Integrating Chemical Analysis and Bioanalysis to Evaluate the Contribution of Wastewater 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

1. Introduction

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

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

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

equivalent concentrations from bioassays (BEQ_{bio}). For example, detected chemicals can often explain a high percentage of estrogenic activity (Leusch et al., 2014; Murk et al., 2002), while only a small fraction of non-specific effects or adaptive stress responses can typically be explained (Tang et al., 2013; Yeh et al., 2014).

In this study, both chemical analysis and bioanalysis were applied to assess the micropollutant burden in small streams upstream and downstream of three WWTPs, with the upstream sites not being affected by inputs of treated wastewater. The water samples were collected under low flow conditions to minimize the impact of diffuse sources. The analyzed chemicals were primarily pharmaceuticals and pesticides, with the other analyzed chemicals including biocides, food additives, illicit drugs, industrial chemicals and estrogens. The biological effects were evaluated using a suite of *in vitro* assays, which represent different cellular toxicity pathways, including xenobiotic metabolism, receptor-mediated effects, adaptive stress responses and cytotoxicity, as well as whole organism assays with algae and zebrafish embryos. Assays indicative of xenobiotic metabolism, such as activation of the aryl hydrocarbon receptor (AhR), and adaptive stress responses, such as the oxidative stress response, can respond to a range of compounds with different modes of action (Martin et al., 2010; US EPA, 2015). In contrast, assays indicative of receptor-mediated effects can provide information about the presence of chemicals with a common specific mode of action. For example, hormone-mediated effects including activation of the estrogen receptor (ER) and activation of the androgen receptor (AR) can detect natural and synthetic hormones, as well as other environmental endocrine disrupting compounds, which are often associated with wastewater (Vethaak et al., 2005). Further, assays indicative of inhibition of photosystem II (PSII) and of acetylcholinesterase (AChE) are more suitable to detect chemicals of an agricultural origin as they can detect PSII inhibiting herbicides (Escher et al., 2008a) and organophosphate and carbamate insecticides (Hamers et al., 2000), respectively.

The current study aimed to assess the impact of wastewater effluent on the micropollutant burden in small streams using a complementary chemical analysis and bioanalysis approach. Four hundred and five chemicals were analyzed and the applied test battery included assays indicative of activation of AhR, activation of the AR, activation of the ER, PSII and algal growth inhibition, AChE inhibition, mutagenicity and adaptive stress responses for oxidative stress, genotoxicity and inflammation, as well as assays indicative of estrogenic activity and developmental toxicity in zebrafish embryos. A mass balance approach was used to calculate the fraction of effluent downstream based on both chemical analysis and bioanalysis, while mixture toxicity modeling was applied to assess whether the detected chemicals were contributing to the biological effect.

2. Materials and Methods

2.1. Sampling

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

2.2. Chemical Analysis

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

LiChrolut EN RP-18 SPE cartridges (Merck, Germany) based on the protocol in Margot et al. (2013). The list of analyzed chemicals is shown in Table S2. Detection limits are provided in ng/L, but the detected chemicals were converted to molar units for mixture toxicity modeling.

2.3. Sample Extraction for Bioanalysis

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

2.4. Bioanalysis

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

2.5. Data Evaluation

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

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

$$BEQ_{bio} = \frac{EC_{10}(ref)}{EC_{10}(sample)} \text{ or } \frac{EC_{IR1.5}(ref)}{EC_{IR1.5}(sample)} \quad (1)$$

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

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

$$REP_i = \frac{EC_{50}(ref)}{EC_{50}(i)} \text{ or } \frac{EC_{IR1.5}(ref)}{EC_{IR1.5}(i)} \text{ or } \frac{EC_{10}(ref)}{EC_{10}(i)} \quad (2)$$

$$\log\ EC_{10\ absolute} = \log AC_{50} + \log\left(\frac{10\%}{\text{Hill top-}10\%}\right) \quad (3)$$

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

$$BEQ_{chem} = \sum_{i}^{n} REP_i \cdot C_i \quad (4)$$

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

$$f_{eff} = \frac{C_{i,\,down} - C_{i,\,up}}{C_{i,\,effluent} - C_{i,\,up}} \text{ or } f_{eff} = \frac{BEQ_{bio,\,down} - BEQ_{bio,\,up}}{BEQ_{bio,\,effluent} - BEQ_{bio,\,up}} \quad (5)$$

3. Results and Discussion

3.1. Chemical Analysis

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

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

3.2. Bioanalysis

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

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

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

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

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

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

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

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

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

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

3.3. Assessing the Fraction of Wastewater Effluent Downstream

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

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

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

4. Conclusions

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

information about the mixture effects of additional chemicals in the samples, while the chemical analysis showed differences in the chemical pollution profiles at the different sampling locations.

Mixture toxicity modeling was performed to assess the contribution of detected chemicals to the observed effect, with the fraction explained varying for the different assays. The lack of effect data for the detected micropollutants in the different assays was a major limitation and future work should focus on fingerprinting the effect of common water pollutants including the question of which chemicals and which biological endpoints a comprehensive effect analysis would encompass.

Further, this study shows that micropollutants, including pharmaceuticals, pesticides and corrosion inhibitors, are being discharged into small streams at nanomolar concentrations, with WWTPs as one of the main sources during low flow conditions. While less contaminated, the upstream sites were far from pristine, with agriculture contributing to the detected chemicals, as indicated by elevated concentrations of some pesticides detected, which was also reflected in the bioanalytical results. To our knowledge we have for the first time expanded a mass balance model (Equation 5) used to determine the fraction of effluent to the overall volume of the creeks from chemical analysis to bioassays. Results on the mass balance between up- and downstream samples in relation to input by effluent were consistent between chemical analysis and bioassays. Both chemical analysis and bioanalysis suggest that improved wastewater treatment technology will not completely reduce the micropollutant burden, which renders additional source control measures also necessary. Further, this study illustrates the relevance of the complementary approach to identify and characterize micropollutant sources.

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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: $(2.58 \pm 0.81)\times10^{-9}$ 24 h: $(2.68 \pm 0.45)\times10^{-9}$ 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

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

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

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

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List of Figures

Figure 1

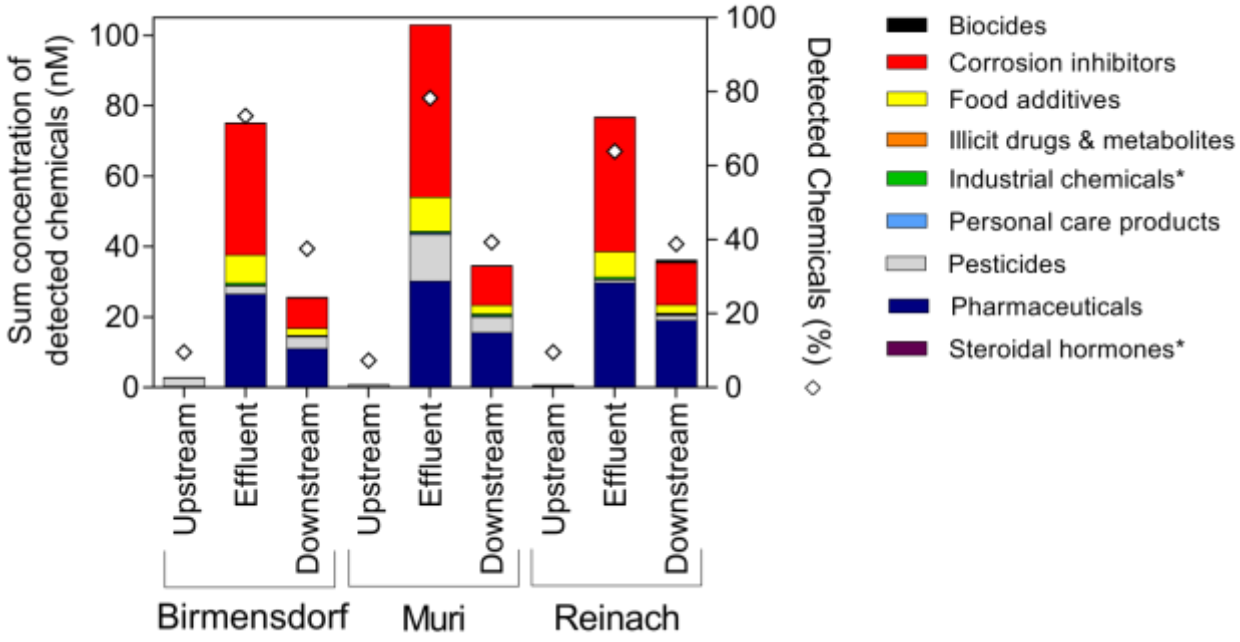

Sum concentration of detected chemicals (nM)
Detected Chemicals (%)
100
80
60
40
20
0
Upstream
Effluent
Downstream
Birmensdorf
Muri
Reinach
Biocides
Corrosion inhibitors
Food additives
Illicit drugs & metabolites
Industrial chemicals*
Personal care products
Pesticides
Pharmaceuticals
Steroidal hormones*

Figure 2

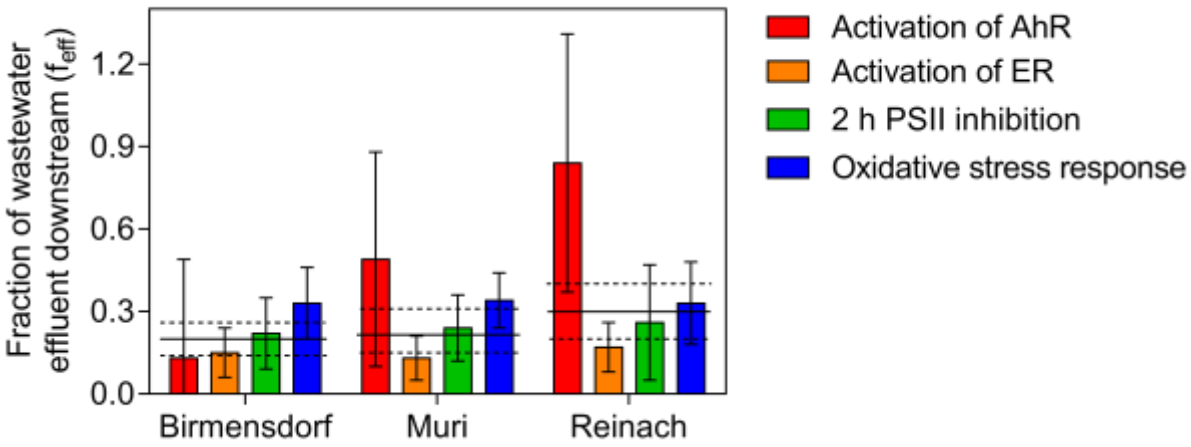
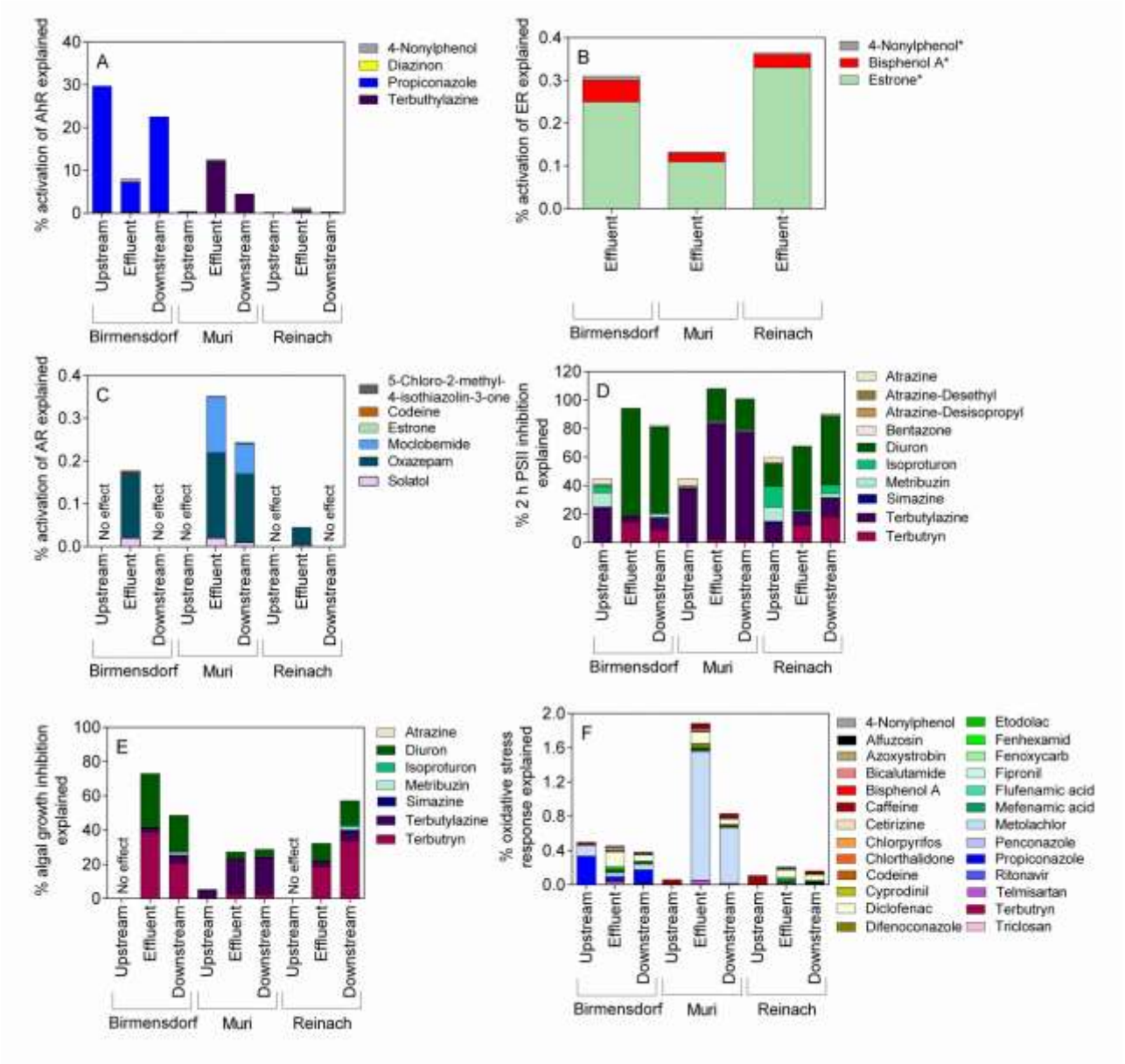

Fraction of wastewater effluent downstream (f_{eff})
1.2
0.9
0.6
0.3
0.0
Birmensdorf
Muri
Reinach
Activation of AhR
Activation of ER
2 h PSII inhibition
Oxidative stress response

Figure 3


A
% activation of AhR explained
4-Nonylphenol
Diazinon
Propiconazole
Terbuthylazine
B
% activation of ER explained
4-Nonylphenol*
Bisphenol A*
Estrone*
C
% activation of AR explained
5-Chloro-2-methyl-4-isothiazolin-3-one
Codeine
Estrone
Moclobemide
Oxazepam
Solatol
No effect
D
% 2 h PSII inhibition explained
Atrazine
Atrazine-Desethyl
Atrazine-Desisopropyl
Bentazone
Diuron
Isoproturon
Metribuzin
Simazine
Terbutylazine
Terbutryn
E
% algal growth inhibition explained
F
% oxidative stress response explained
4-Nonylphenol
Alfuzosin
Azoxystrobin
Bicalutamide
Bisphenol A
Caffeine
Cetirizine
Chlorpyrifos
Chlorthalidone
Codeine
Cyprodinil
Diclofenac
Difenoconazole
Etodolac
Fenhexamid
Fenoxycarb
Fipronil
Flufenamic acid
Mefenamic acid
Metolachlor
Penconazole
Propiconazole
Ritonavir
Telmisartan
Terbutryn
Triclosan
Upstream
Effluent
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Birmensdorf
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