



OPEN

Ingested plastic transfers hazardous chemicals to fish and induces hepatic stress

SUBJECT AREAS:

ECOPHYSIOLOGY

ECOLOGY

CONSERVATION

ENVIRONMENTAL CHEMISTRY

Chelsea M. Rochman¹, Eunha Hoh², Tomofumi Kurobe¹ & Swee J. Teh¹

¹Aquatic Health Program, Department of Anatomy, Physiology and Cell Biology, School of Veterinary Medicine, University of California, Davis, CA 95616, USA, ²Graduate School of Public Health, San Diego State University, CA 92182, USA.

Received
3 May 2013

Accepted
4 November 2013

Published
21 November 2013

Correspondence and
requests for materials
should be addressed to
C.M.R. (cmrochman@
ucdavis.edu)

Plastic debris litters aquatic habitats globally, the majority of which is microscopic (< 1 mm), and is ingested by a large range of species. Risks associated with such small fragments come from the material itself and from chemical pollutants that sorb to it from surrounding water. Hazards associated with the complex mixture of plastic and accumulated pollutants are largely unknown. Here, we show that fish, exposed to a mixture of polyethylene with chemical pollutants sorbed from the marine environment, bioaccumulate these chemical pollutants and suffer liver toxicity and pathology. Fish fed virgin polyethylene fragments also show signs of stress, although less severe than fish fed marine polyethylene fragments. We provide baseline information regarding the bioaccumulation of chemicals and associated health effects from plastic ingestion in fish and demonstrate that future assessments should consider the complex mixture of the plastic material and their associated chemical pollutants.

Small plastic debris is ubiquitous in the aquatic environment, contaminating coastal^{1,2}, deep-sea³, near-shore¹ and open-ocean^{1,4,5} pelagic habitats. Global trends suggest that accumulations are increasing in aquatic habitats^{1,5}, consistent with trends in plastic production— increasing 560 fold in just over 60 years⁶. Production trends in combination with increasing environmental accumulations may lead to greater hazards for wildlife.

Hazards associated with plastic debris include physical components of the material^{7–9}, chemical ingredients^{7,10,11} and sorbed environmental chemicals^{7,10} (e.g. persistent bioaccumulative and toxic substances (PBTs)^{12,13} and metals¹⁴). Upon ingestion, microscopic plastic fragments can translocate into the tissues of mussels¹⁵ and cause increased granulocytomas and decreased lysosomal membrane stability⁹. Based upon the UN Globally Harmonised System, > 50% of plastics are associated with hazardous monomers, additives and chemical byproducts¹¹ (e.g. the carcinogenic polyvinyl chloride (PVC) monomer is the building block for the PVC¹¹ piping that transports our drinking water). PBTs, found on recovered plastic debris globally¹², bioaccumulate in foodwebs¹⁰ and are linked with several adverse effects including endocrine disruption¹⁶, decreased fish populations¹⁷ and reduced species evenness and richness¹⁸.

A concern often raised, that remains poorly understood, is the extent that chemicals associated with plastic debris, via environmental sorption^{12,13} or the manufacturing process^{10,11}, bioaccumulate in animals as a consequence of ingestion. Evidence from laboratory studies include the bioaccumulation of polybrominated diphenyls (PBDEs), a flame-retardant added to plastics, in crickets via ingestion of polyurethane foam¹⁹ and greater concentrations of polychlorinated biphenyls (PCBs) in lugworms fed polystyrene with sorbed PCBs²⁰. In nature, plastics with sorbed chemicals are found globally from coastal areas to the remote habitats of the subtropical gyres¹². Evidence from observational studies in nature have found that birds with plastic in their stomachs have greater concentrations of lower chlorinated PCBs in their tissue than those that do not²¹ and similar congener patterns of PBDEs in their tissues as those found on the ingested plastic²². Of greater concern, is the hazards to wildlife health when they are exposed to the complex mixture of plastic material and plastic-associated chemicals (including the chemical ingredients and those sorbed from nature)²³.

The physical and chemical hazards outlined above combined with the ingestion of plastic by a large range of aquatic organisms across multiple trophic levels²⁴ and the evidence that supports chemical transfer from plastics to wildlife^{19–22} prompted us to measure the bioaccumulation of chemicals and adverse health effects from plastic ingestion in fish. Fish, one of the largest and most diverse groups of animals and of great ecological- and commercial- importance²⁵, are useful as sensitive indicators of effects associated with stressors in aquatic



habitats²⁶. Furthermore, plastic particles are reported in the gut content of several species of fish globally including from pelagic habitats^{27,28}, estuaries^{29–31}, and bays³².

Using Japanese medaka (*Oryzias latipes*), a widely accepted model fish species^{33,34}, we achieved baseline information regarding the bioaccumulation of PBTs and associated health effects in fish via a chronic dietary exposure to low-density polyethylene (LDPE) plastic. Polyethylene has a greater affinity for organic contaminants than other mass-produced polymers¹³, comprises the largest component of plastic production globally (29%⁶) and is one of the most common polymers recovered as aquatic debris³⁵. Fish were exposed to three treatments: a negative control (no LDPE), a virgin-plastic (LDPE virgin pre-production plastic) and a marine-plastic treatment (LDPE deployed in an urban bay). Medaka were exposed to 10% plastic (by weight) mixed into treatment diets and sprinkled at the top of each tank. Diet and plastic dissociated at the surface and thus fish were exposed to plastic similar to the way they are in the wild (i.e. floating in the water column). As such, this translates to 8 ng of plastic per mL of water. Maximum concentrations reported in the North Pacific Subtropical Gyre are 300 ng/mL⁵, and thus the concentrations of plastic used in this experiment may be considered environmentally relevant. Our chemical analyses targeted polycyclic aromatic hydrocarbons (PAHs), PCBs and PBDEs (see Figure 1 for a schematic diagram). All accumulate on plastic debris in marine habitats¹². In addition, PBDEs are additives on several plastics³⁶ and PAHs are a likely byproduct of plastic manufacturing³⁷.

We hypothesized that, after a 2-month exposure, there would be larger concentrations of PBTs in the tissue of fish exposed to marine-plastic and that hepatic stress would be observed, using histopathology, in fish exposed to virgin- and marine-plastic. In addition, because a healthy liver is critical for metabolizing organic contaminants, aided by the CYP1A enzyme³⁸, we hypothesized greater expression of CYP1A, using RT-qPCR, in medaka exposed to virgin- and marine-plastic.

Results

A cocktail of pollutants: plastic and PBTs in treatment diets. In a previous study, we found that polyethylene sorbs greater concentrations of PAHs and PCBs than other mass-produced polymers¹³. Thus, to prepare treatments, LDPE was deployed off of a floating dock in San Diego Bay, CA for three months in preparation for the dietary exposure. 12 PAHs, 27 PCBs and 13 PBDEs were targeted in virgin- and marine- LDPE pellets and 6 PAHs, 10 PCBs and 7 PBDEs were detected and quantified (Figure 1). Concentrations of total PAHs, PCBs and PBDEs on marine-LDPE were $4 \times$, $15 \times$ and $1.4 \times$ greater than on virgin-LDPE pellets respectively (Table 1). For concentrations of individual congeners see Supplementary Table 1.

PAH, PCB and PBDE congeners quantified on LDPE were measured in all treatment diets (negative control, virgin-plastic and marine-plastic). Due to the presence of cod liver oil in all diets, PAHs, PCBs and PBDEs were present in all treatments including the negative control (Table 1). Still, the addition of LDPE deployed in San Diego Bay assured that the marine-plastic treatment diet exposed fish to greater concentrations of PBTs than the negative control and virgin-plastic treatment diets. Moreover, the addition of virgin-LDPE contributed greater concentrations of PAHs and PBDEs to the virgin-plastic than the negative control treatment. In this way, fish were exposed to greater concentrations of PBTs as a direct result of the addition of plastic to their diet. The virgin-plastic treatment diet had $2.4 \times$ greater total PAHs, $2.2 \times$ greater total PBDEs and relatively similar concentrations of total PCBs as the negative control treatment diet. The marine-plastic treatment diet had $2.3 \times$ greater total PAHs, $6 \times$ greater total PCBs and $2.8 \times$ greater total PBDEs relative to the negative control treatment diet (Table 1). Our results show similar concentrations of PAHs reported in the marine-plastic and virgin-plastic treatment diets although concentrations of PAHs were much greater in marine- compared to virgin-LDPE pellets. Due to the complex matrix of the diet, we achieved poor resolution for several PAH congeners. In particular, fluoranthene was found at much greater concentrations on LDPE deployed in San Diego Bay,

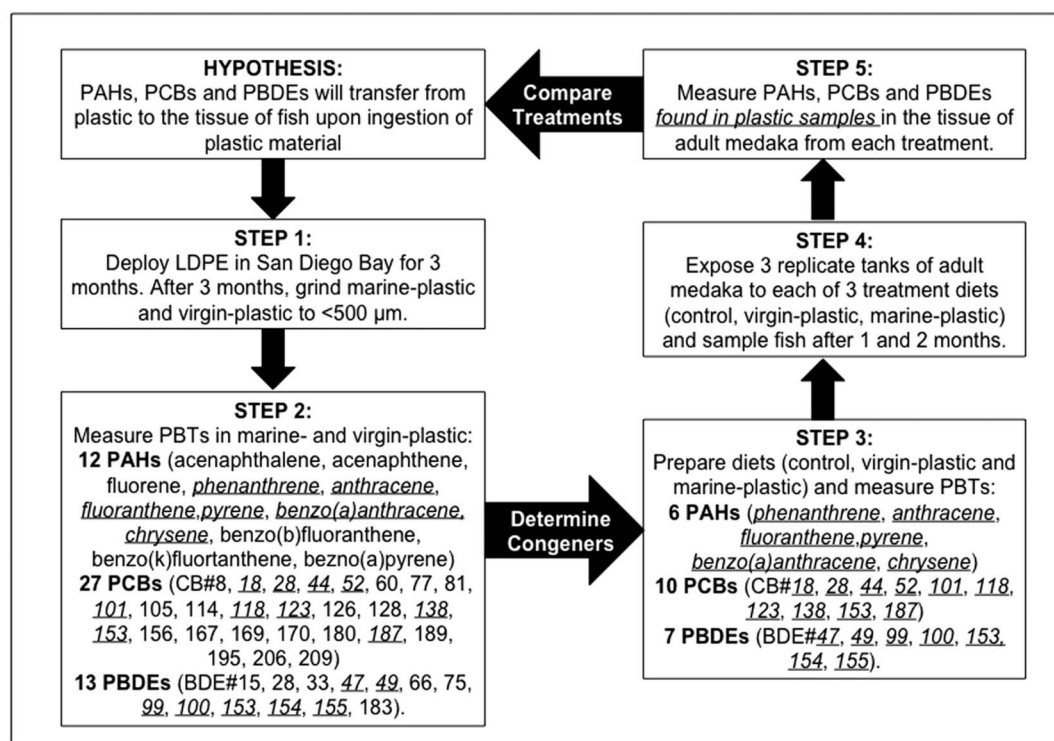


Figure 1 | Schematic diagram of experimental design. The diagram shows how we chose which contaminants to look for based upon our hypothesis.



Table 1 | Concentrations of chemicals (ng/g) in LDPE and diet matrices

	Σ PAHs	Σ PCBs	Σ PBDEs
virgin ground LDPE (ng/g pellet)	32	1.1	1.4
marine ground LDPE (ng/g pellet)	129	17	1.9
control diet (ng/g diet)	24	0.3	1.1
virgin-plastic diet (ng/g diet)	58	0.8	2.4
marine-plastic diet (ng/g diet)	55	5.3	3.1

ΣPAHs: phenanthrene, anthracene, fluoranthene, pyrene, benzo(a)anthracene and chrysene.
 ΣPCBs: CB#18, 28, 44, 52, 101, 118, 123, 138, 153, 187.
 ΣPBDEs: BDE#47, 49, 99, 100, 153, 154, and 155.

which is consistent with results from our previous study¹³, but the poor resolution in the peaks did not allow us to detect this difference among diet samples. Individual congeners measured in all diets can be found in Supplementary Table 1.

Bioaccumulation of PBTs via dietary exposure to treatment diets.

After two months of dietary exposure, general patterns show a greater concentration of PBTs in fish exposed to the marine-plastic treatment. Mean concentrations of total PAHs, PCBs and PBDEs in fish from the marine-plastic treatment were 2.4 ×, 1.2 × and 1.8 × greater respectively than in fish from the negative control treatment (Figure 2). While the total concentrations of PAHs and PCBs were not significantly different among treatments ($P = 0.234$ and $P = 0.118$ respectively; Figure 2; see Supplementary Table S2 for ANOVA tables), concentrations of chrysene ($P = 0.006$) and PCB28 ($P = 0.022$) were significantly greater ($\alpha = 0.05$) in fish exposed to the marine-plastic treatment relative to the virgin-plastic and negative control treatments (Supplementary Tables S1 and S2). Nevertheless, total concentrations of PBDEs ($P = 0.0003$) and all individual PBDE congeners ($P < 0.05$), with the exception of BDE155 ($P = 0.425$), in fish are significantly different among treatments such that fish exposed to the marine-plastic treatment have significantly greater ($\alpha = 0.05$) concentrations of PBDEs than both the virgin-plastic and control treatments (Figure 2, Supplementary Tables S1 and S2). While we observed greater concentrations of PBTs in fish

exposed to marine-plastic, this pattern was only apparent after the full 2-month exposure (Figure 2). There were not significant differences among treatments at the one-month sampling period ($P > 0.05$; Figure 2 and Supplementary Table S3) suggesting that short-term exposures to a 10% plastic diet may not be a significant source of PBTs to aquatic life.

Adverse health effects. Hypotheses regarding adverse effects to fish focused on sublethal effects to the liver induced by the ingestion of plastic. Here, we exposed fish to a low-dose of chemicals sorbed to plastics over a relatively long time period. Thus, as expected, we did not observe large amounts or differences in mortality among treatments. Rates of mortality within each treatment, throughout the 2-month experiment, were 4% from the negative control and virgin plastic treatments and 6% from the marine plastic treatment.

The Aryl hydrocarbon (AhR) receptor is activated upon exposure to several PBTs (including PAHs, PCBs and PBDEs) as a defense mechanism to aid metabolism³⁹. After activation, AhR translocates to the nucleus to dimerize with ARNT, leading to changes in xenobiotic response-related gene transcription⁴⁰. Transcriptional induction of CYP1A is a sensitive and specifically adaptive response in fish after exposure to polycyclic and halogenated aromatic hydrocarbons^{40,41} and plays a role in detoxification and/or metabolic activation (carcinogenesis) of exogenous compounds⁴². Because response can differ among genders when fish are reproductively active, associated with biochemical changes during spawning⁴³, expression among treatments was compared separately for each sex. After one and two months of exposure, 1-factor ANOVAs showed no significant differences in expression of CYP1A (expressed as $2^{-\Delta Ct}$) among treatments for both males ($P = 0.436$ (1 month), $P = 0.324$ (2 month)) and females ($P = 0.254$ (1 month), $P = 0.118$ (2 month)) individually (Figure 3).

Still, fish exposed to virgin- and marine-plastic treatments show signs of stress in their livers, including glycogen depletion, fatty vacuolation and single cell necrosis. Severe glycogen depletion was seen in 74% of fish from the marine-plastic treatment ($n = 19$ fish), 46% of fish from the virgin-plastic treatment ($n = 24$ fish), and 0% of fish from the control treatment ($n = 24$ fish). Fatty vacuolation was seen in 47% of fish from the marine-plastic treatment, 29% of fish

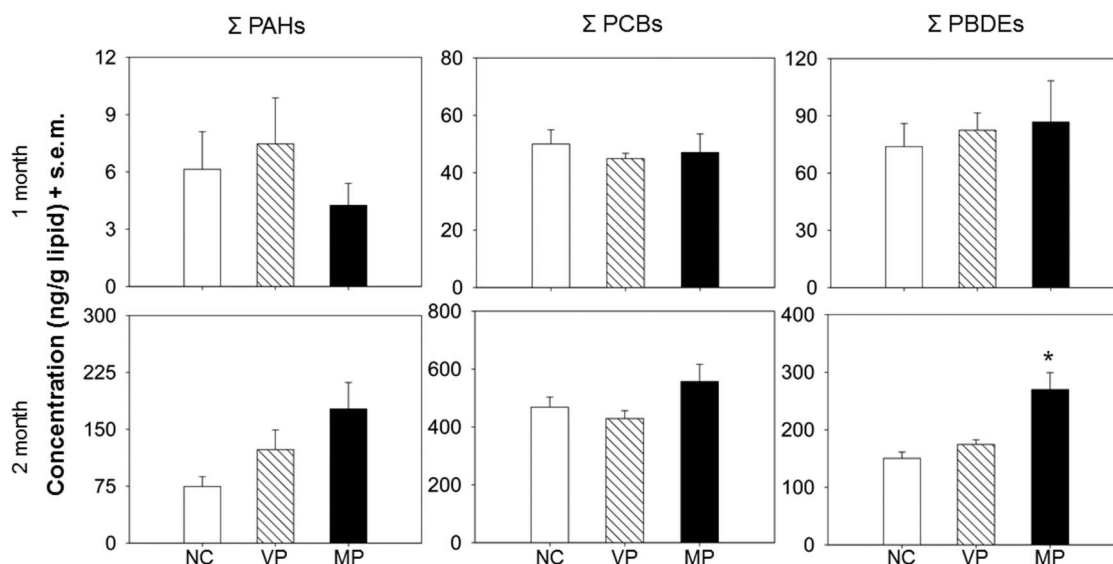


Figure 2 | Body burden of *Oryzias latipes* after the 1- and 2-month exposure. Bar graphs show mean concentrations (ng/g lipid + s.e.m) of total PAHs (left), PCBs (middle) and PBDEs (right) in fish tissue ($n = 3$) after one (top) and two (bottom) months of exposure. White bars represent the negative control (NC), bars with diagonal lines represent the virgin-plastic (VP) and black bars represent the marine-plastic (MP) treatment. A 2-factor ANOVA showed no significant differences between treatments for total PAHs, PCBs or PBDEs after 1 month and for total PAHs and PCBs after 2 months, but showed a significant difference ($P = 0.0003$) between treatments for total PBDEs after 2 months. A post-hoc SNK distinguished the marine-plastic having greater concentrations than the virgin-plastic and control treatment.

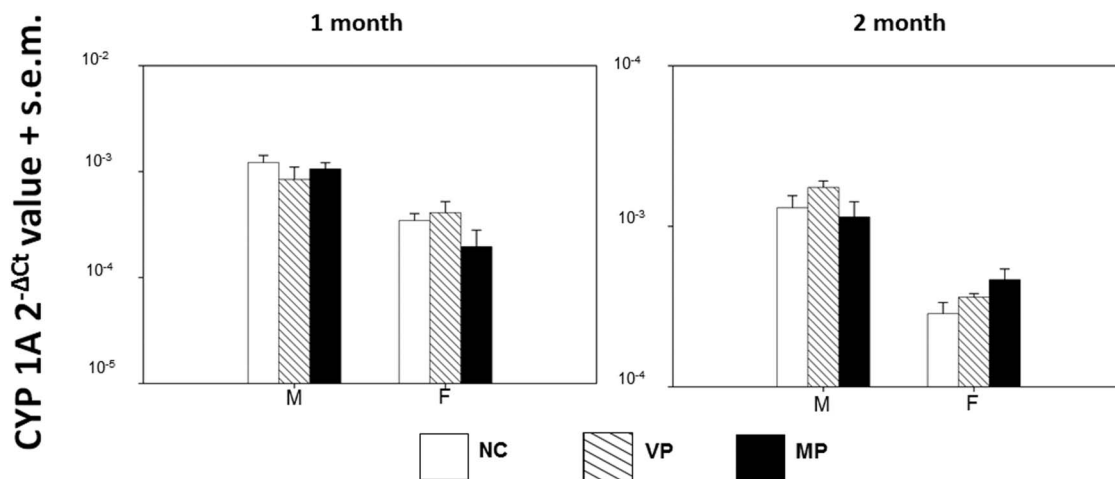


Figure 3 | Hepatic CYP1A expression in medaka. The CYP1A value (y-axis) is given as the mean $2^{-\Delta C_t}$ value + s.e.m. (individual data points normalized to the internal control⁴⁸) for the 1-month (left) and 2-month (right) exposure for males (M) and females (F) separately ($n = 3$). For all graphs the negative control (NC) treatment is depicted by white, the virgin-plastic (VP) treatment by diagonal and the marine-plastic (MP) treatment by black bars. For CYP1A expression, 1-factor ANOVAs showed no significant difference ($P > 0.05$) between treatments for either sex after both time periods.

from the virgin-plastic treatment and 21% of fish from the control treatment. Single cell necrosis was seen in 11% of fish from the marine-plastic treatment and in 0% of fish from the control and virgin-plastic treatment. An eosinophilic focus of cellular alteration, a precursor to a tumor, was seen in one fish from the virgin-plastic treatment (Figure 4b) and a tumor, a hepatocellular adenoma (comprising 25% of the liver), was seen in one fish from the marine-plastic treatment (Figure 4c).

Discussion

One question often asked is whether plastic debris is a vector for PBTs to bioaccumulate in organisms that ingest it. The objective of this work was to address this question using a medaka fish model, as several species of fish in the wild ingest plastic debris^{27–32}. Our model included environmentally relevant factors—LDPE fed to fish contained concentrations of PBTs that sorbed to LDPE from seawater in an urban bay, fish were chronically exposed to plastic similar to the way they are exposed in aquatic habitats²⁹, and fish were exposed to environmentally relevant concentrations of plastic⁵. We corroborated our first hypothesis, concluding that plastic deployed in the marine environment does serve as a vector for the bioaccumulation of PBTs sorbed to plastic, suggesting that plastic debris serves as a vector for the bioaccumulation of PBTs in wildlife.

While our experiment showed a general pattern, whereby concentrations of PAHs, PCBs and PBDEs were greatest in fish exposed to the marine-plastic treatment for 2 months, concentrations among

treatments were only significantly different ($\alpha = 0.05$) for chrysene, PCB28 and all PBDE congeners except BDE155. Because PAHs are easily biodegradable, bioaccumulation concentrations do not always reflect exposure⁴⁴ and thus the weak pattern shown here for PAHs may be expected. Due to the ubiquity of PCBs in the environment, it is difficult to differentiate bioaccumulation via plastic from bioaccumulation via the foodweb²², consistent with the difficulty observed here in differentiating PCB bioaccumulation from plastic versus from cod liver oil. Still, the significant effect shown here for a lower-chlorinated PCB congener (PCB28) is consistent with observations of plastic ingestion by seabirds in the laboratory¹⁰ and in nature²¹, both finding a positive relationship between the bioaccumulation of lower-chlorinated PCB congeners and plastic debris. Moreover, the strong pattern for PBDEs observed here is consistent with previous observations showing a pattern between PBDEs and plastic ingestion in wild seabirds²².

One might expect to see a similar pattern for PCBs and PBDEs due to their similarities in hydrophobicity. The pattern was the same, and we observed greater concentrations of PCBs and PBDEs in fish fed the marine-plastic treatment than the virgin-plastic and negative control treatments; however, the effect size for PCBs ($P = 0.1$) was not large enough to be considered significant at $\alpha = 0.05$. PBDEs are less stable than PCBs, breaking down into their lower brominated congeners, and biomagnifying less than PCBs^{45,46}. Because PBDEs do not biomagnify as well as PCBs, making exposure from prey smaller than for PCBs, contribution of PBDEs from plastic may be easier to

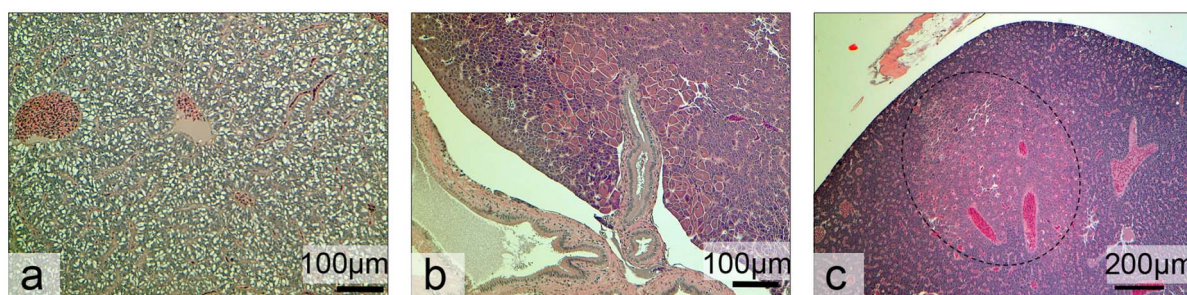


Figure 4 | Liver Histopathology in medaka sampled after 2 months. Micrographs show livers that are glycogen-rich from the control treatment (a) and glycogen-depleted from the virgin-plastic (b) and the marine-plastic treatment (c). An eosinophilic focus of cellular alteration, a precursor to a tumor, was observed in one fish from the virgin-plastic treatment (b). The circle highlights eosinophilic (pinkish coloration) hepatocytes, approximately twice as large as the basophilic (blue coloration) glycogen-depleted hepatocytes. The progression of neoplastic hepatocytes is evidence by the presence of a tumor, a hepatocellular adenoma, in one fish from the marine-plastic treatment (encircled in panel c).



clearly observe than the contribution of PCBs²². Furthermore, the instability of PBDEs may also mean they are more likely to transfer from plastic to an organism than PCBs, whose higher-chlorinated congeners have been shown to have a strong bond to plastic debris¹². Testing this hypothesis may improve understanding of the patterns observed here.

There may have been greater differences among treatments if the diets formulated did not include cod liver oil and were free from contamination of PAHs, PCBs and PBDEs in the absence of plastic. Still, the situation in this laboratory experiment is ecologically relevant to wildlife because of the ubiquity and persistence of PBTs in water, sediments and foodwebs globally⁴⁷. Because there were not statistically significant differences among concentrations of PAHs, PCBs or PBDEs between the virgin-plastic and control treatments, we conclude that the PBTs sorbed to plastic from ambient seawater did transfer from plastic to medaka upon ingestion. Thus, results from this experiment suggest that a chronic exposure of plastic debris in nature may be a significant route of exposure for PBTs in wildlife despite globally contaminated habitats. We suggest that future work test this hypothesis.

Another frequently asked question is how plastic ingestion affects the health of fish. This experiment demonstrates hepatic stress in medaka exposed to plastic, with a greater effect in fish exposed to the combination of plastic and sorbed contaminants. Thus, future assessments regarding hazards associated with plastic in aquatic habitats should consider the complex mixture associated with aquatic plastic debris^{11,23}.

Fish in this experiment were exposed to a complex mixture of chemicals via plastic ingestion, including targeted PAHs, PCBs and PBDEs in addition to constituents of the plastic itself and other non-targeted chemicals (including metals¹⁴) that sorb to LDPE from the marine environment. Not all chemicals activate AhR activity, and some act as inhibitors. For example, fluoranthene (detected in the diets fed to medaka in this study) inhibits AhR activity and can decrease expression of CYP1A⁴⁸. Increasing evidence indicates that multifactorial mechanisms might be involved when organisms are exposed to a complex mixture. Our results suggest that an enhanced and/or inhibitory metabolic compensatory response elicited during this chronic 2-month exposure, with various modes of action, may be the reason for the insignificant differences in AhR activity. For further evidence regarding effects from exposure, we examined the livers of experimental medaka for tissue damage. Because the liver plays a central role in the metabolism and detoxification of xenobiotics⁴⁹, liver damage may hinder induction of such mechanisms including AhR activation.

Using histology, we observed severe glycogen depletion, fatty vacuolation, cellular necrosis, and lesions. Severe glycogen depletion has been observed in fish exposed to organochlorinated xenobiotics, like PCBs, and is attributed to the direct effect of the chemical on carbohydrate metabolism and is likely linked to the energy cost of detoxification⁵⁰. Fatty vacuolation can lead to fatty liver degeneration⁵¹ and has been reported upon exposure to PCBs in rats⁵². Although the number of lesions observed in fish is low, the formation of preneoplastic and neoplastic lesions observed in fish from the virgin- and marine-plastic treatments are likely related to the plastic. No lesions were observed in fish from the control treatment and the formation and promotion of spontaneous tumors is very rare in medaka less than one year of age⁵³.

Overall, we conclude that polyethylene ingestion is a vector for the bioaccumulation of PBTs in fish, and that toxicity resulting from plastic ingestion is a consequence of both the sorbed contaminants and plastic material. Thus, hazards related to plastic debris are not one-sided – supporting the idea that the mixture of plastic and sorbed pollutants associated with plastic debris should be acknowledged in aquatic habitats²³. Future studies should examine the hypothesis that plastics are a multiple stressor in aquatic habitats, shifting

the focus to health effects from the combination of: the type, size and shape of the material, the chemical ingredients and the concentration of chemicals that sorbs to the material from the environment. Research that can prioritize those plastics associated with the greatest number of priority pollutants via their chemical constituents (e.g. polyurethane and polyvinylchloride^{7,11}) or those that sorb the largest concentrations of chemicals from the environment (e.g. PE and PP¹³) is suggested.

Meanwhile the waste we generate globally is accumulating faster than urbanization, 10% of which is plastic⁵⁴, predicted to reach 2.2 billion tons annually by 2025⁵⁴. As such, if we continue business-as-usual, by 2025 the amount of plastic discarded will surpass 220 million tons annually increasing opportunities for exposure by a wide breadth of organisms. Thus, it is time to implement more extensive research that can result in effective policy and management including the invention of materials that are sustainable and safe for people, the environment and wildlife.

Methods

Dietary exposure. Control, virgin- and marine- plastic treatment diets were prepared. To prepare the marine-plastic treatment, virgin LDPE pellets (3 mm diameter) were deployed in the southern part of San Diego Bay. Briefly, 50 g of LDPE pellets in an individual Nitex mesh (1.3 mm) bag was deployed from a PVC frame suspended from a floating dock approximately 0.5 m below the surface at the Coronado Cays Yacht Club. After a 3-month time period, the plastic pellets were collected and stored at -20°C until future analysis and use.

After deployment, pellets were prepared for plastic treatment diets. First, virgin and recovered marine LDPE pellets were rinsed in ultrapure water and dried under a fume hood to remove sediment from the marine samples. In a few cases, large fouling organisms were seen attached to individual pellets; these pellets were excluded. Next, marine- and virgin- LDPE pellets were ground to < 0.5 mm using separate conical burr grinders for conventional use. To avoid procedural contamination each grinder was cleaned by rinsing three times with acetone followed by hexane and grinding was conducted under a fume hood. Immediately before grinding, pellets were dipped in liquid nitrogen, using a stainless steel mesh, to avoid loss of chemical contamination due to heat. After grinding, plastics were sieved in pre-cleaned stainless steel metal sieves to collect fragments < 0.5 mm.

All treatment diets for the 2-month exposure were prepared in our laboratory prior to experimentation. Diets were mixed on pre-cleaned surfaces using separate pre-cleaned dishes for each treatment to avoid procedural contamination and cross-contamination among treatments. In nature, medaka are omnivorous, feeding upon phyto- and zoo- plankton³⁴. In the laboratory, diets are generally synthetic and may be supplemented with brine shrimp nauplia. For this experiment, treatment diets were mixed in the laboratory as follows. The negative control treatment diet contained 0% plastic and the virgin- and marine- plastic treatment diets contained 10% plastic. The ingredients for the negative control diet consisted of 62 g vitamin free casein, 30 g wheat gluten, 54.4 g dextrin, 8 g egg albumin, 10.4 g soy lecithin, 4 g vitamin premix, 6 g mineral premix, 4 g corn oil, 10 g cod liver oil and 7.2 g celufil. Vitamin and mineral mixes were purchased from ICN (Biomedical, Inc., Irvine, CA) and all other ingredients from U.S. Biochemical Corporation (Cleveland, OH). Diets containing plastic consisted of the same ingredients but substituted 20 g of dextrin with LDPE.

Adult medaka (7 month old and approximately 2.5–3 cm in length and 300 mg in weight) were randomly placed into nine 38 L tanks (71 fish per tank) on a 16-hour light-cycle. Water flow-rate and temperature was 720 ml/minute and 22–25°C respectively. Water quality (pH (7.8 ± 0.2), ammonium and nitrite (not detectable), nitrate (7.9 ± 1 ppm), water hardness ($120 \text{ mg L}^{-1} \text{ CaCO}_3$), electrical conductivity (400 μMhos) and alkalinity (100 mg/CaCO_3)) was monitored weekly. After one-month acclimation, three tanks were randomly assigned to each treatment.

During the exposure, fish were fed 2% body-weight per day divided into two portions (~ 3 mg diet, 0.3 mg of which is plastic). To assign portions each week, 20 fish per tank were weighed weekly and average body-weight per tank assessed. Fish were fed twice daily by sprinkling their diet over the surface of each tank. This caused the diet to disperse and thus the plastic particles and diet were independent of each other. Still, we observed fish eating the plastic and identified it in the fecal material and in their GI tract under a microscope. Thus, although we did not force-feed the fish plastic, we are sure that several fish did ingest it as part of their diet. During each 30-minute feeding, water-flow to the tanks was turned off to prevent LDPE-contamination in the recirculating system. Afterward, waste and 30% of the water were siphoned from each tank and floating plastic removed by net.

Tanks were cleaned weekly. To prevent cross-contamination, activated charcoal filters were used and changed twice per month. PBTs were not detected in any of three replicate water samples collected post-experiment. In addition, plastic was never observed in tanks with the negative control treatment. Care, maintenance, handling, and sampling followed protocols approved by the UC-Davis Animal Care and Use Committee. After one and two months, fish were euthanized with MS-222 (tricaine methane sulfonate, Argent Chemical Laboratories Inc., Redmond, WA), weighed, measured and prepared for analyses.



Chemical analysis of PAHs, PCBs and PBDEs. Water, plastic, diet and tissue samples were analyzed for PAHs, PCBs and PBDEs (Figure 1). For information regarding materials, sample preparation, analysis and QA/QC see Supplementary Methods. Concentrations of each congener and the totals of PAHs, PCBs, and PBDEs were quantified in fish. At the one-month sampling period, one pooled 5 g sample (approximately 14 fish) from each tank was analyzed and a 1-factor ANOVA ($n = 3$; $\alpha = 0.05$) analyzed log-transformed concentrations among treatments. At the two-month sampling period, three pooled 5 g samples from each tank were analyzed and a 2-factor ANOVA ($n = 3$; $\alpha = 0.05$) analyzed log-transformed concentrations among treatments with fixed-factor “treatment” and random-factor “tank” nested in “treatment”. When “tank \times treatment” was > 0.250 , this term was pooled. For the majority of the data, a Cochran’s (1951) C-test ($\alpha = 0.05$) showed homogeneity of variances. In some cases variances were heterogeneous, but still analyzed because analysis of variance is relatively robust to heterogeneous variances⁵⁵. SNK tests ($\alpha = 0.05$) distinguished significantly different treatment means. Statistical analyses were performed using GMAV (EICC, University of Sydney).

CYP1A expression. At each sampling, livers of six male and six female medaka (pooled by sex) were sampled from each replicate tank, weighed and stored at -80°C . Total RNA was isolated using TRIzol[®] reagent following the manufacturer’s instruction (Life Technologies, Carlsbad, CA, USA). Concentration of total RNA was quantified by NanoDrop[®] spectrophotometry (NanoDrop Technologies, Wilmington, DE, USA). All RNA samples were treated with DNase I (Sigma, AMPD1-1Kit) to eliminate carryover contamination of genomic DNA. First-strand cDNA synthesis was performed using Superscript-II (Life Technologies) following the manufacturer’s protocol. Primers for reference genes (GAPDH, β actin, and 18SrRNA) and CYP1A for RT-qPCR were purchased from Invitrogen (Supplementary Table 4)^{56,57}. All reactions were performed in triplicate using an AB 7900 HT FAST thermocycler with SYBR-Green Universal Master Mix (Life Technologies). The internal control for each sample was calculated as the geometric mean of the three reference genes (GAPDH, β actin, 18sRNA). The coefficient of variation among internal controls across all samples was $< 5\%$. The amplification efficiencies for the primer and probe set between CYP1A and the three reference genes were within $\pm 5\%$.

The cycle threshold value (C_T) for the reference genes and the gene of interest was determined and C_T values for each sample were calculated as the mean of triplicate reactions. 1-factor ANOVAs ($n = 3$, $\alpha = 0.05$), with fixed factor treatment, analyzed $2^{-\Delta C_T}$ values (individual data points normalized to the internal control⁵⁸) among treatments for each sex and sampling period individually. $2^{-\Delta C_T}$ values were log transformed and homogeneity of variance was verified by a Levene’s test ($\alpha = 0.05$). Statistical analyses were performed using SYSTAT 12 (SYSTAT Software, Chicago, IL).

Histopathology. At the two-month sampling period eight fish (4 males and 4 females) were randomly sampled from each replicate tank for histopathology. Fish were fixed in 10% neutral buffered formalin and dehydrated in a graded ethanol series and embedded in paraffin. Serial trans-sagittal sections (3 μm) were stained with hematoxylin and eosin (H&E) and lateral views screened by a BH-2 Olympus microscope. Five fish from the marine-plastic treatment were sacrificed due to methodological error during processing for histopathological analysis. Livers were screened for a variety of histopathological features and scored on an ordinal ranking system of 0 = none/minimal, 1 = mild, 2 = moderate, and 3 = severe. Glycogen depletion is characterized by smaller hepatocytes and irregular, and poorly demarcated cytoplasmic vacuolation and increased cytoplasmic basophilia. Single-cell necrosis is characterized by eosinophilic (i.e., pink coloration) cytoplasm with nuclear pyknosis and karyorrhexis. Fatty vacuolar degeneration, lipidosis, is characterized by excess lipid, appearing as clear, round, and well-demarcated cytoplasmic vacuoles. Because of the significance of foci of cellular alteration and hepatic tumors in the progression of fish hepatocarcinogenesis, these lesions were counted rather than scored.

- Thompson, R. C. *et al.* Lost at sea: Where is all the plastic? *Science* **304**, 838 (2004).
- Browne, M. A. *et al.* Accumulations of microplastic on shorelines worldwide: sources and sinks. *Environ. Sci. Technol.* **45**, 9175–9179 (2011).
- Barnes, D., Galgani, F., Thompson, R. & Barlaz, M. Accumulation and fragmentation of plastic debris in global environments. *Phil. Trans. R. Soc. B.* **364**, 1985–1998 (2009).
- Law, K. L. *et al.* Plastic accumulation in the North Atlantic Subtropical Gyre. *Science* **329**, 1185–1188 (2010).
- Goldstein, M. C., Rosenberg, M. & Cheng, L. Increased oceanic microplastic debris enhances oviposition in an endemic pelagic insect. *Biol. Lett.* **8**, 817–820 (2012).
- PlasticsEurope, Plastics—the Facts 2012: An analysis of European plastics production, demand and waste data for 2011. <http://www.plasticseurope.org/Document/plastics-the-facts-2012.aspx?FolID=2> (2012), January, 2013.
- Rochman, C. M. *et al.* Classify plastic waste as hazardous. *Nature* **494**, 169–171 (2013).
- Pauly, J. *et al.* Inhaled Cellulosic and Plastic Fibers Found in Human Lung Tissue. *Cancer Epidem. Biomar.* **7**, 419–428 (1998).

- von Moos, N., Burkhardt-Holm, P. & Kohler, A. Uptake and effects of microplastics on cells and tissue of the blue mussel *Mytilus edulis* L. after an experimental exposure. *Environ. Sci. Technol.* **46**, 11327–11335 (2012).
- Teuten, E. L. *et al.* Transport and release of chemicals from plastics to the environment and to wildlife. *Phil. Trans. R. Soc. B* **364**, 2027–2045 (2009).
- Lithner, D., Larsson, A. & Dave, G. Environmental and health hazard ranking and assessment of plastic polymers based on chemical composition. *Sci. Total. Environ.* **409**, 3309–3324 (2011).
- Hirai, H. *et al.* Organic micropollutants in marine plastic debris from the open ocean and remote and urban beaches. *Mar. Pollut. Bull.* **62**, 1683–1692 (2011).
- Rochman, C. M., Hoh, E., Hentschel, B. T. & Kaye, S. Long-term field measurements of sorption of organic contaminants to five types of plastic pellets: Implications for plastic marine debris. *Environ. Sci. Technol.* **47**, 1646–1654 (2013).
- Holmes, L. A., Turner, A. & Thompson, R. C. Adsorption of trace metals to plastic resin pellets in the marine environment. *Environ. Pollut.* **160**, 42–48 (2012).
- Browne, M. A., Dissanayake, A., Galloway, T., Lowe, D. & Thompson, R. Ingested microscopic plastic translocates to the circulatory system of the mussel, *Mytilus edulis* (L.). *Environ. Sci. Technol.* **42**, 5026–5031 (2008).
- Guillette Jr, L. J. *et al.* Development abnormalities of the gonad and abnormal sex hormone concentrations in juvenile alligators from contaminated and control lakes in Florida. *Environ. Health Perspect.* **102**, 680–688 (1994).
- McKinley, A. & Johnston, E. L. Impacts of contaminant sources on marine fish abundance and species richness: a review and meta-analysis of evidence from the field. *Mar. Ecol. Prog. Ser.* **420**, 175–191 (2010).
- Johnston, E. L. & Roberts, D. A. Contaminants reduce the richness and evenness of marine communities: A review and meta-analysis. *Environ. Pollut.* **157**, 1745–1752 (2009).
- Gaylor, M. O., Harvey, E. & Hale, R. C. House crickets can accumulate polybrominated diphenyl ethers (PBDEs) directly from polyurethane foam common in consumer products. *Chemosphere* **86**, 500–505 (2012).
- Besseling, E., Wegner, A., Foekema, E., Van Den Heuvel-Greve, M. & Koelmans, A. A. Effects of microplastic on fitness and PCB bioaccumulation by the lugworm *Arenicola marina* (L.). *Environ. Sci. Technol.* **47**, 593–600 (2013).
- Yamashita, R., Takada, R., Fukuwaka, M. & Watanuki, Y. Physical and chemical effects of ingested plastic debris on short-tailed shearwaters, *Puffinus tenuirostris*, in the North Pacific Ocean. *Mar. Pollut. Bull.* **62**, 2845–2849 (2011).
- Tanaka, K. *et al.* Accumulation of plastic-derived chemicals in tissues of seabirds ingesting marine plastics. *Mar. Pollut. Bull.* **69**, 219–222 (2013).
- Rochman, C. M. Plastics and priority pollutants: a multiple stressor in aquatic habitats. *Environ. Sci. Technol.* **47**, 2439–2440 (2013).
- Cole, M., Lindeque, P., Halsband, C. & Galloway, T. S. Microplastics as contaminants in the marine environment: A review. *Mar. Pollut. Bull.* **62**, 2588–2597 (2011).
- Nelson, J. S. *Fishes of the World*, 4th ed. John Wiley and Sons, Inc., New York (2006).
- McCauley, R. W. Determining the health of fish communities—parallels with human medicine. *J. Great Lakes Res.* **16**, 635–636 (1990).
- Davison, P. & Asch, R. Plastic ingestion by mesopelagic fishes in the North Pacific Subtropical Gyre. *Mar. Ecol. Prog. Ser.* **432**, 173–180 (2011).
- Lusher, A. L., McHugh, M. & Thompson, R. C. Occurrence of microplastics in the gastrointestinal tract of pelagic and demersal fish from the English Channel. *Mar. Pollut. Bull.* **67**, 94–99 (2013).
- Possatto, F. E., Barletta, M., Costa, M. F., Ivar do Sul, J. A. & Dantas, D. Plastic debris ingestion by marine catfish: An unexpected fisheries impact. *Mar. Pollut. Bull.* **62**, 1098–1102 (2011).
- Ramos, J. A. A., Barletta, M. & Costa, M. F. Ingestion of nylon threads by Gerreidae while using a tropical estuary as foraging grounds. *Aquat. Biol.* **17**, 29–34 (2012).
- Dantas, D. V., Barletta, M. & Ferreira da Costa, M. The seasonal and spatial patterns of ingestion of polyfilament nylon fragments by estuarine drums (Sciaenidae). *Environ. Sci. Pollut. Res.* **19**, 600–606 (2012).
- Carpenter, E. & Smith, K. Plastics on the Sargasso Sea surface. *Science* **175**, 1240–1241 (1972).
- Teh, S. J. & Hinton, D. E. Gender-specific growth and hepatic neoplasia in medaka (*Oryzias latipes*). *Aquat. Toxicol.* **41**, 141–159 (1998).
- Shima, A. & Mitani, H. Medaka as a research organism: past, present and future. *Mech. Develop.* **121**, 599–604 (2004).
- Andrady, A. L. Microplastics in the marine environment. *Mar. Pollut. Bull.* **62**, 1596–1605 (2011).
- Meeker, J. D., Sathyanarayana, S. & Swan, S. H. Phthalates and other additives in plastics: human exposure and associated health outcomes. *Phil. Trans. R. Soc. B* **364**, 2097–2113 (2009).
- Karami, S. *et al.* Renal cancer risk and occupational exposure to polycyclic aromatic hydrocarbons and plastics. *J. Occup. Environ. Med.* **53**, 218–223 (2011).
- Livingstone, D. R. The fate of organic xenobiotics in aquatic ecosystems: quantitative and qualitative differences in biotransformation by invertebrates and fish. *Comp. Biochem. Phys. A* **120**, 43–49 (1998).
- Bemanian, V., Male, R. & Goksoyr, A. The aryl hydrocarbon receptor-mediated disruption of vitellogenin synthesis in the fish liver: Cross-talk between AHR- and ER α -signaling pathways. *Comp. Hepatol.* **3**, 1–14 (2004).



40. Fang, C. *et al.* PFOS elicits transcriptional responses of the ER, AHR and PPAR pathways in *Oryzias melastigma* in a stage-specific manner. *Aquatic Toxicol.* **106–107**, 9–19 (2012).
41. Bruschweiler, B. J., Wurgler, F. E. & Fent, K. Inhibitory effects of heavy metals on cytochrome P4501A induction in permanent fish hepatoma cells. *Arch. Environ. Contam. Toxicol.* **31**, 475–482 (1996).
42. Kashiwada, S. *et al.* Age-dependent *in situ* hepatic and gill CYP1A activity in the see-through medaka (*Oryzias latipes*). *Comp. Biochem. Phys. C* **145**, 96–102 (2007).
43. Jimenez, B. D., Oikari, A., Adams, S. M., Hinton, D. E. & McCarthy, J. F. Hepatic enzymes as biomarkers: Interpreting the effects of environmental, physiological and toxicological variables. In Mc-Carthy, J. F. & Shugart, L. E., eds, *Biomarkers of Environmental Contamination*. Lewis, Boca Raton, FL, USA, pp 231–244 (1990).
44. van der Oost, R., Beyer, J. & Vermeulen, N. P. E. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. *Environ. Toxicol. Pharm.* **13**, 57–149 (2003).
45. Mizukawa, K. *et al.* Bioconcentration and biomagnifications of polybrominated diphenyl ethers (PBDEs) through lower-trophic-level coastal marine food web. *Mar. Pollut. Bull.* **58**, 1217–1224 (2009).
46. Mizukawa, K. *et al.* Biomagnification and debromination of polybrominated diphenyl ethers in a coastal ecosystem in Tokyo Bay. *Sci. Tot. Environ.* **449**, 401–409 (2013).
47. Ross, P. S. & Birnbaum, L. S. Integrated human and ecological risk assessment: A case study of persistent organic pollutants (POPs) in humans and wildlife. *Hum. Ecol. Risk Assess.* **9**, 303–324 (2010).
48. Willett, K. L., Wassenberg, D., Lienesch, L., Reichert, W. R. & Di Giulio, R. T. *In Vivo* and *in Vitro* inhibition of CYP1A-dependent activity in *Fundulus heteroclitus* by the polynuclear aromatic hydrocarbon fluoranthene. *Toxicol. Appl. Pharm.* **177**, 264–271 (2001).
49. Olsvik, P. A., Lie, K. K., Saele, O. & Sanden, M. Spatial transcription of CYP1A in fish liver. *BMC Phys.* **7**, 1–10 (2007).
50. Hugla, J. L. & Thome, J. P. Effects of polychlorinated biphenyls on liver ultrastructure, hepatic monooxygenases, and reproductive success in the barbel. *Ecotox. Environ. Safte.* **42**, 265–273 (1999).
51. Wester, P. W. & Canton, J. H. Histopathological study of *Poecilia reticulata* (guppy) after long-term exposure to bis(tri-*n*-butyltin)oxide (TBTO) and di-*n*-butyltin dichloride (DBTC). *Aquat. Toxicol.* **10**, 143–165 (1987).
52. Bruckner, J. V., Khanna, K. L. & Cornish, H. H. Biological responses of the rat to polychlorinated biphenyls. *Toxicol. Appl. Pharm.* **24**, 434–448 (1973).
53. Hawkins, W. E., Overstreet, R. M. & Walker, W. W. Carcinogenicity tests with small fish species. *Aquat. Toxicol.* **11**, 113–128 (1988).
54. The World Bank. What a Waste: A global review of solid waste management. Urban Development Series Knowledge Papers, March 2012.
55. Underwood, A. J. Techniques of analysis of variance in experimental marine biology and ecology. *Annu. Rev. Oceanogr. Mar. Biol.* **19**, 513–603 (1981).
56. Zhang, Z. & Hu, J. Development and validation of endogenous reference genes for profiling of medaka (*Oryzias latipes*) exposed to endocrine disrupting chemicals by quantitative real-time RT-PCR. *Toxicol. Sci.* **95**, 356–368 (2007).
57. Hong, H. N., Kim, H. N., Park, K. S., Lee, S. & Gu, M. B. Analysis of the effects of diclofenac on Japanese medaka (*Oryzias latipes*) using real-time PCR. *Chemosphere* **67**, 2115–2121 (2007).
58. Schmittgen, T. D. & Livak, K. L. Analyzing real-time PCR data by the comparative C_T method. *Nature Protocols* **3**, 1101–1108 (2008).

Acknowledgments

This material is based on work supported by EPA Superfund Region 9, UC Davis Aquatic Health Program, SDSU Division of Research Affairs, and a National Science Foundation Graduate Research Fellowship (Grant No. 2010101195). The American Chemistry Council donated virgin plastic pellets. We thank Coronado Cays Yacht Club for donating dock space. R. Struch assisted with chemical analyses at SDSU, W. Lao and K. Maruya assisted with chemical analysis at SCCWRP, Z. Schakner assisted in the field, M. A. Browne provided statistical advice and S. Serrato, C. Teh, S. Acuna, M. Kawaguchi and I. Flores assisted in the laboratory. We thank K. Maruya for comments on an earlier version of the manuscript.

Author contributions

C.M.R. designed the study, conducted the field deployment and dietary exposure, processed analytical chemistry and qPCR samples, performed data analysis, designed figures and wrote the manuscript. S.J.T. advised on experimental design, processed histology samples, performed data analysis, helped design figures and commented on the manuscript. E.H. advised on chemical and data analyses and commented on the manuscript. T.K. processed qPCR samples, advised on qPCR and data analyses and design of figures and commented on the manuscript.

Additional information

Supplementary information accompanies this paper at <http://www.nature.com/scientificreports>

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Rochman, C.M., Hoh, E., Kurobe, T. & Teh, S.J. Ingested plastic transfers hazardous chemicals to fish and induces hepatic stress. *Sci. Rep.* **3**, 3263; DOI:10.1038/srep03263 (2013).



This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivs 3.0 Unported license. To view a copy of this license, visit <http://creativecommons.org/licenses/by-nc-nd/3.0>