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2 **Assessing the mixture effects in *in vitro* bioassays of chemicals occurring in small**  
3 **agricultural streams during rain events**

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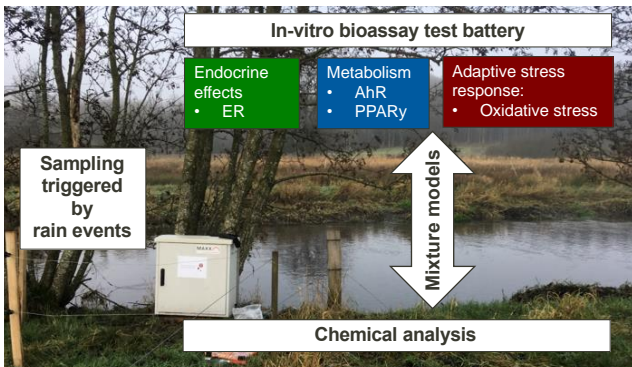
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20 **TOC Art**

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24 **Abstract.** Rain events may impact the chemical pollution burden in rivers. Forty-four small streams  
25 in Germany were profiled during several rain events for the presence of 395 chemicals and five types  
26 of mixture effects in *in vitro* bioassays (cytotoxicity, activation of the estrogen, aryl hydrocarbon and  
27 peroxisome proliferator-activated receptors and oxidative stress response). While these streams were  
28 selected to cover a wide range of agricultural impacts, in addition to the expected pesticides,  
29 wastewater-derived chemicals and chemicals typical for street run-off were detected. The  
30 unexpectedly high estrogenic effects in many samples indicated impact by wastewater or overflow of  
31 combined sewer systems. The 128 water samples exhibited a high diversity of chemical and effect  
32 patterns, even for different rain events at the same site. The detected 290 chemicals explained only a  
33 small fraction (<8 %) of the measured effects. The experimental effects of designed mixtures of  
34 detected chemicals that were expected to dominate the mixture effects of detected chemicals were  
35 consistent with predictions for concentration addition by a factor of two for 94 % of the mixtures.  
36 Overall, the burden of chemicals and effects were much higher than previously detected in surface  
37 water during dry weather with the effects often exceeding effect-based trigger values.

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39

## 40 **Introduction**

41 Surface waters can be impacted by a large number of organic micropollutants, including pesticides,  
42 pharmaceuticals and industrial compounds, which can enter the aquatic environment from both point  
43 sources, such as wastewater effluent discharge, and non-point sources, such as agricultural run-off.  
44 Small streams have large lotic biodiversity, but, in comparison to larger systems, can be  
45 disproportionately affected by chemical pollution due to smaller dilution ratios.<sup>1</sup> Pesticides from  
46 agricultural run-off reduced invertebrate biodiversity in streams in Australia and Europe<sup>2, 3</sup> and  
47 wastewater treatment plant (WWTP) effluents may also impact invertebrates.<sup>4</sup> Further, the ecological  
48 effects of pesticides on small streams generally increase after rainfall events due to run-off from  
49 agricultural areas.<sup>5</sup>

50 Several studies that have evaluated the risk posed by organic chemicals in small streams have focused  
51 on chemical analysis.<sup>6,7</sup> Targeted chemical analysis is traditionally applied to monitor chemical water  
52 quality, but lacks information on effects of non-target chemicals or chemicals at concentrations below  
53 analytical detection limits. Still, these may contribute to the overall effect. *In vitro* bioassays can be  
54 applied for water quality monitoring to detect the mixture effects of chemicals present in a sample.  
55 Combinations of *in vitro* bioassays and chemical analysis have been applied mainly to larger water  
56 bodies,<sup>8-11</sup> with fewer studies addressing smaller streams and mainly under low flow conditions in  
57 dry weather.<sup>12, 13</sup> In contrast, during rainfall events, concentrations of pesticides and their  
58 transformation products have been observed to peak in small rivers.<sup>14,15</sup> Given that substantial effects  
59 in *in vitro* assays have been observed in collected stormwater,<sup>16,17</sup> it is timely to ask the question how  
60 chemicals and their mixtures assessed by an *in vitro* test battery fare during rain events in small  
61 streams.

62 We assessed the chemical burden in small agricultural streams during rainfall events using a battery  
63 of *in vitro* bioassays to identify which mixture effects exceed acceptable levels and which types of  
64 chemicals are driving the observed mixture effects. Water extracts were collected from 44 sites  
65 throughout Germany, with multiple samples collected during different rain events at most sites. The  
66 studied bioassays covered different stages of cellular toxicity pathways, including induction of  
67 xenobiotic metabolism, hormone receptor-mediated effects and adaptive stress responses.  
68 Specifically, this included assays indicative of activation of the aryl hydrocarbon receptor (AhR),  
69 binding to the peroxisome proliferator-activated receptor gamma (PPAR $\gamma$ ), activation of the estrogen  
70 receptor (ER) and oxidative stress response. These bioassays were responsive in surface water and  
71 wastewater,<sup>10, 18, 19</sup> with the endpoints also identified as most the responsive and therefore priority  
72 endpoints for surface water using the multiplexed Attagene assays that cover 69 endpoints.<sup>18, 20, 21</sup>  
73 The effect in the water extracts were compared with bioassay specific effect-based trigger values

74 (EBTs) derived from Environmental Quality Standards (EQS) from the European Union Water  
75 Framework Directive (WFD).<sup>22</sup> In addition to bioanalysis, chemical analysis of 395 chemicals  
76 including pesticides, pharmaceuticals and industrial chemicals was undertaken.

77 Iceberg modelling using the bioanalytical equivalent concentration (BEQ) approach was applied in  
78 the current study to determine the contribution of detected chemicals to the observed effect.<sup>23</sup>  
79 Bioanalytical equivalent concentrations from bioanalysis ( $BEQ_{\text{bio,iceberg}}$ ) relates the effect of the  
80 sample to the effect induced by the assay reference compound, whereas bioanalytical equivalent  
81 concentrations from chemical analysis ( $BEQ_{\text{chem}}$ ) are determined based on the concentration of a  
82 chemical in a sample and its relative effect potency ( $REP_i$ ).  $BEQ_{\text{chem}}$  is similar to the toxic unit (TU)  
83 approach<sup>24, 25</sup> or exposure-activity ratio (EAR) approach,<sup>21</sup> and the different measures can be  
84 converted into each other.<sup>26</sup>

85 The BEQ concept is based on the assumption that the many chemicals in a mixture act in a  
86 concentration additive manner, which was appropriate to predict mixture toxicity in assays indicative  
87 of receptor-mediated effects, adaptive stress responses and cytotoxicity.<sup>17, 27, 28</sup> In the field, stress can  
88 exacerbate the mixture effects and lead to more-than additive effects,<sup>29</sup> but for large number of  
89 chemicals, as in our study, additive mixture models are considered as broadly applicable also in *in*  
90 *vivo* assays.<sup>30</sup>

91  $BEQ_{\text{bio,iceberg}}$  and  $BEQ_{\text{chem}}$  can be compared to determine how much of the effect is explained by  
92 detected chemicals. In previous studies only a small fraction of the sample's effect in assays indicative  
93 of xenobiotic metabolism and adaptive stress responses could be explained by the quantified  
94 chemicals.<sup>8, 10, 18, 31, 32</sup> This is likely due to the thousands of non-quantified chemicals expected to be  
95 present in water samples<sup>33</sup> that may trigger these bioassays. To further explore which and how  
96 chemicals contribute to the known effect (i.e., the "tip of the iceberg"),<sup>34</sup> more than 200 synthetic  
97 mixtures of detected chemicals were run in the bioassays indicative of activation of AhR, binding to  
98 PPAR $\gamma$  and oxidative stress response. In contrast, for hormonal effects, a small number of potent  
99 hormone receptor agonists can typically explain the majority of effects,<sup>35</sup> and therefore no synthetic  
100 mixtures were measured in the assay for the activation of ER.

101

## 102 **Materials and Methods**

103 ***Sampling and sample processing.*** 128 water samples were collected from 44 sites in eleven German  
104 states from April to September 2018 (Table S1 of the Supporting Information) using a modified  
105 sampling device based on the technology introduced by Schulze et al.<sup>36</sup> Rain events causing water  
106 levels to rise by at least 5 cm in the streams triggered sampling. Two different sampling devices were  
107 used. One autosampler (Maxx Maxx Meß- und Probenahmetechnik GmbH, Rangendingen,

108 Germany) collected forty subsamples of 50 mL over a time period of 3 hours 20 minutes during the  
109 rain event with each subsample collected every 5 min (duration of sampling approximately 45 sec).  
110 The other sampling device was also triggered by rising water levels and collected up to 1 L of water  
111 in one bottle as described by Liess and van der Ohe.<sup>37</sup> The combined water samples of each rain event  
112 yielded a volume of up to 1 L or 2 L (less if the sampling device clogged), which was enriched after  
113 filtration using solid-phase extraction (SPE) with HR-X sorbent<sup>38</sup> with SPE process blanks run in  
114 parallel. For details on sampling sites, sampling and sample processing, see SI, Section S1.

115

116 **Chemical analysis.** 395 compounds (Table S2) were analyzed by liquid chromatography coupled to  
117 high resolution mass spectrometry (LC-HRMS) by direct injection as described in Section S2.

118

119 **Bioanalysis.** The extracts were run in four bioassays, AhR CALUX, PPAR $\gamma$  GeneBLAzer, ER $\alpha$   
120 GeneBLAzer and AREc32 (see Table S4). All studied bioassays are mammalian reporter gene assays  
121 and were run in 384-well plates, with detailed methods provided in Neale et al.<sup>32</sup> and König et al.<sup>10</sup>  
122 In addition to the environmental extracts, individual chemicals found at high concentrations or  
123 expected to contribute to the effect were also run in the AhR CALUX (78 chemicals), PPAR $\gamma$   
124 GeneBLAzer (43 chemicals) and AREc32 (87 chemicals) assays (all fingerprinted chemicals listed  
125 in Table S5). For all assays, cell viability in the mammalian cell lines was assessed in parallel to  
126 induction based on cell confluency using an IncuCyte S3 live cell imaging system (Essen BioScience,  
127 Ann Arbor, Michigan, USA).<sup>19</sup> Any concentrations that reduced cell viability by 10% or more (i.e.,  
128 caused 10% or more cytotoxicity) were excluded from further data evaluation.

129

130 **Data evaluation.** Linear concentration-effect curves at effect levels up to 30% were used for data  
131 evaluation, with the concentration causing 10% effect (EC<sub>10</sub>) derived for AhR CALUX, PPAR $\gamma$   
132 GeneBLAzer and ER $\alpha$  GeneBLAzer and the concentration causing an induction ratio of 1.5 (EC<sub>IR1.5</sub>)  
133 determined for AREc32. The concentration causing 10% inhibition (IC<sub>10</sub>) was also evaluated using  
134 linear concentration-effect curves. Detailed information about the applied data evaluation approach  
135 is available in Escher et al.<sup>39</sup> The EC<sub>10</sub> and EC<sub>IR1.5</sub> values were expressed as a relative enrichment  
136 factor (REF) in units of  $L_{\text{water}}/L_{\text{bioassay}}$ , while the EC<sub>10</sub> and EC<sub>IR1.5</sub> values for the individual chemicals  
137 were given in molar units.

138

139 **Iceberg modelling.** Iceberg modelling using both the BEQ and TU approaches was applied in the  
140 current study to determine how much of the observed effect can be explained by quantified chemicals  
141 and how much is due to unknown chemicals (Figure 1). Sample EC values were converted to BEQ<sub>bio</sub>,

142  $iceberg$  using the EC value of the reference compound (Equation 1).  $BEQ_{chem}$  was calculated using  
 143 Equation 2 by summing the  $BEQ_i$  of each quantified and bioanalytically characterized chemical.  $BEQ_i$   
 144 is the product of the concentration of the detected chemical ( $C_i$ ) in molar units and its  $REP_i$ .  $REP_i$  was  
 145 calculated using Equation 3 using the EC value of the detected chemical  $i$  and the EC value of the  
 146 reference compound. Note that  $BEQ_{bio, iceberg}$  was based on the effect of SPE extracts, whereas  
 147  $BEQ_{chem}$  was calculated from  $C_i$  using direct injection into the LC-HRMS, which is acceptable  
 148 because generally good chemical recovery was observed previously for HR-X sorbent.<sup>23</sup> Hydrophilic  
 149 compounds are likely to be poorly recovered by the HR-X sorbent, but these chemicals were not  
 150 expected to contribute significantly to the observed mixture effect due to their typically much lower  
 151 potency (Table S5). The EC values for the detected chemicals were either measured as part of this  
 152 study or collected from the literature and the US EPA Tox21 database.<sup>40</sup> BEQ was expressed as  
 153 benzo[a]pyrene equivalent concentrations (B[a]P-EQ) for AhR CALUX, rosiglitazone-EQ for  
 154 PPAR $\gamma$  GeneBLAzer, 17 $\beta$ -estradiol equivalent concentrations (EEQ) for ER $\alpha$  GeneBLAzer and  
 155 dichlorvos-EQ for AREc32.

$$157 \quad BEQ_{bio,iceberg} = \frac{EC_y (ref)}{EC_y (sample)} \quad (1)$$

$$160 \quad BEQ_{chem} = \sum_{i=1}^n BEQ_i = \sum_{i=1}^n REP_i \cdot C_i \quad (2)$$

$$163 \quad REP_i = \frac{EC_y (ref)}{EC_y (i)} \quad (3)$$

167 The sample  $IC_{10}$  values were converted to  $TU_{cytotoxicity(bio, iceberg)}$  using Equation 4 based on Müller et  
 168 al.<sup>13</sup>  $TU$  based on chemical analysis ( $TU_{cytotoxicity(chem)}$ ) was calculated using the detected chemical  
 169 concentration and the  $IC_{10}$  value of the detected chemical  $i$  (Equation 5).  $IC_{10}$  values for analyzed  
 170 chemicals were measured in the current study or collected from the US EPA Tox21 database (Escher  
 171 et al. submitted). While not commonly applied for *in vitro* bioassays, TUs from chemical analysis are  
 172 often calculated for whole organisms, such as algae, daphnia and fish.<sup>25</sup>

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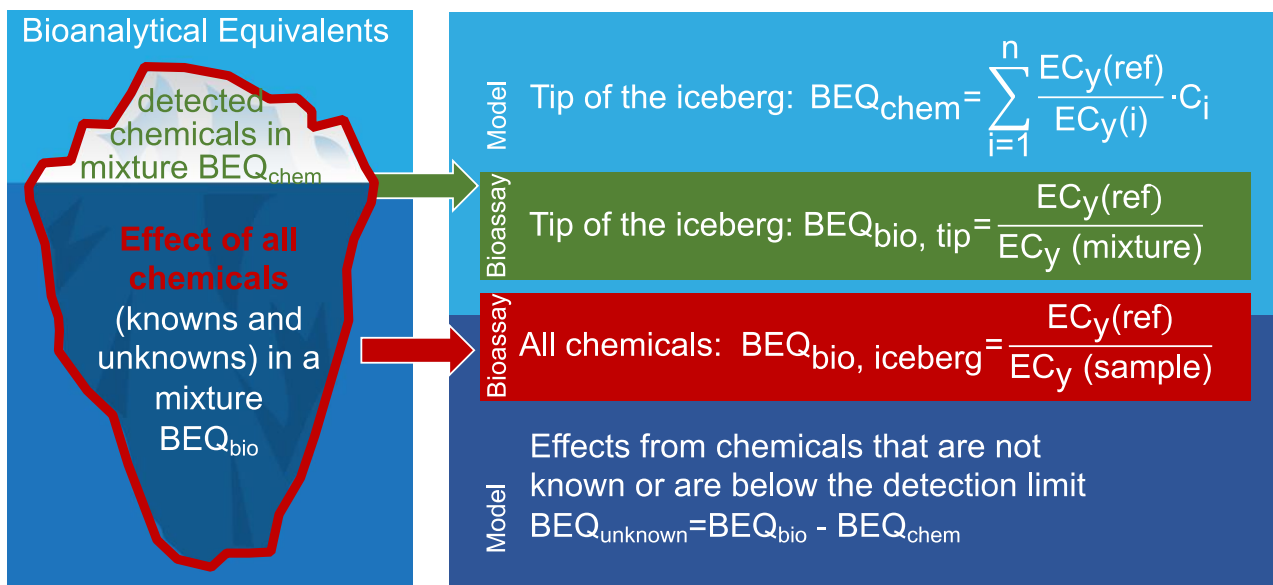
$$TU_{\text{cytotoxicity}(\text{bio, iceberg})} = \frac{1}{IC_{10}(\text{sample})} \quad (4)$$

$$TU_{\text{cytotoxicity}(\text{chem})} = \sum_{i=1}^n \frac{C_i}{IC_{10}(i)} \quad (5)$$

The percent contribution of individual detected chemicals  $i$  to the known fraction of effect (e.g.,  $BEQ_{\text{chem}}$  or  $TU_{\text{cytotoxicity}(\text{chem})}$ ) was calculated using Equations 6 and 7.

$$\% \text{ contribution of } i \text{ to } BEQ_{\text{known}} = \frac{REP_i \cdot C_i}{BEQ_{\text{chem}}} \cdot 100\% \quad (6)$$

$$\% \text{ contribution of } i \text{ to } TU_{\text{known}} = \left( \frac{C_i}{IC_{10}(i)} \cdot \frac{1}{TU_{\text{cytotoxicity}(\text{chem})}} \right) \cdot 100\% \quad (7)$$



189  
190 **Figure 1:** Bioanalytical equivalent concentrations from chemical analysis ( $BEQ_{\text{chem}}$ ) are compared to  
191 the bioanalytical equivalent concentrations from bioanalysis ( $BEQ_{\text{bio, iceberg}}$ ) using iceberg modelling.

192 The contribution of detected chemicals to  $BEQ_{chem}$  (e.g., “tip of the iceberg”) is determined both by  
193 modelling and using designed mixture experiments ( $BEQ_{bio, tip}$ ). Y stands for the effect measure, e.g.,  
194  $y=10$  for 10%,  $EC_{10}$ , or  $IR_{1.5}$  for  $EC_{IR1.5}$ .

195

196 **Tip of the iceberg mixtures.** Chemicals that dominated  $BEQ_{chem}$  were mixed in the ratios of  
197 concentrations they were detected in the samples. For activation of AhR 17 chemicals (1H-  
198 benzotriazole, 2-benzothiazolesulfonic acid, 2-hydroxybenzothiazole, 2,6-dichlorbenzamide, 5-  
199 methyl-1H-benzotriazole, 7-diethylamino-4-methylcoumarin, chlorotoluron, diflufenican, diuron,  
200 epoxiconazole, genistein, iminostilbene, isoproturon, MCPA, metamitron, pindolol, propylparaben)  
201 were mixed in 107 combinations of detected concentrations. Pindolol and 2,6-dichlorbenzamide were  
202 added because they had shown a positive response in the Tox21 database but our experiments showed  
203 no activity. Logistic reasons prohibited preparing matching mixtures for all water samples, but 107  
204 of 128 mixtures were prepared. For PPAR $\gamma$ , we mixed 17 other chemicals (2-benzothiazolesulfonic  
205 acid, 2-hydroxybenzothiazole, 2,4-dichlorophenoxyacetic acid, 3,5,6-trichloro-2-pyridinol, 7-  
206 diethylamino-4-methylcoumarin, bezafibrate, chloridazon, desethylterbutylazine, diclofenac,  
207 losartan, MCPA, naproxen, prosulfocarb, prothioconazole-desthio, quinoxifen, thiacloprid amide,  
208 triphenylphosphate) in 76 mixtures ratios as they were detected and one chemical (prothioconazole-  
209 desthio) turned out to be inactive during mixture experiments. For AREc32, 16 chemicals (2-  
210 benzothiazolesulfonic acid, 2-hydroxybenzothiazole, 2,4-dinitrophenol, 7-diethylamino-4-  
211 methylcoumarin, benalaxyl, desphenyl-chloridazon, dimethenamid, ethofumesate, flufenacet,  
212 genistein, iminostilbene, metazachlor, metolachlor, pethoxamid, propylparaben, triphenylphosphine  
213 oxide), one of which (benalaxyl) turned out to be inactive, were mixed in 44 mixture ratios. In  
214 addition, an equipotent mixture was prepared for all assays.

215 The stock solutions of the mixtures were prepared in DMSO from DMSO stocks of single compounds  
216 using a Tecan D300e Digital Dispenser (Tecan, Crailsheim, Germany). The effect concentrations of  
217 the mixtures  $EC_y(\text{mixture})$  were reported in total molar concentration (of all 17 or 16 chemicals  
218 including the inactive ones) and converted to simulated REF by dividing by the total molar  
219 concentrations of these compounds in the water samples to yield  $EC_y(\text{mixture})$  in units of REF. The  
220  $BEQ_{bio, tip}$  of the designed mixtures (Equation 8) were then compared with  $BEQ_{chem}$  and  $BEQ_{bio, iceberg}$ .

221

222

$$BEQ_{bio, tip} = \frac{EC_y(\text{ref})}{EC_y(\text{mixture})}$$

223

(8)

224



225 The index on prediction quality (IPQ, Equations 9 and 10) serves as a measure of how well  
226 experimental ( $BEQ_{bio,tip}$ ) and predicted mixture effect ( $BEQ_{chem,tip}$ ) agree, with an IPQ of 0 indicating  
227 optimal agreement.<sup>27, 41</sup>

228

229 For  $BEQ_{bio,tip} > BEQ_{chem,tip}$ :  $IPQ = \frac{BEQ_{chem,tip}}{BEQ_{bio,tip}} - 1$   
230 (9)

231 For  $BEQ_{chem,tip} > BEQ_{bio,tip}$ :  $IPQ = 1 - \frac{BEQ_{chem,tip}}{BEQ_{bio,tip}}$   
232 (10)

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234

## 235 **Results and Discussion**

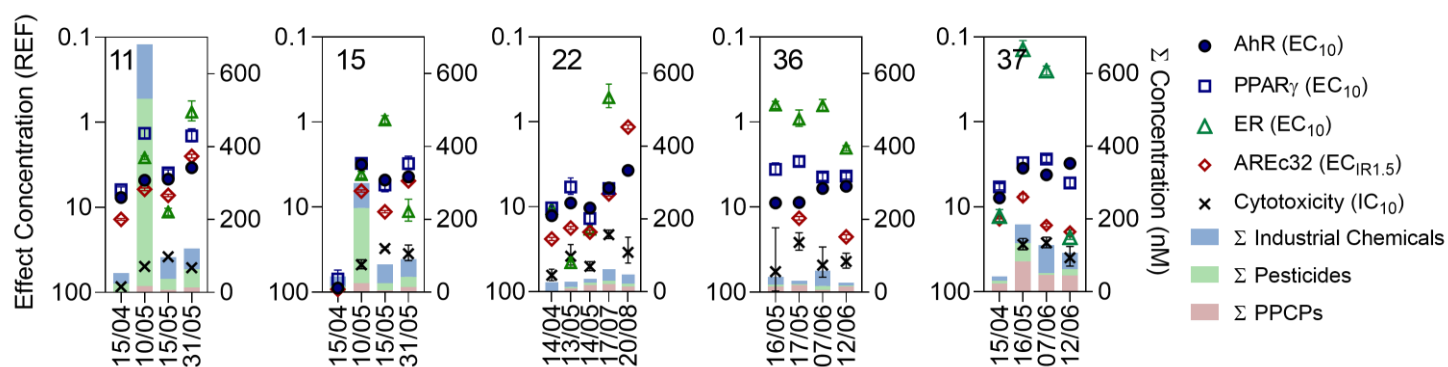
236 **Chemical analysis.** 290 of the analyzed 395 chemicals were detected in at least one water sample  
237 (Table S2), with 10 to 144 chemicals detected per site. The industrial compound 2-  
238 benzothiazolesulfonic acid was most frequently detected and was found in 124 of the 128 samples  
239 (97% detection frequency). It is used in the production of rubber, is also a transformation product of  
240 mercaptobenzothiazole and its derivatives and has been previously detected in wastewater and surface  
241 water.<sup>10, 42</sup> It was also one of the most commonly detected chemicals in the Danube River.<sup>31</sup> In street  
242 run-off the concentrations of 2-benzothiazolesulfonic acid were up to 50  $\mu\text{g/L}$  and thus 10 times  
243 higher than in wastewater or surface water, where it was present in similar concentration ranges as in  
244 the current study.<sup>43</sup> The chemical found at the highest concentration, with up to 126.2  $\mu\text{g/L}$  (average  
245 concentration 11.2  $\mu\text{g/L}$ ), was oxypurinol, which is the pharmaceutical metabolite of the anti-gout  
246 pharmaceutical allopurinol, and has previously been found at concentrations up to 22.6  $\mu\text{g/L}$  in  
247 German surface water.<sup>44</sup> The chemical profile also varied between sites and over time, with some  
248 sites dominated by pesticides and others containing higher concentrations of pharmaceuticals and  
249 personal care products (PPCPs) (Figure 2, Figure S1). A thorough evaluation of the chemical analysis  
250 is beyond the scope of the present study, which focuses on bioassays.

251

252 **Bioanalysis.** The observed effect in the activation of AhR, binding to PPAR $\gamma$ , activation of ER,  
253 oxidative stress response and cytotoxicity varied both between sites and within the same site over  
254 time (Figure 2 and Figure S2, see Table S6 for all EC values). For example, estrogenic activity varied  
255 by almost a factor of one hundred in Site 22 between different rain events (Figure 2). Activation of  
256 ER was often the most responsive endpoint, followed by the responses of assays indicative of

257 xenobiotic metabolism, activation of AhR and binding to PPAR $\gamma$ . The oxidative stress response assay  
 258 was in many sites the least responsive.

259 While the studied small streams were in agricultural areas, five of the 44 sites (5, 26, 29, 35, 37) were  
 260 directly impacted by municipal WWTP effluents and three others (sites 21, 22, 23) by industrial  
 261 WWTPs (Table S1). Several other sites showed typical markers of wastewater, including the  
 262 pharmaceutical carbamazepine and artificial sweeteners sucralose and saccharin (Table S1). A subset  
 263 of these sites often had EC<sub>10</sub> values less than one (i.e., effect observed after dilution) in the activation  
 264 of ER assay pointing towards wastewater discharge (e.g., sites 13, 31 and 36). This suggests that  
 265 water from water retention basins or combined sewer systems, where capacities were exceeded during  
 266 rainfall events, entered streams or diffuse effluents from small upstream urban areas (Table S1)  
 267 contributed to the effects.



268  
 269  
 270 **Figure 2:** EC values for activation of AhR, binding to PPAR $\gamma$ , activation of ER and oxidative stress  
 271 response (AREc32) for selected sites (11, 15, 22, 36, 37), with sum concentration of industrial  
 272 compounds, pesticides and pharmaceuticals and personal care products (PPCPs) (nM). Cytotoxicity  
 273 IC<sub>10</sub> values are for the AhR CALUX, with IC<sub>10</sub> values for the other assays provided in Table S6.

274  
 275 The level of activation of AhR and binding to PPAR $\gamma$  was similar to that previously observed in the  
 276 German Ammer River, with EC<sub>10</sub> REF values ranging between 2.0 to 35 and 1.1 to 90, respectively.<sup>13</sup>  
 277 In contrast, estrogenic activity in the small streams was often higher than the observed effect in the  
 278 Ammer River,<sup>13</sup> with many of the samples showing activity similar to wastewater effluent.<sup>10, 18</sup> The  
 279 oxidative stress response was in a similar range as detected previously in streams and rivers in  
 280 Australia, Germany and Switzerland.<sup>12, 13, 18</sup>

281  
 282 **Comparison of measured effects in the water samples with effect-based triggers (EBT).** The surface  
 283 water extract EC values were converted to BEQ<sub>bio, iceberg</sub> values in units of ng or  $\mu$ g of reference

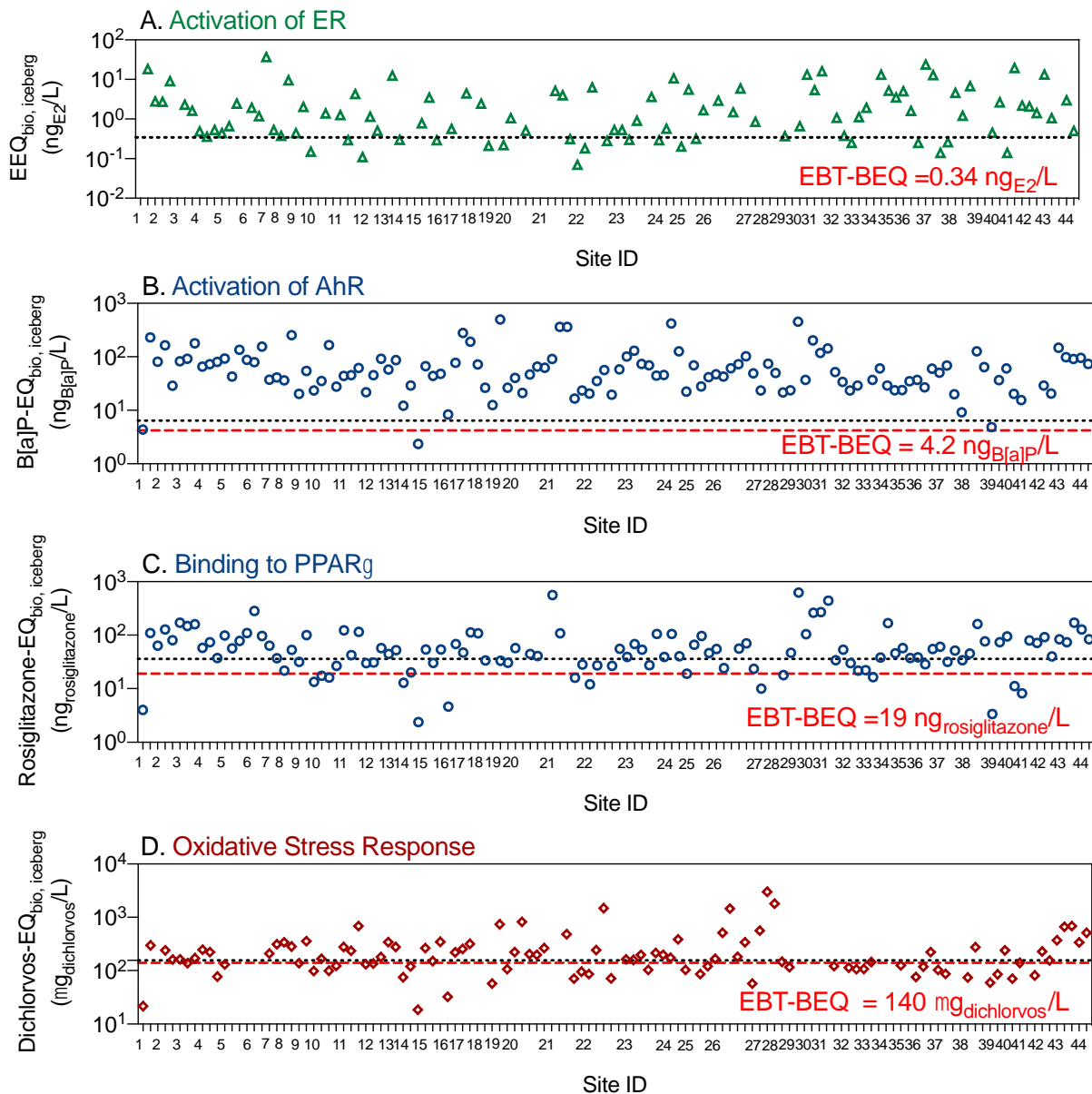
284 compound per liter and were compared with preliminary surface water EBTs derived from the EU  
285 Water Framework Directive.<sup>22</sup> The preliminary EBTs, which were derived by reading across from  
286 the current environmental quality standards in the Water Framework Directive and applying a mixture  
287 factor where necessary, were updated with the newly available single chemical effect data (Table S5,  
288 no update of EBT for ER $\alpha$  GeneBLAzer) using the template provided by Escher et al.<sup>22</sup>  
289 The EEQ of 79% of samples (Table S6) exceeded EEQ-EBT of 0.34 ng<sub>EE2</sub>/L for ER $\alpha$  GeneBLAzer<sup>22</sup>  
290 (Figure 3A), which was an unexpectedly high percentage, given that the sampling sites were selected  
291 with a focus on agricultural impact. However, chemicals usually associated with treated or untreated  
292 wastewater were detected at several sites (Table S1), which is consistent with the high EEQs.  
293 Previously, the EBT-EEQ had been able to differentiate clearly between wastewater and surface water  
294 with surface water rarely exceeding the EBT-EEQ.<sup>22</sup> The elevated estrogenic activity could be related  
295 to lower retention times in the WWTP and thus lower treatment efficacy and diffuse input of urban  
296 stormwater contamination from combined sewer systems. Rain events can also lead to dilution but  
297 since we only sampled during rain events, not the periods before and after the event, we cannot judge  
298 if dilutions by rain occurred. For example, sites 5, 21, 26, 29 and 35 were impacted by wastewater  
299 (Table S1) and all exceeded the activation of EBT-EEQ. In contrast, sites 22 and 37 also had WWTPs  
300 upstream of the respective sampling sites, but only exceeded the EBT during some rainfall events.  
301 The EBT-B[a]P-EQ for AhR CALUX was published as 6.4 ng<sub>B[a]P</sub>/L<sup>14</sup> but this value was only based  
302 on four experimental EC<sub>10</sub> values. Using nine additional EC<sub>10</sub> values (Table S5) brought the EBT-  
303 B[a]P-EQ to 4.3 ng<sub>B[a]P</sub>/L, indicating the robustness of the initial derivation. The EC<sub>10</sub> in Table S6  
304 were converted to B[a]P-EQ with Equation 1 using the EC<sub>10</sub> for B[a]P of 212 ng<sub>B[a]P</sub>/L. 98% of the  
305 samples' B[a]P-EQ exceeded this EBT-B[a]P-EQ (Figure 3B). Prior experience with AhR CALUX  
306 in water samples is limited, but WWTP effluents<sup>19</sup> and wastewater-impacted rivers<sup>13</sup> had similarly  
307 high B[a]P-EQ values as many of the present water samples, while small streams unimpacted by  
308 wastewater had lower B[a]P-EQ levels.<sup>13</sup>  
309 The EBT-rosiglitazone-EQ for PPAR $\gamma$  GeneBLAzer was previously 36 ng<sub>rosiglitazone</sub>/L,<sup>22</sup> but was only  
310 based on data for three chemicals. With now six active chemicals the revised EBT-rosiglitazone-EQ  
311 amounted to 19 ng<sub>rosiglitazone</sub>/L. Only 13% of the samples (Table S6) were compliant, with the  
312 remainder exceeding this EBT (Figure 3C). This is in contrast to a previous study, where only  
313 untreated wastewater exceeded the preliminary EBT for PPAR $\gamma$ , whereas surface water samples from  
314 the Danube River were compliant.<sup>10</sup> In another small stream, this revised EBT-rosiglitazone-EQ was  
315 able to clearly differentiate between unimpacted stretches and tributaries of the river and WWTP  
316 effluent or thereby impacted stretches of the river.<sup>13</sup>

317 The EBT-dichlorvos-EQ for AREc32 remained virtually constant with  $140 \text{ ng}_{\text{dichlorvos}}/\text{L}$  despite the  
 318 database increasing from 11 to 21 chemicals. 60% of the samples exceeded this EBT-dichlorvos-EQ  
 319 (Table S6, Figure 3D). Again, this EBT had previously differentiated well between more polluted  
 320 water (wastewater and urban stormwater) and river water<sup>22</sup> and in another small stream study during  
 321 dry weather, all sites, including those impacted by WWTP effluent were below the EBT-dichlorvos-  
 322 EQ.<sup>13</sup>

323 This comparison with EBT-BEQs as well as with previous samples from wastewater and surface  
 324 water suggests that many of the sites have a high chemical mixture burden, particularly concerning  
 325 chemicals that activate AhR and ER.

326

327



328

329 **Figure 3:** Comparison of water extract  $BEQ_{bio, iceberg}$  values (ordered by site ID (Table S1)) with the  
330 preliminary effect-based trigger values (EBT) from Escher et al.<sup>22</sup> (dotted black lines) and the  
331 revised EBTs (red dashed lines).

332

333 ***Which chemicals are driving the effects in the water extracts?*** To better understand which chemicals  
334 are driving the observed effects, chemicals detected in the water extracts at high concentrations or  
335 expected to contribute to the effect in assays indicative of activation of AhR, binding to PPAR $\gamma$  and  
336 the oxidative stress response were fingerprinted. We omitted fingerprinting of single chemicals in the  
337 activation of ER assay because a small number of potent chemicals, namely natural and synthetic  
338 steroidal hormones, typically explain most of the effect in this endpoint.<sup>45,46</sup> Bioanalysis is sufficient  
339 to characterize estrogenicity in water samples as the ratio of bioactive estrogens is typically fairly  
340 constant in surface waters.<sup>35</sup> A wider range of chemicals are active in assays indicative of induction  
341 of xenobiotic metabolism and adaptive stress responses.<sup>47</sup> The  $IC_{10}$  and  $EC$  values for all chemicals  
342 measured in the current study or taken from literature are provided in Table S5.

343 For activation of AhR, effect measurements were available for 316 of the 395 analyzed chemicals  
344 (80%) using both experimental data and the Tox21 database. Of the 290 detected chemicals, effect  
345 data was available for 236 chemicals (81%), but most were not active (Table S5, Figure S3).  $EC_{10}$   
346 values were available for 40 chemicals detected in the water extracts for the activation of the AhR  
347 assay. Nineteen of these values were from the Tox21 database, which used a different activation of  
348 AhR assay (rat cell line in the current study versus human cell line in Tox21 database). However,  
349  $EC_{10}$  values for common chemicals run in both assays were generally within one order of magnitude  
350 (Figure S4), so both datasets were used to determine the effect based on chemical analysis,  $BEQ_{chem}$   
351 (Table S7).

352 On average, 2-benzothiazolesulfonic acid explained 29.2% of the  $B[a]P-EQ_{chem}$  in the water extracts  
353 (between 0 to 98.2% explained), followed by the herbicide diuron (average 14.9%) (Figure 4A). The  
354 average contribution to  $B[a]P-EQ_{chem}$  is presented in Figure 4, but the contribution of each chemical  
355 to  $B[a]P-EQ_{chem}$  varied greatly for the different water extracts because the presence and  
356 concentrations of the individual chemicals varied considerably (Table S2) resulting in a wide range  
357 of  $B[a]P-EQ_i$  (Figure S5). For example, the industrial compound 7-diethylamino-4-methylcoumarin  
358 explained on average 4.8% of  $B[a]P-EQ_{chem}$  but contributed to over 95% of  $B[a]P-EQ_{chem}$  in all water  
359 extracts from the wastewater-impacted Site 37. 2-Benzothiazolesulfonic acid was one of the least  
360 potent chemicals in AhR CALUX ( $REP_i$   $5.67 \times 10^{-6}$ ), but it was present in all but two of the water  
361 extracts and was found at high concentrations (up to 6.4  $\mu\text{g/L}$ ). Therefore, not only highly potent  
362 chemicals but also chemicals present at high concentrations will contribute to the effect.

363 When comparing B[a]P-EQ<sub>chem</sub> to B[a]P-EQ<sub>bio,iceberg</sub>, only between 0.0004 to 2.79% of the effect  
364 could be explained by detected chemicals (Table S7). Previous studies have found between 0.2 to  
365 71% of activation of AhR that could be explained by the quantified chemicals in surface water.<sup>12, 31</sup>  
366 These studies only had EC values for three to four of the detected chemicals, compared to 40 detected  
367 bioactive chemicals in the current study. AhR is mainly activated by hydrophobic organics such as  
368 polycyclic aromatic hydrocarbons. These bind to suspended particulate matter and would not be  
369 expected in the water sample filtered with a 0.7 µm filter but residual smaller particles and colloids  
370 may pass and be enriched by SPE, contributing to the unknown fraction of B[a]P-EQ<sub>bio,iceberg</sub>. For  
371 these particles, a source in addition to road run-off, agricultural run-off and WWTP effluent will also  
372 be atmospheric deposition.<sup>48</sup>

373 Effect measurements were available for 310 out of the 395 analyzed chemicals for PPAR $\gamma$   
374 GeneBLAzer, with data available for 232 of the detected chemicals (80%) (Table S5). However, only  
375 9% of the detected chemicals tested in PPAR $\gamma$  GeneBLAzer were active, with REP<sub>i</sub> values available  
376 for 20 chemicals (Figure S3). Diclofenac explained on average around a third (35.4%) of  
377 rosiglitazone-EQ<sub>chem</sub>, followed by 2-benzothiazolesulfonic acid (average 25.3%) and the herbicide  
378 MCPA (average 12.4%) (Figure 4B, Figure S6). Diclofenac was among the most potent chemicals  
379 measured in the PPAR $\gamma$  GeneBLAzer assay in the current study (REP<sub>i</sub>  $5.42 \times 10^{-4}$ ) and was also found  
380 at high concentrations (up to 1.3 µg/L). However, rosiglitazone-EQ<sub>chem</sub> could only explain up to  
381 1.66% of rosiglitazone-EQ<sub>bio,iceberg</sub> (average 0.18%) (Table S8). Detected chemicals have previously  
382 shown to explain a low fraction of the effect (<1%) in the PPAR $\gamma$  GeneBLAzer assay in surface water  
383 and wastewater<sup>10</sup> and spiked surface water.<sup>23</sup>

384 Bioassay data were available for either the AREc32 or ARE GeneBLAzer assays for 309 of the 395  
385 chemicals analyzed. If both were available, only AREc32 was reported. Of the 290 detected  
386 chemicals, effect data was available for 233 chemicals (80%), with 52 of the detected chemicals active  
387 in the AREc32 (29 chemicals) or ARE GeneBLAzer assays (23 chemicals) (Table S5, Figure S3).  
388 The ARE GeneBLAzer data were collected from the US EPA Tox21 database and was expressed as  
389 an EC<sub>10</sub> rather than an EC<sub>IR1.5</sub>. The EC<sub>IR1.5</sub> and EC<sub>10</sub> values for common chemicals were generally  
390 within an order of magnitude (Figure S7), but the REP<sub>i</sub> values for chemicals run in ARE GeneBLAzer  
391 were calculated using the dichlorvos EC<sub>10</sub> value from the Tox21 database.

392 2-Benzothiazolesulfonic acid explained 35.4% of dichlorvos-EQ<sub>chem</sub> on average, followed by  
393 industrial compound 2,4-dinitrophenol (average 12.0%) and herbicide metolachlor (average 7.2%)  
394 (Figure 4C, Figure S8). Metolachlor was previously found to contribute to dichlorvos-EQ<sub>chem</sub> for the  
395 oxidative stress response in wastewater effluent and surface water downstream of a WWTP in  
396 Switzerland.<sup>12</sup> On average, only 0.28% of dichlorvos-EQ<sub>bio,iceberg</sub> could be explained by dichlorvos-

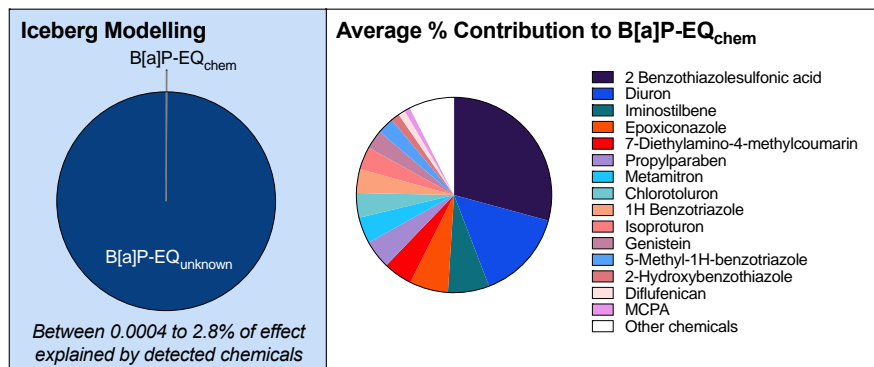
397 EQ<sub>chem</sub> (Table S9). This is similar to previously observed for surface water and wastewater.<sup>12, 27, 31</sup> In  
398 one sample, 8b, 8% of dichlorvos-EQ<sub>bio,iceberg</sub> was explained by the potent herbicide pethoxamid (REP;  
399 2.66), which was detected at 13.1 µg/L.

400 While many different chemicals contributed to the BEQ<sub>chem</sub> in the three assays, 2-  
401 benzothiazolesulfonic acid explained between 25.3 to 35.4% of BEQ<sub>chem</sub> on average in the three  
402 assays. While 2-benzothiazolesulfonic acid was not particularly potent in any of the assays, the  
403 widespread presence and high concentrations (average concentration 1.1 µg/L) meant it was a  
404 dominant contributor to BEQ<sub>chem</sub>. This suggests that future water quality monitoring studies should  
405 include 2-benzothiazolesulfonic acid, especially as it is also a marker of street run-off and as such  
406 complements the traditional wastewater markers such as estrogenic hormones or pesticides as  
407 markers for agricultural inputs.

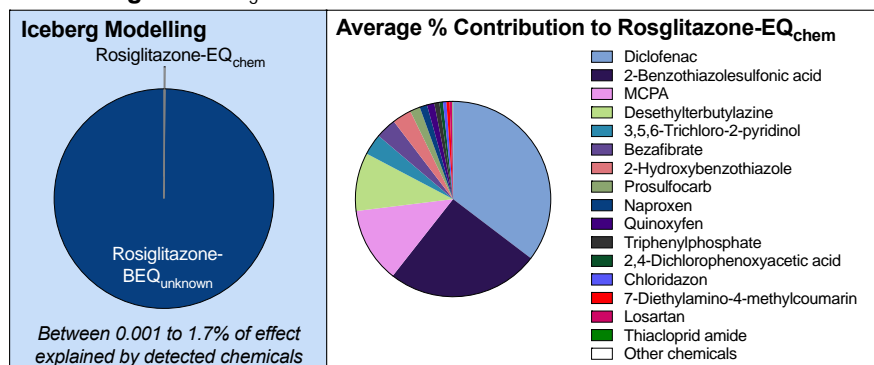
408 Other studies have also used *in vitro* or *in vivo* data to prioritize chemicals of concern. Focusing on  
409 assays included in the US EPA Tox21 database, Corsi et al.<sup>21</sup> found that the industrial compounds 4-  
410 nonylphenol and bisphenol A and the herbicides metolachlor and atrazine were among the chemicals  
411 identified as of greatest concern in water samples collected from the Great Lakes tributary.  
412 Metolachlor was also identified as a contributor to dichlorvos-EQ<sub>chem</sub> for oxidative stress response in  
413 the current study. Further, many of the chemicals contributing to BEQ<sub>chem</sub>, including the  
414 pharmaceuticals bezafibrate and diclofenac and the herbicides prosulfocarb and metolachlor, also  
415 ranked highly in a list of 214 chemicals present in European surface waters that potentially pose an  
416 acute hazard to fish, algae or crustaceans.<sup>49</sup>

417 Iceberg modeling of cytotoxicity is described and discussed in the SI, Section S5. Overall, a  
418 substantially higher fraction of cytotoxicity than of activation of specific effects could be explained  
419 because a larger number, i.e., 102, detected chemicals had experimental cytotoxicity IC<sub>10</sub>: 0.2 to  
420 122% for AhR CALUX, 0.2 to 22% for PPAR $\gamma$  GeneBLAzer and 0.02 to 8.8 % for AREc32 (Figure  
421 S10).

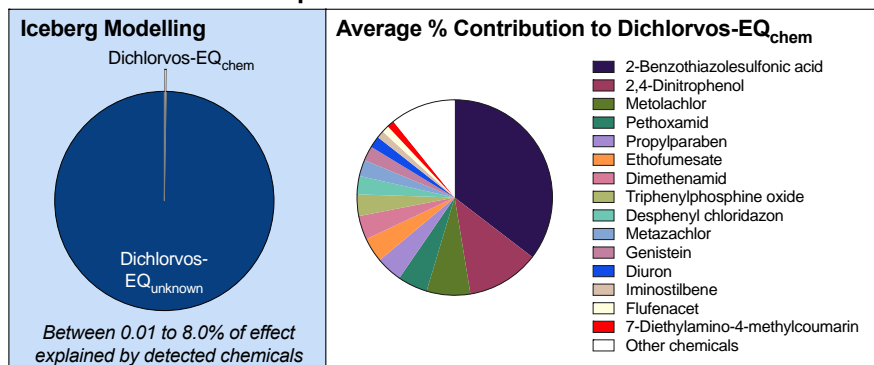
### A. Activation of AhR



### B. Binding to PPAR $\gamma$



### C. Oxidative Stress Response



422

423 **Figure 4:** Average fraction of BEQ<sub>chem</sub> that explained BEQ<sub>bio,iceberg</sub> (left) and top 15 to 16 chemicals  
 424 contributing on average to BEQ<sub>chem</sub> (right) for assays indicative of activation of (A) AhR, (B)  
 425 binding to PPAR $\gamma$  and (C) oxidative stress response.

426

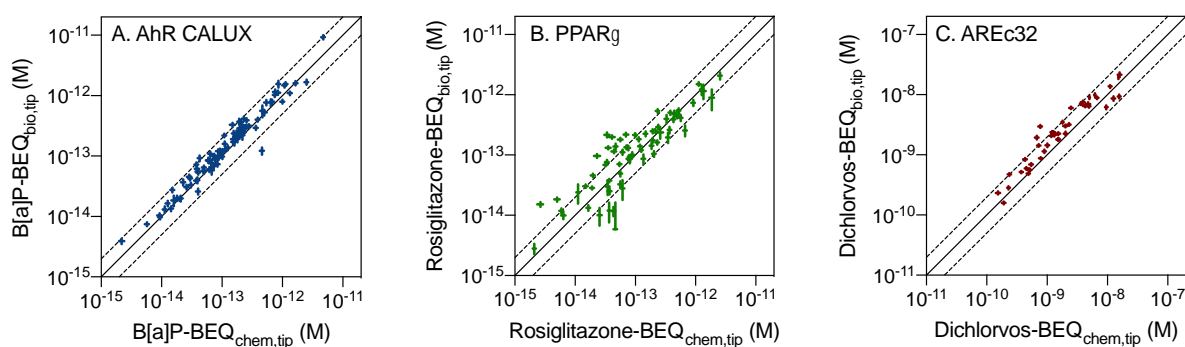
427 ***Equipotent mixtures of the detected chemicals.*** The concentration-response curve for activation of  
 428 AhR of the equipotent mixture of the 15 chemicals that contributed most to the BEQ<sub>chem</sub> agreed well  
 429 with the prediction for concentration addition (Figure S11A) with an index of prediction quality (IPQ)  
 430 of -0.11. This means that the chemicals detected are acting according to the mixture concept of  
 431 concentration addition in mixtures. The equipotent mixture of PPAR $\gamma$  GeneBLAzer (Figure S11B)  
 432 was much more potent than predicted for concentration addition with an IPQ of 3.69. This is  
 433 especially surprising because the mixtures with the concentration ratios as detected in the water



434 samples were generally much closer to IPQ 0. The equipotent mixture of AREc32 (Figure S11C) had  
435 an IPQ of 0.46, which means that the experimental effect was higher than the predicted mixture effect.  
436 Various 5- to 10-component equipotent mixtures run in the AREc32 assay had IPQs around 0  
437 confirming concentration addition but some mixtures had IPQ up to 1 indicating some variability.<sup>27</sup>

438

439 **Tip of the iceberg mixtures.** Since B[a]P-EQ<sub>chem</sub> explained only a very small fraction of the B[a]P-  
440 EQ<sub>bio</sub> (Figure S12A), it was checked by designed mixture experiments of chemicals in the detected  
441 concentration ratios of water samples if the detected chemicals act together according to concentration  
442 addition. The 107 reconstituted mixtures in AhR contained between 3 and 14 components in the  
443 detected concentration ratios. The selected 17 chemicals explained on average 93% of the overall  
444 BEQ<sub>chem</sub> (min 26 %, max 99.9%). The concentration-response curves for activation of AhR are  
445 depicted in Figure S13 together with the predictions for CA. The EC<sub>10</sub> values were converted to  
446 BEQ<sub>bio,tip</sub> and compared with BEQ<sub>chem,tip</sub> (Table S7, Figure 5A and S12B). With few exceptions, the  
447 agreement was within a factor of two, which is also reflected by the IPQ values (Table S7), which  
448 had a mean of 0.24 (95% CI 0.14 to 0.33, Figure S12C), indicating a slightly higher effect of the  
449 experimental mixture BEQ<sub>bio,tip</sub> than of the predicted BEQ<sub>chem,tip</sub>. This small systematic deviation may  
450 be caused by the two chemicals that were inactive in the mixture experiments, whereas they had been  
451 reported active in Tox21. They may have been below their threshold of effect alone but contributed  
452 to the mixture effect.



453

454 **Figure 5:** Agreement between BEQ<sub>bio,tip</sub> and BEQ<sub>chem,tip</sub> for (A) activation of AhR, (B) binding to  
455 PPAR $\gamma$  and (C) oxidative stress response. No symbols are shown, the lines at the points are the  
456 error bars (standard error), the full line is the 1:1 relationship and the dashed lines indicate 2:1 and  
457 1:2 ratios.

458

459 The 76 mixtures of the 17 chemicals with the highest predicted rosiglitazone-EQ<sub>chem</sub> in concentration  
460 ratios of the water samples (Table S8, concentration-response curves (CRCs) in Figure S14) yielded  
461 IPQs ranging from -6.9 to 5.4, with a mean of 0.32 but the 95% CI only ranged from -0.04 to 0.70,

462 which indicates that the majority of IPQs is above 0, indicating more potent mixtures than expected  
463 (Figure S15C). The relationship between rosiglitazone-EQ<sub>chem,tip</sub> and rosiglitazone-EQ<sub>bio,tip</sub> showed  
464 more variability than in AhR CALUX but the values are within a factor of two around the one-to-one  
465 line (Figure 5B). The higher variability between prediction and measurement is caused by the  
466 generally higher variability of individual data points in the CRCs of this assays, which is due to a  
467 larger background signal and hence lower signal-to-noise ratio.

468 The deviation from the relationship between dichlorvos-EQ<sub>bio,tip</sub> and dichlorvos-EQ<sub>chem,tip</sub> was well  
469 within a factor of two (CRCs in Figure S16, Figure 5C, Table S9) but directed towards higher  
470 experimental effects similar to AhR. Hence the deviation towards higher potency experimentally as  
471 compared to the mixture model of concentration addition appears to be small but consistent and might  
472 be caused by some imprecision of the single chemicals' EC<sub>10</sub> values or the one inactive chemical  
473 benalaxyl. The IPQ values of the 44 mixtures (Table S9) ranged from -0.69 to 3.5 with a mean of  
474 0.51 (95% CI 0.30 to 0.59, Figure S17C).

475 In summary, over all the 227 mixtures the mixture components appeared to act fairly close to  
476 concentration-additive in all three *in vitro* bioassays, confirming that the BEQ concept is applicable  
477 to these bioassays and types of samples. The IPQs were close to 0 with a tendency to positive values  
478 for AhR CALUX (Figure S12C) and AREc32 (Figure S17C), even more for PPAR $\gamma$  GeneBLAzer  
479 (Figure S15C), which points to experimental effects being slightly higher than predicted, but the IPQ  
480 values did not shown any correlation to the composition of any of the mixtures.

481

482 **Outlook.** It has been demonstrated previously that a complete pesticide screening is required to  
483 estimate the surface water quality of small streams<sup>50</sup> and, while individual pesticides might exceed  
484 chemical-specific water quality criteria, it is really the mixture effect that needs to be considered to  
485 understand ecological effects<sup>51</sup> and risk.<sup>7</sup> Pesticides drive the risk predicted with the method of multi  
486 substance potentially affected fraction (msPAF) even in wastewater impacted streams at low-flow  
487 conditions.<sup>52</sup>

488 But the situation might change dramatically during rain events as described here, where we recorded  
489 a high spatial and temporal variability. While further studies on exceedance of chemical-specific  
490 water quality criteria and the ecological impact and *in vivo* toxicity of the described rain events are  
491 forthcoming, the focus on present study was on the *in vitro* assays and biological endpoints most  
492 commonly impacted by water-borne pollutants.

493 We demonstrated that non-pesticide chemicals and even typical wastewater-derived chemicals were  
494 found at sites assumed prior to the study to be largely free from wastewater effects. All observed *in*  
495 *vitro* effects were dominated by street run-off chemicals such as 2-benzothiazolesulfonic acid.

496 Previous effect studies on stormwater demonstrated that effect levels were similarly high as WWTP  
497 effluent and all urban stormwater samples investigated showed estrogenic effects.<sup>17</sup> Rain events  
498 clearly pose a threat to water quality in small streams and analysis of pesticides alone cannot  
499 adequately judge the toxicological impact unless analytical monitoring is complemented by  
500 bioassays.

501

## 502 **ASSOCIATED CONTENT**

### 503 **Supporting information**

504 The supporting information is available free of charge at <https://pubs.acs.org/doi...>

505 Additional information on chemical analysis, bioassays, iceberg modelling of effects and  
506 cytotoxicity, equipotent mixture experiments, designed mixture experiments (pdf). Excel file  
507 with all experimental data.

508

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551 **Author Contributions**

552 MaL lead the sampling study; WB and RBS contributed conceptually and to site selection and  
553 sampling, TS, ML developed the sampling device; TS designed and programmed the target screening  
554 software, MaL, MoL, LL, RBS, VS, PV and OW contributed to monitoring coordination, site  
555 selection, sampling and evaluation of wastewater influence; EC and MKr performed the chemical  
556 analysis and data evaluation; RG, MoL and VS, extracted all samples; MKö and RS performed the  
557 bioassay experiments; GB performed and evaluated the tip of the iceberg mixture experiments; BE  
558 conceived the bioassay study, developed all data evaluations and models; PN evaluated all bioassay  
559 data, performed the iceberg modeling; PN and BE wrote the manuscript; all authors reviewed the  
560 manuscript.

561 All authors have given approval to the final version of the article.

562

563 **Notes**

564 The authors declare no competing financial interest.

565

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578

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