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Effects of Leachates from UV-Weathered Microplastic in Cell-Based Bioassays

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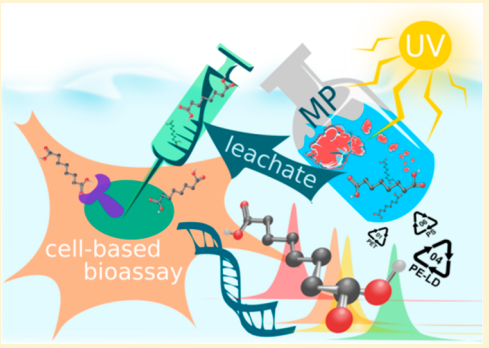
Supporting Information

ABSTRACT: Standard ecotoxicological testing of microplastic does not provide insight into the influence that environmental weathering by, e.g., UV light has on related effects. In this study, we leached chemicals from plastic into artificial seawater during simulated UV-induced weathering. We tested largely additive-free preproduction polyethylene, polyethylene terephthalate, polypropylene, and polystyrene and two types of plastic obtained from electronic equipment as positive controls. Leachates were concentrated by solid-phase extraction and dosed into cell-based bioassays that cover (i) cytotoxicity; (ii) activation of metabolic enzymes via binding to the arylhydrocarbon receptor (AhR) and the peroxisome proliferator-activated receptor (PPARγ); (iii) specific, receptor-mediated effects (estrogenicity, ERα); and (iv) adaptive response to oxidative stress (AREc32). LC-HRMS analysis was used to identify possible chain-scission products of polymer degradation, which were then tested in AREc32 and PPARγ. Explicit activation of all assays by the positive controls provided proof-of-concept of the experimental setup to demonstrate effects of chemicals liberated during weathering. All plastic leachates activated the oxidative stress response, in most cases with increased induction by UV-treated samples compared to dark controls. For PPARγ, polyethylene-specific effects were partially explained by the detected dicarboxylic acids. Since the preproduction plastic showed low effects often in the range of the blanks future studies should investigate implications of weathering on end consumer products containing additives.



INTRODUCTION

Pollution of the aquatic environment by plastic debris has become ubiquitous over the last decades and fits the profile of a planetary boundary threat.[1] Plastic material in the environment is impacted by weathering processes such as UV light-induced degradation, mechanical stress, temperature and salinity changes, as well as biological influences exerted by superficial biofilms and fauna.[2−5] Weathering causes fragmentation, leading to formation of microplastic (<5 mm),[6] and to the liberation of additives, related degradation products, and products of polymer chain-scission reactions as free chemicals.[2]

Many studies have investigated the potential effects of microplastic by addressing the physical presence and impact of the particles themselves. Negative effects on organisms from different trophic levels such as algae, daphnia, and fish have been reported for laboratory studies using pristine microplastic particles.[7−9] Furthermore, plastic debris has the potential to serve as a source and sink of persistent organic pollutants (POPs)[10] which may facilitate the transport of such substances, often referred to as the "vector effect".[11] Depending on the polymer's intended use, additives such as UV stabilizers and flame retardants are added to preproduction polymers during manufacturing.[12] Once released to the environment, plastic debris may act as a source of these additives and hence has the potential to negatively impact organisms.[13] The high sorptive capacity for hydrophobic organic contaminants such as Polychlorinated Biphenyls (PCBs)[14,15] and Polycyclic Aromatic Hydrocarbons (PAHs)[16] renders polymers also a sink for these compounds.

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Considered in total, it is apparent that under the influence of environmental weathering, plastic materials, including the polymer carbon backbone chains and associated chemicals, will ultimately release a complex mixture of chemicals that includes many unknown degradation products. Unraveling the potential effects of weathering plastic and the associated chemicals is a high research priority for planetary health.[1]

Previous studies have described acute toxicity of leachable fractions of various plastic types toward *Daphnia magna*[17,18] and the marine copepod *Nitocra spinipes*.[19] Li et al. (2016)[20] revealed larval toxicity and settlement inhibition to the barnacle *Amphibalanus amphitrine* during a 24 h exposure scenario in leachates from seven recyclable commercial plastic products. Cytotoxic end points like cell growth, survival and colony-forming capability were negatively affected by plastic leachates from biomedical devices tested in the human cell line L929 after 1 h of exposure.[21] Coffin et al. (2018)[22] detected estrogenic effects and binding to the arylhydrocarbon receptor (AhR) by chemicals leached from virgin, weathered and field-collected in situ plastic samples from the North Pacific Gyre.

The most important abiotic degradation process for plastic in the environment is UV radiation-initiated autocatalytic radical oxidation.[2,23] Recently, Gewert et al. (2018)[24] identified a set of low molecular weight polymer chain scission products liberated from commercially important polymers exposed to UV light. They were mainly dicarboxylic acids, but also included other oxidized end-groups. Toxicological studies on the chemicals leaching from plastic often lack related chemical analyses as well as consideration of UV light-induced changes of the polymers' chemical composition, a so-called fingerprint, compared to the pristine material.

To improve our understanding how weathering can influence MP-induced effects, we aimed in this study to identify potential activation of cellular signaling pathways by leachates that were generated as a result of artificial UV light-induced weathering of four commercially important polymers (polyethylene (PE), polyethylene terephthalate (PET), polypropylene (PP), and polystyrene (PS)) in artificial seawater (ASW). To demonstrate the sensitivity of our test battery toward substances liberated from the test material, two positive controls from electronic waste and a computer keyboard known to contain pollutants and/or additives were included. A test battery of four cell-based bioassays was chosen to cover relevant biological end points. They were selected based on the available analytical data of e-waste as a positive control for which high concentrations of, among others, PCBs, Polybrominated Diphenyl Ethers (PBDEs), and Bisphenol A (BPA) were measured in previous studies.[25,26] Another material selection criterion referred to prominent plasticizers and additives often added to preproduction polymers in the plastic industry to customize the material for its intended use.[12]

Concentrated leachates were dosed into cell-based bioassays covering (i) cytotoxicity; (ii) activation of metabolic enzymes via binding to the AhR[27] and the peroxisome proliferator-activated receptor gamma (PPARγ);[28,29] (iii) specific, receptor-mediated effects (estrogenicity);[30] and (iv) adaptive stress responses exemplified by the oxidative stress response.[31,32] Liquid chromatography coupled to high-resolution mass spectrometry (LC-HRMS) was used to identify potential degradation products, i.e., carboxylic acids, in the leachates. To better account for PPARγ results, we tested the hypothesis that dicarboxylic acids, previously identified as degradation products of UV-weathered PE,[24] could explain the observed explicit induction of PPARγ, by dosing reference mono- and dicarboxylic acids with a range of carbon chain lengths (C5−C18) into the PPARγ assay. In a last step, we applied a concentration addition model to compare the observed bioanalytical effects to the chemical analytical data.

MATERIALS AND METHODS

Test Material and Chemicals. The polymers PE, PET, PP, and PS, purchased from *Goodfellow* (Hamburg, Germany), were chosen as test polymers due to their high production tonnage in European commerce and industrial importance.[33] According to the distributor's information, these pellets were "additive free", only containing antioxidants and trace levels of an unknown pigment in the case of PS to make it look more glass-like when molded (*Goodfellow*, personal communication). Analytical data on degradation products from the identical material was published by Gewert et al. (2018).[24] The pellet test material was milled to <350 μm by the company Messer GmbH (Bad Soden, Germany) to ensure a high surface-to-volume ratio (Figure S1, Supporting Information (SI)). In order to demonstrate that the method was applicable to detect substances leaching from weathering microplastic in cell-based bioassays, we chose two positive controls: a homogenized sample of shredded electric cable plastic waste (e-waste, EW) sampled at a Norwegian electric cable waste-handling facility[25] and a new computer keyboard (keyboard, KB) likely containing flame retardants. Analytical data on BPA and flame retardants of the e-waste was previously published by Morin et al. (2015)[25] and Morin et al. (2017),[26] respectively. Mono- and dicarboxylic acids (α,ω position) of carbon chain lengths of C5, C7−C12, C14, C16, and C18 were purchased from Sigma-Aldrich (Steinheim, Germany) (detailed information SI Table S1). Methanol (Honeywell, Riedl de Haën, Seelze, Germany), ethyl acetate (Honeywell, Riedl de Haën, Seelze, Germany), and water (Fisher Chemical, Schwerte, Germany) were of LC grade.

Weathering. A detailed description on the weathering setup is given by Gewert et al. (2018).[24] In short, triplicates of 50 g of each test material, suspended in 200 mL (i.e., liquid−solid ratio of four) of ASW (Instant Ocean Sea salt, Blacksburg, Virginia U.S.A.) in quartz glass vessels were weathered by intense UV A+B light irradiation (OSRAM Supratec HTC400−241 R7s UVA/UVB lamp), combined with horizontal rotation of the vessels around the lamp. Six vessels were weathered at a time using a custom-made wheel, which rotates the quartz glass vessels around the UV lamp to ensure equal UV exposure and to provide gentle mixing of the particles (SI Figure S2). The samples were weathered for 96 h. UV treatments (UV) were done in triplicates with corresponding dark controls (DC, identical setup but wrapped in aluminum foil, n = 3) of one polymer simultaneously. During the weathering process the temperature was kept between 20 and 30 °C by an air flow cooling system. The 96-h UV treatment in the rotating vessels simulated about 410 days of Middle European sun exposure.[24] Procedural blanks were generated by completing the weathering protocol with ASW without microplastic. A detailed description of the lamp properties and solar simulation equivalence calculation can be found in the SI (Section S1. Experimental setup).

Solid-Phase Extraction. After weathering, the microplastic/leachate water mixture was filtered over a 40 μm steel filter to remove the particles. The chemicals present in the

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Environ. Sci. Technol. 2019, 53, 9214−9223

leachate water were enriched on solid-phase extraction (SPE) cartridges (HLB Plus Oasis 225 mg, Waters GmbH, Eschborn, Germany, conditioned with 5 mL of ethyl acetate/methanol (1:1, v:v), 5 mL methanol and 5 mL of Milli-Q water), dried, and stored at room temperature until analysis. Elution was performed using 10 mL ethyl acetate and 10 mL methanol, and the extracts were combined. Additionally, the extracts were filtered (GF/F Whatman) to remove residues of the artificial seawater salt that precipitated during elution. Three SPE blanks using 200 mL of LC grade water that was enriched and eluted were generated to identify potential background effects. Samples were then blown down to dryness under nitrogen, redissolved in 1 mL methanol and stored at −20 °C. An aliquot of 50 μL was taken from each sample, blown down to dryness and stored at −20 °C for chemical analysis. A detailed SPE protocol can be found in the SI (Section S2. SPE protocol).

Cell-Based Bioassays. To measure the activation of xenobiotic metabolism signaling pathways, the AhR-CALUX assay described by Brennan et al. (2015)[27] and performed according to Nivala et al. (2018)[34] and the PPARγ-bla GeneBLAzer assay[28,29] following the method by Neale et al. (2017)[28] were applied. The activation of oxidative stress response was investigated with the AREc32 assay[31] according to Neale et al.[28] and Escher et al.[32] Potential endocrine disruption was measured with the ERα-bla GeneBLAzer assay for estrogenicity[30] according to the procedure described by König et al. (2017)[35] (SI Table S2). Testing the concentrated plastic leachates was conducted as follows: An aliquot of the sample was blown down to dryness and redissolved in the assay medium (DMEM with GlutaMAX or Opti-MEM, respectively, Thermo Fisher, Waltham, U.S.A.) to prevent exposing the cells to solvents. Cells were seeded in 384 well-plates with a Biotek dispenser, samples were diluted and dosed with a liquid handling system (Hamilton Microlab Star, Bonaduz, Schwitzerland) to guarantee precise dosing and repeatability.[28] Directly before dosing and after 24 h of exposure, the confluency of the cells in all wells in the cell plates was measured using an IncuCyte S3 live cell imaging system (Essen BioScience, Ann Arbor, Michigan, U.S.A.).[34] After 24 h the reporter gene product was quantified after adding the appropriate substrates and measuring fluorescence or luminescence using a microplate reader (Infinite M1000 Pro, Tecan, Grödig/Salzburg, Germany). A first high-concentration dosing (of relative enrichment factors of up to 167 of the extracts, see "Data Evaluation") combined with serial dilution was performed for the detection of cytotoxicity and for range finding. This first experiment was followed by another serial dilution (for the leachates) or a linear dilution (for the carboxylic acids) in a noncytotoxic concentration range for confirmation of the first measurement and to increase robustness and statistical power. The generation of the dilution series was performed on a dilution plate followed by the cell exposure, conducted in technical duplicates. If the data sets deviated from each other, measurements were repeated to confirm dose−response curves and to reduce uncertainty. The deviating data were not included in the final evaluation of the dose−response curves.

Instrumental Analysis. The dried aliquots of the concentrated leachates were taken up in 100 μL of methanol/Milli-Q water (1/1) and analysis of dicarboxylic acids was performed using an UltiMate 3000 Rapid Separation Liquid Chromatography system (Dionex, Germering, Germany) coupled to a Q Exactive HF Hybrid quadrupole-Orbitrap mass spectrometer (Thermo Scientific, Bremen, Germany). The method was adopted from Gewert et al. (2018)[24] as specified in the SI (Section S3. Instrumental Analysis). A six point calibration of pure substances in methanol/Milli-Q water (1/1) was used for quantification of dicarboxylic acids, applying TraceFinder 4.1 (Thermo Scientific). Method detection limits (MDL) and method quantification limits (MQL) were based on dicarboxylic acid concentrations detected in the dark and UV-treated procedural blanks. MDL was calculated as the mean blank concentration of a given carboxylic acid (n = 3) plus three times standard deviation. Analogously, the MQL was calculated as the mean plus nine times standard deviation. If peaks were detected but not quantifiable (i.e., < MQL), then half of the MQL was used for further computation as detailed in SI Table S3.[36]

Mono- and Dicarboxylic Acids in PPARγ and AREc32. Following previous analytical results,[24] we measured the effect of mono- and dicarboxylic acids of various chain lengths (SI Table S1) separately in the two most responsive assays, PPARγ and AREc32, to identify their potential to activate these signaling pathways. Chemicals were dissolved directly in assay medium or via a methanolic spike solution to facilitate dissolution at highest medium solubility. Methanol concentration in the assay medium was kept under 0.1%.

Data Evaluation. The sample concentrations in the bioassays were calculated as the product of the enrichment factor of the extraction (EF_{SPE}) and the bioassay dilution factor ($DF_{bioassay}$), which results in the relative enrichment factor (REF) (see eq 1):

$$REF_{Leachate} = EF_{SPE} \times DF_{bioassay}\left[\frac{L_{water}}{L_{bioassay}}\right] \quad (1)$$

The three receptor-based bioassays (AhR, PPARγ and ERα) were run with corresponding reference compounds (SI Table S2) that elicit high responses in the assay to calculate dose−response curves given as percent (%) response relative to the maximum effect of the reference compound (SI Figures S6−S12). Agonistic responses were determined as the effect concentration (EC) causing 10% response (EC_{10}) over the control cells.

For the adaptive stress response, which is based on the regulation of an antioxidant responsive element (ARE) by transcription factors and for which the dose−response curves would not show leveling off, the response is given as the induction ratio (IR) of 1.5, i.e., 50% over the controls ($EC_{IR1.5}$).

Using GraphPad Prism Sofware Inc. (version 8.0.0), cytotoxicity was calculated as percent decrease in cell viability compared to unexposed control cells.[34] According to Escher et al. (2018),[37] all concentrations above 10% decrease of cell viability (inhibitory concentration, IC_{10}) were removed from the analyses of reporter gene activation to circumvent false positive detections due to a so-called cytotoxicity-associated "burst".[38] The slope and the standard error (SE) of the slope for reporter gene activation were calculated using log−logistic and linear models to calculate EC_{10} and $EC_{IR1.5}$ values. Previous studies have shown that 10% induction is statistically significantly different from the control and can thus be interpreted as a sample-specific effect relative to the control.[37]

Statistical Assessment. EC data can be counterintuitive to describe the dependence of low EC levels and large effect

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Environ. Sci. Technol. 2019, 53, 9214−9223

sizes. Therefore, the above-mentioned EC_{10}, $EC_{IR1.5}$ and IC_{10} values (in the units REF) derived from the bioassays were plotted as the inverse value (in the units 1/REF) on a log scale with effect units (EU_{bio}) (eq 2) used in the case of activation of specific effects and toxic units (TU_{bio}) for cytotoxicity (eq 3). Analogously to the EU_{bio} for unknown mixtures, we define $EU_{bio(i)}$ for a single compound (i) as the inverse $EC_{10(i)}$ derived from the bioassays (eq 4). Bioanalytical equivalent concentrations are presented in Section S4. Bioanalytical equivalent concentrations and in Tables S4−S6.

$$EU_{bio} = \frac{1}{EC_{10}} \text{ or } \frac{1}{EC_{IR1.5}} \quad (2)$$

$$TU_{bio} = \frac{1}{IC_{10}} \quad (3)$$

$$EU_{bio(i)} = \frac{1}{EC_{10(i)}} \text{ or } \frac{1}{EC_{IR1.5(i)}} \quad (4)$$

With the available data, assumptions for a robust linear regression model were violated hampering extended statistical analyses. Therefore, the mean, standard deviation and the 95% confidence interval were calculated for qualitative comparison between the samples in those cases that all triplicates resulted in a measurable effect. If not stated otherwise, then values in the "results" section are the calculated means. Due to the low number of replicates (n = 3), further statistical computation was not meaningful. From single compound EC_{10} data of mono- and dicarboxylic acids a linear least-squares regression was calculated to test for correlation between molecular mass and EU_{bio} using RStudio (version 1.1.456). Validity of the model assumptions was examined using qqplot and checking for normality of the residuals. The difference of the slope from zero was considered significant with α = 0.05.

The EC_{10} values derived from the single compound testing of dicarboxylic acids and the measured sample concentrations of detectable dicarboxylic acids were applied to a mixture toxicity model. Escher et al. (2013)[39] have previously demonstrated that concentration addition applies for the reporter gene assay AREc32 and other end points.[40] Hence, we defined effect units derived from chemical analysis ($EU_{chem(i)}$). $EU_{chem(i)}$ was calculated analogously to toxic units[41,42] as the ratio of measured concentrations c_i of a chemical i and its EC_y value (here: EC_{10}, eq 5). It can be used to explain effects measured in bioassays (here: EU_{bio}) by a certain contribution of n detected chemicals i as the sum of $EU_{chem(i)}$ given as EU_{chem} (eq 6) and to identify the fraction of effect unexplained by the known chemicals (EU_{bio}−EU_{chem}) by so-called iceberg modeling.[28,37]

$$EU_{chem(i)} = \frac{c_i}{EC_{10(i)}} \quad (5)$$

$$EU_{chem} = \sum_{i=1}^{n} EU_{chem(i)} = \sum_{i=1}^{n} \frac{c_i}{EC_{10(i)}} \quad (6)$$

RESULTS

For all assays, the positive controls e-waste and keyboard showed clear induction of the respective signaling pathway (Figure 1A−C), sometimes exceeding 100% effect of the reference compound (SI Figure S7, e.g., AhR sample KB 1−3, KB_DC 1−3). Furthermore, the plastic-free blanks (DC and UV-treatments) resulted in detectable effects for most of the assays for at least one replicate in AhR, AREc32, and PPARγ (Figure 1A−C). For the ERα assay, only e-waste and keyboard resulted in measurable EC_{10} values (Figures S3 and S10). Due to the absence of detectable effects for our test material, the ERα assay was excluded from the subsequent discussion. Cytotoxicity was observed for some of the test polymers (Figure S4).

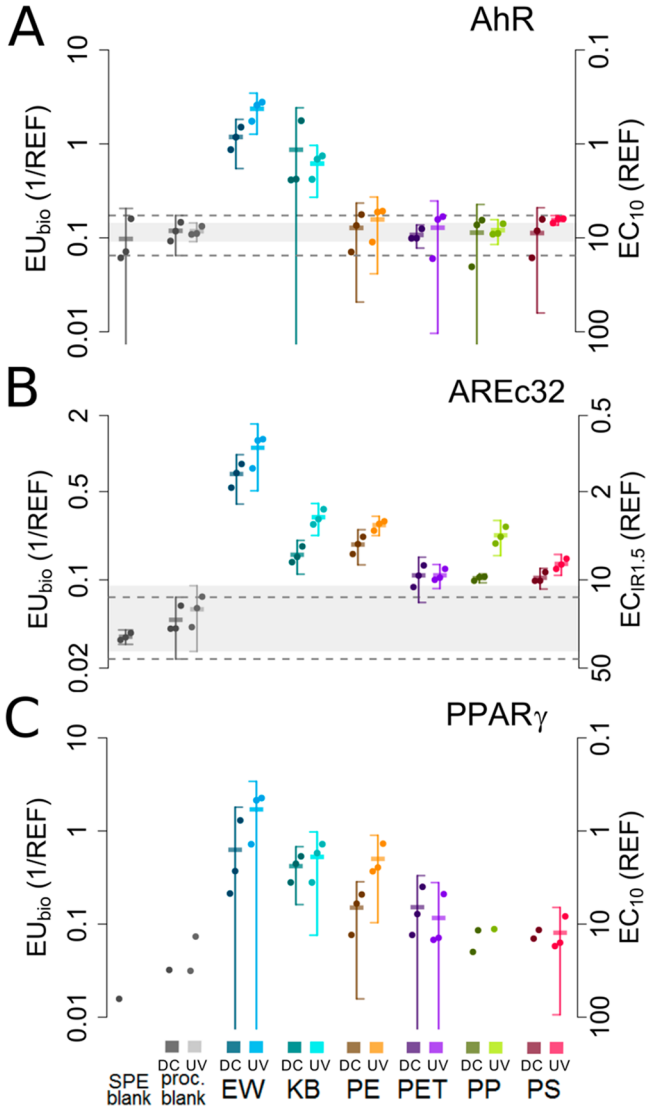

Figure 1. Bioanalytical effect units (EU_{bio}, eq 2) defined as the inverse EC_{10} (1/REF on left y-axis, REF on right y-axis), measured for SPE blanks, procedural blanks, two positive controls (EW and KB) and the four test polymers PE, PET, PP, and PS in the cell-based bioassays AhR (A), AREc32 (B) and PPARγ (C). Dark controls (DC) and UV-treated (UV) samples are presented juxtaposed in darker and lighter shades of the different colors. The squares represent the mean, whiskers the upper and lower range of the 95% confidence interval. The dashed lines and the shaded area represent the minimum and maximum ranges of the 95% confidence interval of the procedural DC and UV-treated blanks to highlight differences from the respective control. For PPARγ, no prediction intervals were included because only one to two replicates resulted in measurable EC_{10} values.

AhR. The AhR signaling pathway was clearly activated by the positive controls, with effects by more than a factor three higher for e-waste (EU_{bio} (EW_{DC}) = 1.19, EU_{bio} (EW_{UV}) = 2.37) than for keyboard (EU_{bio}(KB_{DC}) = 0.87, EU_{bio} (KB_{UV}) =

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Environ. Sci. Technol. 2019, 53, 9214−9223

0.62) (Figure 1 A, Table S4, and SI Figure S7). All test polymers showed low activation of the AhR that did not differ from their corresponding DC or UV procedural blanks indicated by the overlapping 95% confidence band. EU_{bio} of the procedural blanks (EU_{bio} ($blank_{DC}$) = 0.12, EU_{bio} ($blank_{UV}$) = 0.12), and the test polymers (EU_{bio} (all test $polymers_{DC}$) = 0.11−0.13, EU_{bio} (all test $polymers_{UV}$) = 0.12−0.16) were more than a factor of 10 lower than the EU_{bio} values of the DC and UV-treated e-waste. All three replicates of the SPE blanks showed induction of AhR with the lowest mean EU_{bio} of all tested samples in this assay (EU_{bio}(SPE blank) = 0.097) (Figure 1 A). All samples caused cytotoxicity with pronounced effects of the e-waste leachate (DC and UV) (SI Figure S4A).

AREc32. The AREc32 assay, responsive to many chemicals that cause oxidative stress,[39] was activated by all blanks (SPE and procedural blanks), though only with low EU_{bio} (EU_{bio} (SPE and procedural blanks) = 0.04−0.06, Figure 1B, Table S5, and SI Figure S8). The SPE water blanks were at the lower end of the procedural blank levels. All positive controls and the test polymers induced oxidative stress that was above the 95% confidence interval of the respective blanks. Highest effects could be observed for e-waste (EU_{bio} (EW_{DC}) = 0.69, EU_{bio} (EW_{UV}) = 1.11). With the exception of PET, all UV-treated samples showed generally higher EU_{bio} values than their corresponding dark controls. The most pronounced difference between the treatments could be observed for the keyboard (EU_{bio} (KB_{DC}) = 0.16, EU_{bio} (KB_{UV}) = 0.31) and PP (EU_{bio} (PP_{DC}) = 0.10, EU_{bio} (PP_{UV}) = 0.23) where effects for UV treatments were more than a factor two higher than the dark controls, and confidence bands did not overlap. No cytotoxicity was detected for the blanks (SI Figure S4B) even at the highest tested REF of 167 (SI Figure S8). For PP and PS, cytotoxicity could only be measured for UV-treated samples (SI Figure S4B).

PPARγ. Most strikingly, the UV-treated PE (EU_{bio} (PE_{UV}) = 0.50) showed induction levels of PPARγ comparable to the UV-treated positive control keyboard (EU_{bio} (KB_{UV}) = 0.53) (Figure 1C, Table S6, and SI Figure S9). For PE, the UV-treated samples showed a more than three times higher induction than their corresponding dark controls (EU_{bio} (PE_{DC}) = 0.15) which is the most pronounced difference between UV vs DC treatments in all tested assays. Only one SPE blank showed a low EU_{bio} (SPE blank) = 0.016. One dark control and two UV-treated procedural blanks showed activity of EU_{bio} ($blank_{DC}$) = 0.032 and EU_{bio} ($blank_{UV}$) = 0.031−0.073. No EC_{10} value could be determined for several samples of PP and PS. E-waste displayed the strongest activation of the PPARγ signaling pathway across all samples (EU_{bio} (EW_{DC}) = 0.63, EU_{bio} (EW_{UV}) = 1.70). The remaining test polymers resulted in EU_{bio} values that were in the upper range of the procedural blanks.

Analytics. No monocarboxylic acids could be detected with the LC-HRMS setup applied. Furthermore, not all dicarboxylic acid standards were ionizable and thus, only octanedioic acid, nonanedioic acid, decanedioic acid, undecanedioic acid, dodecanedioic acid and tetradecanedioic acid could be analyzed in the leachates (SI Table S3). Their MDLs and MQLs are listed in SI Table S3. Since no recovery experiments targeting these compounds were conducted, the given concentrations should be regarded as semiquantitative. Dicarboxylic acids could be detected above the MDL in the e-waste (UV and DC), the keyboard (DC), PE (UV and DC), PET (DC), PP (DC and UV), and PS (DC and UV). They were quantifiable only in the e-waste (DC and UV), PE (DC and UV), PP (DC), and PS (UV) leachates with dodecanedioic and tetradecanedioic acid as the most frequently quantified dicarboxylic acids (SI Table S3). PE showed the highest concentration of tetradecanedioic acid with differences between DC and UV treatment up to a factor three (PE_{DC} = 0.47−0.60 μM, PE_{UV} = 1.34−1.39 μM).

Mono- and Dicarboxylic Acids. The investigated mono- and dicarboxylic acids were inactive in the AREc32 assay (SI Figure S11) and the AREc32 was therefore not further considered. With increasing chain length (i.e., molecular weight M), the carboxylic acids showed linearly increasing $EU_{bio(i)}$ (decreasing $EC_{10(i)}$) in the PPARγ signaling pathway (Figures 2 and S12). However, a slope that is statistically significantly different from zero was only observed for the monocarboxylic acids (F = 15.39, df = 8, R^2 = 0.66, p < 0.01). The monocarboxylic acids resulted in $EU_{bio(i)}$ values of one and two orders of magnitude higher than the corresponding dicarboxylic acids (SI Table S7). The slope of the dicarboxylic acids was mainly driven by the high $EU_{bio(C14di)}$ of tetradecanedioic acid (M = 258.4 g/mol) to induce PPARγ while short-chained dicarboxylic acids showed low activation of PPARγ (Figure 2).

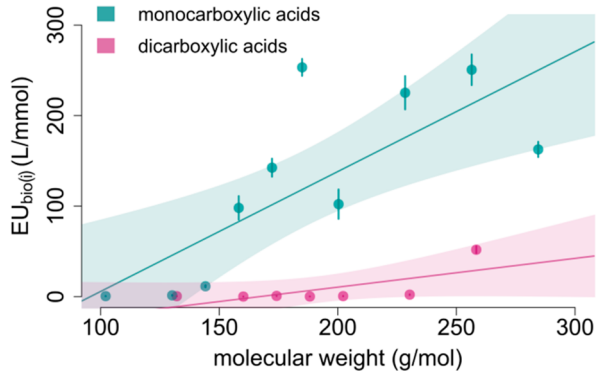

Figure 2. Effect units ($EC_{bio(i)}$ = $1/EC_{10(i)}$) elicited in the PPARγ assay by mono- and dicarboxylic acids of increasing chain length (C5, C7−C12, C14, C16, C18). Monocarboxylic acids showed a significant correlation between molecular weight and target activation (R^2 = 0.66, p < 0.01), which was nonsignificant for the dicarboxylic acids (solid line represents the respective regression line with the shaded area as the 95% confidence band).

Iceberg Modeling. The $EU_{chem(i)}$ values, derived from single compound testing in PPARγ and the respective measured concentrations were summed up in a mixture model based on concentration addition (eq 6) (SI TableS3). They accounted for up to 42% of the observed EU_{bio} values in the case of PE as indicated by proximity of these samples to the 1:1 line (Figure 3). The positive controls showed high EU_{bio} but associated low EU_{chem} which located them more distant from the 1:1 line. The smallest percentage of effects explained by the iceberg model with simultaneous frequent detection of dicarboxylic acids could be observed for the e-waste and keyboard with partly under 1% and 2%, respectively. The concentrations of tetradecanedioic acid increased linearly with increasing EU_{bio} (SI Figure S5).

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Environ. Sci. Technol. 2019, 53, 9214−9223

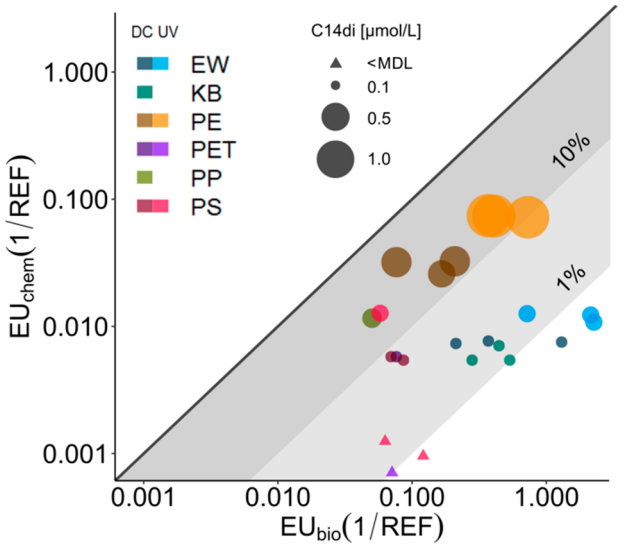

Figure 3. EUs derived from chemical analysis and single compound testing (EU_{chem}) plotted against the bioanalytical effect units (EU_{bio}) for the PPARγ assay (log scale). The 1:1 line indicates that 100%, dark and light shaded area that 10% and 1%, respectively, of the observed effect can be attributed to the analytically determined chemicals. Colors represent the different samples each as the dark control (DC, darker shading) and the UV treatment (UV, lighter shading). The bubble size corresponds to the relative concentration of tetradecanedioic acid, the main driver of the mixture effect. Triangles represent data where tetradecanedioic acid was < MDL.

DISCUSSION

AhR. The low EU_{bio} values of the SPE blanks suggest a limited effect of the sample processing (enrichment, elution, and concentration) on the induction of AhR since two out of three replicates were located at the lower end of the procedural blanks. The induction of AhR by the plastic-free blanks may stem from impurities in the ASW or from the UV-weathering experiment (Figure 1A). According to the manufacturer, the investigated preproduction resin pellets were largely additive-free. A nonsystematic search for typical additives in the full scan data showed the presence of selected additives above blank level only for the e-waste and the keyboard. Therefore, the test polymers were expected to result in low induction in this assay, which was confirmed by their EU_{bio} values that were in the range of the confidence intervals of the corresponding blanks. Potential plastic additives such as brominated flame retardants[43] are known AhR inducers,[44] however, these are often added during processing and not as primary ingredient[12] and hence were likely absent in the leachates of our test polymers. The low induction of the xenobiotic metabolism by our test polymers may further indicate the absence of the most prominent phenols and plasticizers used as monomers and additives in synthetic polymers such as BPA, *n*-nonylphenol and diethylhexyl phthalate (DEHP) since these are known agonists of the AhR.[45,46]

Most importantly, the positive controls e-waste and keyboard provided a proof-of-concept that our test system was capable of detecting effects of chemicals liberated from (weathering) plastic in cell-based bioassays. The concentration of BPA in the e-waste that was tested here was 188 ± 125 mg_{BPA}/kg.[25] If we assume chemical equilibrium between the e-waste and the ASW (Section S5. Mass balance model and eq S3), we can apply a simple two-phase mass-balance model (eqs S3 and S6) to estimate the aqueous leachate concentration of BPA as 2.0 ± 1.3 µmol/L. This rough estimation is only a factor two higher than the BPA leachate water concentrations measured at Norwegian landfills of up to 0.9 µmol/L,[25] demonstrating the environmental relevance of our positive control as a worst case scenario of plastic leachates. Applying the iceberg model for a single compound (here BPA) (eq 5) we can derive an $EU_{chem(i)}$ value of 0.02 (AhR $EC_{10(BPA)}$ = 0.1 mM) which corresponds to a marginal effect contribution of BPA for e-$waste_{DC}$ between 1.3 and 2.3% and e-$waste_{UV}$ with 0.7−1.2%. That means that the mixture effect of many other chemicals in the e-waste account for the observed biological response. Several brominated flame retardants were also reported in this e-waste sample[26] that could have contributed to the observed effects. An important fact throughout all assays is that e-waste showed high EU_{bio} values of around 1 or sometimes higher which means that the experimentally generated leachate water was diluted for the targeted effect range while sample EU_{bio} values below 1 indicate enrichment.

In contrast to our AhR results, Coffin et al. (2018)[22] detected, although statistically not significantly, higher AhR induction by leachates from UV-irradiated consumer plastic than for untreated consumer plastic. Their observations were supported by chemical analyses that suggested enhanced desorption of AhR-active substances such as PCBs and BPA due to the UV treatment.[22]

AREc32. The EU_{bio} values of the SPE blanks were in the lower range of the procedural blanks (DC and UV), indicating, similarly to the AhR results, a certain background effect of the ASW and the experimental procedure on the induction of AREc32 (Figure 1 B). In return, it could also mean that the SPE processing may already introduce AREc32-activating substances either from the LC grade water, the SPE cartridges or the processing itself. Previous studies reported similar $EC_{IR1.5}$ values for solid-phase extracted ultrapure water samples of REF > 20 (EU_{bio} < 0.05) in the AREc32 assay.[32] Our presumably low background contamination is supported by the absence of cytotoxic effects for all blanks (Figure S4B).

Substances that stem from degrading plastic may potentially have caused the oxidative stress response in this assay. The apparent influence of the UV treatment on the ARE induction may indicate that substances were liberated at higher levels from the test polymers and the positive controls during artificial UV irradiation than from the dark controls. This UV-dependent effect is in accordance with a leaching study by Bandow et al. (2017)[47] that detected more explicit leaching of both inorganic and organic compounds in UV-A-irradiated than in merely thermo-oxidized polymer samples.[47] Small reactive molecules have the capacity to induce oxidative stress.[48] Gewert et al. (2018)[24] tentatively identified low-molecular weight fragments with oxidized end groups as degradation products of PE, PET, PP, and PS, applying the identical UV aging setup used here. We hypothesize that these degradation products may potentially be responsible for the observed oxidative stress response. There exist mechanisms for potential cross-talk between the AhR and ARE signaling pathway,[49] however, bifunctional inducers such as certain dioxins or PAHs that are capable of simultaneous activation[50] were probably absent in our test polymer leachates since AREc32 was clearly activated but not AhR. Interestingly, the induction of AREc32 of DC samples indicates that even under dark conditions chemicals that cause oxidative stress are liberated from the test polymers.

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Environ. Sci. Technol. 2019, 53, 9214−9223

PPARγ. On the one hand, the few measurable EC_{10} values of the PPARγ blanks led to some uncertainty when comparing our test polymers to the blanks as done previously for AhR and AREc32. On the other hand, the low detection frequency of the blanks stands for low background contamination of our method and increases the robustness of the response caused by the investigated polymers. The polymers PE (DC only), PET, PP, and PS showed induction ratios comparable to the upper range of the blanks and hence did not allow us to distinguish between samples and blanks. Contrarily, the explicit induction of the UV-treated PE may indicate the presence of degradation products that are capable of specifically activating PPARγ. Candidates are dicarboxylic acids that were previously identified as chain scission products of degrading PE by Gewert et al. (2018).[24] Since fatty acids (FAs) are natural ligands of the PPARγ[51] it is likely that the dicarboxylic acids show similar activity toward this receptor. It is also conceivable that the induction of PPARγ occurred by other known plastic-associated agonists like DEHP.[52] Lovekamp-Swan and Davis (2003)[53] hypothesized that the active metabolite of DEHP, monoethylhexyl phthalate, activates the PPARγ. The fact that the migration potential of phthalates from a PE-based end consumer product (i.e., a shopping bag) was marginally affected by artificial UV exposure[54] renders this class of substances less plausible candidates for the observed induction of PPARγ. Furthermore, it is unlikely that plasticizers like DEHP were added to the PE virgin pellets, as these are typically added to melted virgin pellets during molding.[55]

Mono- and Dicarboxylic Acids. FA derivatives are known ligands of the PPAR family.[51,56−60] Our observed positive correlation between FAs of different carbon chain lengths and their potential to activate PPARγ is supported by observations by Wolf et al. (2008)[61] who described a similarly increasing induction capacity of perfluoroalkyl acids toward PPARα with increasing carbon chain length, up to C9. It should be noted that FAs can act as ligands for all three subtypes α, δ, and γ[62] with PPARγ showing the most restricted FA binding profile.[59]

The effect correlation with carbon chain length and the discrepancy between mono- and dicarboxylic acids may mainly be driven by toxicokinetic processes since (1) a linear relationship between lipid permeability and carbon chain length was observed for monocarboxylic acids,[63] (2) dicarboxylic acids have shown lower abilities to permeate lipid bilayer membranes than their corresponding monocarboxylic acid,[64] and (3) long-chain FAs may be more resistant to metabolism.[65] A toxicodynamic explanation for our observations may be a more effective activation of PPARγ by long-chain FAs.[66] Similar to our finding, Intrasuksri et al. (1988)[65] detected higher PPAR induction potency for FAs in decreasing order from oleic acid (C16) > octanoic acid > octanedioic acid. The neutral form can only passively permeate through the membrane which is impermeable for the anionic form of the fatty acids.[67]

Iceberg Modeling. For the iceberg modeling, we need to consider the analytical results. The frequent but low detected quantities of dicarboxylic acids of different carbon chain lengths throughout the blanks may be regarded as background contamination of unknown source in our experimental setup or the laboratory itself (SI Table S3). We accounted for this background by setting the calculated MDLs and MQLs as quality criteria. While the dicarboxylic acids found in the e-waste and keyboard may stem from impurities and additives, their presence in the PS remain unexplained.

Applying the iceberg model, the observed effects in the PPARγ bioassay (EU_{bio}) were partly explained by the mixture effects of the quantified polymer degradation products, the dicarboxylic acids, present in the leachates. Tetradecanedioic acid was the main mixture risk driver of the detected PPARγ induction of the PE extracts, due to three reasons: (1) It was the most potent PPARγ inducer among the dicarboxylic acids in the single compound testing (Figure 2), (2) it was detected at high quantities exclusively in the PE samples with a factor of around three higher liberation for the UV treatment than for the dark control (Table S3), which is (3) in accordance with the observed bioanalytical effects of the related extracts causing three times higher effects as well (Figures 3, and S5).

For PE, the substantial contribution of EU_{chem} to EU_{bio} of, in some cases, over 40% (Figure 3) is an important explanatory parameter for the PPARγ gene pathway activation. Since Albertsson et al. (1995)[68] identified over 60 PE degradation products, predominantly monocarboxylic acids, we expect those to be present in our PE leachates as well, although they remained undetected by our analytical method. Presumptively, they were not ionizable by our method since derivatization is often a prerequisite for chemical analysis of FAs.[69] It is hence very likely that the identified compounds did not cause the effect alone, but that the mixture effect of all chemicals that are present in the leachate is relevant.[28] It should be noted that the identified dicarboxylic acids, as potential products of UV-weathered PE, could account for a certain effect contribution in PPARy. They could not explain the observed induction of AREc32. We observed higher induction of AREc32 by the UV-treated samples (Figure 1B), still, the mono- and dicarboxylic acids, were largely inactive when tested as single compounds in the AREc32 assay (SI Figure S11). As a consequence, unknown substances might be responsible for the effects in AREc32 which is supported by UV-independent induction of AREc32 by the dark treated samples. The distance of e-waste and keyboard from the 1:1 line in the lower 1% area (Figure 3) indicates that unknown compounds accounted for a larger fraction of sometimes over 99% of the observed effects.

Implications. This study investigated the influence of UV-induced weathering on the liberation of unknown chemical mixtures from largely additive-free preproduction pellets and their effects in cell-based bioassays, addressing a range of cellular response pathways. Compared to measured concentrations of plastic debris in an urban river of up to 0.121 g/L[70] our applied plastic mass concentration for the leaching experiment was 250 g/L. In many cases our UV-treated positive control e-waste resulted in EU_{bio} values >1 (1/REF). That means that the generated leachate water tested in the bioassays was diluted to target the observed effect range. Accounting for this dilution our observed effects were at concentrations of two to three orders of magnitude above high-end plastic concentrations in the environment. Our intention was to reflect the extreme case, to aim for measurable effects. Still, we could address environmental concentrations in the case of the e-waste for which environmental leachate water concentrations showed high levels of contaminants[25,26] as demonstrated for BPA. Under environmental conditions, substances leaching from plastic material may undergo transformation or microbial degradation. These processes will impact their fate and ecotoxicological relevance, but were not subject of this study. Generally, the observed effects of our test polymers were in the lower range compared to our contaminated positive controls. Therefore, future studies

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should focus on more realistic end consumer products, usually containing additives, and their relevance for the aquatic environment to act as a source of leaching and degrading compounds potentially of concern.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.9b02400.

Additional information includes details on (S1) the experimental setup, (S2) the SPE protocol, (S3) instrumental analysis, (S4) the bioanalytical equivalents, and (S5) the mass balance model; (Tables S1−S7) specifications on the mono- and dicarboxylic acids, the bioanalytical test battery, all results of the assays AhR, AREc32, PPARγ, the concentrations of dicarboxylic acids detected in the leachates, and the test results of mono- and dicarboxylic acids for PPARγ; (Figures S1−S5) the test material, the weathering setup, the bioanalytical effect units measured in ERα and bioanalytical toxic units for all assays, and the correlation of tetradecanedioic acid and the EU_{bio} of PPARγ; (Figures S6−S12) the concentration−response curves for the reference compounds of the different assays, for the leachates tested in AhR, AREc32, PPARγ, and ERα and the carboxylic acids tested in AREc32 and PPARγ (PDF)

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Notes

The authors declare no competing financial interest.

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