Assessing the impact of two conventional wastewater treatment plants on small streams with effect-based methods

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60% of surface water bodies in Europe do not meet the requirements for good ecological and chemical status and in Germany the situation is even worse with over 90% of surface waters. In addition to hydromorphological degradation, intensive land use and invasive species, chemical pollution is primarily considered to be responsible for the inadequate ecological status of the water bodies. As a quantitatively important source of micropollutants in particular, wastewater treatment plants (WWTPs) represent an important entry path for chemical stressors. It is therefore important to analyze the effectiveness of the WWTPs in eliminating micropollutants and other chemical stressors to mitigate the negative impacts of the treated wastewater (WW) in the aquatic ecosystems. Accordingly, in this study, we evaluated the impacts of two conventional, medium-sized WWTPs on their small receiving water systems in the southwestern region of Hessen in Germany during two sampling campaigns (spring and fall) using effect-based methods (EBM). The results showed that the untreated WW had a very high baseline toxicity and also high endocrine and mutagenic activities. Conventional WW treatment, consisting of mechanical and biological treatment with nitrification, denitrification and phosphate precipitation, reduced baseline toxicity by more than 90% and endocrine activities by more than 80% in both WWTPs. Despite these high elimination rates, the remaining baseline toxicity, the endocrine, dioxin-like and mutagenic activities of the conventionally treated WW was so high that negative effects on the two receiving waters were to be expected. This was confirmed in active monitoring with the amphipod *Gammarus fossarum* and the mud snail *Potamopyrgus antipodarum*, as mortality of both species increased downstream of the WWTPs and reproduction in *P. antipodarum* was also affected. These results indicate that advanced WW treatment is needed to more effectively eliminate chemical stressors to prevent negative impacts of treated WW particularly in small receiving waters.

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4
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16 ## Abstract
17
18 60% of surface water bodies in Europe do not meet the requirements for good ecological and
19 chemical status and in Germany the situation is even worse with over 90% of surface waters. In
20 addition to hydromorphological degradation, intensive land use and invasive species, chemical
21 pollution is primarily considered to be responsible for the inadequate ecological status of the water
22 bodies. As a quantitatively important source of micropollutants in particular, wastewater treatment
23 plants (WWTPs) represent an important entry path for chemical stressors. It is therefore important
24 to analyze the effectiveness of the WWTPs in eliminating micropollutants and other chemical
25 stressors to mitigate the negative impacts of the treated wastewater (WW) in the aquatic
26 ecosystems. Accordingly, in this study, we evaluated the impacts of two conventional, medium-
27 sized WWTPs on their small receiving water systems in the southwestern region of Hessen in
28 Germany during two sampling campaigns (spring and fall) using effect-based methods (EBM).
29 The results showed that the untreated WW had a very high baseline toxicity and also high
30 endocrine and mutagenic activities. Conventional WW treatment, consisting of mechanical and
31 biological treatment with nitrification, denitrification and phosphate precipitation, reduced
32 baseline toxicity by more than 90% and endocrine activities by more than 80% in both WWTPs.
33 Despite these high elimination rates, the remaining baseline toxicity, the endocrine, dioxin-like
34 and mutagenic activities of the conventionally treated WW was so high that negative effects on
35 the two receiving waters were to be expected. This was confirmed in active monitoring with the
36 amphipod *Gammarus fossarum* and the mud snail *Potamopyrgus antipodarum*, as mortality of
37 both species increased downstream of the WWTPs and reproduction in *P. antipodarum* was also
38 affected. These results indicate that advanced WW treatment is needed to more effectively

39 eliminate chemical stressors to prevent negative impacts of treated WW particularly in small
40 receiving waters.
41
Keywords
43
44 Endocrine disruption, baseline toxicity, dioxin-like activity, mutagenicity, active monitoring,
45 *Potamopyrgus antipodarum, Gammarus fossarum*
46
Introduction
48
49 In many areas worldwide, the ecological status of small streams is impacted by morphological
50 degradation (e.g., channelization and straightening), agricultural land use in the catchment, and a
51 high load of treated wastewater (WW) as the major source of aquatic pollution (Reemtsma et al.,
52 2006; Stalter et al., 2013; Ternes et al., 2017). Next to other anthropogenic chemicals entering the
53 water cycle via municipal and industrial wastewater treatment plants (WWTPs), micropollutants,
54 which include pharmaceuticals, personal care products, biocides, pesticides and endocrine
55 disrupting chemicals (EDCs), among others, have been identified as a main driver that, even at
56 low concentrations, can affect ecosystems and human health (Khan et al., 2022; Yang et al., 2022).
57 These stressors have led to significant declines in aquatic biodiversity, which in turn has had a
58 profound impact on the ecological integrity of many aquatic ecosystems (Enns et al., 2023; Jähnig
59 et al., 2021). Currently, 60% of the surface water bodies in Europe do not meet the requirements
60 of a good ecological and chemical status according to the European Water Framework Directive
61 (European Commission, 2019). In Germany the situation is even worse because more than 90% of
62 surface waters have not yet reached a good ecological status (German Environment Agency, 2017).
63 Municipal WWTPs have been implemented to reduce high nitrogen, phosphorus, and organic
64 matter levels. However, many studies have already shown that the elimination rate for
65 micropollutants often remains poor (Giebner et al., 2018; Gosset et al., 2021; Long & Bonefeld-
66 Jørgensen, 2012). These pollutants are thus continuously released in trace amounts (typically from
67 ng/L to µg/L) into receiving watercourses (Wolf et al., 2022). Particularly, estrone, 17β-estradiol,
68 17α-ethinylestradiol and bisphenol A have been identified by various studies both in the influent
69 and in the effluent of WWTPs (Körner et al., 2000; Kusk et al., 2011). Besides other compounds,
70 such as polycyclic aromatic hydrocarbons (PAHs) which could be aryl hydrocarbon receptor
71 (AhR) agonists and have a high mutagenic potential, have been measured in industrial and
72 domestic discharges entering rivers directly or indirectly via sewage effluents (Denison & Nagy,
73 2003; Reifferscheid et al., 2011). Additionally, biologically highly active chemicals such as
74 pharmaceuticals and pesticides have been detected in the effluents from WWTPs on a regular basis
75 (Lopez et al., 2022; Ternes et al., 2015, 2017).
76 Due to the prominent role of WWTPs as a source of micropollutants in surface waters, there are
77 currently significant efforts to improve the elimination of micropollutants in WW treatment.
78 Oxidative processes such as ozonation and filtration processes with activated carbon have proven

79 to be particularly promising (Prasse et al., 2015). Also, in our study area, the Hessian Ried in the
80 south of Frankfurt am Main, Germany, WW-borne micropollutants have been detected not only in
81 high concentration in the receiving surface waters but also in groundwater, due to the specific
82 geological and hydrological situation with a high infiltration rate. With the "Hessian Ried Trace
83 Substance Strategy", the Hessian state government is currently implementing various measures to
84 reduce micropollutant contamination in surface waters and groundwater and thereby minimize the
85 ecological consequences and risks to drinking water resources. A central measure is the upgrading
86 of selected municipal WWTPs with an advanced WW treatment step (so-called 4th treatment step)
87 to achieve improved micropollutant elimination (HMUKLV, 2018a).
88 As part of a scientific accompanying program, we will examine the effectiveness of this measure,
89 which will be implemented in the next years, with effect-based methods (EBM). The aim of our
90 work is to describe the situation before and after the implementation of the 4th treatment step with
91 a combination of ozonation, followed by activated carbon filtration in two WWTPs. In a first step,
92 we characterized both the impact profile of the treated WW and the effects of WW discharge on
93 the receiving surface waters and thus established a baseline for subsequent investigations after the
94 implementation of the WWTP upgrade. We hypothesize that due to the insufficient elimination of
95 micropollutants (1) a broad spectrum of baseline toxic and specific toxic activities can be detected
96 in conventionally treated WW using EBMs and (2) the corresponding effects can also be detected
97 in the receiving surface water downstream the WWTP effluents. As EBMs we use a battery of *in*
98 *vivo* and *in vitro* bioassays: The baseline or unspecific toxicity is assessed with the Microtox assay
99 with *Aliivibrio fischeri*, mutagenicity with the Ames fluctuation assay (*Salmonella typhimurium*),
100 endocrine and dioxin-like activities with recombinant yeast reporter gene assays *(Saccharomyces*
101 *cerevisiae)*, and reproduction and survival in an active biological effect monitoring with the stream
102 amphipod *Gammarus fossarum* and the mudsnail *Potamopyrgus antipodarum* in two sampling
103 campaigns (spring and fall) to take into account possible seasonal effects.
104
105 **Materials & Methods**
106
107 **Characterization of the wastewater treatment plants Mörfelden-Walldorf and Bickenbach**
108
109 The medium-sized WWTPs Mörfelden Walldorf (MW) and Bickenbach (B) are located in the
110 southwestern region of Hessen in Germany and have approximately 48,000 and 32,000 population
111 equivalents (PE), respectively. The WW is currently processed with conventional treatment
112 (mechanical, biological treatment, nitrification, denitrification, phosphate precipitation) in both
113 WWTPs. The receiving watercourses for the treated wastewater are the Geräthsbach (waterbody
114 code 239818) for the WWTP MW, and the Landbach (waterbody code 239628) for the WWTP B.
115 They are classified as small streams in riverine floodplains (type 19) and exhibit a poor chemical
116 status, a strongly or completely altered morphological structure, as well as a moderate and poor
117 ecological status respectively (HLNUG, 2022). Additionally, the mean flow (MQ) in these streams

118 is 125 L/s (Geräthsbach) and 238 L/s (Landbach) (HLNUG, 2022). Also, it is important to mention
119 that 10 km upstream WWTP MW, there is another WWTP in Langen (Figure 1).
120

Collection and storage of the wastewater samples

123 For the study, four sampling sites were chosen for each of the two WWTPs (Table 1).

124 In two sampling campaigns in April/May 2022 (spring) and October/November 2022 (fall), 2 L
125 water samples were taken at the four sampling sites at each WWTP at the beginning and at the end
126 of the four weeks active biomonitoring period so that two water samples were obtained and
127 analyzed per season. During the sampling campaign in fall 2022, the WWTP in Langen, which is
128 located 10 km upstream of WWTP MW at the Geräthsbach, was also taken into account (sample
129 code BT-L), because the first collection campaign had shown that the Gerätehsbach already
130 showed high activities in the EBM upstream the WWTP MW. At each sampling site, physico-
131 chemical parameters such as temperature, pH, dissolved oxygen, and conductivity were measured
132 in the field with a portable multi-meter (HQ40d, Hach, Germany). The water samples were
133 collected in glass vessels precleaned with deionized water, acetone, and ethanol and heated to
134 200°C before use. The samples were transported and stored at 7°C in darkness. Phosphate,
135 ammonium, nitrite, and nitrate concentrations were determined using spectroquant test kits
136 (Merck, Darmstadt, Germany) immediately after returning the samples to the laboratory.
137

Sample preparation

140 Water samples were filtered through glass microfiber filters (VWR International GmbH, No. 692,
141 European Cat. No. 516-0885, 90 mm, particle retention: 1.5 µm, Darmstadt, Germany). Then, 2000
142 mL of each water sample was solid-phase extracted (SPE) with OASIS HLB cartridges (6cc, 200
143 mg, Waters, Milford, MA, USA) according to Giebner et al. (2018) and transferred to a final
144 volume of 400 µL DMSO so that the final extracts were 5,000-fold concentrated compared to the
145 aqueous samples. Extracts were stored in glass vials at -25°C until further processing. To check a
146 potential contamination of the samples during the sample preparation, 2000 mL of ultrapure water
147 was extracted in parallel (SPE blank) (Giebner et al., 2018).
148

Test systems

150

In vitro assays

152

Baseline toxicity test

154

155 The Microtox assay or bioluminescence inhibition test with the bacterium *Aliivibrio fischeri* was
156 conducted to assess the baseline toxicity in the extracts. Based on the International Organization
157 for Standardization (ISO) guideline 11348-3 (ISO, 2007) the assay was performed in 96-well

158   microplates according to Völker et al. (2017). Samples were analyzed in dilution series in a saline
159   buffer with seven consecutive steps (spacing factor 2 between concentrations), resulting in a 25-
160   fold to 0.2-fold final concentration (Völker et al., 2017). Negative and solvent controls, SPE blank,
161   reference compound (3,5-dichlorophenol), and SPE extracts were serially diluted (1:2) in a saline
162   buffer. 100 mL sample was added to 50 mL of *A. fischeri* solution (not exceeding 1% DMSO in
163   the final medium volume). To detect inhibition, luminescence was measured prior to sample
164   addition and after 30 min incubation using a microplate reader (Infinite 200 Pro, Tecan,
165   Crailsheim, Germany) as described by Völker et al. (2017). The inhibition of luminescence is
166   expressed as the arithmetic mean value of the 50% effect concentration (EC_{50}) of three independent
167   experiments, referring to the relative enrichment factor (REF) of the water sample. Nontoxic water
168   samples exhibit an EC_{50} threshold value of 300 (REF).
169
170   **Recombinant yeast reporter gene assays**
171
172   The Yeast Estrogen Screen (YES), Yeast Androgen Screen (YAS) Yeast Anti-Estrogen Screen
173   (YAES), Yeast Anti-Androgen Screen (YAAS), and Yeast Dioxin Screen (YDS) were used to
174   assess the receptor-mediated agonistic and antagonistic endocrine activity of the extracted water
175   samples as well as the activation of the aryl hydrocarbon receptor assay according to
176   Brettschneider et al. (2019a, b), Giebner et al. (2018) and Schneider et al. (2020). These assays use
177   genetically modified strains of *Saccharomyces cerevisiae*, which contain the human estrogen
178   receptor α, androgen and aryl hydrocarbon receptor, respectively. The YAES and YAAS were
179   performed with native samples because SPE enrichment of water samples from surface waters and
180   WWTPs leads to a strong loss of anti-estrogenic and anti-androgenic activities (Brettschneider et
181   al., 2019a; Giebner et al., 2018; Schneider et al., 2020) .The YES, YAS and YDS were performed
182   in three independent experiments with every sample, the YAES and YAAS in two independent
183   experiments, with eight technical replicates per sample. The measured activities are expressed as
184   equivalent concentrations (EQ) for the positive substances 17β-estradiol (YES), testosterone
185   (YAS), 4-hydroxytamoxifen (YAES), flutamide (YAAS) and β- naphthoflavone (YDS) and were
186   corrected regarding dilution and enrichment so that equivalent concentrations relate back to the
187   native water sample.
188
189   **Ames fluctuation test**
190
191   The Ames fluctuation assay (ISO, 2012) was performed to assess the mutagenic potential of the
192   SPE samples, using *Salmonella typhimurium* strains YG1041 and YG1042 (Hagiwara et al., 1993)
193   with and without metabolic activation by rodent liver enzymes (S9-mix; Envigo CRS, Roßdorf,
194   Germany).
195   The test is based on the inability of genetically modified *S. typhimurium* strains to synthesize the
196   essential amino acid histidine and, therefore, not being able to survive in a histidine-free medium
197   (Maron & Ames, 1983). The bacteria are exposed to the SPE extracts in a 16.7-fold final sample

198   concentration (0.2% v/v solvent) and incubated for 72 hours at 37°C in a histidine-free medium.
199   After this time, a color change of the medium indicates the survival of the bacteria by regaining
200   the ability to synthesize histidine because of a back mutation.
201   YG1041 and YG1042 strains without the S9 mix were tested with 2-nitrofluorene as a positive
202   control. Conversely, these strains with S9-mix were tested with 2-amino anthracene as a positive
203   control. The mutagenic potential of the samples is assessed via the percentage of reverted wells,
204   subtracted by the mutants of negative historical controls with samples exceeding the threshold of
205   20.8% revertant wells are considered as mutagenic. Every sample was tested with both strains,
206   with and without the S9-mix. If an effect was observed, the test was consequently conducted two
207   times.
208
209   *In vivo* **assays**
210
211   The *in vivo* assays were applied as an active in-situ monitoring at the upstream and downstream
212   samplings sites of both WWTPs as described by Brettschneider et al. (2019a) and Harth et al.
213   (2018).
214
215   **Active monitoring with *Gammarus fossarum***
216
217   The gammarids for the active monitoring were collected at the upper reach of the river Urselbach
218   (Hessen, Germany, 50°13'30.1"N 8°31'06.1"E) one week before the start of the spring and fall
219   campaigns. At the upstream and downstream samplings sites of both WWTPs, two stainless steel
220   cages each containing three enclosures (12.5 cm x 6 cm) were fixed on the riverbed in the direction
221   of flow. The enclosures were capped with a net (mesh size 1.0 mm) at the ends to ensure a water
222   flow. Each enclosure, representing a replicate, contained ten gammarids, resulting in a total of 60
223   gammarids per site which were exposed *in situ* for four weeks. Black alder leaves (*Alnus glutinosa*)
224   were provided as food in the enclosures *ad libitum* and two small nets (polytetrafluorethylene, 8.2
225   cm x 3.3 cm) served as hiding places to prevent cannibalism.
226   After four weeks of exposure, the cages were taken to the laboratory, and the gammarids were
227   fixed in 70% ethanol and stored separately in Eppendorf tubes. As biological endpoints the
228   mortality and fecundity index (number of eggs divided by the body length) were assessed.
229   For all measurements, a stereomicroscope (Olympus SZ61 R, Olympus Corporation, Tokio, Japan)
230   with a digital camera (JVC Digital Camera KY-F75U, Victor Company of Japan Ldt., Yokohama,
231   Japan) and the Software Diskus (Version 4.50.1458, Carl H. Hilgers, Königswinter, Germany) was
232   used.
233
234   **Active monitoring with *Potamopyrgus antipodarum***
235
236   The mudsnails were collected at the rivers Lumda and Horloff (Hessen, Germany) in spring and
237   at the rivers Gambach and Waschbach (Hessen, Germany) in fall. Snails with a shell length

238 between 3.5 and 4.5 mm were used, following the protocol by Brettschneider et al. (2019a). At the
239 upstream and downstream samplings sites of both WWTPs, two stainless steel cages containing
240 three smaller steel cages (4.5 cm × 3.5 cm, mesh size 0.7 mm) were fixed on the riverbed in the
241 direction of flow. Ten snails were placed in each small cage, representing the six replicates and
242 resulting in a total of 60 snails which were exposed *in situ* for four weeks. Organic carrot cubes
243 served as food supply and were refilled after two weeks.
244 As biological endpoints the mortality of the snails, the shell length and the number of embryos of
245 the surviving snails were determined using a stereomicroscope with an ocular micrometer (SMZ-
246 168, Motic, Xiamen, China). The active monitoring with *P. antipodarum* followed the principle
247 of the OECD guideline 242 (OECD, 2016) to assess the impact of reproductive toxicants, including
248 EDCs, on this species.
249
250 **Statistical analyses**
251
252 Statistical analyses were performed using GraphPad Prism version 5.03 for Windows (GraphPad
253 Software, San Diego, California, USA). Normal distribution was tested using the D'Agostino and
254 Pearson test. Continuous data were analyzed with t-tests for normally distributed data and Welch's
255 correction for unequal variances. All data were tested as unpaired data and with 95% confidence
256 interval. Quantal data (mortality) were analyzed with Fisher's exact test. The level of significance
257 was defined as $\alpha = 0.005$ and indicated at the graphs with asterisks as follows: ⬇= $p < 0.05$; ⬇⬇=
258 $p < 0.01$ and ⬇⬇⬇= $p < 0.001$
259
260 # Results
261
262 **Physicochemical parameters**
263
264 The conventional treatment process in both WWTPs removed more than 90% of the concentrations
265 of ammonium and phosphate and around 33% of the conductivity, independent of the season.
266 Moreover, the oxygen saturation increased significantly during the fall campaign at the WWTP
267 Mörfelden-Walldorf (MW) by 79% and in spring at the WWTP Bickenbach (B) by 89%. The
268 nitrification in the biological treatment step not only significantly reduced the ammonium
269 concentrations, but the nitrate concentrations increased significantly by factor 9.6 (spring) to 19.6
270 (fall) in MW and by factor 2.5 (spring) to 2.9 times (fall) in B. Considering the sum concentrations
271 of ammonium-N, nitrite-N and nitrate-N, inorganic N was removed by 90% and 94 % in MW and
272 97 and 94% in B in spring and fall, respectively.
273 Regardless of the effectiveness of both WWTPs in reducing the concentrations of phosphate and
274 inorganic nitrogen, they still had a strong impact on the physicochemical parameters in the
275 receiving streams, which carried between 78% and 92% treated WW downstream of the
276 discharger. The oxygen saturation decreased in the receiving streams during both seasons. The
277 conductivity and the nitrate concentrations exceeded the reference value of 800 µS/cm and 0.05

278 mg/L, respectively for river type 19 downstream of both WWTPs during the spring and fall
279 campaign (Halle & Müller, 2014). Despite the efficient ammonium removal in both WWTPs, the
280 ammonium concentrations in the receiving waters exceeded the reference value of 0.4 mg/L by up
281 to factor 2.8 (MW in spring). Conversely, the downstream concentrations for nitrate and phosphate
282 are below the reference value during spring and fall in both WWTPs (10 and 0.05 mg/L,
283 respectively) (German Environment Agency, 2017; Halle & Müller, 2014). Finally, the mean
284 values of the pH (not shown in the table 2) meet the requirements for good chemical status.
285
286 ***In vitro* assays**
287
288 **Baseline toxicity test**
289
290 The results of the Microtox assay are shown in Figure 2. The baseline toxicity increased in the
291 receiving streams downstream of both WWTPs compared with the upstream sites significantly,
292 except at WWTP MW in fall. Furthermore, all sample sites, except upstream of WWTP B in both
293 seasons showed a significant baseline toxicity. The baseline toxicity was removed by 90% in the
294 WWTPs MW and B in spring and by 96% and 97% in fall. After including the effluent from the
295 WWTP Langen (BT-L in Fig. 2A; located upstream of the WWTP MW) in the analyzes in autumn,
296 it became apparent that the basic toxicity, which was already significantly increased at the
297 upstream site of Mörfelden-Walldorf, can be attributed to the discharges from the WWTP Langen.
298
299 **Estrogenic and anti-estrogenic activity**
300
301 The estrogenic activity as determined with the YES is shown in Figure 3. All samples exhibited a
302 significant estrogenic activity above the limit of quantification (LOD = 0.148 ng E-EQ/L). Except
303 the fall campaign at the WWTP MW with a decrease by 6%, the estrogenic activities at the
304 downstream sample sites increased significantly at both WWTPs when compared to the upstream
305 sites in the receiving streams. In the case of the WWTP MW, the increment was around 22% in
306 spring, while at the WWTP B the estrogen activity increased by 60% in the receiving stream in
307 both seasons, despite the fact that the WWTPs removed more than 90% of the estrogenic activity
308 during the biological treatment.
309 The anti-estrogenic activities in the YAES are presented in Figure 4. The WWTPs did not increase
310 the anti-estrogenic activity in the receiving streams significantly. Both WWTPs had a high removal
311 efficiency of anti-estrogenic activities of over 80% in MW and 90% in Bickenbach in both seasons.
312
313 **Androgenic and anti-androgenic activity**
314
315 Androgenic and anti-androgenic activities were analyzed in 5000-fold concentrated SPE extracts
316 and native water samples, respectively. While no cytotoxic effects occurred in the native water
317 samples, the observed cytotoxicity in the SPE extracts necessitated the analysis of diluted extracts.

318 With the exception of the MT samples, no other sample from the two WWTPs and in the receiving
319 streams showed an androgenic and anti-androgenic activity with a LOD of 25.6 ng testosterone-
320 EQ/L and 771 µg flutamide-EQ/L, respectively during the spring and fall campaigns.
321
322 **Dioxin-like activity**
323
324 Figure 5 shows the agonistic activity at the AhR. The MT samples were also cytotoxic for the yeast
325 cells in the YDS, so that the dioxin-like activity hat to be assessed in serial dilutions. However, in
326 the case of Bickenbach, even with a dilution series, it was not possible to calculate the dioxin-like
327 activity of the MT samples (Fig. 5B).
328 All sample sites showed dioxin-like activity above the limit of detection (LOD = 33.8 ng β-
329 naphthoflavone-EQ/L). Dioxin-like activity increased significantly in the receiving streams due to
330 the effluents from the two WWTPs at the downstream sites compared to the upstream sites, with
331 the exception of the fall campaign at the WWTP MW (Fig. 5A). The increases were 21% and 11%
332 in MW and 85% and 72% in B during the spring and fall campaigns, respectively. The calculation
333 of the removal efficiency of dioxin-like activity was only possible for the WWTP MW because of
334 the cytotoxicity even with highly diluted MT samples of the WWTP B. In MW, elimination rates
335 of 60% and 53% were observed.
336
337 **Ames fluctuation test**
338
339 The results of the Ames fluctuation test using the strains YG1041 and YG1042 with S9-mix are
340 documented in Table 3. The tests with both strains without the S9-mix resulted in negative results
341 for all samples (less than 20.8% revertant colonies; data not shown). Due to their high cytotoxicity,
342 the MT samples from both WWTPs could not be analyzed with the Ames fluctuation test. All
343 analyzed wastewater samples from the WWTPs MW, B and Langen proved to be mutagenic in the
344 tests with the strains YG1041 and YG1042 with S9 mix, as did the samples taken from the
345 receiving water below the sewage treatment plants. The upstream (US) samples in the receiving
346 water of the WWTP MW were also mutagenic with both strains, while the US sample at WWTP
347 B was not mutagenic in the spring campaign, but mutagenic with the YG1042 strain with S9-mix
348 in the fall campaign.
349
350 **Active monitoring with *Gammarus fossarum***
351
352 The mortality in *Gammarus fossarum* during the 4 weeks active monitoring period in the receiving
353 rivers of both WWTPs in spring and fall is shown in figure 6. In both rivers the mortality in the
354 upstream site was higher in fall than in spring. There was also a significantly higher mortality in
355 the downstream sites ($p < 0.05$) compared to the upstream sites (except WWTP B in fall) with an
356 increase of 66% and 77% at WWTP MW and B, respectively.
357

358 The fecundity index was assessed for the spring campaign but did not show any significant
359 difference between the downstream and corresponding upstream site. For the fall campaign, the
360 fecundity index could not be calculated because the gammarid populations were outside their
361 reproductive phase.

362

363 **Active monitoring with *Potamopyrgus antipodarum***

364

365 The mortality and number of embryos in *Potamopyrgus antipodarum* during both campaigns are
366 shown in Figures 7 and 8. The mortality increased significantly at the downstream sites at both
367 WWTPs in fall, while there was no effect on mortality during the spring campaign.

368

369

370

371 The embryo numbers as the parameter for reproduction in *P. antipodarum* increased at the
372 downstream sites of both WWTPs in the spring campaign, although this increase was not
373 statistically significant. In contrast, the embryo numbers at the downstream site of the WWTP MW
374 were on the same level as upstream in the fall campaign but increased significantly ($p < 0.01$) at
375 the WWTP B in the same period.

376

377 **Discussion**

378

379 *Baseline toxicity*

380

381 The bioluminescence of *Aliivibrio fischeri* is directly proportional to their metabolic activity, so
382 any disruption by toxic substances results in a decreased luminescence. Considering this, a marked
383 inhibition of the bioluminescence by the raw WW (MT) in both WWTPs was observed (EC_{50}
384 $<6.45\pm 4.16$ REF). This observation is in accordance with previous reports (Macova et al., 2011;
385 Völker et al., 2017). Both conventional WWTPs in B and MW eliminated the baseline toxicity of
386 the raw WW during biological treatment by more than 90%. This elimination performance is also
387 comparable to the findings of other studies, which report elimination rates between 86% and 94%
388 (Völker et al., 2017; Yu et al., 2014).

389

390 Despite the considerable elimination of baseline toxicity during the WW treatment process in both
391 WWTPs, the effluents had still a major impact on the receiving waterbodies, so that the
392 downstream sites during the spring and fall campaign were under the non-toxic limit in the
393 Microtox assay. At MW the baseline toxicity increased by 75% in spring and 12% in fall at DS
394 compared to US, while the corresponding values in B were 68% in spring and 44% in fall. These
395 results suggest that the level of baseline toxicity was higher during spring than in the fall campaign
396 in both WWTPs, which is in line with previous studies that concluded that the toxicity of effluents
397 was usually higher in spring and summer (Vasquez & Fatta-Kassinos, 2013). This can be explained

398   by the fact that during the spring, there is an increase in agricultural activity. Also, due to the
399   increase in runoff, some pesticides or fertilizers may be washed into the water bodies and may
400   contribute to the higher activity in the Microtox assay as a bioassay detecting the unspecific
401   toxicity of a wide range of substances (Tang et al., 2013).
402
403   ***Endocrine activity***
404
405   The endocrine profile of the influent samples indicates that WW contains compounds with
406   estrogenic, androgenic, and anti-estrogenic activities. However, the conventional treatment
407   process in both WWTP efficiently reduced the estrogenic and androgenic activities by more than
408   90%. This finding is in line with the results of other studies which report an elimination of
409   estrogenic and androgenic activities between 88% and 95% (Archer et al., 2020; Arlos et al., 2018;
410   Giebner et al., 2018). In the case of anti-estrogenic activity, the efficiency at the WWTPs MW
411   (above 80%) and B (above 90%) was even higher compared to the results of Giebner et al. (2018)
412   reporting a reduction of anti-estrogenic activity during conventional treatment of only 15%.
413
414   Although the estrogenic activity of the WW was reduced by more than 90% in both WWTPs, the
415   residual activity was sufficient to adversely affect the receiving waters. This is particularly
416   noticeable at the Landbach, the receiving water of WWTP B, where the estrogenic activity
417   increased most significantly downstream of the WWTP ($p < 0.001$). At the Geräthsbach, the
418   estrogenic activity increased significantly only in spring downstream of the WWTP MW ($p < 0.01$)
419   and in autumn it even decreased significantly ($p < 0.05$). These results are in line with results in a
420   survey of 36 Danish WWTPs and their effluents (Danish Ministry of the Environment, 2005). In
421   almost 70% of the water courses receiving effluents from WWTPs, the level of estrogenic activity
422   downstream the discharge point was higher than the activity upstream of the WWTP effluent. Also
423   the study conducted by Aerni et al. (2004) who compared the estrogenic effect upstream and
424   downstream of five Swiss and French WWTPs support our findings. In all the samples sites, the
425   river upstream of the point of discharge of each WWTP had not estrogen activity (<LOQ) while
426   in the effluent the activity was on average between 0.4-5.5 ng E-EQ/L.
427
428   The reason for the lower impact of the WWTP MW on the estrogenic activity in the Geräthsbach
429   compared to the WWTP B and the Landbach is that the Geräthsbach is already influenced by the
430   WWTP Langen upstream of WWTP MW. Accordingly, the WWTP MW is not the only and first
431   source of contamination. This pattern was observed during the spring sampling campaign for
432   almost all analyzed activities (baseline toxicity, estrogenic and dioxin-like activity, mutagenicity).
433   The WWTP Langen is located 10 km upstream of MW and is with 54,600 population equivalents
434   even larger than MW (HMUKLV, 2018b). For this reason, we decided to take an additional sample
435   at the effluent of WWTP Langen during the fall campaign. The result for the estrogenic activity
436   supports the assumption that the effluent from the WWTP Langen is responsible for the higher
437   activity in the US site of the Geräthsbach compared to the Landbach.

438

439   We observed that the estrogenic activity in both WWTPs effluents was significantly higher during
440   the fall campaign than in spring ($p < 0.001$). Also this finding is in line with the Danish survey at
441   36 WWTPs which reports the highest levels of estrogenic activity in summer and fall and the
442   lowest during winter and spring (Danish Ministry of the Environment, 2005). A possible
443   explanation is the higher precipitation in winter and spring so that the concentrations of
444   estrogenically active compounds in the streams are lower than in the dry summer and autumn
445   months. Also, the fall season provides additional phyto-estrogenic material from the leaves
446   (Janeczko, 2021), increasing the estrogenic load in the water bodies.

447

448   In terms of agonistic and antagonistic androgen activity, values under the LOD (25.6 ng T-EQ /L
449   and 771.06 µg Flu-EQ /L) were detected in both effluents. The lack of a measurable androgenic
450   and anti-androgenic activity is supported by previous studies (Brzezinska et al., 2023; Li et al.,
451   2010). The same was observed for the anti-estrogenic activity, where no significant difference was
452   found between upstream and downstream sample points (values close to the LOD=1,84 mg OHT-
453   EQ/L). This finding contrasts the report by Brzezinska et al. (2023) where an antagonistic activity
454   up to100 mg OHT/L was found in the effluent of the WWTP Lodz in Poland.

455

456   **Dioxin-like activity**

457

458   The raw WW at the WWTP MW contained compounds with an agonistic activity at the aryl-
459   hydrocarbon receptor, resulting in a high dioxin-like activity. Although the conventional biological
460   treatment decreased these activities by more 60% in spring and 53% in fall, this decrease is lower
461   than in the study of Magdeburg et al., (2014) reporting an elimination of dioxin-like activity of
462   more than 81%. For WWTP B, the elimination of dioxin-like activity through conventional WW
463   treatment could not be determined because the raw WW samples were cytotoxic after mechanical
464   treatment or the enzymes responsible for luminescence were directly inhibited by the samples
465   (Reifferscheid et al., 2011). Völker et al. (2016) found a similar result for the raw WW of a WWTP
466   in their study.

467

468   Despite the significant reduction of dioxin-like activity in both WWTPs, the remaining activity
469   was sufficient to significantly increase the activity in the Geräthsbach and Landbach, except for
470   the autumn sample from the Geräthsbach, where the activity only increased slightly but not
471   statistically significantly by 12.6%. The measured activities in both rivers are comparable to the
472   rivers Horloff (up to 1150 ng β-NF-EQ/L) and Nidda (up to 1180 ng β-NF-EQ/L) in the north of
473   Frankfurt am Main, which also receive effluents form WWTPs but higher compared to rivers not
474   impacted by WWTP effluents with activities less than 200 ng β-NF-EQ/L (Brettschneider et al.,
475   2019b, 2023).

476

477 The seasonal comparison shows significantly higher dioxin-like activities (p < 0.001) at all
478 sampling points in MW in spring compared to autumn, while for the WWTP B there is only a
479 corresponding trend without significant differences. Although both WWTPs contribute to the high
480 dioxin-like activity in the rivers, other sources are relevant. The dioxin-like activity in the water
481 phase and in the sediments of rivers is primarily caused by polycyclic aromatic hydrocarbons
482 (PAHs), which enter the waters also via atmospheric deposition and surface runoff from traffic
483 areas (Boxall & Maltby, 1997; Maltby et al., 1995).

484

485 **Mutagenicity (Ames fluctuation test)**

486

487 The investigations at both WWTPs showed high mutagenic activities for all BT effluent samples
488 and for all DS samples taken downstream of the WWTPs in the two rivers, using the strains
489 YG1041 and YG1042 with S9 mix, while the same samples were negative in the tests without S9
490 mix. Due to the high cytotoxicity, the MT samples could not be evaluated. Only the spring sample
491 from the Landbach upstream of WWTP B was negative in all Ames tests.

492

493 These findings show that the conventionally treated WW contains substances that are only
494 converted into mutagenic compounds through metabolic activation, as typically occurs by
495 enzymes in the liver of vertebrates or the midgut gland of invertebrates. Furthermore, it shows that
496 conventional WW treatment is not sufficient to eliminate these compounds. The high mutagenic
497 activity at the sampling points in both rivers influenced by the discharge of treated WW
498 demonstrates the urgent need for action to reduce this undesirable effect through advanced WW
499 treatment. Giebner et al. (2018) were able to show that activated carbon filtration in particular is
500 suitable for removing mutagenic substances from WW, while oxidative processes such as
501 ozonation can lead to the formation of alkylating agents, nitrosamines and other mutagenic
502 substances in the treated WW (Mestankova et al., 2014; Schmidt & Brauch, 2008; Tsutsumi et al.,
503 2010).

504

505 **Active monitoring with *Gammarus fossarum* and *Potamopyrgus antipodarum***

506

507 To investigate potential pollution effects on the invertebrate community, we conducted an active
508 monitoring study with the crustacean *Gammarus fossarum* and the gastropod *Potamopyrgus*
509 *antipodarum*. Both species have widely been used for ecotoxicological studies, including the
510 assessment of WWTP effluents and their impacts on the biocenosis in receiving waters
511 (Brettschneider et al., 2019a, 2023; Harth et al., 2018; Schneider et al., 2020). *G. fossarum* is an
512 indicator species of a low-mountain range biocenosis (LANUV, 2015), is particularly sensitive to
513 anthropogenic pressures on water bodies and plays an important role in the food web as a shredder
514 and food for macroinvertebrates, fish, or birds (Besse et al., 2013). *P. antipodarum* is an
515 euryoecious snail inhabiting small creeks, streams, lakes, and estuaries which is particularly
516 sensitive to endocrine active chemicals (Duft et al., 2007).

517

518 Given the prominent ecological function of *G. fossarum* in the aquatic community of low-
519 mountain range rivers, the significant increased mortality downstream of the WWTP MW to 59%
520 in spring and 81% in fall is worrying. Although the same tendency was also observed downstream
521 of the WWTP B in both seasons, the mortality levels were lower and increased only significantly
522 during the spring campaign. These finding are in line with results of other active monitoring studies
523 such as Brettschneider et al. (2019a), who found increased mortality rates of 42% and 68% in *G.*
524 *fossarum* downstream of WWTPs with 43,800 PE and 78,000 PE, respectively.

525

526 Also, the mortality of *P. antipodarum* increased significantly downstream of both WWTPs in fall,
527 while there was no significant change in mortality in spring. This finding corresponds to previous
528 studies on other WW-influenced rivers, in which a significant increase in the mortality of *P.*
529 *antipodarum* occurred downstream of WWTPs, especially in autumn, while the effects were
530 significantly smaller in spring (Brettschneider et al. 2019a). In addition, in previous studies in
531 which both species were used together for active monitoring, mortality was generally lower for *P.*
532 *antipodarum* than for *G. fossarum*, possibly because the snail can avoid exposure peaks by
533 temporarily closing its shell with the operculum (Brettschneider et al., 2023).

534

535 While no influence on reproduction could be determined for *G. fossarum*, the number of embryos
536 for *P. antipodarum* increased downstream of the two WWTPs, except for the autumn campaign in
537 the Landbach. Since it is known that *P. antipodarum* responds to exposure to substances with
538 estrogenic activity by increasing embryo production (Duft et al., 2003, 2007; Jobling et al., 2004)
539 and the estrogenic activity determined by the YES was significantly increased at the study sites
540 located downstream the WWTPs, this indicates that the estrogenic exposure is the responsible
541 factor for the higher embryo numbers. The very high toxicity below the WWTP MW in autumn,
542 which is also reflected in the highest mortalities in *G. fossarum* and *P. antipodarum*, possibly
543 masks the estrogenic effect on the number of embryos in the snail. Also in other studies, the
544 number of embryos in *P. antipodarum* increased below WWTP effluents or when exposed to
545 conventionally treated WW (Brettschneider et al., 2019a; Schneider et al., 2020; Stalter et al.,
546 2010).

547

548 Together, these findings demonstrate that the effluents from conventional WWTPs negatively
549 affect key life cycle parameters of representative macroinvertebrate species in the water bodies.

550

551 **Conclusions**

552

553 The findings of this study showed that the EBM are an efficient tool to analyze the multiple effects
554 that complex mixtures, such as WW, has in the aquatic environment. These methods revealed that
555 although both WWTPs had high toxicity removal rates, remaining baseline toxicity, endocrine
556 activity (especially of estrogenic activity) and dioxin-like activity, had a negative impact on both

557   streams. In the specific case of mutagenic effects, high activities were found in both WWTPs,
558   where the effluents had high mutagenic activity compared with the sample taken upstream. The
559   negative impact of the conventionally treated WW on the receiving streams was not only
560   demonstrated by the *in vitro* test battery but also in the active *in vivo* biomonitoring.
561   In summary, both hypotheses are supported by our findings: conventionally treated WW showed
562   a wide range of activities in assays addressing specific modes of toxicological action but also a
563   high baseline toxicity. These effects were also measured in the receiving surface waters
564   downstream the WWTPs. Therefore, our study demonstrated that conventional WW treatment is
565   insufficient to prevent negative impacts on the receiving aquatic ecosystem. This implies that
566   further advanced WW treatment is urgently needed to guarantee safe effluents in these streams.
567

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569

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Table 1(on next page)

Description of sampling sites

Table 1 . Sampling sites at the WWTPs Mörfelden-Walldorf and Bickenbach and in the receiving rivers.

1

Sampling site [code]	Characteristic
Upstream [US-MW]	Geräthsbach 20 m upstream of the effluent of WWTP Mörfelden-Walldorf (49°58'14.6"N 8°32'59.3"E)
Mechanical treatment [MT-MW]	24 hours composite sample after mechanical treatment at WWTP Mörfelden-Walldorf
Biological treatment [BT-MW]	24 hours composite sample after biological treatment and phosphate precipitation at WWTP Mörfelden-Walldorf
Downstream [DS-MW]	Geräthsbach 20 m downstream of the effluent of WWTP Mörfelden-Walldorf (49°58'14.7"N 8°32'56.6"E)
Upstream [US-B]	Landbach 20 m upstream of the effluent of WWTP Bickenbach (49°45'33.9"N 8°35'52.0"E)
Mechanical treatment [MT-B]	72 hours composite sample after mechanical treatment at WWTP Bickenbach
Biological treatment [BT-B]	72 hours composite sample after biological treatment and phosphate precipitation at WWTP Bickenbach
Downstream [DS-B]	Landbach 20 m downstream of the effluent of WWTP Bickenbach (49°45'33.8"N 8°35'49.4"E

2

Table 2(on next page)

Physicochemical parameters at the sampling sites

Table 2. Mean values of physicochemical parameters during the spring and fall campaign. All data were measured on two occasions (4 weeks difference) during each campaign. BT-L data during the spring campaign was not measured.

1

Parameter	Season	Sample sites								
		BT-L	US-MW	MT-MW	BT-MW	DS-MW	US-B	MT-B	BT-B	DS-B
Oxygen saturation [%]	Spring	--	99.1	65.0	68.5	78.7	92.6	9.00	82.5	65.0
	Fall	104	83.2	20.0	93.6	77.7	85.8	58.3	61.2	68.1
Conductivity [μS/cm]	Spring	--	781	1190	772	817	493	1560	952	967
	Fall	1080	976	1270	934	1000	532	1500	1040	932
Phosphate P [PO_4^{3-}-P mg/L]	Spring		0.04	1.44	0.04	0.03	0.05	0.68	0.02	0.02
	Fall	0.03	0.03	0.87	0.04	0.02	0.02	0.67	0.02	0.02
Ammonium-N [NH_4^+-N mg/L]	Spring		0.03	34.5	1.81	1.11	0.02	52.5	0.26	0.15
	Fall	0.61	0.19	44.7	0.05	0.18	0.11	23.2	0.29	0.32
Nitrite-N [NO_2^--N mg/L	Spring		0.02	0.07	0.06	0.07	0.02	0.03	0.10	0.03
	Fall	0.05	0.05	0.04	0.01	0.03	0.02	0.04	0.04	0.04
Nitrate-N [NO_3.-N mg/L	Spring		1.35	0.16	1.53	1.08	0.45	0.46	1.13	1.03
	Fall	2.21	1.72	0.13	2.55	1.78	0.51	0.32	0.93	0.67

2

Table 3 (on next page)

Overview of the results of the mutagenicity test

Table 3 : Mutagenicity at the WWTPs Mörfelden-Walldorf and Bickenbach in the Ames fluctuation test with Salmonella typhimurium strains YG1041 and YG1042 with S9 mix. Mutagenic samples (> 20.8% revertant colonies) are labelled in red, non-mutagenic samples in green. n.a.: not analyzed due to high cytotoxicity.

YG1041+S9 mix				
Season	WWTP	Sample site	Test response	Revertant percentage mean (%)
Spring	Mörfelden Walldorf	US	+	39.3
		MT	n.a.	n.a.
		BT	+	49.7
		DS	+	56.5
	Bickenbach	US	-	4.8
		MT	n.a.	n.a.
		BT	+	49.2
		DS	+	52.3
Fall	Mörfelden Walldorf	BT-L	+	52.8
		US	-	22.1
		MT	n.a.	n.a.
		BT	+	57.5
		DS	+	54.4
	Bickenbach	US	-	15.3
		MT	n.a.	n.a.
		BT	+	47.1
		DS	+	53.3

YG1042+S9 mix				
Season	WWTP	Sample site	Test response	Revertant percentage mean (%)
Spring	Mörfelden Walldorf	US	+	41.9
		MT	n.a.	n.a.
		BT	+	47.1
		DS	+	41.4
	Bickenbach	US	-	20.5
		MT	n.a.	n.a.
		BT	+	45.0
		DS	+	42.4
Fall	Mörfelden Walldorf	BT-L	+	54.9
		US	+	32.5
		MT	n.a.	n.a.
		BT	+	52.8
		DS	+	51.3
	Bickenbach	US	+	36.2
		MT	n.a.	n.a.
		BT	+	51.8
		DS	+	56.0

1

2

Figure 1

Map of the sampling locations

Figure 1. **Map of the sampling location of the two WWTPs in Germany**.
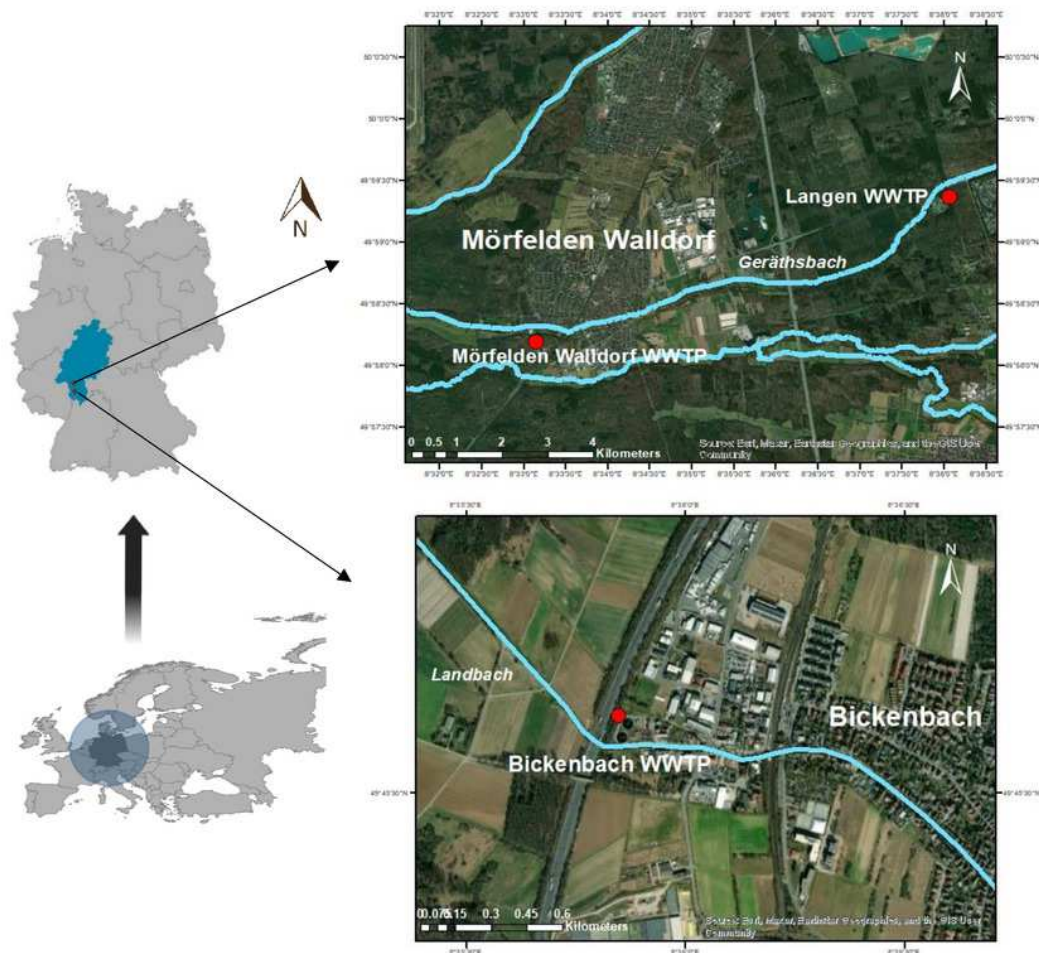
Figure 2

Baseline toxicity of water samples at the WWTPs

Baseline toxicity of water samples at the WWTPs Mörfelden-Walldorf (A) and Bickenbach (B) and in the receiving streams. Toxicity was determined by the Microtox assay and is expressed as EC50 values, referring to the relative enrichment factor (REF) of the water. Low values indicate higher toxicity. Dashed line: non toxic limit (NTL). Significant differences refer to downstream values (DS) compared to upstream values (US) (unpaired t-test with Welch's correction for unequal variances; ★ $p < 0.05$, ★★ $p < 0.01$, ★★★ $p < 0.001$). Numbers in blue indicate the mean decrease in % of measured baseline toxicity during the biological treatment.
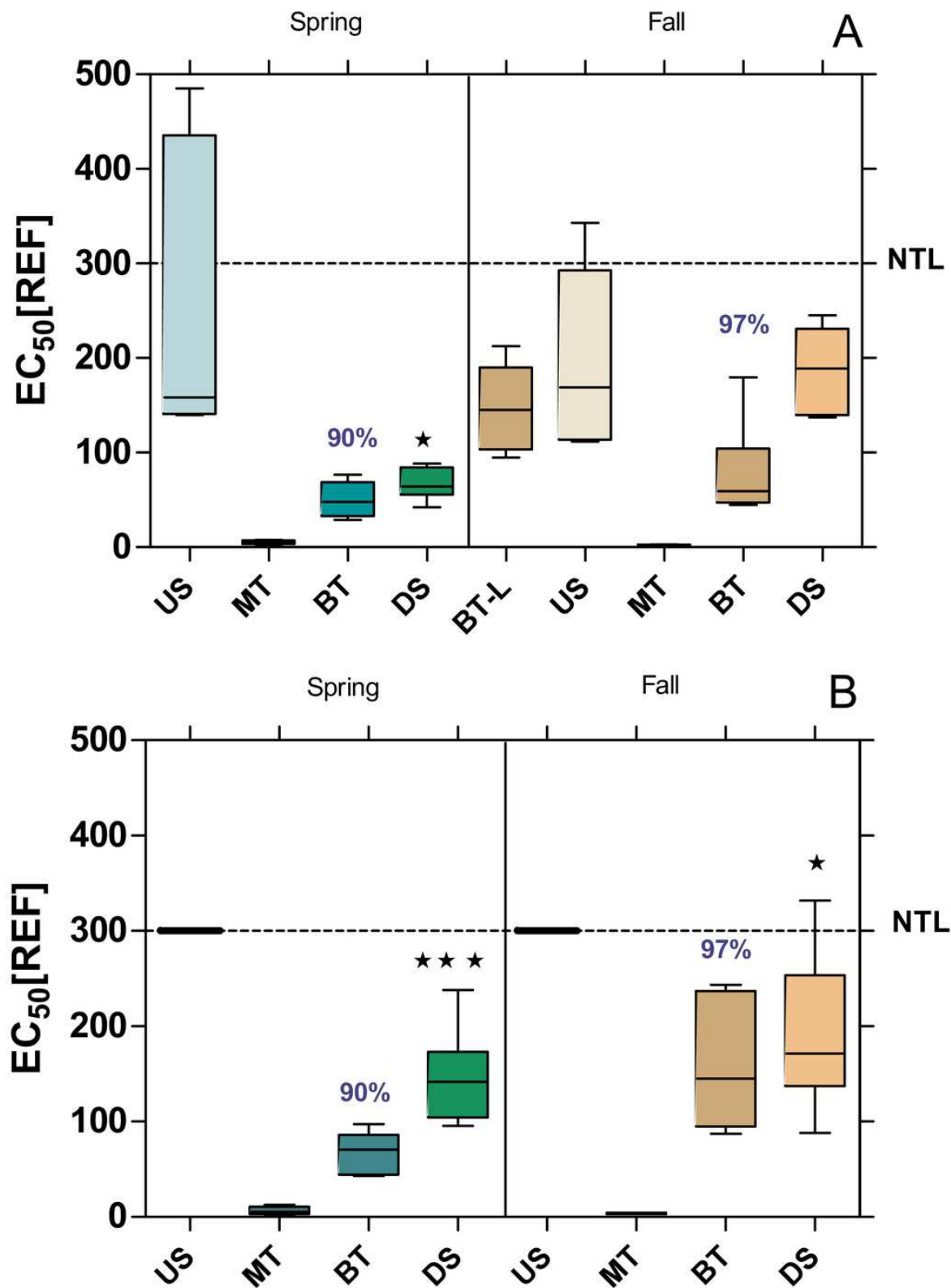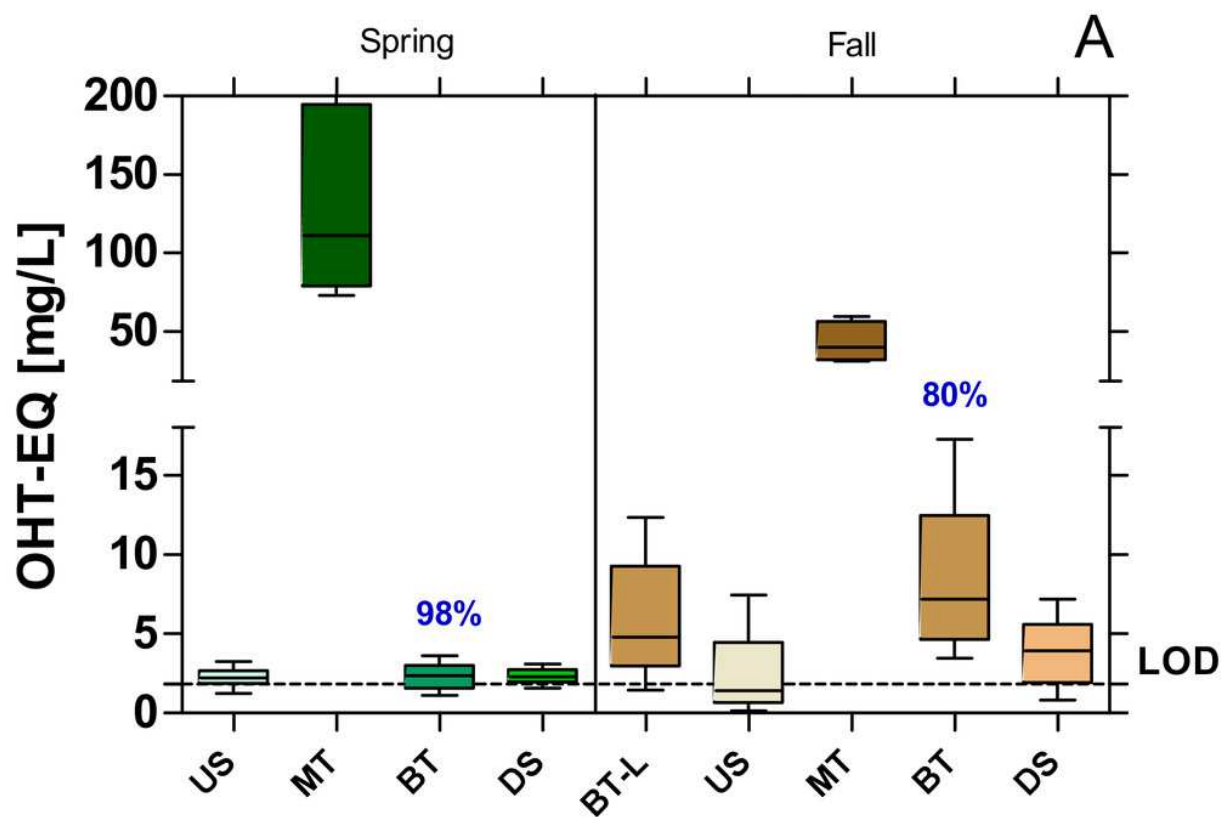
Figure 3

Estrogenic activity

Figure 3 . **Estrogenic activity at the WWTPs Mörfelden-Walldorf (A) and Bickenbach (B) and the receiving streams, determined with the Yeast Estrogen Screen (YES).** *Activities are expressed as 17β-estrogen equivalent concentrations (E-EQ). Dashed line: limit of quantification (LOD: 0.148 ng E-EQ/L). Significant differences refer to downstream values (DS) compared to upstream values (US) (unpaired t-test with Welch's correction for unequal variances; ★ p < 0.05, ★★ p < 0.01, ★★★ p < 0.001). Numbers in blue indicate the mean decrease in % of measured estrogenic activity during the biological treatment*
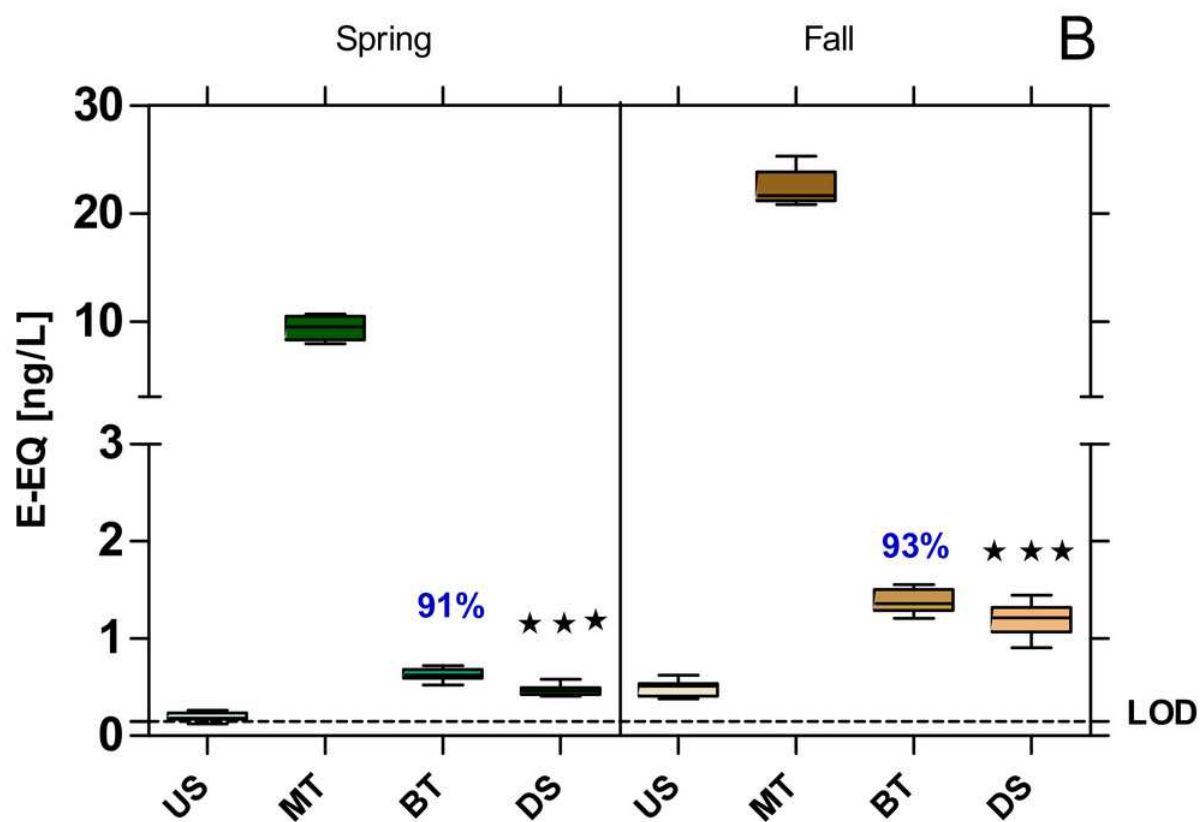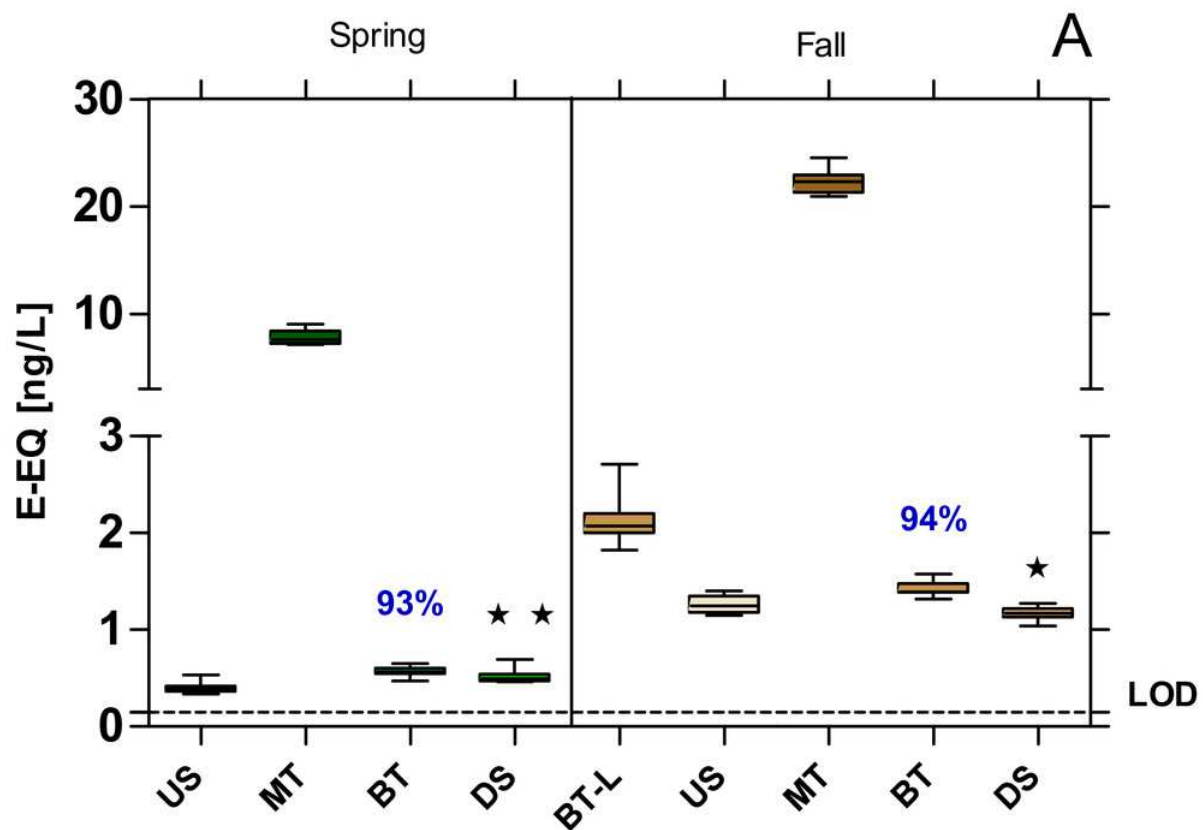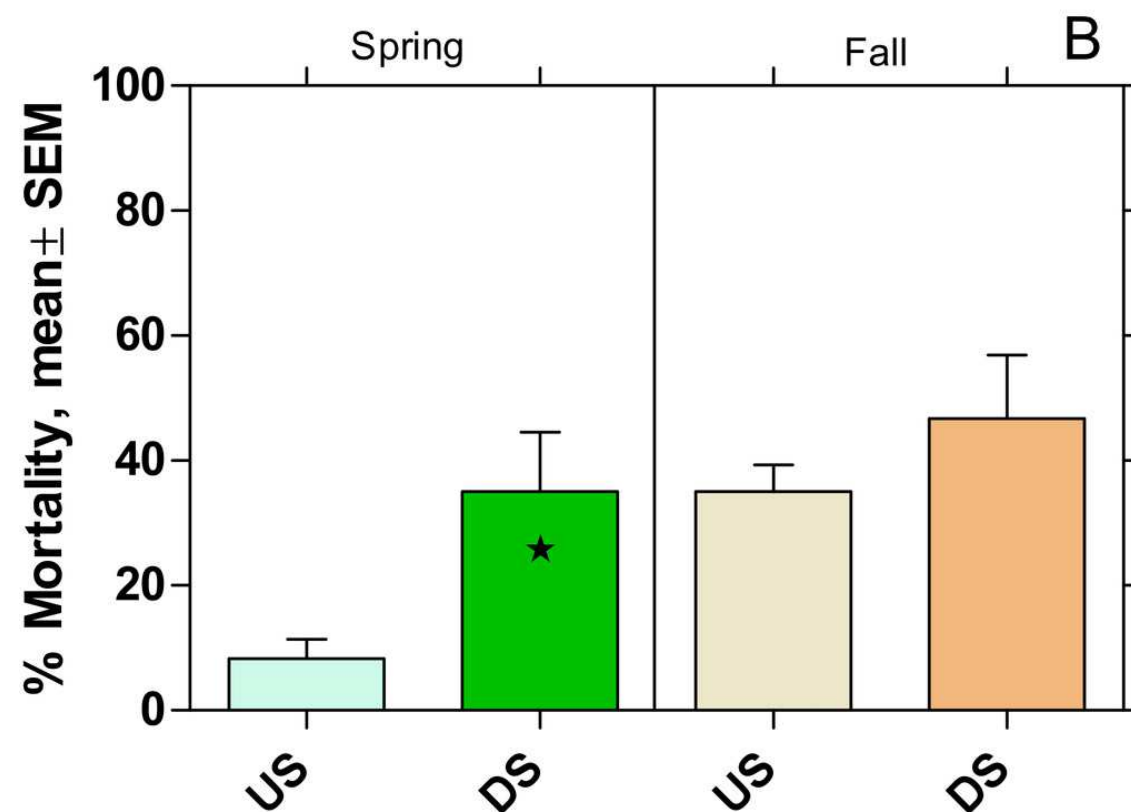
Figure 4

Anti-estrogenic activity

Figure 4. **Anti-estrogenic activity at the WWTPs Mörfelden-Walldorf (A) and Bickenbach (B) and the receiving rivers, determined with the Yeast Anti Estrogen Screen (YEAS).** *Activities are expressed as hydroxy-tamoxifen equivalent concentrations (OHT-EQ). Dashed line: limit of quantification (LOD: 1.84 mg OHT-EQ/L). None of the downstream values (DS) exhibits statistically significant differences to upstream values (US) (unpaired t-test with Welch's correction for unequal variances; p > 0.05). Numbers in blue indicate the mean decrease in % of measured estrogenic activity during the biological treatment*
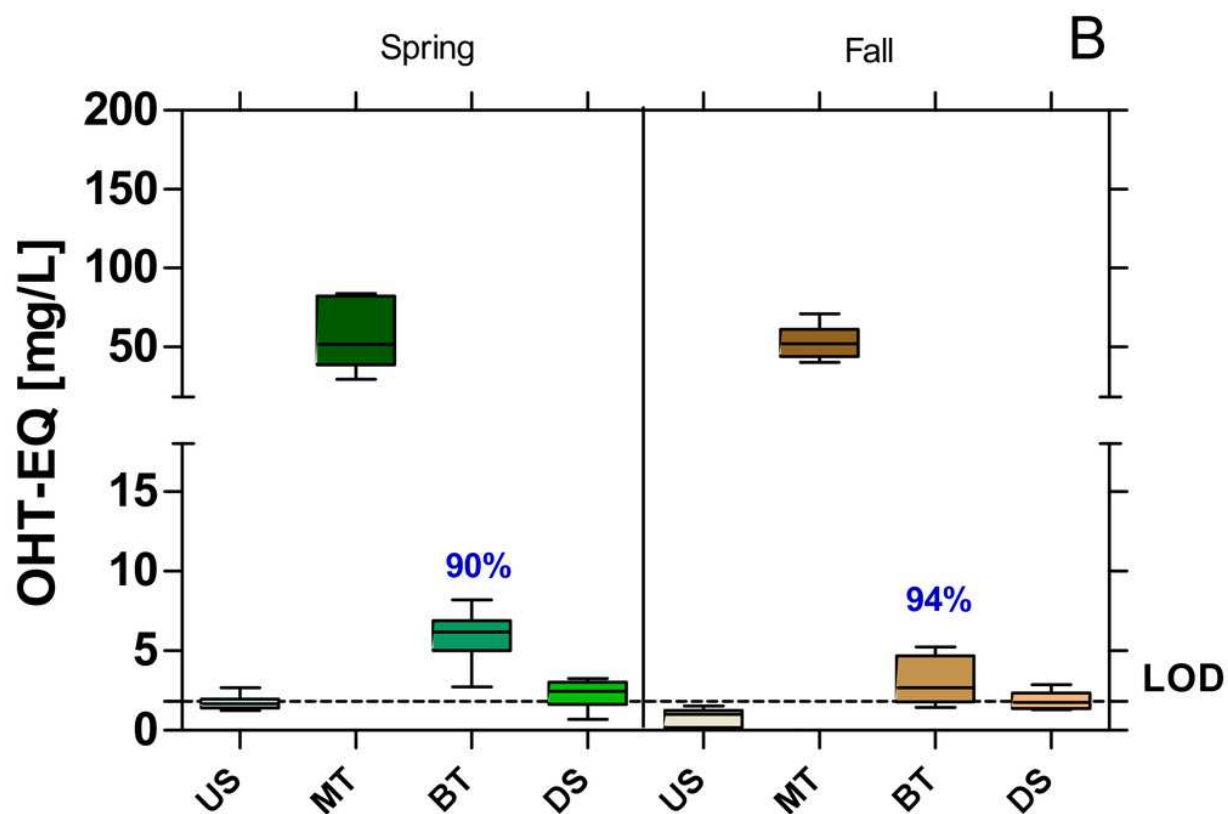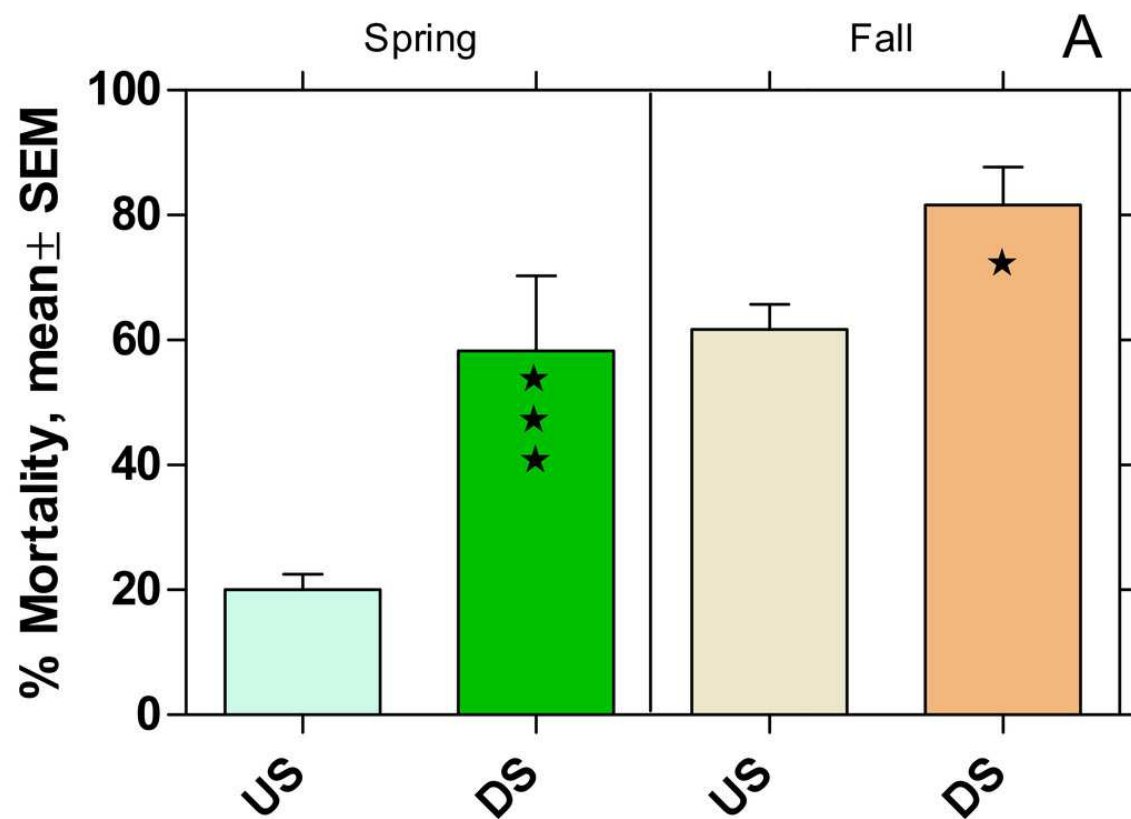
Figure 5

AhR agonistic activity

*Figure 5. **AhR agonistic activity at the WWTPs Mörfelden-Walldorf (A) and Bickenbach (B) and in the receiving rivers, determined with the Yeast Dioxin Screen (YDS).** Activities are expressed as β-naphthoflavone equivalent concentrations (β-NF-EQ). Dashed line: limit of quantification (LOD: 33.8 ng β-NF-EQ/L). Significant differences refer to downstream values (DS) compared to upstream values (US) (unpaired t-test with Welch's correction for unequal variances; ★ p < 0.05, ★★★ p < 0.001). Numbers in blue indicate the mean decrease in % of measured estrogenic activity during the biological treatment; skull and crossbones symbol indicate cytotoxic samples*
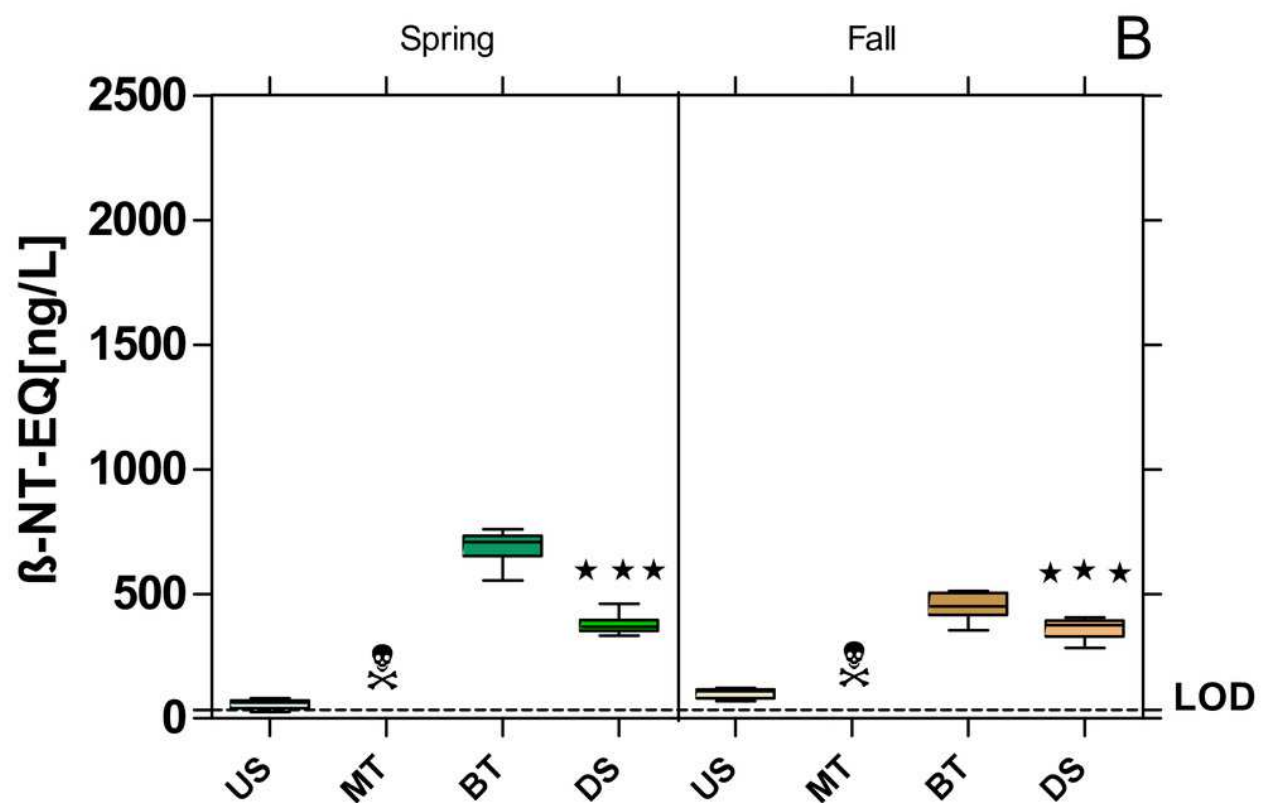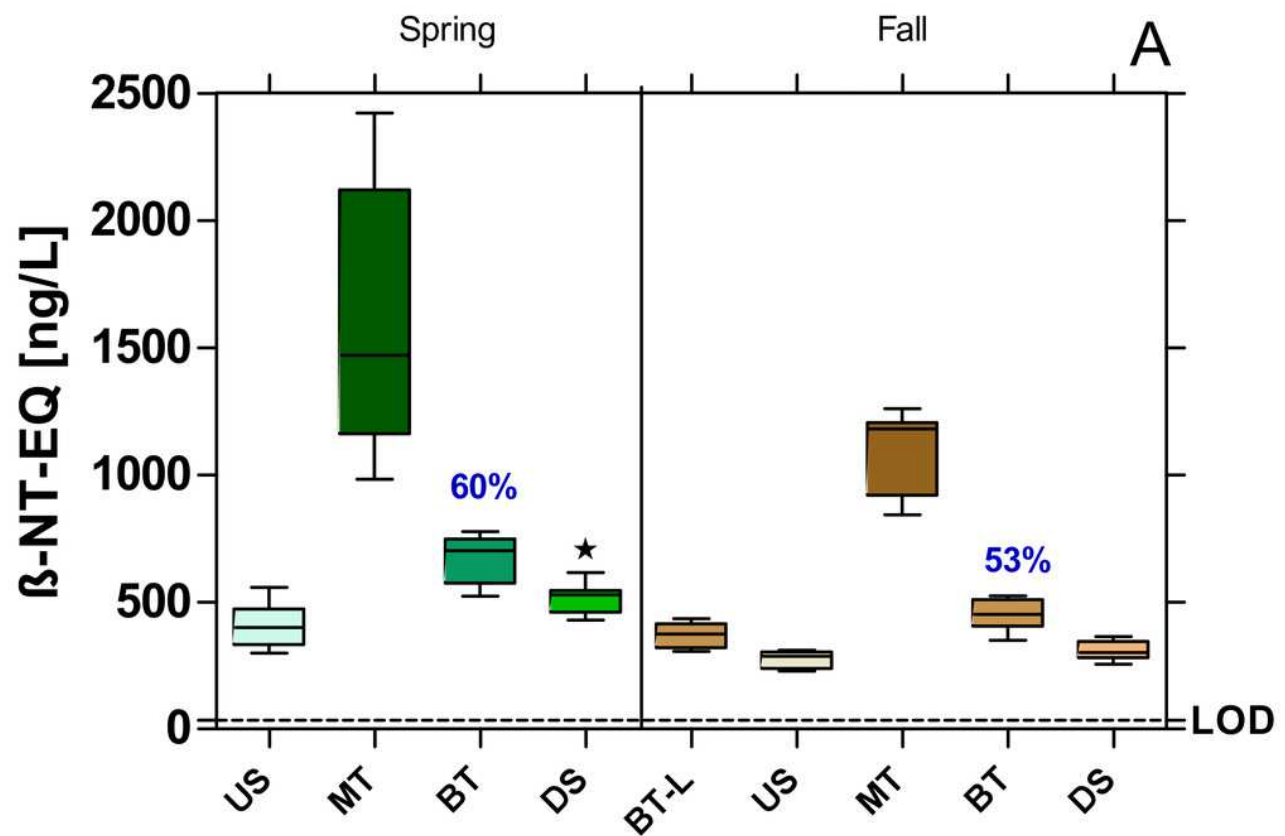
Figure 6

Mortality of *Gammarus fossarum*

Figure 6. Mortality of *Gammarus fossarum* in the active monitoring campaign over one month in the upstream (US) and downstream (DS) site in the receiving rivers of the WWTPs Mörfelden-Walldorf (A) and Bickenbach (B). Significant differences refer to DS compared to US (Fisher's exact test; ★ $p < 0.05$, ★★★ $p < 0.001$).
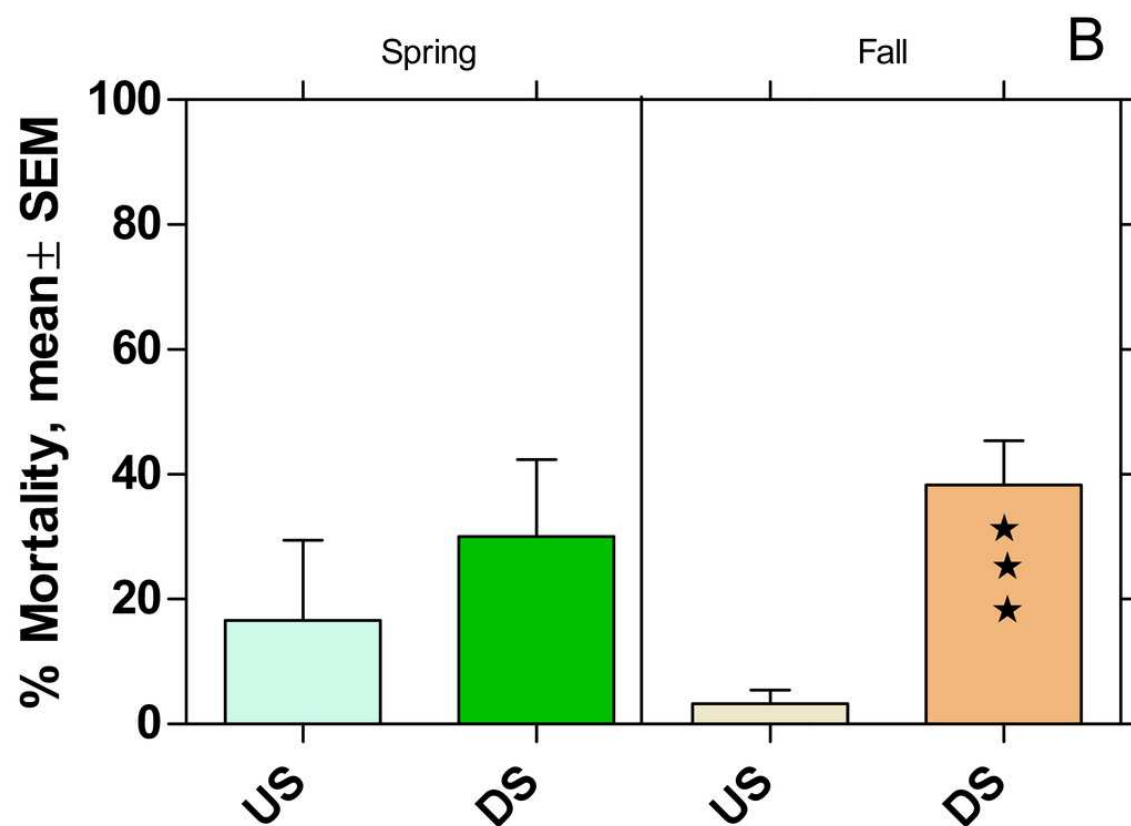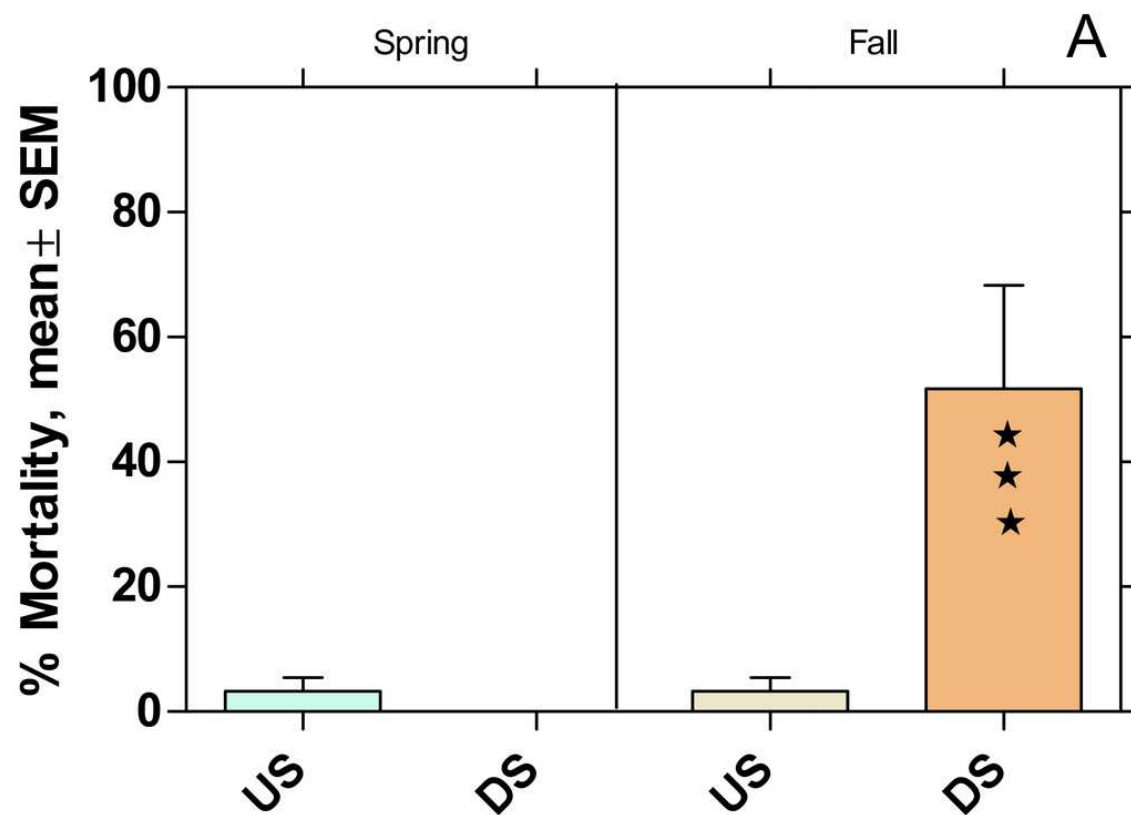
Figure 7

Mortality of *Potamopyrgus antipodarum*

Figure 7. Mortality of *Potamopyrgus antipodarum* in the active monitoring campaign over one month in the upstream (US) and downstream (DS) site in the receiving rivers of the WWTPs Mörfelden-Walldorf (A) and Bickenbach (B).

Significant differences refer to DS compared to US (Fisher's exact test; ★★★ p < 0.001).

Figure 8

Number of embryos in *Potamopyrgus antipodarum*

Figure 8. Number of embryos in *Potamopyrgus antipodarum* in the active monitoring campaign over one month in the upstream (US) and downstream (DS) site in the receiving rivers of the WWTPs Mörfelden-Walldorf (A) and Bickenbach (B). Significant differences refer to DS compared to US (unpaired t-test with Welch's correction for unequal variances; ★★ p < 0.01).