Environment Agency

Evidence

Assessing the impact of exposure to microplastics in fish

Report – SC120056

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Miranda Kavanagh

Director of Evidence

Executive summary

Plastic wastes are rapidly accumulating in landfill and in natural habitats, especially the marine environment, where they create a potential hazard for wildlife. The full environmental impacts of plastic waste are not understood, but the United Nations Environment Programme (UNEP) estimates that marine plastic wastes cause the deaths of hundreds of thousands of aquatic vertebrates each year. These deaths result partly from entanglement or physical damage caused by macroplastics, but there are also concerns that ingested plastic fragments (microplastics) may block the digestive system and cause starvation. Furthermore, it is thought that persistent organic pollutants may partition to plastics and be transported into the food chain. To address these concerns laboratory exposures were conducted to assess the biological effects of ingested plastic particles.

In a first experiment, larval three-spined stickleback (*Gasterosteus aculeatus*) were exposed for 7 days to suspended 1.0 µm fluorescent polystyrene spheres at a density of 10, 100 and 1,000 mg/l. Fluorescent imaging confirmed that the larvae ingested the plastics and that the quantity of fluorescence within the digestive tract was proportional to the density of plastics added to the water. There was no evidence that ingestion of the plastics impacted larval survival, but a negative relationship between quantity of plastic added to the beaker and condition factor was observed ($r = -0.380$; $p<0.001$, $n = 144$); condition factors in the low, medium and high exposures were 14.6% ($p<0.05$), 18.9% ($p<0.01$) and 31.6% lower ($p<0.01$), respectively, when compared to the controls. An increase in expression of the gene encoding the detoxification enzyme CYP1A was also observed in larvae exposed to the medium and high densities of plastic ($p<0.05$).

In a second experiment, adult three-spined stickleback were exposed for 7 days to *Artemia* that had been exposed to 1.0 µm or 10 µm fluorescent plastic spheres. High and low dose groups were generated by feeding with either 100% or 10% contaminated *Artemia*, respectively. The low dose groups and a control group were fed 90% and 100% non-contaminated *Artemia*, respectively. Excretion of the plastics was monitored in a sub-group of fish that were fed non-contaminated *Artemia* for a further 14 days after exposure. Estimates of fluorescent intensity in faecal samples indicated that uptake and excretion of the plastics was rapid; plastics were found in the faeces within 1 day of initiating exposure, but within 2 days of ceasing exposure a 71% decrease in fluorescence was observed, implying that the plastics are not retained within the digestive tract for prolonged periods. There was no evidence for translocation of the plastics from the digestive tract to the circulatory system as reported in other studies. Furthermore, there was no evidence that ingestion of the plastic-contaminated *Artemia* impacted on adult fish survival, body size or condition or expression of CYP1A ($p>0.05$).

In a third experiment, larval stickleback were fed for 14 days on *Artemia* that had been exposed to graded concentrations of bisphenol-A (BPA) in the presence or absence of 0.5 µm fluorescent polystyrene spheres. Ingestion of the microplastics (MPs) and/or BPA-exposed *Artemia* did not impact survival, growth or body condition of the fish larvae; however, a positive relationship between BPA concentration and CYP1A expression was observed ($r = 0.415$, $p<0.05$, $n = 31$) in fish fed *Artemia* that had been exposed to BPA in the presence of MPs. This relationship was not evident for fish fed *Artemia* exposed to BPA in the absence of the MPs, indicating that MPs were indeed partitioning the BPA and transporting it through the food chain.

Collectively, these experiments demonstrate that fish will actively take up microplastics from the water column, as well as ingesting them via their diet. Although ingestion of the micron-sized plastics does not appear to adversely impact the survival or health of

adult fish, at least in the short term, there is evidence to support negative changes in the body condition of larval fish. Furthermore, there was evidence that MPs have the potential to partition an organic pollutant and act as a vector to transport this chemical into the food chain. These results highlight the need for longer-term studies that can more fully evaluate the environmental impacts of plastic ingestion for aquatic organisms.

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1 Introduction

Each year approximately 245 million tonnes (Mt) of plastic are used globally. This production volume coupled with high durability has led to widespread accumulation of discarded plastic in landfills and as litter in terrestrial and aquatic habitats worldwide (Derraik 2002). In 1997, a mass patch of waste material, twice the size of Texas, was discovered in the Pacific Ocean; plastics (estimated 100Mt) comprised up to 80% of the floating waste material (Derraik 2002, Barnes et al. 2009, Ryan et al. 2009). In 2010, a mass of plastic waste was discovered in the North Atlantic (European Commission (DG Environment) 2011) and a further three spots are predicted in the South Pacific, South Atlantic and Indian Oceans. Despite plastics being an internationally recognised pollutant with legislation in place aimed to curb the amount of plastic debris entering the marine environment (Gregory 2009, OSPAR 2009) there are fears that plastic waste will further increase in the future as demands for plastic materials continue to increase (Andrady 2011).

Once in the environment, plastic debris progressively fragments into smaller pieces; plastics undergo minimal biological degradation but are progressively broken down to smaller fragments by mechanical and photo-degradation. These minute fragments of plastic debris, termed microplastics (MPs; particles of plastics with dimensions <5 mm; Andrady 2011) can comprise as much as 85% of stranded plastic debris (Browne et al. 2007). Furthermore, there is evidence that manufactured MPs, used in consumer products, are being introduced directly into the oceans via run-off (Maynard 2006). These include the micron-sized plastic particles typically used as exfoliants in cosmetic formulations (Gregory 1996, Fendall and Sewell 2009), those generated in the ship-breaking industry (Reddy and Shaik 2006) and industrial abrasives in synthetic ‘sandblasting’ media (beads of acrylic plastics and polyester).

A recent report also suggests that MPs are entering the environment through sewage effluents contaminated by fibres from washing clothes (Browne et al. 2011). This has raised concerns internationally about the potential impact of exposure to these MPs for marine organisms.

Plastic debris has been reported in the stomachs of over 180 species, including fish, turtles, marine mammals and birds (Derraik 2002, Thompson et al. 2004, Ryan et al. 2009, Boerger et al. 2010, Davison and Asch 2011, Foekema et al. 2013, Lusher et al. 2013, Verlis et al. 2013), where it is reported to block the digestive tract resulting in physical damage (Gregory 2009) or fill the stomach, resulting in starvation and even potential death of the animal (Carpenter et al. 1972, Bjorndal et al. 1994). However, additional to this potential for physical effects, there are concerns that micro- and nano-sized particles may also translocate from the gut cavity to the circulatory system where they could be taken up and stored in tissues or cells (Browne et al. 2008).

Plastics are typically considered as biochemically inert (Teuten et al. 2009, Roy et al. 2011); however, additives that are commonly incorporated into plastics during manufacture to change their properties or extend the life of the plastic (Barnes et al. 2009, Talsness et al. 2009, Lithner et al. 2011) include endocrine-disrupting chemicals such as polybrominated diphenyl ethers, nonylphenol, phthalates and the constituent monomer bisphenol-A. If these chemicals leach out of the stored plastics they could potentially affect the reproductive physiology of the animal. Furthermore, there are concerns that due to their hydrophobicity persistent organic pollutants (POPs) that occur universally in sea water at very low concentrations will partition to MPs and potentially biomagnify through the food web (Endo et al. 2005, Teuten et al. 2007, Moore 2008, Cole et al. 2011). Recent studies have shown that significant levels of

POPs such as PAH, PCB and DDT concentrate on plastic particles (Rios et al. 2007, Karapanagioti et al. 2011, Heskett et al. 2012), supporting earlier reports of a correlation between amounts of ingested plastics and concentrations of PCBs, DDE, DDT and dieldrin in adult fat tissue in the great shearwater (*Puffinus gravis*) (Ryan et al. 1988).

This project describes a series of three preliminary experiments designed to inform on the potential health effects, for aquatic vertebrates, arising from ingestion of micron-sized plastic particles.

Experiment I: Exposure of larval stickleback to microplastic spheres

The first experiment evaluated the potential health effects arising from direct ingestion of suspended MPs by larval three-spined stickleback; fish larvae feed on microscopic plankton and could potentially mistake MPs for a food source. The larvae (4 days post hatch; dph) were exposed for 7 days to micron-sized green fluorescing polystyrene plastic spheres to assess uptake of the MPs and the relationship between quantity of plastic ingested and body size and condition. As there is some evidence that ingested plastics may leach chemicals that are incorporated into the plastics during their manufacture (Tanaka et al. 2013), expression of the gene encoding CYP1A was also measured in larvae; CYP1A is a detoxification enzyme used as a biomarker to indicate exposure to organic pollutants. Furthermore, as polystyrene plastics are reported to leach styrene oligomers which are weakly oestrogenic (Bang et al. 2012), expression of the gene encoding the oestrogenic biomarker vitellogenin was also measured.

Experiment II: Trophic transfer of microplastic spheres

As adult life-stages of predatory fish are more likely to be exposed to MPs via their diet, a second experiment was conducted in which adult sticklebacks were fed, for 7 days, on a diet of MP-contaminated zooplankton (*Artemia*) and effects on body size and condition evaluated. Additionally, faecal samples were removed daily to assess uptake and egestion of the plastic particles during both the exposure period and during a subsequent 14-day depuration period in which the fish were fed non-contaminated *Artemia*. As in experiment I, levels of both CYP1A and vitellogenin were measured to investigate exposure to organic pollutants and oestrogen.

Experiment III: Microplastics as potential transport vectors for organic pollutants

In the final experiment, stickleback larvae were fed for 14 days with *Artemia* that had been exposed to an organic pollutant (bisphenol-A), in the presence or absence of MPs, to evaluate the potential for MPs to adsorb an organic pollutant and transport it through the food chain.

2 Materials and methods

2.1 Test organisms

The three-spined stickleback used in these experiments were sourced from a first-generation laboratory stock held at the Institute of Marine Sciences, University of Portsmouth; the founder stock originated from a brackish population at Farlington Marshes. The adults were maintained within an aerated artificial seawater (ASW; 3.0 parts per thousand (ppt)) re-circulating system at 18°C with a 16:8 hour light:dark photoperiod and were fed frozen blood worm (Tropical Marine Centre, UK) at least three times daily.

The breeding stock used to supply the larvae for experiments I and III were held under the same conditions as the main stock with the exception that salinity was maintained at 0.5 ppt. Immediately prior to the onset of experiments I and III, sexually mature males were transferred to breeding tanks and were left overnight to establish nests. Two females were added to each tank containing a nest and the fish were left to spawn naturally for up to 2 hours. Nests containing eggs were collected, the eggs removed, rinsed in 0.5 ppt ASW, separated and checked for fertilisation success. Viable embryos from a minimum of three nests were pooled and randomly allocated to beakers containing 300 ml 0.5 ppt ASW. Embryo survival was assessed daily until hatch and any dead/abnormal embryos were removed daily. From 3 days post hatch (dph), larvae were fed twice daily with a suspension of frozen rotifers (Tropical Marine Centre, UK). From 6 dph, the larvae for experiment III were switched to a diet of freshly hatched (<24 hours post hatch, hph) *Artemia* (Ocean Nutrition, Aquatics online, UK).

2.2 Water supply and test apparatus

The ASW for the stock and experimental fish was prepared using reverse-osmosis water with the addition of marine salts (Tropical Marine Centre, UK) to a salinity of either 0.5 ppt or 3.0 ppt. Water temperatures were monitored daily and ranged between 17.9 and 19°C in experiments I and III and between 16.7 and 19°C for experiment II, while pH levels were checked at least twice weekly and ranged between 7.26 and 7.95 for all experiments. Dissolved oxygen concentrations were checked at least twice weekly and remained >70% of the air saturation value for all experiments. The larval exposures were conducted in 1 l glass beakers (working volume 300 ml) and the adult exposures in 10 l aerated glass aquaria (working volume 6 l).

2.3 Microplastics and test chemicals

Fluro-MaxTM green fluorescent polymer microspheres (1.0 µm and 9.9 µm; lots no. 40831 and 41359, respectively) were purchased from ThermoScientific (Fremont, CA, USA); 0.5 µm (lot no. 23189) green fluorescing polymer microspheres were purchased from Duke Scientific Corp (Palo Alto, CA, USA). Bisphenol-A (BPA; lot no. MKAA2480V) was purchased from Sigma-Aldrich (UK). A concentrated stock solution of BPA was prepared by dissolving 5.6 mg in 1 ml of absolute ethanol; 0.592 µl was then added to a 1 l solvent-cleaned glass beaker and the ethanol allowed to evaporate before adding 1 l of ASW (20 g/l) to give a final concentration of 3,200 µg/l. After it had

been shaken vigorously for 5 minutes, the solution was left overnight to allow solubilisation of the BPA.

2.4 Experiment I: Exposure of larval stickleback to microplastic spheres

Larvae (4 dph) were randomly allocated to 12 beakers (n = 14 larvae/beaker) containing 200 ml ASW (0.5 ppt); larvae were fed twice daily with a suspension of frozen rotifers. At 7 dph, numbers were adjusted in each beaker (n = 12 larvae/beaker) to account for low levels of mortality observed during acclimation. The beakers were randomly assigned to one of four treatment groups (n = 3 beakers/treatment); control, low (L), medium (M) or high (H) and exposure initiated by removing 75% of the water and replacing with 150 ml of ASW (0.5 ppt) spiked with MPs (1 µm) to give nominal exposure densities of 0, 5, 50 and 500 mg MP/l. On days 2, 4 and 6 of exposure further 75% water changes were conducted; the replacement water was spiked with respective densities of 1 µm MPs to maintain the nominal exposure densities. Samples of water and detritus were removed from each beaker immediately prior to the water changes for subsequent measurement of fluorescence. During the exposure larvae were fed twice daily with a suspension of frozen rotifers.

2.5 Experiment II: Trophic transfer of microplastic spheres

Prior to initiating experiment II, preliminary exposures were conducted to confirm uptake of the MPs by *Artemia*. *Artemia* cysts (1 g/l; Brine Shrimp Eggs, Ocean Nutrition, Belgium) were hatched in aerated ASW (20 g/l; 25°C) and after 24 hours, the hatched *Artemia* were harvested and re-suspended in fresh ASW. One millilitre of *Artemia* suspension (~150 fry/ml) was then added to beakers containing 60 ml of aerated ASW spiked with 0 or 6 µl of 1.0 µm or 10 µm MPs; final density of 0.106 mg MP/ml. *Artemia* were exposed for a total of 72 hours and subsamples (200 µl) removed after 8, 24, 48 and 72 hours of exposure to confirm uptake of the plastics. The *Artemia* were immobilised by adding 200 µl 50% ethanol and examined under a fluorescent microscope to confirm uptake of the MPs.

Based on the findings of the preliminary *Artemia* exposure, the MP-contaminated *Artemia* for the fish exposure were prepared using freshly harvested *Artemia* that were cultured in fresh ASW for 24 hours post hatch prior to being transferred to 24 hour exposure vessels (at a density of 30 fry/ml) containing either fresh ASW or ASW spiked with MPs (0.106 mg MP/ml). A subsample of *Artemia* was removed from each exposure to confirm uptake of the MPs via fluorescent microscopy.

Adult three-spined stickleback were randomly allocated to 40 glass aquaria containing 6 l of ASW (3.0 ppt); a total of four fish were added to each aquaria. The fish were acclimated to the test conditions for 3 days and during this period fed three times daily with non-contaminated *Artemia* (approximately 3,000 *Artemia* per feed); prior to feeding the *Artemia* were rinsed with reverse osmosis (RO) water to remove excess salt and any MPs that had not been ingested. At the onset of exposure, the aquaria were randomly assigned to each of five treatments (eight aquaria per treatment); control (fed non-contaminated *Artemia*); 1 High or 10 High (fed *Artemia* that had been exposed for 24 hours to either 1 µm or 10 µm sized MPs, respectively); 1 Low or 10 Low (fed 10% of the respective contaminated *Artemia* and 90% non-contaminated *Artemia*). After 7 days, all treatments were maintained for a further 14 days on a diet of non-contaminated *Artemia*. During the exposure and the depuration periods 70% water

changes were performed every third day. Faecal samples were removed from each aquarium every morning to monitor egestion of the fluorescent MPs. Fish were subsampled (1 fish per aquarium) on days 4 and 7 of the exposure period and after 7 and 14 days of depuration.

2.6 Experiment III: Microplastics as potential transport vectors for organic pollutants

Larvae (10 dph) were randomly allocated to 16 beakers (n = 4 larvae/beaker) containing 300 ml ASW (0.5 ppt) and the beakers were assigned in duplicate to one of eight treatment groups; control (-MP), control (+MP), 32 (-MP), 32 (+MP), 320 (-MP), 320 (+MP), 3200 (-MP) and 3200 (+MP). The treatments were created by feeding for 14 days with *Artemia* that had been exposed (from 24 to 48 hph) to graded concentrations of BPA (0, 32, 320 or 3,200 µg BPA/l in ASW 20 g/l), in the absence (-MP) or presence of (+MP) 0.5 µm MPs. For each MP treatment, the plastics were exposed to the graded BPA concentrations for 24 hours prior to addition of the *Artemia* to allow sorption of the chemicals to the plastics. Prior to feeding the *Artemia* were rinsed with RO water to remove excess salt, BPA and any MPs that had not been ingested. Subsamples of *Artemia* were collected daily to confirm uptake of the MPs via fluorescent microscopy. During the exposure, faecal samples were removed daily to monitor egestion of the fluorescent MPs.

2.7 Fish sampling

Fish were sacrificed in a lethal dose (500 mg/l) of MS222 (3-aminobenzoic acid ethyl ester, methanesulfonate salt; Sigma, UK) buffered with sodium bicarbonate (Sigma, UK) to pH 7.4. Total lengths and wet body weight were recorded to the nearest 0.5 mm and 0.01 mg, respectively, for the larvae, and to the nearest 1 mm and 0.01 g, respectively, for the adults. Condition factors were derived by expressing the cube of length as a percentage of the wet body weight. Blood samples were removed from the caudal peduncle of the adult fish (experiment II) using heparinised micro haematocrit tubes to assess evidence for translocation of the MPs into the circulatory system. Whole larvae (from two replicate beakers per treatment for experiment I), or tissue samples (i.e. liver samples from the adult fish for experiment II or anterior proportion of the larvae up to and including the operculum region for experiment III) from each fish were then removed, fixed in RNAlater® and stored at -20°C for subsequent isolation of RNA. Larvae from the third replicate beaker for experiment I were fixed in buffered formalin for subsequent quantification of fluorescence.

2.8 Gene expression

Total RNA was isolated from each tissue sample (for experiment I, two 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 (A260nm; 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.

For each experiment, real-time polymerase chain reaction (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 (IGF1; 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 melting temperature (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 comparative C_T method. There was no evidence that the MP (or BPA exposure) 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 analyses expression of vitellogenin C was found to be 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 (vitellogenin B, CYP1A and IGF1). Duplicate data for each amplified gene of interest were averaged and relative expression calculated using the comparative C_T method with normalisation against expression of 18S rRNA in each sample.

2.9 Fluorescent determinations

Uptake of the fluorescent particles by the individual larvae (experiment I) and *Artemia* (experiments II and III) was confirmed using a Zeiss Lumar V12 stereo microscope with an AxioCam MRm camera and AxioVision software. Green fluorescence was detected using a Green Fluorescent Protein (GFP) filter (excitation 485 nm; emission 520 nm) and an exposure of 6.8 sec for larvae (magnification x21) and 4 sec for the *Artemia* (magnification x100). Fluorescence in water, faecal and fish tissues (relative to the respective control samples) was determined using a POLARstar OPTIMA (BMG Labtech) plate reader with a GFP filter (excitation 485 nm; emission 520 nm).

2.10 Statistical analysis

All data are expressed as the mean plus or minus standard error (±SE) of the mean; data were pooled within each treatment group after confirming that replicates did not differ significantly. To investigate effects of exposure to the MPs or BPA, data were compared to the relevant controls using SPSS Statistics version 20. For experiments I and II, data meeting the assumptions of normality and homogeneity of variance were analysed using univariate analysis of variance followed by a comparison to the control using a two-sided Dunnett t-test. For experiment III, plastics and BPA concentrations were included as factors in the univariate analysis of variance. Relationships between variables were calculated using Pearson correlation (two-tailed) coefficient.

Table 2.1 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
IGF1 (ENSGACG00000020042)	ACTGTGCACCTCCAAAGACC	CTGCACTGCGGTACTAACCA

3 Results

3.1 Experiment I

Fluorescence could not be detected (using the plate reader) within the water samples removed from each beaker. However, fluorescence detected in the detritus sampled from the bottom of the beakers was 1.76 ± 0.14, 8.53 ± 0.88 and 65.16 ± 7.37-fold higher, for the low, medium and high exposure groups, respectively, than fluorescence measured in the control beakers, indicating that the plastics were not remaining in suspension. The larvae were observed to feed on the plastics and fluorescence was 1.23, 1.50 and 1.91-fold higher in larvae from the low, medium and high exposures, respectively, than fluorescence measured in the control larvae (Figure 3.1).

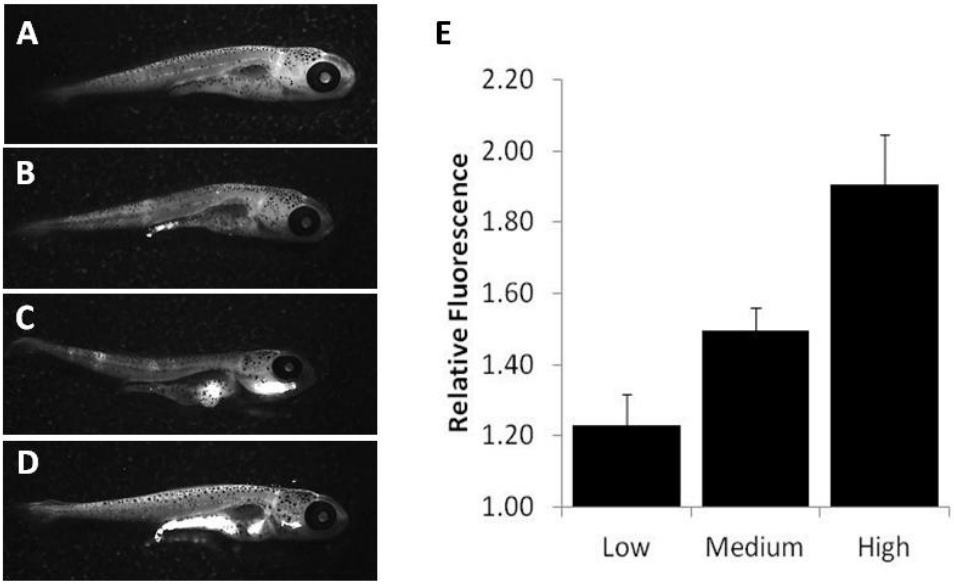

Figure 3.1 Images of three-spined stickleback larvae held under either control (A) conditions or exposed for 7 days to low (B), medium (C) or high (D) densities of green fluorescing microplastic spheres showing a clear accumulation of the fluorescing plastics in the digestive tracts of the exposed larvae. Figure E shows the mean (±SE) fluorescent intensity, relative to the controls, measured in larvae from each treatment.

Ingestion of the MPs did not impact survival of the larvae during the 7-day exposure. A positive relationship between quantity of plastics added and total larval length was observed ($r = 0.419$; $p<0.001$, $n = 144$); the larvae in the medium and high exposure groups were 5.1% ($p<0.05$) and 9.7% ($p<0.01$) longer, respectively, than those in the control group (Figure 3.2A). However, there was no evidence that exposure to the plastics resulted in a proportional increase in body weight (Figure 3.2B) and while the expected positive relationship between body length and weight was observed in larvae sampled from both the control ($r = 0.408$; $p<0.05$, $n = 36$) and low ($r = 0.512$; $p<0.01$, $n = 36$) exposure groups this relationship was not evident in the medium ($r = 0.172$; $p>0.05$, $n = 36$) and high ($r = 0.222$; $p>0.05$, $n = 36$) exposure groups. As a consequence a negative relationship between quantity of plastics added to the beaker and condition factor was observed ($r = -0.380$; $p<0.001$, $n = 144$); condition factors in

the low, medium and high exposures were 14.6% ($p<0.05$), 18.9% ($p<0.01$) and 31.6% lower ($p<0.01$), respectively, when compared to the controls (Figure 3.2C).

There was no evidence that exposure to the plastics altered expression of the gene encoding vitellogenin ($p>0.05$), relative to the controls. However, increases in CYP1A expression were observed in the medium (1.91 ± 0.215; $p<0.05$) and high (1.92 ± 0.363; $p<0.05$) exposed larvae, relative to the controls (Figure 3.2D).

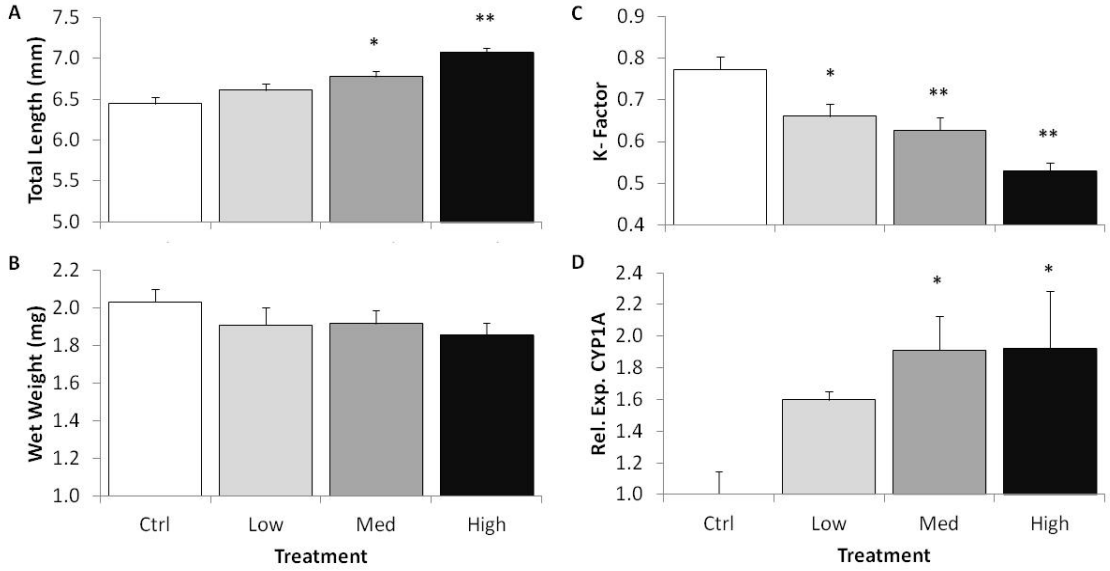

Figure 3.2 Mean total body length (A), wet body weight (B), condition factor (C) and relative expression of the CYP1A gene (D) in larval three-spined stickleback maintained from 7 to 14 days post hatch either under control conditions or in the presence of low, medium or high densities of 1 µm polymer spheres. Each column represents the mean ±SE. Significant differences between the control and exposure groups are denoted as * $p<0.05$ or ** $p<0.01$

3.2 Experiment II

The preliminary exposures with *Artemia* confirmed the suitability of this organism as a vector for trophic transfer of both the 1.0 µm and 10 µm green fluorescing particles. Consistent with reports that *Artemia nauplii* do not develop a complete digestive tract until the 2nd larval moult, there was no evidence for uptake of the plastic particles in *Artemia* (instar I) sampled 8 hours post-exposure. However, from 24 hours of exposure (instar II) fluorescent particles could be observed within the digestive tract (Figure 3.3). Comparison of the *Artemia* sampled from the control and MP treatments at each time-point suggest that ingestion of the plastic particles did not negatively impact *Artemia* survival or development.

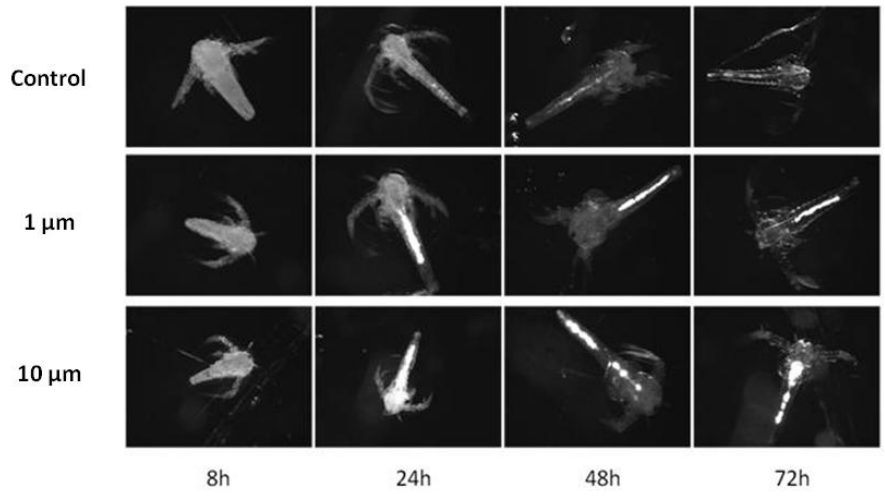

Figure 3.3 *Artemia nauplii* that had been maintained under either control conditions (from <24 hours post hatch) or exposed to 1.0. A clear accumulation of the fluorescing plastics could be observed in the digestive tracts of the exposed *Artemia* after 24, 48 and 72 hours of exposure

Faecal samples were removed daily from each exposure vessel to determine whether the fish were egesting (and consequently ingesting) the MP-contaminated *Artemia*. Due to the natural auto-fluorescence of the *Artemia*, fluorescence was detectable in the faeces removed from the control fish (11.4 ± 0.36 AU, n = 125) throughout the study. However, fluorescence in faeces collected from fish fed the MP-contaminated *Artemia* was consistently higher than fluorescence measured in faeces collected from the control fish (Figure 3.4). These higher levels of fluorescence were detected in all of the MP treatment groups from only 1 day of exposure and consistent with expectation fluorescence was higher in fish fed 100% contaminated *Artemia* (high), compared to fish fed 10% (low) contaminated *Artemia* (Figure 3.4). On day 9 of the study (2 days after ceasing exposure to the MP-contaminated *Artemia*) a 57–62% decrease in fluorescence was observed in fluorescence collected from the high exposure groups (Figure 3.4) and fluorescence further decreased to background levels for the remainder of the depuration period. However, visual examination of the faecal samples revealed that the fish were still excreting small numbers of fluorescent MPs even after 14 days of feeding on non-contaminated *Artemia*.

Ingestion of the MPs did not affect survival, length, weight or body condition of the adult fish during the 7-day exposure. There was also no evidence that uptake of the plastic spheres via the diet altered expression of the gene encoding vitellogenin ($p>0.05$) or CYP1A ($p>0.05$), relative to the controls (data not shown). Body length, weight and body condition was also comparable between treatment groups in fish sampled 7 and 14 days after ceasing exposure ($p>0.05$; data not shown).

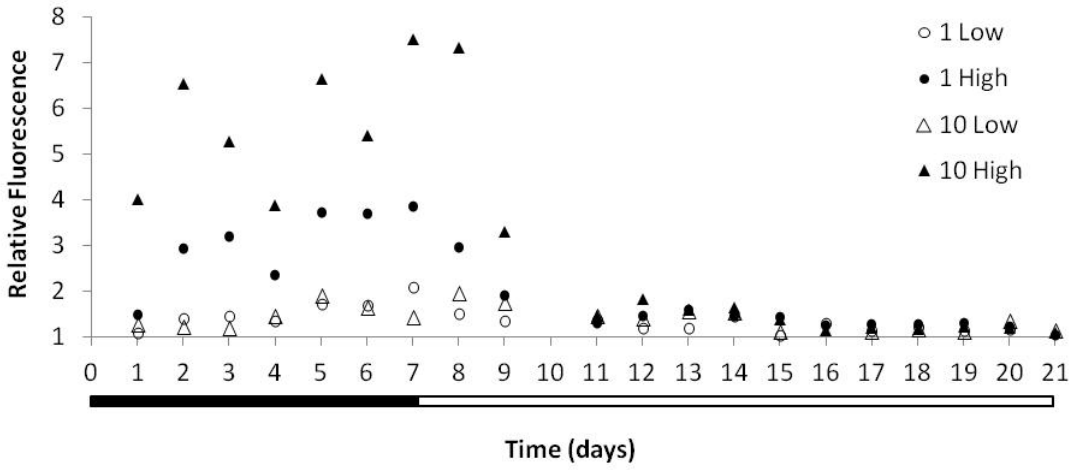

Figure 3.4 Fluorescence, relative to controls, in faecal samples collected from adult three-spined stickleback fed for 7 days on a 10% (low) or 100% (high) diet of plastic-contaminated *Artemia* (exposed for between 48 and 72 hours post hatch (hph) to 1.0 µm or 10 µm green fluorescing microplastic spheres). The control and low exposure groups were fed 100% and 90%, respectively, non-exposed *Artemia* (<72 hph). After the 7-day exposure period (denoted by the black bar under the x-axis) all fish were maintained for a further 14 days (denoted by the white bar under the x-axis) on a diet of non-exposed *Artemia* (<72 hph).

3.3 Experiment III

There was no evidence that exposure to BPA and/or the plastic spheres affected survival of the *Artemia*. However, visual examination of the *Artemia* did suggest that, as BPA concentration increased, the quantity of fluorescence in the digestive tract of the *Artemia* exposed to both BPA and MPs decreased, which may indicate a negative influence of BPA on feeding activity of the *Artemia*. Consistent with this, the fluorescence measured in faeces collected from fish fed the *Artemia* exposed to 32, 320 and 3200 µg/l BPA (+MP) was 35, 51 and 29% lower than that measured in the faeces collected from fish fed *Artemia* exposed to MPs in the absence of BPA (Figure 3.5), although for all treatments fluorescence did remain higher than in faeces collected from fish fed the non-MP-contaminated diets.

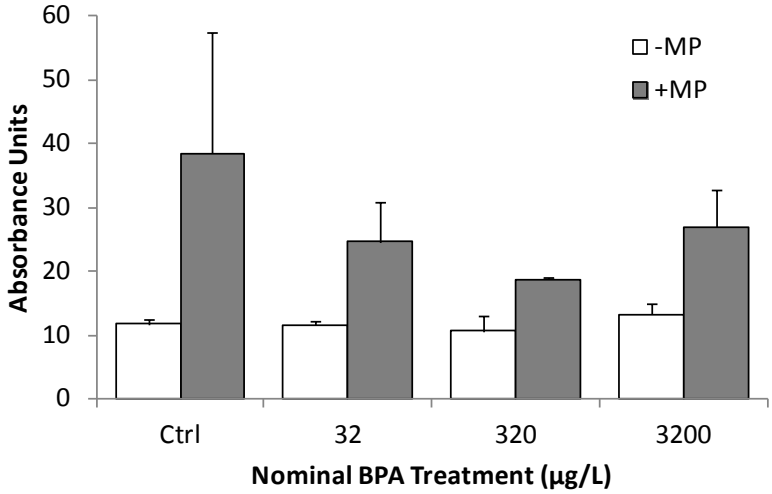

Figure 3.5 Fluorescence in faecal samples collected from larval three-spined stickleback fed for 14 days on a diet of *Artemia* that had been either held as controls (no bisphenol-A; BPA) or exposed to graded concentrations of BPA (nominal concentrations shown on the x-axis) in the presence (grey bars) or absence (white bars) of 0.5 µm green fluorescing microplastic spheres. Each bar represents the mean ±SE for the duplicate vessels for each treatment.

Ingestion of *Artemia* that had been exposed to MPs and/or BPA did not affect fish survival, weight, length and condition, or expression of the vitellogenin gene. Furthermore, there was no evidence that ingesting the MP and/or BPA-exposed *Artemia* influenced expression of CYP1A, when compared to the relevant controls. However, a positive relationship between BPA concentration and relative expression of CYP1A was observed in the fish fed *Artemia* exposed to BPA in the presence of MPs ($r = 0.415$, $p<0.05$, $n = 31$), that was not evident in fish fed BPA-exposed *Artemia* in the absence of MPs ($r = -0.212$, $p>0.05$, $n = 29$).

4 Discussion

Plastics have become a ubiquitous environmental contaminant and each year large numbers of aquatic vertebrates are reported to die as a consequence of ingesting macroplastics or becoming entangled in plastic debris. Until now relatively little has been known about the potential hazard posed by MPs; however, the results of this investigation suggest that MPs may pose a hazard to early life-stages of fish. Larval fish were observed to actively feed upon the MPs and to accumulate large quantities of fluorescing particles within their digestive system. This ingestion resulted in impacts for health with body condition decreasing by 14% to 32% after 7 days of exposure depending on exposure densities.

It has recently been shown that ingestion of MPs can negatively impact algal feeding in zooplankton (<10 µm sized particles; Cole et al. 2013) and decrease body weight of lugworms (400–1,300 µm sized particles; Besseling et al. 2012), but to date, only one other study has considered the effects of direct exposure to MPs on the health of an aquatic vertebrate (Oliveira et al. 2013). In that study there was no evidence that a 96-hour exposure to MPs (1–5 µm) at densities of 18.4 and 184 µg/l impacted survival of juvenile common goby (Oliveira et al. 2013); effects on body size were not measured. In our study, there was also no evidence for an impact of MPs on survival of the three-spined stickleback larvae during the 7-day exposure; however, a negative effect on body condition was observed for all exposure groups. Surprisingly, the reduction in larval condition factor appeared to result from a small but significant increase in body length, rather than a direct effect on body weight.

The mechanism underlying the MP-induced increase in body length is unknown. There are reports that polystyrene can leach styrene oligomers which are weakly oestrogenic (Bang et al. 2012); 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 stickleback (Hahlbeck et al. 2004). However, the lack of evidence for an effect of MP exposure on expression of vitellogenin mRNA would suggest that biologically active concentrations of oestrogenic compounds were not leaching from the plastics. Furthermore, there was no evidence for an effect of MP exposure on expression of IGF1, further indicating that the increased growth was unlikely to be a direct consequence of perturbation of the endocrine system. However, a relationship between quantity of MP ingested and expression of the CYP1A gene was observed. This may indicate leaching of organic compounds from the MPs; CYP1A is an important enzyme involved in detoxification of xenobiotics (Široká and Drastichová 2004). As cytochrome P450s also play a role in endogenous metabolism, the MP-induced increase in CYP1A may have indirectly increased endogenous sterol biosynthesis and thus increased energy for somatic growth, although further work would be necessary to confirm this. Irrespective of the mechanism underlying the effects of MPs on somatic growth, it is important to note that as body weight did not increase proportionally to body length it is unlikely that the growth-promoting effects of the MPs would be sustainable in the longer term and continued exposure to MPs would be likely to result in starvation-induced mortality in the longer term.

In the second experiment, uptake of the MPs via ingestion of MP-contaminated *Artemia* (trophic transfer) was investigated. Consistent with a previous study in which a range of zooplankton were demonstrated to have the capacity to ingest 1.7–30.6 µm polystyrene beads (Cole et al. 2013), the *Artemia* were observed to ingest both the 1.0 µm and 10 µm polystyrene bead plastic particles once they had undergone their first moult (<48 hph) without any discernible effects on survival or development relative to the control *Artemia*, confirming their suitability as a vector for our studies. In contrast to

the effects observed in the larvae, however, there was no evidence for an effect of MP ingestion, via the diet, on body size and condition of adult three-spined stickleback. Analysis of faeces samples collected from the fish fed the MP-contaminated *Artemia* confirmed that the fish were ingesting (and egesting) the MP-contaminated *Artemia*. However, although small numbers of MP particles could still be observed in the faeces up to 14 days after ceasing exposure, more than 40% of MPs were egested within 48 hours, which is consistent with reports that MPs are also rapidly egested by zooplankton, lugworms and mussels (Browne et al. 2008, Besseling et al. 2012, Cole et al. 2013).

Given the rapid egestion of the MPs, the lack of an effect of MPs on somatic growth, body weight or even body condition of the adults is therefore not surprising. However, there was also no evidence for an effect of ingested MPs on CYP1A mRNA expression in the adults, which may indicate that the adults were not exposed to sufficient MPs to induce a response. It should be noted that in the larval fish, CYP1A was isolated from whole body homogenates, whereas for the adults CYP1A was isolated from the liver. There is some evidence that intestinal expression of CYP1A in mammals can be regulated by the Toll-like receptor 2 (TLR2) which is involved in pathogen recognition (Do et al. 2012). Orthologs of TLR2 have been identified in teleost fish and it is possible that the increased expression of CYP1A mRNA observed in the larvae was a consequence of microbes adhered to the MPs (Zettler et al. 2013) inducing an immune response. This would be an interesting area of research for future studies.

It is also important to note that the size of the plastics relative to the size of the animal is likely to be an important consideration when considering the effects of the plastics (Besseling et al. 2012); it is possible that induction of CYP1A in the larvae was an indirect consequence of altered metabolism caused by blockages in the gut that did not occur in the adults due to their greater relative size. Furthermore, despite reports that MPs may translocate from the digestive system to other organs within the organism where they have the potential to induce biological effects (Browne et al. 2008), we found no clear evidence for translocation of the MPs to the circulatory system of the adult fish. Thus, at least under the exposure scenario described here, ingestion of zooplankton contaminated with 1–10 µm plastics does not appear to pose a significant hazard in this context. Although these results indicate that ingestion of small (1–10 µm) MPs is unlikely to present a significant biological hazard for larger-bodied fish in the short term, further investigations using both larger plastics and longer exposure periods are necessary before it can be concluded that trophic transfer of small MPs does not pose a threat.

Additional to the potential for ingested MPs to induce biological effects, there are also concerns that MPs may act as vectors to transport organic pollutants through the food chain (Teuten et al. 2009). Hydrophobic organic contaminants have been shown to have an affinity to adsorb to plastics and have been detected on plastic pellets collected from the marine environment (Mato et al. 2001, 2002, Endo et al. 2005, Rios et al. 2007, Teuten et al. 2007, 2009, Bakir et al. 2012, 2014, Antunes et al. 2013). Furthermore, there is some evidence for plastic-mediated transfer of organic contaminants to organisms (Ryan et al. 1988, Teuten et al. 2007, Besseling et al. 2012), although little is known about the potential for accumulation of these contaminants through the food chain. In this experiment, fish larvae were exposed via their diet to live *Artemia* that had been exposed to graded concentrations of BPA in the presence or absence of MPs. A positive relationship between nominal BPA concentration and expression of the CYP1A gene was observed in fish fed *Artemia* that had been exposed to BPA in the presence of the MPs, but no such relationship was observed for fish fed *Artemia* exposed to BPA in the absence of MPs. As the fish were not directly exposed to the BPA but only indirectly exposed through feeding on the *Artemia* that had ingested the BPA-contaminated MPs, this would suggest that the MPs can act as vectors to transport BPA through the food chain and that BPA is desorbing

from the plastics at concentrations sufficient to result in a detectable response to exposure as indicated by CYP1A expression.

Collectively, these experiments demonstrate that fish will actively take up microplastics from the water column, as well as ingesting them via their diet. Although ingestion of the micron-sized plastics does not appear to adversely impact the survival or health of adult fish, at least in the short term, there is evidence to support negative changes in the body condition of larval fish. Furthermore, there was evidence that MPs have the potential to partition an organic pollutant and act as a vector to transport this chemical into the food chain. These results highlight the need for longer-term studies that can more fully evaluate the environmental impacts of plastic ingestion for aquatic organisms.

References

Andrady, A.L. and Neal, M.A. (2009) Applications and societal benefits of plastics. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 364(1526), 1977–1984.

Andrady, A. L. (2011). Microplastics in the marine environment. Marine Pollution Bulletin, 62(8), 1596-1605.

Antunes, J.C., Frias, J.G.L., Micaelo, A.C. and Sobral, P. (2013) Resin pellets from beaches of the Portuguese coast and adsorbed persistent organic pollutants. *Estuarine, Coastal and Shelf Science*, 130, 62–69.

Bakir, A., Rowland, S.J. and Thompson, R.C. (2012) Competitive sorption of persistent organic pollutants onto microplastics in the marine environment. *Marine Pollution Bulletin*, 64(12), 2782–2789.

Bakir, A., Rowland, S.J. and Thompson, R.C. (2014) Transport of persistent organic pollutants by microplastics in estuarine conditions. *Estuarine, Coastal and Shelf Science,* 140, 14–21.

Bang, D.Y., Kyung, M., Kim, M.J., Jung, B.Y., Cho, M.C., Choi, S.M., Kim, Y.W., Lim, S.K., Lim, D.S., Won, A.J., Kwack, S.J., Lee, Y., Kim, H.S. and Lee, B.M. (2012) Human risk assessment of endocrine-disrupting chemicals derived from plastic food containers. *Comprehensive Reviews in Food Science and Food Safety*, 11(5), 453–470.

Barnes, D.K., Galgani, F., Thompson, R.C. and Barlaz, M. (2009) Accumulation and fragmentation of plastic debris in global environments*. Philosophical Transactions of the Royal Society B: Biological Sciences*, 364(1526), 1985–1998.

Besseling, E., Wegner, A., Foekema, E.M., van den Heuvel-Greve, M.J. and Koelmans, A.A. (2012) Effects of microplastic on fitness and PCB bioaccumulation by the lugworm *Arenicola marina* (L.). *Environmental Science & Technology*, 47(1), 593–600.

Bjorndal, K.A., Bolten, A.B. and Lagueux, C.J. (1994) Ingestion of marine debris by juvenile sea turtles in coastal Florida habitats. *Marine Pollution Bulletin*, 28(3), 154–158.

Boerger, C.M., Lattin, G.L., Moore, S.L. and Moore, C.J. (2010) Plastic ingestion by planktivorous fishes in the North Pacific Central Gyre. *Marine Pollution Bulletin*, 60(12), 2275–2278.

Browne, M.A., Galloway, T. and Thompson, R. (2007) Microplastic – an emerging contaminant of potential concern? *Integrated Environmental Assessment and Management*, 3(4), 559–561.

Browne, M.A., Dissanayake, A., Galloway, T.S. and Lowe, D.M. (2008) Ingested microscopic plastic translocates to the circulatory system of the mussel, *Mytilus edulis*. *Environmental Science & Technology*, 42(13), 5026–5031.

Browne, M.A., Crump, P., Niven, S.J., Teuten, E., Tonkin, A., Galloway, T. and Thompson, R. (2011) Accumulation of microplastic on shorelines worldwide: sources and sinks. *Environmental Science & Technology*, 45(21), 9175–9179.

Carpenter, E.J., Anderson, S.J., Harvey, G.R., Miklas, H.P. and Peck, B.B. (1972) Polystyrene spherules in coastal waters. *Science*, 178(4062), 749–750.

Cole, M., Lindeque, P., Halsband, C. and Galloway, T.S. (2011) Microplastics as contaminants in the marine environment: a review. *Marine Pollution Bulletin*, 62(12), 2588–2597.

Cole, M., Lindeque, P., Fileman, E., Halsband, C., Goodhead, R., Moger, J. and Galloway, T.S. (2013) Microplastic ingestion by zooplankton. *Environmental Science & Technology*, 47(12), 6646–6655.

Davison, P. and Asch, R.G. (2011) Plastic ingestion by mesopelagic fishes in the North Pacific Subtropical Gyre. *Marine Ecology Progress Series*, 432, 173–180.

Derraik, J.G. (2002) The pollution of the marine environment by plastic debris: a review. *Marine Pollution Bulletin*, 44(9), 842–852.

Do, K.N., Fink, L.N., Jensen, T.E., Gautier, L. and Parlesak, A. (2012) TLR2 controls intestinal carcinogen detoxication by CYP1A1. *PloS one*, 7(3), e32309.

Endo, S., Takizawa, R., Okuda, K., Takada, H., Chiba, K., Kanehiro, H., Ogi, H., Yamashita, R. and Date, T. (2005) Concentration of polychlorinated biphenyls (PCBs) in beached resin pellets: variability among individual particles and regional differences. *Marine Pollution Bulletin,* 50, 1103–1114.

European Commission (DG Environment) (2011) *Plastic waste in the environment*. Revised final report.

Fendall, L.S. and Sewell, M.A. (2009) Contributing to marine pollution by washing your face. Microplastics in facial cleansers. *Marine Pollution Bulletin,* 58(8), 1225–1228.

Foekema, E.M., De Gruijter, C., Mergia, M.T., van Franeker, J.A., Murk, A.J. and Koelmans, A.A. (2013) Plastic in North Sea fish. *Environmental Science & Technology*, 47(15), 8818–8824.

Gregory, M. (1996) Plastic 'scrubbers' in hand cleansers: a further (and minor) source for marine pollution identified. *Marine Pollution Bulletin,* 32(12), 867–871.

Gregory, M.R. (2009) Environmental implications of plastic debris in marine settings – entanglement, ingestion, smothering, hangers-on, hitch-hiking and alien invasions. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 364(1526), 2013–2025.

Hahlbeck, E., Griffiths, R. and Bengtsson, B.E. (2004) The juvenile three-spined stickleback (*Gasterosteus aculeatus*) as a model organism for endocrine disruption: I. Sexual differentiation. *Aquatic Toxicology*, 70(4), 287–310.

Heskett, M., Takada, H., Yamashita, R., Yuyama, M., Ito, M., Geok, Y.B. and Mermoz, J. (2012) Measurement of persistent organic pollutants (POPs) in plastic resin pellets from remote islands: toward establishment of background concentrations for International Pellet Watch. *Marine Pollution Bulletin*, 64(2), 445–448.

Karapanagioti, H.K., Endo, S., Ogata, Y. and Takada, H. (2011) Diffuse pollution by persistent organic pollutants as measured in plastic pellets sampled from various beaches in Greece. *Marine Pollution Bulletin,* 62, 312–317.

Katsiadaki, I., Williams, T.D., Ball, J.S., Bean, T.P., Sanders, M.B., Wu, H., Santos, E.M., Brown, M.M., Baker, P., Ortega, F., Falciani, F., Craft, J.A., Tyler, C.R., Viant, M.R. and Chipman, J.K. (2010) Hepatic transcriptomic and metabolomic responses in the stickleback (*Gasterosteus aculeatus*) exposed to ethinyl-estradiol. *Aquatic Toxicology*, 97(3), 174–187.

Lithner, D., Larsson, Å. and Dave, G. (2011) Environmental and health hazard ranking and assessment of plastic polymers based on chemical composition. *Science of the Total Environment*, 409(18), 3309–3324.

Lusher, A.L., McHugh, M. and Thompson, R.C. (2013) Occurrence of microplastics in the gastrointestinal tract of pelagic and demersal fish from the English Channel. *Marine Pollution Bulletin*, 67(1), 94–99.

Mato, Y., Isobe, T., Takada, H., Kanehiro, H., Ohtake, C. and Kaminuma, T. (2001) Plastic resin pellets as a transport medium for toxic chemicals in the marine environment. *Environmental Science & Technology*, 35(2), 318–324.

Mato, Y., Takada, H., Zakaria, M. P., Kuriyama, Y., & Kanehiro, H. (2002). Toxic chemicals contained in plastic resin pellets in the marine environment: Spatial difference in pollutant concentrations and the effects of resin type. *Environmental Science* (Japan).

Maynard, A. (2006) *Nanotechnology: a research strategy for addressing risk* [online]. Woodrow Wilson International Center for Scholars Project on Emerging Nanotechnologies. Available at: http://www.nanotechproject.org/file_download/files/PEN3_Risk.pdf

Moore, C.J. (2008) Synthetic polymers in the marine environment: a rapidly increasing, long-term threat. *Environmental Research*, 108(2), 131–139.

Oliveira, M., Ribeiro, A., Hylland, K. and Guilhermino, L. (2013) Single and combined effects of microplastics and pyrene on juveniles (0+ group) of the common goby *Pomatoschistus microps* (Teleostei, Gobiidae). *Ecological Indicators*, 34, 641–647.

OSPAR (2009) *Marine litter in the North-East Atlantic Region: assessment and priorities for response*. London, 127 pp.

Reddy, M.S. and Shaik, B. (2006) Description of the small plastics fragments in marine sediments along the Alang-Sosiya ship-breaking yard, India. *Estuarine, Coastal and Shelf Science,* 68(3–4), 656–660.

Rios, L.M., Moore, C. and Jones, P.R. (2007) Persistent organic pollutants carried by synthetic polymers in the ocean environment. *Marine Pollution Bulletin,* 54(8), 1230–1237.

Roy, P.K., Hakkarainen, M., Varma, I.K. and Albertsson, A.C. (2011) Degradable polyethylene: fantasy or reality. *Environmental Science & Technology*, 45(10), 4217–4227.

Ryan, P.G., Connell, A.D. and Gardner, B.D. (1988) Plastic ingestion and PCBs in seabirds: is there a relationship? *Marine Pollution Bulletin*, 19(4), 174–176.

Ryan, P.G., Moore, C.J., van Franeker, J.A. and Moloney, C.L. (2009) Monitoring the abundance of plastic debris in the marine environment. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 364(1526), 1999–2012.

Široká, Z. and Drastichová, J. (2004) Biochemical marker of aquatic environment contamination-cytochrome P450 in fish. A review. *Acta Veterinaria Brno*, 73(1), 123–132.

Tanaka, K., Takada, H., Yamashita, R., Mizukawa, K., Fukuwaka, M. A., & Watanuki, Y. (2013). Accumulation of plastic-derived chemicals in tissues of seabirds ingesting marine plastics. Marine pollution bulletin, 69(1), 219-222.

Talsness, C.E., Andrade, A.J., Kuriyama, S.N., Taylor, J.A. and vom Saal, F.S. (2009) Components of plastic: experimental studies in animals and relevance for human health. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 364(1526), 2079–2096.

Teuten, E.L., Rowland, S.J., Galloway, T.S. and Thompson, R.C. (2007) Potential for plastics to transport hydrophobic contaminants. *Environmental Science & Technology,* 41 (22), 7759–7764.

Teuten, E.L., Saquing, J.M., Knappe, D.R., Barlaz, M.A., Jonsson, S., Björn, A. and Takada, H. (2009) Transport and release of chemicals from plastics to the environment and to wildlife. *Philosophical Transactions of the Royal Society B: Biological Sciences*, 364(1526), 2027–2045.

Thompson, R.C., Olsen, Y., Mitchell, R.P., Davis, A., Rowland, S.J., John, A.W., McGonigle, D. and Russell, A.E. (2004) Lost at sea: where is all the plastic? *Science*, 304(5672), 838–838.

Verlis, K.M., Campbell, M.L. and Wilson, S.P. (2013) Ingestion of marine debris plastic by the wedge-tailed shearwater *Ardenna pacifica* in the Great Barrier Reef, Australia. *Marine Pollution Bulletin*, 72(1), 244–249.

Williams, T.D., Wu, H., Santos, E.M., Ball, J., Katsiadaki, I., Brown, M.M., Baker, P., Ortega, F., Falciani, F., Craft, J.A., Tyler, C.R., Chipman, J.K. and Viant, M.R. (2009) Hepatic transcriptomic and metabolomic responses in the stickleback (*Gasterosteus aculeatus*) exposed to environmentally relevant concentrations of dibenzanthracene. *Environmental Science & Technology*, 43(16), 6341–6348.

Zettler, E.R., Mincer, T.J. and Amaral-Zettler, L.A. (2013) Life in the ‘plastisphere’: microbial communities on plastic marine debris. *Environmental Science & Technology*, 47(13), 7137–7146.

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