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Application of cell-based bioassays to evaluate treatment efficacy of conventional and intensified treatment wetlands

Jaime Nivala,[a§] Peta A. Neale,[b§] Tobias Haasis,[c] Stefanie Kahl,[d] Maria König,[e] Roland A. Müller,[a] Thorsten Reemtsma,[d] Rita Schlichting,[e] Beate I. Escher[c,e]*

[a]UFZ – Helmholtz Centre for Environmental Research, Centre for Environmental Biotechnology, 04318 Leipzig, Germany

[b]Australian Rivers Institute, Griffith School of Environment, Griffith University, Southport QLD 4222, Australia

[c]Eberhard Karls University Tübingen, Environmental Toxicology, Center for Applied Geosciences, 72074 Tübingen, Germany

[d]UFZ – Helmholtz Centre for Environmental Research, Department of Analytical Chemistry, 04318 Leipzig, Germany

[e]UFZ – Helmholtz Centre for Environmental Research, Department Cell Toxicology, 04318 Leipzig, Germany

[§]both authors contributed equally to this MS.

*corresponding author: beate.escher@ufz.de; Ph: +49 341 235 1244

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24 **Abstract**

25 Constructed wetlands are commonly used for wastewater treatment when centralized sewage
26 treatment is not feasible. Many studies have focused on the removal of micropollutants by treatment
27 wetlands, but little is known about how well they can remove biological activity. Here we studied
28 the removal efficacy of conventional and intensified treatment wetland designs using both chemical
29 analysis of conventional wastewater parameters and treatment indicator chemicals (caffeine,
30 ibuprofen, naproxen, benzotriazole, diclofenac, acesulfame, carbamazepine) as well as a panel of *in*
31 *vitro* bioassays indicative of different stages of cellular toxicity pathways, such as xenobiotic
32 metabolism, receptor-mediated effect and adaptive stress responses. Water samples collected before
33 and after seven treatment wetlands were compared against the adjacent municipal wastewater
34 treatment plant. The intensified treatment wetlands generally removed micropollutants and
35 biological activity to a greater extent than the conventional wastewater treatment plant, whereas the
36 conventional horizontal subsurface flow wetland showed poor removal of all indicators.
37 Carbamazepine was not well removed by any of the studied systems as expected from reported
38 recalcitrance in aerobic environments. Estrogenic activity, which is a commonly used biological
39 endpoint indicator for wastewater treatment, was removed very well by the intensified wetlands (97
40 to 99.5%) with similar or slightly lower removal efficacy for all other biological endpoints. The
41 results highlight the importance of applying indicator bioassays complementary to indicator
42 chemical analysis for monitoring treatment efficacy. The high removal efficacy of biological effects
43 as a measure of total effect-scaled concentrations of chemicals provides further support to the use of
44 intensified wetlands for wastewater treatment.

45

46

47 **Keywords**: aerated treatment wetland, ecotechnology, micropollutants, *in vitro* bioassays;
48 bioanalytical equivalent concentration

49

1. Introduction

Treatment wetlands are one of many decentralized wastewater treatment technologies that can be used for water quality improvement in areas for which centralized sewage treatment is not an option. Further, treatment wetlands can also be applied as a polishing step after conventional wastewater treatment.[1] Treatment wetlands are based on ecological and natural principles and offer many advantages over other decentralized wastewater treatment technologies. Classic designs are simple to operate, low-cost, and can be constructed out of local materials,[2] leading to the widespread use of wetlands for water quality improvement around the world.

Across the treatment wetland technology gradient from passive to intensified systems, there are trade-offs between system footprint and energy requirements. A decrease in footprint typically comes at a cost of increased electricity consumption and more complex design and operational requirements.[3] However, intensified treatment wetlands are able to degrade pollutants present in wastewater 10- to 1,000-fold faster than completely passive wetland designs.[4]

The first research on micropollutant removal by treatment wetlands started ten years ago in Europe[5-7] and has gained a lot of momentum in recent years. Most studies on micropollutant removal in treatment wetlands focus on Free Water Surface (FWS) and Horizontal Subsurface Flow (HSSF) treatment wetland designs. A review by Verlicchi and Zambello[8] reported variable treatment efficacy for wetlands used for primary, secondary, and tertiary treatment, but identified many individual micropollutants that are well-removed (>75%) in FWS and HSSF wetlands for secondary treatment of domestic wastewater. Many current studies are conducted on laboratory-scale systems under controlled conditions,[9-12] sometimes using synthetic wastewater that does not contain the contaminants of concern at realistic concentrations.[13] Recent studies investigating removal of micropollutants in full-scale treatment systems do exist, but reported only the performance of HSSF[14,15] and FWS[16-18] designs.

While most studies on wetland treatment focus on individual chemicals, wastewater contains a complex mixture of micropollutants, including pharmaceutical and personal care products

76 (PPCP), food additives and industrial chemicals, as well as their transformation products.[19] In a
77 previous study on six treatment wetland on the same site, seven micropollutants were determined
78 over a whole-year period as treatment indicators.[20] These indicators were chosen as to reflect the
79 removal of micropollutants of different biodegradability, from easily degradable (caffeine) to
80 persistent under oxic conditions (carbamazepine).[20,21] The same set of indicators was also used in
81 this study to monitor the performance of the treatment wetlands in removing micropollutants.

82 Chemical analysis alone does not provide any information about the potency of the mixture
83 of detected chemicals and the (waste-) water itself. *In vitro* bioassays can be applied complementary
84 to chemical analysis as they provide information about the effect of all active compounds in a
85 sample. While bioassays have been applied widely to drinking water, surface water and
86 wastewater[22-24] and to assess the treatment efficacy of conventional wastewater treatment plants
87 (WWTP) and advanced water treatment plants,[25-27] there has been limited application to assess the
88 treatment efficacy of constructed wetlands. Most of these studies only focussed on estrogenicity.[28,29]
89 In the most comprehensive study to date, Ávila *et al.*[30] applied bioassays indicative of dioxin-like
90 activity, estrogenicity and apical effects in whole organisms to evaluate the removal of spiked
91 micropollutants in a hybrid constructed wetland, with approximately 70-100% removal of
92 biological activity observed.

93 To ensure that most biological effects elicited by typical wastewater contaminants are
94 detected during monitoring, it is important to assemble a bioanalytical test battery that covers
95 different stages of cellular toxicity pathways, including xenobiotic metabolism, receptor-mediated
96 effects, adaptive stress responses and cytotoxicity.[31] Cellular toxicity pathways are an important
97 stage in adverse outcome pathways, though an effect at the cellular level may not necessarily lead to
98 higher order effects.[32] Test batteries indicative of different stages of cellular toxicity pathways have
99 been successfully applied to drinking water, surface water and wastewater.[22,33,34]

100 In the current study a bioanalytical test battery was applied to assess the ability of seven
101 pilot-scale conventional and intensified wetland systems to remove biological activity. This was

102 complemented with chemical analysis of seven indicator micropollutants representative for a larger
103 number of compounds and from high to low biodegradability,[21] and conventional wastewater
104 parameters. The treatment efficacy of the constructed wetlands was also compared to a conventional
105 WWTP located adjacent to the wetlands that drew from the same raw municipal wastewater.
106 Previous studies showed variable removal of micropollutants over different seasons,[35,36] and in the
107 precedent study on the same wetlands,[20] the removal of nutrients, bulk organic matter and
108 micropollutants also showed seasonal variability. Therefore, the bioanalytical assessment in the
109 present study was conducted in summer (July) and autumn (November) to explore seasonal
110 differences, which could be due to differences in plant growth and microbial activity.

111 The applied test battery included assays indicative of xenobiotic metabolism, receptor-
112 mediated effects and adaptive stress responses. Two assays indicative of xenobiotic metabolism
113 were included in the test battery, the AhR CALUX for activation of the aryl hydrocarbon receptor
114 (AhR) and the PPARγ-*bla* for binding to the peroxisome proliferator-activated receptor gamma
115 (PPARγ). A wide range of environmental chemicals and water samples has been shown to activate
116 AhR and PPARγ.[22,24,31,37] To evaluate hormonal activity, a suite of bioassays indicative of
117 activation of the estrogen receptor (ER), androgen receptor (AR), glucocorticoid receptor (GR) and
118 progesterone receptor (PR) were also included. Receptor-mediated effects are indicative of specific
119 modes of action and have been widely applied to evaluate wastewater and recycled water treatment
120 efficacy.[1,38] As wastewater can contain a mixture of both hormone agonists and antagonists,
121 inhibition of ER, AR, GR and PR were assessed in parallel. Finally, two assays indicative of
122 adaptive stress responses were included, AREc32 for the oxidative stress response and NF-κB-*bla*
123 for the NF-κB response. Adaptive stress response pathways help to re-establish homeostasis after
124 damage to cells by stressors,[39] with previous studies showing a range of water types can activate the
125 oxidative stress response and the NF-κB response.[33,40] Further, cell viability was assessed in parallel
126 for all assays to ensure that cytotoxicity did not interfere with the observed effect. Through the use

127 of the assembled test battery, the current study is the first to comprehensively evaluate the treatment
128 efficacy of conventional and intensified wetlands based on biological activity.
129

130 **2. Materials and Methods**

131 *2.1 Sample collection*

132 Water samples were collected from the wetland systems at the ecotechnology research facility at
133 Langenreichenbach, located near Leipzig, Germany using a previously described sampling
134 methodology based on grab sampling.[20] Kahl *et al.*[20] reported that grab sampling at this same
135 research site with samples averaged over 12 samples taken at 2-h intervals showed negligible
136 variation (4 – 9%) over one week in effluent concentrations from the H50p treatment system for
137 selected micropollutants diclofenac, ibuprofen, and naproxen. Variations were higher within a day
138 (up to 30%) or between consecutive days (up to 40%),[20] but for this study where we mainly
139 evaluated the suitability of bioassays for monitoring tretment efficiacy, so that a grab sample will
140 still be representative of treatment efficacy.
141 Further information about the facility can be found in Nivala *et al.*[41] The constructed wetlands
142 included conventional and intensified designs. The baseline technology is a conventional horizontal
143 flow wetland with 50 cm saturated depth (H50p). The intensified wetland systems tested include a
144 saturated vertical flow wetland with aeration (VAp), a saturated horizontal flow wetland with
145 aeration (HAp), and a reciprocating system (R). One two-stage wetland system was tested, which
146 consisted of the vertical flow aerated wetland followed by an unsaturated sand filter (VAp+VSp).
147 The intensified systems HM and HMc are duplicate horizontal flow aerated wetlands, but in HM
148 aeration was shut off between 40 and 70% of the fractional distance along the direction of flow.
149 Within the HM system, internal water samples were also collected at the locations where aeration
150 was shut off (HM40) and turned back on (HM70). Further information about the studied wetlands
151 can be found in Table 1. With the exception of the reciprocating system, R, all wetlands were
152 planted with common reed (*Phragmites australis*). At the time the study began, all of the systems

153 had been running in steady state for a number of years (see Table 1). The two-stage system
154 VAp+VSp was put into operation in 2016, but the individual systems had been in operation for
155 years prior to the start of the current study. The input for all wetlands was effluent from a septic
156 tank, SEP (out), in which municipal wastewater received primary treatment. Samples were also
157 collected from the influent and effluent of the adjacent municipal WWTP (16,000 population
158 equivalent; anaerobic and aerobic activated sludge treatment with biological and chemical
159 phosphorus precipitation). The WWTP samples were named WWTP (in), and WWTP (out). Two
160 sampling campaigns were conducted in order to capture seasonal differences, with samples
161 collected on the 26th July and 8th November 2016. Two times two-litre grab samples were collected
162 from each system for bioanalysis. The samples were filtered with glass fibre filters prior to
163 enrichment using Oasis HLB solid phase extraction (SPE) cartridges. After extraction, the
164 cartridges were eluted with methanol and blown down under a gentle nitrogen stream, with the
165 duplicates combined to one extract with a final enrichment factor of 1000. Water samples for
166 micropollutant analysis were collected in 50-mL or 100-mL amber glass bottles. The sample
167 collection procedure for conventional wastewater parameters is described in detail elsewhere.[41]

168

169 *2.2 Chemical analysis*

170 Seven common municipal wastewater-based micropollutants, acesulfame, benzotriazole, caffeine,
171 carbamazepine, diclofenac, ibuprofen and naproxen, were analysed using high performance liquid
172 chromatography tandem mass spectrometry (HPLC-MS/MS) using the method described by Kahl et
173 al.[20] Isotope-labelled internal standards of each analyte were used for quantification. In short,
174 samples were directly injected, though only after dilution in the case of WWTP (in), SEP (out),
175 H50p, after addition of the internal standards and filtration (syringe filters, regenerated cellulose
176 membrane). The seven micropollutants served as indicator compounds, representing different
177 degree of biodegradability under aerobic conditions. Further information on the compound choice
178 and biodegradability can be found in Kahl *et al.*[20] A range of conventional wastewater parameters,

179 including electrical conductivity, dissolved oxygen, carbonaceous biochemical oxygen demand
180 ($CBOD_5$), total organic carbon (TOC), total nitrogen (TN), ammonium-nitrogen (NH_4-N) and
181 nitrate-nitrogen (NO_3-N), were also analysed in the treatment wetland samples (Electronic
182 Supplementary Information (ESI), Table S1), with information about the analysis methods in Kahl
183 *et al.*[20]

184 Micropollutant removal was quantified with Equation 1 using influent and effluent
185 concentrations ($C_{influent}$ and $C_{effluent}$, respectively). The influent to all treatment wetland systems was
186 septic tank effluent, SEP (out). Removal was calculated using half of the analytical limit of
187 detection (LOD) if $C_{effluent}$ was below the LOD. The LODs were taken from Kahl *et al.*[20] and are
188 listed also in the ESI, Table S2.

$$\text{Removal (\%)}=\frac{C_{influent} - C_{effluent}}{C_{influent}}\times 100$$

189 (1)

190 *2.3 Bioanalysis*

191 Eight *in vitro* bioassays covering 12 different endpoints were applied in the current study and are
192 summarised in Table 2. The concentration-effect curves for the assays' positive reference
193 compounds are shown in Figure S1 of the ESI. Detailed descriptions of the applied bioassays are
194 available in König *et al.*[33] and Neale *et al.*[31] Cell viability was assessed in parallel to induction for
195 all assays. Cell viability in AREc32 and AhR CALUX was measured using PrestoBlue, a cell
196 permeable resazurin-based solution. For all other assays, the ToxBLAzer DualScreen Kit was used,
197 which combines the ratiometric reporter gene readout of β-lactamase activity with a third
198 fluorometric readout to estimate cytotoxicity. Live-cell analysis using an IncuCyte S3 live cell
199 imaging system (Essen BioScience, Ann Arbor, Michigan, USA) was also applied to assess cell
200 viability in the AhR CALUX assay. Phase contrast images were acquired 24 h after dosing (48 h
201 after seeding). Quantitative analysis of cell confluency was performed using the IncuCyte S3
202 software. Confluency is a good surrogate for cell proliferation, if changes in cell morphology are

203 minor. A comparison of the two techniques to determine cytotoxicity is provided Figure S2 of the
204 ESI, with IncuCyte recommended for measuring cell viability in future studies.

205

206 *2.4 Data evaluation*

207 All bioassay data were evaluated using linear concentration-effect curves at the low-effect linear
208 portion of the otherwise nonlinear concentration-effect curves. The effect concentration causing
209 10% effect (EC_{10}) was determined for assays indicative of xenobiotic metabolism and receptor-
210 mediated effects where a maximum effect could be reached, while an effect concentration causing
211 an induction ratio of 1.5 ($EC_{IR1.5}$) was derived for the adaptive stress response assays. To ensure the
212 concentration-effect curves were linear, effects greater than 40% or induction ratios (IR) greater
213 than 5, as well as concentrations causing more than 10% cytotoxicity, were excluded. For assays
214 run in antagonist mode, the effect concentration causing a suppression ratio of 0.2 ($EC_{SR0.2}$) was
215 also derived from the linear portion of the concentration-effect curves excluding concentrations
216 causing more than 1% cytotoxicity. Further information about the applied data evaluation can be
217 found in Escher *et al.*[22] and Neale *et al.*[31] The EC values were expressed in units of relative
218 enrichment factor (REF), which incorporates sample enrichment by SPE and dilution in the assay.[42]

219 To translate the effect of a sample in a specific bioassay to the concentration of a reference
220 compound that would elicit the same response, the EC values were converted to bioanalytical
221 equivalent concentrations from bioanalysis (BEQ_{bio}). BEQ_{bio} was calculated using Equation 2 with
222 the EC value of the sample and the corresponding EC value of the reference compound (ref).

223

$$BEQ_{bio} = \frac{EC_{10}\ (ref)}{EC_{10}\ (sample)} \text{ or } \frac{EC_{IR1.5}\ (ref)}{EC_{IR1.5}\ (sample)} \text{ or } \frac{EC_{SR0.2}\ (ref)}{EC_{SR0.2}\ (sample)}$$

224 (2)

225

226 BEQ_{bio} before treatment ($BEQ_{bio,\ influent}$) and after treatment ($BEQ_{bio,\ effluent}$) were used to assess the
227 removal efficacy of the studied wetlands, as well as the conventional WWTP (Equation 3). Errors
228 were calculated as described by König *et al.*[33]

229

$$BEQ_{bio}\ \text{Removal}\ (\%) = \frac{BEQ_{bio,\ influent} - BEQ_{bio,\ effluent}}{BEQ_{bio,\ influent}} \times 100$$

230 (3)

231

232 **3. Results and Discussion**

233 *3.1 Chemical analysis*

234 A range of conventional wastewater parameters were assessed, with the results provided in Table
235 S1. Effluent water temperatures in the treatment wetlands ranged from 19.3 – 22.7°C in July and
236 from 9.6 – 11.0°C in November. The organic load of the influent wastewater in July was higher
237 than in November, which was marked by elevated $CBOD_5$ and TOC concentrations in July
238 (761 mg/L and 499 mg/L, respectively) compared to November (304 mg/L and 242 mg/L,
239 respectively). Effluent $CBOD_5$ concentrations from the treatment wetlands were under 10 mg/L for
240 all systems in July except for H50p, which exhibited an effluent concentration of 59 mg/L. Effluent
241 $CBOD_5$ concentrations from the treatment wetlands in November were less than 2 mg/L, except for
242 H50p, which was 30 mg/L. The redox potential in the effluent from each wetland system increased
243 compared to the wetland influent (SEP (out)). The positive redox values in the effluent of the
244 intensified wetlands (+64.1 to +256 mV) indicated aerobic conditions, while in H50p, reducing
245 conditions persisted (-184 mV in July; -208 mV in November). The ammonium-nitrogen (NH_4-N)
246 and total nitrogen (TN) concentrations in the wetland influent were 83 mg/L and 97 mg/L,
247 respectively, in July and 62 mg/L and 72 mg/L, respectively, in November. As a result of the
248 oxidizing conditions in the intensified wetlands (VAp, VAp+VSp, HAp, R, HMc and HM), NH_4-N
249 was also well removed, with effluent NH_4-N concentrations of 1.4 mg/L or lower, regardless of

250 water temperature. The moderately aerobic conditions observed in VAp and R, as evidenced by
251 dissolved oxygen concentrations in July (VAp: 5.5 mg/L; R: 2.0 mg/L) and November (VAp: 8.1
252 mg/L; R: 5.2 mg/L), resulted in low effluent TN concentrations (July, VAp: 27 mg/L; R: 12 mg/L;
253 November, VAp: 33 mg/L; R: 22 mg/L) compared to the other wetlands. The horizontal flow
254 aerated wetlands HAp and HMc exhibited effluent TN concentrations ranging from 40 – 51 mg/L.
255 The conventional horizontal flow wetland H50p did not exhibit notable removal of TN or NH_4-N
256 but efficiently removed nitrate, due to the lack of oxidising conditions, which is consistent with
257 previous studies on this treatment system.[20,43]

258 As expected from their widespread use, all seven indicator micropollutants were detected in
259 the outlet of the septic tank feeding the constructed wetlands, as well as the influent to the WWTP
260 in the µg/L range (Figure 1 and ESI, Table S2). The food additives caffeine and acesulfame were
261 found at the highest concentrations in the inlet to the WWTP and the outlet of the septic tank. Both
262 caffeine and acesulfame have previously been detected in wastewater influent in Germany in the
263 µg/L concentration range.[44,45] Despite the high concentration of caffeine in the water feeding the
264 wetlands, it was rarely detected in the treated effluent (ESI, Table S2) due to its high
265 biodegradability under all redox conditions.

266 Similarly, the concentrations of pharmaceuticals ibuprofen and naproxen, which are readily
267 to moderately biodegradable under aerobic conditions, were also often below the LOD after
268 treatment (ESI, Table S2). In contrast, the corrosion inhibitor benzotriazole and pharmaceuticals
269 carbamazepine and diclofenac were detected in the effluent of all wetland systems, as well as in the
270 effluent of the conventional WWTP (ESI, Table S2). This corresponded well with the reported
271 negligible to low biodegradability of these compounds.[21]

272

273 *3.2 Bioanalysis*

274 EC values in units of REF are provided in Table S3, with the concentration-effect curves shown in
275 Figures S3 to S14. BEQ_{bio} values in units of ng/L are provided in Table 3, though it should be noted

276 that it was not possible to derive BEQ_{bio} for inhibition of ER, inhibition of GR and activation of PR
277 as either no effect was observed up to the maximum REF or cytotoxicity masked the effect. All
278 samples were active in the assays indicative of activation of AhR, binding to PPARγ, oxidative
279 stress response and NF-κB response, which fits with previous observations in samples of raw and
280 treated wastewater.[22,46] A wide range of chemicals are active in assays indicative of xenobiotic
281 metabolism and adaptive stress responses, with Martin *et al.*[37] showing that 52% of 320
282 environmental chemicals induced the Nrf2 ARE reporter gene, with 46% and 17% of the studied
283 chemicals activating PPARγ and AhR, respectively. The NF-κB response was the most responsive
284 assay in the current study, with effects in SEP (out) and H50p observed after 50 times dilution (REF
285 0.02). Wastewater effluent has previously been shown to activate the NF-κB response at low REF,[40]
286 though the causative chemicals are currently unknown, with only 3% of chemicals in the US EPA
287 ToxCast database inducing a response.[47]

288 A suite of assays indicative of activation and inhibition of hormonal activity were also
289 applied, though many of the influent and effluent samples were very cytotoxic, leaving a small
290 window for detection of the effect. Activation of ER was the most responsive, followed by
291 activation of GR, which fits with previous observations from wastewater and surface water.[1,22] It
292 should be noted that activation of GR could only be quantified in the effluent from the intensified
293 wetlands, with cytotoxicity masking the effect in WWTP (in), SEP (out) and H50p. In contrast,
294 activation of AR could only be detected in the influent to the WWTP. While SEP (out) had no
295 agonistic or antagonistic activity on the AR (or activity was masked by cytotoxicity), the effluent
296 from several of the intensified wetlands showed antagonistic effects on AR. Generally, androgenic
297 activity is more common in wastewater, while anti-androgenic activity is more frequently detected
298 in environmental waters.[22] However, other studies have found anti-androgenic activity in
299 wastewater effluent[48] and many environmental estrogenic compounds can also act as anti-
300 androgenic compounds.[49] None of the samples caused inhibition of ER or GR at non-cytotoxic

301 concentrations, while weak inhibition of PR was detected in the effluent of the intensified wetlands
302 in November.
303
304 *3.3. How well did the studied wetlands reduce the chemical concentration and biological activity?*
305 The treatment efficacy of the conventional WWTP and pilot-scale constructed wetlands was
306 assessed using both indicator chemicals and bioanalysis. The intensified wetlands generally showed
307 greater removal of the indicator micropollutants compared to the conventional wetland design
308 H50p, with micropollutant removal in the intensified wetlands similar to or better than the
309 conventional WWTP (Table 4, Figure S15). The lack of oxygen in H50p meant that anoxic, nitrate-
310 reducing conditions dominated and removal efficacy was lower, which agrees with findings of the
311 preceding study[20] and other studies that found less degradation of PPCPs in wetland sediment under
312 anaerobic conditions compared to aerobic conditions.[50] Biodegradation is expected to be a more
313 important removal mechanism than sorption to sediment/soil or plants, as the majority of the
314 indicator micropollutants are polar or even charged (diclofenac, ibuprofen, naproxen) and have
315 octanol-water partition coefficients (log K_{ow}) of their neutral species of less than 4 indicating low
316 hydrophobicity and high mobility in aquatic environments (Table S2).
317 The concentration of ibuprofen was reduced to below the LOD after treatment in all
318 intensified wetlands, while only 61 and 14% was removed by H50p in July and November,
319 respectively. Ibuprofen has previously been shown to be well removed by aerobic degradation
320 processes,[30] explaining the high removal in the intensified systems.
321 Carbamazepine was poorly removed in the conventional WWTP and all wetland systems
322 except H50p in the July sampling, where removal was 48% (Table 4). Carbamazepine was
323 previously reported to be poorly removed in a range of constructed wetland types and conventional
324 WWTP due its recalcitrant nature.[35,36,51] The limited removal observed in H50p, although only in
325 July, could be due to reductive transformation processes enabled under anoxic nitrate-reducing
326 conditions.[20,52]

327 Acesulfame was removed by more than 90% with exception of H50p (Table 4). This is
328 consistent with previous findings in these systems,[20] as well as in several WWTPs and sand
329 filtration of surface water.[53]

330 The intensified wetlands were also unable to remove diclofenac effectively, with the
331 exception of VAp + VSp and HAp in July (96% and 85% removal, respectively). Diclofenac
332 removal by WWTPs can be highly variable, and the influence of operational conditions is not yet
333 fully understood.[21,54] However, results of a previous study on these wetlands suggested that
334 diclofenac removal in treatment wetlands was most effective when high dissolved oxygen
335 concentrations were present in conjunction with low concentrations of organic carbon.[20] This is a
336 plausible explanation for why diclofenac was most efficiently removed in the two-stage system
337 VAp+VSp. The change in season from summer (effluent water temperatures of the wetland systems
338 ranging from 19.3 – 21.3°C) to autumn (effluent water temperatures of the wetland systems ranging
339 from 9.6 – 11.0°C) had little effect on the removal of the indicator micropollutants in the intensified
340 systems, though it did influence the removal efficacy of H50p, with decreasing removal of all
341 compounds compared to July (Figure S15). Increased removal in warmer months has also been
342 observed previously[35] and can be related to increased microbial activity in warmer conditions.

343 Removal of biological activity could only be assessed for five of the studied endpoints,
344 activation of AhR, binding to PPARγ, activation of ER, oxidative stress response and NF-κB
345 response, as cytotoxicity masked induction in the WWTP (in) and SEP (out) samples for the other
346 assays. Similar to the indicator micropollutants, the ability of intensified systems to remove
347 biological activity was comparable to or greater than the removal efficacy of the conventional
348 WWTP for all assays, while the conventional system H50p had the lowest removal efficacy
349 (Figure 2, Table S4).

350 In all six intensified wetlands no influence of the two sampling seasons summer (July) and
351 late autumn (November) on removal of biological activity was observed for any biological
352 endpoint. In contrast to the indicator micropollutants, the removal efficacy of H50p was comparable

353 between the two sampling events, suggesting that the difference in temperature and plant growth
354 did not have a significant impact on the removal of compounds causing biological effects. Some of
355 the active compounds may be more hydrophobic, potentially making sorption a more relevant
356 removal process, with seasonality less likely to have an effect. Estrogenic activity was very well
357 removed by the intensified wetlands, with 97 to 99.5% reduction in BEQ_{bio} after treatment. Several
358 studies have also found good removal of estrogenic activity in wetlands treating municipal
359 wastewater[28] and agricultural wastewater.[55]

360 The smallest reduction in BEQ_{bio} was observed for compounds that activated AhR, with
361 between 74 to 87% reduction for the intensified systems. However, it should be noted that this was
362 considerably better than removal by the conventional WWTP, where BEQ_{bio} for activation of AhR
363 was only reduced by around 50%. Similarly, between 46 to 69% of AhR activity was removed in a
364 water reclamation plant after activated carbon filtration and ozonation.[56] Ávila *et al.*[30] applied an
365 AhR yeast assay to assess the ability of a hybrid constructed wetland to remove dioxin-like activity
366 in wastewater spiked with micropollutants and found complete removal of activity after vertical
367 flow and horizontal flow wetlands, though activity increased again after treatment in the FWS
368 wetland. Since the AhR is a very promiscuous receptor that binds a diversity of chemicals,[57] it is not
369 possible to explain what types of chemicals were dominating the effect removal and what types of
370 chemicals caused the differences between the WWTP and the wetlands. However one feature that
371 AhR ligands have in common is that they are typically fairly large, neutral and hydrophobic
372 chemicals, which contrasts the physicochemical properties of the indicator chemicals that were
373 included in chemical analysis.

374 Few studies have applied bioassays to assess the treatment efficacy of constructed wetlands
375 and most focussed on estrogenicity. Therefore, it was not possible to compare the results for the
376 other assays in similar wetland systems because to our knowledge no such experiments were
377 previously conducted. However, Bain *et al.*[1] found between 69 to 100% reduction in PPARγ
378 activity in three WWTPs, with the greatest removal found for a WWTP which included a

379 constructed wetland for excess nutrient removal as the final step in the treatment train. Between 77
380 to 95% of PPARγ activity was removed by the intensified wetlands in the current study, with the
381 two-stage VAp+VSp being the most effective system.

382 Further, the reduction in BEQ_{bio} for the oxidative stress response was considerably greater in
383 the intensified wetlands (86 to 96%) than previously reported for a conventional WWTP (40%
384 reduction).[58] The NF-κB assay has not previously been applied to assess WWTP efficacy, but over
385 90% reduction in BEQ_{bio} was observed in all intensified wetlands.

386 To explore the effect of aeration on removal, the removal of BEQ_{bio} was compared for
387 identical intensive horizontal flow systems, HM and HMc, with aeration turned off from 40 to 70%
388 fractional distance in HM (Figure 3). Over 90% of BEQ_{bio} for the NF-κB response was removed
389 prior to aeration being shut off, with further treatment having little impact on the biological activity.
390 Further, the majority of biological activity was removed within 40% distance in direction of flow
391 for activation of AhR, binding to PPARγ and oxidative stress response, with some additional
392 decrease in effect in the zone that was slightly less aerobic but not fully anoxic, i.e. from HM40 to
393 HM70. If removal was calculated between HM40 and HM70, it was 46% (July) and 39% (Nov) for
394 AhR, and the step from HM70 to HM did not lead to any additional removal. For PPARγ the
395 incremental relative removal between HM40 and HM70 was 35% (July) and 51% (Nov), and the
396 step from HM70 to the effluent HM removed 30 % (July) and 15% (Nov). If removal was
397 calculated for each step separately for AREc32, the removal between HM40 and HM70 was 66%
398 (July) and 37% (Nov), and the step from HM70 to HM removed no effect (July) and 31% (Nov).

399 This fits with previous findings by Ávila *et al.*[30] that the majority of the biological activity in
400 a hybrid wetland system was removed by aerobic treatment processes. Overall, there was little
401 difference in the absolute removal efficacy of HM and HMc (Figure 2), suggesting that the change
402 in aeration did not significantly alter removal of biological activity. While the indicator
403 micropollutants were not analysed in the HM40 and HM70 samples, comparison of the

404 micropollutant concentrations in the effluent of HM and HMc also shows little difference
405 (Figure 1).

406 Overall, the indicator micropollutants and bioanalysis both show that the studied intensified
407 wetlands have a similar or greater capacity to reduce pollutant load and biological activity as the
408 conventional WWTP. Further, conventional horizontal treatment wetlands under conditions of high
409 organic loading and with low dissolved oxygen concentrations did neither efficiently remove
410 biological activity nor the indicator micropollutants eliminable via oxidative pathways.

411

412 *3.4 Does wetland treated effluent pose a risk to the receiving environment?*

413 To evaluate the potential risks to the receiving environment, the chemical concentrations in the
414 treated effluent were compared with proposed environmental quality standards (EQS). Of the seven
415 indicator micropollutants, proposed average annual EQS were available for five chemicals.[59] All
416 wetland treatment processes reduced the concentration of naproxen below the proposed EQS of
417 1.7 µg/L, while none of the measured benzotriazole concentrations, even in the wastewater influent,
418 exceeded the proposed EQS of 19 µg/L. In contrast, the concentration of carbamazepine was higher
419 than the proposed EQS of 2.0 µg/L after treatment in July, with the exception of conventional
420 WWTP and H50p, which typically had the poorest removal of the other indicator micropollutants
421 and biological activity. Further, none of the treatments reduced the concentration of diclofenac
422 below the proposed EQS of 0.05 µg/L, which is lower than the LOD in this study (0.12 µg/L). The
423 proposed EQS for ibuprofen, 0.01 µg/L, was also lower than the LOD in the current study
424 (0.81 µg/L). While the poor removal of carbamazepine as well as diclofenac by all treatment
425 processes may potentially be problematic, it should be noted that further dilution of the treated
426 effluent in the freshwater environment is expected, with processes such as photodegradation
427 sorption, or (bio-) transformation in the subsurface likely to reduce the concentrations of diclofenac
428 and carbamazepine even further.[21,36,50]

429 Benchmarking the bioanalytical results is more difficult as effect-based trigger values for
430 surface water have not been derived for the studied assays. As the effect in ERα GeneBLAzer was
431 expressed in 17β-estradiol equivalent concentrations (EEQ), the results can be compared with the
432 proposed European Union 17β-estradiol EQS of 0.4 ng/L, though this only represents a single
433 chemical and does not take into account the mixture effects. With the exception of H50p, all studied
434 wetlands reduced the effluent concentrations to between 0.14 – 0.68 ng/L EEQ, with treatment by
435 HAp and VAp+VSp reducing the effluent concentration to below 0.4 ng/L EEQ in both July and
436 November. Further, Jarošová *et al.*[60] derived safe concentrations of estrogenic equivalents (EEQ-
437 SSE) for municipal effluents using a range of *in vitro* bioassays. While ERα GeneBLAzer was not
438 included in the study, the EEQ in the current study were in the range of proposed short-term
439 exposure EEQ-SSE (0.5 to 2 ng/L EEQ), with only effluent from HAp and VAp+VSp in the range
440 of the long-term exposure EEQ-SSE (0.1 to 0.4 ng/L EEQ). Work is currently ongoing to derive
441 effect-based trigger values for a wider range of endpoints relevant for environmental waters.
442

443 **4. Conclusions**

444 In general, the removal efficacy of the biological effects aligned well with the removal efficacy of
445 the well-degradable indicator chemicals but not with recalcitrant indicator chemicals such as
446 carbamazepine. Using a comprehensive battery of bioassays, this study shows for the first time that
447 intensified wetlands can remove biological activity to a greater extent than conventional wastewater
448 treatment. In contrast, the conventional horizontal flow wetland H50p performed much poorer,
449 which was also confirmed by chemical analysis and was consistent with earlier chemical analysis of
450 seven polar indicator chemicals.[20] Estrogenicity is commonly used as a biological endpoint
451 indicator for wastewater treatment, and all intensified wetlands were able to remove estrogenic
452 activity very well (97 to 99.5%). While improved removal of indicator micropollutants was
453 observed for H50p in July, there was no difference in removal of biological activity with season.
454 This suggests that the indicator micropollutants, which were selected based on their

455 biodegradability, cannot be used to predict the removal of overall biological effects, emphasising
456 the importance of applying bioanalysis complementary to chemical analysis.

457 Here we evaluated a wide range of bioassays but for future monitoring studies and
458 investigations of temporal effects as well as different treatment conditions we propose a set of
459 indicator bioassays as treatment performance indicators. Suitable for this purpose of surveillance
460 monitoring in future studies would be a test battery that comprises bioassays for activation of AhR,
461 activation of ER and the oxidative stress response because they yield different pictures of removal
462 and they cover three different stages of the cellular toxicity pathway. In addition, and similar to
463 what is proposed for surface water quality monitoring,[31] one could add one or more bioassays with
464 whole organisms, such as the fish embryo test or an algal toxicity assay, to assure that all bioactive
465 chemicals are captured by the bioassays.

466 Future studies on treatment wetland systems with the battery of indicator bioassays and
467 indicator chemicals should include frequent and regular sampling on full-scale systems over the
468 course of at least one year in order to encompass stochastic variability of influent and effluent
469 wastewater, as well as any seasonal variations in removal of biological effects. A more extensive
470 internal sampling in saturated treatment wetland systems along the flow path could also help to
471 optimise the design of future treatment wetland systems.

472

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485

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608

609

610 **Table 1**: Description of design and system types of the studied treatment wetlands.

Site ID	Design	System type	Design flow (L/d)	Area (m^2)	Effective depth (cm)**	Operation start date
H50p	Conventional	Horizontal flow, planted	200	5.6	50	2010
VAp	Intensified	Vertical flow with aeration, planted	576	6.2	85	2010
VAp + VSp	Intensified, two-stage system	Vertical flow with aeration (saturated) followed by an unsaturated vertical flow, planted	576	6.2 (each cell)	85	2016*
HAp	Intensified	Horizontal flow with aeration, planted	576	5.6	100	2010
R	Intensified	Reciprocating	1440	13.2	95	2011
HMc	Intensified	Horizontal flow with aeration, planted	576	5.6	90	2014
HM	Intensified	Horizontal flow with no aeration from 40 – 70% fractional distance, planted	576	5.6	100	2014

611 *VSp operational since 2012, but only used in combination with VAp since 2016

612 ** Effective depth denotes depth of wetland system actively involved in treatment

613 **Table 2**: Overview of applied bioassays.

Endpoint	Assay	Method reference	Positive reference compound	EC	Positive reference compound EC value
Activation of aryl hydrocarbon receptor (AhR)	AhR CALUX	Brennan *et al.*[61]	2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD)	EC_{10}	$(5.92 \pm 0.16) \times 10^{-13}$ M
Binding to peroxisome proliferator-activated receptor gamma (PPARγ)	PPARγ-*bla*	Neale *et al.*[31]	Rosiglitazone	EC_{10}	$(5.68 \pm 0.37) \times 10^{-10}$ M
Activation of estrogen receptor (ER)	ERα GeneBLAzer	König *et al.*[33]	17β-Estradiol	EC_{10}	$(9.87 \pm 0.60) \times 10^{-12}$ M
Inhibition of estrogen receptor (ER)	ERα GeneBLAzer	König *et al.*[33]	Tamoxifen	$EC_{SR0.2}$	$(2.46 \pm 0.18) \times 10^{-5}$ M
Activation of androgen receptor (AR)	AR GeneBLAzer	König *et al.*[33]	Metribolone (R1881)	EC_{10}	$(4.10 \pm 0.43) \times 10^{-11}$ M
Inhibition of androgen receptor (AR)	AR GeneBLAzer	König *et al.*[33]	Cyproterone acetate	$EC_{SR0.2}$	$(2.45 \pm 0.42) \times 10^{-8}$ M
Activation of glucocorticoid receptor (GR)	GR GeneBLAzer	König *et al.*[33]	Dexamethasone	EC_{10}	$(2.08 \pm 0.05) \times 10^{-10}$ M
Inhibition of glucocorticoid receptor (GR)	GR GeneBLAzer	König *et al.*[33]	Mifepristone (RU486)	$EC_{SR0.2}$	$(3.98 \pm 0.28) \times 10^{-10}$ M
Activation of progesterone receptor (PR)	PR GeneBLAzer	König *et al.*[33]	Promegestone	EC_{10}	$(1.81 \pm 0.08) \times 10^{-11}$ M
Inhibition of progesterone receptor (PR)	PR GeneBLAzer	König *et al.*[33]	Mifepristone (RU486)	$EC_{SR0.2}$	$(3.00 \pm 0.45) \times 10^{-10}$ M
Oxidative stress response	AREc32	Escher *et al.*[46,] Wang *et al.*[62]	tert-Butylhydroquinone (tBHQ)	$EC_{IR1.5}$	$(1.56 \pm 0.03) \times 10^{-6}$ M
NF-κB response	NF-κB-*bla*	König *et al.*[33]	Tumor necrosis factor Alpha (TNFα)	$EC_{IR1.5}$	11.1 ± 0.21 ng/L

614

615 **Table 3**: BEQ_{bio} values for the studied bioassays (ng/L).

		Activation of AhR	**Binding to PPARγ**	**Activation of ER**	**Activation of AR**	**Inhibition of AR**	**Activation of GR**	**Inhibition of PR**	**Oxidative Stress Response**	**NF-κB Response**
WWTP (in)	*July*	(2.53±0.12) ×10^{-1}	(7.19±0.87) ×10^{2}	(2.35±0.17) ×10^{1}	(2.38±0.27) ×10^{1}	Cytotoxic	Cytotoxic	Cytotoxic	(9.23±0.46) ×10^{5}	(2.22±0.14) ×10^{2}
	November	(2.70±0.11) ×10^{-1}	(9.36±0.77) ×10^{2}	(1.05±0.01) ×10^{1}	(1.42±0.18) ×10^{1}	Cytotoxic	Cytotoxic	Cytotoxic	(8.66±0.38) ×10^{5}	(3.09±0.11) ×10^{1}
WWTP (out)	*July*	(1.18±0.05) ×10^{-1}	(8.31±0.66) ×10^{1}	(4.20±0.29) ×10^{-1}	Cytotoxic	Cytotoxic	(1.71±0.06) ×10^{1}	Cytotoxic	(1.37±0.04) ×10^{5}	(3.42±0.15) ×10^{1}
	November	(1.30±0.05) ×10^{-1}	(1.34±0.14) ×10^{2}	(7.05±0.52) ×10^{-1}	Cytotoxic	Cytotoxic	(1.94±0.09) ×10^{1}	Cytotoxic	(1.69±0.04) ×10^{5}	(1.21±0.08) ×10^{2}
SEP (out)	*July*	(8.32±0.50) ×10^{-1}	(5.77±0.58) ×10^{2}	(3.12±0.22) ×10^{1}	Cytotoxic	(3.35±0.72) ×10^{4}	Cytotoxic	Cytotoxic	(1.07±0.03) ×10^{6}	(5.62±0.27) ×10^{2}
	November	(4.44±0.18) ×10^{-1}	(7.65±0.62) ×10^{2}	(1.97±0.05) ×10^{1}	Cytotoxic	(2.81±0.63) ×10^{4}	Cytotoxic	Cytotoxic	(8.48±0.30) ×10^{5}	(5.10±0.30) ×10^{2}
H50p	*July*	(2.93±0.13) ×10^{-1}	(4.61±0.42) ×10^{2}	(2.75±0.21) ×10^{1}	Cytotoxic	Cytotoxic	Cytotoxic	Cytotoxic	(4.95±0.15) ×10^{5}	(5.63±0.23) ×10^{2}
	November	(1.65±0.11) ×10^{-1}	(5.91±0.81) ×10^{2}	(1.15±0.08) ×10^{1}	Cytotoxic	Cytotoxic	Cytotoxic	(7.46±1.25) ×10^{1}	(4.07±0.12) ×10^{5}	(7.37±0.38) ×10^{2}
VAp	*July*	(2.13±0.10) ×10^{-1}	(7.58±0.58) ×10^{1}	(6.52±0.44) ×10^{-1}	Cytotoxic	(1.07±0.29) ×10^{4}	(1.88±0.06) ×10^{1}	Cytotoxic	(1.47±0.04) ×10^{5}	(4.00±0.21) ×10^{1}
	November	(1.02±0.05) ×10^{-1}	(6.54±0.55) ×10^{1}	(4.61±0.30) ×10^{-1}	>50	(4.67±2.38) ×10^{3}	(1.17±0.04) ×10^{1}	(5.93±1.07) ×10^{0}	(1.19±0.03) ×10^{5}	(4.15±0.22) ×10^{1}
VAp + VSp	*July*	(2.08±0.10) ×10^{-1}	(3.65±0.28) ×10^{1}	(1.41±0.09) ×10^{-1}	>50	(1.58±0.44) ×10^{3}	(4.58±0.14) ×10^{0}	(5.86±0.92) ×10^{0}	(8.87±0.28) ×10^{4}	(2.49±0.08) ×10^{1}
	November	(7.72±0.35) ×10^{-2}	(3.54±0.32) ×10^{1}	(1.48±0.10) ×10^{-1}	>50	(1.97±0.50) ×10^{3}	(1.99±0.08) ×10^{0}	(4.62±0.74) ×10^{0}	(5.31±0.17) ×10^{4}	(6.13±0.26) ×10^{0}
HAp	*July*	(1.25±0.07) ×10^{-1}	(5.14±0.40) ×10^{1}	(2.08±0.15) ×10^{-1}	Cytotoxic	(5.00±1.46) ×10^{3}	(7.95±0.55) ×10^{0}	Cytotoxic	(9.09±0.26) ×10^{4}	(2.22±0.08) ×10^{1}
	November	(7.62±0.30) ×10^{-2}	(6.24±0.58) ×10^{1}	(2.19±0.15) ×10^{-1}	>50	(1.14±0.36) ×10^{3}	Cytotoxic	(4.79±0.81) ×10^{0}	(7.87±0.25) ×10^{4}	(1.87±0.09) ×10^{1}
R	*July*	(1.41±0.05) ×10^{-1}	(7.62±0.60) ×10^{1}	(6.84±0.45) ×10^{-1}	Cytotoxic	(1.31±0.32) ×10^{3}	(1.74±0.06) ×10^{1}	Cytotoxic	(1.08±0.04) ×10^{5}	(5.53±0.30) ×10^{1}
	November	(1.00±0.04) ×10^{-1}	(9.91±0.77) ×10^{1}	(5.63±0.39) ×10^{-1}	>50	(6.59±1.30) ×10^{2}	(1.79±0.05) ×10^{1}	(7.07±1.10) ×10^{0}	(8.70±0.26) ×10^{4}	(3.80±0.41) ×10^{1}

HMc	*July*	(1.10±0.05) $\times10^{-1}$	(1.31±0.10) $\times10^{2}$	(3.76±0.28) $\times10^{-1}$	Cytotoxic	Cytotoxic	Cytotoxic	Cytotoxic	(1.04±0.03) $\times10^{5}$	(1.51±0.07) $\times10^{1}$
	November	(6.99±0.30) $\times10^{-2}$	(1.13±0.09) $\times10^{2}$	Cytotoxic	Cytotoxic	Cytotoxic	Cytotoxic	(1.02±0.18) $\times10^{1}$	(6.13±0.18) $\times10^{4}$	(1.40±0.09) $\times10^{1}$
HM	*July*	(1.16±0.06) $\times10^{-1}$	(9.52±0.71) $\times10^{1}$	(2.99±0.22) $\times10^{-1}$	Cytotoxic	Cytotoxic	(8.97±0.55) $\times10^{0}$	Cytotoxic	(9.06±0.28) $\times10^{4}$	(2.48±0.09) $\times10^{1}$
	November	(8.37±0.35) $\times10^{-2}$	(1.42±0.10) $\times10^{2}$	(5.16±0.43) $\times10^{-1}$	Cytotoxic	Cytotoxic	(6.63±0.33) $\times10^{0}$	Cytotoxic	(3.82±0.17) $\times10^{4}$	(1.35±0.10) $\times10^{1}$
HM40	*July*	(1.79±0.07) $\times10^{-1}$	(2.10±0.16) $\times10^{2}$	Cytotoxic	Cytotoxic	Cytotoxic	(2.11±0.11) $\times10^{1}$	Cytotoxic	(2.42±0.11) $\times10^{5}$	(3.48±0.12) $\times10^{1}$
	November	(1.25±0.05) $\times10^{-1}$	(3.42±0.28) $\times10^{2}$	Cytotoxic	Cytotoxic	Cytotoxic	Cytotoxic	(1.40±0.26) $\times10^{1}$	(8.82±0.73) $\times10^{4}$	(1.06±0.04) $\times10^{1}$
HM70	*July*	(9.62±0.47) $\times10^{-2}$	(1.36±0.10) $\times10^{2}$	Cytotoxic	Cytotoxic	Cytotoxic	Cytotoxic	Cytotoxic	(8.15±0.31) $\times10^{4}$	(2.71±0.17) $\times10^{1}$
	November	(7.60±0.37) $\times10^{-2}$	(1.67±0.14) $\times10^{2}$	Cytotoxic	Cytotoxic	Cytotoxic	Cytotoxic	(2.17±0.54) $\times10^{1}$	(5.55±0.21) $\times10^{4}$	(1.91±0.08) $\times10^{1}$

NB: All samples were cytotoxic or had no effect in ERα GeneBLAzer (antagonist mode), GR GeneBLAzer (antagonist mode) and PR GeneBLAzer (agonist mode).

618 **Table 4**: Removal efficacy of the indicator micropollutants by conventional and intensified treatment wetlands, as well as conventional WWTP

619 calculated using Equation1 and the chemical concentrations reported in the ESI, Table S2.

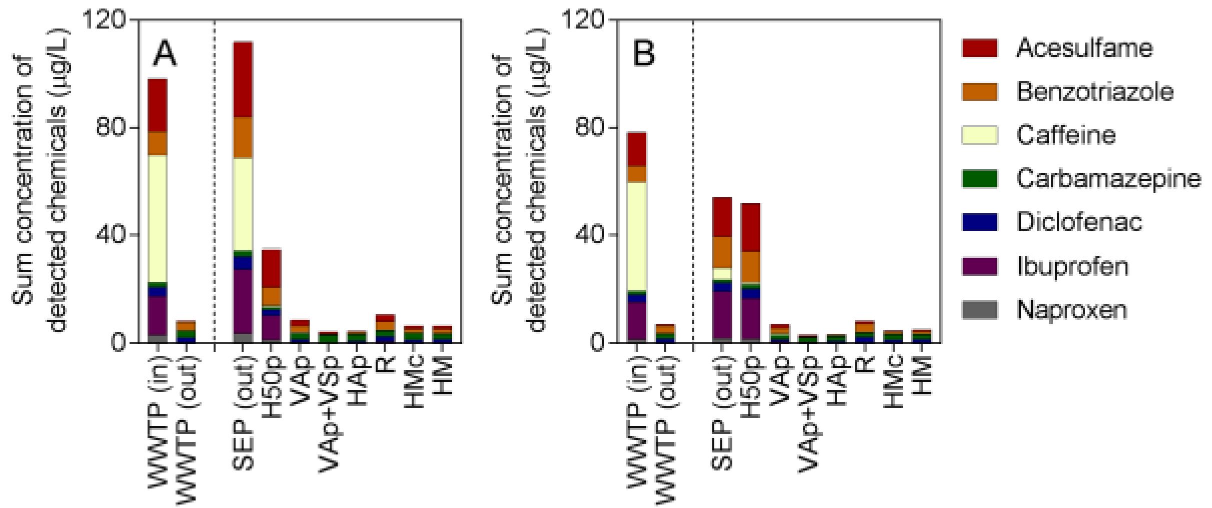
		WWTP	**H50p**	**VAp**	**VAp + VSp**	**HAp**	**R**	**HMc**	**HM**
Caffeine	*July*	>99%	99%	99%	>99%	>99%	>99%	>99%	>99%
	November	>99%	80%	90%	>96%	>96%	94%	93%	>96%
Ibuprofen	*July*	>94%	61%	>97%	>97%	>97%	>97%	>97%	>97%
	November	>94%	13%	>95%	>95%	>95%	>95%	>95%	>95%
Naproxen	*July*	98%	69%	94%	>98%	>98%	93%	>98%	98%
	November	>95%	17%	94%	>97%	>97%	94%	>97%	>97%
Benzotriazole	*July*	64%	55%	84%	97%	96%	79%	90%	89%
	November	54%	0%	81%	98%	97%	72%	93%	90%
Diclofenac	*July*	44%	57%	79%	96%	85%	60%	74%	74%
	November	44%	-14%	72%	88%	76%	36%	62%	56%
Acesulfame	*July*	97%	50%	92%	97%	98%	91%	96%	96%
	November	95%	-21%	91%	96%	98%	93%	98%	96%
Carbamazepine	*July*	-47%	48%	-4%	-27%	-16%	4%	-11%	3%
	November	-25%	-31%	-28%	-31%	-36%	-20%	-55%	-41%

620

621 **Figure 1**: Sum of detected chemicals in units of µg/L in A) July and B) November 2016. SEP(out)

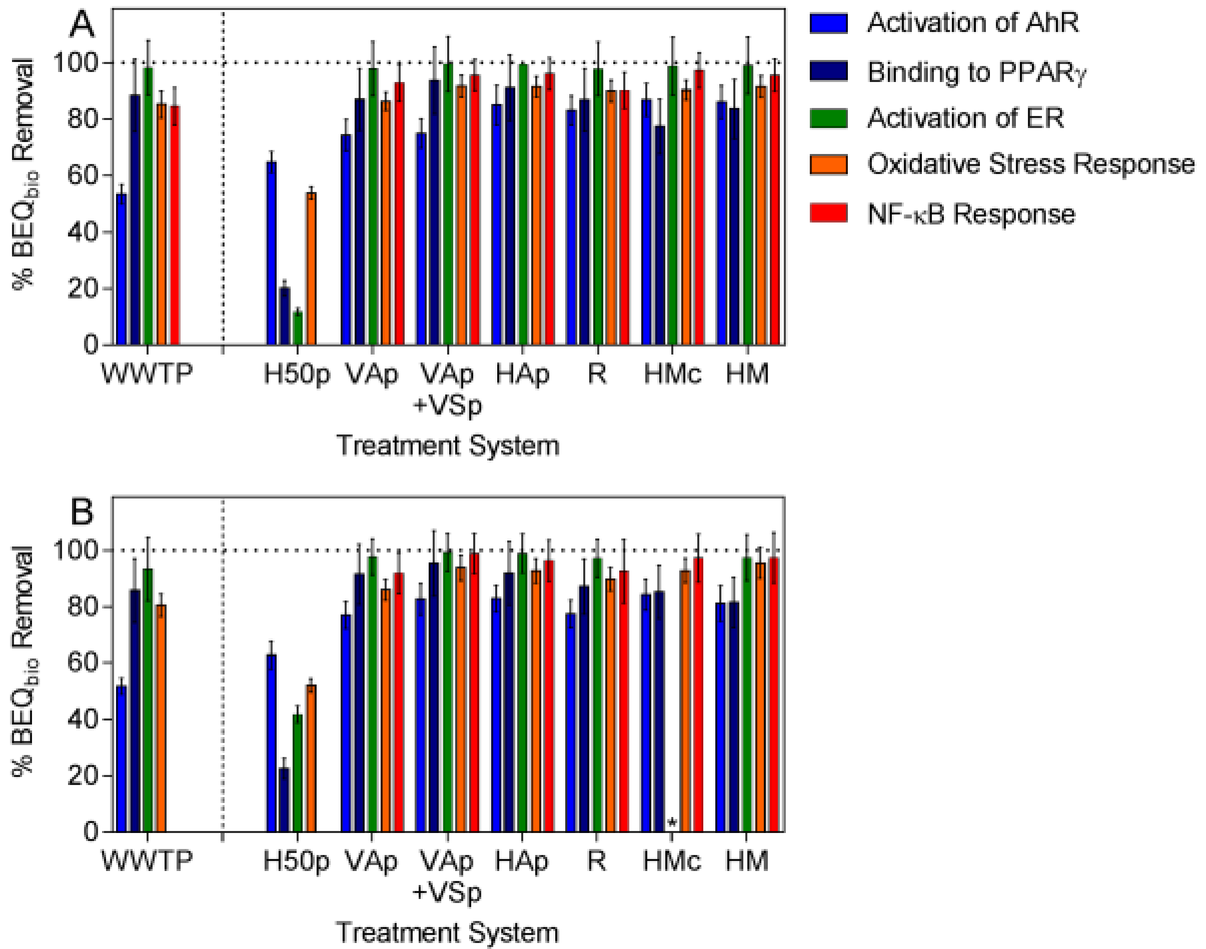
622 denotes the influent to the treatment wetlands. Measured concentrations are also provided in Table

623 S2 of the ESI.



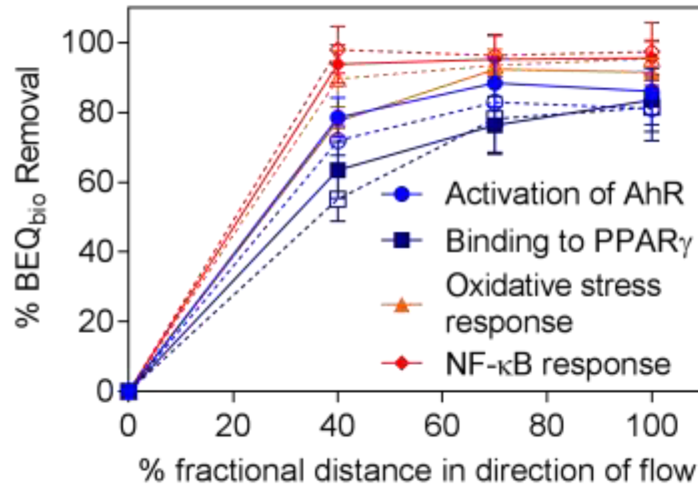
624

625 **Figure 2**: Removal of BEQ_{bio} (%) after treatment in A) July and B) November for assays indicative
626 of activation of AhR, binding to PPARγ, activation of ER, oxidative stress response and NF-κB
627 response. *removal could not be calculated for HMc due to cytotoxicity. The associated data are
628 given in the ESI, Table S4. Error bars were calculated using error propagation from errors derived
629 by concentration-effect curve modeling.



630

631 **Figure 3**: Removal of BEQ_{bio} (%) along the flow path in HM for assays indicative of activation of
632 AhR, binding to PPARγ, activation of ER, oxidative stress response and NF-κB response (Equation
633 3). Samples collected in July indicated by closed symbols and solid lines; samples collected in
634 November indicated by open symbols and dashed lines. Error bars were calculated using error
635 propagation.



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