a contaminated live diet. In this investigation adult three-spined sticklebacks were fed for 7 days on a controlled diet of live *Artemia sp*. previously exposed to 1 or 9.9 µm fluorescent plastic spheres and the potential health effects associated with ingested microplastics assessed. *Artemia sp*. rapidly accumulated the microplastics in their gastrointestinal tract but survival and growth were not impacted over the 72h exposure period. Examination of stickleback faecal pellets confirmed that they were ingesting the microplastic contaminated *Artemia sp.*, but that up to 75% of microplastics were egested within 48h of ceasing exposure. There was no evidence that ingestion of the microplastics affected stickleback survival, length ($p > 0.05$, except sampling day 7 where the 1 µm low treatment showed to be different from the control ($p = 0.041$)), weight ($p > 0.05$) and body condition ($p > 0.05$) for all sampling days. There was also no evidence for altered expression of the gene encoding the detoxification enzyme cytochrome P450 1A (CYP1A) ($p = 0.711$) for fish sampled at the end of the 7 day exposure period to the contaminated diets, suggesting that the plastics were not retained in the gastrointestinal tract for sufficient time to leach biologically active concentrations of organic contaminants. There was also no evidence for translocation of the plastics from the gastrointestinal tract to the circulatory system. Collectively these results indicate that short-term dietary exposure to uncontaminated polystyrene microspheres, at the sized used in this study, are unlikely to pose an acute risk for the health of aquatic invertebrates or fish.

3.2 Introduction

Due to their high abundance in aquatic ecosystems and their wide range of colours, shapes and sizes concerns have been raised that plastics may be confused by wildlife as a food source and become accidentally or directly ingested (Boerger *et al.*, 2010); Indeed there are reports of plastics being found in the intestinal tracts of a broad range of species (Derraik, 2002) including worms (Besseling *et al.*, 2013), fish (Boerger *et al.*, 2010, Davison and Asch, 2011, Foekema *et al.*, 2013, Lusher *et al.*, 2013, Carson, 2013, Sanchez *et al.*, 2014), birds (Verlis *et al.*, 2013), sea turtles (Bjorndal *et al.*, 1994) and marine mammals (Eriksson and Burton, 2003). Indirect ingestion of microplastics via trophic transfer may also play a role in the ingestion and accumulation of plastic particles; First evidence for a trophic transfer of plastic was reported for regurgitations of terns (*Sterna hirundo*) that were collected on Long Island, New York, in 1971 which contained polystyrene pellets (Hays and Cormons, 1974). Microplastics were also recorded from the scats of fur seals (*Arctocephalus* spp.) which were believed to originate from lantern fish (*Electrona subaspera*) (Eriksson and Burton, 2003). More recently, laboratory studies have demonstrated the trophic transfer of microplastics from algae (*Scenedesmus sp.*) to zooplankton (*Daphnia magna*) to fish (*Carassius carassius*) (Cedervall *et al.*, 2012), from zooplankton to mysid shrimps (Setälä *et al.*, 2014) and from mussels (*Mytilus edulis*) to crabs (*Carcinus maenas*) (Farrell and Nelson, 2013). The transfer of plastic particles between trophic levels is of concern since it has the potential to lead to the accumulation along the food chain, resulting in the ingestion of highly contaminated prey by top predators.

Feeding on contaminated prey could also lead to the ingestion of relatively small microplastics which result in different effects compared to larger items; Ingestion of relatively large plastic items that may be directly ingested from the water column has the potential to cause negative effects through blockage of the gastrointestinal tract (GI) and reduced food ingestion resulting in starvation and potentially death of the animal (Gregory, 2009, Carpenter *et al.*, 1972, Bjorndal *et al.*, 1994, Rothstein, 1973, Zitko and Hanlon, 1991). However, relatively small plastic items that get ingested via trophic transfer might lead to different effects; Concerns have been raised regarding the potential for ingested microplastics to translocate from the gastrointestinal tract into the circulatory system or other tissues. Evidence for translocation has been provided for rats (Hussain *et al.*, 2001, Jani *et al.*, 1992, Jani *et al.*, 1989, Carr *et al.*, 1996) and aquatic

organisms (Browne *et al.*, 2008, Farrell and Nelson, 2013, von Moos *et al.*, 2012, Brennecke *et al.*, 2015). These translocated particles could potentially negatively impact cell membrane properties by disturbing the activity of membrane proteins (Rossi *et al.*, 2013) and even accumulate in tissues, as the lack of enzymatic pathways to digest plastics classifies it as bio-inert (Andrady, 2011). To date translocation of ingested plastics in marine organisms has been reported in the blue mussel *Mytilus edulis* (Browne *et al.*, 2008, von Moos *et al.*, 2012) and the crabs *Carcinus maenas* and *Uca rapax* (Farrell and Nelson, 2013, Brennecke *et al.*, 2015). It was reported that smaller particles translocate in greater numbers (up to 60% more) compared to larger particles (Browne *et al.*, 2008), highlighting that trophic transfer as a vector for small particles is of concern. Plastic particles were found in the circulatory system (haemolymph) of mussels as soon as 3h after start of the exposure (Browne *et al.*, 2008, von Moos *et al.*, 2012). Whereas no negative effects of these translocated particles on cell viability assays for neutral red uptake by haemocytes and phagocytosis capability were reported by Browne *et al.* (2008), the study by von Moos *et al.* (2012) reported a reduced lysosomal membrane stability from 6h of exposure but also no effects on endpoints lipofuscin accumulation (for oxyradical damage), neutral lipid content (disturbance of lipid metabolism) and condition index. However, the feeding techniques of *Mytilus edulis* and *Carcinus maenas* are different to those of sticklebacks; *Mytilus edulis* is a filter feeding invertebrate which's diet consists of small suspended food particles from the water column (Bayne *et al.*, 1988). Hence, the gastrointestinal system is specialised on particular food and shows intracellular digestion of small food particles (McVeigh *et al.*, 2006) which might promote microplastic translocation. *Carcinus maenas* on the other hand does not swallow its prey in whole but ingests caught prey in small pieces, previously processed by its chelae and mouth parts (Crothers, 1968). Due to this feeding technique, the ripped open parts of the gastrointestinal tract of the prey can release plastic particles very early during the digestion process, prolonging the possible length of interaction of plastic with the gastrointestinal tract during food digestion but also releases plastics into the water column, opening up further exposure routes like the gills (Farrell and Nelson, 2013). Hence, sticklebacks that swallow their prey in whole might show different results concerning the translocation of plastic particles from the gastrointestinal tract, compared with animals that chew or dismantle their food prior to ingestion.

The potential trophic transfer of small microplastics and the resulting effects including possible translocation to the circulatory system are of concern. Therefore, the aim of this study was to assess the potential health effects resulting from ingestion of a microplastic contaminated diet through an aquatic filter-feeding invertebrate (*Artemia sp*.) on a predatory fish species (three-spined stickleback; *Gasterosteus aculeatus*). *Artemia sp.* were exposed to graded densities of fluorescent polystyrene microspheres for up to 72h to assess ingestion of the microplastics from the water column and effects on survival. Adult sticklebacks were fed for 7 days on live diets of *Artemia sp.* that had been cultured in the presence or absence of the fluorescent microplastic spheres. After 7 days of exposure, the sticklebacks were transferred to clean aquaria and maintained for a further 14 days on a diet of non-plastic contaminated *Artemia sp*.. Faeces samples were collected daily from the aquaria as a non-invasive method to monitor egestion of the plastic spheres. Body lengths and weights of the sticklebacks were measured during both the exposure and depuration periods, to determine the effects of plastic ingestion on body condition. Blood samples, were examined under a fluorescent microscope to assess evidence for translocation of the plastics to the circulatory system. In addition, hepatic expression of the gene encoding the detoxification enzyme CYP1A was measured to inform on whether toxic additives were leaching from the plastic.

3.3 Material and methods

3.3.1 Test organisms

Artemia sp. and *Gasterosteus aculeatus* were maintained as described in Chapter 2, section 2.1.

3.3.2 Water supply and test apparatus

The water supply apparatus was set up as described in Chapter 2, section 2.1.1.2 Additionally, fish were transferred to clean exposure tanks containing fresh artificial sea water (ASW) after day 7 of the exposure when the depuration period started. Water temperatures were monitored daily throughout the experiment and ranged between 16.7 and 19°C, while pH levels were checked twice weekly and ranged between 7.26 and 7.95. Dissolved oxygen concentrations were checked twice weekly and remained over 70% of the air saturation value for the experiment.

3.3.3 Microplastic spheres

See Chapter 2, section 2.2.

3.3.4 Preliminary investigations

Initial investigations were conducted to confirm that *Artemia sp*. would take up the plastic spheres from the water column. One millilitre of *Artemia* nauplii suspension (approx. 150 nauplii ml^{-1}) was added to beakers containing 60 ml of aerated ASW (20 ppt). One beaker was maintained as a control (no microplastics). The remainder was spiked with 6 µl of 1 µm or 9.9 µm microplastics (final density of 0.106 mg microplastic ml^{-1}). After 8, 24, 48 and 72 hours of exposure, triplicate sub samples (200 µl) of *Artemia sp*. were removed from each beaker and transferred to a clear 96-well plate and examined as described in Chapter 2, section 2.3.2.

3.3.5 Trophic transfer experiment

Adult three-spined sticklebacks were randomly allocated to 40 aerated glass aquaria containing 6 L of 3.0 ppt ASW (n = 4 fish per aquarium). The fish were acclimated to the test conditions for 3 days and during this period fed three times daily with non-contaminated *Artemia sp*. (approximately 2800 *Artemia sp.* per feed). At the onset of the exposure, the aquaria were randomly assigned to each of five treatments (8 aquaria were assigned to each treatment with 4 fish each; 32 fish per treatment). The five treatment groups were defined as a control (fed non-contaminated *Artemia sp.*), 1 µm high or 9.9 µm high (fed on a diet of 100% *Artemia sp.* that had been exposed for 24 hours to either 1 µm or 9.9 µm sized microplastics, respectively) and 1 µm low or 9.9 µm low (fed 10% of the respective contaminated *Artemia sp*. and 90% non-contaminated *Artemia sp.*). The daily batches of non-contaminated or plastic-contaminated *Artemia sp*. were prepared by culturing *Artemia* nauplii for 24 hours (at a density of 30 fry ml^{-1}) in either fresh ASW or ASW spiked with 1 µm or 9.9 µm micro spheres (0.106 mg spheres ml^{-1}). After 24h of exposure, triplicate sub samples (100 µl) of *Artemia sp.* were removed to confirm ingestion of the spheres via fluorescent microscopy. A further 200 ml of each *Artemia sp.* culture was removed, sieved and rinsed to remove excess salt and plastic spheres and transferred to a solvent cleaned beaker with 100 ml of 3.0 ppt ASW. This procedure was repeated for each feed.

After the 7 day exposure period (Figure 3.1), all fish were transferred to clean aquaria and maintained for a further 14 day period on a diet of non-contaminated *Artemia sp*. to assess clearance of the spheres from the gastrointestinal tract. Throughout both the exposure and depuration period faecal samples were removed from each aquarium every morning using plastic Pasteur pipettes and transferred to clear, flat bottom 96-well plates to assess evidence for the presence of fluorescent plastic spheres in the faeces.

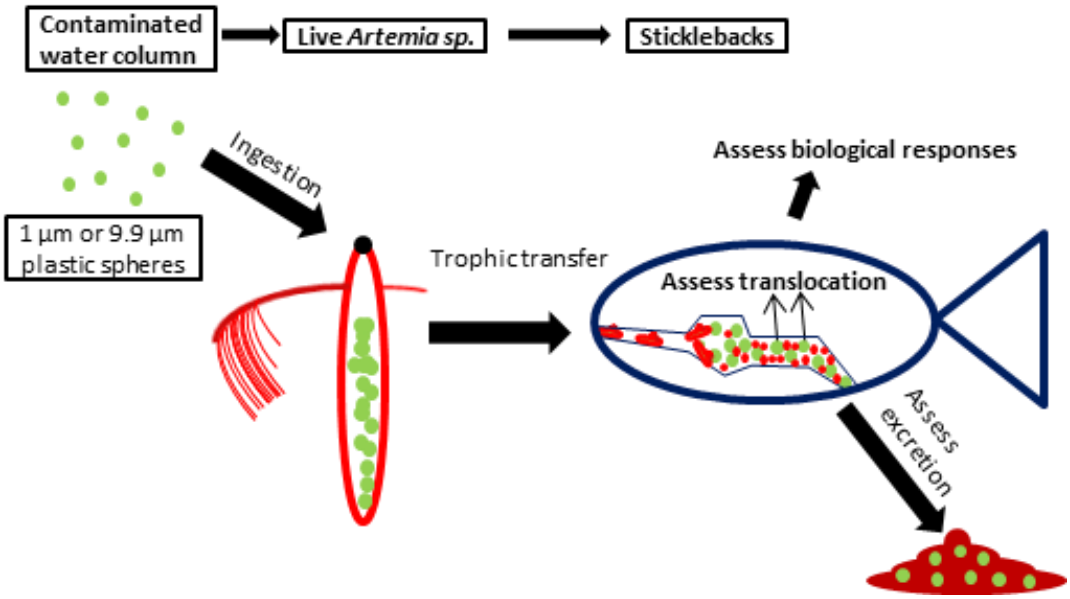

Figure 3.1: Graphical illustration of the trophic transfer experiment. *Artemia sp*. (48 hours post hatch (hph)) were exposed for 24h to a water column spiked with 0.106 mg spheres ml^{-1} of either 1 µm or 9.9 µm polystyrene spheres. Contaminated *Artemia sp.* were then cleaned before they were used as live diet for adult sticklebacks. Sticklebacks were exposed for a total of 7 days to the contaminated diet before they were transferred to a diet consisting of just uncontaminated *Artemia sp.*. Faeces samples were taken daily as an indirect measurement for ingestion of contaminated *Artemia sp.* and to assess plastic elimination over the 14 day clearing time (day 7 - 21). Subsamples of sticklebacks were taken after 4, 7, 14 and 21 days to assess biological effects and possible translocation of ingested plastic spheres.

3.3.6 Fish sampling and analysis

On days 4, 7, 14 and 21 of the experiment, one fish per aquaria was removed and sacrificed as described in Chapter 2, section 2.3.1. Additionally to sampling procedures described in Chapter 2, section 2.3.1, blood samples were removed from the caudal peduncle of the adult fish using heparinised micro haematocrit tubes (Heparinised Microcapillary, Brand GmbH&Co KG) to assess evidence for translocation of the spheres into the circulatory system and an immune response towards possibly

translocated plastic. Blood smears were prepared for subsequent analysis under a fluorescent microscope. Whereas one blood smear was stained with a Wright-Gimsa stain (Sigma Aldrich) to be used for a differential blood cell count (immune response) using a light microscope and 100x magnification, another one was kept unstained for the analysis of translocated plastic spheres as described in section 3.3.7.

3.3.7 Fluorescent determinations

To assess the presence of the fluorescent plastic spheres in the subsampled *Artemia sp.* (confirmation of the contamination of the diet for sticklebacks) and daily faeces samples (confirmation for the ingestion of the contaminated diet and quantification of elimination of ingested particles) fluorescent determinations were carried out as described in Chapter 2, section 2.3.2. Additionally, faeces was analysed with a microplate reader (POLARstar OPTIMA) to compare fluorescence intensity of faeces to assess elimination of the ingested plastic spheres. For these analysis faeces samples were kept in the 96-well plates in which they were stored. Blood smears and histological sections (to assess ingestion, retention and a possible translocation of ingested particles to the circulatory system) were assessed using a Zeiss LUMAR.V12 stereo microscope with an AxioCam MRm camera and AxioVision software (ZEISS, Germany) and a Green Fluorescent Protein (GFP) filter (excitation 485 nm; emission 520 nm) at 80x magnification.

3.3.8 Histology

Histological analysis were performed as outlined in Chapter 2, section 2.3.3.

3.3.9 Gene expression

The molecular work was performed by Dr. Karen Thorpe. Material and Methods for this work can be found in Appendix I. I was provided with raw data from this molecular work for subsequent statistical analysis.

3.3.10 Statistical analysis

Statistical analysis were carried out as detailed in Chapter 2, section 2.3.5. Additionally, Two-way ANOVAs, with time and treatment as factors to test for effects of exposure

time and treatment type on wet weight, length and condition index K were performed. One-way ANOVAs followed by Tukey's comparison revealed non sex specific responses which is why data for both sexes were pooled for analysis.

3.4 Results

3.4.1 Ingestion of fluorescent plastic spheres by *Artemia sp*.

The preliminary exposures with *Artemia sp*. confirmed the suitability of this organism as a vector for trophic transfer of both the 1 and 9.9 µm fluorescing spheres. There was no evidence for ingestion of the spheres in *Artemia sp.* (instar I) sampled 8h post exposure. However, after 24, 48 and 72h of exposure, fluorescent spheres could be observed within the gastrointestinal tract (Figure 3.2). The initial particle count in the contamination medium was $1.09x10^{8}$ and $1.09x10^{5}$ spheres for the 1 and 9.9 µm spheres, respectively. Mean number of plastic spheres were as high as 126 spheres *Artemia sp.*$^{-1}$ (n = 10) for the 9.9 µm treatments at 72h post hatch; the 1 µm spheres were too small to be counted. *Artemia sp.* sub samples which were removed from both the control and microplastic treatments at each time-point were motile and the developmental stage of *Artemi*a *sp*. (instar V after 72h) was comparable between all treatments.

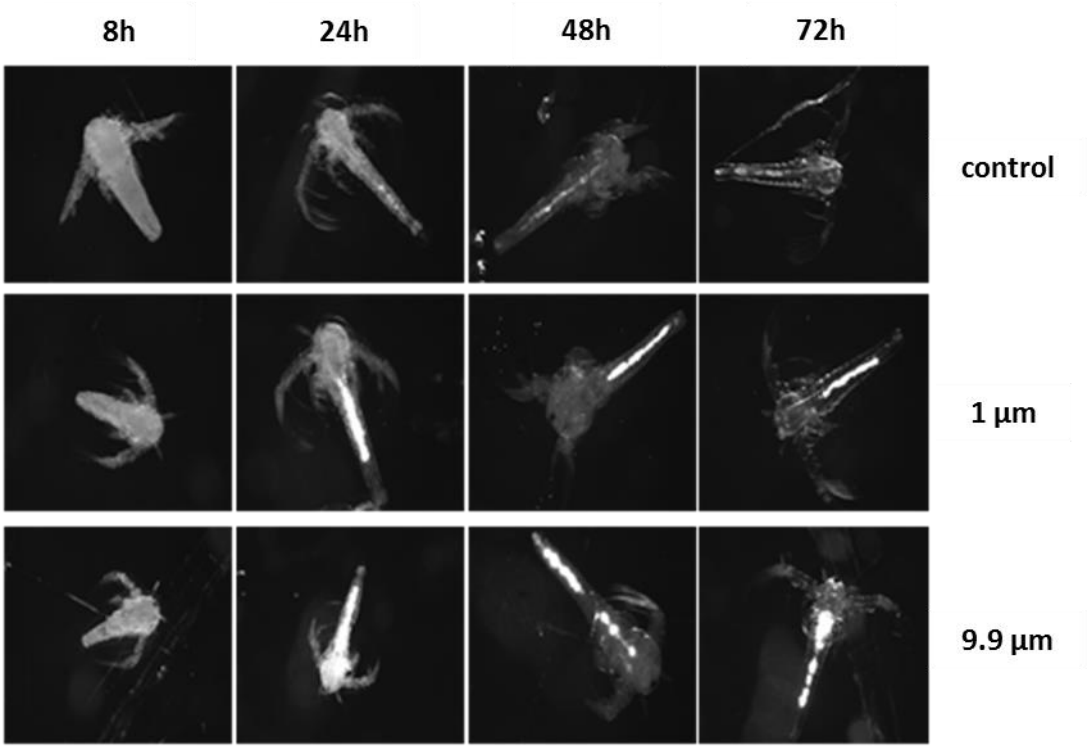

Figure 3.2: Ingestion of plastic spheres by *Artemia sp*. during the preliminary time trial. *Artemia sp*. (0 hours post hatch (hph)) were exposed for 24h to clean artificial sea water (ASW) (control), or ASW contaminated with 1 µm or 9.9 µm polystyrene plastic spheres (0.106 mg spheres ml^{-1}). Subsamples were taken after 8, 24, 48 and 72h to assess plastic ingestion by the *Artemia sp.*. No sign for plastic ingestion was observed after 8h of exposure (8 hph) but first evidence for ingested spheres was found at 24h of exposure (24 hph). Plastic particles accumulated in the gastrointestinal tract of the *Artemia sp.* (indicated by fluorescence) and greater number of spheres could be found with increasing exposure time. No contamination of the control treatment was found and detected fluorescence derived from the auto fluorescence of the *Artemia sp.*.

3.4.2 Ingestion and egestion of the plastic spheres in the three-spined stickleback

Faecal samples were removed daily from each aquarium to determine whether the fish were egesting (and therefore ingesting) the contaminated *Artemia sp.* diet. Due to the natural auto-fluorescence of *Artemia sp.*, fluorescence was detectable in the faeces removed from the control fish (11.4 ± 0.36 Arbitrary units (AU), n = 125) throughout the study. After one day of exposure, levels of fluorescence were consistently higher in faecal samples removed from each of the microplastic treatment groups relative to the controls; Compared to control fluorescence treatments showed 2.1 and 2.6-fold higher fluorescence in fish fed 10% contaminated *Artemia sp.* (low) and 5.8 and 12.8-fold higher fluorescence for fish fed 100% (high) contaminated *Artemia sp.* for 1 and 9.9 µm spheres, respectively during the first week of the experiment (Figure 3.3). Within 24h of ceasing exposure to the microplastic contaminated diet the fluorescence measured in faeces collected from the low treatments (fish fed 10% contaminated *Artemia sp.*) was comparable to the background levels of fluorescence measured in the faeces of the control fish (Figure 3.3). The fluorescence measured in the faeces of fish fed with 100% microplastic contaminated *Artemia sp.* was still high, relative to the controls, at both 24h (18.9 and 55.8-fold higher for the 1 and 9.9 µm particle treatments, respectively) and 48h (3.3 and 18.0-fold higher for the 1 and 9.9 µm particle treatments, respectively) after ceasing exposure. However, relative fluorescence values for faeces samples were observed to drop 4.0 and 2.7-fold and 3.5 and 1.3-fold within 48h after ceasing the exposure for 1 and 9.9 µm spheres of high and low treatments, respectively. After 14 days of being maintained on a non-contaminated diet, fluorescence measured in the faeces was comparable across the treatment groups and the control. However, visual examination of the faecal samples revealed that the fish were still egesting small numbers of fluorescent microplastics with mean particle numbers of 12 ± 6.7 (n = 8) and 6 ± 3.4 (n = 8) in the 21 day faecal samples removed from the 1 and 9.9 µm low treatments respectively and 11 ± 7.1 (n = 8) and 9 ± 5.1 (n = 8) for the 1 and 9.9 µm high treatments, respectively (Figure 3.4).

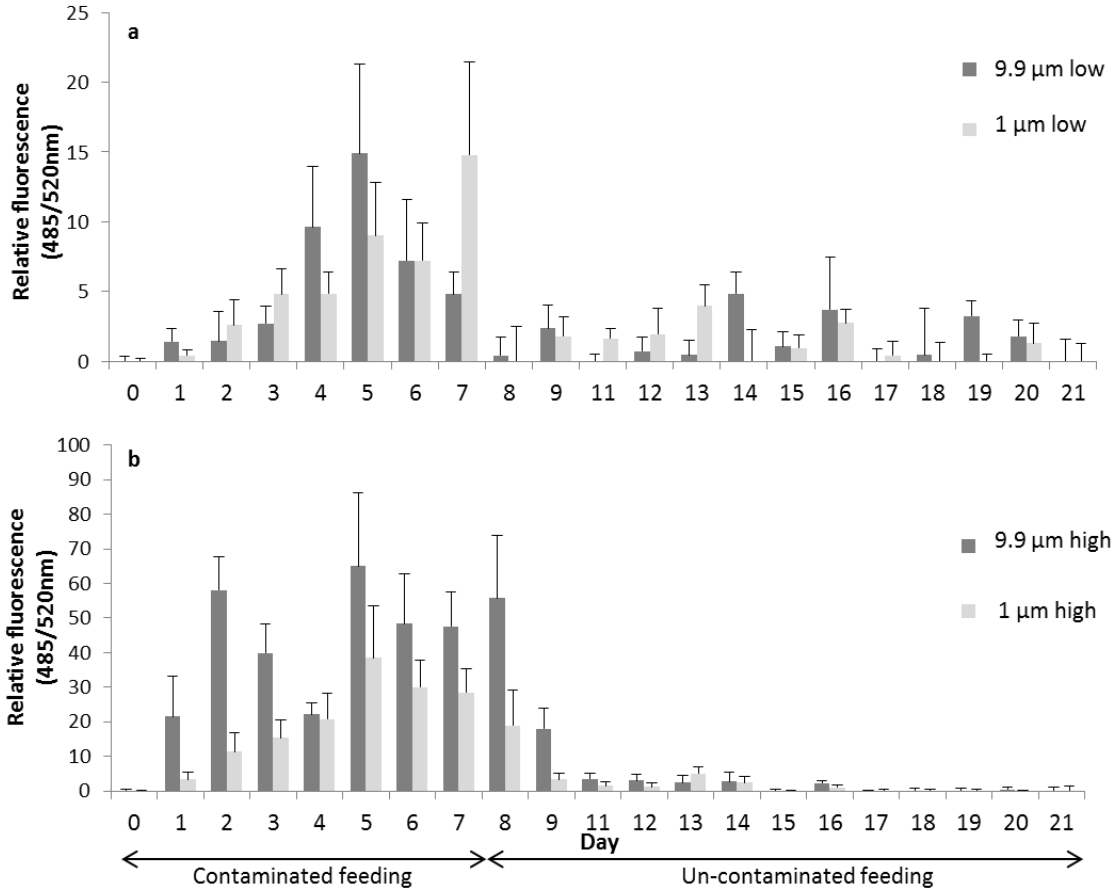

Figure 3.3: Fluorescence, relative to controls, in faecal samples collected from adult three-spined stickleback fed for 7 days on a a = 10% (low) or b = 100% (high) diet of plastic-contaminated *Artemia sp*. (exposed for 24h (between 48 and 72 hours post hatch (hph)) to 1.0 µm or 9.9 µm green fluorescing microplastic spheres). The control and low exposure groups were fed 100% and 90%, respectively, non-exposed *Artemia sp*. (<72h hph). This was followed by a 14 days clearing time during which just uncontaminated *Artemia sp.* were fed. Results are expressed as means ± standard error of the mean.

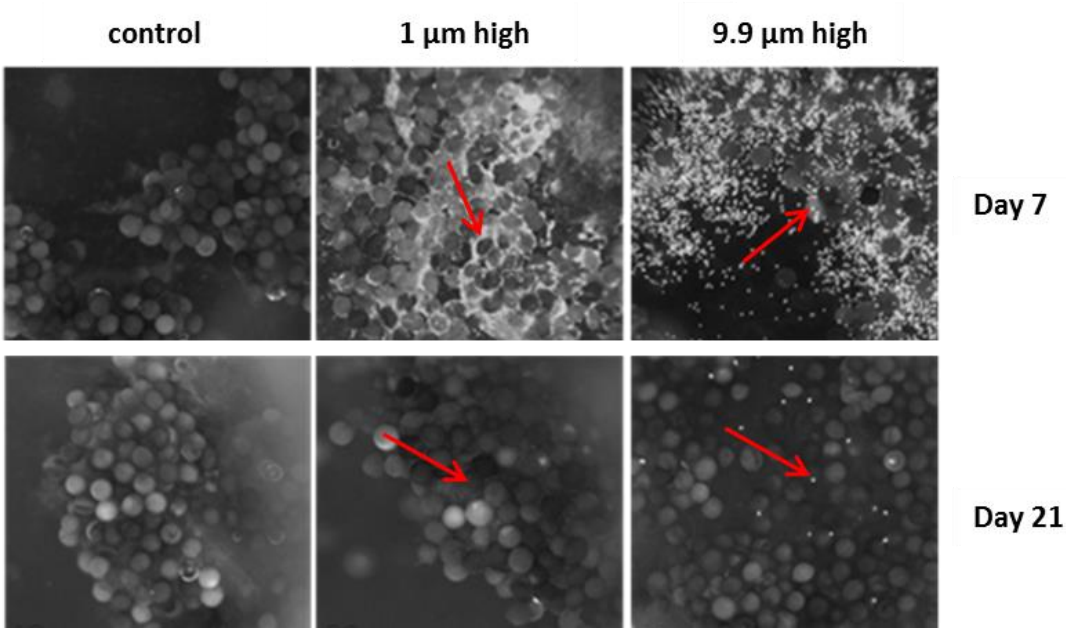

Figure 3.4: Fluorescence in faeces samples collected on day 7 and 21 of the experiment from fish exposed to 100% contaminated *Artemia sp.* (high) that had been previously exposed for 24h to 1 µm or 9.9 µm polystyrene plastic spheres. Great numbers of both particle sizes were found on day 7, indicating the ingestion of the contaminated diet and the egestion of ingested plastic particles. After the 14 day clearing period during which uncontaminated *Artemia sp.* were fed, just few plastic spheres were found (day 21), indicating that even though spheres were retained in the gastrointestinal tract (GI) their number was very low. No contamination of control samples was found. Plastic contamination is indicated with red arrows. Round structures in faeces pellets represent unhatched *Artemia sp.* cysts.

3.4.3 Histological analysis

As expected, no plastic spheres were found in sections of fish from the control treatment for all sampling days (n = 32, using a total of 320 sections (10 sections per fish)). However, no additional evidence for the ingestion of the contaminated *Artemia sp*. and accumulation of plastic spheres in the gastrointestinal tract of fish from the plastic treatments could be provided by the used paraffin based histological analysis. None of the analysed sections (n = 128 fish with a total of n = 1280 sections (10 sections per fish), which were taken across the length of the stomach and intestine) were found to contain plastic spheres.

3.4.4 Biological effects

Ingestion of the microplastics did not affect survival. No difference in weight and condition index K was found over the duration of the exposure period (Table 3.1). Subsamples of fish on day 7 revealed a difference in length between treatment groups and the control ($F_{4,35}$ = 2.797, p = 0.041). A Dunnett´s test revealed that sampled fish from the 1 µm low treatment had a longer standard length compared to the control treatment (p = 0.045). Additionally, a Tukey HSD Post-Hoc test showed that the standard length of the 1 µm low treatments was also longer than the one of the 9.9 µm low treatment (p = 0.037). For all other sampling points no effects on total and standard length were found for the adult fish over all treatments (Table 3.1). Also a Two-way ANOVA with day and treatment as fixed factors did not show any interaction of exposure time and treatment over all sampling days for weight ($F_{12,140}$ = 0.961, p = 0.489), total length ($F_{12,140}$ = 0.949, p = 0.500), standard length ($F_{12,140}$ = 1.101, p = 0.364) and condition index K ($F_{12,140}$ = 1.101, p = 0.364).

There was no evidence for the translocation of plastic spheres to the circulatory system; No plastic spheres could be found in blood smears for all treatments over the duration of the experiment, except two smears from day 4 and 14, one from a 1 µm high and one from a 9.9 µm high treatment, which showed a single fluorescent plastic sphere. However, these single spheres were suspected to be accidental contamination of the smear during sample preparation. Subsamples of stained blood smears used for differential blood cell count did not provide evidence for an increased leucocyte count

(Figure 3.5). On basis of this finding it was decided to cancel the examination of the remaining blood smears.

Table 3.1: Statistical results of analysed biological endpoints growth (total, standard length and weight) and the condition index K of sub samples of fish that had been exposed for 4 or 7 days to a plastic contaminated *Artemia sp*. diet as well as during the following depuration period (days 14 and 21) during which just uncontaminated *Artemia sp*. were fed.

Sampling day	Weight	Total length	Standard length	Condition index
4	$F_{4,35} = 0.083, p = 0.987$	$F_{4,35} = 0.205, p = 0.934$	$F_{4,39} = 0.211, p = 0.930$	$F_{4,35} = 0.711, p = 0.590$
7	$F_{4,35} = 1.055, p = 0.393$	$F_{4,35} = 1.893, p = 0.134$	$F_{4,35} = 2.797, p = 0.041$	$F_{4,35} = 1.203, p = 0.327$
14	$F_{4,35} = 1.111, p = 0.367$	$F_{4,35} = 1.039, p = 0.401$	$F_{4,39} = 1.281, p = 0.296$	$F_{4,35} = 0.216, p = 0.928$
21	$F_{4,35} = 1.049, p = 0.369$	$F_{4,35} = 0.579, p = 0.680$	$F_{4,35} = 0.475, p = 0.754$	$F_{4,35} = 1.587, p = 0.199$

		Count No.						
		1		2		3		%
	Treatment	Red	White	Red	White	Red	White	of white
Day 4	control	91	0	60	0	103	0	0
	control	191	1	54	0	107	0	0.28
	1 µm low	140	0	190	1	158	0	0.20
	1 µm low	100	0	233	0	94	0	0
	1 µm high	172	1	122	0	117	0	0.24
	1 µm high	144	0	263	1	71	0	0.21
	9.9 µm low	191	1	132	1	188	0	0.39
	9.9 µm low	255	0	117	2	127	0	0.40
	9.9 µm high	103	0	133	0	70	0	0
	9.9 µm high	179	2	216	2	257	2	0.92

		1		2		3		%
	Treatment	Red	White	Red	White	Red	White	of white
Day 7	control	99	1	223	0	107	1	0.47
	control	134	0	115	0	116	0	0
	1 µm low	297	0	203	0	332	0	0
	1 µm low	126	0	136	1	138	0	0.25
	1 µm high	169	0	198	1	111	2	0.63
	1 µm high	131	1	307	0	130	3	0.70
	9.9 µm low	151	2	211	4	143	2	1.58
	9.9 µm low	196	0	91	0	186	0	0
	9.9 µm high	260	0	154	0	238	1	0.15
	9.9 µm high	50	1	98	0	106	2	1.18

		1		2		3		%
	Treatment	Red	White	Red	White	Red	White	of white
Day 14	control	112	1	149	0	93	0	0.28
	control	136	0	291	0	128	0	0
	1 µm low	99	3	198	0	167	2	1.08
	1 µm low	113	5	151	0	71	2	2.09
	1 µm high	93	0	290	1	189	0	0.17
	1 µm high	151	2	182	0	86	0	0.48
	9.9 µm low	118	2	174	1	205	10	2.62
	9.9 µm low	167	4	195	2	157	1	1.35
	9.9 µm high	205	1	180	1	212	1	0.50
	9.9 µm high	171	0	135	2	133	1	0.68

		1		2		3		%
	Treatment	Red	White	Red	White	Red	White	of white
Day 21	control	138	2	152	0	122	0	0.49
	control	171	0	186	0	84	0	0
	1 µm low	100	0	96	0	125	1	0.31
	1 µm low	227	1	167	0	101	1	0.40
	1 µm high	142	0	326	0	151	0	0
	1 µm high	115	0	151	1	117	1	0.52
	9.9 µm low	131	0	105	0	125	0	0
	9.9 µm low	147	2	119	0	103	1	0.81
	9.9 µm high	109	0	78	0	104	0	0
	9.9 µm high	137	0	207	1	117	0	0.22

Figure 3.5: Results of the differential blood cell count from Wright-Giemsa stained blood smears using 100x magnification. Each subsample day (Day 4, 7, 14 and 21) blood samples were taken from the caudal peduncle of the adult fish using heparinised micro haematocrit tubes and blood smears prepared for analysis. For each treatment 2 blood smears were chosen and a differential blood cell count performed for 3 representative areas (Count No.) of each blood smear. Red = Total number of haematocytes. White = Total number of Leucocytes. Numbers are total numbers of counted blood cells.

There was also no evidence that ingestion of the plastic spheres via the diet modified hepatic expression of CYP1A ($F_{4,34}$ = 0.535, p = 0.711; Figure 3.6), relative to the controls.

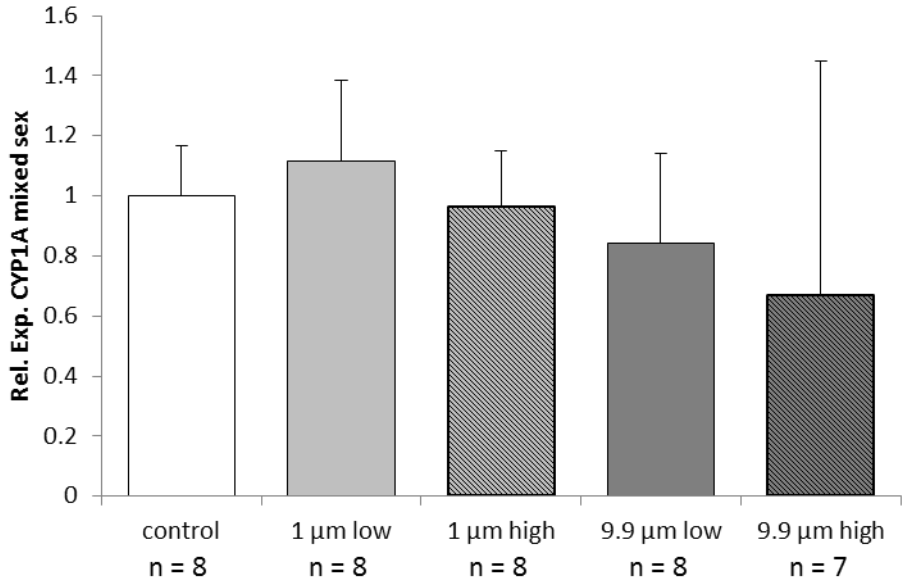

Figure 3.6: Relative expression of the gene encoding cytochrome P450 1A (CYP1A) from adult three-spined stickleback fed for 7 days on a 10% (low) or 100% (high) diet of plastic-contaminated *Artemia sp*. (exposed for 24h (between 48 and 72 hours post hatch (hph)) to 1 µm or 9.9 µm green fluorescing microplastic spheres). The control and low exposure groups were fed 100% and 90%, respectively, non-exposed *Artemia sp*. (< 72h hph).

3.5 Discussion

The results from this investigation imply that, while filter feeding zooplankton may act as vectors for transferring micro sized plastic particles through the aquatic food chain, the biological consequences of such trophic transfer may be negligible. *Artemia sp.* rapidly accumulated large quantities of micro spheres within their gastrointestinal tract with no measurable impact on their survival or development in the short-term. The fish actively preyed upon the contaminated *Artemia sp.* and accumulated large quantities of fluorescent particles within their gastrointestinal tract but these were rapidly egested and there was no evidence for negative impacts on the fish either during or post exposure.

Consistent with previous reports for zooplankton species (Collignon *et al.*, 2014, Cole *et al.*, 2013, Frias *et al.*, 2014), *Artemia sp*. nauplii (72h post hatch) accumulated high

quantities of the 1 and 9.9 µm microplastic spheres within their gastrointestinal tract. No ingestion of the plastic particles was observed in earlier life stages of *Artemia sp.* (instar I; (< 24h post hatch) as *Artemia sp.* do not develop an open gastrointestinal tract until the second larval moult (instar II) (Sorgeloos *et al.*, 2001). There was no evidence that accumulation of the plastic spheres in the intestinal tract negatively impacted *Artemia sp.* survival which is consistent with earlier studies in which ingestion of micro spheres in the same size range did not negatively impact the survival of planktonic organisms in the short-term (up to 96h) (Lee *et al.*, 2013c). These results suggest that ingested virgin 1 and 9.9 µm plastic spheres do not pose an acute risk to zooplanktonic organisms. Accordingly, organisms with ingested plastic particles stay alive and can be eaten by biota of higher trophic levels which can lead to the transfer of plastic particles along the food chain (Cedervall *et al.*, 2012).

In this study, faeces samples were removed daily as a non-invasive, indirect assessment for the ingestion of micro spheres by the fish. Higher contamination levels of faeces samples during the exposure time to contaminated diets provided evidence for the trophic transfer of plastic spheres from *Artemia sp.* to sticklebacks (Figure 3.3). The level of contamination was proportional to treatment level over the course of the first 7 days, demonstrating a dose dependent ingestion of spheres from the contaminated diets. The observed rapid egestion of the plastic particles (25 to 75%, mean egestion 58% within 48h) could indicate that plastics were not released from the food source. However, since *Artemia sp.* are widely used as live food for aquaculture and laboratory stocks (Léger *et al.*, 1986), small crustaceans are part of a sticklebacks natural diet (Hynes, 1950, Allen and Wootton, 1984) and no undigested remains were found in faeces samples, it is assumed that *Artemia sp.* were successfully digested. Therefore, plastic spheres were released from the ingested diet and had the potential to interact with the GI. The low levels of plastic contamination which could still be found up to 14 days after feeding on non-contaminated *Artemia sp.* indicated that a small proportion of the ingested particles can be retained for extended periods in the gastrointestinal tract. Contamination of the faecal samples from egested plastic spheres from previous days by *Artemia sp.* and sticklebacks was minimised by the daily removal of faeces and detritus from the bottom of the tank and regular water changes. In addition, fish for the 14 day clearing period were transferred to new aquaria with clean ASW and fed with just uncontaminated *Artemia sp.*. The rapid egestion of ingested small plastic spheres reduces the likelihood of plastics to induce effects through the interaction with the

gastrointestinal lining and also limit the risk of biomagnification through tropic transfer to an organism of a higher trophic level. Additionally, the rapid egestion indicates that there was no risk for the blockage or obstruction of the GI of adult sticklebacks by the ingested spheres. However, since high numbers of plastic spheres were found to be incorporated in faecal pellets, the ingestion by detritus feeders is likely. Detritus feeders are organisms of a lower trophic level than sticklebacks, like the lugworm *Arenicola marina* and are known to ingest plastic particles from sediments (Besseling *et al.*, 2013, Wright *et al.*, 2013). A reverse trophic transfer from an organism of a higher trophic level to an organism of a lower trophic level through contaminated faeces pellets could pose an additional exposure route to detritus feeders.

Histological samples to assess the presence of ingested spheres in the GI and translocation of those particles into epithelial cells of the intestine could not provide supporting information to the faeces samples and blood smears. Even though previous studies (Sussarellu *et al.*, 2014, Browne *et al.*, 2008) successfully applied paraffin based techniques to create histological samples containing fluorescent polystyrene plastic spheres, the embedding process either dissolved or washed away the plastic spheres from the samples. Accordingly, conventional histological techniques with paraffin embedding are an unsuitable approach.

The translocation of micro and nanoplastic particles is of concern since it can lead to the accumulation of plastic in tissues and has the potential to lead to additional biological effects like a negative impact on the lipid metabolism and inflammatory responses (Cedervall *et al.*, 2012, von Moos *et al.*, 2012). However, translocation of microplastic for marine organisms has so far only been shown for invertebrates; A study conducted by von Moos *et al.* (2012) showed transport of plastic particles to the lysosomal system of *Mytilus edulis* (L.) as soon as 3h after initiating exposure. The used polarized light microscopy technique could not provide data for the size of translocated particles ranging from > 0 - 80 µm. Browne *et al.* (2008) reported the translocation of 3 and 9.6 µm sized plastic spheres to the haemolymph of *Mytilus edulis* (L.) as soon as 3 days after initiating exposure and found that the smaller sized particles were more frequently translocated. Evidence for the importance of particle size for translocation was provided for terrestrial vertebrates; Studies on rats found a strong decrease in the percentage of translocated particles compared to the exposure volume with increasing size of the particles (Jani *et al.*, 1996, Jani *et al.*, 1990) showing, that particles ranging from 0.05 –

0.5 µm were translocated from the gastrointestinal tract into tissues but 1 and 3 µm particles were just very poorly or not at all translocated. This indicates that a faster translocation of smaller particles is applicable and suggests that larger particles take longer to be translocated, up to the point where they are too big to be physically taken up. In this study no evidence for translocation of the plastic spheres to the circulatory system was found as shown by the examination of blood smears. Using a longer exposure window to the plastic spheres as Browne *et al.* (2008), whilst using similar sized plastic spheres, the particle size seemed to be the limiting factor for translocation in this study. This might be due to the different physiology of the gastrointestinal tract; The filter feeding blue mussel *Mytilus edulis* as it was used by Browne *et al.* (2008) and Moos *et al.* (2012), is able to digest food particles via an intracellular digestion that exists beside the enzymatic digestion (McVeigh *et al.*, 2006). During this digestive process whole food particles are taken up by phagocytosis into epithelium cells of the gastrointestinal tract and processed before nutrients are passed on to the hemolymph (where plastic particles were reported by Browne *et al.* (2008)) and waste particles are excreted back into the lumen of the gastrointestinal tract. Involved in this process is the lysosomal system (McVeigh *et al.*, 2006) (where plastic particles were reported to be found from von Moos *et al.* (2012)). Even though intracellular digestion is important in fish larvae (Watanabe, 1982), adult teleosts with a fully developed gastrointestinal tract digest food extracellular via enzymatic digestion in the stomach and absorb amino acids, lipids and carbohydrates (Bakke-McKellep *et al.*, 2000). Additionally, water-borne exposures like they were used by Browne *et al.* (2008) and von Moos *et al.* (2012) allow multiple exposure routes via exposed tissues like gills as well as ingestion, whereas a dietary exposure just targets ingested plastics. The single plastic spheres which were found in some of the blood smears in the presented study are believed to be a contamination during the sample preparation and other studies have previously shown the likelihood of cross contaminations when working with microscopic plastic particles (Foekema *et al.*, 2013). However, the translocation of low numbers of small plastic spheres might have been missed due to the here used magnifications and the fluorescent microscopy which might have been too insensitive to pick up very low numbers of translocated particles.

It is suspected that plastics can leach incorporated chemicals like additives and plasticisers that might induce biological responses (Teuten *et al.*, 2009, Oehlmann *et al.*, 2009). Uncontaminated polystyrene pellets have been reported to contain high levels of

parental polycyclic aromatic hydrocarbons (PPAHs) at concentrations of 79 - 97 ng g^{-1} pellet and have been shown to leach some of these chemicals in sea water (Rochman *et al.*, 2013c). Additionally, polystyrene is known to leach styrene monomers (Ahmad and Bajahlan, 2007, Miller *et al.*, 1994, Withey, 1976). Other polymers like polyethylene were reported with lower quantities of such PPAHs (3 - 6 ng g^{-1} on HDPE and not detectable to 13 ng g^{-1} in LDPE), highlighting the higher potential for polystyrene to induce adverse effects through the leaching of plastic derived chemicals. The liver has been identified as a good target organ for the detection of upregulated CYP1A in fish (Billiard *et al.*, 2004) and it has been previously shown that cytochrome P450 is involved in the hydroxylation of alkanes (Blasig *et al.*, 1984) and might be therefore also used as a biomarker for the exposure to plastic particles. PAHs have been reported to induce elevated expression of CYP1A in the liver of adult zebrafish (*Danio rerio*) at an exposure concentration of 162 µg L^{-1} benzo-a-pyrene after a 48h water borne exposure (Gerger and Weber, 2015). However, CYP1A expression which was used as a biomarker for upregulated detoxification processes was not affected. The findings of a non-upregulated, relative expression of CYP1A could suggest that the residence time of the virgin plastic spheres was too short (up to 75% egested within 48h) to cause the leaching of biologically active amounts of plastic derived chemicals. Differences in the relative expression of CYP1A between treatment groups might also have been missed due to the applied sampling regime. Differences in the levels of relative expression of CYP1A (up to 60-fold increase) in *Fundulus heteroclitus* were detected right after a 6h exposure period to 5 mg L^{-1} benzo-a-pyrene, whereas relative expression levels were comparable to controls at the other sampling points (6, 18, 24 and 30h post exposure) (Wang *et al*., 2010). These findings highlight the importance of sampling time for the relative expression levels of CYP1A and indicate that PAH induced upregulation of CYP1A expression might have been missed due to the here used sampling regime.No effects on growth (length and weight) and condition index were observed for fish sampled during and after the exposure. The absence of effects measured with these biomarkers may be due to the rapid egestion of the plastic spheres. The here used spheres size which did not cause blockages or obstructions of the GI allowed normal ingestion and digestion of food as shown by the active predation on *Artemia sp.* and daily egested faeces. According to the here used molecular biomarker no energy had to be invested into upregulated detoxification processes.

In conclusion, the ingestion of uncontaminated polystyrene micro spheres via a trophic transfer from zooplankton to fish is considered to not pose a risk in the short term, measured with the here used biomarkers for adult sticklebacks. However, biological effects, previously shown for other species (von Moos *et al.*, 2012, Wright *et al.*, 2013) or suggested following translocation (Cedervall *et al.*, 2012) might be observed with different particle sizes. It might therefore be that the size of the ingested particles via trophic transfer plays an essential role to induce biological effects. Small particles that are subject to translocation induce negative effects on cellular and molecular levels (Cedervall *et al.*, 2012, von Moos *et al.*, 2012) whereas big plastic particles could lead to deleterious short term effects like blockages of the GI (Carpenter *et al.*, 1972, Rothstein, 1973). Uncontaminated ingested particles that are too small to cause internal blockages and too big to be translocated, pose a minor threat to the organism since they are mainly rapidly egested. For future experiments not only a prolonged exposure period to the contaminated food source but also the assessment of particle characteristics like size, shape and degradation state may be used to investigate the effects of microplastics on fish. Additionally, more sensitive endpoints and molecular biomarkers should be applied to test for changes in energy reserves and for effects that might have been missed in this study.

Chapter 4

Effects of exposure to microplastics in young larvae (7 dph) of the three-spined stickleback (*Gasterosteus aculeatus*)

The preliminary exposure and first experiment were conducted by an undergraduate project student at the University of Portsmouth (Chris Payne) under the supervision of Dr. Karen L. Thorpe. I assisted with these exposures. The molecular work for experiment 1 was conducted by Dr. Karen L. Thorpe. I conducted all of the experimental work described for experiment 2.

The analysis and interpretation of the data from all experimental procedures outlined in this chapter is my own.

4.1 Abstract

Young larval stages of fish are especially vulnerable to pollutants due to limited energy reserves and the transition from utilising yolk sac energy reserves to exogenous feeding. During this transition larval fish have special dietary requirements and are under pressure to accumulate energy for early allometric growth of body parts involved in predator avoidance and feeding. Deviation from these requirements has the potential to impact growth and condition of the fish. Ingestion of plastic by fish larvae has been previously reported but little is known about the effects of such ingested plastic. In this chapter, experiments were conducted to determine whether three-spined stickleback (*Gasterosteus aculeatus*) larvae would accidentally ingest microplastic spheres from the water column and to assess the effects of any ingestion. Larvae (7 dph) were exposed to graded densities (10.6, 106 and 1060 mg L^{-1}) of 1 µm spheres for a preliminary study and (5.3, 53 and 530 mg L^{-1}) of 1 µm or 9.9 µm sized fluorescent polystyrene microspheres, via the water column, for exposures 1 and 2, respectively. The 7 day exposures indicated that larvae ingested plastics in a dose dependent manner (p = 0.005). In the preliminary study, ingestion of 1 µm microplastic positively affected growth (length (p < 0.001) and wet weight (p = 0.001)) but had a negative effect on condition factor K (p = 0.024). Exposure of larvae to the lower concentrations showed a positive effect on total length (p < 0.001) and negative effect on condition index K (p < 0.001) but no effects on wet weight (p = 0.118) for the 1 µm spheres and a negative effect on condition index K (p = 0.001) but not length (p = 0.292) and weight (p = 0.720) for the 9.9 µm spheres. An increase in relative expression of cytochrome P450 1A (CYP1A) for fish from experiment 1 (1 µm spheres) was observed (p = 0.029) but exposure did not affect relative expression of vitellogenin B (VTG B) (p = 0.185). These results suggest that ingested polystyrene microspheres have the potential to negatively affect young life stages of fish in the short-term through a reduction in condition and upregulated detoxification processes.

4.2 Introduction

As the smallest self-supporting vertebrates (Wieser, 1995), young fish larvae are an especially vulnerable life stage that has to cope with high predatory pressure and early autonomous feeding. To escape predators and to improve predation on food (Bailey and Houde, 1989) larvae can show rapid growth; Dry body mass has been observed to increase 100-fold within 3 weeks in newly hatched larvae of turbot, mackerel, anchovy, herring and cod (Nellen, 1986). Such early growth is mainly due to allometric development of the tail and parts of the head involved in predator avoidance and feeding (Osse *et al.*, 1997, Fuiman, 1983). Whereas the yolk sac provides young fish larvae with substrates for energy production over the first few days, exogenous feeding soon becomes their only mean of energy accumulation (Heming and Buddingtion, 1988). The time of complete yolk sac absorption and the change to exogenous feeding is one of the most critical steps in larval development where over 90% of larval mortality can occur (Houde, 2008). Due to little to no energy reserves after yolk sac absorption, these young life stages are especially susceptible to energy depletion (Huebert and Peck, 2014). Lower food availability has been linked to reduced growth in larval striped bass, (*Morone saxatalis*) (Wright and Martin, 1985) and short time starvation can lead to a so called “point of no return” after which malnutritioned larvae are unable to recover, even if suitable prey becomes available (Rana, 1985, Blaxter and Hempel, 1963). Additionally, stress can negatively affect larval growth through a reduction of appetite and food intake, food assimilation and a reduced metabolic rate (Wendelaar Bonga, 1997).

High levels of plastics in important nursery environments like rivers and estuaries (see Chapter 1, section 1.2) pose a potential threat to these young life stages since the wide range of different sizes, shapes and colours of plastic particles (Shaw and Day, 1994), combined with their broad vertical distribution in the water column ranging from surface floating plastics (Cózar *et al.*, 2014) to the sea floor (Bergmann and Klages, 2012) makes them available for ingestion by a wide range of species. Plastic is suspected to be confused as prey and become attacked and ingested by marine organisms (Shaw and Day, 1994, Carson, 2013). Due to their reliance on small food particles and planktonic organisms, large (Fossi *et al.*, 2014) and small (Cole *et al.*, 2013) filter feeding organisms but also young life stages of non-filter feeding species (Hjelmeland *et al.*, 1988) are susceptible to the ingestion of microplastics. Young fish

larvae that change their feeding strategy from utilising their yolk sac energy reserves to exogenous feeding rely on small organisms or food particles that are relatively immobile and therefore easy to catch and ingest (Huebert and Peck, 2014). Hence, floating microplastics are an easy prey and can become ingested (Hoss and Settle, 1990, Possatto *et al.*, 2011, Mazurais *et al.*, 2014, Hjelmeland *et al.*, 1988). However, even plastic fragments that are below the visual detection limits of fish larvae (30 - 40μm) (Olsen *et al.*, 2007) may become accidentally ingested. Many fish, including sticklebacks feed on their prey through a suction method where rapid opening of the mouth, followed by a slower closure, creates a suction effect through which the prey are ingested from the water column (Wootton, 1984). This feeding technique allows for small plastic particles that normally would not be ingested to mix with the larval diet, causing them to be drawn into the mouth of the larvae and subsequently ingested.

Ingestion of plastic can lead to the blockage of the gastrointestinal tract (GI), resulting in a lower food ingestion and energy depletion in the long term and even death (Gregory, 1991). Wright *et al.* (2013) observed a reduction in energy reserves of up to 50% in deposit feeding marine worms following an exposure to 130 μm un-plasticised polyvinylchloride particles for 28 days, which was thought to be due to prolonged gastrointestinal residence times of food and reduced feeding activity, caused by the ingested plastic (Wright *et al.*, 2013). Another study, also exposing *Arenicola marina* (this time to sediment contaminated with 400 - 1300 μm polystyrene spheres for 28 days) found a negative effect on feeding activity and a loss in weight (Besseling *et al.*, 2013). After depleting yolk energy reserves and switching to exogenous feeding, young fish larvae are susceptible to starve due to low energy reserves (Huebert and Peck, 2014). Hence, reduced energy assimilation due to plastic ingestion is likely to impact on larval growth rate, as exogenous feeding/energy accumulation has been suggested to have a possible interaction with larval size (Augustine *et al.*, 2011). Just very limited data is available regarding the effects of microplastics on growth development of teleost fish. However, exposure of 8 dph seabass larvae (*Dicentrarchus labrax*) to a diet contaminated with 0, 103, 104 or 105 fluorescent microbeads g^{-1} of food (mix of 10 - 45 μm) for 18 days found a 25% decreased growth rate following the exposure (Mazurais *et al.*, 2014). Additionally, exposure of larvae of the sea urchin *Tripneustes gratilla* to 0, 1, 10, 100 and 300 spheres ml^{-1} of 10 - 45 μm polyethylene plastic spheres indicated that plastic particles were ingested in a dose dependent manner and resulted in a small non dose dependent effect on larval growth (Kaposi *et al.*, 2013). Also ingestion of

plastic particles in birds has been observed to affect food intake and thus energy accumulation (Ryan, 1990) and to cause hepatic stress in exposed fish (Rochman *et al.*, 2013b).

The presence of high levels of microplastic sized plastic fragments in aquatic nursery environments and their potential to become ingested is of concern since larval stages are especially vulnerable to environmental and anthropogenic stress due to low energy reserves. Therefore, the aim of this chapter was to investigate the potential of different sized polystyrene plastic spheres at different concentrations to induce biological effects in young fish larvae. Ingestion of plastic spheres was confirmed using fluorescent microscopy of larvae and levels of ingestion evaluated using relative fluorescent intensity. To evaluate if exposure had any effects on biological endpoints the length, wet weight and condition index K were compared to a control group. Additionally, relative expression of the detoxification enzyme CYP1A was investigated to test for detoxification processes to alkanes on a molecular level. To test for exposure to oestrogenic, plastic derived chemicals the relative expression of VTG B was assessed.

4.3 Material and methods

An initial range finding experiment (preliminary experiment) was conducted to determine the concentrations of plastic particles to be used for the main experiments. For this, 10.6, 106 and 1060 mg L^{-1} of 1 µm fluorescent polystyrene microspheres were used. This was followed by two experiments to assess the biological effects of the ingested plastics in larvae exposed to graded densities (5.3, 53 and 530 mg L^{-1}) of 1 µm (experiment 1) and 9.9 µm (experiment 2) polystyrene spheres. Length weight and condition index K were evaluated for all experiments as measurements for biological effects as described in Chapter 2, section 2.3.1. Additionally, relative expression of CYP1A (biomarker for exposure to organic pollutants) and the egg yolk protein VTG B (biomarker for exposure to oestrogenic pollutants) were measured for experiment 1. Details for the material and methods for the molecular work can be found in Appendix I.

4.4 Test organisms

F_2 generation stickleback eggs were obtained from the spawning of three separate breeding pairs in aquaria at the Institute of Marine Sciences, University of Portsmouth and maintained prior to the exposure as described in Chapter 2, section 2.1.1.1. Shortly before hatching, eggs were randomly transferred between 12, one litre beakers, with 40 ml (preliminary experiment) or 200 ml (main experiments) of AFW. Beakers were maintained under the same conditions as the 96-well plates. Live and dead eggs were recorded daily, with any dead eggs removed. Pre exposure the larvae were fed defrosted rotifers (Tropical Marine Centre, UK) twice daily from 2 days post hatch until the initiation of the experiment.

4.4.1 Microplastic spheres

For the preliminary experiment and experiment 1, 1 µm sized fluorescent polystyrene spheres were used. For experiment 2, 9.9 µm sized fluorescent polystyrene spheres were used. For details of the used spheres see Chapter 2, section 2.2.

4.4.2 Water supply apparatus

See Chapter 2, section 2.1 for details of the water supply apparatus. Water temperatures were monitored daily throughout the experiments and ranged between 17.9 and 19°C (preliminary experiment and experiment 1) and 17.5 and 18.5°C (experiment 2), while pH levels were checked twice weekly and ranged between 7.26 and 7.95 (preliminary experiment and experiment 1) and 7.05 and 7.52 (experiment 2). Dissolved oxygen concentrations were checked twice weekly and remained over 70% of the air saturation value for the duration of all experiments.

4.4.3 Fish exposure

All exposures (preliminary, experiment 1 and experiment 2) began as the stickleback larvae reached an age of 7 dph. The designated hatching day was the day the majority of larvae had hatched, which was day 1 for this test.

Larvae for the preliminary exposure were maintained in 40 ml of AFW and exposed for a total of 7 days. One treatment was maintained as a control free of any plastic spheres.

The other 3 treatments were exposed to 1 µm microplastic beads with a logarithmic ascending dosage. A Low treatment was exposed to 10.6 mg L^{-1}, a Medium treatment to 106 mg L^{-1} and a High treatment to 1060 mg L^{-1} plastic spheres. A total of 12 beakers were used with 7 fish $beaker^{-1}$ and 3 replicates $treatment^{-1}$.

Larvae for experiment 1 were maintained in 200 ml of AFW and exposed for a total of 7 days. One treatment was maintained as a control free of any plastic spheres. The other 3 treatments were exposed to 1 µm microplastic beads with a logarithmic ascending dosage. A Low dose treatment was exposed to 5.3 mg L^{-1}, a Medium dose treatment was exposed to 53 mg L^{-1} and a High dose treatment was exposed to 530 mg L^{-1} of polystyrene plastic spheres. A total of 12 beakers were used with 12 fish $beaker^{-1}$ and 3 replicates $treatment^{-1}$.

Larvae for experiment 2 were maintained in 200 ml of AFW and exposed for a total of 7 days. One treatment was maintained as a control free of any plastic spheres. The other 3 treatments were exposed to 9.9 µm microplastic beads with a logarithmic ascending dosage. A Low dose treatment was exposed to 5.3 mg L^{-1}, a Medium dose treatment was exposed to 53 mg L^{-1} and a High dose treatment was exposed to 530 mg L^{-1} of polystyrene plastic spheres. A total of 12 beakers were used with 8 fish $beaker^{-1}$ and 3 replicates $treatment^{-1}$.

4.4.4 Sampling

On day 7 of the experiment (larval age 14 dph), larvae were sacrificed as described in Material and Methods (Chapter 2) section 2.3. Three fish per beaker were fixed in RNAlater (Sigma Aldrich, UK) and stored at -80°C for subsequent isolation of RNA (experiment 1). The remaining fish were fixed in 10% buffered formalin for fluorescent microscopy to evaluate presence of ingested microspheres in the GI (preliminary and experiments 1 and 2).

4.4.5 Fluorescent determinations

To provide evidence for the ingestion of plastic from the water column and investigate dose dependent effects on the ingestion of plastic from the water column, presence of fluorescent plastic beads within the larva was determined. For each sample, presence or absence of fluorescent microbeads was recorded by placing individual larvae on a

microscope slide and examine it with a Zeiss LUMAR.V12 stereo microscope with an AxioCam MRm camera and AxioVision software (ZEISS, Germany) and a Green Fluorescent Protein (GFP) filter (excitation 485 nm; emission 520 nm) at 20x magnification. To compare fluorescence of larvae between treatments, larvae were placed in clear, flat bottom 96-well plates (one larvae per well) and read with a POLARstar OPTIMA microplate reader. The fluorescence comparison using the microplate reader was just carried out for experiment 1. Additionally, to monitor egestion of the ingested plastic spheres, detritus samples were collected and analysed with a Zeiss LUMAR.V12 stereo microscope with an AxioCam MRm camera and AxioVision software (ZEISS, Germany) and a Green Fluorescent Protein (GFP) filter (excitation 485 nm; emission 520 nm) at 80x magnification. Analysis of detritus samples was just carried out for experiment 2.

4.4.6 Gene expression

Molecular analysis was carried out by Dr Karen L. Thorpe for larvae from experiment 1. Material and methods for this molecular work can be found in Appendix I.

4.4.7 Statistical analysis

All statistical analysis was carried out as detailed in Chapter 2, section 2.3.5.

4.5 Results

4.5.1 Effects on mortality

The mortality of one exposed larvae was observed during the preliminary study (98.8% survival). No mortality was observed for exposed larvae from experiment 1 (100% survival), whereas 9 larvae died during experiment 2 (89.7% survival). Of these dead fish in experiment 2, four were found in the Control treatment and five in the Medium treatment. Not more than one dead larva was found per treatment per day and mortality occurred across replicates of the affected treatments.

4.5.2 Ingestion of plastic spheres from the water column

Consistent with expectations, no plastic particles were found in larvae of the Control group of the preliminary and the two main experiments. Examination of sampled larvae from the preliminary experiment with a fluorescent microscope suggested that all fish from plastic treatments had ingested the fluorescent plastic spheres and that the quantity ingested appeared to be related to dose (Figure 4.1). The same effect on plastic ingestion was suggested for both main experiments (Figure 4.2). Further evidence, to this visual examination for a dose dependent ingestion of the spheres, was provided by fluorescent plate reader results (Figure 4.3). Fluorescence of Low treatments were 1.04 ± 0.17 fold higher, Medium treatments were 1.17 ± 0.27 folds higher and High treatments groups were 1.30 ± 0.42 folds higher when compared to the Control group and showed to be different between exposure groups ($H(3, N = 69) = 27.629$, $p<0.001$). However, pairwise comparison of treatments with the Control and between plastic treatments revealed that the Low treatment did not have a higher fluorescence when compared to the Control ($H(1, N = 35) = 0.393$, $p = 0.531$). The Medium ($H(1, N = 35) = 11.557$, $p = 0.001$) and the High ($H(1, N = 35) = 10.893$, $p = 0.001$) treatments, however, showed to have a higher larval fluorescence when compared to the Control. Larvae from the Low treatment group were found to have a lower fluorescence when compared to the Medium ($H(1, N = 36) = 6.247$, $p = 0.012$) and High ($H(1, N = 36) = 8.289$, $p = 0.004$) treatments. No difference in fluorescence was found between the Medium and the High treatments ($H(1, N = 36) = 2.027$, $p = 0.155$). Whereas these pairwise comparisons did not support a clear treatment related ingestion of plastic spheres, a Pearson´s correlation confirmed that higher treatment exposure levels were correlated to higher fluorescent levels of examined larvae ($r = 0 .554$, $p < 0.001$, $n = 71$) (Figure 4.4).

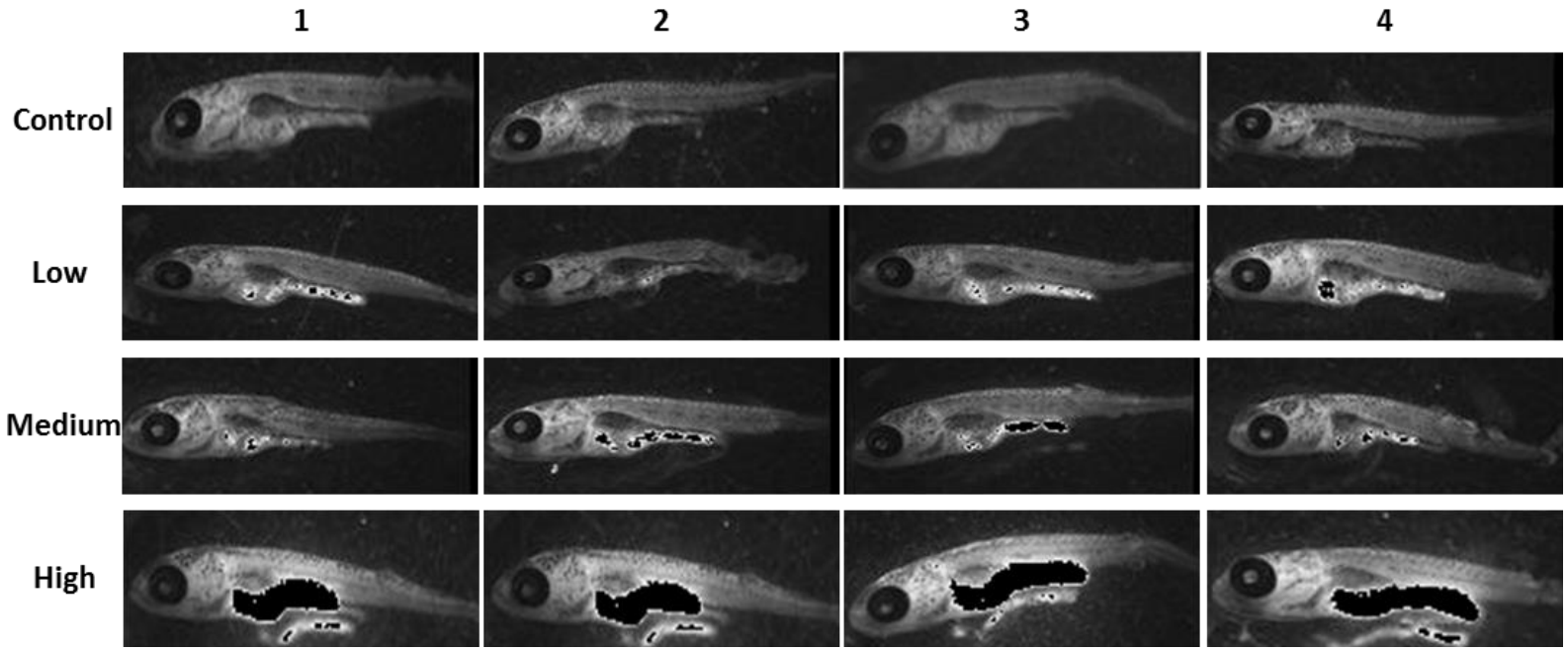

Figure 4.1: Pictures of larvae from the preliminary experiment taken with a fluorescent microscope after 7 days of exposure to a plastic contaminated water column.Fluorescence in the gastrointestinal tract (GI) of larvae indicates a dose dependent ingestion of the plastic spheres from the water column. Control (0 mg L^{-1}), Low (10.6 mg L^{-1}), Medium (106 mg L^{-1}) and High (1060 mg L^{-1}) treatment. Pictures of four fish (numbers 1 - 4) were taken per treatment. Fluorescence is indicated by bright white and black colour in the GI of larvae.

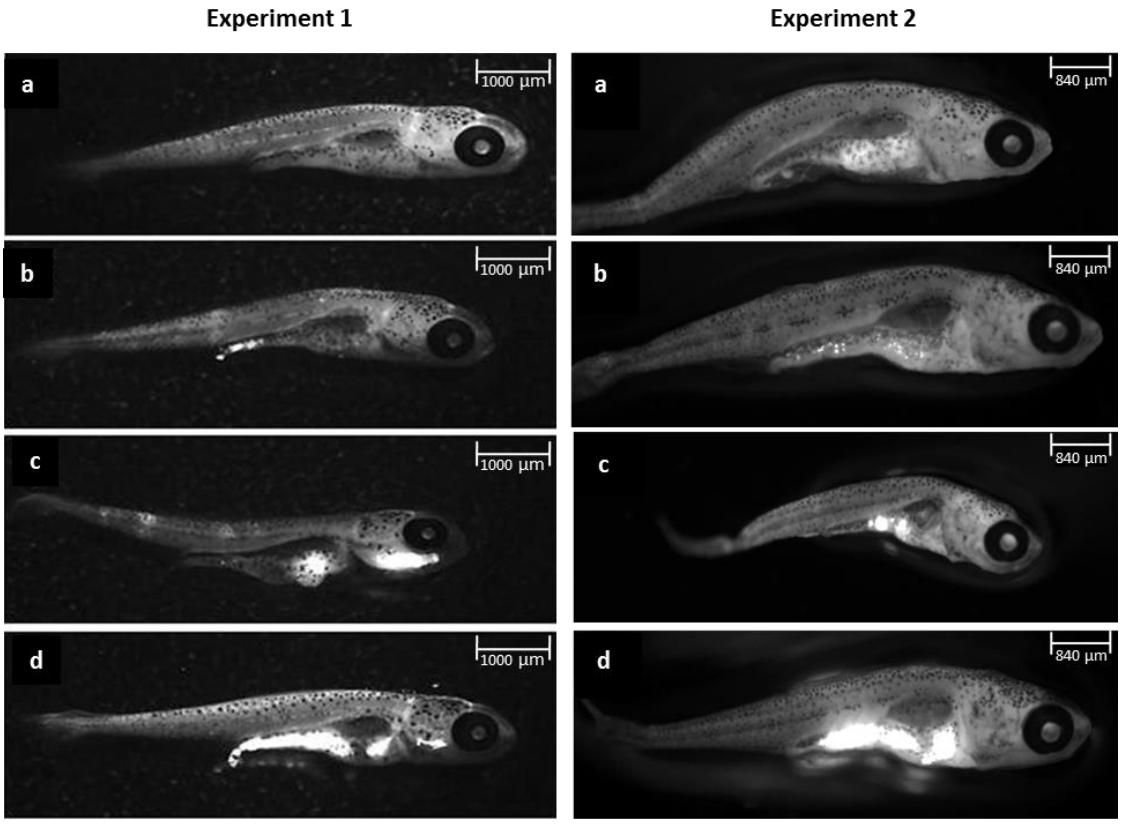

Figure 4.2: Pictures of larvae taken with a fluorescent microscope after 7 days of exposure to a plastic contaminated water column. Fluorescence in the gastrointestinal tract (GI) of larvae indicates a dose dependent ingestion of the plastic spheres from the water column. a = Control (0 mg L^{-1}), b = Low (5.3 mg L^{-1}), c = Medium (53 mg L^{-1}) and d = High (530 mg L^{-1}) treatment. Fluorescence of Control fish derived from the auto fluorescence of the GI.

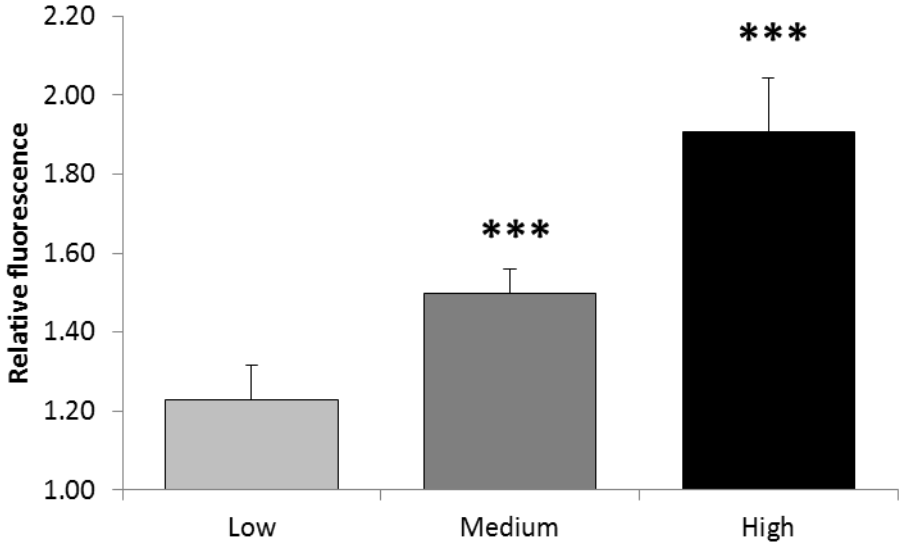

Figure 4.3: Mean (± SEM) fluorescent intensity, relative to the Controls, measured in larvae from each treatment after a 7 day exposure to a logarithmic ascending dosage of fluorescent plastic spheres (1 µm) measured with a fluorescent plate reader Low (5.3 mg L^{-1}), Medium (53 mg L^{-1}) and High (530 mg L^{-1}). Larvae for this analysis derived from experiment 1. ***=$p < 0.001$.

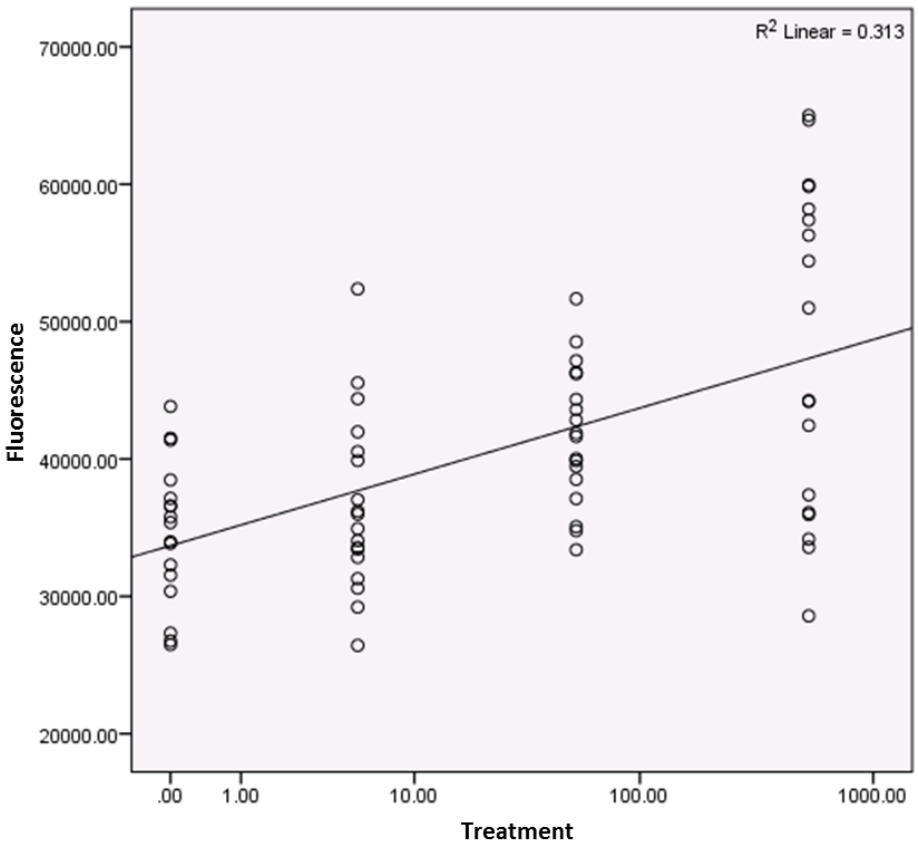

Figure 4.4: Pearson´s correlation between larval fluorescence and exposure concentration of larvae sampled after a 7 day exposure to 0, 5.3, 53 and 530 mg L^{-1} fluorescent plastic spheres. Fluorescence of larvae was determined using a fluorescent plate reader and values (y-axis) correlated with treatment groups (x-axis). A Person´s correlation revealed a dose dependent intensity of fluorescence (r = 0 .554, p < 0.001, n = 71).

In line with the full body examinations of sampled larvae with a fluorescent microscope and the measurements of the fluorescent plate reader that indicated a dose dependent ingestion of plastic spheres, visual examination of detritus samples indicated a dose dependent egestion of plastic spheres (Figure 4.5).

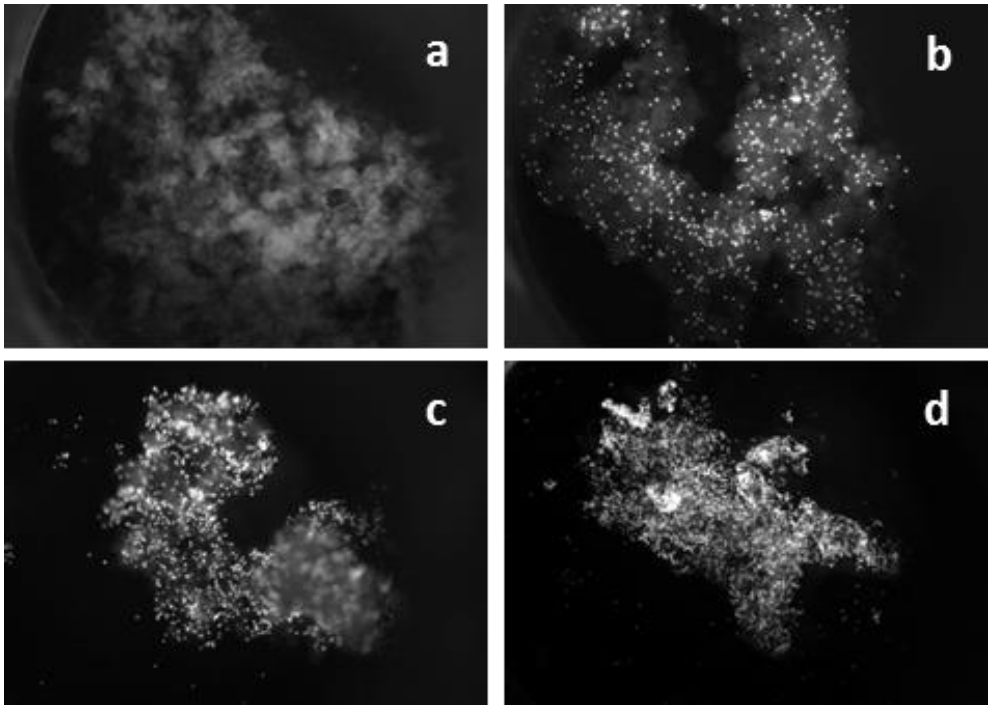

Figure 4.5: Pictures of detritus samples, taken with a Zeiss LUMAR.V12 stereo microscope with an AxioCam MRm camera and AxioVision software (ZEISS, Germany) and a Green Fluorescent Protein (GFP) filter (excitation 485 nm; emission 520 nm) at x80 magnification. a = Control (0 mg L^{-1}), b = Low (5.3 mg L^{-1}), c = Medium (53 mg L^{-1}) and d = High (530 mg L^{-1}). Detritus samples mostly comprised out of faeces. Fluorescence indicated a dose dependent egestion of plastic particles. Detritus samples derived from experiment 2.

4.5.3 Biological effects

4.5.3.1 Effects on total length

Results from the preliminary experiment revealed that the 7 day exposure to the 1 µm plastic spheres induced a higher total length ($H(3, N = 83) = 20.834$, $p < 0.001$) (Figure 4.9a). Pairwise comparison of treatment groups with the Control revealed that a higher total length could be found for fish exposed to the Low ($H(1, N = 24) = 6.607$, $p = 0.010$), Medium ($H(1, N = 41) = 10.032$, $p = 0.002$) and High ($H(1, N = 42) = 18.024$, $p < 0.001$) concentration of plastic spheres. The observed increase in length seemed to be positively correlated to the level of microplastics ($r = 0.339$, $p = 0.002$, $n = 83$) (Figure 4.6a).

Similar to these results also the exposure to 1 µm plastic spheres in experiment 1 induced an increased total length ($H(3, N = 144) = 30.607$, $p < 0.001$) (Figure 4.10a). Pairwise comparison between treatment groups, however, revealed no differences between the Control and the Low treatment ($H(1, N = 72 = 1.978$, $p = 0.160$) but between the Control and the Medium ($H(1, N = 72) = 25.451$, $p = 0.004$) and High ($H(1, N = 72) = 25.451$, $p < 0.001$) treatments. Like for the preliminary study also the lower concentrations seemed to affect an increase in length in a positively, dose dependent manner ($r = 0.419$, $p < 0.001$, $n = 144$) (Figure 4.6b). Additionally, a Pearson´s correlation revealed that the dose dependent increase of fluorescence of fish larvae measured with the fluorescent plate reader was correlated with increased length ($r = 0.984$, $p < 0.001$, $n = 12$).

Unlike for the exposures to the 1 µm spheres, no effect on total length could be observed when larvae were exposed to the 9.9 µm spheres in experiment 2 ($H(3, N = 87) = 3.728$, $p = 0.292$) (Figure 4.10a). However, even though no differences were found, relative to the control, a positive dose dependent effect on total length was still observed for experiment 2 ($r = 0.339$, $p = 0.002$, $n = 83$) (Figure 4.6c).

Overall, the highest concentration induced the most pronounced effects on total length when compared to experiment 1 and 2. Comparing the two particles sizes revealed that the 1 µm sized plastic spheres had the potency to induce stronger effects when compared to the 9.9 µm spheres.

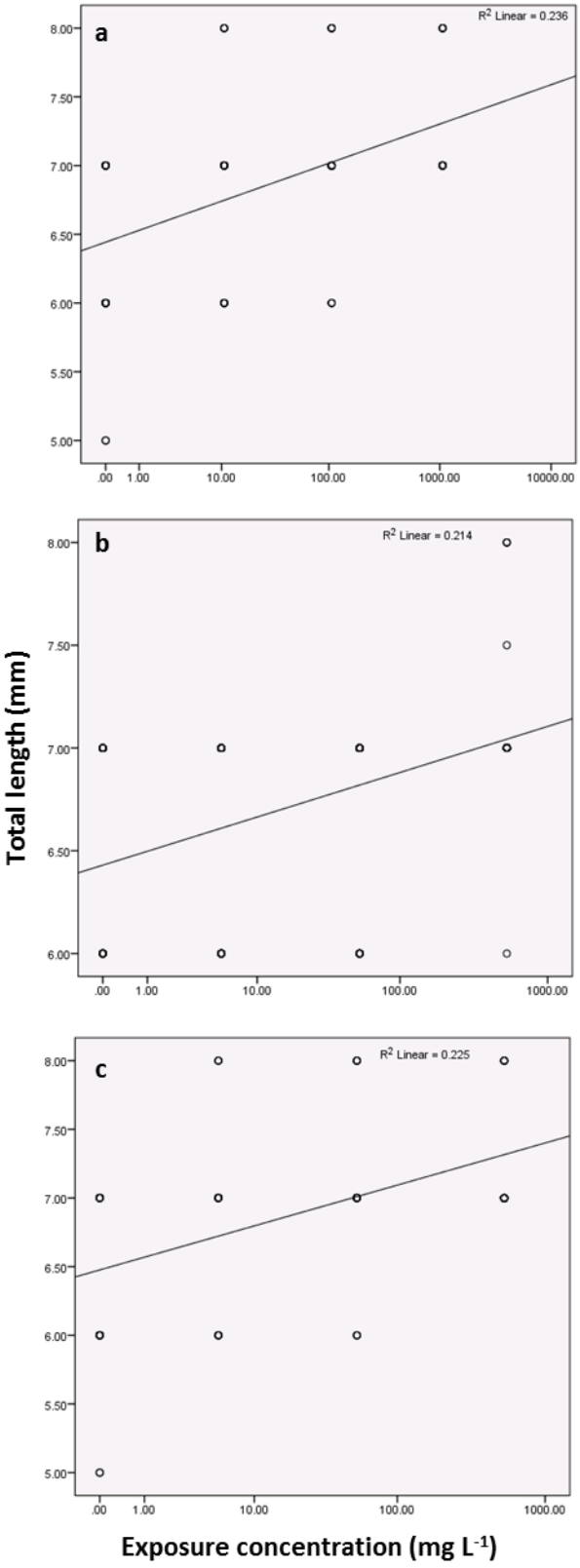

Figure 4.6: Correlations of total length with exposure concentration of larvae sampled after 7 days of exposure to a plastic contaminated water column with a = 1 µm spheres at 0, 10.6, 106 and 1060 mg L^{-1}; b = 1 µm spheres at 0, 5.3, 53 and 530 mg L^{-1}; c = 9.9 µm spheres at 0, 5.3, 53 and 530 mg L^{-1}.

4.5.3.2 Effects on wet weight

Exposure to 1 µm spheres in the preliminary experiment had an effect on wet weight of exposed fish ($F_{3,79}$ = 6.474, p = 0.001) (Figure 4.9b). A Dunnett´s Post-Hoc test revealed that higher wet weights could be found for the Medium (p = 0.001) and High (p = 0.004) treatments when compared to the Control. There was a weak correlation for a dose dependent, positive increase in weight (r = 0.243, p = 0.27, n = 83) (Figure 4.7a).

Unlike the found similarities in total length, exposure to 1 µm plastic spheres in experiment 1 had no effect on measured wet weights ($F_{3,140}$ = 0.992, p = 0.399) (Figure 4.10b). Additionally, no correlation was found between exposure dosages and measured wet weight for experiment 1 (r = -0.099, p = 0.238, n = 144) (Figure 4.7b).

Similar to results from experiment 1, no effects on wet weight of sampled larvae ($F_{3,83}$ = 0.447, p = 0.720) was found for larvae exposed to the 9.9 µm spheres in experiment 2 (Figure 4.10b). However, there was a weak but significant, positive correlation between exposure concentration and measured wet weight for experiment 2 (r = 0.233, p = 0.035, n = 83) (Figure 4.7c).

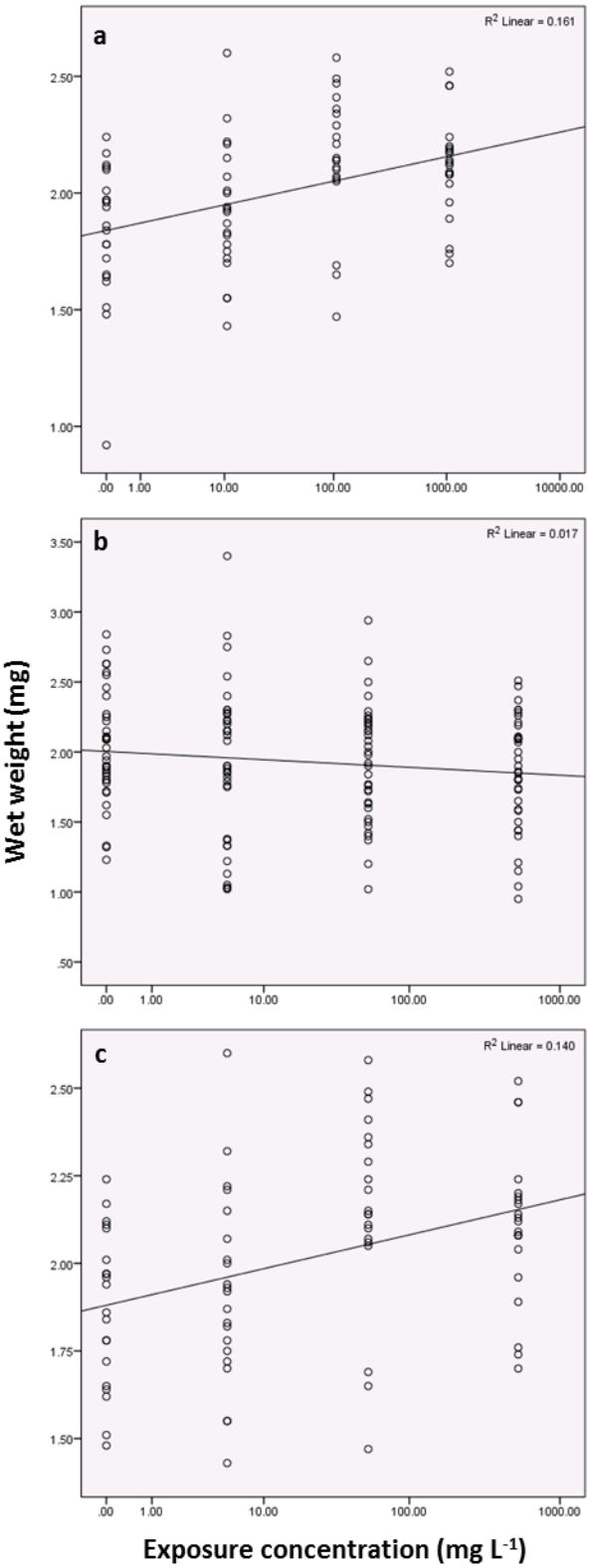

Figure 4.7: Correlations of wet weight with exposure concentration of larvae sampled after 7 days of exposure to a plastic contaminated water column with a = 1 µm spheres at 0, 10.6, 106 and 1060 mg L^{-1}; b = 1 µm spheres at 0, 5.3, 53 and 530 mg L^{-1}; c = 9.9 µm spheres at 0, 5.3, 53 and 530 mg L^{-1}.

4.5.3.3 Effects on condition index K

The condition index K showed significant differences for the preliminary exposure to the 1 µm plastic spheres (H(3, N = 83)= 9.422, p = 0.024) (Figure 4.9c). Pairwise comparison of the plastic treatments with the Control revealed that larvae from the Low (H(1, N = 42) = 5.242, p = 0.022) and High (H(1, N = 42) = 7.942, p = 0.005) but not the Medium ($F_{1,39}$ = 3.414, p=0.072) treatment had reduced condition indices. A weak but significant negative correlation was found between exposure levels and condition index K (r = -0.231, p = 0.037, n = 83) (Figure 4.8a).

Similar results for a reduced condition of exposed fish were found for experiment 1 ($F_{3,140}$ = 13.372, p < 0.001) (Figure 4.10c). A Dunnett´s post hoc test revealed decreased condition indices between the Control and the Low (p = 0.02), Medium (p = 0.01) and High (p < 0.001) treatments. A negative correlation was found between exposure concentration and condition index K (r = -0.381, p < 0.001, n = 143) (Figure 4.8b).

Like the preliminary exposure and experiment 1, also the 9.9 µm sized spheres used in experiment 2 showed to have an effect on larval condition (H(3, N = 87) = 16.007, p = 0.001) (Figure 4.10c). Pairwise comparison of treatments with the Control revealed that there was no difference between the Low treatment and the Control group (H(1, N = 44) = 0.333, p = 0.564) but a reduced condition index for the Medium (H(1, N = 39) = 6.976, p = 0.008) and High (H(1, N = 44) = 6.242, p = 0.012) treatments. A Pearson´s correlation revealed a weak but significant, negative correlation between increasing exposure levels and condition index K (r = -0.241, p = 0.028, n = 83) (Figure 4.8c).

All exposures indicated that ingestion of the plastic spheres had a negative effect on the condition index K.

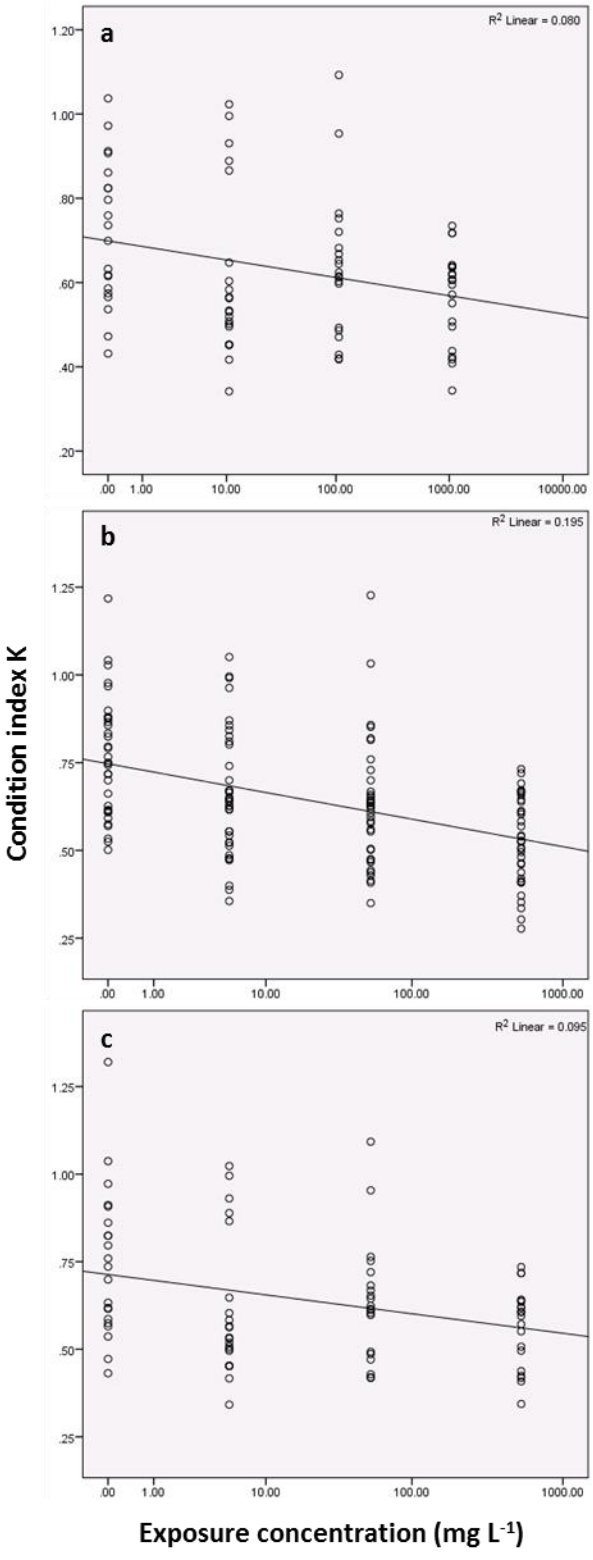

Figure 4.8: Correlations of condition index K with exposure concentration of larvae sampled after 7 days of exposure to a plastic contaminated water column with a = 1 µm spheres at 0, 10.6, 106 and 1060 mg L^{-1}; b = 1 µm spheres at 0, 5.3, 53 and 530 mg L^{-1}; c = 9.9 µm spheres at 0, 5.3, 53 and 530 mg L^{-1}.

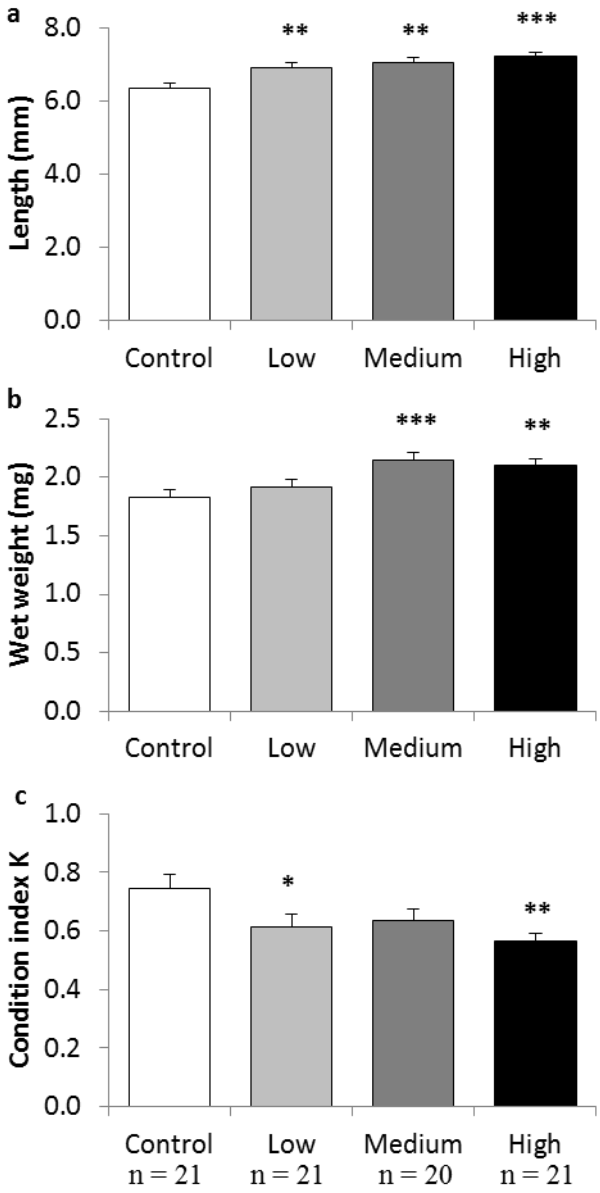

Figure 4.9: Biological endpoints of the preliminary study measured with a = total length, b = wet weight and c = condition factor K of sampled larvae after a 7 day exposure to a logarithmic ascending dosage of 1 µm plastic spheres (Control = 0 mg L^{-1}, Low = 10.6 mg L^{-1}, Medium = 106 mg L^{-1} and High = 1060 mg L^{-1}) from the preliminary experiment (exposed to 1 µm spheres). An increase in length was observed for the Medium (p = 0.002) and High (p < 0.001) treatments, an increased wet weight for the Medium (p = 0.001) and High (p = 0.004) and a reduction in condition index K for the Low (p = 0.022) and High (p = 0.005) treatments when compared to the Control group. Each column represents the mean ± SEM.

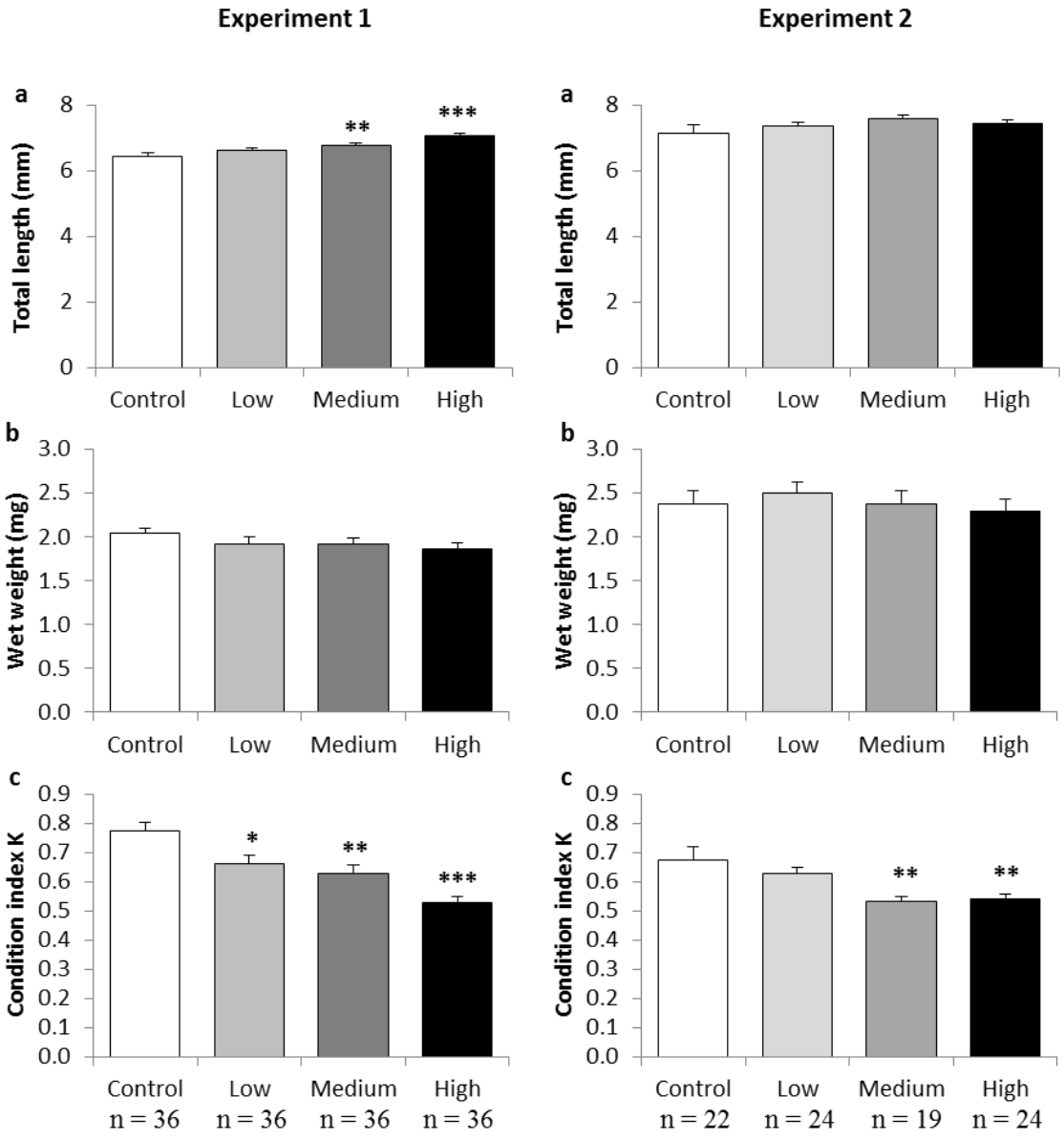

Figure 4.10: Biological endpoints of the two main experiments measured with a = total length, b = wet weight and c = condition factor K of sampled larvae after a 7 day exposure to a logarithmic ascending dosage of 1 µm (experiment 1) or 9.9 µm (experiment 2) plastic spheres (Control = 0 mg L^{-1}, Low = 5.3 mg L^{-1}, Medium = 53 mg L^{-1} and High = 530 mg L^{-1}). An increase in length was observed for the Medium (p = 0.004) and High (p < 0.001) treatments of experiment 1 and a reduction in condition index K for Medium (p = 0.008) and High (p = 0.012) treatments of experiment 2 and Low (p = 0.02), Medium (p = 0.01) and High (p < 0.001) plastic treatments of experiment 1. Each column represents the mean ± SEM.

4.5.4 Gene expression

Real time qPCR analysis of relative expression of the gene encoding CYP1A was found to be differently expressed between treatments ($F_{3,20}$ = 3.698, p = 0.029) (Figure 4.11a). A Dunnett´s Post-Hoc test revealed that there was no difference between the Low treatment and the Control (p = 0.177, n = 12) but for the Medium (p = 0.025, n = 12) and the High (p = 0.023, n = 12) treatments when compared to the Control. Additionally, a correlation between plastic exposure levels and relative expression of CYP1A was found (r = 0.461, p = 0.023, n = 24). There was no evidence that exposure induced an increase of relative expression for VTG B (H(3, N = 24) = 4.820, p = 0.185) (Figure 4.11b) in larval fish following the 7 day exposure.

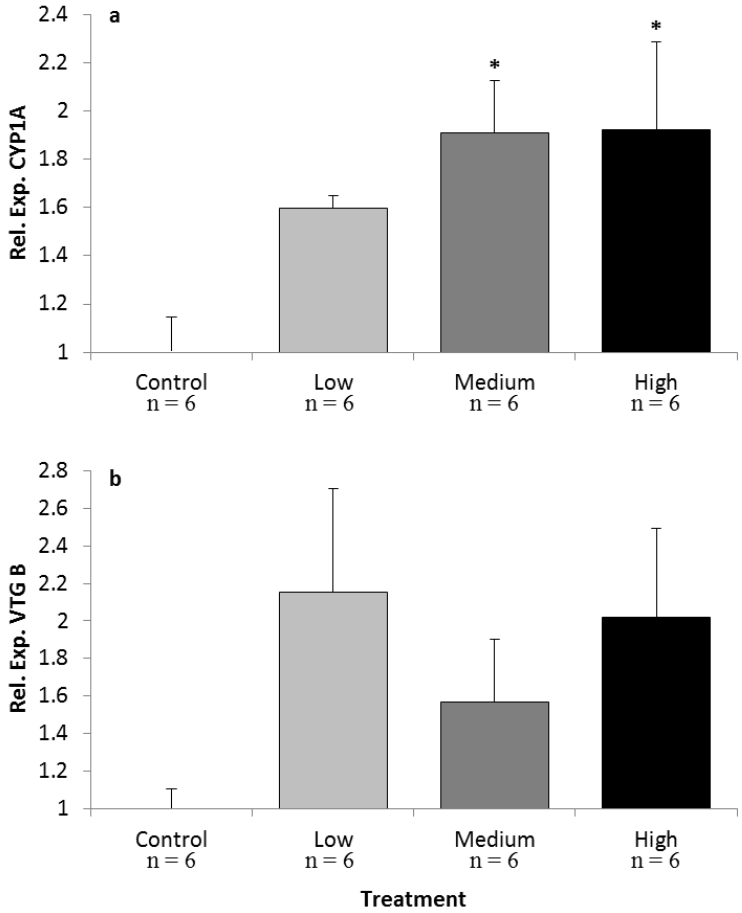

Figure 4.11: Relative expression of cytochrome P450 1A (CYP1A (a)) and vitellogenin B (VTG B (b))following a 7 day exposure to a logarithmic ascending dosage (Control = 0 mg L^{-1}, Low = 5.3 mg L^{-1}, Medium = 53 mg L^{-1} and High = 530 mg L^{-1}) of 1 µm polystyrene plastic spheres. Upregulated relative expression of CYP1A was found for the medium (p = 0.025) and high treatments (p = 0.023) whereas no differences were found for the expression of VTG B. Fish used for this analysis derived from experiment 1. Each column represents the mean ± SEM.

4.6 Discussion

This study raises concern that exposure to a plastic contaminated water column can have negative effects on the condition of young fish larvae. Ingestion of plastic particles was observed in all experiments for all exposure concentrations of both sphere sizes, indicating that plastic ingestion will increase as pollution levels rise. The higher concentrations of the 1 µm plastic spheres used in the preliminary study had a positive impact on length ($p < 0.001$) and wet weight ($p = 0.001$) but had a negative effect on condition factor K ($p = 0.024$). Findings from experiment 1, using lower concentrations of the 1 µm plastic spheres confirmed the findings for a positive effect on length ($p < 0.001$) and negative effect on condition index K ($p < 0.001$). However, no effect on wet weight was observed ($p = 0.118$). Experiment 2 using the larger 9.9 µm sized plastic spheres found similar results for a reduced condition index K ($p = 0.001$) but did not show an effect on length ($p = 0.292$) and weight ($p = 0.720$), indicating that the smaller spheres had a higher potency to induce effects for these measured biomarkers. For all experiments, a negative, dose dependent effect for the condition index K could be observed. Additionally, up regulation of expression of CYP1A in larvae from experiment 1 indicated an induction of elevated detoxification processes in the Medium ($p = 0.025$) and High ($p = 0.023$) treatments when compared to the Control. No effects for the relative expression of VTG B were found ($p = 0.185$), suggesting that no biologically active concentrations of oestrogenic chemicals leached from the plastic spheres.

The survival of larvae from both experiments using the 1 µm plastic spheres was not affected by the exposure over the duration of 7 days which is consistent with findings from other studies (Kaposi *et al.*, 2013, Besseling *et al.*, 2013, Mazurais *et al.*, 2014). The mortality of the larvae observed in experiment 2 is thought to be due to stress after water changes since just single mortalities across replicates occurred.

Larvae from all exposures showed a dose dependent ingestion of the plastic spheres which is consistent with findings for other marine larvae (Kaposi *et al.*, 2013) and suggests that increasing pollution levels can lead to elevated ingestion rates in fish larvae. Additionally, detritus samples from experiment 2 indicated a dose dependent egestion of plastic spheres. However, since faeces pellets of such young fish are very small, samples consisted of faeces and detritus from the bottom of the exposure vessels.

Since static water conditions were used, the density of water at 18°C is 0.9986 g cm^{-3} (ASCE-library) and the density of the used plastic spheres is 1.05 g cm^{-3} (see Chapter 2, section 2.2), spheres were expected to slowly sink to the bottom of the exposure vessels. Therefore, the observed dose dependent fluorescence in detritus samples may not only be due to a dose dependent egestion but also due to the actual exposure concentrations.

The observed dose dependent increase in total length, which was observed for the preliminary experiment and experiment 1 but not for experiment 2, stands in contrast to expectations and results from other studies; Ingestion of plastic particles that do not contain any nutritional value and which are considered biochemically inert (Teuten *et al.*, 2009, Lithner *et al.*, 2011, Roy *et al.*, 2011) can induce a false sense of satiation due to the accumulation of indigestible plastic in gastrointestinal tract. A likely consequence of this is a reduced feeding rate and thus energy accumulation (Ryan, 1990). Since young fish larvae invest the majority of their energy in growth, this reduction in energy accumulation is likely to result in reduced growth. Accordingly, Mazurais *et al.* (2014) who exposed 8 dph seabass larvae (*Dicentrarchus labrax*) to a diet contaminated with 0, 103, 104 or 105 fluorescent microbeads g^{-1} of food (mix of 10 - 45 μm) for 18 days found a 25% decreased growth rate following the exposure. Also Kaposi *et al.* (2013) found a decrease in post-oral arm length in *Tripneustes gratilla* larvae that had been exposed to the highest concentration (0, 1, 10, 100 and 300 spheres ml^{-1}) of 10 - 45 μm polyethylene plastic. One possible explanation for the observed dose dependent increase in length could be the leaching of biologically active concentrations of plastic derived chemicals. Polystyrene has been reported to leach bioactive compounds like alkylphenols (Soto *et al.*, 1991) and styrene (Ahmad and Bajahlan, 2007). Styrene exposure has been linked to increased levels of the human growth hormone (HGH) through neuroendocrine disrupting effects (Mutti *et al.*, 1984), an effect that cannot be observed for other plastics like polyethylene which was used for Kaposi *et al.* (2013) and Mazurais *et al.* (2014). Alkylphenols and styrene have been found to display oestrogenic properties (Bang *et al.*, 2012) and oestrogens are known to be involved in regulation of fish growth and exposure to oestrogens has been shown to have a growth-promoting effect in larval sticklebacks (Hahlbeck *et al.*, 2004a). It has been previously reported that cytochrome P450 is involved in the hydroxylation of alkanes (Blasig *et al.*, 1984) and is thought to be one of the main enzymes involved in the biotransformation of xenobiotics in fish (Boon *et al.*, 2002). Hence, the increased relative expression of CYP1A in the Medium and High treatment groups of experiment 1 could indicate an

exposure to plastic derived chemicals. Even though no differences in the relative expression of VTG B was found, plastic treatments showed a trend for elevated expression levels, which indicates that fish were exposed to low concentrations of weakly estrogenic chemicals. The found relationship between plastic exposure levels and relative expression of the CYP1A may indicate a dose related leaching of plastic derived chemicals. However, the low fold changes in the relative expression of CYP1A observed in this study suggest that just low concentrations leached from the ingested spheres. In comparison, a 12 day exposure of Chinese rare minnow (*Gobiocypris rarus*) to as little as 4 µg L^{-1} of benzo-a-pyrene resulted in a 10-fold change in the relative expression levels of CYP1A. The exposure to 100 µg L^{-1} benzo-a-pyrene resulted in a 38-fold increase in the relative expression of CYP1A when compared to controls (Yuan *et al*., 2013). Objections that the sampling regime might have missed higher relative expression levels of CYP1A (Wang *et al*., 2010; see Chapter 3, section 3.5) have to be considered as well. However, continuous exposure of larvae of red sea bream (Pagarus major) to benzo-a-pyrene showed a continuous increase in the levels of CYP1A expression from 24h to 120h post hatch at all exposure concentrations (0.1, 0.5 and 1 µg L^{-1}) (Wang *et al*., 2009). This indicates that continuous exposure of young fish larvae has the potential to lead to greater relative expression levels of CYP1A over time. Even though the underling processes remain unclear, plastic promoted growth combined with lower energy accumulation levels due to ingested plastic, combined with the elevated energetic costs of upregulated, CYP1A mediated, detoxification processes may lead to the rapid exhaustion of the larvae´s limited energy reserves.

Indeed, signs for the depletion of energy reserves were observed for all experiments, indicated by a dose dependent reduction in condition index K. The condition index K ($K = 100*(W/L^b)$) (see Chapter 2, section 2.3.1) as calculated after Fulton (Ricker, 1975) provides a useful and one of the most widely used tools to evaluate a weight-length ratio based condition in fish (Jones *et al.*, 1999). However, when calculating the condition index K, caution has to be taken since the assumption of isometric growth of the organism is of importance because length is raised to the 3rd power (b = 3) (Stevenson and Woods, 2006). Since fish and especially larvae and juveniles, show allometric growth (Osse *et al.*, 1997), calculated condition indices after Fulton should be assessed with care; If b, deviates from this assumption ($b \neq 3$) results for K might not be reliable. For this study, a length-weight regression found that exposed larvae showed a positive allometric growth ($b > 3$). For values of $b > 3$, there is a significant positive relationship

between K and fish length, indicating that K will increase with increasing length (Anderson and Gutreuter, 1983) (b < 3 results in a decrease in K with increasing length (Cone, 1989)). The calculated reduced condition index K might therefore underestimate the actual loss in condition of exposed larvae. However, the decline in condition which could be observed at concentrations of 5.3 µg L^{-1} (1 µm spheres) and 53 µg L^{-1} (9.9 µm spheres) is consistent with other studies that found a negative effect on condition or energy reserves due to plastic exposure (Wright *et al.*, 2013, Ryan, 1988, Spear *et al.*, 1995, Besseling *et al.*, 2013, Oliveira *et al.*, 2013).

Whereas the preliminary study showed an increase in wet weight for the Medium and High treatments, both main experiments did not affect the wet weight of exposed larvae. The increased weight found in the preliminary study is thought to be caused by the presence of high numbers of ingested plastic particles. This is supported by the dose dependent ingestion of plastic spheres from the water column which then resulted in a dose dependent increase in weight for larvae from the preliminary experiment. The lower concentrations used in the main experiments are believed to have resulted in an overall lower number of ingested plastic spheres which did not result in weight affecting numbers of ingested plastic spheres in the gastrointestinal tract.

For the measured biomarkers length and condition index K, the smaller sized plastic spheres (1 µm) showed to have a higher potency to cause more severe effects compared to the larger plastic spheres (9.9 µm). This effect might be explained by the leaching of plastic derived chemicals which has been demonstrated to take place over the plastic´s surface and is known to slow down once chemical compounds have to migrate from the inner polymer structure to the surface (Teuten *et al.*, 2009). Since the same volume of plastic spheres was used to create treatments for both experiments, fish from experiment 1 were exposed to higher numbers of smaller spheres compared to experiment 2. Hence, the higher combined surface area/volume ratio of the smaller particles may have promoted the leaching of elevated levels of chemicals from the polymer structure compared to larger particles. This hypothesis could be tested by a leaching experiment where an equal mass of the 1 µm or 9.9 µm spheres are exposed to physiological conditions in the GI of fish. Extracts from the leachates could be then analysed via GC-MS to quantify the amount of leached chemicals.

In conclusion, these data indicate that exposure of young fish larvae to a water column contaminated with small plastic particles has the potential to induce growth in length through unknown chemical processes. Further experiments are needed to provide insight into the underlying processes of this growth induction. Analysis of insulin like growth factors could provide valuable information for GH related growth processes induced by plastic exposure. Additionally, different plastic types could be used to determine if the effects on growth were related to plastic derived chemicals specific to polystyrene. However, simultaneous to the induced growth, plastic ingestion was found to lead to a reduced condition index, suggesting reduced energy reserves. Such effects on energy reserves are critical for fish larvae since they could lead to energy depletion in the relative short term due to low energy reserves of this young life stage. Depleted energy reserves could fail to support the here detected, CYP1A mediated, elevated detoxification processes, leading to toxic effects of plastic derived and environmental xenobiotics.

Chapter 5

Comparison of the biological activity of artificially weathered and non-weathered plastic bag fragments in three-spined stickleback (*Gasterosteus aculeatus*) exposed via their diet

The artificial weathering of the plastic bags used for this study was done by Dr Zhongyi Zhang (School of Engineering, University of Portsmouth).

5.1 Abstract

In Europe, more than 98 billion single-use plastic bags are used annually from which an estimated 4.5 billion end up in the environment. During their lifetime in the environment plastic bags degrade through chemical and mechanical factors like photodegradation and abrasion, leading to micro sized fragments which become available for ingestion by organisms of many trophic levels. Even though, the ingestion of plastic by marine biota is widely reported, the biological activity of ingested degraded and non-degraded plastics remains widely unclear. In this study male three-spined sticklebacks were exposed for 28 days, via their diet, to degraded or non-degraded micro sized fragments of two types of plastic bags. The contaminated diet was created by spiking blood worms with 0.5% of weathered or non-weathered fragments of the respective plastic bag type. Nile red staining and fluorescent microscopy of faeces samples confirmed the daily egestion (and therefore ingestion) of the plastics for all plastic treatments. No plastics were observed in the faeces of fish fed blood worms that had not been spiked with plastics (controls). Analysis of presence of digesting food in the gastrointestinal tract during gastrointestinal dissections provided evidence for a prolonged gastric evacuation time of plastic treatments due to higher retention of digesting food matter, when compared to control fish. Fish from one degraded plastic treatment were found with an elevated liver weight (p = 0.032) and fish from both degraded treatments were found with higher wet (p = 0.021) and gutted weights (p = 0.022) when compared to the control. No mortalities were observed over the duration of the experiment and no effects on length (p = 0.153) and condition index K (p = 0.09) were found, indicating limited effects of exposure in the short-term. Additionally, the release of plastic derived chemicals under simulated stomach pH conditions was detected via GC-MS. These analysis indicated that a total of 28 chemicals leached from the plastic bag fragments, whereas four of these chemicals might have been endocrine disrupting xenoestrogens. Collectively, these results provide evidence that ingested, weathered plastic fragments have a higher potency to induce biological effects and results suggest that these effects might be due to the leaching of plastic derived chemicals, possibly affecting energy metabolism and lead to increased liver weight.

5.2 Introduction

Packaging (i.e. plastic bags) is the largest sector for plastic appliances in Europe (PlasticsEurope, 14/15), accounting for nearly 40% of the total annual plastic demand in Europe (46.3 million tonnes in 2013). Numbers of consumed single-use plastic bags made from high density polyethylene (HDPE) was close to 88 billion bags in 2010 (BIOIntelligenceService, 2011). Due to their light weight and low price single use plastic bags are disposed of carelessly or can get blown away from dumping sites and it was estimated that 4.5 billion single-use plastic bags ended up in the environment as litter in 2010 (BIOIntelligenceService, 2011). As a result, plastic bags were amongst the three most abundant debris classes found on beaches worldwide in 2010 (BIOIntelligenceService, 2011).

During its life in the environment plastic bags, like other plastic waste, break down into smaller fragments by degradation, leading to micro and nano sized particles (Andrady, 2011). The process of degradation can be driven by many factors (Singh and Sharma, 2008) varying strongly between the location and their environmental factors such as heat, UV exposure and mechanical stress (Andrady, 1990). In the marine environment, degradation of plastic debris on beaches is believed to show the most accelerated weathering (Pegram and Andrady, 1989) due to the exposure to high temperatures (Shaw and Day, 1994) and high UV radiation (Andrady, 2011). Even though this process can take centuries for some types of plastics to be completely decomposed (Derraik, 2002), plastic bags can take as little as 10 to 30 years to be broken down significantly in the water column (BIOIntelligenceService, 2011), indicating their rapid degradation in the environment. The resulting, small fragments have the potential to interact with aquatic life; Ingestion of small plastic items has been reported for a wide range of species including zooplankton (Cole *et al.*, 2013), mussels (Browne *et al.*, 2008), worms (Besseling *et al.*, 2013), fish (Foekema *et al.*, 2013, Lusher *et al.*, 2013) and birds (Verlis *et al.*, 2013). However, ingestion of degraded plastic fragments is of concern since cracks in the polymer structure as outcome of the degradation process do not only result in embrittlement and smaller fragments but has also the potential to lead to additional leaching of additives due to a higher surface area (Teuten *et al.*, 2007).

Whereas the leaching of incorporated chemicals from the centre of the polymer slows down over time as the material reaches its glass transition state (Ejlertsson *et al.*, 2003),

damaged polymer structures do not only have an increased surface area over which chemicals can be leached but also enable chemicals from the centre of the polymer to leach from the matrix over newly exposed surfaces (Teuten *et al.*, 2009). Accordingly, an exposure to degraded plastic may cause different/additional effects, compared to its non-degraded state. Many plastic polymers are known to have high concentrations of bioactive monomer additives incorporated into the plastics matrix to change its characteristics (Moore, 2008). The composition and quantities of these additives like plasticisers, UV stabiliser, phthalates to soften polymer structures and also flame retardants and colorants depends on the purpose of the final product. It is estimated that plastic fillers, reinforcements and additives can account for 50% to 67% of the plastic product by weight, depending on the plastic type (Colton *et al.*, 1974, Giam *et al.*, 1984). Even though data for aquatic environments is rare, the potential of degrading plastics to leach plasticisers and additives has been described for landfill models (Bauer and Herrmann, 1997, Teuten *et al.*, 2009), indicating the risk of such chemicals to enter the environment. Incorporated, hydrophobic chemical compounds are less likely to leach from the polymer structure, whereas more hydrophilic compounds like phenols can be more easily released to aquatic environments (Teuten *et al.*, 2009). Bisphenol A for example has been found to leach from plastics to water in quantities of up to 139 μg g^{-1} plastic (Yamamoto and Yasuhara, 1999). The leaching of plastic derived chemicals is of concern since they can have adverse effects and many phenolic and alkylphenolic compounds like bisphenol A and nonylphenol on aquatic life are described as xenoestrogenic due to their potential to act as endocrine disruptors in aquatic organisms (Kwak *et al.*, 2001, Oehlmann *et al.*, 2009).

The aim of this chapter was to investigate the potential health effects arising from ingested microplastic sized fragments of weathered and non-weathered, conventional HDPE single use plastic bags from two major UK food retailers. Adult three-spined sticklebacks were exposed to plastic contaminated diets for 28 days during which faeces samples were taken to monitor egestion and therefore ingestion of plastics. To evaluate the effects of ingested artificially weathered and non-weathered plastic bag fragments the biological endpoints length, wet weight and gutted weight, liver weight, condition index K and hepatosomatic index were compared. Additionally, gastrointestinal tracts (Oesophagus, stomach, intestine; GI) were dissected to assess plastic ingestion as well as size and number of up taken particles and determine the potential risk for accumulation or blockage of the GI by plastic particles. Finally, an extraction of

chemicals from plastic fragments was performed, followed by GC-MS analysis to identify leached chemicals from the plastic bags under artificial stomach pH conditions. Results were used to determine possible exposure to plastic derived chemicals.

5.3 Material and methods

Since the weathering of plastic items under the influence of UV exposure leads to the degradation and embrittlement of synthetic polymers (Decker, 1984, Andrady *et al.*, 1998) (see Chapter 1, section 1.2.3), weathered plastic used for the experiment will be referred to as degraded from hereon.

5.3.1 Test organisms

The three-spined sticklebacks used in this study were approximately 14 months old at the start of the experiment and maintained as described in Chapter 2, section 2.1.1.2. Fish were selected for male fish which was confirmed histologically (see sections 5.3.6.4 and 5.4.1). The initial live wet weight for fish from each replicate was determined at day -4 of the study (Control = 691 ± 43 mg (n = 12), AnD = 642 ± 45 mg (n = 12), AD= 637 ± 42 mg (n = 12), BD= 670 ± 3 mg (n = 12) and BnD= 668 ± 8 mg (n = 12)). For the definition of treatment abbreviations see section 5.3.3.1. No differences were found for the day -4 live wet weight between any exposure tank ($F_{9,51}$ = 0.422, p = 0.788).

5.3.2 Water supply and test apparatus

See Chapter 2, section 2.1.1.3 for details of the water supply and test apparatus. Additionally, to the material and methods described in Chapter 2, section 2.1.1.3, fish were transferred to new exposure tanks with clean artificial sea water (ASW) on day 13 of the experiment as part of a water change. Water temperatures were monitored daily throughout the experiment and ranged between 16.8 and 19.2°C, while pH levels were checked twice weekly and ranged between 8.07 and 8.72. Dissolved oxygen concentrations were checked twice weekly and remained over 70% of the air saturation value for the experiment.

5.3.3 Preparation of the contaminated diets

5.3.3.1 Preparation of plastic fragments

Four different plastic treatments originating from two plastic bag types of two major UK food retailers were prepared prior to the experiment. Plastic bag origin will be referred to type A for one retailer and type B for the other: Degraded (D) plastic bags that had been exposed to 500 hours of UV light in an accelerated weathering station and non-degraded (nD) plastic bags (no UV exposure) were finely chopped up using a microtome blade to approximately 2 mm long and 1 mm wide fragments. Mean measurements for the fragments were AD = 1.6 ± 0.3 mm (n = 30), BD = 1.6 ± 0.3 mm (n = 30), AnD = 2.1 ± 0.2 mm (n = 30), BnD = 2.6 ± 0.5 mm (n = 30). The degraded plastics were found to be more brittle than the non-degraded plastics resulting in smaller fragments and examination via microscopy, revealed the presence of very small plastic particles (< 0.1 mm) that derived from the chopping process.

5.3.3.2 Preparation of the fish´s diet

Since larger (> 0.1 mm) and smaller (< 0.1 mm) plastic pieces could not be separated, the chopped up plastics including the small fragments were mixed with 50% blended and 50% non-blended, defrosted and drained blood worms (Tropical Marine Centre, UK) at a concentration of 5 mg of plastic per 1 g of food in a ceramic dish to create a paste. The resulting blood worm plastic paste was then filled into plastic straws using a 2 ml syringe without needle, re-frozen at -20°C and kept under these conditions until they were used as food (Figure 5.1).

One millimetre of the contaminated frozen fish food from each treatment was taken as a subsample to test for presence of plastic fragments. The samples were placed on microscope slides and thawed before they were stained with a nile red (9-diethylamino-5H-benzo [a] phenoxazine-5–one), Arcos Organics) solution and analysed for plastic content under a fluorescent microscope at 20x magnification. Nile red staining was performed using a 0.01 g ml^{-1} working solution of nile red, dissolved in 100% ethanol. Food samples were stained by adding 200 µl of working solution on the defrosted sample before it was squashed with a cover slip. After 5 min the slide was examined with a Zeiss LUMAR.V12 stereo microscope with an AxioCam MRm camera and AxioVision software (ZEISS, Germany) and a Green Fluorescent Protein (GFP) filter (excitation 485 nm; emission 520 nm) at 20x magnification. Nile red has been previously applied to visualise small plastic particles with the help of microscopes (Andrady, 2011).

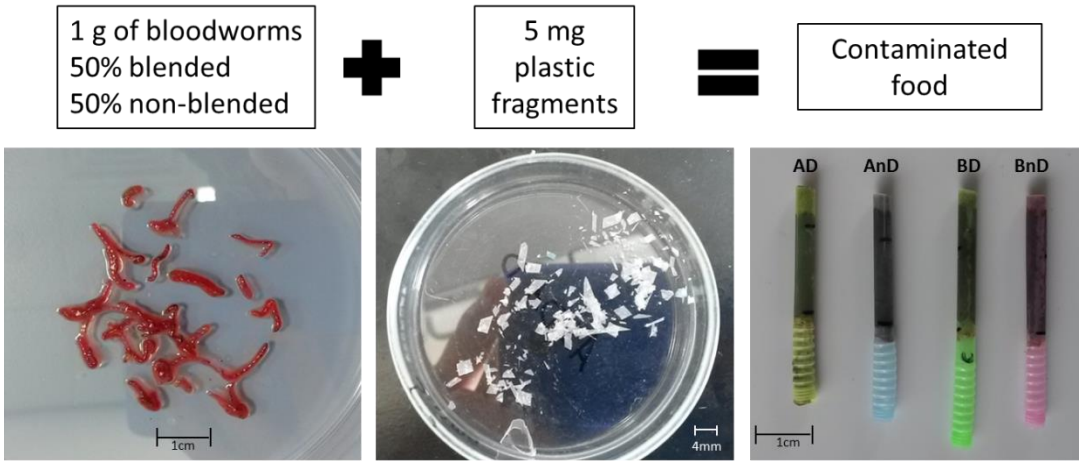

Figure 5.1: Preparation of the contaminated food for the dietary exposure using degraded (D) and non-degraded (nD) fragments of two plastic bag types (A and B). Fifty percent of blood worms were blended and 50% were kept non-blended to create a paste before the chopped up plastic bag were added at a ratio of 1 g of defrosted blood worms per 5 mg plastic and mixed well. The resulting plastic/blood worm past was then filled in colour coded plastic straws, frozen and kept at -20°C until needed.

5.3.4 Fish exposure

Adult male sticklebacks were randomly assigned to 10 aerated glass aquaria (n = 6 fish tank^{-1}). Exposure tanks contained 6 L of 3.0 ppt ASW and sticklebacks were acclimated to test conditions for 3 days and fed frozen blood worms three times daily during this period. The five treatment groups were set up in duplicates and were defined as; One control (C) treatment which was fed uncontaminated, frozen bloodworm pellets. Two degraded (D) treatments which were fed frozen blood worm pellets, contaminated with degraded states of plastic bag types A or B, referred to as AD and BD from hereon. Two non-degraded (nD) treatments which were fed frozen bloodworm pellets, contaminated with non-degraded states of plastic bag types A and B, referred to as AnD and BnD from heron. Fish were fed three times daily with 0.22 g (frozen weight) (approximatively 5% of body mass) of the artificial diet per feed (0.66 g per day) for a total of 28 days. Throughout the exposure period daily faeces samples were removed from the exposure tanks to assess evidence for the presence and evaluate numbers of egested plastic particles.

5.3.5 GC-MS analysis

GC-MS analysis of plastic samples was conducted to identify types of leaching chemicals from the chopped up degraded (D) and non-degraded (nD) plastic bags. Prior to the analysis all glassware was solvent cleaned by rinsing it first with methanol (Fisher Scientific) for three times and then *n*-hexane (Fisher Scientific) for three times. The pH conditions in the fish's stomach (pH conditions in the stomach can range from 2.5 to 5 in the three-spined stickleback (Hale, 1965)) were recreated via an acidic medium which was prepared by gradually adding hydrochloric acid (Fisher Scientific) to reverse osmosis water. This approach was set up to simulate stomach pH during the digestion process of the diet. Chopped up degraded or non-degraded plastic (100 mg) was then added to 200 ml of this artificial medium (pH 2.6), in a 300 ml Erlenmeyer flask (Fisher brand) and sealed with aluminium foil. One flask was used per treatment (C, AD, AnD, BD, BnD). Flasks were then placed on a shaker and shook at 110 rpm for 48h in the dark at 18°C. After 48h chemicals were extracted from the media by adding 5 ml of *n*-hexane, shaking and pipetting out the separating hexane phase in to a clean 25 ml glass vials, using glass pipettes, sealed with aluminium foil and closed with a screw cap. Three millilitres of the recovered hexane were evaporated to near dryness under a

nitrogen flow and reconstituted in *n*-hexane (1 ml) before being transferred to amber GC-MS glass vials. An Agilent GC-MS (6890N GC) equipped with split/splitless injector, fitted with a HP-5MS UI capillary column (30 m long, 0.25 mm i.d. x 0.25 μm film thickness) and connected to a mass selective detector (Agilent 5975) was used in scan mode to scan the extract. Samples were injected (2 μl) in the splitless mode at an injection temperature of 290°C. The column oven was initially held at 50°C for 3.2 min, before increasing the temperature in a series of steps (150°C at 30°C min^{-1}; 238°C at 2°C min^{-1}; 272°C at 3 C min^{-1}; to 300°C at 70°C min^{-1} to 300°C where it was held for 2.73 min). Helium was used as a carrier gas at a constant flow rate of 1 ml min^{-1}. Identification and analysis of leached chemicals was carried out by individual retention times of the chemical compounds which were compared with the internal library and mass spectra acquired using electron ionization (EI) at 70 eV.

5.3.6 Fish sampling and analysis

After 4 weeks fish were sacrificed as described in Chapter 2, section 2.3. Liver, gastrointestinal tract and gonads were sampled from each fish. Whereas the liver was stored in RNAlater, the gastrointestinal tract and the gonads were fixed in 10% buffered formalin. Wet and gutted weight, liver weight and weight of the gastrointestinal tract were weighed to the nearest 0.01 mg and total and standard length measured to the nearest 1 mm.

5.3.6.1 Gastrointestinal (GI) examinations

To assess if the ingestion of the contaminated diets had any effects on food digestion, pictures were taken using a stereo microscope (Leica S6D) with attached camera (JVC KY-F1030U) using imaging software (KY link) to record the appearance of the GI sample and to assess accumulation of processed food prior to the dissection of the GI tracts. Accumulation of processed food was differentiated, depending on the location of food matter present within the GI which was divided into oesophagus, stomach and intestine. A scoring system was applied where presence of processed food in a compartment was scored with 1, whereas absence of food was scored with 0. Accordingly, a maximum score of 3 could be achieved if processed food was present in each compartment of the GI (Oesophagus, stomach and intestine). The values from the scoring system were used for statistical analysis.

5.3.6.2 Gastrointestinal dissections

Dissections were performed to assess presence, quantities and size of ingested plastic fragments in the GI (Figure 5.2). After fixing in 10% buffered formalin, GI tracts were transferred to 70% ethanol (Fisher Scientific, analytical grade) 24h prior to examination. GI tracts were removed from the glass vials and placed in glass petri dishes for further procedures. Sample length (Oesophagus + stomach + intestine) and separated stomach length were determined to the nearest mm. Oesophagus, stomach and intestine were separated using a microtome blade (Thermo Scientific MB35 Premier) and examined separately. Microtome blades were used to carefully open up the compartments of the GI tract and the content examined for plastic; Plastic found was cleared from processed food if necessary, counted and measured to the nearest 0.1 mm under a dissecting microscope (Leica Zoom 2000) at 10.5x magnification before being weighed to the nearest 0.01 mg using a fine balance (Fisherbrand PS-100). Additionally, the stomach walls were carefully scraped using a small spatula to analyse it for microscopic plastic particles. The scraped off matter was transferred to a microscope slide (Thermo Scientific), stained with nile red (Arcos Organics), covered with a 22x50 cover glass (Thermo Scientific) and stored in the dark until examination with a Zeiss LUMAR.V12 stereo microscope with an AxioCam MRm camera and AxioVision software at 80x magnification.

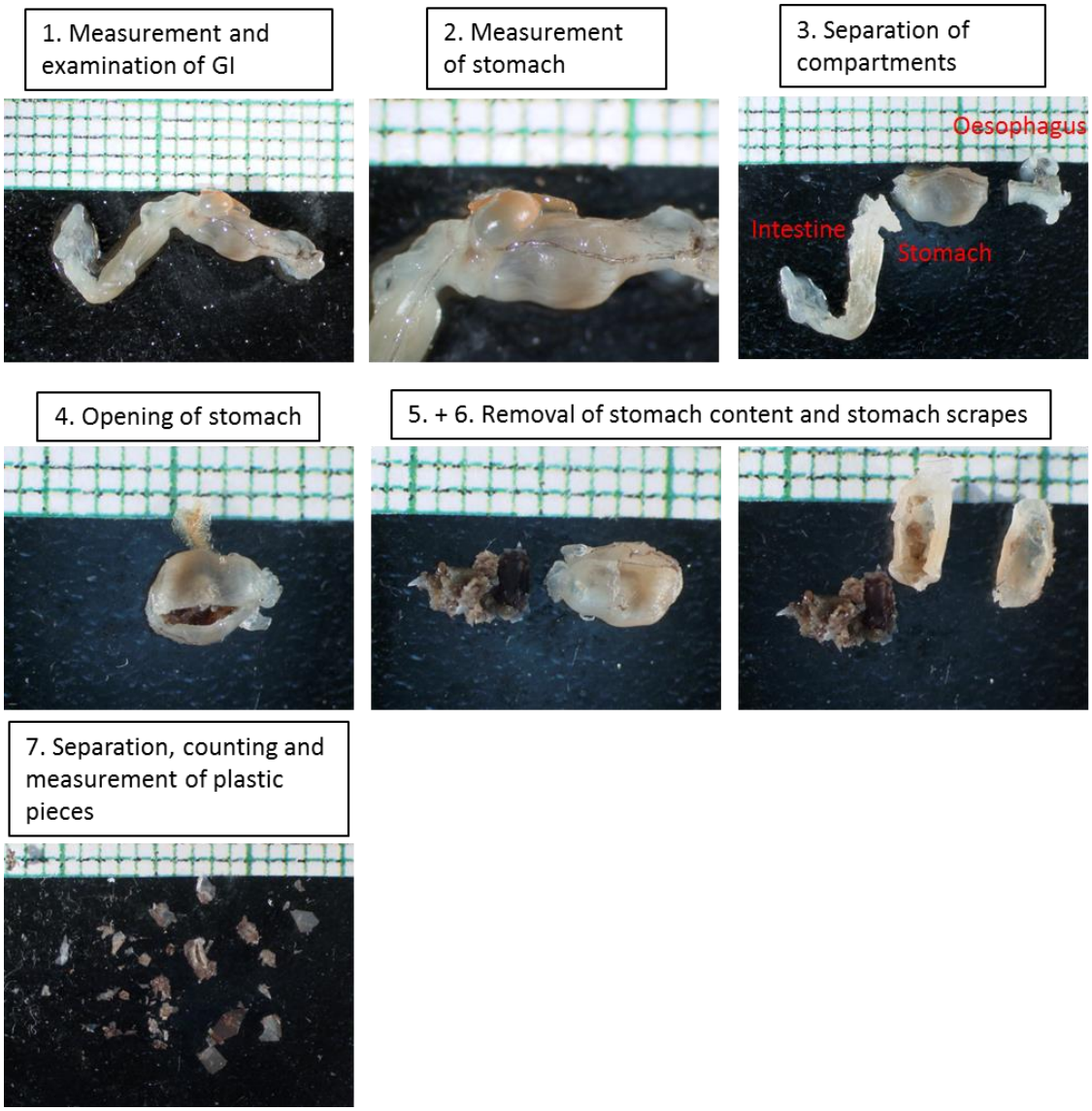

Figure 5.2: Dissection of gastrointestinal tracts (GI) of sampled fish after 28 days of exposure to a plastic contaminated diet. The figure displays an example of a step by step procedure of the GI dissection and content analysis. All procedures were performed under a dissecting microscope at 10.5x magnification, using graph paper to determine the size of plastic particles to the nearest 0.1 mm. After the initial measurements (1 and 2) the GI was separated into the single compartments (Oesophagus, stomach and intestine) which were opened and assessed for plastic content separately. Additionally, stomach scrapes were performed to assess presence of microscopic plastic particles.

5.3.6.3 Analysis of faecal matter

Daily taken faeces samples were pooled per tank, since their origin could not be determined to a specific fish. Faeces pellets were examined for the presence of egested plastics, and the number and size of egested plastics determined. Faeces samples were stored in clear, flat bottom 96-well plates (Costar) before they were transferred on to microscope slides (Thermo Scientific) and stained with 200 µl of nile red solution. Stained faeces samples were kept in the dark for less than 24h until further examination using a Zeiss LUMAR.V12 stereo microscope with an AxioCam MRm camera and AxioVision software using a Green Fluorescent Protein (GFP) filter (excitation 485 nm; emission 520 nm) at x20 magnification (Figure 5.3). All plastic particles were counted and divided into two categories (big > 1 mm or small < 1 mm).

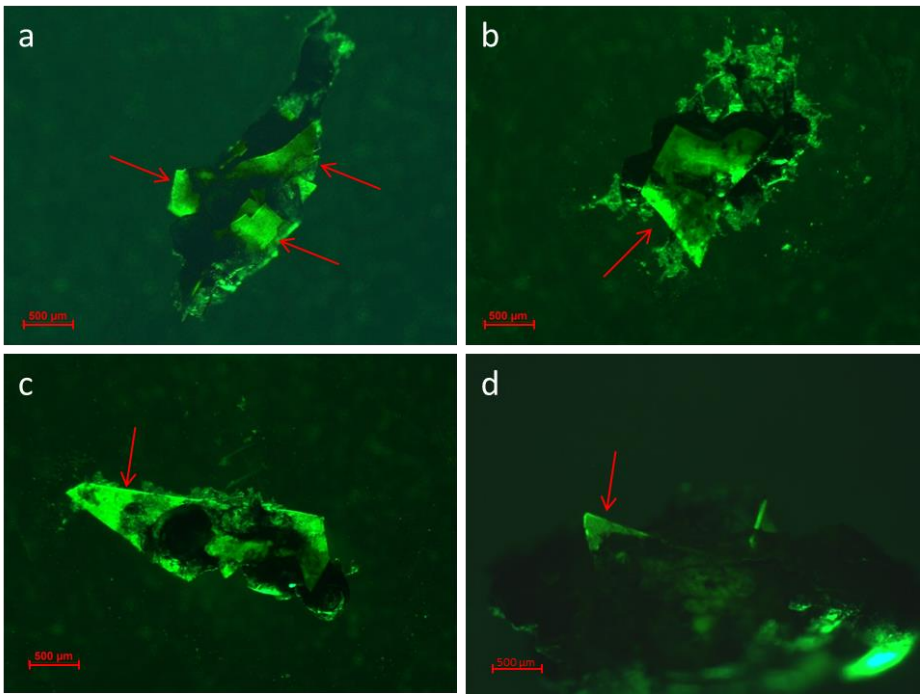

Figure 5.3: Example pictures of nile red stained faeces samples that were collected from a = Type A degraded, b = Type B degraded, c = Type A non-degraded and d = Type B non-degraded treatment exposure tank during the 28 day exposure. Faeces pellets were stained with a nile red solution (0.01 g ml^{-1}) prior to analysis under a Zeiss LUMAR.V12 stereo microscope with attached AxioCam MRm camera and AxioVision software using a Green Fluorescent Protein (GFP) filter (excitation 485 nm; emission 520 nm) and x50 magnification. Dark matter represents unstained faeces composed of digested blood worms. The red arrows show the green fluorescing plastic fragments.

5.3.6.4 Gonadal sex determination

For histological methods of the gonadal sex determination see Chapter 2, section 2.3.3. Just male fish were used for further analysis of endpoints to measure a sex specific response towards the exposure, since plastic can leach additives that can induce oestrogenic effects (Soto *et al.*, 1991, Sonnenschein and Soto, 1998) but also plasticisers which can reduce testosterone levels (Foster, 2006). Accordingly, exposure to plastic fragments might induce sex specific responses due to endocrine disruption of sexual hormone levels.

5.3.7 Statistical analysis

All statistical analysis were carried out as detailed in Chapter 2, section 2.3.5.

5.4 Results

5.4.1 Mortality, behaviour and sex determination

There was no evidence that ingestion of the plastic contaminated food negatively impacted the health of the fish; survival was 100% and fish from all treatments fed actively throughout the experiment and did not display any obvious signs of stress (body colour and general activity levels were comparable to the control fish).

Histological analysis revealed that 12 of the 60 fish were female; data from these females were excluded from further analysis, leaving final sample sizes of 8, 12, 9, 10 and 9 fish group^{-1} for the C, AnD, AD, BnD and BD treatments, respectively.

5.4.2 Presence of plastic particles in the GI

Consistent with expectation no plastic was found in the GI tract of fish from the control treatment (n = 8). There were also no plastics found in the oesophagus of fish from any of the plastic treatment groups (n = 40). However, plastics were observed in the remainder of the GI tract for all fish fed the AnD and BD contaminated diets and for 89% of fish fed the AD contaminated diets and 70% of fish fed the BnD contaminated diets. For those fish found to have plastics in their GI tracts, further analysis revealed that the plastics were only present in the stomach compartment of the GI tract for 58%,

50%, 43% and 78% of the fish fed the AnD, AD, BnD and BD contaminated food, respectively. Plastics could be found in both the stomach and intestine of the GI tract for 25%, 38%, 57% and 22% of the fish fed the AnD, AD, BnD and BD contaminated food, respectively. In 17% of the fish fed the AnD contaminated food and 12% of those fed the AD contaminated food plastics were only found in the intestine.

It was observed that the fish fed the non-degraded plastics (AnD and BnD) had lower numbers (46.8 ± 9.9 and 15.2 ± 6.1 pieces of plastic, respectively) of plastic fragments within their GI tract than those fed the weathered plastics (AD and BD; 72.7 ± 18.4 and 79.2 ± 22.1 pieces, respectively; $H(3, N = 34) = 8.886$, $p = 0.031$). However, the fragments of non-weathered plastics (AnD and BnD) were observed to be larger (2.2 ± 0.18 mm and 2.8 ± 0.17 mm, respectively) when compared to those that had been weathered (AD and BD; 0.8 ± 0.2 mm and 1.1 ± 0.0 mm, respectively; $H(1, N = 1935) = 4886.719$, $p < 0.001$; Figure 5.4). When the mean total surface area of the plastics recovered from the GI tracts was compared there was no evidence for differences between the non-degraded ($F_{1,17} = 0.220$, $p = 0.645$) and degraded ($F_{1,15} = 0.550$, $p = 0.470$) plastics of Type A and B, indicating that they were exposed to comparable quantities of plastic (Table 5.1). Similarly, there were no differences in weight between the recovered non-weathered and weathered plastics for the Type A ($H(1, N = 19) = 1.542$, $p = 0.214$) and Type B ($F_{1,15} = 0.222$, $p = 0.645$) bags (Table 5.1).

For most fish, the plastics found in the GI tracts were embedded in the processed food, but in 17, 30 and 22% of the fish exposed to the AnD, BnD and BD contaminated diets plastics could be found in the GI tract in the absence of processed food. Higher numbers of plastic fragments could be found when processed food was present (AnD = 33.7 ± 7.9, AD = 62.2 ± 19.7, BnD = 11.9 ± 5.2 and BD = 70.3 ± 21.0 items fish^{-1} (n = 33)), whereas fish that had already egested processed food from the GI were found with low numbers of plastic particles (2.7 ± 1.1 items fish^{-1} (n = 7)).

When comparing the average size of plastic fragments used for the preparation of the contaminated diets (AD = 1.6 ± 0.3 mm (n = 30), BD = 1.6 ± 0.3 mm (n = 30), AnD = 2.1 ± 0.2 mm (n = 30), BnD = 2.6 ± 0.5 mm (n = 30)) with those of plastic fragments found during the GI dissections (AD: 0.8 ± 0.0 mm (n = 658), BD: 1.1 ± 0.0 mm (n = 712), AnD: 2.1 ± 0.1 mm (n = 461), BnD: 2.8 ± 0.2 mm (n = 105)) it was observed that the mean particle size of degraded fragments had reduced more than those of non-

degraded treatments. This observation was significant for the AD treatment ($H(1, N = 688) = 12.758$, $p < 0.001$). This suggest that the degraded plastic bag fragments had broken up into smaller fragments either during food preparation or digestion and provide evidence for degraded polymer structures of artificially weathered plastic fragments.

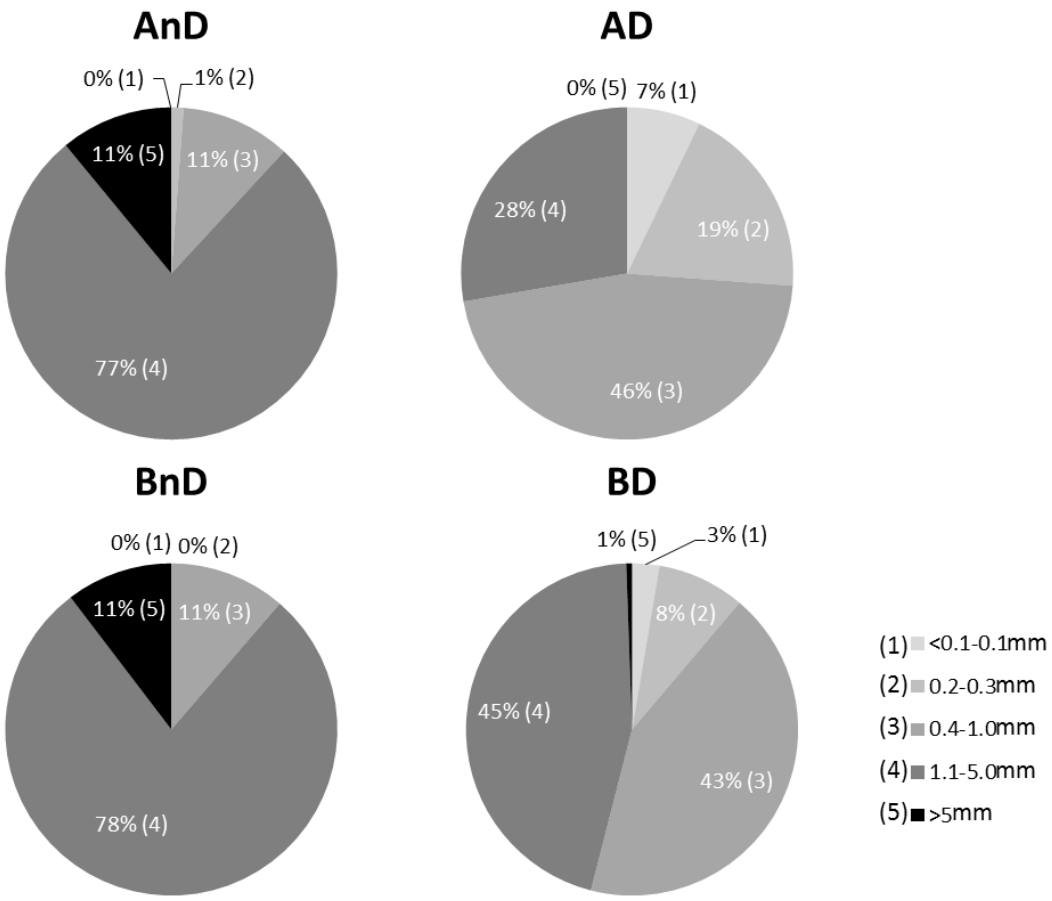

Figure 5.4: Size distribution of plastic particles found in the gastrointestinal (GI) tracts of fish that had been fed diets contaminated with fragments of degraded (D) and non-degraded (nD) HDPE bag types (A and B) for 28 days. All plastic particles were separated, counted and measured to the nearest 0.1 mm and divided into size classes. For each treatment, the pie chart shows the percentage of plastics found for each size class relative to the total number of fragments found in the GI tract.

Table 5.1: Number, size and weight of ingested plastic fragments observed in the gastrointestinal tracts of fish maintained on a non-contaminated diet (control; C), or a diet contaminated with fragments of type A non-degraded (AnD) or degraded (AD) plastic bags, or type B non-degraded (BnD) or degraded (BD) plastic bags. Data displayed in the table is the mean ± SEM. Sto = stomach, Int = intestine. No plastic was found in the oesophagus.

Treatment	No. of plastic pieces Sto	No. of plastic pieces Int	Particle size min. (mm)	Particle size max. (mm)	Total surface area (mm^2)	Plastic weight (mg)
C	0.0 ± 0.0	0.0 ± 0.0	N/A	N/A	N/A	N/A
AnD	33.7 ± 7.9	13.1 ± 8.9	0.7 ± 0.2	4.8 ± 0.5	80.7 ± 16.8	1.6 ± 0.4
AD	62.2 ± 19.7	10.4 ± 8.9	0.2 ± 0.1	1.7 ± 0.3	58.7 ± 16.1	1.3 ± 0.7
BnD	11.9 ± 5.2	3.3 ± 2.2	1.6 ± 0.9	4.1 ± 1.3	46.9 ± 20.2	0.9 ± 0.4
BD	78.1 ± 21.7	1.1 ± 0.7	0.5 ± 0.3	2.8 ± 0.5	90.5 ± 27.4	0.98 ± 0.3

5.4.3 Presence of plastic particles in faecal samples

There was no evidence of plastic contamination within the faeces samples collected from the control treatment over the duration of the experiment. However, 24h after initiating the exposure plastics could be found in the faeces collected from fish fed the degraded plastic contaminated diets. First presence of plastic in faeces samples of non-degraded treatments was found 48h after initiating the exposure (Figure 5.5). After this, presence of plastic in faeces samples was constant for the duration of the experiment for the AnD, AD and BD treatments. Faeces samples from the BnD treatment from day 4 and 5, however, were found with no plastic. It was observed that fish from the degraded plastic treatments (AD and BD) egested higher numbers of smaller (< 1 mm) plastic fragments (169.6 ± 15.2 items sample^{-1} and 108.1 ± 12.4 items sample^{-1}, respectively) compared to non-degraded plastic treatments (AnD and BnD) which egested lower numbers of larger (> 1 mm) plastic fragments (14.4 ± 1.7 items sample^{-1} and 13.8 ± 1.3 items sample^{-1}, respectively; $H(1, N = 232) = 107.485$, $p < 0.001$). These findings are consistent with observations from the GI dissections and preparations of the contaminated diets where degraded plastics were found in higher numbers and smaller sizes compared to the non-degraded plastics. Further investigation into the numbers of egested particles showed that there were no differences between replicates of the same treatment for AnD ($F_{1,52} = 0.100$, $p = 0.922$), AD ($F_{1,56} = 0.118$, $p = 0.733$) and BD ($H(1, N = 58) = 1.007$, $p = 0.316$), except for the BnD treatment which showed a difference in the number of egested particles between replicates ($H(1, N = 58) = 8.000$, $p = 0.005$). Comparison between degraded or non-degraded treatments revealed that fish from the BD treatment egested higher numbers of plastic compared to the AD treatment ($H(1, N = 116) = 11.274$, $p = 0.001$), whereas numbers of egested particles was not different between the AnD and BnD treatments ($H(1, N = 116) = 0.032$, $p = 0.857$). Plastic egestion from fish was not uniform over the duration of the experiment and spikes and drops in numbers of egested plastic particles could be observed (Figure 5.5).

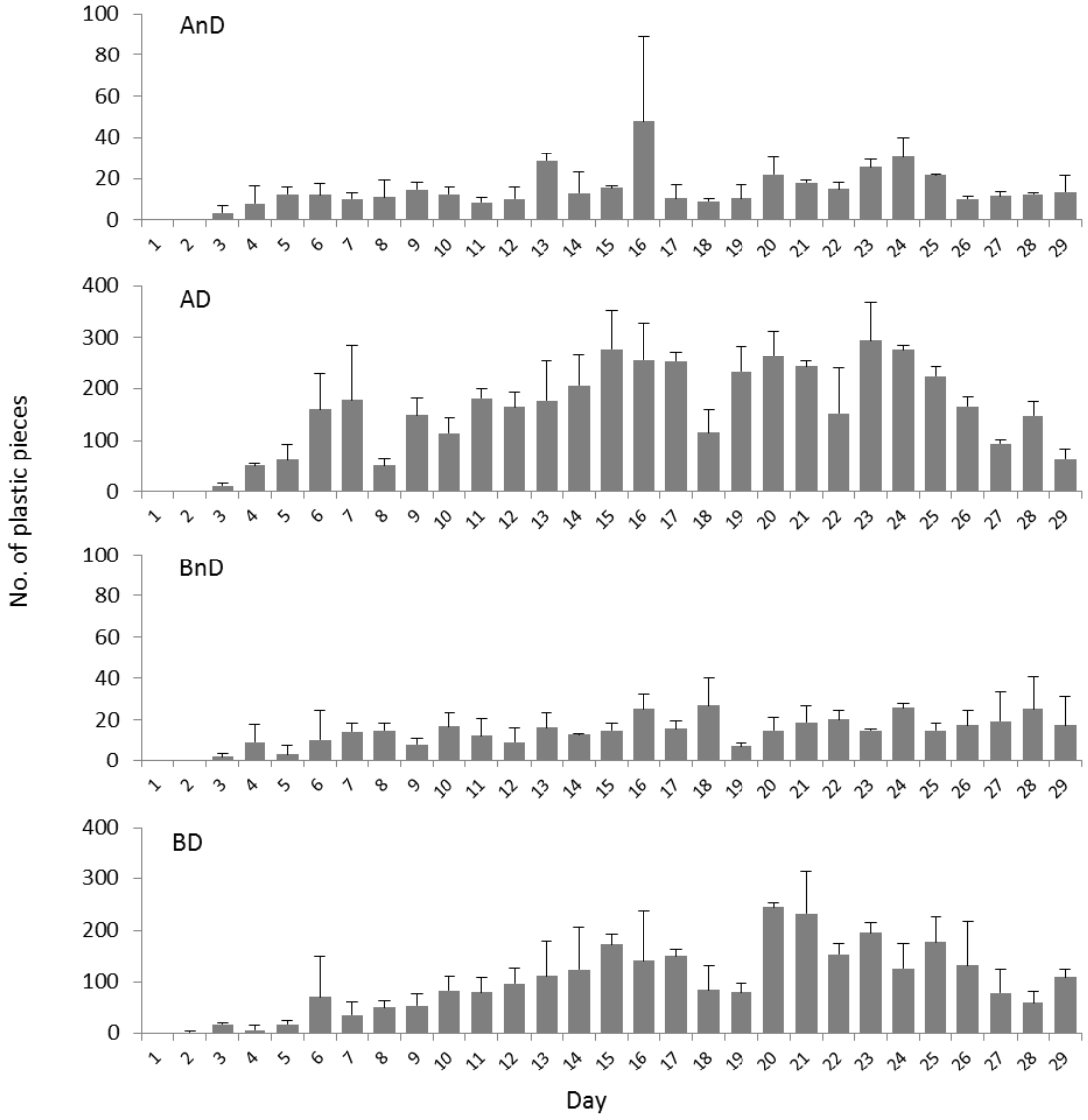

Figure 5.5: Number of plastic fragments present in daily faeces samples collected from the degraded (D) and non-degraded (nD) treatment groups of two carrier bag types A and B. Faeces were also collected daily from the control groups but were confirmed to be free from plastic contamination. Faeces samples were stained with nile red, squashed on a microscope slide and examined under a fluorescent microscope at x50 magnification, using a green fluorescence protein (GFP) filter. Total numbers of plastic pieces was recorded per replicate and the mean calculated. Displayed values are the mean ± SEM. Y-Axis labelling differs between graphs due to the higher numbers of egested plastic particles that were found in faeces of degraded treatments compared to faeces of non-degraded treatments.

5.4.4 Biological effects

No evidence for differences in standard length ($F_{4,43}$ = 1.768, p = 0.153) of the sampled fish was found between all treatment groups (Figure 5.6a). Significant differences in wet weight ($F_{4,43}$ = 3.248, p = 0.021) were observed. A Dunnett`s Post Hoc tests revealed that the degraded treatments (AD and BD) had higher wet weights (p = 0.039 and p = 0.017, respectively) when compared to the control (Figure 5.6b). No differences in wet weight were found for AD or BD when compared to non-degraded treatments (AnD and BnD) with a Tukey Post Hoc test (p = 0.994 and p = 0.369 or p = 0.933 and p = 0.203, respectively). However, a difference in measured GI weight ($F_{4,43}$ = 2.822, p = 0.036) between treatment groups was found, which was due to large accumulations of processed food (see section 5.4.5). Even though a Dunnett´s Post Hoc test revealed that just the AnD treatment had an elevated GI weight (p = 0.012) when compared to the control but not the other treatments (AD: p = 0.108, BnD: p = 0.703 and BD: p = 0.189; Figure 5.8a), the wet weight was not assumed to be a reliable indicator for the fish´s weight. Instead, the gutted weight in combination with the standard length was used to calculate the condition index K and liver weight in combination with gutted weight was used to calculate the hepatosomatic index (HIS; see section 5.4.6). A difference in gutted weight was found between all treatments ($F_{4,43}$ = 3.169, p = 0.023). A Dunnett´s Post Hoc test revealed differences for the AD and BD treatments (p = 0.04 and p = 0.017, respectively) when compared to the control (Figure 5.6c). No differences between AD or BD and non-degraded (AnD and BnD) treatments were found (p = 0.982 and p = 0.885 or p = 0.371 and p = 0.200, respectively) using a Tukey Post Hoc test. Analysis of the calculated condition index K using gutted weight and standard length revealed that there was no difference between all treatment groups ($F_{4,43}$ = 2.157, p = 0.090) (Figure 5.6d).

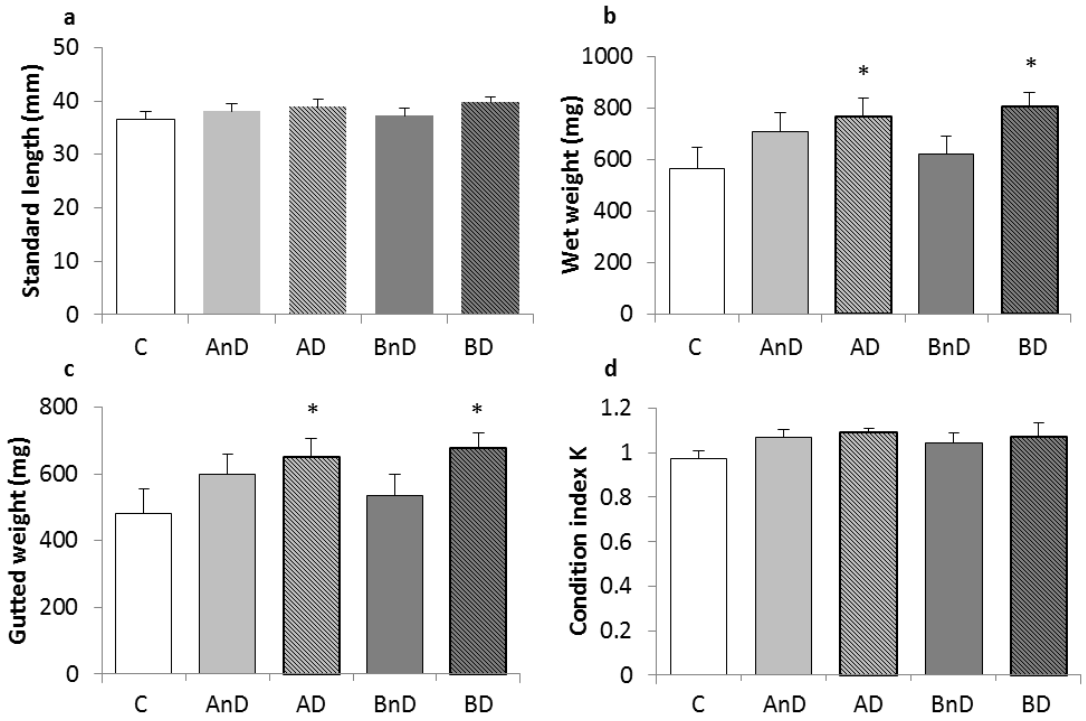

Figure 5.6: Biological effects, measured with standard length (a), wet weight (b), gutted weight (c) and condition index K (d) following a 28 day exposure to diets contaminated with degraded (D) or non-degraded (nD) fragments of two plastic bag types (A and B). The sample size for each treatment for all analysed endpoints was n = 12. No effects were found for standard length and condition index K. Elevated wet weights and gutted weights were observed for the degraded treatments BD (p = 0.017 and p = 0.017, respectively) and AD (p = 0.039 and p = 0.04, respectively). Displayed values are the mean ± SEM.

5.4.5 Retention of processed food

Examination of pictures taken from the GIs before dissection showed that 50% of control fish had processed food still present in the GI. Of these fish 0% had food matter present in the stomach. Evaluating pictures from the non-degraded (AnD and BnD) and degraded (AD and BD) plastic treatments showed that all groups exposed to the contaminated diets had higher amounts of retained processed food (83% and 70%, 89% and 89%, respectively) still present in the GI. Even though an overall difference in retained food in the GI was found between plastic treatments and the control (H(4, N = 48) = 12.324, p = 0.015), pairwise comparisons of treatments showed that not the BnD treatment (H(1, N = 18) = 2.889, p = 0.089) but the other plastic treatments (AnD (H(1, N = 20) = 8.274, p = 0.012), AD (H(1, N = 17) = 7.774, p = 0.011) and BD (H(1, N = 17) = 9.067, p = 0.003); Figure 5.8b) had a higher amount of retained food present in the GI. Of fish that were found with retained processed food in the GI, 100% and 71% of the non-degraded treatments (AnD and BnD) and 88% and 100% of the degraded treatments (AD and BD) were found with retained food present in the stomach. This higher retention of processed food in the stomach of plastic exposed fish was found to be significant for all plastic treatments when compared to the control (AnD (H(1, N = 14) = 13.000, p < 0.001), AD (H(1, N = 11) = 10.000, p = 0.002), BnD (H(1, N = 9) = 8.000, p = 0.005) and BD (H(1, N = 12) = 11.000, p = 0.001). There was no difference in the amount of retained processed food between plastic treatments (H(3, N = 40) = 1.845, p = 0.605). Since GI dissections revealed that most of plastic in the GI was associated with processed food a Pearson´s correlation was performed which showed a positive correlation between the amount of retained food and the total surface area of plastic in the GI (r = 0.623 n = 35, p < 0.001; Figure 5.7).

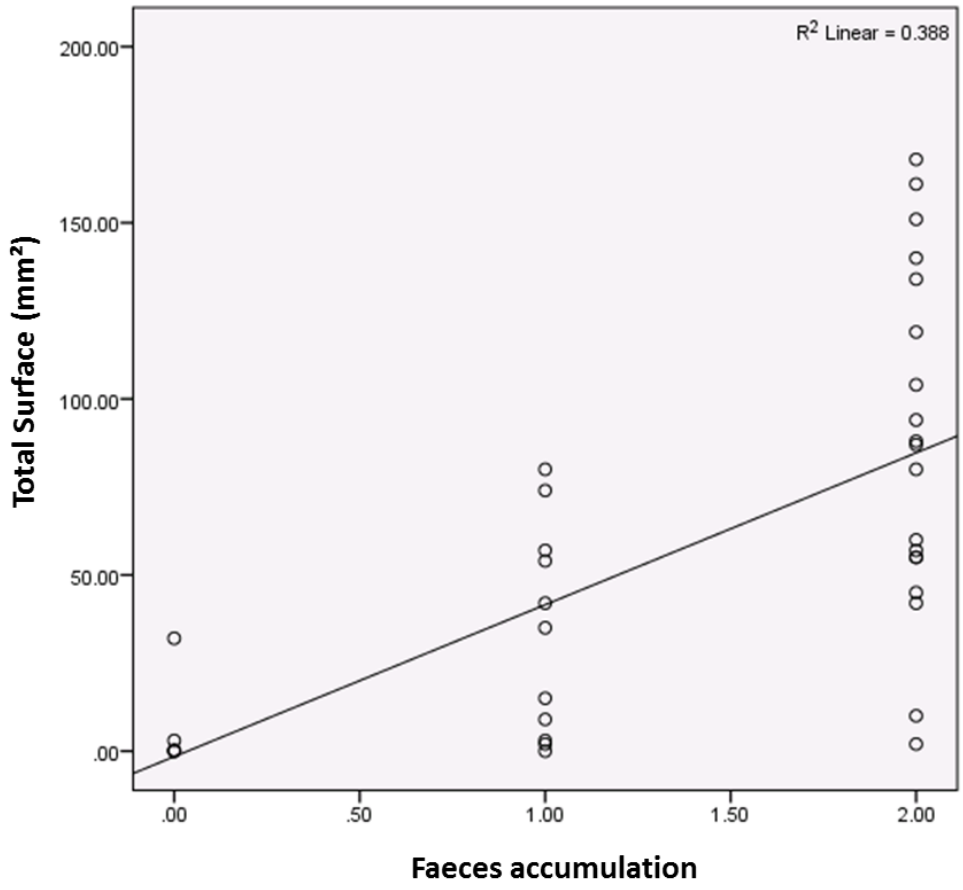

Figure 5.7: Graph for Pearson´s correlation to test for a correlation between total plastic surface area and the amount of retained food in the gastrointestinal tract (GI) of fish that had been exposed for 28 days to a diet that was contaminated with degraded or non-degraded fragments of plastic carrier bags. The values for the faeces accumulation (x-axis) derived from the applied scoring system which rated presence of processed food in a compartment of the GI with 1 point, whereas absence was rated with 0 points. Since no ingested food was found in the oesophagus a maximum score of 2 was achieved (processed food present in stomach and intestine). These values were then correlated with the total surface area of the plastic that was found during GI dissections (y-axis).

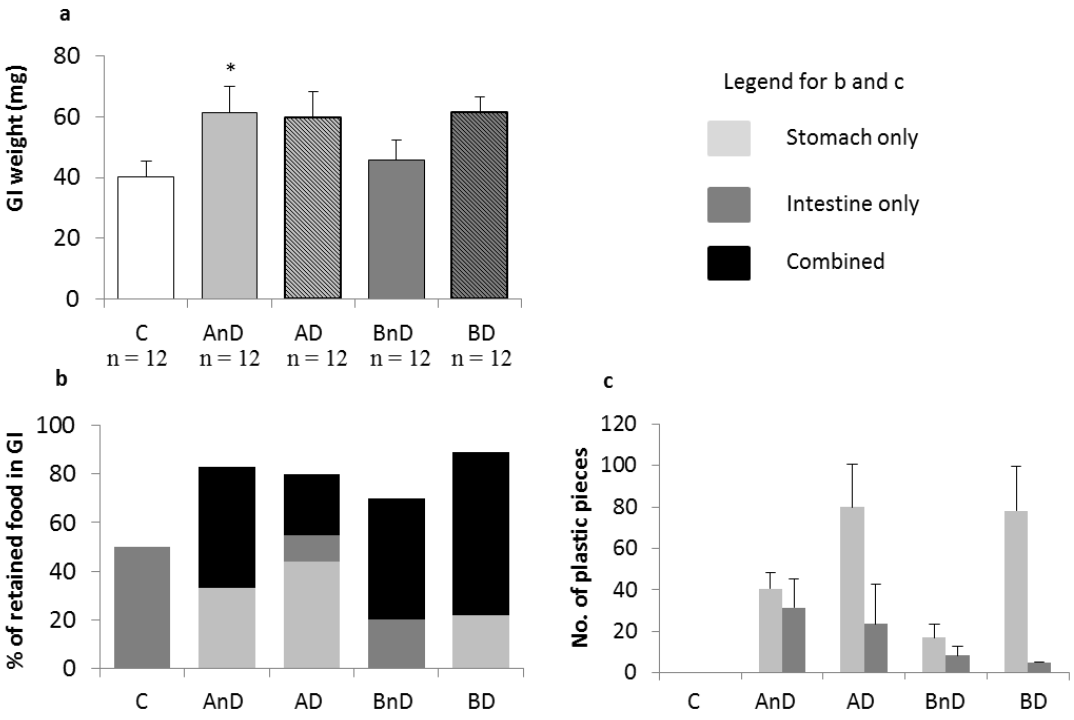

Figure 5.8: Presence of plastic in compartments of the gastrointestinal tract (GI) and effects on food retention and stomach weight in fish samples after 28 days of exposure to a plastic contaminated diet with degraded (D) or non-degraded (nD) fragments of two plastic bag types A and B. a = gastrointestinal weights of sampled fish. The AnD treatment showed an elevated gastrointestinal weight compared to the control treatment (p = 0.012). b = % of retained food found in the compartments of the GI (stomach only, intestine only or in both compartments combined) of sampled fish from each treatment. The non-plastic control fish were the only ones to be found without food matter present in the stomach whereas all plastic treatments were found with food present in the stomach and stomach and intestine combined. c = Number of plastic pieces found in the compartments of the gastrointestinal tract (stomach and intestine) during the dissections. No plastic was found in gastrointestinal tracts of the control treatment and degraded treatments showed a higher number of retained particles compared to non-degraded treatments. Values presented in a and c are the mean ± SEM.

5.4.6 Effects on liver weight

The analysis of measured liver weight revealed a difference between all treatments ($F_{4,43}$ = 2.637, p = 0.047; Figure 5.9a). However, no differences were found for the hepatosomatic index (HSI) ($F_{4,43}$ = 2.283, p = 0.076; Figure 5.9b). Further analysis with a Dunnett´s comparison showed that just the BD treatment had a higher liver weight when compared to the control group (p = 0.032). Additionally, Pearson`s correlations were computed to assess the relationship between liver weight and gutted weight and liver weight and weight of the GI between all treatments. A positive correlation was observed for all treatments between liver weight and gutted weight (r = 0.711, n = 48, p < 0.001; Figure 5.10a).

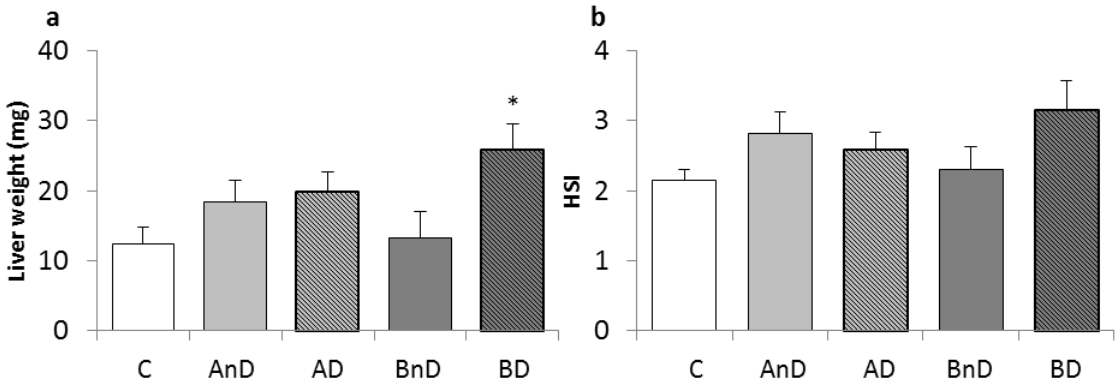

Figure 5.9: Liver weight and hepatosomatic index (HSI) of sampled fish after 28 days of exposure to a diet contaminated with degraded (D) and non-degraded (nD) fragments of two plastic bag types A and B. Sample size for both measured enpoints was n = 12 per treatment. a = Liver weight. Fish exposed to the BD fragments showed elevated liver weights (p = 0.032) when compared to fish fed with uncontaminated diet (control (C)). b = HSI calculated using liver weight and gutted weight ((liver weight (mg)/gutted weight (mg))*100). No differences were found for the HSI between plastic exposed fish and the control treatment. All displayed values are the mean + SEM.

5.4.7 GC-MS analysis

The GC-MS analysis detected a total of 28 different chemical compounds (Table 5.2). The number of chemical compounds leached from the different treatments were AnD = 11, AD = 4, BnD = 12, BD = 10. Since no standards were used to verify potentially detected substances, the presented results give a best fit of analysed molecules with the GC-MS library. Even though peak qualities were high for most of the listed compounds, the presented detected chemicals have to be seen as an indicator for potentially leached chemicals. Nineteen of the 28 compounds were classified by the GC-MS library as hydrocarbons with varying chain lengths. From the remaining 8 compounds, four are suspected to belonged to different chemical groups (4-Hydroxymandelic acid,ethyl ester,di-TMS, Dihydrocoumarin,4,4,5,7,8-pentamethyl, Thiemo[3,2-c]pyridine,3-bromo and Butylated Hydroxytoluene) and 4 suspected to be phenolic compounds. The suspected phenolic compounds, were Phenol,2,4-bis(1,1-dimethylethyl), Phenol,2-bromo-4-(1,1-dimethylethyl), Phenol,m-tert-butyl and Phenol,p-tert-butyl and belong to the family of alkylphenols. All of these potentially leached alkylphenols are known to exhibit endocrine disrupting effects (Olsen *et al.*, 2002, Routledge and Sumpter, 1997, EU, 2008, Tollefsen *et al.*, 2008). The two degraded treatments were found to have potentially leached up to three of these alkylphenolic compounds (AD = 3, BD = 2) whereas the non-degraded plastic bag fragments had potentially released one or two of the detected alkylphenolic compounds (AnD = 1 and BnD = 2). For both plastic bag types the non-degraded treatments are suspected to have leached more alkanes compared to the degraded treatments. However, due to the fact that no standards were used for the GC-MS analysis, no evaluation of the quantities of leached chemicals can be made.

Table 5.2: Candidate chemicals (based on GC-MS library matches) detected via gas chromatography using the acidified water approach. Fragments of plastic bags from each treatment were treated for 48h with acidified water (pH 2.6) to mimic acidic conditions in the stomach of fish. After 48 hours the acidified medium was treated with *n*-hexane to extract chemicals from the water which was then analysed for leached chemicals via gas chromatography. Numbers in brackets are the match values (peak quality) in percent for the detected chemicals.

No.	Chemical compound	AD	AnD	BD	BnD	Control
	Alkanes					
1	Cyclododecane			**X (91)**		
2	Dihydrocoumarin,4,4,5,7,8-pentamethyl		**X (55)**			
3	Docosane			**X (90)**		
4	Eicosane		**X (95)**		**X (97)**	
5	Heptadecane			**X (86)**		
6	Heptadecane,3-methyl				**X (90)**	
7	Hexadecane		**X (99)**	**X (95)**	**X (98)**	
8	Hexatriacontane		**X (64)**			
9	Nonadecane			**X (86)**		
10	Nonadecane,9-methyl				**X (64)**	
11	Octacosane			**X (91)**		
12	Octadecane		**X (98)**		**X (98)**	
13	Octadecane-1-chloro		**X (64)**			
14	Pentadecane				**X (96)**	
15	Pentadecane,3-methyl		**X (91)**			
16	Pentatriacontane				**X (68)**	
17	Tetradecane		**X (98)**	**X (94)**	**X (98)**	
18	Tetratriacontane			**X (78)**		
19	Tridecane,5-methyl		**X (72)**			
20	Undecane,4-cyclohexyl				**X (68)**	
	Phenols					
21	Phenol,2,4-bis(1,1-dimethylethyl)	**X (89)**		**X (90)**	**X (95)**	
22	Phenol,2-bromo-4-(1,1-dimethylethyl)	**X (93)**				
23	Phenol,m-tert-butyl	**X (90)**	**X (93)**			
24	Phenol,p-tert-butyl			**X (91)**	**X (93)**	
	Miscellaneous					
25	4-Hydroxymandelic acid,ethyl ester,di-TMS	**X (64)**				
26	Butylated Hydroxytoluene		**X (93)**			
27	Dihydrocoumarin,4,4,5,7,8-pentamethyl		**X (55)**			
28	Thiemo[3,2-c]pyridine,3-bromo				**X (68)**	
		n=4	n=11	n=10	n=12	n=0

5.5 Discussion

The findings from this investigation indicate a potential health hazard for small aquatic vertebrates through the ingestion of plastic bag fragments. Even though mortality and behaviour of fish were not affected, fish from plastic treatments were found with prolonged GI residence times of digesting food (p = 0.015), likely caused by accumulations of high numbers of ingested plastic particles. Such a prolonged GI residence time of food has the potential to lead to reduced food ingestion, resulting in a lower energy accumulation and therefore energy depletion in the long term. Additionally, the 28 day exposure resulted in elevated gutted weights (p = 0.040, and p = 0.017) for fish that had been exposed to diets contaminated with degraded fragments of both carrier bag types (A and B, respectively). Exposure also led to an elevated liver weight of one of the degraded treatments (BD; p = 0.032). GC-MS analysis identified the potential leaching of three oestrogenic and one antiandorgenic alkylphenols under simulated stomach pH conditions.

As expected, no effect on mortality of the exposed fish was found which indicated that the exposure did not affect survival in the short term. This is consistent with previous studies exposing fish to a plastic contaminated diet (Rochman *et al.*, 2013b, Rochman *et al.*, 2014) and highlights that even high numbers of ingested plastic particles that do not block the GI, do not pose an acute risk to induce mortality in fish.

No differences in behaviour were observed between treatment groups. All fish were found to feed actively throughout the experiment and did not avoid ingesting the plastic contaminated diet, as indicated by the presence of plastic in faecal samples and GIs which further supports reports that fish can ingest plastic particles (Choy and Drazen, 2013, Lusher *et al.*, 2013, Foekema *et al.*, 2013). This indicates that no filing effects were induced by the ingested plastic over the here observed time frame which could have led to a reduced food ingestion and thus have potential effects on energy assimilation (Ryan, 1988).

Gastrointestinal transit times of food contaminated with the smaller degraded fragments were lower than that compared to food contaminated with the larger non-degraded plastic fragments which is consistent with findings that smaller, indigestible food particles are more rapidly processed than larger ones (dos Santos and Jobling, 1991). Ingestion of plastic was found to induce prolonged GI residence times (indicated by

higher amounts of retained food in the GI) and gastric evacuation times (indicated by higher presence of retained food in the stomach compartment) of processed food in the GI of all plastic treatments. Sticklebacks were fed the last time 24h prior to sampling and were expected to have completed gastric emptying and egested most of the up taken food at the time of sampling; Rajasilta (1980) observed a digestion rate between 7.4 ± 2.5 and 15.2 ± 2.8 % h^{-1}, depending on the composition of the diet at 18°C in sticklebacks (Rajasilta, 1980) and a gastric evacuation study by Vinagre *et al.* (2007) presented data that suggested total gastric emptying after 16h after a meal in *Solea solea* and *Solea senegalensis* (both predatory fish) at 20°C (Vinagre *et al.*, 2007). Complete gastric emptying was found for control fish after 24h whereas prolonged gastric emptying times of plastic treatments could be observed as indicated by elevated levels of retained processed food in the stomach for 75% of fish of all plastic treatments. A positive correlation between the amount of retained food and the total surface area of plastic in the GI indicated that the presence of ingested plastic lead to the observed prolonged residence times of processed food. This observed retention could be explained by obstructions caused by plastic (Pierce *et al.*, 2004) but also a more solid composition of the digesting food (Read and Houghton, 1989) due to the hindered excretion of gastric enzymes (Day, 1980) and the plastic itself. Signs for obstructions were seen during the faecal analysis; Faeces samples indicated a possible temporary blockage or obstruction of the GI, indicated by a non-uniform egestion of plastic pieces. However, since faeces samples for fluorescent analysis were pooled per exposure tank, it is difficult to conclude that blockages or obstructions occurred in individual fish. Prolonged retention of food might lead to satiation effects, resulting in lower food consumption (Derraik, 2002, Thompson, 2006, Ryan, 1988), energy assimilation and therefore energy depletion in the long term (Cole *et al.*, 2015). However, prolonged residence time can also lead to an increased leaching of chemicals, not only due to a longer exposure window but also an increased accumulation of ingested plastic particles. To test for plastic related effects on enzyme excretion and energy absorption, future studies might consider to test the calorific value of produced faeces and compare those to control fish (Lutz, 1990).

There was a positive effect on gutted weight for fish from both degraded plastic treatments. These unexpected results stand in contrast to findings of plastic exposures in other studies like Besseling *et al.* (2013) who found a positive correlation between weight loss and plastic concentration in *Arenicola marina* (Besseling *et al.*, 2013). This

observed effect might be explained by the leaching of plastic derived chemicals. The leaching of plastic derived chemicals takes place over the plastic´s surface and is known to slow down once chemical compounds have to migrate from the inner polymer structure to the surface (Ejlertsson *et al.*, 2003). Hence, a high surface area/volume ratio of particles but also cracked open polymer structures that expose the inner matrix of the polymer could promote the leaching of elevated levels of chemicals from the polymer structure. The liver as an important site of detoxification processes can indicate the exposure to higher pollutant levels through an increase in liver weight (Slooff *et al.*, 1983). The BD treatment, which had the highest total surface area of degraded fragments in the GI, was the only treatment to show significantly elevated liver weights. Even though the AnD treatment had the second highest total surface area, the non-degraded polymer structures seemed to release less chemicals compared to the AD treatment which had a higher liver weight but a lower total surface area. Hence, the observed effects on weight for the degraded treatments of both plastic bag types are thought to be due to the release of bioactive additives. The GC-MS analysis suggested that 4 alkylphenolic compounds leached from the plastic fragments. These chemicals which were proposed as a best fit by the GC-MS library are all suspected to have endocrine disrupting properties; Brominated phenols like the proposed Phenol,2-bromo-4-(1,1-dimethylethyl) but also the detected Phenol,m-tert-butyl and Phenol,p-tert-butyl are suspected to exhibit oestrogen-like activity by binding to the oestrogen receptor (ER) with affinities 10,000, 1,000,000 and 1,500,000-fold less, respectively, compared to 17β-estradiol (Olsen *et al.*, 2002, Routledge and Sumpter, 1997, EU, 2008). However, even though the single compounds are just moderate to weakly oestrogenic, mixtures of oestrogenic compounds are thought to have additive effects (Kortenkamp, 2007, Thorpe, 2001, Thorpe *et al.*, 2001, Thorpe *et al.*, 2003). Accordingly, mixtures of bisphenol A (BPA) and nonylphenol (NP) have been shown to have higher oestrogenic potency compared to the single components in swordtail fish (*Xiphophorus helleri*) (Kwak *et al.*, 2001). In addition, the potentially leached2,4-di-tert-butylphenol, a degradation compound of phenolic antioxidants and a by-product of tris (2,4-di-tert-butylphenyl) phosphate, used to produce polyethylene (Bach *et al.*, 2013) is suspected to act as an androgen antagonist in rainbow trout (Tollefsen *et al.*, 2008). Exposure to antiandrogens is known to reduce testosterone levels in fish (Sharpe *et al.*, 2004). Oestrogenic phenolic compounds are suspected to have obesogenic properties by promoting adipogenesis, adipocyte differentiation and disturbance of the lipid

metabolism (Grün and Blumberg, 2009). They also disrupt the homeostasis of energy balance, glucose and lipid metabolism, and control of adipogenesis through the interaction with oestrogen and androgen receptors leading to sex steroid dysregulations or by inducing neuroendocrine effects, leading to the central integration of energy balance (Kelishadi *et al.*, 2013). In fish exposure to xenoestrogens (NP, 4-tert-octylphenol (t-OP) or BPA) has been shown to disturb lipid metabolism through the upregulation of peroxisome proliferator activated receptors and fatty acid synthase (which are responsible for lipid accumulation) and downregulation of hormone-sensitive lipase (which plays a pivotal role in fat mobilization), leading to lipid accumulation of exposed fish (Maradonna *et al.*, 2015). Like the tested xenoestrogens by Maradonna *et al.* (2015), also the here potentially leached xenoestrogens exhibit oestrogenic action through the binding to ER. Therefore, the observed increased gutted weight of the two degraded treatments (AD and BD) could indicate the action of obesogenic chemicals. Since just male fish were analysed, the mixture of multiple oestrogenic and one anti-androgenic chemicals might have disturbed oestrogen-androgen balance through oestrogenic action by binding to the ER and decrease of testosterone levels, respectively.

No differences for length and condition index K were observed which indicates that exposure did not affect these endpoints over the here investigated time frame. Since sexually mature fish were used for this experiment and experimental conditions (illumination and temperature) were set for summer time, growth in length was expected to be marginally affected; Sticklebacks are long-day breeders, in which spring and summer conditions (increasing length of the day and increasing water temperature) stimulate spawning (Baggerman, 1990). Since fish are known to decrease growth significantly during reproductive season due to energy allocation to processes related to reproduction (Munro, 1990), fish from this investigation were not expected to display significant changes in length. Indeed, the histological gender determination showed that exposed females (all females were excluded from the analysis) had developed oocytes present in their gonads. Even though differences in weight were found, no change in condition was induced by the exposure to the plastic contaminated diets.

Collectively, results from this investigation suggest that an ingestion of a diet contaminated with fragments of plastic bags can have negative effects on food digestion and energy metabolism in fish. Both, degraded and non-degraded plastics lead to

prolonged gastric evacuation and GI residence times of digesting food, potentially resulting in a reduced food ingestion and energy depletion in the long term. Whereas one degraded treatment (BD) induced a higher liver weight, both degraded treatments induced higher gutted weights, indicating that degraded polymer structures have a higher potential to leach potentially biologically active concentrations of endocrine disrupting chemicals compared to non-degraded plastics. This increase in weight could be the result of the disruption of the energy balance through xenoestrogens with obesogenic mode of action. This potential disturbance in energy metabolism has the potential to result in a deviation of energy resources away from growth or reproductive processes towards energy storage and might therefore reduce growth and reproductive success. Further research is needed to assess the effects of the observed effects on food digestion and suggested disturbance in energy metabolism in the long term. Additional molecular endpoints to test for changes in oestrogen/androgen ratios and/or alterations in other processes regulated by these hormones (including growth, bone morphogenesis, insulin signalling, neural development, cell division and apoptosis) (Oehlmann *et al.*, 2009) should be investigated following a plastic exposure. Molecular endpoints to test for exposure to oestrogens and antiandrogens should be included to give further evidence for the exposure to biologically active concentrations of plastic derived chemicals. Additionally, testing for obesity induced by a potential leaching of obesogenic chemicals could be assessed using nile red staining (Tingaud-Sequeira *et al.*, 2011) and tissue lipid content (Bligh and Dyer, 1959).

Chapter 6

Assessing the potential for microplastic to act as a vector of biologically active concentrations of absorbed xenobiotics via trophic transfer to fish larvae

Data presented for experiment 1 were produced by an undergraduate project student at the University of Portsmouth (Harry Bannister) under the supervision of Dr. Karen L. Thorpe. I assisted with the exposure and provided training for the involved analytical techniques. The molecular work for experiment 1 was conducted by Dr. Karen L. Thorpe. I conducted all of the experimental work described for experiment 2.

The analysis and interpretation of the data from all experimental procedures outlined in this chapter is my own.

6.1 Abstract

Plastics can adsorb and transport high levels of environmental chemical pollutants from the water column. Upon ingestion, these adsorbed pollutants can show increased desorption under physiological conditions, potentially exposing the organism to harmful levels of chemicals. It remains, however, unclear if plastics can act as a vector for chemical pollutants, ingested via trophic transfer and can induce effects in predators. To investigate if plastic ingested via trophic transfer can act as a vector for chemical pollutants to fish, young (10 dph) three-spined stickleback (*Gasterosteus aculeatus*) larvae were exposed in a series of two experiments; Larvae were exposed for 14 days (experiment 1) or 17 days (experiment 2) to a zooplankton diet (*Artemia sp*.) which had been cultured in graded concentrations of bisphenol A (BPA) (0, 32, 320 and 3200 µg L^{-1}) with or without the addition of 0.1 µl ml^{-1} of 0.5 µm (experiment 1) or 9.9 µm (experiment 2) fluorescent plastic spheres for 24h. Exposure to the highest BPA concentration (3200 µg L^{-1} BPA) with the addition of microplastic induced mortality in 26% of exposed larvae of experiment 2. This effect was not found for the highest BPA concentration without the addition of plastic or the smaller plastic spheres used in experiment 1. No effects on growth, measured with length (p = 0.991 and p = 0.823), weight (p = 0.908 and p = 0.821) and condition index K (p = 0.220 and p = 0.407) were observed for experiment 1 and 2, respectively. Relative expression of the detoxification enzyme cytochrome P450 1A (CYP1A) was found to be upregulated for the highest BPA concentration with the addition of microplastics for experiment 1 (p = 0.028) and experiment 2 (p = 0.027). These results provide evidence that microplastics contaminated with BPA, ingested via trophic transfer, have the potential to lead to toxic effects in young fish larvae.

6.2 Introduction

Environmental chemical pollutants have been reported to accumulate on plastics (Mato *et al.*, 2000, Rios *et al.*, 2007, Ogata *et al.*, 2009, Rochman *et al.*, 2013a). Concentrations of PCBs adsorbed to polyethylene pellets has been reported to be as high as 10,000 ng g^{-1} of plastic (Ogata *et al.*, 2009). Commonly adsorbed chemicals to plastic are polychlorinated biphenyls (PCBs) and polycyclic aromatic hydrocarbons (PAHs) (Ogata *et al.*, 2009). These chemical classes contain substances that are suspected to have endocrine disrupting effects through oestrogenic action (Fertuck *et al.*, 2001, Matthews and Zacharewski, 2000). Non-polar, chemical pollutants like polychlorinated biphenyls **(**PCBs) and polycyclic aromatic hydrocarbons (PAHs) in aquatic environments tend to partition to soils and sediments (Morel and Gschwend, 1987, Kile *et al.*, 1995) whereas the organic matter in the soil/sediment (SOM) acts as partitioning medium and the mineral matter acts as adsorbent (Chiou *et al.*, 1979, Chiou *et al.*, 1981, Chiou *et al.*, 1983). However, there is evidence that plastics have higher binding rates of non-polar chemicals compared to sediments and adsorb chemicals from the water column with linear distribution coefficient K_d up to 280 times higher for plastic than sediment (K_d plastic 38,100 ± 5600 compared to K_d sediment 135 ± 16 for the sorption of phenanthrene to polyethylene) (Teuten *et al.*, 2007). This might lead to potentially heavily contaminated plastic; In laboratory experiments, chemicals (e.g. PAHs and metals) can reach equilibrium on plastic in less than 72 h (Teuten *et al.*, 2007, Holmes *et al.*, 2012).

Concerns are raised, since over marine 557 species are known to ingest and be affected by plastics, including fish larvae (Hoss and Settle, 1990, Kühn *et al.*, 2015, Mazurais *et al.*, 2014). Young fish larvae are an especially vulnerable life stage, prone to energy depletion due to their low energy reserves after exhaustion of yolk sac energy reserves (see Chapter 4, section 4.2). Zooplankton which has been reported to ingest microplastic (Cole *et al.*, 2013, Setälä *et al.*, 2014, Frias *et al.*, 2014) is of essential importance for the food web, especially for young fish larvae which depend on it as a food source. Evidence for a trophic transfer of plastics from zooplankton to fish has been provided (see Chapter 3) (Cedervall *et al.*, 2012). As a consequence, there is a potential for fish larvae to ingest polluted microplastic via the diet and become exposed to elevated levels of pollutants (Teuten *et al.*, 2009). Contaminated plastic that is directly ingested from the water column is known to show increased desorption of accumulated pollutants

from the plastic under simulated physiological conditions of the gastrointestinal tract (GI) (Bakir *et al.*, 2014a). Accordingly, accumulation of persistent bioaccumulative and toxic substances (PBTs) has been found in fish tissue after exposure to contaminated plastic pellets which can be followed by adverse effects like glycogen depletion, fatty vacuolation and single cell necrosis (Rochman *et al.*, 2013b). However, it remains unclear if the ingestion of contaminated plastic via trophic transfer can lead to desorption of adsorbed chemicals in sufficient concentrations to induce biological effects.

Of the annual bisphenol A (BPA, 4,4'-isopropylidine diphenol, CAS Registry No. 80-05-7) production of approximately 7 billion pounds (Erler and Novak, 2010) about 1 billion pounds of BPA are released into the environment annually (Erler and Novak, 2010) through landfill waste, as well as BPA containing sewage and effluent (Crain *et al.*, 2007). Concentrations of BPA were found at 0.18 µg L^{-1} to 4.3 mg L^{-1} in leachates from municipal waste disposal sites (Teuten *et al.*, 2009) and in the order of g day^{-1} for effluents of waste water treatment plants (Sánchez-Avila *et al.*, 2009). BPA is a moderately hydrophobic compound (Octanol-Water Partition Coefficient (K_{ow}) of $logK_{ow}$ 3.4 (Cousins *et al.*, 2002) and it is estimated that 50% of dissolved BPA will bind to sediments and soils (Anupama-Niar and Sujatha, 2012). Accordingly, there is also the potential for plastic fragments to be contaminated with BPA. Exposure is of concern since BPA is known to exhibit endocrine disrupting effects in vertebrates at environmentally relevant concentrations (Crain *et al.*, 2007). Through the direct binding to the oestrogen receptor (ER) (Crain *et al.*, 2007, Harris *et al.*, 1997), BPA can induce oestrogenic responses in young life stages of fish, leading to alterations in growth, endocrine disruption and act as androgen receptor antagonist (Sun *et al.*, 2014).

The aim of this investigation was to determine if the ingestion of a live, contaminated *Artemia sp*. diet over a 14 or 21 day exposure period (experiment 1 or 2, respectively) can induce biological effects in 10 dph stickleback larvae. For this, *Artemia sp*. were cultured in graded concentrations of BPA (0, 32, 320 and 3200 µg L^{-1}) in the absence or presence of microplastics (0.5 or 9.9 µm at 0.1 mg ml^{-1} experiment 1 or 2, respectively) for 24h before being used as live food. Subsamples of *Artemia sp*. were taken daily to confirm plastic ingestion and to test for motility and activity. Faeces samples were removed every second day to assess egestion and therefore ingestion of plastic particles via trophic transfer by the stickleback larvae. Additionally, fluorescent microscopy was

used to assess the presence of ingested spheres in the GI of stickleback larvae and assess for effects on food retention. Biological endpoints to measure the effects of exposure were survival, growth (length and weight) and condition index K. In addition, to assess evidence for the exposure to plastics derived xenobiotics, relative expression of the gene encoding the detoxification enzyme CYP1A was measured.

6.3 Material and Methods

In this Chapter, two experiments were performed. Both experiments used the same general experimental set up for the preparation of the contaminated diets and the fish exposure. However, the used sizes of tested plastic spheres, exposure duration and number of exposed fish varied between exposures. Exposure duration and sphere size for experiment 2 were increased to test for effects of a prolonged exposure and larger spheres on fish larvae, compared to the shorter exposure time to smaller spheres of experiment 1. Additionally, the sample size for experiment 2 was increased to provide more statistical power.

Larvae in experiment 1 were maintained in 500 ml of AFW and exposed for a total of 14 days. Two treatments were maintained as controls. One was fed a live *Artemia sp*. diet free of any plastic spheres and BPA (Control) whereas the other was fed a live *Artemia sp*. diet contaminated with plastic spheres but free of BPA (Control+MP). The other 6 treatments were exposed to a live *Artemia sp*. that had been cultured in ascending BPA concentrations (32, 320 and 3200 µg L^{-1}) in the presence or absence of 0.5 µm fluorescent polystyrene plastic spheres.

Larvae in experiment 2 were maintained in 500 ml of AFW and exposed for a total of 21 days. Two treatments were maintained as controls. One was fed a live *Artemia sp*. diet free of any plastic spheres and BPA (Control) whereas the other was fed a live *Artemia sp*. diet contaminated with plastic spheres but free of BPA (Control+MP). The other 7 treatments were exposed to a live *Artemia sp*. that had been cultured in ascending BPA concentrations (32, 320 and 3200 µg L^{-1}) in the presence or absence of 9.9 µm fluorescent polystyrene plastic spheres.

6.3.1 Test material

See Chapter 2, section 2.2 for information about the used plastic spheres.

Bisphenol A (BPA; lot no. MKAA2480V) was purchased from Sigma-Aldrich (UK).

All used glass equipment was washed with RO water and rinsed in ethanol prior to test solution preparation. The BPA stock solutions for both experiments were prepared by dissolving 5.4 mg BPA powder into 1 ml ethanol. Of this stock solution, 0.592 µl was then pipetted into a glass bottle and left for the ethanol to evaporate. Then 1 L of artificial sea water (ASW) (20 ppt) was added and mixed well to create the 3200 µg L^{-1} test solution. The other test solutions (320 µg L^{-1} and 32 µg L^{-1}) were created by 10-fold dilution of the 3200 µg L^{-1} solution in ASW (20 ppt). For the control treatments, 0.592 µl of ethanol was added to a glass bottle and left to evaporate before 1 L of ASW water (20 ppt) was added. All solutions were stored in the dark for the duration of the experiment.

6.3.2 Test organisms

Artemia sp. cysts were purchased from Ocean Nutrition (Aquatics online, UK) and cultured daily at a concentration of 1 g cysts L^{-1} of ASW (20 ppt) at 25˚C with continuous illumination and aerated vigorously to keep cysts in suspension. For each fresh culture, after 24h, *Artemia sp*. were harvested into a 2 litre beaker and matured under culture conditions for a further 24h since preliminary experiments showed that *Artemia sp*. do not ingest plastic spheres of 9.9 µm before 8 hph (see Chapter 3, section 3.4.1).

F_2 generation stickleback eggs were obtained from the spawning of three (experiment 1) and four (experiment 2) separate breeding pairs in aquaria at the Institute of Marine Sciences, University of Portsmouth (see Chapter 2, section 2.1.1.1) and maintained as described in Chapter 2, section 2.1.1.2. As the first larvae started to hatch, unhatched, healthy eggs and hatched larvae were equally transferred by random between 16 (experiment 1) or 24 (experiment 2) 1 L beakers containing 500 ml of AFW (0.5 ppt). Live and dead eggs were recorded daily, with any dead eggs removed. Pre exposure the larvae were maintained as detailed in Chapter 2, section 2.1.1.2. During this time, temperature was checked daily whereas pH and dissolved oxygen (DO) were measured every second day.

6.3.3 Water supply apparatus

Throughout the experiment fish were held in 1 L non-aerated glass beakers (working volume 500 ml) containing AFW (0.5 ppt). A static renewal system was used with 80% of the water in each beaker being removed and replaced every second day. Water temperatures were monitored daily throughout the experiments and ranged between 17.9 and 19°C (experiment 1) and 16.2 and 18°C (experiment 2), while pH levels were checked twice weekly and ranged between 7.26 and 7.95 (experiment 1) and 8.21 and 8.90 (experiment 2). DO concentrations were checked twice weekly and remained over 70% of the air saturation value for both experiments.

6.3.4 Preparation of contaminated diets

Contaminated diets for both experiments were prepared by adding 60 ml of the respective BPA solution to 100 ml beakers, one per treatment. Plastic treatments had an additional 6 µl of plastic spheres added to the beaker. Beakers were maintained for 24h in 2 glass tanks, one for microsphere treatments and one for non-microsphere treatments to avoid cross contamination. The tanks had RO water added to the bottom and were covered with a glass lid to minimise evaporation of the BPA solution. This set up was held at 25°C with continuous illumination (Figure 6.1a). After 24h, exposure beakers were moved to new glass tanks, 2.5 ml of 24 hph *Artemia sp*. culture was added to each treatment beaker and exposed for 24h. During this 24h exposure the beakers were gently aerated (Figure 6.1b). Prior to feeding, sub samples (200 µl) of *Artemia sp*. were removed and assessed for swimming behaviour under a dissecting microscope (Zoom 2000, Leica) at 12.5x magnification. After immobilisation in ethanol, plastic presence in *Artemia sp*. was assessed under a fluorescent microscope (see Chapter 2, section 2.3.2).

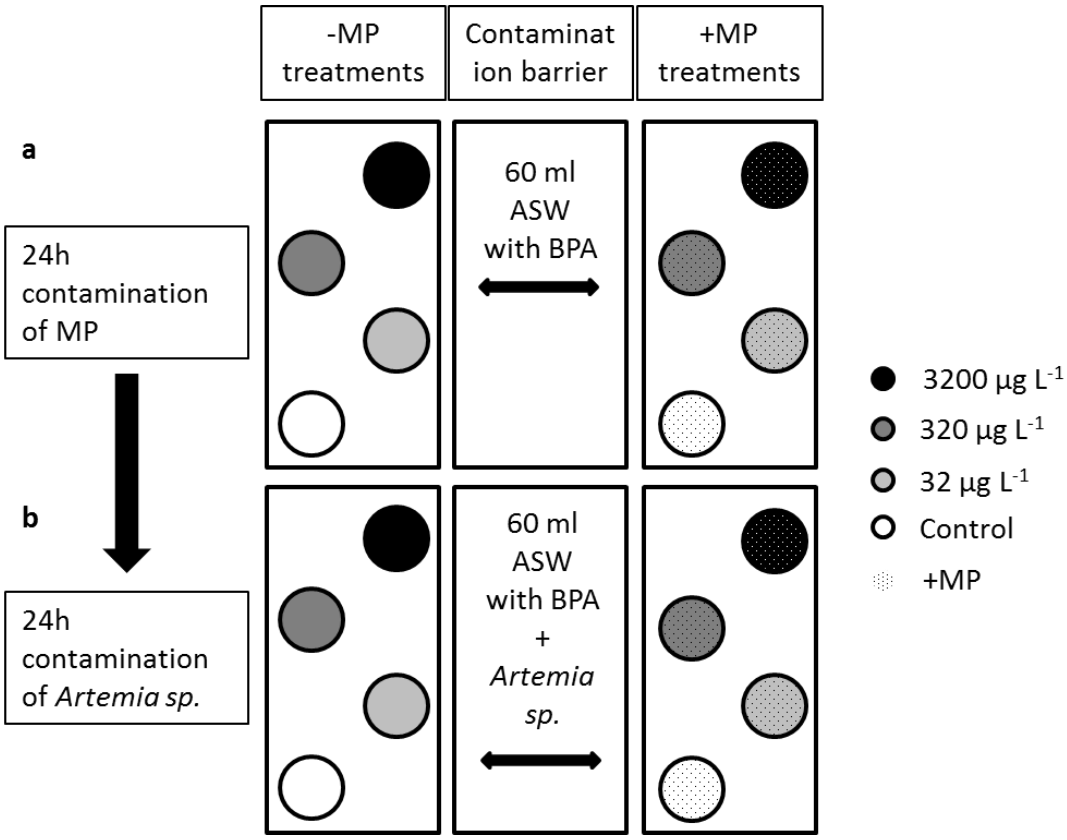

Figure 6.1: Setup for the contamination of polystyrene plastic spheres with graded solutions of BPA (0, 32, 320 and 3200 µg L^{-1}) and subsequent exposure of *Artemia sp.* to graded BPA solutions with or without previously contaminated plastic spheres. a = Graded concentrations of BPA solution were incubated with or without the addition of polystyrene plastic spheres (MP; 0.1 mg ml^{-1}) for 24h before the incubation vessels were transferred to set up b. b = 24 hours post hatch old *Artemia sp.* were added to the exposure vessels and incubated for another 24h. During this 24h incubation period gentle aeration was added to each beaker. After 24h of incubation in set up b the *Artemia sp.* were sieved and washed before being used as live diet for the stickleback larvae.

6.3.5 Fish exposure

Exposure to fish began as the stickleback larvae reached an age of 10 dph. The designated hatching day was the day the majority (80%) of larvae had hatched, which was day 1 for this test. Unequal numbers of larvae per exposure beaker (Control (n = 7), 32 μg L^{-1} BPA (n = 8), 320 μg L^{-1} BPA (n = 7), 3200 μg L^{-1} BPA (n = 7), Control + 0.1 μg ml^{-1} microplastic (n = 7), 32 μg L^{-1} BPA + 0.1 μg ml^{-1} microplastic (n = 8), 320 μg L^{-1} BPA + 0.1 μg ml^{-1} microplastic (n = 8), 3200 μg L^{-1} BPA + 0.1 μg ml^{-1} microplastic (n = 8)) and 2 replicates per treatment were used for experiment 1. An equal number of larvae per exposure beaker (n = 9) with 3 replicates per treatment was used for experiment 2. From here on, abbreviations for treatments will be used when referring the above named treatment groups (e.g. 32 μg L^{-1} BPA will be referred to as 32BPA whereas treatments containing microplastic in addition to the BPA solutions will be referred to as +MP (e.g. 32BPA+MP)). The total planned exposure time was 14 (experiment 1) and 21 (experiment 2) days. Fish were fed twice daily with contaminated *Artemia sp*., corresponding to the treatment group. For each feed 30 ml was taken from each *Artemia sp*. treatment beaker and filtered through a 125 μm sieve. Four different sieves were used, one for each Control, Control+MP, BPA and BPA+MP to avoid contamination. For each treatment, *Artemia sp.* were washed with tap water to remove any excess BPA and microplastics and then rinsed into separate beakers for each treatment with 20 ml of AFW (0.5 ppt). Treatment assigned plastic pipettes were then used to feed 5 ml of *Artemia sp*. per replicate. All equipment was then washed thoroughly to avoid contamination of treatments.

6.3.6 Sampling

Each day before the first feed, *Artemia sp*. samples were taken from each treatment, transferred to a 96-well plate and examined with a stereomicroscope (Leica Zoom 2000) at 10.5x magnification to confirm that they were still motile and had comparable activity levels across treatments. After these observations, *Artemia sp*. were immobilized and examined as detailed in Chapter 2, section 2.3.2.

According to plan, fish from experiment 1 were sampled after 14 days of exposure. For experiment 2 the originally planned exposure period of 21 days had to be shortened due to the larvae in the highest BPA+MP treatment (3200BPA+MP) exhibiting signs of

toxicity; All larvae were sampled on day 17 of the exposure. Larvae for both experiments were sacrificed as described in Chapter 2, section 2.3.1. The head region including the liver of each larva was removed, fixed in RNAlater (Sigma Aldrich, UK) and stored at -80°C for subsequent isolation of RNA. The remainder of the fish was fixed in 10% buffered formalin for fluorescent microscopy to evaluate presence of microspheres in the GI.

6.3.7 Histological analysis

Since previous histological analysis using paraffin embedded samples could not provide further evidence for the ingestion of fluorescent particles due to complications during sample preparation (See Chapter 3), a resin based histological technique was chosen to analyse presence of plastic spheres in the GI of fish from experiment 1. Larvae were prepared after the following protocol:

Posterior parts of larvae that had been previously fixed in 10% buffered formalin were dehydrated and infiltrated with resin:

50% ethanol - 15 min

70% ethanol - 15 min

90% ethanol - 10 min

100% ethanol - 10 min

100% resin - 2 hours

100% resin - 1 hour

After dehydration and infiltration a polymerisation step was performed. Gelatine capsules (size 00) which were filled with London Resin (LR) White (Electron Microscopy Sciences, USA) and fish bodies placed inside the resin with a longitudinal orientation to the capsules. The capsules were then sealed using the appropriate lids and the polymerisation step was performed at 65°C in a curing oven for 48h. During the

polymerisation step, glass knives were prepared for subsequent sample sectioning. Glass knives were made using a LKB knife maker Type 7801B. Glass strips (400 mm x 25 mm x 6.4 mm) were clamped into place and scored with a tungsten wheel at 25 mm intervals. These were then fractured to produce squares (25 mm x 25 mm). Each square was then rotated 90° counter clockwise, clamped into place and then scored and fractured to produce 2 glass knives. The knives were then examined for a clean break and a good cutting edge. The sharp left edge of the knife was used for the sectioning and the less sharp right side was used for trimming of the resin block containing the sample. Polymerised, LR White embedded samples were trimmed and sectioned by carefully removing the gelatine capsules and trimming of the resin blocks before samples were sectioned using a glass knife on an Ultracut E Microtome. Initial trimming was done to form a prism shape. This reduced the cutting area for sections to decrease the strain on the knife edge and help to produce better quality sections. Fish tissues were cut cross sectional with a 2 μm section thickness, transferred to a water bath and then floated onto glass slides before being placed on a hot plate to dry. Prepared slides were then examined as described in Chapter 2, section 2.3.2 to detect green fluorescence in the prepared sections.

6.3.8 Fluorescent determinations

Presence of plastic spheres in larval and faeces samples were assessed according to Chapter 2 section 2.3.2.

LR White embedded sections were analysed using a Zeiss LUMAR.V12 stereo microscope with an AxioCam MRm camera and AxioVision software (ZEISS, Germany) and a Green Fluorescent Protein (GFP) filter (excitation 485 nm; emission 520 nm) at 80x magnification. However, since also this technique used for samples of experiment 1 could not provide evidence for plastic presence in the GI of exposed larvae, larvae from experiment 2 were assessed for the presence of microspheres in the GI using fluorescent microscopy without previous histological preparations. Larvae were prepared by carefully cutting open the body of the fish using disposable steel microtome blades (Thermo Scientific MB35 Premier) under a stereo microscope (Leica M210F), the GI was removed and cleaned of surrounding tissue. Pictures of the whole GI and close ups were taken with an attached camera (DFC310FX) and imaging software (Leica application suite V.4.5.0) and Green Fluorescent Protein (GFP) filter

(excitation 485 nm; emission 520 nm) at 12.5x and 80x magnification respectively to assess microsphere presence in the GI (Figure 7.2 a-e).

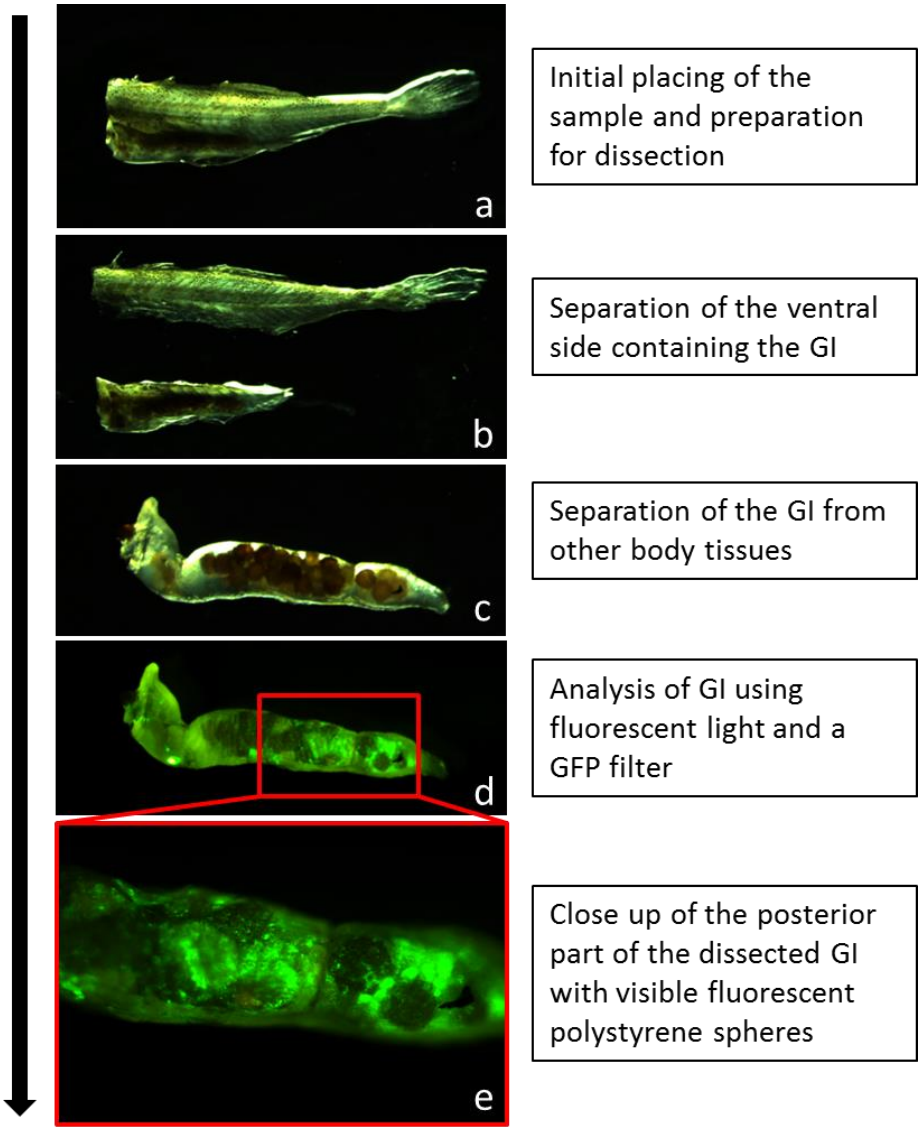

Figure 6.2: Stepwise flow diagram of an example dissection to separate the gastrointestinal tract (GI) for fluorescent analysis with stereo microscope (Leica M210F), attached camera (DFC310FX), imaging software (Leica application suite V.4.5.0) and Green Fluorescent Protein (GFP) filter (excitation 485 nm; emission 520 nm) at 12.5x and 80x magnification respectively. a = the remainder of the sampled fish (head region including liver was separated and fixed for molecular analysis) to be used for the dissection of the GI. b = separation of the ventral site containing the GI. c = separation of the intestine from other body tissues and examination of the GI for retained faeces. Round structures are unhatched *Artemia* cysts. d = fluorescent analysis of GI content for plastic presence. e = close up of a plastic contaminated area of the GI, fluorescence indicates presence of ingested plastic spheres.

6.3.9 Assessment for retained faeces in the GI

For experiment 2 pictures taken during the dissection and analysis for presence of fluorescent plastic spheres in the GI were also used to determine the amount of retained food in the GI. Pictures were evaluated using a scoring system for which processed food present in either the stomach or intestine were assigned a score of 1, those with food present in both a score of 2 and those with no processed food present were assigned a score of 0. Achieved values were then compared to assess presence of processed food in stomach and intestine between treatments.

6.3.10 Gene expression analysis

For material and methods of the molecular analysis performed by Dr Karen L. Thorpe for experiment 1 see Appendix I.

See Chapter 2, section 2.3.4 for details of the molecular work performed for experiment 2.

Molecular work for experiment 1 was performed at the University of Portsmouth and molecular work for experiment 2 was performed at the University of York. Due to the differences in equipment, different protocols were used.

6.3.11 Statistical analysis

All statistical analysis was carried out as detailed in Chapter 2, section 2.3.5.

6.4 Results

6.4.1 Contamination of the live diet

As expected, the examination of daily *Artemia sp*. sub samples confirmed the ingestion of plastic spheres by *Artemia sp*. for all plastic treatments (n = 72) whereas no plastic was found for sub samples of the non-plastic treatments (n = 72) over the duration of the experiment (Experiment 1: Figure 6.3 and Experiment 2: Figure 6.4). No effects of the BPA and BPA+MP exposure were found for the 24h exposure since the visual

examination of *Artemia sp*. showed that *Artemia sp*. from all exposure groups of both experiments were still motile and were found with comparable activity levels.

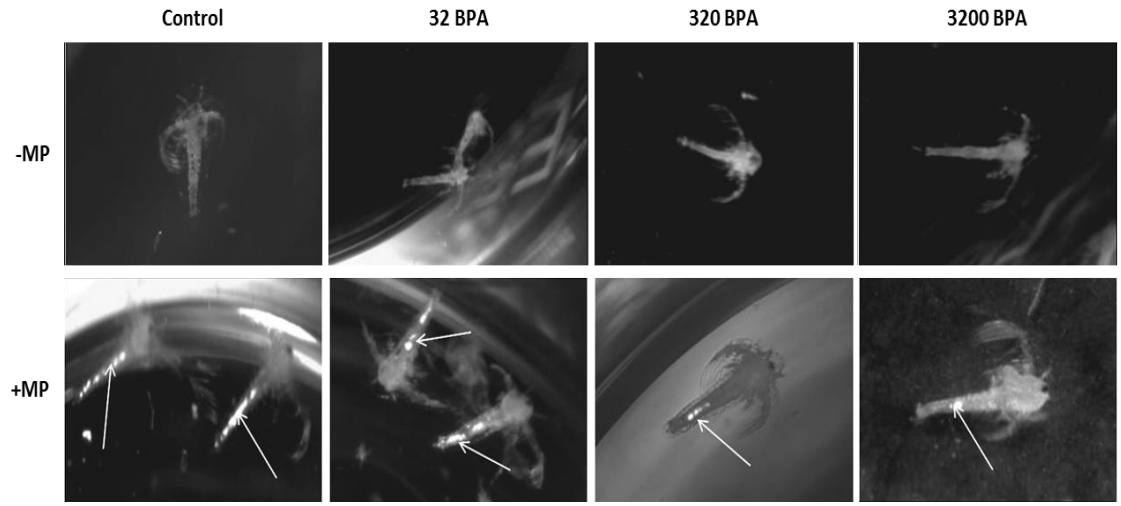

Figure 6.3: Pictures of subsampled *Artemia sp*. that had previously been exposed for 24h to graded concentrations of bisphenol A (BPA) (0, 32, 320 and 3200 µg L^{-1}) in the presence (+MP) or absence (-MP) of 0.5 µm fluorescent plastic spheres (0.1 mg ml^{-1}). A Zeiss LUMAR.V12 stereo microscope with a Green Fluorescent Protein (GFP) filter (excitation 485 nm; emission 520 nm) was used with an AxioCam MRm camera and AxioVision software to detect green fluorescence in the *Artemia sp.* (80x magnification). A clear accumulation of the fluorescing plastics could be observed in the gastrointestinal tracts of the exposed *Artemia sp*. after 24 hours of exposure, indicated by white arrows.

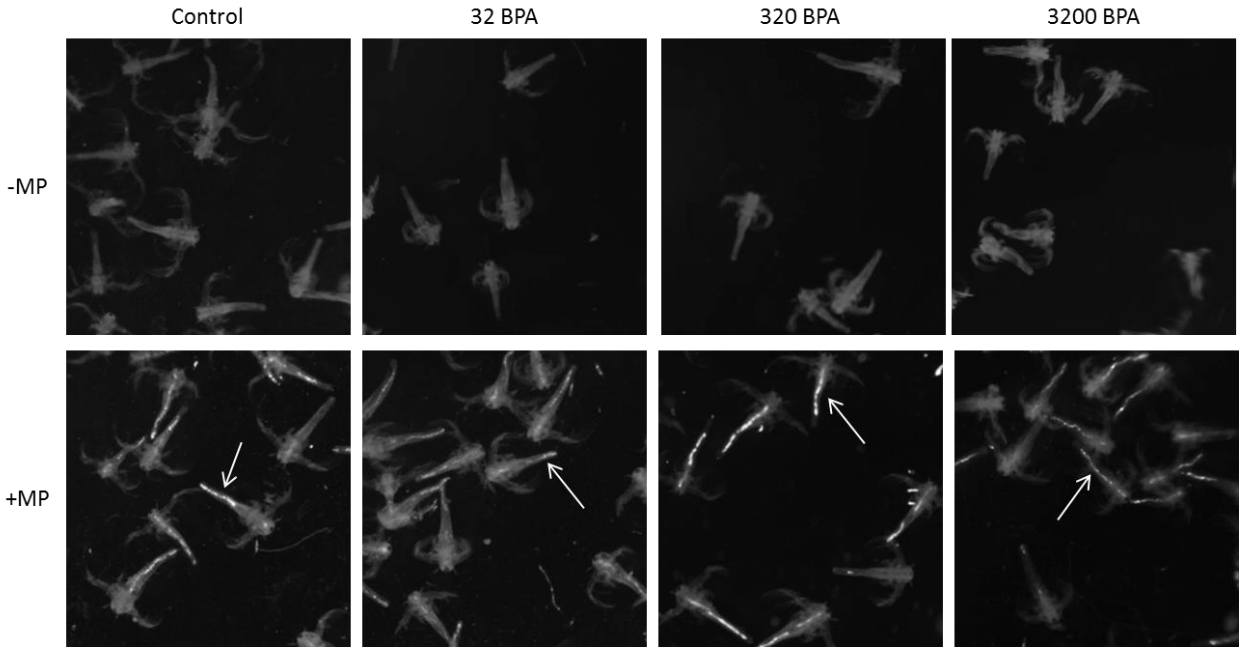

Figure 6.4: Plastic contamination in *Artemia sp*. from experiment 2 that had previously been exposed for 24h to graded concentrations of bisphenol A (BPA) (0, 32, 320 and 3200 µg L^{-1}) in the presence (+MP) or absence (-MP) of 9.9 µm fluorescent plastic spheres (0.1 mg ml^{-1}). A Zeiss LUMAR.V12 stereo microscope with a Green Fluorescent Protein (GFP) filter (excitation 485 nm; emission 520 nm) was used with an AxioCam MRm camera and AxioVision software to detect green fluorescence in the *Artemia sp.* (80x magnification). A clear accumulation of the fluorescing plastics could be observed in the gastrointestinal tracts of the exposed *Artemia sp*. after 24 hours of exposure, indicated by white arrows.

6.4.2 Plastic presence in faeces samples

Examination of faeces samples from experiment 2 of the plastic treatments (n = 84) confirmed that fish were egesting plastic particles and therefore provided evidence for the ingestion of the contaminated *Artemia sp*. diets. No plastic was found in faeces samples from the non-plastic treatments (n = 84) (Figure 6.5).

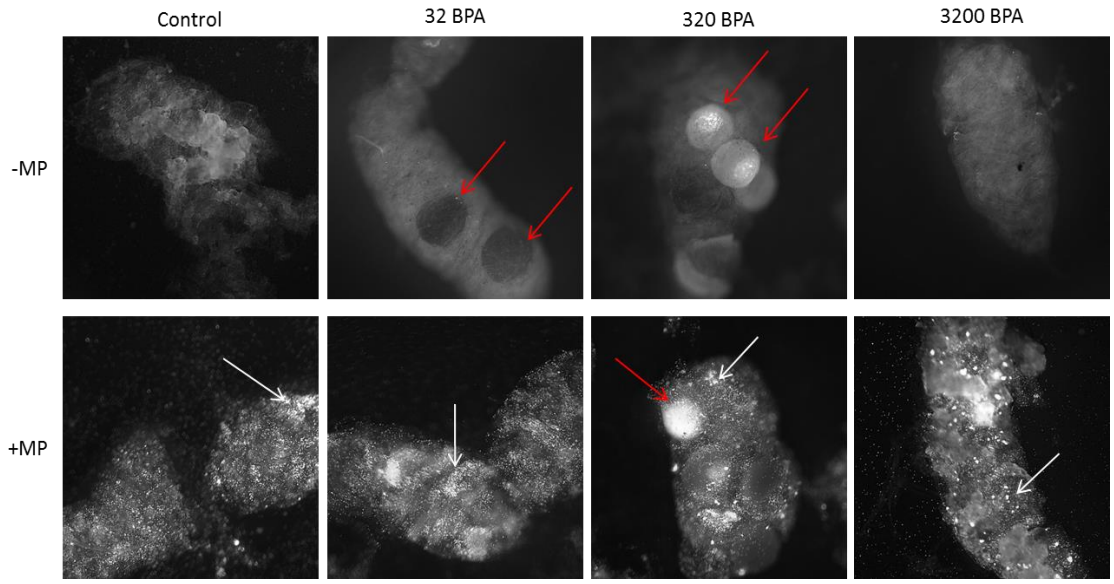

Figure 6.5: Plastic contamination in faeces collected from fish fed *Artemia sp*. that had previously been exposed for 24h to graded concentrations of bisphenol A (BPA) (0, 32, 320 and 3200 µg L^{-1}) in the presence (+MP) or absence (-MP) of 9.9 µm fluorescent plastic spheres (0.1 mg ml^{-1}). Faeces pellets were examined for plastic presence with a fluorescent microscope using a green fluorescence protein (GFP) filter. No plastic contamination was found for faeces pellets from the –MP treatments, whereas contamination with fluorescent polystyrene spheres could be observed for all +MP treatments. Larger round structures represent unhatched *Artemia* cysts, indicated by red arrows. White arrows indicate an example for the presence of plastic spheres. A Zeiss LUMAR.V12 stereo microscope with a Green Fluorescent Protein (GFP) filter (excitation 485 nm; emission 520 nm) was used with an AxioCam MRm camera and AxioVision software to detect green fluorescence in the faeces samples (20x magnification).

6.4.3 Presence of plastic in fish larvae

Examination of dissected GIs of stickleback larvae from experiment 2 revealed that all larvae exposed to the plastic contaminated *Artemia sp*. diets (n = 98) had plastic spheres present in the GI, whereas no plastic spheres were found in GI from non-plastic treatments (n = 105) (Figure 6.6). These results confirm findings from the faeces analysis that fish had ingested the plastic contaminated diet. It was observed that beside of the ingestion of *Artemia sp*., fish larvae from all treatments had also ingested unhatched cysts, which is consistent with findings from the faeces samples.

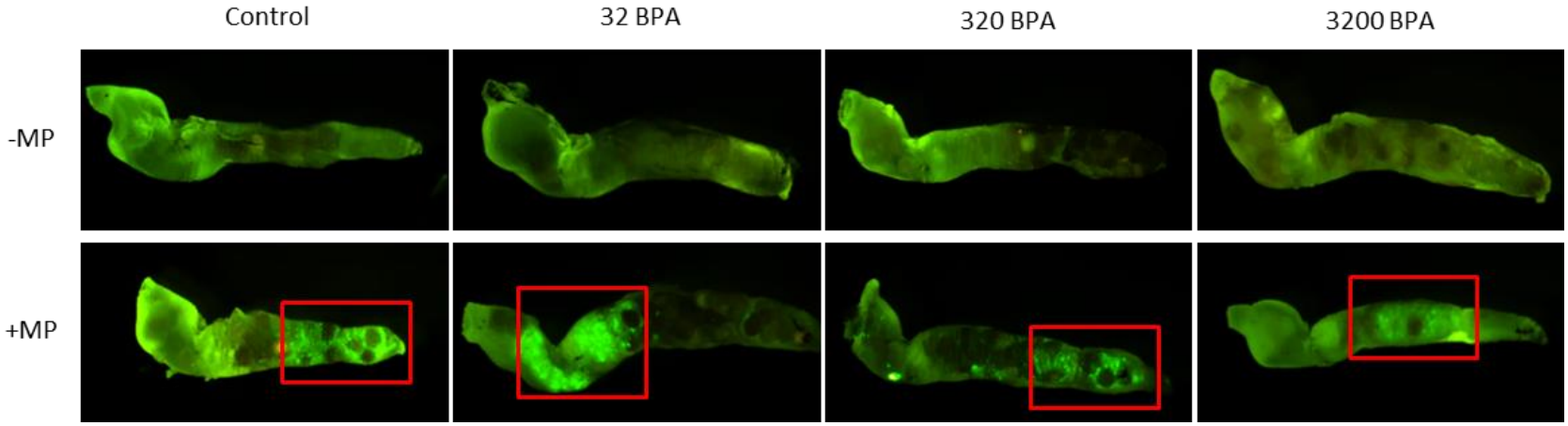

Figure 6.6: Presence of fluorescent polystyrene spheres (MP) in dissected gastrointestinal tracts of sticklebacks that were exposed for 17 days to *Artemia sp.* that had previously been exposed for 24h to graded concentrations of bisphenol A (BPA) (0, 32, 320 and 3200 µg L^{-1}) in the presence (+MP) or absence (-MP) of 9.9 µm fluorescent plastic spheres (0.1 mg ml^{-1}). Dissection of gastrointestinal tracts was performed as explained in Figure 6.2. No plastic contamination of gastrointestinal tract content was observed for –MP treatments, whereas fluorescent plastic spheres could be found in gastrointestinal tracts of all +MP treatments. Larger round structures represent unhatched *Artemia sp.* cysts. Red boxes highlight the areas of major fluorescence deriving from ingested plastic spheres. A (Leica M210F) with an attached camera (DFC310FX) and imaging software (Leica application suite V.4.5.0) and Green Fluorescent Protein (GFP) filter (excitation 485 nm; emission 520 nm) was used to detect fluorescence in dissected gastrointestinal tracts (12.5x magnification)

6.4.4 Effects on food digestion

Examination of pictures taken from separated GIs (see Figure 6.2c) indicated differences in the retention of processed food between and within non-plastic and plastic treatments. Evaluation of pictures showed that non-plastic treatments had higher amounts of retained food present exclusively in the stomach, compared to plastic treatments ($H(1, N = 8) = 3.938$, $p = 0.047$; Figure 6.7). No differences were found when comparing non-plastic and plastic treatments for the presence of processed food present exclusively in the intestine ($H(1, N = 8) = 0.333$, $p = 0.564$; Figure 6.7), in the stomach and intestine combined ($H(1, N = 8) = 0.750$, $p = 0.386$; Figure 6.7) and for fish without processed food in the GI ($H(1, N = 8) = 2.215$, $p = 0.137$; Figure 6.7). However, comparing the overall presence of retained food in all compartments of the GI using the scoring system revealed that non-plastic treatments had lower amounts of processed food present in the GI when compared to plastic treatments ($H(1, N = 204) = 5.491$, $p = 0.019$; Figure 6.8). However, this observed effect did not seem to apply for the two highest treatment groups (3200BPA and 3200BPA+MP) when compared to their respective treatment groups (non-plastic and plastic treatments). Pairwise comparison revealed that non-plastic treatments (Control, 32BPA and 320BPA) had a lower presence of retained food in the GI when compared to the 3200BPA treatment ($H(1, N = 54) = 7.987$, $p = 0.005$, $H(1, N = 51) = 4.592$, $p = 0.032$ and $H(1, N = 54) = 5.161$, $p = 0.023$), respectively). Pairwise comparisons for the plastic treatments revealed that the BPA+MP treatments (Control+MP, 32BPA+MP and 320BPA+MP) had a higher presence of retained food in the GI when compared to the 3200BPA+MP treatment ($H(1, N = 46) = 13.010$, $p < 0.001$, $H(1, N = 45) = 12.317$, $p < 0.001$ and $H(1, N = 46) = 8.388$, $p = 0.004$), respectively). When excluding data from the 3200BPA and 3200BPA+MP treatments due to their contradictive presence of retained food and re-analysing the level of retained food in the GI between non-plastic (Control, 32BPA and 320BPA) and plastic treatments (Control+MP, 32BPA+MP and 320BPA+MP) a stronger effect for the higher retention of processed food in the GI of plastic treatments was found ($H(1, N = 158) = 17.801$, $p < 0.001$).

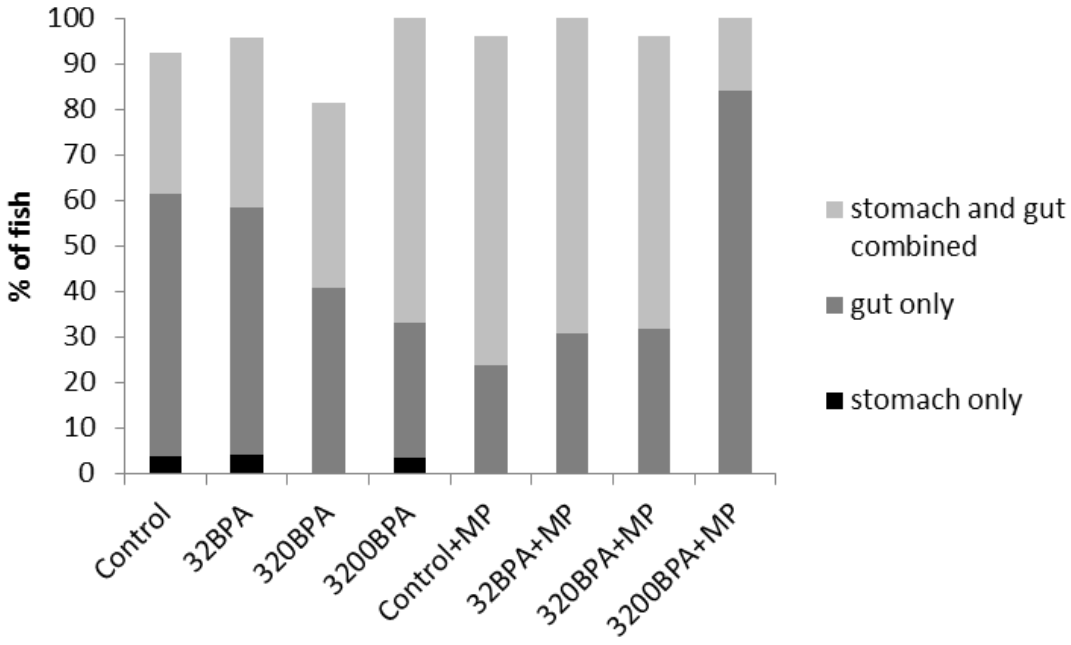

Figure 6.7: Presence of processed food in the compartments of the gastrointestinal tract of fish that had been exposed for 17 days to a diet of *Artemia sp*. that had previously been exposed for 24h to graded concentrations of bisphenol A (BPA) (0, 32, 320 and 3200 µg L^{-1}) in the presence (+MP) or absence of 9.9 µm fluorescent plastic spheres (0.1 mg ml^{-1}). The sample sizes were: Control: n = 27, 32BPA: n = 24, 320BPA: n = 27, 3200BPA: n = 27, Control+MP: n = 27, 32BPA+MP: n = 26, 320BPA+MP: n = 27, 3200BPA+MP: n = 20.

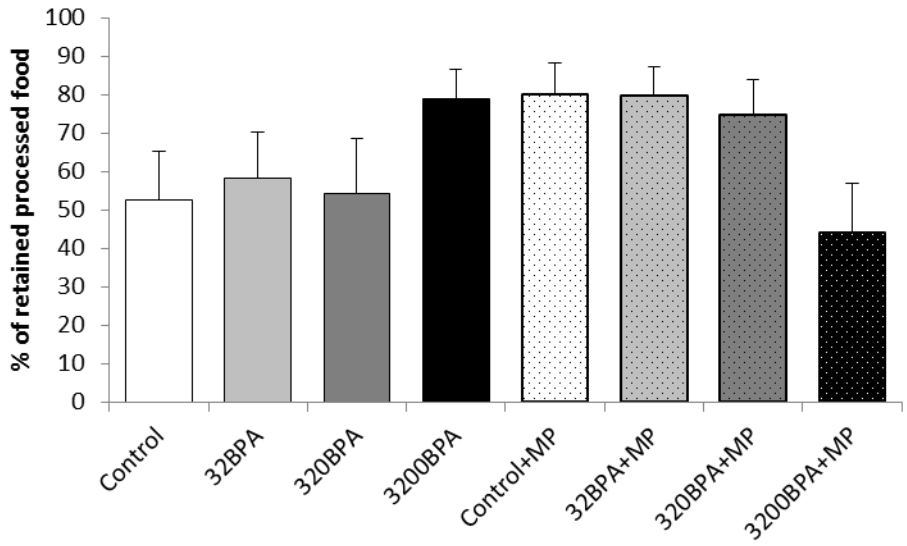

Figure 6.8: Amount of retained food in gastrointestinal tracts of sampled fish that were exposed to *Artemia sp*. that had previously been exposed for 24h to graded concentrations of bisphenol A (BPA) (0, 32, 320 and 3200 µg L^{-1}) in the presence (+MP) or absence of 9.9 µm fluorescent plastic spheres (0.1 mg ml^{-1}). The percentage of retained faeces was evaluated using dissected gastrointestinal tracts (GI) (see Figure 6.2). The sample sizes were: Control: n = 27, 32BPA: n = 24, 320BPA: n = 27, 3200BPA: n = 27, Control+MP: n = 27, 32BPA+MP: n = 26, 320BPA+MP: n = 27, 3200BPA+MP: n = 20. The percentage of retained food in GIs of non-plastic treatments was generally lower compared to +MP treatments with the exception of the 3200 µg L^{-1} treatments where a reversed trend was found. Displayed values are the mean ± SEM.

6.4.5 Biological effects

No mortalities were observed for larvae exposed in experiment 1. For experiment 2 one mortality was observed in the 32BPA+MP treatment (on day 6) and 3 mortalities for the 32BPA treatment (1 mortality on day 4, which was a deformed larvae since the start of the experiment and 2 mortalities on day 15 which were related to stress after a water change). On day 16 of the study, mortality was observed in two of the three replicates of the 3200BPA+MP treatment. Replicate A had a mortality of 4 (44%) and replicate B of 3 (33%) larvae (n = 9 per replicate). Due to this mortality the exposure was terminated on day 17 instead of day 21.

No effects of exposure for experiment 1 and 2 were observed for the measured biomarkers length ($F_{7,52}$ = 0.162, p = 0.991 and $F_{7,197}$ = 0.515, p = 0.823, respectively; Figure 6.9a), weight ($F_{7,52}$ = 0.383, p = 0.908 and $F_{7,197}$ = 0.517, p = 0.821, respectively; Figure 6.9b) and the condition index K ($F_{7,52}$ = 1.412, p = 0.220, and $F_{7,197}$ = 1.036, p = 0.407, respectively; Figure 6.9c). However, further analysis revealed that fish exposed in experiments 1 had a higher condition index compared to fish exposed in experiment 2 ($F_{1,249}$ = 465.923, p < 0.001). The comparison of treatments from both experiments, using a One-way ANOVA with Tukey HSD post hoc test, revealed a lower condition index between multiple treatments (Table 6.1).

Table 6.1: Comparison of the condition index K between treatments of experiment 1 and 2 in which larvae were exposed to *Artemia sp*. that had previously been exposed for 24h to graded concentrations of bisphenol A (BPA) (0, 32, 320 and 3200 µg L^{-1}) in the presence (+MP) or absence of 0.5 µm (experiment 1) or 9.9 µm (experiment 2) fluorescent plastic spheres (0.1 mg ml^{-1}). Values displayed are *p*-values derived from a One-way ANOVA with a Tukey HSD post hoc test. Red coloured values denote a significant difference at the 0.05 level.

	Experiment 2							
	Control	32BPA	320BPA	3200BPA	Control +MP	32BPA +MP	320BPA +MP	3200BPA +MP
Experiment 1								
Control	1.000	0.980	0.987	0.981	0.973	0.996	1.000	0.890
32BPA	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.998
320BPA	0.745	0.104	0.117	0.101	0.087	0.183	0.412	0.045
3200BPA	0.549	0.048	0.054	0.046	0.039	0.091	0.244	0.019
Control+MP	0.993	0.512	0.522	0.510	0.470	0.681	0.907	0.301
32BPA+MP	0.904	0.191	0.214	0.186	0.162	0.317	0.615	0.087
320BPA+MP	0.711	0.078	0.088	0.074	0.063	0.145	0.361	0.031
3200BPA+MP	0.641	0.059	0.066	0.055	0.047	0.112	0.299	0.023

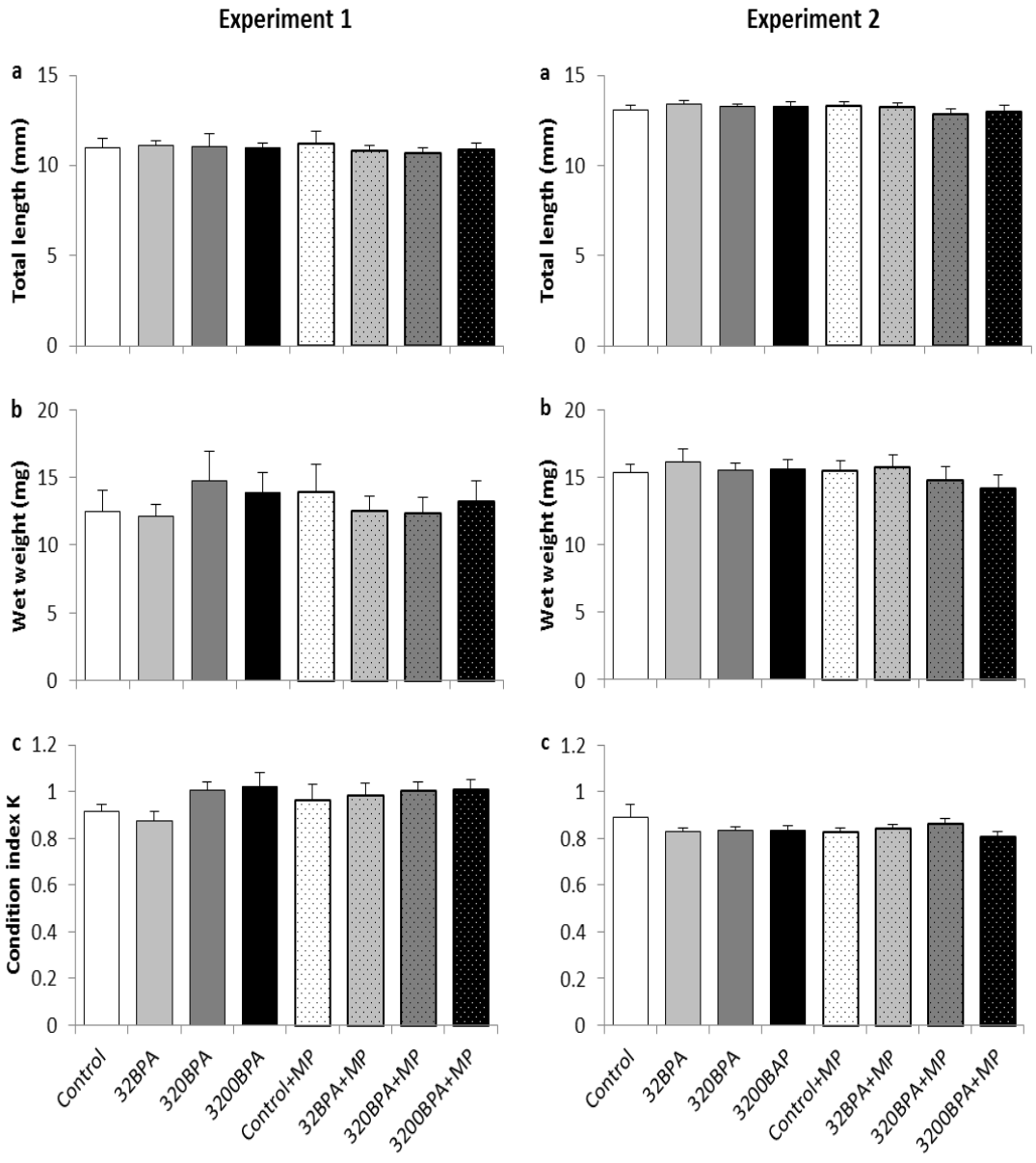

Figure 6.9: Biological effects measured with a = Total length, b = Wet weight and c = Condition index K of sampled larvae exposed to *Artemia sp*. that had previously been exposed for 24h to graded concentrations of bisphenol A (BPA) (0, 32, 320 and 3200 µg L^{-1}) in the presence (+MP) or absence of 0.5 µm (experiment 1) or 9.9 µm (experiment 2) fluorescent plastic spheres (0.1 mg ml^{-1}). Larvae were exposed for 14 (experiment 1) or 17 days (experiment 2). Sample sizes were: Control: n = 14, 32BPA: n = 16, 320BPA: n = 14, 3200BPA: n = 14, Control+MP: n = 14, 32BPA+MP: n = 16, 320BPA+MP: n = 16, 3200BPA+MP: n = 16 for experiment 1 and Control: n = 27, 32BPA: n = 24, 320BPA: n = 27, 3200BPA: n = 27, Control+MP: n = 27, 32BPA+MP: n = 26, 320BPA+MP: n = 27, 3200BPA+MP: n = 20 for experiment 2. Data is expressed as the mean ± SEM.

6.4.6 Gene expression

For experiment 1 and 2 the highest relative expression of the gene encoding CYP1A was found for the 3200BPA+MP treatment groups (Figure 6.10 and 6.11, respectively). Fish exposed to experimental conditions of experiment 1 revealed an upregulated relative expression level when all treatments were compared ($H(1, N = 59) = 16.144$, $p = 0.024$). Pairwise comparison revealed differences between the 3200BPA+MP treatment and the Control, 32BPA, 3200BPA and the 320BPA+MP treatments ($H(1, N = 15) = 4.835$, $p = 0.028$, $H(1, N = 15) = 7.714$, $p = 0.005$, $H(1, N = 15) = 9.054$, $p = 0.003$ and $H(1, N = 16) = 4.864$, $p = 0.027$, respectively). No overall difference in the levels of the relative expression of CYP1A were found for fish that were exposed to the experimental conditions of experiment 2 ($F_{7,34} = 1.985$, $p = 0.086$). However, a Tukey Post Hoc test revealed that the 3200BPA+MP treatment had an elevated relative expression when compared to the Control ($p = 0.027$) but not the other treatment groups ($p > 0.05$). No differences in relative expression levels of VTG B were found between treatments of experiment 1 ($F_{7,50} = 0.526$, $p = 0.811$; Figure 6.10b).

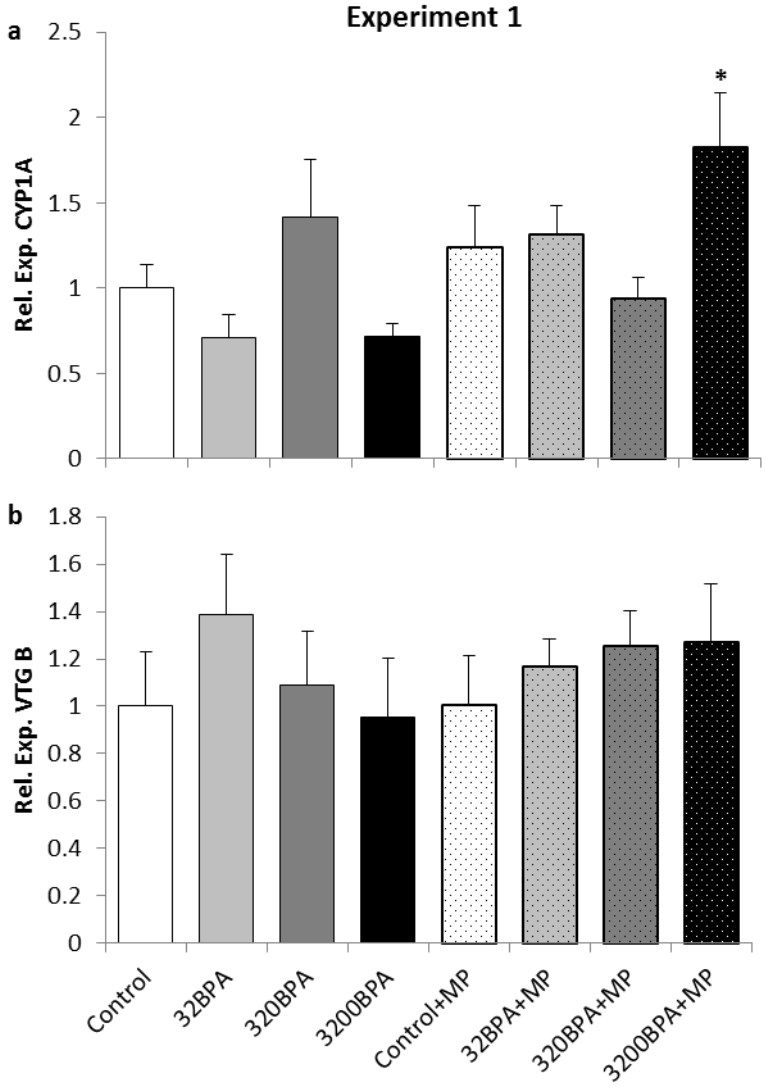

Figure 6.10: Results of the molecular analysis from experiment 1. Relative expression of a = cytochrome P450 1A (CYP1A) and b = vitellogenin B (VTG B) of larvae that had been exposed for 14 days to *Artemia sp*. that had previously been exposed for 24h to graded concentrations of bisphenol A (BPA) (0, 32, 320 and 3200 µg L^{-1}) in the presence (+MP) or absence of 0.5 µm fluorescent plastic spheres (0.1 mg ml^{-1}). Sample sizes were: Control: n = 7, 32BPA: n = 7, 320BPA: n = 7, 3200BPA: n = 7, Control+MP: n = 7, 32BPA+MP: n = 8, 320BPA+MP: n = 8, 3200BPA+MP: n = 8 for the CYP1A analysis and Control: n = 7, 32BPA: n = 7, 320BPA: n = 6, 3200BPA: n = 7, Control+MP: n = 7, 32BPA+MP: n = 8, 320BPA+MP: n = 8, 3200BPA+MP: n = 8 for VTG B analysis. Data has been normalised to the housekeeping gene (18S rRNA) using the δδCt method and is expressed as the mean ± SEM.

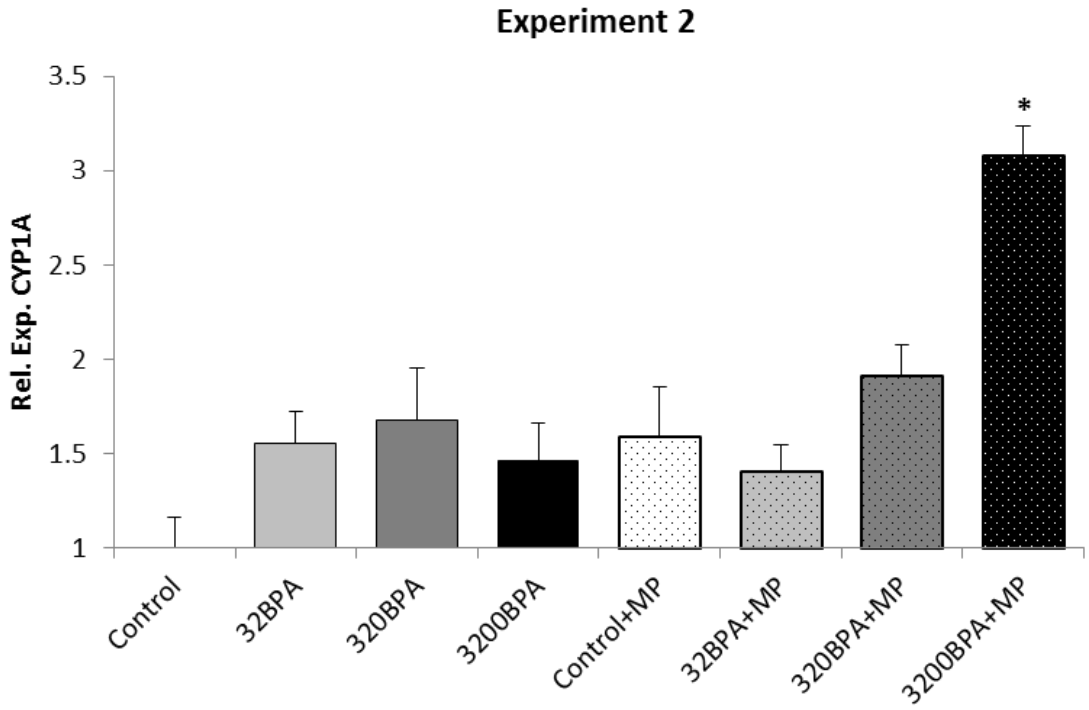

Figure 6.11: Relative expression of Cytochrome P450 1A (CYP1A) of stickleback larvae following a 17 day exposure to *Artemia sp*. that had previously been exposed for 24h to graded concentrations of bisphenol A (BPA) (0, 32, 320 and 3200 µg L^{-1}) in the presence (+MP) or absence of 9.9 µm fluorescent plastic spheres (0.1 mg ml^{-1}). Sample sizes were: Control: n = 5, 32BPA: n = 6, 320BPA: n = 4, 3200BPA: n = 6, Control+MP: = 5, 32BPA+MP: n = 6, 320BPA+MP: n = 4, 3200BPA+MP: n = 6. Data has been normalised to the housekeeping gene (18S rRNA) using the δδCt method and is expressed as the mean ± SEM.

6.5 Discussion

The findings from these investigations provide evidence that microplastics can act as vectors to transport pollutants into the food chain and that filter feeding zooplankton can act as vector for the trophic transfer of BPA contaminated, 0.5 and 9.9 µm sized plastic spheres to young fish larvae. Larvae ingested the contaminated *Artemia sp*. and were found to successfully egest plastic spheres after digestive processes indicating no blockages of the GI. Exposure to the highest BPA+MP treatment induced toxic effects in larvae from experiment 2. The absence of toxic effects in experiment 1 is thought to be due to the higher condition of exposed larvae in the 3200BPA+MP treatment, indicating higher energy reserves for detoxification processes. The exposure to xenobiotics was confirmed for both experiments in the 3200BPA+MP treatments as elevated relative expression levels of CYP1A were detected. Ingestion of the contaminated diets did not result in differences of length, weight and condition index K for both experiments, indicating no measurable effects on these biological endpoints. Ingestion of plastic contaminated *Artemia sp*. was observed to lead to higher retention of food in the GI when compared to non-plastic treatments, indicating a potential to result in filling effects and reduced food ingestion in the long term.

In line with previous reports for the ingestion of microplastic by zooplanktonic organisms (Cole *et al.*, 2015, Lee *et al.*, 2013c, Setälä *et al.*, 2014, Cole *et al.*, 2013) and findings of Chapter 3, the here used *Artemia sp*. were found to ingest the plastic spheres from the water column. As expected, there were no effects observed on motility, activity and survival of *Artemia sp*. exposed to the here used BPA concentrations in the presence or absence of plastic spheres over the duration of 24h. This is consistent with findings of single exposures (BPA or microplastics) of zooplanktonic organisms; *Daphnia magna* was not negatively affected at a BPA concentration of 3160 µg L^{-1} over a 21 day chronic reproduction test (Caspers, 1998) and also ingested plastic spheres were reported to not negatively impact the survival of zooplanktonic organisms over the here used time frame (Lee *et al.*, 2013c, Cole *et al.*, 2015). To my knowledge, no data on the combined effects are available. However, observations of this study suggest that there are limited to no effects on motility, activity and survival of 48 hph *Artemia sp.* for the here used experimental conditions. Therefore, *Artemia sp.* and other zooplanktonic organisms may have the potential to act as vectors for contaminated plastic particles to organisms of a higher trophic level.

Faeces samples collected from experiment 2 showed a constant contamination with plastic spheres for plastic treatments. This finding is important, since free plastic spheres in faeces pellets do not only suggest an egestion of ingested plastic spheres like it has been reported for other larval organisms (Kaposi *et al.*, 2013, Mazurais *et al.*, 2014) but also indicates the successful digestion of the contaminated *Artemia sp*. diet, resulting in the release of plastic spheres which were then able to interact with the GI of fish larvae. Accordingly, the larvae were exposed to BPA contaminated plastic spheres which had the potential to desorb their chemical burden under the physiological conditions in the GI (Bakir *et al.*, 2014a). Findings for the ingestion and successful digestion of ingested *Artemia sp*. from the analysis of faeces samples were supported by GI dissections and analysis with a fluorescent microscope. GI dissections provided further evidence for the ingestion of contaminated *Artemia sp*. through the presence of fluorescent plastic spheres in the GI of larvae which were found to be dispersed through the processed food. Additionally, examination of intestine content and faeces samples showed the ingestion and egestion of unhatched *Artemia sp.* cysts which have an approximate, rehydrated diameter between 243.1 and 285.4 µm (Abatzopoulos *et al.*, 2006). Hence an internal blockage of the GI is very unlikely to be caused by the plastic particles of the here used sizes. However, the presence of the larger, spherical cysts indicated that there is a potential for larvae to ingest bigger plastic particles which might lead to accumulations and blockages of the GI (Pierce *et al.*, 2004).

GI dissections of larvae also revealed a trend for an increased presence of processed food of fish exposed to the plastic treatments and might therefore indicate a plastic induced prolonged digestion of ingested food due to indigestible matter in form of plastic spheres (Read and Houghton, 1989). Non-plastic treatments generally showed a lower retention of processed food in comparison to plastic treatments. However, the two highest BPA treatments (3200BPA and 3200BPA+MP) seemed to show a contradictive effect on food retention compared to non-plastic and plastic treatments, respectively. The observed higher presence of food in fish from the 3200BPA compared to other non-plastic treatments might be explained by slower swimming *Artemia sp*. that were easier to capture by the larvae. Exposure to contaminants is suspected to reduce locomotion behaviour in *Artemia salina* (Venkateswara Rao *et al.*, 2007). Even though no effects on locomotion were observed for the *Artemia sp*. subsamples after the 24h contamination period, the osmotic stress after being transferred from the culture medium (20 ppt) to the fresh water conditions in the exposure vessels (0.5 ppt) following the high levels of

BPA exposure might have resulted in reduced locomotion. This impaired locomotion would also apply to *Artemia sp*. of the 3200BPA+MP treatment. However, the observed low presence of processed food in the 3200BPA+MP treatment, compared to other plastic treatments, might be due to a reduced food ingestion due to toxic effects; Loss of appetite may be an example of a negative side effect of the stress response in fish (Wendelaar Bonga, 1997). It is suggested that repair mechanisms that are induced by physiological changes may result in a reduced ability to process food and consequently lead to a loss in appetite and a reduced food ingestion in fish (Heath, 1995). As a result, fish can show a substantially reduced food ingestion when exposed to pollutants (Wendelaar Bonga, 1997).

The observed lack of effects on length, weight and condition index K for exposed fish indicates no measurable effects of these biomarkers following the ingestion of the BPA contaminated plastic spheres via trophic transfer by young fish larvae over the here investigated time frame. To my knowledge, no data are available regarding the effects of ingested, BPA contaminated plastic particles on length, weight and condition of fish larvae. However, data for the exposure to the single compounds (plastic or BPA) are available. Exposure to plastic particles has been reported to result in reduced length of aquatic invertebrates and vertebrates (Besseling *et al.*, 2014, Mazurais *et al.*, 2014, Kaposi *et al.*, 2013) and have negative effects on weight in worms (Besseling *et al.*, 2013) and birds (Spear *et al.*, 1995). Plastic ingestion has also been linked to decrease energy reserves (Wright *et al.*, 2013) and lead to reduced energy accumulation (Ryan, 1988), indicating negative effects on condition. However, this study differs from the above mentioned field and laboratory studies by using a trophic transfer exposure. As indicated in Chapter 3, trophic transfer exposures may have a lower or no impact on length, weight and condition index compared to water borne or dietary exposures due to the fact that the contaminated diet has to be digested previous to the release of incorporated plastic. Accordingly, exposure to the ingested plastic might be limited. In line with the findings from Chapter 3, this trophic transfer exposure did not result in measurable, plastic induced effects on length, weight and condition index. Even though effects of exposure to BPA on growth and development of aquatic vertebrates varies in the literature (Staples *et al.*, 2002), the lowest concentrations of BPA that have been reported to cause chronic effects on growth of fish range from 1280 to 11,000 µg L^{-1} (Yokota *et al.*, 2000, BayerAG, 1999, Caunter, 1999). A review comparing studies investigating the effects of BPA exposure on aquatic life found that exposure to BPA

does not reduce growth of vertebrates, invertebrates, and algae at concentrations below 400 µg L^{-1} (Staples *et al.*, 2002). Consistent with these findings, also exposure to BPA contaminated plastic spheres did not affect growth for the studies in this chapter. This suggests that just low concentrations of BPA desorbed form the ingested plastic spheres during the digestion process, which is supported by the unaltered expression of VTG B. However, the upregulated relative expression of CYP1A in the 3200BPA+MP treatments of both experiments might indicate the exposure to biologically active concentrations of xenobiotics. As the BPA concentrations used to contaminate the plastic spheres was the only variable between plastic exposure groups, this higher relative expression is likely to be caused by exposure to desorbed BPA from the plastic spheres. This finding, however, is contradictory to reports that have documented a negative effect of BPA exposure on the expression levels of CYP1A in fish (Arukwe *et al.*, 2000, Olsvik *et al.*, 2009). Whereas the underlying processes for this observed BPA related induction of CYP1A remain unclear, ingested plastic spheres might have an influence on the observe effects. Even though, BPA is suspected to have induced the upregulated expression of CYP1A, the exposure concentration of BPA to fish was not believed to be high enough to induce short term effects on length, weight and condition index. This is in line with Staples *et al.* 2002 who stated that biochemical biomarkers provide insight into mechanisms of action but might not correlate with apical endpoints related to survival and growth (Staples *et al.*, 2002). However, since larval fish have just very limited energy reserves a decrease in growth would be expected in the long term due to upregulated detoxification processes mediated via the expression of CYP1A, which has been linked to increased energetic costs in fish (Al-Hameedi, 2009).

Exposure concentrations of fish to BPA derived from the contaminated plastic spheres are not known for this study since no chemical determinations were undertaken to quantify the adsorption of BPA to the polystyrene (PS) model plastic and desorption of accumulated BPA under physiological conditions in the GI of larvae. However, adsorption of chemicals from aqueous phases onto plastic has been reported (Teuten *et al.*, 2007, Mato *et al.*, 2000, Rios *et al.*, 2007, Rochman *et al.*, 2013a). Short exposure times of 72h have been reported to lead to equilibrium of chemicals (e.g. PAHs and metals) on plastic (Teuten *et al.*, 2007, Holmes *et al.*, 2012). However, physical properties of the polymer, like surface area (Teuten *et al.*, 2007) as well as diffusivity and crystallinity (Mato *et al.*, 2000) influence the amount of adsorbed chemicals. Adsorption of PCBs to PE microspheres (10 - 180 µm) was found to be 1 - 2 orders of

magnitudes lower compared to PS nanospheres (70 nm). Additionally, PS has been reported with higher adsorption rates of PAHs compared to PP (Rochman *et al.*, 2013a, Rochman *et al.*, 2013c). Accordingly, polystyrene can accumulate high levels of chemical contaminants (Rochman *et al.*, 2013c, Velzeboer *et al.*, 2014). The release of absorbed chemicals by plastic and adverse effects of such desorbed chemicals has been reported (Teuten *et al.*, 2009, Rochman *et al.*, 2014, Rochman *et al.*, 2013b). Additionally, desorption of accumulated chemicals from the plastic has been reported to be enhanced under physiological conditions (Bakir *et al.*, 2014a). Future studies are needed to determine the quantity of absorbed BPA to the used polystyrene spheres and test for desorption rates under physiological conditions in the GI of the model organism in order to make predictions about the exposure concentration.

Previous reports indicated that microplastic ingestion had no effects on survival of exposed organisms (Kaposi *et al.*, 2013, Besseling *et al.*, 2013, Mazurais *et al.*, 2014). Similar, also multiple BPA exposures from 14 to more than 400 days to BPA concentrations ranging from 100 to 3160 µg L^{-1} indicated no effect on survival (Staples *et al.*, 2002). However, combined effects of BPA with particular matter has been reported to induce additional effects; Zebrafish embryos (*Danio rerio*) that were exposed to a water column contaminated with BPA (5 mg L^{-1}) alone did not show any effects on survival at 96h post fertilisation (hpf), exposure to a mixture with titanium dioxide nanoparticles (TiO_2-NP; 10 mg L^{-1}) that had been previously found to accumulate BPA from an aqueous solution resulted in a 100% mortality at 84 hpf (Yan *et al.*, 2014). No mortality was observed for larvae exposed in experiment 1 which is consistent with findings from the single exposures to microplastics or BPA. However, mortality was observed for larvae exposed to the 3200BPA+MP treatment of experiment 2. Molecular work of both exposures indicated the exposure to biologically active concentrations of xenobiotics in the 3200BPA+MP treatments. However, since a greater surface area has been described to have a positive effect on adsorption of chemicals (Teuten *et al.*, 2007), the 9.9 µm plastic spheres used in experiment 2 were expected to have absorbed less chemicals due to their smaller surface area compared to the 0.5 µm plastic spheres. The observed effect on mortality is therefore linked to the lower condition of larvae exposed in experiment 2. A lower condition index has been linked to lower energy reserves in fish (Herbinger and Friars, 1991, Chellappa *et al.*, 1995) which might have limited the larvae´s capability to support increased energy requirements for CYP1A mediated detoxification processes (Al-Hameedi, 2009).

In addition to previous studies that suggested that plastic particles can transport and release organic pollutants following a direct ingestion (Teuten *et al.*, 2009, Rochman *et al.*, 2014, Rochman *et al.*, 2013b, Besseling *et al.*, 2013), the results from this chapter indicate that also the indirect ingestion of such contaminated plastic can result in adverse effects in fish. Zooplanktonic organisms were found to have the potential to act as vectors of high numbers of contaminated plastic particles from the water column to organisms of a higher trophic level. During this process, these contaminated particles were found to have the potential to retain biologically active concentrations of absorbed xenobiotics, indicated by an elevated relative expression of CYP1A for both exposures. No acute effects on growth and condition were observed over the here investigated timeframe. However, the ingested plastic was found to induce an increased retention of food in the GI of exposed fish, possibly leading to energy depletion in the long term. Additionally, exposure to contaminated plastic has the potential to induce toxic effects in poorly conditioned fish larvae.

Chapter 7

Discussion

7.1 Overview

Due to their distribution and abundance in aquatic environments, microplastic have been classified as a pollutant in their own right (Ryan *et al.*, 2009, Thompson *et al.*, 2004). Ingestion of microplastic and first evidence for negative effects of ingested microplastic has been reported for a wide range of species (Lusher, 2015). However, the research on the effects of microplastics is still described to be in its infancy (Thompson, 2015, Lusher, 2015, Kühn *et al.*, 2015) and considered a less well studied part of marine debris (Doyle *et al.*, 2011). To contribute to the current lack of knowledge, the primary aim of this thesis was to determine whether the ingestion of microplastics has the potential to result in negatively health effects in the three-spined stickleback. A series of in vivo exposures were conducted using a small predatory teleost, the three-spined stickleback (*Gasterosteus aculeatus*), to compare different routes of dietary ingestion of the microplastics, i.e. trophic transfer (Chapter 3 and 6), dietary (Chapter 5) and water borne (Chapter 4) and different sizes and types of microplastic. Whereas all chapters investigated the ingestion, egestion and biological effects of ingested microplastic, the single chapters focussed on different aims: Chapter 3 focussed on the retention and possible translocation of small polystyrene spheres (1 or 9.9 µm), ingested via trophic transfer in adult fish and Chapter 4 investigated the effects of a water column contaminated with polystyrene plastic spheres (1 or 9.9 µm) on larval fish. Chapter 5 assessed the effects of exposure to environmentally relevant microplastic (HDPE plastic bag fragments) on adults and investigated if degradation of the material increases its potential to induce biological effects. Finally, Chapter 6 determined whether a trophic transfer of polystyrene spheres (0.5 or 9.9 µm) that had previously been exposed to BPA have a higher potential to induce biological effects in larval fish, compared to non-contaminated plastic spheres and might therefore have the potential to act as vectors of absorbed organic pollutants to the food chain.. Results from all chapters indicated that adult and larval sticklebacks will ingest high numbers of microplastics via all investigated exposure routes. In adult sticklebacks the ingestion of 1 and 9.9 µm polystyrene spheres via trophic transfer did not lead to measurable biological effects or translocation to the circulatory system due to the rapid evacuation of ingested plastic spheres via the faeces and the size of used spheres, respectively (Chapter 3). As the size of ingested microplastic via trophic transfer is regulated by the size of particles that are ingested by the prey, these findings highlight that effects like blockages and obstructions of the GI are less likely compared to directly ingested plastic. However,

negative effects of microplastic ingestion were observed for larval fish that had accidentally ingested 1 µm or 9.9 µm polystyrene spheres from the water column. Ingestion lead to a dose dependent increase in length, decrease in condition and increase in relative expression of CYP1A for larvae exposed to 1 µm spheres and a dose dependent decrease in condition for larvae exposed to the 9.9 µm spheres (Chapter 4). This highlights that smaller plastic spheres had a higher potential to induce biological effects when compared to the larger spheres. Chapter 5 reported the potential health risks that arise from the ingestion of microplastics derived from plastic bags through prolonging effects on food digestion. Additionally, it was highlighted that degraded polymer structures have an increased potential to induce biological effects, suspected to be caused by the leaching of incorporated chemicals which lead to increased liver weights and gutted weights. Finally, the potential health risk for the ingestion of contaminated microplastic was demonstrated in Chapter 6. Results suggested that polystyrene spheres of 0.5 and 9.9 µm may act as vectors for adsorbed pollutants from the water column via trophic transfer to fish larvae where they induced upregulated detoxification processes (CYP1A) for both particles sizes and induced toxic effects for the 9.9 µm spheres.

7.2 Exposure routes

Filter feeding zooplankton has been previously reported to ingest small plastic particles from the water column (Cole *et al.*, 2013, Cole *et al.*, 2015, Setälä *et al.*, 2014, Desforges *et al.*, 2015, Cedervall *et al.*, 2012). As many fish species actively feed on zooplankton, the ingestion of contaminated prey could be an important exposure route to small plastic particles. Adult and larval sticklebacks were found to ingest plastic from a contaminated live diet but no measurable biological effects were found to be induced by the virgin microplastics. The absence of effects highlights that even though trophic transfer can be an exposure route to microplastics, the relatively small size of ingested plastics poses a low risk for physical effects like blockages or obstructions of the GI (Chapter 3 and 6). Small plastic spheres still have the potential to cause negative effects due to the leaching of additives or desorption of accumulated chemicals (Chapter 6). Additionally, the translocation of small plastic particles is of concern. Even though no evidence for translocation was found in this thesis, other studies have highlighted the potential for small microplastics to translocate from the GI (Browne *et al.*, 2008, von Moos *et al.*, 2012). Accordingly, future studies should focus on the effects of plastic

derived chemicals and translocated particles when investigating the risks of microplastic ingested via trophic transfer.

Larval stages of fish are the smallest self-supporting vertebrates (Wieser, 1995) and can show mortality rates of over 90% when switching to exogenous feeding due to the depletion of yolk sac energy reserves (Houde, 2008). Hence, early energy assimilation is critical for fish larvae (Rana, 1985, Blaxter and Hempel, 1963). Accordingly, the ingestion of indigestible plastic with no nutritional value could pose a threat to larval fish. Accidental ingestion by stickleback larvae was found to be related to dose of the microplastic contamination in the water column. Hence, the ingestion from the water column has to be considered an important exposure route for high numbers of microplastics by fish larvae, especially considering the already high pollution levels of aquatic nursery environments (Lechner *et al.*, 2014). Direct ingestion of microplastic (Chapter 4 and 5) might have a higher significance to induce physical effects as it can lead to the ingestion of larger plastics which were found to affect food digestion (Chapter 5) and have the potential to block the GI.

Whereas these data provide evidence for the accidental ingestion of small microplastic spheres by multiple ontogenetic life stages of sticklebacks via trophic transfer and the water column, no statement about the quantity of ingested particles were made. Especially for trophic transfer exposures, the transfer coefficients calculated from plastic concentrations in water to zooplankton and then fish could be of interest to make predictions about a possible accumulation along the food chain. However, in this thesis, exposure to a plastic contaminated water column was found to have a higher potency to induce biological effects, compared to an exposure via trophic transfer. This might be due to the ingestion of higher numbers of polystyrene spheres, the potentially prolonged exposure time to the plastic (since they were not incorporated in a live diet) as well as additional exposure routes via the gills and skin.

7.3 Effects of environmentally relevant plastic

In the environment, organisms are exposed to plastic that has been exposed to environmental factors like UV radiation, heat and mechanical processes that degrade their polymer structure (Andrady, 2011, Cooper and Corcoran, 2010). Cracks in the polymer structure due to the environmental degradation of the material expose the inner

matrix, leading to additional leaching of incorporated chemicals (Ejlertsson *et al.*, 2003) and increase the surface area over which the leaching of chemicals takes place. Results of Chapter 5 provided evidence that degraded polymer structures might have an increased potential to leach biologically active concentrations of incorporated chemicals, which was suggested by increased liver weight and gutted weight through a potential disturbance of the energy metabolism. Accordingly degraded plastic is of greater concern than non-degraded plastic for chemically induced effects and future studies should focus on the effects of leached chemicals when investigating the potential health risks of degraded plastic.

Additionally, plastic accumulates environmental pollutants through adsorption which can reach high concentrations (Mato *et al.*, 2000, Rios *et al.*, 2007, Ogata *et al.*, 2009). Results presented in Chapter 6 proposed that plastic has the potential to act as a vector for biologically active concentrations of adsorbed chemicals from the water column to fish via trophic transfer which have the potential to lead to toxic effects in the short term. These findings highlight that adsorbed chemicals of environmental plastic are of concern in the induction of biological short term effects. Additionally, microplastic ingested via trophic transfer has to be recognised as an exposure route of concern for the exposure to environmental pollutants, especially considering the high surface area of small sized plastics.

7.4 Biological significance of used biomarkers

Since the investigation of effects of microplastic on aquatic life is a relatively new research area, there is a lack of endpoints specific to the effects of plastic. Therefore, the majority of the endpoints used in this thesis were holistic endpoints, to inform on the health effects of exposure, rather than endpoints focussing on single responsive pathways. To assess the sensitivity of the three-spined stickleback to microplastic, a combination of several biomarkers were used, revealing their biological significance depending on the life stage of the fish, exposure routes and chemical contamination.

No effects on mortality, length and condition index were observed for adult fish exposed to virgin plastic spheres via trophic transfer (Chapter 3) and degraded and non-degraded plastic bag fragments via the diet (Chapter 5), indicating a low toxicity of microplastics. However, since just short exposure periods and low numbers of fish were

used, further investigations are needed to confirm these findings. The effect on wet weight of fish exposed to degraded fragments of carrier bags was suspected to be the result of leached chemicals and indicates potentially negative effects on the health. Accordingly, this should be further investigated by single and combined exposures of the alkylphenols that were suggested to have leached from the plastic bag fragments by the GC-MS.

Larval fish showed a higher potential to be negatively affected by plastic exposure. A negative effect on the condition and a positive effect on length were observed for larvae exposed to a microplastic contaminated water column (Chapter 4). Additionally, the exposure to BPA contaminated plastic spheres via trophic transfer induced mortality (Chapter 6). The finding for an increased length to follow the exposure to plastic particles stands against findings from other studies. These differences can be explained by the different polymer types used compared to other studies. The used polystyrene spheres are expected to have leached different chemicals compared to polyethylene for which ingestion has been reported to induce negative effects on growth (Mazurais *et al.*, 2014, Kaposi *et al.*, 2013). Additionally, the reduced condition index which was found for the same experiment highlights that ingested microplastic can negatively affect energy reserves. The observed mortality for larval fish in Chapter 6 highlights that even though virgin and degraded plastics have a low toxicity, adsorbed chemicals can induce toxic effects.

These findings highlight the need for further research with juvenile and larval fish but also show the need for more sensitive biomarkers for future short term exposures. Molecular endpoints related to growth should be applied to provide further evidence for the observed effects. The GH and its induction of insulin-like growth factors (IGF-1 and IGF-2) are growth inducing factors in teleosts (Reinecke *et al.*, 2005, Nordgarden *et al.*, 2006, Funkenstein *et al.*, 1989) and should be therefore prime target genes. Additionally, assessment for lipid (Bligh and Dyer, 1959), carbohydrate (Carroll *et al.*, 1956) and protein (Gornall *et al.*, 1949) content should be assessed to provide supporting evidence for the condition index K. The combination of these biomarkers has been applied successfully in a previous study to measure energetic costs towards exposure to xenobiotics (Pini, 2014). Additionally, water content analysis of the organism should be carried out since multiple studies have linked starvation and weight

loss to increased water content in fish (Groves, 1970, Idler and Bitners, 1958, Niimi, 1972).

Plastic spheres ingested via trophic transfer (Chapter 6) and plastic bag fragments ingested via the diet (Chapter 5) were found to have prolonging effects on food digestion in larval and adult fish, respectively. Whereas these observed effects did not lead to a measurable reduction on energy reserves using the condition index K in the short term, negative effects on energy reserves are expected in the long term. Energy depletion as a result of prolonged food digestion has the potential to lead to negative effects on growth and condition of the fish, likely leading to a reduced fitness of the organism. Accordingly, effects on food digestion can be an important endpoint for future studies. However, further investigations are needed to explore the effects of the observed effects on food digestion on energy reserves in the long term. To assess if the observed effects on food digestion can have an impact on energy reserves, plastic related effects on enzyme excretion and energy absorption could be determined by measuring the calorific value of produced faeces (Lutz, 1990). Additionally, assessment of lipid, carbohydrate and protein content of the organism could be applied to investigate the effects of a slower food digestion on energy reserves.

Relative expression of CYP1A was found to be upregulated as a result of microplastic exposure for larval fish exposed via the water column (Chapter 4) and trophic transfer to BPA contaminated plastic spheres (Chapter 6). This finding reveals that biologically active concentrations of incorporated and adsorbed chemicals can be released by the plastic. CYP1A as a biomarker for exposure to xenobiotics provides a useful tool to test for such chemicals and should be applied by future studies. However, it has to be noted that even though the detected fold changes in the relative expression of CYP1A were significant for some chapters, the low fold change suggests exposure to just low concentrations of plastic derived chemicals. As this effect is suspected to be due to the leaching of PAHs and virgin polystyrene has been reported to contain high levels of PAHs (which can induce upregulation of CYP1A expression through the binding to the AHR receptor in teleosts (Billiard *et al*., 2002, Billiard *et al*., 2004)) compared to other plastic types (Rochman *et al*., 2013c) the already low fold change of relative expression of CYP1A might not be observed for other plastic types. No effect on the relative expression of VTG was found in Chapter 4, which limits its significance as a biomarker to detect the leaching of low levels of weakly oestrogenic chemicals in larval fish.

Accordingly, future studies should consider the use of more sensitive oestrogen biomarkers like zona radiata protein (Zrp) as Zrp-beta has been reported to be more sensitive towards exposure to oestrogens than VTG (Arukwe *et al.*, 1997).

7.5 Environmental relevance of the used plastic levels

It should be noted that all presented experiments and the obtained results were not based on environmentally relevant concentrations of microplastics. Even though high levels of plastic particles can be found in aquatic environments (see Chapter 1, section 1.2.4) the here used levels of plastic spheres exceeded those found in the environment. This is in line with numerous peer reviewed studies using fluorescent microsphere model plastics to investigate ingestion and effects of microplastics on aquatic organisms (von Moos *et al.*, 2012, Farrell and Nelson, 2013, Wegner *et al.*, 2012, Oliveira *et al.*, 2013, Setälä *et al.*, 2014). Also the study using fragments of plastic bags (Chapter 5) exceeded numbers of environmentally relevant concentrations but was still lower compared to a dietary exposures by Rochman *et al.* (2013). Rochman *et al.* (2013) used 10% LDPE particles mixed with the diet, whereas the percentage for the dietary exposure presented in Chapter 5 was 0.5% of plastic fragments mixed with the diet. Even though it is unlikely that environmental plastic pollution will reach the here used levels, the pollution of aquatic environments is constantly increasing; In the US alone about 100 tons of microplastic are introduced into the ocean annually (Gouin *et al.*, 2011). Additionally, current sampling methods exclude microplastic fragments in the low micrometre range, leaving an incomplete picture of the distribution and contamination levels of this type of microplastic which can be 1000 to 100,000 times higher than reported values (Norén, 2007). Results presented in this thesis have to be seen as proof of principle for the potential effects of ingested plastic polymers themselves, plastic derived chemicals and absorbed and released chemicals by plastic. Studies like this thesis are needed to provide a better insight to assess the potential negative biological effects of microplastics and reveal which endpoints might be of interested for future studies based on environmentally relevant levels of microplastics.

7.6 Future work

Due to the limited knowledge of effects of plastic ingestion on aquatic organisms the aim of this thesis was to provide evidence for general effects induced by a plastic

ingestion in the short term, rather than an in depth investigation into a specific responsive pathway towards a single polymer type or a plastic associated chemical. Therefore, the majority of analysed endpoints focused primarily on holistic endpoints to assess effects on an organism level. Some endpoints on a molecular and cellular level of biological organisation were carried out to provide initial evidence for the potential pathways that lead to the observed effects on an organism level. As already highlighted in this chapter, further experiments are needed to provide additional evidence for the here observed effects, not only in fish but also other marine organisms in an effort to understand the threat that microplastic pollution poses to wildlife. Since results indicated that physical characteristics like shape, size and condition of the polymer matrix but also plastic derived and absorbed chemicals can induce negative effects, future research should consider investigating the interaction of those factors. Especially for chemical compounds additive, potentiated, synergistic or antagonistic interactions might lead to yet unobserved effects. Further experiments are needed to determine the types and quantities of leached chemicals from uncontaminated plastic and levels of accumulated and desorbed chemicals to address the missing link between exposure and observed effects for Chapters 4 and 6. The need for long term exposures to provide information about the long term effects of plastic induced effects on the used biomarkers has been highlighted. Finally, future experiments might consider the use of environmentally relevant concentrations of plastic to test if the effects of ingested plastic presented in this thesis can be observed under the present pollution levels.

7.7 Conclusion

The results in this thesis demonstrate that adult and larval sticklebacks will ingest microplastic from the water column, the diet and via trophic transfer. The physical properties of ingested microplastic had no effect on survival or health in the short term but results suggest that microplastics can leach biologically active concentrations of incorporated chemicals and may act as vector to transport organic pollutants into the food chain.

Whereas further investigations are needed to determine types and quantities of leached and desorbed chemicals and affected pathways, plastic associated chemicals were identified to have the potential to lead to negative effects on survival and health in the short term.

This study highlights that ingestion of microplastic by aquatic biota is of concern and the need for long term studies to investigate the effects of plastic ingestion of the used biomarkers and fully evaluate the environmental impacts of plastic ingestion for aquatic organisms.

Appendix I

Material and Methods for the molecular work performed by Dr. Karen L. Thorpe for Chapters 3, 4 and 6.

Total RNA was isolated from larvae (2 larvae were pooled for each preparation) using Qiagen RNeasy Mini Kit (including the RNase-free DNase set) as described by the manufacturer (Qiagen). Total RNA concentration was estimated from absorbance at 260 nm (A260 nm; Nanodrop 1000; Thermo Scientific) and RNA quality verified by electrophoresis (1.5% agarose gel) and by A260 nm/A280 nm ratios > 2.0. cDNA was synthesised from 1 μg total RNA using qScript cDNA synthesis kits (Quanta Biosciences) according to manufacturer instructions. Real-time PCR (RT-QPCR) was initially conducted using pooled cDNA from each treatment for each of three reference genes (18S rRNA, beta-tubulin and ribosomal protein L8, (Katsiadaki *et al.*, 2010)) and four target genes (vitellogenin B and vitellogenin C, (Katsiadaki *et al.*, 2010); cytochrome P450 1A (CYP1A), (Williams *et al.*, 2009); and insulin growth factor-1 (IGF-1; designed using Primer 3). Primers (see Table 1) were synthesised by Invitrogen (Life Technologies, UK). Specificity of primer sets was confirmed by the observation of single amplification products of the expected size and Tm. The pooled cDNA samples were diluted 1:10 and RT-QPCR performed using LabTAQ™ Green (Lab Tech International Ltd, Uckfield, UK) on an Eco Illumina® (San Diego, CA, USA) real-time PCR cycler. The PCR reactions were performed with an initial incubation at 95°C for 2 min, followed by 40 cycles of 95°C for 5 sec and 60°C for 30 sec. Following the final cycle, the reactions underwent a 15 sec 95°C denaturing step followed by a 15 sec, 55°C hybridisation step before PCR product melt curves were determined during a further temperature increase to 95°C. The control group of animals (not exposed to MPs) were used as the reference sample. The relative expression of each gene was calculated using the ΔΔCt method. There was no evidence that the microplastics influenced expression of any of the reference genes, but as 18S rRNA was most highly expressed this was selected as a reference gene for normalisation. Based on these analysis expression of vitellogenin C was found to too low to be reliably detected and so was excluded from further analysis. The PCR reactions were then repeated for independent samples, using the conditions described above (with the number of cycles reduced to 35), to determine relative expression of each gene (VTG B, CYP1A and IGF-1). Duplicate data for each amplified gene of interest were averaged and relative expression calculated using the ΔΔCt method with normalisation against expression of 18S rRNA in each sample.

Table 1: Nucleotide sequences for real-time PCR primers.

Transcript (gene)	Forward primer (5'-3')	Reverse primer (5'-3')
18S rRNA (ENSGACG00000021687)	CGGCTACCACATCCAAGGAA	TCCTGTATTGTTATTTTTCGTCACTACCT
Beta-tubulin (ENSGACG00000003471)	AACCAGATCGGCGCAAAGT	ACCCGATGCCTCATTGTAGTAGAC
RPL8 (ENSGACG00000002035)	CGACCCGTACCGCTTCAAGAA	GGACATTGCCAATGTTCAGCTGA
Vitellogenin B (ENSGACG00000009711)	CGCATGAAGATTACCTGGGAAA	AATCTCGTTGTGTAGCGGGAAA
Vitellogenin C (ENSGACG00000009490)	TGACACTATCGTCAACCTTGTGAGA	CGCCATGGATGCTAGACTCTTC
CYP1A (ENSGACT00000019429)	ACGTGCAGATGTCAGACGAG	TTGGGTTTGTCGGAGAGAAG
IGF-1 (ENSGACG00000020042)	ACTGTGCACCTCCAAAGACC	CTGCACTGCGGTACTAACCA

Abbreviations

Abbreviations, symbols and acronyms used in the text are defined below, excluding those in equations, which are defined in situ.

+MP	With the addition of microplastic
3200BPA	3200 µg L^{-1} bisphenol A
320BPA	320 µg L^{-1} bisphenol A
32BPA	32 µg L^{-1} bisphenol A
A	Carrier bag type A
AChE	Acetylcholinesterase
AD	Carrier bag type A degraded
AFW	Artificial fresh water
AnD	Carrier bag type A non-degraded
ANOVA	Analysis of variances
ASCE	American Society of Civil Engineers
ASW	Artificial sea water
AU	Arbitray Units
B	Carrier bag type B
BAföG	Bundesausbildungsförderungsgesetz
BCFs	Bioconcentration factors
BD	Carrier bag type B degraded

BFRs	Brominated flame retardants
BnD	Carrier bag type B non-degraded
BPA	Bisphenol A (4,4'-isopropylidine diphenol)
BPF	The British Plastic Federation
C	Control
CA	California
CAS	Chemical Abstracts Service
cDNA	Complementary deoxyribonucleic acid
CH	Switzerland
CO_2	Carbon dioxide
CYP1A	Cytochrome P450 1A
D	Degraded
DEHP	Di-(2-ethylhexyl)-phthalate
DNA	Deoxyribonucleic acid
DO	Dissolved oxygen
dph	Days post hatch
EI	Electron ionization
ER	Oestrogen receptor

EU-27+CH/NO European Union member states (27 in 2012 before the accession of Croatia on 1 July 2013) plus Switzerland and Norway

GC-MS	Gas chromatography–mass spectrometry
GFP	Green Fluorescent Protein
GH	Growth hormone
GI	Gastrointestinal tract
GST	Glutathione *S*-transferase
H&E	Hematoxylin and eosin
HDPE	High density polyethylene
HGH	Human growth hormone
HIS	Hepatosomatic index
hpf	Hours post fertilisation
hph	Hours post hatch
HSD	Honestly Significant Difference
i.e.	That is
IDH	Isocitrate dehydrogenase
IGF	Insulin like growth factor
IMS	Institute of Marine Sciences
IUCN	International Union for Conservation of Nature
K_d	Linear distribution coefficient
K_{ow}	Octanol-Water Partition Coefficient
LC	Lethal concentration

LD	Lethal dose
LDPE	Low density polyethylene
LPO	Lipid peroxidation
LR	London Resin
max.	Maximum
M-cells	Microfold cells
min.	Minimum
MP	Microplastic
-MP	Without the addition of microplastic
MS222	3-aminobenzoic acid ethyl ester, methanesulfonate salt
N/A	Not applicable
nD	Non-degraded
nile red	9-diethylamino- 5H-benzo [a] phenoxazine-5–one
NO	Norway
No.	Number
NP	Nonylphenol
PAHs	Polycyclic aromatic hydrocarbons
PBDEs	Polybrominated diphenyl ethers
PBTs	Persistent bioaccumulative and toxic compounds
PCBs	Polychlorinated biphenyl

PCR	Polymerase chain reaction
PE	Polyethylene
PET	Polyethylenterephthalat
pH	Numeric scale used to specify the acidity or alkalinity of an aqueous solution
PMMA	Polymethylmethacrylate
POPs	Persistent organic pollutants
PP	Polypropylene
PPAHs	Parental polycyclic aromatic hydrocarbons
ppt	Parts per thousand
PS	Polystyrene
PVC	Polyvinylchlorid
Rel. Exp.	Relative expression
RNA	Ribonucleic acid
RO	Reverse osmosis
rt q-PCR	Quantitative real time polymerase chain reaction
SEM	Standard error of the mean
SOM	Organic matter in the soil/sediment
Sto	Stomach
TiO_2-NP	Titanium dioxide nanoparticles

t-OP	4-tert-octylphenol (t-OP) 4-tert-octylphenol
UK	United Kingdom
UNESCO	United Nations Educational, Scientific and Cultural Organization
US	United states of America
USA	United States of America
USEPA	United States Environmental Protection Agency
UV	Ultra violet
VTG	Vitellogenin
VTG B	Vitellogenin B
Zrp	Zona radiate protein
δδCt	Delta Delta C(T)

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