

Benchmarking the in Vitro Toxicity and Chemical Composition of Plastic Consumer Products

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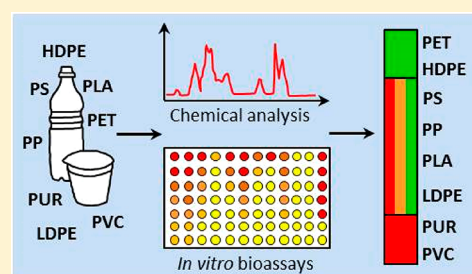
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Supporting Information

ABSTRACT: Plastics are known sources of chemical exposure and few, prominent plastic-associated chemicals, such as bisphenol A and phthalates, have been thoroughly studied. However, a comprehensive characterization of the complex chemical mixtures present in plastics is missing. In this study, we benchmark plastic consumer products, covering eight major polymer types, according to their toxicological and chemical signatures using in vitro bioassays and nontarget high-resolution mass spectrometry. Most (74%) of the 34 plastic extracts contained chemicals triggering at least one end point, including baseline toxicity (62%), oxidative stress (41%), cytotoxicity (32%), estrogenicity (12%), and antiandrogenicity (27%). In total, we detected 1411

features, tentatively identified 260, including monomers, additives, and nonintentionally added substances, and prioritized 27 chemicals. Extracts of polyvinyl chloride (PVC) and polyurethane (PUR) induced the highest toxicity, whereas polyethylene terephthalate (PET) and high-density polyethylene (HDPE) caused no or low toxicity. High baseline toxicity was detected in all “bioplastics” made of polylactic acid (PLA). The toxicities of low-density polyethylene (LDPE), polystyrene (PS), and polypropylene (PP) varied. Our study demonstrates that consumer plastics contain chemical compounds that are toxic in vitro but remain largely unidentified. Since the risk of unknown compounds cannot be assessed, this poses a challenge to manufacturers, public health authorities, and researchers alike. However, we also demonstrate that products not inducing toxicity are already on the market.



1. INTRODUCTION

To date, humankind has produced 8300 million metric tons of plastics with an exponentially growing production.¹ From a material perspective, plastics are cheap and versatile materials and, thus, an integral part of our everyday lives. From a chemical perspective, plastic products are complex mixtures of one or more polymers, fillers, and multiple additives, such as plasticizers, flame retardants, stabilizers, antioxidants, and pigments to improve the material's functionality.² In addition to these additives, other chemicals are present in plastics, including unreacted monomers, starting substances, and nonintentionally added substances (NIAS, impurities and side or breakdown products).³

As most of these chemicals are not covalently bound to the polymer, they can be released at all stages of the plastics' life-cycle via migration to liquids or solids or via volatilization. This can result in a transfer of chemicals in the packed goods (e.g., foodstuff), as well as human (e.g., indoor air and household dust) and natural environments (e.g., water bodies). Accordingly, plastic materials are an important source of human exposure to chemicals.⁴ Well-known examples include

the plastic monomer bisphenol A (BPA) and phthalate esters used as plasticizers.⁵ Their metabolites have been detected in >92 and >98% of the US general population, respectively,^{6–8} indicating ubiquitous exposure.

While exposure, hazard, and epidemiological data on few, prominent plastic-associated chemicals, such as BPA, is abundant,⁹ it remains challenging to assess the chemical safety of plastics because (1) they comprise a diverse and heterogeneous group of polymers and (2) each product has an individual and complex chemical composition, which (3) often includes unknown compounds. Today, more than 5300 polymer formulations are commercial available¹⁰ and more than 4000 known chemicals are associated with plastic packaging alone.⁴ This chemical complexity puts into question current approaches to assess the safety of plastics, especially with regards to food contact materials (FCMs).¹¹ While the

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risk of starting substances and additives is evaluated prior to the authorization of FCMs in many countries,¹² this approach disregards unexpected and unknown compounds present in the final product (e.g., NIAS), as well as mixture toxicity.¹³

To address these limitations, *in vitro* and *in vivo* bioassays can be used to assess the toxicity of the whole migrate leaching from the final product.^{14,15} Compared to the chemical analysis of selected target compounds, bioassays integrate the toxicity of mixtures leaching from plastics including known chemicals with unknown toxicity and truly unknown compounds. Further, the chemicals causing toxicity can be identified when coupling bioassays to chemical analysis.^{16,17}

Previous studies have demonstrated that plastic FCMs induce *in vitro* and *in vivo* toxicity.¹⁴ Since these studies focused on few end points and products, a comprehensive toxicological characterization of plastics is missing. Thus, our study aims at comparing the toxicological and chemical profiles of a range of everyday consumer products made of petroleum-based commodity and bio-based polymers. We hypothesized that the toxicity present in plastics can be benchmarked based on the polymer type. Further, we tested the hypothesis that their chemical signature predicts the toxicity. Finally, we aimed at identifying and prioritizing the chemicals leaching from plastics.

We selected 34 plastic consumer products from the market covering FCMs and non-FCMs made of high-density and low-density polyethylene (HDPE, LDPE), polystyrene (PS), polypropylene (PP), polyethylene terephthalate (PET), polyvinyl chloride (PVC), polyurethane (PUR), and the bio-based polylactic acid (PLA). We extracted these products and analyzed the extracts' baseline toxicity, oxidative stress induction, cytotoxicity, and endocrine activity *in vitro*. In addition, we performed nontarget, high resolution gas chromatography–mass spectrometry (GC-QTOF-MS) to characterize the chemicals present in plastics and used ToxCast data to prioritize them.

2. MATERIALS AND METHODS

2.1. Sample Selection and Polymer Identification.

We selected 34 plastic products (Table 1) covering the polymer types with the highest market share (PP > LDPE > HDPE > PVC > PUR > PET > PS).¹⁸ These petroleum-based materials include plastics with high (e.g., PVC) and low additive content (e.g., PET). In addition, we included PLA as bio-based, biodegradable plastics because these materials are potential replacements for petroleum-based plastics.¹⁹ We selected four or five items per polymer type. Wherever possible, we included packaging products as this sector has the highest plastic demand.¹⁸ We selected high consumption product classes based on their share in municipal waste (containers > plastic wraps > bags and sacks > soft drink bottles).²⁰ The samples include 20 products with and 14 without food contact. The ratio of FCMs and non-FCMs is different for the polymer types (PS only FCM, PUR only non-FCM). We purchased the products in local retailer stores and confirmed their polymer types (most contained a recycling code) using Fourier-transform infrared spectroscopy (FTIR, PerkinElmer, Spectrum Two, Waltham, Massachusetts). The spectra of the samples can be accessed under DOI: [10.5281/zenodo.3263830](https://doi.org/10.5281/zenodo.3263830). They were compared to reference spectra from our own library and the literature using the software SpectraGryph.²¹

Table 1. Plastic Products Analyzed in This Study

sample	plastic product	FCM ^a
HDPE 1	refillable drinking bottle	yes
HDPE 2	yogurt drink bottle	yes
HDPE 3	bin liner	no
HDPE 4	shower gel bottle	no
LDPE 1	lemon juice bottle	yes
LDPE 2	plastic wrap	yes
LDPE 3	freezer bag	yes
LDPE 4	hair conditioner bottle	no
PS 1	yogurt cup	yes
PS 2	fruit tray	yes
PS 3	vegetable tray	yes
PS 4	plastic cup	yes
PP 1	refillable drinking bottle	yes
PP 2	yogurt cup	yes
PP 3	gummy candy packaging	yes
PP 4	handkerchief packaging	no
PP 5	shampoo bottle	no
PET 1	soft drink bottle	yes
PET 2	yogurt cup	yes
PET 3	oven bag	yes
PET 4	vegetable tray	yes
PET 5	shampoo bottle	no
PVC 1	plastic wrap	yes
PVC 2	place mat	no
PVC 3	pond liner	no
PVC 4	floor covering	no
PUR 1	scouring pad	no
PUR 2	kids bath sponge	no
PUR 3	acoustic foam	no
PUR 4	shower slippers	no
PLA 1	yogurt cup	yes
PLA 2	vegetable tray	yes
PLA 3	shampoo bottle	no
PLA 4	coffee cup lid	yes

^aFCM: Food contact material.

2.2. Plastic Extraction. Whenever feasible, we used glass or polytetrafluoroethylene consumables to avoid sample contamination and rinsed all materials twice with acetone (pico-grade, LGC Standards) and annealed them at 200 °C for ≥3 h. The content was removed from packaging samples, and the products were rinsed thoroughly with ultrapure water until residues were completely removed. All samples were cut into 0.5–0.8 × 2 cm pieces and foamy products additionally to a thickness of 0.5 cm. Three grams of each were placed in one or two amber glass vials, depending on their volume. After the addition of 20 mL of methanol (99.9% LC-grade, Sigma-Aldrich), samples were extracted by sonication in an ultrasound bath for 1 h at room temperature. We selected methanol because this was the only solvent that did not dissolve any of the polymers. The methanol was transferred into clean glass vials, and 20 μL of the methanol extracts were retained for chemical analysis. After 200 μL of dimethyl sulfoxide (DMSO, Uvasol, Merck) was added as a keeper, samples were evaporated under a gentle stream of nitrogen to a final volume of 200 μL and stored at –20 °C prior to *in vitro* analysis. Two procedural blanks (PB 1/2) consisting of amber glass vials not containing any sample but only methanol were treated identically to control for a potential contamination. To contextualize the bioassay results, we use “plastic equivalents”

in such that “1 mg plastic” implies the toxicity extracted from 1 mg of plastic material. Accordingly, 1 μL sample extract corresponds to 15 mg plastic (exception PS 2: 1 μL = 7.5 mg plastic).

2.3. Bioassays. All bioassays were conducted in 96-well microtiter plates with negative controls, solvent controls (DMSO), PB 1/2, and a solvent blank (SB, 20 mL of pure methanol used for sample extraction evaporated to 200 μL of DMSO). Samples, solvent controls, and blanks were diluted 100-fold (baseline toxicity), 200-fold (oxidative stress response), or 480-fold (endocrine activity) with medium, resulting in a maximum final solvent concentration of 1%, 0.5%, or 0.2% (v/v), respectively. Since DMSO solvent controls did not exhibit any effects compared to negative controls in these concentrations, the results for both controls were pooled. Throughout the experiments, none of the controls and blanks induced toxicity. Thus, there was no contamination during sample extraction and analysis (Figure S1).

2.3.1. Baseline Toxicity. The Microtox assay with the bioluminescent bacterium *Aliivibrio fischeri* was performed according to an international guideline²² miniaturized to a 96-well plate format.²³ In brief, extracts and controls including the reference compound 3,5-dichlorophenol (Table S1 and Figure S2) were analyzed in serial dilutions (1:2 in saline buffer). For extracts, these eight concentrations correspond to 0.18–22.5 mg plastic, except for PS 2 (0.09–11.25 mg plastic), PVC 1 and PLA 3 (further diluted to 2.7 μg plastic). Fifty microliters of *A. fischeri* suspension was added to 100 μL sample. Luminescence was measured prior to and 30 min after sample addition using a Spark 10M microplate reader (Tecan, Crailsheim, Germany).

In accordance with the ISO guideline,²² the results were corrected for the luminescence in the blanks (empty wells) and for the change in luminescence in negative controls over 30 min, resulting in a relative luminescence inhibition (%). Dose–response relationship curves were derived for each sample using a four-parameter logistic model with the lower and upper plateau constrained to 0 and 100% luminescence inhibition, respectively. Results, from three to five independent experiments with two technical replicates each, are expressed as effect concentration ($\text{EC}_{20} \pm \text{SEM}$, mass of plastic inducing a 20% luminescence inhibition) and mean effect size $\pm \text{SEM}$ (luminescence inhibition induced by 22.5 mg plastic). In case an EC_{20} could not be derived, we used an EC_{20} of 25 mg plastic indicating that the EC_{20} is larger than the highest analyzed concentration.

2.3.2. Oxidative Stress Response. We used the AREc32 assay to investigate the induction of an oxidative stress response in the Nrf2/ARE pathway.²⁴ The AREc32 cell line was obtained from Signosis, Inc. (catalog number SL-0010-NP, Santa Clara, CA, USA) and checked for the absence of mycoplasma contamination (MycAlert PLUS Mycoplasma Detection Kit, Lonza, Walkersville, USA). The assay was performed as described previously²⁵ with minor modifications. In brief, 12 000 cells well^{-1} were seeded in 96-well plates. After 24 h, 100 μL medium well^{-1} was replaced by medium containing eight concentrations of the samples serially diluted 1:2 (0.06–7.5 mg plastic except PS 2, 0.03–3.8 mg plastic) or the reference compound *tert*-butylhydroquinone (Table S1 and Figure S2). After 24 h, cell viability and luciferase activity were determined. The former was performed visually by brightfield microscopy (Zeiss, Axiovert 40C)²⁶ as this was more sensitive

than the resazurin assay. If morphological changes (abundance of spherical or dead cells) were apparent, the respective treatment was considered cytotoxic and excluded from further analysis. The luciferase activity was determined immediately after adding 100 μL of 0.015% w/v beetle luciferin potassium salt (Promega, E1601) using a Spark 10M microplate reader. Each sample was analyzed in three to four independent experiments with duplicates each.

We derived dose–response relationships for the induction ratios (IR) using a four-parameter logistic model (lower plateau constrained to 1) to interpolate the plastic mass producing an IR of 2 over the control ($\text{EC}_{\text{IR}2}$). In case an $\text{EC}_{\text{IR}2}$ could not be derived, we used an $\text{EC}_{\text{IR}2}$ of 8 mg plastic, indicating that the $\text{EC}_{\text{IR}2}$ is larger than the highest analyzed concentration. The IR at the highest noncytotoxic concentration is also reported.

2.3.3. Endocrine Activity. We used yeast-based reporter-gene assays to investigate the induction of agonistic activity at the human estrogen receptor α (hER α)²⁷ and antagonistic activity at the human androgen receptor (hAR).²⁸ The Yeast Estrogen Screen (YES) and the Yeast Antiandrogen Screen (YAAS) were performed as previously described with minor modifications.²⁹ In brief, samples were diluted 480-fold in medium resulting in a final sample concentration of 3.75 mg plastic equivalents well^{-1} . Samples that induced $\geq 20\%$ cytotoxicity were excluded and reanalyzed in seven additional 1:2 serial dilutions (lowest concentration in the YES, PLA 3 = 3.7 μg plastic, PS 2 = 29.3 μg , PVC 2/PLA 1 = 58.6 μg , and in the YAAS, PLA 3 = 3.7 μg plastic, PP 2 = 14.6 μg , PP 3/PP 5/PVC 2/PLA 1 = 29.3 μg). 17 β -estradiol and flutamide served as reference compounds for the YES and YAAS, respectively (Table S1 and Figure S2). To determine the antagonistic activity in the YAAS, 10 nmol L^{-1} testosterone, inducing $\sim 75\%$ activity, was added. The initial cell density was adjusted to 25 formazin attenuation units (FAU) for YES and 100 FAU for YAAS. After 20 h incubation, we determined the cell density as absorbance at 595 nm on a Spark 10M instrument. After transferring 30 μL well^{-1} to a new 96-well plate, 50 μL of *lacZ* buffer containing 1.5 mmol L^{-1} 4-methylumbelliferyl β -D-galactopyranoside (MUG, Merck, CAS 6160-78-7) and 1 mmol L^{-1} dithiothreitol (Sigma-Aldrich, CAS 3483-12-3) was added. The fluorescence (excitation = 360 nm, emission = 465 nm) was determined after 40 min incubation at 30 °C using a Spark 10M instrument. We also analyzed all samples for autofluorescence prior to the MUG addition and did not observe any. All noncytotoxic samples were analyzed in three independent experiments with eight replicates, each.

Data was processed as previously described to derive the relative cytotoxicity, as well as relative estrogenic and antiandrogenic activities.³⁰ The limit of detection (LOD) of each experiment was calculated as three times the standard deviation (SD) of pooled negative and solvent controls. Significant differences were calculated for effects > LOD.

Dose–response relationships for cytotoxicity and relative endocrine activity were calculated using a four-parameter logistic function constrained to bottom level of zero (0% cytotoxicity/activity) and for cytotoxicity also a top level of 100%. The respective plastic equivalents inducing 20% cytotoxicity (effect concentration, EC_{20}) were interpolated from the dose–response curves. For the endocrine activity, the EC_{50} was used. To ensure comparability of independent experiments only those experiments were considered in which the dose–response relationship of the reference compound

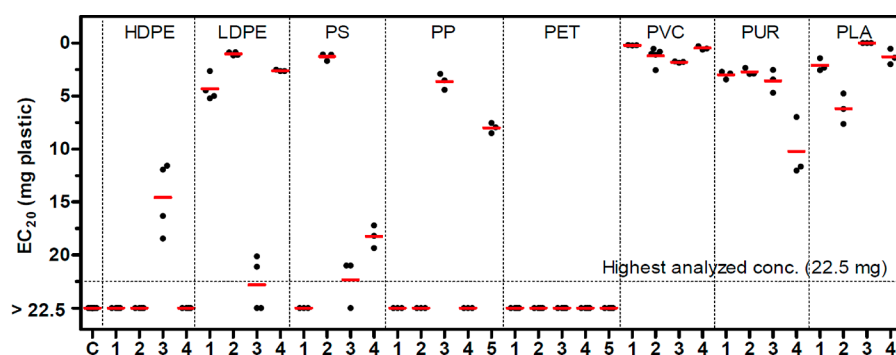


Figure 1. Baseline toxicity of plastic extracts in the Microtox assay. Data is presented as mean EC_{20} for bioluminescence inhibition (lines) from three to five independent experiments (dots) performed with duplicates. The >22.5 indicates that the extracts of 22.5 mg plastic (highest analyzed concentration) did not inhibit the bioluminescence by $>20\%$.

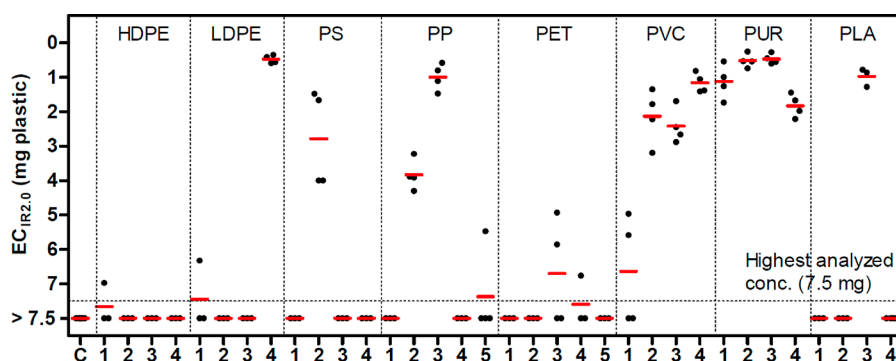


Figure 2. Oxidative stress response induced by plastic extracts in the AREc32 assay. Data is presented as mean EC_{IR2} (lines) from three to four independent experiments (dots) performed with duplicates. The >7.5 indicates that extracts from 7.5 mg plastic (highest analyzed concentration) did not produce an induction ratio of 2 (IR2).

had a $r^2 > 0.9$, a minimal relative luminescence unit >4500 , and a maximal $>50\,000$, as well as an EC_{50} next to 6×10^{-11} mol L^{-1} 17β -estradiol (YES) or 2×10^{-5} mol L^{-1} flutamide (YAAS, Table S1).

2.4. Chemical Analysis. Methanolic extracts were analyzed using an Agilent 7890B gas chromatograph with electron ionization and an Agilent 7200 QTOF mass spectrometer (1 μL injection volume, see SI for details). Chromatograms were automatically integrated using Masshunter (selecting peaks with an area $\geq 1\%$ of the largest peak, “features”) and compounds identified by comparison of the mass spectra with the NIST 14 library (score ≥ 70) using a nontargeted approach. We refer to the latter chemicals as tentatively identified as we did not use authentic standards to confirm their identity. This corresponds to level 2 of confirmation (probable identification).³¹ We removed all tentatively identified compounds found in both PBs from our samples. For each sample and PB, we calculated the sum of all peak areas as indicator for the total abundance of chemicals, the total peak count (features) as indicator for the number of compounds and the relative number of unidentified peaks (score < 70). The raw data from GC-QTOF-MS/MS analysis can be accessed under DOI: [10.5281/zenodo.3263830](https://doi.org/10.5281/zenodo.3263830).

2.5. Data Analysis. We used GraphPad Prism 5 and 7 (GraphPad Software, San Diego, CA) for nonlinear regressions and statistical analyses. To compare two treatments, we used Mann–Whitney tests. A $p < 0.05$ was considered statistically significant.

Out of the tentatively identified chemicals from the GC-QTOF-MS analysis, we selected the five peaks with the largest

areas that did not occur in the blanks and queried their CAS numbers in PubChem³² using R³³ to extract information on the compounds’ industrial function according to the Toxic Substances Control Act (TSCA).³⁴ In addition, we cross-referenced the CAS numbers of all compounds with the database of “Chemicals associated with Plastic Packaging” (CPPdb List A and B)⁴ to identify the origin (likely and possibly originating from plastics).

We downloaded the most recent ToxCast database (INVITRODB_V3_SUMMARY from the US EPA)³⁵ and cross-referenced the CAS numbers of all compounds with `oldstyle_ac50_Matrix_180918.csv` to filter for tested and active chemicals. We selected the high-throughput assays matching our end points (Table S2) and extracted the respective activity values 50 (AC_{50} , concentration at 50% of maximum activity) for our compounds. Taking a worst-case approach, we calculated the ratios of the lowest available AC_{50} and the largest peak area for each end point. We used the ten compounds with the lowest ratio from each end point to compile a joint list of priority chemicals.

To benchmark toxicity in a heat map, we normalized each effect concentration or level (Tables S3, S4, and S5) to the lowest (0%) and highest value (100%) in the data set. We did the same for data from chemical analysis (Table S6; total peak area, number of all detected peaks, and percent of unidentified peaks).

We performed cluster analyses to compare the toxicological (Microtox EC_{20} , AREc32 EC_{IR2} , and YES/YAAS % relative activity) and chemical signatures of the samples. For the latter, we converted the data from the Agilent instrument to an

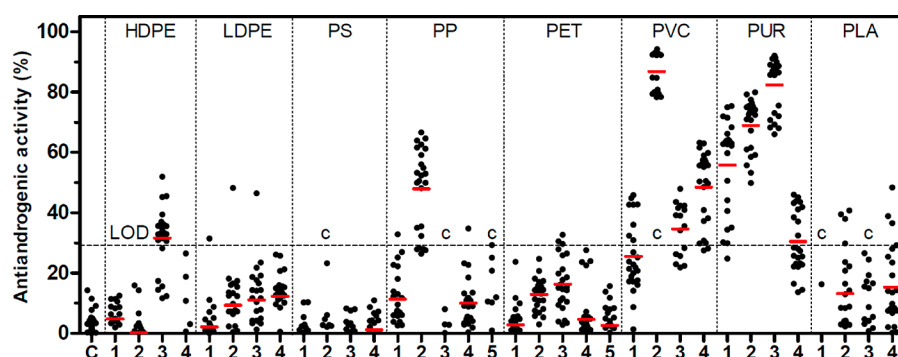


Figure 3. Relative antiandrogenic activity given as relative human androgen receptor inhibition of extracts from 3.75 mg plastic or, if cytotoxic (c), for the highest noncytotoxic concentration (Table S5). Data ($n = 24$, dots) is presented with means (lines). Mean effects $>$ LOD were considered significant.

mzML format using MSConvertGUI³⁶ and processed the data using MZmine 2.33³⁷ to generate a joint peak list containing the peak areas of all masses detected in the samples. We calculated the Euclidean distance between samples and clustered them hierarchically using the “complete linkage” method with the “dist” and “hclust” functions in R.³³

3. RESULTS

3.1. Baseline Toxicity. The inhibition of bioluminescence in *A. fischeri* is more sensitive than other end points for nonspecific toxicity, such as cytotoxicity in mammalian cells.³⁸ We observed baseline toxicity for two-third of the 34 plastic extracts (Figure 1). All PVC, PUR, and PLA, as well as three out of four LDPE products inhibited bioluminescence with a high efficiency (low EC_{20}) and effect level (Table S3 and Figure S3). In contrast, none of the PET extracts induced an effect. The baseline toxicity of HDPE, PS, and PP extracts varied with the product.

3.2. Oxidative Stress Response. The AREc32 assay is used to investigate the induction of the Nrf2-ARE regulated oxidative stress response in a human cell line.²⁴ Fourteen plastic extracts activated this pathway (Figure 2), including all PVC and PUR samples. While PUR extracts ($EC_{IR2} = 0.47$ – 1.82 mg plastic) were more efficient than PVC extracts ($EC_{IR2} = 1.16$ – 5.27 mg plastic), the effect level was higher for PVC (IR = 2.58–13.6) than for PUR samples (IR = 2.75–3.88, Table S4 and Figure S4). In addition, one LDPE, PLA, PET, and PS sample each, as well as two PP samples, induced an oxidative stress response. Here, LDPE 4 induced the highest effect (IR = 37.0) with a high potency ($EC_{IR2} = 0.48$ mg plastic).

3.3. Endocrine Activity. To investigate whether plastics contain estrogen receptor agonists or androgen receptor antagonists, we analyzed the samples in reporter gene assays. Four extracts (HDPE 3, PS 1, PVC 2, and PVC 4) activated the estrogen receptor above the LOD (2.33% relative estrogenic activity). However, the estrogenic activity was low for all samples (Table S5 and Figures S5 and S6), except for a place mat (PVC 2). This sample induced the strongest estrogenic activity with up to 27% (at 0.94 mg plastic, Table S5 and Figure S6).

Compared to that, the extracts’ antiandrogenic activity (LOD = 29.18%) was more pronounced, with 9 out of the 34 samples inhibiting the androgen receptor by 30–87% (Figures 3 and S7 and Table S5). Here, all PUR extracts, three PVC extracts, and one extract from PP and HDPE were

antiandrogenic. As for estrogenic activity, the place mat (PVC 2) induced the strongest effect ($EC_{50} = 0.97$ mg plastic, 87% receptor inhibition, Table S5 and Figure S7).

3.4. Cytotoxicity. In total, nine extracts were cytotoxic to the cells used in the AREc32 assay (Table S4). Here, PS 2 and PUR 1–3 were most potent with a highest noncytotoxic concentration of ≤ 1.88 mg plastic. In yeast cells, four extracts (PS 2, PVC 2, PLA 1, and 3) were cytotoxic (Table S5, $EC_{20} = 0.05$ – 3.59 mg plastic). In addition, PP 3 and 5 were cytotoxic in the YAAS but not in the YES. The extract of a PLA shampoo bottle (PLA 3) was most potent.

3.5. Comparison of Food and Non-food Contact Materials. To investigate whether FCMs contain a lower toxicity than non-FCMs, we pooled the data from the 20 products with and the 14 products without food contact. We did not observe a significant difference for baseline toxicity and estrogenicity (Figure S8). In contrast, non-FCMs induced a significantly higher oxidative stress response and antiandrogenicity. However, this was not generally true as some individual FCMs were more toxic than non-FCMs made of the same plastic type (e.g., in case of PP, PET, and PVC). Furthermore, we observed a high toxicity for specific food contact articles, including a food wrap (PVC 1, baseline toxicity and antiandrogenicity), a yogurt cup, a food tray, and a coffee cup lid (PLA 1, 2, and 4, baseline toxicity), a gummy candy packaging (PP 3, oxidative stress response), and another yogurt cup (PP 2, antiandrogenicity).

3.6. Nontarget Chemical Screening. To get an overview of the chemical content of the plastic extracts, we ranked them according to the total peak count and area derived from the GC-QTOF-MS data. Overall, we detected between 0 and 194 features per sample. PVC 3 had the largest total peak count and area (Table S6). In total, 15 extracts contained more than 40 peaks, including all PVC, three PUR and three PP products. On the lower end of the spectrum, the PET samples contained a maximum of five features and small total peak areas. Four PVC and two PLA products ranked among the samples with the ten largest total peak areas.

In total, we detected 1411 features. We searched their mass spectra in the NIST database to tentatively identify them. Here, 362 spectra matched a known chemical with a score ≥ 70 (26% of all compounds, Table S7) corresponding to 260 unique compounds. These represent 18% of all detected chemicals. Out of the 260 unique chemicals, 60 were detected in more than one sample, including 12 compounds that were present in more than three samples (Table S8). Butylated

Table 2. High Priority Chemicals in Plastics According to Toxicity (ToxCast data) and Abundance in the Samples (Peak Area)^a

CAS	name	lowest AC ₅₀ value from ToxCast (μM)				origin	detected in samples
		OX	AA	E	CT		
10482-56-1	α-terpineol	39.5	NA	1.64	0.06	C	LDPE 1/4
112-62-9	9-octadecenoic acid (Z)-, methyl ester	ND	NA	46.3	1.64 × 10 ⁻³	P	LDPE 2, PVC 2
112-63-0	9,12-octadecadienoic acid (Z,Z)-, methyl ester	27.8	53.9	6.93	24.9	p	LDPE 1
112-80-1	oleic acid	100	4.59	33.4	1.00 × 10 ⁻⁵	P	LDPE 1, PLA 3/4
115-99-1	linalyl formate	60.4	NA	NA	0.17	C	LDPE 1/4
119-61-9	benzophenone	112	NA	5.35	0.24	P	PP 5, PVC 1/3
120-46-7	dibenzoylmethane	4.16	45.3	7.22	52.9	P	PVC 3
128-37-0	butylated hydroxytoluene	49.2	0.11	21	0.08	P	PP 3/5, PVC 2/3, PUR 1/2/4
13466-78-9	3-carene	53.0	NA	92.3	NA	C	HDPE 4, LDPE 1/4
143-07-7	dodecanoic acid	106	14.0	6.85	18.8	P	PLA 3/4
149-57-5	hexanoic acid, 2-ethyl-	NA	52.8	NA	0.83	P	PUR 2
2425-77-6	1-decanol, 2-hexyl-	91.0	NA	14.9	46.1	P	PP 5
26896-20-8	neodecanoic acid	ND	22.21	87.0	ND	P	PVC 3
29761-21-5	isodecyl diphenyl phosphate	18.9	45.2	9.39	1.13 × 10 ⁻⁵	P	PVC 2/3/4
5392-40-5	citral	68.7	NA	22.2	1.64 × 10 ⁻³	P	LDPE 4
554-12-1	methyl propionate	ND	NA	NA	0.22	C/p	PLA 1/2
55406-53-6	iodopropynyl butylcarbamate	3.20	9.19	24.5	1.86	P	PLA 3
57-10-3	n-hexadecanoic acid	NA	NA	37.5	70.4	P	PLA 3/4
57-11-4	octadecanoic acid	NA	12.1	2.30	11.1	P	PLA 4
77-90-7	tributyl acetylcitrate	57.3	38.4	NA	NA	P	PP 3/4, PVC 3/4
7785-70-8	α-pinene	NA	NA	0.73	NA	C	LDPE 4
78-40-0	triethyl phosphate	NA	NA	90.5	1.50 × 10 ⁻⁵	P	PUR 3
80-54-6	lilial	24.7	NA	25.4	0.02	P	PP 5
84-76-4	1,2-benzenedicarboxylic acid, dinonyl ester	ND	3.40	NA	NA	P	PVC 4
84-77-5	didecyl phthalate	ND	17.3	NA	NA	P	PVC 3
85-68-7	benzyl butyl phthalate	45.1	36.8	6.41	1.65 × 10 ⁻³	P	PVC 4
99-87-6	p-cymene	NA	NA	NA	3.68 × 10 ⁻³	P	LDPE 4

^aCompounds listed in Table S10 were classified as plastic-associated (P). The other compounds were likely associated with plastics (p) or the packed content (C). Note, OX, oxidative stress; AA, antiandrogenicity; E, estrogenicity; CT, cytotoxicity; NA, not active; ND, not determined; one compound (76-25-5) was removed as implausible.

hydroxytoluene (7 detects), 1,7-di-iso-propylnaphthalene (6), methyl isostearate (6), and methyl di-*t*-butyl hydroxyhydrocinnamate (6) were most common. Interestingly, some chemicals were specific to a certain polymer type with styrene and one benzene present in all PS samples (Table S8 and S9).

3.7. Origin and Functionality of the Detected Chemicals. Regarding their functionality, most of the tentatively identified compounds are classified as food additives and contaminants (13.2%), intermediates (9.9%), solvents (8.6%), process regulators and aids (8.3%), surface-active substances (6.3%), as well as lubricants and lubricant additives (6.3%) according to TSCA (Table S10). Regarding their origin, we cross-referenced our data set with the “Chemicals associated with Plastic Packaging database”⁴ and found 57 compounds likely or potentially associated with plastic packaging (see Table S9 for details). These chemicals include monomers (styrene in all PS samples) and additives, such as flame retardants (e.g., triethyl phosphate in sample PUR 3), UV filters (e.g., benzophenone in PP 5, PVC 1/3), and antioxidants (e.g., butylated hydroxytoluene in PP 3/5, PVC 2/3, PUR 1/2/4). Further, we identified the plasticizers decanedioic acid, dibutyl ester (PP 3), tributyl acetylcitrate (PP 3/4, PVC 3/4), bis(2-ethylhexyl) phthalate (DEHP in PVC 2), and didecyl phthalate (DIDP in PVC 3). We also detected seven known NIAS, including 9-octadecenamamide (PS 2, PP 4, PVC 2, PUR 2, PLA 3), di-*tert*-butylphenol (HDPE 3, LDPE 2/3), a derivative of benzenepropanoic acid (HDPE 2,

LDPE 2/3, PP 3/5, PUR 3), and a di-*tert*-butyl-oxaspirodecadienedione (LDPE 3).

3.8. Toxicity of the Detected Chemicals. We cross-referenced the 260 tentatively identified compounds with in vitro toxicity data from ToxCast. Sixty chemicals (23%) were analyzed in at least one assay for estrogenicity, antiandrogenicity, oxidative stress response, or cytotoxicity (see Tables S11 and S2 for assay information). Thirty-one and 24 chemicals were estrogenic or antiandrogenic in at least one ToxCast assay, respectively. Twenty-five and 52 compounds induced oxidative stress or cytotoxicity, respectively. Regarding the polymers, LDPE (13 chemicals), PVC (11), and PLA (7) contained the most known estrogenic compounds, and PVC (11) and PLA (5), as well as LDPE, PP, and PUR (4), the most antiandrogenic compounds. Chemicals inducing oxidative stress or cytotoxicity were most present in LDPE (11), PVC (9), and PP (6), as well as LDPE (31), PLA (16), and PP (15), respectively.

We compared the lowest AC₅₀ values of each compound with its highest peak area in the plastic samples (see Table S11). We use the latter as proxy for the abundance of the chemical in the sample. However, this approach has major limitations because the peak area depends on other parameters than concentration, including volatility and ionizability. We used the ratio of AC₅₀ to peak area to prioritize the top ten compounds per end point. In total, 27 compounds had a low ratio of toxicity to abundances (Table 2). On the basis of the

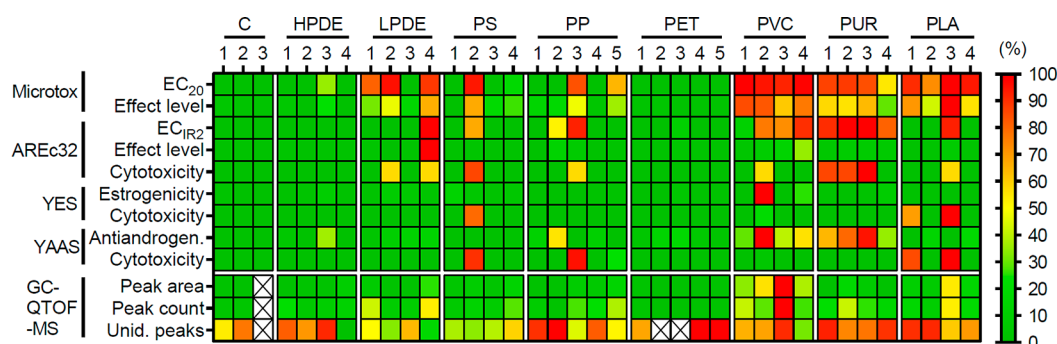


Figure 4. Toxicological and chemical signatures of plastics based on the results of all bioassays and GC-QTOF-MS data (total peak area, number of all detected peaks (peak/feature count), ratio of unidentified peak (unid. peaks)). Controls (C) include procedural blank 1 (1) and 2 (2), as well as the solvent blank (3). Note: EC_{20} , effect concentration inducing 20% baseline toxicity; EC_{IR2} , effect concentration with an induction ratio of 2 over the negative control.

ToxCast data, α -pinene, α -terpineol, and octadecanoic acid were most estrogenic ($AC_{50} < 3 \mu M$). Butylated hydroxytoluene and 1,2-benzenedicarboxylic acid, dinonyl ester were the most potent antiandrogens with AC_{50} values $< 4 \mu M$. Iodopropynyl butylcarbamate, dibenzoylmethane, and phosphoric acid, isodecyl diphenyl ester induced oxidative stress at the lowest AC_{50} ($< 20 \mu M$, Table S11). Oleic acid, isodecyl diphenyl phosphate, and triethyl phosphate were most cytotoxic ($AC_{50} < 2 \times 10^{-5} \mu M$). Interestingly, 21 out of the 27 compounds affected more than one in vitro end point. Moreover, 21 priority compounds originated from plastics and five were associated with the packed content.

3.9. Comparing the Toxicological and Chemical Signatures of Plastics. A comparison of the toxicological signatures of the products highlights that PVC and PUR affected most end points (Figure 4). PLA was similarly effective, especially regarding the induction of baseline toxicity and cytotoxicity. In contrast, HDPE and PET induced the lowest toxicity across all assays. The signatures of products made from LDPE, PS, and PP are more heterogeneous. Here, some samples were toxic in a range of assays, whereas other products from the same polymer type were not. We performed a cluster analysis to test the hypothesis that the polymer type predicts the toxicity of a material. The samples clustered in three main groups that correspond well to a low, medium, and high toxicity across all assays (Figure S9). All HDPE and PET samples clustered in the low and PUR samples in the high toxicity group. Accordingly, the polymer type may be predictive for the toxicity of these materials. All other polymer types spread across different toxicity clusters indicating that a generalization regarding their toxicity is not possible.

We used the same approach to compare the chemical signatures of the samples and observed no clear patterns regarding the number of detected features and the total peak area (Figure 4). However, this analysis was dominated by PVC 3, which contained by far the most compounds in the highest abundance. The number of unidentified peaks was high across all samples except for most LDPE and PS products. A cluster analysis using the full mass spectral data, including the unidentified peaks, classified the samples according to increasing chemical complexity but did not return distinct clusters (Figure S9). Here, most but not all products made from either PET, HDPE (low complexity), or PS (medium complexity) were chemically very similar. For the other polymer types, chemical signatures clustered widely indicating a low similarity of samples made of the same polymer.

While some products from the low and high toxicity cluster were found to be of low and high chemical complexity, there are some exceptions to this trend. For instance, PUR 1 clustered with the nontoxic samples based on its chemical signature but was highly toxic. Vice versa, the nontoxic HDPE 4 was chemically more similar to the very toxic samples. While there was a general trend for an increased toxicity with higher chemical complexity, chemical, and toxicological signatures do not match. Accordingly, it is not possible to predict the toxicity of a polymer based on chemical analysis.

4. DISCUSSION

4.1. Common Plastic Products Contain Chemicals Inducing In Vitro Toxicity.

In previous studies, bioassays have been applied to assess the toxicity leaching from diverse FCMs.¹⁴ However, this is mainly restricted to certain materials and toxicological end points and based on the analysis of packed food or leachates from migration studies. Thus, a comprehensive assessment of the toxicity present in plastic products covering all commodity polymers is absent. In our study, the majority of plastics contained chemicals inducing unspecific toxicity, including baseline toxicity, oxidative stress, and cytotoxicity. Twenty-one out of 34 samples induced baseline toxicity, which in case of the most potent samples translated to cytotoxicity in the other bioassays. Little information is available on unspecific toxicity leaching from plastics. Szczepańska et al.^{39,40} reported a strong baseline toxicity migrating from two PE FCMs, as well as baby toys (diverse polymers). In line with our findings, PET-bottled water did not induce baseline toxicity in the Microtox assay⁴¹ or cytotoxicity in MCF7 and PALM cells,⁴² as well as HePG2 cells.⁴³ This implies that PET does not contain chemicals inducing unspecific toxicity. The results on the cytotoxicity of water stored in PET and PVC bottles in murine fibroblasts (L-929) are conflicting.⁴⁴ So far, there is no data on plastics containing chemicals triggering an oxidative stress response. While previous reports are sporadic, our results imply that chemicals inducing unspecific toxicity are prevalent in plastic products, especially in those made from PVC, PUR, and PLA.

Our results also show that plastics contain endocrine disrupting chemicals. Here, antiandrogenicity (9 products) was more frequent and potent than estrogenicity (4). Compared to unspecific toxicity, more data is available on the endocrine activity of plastics, mainly on bottled water packed in PET.¹⁴ Estrogenicity has been detected in plastics used as food packaging, medical supplies and labware,^{45–48}

casings of consumer electronics,¹⁷ baby teething toys,⁴⁹ and pet toys.⁵⁰ Antiandrogenicity was reported in FCMs⁴⁸ and baby products.^{49,40} Studies with reporter-gene assays compared the endocrine activity of multiple plastic FCMs and confirm our findings that PET does neither contain estrogenic nor antiandrogenic compounds.^{47,48} Similar to our study, estrogenicity was less common in PE, PP, and PS⁴⁷ than antiandrogenicity.⁴⁸ In contrast, Yang et al.⁴⁶ reported a widespread estrogenicity leaching from multiple plastic products. Here, 72% of the 455 samples induced a proliferative response in the E-Screen, including products made of PLA, PET, HDPE, PP, and PS. Since our extraction conditions are much harsher than Yang et al.'s, there are only two alternative explanations for the conflicting observations: Either the YES is prone to false-negatives (e.g., because of its lower sensitivity) or the E-Screen is sensitive to false-positives (e.g., because the proliferative response is not exclusively mediated via hER α).

4.2. Toxicity is Less Prevalent in FCMs but Not Absent. Our results indicate that plastic products not intended for food contact induce a higher oxidative stress response and antiandrogenicity compared to FCMs. This may reflect the stricter regulation of chemicals used in FCMs.¹² However, concerns have been raised over the safety of FCMs,^{51,14} especially with regards to the migration of unknown chemicals. Along the same line, our study shows that some plastic FCMs contain compounds inducing oxidative stress, estrogenicity, and antiandrogenicity. Importantly, both, FCMs and non-FCMs induced a similar level of baseline toxicity. This underpins the concerns over the adequacy of the current approach for safety assessment of FCMs and implies that bioassays might be more appropriate to assess their safety. Importantly, plastics not intended for food contact can be relevant sources of chemical exposures, too. Humans may be exposed via ingestion (e.g., mouthing behavior), dermal exposure, and inhalation if the chemicals readily migrate. In addition, these chemicals may also affect wildlife, especially in habitats that accumulate plastic litter.

4.3. Plastics Contain a Complex Mixture of Low Molecular Weight Chemicals. Using a nontargeted screening with GC-QTOF-MS, we detected 1411 features in total. While the chemical composition varied with the polymer and the individual product, we detected >40 compounds in 15 samples. This shows that plastic products contain a large number and wide variety of low molecular weight chemicals. So far, the few studies that have used nontarget approaches mainly focus on individual polymers or products. As an example, Dorival-García et al.⁵² used GC-Orbitrap-MS and detected 32 and identified 20 compounds in PE-based single-use bags for cell-cultivation. Vera et al.⁵³ analyzed 26 FCM films made from PP and tentatively identified 74 chemicals. However, as in case with other studies, the total number of detected peaks was not reported. This makes it difficult to evaluate the extent to which the chemical composition of plastics is (un)known. Here, we tentatively identified 260 chemicals out of 1411 features. This demonstrates that most of the chemicals present in plastics (82%) cannot be identified using the NIST database and, thus, remain unknown. Since the health risks of unknown compounds cannot be assessed, this poses a challenge for plastic manufacturers, public health authorities, and researchers alike.

4.4. Toxicological Prioritization of Chemicals in Plastics Is Possible but Remains Fragmentary. Focusing on the tentatively identified compounds, we show that at least

57 chemicals originate from the plastic products in which they are used as monomers, intermediates, solvents, process regulators, and additives. We also detected seven known NIAS. However, the identification of the compounds' origin and function was challenging and hampered by the lack of publicly available data. Accordingly, there is a need to create better chemical inventories for plastics, including NIAS, which will also facilitate the characterization of human exposures to plastic-associated chemicals.

We used ToxCast data to prioritize the detected compounds according to their in vitro toxicity and retrieved high-throughput data for 23% of the chemicals. This highlights that toxicological data is unavailable for most of the known chemicals. Accordingly, we speculate that these 60 compounds are unlikely to explain the toxicity we observed in the plastic extracts. A prioritization resulted in 21 plastic-associated chemicals with high in vitro toxicity, based on ToxCast data, and high abundance in our samples. These include well-known additives (e.g., benzophenone, butylated hydroxytoluene, triethyl phosphate), as well as several compounds that have not received scientific attention but might be toxicologically relevant. For instance, the isomers of decanoic acid that we detected in a range of plastics are estrogenic and antiandrogenic according to ToxCast. Accordingly, this prioritization exercise can help generating hypotheses for future toxicological and epidemiological research.

4.5. Some Polymers Contain More Toxic Chemicals than Others. On the basis of our data, PVC and PUR products contained chemicals inducing the highest toxicity at most end points. In contrast, products made from PET and HDPE induced, if at all, the lowest in vitro effects. As this was true for all samples from those polymer types, we conclude that PVC and PUR generally contain more toxic chemicals than other polymers. This is supported by previous studies with aquatic invertebrates. Here, migrates from PVC and PUR induced the highest acute toxicity compared to other commodity plastics in the freshwater cladoceran *Daphnia magna*,⁵⁴ the marine copepod *Nitocra spinipes*,⁵⁵ and the barnacle *Amphibalanus amphitrite*.⁵⁶ PVC and PUR are known to require large numbers and quantities of additives and have been ranked most hazardous based on their chemical composition.⁵⁷ Notably, all PLA products induced strong baseline toxicity similar to PVC and PUR. This demonstrates that this bio-based and biodegradable material, despite being marketed as better alternative, is not necessarily safer than conventional plastics (see ref 58 for review).

For the other commodity plastics, LDPE, PS, and PP, a generalization based on toxicological and chemical signatures is not possible because certain products triggered a range of toxicological end points, whereas others did not. This implies that the toxicity of these products depends on their individual chemical composition, which remains unknown to the public. On a positive note, this also implies that alternative polymer formulations are available on the market not containing the chemicals that induced the toxicity investigated in this study.

4.6. Limitations and Future Directions. Given the diversity of plastics, our analysis of four to five products per polymer type is certainly not representative. Nonetheless and to the best of our knowledge, it represents the most comprehensive study of the toxicity and chemicals present in plastics available, so far. The same is true regarding the in vitro end points we investigated. We selected assays that are well-established, robust, and in parts, standardized. We focused on

baseline toxicity, oxidative stress, and cytotoxicity, as well as endocrine activity because these are potentially relevant for human health. However, it is important to highlight that our aim was not to draw conclusions regarding the health impacts of plastics but rather to benchmark materials based on their intrinsic toxicity. Along the same line, we extracted plastics as worst-case scenario instead of migration testing with softer solvents (e.g., water). Thus, we expect to see different toxicological and chemical signatures when using more realistic migration conditions. The chemical screening with GC-QTOF-MS is certainly limited because it is selective to semivolatile and nonpolar organic compounds. Accordingly, nonvolatile and polar compounds will be underrepresented in our data. We decided to use GC-QTOF-MS because comprehensive spectral libraries for compound identification are available. However, the NIST database may be limited in their coverage of plastic-associated chemicals, especially NIAS, and the rate of false identifications might be high. A confirmation of compounds of interest using authentic standards can be used to resolve the latter. The same may be true for the ToxCast data, which in addition might be prone to false-positives and -negatives, as recently discussed for PPAR γ and RXR α .⁵⁹ The only viable strategy to address the limitations of both databases is to perform effect-directed analysis to identify the compounds causing the toxicity present in plastics. In a larger context, we need to approach the challenges of assessing the risks of plastic materials from a new perspective: Acknowledging their chemical complexity is the first step towards developing new scientific and regulatory approaches to improve their safety.

■ ASSOCIATED CONTENT

5 Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.9b02293.

Methodology on chemical analysis, further in vitro toxicity data (baseline toxicity, oxidative stress, cytotoxicity, estrogenicity, antiandrogenicity) of reference compounds and samples, toxicity of FCMs vs non-FCMs, total peak number and area observed in plastic extracts, compounds tentatively identified by NIST database search, list of all compounds and their functionality, as well of those only present in more than three samples and of those associated with plastics, tentatively identified chemicals that induce antiandrogenicity, estrogenicity, oxidative stress response, or cytotoxicity based on ToxCast data, ToxCast assays used to retrieve AC₅₀ toxicity values, and hierarchical clustering of plastic extracts according to toxicity and chemical data (PDF)

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L.Z., C.V., and M.W. conceived the study, L.Z. performed the experiments, G.D. performed the chemical analyses, L.Z. and M.W. analyzed the data and wrote the manuscript, and all authors provided comments on the manuscript.

Notes

The authors declare no competing financial interest.

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