Ecotoxicological characterization of micropollutants and wastewater samples from conventional biological and advanced wastewater treatment

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Abbreviations

µg	microgram
•OH	hydroxyl radical
ABC	adenosine triphosphate binding cassette
AChE	acetylcholinesterase
AhR	aryl hydrocarbon receptor
AHTN	7-acetyl-1,1,3,4,4,6-hexamethyl-1,2,3,4-tetrahydro naphthalene
AOP	adverse outcome pathway
ATP	adenosine triphosphate
AWWT	advanced wastewater treatment
BAC	biologically activated carbon
BAT	best available technology
BBP	benzyl butyl phthalate
BF	biological filtration
β-NF	beta-naphthoflavone
BMU	Bundesministerium für Umwelt, Naturschutz und nukleare Sicherheit
BOD	biological oxygen demand
BPA	bisphenol A
BPS	bisphenol S
BLAST	basic local alignment search tool
ceCYP-35A3	*Caenorhabditis elegans* cytochrome P450 isoform 35A3 (protein)
cep-1	transcription factor (*Caenorhabditis elegans*), human p53 ortholog
COD	chemical oxygen demand
CYP	cytochrome P450
daf-16	abnormal dauer formation 16 (gene)
DART	developmental and reproductive toxicity
DDT	dichlordiphenyltrichlorethan
DEB	dynamic energy budget
DEHP	di(2-ethylhexyl)phthalate
DEP	diethyl phthalate
DNA	deoxyribonucleic acid
DOC	dissolved organic carbon
E1	estrone
E2	17β-estradiol
EC	effect concentration
EC_{50}	median effect concentration
EDA	effect directed analysis
EDC	endocrine disrupting chemical
EE2	17α-ethinylestradiol
EFF	wastewater treatment plant effluent

EQS	environmental quality standard
EPA	Environmental Protection Agency
EROD	ethoxyresorufin-O-deethylase
F_1	filial generation 1
F_2	filial generation 2
GAC	granulated activated carbon
gcs-1	glutamylcysteine synthetase (gene)
GST	glutathione-s-transferase (protein)
GW	groundwater
hCYP-2C8	human cytochrome P450 isoform 2C8 (protein)
HHCB	1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethylcyclopenta-γ-2-benzopyran
HRT	hydraulic retention time
HSP	heat shock protein
hus-1	DNA damage checkpoint protein
ICI 182780	fulvestrant
INF	wastewater treatment plant influent
ISO	International Organization for Standardization
L1	larval stage 1 (*Caenorhabditis elegans*)
L2	larval stage 2 (*Caenorhabditis elegans*)
L3	larval stage 3 (*Caenorhabditis elegans*)
L4	larval stage 4 (*Caenorhabditis elegans*)
LC	liquid chromatography
LCA	life cycle assessment
LOEC	lowest observed effect concentration
LOQ	limit of quantification
LVSPE	large volume solid phase extraction
MEC	measured environmental concentration
MoA	mode of (toxic) action
n.a.	not applicable
n.d.	not determined
NC	negative control
NDMA	N-Nitrosodimethylamine
NHR	nuclear hormone receptor
NOM	natural organic matter
O_3	ozone (in the context of wastewater ozonation)
p53	human phosphoprotein 53 (tumor suppressor protein)
PAC	powdered activated carbon
PAH	polycyclic aromatic hydrocarbon
PC	positive control
PCB	polychlorinated biphenyl
PGP	P-glycoprotein

PNEC	predicted no effect concentration
POP	persistent organic pollutant
PPCP	pharmaceuticals and personal care products
P_{total}	total phosphorus
QSAR	quantitative structure activity relationships
RNAi	ribonucleic acid interference
ROS	reactive oxygen species
RTG	rainbow trout gonad
SC	solvent control
SDR	short-chain dehydrogenase
SEM	standard error of the mean
SKN-1	transcription factor skinhead-1
SMX	sulfamethoxazole
SOD	superoxide dismutase
SPE	solid phase extraction
SW	surface water
TCC	triclocarban
TCS	triclosan
TIE	toxicity identification evaluation
TN	total nitrogen
TP	transformation product
TSS	total suspended solids
UGT	uridine 5'-diphospho-glucuronosyltransferase
WET	whole effluent toxicity
WFD	water framework directive
WW	wastewater
WWTP	wastewater treatment plant
YAAS	yeast anti-androgen screen
YAES	yeast anti-estrogen screen
YAS	yeast androgen screen
YES	yeast estrogen screen
YG7108	*Salmonella typhimurium* YG7108 strain

Abstract

Clean water is fundamental to human health and ecosystem integrity. However, water quality deteriorates due to novel anthropogenic pollutants present at microgram per liter concentrations in urban water cycles (termed micropollutants). Wastewater treatment plants (WWTP) have been identified as major point sources for aquatic (micro-)pollutants. Chemical and ecotoxicological analyses have shown that conventional biological WWTPs do not fully remove micropollutants and associated toxicities, which is often because of mobile, polar and/or recalcitrant compounds. To minimize possible environmental risks, advanced wastewater treatment (AWWT) technologies could be a promising mitigation measure. Multiple processes are therefore being developed and evaluated such as ozonation and ozonation followed by granulated activated carbon (GAC) or biological filtration.

Assessing the performance of these combined AWWTs was the focus the TransRisk project. Within this project, this thesis accomplished four major goals. Firstly, the preparation of (waste)water samples was optimised for *in vitro* bioassays. Acidification, filtration and solid phase extraction (SPE) were tested for their impact on environmentally relevant *in vitro* endocrine activities, mutagenicity, genotoxicity and cytotoxicity. Significantly different outcomes of these assays were detected comparing neutral and acidified samples. Sample filtration had a lesser impact, but in some cases retention of particle-bound compounds could have caused significant toxicity losses. Out of three SPE sorbents the Telos C18/ENV at sample pH 2.5 extracted highest toxicity, some undetected in aqueous samples. These results indicate that sample preparation needs to be optimised for specific sample matrices and bioassays to avoid false-positive or -negative detects in effect-based analyses.

Secondly, the above listed *in vitro* toxicities were monitored in a protected region for drinking water production in South-West Germany (2012−2015). Out of 30 sampling sites surface water and groundwater were the least polluted. Nonetheless, a few groundwater samples induced high anti-estrogenic activity that prompted further monitoring. The latter included a waterworks in which no toxicity was detected. Hospital wastewater also had elevated *in vitro* toxicities and hospitals are, thus, relevant intervention points for source control. The biological WWTPs were effective in removing most of the detected toxicity, and the selected bioassays proved to be pertinent tools for water quality assessment and prioritisation of pollution hotspots.

Thirdly, the *in vivo* bioassay ISO10872 based on *Caenorhabditis elegans* (*C. elegans*) was adapted for this thesis. Using this model, a median effect concentration (EC_{50}) for reproductive toxicity of the polycyclic aromatic hydrocarbon β-naphthoflavone (β-NF) of 114 µg/L was computed which is slightly lower than reported in the scientific literature. β-NF induced *cyp-35A3*::GFP (a biomarker in transgenic animals) in a time and concentration dependent manner (≤ 21.3–24 fold above controls). β-NF spiked wastewater samples supported earlier hypotheses on particle-bound pollutants. Reproductive toxicity (96 h) and *cyp-35A3* induction (24 h) of biologically treated and/or ozonated wastewater extracts and growth promoting effects of GAC/biologically filtered ozonated wastewater extracts were observed. This suggested the presence of residual bioactive/toxic chemicals not included in the targeted chemical analysis. It also highlighted the importance of integrating multiple (apical and molecular) endpoints in wastewater assessments.

Fourthly, five *in vitro* and the adapted *C. elegans* bioassay were integrated into a wastewater quality evaluation (developed within TransRisk). Out of the five AWWT options, ozonation (at 1 g $O_{3,applied}$/g DOC, HRT ~ 18 min) combined with non-aerated GAC filtration was rated most effective for toxicity removal. All five AWWTs largely removed estrogenic and (anti-)androgenic activities, but not anti-estrogenic activity and mutagenicity, which even increased during ozonation. This has been observed in related studies and points towards toxic transformation products. These results also emphasized the need for implementing an effective post-treatment for ozonation. The results from a parallel *in vivo* study with *Lumbriculus variegatus* and *Potamopyrgus antipodarum* conducted on site at the WWTP (using flow through systems) were in accordance with the *C. elegans* results. In this context, *C. elegans* can be further implemented as sensitive, feasible and ecologically relevant model.

In conclusion, this thesis shows how optimised sample preparation, long-term (*in vitro)* environmental monitoring, sensitive and ecologically relevant (*in vivo*) bioassays as well as innovative evaluation concepts, are pivotal in improving the removal of micropollutants and their toxicities with AWWTs. Future research should further develop and evaluate measures at sewer systems, conventional biological, tertiary and other advanced treatment technologies, as well as sociopolitical strategies (e.g., source control or natural conservation) and restoration projects. The effect-based tools optimised in this thesis will support assessing their success.

1 General introduction

1.1 Urban water cycle and the emission of wastewater

Along human history natural water cycles are becoming increasingly interconnected with anthropogenic activities, infrastructures and emissions. While in 2014 54% of the world population resided in urban areas, this rate was predicted to increase to 65% until 2050 (Brooks et al. 2020, World Health Organization 2016). This growth will also lead to massive expansion and intensification of water usage and demand, conveying key challenges to global water and wastewater management. These challenges are associated with other major environmental concerns such as climate change, resource depletion and habitat/biodiversity impacts (e.g., Rockström et al. 2009, Stamm et al. 2016).

Water represents the largest mass influx into urban areas (Philip et al. 2011). Due to the high demand on water, 4.5×10^{12} m^3 of water is withdrawn from freshwater resources every year. This approximates to one third of the total existing amount (Jackson et al. 2001). Fresh water is mainly extracted from groundwater and surface water, whereby its extraction relies on various qualitative, quantitative, economic and regulatory aspects. To produce drinking water it is essential to remove water-borne pathogens and contaminants. Depending on the initial water quality this requires basic (e.g., boiling for disinfection) to advanced (e.g., reverse osmosis) treatment processes. The application range of (treated) water is extremely versatile. Households mostly use tap and bottled water, while consuming 7% of the total extracted water. Industry needs 22%, while the largest amount (65%) is consumed by agriculture (Jackson et al. 2001). Figure 1 illustrates main water usage sites and wastewater fluxes. Wastewater is generated where water usage is accompanied by degradation of the provided quality. Wastewater in urban areas is generally collected via drainage networks and sewer systems that merge into wastewater treatment plants (WWTP). In WWTPs influent wastewater is treated by elaborate processes (multi-barrier principle), because WWTP effluents have to fulfill regulated quality standards to be permissible for discharge into receiving water bodies. The main goal of wastewater treatment thereby is that no biological/ecological risks are posed to humans and the environment (Tchobanoglous and Burton 1991). Once this requirement is fulfilled the water cycle can begin anew.

Figure 1. Urban water cycle scheme indicating major entities including inputs (green lines), flows (blue lines), outputs (brown lines) and impacting factors (grey lines, Philip et al. 2011)

1.2 Wastewater treatment plants as point sources for aquatic pollution

In 2012 approximately half of European surface waters did not match a “good ecological status” (European Commission 2012, European Parliament and Council 2000). Large parts of surface water pollution is caused by WWTPs (European Commission 2012, Malaj et al. 2014, Pal et al. 2010, Triebskorn et al. 2017, 2019). Their contribution will likely increase along the ongoing urbanization and growing world population (compare 1.1). Raw (untreated) municipal wastewater generally consists of 99% water and 1% waste. The waste fraction contains nutrients, (dissolved) organic matter, bacteria and other particulate matter (including nano- and microplastic) as well as toxic pollutants (e.g., Bruni et al. 2019, Tchobanoglous and Burton 1991, Triebskorn et al. 2019, Stamm et al. 2016). Due to its hazard

potential wastewater is treated by series of physical, chemical and biological WWTP processes that categorize into primary, secondary and where applicable tertiary and advanced treatment. Each of these stages (further) reduces the content of specific wastewater constituents. Preliminary treatment excludes grease, oil, debris, grit and odor before primary treatment separates out floating and settling materials (e.g., in primary clarifiers). Secondary treatment mainly reduces organic matter and suspended solids; most commonly by activated sludge processes (Prasse et al. 2015). Tertiary treatment aims at enhanced denitrification and removal of phosphorus, bacteria and virus, by coagulation, flocculation, precipitation and/or filtration. Waste sludge is mostly withdrawn during primary settling and/or the biological stage. It has to undergo separate treatment in digesters, centrifuges or presses that prepare it for final disposal (e.g., by incineration) or reuse (compare 'peak phosphorous', Cordell et al. 2009). Treated wastewater either becomes discharged into surface waters or marine environments or may be allocated for reuse depending on its final quality (e.g., in industry, irrigation or prospectively reclaimed water, Tchobanoglous and Burton 1991).

Wastewater quality has been characterized by physical parameters, such as odor, turbidity or TSS (compare figure 16), chemical parameters such as pH, salinity, nitrogen (e.g., total nitrogen (TN) computed as sum concentration of NH_4^+, NO_2^- and NO_3^-), phosphorus (e.g., total phosphorus (P_{total})), sulfate/sulfides (SO_4^{2-}/S^{2-}), biological/chemical oxygen demand (BOD/COD), dissolved organic carbon (DOC), often extended by adsorbable organic halides (AOX) or benzene, toluene, xylenes (BTEX), and biological parameters such as bacteria, viruses or parasites. Several of these parameters constitute, contribute or indicate aquatic pollution, if released at excessive amounts into water bodies. Most countries thus regulate their discharge through setting permissible limits (e.g., European Council 1991, US EPA 2002). Other parameters such as the BOD_5, which refers to the BOD of a wastewater sample within a 5 day incubation period, are used as quantitative indicators of the contained biodegradable compounds. Despite that these parameters represent "sums" of relevant physicochemical attributes/chemicals specific (micro)pollutants may not be captured. For this reason, several regulations have included so called priority pollutants based on their toxicity and other environmentally relevant criteria such as persistence (European Parliament and

Council 2000, US EPA 1972). Priority pollutants that have to be monitored such as heavy metals, polycyclic aromatic hydrocarbon (PAH), different pesticides, endocrine disrupting chemicals (EDCs) and industrial chemicals, whose usage has to be restricted over the next decades. Moreover, if compliance with directives (e.g., Urban Waste Water Directive, European Council 1991) is not fulfilled, such as permissible discharge limits, immediate actions should be taken (e.g., technical WWTP and/or source control measures). In addition, several contaminants have been placed onto watch lists to become priority pollutants such as of the water framework directive (WFD, European Parliament and Council 2013), or are part of additional regulation such as for industrial wastewaters (e.g., indirect emissions).

Nonetheless, the vast majority of known and emerging pollutants remains unregulated as well as incompletely removed during conventional biological wastewater treatment (compare 1.2.1). This circumstance has defined WWTP as major point sources for (micro)pollutants and potentially associated risks in aquatic ecosystems. Several of these pollutants were repeatedly made responsible for adverse effects on different aquatic species (1.3) and regionally conflicted with environmental quality standards (EQS, a presently non-legislative ecotoxicological complementation to chemical permissible limits). Although EQS proved to be useful in protecting aquatic organisms to certain extend, they have been defined for only a few compounds up to present (Escher et al. 2018, Malaj et al. 2014, Tousova et al. 2017). In addition to these challenges, most pollutants do not fully mineralize during different wastewater treatments, triggering the emission of unknown TPs. TPs may show higher toxicity than their parental compound (Boxall et al. 2004, Cao et al. 2020b, Cwiertny et al. 2014, Knoop et al. 2018, Larcher et al. 2012, Ma et al. 2019, Schlüter-Vorberg et al. 2015). The overall situation has thus initiated numerous research projects on the detection and characterization of wastewater-borne (micro)pollutants/TPs (1.2.1) and has strongly challenged WWTP to further reduce emissions such as by technical upgrades (1.2.2).

1.2.1 Environmental behavior and biological impacts of wastewater-borne (micro)pollutants

Concerns in the late 1960s focused on WWTP as causes for eutrophication and declining fish populations (e.g., Karlson et al. 2002, Pihl et al. 1991). Thereafter, the discharge of so called macro-pollutants (including nutrients, organic matter, salts

and acids) was brought under control (Schwarzenbach et al. 2006). In the 1980s the attention shifted to hazardous organic chemicals. Persistent organic pollutants (POPs), such as PAH or polychlorinated biphenyls (PCBs), but also heavy metals, were widely investigated and regulated (UNEP 2005). Through ugrading WWTPs (e.g., enhancing biodegradation and sludge adsorption) and source control measures (e.g., at industrial sites) many POPs were reduced to levels of lesser concern (Prasse et al. 2015, Völker et al. 2019). In the last two decades instrumental chemistry has advanced in a way that organic pollutants occurring at trace concentrations could be reliably detected. According to their concentration in the nanogram to microgram per liter range these pollutants were termed “micro-pollutants” (Kümmerer 2011). Micropollutants have been classified (Kümmerer 2011, Ribeiro et al. 2015) according to:

- Their anthropogenic function such as pharmaceuticals and personal care products (PPCPs), biocides, nutrient-related or industrial chemicals (such as brominated flame retardants or surfactants).
- Their chemical structures such as phenols, phthalates or polyhalogenated compounds involving perfluorinated compounds or brominated diphenhyl ethers.
- Their mode of action (MoA) such as EDCs or anti-neoplastics.

Many micropollutants show (pseudo)persistent characteristics, a high mobility in the water cycle and/or low sorption rate (De Baat et al. 2020, Reemtsma et al. 2016, Richardson 2009). Several micropollutants were thus detected at nanogram per liter concentrations in groundwater (Heberer 2002) and raw drinking water (Benotti et al. 2009). Nonpolar micropollutants often show high biodegradability, their hydrophobic properties however also facilitates accumulation to sediments and/or in aquatic biota (Brown et al. 2007, Fick et al. 2010). Munz et al. (2018) detected this phenomena for 63 (semi)polar micropollutants. Bioaccumulation phenomena generally imply further risks of trophic transfers (e.g., Park et al. 2009, Stylianou et al. 2018). Albeit significant hazards to humans could so far be prevented (such as by multi-barrier principle in the production of drinking water), ecotoxicological effects and ecological impacts are well documented in the scientific literature (e.g., Stamm et al. 2016, 1.3). The following passages shall summarize a few relevant examples hereof.

EDCs, environmental hormones and WWTP discharges containing these compounds have been associated with reproductive impairments in wild fish and other species (Jobling et al. 1998, 2002, Sumpter 2005, Tetreault et al. 2011, Wagner et al. 2017). Although suspected causative agents, such as estrone (E1), estradiole (E2) or ethinylestradiole (EE2) are generally effectively removed during activated sludge treatments (e.g., to 83%, 99% and 78% for E1, E2 and EE2, Ternes et al. 1999), these compounds nonetheless indicated high potencies at very low concentrations. EE2 for instance disturbed egg fertilization and sex ratios of fat head minnows at a lowest observed effect concentration (LOEC) of 0.32 ng/L (Parrott and Blunt 2005). It was further shown to cause *in situ* feminization of males at longer-term exposure to 5–6 ng/L (Kidd et al. 2007). Thus their measured environmental concentrations (MECs) often range above their reported effect concentrations (EC, Quednow and Püttmann 2008). Other EDCs such as the industrial chemical nonylphenol (priority pollutant WFD) caused feminization in fish at a LOEC of 8.2 µg/L. Despite its ban in several countries nonylphenol was detected at up to 4.1 µg/L in surface waters and 1 mg/kg in river sediments (Soares et al. 2008, World Health Organization 2002). The UV filter octinoxate (or 2-ethylhexyl-4-methoxycinnamate) was recently added to the WFD watch list (Ramos et al. 2015, European Parliament and Council 2013). Its accumulation in sediments (at µg/kg) and in aquatic biota (up to several hundred µg/kg) is thought to be associated with toxicological effects (Kaiser et al. 2012). Markedly, only little is known about its occurrence and behavior in WWTPs (compare 1.2.2).

Low ECs were also observed for different pharmaceuticals, such as carbamazepine, diclofenac or metoprolol. Diclofenac was reported to induce tissue-specific damage in rain bow trout at a LOEC of 1 µg/L (Triebskorn et al. 2007). Albeit WWTP influent concentrations of diclofenac and carbamazepine are relatively low, both compounds indicated poor removal rates during activated sludge treatment (Abbas et al. 2018, Calisto and Esteves 2009) and were detected in surface waters in the microgram per liter range (Gros et al. 2006, Zhou et al. 2009). High plasma concentrations of diclofenac were quantified in rainbow trout exposed to diluted treated wastewater (Brown et al. 2007). High bioconcentration factors were quantified for other pharmaceuticals (Burns et al. 2010, Fick et al. 2010). Antibiotics, excreted by humans or livestock represent another problematic group of pharmaceuticals which

stimulated (multi-)resistant mechanisms in sludge bacteria (Bruni et al. 2019, Rizzo et al. 2013, Triebskorn et al. 2017). Accordingly azithromycin, clarithromycin and erythromycin, detected in treated wastewaters, surface water and groundwater (up to the microgram per liter range), were included into the mentioned recent WFD watch list (European Parliament and Council 2013).

Biocides are another frequently detected pollutant group in aquatic compartments (e.g., Abbas et al. 2018). Pesticides of different classes normally occur at low concentrations in WWTP influents (e.g., < 1 µg/L, Köck-Schulmeyer et al. 2013). Many pesticides are recalcitrant towards conventional biological wastewater treatment (Köck-Schulmeyer et al. 2013, Sadaria et al. 2016). They are also widely distributed in natural waters due to their extensive agricultural application. In addition certain neonicotinoids indicated diffusion into surface waters from groundwater (Morrissey et al. 2015). Many pesticides and their formulations/TPs have been associated with toxicity, including carcinogenicity, reproductive toxicity, hepatotoxicity and/or neurotoxicity in different non-target species (Cao et al. 2020a, Dabrowski et al. 2014, Sanchez-Bayo and Hyne 2014). Although newer generations of pesticides are equipped with comparably short environmental half-lifes, ecological and human health risks prevail. For instance several neonicotinoids discharged from WWTPs were observed to exceed regulatory threshold. However, adverse effects on aquatic invertebrates also occurred below threshold concnetrations (Münze et al. 2017). Many pesticides have thus to be stringently regulated or had to be phased out in the past (European Parliament and Council 2013, US EPA 2011). Other biocides, such as antifouling agents, are often underrepresented in regulative assessments, despite their high production volumes and potential toxicity (e.g., Dafforn et al. 2011).

Foodstuff-related chemicals, such as artificial sweeteners, are often used as chemical indicator substances due to their aquatic persistence and mobility (e.g., Abbas et al. 2018, Seitz and Winzenbacher 2017). For a number of these chemicals even health risks were reported. It was for instance observed that the food additive “E 321” butylhydroxytoluol (2,6-di-tert-butyl-4-methylphenol, BHT) produces a metabolite that apparently caused DNA damage in rat and mice (Fries and Püttmann 2004). BHT entering the aquatic environment through WWTPs was detected in surface water (< 1.6 µg/L), groundwater (< 2.2 µg/L) and wastewater

(< 0.3 µg/L, Barbosa et al. 2016). It was thus also placed on the WFD watch list (European Parliament and Council 2013). In general, more (eco)toxicological data has been obtained on BHT, its TP(s) and other nutrition-related chemicals, to stringently exclude risks to humans and the environment.

Although older generations of POPs do not (regularly) exceed permissible thresholds any more, WWTP effluents are considered as prevailing source of certain PAH and PCBs (Abdel-Shafy and Mansour 2016, Forsgren 2015). Their emission is probably linked to their on-going industrial application and high production volumes (e.g., Bergqvist et al. 2006, Blanchard et al. 2004). Many hydrophobic POPs "escape" wastewater treatment bound to residual TSS (e.g., Marttinen et al. 2003, McLaggan et al. 2012). In surface waters they often accumulate in sediment sinks that are documented to exert toxic effects to bottom-dwelling organisms (e.g., Ahlf et al. 2002). Due to this fact sediments were integrated as one of the monitoring targets (besides the water phase and aquatic biota) of the WFD (European Parliament and Council 2008). Other than point sources, diffuse emissions including those from registered contamination sites or resulting from man-made disasters such as oil spills (Kim et al. 2019c), represent relevant sources of hazardous POPs.

1.2.2 Advanced wastewater treatment technologies for the additional removal of (micro)pollutants and residual toxicity

In response to the growing awareness about residual (micro)pollutants and toxicity in conventional-biological WWTP effluents, advanced wastewater treatment (AWWT) technologies were proposed as mitigation measure (e.g., Barbosa et al. 2016, Rizzo et al. 2019, Völker et al. 2019). Predominant AWWT technologies under investigation are advanced oxidation processes (such as ozonation or UV treatments), adsorptive technologies (such as powdered or granulated activated carbon), pressure-driven membranes (such as ultrafiltration or reverse osmosis), ion exchangers and air stripping techniques (Tchobanoglous and Burton 1991).

Advanced oxidation processes are classified by producing reactive oxidizing species. These specimens are mainly hydroxyl radicals (•OH) such as formed from hydrogen peroxide (H_2O_2) or from ozone (O_3). The latter two are directly introduced into the wastewater matrix. Hydroxyl radicals and ozone unspecifically react with wastewater constituents leading to stepwise degradation/transformation of the

attacked compounds/TPs (Schindler-Wildhaber et al. 2015). Advanced oxidation processes can be installed as pretreatment step for enhancing micropollutant biodegradability in subsequent treatment stages or as post-treatment step for oxidizing residual micropollutants and DOC (e.g., Bougrier et al. 2007, Rizzo 2011, Yeom et al. 2002). Under optimised conditions advanced oxidation processes, such as (catalytic) ozonation, (photo-)Fenton processes, wet peroxide/air or electrochemical oxidation and photocatalysis, indicated moderate to high (compound- and matrix-specific) removal rates (e.g., Klavarioti et al. 2009, Knopp et al. 2016, Ribeiro et al. 2015). Due to this potential ozonation has already been implemented at the national scale in a few countries (Gottschalk et al. 2009, Barbosa et al. 2016). Certain wastewater constituents delimit the effectivity of advanced oxidation processes such as high natural organic matter (NOM) content scavenging reactive oxidizing species introduced into the wastewater matrix. Other drawbacks are the generation of secondary wastes (most Fenton processes), demanding process parameters (e.g., low pH for conventional Fenton processes or elevated temperatures for wet peroxide oxidation), dependency on ambient conditions (e.g., solar light for photocatalysis), and low mineralization potential (e.g., ozonation at economic ozone doses and realistic HRTs as limited by DOC content). Moreover, advanced oxidation processes can generate TPs that obligate post-treatment. Many TPs can derive from a single oxidized compound, while the total number and physicochemical properties of the resulting TPs remains difficult to predict at present. TPs are currently classified based on their origin such as the mentioned technical TPs (from AWWT). Furthermore, natural TPs involve human/bacterial metabolites or solar degradation products (Escher and Fenner 2011). Prominent examples of TPs formed during ozonation are bromate (generated in the presence of bromide) and brominated TPs (in the presence of organic matter) as well as N-Nitrosodimethylamine (NDMA, Schindler-Wildhaber et al. 2015, Wu et al. 2019).

Adsorptive wastewater treatment by granulated or powdered activated carbon (PAC) is known for its effective removal of non-polar to moderately polar micropollutants and their toxicity (Prasse et al. 2015). Due to its granular structure GAC is packed into bed filters, whereas PAC is added to the wastewater and removed by subsequent filtration after defined contact times (Boehler et al. 2012).

(Micro)pollutant removal and the large capacity of these technologies base on the porous structure of charcoal, exhibiting a large surface that allows for hydrophobic interactions and chemical binding. Both processes have several advantages, such as a comparably low energy demand (PAC ≥ GAC) and a high removal effectivity (often PAC ≥ ozonation, e.g., Altmann et al. 2014). Moreover, the micropollutant removal normally proceeds without the generation of TPs. Their major disadvantage is that there capacity can become saturated, once all accessible binding palces are occupied. Then, these techniques need to be regenerated such as by heating them up to very high temperatures. They also suffer from variable sorption rates towards different compounds (Prasse et al. 2015). Biologically activated carbon (BAC) is thus being tested as an alternative that demonstrated to effectively decrease concentrations of DOC, nitrogen and of a number of recalcitrant micropollutants (Chen et al. 2017, Reungoat et al. 2012). BAC systems use fixed beds filled with GAC to support the growth of bacteria, which degrade the respective (micro)pollutants. Due to their performances in terms of micropollutant and toxicity retention all three technologies (GAC, PAC and BAC) have been implemented at various scales at WWTPs (e.g., Boehler et al. 2012, Mailler et al. 2014, table 4). However, for all activated carbon processes, highly polar chemicals may not be fully removed (Prasse et al. 2015, Reemtsma et al. 2016, Völker et al. 2019). Other biologically-activated filter materials are also being examined such as expanded clay in the biological filters (BFs) used in the TransRisk project (Knopp et al. 2016, 1.5).

Membrane based technologies have widely been utilized such as in reverse osmosis to desalinate sea water or in membrane bioreactors as an alternative to activated sludge processes (Schneider et al. 2020, Tchobanoglous and Burton 1991). Lately, ultrafiltration, microfiltration or nanofiltration that are distinguished by different standard pressures and pore sizes were tested as promising AWWT technologies (e.g., for polishing final effluents or indirect potable reuse). Thereby the molecular weight cut-off and other membrane criteria determine the rates and types of retained compounds/particles (Oulton et al. 2010). Promising variations of these technologies are being developed, such as forward osmosis and membrane distillation (Tchobanoglous and Burton 1991). Although processes, such as reverse osmosis and nanofiltration can produce particle-free effluents of very high quality

(Stalter 2010), major disadvantages are their high energy demand and production of a (highly) enriched retentates that requires independent costly treatment (Perez-Gonzalez et al. 2012). Membrane technologies are in a number of cases combinable with certain biotechnological applications. Enzymes, such as laccases, lignin peroxidase, manganese peroxidase, purified from funghi, bacteria or plants respectively are therefore immobilized on membranes. In addition, these enzymes can be dissolved in bioreactors, freely or mounted onto carriers (e.g., Demarche et al. 2012). In nature, they degrade/modify certain chemicals, organic matter or biomass. These functions can be also be used to degrade several hormones, phenols, plasticizers, PAH (Demarche et al. 2012, Kim and Nicell 2006), EDCs, antibiotics (Becker et al. 2017) and other pharmaceuticals in different wastewater matrices. As enzyme technologies are only recently being transferred to the treatment of wastewaters, their potential seems by far not exceeded (such as regarding the full range of micropollutant degradations and large-scale technical application).

AWWT technologies may be combined with each other, as far as economically feasible and once an individual technology alone does not provide the demanded wastewater quality. This is mostly realized by additional post-treatment such as in the combination of ozonation with sand-, activated carbon or biological filtration (Knopp et al. 2018, Prasse et al. 2015, Völker et al. 2019) and other promising process combinations (e.g., Knopp et al. 2018, Yang et al. 2017). The latter may for instance be realized by “hybrids” of technologies such as in biological membrane assisted carbon filtration (e.g., van Hege et al. 2002). AWWT have been benchmarked for their efficacy by means of chemical, ecotoxicological and microbiological analyses (compare 1.3–1.5 and 2.4). A prominent example in this regard is given by the combination of ozonation with sandfiltration or activated carbon filtrations, which demonstrated high removal rates for a large set of micropollutants, TPs as well as toxicity that withstood or was generated during ozonation (da Costa et al. 2014, Magdeburg et al. 2014, Knopp et al. 2016, Stalter et al. 2010, Wu et al. 2019, table 3−4). Other wastewater treatment systems currently under investigation as independent or post/tertiary treatments are biologically-activated filtrations (including riverbank filtration or retention soil filters), constructed wetlands and/or aquifer recharge (e.g., Li et al. 2014, Zhang et al.

2014). Although many of these technologies and treatment processes are considered promising in further reducing wastewater-borne micropollutants and toxicity (e.g., Bundschuh et al. 2011b, Hicks et al. 2016), more interdisciplinary research is needed for their holistic evaluation and comparison.

1.3 Ecotoxicological characterization of water and wastewater quality by *in vitro* and *in vivo* bioassays

Ecotoxicological methods are essential to environmental risk assessments and monitoring, as they study the biological responses of (micro)pollutants or environmental samples (e.g., Backhaus et al. 2019, Brack et al. 2017). Bioassays are mostly combined with chemical analyses to classify/identify the drivers and conditions of (micro)pollutant-based effects (e.g., Maier et al. 2016, Neale et al. 2017, Sonne et al. 2018, Stamm et al. 2016). Challenges arise from various aspects, such as the large number of contaminants in urban water cycles, their occurrence at mostly low concentrations, selecting the appropriate sampling, sample preparation and detection methods as well as breaking down the overall complexity and variability of aquatic ecosystems (Eggen et al. 2004, Stalter et al. 2013, figure 1–2). These circumstances also have to be considered when planning, conducting and evaluating environmentally relevant investigations on AWWT.

Sampling guidelines are also compiled and adapted to achieve this goal (Prasse et al. 2015, Völker et al. 2019) such as for different environmental compartments and sampling modes (e.g., grab versus composite samples or extraordinary conditions such as heavy rain falls or proximity to pollution hot spots). In all cases, proper sampling and sample handling is crucial to minimize deviation from *in situ* conditions (Baker and Kasprzyk-Hordern 2011, Hillebrand et al. 2013). Albeit immediate testing is imperative, storage periods of several days or weeks leading to degradation of target compounds can sometimes not be avoided. Sample freezing for instance is regularly applied to slow down physicochemical and microbial processes that take place during storage. Microorganisms are further targeted by filtration (e.g., at pore sizes < 0.2 µm) as well as by the addition of acids or preservatives. In accordance, suitable storage vessels (e.g., amber glass for organic pollutants) and conditions (e.g., storage temperature) have to be defined. Each of these techniques beholds advantages and disadvantages that have to be balanced with a higher environmental relevance of field investigations (compare below).

Untreated wastewater samples may serve for whole effluent toxicity (WET) estimations (Norberg-King et al. 2018). Dilution series must be prepared to investigate ECs related to different endpoints. Sample preparation may also

increase sensitivity and feasibility (compare effect directed analysis, EDA, or toxicity identification evaluations, TIE). Other bioassays do not require this steps due to their high sensitivity (e.g., Backe and Field 2012, Völker et al. 2019, Escher et al. 2014, 2018). Sample preparation is moreover practiced to reduce matrix interferences and for reasons of sample stabilization. Sample extraction and enrichment are common sample preparation approaches that involve techniques such as ultrasonic, solvent, soxhlet, liquid-liquid or SPE, freeze-drying, passive sampling and purge-trap-methods (De Baat et al. 2020, Prasse et al. 2015, Zwart et al. 2018). Many of these techniques are known to behold specific selectivity and effectivity towards different chemicals and environmental matrices (compare 2.1 and Abbas et al. 2019).

Ecotoxicological methods comprise field (*in situ*), semi-field (such as mesocosms) and laboratory studies using *in vivo* (whole organisms) and *in vitro* (cells) bioassays. In *in vitro* an *in vivo* bioassays different organisms/cells become exposed to individual compounds, synthetic mixtures or environmental samples (e.g., Klaassen 2007, Escher et al. 2017, Stamm et al. 2016). Bioassays are thereby capable of integrating and detecting the effects of multiple pollutants also referred to as mixture toxicity. Therefore, bioassays can be used as proxy of environmental toxicities, albeit full assessment of the latter generally requires (additional) higher tier testing (figure 2). Moreover, for optimal test strategies a well-defined set of parameters need to be considered such as organismal sensitivities, targeted endpoints, expected contaminant concentrations and other exposure related conditions (Prasse et al. 2015). To assess a widest feasible range of ecotoxicological effects *in vivo* bioassays should ideally be conducted using species from different taxonomic groups, trophic levels (such as producers or primary/secondary consumers) or specific habitats (such as aquatic or benthic/sediment dwelling, Rizzo 2011, Schlüter-Vorberg et al. 2017, Wernersson et al. 2015). Ecotoxicological assessments thus often include invertebrates such as the water flea *Daphnia magna* (*D. magna,* Organisation for Economic Cooperation and Development, OECD 2012), plants such as the duckweed *Lemna minor* (*L. minor*, OECD 2006), and fish species such as *Danio rerio* (*D. rerio*, zebrafish, ISO 1996) or *Oncorhynchus mykiss* (*O. mykiss*, rainbow trout, OECD 1992). These tests are often adapted or modified such as in case of the fish early-life stage test. *In vivo* assays are often combined with *in vitro* test systems in so called batteries of bioassays (compare below).

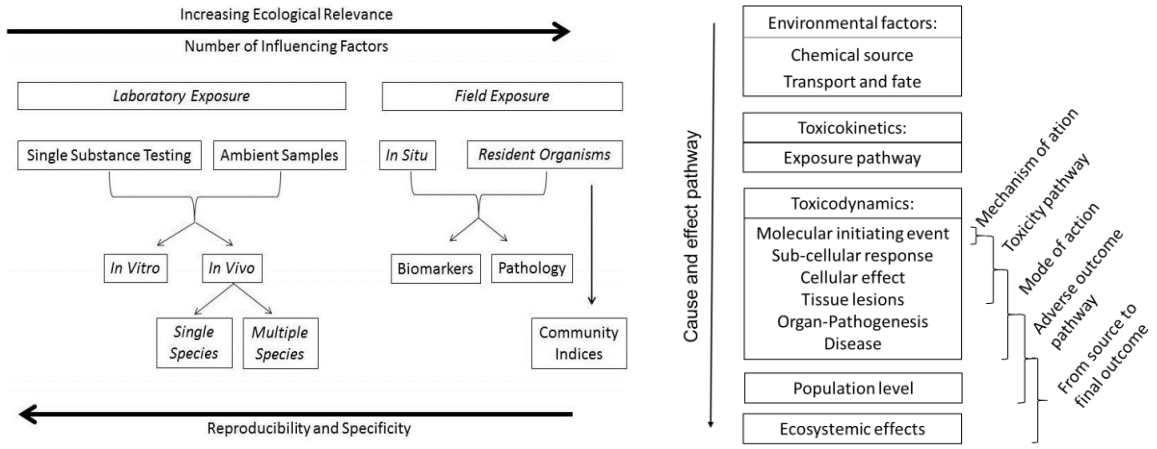


Figure 2. Ecotoxicological test procedures including their estimated ecological relevance, influencing factors and reproducibility/specificity (left, Connon et al. 2012). Each test examines different patches of toxicological cause and effect pathways (right).

In vitro bioassays utilize bacterial or eukaryotic cells (including recombinant cell lines) to screen for non-specific toxicity (e.g., cytotoxicity) as well as specific toxicity pathways / MoA (e.g., Escher et al. 2020, Rehberger et al. 2018, 2.2). *In vitro* systems are known for their high reproducibility and feasibility (figure 2) and are thus used for large-scale initiatives in predictive toxicology such as the Tox21 or ToxCast database (Brooks et al. 2020). Nonetheless they incorporate a lower predictive power regarding population-relevant effects than *in vivo* bioassays. Thus they are mainly used in mechanistic studies (e.g., on receptor interactions or cellular stress / metabolic responses) and MoA-based screenings of environmental samples as well as for substituting animal testing (e.g., Burgess et al. 2013, Martin et al. 2010, Escher et al. 2014, Norberg-King et al. 2018). They also became important (prospective and retrospective) monitoring tools including water and wastewater regulations (e.g., European Parliament and Council 2000, Leusch et al. 2017, Wernersson et al. 2015). A recent sound concept in this regard are effect based trigger values used as ecotoxicological (MoA-specific) complementation to EQS (Daniels et al. 2019, Escher et al. 2018, Itzel et al. 2019).

In vivo and *in vitro* bioassays have fundamentally shaped the current (eco)toxicological knowledge and large knowledge base on hazardous substances in the urban water cycle. A database query in the Web of Science (Clarivate Analytics, USA) searching for the term “wastewater toxicity” for instance indexed 5,547 publications referring to the topic and 410 publications containing these words

in the title of the respective studies (Web of Science 2017). Of these publications 601 and 41 respectively were published only in 2016, while some of the earliest date back to the 1970s (e.g., Esvelt et al. 1973). The number of relevant publications increases once keywords synonymous or related to toxicity (such as “adverse effects”, “negative impacts”, “hazards”, “risks”, “detoxification”) or to wastewater (such as “WWTP discharges”, “sewage treatment” or “municipal effluents”) are included or if specific bioassays, endpoint, pollutant groups and treatment technologies are searched.

This exemplary database query indicates the broad research on WWTP discharges including their environmental risks. Only a fraction of these studies identified plausible causative agents of the observed effects. This circumstance is particularly complex because of the vast number of existing and newly developed chemicals that are presently not or insufficiently assessed**.** The authors of these studies thus generally concluded towards unknown compounds and sample constituents not covered by target chemical analyses and/or their cumulative effects to be responsible (e.g., Abbas et al. 2018, Backhaus and Karlsson 2014, Maier et al. 2016, Thrupp et al. 2018). Through these studies it also became apparent that neither the reduction of target (micro)pollutants, nor of the DOC content, may correlate to the removal of toxicity. Effect-based methods (comprising *in vitro*, *in vivo* and *in situ*) are thus imperative to complement on chemical analyses (compare above) and to establish the missing line(s) of evidence (Backhaus et al. 2019, De Baat et al. 2020, Leusch et al. 2014). Moreover, the development of new methods and optimisation of current test strategies as well as further integration into (waste)water-related regulations should be urged (e.g., Pal et al. 2010, Reemtsma et al. 2016, Rizzo 2011, Schwarzenbach et al. 2006, Völker et al. 2019).

1.4 *Caenorhabditis elegans* in ecotoxicological research

1.4.1 Ecology and establishment as biological model organism

Caenorhabditis elegans (*C. elegans*) belongs to the diverse animal phylum Nematoda (roundworms). It was firstly described by Maupas (1899) who discovered it in humus rich soils in Algeria. In later years, it was also identified in freshwater habitats (i.e., Hirschmann 1952). *C. elegans* generally prefers temperate regions and, as a bacterivore, microorganism-rich habitats such as rotting fruits and plants (Hope 1999). Like other nematodes, it plays an important role in soil and benthic food webs (Yeates et al. 1993, Traunspurger 1997), wherein it is eaten by predating nematodes and omnivore insects. If present in unfavorable environments (compare 1.4.2 and 1.4.4) it can assume a so called dauer state. Dauer larvae ingested by invertebrates were observed to be (passively) disseminated to more distant locations (Félix and Braendle 2010). First laboratory isolates were gained early on (Nigon 1949). Since then, the growing knowledge on *C. elegans* reflects in more than 15,000 published articles up to 2010, including its complete cell lineage, fully sequenced genome and other major discoveries (e.g., Chalfie 2009, Félix and Braendle 2010). Its genetic and functional correlations to humans (Corsi 2006, Kim et al. 2019c, Leung et al. 2008) were used to elucidate biochemical pathways of human diseases such as Alzheimer, Diabetes or obesity (Tejeda-Benitez and Olivero-Verbel 2016a). Similar to *Drosophila melanogaster* it implies typical advantages of a biological model, including a well-described morphology, small transparent body (well observable with differential interference contrast (DIC) microscopy), fast reproduction cycle, large brood size, facile cultivation, low maintenance costs (e.g., frozen stocks that can be kept for months), rather invariant development, amenability to genetic crosses and an extensive spectrum of methods (e.g., Altun and Hall 2009, Hope 1999, Sulston and Horvitz 1977). Despite these advantages a few experimental limitations exist, such as a rather impermeable cuticule, and similar to other laboratory models, the reconstruction/extrapolation of *in situ* environmental conditions, has to be considered when applying *C. elegans* as ecotoxicological model. Therefore, well-defined and adapted culturing practices (Hunt 2016) as well as result verification in the light of higher tier studies (compare figure 2 and chapter 2.3) have been recommended as promising way forward.

1.4.2 Development and reproduction

C. elegans exists as hermaphrodites (XX) and males (X0) incorporating five pairs of autosomes with ~18,000 genes. Hermaphrodites mainly self-fertilize producing genetically identical offspring. Males arise by spontaneous non-disjunction at a rate of 0.1%. Mating increases the genetic variability, including a population proportion of males of up to 50% (Altun and Hall 2009). Adults can grow to an average length of 0.8–1.1 mm during a short life cycle of about 3.5 days (figure 3). The hermaphrodite life span counts 12–20 days under standard laboratory conditions (optimal feeding, 20° C). *C. elegans* can survive 4–8 times longer as dauer larvae (Cassada and Russell 1975, Golden and Riddle 1984, figure 3). Under favorable conditions adult hermaphrodites may lay up to 280–300 eggs in total (Byerly et al. 1976), but the number of fertilized eggs can increase to 1200–1400 upon mating (Hodgkin 1988). Byerly et al. (1976) observed the life cycle to be temperature dependent and a continuous temperature of > 25°C causes sterility.

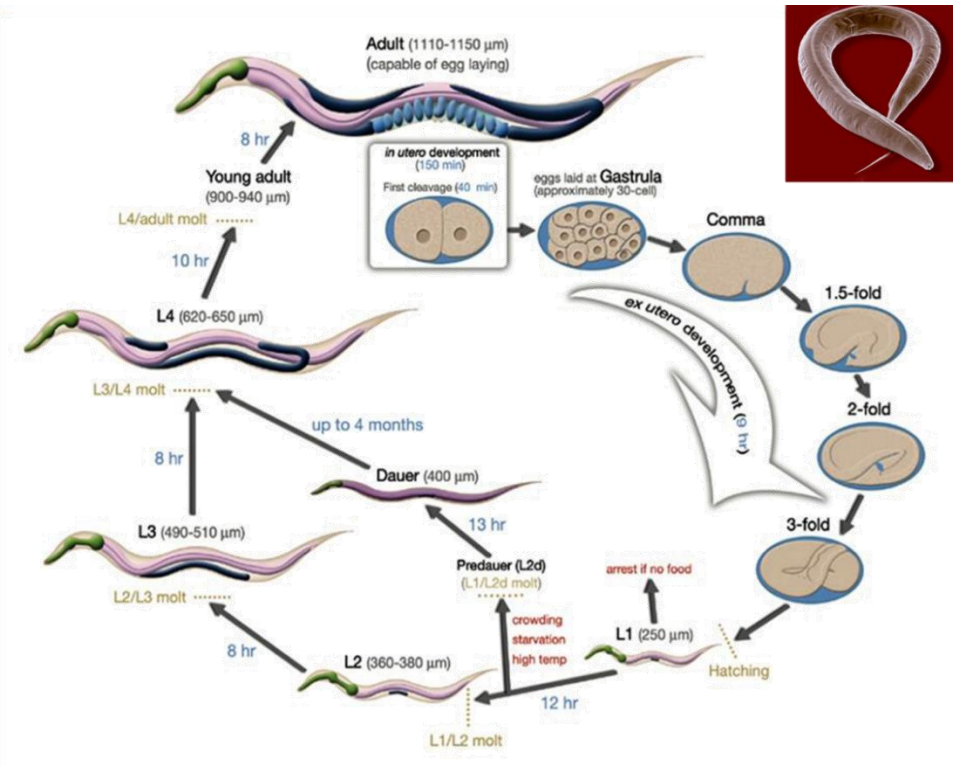


Figure 3. *C. elegans* life cycle (Altun and Hall 2009) and scanning electron microscope image of an adult hermaphrodite (J. Berger, Max-Planck-Institute for Developmental Biology, Germany). Illustration indicates pharynx (green), intestine (pink), proximal and distal gonad (dark blue), uterus with developing eggs (light blue) and developmental data.

The genetics of *C. elegans* proved to be relatively amenable, which could hint on alternative ecological specializations (Félix and Braendle 2010, 1.4.4). Adult hermaphrodites count 959 somatic nuclei and approximately 2000 germ cells. Males incorporate 1031 somatic nuclei and about 1000 germ cells (Alberts et al. 2002). Somatic cells differentiate into various tissues and compartments, such as neurons, muscles, hypodermal cells, reproduction system, intestine, pharynx, excretory system and glands (Sulston and Horvitz 1977). *C. elegans* also implies a sensory system that reacts to various environmental signals (1.4.4). In addition, *C. elegans* shows a wide range of behavioral traits such as feeding, defecation, egg laying, locomotion, learning as well as social behavior (e.g., Rankin 2002, De Bono 2003).

1.4.3 Xenobiotic metabolism

C. elegans has a versatile xenobiotic metabolism that reflects its evolutionary adaption to heterogeneous habitats and various external stressors. This involves traits such as its sensory system that can trigger avoidance behaviors towards contaminated food or pathogenic bacteria (Sambongi et al. 1999, Lindblom and Dodd 2006). Primary exposure sites for environmental chemicals, small particles and other ingestible materials are pharynx and intestine. In contrast, the permeability of its cuticle seems rather low (Chisholm and Xu 2012). In the intra-cellular space xenobiotics are recognized by ligand activated transcriptional factors, such as nuclear hormone receptors (NHRs) or the maternally-inherited skinhead-1 (SKN-1), that regulate different detoxification genes (figure 4). DNA sequencing and homology analyses thereby revealed 284 genes coding for NHRs; five times more than in vertebrates. However, the majority of *C. elegans* NHRs are currently considered orphan (An and Blackwell 2003, Antebi 2006, Inoue et al. 2005, Mendelski et al. 2019, Taubert et al. 2011).

Similar to most organisms, detoxification in *C. elegans* proceeds through three phases. Phase 1 (transformation) is characterized by the modification of xenobiotics through cytochromes P450 (CYPs) and short-chain dehydrogenases (SDRs). Pan et al. (2016) report on 83 *C. elegans* and 57 human *cyp* genes, while the overall evolutionary diversity of *cyps* estimates to 39,417 isoforms in 236 species. CYPs generally change the polarity of target molecules by adding chemical groups (see 2.3.2 for examples). Phase 2 (conjugation) genes, such as glutathione-s-transferase

(GST) or UDP-glucuronosyl-transferase (UGT) further increase the solubility of their targets. They are also responsible for directing their excretion, which is performed by transport proteins. The latter belong to phase 3 (excretion) and include adenosine triphosphate (ATP)-binding cassette (ABC) transporters, such as P-glycoproteins (PGP 1–4), and other efflux pumps (Lindblom and Dodd 2006). Modified xenobiotics that are excreted into the gut lumen are subsequently removed by defecation.

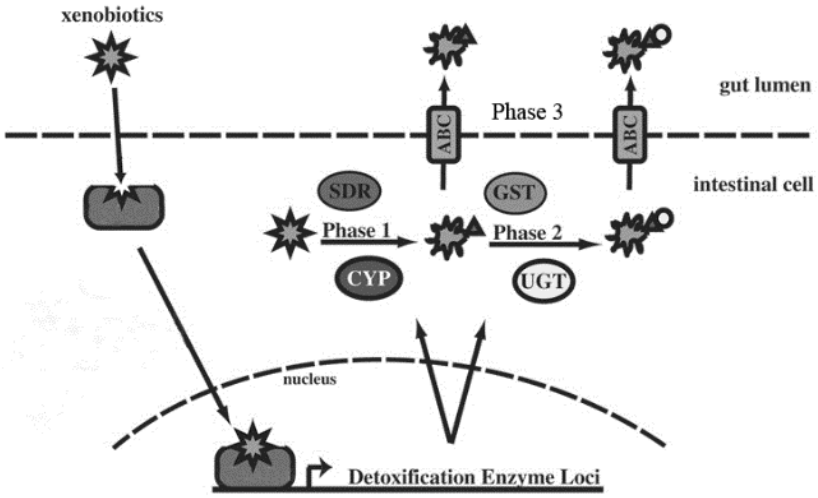


Figure 4. Detoxification in an intestinal cell of *C. elegans* (Lindblom and Dodd 2006)

Toxic effects of xenobiotics often go in hand with the formation of reactive intermediates, such as reactive oxygen species (ROS). Several detoxification genes protect *C. elegans* against ROS, such as GSTs, catalases, superoxide dismutases (SODs) or glutamylcysteine synthetase (e.g., *gcs-1*, An and Blackwell 2003, Inoue et al. 2005). Other (chemical) stress related genes are heat shock proteins (HSPs), metallothioneins (e.g., *mtl-1* and *mtl-2*) or abnormal dauer formation 16 (DAF-16, Freedman et al. 1993, Morimoto 1998). The human *p53* ortholog *cep-1* (Rodriguez et al. 2013) is also known to participate in protective and regulatory mechanisms associated with chemical/stress responses. The complex metabolic interplay of these genes is the basis for cellular integrity and homeostasis. If detoxification capacities are exceeded damage may propagate and irreversible effects may occur.

1.4.4 Environmental toxicology

Early studies on environmental factors and cues investigated responses to food sources, temperatures (e.g., Klass 1977), heavy metals (e.g., Popham and Webster 1979), gamma-radiation (Yeargers 1981), UV light (Hartman and Herman 1982) and

pharmaceuticals (e.g., Höss and Weltje 2007). These studies prepared the ground for *C. elegans* as a toxicological model (Leung et al. 2008, Hägerbäumer et al. 2015, Williams et al. 2017, Wilson and Kakouli-Duarte 2009) used for the assessment of anthropogenic chemicals, technical materials and environmental samples including sediments, soils, sludge or waste (table 2). *C. elegans* has also been involved in microcosm studies and community analyses (e.g., Hägerbäumer et al. 2015, Mueller et al. 2020a, Wang et al. 2015, Wilson and Khakouli-Duarte 2009, table 2). The nematode thereby proved to be a versatile tool for answering toxicokinetic (Burns et al. 2010, Chen et al. 2016, 2018, Kim et al. 2019a, Liu et al. 2019, Mueller et al. 2020b, Offermann et al. 2009, Ristau et al. 2015, Roh et al. 2014, 2016, Spann et al. 2015, Stylianou et al. 2018) and toxicodynamic research questions (compare below).

C. elegans studies have examined a panoply of apical endpoints including mortality (e.g., Coomans and Vanderhaeghen 1985, Williams and Dusenbery 1990), life span, reproduction (e.g., Anderson et al. 2001, Leung et al. 2010), fertility/fecundity (e.g., van Kessel 1989, Popham and Webster 1979), population growth rate (e.g., Ohba and Ishibashi 1984), growth (e.g., van Kessel 1989, Ohba and Ishibashi 1984), morphology (e.g., Ohba and Ishibashi, 1984, Popham and Webster 1979) and development (e.g., Coomans and Vanderhaeghen 1985, Ohba and Ishibashi 1984). Most apical endpoints incorporate midpoints or secondary endpoints such as life span, larval arrest or morphological defects for development (e.g., Tejeda-Benitez and Olivero-Verbel 2016a) and reproductive delays, decreased progeny or reduced fertility for reproduction (e.g., Tejeda-Benitez and Olivero-Verbel 2016a). Behavioral endpoints include feeding, locomotion (e.g., Williams and Dusenbery 1990, Boyd et al. 2003, Anderson et al. 2004), chemotaxis, avoidance and motility (e.g., Bargmann 2006, Li et al. 2020, Sambongi et al. 1999). These are complemented by cellular and molecular endpoints, such as apoptosis, disruption of membranes, mitochondria, chromosomal aberrations, cell cycle inhibitions, reversible and irreversible DNA damage (e.g., Allard et al. 2013, Behl et al. 2016, Leung et al. 2010), gene expression profiling (e.g., Peter et al. 1996, de Pomerai et al. 2008, Reichert and Menzel 2005, 2.3.3), energy budget (e.g., McLaggan et al. 2012). Other complementations are given by interactions of toxicity pathways such as in behavioral neurotoxicity (e.g., Williams and Dusenbery 1990, Liu et al. 2020).

1.5 Integration of this thesis into the present state of research

This thesis was embedded in the research project TransRisk dealing with the "characterization, communication and minimization of risks from novel pollutants and pathogens in the water cycle" (www.transrisk-projekt.de). Anthropogenic (micro-)pollutants vastly contribute to aquatic pollution. The characterization of their realistic hazards and feasible mitigation options has become imperative. Due to their incomplete removal in conventional biological WWTPs, AWWT technologies are being implemented to (further) reduce their emission. This thesis particularly aims at the ecotoxicological evaluation of the effectivity of the AWWT processes ozonation and ozonation coupled to GAC/biological post-filtration, which was targeted in the following key challenges and knowledge gaps:

1) Sample preparation is pivotal for the accurate detection and quantification of micropollutants and toxicity (Prasse et al. 2015, Völker et al. 2019). Nonetheless, current methods are mainly optimised for chemical analyses (Escher et al. 2005, Prasse et al. 2015), but not for effect-based detections (Bistan et al. 2012, Stalter et al. 2016, Wagner and Oehlmann 2010). In our study we optimised three major methods - acidification, filtration and SPE - for *in vitro* analysis. In earlier studies, acidification was capable of modifying pollutants in different water matrices (Baker and Kasprzyk-Hordern 2011, US EPA 2010, Vanderford et al. 2011), filtration altered specific *in vitro* activities compared to untreated samples (Dagnino et al. 2010, Janex-Habibi et al. 2009, Routledge 2003) and SPE selectively enriched (micro)pollutants. This situation can cause under- or over-estimations of actual hazards. We optimised these techniques using eleven *in vitro* bioassays and different (waste)water matrices. Based thereon specific preparation procedures and optimised methods were recommended (3.1 and Abbas et al. 2019).

2) A long-term environmental monitoring was conducted in a protected region in Baden-Württemberg, Germany, using *in vitro* bioassays screening for endocrine activities, mutagenicity and genotoxicity. Up to 33 sites representative for the water cycle were sampled during five campaigns (2012–2014) including wastewater, surface water, groundwater and drinking water. *In vitro* bioassays were accompanied by a chemical monitoring of 92 chemical indicators (Anna Bollmann unpublished results, Seitz and Winzenbacher 2017). Main objectives were to

determine the types and levels of ecotoxicological *in vitro* potentials, to localize eventual pollution hot spots, to indicate environmental and/or human hazards as well as to generate data sets for before-and-after-comparisons of WWTP upgrades (2.2). The obtained results further supported regional stakeholders (e.g., waterworks and municipalities) in their sustainable (waste)water management plans.

3) An *in vivo* bioassay based on ISO10872 and the model *C. elegans* (Haegerbaeumer et al. 2019, Höss et al. 2012, Wilson and Khakouli-Duarte 2009) was adapted for this thesis. This involved apical (reproduction and growth) and molecular endpoints (CYP-35A3 related xenobiotic metabolism in a transgenic strain, Menzel et al. 2007). Proof-of-principle experiments were conducted using the reference PAH β-NF (Forsgren 2015, Leung et al. 2010) and different (waste)water samples (aqueous, spiked and extracted) comprising the described conventional biological and AWWTs. An influence of TSS was also analysed, because particle-bound pollutants may alter bioavailability (Offermann et al. 2009, Spann et al. 2015, Stylianou et al. 2018, table 2). Cumulative effects due to background contaminations were hypothesized (Backhaus and Karlsson 2014, Thrupp et al. 2018). The adapted bioassay allowed for the combined assessment of developmental and reproductive toxicity (DART) and CYP-35A3 induction (2.3 and Abbas et al. 2018). Novel test systems and strategies have been rated highly important, because standard bioassays may be insensitive in detecting relevant water quality deficiencies (Berger et al. 2016, Schwarzenbach et al. 2006, Sonne et al. 2018, Wigh et al. 2018).

4) The AWWTs ozonation and ozonation with GAC/biological post-filtration were evaluated for their (micro)pollutant/toxicity removal capacity. The ecotoxicological part of this evaluation was performed with a battery of *in vitro* and on-site *in vivo* assays previously applied in related wastewater assessments (e.g., da Costa et al. 2014, Gartiser et al. 2010, Giebner et al. 2018, Magdeburg et al. 2012, 2014, Maltby et al. 2000, Stalter et al. 2010, 2011, Triebskorn et al. 2017). This thesis thereby examined (the above mentioned) five *in vitro* and *C. elegans* bioassay for their usefulness at the laboratory-scale. The detections made by these assays were integrated into a ‘wastewater quality evaluation matrix’ (developed in TransRisk, Ternes et al. 2017) that rated ozonated coupled to GAC filtration as most effective AWWT option (out of five, 2.4). The evaluation concept may readily be transferred to future evaluations of WWTPs.

2 General discussion

2.1 Optimising sample preparation for *in vitro* bioassays

Sample preparation methods can bias the quantification of the original toxicity of environmental samples (e.g., Neale et al. 2018). Main reasons for this are modifications/losses of sample constituents during sample processing/storage and/or interfering environmental factors (Daughton 2003, Maruya et al. 2016). This chapter discusses ways to reduce such artifacts at different preparational/analytical steps of an *in vitro* analysis. Bioassay-based optimisations have scarcely been performed despite their apparent relevance for realistic hazard assessment and monitoring. Characteristic challenges in this endeavor were previously studied by Bistan et al. (2012), Macova et al. (2010), Neale et al. (2015) and Stalter et al. (2016). Based thereon the present optimisation focuses on the influence of sample acidification, filtration and SPE on the outcome of eleven *in vitro* bioassays testing 17 (waste)water samples representative for the urban water cycle.

2.1.1 Sample acidification

Acidification is commonly used for stabilization of (non-)target compounds during sample storage. The present experiments focused on possible ecotoxicological discrepancies between neutral (untreated) and acidified samples that would emerge during a 24 h storage period. Significant differences occurred regarding different endocrine activities and cytotoxicity. The degree of this discrepancy was higher at certain sampling points (e.g., highest for raw wastewater) and in a number of *in vitro* bioassays (e.g., highest in the yeast anti-estrogen screen, YAES). However, no obvious reason or trend became apparent, such as sample toxicity or the specificities of the respective *in vitro* bioassay. From these findings it was thus concluded that 1) the added sulphuric acid can modify (bioactive) sample constituents (e.g., in an unspecific manner) and bioassay outcomes, 2) Possible sample modifications by microbes contained in neutral samples may or may not have a more severe effect on the outcome of an *in vitro* bioassay than those mentioned under 1). Both situations (1 and 2) thus demand further research to fully clarify the advantages/disadvantages of testing native or acidified samples, and to allow their analytical comparison to other sample stabilization techniques (such as sample filtration or sample freezing).

To this end, neutral samples were recommended for ecotoxicological assessments, because higher toxicity was detected. It is also known that the chemical composition of samples may be altered by pH shifts and acid-based hydrolyses (Baker and Kasprzyk-Hordern 2011, Stalter et al. 2016, Prasse et al. 2015, Vanderford et al. 2011). Such an alteration was possibly observed by Bollmann et al. (unpublished results) who performed a non-target screening on a wastewater sample (that was also tested in this thesis, Abbas et al. 2019). In this screening the researchers detected an overlap of compounds of only 72−75 % between the neutral and acidified sample aliquot. In addition, acidified samples require further preparation steps, such as neutralisation prior to bioassays, not to compromise bioassays test organisms.

2.1.2 Sample filtration

Filtration has several advantages such as isolation of particulate matter and/or sample sterilization (e.g., Janex-Habibi et al. 2009, McLaggan et al. 2012). Earlier bioanalytical comparisons also illustrated potential drawbacks such as significant losses of detectable endocrine activity. These losses seemed to originate from the retention of TSS-bound compounds (also hypothesized by Abbas et al. 2018) which was also suggested in earlier studies (Dagnino et al. 2010, Janex-Habibi et al. 2009, Routledge 2003, Shieh et al. 2016) that also described hydrophobic (micro)pollutants to adsorb to filter membranes. In this context Ng and Cao (2015) published the article “What Exactly Are You Filtering Out?” wherein they suggest to better adjust filter materials to the aims of an investigation.

In several samples the present *in vitro* bioassays detected higher endocrine activities in filtered samples than the corresponding unfiltered samples. This was explained as the *in vitro* test systems applied in this thesis can be activated and/or inhibited by agonistic or antagonistic compounds respectively. Such interactions in wastewater samples have been reported earlier (Ihara et al. 2014, Itzel et al. 2019, Rao et al. 2014, Zwart et al. 2018). Thus a selective retention of (particle-associated) antagonists during filtration could have allowed an increased receptor binding of agonist in the respective filtrates, and vice versa. In addition, agonist/antagonists could have been “washed off” from their particle-bound states during filtration. In this way they could further have altered their ratio in the filtrates. Despite these findings, most samples showed similar activity levels regarding unfiltered and filtered

samples, which suggested that most environmental hormones and EDCs remained dissolved in the aqueous phase of the respective samples (e.g., due to their hydrophilicity /polarity, 1.2.1).

Filtration unpredictably modified the composition and *in vitro* activities/toxicity of (waste)water samples. This situation may lead to misinterpretations of bioassay results including under- and over-estimations of actual hazards (e.g., Burton 2000, Dagnino et al. 2010). For the effective application of filtration in ecotoxicology further compound, sample type and bioassay specific optimisations are recommended.

2.1.3 Solid phase extraction

Micropollutants detection often requires sample enrichment, such as by SPE, to increase quantification limits (LOQs) and for reducing matrix interferences (Prasse et al. 2015, Wagner and Oehlmann 2011, Wagner et al. 2013). SPE methods have been optimised for the recovery of different pollutant classes (Baker and Kasprzyk-Hordern 2011, Leusch et al. 2012, de Alda and Barcelo 2001) focusing on methodical parameters, such as sample pretreatment, SPE sorbents or different extraction modes (Pietrogrande and Basaglia 2007). These optimisations also involved different evaluation methods such as multivariate statistics (e.g., Harju et al. 2015, Polo et al. 2005, Yang et al. 2014). Most studies used defined standard solutions, while a few further validated their results using complex environmental samples. The present investigation focused on benchmarking six SPE methods for their recovery of endocrine activity, genotoxicity and cytotoxicity from real (waste)water samples. These methods combined three SPE sorbents (Oasis HLB, Supelco ENVI-Carb+ and Telos C18/ENV) with two extraction pH (7 and 2.5). All SPE sorbents were eluted with a methanol:acetone (1:1) mixture (figure 5). Moreover, a multivariate evaluation via the Pareto algorithm was integrated (compare Abbas et al. 2019).

The multivariate optimisation approach demonstrated that the highest endocrine activities and genotoxicity were recovered by the Telos C18/ENV sorbent. Highest cytotoxicity was obtained by the Oasis HLB sorbent. By the optimised SPE method certain endocrine activities, such as estrogenicity, (anti-)androgenicity and dioxin-like (AhR) activity, were only detected after sample enrichment. In line with these results the Telos C18/ENV and Oasis HLB indicated effective recoveries of

cytotoxicity (Escher et al. 2005, Stalter et al. 2011, 2016) and estrogenicity (Bistan et al. 2012, Wagner and Oehlmann 2011) in other optimisation studies. Regarding the putative causative agents of these activities/toxicity the composite material of the Telos C18/ENV and the amphipathic material of the Oasis HLB were previously described to extract compounds with various physicochemical properties (e.g., Leusch et al. 2012). It is also thought that the applied *in vitro* bioassays can be activated by heterogeneous chemical compounds. In addition, Stalter et al. (2016) suggested that the ENV material of the Telos C18/ENV and the Oasis HLB seem capable of adsorbing polar cytotoxic compounds. Wagner and Oehlmann (2011) suspected non-polar chemicals to act as causative agents of the estrogenicity that they had extracted from bottled mineral water using a C18 material. It remains to be clarified whether (and how) these results apply to the present recovery rates. Most extractions in this study were more effective at an acidic sample enrichment pH (Abbas et al. 2019). The multivariate Pareto evaluation however favored a neutral sample extraction pH and SPE by the Telos C18/ENV as optimal method. This difference resulted from the integration of the parameter cytotoxicity. The latter was rated as potential indicator of a higher overall pollutant load including environmental hormones and EDCs (compare figure 6 and further discussion).

More effective extraction at acidic pH were also obtained by Misik et al. (2011) and Stalter et al. (2016) for the endpoints mutagenicity and cytotoxicity respectively. In their study Stalter et al. (2016) compared different SPE sorbents applied to disinfected drinking water, whereas Misik et al. (2011) had extracted biologically-treated and ozonated wastewater using a C18 sorbent. In the general context of SPE of bioactive/toxic compounds Escher et al. (2005) mention that short-term acidification may increase the recovery of weak acids and ionized (micro-)pollutants, while weak bases or zwitterions might be lost. Stalter et al. (2016) however also argue that short-term acidification (compare 2.1.1 and Abbas et al. 2019) can already degrade SPE matrices and/or modify dissolved organic matter constituents in aqueous samples.

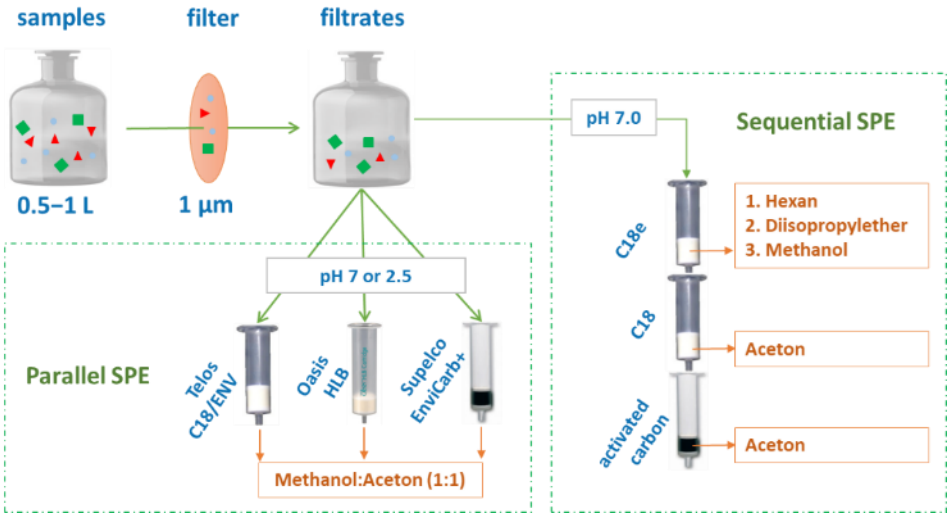


Figure 5. Two SPE methods designed as parallel and sequential protocols optimised for *in vitro* bioassays in the course of this thesis and within the TransRisk project.

These results point out the necessities in further optimising SPE methods for *in vitro* bioassay-based (as well as chemical) assessments and monitoring. Therefore, several optimisation parameters and experimental set ups described in the scientific literature seem promising. Different SPE sorbents and extraction modes (e.g., comparing eluting solvents of different polarities) improved the recovery of target compounds and TPs (e.g., Chang et al. 2009, Kern et al. 2009, Stalter et al. 2016). Bollmann et al. (unpublished results) connected three SPE cartridges (C18e, C18 and activated carbon) in a serial manner ('Sequential SPE' in figure 5) for the extraction of different (waste)water samples. Using the eluting solvents hexan, diisopropyl ether, methanol and acetone different extractions were performed. These could be used for separate (sample pre-fractionation) or combined ("total extract") analysis. In addition, these extracts were tested in the previously described *in vitro* bioassays. The sequential methods performed equally well than the 'Parallel SPE' method (figure 5) for most of the endocrine activities and cytotoxicity. Merging the individual solvent fractions into a "total extract" has not been compared.

Another SPE parameter that proved to be particularly relevant for bioassay analyses is the final enrichment factor at which an extract is tested. SPE enrichment factors were compared in previous studies (Escher et al. 2014, Macova et al. 2010, Tang et al. 2013). Escher et al. (2009, 2014) used 103 *in vitro* bioassays for "Benchmarking Organic Micropollutants in Wastewater" by applying a concept that compared the estrogenicity, genotoxicity and cytotoxicity of extracted wastewater

samples along the enrichment factor required to produce an effect (compare figure 6 in this context). Extracted samples also built the basis for EDA to identify possible causative agents (Burgess et al. 2013, Brack et al. 2017, Neale et al. 2017, Schulze et al. 2017, Tang et al. 2014). Samples with low contamination level such as groundwater and certain endpoints without toxicity thresholds such as genotoxicity often require high sample enrichment factors (up to 100, Keiter et al. 2006, Stalter et al. 2016). This circumstance may further be required for extracting surface waters or wastewater from AWWT stages, due to their generally low contamination levels.

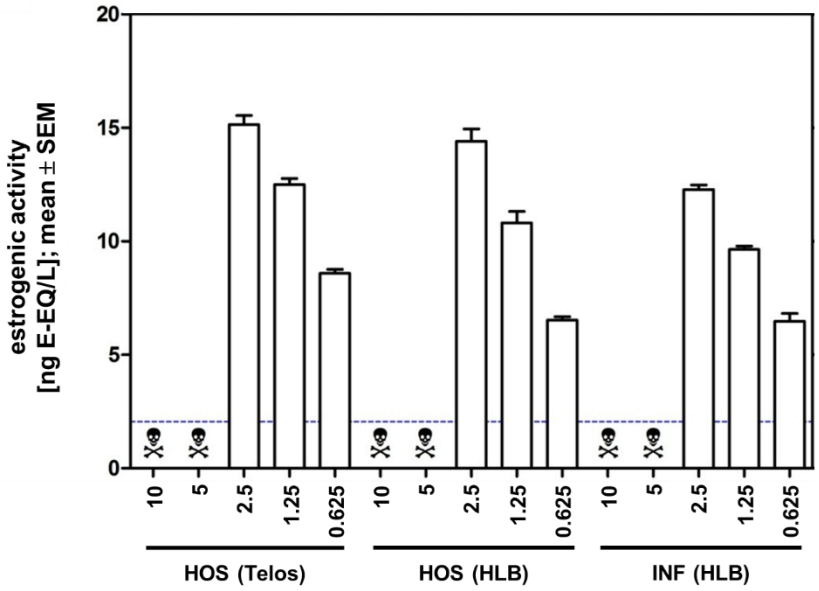


Figure 6. Estrogenic activity in extracted hospital wastewater (HOS) and a WWTP influent (INF) quantified as mean 17β-estradiol equivalents (ng E-EQ/L) ± standard error of the mean (SEM). Telos C18/ENV ("Telos") and Oasis HLB ("HLB") were applied as SPE sorbents. Different sample concentration factors (0.625−10 fold) were used to investigate concentration response relationships. In 5−10 fold concentrated samples cytotoxicity occurred (☠). Dashed line = limit of quantification. Unpublished result jointly obtained by I. Schneider and A. Abbas.

Nevertheless, exhaustive sample concentration may bear the risk of introducing artifacts such as from the (co)enrichment of matrix components and/or leaching materials (Leusch et al. 2014, Macova et al. 2010). Control samples such as procedural blanks are thus imperative for any bioanalysis involving SPE extracts (Stalter et al. 2016). High concentration factors (below toxic concentrations of keeping solvents) often indicate unspecific toxicity masking other endpoints (Escher et al. 2020). In contrast, at (very) low concentration factors the investigated effects

may be diluted out. In addition, interpolation from lower to higher enrichment factors (and vice versa) was rated rather unfeasible due to mostly non-linear concentration response relationships (figure 6). For analyzing SPE extracts the performance of preliminary range-finding experiments has thus been recommended (Tang et al. 2014, Escher et al. 2014).

Further optimisation potential was also allocated to loading modes and sample volumes. In a study by Macova et al. (2011) extraction of higher sample loading volumes (0.5–4 L) lowered the detection limit for cytotoxicity (*Alliovibrio fisheri*), estrogenicity (E-Screen), dioxin-like activity (AhR-CAFLUX) and genotoxicity (umu test) by up to 8 to 10 fold. As previously stated higher sample loading volumes may go in hand with an increased risk of artefacts and may require extended sample preparation, such as additional sample filtration. Schulze et al. (2017) developed an automated on site SPE device that can process sample volumes up to 50 L. In their experiments such sample volumes enhanced the recovery and enrichment of (ultra-)low concentrated (micro)pollutants and different *in vitro* activities, such as mutagenicity or (anti-)estrogenic activities. This technique (also termed large volume SPE, LVSPE) seems particularly useful for surface water and groundwater monitoring as well as investigations requiring a high number of parallel experiments on the same sample/extract. LVSPE also requires elaborate equipment and handling, such as the preparation of special circulation blanks. Automated and/or on-site SPE methods were also implemented with beneficial outcomes in several ecotoxicological studies (e.g., Henneberg et al. 2014, Prieto-Rodriguez et al. 2013).

Additional potential lies in optimising SPE methods for an enhanced recovery of volatile, (highly) polar and particle-associated compounds that are mostly lost during conventional sample processing and/or standard SPE procedures (compare below). Significantly different test results were for instance obtained in the E-Screen, once a keeping solvent was added before full evaporation of the eluting solvent (Wagner and Oehlmann 2010). This measure indicated the presence of estrogenic compounds that volatilized during evaporation without adding a keeping solvent. In an investigation by Stalter et al. (2016) solvent evaporation of wastewater extracts without solvent exchange (dimethyl sulfoxide, DMSO) diminished the recovery of cytotoxicity. These studies show how simple sample preparation steps can significantly improve the effectivity of standard SPE methods. However, according

to Stalter et al. (2016) and others (e.g., Benner and Ternes 2009, De Baat et al. 2020), the effective extraction of volatile, (highly) polar and unknown compounds however remains one of the main challenges in optimising the preparation of water and wastewater samples.

In perspective, optimised SPE methods offer specific advantages for *in vitro* bioassay-based environmental assessments and monitoring. Among their important benefits are an improved preservation, enrichment, detection and identification of micropollutants and toxicity (compare 2.2−2.4). SPE methods have previously facilitated the classification of (putative) causative agents (Itzel et al. 2020) exhibiting estrogenicity (Allinson et al. 2010, Ma et al. 2005, Routledge 2003, Zhao et al. 2015), anti-estrogenicity (Tang et al. 2014), dioxin-like (Allinson et al. 2011, Ma et al. 2005) and retinoic acid-like activities (Allinson et al. 2011, Sawada et al. 2012), genotoxicity (Keiter et al. 2006) or cytotoxicity (Allinson et al. 2010, Ma et al. 2005). If the same SPE extracts are applied in parallel in selected *in vitro* and *in vivo* test systems this can provide valuable insights regarding observed physiological effects (e.g., Hugget et al. 2003, Schneider et al. 2020). Another way forward will be to compare optimised SPE methods with particular sampling or other sample extraction techniques (such as SPME, passive samplers or purge-and-trap methods) and to broaden the knowledge on their benefits, limitations and further options for optimisation.

2.2 *In vitro* bioassays as environmental monitoring tools – case study of a water protection region in Southwest Germany

In vitro bioassays are important tools for ecotoxicological assessments and environmental monitoring. They are routinely applied to investigate unspecific toxicity as well as specific MoAs. MoA based bioassays can serve as indicators of different bioactive/toxic contaminants such as herbicides or EDCs (e.g., Escher et al. 2014, Wagner et al. 2017). The *in vitro* activity of these pollutants has often been verified using *in vivo* test systems. For certain EDCs the induction of vitellogenin served as biomarker for reproductive toxicity (e.g., Huang et al. 2016, Stalter et al. 2015). In this way *in vitro* and *in vivo* results were associated with suspected or identified compounds (compare EDA). Nevertheless, once the selected assays lack specificity and sensitivity respective toxicants may remain undetected. Clarification of causative agents thus resembles a complex task. *In vitro* bioassays for baseline or reactive toxicity (such as cytotoxicity) are known to integrate compounds with multiple MoAs (e.g., Escher et al. 2014, 2020, Riss and Moravec 2004, Tang et al. 2014). Although cytotoxicity has helped to draw correlations to *in vivo* effects (e.g., Stalter et al. 2015), several studies involving iceberg experiments demonstrated that most cytotoxic agents remained unknown (e.g., Neale et al. 2014, Tang et al. 2014). However, cytotoxicity assays proved to be beneficial tools in (high-throughput and preliminary) environmental screenings and prioritisation of pollution sites.

In the present thesis an environmental monitoring was conducted that involved six sampling campaigns at up to 30 sampling sites within a water protection region in Southwest Germany (compare Seitz and Winzenbacher 2017). Water and wastewater samples from relevant sites were screened for different endocrine activities (according to Routledge and Sumpter 1996, Stalter et al. 2011, Wagner et al. 2013), mutagenicity (based on ISO11350), genotoxicity (according to ISO13829) and cytotoxicity (occurring in these cellular test systems). The *in vitro* investigation was accompanied by a detailed chemical monitoring program (Anna Bollmann unpublished results, Seitz and Winzenbacher 2017) to investigate potential correlations with respective chemical indicators. In the course of this monitoring, high endocrine activities were detected in hospital, raw and to lesser extend biologically-treated wastewater with predominantly (anti-)estrogenic and partially androgenic activities.

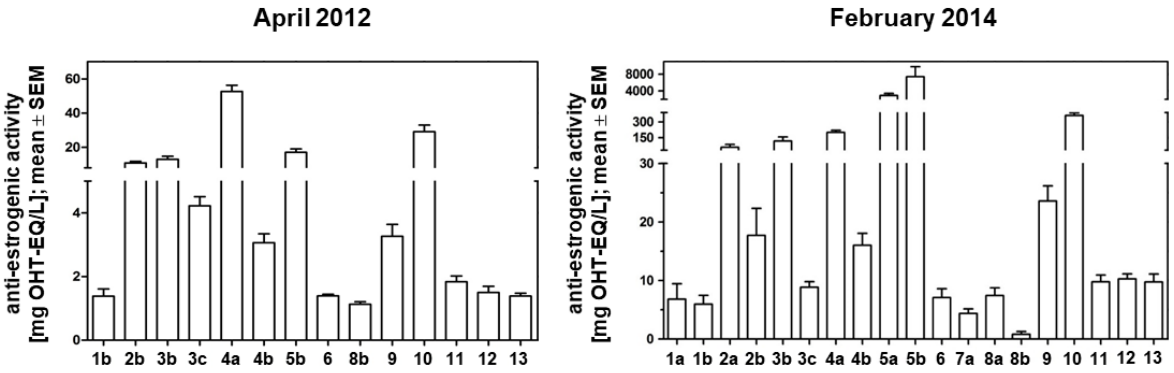


Figure 7. Anti-estrogenic activities in aqueous samples quantified as mean 4-hydroxytamoxifen equivalents (mg OHT-EQ/L) ± SEM. A total of 14−19 sampling sites were analyzed in April 2012 and February 2014 respectively including WWTP influents (1a, 2a, 4a), effluents (1b, 2b, 3b, 4b), a filtered WWTP effluent (3c), hospital wastewaters (5a, 5b), surface waters (6, 12, 13), samples from a rain water collection / retention / infiltration basin (7a, 8b, 8b) as well as groundwater hotspots (9−11). Results jointly obtained by I. Schneider and A. Abbas in collaboration with the TransRisk project partners.

In contrast, genotoxic and mutagenic potentials were rarely detected in raw and treated wastewater. Extracted hospital wastewater however indicated both genotoxicity and mutagenicity at increased rates. None of these toxicities including cytotoxicity was detected in surface water or groundwater throughout the entire monitoring. These results were in line with the scientific literature, whereby cytotoxicity and genotoxicity were mainly detected in extracted wastewater or (highly) polluted surface water (e.g., Baumstark-Khan et al. 2005, Escher et al. 2014, Macova et al. 2011). The fact that groundwater generally exhibits no or insignificant toxicity unless affected by landfills or contamination sites (e.g., Baun et al. 2000) was also supported by the present monitoring results. *In vitro* activities/toxicities were further investigated in the context of WWTP efficiencies. Their removal efficiencies were compared to identify and prioritize plausible pollution sources in the model region. All surveyed WWTPs (Langenau, Halzhausen, Steinhäule) indicated an effective removal of estrogenic, androgenic, genotoxic and mutagenic potentials (figure 8). This is of particular relevance as a few of the receiving streams of WWTPs show high wastewater fractions and flow through water protection zones of increased vulnerability.

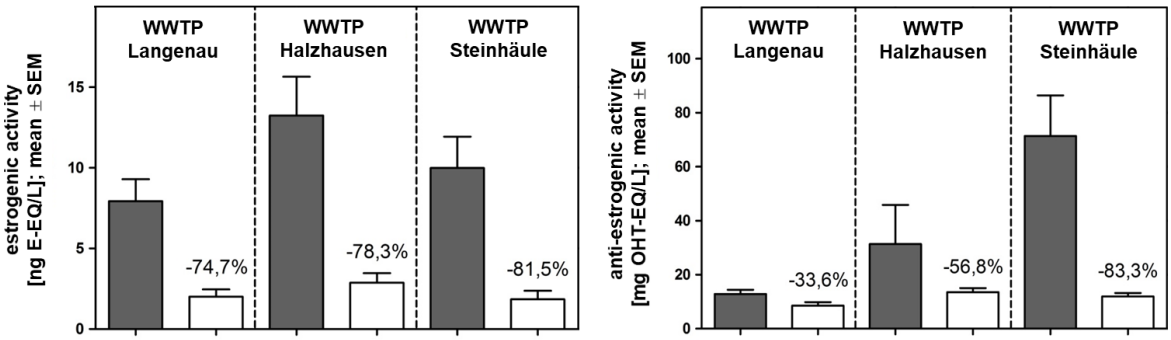


Figure 8. Estrogenic (left) and anti-estrogenic (right) activities in raw (dark grey bars) and biologically-treated (white bars) wastewater samples from three WWTPs (using activated sludge) presented as mean 17β-estradiol (ng E-EQ/L) or 4-hydroxytamoxifen (mg OHT-EQ/L) equivalents ± SEM, respectively. Results base on five monitoring campaigns (2012–2014, n = 40−48). Negative percent values (above white bars) represent the estimated removal rate towards the respective activity. Results jointly obtained by I. Schneider and A. Abbas.

The extensive reduction of these *in vitro* activities was reported for other WWTPs (e.g., Allinson et al. 2011, European Commission 2012, Giebner et al. 2018, Sawada et al. 2012). Their retention indicates the biodegradability and/or sludge adsorption of the respective (micro)pollutants during activated sludge treatments (Margot et al. 2013, Leusch et al. 2014, Dagnino et al. 2010). Notwithstanding the suitability of the applied *in vitro* bioassays for evaluating WWTP efficiencies, care should be taken when different test systems probing for the same endpoint are compared (e.g., AYES, E-screen, ER-calux, ER-GeneBlazer or YES for estrogenicity) and/or once different sample preparation methods were used to obtain a result (e.g., Magdeburg et al. 2014, Jia et al. 2015). Unlike the other *in vitro* potentials, anti-estrogenic activity seemed less effectively removed at a few investigated WWTPs (figure 8). Residual anti-estrogenic activity in treated wastewater was also observed by Ihara et al. (2014), Itzel et al. (2020) and Rao et al. (2014), who stated the need for further research on this endpoint in water and wastewater assessments.

Markedly, elevated anti-estrogenic activities were recurrently recognized in one of the groundwater hotspots (sample 10 in figure 7). These hotspots are known to be impacted by a nearby landfill (Wolfram Seitz, personal communication) so that they were previously prioritized for chemical monitoring. The present ecotoxicological monitoring of these hotspots included the examination of their most proximate water intakes and wells used for the extraction of raw drinking water (figure 9). Three

important aims were pursued in this investigation: 1) the detection of relevant *in vitro* activities, 2) to ensure an effective water treatment, if these activities would occur and 3) to narrow down putative causative agents in groundwater (e.g., if natural or anthropogenic chemicals) and their potential sources, such as WWTPs or agricultural run offs.

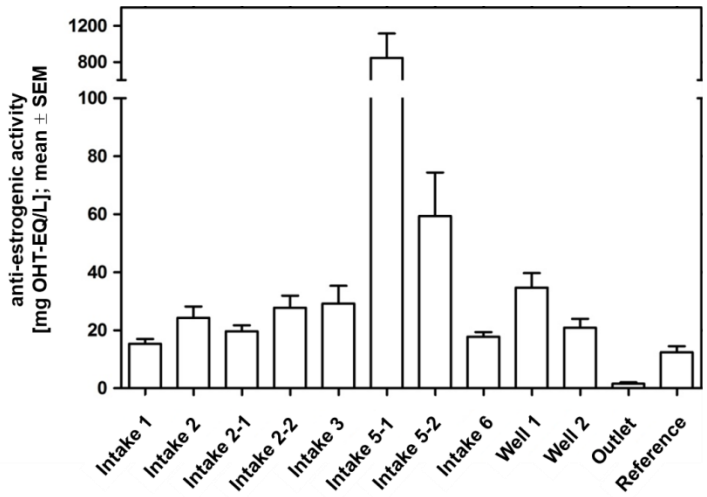


Figure 9. Anti-estrogenic activities presented as mean 4-hydroxytamoxifen equivalents (mg OHT-EQ/L) ± SEM of aqueous grab samples taken from groundwater intakes (1−6) and groundwater wells (1−2) in the model region in October 2013 (n = 8). Outlet = polished groundwater from a local waterworks. Reference = raw groundwater from large depth. Results jointly obtained by I. Schneider and A. Abbas in collaboration with the TransRisk project partners.

As an outcome, one groundwater intake (sample 5-1 and 5-2), but none of the other prioritized sampling sites indicated any relevant anti-estrogenic activity. Importantly, no activity was detected in drinking water from a supply pipe (sample ‘outlet’) and in groundwater used for the production of drinking water after minor treatment (sample ‘well 1’ and ‘well 2’). These results were obtained using aqueous grab samples of the sampling campaign in October 2013 (figure 9). Samples taken during the subsequent monitoring (February 2014) from the same sampling sites served for follow up examination. For possible effect confirmation these samples were prepared by SPE. The high anti-estrogenic activity previously detected in sample 5-1 (figure 9) could not be confirmed (remaining at the level of the LOQ, results not shown). Moreover, the parallel chemical monitoring at these sampling sites also did not showed any relevantly increased concentrations of (micro)pollutants.

Up to present, only a small number of chemicals are known for anti-estrogenic effects. One chemical thereof that was included in the chemical monitoring program was 1H-benzotriazole (Abbas et al. 2018). Benzotriazole was reported for *in vitro* anti-estrogenicity by Harris et al. (2007) as well as to be responsible for different *in vivo* effects (Seeland et al. 2012). However, MECs of 1H-benzotriazol at all monitored sites (e.g., 30−90 ng/L in the groundwater hotspots) were far below its reported ECs (e.g., several milligram per liter in the YAES). Nonetheless, Harris et al. (2007) also computed a predicted no effect concentration (PNEC) of 60 µg/L based on further literature data. Moreover, other antagonist of the human estrogen receptor capable of activating the YAES could have been present in the analyzed groundwater samples. Known or suspected anti-estrogens in the medical and ecotoxicological literature are for instance the anti-neoplastic drugs fulvestrant (ICI 182780, e.g., Hu et al. 1993), raloxifene (Matsumoto et al. 2004), tamoxifen and a number of its derivatives/TPs (Knoop et al. 2018, Sohoni and Sumpter 1998, Stalter et al. 2011, Zhao et al. 2011) as well as the insecticide hexachlorcyclohexan (Li et al. 2008), several flame retardants (Di Benedetto 2009, Martin Wagner personal communication, Zhang et al. 2014), the disinfectants hexachlorophene, pentachlorophenol and the vitamin K3 (Jung et al. 2004), the PAHs dibenz[a,h]-anthracene, 6-hydroxy-chrysene, 2,3-benzofluorene and benzo(a)pyrene (Tran et al. 1996). However, these compounds were not expected to occur at all or at relevant concentrations in the analyzed samples. Whereas potential mixture toxicity effects remained to be investigated. A number of natural compounds have been discussed to imply anti-estrogenic potentials or to be capable of influencing the outcome of *in vitro* bioassay (e.g., Janosek et al. 2007, Neale et al. 2015). As substances such as humic or fulvic acids represent natural soil constituents, they seem (more) likely to occur at milligram per liter concentrations in groundwater. Follow up investigations thus seem essential to clarify on the nature of the anti-estrogenic activities in aqueous and extracted samples. Unlike for groundwater, anti-estrogenic pharmaceuticals might explain the high anti-estrogenicity detected in hospital wastewater (sample 5a and 5b, figure 7). These compounds are regularly administered in oncology departments such as in the hospital referring to sample 5a. Patients undergoing hormone therapies may furthermore emit these pharmaceuticals into sewage collected from households.

Hospital wastewater moreover exhibited high anti-androgenic activity. This activity may derive from anti-androgenic drugs used in cancer therapy. Regularly administered drugs in this regard are bicalutamide (e.g., Blackledge 1993), flutamide (e.g., Grover et al. 2011, Sohoni and Sumpter 1998, used as positive control in the YAAS) and its metabolite hydroxyflutamide (e.g., Kusk et al. 2011), nilutamid (e.g., Gaillard 1996), enzalutamid (e.g., Gordon et al. 2017) and 4-hydroxytamoxifen (compare anti-estrogens above). Zwart et al. (2018) detected the drugs amitriptyline (anti-depressant) and celecoxib (anti-inflammatory drug) in surface water, which may also be administered in the analyzed hospital. Both compounds exhibited anti-androgenic properties. However, their individual concentrations might not reach EC in the YAAS. Other anthropogenic chemicals with experimented anti-androgenic potential are 1H-benzotriazole (Fent et al. 2014), the plasticizers bisphenol A (BPA, e.g., Li et al. 2008, Sohoni and Sumpter 1998) and benzyl butyl phthalate (BBP, Li et al. 2010, Sohoni and Sumpter 1998), the industrial chemicals nonylphenol and octylphenol (e.g., Li et al. 2010) as well as several pesticides, such as a degradation product of dichlordiphenyltrichlorethane (DDT, e.g., Laudet and Gronmeyer 2002, Sohoni and Sumpter 1998), r-hexachlorcyclohexan, hexachlorobenzene (Li et al. 2008), iprodione, linuron, metabolites of vinclozolin, methoxyclor and procymidone (Rempel and Schlenk 2008, Urbatzka et al. 2007). Henry and Fair (2013) investigated anti-androgenic activities for perfluorooctane sulfonate, perfluorooctanoic acid and triclosan. An EDA by Urbatzka et al. (2007) identified a number of these compounds in surface waters. The authors further concluded towards unknown anti-androgenic causative agents. Regarding the present samples non-pharmaceutical compounds such as identified by Muschket et al. (2018) and Urbatzka et al. (2007) or quantified in the course of this thesis (1H-benzotriazole, compare above) seem unlikely to occur in hospital wastewater or only at very low concentrations including their potential release from sources such as polycarbonate (BPA) or polyvinylchloride (BBP) plastic materials. It also remains to be investigated to which extend NOM constituents that were supposed to interfere with the human androgen receptor (Bittner et al. 2012) may occur in hospital wastewater (at relevant concentrations). However, due to elevated concentrations and known potencies of the mentioned anti-androgenic pharmaceutical they remained the main suspects. As all of the investigated hospitals connect to WWTPs precautionary measures seem advisable

to verify their elimination during wastewater treatment as well as to prevent their excessive discharge into the environment, such as during heavy rain events. This may similarly pertain the genotoxic and mutagenic potentials detected in hospital wastewater.

The presented *in vitro* bioassay results support the scientific consensus on their usefulness in environmental monitoring and ecotoxicological assessments. Although extrapolations from *in vitro* to *in vivo* systems can be attached to uncertaincy (Henneberg et al. 2014, Huang et al. 2016, Prasse et al. 2015, Rehberger et al. 2018, Schneider et al. 2020), *in vitro* bioassays are nonewithstanding beneficial tools for the detection of hazard potentials and MoA-based classification of (micro)pollutants and (waste)water samples. Due to this they were furthermore integrated in a (waste)water quality evaluation concept developed in the course of the TransRisk project (2.4). In addition, several under-investigated endpoints / MoAs are presently implemented in *in vitro* bioassay based wastewater assessments (Daniels et al. 2019, Escher et al. 2014). These assays amongst others indicated the presence of mostly undetected EDCs (e.g., glucocorticoids or progestogens) calling for further research.

2.3 *In vivo* bioassays for the ecotoxicological assessment of wastewater – the case of *Caenorhabditis elegans*

2.3.1 Development and reproduction as human and environmentally relevant endpoints

C. elegans is a well established DART model in human toxicology (Avila et al. 2011, Baker 2016, Harlow et al. 2016, 2018, Leung et al. 2008, Williams et al. 2017). This includes a high predictive power (Allard et al. 2013, Hunt 2016, Kim et al. 2020, Wittkowski et al. 2019). In this context, Racz et al. (2017) analyzed 31 human reproductive toxicants with 27 also showing DART in *C. elegans*. Harlow et al. (2016) reported on a high positive (89%) and lower negative (25%) predictivity regarding the estimated mammalian activity of 72 pesticides. The low negative predictivity could however be the result of recording only one endpoint (egg viability), whereas incorporating other endpoints may have likely identified further matches. Due to their relevance to humans as well as wild life DART endpoints are integral part of regulatory (and non-regulatory) toxicity assessments (Wernersson et al. 2015, Wilson and Khakouli-Duarte 2009, Wittkowski et al. 2019).

In this light, many DART inducing chemicals, including different aquatic micropollutants, were examined using *C. elegans* (compare below selection):

- The metals aluminium, barium, cadmium, chromium, lead, mercury, nickel, palladium and platinum all exhibited DART in *C. elegans*, as well as excessive concentrations of the essential metals cobalt, copper, iron, manganese, potassium and zinc or the metalloid arsenic (e.g., Avila et al. 2011, Schertzinger et al. 2017, Tejeda-Benitez et al. 2016a, 2016b, Wang et al. 2008a). Thereby *C. elegans* indicated similar sensitivity than *D. magna* and the redworm *Eisenia fetida* regarding several of the above cited metals (Queirós et al. 2019).
- Pesticides represent another relevant group investigated for DART involving various MoAs (Harlow et al. 2016, Tejeda-Benitez and Olivero-Verbel 2016a, table 2). Severe damage often occurred at low ECs such as for chlorpyrifos (at 3−100 µg/L (Roh and Choi 2008, Ruan et al. 2012), fenoxycarb and spirotetramat (at 1.5 and 0.75 µg/L respectively, Xiong et al. 2017), epoxiconazole (LOEC of 0.1 µg/L, Li et al. 2016) or lindane (at 1–100 ng/L, Yu et al. 2020). DART of other pesticides thereby included transgenerational effects

(e.g., Lopes et al. 2008), interference with biochemical signaling pathways (e.g., Li et al. 2016) or cumulative effects (Martin et al. 2009).

- Several PPCPs were reported for DART in *C. elegans* such as EE2 (Höss and Weltje 2007), nicotine (e.g., Smith et al. 2013), piperazine analogs (Racz et al. 2017), the withdrawn anti-obesity drug sibutramine (Aitlhadj and Stürzenbaum 2013), tamoxifen (Höss and Weltje 2007), the anticancer drug 5-fluorouracil (ECs ≥ 0.6 mg/L, Kumar et al. 2010, table 2), the banned weight loss agents clenbuterol and animal feed additive ractopamine (ECs ≥ 0.1–10 µg/L Zhuang et al. 2014), carbamazepine (ECs at [mg/L], Olga Kolychalow, personal communication), the antibiotic sulfamethoxazole (SMX, at environmentally relevant ECs, Yu et al. 2011, Liu et al. 2013, table 2) or the antimicrobial agents triclocarban and triclosan (LOECs of 10–100 µg/L respectively, Lenz et al. 2017, table 2). The ECs of triclocarban and triclosan were thereby similar than to those of *D. renio* embryos (Sreevidya et al. 2018).
- Several environmental hormones and EDCs were positively assessed for DART in *C. elegans.* Albeit *C. elegans* is known not to incorporate an entire endocrine system, several hormonal pathways have been assumed (Höss and Weltje 2007). Related adverse effects were documented for nonylphenol (Cao et al. 2020a), di(2-ethylhexyl)phthalate (DEHP, Roh et al. 2007, Pradhan et al. 2018), its substitute diethyl phthalate (DEP, Pradhan et al. 2018), BPA (Zhou et al. 2016) and its substitute bisphenol S (BPS, Chen et al. 2016, Mersha et al. 2015). García-Espiñeira et al. (2018) showed that 11.4 and 114 µg BPA / L increased body length or brood size. The authors suggested a non-linear-concentration-response-relationship such as established for EDCs in other species. Camacho et al. (2018) further examined an epigenetic mechanism that could have triggered reproductive dysfunctions inherited over five generations (table 2). Mersha et al. (2015) suggested a similar MoA for the structurally related BPS. In this context Chen et al. (2016, 2019) proposed DNA damage (BPS, Chen et al. 2016) and interference with mitochondrial cholesterol transport (BPA, Chen et al. 2019) as possible molecular initiating events (compare AOP concept, 2.3.3).
- Industrial chemicals included 2-(thiocyanomethylthio)-benzothiazol (Allard et al. 2013), different flame retardants (Behl et al. 2016, Liu et al. 2019), octachlorostyrene (Kim and Choung 2009), tributyltin (Cheng et al. 2014) and vinyl chloride (Nam and An 2010). DART was also rated more sensitive than

lethality in assessing the toxicity of PAHs (Leung et al. 2010, Sese et al. 2009). Nonetheless, Ura et al. (2002) computed an 24 h LC_{50} of 50 µg/L for B[a]P. Sese et al. (2009) reported a comparable LC_{50} of 80 µg/L (and an EC_{50} for reproduction of 59 µg/L). The sensitivity of *C. elegans* towards B[a]P was similar to *D. magna* (Sese et al. 2009). Haegerbaeumer et al. (2018) detected an even lower LC_{50} of 12.8 µg/Lafter 48 h exposure of L1 animals to B[a]P. Toxicokinetic experiments with fluoranthene indicated a rapid bioaccumulation and food particles to significantly contribute to the DART of this PAH (Matthai 2009).

In the course of this thesis β-NF was analyzed for DART in *C. elegans* (e.g., figure 10–12). A LOEC of 100 µg/L and an EC_{50} of 140 µg/L was determined for brood size reduction (96 h). These ECs were comparable to previous observations (ECs ≥ 273 µg/L, Leung et al. 2010). Markedly, adult hermaphrodites exposed to high β-NF concentrations exhibited an increased bagging rate (figure 10). Mosser et al. (2011) and several of the cited authors described the Bag phenotype as good indicator of stress. Its feasibility as (eco)toxicological endpoint however remains to be clarified. Regarding the possible MoAs of β-NF not much has been proposed, but it was experimented that β-NF does not bind to the ortholog of the human AhR in *C. elegans* (Powell-Coffman et al. 1998). β-NF and PAHs may thus further be examined within ecotoxicological concepts discussed under 2.3.2–2.3.3.

In this thesis DART was further investigated for groundwater, surface water and wastewater from different WWTP using conventional biological and AWWT processes (Abbas et al. 2018, figure 11 and 17, table 3). The impacts of these samples on reproduction/growth indicated the presence of residual causative agents in WWTP effluents (discussed in further detail in Abbas et al. 2018). Thereby, DART endpoints proved to be beneficial in comparing the effectiveness of different WWTP processes (2.4). These estimations are required for reducing WWTP emissions (such as through WWTP upgrade or centralisation) and for reaching the goals of the WFD (European Parliament and Council 2008, 1.2.2). It also became apparent that chemical target analysis, including several compounds affecting the reproduction and growth of *C. elegans*, did not explain the detected effects (see ‘Online Resource 3’ in Abbas et al. 2018). Chemical analyses should thus be better adjusted to ecotoxicological effects and research questions (e.g., Maier et al. 2016, Neale et al. 2017, 2018). This adjustment should acknowledge (micro)pollutants

known for their presence in wastewater as well as DART in *C. elegans*. Even if their MECs generally occur below their reported EC, cumulative effects may not be excluded in advance. In general, ecosystem complexity and matrix effects that can interfere with bioassay outcomes (e.g., Haitzer et al. 1999, Höss et al. 2001) should be better involved in the assessment and monitoring of (waste)water.

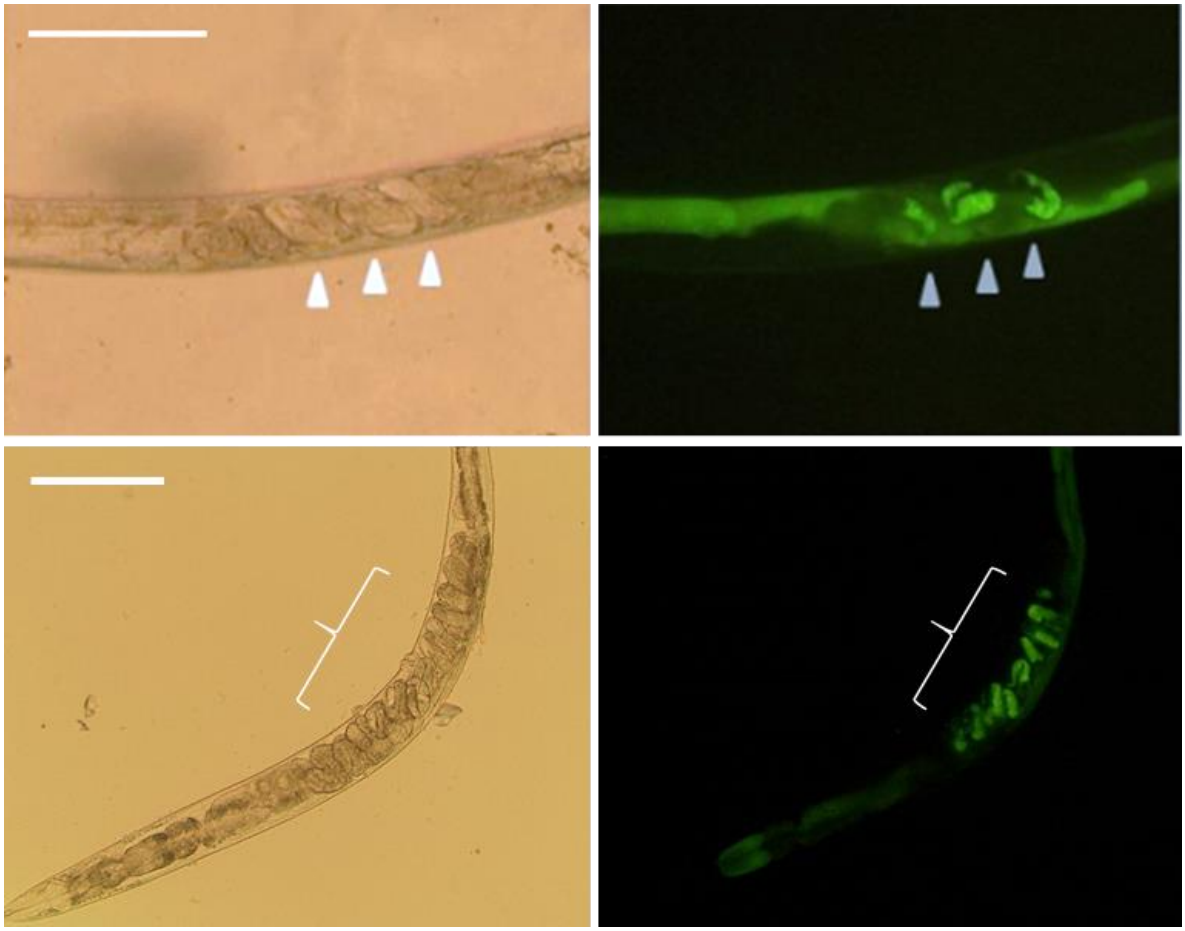


Figure 10. Expression of *cyp-35A3*::GFP in transgenic *C. elegans* of the Bag phenotype. Arrows/Brackets pointing at internally hatching larvae in adult hermaphrodites. Images (left: DIC and right: GFP) recorded after 48 h exposure to β-NF (1 mg/L). Size bar = 100 µm. Unpublished results jointly obtained by L. Valek and A. Abbas.

Hitchcock et al. (1997, 1998) investigated municipal and industrial wastewater using *C. elegans*. The authors concluded that discharges should undergo intensified *in vivo* monitoring as well as source control. Wang et al. (2008, 2010) performed two TIE on a paper recycling mill effluent whereby *C. elegans* was sensitive towards several TIE sample manipulations. McLaggan et al. (2012) used transgenic strains and DART endpoints to study different pollutants (such as PAHs, PCBs, EDCs and metals) extracted from sewage sludge. Wang et al. (2015) adapted the “transgenic

approach” by combining molecular, apical, behavioral and community endpoints to investigate a natural wetland (focusing on nitrogen and phosphorous). In a study of Clavijo et al. (2016) the authors determined that physicochemical and bacteriological water parameters correlated to < 62 % of the observed growth inhibitions (compare ‘immunotoxicology’ under 2.3.3). Kim et al. (2019c) detected DART in model crude oil samples apparently triggering the nucleotide excision repair pathway (table 2, 2.3.3). The authors suggested the PAH C3-naphthalene as major toxicity contributor and validated their results using *D. renio* (Kim et al. 2020). In addition, DART endpoints have been employed to assess the quality of river sediments (e.g., Harris et al. 2020, Höss et al. 2012, Menzel et al. 2009). For several sediment samples Duft (2004) observed a higher sensitivity and feasibility of *C. elegans* over *Chironomus riparius*. However, the author suggested that both assays may complement each other as well as comparisons to other sensitive species in respective bioassay batteries. Soil quality/toxicity has also been assesses using *C. elegans* DART endpoints (e.g., Graves et al. 2005, Höss et al. 2009, 2015, Höss and Römbke 2019, Peredney and Williams 2000, Roh et al. 2007, Wilson and Khakouli-Duarte 2009).

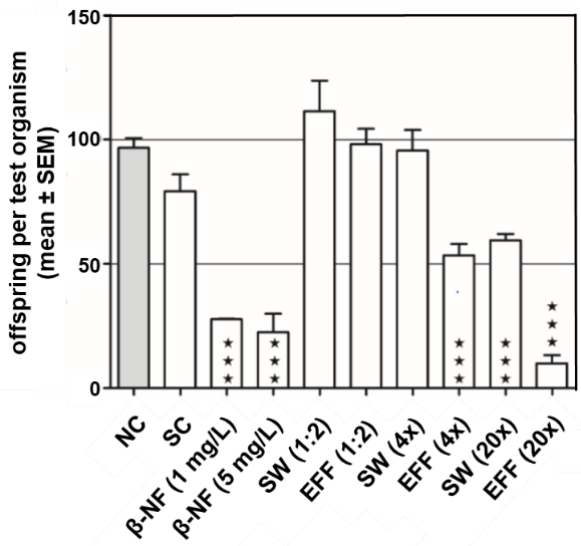


Figure 11. Reproductive toxicity of β-NF (1−5 mg/L), surface water (SW) and WWTP effluent (EFF) samples to *C. elegans*. Reproduction (brood size) expressed as mean offspring number per test organism ± SEM. Aqueous (1:2) and extracted (4−20x) samples tested as 0.5, 4 and 20 fold concentrations respectively. Significant differences (*** $p < 0.001$) tested by 1-way ANOVA against negative control (NC, S-media). Solvent control (SC) = 0.4% DMSO. n = 25−40 per treatment group. Results jointly obtained by L. Valek and A. Abbas.

Based on these results the development and reproduction of *C. elegans* provides sensitive and environmentally relevant endpoints for the assessment and monitoring of different environmental compartments. By implementing thorough experimental designs (such as involving a 'good *C. elegans* culture practice', Hunt 2016), and test strategies, such as combined with chemical analyses (e.g., Abbas et al. 2018, McLaggan et al. 2012, Wang et al. 2008b, 2015) as well as mutant and transgenic strains (compare 2.3.3), hypotheses such as on probable causative agents (Harris et al. 2020) can be tested. DART-related endpoints are furthermore important for cross-references to other species and for high throughput screens (HTS) that are witnessing a growing relevance in (eco)toxicology (Avila et al. 2011, Boyd et al. 2010, 2012, Brooks et al. 2020, Hunt 2016).

2.3.2 CYP-35As as biomarker of (micro)pollutant exposure and contaminated environmental samples

Biomarkers are important tools to indicate the physiological and metabolic state and its modification (Peakall 1994, Walker 1998). Protein expression is a frequent biomarker for intracellular exposures to xenobiotics (Wilson and Khakouli-Duarte 2009, 1.4.3). Effect biomarkers detect (sub)cellular effects such as oxidative stress, DNA damage/repair, apoptosis and/or activation of detoxification (table 2, figure 15). A few biomarkers are intermediates between exposure and effect biomarkers. An example hereof are DNA damage associated genes that are expressed upon reversible and irreversible DNA manipulations. The biological relevance of changes in gene expression, however, mainly depends on the further manifestation of cellular effects and their propagation to higher levels of organization (Feder and Walser 2005, Peakall 1994, Walker 1998). Detoxification pathways in *C. elegans* (and other species) are frequently investigated through CYPs (Gotoh 1998, Harlow et al. 2018, Nelson et al. 1996, 1.4.3). Members of the *C. elegans cyp-35A* subfamily (n = 5) are highly inducible by various xenobiotics such as PAH, PCBs and dioxin-like pollutants (table 1–2, figure 12). Although biotransformation of organic chemicals by CYPs generally initiates their detoxification, they can also contribute to toxicity through bioactivation (e.g., Leung et al. 2010, Schäfer et al. 2009). Accordingly, knockdown/knockout of CYPs often reduced the toxicity of the respective pollutants and/or led to rescued phenotypes (Eom et al. 2014, Harlow et al. 2016, 2018, Jones et al. 2015, Min et al. 2015, Roh and Choi 2011, Schäfer et al. 2009).

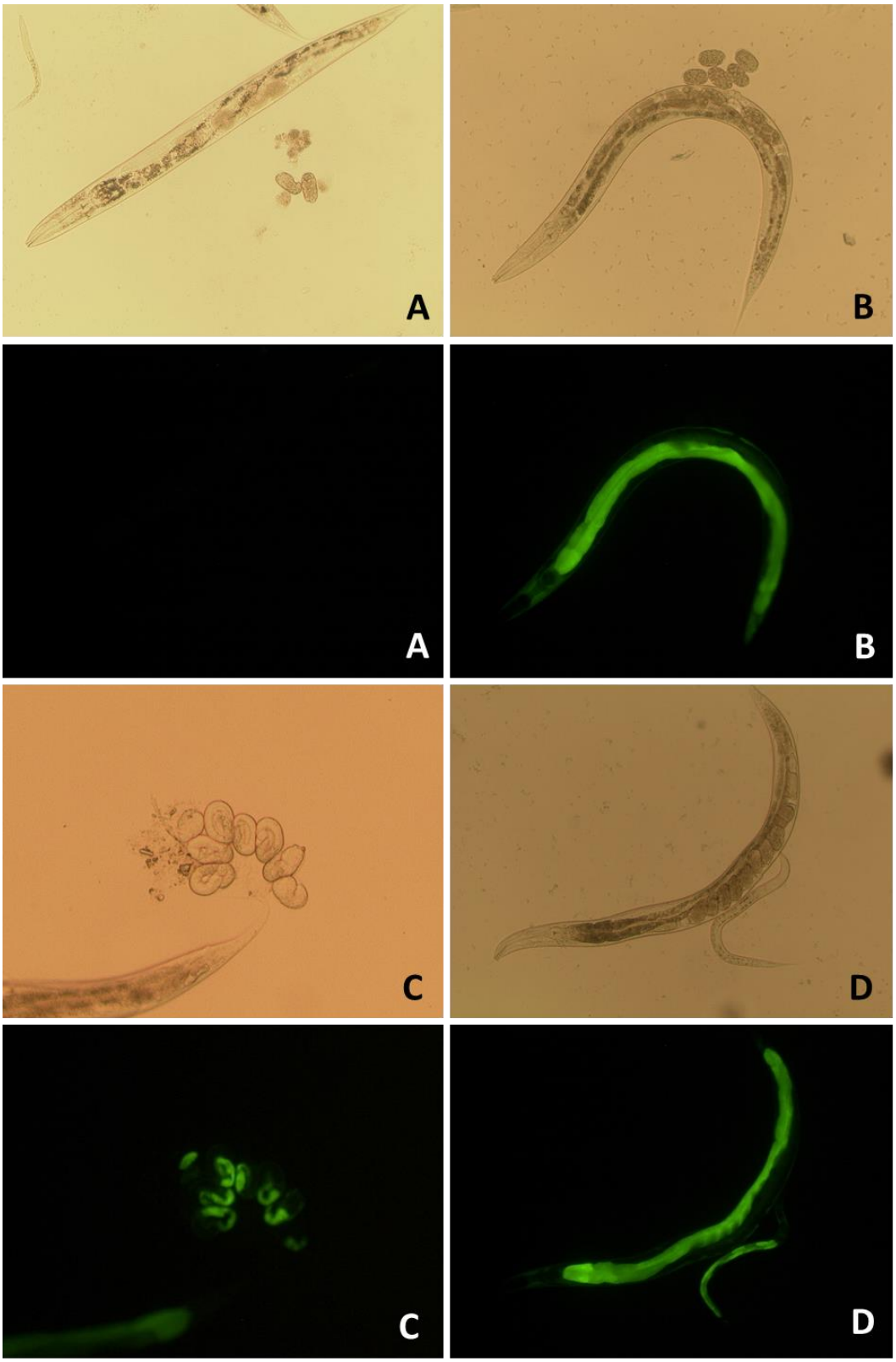
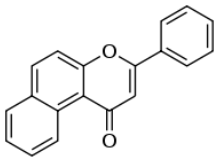
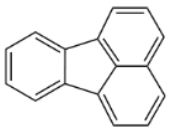
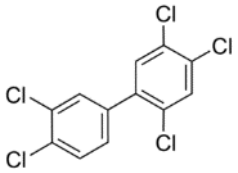
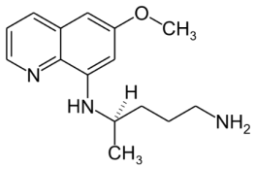
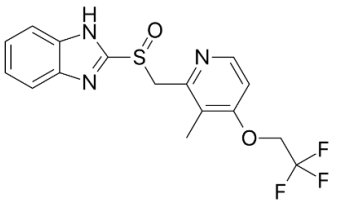
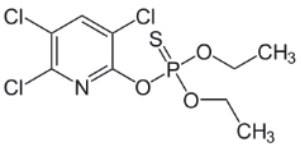
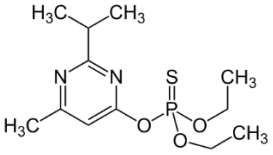
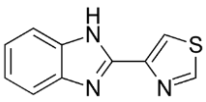
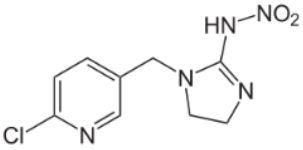
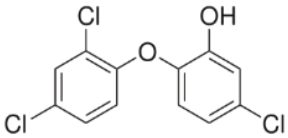
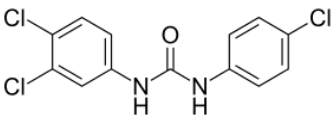
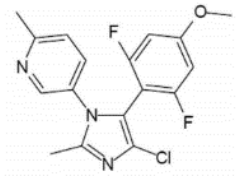
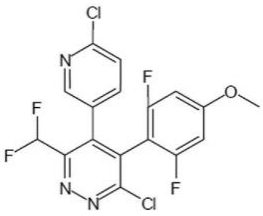
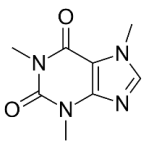


Figure 12. Expression of *cyp-35A3*::GFP in transgenic *C. elegans*. No biomarker induction was detected in unexposed control animals (A) or freshly laid eggs after 24 h of exposure to β-NF (B). A strong GFP signal was captured at the late embryonic (C), larval (D) and adult stage (B–D). Magnification adjusted to the respective developmental stage. DIC = panel one and three. GFP = panel two and four. Results obtained by L. Valek and A. Abbas.

In this thesis *cyp-35A2*::GFP and *cyp-35A3*::GFP served as biomarkers of exposure to the reference PAH β-NF (Menzel et al. 2001, 2007) as well as environmental samples possibly containing *cyp-35A* inducers. β-NF induced both isoforms in a concentration-dependent manner at subacute exposures. For *cyp-35A3*::GFP, maximal expression levels were reached after 8 h, whereas the earliest induction was recorded after 1 h of exposure to 5 mg/L. An earlier detection was methodically unfeasible due to the time for translating and post-modifying the GFP (estimated to 1 h by David et al. 2003). PCR methods could be used to spare this time lag (Valek 2013) and to quantify the expression levels of multiple CYPs in parallel. However, these methods do not allow for microscopic (live) imaging such as performed with the *cyp-35A3*::GFP and other transgenic strains. By fluorescence microscopy a predominant expression location of *cyp-35A3* was confirmed for the intestine (figure 12 and Abbas et al. 2018) similar to the observation by Menzel et al. (2007). The *C. elegans* intestine is its primary detoxification organ and also the main exposure route for various chemicals and ingested particles (Offermann et al. 2009, Wilson and Khakouli-Duarte 2009, Stylianou et al. 2018). Based on other studies overlapping substrates and substituting roles for *cyp-35A* subfamily members were suggested (Harlow et al. 2018, Inokuchi et al. 2014, Jones et al. 2015, Roh et al. 2014). This highlights the importance of detecting multiple CYP isoforms in ecotoxicogenomic studies. The induction of *cyp-35A3*::GFP was furthermore tracked throughout different developmental stages (figure 12). This result was in line with previous observations by Menzel et al. (2007). Interestingly, *cyp-35A3*::GFP induction was also found in internally hatching larvae of the Bag phenotype (figure 10). In the literature *cyp-35A3* inducers were mostly tested at concentrations in the milligram per liter range (table 1). A few studies also analyzed concentration response relationships for CYP inducing compounds such as β-NF (Menzel et al. 2002). Therein, Menzel et al. (2002) reported on a LOEC 0.1 µg β-NF / L for *cyp-35A3* induction. In comparison, an EC_{50} (8−24 h) of 72−79 µg/L β-NF was determined in this thesis. CYP induction concentrations in the microgram per liter range were also detected for triclocarban (> 170 µg/L, Inokuchi et al. 2014) and chlorpyrifos (60−300 µg/L, 2−8 h, Roh et al. 2016) speaking for the sensitivity of this biomarker. Similar to β-NF (DART at > 100 µg/L), the low induction concentrations of triclocarban conincided with high DART. In case of chlorpyrifos *cyp-35A3* induction concentrations even came close to the LC_{50}(24 h) of 300 µg/L. In addition,

prolonged exposure (96 h) at 10−100 µg/L was concomitant with severe developmental and reproductive defects (Roh and Choi 2008). Similar ECs of DART and *cyp-35A3* induction might indicate its involvement in bioactivation (compare putative toxicodynamic and AOP concepts discussed in figure 15 and under 2.3.3).

Table 1. Selected *C. elegans cyp-35A3* inducers in the scientific literature (reference given in brackets). Two out of four inducers reported by Harlow et al. (2016) are shown. 3D-models of compounds available at PubChem (Kim et al. 2016) and other databases

Selected *cyp-35A3* inducers		
 β-NF (PAH) (Menzel et al. 2001, 2007)	 fluoranthene (PAH) (Menzel et al. 2001)	 PCB52 (Menzel et al. 2001)
 (R)-primaquine (Menzel et al. 2001)	 lansoprazole (Menzel et al. 2001)	 benzene (Eom et al. 2014)
 chlorpyrifos (Roh et al. 2014, 2016)	 diazinon (Vinuela et al. 2010)	 thiabendazole (Jones et al. 2013, 2015)
 imidacloprid (Jones et al. 2013)	 triclosan (Inokuchi et al. 2014)	 triclocarban (Inokuchi et al. 2014)
 imidazole fungicide (Harlow et al. 2016)	 pyridazine fungicide (Harlow et al. 2016)	 caffeine (Min et al. 2015)

Biomarkers of exposure represent relevant tools for toxicokinetic analyses (Offermann et al. 2009, Roh et al. 2016, Stylianou et al. 2018, table 2, figure 13). Toxicokinetic information such as determining bioavailable concentrations of (micro)pollutants are important such as for defining toxicity thresholds in ERA. Bioavailable concentrations also depend on the degradation and detoxification rates of the respective compounds (e.g., Roh et al. 2016, Spann et al. 2015). In this thesis it was hypothesized that decreasing internal concentrations of β-NF may coincide with a down-regulation of *cyp-35A3::*GFP. The down-regulation of *cyp-35A3::*GFP apparently depends on various exogenous factors (e.g., nominal β-NF concentrations and exposure duration/intervals) and internal conditions (e.g., exposure routes or bioaccumulation behavior). Although in the conducted experiments internal concentrations were not directly measured (e.g., compare Chen et al. 2016 for BPA/BPS), it was found that *cyp-35A3*::GFP levels gradually decreased over 72 h after β-NF exposure at 1 mg/L for 24 h (figure 13). In perspective, such experiments will have to be complemented by examining the above mentioned exogenous and endogenous factors. The potential effect of these parameters on downstream effects such as DART are thereby of particular interest. Moreover, the degradation kinetics of GFP itself may have to be involved such as by comparing reports on the half-life of the fluorescence protein (e.g., 26 h, Corish and Tyler-Smith 1999) with the present conditions.

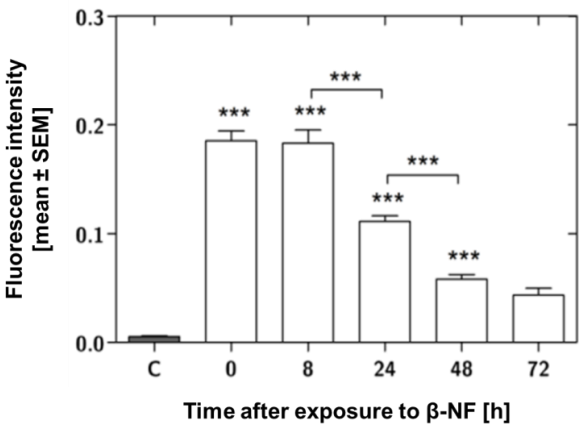


Figure 13. Down-regulation (0−72 h) of *cyp-35A3*::GFP in transgenic *C. elegans* after 24 h exposure to 1 mg β-NF/L. Negative control (C) = unexposed animals. Significant differences tested against C (if not noted elsewise) as described by Valek (2013)

Biomarkers are furthermore used for assessing environmental samples (table 2). Ecotoxicogenomic studies involving *cyp-35A*s were for instance performed on

contaminated surface water (Kumar et al. 2015), river sediments (Menzel et al. 2009) and soils (Anbalagan et al. 2012, 2013, Roh et al. 2007). Thereby, different (clusters of) up- or downregulated genes correlated to pollution levels and/or DART. In this thesis, one biologically treated WWTP effluent and corresponding ozonated wastewater (analysed in this thesis) significantly induced *cyp-35A3*::GFP (table 3–4 and Abbas et al. 2018). Because the ozonated WWTP effluent induced *cyp-35A3*::GFP to higher extend a possible explanation was given in the context of oxidative TPs (such as corroborated for other aquatic species, da Costa et al. 2014, Stalter et al. 2010, Magdeburg et al. 2012, Giebner et al. 2018). The presumed TPs might thereby either resemble newly generated and/or previously contained *cyp-35A3* inducers with higher bioavailability/potency. The causative agents for this effect could not be evaluated, because, except for caffeine, none of the *cyp-35A3* inducers cited in table 1 was included in the chemical target monitoring of this thesis. In addition, the MEC of caffeine (see 'Online Resource 3' in Abbas et al. 2018) ranged far below its reported induction concentration (Min et al. 2015). This was also found regarding the concentrations of other wastewater-borne *cyp-35A3* inducers in table 1 (e.g., Forsgren 2015). The MECs of chlorpyrifos and triclocarban (< 0.52 µg/L, Norberg-King 2001 and 0.17 µg/L, Heidler et al. 2006, respectively) were approximately 100 fold lower than their ECs (60−300 µg/L, Roh et al. 2016 and > 170 µg/L, Inokuchi et al. 2014, respectively). Therefore, hypotheses on potential cumulative effects of unknown and/or low concentrated *cyp-35A3* inducers as well as particle bound (micro)pollutants (such as based on their log K_{ow} values) were discussed (Abbas et al. 2018). These findings further highlighted the need to carefully and case-specifically amend chemical (target) and ecotoxicological analyses (e.g., Chakrabarti et al. 2015, Neale et al. 2018, Tang et al. 2014).

For further establishing CYPs as biomarkers the identification of their inducers and substrate spectra should go in hand with research on their intra- and interspecies relationships. Such information can for instance be obtained from gene ontology studies, by quantitative structure activity relationships (QSAR, e.g., Escher and Hermens 2002, Ristau et al. 2015) and other (experimental) approaches (e.g., Harlow et al. 2018). Due to the evolutionary conservation of CYPs many of their biological functions and toxicological responses (highly) correlate between species. This conservation is apparent at the genetic level. According to a BLAST research

cyp-35A3 (ceCYP-35A3) shares 100% DNA sequence homology with CYPs orthologs in three other nematodes (*Caenorhabditis brenneri, Caenorhabditis briggsae* and *Caenorhabditis remanei*), but also with one CYP in the flower *Camellia japonica* (Ensembl 2018, Wormbase 2018). The human ortholog *cyp-2C8* (hCYP-2C8) displays 93.7% sequence homology with ceCYP-35A3, which is higher than for the *Drosophila melanogaster* ortholog (*cyp18a1-PB*, 90.7%). Functional homologies were further ascribed to human CYP-2J2, -2R1 and -2U1 using the 'Protein Analysis Through Evolutionary Relationships Classification System' (Mi et al. 2013). However, the human CYP1 class and CYP1-like metabolism is apparently missing in *C. elegans*. Nonetheless, *C. elegans* shares a variety of CYP-based metabolites with humans (Harlow et al. 2018). Similar to other proteins, structures of CYPs have been modelled and reconstructed from crystal structures (Totah and Rettie 2005). From this data *in silico* methods can generate 3D-models (e.g., figure 14) which are used to compute binding affinities of experimented and probable substrates (Marcus Weber, personal communication). No model of ceCYP-35A3 seemed presently available in the literature.

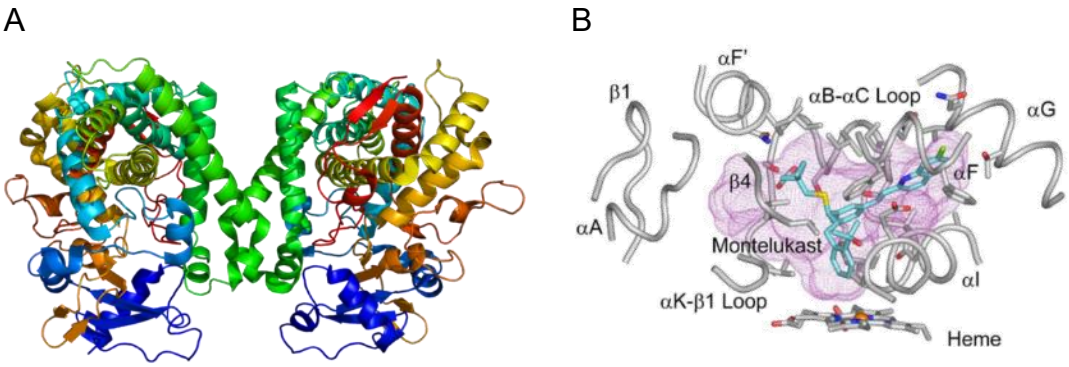


Figure 14. *In silico* models of hCYP-2C8 (human ceCYP-35A3 ortholog). Ribbon diagram (A) of its asymmetric unit wherein colors represent different domains (Berman et al. 2000). Stereo-image (B) of the active site (Johnson and Stout 2005) including exemplary substrate (center) and heme cofactor (bottom).

Substrate spectra of CYPs can include structurally diverse compounds (e.g., Johnson and Stout 2005, table 1). Markedly, for *cyp-35A2* Anbalagan et al. (2012) even reported on an apparent induction by heavy metals. It however remained to be clarified, if this induction was associated with real catalytic, (co-)regulative or unspecific toxicity. Metabolic activities of hCYP-2C8 (ceCYP-35A3 ortholog) have widely been investigated for various drugs and toxicants (e.g., Backman et al. 2016,

Johnson and Stout 2005, Totah and Rettie 2005). hCYP-2C8 is highly expressed in the liver also representing a main detoxification organ (Backman et al. 2016). Different natural compounds are known hCYP-2C8 substrates/inducers such as certain terpenes, the natural flavone eupatilin (structurally related to β-NF) or ethanol. They also comprise caffeine and lansoprazole (Backman et al. 2016), which have been reported as ceCYP-35A3 inducers (table 1), as well as chlorpyrifos (Abass et al. 2009). No literature was found on β-NF, fluoranthene or PCB52 as hCYP-2C8 effectors. From the ethoxyresorufin-O-deethylase (EROD) assay it is however known that β-NF, several other PAH and dioxin-like toxicants induce hCYP-1A1 in fish (e.g., Kais et al. 2018, Maier et al. 2016, Mohammadi-Bardbori 2014, table 4). β-NF is described to be targeted by hCYP-1A2 (Chakrabarti et al. 2008) which also metabolizes caffeine (Omiecinski et al. 1999). The fact that homologous and non-homologous CYPs (such as hCYP-1A1 and ceCYP-35A3) can have overlapping substrate spectra further illustrates their evolutionary relationships and resulting complex metabolic interplay. Future research will further elaborate the usefulness of CYPs as human and environmental biomarkers and their integration into state of the art ecotoxicogenomic investigations (Chakrapani et al. 2008, Fajardo et al. 2020, Harlow et al. 2016, Inokuchi et al. 2014, Min et al. 2015, 2.3.3).

2.3.3 Functional ecotoxicogenomics, mechanistic ecotoxicology and novel endpoints for (micro)pollutant and (waste)water quality assessment

A growing number of mechanistic studies are carried out with *C. elegans* deciphering (eco)toxicological responses to anthropogenic and natural chemicals as well as contaminated environmental samples. The complex interplay of these responses are often studied by *omic* approaches (referring to the genome, proteome and metabolome) to detect and describe the involved physiological processes and toxicological pathways. Genome wide expression screens have been established in ecotoxicogenomics to profile the entirety of up- or down-regulated genes (compare 2.3.2). In general, these screens base on the assumption that chemicals with shared MoA (repeatedly) induce related expression patterns that can be contrasted with homeostatic conditions and other types of environmental stressors. A main aim is to transfer this information to predictive ecotoxicology (e.g., Brooks et al. 2020, Steinberg et al. 2008, Wilson and Khakouli-Duarte 2009). Furthermore, genomic

and biomarker assessments may provide higher sensitivity and pace than apical and life cycle parameters in reflecting pollutant induced changes. This facilitates more detailed comparisons of acute versus chronic exposures and resulting effects (e.g., Alda Álvarez et al. 2006, Cui et al. 2007, Fajardo et al. 2020, Ruan et al. 2009).

Global gene expression patterns upon exposures to wastewater-borne (micro)pollutants were studied in the context of environmental hormones (17β-estradiol and progesterone, Custodia et al. 2001), the PAHs β-NF and fluoranthene, the herbicide atrazine, the pharmaceutical clofibrate, the EDCs diethylstilbestrol (Reichert and Menzel 2005) and DEHP (Roh et al. 2007), PCB52 (Menzel et al. 2007), the industrial chemical octachlorostyrene (Kim and Choung 2009), the synthetic polycyclic musks 7-acetyl-1,1,3,4,4,6-hexamethyl-1,2,3,4-tetrahydro naphthalene (AHTN) and 1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethylcyclopenta-γ-2-benzopyran (HHCB, Mori et al. 2007), heavy metals (Cd, Hg and Pb with detection thresholds as low as at 48−82 ng/L, Tominaga et al. 2008, Cui et al. 2007), the organophosphate pesticide dichlorvos (Lewis et al. 2013), benzene (Eom et al. 2014, table 2) and caffeine (Min et al. 2015, table 2). Many of these genome wide screens suggested an involvement of CYPs (including the *cyp-35A* subfamily) in the respective toxicological/metabolic responses/pathways (Custodia et al. 2001, Roh et al. 2007, Schäfer et al. 2009). Kim and Choung (2009) detected transgenerational alterations of gene expression after exposure of the parental generation. Lewis et al. (2009) moreover observed a relatively rapid recovery of overall gene expression (< 26 h) after subacute exposures (2 or 8 h) to dichlorvos. When Reichert and Menzel (2005) tested several wastewater-borne pollutants (in the milligram per liter range) the number of induced (n ≤ 203 at 2−42 fold higher than the control) or repressed (n ≤ 153 at 2−7 fold lower than the control) genes followed the order fluoranthene > clofibrate > atrazine > β-NF > diethylstilbestrol or atrazine > fluoranthene > diethylstilbestrol > β-NF > clofibrate respectively. β-NF induced 40 genes in total including carboxylesterases, collagen genes, CYPs (with β-NF as strongest inducer), cytochrome b5, GST-20, UGTs, 11 genes belonging to different metabolic pathways (such as the metallothionein *mtl-1*) and 15 unknown genes. Two β-NF down-regulated genes were detected including C-type lectin (related to immune defense). Overall, these and earlier experiments (Menzel et al. 2001) supported the described role of these genes in the xenobiotic defense of *C. elegans*.

Natural compounds have also been examined. Humic substances were for instance investigated using a surface water reverse osmosis isolate (NOM concentration of 6.4 mg/L) and the synthetic humic-like substance HS1500 (at 7.6 mg/L) wherein CYPs and various other genes were found to be differentially expressed. Albeit potential MoAs of humic substances remained unclear, screening natural compounds is particularly important for investigating environmental samples. Regarding the latter, Menzel et al. (2009) examined surface water sediments that exhibited different contamination degrees (Elbe > Rhine > Danube) with heavy metals, PAHs, PCBs and other POPs (such as octachlorostyrene) resembling the main representatives. All samples induced genes involved in certain metabolic processes, while a few samples also showed DART (using 25−50% pore water), *in vitro* genotoxicity and estrogenicity, that in many cases correlated to the respective gene expression profiles. PAH contaminated soils (after remediation) were also screened in a study by Fajardo et al. (2020), who also found growth, reproduction and survival to be significantly impacted.

In general, several methodical aspects have to be considered when performing and evaluating genomic screens (that apply to the entire field of ecotoxicogenomics):

- Robust design (matched with previous data and experimental standards).
- Data normalization (respecting baseline expression and methodical LOQs).
- Inclusion of multivariate statistics (as foundation for gene ontology approaches).
- Functional validation of results (by follow up experiments on downstream effects ruling out unspecific responses / false positives, compare below).

Furthermore, a number of biological/ecotoxicological challenges lie ahead in further establishing genomic screens as robust assessment tools and reliable prediction instruments for (defined and complex/dynamic) environmental stressors such as:

- Detailed comparisons of regular and irregular expression patterns (based on standardized maps/databases such as Davis et al. 2017, EBI 2018, Howe et al. 2017 and Kim et al. 2001). Respective data sets should enable the differentiation between responses such as specific versus unspecific or natural versus (micro)pollutant induced. Accordingly, they may comprise suitable PCs and/or interrogation of relevant ontogenetic/population and environmental factors that could cause variation in expression patterns/levels (e.g., Steinberg et al. 2008, Escher et al. 2017, Roh et al. 2016).

- Comprehensive involvement of multiple regulation mechanisms on the DNA, transcriptional, RNA, translational or protein level (such as epigenetics or miRNAs, e.g., Camacho et al. 2018, Lundby et al. 2016, Weinhouse et al. 2018, Zhao et al. 2016) that could be impeded by xenobiotic exposure (Reinke et al. 2013, Steinberg et al. 2008, Taki et al. 2014), while escaping detection in regular genomic screens.
- Cross references to aquatic organisms such as *Daphnia* or fish species (e.g., Colbourne et al. 2015, Harris et al. 2020, Kim et al. 2019c, 2020, Steinberg et al. 2008).

Various “non-genome wide” screens have been carried out focusing on individual or multiple genes in *C. elegans*, while often applying DNA/RNA sequencing and/or the respective mutant/transgenic strains (e.g., Cong et al. 2020, Mendelski et al. 2019). Due to their reduced methodical effort compared to whole genome assays, these studies generally incorporate an extended set of investigated endpoints (compare table 2 below). The systematic selection of potentially affected endpoints is crucial for ruling out (false) negative results and for examining “downstream” (adverse) effects at higher organizational levels (Harlow et al. 2016). Up to present, mechanistic investigations with *C. elegans* illustrated how biomarker and genomic screens (molecular endpoints) combined with other endpoints (such as biochemical, metabolic, neurological, behavioral or physiological) can be powerful tools to characterize potential MoAs of (micro)pollutants as well as environmental samples.

Table 2. Selected (non-genome wide) studies on effects and apparent MoAs of environmental chemicals/samples in *C. elegans*. Studies using multiple endpoints and biomarkers were listed chronologically (latest on top). Certain chemicals were cited by their abbreviation (as given in text) or summarized by pollutant classes (e.g., pesticides). Arrows ("↑" and "↓") indicate significantly up-/down-regulated genes or stimulation/inhibition of the respective endpoint, while delta symbol ("Δ") indicates a relevant/significant alteration. Environmental relevance was estimated regarding selected compartments such as soil, groundwater (GW), surface water (SW), sediments, wastewater including industrial/hospital wastewater (WW), marine waters (MW) or drinking water (DW) as well as specific fields of ecotoxicology (excepting human toxicology).

Compound / sample type	Investigated endpoints	Toxicity mechanism / MoA	Specific remarks	Aquatic relevance	Reference
Present study:					
β-NF	Apical (brood size ↓) and molecular (*cyp-35A2* ↑, *cyp-35A3* ↑)	Involvement of CYPs in the reproductive toxicity of β-NF	Particulates assumed as main exposure route	WW, TSS, sediments	Abbas et al. 2018 and 2.3
EFF-1	Apical (brood size ↓)	Effects unexplained by chemical target analysis. Putative cumulative effects and potential role of TSS.	Chemical target analyses (n = 111)	WW, SW, TSS	
EFF-4	Molecular (*cyp-35A3* ↑)			WW, SW, TSS	
EFF+O_3	Apical (growth ↑, insignificant) and molecular (*cyp-35A3* ↑)			Oxidative TPs, AWWT	
(Micro)pollutant studies:					
Chlorpyrifos (compare below) **and its TPs**	Apical (DART↑), behavioral (locomotion Δ), cellular (neuronal integrity ↓), biochemical (ROS ↑), molecular (*tph-1*::GFP↑)	Chlorpyrifos (20–50 µg/L) and generated TPs (0–10h) triggering multiple (neuro-)toxicity pathways	Higher toxicity of proposed photolytic TPs than non-irradiated standard solution	SW, WW, TPs	Cao et al. (2020b)
Lindane	Apical (DART↑), behavioral (locomotion Δ), intestinal membrane permeability↑, biochemical (ROS ↑), molecular (*sod-5* ↑, *isp-1* ↑, *mtm-6* ↓, *opt-2* ↓)	Significant correlation of oxidative stress response and intestinal membrane permeability with apical effects	LOECs ≥ 1 ng/L	Soil, SW, GW	Yu et al. (2020)

Table 2 (continued)

Tetrabromo-BPA	Apical (DART↑), behavioral (locomotion∆), neurotoxicity (neuronal damage), biochemical (ROS), molecular (20 genes incl. *cyp-35A2*)	Trans-generational neurotoxicity, whereby gene expression was more altered at low test concentrations	Locomotion and gene expression affected in G_1 and G_2 (1–100 µg/L)	WW, soil	Liu et al. (2020)
		Pulsed exposure at environmentally relevant concentrations triggered higher toxicity as well as adaption	LOECs at 1–10 µg/L		Liu et al. (2019)
BPA (compare below)	Apical (DART↑), cellular (germline nuclear loss↑, apoptosis↑), biochemical (intracellular↓ and mitochondrial↓ cholesterol level)	Apparent interference of BPA (at [µg/L]) with mitochondrial cholesterol transport as mediated by steroidogenic acute regulatory protein	Rescue phenotypes by mutational analysis and potential endocrine effects of cholesterol	WW, SW	Chen et al. (2019)
Methampheta-mine, ketamine	Apical (DART↑), behavioral ∆, biochemical (neurotransmitter ∆), molecular (oxidative stress related genes ↑)	Behavioral impairment (≥ 50 ng/L) reflected in altered neurotransmitter content and probable association of oxidative stress response with DART	ECs at MECs incl. risk estimation	WW, SW	Wang et al. (2019)
Hexabromo-cyclododecane (flame retardant)	Apical (DART↑), behavioral ∆, biochemical (ROS↑), cellular (apoptosis↑), molecular (16 genes ∆ incl. *cyp-35A2* ↑)	Apparent protective role of *sod-3* and *cep-1* as well as N-acetyl-L-cysteine / ascorbate against supposedly ROS derived downstream effects	Detection of altered gene expression at a LOEC of 1.28 µg/L	WW, SW, soil	Wang et al. 2018
Triclosan, triclocarban (compare below and Sreevidya et al. 2018)	Apical (DART↑), behavioral (locomotion ∆), biochemical (ROS↑) and molecular (*daf-16*::GFP↑)	Metabolic shifts in carbohydrates, amino acids (energy budget) and tyrosine, serine, polyamines (neurotransmitter / stress response)	Several phenotypes could be affected by triclosan exposure	WW, SW	Kim et al. (2019b)
	Apical (lethality ↑, DART ↑, lifespan ↓), biochemical (oxidative stress) and molecular (*daf-16, xol-1*)	*daf-16*::GFP nuclear relocalization and *xol-1*::GFP confirming oxidative stress response and DART (respectively)	Authors suggest further research on endocrine disrupting potential		Lenz et al. (2017)

Table 2 (continued)

Chlorpyrifos	Molecular (*cyp-35A2−3*) and biochemical (AChE ↓). Study includes summary on previously investigated endpoints in the literature.	Metabolisation by CYPs and AChE inhibition by chlorpyrifos bio-TP. Internal concentration and time dependencies	Involves toxicokinetic parameters and realistic/low exposure concentrations	Soil, SW, GW	Roh et al. (2016, 2014)
72 pesticides	Apical (egg viability/hatching ↓) and molecular (CYPs ↑)	DART in relation to humans. Functional involvement of CYPs	Comparison of positive/negative predictive power (incl. RNAi)	Soil, SW, GW	Harlow et al. (2016), also see table 1
BPA	Apical (growth ↓, reproduction ↓), behavioral (locomotion ∆), , cellular (apoptosis ↑), biochemical (lipofuscin accumulation, ROS production), molecular (incl. *cyp-35A2* ↑)	Cell apoptosis leading to DART and neurobehavioral toxicities	LOECs in the lower microgram per liter range	WW, SW	Zhou et al. (2016)
Caffeine	Apical (brood size ↓, embryonic lethality ↑, larval arrests ↑) and molecular (global gene expression ↑↓)	Toxicological role of CYPs with focus on *cyp-35As* (confirmed by RNAi)	Readily degradable by biological WWT	WW	Min et al. (2015), also see table 1
Thiabendazole	Apical (egg number ↓) and molecular (CYP activation by NHR-176)	Potential involvement of bio-TPs in reproductive toxicity	Classification of transcriptional regulators	Chemical analysis (involving TPs)	Jones et al. (2013, 2015), also see table 1
NDMA, dibromoacetic acid	Apical (life span ↑, reproduction, body size ↑, thermal stress resistance), biochemical (oxidative capacity, lipid peroxidation) and molecular (17 genes incl. *cyp-35A2* ↓)	Protective stress response or energy relocation	Low dose experiment	WW, SW	Babersch ke et al. (2014)
Triclosan, triclocarban (compare above and Sreevidya et al. 2018)	Apical (DART ↑) and molecular (79 CYPs)	19 (triclosan, 5 mg/L) and 10 (triclocarban, 0.17 mg/L) up-regulated CYPs potentially involved in the observed DART	Utilization of a DNA microarray chip customized for CYPs	WW, SW	Inokuchi et al. (2014)

Table 2 (continued)

Benzene	Apical (survival, reproduction ↓), behavioral (locomotion Δ) and molecular (global gene expression ↑↓ incl. *cyp-35A*s)	Distinct roles of *cyp-35A2* and *cep-1* in benzene-induced behavioral responses and reproductive toxicity	Computational behavior analysis as integrative evaluation tool	WW, DW	Eom et al. (2014), also see table 1
Clenbuterol, ractopamine	Apical (DART ↑), behavior (locomotion Δ), biochemical (autofluorescence ↑, ROS ↑), molecular (gene expression ↑↓)	Insulin/IGF influenced life span reduction. Decreased ROS and DART by *sod-2* overexpression	Acute and prolonged exposure. Low dose effects (e.g., 10 µg/L)	WW (depending on concen-tration)	Zhuang et al. (2014)
SMX	Apical (reproduction ↓, growth ↑, life span ↑), behavioral (thermal resistance, pharynxal pumping ↑), biochemical (lipid peroxidation ↑), molecular (16 genes ↑↓)	Lipid peroxidation and up-regulated *hsp-16.1* indicated oxidative stress; possibly associated with mitohormesis affecting life span and pumping frequency affecting growth	Explanation on bacterivorous *C. elegans* as *r*-strategists	WW, SW, Soil	Liu et al. (2013)
Nicotine	Behavior (nicotine preference ↑, locomotion), biochemical (gustatory plasticity ↓), molecular (*bas-1, cat-2, lev-1, tph-1 unc-29*)	Suggested essential role for serotonin signaling pathway	Mutant analysis after acute pre-exposure and new locomotion assay	WW (depending on concentration)	Matsuura et al. (2013)
Fenitrothion	Apical (DART ↑), behavioral (immobility ↑), biochemical (AChE activity ↓), molecular (*cyp-35A2* ↑)	Role for *cyp-35A2* in the observed toxic effects suggested by RNAi and mutant analysis	Quantification of fenitrothion degradation in exposure medium	SW (depending on concentration)	Roh and Choi (2011)
5-fluorouracil	Apical (DART ↑), cellular (apoptosis ↑, cell cycle arrest ↑), molecular (*lin-29*, *ung-1*)	Cell cycle arrests followed by germline apoptosis. Repression of *lin-29* transcription factor related to vulval development and egg laying	Partially diminished effects on embryo hatching upon knockdown of *ung-1*	WW (depending on concentration)	Kumar et al. (2010)
Five pesticides	Apical (DART ↑) and behavioral (locomotion Δ)	Neurotoxic pesticides affected locomotion, while pesticides targeting insect growth diminished *C. elegans* reproduction	Comparison of acute versus chronic exposure (sensitivity 24 h > 72 h)	Soil, SW, GW	Ruan et al. (2009)
Levamisole (pesticide)	Population (males Δ), apical (survival ↓↑, fecundity ↓↑), metabolic (adaption costs)	Multi-generational (n = 20) population/apical effects as potential result of reduced motility (encounters↓ thus outcrossing rate↓)	Investigation of rapid adaption and ecological relevance	Soil, SW, GW	Lopes et al. (2008)

Table 2 (continued)

Environmental studies:					
Sediment extracts from superfund site	Apical (DART↑), biochemical (ATP level↓), EROD activity (bacteria and transgenic strain) ↑	Basal zebrafish *cyp-1A* expression, PAH-biotransformation, downstream effects in transgenic *C. elegans*	Cross-species method indicating protective role of *cyp-1A*	SW, GW, sediments	Harris et al. (2020)
Acidic and basic culturing environment	Apical (DART↑) and molecular (transcriptome Δ)	Activation of cuticle synthesis, structure-related (pH = 4.33) and xenobiotic defense (pH < 4.33) genes	Evaluation of potential climate change driven acidification effects	MW, SW, soils (through acid rain)	Cong et al. (2020)
Micro-polystyrene particles	Apical (lifespan↓), behavioral (defecation Δ), cellular (motor neuron GFP reporter Δ), molecular (biomarker including *skn-1* Δ)	Activation of xenobiotic defense and defecation pathways (ultimately impacting AVL and DVB neurons as well as life span)	Particle size (1–5 µm) and concentration (~10^7–10^{10} particles/m^2) dependency	SW, MW, WW	Shang et al. (2020)
High-density polyethylene particles	Apical (DART↑), molecular (pathway-specific biomarker Δ)	Involvement of nucleotide excision repair and TGF-β signaling in toxicity pathway and proposition of two AOPs	Correlation to zebrafish (experimental) and humans (*in silico*)	SW, MW, WW	Kim et al. (2020)
Nano-polystyrene particles	Apical (DART↑), behavioral (locomotion↓), biochemical (ROS↑), metabolic markers Δ	Perturbation of energy budget related metabolites and ROS production suggests relation to DART/locomotion	Correlation of results with particle properties and uptake kinetics	SW, MW, WW	Kim et al. (2019a)
Model crude oil from spill event (water soluble fraction)	Apical (reproduction↓) and molecular (selected NER pathway genes↑)	Activation of NER pathway and identification of the PAH C3-naphthalene as main toxicity contributor (confirmed by RNAi)	Passive dosing method, validation of results in *D. renio* and comparison to human blood biomarker	MW, soil, GW (e.g., if similarly polluted)	Kim et al. (2019c)
Bile acid metabolites of synthetic and natural origin	Apical (DART↑), behavioral (locomotion Δ), molecular (transcriptome Δ, KO-mutants Δ)	Potential endocrine effects of a bacterial manure-borne bile acid metabolite could be mediated by putative androgen receptor NHR-69	Model system with *Pseudomonas* and metabolisation in soil/sand microcosm	Soil, SW (e.g., if affected by agricultural manure)	Mendelski et al. (2019)
Metal contaminated soil leachate	Apical (lethality, life span↓), molecular (33 genes incl. *cyp-35A2* ↑), metabolism (fatty acid content ↑)	Stress response and impacted fat metabolism with chronic downstream effects on life span	Metal and PCA to classify water soluble and bioavailable fraction	Soil, GW	Rai et al. (2019)

Table 2 (continued)

Surface water upstream of a large river dam	Apical (lifespan, growth, intestinal permeability ↑), behavior (locomotion ↓), biochemical (ROS ↑), molecular (*sod-2* ↑, *sod-5* ↑, *clk-1* ↑, *mev-1* ↓)	Involvement of *sod-2/-5* in ROS associated (intestinal/behavioral) toxicity of a backwater sample (suggested by mutational analysis)	Aqueous and particulate phase of samples induced comparable toxicity	SW, (GW), DW, TSS	Xiao et al. (2018)
River sediments (aqueous and solvent extracts)	Apical (survival ↓, growth ↓), behavior (locomotion, body bends Δ), molecular (*cyp-34A9* ↑, *gst-1* ↑, *gpx-1* ↑, *hsp-6* ↑, *hsp-16.2* ↑, *hsp-70* ↑, *mlt-1* ↑, *mlt-2* ↑, *sod-1* ↑, *sod-4* ↑)	Toxic profiles for different samples	Effect correlation to heavy metal and/or PAH content (thresholds and PCA analysis)	Sediments, SW, DW, WW	Tejeda-Benitez et al. (2016b, 2018)
Soil samples near wetlands and farmlands	Community analysis (Δ), apical (mortality ↑, reproduction ↓), molecular (*hsp-70* ↓, *hsp-90* ↑)	Multi-level disturbances at allocated to N- and P-contamination (from non-point sources such as nearby farms)	Included multivariate statistics and 4 soil parameters	Soils, GW	Wang et al. (2015)
Mining-affected SW	Apical (lethality) and molecular (35 genes ↑↓ incl. *cyp-35A2* ↓)	Deviating MoA for heavy metal. Reconstituted and native SW. Role of DOC, metal speciation or undetected pollutants	No unpolluted reference site (or spiked SW) included. Heavy metal analysis	SW, GW, (DW)	Kumar et al. (2015)
Dispersed crude oil (including cleanup dispersant)	Cellular (apoptosis ↑) and molecular (13 specific genes ↑↓)	*Cep-1* mediated germ cell apoptosis (e.g., suppressed apoptosis in *cep-1* loss of function mutant) as crucial driver of the observed DART	Expression patterns correlated to exposure levels	MW, soil, GW (e.g., if similarly polluted)	Polli et al. (2014)
Mining affected sediment and SW samples	Apical (growth ↓) and molecular (*mlt-1* expression and different hypersensitive mutants)	SW toxicity was attributed to osmotic stress, whereas sediment toxicity to metals/metalloids	Includes chemical and limnological analysis	SW, sediments	Turner et al. (2013)
Industrial WW	Apical (life span ↓, dauer formation, reproduction ↓), behavior (locomotion ↓), biochemical (autofluorescence ↑), molecular (*daf-2*)	Toxic effects of different metal combinations (partially independent of the insulin-like pathway)	Heavy metals as main drivers of life span reduction suggested by the TIE approach	WW, SW	Wang et al. (2010, 2008
Landfill impacted soil samples	Apical (mortality, growth, reproduction) and molecular (stress-related gene expression Δ)	Toxicity and connected stress protein induction (incl. *cyp-35A2*) of target compound DEHP not reflected in DEHP-contaminated soil samples	Including reference site and quantification of DEHP concentrations	Soil, GW	Roh et al. (2007)

In perspective, the vast amount of information enclosed in these studies may be examined for their integration into recently shaping concepts such as ‘multilevel biomarker analysis in environmental toxicology and risk assessment’ (Choi 2008), ‘adverse outcome pathways’ (Angrish et al. 2018, Leist et al. 2017, Kim et al. 2020), ‘multi-stressor response profiles’ (Prasse et al. 2015), high-throughput toxicology frameworks (e.g., Schroeder et al. 2016), exposomics (Escher et al. 2017) and others (Brooks et al. 2020). Several of these concepts show overlapping features that aim at establishing or underpinning toxicological cause and effect relationships between chemical stressors (including environmental samples) and (adverse) physiological effects (such as approached in figure 15).

In case of the AOP concept these relationships are looked for between ‘molecular initiating events’/‘key events’ and the ‘dynamic energy budget’ (DEB). Exhaustion of DEB might be followed by associated adverse events (e.g., Connon et al. 2012, Escher et al. 2017, Jager and Ashauer 2018). This approach mainly aims at utilizing the generated quantitative input on the individual level for predictive ecotoxicology on the population level (e.g., OECD 2018). The DEB model was tested as part of modelling approaches such as DEBtox and others. Up to present a number of AOPs have been elaborated for *C. elegans* (Alda Álvarez et al. 2006, Fueser et al. 2018, Jager et al. 2005, 2014, Jeong et al. 2018, Kim et al. 2020, Margerit et al. 2016, Swain et al. 2010, Wren et al. 2011).

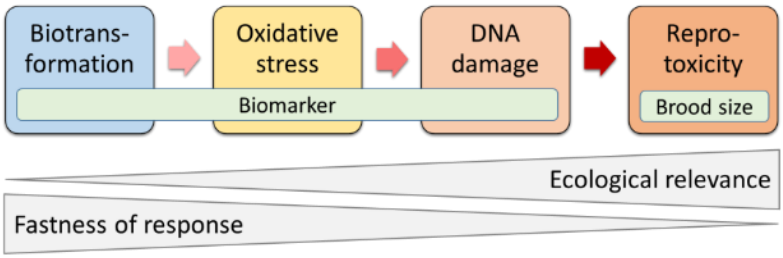


Figure 15. Mechanistic scheme of a hypothetical DART pathway in *C. elegans*. Therein DART (such as occurring upon exposure to β-NF) manifests at the cellular/tissue level and propagates to the physiological level through characteristic toxicological events (large boxes). These can be methodically detected via different endpoints/approaches (green boxes) which beholds a decreasing ‘fastness of response’ and increasing ‘ecological relevance’ as indicated.

In general, AOPs further illustrate the complexity of toxicological pathways and (micro)pollutant interactions, whereby (through continuous research and optimisation, compare Lagadic et al. 2020) offering an integrative toolbox for an enhanced understanding and utilization such as in ERA (e.g., Ashauer and Jager 2018, Brooks et al. 2020, Society for Advancement of AOPs 2018). Similar to the field of ecotoxicogenomics important challenges lie ahead regarding mechanistic studies and ecotoxicological concepts with *C. elegans*. Tackling these challenges will further catalyze the establishment of *C. elegans* as relevant and sensitive model organism in chemical and ERA such as referring to urban water cycles. Based on the literature research and experimental results gathered in this thesis (e.g., 1.4, 2.3 and Abbas et al. 2018) its established and prospective usefulness seems mainly reflected in the following arenas:

1) Molecular biomarker-based indication, classification and evaluation of anthropogenic/chemical stressors in complex environmental samples and their toxicological/functional characterisation including genome wide expression "fingerprints". Particular focus is placed on major dysregulated physiological processes/pathways such as development and reproduction while respecting environmental conditions/factors (e.g., Allard et al. 2010, Kumar et al. 2015, table 2). Conducting chemical analyses of water and wastewater samples should be adapted to (known and hypothetical) chemical stressors to *C. elegans* (Abbas et al. 2018).

2) Enhanced detection of DART and other apical/physiological endpoints with high environmental and human relevance. These evaluations should be optimised by involving mutant and transgenic *C. elegans* strains in combination with HTS to achieve increased statistical power, feasibility and sensitivity (e.g., Allard et al. 2013, Avila et al. 2011, Boyd et al. 2010, Hunt 2016, Lundby et al. 2016, Schroeder et al. 2016, Turner et al. 2013, Xiong et al. 2017, Yu et al. 2020). Mechanistic linkages (compare Anderson and Wild 1994, Angrish et al. 2018, figure 15, table 2 and genome wide screens under 2.3.3) should also facilitate hazard assessment (compare AOP and other cited concepts/studies). Moreover, endpoints/methods (adapted from other fields of research) should continuously be adapted such as under points 2.1−2.5).

2.1) Molecular and cell biology such as DNA damage/repair detection via the *xol-1*::GFP strain (Allard et al. 2013, Lenz et al. 2017, Parodi et al. 2015), the *xpa-1* deficient strain (Leung et al. 2010), the *hus-1*::GFP strain (Hofmann et al. 2002) or via PCR (e.g., Leung et al. 2010, Neher and Stürzenbaum 2006, Zuo et al. 2017). Furthermore, detection of cell cycle arrests (e.g., Cheng et al. 2014, Kumar et al. 2010), mitochondrial integrity (e.g., Behl et al. 2016), epigenetic consequences (e.g., Camacho et al. 2018, Lundby et al. 2016, Weinhouse et al. 2018) or germ line apoptosis via a *ced-1*::GFP strain (e.g., Allard et al. 2013). In addition, immunostaining and other methods (e.g., Parodi et al. 2015, Chen et al. 2016, 2019) may complement the listed molecular/cellular endpoints.

2.2) Neurotoxicology and behavioral biology (e.g., Aitlhadj and Stürzenbaum 2013, Avila et al. 2011, Bargmann 2006, Boyd et al. 2010, Cao et al. 2020b, Gerhardt et al. 2002, Jones and Candido 1999, Ju et al. 2014, Leung et al. 2008, Liu et al. 2019, Matsuura et al. 2013, Roh and Choi 2011, Tejeda-Benitez and Olivero-Verbel 2016a, Tseng et al. 2013).

2.3) Immunotoxicology. Whereby 'microbial pathogens' (Bruni et al. 2019, Darby 2005, van der Hoeven et al. 2011), 'bacteriological parameters' (Clavijo et al. 2016, Stylianou et al. 2018) and 'immunological markers' (e.g., Lewis et al. 2013, Kumar et al. 2015, Merkx-Jacques et al. 2013, Reichert and Menzel 2005) could play a relevant role in (waste)water assessments. A recent example by Bruni et al. (2019) showed significant effects in *C. elegans* exposed to *Micrococcus luteus* and *Acinetobacter iwoffii* isolated from wastewater samples (both strains carrying antibiotic resistances). Further and/or initial activation/weakening of the immune defense could occur through (micro)pollutant co-exposure (compare Stylianou et al. 2018 for *C. elegans* or Schlüter-Vorberg et al. 2017 for *D. magna*). In this context WWTP, such as using gravitational systems instead of micro-sieves or UV-based disinfection, may be investigated for potential residual emission of *C. elegans* pathogens (or parasites). Because of the absence of an adaptive immunity in *C. elegans* more research is also needed to correlate these results to other species beholding (more) complex immune systems.

2.4) Biochemistry and metabolomics. Including endpoints/methods such as quantifying ATP levels (e.g., Jones et al. 2011, Lagido et al. 2008, 2009, McLaggan et al. 2012), dauer formation (e.g., Fielenbach and Antebi 2008, Lant and Storey

2010, Wang et al. 2010), intestinal autofluorescence (e.g., Pincus et al. 2016, Wang et al. 2008b), metabolic activity in connection with different metabolites (e.g., Kim et al. 2019b, Pradhan et al. 2018), oxygen consumption (e.g., Bodhicharla et al. 2014, Han et al. 2018, Schouest et al. 2009, Zuo et al. 2017), oxidative stress (e.g., Liu et al. 2013, Roh et al. 2018, Tseng et al. 2013, Wannous 2011, Xiao et al. 2018, Zhuang et al. 2014) and other biochemical/metabolic parameters.

2.5) Evolutionary developmental biology (e.g., Dutilleul et al. 2014).

3) Comparisons/Correlation of results assembled under point 1–2) to environmental conditions (such as given at multiple sampling sites) and other (nematode) species (Begasse et al. 2015, Boyd and Williams 2003, Haitzer et al. 1999, Höss et al. 2001, 2017, Hägerbäumer et al 2015, Haegerbaeumer et al. 2018, Kim et al. 2020, Maltby et al. 2000, Queirós et al. 2019, Wilson and Khakouli-Duarte 2009) for obtaining a more comprehensive picture about ‘ecological realism and relevance’ (Escher et al. 2017, Fischer et al. 2013, Jager and Ashauer 2018, Stamm et al. 2016) and for supporting weight-of-evidence frameworks (as far as applicable).

2.4 Integrated wastewater quality evaluation – case study of a pilot WWTP equipped with AWWT technologies

Wastewater-borne (micro)pollutants largely contribute to the pollution of SWs and risks to aquatic ecosystems (1.2). Despite their large number current regulative monitoring focuses on a limited number of (micro)pollutants and physicochemical sum parameters such as TN, P_{total}, COD, BOD_5, DOC and TSS (compare Abbas et al. 2018). The reduction of organic (micro)pollutants is assumed to behave proportionally to a decreasing COD and BOD_5. This holds true for many micropollutants particularly biodegradable and/or TSS-adsorbing compounds. However, a substantial fraction remains unaffected by conventional-biological treatment and becomes emitted into receiving water bodies representing (unpredicted) ecological risks (Link et al. 2017, Loos et al. 2013, Malaj et al. 2014, Pal et al. 2010, Schwarzenbach et al. 2006, Stalter et al. 2013). AWWT technologies have important advantageous in further reducing these emissions (Bui et al. 2016, Prasse et al. 2015, Rizzo et al. 2019, Schwarzenbach et al. 2006, Völker et al. 2019). They are thus currently installed and evaluated at different scales such as within the TransRisk project.

Ecotoxicological methods are essential for evaluating these technologies as the detection of contaminant removal rates by chemical methods alone would be unfeasible respecting their high number, generally low concentrations and potentially associated toxicities (e.g., Brack et al. 2017, Prasse et al. 2015, Reemtsma et al. 2016). In addition, challenges such as the generation of oxidative TPs during ozonation or UV radiation (1.2.2) necessitates bioassays to analyze the removal/generation of toxicity/hazardous compounds/TPs. This is particularly important, because the majority of TPs are presently unknown, difficult to detect (due to the lack of chemical standards / reference substances) and/or hard to predict (due to the complexity and variability of wastewater matrices).

The TransRisk evaluation concept mainly focuses on the reduction of chemical, ecotoxicological and microbial indicators by different AWWT options (Ternes et al. 2017, www.transrisk-projekt.de). The presented case study refers to a full-scale WWTP connected to a pilot scale ozonation and GAC/BF post-filtration systems (figure 16 and Abbas et al. 2018). During the presented case study (April−May 2014)

an average ozone dose of 1 g($O_{3,applied}$)/g(DOC) and HRT of ~18 min were applied. GAC (internal surface 1,200 m^2/g, grain size 1−4.8 mm) and BFs (extended clay, grain size 1−5 mm) ran in a non-aerated and aerated mode (using ambient air). Further process and WWTP parameters are described in Abbas et al. (2018), Knopp et al. (2016) and Ternes et al. (2017). Effect-based assessments were conducted based on selected *in vitro* (endocrinicity and mutagenicity) and *in vivo* (reproductive toxicity) endpoints. According to the change of these detections the efficiency of the respective AWWT option was rated (relative to the conventionally treated WWTP effluent). The *C. elegans in vivo* bioassay (ISO10872) implemented in the present case study was selected from the bioassay battery applied in the TransRisk project.

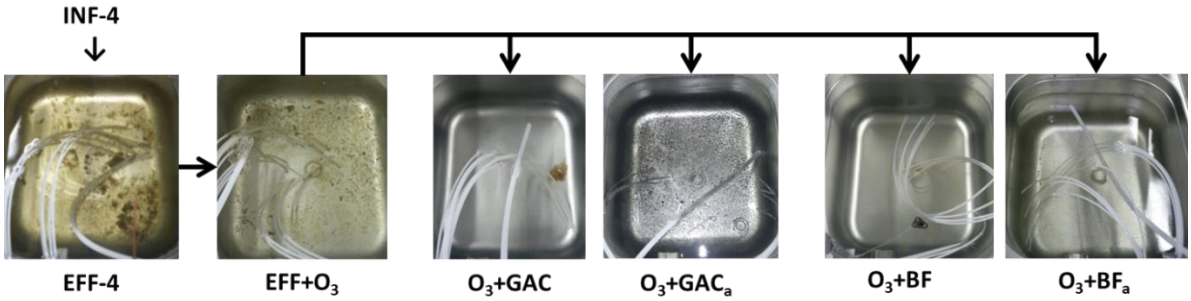


Figure 16. Wastewater collection basins at the pilot WWTP in Hessen, Germany installed during on site testing. Process flow from the WWTP influent (INF-4) to the effluent (EFF-4, activated sludge), ozonated effluent (EFF+O_3) and GAC/BF post-treatments (O_3+GAC, O_3+GAC_a, O_3+BF, O_3+BF_a) as indicated. Treatment degree was visually reflected by decreasing TSS content and turbidity (from left to right). Sedimented particles in the O_3+GAC_a were small GAC particles transported via the supply tubes. Samples taken as 24 h composites and prepared on site by SPE. Photographs taken by I. Schneider and A. Abbas.

The latter furthermore included *D. magna* (OECD 2012), *Lemna minor* (OECD 2006), the blackworm *Lumbriculus variegatus* (OECD 2007) and the mud snail *Potamopyrgus antipodarum* (OECD 2016). These test organisms were investigated in parallel studies by Schneider et al. (2020) and Schlüter-Vorberg et al. (2017) respectively. They proved to be robust sentinel species for characterizing wastewaters (Gartiser et al. 2010, Giebner et al. 2018, Magdeburg et al. 2012, Maltby et al. 2000, Kontana et al. 2009, Rizzo 2011, Stalter et al. 2010). Standardized test systems may however not indicate these properties throughout all wastewater investigations leading to variable bioassay results and compromised

sensitivities (Berger et al. 2016, Wigh et al. 2018, Völker et al. 2017, 2019). Thus the present aim was to examine an integration of the results obtained with *C. elegans* into the wastewater evaluation matrix and to deduct its usefulness to wastewater investigations (such as discussed under 2.3). Similar to *P. antipodarum* (endpoint: number of embryos), *D. magna* (endpoint: number of offspring) and *L. variegatus* (endpoint: total number of worms), brood size was selected as life-cycle parameter of *C. elegans* and population-relevant endpoint (compare 2.3.1).

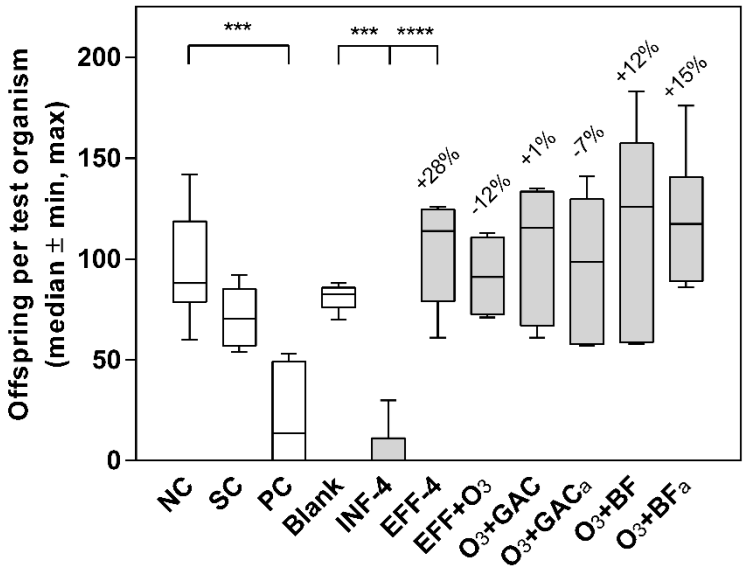


Figure 17. Impacts of WWTP influent (INF-4), effluent (EFF-4), ozonated effluent (EFF+O_3) and ozone post-filtrations (O_3+GAC, O_3+GAC_a, O_3+BF, O_3+BF_a) on the reproduction (brood size) of *C. elegans*. Samples were taken in April 2014 and analyzed at 10x concentrations. Results pooled from two experiments (n = 60). Percent values above bars indicate the percent increase/decrease in brood size relative to the previous treatment stage (except for the EFF-4 which refers to the blank). Significant differences (*** $p < 0.001$, **** $p < 0.0001$) were tested by one-way ANOVA with Bonferroni's multiple comparison test. NC = M9 medium. PC = BAC (5 mg/L). SC = 0.2% DMSO in M9 medium. Blank = SPE blank prepared from analytically-pure groundwater. Further abbreviations/details given in text.

In the *C. elegans* analysis seen in figure 17 (from April 2014) the WWTP influent (INF-4) led to a strong reproductive decline, while the brood size of *C. elegans* exposed to the WWTP effluent (EFF-4) returned to a level that was slightly higher than NC and blank. This result indicated an effective removal of reproductive toxicants during activated sludge treatment. Compared to the EFF-4, the brood size in the EFF+O_3 (ozonated effluent) decreased by 12%. Although this decrease was statistically insignificant, it might have been caused by oxidative TPs generated

during ozonation such as corroborated with other aquatic species (da Costa et al. 2014, Magdeburg et al. 2012, Stalter et al. 2010, Giebner et al. 2018). In the post-filtration stages this reduction diminished (O_3+GAC, O_3+GAC_a) or higher brood sizes compared to the EFF-4 were detected (O_3+BF, O_3+BF_a). The latter result was confirmed at a later sampling date (figure 4A in Abbas et al. 2018) as well as for the endpoint growth (figure 4B in Abbas et al. 2018). Several reasons were considered regarding the enhanced reproduction/growth in the GAC/BF stages. A plausible explanation would be that the quality of the treated wastewater was beneficial (rather than harmful) to *C. elegans*. However, due to the remaining DOC (compare 'Online Resource 2' in Abbas et al. 2018) and residual endocrine activity after advanced treatment (compare figure 2 and 5 in Schneider et al. 2020) the presence of recalcitrant EDCs and/or bioactive natural compounds affecting the growth/reproduction of *C. elegans* (see Höss et al. 2001, Höss and Weltje 2007 or Vingskes and Spann 2018 for examples) may not be excluded *a priori*.

The *in vitro* results incorporated in the present study were obtained from bioassays for (anti)estrogenic (YES and YAES), (anti)androgenic (YAS and YAAS) and mutagenic (Ames fluctuation test) potentials of the wastewater samples (collected during February−April 2014). Similar to the included *in vivo* test systems these assays/endpoints have been beneficially utilized in related wastewater assessments (Chen et al. 2017, Escher et al. 2009, 2018, Filby et al. 2010, Giebner et al. 2018, Magdeburg et al. 2012, Margot et al. 2013, Schindler-Wildhaber et al. 2015, Stalter et al. 2010, Triebskorn et al. 2017, Välitalo et al. 2017, Völker et al. 2017). Using these assays, the conventional biological effluent indicated characteristic activity/toxicity levels (see Abbas et al. 2019 for further references). Residual estrogenic activity has frequently been observed after activated sludge treatment. Estrogenic activity detected in the EFF-4 was effectively reduced by ozonation, while no further increase occurred in the GAC/BF stages (figure 5A in Schneider et al. 2020). Only minor anti-estrogenic activity was detected in the WWTP effluent. This activity seemed moderately increased by ozonation, similar to previous observations in the course of this thesis (Abbas et al. 2019). It was hypothesized that both phenomena might be the results of shifted ratios of agonistic and antagonistic activities (e.g., Ihara et al. 2014, Rao et al. 2014) and/or anti-estrogenic TPs (e.g., Itzel et al. 2020, Knoop et al. 2018). Albeit GAC filtration reduced the

antagonistic activity to the level of the EFF-4, it remained elevated in the BFs (figure 5B in Schneider et al. 2020). Plausible causative agents remained to be elucidated.

Table 3. Wastewater quality evaluation matrix based on the TransRisk project. In the present case five *in vitro* (YES, YAES, YAS, YAAS and Ames, compare Schneider et al. 2020) and one *in vivo* (ISO10872, figure 17) bioassay were used to estimate the efficacies of the five described AWWT processes (sampled during February−April 2014). Efficacies were scored based on the [%]-change in biological activity/toxicity relative to the WWTP effluent (note that negative percent values equal to an increase). Scores were weighted according to the precluded environmental relevance of the respective endpoint (compare main text). All samples were analyzed at 10 fold concentration (SPE extracts)

		Treatment stage				
		EFF+O_3	**O_3+GAC**	**O_3+GAC$_a$**	**O_3+BF**	**O_3+BF$_a$**
Bioassay	**YES**					
	change [%]	91.6	90	89.1	90	89.5
	score	+2	+2	+2	+2	+2
	YAES					
	change [%]	-217	-6.53	-39.4	-317	-288
	score	-2	0	-1	-2	-2
	YAS					
	change [%]	21.2	63	41.2	41.5	79.3
	score	+1	+1	+1	+1	+1
	YAAS					
	change [%]	81.2	91.3	84	84	73.2
	score	+2	+2	+2	+2	+1
	Ames (YG7108)					
	change [%]	-6350	-1000	-850	-3999	-3650
	score	-3	-3	-3	-3	-3
	***C. elegans* (brood size)**					
	change [%]	12	-0.8	7.2	-12.3	-14.7
	score	0	0	0	0	0
	alternative score	-3	0	0	(+3/-3)	(+3/-3)
	Final score	**0**	**2**	**1**	**0**	**-1**
	Alternative final score	-3	2	1	(3/-3)	(2/-4)

Androgenic and anti-androgenic activities were detected at relatively high levels in the WWTP effluent. Anti-androgenic activities also remained at moderate levels after O_3 and in the post-filtration stages (compare Itzel et al. 2020), while androgenic activities were more effectively reduced (figure 5C and 5D in Schneider et al. 2020). Further experiments should thus be performed to confirm the occurrence of

recalcitrant (anti)androgenic activities in these wastewaters. Moreover, low mutagenicity levels in the WWTP effluent were significantly increased after ozonation. It might be speculated, if and how far this mutagenicity/genotoxicity might have been involved in the reproductive decline observed for this treatment group with *C. elegans* (figure 17). Regarding the post-filtrations systems, GAC filtration more effectively decreased the detected mutagenicity compared to BF (5.4−5.5 fold versus 2.4−2.7 fold respectively), but the residual toxicity was still significant. Due to the high human and environmental relevance of the endpoint mutagenicity, confirmed and reoccurring mutagenicity in WWTP discharges from conventional-biological as well as advanced treatments should be further investigated.

The described *in vivo* (figure 17) and *in vitro* (figure 5 in Schneider et al. 2020) results were taken as an input for the evaluation matrix computed in table 3. Depending on their environmental relevance differently weighted scores (TransRisk workshop in 2015) were ascribed according to the respective detection levels and their changes upon wastewater treatment. *In vitro* bioassays probing for agonistic (YES/YAS) and antagonistic (YAES/YAAS) endocrine activities were allocated with scores from -2 to +2 depending on their removal degree (20−80% = 1 and > 80% = 2), increase (-20 to -100% = -1 and < -100% = -2) or stagnancy (-20 to 20% = 0) in activity during the respective wastewater treatment stage. For the Ames fluctuation test these scores ranged from -3 (< -20% change) to 3 (> 20% change), due to the generally higher risk associated with mutagenic compounds. Changes in the brood size of *C. elegans* were assigned the lowest/highest scores ranging from -6 (> 20% change) to 6 (< -20% change) as adverse effects at the whole organism level were ascribed the highest biological/ecological relevance. In addition, an ‘alternative score’ was examined for the latter endpoint (table 3). The respective weighting involved the scores -3 and 3 for changes in brood sizes from 10 to 20% and -20 to -10% respectively. This rating was examined in the light of the discussed sensitivity of *C. elegans* (2.3) and for its implications on the evaluation matrix (discussed below).

The overall rating (‘final score’, table 3) of the effectivities of the five AWWT options follows the order: O_3+GAC > O_3+GAC_a ~ O_3+BF ~ EFF+O_3 > O_3+BF_a. This rating indicated a slightly better performance of the GAC filtrations compared to the BFs. It also showed that aeration did not measurably improve the performance of these

two post filtration systems. The score of the aerated BF was lowest and lower than of ozonation alone. These results reflect in the analytical chemistry part of the TransRisk project (Abbas et al. 2018, Knopp et al. 2016, Ternes et al. 2017). Moreover, comparably high effectivities of activated carbon treatments (GAC, PAC or BAC) as post-filtration systems (but also as independent AWWT) have been documented in the literature (1.2.2). Together with sandfiltration, these technologies have been recommended post-treatments for ozonation (Magdeburg et al. 2014, Knopp et al. 2016, Prasse et al. 2015, Stalter et al. 2010, Reemtsma et al. 2016, 1.2.2).

In the performed case study it may be considered that the presented evaluation matrix allows for a best/worst theoretically-achievable 'final score' of 17/-17 respectively (table 3). In this context all five treatment options achieved an intermediate positioning (-1 to 2) and in this case were to be rated ineffective. To be exact the latter derived from high (e.g., estrogenic and anti-androgenic activities) and moderate (e.g., androgenic activity and mutagenicity) removal rates as well as activities/toxicities that increased (e.g., anti-estrogenicity) or remained stagnant respecting the ascribed thresholds (*C. elegans* brood size). This result seems expectable due to different reasons:

1) Most estrogenic compounds have been reported to be effectively broken down during ozonation (Abbas et al. 2019, Ma et al. 2005, Maletz et al. 2012, Reungoat et al. 2012). Ozonation was also the main driver of the high removal rates observed for all five AWWT options in the present evaluation.
2) Although anti-estrogens (2.2) were reported to occur in wastewater, not much is known about their concentrations and removal. This means many might not be fully degraded/retained and enter surface waters through WWTPs. This should also be regarded when testing (treated) wastewater for agonistic/antagonistic effects (compare Abbas et al. 2019, Leusch et al. 2017, Rao et al. 2014).
3) Residual androgenic compounds remained speculative as generally higher removal rates have been reported for activated sludge treatment as well as ozonation (Abbas et al. 2019, Rao et al. 2014, Stalter et al. 2011).
4) High removal rates of anti-androgenic activities may however induce changes in agonistic/antagonistic interactions that could lead to higher detections of androgenic activities in the AWWT stages.

5) Elevated levels of mutagenicity are occasionally observed in connection with ozonation. Different studies suspected this toxicity to derive from generated or potentiated mutagenic/genotoxic TPs (e.g., Jia et al. 2015, table 4). Because of the increased environmental relevance repeated detections of mutagenicity generally call for further research such as on potential causative agents and further optimisation of AWWTs (and other measures, compare 3.).
6) Impacts on the brood size of *C. elegans* ranged within the ±20% threshold for all five AWWT options (allocating scores of "0"). A hypothetical threshold of ±10% would lead to an 'alternative final score' of -3 (instead of 0) for ozonation, which may better reflect potential adverse effects. The rating of the treatment options O_3+GAC and O_3+GAC_a remains unchanged. The O_3+BF and O_3+BF_a treatments would be rated as +3 or -3 depending on the exact nature of these effects: positive (wastewater quality higher than in the EFF/NC) or adverse (such as resulting from residual EDCs, compare above discussion of figure 17).

The results of two *in vivo* on-site studies conducted with *D. magna* and *P. antipodarum* at the pilot WWTP (Schlüter-Vorberg et al. 2017, Schneider et al. 2020) well relate to the results gathered with *C. elegans*. In these studies the 'number of embryos' of *P. antipodarum* also slightly increased in the WWTP effluent compared to the NC. For both species a noticeable and in case of *P. antipodarum* significant reproductive decline was detected after ozonation (EFF+O_3). This apparent reproductive toxicity was removed in the O_3+GAC treatment supporting the evaluated effectivity of this AWWT option (table 3) and previous observations in related studies (table 4). The O_3+BF treatment decreased this toxicity (to *P. antipodarum*) to slightly lesser extent, while the O_3+GAC_a and O_3+BF_a treatments did not (figure 3 in Schneider et al. 2020). A similar picture was obtained based for the endpoint 'number of offspring' of *D. magna.* However, BF as well as aeration both led to higher offspring numbers than GAC and non-aerated treatments. Regarding the test species *L. variegatus*, also implemented in TransRisk, the WWTP effluent (EFF-4) adversely affected the reproduction of worms. In the ozonated effluent (EFF+O_3) the number of worms was significantly elevated indicating a positive/detoxifying effect of ozonation, while both GAC filtrations performed similarly well and again better than the BFs.

Table 4. Ecotoxicological effects of corresponding WWTP effluents (EFF), ozonated effluents (EFF+O_3) and three types of post-treatment: A) activated carbon (O_3+AC), B) biological treatment (O_3+B) and C) BAC as "hybrid" of A) and B) (O_3+BAC) reported in the literature. Arrows "↓" = reduction, "↔" = stagnancy and "↑" = increase in bioactivity/toxicity/expression level compared to the previous treatment stage: in case of the EFF this is given by the WWTP influent or (as the INF often indicated cytotoxicity or mortality respectively) by the NC (indicated with superscript "c"). Red, yellow or green fields indicate high, moderate or low/no residual bioactivity, toxicity or modified expression level respectively. Results may refer to different test systems, exposure conditions (e.g., flow-through systems, aqueous or extracted samples), process parameters (e.g., ozone doses and implementation scales) and other characteristics as described in the respective references. If a table cell is split into multiple cells this reflects results from different experiments/studies. "Repro" = reproduction. "Histo" = histopathology. "om" = *O. mykiss.* "dr" = *D. rerio.* "n.a." = not applicable. "n.d." = not determined.

A	Treatment stage / Endpoint/Species		EFF		EFF+O_3		O_3+AC		Reference
In vitro	Estrogenicity		↓	↑^{c}	↓	↔	↔	↓	Giebner et al. (2016), Dopp et al. (2021)
	Anti-estrogenicity		↓		↓	↑	↑	↓	
	Androgenicity		↓		↔		↔		
	Anti-androgenicity		n.d.		↓		n.a.		Stalter et al. (2011)
	Dioxin-like activity		↓		↓		↓		Maier et al. (2016)
	Mutagenicity, Genotoxicity		↓	n.d.	↓	↑	↔	↓	Chen et al. (2017), Giebner et al. (2016)
	Photosynthesis inhibition		↓		↓		↔		Stapf et al. (2017), Dopp et al. (2021)
	Cell growth inhibition		↓		↓		↔		
	AchE inhibition		↓		↓		↔		
	Cytotoxicity		↓	↓^{c}	↓	↔	n.d.	↑	
	Cell morphology		↑^{c}		↑		↓		Jekel and Ruhl (2016)
	ROS		↑^{c}		↔		↓		
In vivo	*L. minor* growth		↔^{c}		↔		↔		Schlüter-Vorberg et al. (2017)
	P. antipodarum repro		↓		↔		↔		Giebner et al. (2018)
	O. mykiss liver-histo		↓		n.d.		↓	↓	Triebskorn (2017)
	Biomarker	drVTG	↑^{c}		↓		n.a.		Sun et al. (2017)
		omVTG	↑^{c}	↔^{c}	↓	n.d.	n.a.	↑	Magdeburg et al. (2014), Stalter et al. (2010), Triebskorn (2017)
		omCYP-1A1	↑^{c}		n.d.		↓		Maier et al. (2016)
		ceCYP-35A3	↑		↑		n.d.		Abbas et al. 2018

Table 4. (Continued)

B	Treatment stage / Endpoint/Species	EFF	EFF+O_3	O_3+B	Reference
In vitro	Estrogenicity	↓ | n. a. | ↑[c]	↓ | ↓↑ | ↑	↓ | ↔ | ↔	Itzel et al. (2020), Dopp et al. (2021)
	Anti-estrogenicity	↓	↔	↑	
	Androgenicity	↓	↔	↔	
	Anti-androgenicity	↓ | ↑	↑	↔	
	Mutagenicity, Genotoxicity	n.d.	↓	↓	Wu et al. (2014)
	Bioluminescence (*V. fisheri*)	n.d. | n.a.	↓ | ↑	↑ | ↑	Li et al. (2015), Październ et al. (2017)
	Cytotoxicity	↓[c] | ↓[c]	↓ | ↓	↔ | ↓	Dopp et al. (2021)
C	Treatment stage / Endpoint/Species	EFF	EFF+O_3	O_3+BAC	Reference
In vitro	Estrogenicity	n.d.	↓ | ↓ | ↓	↔ | ↓ | ↔	Reungoat et al. (2012)
	Bioluminescence (*V. fisheri*)		↓ | ↓ | ↓	↓ | ↓ | ↓	
	Genotoxicity	n.a.	↓	↓	Reaume et al. (2014)

In summary, for most of the detected toxicities the activated sludge treatment at the inestigateed WWTP constituted an effective treatment barrier. When the activated sludge treatment shows a (very) high performance the (full) capacity of tertiary and AWWT options may be difficult to estimate, since there might be only little residual toxicity to be removed/evaluated (Schlüter-Vorberg et al. 2017, Völker et al. 2017). In addition, it could be argued that an extended selection of robust and sensitive *in vitro* and *in vivo* bioassays may have revealed undetected effects, which should be integrated into future wastewater quality evaluations not to overlook significant biological risks (compare Berger et al. 2016, Escher et al. 2014 for DW, Schwarzenbach et al. 2006, Sonne et al. 2018, Stamm et al. 2016, Wigh et al. 2018). However, extensive on-site as well as laboratory-scale bioassay batteries may be unfeasible and costly to perform, thus further bioassay optimisation, prioritisation, complementation and case-specific application has been advised for providing high environmental relevance of the analyses (Berger et al. 2016, Bunzel et al. 2013, Brodin et al. 2014, Rizzo 2011, Stamm et al. 2016).

In general, residual toxicity in advanced treated wastewater (e.g., Bundschuh et al. 2011a, 2011b, Reungoat et al. 2012, table 4) stresses the identification of respective causative agents, detection of their MEC and and characterisation of their potential ecotoxicity. Chemical target analysis has often been of minor success in elucidating these effects and in computing realistic EQS that are to be transferred to regulation bodies. This inspires to further improve chemical analyses such as by detecting (a higher number of) suspected toxicants that are adapted to environmentally relevant endpoints (Escher et al. 2018, Maier et al. 2016, Sonne et al. 2018, Stamm et al. 2016, Tang et al. 2014, Vasquez and Fatta-Kassinos 2013). In perspective, continued research should be addressed on:

- Best available technologies (BATs, including most effective process combinations).
- Optimal evaluation strategies, ideally involving performance reviews before-and-after technical upgrades and *in situ* comparisons up-and-downstream of WWTPs (including uncontaminated and contaminated reference sites, Ashauer 2016, Brettschneider et al. 2019, Bundschuh et al. 2011a, 2011b, Henneberg et al. 2014, Hicks et al. 2016, Maier et al. 2016, Triebskorn et al. 2017, Wilhelm et al. 2017).
- Farsighted risk management (acknowledging site specificity and LCA).

3. Conclusions and outlook

Optimising sample preparation for *in vitro* bioassays (2.1 and Abbas et al. 2019)

- Sample preparation methods such as acidification, filtration and SPE significantly influenced the detected ecotoxicologically relevant *in vitro* endpoints. These findings hinted on according shifts in the chemical composition of the prepared (waste)water samples.
- Acidified samples mostly showed higher or lower *in vitro* activities compared to neutral samples that could not be further explained. Sample filtration often led to losses in *in vitro* activities, but in most cases the investigated *in vitro* activity remained unaffected. However, in case of antagonistic activities (anti-estrogenic and anti-androgenic) several samples indicated increased activities after filtration, which might have resulted from altered ratios of agonistic/antagonistic compounds in the samples.
- Ecotoxicological optimisation of SPE methods (up to present mainly performed for chemical analyses) proved to be a valuable approach for the effective recovery of biological activity/toxicity from different water and wastewater samples and thus the reduction of false negative results (e.g., undetected in aqueous samples). The commercial SPE column Telos C18/ENV thereby demonstrated higher effectivity than the Oasis HLB and the Supelco ENVI-Carb+. Moreover, a sample extraction pH of 7 was favored over a pH of 2.5 (short-term acidification) as corroborated by multivariate statistics using the Pareto algorithm.

***In vitro* bioassays as environmental monitoring tools (2.2)**

- MoA-based *in vitro* bioassays such as integrated and investigated in the long-term environmental monitoring campaign of the water protection model region in Southwest Germany demonstrated to be beneficial tools for the detection and prioritisation of cytotoxicity, genotoxicity, mutagenicity and different endocrine activities in aqueous and extracted (waste)water samples.
- Several endocrine activities were recurrently detected in hospital/raw and to lesser extend in biologically-treated wastewater. The monitoring indicated their effective removal during the activated sludge treatments at the investigated

WWTPs. Surface water and groundwater samples (used for the production of drinking water) were also found not to exhibit any of the analyzed biological activities/toxicity at significant levels, except for the anti-estrogenicity at one sampling site (compare below).

- Significant anti-estrogenic activity was frequently detected in one out of three groundwater hotspots. These hotspots were previously characterized as part of the long-term chemical monitoring of the model region. Potential anti-estrogenic causative agents, however, remained unknown. Importantly, only one groundwater intake, but none of the other intakes/wells, nor a drinking water supply pipe, showed any significant anti-estrogenicity. This potential hotspot should thus undergo extended chemical and toxicological monitoring.
- Regarding the high anti-estrogenic/-androgenic activity detected in hospital wastewater, several known compounds came into question. Main suspects were pharmaceutical used in anti-cancer therapies. As the sampled hospital connects to one of the investigated WWTPs it would be interesting to clarify, if the reduced activity detected in the WWTP influent resulted from the dilution and/or degradation/adsorption of these compounds in the sewer system and at which concentration they are contained in the WWTP effluent.

Adapted *C. elegans* bioassay and usefulness for the ecotoxicological characterization of water and wastewater samples (2.3 and Abbas et al. 2018)

- The sensitivity and ecological relevance of *C. elegans* was exploited for examining the reference PAH β-NF and different (waste)water samples. β-NF exhibited high DART with an EC_{50} of 114 µg/L (brood size, 96 h). One biologically-treated WWTP effluent indicated residual high levels of reproductive toxicity. These investigations were extended for the AWWTs ozonation and GAC/BF-post-filtration, whereby no reproductive toxicity was detected (including the respective WWTP effluent).
- Similar to our filtration results in 2.1 and Abbas et al. 2019 the result obtained with *C. elegans* and unspiked versus β-NF-spiked (waste)water samples indicated a role for TSS (> 1 µm) in the toxicity of this PAH (predicted log K_{ow} of 4.7) as well as its putative cumulative effects with other sample constituents.
- The additional developmental endpoint ‘larval length’ indicated significantly promoted growth after exposure to three extracted WWTP effluents. Although

plausible causative agents of this effect remained speculative (e.g., if natural or anthropogenic chemicals), it demonstrated the importance of integrating multiple endpoints into wastewater related assessments (compare below).

- A molecular biomarker of exposure was integrated into the *C. elegans* bioassay by the transgenic *cyp-35A3*::GFP strain. This CYP subfamily is induced by various (micro)pollutants such as PAH, PCBs and different pesticides and pharmaceuticals. The present experiments detected a developmental-stage-, tissue- and concentration-dependent induction of *cyp-35A3*::GFP by β-NF. High expression levels in adult hermaphrodites were reached after subacute exposures (e.g., EC_{50} of 71.5 µg/L after 8 h). Furthermore, *cyp-35A3*::GFP was induced by a conventional-biological and to higher extend by the corresponding ozonated WWTP effluent. It was hypothesized that this could have been caused by oxidative TPs or potentiation of *cyp-35A3* inducing compounds. However, the removal of this effect by the GAC/BF-post-filtration-systems remained to be clarified.
- An accompanying chemical analysis quantified several known effectors of the reproduction/growth of *C. elegans*. The conventional-biological and AWWT stages at the pilot WWTP effectively removed the majority of these known effectors as well as most substances included into the target chemical analysis (in most cases below LOQs). Their overall reduction however did not eliminate the reproductive toxicity in one WWTP effluent sample, nor the growth enhancing effect detected in the GAC/BF-post-filtration systems. This and other effects in treated wastewater generally raise the question on the chemical identity of remaining causative agents and calls for a tighter linkage of chemical and ecotoxicological analysis.
- Based on a literature survey, ecotoxicogenomic and mechanistic studies performed with *C. elegans* demonstrate a high potential in characterizing multiple adverse impacts of anthropogenic (micro)pollutants and contaminated environmental samples at different levels of biological organization. Several studies thereby disclosed and describe correlations of molecular/cellular endpoints (e.g., DNA damage or apoptosis) and effects on the physiological level (e.g., DART or survival). The outcome of these studies may thus be beneficially used as input into broader conceptual frameworks such as AOPs and ERA (including high-throughput approaches). In this context it furthermore seems

promising to assess less frequently examined endpoints (e.g., neurotoxicity or metabolic toxicity) that might affect population/community relevant endpoints (e.g., by exhausting ATP/energy resources or by inducing starvation responses).

Integrated wastewater quality evaluation and perspectives on multifocal reduction of wastewater-borne emissions (2.4)

- Bioassays have been beneficially implemented for evaluating the effectivity of conventional-biological and advanced WWTP processes. The present innovative evaluation concept followed an integrative approach involving chemical, (eco-)toxicological and microbial assessments for far-sighted and environmentally-relevant risk management.
- In the present effect-based evaluation, pilot scale ozonation combined with (non-aerated) GAC post-filtration (O_3+GAC) proved to be the most effective treatment option out of five AWWT processes (namely ozonation, ozonation plus non-aerated or aerated GAC- or BF-post-filtration). This result was obtained using five *in vitro* and one *in vivo* test system analysing different MoA-based and environmentally relevant endpoints respectively. Despite the high removal effectivity of the O_3+GAC process towards most effects, mutagenicity and anti-estrogenic activity could not be completely eliminated calling for further investigation.
- The present bioassay battery demonstrated to be useful in evaluating AWWTs. In future studies further endpoints and “non-standard” bioassays should be examined for detecting additional (potentially neglected) effects. As “gold standard” long-term environmental monitoring of key indicator taxa in receiving water bodies before and after WWTP upgrades should be performed (fulfilling both principles ‘ecological relevance’ and ‘environmental context’).
- Other conventional, tertiary and advanced wastewater treatment processes as well as non-technical mitigation measures have been researched and evaluated as valuable additions and/or alternative solutions for further reducing WWTP emissions. By interdisciplinary and participatory approaches involving relevant stakeholders (e.g., municipalities, federal agencies, affiliated industries, educators and the general public) discussion and planning of sustainable (waste)water management becomes more realistic and compliant with regulations (e.g., the WFD or Urban Waste Water Directive).

Therefore the following outlook highlights a few important management paradigms:

- Optimising conventional biological treatment by refining process parameters (e.g., HRTs or sludge ages), tertiary treatments (e.g., mechanical/biological post-filtrations, compare Triebskorn 2017, Völker et al. 2016, 2019) or further improvement of rain/mixed water retention basins.
- Closing smaller/inefficient WWTPs and centralization within larger WWTPs that offer better treatment of (micro)pollutants, toxicity and relevant parameters. Extending the connectivity of remote areas to sewer systems/WWTPs. Sewer systems should be increasingly screened for leakages and combined overflow systems installed where beneficial (Musolff et al. 2010, Phillips et al. 2012).
- Considering cost-benefit analyses and life cycle assessment (LCA) of WWTPs (e.g., Corominas et al. 2013, Papa et al. 2013, Pedrazzani et al. 2018) for creating a broader knowledge and decision base regarding investment and maintenance costs, energy and resource demands, infrastructural requirements, greenhouse gas emissions, ecological footprints and other important sustainability aspects.
- Source control measures such as at households (e.g., through more “eco-friendly” products and controlled discharge of wastes including expired pharmaceuticals), industries/public institutions (e.g., increased on-site pretreatment of hospital/industrial wastewater, Lienert et al. 2011) and agricultural land (e.g., buffer zones against nutrient-/pesticide-rich runoffs, Reichenberger et al. 2007, or transitions towards more ‘ecological agriculture’)
- In addition to WWTP-related measures for improving surface water quality, the overall drivers and factors of ecosystemic risks (such as hydromorphological and habitat degradation, invasive species and diffuse pollution sources) ought to be evaluated and tackled for sustainable water and wastewater management. This was one of the outcomes of a federal study (BMU 2013) that assessed multiple measures towards their impact on reaching the “good ecological status” of European surface waters. Thereby WWTP upgrades were predicted to contribute to 10%, educational programs (compare above mentioned participatory approach) to 19%, pollution prevention strategies (including source control) to 25% and river restoration campaigns to 46% to achieving this status (once performed in a site-specific and context-dependent manner).

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Annex

A.1 What you extract is what you see: Optimising the preparation of water and wastewater samples for *in vitro* bioassays

Aennes Abbas*, Ilona Schneider*, Anna Bollmann, Jan Funke, Jörg Oehlmann, Carsten Prasse, Ulrike Schulte-Oehlmann, Wolfram Seitz, Thomas Ternes, Marcus Weber, Henning Wesley, Martin Wagner

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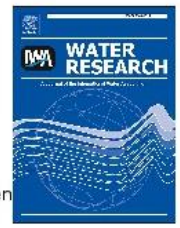

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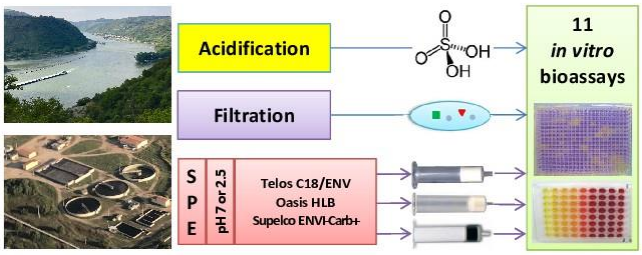

What you extract is what you see: Optimising the preparation of water and wastewater samples for *in vitro* bioassays

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Abstract

The assessment of water quality is crucial for safeguarding drinking water resources and ecosystem integrity. To this end, sample preparation and extraction is critically important, especially when investigating emerging contaminants and the toxicity of water samples. As extraction methods are rarely optimised for bioassays but rather adopted from chemical analysis, this may result in a misrepresentation of the actual toxicity.

In this study, surface water, groundwater, hospital and municipal wastewater were used to characterise the impacts of common sample preparation techniques (acidification, filtration and solid phase extraction (SPE)) on the outcomes of eleven *in vitro* bioassays. The latter covered endocrine activity (reporter gene assays for estrogen, androgen, aryl-hydrocarbon, retinoic acid, retinoid X, vitamin D, thyroid receptor), mutagenicity (Ames fluctuation test), genotoxicity (umu test) and cytotoxicity. Water samples extracted using different SPE sorbents (Oasis HLB, Supelco ENVI-Carb+, Telos C18/ENV) at acidic and neutral pH were compared for their performance in recovering biological effects.

Acidification, commonly used for stabilisation, significantly altered the endocrine activity and toxicity of most (waste)water samples. Sample filtration did not affect the majority of endpoints but in certain cases affected the (anti-)estrogenic and dioxin-like activities. SPE extracts (10.4× final concentration), including WWTP effluents, induced significant endocrine effects that were not detected in aqueous samples (0.63× final concentration), such as estrogenic, (anti-)androgenic and dioxin-like activities. When ranking the SPE methods using multivariate Pareto optimisation an extraction with Telos C18/ENV at pH 7 was most effective in recovering toxicity. At the same time, these extracts were highly cytotoxic masking the endpoint under investigation. Compared to that, extraction at pH 2.5 enriched less cytotoxicity.

In summary, our study demonstrates that sample preparation and extraction critically affect the outcome of bioassays when assessing the toxicity of water samples. Depending on the

water matrix and the bioassay, these methods need to be optimised to accurately assess water quality.

Keywords

Activated carbon, advanced treatment, endocrine disrupting chemicals, micropollutants, ozonation, transformation products, tertiary treatment

Abbreviations

9-cis-RA	9-*cis* retinoic acid
4-NOPD	4-nitro-*o*-phenylenediamine
4-NQO	4-nitroquinoline N-oxide
AhR	aryl-hydrocarbon receptor
Ames	bacterial reverse mutation test
ANOVA	analysis of variance
at-RA	all-*trans* retinoic acid
CAS	Chemical Abstracts Service
CPRG	chlorophenol red-β-D-galactopyranoside
DIN	German Institute of Standardisation (Deutsches Institut für Normung)
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DOC	dissolved organic carbon
E_2	17β-estradiol
EC	European Commission
EC_{50}	Median effect concentration
EDCs	endocrine disrupting chemicals
EFF	effluent
FB	filtration basin
Flu	flutamide
GW	groundwater
hAR	human androgen receptor
hERα	human estrogen receptor α

HOS	hospital
IB	infiltration basin
INF	influent
IR	induction rate
ISO	International Standard Organisation
lacZ	bacterial gene coding β-galactosidase
LOQ	limit of quantification
MS	microsieve
n.a.	not analysed
NF	nitrofurantoin
β-NF	β-naphthoflavone
n.s.	not significant
OD	optical density
OHT	4-hydroxytamoxifen
ONPG	*o*-nitrophenyl β-D-galactopyranoside
PTFE	polytetrafluorethylene
RARα	retinoic acid receptor α
RXRα	retinoid X receptor α
SOS	inducible bacterial DNA repair system
SPE	solid phase extraction
SW	surface water
T	testosterone
T_3	3,3′,5-triiod-L-thyronine
TA100	recombinant strain of *Salmonella typhimurium*
TA98	recombinant strain of *Salmonella typhimurium*
TRα	thyroid receptor α
TSS	total suspended solids
umu	bacterial test for the determination of genotoxicity
umuC	bacterial ultra violet mutagenesis gene C
US EPA	United States Environmental Protection Agency
uvrB	gene of a bacterial DNA repair system
VDR	vitamin D receptor
WWTP	wastewater treatment plant
YAAS	yeast anti-androgen screen

YAES	yeast anti-estrogen screen
YAS	yeast androgen screen
YDS	yeast dioxin screen
YES	yeast estrogen screen

57

1 Introduction

Anthropogenic micropollutants typically occur at nanogram to microgram per litre concentrations in urban water cycles. Micropollutants may pose a risk to ecosystems as they have been associated with negative impacts on aquatic biota (Malaj et al. 2014, Prasse et al. 2015). Micropollutants are found amongst pharmaceuticals, personal care products, industrial chemicals, pesticides and biocides (Kümmerer 2011) that are emitted from different anthropogenic sources. These sources can be diffuse, such as agricultural runoffs, or point sources, such as wastewater treatment plant (WWTP) discharges. Several studies have demonstrated an incomplete removal of micropollutants and relevant toxicity after conventional wastewater treatment using activated sludge (Prasse et al. 2015). Therefore, advanced wastewater treatment technologies utilising chemical oxidation or adsorption are being developed to increase the removal of micropollutants and toxicity (Miklos et al. 2018, Rizzo 2011). *In vitro* bioassays play a crucial role for the ecotoxicological assessment of water and wastewater quality because they determine the joint toxicity caused by complex samples, often regarding a specific mode of action (Escher et al. 2014, 2018, Leusch et al. 2017). Bioassays are routinely used in monitoring campaigns and sufficiently advanced to be integrated into water and wastewater regulations (Brack et al. 2017, Escher et al. 2018).

Environmental water and wastewater samples represent complex mixtures of known and unknown chemicals (Schwarzenbach et al. 2006) and are characterised by a variable composition with respect to matrix parameters (e.g., suspended solids or dissolved organic carbon (DOC)). The toxicity of the samples is mainly determined by the type and concentration of the active, anthropogenic or natural compound(s) and their cumulative effects. However, the sample matrix can also affect the outcome of a bioassay (Janošek et al. 2007, Neale et al. 2015). In addition, samples can undergo physicochemical and biological processes that can transform or degrade the active compounds and may, therefore, modulate the biological effects under investigation.

Because of their ability to reduce matrix effects, to preserve and to concentrate dissolved organic chemicals in aqueous samples, different extraction methods, such as solid phase extraction (SPE), are used in chemical and ecotoxicological studies (Prasse et al. 2015). While sample preparation and extraction methods are commonly optimised for chemical analysis, i.e., to maximise the recovery of specific target compounds, this is rarely done in bioassay studies (Bistan et al. 2012, Neale et al. 2018, Schulze et al. 2017) because the "true" toxicity to recover remains unknown. Thus, standard extraction procedures adapted from chemical analysis are mainly used. Comparative studies have indicated that such chemical "standard" methods can be ineffective in extracting unknown, active compounds from water samples (Hendriks et al. 1994, Wagner and Oehlmann 2011). Because this can lead to an underestimation or false negative results, optimising sample preparation and extraction to recover a maximum of toxicity should be imperative for bioassay studies.

The aim of our study was to assess the impacts of common samples preparation methods on the detection of environmentally-relevant endocrine activities, genotoxicity and cytotoxicity in water and wastewater samples. These samples consisted of surface water, groundwater, hospital wastewater, raw (untreated), conventionally-treated and ozonated wastewater. These samples consisted of grab as well as composite samples with low to high contamination degrees to allow for an optimal comparison of SPE methods. The toxicity of untreated aqueous samples and samples that were acidified (24 h at pH 2.0) or filtered (1 µm pore size) was compared in eleven *in vitro* bioassays. Furthermore, the effectiveness of six SPE methods was compared by extracting samples with three SPE sorbents at acidic and neutral sample pH (2.5 and 7 right before loading). Aqueous and extracted samples were analysed using bioassays for nine human hormone receptors, the umu test and the Ames fluctuation test. The outcome of these bioassays was evaluated by a multivariate Pareto optimisation to identify the most effective sample extraction method.

2 Material and methods

2.1 Characterisation of sampling sites

Sampling locations were selected according to their relevance and representativeness regarding the water cycle in a model region in Baden-Württemberg (Germany, Table 1, samples 1–14, see Seitz and Winzenbacher 2017 for details). Samples comprised influents and effluents of three municipal WWTPs (WWTP 1–3) with activated sludge treatment, two hospital wastewaters, three rivers (surface water), influent and effluent of a filtration basin, two storm water sedimentation tanks, one storm water overflow tank (with infiltration basin), and three groundwater monitoring wells (hotspots). Additional wastewater samples were taken from a pilot WWTP (WWTP 4) in Hessen, Germany (Knopp et al. 2016), equipped with advanced treatment technologies, including a full-scale ozonation of conventionally treated effluent (activated sludge) filtered using a microsieve (MS, filtration at mesh size: 10 µm) to reduce total suspended solids (TSS, Table 1, samples 15–19). The ozonation was performed with 0.33 g O_3/g DOC.

2.2 Collection of water and wastewater samples

Wastewater samples (influent and effluent) from the municipal WWTPs in Baden-Württemberg (sampling period: April (B), July (C, D) and December (E) 2012) and the pilot WWTP in Hessen (sampling period: March (A), April (B), July 2012 (C, D) and December (E) 2012, January (F) 2013) were collected as grab (samples 1, 6, 8–14, 18) or 24 h composite samples (samples 2–5, 7, 15–17, Table 1). The results of corresponding samples (e.g., influents or effluents) were compared to each other, only, with exception of the event-driven sampling of samples 6 and 7 (FB-IN and FB-OUT, Table 1). For the collection of 24 h composite samples, wastewater was continuously pumped through polytetrafluoroethylene (PTFE) tubes into 5 L glass bottles. Bottles were kept at 4°C in darkness during sampling. Hospital effluents, surface waters, samples from storm water sedimentation and an overflow

tank (with infiltration basin) as well as groundwater hotspots were grab samples (sampling period: April (B), July (C, D) and December (E) 2012). All samples were stored at 4°C in pre-cleaned, amber glass bottles with PTFE lids and analysed (aqueous samples for acidification and filtration experiments) or further processed (comparison of SPE methods) within 48 h after sampling.

2.3 Sample preparation

2.3.1 Acidification for testing aqueous samples

One aliquot (40 mL) of the aqueous (waste)water sample was kept at the original pH, another aliquot (40 mL) was acidified with sulphuric acid (5 mol/L, purity "pro analysi") to pH 2.0 directly after sampling. After storage for 24 h at 4°C in the dark, acidified samples were neutralised with sodium hydroxide (1 mol/L, purity "pro analysi") to pH 7 prior to analysing the aqueous samples in the bioassays (in contrast to short-term acidification for SPE, 2.3.3).

2.3.2 Filtration for testing aqueous samples

One aliquot of the (waste)water sample remained unfiltered while another aliquot was filtered using glass fibre filters (Whatman GF6, pore size 1 µm) to reduce TSS. Selected filtered and unfiltered aqueous samples were tested as aqueous samples (not SPE extracts) in the *in vitro* assays (2.4). The glass fibre filters containing the retentate were suspended in ultrapure water (10 min in an ultrasonic bath) and the obtained aqueous suspensions were analysed for endocrine activity retained on the filters. A filter control was run and analysed in parallel: ultra-pure water was filtered and an empty glass fibre filter was suspended as well. Additionally, the influence of a microsieve (mesh size: 10 µm) on endocrine and genotoxic activity of conventionally treated effluent after final sedimentation at WWTP 4 was investigated by taking wastewater samples before and after the microsieve. A microsieve control was analysed as well (data not shown): fragments of the microsieve were incubated in

ultra-pure water and in methanol for 70 d and the resulting suspensions were tested in the *in vitro* bioassays.

2.3.3 Solid phase extraction

Three commonly used types of SPE sorbents were tested for the recovery of endocrine, genotoxic, and mutagenic activities: Oasis HLB (200 mg), Kinesis Telos C18/ENV (500 mg C18, 200 mg ENV) and Supelco ENVI-Carb+ (200 mg). Prior to sample loading, the cartridges were conditioned as follows: Oasis HLB and Telos C18/ENV were conditioned consecutively with 1 x 2 mL heptane, 1 x 2 mL acetone, 3 x 2 mL methanol (LC-MS Optigrade) and 4 x 2 mL ultrapure water. Supelco ENVI-Carb+ cartridges were turned (top to bottom) before they were conditioned with 1 x 2 mL acetone and 1 x 2 mL methanol. Afterwards, the columns were turned again (loading direction) and conditioned with 1 x 2 mL acetone, 3 x 2 mL methanol and 4 x 2 mL ultrapure water. For each sample, 500 mL sample was extracted at two pH values, neutral (pH 7) and acidified with sulphuric acid (3.5 mol/L) to pH 2.5.

SPE was performed within 48 h after collection and directly after acidification. The columns were dried under a stream of nitrogen and stored at -20°C. Samples extracted at neutral pH were eluted with 5 x 2 mL acidified methanol and 5 x 2 mL acetone, each containing 0.2% formic acid. Acidified samples were consecutively eluted with 5 x 2 mL methanol and 5 x 2 mL acetone at neutral pH. After adding 100 µL dimethyl sulfoxide (DMSO), the combined methanol-acetone extract was concentrated to 100 µL final volume under a gentle nitrogen stream. The extracts (5000-fold concentrated compared to the aqueous sample) were stored at -20°C until testing. A SPE blank was prepared in parallel to each sampling campaign to control for contamination by loading each column type with ultrapure water and extracting them with neutral and acidified methanol and acetone, respectively.

2.4 *In vitro* bioassays

2.4.1 Recombinant yeast screens for endocrine activities

In this study, nine recombinant yeast-based reporter-gene assays were used to detect endocrine activities: Yeast Estrogen Screen (YES, human estrogen receptor α (hERα)), Yeast Anti-Estrogen Screen (YAES), Yeast Androgen Screen (YAS, human androgen receptor (hAR)), Yeast Anti-Androgen Screen (YAAS) first described by Routledge and Sumpter (1996) and Sohoni and Sumpter (1998), Yeast Dioxin Screen (YDS, aryl-hydrocarbon receptor (AhR, Miller 1997)), as well as yeast two-hybrid assays for retinoic acid receptor α (RARα), retinoid X receptor α (RXRα), vitamin D receptor (VDR) and thyroid receptor α (TRα) introduced by Inoue et al. (2009). We used yeast-based assays rather than mammalian cell lines because they are robust in terms of cytotoxicity, because they have been validated by ISO (ISO 19040-1:2018) and to compare the results to our previous work.

All bioassays have the same principle: The activation of the respective receptor by chemicals present in the sample triggers the expression of β-galactosidase, which cleaves the chromogenic substance chlorophenol red-β-D-galactopyranoside (CPRG; CAS 99792-79-7, Sigma-Aldrich, Germany). The intensity of the colour change (yellow to red) is proportional to the agonistic activity of the sample and is measured with a photometer (Multiskan Ascent, Thermo Fisher Scientific, Braunschweig, Germany) at a wavelength of 540 nm (OD_{540}). To screen for antagonistic activities (YAES and YAAS), a known agonist is added. Thus, antagonistic compounds reduced the reporter gene activity induced by the agonist.

All bioassays were conducted in 96-well microtiter plates (f-form, VWR Darmstadt, Germany) as described previously (Völker et al. 2016, Wagner et al. 2013, Stalter et al. 2011, Wagner and Oehlmann 2009). In brief, aqueous samples were analysed in eight replicates with a dilution factor of 1.6 (i.e., 0.625-fold final sample concentration). SPE extracts were diluted 480-fold resulting in a 10.4-fold final sample concentration (0.2% v/v solvent

concentration, eight replicates). This enrichment factor was used for all SPE extracts (compare 2.2 and Table 1). After 18–22 h incubation (depending on the assay) at 30°C and 1200 rpm, cell number (absorbance at 595 nm, OD_{595}, to detect cytotoxic effects) and reporter-gene activity (OD_{540}) were determined photometrically. In each assay and experiment, concentration-response curves for the appropriate reference compound were generated (see Table S1 and Figures S1–S5 for details).

The OD_{540} was corrected for the respective cell density (OD_{595}). If > 20% cytotoxicity occurred (see 2.5) results were not used. The corrected absorbance was normalised to the negative/solvent controls (0%) and the maximum activity of the reference compound (100%) to calculate relative activities (%). For the antagonist assays, a control without agonist was used to represent 100% receptor inhibition.

The limit of quantification (LOQ) was calculated for each bioassay and experiment using the mean activity of the negative control and adding threefold it's standard deviation. As the LOQs varied between bioassays and experiments, they were not shown for the sake of clarity. However, in general only results above the LOQs were considered. In a few cases, such as estrogenic activity, lower activities were shown because of their ecotoxicological relevance (low effect threshold) and for comparing WWTP effectivities.

2.4.2 Genotoxicity assay (umu test)

Genotoxic effects were assessed using the umu test (ISO 13829) with the genetically modified *Salmonella typhimurium* strain TA1535 (pSK1002). The umu test detects primary reversible or irreversible DNA damages that induce the expression of the DNA SOS-repair system associated with the UV mutagenesis gene C (umuC gene). Genotoxic substances in the samples lead to an expression of β-galactosidase from the umuC-*lacZ* construct. The reporter-gene activity is determined by the cleavage of the chromogenic substance *o*-nitrophenyl β-D-galactopyranoside (ONPG, CAS 369-07-3, Sigma-Aldrich, Germany). The umu test was

238 conducted as described by Magdeburg et al. (2014). In brief, aqueous samples were analysed
239 after sterile filtration (injection filter with PTFE membrane: pore size 0.2 µm, neoLab,
240 Germany) with a dilution factor of 1.7 and SPE extracts in a 20-fold final sample
241 concentration (0.4% v/v solvent) in eight replicates. Ten concentrations between 5−2000 µg/L
242 final concentration in the well of 4-nitroquinoline N-oxide (4-NQO; CAS 56-57-5, Sigma-
243 Aldrich, Germany) were used as genotoxic reference compound (Table S1). Cytotoxicity
244 (OD_{595}) and genotoxicity (OD_{414}) were determined photometrically. The OD_{414} was corrected
245 for the respective cell density (OD_{595}) if no cytotoxicity occurred (see 2.5). A linear
246 regression line was generated using the corrected OD_{414} of the reference compound (Figure
247 S6). The induction rate (IR) was calculated using the corrected OD_{414} of the samples. An
248 IR ≥ 1.5 is considered potentially genotoxic.
249
250 **2.4.3 Mutagenicity assay (Ames fluctuation test)**
251 Mutagenic effects (i.e., irreversible DNA damage) were analysed using the Ames fluctuation
252 test (ISO/DIN 11350) with two genetically modified strains of *Salmonella typhimurium*
253 (TA98 and TA100). The assay detects the induction of point mutations in special marker
254 genes coding for enzymes involved in histidine biosynthesis as frameshift mutations (TA98)
255 and base pair substitutions (TA100). To increase sensitivity, the strains TA98 and TA100
256 have a mutation in the *uvrB* DNA repair gene. In the absence of mutagens, the strains do not
257 grow in histidine-free medium and a reverse mutation in the marker genes enables histidine
258 synthesis and thus growth. This leads to a pH change in the assay medium that is determined
259 photometrically at a wavelength of 414 nm.
260 The Ames test was conducted as described by Magdeburg et al. (2014). In brief, aqueous
261 samples were tested after sterile filtration (see 2.3.2) with a dilution factor of 1.25 and SPE
262 extracts in a 10-fold final sample concentration (0.2% v/v solvent). Mutagenic reference
263 compounds were used as positive controls (TA98: 10 mg/L final concentration in the well 4-

nitro-*o*-phenylenediamine (4-NOPD, CAS 99-56-9, Sigma Aldrich, Germany, Table S1); TA100: 0.25 mg/L final concentration in the well nitrofurantoin (NF; CAS 67-20-9, Sigma Aldrich, Germany, Table S1). The mutagenic activity of the sample was determined photometrically with a cut-off value at a wavelength of 414 nm by counting the number of wells that shifted from purple (negative) to yellow (positive).

2.5 Data analysis

In this study, cytotoxicity was defined as a cell number in the sample of ≤ 80% compared to the negative control (solvent control) analysed in parallel in each experiment.

Statistical analyses were performed using GraphPad Prism (version 5.03, GraphPad Software Inc., San Diego, California, USA). Datasets were analysed using the D'Agostino and Pearson omnibus normality test for Gaussian distribution and the Bartlett's test for homogeneity of variances. In case of a normal distribution and equal variances significant differences between the datasets were determined using a one-way ANOVA with Dunnett's post-test. If the datasets were not normally distributed, the nonparametric Kruskal-Wallis test with Dunn's post-test was used. An unpaired t-test was used to determine significant differences between neutral and acidified samples and unfiltered and filtered samples. A p-value ≤ 0.05 was considered significant.

The mathematical part of the methodological optimisation was carried out using a Pareto strategy (Ehrgott 2000) further adapted for the multivariate optimisation, similar to the use of colour coding in *in silico* toxicology (Durmaz et al. 2015). The main optimisation criterion was to assess sample preparation methodologies that achieved the highest measured biological activity in six different parameters. Pareto thereby classified a preparation method as non-optimal, if another preparation method exists that delivers "better" values regarding *all* parameters (YES, YAS, etc.) and *all* tested samples. Non-optimal preparation methods are

289 excluded from the list leading to a ranked set of Pareto-optimal sample preparation methods.

290 The applied strategy also tackled scenarios with missing data.

3 Results and discussion

3.1 Sample acidification for testing aqueous samples

Analytical chemists use acid as a standard method to stabilise aqueous samples and prevent the biodegradation of (micro)pollutants (Prasse et al. 2015). Stabilisation is thought to occur by deactivating microorganisms (Baker and Kasprzyk-Hordern 2011, US EPA 2010) that may use target analytes as substrates. Therefore, the procedure is often adopted in ecotoxicology for conserving the toxicity of samples but often without studying its effectiveness.

The present results show that sample acidification and storage over 24 h significantly affected the endocrine activities and mutagenicity of aqueous samples compared to the samples kept at neutral pH (Figure 1, full data sets in Table S2). Focusing on a change of the endocrine activities or mutagenicity of ≥ 10%, untreated wastewater was most affected by acidification (Table S3) whereby 50% of the assays (n = 22) showed decreased activities between -13 and -94%. In case of the influent and effluent of the filtration basin 32% of the bioassays (n = 22) indicated altered activities between -13% and -37%. Groundwater (9%, n = 33), ozonated wastewater (9%, n = 11) and surface water (3%, n = 33) were least affected (Table S3).

Regarding the different bioassays, the activities in the YAES, RXR and Ames TA100 assays were most affected by acidification (Table S4). 65% of the YAES experiments showed decreased (-13 to -32%) or increased (+15 to +34%) activities (Figure 1A). The Ames TA100 was affected in 24% of the experiments with decreasing (-13 to -77%) as well as increasing mutagenicity (+17%) compared to neutral samples (Figure 1C, Table S4). Acidification caused the highest decrease of mutagenicity in the Ames TA98 with -94% followed by the RAR assay with -88% (Figure 1B). In the remaining bioassays, low endocrine or genotoxic activities were detected. Thus, no conclusion of the influence of acidification on these endpoints was possible (Figure S7, Table S2).

In summary, sample acidification led to a decrease (-13 to -94%) of activity in 81% and to an increase (+10 to +34%) of activity in 19% of the cases (n = 32). This indicates that sample

317 acidification significantly affects the outcomes of bioassays. Two hypotheses may explain the
318 changes in toxicity: 1) In acidified samples, acids may interfere with active chemicals or 2) in
319 neutral samples, microbial activity may degrade or transform the active chemicals.
320 Basically, the key question is whether the neutral (hypothesis 1) or the acidified sample
321 (hypothesis 2) represent the "true" toxicity. For chemical analysis, there is consensus that
322 acidification stabilises most compounds and prevents microbial degradation (Baker and
323 Kasprzyk-Hordern 2011, Vanderford et al. 2011, US EPA 2010). However, our data implies
324 that besides few exceptions the *in vitro* activity is lower at acidic compared to neutral pH
325 (Figure 1, Table S2). Accordingly, samples at a neutral pH may better represent the actual
326 toxicity. If this hypothesis holds true, an acidification of samples would either reduce the
327 concentration of active chemicals by increasing adsorption to suspended matter (Baker and
328 Kasprzyk-Hordern 2011) or by increasing hydrolysis (Prasse et al. 2015).
329 Alternatively, it can be assumed that the higher activity in neutral samples is an artefact
330 caused by a change in sample composition. Here, continuous microbial activity may
331 deconjugate compounds resulting in a higher biological activity. This occurs during biological
332 wastewater treatment (Andersen et al. 2003, Koh et al. 2008, Wu et al. 2017). However, an
333 on-going microbial degradation of active compounds would counteract this process (Giebner
334 et al. 2018).
335 In reality, the toxicity of an aqueous sample may change at either neutral or acidic pH. As this
336 depends on the chemical and biological composition of a sample, it is difficult to generalise
337 which condition best represents the actual toxicity. Based on the present data, we argue that a
338 neutral pH comes closest to reality, as the sample is minimally processed. In addition, a
339 higher biological activity will result in a more protective water quality assessment if one
340 accepts that the risks of false-positives outweighs the risk of false-negatives.
341

342 **3.2 Sample filtration for testing aqueous samples**

Sample filtration is beneficial to stabilise compounds (Baker and Kasprzyk-Hordern 2011), to avoid clogging of SPE cartridges, to remove TSS (Janex-Habibi et al. 2009) and to sterilise samples (Gehrmann et al. 2018). In the present study, unfiltered and corresponding glass fibre filtered (pore size 1 µm) aqueous samples as well as aqueous suspension of the filter retentates were compared to investigate the impacts of filtration on the toxicity. These comparisons further included a microsieve (pore size 10 µm) installed at one WWTP, which had a minimal effect on the toxicity (full data set in Table S5).

Focusing on a change of the different endocrine activities or mutagenicity of ≥ 10% again, the untreated wastewater was affected at most by filtration (Tables S5 and S6). Here, the toxicity was decreased by -20 and -54% and increased by +28 and +61% in 22% of the bioassays (n = 18, Figure 2A, 2B). For surface water, activities were altered in 14% (n = 7) of the bioassays with one affected endpoint (Figure S8). Conventionally treated wastewater and groundwater were less or not affected by filtration (Figures 2C and S8, Table S6).

Filtration had the strongest impact on the YAES (50% of the assays, n = 8; Table S7) followed by the YES and YAAS (25%, n = 8 each) and YDS (13%, n = 8). The effects observed in the other bioassays were too low to evaluate the influence of filtration on these endpoints (Figures 2 and S8, Table S5).

The aqueous suspension of the filter retentates also showed relevant changes in endocrine activities ≥ 10% in 19% (n = 36) of the yeast-based assays. The retentates were anti-estrogenic (57%, n = 7) and anti-androgenic (43%, n = 7) with activities from 21–80% (YAES) and 30–45% (YAAS, Table S5). In two samples, the endocrine activity in the filtered sample was significantly ($p \leq 0.001$) lower than in the unfiltered sample. As the retentate was also active, the activity was retained by filtration. In two cases, significantly higher ($p \leq 0.001$) activities were detected in the filtered compared to the unfiltered samples. Here, the retentate was active as well. In two YAES experiments, the endocrine activities in the filtered and unfiltered samples were on a comparable high level (84– 91%) and the retentate

was active as well (46 and 80%). One sample was not anti-androgenic as filtered and unfiltered water, but as filter retentate (45%, Figure S8, Table S5).

In summary, sample filtration led to a decrease (-18 to -54%) of activity in 33% and to an increase (+13 to +61%) of activity in 67% of the cases (n = 9) and, thus, has a significant impact on the bioassay results. The retention of particle-associated hormones and endocrine disrupting chemicals (EDCs) may explain this observation. This is supported by the detection of significant endocrine activities in the filter retentates and previous observations (Dagnino et al. 2010, Routledge 2003, Shieh et al. 2016).

Interestingly, few filtered samples had significantly higher endocrine activities than the corresponding unfiltered samples. For the WWTP effluent filtered by a microsieve we detected an approximately 2-fold increase in anti-estrogenic activity (Table S5). This may be the result of an altered ratio of agonistic and antagonistic activities (Ihara et al. 2014, Rao et al. 2014) or the leaching of "new" compounds by the filter materials (filter controls confirmed this was not the case). In the present case, dissimilar affinities towards filter materials and/or suspended solids (Ng and Cao 2015, Wangmo et al. 2018) could have resulted in a retention of antagonistic and thus increased agonistic activities in the filtrate and vice versa.

In conclusion, the application of sample filtration should be well-adjusted to the aims of a study, the characteristics of investigated (waste)water samples and bioassay specificities, as this is crucial to avoid misestimating the *in vitro* toxicity (Dagnino et al. 2010, EC 2003). In the present study, this was amongst others observed when evaluating the removal of (anti-)estrogenic and dioxin-like activities at WWTP 1 (Figure 2). Depending on whether the filtered or unfiltered samples are considered, one can conclude that the treatment in WWTP 1 either increases or decreases the toxicity.

3.3 Comparison of aqueous and extracted samples

394 Comparing the toxicity of aqueous samples and corresponding SPE extracts is rarely done but
395 has a number of advantages, such as the possibility to calculate recovery rates and evaluate
396 the environmental relevance of obtained results (Giebner et al. 2018, Muschket et al. 2017,
397 Tousova et al. 2017, Wangmo et al. 2018).
398 In the present case, most aqueous samples induced minimal estrogenic, anti-androgenic and
399 retinoic acid-like activities (Figure 3, Tables S8, S9, S10). However, anti-estrogenic activities
400 between 21 and 91% were detected in all aqueous samples (Figure 3B). The activities were
401 < 19% in the other bioassays (Figures 3D and S9, Table S8). In extracted samples, the
402 estrogenic activity (≤ 8%, n = 35) was generally as low as in the corresponding aqueous
403 samples (≤ 13%, n = 8; Figures 3 and 4, Table S9). The minor estrogenic activity detected in
404 most samples in this study is in line with other studies on biological (Jalova et al. 2013, Keiter
405 et al. 2006, Metcalfe et al. 2013) and advanced wastewater treatment (Ma et al. 2005, Maletz
406 et al 2013).
407 The anti-estrogenic activity of the extracts was variable and, depending on the SPE method, in
408 parts very high (13–89%, n = 35) and comparable to the corresponding aqueous samples
409 (Figures 3B and 4). This indicated that the causative compounds were either only partially
410 recovered or that the anti-estrogenicity of the aqueous samples is caused by the matrix (Neale
411 et al. 2015). Interestingly, the high anti-estrogenic activities in the extracts point towards
412 potential masking effects, whereby receptor antagonists reduce the detection of agonistic
413 activity in water sample. This phenomenon has also been discussed by other authors (Giebner
414 et al. 2018, Gehrmann et al. 2018, Ihara et al. 2014, Rao et al. 2014, Stalter et al. 2011). In
415 addition, groundwater was significantly anti-estrogenic (Figure 3B, Table S8 and S9). This
416 calls for further clarification regarding the presence of EDCs in groundwater.
417 In contrast, the anti-androgenic activity was low in most aqueous samples (≤ 5%, n = 7) but
418 higher in the extracts (9–89%, n = 30, Figures 3C and 4, Table S9) indicating a successful
419 extraction. Except for hospital wastewater, which may contain anti-androgenic

pharmaceuticals (Sohoni and Sumpter 1998, Stalter et al. 2011), the majority of aqueous samples exhibited only low androgenic and anti-androgenic activities (Figures 3C and S9, Table S8). The androgenic activities remained low in the corresponding extracts, whereas anti-androgenic activities were detected at moderate to high levels. As in case of the anti-estrogenic activity, androgen receptor antagonists may mask the androgenic activity. Such interactions were described for WWTP effluents (Leusch et al. 2017, Rao et al. 2014) and ozonated hospital wastewater (Gehrmann et al. 2018). The high removal of these activities reported for activated sludge treatment (Rao et al. 2014) and ozonation (Stalter et al. 2011) were not observed in this study.

The highest RAR activity was detected in aqueous hospital and untreated wastewater (HOS: 93%, INF-1: 23%) and corresponding extracts, depending on the SPE-method (HOS: 14–91%, INF-1: 0–54%; Figures 3E and 4, Table S9). This implies that the active compounds were only partially extracted. Only hospital and untreated wastewater induced RAR activities, which was removed in the effluent (Figure 3E, Tables S8 and S9). RXR activities were detected in extracted WWTP effluent and ozonated effluent (Figure S9, Table S8). So far, only few studies reported RAR and RXR activities in water (Inoue et al. 2009) and wastewater (Allinson et al. 2011, Inoue et al. 2011). In the experiments by Sawada et al. (2012) and Cao et al. (2009) these activities readily degraded during activated sludge treatment and lab-scale ozonation, respectively. Likewise, only a few studies exist on VDR- and TR-like activities in (waste)water samples (Escher et al. 2014, Inoue et al. 2011, Kusk et al. 2011, Leusch et al. 2017). In any case, activity levels in the present aqueous/extracted samples were negligible.

Moderate dioxin-like activities were detected in a number of extracted but none of the aqueous samples (Table S8). Highest activities were observed in raw, treated and hospital wastewater. Lowest activities were observed for ozonated wastewater and groundwater. Its removal during biological and advanced wastewater treatment has been observed in several

446 (Allinson et al. 2011, Loos et al. 2012, Stalter et al. 2011) but not all studies (Jia et al. 2015,
447 Rao et al. 2014, Reungoat et al. 2010) supporting its detection in the present WWTP effluents.
448 While none of the aqueous samples (n = 6) was active in the umu assay, 33% (n = 27) of the
449 extracts were potentially genotoxic (Figure 3F, Tables S8 and S9). Low to moderate
450 genotoxicity was detected in extracted hospital, raw and treated wastewater but in none of the
451 other samples. Other studies observed genotoxicity in extracted WWTP effluents (Macova et
452 al. 2011, Keiter et al. 2006, Escher et al. 2014). These potentials generally decreased upon
453 ozonation (Cao et al. 2009, Misik et al. 2011).
454
455 **3.4 Identifying the optimal SPE method**
456 Similar to analytical chemistry (Baker and Kasprzyk-Hordern 2011, Maruya et al. 2016, Polo
457 et al. 2005), SPE of (waste)water samples is advantageous for *in vitro* bioassays. Extraction
458 prevents the microbial degradation of untreated samples and improves the detection of
459 toxicological effects caused by low (micro)pollutant concentrations (Escher et al. 2005,
460 Janošek et al. 2007, Macova et al. 2011, Neale et al. 2015, 2018). SPE can also minimise
461 matrix interferences by reducing natural organic matter and excluding ions, nutrients or acids
462 (Neale and Escher 2014, Prasse et al. 2015, Escher et al. 2018).
463 In contrast to chemical analysis of target compounds, the recovery of toxicity by SPE cannot
464 be evaluated because the causative chemicals and mixture effects remain unknown. Thus, this
465 study aimed at maximising the extraction of toxicity by comparing two mixed-mode
466 hydrophilic/hydrophobic (Oasis HLB and Supelco ENVI-Carb+) and one composite (Telos
467 C18/ENV) SPE sorbents. These SPE sorbents enrich a broad and heterogeneous spectrum of
468 chemicals (Köke et al. 2018, Leusch et al. 2012, Neale et al. 2018). Extracting both neutral
469 and acidified samples, six different SPE methods were evaluated by a semi-quantitative
470 (3.4.1–3.4.4) approach followed by multivariate statistics (3.4.5).
471

472 **3.4.1 Blanks**

473 In parallel to the extraction of the samples, a SPE blank was prepared to control for potential
474 contaminants in reference waters and used materials (Kolkman et al. 2013, Neale et al. 2018,
475 Schulze et al. 2017). Each cartridge type was loaded with ultrapure water and extracted as
476 described in 2.3.3. The extracts of the 60 SPE blanks were negative in all bioassays except in
477 two cases (3%): Supelco ENVI-Carb+ at pH 7 and pH 2.5 in the YAAS. Here, the activities
478 were 2% and 3% higher than the limit of quantification. In addition, a DMSO sample was
479 included in parallel to the SPE extracts in each *in vitro* bioassay as a solvent control. These
480 solvent controls did not induce an effect in the bioassays.

481

482 **3.4.2 Cytotoxicity**

483 Cytotoxicity is often used as indicator of the reactive toxicity of environmental samples and
484 their overall (micro)pollutant load. It, thus, represents an important endpoint which is
485 integrated into several water quality assessments (Escher et al. 2014, 2018, Leusch et al. 2014,
486 Välitalo et al. 2017). However, depending on the investigated endpoint, cytotoxicity can also
487 prevent or mask the detection of specific toxicity (see 4).
488 In the present study, none of the aqueous samples induced cytotoxic effects (Figure 4, Tables
489 S8 and S9). Cytotoxicity was, however, frequently detected in SPE extracts (Figure 4).
490 Untreated wastewater induced cytotoxicity in 50% (HOS) and 38% (INF-1) of sample
491 extracts (n = 60, each) tested in ten *in vitro* bioassays (Table 2). For conventionally treated
492 wastewater (EFF-1, EFF-4, EFF-4-MS, n = 54–60) cytotoxicity was observed in ≤ 25% of
493 extracts (Table 2). The occurrence of cytotoxicity in extracted ozonated wastewater (sample
494 EFF-4-MS-O_3, n = 54) and groundwater (sample GW-1, n = 60) was 35 and 2%, respectively
495 (Table 2).
496 The choice of the SPE method had a substantial influence on the detection of cytotoxicity: the
497 extracts of the Oasis HLB and the Telos C18/ENV (neutral pH) were cytotoxic in 32% and

50% of the bioassays (n = 78 each, Table 2). At acidified pH, these extracts induced similar cytotoxicity with 15 and 13%, respectively (n = 78 each, Table 2). Samples extracted with the Supelco ENVI-Carb+ at neutral pH were more cytotoxic (12%) compared to the corresponding samples that were extracted at acidified pH (not cytotoxic effects, n = 78 each, Table 2).

In general, samples extracted at neutral pH induced higher cytotoxicity than acidified samples (Figure 4) and Telos C18/ENV extracts were more cytotoxic than those of Oasis HLB and Supelco ENVI-Carb+. Thus, extraction at neutral pH with Telos C18/ENV was the method where the highest cytotoxicity was detected (Figure 4). Escher et al. (2005) found an extraction at pH 3 (using the Oasis HLB) to be more effective than pH 7 and pH 11 in a study on spiked urine samples. Stalter et al. (2011) observed this for acidified biologically-treated and ozonated wastewater. Both studies suggest that compounds with acidic moieties to be responsible for the recovered cytotoxicity. This is in contrast to the present results, which suggest that the cytotoxicity in a broad range of bioassays is extracted more effectively at neutral pH.

In a recent study by Stalter et al. (2016) the Telos ENV (without C18 sorbent) followed by the Oasis HLB recovered most cytotoxicity amongst nine other SPE sorbents from disinfected drinking water (acidified before extraction). Polar compounds adsorbed by the ENV as well as the HLB sorbent material were suspected as main causative agents. Although Stalter et al. (2016) did not compare an extraction at neutral pH the results support the effectivity of the Telos C18/ENV and Oasis HLB observed in the present study. Along the same line, a multilayer SPE based on Oasis HLB induced more cytotoxicity than a single sorbent method in a study by Neale et al. (2018).

Conventional wastewater treatment decreased the occurrence of cytotoxicity from 38% of the extracts to 7% in case of WWTP 1 (Table 2). In contrast, ozonation increased the number of cytotoxic extracts from 24 to 35% (Table 2). This observation supports earlier hypotheses on

524 the formation of toxic transformation products (TPs) during ozonation (Jia et al. 2015,
525 Lundström et al. 2010, Magdeburg et al. 2014). In contrast to the WWTP samples, only 2% of
526 groundwater extracts were cytotoxic. This is in agreement with the high water quality
527 monitored at GW sampling sites 1–3 (Seitz and Winzenbacher 2017) as well as the rare
528 detection of cytotoxicity in groundwater, unless influenced by landfill leachates, industrial or
529 other contaminated sites (Baumstark-Khan et al. 2005, Baun et al. 2000).
530
531 **3.4.3 Endocrine endpoints**
532 Pooling the results according to water sample type, the highest mean estrogenic activity was
533 found in conventionally treated wastewater (EFF-1, EFF-4, EFF-4-MS) extracted with Telos
534 C18/ENV (pH 2.5) with 5% (n = 4) relative activity and Oasis HLB (pH 2.5) with 5% (n = 4)
535 relative activity (Table S11, Figure S10). Samples extracted at neutral pH with the same SPE
536 sorbents induced lower estrogenic activities (3%, n = 2; 2%, n = 3). Extracts produced with
537 Supelco ENVI-Carb+ showed low estrogenic activity regardless of the adjusted pH.
538 With regard to the anti-estrogenic activity of conventionally treated (EFF) and ozonated
539 (EFF-O_3) wastewater as well as groundwater (GW) both sorbents, Oasis HLB and Telos
540 C18/ENV showed similar effectivity when samples were extracted at pH 2.5 (Figures 4 and
541 S10, Tables S8 and S11). For conventionally treated wastewater (EFF) and groundwater
542 (GW) extracted at neutral pH with the same sorbents the mean anti-estrogenic activity was
543 higher. The highest mean anti-estrogenic activity was found in samples extracted with
544 Supelco ENVI-Carb+ at neutral pH (62–87%, n = 1–2).
545 In case of the anti-androgenic activity of all sample types, acidified samples extracted with
546 Oasis HLB and Telos C18/ENV produced similar results again (Figures 4 and S11). Because
547 of high cytotoxicity, the activities of neutrally extracted samples could not be analysed.
548 Treated wastewater and groundwater extracted with Supelco ENVI-Carb+ at both pH values
549 induced lower anti-androgenic activities than the other SPE methods. As the activity in the

other bioassays was minor, no comparison of the SPE methods on these endpoints was possible (Figures S11–S14).

Based on the above results the Telos C18/ENV sorbent followed by the Oasis HLB recovered highest endocrine activities from the majority of (waste)water samples. However, the Supelco ENVI-Carb+ sorbent was more effective in recovering androgenic activities. This is in part reflected in previous studies. In a study on bottled mineral water, a C18 material recovered higher estrogenic activity compared to the Oasis HLB and Supelco ENVI-Carb+ (Wagner and Oehlmann 2011). The authors argue that non-polar chemicals are responsible for this effect. In the present study, most estrogenicity was recovered by the Telos C18/ENV (involving a similar C18 material), while Oasis HLB achieved comparable levels.

Except for estrogenicity, endocrine activities were more effectively recovered at pH 2.5. However, the more frequent detection of cytotoxicity in pH 7 extracts might have masked the respective activities. Despite the effective extraction of endocrine activities, it remained insufficient from some (waste)waters and endpoints (Figures 3 and S9, Table S8). This includes the anti-estrogenicity, which was enriched from several but not all samples. The difficulty in extracting anti-estrogenic activity has been observed and discussed in previous studies (Giebner et al. 2018).

3.4.4 Genotoxicity and mutagenicity

The highest genotoxicity (IR 4.37) was detected in the Telos C18/ENV pH 2.5 extract of untreated hospital wastewater (HOS, Tables S8 and S9, Figure S14). Seven extracts (100%) of the Oasis HLB and Telos C18/ENV sorbents at both pH 7 and 2.5 of the conventionally treated wastewater of the pilot WWTP 4 (EFF-4 and EFF-4-MS) were genotoxic with induction rates between 1.50 and 1.87. The extracts of a WWTP 1 (INF-1 and EFF-1), except one extract produced with Oasis HLB, pH 2.5, and groundwater (GW-1) did not induce

575 genotoxicity. All extracts produced with Supelco ENVI-Carb+ (pH 7 and pH 2.5) were not
576 active, either.
577 Genotoxicity was enriched from four out of six sampling sites (Figure S14, Tables S8 and S9)
578 but IRs remained only moderately increased compared to the corresponding aqueous samples
579 (except for hospital wastewater). One reason for this could be that genotoxicity of
580 (waste)water samples is generally detected at higher sample enrichment factors (e.g., 100-
581 fold, Keiter et al. 2006, Schulze et al. 2017, Stalter et al. 2016) or at contamination hotspots
582 (Baumstark-Khan et al. 2005, Baun et al. 2000).
583 In line with the efficiency of the Telos C18/ENV pH 2.5 method, Magdeburg et al. (2014)
584 extracted genotoxicity and mutagenicity from wastewater (biological and advanced treatment)
585 using the Oasis HLB at pH 2. Although the authors did not compare different SPE methods,
586 their results seem in agreement with the present results. Mutagenicity and cytotoxicity were
587 also higher in biologically-treated and ozonated wastewater extracted at pH 2 (instead of pH
588 7) using a C18 sorbent (Misik et al. 2011). For the other investigated *in vitro* endpoints, no
589 SPE optimisation study was found in the literature.
590

591 **3.4.5 What is the best SPE method?**

592 Regarding the results of five types of water samples tested with five *in vitro* bioassays the
593 most effective SPE method for the extraction of endocrine activities was Telos C18/ENV pH
594 7 (7x), followed by Telos C18/ENV pH 2.5 and Supelco ENVI-Carb+ pH 7 (each 5x), Oasis
595 HLB pH 7 (4x), Oasis HLB pH 2.5 (2x) and Supelco ENVI-Carb+ pH 2.5 (1x, Table 3). To
596 statistically distinguish between optimal (and non-optimal) SPE methods a multivariate
597 optimisation based on Pareto was implemented (Durmaz et al. 2015, Ehrgott 2000). Pareto
598 computed sample type and bioassay specific "Pareto optimal" methods.
599 The Pareto results are exemplified for conventionally treated wastewater (EFF-4) in five *in*
600 *vitro* bioassays, whereby Pareto is based on the activity percentiles (Table S12) for ranking

601 the SPE methods (Table S13). The best extraction methods ("Pareto best") were Telos
602 C18/ENV pH 7 followed by Oasis HLB pH 7 and Telos C18/ENV pH 2.5 (see Table S13 for
603 detailed results). The ranking of these methods was computed as follow: Instead of looking at
604 the "best" extraction results within a certain matrix, the "worst" results were classified as
605 "false negative responders". The Supelco ENVI-Carb+ method at pH 2.5 was three times
606 "Pareto-worst" as it extracted the lowest activity in a maximal number of bioassays. All other
607 methods performed better. When an extract was cytotoxic, the result was marked with the
608 label "cytotoxic" instead of providing a value. The Pareto algorithm is capable of evaluating
609 data sets with a limited number of such results. In case of an excessive degree of cytotoxicity
610 (HOS and INF-1), the corresponding SPE method was, however, not listed in the respective
611 ranking matrix and the level of relevance decreases for this parameter. This means that the
612 ranking for this parameter is not reaching the "worst" class anymore. This evaluation
613 procedure was performed for all data sets referring to the different samples, SPE methods and
614 *in vitro* bioassays to obtain the following overall ranking of "Pareto optimal" SPE methods:
615 Regarding the five sample types, the method Telos C18/ENV at pH 7 was four times "Pareto
616 best", followed by Oasis HLB pH 7 and pH 2.5 (each 2x, Tables 3 and S14). In terms of the
617 five bioassays, the methods Telos C18/ENV at pH 2.5 and Supelco ENVI-Carb+ at pH 7 were
618 two times "Pareto best", respectively (details in Table S14).
619 Accordingly, the method Telos C18/ENV at pH 7 was "Pareto best" regarding the effectivity
620 in extracting different types of water and wastewater samples with respect to the highest
621 endocrine activities (Table 3). Higher recoveries at neutral pH (over acidic and basic pH)
622 were also observed by Tousova et al. (2017) for several endpoints also investigated in this
623 study. The authors, however, used other sorbents for large volume SPE of surface waters.
624 Summing up the results of the *in vitro* bioassays and Pareto optimisation, the methods Telos
625 C18/ENV pH 7 and Oasis HLB pH 7 were optimal to enrich endocrine activities but also the
626 highest cytotoxicity (Table 2). The corresponding methods at pH 2.5 showed good results as

well as lower cytotoxicity (Tables 2 and S14). The final recommendation for most effective recovery of *in vitro* toxicity from diverse (waste)waters is, thus, to use the Telos C18/ENV method at a sample pH of 7.

4 Challenges in optimising sample preparation for bioassays

Despite the advantages of optimising the sample preparation for bioassay analyses (Muschket et al. 2017, Neale et al. 2018, Ternes et al. 2017), a number of important challenges remain.

The first challenge is that the "true" toxicity of a sample (at a given sampling site and time) remains unknown. The reason for this is that for complex environmental samples, the causative compounds, potential mixture effects and confounding factors (e.g., matrix effects) are largely unspecified. Accordingly, each step of sampling and sample preparation and storage may change the chemical composition of a sample and its toxicity. Active compounds may be added (via contaminated materials) or removed (via adsorption to materials) during sampling, added or removed during transport and storage (via microbial activity) and added or removed during sample preparation.

Second, the differentiation between toxicity caused by anthropogenic pollutants and naturally occurring compounds, often referred to a matrix effects, remains challenging. For instance, our approach in maximising the recovery of toxicities may come at the costs of also maximising matrix effects. One such example is the co-extraction of DOC that may induce artefacts in bioassays for receptor antagonism (Neale and Escher 2014). Several confounding factors resulting in false-positive or negative result need to be considered when interpreting bioassay data (discussed in Giebner et al. 2018). However, sample preparation may not be the appropriate tool to address these. Instead, post-extraction analysis (such as effect-directed analysis) can be a way to separate the toxicity caused by anthropogenic and natural compounds.

The third challenge is the selectivity of sample extraction: While SPE methods with broad selectivity exist, an extraction of chemicals is always selective, resulting in a loss of compounds with low affinity to the sorbent (Köke et al. 2018, Neale et al. 2018, Niss et al. 2018, Stalter et al. 2016). Accordingly, the toxicity of an extract will never fully represent the toxicity of the extracted sample. Thus, the question is rather how much loss in toxicity during

656 extraction is acceptable. One way of addressing this is to compare the toxicity of extracts to
657 aqueous samples (Dagnino et al. 2010, EC 2003). Another way is to optimise the recovery of
658 toxicity. Both strategies were adopted in this study to identify the best extraction method.
659 The forth challenge arises from cytotoxicity masking the effect under investigation, which is
660 often the case at high concentration factors. While cytotoxicity can be considered an
661 important toxicological endpoint by itself outweighing the specific effect is masks, it is most
662 commonly rather regarded an obstacle that needs to be removed. This can be achieved by
663 diluting a sample to a non-cytotoxic concentration (Inoue et al. 2009, 2011, Leusch et al.
664 2017, Neale et al. 2018, Välitalo et al. 2017). However, this also dilutes the effect of interest.
665 Alternative approaches, such as minimising the dilution of aqueous samples (Niss et al. 2018)
666 or reducing exposure times in the bioassay as well as cleaning up the cytotoxicity (e.g., by
667 fractionation), have so far not been widely adopted.
668 These challenges are connected to a range of SPE parameters. Thus, the sorbent (Chang et al.
669 2009, Escher et al. 2005, Stalter et al. 2016), sample volumes (Macova et al. 2011, Schulze et
670 al. 2017), eluting solvents (Lu et al. 2010, Välitalo et al. 2017, Yang et al. 2014), fractionation
671 steps (Leusch et al. 2017, Välitalo et al. 2017) and operating modes such as large volume or
672 multilayer SPE (Köke et al. 2018, Schulze et al. 2017) can be optimised.
673 Acknowledging that it is impractical to perform an optimisation for every sample and every
674 bioassay, a range of case studies for different matrices can be used to evaluate whether
675 specific sample preparation methods perform generally better than others. We have taken such
676 approach in the present study and conclude that the Telos C18/ENV method at neutral sample
677 pH performs best in recovering multiple endocrine activities and cytotoxicity from aqueous
678 samples.

5 Conclusions

1. Acidification of aqueous (waste)water samples significantly alters a range of *in vitro* toxicities, including anti-estrogenic, anti-androgenic and retinoic acid-like activities as well as mutagenicity. Sample filtration has a minor impact on the samples' toxicity.
2. Compared to aqueous samples, solid phase extraction enriches most *in vitro* toxicities. However, some activities (e.g., anti-estrogenicity) remain poorly extractable.
3. When comparing six SPE methods, the choice of the optimal method depends on the matrix as well as the *in vitro* endpoint.
4. In general, an extraction using Telos C18/ENV at a sample pH of 7 was most effective in recovering *in vitro* toxicity from (waste)water samples. However, these methods also co-extract a high cytotoxicity masking other endpoints. Using the same method at a sample pH of 2.5 reduced the extraction of cytotoxicity.
5. Sample preparation needs to be optimised when analysing the toxicity of water samples. While this is a resource-consuming task involving multiple methodological parameters, water quality can only be accurately assessed when the recovery of the toxicity of a sample is maximal.

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1008

1009 **Tables**

1010 **Table 1:** Overview of the investigated samples; WWTP: wastewater treatment plant. Details

1011 on samples 1–14 can be found in Seitz and Winzenbacher (2017).

Sample No.	Type of sample	Sample acronym	Sampling mode
1	untreated wastewater (hospital effluent)	HOS	grab
2	untreated wastewater (WWTP 1 influent)	INF-1	composite
3	conventionally treated wastewater (WWTP 1 effluent)	EFF-1	composite
4	conventionally treated wastewater (WWTP 2 effluent)	EFF-2	composite
5	conventionally treated wastewater (WWTP 3 effluent)	EFF-3	composite
6	conventionally treated wastewater (WTTP 4 influent of a filtration basin)	FB-IN	grab
7	conventionally treated wastewater (WTTP 4 effluent of a filtration basin)	FB-OUT	composite
8	surface water of an infiltration basin	IB (SW)	grab
9	surface water 1 (river)	SW-1	grab
10	surface water 2 (river)	SW-2	grab
11	surface water 3 (river)	SW-3	grab
12	groundwater 1 (hotspot)	GW-1	grab
13	groundwater 2 (hotspot)	GW-2	grab
14	groundwater 3 (hotspot)	GW-3	grab
15	conventionally treated wastewater (pilot WWTP)	EFF-4	composite
16	ozonated conventionally treated wastewater (before microsieve, pilot WWTP)	EFF-4-O_3	composite
17	conventionally treated wastewater (after microsieve, pilot WWTP)	EFF-4-MS	composite
18	ozonated microfiltered conventionally-treated wastewater (pilot WWTP)	EFF-4-MS-O_3	composite
19	tap water (pilot WWTP)	TAP	grab

1012

1013 **Table 2:** Occurrence of cytotoxicity (%) during the analysis of all sample extracts in ten *in*
1014 *vitro* bioassays (except EFF-4-MS (F) and EFF-4-MS-O_3 (F): n = 9) pooled according to SPE
1015 method. Corresponding samples were taken on the same sampling dates in July (D) 2012 and
1016 in January (F) 2013.

sample	Oasis HLB		Telos C18/ENV		Supelco ENVI-Carb+		Sample
	pH 7	pH 2.5	pH 7	pH 2.5	pH 7	pH 2.5	mean
HOS	80	70	100	50	0	0	50 (n = 60)
INF-1	60	50	70	50	0	0	38 (n = 60)
EFF-1	0	0	30	0	10	0	7 (n = 60)
EFF-4	0	0	0	0	0	0	0 (n = 60)
EFF-4-MS (D)	0	0	50	0	0	0	8 (n = 60)
EFF-4-MS (F)	44	0	56	0	44	0	24 (n = 54)
EFF-4-MS-O_3 (F)	78	0	100	0	33	0	35 (n = 54)
GW-1	0	0	0	0	10	0	2 (n = 60)
Method mean	32 (n = 78)	15 (n = 78)	50 (n = 78)	13 (n = 78)	12 (n = 78)	0 (n = 78)	

1017

1018 **Table 3:** Most effective SPE methods for the extraction of estrogenic (YES), anti-estrogenic
1019 (YAES), androgenic (YAS), anti-androgenic (YAAS) and dioxin-like (YDS) activity from
1020 water and wastewater samples (inner table, based on Table S8). In addition, "Pareto best"
1021 methods for each bioassay and sample type were computed. Double/triple listings represent
1022 equally effective methods. Hospital wastewater (HOS) and one WWTP influent (INF-1) were
1023 not analysed due to excessive cytotoxicity. Brackets: activity ≤ 10%; "-": no endocrine
1024 activity/cytotoxicity

Bioassay / Sample type	YES	YAES	YAS	YAAS	YDS	Pareto best: sample type
EFF-1	(Oasis 2.5)	Supelco 7	(Oasis 7)	Oasis 2.5	Telos 7	Oasis 2.5 Telos 7
EFF-4	(Telos 2.5)	Telos 7	(Oasis 7)	Telos 7	Telos 7	Oasis 7 Telos 7 Telos 2.5
EFF-4-MS	(Telos 2.5)	Oasis 7	(Supelco 7)	Oasis 7	Telos 7	Telos 7
EFF-4-MS-O_3	-	Supelco 7	(Supelco 2.5)	Telos 2.5	(Telos 2.5)	Supelco 7
GW-1	(Telos 7)	Telos 7	(Supelco 7)	Telos 2.5	(Supelco 7)	Oasis 7 Oasis 2.5 Telos 7
Pareto best: bioassay	Telos 2.5	Supelco 7	Supelco 7	Telos 2.5 Supelco 2.5	Telos 7	**Telos 7**

1025

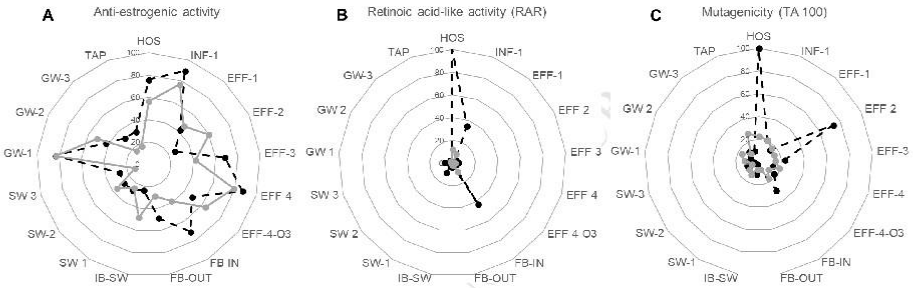

Figure 1: Impact of acidification. Anti-estrogenic activity (A), retinoic acid-like activity (RAR, B) and mutagenicity (Ames TA 100, C) of neutral (black) and acidified (grey) aqueous water and wastewater samples (mean in %). Corresponding samples (INF-1/EFF-1, EFF-4/EFF-4-O_3 and FB-IN/FB-OUT) were taken on the same sampling date in March 2012 and April 2012, respectively.

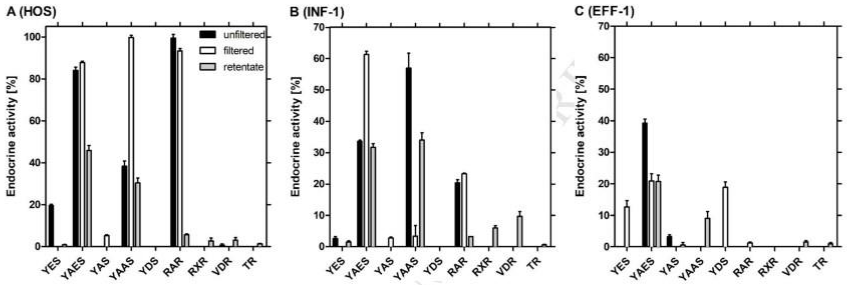

Figure 2: Impact of filtration. Endocrine activity (%, mean ± SEM) of unfiltered (black bars) and filtered (white bars) wastewater samples and the aqueous suspensions of the filter retentate (grey bars). A: untreated hospital wastewater (HOS), B: untreated municipal wastewater of WWTP 1 (INF-1), C: conventionally treated effluent of WWTP 1 (EFF-1). YES: estrogenic, YAES: anti-estrogenic, YAS: androgenic, YAAS: anti-androgenic, YDS: dioxin-like, RAR: retinoic acid-like, RXR: retinoid-X-like, VDR: vitamin D-like, TR: thyronine-like. Corresponding samples (INF-1/EFF-1) taken on the same sampling date in July 2012.

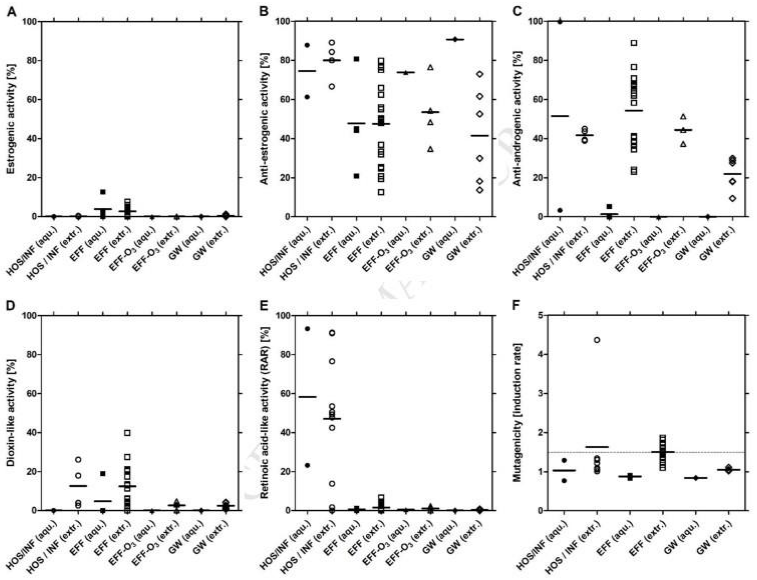

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Figure 3: Comparison of aqueous and extracted samples. Estrogenic (A), anti-estrogenic (B), anti-androgenic (C), dioxin-like (D) and retinoic acid-like (RAR, E) activity in % and genotoxicity as induction rate (umu, F) of the pooled data of aqueous (aqu.) water and wastewater samples (0.63-fold final concentration) and of the corresponding 10.4-fold concentrated SPE extracts (extr.). Symbols: mean activity of the individual sample, line: mean of all samples of one sample type, filled symbol: aqueous sample, clear symbol: SPE extract, HOS: untreated hospital wastewater, INF: untreated influent, EFF: conventionally treated effluent, EFF-O_3: ozonated conventionally treated wastewater, GW: groundwater. Corresponding samples were taken within the same sampling period in July 2012 and January 2013.

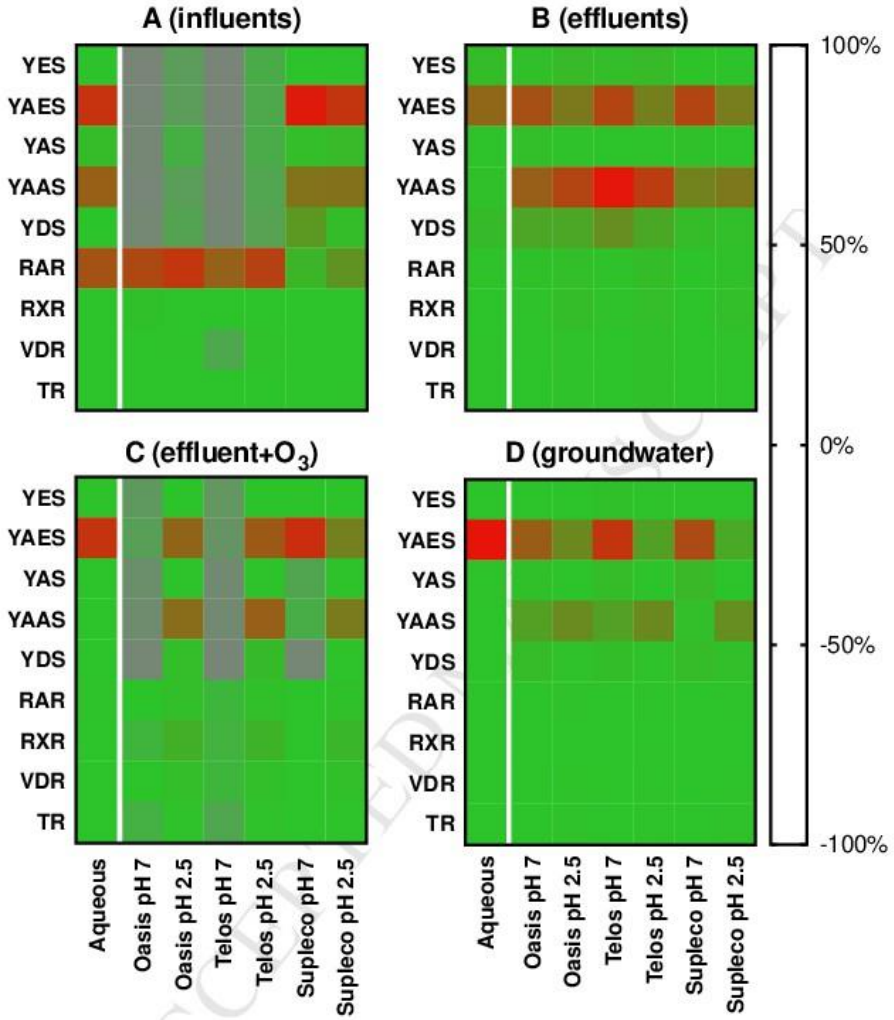

Figure 4: Comparison of the six SPE methods. Endocrine activity (0% to 100%) and cytotoxicity (0% to -100%) of aqueous samples and the corresponding SPE extracts (0.63 and 10.4-fold final concentration, respectively) of wastewater treatment plant influents (A), effluents (B), ozonated effluent (C) and groundwater (D). Six SPE methods were compared: Oasis HLB, Telos C18/ENV and Supelco ENVI-Carb+ extraction at pH 7 and pH 2.5. The results were pooled from the different samples according to water type. Green: 0.0% endocrine activity/cytotoxicity, red: 100% endocrine activity, grey: 100% cytotoxicity.

What you extract is what you see: Optimising the preparation of water and wastewater samples for *in vitro* bioassays

Aennes Abbas & Ilona Schneider, Anna Bollmann, Jan Funke, Jörg Oehlmann, Carsten Prasse, Ulrike Schulte-Oehlmann, Wolfram Seitz, Thomas Ternes, Marcus Weber, Henning Wesely, Martin Wagner

HIGHLIGHTS

- Acidification of (waste)water samples significantly affects their *in vitro* toxicity
- Filtration does not affect the toxicity of most (waste)water samples
- All six SPE methods recovered *in vitro* toxicity, depending on endpoints/matrices
- Best SPE methods were identified for each matrix and endpoint
- Multivariate optimisation identified Telos C18/ENV (pH7) as overall best SPE method

Supplementary information (paper A.1)

1 Material and methods

Table S1: Overview of the bioassays used in this study, including endpoints (in brackets), concentration range [mol L^{-1}] of the respective reference compound (positive control), background agonists and EC_{50} values.

In vitro bioassay	Positive control	Concentration range [mol L^{-1}]	EC_{50}-values
YES (estrogenicity)	17β-estradiol (E_2) (CAS: 50-28-2)	1.0×10^{-12} - 1.0×10^{-08}	1.23×10^{-10}
YAES (anti-estrogenicity)	4-Hydroxytamoxifen (OHT, CAS: 68392-35-8) background agonist: 0.1 nmol/L 17β-estradiol (E_2)	1.25×10^{-06} - 8.0×10^{-05}	6.53×10^{-06}
YAS (androgenicity)	Testosterone (T, CAS: 58-22-0)	3×10^{-11} - 1.0×10^{-07}	4.36×10^{-09}
YAAS (anti-androgenicity)	Flutamide (Flu, CAS: 13311-84-7) background agonist: 3 nmol/L testosterone	7.81×10^{-07} - 5.0×10^{-05}	3.13×10^{-06}
YDS (dioxin-like)	β-Naphthoflavone (β-NF, CAS: 6051-87-2)	1.0×10^{-09} - 1.0×10^{-05}	1.19×10^{-07}
RAR (vitamin A-like)	All-trans retinoic acid (at-RA, CAS: 302-79-4)	1.0×10^{-09} - 3.0×10^{-06}	3.14×10^{-08}
RXR (vitamin A-like)	9-cis retinoic acid (9-cis-RA, CAS: 5300-03-8)	1.0×10^{-08} - 1.0×10^{-05}	4.50×10^{-07}
VDR (vitamin D-like)	1α,25-Dihydroxyvitamin D3 (Calcitriol, CAS: 322222-06-3)	1.0×10^{-10} - 3.0×10^{-07}	5.28×10^{-08}
TR (thyronine-like)	3,3′,5-Triiod-L-thyronine (T_3, CAS: 6893-02-3)	1.0×10^{-09} - 3.0×10^{-06}	2.23×10^{-07}
Ames (TA98)	4-Nitro-*o*-phenylenediamine (4-NOPD, CAS: 99-56-9)	10 mg/L	-
Ames (TA100)	Nitrofurantoin (NF, CAS: 67-20-9)	0.25 mg/L	-
Umu-test (genotoxicity)	4-Nitroquinoline N-oxide (4-NQO, CAS 56-57-5)	5.0 - 2000 µg/L	-

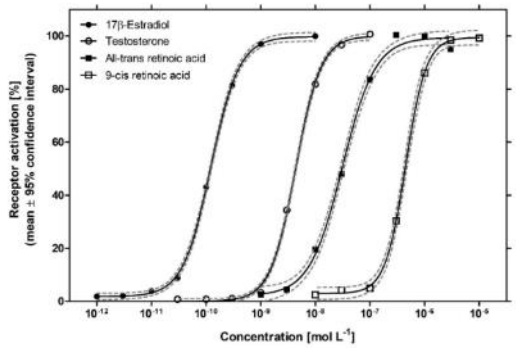

Figure S1: Receptor activation (%, mean ± 95% confidence interval) as concentration-response relationships of six (RAR, RXR) and seven (YES, YAS) experiments at the human estrogen (left), androgen (second left), retinoic acid receptor (third left) and retinoid X (right) receptor (YES: 17β-estradiol; YAS: testosterone; RAR: all-trans retinoic acid; RXR: 9-cis retinoic acid).

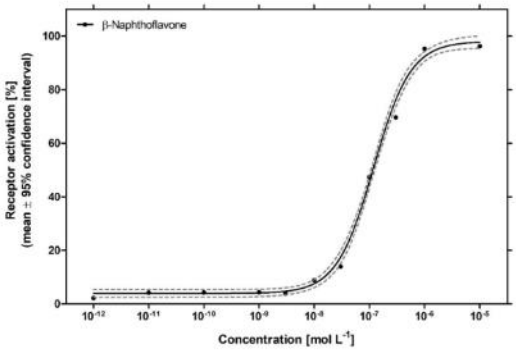

Figure S2: Receptor activation (%, mean ± 95% confidence interval) as concentration-response relationship of seven experiments at the human aryl-hydrocarbon receptor (YDS: β-Naphthoflavone).

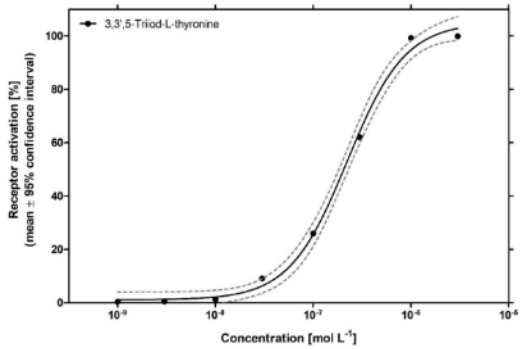

Figure S3: Receptor activation (%, mean ± 95% confidence interval) as concentration-response relationship of six experiments at the human thyronine receptor (TR: 3,3',5-triiod-L-thyronine).

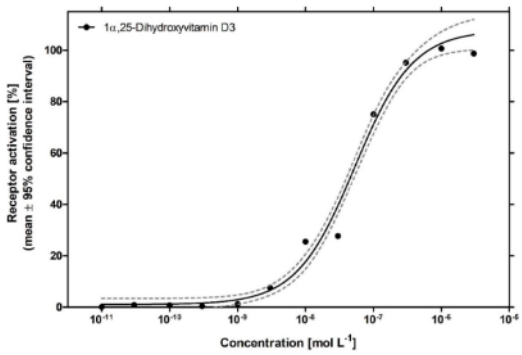

Figure S4: Receptor activation (%, mean ± 95% confidence interval) as concentration-response relationship of six experiments at the human vitamin D receptor (VDR: 1α,25-dihydroxyvitamin D3).

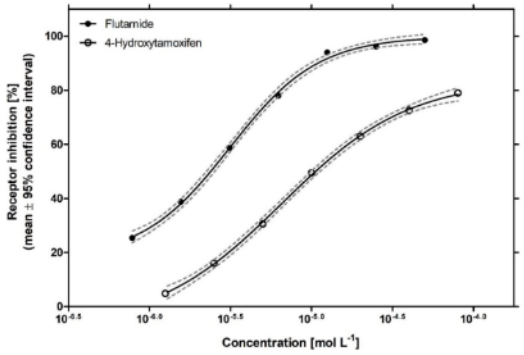

Figure S5: Receptor inhibition (%, mean ± 95% confidence interval) as concentration-response relationships of seven experiments, each, at the human androgen (left) and estrogen (right) receptor (YAAS: flutamide; YAES: 4-hydroxytamoxifen)).

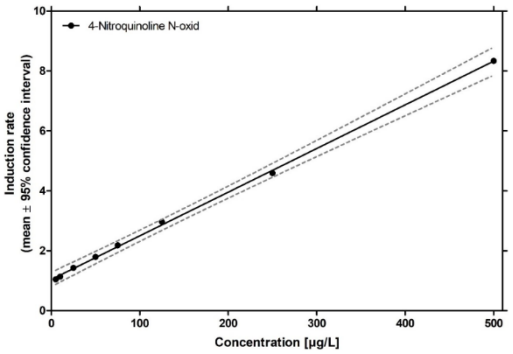

Figure S6: Induction rate (mean ± 95% confidence interval) of the positive control as linear regression of seven experiments of the umu test (4-nitroquinoline N-oxid)

2 Results and discussion

2.1 Sample acidification

Table S2: Estrogenic (YES), anti-estrogenic (YAES) and androgenic (YAS) activity (%; mean ± SEM) of neutral and acidified (pH 2) aqueous samples. Significant differences between neutral and acidified samples are marked with asterisks: ★ $p \leq 0.05$, ★★ $p \leq 0.01$, ★★★ $p \leq 0.001$ (unpaired t-test), n.s.: not significant. Corresponding samples were taken on the same sampling dates in March (A) and April (B) 2012.

sample	*in vitro* bioassay								
	YES		significance	YAES		significance	YAS		significance
	neutral	pH 2	neutral/pH 2	neutral	pH 2	neutral/pH 2	neutral	pH 2	neutral/pH 2
HOS (B)	1.38 ± 0.22	1.80 ± 0.96	n.s.	75.8 ± 1.88	56.5 ± 1.30	★★★	25.7 ± 0.52	9.61 ± 0.52	★★★
INF-1 (B)	0.0	0.0	n.s.	89.8 ± 0.63	76.9 ± 1.08	★★★	19.4 ± 0.82	16.5 ± 0.50	★★
EFF-1 (B)	0.0	0.0	n.s.	41.8 ± 1.89	46.5 ± 1.82	n.s.	0.0	0.84 ± 0.24	★★★
EFF-2 (B)	0.0	0.0	n.s.	26.1 ± 2.66	60.0 ± 0.80	★★★	0.0	0.35 ± 0.32	★★
EFF-3 (B)	0.0	0.0	n.s.	68.4 ± 1.61	41.9 ± 2.10	★★★	0.0	1.07 ± 0.20	★★★
EFF-4 (A)	2.18 ± 0.36	1.92 ± 0.38	n.s.	86.9 ± 1.50	79.0 ± 1.30	★★	0.0	0.80 ± 0.41	★★★
EFF-4-O_3 (A)	0.08 ± 1.10	0.39 ± 1.08	n.s.	48.3 ± 1.28	63.5 ± 0.71	★★★	0.0	1.47 ± 0.12	★★★
FB-IN (B)	1.41 ± 0.24	1.08 ± 0.12	n.s.	71.0 ± 2.19	38.6 ± 0.99	★★★	30.3 ± 1.54	15.7 ± 1.46	★★★
FB-OUT (B)	0.0	0.0	n.s.	48.9 ± 1.49	28.8 ± 1.22	★★★	0.19 ± 0.07	0.16 ± 0.21	n.s.
IB (SW) (B)	0.0	0.0	n.s.	23.6 ± 1.07	48.3 ± 1.49	★★★	0.12 ± 0.05	0.55 ± 0.08	★★★
SW-1 (B)	0.0	0.0	n.s.	27.1 ± 0.61	24.9 ± 1.49	n.s.	0.0	0.0	n.s.
SW-2 (B)	0.0	0.0	n.s.	27.8 ± 2.32	35.6 ± 0.77	★★	0.0	0.44 ± 0.29	★★
SW-3 (B)	2.11 ± 1.87	0.0	n.s.	26.9 ± 1.12	12.8 ± 1.43	★★★	0.0	0.39 ± 0.25	★★★
GW-1 (B)	0.0	0.0	n.s.	83.6 ± 1.47	83.1 ± 1.25	n.s.	0.0	0.98 ± 0.11	★★★
GW-2 (B)	0.0	0.0	n.s.	42.9 ± 2.32	51.5 ± 1.73	★★	0.0	0.0	n.s.
GW-3 (B)	0.0	0.0	n.s.	31.7 ± 1.69	16.4 ± 0.31	★★★	0.0	0.61 ± 0.13	★★★
TAP (A)	2.21 ± 0.63	0.52 ± 0.43	★	31.5 ± 3.96	17.5 ± 4.65	★	0.0	1.14 ± 0.18	★★★

Table S2 continued: Anti-androgenic (YAAS), dioxin-like (YDS) and retinoic acid-like (RAR) activity (%; mean ± SEM) of neutral and acidified (pH 2) aqueous samples. Significant differences between neutral and acidified samples are marked with asterisks: ★ $p \leq 0.05$, ★★ $p \leq 0.01$, ★★★ $p \leq 0.001$ (unpaired t-test), n.s.: not significant. Corresponding samples were taken on the same sampling dates in March (A) and April (B) 2012.

sample	*in vitro* bioassay								
	YAAS		significance	YDS		significance	RAR		significance
	neutral	pH 2	neutral/pH 2	neutral	pH 2	neutral/pH 2	neutral	pH 2	neutral/pH2
HOS (B)	0.0	0.0	n.s.	46.4 ± 2.00	5.22 ± 0.98	★★★	100 ± 2.29	12.2 ± 4.47	★★★
INF-1 (B)	0.0	0.0	n.s.	16.3 ± 2.77	1.92 ± 0.67	★★★	35.1 ± 0.95	8.68 ± 1.36	★★★
EFF-1 (B)	0.0	0.0	n.s.	0.0	0.0	n.s.	3.92 ± 1.10	1.22 ± 0.44	★
EFF-2 (B)	5.38 ± 4.70	0.0	n.s.	0.0	0.0	n.s.	4.16 ± 0.35	1.68 ± 0.72	★★
EFF-3 (B)	8.51 ± 3.64	7.95 ± 4.10	n.s.	0.0	0.0	n.s.	5.17 ± 1.85	2.47 ± 0.43	n.s.
EFF-4 (A)	21.3 ± 1.38	17.0 ± 3.82	n.s.	0.0	0.0	n.s.	3.61 ± 0.31	0.71 ± 0.33	★★★
EFF-4-O_3 (A)	0.0	0.0	n.s.	0.0	0.0	n.s.	1.58 ± 0.86	0.68 ± 0.51	n.s.
FB-IN (B)	0.0	0.0	n.s.	37.0 ± 1.08	4.96 ± 1.00	★★★	42.7 ± 2.35	8.49 ± 0.44	★★★
FB-OUT (B)	0.67 ± 1.42	0.0	★★★	0.0	0.0	n.s.	2.60 ± 0.32	1.67 ± 0.47	n.s.
IB (SW) (B)	0.0	0.0	n.s.	0.0	0.0	n.s.	3.59 ± 0.28	1.72 ± 0.35	★★★
SW-1 (B)	0.0	0.0	n.s.	0.0	0.0	n.s.	10.0 ± 4.96	0.27 ± 0.16	n.s.
SW-2 (B)	0.0	0.0	n.s.	0.0	0.0	n.s.	1.52 ± 0.45	0.75 ± 0.79	n.s.
SW-3 (B)	0.0	0.0	n.s.	0.0	0.0	n.s.	2.85 ± 1.49	0.0	★
GW-1 (B)	10.5 ± 4.75	16.3 ± 5.70	n.s.	0.0	0.0	n.s.	6.97 ± 2.48	1.28 ± 0.29	n.s.
GW-2 (B)	0.0	0.0	n.s.	0.0	0.0	n.s.	2.74 ± 0.30	0.78 ± 0.50	★★
GW-3 (B)	0.0	0.0	n.s.	0.0	0.0	n.s.	3.50 ± 0.84	2.62 ± 0.73	n.s.
TAP (A)	0.91 ± 4.81	7.19 ± 4.80	n.s.	0.0	0.0	n.s.	1.71 ± 0.14	0.0	★★★

Table S2 continued: Retinoid X-like (RXR), vitamin D-like (VDR) and thyronin-like (TR) activity (%; mean ± SEM) of neutral and acidified (pH 2) aqueous samples. Significant differences between neutral and acidified samples are marked with asterisks: ★ $p \leq 0.05$, ★★ $p \leq 0.01$, ★★★ $p \leq 0.001$ (unpaired t-test), n.s.: not significant. Corresponding samples were taken on the same sampling dates in March (A) and April (B) 2012.

sample	*in vitro* bioassay								
	RXR		significance	VDR		significance	TR		significance
	neutral	pH 2	neutral/pH 2	neutral	pH 2	neutral/pH 2	neutral	pH 2	neutral/pH 2
HOS (B)	25.0 ± 7.01	0.0	★★★	4.19 ± 1.10	0.0	★★★	0.38 ± 0.28	0.47 ± 0.28	n.s.
INF-1 (B)	32.2 ± 4.50	0.0	★★★	1.99 ± 1.36	0.0	★★	0.86 ± 0.24	0.0	★★
EFF-1 (B)	0.0	0.0	n.s.	0.0	0.0	n.s.	0.0	0.0	n.s.
EFF-2 (B)	0.0	0.0	n.s.	0.0	0.0	n.s.	0.0	0.0	n.s.
EFF-3 (B)	0.0	10.2 ± 3.53	n.s.	0.0	0.0	n.s.	0.0	0.0	n.s.
EFF-4 (A)	0.46 ± 0.15	0.88 ± 0.32	n.s.	0.36 ± 0.19	0.21 ± 0.13	n.s.	0.0	0.0	n.s.
EFF-4-O_3 (A)	0.0	9.74 ± 4.36	★★	0.0	0.0	n.s.	0.0	0.09 ± 0.21	n.s.
FB-IN (B)	36.8 ± 7.32	0.0	★★★	0.94 ± 1.18	0.0	★	0.08 ± 0.27	0.0	★★★
FB-OUT (B)	0.0	0.0	n.s.	0.0	0.0	n.s.	0.22 ± 0.24	0.0	★
IB (SW) (B)	13.1 ± 8.06	0.0	n.s.	0.45 ± 0.43	0.0	★	0.50 ± 0.27	0.0	★★
SW-1 (B)	0.0	0.0	n.s.	0.0	0.0	n.s.	0.0	0.0	n.s.
SW-2 (B)	0.0	0.0	n.s.	0.0	0.0	n.s.	0.0	0.0	n.s.
SW-3 (B)	0.0	9.74 ± 4.07	★	0.0	4.63 ± 1.50	★★★	0.0	0.0	n.s.
GW-1 (B)	50.5 ± 7.02	32.7 ± 2.18	★	0.94 ± 0.53	0.49 ± 0.27	n.s.	1.43 ± 0.15	0.0	★★★
GW-2 (B)	0.0	0.0	n.s.	0.0	0.0	n.s.	0.0	0.0	n.s.
GW-3 (B)	0.0	18.7 ± 3.05	★★★	0.0	0.50 ± 0.25	★★★	0.0	0.14 ± 0.27	★★
TAP (A)	0.25 ± 0.27	0.25 ± 0.27	n.s.	0.19 ± 0.11	0.22 ± 0.11	n.s.	0.0	0.0	n.s.

Table S2 continued: Genotoxicity (umu: induction rate as mean ± SEM]) and mutagenicity (Ames, % as mean]) of neutral and acidified (pH 2) aqueous samples; umu: potential genotoxicity if induction rate is ≥ 1.5, Ames: potential mutagenicity if mean is ≥ 20.8%. Significant differences between neutral and acidified samples are marked with asterisks: ★ $p \leq 0.05$, ★★ $p \leq 0.01$, ★★★ $p \leq 0.001$ (umu: unpaired t-test, Ames: Fisher's exact test), n.s.: not significant. Corresponding samples were taken on the same sampling dates in March (A) and April (B) 2012.

sample	*in vitro* bioassay								
	Umu		significance	Ames TA98		significance	Ames TA100		significance
	neutral	pH 2	neutral/pH 2	neutral	pH 2	neutral/pH 2	neutral	pH 2	neutral/pH 2
HOS (B)	0.96 ± 0.02	0.92 ± 0.04	n.s.	100	6.25	★★★	100	22.9	★★★
INF-1 (B)	0.92 ± 0.03	0.98 ± 0.04	n.s.	6.25	4.17	n.s.	20.8	20.8	n.s.
EFF-1 (B)	0.85 ± 0.03	0.74 ± 0.02	★★	0.0	8.33	n.s.	14.6	16.7	n.s.
EFF-2 (B)	0.95 ± 0.06	0.94 ± 0.05	n.s.	66.7	0.0	★★★	72.9	14.6	★★★
EFF-3 (B)	0.87 ± 0.04	0.87 ± 0.03	n.s.	6.25	4.17	n.s.	22.9	14.6	n.s.
EFF-4 (A)	0.80 ± 0.02	1.11 ± 0.01	★★★	4.17	6.25	n.s.	12.5	18.8	n.s.
EFF-4-O_3 (A)	0.86 ± 0.03	0.89 ± 0.04	n.s.	6.25	8.33	n.s.	16.7	10.4	n.s.
FB-IN (B)	0.83 ± 0.02	0.94 ± 0.03	★★	6.25	6.25	n.s.	29.2	16.7	n.s.
FB-OUT (B)	1.21 ± 0.03	0.87 ± 0.03	★★★	4.17	4.17	n.s.	6.25	6.25	n.s.
IB (SW) (B)	0.98 ± 0.02	1.20 ± 0.05	★★★	0.0	10.4	n.s.	10.4	6.25	n.s.
SW-1 (B)	1.01 ± 0.07	0.95 ± 0.02	n.s.	2.08	10.4	n.s.	2.08	12.5	n.s.
SW-2 (B)	1.15 ± 0.05	0.63 ± 0.03	★★★	6.25	4.17	n.s.	10.4	10.4	n.s.
SW-3 (B)	0.91 ± 0.05	0.99 ± 0.06	n.s.	4.17	2.08	n.s.	10.4	6.25	n.s.
GW-1 (B)	0.96 ± 0.04	0.98 ± 0.05	n.s.	4.17	4.17	n.s.	4.17	12.5	n.s.
GW-2 (B)	1.21 ± 0.04	0.61 ± 0.03	★★★	2.08	2.08	n.s.	8.33	16.7	n.s.
GW-3 (B)	0.87 ± 0.02	0.85 ± 0.02	n.s.	8.33	0.0	n.s.	12.5	10.4	n.s.
TAP (A)	0.86 ± 0.02	1.86 ± 0.08	★★★	2.08	4.17	n.s.	10.4	27.1	n.s.

Table S3: Percentage of *in vitro* bioassays (n = 11 per sample, umu test excluded) in which acidification caused a change in activity of ≥ 10% within one sample type. Corresponding samples were taken on the same sampling dates in March (A) and April (B) 2012.

Type of sample	[%]	
untreated wastewater (HOS (B), INF-1 (B))	50.0	n = 22
influent and effluent of a filtration basin (FB-IN (B), FB-OUT (B))	31.8	n = 22
surface water of an infiltration basin (IB (SW) (B))	18.2	n = 11
tap water (TAP (A))	18.2	n = 11
conventionally treated wastewater (EFF-1 (B), EFF-2 (B), EFF-3 (B), EFF-4 (A))	11.4	n = 44
groundwater (hotspots; GW-1 (B), GW-2 (B), GW-3 (B))	9.1	n = 33
ozone-treated wastewater (EFF-4-O_3 (A))	9.1	n = 11
surface water (SW-1 (B), SW-2 (B), SW-3 (B))	3.0	n = 33

Table S4: Percentage of the number of analysed samples (n = 17 per bioassay) in which acidification caused a change in the activity of ≥ 10% in one *in vitro* bioassay.

Type of *in vitro* bioassay	[%]
YAES	64.7
RXR	41.2
Ames TA100	23.5
RAR	17.6
YDS	17.6
YAS	11.8
Ames TA98	11.8
YES	0.0
YAAS	0.0
VDR	0.0
TR	0.0

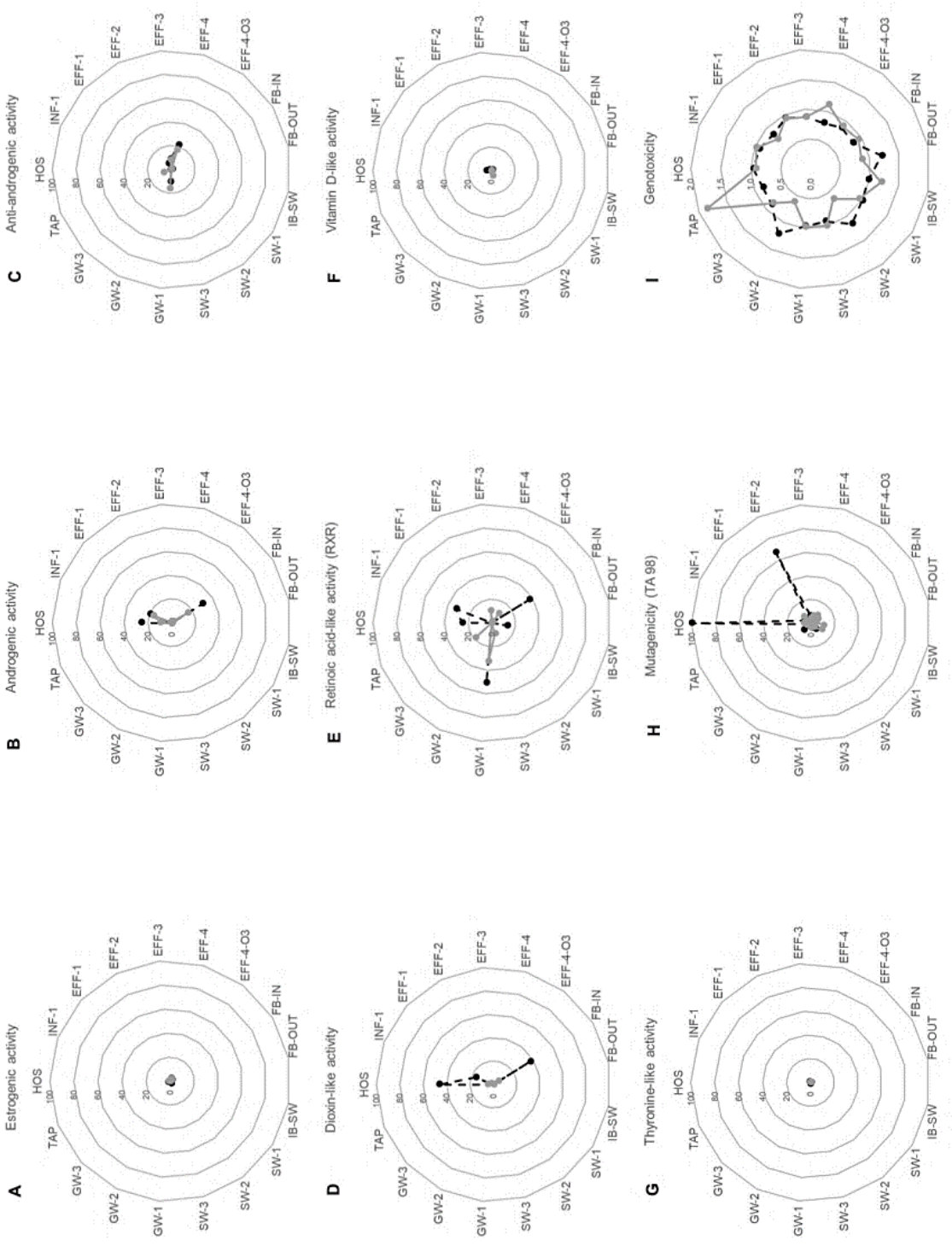

Figure S7: Estrogenic (A), androgenic (B), anti-androgenic (C), dioxin-like (D), retinoic acid-like (RXR, E), vitamin D-like (F) and thyronine-like (G) activity, mutagenicity (TA98, H) and genotoxicity (I) in % of neutral (black) and acidified (grey) aqueous water and wastewater samples. Corresponding samples were taken on the same sampling dates in March and April 2012, respectively.

2.2 Sample filtration

Table S5: Endocrine activity (%; mean ± SEM), genotoxicity (umu: induction rate [mean ± SEM]) and mutagenicity (Ames [%; mean]) of unfiltered and filtered samples (Whatman GF6) and aqueous suspensions of the filter retentate; n.a.: not analysed. Umu: genotoxic if induction rate is ≥ 1.5; Ames: mutagenic if mean is ≥ 20.8%. Significant differences between unfiltered and filtered samples are marked with asterisks: ★ $p \leq 0.05$, ★★ $p \leq 0.01$, ★★★ $p \leq 0.001$ (endocrine activity and umu: unpaired t-test, Ames: Fisher's exact test), n.s.: not significant. Samples taken in March (A), middle of July (C), end of July (D) 2012 and December (E) 2012.

sample	*in vitro* bioassay	unfiltered	filtered	aqueous suspension	significance unfiltered/filtered
HOS (C)	YES	19.6 ± 0.61	0.0	0.75 ± 0.24	★★★
	YAES	84.1 ± 1.47	87.8 ± 0.68	45.9 ± 2.33	★
	YAS	0.0	5.28 ± 0.37	0.0	★★★
	YAAS	38.3 ± 2.49	99.7 ± 1.07	30.3 ± 2.34	★★★
	YDS	0.0	0.0	0.0	n.s.
	RAR	99.4 ± 1.78	93.3 ± 1.18	5.66 ± 0.33	★
	RXR	0.0	0.0	2.70 ± 1.31	n.s.
	VDR	0.64 ± 0.64	0.0	3.11 ± 1.22	★★★
	TR	0.0	0.0	1.32 ± 0.22	n.s.
	Umu	1.10 ± 0.11	1.29 ± 0.12	0.85 ± 0.05	n.s.
INF-1 (C)	YES	2.56 ± 0.67	0.0	1.48 ± 0.36	★★★
	YAES	33.6 ± 0.35	61.3 ± 1.03	31.7 ± 1.15	★★★
	YAS	0.0	2.74 ± 0.33	0.0	★★★
	YAAS	57.0 ± 4.70	3.31 ± 3.46	34.0 ± 2.32	★★★
	YDS	0.0	0.0	0.0	n.s.
	RAR	20.4 ± 0.97	23.2 ± 0.27	3.21 ± 0.07	★
	RXR	0.0	0.0	5.93 ± 0.75	n.s.
	VDR	0.0	0.0	9.69 ± 1.50	n.s.
	TR	0.0	0.0	0.53 ± 0.21	n.s.
	Umu	0.77 ± 0.04	0.77 ± 0.04	0.79 ± 0.05	n.s.

Table S5 continued.

sample	*in vitro* bioassay	unfiltered	filtered	aqueous suspension	significance unfiltered/filtered
EFF-1 (C)	YES	0.0	12.7 ± 1.98	0.0	★★★
	YAES	39.2 ± 1.32	20.9 ± 2.32	20.7 ± 2.06	★★★
	YAS	3.21 ± 0.58	0.0	0.56 ± 0.78	★★
	YAAS	0.0	0.0	9.04 ± 2.12	n.s.
	YDS	0.0	18.9 ± 1.69	0.0	★★★
	RAR	0.0	1.23 ± 0.27	0.0	★★★
	RXR	0.0	0.0	0.0	n.s.
	VDR	0.0	0.0	1.51 ± 0.51	n.s.
	TR	0.0	0.0	1.01 ± 0.33	n.s.
	Umu	0.75 ± 0.03	0.83 ± 0.06	0.73 ± 0.04	n.s.
EFF-1 (E)	YES	0.0	0.0	n.a.	n.s.
	YAES	29.0 ± 2.40	22.6 ± 1.43	n.a.	★
	YAS	0.0	1.20 ± 0.49	n.a.	★★★
	YAAS	0.0	0.0	n.a.	n.s.
	YDS	0.0	0.0	n.a.	n.s.
	RAR	0.0	0.0	n.a.	n.s.
	RXR	n.a.	n.a.	n.a.	n.a.
	VDR	n.a.	n.a.	n.a.	n.a.
	TR	12.1 ± 0.41	9.74 ± 1.53	n.a.	n.s.
	Umu	n.a.	n.a.	n.a.	n.a.
EFF-4 (A)	YES	0.0	0.0	n.a.	n.s.
	YAES	60.6 ± 1.91	59.2 ± 1.23	n.a.	n.s.
	YAS	0.0	0.0	n.a.	n.s.
	YAAS	0.0	0.0	n.a.	n.s.
	YDS	0.0	0.0	n.a.	n.s.
	RAR	2.74 ± 0.30	9.16 ± 4.01	n.a.	n.s.
	RXR	0.0	0.0	n.a.	n.s.
	VDR	0.0	1.64 ± 2.11	n.a.	n.s.
	TR	0.0	0.15 ± 0.11	n.a.	n.s.
	Umu	1.05 ± 0.04	0.87 ± 0.03	n.a.	★★
	Ames TA98	4.17	0.0	n.a.	n.s.
	Ames TA100	14.6	18.8	n.a.	n.s.

Table S5 continued.

sample	*in vitro* bioassay	unfiltered	filtered	aqueous suspension	significance unfiltered/filtered
SW-1 (E)	YES	0.0	0.0	n.a.	n.s.
	YAES	17.7 ± 0.97	31.0 ± 2.11	n.a.	★★★
	YAS	0.0	0.12 ± 0.58	n.a.	n.s.
	YAAS	0.0	0.0	n.a.	n.s.
	YDS	0.0	0.0	n.a.	n.s.
	RAR	0.0	0.0	n.a.	n.s.
	RXR	n.a.	n.a.	n.a.	n.a.
	VDR	n.a.	n.a.	n.a.	n.a.
	TR	9.81 ± 0.91	9.07 ± 0.81	n.a.	n.s.
	Umu	n.a.	n.a.	n.a.	n.a.
GW-1 (C)	YES	0.0	0.0	2.17 ± 1.36	n.s.
	YAES	85.6 ± 0.47	90.7 ± 0.52	79.5 ± 0.57	★★★
	YAS	3.00 ± 0.63	0.43 ± 1.44	0.0	n.s.
	YAAS	0.0	0.0	45.2 ± 9.34	n.s.
	YDS	0.0	0.0	0.0	n.s.
	RAR	0.17 ± 0.21	0.0	1.33 ± 0.19	★
	RXR	0.0	0.0	5.25 ± 0.92	n.s.
	VDR	0.0	0.0	2.95 ± 0.96	n.s.
	TR	0.0	0.0	1.99 ± 0.69	n.s.
	Umu	0.82 ± 0.04	0.84 ± 0.04	0.85 ± 0.05	n.s.
EFF-4 (D)/ EFF-4-MS (D)	YES	0.0	0.0	n.a.	n.s.
	YAES	45.1 ± 0.81	80.8 ± 0.53	n.a.	★★★
	YAS	0.05 ± 0.69	0.0	n.a.	n.s.
	YAAS	0.0	5.24 ± 4.52	n.a.	n.s.
	YDS	0.0	0.0	n.a.	n.s.
	RAR	0.40 ± 0.16	0.65 ± 0.14	n.a.	n.s.
	RXR	0.0	0.0	n.a.	n.s.
	VDR	0.64 ± 0.75	0.0	n.a.	n.s.
	TR	0.96 ± 0.34	0.0	n.a.	n.s.
	Umu	0.89 ± 0.04	0.91 ± 0.05	n.a.	n.s.

Table S6: Percentage of *in vitro* bioassays (except umu test) in which filtration caused a change in activity of ≥ 10% within one sample type. Corresponding samples were taken on the same sampling dates in March (A), in the middle of July (C), at the end of July (D) and in December 2012.

Type of sample	[%]	
untreated wastewater (HOS (C), INF-1 (C))	22.2	n = 18
surface water (SW-1 (E))	14.3	n = 7
conventionally treated wastewater (EFF-1 (C), EFF-1 (E), EFF-4 (A), EFF-4 (D))	11.1	n = 36
groundwater (hotspot; GW-1 (C))	0.0	n = 9

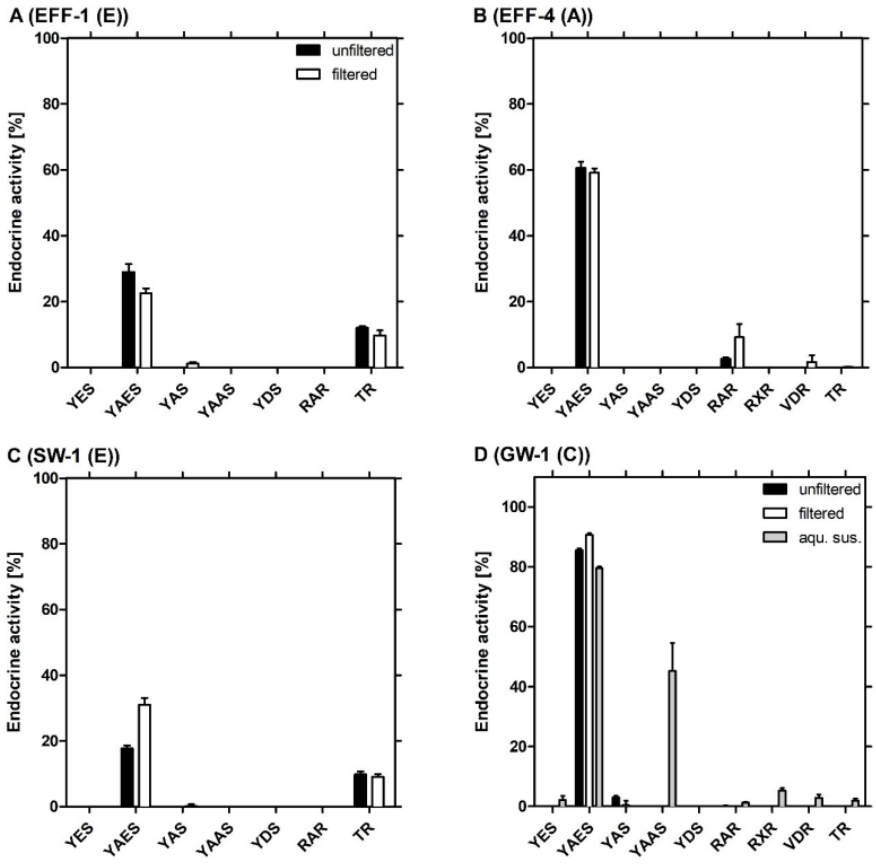

Figure S8: Endocrine activity (%, mean ± SEM) of unfiltered (black bars) and filtered (white bars) water and wastewater samples and the aqueous suspensions of the filter retentate (grey bars) of conventionally treated wastewater (A and B: effluents of two WWTPs (EFF-1 and EFF-4)), surface water (C: SW-1) and groundwater (D: GW-1). YES: estrogenic; YAES: anti-estrogenic; YAS: androgenic; YAAS: anti-androgenic; YDS: dioxin-like, RAR: retinoic acid-like, RXR: retinoid X-like, VDR: vitamin D-like, TR: thyronine-like. No aqueous suspension of the filter retentate was analysed in A, B and C. No RXR and VDR assays were performed in A and C. Corresponding samples were taken on the same sampling dates in March (A), July (C) and December (E) 2012.

Table S7: Percentage of the number of analysed samples in which filtration caused a change in the activity of ≥ 10% within one type of *in vitro* bioassay.

Type of *in vitro* bioassay	[%]	
YAES	50.0	n = 8
YES	25.0	n = 8
YAAS	25.0	n = 8
YDS	12.5	n = 8
YAS	0.0	n = 8
RAR	0.0	n = 8
RXR	0.0	n = 6
VDR	0.0	n = 6
TR	0.0	n = 6
Ames TA98	0.0	n = 1
Ames TA100	0.0	n = 1

2.3 Solid Phase Extraction

Table S8: Endocrine activity (%, mean ± SEM) and genotoxicity (umu: induction rate, mean ± SEM) of aqueous samples and 10-fold concentrated SPE extracts, ☠: cytotoxic. Umu: potential genotoxicity if induction rate is ≥ 1.5. Corresponding samples were taken on the same sampling date in the middle of July (C) 2012, the end of July (D) 2012 and in January (F) 2013

sample	*in vitro* bioassay	aqueous	Oasis HLB		Telos C18/ENV		Supelco ENVI-Carb+	
			pH 7	pH 2.5	pH 7	pH 2.5	pH 7	pH 2.5
HOS (C)	YES	0.0	☠	☠	☠	☠	0.0	0.35 ± 0.26
	YAES	87.8 ± 0.68	☠	☠	☠	☠	89.1 ± 0.74	66.6 ± 1.41
	YAS	5.28 ± 0.37	☠	☠	☠	☠	0.0	6.67 ± 0.83
	YAAS	99.7 ± 1.07	☠	☠	☠	☠	38.9 ± 5.40	39.4 ± 4.95
	YDS	0.0	☠	☠	☠	☠	26.1 ± 0.67	2.58 ± 0.22
	RAR	93.3 ± 1.18	76.6 ± 1.22	91.3 ± 1.39	☠	91.0 ± 2.12	13.8 ± 0.65	47.8 ± 0.98
	RXR	0.0	2.75 ± 0.63	☠	☠	0.0	0.0	0.0
	VDR	0.0	☠	0.77 ± 0.26	☠	0.0	0.0	0.0
	TR	0.0	☠	0.0	☠	0.0	0.0	0.0
	Umu	1.29 ± 0.12	☠	☠	☠	4.37 ± 0.19	1.22 ± 0.04	1.01 ± 0.04
INF-1 (C)	YES	0.0	☠	☠	☠	☠	0.0	0.0
	YAES	61.3 ± 1.03	☠	☠	☠	☠	84.3 ± 2.75	80.0 ± 2.44
	YAS	2.74 ± 0.33	☠	☠	☠	☠	5.33 ± 0.70	3.47 ± 0.40
	YAAS	3.31 ± 3.46	☠	☠	☠	☠	43.7 ± 4.31	45.0 ± 2.18
	YDS	0.0	☠	☠	☠	☠	17.9 ± 0.23	3.95 ± 0.14
	RAR	23.2 ± 0.27	49.3 ± 1.01	53.5 ± 0.97	50.6 ± 1.19	42.5 ± 0.34	0.0	1.67 ± 0.27
	RXR	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	VDR	0.0	0.0	0.0	☠	1.20 ± 0.60	0.0	0.0
	TR	0.0	0.0	0.0	0.0	0.0	0.0	0.72 ± 0.26
	Umu	0.77 ± 0.04	☠	1.34 ± 1.34	☠	1.06 ± 0.02	1.30 ± 0.06	1.09 ± 0.03

Table S8 continued.

sample	*in vitro* bioassay	aqueous	Oasis HLB		Telos C18/ENV		Supelco ENVI-Carb+	
			pH 7	pH 2.5	pH 7	pH 2.5	pH 7	pH 2.5
EFF-1 (C)	YES	12.7 ± 1.98	0	7.60 ± 0.41	3.93 ± 0.58	5.04 ± 0.47	0.08 ± 0.19	0.0
	YAES	20.9 ± 2.32	19.3 ± 2.86	50.5 ± 0.99	49.5 ± 2.18	12.6 ± 3.22	55.0 ± 0.49	25.4 ± 0.43
	YAS	0.0	1.55 ± 0.45	0.81 ± 0.55	☠	0.0	1.43 ± 0.46	0.0
	YAAS	0.0	24.1 ± 2.84	70.8 ± 1.37	☠	66.6 ± 2.14	36.3 ± 4.65	41.2 ± 2.30
	YDS	18.9 ± 1.69	4.30 ± 0.42	17.4 ± 0.46	20.8 ± 0.54	3.66 ± 0.39	2.07 ± 1.14	0.0
	RAR	1.23 ± 0.27	0.0	0.0	0.0	0.83 ± 0.20	0.0	0.60 ± 0.39
	RXR	0.0	0.0	0.0	2.07 ± 2.71	0.12 ± 0.71	0.0	0.0
	VDR	0.0	0.0	0.0	0.0	0.91 ± 0.15	0.0	0.25 ± 0.19
	TR	0.0	0.0	0.0	0.0	0.42 ± 0.14	0.0	0.44 ± 0.13
	Umu	0.83 ± 0.06	1.10 ± 0.04	1.62 ± 0.07	☠	1.36 ± 0.03	☠	1.17 ± 0.04
EFF-4 (D)	YES	0.0	3.05 ± 0.26	5.08 ± 0.38	2.07 ± 0.49	5.91 ± 0.38	0.66 ± 0.29	0.0
	YAES	45.1 ± 0.81	66.0 ± 0.58	48.6 ± 1.83	79.8 ± 0.77	56.0 ± 2.36	76.7 ± 1.36	50.0 ± 1.75
	YAS	0.05 ± 0.69	4.59 ± 1.10	1.41 ± 0.33	0.0	0.0	0.0	0.01 ± 0.34
	YAAS	0.0	61.9 ± 2.71	66.5 ± 1.50	88.9 ± 1.25	76.6 ± 2.50	40.6 ± 3.08	38.2 ± 2.69
	YDS	0.0	20.3 ± 0.78	13.6 ± 0.64	21.2 ± 1.54	13.0 ± 1.10	4.56 ± 1.43	1.12 ± 1.32
	RAR	0.40 ± 0.16	0.0	2.86 ± 0.53	0.47 ± 0.68	2.74 ± 0.42	0.0	2.44 ± 0.65
	RXR	0.0	0.0	3.61 ± 2.85	0.0	1.87 ± 0.65	0.0	1.21 ± 0.82
	VDR	0.64 ± 0.75	0.0	0.97 ± 0.12	0.0	1.92 ± 0.31	0.0	1.60 ± 0.28
	TR	0.96 ± 0.34	0.0	1.86 ± 0.14	0.01 ± 0.58	1.96 ± 0.23	0.0	0.0
	Umu	0.89 ± 0.04	1.54 ± 0.03	1.82 ± 0.02	1.50 ± 0.08	1.87 ± 0.03	1.43 ± 0.09	1.25 ± 0.07

Table S8 continued.

sample	*in vitro* bioassay	aqueous	Oasis HLB		Telos C18/ENV		Supelco ENVI-Carb+	
			pH 7	pH 2.5	pH 7	pH 2.5	pH 7	pH 2.5
EFF-4-MS (D)	YES	0.0	3.09 ± 0.60	3.20 ± 0.38	☠	4.77 ± 0.37	1.24 ± 0.17	0.78 ± 0.33
	YAES	80.8 ± 0.53	76.3 ± 1.46	33.3 ± 1.85	☠	48.0 ± 2.33	62.4 ± 1.37	31.9 ± 2.34
	YAS	0.0	0.43 ± 0.56	0.29 ± 0.36	☠	0.71 ± 0.71	2.44 ± 1.14	0.0
	YAAS	5.24 ± 4.52	68.0 ± 2.80	63.1 ± 2.89	☠	67.3 ± 2.39	23.0 ± 1.56	34.5 ± 1.56
	YDS	0.0	20.6 ± 0.27	6.21 ± 1.21	39.8 ± 2.09	11.4 ± 1.66	5.85 ± 0.16	4.26 ± 1.46
	RAR	0.65 ± 0.14	0.25 ± 0.16	2.72 ± 0.35	0.0	3.59 ± 0.50	0.0	0.94 ± 0.24
	RXR	0.0	2.06 ± 0.28	0.59 ± 0.44	0.0	0.0	0.0	0.0
	VDR	0.0	0.22 ± 0.14	1.74 ± 0.29	0.0	1.88 ± 0.35	0.0	0.87 ± 0.47
	TR	0.0	0.0	0.0	0.0	0.55 ± 0.24	0.0	0.0
	Umu	0.91 ± 0.05	1.61 ± 0.08	1.72 ± 0.11	☠	1.73 ± 0.07	1.45 ± 0.06	1.35 ± 0.04
EFF-4-MS (F)	YES	2.39 ± 1.16	☠	4.49 ± 0.11	☠	4.77 ± 0.12	0.0	0.83 ± 0.25
	YAES	44.2 ± 4.45	75.1 ± 1.17	20.8 ± 0.83	☠	24.7 ± 0.69	☠	36.8 ± 1.66
	YAS	0.0	☠	0.44 ± 0.13	☠	0.22 ± 0.08	☠	0.71 ± 0.15
	YAAS	0.0	☠	58.3 ± 0.81	☠	64.6 ± 0.69	☠	40.4 ± 2.21
	YDS	0.0	☠	20.4 ± 0.38	☠	27.5 ± 0.65	☠	2.44 ± 0.09
	RAR	0.0	4.06 ± 0.33	4.67 ± 0.12	3.22 ± 0.19	6.71 ± 0.12	0.0	0.90 ± 0.18
	RXR	0.0	0.0	9.68 ± 1.12	0.0	9.88 ± 1.91	0.0	8.36 ± 0.64
	VDR	0.0	0.0	0.92 ± 0.19	0.0	0.95 ± 0.24	0.0	1.23 ± 0.19
	TR	0.0	0.0	1.02 ± 0.08	0.0	1.51 ± 0.04	0.0	0.27 ± 0.09

Table S8 continued.

sample	*in vitro* bioassay	aqueous	Oasis HLB		Telos C18/ENV		Supelco ENVI-Carb+	
			pH 7	pH 2.5	pH 7	pH 2.5	pH 7	pH 2.5
EFF-4-MS-O_3 (F)	YES	0.04 ± 0.35	☠	0.0	☠	0.0	0.0	0.0
	YAES	73.9 ± 1.13	☠	48.4 ± 0.48	☠	54.3 ± 0.53	76.5 ± 0.86	34.7 ± 0.60
	YAS	0.0	☠	0.08 ± 0.09	☠	0.01 ± 0.08	☠	0.27 ± 0.12
	YAAS	0.0	☠	44.4 ± 1.32	☠	51.4 ± 1.05	☠	37.3 ± 2.62
	YDS	0.0	☠	3.03 ± 0.17	☠	4.76 ± 0.18	☠	0.12 ± 0.05
	RAR	0.46 ± 0.31	0.0	2.26 ± 0.25	☠	1.57 ± 0.22	0.0	1.23 ± 0.17
	RXR	0.0	☠	10.5 ± 0.85	☠	8.69 ± 0.64	0.0	6.74 ± 0.79
	VDR	0.0	0.0	2.22 ± 0.15	☠	1.70 ± 0.24	0.0	1.73 ± 0.16
	TR	0.0	☠	0.72 ± 0.10	☠	0.78 ± 0.10	0.0	0.31 ± 0.06
GW-1 (C)	YES	0.0	0.0	0.0	1.17 ± 0.35	0.03 ± 0.20	0.40 ± 0.22	0.05 ± 0.46
	YAES	90.7 ± 0.52	52.6 ± 0.97	29.9 ± 0.96	73.0 ± 0.43	18.2 ± 1.75	61.6 ± 1.65	13.7 ± 2.07
	YAS	0.43 ± 1.44	1.28 ± 0.57	0.0	3.45 ± 0.69	0.0	6.15 ± 0.46	0.0
	YAAS	0.0	18.2 ± 1.84	28.8 ± 2.09	17.9 ± 0.86	29.9 ± 1.24	9.40 ± 2.61	27.5 ± 2.51
	YDS	0.0	4.09 ± 0.91	0.91 ± 0.69	2.75 ± 0.30	0.72 ± 1.08	4.22 ± 0.65	1.83 ± 0.23
	RAR	0.0	0.0	0.83 ± 0.20	0.0	0.25 ± 0.16	0.0	0.46 ± 0.23
	RXR	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	VDR	0.0	0.0	0.49 ± 0.12	0.0	0.37 ± 0.06	0.0	0.34 ± 0.21
	TR	0.0	0.0	0.87 ± 0.18	0.0	0.47 ± 0.11	0.0	0.82 ± 0.14
	Umu	0.84 ± 0.04	1.03 ± 0.02	1.11 ± 0.03	1.05 ± 0.01	1.04 ± 0.02	☠	1.02 ± 0.01

Table S9: Minimum and maximum of endocrine activity (%; mean ± SEM) and genotoxicity (umu: induction rate, mean ± SEM) of selected *in vitro* bioassays of aqueous samples and 10-fold concentrated SPE extracts, n.a.: not analysed. Umu: potential genotoxicity if induction rate is ≥ 1.5. Corresponding samples were taken on the same sampling dates in the middle of July (C) and at the end of July (D) 2012 and in January (F) 2013.

sample	*in vitro* bioassay									
	YES		YAES		YAAS		RAR		Umu	
	aqueous	extract	aqueous	extract	aqueous	extract	aqueous	extract	aqueous	extract
HOS (C)	0.0	0.0 – 0.35 ± 0.26 (n = 2)	87.8 ± 0.68	66.6 ± 1.41 – 89.1 ± 0.74 (n = 2)	99.7 ± 1.07	38.9 ± 5.40 – 39.4 ± 4.95 (n = 2)	93.3 ± 1.18	13.8 ± 0.65 – 91.3 ± 1.39 (n = 5)	1.29 ± 0.12	1.01 ± 0.04 – 4.37 ± 0.19 (n = 3)
INF-1 (C)	0.0	0.0 (n = 2)	61.3 ± 1.03	80.0 ± 2.44 – 84.3 ± 2.75 (n = 2)	3.31 ± 3.46	43.7 ± 4.31 – 45.0 ± 2.18 (n = 2)	23.2 ± 0.27	0.0 – 53.5 ± 0.97 (n = 6)	0.77 ± 0.04	1.06 ± 0.02 – 1.34 ± 1.34 (n = 4)
EFF-1 (C)	12.7 ± 1.98	0.0 – 7.60 ± 0.41 (n = 6)	20.9 ± 2.32	12.6 ± 3.22 – 55.0 ± 0.49 (n = 6)	0.0	24.1 ± 2.84 – 70.8 ± 1.37 (n = 5)	1.23 ± 0.27	0.0 – 0.83 ± 0.20 (n = 6)	0.83 ± 0.06	1.10 ± 0.04 – 1.62 ± 0.07 (n = 4)
EFF-4 (D)	0.0	0.0 – 5.91 ± 0.38 (n = 6)	45.1 ± 0.81	48.6 ± 1.83 – 79.8 ± 0.77 (n = 6)	0.0	38.2 ± 2.69 – 88.9 ± 1.25 (n = 6)	0.40 ± 0.16	0.0 – 2.86 ± 0.53 (n = 6)	0.89 ± 0.04	1.25 ± 0.07 – 1.87 ± 0.03 (n = 6)
EFF-4-MS (D)	0.0	0.78 ± 0.33 – 4.77 ± 0.37 (n = 5)	80.8 ± 0.53	31.9 ± 2.34 – 76.3 ± 1.46 (n = 5)	5.24 ± 4.52	23.0 ± 1.56 – 68.0 ± 2.80 (n = 5)	0.65 ± 0.14	0.0 – 3.59 ± 0.50 (n = 6)	0.91 ± 0.05	1.35 ± 0.04 – 1.73 ± 0.07 (n = 5)
EFF-4-MS (F)	2.39 ± 1.16	0.0 – 4.77 ± 0.12 (n = 4)	44.2 ± 4.45	20.8 ± 0.83 – 75.1 ± 1.17 (n = 4)	0.0	40.4 ± 2.21 – 64.6 ± 0.69 (n = 3)	0.0	0.0 – 6.71 ± 0.12 (n = 6)	n.a.	n.a.
EFF-4-MS-O_3 (F)	0.04 ± 0.35	0.0 (n = 4)	73.9 ± 1.13	34.7 ± 0.60 – 76.5 ± 0.86 (n = 4)	0.0	37.3 ± 2.62 – 51.4 ± 1.05 (n = 3)	0.46 ± 0.31	0.0 – 2.26 ± 0.25 (n = 5)	n.a.	n.a.
GW-1 (C)	0.0	0.0 – 1.17 ± 0.35 (n = 6)	90.7 ± 0.52	13.7 ± 2.07 – 73.0 ± 0.43 (n = 6)	0.0	9.40 ± 2.61 – 29.9 ± 1.24 (n = 6)	0.0	0.0 – 0.83 ± 0.20 (n = 6)	0.84 ± 0.04	1.02 ± 0.01 – 1.11 ± 0.03 (n = 5)

Table S10: Pooled data ((waste)water type and SPE-extracts) of endocrine activity (%; mean ± SEM) and genotoxicity (umu: induction rate, mean ± SEM) of aqueous samples and 10-fold concentrated SPE extracts, n.a.: not analysed. Umu: potential genotoxicity if induction rate is ≥ 1.5. Corresponding samples were taken on the same sampling dates in the middle of July (C) and at the end of July (D) 2012 and in January (F) 2013.

sample	*in vitro* bioassay									
	YES		YAES		YAS		YAAS		YDS	
	aqueous	extract	aqueous	extract	aqueous	extract	aqueous	extract	aqueous	extract
HOS (C) / INF-1 (C)	0.0 (n = 2)	0.09 ± 0.09 (n = 4)	74.6 ± 13.3 (n = 2)	80.0 ± 4.84 (n = 4)	4.01 ± 1.27 (n = 2)	3.87 ± 1.45 (n = 4)	51.5 ± 48.2 (n = 2)	41.8 ± 1.53 (n = 4)	0.0 (n = 2)	12.6 ± 5.67 (n = 4)
EFF-1 (C) / EFF-4 (D) / EFF-4-MS (D and F)	3.77 ± 3.03 (n = 4)	2.70 ± 0.51 (n = 21)	47.8 ± 12.4 (n = 4)	47.6 ± 4.50 (n = 21)	0.01 ± 0.01 (n = 4)	0.79 ± 0.26 (n = 19)	1.31 ± 1.31 (n = 4)	54.3 ± 4.29 (n = 19)	4.73 ± 4.73 (n = 4)	12.4 ± 2.28 (n = 21)
EFF-4-MS-O_3 (F)	0.04 (n = 1)	0.0 (n = 4)	73.9 (n = 1)	53.5 ± 8.70 (n = 4)	0.0 (n = 1)	0.12 ± 0.08 (n = 3)	0.0 (n = 1)	44.4 ± 4.07 (n = 3)	0.0 (n = 1)	2.64 ± 1.35 (n = 3)
GW-1 (C)	0.0 (n = 1)	0.28 ± 0.19 (n = 6)	90.7 (n = 1)	41.5 ± 9.95 (n = 6)	0.43 (n = 1)	1.81 ± 1.03 (n = 6)	0.0 (n = 1)	22.0 ± 3.31 (n = 6)	0.0 (n = 1)	2.42 ± 0.62 (n = 6)
	RAR		RXR		VDR		TR		Umu	
	aqueous	extract	aqueous	extract	aqueous	extract	aqueous	extract	aqueous	extract
HOS (C) / INF-1 (C)	58.3 ± 35.1 (n = 2)	47.1 ± 9.64 (n = 11)	0.0 (n = 2)	0.28 ± 0.28 (n = 10)	0.0 (n = 2)	0.22 ± 0.15 (n = 9)	0.0 (n = 2)	0.07 ± 0.07 (n = 10)	1.03 ± 0.26 (n = 2)	1.63 ± 0.46 (n = 7)
EFF-1 (C) / EFF-4 (D) / EFF-4-MS (D and F)	0.57 ± 0.26 (n = 4)	1.54 ± 0.38 (n = 24)	0.0 (n = 4)	1.64 ± 0.64 (n = 24)	0.16 ± 0.16 (n = 4)	0.56 ± 0.14 (n = 24)	0.24 ± 0.24 (n = 4)	0.34 ± 0.13 (n = 24)	0.88 ± 0.02 (n = 3)	1.50 ± 0.06 (n = 15)
EFF-4-MS-O_3 (F)	0.46 (n = 1)	1.01 ± 0.45 (n = 5)	0.0 (n = 1)	6.48 ± 2.29 (n = 4)	0.0 (n = 1)	1.13 ± 0.47 (n = 5)	0.0 (n = 1)	0.45 ± 0.18 (n = 4)	n.a.	n.a.
GW-1 (C)	0.0 (n = 1)	0.26 ± 0.14 (n = 6)	0.0 (n = 1)	0.0 (n = 6)	0.0 (n = 1)	0.2 ± 0.09 (n = 6)	0.0 (n = 1)	0.36 ± 0.17 (n = 6)	0.84 (n = 1)	1.05 ± 0.02 (n = 5)

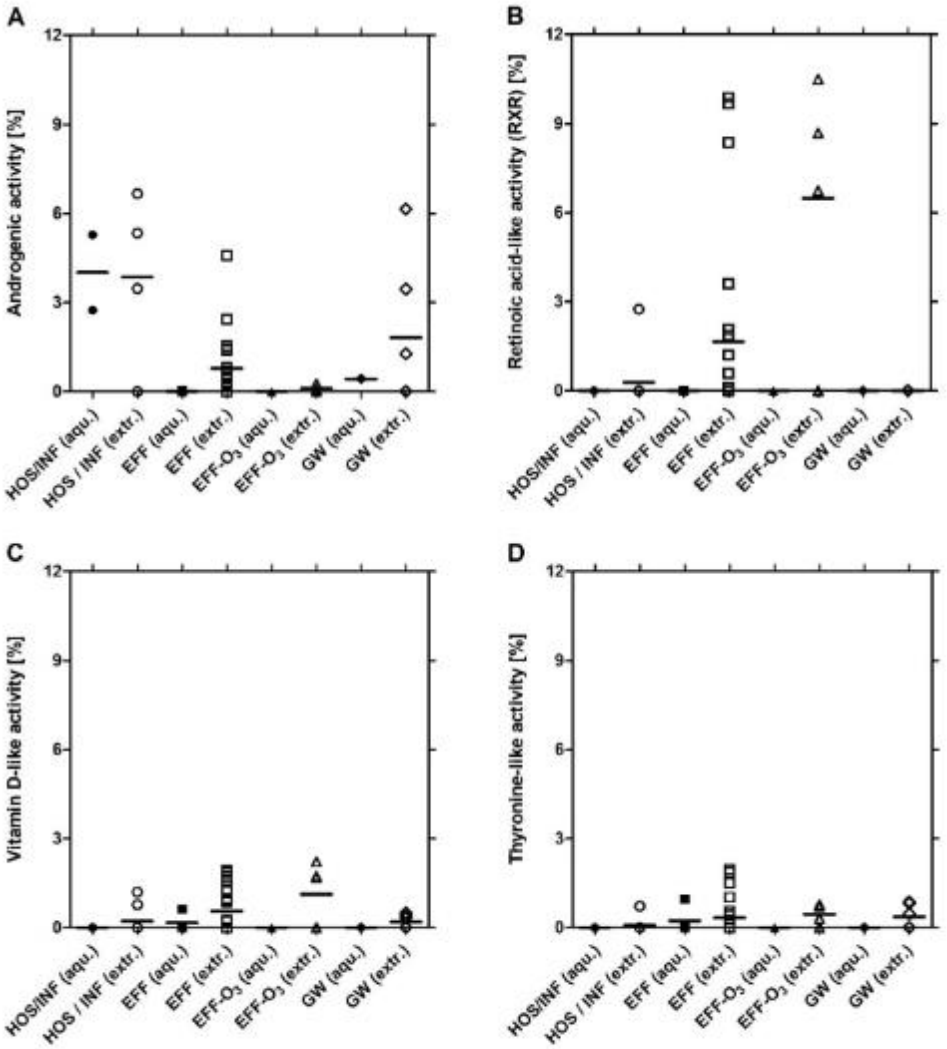

Figure S9: Androgenic (A), retinoid X-like (RXR, B), vitamin D-like (C) and thyronine-like (D) activity in % of the pooled data of aqueous (aqu.) water and wastewater samples and of the corresponding pooled 10-fold SPE extracts (extr). Symbols: activity of the individual sample, line: mean of all samples of one water type, filled symbol: aqueous sample, clear symbol: SPE extract, HOS: hospital effluent (untreated wastewater), INF: influent (untreated wastewater), EFF: effluent (conventionally treated wastewater), EFF-O_3: ozonated conventionally treated wastewater, GW: groundwater. The corresponding samples were taken on the same sampling dates in July 2012 and January 2013.

Table S11: Pooled data ((waste)water type) of endocrine activity (%; mean ± SEM) and genotoxicity (umu: induction rate, mean ± SEM) of aqueous samples and 10-fold concentrated SPE extracts, ☠: cytotoxic. Umu: potential genotoxicity if induction rate is ≥ 1.5. Corresponding samples were taken on the same sampling date in the middle of July (C) 2012, the end of July (D) 2012 and in January (F) 2013

sample	*in vitro* bioassay	aqueous	Oasis HLB		Telos C18/ENV		Supelco ENVI-Carb+	
			pH 7	pH 2.5	pH 7	pH 2.5	pH 7	pH 2.5
HOS (C) / INF-1 (C)	YES	0.0 (n = 2)	☠	☠	☠	☠	0.0 (n = 2)	0.18 ± 0.18 (n = 2)
	YAES	74.6 ± 13.3 (n = 2)	☠	☠	☠	☠	86.7 ± 2.40 (n = 2)	73.3 ± 6.70 (n = 2)
	YAS	4.01 ± 1.27 (n = 2)	☠	☠	☠	☠	2.67 ± 2.67 (n = 2)	5.07 ± 1.60 (n = 2)
	YAAS	51.5 ± 48.2 (n = 2)	☠	☠	☠	☠	41.3 ± 2.40 (n = 2)	42.2 ± 2.80 (n = 2)
	YDS	0.0 (n = 2)	☠	☠	☠	☠	22.0 ± 4.10 (n = 2)	3.27 ± 0.69 (n = 2)
	RAR	58.3 ± 35.1 (n = 2)	63.0 ± 13.7 (n = 2)	72.4 ± 18.9 (n = 2)	50.6 (n = 1)	66.8 ± 24.3 (n = 2)	6.90 ± 6.90 (n = 2)	24.7 ± 23.1 (n = 2)
	RXR	0.0 (n = 2)	1.38 ± 1.38 (n = 2)	0.0 (n = 1)	0.0 (n = 1)	0.0 (n = 2)	0.0 (n = 2)	0.0 (n = 2)
	VDR	0.0 (n = 2)	0.0 (n = 1)	0.39 ± 0.39 (n = 2)	☠	0.60 ± 0.60 (n = 2)	0.0 (n = 2)	0.0 (n = 2)
	TR	0.0 (n = 2)	0.0 (n = 1)	0.0 (n = 2)	0.0 (n = 1)	0.0 (n = 2)	0.0 (n = 2)	0.36 ± 0.36 (n = 2)
	Umu	1.03 ± 0.26 (n = 2)	☠	1.34 (n = 1)	☠	2.72 ± 1.66 (n = 2)	1.26 ± 0.04 (n = 2)	1.05 ± 0.04 (n = 2)

Table S11 continued.

sample	*in vitro* bioassay	aqueous	Oasis HLB		Telos C18/ENV		Supelco ENVI-Carb+	
			pH 7	pH 2.5	pH 7	pH 2.5	pH 7	pH 2.5
EFF-1 (C) / EFF-4 (D) /	YES	3.77 ± 3.03 (n = 4)	2.05 ± 1.02 (n = 3)	5.09 ± 0.92 (n = 4)	3.0 ± 0.93 (n = 2)	5.12 ± 0.27 (n = 4)	0.50 ± 0.29 (n = 4)	0.40 ± 0.23 (n = 4)
EFF-4-MS (D) / EFF-4-MS (F)	YAES	47.8 ± 12.4 (n = 4)	59.2 ± 13.5 (n = 4)	38.3 ± 6.99 (n = 4)	64.7 ± 15.2 (n = 2)	35.3 ± 10.1 (n = 4)	64.7 ± 6.37 (n = 3)	36.0 ± 5.21 (n = 4)
	YAS	0.01 ± 0.01 (n = 4)	2.19 ± 1.24 (n = 3)	0.74 ± 0.25 (n = 4)	0.0 (n = 1)	0.23 ± 0.17 (n = 4)	1.29 ± 0.71 (n = 3)	0.18 ± 0.18 (n = 4)
	YAAS	1.31 ± 1.31 (n = 4)	51.3 ± 13.7 (n = 3)	64.7 ± 2.65 (n = 4)	88.9 (n = 1)	68.8 ± 2.67 (n = 4)	33.3 ± 5.30 (n = 3)	38.6 ± 1.50 (n = 4)
	YDS	4.73 ± 4.73 (n = 4)	15.1 ± 5.38 (n = 3)	14.4 ± 3.07 (n = 4)	27.3 ± 6.27 (n = 3)	13.9 ± 4.97 (n = 4)	4.16 ± 1.11 (n = 3)	1.96 ± 0.92 (n = 4)
	RAR	0.57 ± 0.26 (n = 4)	1.08 ± 1.0 (n = 4)	2.56 ± 0.96 (n = 4)	0.92 ± 0.77 (n = 4)	3.47 ± 1.23 (n = 4)	0.0 (n = 4)	1.22 ± 0.41 (n = 4)
	RXR	0.0 (n = 4)	0.52 ± 0.52 (n = 4)	3.47 ± 2.22 (n = 4)	0.52 ± 0.52 (n = 4)	2.97 ± 2.34 (n = 4)	0.0 (n = 4)	2.39 ± 2.01 (n = 4)
	VDR	0.16 ± 0.16 (n = 4)	0.06 ± 0.06 (n = 4)	0.91 ± 0.36 (n = 4)	0.0 (n = 4)	1.42 ± 0.28 (n = 4)	0.0 (n = 4)	0.99 ± 0.29 (n = 4)
	TR	0.24 ± 0.24 (n = 4)	0.0 (n = 4)	0.72 ± 0.45 (n = 4)	0.0 (n = 4)	1.11 ± 0.37 (n = 4)	0.0 (n = 4)	0.18 ± 0.11 (n = 4)
	Umu	0.88 ± 0.02 (n = 3)	1.42 ± 0.16 (n = 3)	1.72 ± 0.06 (n = 3)	1.50 (n = 1)	1.65 ± 0.15 (n = 3)	1.44 ± 0.01 (n = 2)	1.26 ± 0.05 (n = 3)

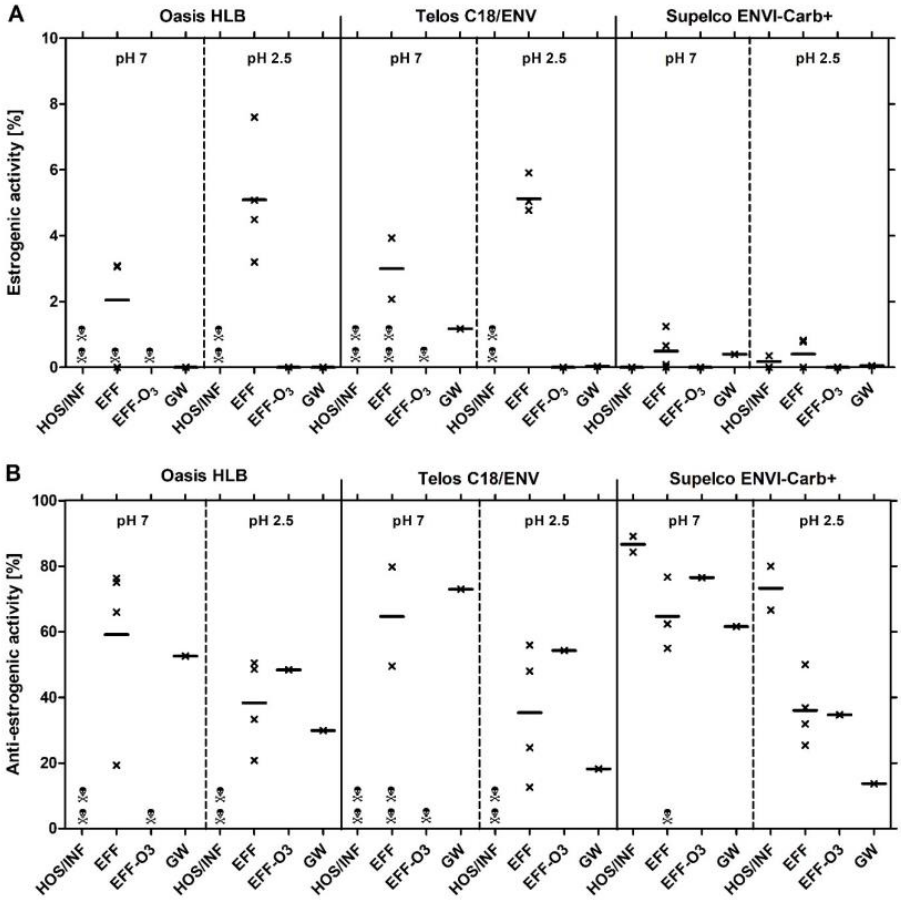

Figure S10: Estrogenic (A) and anti-estrogenic (B) activity in % of SPE extracts of water and wastewater samples using three different SPE columns and two different pH values. The results were pooled from the different samples according to water type. Symbols: activity of the individual sample, line: mean of all samples of one water type, ☠: cytotoxic, HOS: hospital effluent (untreated wastewater), INF: influent (untreated wastewater), EFF: effluent (conventionally treated wastewater), EFF-O_3: ozonated conventionally treated wastewater, GW: groundwater.

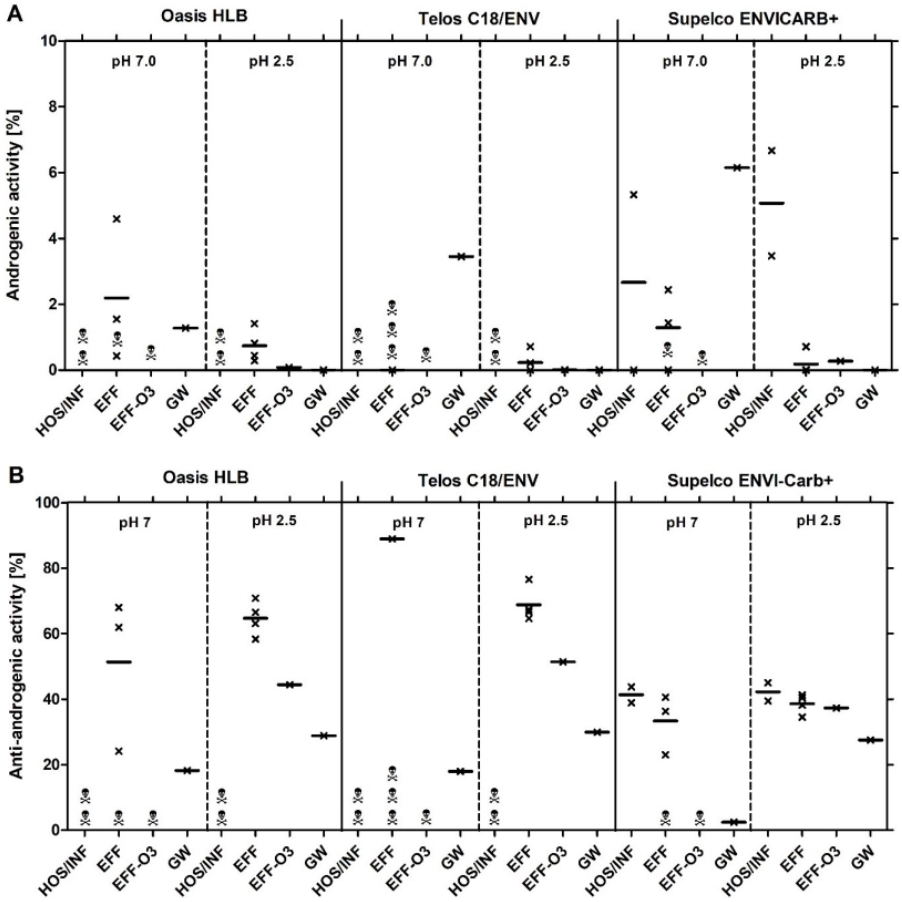

Figure S11: Androgenic (A) and anti-androgenic (B) activity in % of SPE extracts of water and wastewater samples using three different SPE columns and two different pH values. The results were pooled from the different samples according to water type. Symbols: activity of the individual sample, line: mean of all samples of one water type, ☠: cytotoxic, HOS: hospital effluent (untreated wastewater), INF: influent (untreated wastewater), EFF: effluent (conventionally treated wastewater), EFF-O_3: ozonated conventionally treated wastewater, GW: groundwater.

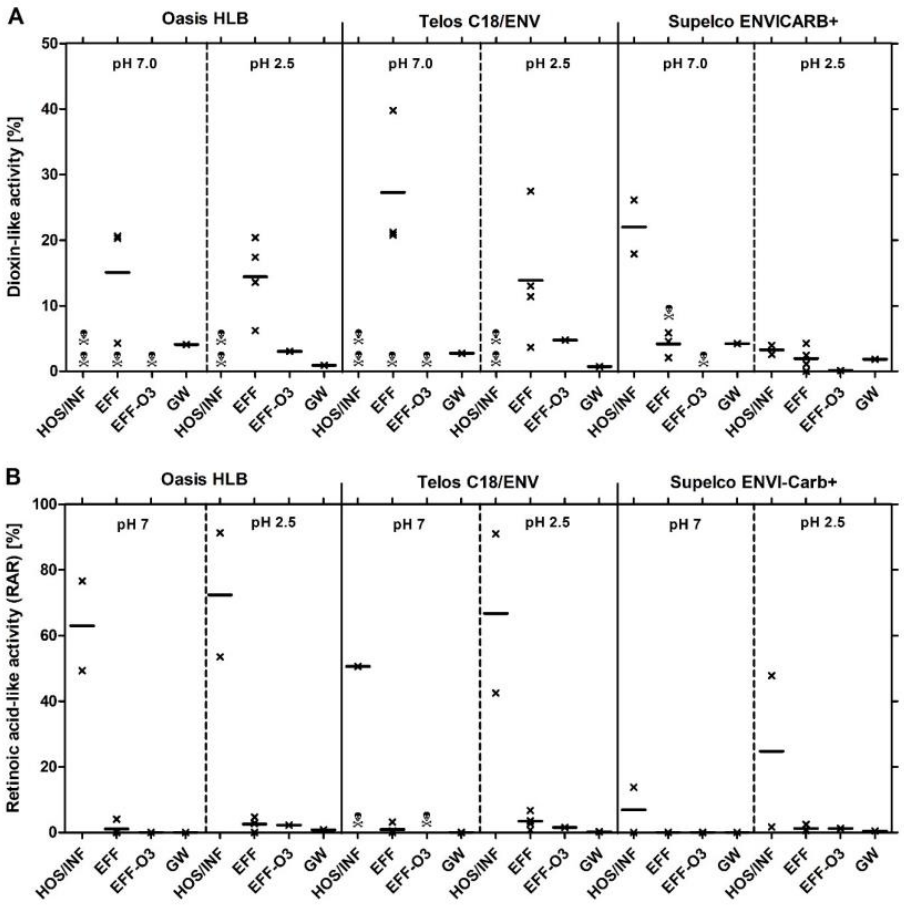

Figure S12: Dioxin-like (A) and retinoic acid-like (RAR, B) activity in % of SPE extracts of water and wastewater samples using three different SPE columns and two different pH values. The results were pooled from the different samples according to water type. Symbols: activity of the individual sample, line: mean of all samples of one water type, ☠: cytotoxic, HOS: hospital effluent (untreated wastewater), INF: influent (untreated wastewater), EFF: effluent (conventionally treated wastewater), EFF-O_3: ozonated conventionally treated wastewater, GW: groundwater.

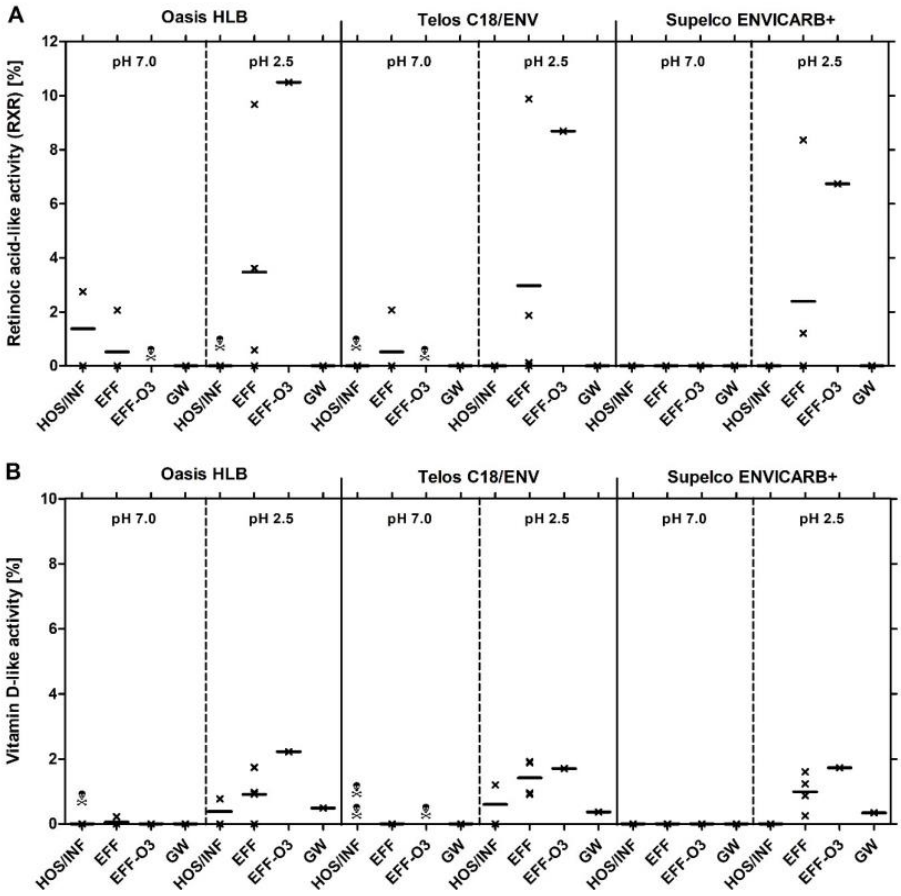

Figure S13: Retinoid X-like (RXR, A) and vitamin D-like (B) activity in % of SPE extracts of water and wastewater samples using three different SPE columns and two different pH values. The results were pooled from the different samples according to water type. Symbols: activity of the individual sample, line: mean of all samples of one water type, ☠: cytotoxic, HOS: hospital effluent (untreated wastewater), INF: influent (untreated wastewater), EFF: effluent (conventionally treated wastewater), EFF-O_3: ozonated conventionally treated wastewater, GW: groundwater.

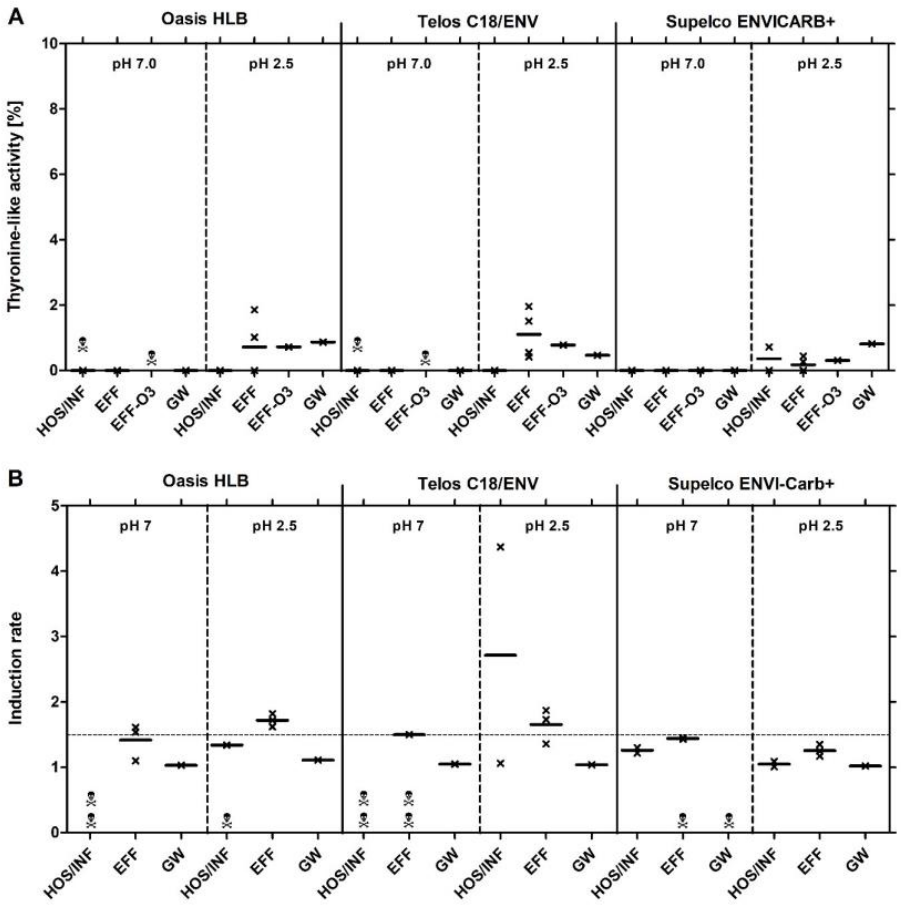

Figure S14: Thyronine-like (A) activity in % and induction rate (umu, B) of SPE extracts of water and wastewater samples using three different SPE columns and two different pH values. The results were pooled from the different samples according to water type. Symbols: activity of the individual sample, line: mean of all samples of one water type, ☠: cytotoxic, umu: potential genotoxicity if induction rate is ≥ 1.5, HOS: hospital effluent (untreated wastewater), INF: influent (untreated wastewater), EFF: effluent (conventionally treated wastewater), EFF-O_3: ozonated conventionally treated wastewater, GW: groundwater.

2.4 Pareto optimisation and ranking

Table S12: Endocrine activity (%) of conventionally treated wastewater (sample EFF-4 (D)) as aqueous sample and 10-fold concentrated SPE extracts from six different methods: three solid phase extraction (SPE) columns (Oasis HLB, Telos C18/ENV and Supelco ENVI-Carb+) were used at two pH values (pH 7 and pH 2.5) and tested in five recombinant yeast screens (YES, YAES, YAS, YAAS and YDS).

method / bioassay	Oasis HLB		Telos C18/ENV		Supelco ENVI-Carb+		aqueous
	pH 7.0	pH 2.5	pH 7.0	pH 2.5	pH 7.0	pH 2.5	sample
YES	3.05	5.08	2.07	5.91	0.66	0.0	0.0
YAES	66.0	48.6	79.8	56.0	76.7	50.0	45.1
YAS	4.59	1.41	0.0	0.0	0.0	0.01	0.05
YAAS	61.9	66.5	88.9	76.6	40.6	38.2	0.0
YDS	20.3	13.6	21.2	13.0	4.56	1.12	0.0

Table S13: Pareto ranking (1st rank = "best", 6th rank = "worst") of the six different SPE methods according to their effectivity in extracting different endocrine activities from conventionally treated wastewater (sample EFF-4 (D), Table S12). Oasis: Oasis HLB, Telos: Telos C18/ENV, Supelco: Supelco ENVI-Carb+, 7: pH 7, 2.5: pH 2.5. In case of the YAS no 5th and 6th rank existed.

ranking / bioassay	best	2nd	3rd	4th	5th	worst
YES	Telos 2.5	Oasis 2.5	Oasis 7	Telos 7	Supelco 7	Supelco 2.5
YAES	Telos 7	Supelco 7	Oasis 7	Telos 2.5	Supelco 2.5	Oasis 2.5
YAS	Oasis 7	Oasis 2.5	Supelco 2.5	Telos 7 Telos 2.5 Supelco 7	–	–
YAAS	Telos 7	Telos 2.5	Oasis 2.5	Oasis 7	Supelco 7	Supelco 2.5
YDS	Telos 7	Oasis 7	Oasis 2.5	Telos 2.5	Supelco 7	Supelco 2.5

Table S14: Pareto ranking (best, 2nd–4th best) of SPE methods of all water types according to their effectivity in extracting different types of water and wastewater samples with respect to the highest endocrine activities. Hospital wastewater (HOS) and WWTP influent (INF-1) was not ranked due to excessive cytotoxicity. Oasis: Oasis HLB, Telos: Telos C18/ENV, Supelco: Supelco ENVI-Carb+, 7: pH 7, 2.5: pH 2.5. Corresponding samples were taken on the same sampling dates in the middle of July (C) and at the end of July (D) 2012 and in January (F) 2013.

ranking / sample type	best	2nd	3rd	4th
EFF-1 (C)	Oasis 2.5 Telos 7	Oasis 7 Supelco 7	Telos 2.5 Supelco 2.5	–
EFF-4 (D)	Oasis 7 Telos 7 Telos 2.5	Supelco 7	Oasis 2.5 Supelco 2.5	–
EFF-4-MS (D)	Telos 7	Oasis 7 Telos 2.5	Oasis 2.5	Supelco 7
EFF-4-MS-O_3 (F)	Supelco 7 (no ranking for Oasis 7, Telos 7)	Oasis 2.5 Telos 2.5	Supelco 2.5	–
GW-1 (C)	Oasis 7 Oasis 2.5 Telos 7	Telos 2.5 Supelco 7 Supelco 2.5	–	–
bioassay	best	2nd	3rd	4th
YES	Telos 2.5	Oasis 2.5 Supelco 2.5	Telos 7 Supelco 7	Oasis 7
YAES	Supelco 7	Telos 7	Oasis 7 Supelco 2.5	Oasis 2.5 Telos 2.5
YAS	Supelco 7	Oasis 7	Supelco 2.5	Oasis 2.5 Telos pH 7 Telos 2.5
YAAS	Telos 2.5 Supelco 2.5	Oasis 7 Oasis 2.5 Telos 7	Supelco 7	–
YDS	Telos 7	Supelco 7	Telos 2.5 Supelco 2.5	Oasis 7 Oasis 2.5

A.2 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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Individual contributions of the first author and co-authors:

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2. Performance of experiments and assays

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3. Compilation of data sets and tables/figures:

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5. Drafting of the Manuscript:

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Author accepted manuscript (paper A.2)

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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Keywords: municipal effluents, contaminants of emerging concern, CYP biomarker, persistent organic pollutants (POPs), toxic effects, transformation products, *in vivo* bioassay, ozonation

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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

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

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

2 Materials and methods

2.1 Conventional wastewater treatment plants

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

2.2 Pilot wastewater treatment plant equipped with advanced treatment technologies

The pilot WWTP was fed by the conventionally treated wastewater of WWTP-4 and included an ozonation (O_3) coupled to GAC or BF (Fig. 1; Knopp et al. 2016). The WWTP effluent was filtered by a 10 µm microscreen to reduce suspended solids prior to O_3. 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,

224 0.1% final concentration) was supplemented to all wells. The resulting bacterial
225 suspension was used as negative control (NC). For testing SPE extracts: depending on
226 the final concentration factor, 10x or 25x, an extract volume of 1 µL (1:500) or 2.5 µL
227 (1:200) of the 5000x SPE extracts (2.3) was added respectively. For testing aqueous
228 samples: 0.5 mL of sample was added (1:2). Addition of samples/extracts represented
229 the starting point (t_0) of the bioassays. Microtiter plates were incubated at 20 °C in the
230 dark for 1–96 h depending on the endpoint (2.6.1 and 2.6.2). Highest final SPE
231 enrichment factor tested (25x) represented a DMSO concentration of 0.5% (v/v). At this
232 solvent concentration no adverse effects on *C. elegans* were reported (Boyd et al. 2010).
233 In prescreening experiments 10x concentrated samples were tested (3.1). For samples
234 from WWTP 1–3 a 25x concentration factor was applied (3.2). However, for these
235 samples mortality occurred in the INF 1–3 (data not shown) thus 1:2 dilutions were
236 prepared. Accordingly, WWTPs 1–3 were tested in 12.5x concentrations. Samples from
237 WWTP-4 were tested in 25x concentrations (3.3).

238 **2.6.1 Endpoint brood size and larval length**

239 Benzylcetyldimethylammonium chloride (BAC-C16, 5 mg/L, CAS 122-18-9, Alfa Aesar,
240 95%) was used as additional positive control (PC) for reprotoxicity and inhibition of growth
241 (Höss et al. 2012). The duration of the respective bioassays was 96 h. At their termination
242 (t_{end} = 96 h) adult and larval nematodes were sacrificed by heat shock (15 min at 80°C)
243 and stained with rose bengal (CAS 632-69-9, AppliChem) for microscopic evaluation
244 (30x). For the endpoint brood size (reproduction) 10 individuals were exposed in 3
245 replicates each per experiment. Total n per treatment group are indicated in figure
246 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

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

430 Unspiked surface water and effluent of WWTP-1 did not cause any significant *cyp-*
431 *35A3*::GFP induction (Fig. 5 and Online Resource 7). Spiking with β-NF, however,
432 resulted in an effective induction, which was higher in the aqueous effluent compared to
433 the surface water sample. This is in accordance with the results observed for reproduction
434 and might be explained by joint effects caused by low concentrations of multiple CYP-
435 inducers in the effluent, which do not induce expression without β-NF and/or natural
436 factors affecting the latter. Another factor might have contributed: β-NF has a log K_{ow} of
437 4.7 (estimated using US EPA's EPISuite) and will adsorb to particles, such as from TSS
438 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

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

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

4.3 Advanced wastewater treatment technologies

An early ecotoxicological contribution to the research on advanced wastewater treatment technologies was performed with *C. elegans* (Hitchcock et al. 1998). The authors 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

615 Fig. 4 Impacts of extracted groundwater (GW, SPE blank), wastewater treatment plant
616 influent (INF-4), effluent (EFF-4) and advanced treatments on the brood size (A) and
617 length of larvae (B) of *C. elegans.* Advanced treatments comprised of ozonation
618 (EFF+O_3) and ozonation followed by aerated and non-aerated granular activated carbon
619 filtration (O_3+GAC, O_3+GAC_a) or biofiltration (O_3+BF, O_3+BF_a). Samples (grey bars) were
620 analyzed in 25x concentrations. Results pooled from four experiments for brood size (n =
621 95 per treatment group) and one experiments for larval length (n = 60 per treatment
622 group). Significant differences (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) were tested against
623 NC and GW by Kruskal-Wallis test with Dunn's post-test. NC (white bar) = M9 medium.
624 PC (white bar) = BAC (5 mg/L)

625 Fig. 5 A) *cyp-35A3*::GFP expression in transgenic *C. elegans* after 8 h exposure to 1 mg/L
626 β-naphthoflavone (β-NF). Exposed adult hermaphrodites showed a strong GFP signal
627 along their intestine, as detected by fluorescence microscopy (100x). Images (NC, β-NF)
628 show an overlay of differential interference contrast microscopy (DIC) and GFP channel.
629 NC = M9 medium. Bar = 200 µm. B and C) Impacts of aqueous and extracted ultrapure
630 water (UPW), surface water (SW), wastewater treatment plant effluent (EFF-1, EFF-4)
631 and ozonated effluent (EFF+O_3) on *cyp-35A3*::GFP expression. Aqueous (white bars)
632 and extracted (grey bars) samples were analyzed in 0.5x and 10x concentrations
633 respectively after 24 h exposure. Spiked aqueous samples (marked by superscript s)
634 contained 0.5 mg/L β-NF. Results pooled from two experiments (n = 10 per treatment
635 group, respectively). Significant differences (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p <$
636 0.0001) tested unspiked against spiked samples (B) and against controls (B, C) by one-
637 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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Supplementary information (paper A.2)

Online Resource 1: Tab. Characteristics of wastewater treatment plants (WWTPs) 1–4, including population equivalents (PE), connected PE (in brackets), WWTP capacity and amount of treated wastewater (WW), hydraulic retention time (HRT) in the conventional-biological stage, average sludge age in the activated sludge treatment, approximate wastewater fraction in receiving water body and further specifications. "n.d." = not detected

WWTP	PE	Treated WW [m^3/a]	HRT [h]	Sludge age [d]	Receiving water body	Further specifications
1	440,000 (n.d.)	38.8 x 10^6	19	14	~ 6 % wastewater	Catchment area in metropolitan area, including commercial districts and hospitals. Receiving surface water used as drinking water resource.
2	16,000 (10,000)	1.5 x 10^6	9–16	14	~ 50 % wastewater	Receiving water body with high groundwater infiltration.
3	16,600 (14,000)	2.5 x 10^6	9–15	11	~ 18 % wastewater	Periodically high proportion of external WW (from a hospital).
4	50,000 (42,000)	2.3 x 10^6	45	12–18	n.d.	WWTP connected to a pilot WWTP with advanced WW treatment. Catchment area with commercial and hospital wastewater discharges.

Online Resource 2: Tab. Selected wastewater and process parameters of WWTP-4. Parameters included: pH, electric conductivity, spectral absorption coefficient at 254 nm (UV_{254}), dissolved organic carbon (DOC), chemical oxygen demand (COD), filtered chemical oxygen demand ($COD_{0.45\mu m}$), ammonium (NH_4^+), nitrate (NO_3^-), nitrite (NO_2^-), total phosphorous (P_{total}), hydraulic retention time of ozone (O_3-HRT), ozone dose (D), specific ozone dose (d), ozone consumption (Z), specific ozone consumption (z), filtration speed (v_F) and empty bed contact time (EBCT). Parameters were determined in 24 h composite (CS) or grab samples (GS) from April 14th, 2015. A few grab samples were taken on April 16th, 2015. The average of both samples is reported (GS_2). "n.a." = not applicable. "n.d." = not detected. LOQ are indicated with "<"

Sample type	Parameter	Sampling point						
		INF-4	EFF-4	EFF+O_3	O_3+GAC	O_3+GAC_a	O_3+BF	O_3+BF_a
GS_2	pH	7.59	7.33	n.d.	n.d.	n.d.	n.d.	n.d.
GS_2	Conductivity [µS/cm]	1601	1213	n.d.	n.d.	n.d.	n.d.	n.d.
CS	SAC_{254}	0.66	0.22	0.09	0.06	0.05	0.08	0.07
GS_2	UV_{254}	0.67	0.22	0.08	0.25	0.05	0.06	0.06
CS	DOC [mg/L]	96.5	13.4	12.2	8.3	7.6	9.7	9.0
GS_2	DOC [mg/L]	105	13.5	13.1	8.1	7.9	9.2	8.9
CS	COD / $COD_{0,45\mu m}$ [mg/L]	938 / 309	32.4	27.7	18.1	16.8	21.9	19.7
GS_2	COD / $COD_{0,45\mu m}$ [mg/L]	935 / 325	33.1	27.8	17.7	17.2	20.3	19.9
GS	P_{total} [mg/L]	13.0	0.65	0.64	0.66	0.76	0.63	0.81
GS	NH_4^+ [mg/L]	67.5	0.12	0.17	< 0.015	< 0.015	< 0.015	< 0.015
GS	NO_2^- [mg/L]	0.04	0.15	< 0.015	< 0.015	< 0.015	< 0.015	< 0.015
GS	NO_3^- [mg/L]	0.5	1.64	1.89	2.32	2.44	2.25	2.36
GS	O_3-HRT [min]	n.a.	n.a.	16.7	n.a.	n.a.	n.a.	n.a.
GS	D [g/m³]	n.a.	n.a.	13.8	n.a.	n.a.	n.a.	n.a.
GS	d [g(O_3)/g(DOC)]	n.a.	n.a.	1.0	n.a.	n.a.	n.a.	n.a.
GS	Z [g/m³]	n.a.	n.a.	13.5	n.a.	n.a.	n.a.	n.a.
GS	z [g(O_3)/g(DOC)]	n.a.	n.a.	1.0	n.a.	n.a.	n.a.	n.a.
GS	v_F [m/h]	n.a.	n.a.	n.a.	4.8	5.5	4.7	5.5
GS	EBCT [min.]	n.a.	n.a.	n.a.	25.3	23.7	29.2	25.8

Online Resource 3: Tab. Concentrations of micropollutants and their transformation products (indicated in *italics*) in [µg/L]. Results are presented for December 2012 (SW), February 2014 (WWTP 1-3) and April 2015 (WWTP-4) for samples tested on *C. elegans* (see 2. in the main manuscript). Analytical LOQ was 0.025 µg/L as indicated, if not noted elsewise. Concentrations detected below the LOQ are marked with "<". Samples were measured as duplicates, while the second measurement was performed as standard addition (n = 5 concentrations of added standard). For (1H-)benzotriazole the median concentration of seven sampling campaigns (April 2012 to February 2014) is used (marked with "[ ]") due to an outlier. "n.d." = not detected. Compounds tested positively for (repro)toxicity and/or growth promotion in the *C. elegans* literature are depicted at the end of the table

Sampling point: / Compound/Group:	SW	INF-1	EFF-1	INF-2	EFF-2	INF-3	EFF-3	INF-4	EFF-4	EFF+O_3	O_3+GAC	O_3+GAC$_a$	O_3+BF	O3+BF$_a$
Pharmaceuticals and contrast media														
1-Adamantylamine	< 0.025	0.12	0.17	0.12	0.12	0.12	0.1	0.23	0.43	0.05	0.05	0.05	0.05	0.03
Acyclovir	n.d.	0.32	< 0.025	0.03	< 0.025	0.5	< 0.025	2.37	0.2	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025
Carboxyacyclovir	n.d.	8.2	6.8	1.8	8.5	1.9	5.1	2.2	20	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025
Bezafibrate	< 0.025	0.36	0.07	0.12	0.06	0.53	0.06	1.9	0.33	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025
Bisoprolol	< 0.025	0.43	0.23	0.75	< 0.025	< 0.025	0.31	1.5	1.2	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025
Carbamazepine (CBZ)	< 0.025	0.45	0.65	0.45	0.44	0.36	0.45	1.36	1.47	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025
10,11-dihydro-10,11-dihydroxy-CBZ	0.04	1.5	1.3	1.4	1.2	0.97	0.92	5.3	4.0	0.37	0.13	0.13	0.36	0.38
CBZ-Epoxid	< 0.025	0.06	0.08	0.06	0.07	0.04	0.05	0.05	0.13	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025
Clarithromycin	0.04	0.16	0.21	0.08	0.42	0.34	0.29	0.68	0.36	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025
Diatrizoic acid	0.1	5.6	5.7	0.52	0.15	1.1	1.7	7.0	7.9	6.6	5.7	5.6	6.1	5.8
Diclofenac	0.05	2.6	3.1	2.3	3.2	1.8	1.8	8.9	5.8	< 0.10	< 0.10	< 0.10	< 0.10	< 0.10
4-Hydroxydiclofenac	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	2.01	1.68	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025
Erythromycin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.96	0.76	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
Dehydrato-erythromycin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.12	0.12	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025
Fenofibric acid (fenofibrate)	0.03	0.77	0.09	1.4	0.76	< 0.025	0.1	0.9	0.19	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025
Gabapentin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	19.1	8.08	1.77	1.54	1.24	1.79	1.63
Ibuprofen	< 0.025	20	< 0.025	< 0.025	0.14	13	< 0.025	39	0.07	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025
Carboxyibuprofen	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	12.9	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025
1-Hydroxyibuprofen	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	5.85	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025
2-Hydroxyibuprofen	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	64.3	0.339	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025
3-Hydroxyibuprofen	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	8.91	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025

(Tab. continued)

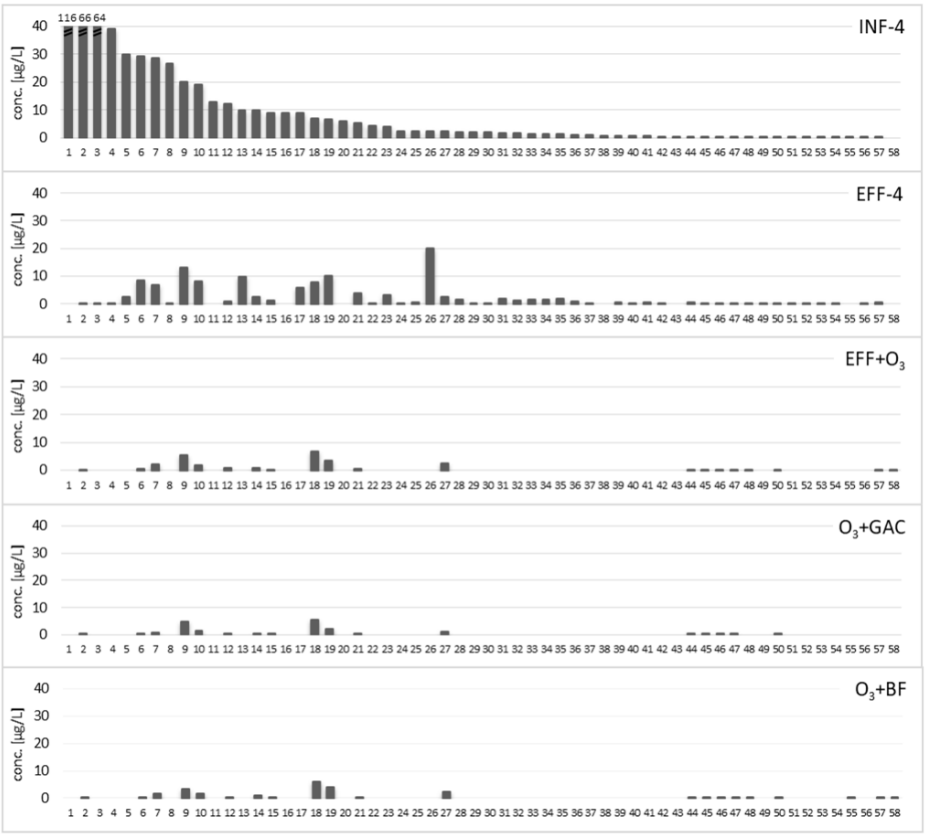
Sampling point: / Compound/Group:	SW	INF-1	EFF-1	INF-2	EFF-2	INF-3	EFF-3	INF-4	EFF-4	EFF+O_3	O_3+GAC	O_3+GAC_a	O_3+BF	O3+BF_a
Pharmaceuticals and contrast media														
Iohexol	0.11	29	11	0.07	< 0.025	5.9	5.5	8.9	1.2	0.3	0.09	0.14	0.2	0.22
Iomeprol	0.4	76	48	1.6	1.2	22	3.1	29	6.9	2.3	0.61	0.93	1.9	1.6
Iopamidol	0.12	7.1	5.9	14	0.09	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025
Iopromide	0.19	33	9.5	4.4	< 0.025	5.6	3.4	9.8	2.7	0.88	0.23	0.37	1.1	0.88
Metoprolol	0.09	1.7	1.5	2.5	4.2	1.7	1.7	1.2	1.8	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025
Naproxen	< 0.025	0.98	0.08	0.6	0.19	0.59	0.24	4.2	0.22	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025
Oxazepam	< 0.025	0.09	0.09	0.06	0.05	0.08	0.06	0.19	0.33	0.03	< 0.025	< 0.025	0.03	0.03
Paracetamol	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	26.5	0.03	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025
Primidone	< 0.025	0.21	0.21	0.25	0.13	0.11	0.12	0.14	0.18	0.05	0.07	0.07	0.04	0.04
Phenylethylmalondiamide	< 0.025	0.31	0.35	0.21	0.23	0.2	0.21	0.23	0.27	0.04	0.04	0.03	0.04	0.03
Propranolol	< 0.025	0.06	0.05	0.07	0.05	0.08	0.06	0.06	0.08	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025
Phenazone	< 0.025	< 0.025	0.19	0.03	0.12	0.04	0.2	0.04	0.37	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025
4-Acetamidoantipyrin	0.1	11	0.16	12	1.4	9	2.9	30	2.6	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025
4-Formylaminoantipyrin	0.09	4.2	4.7	4.9	3.7	4.1	3.4	10	9.8	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025
Ritalinic acid (methylphenidate)	< 0.025	0.1	< 0.025	0.12	0.06	0.06	0.06	0.07	0.06	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025
Roxithromycin	< 0.025	0.08	0.09	0.11	0.18	0.04	0.03	0.48	0.38	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025
Sotalol	< 0.025	0.3	0.28	0.6	0.36	0.45	0.4	1.6	2	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025
Sulfamethoxazole	< 0.025	0.25	0.79	< 0.025	0.15	0.16	0.17	0.76	0.44	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025
N-Acetylsulfamethoxazol	< 0.025	0.88	0.19	0.47	0.29	0.62	0.04	1.86	0.35	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025
Tramadol	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.37	1.42	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025
Tramadol-N-Oxid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	< 0.025	< 0.025	0.0375	< 0.025	< 0.025	0.04	0.04
Trimethoprim	< 0.025	0.16	0.45	0.04	0.18	< 0.025	0.17	0.17	0.23	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025
Nutrition-related chemicals														
Caffeine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	116	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
Acesulfame	0.69	n.b	n.b	16	14	13	12	20	13	5.3	5.1	4.3	2.9	4.1
Cyclamate	0.12	n.b	n.b	0.08	0.16	0.08	0.01	66	0.08	0.05	0.05	0.03	0.04	0.03
Saccharin	< 0.025	n.b	n.b	32	< 0.025	2.3	< 0.025	12	0.98	0.68	0.03	0.05	0.09	0.06
Sucralose	0.07	n.b	n.b	3.7	1.6	0.1	1.4	6.7	10	3.6	2.3	2	4.4	3.9

(Tab. continued)

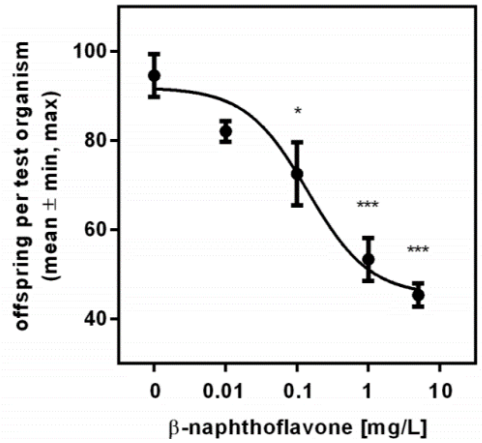
Compound/Group: / Sampling point:	SW	INF-1	EFF-1	INF-2	EFF-2	INF-3	EFF-3	INF-4	EFF-4	EFF+O_3	O_3+GAC	O_3+GAC_a	O_3+BF	O3+BF_a
Industrial chemicals and biocides														
Benzothiazole														
2-Mercaptobenzothiazole	< 0.025	2.9	0.45	1.2	0.46	0.55	0.24	0.55	0.46	< 0.05	< 0.05	< 0.05	< 0.05	< 0.05
2-(Methylthio)-benzothiazole	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025
1,3-benzothiazol-2-amine	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025	0.13	0.29	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025
2-Hydroxybenzothiazole	0.12	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025	n.d.	< 0.025	< 0.025	< 0.050	< 0.025	< 0.050	< 0.050
Benzothiazol-6-carboxylic acid	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025	< 0.25	< 0.25	< 0.25	< 0.25	< 0.25	< 0.25	< 0.25
2-(Thiocyanomethylthio)-benzothiazole (TCMTB)	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025
Benzotriazole and Tolyltriazole														
(1H-)Benzotriazole	0.35	12	6.1	[5.1]	[5.8]	[4.8]	[5.3]	29.2	8.4	0.43	0.06	0.08	0.36	0.39
1-Hydroxybenzotriazole	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	2.3	0.56	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025
4-Hydroxy-1H-benzotriazole	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.79	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025
Tolyltriazole	0.15	2.1	1.5	2.2	0.8	1.8	1.7	3.9	3	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025
Melamine	0.86	0.25	0.23	0.33	0.37	0.2	0.32	2.2	2.6	2.5	1.1	1.2	2.3	2.3
Hexamethoxymethylmelamine	0.06	0.04	0.04	0.17	0.09	0.16	0.14	0.2	0.31	0.05	0.03	0.03	0.06	0.05
Chloridazon	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025
Desphenyl-chloridazon	0.05	0.12	0.13	< 0.025	0.14	0.27	0.4	0.05	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025	0.05
Methyl-Desphenyl-chloridazon	< 0.025	0.03	0.04	< 0.025	< 0.025	0.09	0.12	< 0.25	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025
N,N-Diethyl-meta-toluamide (DEET)	< 0.025	0.18	0.11	0.08	0.06	0.06	0.04	0.04	0.58	0.09	< 0.025	< 0.025	0.06	0.06
N,N-dimethylsulfamide (dichlofluanid and tolylfluanid)	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025	< 0.025	0.22	0.20	0.09	0.08	0.07	0.09	0.08

Compounds detected below the LOQ at all sampling points	Atenolol, aspartame, betaxolol, chloramphenicol, clenbuterol, *clofibric acid*, crotamiton, dapsone, diazepam, doxicyclin, etofibrate, fenofibrate, fenoprofen, gemfibrozil, indometacin, iotalamic acid, ketoprofen, metronidazol, oxytetracycline, pentoxifylline, phenacetin, pindolol, propyphenazone, ronidazole, sulfadiazine, sulfadimidine, sulfamerazine

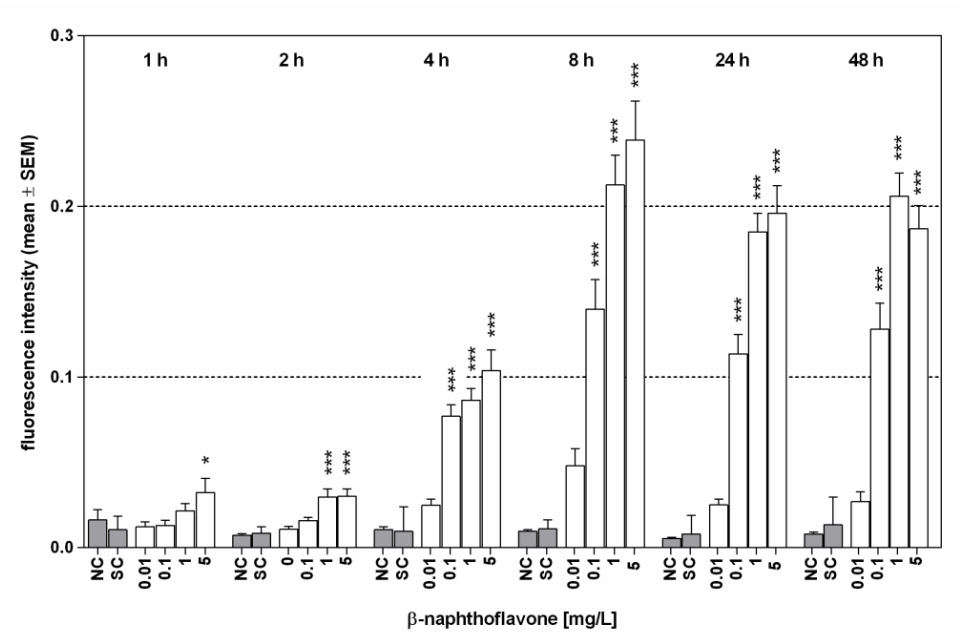
Compounds tested positively for (repro)toxicity in *C. elegans* (ECs indicated in brackets, if available)	Adamantylamine (EC = 302 mg/L, Kao et al. 2016), TCMTB (EC = 2.3 mg/L, Allard et al. 2013), caffeine (EC_{50} = 1.9 g/L, Boyd et al. 2010), carbamazepine (EC_{10}(120 h) = 8.1 mg/L, EC_{50}(120 h) = 132 mg/L, Olga Kolychalow, personal communication), DEET (Hartman and Freedman 2005), saccharin (Sofia Allison, personal communication), sulfamethoxazole (LOEC = 25.3 mg/L, Liu et al. 2013)
Compounds tested positively for growth promotion in *C. elegans* (ECs indicated in brackets, if available)	Sulfamethoxazole (LOEC = 25.3 mg/L, Liu et al. 2013)



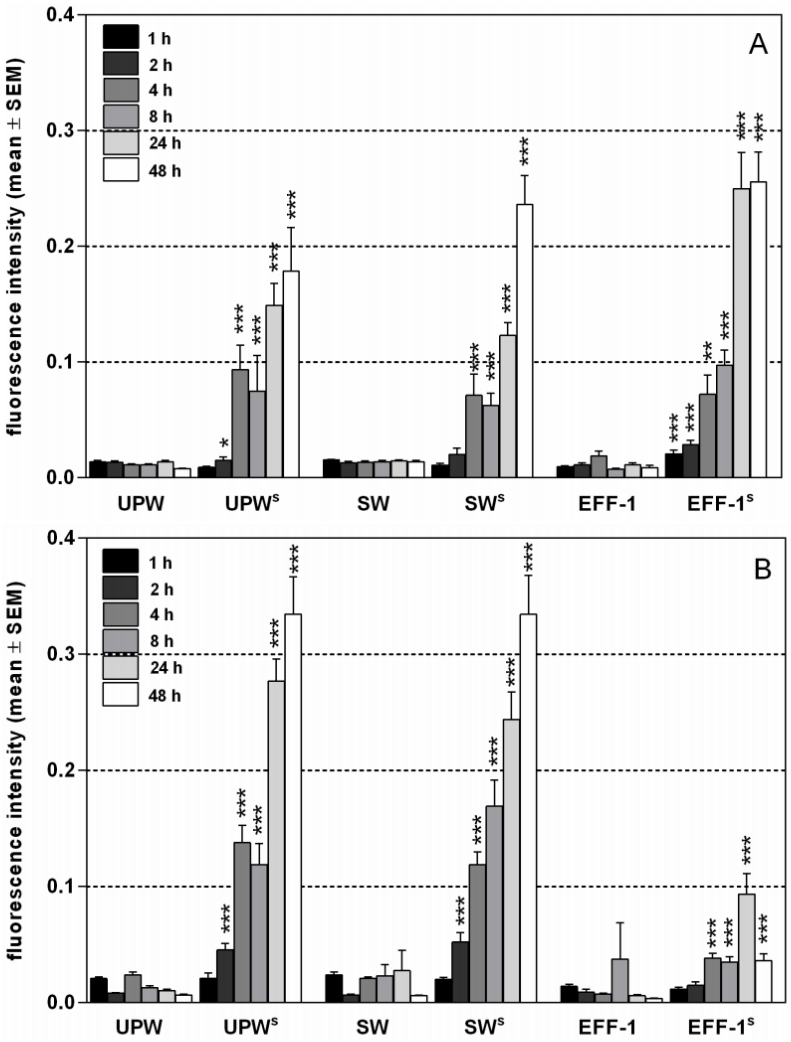
Online Resource 4: Fig. Micropollutant removal during conventional and advanced wastewater treatment at WWTP-4. Concentrations (conc., in [µg/L], y-axes) of micropollutants and transformation products (n = 58) were quantified for samples from April 2015. WWTP stages are WWTP influent (INF-4), WWTP effluent (EFF-4), ozonated effluent (EFF+O_3), granulated activated carbon filtration or biofiltration of ozonated WWTP effluent (O_3+GAC, O_3+BF). Post-filtrations by GAC and BF are given as mean of the aerated and non-aerated systems due to equivalent removal efficiencies (compare 3.5 and 4.4 in the main manuscript). Compound annotations (Transformation products indicated in *italics*): caffeine (1), cyclamate (2), *2-hydroxyibuprofen* (3), ibuprofen (4), *4-acetamidoantipyrin* (5), (1H-)benzotriazole (6), iomeprol (7), paracetamol (8), acesulfame (9), gabapentin (10), *carboxyibuprofen* (11), saccharin (12), *4-formylaminoantipyrin* (13), iopromide (14), iohexol (15), *3-hydroxyibuprofen* (16), diclofenac (17), diatrizoic acid (18), sucralose (19), *1-hydroxyibuprofen* (20), *10,11-dihydro-10,11-dihydroxycarbamazepin* (21), naproxen (22), tolyltriazole (23), acyclovir (24), *1-hydroxybenzotriazol* (25), *carboxyacyclovir* (26), melamine (27), *4-hydroxydiclofenac* (28), bezafibrate (29), *N-acetylsulfamethoxazol* (30), sotalol (31), bisoprolol (32), tramadol (33), carbamazepine (34), metoprolol (35), erythromycin (36), *fenofibric acid* (37), *4-hydroxy-1H-benzotriazol* (38), sulfamethoxazole (39), clarithromycin (40), *2-(methylthio)-benzothiazol*e (41), roxithromycin (42), *2-hydroxybenzothiazol* (43), 1-adamantylamine (44), primidone (45), N,N-dimethylsulfamide (46), *hexamethoxymethylmelamine* (47), oxazepam (48), trimethoprim (49), PEMA (phenylethylmalondiamide) (50), *dehydrato-erythromycin* (51), *ritalinic acid* (52), propranolol (53), *carbamazepine-epoxid* (54), *desphenyl-chloridazon* (55), phenazone (56), N,N-diethyl-meta-toluamide (DEET) (57), *tramadol-N-oxid* (58)



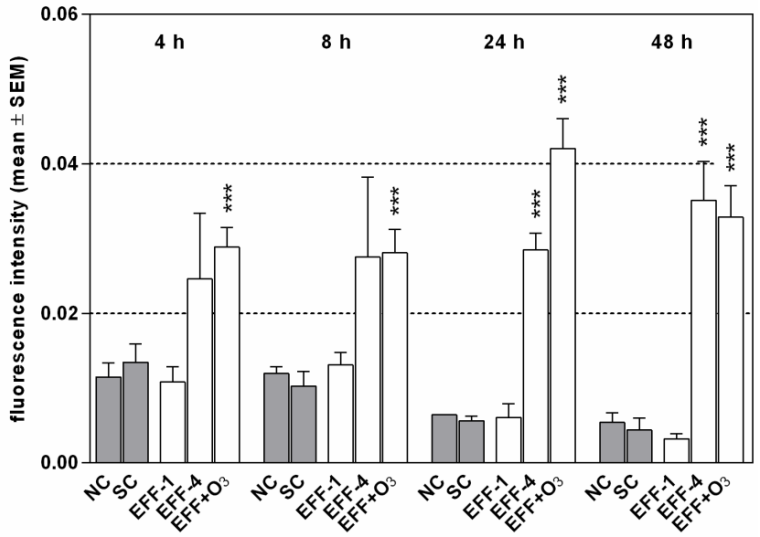
Online Resource 5: Fig. Concentration-response-curve of β-naphthoflavone for the endpoint brood size of *C. elegans*. The mean number of offspring per adult was quantified after 96 h of exposure to β-NF (0.01–5.0 mg/L) commencing at the L1 stage. Significant differences (* = $p < 0.05$, *** = $p < 0.001$) were tested against the control (M9 media, indicated as 0 mg/L β-naphthoflavone) by one-way ANOVA with Tukey's post-hoc analysis. The median effective concentration EC_{50} (0.14 mg/L) was derived using a logistic regression model ($y = 45.3 + 46.6 / (1+10^{lg(x)+0.85})$, $R^2 = 0.74$)



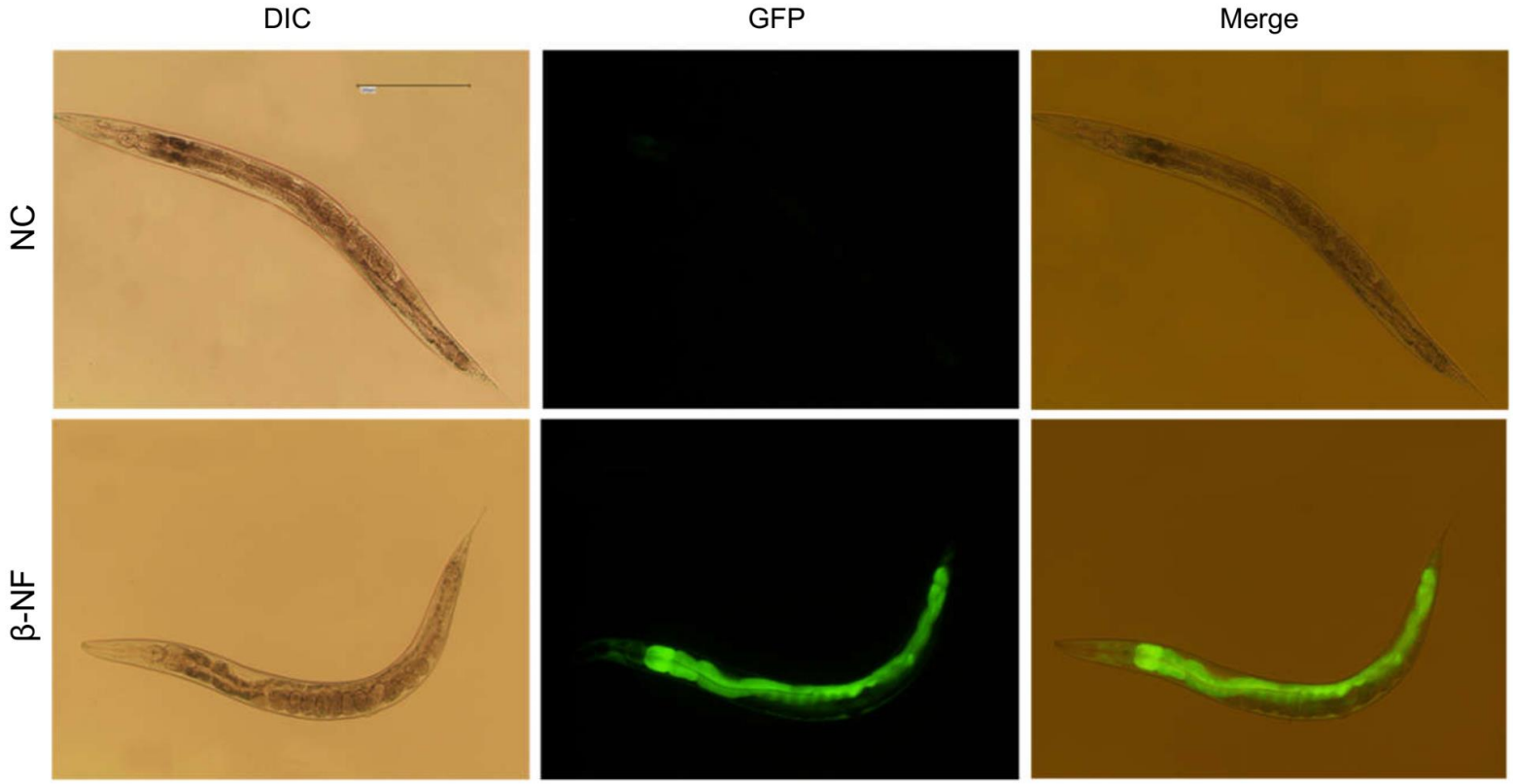
Online Resource 6: Fig. Impacts of β-naphthoflavone on *cyp-35A3*::GFP expression in transgenic *C. elegans*. Samples were analyzed after 1–48 h exposure of adult hermaphrodites to 0.01–5.0 mg/L β-naphthoflavone. Results pooled from two experiments (n = 25 per treatment). Significant differences (* $p < 0.05$, *** $p < 0.001$) were tested against the controls of each time point by one-way ANOVA followed by Dunett's comparison test. NC = M9 medium. Solvent control (SC) = 0.2 % DMSO in M9 medium. Median effective concentrations EC_{50} for the 8 h (71.5 µg/L) and 24 h (78.6 µg/L) time point were derived using logistic regression models (8 h: $y = -0.0039 + 0.26 / (1+10^{(-1.15-lg(x))*0.67})$, $R^2 = 0.68$ and 24 h: $y = 0.0046 + 0.19 / (1+10^{(-1.11-lg(x))*1.03})$, $R^2 = 0.69$)



Online Resource 7: Fig. Impacts of aqueous (A) and extracted (B) ultrapure water (UPW), surface water (SW) and wastewater treatment plant effluent (EFF-1) on *cyp-35A3*::GFP expression in transgenic *C. elegans.* Aqueous and extracted samples were analyzed in 0.5 and 10-fold concentrations (respectively) after 1–48 h of exposure of adult hermaphrodites. Spiked aqueous samples (marked by superscript s) contained 0.5 mg/L β-naphthoflavone. Results pooled from two experiments (n = 15 per treatment group). Significant differences (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) were tested aqueous against spiked samples of each time point (A, B) by unpaired t-test



Online Resource 8: Fig. Impacts of extracted wastewater treatment plant effluents (EFF-1, EFF-4) and ozonated effluent (EFF+O_3) on *cyp-35A3*::GFP expression in transgenic *C. elegans.* Extracted samples (white bars) were analyzed in 10-fold concentrations after 4–48 h of exposure of adult hermaphrodites. Results pooled from two experiments (n = 10 per treatment group). Significant differences (*** $p < 0.001$) tested against controls of each time point by one-way ANOVA followed by Dunett's comparison test. NC = M9 medium. Solvent control (SC) = 0.2% DMSO in M9 medium



Online Resource 9: Fig. 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 fluorescence signal resulting from green fluorescence protein (GFP) along their intestine, as detected by fluorescence microscopy (100x magnification). DIC = Differential interference contrast microscopy. Merge = Overlay of DIC and GFP channel. NC = M9 medium. Bar = 200 µm

A.3 Post-treatment of ozonated wastewater with activated carbon and biofiltration compared to membrane bioreactors: Toxicity removal *in vitro* and in *Potamopyrgus antipodarum*

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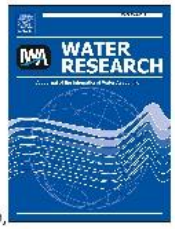

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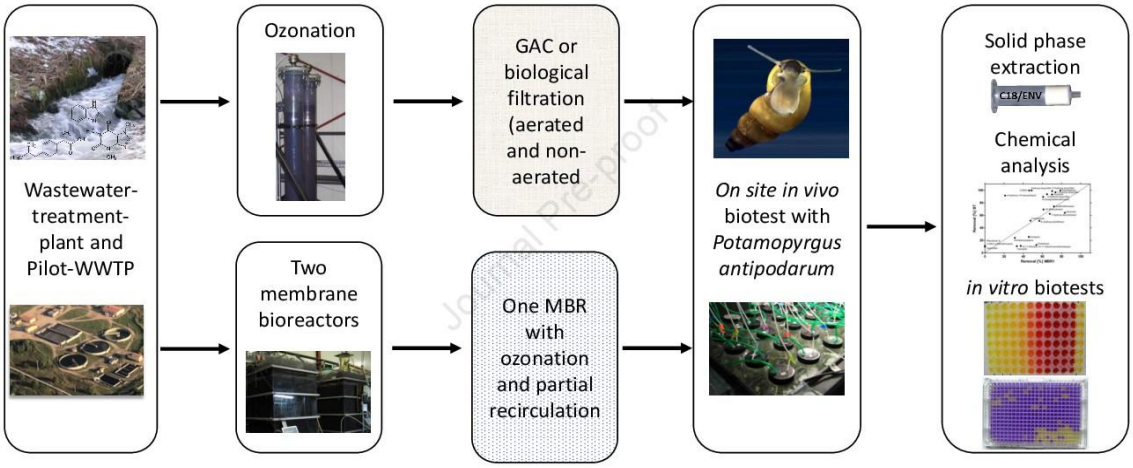

Post-treatment of ozonated wastewater with activated carbon and biofiltration compared to membrane bioreactors: Toxicity removal *in vitro* and in *Potamopyrgus antipodarum*

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Abstract

Wastewater treatment plants are major point sources of (micro)pollutant emissions and advanced wastewater treatment technologies can improve their removal capacity. While abundant data on individual advanced treatment technologies is available, there is limited knowledge regarding the removal performance of ozonation combined with multiple post-treatments and stand-alone membrane bioreactors. This is especially true for the removal of *in vitro* and *in vivo* toxicity.

Therefore, we investigated the removal of 40 micropollutants and toxicity by a pilot-scale ozonation with four post-treatments: non-aerated and aerated granular activated carbon and biological filtration. In addition, two stand-alone membrane bioreactors fed with untreated wastewater and one MBR operating with ozonated partial flow recirculation were analysed. Aqueous and extracted samples were analysed *in vitro* for (anti)estrogenic, (anti)androgenic and mutagenic effects. To assess *in vivo* effects, the mudsnail *Potamopyrgus antipodarum* was exposed in an on-site flow-through system.

Multiple *in vitro* effects were detected in conventionally treated wastewater including estrogenic and anti-androgenic activity. Ozonation largely removed these effects, while anti-estrogenic and mutagenic effects increased suggesting the formation of toxic transformation products. These effects were significantly reduced by granular activated carbon being more effective than biological filtration. The membrane bioreactor performed similarly to the conventional treatment while the membrane bioreactor with ozonation had a comparable removal performance like ozonation.

Conventionally treated wastewater increased the growth of *P. antipodarum*. Ozonation reduced the reproduction indicating a potential formation of toxic transformation products. In the post-treatments, these effects were compensated or remained unaffected. The effluents of the membrane bioreactors induced reproductive toxicity.

Our results show that ozonation is effective in further reducing toxicity and micropollutant concentrations. However, the formation of toxicity requires a post-treatment. Here, ozonation coupled to granular activated carbon filtration seemed the most promising treatment process.

44 **Keywords**

45 reporter-gene assays, endocrine disrupting chemicals, sewage, advanced wastewater treatment, on-
46 site testing, transformation product

47 **Abbreviations**

4-NOPD	4-nitro-o-phenylenediamine
a	aerated (with ambient air)
Ames	bacterial reverse mutation test
ANOVA	analysis of variance
AOP	advanced oxidation process
AWWT	advanced wastewater treatment
BF	biofilter
BSA	bovine serum albumin
BT	biological treatment
C	carbon
CAS	Chemical Abstracts Service
COD	chemical oxygen demand
D	ozone dose
DIN	German Institute of Standardisation (Deutsches Institut für Normng)
d	specific ozone dose
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DOC	dissolved organic carbon
E_2	17β-estradiol
EBCT	empty bed contact time
EC_{50}	Median effect concentration
EE_2	17α-ethinylestradiol
EQS	environmental quality standards
FI	fecundity index
Flu	flutamide
GAC	granular activated carbon
hAR	human androgen receptor
hERα	human estrogen receptor α
H_2SO_4	sulphuric acid
H_3PO_4	phosphorus acid
HPLC	high pressure liquid chromatography
HRT	hydraulic retention time
ISO	International Standard Organisation
LC	liquid chromatography

LOQ	limit of quantification
MBR	membrane bioreactor
MS	mass spectrometry
n.a.	not analysed
Na_2SO_4	sodium sulphate
n.c.	not calculable
NC	negative control
n.d.	not detected
NF	nitrofurantoin
NH_4-N	ammonium
NO_2-N	nitrite
NO_3-N	nitrate
n.s.	not significant
O_2	oxygen
O_3	ozone
OD	optical density
OECD	Organisation for Economic Co-operation and Development
OHT	4-hydroxytamoxifen
P_{total}	total phosphor
PAC	powdered activated carbon
P. antipodarum	*Potamopyrgus antipodarum*
PC	positive control
PO	propylene oxide
PT	primary treatment
PTFE	polytetrafluoroethylene
RR	recirculation rate
rpm	round per minute
SAC_{254}	spectral absorption coefficient at a wavelength of 254 nm
SC	solvent control
SD	standard deviation
SEM	standard error of the mean
SI	supplementary information
SPE	solid phase extraction
T	testosterone
TA100	recombinant strain of *Salmonella typhimurium*
TA98	recombinant strain of *Salmonella typhimurium*

TP	transformation product
UV	ultra violet
V_F	filter velocity
w/o	without
WWTP	wastewater treatment plant
YAAS	Yeast anti-androgen screen
YAES	Yeast anti-estrogen screen
YAS	Yeast androgen screen
YES	Yeast estrogen screen
YG7108	recombinant strain of *Salmonella typhimurium*
Z	ozone consumption
z	specific ozone consumption

1 Introduction

Municipal wastewater treatment plants (WWTPs) are main entry points for the emission of chemicals to aquatic ecosystems, including pollutants of emerging concern (Loos et al. 2013) and micropollutants (Schwarzenbach et al. 2006). WWTPs are known to incompletely remove different micropollutants during conventional, biological wastewater treatment, such as using activated sludge. Reasons for this are low biodegradability and/or high polarity of chemicals (Knopp et al. 2016). Certain micropollutants have been detected throughout the water cycle including nanogram per liter concentrations in drinking water (Benotti et al. 2009) and have been characterised as relevant risk to ecosystem integrity and drinking water resources (Malaj et al. 2014). Chemical contamination resulted in the establishment of environmental quality standards (EQS) in many countries, including their integration into different (waste)water policies (e.g., European Parliament and Council 2008, 2013) and the implementation of technical mitigation measures.

One major measure is the development and implementation of advanced wastewater treatment (AWWT) technologies (Bui et al. 2016). Key AWWT include advanced oxidation processes (AOPs, e.g., ozonation in combination with UV radiation), activated carbon treatments (e.g., granular activated carbon (GAC) or powdered activated carbon (PAC)) or pressure-driven membranes (e.g., reverse osmosis). These technologies demonstrated additional removal of (micro)pollutants from biologically treated wastewater. However, each technology has certain weaknesses such as the formation of potentially toxic transformation products (TPs) during AOP or an insufficient sorption of polar chemicals to activated carbon (Rizzo 2011). Accordingly, the addition of a post-treatment (i.e., filtration after ozonation) and optimised parameter settings (e.g., ozone (O_3) doses and hydraulic retention times (HRTs)) have been recommended (Völker et al. 2019). The present study investigates an innovative process combination for the further reduction of relevant (micro)pollutants and toxicity. The focus was the upgrade of a municipal WWTP with activated sludge treatment in Hesse, Germany with a pilot-scale ozonation in combination with subsequent non-aerated and aerated GAC/biofilter (BF) (Figure 1). Ozonation was chosen because the chemical oxidation induces a transformation of (micro)pollutants in the wastewater and, thus,

increases the accessibility to and degradation in the biological treatment. These transformation processes and the resulting TPs can result in the formation of *in vitro* and *in vivo* toxicity (Völker et al. 2019). Therefore, ozonation was combined with GAC and biofilter as adsorptive techniques to reduce these effects. This is novel because commonly GAC filtration is used as a post-treatment technology for activated sludge treatments but not in combination with other AWWT technologies.

Membrane bioreactors (MBRs) present a stand-alone technology to treat raw wastewater, such as hospital wastewater (Bui et al. 2016, Skouteris et al.2012, Verlicchi et al. 2010). The benefits of using MBRs are amongst others that a final sedimentation is not needed and that a higher solid content in the MBR results in smaller construction volumes and higher sludge ages that may positively affect micropollutant removal. Again, little is known regarding their performance in reducing toxicity (Gehrmann et al. 2018, Maletzt et al. 2013, Snyder et al. 2007). Thus, two MBRs fed with untreated wastewater, one incorporating a partial flow recirculation of ozonated wastewater, were examined (Figure 1) focusing on the combination of oxidation and biological treatment. The aim was to test whether higher removal rates can be achieved with the lowest ozone concentration. Such an approach has not yet been investigated. Another benefit of the implementation of the recirculation concept was that it does not require an expansion of existing activated sludge treatment and, thus, lowers the operating costs.

As multiple AWWT technologies and combinations thereof are available, it is important to compare their performance in removing chemicals and toxicity. So far, most previous studies investigated only a single AWWT technology, often alone or less frequently in combination with one post-treatment (e.g., ozonation combined with sand filtration). In addition, most studies are performed at different WWTPs complicating the comparison of technological performance and efficiency of multiple technologies. Studies comparing multiple process combinations at the same plant are rather scarce (Stalter et al. 2010, Völker et al. 2016). However, such studies are needed to assess the benefits of conventional and AWWT technologies.

To evaluate the efficiency of AWWT technologies, chemical and ecotoxicological analysis are complementary because the former allows for determining the removal of priority compounds while the latter enables the assessment of toxicity removal caused by an overall mixture of chemicals (Cao et al. 2009). This combination is particularly important because the removal of target compounds does not *per se* correlate to toxicity removal (Magdeburg et al. 2014). Case-specific combinations of bioassays and chemical analyses were thus rated as 'gold standard' (Ternes et al. 2017).

In the current study, we used multiple *in vitro* bioassays and one *in vivo* bioassay with the New Zealand mudsnail *Potamopyrgus antipodarum* and quantified 28 representative micropollutants and twelve standard wastewater parameters. The performance of a full scale conventional biological wastewater treatment (BT) combined with a subsequent pilot scale ozonation (BT+O_3) followed by GAC filtration or BF as well as two stand-alone MBRs, one MBR with partial flow ozonation (MBR1, MBR1+O_3 and MBR2, respectively) were investigated. The evaluation focused on the removal of target chemicals and toxicity compared to the activated sludge treatment (O_3, GAC, BF) or raw wastewater (MBRs). In this context, three hypotheses were tested: 1) Increasing the ozone dose and HRT increases the removal of micropollutants and *in vitro* toxicities; 2) Ozonation generates toxic TPs that adversely affect different *in vitro* and/or *in vivo* endpoints while a post-treatment reduces these effects; 3) The MBRs remove chemicals and toxicity with a performance comparable to an activated sludge treatment with a partial flow ozonation further increasing the performance. The aim of this work was to compare the toxicity and micropollutant removal of the multiple combinations of AWWT technologies implemented at the same WWTP and provide recommendations on which technologies perform best.

2.1 Characterisation of the pilot WWTP with ozonation and post-treatments

The pilot plant investigated in this study received wastewater from a full-scale WWTP in South Hesse, Germany (Knopp et al. 2016, Table S1). The latter has about 40,000 population equivalents and an average discharge of 6,400 m^3/d composed of ~70% municipal and ~30% industrial sources. The primary treatment (PT) consists of a mechanical screen and grit removal (raw effluent). The secondary treatment is a biological activated sludge process with denitrification, nitrification and phosphorus removal (chemical precipitation) and final clarifiers. In the pilot WWTP the wastewater from this secondary treatment was filtered with a micro-sieve (10 µm, Rodisc, Huber SE, Berching, Germany) to further reduce total suspended solids before complete treatment in ozone system 1 (Figure 1, Table S2). This system (Xylem Water Solutions, Herford, Germany) consisted of two 0.113 m^3 bubble columns (height: 3.6 m, ∅: 0.2 m) connected in series and one 0.049 m^3 equalisation tank (height: 1.5 m, ∅: 0.2 m). One bubble column was run in counter-current, the other one was run in direct-current. The applied ozone dose was 10.1 g/m^3 (n = 22), the specific ozone consumption was 0.93 g O_3/g DOC (n = 22) and the hydraulic retention time (HRT) was 17.9 min (n = 22, Table S3). After full-scale ozonation the wastewater was treated in four parallel post-treatments: two GAC filters (grain size 1.0–4.75 mm, internal surface 1,200 m^2/g, Epibon A, Donau Carbon, Frankfurt/Main, Germany) and two BFs (grain size 1–5 mm, AR1/5-580, ARGEX NV, Belgium) using extended clay as non-adsorptive carrier. The post-treatments were identical in dimension (height: 4.0 m, ∅: 0.19 m). One GAC filter and one BF were aerated with ambient air (velocity: ~4.0 m/h) while the other ones remained non-aerated. The empty bed contact time of all filters ranged from 26.7 to 36.4 min with a filter velocity of about 3.33 to 4.96 m/h (Table S4) achieving a net specific throughput of approximately 7,500–10,000 m^3/m^3 bed volume.

The two pilot-scale MBRs (BIO-CEL BC-10-10-PVC, MICRODYN-NADIR, Wiesbaden, Germany) were fed with mechanically treated raw wastewater from the full-scale WWTP (Figure 1, Table S2). Both MBRs had a volume of about 1.6 m^3, each, and were operated in parallel. They consisted of an aerated tank with a submerged membrane (0.04 µm) and a denitrification reactor.

Wastewater from MBR1 was ozonated in ozone system 2 (Xylem Water Solutions, Herford, Germany) consisting of one bubble column (height: 1.5 m, ∅: 0.2 m, volume: 0.049 m^3) and an equalisation tank (height: 0.9 m, ∅: 0.2 m, volume: 0.03 m^3). The applied ozone dose was 6.78 g/m^3 (n = 5), the specific ozone consumption was 0.96 g O_3/g DOC (n = 5) and the HRT was 26.1 min (n = 5, Table S3). A defined fraction of the ozonated wastewater was recirculated into MBR1 with a recirculation rate of 2.02 (n = 5). The sludge retention time was 55 days. MBR2 served as reference and its wastewater was neither ozonated nor recirculated. Further technical details and process parameters are described in the supplementary information (Tables S1–S4).

2.2 Optimal ozone dose and hydraulic retention time

Prior to the on-site experiment with *P. antipodarum* (2.3), an experiment to determine the optimal ozone dose and HRT was performed. Conventionally treated wastewater from the municipal WWTP was ozonated using four increasing ozone doses (0.18–0.51 g $O_{3,\ applied}$/g DOC) at a constant HRT of 12.6 min as well as a constant ozone dose of 0.53 g $O_{3\ applied}$/g DOC using five HRTs (4.6–15.1 min). Three 24 h composite samples were taken from each adjusted ozone dose and HRT. These wastewater samples were extracted (2.4) and analysed in five *in vitro* bioassays (2.5).

2.3 On-site *in vivo* experiment with *Potamopyrgus antipodarum*

P. antipodarum was collected in the stream Lumda in Hesse, Germany (50°38'52.64" N, 8°53'49.28" E) and acclimatised in the laboratory to culture medium at 16.0°C and a light-dark-regime of 16:8 h for four weeks. Animals with shell heights between 3.4 and 4.0 mm were used for the experiment (mean ± SD: 3.66 ± 0.16 mm, n = 50). The endpoints reproduction (number of embryos), growth (shell height) and biomarkers for energy reserves (protein, lipid and glycogen content) were analysed.

The on-site experiment was carried out in a continuous flow-through system directly at the pilot WWTP based on OECD guideline 242 (OECD 2016). Wastewater from nine points representing different treatment stages and degrees were tested (Figure 1): after conventional BT, after ozone

system 1 ($BT+O_3$), after non-aerated GAC filtration, after aerated GAC filtration (GAC_a), after non-aerated BF, after aerated BF (BF_a), after MBR1 and MBR2 and after ozone system 2 ($MBR1+O_3$). The PT was not investigated because other studies reported on high mortality upon exposure to raw wastewater (Giebner et al. 2018, Smital et al. 2011).

Peristaltic pumps (Otto Huber, Böttingen, Germany) constantly pumped the undiluted wastewater through polytetrafluoroethylene (PTFE) tubes from the nine treatment stages to 10 L high-grade stainless-steel reservoirs allowing residual ozone to gas out. From these reservoirs, smaller peristaltic pumps (IPC 24, Ismatec, Wertheim-Mondfeld, Germany) pumped the wastewater constantly through PTFE tubes into the exposure vessels containing the test organism. The exposure vessels were placed in random order in a tank filled with water nearly up to the passive overflows of the exposure vessels. Water temperature was adjusted to 16°C using four heating elements and an external cooling unit (Julabo, Seelbach, Germany). A negative control group (NC) with culture medium (OECD 2016) and a positive control group (PC) with culture medium containing 25.0 ng/L 17 α-ethinylestradiol (EE_2) ran in parallel to the wastewater treatments in a flow-through system as well. Fresh culture medium of the NC and PC was prepared regularly (Table S5). Each test vessel (1 L) was filled with 600 mL medium or wastewater and had a 6-fold volume water exchange rate per day. All vessels were aerated with ambient air filtered with a 0.2 µm laboratory injection filter.

Twenty-five mudsnails were exposed in each replicate (four replicates per treatment group) and fed every third day with 0.25 mg fine powdered fish feed (Tetra Phyll) per snail and day. After 28 days of exposure under a light:dark regime of 16:8 h, the mudsnails were frozen in liquid nitrogen and stored at −80°C until analysis. For the analyses, the mudsnails were defrosted, shell height was measured to the nearest 0.1 mm and shells were cracked and carefully removed to determine the total number of embryos in the brood pouch. In addition, aqueous grab samples of the NC and the PC medium and aqueous 24 h composite samples and 5000-fold enriched samples of the different wastewaters were tested *in vitro* (see 2.4–2.5, Table S5). Protein, glycogen and lipid content as biomarkers for energy reserves were determined as described in the Supplementary Information (S1.3, Figures S1–S3, Tables S6–S8). In brief, each mudsnail was weighed (accuracy ±0.01 mg)

and homogenised in 300 µL sodium sulphate solution (Na_2SO_4; 2.0%) for three minutes under 30 turns per second using a grinding ball and a swing mill (MM 400, Retsch GmbH, Haan, Germany). The protein content was determined as described in Bradford (1976). Glycogens and lipids were separated as described by van Handel (1965) and determined using hot anthrone and vanillin reactions (van Handel 1985a, b). The protein, glycogen and lipid content of the samples was calculated in µg/mg mudsnail and then converted to an energy content of the lipid reserve in J/mg mudsnail using the specific calorific value (Berg et al. 2007).

2.4 Wastewater sample preparation: Solid phase extraction (SPE)

The SPE column Telos C18/ENV, 500 mg+200 mg/6 mL (Kinesis Ltd., St. Neots, Great Britain) was used for extracting the wastewater samples because they were optimal for the enrichment of endocrine activity and mutagenicity from wastewater (Abbas et al. 2019). The SPE columns were conditioned consecutively with 1 x 2.0 mL heptane, 1 x 2.0 mL acetone, 3 x 2.0 mL methanol and 4 x 2.0 mL ultra-pure water. SPE was performed within 48 h after sample collection. Each wastewater sample was collected as 24 h composite sample (Table S5). After filtration with GF 6 filters (Whatman, GE Healthcare Life Sciences, Chalfont St. Giles, England), 500 mL of each sample were acidified to pH 2.5 with sulphuric acid (3.5 mol/L) directly before enrichment and extracted. The columns were dried under N_2 and eluted with methanol and acetone at neutral conditions (5 x 2.0 mL, respectively). After adding 100 µL dimethyl sulphoxide (DMSO) each methanol-acetone extract was concentrated to 100 µL final volume under a gentle N_2 stream. All DMSO extracts (5,000-fold concentrated compared to the aqueous sample) were stored at −20°C until testing. A SPE blank (solvent control, SC) was prepared by extracting 500 mL ultra-pure water. SPE blanks were identically prepared in parallel to the enrichment of samples from each sampling campaign.

2.5 *In vitro* bioassays for endocrine activities and mutagenicity

2.5.1 Recombinant yeast screens for endocrine activities

Four recombinant yeast-based reporter gene assays were used to detect endocrine activities in wastewater samples: Yeast Estrogen Screen (YES, human estrogen receptor α (hERα)), Yeast Anti-Estrogen Screen (YAES), Yeast Androgen Screen (YAS, human androgen receptor (hAR)) and Yeast Anti-Androgen Screen (YAAS) as first described by Routledge and Sumpter (1996) and Sohoni and Sumpter (1998). The YES and YAS are used to study compounds activating the hERα and hAR (receptor agonists) while the YAES and YAAS detect chemicals blocking the respective receptors (antagonists). All bioassays were performed in 96-well microtiter plates (f-form, VWR Darmstadt, Germany) as previously described by Völker et al. (2016). In brief, aqueous samples were analysed in a 0.63-fold final sample concentration (1.6-fold dilution). SPE extracts were analysed with a dilution factor of 480 resulting in a 10.4-fold final sample concentration (0.2% v/v solvent concentration). All samples were analysed in eight replicates. Negative controls (NC) using ultra-pure water (aqueous samples), solvent controls with DMSO (SC, for SPE extracts) and PCs were analysed in each experiment (see Figures S4 and S5 and Table S9 for details). The incubation times at 30°C and 1200 rpm depended on the bioassay and were between 18 and 22 h. Results were not used if > 20% cytotoxicity compared to the NC/SC occurred. Relative endocrine activities were calculated by normalising the reported gene activity to the NC/SC (0%) and the maximum activity of the reference compound (100%). A control without agonist was used for the antagonistic assays to represent 100% receptor inhibition. Selected SPE extracts, particularly those that were cytotoxic, were tested with dilution factors of 1:2 to 1:16 to generate concentration-response-relationships (Figure S6).

2.5.2 Recombinant bacterial test for mutagenicity (Ames fluctuation test)

The Ames fluctuation test (ISO DIN 11350, 2012) was used to identify mutagenic activity (i.e., irreversible DNA damages) with three genetically-modified strains of the bacterium *Salmonella typhimurium* (TA98, TA100 and YG7108) as described by Magdeburg et al. (2014). In brief, SPE extracts were tested in a 10.4-fold final sample concentration (0.2% v/v solvent). Mutagenic reference compounds were used as PC (Table S9). A SC (DMSO) ran in parallel to the extracts in

each experiment. Cell density was measured photometrically to determine cytotoxic effects. By counting the number of wells that shifted from purple (negative) to yellow (positive) the mutagenic activity of the sample was determined photometrically.

2.6 Chemical analysis

Chemical analysis of wastewater samples was carried out once per week (four times) during the 28 days on-site experiment (2.3). The selection criteria of the 28 micropollutants were amongst others their high polarity and no/low reduction by conventional and/or AWWT technologies, the formation of stable TPs, their ecotoxicological relevance, their detection frequency in aqueous environments and their use as wastewater tracer. Thus, an analysis of these micropollutants and their corresponding TPs was conducted by high performance liquid chromatography (HPLC; Thermo Dionex UltiMate 3000 RSLC, Thermo Fisher Scientific Inc., Waltham, USA) coupled via an electrospray interface with a mass spectrometry (MS) system (MS/MS; Sciex Qtrap 5500, AB Sciex, Framingham, USA) without sample enrichment (Seitz and Winzenbacher 2017). The injection volume was 100 µl. Ultrapure water (Purelab Ultra, Elga, Celle, Germany) was used for dilution or as eluent. Furthermore, the LC/MS grade formic acid (Fluka, MS grade, 98%), ammonium formate (Sigma-Aldrich, > 99.995%) and acetonitrile (Carl Roth, LC-MS grade, > 99.95%) were used. Separation was achieved on a Kinetex 2.6 µm C18 column (100 × 4.6 mm, Phenomenex Inc., Torrance, USA) at a flow rate of 0.6 mL/min with a pre-column (Security Guard KIT KJO-4282, Phenomenex, Torrance, USA). Mass spectrometry was carried out in positive/negative polarity switching electrospray ionization mode. The limit of quantification (LOQ) was 0.025 µg/L. The chemical analysis was done using the following standard methods DIN 38407-36 (2014) and DIN 38407-47 (2015).

2.7 Measurement of physical-chemical wastewater parameters

The following water parameters were determined directly at the pilot WWTP using standardised cuvette tests (Hach Lange, Düsseldorf, Germany): chemical oxygen demand (COD), dissolved

organic carbon (DOC), nitrite (NO_2-N), nitrate (NO_3-N), ammonium (NH_4-N), total phosphor (P_{tot}) and spectral absorption coefficient at 254 nm (SAC_{254}) (Table S10). In addition, the following water parameters were measured directly in the exposure vessels as requested by OECD (2016): pH, conductivity, oxygen saturation and oxygen concentration using potentiometric electrodes (Multi 340i/SET, WTW Weilheim, Germany), nitrite (NO_2-N), nitrate (NO_3-N), ammonium (NH_4-N) and total hardness using rapid test kits (Aquamerck, Merck, Darmstadt, Germany, Table S11). Temperature was measured in the tank with two data loggers that recorded the temperature every 15 min.

2.8 Statistical analysis

Statistical analyses were performed using GraphPad Prism (version 5.03, GraphPad Software, San Diego, California, USA). Mortality data were analysed using Fisher's exact test. Gaussian distribution was tested with the D'Agostino and Pearson omnibus normality test and homogeneity of variances with the Bartlett's test. In case of a normal distribution and equal variances, significant differences between the datasets were analysed using a one-way ANOVA with Bonferroni's post-test (glycogen and total energy content). If the datasets were not normally distributed, the nonparametric Kruskal-Wallis test with Dunn's post-test was used (shell height, total number of embryos and energy contents as protein and lipid). Significant differences between treatments were marked with asterisks: $p < 0.05$: ★, $p < 0.01$: ★★, $p < 0.001$: ★★★.

3 Results

3.1 Optimal ozone dose and hydraulic retention time

3.1.1 Optimal ozone dose

The mean estrogenic and anti-estrogenic activity of the BT was 7.31 ± 0.21% and 61.7 ± 0.55%, respectively. With increasing ozone dose, the estrogenic activity decreased by 94.0% to 0.44 ± 0.07% at the highest ozone dose whereas the anti-estrogenic activity increased by 29.1% to 79.6 ± 1.37% (Figure 2A, Table S12). No androgenic activity was detected in the BT and at all ozone doses (Figure 2B). In contrast, the anti-androgenic activity in the BT was 76.1 ± 0.72%. With increasing ozone dose, the anti-androgenic activity decreased by 35.1% to 49.3 ± 0.73% at the highest ozone dose (Figure 2B, Table S12).

None of the treatments was mutagenic in the Ames TA98 strain (Figure 2C). However, the Ames TA100 strain indicated a potential mutagenicity in the BT (21.2 ± 2.59%) which increased by 67.1% with increasing ozone dose to maximal 35.4 ± 2.10% (Table S12).

3.1.2 Optimal hydraulic retention time

The mean estrogenic activity of the BT was 3.58 ± 0.12%. Ozonation reduced the estrogenic activity by 81.3 to 95.7% independent of the HRT (Figure 2D). The mean anti-estrogenic activity of the BT was 71.0 ± 0.45% and decreased by 12.9% at the lowest HRT to 61.9 ± 0.91%. With increasing HRTs the anti-estrogenic activity first increased before it remained constant within the same range like the BT (Table S13). Again, no androgenic activity was detected in the BT and at all tested HRTs (Figure 2E). However, the anti-androgenic activity of the BT was 70.9 ± 0.80% and decreased by 43.6% to 39.9 ± 2.21% at the lowest HRT. With increasing HRTs, the anti-androgenic activity first increased to 60.7 ± 0.88% before it decreased to 40.7 ± 0.93% at highest HRT (–42.6% compared to the BT, Table S13).

Again, none of the treatments was mutagenic in the Ames TA98 strain (Figure 2F). In contrast, the Ames TA100 indicated potential mutagenicity in the BT (21.5 ± 1.64%). This effect increased by 93.5% at higher HRTs to maximal 41.7 ± 3.18% (Table S13).

3.2 On-site *in vivo* experiment with *Potamopyrgus antipodarum*

3.2.1 Mortality

The mortality of *P. antipodarum* at the end of the 28 days of exposure was low in all controls and the treatment groups. The highest mortality was observed in the PC (3.0 ± 1.92%) and in the non-aerated GAC filter (3.0 ± 3.0%, Table S14). The mortality in the NC was 1.0 ± 1.0%. Thus, the validity criteria of the OECD guideline (maximal 20% mortality) was met (OECD 2016).

3.2.2 Growth and reproduction

At the end of the experiment, the shell heights of the mudsnails were maximal in the BT (3.98 ± 0.23 mm) and differed slightly but significantly ($p < 0.05$) from the NC (3.82 ± 0.17, Figure 3A, Table S14). *P. antipodarum* exposed to water from all AWWTs did not grow less compared to the BT except those exposed to effluent from MBR2 (3.84 ± 0.21, $p < 0.05$).

Exposure to 25 ng/L EE_2 used as PC (27.7 ± 5.36 embryos per female) induced the reproduction by 17.0% compared to NC (23.7 ± 5.27 embryos per female, Figure 3B, Table S14). The total number of embryos exposed to the BT (28.1 ± 6.00) was on the same level as the PC but not significantly higher than in the NC. Ozonation led to a significant reduction (–21.9%, $p < 0.01$) in the number of embryos per female (21.9 ± 5.94) compared to the BT. The reproduction in the subsequent treatments (GAC, GAC_a, BF, BF_a) was below the level of the BT. The number of embryos in animals from the aerated treatments differed significantly (GAC_a: –18.7%, $p < 0.05$ and BF_a: –24.0%, $p < 0.001$) and were lower than the non-aerated treatments (GAC: –2.07% and BF: –10.7%). The exposure to wastewater after the MBRs caused significant reductions (MBR1: –29.9%, $p < 0.01$; MBR1+O_3: –19.6%, $p < 0.01$; MBR2: –56.0%, $p < 0.001$) in the total number of embryos compared to BT.

3.2.3 Biomarkers for energy reserves (glycogen, protein and lipid content)

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The highest mean protein content reflecting the energy state of the mudsnails was determined in the non-aerated BF (0.31 ± 0.07 J/mg tissue, Figure 4A, Table S15). The lowest protein content was found in the MBR2 (0.23 ± 0.08 J/mg). However, no significant differences were detected.

The glycogen content was highest (+29.2%, $p < 0.05$, Figure 4B, Table S15) in animals from the non-aerated GAC filter (0.24 ± 0.08 J/mg) and significantly higher compared to the BT (0.19 ± 0.04 J/mg) and lowest in *P. antipodarum* from the MBR1 (0.15 ± 0.05 J/mg).

The lipid contents of the mudsnails in the PC (0.96 ± 0.42 J/mg) and BT (0.95 ± 0.73 J/mg) were significantly lower (–39.8%, $p < 0.01$ and -40.1%, $p < 0.05$) compared to the NC (1.59 ± 0.54 J/mg, Figure 4C, Table S15). The highest lipid content was determined in animals from the non-aerated BF (2.05 ± 0.31 J/mg) and differed together with aerated GAC filter treatment (1.52 ± 0.51 J/mg) significantly from the BT (+115%, $p < 0.001$ and +59.7%, $p < 0.05$, respectively).

The total energy content in mudsnails from the PC (1.44 ± 0.43 J/mg) and the BT (1.38 ± 0.77 J/mg) were lowest with significant differences (–30.6%, $p < 0.001$ and –33.2%, $p < 0.001$) compared to the NC (2.07 ± 0.56 J/mg, Figure 4D, Table S16). The total energy content of the mudsnails exposed to water from the AWWT were higher than in the BT with significant differences in the GAC_a (1.94 ± 0.36 J/mg, +40.2%, $p < 0.01$), the BF (2.54 ± 0.35 J/mg, +83.7%, $p < 0.001$) and BF_a (1.87 ± 0.47 J/mg, +35.2%, $p < 0.05$).

3.2.4 *In vitro* bioassays for endocrine and mutagenic activity

The extracts of the PT were cytotoxic in all *in vitro* assays (Figures 6, 7) and, thus, not considered.

Recombinant yeast screens for endocrine activity

The aqueous samples of the PC spiked with 25 ng/L EE_2 had a mean estrogenic activity of 28.2 ± 0.47 ng ethinylestradiol-equivalents/L that corresponds to a receptor activation of 26.1 ± 0.78%.

The aqueous PT samples were neither estrogenic (1.60 ± 0.27%) nor anti-androgenic (1.03 ± 0.41%) but induced a high anti-estrogenic (95.0 ± 0.71%) and androgenic (38.2 ± 2.30%)

activity (Figure S8, Table S17). In the BT the anti-estrogenic and androgenic activities were reduced to 57.4 ± 2.83% (–39.6%) and 0.06 ± 0.03% (–99.8%), respectively. The mean endocrine activities in all AWWT (BT+O_3, GAC, GAC_a, BF and BF_a) and MBR systems (MBR1, MBR1+O_3 and MBR2) were on a comparable level to BT.

The SPE extracts of the BT indicated a mean estrogen activity of 16.9 ± 1.60% (Figure 5A, Table S18). Ozonation reduced the estrogenic activity by 96.5% to 0.59 ± 0.11%. The following GAC filter and BF showed a reduction of the estrogen activity compared to the BT by 95.1 to 95.9% as well. For the MBR systems this reduction ranged between 81.7% in MBR2 and 97.4% in MBR1+O_3.

Ozonation of the BT increased the anti-estrogenic activity of the extracts by 163% from 14.1 ± 1.53% to 37.2 ± 1.43% (Figure 5B, Table S18). Post-filtration reduced this anti-estrogenic activity by 5.03 to 49.9% but the activity was still higher compared to the BT (+31.8% (GAC), +65.7% (GAC_a), +150% (BF) and +144% (BF_a)). The wastewater of the MBR1, MBR1+O_3 and MBR2 indicated a higher anti-estrogen activity compared to the BT with an increase by 162, 93.3 and 201%, respectively and a maximal activity of 42.6 ± 2.95% in MBR2.

The mean androgenic activity (Figure 5C, Table S18) of the BT extracts was 1.76 ± 0.31% and was reduced by 10.1 to 84.0% in all AWWT (BT+O_3, GAC, GAC_a, BF and BF_a) and MBR systems (MBR1, MBR1+O_3 and MBR2).

A mean anti-androgenic activity (Figure 5D, Table S18) of 72.1 ± 2.05% was determined in the SPE extracts of the BT. Compared to this treatment the AWWT (BT+O_3, GAC, GAC_a, BF and BF_a) and MBR systems (MBR1, MBR1+O_3 and MBR2) reduced the anti-androgenic activity by 7.68 to 72.6%.

Ames fluctuation test for mutagenicity

No mutagenic activity was detectable in the BT in the Ames strain YG7108 (Figure 6, Table S18). Ozonation of the BT induced a high mutagenicity of 93.2 ± 1.29%. Water treated with GAC and GAC_a was not mutagenic in contrast to the BF and BF_a with 50.8 ± 2.29% and 52.9 ± 4.87%,

410 respectively. No mutagenicity was detected in MBR1 and MBR2 whereas MBR1+O_3 showed a

411 mutagenicity of 67.5 ± 4.62%.

412

413 **3.3 Chemical analysis**

414 The chemical analysis was conducted in parallel to the ecotoxicological investigations and included
415 28 micropollutants mainly belonging to the group of pharmaceuticals such as radio-opaque
416 substances, anticonvulsants, antibiotics (including metabolites such as of carbamazepine, diclofenac
417 or ibuprofen) as well as nutrition-related chemicals (caffeine), herbicides (mecoprop) and industrial
418 chemicals (benzotriazole and tolyltriazole). In the PT, caffeine was detected at the highest
419 concentration of 162 ± 23.2 µg/L followed by carboxy-ibuprofen (74.7 ± 6.27 µg/L), 2-hydroxy-
420 ibuprofen (47.3 ± 4.97 µg/L) and 1H-benzotriazole (25.0 ± 0.71 µg/L). The concentrations of the
421 other substances were between 0.025 and 14.4 µg/L (Table S19). The BT reduced the
422 concentrations of 15 out of 28 chemicals by more than 50% (highest reduction, -99.8% for caffeine
423 and carboxy-ibuprofen). For nine chemicals, the reduction was low (< –25%). For carbamazepine
424 and carboxy-acyclovir a concentration increase was detected.
425 Ozonation led to a further reduction of 21 substances ranging from –11.1% (iopamidol) and –99.1%
426 (carboxy-acyclovir)) compared to the BT (Figure 7A, Table S19). The concentrations of 18
427 substances decreased by more than 50%. For another three compounds, the concentrations
428 decreased by between 10 and 50%. Two TPs (3-hydroxy-ibuprofen and tramadol-N-oxide)
429 indicated higher concentrations in the BT+O_3 than in the BT.
430 The post-treatments further reduced the concentrations of most target substances (Figures S9 and
431 S10, Tables S19 and S20). For certain compounds for which ozonation did not achieve a complete
432 removal (e.g., 3-hydroxy-ibuprofen, diclofenac, sulfamethoxazole), a post filtration led to an overall
433 removal of 75.0 to 90.7% compared to the BT+O_3. For a small set of compounds (2-hydroxy-
434 ibuprofen, 4-hydroxy-1H-benzotriazole, carboxy-acyclovir, paracetamol), a moderate additional
435 removal between 31.1 and 42.9% occurred in the GAC filters and BFs compared to the BT+O_3.
436 GAC filters showed a higher removal rate for seven compounds including 1H-benzotrialzole,

437 amidotrizoic acid, iomeprole, iopromide, tolytriazole and tramadol N-oxide compared to the BFs.

438 Certain compounds such as caffeine or mecoprop could however not be further removed by the
439 GAC filters and BFs compared to the BT+O_3.
440 MBR1 and MBR2 had slightly lower removal efficacies regarding the 28 chemicals than the BT
441 (Figures 7B and S11, Tables S20–S21). The ozonation increased the removal in the MBR1 with
442 efficiencies comparable to the BT+O_3. However, the concentration of carboxy-acyclovir increased
443 in the BT (+367%), MBR1 and MBR2 (+146 and +343%, respectively) as well as MBR1+O_3
444 (+39.3%).
445 The results for the water parameters can be found in the Supplementary Information (S2.4, Tables
446 S22–S29).

448 **4.1 Optimal ozone dose and hydraulic retention time**

449 **4.1.1 Optimal ozone dose**

450 In line with previous research, an additional ozonation of conventionally treated wastewater
451 efficiently reduced the estrogenic activity (Völker et al. 2019). The removal of estrogenicity
452 increased with ozone dose and doses ≥ 0.44 g O_3/g DOC were most effective (Figure 2A, supports
453 hypothesis 1). Interestingly, we observed a marked increase of the anti-estrogenic activity with
454 higher ozone dosage (falsifies hypothesis 1), a phenomenon that has been reported previously
455 (Giebner et al. 2018, Gehrmann et al. 2018, Itzel et al. 2020, Stalter et al. 2011). One potential
456 reason is the removal of estrogens masking the anti-estrogenicity (Ihara et al. 2014, Leusch et al.
457 2017, Ma et al. 2005, Rao et al. 2014) or the formation of anti-estrogenic TPs during ozonation
458 (compare hypothesis 2, Knoop et al. 2018).

459 In contrast to previous studies that reported an effective removal of anti-androgenic activity in
460 biologically treated (Rao et al. 2014) and ozonated (Stalter et al. 2011) wastewater, we detected a
461 high anti-androgenicity in the BT as well as the BT+O_3 samples (Figure 2B) that was not fully
462 removed by the applied ozone doses. Treatment with the highest dose (0.51 g $O_{3\ applied}$/g DOC) led
463 to a 35.1% reduction. This indicated the presence of relatively stable anti-androgenic substances
464 (Itzel et al. 2020).

465 The Ames TA100 was more suitable for detecting mutagenicity than the Ames TA98 (Figure 2C).
466 Again, this is in line with previous research (Völker et al. 2019). The mutagenicity (TA100)
467 increased at higher ozone doses indicating the formation of mutagenic TPs. Higher mutagenicity in
468 ozonated wastewater was previously reported (Chen et al. 2017, Giebner et al. 2018, Jia et al. 2015,
469 Magdeburg et al. 2014). These findings underline the importance of implementing ozonation post-
470 treatments (4.4).

471 With regards to determining the optimal ozone dose, it becomes obvious that a balance needs to be
472 found between the removal of estrogenic and anti-androgenic compounds on the one, and the

formation of anti-estrogenic and mutagenic chemicals on the other side. Here, a dose of 0.33 g O_3/g DOC might represent a good compromise.

4.1.2 Optimal hydraulic retention time

The experiment with a high ozone dose and different HRTs supports the results of the previous experiment: The mean estrogenic activity was reduced in ozonated wastewater compared to the BT for all HRTs (Figure 2D). The anti-estrogenic activity decreased at the lowest HRT but remained at the level of BT at higher HRTs. The results support the idea of a generation of anti-estrogenic TPs during ozonation because the estrogenic activity was on a comparable low level at all HRTs.

Again, the anti-androgenic activity was high in BT (Figure 2E) and was reduced most at the shortest and longest HRT. The lower removal in the intermediate HRTs might be explained by anti-androgenic TPs (hypothesis 2). The mutagenicity detected in the Ames TA100 in the BT increased at particular longer HRTs (Figure 2F). This observation further substantiates the formation of mutagenic TPs during ozonation (hypothesis 2).

4.2 *In vivo* effects in *Potamopyrgus antipodarum*

4.2.1 Growth and reproduction

P. antipodarum were larger when exposed to water from BT compared to the NC (Figure 3A) which may be the result of a better nutrient supply in the BT containing additional organic matter. Furthermore, a significantly lower shell height was detected in the MBR2 compared to the BT which may indicate a lower removal of general toxicity in MBR2.

The reproduction of *P. antipodarum* was increased in the BT and the PC (Figure 3B) compared to the NC. One reason for this could be a better nutrition (compare above). Here, several studies showed that gastropods with a better nutrient supply produced a higher number of eggs (Augusto et al. 2012, Keas & Esch 1997, Ter Maat et al. 2007). Another reason might be the presence of residual endocrine disrupting substances (Duft et al. 2007, Stalter et al. 2011, Stange et al. 2012) in

wastewater. The detected *in vitro* estrogenic activity in the BT (Figure 5A) on the human estrogen receptor may tentatively point towards such chemicals.

The fecundity index (FI, Ladewig et al. 2006, Schneider et al. 2015) was used to further elaborate on these hypotheses. The FI is calculated as the ratio of number of embryos and the shell height of each individual. The FI of the PC and BT were not significantly higher compared to the NC (Figure S7, Table S14) which illustrates that the mudsnails carried a normal number of embryos according to their size. Hence, the higher number of embryos in the BT and the PT could not definitely be related to a higher shell height due to a better nutrient supply or to the detected estrogenic activity.

The reproduction decreased in snails exposed to ozonated wastewater (BT+O_3) and to water from the post-treatments GAC_a, BF_a as well as from MBR1, MBR1+O_3 and MBR2. Here, the significantly decreased FI indicated a reproductive toxicity compared to the BT (Figure S7, Table S14). The reproductive toxicity could be induced by unspecific toxicity of the ozonated wastewater and/or toxic TPs (Völker et al. 2019). In a study by Giebner et al. (2018) the total number of embryos of *P. antipodarum* also decreased after the AWWT ozonation and activated carbon treatment. The authors assumed that the decreased reproduction was caused by a general toxicity of the wastewater. Interestingly, the reproductive toxicity in snails exposed to water from MBR2 implies that it does not remove toxicity as good as a conventional BT (falsifies hypothesis 3).

4.2.2 Biomarkers for energy reserves (glycogen, protein and lipid content)

Glycogen, protein and lipid content have not been previously analysed in *P. antipodarum* exposed to wastewater. They are of interest because the energy content has an influence on reproduction of gastropods (Gust et al. 2011). In the present study, differences in biomarker sensitivity were observed in the order of lipid > glycogen > protein content after the exposure to the different wastewaters (Figure 4). Gust et al (2011) reported that glycogen was the preferred energy invested in the reproduction of *P. antipodarum* followed by lipids. In this study, exposure to differently treated wastewater did not affect the protein content but the glycogen content of the mudsnails

exposed to water from GAC. This may indicate a better nutrition. The lipid contents were reduced by exposure to water from BT and GAC_a. For BT, this does not support our hypothesis of a better nutrition. For GAC_a, this implies an energy depletion which might have been resulted in a lower reproduction. In snails exposed to water from the BF, the lipid content was increased but did not result in a higher reproduction. The total energy content mirrors that picture because lipids are the dominant energy storage in *P. antipodarum*.

4.2.3 *In vitro* endocrine activity and mutagenicity

The aqueous samples taken in parallel to the *in vivo* experiment did not induce any relevant estrogenic and anti-androgenic activities in any sample (Figure S8, Table S17). Accordingly, the removal capacity could not be evaluated for these two parameters. In contrast, high anti-estrogenic and androgenic activities were detected in PT. The androgenic activity was almost completely removed in the BT whereas the anti-estrogenic activity was substantially reduced but remained on a relatively high level throughout all AWWT technologies (Figure S8, Table S17). Hence, the cleaning capacity of the BT seemed not sufficient in removing the latter, which has been suggested in earlier studies on the present (Abbas et al. 2019) and on other activated sludge treatments (Harth et al. 2018, Ihara et al. 2014, Rao et al. 2014, Tang et al. 2014).

Regarding the 10.4-fold concentrated extracts, the estrogenic activity in the BT was almost completely removed by ozonation (Figures 6, Table S18). Accordingly, an additional removal by the post-treatments could not be assessed. In contrast, the anti-estrogenic activity increased markedly in $BT+O_3$. The BF and BF_a did not reduce the anti-estrogenic activity whereas GAC and GAC_a were more effective. One explanation might be that the activated carbon is better in adsorbing more polar ozonation TPs than the more non-polar BF.

Ozonation led to reduction of the anti-androgenic activity but it remained on a relatively high level compared to previous reports (Gehrmann et al. 2018, Itzel et al. 2020) indicating an incomplete oxidative removal of anti-androgenic compounds. Subsequent filtration incompletely reduced this

activity whereby both GAC filters were more effective than the BF. This result was consistent with the result for the anti-estrogenic activity.

Compared to the BT, the MBRs were much more effective in reducing estrogenic (MBR1 and 2) and anti-androgenic activity (MBR1) whereas they release a much higher anti-estrogenic activity. An almost total reduction of estrogenic activity and simultaneous increase of anti-estrogenic activity in the MBR1+O_3 is consistent with the observation for the BT+O_3 (compare above) indicating an incomplete removal of substances with anti-estrogenic activity.

The results of the Ames test with the strain YG7108 (Figure 6) support previous hypotheses on mutagenic TPs generated during ozonation (BT+O_3 and MBR1+O_3). Interestingly, water treated with BF was also mutagenic. Here, the causes remain unknown. Again, the GAC treatments did not generate mutagenic activity. These results again indicated a higher performance of the GAC filters compared to the BFs.

4.3 Removal of micropollutants

Twenty-eight micropollutants and twelve wastewater parameters were analysed in parallel to the on-site experiment with *P. antipodarum* to evaluate the performance of the AWWT technologies. The BT effectively reduced the COD, DOC, NH_4-N, P_{total} and SAC_{254}. These parameters were only minimally affected by ozonation, except for the SAC_{254}. GAC and BF achieved an additional reduction of the COD, DOC and SAC_{254} whereby GAC was more effective than BF (Tables S22–S29).

The MBR systems decreased most of these parameters, except for NO_3-N, NH_4-N and P_{total} at comparable or higher effectivity than the BT. MBR1 had a slightly higher effectivity than MBR2, which may have been the result of the recirculated ozonated wastewater from the MBR1+O_3. Generally, the MBR1+O_3 only showed a comparable (SAC_{254}) or better (COD, DOC, NO_2-N) removal than the BT+O_3 (hypothesis 3).

With a few exceptions, the concentrations of 28 micropollutants and TPs decreased with increasing treatment degree. Carboxy-acyclovir was for instance found at higher concentration in the BT and MBRs compared to the PT because it is formed from acyclovir during biological treatment (Prasse et al. 2012). Ozonation decreases the concentration of carboxy-acyclovir with an additional removal in the subsequent post-treatments. In general, ozonation resulted in an additional removal of target compounds compared to the conventional treatment (Figure 7) with the exception of 3-hydroxy-ibuprofen, 4-hydroxy-1H-benzotriazol, 4-nitro-sulfmethoxazole, carboxy-ibuprofen, caffeine, paracetamol and mecoprop. This is in line with a multitude of previous studies demonstrating the performance of ozone treatments in further reducing micropollutants (Prasse et al. 2015).

A post-treatment with GAC further reduced the concentrations of compounds detected after ozonation (Table S19). In most cases, this reduction was to levels below the LOQ for both, non-aerated and aerated GAC filtration. This demonstrates that a combination of ozonation and activated carbon post-treatments is very effective in removing micropollutants. The two BF systems also reduced the concentrations of micropollutants further with no marked difference between non-aerated and aerated BF. They were, however, less effective in removing some compounds (e.g., iopromide) than the GAC systems (Table S20).

The MBR systems had a very similar performance in removing target chemicals like the conventional activated sludge treatment (Figure 7). This is in line with previous studies (Bertanza et al. 2017, Maletz et al. 2013). The combination of an MBR with ozonation further improved the reduction of recalcitrant chemicals (Table S20). Accordingly, MBRs can be a suitable alternative for a conventional treatment in specific situations (e.g., lack of space).

4.4 What is the optimal wastewater treatment from an ecotoxicological point of view?

Residual ecotoxicological effects and micropollutants were detected in the present full-scale WWTP using an activated sludge treatment. This highlights the need for alternative and/or AWWT treatment options and/or optimisation of the activated sludge treatment. Here, ozonation was

effective in reducing the estrogenic activity but did not remove or even increased the anti-estrogenic activity, anti-androgenic activity and mutagenicity. We also observed a reduction in growth and reproduction of *P. antipodarum* exposed on-site to ozonated wastewater. These findings support the idea that ozonation is effective in removing some specific toxicities while it generates toxic TPs that induce other adverse effects (Völker et al. 2019, hypothesis 2). Accordingly, a post-treatment is needed to reduce these effects. Here, GAC filtration was more effective than the BFs in reducing the residual/generated *in vitro* toxicity. The same was true for some micropollutants. No specific differences were observed for aerated versus non-aerated systems. As all post-treatments were fed with the same wastewater, we conclude that a GAC post-treatment is preferable to BF when improving the toxicity/chemical removal of ozonated wastewater. However, other considerations (e.g., energy demand, available space, carbon footprint) need to be taken into account when deciding on a suitable post-treatment.

MBR systems can be a promising alternative to conventional activated sludge processes (Bui et al. 2016). In the present study, MBR1 but not MBR2 had a similar removal performance for toxicity and micropollutants like the BT (hypothesis 3). Raw wastewater treated in MBR2 induced a marked reproductive toxicity in *P. antipodarum*. Thus, a combination with ozonation (MBR1) might be preferable. However, the latter treatment generated a high mutagenicity which was removed by recirculating the ozonated water in the MBR. Accordingly, a combination of MBR and ozonation technologies might represent a promising option for specific situations, such as little available space for WWTP in urban settings.

633 **5 Conclusions**

624 • To determine optimal ozone doses and HRTs, maximum removal rates and generation of *in*
625 *vitro* toxicity have to be balanced. An ozone dose of 0.33 g O_3 /g DOC and an HRT of 12.6 min
626 seemed optimal.

627 • While ozonation was effective in further reducing toxicity and micropollutants it also formed
628 toxic TPs. Thus, post-treatment is needed. Activated carbon and biological post-filtration
629 (further) reduced most of the effect with GAC being more effective than BF.

630 • MBR systems as alternatives to an activated sludge treatment were similarly effective like the
631 BT and even performed better (e.g., removal of estrogenicity). MBR+O_3 improved the removal
632 performance but also generated mutagenicity. The latter was reduced by recirculation to the
633 MBR which might represent a promising option.

634 • A significant anti-estrogenic activity remained in all AWWTs which should be further
635 investigated.

636 • Conventionally treated wastewater affected growth and reproduction of *P. antipodarum* (better
637 nutrient supply or exposure of estrogenic chemicals). Ozonation reduced the reproduction
638 indicating a potential formation of toxic TPs. In the post-treatments these effects were
639 compensated or remained unaffected. All MBR treatments induced reproductive toxicity.

640 • Ozonation of conventionally treated wastewater reduced micropollutants and improved
641 wastewater parameters. Post-treatment with GAC/BF resulted in an additional reduction. MBRs
642 were comparable to BT while MBR+O_3 was similarly effective like BT+O_3.

643 • For an optimised effect-based assessment of wastewater quality of conventional and AWWT
644 sensitive and environmentally relevant *in vitro* and *in vivo* endpoints as well as an adapted
645 chemical analysis are needed. In addition, further parameters (e.g., energy demand, carbon
646 emission), alternative technical options (e.g., optimising activated sludge treatments) and
647 socioeconomic factors (i.e., source control) have to be considered.

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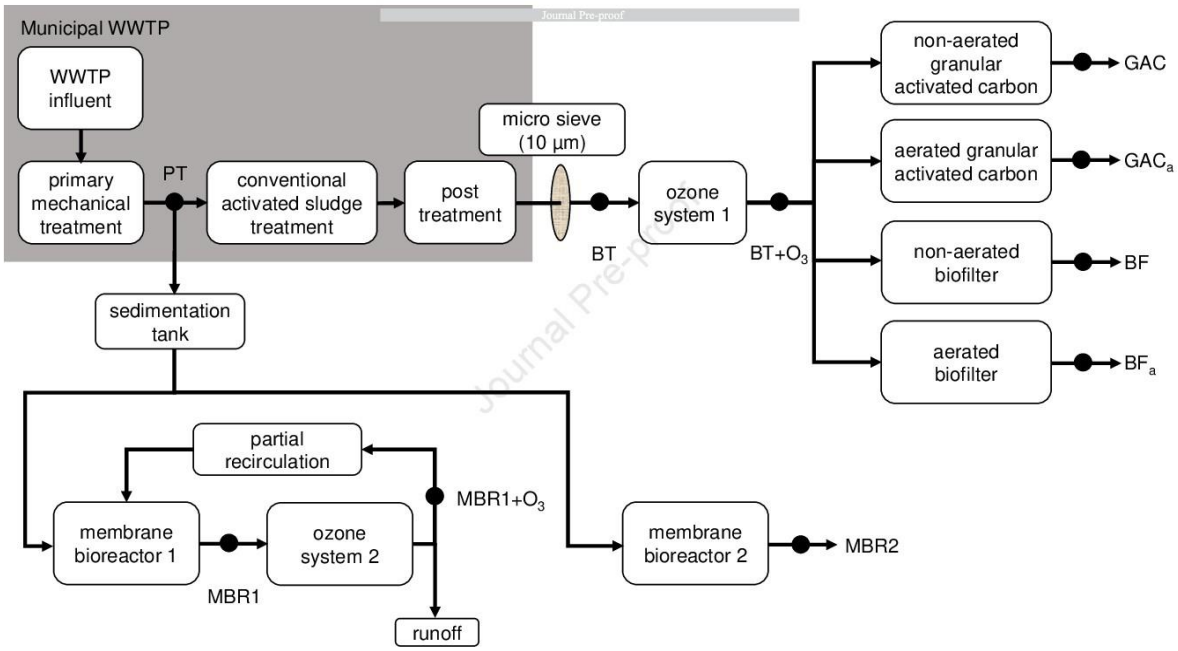

Municipal WWTP
WWTP influent
primary mechanical treatment
PT
conventional activated sludge treatment
post treatment
micro sieve (10 µm)
BT
ozone system 1
BT+O3
non-aerated granular activated carbon
GAC
aerated granular activated carbon
GACa
non-aerated biofilter
BF
aerated biofilter
BFa
sedimentation tank
partial recirculation
MBR1+O3
membrane bioreactor 1
ozone system 2
MBR1
runoff
membrane bioreactor 2
MBR2

Figure 1: Process design of the WWTP and AWWT. Process design of the municipal wastewater treatment plant (WWTP) and the pilot-scale advanced wastewater treatment technologies (AWWT). Sampling points are marked with black dots. PT: after primary treatment, BT: after conventional biological treatment, BT+O_3: biological treatment after ozonation, GAC: non-aerated granular activated carbon, GAC_a: granular activated carbon aerated with ambient air, BF: non-aerated biofilter, BF_a: biofilter aerated with ambient air, MBR1/2: membrane bioreactor 1/2, MBR1+O_3: membrane bioreactor 1 after ozonation.

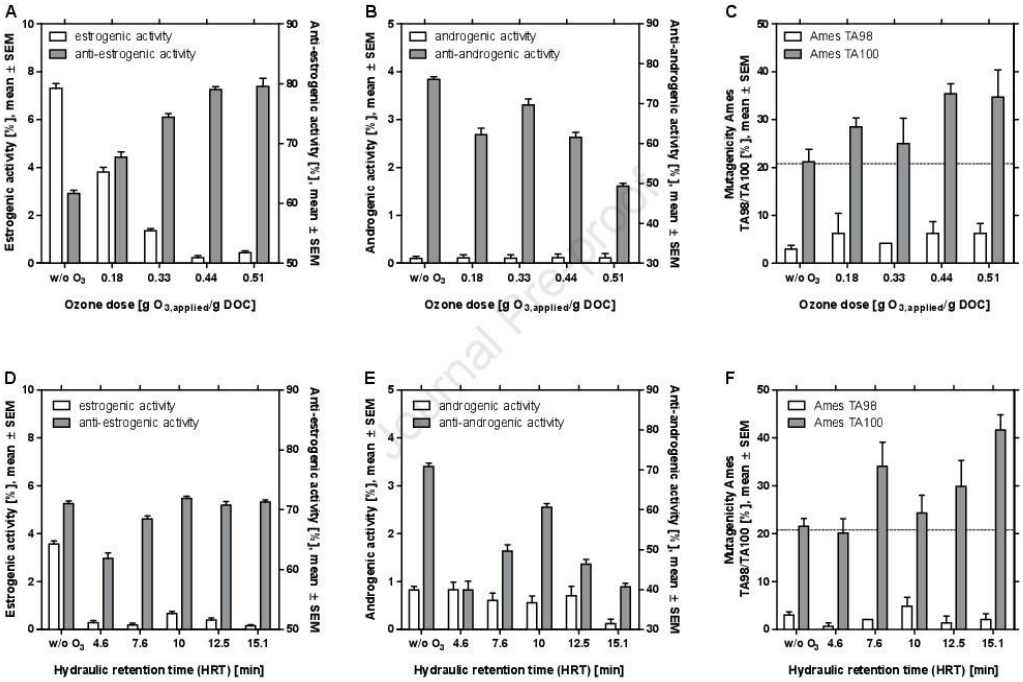

A
estrogenic activity
anti-estrogenic activity
Estrogenic activity [%], mean ± SEM
Anti-estrogenic activity [%], mean ± SEM
w/o O3
0.18
0.33
0.44
0.51
Ozone dose [g O3,applied/g DOC]
B
androgenic activity
anti-androgenic activity
Androgenic activity [%], mean ± SEM
Anti-androgenic activity [%], mean ± SEM
Ozone dose [g O3,applied/g DOC]
C
Ames TA98
Ames TA100
Mutagenicity Ames TA98/TA100 [%], mean ± SEM
Ozone dose [g O3,applied/g DOC]
D
estrogenic activity
anti-estrogenic activity
Estrogenic activity [%], mean ± SEM
Anti-estrogenic activity [%], mean ± SEM
w/o O3
4.6
7.6
10
12.5
15.1
Hydraulic retention time (HRT) [min]
E
androgenic activity
anti-androgenic activity
Androgenic activity [%], mean ± SEM
Anti-androgenic activity [%], mean ± SEM
Hydraulic retention time (HRT) [min]
F
Ames TA98
Ames TA100
Mutagenicity Ames TA98/TA100 [%], mean ± SEM
Hydraulic retention time (HRT) [min]

Figure 2: Optimal ozone dose and hydraulic retention time. Estrogenic and anti-estrogenic activity (A, D), androgenic and anti-androgenic activity (B, E) and mutagenicity (C, F) in % (mean ± SEM) of conventional biological treated wastewater (without ozone; A, B: n = 93–96; D, E: n = 117–120; C, F: n = 12–15) and ozonated wastewater (three SPE extracts per ozone dose (A, B: n = 16–24, C: n = 3) and hydraulic retention time (D, E: n = 21–24; F: n = 3)). A, B, C: multiple ozone dose (0.18–0.51 g $O_{3,\ applied}$/g DOC) at a constant hydraulic retention time of 12.6 min; D, E, F: multiple hydraulic retention times (4.6–15.1 min) at a constant ozone dose of 0.53 g $O_{3,\ applied}$/g DOC. w/o O_3: without ozone.

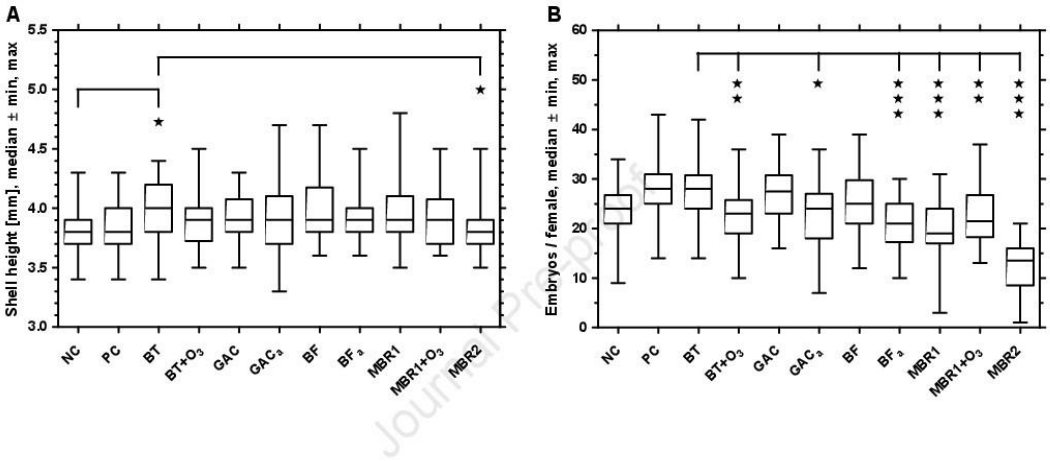


Figure 3: Growth and reproduction. Size (A) and reproduction (B) of *Potamopyrgus antipodarum* after 28 days of exposure to the negative control (NC), the positive control (PC), the conventional biological treatment (BT) and the eight advanced treatment technologies. BT+O_3: after ozone system 1, GAC: after non-aerated granular activated carbon treatment, GAC_a: after aerated granular activated carbon treatment, BF: after non-aerated biofilter treatment, BF_a: after aerated biofilter treatment, MBR1/2: after membrane bioreactor 1/2, MBR1+O_3: after ozone system 2. Significant differences to BT are indicated with asterisks: ★ $p < 0.05$, ★★ $p < 0.01$, ★★★ $p < 0.001$ (Kruskal-Wallis with Dunn's post-test), n = 35–40.

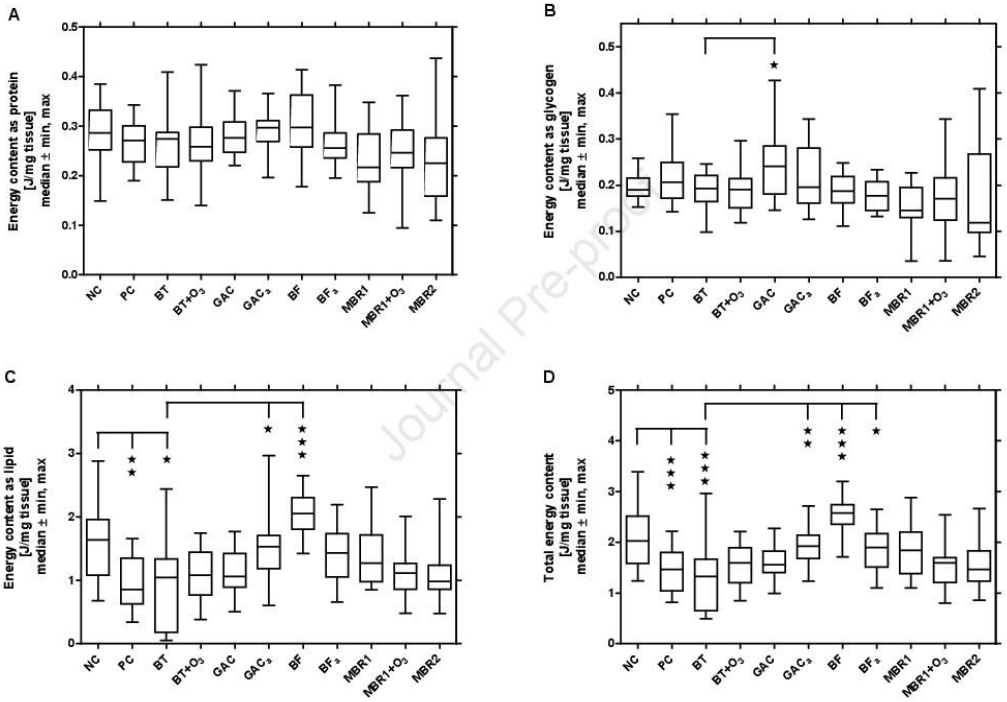


Figure 4: Biomarkers for energy reserves. Energy content as protein (A), glycogen (B), lipid (C) and total energy content (D) in J/mg tissue of *Potamopyrgus antipodarum* after 28 days exposure to water from the negative control (NC), the positive control (PC), the conventional biological treatment (BT) and the eight advanced treatment technologies in an on-site flow-through system. BT+O_3: after ozone system 1, GAC: after non-aerated activated granular carbon treatment, GAC_a: after aerated activated granular carbon treatment, BF: after non-aerated biofilter treatment, BF_a: after aerated biofilter treatment, MBR1/2: after membrane bioreactor 1/2, MBR1+O_3: after ozone system 2. Significant differences to NC and BT, are indicated with asterisks: ★ $p < 0.05$, ★★ $p < 0.01$, ★★★ $p < 0.001$ (One-way ANOVA with Bonferroni's post-test (B, D) or Kruskal-Wallis with Dunn's post-test (A, C)), n = 17–20.

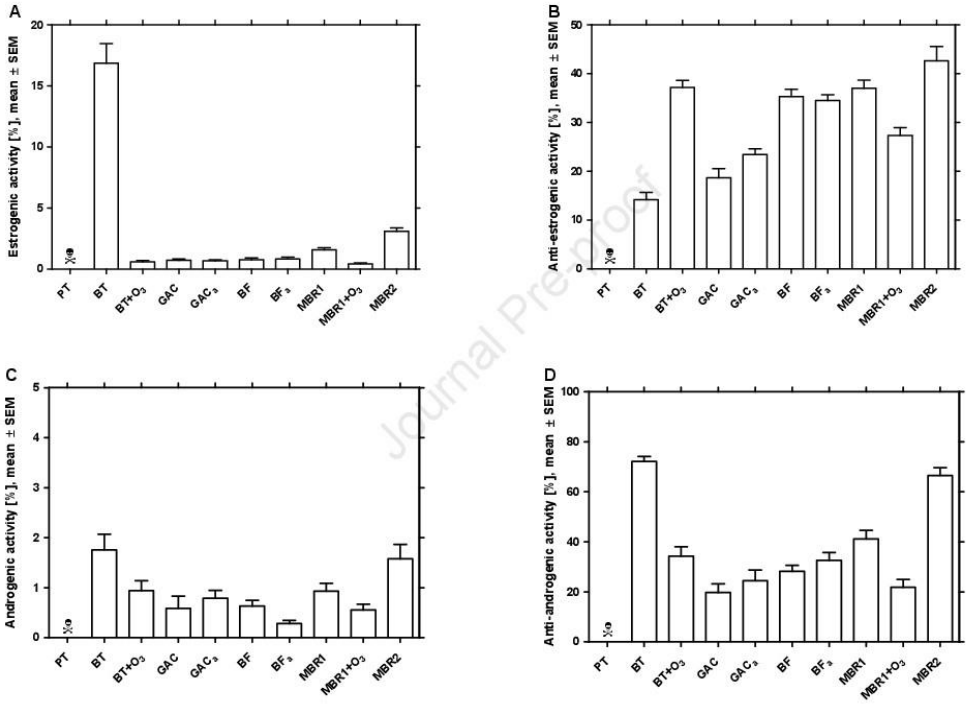


Figure 5: Endocrine activities of the on-site biotest. Estrogenic (A), anti-estrogenic (B), androgenic (C) and anti-androgenic activity (D) in SPE extracts produced from 24 h composite samples taken in parallel to the *in vivo* experiment. PT: after primary treatment, BT: after conventional biological treatment, BT+O_3: after ozone system 1, GAC: after non-aerated granular activated carbon treatment, GAC_a: after aerated granular activated carbon treatment, BF: after non-aerated biofilter treatment, BF_a: after aerated biofilter treatment, MBR1/2: after membrane bioreactor 1/2, MBR1+O_3: after ozone system 2, ☠: cytotoxic, n = 32.

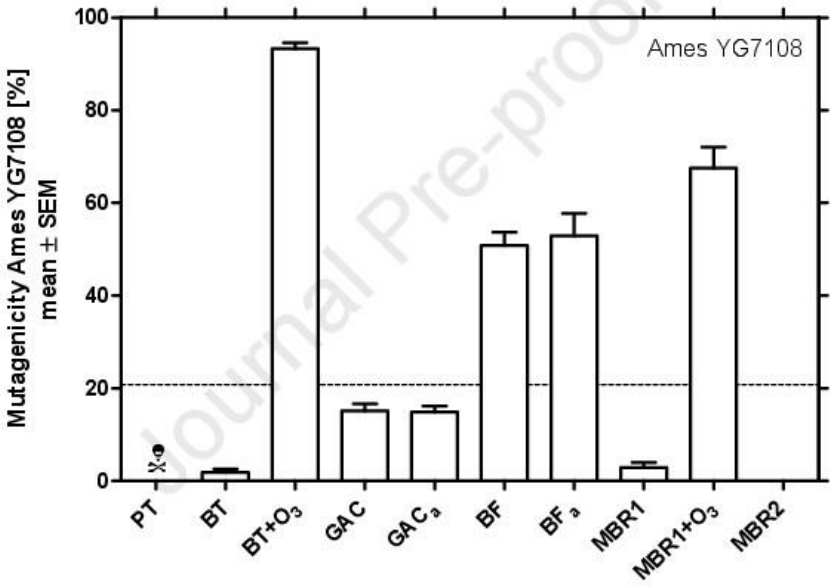
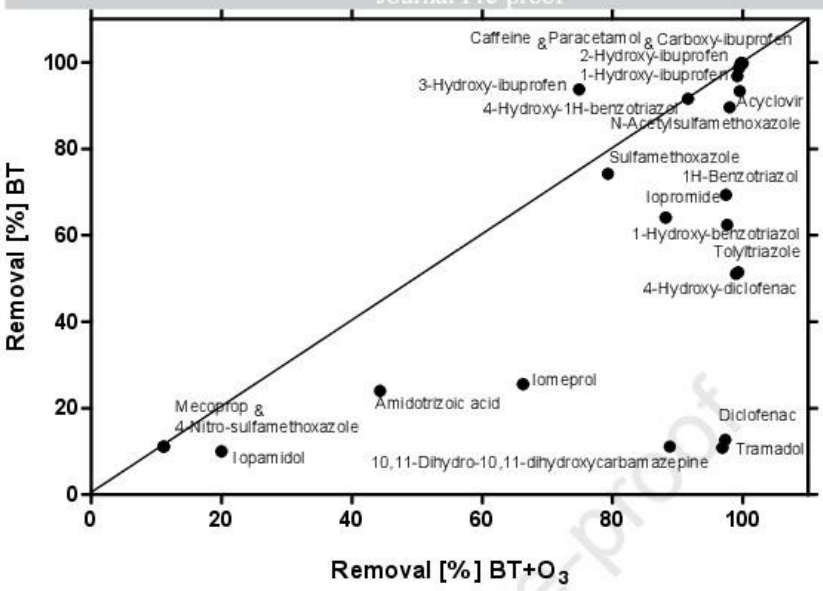


Figure 6: Mutagenicity of the on-site biotest. Mutagenicity in the Ames strain YG7108 in SPE extracts produced from 24 h composite samples taken in parallel to the *in vivo* experiment. PT: after primary treatment, BT: after conventional biological treatment, BT+O_3: after ozone system 1, GAC: after non-aerated granular activated carbon treatment, GAC_a: after aerated granular activated carbon treatment, BF: after non-aerated biofilter treatment, BF_a: after aerated biofilter treatment, MBR1/2: after membrane bioreactor 1/2, MBR1+O_3: after ozone system 2, ☠: cytotoxic, n = 8.



B

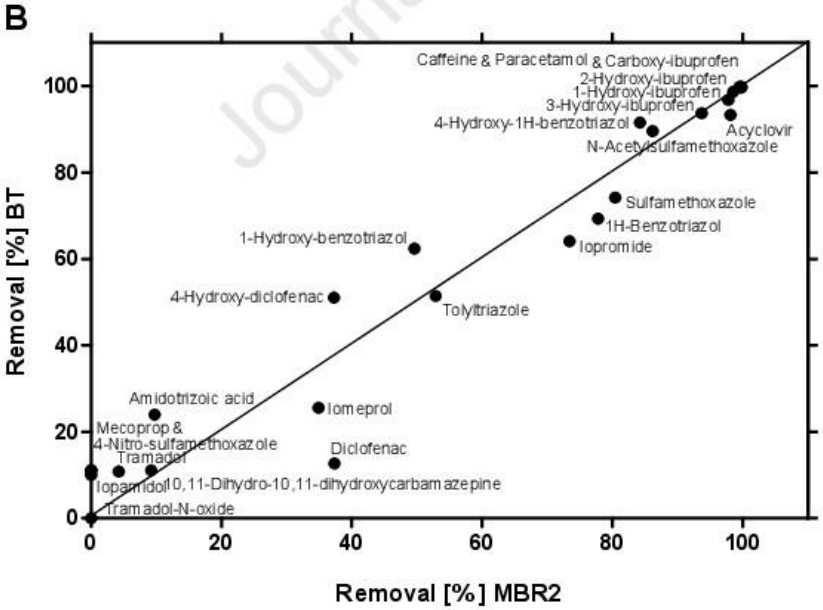


Figure 7: Chemical analysis. Removal of micropollutants by the conventional biological treatment (BT), by the ozonation (BT+O_3, A) and by the membrane bioreactor 2 (MBR2, B) compared to the primary treatment. n = 1–4.

Post-treatment of ozonated wastewater with activated carbon and biofiltration compared to membrane bioreactors: Toxicity removal *in vitro* and in *Potamopyrgus antipodarum*

Ilona Schneider, Aennes Abbas, Anna Bollmann, Andrea Dombrowski, Gregor Knopp, Ulrike Schulte-Oehlmann, Wolfram Seitz, Martin Wagner, Jörg Oehlmann

HIGHLIGHTS

- Comprehensive comparison of toxicity and micropollutant removal by advanced wastewater treatment
- Ozonation reduces estrogenicity and micropollutants but forms anti-estrogenicity and mutagenicity
- Post-treatment with granular activated carbon is more effective than biofilters
- Membrane bioreactors are as effective as conventional biological wastewater treatment
- Effluents of ozonation and membrane bioreactors induce reproductive toxicity in *P. antipodarum*

Supplementary information (paper A.3)

S1 Material and methods

S1.1 Technical parameters of the municipal and the pilot wastewater treatment plant

Table S1: Technical parameters of the municipal wastewater treatment plant.

conventional activated sludge:	11,750 m^3
volume of the pipelines:	100 m^3
average dry weather flow rate:	3750 m^3/d
hydraulic retention time:	75.8 h (3.16 d)

Table S2: Technical parameters of the pilot wastewater treatment plant.

	volume [m^3]	**flow rate [m^3/h]**	**retention time [h]; [d]**
Micro sieve	5	2.32	2.15; 0.090
Ozone system 1	0.212	0.709	0.299; 0.012
Surge tank 1 (ozone system 1)	0.150	0.709	0.212; 0.009
Surge tank 2 (ozone system 1)	0.150	0.650	0.231; 0.010
Granular activated carbon filter (non-aerated)	0.110	0.131	0.840; 0.035
Granular activated carbon filter (aerated)	0.110	0.085	1.30; 0.054
Biofilter (non-aerated)	0.110	0.124	0.886; 0.037
Biofilter (aerated)	0.110	0.128	0.858; 0.036
Reservoir with wastewater of the primary treatment	3.86	7.74 (186 m^3/d)	0.499; 0.021
Previous tank before MBR	2.0	0.684 (16.4 m^3/d)	2.92; 0.122
MBR1	1.64	0.122	13.5; 0.561
MBR2	1.64	0.043	38.4; 1.60
Surge tank 1 (ozone system 2)	0.100	0.117 (2.81 m^3/d)	0.853; 0.036
Ozone system 2	0.049	0.117 (2.81 m^3/d)	0.416; 0.017

S1.2 Process parameters of the pilot wastewater treatment plant

Table S3: Process parameters (mean ± SD) of ozone system 1 and 2 during the test period of 28 days. D: ozone dose, d: specific ozone dose, DOC: dissolved organic carbon, HRT: hydraulic retention time, O_3: ozone, RR: recirculation rate, Z: ozone consumption, z: specific ozone consumption.

	HRT [min]	D [g/m³]	d [g O_3/g DOC]	Z [g/m³]	z [g O_3/g DOC]	RR
Ozone system 1	17.9 ± 0.38 (n = 22)	10.1 ± 1.35 (n = 22)	0.95 ± 0.21 (n = 22)	9.86 ± 1.35 (n = 22)	0.93 ± 0.20 (n = 22)	-
Ozone system 2	26.1 ± 1.36 (n = 5)	6.78 ± 0.35 (n = 5)	-	-	0.96 ± 0.08 (n = 5)	2.02 ± 0.10

A recirculation rate of 2.0 means that for example 100 L wastewater from ozone system 2 was recirculated to 50 L wastewater of the primary treatment that in the end the ozone system 2 was fed with 150 L of a mixture of both wastewaters.

Table S4: Filter velocity (V_F) and empty bed contact time (EBCT) (mean ± SD, respectively) of the advanced treatment processes after ozone system 1 during the test period of 28 days. GAC: non-aerated granular activated carbon treatment, GAC_a: aerated granular activated carbon treatment, BF: non-aerated biofilter treatment, BF_a: aerated biofilter treatment.

	GAC	GAC_a	BF	BF_a
V_F [m/h]	4.92 ± 0.08 (n = 31)	3.33 ± 0.64 (n = 8)	4.96 ± 0.14 (n = 10)	4.94 ± 0.06 (n = 11)
EBCT [min]	28.3 ± 0.57 (n = 31)	36.4 ± 5.66 (n = 8)	27.4 ± 0.99 (n = 10)	26.7 ± 0.28 (n = 11)

Table S5: Overview of the sampling dates of the medium of the negative control (NC) and the positive control (PC) and the wastewater treatments. BT: after conventional biological treatment, BT+O_3: after ozone system 1, GAC: after non-aerated granular activated carbon treatment, GAC_a: after aerated granular activated carbon treatment, BF: after non-aerated biofilter treatment, BF_a: after aerated biofilter treatment, MBR1/2: after membrane bioreactor 1/2, MBR1+O_3: after ozone system 2, *: fresh medium was prepared.

Sample acronym	Sampling date in 2014	Sampling mode
NC	27.01.*, 28.01., 30.01., 02.02., 04.02., 05.02.*, 06.02., 07.02., 08.02.*, 09.02., 10.02., 11.02., 12.02.*, 13.02., 14.02.*, 15.02., 16.02., 17.02.*, 18.02., 19.02., 20.02., 21.02.*, 22.02., 23.02., 24.02.	grab
PC	27.01.*, 28.01., 30.01., 02.02., 04.02., 05.02.*, 06.02., 07.02., 08.02.*, 09.02., 10.02., 11.02., 12.02.*, 13.02., 14.02.*, 15.02., 16.02., 17.02.*, 18.02., 19.02., 20.02., 21.02.*, 22.02., 23.02., 24.02., 25.02.	grab
BT	28./29.01., 04./05.02., 11./12.02., 18./19.02.	24 h composite
BT+O_3	28./29.01., 04./05.02., 11./12.02., 18./19.02.	24 h composite
GAC	28./29.01., 04./05.02., 11./12.02., 18./19.02.	24 h composite
GAC_a	28./29.01., 04./05.02., 11./12.02., 18./19.02.	24 h composite
BF	28./29.01., 04./05.02., 11./12.02., 18./19.02.	24 h composite
BF_a	28./29.01., 04./05.02., 11./12.02., 18./19.02.	24 h composite
PT	29./30.01., 04./05.02., 11./12.02., 18./19.02.	24 h composite
MBR1	29./30.01., 04./05.02., 11./12.02., 18./19.02.	24 h composite
MBR1+O_3	29./30.01., 04./05.02., 11./12.02., 18./19.02.	24 h composite
MBR2	29./30.01., 04./05.02., 11./12.02., 18./19.02.	24 h composite

S1.3 Measurement of biomarkers for energy reserves (protein, glycogen and lipid content)

The measurement of the protein content was done according to Bradford (1976). 50.0 µL of the homogenate were mixed with 1.5 mL Bradford reagent (AppliChem GmbH, Darmstadt, Germany) using a vortex and incubated at room temperature for five minutes. Five increasing concentrations of a bovine serum albumin solution (BSA; 0.1%) mixed with Bradford reagent and incubated as well served as a linear standard calibration curve (Tables S6 and S7, Figure S1). The absorption was measured at a wavelength of 595 nm using a photometer (BioSpectrometer, Eppendorf, Hamburg, Germany). The protein content of the samples was calculated in µg/mg tissue and then converted to an energy content of the protein reserve in J/mg tissue using the specific calorific value of 17.0 kJ/g (Berg et al. 2007).

The separation of the glycogen and lipids was done using the micro-separation method as described in van Handel (1965). 100 µL of the homogenate were mixed with 1.6 mL of a chloroform-methanol solution (1:1) and centrifuged (centrifuge 5702, Eppendorf, Hamburg, Germany) for two minutes at 3,000 rpm. The generated pellet at the ground contained the glycogen. The separated chloroform-methanol supernatant containing the lipids was mixed with 0.6 mL demineralised water and centrifugalised for two minutes at 3,000 rpm. The upper water-methanol fraction was discharged, the lower chloroform fraction contained the lipids.

The glycogen content was measured using hot anthrone reaction (van Handel 1965, 1985a). The glycogen pellets were mixed with 5.0 mL anthrone reagent and incubated in a water bath (Grand Instruments, Cambridge, England) at 95.0°C for 17 minutes. Six increasing concentrations of a glucose solution (0.1%), mixed with anthrone reagent and incubated as well, served as a linear standard calibration curve (Tables S6 and S8, Figure S2). The absorption was measured at a wavelength of 625 nm using a photometer (BioSpectrometer, Eppendorf, Hamburg, Germany). The glycogen content of the samples was calculated in µg/mg tissue and converted to an energy content of the glycogen reserve in J/mg tissue using the specific calorific value of 17.0 kJ/g (Berg et al. 2007).

The lipid content was measured using the vanillin reaction (van Handel 1965, 1985b). After evaporation of the chloroform in a water bath (95.0°C) lipids were mixed with 200 µL sulphuric acid (H_2SO_4; 95%) and incubated at 95.0°C for 10 minutes. After adding 5.0 mL of the vanillin reagent the samples were mixed with a vortex and incubated for five minutes at room temperature. Five increasing concentrations of a colza solution (0.1%) were treated like the samples and served as a linear standard calibration curve (Tables S6 and S8, Figure S3). The absorption was measured at a wavelength of 625 nm using a photometer (BioSpectrometer, Eppendorf, Hamburg, Germany). The lipid content of the samples was calculated in µg/mg tissue and then converted to an energy content of the lipid reserve in J/mg tissue using the specific calorific value of 37.0 kJ/g (Berg et al. 2007).

Table S6: Solvents needed for the determination of the protein, glycogen and lipid content. BSA: bovine serum albumin, H_2SO_4: sulphuric acid, H_3PO_4: phosphorus acid, Na_2SO_4: sodium sulphate solution.

Sodium sulphate solution (2.0%)	2.0 g Na_2SO_4 + 100 mL demineralised water
Bovine serum albumin solution (0.1%)	100 mg BSA + 100 mL Na_2SO_4 solution
Chloroform-methanol solution	mix 1:1
Anthrone reagent	150 mL cooled demineralised water
	add 385 mL sulphuric acid (H_2SO_4) stepwise
	dissolve 750 mg anthrone
	storage in the refrigerator
Vanillin reagent	100 mL heated demineralised water
	dissolve 600 mg vanillin
	add 400 mL phosphorus acid (H_3PO_4)
	storage in brown glass bottles in the dark

Table S7: Determination of the protein content: pipette scheme for the standard calibration curve with the five increasing concentrations of a bovine serum albumin solution (BSA; 0.1%) and sodium sulphate solution (Na_2SO_4).

No.	BSA solution (0.1%) [µL]	Na_2SO_4 solution (2.0%) [µL]
1	0.0	50.0
2	12.5	37.5
3	25.0	25.0
4	37.5	12.5
5	50.0	0.0

Table S8: Determination of the glycogen and lipid content: volumes [µL] of the glucose and colza solutions needed for the five, respectively six, increasing concentrations for the standard calibration curves.

No.	Glucose solution (0.1%) [µL]	Colza solution (0.1%) [µL]
1	0.0	0.0
2	25.0	50.0
3	50.0	100
4	100	200
5	150	400
6	200	-

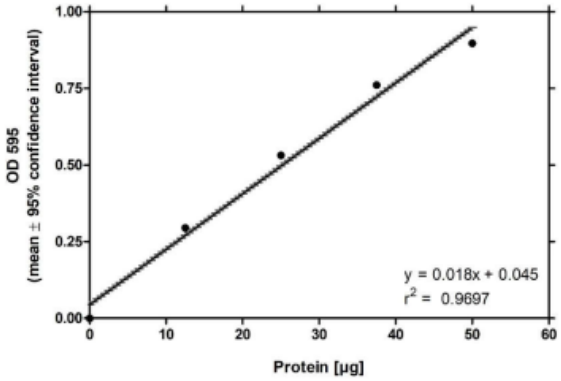


Figure S1: Optical density at a wavelength of 595 nm (OD 595) of five protein concentrations [µg] as linear regression (mean ± 95% confidence interval) of seven experiments.

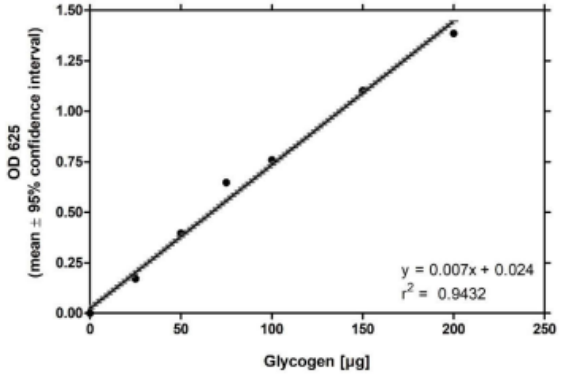


Figure S2: Optical density at a wavelength of 625 nm (OD 625) of seven glycogen concentrations [µg] as linear regression (mean ± 95% confidence interval) of seven experiments.

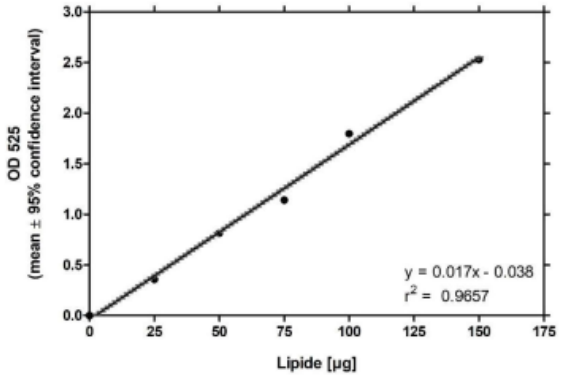


Figure S3: Optical density at a wavelength of 525 nm (OD 525) of six lipid concentrations [µg] as linear regression (mean ± 95% confidence interval) of seven experiments.

S1.4 *In vitro* bioassays for endocrine and mutagenic activity

Table S9: Overview of the bioassays used in this study, including endpoints, concentration range of the respective reference compound (positive control), background agonists and EC_{50} values.

***In vitro* bioassay**	**Positive control**	**Concentration range [mol L^{-1}]**	**EC_{50} [mol L^{-1}]**
YES (estrogenicity)	17β-estradiol (E_2, CAS: 50-28-2)	1.0×10^{-12} - 1.0×10^{-08}	1.25×10^{-10}
YES (estrogenicity)	17α-ethinylestradiol (EE_2, CAS: 57-63-6)	1.0×10^{-12} - 1.0×10^{-08}	1.32×10^{-10}
YAES (anti-estrogenicity)	4-hydroxytamoxifen (OHT, CAS: 68392-35-8) background agonist: 0.1 nmol/L 17β-estradiol (E_2)	1.25×10^{-06} - 8.0×10^{-05}	1.09×10^{-05}
YAS (androgenicity)	testosterone (T, CAS: 58-22-0)	3.0×10^{-11} - 1.0×10^{-07}	4.54×10^{-09}
YAAS (anti-androgenicity)	flutamide (Flu, CAS: 13311-84-7) background agonist: 3 nmol/L testosterone	7.81×10^{-07} - 5.0×10^{-05}	3.37×10^{-06}
Ames TA98 (mutagenicity)	4-nitro-*o*-phenylenediamine (4-NOPD, CAS: 99-56-9)	10 mg/L	-
Ames TA100 (mutagenicity)	nitrofurantoin (NF, CAS: 67-20-9)	0.25 mg/L	-
Ames YG7108 (mutagenicity)	propylene oxide (PO, CAS 75-56-9)	0.2%	-

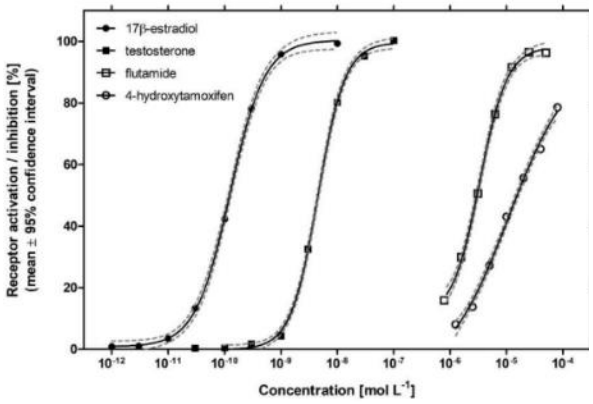


Figure S4: Receptor activation (YES, YAS) and inhibition (YAES, YAAS) as concentration-response relationships of six (YAES, YAAS) and seven (YES, YAS) experiments at the human estrogen and androgen receptor (YES: 17β-estradiol; YAS: testosterone; YAAS: flutamide; YAES: 4-hydroxytamoxifen)

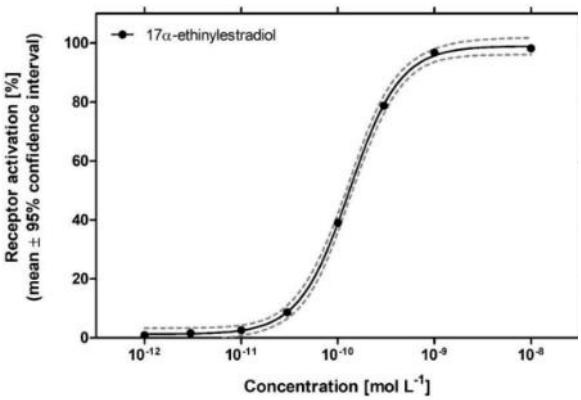


Figure S5: Concentration response relation-ships of 17α-ethinylestradiol in four YES experiments

S1.5 Measurement of water parameters

Table S10: Methods and measurement ranges of the water parameters measured directly in the effluents of the nine wastewater treatment reactors.

physical-chemical parameter	method	range
chemical oxygen demand (COD)	DIN ISO 15705-H45 HACH-LANGE cuvette test LCK414 and LCK514	5–60 mg O_2/L and 100–2000 mg O_2/L
dissolved organic carbon (DOC)	HACH-Lange cuvette test LCK385	3–30 mg C/L
dissolved organic carbon (DOC)	DIN 1484	0.5–100 mg C/L)
nitrite (NO_2-N)	cuvette test corresponding to EN ISO 26777, DIN 38405 D10 HACH-Lange cuvette test LCK341	0.015–0.6 mg NH_4-N/L
nitrate (NO_3-N)	cuvette test corresponding to ISO 7890-1-2-1986, DIN 38405 D9-2 HACH-Lange cuvette test LCK339 and LCK340	0.23–13.5 mg NO_3-N/L and 5–35 mg NO_3-N/L
ammonium (NH_4-N)	cuvette test corresponding to ISO 7150-1, DIN 38406 E5-1 HACH-LANGE cuvette test LCK303 and LCK 304	2.0–47 mg NH_4-N/L and 0.015–2 mg NH_4-N/L
total phosphor	cuvette test corresponding to ISO 6878-1-1986, DIN 38405 D11-4 HACH-Lange cuvette test LCK339 and LCK340	0.23–13.5 mg NO_3-N/L and 5–35 mg NO_3-N/L
Spectral absorption coefficient at 254 nm (SAC_{254})	Determination of the decrease of light of a filtered sample at a wavelength of 254 nm following the principle of Beer-Lambert law	

Table S11: Methods and measurement ranges of the water parameters measured directly in the exposure vessels.

physical-chemical parameter	method	range
temperature [°C]	TetraCon-325, Multi 340i / SET, WTW Weilheim	---
pH	SenTix 41, Multi 340i / SET, WTW Weilheim	---
conductivity [µS/cm]	TetraCon-325, Multi 340i / SET, WTW Weilheim	---
oxygen content [mg/L]	OxiCal-SL, Multi 340i / SET, WTW Weilheim	---
oxygen saturation [%]	OxiCal-SL, Multi 340i / SET, WTW Weilheim	---
nitrite [mg/L]	nitrite-test, Aquamerck, MERCK Darmstadt	0.025–0.5 mg/L
nitrate [mg/L]	nitrate-test, Aquamerck, MERCK Darmstadt	10–150 mg/L
ammonium [mg/L]	ammonium-test, Aquamerck, MERCK Darmstadt	0.5–10 mg/L
total hardness [°d]	total hardness-test Merckoquant, MERCK Darmstadt	< 3 – > 21°d

S2 Results and discussion

S2.1 Optimal ozone dose and hydraulic retention time

Table S12: Estrogenic (YES), anti-estrogenic (YAES), androgenic (YAS) and anti-androgenic (YAAS) activity and mutagenicity (Ames TA98, Ames TA100) in % (mean ± SEM) from three SPE-extracts each produced from 24 h composite samples of conventionally treated wastewater (BT) and ozonated wastewater with ozone dose of 0.18–0.51 g $O_{3,\ applied}$/g DOC at a constant hydraulic retention time (HRT) of 12.6 min. The change of endocrine activity and mutagenicity compared to the conventional biological treatment (Δw/o O_3) is given in %.

	ozone dose [g $O_{3,\ applied}$/g DOC]								
	w/o O_3	**0.18**	**Δ w/o O_3 [%]**	**0.33**	**Δ w/o O_3 [%]**	**0.44**	**Δ w/o O_3 [%]**	**0.51**	**Δ w/o O_3 [%]**
YES	7.31 ± 0.21 (n = 96)	3.81 ± 0.19 (n = 24)	–47.8	1.35 ± 0.08 (n = 24)	–81.5	0.23 ± 0.09 (n = 16)	–96.9	0.44 ± 0.07 (n = 24)	–94.0
YAES	61.7 ± 0.55 (n = 93)	67.7 ± 0.87 (n = 23)	+9.85	74.4 ± 0.60 (n = 22)	+20.7	79.1 ± 0.45 (n = 21)	+28.2	79.6 ± 1.37 (n = 20)	+29.1
YAS	0.10 ± 0.04 (n = 96)	0.11 ± 0.07 (n = 24)	+9.28	0.11 ± 0.07 (n = 24)	+6.39	0.12 ± 0.07 (n = 24)	+18.7	0.11 ± 0.09 (n = 24)	+10.0
YAAS	76.1 ± 0.72 (n = 95)	62.2 ± 1.64 (n = 24)	–18.2	69.7 ± 1.45 (n = 24)	–8.39	61.6 ± 1.28 (n = 24)	–19.0	49.3 ± 0.73 (n = 24)	–35.1
Ames TA98	2.95 ± 0.79 (n = 12)	6.25 ± 4.17 (n = 3)	-	4.17 ± 0.00 (n = 3)	-	6.25 ± 2.41 (n = 3)	-	6.25 ± 2.08 (n = 3)	-
Ames TA100	21.2 ± 2.59 (n = 12)	28.5 ± 1.85 (n = 3)	+34.5	25.0 ± 5.25 (n = 3)	+18.1	35.4 ± 2.10 (n = 3)	+67.1	34.7 ± 5.67 (n = 3)	+63.9

Table S13: Estrogenic (YES), anti-estrogenic (YAES), androgenic (YAS) and anti-androgenic (YAAS) activity and mutagenicity (Ames TA98, Ames TA100) in % (mean ± SEM) from three SPE-extracts each produced from 24 h composite samples of conventionally treated wastewater (BT) and ozonated wastewater with hydraulic retention times (HRTs) of 4.6–15.1 min at a constant ozone dose of 0.53 g $O_{3,\ applied}$/g DOC. The change of endocrine activity and mutagenicity compared to the conventional biological treatment (Δw/o O_3) is given in %.

	hydraulic retention time [min]										
	w/o O_3	**4.6**	**Δ w/o O_3 [%]**	**7.6**	**Δ w/o O_3 [%]**	**10.0**	**Δ w/o O_3 [%]**	**12.5**	**Δ w/o O_3 [%]**	**15.1**	**Δ w/o O_3 [%]**
YES	3.58 ± 0.12 (n = 119)	0.29 ± 0.07 (n = 24)	–91.8	0.19 ± 0.07 (n = 21)	–94.6	0.67 ± 0.10 (n = 24)	–81.3	0.40 ± 0.09 (n = 23)	–88.8	0.16 ± 0.04 (n = 22)	–95.7
YAES	71.0 ± 0.45 (n = 117)	61.9 ± 0.91 (n = 23)	–12.9	68.5 ± 0.55 (n = 23)	–3.65	71.9 ± 0.38 (n = 23)	+1.21	70.8 ± 0.62 (n = 21)	–0.32	71.3 ± 0.34 (n = 23)	+0.35
YAS	0.82 ± 0.08 (n = 120)	0.83 ± 0.16 (n = 24)	+0.96	0.60 ± 0.15 (n = 23)	–26.5	0.56 ± 0.14 (n = 24)	–32.0	0.71 ± 0.20 (n = 24)	–14.1	0.12 ± 0.09 (n = 24)	–85.2
YAAS	70.9 ± 0.80 (n = 117)	39.9 ± 2.21 (n = 24)	–43.6	49.7 ± 1.58 (n = 24)	–29.9	60.7 ± 0.88 (n = 24)	–14.4	46.4 ± 1.15 (n = 23)	–34.6	40.7 ± 0.93 (n = 24)	–42.6
Ames TA98	3.05 ± 0.67 (n = 15)	0.69 ± 0.69 (n = 3)	-	2.08 ± 0.00 (n = 3)	-	4.86 ± 1.84 (n = 3)	-	1.39 ± 1.39 (n = 3)	-	2.08 ± 1.20 (n = 3)	-
Ames TA100	21.5 ± 1.64 (n = 15)	20.1 ± 3.02 (n = 3)	–6.55	34.1 ± 5.02 (n = 3)	+58.2	24.3 ± 3.68 (n = 3)	+13.0	29.9 ± 5.44 (n = 3)	+38.7	41.7 ± 3.18 (n = 3)	+93.5

S2.2 *In vivo* on-site experiment with *Potamopyrgus antipodarum*

S2.2.1 Mortality, growth and reproduction

Table S14: Mortality in % (mean ± SEM), shell height in mm (mean ± SD), total number of embryos (mean ± SD) and fecundity index (mean ± SD) of *Potamopyrgus antipodarum* after 28 days of exposure to the negative control (NC), the positive control (PC), the conventional biological treatment (BT) and the eight advanced treatment technologies. BT+O_3: after ozone system 1, GAC: after non-aerated granular activated carbon filter treatment, GAC_a: after aerated granular activated carbon filter treatment, BF: after non-aerated biofilter treatment, BF_a: after aerated biofilter treatment, MBR1/2: after membrane bioreactor 1/2, MBR1+O_3: after ozone system 2. The change of the shell height and the total number of embryos compared to the negative control (ΔNC) or the conventional biological treatment (ΔBT) is given in %. Significant differences compared to ΔNC and ΔBT are marked with asterisks: ★ $p \leq 0.05$, ★★ $p \leq 0.01$, ★★★ $p \leq 0.001$ (Kruskal-Wallis with Dunn's post-test), n.s.: not significant.

treatment	mortality [%]	shell height [mm]	Δ [%]	total number of embryos	Δ [%]	fecundity index	Δ [%]
NC	1.00 ± 1.00 (n = 100)	3.82 ± 0.17 (n = 40)	-	23.7 ± 5.27 (n = 40)	-	6.17 ± 1.21 (n = 40)	-
PC	3.00 ± 1.92 (n = 100)	3.87 ± 0.21 (n = 37)	ΔNC +1.31 (n.s.)	27.7 ± 5.36 (n = 40)	ΔNC +17.0 (n.s.)	7.07 ± 1.23 (n = 37)	ΔNC +14.6 (n.s.)
BT	2.00 ± 1.16 (n = 100)	3.98 ± 0.23 (n = 39)	ΔNC +4.30 (★)	28.1 ± 6.00 (n = 40)	ΔNC +18.7 (n.s.)	7.02 ± 1.25 (n = 39)	ΔNC +13.8 (n.s.)
BT+O_3	1.00 ± 1.00 (n = 100)	3.90 ± 0.21 (n = 40)	ΔBT –1.98 (n.s.)	21.9 ± 5.94 (n = 40)	ΔBT –21.9 (★★)	5.61 ± 1.43 (n = 40)	ΔBT –20.1 (★★★)
GAC	3.00 ± 3.00 (n = 100)	3.90 ± 0.20 (n = 40)	ΔBT –1.98 (n.s.)	27.5 ± 5.30 (n = 40)	ΔBT –2.07 (n.s.)	7.01 ± 1.10 (n = 40)	ΔBT –0.11 (n.s.)
GAC_a	1.33 ± 1.33 (n = 100)	3.92 ± 0.28 (n = 35)	ΔBT –1.56 (n.s.)	22.8 ± 6.19 (n = 35)	ΔBT –18.7 (★)	5.79 ± 1.36 (n = 35)	ΔBT –17.5 (★★)
BF	2.00 ± 1.16 (n = 100)	3.94 ± 0.25 (n = 40)	ΔBT –1.05 (n.s.)	25.1 ± 6.25 (n = 40)	ΔBT –10.7 (n.s.)	6.34 ± 1.44 (n = 40)	ΔBT –9.63 (n.s.)
BF_a	1.00 ± 1.00 (n = 100)	3.93 ± 0.19 (n = 40)	ΔBT –1.43 (n.s.)	21.3 ± 5.04 (n = 40)	ΔBT –24.0 (★★★)	5.43 ± 1.24 (n = 40)	ΔBT –22.6 (★★★)
MBR1	0.00 ± 0.00 (n = 100)	3.93 ± 0.27 (n = 40)	ΔBT –1.36 (n.s.)	19.7 ± 5.98 (n = 40)	ΔBT –29.9 (★★★)	5.01 ± 1.48 (n = 40)	ΔBT –28.7 (★★★)
MBR1+O_3	1.00 ± 1.00 (n = 100)	3.92 ± 0.24 (n = 40)	ΔBT –1.68 (n.s.)	22.6 ± 6.20 (n = 40)	ΔBT –19.6 (★★)	5.73 ± 1.40 (n = 40)	ΔBT –18.3 (★★)
MBR2	1.00 ± 1.00 (n = 100)	3.84 ± 0.21 (n = 40)	ΔBT –3.57 (★)	12.4 ± 5.35 (n = 40)	ΔBT –56.0 (★★★)	3.20 ± 1.34 (n = 40)	ΔBT –54.4 (★★★)

S2.2.2 Biomarkers for energy reserves (glycogen, protein and lipid content)

Table S15: Energy content as protein, glycogen and lipid in J/mg tissue (mean ± SD) of *Potamopyrgus antipodarum* after 28 days of exposure to the negative control (NC), the positive control (PC), the conventional biological treatment (BT) and the eight advanced treatment technologies. BT+O_3: after ozone system 1, GAC: after non-aerated activated carbon filter treatment, GAC_a: after aerated activated carbon filter treatment, BF: after non-aerated biofilter treatment, BF_a: after aerated biofilter treatment, MBR1/2: after membrane bioreactor 1/2, MBR1+O_3: after ozone system 2. The change of the protein, glycogen and lipid content compared to the negative control (ΔNC) or the conventional biological treatment (ΔBT) is given in %. Significant differences compared to ΔNC and ΔBT are marked with asterisks: ★ $p \leq 0.05$, ★★ $p \leq 0.01$, ★★★ $p \leq 0.001$ (one-way ANOVA with Bonferroni's post-test (energy content as glycogen) or Kruskal-Wallis with Dunn's post-test (energy content as protein and lipid)), n.s.: not significant.

treatment	protein [J/mg]	Δ [%]	glycogen [J/mg]	Δ [%]	lipid [J/mg]	Δ [%]
NC	0.29 ± 0.06 (n = 19)	-	0.20 ± 0.03 (n = 19)	-	1.59 ± 0.54 (n = 20)	-
PC	0.26 ± 0.04 (n = 20)	ΔNC –8.74 (n.s.)	0.22 ± 0.06 (n = 20)	ΔNC +10.5 (n.s.)	0.96 ± 0.42 (n = 19)	ΔNC –39.8 (★★)
BT	0.26 ± 0.06 (n = 19)	ΔNC –9.56 (n.s.)	0.19 ± 0.04 (n = 20)	ΔNC –3.98 (n.s.)	0.95 ± 0.73 (n = 20)	ΔNC –40.1 (★)
BT+O_3	0.26 ± 0.06 (n = 20)	ΔBT +0.42 (n.s.)	0.19 ± 0.04 (n = 20)	ΔBT –0.16 (n.s.)	1.10 ± 0.41 (n = 19)	ΔBT +15.0 (n.s.)
GAC	0.28 ± 0.04 (n = 20)	ΔBT +6.26 (n.s.)	0.24 ± 0.08 (n = 20)	ΔBT +29.2 (★)	1.09 ± 0.33 (n = 19)	ΔBT +13.9 (n.s.)
GAC_a	0.28 ± 0.05 (n = 19)	ΔBT +7.94 (n.s.)	0.21 ± 0.07 (n = 20)	ΔBT +13.7 (n.s.)	1.52 ± 0.51 (n = 20)	ΔBT +59.7 (★)
BF	0.31 ± 0.07 (n = 20)	ΔBT +17.9 (n.s.)	0.19 ± 0.04 (n = 20)	ΔBT –1.11 (n.s.)	2.05 ± 0.31 (n = 20)	ΔBT +115 (★★★)
BF_a	0.26 ± 0.04 (n = 20)	ΔBT –0.08 (n.s.)	0.18 ± 0.04 (n = 20)	ΔBT –4.78 (n.s.)	1.43 ± 0.47 (n = 20)	ΔBT +49.8 (n.s.)
MBR1	0.23 ± 0.07 (n = 19)	ΔBT –10.3 (n.s.)	0.15 ± 0.05 (n = 20)	ΔBT –19.0 (n.s.)	1.40 ± 0.51 (n = 18)	ΔBT +46.4 (n.s.)
MBR1+O_3	0.25 ± 0.07 (n = 20)	ΔBT –5.00 (n.s.)	0.17 ± 0.07 (n = 20)	ΔBT –7.80 (n.s.)	1.10 ± 0.38 (n = 18)	ΔBT +14.8 (n.s.)
MBR2	0.23 ± 0.08 (n = 20)	ΔBT –12.7 (n.s.)	0.18 ± 0.11 (n = 20)	ΔBT –6.58 (n.s.)	1.14 ± 0.48 (n = 19)	ΔBT +18.9 (n.s.)

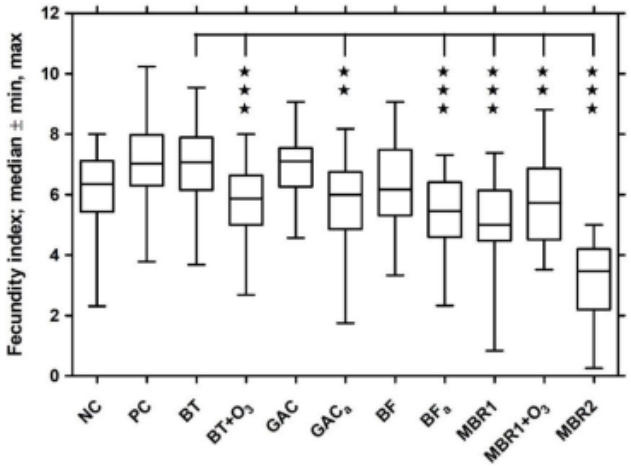


Figure S6: Fecundity index of *Potamopyrgus antipodarum* after 28 days of exposure to the negative control (NC), the positive control (PC), the conventional biological treatment (BT) and the eight advanced treatment technologies. BT+O_3: after ozone system 1, GAC: after non-aerated granular activated carbon treatment, GAC_a: after aerated granular activated carbon treatment, BF: after non-aerated biofilter treatment, BF_a: after aerated biofilter treatment, MBR1/2: after membrane bioreactor 1/2, MBR1+O_3: after ozone system 2. Significant differences to BT are indicated with asterisks: ★★ $p < 0.01$, ★★★ $p < 0.001$ (Kruskal-Wallis with Dunn's post-test), n = 35–40.

Table S16: Total energy content (protein + glycogen + lipid) in J/mg tissue (mean ± SD) of *Potamopyrgus antipodarum* after 28 days of exposure to the negative control (NC), the positive control (PC), the conventional biological treatment (BT) and the eight advanced treatment technologies. BT+O_3: after ozone system 1, GAC: after non-aerated activated carbon filter treatment, GAC_a: after aerated activated carbon filter treatment, BF: after non-aerated biofilter treatment, BF_a: after aerated biofilter treatment, MBR1/2: after membrane bioreactor 1/2, MBR1+O_3: after ozone system 2. The change of the total energy compared to the negative control (ΔNC) or the conventional biological treatment (ΔBT) is given in %. Significant differences compared to ΔNC and ΔBT are marked with asterisks: ★ $p \leq 0.05$, ★★ $p \leq 0.01$, ★★★ $p \leq 0.001$ (one-way ANOVA with Bonferroni's post-test), n.s.: not significant.

treatment	total energy [J/mg]	Δ [%]
NC	2.07 ± 0.56 (n = 19)	-
PC	1.44 ± 0.43 (n = 19)	ΔNC –30.6 (★★★)
BT	1.38 ± 0.77 (n = 19)	ΔNC –33.2 (★★★)
BT+O_3	1.55 ± 0.42 (n = 19)	ΔBT +12.1 (n.s.)
GAC	1.61 ± 0.33 (n = 19)	ΔBT +16.4 (n.s.)
GAC_a	1.94 ± 0.36 (n = 19)	ΔBT +40.2 (★★)
BF	2.54 ± 0.35 (n = 20)	ΔBT +83.7 (★★★)
BF_a	1.87 ± 0.47 (n = 20)	ΔBT +35.2 (★)
MBR1	1.82 ± 0.53 (n = 17)	ΔBT +31.6 (n.s.)
MBR1+O_3	1.52 ± 0.42 (n = 18)	ΔBT +9.47 (n.s.)
MBR2	1.55 ± 0.47 (n = 19)	ΔBT +12.1 (n.s.)

S2.2.3 *In vitro* bioassays for endocrine activity and mutagenicity

Table S17: Estrogenic (YES), anti-estrogenic (YAES), androgenic (YAS) and anti-androgenic (YAAS) activity of the aqueous samples from four 24 h composite samples per treatment. PT: after primary treatment, BT: after conventional biological treatment, BT+O_3: after ozone system 1, GAC: after non-aerated granular activated carbon filter treatment, GAC_a: after aerated granular activated carbon filter treatment, BF: after non-aerated biofilter treatment, BF_a: after aerated biofilter treatment, MBR1/2: after membrane bioreactor 1/2, MBR1+O_3: after ozone system 2. The change of endocrine activity compared to the conventional biological treatment (ΔBT) is given in %. n.c.: not calculable.

	YES	Δ BT [%]	YAES	Δ BT [%]	YAS	Δ BT [%]	YAAS	Δ BT [%]
PT	1.60 ± 0.27 (n = 8)	–58.6	95.0 ± 0.71 (n = 16)	–39.6	38.2 ± 2.30 (n = 56)	–99.8	1.03 ± 0.41 (n = 40)	n.c.
BT	0.66 ± 0.11 (n = 32)	-	57.4 ± 2.83 (n = 32)	-	0.06 ± 0.03 (n = 40)	-	0.00 ± 0.00 (n = 32)	-
BT+O_3	0.25 ± 0.07 (n = 32)	–62.1	59.5 ± 2.89 (n = 32)	+3.62	0.03 ± 0.02 (n = 40)	–53.0	0.20 ± 0.20 (n = 32)	n.c.
GAC	0.33 ± 0.07 (n = 32)	–50.0	66.1 ± 3.04 (n = 32)	+15.1	0.02 ± 0.01 (n = 40)	–74.0	0.00 ± 0.00 (n = 32)	n.c.
GAC_a	0.24 ± 0.07 (n = 32)	–63.2	71.2 ± 2.77 (n = 32)	+24.0	0.05 ± 0.03 (n = 40)	–11.4	0.00 ± 0.00 (n = 32)	n.c.
BF	0.28 ± 0.07 (n = 32)	–57.6	60.5 ± 3.03 (n = 32)	+5.37	0.02 ± 0.02 (n = 40)	–73.2	0.26 ± 0.25 (n = 32)	n.c.
BF_a	0.33 ± 0.09 (n = 32)	–50.4	64.9 ± 2.86 (n = 32)	+13.1	0.02 ± 0.02 (n = 40)	–70.9	0.00 ± 0.00 (n = 32)	n.c.
MBR1	0.21 ± 0.05 (n = 24)	–68.6	61.9 ± 2.71 (n = 32)	+7.88	0.09 ± 0.03 (n = 40)	+40.3	0.84 ± 0.43 (n = 31)	n.c.
MBR1+O_3	0.23 ± 0.06 (n = 24)	–65.7	63.1 ± 2.90 (n = 32)	+9.91	0.07 ± 0.03 (n = 40)	+21.5	0.89 ± 0.57 (n = 31)	n.c.
MBR2	0.33 ± 0.07 (n = 32)	–50.2	66.2 ± 2.94 (n = 32)	+15.3	0.04 ± 0.02 (n = 40)	–42.0	0.39 ± 0.28 (n = 32)	n.c.

Table S18: Estrogenic (YES), anti-estrogenic (YAES), androgenic (YAS) and anti-androgenic (YAAS) activity and mutagenicity (Ames YG7108) from four SPE extracts each produced from 24 h composite samples. PT: after primary treatment, BT: after conventional biological treatment, BT+O_3: after ozone system 1, GAC: after non-aerated granular activated carbon filter treatment, GAC_a: after aerated granular activated carbon filter treatment, BF: after non-aerated biofilter treatment, BF_a: after aerated biofilter treatment, MBR1/2: after membrane bioreactor 1/2, MBR1+O_3: after ozone system 2. The change of endocrine activity and mutagenicity compared to the conventional biological treatment (ΔBT) is given in %. ☠: cytotoxic.

	YES	Δ BT [%]	YAES	Δ BT [%]	YAS	Δ BT [%]	YAAS	Δ BT [%]	Ames YG7108
PT	☠	-	☠	-	☠	-	☠	-	☠
BT	16.9 ± 1.60 (n = 32)	-	14.1 ± 1.53 (n = 32)	-	1.76 ± 0.31 (n = 32)	-	72.1 ± 2.05 (n = 32)	-	1.82 ± 0.73 (n = 8)
BT+O_3	0.59 ± 0.11 (n = 32)	–96.5	37.2 ± 1.43 (n = 32)	+163	0.94 ± 0.20 (n = 32)	–46.3	34.3 ± 3.79 (n = 32)	–52.5	93.2 ± 1.29 (n = 8)
GAC	0.73 ± 0.10 (n = 32)	–95.7	18.6 ± 1.90 (n = 32)	+31.8	0.59 ± 0.24 (n = 32)	–66.5	19.8 ± 3.44 (n = 32)	–72.6	15.1 ± 1.56 (n = 8)
GAC_a	0.69 ± 0.08 (n = 32)	–95.9	23.4 ± 1.15 (n = 32)	+65.7	0.79 ± 0.16 (n = 32)	–55.1	24.5 ± 4.25 (n = 32)	–66.0	14.8 ± 1.33 (n = 8)
BF	0.78 ± 0.13 (n = 32)	–95.4	35.3 ± 1.51 (n = 32)	+150	0.63 ± 0.11 (n = 32)	–63.9	28.3 ± 2.39 (n = 32)	–60.8	50.8 ± 2.92 (n = 8)
BF_a	0.83 ± 0.15 (n = 32)	–95.1	34.5 ± 1.21 (n = 32)	+144	0.28 ± 0.07 (n = 32)	–84.0	32.7 ± 3.02 (n = 32)	–54.7	52.9 ± 4.87 (n = 8)
MBR1	1.58 ± 0.16 (n = 32)	–90.6	37.0 ± 1.66 (n = 32)	+162	0.93 ± 0.16 (n = 32)	–47.0	41.2 ± 3.45 (n = 32)	–42.9	2.84 ± 1.15 (n = 8)
MBR1+O_3	0.44 ± 0.09 (n = 32)	–97.4	27.3 ± 1.64 (n = 32)	+93.3	0.55 ± 0.12 (n = 32)	–68.7	21.8 ± 3.17 (n = 32)	–69.7	67.5 ± 4.62 (n = 8)
MBR2	3.09 ± 0.29 (n = 32)	–81.7	42.6 ± 2.95 (n = 32)	+201	1.58 ± 0.29 (n = 32)	–10.1	66.6 ± 3.07 (n = 32)	–7.68	0.00 ± 0.00 (n = 8)

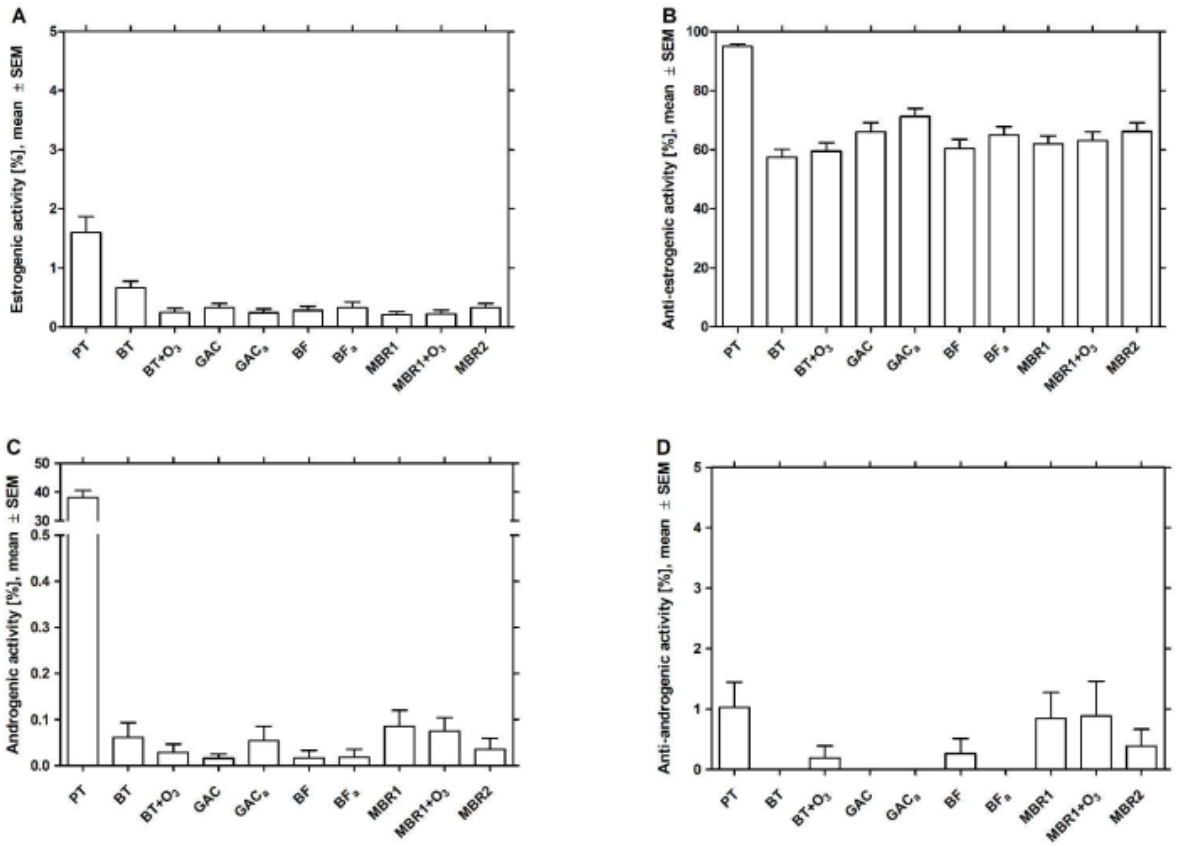


Figure S7: Estrogenic activity (A), anti-estrogenic activity (B), androgenic activity (C) and anti-androgenic activity (D) of the aqueous samples from four 24 h composite samples per treatment. PT: after primary treatment, BT: after conventional biological treatment, BT+O_3: after ozone system 1, GAC: after non-aerated granular activated carbon filter treatment, GAC_a: after aerated granular activated carbon filter treatment, BF: after non-aerated biofilter treatment, BF_a: after aerated biofilter treatment, MBR1/2: after membrane bioreactor 1/2, MBR1+O_3: after ozone system 2. n = 8–32 (A), n = 16–32 (B), n = 40–56 (C), n = 31–40 (D).

S2.3 Chemical analysis

Table S19: Concentrations in µg/L (mean ± SEM) of chemicals from four 24 h composite samples in the primary treatment (PT), the conventional biological treatment (BT), the non-aerated granular activated carbon filter treatment (GAC) and the aerated granular activated carbon filter treatment (GAC_a). The change of the concentration compared to the primary treatment (Δ PT) is given in %. n.d.: not detected.

	PT	BT	Δ PT [%]	BT+O_3	Δ PT [%]	GAC	Δ PT [%]	GAC_a	Δ PT [%]
10,11-Dihydro-10,11-dihydroxycarbamazepine	3.15 ± 0.350 (n = 4)	2.80 ± 0.252 (n = 3)	–11.1	0.353 ± 0.015 (n = 3)	–88.8	0.025 ± 0.000 (n = 4)	–99.2	0.025 ± 0.000 (n = 4)	–99.2
1H-Benzotriazol	25.0 ± 0.707 (n = 4)	7.68 ± 0.544 (n = 4)	–69.3	0.650 ± 0.047 (n = 4)	–97.4	0.026 ± 0.001 (n = 4)	–99.9	0.319 ± 0.294 (n = 4)	–98.7
1-Hydroxy-benzotriazol	1.31 ± 0.169 (n = 4)	0.493 ± 0.063 (n = 4)	–62.4	0.031 ± 0.006 (n = 4)	–97.6	0.025 ± 0.000 (n = 4)	–98.1	0.025 ± 0.000 (n = 4)	–98.1
1-Hydroxy-ibuprofen	5.83 ± 0.335 (n = 4)	0.187 ± 0.017 (n = 3)	–96.8	0.050 ± 0.025 (n = 3)	–99.1	0.063 ± 0.022 (n = 4)	–98.9	0.063 ± 0.022 (n = 4)	–98.9
2-Hydroxy-ibuprofen	47.3 ± 4.97 (n = 4)	0.618 ± 0.047 (n = 4)	–98.7	0.265 ± 0.083 (n = 4)	–99.4	0.175 ± 0.043 (n = 4)	–99.6	0.175 ± 0.043 (n = 4)	–99.6
3-Hydroxy-ibuprofen	5.98 ± 2.42 (n = 4)	0.375 ± 0.072 (n = 4)	–93.7	1.50 ± 1.17 (n = 4)	–74.9	0.375 ± 0.072 (n = 4)	–93.7	0.375 ± 0.072 (n = 4)	–93.7
4-Hydroxy-1H-benzotriazol	0.520 ± 0.047 (n = 4)	0.044 ± 0.019 (n = 4)	–91.6	0.044 ± 0.019 (n = 4)	–91.6	0.025 ± 0.000 (n = 4)	–95.2	0.025 ± 0.000 (n = 4)	–95.2
4-Hydroxy-diclofenac	2.38 ± 0.229 (n = 4)	1.16 ± 0.148 (n = 3)	–51.0	0.025 ± 0.000 (n = 3)	–98.9	0.025 ± 0.000 (n = 4)	–98.9	0.025 ± 0.000 (n = 4)	–98.9
4-Nitro-sulfamethoxazole	0.038 ± 0.007 (n = 4)	0.033 ± 0.008 (n = 3)	–11.1	0.033 ± 0.008 (n = 3)	–11.1	0.038 ± 0.007 (n = 4)	±0.0	0.038 ± 0.007 (n = 4)	±0.0
Acyclovir	6.75 ± 0.771 (n = 4)	0.450 ± 0.069 (n = 4)	–93.3	0.031 ± 0.006 (n = 4)	–99.5	0.025 ± 0.000 (n = 4)	–99.6	0.025 ± 0.000 (n = 4)	–99.6
Amidotrizoic acid	4.19 ± 2.97 (n = 4)	3.19 ± 1.91 (n = 4)	–24.0	2.33 ± 1.28 (n = 4)	–44.4	1.37 ± 0.302 (n = 4)	–67.4	1.43 ± 0.325 (n = 4)	–65.8
Carbamazepine	1.20 ± 0.147 (n = 4)	1.43 ± 0.067 (n = 3)	+19.4	0.025 ± 0.000 (n = 3)	–97.9	0.025 ± 0.000 (n = 4)	–97.9	0.025 ± 0.000 (n = 4)	–97.9
Carboxy-acyclovir	1.04 ± 0.178 (n = 4)	4.85 ± 1.04 (n = 4)	+367	0.044 ± 0.019 (n = 4)	–95.8	0.025 ± 0.000 (n = 4)	–97.6	0.025 ± 0.000 (n = 4)	–97.6
Carboxy-ibuprofen	74.7 ± 6.27 (n = 4)	0.150 ± 0.117 (n = 4)	–99.8	0.035 ± 0.006 (n = 4)	–100	0.025 ± 0.000 (n = 4)	100	0.025 ± 0.000 (n = 4)	100
Caffeine	162 ± 23.2 (n = 4)	0.312 ± 0.229 (n = 3)	–99.8	0.352 ± 0.224 (n = 3)	–99.8	0.335 ± 0.180 (n = 4)	–99.8	0.406 ± 0.190 (n = 4)	–99.7
Dehydrato-erythromycin	n.d.	0.120 ± 0.000 (n = 1)	-	0.050 ± 0.000 (n = 1)	-	0.050 ± 0.000 (n = 1)	-	0.052 ± 0.000 (n = 1)	-
Diclofenac	5.08 ± 0.431 (n = 4)	4.43 ± 0.067 (n = 3)	–12.7	0.137 ± 0.112 (n = 3)	–97.3	0.025 ± 0.000 (n = 4)	–99.5	0.025 ± 0.000 (n = 4)	–99.5
Erythromycin	n.d.	0.330 ± 0.000 (n = 1)	-	0.025 ± 0.000 (n = 1)	-	0.025 ± 0.000 (n = 1)	-	0.025 ± 0.000 (n = 1)	-
Iomeprol	9.09 ± 5.88 (n = 4)	6.77 ± 3.14 (n = 3)	–25.6	3.07 ± 1.04 (n = 3)	–66.3	0.173 ± 0.033 (n = 4)	–98.1	0.363 ± 0.059 (n = 4)	–96.0
Iopamidol	0.042 ± 0.008 (n = 3)	0.038 ± 0.013 (n = 2)	–10.0	0.033 ± 0.008 (n = 3)	–20.0	0.042 ± 0.008 (n = 3)	±0.0	0.042 ± 0.008 (n = 3)	±0.0
Iopromide	8.16 ± 3.87 (n = 4)	2.93 ± 1.51 (n = 3)	–64.1	0.966 ± 0.538 (n = 3)	–88.2	0.025 ± 0.000 (n = 4)	–99.7	0.054 ± 0.018 (n = 4)	–99.3
Mecoprop	0.038 ± 0.007 (n = 4)	0.033 ± 0.008 (n = 3)	–11.1	0.033 ± 0.008 (n = 3)	–11.1	0.038 ± 0.007 (n = 4)	±0.0	0.038 ± 0.007 (n = 4)	±0.0
N-Acetyl-sulfamethoxazole	1.25 ± 0.132 (n = 4)	0.130 ± 0.010 (n = 3)	–89.6	0.025 ± 0.000 (n = 3)	–98.0	0.025 ± 0.000 (n = 4)	–98.0	0.025 ± 0.000 (n = 4)	–98.0
Paracetamol	14.4 ± 2.23 (n = 4)	0.044 ± 0.019 (n = 4)	–99.7	0.044 ± 0.019 (n = 4)	–99.7	0.025 ± 0.000 (n = 4)	–99.8	0.025 ± 0.000 (n = 4)	–99.8
Sulfamethoxazole	1.30 ± 0.108 (n = 4)	0.335 ± 0.065 (n = 4)	–74.2	0.269 ± 0.244 (n = 4)	–79.3	0.025 ± 0.000 (n = 4)	–98.1	0.025 ± 0.000 (n = 4)	–98.1
Tolyltriazole	4.78 ± 0.58 (n = 4)	2.32 ± 0.341 (n = 4)	–51.5	0.032 ± 0.006 (n = 4)	–99.3	0.025 ± 0.000 (n = 4)	–99.5	0.025 ± 0.000 (n = 4)	–99.5
Tramadol	0.998 ± 0.043 (n = 4)	0.890 ± 0.061 (n = 3)	–10.8	0.031 ± 0.006 (n = 4)	–96.9	0.025 ± 0.000 (n = 4)	–97.5	0.025 ± 0.000 (n = 4)	–97.5
Tramadol-N-oxide	0.025 ± 0.000 (n = 4)	0.025 ± 0.000 (n = 3)	±0.0	0.047 ± 0.004 (n = 3)	+88.0	0.025 ± 0.000 (n = 4)	±0.0	0.025 ± 0.000 (n = 4)	±0.0

Table S20: Concentrations in µg/L (mean ± SEM) of chemicals from four 24 h composite samples in the primary treatment (PT), the non-aerated biofilter treatment (BF), the aerated biofilter treatment (BF_a), membrane reactor 1 (MBR1) and membrane reactor 1 after ozone system 2 ($MBR1+O_3$). The change of the concentration compared to the primary treatment (Δ PT) is given in %. n.d.: not detected.

	PT	BF	Δ PT [%]	BF_a	Δ PT [%]	MBR1	Δ PT [%]	$MBR1+O_3$	Δ PT [%]
10,11-Dihydro-10,11-dihydroxycarbamazepine	3.15 ± 0.350 (n = 4)	0.353 ± 0.072 (n = 4)	–88.8	0.338 ± 0.043 (n = 4)	–89.3	1.98 ± 0.588 (n = 4)	–37.3	0.496 ± 0.308 (n = 4)	–84.3
1H-Benzotriazol	25.0 ± 0.707 (n = 4)	0.650 ± 0.087 (n = 4)	–97.4	0.608 ± 0.069 (n = 4)	–97.6	9.73 ± 6.76 (n = 4)	–61.1	1.17 ± 0.700 (n = 4)	–95.3
1-Hydroxy-benzotriazol	1.31 ± 0.169 (n = 4)	0.025 ± 0.000 (n = 4)	–98.1	0.025 ± 0.000 (n = 4)	–98.1	0.423 ± 0.127 (n = 4)	–67.7	0.099 ± 0.074 (n = 4)	–92.5
1-Hydroxy-ibuprofen	5.83 ± 0.335 (n = 4)	0.063 ± 0.022 (n = 4)	–98.9	0.063 ± 0.022 (n = 4)	–98.9	1.53 ± 1.46 (n = 4)	–73.7	0.090 ± 0.044 (n = 4)	–98.5
2-Hydroxy-ibuprofen	47.3 ± 4.97 (n = 4)	0.183 ± 0.039 (n = 4)	–99.6	0.175 ± 0.043 (n = 4)	–99.6	14.1 ± 13.6 (n = 4)	–70.2	0.560 ± 0.381 (n = 4)	–98.8
3-Hydroxy-ibuprofen	5.98 ± 2.42 (n = 4)	0.375 ± 0.072 (n = 4)	–93.7	0.375 ± 0.072 (n = 4)	–93.7	2.10 ± 1.77 (n = 4)	–64.9	1.50 ± 1.17 (n = 4)	–74.9
4-Hydroxy-1H-benzotriazol	0.520 ± 0.047 (n = 4)	0.025 ± 0.000 (n = 4)	–95.2	0.025 ± 0.000 (n = 4)	–95.2	0.411 ± 0.363 (n = 4)	–21.1	0.051 ± 0.026 (n = 4)	–90.1
4-Hydroxy-diclofenac	2.38 ± 0.229 (n = 4)	0.025 ± 0.000 (n = 4)	–98.9	0.025 ± 0.000 (n = 4)	–98.9	1.03 ± 0.526 (n = 4)	–56.8	0.153 ± 0.122 (n = 4)	–93.5
4-Nitro-sulfamethoxazole	0.038 ± 0.007 (n = 4)	0.038 ± 0.007 (n = 4)	±0.0	0.038 ± 0.007 (n = 4)	±0.0	0.038 ± 0.007 (n = 4)	±0.0	0.038 ± 0.007 (n = 4)	±0.0
Acyclovir	6.75 ± 0.771 (n = 4)	0.025 ± 0.000 (n = 4)	–99.6	0.025 ± 0.000 (n = 4)	–99.6	2.02 ± 1.93 (n = 4)	–70.1	0.091 ± 0.066 (n = 4)	–98.6
Amidotrizoic acid	4.19 ± 2.97 (n = 4)	2.23 ± 1.26 (n = 4)	–46.9	2.37 ± 1.33 (n = 4)	–43.5	2.89 ± 1.66 (n = 4)	–31.2	2.22 ± 2.09 (n = 4)	–47.0
Carbamazepine	1.20 ± 0.147 (n = 4)	0.025 ± 0.000 (n = 4)	–97.9	0.025 ± 0.000 (n = 4)	–97.9	0.668 ± 0.184 (n = 4)	–44.4	0.124 ± 0.099 (n = 4)	–89.7
Carboxy-acyclovir	1.04 ± 0.178 (n = 4)	0.025 ± 0.000 (n = 4)	–97.6	0.025 ± 0.000 (n = 4)	–97.6	2.55 ± 0.591 (n = 4)	+146	1.45 ± 1.42 (n = 4)	+39.3
Carboxy-ibuprofen	74.7 ± 6.27 (n = 4)	0.028 ± 0.003 (n = 4)	–100	0.029 ± 0.004 (n = 4)	–100	38.3 ± 38.2 (n = 4)	–48.8	0.094 ± 0.069 (n = 4)	–99.9
Caffeine	162 ± 23.2 (n = 4)	0.338 ± 0.200 (n = 4)	–99.8	0.489 ± 0.279 (n = 4)	–99.7	87.1 ± 86.3 (n = 4)	–46.2	0.491 ± 0.224 (n = 4)	–99.7
Dehydrato-erythromycin	n.d.	0.050 ± 0.000 (n = 1)	-	0.050 ± 0.000 (n = 1)	-	n.d.	-	n.d.	-
Diclofenac	5.08 ± 0.431 (n = 4)	0.025 ± 0.000 (n = 4)	–99.5	0.025 ± 0.000 (n = 4)	–99.5	2.35 ± 0.718 (n = 4)	–53.7	0.269 ± 0.244 (n = 4)	–94.7
Erythromycin	n.d.	0.025 ± 0.000 (n = 1)	-	0.025 ± 0.000 (n = 1)	-	n.d.	-	n.d.	-
Iomeprol	9.09 ± 5.88 (n = 4)	2.45 ± 0.689 (n = 4)	–73.0	2.30 ± 0.785 (n = 4)	–74.8	4.93 ± 2.14 (n = 4)	–45.8	0.718 ± 0.153 (n = 4)	–92.1
Iopamidol	0.042 ± 0.008 (n = 3)	0.042 ± 0.008 (n = 3)	±0.0	0.042 ± 0.008 (n = 3)	±0.0	0.042 ± 0.008 (n = 3)	±0.0	0.042 ± 0.008 (n = 3)	±0.0
Iopromide	8.16 ± 3.87 (n = 4)	0.781 ± 0.397 (n = 4)	–90.4	0.515 ± 0.397 (n = 4)	–93.7	1.36 ± 0.466 (n = 4)	–83.4	0.369 ± 0.115 (n = 4)	–95.5
Mecoprop	0.038 ± 0.007 (n = 4)	0.038 ± 0.007 (n = 4)	±0.0	0.038 ± 0.007 (n = 4)	±0.0	0.038 ± 0.007 (n = 4)	±0.0	0.038 ± 0.007 (n = 4)	±0.0
N-Acetyl-sulfamethoxazole	1.25 ± 0.132 (n = 4)	0.025 ± 0.000 (n = 4)	–98.0	0.025 ± 0.000 (n = 4)	–98.0	0.490 ± 0.304 (n = 4)	–60.8	0.113 ± 0.079 (n = 4)	–90.9
Paracetamol	14.4 ± 2.23 (n = 4)	0.025 ± 0.000 (n = 4)	–99.8	0.025 ± 0.000 (n = 4)	–99.8	3.04 ± 3.02 (n = 4)	–78.8	0.030 ± 0.005 (n = 4)	–99.8
Sulfamethoxazole	1.30 ± 0.108 (n = 4)	0.025 ± 0.000 (n = 4)	–98.1	0.025 ± 0.000 (n = 4)	–98.1	0.362 ± 0.246 (n = 4)	–72.1	0.044 ± 0.019 (n = 4)	–96.6
Tolyltriazole	4.78 ± 0.58 (n = 4)	0.029 ± 0.004 (n = 4)	–99.4	0.027 ± 0.002 (n = 4)	–99.4	2.50 ± 1.47 (n = 4)	–47.6	0.371 ± 0.253 (n = 4)	–92.2
Tramadol	0.998 ± 0.043 (n = 4)	0.025 ± 0.000 (n = 4)	–97.5	0.025 ± 0.000 (n = 4)	–97.5	0.665 ± 0.213 (n = 4)	–33.3	0.190 ± 0.140 (n = 4)	–81.0
Tramadol-N-oxide	0.025 ± 0.000 (n = 4)	0.043 ± 0.008 (n = 4)	+71.0	0.042 ± 0.007 (n = 4)	+68.0	0.025 ± 0.000 (n = 4)	±0.0	0.028 ± 0.003 (n = 4)	+10.0

Table S21: Concentrations in µg/L (mean ± SEM) of chemicals from four 24 h composite samples in the primary treatment (PT), the conventional biological treatment (BT) and membrane reactor 2 (MBR2). The change of the concentration of MBR2 compared to the primary treatment (Δ PT) is given in %. n.d.: not detected.

	PT	BT (for comparison)	MBR2	Δ PT [%]
10,11-Dihydro-10,11-dihydroxycarbamazepine	3.15 ± 0.350 (n = 4)	2.80 ± 0.252 (n = 3)	2.86 ± 0.723 (n = 4)	–9.21
1H-Benzotriazol	25.0 ± 0.707 (n = 4)	7.68 ± 0.544 (n = 4)	5.55 ± 1.12 (n = 4)	–77.8
1-Hydroxy-benzotriazol	1.31 ± 0.169 (n = 4)	0.493 ± 0.063 (n = 4)	0.660 ± 0.190 (n = 4)	–49.6
1-Hydroxy-ibuprofen	5.83 ± 0.335 (n = 4)	0.187 ± 0.017 (n = 3)	0.128 ± 0.013 (n = 4)	–97.8
2-Hydroxy-ibuprofen	47.3 ± 4.97 (n = 4)	0.618 ± 0.047 (n = 4)	0.700 ± 0.172 (n = 4)	–98.5
3-Hydroxy-ibuprofen	5.98 ± 2.42 (n = 4)	0.375 ± 0.072 (n = 4)	0.375 ± 0.072 (n = 4)	–93.7
4-Hydroxy-1H-benzotriazol	0.520 ± 0.047 (n = 4)	0.044 ± 0.019 (n = 4)	0.082 ± 0.022 (n = 4)	–84.2
4-Hydroxy-diclofenac	2.38 ± 0.229 (n = 4)	1.16 ± 0.148 (n = 3)	1.49 ± 0.461 (n = 4)	–37.3
4-Nitro-sulfamethoxazole	0.038 ± 0.007 (n = 4)	0.033 ± 0.008 (n = 3)	0.038 ± 0.007 (n = 4)	±0.0
Acyclovir	6.75 ± 0.771 (n = 4)	0.450 ± 0.069 (n = 4)	0.127 ± 0.020 (n = 4)	–98.1
Amidotrizoic acid	4.19 ± 2.97 (n = 4)	3.19 ± 1.91 (n = 4)	3.78 ± 2.45 (n = 4)	–9.78
Carbamazepine	1.20 ± 0.147 (n = 4)	1.43 ± 0.067 (n = 3)	1.14 ± 0.359 (n = 4)	–4.92
Carboxy-acyclovir	1.04 ± 0.178 (n = 4)	4.85 ± 1.04 (n = 4)	4.60 ± 1.18 (n = 4)	+343
Carboxy-ibuprofen	74.7 ± 6.27 (n = 4)	0.150 ± 0.117 (n = 4)	0.158 ± 0.053 (n = 4)	–99.8
Caffeine	162 ± 23.2 (n = 4)	0.312 ± 0.229 (n = 3)	0.794 ± 0.322 (n = 4)	–99.5
Dehydrato-erythromycin	n.d.	0.120 ± 0.000 (n = 1)	n.d.	-
Diclofenac	5.08 ± 0.431 (n = 4)	4.43 ± 0.067 (n = 3)	3.18 ± 0.989 (n = 4)	–37.3
Erythromycin	n.d.	0.330 ± 0.000 (n = 1)	n.d.	-
Iomeprol	9.09 ± 5.88 (n = 4)	6.77 ± 3.14 (n = 3)	5.92 ± 3.50 (n = 4)	–34.9
Iopamidol	0.042 ± 0.008 (n = 3)	0.038 ± 0.013 (n = 2)	0.042 ± 0.008 (n = 3)	±0.0
Iopromide	8.16 ± 3.87 (n = 4)	2.93 ± 1.51 (n = 3)	2.17 ± 1.37 (n = 4)	–73.4
Mecoprop	0.038 ± 0.007 (n = 4)	0.033 ± 0.008 (n = 3)	0.038 ± 0.007 (n = 4)	±0.0
N-Acetyl-sulfamethoxazole	1.25 ± 0.132 (n = 4)	0.130 ± 0.010 (n = 3)	0.173 ± 0.041 (n = 4)	–86.2
Paracetamol	14.4 ± 2.23 (n = 4)	0.044 ± 0.019 (n = 4)	0.025 ± 0.000 (n = 4)	–99.8
Sulfamethoxazole	1.30 ± 0.108 (n = 4)	0.335 ± 0.065 (n = 4)	0.254 ± 0.069 (n = 4)	–80.4
Tolyltriazole	4.78 ± 0.58 (n = 4)	2.32 ± 0.341 (n = 4)	2.25 ± 0.452 (n = 4)	–52.9
Tramadol	0.998 ± 0.043 (n = 4)	0.890 ± 0.061 (n = 3)	0.955 ± 0.217 (n = 4)	–4.26
Tramadol-N-oxide	0.025 ± 0.000 (n = 4)	0.025 ± 0.000 (n = 3)	0.025 ± 0.000 (n = 4)	±0.0

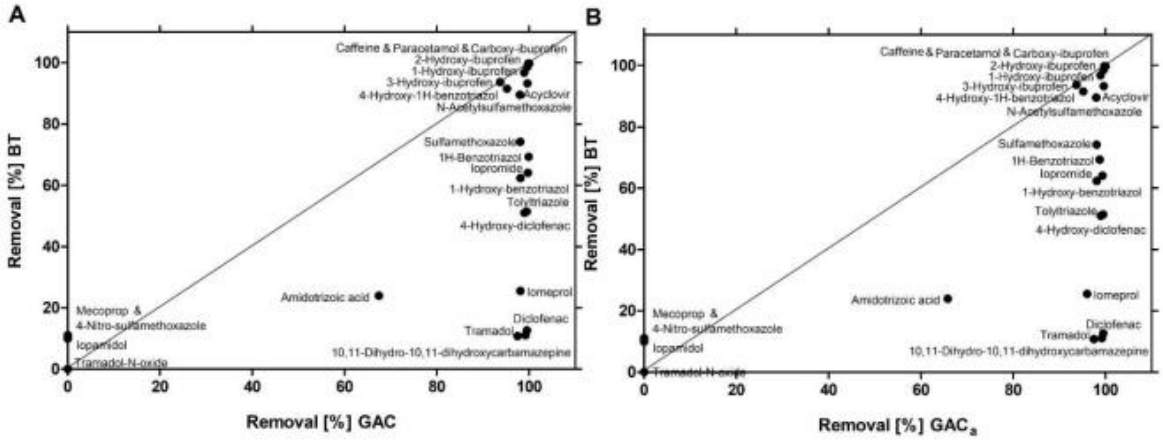


Figure S8: Removal of chemicals in the conventional biological treatment (BT) compared to the non-aerated granular activated carbon filter treatment (GAC, A) and the aerated granular activated carbon filter treatment (GAC_a, B). n = 1–4.

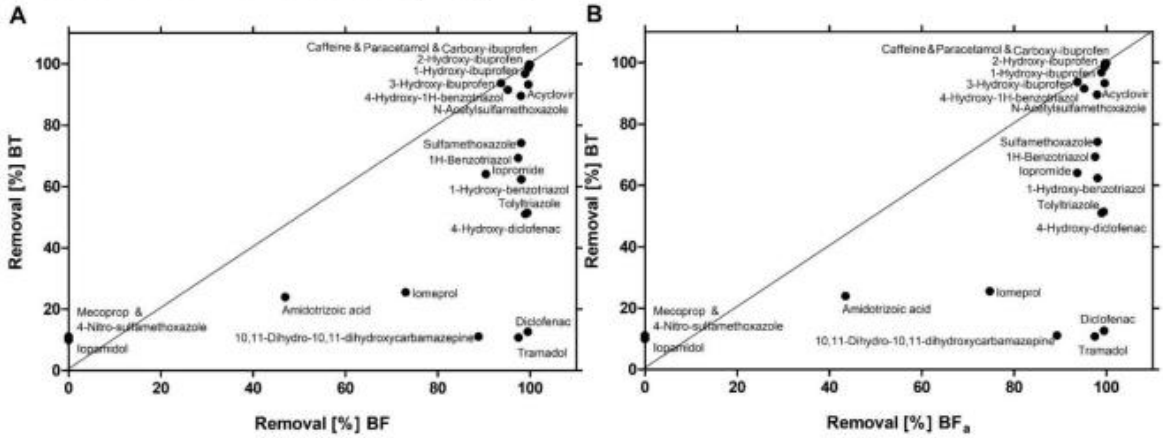


Figure S9: Removal of chemicals in the conventional biological treatment (BT) compared to the non-aerated biofilter treatment (BF, A) and the aerated biofilter treatment (BF_a,B). n = 1–4.

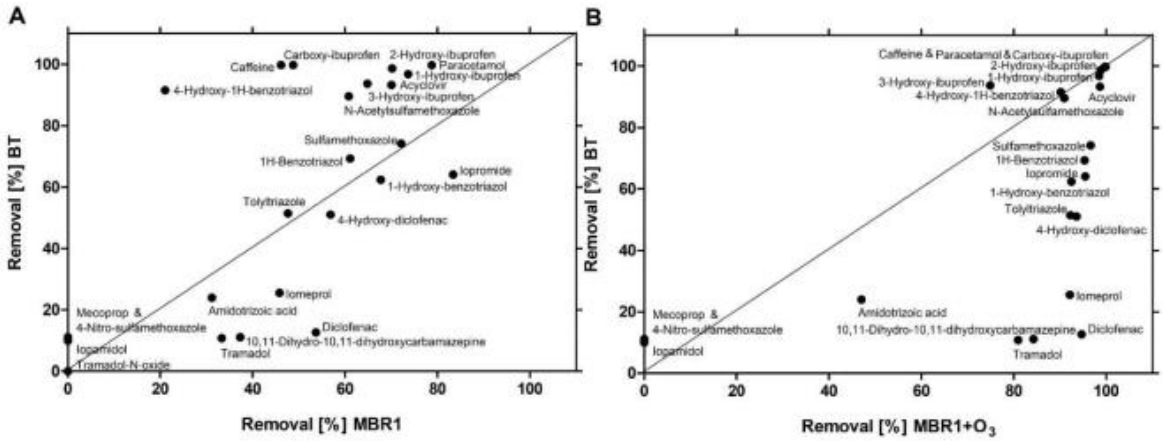


Figure S10: Removal of chemicals in the conventional biological treatment (BT) compared to the membrane bioreactor 1 (MBR1, A) and the membrane bioreactor 1 after ozone system 2 ($MBR1+O_3$, B). n = 1–4.

S2.4 Physical-chemical parameters

During the 28 days on-site experiment with *Potamopyrgus antipodarum* standardised physical-chemical parameters (pH, conductivity, oxygen saturation, oxygen content, NO_2-N, NO_3-N, NH_4-N and total hardness) were periodically (n = 4) determined directly in the exposure vessels using test kits as well as in the course of the almost daily (n = 22) surveillance of wastewater parameters at the WWTP (COD, DOC, NO_2-N, NO_3-N, NH_4-N, P_{total} and SAC_{254}, compare 2.9). Temperature was measured in the tank. Detailed results are provided in Tables S22-S29 and summarised in the following.

The temperature (set: 16.0 ± 1.0°C; measured: 16.3 ± 0.63°C, range: 14.2–19.6°C; n = 5544) in the tank and the parameters determined in each exposure vessel met the validity criteria according to OECD (2016) regarding the pH (set: 8.0 ± 0.5, should not fall below pH 6.5, measured: 6.69–8.28, n = 44), oxygen content (set: > 6.0 mg/L, measured: 7.30–13.5 mg/L, n = 44) and oxygen saturation (set: > 60.0%, measured: 72.6–137%, n = 44). The recommended value for the conductivity was also met in the NC and PC (set: 770 ± 100 µS/cm, measured: 700–800 µS/cm, n = 8). The conductivity in the exposure vessels of the nine tested wastewater treatments was minimal 947 µS/cm and maximal 1425 µS/cm (n = 36). The total hardness was between 4°dH and 7°dH (n = 8) in the NC and PC and between 15°dH and 20°dH (n = 36) in the wastewater treatment groups.

The concentrations of nitrite (NO_2-N) ranged between 0.005 mg/L and > 0.1 mg/L in all exposure vessels. The concentrations of ammonium (NH_4-N) were < 0.05 mg/L in all treatments, except once (day 24) in MBR2 (1.5 mg/L). The concentration of nitrate (NO_3-N) was maximal 1.0 mg/L in NC and PC and maximal 10 mg/L in the BT, BT+O_3 and the post-filtration systems. The MBR1, MBR1+O_3 and MBR2 showed the highest nitrate concentrations between 40 mg/L and 80 mg/L at the end of the experiment.

The results of the physical-chemical analysis as part of the regulative WWTP monitoring were in line with those from the test kits regarding the overlapping nitrogen parameters. In both cases NO_2-N, NO_3-N and NH_4-N exhibited typical concentration trends for activated sludge treatments (that include nitrification-denitrification) and for the present AWWT. COD, DOC, P_{total} and SAC_{254} likewise displayed typical concentration tendencies as reported in the scientific literature (further discussed under 4.3).

Table S22: Chemical oxygen demand (COD) in mg/L measured directly in the ten effluents of the reactors on the pilot wastewater treatment plant during 28 days of exposure. PT: after primary treatment, BT: after conventional biological treatment, BT+O_3: after ozone system 1, GAC: after non-aerated granular activated carbon filter treatment, GAC_a: after aerated granular activated carbon filter treatment, BF: after non-aerated biofilter treatment, BF_a: after aerated biofilter treatment, MBR1/2: after membrane bioreactor 1/2, MBR1+O_3: after ozone system 2, n.a.: not analysed.

Time [d]	PT	BT	BT+O_3	GAC	GAC_a	BF	BF_a	MBR1	MBR1+O_3	MBR2
1	123	15.0	12.5	n.a.	n.a.	n.a.	n.a.	12.1	10.9	22.5
2	311/197	17.2	16.9	<5.00	8.90	11.3	12.0	8.92	6.73	12.8
3	611/312	23.3	22.6	11.2	11.8	16.4	16.0	n.a.	n.a.	n.a.
4	314	23.6	20.7	n.a.	n.a.	n.a.	n.a.	20.7	15.8	20.4
5	323	25.6	21.7	n.a.	n.a.	n.a.	n.a.	21.9	17.0	22.2
8	333	29.7	24.2	n.a.	n.a.	n.a.	n.a.	16.9	13.3	22.1
9	364/633	29.9	24.5	6.30	14.5	21.2	21.1	16.8	11.8	22.6
10	324	29.7	25.8	n.a.	n.a.	n.a.	n.a.	17.5	14.1	23.6
11	n.a.	27.7	23.6	n.a.	n.a.	n.a.	n.a.	n.a.	14.9	11.1
12	624/314	27.8	24.6	11.1	14.3	19.5	20.9	n.a.	n.a.	29.3
15	664/320	26.1	22.6	n.a.	n.a.	n.a.	n.a.	21.7	16.8	20.9
16	559/291	32.6	29.0	17.1	18.0	22.6	20.8	17.3	14.2	23.3
17	270	28.2	23.4	n.a.	n.a.	n.a.	n.a.	14.1	10.8	21.3
18	359	29.4	24.2	12.9	13.0	19.5	20.3	15.1	11.9	22.1
19	282/97.9	19.7	16.4	n.a.	n.a.	n.a.	n.a.	10.2	8.66	18.0
22	234	25.2	21.4	n.a.	n.a.	n.a.	n.a.	12.2	10.2	19.2
23	642/338	26.3	23.1	10.9	<5.00	17.9	18.6	14.4	10.2	19.7
24	250	28.0	24.4	n.a.	n.a.	n.a.	n.a.	14.5	12.2	21.7
25	397	28.1	24.1	14	15.9	17.5	20.4	14.5	11.7	22.0
26	282	30.2	26.5	n.a.	n.a.	n.a.	n.a.	19.1	16.1	23.5
29	320	30.5	25.9	n.a.	n.a.	n.a.	n.a.	16.3	13.7	22.5
30	638/349	29.2	25.4	13.7	14.8	21.3	20.7	16.5	11.2	24.1

Table S23: Dissolved organic carbon (DOC) in mg/L measured directly in the ten effluents of the reactors on the pilot wastewater treatment plant during 28 days of exposure. PT: after primary treatment, BT: after conventional biological treatment, BT+O_3: after ozone system 1, GAC: after non-aerated granular activated carbon filter treatment, GAC_a: after aerated granular activated carbon filter treatment, BF: after non-aerated biofilter treatment, BF_a: after aerated biofilter treatment, MBR1/2: after membrane bioreactor 1/2, MBR1+O_3: after ozone system 2, n.a.: not analysed.

Time [d]	PT	BT	BT+O_3	GAC	GAC_a	BF	BF_a	MBR1	MBR1+O_3	MBR2
1	39.8	6.71	6.83	n.a.	n.a.	n.a.	n.a.	6.25	6.25	10.9
2	71.0	8.89	8.22	3.01	4.76	6.68	7.41	6.41	6.07	9.43
3	101	9.85	9.42	5.41	6.14	8.13	8.07	n.a.	n.a.	n.a.
4	96.0	9.43	9.36	n.a.	n.a.	n.a.	n.a.	8.80	7.44	8.98
5	101	10.7	10.8	n.a.	n.a.	n.a.	n.a.	9.23	8.45	9.76
8	101	12.0	11.6	n.a.	n.a.	n.a.	n.a.	7.31	6.89	9.81
9	106	11.9	11.4	4.36	6.98	9.40	9.42	8.00	6.25	9.18
10	105	12.4	11.7	n.a.	n.a.	n.a.	n.a.	8.07	7.39	10.0
11	n.a.	12.0	11.4	n.a.	n.a.	n.a.	n.a.	6.18	7.08	n.a.
12	98.5	11.3	11.2	5.91	7.03	8.85	9.25	n.a.	n.a.	12.7
15	96.5	10.7	10.3	n.a.	n.a.	n.a.	n.a.	8.57	7.86	8.46
16	83.5	11.4	11.6	7.42	8.29	9.8	10.5	7.16	6.54	9.12
17	82.5	11.3	16.7	n.a.	n.a.	n.a.	n.a.	6.45	5.93	8.74
18	100	11.7	11.1	6.17	6.51	8.85	8.91	9.25	6.22	9.38
19	33.9	7.80	8.05	n.a.	n.a.	n.a.	n.a.	5.85	4.78	7.63
22	76.0	10.1	9.70	n.a.	n.a.	n.a.	n.a.	6.14	5.54	9.02
23	98.5	10.8	10.6	5.96	<3.00	8.6	8.4	6.42	5.65	7.96
24	83.5	11.0	10.8	n.a.	n.a.	n.a.	n.a.	6.57	8.25	8.64
25	109	11.5	11.0	6.59	6.83	9.68	8.63	6.08	5.68	8.07
26	86.0	11.7	11.3	n.a.	n.a.	n.a.	n.a.	7.72	7.21	8.81
29	102	12.8	11.7	n.a.	n.a.	n.a.	n.a.	9.03	6.56	9.41
30	104	12.4	11.8	6.75	7.13	9.84	9.73	6.90	6.43	8.57

Table S24: Ammonium (NH_4-N) in mg/L measured directly in the ten effluents of the reactors on the pilot wastewater treatment plant during 28 days of exposure. PT: after primary treatment, BT: after conventional biological treatment, BT+O_3: after ozone system 1, GAC: after non-aerated granular activated carbon filter treatment, GAC_a: after aerated granular activated carbon filter treatment, BF: after non-aerated biofilter treatment, BF_a: after aerated biofilter treatment, MBR1/2: after membrane bioreactor 1/2, MBR1+O_3: after ozone system 2, n.a.: not analysed.

Time [d]	PT	BT	BT+O_3	GAC	GAC_a	BF	BF_a	MBR1	MBR1+O_3	MBR2
1	23.8	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	<0.015	<0.015	0.019
2	64.5	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	0.045	0.035	0.048
3	100	0.306	0.321	0.032	<0.015	0.23	<0.015	n.a.	n.a.	n.a.
4	100	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	0.184	0.133	0.139
5	98.5	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	0.352	0.226	1.33
8	66.0	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	0.018	0.028	0.205
9	96.5	0.176	0.218	0.026	<0.015	0.212	0.018	0.058	0.047	0.117
10	102	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	0.064	0.062	1.24
11	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
12	96.0	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	0.339
15	67.0	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	10.8	7.39	0.051
16	55.0	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	0.046	0.028	0.042
17	100	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	0.043	0.042	1.32
18	n.a.	0.489	0.518	0.122	<0.015	0.361	0.017	n.a.	n.a.	n.a.
19	28.0	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	<0.015	0.022	<0.015
22	55.0	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	0.041	0.047	0.025
23	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
24	86.5	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	0.106	0.111	18.9
25	n.a.	0.115	0.144	0.028	<0.015	0.087	0.226	n.a.	n.a.	16.2
26	97.0	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	6.12	5.11	26.7
29	90.5	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	4.02	3.88	39.9
30	n.a.	0.139	0.186	0.028	<0.015	0.088	0.019	n.a.	n.a.	39.7

Table S25: Nitrite (NO_2-N) in mg/L measured directly in the ten effluents of the reactors on the pilot wastewater treatment plant during 28 days of exposure. PT: after primary treatment, BT: after conventional biological treatment, BT+O_3: after ozone system 1, GAC: after non-aerated granular activated carbon filter treatment, GAC_a: after aerated granular activated carbon filter treatment, BF: after non-aerated biofilter treatment, BF_a: after aerated biofilter treatment, MBR1/2: after membrane bioreactor 1/2, MBR1+O_3: after ozone system 2, n.a.: not analysed.

Time [d]	PT	BT	BT+O_3	GAC	GAC_a	BF	BF_a	MBR1	MBR1+O_3	MBR2
1	0.084	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	<0.015	<0.015	0.021
2	0.024	0.345	<0.015	<0.015	<0.015	0.186	<0.015	0.062	<0.015	0.051
3	0.031	0.336	<0.015	0.081	<0.015	0.182	<0.015	n.a.	n.a.	n.a.
4	0.035	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	0.403	0.020	0.138
5	0.026	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	0.999	0.056	0.279
8	0.032	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	0.076	<0.015	0.087
9	0.045	0.237	<0.015	<0.015	<0.015	0.162	<0.015	0.132	<0.015	0.220
10	0.027	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	0.328	<0.015	0.600
11	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	0.07	<0.015	n.a.
12	0.029	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	0.406
15	0.032	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	0.342	0.049	0.085
16	0.027	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	0.027	<0.015	0.080
17	0.031	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	0.115	<0.015	0.238
18	n.a.	0.538	<0.015	0.248	<0.015	0.318	<0.015	n.a.	n.a.	n.a.
19	0.157	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	0.024	<0.015	0.017
22	0.026	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	0.052	<0.015	0.039
23	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
24	0.28	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	0.087	<0.015	0.111
25	n.a.	0.238	<0.015	0.032	<0.015	0.183	<0.015	n.a.	n.a.	n.a.
26	0.023	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	0.013	0.023	0.060
29	0.03	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	0.134	0.039	0.061
30	n.a.	0.265	<0.015	0.016	<0.015	0.234	<0.015	n.a.	n.a.	n.a.

Table S26: Nitrate (NO_3-N) in mg/L measured directly in the ten effluents of the reactors on the pilot wastewater treatment plant during 28 days of exposure. PT: after primary treatment, BT: after conventional biological treatment, BT+O_3: after ozone system 1, GAC: after non-aerated granular activated carbon filter treatment, GAC_a: after aerated granular activated carbon filter treatment, BF: after non-aerated biofilter treatment, BF_a: after aerated biofilter treatment, MBR1/2: after membrane bioreactor 1/2, MBR1+O_3: after ozone system 2, n.a.: not analysed.

Time [d]	PT	BT	BT+O_3	GAC	GAC_a	BF	BF_a	MBR1	MBR1+O_3	MBR2
1	<0.230	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	29.8	29.6	29.2
2	0.295	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	36.6	36.2	32.8
3	0.258	1.65	2.28	2.90	3.03	2.41	2.76	n.a.	n.a.	n.a.
4	0.441	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	58.6	59.0	56.8
5	0.332	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	65.4	65.6	68.0
8	0.348	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	49.0	50.0	66.2
9	0.400	1.64	1.99	2.15	2.38	1.91	2.14	53.2	53.0	60.2
10	0.367	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	68.0	69.0	67.8
11	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	71.2	70.6	n.a.
12	0.284	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	63.6
15	0.293	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	42.4	51.5	50.6
16	0.321	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	47.2	48.4	47.6
17	0.421	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	36.8	35.8	32.2
18	n.a.	2.70	3.67	3.88	4.22	3.37	4.06	n.a.	n.a.	n.a.
19	0.232	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	40.4	40.6	32.4
22	0.320	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	37.2	37.6	27.6
23	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
24	0.412	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	50.2	51.2	24.0
25	n.a.	1.63	1.99	2.42	2.55	1.96	2.21	n.a.	n.a.	n.a.
26	0.288	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	27.2	29.0	7.56
29	0.340	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	25.2	25.6	6.99
30	n.a.	1.54	2.35	2.49	2.57	2.11	2.34	n.a.	n.a.	n.a.

Table S27: Total phosphorus (P_{total}) in mg/L measured directly in the ten effluents of the reactors on the pilot wastewater treatment plant during 28 days of exposure. PT: after primary treatment, BT: after conventional biological treatment, BT+O_3: after ozone system 1, GAC: after non-aerated granular activated carbon filter treatment, GAC_a: after aerated granular activated carbon filter treatment, BF: after non-aerated biofilter treatment, BF_a: after aerated biofilter treatment, MBR1/2: after membrane bioreactor 1/2, MBR1+O_3: after ozone system 2, n.a.: not analysed.

Time [d]	PT	BT	BT+O_3	GAC	GAC_a	BF	BF_a	MBR1	MBR1+O_3	MBR2
1	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
2	8.29	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	6.04	6.07	6.48
3	n.a.	0.795	0.807	0.735	0.825	0.766	0.788	n.a.	n.a.	n.a.
4	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
5	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
8	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
9	11.9	0.432	0.437	0.260	0.483	0.432	0.464	5.90	5.95	7.10
10	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
11	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
12	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
15	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
16	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
17	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
18	n.a.	0.635	0.619	0.456	0.508	0.580	0.558	n.a.	n.a.	n.a.
19	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
22	8.76	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	6.64	6.51	6.98
23	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
24	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
25	n.a.	0.485	0.476	0.557	0.496	0.438	0.576	n.a.	n.a.	n.a.
26	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
29	12.2	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	7.14	7.16	7.44
30	n.a.	0.544	0.576	0.638	0.58	0.610	0.571	n.a.	n.a.	n.a.

Table S28: Spectral absorption coefficient at 254 nm (SAC_{254}) in cm^{-1} measured directly in the ten effluents of the reactors on the pilot wastewater treatment plant during 28 days of exposure. PT: after primary treatment, BT: after conventional biological treatment, BT+O_3: after ozone system 1, GAC: after non-aerated granular activated carbon filter treatment, GAC_a: after aerated granular activated carbon filter treatment, BF: after non-aerated biofilter treatment, BF_a: after aerated biofilter treatment, MBR1/2: after membrane bioreactor 1/2, MBR1+O_3: after ozone system 2, n.a.: not analysed.

Time [d]	PT	BT	BT+O_3	GAC	GAC_a	BF	BF_a	MBR1	MBR1+O_3	MBR2
1	0.305	0.112	0.042	n.a.	n.a.	n.a.	n.a.	0.126	0.093	0.217
2	0.474	0.120	0.049	0.014	0.027	0.041	0.041	0.108	0.055	0.190
3	0.732	0.144	0.059	0.023	0.030	0.050	0.050	n.a.	n.a.	n.a.
4	0.706	0.162	0.068	n.a.	n.a.	n.a.	n.a.	0.157	0.104	0.218
5	0.715	0.181	0.067	n.a.	n.a.	n.a.	n.a.	0.170	0.110	0.225
8	0.745	0.186	0.071	n.a.	n.a.	n.a.	n.a.	0.139	0.062	0.223
9	0.796	0.193	0.082	0.022	0.037	0.069	0.070	0.131	0.017	0.224
10	0.704	0.200	0.078	n.a.	n.a.	n.a.	n.a.	0.149	0.083	0.242
11	n.a.	0.194	0.079	n.a.	n.a.	n.a.	n.a.	0.131	0.057	n.a.
12	0.723	0.195	0.076	0.033	0.040	0.072	0.071	n.a.	n.a.	0.292
15	0.732	0.187	0.074	n.a.	n.a.	n.a.	n.a.	0.177	0.108	0.217
16	0.609	0.190	0.079	0.038	0.038	0.065	0.066	0.125	0.058	0.214
17	0.623	0.195	0.080	n.a.	n.a.	n.a.	n.a.	0.121	0.058	0.215
18	0.935	0.200	0.085	0.038	0.040	0.071	0.074	0.126	0.055	0.226
19	0.283	0.140	0.049	n.a.	n.a.	n.a.	n.a.	0.090	0.044	0.181
22	0.586	0.171	0.071	n.a.	n.a.	n.a.	n.a.	0.111	0.055	0.189
23	0.769	0.179	0.067	0.030	0.012	0.055	0.058	0.116	0.053	0.201
24	0.589	0.190	0.077	n.a.	n.a.	n.a.	n.a.	0.127	0.062	0.210
25	1.043	0.221	0.106	0.069	0.070	0.095	0.097	0.162	0.104	0.250
26	0.676	0.205	0.080	n.a.	n.a.	n.a.	n.a.	0.147	0.082	0.228
29	0.678	0.212	0.082	n.a.	n.a.	n.a.	n.a.	0.132	0.074	0.219
30	0.843	0.216	0.088	0.045	0.048	0.076	0.078	0.125	0.057	0.229

Table S29: Physical-chemical parameters of the negative control (NC), the positive control (PC), the conventional biological treatment (BT) and the eight advanced treatment technologies measured directly in the exposure vessels during 28 days of exposure. BT+O_3: after ozone system 1, GAC: after non-aerated granular activated carbon filter treatment, GAC_a: after aerated granular activated carbon filter treatment, BF: after non-aerated biofilter treatment, BF_a: after aerated biofilter treatment, MBR1/2: after membrane bioreactor 1/2, MBR1+O_3: after ozone system 2, NO_2-N: nitrite, NO_3-N: nitrate, NH_4-N: ammonium.

	pH	conductivity [µS/cm]	oxygen [%] / [mg/L]	hardness [°dH]	NO_2-N [mg/L]	NO_3-N [mg/L]	NH_4-N [mg/L]
day 4							
NC	7.88	782	94.0 / 9.55	6	0.005	< 0.5	< 0.05
PC	7.85	783	92.1 / 9.28	6	0.0	< 0.5	< 0.05
BT	7.06	966	72.6 / 7.30	17	> 0.1	5	< 0.05
BT+O_3	7.00	957	129 / 13.0	16	> 0.1	1-5	< 0.05
GAC	7.30	965	112 / 11.3	15	> 0.1	1-5	< 0.05
GAC_a	7.58	970	93.3 / 9.41	16	0.03	0.5-1	< 0.05
BF	7.48	970	123 / 12.3	15	> 0.1	1	< 0.05
BF_a	7.58	971	93.1 / 9.34	15	0.04	1	< 0.05
MBR1	6.70	1280	92.7 / 9.25	19	> 0.1	40	< 0.05
MBR1+O_3	6.74	1284	105 / 10.5	19	> 0.1	20	< 0.05
MBR2	6.69	1251	91.0 / 9.13	19	> 0.1	20	< 0.05
day 10							
NC	8.11	800	92.7 / 9.40	6	0.005	0.0	< 0.05
PC	7.84	796	92.6 / 9.11	7	0.005	0.0	-
BT	8.00	1220	90.7 / 8.91	19	> 0.1	0-10	< 0.05
BT+O_3	7.60	1223	137 / 13.5	18	0.1	0	< 0.05
GAC	7.59	1221	113 / 11.1	19	0.1	0-10	< 0.05
GAC_a	7.95	1212	94.4 / 9.28	19	0.02	0-10	< 0.05
BF	7.45	1223	112 / 11.0	19	> 0.1	0-10	< 0.05
BF_a	7.86	1218	93.6 / 9.21	19	0.02	0-10	< 0.05
MBR1	7.40	1407	91.8 / 9.01	20	> 0.1	50	-
MBR1+O_3	7.26	1405	121 / 11.9	20	0.06	50	-
MBR2	6.93	1425	77.9 / 7.65	20	0.06	50	-
day 17							
NC	8.28	763	90.2 / 8.88	5	0.012-0.02	< 0.5	< 0.05
PC	8.0	761	83.1 / 8.2	4	0.012	< 0.5	< 0.05
BT	7.81	1012	87.4 / 8.62	18	> 0.1	5	< 0.05
BT+O_3	7.80	1007	97.7 / 9.67	18	> 0.1	5	< 0.05
GAC	7.78	1003	103 / 10.2	18	> 0.1	5	< 0.05
GAC_a	7.81	1002	90.1 / 8.91	18	0.06-0.08	1-5	< 0.05
BF	7.71	1006	111 / 11.2	18	> 0.1	5	< 0.05
BF_a	7.81	1000	91.7 / 9.06	18	0.05-0.06	1-5	< 0.05
MBR1	7.70	1082	101 / 9.94	19	> 0.1	40-80	< 0.05
MBR1+O_3	7.60	1086	115 / 11.4	19	> 0.1	40	< 0.05
MBR2	7.38	1096	79.5 / 7.86	19	> 0.1	40	< 0.05
day 24							
NC	8.14	701	92.1 / 9.11	4	0.012	< 0.5	< 0.05
PC	7.90	700	83.4 / 8.22	5	0.012	1.0	< 0.05
BT	7.70	947	83.0 / 8.22	16	0.08	1-5	< 0.05
BT+O_3	7.63	975	97.4 / 9.63	16	> 0.1	1-5	< 0.05
GAC	7.78	976	97.9 / 9.65	16	0.1	1-5	< 0.05
GAC_a	7.90	970	93.2 / 9.19	16	0.05	1-5	< 0.05
BF	7.85	974	100 / 9.85	16	> 0.1	1-5	< 0.05
BF_a	7.96	976	92.9 / 9.18	16	0.04	1-5	< 0.05
MBR1	7.70	1141	91.0 / 9.07	18	> 0.1	40-80	< 0.05
MBR1+O_3	7.56	1147	95.3 / 9.42	17	> 0.1	40-80	< 0.05
MBR2	7.57	1253	83.2 / 8.24	17	> 0.1	40	1.5

A.4 Zusammenfassung (German summary)

Sauberes Wasser ist eine grundlegende Voraussetzung unseres Lebens. Abwasser-bürtige Emissionen in Form von anthropogenen Mikroschadstoffen gefährden die Resource Wasser und die Integrität des Ökosystems auf verschiedenen Skalen (z.B. Link et al. 2017, Malaj et al. 2014, Pal et al. 2010, Stalter et al. 2013). Bestimmte Kontaminanten und deren Transformationsprodukte verteilen sich weitläufig im Wasserkreislauf und werden kontinuierlich emittiert. Bestimmte Stoffe gelangen so in geringen Konzentrationen bereits ins Trinkwasser (Benotti et al. 2009). Die ökotoxikologische Untersuchung und das Umweltmonitoring von (Mikro-)Schadstoffen ist somit eine essentielle Aufgabe, die in diversen Wasser- und Abwasserdirektiven verankert wurde (z.B. EC 1991, 1998, 2008, US EPA 1972, 2002). Unter anderem widmen sich dieser Aufgabe interdisziplinäre Forschungsprojekte und soziopolitische Plattformen zur Reduktion von Mikroschadstoffen und für ein nachhaltiges Abwassermanagement (z.B. Rizzo 2019). Kläranlagen sind Haupteinleiter für aquatische (Mikro-)Schadstoffe (EEA 2012). In den letzten Jahrzehnten wurden Kläranlagen optimiert und an vielen Standorten um Prozesse wie die Tertiärbehandlung erweitert (Prasse et al. 2015, Reemtsma et al. 2016). Nichtsdestoweniger führt die konventionelle biologische Abwasserbehandlung nur bei einem Bruchteil der bekannten und bedenklichen Substanzen zu einer vollständigen Mineralisierung. Ein Grund hierfür kann eine geringe biologische Abbaubarkeit bestimmter synthetischer Verbindungen sein oder auch eine mangelnde Adsorption an Klärschlammpartikel.

Die wachsende Weltbevölkerung und Verstädterung erhöht die Gesamtmengen des zu behandelnden Abwassers. Ferner werden dank verbesserter Analytik fortwährend neue Mikroschadstoffe nachgewiesen (z.B. Rockström et al. 2009, Stamm et al. 2016, Philip et al. 2011). Gegenwärtig wird daher das Potenzial verschiedener Technologien der weiterführenden Abwasserbehandlung untersucht, um eine zusätzliche Reduktion von Mikroschadstoffen und anderer Abwasser-bürtiger Emissionenen zu erreichen (Tchobanoglous and Burton 1991). Die vorliegende Arbeit war in das Forschungsprojekt TransRisk eingebettet, welches sich mit der "Charakterisierung, Kommunikation und Minimierung von Risiken durch neuartige Schadstoffe und Krankheitserreger im Wasserkreislauf" beschäftigt (www.transrisk-projekt.de). Die nachfolgenden ökotoxikologischen

Herausforderungen, verbunden mit anthropogenen Mikroschadstoffen und den weiterführenden Abwasserbehandlungstechnologien Ozonung und Ozonung gefolgt von körniger Aktivkohle- und Bio-Filtration (Pilot-Maßstab), werden darin detailiert untersucht.

Die Vorbereitung von (Ab-)Wasserproben ist von zentraler Bedeutung für die akkurate und repräsentative ökotoxikologische Bewertung. In der vorliegenden Arbeit wurde untersucht, welche Auswirkung routinemäßig angewandte Vorbereitungstechniken - Ansäuerung (pH 2), Filtration (1 µm Porengröße) und Festphasenextraktion (SPE) - auf den Nachweis umweltrelevanter endokriner Aktivitäten, Mutagenität, Genotoxizität und Zytotoxizität haben. In früheren Studien zeigte sich, dass diese Methoden in der Lage sind, die Konzentration bioaktiver Verbindungen zu verändern und/oder den Nachweis der Toxizität in (Ab-)Wasserproben signifikant zu beeinflussen (z.B. Baker und Kasprzyk-Hordern 2011, Maruya et al. 2016, Neale et al. 2018, Vanderford et al. 2011). Die vorliegende Untersuchung konzentrierte sich auf unbehandeltes, biologisch-behandeltes, ozoniertes und Krankenhaus-Abwasser sowie Grundwasser.

Die Probenansäuerung, die im Allgemeinen zur Inaktivierung von Mikroorganismen angewendet wird, schien zur Stabilisierung der untersuchten In-vitro-Effekte ungeeignet. Letztere waren nach 24 Stunden Lagerung in den meisten angesäuerten Proben im Vergleich zu ihren neutralen Äquivalenten signifikant verändert. Es wurde kein Zusammenhang mit den Aktivitäts-/Toxizitäts-Leveln oder den Sensitivitäten der Bioassays beobachtet. Effekte wie die säurekatalysierte Hydrolyse (Prasse et al. 2015) oder ein mikrobieller Abbau in neutralen Proben (Giebner et al. 2018) konnten anhand der vorliegenden Daten jedoch nicht ausgeschlossen werden. In Folgeexperimenten soll nun geklärt werden, welcher Probentyp (angesäuert/neutral) einer Toxizität im Umweltkontext am Nächsten kommt.

Die Probenfiltration ist für etablierte Vorteile, wie die Reduktion von Schwebstoffen, Mikroorganismen oder des Verstopfens von SPE-Kartuschen, sowie die Stabilisierung bestimmter bioaktiver Verbindungen bekannt (Baker und Kasprzyk-Hordern 2011, Gehrmann et al. 2016, Janex-Habibi et al. 2009). In der vorliegenden Arbeit wurden daher mögliche Auswirkungen der Filtration (Glasfaserfilter mit einer

Porengröße von 1 µm) verglichen, wobei die meisten In-vitro-Effekte unverändert blieben. Mehrfach zeigten gefilterte Proben jedoch auch signifikante Aktivitätsverluste an, welche Schwebstoff-gebundenen Verbindungen zugeschrieben wurden. Ferner wurden in bestimmten Fällen höhere endokrine Aktivitäten im Filtrat nachgewiesen, was ein verschobenes Verhältnis von Agonisten und Antagonisten als Ursache haben könnte (Ihara et al. 2014, Rao et al. 2014).

Derzeitige SPE-Methoden sind vor allem für die chemische Analytik ausgelegt (z.B. Escher et al. 2005, Prasse et al. 2015), während der Anreicherung der Toxizität weit weniger Aufmerksamkeit gewidmet wurde (Bistan et al. 2012, Stalter et al. 2016, Wagner und Oehlmann 2010, Neale et al. 2018). Dies ist jedoch entscheidend für die akkurate und repräsentative Bewertung und das Monitoring von Mikroschadstoffen. Da letztere meist in niedrigen Umweltkonzentrationen auftreten, ist eine Probenanreicherung unter anderem erforderlich, um Bestimmungsgrenzen von Bioassays zu überwinden. Darüber hinaus führen SPE-Methoden zu einem Ausschluss von Verunreinigungen aus Umweltmatrizen, was das Risiko experimenteller Artefakte weiter reduziert (Macova et al. 2010, Neale et al. 2015, Prasse et al. 2015). Die vorliegende Optimierungsstudie untersuchte drei SPE-Sorbentien (Oasis HLB, Supelco ENVI-Carb+, Telos C18/ENV) und zwei pH-Werte zur Probenextraktion (pH 7 und 2,5). Für die meisten Endpunkte lieferte der Telos C18/ENV Sorbent, gefolgt vom Oasis HLB Sorbent, die besten Ergebnisse. Einige Ausnahmen wurden ebenfalls deutlich, wie der Supelco ENVI-Carb+ Sorbent zum Extrahieren von ozoniertem Abwasser (pH 7). Die höchste Zytotoxizität, welche in der Regel die Gesamt-Schadstoffbelastung einer Probe widerspiegelt, wurde bei einem Proben-pH von 7 extrahiert. Allerdings erleichterte ein pH von 2,5 den Nachweis der meisten endokrinen Aktivitäten, wodurch eine Probenverdünnung überflüssig wird. Die optimierte SPE-Methode wurde anschließend in einem Bewertungskonzept zur Abwasserqualität eingesetzt (Ternes et al. 2017).

Das Umweltmonitoring von Mikroschadstoffen und möglicher Effekte sind eine wesentliche ökotoxikologische Aufgabe. In-vitro-Bioassays sind wichtige Werkzeuge in diesem Prozess, da sie In-vivo-Methoden und Untersuchungen im Freiland ergänzen können. Im Rahmen dieser Arbeit wurde ein langfristiges In-vitro-Monitoring (2012−2015) in einer Wasserschutzregion in Südwestdeutschland durchgeführt. Über 30 Probenahmestellen, welche verschiedene Zonen des

Wasserkreislaufs widerspiegeln, wurden untersucht. Signifikante und wiederholt auftretende Effekte erlaubten die Klassifizierung und Priorisierung potenzieller Hotspots sowie der Leistung von Kläranlagen. Während in Oberflächengewässern und Grundwasser keine bis minimale Kontaminationen festgestellt wurden, wurden starke Effekte in Krankenhaus-Abwässern und im Rohabwasser gefunden (hauptsächlich östrogene und antiöstrogene, sowie in geringerem Maße androgene Aktivitäten). Die meisten Kläranlagen, darunter mehrere in Wasserschutzzonen, reduzierten diese Effekte auf ein unbedenkliches Niveau (z.B. Langenau, Halzhausen, Steinhäule). Die Reduktion dieser Aktivitäten während des Belebtschlammverfahrens wurde in anderen Studien beobachtet (z.B. Allinson et al. 2011, EC 2012, Sawada et al. 2012). Dies gilt ebenso für die weniger effektiv entfernte antiöstrogene Aktivität (Ihara et al. 2014, Rao et al. 2014).

Einer der Grundwasserhotspots wies wiederholt hohe antiöstrogene Aktivitäten auf. Aufgrund der Trinkwasser-Relevanz dieses Umweltkompartiments wurde ein zusätzliches Monitoring an verschiedenen Probenahmestellen in unmittelbarer Nähe des Hotspots durchgeführt. Diese Standorte umfassten mehrere Grundwasser-entnahmestellen, Brunnen und eine Probe eines Trinkwasserauslasses aus einem nahegelegenen Wasserwerk. Darüber hinaus wurden die Ergebnisse früherer und einer parallelen chemischen Untersuchung in der Modellregion zum Hotspot berücksichtigt (Anna Bollmann unveröffentlichte Ergebnisse, Seitz und Winzenbacher 2017). Das erweiterte Monitoring zeigte, dass sich alle Standorte hinsichtlich der untersuchten In-vitro-Aktivitäten und Toxizitäten unauffällig verhielten. Dieses Ergebnis stimmte mit dem der chemischen Analytik überein. Das fortgesetzte Monitoring der Antiöstrogenität im Grundwasserhotspot und die Untersuchung möglicher Ursachen (z.B. natürliche oder anthropogene Chemikalien) sollte aufgrund der erhöhten Relevanz dieses Umweltkompartiments in Betracht gezogen werden.

Es könnte ebenso von Vorteil sein, Folgeuntersuchungen zur Antiöstrogenität und Antiandrogenität im Krankenhausabwasser durchzuführen, denn das beprobte Krankenhaus (www.uniklinik-ulm.de) könnte unter anderem antiöstrogene (Hu et al. 1993, Knoop et al. 2018, Li et al. 2008, Sohoni und Sumpter 1998) und antiandrogene (Gordon et al. 2017, Grover et al. 2011, Kusk et al. 2011, Sohoni und Sumpter 1998) Chemotherapeutika und andere Arzneimittel in eine

angeschlossene Kläranlage emittieren. Allerdings wurden in dieser Kläranlage keine erhöhte Antiöstrogenität oder Antiandrogenität detektiert.

Die erhöhte Genotoxizität und Mutagenität des Krankenhausabwassers rät ferner zu einer ereignisorientierten Beprobung im Hinblick auf einen überdurchschnittlich hohen Eintrag während Starkregenereignissen. Für derartige Umweltmonitorings und Folgeuntersuchungen bietet die verwendete Batterie an In-vitro-Bioassays eine geeignete Methodik, die unter anderem mit der Effekt-dirigierten-Analyse kombiniert werden kann (z. B. Burgess et al. 2013, Brack et al. 2017).

In-vivo-Bioassays werden im Allgemeinen für ökotoxikologische Fragestellungen verwendet, welche den gesamten Organismus betreffen. Im vorliegenden Fall wurde der biologische Modellorganismus *Caenorhabditis elegans* (*C. elegans*, z.B. Chalfie 2009, Hirschmann 1952, Maupas 1899, Weinhouse et al. 2018) als vielversprechendes Werkzeug zur Charakterisierung der Qualität von Wasser- und Abwasserproben untersucht. Die umwelt- und human-relevanten Endpunkte Reproduktion und Wachstum (Allard et al. 2013, Harlow et al. 2016, Williams et al. 2017) wurden dabei mit einem Biomarkeransatz kombiniert (rekombinanter *cyp-35A3*::GFP-Stamm, Menzel et al. 2007), um mögliche Entwicklungs- und Reproduktionstoxizität (DART) bzw. Cytochrom P450 (Isoform 35A3) induzierenden Verbindungen in diesen Proben zu detektieren. Zu diesem Zweck wurde ein etablierter Nematoden-Bioassay, das International Standard Organization (ISO)-Protokoll 10872 (ISO 1996), adaptiert. Neben der standardisierten und regelmäßigen Anwendung dieses Assays in der Umweltforschung (z.B. Höss et al. 2012), zeigten verschiedene Mikroverunreinigungen DART in *C. elegans* bei Konzentrationen im unteren Mikrogramm pro Liter Bereich (z.B. Haegerbäumer et al. 2017, Li et 2016, Zhuang et al. 2014). Diese Studien verdeutlichen die Durchführbarkeit und Sensitivität von *C. elegans* als ökotoxikologisches Testsystem.

Die vorliegende Arbeit befasst sich mit dem polyzyklischen aromatischen Kohlenwasserstoff β-Naphthoflavon (β-NF), welches als Referenzsubstanz eingesetzt wurde (Leung et al. 2010, Menzel et al. 2001, 2007) sowie mit Abwasserproben aus konventionell-biologischer und weiterführender Abwasserbehandlung (vergleiche zuvor genannte TransRisk-Technologien). Für

das Abwasser-bürtige β-NF (Abdel-Shafy und Mansour 2016, Forsgren 2015) wurde mit dem weiterentwickelten Bioassay eine Median-Effektkonzentration (EC_{50}) für die Brutgröße (96 h) von 114 µg/L berechnet. Mit Hilfe dieses Bioassays wurden anschliessend Experimente mit wässrigen und extrahierten β-NF-dotierten Oberflächenwasser- und Abwasserproben durchgeführt. Diese Experimente bestätigten die Durchführbarkeit und Sensitivität des angepassten Testsystems. Sie wiesen ferner auf mögliche kumulative Effekte mit anderen Probenbestandteilen und einen Einfluss von Schwebstoffen (> 1 µm) auf die Reproduktionstoxizität von β-NF hin (aufgrund einer möglichen Partikelassoziation). Signifikante DART wurde zudem für einen biologisch-behandelten Kläranlagenablauf beobachtet (4−20x SPE-Extrakte, 96 h). Für die Ozonung und Ozonung in Kombination mit GAC- oder Biofiltration wurde keine Reproduktionstoxizität nachgewiesen (einschließlich des entsprechenden Kläranlagenablaufs). Drei extrahierte Kläranlagenabläufe sowie drei aus vier GAC- bzw. biologischen Nachfiltrationssysteme zeigten darüber hinaus einen signifikanten Wachstumseffekt auf *C. elegans*-Larven (96 h). Mögliche natürliche (vergleiche Höss et al. 2001) bzw. anthropogene (vergleiche Höss und Weltje 2007) Verursacher dieses Effekts wurden nicht untersucht.

Mit dem Biomarker-Ansatz konnten verschiedene ökotoxikologisch-relevante Beobachtungen gemacht werden. *cyp-35A3*::GFP wurde durch β-NF in Abhängigkeit der Expositionskonzentration und -Dauer (0,01−5 mg β-NF / L, 1−48 h) sowie des Gewebes und des Entwicklungsstadiums (intestinale Expression unmittelbar nach dem Schlüpfen der Eier) induziert. Die höchsten Expressionsraten wurden nach 8 h Exposition gegenüber 1−5 mg β-NF / L (21,3−24 fach über der Kontrolle) mit einem EC_{50}-Wert von 71,5 µg/L beobachtet. Der zuvor vermutete Einfluss von Schwebstoffen auf die Bioverfügbarkeit von β-NF im dotierten Abwasser konnte in diesen Experimenten bestätigt werden. Zusätzlich wurde *cyp-35A3*::GFP signifikant durch einen extrahierten Kläranlagenablauf und dazugehörigen ozonisierten Kläranlagenablauf induziert, während letzterer den Biomarker in höherem Maße induzierte. Es wurde daher angenommen, dass beide Proben *cyp-35A3*-induzierende Verbindungen enthielten, welche nach deren Reaktion mit Ozon potenziert und/oder bioverfügbarer wurden. Nachfolgende Untersuchungen sollen klären, ob dieser Effekt durch die GAC- und Biofiltrationssysteme eliminiert wird.

Eine parallele chemische Analyse, die mehrere bekannte Effektoren auf die Reproduktion und das Wachstum von *C. elegans* integrierte (z.B. Allard et al. 2013, Boyd et al. 2010, Liu et al. 2013), zeigte keine Korrelation zu den detektierten Effekten. Dieser Umstand legt nahe, zukünftige begleitende chemische Analysen hinsichtlich bekannter und zu erwartender Effektoren anzupassen. Diese Anpassung wird der Frage nach der Identität möglicher Verursacher der Effekte dienlich sein. Die erzielten Ergebnisse bestätigten frühere Untersuchungen zur Eignung und Sensitivität von *C. elegans* für die Untersuchung von (Mikro-)Schadstoffen und Umweltproben (z.B. Hitchcock et al. 1997, 1998, McLaggan et al. 2012, Roh et al. 2007, Wang et al. 2010, 2015, Xiao et al. 2018, Xiong et al. 2017, Yu et al. 2020). Frühere Untersuchungen an *C. elegans*, welche meist mehrere Endpunkte auf verschiedenen Ebenen der biologischen Organisation miteinbezogen, waren zudem Teil von Hochdurchsatz-Screenings (z.B. Boyd et al. 2010, Lundby et al. 2016), Umweltrisikobewertungen höherer Ebenen (z.B. Hägerbäumer et al. 2015, Haegerbäumer et al. 2017, Wilson und Khakouli-Duarte 2009), sowie der Konzeption von Adverse Outcome Pathways (Jager und Ashauer 2018). Perspektivisch werden diese Unterfangen zu einem besseren Verständnis der Toxizitätsmechanismen und Umweltrelevanz anthropogener (Mikro-)Schadstoffe beitragen.

Abschliesend wurde in dieser Arbeit eine Bewertung der Abwasserqualität im Rahmen eines in TransRisk entwickelten integrierten Bewertungskonzepts durchgeführt (Ternes et al. 2017). Dieses Konzept wurde in einer Pilot-Kläranlage mit den weiterführenden Abwasserbehandlungs-Verfahren Ozonung und Ozonung in Verbindung mit GAC- oder biologischer Nachfiltration (jeweils als belüfteter oder unbelüfteter Filter) etabliert. Die Ozonung im Pilotmaßstab wurde mit konventionell-behandeltem Abwasser (Belebtschlammverfahren) aus einer kommunalen Kläranlage gespeist. Der ökotoxikologische Teil der Bewertung umfasste die beschriebene und optimierte SPE-Methode (Abbas et al. 2019), ausgewählte In-vitro-Bioassays (siehe oben), sowie verschiedene In-vivo-Testsysteme (Schneider et al. 2020, Schlüter-Vorberg et al. 2017). Die Auswahl an In-vivo-Bioassays, die hauptsächlich als Durchfluss-On-site-Systeme installiert wurden, erwiesen sich als nützliches und sensitives Werkzeug in früheren Kläranlagenuntersuchungen (z.B.

da Costa et al. 2014, Gartiser et al. 2010, Giebner et al. 2018, Magdeburg ua 2012, 2014, Maltby et al. 2000, Stalter et al. 2010, 2011).

Ein besonderes Ziel dieser Arbeit war es zu vergleichen, ob der berichtete Nutzen des entwickleten *C. elegans* Bioassays auf die Untersuchung verschieden behandelter Abwässer aus einer Pilotkläranlage übertragen werden kann und diese Ergebnisse für ein integriertes Bewertungskonzept herangezogen werden können. Die Ergebnisse von fünf In-vitro-Bioassays zur Untersuchung von In-vitro-(Anti-)Östrogenität, -(Anti-)Androgenität und Mutagenität, sowie der populationsrelevante Endpunkt Reproduktion von *C. elegans,* wurden hierbei in eine "Bewertungsmatrix der Abwasserqualität" integriert, um die effektivste weiterführende Abwasserbehandlung auszumachen. In der berechneten Matrix (Daten von April−Mai 2014, durchschnittliche Ozondosis: 1 g $O_{3,applied}$ / g DOC, HRT ~ 18 min, 10x SPE-Extrakte aus 24 h-Mischproben) wurde die Ozonung in Kombination mir der unbelüfteten GAC-Filtration als effektivste der fünf Optionen ermittelt. Es konnte ferner gezeigt werden, dass östrogene und (anti-)androgene Aktivitäten zwar durch die weiterführenden Abwasserbehandlungstechnologien stark reduziert, mutagene und antiöstrogene Effekte jedoch nicht vollständig eliminiert werden konnten oder sogar erhöht vorlagen. Diese Beobachtung könnte mit früheren Hypothesen zur Erzeugung toxischer Transformationsprodukten übereinstimmen (da Costa et al. 2014, Jia et al. 2015, Knoop et al. 2018, Magdeburg et al. 2014, Stalter et al. 2010). Geringfügig-nachteilige Auswirkungen der Ozonung auf die Reproduktion von *C. elegans* (11,7% verringerte Brutgröße im Vergleich zum Kläranlagenablauf) traten in beiden GAC-gefilterten Proben nicht auf. Beide Proben die der Biofiltration entnommen wurden, zeigten wie zuvor festgestellt einen wachstumsfördernden Effekt (12,5 bzw. 15,1% Zunahme).

In Übereinstimmung mit diesen (und früheren) mit *C. elegans* erzielten Ergebnissen, erscheint die weitere Etablierung dieses Nematoden als vielseitiger und sensitiver Modellorganismus für ökotoxikologische Zwecke vielversprechend (einschliessnlich Wasser- und Abwasser-bezogener Untersuchungen). *C. elegans* könnte hierbei zur Erforschung zusätzlicher bzw. alternativer Testsysteme mit erhöhter Sensitivität und/oder Umweltrelevanz maßgeblich beitragen, wie sie in der wissenschaftlichen Literatur empfohlen wurden (Berger et al. 2016, Schwarzenbach et al. 2006, Völker et al. 2017, Wigh et al. 2016, 2017, 2018, Xiong et al. 2017).

Im Allgemeinen erwies sich die Kombination von In-vitro- und (vorzugsweise On-site-)In-vivo-Testsystemen als geeigneter Ansatz für die Bewertung der Abwasserqualität. Idealerweise sollten diese Bewertungen mit einem langfristigen Umweltmonitoring an Vorflutern und betroffenen Gewässern einhergehen (z.B. Auswirkungen auf Indikatortaxa) sowie ferner sozialpolitische Maßnahmen zur Verringerung relevanter Mikroschadstoffe berücksichtigen (z.B. Source control, European Parlament and Council 2013, Lienert et al. 2011, Reichenberger et al. 2007). Ergänzt werden sollten diese Bemühungen mit der Untersuchung weiterer Optimierungspotenziale im Zusammenhang mit der Abwasser-Kanalisation, konventionell-biologischen, tertiären Behandlungen (z.B. Musolff et al. 2010, Phillips et al. 2012, Triebskorn 2017, Völker et al. 2016) und anderer weiterführenden Abwasserbehandlungen (z.B. Barbosa et al. 2016, Becker et al. 2017, Tchobanoglous and Burton 1991, van Hege et al. 2002). Diese Strategien sollten idealerweise die Gesamtheit relevanter Stressoren aquatischer Ökosysteme berücksichtigen (z.B. BMU 2013, EEA 2012, Philip et al. 2011).

A.5 Acknowledgement

Included in print version / not included in online version.

A.6 CV

Included in print version / not included in online version.

A.7 Publication list

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