



Heriot-Watt University
Research Gateway

Assessment of microplastic-sorbed contaminant bioavailability through analysis of biomarker gene expression in larval zebrafish

Citation for published version:

Sleight, VA, Bakir, A, Thompson, RC & Henry, TB 2017, 'Assessment of microplastic-sorbed contaminant bioavailability through analysis of biomarker gene expression in larval zebrafish', *Marine Pollution Bulletin*, vol. 116, no. 1-2, pp. 291-297. <https://doi.org/10.1016/j.marpolbul.2016.12.055>

Digital Object Identifier (DOI):

[10.1016/j.marpolbul.2016.12.055](https://doi.org/10.1016/j.marpolbul.2016.12.055)

Link:

[Link to publication record in Heriot-Watt Research Portal](#)

Document Version:

Publisher's PDF, also known as Version of record

Published In:

Marine Pollution Bulletin

Publisher Rights Statement:

0025-326X/© 2016 The Authors. Published by Elsevier Ltd. This is an open access article under the CC BY license (<http://creativecommons.org/licenses/by/4.0/>)

General rights

Copyright for the publications made accessible via Heriot-Watt Research Portal is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy

Heriot-Watt University has made every reasonable effort to ensure that the content in Heriot-Watt Research Portal complies with UK legislation. If you believe that the public display of this file breaches copyright please contact open.access@hw.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.



Assessment of microplastic-sorbed contaminant bioavailability through analysis of biomarker gene expression in larval zebrafish



Victoria A. Sleight^{a,b,c,*}, Adil Bakir^{a,d,e}, Richard C. Thompson^a, Theodore B. Henry^{a,c,f,g}

^a School of Marine Science and Engineering, Marine Biology and Ecology Research Centre, University of Plymouth, Plymouth PL4 8AA, UK

^b British Antarctic Survey, Natural Environment Research Council, High Cross, Madingley Road, Cambridge, CB3 0ET, UK

^c Institute of Life and Earth Sciences, School of Energy, Geoscience, Infrastructure and Society, Heriot-Watt University, Edinburgh EH14 4AS, UK

^d Petroleum and Environmental Geochemistry Group, School of Geography Earth and Environmental Sciences, University of Plymouth, Drake Circus, Plymouth, Devon PL4 8AA, UK

^e School of Earth & Environmental Sciences, University of Portsmouth, Burnaby Road, Portsmouth PO1 3QL, UK

^f Center for Environmental Biotechnology, University of Tennessee, Knoxville, TN 37996, USA

^g Department of Forestry, Wildlife and Fisheries, University of Tennessee, Knoxville, TN 37996, USA

ARTICLE INFO

Article history:

Received 23 September 2016

Received in revised form 21 November 2016

Accepted 18 December 2016

Available online 12 January 2017

Keywords:

Danio rerio

Microplastics

Phenanthrene

17 α -ethinylestradiol

Sorption

Bioavailability

ABSTRACT

Microplastics (MPs) are prevalent in marine ecosystems. Because toxicants (termed here “co-contaminants”) can sorb to MPs, there is potential for MPs to alter co-contaminant bioavailability. Our objective was to demonstrate sorption of two co-contaminants with different physicochemistries [phenanthrene (Phe), $\log_{10}K_{ow} = 4.57$; and 17 α -ethinylestradiol (EE2), $\log_{10}K_{ow} = 3.67$] to MPs; and assess whether co-contaminant bioavailability was increased after MP settlement. Bioavailability was indicated by gene expression in larval zebrafish. Both Phe and EE2 sorbed to MPs, which reduced bioavailability by a maximum of 33% and 48% respectively. Sorption occurred, but was not consistent with predictions based on co-contaminant physicochemistry (Phe having higher $\log_{10}K_{ow}$ was expected to have higher sorption). Contaminated MPs settled to the bottom of the exposures did not lead to increased bioavailability of Phe or EE2. Phe was 48% more bioavailable than predicted by a linear sorption model, organism-based measurements therefore contribute unique insight into MP co-contaminant bioavailability.

© 2016 The Authors. Published by Elsevier Ltd. This is an open access article under the CC BY license (<http://creativecommons.org/licenses/by/4.0/>).

1. Introduction

Plastic debris is accumulating in the environment on a global scale and the potential for negative effects on ecosystems has become a priority concern for environmental science and policy (Gregory, 2009). Around 300 million tonnes of plastic are produced every year (Browne et al., 2011; Shim and Thomposon, 2015), and disposal of many of these plastic products leads directly or indirectly to their arrival in aquatic environments. Plastic debris is present in numerous shapes and sizes and particles <5 mm (MPs) in size are reported as the most abundant sizes detected in large-scale assessments of the oceans (Eriksen et al., 2014). Over the last decade, investigations have reported the presence of MPs in sediments (Reddy et al., 2006) and in pelagic zones (Collignon et al., 2012); however, the ecological implications of MPs are unknown. One concern is the potential for MPs to transport harmful pollutants (termed here “co-contaminants”) sorbed from marine environments (Holmes et al., 2012; Mato et al., 2001; Rochman et

al., 2013a) into the food chain (Teuten et al., 2007). Although co-contaminant concentrations can be up to a million times higher on the surface of MPs compared to surrounding seawater (Mato et al., 2001), and many of these MPs with sorbed co-contaminants can be transported to sediment surfaces, there is little information on the bioavailability of co-contaminants to organisms.

Numerous organisms ingest MPs and there is potential that co-contaminants sorbed from the environment are desorbed upon entering the different conditions (pH, ion concentration, etc.) within the lumen of the gastrointestinal tract. Seabirds that have ingested MPs have been reported to have elevated amounts of polychlorinated biphenyls (PCBs) and other persistent organic contaminants (Colabuono et al., 2010; Tanaka et al., 2013), although whether the increased amounts of these contaminants is a consequence of desorption from ingested MPs is unknown. A recent article suggests that MPs ingested by seabirds act as passive samples of the persistent organic pollutants (POPs) that have accumulated within the animal rather than contributing to the accumulation of POPs (Herzke et al., 2016). Recently MPs have been shown to alter the bioavailability of pyrene and PCBs in vertebrates and invertebrates (Avio et al., 2015; Besseling et al., 2013; Oliveira et al., 2013; Rochman et al., 2013b). Previous studies measured the concentration of co-contaminants in different tissues after MP/co-contaminant exposures (Besseling et al., 2013; Oliveira et al., 2013), and some

* Corresponding author at: British Antarctic Survey, Natural Environment Research Council, High Cross, Madingley Road, Cambridge CB3 0ET, UK.
E-mail address: viceig15@bas.ac.uk (V.A. Sleight).

previous work also used gene expression to indicate toxicological effects of MP/co-contaminant exposure (Avio et al., 2015; Rochman et al., 2013b). In order to understand and predict the risks of MPs and MP-sorbed co-contaminants in the environment, more data is required on the bioavailability of sorbed co-contaminants.

The term bioavailability is defined as the extent to which a compound enters tissue and reacts with biological molecules (Semple et al., 2004). Bioavailability can be difficult to assess as there are numerous potentially interacting factors including temperature, interactions with other chemicals, pH, salinity, and organism-specific variables (e.g. digestive system function, respiration, etc.). A relevant means of measuring bioavailability is to use a characterized organism response to a specific substance to indicate the level of bioavailability. Changes in expression of biomarker genes have been used to assess bioavailability of endocrine disrupting substances [e.g. 17 α -ethinylestradiol (EE2)] (Park et al., 2010) and metals (e.g., Hg²⁺) (Henry et al., 2013) sorbed to engineered nanomaterials, and a similar approach will be useful for investigating bioavailability of co-contaminants associated with MPs.

Bioavailability of co-contaminants sorbed to MPs is likely to be related to the physicochemical properties of MPs and the co-contaminants. Various contaminants, with different physicochemical properties, have been shown to interact with MPs in both the environment and laboratory, and co-contaminants that are more hydrophobic appear to be more strongly sorbed and likewise less likely to desorb (Bakir et al., 2012; Frias et al., 2010; Teuten et al., 2009). Phenanthrene [Phe, (\log_{10} of the octanol:water partition coefficient ($\log_{10} K_{ow}$) = 4.57 (Miller et al., 1985)] is a simple three ringed polycyclic aromatic hydrocarbon (PAH) that has been frequently reported as a co-contaminant sorbed to MPs (Mato et al., 2001). PAHs are of concern due to their persistence in the environment as well as their carcinogenic and mutagenic properties (Jennings, 2012; Teranishi et al., 1975). Investigation of sorption of Phe to unplasticized polyvinyl chloride (uPVC, 200–250 μ m) resulted in determination of a distribution coefficient, K_d value, for Phe onto uPVC of 2285 ± 693 L kg⁻¹ (Bakir et al., 2012) and desorption rate of 1.04 ± 0.05 K(day⁻¹) (Teuten et al., 2007). Furthermore, gut surfactants have been shown to increase the desorption rate of Phe in vitro, 2.29 ± 1.40 K(day⁻¹) (Teuten et al., 2007; Voparil and Mayer, 2000), but desorption and bioavailability of Phe sorbed to MPs has not been determined in vivo. In contrast to Phe, 17 α -ethinylestradiol (EE2, a synthetic oestrogenic substance) is less hydrophobic ($\log_{10} K_{ow}$ = 3.67 (Hansch et al., 1995)) and sorption to MPs may be relatively lower and have a lower effect on EE2 bioavailability. Although EE2 sorption with MPs has not been investigated, the bioavailability of EE2 is reduced in zebrafish in the pelagic scenario upon association with fullerene agglomerates [(C₆₀)_n, an engineered nanomaterial] and the EE2 did not become bioavailable when zebrafish ingested the agglomerates (Park et al., 2011, 2010).

The aim of the present study was to use changes in co-contaminant bioavailability to assess sorption to MPs in the water column (pelagic scenario), and, subsequently, to determine if MPs could transport co-contaminants to the bottom (benthic scenario) and increase their local bioavailability to benthic organisms. Two model co-contaminants (Phe and EE2) with different physicochemistries were selected. uPVC was selected as a model polymer because it is reported globally in the marine environment (Andrady, 2011), and because preliminary trials with unplasticized PVC showed that the MPs sank to the bottom. A uPVC size of 200–250 μ m was selected and provided a high surface area for sorption (Teuten et al., 2009), is comparable with previous work (Bakir et al., 2012) and is representative of small MPs found in the environment (Thompson et al., 2004). The bioavailability of Phe and EE2 were assessed by measuring changes in expression of biomarker gene transcripts for these substances in larval zebrafish, *Danio rerio*. It was not the objective of this study to investigate toxicity or bioaccumulation of Phe, EE2, uPVC or the mixture toxicity of these substances.

2. Materials and methods

2.1. Experimental fish

Zebrafish (*Danio rerio*) were provided by the Zebrafish Research Facility at Plymouth University (Plymouth, UK). Animal welfare regulations of the University of Plymouth and the UK Home Office were followed for all experimental procedures with zebrafish. Fish water quality and experimental conditions were: temperature, 27–29 °C; photoperiod, 12 h light; dissolved oxygen, 7.5–8.0 mg/L; pH, 7.2–7.4; total ammonia nitrogen, <0.09; nitrite, <0.18; and nitrate, <22 mg/L. Adult fish were spawned to obtain embryos of the same age (within 1 h fertilization), and larval fish hatched at ~72 h post fertilization (hpf). The duration of fish exposure was 96 h, and all larvae were 168 hpf when they were sacrificed at the end of the experiments.

2.2. Preparation of exposure solution and microplastic characterisations

Stock solutions of Phe and EE2 (Sigma-Aldrich, U.K.) were prepared by dissolution in ethanol (Phe:EtOH, 1 mg/mL stock solution; EE2:EtOH 20 mg/ml stock solution) before addition to fish water to achieve the target nominal concentration. All exposures contained $\leq 0.01\%$ ethanol. All exposure solutions were made in thoroughly-mixed 1-L batches, and added in aliquots to corresponding exposure beakers.

uPVC (Goodfellow, Huntington, UK) was sieved to a size range of 200–250 μ m. Consistent with previous MP sorption studies, uPVC concentration was 400 mg/L for all experiments (Bakir et al., 2012; Teuten et al., 2007). Beakers containing the exposure solutions and uPVC were covered with Parafilm® and kept dark for 5 days at 20 °C with continual orbital rotation (180 rpm). The uPVC was removed from designated treatments (see below) by filtration of the preparation through cellulose filters (Whatman, membrane filter cellulose nitrate, 0.45 μ m), and filtrate was poured back into the original beaker. All solutions were kept in original beakers and transferred to the zebrafish research facility for 24 h prior to exposure to reach optimum rearing temperature, 27 °C \pm 1 °C.

The concentration of uPVC particles was evaluated over time during 96 h to determine changes in numbers of particles within the water column of the exposure chamber. A 500 μ l water sample was collected ($n = 3$) twice daily from the center of the water column by pipette, each sample was dispensed onto colored filter paper (to allow easy identification of white uPVC particles), and particles were counted upon examination with a stereo microscope ($\times 45$). uPVC particles were characterized by stereo microscopy (SM) and scanning electron microscopy (SEM). For size measurements by SM (Olympus SZX7, $\times 45$), samples were mounted onto slides and analyzed using Infinity Analyse software. SEM (JEOL JSM-6610LV) was used to investigate surface topography of particles according to standard procedure (Ashton et al., 2010) - samples were mounted onto disks of adhesive tape and coated with a nano-layer of gold (EMITECH K550) to aid substrate conductivity.

2.3. Experimental design

For each experiment there were three replicate beakers and 35 larvae in each beaker. All experiments were static, 96-h exposures, and each experiment included a positive co-contaminant control treatment (no uPVC) as well as negative controls: fish water, vehicle (EtOH), and uPVC treatments. At the end of exposure, larvae within a beaker were pooled together (one replicate) and frozen (–80 °C) prior to gene expression analysis (see below).

2.4. Experiment 1: dose response

To establish relationships for Phe concentration and *cyp1a* expression, and for EE2 concentration and *vtg* expression, five and six

concentrations were used respectively (Phe: 0, 0.1, 0.2, 0.3 and 0.5 mg/L. EE2: 0, 0.001, 0.01, 0.1, 0.5 and 1 $\mu\text{g/L}$). The Phe experiment was repeated twice. A volume of 200 mL exposure solution (prepared as described above) was added to a 400-mL glass beaker. The concentrations of each substance were selected as they are the highest non-lethal concentrations which allowed for high gene expression whilst avoiding mortality (Gündel et al., 2012; Henry et al., 2009).

2.5. Experiment 2: sorption of co-contaminants to uPVC MPs in the water column – pelagic scenario

Based on results from Experiment 1, a concentration of 0.5 mg/L Phe and 1 $\mu\text{g/L}$ EE2 was selected for further experimentation. uPVC particles (400 mg/L) were suspended in the aqueous phase by a magnetic stirrer (300 rpm), and mixing of the MPs with co-contaminants occurred for 5 d prior to initiating the bioavailability tests. Larvae were contained in a custom-built glass chamber with a double layer of (150 μm) stainless steel mesh (Fig. 1). Whilst in the chamber the larvae were not in

contact with the uPVC but were in contact with the exposure solution. This allowed us to investigate the bioavailability of the co-contaminant that was not sorbed to the MP without being confounded by direct contact (and possible facilitated desorption) between larvae and MPs. Continual mixing of the solution inside the chamber was aided by an air-line through the center of the chamber. In order to accommodate a chamber and stirrer, a volume of 350 mL exposure solution (prepared as described above) was added to a 400-mL glass beaker.

2.6. Experiment 3: bioavailability of co-contaminants sorbed to uPVC upon accumulation of MPs on the bottom – benthic scenario

Concurrent with Experiment 2, the contaminant concentrations were 0.5 mg/L Phe and 1 $\mu\text{g/L}$ EE2. A volume of 300 mL exposure solution (prepared as described above) was added to a 400-mL glass beaker, and all uPVC exposures had two experimental conditions to investigate bioavailability. The first condition had uPVC and contaminant mixed for 5 days (MP + Contaminant), the second condition had uPVC removed (filtration) after mixing (as described above) to investigate the level of expression induced (bioavailability) by contaminant left in solution (MP Filtered). Experiment 3 represented MPs in benthic, sedimentary habitats. During the exposures uPVC particles (400 mg/L) settled onto the bottom of the beakers and zebrafish larvae were observed to rest on top of the uPVC for the first 72 h, in the final 24 h of exposure larvae were observed to be free swimming (Fig. 1).

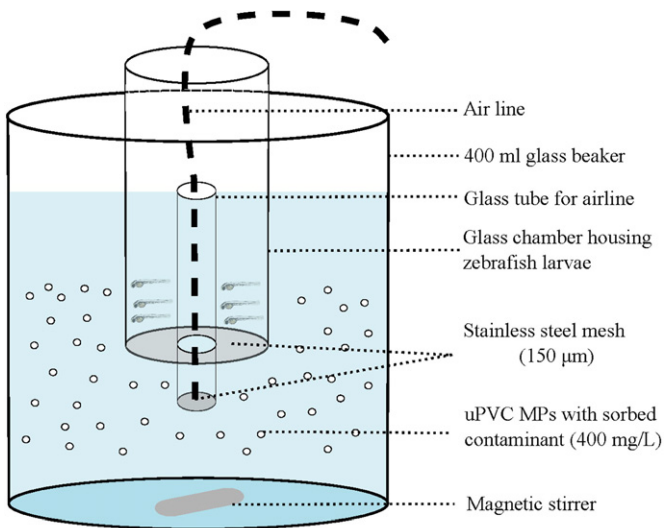
2.7. Gene expression quantification

After sample homogenization (3–5 s of sonication) and tissue disaggregation (QiaShredder column, Qiagen), total RNA was extracted (RNeasy MiniKit for animal tissue, Qiagen, West Crawley, UK) from zebrafish larvae (35 fish in each sample) as previously described (Henry et al., 2009; Park et al., 2010; Reinardy et al., 2013). A DNase treatment (15-min, Qiagen) was used to remove sample contamination by genomic DNA, and 30 μL of water (RNase/DNase free) was used for elution of RNA. The concentration and quality of RNA were determined by spectrophotometer (NanoDrop, ND-1000 Spectrophotometer). Each sample was diluted to 100 $\text{ng } \mu\text{L}^{-1}$ total RNA, and cDNA (800 ng) was synthesized according to manufacturer instructions [ImProm-II™ Reverse Transcription System, Promega; hexanucleotide primers and deoxynucleotide mix (Sigma-Aldrich), and thermocycler conditions: annealing (10 min, 25 °C) extension (20 min, 42 °C), and heat-inactivating reverse transcriptase (10 min, 70 °C; GeneAmp® PCR System, 9700, Applied Biosystems)]. cDNA was stored at –80 °C prior to gene expression analysis.

Primers for amplification of zebrafish transcripts of *cyp1a* (NCBI Reference Sequence: NM_131879.1) were designed with Primer Blast (NCBI) or from our previous work for *vfg* (NCBI Reference Sequence: NM_0010044897.2) and β -actin (NCBI Reference Sequence: NM_131031.1) (Henry et al., 2009; Park et al., 2010; Reinardy et al., 2013). Primers were designed such that amplicons spanned one intron junction and avoided secondary structure, self-annealing, complementarity, and potential hairpins [primer characteristics also evaluated by DNA calculator (Sigma-Aldrich) and OligoCalc (Northwestern University, USA)]. Verification of amplicon size for *vfg*, *cyp1a* and β -actin was carried out on a 2% agarose gel after PCR amplification (Table 1.).

Quantitative PCR (Q-PCR) was carried out as per previous work in our laboratory (Henry et al., 2009; Park et al., 2010; Reinardy et al., 2013), briefly, RNase-free water was used to reconstitute lyophilised primers (Eurofins MWG Operon, Ebersberg, Germany) to 100 μmol that were mixed with SYBR Green JumpStart Taq ReadyMix to give a final reaction concentration of 375 nmol in 20 μL total volume. Fluorescence was detected (StepOne Real-Time PCR System, Applied Biosystems, Warrington, UK) over 40 cycles with the following cycling conditions: denaturing (94 °C, 15 s), primer-specific annealing (55–60 °C, 1 min), and extension (72 °C, 1 min). All samples and standards

A.) Pelagic scenario



B.) Benthic scenario

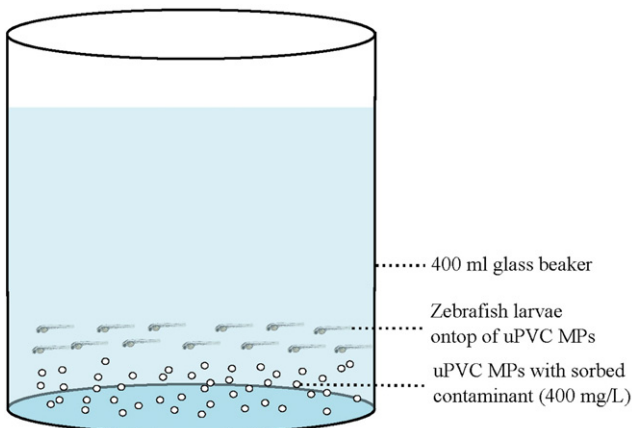


Fig. 1. Schematic diagrams of experimental exposures. A.) Aqueous exposure representing microplastics (contaminated with Phe or EE2) in pelagic environments, stirrer maintained uPVC in suspension and particles did not enter the chamber. An airline acted as an air lift pump to aid mixing of exposure water into the chamber (Experiment 2). B.) Sedimentation exposure representing microplastics (contaminated with Phe or EE2) in benthic habitats, uPVC settled to the bottom of beaker and larvae were observed to rest on top of particles (Experiment 3). Larvae in all exposures were aged up to 168 hpf (by the end of 96 h exposures) and therefore did not have developed mouthparts and were unable to uptake MPs via ingestion.

Table 1

Zebrafish (*Danio rerio*) gene specific primers for cytochrome P450 1a (*cyp1a*) vitellogenin (*vtg*), and the reference gene β -actin. Reference sequence numbers from NCBI, and product length in base pairs (bp).

Gene	Primer	Nucleotide sequence (5'-3')	Amplicon size (bp)
<i>cyp1a</i>	Forward	AGG ACA ACA TCA GAG ACA TCA CCG	174
	Reverse	GAT AGA CAA CCG CCC AGG ACA GAG	
<i>vtg</i>	Forward	ATC AGT GAT GCA CCT GCC CAG ATT G	116
	Reverse	ACG CAA GAG CTG GAC AAG CTG AA	
β -actin	Forward	ACA CAG CCA TGG ATG AGG AAA TCG	138
	Reverse	TCA CTC CCT GAT GTC TGG GTC GT	

were run in triplicate, and a standard curve was prepared on each Q-PCR plate for each gene transcript with 10-fold serial dilutions of cDNA (from pooled samples that had high *cyp1a* or *vtg* expression). The required efficiency of each Q-PCR plate was 90–110% for each gene transcript based on the slope of the standard curve.

Relative quantification of gene expression was by the comparative C_T method, in which the change in expression of the gene of interest is normalized to that of an internal housekeeping gene (Park et al., 2006). Evaluation of β -actin C_T values across samples showed that there was no effect of treatment on β -actin expression (i.e., β -actin acceptable as housekeeping gene). Normalized C_T values (ΔC_T) were obtained by subtracting the C_T value of *cyp1a* or *vtg* from that of β -actin in the same sample. Differences between average ΔC_T of control group and ΔC_T of each sample in all tested groups were expressed as $\Delta\Delta C_T$. The relative fold differences ($2^{\Delta\Delta C_T}$) of target gene expression in exposed samples were compared by treatment concentration for experiment one and by different treatments for experiments two and three.

2.8. Statistical analysis

Statistical analyses were carried out using Minitab 16 software. Dose-response (Experiment 1) data were checked for normality (Kolmogorov-Smirnov's test; $P > 0.05$) and analyzed using a linear regression. All bioavailability data (Experiments 2 and 3) were tested for normality and homogeneity of variance using Kolmogorov-Smirnov's and Bartlett's tests respectively ($P > 0.05$). Fold change was analyzed using a one-way general linear model (GLM) analysis of variance (ANOVA) followed by a post-hoc Tukey's test for multiple comparisons.

3. Results and discussion

During all exposures, zebrafish larvae developed normally, there were no changes in behaviour, and no fish died during any of the experiments. The expression of β -actin did not differ among treatments, including vehicle (EtOH) controls, and this gene transcript was therefore considered appropriate as an internal reference for all experiments. A positive linear relation between co-contaminant concentration and target gene transcript expression was significant for both EE2 with *vtg* ($P = 0.0001$) and Phe with *cyp1A* ($P = 0.012$) respectively (Fig. 2). The concentration-related induction of *vtg* with EE2 was consistent with our previous work (Henry et al., 2009; Park et al., 2010; Reinardy et al., 2013), and other reports (Bowman et al., 2000; Solé et al., 2000). Induction of *cyp1A* by Phe in zebrafish larvae has not been investigated previously by other researchers; however, we have previously demonstrated a similar level of *cyp1A* induction in our laboratory. Relatively low (compared to other *cyp1A* inducers) level of induction of *cyp1A* by Phe is consistent with previous reports (Fent and Bättscher, 2000). The positive relations between co-contaminant concentration and target gene expression indicated that measurement of the expression of these genes is a useful approach for evaluation of changes in co-contaminant bioavailability during sorption reactions with particles as we have demonstrated previously (Henry et al., 2013; Park et al., 2011).

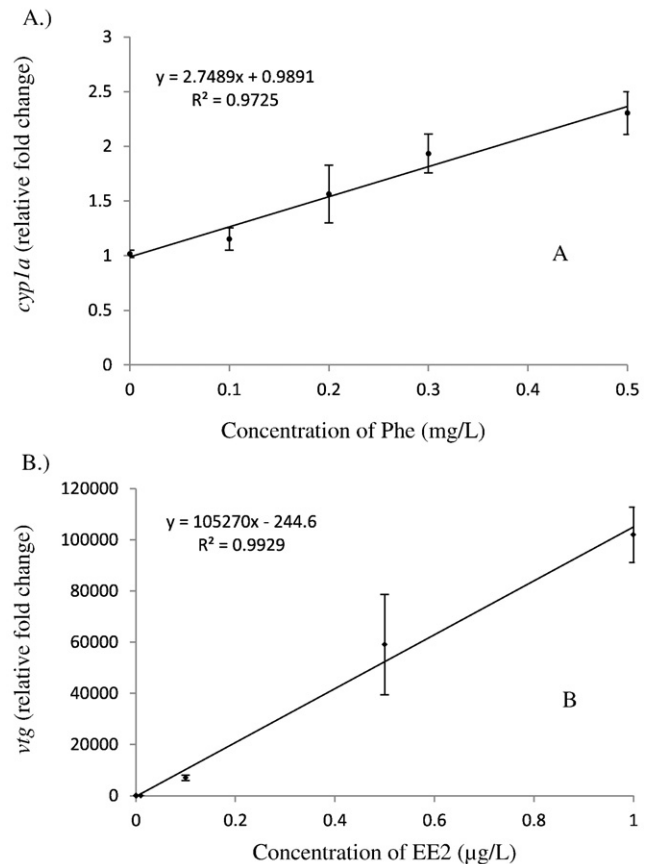


Fig. 2. Experiment 1, Characterization of dose response relationships. Larvae were exposed for 96 h, relative fold changes in expression were calculated by $\Delta\Delta C_T$ method with the gene of interest normalized to zebrafish β -actin gene (mean \pm S.E. $n = 3$). A.) Fold changes in zebrafish cytochrome P450 1a gene (*cyp1a*) expression relative to concentration of Phe, linear regression indicated relationship is significant $P = 0.012$. B.) Fold changes in zebrafish vitellogenin (*vtg*) expression relative to concentration of EE2, linear regression indicated relationship is significant $P = 0.0001$.

Measurements of the size and shape of uPVC particles used in Experiments 2 and 3 indicated that particles were consistent with manufacturer's specifications (Fig. 3). SEM analysis revealed detailed surface topography of uPVC particles, and, compared to images of micro-sized polyethylene previously published (Corcoran et al., 2009), the texture of the uPVC particles of the present study appeared to be more irregular and coarse. Particles were confirmed to be 200–250 μm in diameter, and the surface area computed for spherical particles ($4\pi r^2$) of this diameter range was $1.26\text{--}1.96 \times 10^5 \mu\text{m}^2$. The assumption of spherical shaped particles for determination of surface area by computation has been used previously (Goldstein et al., 2013; Nuelle et al., 2014; Rocha-Santos and Duarte, 2014). Researchers reported the majority of particles collected from the North Pacific by manta trawl to be of a similar size range to those of the present study ($1 \times 10^6 \mu\text{m}^2$) (Goldstein et al., 2013). Based on the computed surface area and total particle concentration ($8 \times 10^4 \text{ particles L}^{-1}$; Fig. 4) the estimated total uPVC surface area for contaminant sorption in Experiments 2 (300 mL) and 3 (350 mL) was $3\text{--}5.5 \times 10^9 \mu\text{m}^2$. These calculations do not take into account detailed surface topography, and therefore likely considerably underestimate the surface area available for sorption of co-contaminants. Nevertheless, the total surface area available for sorption of EE2 and Phe was consistent (i.e., same particle sizes and concentrations) and therefore differences in bioavailability between these co-contaminants are not likely to be related to the differences in surface area available for sorption.

Custom-built chambers were effective at maintaining a relatively homogeneous aqueous dispersion of MPs and prevented direct contact

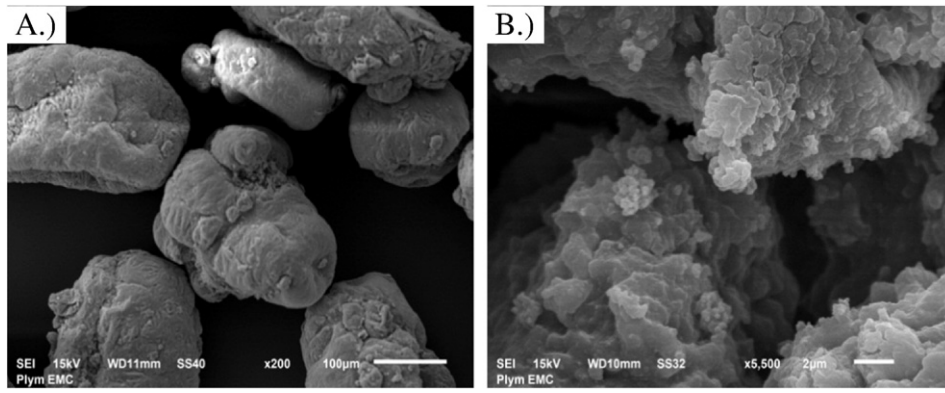


Fig. 3. Characterization of uPVC particles. A.) SEM image of uPVC (scale bar = 100 µm; magnification = ×200) B.) SEM image of same uPVC showing more detailed particle topography (scale bar = 2 µm; magnification = ×5500).

between larvae and MPs in **Experiment 2** (pelagic scenario). The number of particles in the water column decreased over time ($R^2 = 57\%$, $F_{1,22} = 29.16$, $P = 0.0001$, **Fig. 4**), and, at 96 h, the number of particles was 60% of the initial particle concentration. Although 40% of the particles were sedimented from the water column over 96 h, if the chamber is not used, 100% of the particles are lost from the water column and the resulting heterogeneity of exposure can increase the variation of the analysis endpoints. The improvement observed in the number of MPs held in the water column within the chamber is consistent with that observed for engineered nanoparticles in a chamber of similar design (Boyle et al., 2015). Use of the chamber in that study considerably enhanced the reproducibility of nanoparticle toxicity in larval zebrafish compared to exposures without the chamber (i.e., in which particles were allowed to sediment out of the water column). The need to maintain homogenous aqueous dispersions of MPs has not been adequately addressed in previous studies that tested MP effects on aquatic organisms, and is a potential explanation for differences among reports of the effects and behaviour of MPs in previous studies.

Compared to positive controls (i.e., co-contaminant exposures without MPs) expression of *cyp1A* and *vtg* were reduced when MPs were present in the water column indicating reduced co-contaminant bioavailability consistent with co-contaminant sorption to MPs, however the reduction was only significant for one of the eight experimental groups (**Fig. 5**). Based on changes in expression of *cyp1A* and *vtg*, the presence of MPs reduced bioavailability of Phe by only $33\% \pm 6$ SE ($n = 3$) whereas with EE2 bioavailability was reduced by up to $48\% \pm 10$ SE ($n = 3$) (**Fig. 5a, b**). The MPs and their characteristics (e.g., surface area charge etc.) are expected to be the same for all co-

contaminant exposures and therefore the differences in relative bioavailability are either related to differences in our ability to detect changes in bioavailability of each co-contaminant, or by differences in their physicochemistry that relate to sorption with the MPs. The concentrations of EE2 and Phe differed (1 µg/L and 500 µg/L respectively) and this was because the response profile of the biomarker genes differed for these substances (i.e., expression of *vtg* is considerably more sensitive than expression of *cyp1A*). It is possible that greater sorption of EE2 to MPs indicated by lower relative bioavailability (*vtg*

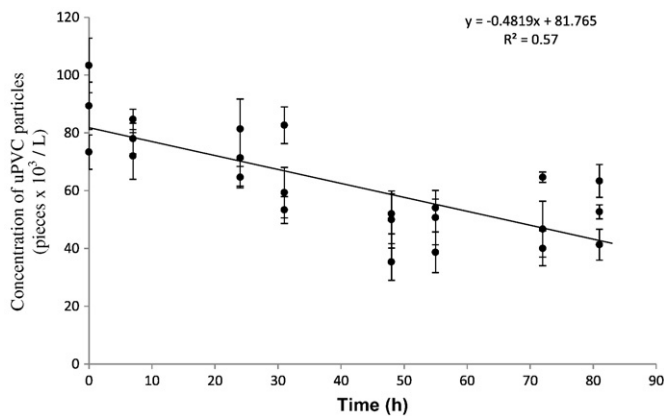


Fig. 4. Characterization of microplastic chamber exposures (in **Experiment 2**) with change in average concentration of uPVC (200–250 µm) particles over time ($F_{1,22} = 29.16$, $P = 0.0001$, error bars = ± technical triplicate S.E.).

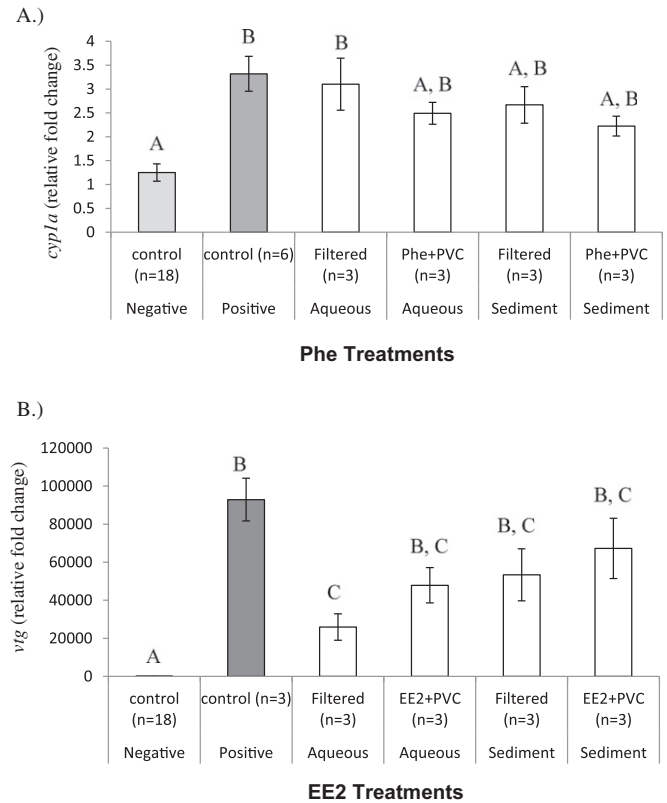


Fig. 5. **Experiments 2** and **3.** Relative fold changes in gene expression were calculated by $\Delta\Delta C_T$ method with gene of interest expression normalized to zebrafish β -actin gene (mean ± S.E. $n = 3-18$). Grey bars = mean average of controls between **Experiments 2** & **3.** Significance indicated by letters above error bars ($n =$ at least 3 as indicated in bar label). A.) Fold changes in zebrafish cytochrome P450 1a gene (*cyp1A*) expression in larvae exposed (96 h) to each of the Phe experimental treatments ($F_{5,30} = 9.14$, $P = 0.0001$). B.) Fold changes in zebrafish vitellogenin gene (*vtg*) expression in larvae exposed (96 h) to each of the EE2 experimental treatments ($F_{5,27} = 44.47$, $P = 0.0001$).

expression) is a consequence of the lower amount of EE2 present (i.e., lower concentration) compared to Phe.

Filtration removal of the MPs after co-contaminant sorption resulted in different patterns of bioavailability between Phe and EE2 when zebrafish were exposed to the filtrate. For Phe, the expression of *cyp1A* was highest (3.3 ± 0.4 SE ($n = 3$) fold) in the positive control (i.e. Phe without MPs), and, in the chamber exposure simulating MPs in pelagic environments (i.e., aqueous MPs), Phe bioavailability appeared to be higher (although observation was not statistically significant) when MPs were removed compared to when MPs were left in the exposure (Fig. 5a). The same reduction in Phe bioavailability was observed in the exposures that simulated MPs in benthic environments (i.e., sedimented MPs). In contrast, the opposite pattern was observed for EE2 by assessment of vtg expression. EE2 bioavailability was lower when the MPs were removed compared to when they were left in the exposure, in the pelagic and benthic scenarios. The consistent opposite trends observed between Phe and EE2 are most likely to be due to the different physicochemistry properties of the co-contaminants. Analytical chemistry was not used to characterize sorption of EE2 to uPVC particles due to difficulty in detecting such low concentrations; however, previous studies have demonstrated EE2 has a strong sorption affinity to plastic (Han et al., 2012; Walker and Watson, 2010). Based on our bioavailability (gene expression) data, EE2 appeared more strongly associated with the MPs than Phe, and when the contaminated MPs were removed (i.e., filtered exposures) much of the bioavailable EE2 was also removed ($F_{5,27} = 44.47$, $P = 0.0001$). The stronger MP association of EE2 compared to Phe is contrary to what would be expected based on physicochemistry (i.e., Phe has higher K_{ow} and lower water solubility than EE2), which suggests that the bioavailability of co-contaminants sorbed to MPs cannot be predicted based on co-contaminant physicochemistry alone.

Although sorption of Phe and EE2 to MPs is related to contaminant physicochemistry, it was shown that sorption alters co-contaminant bioavailability in a manner that cannot be predicted by analytical chemistry alone. The sorption of Phe to uPVC MPs was previously characterized in our laboratory (Bakir et al., 2012), and the following sorption equation was obtained:

$$y = 0.51x - 0.14$$

(where y = concentration of Phe left in the pelagic scenario and x = the initial concentration of Phe).

The Phe sorption equation predicts that if a 500 $\mu\text{g/L}$ initial concentration of Phe was added to water containing uPVC particles then, after sorption, 255 $\mu\text{g/L}$ would be left in aqueous solution. If 255 $\mu\text{g/L}$ was the only bioavailable source of Phe, then, according to the dose-response equation for Phe-*cyp1A*, a *cyp1A* fold change of 1.68 would be expected. However, when MPs were added to 500 $\mu\text{g/L}$ Phe, a *cyp1A* fold change of up to 2.5 ± 0.2 SE ($n = 3$) was observed in larval zebrafish, which is $48\% \pm 14$ SE ($n = 3$) higher than what is predicted by the sorption equation (above, Supplementary Fig 1). The determination of greater Phe bioavailability based on measurement of *cyp1A* expression in larval zebrafish indicates that methods of analytical chemistry may not completely determine the bioavailable fraction of co-contaminants.

The concentrations of MPs and co-contaminants used in the present study were orders of magnitudes higher than what is found in the environment (Aris et al., 2014; Long et al., 1995; Lusher et al., 2014), and co-contaminant concentrations were deliberately tested at higher than environmentally relevant amounts. Results provide evidence that sorption of these substances to MPs occurs (confirming previous results with other substances), but that sorption to MPs may not be as much as determined by analytical chemistry or predicted by contaminant physicochemistry. One reason for the observed higher Phe bioavailability than that predicted by the linear sorption model could be that Phe partitioned to clean fish once they were added to the system. In the environment, MPs with sorbed co-contaminants will likely be dispersed

to new environments and dis-equilibrium conditions will lead to potential changes in bioavailability of sorbed co-contaminants. There is evidence that nanoparticles (NPs) increase the bioavailability of mercury to larval zebrafish. When NPs with sorbed Hg^{2+} sedimented to the bottom of beakers, Hg^{2+} exposure in fish at the bottom of the beaker increased (Henry et al., 2013). The present study did not provide similar evidence of elevated bioavailability of Phe or EE2 in fish exposed to sedimented MPs despite the considerably higher than environmentally relevant concentrations of MPs and co-contaminants tested. It is possible that other benthic exposure scenarios (e.g., benthic organisms in which ingestion of MPs with sorbed co-contaminants) could demonstrate that sedimentation of contaminant-sorbed MPs to the benthos enhances co-contaminant bioavailability, but - at environmentally relevant concentrations - these effects are expected to be minimal.

Author contributions

V.A.S. designed the study, conducted all exposures and qPCR analyses, performed data analysis and interpretation, produced the figures and wrote the manuscript. A.B. contaminated the MPs in line with previous work, provided advice on data interpretation and commented on the manuscript. R.C.T. initially conceived the study, provided advice on data interpretation and commented on the manuscript. T.B.H. initially conceived the study, provided advice on experimental design and data interpretation and helped to write the manuscript.

Acknowledgements

Thank you to Stanley McMahon for fish maintenance and husbandry in the Zebrafish Research Facility, Plymouth, and Andrew Atfield and Dr. Helena Reinardy for laboratory assistance.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.marpolbul.2016.12.055>.

References

- Andrady, A.L., 2011. Microplastics in the marine environment. *Mar. Pollut. Bull.* 62: 1596–1605. <http://dx.doi.org/10.1016/j.marpolbul.2011.05.030>.
- Aris, A.Z., Shamsuddin, A.S., Praveena, S.M., 2014. Occurrence of 17 α -ethynylestradiol (EE2) in the environment and effect on exposed biota: a review. *Environ. Int.* 69: 104–119. <http://dx.doi.org/10.1016/j.envint.2014.04.011>.
- Ashton, K., Holmes, L., Turner, A., 2010. Association of metals with plastic production pellets in the marine environment. *Mar. Pollut. Bull.* 60, 2050–2055.
- Avio, C.G., Gorb, S., Milan, M., Benedetti, M., Fattorini, D., d'Errico, G., Paoletto, M., Bargelloni, L., Regoli, F., 2015. Pollutants bioavailability and toxicological risk from microplastics to marine mussels. *Environ. Pollut.* 198C:211–222. <http://dx.doi.org/10.1016/j.envpol.2014.12.021>.
- Bakir, A., Rowland, S.J., Thompson, R.C., 2012. Competitive sorption of persistent organic pollutants onto microplastics in the marine environment. *Mar. Pollut. Bull.* 64: 2782–2789. <http://dx.doi.org/10.1016/j.marpolbul.2012.09.010>.
- Besseling, E., Wegner, A., Foekema, E.M., van den Heuvel-Greve, M.J., Koelmans, A.A., 2013. Effects of microplastic on fitness and PCB bioaccumulation by the lugworm *Arenicola marina* (L.). *Environ. Sci. Technol.* 47:593–600. <http://dx.doi.org/10.1021/es302763x>.
- Bowman, C.J., Kroll, K.J., Hemmer, M.J., Folmar, L.C., Denslow, N.D., 2000. Estrogen-induced vitellogenin mRNA and protein in sheepshead minnow (*Cyprinodon variegatus*). *Gen. Comp. Endocrinol.* 120:300–313. <http://dx.doi.org/10.1006/gcen.2000.7565>.
- Boyle, D., Boran, H., Atfield, A.J., Henry, T.B., 2015. Use of an exposure chamber to maintain aqueous phase nanoparticle dispersions for improved toxicity testing in fish. *Environ. Toxicol. Chem.* 34 (3), 583–588.
- Browne, M.A., Crump, P., Niven, S.J., Teuten, E., Tonkin, A., Galloway, T., Thompson, R., 2011. Accumulation of microplastic on shorelines worldwide: sources and sinks. *Environ. Sci. Technol.* 45:9175–9179. <http://dx.doi.org/10.1021/es201811s>.
- Colabuono, F.L., Taniguchi, S., Montone, R.C., 2010. Polychlorinated biphenyls and organochlorine pesticides in plastics ingested by seabirds. *Mar. Pollut. Bull.* 60, 630–634.
- Collignon, A., Hecq, J.-H., Glagani, F., Voisin, P., Collard, F., Goffart, A., 2012. Neustonic microplastic and zooplankton in the north western Mediterranean Sea. *Mar. Pollut. Bull.* 64:861–864. <http://dx.doi.org/10.1016/j.marpolbul.2012.01.011>.
- Corcoran, P.L., Biesinger, M.C., Grifi, M., 2009. Plastics and beaches: a degrading relationship. *Mar. Pollut. Bull.* 58, 80–84.

- Eriksen, M., Lebreton, L.C.M., Carson, H.S., Thiel, M., Moore, C.J., Borroero, J.C., Galgani, F., Ryan, P.G., Reisser, J., 2014. Plastic pollution in the World's oceans: >5 trillion plastic pieces weighing over 250,000 tons afloat at sea. *PLoS One* 9, e111913. <http://dx.doi.org/10.1371/journal.pone.0111913>.
- Fent, K., Bättscher, R., 2000. Cytochrome P4501A induction potencies of polycyclic aromatic hydrocarbons in a fish hepatoma cell line: demonstration of additive interactions. *Environ. Toxicol. Chem.* 19, 2047–2058.
- Frias, J.P.G.L., Sobral, P., Ferreira, A.M., 2010. Organic pollutants in microplastics from two beaches of the Portuguese coast. *Mar. Pollut. Bull.* 60, 1988–1992.
- Goldstein, M.C., Titmus, A.J., Ford, M., 2013. Scales of spatial heterogeneity of plastic marine debris in the northeast pacific ocean. *PLoS One* 8, e80020. <http://dx.doi.org/10.1371/journal.pone.0080020>.
- Gregory, M.R., 2009. Environmental implications of plastic debris in marine settings—entanglement, ingestion, smothering, hangers-on, hitch-hiking and alien invasions. *Philos. Trans. R. Soc. Lond. Ser. B Biol. Sci.* 364, 2013–2025.
- Gündel, U., Kalkhof, S., Zitzkat, D., von Bergen, M., Altenburger, R., Küster, E., 2012. Concentration-response concept in ecotoxicoproteomics: effects of different phenanthrene concentrations to the zebrafish (*Danio rerio*) embryo proteome. *Ecotoxicol. Environ. Saf.* 76:11–22. <http://dx.doi.org/10.1016/j.ecoenv.2011.10.010>.
- Han, J., Qiu, W., Meng, S., Gao, W., 2012. Removal of ethinylestradiol (EE2) from water via adsorption on aliphatic polyamides. *Water Res.* 46:5715–5724. <http://dx.doi.org/10.1016/j.watres.2012.08.001>.
- Hansch, C., Leo, A., Hoekman, D.H., 1995. Exploring QSAR: fundamentals and applications in chemistry and biology. *Am. Chem. Soc.*
- Henry, T.B., McPherson, J.T., Rogers, E.D., Heah, T.P., Hawkins, S.A., Layton, A.C., Saylor, G.S., 2009. Changes in the relative expression pattern of multiple vitellogenin genes in adult male and larval zebrafish exposed to exogenous estrogens. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* 154, 119–126.
- Henry, T.B., Wileman, S.J., Boran, H., Sutton, P., 2013. Association of Hg²⁺ with aqueous (C60) n aggregates facilitates increased bioavailability of Hg²⁺ in zebrafish (*Danio rerio*). *Environ. Sci. Technol.* 47:9997–10004. <http://dx.doi.org/10.1021/es4015597>.
- Herzke, D., Anker-Nilssen, T., Nøst, T.H., Götsch, A., Christensen-Dalsgaard, S., Langset, M., Fangel, K., Koelmans, A.A., 2016. Negligible impact of ingested microplastics on tissue concentrations of persistent organic pollutants in northern fulmars off coastal Norway. *Environ. Sci. Technol.* 50:1924–1933. <http://dx.doi.org/10.1021/acs.est.5b04663>.
- Holmes, L.A., Turner, A., Thompson, R.C., 2012. Adsorption of trace metals to plastic resin pellets in the marine environment. *Environ. Pollut.* 160:42–48. <http://dx.doi.org/10.1016/j.envpol.2011.08.052>.
- Jennings, A.A., 2012. Worldwide regulatory guidance values for surface soil exposure to carcinogenic or mutagenic polycyclic aromatic hydrocarbons. *J. Environ. Manag.* 110:82–102. <http://dx.doi.org/10.1016/j.jenvman.2012.05.015>.
- Long, E.R., Macdonald, D.D., Smith, S.L., Calder, F.D., 1995. Incidence of adverse biological effects within ranges of chemical concentrations in marine and estuarine sediments. *Environ. Manag.* 19:81–97. <http://dx.doi.org/10.1007/BF02472006>.
- Lusher, A.L., Burke, A., O'Connor, I., Officer, R., 2014. Microplastic pollution in the North-east Atlantic Ocean: Validated and opportunistic sampling. *Mar. Pollut. Bull.* 88:325–333. <http://dx.doi.org/10.1016/j.marpolbul.2014.08.023>.
- Mato, Y., Isobe, T., Takada, H., Kanehiro, H., Ohtake, C., Kaminuma, T., 2001. Plastic resin pellets as a transport medium for toxic chemicals in the marine environment. *Environ. Sci. Technol.* 35, 318–324.
- Miller, M.M., Wasik, S.P., Huang, G.L., Shiu, W.Y., Mackay, D., 1985. Relationships between octanol-water partition coefficient and aqueous solubility. *Environ. Sci. Technol.* 19:522–529. <http://dx.doi.org/10.1021/es00136a007>.
- Nuelle, M.-T., Dekiff, J.H., Remy, D., Fries, E., 2014. A new analytical approach for monitoring microplastics in marine sediments. *Environ. Pollut.* 184:161–169. <http://dx.doi.org/10.1016/j.envpol.2013.07.027>.
- Oliveira, M., Ribeiro, A., Hylland, K., Guilhermino, L., 2013. Single and combined effects of microplastics and pyrene on juveniles (0+ group) of the common goby *Pomatoschistus microps* (Teleostei, Gobiidae). *Ecol. Indic.* 34:641–647. <http://dx.doi.org/10.1016/j.ecolind.2013.06.019>.
- Park, J.-W., Hecker, M., Murphy, M.B., Jones, P.D., Solomon, K.R., Van Der Kraak, G., Carr, J.A., Smith, E.E., du Preez, L., Kendall, R.J., Giesy, J.P., 2006. Development and optimization of a Q-RT PCR method to quantify CYP19 mRNA expression in testis of male adult *Xenopus laevis*: comparisons with aromatase enzyme activity. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* 144:18–28. <http://dx.doi.org/10.1016/j.cbpb.2006.01.003>.
- Park, J.-W., Henry, T.B., Menn, F.-M., Compton, R.N., Saylor, G., 2010. No bioavailability of 17 α -ethinylestradiol when associated with nC60 aggregates during dietary exposure in adult male zebrafish (*Danio rerio*). *Chemosphere* 81:1227–1232. <http://dx.doi.org/10.1016/j.chemosphere.2010.09.036>.
- Park, J.-W., Henry, T.B., Ard, S., Menn, F.-M., Compton, R.N., Saylor, G.S., 2011. The association between nC60 and 17 α -ethinylestradiol (EE2) decreases EE2 bioavailability in zebrafish and alters nanoaggregate characteristics. *Nanotoxicology* 5:406–416. <http://dx.doi.org/10.3109/17435390.2010.525329>.
- Reddy, M.S., Adimurthy, S., Ramachandriah, G., 2006. Description of the small plastics fragments in marine sediments along the Alang-Sosiya ship-breaking yard, India. *Estuar. Coast. Shelf Sci.* 68, 656–660.
- Reinardy, H.C., Scarlett, A.G., Henry, T.B., West, C.E., Hewitt, L.M., Frank, R.A., Rowland, S.J., 2013. Aromatic naphthenic acids in oil sands process-affected water, resolved by GCxGC-MS, only weakly induce the gene for vitellogenin production in zebrafish (*Danio rerio*) larvae. *Environ. Sci. Technol.* 47:6614–6620. <http://dx.doi.org/10.1021/es304799m>.
- Rocha-Santos, T., Duarte, A.C., 2014. A critical overview of the analytical approaches to the occurrence, the fate and the behavior of microplastics in the environment. *TrAC Trends Anal. Chem.* 65:47–53. <http://dx.doi.org/10.1016/j.trac.2014.10.011>.
- Rochman, C.M., Hoh, E., Hentschel, B.T., Kaye, S., 2013a. Long-term field measurement of sorption of organic contaminants to five types of plastic pellets: implications for plastic marine debris. *Environ. Sci. Technol.* 47:1646–1654. <http://dx.doi.org/10.1021/es303700s>.
- Rochman, C.M., Hoh, E., Kurobe, T., Teh, S.J., 2013b. Ingested plastic transfers hazardous chemicals to fish and induces hepatic stress. *Sci. Rep.* 3, 3263.
- Semple, K.T., Doick, K.J., Jones, K.C., Burauel, P., Craven, A., Harms, H., 2004. Peer reviewed: defining bioavailability and bioaccessibility of contaminated soil and sediment is complicated. *Environ. Sci. Technol.* 38:228A–231A. <http://dx.doi.org/10.1021/es040548w>.
- Shim, W.J., Thomposon, R.C., 2015. Microplastics in the ocean. *Arch. Environ. Contam. Toxicol.* 1–4.
- Solé, M., Porte, C., Barceló, D., 2000. Vitellogenin induction and other biochemical responses in carp, *Cyprinus carpio*, after experimental injection with 17 α -ethinylestradiol. *Arch. Environ. Contam. Toxicol.* 38, 494–500.
- 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. *Mar. Pollut. Bull.* 69, 219–222.
- Teranishi, K., Hamada, K., Hiromu, W., 1975. Quantitative relationship between carcinogenicity and mutagenicity of polyaromatic hydrocarbons in Salmonella typhimurium mutants. *Mutat. Res. Mutagen. Relat. Subj.* 31:97–102. [http://dx.doi.org/10.1016/0165-1161\(75\)90071-0](http://dx.doi.org/10.1016/0165-1161(75)90071-0).
- Teuten, E.L., Rowland, S.J., Galloway, T.S., Thompson, R.C., 2007. Potential for plastics to transport hydrophobic contaminants. *Environ. Sci. Technol.* 41, 7759–7764.
- Teuten, E.L., Saquing, J.M., Knappe, D.R.U., Barlaz, M.A., Jonsson, S., Björn, A., Rowland, S.J., Thompson, R.C., Galloway, T.S., Yamashita, R., Ochi, D., Watanuki, Y., Moore, C., Viet, P.H., Tana, T.S., Prudente, M., Boonyatumanond, R., Zakaria, M.P., Akkavong, K., Ogata, Y., Hirai, H., Iwasa, S., Mizukawa, K., Hagino, Y., Imamura, A., Saha, M., Takada, H., 2009. Transport and release of chemicals from plastics to the environment and to wildlife. *Philos. Trans. R. Soc. Lond. Ser. B Biol. Sci.* 364, 2027–2045.
- Thompson, R.C., Olsen, Y., Mitchell, R.P., Davis, A., Rowland, S.J., John, A.W.G., McGonigle, D., Russell, A.E., 2004. Lost at sea: Where is all the plastic? *Science* 304 (80), 838.
- Voparil, I.M., Mayer, L.M., 2000. Dissolution of sedimentary polycyclic aromatic hydrocarbons into the lugworm's (*Arenicola marina*) digestive fluids. *Environ. Sci. Technol.* 34:1221–1228. <http://dx.doi.org/10.1021/es990885i>.
- Walker, C.W., Watson, J.E., 2010. Adsorption of estrogens on laboratory materials and filters during sample preparation. *J. Environ. Qual.* 39:744–748. <http://dx.doi.org/10.2134/jeq2009.0017>.