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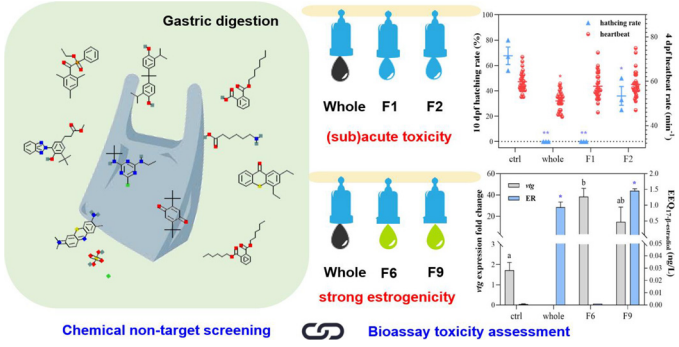
Bioassay guided analysis coupled with non-target chemical screening in polyethylene plastic shopping bag fragments after exposure to simulated gastric juice of Fish

Qiqing Chen[a,*], Mauricius Marques dos Santos[b,c], Philip Tanabe[d], Gary T. Harraka[d], Jason T. Magnuson[d], Victoria McGruer[d], Wenhui Qiu[e], Huahong Shi[a], Shane A. Snyder[b,c], Daniel Schlenk[d,f,**]

[a] State Key Laboratory of Estuarine and Coastal Research, East China Normal University, Shanghai, 200241, China
[b] Nanyang Environment & Water Research Institute (NEWRI), Nanyang Technological University, 1 Cleantech Loop, CleanTech One, #06-08, 637141, Singapore
[c] Department of Chemical & Environmental Engineering, University of Arizona, 1133 E James E Rogers Way, Harshbarger 108, Tucson, AZ, 85721-0011, USA
[d] Department of Environmental Sciences, University of California, Riverside, California 92521, United States
[e] State Environmental Protection Key Laboratory of Integrated Surface Water- Groundwater Pollution Control, School of Environmental Science and Engineering, Southern University of Science and Technology, Shenzhen 518055, China
[f] Institute of Environmental Health, College of Environmental and Resource Sciences, Zhejiang University, Hangzhou 310058, China

GRAPHICAL ABSTRACT



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ABSTRACT

In this study, fragments of polyethylene plastic bags were treated with simulated gastric juice of fish for 16 h. Following solid-phase extraction, methanol eluents caused acute toxicity to embryos and larvae of Japanese medaka. Chromatographic fractions (polar to more non-polar with numbers increasing) of the extract were evaluated for toxicity and estrogenic activity using medaka and an estrogen receptor (ER) cell-line. Fractions 6 and 9 had the highest estrogenic effects with relative hydrophobic chemicals. The *vtg* expression in fraction 6 was 22-fold higher than control, and the ER cellular response in fraction 9 was 8.5-fold higher than controls. Following non-target screening (NTS), several novel phthalates and phenols were identified in the above two fractions. Fractions 1 and 2 appeared to be primarily responsible for the acute toxicity observed with the whole extract. The hatching rate decreased to 36 % in fraction 2, and was not observed following exposure to fraction 1. NTS of these fractions indicated 635 and 808 entities, respectively, most without toxicity information. These

* Corresponding author.
** Corresponding author at: Department of Environmental Sciences, University of California, Riverside, CA 92521, United States.
E-mail addresses: chenqiqing@sklec.ecnu.edu.cn (Q. Chen), dschlenk@ucr.edu (D. Schlenk).

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results indicate plastic leachates from gastric juices of fish are complex mixtures of many compounds that can have acute reproductive and sublethal endocrine impacts in fish.

1. Introduction

In 2017, annual plastic production (348 million tons) surpassed the total mass of human beings on the planet. Plastic packaging constitutes 39.6 % of total plastic production (Steffen et al., 2015; EuropePlastic, 2018). Much of this plastic is transported to surface waters and consumed by wildlife (Menezes et al., 2019; Jabeen et al., 2017; Gatidou et al., 2019; Li et al., 2020; Su et al., 2019). After consumption, gastric digestion has been shown to release numerous additives from the plastic matrix into fluids that are absorbed into the gastrointestinal tracts of biota (Tanaka et al., 2015, 2018; Guo et al., 2020; Liu et al., 2020), and these pollutants may even be transferred to organisms at higher trophic levels (Tanaka et al., 2018).

Various kinds of additives have been detected in plastics, such as plasticizers, bisphenols, as well as persistent bioaccumulative toxic substances (PBTs) (Guo et al., 2020; Zhang et al., 2018; Chen et al., 2019a, 2018; Jang et al., 2016; Chen et al., 2019b). Plasticizers are used for improving the durability and flexibility of polymeric films (Bhunia et al., 2013; Hahladakis et al., 2018). Phthalates, acetyltributyl citrate, and heptyl adipate are all widely used plasticizers (Sablani and Rahman, 2007). Antioxidants are also commonly used in plastic packaging, which are embedded to delay the oxidative degradation induced by reactive free radicals that are generated under light, heat, or mechanical abrasion (Bhunia et al., 2013; Sablani and Rahman, 2007). Typical antioxidants include arylamines, phenolics, and organophosphites, such as bisphenol A (BPA), Irgafos, Irganox, Cyanox series (Bhunia et al., 2013; Kattas et al., 2000). Other known constituents of plastics include colorant pigments, heat stabilizers, slip agents, residual, and unreacted oligomers (Hahladakis et al., 2018).

The most commonly used plasticizers and antioxidants in polyethylene are phthalates, adipate, BPA, DEHA, and alkylphenols, whose migration has been widely documented in literature (Beldi et al., 2012; Fasano et al., 2012; Jeon et al., 2007). Moreover, polyethylene has been widely used to produce shopping bags, and many studies have reported that their embedded additives can leach out under different conditions (Suhrhoff and Scholz-Böttcher, 2016; Mattila et al., 2011; Alam et al., 2018; Simoneit et al., 2005). Previous studies have reported that the detected additive and oligomer amounts reach $0.1-0.23\%$ of the mass of polyethylene shopping bags with multiple compounds detected (Suhrhoff and Scholz-Böttcher, 2016; Simoneit et al., 2005). Examples include phthalates, Irgafos 168 and 1076, tris(2,4-di-tert-butylphenyl) phosphate, chain *n*-alkanes, acetyl tri-*n*-butyl citrate, dehydroabietic acid, heavy metals, organometallic compounds, and many additives' oxidation and degradation products (Suhrhoff and Scholz-Böttcher, 2016; Alam et al., 2018; Simoneit et al., 2005).

Plastic additives have been shown to have multiple adverse effects on marine organisms (Hermabessiere et al., 2017; Guo and Wang, 2019). Many additives, such as phenols and phthalates, have been shown to mimic estrogen and disrupt endocrine pathways in wildlife (Olujimi et al., 2010; Boas et al., 2012). Given the diversity of plastic items and their constituents, not all plastic items have the same levels or types of additives. In a previous study, our results showed that polyethylene plastic bag leachates from simulated bird and fish gastric juices had the highest concentrations of 12 additives targeted for chemical analysis and exhibited the highest estrogen receptor activity among 16 plastic items commonly found in natural organisms (Coffin et al., 2019). When comparing the estrogenic activities predicted from the quantified targeted chemicals with the real biological activities, only 17 % of the activity contribution can be explained (Coffin et al., 2019), suggesting that additional chemicals with estrogen ligand activities were present in the leachate. According to Groh et al. (2019), at least 906 likely chemicals and 3377 possible chemicals have been reported to be present on plastic packaging (Groh et al., 2019).

The combination of effects directed analyses (EDA) with non-target screening (NTS) has been shown to identify causative toxic agents in extracts of surface water, wastewater effluents, and sediments (Brack et al., 2007, 2018). In the current study, we chromatographically separated simulated gastric fluid leachates of polyethylene shopping bag fragments into fractions. Sequentially, the whole extract and the fractions underwent *in vivo* and *in vitro* evaluations for estrogenic activity and acute toxicity. Biologically active fractions then underwent NTS to identify chemical constituents. Results indicate complex mixtures of multiple novel compounds without toxicological evaluation may contribute to the biological effects previously observed with this specific type of plastic.

2. Materials and methods

2.1. Chemicals and materials

Sea salt was purchased from the Instant Ocean (Spectrum, USA). Shopping plastic bags were bought from a local supermarket (reusable type, USA). All other chemical reagents used in the study were of analytical grade (Sigma-Aldrich, USA). All the glassware were thoroughly rinsed with methanol three times to avoid organic contamination and then rinsed by Milli-Q water and dried at 60 °C before use. Procedural blanks were conducted in parallel throughout the process.

2.2. Organisms and cells used for bioassays

Japanese medaka (*Oryzias latipes*) was used to screen plastic leachate toxicity in the present study. The medaka was cultured at the University of California, Riverside (AUP # 20,140,002), and housed in medium-hard water at 28 °C with a photoperiod of 14:10 h of light: dark. Fish adults were fed twice daily with brine shrimp (*Artemia nauplii*), and experimental embryos were collected before 4 hpf (Coffin et al., 2018). Human breast carcinomaVm7Luc4E2 (ER) cells were donated by Dr. Michael Denison (University of California, Davis). The cells were incubated in the ER growth media of Roswell Park Memorial Institute (RPMI)-1640 (Mediatech Inc., USA) and 10 % dialyzed fetal bovine serum (Invitrogen, USA) at 37 °C before assay. The assay media was phenol-red free Dulbecco's minimum essential medium with 5% charcoal-stripped fetal bovine serum and 2% Glutamax (Sigma-Aldrich, USA) (Coffin et al., 2019).

2.3. Polyethylene plastic packaging fragment digestion and leachates separation

White colored polyethylene plastic bags were bought from a local supermarket, and the composition was identified as polyethylene using Fourier Transform Infrared spectroscopy (FTIR) with a matching rate > 70 % (Nicolet iN10, Thermal Fisher Scientific, USA) (Fig. S1A). Meanwhile, we also found one additive, calcium carbonate, on the plastic surface (Fig. S1B). This additive is usually used as a pigment or modifier embedded in plastics (Murphy, 2001).

Since previous studies indicated simulated fish digestive leachate from this item possessed significant biological and chemical activities for the estrogen receptor, similar methods were used in this study (Bigg, 1985; Jackson et al., 1987). In brief, 2 g of pepsin, 32 g of sea salt were added into 1 L of Milli-Q water in a glass container. Then, shopping

plastic bags (Fig. S2) were arbitrarily cut into small square fragments (~5 cm length) and put into a glass container. Next, the containers were shaken at 100 rpm (1575 R, VWR Scientific, USA) at 28 °C for 16 h (to simulate fish gut retention time) (Turner et al., 2001).

The amount of plastic fragments used in 1 L extraction solution was 7 g (0.007 g/g). The reported plastic mass concentration in fish samples was 5 ± 8 mg for *Symbolophorus californiensis* (Boerger et al., 2010). The gastrointestinal (GI) tract of fish usually falls in the range of 9–14 % of body weight (Ray and Ringø, 2014). The calculated weight of *S. californiensis* was around 10 g according to its length (Length-Weight relationship for Symbolophorus californiensis, 2020). Thus, the estimated plastic concentration in fish is approximately 0.005 ± 0.007 g/g, which has a similar plastic burden to that used in the present study.

2.4. SPE extraction and RP-HPLC fractionation for plastic extracts

Solid-Phase Extraction (SPE) was used to remove and concentrate additives from the plastic leachates from the simulated gastric juice. Sep-Pak C18 cartridges (6cc (1 g), Waters, USA) were rinsed with 10 mL of methanol and then conditioned with 10 mL of Milli-Q water. Then, each cartridge was loaded with 500 mL of the plastic leachate samples with a flow rate of one drop per second. The cartridges were later washed with Milli-Q water and then eluted with 8 mL of methanol at a flow rate of one drop per 3 s. Finally, two cartridge samples were pooled as one sample, and the extracts were blown to dryness with nitrogen gas and resuspended in methanol. These methanol samples were then separated into two parts: one-tenth was replaced by dimethyl sulfoxide (DMSO) for bioassay analysis, and the leftover remained in methanol for chemical fractionation and quantification.

For the chemical fractionation, the extract from SPE underwent chromatographic separation using High-Performance Liquid Chromatography (HPLC, LC-2030, Prominence-*i*, Shimadzu, Japan) coupled with a Fractionation Collector (CF-1, Spectra/Chrom, Fisher Scientific). Plastic extracts (40 µL) were injected into a C18 column (4.6 mm I.D. × 150 mm, Shiseido, Japan), according to Reineke et al. (Reineke et al., 2002) with modifications. The plastic leachate samples were separated using an isocratic solvent mixture of 50 % water and 50 % methanol over 30 min. A 50 % mixture was arbitrarily based on previous studies with sediment extracts (Schlenk et al., 2005) and wastewater extracts (Sapozhnikova et al., 2005). Fractions were collected every 3 min, with a flow of 1 mL/min resulting in ten fractions. The column temperature was 40 °C. For the subfractionation of fraction 1 and fraction 2, the same HPLC program was used, but fractions were taken every 60 s, resulting in three subfractions per fraction. All the fractions and subfractions were blown to the water phase, and the same volumes of mobile phase (50 % water and 50 % methanol) were collected as solvent control. Based on biological activities, three fourth of the samples were used for bioassay analysis, and the remaining one fourth of the sample was evaporated under nitrogen gas with temperature below 40 °C to dryness for further chemical analysis.

2.5. Plastic extract fractions toxicity to medaka embryos and larvae

For the plastic extract fractions toxicity studies, ten healthy fertilized embryos were selected and transferred to Petri-dishes for exposure, and the final concentration was 1× (relative to ambient concentration). The mortality percentage and heartbeat rate of embryos were recorded after 4 day post fertilization (dpf) and 5dpf for each subfraction (Fig. S3) or fraction. The hatching rate of medaka larvae was recorded after 10 dpf. All exposure solutions were aerated to reach oxygen saturation and replaced 80 % daily.

2.6. Fish mRNA expression analysis

The development status of medaka embryos and larvae were observed with an inverse microscope (SZH10, Olympus, Japan). The hatched medaka larvae were continually exposed until 14 dpf with different plastic leachate fractionation solutions, along with negative control, solvent control, and positive control (containing 500 ng/L of 17-beta estradiol). On the last day of exposure, the fish were anesthetized with 1 g/L MS-222, and their whole bodies were flash-frozen in liquid nitrogen and stored at −80 °C until mRNA analysis.

Quantitative reverse transcriptase-polymerase chain reaction (*q-RT-PCR*) was carried out according to a previously described method (Braunig et al., 2015). First, RNA was extracted from the whole body of Japanese medaka larvae using an RNeasy Mini Kit (Qiagen, Germany). The RNA quality was assessed by the 260/280 nm ratio on a Nanodrop spectrophotometer and verified by its appearance on 1% agarose-formaldehyde gels. For each sample, the cDNA was synthesized from 200 ng/mL of RNA using a reverse transcription system kit following the manufacturer's instruction (Promega, USA). The primer sequences of the target genes of *vtg*, and the reference gene of *β-actin* were designed using the gene bank of NCBI (Table S1), and each gene was tested in three replicates and repeated three times. The reverse PCR experiment was performed as follows: the amplification reaction mixture contained reverse transcription product 1 µL, SybGreen PCR mix 8 µL, each primer (10 pmol/µL) 1 µL, and ultrapure water 5 µL. The reaction mixture underwent 2 min at 95 °C followed by 40 cycles of 10 s at 95 °C, and 30 s at 60 °C, and 5 s at 54 °C in a thermal cycler (CFX-6, Bio-Rad, USA). Fold changes were determined using $2^{\triangle\triangle Ct}$ and normalized the β-actin transcript levels.

2.7. ER bioassay

Cell viability was assessed using the 3-(4,5-dimethylthiazd-2-yl)-2,5- diphenyltetrazolium bromide (MTT) bioassay (Van Meerloo et al., 2011). Sample concentrations that elicited 80 % survival or greater in-well were deemed acceptable for receptor-binding activity measurements. The performance of the ER assay has been explained in detail in a previous study (Coffin et al., 2018). ER cells were then plated at a concentration of 2 × 10^4 cells/ 100 µL in 96-well plates and incubated at 37 °C for 24 h. Then, 10 µL of the leachate in DMEM was added in triplicate at several concentrations, ranging from 1× to 50× (relative to the ambient concentration before SPE) and incubated for 24 h. Cells were lysed, and the luciferase activity of cells was measured in a luminometer with automatic injection of 50 µL of luciferase assay reagent to each well. The relative light units measured were compared to the E2 standard curve following background activity subtraction. The EC_{50} of the positive control 17-*beta*-estradiol was 6.3 ng/L (22.98 pM). The Limit of Detection was 0.64 ng/L, and the Limit of Quantification was 1.28 ng/L.

2.8. Non-target screening

All extracts were diluted to 100× with methanol and analyzed in three technical replicates. Liquid Chromatography-High Resolution Mass Spectrometry (LC-HRMS) analysis was conducted on both positive and negative ionization modes with samples analyzed in random order on an Agilent 1290 Infinity II LC and a 6550 Q-TOF instrument. The separation was performed on an Agilent InfinityLab Poroshell 120 EC-C18, 2.1 × 100 mm, 1.9 µm column, with a 0.4 mL/min mobile phase flow, detailed conditions are provided on Table S2. Components were grouped and extracted according to respective ion clusters with a threshold abundance of 20,000 counts for peak identification using Agilent MassHunter Workstation Profinder (version 10.0). Statistical analysis and data interpretation were made using Agilent MassHunter Workstation Mass Profiler Professional (version 15.1). Potential EDC's and other potential additives were screened against available personal compound database and library (PCDL) for extractables and leachables that includes: (1) stabilizers, accelerators, intermediates, residual monomers, phthalates, lubricants, slip agents, photoinitiators, plasticizers, dyes, and cosmetic additives; (2) antioxidants, UV stabilizers and

their breakdown or degradation products; (3) food packaging contaminants and printing ink components and their breakdown or degradation products; (4) PFCs, PAHs, nitrosamines, and silicones. Tentative identification was achieved through a tiered workflow that considers different identification confidence levels (Schymanski et al., 2014). Probable structures were assigned by library spectrum match and diagnostic evidence (MS/MS) data generated by all ions fragmentation. Accurate mass error (ppm) of less than +/- 5 ppm, library match score > 85 (related to isotopic cluster distribution, number of assigned ions, observable adducts, accurate mass difference) and presence of all ions MS/MS fragmentation pattern were established as probable structure assignment criteria. Digestion and extraction procedural controls and instrument blanks were analyzed using the same procedures. Entities present both in samples (Fig. S4) with an abundance fold change of < 5 than procedural controls were excluded from data analysis.

For semi-quantitative analysis of identified entities, peak areas were normalized by reference mass ion intensities to account for possible ion suppression/enhancement and are presented as parts per thousand (‰) of total areas of all fractions (Table S3&S4).

2.9. Statistics

Statistical analysis was performed by using SPSS software (version 20.0, IBM, USA). The data were first verified for normality with the Shapiro-Wilk method and then compared by the one-way analysis of variance (ANOVA), and significant differences were indicated by using Duncan's multiple range test with $\alpha = 0.05$. Data are presented as mean ± SEM. Data with * represents $p < 0.05$ *vs.* the control values, and the bars with different letters mean they are statistically different from each other ($p < 0.05$).

3. Results and discussion

3.1. Estrogenic activity in different plastic leachate fractions

The estrogenic activity (EA) value of the unfractionated plastic extract in the ER assay was significantly higher than that of control (equivalent to 0.94 ± 0.28 ng/L E2, $p = 0.028$) (Fig. 1). This result is in accordance with our former study that showed plastic bag extracts also had EA after simulated gastric digestion, with higher activity than other plastic products (Coffin et al., 2019).

EA was highest in the F9 fraction, which had compounds (listed in the Table S3) of an approximate LogKow of 6.1. The LogKow value for F9 was an average value for all the detected compounds in the F9 fraction (the LogKow values for chemicals were obtained from the PubChem database (PubChem, 2020). Estradiol equivalent value for the F9 fraction was 1.46 ± 0.11 ng/L E2 in the ER assay, which was significantly higher than that of other fractions, including the negative control. Similarly, *vtg* mRNA expression in the medaka larvae was 8.5 fold higher than that of the negative control ($p = 0.4$). With NTS methodology, we found that the F9 fraction contained 34 entities, including five phthalates; pentyl isopentyl phthalate (PIPP), diheptyl phthalate (DHEPP), dihexyl phthalate (DHP), 2-ethylhexyl hexyl phthalate (HEHP), and butyl octyl phthalate (BOP) (Table S3). DHP is a commercial phthalate ester with an estrogenic activity of 1 nM (Williams et al., 2017). HEHP is a monomer of DEHP, which has been reported to be one of the most widely used plasticizers in the international plastics industry (Erythropel et al., 2014; Chen et al., 2014). Two phenols (bisphenol G and 2,3-dioctylphenol) and 2-ethylhexyl benzoate were identified in the F9. The EC_{10} value of bisphenol G was shown to be 1.9 E-05 M in the estrogenic Yeast Estrogen Screen (YES) assay, and 2,3-Dioctylphenol may also have estrogenic activity, but affinity values were not known (Dvorakova et al., 2018; R.T., 2007). Other UV absorbers, antioxidants, and intermediates and their estrogenic effects are provided in Table 1.

Overall, in the F9 fraction, several novel alkylphenols and phthalates were detected by the NTS. But some other compounds present in this fraction may also exert endocrine disrupting effects. For example, some UV absorbents and antioxidants that may also have slight estrogenic effects according to the Toxcast model (Williams et al., 2017) (Table 1). Additional studies are needed to characterize the identified compounds and determine the concentrations needed to generate estrogenic effects. The diversity of probable structures assigned in each fraction can be partly attributed to the presence of non-intentionally added substances (NIAS), that constitute a group of chemicals not directly applied but introduced or formed during the production process (Martínez-Bueno et al., 2017). The plastic bag used in this study contains at least 20 % of recycled polyethylene plastic as marked on the bags, and the plastic recycling process can bring in many other pollutants.

Unlike F9, the estrogenic effects in F6 were mainly reflected in the *in vivo* assay. Expression of *vtg* transcripts in medaka larvae showed significant upregulation (22-fold) in the F6 fraction compared to the negative control. However, the *in vitro* ER assay had limited activity in the F6 fraction (Fig. 1). Through NTS, 65 entities were found in the F6 fraction (Table S3). Among the identified chemicals, the antimicrobial agent terbuthylazine (TERBA) and the antioxidant 2,5-di-tert-butylbenzoquinone were shown to have AC50 values of 3.18 and 21.03 nM in the *in vitro* ERα_LUC_VM7_Agonist assay (Williams et al., 2017), respectively (Table 1). In addition, two phthalic acid ester (PAEs) monomers, namely, monoisononyl phthalate and monoheptyl phthalate were also observed which may be a result of ester degradation. Two other chemicals with ER activation were observed in the F6 fraction (ethoxylated trimethylolpropane triacrylate and hexyl cinnamaldehyde), with AC50 values of 6.75 and 0.00983 nM respectively (Table 1) (Williams et al., 2017; Kjeldsen et al., 2013).

The combination of several estrogenic compounds together may

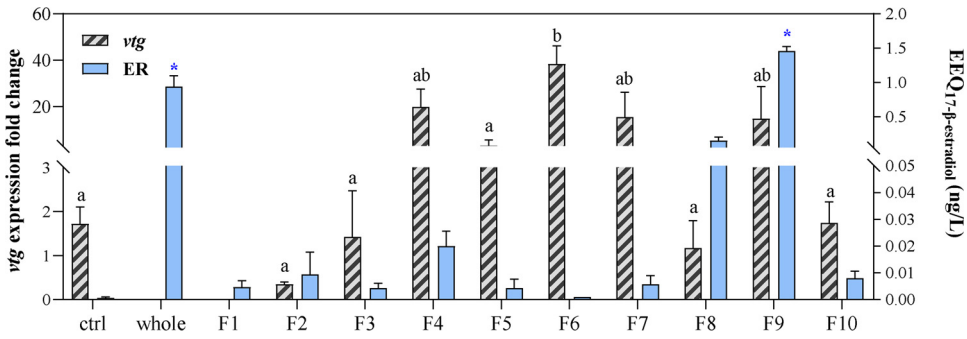

Fig. 1. *In vivo* (medaka *vtg* expression) and *in vitro* (ER cell proliferation) estrogenic activity responses to the plastic leachate exposure. Grey bars represent the expression fold change with mean ± SEM, and blue bars represent estradiol (E2) equivalent (EEQ) values mean ± SEM. Different letters denote statistically significant differences among groups ($p < 0.05$) in the *in vivo* assay. Asterisks denote statistically significant differences relative to control ($p < 0.05$) in the *in vitro* assay. The estrogenic effects of *vtg* mRNA expression was not obtained in the whole extract and F1 samples, because none of the fish embryos exposed to these groups hatched. Ctrl: negative control; whole: the whole extract of the plastic leachate. F1-10: fractions 1-10.

Table 1
The toxicity levels of identified chemicals having demonstrated estrogenic activity in Toxcast and their LC_{50} values in the T.E.S.T. software (Williams et al., 2017; Martin, 2016). Note: ERα bioassay AC_{50} values are obtained from the Toxcast database. LC_{50} values are 48 h *Daphnia magna* acute toxicity results acquired from the T.E.S.T. software, which are only used to reflect the acute toxicity of these chemicals. Numbers in italic are real experimental values, and numbers in normal font are prediction values. The color gradation from red-white-blue represents toxicity from high-medium-low. T.E.S.T.: Toxicity Estimation Software Tool.

Chemicals				ERα bioassay		LC_{50}
Category	chemical number	logKow	Fraction	AC_{50}(nM)	Toxcast model (Williams et al., 2017)	(mg/L)
phthalate	2,4,6-Trimethylbenzoylphenylphosphinic acid ethyl ester	3.900	F1	inactive	potency	0.0452
phthalate	Monoisononyl phthalate	5.600	F6	NA	NA	12.120
phthalate	Monoheptyl phthalate	4.800	F6	NA	NA	15.110
phthalate	PIPP / Pentyl isopentyl phthalate (Isopentyl pentyl phthalate)	5.700	F9	NA	NA	3.8
phthalate	DHEPP / Diheptyl phthalate (DHP)	8.000	F9	inactive	constant	2.97
phthalate	DHP / Dihexyl phthalate (DHXP) (DnHP)	6.820	F9	0.00964	pathway	4.73
phthalate	HEHP / 2-Ethylhexyl hexyl phthalate	6.700	F9	NA		3.38
phthalate	BOP / Butyl octyl phthalate	6.9	F9	inactive	potency	4.09
bisphenols	Bisphenol G	6.300	F9	NA		1.11
bisphenols	2,3-Dioctylphenol	9.700	F9	NA		0.2
colorant/bisphenols	2,4-Dinitrophenol	1.670	F1,F2	inactive	potency	*4.56*
colorant	Toluidine Blue (Tolonium)	NA	F1	NA	NA	NA
antimicrobial	BIT / Benzisothiazolinone	3.400	F2	inactive	constant	32.68
antimicrobial	Fenuron (N,N-Dimethyl-N-phenylurea)	3.130	F2	inactive	constant	29.95
antimicrobial	Terbuthylazine (TERBA)	3.400	F6	0.057	pathway	11.040
catalyst	2,4-Diethylthioxanthone	5.100	F1 F2	very weak active	potency	NA
catalyst	2-Chlorthioxanthone	4.600	F1 F2	inactive	potency	NA
antioxidant	2,5-DBQ / 2,5-Di-tert-butylbenzoquinone	3.400	F1,F2,F6,F9	21.03	Hill	3.56
antioxidant	Isopropyl diphenylamine	4.600	F1	NA	NA	0.84
antioxidant	NDPHA / Nitrosodiphenylamine	3.130	F1,F2	42.65	Hill	*7.84*
antioxidant	Irgafos 168 (Antioxidant 168)	15.500	F9	inactive	potency	NA
antioxidant	BHA / 3-Tert-butyl-4-hydroxyanisole (2-tert-Butyl-4-methoxyphenol)	3.200	F9	0.0139	pathway	2.69
intermediate	4-tert-Butylcyclohexyl acrylate	4.000	F1	NA	NA	NA
intermediate	Aminocaproic acid	−2.950	F1,F2	inactive	potency	37.61
intermediate	3,5,5-Trimethylcyclohexenone (Isophorone)	1.700	F2	inactive	constant	*120.12*
intermediate	Benzophenone	3.180	F9	0.040	pathway	6.72
intermediate	Anthraquinone	3.390	F9	12.050	Gain-loss	2.85
UV absorber	Tinuvin 1130	4.700	F2	NA	NA	1.17
UV absorber	Padimate A	4.300	F9	inactive	constant	5.19
UV absorber	Octyl methoxycinnamate	5.300	F9	6.810	Hill	4.41
others	2-Naphthylamine	2.280	F1,F2	49.15	Hill	3.73
others	1,3-Diisopropylbenzene	4.500	F1,F2	inactive	constant	0.84
others	1,4-Bis(2-[(2-methyl-2-propanyl)peroxy]-2-propanyl)benzene	4.500	F1,F2,F9	NA	NA	NA
others	2-Heptenoic acid	2.100	F1	NA	NA	31.51
others	3-Aminoacetophenone	0.830	F1,F2	inactive	potency	6.52
others	Caprolactam cyclic dimer	0.600	F1,F2	NA	NA	260.32
others	Coumaric acid	1.790	F1,F2	NA	NA	9.57
others	N-Me-DMPEA	1.300	F1,F2	NA	NA	31.21
others	Phenylacrylic acid (Cinnamic acid)	2.130	F1,F2	inactive	constant	26.05
others	Piperidine	0.840	F1 F2	28.83	potency	101.59
others	1,1′-Carbonylbispiperdine	1.400	F2	NA	NA	81.94
others	1-Amino-2-naphthol	1.700	F2	NA	NA	6.23
others	2,2-(Tridecylazanediyl)diethanol	5.000	F2,F9	NA	NA	15.78
others	3-[1 − 4-Cyano-1,2,3,4-tetrahydronapthyl)]propanenitrile	2.400	F2	NA	NA	0.51
others	BBOT / 2,5-bis(5-tert-Butyl-2-benzoxazolyl)thiophene	8.000	F2	inactive	potency	0.0957
others	Nonaethylene glycol	−2.300	F2	inactive	potency	546.82
others	TPO / Diphenyl(2,4,6-trimethylbenzoyl)phosphine oxide	5.000	F2	56.29	Hill	0.036
others	Tribromomethyl phenyl sulfone	3.900	F2	NA	NA	0.36
others	TRPGDA / Tri(propylene glycol) diacrylate	2.200	F2	inactive	constant	NA
others	Benzil	3.380	F6	very weak	potency	7.780
others	Ethoxylated trimethylolpropane triacrylate	2.200	F6	6.75	Gain-loss	NA
others	Hexyl cinnamaldehyde	4.800	F6	0.00983	pathway	1.290
others	Myristamine oxide	6.400	F6	inactive	potency	NA
others	Triethylene glycol bis(2-ethylhexanoate)	5.400	F6	inactive	constant	3.210
others	2-Ethylhexyl benzoate	4.500	F9	45.500	Hill	2.73
others	Stearamide (Octadecanamide)	6.800	F9	inactive	potency	1.030
others	Ricinolic acid (Ricinoleic acid)	5.700	F9	inactive	constant	1.850
others	N,N'-Ethylenebis(stearamide)	15.700	F9	inactive	potency	0.760
others	Ethylene azelate	2.000	F9	NA	NA	82.820
others	BHT-quinone methide (2,6-di-tert-butyl-4-methylene-2,5-cyclohexandienone)	4.600	F9	NA	NA	0.820
others	4-Phenylbenzophenone (4-Benzoylbiphenyl)	4.900	F9	weak active	potency	0.890
others	3,5-di-tert-butyl-4-hydroxyacetophenone	4.600	F9	NA	NA	0.730
others	1,3:2,4-bis(3,4-dimethylbenzylidene)sorbitol	2.800	F9	inactive	Gain-loss	NA

elicit greater responses *in vivo* relative to *in vitro* activities, such as the case for the F6 fraction (Fig. 1). Previous studies have reported similar phenomenon. Mixtures of phenol ethoxylates with both diuron and bifenthrin, showed higher *vtg* mRNA expression in male fathead minnow compared to treatments with the individual compounds (Crago et al., 2015). This may be due to enhanced biotransformation of other non-estrogenic compounds to active metabolites. For example, alkylphenol pretreatment induced the demethylation of the herbicide, and diuron formed the metabolite 3,4-dichlorophenyl-*N*-methylurea, which had a stronger estrogenic signal than diuron in juvenile male tilapia (Felicio et al., 2016).

Alternatively, bioassay markers may have different sensitivities to the direct activation of the estrogen receptors, especially with the presence of other compounds. For example, several compounds have been shown to enhance endogenous estradiol levels (Crago et al., 2015; Felicio et al., 2016). And, in some cases compounds (*i.e.*, 17α-ethynylestradiol (EE2)) have been shown to have higher *in vivo* (zebrafish VTG protein expression) estrogenic activity compared to *in vitro* (MVLN assay) responses (Van den Belt et al., 2004). Thus, *in vivo* estrogenic assays may be more sensitive than *in vitro* tests, due to metabolism and other non-ER targets that can enhance estrogenicity besides direct activation of ER (Van den Belt et al., 2004). Although *in vitro* assays can be applied as screening assays for qualitative assessment of estrogenicity because of high throughput logistics, the *in vivo* assays may be needed for an accurate hazard assessment for wildlife. It is noteworthy that the sum of biological responses from the whole extract was less than individual fractions. This phenomenon can be due to the presence of antagonistic compounds in the complicated whole extracts (Šauer et al., 2018; Hashmi et al., 2020), or due to the lowered bioavailaibility of chemicals in the whole extract with the presence of plastic oligomers (Chen et al., 2019b).

There are various sources of estrogen-like chemicals in the plastic bags. On the one hand, different kinds of additives are often added in the manufacturing of polyethylene shopping bags. On the other hand, the plastic bag used in this study contains at least 20 % of recycled polyethylene plastic as marked on the bags, and the plastic recycling process can bring in many other pollutants. First, halogens can accumulate in the cement kiln system of plastic waste recycling processes (Hahladakis et al., 2018; UNEP, 2015). We did find many chemicals with Cl and Br atoms in the NTS. Second, recycled plastics are usually transported over long distances, during which many pollutants can also adhere to plastics (PlasticsEurope, 2010). Third, it has been reported that due to multiple extrusion steps during the waste plastic recycling, multiple contaminants can be introduced into the recycled plastic products (Peres et al., 2016).

3.2. Acute and sub-acute toxicity of plastic leachate (sub)fractions to medaka

Lethality was measured in medaka embryos 4 dpf following exposure and indicated there were no significant differences between fraction treatments and negative/solvent controls (Fig. 2A). Similarly, cytotoxicity was not observed in the ER bioassays for either whole extracts or fractions after 24 h exposure.

In contrast, heart rate (5 dpf) decreased significantly from 60 ± 5 beats/min to 51 ± 4 beats/min when comparing the whole plastic extracts with control ($p < 0.05$) (Fig. 2B). Besides, significant reduction of hatching rate (10 dpf) was observed following exposure, with the whole extract, F1 fraction, and F2 fraction relative to control, respectively (Fig. 2C). Japanese medaka embryos failed to hatch after the whole extract and the F1 fraction exposure. There were 4273 entities in the whole extract, with 64 % of the compounds concentrated in the first two fractions, (776 entities in the F1 fraction, and 1071 entities in the F2 fraction) (Fig. 2D).

In the F1 and F2 fractions, although few chemicals possessed estrogenic activity, some might have had toxic effects in aquatic organisms. Here we used the LC_{50} values for *Daphnia magna* obtained from the T.E.S.T. database (Martin, 2016), with which data to reflect the acute toxicity of the detected compounds (Martin, 2016). For example, several compounds had 48 h LC_{50} values less than 1 mg/L (2,4,6-trimethylbenzoylphenylphosphinic acid ethyl ester, isopropyl diphenylamine, 1,3-diisopropylbenzene, 3-[1 − 4-Cyano-1,2,3,4-tetrahydronapthyl)] propanenitrile, BBOT, TPO, and tribromomethyl phenyl sulfone) were detected, deserves our further attention (Table 1).

Also, several other novel compounds were identified in the first two fractions, including chemical initiators (2,4-diethylthioxanthone (DETX) and 2-chlorthioxanthone), colouring pigments (toluidine blue and 2,4-dinitrophenol), polymer synthesis intermediates (4-*tert*-butylk cyclohexyl acrylate, aminocaproic acid, and NDPHA (nitrosodiphenylamine)), antioxidants (2-naphthylamine, 2,5-DBQ (2,5-Di-*tert*-butylbenzoquinone), isopropyl diphenylamine), and the UV-

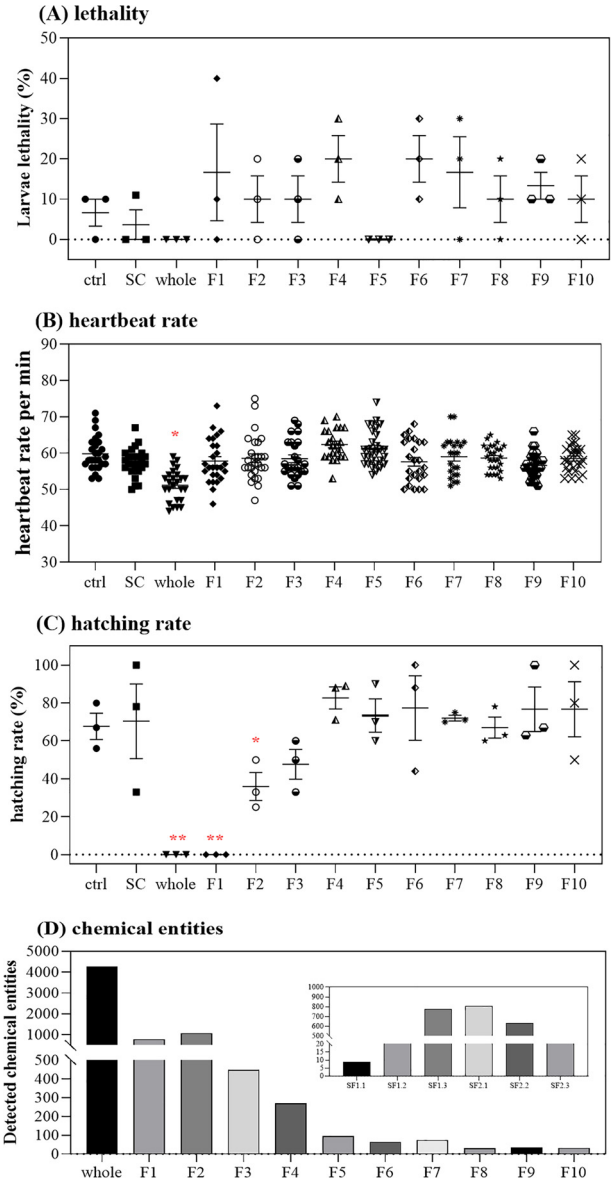

Fig. 2. Acute and sub-acute toxicity endpoints for Japanese medaka after plastic leachate exposure and chemical entities in the leachate. (A) lethality of medaka embryo on 4 dpf; (B) heartbeat rate of medaka embryo on 5 dpf; (C) medaka larvae hatching rate on 10 dpf; (D) chemical entities detected by non-target screening in different fractions and subfractions. ctrl: negative control; SC: solvent control; whole: the whole extract of the plastic leachate. F1-10: fractions 1-10; SF: sub-fractions.

absorbent, tinuvin 1130. However, these compounds had relatively lower acute toxicity based on LC50 values. Antioxidants are usually embedded into plastics to inhibit polymer oxidative degradation when exposed to ultraviolet (UV) light (Bhunia et al., 2013; Hahladakis et al., 2018). Soluble azocolorants, which can provide a variety of colours for plastic packaging (Groh et al., 2019). The two colorants detected in the study were blue (toluidine blue) and yellow (2,4-dinitrophenol), which was consistent with the green appearance on the shopping bags (Fig. S2). Catalyst residues, such as initiators, may remain on the plastic after manufacturing even though most of the initiators can be neutralized by catalyst deactivators (Hahladakis et al., 2018; Groh et al., 2019). 2-naphtylamine (a potent carcinogen to humans) (Bie et al., 2017), and aminocaproic acid (a hydrolytic enzyme inhibitor which can inhibit the phase I biotransformation in biota (Purwin et al., 2017)) were also found in the F1 fraction (Fig. 3).

The F1 and F2 fractions had the Top 1 and Top 2 chemical entity numbers among the ten fractions, with 808 and 635 entities, respectively, which seems to be responsible for their high acute toxicity in bioassays. However, the toxicity for different fractions was not completely dependent on the overall number of chemicals. For example, the SF2.1 subfraction had 808 entities, but it exerted much lower lethality (10 ± 10 %) and did not alter heart rate (60 ± 6 times/min) nor hatching rates (31 ± 18 %) (Fig. S3). Similarly, SF2.2 had the second most entities among subfractions with 635 entities but did not cause lethality or sub-acute toxicity. This may be because that most of the entities found in the fractions are ethylene (C_2H_4) derived polymers that make up the plastic (Fig. S4), and the toxic effects shall be mainly determined by the properties of the pollutants.

3.3. Environmental significance

In this study, we find that the toxicity of plastics may not only be solely due to its physical impairment. Multiple estrogenic active substances exist within plastics and may cause estrogenic effects in some leaching scenarios. Of additional concern was the huge number of largely uncharacterized chemicals in plastics, with more than 4,700

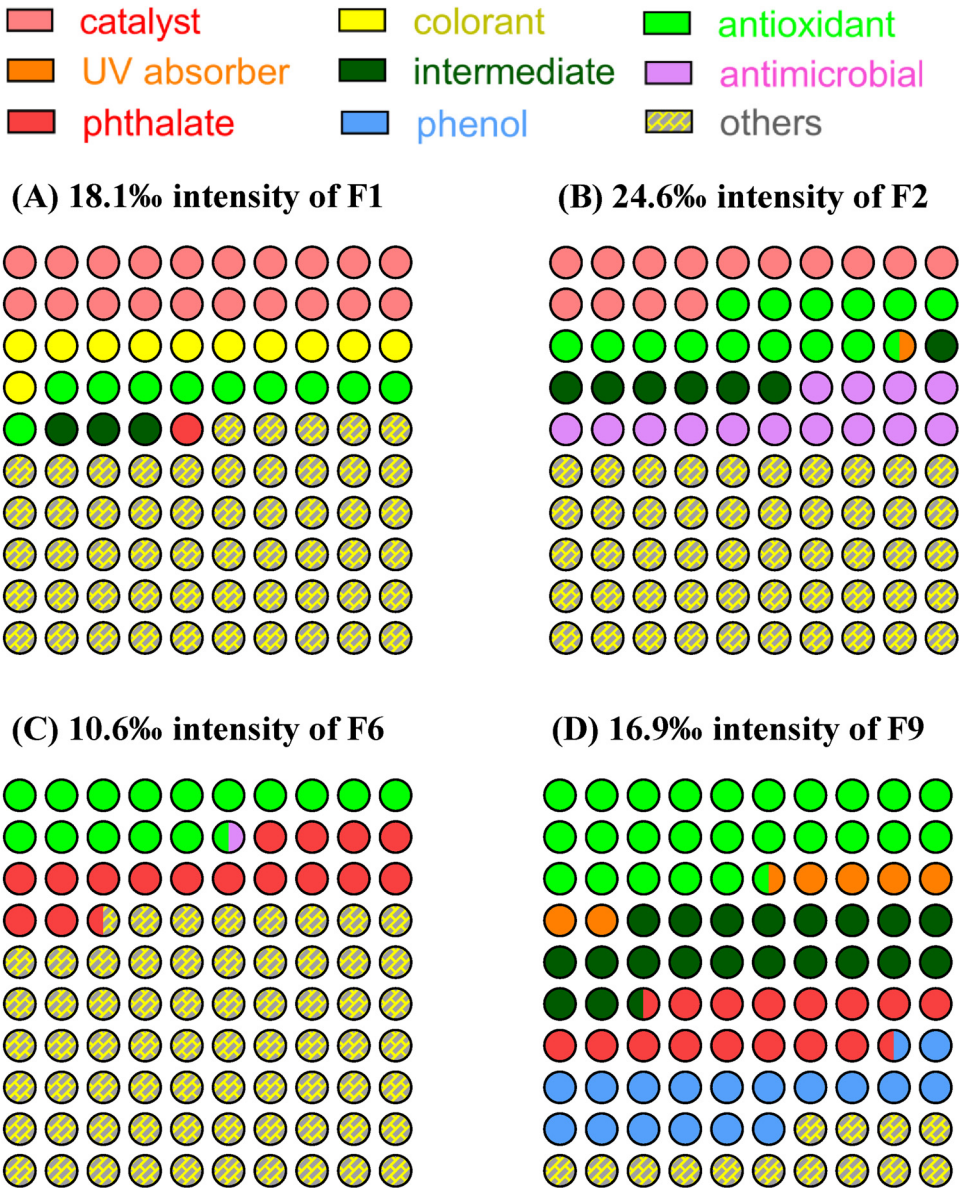

Fig. 3. The identified chemical entity compositions based on intensities in four fractions. F1: fraction 1; F2: fraction 2; F6: Fraction 6; F9: fraction 9. Every circle in the figure characterizes 1% intensity of the total identified chemical intensities in each fraction (each identified chemical has more than 5-fold higher intensity than that in the procedural control). The total chemical entity intensity constitutes 18.1‰, 24.6‰, 10.6‰, and 16.9‰ of the total fraction intensities, respectively. The identified chemical entity compositions based on entity numbers can be found in Figure S5.

entities found on this single-use plastic item. Even though many of the chemicals were chains having the repeat unit [CH_2], we still identified a variety of catalysts, dyes, antioxidants, intermediates, and antibacterial agents in different fractions. Besides, plasticizers, bisphenols, and other estrogenic chemicals were identified in fractions with higher logKow values suggesting absorption from the gastrointestinal tracts of fish may be rapid. The more polar substances (especially existing in the first two fractions) also migrated into the surrounding environment following simulated digestion easily. Absorption and resulting toxicity of most of these materials in biota are generally unknown, and given the detection of these substances in the digest, additional studies are warranted. As this study only targeted estrogenic and developmental responses in fish, additional biological targets may also need to be examined to better characterize the potential risks of plastics to biota.

CRediT authorship contribution statement

Qiqing Chen: Methodology, Writing - original draft, Formal analysis, Investigation, Writing - review & editing. **Mauricius Marques dos Santos:** Methodology, Writing - original draft, Formal analysis, Writing - review & editing. **Philip Tanabe:** Methodology, Resources, Writing - review & editing. **Gary T. Harraka:** Investigation, Writing - review & editing. **Jason T. Magnuson:** Investigation, Writing - review & editing. **Victoria McGruer:** Methodology, Writing - review & editing. **Wenhui Qiu:** Methodology, Writing - review & editing. **Huahong Shi:** Writing - review & editing. **Shane A. Snyder:** Supervision, Writing - review & editing. **Daniel Schlenk:** Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jhazmat.2020.123421.

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