Ecotoxicological impacts of surface water and wastewater from conventional and advanced treatment technologies on brood size, larval length and cytochrome P450 (35A3) expression in *Caenorhabditis elegans*

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Abstract

Anthropogenic micropollutants and transformation products (TPs) negatively affect aquatic ecosystems and water resources. Wastewater treatment plants (WWTP) represent major point sources for (micro)pollutants and TP in urban water cycles.

The aim of the current study was to assess the removal of micropollutants and toxicity during conventional and advanced wastewater treatment. Using wild type and transgenic *Caenorhabditis elegans* the endpoints reproduction, growth and cytochrome P450 (CYP) 35A3 induction (via *cyp-35A3*::GFP) were assessed. Samples were collected at four WWTPs and a receiving surface water. One WWTP included the advanced treatments: ozonation followed by granular activated carbon (GAC) or biological filtration (BF), respectively. Relevant micropollutants and WWTP parameters (n = 111) were included.

Significant reproductive toxicity was detected for one WWTP effluent (31–83% reduced brood size). Three of four effluents significantly promoted the growth of *C. elegans* larvae (49–55% increased lengths). This effect was also observed for the GAC (34–41%) and BF (30%) post-treatments. Markedly, significant *cyp-35A3*::GFP induction was detected for one effluent before and after ozonation, being more pronounced for the ozonated samples (5 and 7.4 fold above controls).

While the advanced treatments decreased the concentrations of most micropollutants, the observed effects may be attributed to effects of residual target compounds and/or compounds not included in the target chemical analysis. This highlights the need for an integrated assessment of (advanced) wastewater treatment covering both, biological and chemical parameters.

1 Introduction

The nematode *Caenorhabditis elegans* is one of the main model organisms in biology. *C. elegans* has a versatile and well characterized physiology, with several biochemical pathways conserved to those in humans (Leung et al. 2008). In addition, *C. elegans* implies a short lifespan (12–20 d), fast reproductive cycle (3 d at 20 °C) and facile cultivation. Based on its ecological relevance (Félix and Braendle 2010), the widespread particle feeder is increasingly used in ecotoxicology (Hägerbäumer et al. 2015; Leung et al. 2008), comprising a wide range of methodologies as well as molecular, apical and community endpoints (Wilson and Khakouli-Duarte 2009). Since the late 1990s mutant and transgenic strains, which became readily available for *C. elegans*, have been utilized in ecotoxicology (e.g., Peter et al. 1996). These strains contain gene knockouts, artificial mutations such as causing hypersensitivity to certain xenobiotics and/or recombinant reporter genes, such as green fluorescent protein (GFP), coupled to target genes of ecotoxicological interest (e.g., Wilson and Khakouli-Duarte 2009; Xiong et al. 2017). The cytochrome P450 (CYP) gene family counts more than 80 candidates in *C. elegans*. CYPs fulfill essential cellular functions, such as phase I detoxification (Lindblom and Dodd 2006). In ecotoxicogenomics, gene expression profiling of CYPs thus became an established biomarker (Reichert and Menzel 2005; Wilson and Khakouli-Duarte 2009). Menzel et al. (2001, 2007) showed that exposure to xenobiotics induced the expression of specific sets of CYPs. *cyp-35A3* (human CYP2-like) investigated in this study is induced by the polycyclic aromatic hydrocarbons (PAH) β-naphthoflavone (β-NF), and fluoranthene, the polychlorinated biphenyl (PCB) 2,2',5,5'-tetrachlorobiphenyl (PCB52), the pharmaceuticals primaquine and lansoprazol (Menzel et al. 2001), benzene (Eom et

al. 2014), the insecticides chlorpyrifos, diazinon (Roh et al. 2014) and imidacloprid, the anthelmintic thiabendazole (Jones et al. 2013), the antimicrobials triclosan and trichlocarban (Inokuchi et al. 2014), as well as caffeine (Min et al. 2015). The rationale for selecting *cyp-35A3* in this study was that several of these compounds induced *cyp-35A3* at higher levels than most other CYPs. This responsiveness seems to be a common feature of all members of the *cyp-35A* subfamily (Menzel et al. 2001; Min et al. 2015). Because several *cyp-35A* inducers represent known environmental pollutants, members of this gene subfamily have been integrated into ecotoxicogenomics studies on environmental samples, such as contaminated soil (Anbalagan et al. 2013) and river sediments (Menzel et al. 2009). In general, its fully sequenced genome renders *C. elegans* an ideal model for (eco)toxicogenomics studies (Reichert and Menzel 2005) that is applied for the testing of chemicals, technical materials and in environmental risk assessment (ERA; Hägerbäumer et al. 2015; Leung et al. 2008; Wilson and Khakouli-Duarte 2009).

WWTPs represent major point sources for (micro)pollutants in aquatic ecosystems (e.g., Loos et al. 2013). Discharges from conventionally treated wastewater (activated sludge treatment) are associated with multiple adverse effects on sensitive aquatic species (Prasse et al. 2015), including *C. elegans* (Hitchcock et al. 1997). These discharges contain complex mixtures of various pollutant classes, such as PAHs. PAHs belong to the group of persistent organic pollutants (POP) that despite their reduced emission in the last few decades are regularly detected in WWTP effluents, surface water and river sediments (Forsgren 2015). As a consequence, fluoranthene was listed as priority pollutant by the US EPA and in the EU water framework directive (WFD) representing

99 other hazardous PAHs (European Commission 2000). PAHs are known for their
100 genotoxicity in various species. Unlike other PAHs, β-NF is not carcinogenic and seemed
101 not to cause DNA damage to *C. elegans* (Leung et al. 2010). Nonetheless, β-NF caused
102 significant reproduction toxicity and growth inhibitions (Leung et al. 2010; Menzel et al.
103 2001).

104 With the improvement of analytical methods novel anthropogenic chemicals, including
105 pharmaceuticals, biocides, nutrient related or industrial chemicals, have been detected in
106 WWTP effluents and receiving water bodies. Despite a growing knowledge base, the
107 majority of natural and anthropogenic chemicals in wastewater remain presently unknown
108 (Petrie et al. 2015). Moreover, a significant fraction of these substances, including
109 micropollutants, are not or only incompletely removed during conventional wastewater
110 treatment (Loos et al. 2013). To tackle this, advanced treatment technologies have been
111 developed and implemented, including oxidative treatment technologies (e.g., ozonation
112 or UV + H_2O_2), adsorptive technologies (e.g., granulated or powdered activated carbon
113 (GAC, PAC)) and biotechnology (e.g., immobilized enzymes). Different technologies (and
114 their combinations) effectively increase the removal of residual (micro)pollutants and
115 toxicity. However, they also indicated negative side effects (Prasse et al. 2015).
116 Adsorptive treatment technologies do not remove highly polar chemicals. Oxidative and
117 enzymatic treatments do not fully mineralize a large set of substances. Oxidative
118 treatments thereby generate unknown transformation products (TP) (Magdeburg et al.
119 2012) that can be more toxic than their parental compounds (Sinclair and Boxall 2003).
120 Because of this they require additional post-treatment, such as by sandfiltration (e.g.,
121 Magdeburg et al. 2012). From the research on wastewater treatment processes it also

122 became apparent that the removal of target compounds does not necessarily result in a
123 removal of toxicity.

124 The present study aimed at extending on this knowledge by assessing the removal of
125 (micro)pollutants and toxicity (xenobiotic metabolism) by conventional and advanced
126 wastewater treatment. Samples were collected at four WWTPs of different size classes
127 (small, medium and large) equipped with conventional activated sludge and different
128 advanced treatments. The latter were installed at one WWTP and comprised of an
129 ozonation of the WWTP effluent and sequential GAC filtration or biofiltration (BF). In
130 addition, surface water was sampled downstream of one of the investigated WWTPs. For
131 the analysis of these samples an established *C. elegans* bioassay was adapted from the
132 International Organization for Standardization (ISO) guideline 10872 (Höss et al. 2012).
133 Lab-scale *in vivo* bioassays such as ISO 10872 are valuable tools in assessing the toxicity
134 and biological activity of environmental samples. Their outcome thereby provides
135 valuable indications on the quality of (waste)water and can serve as proxy of potential
136 biological impacts of chemicals. This standardized bioassay has also been used to
137 examine the impacts of various chemicals with different modes of action in other studies
138 (e.g., Ristau et al. 2015; Haegerbaeumer et al. 2018). The guideline comprises the apical
139 endpoints reproduction and growth that respond sensitively to testing environmental
140 samples (Wilson and Khakouli-Duarte 2009). A main objective of this study was to
141 integrate molecular endpoints for xenobiotic metabolism into the assay, which may be
142 more sensitive. *Cyp-35A3*::GFP (Menzel et al. 2007) was selected as biomarker for CYP-
143 35A3 related xenobiotic metabolism in transgenic *C. elegans* (e.g., Min et al. 2015; Roh
144 et al. 2014). Using the PAH and potent *cyp-35A3* inducer β-NF, proof of principle

145 experiments were carried out on surface water and wastewater prepared by different
146 techniques. These experiments aimed at determining the assay sensitivity and
147 characterizing the impact of the sample matrix, such as from total suspended solids (TSS)
148 content or background (micro)pollutant concentrations. Based on these results, 15
149 relevant sampling points, representative for the urban water cycle, were analyzed. Special
150 focus was put on the comparison of conventional and advanced treatments, the
151 respective micropollutant removal efficacies and the occurrence of residual
152 micropollutants and/or toxicity in WWTP discharges and receiving surface water. Two
153 main hypotheses were tested: 1) Advanced wastewater treatment is more effective in
154 removing (micro)pollutants and toxicity. 2) The removal of target compounds does not *per*
155 *se* translate to a removal of toxicity. For quantification of (micro)pollutants and TPs the
156 concentrations of 92 chemical indicator substances (Seitz and Winzenbacher 2017) and
157 19 WWTP parameters (Knopp et al. 2016) were determined.

158 ## 2 Materials and methods

159 ### 2.1 Conventional wastewater treatment plants

160 Three WWTPs and one surface water were sampled in the state Baden-Württemberg,
161 Germany, in December 2012, October 2013 and February 2014. The considered region
162 comprises a water protection area of 513 km^2 that provides drinking water for
163 approximately 3.5 million inhabitants. WWTP-1 (440,000 population equivalents, PE) is
164 located near this area (3.5 km), 12 km upstream of the SW sampling site. WWTP-2
165 (16,000 PE) and WWTP-3 (16,600 PE) are situated within the water protected area. The
166 SW was sampled from the Danube (near Leipheim), one of the largest rivers in Germany.
167 At the sampling point, a wastewater fraction of approximate 6% was measured (Seitz and
168 Winzenbacher 2017). WWTP-4 (50,000 PE) is located in the state of Hessen, Germany.
169 Samples were taken in March and April 2015. All WWTPs (1–4) use conventional
170 treatment based on activated sludge, but differ in their catchment areas, corresponding
171 wastewater quality, receiving surface waters and other specifications (Online Resource
172 1; Knopp et al. 2016; Seitz and Winzenbacher 2017). Samples were collected at WWTP
173 influents (INF 1–4) and effluents (EFF 1–4) according to 2.3.

174 ### 2.2 Pilot wastewater treatment plant equipped with advanced treatment
175 technologies

176 The pilot WWTP was fed by the conventionally treated wastewater of WWTP-4 and
177 included an ozonation (O_3) coupled to GAC or BF (Fig. 1; Knopp et al. 2016). The WWTP
178 effluent was filtered by a 10 µm microscreen to reduce suspended solids prior to O_3.
179 Samples were taken according to 2.3 from the influent (INF-4), after activated sludge

180 treatment (EFF-4), after the ozonation (EFF+O_3), GAC (O_3+GAC) and BF (O_3+BF). GAC
181 and BF were operated in parallel in an unaerated (O_3+GAC and O_3+BF) and aerated
182 (O_3+GAC_a and O_3+BF_a) mode using compressed ambient air. Details on process
183 parameters can be found in Online Resource 2.

184 **2.3 Sampling and sample preparation**

185 Wastewater samples (1–5 L) were collected as 24 h composite samples. Surface water
186 samples were collected as 1 L grab samples. Aqueous samples were kept in amber glass
187 bottles at 4 °C until testing (max. 3 d after sampling) or extracted on site directly after
188 sampling by an optimized solid phase extraction (SPE) method (Abbas et al., in prep.).
189 The procedure in brief: Prior to SPE, 500 mL of each sample were filtered through
190 Whatman GF6 filters (pore size < 1 µm), acidified with sulfuric acid (3.5 M, picograde) to
191 pH 2.5 and extracted using Telos C18/ENV columns (Kinesis). A SPE blank was included
192 by applying the same procedure to an analytically pure groundwater (GW) sample. SPE
193 columns were eluted with 5 x 2 mL methanol (Carl Roth, Rotisolv, Ultra LC-MS) and
194 5 x 2 mL acetone (Carl Roth, Rotisolv, GC Ultra). 100 µL dimethyl sulfoxide (DMSO,
195 Sigma-Aldrich, 99.5%) was added to each extract. The methanol/acetone was
196 evaporated under a gentle nitrogen stream. This resulted in a 5000 fold increase in solute
197 concentration (5000x). SPE extracts were kept at -20 °C until bioassay analysis.

198 **2.4 Spiking of samples with β-naphthoflavone**

199 Aqueous SW and EFF-1 from December 2012 were spiked to 1 mg/L β-NF (CAS 6051-
200 87-2, Alfa Aesar, > 98%). Ultrapure water (UPW) was used as blank sample (TKA
201 GenPure, Thermo Fisher Scientific). β-NF was selected as a reference compound for

reproductive toxicity, growth inhibition (Leung et al. 2010) and *cyp-35A3* expression (Menzel et al. 2001, 2007). For spiking 1 µL of a 1 mg/mL stock solution in DMSO was added to 1 L of the respective sample (0.1% DMSO final). Aqueous and spiked samples were analyzed as 1:2 dilution, resulting in a final β-NF concentration of 0.5 mg/L for the spiked samples. In addition, aqueous (UPW, SW and EFF-1) and spiked (UPW^s, SW^s and $EFF\text{-}1^s$) samples were subjected to SPE (according to 2.3).

2.5 *C. elegans* strains and maintenance

The *C. elegans* N2 strain, variety Bristol was obtained from the Caenorhabditis Genetic Center (CGC, Minneapolis, USA). The transgenic strain expressing the *cyp-35A3*::GFP construct was kindly provided by Dr. Ralph Menzel (Humboldt Universität zu Berlin, Germany). *C. elegans* were maintained on agar plates containing nematode growth medium (NGM). The *Escherichia coli* OP50 strain (uracil-deficient, obtained from the CGC) was used as food source. *C. elegans* stock plates (prepared according to ISO 10872) were kept at 20 ± 1 °C in the dark. Fresh stock plates were prepared 3–5 d prior to bioassay analysis.

2.6 Adapted *C. elegans* bioassay

ISO 10872 was adapted as follows: For the endpoints brood size and larval length synchronized L1 larvae were transferred into 24 well microtiter plates (n = 5–10 per replicate, compare 2.6.1 and 2.6.2). Each well contained 0.8 mL M9 medium. After transfer of L1 larvae 400 or 401–402.5 µL M9 were removed for testing aqueous samples or SPE extracts respectively. 100 µL of an OP50 suspension (500 FAU, final concentration) in M9 including cholesterol (CAS 57-88-5, Sigma-Aldrich, > 92.5% GC,

0.1% final concentration) was supplemented to all wells. The resulting bacterial suspension was used as negative control (NC). For testing SPE extracts: depending on the final concentration factor, 10x or 25x, an extract volume of 1 µL (1:500) or 2.5 µL (1:200) of the 5000x SPE extracts (2.3) was added respectively. For testing aqueous samples: 0.5 mL of sample was added (1:2). Addition of samples/extracts represented the starting point (t_0) of the bioassays. Microtiter plates were incubated at 20 °C in the dark for 1–96 h depending on the endpoint (2.6.1 and 2.6.2). Highest final SPE enrichment factor tested (25x) represented a DMSO concentration of 0.5% (v/v). At this solvent concentration no adverse effects on *C. elegans* were reported (Boyd et al. 2010). In prescreening experiments 10x concentrated samples were tested (3.1). For samples from WWTP 1–3 a 25x concentration factor was applied (3.2). However, for these samples mortality occurred in the INF 1–3 (data not shown) thus 1:2 dilutions were prepared. Accordingly, WWTPs 1–3 were tested in 12.5x concentrations. Samples from WWTP-4 were tested in 25x concentrations (3.3).

2.6.1 Endpoint brood size and larval length

Benzylcetyldimethylammonium chloride (BAC-C16, 5 mg/L, CAS 122-18-9, Alfa Aesar, 95%) was used as additional positive control (PC) for reprotoxicity and inhibition of growth (Höss et al. 2012). The duration of the respective bioassays was 96 h. At their termination (t_{end} = 96 h) adult and larval nematodes were sacrificed by heat shock (15 min at 80°C) and stained with rose bengal (CAS 632-69-9, AppliChem) for microscopic evaluation (30x). For the endpoint brood size (reproduction) 10 individuals were exposed in 3 replicates each per experiment. Total n per treatment group are indicated in figure captions. For a comparative analysis in selected experiments, 5 individuals in 5 replicates

were used (adapted from ISO 10872). The offspring of each replicate was counted after 96 h and presented as mean number of offspring per adult hermaphrodite. For determining larval lengths (endpoint growth), 20 randomly picked larvae from each replicate were measured. Data of the replicates were pooled if no statistical difference occurred.

2.6.2 Endpoint *cyp-35A3*::GFP expression

β-NF served as reference substance for the expression of *cyp-35A3*::GFP in transgenic *C. elegans* (Menzel et al. 2007). For the exposure to β-NF, wastewater samples and SPE extracts adult specimens were used. The procedure was analogous to the endpoints in 2.6.1 except shorter exposure times (1–48 h). *cyp-35A3*::GFP expression levels were evaluated for a minimum of 10 adults per treatment group using fluorescence microscopy. Individuals were mounted onto microscopy slides and immobilized by a drop of sodium azide (Sigma-Aldrich, 10 mM). GFP localization and fluorescence intensities were determined using an Olympus BX50 microscope at 100x magnification, an excitation wavelength of 470–490 nm and emission wavelength of 515 nm. Images were taken with a digital imaging system (Discus software) and processed with ImageJ (National Institute of Health, USA). Background fluorescence was subtracted based on the average GFP signal of unexposed (NC) organisms.

2.7 Chemical analysis and WWTP parameters

Water and wastewater samples were analyzed for selected WWTP parameters and micropollutants (Online Resource 2–3). Quantification of micropollutants was performed by HPLC (Thermo Dionex UltiMate 3000 RSLC) and electrospray MS/MS detection

(Sciex Qtrap 5500) as described by Seitz and Winzenbacher (2017). WWTP parameters were determined according to regulatory standards (as described by Knopp et al. 2016). A defined set of process parameters (n = 7) was documented for the advanced wastewater treatment technologies (Online Resource 2).

2.8 Statistical analysis

Statistical analysis was performed using GraphPad Prism, version 5.0–7.0 (GraphPad Software, San Diego, USA) and Microsoft Excel 2010 (Microsoft, Redmond, USA). Statistically significant differences between treatments were analyzed as indicated in figure captions. β-NF concentration response curves were computed based on the reprotoxicity and *cyp-35A3*::GFP expression levels of 0.01, 0.1, 1 and 5 mg/L β-NF after 96 h and 1–48 h of exposure, respectively. Logistic regression models were used to derive the median effective concentrations EC_{50} (Online Resource 5–6).

3 Results

3.1 Aqueous and β-naphthoflavone spiked surface water and wastewater

In previous studies, β-NF affected the reproduction and growth of *C. elegans* at exposure concentrations of > 273 µg β-NF/L (Leung et al. 2010; Reichert and Menzel 2005). In the present experiments β-NF caused a concentration-dependent decrease in brood size with the lowest observed effect concentration (LOEC) of 100 µg/L and an EC_{50} of 140 µg/L (Online Resource 5). Based on this proof of principle experiments were conducted using the reference compound β-NF as well as aqueous surface water (SW) and WWTP effluent (EFF-1). Aqueous samples, including an ultrapure water control (UPW), were spiked to 1 mg/L β-NF and tested as 1:2 dilutions. Average offspring numbers were 98.6 ± 8.1 juveniles per adult in the UPW control. The SW did not induce reprotoxicity, but slightly increased the reproduction by 10% compared to the UPW (Fig. 2). The same was true for the 10x concentrated SW extract. In contrast, the aqueous WWTP effluent (EFF-1) significantly reduced reproduction by 83% compared to the control. The 10x concentrated extract of EFF-1 induced a 31% reduction in brood size compared to the extracted ultrapure water. This reprotoxicity was however not as pronounced as for the aqueous sample. As expected, the presence of 0.5 mg/L β-NF in spiked samples significantly reduced brood sizes. For the spiked ultrapure water (UPW^s) reproduction was 46% lower than in the unspiked reference. Along that line, exposure to spiked surface water (SW^s) resulted in a 40% smaller brood size compared to SW. The spiked WWTP effluent induced more than 90% mortality thus reproduction was not assessed. The extracts of spiked UPW and SW significantly reduced the reproduction to levels comparable to the

304 aqueous spiked samples. Despite a 10x concentration factor, the spiked WWTP effluent
305 sample induced lower reprotoxicity than the aqueous EFF-1^{s}.

306 **3.2 Conventional wastewater treatment**

307 The impacts of influent and effluent samples from three WWTPs applying conventional
308 activated sludge treatment on the brood size and larval length of *C. elegans* were
309 investigated. Samples were analyzed in 12.5x concentrations. Regarding the endpoint
310 brood size (Fig. 3A), a high variability in the influent samples was observed. Mean
311 offspring numbers for INF-1, INF-2 and INF-3 were 19, 11 and 14% lower than in the GW
312 control (85.6 ± 7.9 juveniles per adult), respectively. For the effluent samples variability
313 was lower and for EFF 1–2 comparable to those of NC and GW. Here, the mean offspring
314 numbers in EFF-1, EFF-2 and EFF-3 were increased by 40, 45 and 80% respectively
315 compared to GW. Larval lengths were quantified to detect possible impacts on *C. elegans*
316 growth (Fig. 3B). Larvae of NC and GW had grown to a mean length of 391 ± 14.2 µm
317 and 336 ± 11.9 µm, respectively. Length distributions of EFF-1, EFF-2 and EFF-3 were
318 broader than for GW and larvae were observed to be significantly longer (mean lengths
319 of 515 ± 21.5 µm, 495 ± 16.5 µm and 517 ± 17.4 µm, respectively). Larval growth was not
320 determined for the influent samples.

321 **3.3 Advanced wastewater treatment technologies**

322 The samples from the conventional and subsequent advanced wastewater treatments at
323 WWTP-4 were analyzed for their effects on brood size and larval lengths. These samples
324 were tested as 25x concentrated extracts as no significant mortality occurred (compare
325 2.6). A high reprotoxicity was induced by the INF-4 sample with an average offspring

number 98% lower than in the GW control (68.2 ± 9.8, Fig. 4A). The samples from the subsequent treatments EFF-4 and EFF+O_3 were not reprotoxic but increased the average offspring number by 11.6% and 19.4% compared to GW, respectively ($p > 0.05$). For O_3+GAC, O_3+GAC_a, O_3+BF and O_3+BF_a an increase of average offspring numbers was observed (17.8, 26.9, 30.6 and 42% compared to GW, respectively), which was not significant. Similarly, the larvae length tends to increase (Fig. 4B). Here, larvae exposed to the conventionally treated effluent (EFF-4) had an average length of (389 ± 17.4 µm) that was slightly but not significantly higher than in the NC (345 ± 15 µm) and GW (350 ± 15.7 µm). For EFF+O_3 (422 ± 23.8 µm) a further non-significant increase was observed. In the O_3+GAC (494 ± 26.5 µm), O_3+GAC_a (469 ± 25.4 µm) and O_3+BF_a (456 ± 23 µm) treatments larvae were significantly larger compared to NC and GW. The length of larvae exposed to O_3+BF (347 ± 16.4 µm) was at the level of GW. These results were qualitatively confirmed throughout multiple experiments (n = 6).

3.4 *cyp-35A3*::GFP induction in transgenic *C. elegans*

To evaluate potential impacts of water and wastewater samples on the xenobiotic metabolism of *C. elegans* the P*cyp-35A3*::GFP transgenic strain was used (Menzel et al. 2007). CYP-35A3 served as biomarker for the exposure to PAH, PCB and other *cyp-35A3* inducing compounds. First, it was investigated whether the reference compound β-NF induces *cyp-35A3*::GFP expression. A concentration- and time-dependent increase (0.01–5 mg β-NF/L, 1–48 h) in GFP signal was observed (Online Resource 6). EC_{50} values of 71.5 and 78.6 µg/L were reached after 8 and 24 h respectively. The highest expression levels (21.3 and 24 fold above the control) were reached after 8 h of exposure

348 to 1 and 5 mg/L β-NF, respectively. *cyp-35A3*::GFP expression responded fast to an
349 exposure to 5 mg/L β-NF (after 1 h). From 4 h onwards, the LOEC was 0.1 mg/L β-NF.

350 Based on these results the sensitivity of *cyp-35A3*::GFP expression towards different
351 aqueous, spiked and enriched water and wastewater samples was compared (Fig. 5 and
352 Online Resource 7). None of the aqueous samples (UPW, SW, EFF-1) significantly
353 induced *cyp-35A3*::GFP. Similar to their aqueous equivalents, exposure to 10x
354 concentrated extracts of these samples did not significantly induce *cyp-35A3*::GFP at any
355 exposure time. In contrast, the β-NF-spiked aqueous samples (UPW^s, SW^s and $EFF\text{-}1^s$)
356 significantly induced the expression. Similar to β-NF, this increase was time-dependent
357 (1–48 h) and maximal expression levels were reached after 24–48 h. The earliest
358 significantly increased expression was detected after 1 h of exposure to $EFF\text{-}1^s$. The
359 exposure to the extracted spiked samples UPW^s and SW^s led to slightly higher CYP-
360 35A3::GFP levels compared to the aqueous spiked samples. Interestingly, *cyp-35A3*
361 expression induced by $EFF\text{-}1^s$ extracts was significantly lower than for the aqueous EFF-
362 1^s sample (Fig. 5B).

363 With regard to advanced wastewater treatment technologies, the effluents of conventional
364 WWTPs (EFF-1, EFF-4) were compared to ozonation (EFF+O_3, Fig. 5C). Samples were
365 analyzed as 10x extracts for multiple exposure times (4–48 h, Online Resource 8). Again,
366 EFF-1 did not cause any significant *cyp-35A3*::GFP induction. In contrast, EFF-4 and its
367 subsequent treatment by ozonation (EFF+O_3) significantly increased *cyp-35A3*::GFP
368 expression. The induction by EFF+O_3 (7.4 fold above the control level, at 24 h) was
369 significantly higher than by EFF-4 (5 fold above the control level, at 24 h).

3.5 Chemical analysis and WWTP parameters

The experiments with *C. elegans* were accompanied by a detailed chemical analysis of (micro)pollutants and WWTP parameters (Online Resource 2–4). Focusing on WWTP-4, DOC, conductivity, UV_{254}, NH_4^+ and P_{total} were removed with rates characteristic for conventional biological and advanced wastewater treatment (Knopp et al. 2016). For instance, the advanced technologies (EFF-4 vs. EFF+O_3 and EFF+O_3 vs. O_3+GAC/GAC_a, O_3+BF/BF_a) demonstrated additional removal rates in terms of these parameters although to a different extent. The DOC was reduced by only 9% from EEF-4 to EFF+O_3, but further 32, 37, 21 and 26% by O_3+GAC, O_3+GAC_a, O_3+BF, O_3+BF_a, respectively.

Out of the 92 target compounds, 57 substances and TPs were detected above the LOQ in the INF-4 and 50 in the EFF-4. The concentrations of 14 of these compounds were reduced by > 90%, of 10 by 50–90% and of 14 by < 50%. Further 19 compounds occurred at higher concentrations in the effluent than in the influent, whereby the concentration of 13 was increased by > 25%. Carboxy-acyclovir (main TP of acyclovir), acesulfame, sucralose, 4-formylaminoantipyrin (TP of phenazone) and benzotriazole occurred at the highest concentration in the effluent (20, 13, 10, 9.8, 8.4 µg/L, respectively). Ozonation effectively reduced the concentration of the majority of substances. From 50 substances above the LOQ in the EFF-4 only 20 were detected in the EFF+O_3. The concentrations of only 5 substances decreased by less than 50%, including diatrizoic acid, acesulfame, sucralose, melamine and iomeprol (Online Resource 3). The four post-treatments resulted in a low (BFs) to moderate (GAC filtrations) additional removal. An average removal rate of 36, 39, 11 and 18% (O_3+GAC, O_3+GAC_a, O_3+BF, O_3+BF_a compared to

392 EFF+O_3) was determined. Diatrizoate had the highest concentrations after post-treatment

393 5.6–6.1 µg/L, followed by acesulfame (4.1–5.1 and sucralose (2–4.4 µg/L).

4 Discussion

4.1 β-naphthoflavone and spiked environmental samples

The detected reprotoxicity of the reference substance β-NF (3.1) was higher than reported in the literature (Leung et al. 2010; Reichert and Menzel 2005). Regarding the biomarker CYP-35A3 an intestinal expression of *cyp-35A3*::GFP (Online Resource 9 and Menzel et al. 2007) was confirmed for all β-NF ECs (0.1–5 mg/L). The intestine of *C. elegans* is known as its detoxification organ, which may hint on the physiological role of CYP-35A3 and/or mode of action of β-NF. EC_{50} values of 71.5 and 78.6 µg/L for the 8 and 24 h time point respectively were recorded (Online Resource 6). These ECs indicated a slightly higher sensitivity of the biomarker compared to the endpoint reproduction (EC_{50} = 140 µg/L, 96 h). Markedly, β-NF strongly induced all *cyp*-35A subfamily members and several other CYPs (Menzel et al. 2001). Menzel et al. (2005) knocked down *cyp*-35A subfamily members, which decreased the reproductive toxicity of PCB52 and fluoranthene. Inokuchi et al. (2014) suggested a role for CYPs (including CYP-35A3) in the tolerance against triclosan and trichlocarban. Roh et al. (2014) supposed an involvement of CYP-35A3 in the metabolic toxicity of chlorpyrifos. Accordingly, the reprotoxicity of β-NF (and its potential metabolites) may be mediated via CYP-35As.

The potential impact of the sample matrix on the β-NF effects was examined by spiking surface water and wastewater samples. Spiked surface water induced a high reprotoxicity similar to the spiked ultrapure water control. For the unspiked surface water sample no reprotoxicity was detected. This indicated that no reprotoxicity is present and that the surface water matrix does not interfere with the β-NF toxicity. This is further supported by

the detected low micropollutant concentrations (Online Resource 3; Seitz and Winzenbacher 2017). The effluent of WWTP-1 decreased the brood size by 83% and spiking further increased this effect to 100% (Fig. 2). This suggests a joint effect of β-NF and other reprotoxic wastewater constituents including natural factors that may affect these toxicities. Mixture toxicity was previously suggested for wastewater contaminants in *C. elegans* (Hitchcock et al. 1997). The fact that there was no difference in the reprotoxicity induced by the spiked aqueous and extracted ultrapure water and surface water (Fig. 2) suggested a low recovery rate towards β-NF, which may not effectively elute from the SPE sorbent due to its hydrophobicity. In contrast, the extracted effluent sample (EFF-1) induced toxicity indicating that other reprotoxic compounds than β-NF were extractable. However, the reprotoxicity in the extracted EFF-1 and EFF-1^{S} was lower than in their aqueous equivalents, which may attribute to particle associated reprotoxicity filtered out during SPE pre-filtration (compare below) and/or the absence of non-extractable natural factors (compare above).

Unspiked surface water and effluent of WWTP-1 did not cause any significant *cyp-35A3*::GFP induction (Fig. 5 and Online Resource 7). Spiking with β-NF, however, resulted in an effective induction, which was higher in the aqueous effluent compared to the surface water sample. This is in accordance with the results observed for reproduction and might be explained by joint effects caused by low concentrations of multiple CYP-inducers in the effluent, which do not induce expression without β-NF and/or natural factors affecting the latter. Another factor might have contributed: β-NF has a log K_{ow} of 4.7 (estimated using US EPA's EPISuite) and will adsorb to particles, such as from TSS in wastewater. Higher TSS can thus partition more bioavailable β-NF into the particulate

phase of wastewater compared to surface water. As ingestion of contaminated food particles is the main exposure route for several pollutants in *C. elegans* (Offermann et al. 2009), the interaction of β-NF and wastewater-borne particles may thus explain the higher toxicity observed in the aqueous sample. In addition, this was not the case for extracted samples in which particulate matter larger than 1 µm and sample impurities were generally removed prior to or during extraction respectively. These results underline the importance to consider contaminated suspended solids in ecotoxicological evaluations of WWTP discharges (Burton et al. 2000) for which particle-feeding species such as *C. elegans* may offer several advantages.

4.2 Conventional wastewater treatment

Hitchcock et al. (1997) observed high levels of mortality when exposing *C. elegans* to WWTP effluent samples from conventional activated sludge treatment. In the present study mortality occurred in most of the 25x WWTP influent, but not effluent samples of WWTPs 1–3 (data not shown). However, aqueous and extracted effluent samples of WWTP-1 (from December 2012) exhibited a respective 31–83% decrease in brood size (Fig. 2). Similar (repro)toxicity has been reported for other species exposed to conventionally treated WWTP effluents (e.g., Giebner et al. 2016; Magdeburg et al. 2012). In contrast, none of the extracted effluent samples of WWTPs 1–3 from October 2013 and February 2014 induced significant (repro)toxicity (Fig. 3). The corresponding influent samples however exhibited moderate to high levels of reprotoxicity. Growth was selected as additional endpoint (Höss et al. 2012). *C. elegans* larvae exposed to the effluents from WWTPs 1–3 were significantly longer compared to the NC and GW control. The lengths of the majority of these larvae herby corresponded to the L3 instead of the L1 stage, which

462 suggests that the samples strongly promoted the growth of *C. elegans*. Such effects have
463 been observed for other conventionally treated effluents and model invertebrates as well
464 (e.g., Völker et al. 2017) where they were caused by residual nutrients (compare 4.3).

465 The extracted effluent from WWTP-1 did apparently not induce *cyp-35A3* to any
466 significant extend. In contrast, the extracted effluent from WWTP-4 caused a significantly
467 elevated expression, implying this WWTP emits CYP inducers. Generally, known *cyp-*
468 *35A3* inducing (micro)pollutants, such as β-NF, fluoranthene, PCB52, chlorpyrifos or
469 thiabendazole, have been detected in treated wastewaters in the microgram per liter
470 range (e.g., Quevauviller et al. 2006; Peris-Vicente et al. 2016). Diazinon, imidacloprid
471 and lansoprazol ranged at the nanogram per liter scale (e.g., Loos et al. 2013). Caffeine
472 is the only known *cyp-35A3* inducer analyzed in this study (3.4) and was detected in the
473 EFF-4 and EFF+O_3 below the LOQ (< 0.05 µg/L). For *cyp-35A3* expression experiments
474 most of these compounds were tested in the lower milligram per liter range, thus far above
475 their reported wastewater concentrations. However, hydrophobic *cyp-35A3* inducing
476 compounds, such as triclosan and trichlocarban, benzene and the mentioned PCBs and
477 PAHs, readily adsorb to sludge (McLaggan et al. 2012; Chalew and Halden 2010). This
478 indicated that the particulate phase of environmental samples should be considered when
479 estimating realistic exposure concentrations of these compounds.

480 **4.3 Advanced wastewater treatment technologies**

481 An early ecotoxicological contribution to the research on advanced wastewater treatment
482 technologies was performed with *C. elegans* (Hitchcock et al. 1998). The authors
483 observed that the toxicity of an acid-based dye wastewater increased along the duration

484 of ozonation. The effect was attributed to the generation of toxic TPs during ozonation.
485 This hypothesis has been corroborated using several aquatic species exposed to
486 ozonated wastewater (Magdeburg et al. 2012; Giebner et al. 2016). In contrast to these
487 studies neither conventionally nor advanced treated wastewater at WWTP-4 negatively
488 affected the reproduction of *C. elegans* (Fig. 4A). Accordingly, the removal of toxicity by
489 the post-treatments (such as postulated in hypothesis 1) in the Introduction) could not be
490 assessed. This is in accordance with other model species, which were not sufficiently
491 sensitive for the evaluation of advanced wastewater treatment (Völker et al. 2017). Mutant
492 and transgenic of *C. elegans* strains, such as the mentioned hypersensitive mutant (e.g.,
493 Xiong et al. 2017), may thus represent promising alternative tools for assessing the
494 toxicity of (highly) treated wastewaters and micropollutant effects at (very) low
495 concentrations. Another explanation for the observation at WWTP-4 might be the general
496 variability of the wastewater matrix. (Micro)pollutants and natural compounds in WWTP
497 influents and effluents can vary significantly depending on the catchment area and WWTP
498 characteristics respectively (e.g., WWTP-1 and WWTP-4, Online Resource 1). Moreover,
499 toxic oxidation products amongst (highly) polar compounds may be lost during SPE of
500 ozonated (waste)water samples (Stalter et al. 2016).

501 In comparison, the endpoint larval growth was affected by the advanced wastewater
502 treatment stages with a significantly increased larvae length in the activated charcoal
503 treatments and the aerated biofilter (Fig. 4B). The largest increase was observed for the
504 O_3+GAC. Different anthropogenic compounds (Höss and Weltje 2007) and natural
505 organic matter (NOM) constituents (Höss et al. 2001) demonstrated to affect *C. elegans*
506 reproduction and/or growth. As most of these compounds are effectively removed during

activated sludge treatments (e.g., nonylphenol) or hardly enriched by the applied SPE method (e.g., inorganic trace nutrients or macromolecular NOM) the causes of the observed effect remain speculative.

A significant impact of the advanced wastewater treatment ozonation was detected utilizing *cyp-35A3*::GFP. The extracted effluent from WWTP-4 (EFF-4) led to significant inductions of *cyp-35A3*::GFP. Markedly, the induction levels of EFF-4 were higher after ozonation (Fig. 5C, Online Resource 8). As observed for other species (Magdeburg et al. 2012), this increased CYP expression may have been the result of toxic/bioactive TPs generated by the oxidative treatment. This result further speaks for the usefulness of *C. elegans* mutant/transgenic strains in wastewater quality assessments. Unfortunately, we did not investigate the fate of this biological activity in the post-treatments and it remains to be determined whether the CYP induction is removed here.

4.4 Micropollutant removal

The concentrations of most target compounds, DOC and other relevant wastewater parameters decreased in the conventional biological and the advanced treatment stages (3.5). This confirmed the additional reduction capacity of ozonation and the GAC/BF post-treatments such as postulated in hypothesis 1) in the Introduction. The causes of the observed effects of the respective wastewater samples on *C. elegans* (3.1–3.4) however remain to be clarified.

Chemical indicators analyzed in this study (Online Resource 3–4) for which toxicological data was available in the *C. elegans* literature mainly ranged amongst pharmaceuticals, which may attribute to its growing application in biomedical research (Leung et al. 2008).

529 Certain of the chemical indicators indicated (repro)toxicity, including 1-adamantylamine
530 (Kao et al. 2016), 2-(thiocyanomethylthio)-benzothiazol (Allard et al. 2013), caffeine (Boyd
531 et al. 2010), carbamazepine (Olga Kolychalow, personal communication), DEET
532 (Hartman and Freedman 2005), as well as depressed fertility, such as saccharin (Sofia
533 Allison, personal communication) or growth promotion, such as sulfamethoxazole (Liu et
534 al. 2013). Nonetheless, none of these compounds seemed individually responsible for the
535 effects observed in this study, because their concentrations (Online Resource 3) were
536 lower than their reported ECs. A few chemical indicators were tested positively for
537 biochemical or molecular endpoints in *C. elegans* which occurred in the microgram per
538 liter range in the wastewater samples from conventional treatment, such as diclofenac or
539 sotalol (Petersen et al. 2004) as well as the advanced wastewater treatment stages, such
540 as acesulfame or gabapentin (Caylor et al. 2013). It should also be considered that the
541 concentrations of chemical indicators measured in this and most of the cited studies
542 referred to the aqueous phase of the respective wastewater samples. In contrast, their
543 accumulation to sludge particles (Chalew and Halden 2010; McLaggan et al. 2012) and
544 potential mixture toxicity effects (e.g., additive or synergistic) have rarely been compared.
545 However, it is also likely, that the chemical analysis of target micropollutants did not cover
546 the toxicologically relevant compounds (e.g., Tang et al. 2014), supporting hypothesis 2)
547 postulated in the Introduction. This further highlights the need to combine biological and
548 chemical methods to assess the effectiveness of (advanced) wastewater treatment.

549

5 Conclusions

The technical removal of anthropogenic micropollutants and transformation products from WWTP discharges is pivotal for improving water quality and mitigating potential ecological risks (European Commission 2000). Assessing the effectiveness of wastewater treatment in removing chemicals and toxicity is a pre-requisite to the success of this measure. For this, efficient and sensitive methods have been developed and implemented (e.g., Wernersson et al. 2015). Along that line, this study aimed at adapting a well-established *C. elegans* bioassay for combining apical (growth and reproduction) and molecular (CYP-35A3 related xenobiotic metabolism) endpoints.

The bioassay was validated using β-NF as reference compound and different sample matrices. β-NF dose-dependently induced reproductive toxicity and *cyp-35A3* expression at concentrations > 100 µg/L. The matrix wastewater effluent was discussed to have modulated the β-NF effects either because of sorption to suspended solids or the presence of other toxic compounds as well as natural factors affecting the latter. Furthermore, a comparison of aqueous and extracted samples demonstrated that *cyp-35A3*-inducing compounds were not completely extractable. These results support earlier scientific consent about case-specific sample preparation in wastewater quality assessments.

In this study, wastewater from four conventional WWTPs was assessed to investigate efficiencies of the activated sludge treatments in removing (micro)pollutants and toxicity. One effluent significantly inhibited the reproduction of *C. elegans* indicating the presence of residual toxicity. Three effluents significantly promoted larval growth due to unknown

causes. The forth effluent significantly induced the biomarker *cyp-35A3*::GFP. The variety of effects observed in the different WWTPs demonstrates the importance of integrating multiple biological endpoints and chemical analysis when assessing their removal capacities.

This approach is even more relevant when evaluating advanced wastewater treatment technologies. At WWTP-4 they consisted of a pilot scale ozonation and ozonation followed by granular activated carbon filtration or biofiltration. Because the conventionally treated effluent did not affect the reproduction of *C. elegans*, it was not possible to evaluate the performance of the post-treatments in removing reprotoxicity. However, the post-treatment with granular activated carbon filtration and aerated biofiltration significantly promoted larval growth. The conventionally treated effluent significantly induced *cyp-35A3*::GFP expression, which was further increased by ozonation. As reported by previous studies, this might be the cause of toxic transformation products generated during oxidative treatment. It however remained to be investigated whether this effect persisted in the post-treatments (GAC/BF). Because the advanced treatments decreased the concentrations of most chemical indicators below the LOQs, the observed effects might be attributed to effects of chemical indicators that were not (fully) eliminated and/or compounds not covered by the target chemical analysis. This highlights the need for an integrated assessment of (advanced) wastewater treatment covering both, biological and chemical parameters.

Figure captions

Fig. 1 Process scheme of WWTP-4. The first part of the WWTP (left) operates a conventional biological treatment process. The second part (right) is a pilot WWTP with advanced wastewater treatment technologies: Ozonation connected to aerated and non-aerated granular activated carbon (GAC) filtration or biofiltration. Grey dots indicate sampling points (24 h composites)

Fig. 2 Impacts of aqueous and extracted ultrapure water (UPW), surface water (SW) and wastewater treatment plant effluent (EFF-1) on the brood size of *C. elegans.* Aqueous (white bars) and extracted (grey bars) samples were analyzed in 0.5x and 10x concentrations, respectively. Spiked aqueous samples (marked by superscript s) contained 0.5 mg/L β-naphthoflavone. Results pooled from two experiments (n = 40–120 per treatment). Significant differences (** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$) tested unspiked against spiked samples (if not noted elsewise) by one-way ANOVA with Tukey's post-hoc analysis. ☠ > 90% mortality

Fig. 3 Impacts of extracted groundwater (GW, SPE blank), wastewater treatment plant influent (INF 1–3) and effluent (EFF 1–3) on the brood size (A) and length of larvae (B) of *C. elegans*. Samples (grey bars) were analyzed in 12.5x concentrations. Results pooled from three experiments for brood size (n = 45 per treatment group) and two experiments for larval lengths (n = 120–125 per treatment group). Significant differences (** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$) were tested against NC and GW (A, B) as well as INFs against EFFs (A) by Kruskal-Wallis test with Dunn's post-test. NC (white bar) = M9 medium. PC (white bar) = BAC (5 mg/L). ns = not significant

Fig. 4 Impacts of extracted groundwater (GW, SPE blank), wastewater treatment plant influent (INF-4), effluent (EFF-4) and advanced treatments on the brood size (A) and length of larvae (B) of *C. elegans.* Advanced treatments comprised of ozonation (EFF+O_3) and ozonation followed by aerated and non-aerated granular activated carbon filtration (O_3+GAC, O_3+GAC_a) or biofiltration (O_3+BF, O_3+BF_a). Samples (grey bars) were analyzed in 25x concentrations. Results pooled from four experiments for brood size (n = 95 per treatment group) and one experiments for larval length (n = 60 per treatment group). Significant differences (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) were tested against NC and GW by Kruskal-Wallis test with Dunn's post-test. NC (white bar) = M9 medium. PC (white bar) = BAC (5 mg/L)

Fig. 5 A) *cyp-35A3*::GFP expression in transgenic *C. elegans* after 8 h exposure to 1 mg/L β-naphthoflavone (β-NF). Exposed adult hermaphrodites showed a strong GFP signal along their intestine, as detected by fluorescence microscopy (100x). Images (NC, β-NF) show an overlay of differential interference contrast microscopy (DIC) and GFP channel. NC = M9 medium. Bar = 200 µm. B and C) Impacts of aqueous and extracted ultrapure water (UPW), surface water (SW), wastewater treatment plant effluent (EFF-1, EFF-4) and ozonated effluent (EFF+O_3) on *cyp-35A3*::GFP expression. Aqueous (white bars) and extracted (grey bars) samples were analyzed in 0.5x and 10x concentrations respectively after 24 h exposure. Spiked aqueous samples (marked by superscript s) contained 0.5 mg/L β-NF. Results pooled from two experiments (n = 10 per treatment group, respectively). Significant differences (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$) tested unspiked against spiked samples (B) and against controls (B, C) by one-way ANOVA with Tukey's post-hoc analysis. Dashed lines = limit of quantification. C)

638 NC (white bar) = M9 medium. Solvent control (SC, white bar) = 0.2% DMSO in M9
639 medium. Fluorescence intensity of PC (1 mg/L β-NF) = 0.185 (result not shown).

640

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