

1 Supporting Information for  
2 **Efficient Removal of Estrogenic Activity**  
3 **during Oxidative Treatment of Waters**  
4 **Containing Steroid Estrogens**

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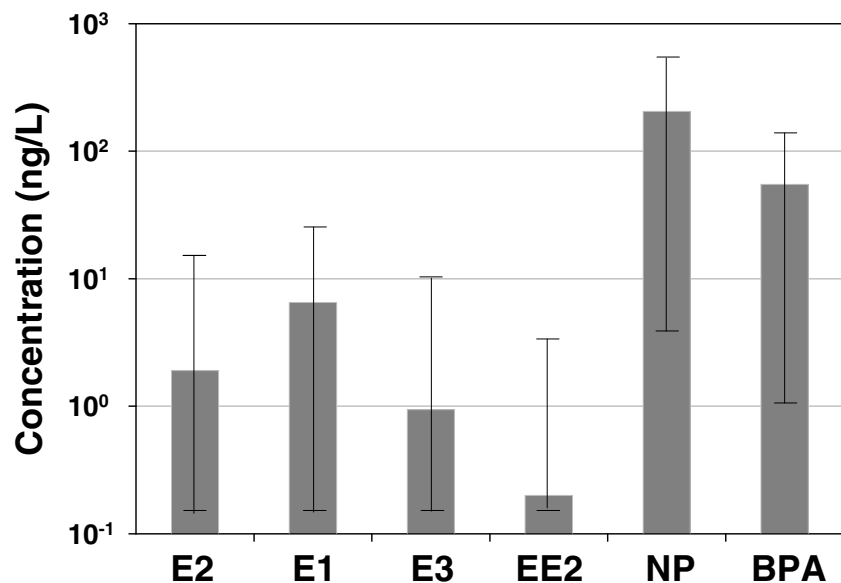
15 7 texts, 2 tables, and 10 figures are available for further information addressing materials,  
16 experimental procedures, and estrogenic chemicals in WWTP effluents.

1 **Text S1. Estrogenic chemicals in effluents of wastewater treatment plant (WWTP)**

2 Municipal wastewater is one of the main routes of emission for most important endocrine  
3 disrupting chemicals (EDCs) into the (aquatic) environment. Of many kinds of EDCs,  
4 steroid estrogens and xenobiotic estrogens such as alkylphenols are two main groups of  
5 chemicals responsible to cause estrogenic effects in aquatic organisms, especially in fish  
6 (S1). To estimate the potential impact of single EDCs on aquatic eco-systems, the  
7 estrogenic equivalent concentrations were computed by multiplying average WWTP  
8 effluent concentrations with their respective specific relative estrogenic potency.

9 Figure S1 shows the average concentrations of steroid estrogens and alkylphenols in  
10 investigated WWTP effluents in several European countries (S2–S10). Median  
11 concentrations of the selected steroid estrogens were 1.9 ng/L for 17 $\beta$ -estradiol (E2,  
12 number of measurement data (no.) = 93), a natural vertebrate estrogen; 6.5 ng/L for estrone  
13 (E1, no. = 93), a principal metabolite of E2, 0.94 ng/L for estriol (E3, no. = 55), an another  
14 metabolite of E2; and 0.2 ng/L for 17 $\alpha$ -ethinylestradiol (EE2, no. = 93), a synthetic steroid  
15 which is the active ingredient in most human contraceptive pills. Median concentrations of  
16 the selected alkylphenols were higher than those of the steroid estrogens with 205 ng/L for  
17 nonylphenol (NP, no. = 52), a breakdown product from some industrial surfactants; 55 ng/L  
18 for bisphenol-A (BPA, no. = 41), a chemical used in the manufacture of plastics and epoxy  
19 resins. It should be noted that the median concentrations in WWTP effluents in Figure S1  
20 can be lower for well functioning nutrient removal plants with increased SRTs (sludge  
21 retention times more than 15 hours).

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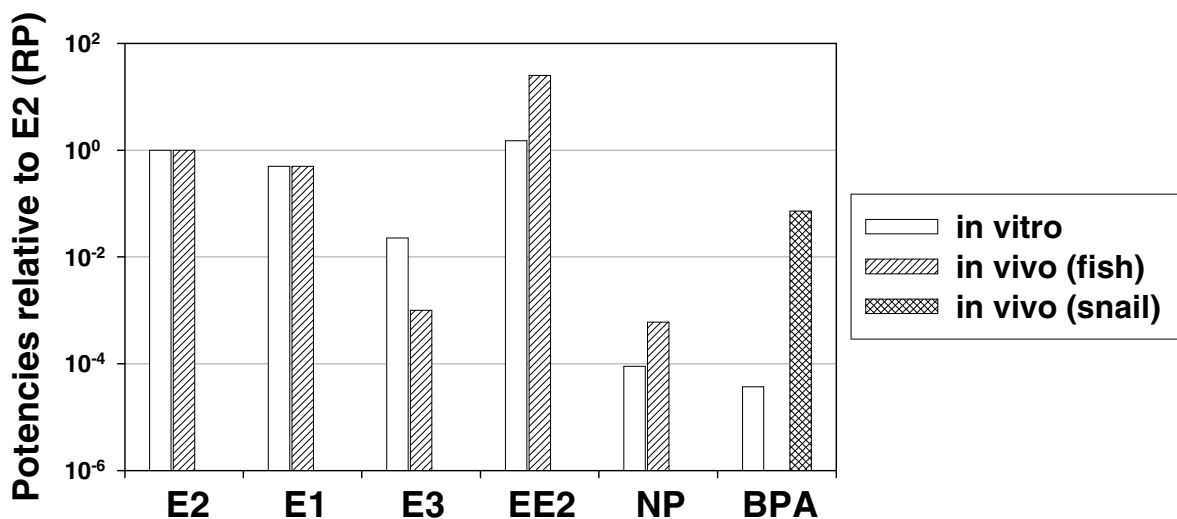
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3 Figure S1. Median concentration of selected estrogenic chemicals in WWTP effluents in  
 4 several European countries. Upper and lower bars represent maximum concentrations and  
 5 limits of detection, respectively. Data for E2, E1, and EE2 were from refs. S3, S4, S5, and  
 6 S8; data for E3 were from refs. S4 and S5; data for NP were from refs. S2, S7, S8, S9, and  
 7 S10; data for BPA were from refs. S6 and S10.

8

9 The estrogenic potencies of EDCs depend on the test system (*in vitro* vs. *in vivo*) and the  
 10 test organisms (e.g. vertebrate such as fish vs. invertebrate such as snails) as shown in  
 11 Figure S2. Generally, steroid estrogens have much higher estrogenic potencies to fish than  
 12 alkylphenols. Among the steroid estrogens, EE2 has the highest *in vivo* potency (median  
 13 effective concentration = 1.38 ng/L), which is 25 times higher than that of E2 (S1).  
 14 Freshwater ramshorn snail showed an EC<sub>10</sub> (effective concentration at 10%) for

1 reproduction at as low as 13.9 ng/L to BPA, indicating that the snails are by a factor of  
2 2000 more sensitive to this estrogenic chemical than fish (S11).  
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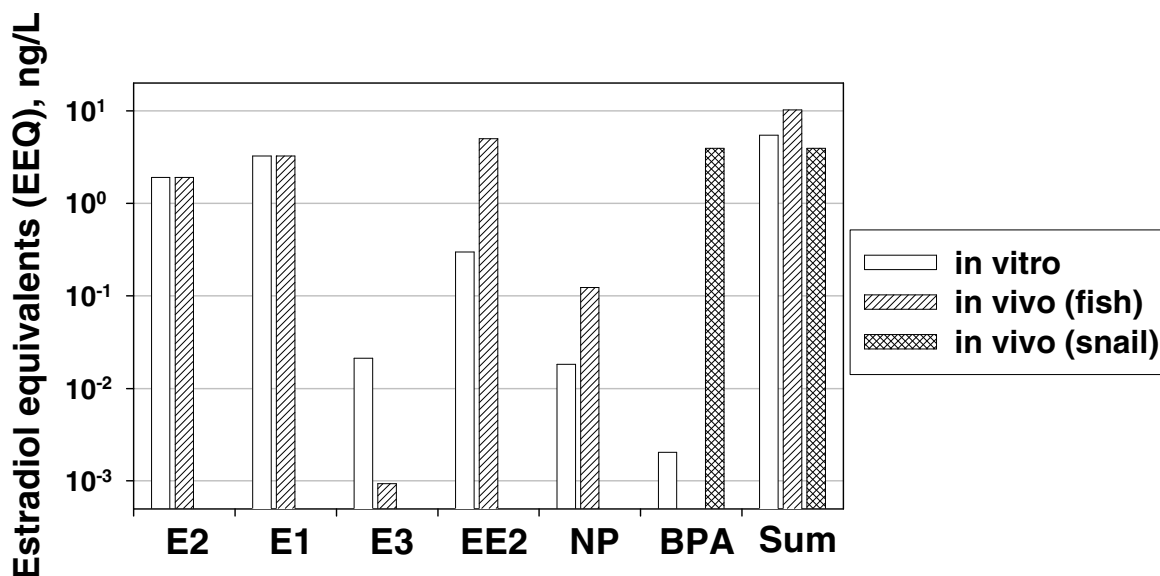
6 Figure S2. Potencies of selected estrogenic chemicals relative to E2 (RPs). The mean  
7 effective concentration of E2 *in vitro* (EC<sub>50</sub>) is 23±3 ng/L. *In vitro* and *in vivo* potencies of  
8 E2, E1, E3, and EE2 were taken from ref. S1. *In vitro* potencies of NP and BPA were taken  
9 from ref. S12. *In vivo* potency of NP was taken from ref. S13. *In vivo* potency of BPA to  
10 snail was taken from ref. S11.

11

12 In Figure S3, the impact of estrogenic compounds in WWTP effluents are compared with  
13 each other based on their estradiol equivalent concentrations (EEQs), which were  
14 calculated from the product of the median concentration presented in Figure S1 and the  
15 specific estrogenic potency presented in Figure S2. On the basis of *in vivo* potency, EE2  
16 could be one of the most important estrogenic chemicals to fish due to its extremely high

1 potency even though its relative low average concentration (typically below 1 ng/L).  
 2 However, BPA could be one of the most important estrogenic chemicals to snails due to its  
 3 high potency as well as relatively high average concentration.

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7 Figure S3. Estradiol equivalent concentrations (EEQs) of each selected estrogenic  
 8 chemicals and their summation (Sum) present in WWTP effluents in several European  
 9 countries.  $EEQ_i$  of each estrogenic chemical was calculated from the product of the  
 10 median concentration presented in Figure S1 and the specific estrogenic potency presented  
 11 in Figure S2 ( $EEQ_i = c_i \cdot RP_i$ ). Sum was calculated as  $\sum_i c_i \cdot RP_i = \sum_i EEQ_i$ .

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1 **Text S2. Chemical reagents**

2 *Chemicals.*

3  $17\alpha$ -ethinylestradiol (EE2),  $17\beta$ -estradiol (E2), and 2-methoxyestradiol (2-methoxy E2)  
4 were purchased from Sigma-Aldrich. 2-bromoethinylestradiol (2-Br EE2), 2-  
5 bromoestradiol (2-Br E2), 4-bromoethinylestradiol (4-Br EE2), 2,4-dibromoethinylestradiol  
6 (2,4-diBr EE2), 2-hydroxyestradiol (2-OH E2), 4-hydroxyestradiol (4-OH E2), 6-  
7 oxoethinylestradiol (6-oxo EE2), 2-fluorestradiol (2-F E2), and 2-iodoestradiol (2-I E2)  
8 were purchased from Steraloids Inc. (Rhode Island, USA). Other chemicals and solvents  
9 were purchased from various commercial suppliers and used as received.

10

11 *Synthesis of chlorinated products of EE2.*

12 2-chloroethinylestradiol (2-Cl EE2), 4-chloroethinylestradiol (4-Cl EE2), and 2,4-  
13 dichloroethinylestradiol (2,4-diCl EE2) were not commercially available and synthesized  
14 by chlorination of EE2 using hypochlorite. 5 mL of 0.2 M EE2 in methanol was mixed with  
15 2.5 mL of aqueous sodium hypochlorite (0.44 M) and allowed to react for a day. The  
16 chlorinated solutions were fractionated by passage through a Nucleosil 100-5 C18 column  
17 (125 mm  $\times$  4 mm). The column was eluted with a 50/50% of water/acetonitrile mixture at  
18 flow rate of 1 mL/min. Retention times of 2-Cl EE2, 4-Cl EE2, and 2,4-diCl EE2 were 5.8  
19 min, 6.7 min, and 11.4 min, respectively. Fractions corresponding to each product peak  
20 (observed via UV) were subsequently isolated using post-column fraction collection. For  
21 each fraction, the acetonitrile was first evaporated under gentle nitrogen stream and the  
22 remaining water was frozen at  $-80^{\circ}\text{C}$ . The frozen samples were brought to dryness by

1 freeze-drying to give 5 – 20 mg of final products. To confirm the structures of the products,  
2 the residual components in the freeze-dried sample were reconstituted in chloroform for <sup>1</sup>H-  
3 NMR analysis and in water/methanol mixture (50/50%) for LC/MS analysis. <sup>1</sup>H-NMR  
4 measurements were performed on a Bruker ASX-400 spectrometer which were carried out  
5 at 297K with samples of typically 2 mg dissolved in 0.7 mL chloroform. LC/MS  
6 measurements were performed using reverse-phase liquid chromatography (Hewlett-  
7 Packard, Waldbronn, Germany) coupled to a triple quadrupole mass spectrometer (TQS  
8 Quantum Discovery, Thermo Finnigan, San Jose, CA, USA) which was operated in a  
9 negative ion mode electrospray. Ionization efficiency was increased by post-column  
10 addition of a 2.5% ammonia solution at 10 mL/min using a micro-HPLC pump. Nitrogen  
11 was used as drying and nebulizer gas. Separation was achieved with a Nucleosil 100-5 C18  
12 column (250 mm × 4 mm) which was eluted with a 50/50% of water/acetonitrile mixture at  
13 a flow rate of 0.2 mL/min. The <sup>1</sup>H-NMR and LC/MS spectral data for EE2 and chlorinated  
14 products of EE2 are summarized in Table S1. In the <sup>1</sup>H-NMR spectra, the regions of  
15 aromatic protons were used to confirm the structures of 2-Cl EE2 and 4-Cl EE2,  
16 respectively. In the LC/MS spectra, a parent peak at a mass number corresponding to M<sup>+</sup>  
17 and peaks of characteristic chlorine isotopes were used to confirm the products.

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1 Table S1. <sup>1</sup>H-NMR and LC/MS data for EE2 and chlorinated products of EE2

Compound	<sup>1</sup> H-NMR (CHCl <sub>3</sub> )			LC/MS
	H-1	H-2	H-4	
EE2	7.16 (d, 8.5) <sup>a</sup>	6.63 (dd, 2.8, 8.5)	6.56 (d, 2.8)	295 (M <sup>+</sup> ) <sup>b</sup>
2-Cl EE2	7.21 (s)		6.73 (s)	329, 331 (M <sup>+</sup> )
4-Cl EE2	7.16 (d, 8.5)	6.86 (d, 8.5)		329, 331 (M <sup>+</sup> )
2,4-diCl EE2				363, 365, 367 (M <sup>+</sup> )

2 <sup>a</sup>Chemical shifts are given in δ values. The parenthesis shows the multiplicity (s = singlet, d  
3 = doublet, dd = doublet of doublets) and *J* values (Hz), <sup>b</sup>Major molecular ion.

4  
5 *Preparation and quantification of oxidants.*

6 Stock solutions of chlorine (1–20 mM) were prepared by diluting a commercial solution  
7 of sodium hypochlorite (10% active chlorine, Riedel-deHaen, Germany) and were  
8 standardized by the measurement of the absorbance of OCl<sup>-</sup> at 292 nm ( $\epsilon = 350 \text{ M}^{-1} \text{ cm}^{-1}$ )  
9 (S14). Chlorine concentrations during some kinetic experiments were measured by the  
10 formation of I<sub>3</sub><sup>-</sup> ( $\epsilon$  at 353nm = 26400 M<sup>-1</sup> cm<sup>-1</sup>) after addition of excess iodide at pH 3  
11 (S14). Stock solutions of bromine (~0.75 mM) were prepared by addition of 1 mM ozone to  
12 a 0.8 mM solution of bromide at pH 4 (10 mM phosphate) (S15). Stock solutions of  
13 hypobromite were standardized by the measurements of the absorbance of OBr<sup>-</sup> at 329 nm  
14 ( $\epsilon = 332 \text{ M}^{-1} \text{ cm}^{-1}$ ) (S16). Ozone was produced with a Fischer 502 ozone generator by  
15 using pure oxygen as feed gas. Ozone stock solutions (~1.5 mM) were produced by  
16 sparging ozone-containing oxygen through Milli-Q water that was cooled in an ice bath and  
17 were standardized spectrophotometrically at 260 nm ( $\epsilon = 3290 \text{ M}^{-1} \text{ cm}^{-1}$ ) (S17). OH  
18 radicals were *in-situ* generated by photolysis of hydrogen peroxide (1 mM) at 254nm.

1 Irradiations were performed in quartz tubes using a merry-go-round photoreactor (DEMA  
2 125, Hans Mangels GmbH, Bornheim-Roisdorf, Germany) equipped with a low-pressure  
3 mercury lamp (Heraeus Noblelight, TNN 15/32) driven at a power of 15 W. Fluence and  
4 fluence rate were determined by chemical actinometry at low optical density using 5  $\mu\text{M}$   
5 aqueous atrazine as an actinometer described by Meunier, *et al* (S18). Direct UV  
6 transformation of EE2 was found to be negligible in the applied experimental conditions  
7 (below 5 %). Chlorine dioxide ( $\sim 7$  mM) was produced by mixing potassium  
8 peroxodisulfate ( $\text{K}_2\text{S}_2\text{O}_8$ ) with sodium chlorite ( $\text{NaClO}_2$ ) according to a method described  
9 by Gates (S19). Stock solutions of chlorine dioxide were standardized  
10 spectrophotometrically based on the molar absorptivity of  $\text{ClO}_2$ ,  $\epsilon = 1230 \text{ M}^{-1} \text{ cm}^{-1}$  at 359  
11 nm (S20). Potassium ferrate of high purity ( $\sim 90\%$ ) was prepared by the method of  
12 Thompson *et al* (S21). Stock solutions of ferrate (0.2–1.5 mM) were freshly prepared by  
13 dissolving solid samples of potassium ferrate ( $\text{K}_2\text{FeO}_4$ ) in Milli-Q water (pH  $\approx 9.2$ ) and  
14 standardized by the measurements of the absorbance of Fe(VI) at 510 nm ( $\epsilon = 1150 \text{ M}^{-1}$   
15  $\text{cm}^{-1}$ ) (S22). Stock solutions of monochloramine ( $\text{NH}_2\text{Cl}$ , 10 mM) were freshly prepared by  
16 mixing solutions of hypochlorite (20 mM) and ammonia (30 mM), which both adjusted  
17 with pH 8.6 with  $\text{H}_2\text{SO}_4$  in a T-mixer (double-two-jet tangential mixing system with a flow  
18 rate of 30 mL/min) to prevent any local excess of  $\text{OCl}^-$  (S14). Monochloramine was  
19 standardized spectrophotometrically based on the molar absorptivity of  $\text{NH}_2\text{Cl}$ ,  $\epsilon = 461 \text{ M}^{-1}$   
20  $\text{cm}^{-1}$  at 243 nm (S14).

21

22 **Text S3. Recombinant yeast estrogen screen (YES)**

1 For quantitative measurements of estrogenic activity, the yeast estrogen screen (YES)  
2 was selected because it has the requisite combination of robustness and estrogenic  
3 selectivity and sensitivity (*S23*, *S24*). This bioassay uses a genetically modified strain of  
4 yeast with an incorporated gene for the human estrogen  $\alpha$ -receptor (hER) and an expression  
5 plasmid containing the hERE (estrogen response element) and a reporter gene to quantify  
6 the activity. hER can form complexes with estrogenic compounds. Upon binding an active  
7 estrogen, the hER binds to the ERE which triggers the expression of the reporter gene lac-Z.  
8 Lac-Z in turn induces the production of the enzyme  $\beta$ -galactosidase followed by its  
9 excretion into the yeast growth medium. The amount of the formed enzyme can be assessed  
10 indirectly by adding a yellow substrate, chlorophenol red- $\beta$ -D-galactopyranoside, which is  
11 cleaved by  $\beta$ -galactosidase, yielding a red end product. The intensity of the color change in  
12 the yeast suspension is a quantitative estimate of the estrogenic activity of the added  
13 compound.

14 All samples for YES were prepared in ethanol. Based on the samples prepared in ethanol,  
15 series of 6–12 dilutions (1 : 1 or 1 : 0.75) were prepared in ethanol to obtain concentration-  
16 response curves in the YES. Of each dilution, 20  $\mu$ L was added to a 96-well microtiter plate.  
17 Besides the samples, each plate contained a standard curve with EE2 in ethanol (final EE2  
18 concentrations dissolved in assay medium were between  $2.5 \times 10^{-9}$  and  $9.77 \times 10^{-12}$  M) and  
19 a row of blanks for ethanol and assay medium itself, respectively. After ethanol was  
20 evaporated to dryness, the assay medium containing yeast cells (200  $\mu$ L of  $9.4 \times 10^8$  yeast  
21 cells/L) was added in each well. The color development (540 nm) was measured after  
22 incubating the microtiter plate for 72 h at 30°C. Inhibition of yeast cell growth was

1 regarded as an acute toxic effect of a tested sample or compound and this was observed as a  
2 reduction in absorbance at 620 nm compared to reference wells (S24). In all tested samples,  
3 yeast cell growth was not impaired. In most cases, measurements of estrogenic activity for  
4 each sample were carried out in three or four replicates. In the case of ozonated samples,  
5 measurements were performed once for each sample.

6

#### 7 **Text S4. Estrogenic activity of transformation mixtures of EE2: EE2 vs. EEEQ**

8 *Oxidation of EE2.* The reaction solutions consisted of Milli-Q purified water spiked with  
9 10  $\mu\text{M}$  EE2 were buffered to pH 8 with 5 mM phosphate buffer. For ozonation, the  
10 solutions contained 5 mM *tert*-butanol additionally as an OH radical scavenger to suppress  
11 oxidation of EE2 by OH radicals. To a series of identical reaction solution (25 mL with 10  
12  $\mu\text{M}$  EE2), stock solutions of each oxidant were added under vigorous stirring to obtain  
13 oxidant doses ranging from 2 to 40  $\mu\text{M}$ . After 1–2 days at room temperature, 1 mL of the  
14 reaction solution was taken for determination of the EE2 concentration by HPLC/UV-  
15 Fluorescence system. Another 4 mL of the reaction solution was taken for solid-phase  
16 extraction and subsequent estrogenic activity measurements. In the case of OH radicals  
17 which are generated *in-situ*, a series of reaction solutions (20 mL) with 10  $\mu\text{M}$  EE2 and 1  
18 mM hydrogen peroxide was irradiated at 254nm ( $\text{H}_2\text{O}_2 + h\nu \rightarrow 2\bullet\text{OH}$ ) for different time  
19 periods ranging from 10 to 300 sec, which correspond to UV fluence ranging from 10 to  
20 400  $\text{mJ}/\text{cm}^2$ . Immediately after the irradiation, 4 mL of the reaction solution was applied to  
21 the solid phase extraction. EE2 concentration in the irradiated solutions was also measured

1 within several hours by the HPLC/UV-Fluorescence system. Direct UV transformation of  
2 EE2 was found to be negligible in the applied experimental conditions (below 5 %).

3 *Determination of EE2.* EE2 concentrations were determined with a Agilent 1100 series  
4 HPLC system equipped with a Nucleosil 100-5 C18 column (125 mm × 4 mm) and a UV  
5 (diode array) or fluorescence detector (HPLC/UV-Fluorescence system). The mobile phase  
6 consisted of 50% 10 mM phosphoric acid and 50% acetonitrile at a flow rate of 0.6 mL/min.

7 *Solid-Phase Extraction (SPE).* Solid phase extractions were performed to transfer EE2  
8 and transformation products of EE2 from water to ethanol phase for subsequent estrogenic  
9 activity measurements by the yeast estrogen screen (YES). Direct use of the (diluted) water  
10 sample in the YES were avoided because interferences were sometimes found in the  
11 estrogenic response presumably caused by salts (i.e. phosphate) or iron (in the case of  
12 ferrate) from the water samples. Before the extraction, the pH of the samples was adjusted  
13 to 3 by 1 M HCl. The samples were then enriched on a mixed solid phase (Merck, VWR,  
14 Dietikon, Switzerland) which is composed of 100 mg of LiChrolut<sup>®</sup> EN  
15 (ethylvinylbenzene-divinylbenzene copolymer) in the bottom and 200 mg of LiChrolut<sup>®</sup>  
16 RP-18 in the top (S25). LiChrolut<sup>®</sup> EN is suitable to extract polar organic compounds from  
17 water (S26, S27). Due to its large specific surface (1200 m<sup>2</sup>/g according to BET) the  
18 adsorption capacity for polar organic substances is excellent, e.g. 500 mg caffeine/g sorbent,  
19 where caffeine is a model substance for polar substances ( $\log K_{ow, \text{caffeine}} = -0.07$ ) (S27).  
20 Thus even very polar transformation products will not be lost. The enriched cartridges were  
21 eluted four times with 1 mL acetone. These extracts were dried down in a gentle nitrogen  
22 stream and the final extract was reconstituted in 2 – 4 mL of ethanol and stored at –20 °C.

1 To confirm whether initial transformation products of EE2 are well extracted by the SPE  
2 (LiChrolut<sup>®</sup> EN/RP-18), 10 μM of EE2 were treated at pH 8 with different doses of  
3 chlorine dioxide (6, 12, 18, 24, 30, and 38 μM) or ferrate (5, 10, 20, 30, and 40 μM). The  
4 HPLC/UV chromatograms were then compared for the treated samples before and after the  
5 SPE. Figures S4 and S5 show the HPLC/UV chromatograms of EE2 (10 μM) treated with  
6 16 μM of chlorine dioxide (Figure S4) and 20 μM of ferrate (Figure S5), respectively, as  
7 representative data. In both cases, more than 7 different peaks were identified as  
8 transformation products of EE2 in the applied oxidant doses. Generally, the HPLC/UV  
9 chromatograms of samples after the SPE were nearly the same as those before the SPE  
10 except that the signals after the SPE were higher than those before SPE due to the  
11 enrichment during the SPE (a factor of 2). The recoveries of the EE2 and its transformation  
12 products were calculated by using eq 2 for the 8 peaks in the case of chlorine dioxide and  
13 for the 9 peaks in the case of ferrate (Table S2). Recoveries were at least 83%, indicating  
14 that EE2 and its initial transformation products from reaction with chlorine dioxide and  
15 ferrate are successfully retained during the SPE with the LiChrolut<sup>®</sup> EN/RP-18.

16

$$17 \quad \text{recovery (\%)} = \frac{\text{peak area after SPE}}{\text{peak area before SPE}} \times \frac{\text{sample volume after SPE}}{\text{sample volume before SPE}} \quad (2)$$

18

19 Initial transformation products of EE2 by ozone or hydroxyl radical are expected to be  
20 well extracted by the LiChrolut<sup>®</sup> EN/RP-18 because they are structurally close to or  
21 sometimes the same with the initial transformation products of EE2 by chlorine dioxide or

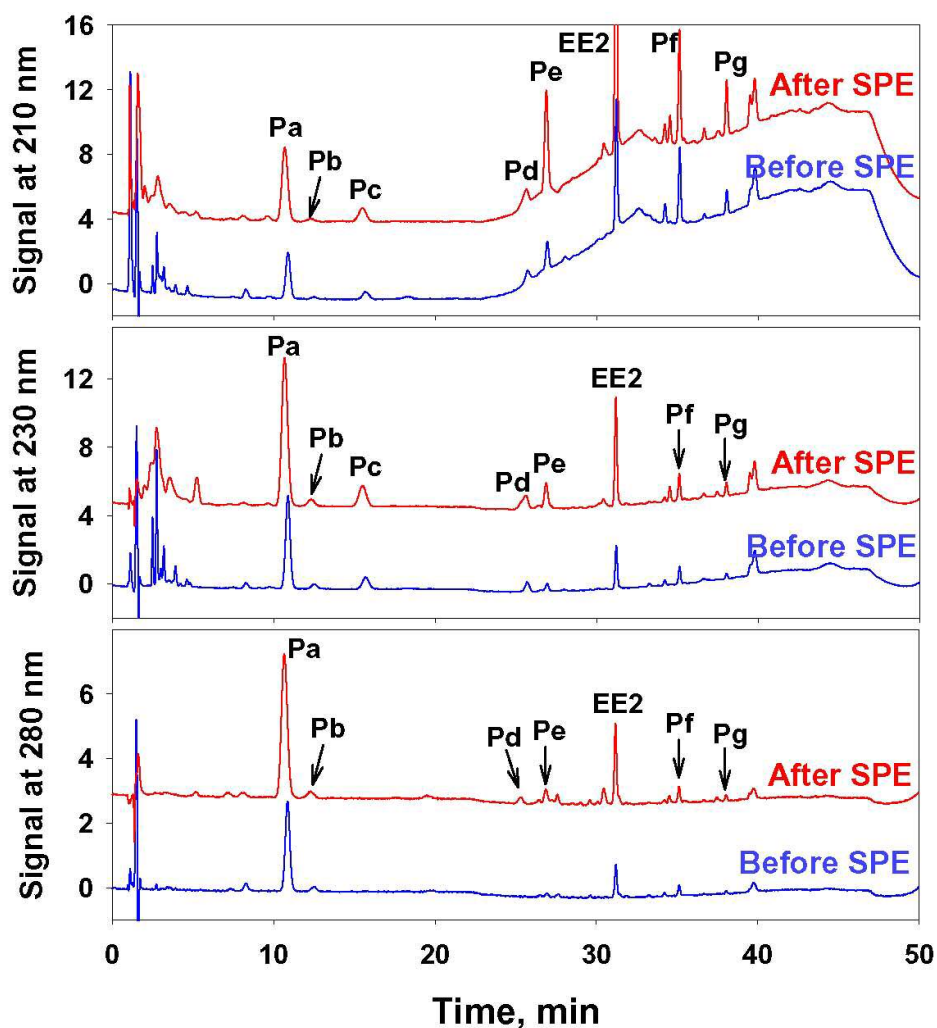
1 ferrate (see the main text). In the case of ozone, Huber, *et al* (S28) applied a freeze-drying  
 2 (lyophilization) method to transfer EE2 and transformation products of EE2 from water to  
 3 ethanol phase for estrogenic activity measurements by the YES. They obtained the same  
 4 result as in the current study. The reduction of the relative estrogenic activity was linearly  
 5 proportional to the decrease of EE2 concentration during sub-stoichiometric oxidation of 10  
 6  $\mu\text{M}$  EE2. Initial transformation products of EE2 by chlorine or bromine can also be well  
 7 extracted because halogenation of EE2 generally produces more hydrophobic products than  
 8 EE2.

9  
 10 Table S2. Recovery of EE2 and its transformation products in the solid phase extraction  
 11 (LiChrolut<sup>®</sup> EN/RP-18).

Chlorine dioxide				Ferrate			
Compound	RT (min) <sup>a</sup>	Recovery (%) <sup>b</sup>	n <sup>c</sup>	Compound	RT (min) <sup>a</sup>	Recovery (%) <sup>b</sup>	n <sup>c</sup>
Pa	10.75 ± 0.10	97 ± 1	5	P1	7.70 ± 0.08	109 ± 15	3
Pb	12.39 ± 0.11	93 ± 7	3	P2	9.15 ± 0.08	100 ± 3	4
Pc	15.58 ± 0.11	97 ± 9	6	P3	9.95 ± 0.07	103 ± 4	4
Pd	25.65 ± 0.04	83 ± 29	4	P4	10.77 ± 0.08	108 ± 7	4
Pe	26.89 ± 0.03	218 ± 28	2	P5	26.85 ± 0.01	98 ± 5	3
EE2	31.20 ± 0.01	100 ± 3	6	P6	27.61 ± 0.02	86 ± 34	4
Pf	35.13 ± 0.01	84 ± 22	3	P7	29.00 ± 0.01	116 ± 21	3
Pg	38.04 ± 0.01	114 ± 3	3	P8	30.42 ± 0.01	206 ± 41	3
				EE2	31.19 ± 0.01	100 ± 3	4

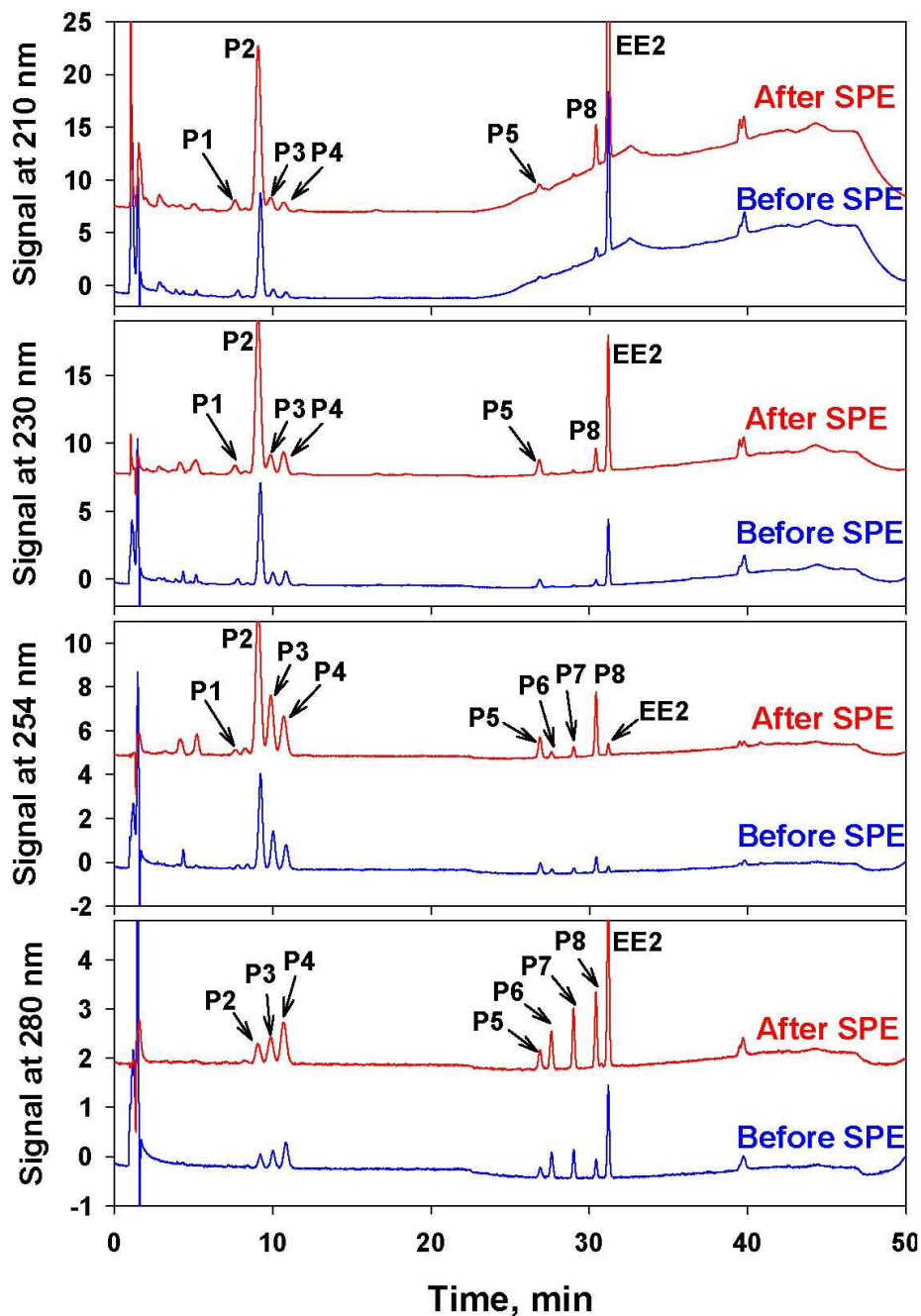
12 <sup>a</sup>RT = retention time in the HPLC/UV chromatograms, <sup>b</sup>calculated by the eq 1, <sup>c</sup>n = number  
 13 of extraction.

14  
 15



1

2 Figure S4. HPLC/UV chromatograms of EE2 samples treated with chlorine dioxide before  
 3 (blue lines) and after the solid phase extraction (red lines). Samples were concentrated by a  
 4 factor of 2 after the SPE. Oxidation conditions:  $[EE2]_0 = 10 \mu M$ ,  $[ClO_2]_0 = 16 \mu M$ , and  $pH$   
 5  $= 8$  (5 mM phosphate), HPLC conditions: flow rate = 1 mL/min, gradient program = 0 – 20  
 6 min with 25 %  $CH_3CN$  (AC) and 75 % 10 mM  $H_3PO_4$  in water ( $H_2O$ ), 20 – 40 min with  
 7 25→70 % AC and 75→30 %  $H_2O$ , 40 – 45 min with 70 % AC and 30 %  $H_2O$ , 45 – 48 min  
 8 with 70→25 % AC and 30→75 %  $H_2O$ , and 48 – 50 min with 25 % AC and 75 %  $H_2O$ .



1

2 Figure S5. HPLC/UV chromatograms of EE2 samples treated with ferrate before (blue  
 3 lines) and after the solid phase extraction (red lines). Samples were concentrated by a factor  
 4 of 2 after SPE. Oxidation conditions:  $[EE2]_0 = 10 \mu\text{M}$ ,  $[Fe(VI)]_0 = 20 \mu\text{M}$ , and  $\text{pH} = 8$  (5  
 5 mM phosphate), HPLC conditions are the same as those in Figure S4.

1 **Text S5. Evaluation of the concentration-effect curves.**

2 The EE2 concentration in the final extracts was measured to obtain enrichment factors.  
3 The enrichment factor in the SPE (enrichment factor<sub>SPE</sub>) was calculated by eq 3, where  
4 [EE2]<sub>sample</sub> refers to the EE2 concentration in the water samples undergoing SPE, and  
5 [EE2]<sub>extract</sub> refers to the EE2 concentration in the final extracts.

6

7 
$$\text{enrichment factor}_{\text{SPE}} = \frac{[\text{EE2}]_{\text{extract}}}{[\text{EE2}]_{\text{sample}}} \quad (3)$$

8

9 In the YES, the extracts were diluted according to eq 4.

10

11 
$$\text{dilution factor}_{\text{YES}} = \frac{\text{volume of extract added to YES}}{\text{total volume of YES}} \quad (4)$$

12

13 The final relative concentration factor (RCF) is calculated by multiplying the enrichment  
14 factor in the SPE with the dilution factor in the YES (eq 5).

15

16 
$$\text{RCF} = \text{enrichment factor}_{\text{SPE}} \times \text{dilution factor}_{\text{YES}} \quad (5)$$

17

18 The concentration-effect curve of each sample was fitted to a symmetric logistic function  
19 (eq 6) using the software Prism (GraphPad, San Diego, CA, USA) by optimizing the  
20 relative concentration factor (RCF) causing a 50% effect (EC<sub>50</sub>).

21

1 
$$\text{Effect (\%)} = \frac{100\%}{1 + 10^{(\log EC_{50} - \log RCF)m}} \quad (6)$$

2

3 The slope of eq 6 (m) was fixed to the slope of EE2 as a standard (ref. 24 for explanations  
4 and procedures in detail), and the estrogenic activity of samples was reported as  
5 ethinylestradiol equivalent concentration, EEEQ (eq 7).

6

7 
$$\text{EEEQ} = \frac{EC_{50}(\text{EE2})}{EC_{50}} \quad (7)$$

8

9 Figure S6 shows the change of concentration-effect curves of 10  $\mu\text{M}$  EE2 in the YES as a  
10 function of different chlorine doses (0–28  $\mu\text{M}$ ), as a representative example. With  
11 increasing chlorine doses, the concentration-effect curve shifts to right, indicating the  
12 reduction of estrogenic activity. From the fitting of the curves with eq 6 and using eq 7,  
13  $EC_{50}$  and EEEQ were obtained, respectively.

14  $EC_{50}$  of each structural derivatives (including initial transformation products of EE2)  
15 were determined by fitting the concentration-effect curve (eq 6) while optimizing the actual  
16 concentration of the compound in the final YES medium (instead of RCF) causing a 50%  
17 effect ( $EC_{50}$ ). Figure S7 shows the concentration-effect curves of selected compounds.

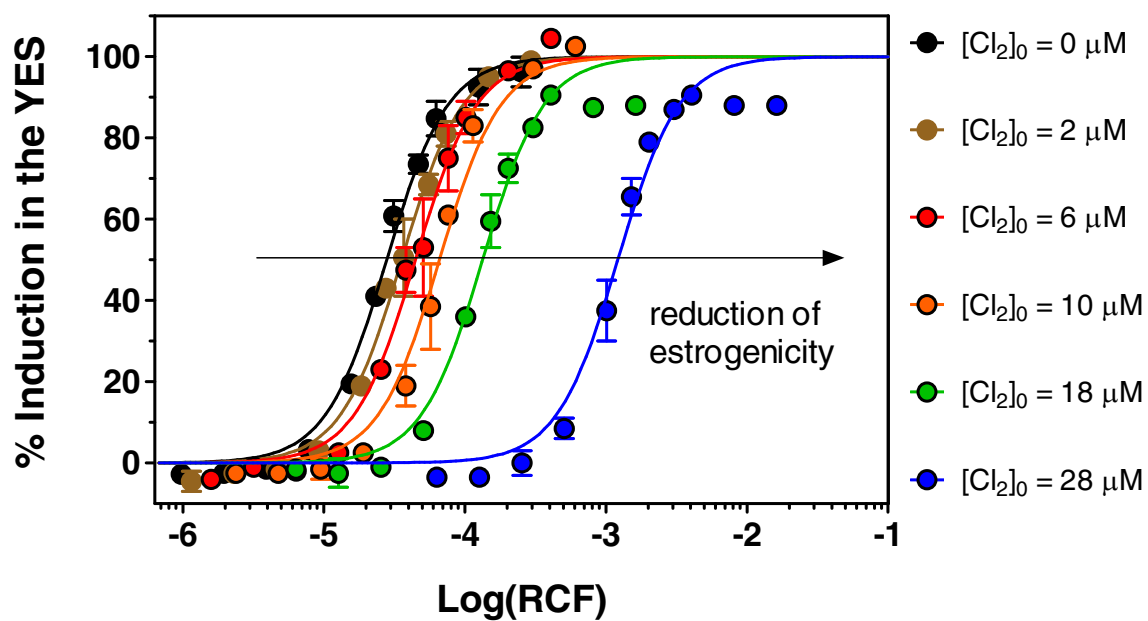
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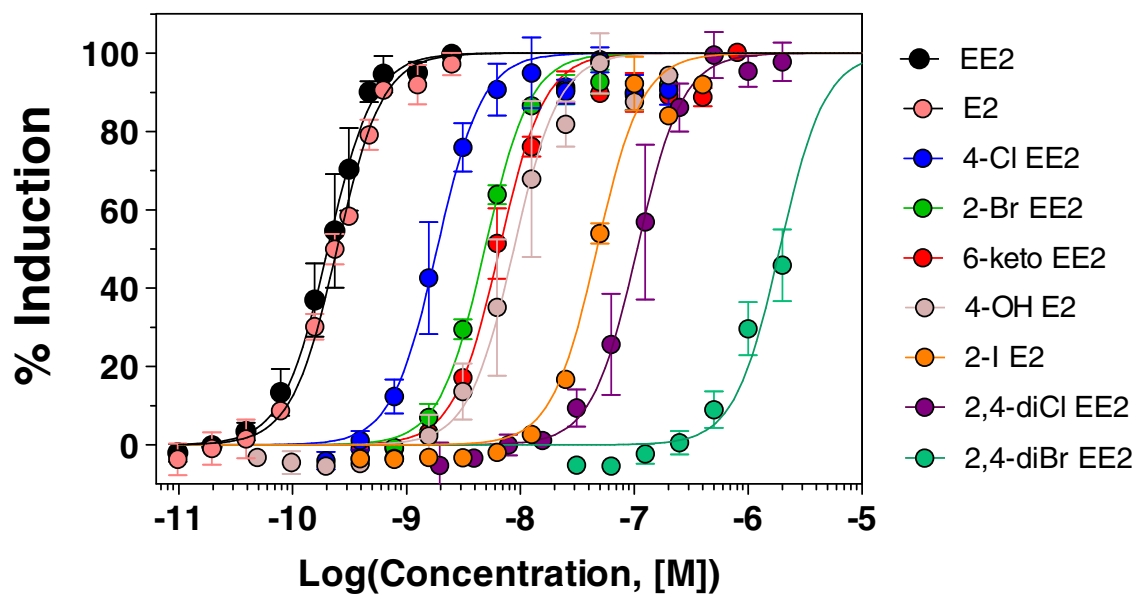
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Figure S6. Changes of the concentration-effect curves in the YES for the EE2 samples treated by different amounts of chlorine. [EE2]<sub>0</sub> = 10 μM, [Cl<sub>2</sub>]<sub>0</sub> = 0–28 μM, pH = 8.0 (5 mM phosphate buffer), T = 23 ± 1 °C, RCF = Relative concentration factor.

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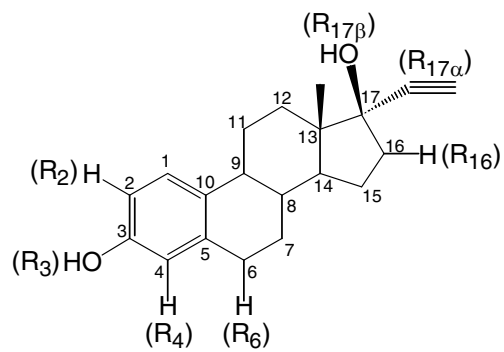
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Figure S7. Concentration-effect curves of selected structural derivatives of EE2 in the YES.

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	R <sub>2</sub>	R <sub>3</sub>	R <sub>4</sub>	R <sub>6</sub>	R <sub>16</sub>	R <sub>17α</sub>	R <sub>17β</sub>
EE2							
E2						H	
E1						H	=O
E3					OH	H	
2-Cl EE2	Cl						
4-Cl EE2			Cl				
2,4-diCl EE2	Cl		Cl				
2-Br EE2	Br						
4-Br EE2			Br				
2,4-diBr EE2	Br		Br				
2-OH E2	OH					H	
4-OH E2			OH			H	
6-oxo EE2				=O			
Mestranol		OCH <sub>3</sub>					
2-F E2	F					H	
2-Br E2	Br					H	
2-I E2	I					H	
2-methoxy E2	OCH <sub>3</sub>					H	

4

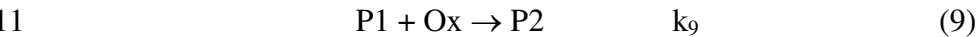
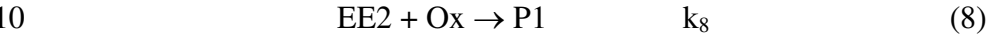
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Figure S8. Structural-derivatives of EE2 tested for relative estrogenic activity.

6

1 **Text S6. Modeling of the change of estrogenic activity during oxidative treatment of**  
2 **an estrogenic compound: kinetics of the transformation of the initial products.** To  
3 understand the correlation between transformation of an estrogenic compound (i.e. EE2)  
4 and removal of its estrogenic activity during oxidative treatment of EE2, a kinetic model  
5 was formulated. In this kinetic model, EE2 is transformed to P1, a primary product, with a  
6 second-order rate constant of  $k_8$  (reaction 8). P1 is further transformed to P2, a final product,  
7 with a second-order rate constant of  $k_9$  (reaction 9). Two moles of oxidant (Ox) is  
8 consumed to transform one mole of EE2 to P2.

9



12

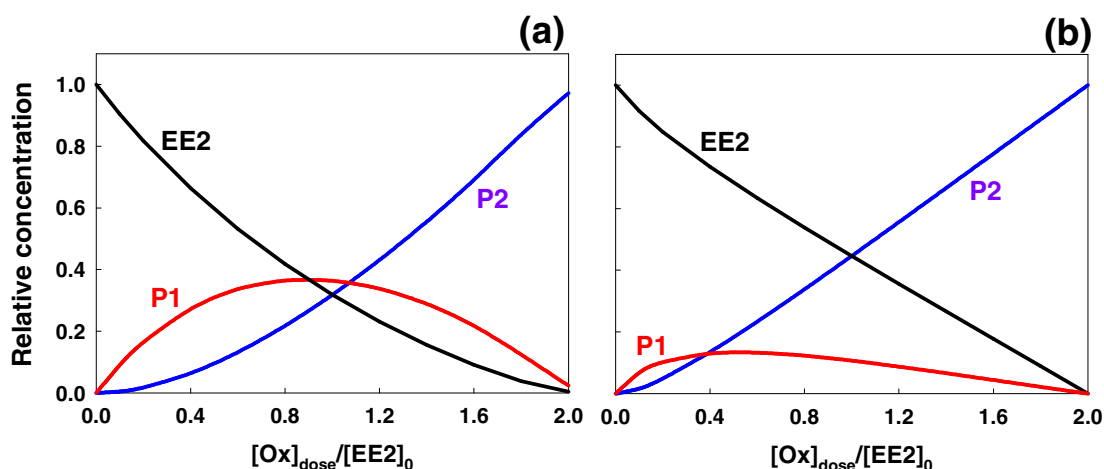
13 Based on this kinetic model, the variation of concentrations of EE2, P1, and P2 as a  
14 function of oxidant doses ( $0 \leq [\text{Ox}]_{\text{dose}}/[\text{EE2}]_0 \leq 2$ ) was simulated using REACT, a kinetic  
15 simulation program. Figure S9 shows the simulation results when two different scenarios  
16 for the kinetic condition of the reactions 8 and 9 ( $k_8$  vs.  $k_9$ ) were applied. As it is expected,  
17 a lower accumulation of P1 is observed when  $5k_8 = k_9$  ( $[\text{P1}]/[\text{EE2}]_0 \leq 0.14$ ) than when  $k_8 =$   
18  $k_9$  ( $[\text{P1}]/[\text{EE2}]_0 \leq 0.37$ ).

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1

2 Figure S9. Simulated variation of concentrations of EE2 and its oxidation products, P1 and  
 3 P2, during oxidative transformation of EE2. Conditions for the kinetic runs: (a)  $k_8 = k_9$ , (b)  
 4  $5k_8 = k_9$ . See the main text for further explanation.

5

6 As a next step, three different scenarios for the relative estrogenic potencies of P1 and P2  
 7 ( $RP_{P1}$  and  $RP_{P2}$ ) compared to EE2 were applied to the two different kinetic simulation  
 8 results. In the first scenario, P1 and P2 have the same potency as the parent compound EE2  
 9 ( $RP_{P1} = RP_{P2} = 1$ ). In the second scenario, P1 has the same potency as EE2 but P2 has ten  
 10 times lower potency than EE2 ( $RP_{P1} = 1$ ,  $RP_{P2} = 0.1$ ). In the third scenario, P1 and P2 have  
 11 ten and a hundred times lower potency than EE2, respectively ( $RP_{P1} = 0.1$ ,  $RP_{P2} = 0.01$ ). To  
 12 determine the estrogenic activity of the sample, which is expressed in EE2 equivalents  
 13 (EEEQs), the  $RP_i$ s were multiplied with the sample concentration of the corresponding  
 14 chemical ( $c_i$ ). The obtained EEEQ<sub>i</sub>s for the single substances were then added to get the  
 15 total EEEQ of the sample (eq 10). The individual EEEQ<sub>i</sub>s can be added because estrogenic  
 16 compounds act according to the same mechanism and thus obey the ‘concentration addition

1 model' of mixture toxicity, as has been demonstrated by various studies covering *in vitro*  
2 and *in vivo* effects (S24 and references therein).

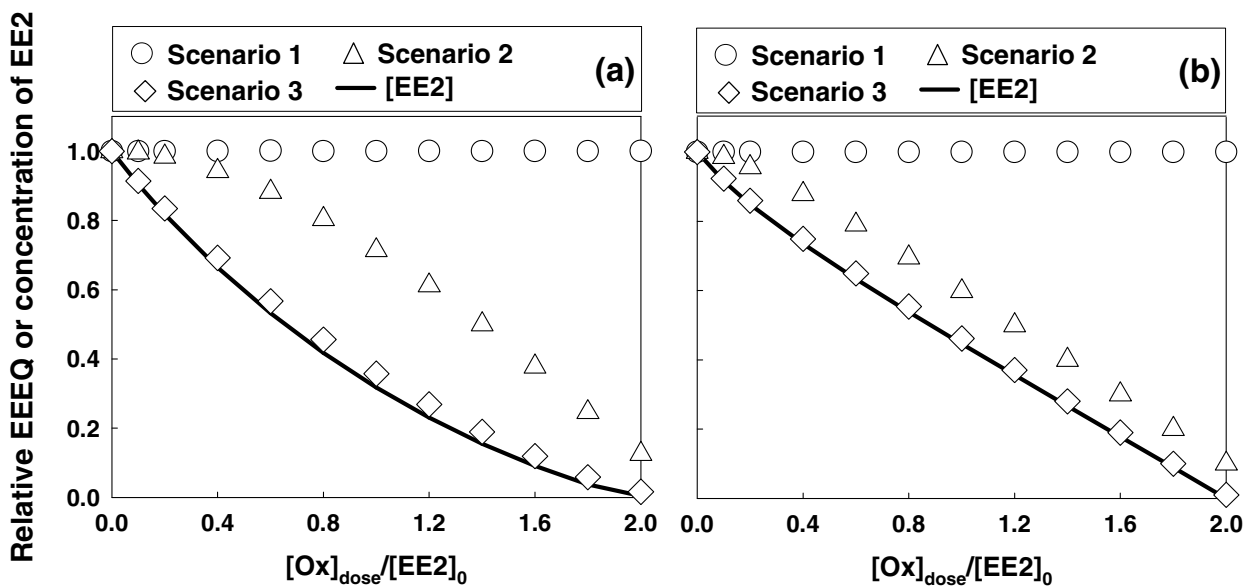
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$$4 \quad \text{EEEQ} = \sum_i \text{RP}_i \cdot c_i = \sum_i \text{EEEQ}_i \quad (10)$$

5

6 Figure S10 shows the simulation results for the decrease of the EE2 concentration and the  
7 decrease of EEEQ at three different scenarios for the relative potency ( $\text{RP}_{\text{P1}}$  vs.  $\text{RP}_{\text{P2}}$ )  
8 which are combined with two different scenarios for the kinetics, (a)  $k_8 = k_9$ , and (b)  $5k_8 =$   
9  $k_9$ . As expected, in the first scenario ( $\text{RP}_{\text{P1}} = \text{RP}_{\text{P2}} = 1$ ), there is no decrease of the relative  
10 EEEQ. In the second scenario, the decrease of the relative EEEQ is slower than the  
11 decrease of the relative EE2 concentration. In the third scenario, the decrease of the relative  
12 EEEQ is directly proportional to the decrease of the relative EE2 concentration under both  
13 kinetic conditions. The difference between the relative EEEQ and the relative EE2  
14 concentration from the kinetic condition of  $5k_8 = k_9$  is smaller than that of  $k_8 = k_9$ . This is  
15 because of a faster further transformation of P1 that has the same estrogenic activity as EE2.  
16 Overall, this kinetic modeling illustrates that the difference between the relative EEEQ and  
17 the EE2 concentration during transformation of EE2 is determined by two factors: 1)  
18 relative potencies (RPs) and 2) kinetics of further transformation of initial transformation  
19 products.

20



1  
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3 Figure S10. Simulated decrease of the relative EEEQ (symbols) and the relative EE2  
4 concentration (lines) during oxidative treatment of EE2. Conditions for the kinetics: (a)  $k_8$   
5 =  $k_9$ , (b)  $5k_8 = k_9$ ; conditions for the relative potency (RP): scenario 1:  $RP_{P1} = RP_{P2} = 1$ ,  
6 scenario 2:  $RP_{P1} = 1$  and  $RP_{P2} = 0.1$ , and scenario 3:  $RP_{P1} = 0.1$  and  $RP_{P2} = 0.01$ .

7

### 8 Text S7. $\Delta MR$

9  $MR$  (molar refractivity) is defined as  $MR = 0.1[(n^2 - 1)/(n^2 + 2)] (MW/d)$  where  $n$  is the  
10 refractive index,  $MW$  is the molecular weight, and  $d$  is the density of a compound. Since  
11 there is rather little variation in  $n$ ,  $MR$  is largely a measure of volume with a small  
12 correction for polarizability (S29). In addition,  $\Delta MR$ , which is defined as  $\Delta MR =$   
13  $MR_{\text{substituent}} - MR_{\text{hydrogen}}$ , was used instead of  $MR$  in this study to set EE2 or E2 as a  
14 reference compound ( $\Delta MR_{\text{EE2 or E2}} = 0$ ).

15

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