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# Integrating chemical analysis and bioanalysis to evaluate the contribution of wastewater effluent on the micropollutant burden in small streams

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3 **Integrating Chemical Analysis and Bioanalysis to Evaluate the Contribution of Wastewater**
4 **Effluent on the Micropollutant Burden in Small Streams**

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**Abstract**

Surface waters can contain a range of micropollutants from point sources, such as wastewater effluent, and diffuse sources, such as agriculture. Characterizing the source of micropollutants is important for reducing their burden and thus mitigating adverse effects on aquatic ecosystems. In this study, chemical analysis and bioanalysis were applied to assess the micropollutant burden during low flow conditions upstream and downstream of three wastewater treatment plants (WWTPs) discharging into small streams in the Swiss Plateau. The upstream sites had no input of wastewater effluent, allowing a direct comparison of the observed effects with and without the contribution of wastewater. Four hundred and five chemicals were analyzed, while the applied bioassays included activation of the aryl hydrocarbon receptor, activation of the androgen receptor, activation of the estrogen receptor, photosystem II inhibition, acetylcholinesterase inhibition and adaptive stress responses for oxidative stress, genotoxicity and inflammation, as well as assays indicative of estrogenic activity and developmental toxicity in zebrafish embryos. Chemical analysis and bioanalysis showed higher chemical concentrations and effects for the effluent samples, with the lowest chemical concentrations and effects in most assays for the upstream sites. Mixture toxicity modeling was applied to assess the contribution of detected chemicals to the observed effect. For most bioassays, very little of the observed effects could be explained by the detected chemicals, with the exception of photosystem II inhibition, where herbicides explained the majority of the effect. This emphasizes the importance of combining bioanalysis with chemical analysis to provide a more complete picture of the micropollutant burden. While the wastewater effluents had a significant contribution to micropollutant burden downstream, both chemical analysis and bioanalysis showed a relevant contribution of diffuse sources from upstream during low flow conditions, suggesting that upgrading WWTPs will not completely reduce the micropollutant burden, but further source control measures will be required.

**Keywords**: wastewater; micropollutant; chemical analysis; bioassays; surface water; mixture modeling

65 **1. Introduction**

66 Surface waters can contain a wide range of micropollutants, including pesticides, pharmaceuticals, 67 personal care products and industrial compounds (Loos et al., 2009; Moschet et al., 2015), which 68 have the potential to adversely impact exposed ecosystems (Malaj et al., 2014; Stalter et al., 2013). 69 To mitigate the effect of micropollutants on the aquatic environment, it is important to identify their 70 sources, which can be either from point sources, such as wastewater effluent discharges, or diffuse 71 sources, such as agriculture (Eggen et al., 2014; Maletz et al., 2013). This can help to inform 72 solutions to reduce the concentration and bioactive fraction of micropollutants, i.e., the 73 micropollutant burden, in surface waters, which may include upgrading wastewater treatment plants 74 (WWTPs) or regulatory changes, such as banning certain chemicals (Schwarzenbach et al., 2006).

75 Water quality monitoring programs, such as requested under the European Union Water 76 Framework Directive (European Commission, 2011; European Commission, 2012), typically focus 77 on chemical analysis, which can provide useful information about the concentration and type of 78 chemicals present in a sample. However, targeted chemical analysis alone has some limitations. It is 79 unable to detect unidentified chemicals and transformation products or account for the mixture 80 effects that can occur between the many compounds present in water. For a comprehensive view of 81 the micropollutant burden, chemical analysis should be combined with bioanalysis. While bioassays 82 cannot identify individual chemicals, they can provide information about the joint effect of all 83 bioavailable active chemicals present in a sample, with more potent chemicals showing a greater 84 effect in the assay (Escher and Leusch, 2012; Prasse et al., 2015; Wernersson et al., 2015). The 85 development of bioanalytical tools for water monitoring requires adequate choice of biological 86 endpoints and quality measures (Altenburger et al., 2015; Busch et al., 2016), with applied 87 bioanalytical test batteries ideally including assays indicative of induction of xenobiotic 88 metabolism, endocrine disruption, reactive modes of action, adaptive stress responses and 89 cytotoxicity (Escher et al., 2014).

90 The complementary approach of chemical analysis and bioanalysis has been applied to 91 monitor water quality and to evaluate WWTP and advanced water treatment plant efficiency 92 (Creusot et al., 2014; Jálová et al., 2013; Margot et al., 2013; Tang et al., 2014). Applying bioassays 93 and chemical analysis in parallel overcomes the limitations associated with the individual 94 approaches and can reveal the presence of potent undetected chemicals and identify chemicals that 95 contribute to the observed effect (Escher and Leusch, 2012). Mixture toxicity modeling can be used 96 to determine the fraction of the observed effect that can be explained by detected chemicals using 97 the bioanalytical equivalent concentration (BEQ) approach (Neale et al., 2015a). Bioanalytical 98 equivalent concentrations from chemical analysis ($BEQ_{chem}$) are calculated using the detected 99 chemical concentration and their relative potency, which can be compared to bioanalytical

100 equivalent concentrations from bioassays ($BEQ_{bio}$). For example, detected chemicals can often
101 explain a high percentage of estrogenic activity (Leusch et al., 2014; Murk et al., 2002), while only
102 a small fraction of non-specific effects or adaptive stress responses can typically be explained (Tang
103 et al., 2013; Yeh et al., 2014).
104 In this study, both chemical analysis and bioanalysis were applied to assess the
105 micropollutant burden in small streams upstream and downstream of three WWTPs, with the
106 upstream sites not being affected by inputs of treated wastewater. The water samples were collected
107 under low flow conditions to minimize the impact of diffuse sources. The analyzed chemicals were
108 primarily pharmaceuticals and pesticides, with the other analyzed chemicals including biocides,
109 food additives, illicit drugs, industrial chemicals and estrogens. The biological effects were
110 evaluated using a suite of *in vitro* assays, which represent different cellular toxicity pathways,
111 including xenobiotic metabolism, receptor-mediated effects, adaptive stress responses and
112 cytotoxicity, as well as whole organism assays with algae and zebrafish embryos. Assays indicative
113 of xenobiotic metabolism, such as activation of the aryl hydrocarbon receptor (AhR), and adaptive
114 stress responses, such as the oxidative stress response, can respond to a range of compounds with
115 different modes of action (Martin et al., 2010; US EPA, 2015). In contrast, assays indicative of
116 receptor-mediated effects can provide information about the presence of chemicals with a common
117 specific mode of action. For example, hormone-mediated effects including activation of the
118 estrogen receptor (ER) and activation of the androgen receptor (AR) can detect natural and
119 synthetic hormones, as well as other environmental endocrine disrupting compounds, which are
120 often associated with wastewater (Vethaak et al., 2005). Further, assays indicative of inhibition of
121 photosystem II (PSII) and of acetylcholinesterase (AChE) are more suitable to detect chemicals of
122 an agricultural origin as they can detect PSII inhibiting herbicides (Escher et al., 2008a) and
123 organophosphate and carbamate insecticides (Hamers et al., 2000), respectively.
124 The current study aimed to assess the impact of wastewater effluent on the micropollutant
125 burden in small streams using a complementary chemical analysis and bioanalysis approach. Four
126 hundred and five chemicals were analyzed and the applied test battery included assays indicative of
127 activation of AhR, activation of the AR, activation of the ER, PSII and algal growth inhibition,
128 AChE inhibition, mutagenicity and adaptive stress responses for oxidative stress, genotoxicity and
129 inflammation, as well as assays indicative of estrogenic activity and developmental toxicity in
130 zebrafish embryos. A mass balance approach was used to calculate the fraction of effluent
131 downstream based on both chemical analysis and bioanalysis, while mixture toxicity modeling was
132 applied to assess whether the detected chemicals were contributing to the biological effect.
133
134

135 **2. Materials and Methods**

136 *2.1. Sampling*

137 Three WWTPs, Birmensdorf, Muri and Reinach, in Switzerland were the focus of the study. The
138 location of the study sites is shown in Figure S1 of the Supplementary Material, with information
139 about treatment processes, catchment size and land use provided in Table S1. All selected sites had
140 an average dry weather flow rate greater than 10 L/s, had no input of treated wastewater upstream
141 and greater than 20% wastewater input downstream during low flow conditions. For further details
142 and exact sampling locations refer to Burdon et al. (2016) and Stamm et al. (2016). Within each site
143 there was comparable land use (Table S1), morphology and river bank vegetation upstream and
144 downstream and all WWTPs applied biological activated sludge treatment. At each site, grab water
145 samples were collected in glass bottles upstream and downstream of the WWTP during low flow
146 conditions, as well as from the effluent stream, on the 19$^{th}$ May 2014. Eighteen liters of water were
147 collected at both the upstream and downstream sites, while 10.5 L of effluent was sampled, with all
148 samples collected within an hour at each site. The upstream samples were collected directly before
149 the WWTP, while the downstream samples were collected approximately 50 to 300 m from the
150 effluent discharge once complete mixing was obtained, which was assessed by conductivity
151 measurements. Nanopure water was used as the process control. All samples were stored in the dark
152 at 4°C or -20°C prior to extraction, with samples stored at 4°C extracted within 96 h of sampling.

153

154 *2.2. Chemical Analysis*

155 400 individual chemicals in the water samples were analyzed using online solid phase extraction
156 (SPE) liquid chromatograph-high resolution tandem mass spectrometry (LC-HR-MS/MS) based on
157 the method described in Huntscha et al. (2012) with some modifications. Briefly, the water samples
158 were thawed at room temperature and filtered with 0.7 µm pore size glass fiber filters (Whatman,
159 United Kingdom). Chemicals sorbed to particulate matter were not considered in this study, with
160 samples for bioanalysis also filtered prior to extraction. Effluent samples were diluted by a factor of
161 4 with nanopure water to minimize matrix effects. After the addition of internal standards to
162 account for any loss during sample preparation, 20 mL aliquots were automatically enriched using
163 SPE, with the pH of the aliquots automatically adjusted to pH 7 with 80 µL of 0.5 M citrate buffer
164 prior to enrichment. The online SPE cartridges were manually packed with 9 mg of Strata X-CW,
165 Strata X-AW and Isolute Env+ at a ratio of 1:1:1.5 (Phenomenex, Switzerland; Biotage, Sweden) at
166 the bottom and 9 mg of Oasis HLB (Waters, US) as the top layer in the enrichment flow direction.
167 Elution was performed in back-flush mode with methanol containing 0.1% formic acid. Five
168 additional estrogenic compounds were analyzed in the effluent samples using a dedicated LC-
169 MS/MS method (Vermeirssen et al., 2005). The samples for estrogenic analysis were enriched using

170 LiChrolut EN RP-18 SPE cartridges (Merck, Germany) based on the protocol in Margot et al.
171 (2013). The list of analyzed chemicals is shown in Table S2. Detection limits are provided in ng/L,
172 but the detected chemicals were converted to molar units for mixture toxicity modeling.
173
174 *2.3. Sample Extraction for Bioanalysis*
175 To extract a wide range of contaminants from the water samples for bioanalysis, multi-layer SPE
176 cartridges were manually filled with three layers: 1) 200 mg of EnviCarb, 2) 350 mg of Strata X-
177 CW, Strata X-AW and Isolute Env+ at a ratio of 1:1:1.5 (Phenomenex, Switzerland; Biotage,
178 Sweden), and 3) 200 mg of Oasis HLB (Waters, US) as the first material in the enrichment flow
179 direction. To extract the large water volume needed for bioanalysis, several cartridges were
180 extracted in parallel to enrich 7.5 L of effluent and 15 L of surface water upstream and downstream,
181 respectively. The same volume of nanopure water was extracted for the process control. The
182 extraction protocol was based on Kern et al. (2009) with some modifications. Briefly, the pH of the
183 water samples was adjusted to 6.5-6.7 prior to filtration with 0.7 µm pore size glass fiber filters (47
184 mm diameter, Whatman, United Kingdom). The SPE cartridges were conditioned with 5 mL
185 methanol followed by 10 mL nanopure water. For each cartridge 1.5 L of upstream or downstream
186 sample or 0.5 L of effluent sample was extracted by passing the sample under vacuum. After
187 drying, the cartridges were inverted to elute them in the opposite flow direction in three steps with 6
188 mL of ethyl acetate/methanol (50:50) containing 0.5% ammonia, 3 mL of ethyl acetate/methanol
189 (50:50) containing 1.7% formic acid and 2 mL of methanol. The combined neutral extracts were
190 evaporated at 40°C under a stream of nitrogen and split into the corresponding volumes for
191 bioanalysis, then evaporated to dryness prior to shipping. The composition of the offline SPE
192 cartridge used for bioanalysis differed from the online SPE cartridges used for chemical analysis
193 due to the higher volume requirements, but for comparison 14 of the analyzed chemicals were also
194 analyzed in the offline SPE extracts after the addition of internal standard. The properties of the
195 analyzed compounds included a range of octanol-water partition coefficients (log $K_{OW}$ -1.0 to 5.0)
196 and speciation. Some variability was observed between the two methods for some compounds in the
197 low pM concentration range, such as atrazine, but overall, the comparison indicates that the
198 chemical concentration in the extracts used for bioanalysis was representative of the analyzed
199 chemical concentration (Figure S2). In addition, samples for the AChE inhibition assay and the
200 combined algae assay were enriched using LiChrolut EN RP-18 SPE cartridges (Merck, Germany)
201 based on the protocol in Margot et al. (2013).
202
203
204

205 *2.4. Bioanalysis*

206 The water extracts were analyzed in 11 bioassays, representing 13 different endpoints. A summary
207 of the applied bioassays can be found in Table 1, with detailed information provided in Section S1.

208

209 *2.5. Data Evaluation*

210 The data were evaluated using linear concentration-effect curves to determine the concentration
211 causing 10% effect ($EC_{10}$) or effect concentration causing an induction ratio of 1.5 ($EC_{IR1.5}$) (Escher
212 et al., 2014). The zebrafish embryo acute toxicity assay was tested at a single concentration and %
213 effect was reported. For the adaptive stress response endpoints no maximum effect can be reached
214 and the effect is reported as an induction ratio (IR). Concentration-effect curves for adaptive stress
215 responses are typically linear up to an IR of 5 and therefore the $EC_{IR1.5}$ is interpolated from a linear
216 regression through IR 1, the control effect (Escher et al., 2012). For endpoints where the effects can
217 be converted to % maximum effect, the concentration-effect curves typically have log-sigmoidal
218 shapes and can be fitted with probit, logit or Weibull models (Scholze et al., 2001). Since log-
219 sigmoidal concentration-effect curves may be considered linear in the lower portion of the curve,
220 responses up to 30 to 40% effect can be plotted using linear concentration-effect curves (Escher et
221 al., 2014). Thus, for consistency, in the current study, all assays were evaluated using linear
222 concentration-effect curves with the equations described in detail in Escher et al. (2014). As the
223 concentration of all chemicals in the sample was unknown, units of relative enrichment factor
224 (REF) were used, which take into account sample enrichment by SPE and dilution in the assay
225 (Escher and Leusch, 2012).

226 For mixture toxicity modeling, $BEQ_{bio}$ was calculated using the EC values of the reference
227 compound (ref) and the matching EC value of the sample (Equation 1). The error associated with
228 $BEQ_{bio}$ was determined using error propagation.

229

$$BEQ_{bio} = \frac{EC_{10}(\text{ref})}{EC_{10}(\text{sample})} \text{ or } \frac{EC_{IR1.5}(\text{ref})}{EC_{IR1.5}(\text{sample})}$$

230 (1)

231

232 To assess the contribution of the detected chemicals to the biological effect, $BEQ_{bio}$ can be
233 compared to $BEQ_{chem}$. This involves calculating the relative effect potency ($REP_i$) of the detected
234 chemicals using the EC value of the reference compound and the EC value of the detected chemical
235 (i) (Equation 2). EC values were collected from the peer-reviewed literature or the US EPA
236 ToxCast database (US EPA, 2015). EC values from the literature were usually provided as $EC_{50}$
237 values, while raw fluorescence data was available in the ToxCast MySQL database, allowing

238 $EC_{IR1.5}$ values to be calculated for the adaptive stress response assays using linear concentration-
239 effect curves. $EC_{10\ absolute}$ values for the activation of 10% maximum effect in the AR assay were
240 derived using the 50% activity concentration ($AC_{50}$) values provided in the ToxCast database and
241 the hill top of the concentration-effect curve (Equation 3).
242

$$REP_i=\frac{EC_{50}(ref)}{EC_{50}(i)} \text{ or } \frac{EC_{IR1.5}(ref)}{EC_{IR1.5}(i)} \text{ or } \frac{EC_{10}(ref)}{EC_{10}(i)}$$

243 (2)

$$\log\ EC_{10\ absolute}=\log AC_{50}+\log\left(\frac{10\%}{\text{Hill top-}10\%}\right)$$

244 (3)
245
246 The detected chemical concentration ($C_i$) in molar units and the calculated $REP_i$ (also derived from
247 molar ratios) were used to determine $BEQ_{chem}$ (Equation 4).
248

$$BEQ_{chem}=\sum_{i}^{n} REP_i \cdot C_i$$

249 (4)
250
251 To assess whether the chemical concentration or biological effects at the downstream sites deviated
252 from what would be expected based on pure physical mixing of upstream water and WWTP
253 effluent, the mean fraction of wastewater effluent downstream ($f_{eff}$) was calculated using $C_i$ or
254 $BEQ_{bio}$ for the upstream, downstream and effluent samples (Equation 5). For chemical analysis only
255 chemicals detected in all three samples (upstream, effluent and downstream) were used to calculate
256 $f_{eff}$, with the mean $f_{eff}$ based on individual chemicals reported for each site. In some cases, there was
257 no substantial difference in the detected concentration in the samples when considering analytical
258 uncertainty, leading to a $f_{eff}$ outside of the range of 0 to 1. These values, which were all in the low
259 ng/L range, were excluded from the $f_{eff}$ calculation. Likewise for $f_{eff}$ derived from $BEQ_{bio}$, only
260 those bioassays that were above the detection limit in all three samples were used. Therefore
261 activation of AR and algal growth inhibition could not be included. The uncertainties associated
262 with the $f_{eff}$ estimates were quantified by error propagation of the measurement uncertainty, as
263 described in detail in Section S2.
264
265

$$f_{eff}=\frac{C_{i,\ down}-C_{i,\ up}}{C_{i,\ effluent}-C_{i,\ up}} \text{ or } f_{eff}=\frac{BEQ_{bio,\ down}-BEQ_{bio,\ up}}{BEQ_{bio,\ effluent}-BEQ_{bio,\ up}}$$

266 (5)

267

268 **3. Results and Discussion**

269 *3.1. Chemical Analysis*

270 Four hundred chemicals were analyzed in the upstream and downstream samples, while a subset of
271 78 of the 400 chemicals were also analyzed in the wastewater effluent. Further, 5 estrogenic
272 chemicals were only analyzed in the effluent samples, as these compounds are often present at or
273 below the analytical limit of quantification in surface water. In total, 191 chemicals were detected at
274 least once, with the detected concentrations provided in Table S3. Overall, 57 chemicals were
275 detected at least once in the upstream samples and 185 chemicals were detected at least once in the
276 downstream samples. Despite the fact that fewer chemicals were analyzed, the highest percentage
277 of detected chemicals and the highest total chemical concentrations were found in the effluent
278 sample at all three sites (Figure 1).

279 Based on the 78 chemicals analyzed in all samples, the sum concentration in the effluent
280 samples ranged from 74.7 nM (16.2 µg/L, 58 compounds) to 103 nM (23.1 µg/L, 62 compounds),
281 while the sum concentration in the downstream samples ranged from 18.5 nM (4.11 µg/L, 63
282 compounds) to 24.6 nM (5.47 µg/L, 65 compounds). The upstream samples had the lowest sum of
283 chemical concentrations and the lowest number of detected chemicals, with sum concentrations
284 ranging from 0.37 nM (0.10 µg/L, 16 compounds) to 2.18 nM (0.67 µg/L, 20 compounds). The
285 most prevalent chemical class in the effluent was corrosion inhibitors, followed by pharmaceuticals.
286 Pharmaceuticals and corrosion inhibitors were also the predominant chemicals detected downstream
287 with pesticide concentrations up to two orders of magnitude lower. The upstream chemical profile
288 differed from the effluent and downstream samples, with pesticides contributing to 43 to 90% of the
289 sum of chemical concentrations upstream (Figure S3). It should be noted that the concentration of
290 many pesticides was similar in both the upstream and downstream samples, but the presence of
291 other chemical classes meant that the contribution of pesticides to the total micropollutant
292 concentration downstream was low (1.3 to 13%). The difference in chemical profiles and sum of
293 concentrations upstream and downstream clearly shows the influence of wastewater effluent, as
294 well as other micropollutant sources (e.g. agriculture), on the receiving streams.

295

296 *3.2. Bioanalysis*

297 The EC values, provided in units of REF, for all samples are shown in Table S4 and Figure S4,
298 along with the concentration-effect curves in Figure S5. To demonstrate the applicability of linear

299 concentration-effect curves, $EC_{10}$ values calculated from linear concentration-effect curves at low
300 effect levels (<40%) were compared to $EC_{10}$ values determined by evaluating the same data using
301 log-sigmoidal concentration-effect curves for the activation of ER (MELN), activation of AR and 2
302 h PSII inhibition assays in Figure S6. Reasonable agreement between the two evaluation methods
303 was observed, supporting the use of linear concentration-effect curves in the current study (Figure
304 S6). Cell viability was measured in parallel to oxidative stress, p53 and NF-κB induction (Figures
305 S7-S9) and only concentrations less than the cytotoxicity $EC_{10}$ were included in the linear
306 concentration-effect assessment to determine $EC_{IR1.5}$. Cytotoxicity masked induction for all samples
307 in the p53 response assay (Figure S8), so it was not possible to derive $EC_{IR1.5}$ values. Previous
308 studies have also found a small window between p53 induction and cytotoxicity for the assay
309 (Neale et al., 2015c; Yeh et al., 2014). Cytotoxicity was also evaluated for the activation of AhR,
310 activation of ER and activation of AR assays (data not shown) and only non-cytotoxic values were
311 reported below.

312 Activation of ER (MELN) and NF-κB response assays were among the most responsive
313 assays, with mutagenicity being the least responsive endpoint; effects were observed in the
314 Birmensdorf and Reinach downstream samples only. The measurement of activation of ER using
315 the human MELN assay identified WWTP effluents as a major source of estrogenic compounds to
316 the aquatic environment at all studied sites. The estrogenic activities measured were similar among
317 the three WWTP effluents and were systematically higher than at the upstream and downstream
318 sites, the latter being clearly impacted by effluent discharges. In addition to activation of ER,
319 activation of AR was also assessed, but was less responsive, with only the effluent samples and
320 Muri downstream having a response in the assay. The MDA-kB2 cell line also contains
321 glucocorticoid receptors (GR) (Wilson et al., 2002), but exposure of the samples in the presence of
322 AR antagonist flutamide abolished the effect of the active samples, indicating that the observed
323 effects are really androgenic and not related to activation of GR. Previous studies have also shown
324 low or no AR activation in water samples compared to ER activation (Leusch et al., 2014; Scott et
325 al., 2014). Further, as some environmental estrogenic compounds are also antiandrogens, this could
326 lead to an underestimation of AR activation (Sohoni and Sumpter, 1998).

327 While this study further confirmed the usefulness of reporter gene estrogenic assays to
328 identify and characterize sources of estrogenic compounds in aquatic systems, it is also important to
329 determine whether fish can be affected after exposures to such levels of estrogenic contamination.
330 For example, Sonavane et al. (2016) recently showed fish-specific estrogenic responses in some
331 environmental samples when comparing human and zebrafish *in vitro* ER activation assays. As a
332 consequence, the samples were also tested in the transgenic *cyp19a1b*-GFP zebrafish embryo assay.
333 At the upstream and downstream sites, where quantifications of the ER activation using the MELN

334 assay were low, no induction of the brain aromatase was observed. Conversely, Muri effluent
335 induced a concentration-dependent induction of the ER-regulated brain aromatase gene showing
336 that the level of estrogenic contamination was sufficient to induce an estrogenic response in the
337 developing brain of fish. In contrast, no estrogenic activity was recorded in zebrafish for the
338 Birmensdorf and Reinach effluents, despite all three effluents having similar ER activation
339 responses in the MELN assay. This was due to the occurrence of mortality caused by the
340 Birmensdorf and Reinach effluents after 96 h exposure at REFs as low as 4.5 and 4.8, respectively.
341 These data highlight the relevance of using whole organism assays complementary to reporter gene
342 assays, as it allows the identification of samples that can induce both estrogenic effects and/or
343 developmental toxicity in fish.

344 All samples had an effect in the zebrafish embryo acute toxicity assay, though there was no
345 significant difference in lethal effect between all sites after 120 h (Figure S10). Due to volume
346 demands, the assay was only run at a single concentration, so EC values could not be determined.
347 The applied REFs ranged from 25 to 50, which were higher than the maximum REF applied in the
348 transgenic c*yp19a1b*-GFP zebrafish assay, where mortality was observed in the Birmensdorf and
349 Reinach effluents at lower REFs (4.4 and 5.8, respectively). The result was also unusual as very
350 little effect was observed at 24 and 48 h, though some sublethal effects, including malformations
351 and reduced blood circulation, were observed at 48 h (Figure S10).

352 The majority of assays showed a clear trend of highest response in the effluent samples, with
353 a reduced effect in the downstream samples and the lowest effect in the upstream samples. This
354 result was expected and fits well with the detected chemical concentrations in Figure 1. One
355 exception was the NF-κB response assay at Muri, where upstream was the most potent sample,
356 followed by downstream and then effluent. The NF-κB response assay has only recently been
357 applied for water quality monitoring (Escher et al., 2014; Neale et al., 2015a) and it is still unclear
358 which environmental compounds activate this assay. For example, of the 191 detected chemicals,
359 135 were included in the ToxCast database and all were either inactive or cytotoxic in the NF-κB
360 response assay (US EPA, 2015). It should be pointed out that the NF-κB response assay in the
361 ToxCast database is based on the ME180 cervical cancer cell line, while the THP-1 monocytic cell
362 line was used in the current study. Thus, before this assay is used further for water quality
363 monitoring, more work is required to better understand which environmental compounds are
364 inducing a response in this assay.

365 In any case, the Muri upstream sample appears to be more potent than the other upstream
366 samples, despite having a comparable sum chemical concentration (0.98 nM, 29 chemicals), with a
367 higher response in the oxidative stress response assay and low level mortality in the *cyp19a1b*-GFP
368 zebrafish embryo assay, which was not observed at the other upstream sites. Further, the algal

369 growth assay is of particular interest at the Muri upstream sample. For all other sites, algal growth
370 inhibition was less sensitive than PSII inhibition and this fits with previous results in the literature
371 where it was shown that PSII inhibiting herbicides typically dominate the biological effect on
372 photosynthesis (Escher et al., 2008a; Tang and Escher, 2014). However, the opposite is observed at
373 Muri upstream, with increased algal growth inhibition, which suggests that the contaminants are not
374 only PSII inhibiting herbicides, but also potentially other herbicides with different modes of action,
375 such as amino acid biosynthesis inhibition, or chemicals that have a non-specific effect on the algae.
376 This example demonstrates the value of applying bioanalytical tools complementary to chemical
377 analysis for water quality monitoring as they are able to indicate the presence of potent unidentified
378 chemicals.

379 As well as containing micropollutants, environmental waters may also contain dissolved
380 organic carbon (DOC), which may interfere with bioassays. For example, co-extracted DOC has
381 previously been shown to cause experimental artefacts in the enzymatic AChE inhibition assay,
382 with concentrations as low as 2 mg of carbon per liter ($mg_C/L$) suppressing the agonist response
383 (Neale and Escher, 2013). The DOC extraction efficiency of LiChrolut EN RP-18 SPE cartridges,
384 which were used for the AChE inhibition assay, has not been assessed previously, but the
385 commonly used Oasis HLB SPE cartridge can co-extract between 40-70% DOC (Neale and Escher,
386 2013). The DOC concentration in the studied water samples ranged from 2.2 to 11.8 $mg_C/L$, with
387 samples enriched 500 to 1000 times by SPE. If only 10% of the DOC was co-extracted by SPE, this
388 would yield a DOC concentration from 220 to 590 $mg_c/L$ in the extracts. After dilution in the assay,
389 the lowest applied REF of 5.2 would still have a co-extracted DOC concentration of 2.6 $mg_C/L$.
390 This suggests that DOC is most likely interfering with the AChE inhibition assay and therefore the
391 assay will not be used further for mixture toxicity modeling. The effect of DOC has been previously
392 assessed for cell based assays, with negligible effect found in agonist mode (Neale and Escher,
393 2014), though DOC can potentially interfere with assays run in antagonist mode (Neale et al.,
394 2015b). Additional sample pre-treatment steps, such as fractionation, may help to reduce
395 experimental artifacts associated with DOC (Ouyang et al., 2016).
396

397 *3.3. Assessing the Fraction of Wastewater Effluent Downstream*

398 The fraction of wastewater effluent downstream ($f_{eff}$) was determined based on both chemical
399 analysis and bioanalysis. Assuming pure mixing without any elimination during the small spatial
400 and temporal range the same $f_{eff}$ would be expected based on both chemical analysis and
401 bioanalysis. For chemical analysis, mean $f_{eff}$ were calculated from 10 to 11 individual chemicals
402 that were detected in the upstream, effluent and downstream samples at each site (Table 2, Figure
403 S11). Based on the individual chemicals at each site, mean $f_{eff}$ ranged from 0.20±0.06 to 0.30±0.10,

404 which indicates a substantial influence of effluent on the downstream site. The chemical classes of
405 the individual chemicals used to calculate mean $f_{eff}$ included pesticides, pharmaceuticals, corrosion
406 inhibitors and food additives.

407 For the $f_{eff}$ calculated from bioassays, the EC values were converted to $BEQ_{bio}$ (Table S5),
408 which provides the concentration of a reference compound in molar units that would have the same
409 response as the sample extract. Figure 2 shows a good agreement between the mean $f_{eff}$ calculated
410 from individual chemicals and $f_{eff}$ calculated from $BEQ_{bio}$ for activation of ER, 2h PSII inhibition
411 and oxidative stress response. For example, $f_{eff}$ for 2 h PSII inhibition ranged from 0.22±0.13 to
412 0.26±0.21, which fits within the range of $f_{eff}$ calculated for individual PSII herbicides (0.15 to 0.45)
413 (Table 2). In contrast, $f_{eff}$ for activation of AhR at Muri and Reinach were higher than mean $f_{eff}$
414 based on chemical analysis, with $f_{eff}$ up to 0.87±0.47 at Reinach. However, there was high
415 uncertainty associated with the calculation of $f_{eff}$ for $BEQ_{bio}$ for the AhR assay (coefficients of
416 variance ranging from 56 to 258%). This was due to the small differences seen between the
417 $BEQ_{bio,up}$ and $BEQ_{bio,down}$ as well as $BEQ_{bio,up}$ and $BEQ_{bio,eff}$ for AhR at all sites resulting in very
418 small numbers for both the nominator and denominator in Equation 5, with associated large errors
419 of the resulting $f_{eff}$.

420 Further, it should be noted that grab samples were used in this study to derive $f_{eff}$. Therefore,
421 potential daily variations in effluent discharge may alter $f_{eff}$, though the fact that $f_{eff}$ was similar for
422 all three sites supports the application of the current approach under low flow conditions.
423 Nevertheless, overall we conclude that there is a reasonable agreement of the mass balance over
424 upstream, downstream and effluent locations for $BEQ_{bio}$ and the chemical analysis. This has been
425 previously observed for chemical analysis, with Fairbairn et al. (2016) finding that the downstream
426 composition could be generally explained by physical mixing of upstream river water and
427 wastewater effluent using a mass balance approach.
428

429 *3.4. Mixture Toxicity Modeling*

430 Mixture toxicity modeling was applied to determine if the detected chemicals were contributing
431 significantly to the observed effect. Between 2 and 26 EC values were found in the literature or
432 ToxCast database for the detected chemicals in the studied assays, respectively. Out of the 191
433 detected chemicals, 135 and 142 chemicals were included in the ToxCast database for the oxidative
434 stress response and activation of AR assays, respectively (Table S6). For the oxidative stress
435 response assay, 26 of the detected chemicals were active, 109 were inactive and no information was
436 available for 56 chemicals, while only 6 were active in the activation of AR assay, with 136 inactive
437 and no information available for 49 chemicals. As discussed above, none of the detected chemicals
438 in the ToxCast database had a response in the NF-κB response assay so it was not possible to

439 determine $BEQ_{chem}$. Large screening datasets with known active and inactive chemicals were not
440 available for the other studied assays, with available EC values instead collected from the literature.
441 The available EC values were used to calculate $REP_i$ values (Table S7), which, along with detected
442 chemical concentrations, were used to calculate $BEQ_{chem}$ (Table 3). $BEQ_{bio}$ and $BEQ_{chem}$ were
443 compared to assess what fraction of the effect could be explained by detected chemicals (Table 3),
444 with the percent contribution of individual detected chemicals shown in Figure 3 for activation of
445 AhR, activation of ER (MELN), activation of AR, 2 h PSII inhibition, algal growth inhibition and
446 oxidative stress response. The contribution of detected chemicals to 24 h PSII inhibition is shown in
447 Figure S12. Overall, the availability of effect data for the analyzed compounds presented a
448 limitation for mixture toxicity modeling.
449 By comparing $BEQ_{bio}$ and $BEQ_{chem}$, up to 30% of AhR activation at the Birmensdorf site
450 could be explained by the fungicide propiconazole, with the herbicide terbuthylazine contributing to
451 12% of effect in the Muri effluent. With the exception of the insecticide diazinon, the literature EC
452 values were determined using an AhR assay based on a mouse model, while an AhR assay based on
453 a rat model was used in the current study. Consequently, differences in species sensitivity and
454 selectivity may potentially limit the utility of mixture toxicity modeling for this assay.
455 Numerous studies have shown that a significant fraction of ER activation in wastewater and
456 surface water can be explained by chemicals including natural and synthetic hormones,
457 alkylphenols and phytoestrogens (Murk et al., 2002; Neale et al., 2015a; Rutishauser et al., 2004).
458 However, only a small fraction of the effect could be explained for the ER activation assay in the
459 current study as the chemical analysis focused primarily on emerging contaminants, such as
460 pharmaceuticals and pesticides, rather than natural and synthetic estrogenic contaminants, with only
461 five estrogenic chemicals analyzed in the effluent samples. Three estrogenic chemicals, estrone,
462 bisphenol A and 4-nonylphenol, were detected in the effluent and could explain only up to 0.4% of
463 the observed effect. Potent estrogenic chemicals 17α-ethinyl estradiol and 17β-estradiol were also
464 analyzed in wastewater effluent, but were not detected above the limit of quantification. The
465 detection limits for 17α-ethinyl estradiol and 17β-estradiol were in the low ng/L range (0.3 and 0.1
466 ng/L, respectively), though the detection limit for 17α-ethinyl estradiol is still over an order of
467 magnitude higher than the proposed environmental quality standard of 0.035 ng/L (European
468 Commission, 2012). The measured $BEQ_{bio}$ values are similar to previously measured $BEQ_{bio}$ values
469 for surface water and wastewater (Jugan et al., 2009) and shows that the activation of ER assay is a
470 sensitive tool to detect the presence of estrogenic compounds and can be used complementary to
471 chemical analysis that is often not sensitive enough for the low effect thresholds. In comparison,
472 few studies have assessed the contribution of the detected chemicals to the activation of AR. Bellet
473 et al. (2012) found that detected steroidal hormones, none of which were analyzed in the current

474 study, could only explain up to 5.5% of AR activity in raw wastewater. In the current study, EC
475 values were available for six of the detected chemicals in the ToxCast database, with only up to
476 0.4% of AR activity explained.

477 The majority of PSII inhibition at 2 h was explained by ten of the detected chemicals, with
478 the herbicides diuron and terbuthylazine mostly contributing to the effect. Previous studies have
479 also shown that detected herbicides can often explain the majority of PSII inhibition (Bengtson
480 Nash et al., 2006; Escher et al., 2011) as only PSII herbicides will have an effect in the assay after
481 the short exposure time. At all three sites, a lower fraction of the effect could be explained in the
482 upstream samples, which could suggest the presence of additional undetected PSII inhibitors.
483 Thirteen further PSII inhibitors, including bromacil and hexazinone, were analyzed in the upstream
484 and downstream samples, but were not detected above the limit of quantification; however, it is
485 possible that the presence of low concentrations of these compounds could still contribute to the
486 effect. Further, herbicide transformation products may also contribute to the effect; however, as can
487 be seen in Table S7, transformation products, such as atrazine-desethyl and atrazine-desisopropyl,
488 are often less potent than their parent compounds. In contrast to PSII inhibition, only up to 73% of
489 algal growth inhibition was explained by 7 detected compounds, which were all PSII inhibiting
490 herbicides. Thus, the presence of other detected herbicides, such as mecoprop and metolachlor, may
491 have contributed to the effect on growth rate, but EC values were not available in the studied assay.
492 EC values for up to 26 of the detected chemicals were available in the ToxCast database, though
493 only 1.9% of the oxidative stress response could be explained. This discrepancy has been observed
494 previously for wastewater, surface water and swimming pool water (Escher et al., 2014; Neale et
495 al., 2015a; Yeh et al., 2014) and demonstrates that many compounds can induce the oxidative stress
496 response. For example, 22.6% of the 7522 analyzed chemicals in the ToxCast database were active
497 in the oxidative stress response assay (US EPA, 2015). As different chemical classes can induce
498 oxidative stress, it was possible to see different effect profiles upstream and downstream. For
499 example, the pharmaceutical diclofenac only contributed to the effect in the effluent and
500 downstream samples, while the fungicide propiconazole contributed more upstream than
501 downstream at the Birmensdorf site. The herbicide metolachlor contributed to 1.5% and 0.7% of the
502 oxidative stress response in the Muri effluent and downstream samples, respectively, with minimal
503 contribution in the Muri upstream sample, indicating that wastewater discharge is the likely source.
504

505 **4. Conclusions**

506 The combination of chemical analysis and bioanalysis proved to be a valuable complementary
507 approach to monitor the micropollutant burden in the aquatic environment. Bioanalysis provided

508 information about the mixture effects of additional chemicals in the samples, while the chemical
509 analysis showed differences in the chemical pollution profiles at the different sampling locations.
510 Mixture toxicity modeling was performed to assess the contribution of detected chemicals to
511 the observed effect, with the fraction explained varying for the different assays. The lack of effect
512 data for the detected micropollutants in the different assays was a major limitation and future work
513 should focus on fingerprinting the effect of common water pollutants including the question of
514 which chemicals and which biological endpoints a comprehensive effect analysis would encompass.
515 Further, this study shows that micropollutants, including pharmaceuticals, pesticides and
516 corrosion inhibitors, are being discharged into small streams at nanomolar concentrations, with
517 WWTPs as one of the main sources during low flow conditions. While less contaminated, the
518 upstream sites were far from pristine, with agriculture contributing to the detected chemicals, as
519 indicated by elevated concentrations of some pesticides detected, which was also reflected in the
520 bioanalytical results. To our knowledge we have for the first time expanded a mass balance model
521 (Equation 5) used to determine the fraction of effluent to the overall volume of the creeks from
522 chemical analysis to bioassays. Results on the mass balance between up- and downstream samples
523 in relation to input by effluent were consistent between chemical analysis and bioassays. Both
524 chemical analysis and bioanalysis suggest that improved wastewater treatment technology will not
525 completely reduce the micropollutant burden, which renders additional source control measures also
526 necessary. Further, this study illustrates the relevance of the complementary approach to identify
527 and characterize micropollutant sources.
528

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769

770 Table 1: Summary of studied bioassays (REF: relative enrichment factor).

| Endpoint | Assay | Method reference | Positive reference compound | EC value | Positive reference compound $EC_{10}$ or $EC_{IR1.5}$ (M) | Maximum REF |
|---|---|---|---|---|---|---|
| Activation of AhR | CAFLUX | Nagy et al. (2002) | 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) | $EC_{10}$ | $(1.24 \pm 0.22)\times10^{-12}$ | 100 |
| Activation of ER (MELN) | MELN | Balaguer et al. (1999); Kinani et al. (2010) | 17β-Estradiol | $EC_{10}$ | $(1.74 \pm 0.44)\times10^{-12}$ | 84.7 |
| Activation of ER (zebrafish) | cyp19a1b-GFP | Brion et al. (2012) | 17β-Estradiol | $EC_{IR1.5}$ | $(1.29 \pm 0.07)\times10^{-10}$ | 17 |
| Activation of AR | MDA-kB2 | Wilson et al. (2002); Pavlíková et al. (2012) | Dihydrotestosterone (DHT) | $EC_{10}$ | $(3.12 \pm 0.50)\times10^{-11}$ | 50 |
| AChE inhibition | AChE inhibition | Ellman et al. (1961); Escher et al. (2008b) | Parathion | $EC_{10}$ | $(1.80 \pm 0.16)\times10^{-8}$ | 333 |
| 2 and 24 h PSII inhibition, algal growth inhibition | Combined algae assay with *Pseudokirchneriella subcapitata* | Escher et al. (2008a) | Diuron | $EC_{10}$ | 2 h: $(2.58 \pm 0.81)\times10^{-9}$<br>24 h: $(2.68 \pm 0.45)\times10^{-9}$<br>Growth: $(1.62 \pm 0.50)\times10^{-8}$ | 267 |
| Mutagenicity (TA98 + S9) | Ames fluctuation test | Reifferscheid et al. (2012) | 2-Aminoanthracene | $EC_{10}$ | $(7.52 \pm 2.57)\times10^{-8}$ | 400 |
| Oxidative stress response | ARE-*bla* | Neale et al. (2015a) | tert-Butylhydroquinone (tBHQ) | $EC_{IR1.5}$ | $(2.44 \pm 0.24)\times10^{-6}$ | 125 |
| p53 response | p53RE-*bla* | Neale et al. (2015c) | Mitomycin | $EC_{IR1.5}$ | $(4.53 \pm 0.15)\times10^{-8}$ | 125 |
| NF-κB response | NF-κB-*bla* | Jin et al. (2015) | Tumor necrosis factor alpha | $EC_{IR1.5}$ | $(2.00 \pm 0.40)\times10^{-2\dagger}$ | 125 |

|  |  |  | (TNFα) |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| Mortality | Zebrafish embryo toxicity test | OECD (2013) | 3,4-Dichloroaniline | - | - | 50 |

771 NB: No EC value could be derived for the zebrafish embryo toxicity test as it was tested at a single concentration only.

772 [†]in units of μg/L

773 Table 2: The fraction of wastewater effluent downstream ($f_{eff}$) for individual chemicals detected at
774 the three sites.

| Chemical | Birmensdorf | Muri | Reinach |
|---|---|---|---|
| 1H-Benzotriazole | 0.22 | | 0.31 |
| 5-Methyl-1H-benzotriazole | 0.23 | 0.23 | 0.32 |
| Atrazine | | 0.21 | |
| Atrazine-desethyl | | 0.45 | |
| Azoxystrobin | | | 0.24 |
| Carbendazim | 0.23 | | 0.36 |
| Caffeine | | 0.21 | |
| Cyprodinil | 0.20 | | 0.03 |
| Diazinon | 0.22 | | 0.30 |
| Diuron | | | 0.35 |
| Epoxyconazole | | 0.13 | |
| Hydrochlorothiazide | | | 0.30 |
| Mecoprop | 0.07 | 0.20 | 0.44 |
| Metolachlor | | 0.23 | |
| Propiconazole | 0.29 | | |
| Simazine | 0.15 | 0.22 | |
| Sucralose | | | 0.30 |
| Sulfamethoxazole | | 0.23 | |
| Tebuconazole | | 0.24 | |
| Telmisartan | 0.15 | | |
| Terbutylazine | 0.25 | 0.23 | 0.35 |
| *Mean ± standard deviation* | *0.20 ± 0.06* | *0.23 ± 0.08* | *0.30 ± 0.10* |
| *Number of individual chemicals (n)* | *10* | *11* | *11* |

775

776 Table 3: $BEQ_{bio}$ and $BEQ_{chem}$ values in molar units (M) for all samples in the different assays with percent effect that can be explained by the detected
777 chemicals (% effect).

| | | **Birmensdorf** | | | **Muri** | | | **Reinach** | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | **Upstream** | **Effluent** | **Down stream** | **Upstream** | **Effluent** | **Down stream** | **Upstream** | **Effluent** | **Down stream** |
| Activation of AhR | $BEQ_{bio}$ | $1.04\times10^{-13}$ | $1.96\times10^{-13}$ | $1.16\times10^{-13}$ | $9.43\times10^{-14}$ | $3.10\times10^{-13}$ | $2.00\times10^{-13}$ | $8.17\times10^{-14}$ | $2.38\times10^{-13}$ | $2.12\times10^{-13}$ |
| | $BEQ_{chem}$ | $3.10\times10^{-14}$ | $1.57\times10^{-14}$ | $2.61\times10^{-14}$ | $3.96\times10^{-16}$ | $3.88\times10^{-14}$ | $8.97\times10^{-15}$ | $1.83\times10^{-16}$ | $3.01\times10^{-15}$ | $5.92\times10^{-16}$ |
| | % effect | 29.7 | 8.0 | 22.5 | 0.4 | 12.5 | 4.5 | 0.2 | 1.3 | 0.3 |
| Activation of ER (MELN) | $BEQ_{bio}$ | $9.48\times10^{-13}$ | $7.28\times10^{-12}$ | $1.88\times10^{-12}$ | $1.65\times10^{-12}$ | $1.55\times10^{-11}$ | $3.49\times10^{-12}$ | $5.59\times10^{-13}$ | $1.53\times10^{-11}$ | $3.13\times10^{-12}$ |
| | $BEQ_{chem}$ | N/A | $2.27\times10^{-14}$ | N/A | N/A | $2.08\times10^{-14}$ | N/A | N/A | $5.56\times10^{-14}$ | N/A |
| | % effect | - | 0.3 | - | - | 0.1 | - | - | 0.4 | - |
| Activation of ER (zebrafish) | $BEQ_{bio}$ | $<9.84\times10^{-12}$ | Mortality | $<9.74\times10^{-12}$ | $<9.74\times10^{-12}$ | $7.89\times10^{-11}$ | $<9.77\times10^{-12}$ | $<7.60\times10^{-12}$ | Mortality | $<9.82\times10^{-12}$ |
| | $BEQ_{chem}$ | N/A | $3.90\times10^{-13}$ | N/A | N/A | $3.72\times10^{-13}$ | N/A | N/A | $1.06\times10^{-12}$ | N/A |
| | % effect | - | - | - | - | 0.5 | - | - | - | - |
| Activation of AR | $BEQ_{bio}$ | $<1.43\times10^{-12}$ | $8.89\times10^{-12}$ | $<1.42\times10^{-12}$ | $<1.42\times10^{-12}$ | $1.07\times10^{-11}$ | $2.97\times10^{-12}$ | $<1.25\times10^{-12}$ | $4.22\times10^{-11}$ | $<1.43\times10^{-12}$ |
| | $BEQ_{chem}$ | $1.60\times10^{-16}$ | $1.57\times10^{-14}$ | $3.32\times10^{-15}$ | $1.10\times10^{-16}$ | $3.80\times10^{-14}$ | $7.26\times10^{-15}$ | $2.09\times10^{-16}$ | $1.71\times10^{-15}$ | $5.01\times10^{-15}$ |
| | % effect | - | 0.2 | - | - | 0.4 | 0.2 | - | 0.04 | - |
| 2 h PSII Inhibition | $BEQ_{bio}$ | $4.38\times10^{-11}$ | $5.53\times10^{-10}$ | $1.58\times10^{-10}$ | $2.61\times10^{-11}$ | $1.11\times10^{-9}$ | $2.87\times10^{-10}$ | $3.35\times10^{-11}$ | $2.84\times10^{-10}$ | $9.83\times10^{-11}$ |
| | $BEQ_{chem}$ | $1.96\times10^{-11}$ | $5.21\times10^{-10}$ | $1.30\times10^{-10}$ | $1.18\times10^{-11}$ | $1.20\times10^{-9}$ | $2.89\times10^{-10}$ | $2.01\times10^{-11}$ | $1.92\times10^{-10}$ | $8.91\times10^{-11}$ |
| | % effect | 44.9 | 94.3 | 82.3 | 45.1 | 108 | 101 | 59.9 | 67.3 | 90.7 |
| 24 h PSII Inhibition | $BEQ_{bio}$ | $4.92\times10^{-11}$ | $7.97\times10^{-10}$ | $2.13\times10^{-10}$ | $3.06\times10^{-11}$ | $2.40\times10^{-9}$ | $7.97\times10^{-10}$ | $5.84\times10^{-11}$ | $4.72\times10^{-10}$ | $1.79\times10^{-10}$ |
| | $BEQ_{chem}$ | $1.68\times10^{-11}$ | $6.96\times10^{-10}$ | $1.60\times10^{-10}$ | $1.30\times10^{-11}$ | $1.39\times10^{-9}$ | $3.32\times10^{-10}$ | $1.78\times10^{-11}$ | $2.72\times10^{-10}$ | $1.28\times10^{-10}$ |
| | % effect | 34.2 | 87.3 | 75.1 | 42.4 | 57.6 | 41.7 | 30.6 | 57.7 | 71.4 |
| Algal growth | $BEQ_{bio}$ | $<1.80\times10^{-10}$ | $1.33\times10^{-9}$ | $4.54\times10^{-10}$ | $3.19\times10^{-10}$ | $6.73\times10^{-9}$ | $1.53\times10^{-9}$ | $<1.80\times10^{-10}$ | $1.24\times10^{-9}$ | $3.46\times10^{-10}$ |

| inhibition | $BEQ_{chem}$ | $3.04\times10^{-11}$ | $9.71\times10^{-10}$ | $2.20\times10^{-10}$ | $1.68\times10^{-11}$ | $1.83\times10^{-9}$ | $4.39\times10^{-10}$ | $2.65\times10^{-11}$ | $3.98\times10^{-10}$ | $1.98\times10^{-10}$ |
|---|---|---|---|---|---|---|---|---|---|---|
| | % effect | - | 73.0 | 48.6 | 5.30 | 27.2 | 28.7 | - | 32.2 | 57.2 |
| Oxidative | $BEQ_{bio}$ | $4.06\times10^{-8}$ | $1.43\times10^{-7}$ | $7.43\times10^{-8}$ | $5.89\times10^{-8}$ | $2.08\times10^{-7}$ | $1.09\times10^{-7}$ | $4.23\times10^{-8}$ | $2.73\times10^{-7}$ | $1.18\times10^{-7}$ |
| Stress | $BEQ_{chem}$ | $2.02\times10^{-10}$ | $6.51\times10^{-10}$ | $2.82\times10^{-10}$ | $3.53\times10^{-11}$ | $3.92\times10^{-9}$ | $9.06\times10^{-10}$ | $4.56\times10^{-11}$ | $5.77\times10^{-10}$ | $1.88\times10^{-10}$ |
| Response | % effect | 0.5 | 0.5 | 0.4 | 0.1 | 1.9 | 0.8 | 0.1 | 0.2 | 0.2 |

778

**List of Figures**

**Figure 1:** Sum chemical concentration detected at each site (nM) with the percentage of analyzed chemicals detected at each site (open diamonds). Four hundred compounds were analyzed in the upstream and downstream sites and 83 compounds were analyzed in the effluents.
*steroidal hormone estrone and two of the industrial chemicals, 4-nonylphenol and bisphenol A, were only analyzed in the effluents.

**Figure 2:** Fraction of wastewater effluent downstream ($f_{eff}$) calculated using $BEQ_{bio}$ values (error bars indicate standard deviation calculated using error propagation) compared to the mean $f_{eff}$ based on individual chemicals (solid black lines, with dashed lines indicating standard deviation).

**Figure 3:** Percent effect explained by individual detected chemicals for A) activation of AhR, B) activation of ER (MELN), C) activation of AR, D) 2 h PSII inhibition, E) algal growth inhibition and F) oxidative stress response. Note the different scales for the y-axes.
*estrogenic compounds were only measured in the effluent samples.

**Figure 1**

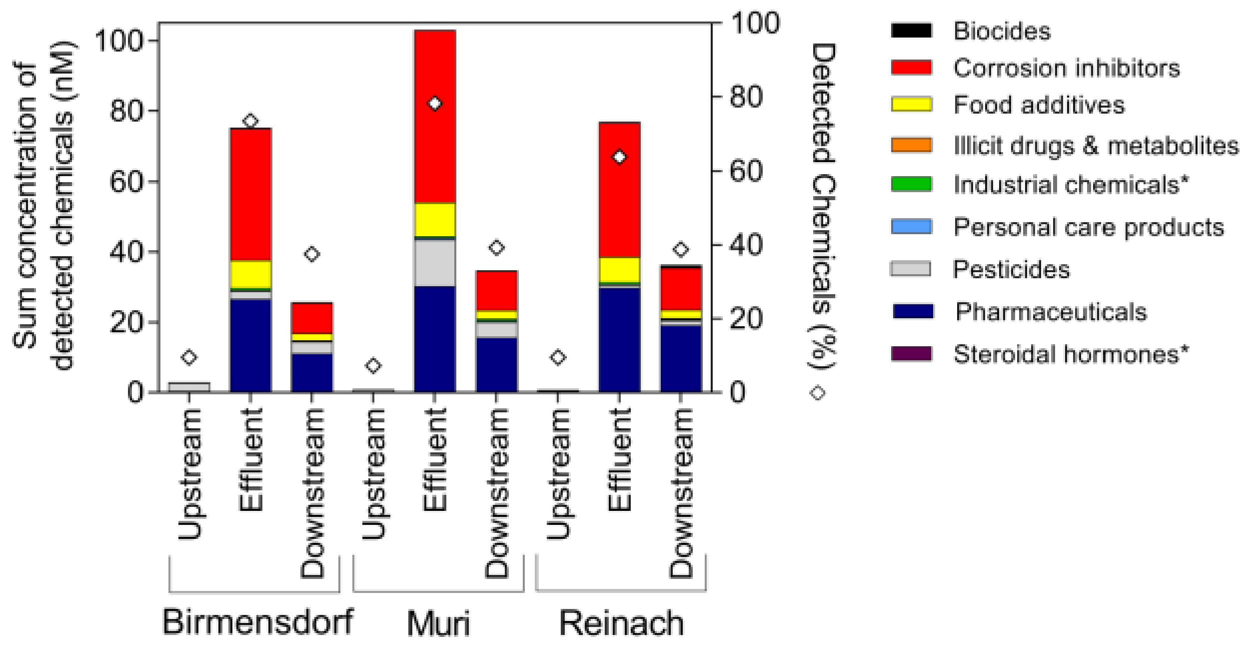

**Figure 2**

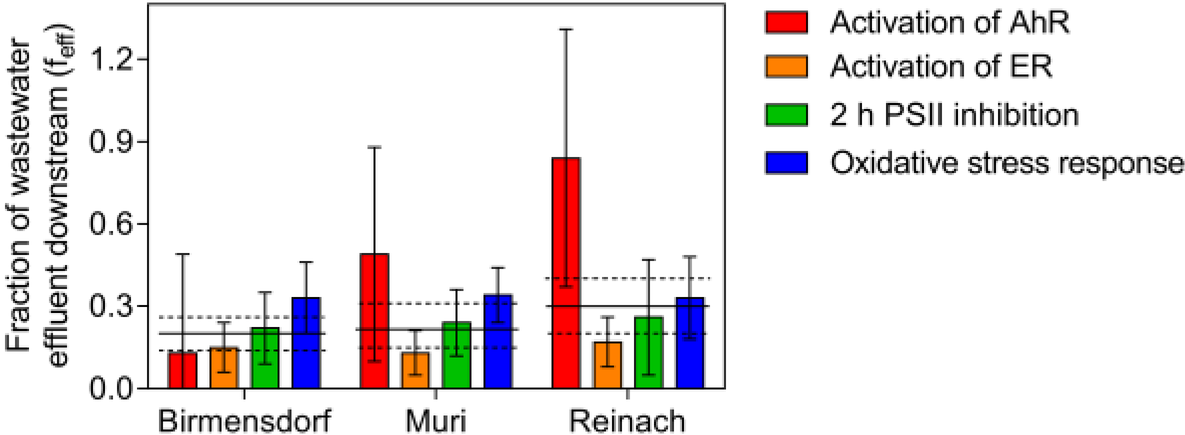

**Figure 3**