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Sample preparation and ecotoxicological assessment of conventional and advanced wastewater treatments with *in vitro* and *in vivo* bioassays

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Table of contents

Abbreviations

µg	microgram
a	aerated
AhR	aryl-hydrocarbon receptor
Ames	bacterial reverse test assessing mutations in the genome
AOC	assimilable organic carbon
AR	androgen receptor
AWWT	advanced wastewater treatment
BAC	biological activated carbon
BDOC	biodegradable dissolved organic carbon
BF	biofilter
BMBF	Federal Ministry of Education and Research (German: Bundesministerium für Bildung und Forschung)
BOD	biological oxygen demand
BPA	bisphenol A
BT	biological treatment
C	control
CALUX	chemically activated luciferase gene expression
CAS	conventional activated sludge
C. carpio	*Cyprinus carpio* (common carp)
C. dubia	*Ceriodaphnia dubia* (water flea)
CHO-9	Chinese hamster ovary cell line
CO_2	carbon dioxide
COD	chemical oxygen demand
COFA	N-(4-carbamoyl-2-imino-5-oxo-imidazolidin)-formamido-N-methoxyacetetic acid (oxidative TP of carboxy-acyclovir)
Cr	chrome
C. riparius	*Chironomus riparius* (non-biting midge)
DEET	*N,N*-diethyl-*m*-toluamide
DEHP	di-2-ethylhexyl phthalate
DEP	diethyl phthalate
D. magna	*Daphnia magna* (large water flea)

DMS	*N,N*-dimethylsulfamide
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DOC	dissolved organic carbon
DOM	dissolved organic matter
D. polymorpha	*Dreissena polymorpha* (zebra mussel)
D. rerio	*Danio rerio* (zebrafish)
D. subspicatus	*Desmodesmus subspicatus* (green algae)
E_1	estrone
E_2	17β-estradiol
EC	European Commission
EDC	endocrine disrupting chemical
EE_2	17α-ethinylestradiol
e.g.	for example (Latin: exempli gratia)
ELISA	enzyme-linked immunosorbent assay
EOAs	environmental organic acids
(h) ER (α)	(human) estrogen receptor (α)
EU	European Union
EU WFD	European Water Framework Directive
Fe	iron (Latin: *ferrum*)
FELST	fish early life stage test
FKZ	support code (German: Förderkennzeichen)
GAC	granular activated carbon
GC	gas chromatography
G. fossarum/pulex	*Gammarus fossarum/pulex* (amphipods)
GH3	rat pituitary cell line
h	hour
HepaRG	human liver carcinoma cell line
HepG2	human hepatocyte cell line
HPLC	high performance liquid chromatography
HRT	hydraulic retention (residence) time
L	litre
LC	liquid chromatography

L. minor	*LemnaLemna minor* (common duckweed)
L. variegatus	*Lumbriculus variegatus* (annelid)
m	metre
m^3	cubic metre
MBR	membrane bioreactor
medERα	estrogen receptor α from the medaka (*Oryzias latipes*)
MEHP	mono-(2-ethylhexyl) phthalate
MEP	monoethyl phthalate
mg	milligram
mm	millimetre
MP	micropollutant
MS	mass spectrometry
n.a.	no activity
NaWaM	sustainable water management (German: Nachhaltiges Wassermanagement)
NC	negative control
n.c.	not calculable
NDMA	*N*-Nitrosodimethylamine
NF	nanofiltration
ng	nanogram
(N-)NH_3	ammonia
NH_4^+	ammonium
(N-)NO_2^-	nitrite
n.s.	not significant
O_3	ozone
OECD	Organisation for Economic Co-operation and Development
OH	hydroxy/hydroxyl
O. latipes	*Oryzias latipes* (Japanese medaka)
O. mykiss	*Oncorhynchus mykiss* (rainbow trout)
O. niloticus	*Oreochromis niloticus* (Nile Tilapia)
PAC	powder activated carbon
PAH	polycyclic aromatic hydrocarbon
P. antipodarum	*Potamopyrgus antipodarum* (mudsnail)
Pb	lead (Latin: *plumbum*)

PC	positive control
PCB	polychlorinated biphenyl
PFASs	perfluoralkyl substances
PFOA	perfluorooctanoic acid
PFOS	perfluorooctansulfonic acid
pg	picogram
pH	Latin: *pondus* /*potentia hydrogenii*
PPCPs	pharmaceuticals and personal care products
P. promelas	*Pimephales promelas* (fathead minnow)
P. subcapitata	*Pseudokirchneriella subcapitata* (green algae)
PT	primary treatment
PTFE	polytetrafluoroethylene
RAR	retinoic acid receptor
RiSKWa	risk management of new pollutants and pathogens in the water cycle (German: Risiko von neuen Schadstoffen und Krankheitserregern im Wasserkreislauf)
RO	reversed osmosis
RXR	retinoic X receptor
S. cerevisiae	*Saccharomyces cerevisiae* (yeast)
SEM	standard error of the mean
SF	sand filtration
SPE	solid phase extraction
SPM	suspended particular matter
TP	transformation product
TR	thyroid receptor
TSS	total suspended solids
UF	ultrafiltration
umuC	bacterial reverse test assessing genotoxicity
UV	ultraviolet
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
YG7108	*Salmonella typhimurium* YG7108 Ames strain
Zn	zinc

List of figures

List of tables

Abstract

In almost all parts of the world the industrialisation grows continuously and thus, the chemical pollution of natural waters has become a major public concern. A major consequence and one of the key environmental problems we are facing today is the increasing contamination of freshwater systems with chemicals. The chemicals are detected in wastewater, surface (river) water, ground water and drinking water ubiquitously in natural waters and not only in industrialised areas. The main point sources for water pollution and the release of these synthetic organic substances of human origin, so called micropollutants (MPs), are wastewater treatment plants (WWTPs). These MPs such as pharmaceuticals, personal care products, disinfectant chemicals, chemicals used in the industry and in households, contraceptives, hormones, food additives, artificial sweeteners, pesticides, biocides, and many emerging contaminants are only incompletely removed by the existing conventional wastewater treatment technologies. The MPs end up in the water cycle and have adverse effects on wildlife aquatic ecosystems and human health even at very low concentrations. Therefore, advanced wastewater treatment (AWWT) technologies, such as ozonation, treatment with activated carbon, biofiltration, membrane bioreactors (MBRs) or exposure to ultraviolet light are investigated as options to upgrade conventional WWTPs. However, several studies show that especially the ozonation of wastewater generates diverse transformation products (TPs) with unknown properties. These TPs could be more toxic than the mother compound. Thus, a post-treatment after the ozonation process is required.

The present thesis was part of the BMBF-funded TransRisk project dealing with “the characterisation, communication, and minimisation of risks of emerging pollutants and pathogens in the water cycle”. One main objective was the investigation of

conventional treated wastewater after a full-scale ozonation with four post-treatments (each non-aerated and aerated granular activated carbon (GAC) filtration and biofiltration) in comparison to a MBR treatment of raw (untreated) wastewater separately and in combination with an additional ozonation on a pilot WWTP. For this purpose, the wastewater samples were characterised with a comprehensive battery of *in vitro* and *in vivo* bioassays. The *in vitro* bioassays were performed to detect endocrine activities (such as (anti)estrogenic and (anti)androgenic activities), genotoxicity, and mutagenicity. The results showed a decreased estrogenic activity due to the conventional wastewater treatment as well as the ozonation, but a distinct increase of the anti-estrogenic activity and the mutagenicity in the ozonated wastewater, possibly caused by new formed TPs, that were reduced after the post-treatments whereas the GAC filtration performed better than the biofiltration. The *in vivo* bioassays included for example the impact of the wastewater on mortality, reproduction, development, and energy reserves of the test organisms. The *in vivo* on-site tests with the mudsnail *Potamopyrgus antipodarum* and with the amphipod *Gammarus fossarum* indicated a major impact of conventional treated wastewater, ozonated wastewater, and MBR treated wastewater. The flow channel experiments in the laboratory with *Gammarus pulex* pointed to a serious impact of an estrogenic effluent on life-history traits of the amphipod. Finally, an ozonation of the wastewater with subsequent GAC filtration represented the most promising option. In addition, chemical analyses of 40 selected MPs, so called tracer substances, performed in parallel to the *in vitro* and *in vivo* bioassays underlined this assumption.

A second main objective was the optimisation of the preparation of water and wastewater samples for ecotoxicological *in vitro* bioassays because common sample preparation techniques are predominantly adapted for chemical analyses. Therefore, the impact of sample filtration, long-term acidification with following neutralisation as

well as the enrichment with solid phase extraction (SPE) in combination with short-term acidification were investigated using amongst others raw (untreated) wastewater, hospital wastewater, conventional treated and ozonated wastewater, surface water, and ground water. Overall, eleven *in vitro* bioassays were performed for the detection of endocrine activities, genotoxicity, and mutagenicity. The results show that sample filtration and acidification/neutralisation significantly affected the outcome of the bioassays especially the anti-estrogenic activity and the mutagenicity whereas the sample filtration had a minor impact than the acidification. Thus, the testing of untreated (waste)water samples is advisable because the sample is minimally processed. Furthermore, the SPE extracts showed in parts high cytotoxic effects whereby no conclusions on the results of the bioassays were possible. However, the enrichment of endocrine activity and mutagenicity was predominantly effective but depended on the used SPE cartridge and the pH value of the (waste)water samples. Based on the results the use of a Telos C18/ENV cartridge and an acidified sample is recommendable. In the end, there is a need to optimise the sample preparation for *in vitro* bioassays to reach their maximum outcome for the best possible assessment of the water quality.

1 General introduction

1.1 Micropollutants in the aquatic environment

The increasing contamination of freshwater systems with chemicals is one of the key environmental problems we are facing today. The chemical pollution of natural waters has become a major public concern in almost all parts of the world (Wilkinson et al. 2022; Jiang et al. 2013; Boxall et al. 2012) because chemicals are found not only in industrialised areas but ubiquitously in natural waters (Altmann et al. 2016, 2014; Loos et al. 2009). Micropollutants (MPs) are synthetic organic substances of human origin that are detected in the aquatic environment across the European Union for example in wastewater, surface (river) water, ground water, and drinking water (Dopp et al. 2021; Rüdel et al. 2020; Arp & Hale 2019; Loos et al. 2013, 2010, 2009; Reemtsma et al. 2006). These emerging contaminants have proven or potential adverse effects and largely unknown long-term effects on aquatic ecosystems and human health (Tran et al 2018; Loos et al. 2009) and they bear the risk of unintended harmful effects on non-target organisms in the (aquatic) environment and on humans (Altmann et al. 2016; Reaume et al. 2015; Maletz et al. 2013; Hernandez-Leal et al. 2011). MPs generated by human activities are for example personal care products (ultraviolet (UV)-filter and flavours), pharmaceuticals, disinfectant chemicals, chemicals used in the industry and in households (for example plasticisers), contraceptives, hormones, food additives, artificial sweeteners, pesticides, biocides (fungicides and insecticides), and many emerging contaminants are removed incompletely by existing wastewater treatment technologies (Tran et al. 2018; Seitz & Winzenbacher 2017; Knopp et al. 2016; Loos et al. 2013, 2010, 2009; Boehler et al. 2012). Furthermore, the better part of these MPs are endocrine disruptors (Hernandez-Leal et al. 2011). The occurrence of endocrine

disrupting chemicals (EDCs) for example increased rapidly worldwide in recent decades and they are frequently found in effluents of wastewater treatment plants (WWTPs) (Bertanza et al. 2011; Mnif et al. 2010). Endocrine effects, attributed to the release of the synthetic estrogen 17α-ethinylestradiol (EE_2), natural estrogens (estrone (E_1) and 17β-estradiol (E_2)) or nonylphenol, on mussels and fish such as intersex, reproductive disruption or feminisation of males have been observed in rivers downstream of municipal WWTPs (Margot et al. 2013). In the European Union there are more than 100,000 registered chemicals and 30,000-70,000 are in daily use (Brack et al. 2018; Dulio et al. 2018; Oehlmann et al. 2014; Loos et al. 2009). Because analytical analyses become more and more sensitive these anthropogenic MPs can be detected in the aquatic environment in the range of microgram per litre to nanogram per litre even in tap water after drinking water treatment because rivers and lakes are used in many places for drinking water supply (Knopp & Cornel 2015; Margot et al. 2013). MPs differ in many of their properties for example regarding polarity, solubility or molecular size. There are two main pathways on which MPs end up in the environment. In industrialised countries more than 90% of the wastewater is treated in centralised urban, industrial or hospital WWTPs. Those WWTPs represent main point sources for water pollution and the release of these MPs into the aquatic ecosystems (Dopp et al. 2021; Bertanza et al. 2011; Hollender et al. 2009) because the degradation and mineralisation of most of the MPs in the conventional treatment is incomplete (Enns et al. 2023). Thus, MPs could have negative effects on aquatic biocoenosis in spite of low concentrations. Consequently, they become an increasing threat to aquatic ecosystems and to the safety of drinking water resources (Enns et al. 2023; Altmann et al. 2016; Knopp & Cornel 2015; Margot et al. 2013; Boehler et al. 2012; Loos et al. 2009). Pharmaceuticals, like the synthetic hormone EE_2, are for example of particular

toxicological concern because they were designed to exert their biological activity at low concentrations (Maletz et al. 2013). However, organic chemicals were detected in the influents and effluents of WTTPs in concentrations ranging from pg/L to the lower ng/L level to several µg/L and, in specific cases, even mg/L and there is concern regarding the individual or mixture low level long-term exposure of these chemicals and the potential adverse health effects on wildlife and humans (Dopp et al. 2021; Seitz & Winzenbacher 2017; Reaume et al. 2015; Altmann et al. 2014; Loos et al. 2013; Margot et al. 2013; Hernandez-Leal et al. 2011, 2010; Hollender et al. 2009; Scheurer et al. 2009; Reemtsma et al. 2006).

Reungoat et al. (2012) detected trace organic chemicals in conventional treated wastewater of three full-scale wastewater reclamation plants with concentration varying from low ng/L up to µg/L levels that showed the incomplete removal of these compounds in WWTPs. Furthermore, the authors note that the concentrations of most of the organic chemicals remained in the same order of magnitude across three different WWTPs in spite of different locations and sampling times. On the one hand these results show how ubiquitously distributed these compounds are in treated WWTP effluents and on the other hand the pattern of a regular consumption in the area around the investigated WWTPs. But even if chemicals occur in pg/L ranges in natural waters they are of relevant (eco)toxicological concern only on the basis of the huge amount of MPs and the difficulty to assess the effects on the aquatic environment when they are present in complex mixtures (Loos et al. 2009, Schwarzenbach et al. 2006). In addition to WTTPs as main point sources, a main diffuse source of MPs is agriculture for example fertilisation of fields with manure that could for example contain veterinary pharmaceuticals, livestock breeding or the use of pesticides (Knopp & Cornel 2015; Altmann et al. 2014; Maletz et al. 2013).

However, priority substances or other organic compounds are not regulated for WWTP effluents, but for surface waters the European Parliament and the Council established a framework for Community action in the field of water policy, the European Water Framework Directive (EU WFD) 2000/60/EC (Loos et al. 2013; Bertanza et al. 2011; EU Directive 2000/60/EC). Thus, for the further improvement of the quality of European water bodies the EU WFD induces an overall policy with respect to hazardous substances to achieve a reliable chemical and biological/ecological status and an overall “good water status” for all European surface waters (Dopp et al. 2021; Itzel et al. 2017; Loos et al. 2009). The first list of priority substances (Annex X to the EU WFD 2000/60/EC) was established in Decision 2455/2001/EC. This first list was replaced and amended in 2008 (EU Directive 2008/105/EC on the protection of groundwater against pollution and deterioration, Annex II) also setting environmental quality standards for the substances in surface water and included 33 (groups of) compounds (Wenzel et al. 2008). In addition, a number of 11 substances were listed as subjects to review for the possible identification as priority (hazardous) substances. The list of priority substances was again amended and replaced in 2013 (EU Directive 2013/39/EU) on environmental quality standards in the field of water policy, Annex I) and already included 45 (groups of) substances. These updates enhance the list with substances for which monitoring is difficult or needs to be intensified for the purpose of the support of risk assessment and the identification of new priority substances such as EDCs, macrolide antibiotics and neonicotinoids (Dopp et al. 2021; Itzel et al. 2017). Therefore, urban conventional WWTPs should be upgraded with advanced wastewater treatment (AWWT) technologies for example ozonation, treatment with activated carbon, biofiltration (BF), membrane bioreactors (MBRs) or exposure to UV-light as stand-alone systems or a combination of these techniques (Bertanza et al. 2011). But the obligations set for the EU are not equally fulfilled by all its Member States

(Reemtsma et al. 2006). For instance, some Member States still ignore the fact that wastewater which has not been properly treated will be carried by the river basin and cause pollution in downstream river sections or marine waters. Therefore, these Member States have not provided the necessary measures to tackle the problem of water pollution for a large number of agglomerations. Furthermore, the Member States also have underestimated the necessary treatment requirements for large cities such as London, Paris, Madrid, Milan, and others. Another example is the identification of sensitive areas and the improvements in terms of the wastewater infrastructure in these sensitive areas and their catchments. Sensitive areas require specific water protection for reasons such as eutrophication but also bathing water zones. However, a high number of areas considered to suffer from eutrophication still have not been identified by the Member States. Several Member States still discharge 58% of their wastewater into sensitive areas without receiving a sufficient treatment. Thus, only about 42% of the agglomerations discharging into sensitive areas provided the required more stringent wastewater treatment. In this context, the total nitrogen concentrations in European rivers, reflecting the nitrogen impact by agriculture as well as the still insufficient nitrogen removal by WWTPs, have remained high despite the efforts to reduce the nitrogen from urban wastewater (Commission of the European Communities 2004).

1.2 Removal of micropollutants during conventional wastewater treatment

The focus of the conventional municipal wastewater treatment was for a long time the removal of organic matter (for example carbon), nutrients (for example nitrogen and phosphorus), pathogens, and coliforms (Loos et al. 2013, Wenzel et al. 2008). The

main component of the biological treatment (BT) is the biotransformation and biodegradation of organic components. For this purpose, these organic components in the wastewater are converted by different biochemical reactions, for example oxidation, reduction or hydrolysis (Knopp & Cornel 2015). In addition, many non-polar chemical compounds are well removed by the sorption to sludge. Another important removal pathway of organic compounds during wastewater treatment is stripping by aeration (volatilisation). But some polar substances are poorly degradable and may be discharged with WWTP effluents into receiving waters and then occur in surface waters. Several polar chemicals (for example nonylphenol and perfluoroalkyl substances) are even formed from precursor compounds in WWTPs (Loos et al. 2013, Reemtsma et al. 2006). Because municipal WWTPs and hospital effluents are main point sources of pharmaceuticals in aquatic ecosystems, the degradation and removal of these substances and their metabolites becomes a more and more important aspect because of their possible adverse effects on wildlife and humans. Pharmaceuticals, for example, are designed for being biologically active at low concentrations in humans and domestic animals. However, elimination of these substances during conventional wastewater treatment is not always sufficient and unintended harmful effects on non-target organisms in the environment could be the consequence (Maletz et al. 2013).

The occurrence of up to 156 polar organic persistent pollutants in the effluent of 90 European WWTPs (treating domestic wastewater of mainly municipal origin and in part dominated by industrial wastewaters), European river waters (122 sampling stations in 27 European countries) and 164 individual ground water samples from 23 European countries was examined by Loos et al. (2009, 2010, 2013). The authors detected pharmaceuticals, pesticides, benzotriazoles, hormones, flame retardants, plasticizers, and endocrine disruptors in all kinds of investigated (waste)water.

In a conventional WWTP treating domestic wastewater, urban runoff, and wastewater of a major hospital and several clinics, more than 70 potentially problematic organic MPs (for example pesticides, pharmaceuticals, endocrine disruptors, and drug metabolites) were regularly detected at different stages of wastewater treatment. Average removals of less than 50% were found for 50 (≙ 71.4%) of the 70 detected compounds in the effluent of the BT. The average concentration of 16 compounds (≙ 22.9%) in the effluent was above 1 µg/L and even 52 compounds (≙ 74.3%) had a concentration above 100 ng/L. The most persistent MPs were amongst others the pharmaceuticals carbamazepine, clindamycin, diclofenac, and metoprolol (Margot et al. 2013). Chemical analyses of municipal and hospital wastewater performed in a study by Seitz and Winzenbacher (2017) show that wastewater from conventional WWTPs still include a plenty of MPs and their transformation products (TPs). The authors investigated 84 anthropogenic compounds belonging to diverse groups (pharmaceuticals, iodinated X-ray contrast media, sweeteners, industrial chemicals (benzotriazoles, melamines and benzothiazoles) and pesticide metabolites) in up to 20 sampling sites (untreated and treated wastewater, wastewater from hospitals, stream (surface) waters, runoff water from roads and groundwater hotspots). Some chemicals are not degraded at all or only very slow like persistent organic pollutants or pharmaceuticals such as carbamazepine and sulfamethoxazole (Schwarzenbach et al. 2006). Several MPs belonging to benzotriazoles, sweeteners, melamines and pesticide metabolites were already detected in surface water and groundwater hotspots and even water suppliers have to deal with MPs contaminating drinking water (Seitz & Winzenbacher 2017; Loos et al. 2010, 2009; Scheurer et al. 2009).

In the end, various studies investigating diverse types of wastewater indicate that a plenty of MPs and their human metabolites and TPs were not completely mineralised with conventional wastewater treatment technologies exerted at the corresponding

point in time and ended up in the water cycle (Rogowska et al. 2020; Kim & Zoh 2016; Margot et al. 2015; Luo et al. 2014).

1.3 Advanced wastewater treatment: a further reduction of micropollutants and toxicity?

AWWT processes (for example ozonation or reverse osmosis) have been used for drinking water production for the removal of trace contaminants but the use of AWWT in the general wastewater treatment is not as common (Lee et al. 2012). However, in the last years the focus of the municipal wastewater treatment to remove organic matter and nutrients changed and nowadays a lot of development effort and research are investigated to AWWTs for the further removal of pathogens, MPs, (eco)toxicity, hormone effects and hazardous substances in sewage effluents that previously was a side benefit (Wenzel et al. 2008, Maletz et al. 2013). AWWT technologies, for example ozonation, treatment with activated carbon, sand filtration (SF), BF or MBRs, are necessary to reduce the release of MPs, that are present in the conventional treated wastewater, into the aquatic environment (Bui et al. 2016; Margot et al. 2013).

1.3.1 Advanced wastewater treatment technologies used in this study

The WWTPs investigated in this study include two processes: First, conventionally treated wastewater with activated sludge (CAS) of a municipal WWTP was ran through a micro sieve (diameter: 10 µm) to reduce total suspended solids (TSS) and was directed to pilot-scale AWWTs (Figure 1). The wastewater passes through a full-scale ozonation that was connected to a total of four post-treatments: both, non-aerated and aerated granular activated carbon (GAC) filtration and non-aerated and aerated BF. In the second process, raw (untreated) sedimented wastewater from the municipal

WWTP fed two stand-alone MBRs. One MBR included a second ozone system with partial recirculation (Figure 1).

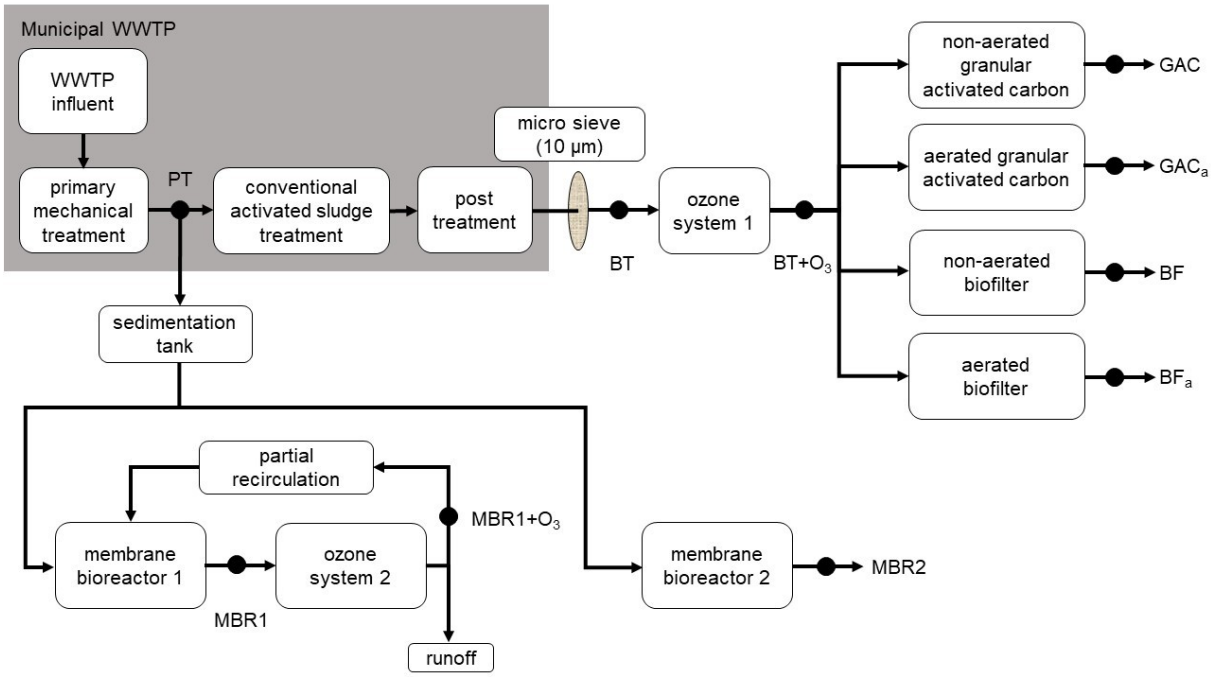

Figure 1: Process design of the municipal wastewater treatment plant (WWTP) and the pilot-scale advanced wastewater treatment (AWWT) technologies. Sampling points are marked with black dots. PT: after primary treatment, BT: after conventional biological treatment, BT+O_3: biological treatment after ozonation, 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 (Schneider et al. 2020, Annex A.2).

1.3.2 How advanced wastewater treatment technologies work

1.3.2.1 Ozonation

In water and wastewater treatment processes ozonation has been widely applied as a method for disinfection and decolouration of (waste)water and drinking water, the removal of odorous or flavouring substances or the reduction of organic parameters and other MPs (for example pharmaceuticals and endocrine disruptors) to minimize

the release of these substances into the aquatic environment (Lim et al. 2022, Altmann et al. 2014; Margot et al. 2013; Hernandez-Leal et al. 2011; Zimmermann et al. 2011; Lee & von Gunten 2010; Hollender et al. 2009). Ozonation means the oxidation of MPs using ozone (O_3) which is generated from ambient air or pure oxygen and that is one of the most potent and commonly used oxidising agents. For this purpose, the gaseous ozone is added to pre-treated wastewater (for example conventional treatment or MBR treatment) in special chemical reaction tanks (Knopp & Cornel 2015). Ozone reacts directly with the MPs as a selective oxidant through its strong oxidative properties by oxidation or indirectly after ozone decomposition and the formation of hydroxyl radicals as non-selective oxidant. Substances with aromatic rings (with double bounds), electron rich functional groups, and tertiary amines are highly reactive with molecular ozone. But as a result of the ozonation process the substances are transformed into other compounds and not completely removed from the wastewater. The identification of the reaction pathways and the new formed TPs is the objective of extensive research (Lim et al. 2022; Gulde et al. 2021; Ikehata & Li 2018; Sharma et al. 2018; Lee & von Gunten 2016, 2010; Hübner et al. 2015, 2014; Scheurer et al. 2012; Zimmermann et al. 2012; Fatta-Kassinos et al. 2011; Benner & Ternes 2009 a,b).

The ozonation system of the pilot plant investigated in the studies by Schneider et al. (2020, Annex A.2), Schlüter-Vorberg et al. (2017), and Knopp et al. (2016) consisted of two bubble columns connected in series with 3.6 m in height, 0.2 m in diameter, and a total volume of 0.113 m^3, an equalization tank and an ozone generator.

Another ozone system configurations are depicted in the studies by Dopp et al. (2021), Reaume et al. (2015), Margot et al. (2013), Lee et al. (2012), Bertanza et al. (2011), and Hollender et al. (2009).

1.3.2.2 Activated carbon

Activated carbon is a (post-)treatment technology for conventional treated wastewater as well as for advanced treated wastewater for example ozonated wastewater but also for drinking water (Altmann et al. 2016). The principle is based on a physical adsorption to a high specific surface area where a gaseous or dissolved substance adsorbs to a solid phase (Knopp & Cornel 2015; Margot et al. 2013). Activated carbon is commonly used for sorption of organic MPs like pesticides or taste or odour compounds (Serrano et al. 2011). The adsorption of a specific substance on activated carbon is determined by its chemical properties (Altmann et al. 2014). There are two forms of activated carbon available: powder activated carbon (PAC) or GAC. The addition of PAC to the wastewater in a contact-tank results in an adsorption of MPs to their surface. After this adsorption sedimentation and filtration steps follow to separate the loaded PAC from the wastewater. Afterwards the loaded PAC can be reused in the contact-tank, it can be applied to the aeration tank with following disposal together with the sewage sludge or it has to be disposed for example by incineration together with the sewage sludge (Knopp & Cornel 2015; Margot et al. 2013; Boehler et al. 2012).

However, GAC is used as a fixed bed in flow-through filter systems. For a long time, GAC has been applied as adsorbent for drinking water with low to moderate concentrations of dissolved organic carbon (DOC) and very low particle concentrations. The loading capacity of GAC depends on its particle size and decreases with increasing diameter. The adsorption kinetics improve with decreasing GAC grain size. Thus, small-sized GAC filter systems have a higher adsorption capacity than filter systems with larger GAC grain size (Altmann et al. 2016; Corwin & Summers 2010; Nowotny et al. 2007). An increasing amount of wastewater streaming through the filter system leads to a degraded performance of the filters and a reduced adsorption capacity. After the adsorption of MPs to the surface the loaded GAC can

be backwashed and thus, reactivated for further usage. The usage of fine GAC fractions as a filter medium led to short backwash intervals or a blocking of the filter (Altmann et al. 2016; Reungoat et al. 2012; Corwin & Summers 2011). A filter velocity of 4–10 m/h or rather a retention time of 10–21 minutes is sufficient to reduce the concentrations of a wide range of common MPs. A post filtration step with PAC/GAC could reduce possible negative effects of the (ozonated) wastewater (Knopp & Cornel 2015).

The non-aerated and aerated GAC filter systems of the pilot plant investigated in the studies by Schneider et al. (2020, Annex A.2), Schlüter-Vorberg et al. (2017), and Knopp et al. (2016) were designed identically. Each filter column was 4 m in total height and 0.19 m in diameter and contained two grit support layers (layer 1: height 0.14 m, diameter 6-8 mm; layer 2: height 0.16 m, diameter 2-4 mm) and a 2.08 m GAC layer. Another GAC filter system configurations are depicted in the studies by Giebner et al. (2018) and Altmann et al. (2016).

1.3.2.3 Biofiltration

BF can be used as a main conventional biological wastewater treatment or as a post-treatment step after a previous conventional or advanced treatment, for example ozonation. In contrast to the physical activated carbon treatment BF is a biological treatment. The biofilter consists of a sessile biomass (biofilm) that grows on a backing material (e.g. sand or expanded clay). BFs can be operated under non-aerated or aerated conditions or under addition of a carbon source and thus be used for specific elimination of carbon or phosphorous and they are especially suitable for nitrification and denitrification processes. Besides, BF lead to a detention of suspended particles (Rocher et al. 2012; Meda & Cornel 2010a). BFs have to be flushed regularly because

of the growing biomass and the inclusion of suspended solids. Only a few studies have been published concerning BF (Knopp & Cornel 2015).

Angelakis and Snyder (2015) published that the size of the filter media affects the performance in systems using BF. Smaller diameter of the BF resulted in a better nutrient removal efficiency. But finally, the mechanisms that are responsible for the performance of BF with non-adsorptive as well as adsorptive properties are not completely understood (Reaume et al. 2015; Reungoat et al. 2011).

Biological aerated filters combine filtration and biological processes in one reactor. These filter systems are modular and consist of several filter units. In general, more than six filter units operate in parallel. They have a high biomass content and high volumetric reaction rates (Meda & Cornel 2010a).

The non-aerated and aerated BF systems of the pilot plant investigated in the studies by Schneider et al. (2020, Annex A.2), Schlüter-Vorberg et al. (2017), and Knopp et al. (2016) were designed identically. Each filter column was 4 m in total height and 0.19 m in diameter and contained two grit support layers (layer 1: height 0.14 m, diameter 6-8 mm; layer 2: height 0.16 m, diameter 2-4 mm) and a 2.08 m expanded clay layer.

Another BF system configurations are depicted in the studies by Giebner et al. (2018), Reaume et al. (2015), Magdeburg et al. (2014), Lee et al. (2012), Reungoat et al. (2011), Meda & Cornel (2010a,b), Stalter et al. (2010a), and Hollender et al. (2009).

1.3.2.4 Membrane bioreactors

Industrial and municipal wastewater are mainly processed by CAS treatment but MBRs have become a viable alternative to CAS and is the fastest growing wastewater treatment system available. MBRs present a stand-alone technology and were installed when AWWT is needed (for example for water reuse), to treat raw wastewater (such as hospital wastewater) or when a compact system is required (Bertanza et al.

2017; Besha et al. 2017). The MBR technique is a combination of the biological sewage treatment with a membrane filtration representing a physical treatment process (Boonnorat et al. 2017). The MBR process includes a suspended growth activated sludge system that uses microporous membranes for solid or liquid separation instead of secondary clarifiers. The typical structure of a MBR system is a stirred anoxic zone where metal salt is added to the primary effluent followed by an aerobic zone that includes submerged membranes and a mixed liquor recycle for denitrification. Some MBR systems use pressure membranes rather than submerged membranes external to the bioreactor (Chapman et al. 2004). Anaerobic MBRs for example can be fitted with flat sheet, hollow fibre or tubular membranes that operate either in the micro- or ultrafiltration (UF) region whereas the use of ceramic membranes is not widely reported (Skouteris et al. 2012). Maletz et al. (2013) described MBR systems where the membranes are directly integrated into the activated sludge to guarantee a microfiltration of the biologically treated sewage. Furthermore, MBR systems could be constructed as parallel lines each treating raw wastewater and which are equipped with hollow fibre membranes or flat sheet membranes (Bertanza et al. 2017; Camacho-Muñoz et al. 2012).

Another MBR system configurations are depicted in the studies by Camacho-Muñoz et al. (2012), Lee et al. (2012), and Serrano et al. (2011).

1.3.3 Removal of micropollutants during advanced wastewater treatment

1.3.3.1 Ozonation

Ozone has been proved to be very effective in oxidising many chemicals of emerging concern in municipal WWTP effluents in general by monitoring the disappearance of the parent compound (Reaume et al. 2015). But the oxidation of these MPs is predominantly incomplete and the MPs are not fully mineralised to inorganic carbon

dioxide (CO_2) and water but only transformed to a multitude of known and unknown smaller substances so called TPs. In many cases, these newly formed TPs have much lower biological activity and in part higher polarities than the parent compounds (Itzel et al. 2019; Knopp & Cornel 2015; Lee et al. 2012; Reungoat et al. 2012, 2010; Zimmermann et al. 2011; Hollender et al. 2009). Some of these TPs are (more) bio-degradable but the chemical structure, properties, toxicity and behaviour in the environment of most of these TPs are mostly unknown and these TPs are of more concern than the parent compounds. Furthermore, TPs are expected to be formed from their reactions with other compounds in the wastewater matrix and not only from the reaction of ozone with MPs (Reaume et al. 2015; Altmann et al. 2014; Lee et al. 2012). Several studies investigated the reduction of the concentrations of common MPs due to ozonation (Schneider et al. 2020, Annex A.2; Itzel et al. 2017; Knopp et al. 2016; Knopp & Cornel 2015; Altmann et al. 2014; Maletz et al. 2013; Margot et al. 2013; Bertanza et al. 2011; Hernandez-Leal et al. 2011; Reungoat et al. 2011, 2010; Wenzel et al. 2008). Compounds including activated aromatic moieties, amine functions or double bounds showed high removal rates even at low ozone concentrations and hydraulic retention times (HRTs). The removal of more resistant MPs to oxidation by ozone increased with increasing ozone dose. However, a few MPs were persistent to a large extend. Thus, several compounds such as beta-blockers, biocides, benzotriazole, and X-ray contrast media were still found after the ozonation process (Schneider et al. 2020, Annex A.2; Magdeburg et al. 2014; Lee et al. 2012; Reungoat et al. 2012; Hollender et al. 2009).

1.3.3.2 Activated carbon

In water and advanced wastewater treatment, the adsorption to activated carbon is an established technology for the removal/biodegradation of a broad spectrum of MPs

from the liquid phase and is widely used in drinking water treatment (Margot et al. 2013; Serrano et al. 2011). In addition, the application of GAC includes a filtration and thus the removal of particular matter. Therefore, suspended solids and phosphorus are effectively removed from the wastewater.

The addition of PAC is sufficient to reduce the concentrations of a wide range of common MPs (Knopp & Cornel 2015). Well-adsorbing organic MPs showed removal rates of more than 80% due to GAC-filtration as well as due to the addition of PAC (Schneider et al. 2020, Annex A.2; Alvarino et al. 2017; Altmann et al. 2016, 2014; Knopp et al. 2016; Magdeburg et al. 2014; Margot et al. 2013; Boehler at al. 2012; Reungoat et al. 2012, 2011, 2010; Hernandez-Leal et al. 2011; Serrano et al. 2011). An increased elimination efficiency of medium and weakly adsorbing MPs was reached by the use of a higher PAC dosage and contact times (Altmann et al. 2016, 2014; Boehler et al. 2012). However, some X-ray contrast media and antibiotics were not well adsorbed to PAC and showed lower elimination rates. Thus, they remained in the wastewater (Magdeburg et al. 2014; Nowotny et al. 2007).

1.3.3.3 Biofiltration

BF is used in the field of municipal wastewater treatment and provides efficient carbon and nitrogen removal by combining both physical and biological purification processes using immersed mineral, plastic or synthetic inert filter media where bacteria can settle and break down the pollutants occurring in the wastewater (Rocher et al. 2012). Besides, BF is approved for the treatment of ozonated wastewater, for example, in drinking water applications, to minimize the concentrations of unknown MP organic oxidation products in the treated (waste)water (Lee et al. 2012). However, only a few studies have been published concerning BF and the elimination of MPs (Knopp & Cornel 2015). The investigations of a BF connected subsequent to an ozonation

system showed that the concentrations of pharmaceuticals and personal care products in the BF effluent were in general similar to the effluent of the ozone contactor indicating that the BF did not provide additional removal of these compounds. Thus, the value of BF is the removal of oxidation products and not a further biodegradation of the investigated substances (Lee et al. 2012). A few studies underline this statement and showed no additional elimination of some of the analysed compounds in the wastewater after the biological post treatment in comparison to the ozonated wastewater (Itzel et al. 2017; Magdeburg et al. 2014; Hollender et al. 2009). But another studies indicated that a BF step after the ozonation further reduced the concentration of the investigated substances (Schneider et al. 2020, Annex A.2; Knopp et al. 2016). However, in comparison to the conventional treated wastewater, studies showed a further reduction of the concentrations of the analysed substances due to the BF process (Hollender et al. 2009). But there are also studies that indicated only a limited improvement of the quality of the wastewater after the BF step (Reungoat et al. 2011).

1.3.3.4 Membrane bioreactors

Only a few studies analysed the removal of organic pollutants in full-scale MBR plants. The technique of MBR systems is a combination of the biological sewage treatment with a membrane filtration representing a physical treatment process (Boonnorat et al. 2017). Indeed, several studies investigated the removal of emerging and priority organic pollutants in MBR technologies but most of them used laboratory-scale pilot plant bioreactors with accurately controlled operating parameters (Camacho-Muñoz et al. 2012). However, MBR treatment of municipal wastewater proved to be more efficient in the removal of MPs compared to the CAS treatment possibly due to higher biomass concentrations and sludge retention times. MBRs showed higher elimination

rates of several pharmaceutical residues and antibiotics that were poorly removed by the CAS treatment (Bertanza et al. 2017). In case of the biodegradation of EDCs CAS and MBR treatments of domestic and industrial wastewater showed similar performances (Bertanza et al. 2011). Also, Boonnorat et al. (2017), Maletz et al. (2013), Lee et al. (2012), and Wenzel et al. (2008) reported on a significantly decrease of the concentrations of most of the investigated MPs occurring in diverse kinds of wastewater due to a MBR treatment. However, specific MPs indicated no significant removal after a MBR treatment whereas other particular MPs showed moderate and high removal rates (Alvarino et al. 2017; Camacho-Muñoz et al. 2012; Serrano et al. 2011).

1.4 Removal of toxicity? The use of bioassays in wastewater quality determination

Wastewater is a complex mixture of many different emerging chemicals and their TPs (Escher et al. 2020; Angelakis & Snyder 2015; Reaume et al. 2015; Loos et al. 2013; Boehler et al. 2012). One important approach to determine the quality of (waste)water are chemical analyses using solid phase extraction (SPE), high performance, ultra performance, or reversed phase liquid chromatography (LC), (tandem) mass spectrometry (MS), and gas chromatography (GC) and several combinations of these techniques (de Oliveira et al. 2020; Perez-Lemus et al. 2019; Fatta et al. 2007). There are three approaches for chemical analyses and the identification of MPs and TPs in (waste)water samples: (1) The target screening is based on the determination of already known MPs and TPs. The identification and the confirmation of these substances is performed by measuring available reference standard solutions. (2) The suspect screening investigates the identification of possible MPs and TPs when a

reference standard is not available and thus, the confirmation of the analytes is not possible. But the molecular formular and structure of the suspected molecules are assembled from the literature or can be predicted utilising computational models and tools. (3) The non-target screening is normally conducted after the performance of the target and suspect screening and involves all remaining components detected in the sample. This analysing technique implies the identification of novel MPs and TPs for which no previous knowledge or prior structural information is available (Hajeb et al. 2022; González-Gaya et al. 2021; Wang et al. 2020; Bletsou et al. 2015; Schymanski et al. 2015). However, the results of these analyses only reflect the detection and concentrations of single known and unknown chemical substances, either their individual removal/degradation or their individual formation/increase. Furthermore, it is not feasible to detect and to assess all known and unknown TPs that are formed for example by ozonation (Reaume et al. 2015; Hjelmborg et al. 2006). Another point of concern are cocktail effects and synergistic interactions of chemicals in mixtures as they occur in already treated wastewater samples. Synergy, as the main concern, implies whether some chemicals are able to enhance the effect of other chemicals jointly leading to a larger effect than predicted. But it was concluded that true synergistic interactions between chemicals are rare and often occur at high concentrations. Therefore, the use of standard models such as the concentration addition model, addressing the cumulative rather than synergistic effects of co-occurring chemicals, are regarded as the most important step in the risk assessment of chemical cocktails (Coors et al. 2018; Cedergreen 2014; Syberg et al. 2008; Ra et al. 2006). However, chemicals that show no effect when they were tested as individual compounds induced considerable effects when they were tested as a mixture of these compounds. For example, “synergy” has been observed in yeast estrogen systems with mixtures of E_2 and xenoestrogens. Thus, the estrogenic activity in wastewater

effluent could be underestimated when the activity of one or a few compounds are considered or if the theoretical estrogenic activity is assumed to be the sum of the activity of each single compound (Mnif et al. 2010; Björkblom et al. 2008; Hjelmborg et al. 2006). A study by Björkblom et al. (2008) showed that the activity of wastewater extracts was much higher than the activity that could have been expected only on the base of the chemical analysis whereat it has to be considered that chemical analyses are not able to detect the whole spectrum of MPs and TPs within the complex wastewater sample and that these unknown MPs and TPs could be responsible for or contribute to the observed effects.

But despite the availability of a huge amount of data, it is still difficult to evaluate the combined effects of trace pollutants in complex matrices such as their additive, greater-than-additive/over-additive (often referred as “synergistic”) and less-than-additive/antagonistic activity (Rider et al. 2018; Bertanza et al. 2011). Thus, chemical analyses alone are not sufficient for the investigation of effects among mixtures of different pollutants and their TPs (Dopp et al. 2019; Hjelmborg et al. 2006).

However, for the determination of the quality of (waste)water the analysis of the toxicity is indispensable. For this purpose, ecotoxicological *in vitro* bioassays and *in vivo* test are another important approach to directly determine the toxicological impacts of MPs in (waste)water more effectively (Reaume et al. 2015; Maletz et al. 2013). These tests cover the impacts of the complex nature of (waste)water on the whole organism including the effects of the complex mixtures of chemicals in the (waste)water and the interaction between these chemicals for example if they exacerbate or attenuate particular effects. For instance, a study by Magdeburg et al. (2014) showed that chemical analysis demonstrated the efficiency of ozonation in oxidising selected compounds whereas *in vitro* and *in vivo* bioassays detected adverse effects in the ozonated wastewater caused by a toxicity of oxidation products. However, a higher

reduction of toxicity compared to the target organic pollutants in extended anaerobic conditions in the biological wastewater treatment was reported by Völker et al. (2017, 2016). Therefore, several studies highlighted that the biological activity and an extensive ecotoxicological evaluation of (waste)water should also be monitored for example for the better evaluation of treatment suitability (Maletz et al. 2013; Bertanza et al. 2011; Stalter et al. 2010 a,b; Mispagel et al. 2009; Fernandez et al. 2008; Svenson et al. 2003). High throughput *in vitro* bioassays with a large array of biological endpoints can support the qualitative and quantitative identification of these chemical mixtures. Today, there is a growing trend of rapid and high-throughput toxicity screening for water using *in vitro* bioassays (Angelakis & Snyder 2015). *In vitro* bioassays, for example on enriched SPE samples, are very sensitive even for the identification and characterisation of low toxic water samples and focus on the impacts produced by particular (micro)pollutants. They cover different modes of toxic action and thus, are suitable tools for the characterisation of the toxicity of different types of (waste)water (Margot et al. 2013; Stalter et al. 2010b). However, *in vitro* bioassays can only indicate a single biological endpoint (for example an endocrine activity or mutagenicity) and single *in vitro* tests are not sufficient in the determination of wastewater quality. Therefore, the results of different studies indicated that a combination of diverse receptor mediated and non-receptor mediated bioassays are required to enable the comprehensive assessment of for example the endocrine disrupting potential of complex (waste)water samples (Maletz et al. 2013). The studies of Reungoat et al. (2012, 2010) and Bertanza et al. (2011) showed that the performed biological assays indicated effects that would have been missed if only chemical analyses had been conducted.

Altogether, the use of a combination of chemical analysis and bioanalytical assays is beneficial to assess the efficiency of a treatment process. Considered individually, both

resources could result in inconsistent conclusions and a combined examination enhances the point of view (Reungoat et al. 2012, 2010).

Another important aspect are *in vivo* bioassays to investigate potential effects of a mixture of compounds and the (chemical) fate of (emerging) chemicals and their TPs in organisms. *In vivo* tests enable the investigation of the impact of the long-term and chronic toxicity of the whole effluent on organisms. Furthermore, *in vivo* tests provide the analysis of the effect of very polar (micro)pollutants present in the wastewater (for example ozonation by-products) that would get lost for example during a SPE enrichment process in preparation for *in vitro* bioassays because these substances are not well extracted (Margot et al. 2013; Stalter et al. 2010a). Mispagel et al. (2009) postulates more studies and research with investigations of for example estrogenic activity that was found in the effluent of rural and regional WWTP in their study and if this activity is sufficient to induce a physiological effect in exposed aquatic organisms for example in native fish. In a pilot study by Margot et al. (2013) a broad range of bioassays were performed and they indicated that most acute toxicity bioassays were not sensitive enough to detect the effects of low MP concentrations in wastewater consisting of conventional treated domestic wastewater, urban runoff, and wastewater of hospitals and clinics. Furthermore, Wigh et al. (2018) mentioned that the toxicity of WWTPs is usually assessed with standardised bioassays and that the assessment of sub-lethal toxic effects needed the development of more adapted tests.

Only a few studies investigated the endocrine activity, toxicity or mutagenicity of wastewater samples in *in vitro* bioassays in the laboratory in combination with the impact of the whole wastewater in long-term *in vivo* tests with algae, higher plants, invertebrates or vertebrates for example with the common duckweed *LemnaLemna minor*, the large water flea *Daphnia magna*, the non-biting midge *Chironomus riparius*, the annelid *Lumbriculus variegatus*, the zebra mussel *Dreissena polymorpha*, the

mudsnail *Potamopyrgus antipodarum*, and the rainbow trout *Oncorhynchus mykiss*. A further benefit is the investigation of the wastewater in *in vivo* tests in on-site tests on the ground of the WWTP under flow-through conditions. Thereby, the whole organism is constantly exposed to the (treated) wastewater that is an important fact for the assessment of possible toxication or detoxication processes for example after the ozonation of the wastewater or diverse post treatments because the loss of substances and compounds is reduced to a minimum (Schneider et al. 2020, Annex A.2; Giebner et al. 2018; Magdeburg et al. 2014; Margot et al. 2013; Stalter et al. 2010a,b).

1.4.1 Conventional wastewater treatment

In general, a conventional treatment of wastewater effectively reduced the toxicity. But there are still detectable effects remaining in the effluents that may represent a risk to the receiving ecosystem. There are several studies investigating different kinds of conventional treated wastewater with diverse bioassays (Völker et al. 2019).

Various endocrine activities such as estrogenic, androgenic, and aryl-hydrocarbon (for dioxins and dioxin-like chemicals) agonistic activity were effectively removed by the conventional treatment but they were still provable in the wastewater in parts in environmentally relevant concentrations. In contrast, it was reported that anti-estrogenic and anti-androgenic activity occurred in the conventional treated wastewater that were not detected in the influent (Stalter et al. 2011). Further studies showed low removal rates of anti-estrogenic and anti-androgenic activity and thus, high remaining activity of both in the treated wastewater (Schneider et al. 2020, Annex A.2; Itzel et al. 2019; Giebner et al. 2018). Moreover, a high non-specific cytotoxicity and an increased and substantial genotoxicity were determined in the conventional treated wastewater as well (Dopp et al. 2021; Magdeburg et al. 2014; Stalter et al. 2011, 2010a). Also, mutagenicity was still detectable after the treatment (Giebner et al. 2018).

However, the growth inhibition as well as the photosynthesis inhibition of the green algae *Desmodesmus subspicatus* and *Pseudokirchneriella subcapitata* were reduced due to the conventional wastewater treatment but still remained in the wastewater (Itzel et al. 2017; Margot et al. 2013). Besides, the growth of the common duckweed *L. minor*, the reproduction and the biomass of the worm *L. variegatus*, the survival rate of the large water flea *D. magna*, and the reproduction of the mudsnail *P. antipodarum* were reduced whereas the mortality of the non-biting midge *C. riparius* was increased (Schlüter-Vorberg et al. 2017; Magdeburg et al. 2012). However, several studies indicated an increased reproduction of the mudsnail *P. antipodarum* exposed to conventional treated wastewater (Schneider et al. 2020, Annex A.2; Stalter et al. 2010a). Furthermore, the wastewater negatively affected the overall survival and different developmental stages of the rainbow trout *O. mykiss* like an increased coagulation of the eggs, a decelerated hatching progress and a decreased hatching success of the larvae, a delayed swim-up of the fish, and an increased vitellogenin content in yolk-sac larvae and juvenile fish. In addition, the body length as well as the biomass of the fish were lower at the end of the experiments (Magdeburg et al. 2014; Margot et al. 2013; Stalter et al. 2010b). Furthermore, conventional treated wastewater increased both, the number of egg-carrying females of the Japanese medaka (*Oryzias latipes*) and the number of eggs (Altmann et al. 2012). In contrast, a reduced egg production of the fathead minnow (*Pimephales promelas*) exposed to wastewater after a conventional treatment was reported as well (Filby et al. 2010; Thorpe et al. 2009). In addition, the mortality of juvenile individuals of two other fish species (the Nile Tilapia (*Oreochromis niloticus*) and the common carp ((*Cyprinus carpio*)) increased with the investigated increasing wastewater fractions of a conventional WWTP (Boonnorat et al. 2017).

1.4.2 Advanced wastewater treatment

1.4.2.1 Ozonation

Ozonation further reduced the estrogenic activity detected in the conventional treated wastewater even at a low ozone dosage (Völker et al. 2019; Margot et al. 2013; Reungoat et al. 2012; Stalter et al. 2011). Thereby, the reduction of the estrogenic activity was not significantly affected by the ozone dosage or diverse increasing retention times (Bertanza et al. 2011; Hashimoto et al. 2007). Besides, the response of the aryl-hydrocarbon receptor, the androgenic and anti-androgenic receptor as well as the genotoxicity were reduced in the ozonated wastewater. In addition, the removal of most of the above-mentioned endocrine activities and the genotoxic effects increased with increasing ozone dosage (Itzel et al. 2019; Reaume et al. 2015; Magdeburg et al. 2014; Reungoat et al. 2011, 2010; Stalter et al. 2011). In contrast, the ozonation process effected a higher production of estradiol and aromatase activity indicating a potential disruption of the steroid synthesis pathway (Maletz et al. 2013). Moreover, Giebner et al. (2018), Magdeburg et al. (2014) and Stalter et al. (2010a) reported on a significantly increased genotoxicity and the occurrence of mutagenicity in the wastewater caused by the ozonation process whereat the mutagenic potential increased with increasing ozone dosage. Furthermore, the androgenic, the anti-estrogenic and the anti-androgenic activity increased in the ozonated wastewater (Itzel et al. 2019, 2018; Stalter et al. 2011). Also, Gehrmann et al. (2018) detected a high remaining anti-estrogenic and anti-androgenic activity in the ozonated wastewater. However, the ozonation reduced the non-specific toxicity as well as cytotoxic effects in the wastewater (Dopp et al. 2021; Reungoat et al. 2012; Stalter et al. 2011) but cytotoxic effects were still observed in the wastewater samples after the ozonation process (Dopp et al. 2021).

The growth inhibition and the photosynthesis inhibition of the green algae *P. subcapitata* were reduced due to the ozonation process (Margot et al. 2013). An overall reduction of inhibitory effects on the green algae *D. subspicatus* was also shown by Itzel et al. (2017). Though, the ozonation increased the growth inhibition of the common duckweed *L. minor*, the mortality of the non-biting midge *C. riparius* and the zebra mussel *D. polymorpha* as well as the overall toxicity tested with the large water flea *D. magna* and the annelid *L. variegatus* showing a decreased reproduction of both species and a decreased biomass of the worms. The reproduction of the mudsnail *P. antipodarum* decreased as well in the ozonated wastewater (Schlüter-Vorberg et al. 2017; Magdeburg et al. 2012; Stalter et al. 2010a). Indeed, the overall survival of the rainbow trout *O. mykiss*, the hatching success of the larvae, and the delay of the swim-up of the fish were significantly improved in the ozonated wastewater. Furthermore, the body length and body mass increased compared to the conventional treated wastewater (Margot et al. 2013). On the contrary, Magdeburg et al. (2014) and Stalter et al. (2010b) reported on adverse effects of the ozonation process on the rainbow trout *O. mykiss* such as a higher mortality of embryos, larvae and adult fish and a substantial retardation in the development of the fish exposed to the ozonated wastewater. The egg coagulation was increased, the hatching success was decreased, and the swim-up was delayed. Furthermore, the ozonation of the wastewater induced a decreased body length and body mass of the fish. However, both studies showed that an increase of the vitellogenin concentration in the juvenile fish as it was detected in the conventional treated wastewater was not observed in the ozonated wastewater (Margot et al. 2013, Stalter et al. 2010b). Furthermore, the number of egg-carrying females of the Japanese medaka *O. latipes* was lower in the wastewater after the ozonation process (Altmann et al. 2012).

1.4.2.2 Activated carbon

The treatment of the wastewater with PAC or the filtration through a GAC filter significantly reduced the estrogenic, androgenic, anti-androgenic, and aryl-hydrocarbon receptor agonistic activity as well as genotoxic and mutagenic effects (Völker et al. 2019; Giebner et al. 2018; Itzel et al. 2018; Magdeburg et al. 2014; Margot et al. 2013; Reungoat et al. 2012; Stalter et al. 2011, 2010a). On the contrary, the anti-estrogenic activity increased in parts distinctly after the treatment with PAC or GAC filtration (Giebner et al. 2018; Stalter et al. 2011). However, Itzel et al. (2018) reported on a further reduction of the anti-estrogenic activity until it was no longer detectable in the GAC-filtered wastewater. The filtration with biological activated carbon (BAC) had no significant effect on the reduction of dioxin and dioxin-like chemicals whereas the genotoxicity and the neurotoxicity were reduced below the limit of detection (Reungoat et al. 2011, 2010). The non-specific toxicity also distinctly decreased by the filtration of the wastewater with BAC or the treatment with PAC (Reungoat et al. 2012, 2011, 2010; Stalter et al. 2011). The PAC treatment of the wastewater indicated a clearly reduction of the growth inhibition and the photosynthesis inhibition of the green alga *P. subcapitata* as well (Margot et al. 2013). In contrast, Stalter et al. (2010a) reported on adverse effects on the common duckweed *L. minor* caused by PAC treated wastewater. Furthermore, the toxic effects on the annelid *L. variegatus* were slightly increased after the PAC treatment (Stalter et al. 2010a) whereas the reproduction of the worms was significantly increased in GAC filtered wastewater (Schlüter-Vorberg et al. 2017). However, the reproduction of the mudsnail *P. antipodarum* was significantly reduced due to the wastewater treatment with PAC or the filtration through a GAC filter (Giebner et al. 2018; Stalter et al. 2010a). Anyway, the survival and the hatching success of the rainbow trout *O. mykiss* were significantly enhanced after the treatment with PAC and no delay of the hatching progress of the larvae or the swim-up of the fish

were observed. Furthermore, no adverse effects on the body mass or body length of the fish were noticed (Magdeburg et al. 2014; Margot et al. 2013).

1.4.2.3 Biofiltration

The estrogenic, anti-androgenic, and aryl-hydrocarbon activity were highly reduced in the wastewater after a SF step. In contrast, the androgenic and especially the anti-estrogenic activity were not reduced or even obviously increased in the sand filtered wastewater (Gehrmann et al. 2018; Giebner et al. 2018; Stalter et al. 2011, 2010a). However, a high remaining anti-androgenic activity was detected by Gehrmann et al. (2018) in the wastewater that passed through a sand filter.

BF processes (with BAC and SF) in parts distinctly reduced the genotoxicity of the wastewater whereas potential mutagenicity was detectable after the SF (Magdeburg et al. 2014; Reaume et al. 2015). However, the non-specific toxicity and the mutagenicity were in parts obviously reduced due to SF (Giebner et al. 2018; Margot et al. 2013; Stalter et al. 2011). Besides, Magdeburg et al. (2012) reported on a significantly increased survival of the large water flea *D. magna* after the SF process whereas the SF slightly increased the toxicity of the annelid *L. variegatus* (Schlüter-Vorberg et al. 2017; Stalter et al. 2010a). The reproduction of the mudsnail *P. antipodarum* significantly decreased in sand filtered wastewater (Giebner et al. 2018; Magdeburg et al. 2012; Stalter et al. 2010a). Moreover, the SF process slightly increased the egg coagulation and decreased the body length and the body mass of the rainbow trout *O. mykiss* as well as the vitellogenin levels in the fish (Stalter et al. 2010b).

A fluidised (moving) bed used as a biological post treatment decreased in parts distinctly the estrogenic, cytotoxic, and genotoxic effects in the wastewater whereas an increased estrogenic activity and cytotoxicity were detected in a polishing pond as

well utilised as a biological post treatment (Dopp et al. 2021; Itzel et al. 2019, 2017). Furthermore, the wastewater of a fluidised-bed reactor indicated high remaining anti-estrogenic and anti-androgenic activity and additionally significantly increased the growth inhibition of an alga (Itzel et al. 2019, 2017).

1.4.2.4 Membrane bioreactors

The MBR treatment significantly decreased the estrogenic and androgenic activity of the wastewater and showed to some extent a higher efficiency in the reduction of the estrogenic activity compared to the conventional treatment. But a residual estrogenic activity was still detectable in the wastewater (Gehrmann et al. 2018; Itzel et al. 2018; Bertanza et al. 2017, 2011; Maletz et al. 2013). The anti-estrogenic and anti-androgenic activity were reduced as well due to the MBR treatment but in parts high levels of both activities were yet provable in the MBR treated wastewater samples (Gehrmann et al. 2018; Itzel et al. 2018). The anti-estrogenic activity even increased after the MBR treatment (Itzel et al. 2018). However, the concentration of substances that have the ability to alter the sex steroid production was successfully reduced due to the MBR treatment (Maletz et al. 2013).

The reproduction of the annelid *L. variegatus* was significantly increased in the MBR treated wastewater (Schlüter-Vorberg et al. 2017). Furthermore, the MBR treatment indicated an increased mortality of two fish species, the common carp *C. carpio* and the Nile Tilapia *O. niloticus* (Boonnorat et al. 2017).

1.5 Integration of the present work into the current state of research, knowledge gaps and goals of this work

The present work was enclosed in the scientific joint research project TransRisk investigating and focussing on the characterisation, communication, and minimisation of risks of emerging pollutants and pathogens in the water cycle (www.transrisk-projekt.de) that was funded by the Federal Ministry of Education and Research (BMBF) (support code (FKZ): 02WRS1275A) within the research focus “NaWaM – Sustainable water management” inside the funding measure “RiSKWa – risk management of new pollutants and pathogens in the water cycle”. The objective of TransRisk was the combination of (eco)toxicological, chemical, and technical approaches for the development of new policies concerning the characterisation and minimisation of risks associated with impacts of organic MPs and pathogens occurring in urban water cycles. The detailed analyse of the resultant risk could afterwards be integrated into an action-oriented risk management concept.

The industrialisation grows continuously all over the world. A consequence of this global growth is amongst other things the production of new chemicals that are used in different areas of the everyday life for example personal care products, pharmaceuticals, pesticides (including insecticides and fungicides), cleaning agents and industrial chemicals. Most of these substances end up as MPs in the water cycle. Conventional WWTPs are expected to clean-up the sewage before it gets back into the global water cycle. But many studies show that the cleaning capacity of conventional WWTPs is not sufficient enough to remove the chemicals from the wastewater and thus conventional WWTP became hotspots of emerging contaminants (Rogowska et al. 2020; Kim & Zoh 2016; Margot et al. 2015; Luo et al. 2014; compare above chapter 1.2). Chemical analyses show that numerous chemical substances are

not fully mineralised in conventional WWTP and MPs are detected in sewage water, surface water and ground water (Seitz & Winzenbacher 2017; Loos et al. 2013, 2010, 2009; Prasse et al. 2012; Reemtsma et al. 2006; unpublished data; compare above chapter 1.2). Several (eco)toxicological studies indicated negative effects of conventional treated wastewater on organisms of the aquatic ecosystem and potential endocrine impacts on human hormone systems (Dopp et al. 2021; Itzel et al. 2019, 2017; Völker et al. 2019; Giebner et al. 2018; Boonnorat et al. 2017; Schlüter-Vorberg et al. 2017; Magdeburg et al. 2014, 2012; Margot et al. 2013; Altmann et al. 2012; Stalter et al. 2011, 2010a, 2010b; compare above chapter 1.4.1). As shown above these bioassays are needed to comprehensively understand the removal of the toxicity of the wastewater. Therefore, AWWT technologies were established to improve the cleaning capacity of WWTPs. AWWT technologies are for example ozonation, treatments with activated carbon, BF, SF, and MBRs. There is abundant knowledge about how single AWWT technologies are operating (Bertanza et al. 2017, 2011; Altmann et al. 2016, 2014; Maletz et al. 2013; Margot et al. 2013; Boehler at al. 2012; Wenzel et al. 2008; Nowotny et al. 2007; Chapman et al. 2004; compare above chapters 1.3.3.1 to 1.3.3.4 and 1.4.2.1 to 1.4.2.4). On the contrary, there is only limited data of the performance of combined multiple AWWT technologies available (Schlüter-Vorberg et al. 2017; Magdeburg et al. 2012; Reungoat et al. 2012; Hernandez-Leal et al. 2011; Stalter et al. 2011, 2010a, 2010b). However, diverse combinations of AWWT technologies emerge to optimise the removal of MPs and toxicity but they have not been assessed so far.

Therefore, one goal of this study was the investigation of the wastewater of a municipal WWTP with multiple subsequent AWWT in a pilot-scale format (Figure 1) to assess their performance of removing MPs and toxicity and find the optimal wastewater treatment (Schneider et al. 2020, Annex A.1, compare below chapter 1.6). Four

different ozone dose and five different HRTs were tested to find the optimal values for the reduction of MPs and toxic effects. After the ozonation process the ozonated wastewater pass through four different post-treatments (non-aerated and aerated GAC filtration and non-aerated and aerated biological filtration) to investigate a further reduction of MPs, newly formed TPs, and toxicity. In addition, sedimented wastewater after the primary mechanical treatment ran into two stand-alone MBRs. One MBR operated with ozone system 2 and a partial flow recirculation (Figure 1). The aim was the comparison of the removal efficiency of MPs and toxicity of these MBR systems with the single conventional wastewater treatment and in combination with ozonation. Aqueous and extracted wastewater from ten different sampling points (Figure 1) were analysed *in vitro* to determine the (anti)estrogenic and (anti)androgenic activity and mutagenicity to detect possible endocrine disrupting potentials and possible impacts on the genome. In addition, chemical analyses were conducted to examine the removal of 40 selected MPs as tracer substances. The investigation of *in vivo* toxicity of nine different wastewater streams (excluding the primary treatment) was performed using the mudsnail *P. antipodarum* and the amphipod *Gammarus fossarum* in an on-site flow-through system on the pilot-scale WWTP to detect improvements of the quality of the wastewater or possible adverse effects on aquatic invertebrates.

Above-mentioned chemical, *in vitro* and *in vivo* investigations require a multitude of aqueous wastewater samples. But the stability of aqueous wastewater samples is limited because of physiochemical (for example exposure to light) or biological processes (for example microbiological degradation). Thus, sample preparation is crucial for the precise detection and quantification of MPs and the determination of (eco)toxicity (Völker et al. 2019; Prasse et al. 2015). Nowadays, there is much knowledge on how to prepare wastewater samples for chemical analyses for example using filtration, acidification, or SPE (Seitz & Winzenbacher 2017; Knopp et al. 2016;

Magdeburg et al. 2014; Loos et al. 2013, 2010, 2009; Margot et al. 2013; Boehler et al. 2012; Lee et al. 2012; Prasse et al. 2012, 2011; Reungoat et al. 2012; Serrano et al. 2011; Fernandez et al. 2009; Björkblom et al. 2008; compare below chapter 3.1, 3.1.1, 3.1.2, and 3.1.3). In several studies, the sample preparation for *in vitro* bioassays is similarly to the chemical methods with only a few differences (Loos et al. 2013). The extraction of the pollutants and the clean-up procedure for the biological analyses was even the same as for the chemical analyses (Bertanza et al. 2011). Thus, the adaption of the methods is optimised for a few specific chemicals (Prasse et al. 2015; Escher et al. 2005) and does not necessarily extract the toxicity (Stalter et al. 2016; Wagner & Oehlmann 2011). In this context, there is only little knowledge how these common preparation and stabilisation techniques affect the outcome of (eco)toxicological *in vitro* bioassays. However, for the assessment of water quality it is important to estimate the toxicity of water and waste water samples and emerging contaminants.

Thus, another goal of this work was to identify an optimal preparation method for aqueous water and wastewater samples for ecotoxicological *in vitro* investigations (Abbas et al. 2019, Annex A.2, compare below chapter 1.6). For this purpose, 18 types of water and wastewater (for example raw (untreated) wastewater, municipal conventionally treated and hospital wastewater, ozonated wastewater, surface water and groundwater) were analysed after filtration, acidification and SPE. Thereby, the performance of three different SPE sorbents (Oasis HLB, Supelco ENVI-Carb+ and Telos C18/ENV) at neutral and acidic pH were compared to identify the best extraction method for the recovering of biological effects. Altogether, aqueous and extracted water and wastewater samples were tested in eleven *in vitro* bioassays for endocrine activity (yeast-based recombinant reporter gene assays aiming the detection of (anti)estrogenic (YES/YAES), (anti)androgenic (YAS/YAAS), aryl-hydrocarbon (AhR) -like (dioxin-like (YDS)), retinoic acid-like (RAR), retinoic X-like (RXR), vitamin D-like

(VDR) and thyroid-like (TR) activity) as well as mutagenicity (Ames fluctuation test) and genotoxicity (*umuC* test), both test systems using genetically modified bacterial strains, and cytotoxicity.

In addition, experiments in the laboratory in artificial indoor flow-channels were performed to assess the impact of a wastewater effluent with known estrogenic activity on life-history traits of the freshwater amphipod *Gammarus pulex* (Schneider et al. 2015, Annex A.4, compare below chapter 1.6). Furthermore, laboratory-scaled bioassays with the model organism *Caenorhabditis elegans* were done to examine the ecotoxicological impacts of surface water and conventional as well as advanced treated wastewater on the reproduction and the development of the worms and additional on the cytochrome P450 (35A3) expression indicating an exposure to polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) or other *cyp-35A3*-inducing compounds (Abbas et al. 2018, Annex A.3, compare below chapter 1.6).

1.6 Publications included in this thesis

The following publications are part of this thesis. The main findings of each publication are summarised.

A.1: Abbas, A., Schneider, I., Bollmann, A., Funke, J., Oehlmann, J., Prasse, C., Schulte-Oehlmann, U., Seitz, W., Ternes, T., Weber, M., Wesely, H. & Wagner, M. (2019): What you extract is what you see: Optimising the preparation of water and wastewater samples for *in vitro* bioassays. Water Research 152, 47–60.

The common sample preparation techniques for aqueous water and wastewater samples (for example filtration, acidification, SPE) are predominantly adopted for chemical analyses and rarely optimised for (eco)toxicological *in vitro* bioassays. This performance includes the risk of misinterpretation of the water quality and the real toxicity of water and wastewater samples.

The results of the study by Abbas et al. (2019, Annex A.1) “What you extract is what you see: Optimising the preparation of water and wastewater samples for *in vitro* bioassays” indicated that sample acidification with sulphuric acid, storage for 24 h and neutralisation with sodium hydroxide significantly affected the endocrine activity and mutagenicity of aqueous samples compared to the samples kept at a neutral pH and thus the outcome of the ecotoxicological *in vitro* bioassays.

Overall, the comparison of different sample preparation techniques such as acidification, filtration or SPE enrichment showed a strong impact on the outcome of endocrine activities and mutagenicity. To avoid the misestimating of the *in vitro* toxicity in the future the implementation of sample preparation should be accurately adapted

to the aims of the study, to the qualities of the investigated water and wastewater samples and to the specifies of the performed *in vitro* bioassays.

A.2: Schneider, I., Abbas, A., Bollmann, A., Dombrowski, A., Knopp, G., Schulte-Oehlmann, U., Seitz, W., Wagner, M. & Oehlmann, J. (2020): Post-treatment of ozonated wastewater with activated carbon and biofiltration compared to membrane bioreactors: Toxicity removal *in vitro* and in *Potamopyrgus antipodarum*. Water Research 185, 116104.

The (aquatic) environment is exposed to increasing amounts of chemical substances generated from globally growing industrialisation. Conventional WWTPs equipped with biological sludge treatment do not have the capacity to remove these substances from the wastewater and finally become a major point source of (micro)pollutant emissions. AWWT technologies such as ozonation, treatments with activated carbon, BF, SF and MBRs are expected to improve the removal capacity of those (micro)pollutants. Predominantly, studies are published that operates with individual AWWT technologies. The study by Schneider et al. (2020, Annex A.2) “Post-treatment of ozonated wastewater with activated carbon and biofiltration compared to membrane bioreactors: Toxicity removal *in vitro* and in *Potamopyrgus antipodarum*” examined the removal performance of ozonation combined with multiple post-treatments and stand-alone MBRs with one MBR operating with ozonation and partial flow recirculation on a pilot WWTP. Several *in vitro* effects were detected in conventionally treated and ozonated wastewater especially estrogenic, anti-estrogenic and anti-androgenic activities as well as potential mutagenicity. Furthermore, the ozone dose and the HRT affected the results of the *in vitro* bioassays.

Overall, the results of the *in vitro* bioassays, the *in vivo* on-site test with *P. antipodarum* and the chemical analysis indicated that ozonation is effective in further reducing most endocrine activities and MP concentrations. However, ozonation led to the formation of unknown TPs indicating potential toxicity and a post-treatment is required. Finally, ozonation with subsequent filtration with GAC was the most effective process to reduce the generated toxicity. Besides, the study highlights the importance of the combination of *in vitro* bioassays, *in vivo* tests and chemical analyses to assess the conventional and AWWT processes.

A.3: Abbas, A., Valek, L., Schneider, I., Bollmann, A., Knopp, G., Seitz, W., Schulte-Oehlmann, U., Oehlmann, J. & Wagner, M. (2018): 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*. Environmental Science and Pollution Research 25, 13868–13880.

As mentioned above, WWTPs play an important role as major point sources of anthropogenic (micro)pollutants and TPs in urban water cycles that negatively affect aquatic ecosystems and water resources. The study by Abbas et al. (2018, Annex A.3) “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*” showed that conventionally treated wastewater induced reproductive and developmental toxicity in *Caenorhabditis elegans* that was not exacerbated by ozonation. The post-treatments filtration with GAC and BF successfully reduced the developmental toxicity. However, the results indicated that the worms tested in conventionally treated as well as in ozonated

wastewater were exposed to PAHs, PCBs, or other *cyp-35A3*-inducing compounds. The chemical analyses indicated that the AWWT technologies decreased the concentrations of most MPs.

Overall, the results demonstrate that an integrated assessment of biological and chemical parameters is necessary for conventional and AWWT in the future.

A.4: Schneider, I., Oehlmann, J. & Oetken, M. (2015): Impact of an estrogenic sewage treatment plant effluent on life-history traits of the freshwater amphipod *Gammarus pulex*. Journal of Environmental Science and Health, Part A: Toxic/Hazardous Substances and Environmental Engineering 50 (3), 272–281.

WWTPs are still a major source of the contamination of amongst others surface waters with for example pesticides, pharmaceuticals, personal care products and EDCs that have the potential to affect local macroinvertebrate communities in the streams. The study by Schneider et al. (2015, Annex A.4) “Impact of an estrogenic sewage treatment plant effluent on life-history traits of the freshwater amphipod *Gammarus pulex*” investigated the impact of the wastewater of a conventional WWTP with known estrogenic activity on different life-history traits of *G. pulex* as a sensitive biological indicator for the assessment of water quality in surface waters in artificial indoor flow-channels under constant conditions to different circulating wastewater concentrations (0%, 33%, 66% and 100%). The amphipods had an increasing body length with increasing wastewater concentrations compared to the control. Moreover, the sex ratio (male to female) shifted in favour to the females, the fraction of brooding females, the mean number of eggs in the brood pouch and the fecundity indices were significantly increased and finally the total number of the offspring increased clearly with increasing wastewater concentrations.

Overall, the results demonstrate that wastewater from conventional WWTPs can affect macroinvertebrate communities in several ways. An additional nutrient supply increased the body length of the amphipods and EDCs had diverse effects on the hormone system of *G. pulex*. The results illustrate that WWTPs with AWWT technologies are needed in the future.

2 Additional results

2.1 On-site *in vivo* experiment with *Gammarus fossarum*

Within the scope of the TransRisk project an on-site test with the amphipod *G. fossarum* was conducted at the pilot WWTP. Detailed information to this pilot WWTP is given in Schneider et al. (2020, Annex A.2) and Knopp et al. (2016).

The performance of the on-site test with *G. fossarum* based on the on-site test with the mudsnail *P. antipodarum* (Schneider et al. 2020, Annex A.2). Detailed information about the material and the methods is given in Annex A.5.

2.2 Results

2.2.1 Mortality

The mortality of *G. fossarum* at the end of the on-site experiment after 30 days of exposure in the negative control (NC) and the positive control (PC) was below 20%. The biological treatment (BT) showed the highest mortality and differed significantly compared to the NC and the eight remaining wastewater treatments. Detailed information is given in Annex A.5.

2.2.2. Growth and reproduction

The body length of the male amphipods was minimal in GAC and maximal in BT+O_3. Significant increases to the BT were observed in BT+O_3, GAC_a, and MBR2.

The body length of female individuals was minimal in GAC and maximal in BT+O_3 as well. A significant increase compared to the BT was only observed in BT+O_3.

The sex-ratio (male to female) was predominantly balanced and varied with no significant differences between the investigated (waste)water.

The percentage of egg-carrying females was minimal in the BT and significantly lower compared to the NC. The treatment BT+O_3 showed the highest number of egg-carrying females and differed significantly compared to the BT.

The minimal and maximal number of eggs per female were determined in GAC_a and MBR2, respectively, with no significant differences compared to the BT. The total number of eggs was minimal in the BT and maximal in BT+O_3.

The fecundity index was minimal in GAC_a and maximal in MBR2 as well with no significant differences between the C and the BT and the eight wastewater treatments. Detailed information is given in Annex A.5.

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

The energy content as protein was minimal in the BT and differed significantly compared to the NC. The remaining eight wastewater treatments showed higher energy contents as protein compared to the BT except BT+O_3.

The energy content as glycogen was minimal in the NC and the PC. The NC differed significantly compared to the maximal result of the BT. The other eight wastewater treatments showed lower energy contents as glycogen compared to the BT with significant differences in the non-aerated and aerated GAC and BF.

The energy content as lipid also was minimal in the NC and PC. Again, the energy content as lipid was maximal in the BT and the eight wastewater treatments showed lower energy contents as lipid compared to the BT with significant differences except BT+O_3.

The total energy content (protein + glycogen + lipid) was minimal in NC and PC. The NC differed significantly compared to the highest result of the BT. The other eight wastewater treatments showed lower total energy contents compared to the BT with

significant differences except BT+O_3 and MBR1+O_3. Detailed information is given in Annex A.5.

2.2.4 *In-vitro* bioassays for endocrine and mutagenic activity

2.2.4.1 Native samples

A low estrogenic activity was detected in the native sample of the PT. The BT reduced the estrogenic activity that was further reduced by ozonation. The estrogenic activity of the non-aerated GAC and BF treatments were on the same level as the BT. The aerated GAC and BF showed a lower estrogenic activity compared to the BT. The MBR systems indicated a higher estrogenic activity compared to the BT.

In contrast to the estrogenic activity, a high anti-estrogenic activity was detected in the native sample of the PT. The BT reduced the anti-estrogenic activity but it still remained on a high level. The activity of the BT+O_3 with both subsequent GAC and BFs and the MBR systems were on a comparable and also high level of the BT.

A medium-ranged androgenic activity was detected in the native sample of the PT as well. Again, the activity was reduced in the BT. The further wastewater treatments showed a higher androgenic activity compared to the BT except MBR1 and MBR1+O_3. A low anti-androgenic activity was only detectable in MBR1 and MBR1+O_3. Detailed information is given in Annex A.5.

2.2.4.2 SPE-extracts

The SPE extracts of the PT were cytotoxic in all *in vitro* bioassays and, thus, not considered.

The SPE extract of the BT showed a low estrogenic activity (Figure 2A). The ozonation reduced this activity and it remained on a relatively low level in the following nonaerated and aerated GAC filters and BFs compared to the BT. The estrogenic activity of MBR1

and MBR2 was also low. The ozonation of MBR1 led to a reduction of the estrogenic activity as well.

The anti-estrogenic activity of the BT was relatively high again (Figure 2B). The ozonation process reduced the activity only in parts. Compared to the BT both GAC filters reduced the anti-estrogenic activity more effectively than both BFs. The anti-estrogenic activity of MBR1 was in the high range of the BT in contrast to MBR1+O_3 with an obviously decreased activity. MBR2 showed the maximal anti-estrogenic activity.

The androgenic activity was very low in all wastewater treatments (Figure 2C).

The BT showed the maximal anti-androgenic activity (Figure 2D). Again, the ozonation reduced the activity only in parts. Comparable to the anti-estrogenic activity, both GAC filters reduced the anti-androgenic activity more effectively than both BFs. In MBR1 a slightly lower anti-androgenic activity than in the BT was detected. Again, the ozonation of MBR1 further reduced this activity. The anti-androgenic activity of MBR2 was in the maximal range of the BT. Detailed information is given in Annex A.5.

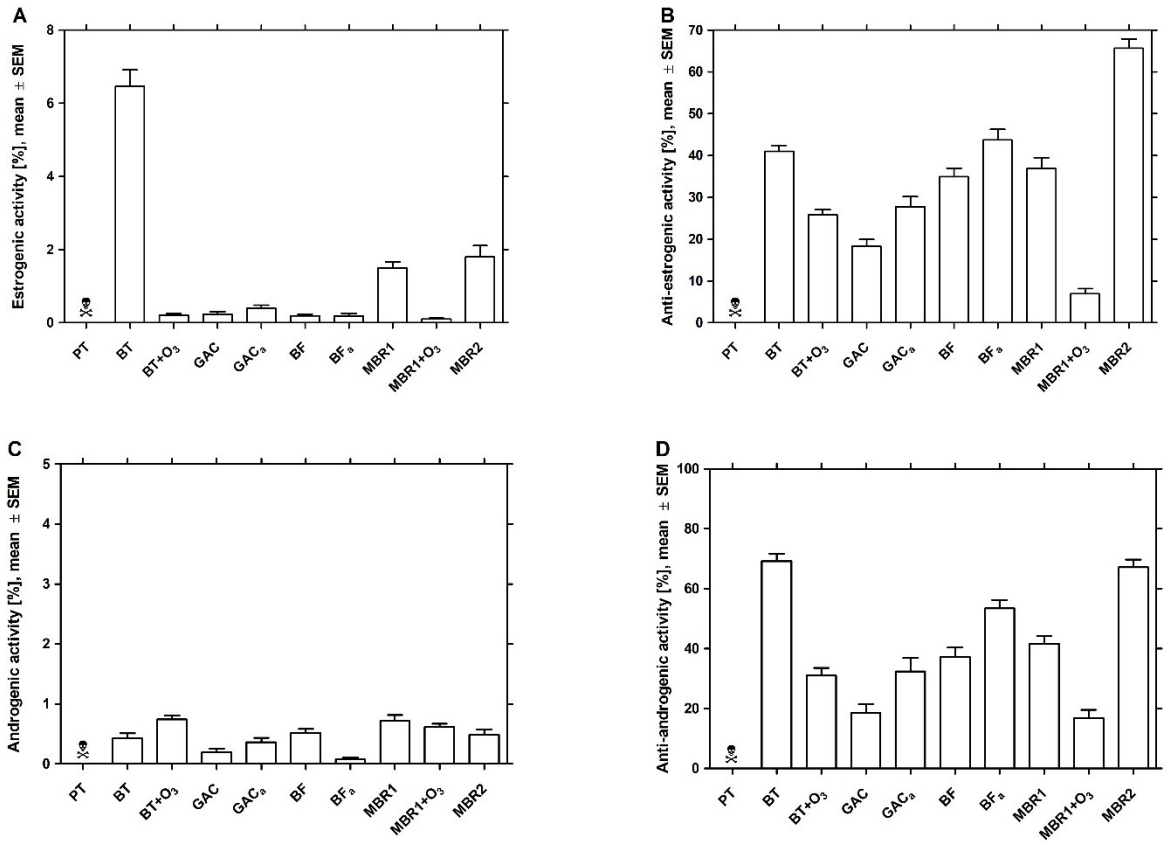

Figure 2: Estrogenic (A), anti-estrogenic (B), androgenic (C), and anti-androgenic activity (D) in three SPE extracts each produced from 24 h composite samples per treatment taken in parallel to the *in vivo* on-site experiment with *Gammarus fossarum*. PT: after primary treatment, BT: after conventional 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 = 24.

There was no potential mutagenicity detectable in the BT (Figure 3). In contrast, the ozonated wastewater showed an extremely high potential mutagenicity. Both GAC filters reduced the potential mutagenicity contrary to both BFs. No potential mutagenicity was detected in MBR1 and MBR2 but again the ozonation induced a severe potential mutagenicity in MBR1+O_3. Detailed information is given in Annex A.5.

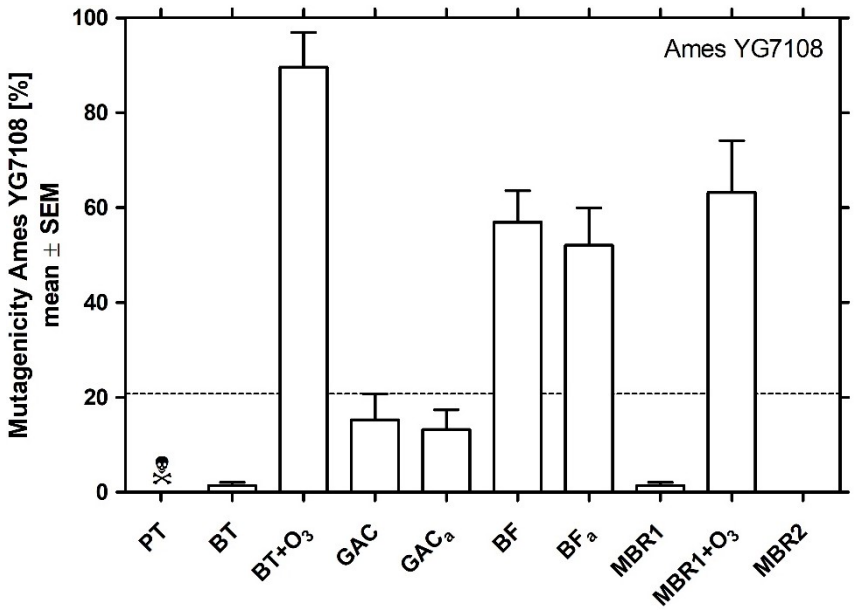

Figure 3: Mutagenicity in the Ames strain YG7108 in three SPE extracts each produced from 24 h composite samples per treatment taken in parallel to the *in vivo* on-site experiment with *Gammarus fossarum*. PT: after primary treatment, BT: after conventional biological treatment, $BT+O_3$: after ozone 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 = 3.

2.2.5 Chemical analysis

The chemical analyses included 28 MPs that covered industrial chemicals (for example tolyltriazole and benzotriazole), pharmaceuticals such as anticonvulsants, antibiotics (including the metabolites of diclofenac, ibuprofen, and carbamazepine) and radio-opaque substances, herbicides such as mecoprop, and nutrition-related chemicals such as caffeine. Some of these compounds were chosen as tracer substances for the removal effectivity of the AWWT technologies because it is known that they are poorly degradable in the conventional wastewater treatment (Seitz & Winzenbacher 2017).

In the PT highest concentrations of caffeine, carboxy-ibuprofen, 2-hydroxy-ibuprofen and 1H-benzotriazole were detected. The concentrations of the other substances were below 17 µg/L.

The BT reduced the concentrations of 16 out of these 28 compounds by more than 50% (Figure 4). The highest reduction rates showed carboxy-ibuprofen (–99.9%), paracetamol (–99.8%), caffeine (–99.7%), 2-hydroxy-ibuprofen (–99.0%) and 1-hydroxy-ibuprofen (–97.9%). Six MPs were reduced by less than 25% in the BT. In contrast, 10,11-dihydro-10,11-dihydroxycarbamazepine (+11.8%;), iopamidol (+20.6%), carbamazepine (+21.7%), mecoprop (+70.4%) and carboxy-acyclovir (+399%) showed an increased concentration in the BT compared to the PT.

The ozonation led to a further reduction of the concentrations of 17 MPs by more than 50% compared to the BT (Figure 4A). A few substances even showed increased concentrations in the ozonated wastewater.

Three post-treatments after the ozonation (GAC, GAC_a and BF) had no remarkable effect on the concentration of 18 MPs compared to BT+O_3. Some compounds showed a higher removal rate in both GAC-filters compared to both BFs. However, the concentration of a few substances increased in both GAC-filters compared to BT+O_3. Furthermore, an increase of the concentrations of 20 MPs occurred in the BF_a.

The MBR2 treatment was as efficient as the BT concerning the removal of the 28 MPs (Figure 4B). However, comparable to the BT, an increased concentration of some substances was detected in the MBR2. The MBR1 treatment showed a slightly higher removal efficiency of the compounds than the BT. The ozonation of MBR1 increased the removal compared to MBR1 with comparable efficiencies to BT+O_3. Detailed information is given in Annex A.5.

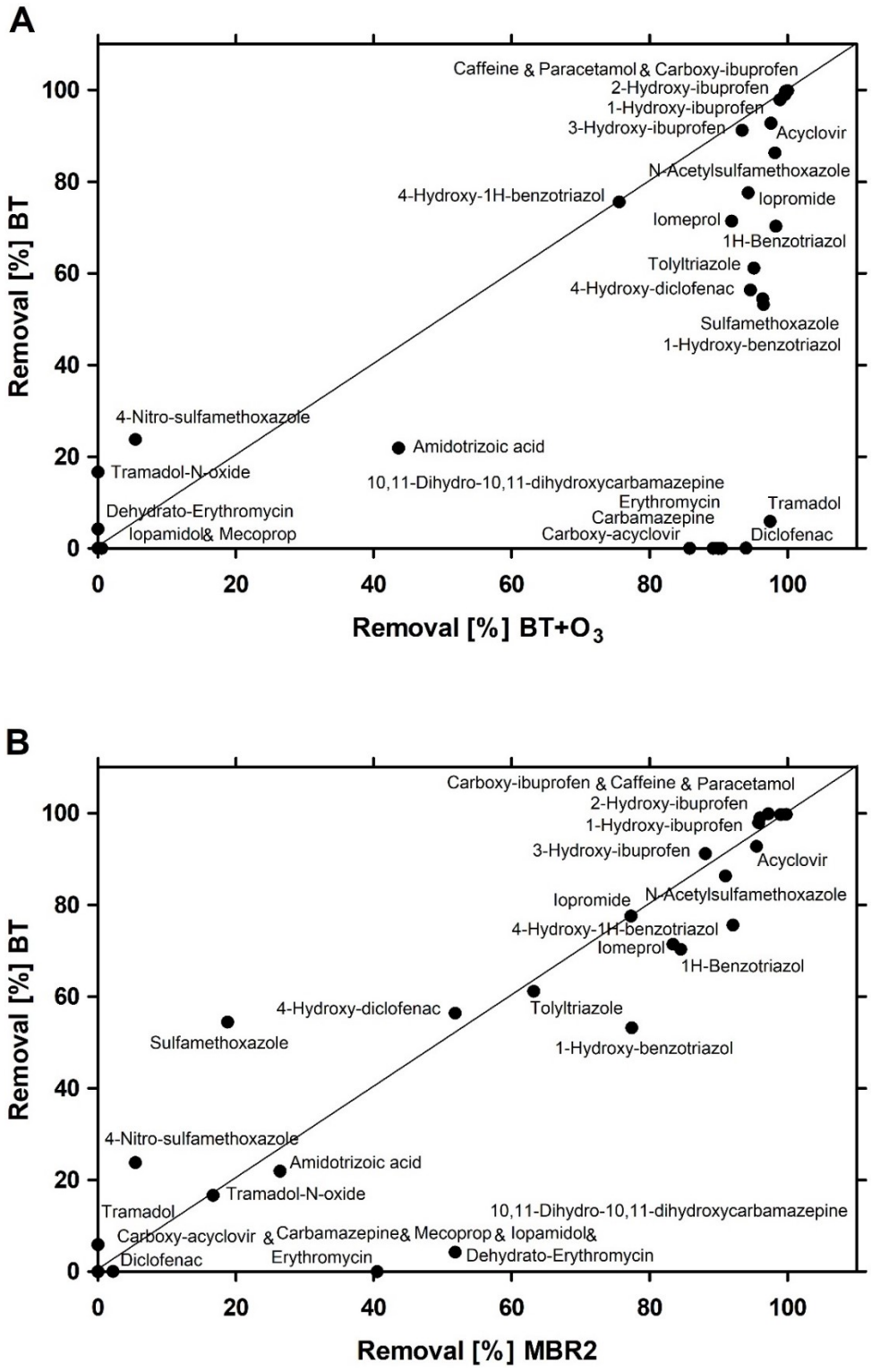

Figure 4: Removal of micropollutants by the conventional biological treatment (BT), the ozonation (BT+O_3, A), and the membrane bioreactor 2 (MBR2, B) compared to the primary treatment. Wastewater was sampled in parallel to the *in vivo* on-site experiment with *Gammarus fossarum*.

3 General discussion

3.1 Preparation of wastewater samples for *in vitro* bioassays

Several (eco)toxicological studies investigated the effect of conventional and advanced treated wastewater using a high number of different *in vitro* bioassays for diverse endpoints (compare above chapter 1.4). But there is only little information about the preparation of the wastewater samples for the utilised *in vitro* bioassays. The clean-up procedure of the wastewater samples and the extraction of the pollutants for the biological analyses was the same as for the chemical analyses in a study by Bertanza et al. (2011) whereas the sample preparation for the applied *in vitro* bioassays by Loos et al. (2013) was analogue to the chemical methods with only a few differences. But there is only little knowledge on the impact that these common (chemical) preparation techniques have on the results of the (eco)toxicological *in vitro* bioassays. The estimation of the (eco)toxicity of water and wastewater samples and the emerging contaminants again is an important aspect for the assessment of the water quality.

3.1.1 Acidification of wastewater samples

The acidification, for example with hydrochloric acid, of water and wastewater samples is a usual procedure before they undergo chemical analyses such as high performance liquid chromatography (HPLC), MS or gas GC with the objective of the stabilisation of the aqueous samples with its included chemicals and the prevention of biological processes for example microbiological degradation (Itzel et al. 2017; Prasse et al. 2015; Margot et al. 2013; Baker and Kasprzyk-Hordern 2011; Vanderford et al. 2011). In (eco)toxicological studies there is no distinct procedure identifiable for the acidification of (waste)water samples before they were investigated in diverse

bioassays. For instance, a buffer solution was added to the wastewater samples to ensure an acidic pH before they were tested *in vitro* for toxicity and estrogenic activity using a photobacterium, an enzyme-linked immunosorbent assay (ELISA), and yeast cells (Mispagel et al. 2009). Wastewater samples were acidified before they were investigated for vitellogenin induction as an endpoint for estrogenic activity in hepatocytes of a fish (Björkblom et al. 2008). (Diluted) hydrochloric acid was used to adjust an acidic pH of the wastewater samples prior to *in vitro* analyses for (anti)estrogenic and (anti)androgenic activity, aryl-hydrocarbon agonistic activity, cytotoxicity, neurotoxicity, mutagenicity, genotoxicity, phytotoxicity, bioluminescence inhibition, and non-specific toxicity using human breast cancer cell lines, rat pituitary cell lines, yeast cells, or bacterial strains (Magdeburg et al. 2014; Reungoat et al. 2012, 2011, 2010; Stalter et al. 2011; Svenson et al. 2003).

The above referred studies show that wastewater samples of diverse origin were acidified predominantly with hydrochloric acid before they were investigated in a wide range of different *in vitro* bioassays unknowingly which impact comes from this treatment steps and how they influence the results of the (eco)toxicological bioassays. In our study by Abbas et al. (2019, Annex A.1) 18 different types of aqueous water and wastewater samples such as raw (untreated) wastewater, conventional treated wastewater of municipal WWTPs, hospital wastewater, ozonated wastewater, surface water, and groundwater were acidified with sulfuric acid (5 mol/L) to pH 2.0 immediately after the sampling, stored for 24 h, and neutralised with natrium hydroxide. Afterwards, the samples were investigated in nine yeast-based recombinant reporter gene *in vitro* bioassays for the detection of endocrine activity (estrogenic, anti-estrogenic, androgenic, anti-androgenic, aryl-hydrocarbon-like, retinoic acid-like, retinoic X-like, vitamin D-like, and thyroid receptor-like) and in two *in vitro* bioassays using bacterial strains for the detection of mutagenicity and genotoxicity. A sterile

filtration (pore size: 0.2 µm) of the (waste)water samples was essential for the bioassays detecting genotoxicity and mutagenicity. Cytotoxicity was analysed in both types of bioassays. Focusing on a change of endocrine activity and mutagenicity of ≥ 10% compared to the neutral samples the results of our study showed in 81.2% of the bioassays a decrease of the activity and an increase of the activity in 18.8% of the bioassays due to the acidification. The most affected kind of (waste)water was the raw (untreated) wastewater indicating decreased activities in 50.0% of the bioassays due to the acidification followed by the influent and the effluent wastewater of a filtration basin whereas groundwater, ozonated wastewater, and surface water were least affected. The activities of the three bioassays detecting anti-estrogenic activity, retinoic X-like activity, and mutagenicity were most altered by the acidification process. Although, the shift of the activities showed no consistent picture. Compared to the corresponding neutral samples the anti-estrogenic activity and the mutagenicity both indicated decreased as well as increased activities. The highest impact caused by the acidification of the water and wastewater samples was observed in a bioassay detecting mutagenicity showing a decreased activity of –93.8% followed by a retinoic acid receptor activity of –87.9%. The observed endocrine, mutagenic, and genotoxic effects in the residual analysed bioassays were too low to assess the impact of the acidification process on these endpoints.

In the end, our data implied that the overall activity of the utilised *in vitro* bioassays is lower in the acidified water and wastewater samples compared to the corresponding ones that were kept at a neutral pH. One reason for the decreased activities may be the reduction of the active chemical compounds in the acidified sample by an increased adsorption to suspended matter (Baker and Kasprzyk-Hordern 2011). Another aspect may be an increasing hydrolysis of the chemical compounds (Prasse et al. 2015) that are responsible for the activity in the used bioassays. But the results of our study did

not definitely clarify which handling of the water and wastewater samples represents the endocrine activity and mutagenicity of the original samples. On the one hand, the acidification of aqueous water and wastewater samples significantly affected the endocrine activity and mutagenicity of the samples compared to the samples kept at a neutral pH. One the other hand microbial activity in the not acidified samples may lead to change in the composition of the sample due to a deconjugation of the active chemical compounds and thus, to an increased biological activity that was observed during conventional wastewater treatment (Wu et al. 2017; Koh et al. 2008; Andersen et al. 2003). In contrast, the biological activity may be decreasing in samples kept at neutral pH because of the ongoing microbial degradation of active compounds in the sample with increasing storage time (Giebner et al. 2018).

Finally, keeping the water and wastewater samples at a neutral pH and the acidification of the samples, each had an impact on the results of the endocrine activity and the mutagenicity and in the following way on the outcome of the investigated ecotoxicological *in vitro* bioassays. As a consequence, a misinterpretation of the original toxicity of water and wastewater samples is possible in both sample handlings. Based on the results of our study (Abbas et al. 2019, Annex A.1) an investigation of samples kept at a neutral pH is advisable because the water and wastewater samples are minimally processed and furthermore, the samples should be analysed as soon as possible to counteract a proceeding microbial degradation.

3.1.2 Filtration of wastewater samples

Comparable to the acidification process, water and wastewater samples are usually filtered before they were investigated in chemical analyses. Diverse membranes or glass fibre filters with varying pore sizes were used for the filtration process (Margot et al. 2013; Hernandez-Leal et al. 2011).

In (eco)toxicological studies wastewater samples are often filtered before they are investigated in *in vitro* bioassays mainly to reduce the included suspended solids and to avoid clogging of the filters during sterilisation (Dopp et al. 2021; Itzel et al. 2018; Mnif et al. 2010). In some cases of *in vitro* bioassays water and wastewater samples have to be sterile before they can be analysed in the bioassay and thus a sterile filtration is essential (Gehrmann et al. 2018). Also, glass fibre filters but as well nitrocellulose, cellulose nitrate, cellulose acetate, or syringe filters as well as polyethylene filter discs with varying pore sizes are used for the filtration of wastewater samples prior to investigations for (anti)estrogenic, (anti)androgenic, pregnane X, glucocorticoid, progesterone, mineralocorticoid, aryl-hydrocarbon agonistic, and dioxin-like activity, as well as cytotoxicity, genotoxicity, mutagenicity, neurotoxicity, phototoxicity, non-specific toxicity, acute toxicity, and bioluminescence inhibition using human cell cultures (hepatocytes, breast cancer, cervical carcinoma, prostate adenocarcinoma, liver carcinoma), hamster ovary cell cultures, yeast cells, bacterial strains, diatom cultures, or ELISA (Dopp et al. 2021; Itzel et al. 2019, 2018; Gehrmann et al. 2018; Reaume et al. 2015; Loos et al. 2013; Reungoat et al. 2012, 2011, 2010; Mnif et al. 2010; Mispagel et al. 2009; Svenson et al. 2003).

The referred studies illustrate that water and wastewater samples of various origin were filtered with different kinds of filter media before they were analysed in diverse *in vitro* bioassays without knowing which effect the filtration steps imply and how they affect the outcome of the (eco)toxicological bioassays.

In our study by Abbas et al. (2019, Annex A.1) 18 different types of aqueous water and wastewater samples for example raw (untreated) wastewater, conventional treated wastewater of municipal WWTPs, hospital wastewater, ozonated wastewater, surface water, and groundwater were filtered through a glass fibre filter with a pore size of 1 µm (Whatman GF6). In the following, the samples were analysed in nine yeast-based

recombinant reporter gene *in vitro* bioassays for the detection of endocrine activity (estrogenic, anti-estrogenic, androgenic, anti-androgenic, aryl-hydrocarbon-like, retinoic acid-like, retinoic X-like, vitamin D-like, and thyroid receptor-like) and in two *in vitro* bioassays using bacterial strains for the detection of mutagenicity and genotoxicity. A sterile filtration (pore size: 0.2 µm) of the (waste)water samples was essential for the bioassays detecting genotoxicity and mutagenicity. In both types of bioassays cytotoxicity was investigated. Focusing on a change of endocrine activity and mutagenicity of ≥ 10% compared to the not filtrated samples the results of our study showed that 33.3% of the investigated bioassays indicated a decrease of the activity whereas 66.7% of the bioassays indicated an increased activity due to the filtration process. Again, the raw (untreated) wastewater was at most affected by the filtration followed by surface water and conventional treated wastewater. Groundwater samples were not affected by filtration. The filtration process had the strongest impact on the bioassay detecting anti-estrogenic activity followed by estrogenic and anti-androgenic activity and aryl-hydrocarbon-like activity. Furthermore, the prepared aqueous suspensions of the filter retentates indicated notably endocrine activities as well only regarding endocrine activities ≥ 10%. Thereby, anti-estrogenic activity was detected in 57.1% of the bioassays whereas 42.9% of the bioassays showed anti-androgenic activities. In the remaining utilised bioassays, only low endocrine activity, mutagenicity, or genotoxicity were detectable. Thus, no conclusion of the sample filtration on these endpoints was possible. However, Svenson et al. (2003) reported that there was no evidence for the adsorption of estrogenic compounds on the particles that were collected by a filtration through 20 µm polyethylene filter discs.

In summary, in some cases the activity in the filtered samples was lower compared to the corresponding unfiltered samples whereat parts of the aqueous suspensions of the filter retentates also showed activity in the used bioassays. Hence, the activity possibly

related to particle associated hormones and EDCs was retained by the filtration process (Shieh et al. 2016; Dagnino et al. 2010; Routledge 2003). In other cases, the activity detected in the filtered sample was higher in comparison to the corresponding unfiltered sample whereas the aqueous suspension of the filter retentate was active as well. Further cases indicated a comparable high level of activity in the unfiltered as well as in the corresponding filtered sample and in addition the suspension of the filter retentates also showed high levels of activity. Even one sample was not active as unfiltered and filtered sample but the suspension of the filter retentate showed activity in one bioassay. The above-mentioned observations may be the result of a potential leaching of substances out of the filter material that caused the noticed activities in the *in vitro* bioassays. Concerning the results of our study this explanation is less applicable because aqueous samples of ultra-pure water run through empty glass fibre filters in parallel to the water and wastewater samples. In addition, aqueous suspensions of pure glass fibre filter material were produced. These samples served as a control group and were analysed in parallel with the water and wastewater samples in the whole range of utilised bioassays. The results of the filter controls indicated no activity in the bioassays. Another reason for the observed activities in the present case may be the result of an alteration of the ratio of compounds inside the samples that have agonistic or antagonistic potential with regard to the respective receptor (Ihara et al. 2014; Rao et al. 2013). Dissimilar affinities towards suspended solids within the sample and/or the filter material (Wangmo et al. 2018; Ng and Cao 2015) could have resulted in a retention of substances with agonistic properties and thus an increased antagonistic activity in the bioassay was detectable. Just as the reverse effect if the retention of compounds with antagonistic properties lead to an increase of the agonistic activity in the relative bioassay.

In the end, the filtration process of water and wastewater samples distinctly affected the endocrine activity as well as the mutagenicity and thus the outcome of the investigated ecotoxicological *in vitro* bioassays. Consequently, comparable to the acidification process of water and wastewater samples, a misestimation of the original toxicity of the samples is quite possible. Furthermore, the assessment of the cleaning capacity of a WWTP and/or single (advanced) treatment steps may be underestimated or overestimated depending on whether the results of the *in vitro* bioassays of the unfiltered (original) or filtered samples as well as the neutral (original) or acidified samples were considered for the basis of valuation. In conclusion, the implementation of the filtration and/or acidification of water and wastewater samples should be well-considered and well-adapted to the aims of the study, to the characteristics of the water and wastewater samples to be analysed as well as to the specificities of the investigated (eco)toxicological *in vitro* bioassays.

3.1.3 Solid phase extraction

The extraction of wastewater samples for the enrichment of organic substances is on the one hand required for chemical analysis to improve the sensitivity of MS-GC screenings and LC-MS screenings as well as the following characterisation of the substances but on the other hand also to increase the sensitivity of effect-based investigations. In addition, the extraction of organic substances effectuates a separation as the case may be disruptive substances such as phosphate, nitrate, and ammonium and as a result matrix effects were minimised (Schmidt et al. 2011). A SPE is suited for the isolation and the concentration of organic constituents of municipal wastewater effluent prior to analysis with (eco)toxicological *in vitro* bioassays (Björkblom et al. 2008). For example, wastewater samples were extracted by a SPE similarly to the chemical methods with only a few differences. Afterwards, these

extracts were analysed in (eco)toxicological *in vitro* bioassays (Loos et al. 2013). However, so called SPE discs could be used for the extraction purpose within the scope of the sample preparation. These SPE discs are suitable to cover the whole spectrum of substances from non-polar compounds to polar compounds. Furthermore, MPs that are bounded to particles inside the wastewater sample are included during the extraction process. Another benefit of the SPE discs is the diameter of 47 mm that allows the prevention of a blockage of the filters while the wastewater samples were extracted. Thus, a representative enrichment of the wastewater samples is allowed (Schmidt et al. 2011). Also, Macova et al. (2010) discussed that a (waste)water sample enrichment by SPE led to increased concentrations of MPs in the extracts and thus, enables a better detection in the used *in vitro* bioassays. Furthermore, the previous SPE also limits the impact of matrix components and metals that are in part separated throughout the extraction process (Margot et al. 2013).

In original and non-concentrated water and wastewater samples effects in (eco)toxicological *in vitro* bioassays are difficult to detect because the concentrations of MPs are typically in the range of micro-, nano- or even picogram per litre. Hence, a sample preparation prior to the bioanalysis is required and an enrichment of water and wastewater samples is recommended (Neale et al. 2018; Gehrmann et al. 2018; Grummt et al. 2013). The performance of a SPE is commonly applied to enrich water samples. Environmental water samples like surface water or drinking water often needed to be enriched up to 100 times to detect an effect. Considering the dilution factor of the water samples in the conducted bioassays the initial enrichment of the water sample by SPE is typically 1000 to 2000-fold (Neale et al. 2018).

Several types of SPE cartridges such as Oasis HLB (predominantly), Strata XL, C18, ENV+, LiChrolut EN-RP18, and Sep-Pack tC18 are used preliminary to investigations for (anti)estrogenic, (anti)androgenic, progesterone, pregnane X, aryl-hydrocarbon

agonistic, mineralocorticoid, glucocorticoid, and dioxin-like activity, disruption of the steroidogenesis pathway, vitellogenin induction, cytotoxicity, genotoxicity, mutagenicity, neurotoxicity, phytotoxicity, non-specific toxicity, acute toxicity, and bioluminescence inhibition using human cell cultures (hepatocytes, breast cancer, cervical carcinoma, prostate adenocarcinoma, liver carcinoma), hamster ovary cell cultures, rat pituitary cell lines, fish hepatocytes, algae, yeast cells, bacterial strains, diatom cultures, or ELISA (Dopp et al. 2021; Itzel et al. 2019, 2018, 2017; Gehrmann et al. 2018; Giebner et al. 2018; Reaume et al. 2015; Magdeburg et al. 2014; Loos et al. 2013; Maletz et al. 2013; Margot et al. 2013; Reungoat et al. 2012, 2011, 2010; Stalter et al. 2011; Mnif et al. 2010; Björkblom et al. 2008; Mispagel et al. 2008; Hashimoto et al. 2007; Svenson et al. 2003). SPE extracts are also used in an *in vivo* reproduction test with a mudsnail (Giebner et al. 2018).

The above-mentioned studies illustrate that there is no consistent practice for the preparation and the enrichment of water and wastewater samples prior to the investigation in (eco)toxicological *in vitro* bioassays. Several types of SPE cartridges were utilised to produce SPE extracts from diverse types of wastewater which were afterwards analysed in large range of *in vitro* bioassays.

In our study by Abbas et al. (2019, Annex A.1) the optimal recovery of endocrine, genotoxic, and mutagenic activity out of different types of wastewater such as raw (untreated) wastewater, hospital wastewater, conventional (biological) treated wastewater, ozonated wastewater, and groundwater was investigated by the use of three generally applied SPE cartridges (Kinesis Telos C18/ENV, Oasis HLB, and Supelco ENVI-Carb+). Thereby, each (waste)water sample was extracted at two pH values: neutral at a pH of 7 and acidified with sulphuric acid to a pH of 2.5. The generated dimethyl sulphoxide (DMSO) extracts were 5000-fold concentrated compared to the original aqueous samples. Throughout the performance of the *in vitro*

bioassays the extracts were diluted by diverse factors resulting in a 10.4-fold final sample concentration for the yeast-based bioassays detecting endocrine activity and in a 20-fold respectively 10-fold final sample concentration in the bioassay using bacterial strains detecting genotoxicity and mutagenicity. The results of our study showed the occurrence of cytotoxicity in all bioassays but the choice of the SPE method had a substantial influence on the detection of the cytotoxic activity. The highest cytotoxicity was detected in the extracts of the Kinesis Telos C18/ENV at neutral pH (50.0%) followed by the Oasis HLB cartridge (32.1%) and the Supelco ENVI-Carb+ (11.5%), both also extracted at neutral pH. On the contrary, extracts produced from acidified samples induced distinctly lower cytotoxic effects (Kinesis Telos C18/ENV: 12.8%, Oasis HLB: 15.4%, Supelco ENVI-Carb+: 0.0%). There are special *in vitro* bioassays for the detection of cytotoxicity but in other *in vitro* bioassays cytotoxic effects indeed provide information about the toxicity of the analysed wastewater sample but the cytotoxicity also masked the primary considered endpoint of the investigation for example endocrine activities, genotoxicity or mutagenicity. One possibility is the dilution of the extract of the wastewater sample until cytotoxic effects no longer occur in the utilised bioassay (Figure 5A). But one disadvantage of a dilution is the fact that the possible endocrine activity, genotoxicity or mutagenicity inside the sample is diluted as well. Therefore, one can conclude a false negative result if the dilution of the extract is too high and the possible endocrine, genotoxic or mutagenic activity lies under the limit of detection of the bioassay (Figure 5A, sample 64). Furthermore, if there is an activity detectable in a diluted sample it is not possible to calculate the activity of the original undiluted sample (activity of the diluted sample multiplied with the dilution factor) because there is no linear relationship between the activity of the sample and the dilution factor. Thus, a serial dilution of the cytotoxic extract has to be produced to be able to compute the activity in the original undiluted

wastewater sample (Figure 5 B,C). But in some cases, a dilution series of the extract could not be applied due the limited sample extract volumes (Stalter et al. 2011).

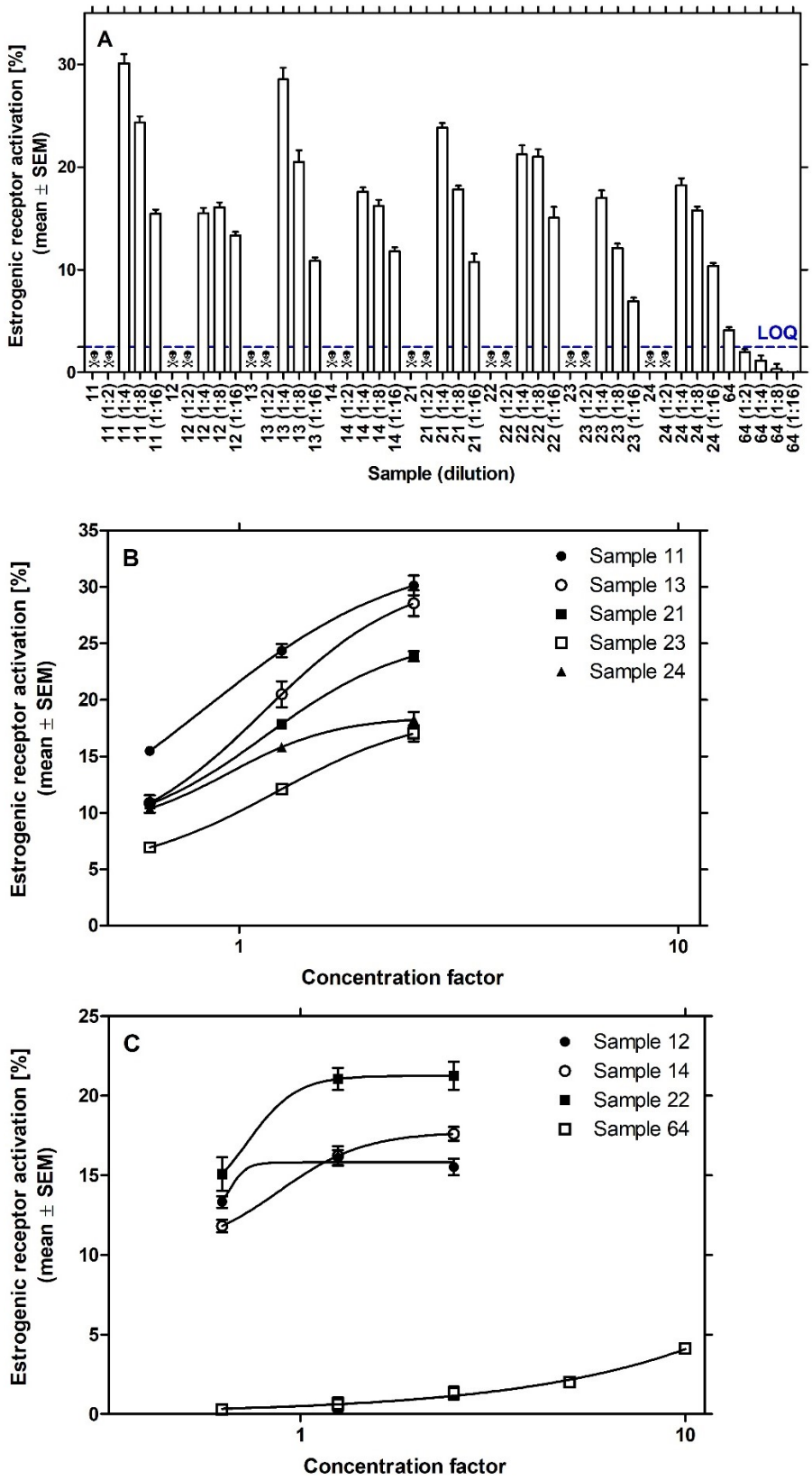

Figure 5: Occurrence of cytotoxicity (☠) in 10-fold SPE extracts of different wastewater samples (hospital wastewater: samples 11, 12, 13, 14; raw (untreated) wastewater: samples 21, 22, 23, 24; conventional biological treated wastewater: sample 64), diverse dilutions of the extracts (1:2 to 1:16) and the estrogenic receptor activation in % (mean ± SEM) of these samples (A). Estrogenic receptor activation in % (mean ± SEM) depending on the concentration factor (0.625, 1.25, 2.5, 5.0, and 10.0) of the serial dilution of each sample to be able compute the estrogenic activity of the original undiluted SPE extract of the wastewater sample (B, C); LOQ: limit of quantification)

Also, Maletz et al. (2013) observed that the SPE extracts of untreated hospital effluents with sewage concentrations equal or greater than one-time (1x) caused significant cytotoxicity to the yeast cells in a bioassay for the detection of estrogenic activity.
Gehrmann et al. (2018) as well reported that the extracted influent samples with an enrichment factor of 20-fold of a WWTP exclusively treating hospital wastewater showed cytotoxic effects in cell cultures and yeast-based *in vitro* bioassays and could not be used for the assessment of estrogenic activity.
Cytotoxic effects of SPE-extracts of several types of wastewater (conventional treated wastewater, ozonated wastewater, after ozonation with following SF, after treatment with PAC and subsequent SF) were investigated in a study by Stalter et al. (2011) using the rat pituitary cell line GH3. The results indicated a maximum toxicity in 10-fold concentrated DMSO extracts in all treatment groups and thus, the extracts were tested in a 5-fold final sample concentration. In contrast, methanol extracts with a 10-fold final sample concentration were analysed in the bioassay because 5-fold concentrated extracts did not induce significant toxic effects after the conventional treatment. In addition, their results also emphasised that the sample preparation and the choice of the method for the SPE enrichment substantially affected the cytotoxic effects and

thus, the outcome of the *in vitro* bioassay. SPE extracts of ozonated wastewater extracted at a neutral pH of 7 and dissolved in DMSO showed a reduced cytotoxicity by 32% compared to the secondary clarifier whereas the DMSO extract of the ozonated wastewater sample that was extracted at an acidified pH of 2 indicated a reduction of the cytotoxicity by 47% compared to the secondary clarifier which implies a difference between the two SPE methods of averaged 24%. The authors inferred that the reduced cytotoxicity of the samples that were extracted at pH 7 compared to those extracted at pH 2 indicated the presence of toxic compounds with acidic moieties particularly after the ozonation process. A significantly reduced mutagenicity with the Ames fluctuation test was observed by Magdeburg et al. (2014) in samples extracted at pH 7 in comparison to extracted samples at a lower pH of 2 implying that the samples contained mutagenic compounds with acidic moieties that need to be protonated prior to the SPE enrichment. The methanol extracts that also were extracted at a pH of 2 were evaporated to dryness before they were tested in the bioassay and they led to a reduction of the cytotoxic effects by 72% after ozonation and by 71% after activated carbon treatment with subsequent SF each compared to the secondary clarifier even though the sample concentration (10-fold) was doubled compared to the DMSO extracts that were 5-fold concentrated (Stalter et al. 2011). The authors argued that the reduced cytotoxicity of the methanol extracts implied that the complete evaporation of the solvent extracts in the test vessels of the bioassay caused a considerable loss of toxic volatile substances. Hence, the toxic effects of the wastewater samples are consistently underestimated especially after the ozonation. Escher et al. (2005) investigated the SPE enrichment of spiked water and urine samples with a mixture of six pharmaceuticals (carbamazepine, diclofenac, EE_2, ibuprofen, propranolol, and sulfamethoxazole) by the use of seven different SPE-cartridges (amongst others Oasis HLB) and different pH values (pH 3, pH 7, pH 9, and pH 11). The SPE extracts were

analysed with the bioluminescence inhibition test with the marine bacterium *Aliibrio fischeri* the chlorophyll fluorescence test with the green unicellular green algae *D. subspicatus*, and the YES with the yeast *Saccharomyces cerevisiae*. The results showed that the recovery rates depended on the pH value and were highest at pH 3 (up to 143%) and decreased with increasing pH (up to 91% at pH 7 and up to 38% at pH 11). Furthermore, the recovery rates differed distinctly inside the used SPE cartridges. The authors recommended using LiChrolut EN/RP-C18 as a solid-phase material and the performance of the extraction at a pH 3 for the proposed screening test battery for the assessment of the cumulative impact of pharmaceuticals and other MPs in urine, wastewater, and environmental samples.

Various samples of disinfected (chlorinated) drinking water were enriched by the use of 11 different SPE cartridges (amongst others Oasis HLB, Telos ENV, LiChrolut, StrataX, and ENV+) by Stalter et al. (2016). The samples were extracted at an acidic pH between pH 1 and pH 3 with eight SPE cartridges and at a neutral pH 7 with three cartridges. The sorbent Telos ENV performed superior for the enrichment of non-volatile disinfection by-products in comparison to the ten other sorbents and showed the highest recovery rates followed by the Oasis HLB, LiChrolut, StrataX, and ENV+ cartridges using the bacterial Microtox assay testing for cytotoxicity and mammalian cells indicating the induction of oxidative stress. Furthermore, the authors highlighted that SPE is unsuitable for the extraction of volatile disinfection by-products and a separate extraction step is needed.

However, the comparison of aqueous and extracted wastewater samples indicated that the estrogenic activity of the extracted samples was generally as low as the corresponding aqueous samples (Abbas et al. 2019, Annex A.1). Thus, either no enrichment of estrogenic active substances was feasible by the used SPE methods or the estrogenic activity was covered by substances with anti-estrogenic potential. The

anti-estrogenic activity detected in the extracted samples was varying depending on the extraction method and in parts very high and comparable to the aqueous samples as well. Hence, these compounds were potentially extractable by the investigated SPE methods. Furthermore, substances with anti-androgenic or genotoxic potential seemed to be extractable by the used SPE methods because the aqueous samples showed no genotoxicity and only low anti-androgenic activity in contrast to the corresponding extracts indicating obviously higher activities. Conspicuously, potentially genotoxic extracts only occurred in extracts produced by the Kinesis Telos C18/ENV and Oasis HLB cartridges and not by the Supelco ENVI-Carb+ columns and any more, the anti-androgenic activity was averaged appreciably higher in the two at first above mentioned cartridges than in the third one. These results are possibly due to the different compositions of the filter materials inside the cartridges.

However, a multivariate optimisation based on Pareto was calculated (Durmaz et al. 2015; Ehrgott 2000) to statistically distinguish between optimal and non-optimal SPE methods regarding the results of the different water and wastewater samples at the two investigated pH values and the various *in vitro* bioassays. The Pareto results showed that the Telos C18/ENV and Oasis HLB cartridges, both at pH 7, were optimal in the effectivity of extracting the different water and wastewater samples and indicated the highest endocrine activities. At the same time, these methods exhibited the highest cytotoxic effects masking the endpoint under investigation. But both cartridges at a pH of 2.5 also showed good results in the extraction of endocrine activity with lower cytotoxicity. Thus, the authors recommended the Telos C18/ENV at pH 2.5 followed by the Oasis HLB at pH 2.5 as well for the enrichment of different types of (waste)water for the investigation of various endpoints of *in vitro* bioassays.

In this context, Oasis HLB cartridges (200 mg) were successfully applied to extract estrogenic as well as androgenic activity (low activities in the aqueous samples

compared to high activities in the SPE extracts) whereas the enrichment of anti-estrogenic activity was not as effective (lower activities in the SPE extracts compared to the aqueous samples) in a study by Giebner et al. (2018).

As a result of a research by Dopp et al. (2021), the SPE-enriched samples using an Oasis HLB cartridge of one WWTP showed no genotoxic effects even though the original aqueous samples were genotoxic in tests with human hepatocytes (HepG2 cells) and human liver carcinoma cells (HepaRG cell line). The proved cytotoxicity and genotoxicity in original aqueous samples in contrast to the SPE enriched samples may be due to the extraction method and the loss of components throughout the extraction process. On the other hand, the aqueous samples showed no cytotoxic effects using a test system with Chinese hamster ovary (CHO-9) cells whereas cytotoxicity was detected in the SPE extracts of all investigated samples indicating the extractability of cytotoxic substances by the used SPE method. In addition, estrogenic activity was successfully enriched by the Oasis HLB cartridge indicated by an estrogen receptor chemically activated luciferase gene expression (ER-CALUX) test with a human breast cancer cell line (Dopp et al. 2021). Furthermore, Strata XL (polymer-based) cartridges were feasible to extract substances or compounds with estrogenic activity, anti-estrogenic activity, and anti-androgenic activity detected with two yeast-based bioassays and a human breast cancer cell line (Gehrmann et al. 2018).

Finally, the observed effects in the utilised *in vitro* bioassays were a result of a mixture of the extractable fraction of the organic pollutants present in the wastewater sample. Potentially toxic or endocrine active by-products formed by ozonation are neglected because these substances are hardly extractable with the conventional SPE methods because of their high polarities and degradability (Stalter et al. 2011). In this context, potentially toxic by-products originating from the oxidation of dissolved organic matter (DOM) such as aldehydes, ketones and other oxygen-rich compounds might not be

enriched during SPE necessary for the investigated *in vitro* tests (Zimmermann et al. 2011). For example, the enrichment of the TPs of propranolol that were formed during the ozonation process was not feasible by SPE due to the high polarities of the oxidation products (Stalter et al. 2010a; Benner & Ternes 2009b). Hence, the polar TPs generated by the ozonation were not considered in toxicity analysis due to the limited extractability and thus, the non-specific toxicity of wastewater after the treatment with ozone is underestimated again. Consequently, further research on the choice of the extraction method is desirable to increase the fraction of extractable polar oxidation products (Stalter et al. 2011). Likewise, Reungoat et al. (2012) reported that very hydrophilic (polar) and volatile substances are not captured by the cartridges used during the SPE enrichment and thus, these compounds are not expected to have a significant effect on the whole mixture toxicity. Besides, Neale et al. (2018) mentioned that the use of *in vitro* bioassays increased for the monitoring of water quality. But for example, water samples from surface water oftentimes need to be enriched to detect an effect in these bioassays. Therefore, a SPE is commonly applied. However, the applied methods are typically optimised for chemical analysis and for the recovery of the target chemicals and not for the effect recovery in *in vitro* bioassays. The authors complain that there is considerably less work with only a few studies analysing a SPE recovery for these bioassays and that there is a lack of experimentally determined recoveries by typically applied enrichment techniques. But the understanding of the recovery of biological effects is essential for the application of bioassays for the monitoring of the water quality and for the regulatory acceptance of these tools. Thus, the authors adjusted pristine surface water samples to a pH of 6.5 and spiked them with a mixture of 579 organic chemicals including pharmaceuticals, pesticides, industrial compounds, and natural and synthetic hormones which covered a wide range of physicochemical properties and extracted them by SPE using multi-layer SPE

cartridges (for example Oasis HLB, Strata X, Isolute Env+, and Supelclean EnviCarb). Non-specific effects were investigated by the measurement of cytotoxicity and the activation of xenobiotic metabolism (for example the activation of the aryl hydrocarbon and pregnane X receptor), hormone receptor mediated effects (such as the activation of the estrogen, androgen, glucocorticoid, and progesterone receptor), adaptive oxidative stress responses, and fish embryo toxicity/mortality. The results clearly showed that the performance of a SPE as a sample preparation method is required for samples of surface water. In addition, the study pointed out that adequate controls (blanks) have to be prepared in parallel to the water samples when the used SPE methods for the *in vitro* bioassays are adapted from chemical analysis because their results indicated that the SPE blanks triggered cytotoxic effects at a high enrichment of the water samples. Also, Grummt et al. (2013) pointed out that the concentrations of most of the emerging chemicals detected in drinking water are several orders of magnitude below the concentrations that were investigated in the standard test systems commonly used in (eco)toxicological research. Thus, new methods have to be found to increase the concentrations of these chemicals. The authors noticed as well that the practice of the enrichment of water samples that is routinely implemented in chemical analysis have to be adapted to the requirements of the bioassays that will be used for example for risk assessment.

The above-mentioned studies show that each extraction method effected changes of the composition of the contaminants in the extracts. Some compounds get lost for example via volatilisation and further compounds are not equally enriched due to their wide range of physicochemical properties. Therefore, the relative enrichment factors refer to the volume of the enriched sample and do not reflect the enrichment of each sample's chemical constitution. This highlights the importance of using optimised and well-defined and well-adjusted methods for the enrichment of the samples (Stalter et

al. 2016). In this context, the used sorbent (Stalter et al. 2016; Chang et al. 2009; Escher et al. 2005), different sample volumes (Schulze et al. 2017; Macova et al. 2011), the solvents utilised for the elution (Välitalo et al. 2017; Yang et al. 2014; Lu et al. 2010), fractionation steps (Leusch et al. 2017; Välitalo et al. 2017), and divers operating modes such as a SPE performance with multilayer cartridges or large volumes of water and wastewater (Köke et al. 2018; Neale et al. 2018; Schulze et al. 2017) are factors that can be optimised.

Summarising the last three subitems water and wastewater samples oftentimes were filtered, acidified or enriched by a SPE before they were analysed in (eco)toxicological *in vitro* bioassays. The impact of these treatment steps on the composition of chemical and biological active ingredients of the (waste)water sample are rarely known. The results clearly show that further research is unconditionally required.

3.2 Removal of micropollutants during conventional and advanced wastewater treatment

The influent wastewater of WWTPs included a plenty of pharmaceuticals and priority organic compounds. Anti-inflammatory drugs such as ibuprofen and salicylic acid were the most frequently detected pharmaceutical compounds and reached the highest concentrations that could be associated with the high consumption of these pharmaceuticals because a medical prescription is not necessary (Camacho-Muñoz et al. 2012).

3.2.1 Conventional wastewater treatment

The main removal pathways of organic pollutants in the wastewater treatment are biodegradation, sorption to the generated sludge, air-stripping, and photo-transformation whereas the last two mechanisms are neglectable in WWTPs. Hydrophobic substances presumably are mainly removed by a retention in the soil while biodegradation processes are more probable for hydrophilic compounds (Camacho-Muñoz et al. 2012; compare above chapter 1.2).

However, benzotriazoles are typical examples of polar and poorly degradable trace pollutants. Benzotriazoles, such as the high production volume chemical benzotriazole, are used in many industrial applications, but also in households for example as anticorrosive additives in dishwashing agents and at airports in deicing fluids. These chemical substances own a high polarity and thus a high solubility in water but they are also moderately persistent against biological and photochemical degradation processes in WWTPs and thus they occur in high concentrations in municipal wastewater and in the aquatic environment (Altmann et al. 2014; Loos et al. 2013; Janna et al. 2011; Reemtsma et al. 2010). Also, several iodinated X-ray contrast media such as iomeprol as well as the antiepileptic drug carbamazepine were found to be almost completely resistant to biological degradation and thus, are frequently found in the aquatic environment in parts in high concentrations as well. Thereby, a variation of the measured iomeprol concentrations was detected that is probably due to locally differing applications of different X-ray contrast media (Hollender et al. 2009; Altmann et al. 2014). Furthermore, many polar drugs and biocides such as the antimicrobials clarithromycin and trimethoprim as well as the analgesic diclofenac were only in parts sorbed or degraded. However, several pharmaceuticals with lower concentration levels in the range of µg/L to ng/L for example paracetamol or ibuprofen were reduced by > 90% in the activated sludge process of the investigated WWTPs (Hollender et al.

2009). Also, Margot et al. (2013) reported that the analgesic paracetamol was completely eliminated in all investigated campaigns of the conventional wastewater treatment. But the detected concentrations of some compounds such as the beta blocker metoprolol, the antibiotic clindamycin, and most of the pharmaceutical metabolites were higher in the effluent of the BT compared to the influent wastewater. These results could be due to firstly the biological cleavage of pharmaceutical conjugates (human metabolites) during the treatment producing again the parent compound, secondly to the formation of bacterial metabolites throughout the BT, thirdly to the release of compounds during the BT that were trapped in faeces particles and fourthly to analytical uncertainties (Margot et al. 2013). In this context, the discharge of municipal wastewater is one main polluter for the entry of perfluoralkyl substances (PFASs) such as perfluorooctanoic acid (PFOA) and perfluorooctansulfonic acid (PFOS) into the aquatic environment. The concentration of PFAS often increase in WWTPs due to the biodegradation of precursor substances during the activated sludge treatment. In general, PFOA is completely discharged into the receiving water whereas about 50% of PFOS is retained in the sewage sludge (Loos et al. 2013).

In our studies (Schneider et al. 2020, Annex A.2; unpublished data: compare 2.2.5 and Annex A.5) the substances carbamazepine and carboxy-acyclovir amongst others showed lower mean concentrations in the influent wastewater and higher mean concentrations after the conventional treatment. Carboxy-acyclovir is a product of the biotransformation of the pharmaceutical and antiviral drug acyclovir in humans as well as in microorganisms in the activated sludge treatment of WWTPs and was detected with concentrations in the range of 247 ng/L to 430 ng/L in the influent and 890 ng/L to 2380 ng/L in the effluent whereas simultaneously the concentration of the mother compound acyclovir decreased from 1800-1990 ng/L in the influent to 121-148 ng/L in the effluent. Even in different environmental samples such as surface water and

drinking water carboxy-acyclovir was proved with concentrations up to 3200 ng/L and 40 ng/L, respectively. Furthermore, actually eight TPs were identified for penciclovir, an antiviral drug as well, after the conventional wastewater treatment (Prasse et al. 2012, 2011). An inverse relationship could be the explanation for the increased concentrations of carbamazepine detected in the wastewater. Conjugated compounds, such as hydroxylated human carbamazepine as a component of the influent, are potentially cleaved by the microorganisms during the conventional treatment and thus, are transformed into the original mother compounds (Besha et al. 2017).

The conventional treatment exhibited large variations of the removal rates of the investigated compounds among the different wastewater sampling campaigns. These variations could in parts be due to the diverse levels of nitrification that were reached in the BT such as the degree of the nitrification of ammonium. A removal of less than 30% in a non-nitrifying sludge was observed for some MPs (for example bisphenol A) compared to more than 60% removal in a treatment with complete nitrification (Margot & Magnet 2011). The higher removal rates of the MPs observed at high nitrification levels could probably be explained by the longer HRT in the reactor that led to a longer period that is available for the degradation processes in addition to the presence of a more divers microbial population with different metabolism mechanisms and at least a higher activity of the nitrifying bacteria (Margot et al. 2013).

Also, Boonnorat et al. (2017) reported on a further decrease of toxic compounds such as diclofenac, carbamazepine, bisphenol A and *N,N*-diethyl-*m*-toluamide (DEET) in the wastewater by increasing the nitrogen concentration. The authors argued that a higher nitrogen content improved the amount and the diversity of the bacteria and particularly the nitrifying bacteria producing enzymes that are an important factor within the degradation of toxic compounds.

The removal rate after the conventional treatment was low for the anticonvulsant carbamazepine (7.6%), the analgesic/anti-inflammatory substance diclofenac (9%), the corrosion inhibitor benzotriazole (24%), the iodinated contrast media iomeprol (25%), iopamidol (21%), and iopromide (29%), the herbicide mecoprop (29%), and the drug metabolite 10,11-dihydro-10,11-dihydroxycarbamazepine (0%). The antibiotic sulfamethoxazole and the analgesic/anti-inflammatory substance ibuprofen showed removal rates in the medium range of 38% and 57%, respectively. However, high removal rates were detected for the food component caffeine (> 92%), the drug metabolite N-acetyl-sulfamethoxazole (93%), and the analgesic/anti-inflammatory substance paracetamol (100%) (Margot et al. 2013).

These research findings are comparable to the results of our studies (Schneider et al. 2020, Annex A.2; unpublished data: compare 2.2.5 and Annex A.5). Low removal rates after the conventional treatment were detected for tramadol (10.8%), 10,11-dihydro-10,11-dihydroxycarbamazepine (11.1%), 4-nitrosulfamethoxazole (11.1%), diclofenac (12.7%), iomeprol (25.6%), iopamidol (10.0%), mecoprop (11.1%), and amidotrizoic acid (24.0%). The decrease of the concentrations of tolyltriazole (51.5%), 1H-benzotriazole (69.3%), 1-hydroxy-benzotriazole (62.4%), 4-hydroxy-diclofenac (51.0%), iopromide (64.1%) and sulfamethoxazole (74.2%) were in a medium range. A high removal rate was achieved for acyclovir (93.3%), 3-hydroxy-ibuprofen (93.7%), 4-hydroxy-1H-benzotriazole (91.6%), and N-acetyl-sulfamethoxazole (89.6%) and an almost complete reduction were indicated by 1-hydroxy-ibuprofen (96.8%), 2-hydroxy-ibuprofen (98.7%), paracetamol (99.7%), carboxy-ibuprofen (99.8%), and caffeine (99.8%).

Finally, the average concentrations of MPs in the wastewater after the conventional treatment is on the one hand impacted by the composition of the raw (untreated) wastewater. The composition of the raw wastewater is on the other hand dependent

on the consumption of these compounds in the watershed of the investigated WWTP. Higher concentrations of specific pharmaceuticals such as iodinated contrast media are probably due to the presence of many hospitals and clinics in the catchment area. An increased concentration of pesticides in the wastewater presumably resulted from wet weather phases and rain events and the arising of run-off water causing the leaching of facades and the discharge of pesticides that were used in gardens and the urban area. Thus, the daily average concentration of the same compound in the influent wastewater showed high variations between different sampling campaigns likely due to variations of the consumptions of these compounds in the watershed of the WWTP. These results highlight the importance of long-term sampling campaigns of at least one year to cover the different consumption habits of the respective substances (Margot et al. 2013). Furthermore, a comparison with other studies investigating different types of wastewater is important for the interpretation of the results of the respective study (Loos et al. 2013).

In the end, next to PFASs and benzotriazoles also diverse pharmaceuticals, personal care products, pesticides, sweeteners, organophosphate ester flame retardants, and X-ray contrast agents were detected in the effluent of WWTPs with conventional treatment (Loos et al. 2013) indicating that this treatment is not sufficient to remove these substances from the wastewater and thus, they could have impacts on the aquatic environment because these substances were already detected in surface water as well as in groundwater (Loos et al. 2010, 2009).

3.2.2 Advanced wastewater treatment

3.2.2.1 Ozonation

In three WWTPs the degree of the removal of the investigated trace organic chemicals that was achieved by ozonation depended on the compounds themselves and the

ozone dose. Some chemicals were efficiently removed independent from the ozone dose whereas the removal rate of other substances was lower and generally dependent on the ozone dose. An increase of the ozone dosage led to an increased removal rate especially for compounds with lower removal rates (Reungoat et al. 2012; 2010).

The removal rate after the ozonation was low for the iodinated contrast media iomeprol (43%), iopamidol (42%), and iopromide (34%), and in a medium range for the drug metabolites 10,11-dihydro-10,11-dihydroxycarbamazepine (47%) and N-acetyl-sulfamethoxazole (50%), the herbicide mecoprop (60%), the analgesic/anti-inflammatory substance ibuprofen (63%), and the corrosion inhibitor benzotriazole (64%). High removal rates were reached for the analgesic/anti-inflammatory substances paracetamol (> 85%) and diclofenac (94%), the antibiotic sulfamethoxazole (93%), the anticonvulsant carbamazepine (97%), and the food component caffeine (> 92%). A few MPs showed removal rates of more than 90% even at the lowest investigated ozone dose (Margot et al. 2013). Also, Magdeburg et al. (2014) reported on medium removal rates in the range of 49% to 55% for iodinated contrast media and high removal efficiencies of more than 90% for carbamazepine, sulfamethoxazole, diclofenac, tramadol, and clarithromycin. Most of these (pharmaceutical) substances such as antibiotics, beta-blockers, analgesic/anti-inflammatory drugs, anticonvulsants, pesticides, and substances with estrogenic activity had a high and quick ozone reactivity because of selective electronic-rich moieties for example activated aromatic systems (such as phenols and anilines), amines, tertiary amino groups, or double bonds (for example olefines) (Margot et al. 2013; Lee & von Gunten 2012; Altmann et al. 2014; Hübner et al. 2012; Lee et al. 2012; Schaar et al. 2010; Reungoat et al. 2010; Hollender et al. 2009). Lower removal rates than expected could be explained to the possible sorption of the substance to aquatic

colloid particles (1 nm to 1 µm) that presumably protect it against the attack of the ozone (Zimmermann et al. 2011; Worms et al. 2010). Another reason for an incomplete removal of very reactive substances could be a reduced exposure to the ozone because of a potential short-circuiting of a small water fraction through the ozone reactor (Margot et al. 2013). MPs that showed medium removal rates (around 50% to 60%) have only a low reactivity with the selective ozone but showed a high reactivity with the unselective oxidant hydroxyl (OH) radical. The formation of OH radicals in the wastewater is mainly due to the reaction of the ozone with the organic wastewater matrix such as organic matter that is enclosed in the effluent. Hence, a variation in the composition of the organic matter by for example adding a coagulant can lead to different amounts of OH radicals that were formed per unit of ozone. In addition, the concentration of OH radicals is dependent on the occurrence of OH radical scavengers (such as carbonate) and the pH. Thus, the reactions of MPs with OH radicals and in the following way the removal of these substances from the wastewater is more affected by the quality of the wastewater than the direct oxidation with ozone. Furthermore, substances with amide functions and human metabolites of pharmaceuticals (such as 10,11-dihydro-10,11-dihydroxycarbamazepine and N-acetyl-sulfamethoxazole) that are mostly hydrolysed, hydroxylated or conjugated forms of the mother compounds (carbamazepine and sulfamethoxazole, respectively) indicated principally a lower removal rate as the mother compounds probably due to the protective effect of the hydroxyl or acetyl group on the reactive moiety. These groups alter the electron density and thus slow down the reaction rate (Margot et al. 2013). MPs with low removal rates (about and under 30%) showed a low reactivity with ozone as well as a low reactivity with OH radicals. Iodinated contrast media (such as iopromide, iomeprol, iohexol and iopamidol) and some anticonvulsants (for example gabapentin) and pesticides are examples for substances with a specific resistance to

the degradation with ozone or hydroxyl radicals. However, Reungoat et al. (2010) reported that hydroxyl radicals are highly reactive with almost any organic molecule and suggested that the observed removal of iopromide was caused by a substantial exposure to hydroxyl radicals. The persistence even for efficient biological wastewater treatment in addition to their resistance to ozonation is especially of environmental concern because the iodinated contrast media and gabapentin are detected in the wastewater in parts distinctly above 1 µg/L (influent concentrations: 3.36 µg/L to 14.5 µg/L, effluent concentrations: 2.54 µg/L to 10.5 µg/L) (Margot et al. 2013).

One opportunity to increase the removal efficiency of persistent MPs is the increase of the ozone dosage (Lee et al. 2012). An ozone dose of 17.6 mg/L O_3 (≙ 2.6 g O_3/g DOC) led to higher removal rates of iopamidol (84%), iohexol (82%), iomeprol (81%), and gabapentin (88%). But on the other hand, the application of higher ozone dosage implicates higher costs and an increased risk of the formation of toxic oxidation by-products such as carcinogenic bromate, formaldehydes, or nitrosamines whereas the formation of bromate also increased with increasing ozone concentrations (Margot et al. 2013; Zimmermann et al. 2011). Indeed, nitrosamines are in parts removable with a subsequent SF (Hollender et al. 2009) but the concentration of bromate was not reduced after the sand filter (Margot et al. 2013) and thus, high bromate concentrations end up in the effluent and possibly reached the aquatic environment. Furthermore, the chemical compounds often are not completely mineralised during the ozonation process but transformed to unknown intermediates with unknown probably (more) toxic properties as it is reported for the antiepileptic drug carbamazepine, the beta-blockers metoprolol and propranolol, and various PAHs. Therefore, the identification of the TPs that are responsible for the increased toxicity is worthwhile (Stalter et al. 2010a). Also, Lee and von Gunten (2010) reported on organic matter, ammonia, nitrite, and bromide included in the wastewater that affected the

transformation efficiency of the MPs during the treatment with ozone. A higher organic carbon concentration in the wastewater for example can have adverse effects such as a higher ozone demand, and the interference between the oxidation of bulk organic matter and target compounds (Altmann et al. 2014; Lee et al. 2012). In the end, the ozone dose is typically adjusted to the concentrations of the DOC in the wastewater and thus, an average ozone dose of 5.7 mg O_3/L (≙ 0.85 g O_3/g DOC) was adequate to achieve an averaged reduction by 80% of the investigated MPs compared to the raw (untreated) wastewater (Altmann et al. 2014; Margot et al. 2013). Also, a low ozone dosage is capable to remove some compounds that are recalcitrant to the ozone itself because in the initial phase of the ozonation the ozone decomposes rapidly due to the reaction with the organic matter in the effluent and generates high amounts of hydroxyl radicals (Reungoat et al. 2012).

Our results (Schneider et al. 2020, Annex A.2; unpublished data: compare 2.2.5 and Annex A.5) showed comparable results as published in the literature. The ozone dosage was in the same range (0.93 g O_3/g DOC) and the overall removal rates after the ozonation process were similar. High removal rates or an almost complete reduction were achieved by most of the investigated substances: 3-hydroxy-ibuprofen (74.9%), sulfamethoxazole (79.3%), iopromide (88.2%), 10,11-dihydro-10,11-dihydroxycarbamazepine (88.8%), 4-hydroxy-1H-benzotriazole (91.6%), carboxy-acyclovir (95.8%), tramadol (96.9%), diclofenac (97.3%), 1H-benzotriazole (97.4%), 1-hydroxy-benzotriazole (97.6%), carbamazepine (97.9%), N-acetyl-sulfamethoxazole (98.0%), 4-hydroxy-diclofenac (98.9%), 1-hydroxy-ibuprofen (99.1%), tolyltriazole (99.3%), 2-hydroxy-ibuprofen (99.4%), acyclovir (99.5%), paracetamol (99.7%), caffeine (99.8%), and carboxy-ibuprofen (100%). Medium and low reduction rates exhibited iomeprol (66.3%), amidotrizoic acid (44.4%), iopamidol (20.0%), 4-nitrosulfamethoxazole (11.1%), and mecoprop (11.1%).

Finally, the removal of MPs is dependent on the chemical structure of the compounds and their chemical moieties affecting their reaction with ozone but also on the operational conditions such as the ozone dose, the wastewater quality (the presence of ozone and hydroxyl radical competitors or scavengers, the pH, the concentration of organic matter and inorganic compounds like nitrite in the effluent, etc.), and the addition of coagulants (Altmann et al. 2014; Margot et al. 2013; Lee & von Gunten 2012). A high removal efficiency of MPs at low ozone dosage is feasible when the substances offer selective electronic-rich moieties, if a low average DOC concentration in the effluent is given, if competitors for ozone consumption (such as nitrite) are almost complete absent and if the pH of the wastewater is neutral that enables a relatively high ozone stability (Hollender et al. 2009).

3.2.2.2 Activated carbon

The removal rate after the treatment with PAC and following UF membranes was in a medium range for the iodinated contrast media iomeprol (54%), iopamidol (49%), and iopromide (47%), the analgesic/anti-inflammatory substance diclofenac (69%), the antibiotic sulfamethoxazole (64%), the drug metabolites 10,11-dihydro-10,11-dihydroxycarbamazepine (52%) and N-acetyl-sulfamethoxazole (> 20%), the herbicide mecoprop (48%), and the food component caffeine (65%). Higher removal rates were detected for the analgesic/anti-inflammatory substance ibuprofen (83%), the anticonvulsant carbamazepine (90%), and the corrosion inhibitor benzotriazole (90%) (Margot et al. 2013). Comparable results were published by Magdeburg et al. (2014). An application of PAC to the wastewater reduced the concentration of carbamazepine and clarithromycin by more than 80% whereas the iodinated contrast media, sulfamethoxazole and diclofenac showed removal rates in the range of 12% to 49%. The substances with a high affinity to PAC and thus, a high removal rate (more than

90%) even at 10 mg/L PAC were predominantly either positively charged or neutral at the pH of the wastewater and covered a broad range of hydrophobicity. A medium affinity to PAC (averaged removal rates between 70% and 90%) was detected for neutral or negatively charged substances. A low PAC affinity (averaged removal rates between 11% and 66%) was shown by substances with also neutral and negative charges but in parts in combination with hydrophilic characteristics (Margot et al. 2013). A low PAC affinity of charged and hydrophilic substances was also reported by Boehler et al. (2012) and Reungoat et al. (2010). Again, the application of a higher PAC dose (60 mg/L) and/or a prolonged contact time (above one or even two hours) could increase the removal efficiency of some MPs with a low PAC affinity such as sulfamethoxazole, mecoprop, iohexol, iomeprol, and iopromide to more than 90%. But the use of a higher PAC dosage generates a production of larger amounts of sludge, causes higher costs and also reduces the applicability for full scale plants (Magdeburg et al. 2014; Maletz et al. 2013). Partly high variations of the removal rate of one compound, especially for compounds with low PAC affinity, or within one PAC dose could thus be explained on the one hand by different PAC dose that were applied and on the other hand by diverse parameters such as the quality of the wastewater (various DOC concentrations in the wastewater leading to a direct competition for the adsorption sides between the organic matter and the MPs or causing a blockage/constriction of the pores (Altmann et al. 2014; Delgado et al. 2012) and operational parameters for example the residence time and the PAC type. In addition, very low concentrations of a substance in the wastewater can induce high uncertainties in the estimation of the removal rate (Margot et al. 2013). However, a PAC dose related to the DOC concentration of the wastewater indicated a good correlation of the removal of the organic MPs independent from the WWTP effluent (Altmann et al. 2014).

Finally, a PAC dose in the range of 10 mg/L to 20 mg/L (DOC: 5-10 mg/L) in an optimised system is expected to result in an advanced removal of a broad spectrum of MPs and even iodinated X-ray contrast media by more than 70% (Margot et al. 2013). MPs with hydrophobic properties such as carbamazepine indicated an affinity and higher adsorption to PAC whereas (highly) polar and hydrophilic substances and compounds such as sulfamethoxazole required a higher PAC dosage or were only partly removed. The differences between the affinity of MPs to PAC and thus the removal rate can be explained by different PAC characteristics or the biodegradation phenomena in the reactor (Alvarino et al. 2017; Margot et al. 2013; Magdeburg et al. 2014; Hollender et al. 2009). Besides, the removal rate of diverse substances decreased over time as a result of a progressive PAC saturation (Serrano et al. 2011) whereas the saturation depends on the ionic charge of the MP occurring at first for the negatively charged compounds (such as diclofenac), secondly for the neutral charged substances (such as carbamazepine) and at last for the positively charged compounds (such as trimethoprim). Also, Reungoat et al. (2010) expected that the adsorption capacity of the activated carbon filter will decrease with time and presumably be exhausted while biological activity will develop in the filter and possibly contribute to the biodegradation of for example by-products formed by ozonation whereat the bacterial community is suggested to adapt to the biodegradation of compounds that are refractory in the conventional treatment (Reungoat et al. 2012). Thus, a periodical addition of new PAC or a PAC replacement is required to ensure the removal efficiency of the adsorption processes (Alvarino et al. 2017). Furthermore, a recycling of the used PAC to the BT to reach a two-step counter current use of the PAC increases the elimination of the investigated MPs up to 90% and reduced the background DOC concentration by 40% to 50% (Boehler et al. 2012).

Our results (Schneider et al. 2020, Annex A.2; unpublished data: compare 2.2.5 and Annex A.5) showed that a filtration with GAC after the ozonation process further decreased the concentration of most of the investigated substances. A medium removal rate was achieved by carboxy-ibuprofen (29.1%), 2-hydroxy-ibuprofen (34.0%), amidotrizoic acid (41.5%), 4-hydroxy-1H-benzotriazole (42.9%), carboxy-acyclovir (42.9%), and paracetamol (42.9%). The concentrations of 3-hydroxy-ibuprofen (75.0%), diclofenac (81.7%), sulfamethoxazole (90.7%), 10,11-dihydro-10,11-dihydroxycarbamazepine (92.9%), iomeprol (94.4%), 1H-benzotriazole (96.0%) iopromide (97.4%) were even highly reduced by the GAC filtration indicating that the ozonation process transformed these substances resulting in a higher adsorption ability to GAC. However, tolyltriazole (22.5%), tramadol (20.0%), 1-hydroxy-benzotriazole (20.0%), acyclovir (20.0%), and caffeine (4.7%) only showed low removal rates.

Overall, an adsorption to activated carbon is complex and difficult to predict because the mechanism includes several types of interactions such as electrostatic interactions between a charged compound and the charges of the surface of the activated carbon, van der Walls interactions as well as hydrogen bonding (Reungoat et al. 2012).

3.2.2.3 Biofiltration

A following SF after the ozonation process only showed a limited effect on the additional removal of MPs of less than 5%. Higher removal rates (> 10%) were primarily detected for compounds such as ibuprofen, that were already well removed in an effective BT, probably basing on the sorption of the substances on the biofilm developed on the sand filter (Margot et al. 2013).

Our studies (Schneider et al. 2020, Annex A.2; unpublished data: compare 2.2.5 and Annex A.5) indicated a more effective reduction of substances due to a subsequent

BF of the ozonated wastewater. A medium removal rate was achieved by 2-hydroxy-ibuprofen (31.1%), 4-hydroxy-1H-benzotriazole (42.9%), carboxy-acyclovir (42.9%), and paracetamol (42.9%). A high removal rate reached 3-hydroxy-ibuprofen (75.0%), diclofenac (81.7%), and sulfamethoxazole (90.7%). However, a low removal rate indicated 1H-benzotriazole (0.0%), 10,11-dihydro-10,11-dihydroxycarbamazepine (0.23%), amidotrizoic acid (4.63%), tolyltriazole (9.30%), iopromide (19.1%), tramadol (20.0%), 1-hydroxy-benzotriazole (20.0%), acyclovir (20.0%), iomeprol (20.1%), and carboxy-ibuprofen (22.0%). The observed reduced concentrations could also be attributed to a transformation of these substances due to the ozonation process into more biodegradable or adsorbable compounds that are removed by the BF.

The BF used in the pilot study by Lee et al. (2012) removed about 50% of the biodegradable dissolved organic carbon (BDOC). Hence, the BF would be able to remove a part but not the whole of the degradation products of pharmaceuticals and personal care products (PPCPs) originated during the ozonation process assuming that PPCPs indicate the same degradation processes as the BDOC. The removal capacity of the BF could be improved by modifying the design or the operation parameters of the BF such as a lower filtration rate or a longer empty bed contact time, both allowing the BF to acclimate to the ingredients of the wastewater, or the usage of different filter media.

The importance of a SF step subsequent to the ozonation process is highlighted by Zimmermann et al. (2011) because the SF is capable to remove oxidation by-products such as assimilable organic carbon (AOC) and cancerogenic *N*-nitrosodimethylamine (NDMA) (Sgroi et al. 2018).

3.2.2.4 Membrane bioreactors

The removal mechanisms of MPs in a MBR treatment represent a complex process that is defined by four pathways: firstly, biotransformation or biological degradation, secondly, sorption processes, thirdly, volatilisation or stripping by aeration, and fourthly, physical retention due to the size exclusion by the membranes or the direct sorption to the membrane. Regarding most of the investigated MPs the removal by volatilisation and physical retention (the molecular size of the MPs is smaller than the pore size of the membrane and there is a limited surface area of the membrane for sorption) is insignificant. Therefore, the major removal mechanisms in MBR systems are sorption processes (adsorption in the aqueous phase due to electrostatic interactions of positively charged groups of the MP with the negative charges on the surface of the microorganism and absorption resulting from hydrophobic interactions between the MP and the lipophilic cell membrane of the microorganisms and the fat fractions of the sludge) with subsequent physical retention by the membrane and biological degradation by microorganisms. Both processes, sorption and biological degradation, are correlated with the external and internal (bio)availability of the substrate/MP to the degrading microorganisms. Non-polar substances are mainly sorbed and retained by the membrane whereas polar compounds are predominantly biologically degraded and sorption processes are limited. Thereby, the removal of organic MPs depends on diverse factors affecting the efficiency of the MBR. The most important factors are the physicochemical properties of the MPs (such as the chemical structure, the molecular weight and diameter, the degree of acidity, and hydrophobic/hydrophilic characteristics), the operational conditions (process parameters such as biomass concentration, temperature, sludge retention time, and HRT), the wastewater characteristics (such as the pH value and the concentration of organic matter), and membrane characteristics (such as pore size, contact angle, and

roughness). In general, compounds with simple aliphatic monocyclic aromatic properties (sterically unprotected molecules allowing the unrestricted access of bacteria and enzymes) and/or electron donating groups are easily biodegradable whereas the biodegradability of polycyclic compounds and substances with electron withdrawing groups is lower. Furthermore, compounds possessing functional groups such as esters, nitriles, and aromatic alcohols may show an increased biodegradability whereas functional groups such as iodide-, nitro-, azo-, sulfo-, halogen, and aromatic amine groups led to a decreased biodegradability. The natural hormones E_1 and E_2 for example have hydrophobic properties and thus, they are first sorbed into the activated sludge and then biological degraded. An increase of the sludge retention time effected an improved removal of several MPs such as EE_2 and sulfamethoxazole whereas no impact on other substances (for example carbamazepine and caffeine) was observed. The pH value of the wastewater has an influence on the removal of the MPs by directly affecting the microorganisms (pH optima of the activity of the microbial enzymes) as well as the solubility of the MPs present in the wastewater. A neutral pH 6-7 is in general optimal for the biological removal performance of the microorganisms. An acidic pH reduced the microbial activity to biodegrade several compounds. But the removal efficiency of ionisable and acidic compounds such as sulfamethoxazole, ibuprofen, and diclofenac is enhanced at pH 5 in comparison to less acidic conditions that is possibly due to the hydrophobicity of these substances at pH 5. Furthermore, the functional groups of the MPs have an impact on their removal efficiency. The charge of these functional groups is dependent on the pH value. For example, negatively charged MPs are not well adsorbed by the negatively charged surface of the microorganisms, the sludge, and parts of the membrane and thus, the removal efficiency is decreased. However, there are several substances such as bisphenol A and carbamazepine that indicated low or high removal capacities independently from

the pH value of the wastewater. Besides, MBR systems are exposed to seasonal temperature variations that have an impact on the removal efficiency of the MPs and the performance of the system. Bertanza et al. (2011) argued that a minimum required sludge age is 10 days at 10°C, and further increases do not lead to noticeable improvements. But in general, the bacterial growth and the degradation rate increase with increasing temperature. The removal rate of some pharmaceuticals such as ibuprofen and diclofenac increased within the summer time (17°C water temperature) in comparison to the winter season (around 7°C). Room temperature (20°C) offers the optimal conditions for the growth of the microorganisms and their metabolic activity and thus, the most of the removal of the MPs occurs. A nearly complete removal of ibuprofen was documented at room temperature whereas are partial removal of sulfamethoxazole and erythromycin and almost no removal of diclofenac and carbamazepine was detected at room temperature. However, some compounds indicated higher removal rates at 10°C (for example also diclofenac) whereas the removal of other substances was highest at 45°C. But overall, high temperatures up to 45°C decreased the removal rate presumably due to amongst others a disrupted metabolic activity of the microorganisms, an impeded adsorption to the membrane, and an increase of membrane fouling. In addition, the presence of the MPs themselves as single substances or in combination could have an impact on the activity of the microorganisms in the activated sludge of the MBR although the degradation of MPs is a distinct capability of such microbial sludge communities. Especially wastewater including high concentrations of MPs originating from special locations such as hospitals effected a greater shift in the microbial structure of the sludge communities and also contributed to the fouling of the membranes. Specific groups of bacterial microorganisms may appear or disappear, they might be clustered together or be

dispersed that in turn potentially affected the removal of the MPs (Gurung et al. 2019; Besha et al. 2017; Camacho-Muñoz et al. 2012; Cirja et al. 2008).

The MBR treatment with a sludge retention time of 60 days indicated high median removal rates amongst others of iopamidol (90.2%), testosterone (95.8%), estrone (97.6%), estriol (98.9%), ibuprofen (99.6%), paracetamol (99.6%), and caffeine (99.8%), but only medium or low median removal rate were reached for example for diclofenac (38.8%) and carbamazepine (1.7%) (Gurung et al. 2019).

The high removal rates of some antibiotic substances such as erythromycin in MBRs could be related to the high levels of nitrification in the systems. Also, a linear correlation between the removal rate of several analgesic and anti-inflammatory substances such as ibuprofen and the nitrification rate was observed (Alvarino et al. 2017; 2014). Also, Boonnorat et al. (2017) reported on an improved removal of toxic compounds such as carbamazepine, diclofenac, bisphenol A and DEET in the MBR treated wastewater by increasing the nitrogen content. Comparable to the mechanisms in the conventional treatment the increased removal could be attributed to a higher diversity and amount of the (heterotrophic) nitrifying bacteria.

The results of our studies (Schneider et al. 2020, Annex A.2; unpublished data: compare 2.2.5 and Annex A.5) showed no or only a low removal rate in the MBR in comparison to the raw (untreated) wastewater of iopamidol (0.0%), 4-nitrosulfamethoxazole (0.0%), mecoprop (0.0%), tramadol (4.26%), carbamazepine (4.92%), 10,11-dihydro-10,11-dihydroxycarbamazepine (9.21%), and amidotrizoic acid (9.78%). A medium removal rate was reached by iomeprol (34.9%), 4-hydroxy-diclofenac (37.3%), diclofenac (37.3%),1-hydroxy-benzotriazole (49.6%), tolyltriazole (52.9%), and iopromide (73.4%). The concentrations of most of the substances were highly reduced: 1H-benzotriazole (77.8%), sulfamethoxazole (80.4%), 4-hydroxy-1H-benzotriazole (84.2%), N-acetyl-sulfamethoxazole (86.2%), 3-hydroxy-ibuprofen

(93.7%), 1-hydroxy-ibuprofen (97.8%), acyclovir (98.1%), 2-hydroxy-ibuprofen (98.5%), caffeine (99.5%), carboxy-ibuprofen (99.8%), and paracetamol (99.8%). In contrast and comparable to the conventional treatment the concentration of carboxy-acyclovir increased (+343%) due to the MBR treatment. This increase could also be attributed to the human or microbial biotransformation of the mother compound acyclovir (Prasse et al. 2012, 2011; compare above chapter 3.2.1). Overall, the efficiency of the removal rates was significantly dependent on both the organic compound and the wastewater treatment technology that was applied (Camacho-Muñoz et al. 2012).

The treatment of the wastewater in an aeration tank of a MBR system with 1 g/L PAC improved the removal of more persistent substances and compounds up to 85% whereas amongst others carbamazepine and erythromycin indicated the highest removal rates. These higher removal rates could be attributed to the positively charged amino groups being a component of these substances (Serrano et al. 2011). The improved removal of (recalcitrant) organic MPs (for example carbamazepine and diclofenac) in such an aerobic hybrid-system could also be attributed to the combination of physical-chemical adsorption processes by the directly dosed PAC and biological degradation processes by the MBR (Alvarino et al. 2017).

The combination of a MBR with an ozonation system and partly recirculation (Schneider et al. 2020, Annex A.2; unpublished data: compare 2.2.5 and Annex A.5) effectuated a high or almost complete removal of most of the investigated substances: 3-hydroxy-ibuprofen (74.9%), tramadol (81.0%), 10,11-dihydro-10,11-dihydroxycarbamazepine (84.3%), carbamazepine (89.7%), 4-hydroxy-1H-benzotriazole (90.1%) N-acetyl-sulfamethoxazole (90.9%), iomeprol (92.1%), tolyltriazole (92.2%), 1-hydroxy-benzotriazole (92.5%), 4-hydroxy-diclofenac (93.5%), diclofenac (94.7%), 1H-benzotriazole (95.3%), iopromide (95.5%), sulfamethoxazole

(96.6%), 1-hydroxy-ibuprofen (98.5%), acyclovir (98.6%), caffeine (99.7%), 2-hydroxy-ibuprofen (98.8%), paracetamol (99.8%), and carboxy-ibuprofen (99.9%). However, the concentrations of iopamidol (0.0%), mecoprop (0.0%), 4-nitrosulfamethoxazole (0.0%), and amidotrizoic acid (47.0%) were not or only partly reduced compared to the raw (untreated) wastewater. Furthermore, the concentration of carboxy-acyclovir increased (by 39.3%) in the MBR treated and ozonated wastewater.

Overall, the removal rates of the MBR with ozonation and partly recirculation were higher compared to the single MBR system. The additional removal of the substances, especially 1-hydroxy-benzotriazole, diclofenac, 4-hydroxy-diclofenac, and 1-hydroxy-benzotriazole, could be explained by the partly recirculation that enables the alternately and periodic attack of the ozone and the biological degradation of the MBR system on the chemical structures of the compounds.

Overall, the treatment of the wastewater with a single MBR system is not sufficient to remove numerous of the investigated emerging MPs effectively from the wastewater whereat individual substances indicated very high removal rates. However, the combination of a MBR system with nanofiltration (MBR-NF), reversed osmosis (MBR-RO), GAC (MBR-GAC) or PAC (MBR-PAC) achieved an efficient removal of the MPs (Besha et al. 2017). Furthermore, it has to be considered that to some extent a great discrepancy and a wide range of the removal efficiency of individual MPs (for example some antibiotics and diclofenac) in the MBR treatment was reported in several literature (Gurung et al. 2019). However, the half of the determined compounds were not significantly removed even after the tertiary wastewater treatment with AWWT technologies (Reemtsma et al. 2006).

In summary, the AWWT technologies are able to further reduce the concentrations of MPs in comparison to the conventional treatment. But each AWWT owns advantages

and disadvantages that should be weighted up carefully. Optionally, a combination of several AWWT technologies could be the most promising process for a maximal reduction of MPs in the wastewater leading to minimal presumably adverse effects to the aquatic environment. Thus, further investigations are needed to completely understand the mechanism that are involved in the removal of the MPs, particularly the role of the adsorption processes (Reungoat et al. 2011).

3.3 Removal of *in vitro* toxicity during conventional and advanced wastewater treatment

Although the usage of (eco)toxicological *in vitro* methods is not a common parameter in the guidelines for the testing of the water quality (Dopp et al. 2021), *in vitro* bioassays are important tools for the monitoring of specific modes of (toxic) action in ecotoxicological studies such as endocrine disrupting activities, mutagenicity, genotoxicity, cytotoxicity, and neurotoxicity. For this purpose, a broad and diverse spectrum of (genetically modified) organisms were applied for example bacteria, yeasts, and cell cultures of fishes, hamsters, rats, and humans.

Most of the research studies mainly investigated the estrogenic activity of different kinds of wastewater and only little work has been done on other receptor activators or antagonists (Mnif et al. 2010).

3.3.1 Conventional wastewater treatment

The conventional wastewater treatment was essential for the removal of estrogenic activity in municipal wastewater. The effluents of WWTPs using direct precipitation without a BT showed approximately the same levels with no significant reduction of the

estrogenic activity in comparison to the influent. Even a higher estrogenic activity in the effluent was found compared to the influent (Svenson et al. 2003).

The extracts of a total number of 75 effluents mainly originating from municipal WWTPs, but some plants were dominated by industrial wastewaters, were investigated by Loos et al. (2013). A portion of 27 sample extracts indicated a varying estrogenic activity that was higher than the detection limit. The detected levels of the estrogenic activity were comparable to the results of previous studies analysing the estrogenic activity of the effluent of European WWTPs with different *in vitro* bioassays. Furthermore, 21 out of 25 extracted wastewater samples showed a low dioxin-like activity that exceeded the detection limit. Thus, the wastewater treatment of these WWTPs was not sufficient enough for a complete elimination of substances causing the observed estrogenic and dioxin-like effects (Loos et al. 2013).

Aqueous samples, suspended particular matter, and sludge fractions of three WWTP receiving wastewater from two different sources, on the one hand with a domestic origin (in part with intense touristic activity) and on the other hand with an industrial origin (such as plastic, detergent, paint, and other chemical waste and textile industry) were analysed in various reporter cell lines to characterise their endocrine-disrupting potential. The aqueous samples showed estrogenic and androgenic activity whereas the suspended particular matter and the sludge extracts indicated aryl-hydrocarbon and pregnane X receptor activity next to estrogenic activity assuming that substances with such activity are of environmental concern. However, no agonistic or antagonistic glucocorticoid, progesterone and mineralocorticoid receptor activity was detected indicating that environmental compounds present in the sewage have a limited spectrum of activity. The estrogenic activity of the aqueous samples and the sludge fraction was linked to the presence of EDCs such as natural and synthetic hormones with a high affinity to the estrogen receptor α (ERα) or alkylphenols with a lower affinity

to the ERα whereas the ligands binding to the androgenic and pregnane X receptor could not be identified. Thus, more chemical analysis will be needed. Furthermore, EDCs existing in the suspended particular matter and the sludge fraction, that are able to activate the aryl-hydrocarbon receptor, are metabolically labile compounds. Besides, a difference in the percentaged efficiency of the wastewater treatment of the investigated WWTPs was assessed that was presumably due to the aerobic or anaerobic nature and the duration of the treatment at each WWTP (Mnif et al. 2010).

A substantial estrogenic activity was detected in the effluent of WWTPs receiving wastewater from the textile industry. A chemical analysis may characterise the substances causing the estrogenic activity (Svenson et al. 2003).

In the raw (untreated) wastewater a high estrogenic activity was detected that was obviously reduced during the conventional wastewater treatment whereat the removal of the estrogenic activity depended on the level of nitrification in the range of –75% without nitrification to –99% with total nitrification. Thus, a conventional wastewater treatment with an entire nitrification process is effective to reduce the release of estrogenic substances into the aquatic environment and therefore the risk of the feminisation of mussel and fish populations. But even a low estrogenic activity measured in the wastewater after the BT is able to affect the fertility of sensitive fish species (Margot et al. 2013; Lahnsteiner et al. 2006; Svenson et al. 2003). To this effect, the concentration of vitellogenin in juvenile fish of the rainbow trout *O. mykiss* exposed to conventional treated wastewater was significantly increased compared to the control group even if the estrogenic activity in the SPE extracts was reduced by 88%. Vitellogenin is an egg yolk precursor naturally generated in mature female fish and can serve as a biomarker for the exposure to exogenous substances with estrogenic activity in juvenile and male fish (Margot et al. 2013).

In a similar approach, a combination of biological and chemical analysis was performed to determine the estrogenic activity of SPE extracts of municipal wastewater effluents with a hepatocyte assay of male three-spined stickleback (*Gasterosteus aculeatus*) by the induction of vitellogenin. The extracts of the wastewater as well as the positive control (E_2) induced the production of vitellogenin indicating that substances with an estrogenic activity remained in the treated wastewater (Björkblom et al. 2008).

Furthermore, substance specific chemical analysis in combination with *in vitro* bioassays revealed that the natural hormone E_1 was the main compound causing the estrogenic activity in the wastewater (Maletz et al. 2013). Estrogenic activity was also reduced by the conventional treatment but it was still detectable in the wastewater in varying intensity in the research studies by Kienle et al. 2022; Dopp et al. (2021), Schneider et al. (2020, 2015, Annex A.2, A.4), Itzel et al. (2019, 2017), Giebner et al. (2018), Margot et al. 2013, Stalter et al. (2011, 2010a), unpublished data: compare 2.2.4 and Annex A.5.

The progesterone-like activity one the one hand was decreased by the conventional wastewater treatment (Kienle et al. 2022) and on the other hand the activity in the effluent was higher than in the influent of the WWTP (Kienle et al. 2011). Furthermore, the anti-progesterone-like activity was effectively reduced in conventional treated wastewater (Kienle et al. 2022).

The detected estrogenic, androgenic, and aryl-hydrocarbon agonistic activity in the influent of two WWTPs were comparatively very high and largely removed by the conventional wastewater treatment but they were still provable in the wastewater. Moreover, anti-estrogenic and anti-androgenic activity were analysed in the conventional treated wastewater that did not appear in the wastewater of the influent. This result could be attributed to the more effective removal of the respective agonistic substances during the conventional wastewater treatment. Thereby, a point of concern

is the detected anti-androgenic activity because next to estrogenic activity anti-androgenic activity is an important factor causing the feminisation of wild fish. The determined concentrations of anti-androgenic activity in the conventional wastewater are presumably sufficient to induce biological responses in fish and hence, they are of environmental relevance (Stalter et al. 2011; Jobling et al. 2009, 2006, 2002). Besides, an anti-androgen activity of 100% receptor inhibition in the influent of a WWTP was reported by Itzel et al. (2019) that was reduced by the conventional wastewater treatment but still caused a residual receptor inhibition between 20% and 60% indicating that compounds with antagonistic activity are rather available for biological degradation processes. But also *in vitro* and *in vivo* anti-estrogenic effects of polycyclic musks in the zebrafish were reported by Schreurs et al. (2004). These effects are caused by the fragrances tonalide and galaxolide which are ubiquitously present in the aquatic environment.

However, androgenic activity and anti-estrogenic activity were effectively removed by the conventional wastewater treatment but they were still provable in the wastewater effluent (Itzel et al. 2019, 2017; Giebner et al. 2018).

Our results also showed an effective removal of estrogenic, androgenic, and anti-androgenic activity due to the conventional wastewater treatment but residual activities remained as well. On the other hand, the anti-estrogenic activity was in fact reduced but remained on a relatively high level indicating a lower availability of substance with anti-estrogenic potential to biological degradation processes (Schneider et al. 2020, Annex A.2; unpublished data: compare 2.2.4 and Annex A.5).

The pregnane X receptor activity for the detection of xenobiotic metabolism was reduced but still detectable in the wastewater after the conventional wastewater treatment (Kienle et al. 2022).

Raw wastewater induced a relatively high algae growth inhibition (non-specific toxicity) as well as a photosynthesis inhibition (specific toxicity). The growth inhibition was clearly reduced after the conventional wastewater treatment possibly due to the removal of biodegradable or adsorbable substances that were responsible for the growth inhibition (Kienle et al. 2022; Margot et al. 2013). On the contrary, the inhibition of the photosynthesis was not strongly reduced after the conventional wastewater treatment implying a low biodegradability of substances such as pesticides and algicides inducing this specific effect on the photosystem (Margot et al. 2013). However, the conventional wastewater treatment reduced the toxicity on the photosynthesis and thus the herbicidal activity reported by Kienle et al. (2022).

Besides, significantly increased growth rates of a green algae at low wastewater fractions after a conventional wastewater treatment in contrast to a significant growth inhibition of the algae in higher wastewater fractions were reported by Itzel et al. (2017). However, the non-specific toxicity (bacterial luminescence inhibition assay) was significantly reduced due to the conventional wastewater treatment (Kienle et al. 2022). Conventional treated wastewater also induced in parts substantial cytotoxic genotoxic and mutagenic activity (Dopp et al. 2021; Giebner et al. 2018; Magdeburg et al. 2014; Stalter et al. 2010a).

In the end, these results show clearly that substances with cytotoxic, mutagenic, genotoxic, and endocrine activity remain in the wastewater after the conventional wastewater treatment and thus, have the potential to affect the aquatic environment. Especially compounds with an estrogenic or an anti-androgenic activity may have a significant impact on aquatic organisms because they are introduced into streams and rivers in environmentally relevant concentrations. Furthermore, the (adverse) effects of compounds with an endocrine activity on aquatic organisms is multiplied in small and medium sized rivers and streams because the dilution factor of the wastewater is

minimal in the receiving water bodies. Also, Betz-Koch et al. (2023) highlighted that small rivers play an important role for freshwater ecosystems due to their high biodiversity (Oehlmann et al. 2014) and noted that a systematic pesticide monitoring in small creeks is rarely conducted.

3.3.2 Advanced wastewater treatment

3.3.2.1 Ozonation

A(n) (almost complete) reduction of the estrogenic activity in the ozonated wastewater compared to the conventional wastewater treatment as well as to the raw wastewater was observed in diverse studies (Kienle et al. 2022; Dopp et al. 2021; Schneider et al. 2020, Annex A.2; Giebner et al. 2018; Itzel et al. 2019, 2018, 2017; Maletz et al. 2013; Margot et al. 2013; Altmann et al. 2012; Reungoat et al. 2012, 2011, 2010; Bertanza et al. 2011; Stalter et al. 2011, 2010a; Hashimoto et al. 2007; unpublished data: compare 2.2.4 and Annex A.5)). Thus, ozonation is supposed to be an appropriate method to reduce the estrogenic burden of the wastewater below environmental relevance (Stalter et al. 2010b). However, Dopp et al. (2021) as well as Gehrmann et al. (2018) reported on an increased estrogenic activity after the ozonation process in single bioassays. Ozonation is capable to transform substances with an estrogenic activity such as the natural hormones E_1 and E_2, the synthetic estrogen EE_2 and the chemical compound bisphenol A (BPA), each exhibiting phenol moieties (Maletz et al. 2013; Stalter et al. 2010a). Phenols are an important and critical functional group interacting with the estrogen receptor and they are known to be especially susceptible to an attack of ozone (Stalter et al. 2011). But receptor mediated effects require a good steric fit (key-lock principle) between the ligand (natural hormone or chemical compound) and the receptor (Reungoat et al. 2012). Thus, a removal of estrogenic activity through ozonation is expected because the original substances and

compounds react with the molecular ozone and lose their steric fit that decreases the interaction with the estrogen receptor and hence, the ability to bind and activate the estrogen receptor (Maletz et al. 2013, Reungoat et al. 2012, Stalter et al. 2011). The reduction of estrogenic activity in the ozonated wastewater also indicated that potential TPs of estrogenic chemicals lose their specific toxicity potential as well (Reungoat et al. 2012). Even low ozone concentrations of 0.2 to 0.4 mg O_3/mg DOC are capable to highly reduce the estrogenic activity of the wastewater of more than 90% (Schneider et al. 2020, Annex A.2; Itzel et al. 2018; Reungoat et al. 2012; Stalter et al. 2011) whereas the effectivity was not significantly affected by the ozone dosage or the HRT in terms of increasing ozone dose and higher HRTs did not lead to increasing removal rates of the estrogenic activity (Schneider et al. 2020, Annex A.2; Gehrmann et al. 2018; Zhang et al. 2012; Bertanza et al. 2011; Hashimoto et al. 2007). However, Itzel et al. (2019) observed an increased reduction of the estrogenic activity with increasing ozone dosage. Nevertheless, a higher ozone dosage achieved a distinctly improvement of the removal of EDCs such as nonylphenol and BPA but only a slightly additional decrease of hormonal activity was observed. This result was attributed to the persistence of endocrine disruptors or to the formation of endocrine active by-products (Bertanza et al. 2011).

But another important aspect next to the detection of the estrogenic activity is the investigation of a possible disruption of the steroid synthesis pathway. Indeed, the ozonation process decreased the overall estrogenic activity in the wastewater but at the same time the treatment with ozone effected an obviously increased production of estradiol and aromatase activity and thus, the ozonation appeared to result in a greater endogenous estrogen production. This effect could be explained by the generation of reactive metabolites throughout the ozonation process (Maletz et al. 2013).

Thus, the AWWTs reduce the release of substances with estrogenic activity in most cases below the environmental quality standard and thereby the risk of the feminisation of fish and mussel populations (Margot et al. 2013). Therefore, a reduction of the estrogenic activity in the wastewater below the environmental quality standard could be a relevant environmental benefit for the aquatic wildlife (Stalter et al. 2011).

Furthermore, no progesterone-like activity was detectable in the ozonated wastewater whereas the anti-progesterone activity also decreased on the whole but sporadically increased (Kienle et al. 2022).

Several studies showed that the anti-estrogenic activity was merely low or not reduced and in parts even intensely increased in the ozonated wastewater compared to the conventional wastewater treatment (Schneider et al. 2020, Annex A.2; Giebner et al. 2018; Itzel et al. 2019, 2018; Stalter et al. 2011; Gehrmann et al. 2018; unpublished data: compare 2.2.4 and Annex A.5)). The occurrence of the anti-estrogenic activity in the ozonated wastewater is on the one hand possibly due to the removal of compounds with an estrogenic activity (compare above) that masked the antagonistic activity. A masking effect occurs if both agonistic and antagonistic substances are available in the wastewater. As a result, the agonistic and antagonistic active compounds compete for the respective receptor (Gehrmann et al. 2018; Itzel et al. 2018). A fractionation (of the SPE-extracts) of the wastewater samples and the anew analysis of these fractions with the *in vitro* bioassays could separate the agonists from the antagonists and confirm or falsify possible masking effects (Itzel et al. 2018; Stalter et al. 2011). On the other hand, the increased anti-estrogenic activity could be the result of the formation of TPs with anti-estrogenic characteristics due to the ozonation process (Itzel et al. 2019; personal communication). This hypothesis is underlined by the results of the study by Schneider et al. (2020, Annex A.2) that indicated increased anti-estrogenic activity with increasing ozone dosage as well as increasing HRTs. However, the slight

removal of anti-estrogenic activity in ozonated wastewater was independent from the ozone dose (Gehrmann et al. 2018). In this context, Knoop et al. (2018) reported that the total anti-estrogenic activity was not reduced investigating the TPs of the anti-estrogen pharmaceutical tamoxifen formed by the ozonation process indicating that TPs are conductive to the total anti-estrogenic activity and hence, compensated the reduction of the mother compound tamoxifen. Some TPs even showed a higher anti-estrogenic activity compared to the mother compound whereas the transformation of tamoxifen to *N*-oxides reduced the anti-estrogenic activity. Furthermore, false positive effects in aqueous wastewater samples could be induced in the antagonistic bioassays due to DOC that is able to sorb E_2 leading to a reduction of the available agonistic concentration and hence, the estrogenic activity (Gehrmann et al. 2018; Neale et al. 2015). However, continuing research on potential adverse effects of substances with anti-estrogenic activity is desirable.

On the one hand, ozonated wastewater also highly induced androgenic activity whereas on the other hand the activity (slightly) decreased due to the ozonation process. The removal of the androgenic activity was less effective at a low ozone dose and increased with increasing ozone dosage. Diverse HRTs did not seem to affect the androgenic activity (Schneider et al. 2020, Annex A.2; Stalter et al. 2011; unpublished data: compare 2.2.4 and Annex A.5)). Indeed, Gehrmann et al. (2018) reported that higher ozone dosage did not result in higher removal rates of androgenic active substances. The androgenic activity also decreased in the ozonated wastewater published by Altmann et al. (2012) and Itzel et al. (2019, 2017). However, a reduction of the androgenic activity was not observed and the activity even slightly increased in the ozonated wastewater (Itzel et al. 2018).

The anti-androgenic activity was differentially affected by the ozonation process. An increase of both, the ozone dose and the HRT at first slightly increased the androgenic

activity in the ozonated wastewater before the activity slightly decreased with further increasing ozone dosage and HRTs (Schneider et al. 2020, Annex A.2; Stalter et al. 2011). Our results also showed a decreased anti-androgenic activity in the ozonated wastewater but the activity still remained on a relatively high level (unpublished data: compare 2.2.4 and Annex A.5). However, the results of the research projects by Itzel et al. (2019, 2018) and Gehrmann et al. (2018) indicated no or only a slight reduction of the anti-androgenic activity after the ozonation process that was independent from the ozone dosage and an in parts high remaining anti-androgenic activity in the wastewater detected by the use of three different *in vitro* bioassays. Even a (slightly) increased anti-androgenic activity in the ozonated wastewater was reported. This phenomenon could also be explained by masking effects due to the competition of agonistic and antagonistic substances towards the respective receptor (Itzel et al. 2018). If the concentration of agonistic compounds is reduced in the wastewater sample the activity of the antagonistic substances at the receptor is predominant and vice versa. Thereby, the release of substances with anti-androgenic activity into the receiving (surface) water could result in ecotoxicological impacts comparable to those of estrogenic compounds such as the sexual disruption in form of feminisation of fish in rivers (Itzel et al. 2019; Gehrmann et al. 2018; Jobling et al. 2009).

Ozonation led to a reduced response of the aryl-hydrocarbon receptor that is activated by dioxins and dioxin-like chemicals (Reungoat et al. 2010; Stalter et al. 2011). Besides, the removal of the activity was minimal at low ozone dosage and increased with increasing ozone dose (Stalter et al. 2011).

The cytotoxic and genotoxic activity was reduced by the ozonation process as well suggesting that the whole of the TPs that were produced throughout the ozonation process were less genotoxic in comparison to their parent compounds (Reaume et al. 2015; Magdeburg et al. 2014; Reungoat et al. 2011, 2010; Stalter et al. 2011). On the

other hand, the reduction of cytotoxic effects could be explained by the evaporation of the samples throughout a SPE extraction because volatile substances substantially contribute to toxic effects, especially after the treatment with ozone (Stalter et al. 2011). However, even a low ozone dosage effectively reduced the genotoxicity. An increasing ozone dose led to an increased removal of the genotoxic activity indicating an effective inactivation of genotoxic substances (Magdeburg et al. 2014). In contrast, Stalter et al. (2010a) and also Magdeburg et al. (2014) reported on an increased genotoxicity in the ozonated wastewater by the use of other test systems for the detection of the genotoxicity. Also, Dopp et al. (2021) reported on differing results investigating three WWTPs. On the one hand, cytotoxic and genotoxic activity were (significantly) reduced due to the ozonation whereas on the other hand both activities were proved in the ozonated wastewater. The remaining genotoxicity after the ozonation can on the one hand be explained by the possible generation of genotoxic organic or inorganic TPs throughout the ozonation process and on the other hand by other genotoxic compounds that were originally present in the wastewater but were resistant to or escaped from the attack of the ozone (Reaume et al. 2015; Stalter et al. 2010a). An increased toxicity after ozonation due to the formation of toxic ozone TPs is also reported by Gerrity et al. (2011) and Cao et al. (2009).

Furthermore, our results showed that the mutagenicity was highly increased due to the ozonation process (Schneider et al. 2020, Annex A.2; unpublished data: compare 2.2.4 and Annex A.5). The results of a study by Kienle et al. (2022) also suggested that ozonation produced mutagenic compounds. Thereby, the appearance of mutagenicity in the ozonated wastewater was demonstrated to be caused by the ozonation and was not due to specific ingredients in the wastewater at the investigated WWTPs (Magdeburg et al. 2014). In addition, the mutagenic activity was increased at higher ozone dosage and HRTs compared to the investigated lower ozone dose and HRTs

(Schneider et al. 2020 Annex A.2; Giebner et al. 2018; Magdeburg et al. 2014). A storage of the ozonated wastewater samples for several days in darkness significantly reduced the mutagenic activity that is possible due to biological degradation or chemical decomposition (Magdeburg et al. 2014) highlighting a correct storage and immediately processing and/or testing of the (waste)water samples.

However, other studies showed that the ozonated wastewater indicated no genotoxicity or mutagenicity despite the formation of bromate (Margot et al. 2013; Kienle et al. 2011) or even a decreased mutagenicity after the ozonation process indicating that the ozonation of wastewater of municipal treatment plants reduced the adverse effects that would be caused if the mutagens were released into aquatic ecosystems. Furthermore, the ozonation did not lead to a decrease of the viability of bacteria and eukaryotic cells (Misik et al. 2011).

The non-specific toxicity determined in the conventional treated wastewater was also reduced after the ozonation of the wastewater independent from the ozone dosage. Thus, the mixture of residual parent compounds and their TPs as well as newly formed oxidation by-products had a common lower toxic potential than the mixture of initial parent compounds but these oxidation products still had a remarkable effect in a non-specific toxicity assay. The decreased non-specific toxicity could be attributed to the formation of more hydrophilic compounds of the organic matter because the ozone reacts preferentially with its most hydrophobic fraction. Hence, a decrease in the hydrophobicity also lead to reduction of the non-specific toxicity of the TPs. Overall, there should be no concern with regard to a possible increase of the non-specific toxicity due to the ozonation process of the treated wastewater and the generation of oxidation by-products. But the used bioassay assessing non-specific toxicity did not give consideration to the generation of TPs with specific and reactive toxic modes of action that presumably still represent a hazard to the environment as well as to human

health. Specific toxicity is typically mediated by a receptor and the TPs originated from the oxidation normally showed a lower affinity to the respective receptor. However, there is not enough knowledge of reactive intermediates that can be formed and their effects (Reungoat et al. 2012, 2011, 2010; Escher & Fenner 2011).

Furthermore, the unspecific as well as the specific toxicity of wastewater effluent that were investigated with a test battery of *in vitro* bioassays covering diverse toxicological endpoints such as baseline toxicity, algae (growth) inhibition, and endocrine disrupting activity were significantly reduced due to the ozonation process. It was assumed that not only compounds were oxidised, but also higher concentrations of non-toxic by-products were formed (Escher et al. 2009; Hollender et al. 2009).

The ozonation of conventional treated wastewater led to a clear and significant reduction of the residual algae growth inhibition (non-specific toxicity) and the residual photosynthesis inhibition (specific toxicity) (Kienle et al. 2022; Margot et al. 2013). The reduced toxicity was attributed to a decreased concentration of relevant pesticides and algicides in the AWWTs which act as photosystem inhibitors in plants and algae and that can have a cumulative effect when they are present in a mixture. More precisely, the results of the chemical analysis of the AWWT treatments showed a distinct correlation between the reduction of the concentrations of four specific herbicides and algicides impeding the photosystem in algae and plants and the simultaneously reduced inhibition of the photosystem in the investigated *in vitro* bioassays. Thus, the ozonation process was able to improve the quality of the wastewater (Margot et al. 2013). In this context, the remaining non-specific toxicity (inhibition of bacterial luminescence) was significantly reduced due to the ozonation process (Kienle et al. 2022). Furthermore, it is important to mention that *in vitro* bioassays used for the detection of unspecific toxicity presumably underestimate the potential hazards of by-products generated throughout the ozonation process since these substances are

expected to have a high polarity and to be easily degradable. Thus, transportation and storage time as well as a performance of a SPE extraction of ozonated wastewater samples might cause a significant loss of toxic by-products (Magdeburg et al. 2014; Stalter et al. 2011).

These results showed that even very low ozone dosage are sufficient for an effective removal of the endocrine activity. However, the ozonated wastewater should be further investigated concerning the formation of metabolites with endocrine activity (Maletz et al. 2013).

Finally, weather the ozonation of the wastewater increased or decreased the toxicity of the wastewater depends on the different wastewater compositions, the specific wastewater matrix that is ozonated, the exposure to grab samples or a chronic exposure, the toxic endpoint under investigation, and the used test organism of the bioassay. For example, the differences in the physiological activity between erythrocytes, haemocytes, and liver cells may explain the different results in the investigated bioassays for the detection of genotoxicity (Reaume et al. 2015; Magdeburg et al. 2014).

3.3.2.2 Activated carbon

The treatment of the wastewater with PAC or GAC (in parts with subsequent UF or SF) is also an appropriate method to remove compounds with estrogenic activity from the wastewater (Giebner et al. 2018; Margot et al. 2013; Stalter et al. 2011, 2010a). The detected remaining estrogenic activity after a filtration of ozonated wastewater with BAC is possibly due to residual estrogenic substances that are hardly biodegradable such as xenoestrogens and/or EE_2 known to be less biodegradable than natural estrogens (Reungoat et al. 2012).

Furthermore, an implementation of PAC (with following SF) effectively decreased the androgenic activity, the anti-androgenic activity, and the aryl-hydrocarbon agonistic activity whereas the anti-estrogenic activity in parts distinctly increased. Overall, substances with endocrine activity are adsorbable to PAC and thus, are removable from the wastewater (Giebner et al. 2018; Stalter et al. 2011).

An application of PAC to the wastewater (with following SF) resulted in a significant reduction of the genotoxicity indicating a sufficient inactivation of genotoxic compounds (Magdeburg et al. 2014; Stalter et al. 2010a). Furthermore, a filtration of the wastewater with GAC effectively reduced the mutagenicity (Giebner et al. 2018).

Both, a BAC filter as well as the addition of PAC to the wastewater (with following SF) effectively removed the non-specific toxicity. In case of using SPE-extracts the decrease of cytotoxicity could be the result of the loss of volatile substances throughout the sample evaporation that are known for the contribution to relevant toxic effects (Reungoat et al. 2011; Stalter et al. 2011). The BAC filter also combines physical-chemical adsorption processes provided by the GAC with biodegradation properties offered by the biofilm layer that developed on the GAC particles (Alvarino et al. 2017) that could be conducive to the removal of the non-specific toxicity.

The adsorption to PAC also significantly reduced the residual algae growth inhibition (non-specific toxicity) and the residual photosynthesis inhibition (specific toxicity) of conventional treated wastewater. The reduced toxicity was also explained by a decrease of the concentrations of pertinent algicides and pesticides due to the AWWTs either acting separately as photosystem inhibitors in plants and algae or as a mixture inducing cumulative effects. In this context, chemical analysis indicated the reduction of the concentrations of four specific algicides and herbicides impeding the photosystem in algae and plants and coevally the used *in vitro* bioassays showed a decreased inhibition of the photosystem.

Finally, also PAC adsorption (with UF) improved the quality of the wastewater (Margot et al. 2013).

Oftentimes, a filtration with (B)AC is installed after the ozonation process of the wastewater. Reungoat et al. (2012) demonstrated that the subsequent filtration step further reduced the non-specific toxicity that could be explained by the assumption that more hydrophobic compounds have a higher toxic activity compared to more hydrophilic ones in combination with the capability of the activated carbon to adsorb more hydrophobic compounds effectively. In addition, the estrogenic activity was highly reduced and the genotoxicity and neurotoxicity were below the level of quantification of the bioassays indicating an effective adsorption of the residual compounds inducing these activities (Reungoat et al. 2012, 2011, 2010). A significant reduction of the genotoxicity to the level of the blank due to a BAC BF was reported by Reaume et al. (2015) as well. The high reduction of genotoxic effects during the BAC BF was attributed to its additional ability to remove organic matter by bio-regeneration and the support of more aerobic bioactivity.

Also, Itzel et al. (2018) showed that a filtration of the ozonated wastewater with GAC further reduced the estrogenic activity as well as the anti-estrogenic activity until they were no longer detectable in the wastewater. Furthermore, the androgenic and anti-androgenic activity were both effectively reduced by the GAC filtration.

However, our results also indicated an overall constant estrogenic activity or a slightly further reduction of the androgenic activity of the already low activities detected in the ozonated wastewater. But the anti-estrogenic as well as the anti-androgenic activity remained on a relatively high level and in two cases the anti-estrogenic activity even increased in the GAC filtered wastewater. In contrast, the mutagenicity was effectively reduced due to the GAC filtration indicating a good absorbability of mutagenic compounds (Schneider et al. 2020, Annex A.2; unpublished data: compare 2.2.4 and

Annex A.5). Thus, the application of a GAC filter after the ozonation process should be further investigated as a potential post treatment.

In comparison to the ozonated wastewater a GAC filtration (distinctly) decreased the mutagenicity as well (Schneider et al. 2020, Annex A.2; Giebner et al. 2018; unpublished data: compare 2.2.4 and Annex A.5). Also, Kienle et al. (2022) reported on a further removal of mutagenic compounds after a GAC filtration.

But it has to be considered that the removal efficiency of activated carbon might be lower compared to the ozonation process in the case of higher background concentrations of DOC in the wastewater because if so, the adsorb ability of pollutants is significantly lower (Stalter et al. 2011, 2010a).

A combination of a GAC filter and a sand filter slightly increased the estrogenic activity whereas the mutagenicity was clearly reduced (Giebner et al. 2018).

Finally, a BAC BF or sand BF is an effective procedure to improve the quality of the (ozonated) wastewater (Reaume et al. 2015).

3.3.2.3 Biofiltration

A sand BF only slightly reduced the non-specific toxicity of the wastewater (Reungoat et al. 2011).

A sand filter is oftentimes installed after the ozonation process as well. The SF led to no or only a minor further reduction of the estrogenic activity because it was already very low after the ozonation (Giebner et al. 2018; Margot et al. 2013; Stalter et al. 2011, 2010a).

The anti-estrogenic activity one the one hand decreased after the SF at one WWTP whereas on the other hand the activity increased at another WWTP. Furthermore, the sand filter effected an increased androgenic activity compared to the ozonated wastewater (Stalter et al. 2011). The anti-estrogenic activity and the anti-androgenic

activity were in parts relatively high in the ozonated wastewater before as well as after the SF and no reduction of these activities was detectable. Overall, the sand filter was not sufficient for an effective removal of these antagonistic activities and did not represent a barrier to those compounds (Gehrmann et al. 2018).

A sand BF further (marginally) reduced the genotoxicity of the ozonated wastewater or showed no further effect on the genotoxic activity of the sand filtered wastewater. The further reduction of the genotoxicity after the sand filter could be explained by its ability to detoxify, degrade, or remove transient genotoxic oxidation products (Reaume et al. 2015; Magdeburg et al. 2014; Stalter et al. 2010a).

The mutagenicity of the ozonated wastewater was insignificantly reduced by the sand filter. In addition, the mutagenic effects were still increased compared to the conventional treatment implying a limited removal capacity of mutagenic oxidation products of the SF (Magdeburg et al. 2014). The mutagenic compounds were not completely removed by the sand filter as well (Kienle et al. 2022). However, the mutagenic activity distinctly decreased due to the SF (Giebner et al. 2018).

The installation of a sand filter after the ozonation of the wastewater further reduced the growth inhibition of an algae. Interestingly, the highest improvement of the growth inhibition of the algae after the SF subsequent to the ozonation process was observed when the conventional treatment was not effective, implying that biodegradable toxic compounds remained in the ozonated wastewater. In contrast, the photosynthesis inhibition was not significantly reduced after the SF subsequent to the ozonation possibly due to compounds with a low biodegradability (Margot et al. 2013).

The non-specific toxicity slightly decreased after the SF in comparison to the ozonated wastewater indicating that a subsequent SF step contributed to the removal of toxic compounds (Stalter et al. 2011).

Our results showed that the estrogenic and androgenic activity in the wastewater after a BF step was as low as in the ozonated wastewater. In contrast, the anti-estrogenic and the anti-androgenic activity remained on a comparable and relatively high level as it was detected in the ozonated wastewater. In addition, the mutagenicity was reduced compared to the ozonated wastewater but remained on a high level as well suggesting a poorly biodegradability of antagonistic and mutagenic substances (Schneider et al. 2020, Annex A.2; unpublished data: compare 2.2.4 and Annex A.5).

A fluidised bed was used as biological post-treatment of the ozonated wastewater. On the one hand cytotoxicity occurred after the post treatment in aqueous samples whereas on the other hand the cytotoxic activity was reduced due to the fluidised bed detected in SPE extracts. The wastewater of a polishing pond indicated increased cytotoxic effects as well as an increased estrogenic activity (Gehrmann et al. 2018).

The wastewater of a fluidised bed reactor effected a significantly decreased growth of a green algae and slightly further reduced the estrogenic activity. However, the androgenic as well as the anti-estrogenic activity increased in one sample series due to the fluidised bed reactor whereas the activity decreased in another sample series. Furthermore, a high anti-androgenic activity was still detectable after the treatment of the wastewater in the fluidised bed reactor (Itzel et al. 2019, 2017).

The mutagenic activity of the ozonated wastewater was removed in fixed bed as well as in moving bed reactors (Kienle et al. 2022).

Finally, a BF step after the ozonation process presumably further reduced the toxicity of the ozonated wastewater due to the biodegradation of potentially formed toxic TPs because a treatment with ozone is expected to increase the biodegradability of the organic matter present in the wastewater (Reaume et al. 2015).

3.3.2.4 Membrane bioreactors

The treatment of wastewater in a MBR indicated a higher efficiency in the reduction of the estrogenic activity compared to the conventional activated sludge treatment (Bertanza et al. 2017, 2011). Also, Maletz et al. (2013) reported on an almost complete decrease of the estrogenic activity in a MBR and additionally the MBR treatment successfully reduced the concentrations of compounds with the ability to alter the production of sex steroids. A MBR treatment highly reduced the estrogenic activity of the wastewater but it was still detectable in the effluent. Furthermore, the androgenic activity was also effectively reduced due to the MBR treatment. Both decreased endocrine activities could be attributed to the high sludge ages of the MBR. On the other hand, the wastewater samples showed an in parts severe remaining or even increased anti-estrogenic activity after the MBR treatment. The anti-androgenic activity was partly reduced by the MBR treatment but the results also indicated a high remaining anti-androgenic activity in the wastewater (Itzel et al. 2018; Gehrmann et al. 2018).

Our studies showed comparable results regarding the estrogenic, androgenic, anti-estrogenic, and anti-androgenic activity in MBR treated wastewater as published by Itzel et al. (2018) and Gehrmann et al. (2018). Furthermore, the endocrine activities of MBR treated and ozonated wastewater revealed assimilable trends (Schneider et al. 2020, Annex A.2; unpublished data: compare 2.2.4 and Annex A.5). These detected activities also could be attributed to masking effects of agonistic and antagonistic substances competing for the respective receptor (compare above) and/or a lower biodegradability of antagonistic substances. However, the mutagenicity distinctly increased after the ozonation process of the MBR treated wastewater as well indicating the formation of toxic TPs (Schneider et al. 2020, Annex A.2; unpublished data: compare 2.2.4 and Annex A.5).

Finally, the focus of the previous studies on endocrine activity in diverse treated kinds of wastewater was the detection and the removal rates of estrogenic activity. But the incidence of anti-estrogenic activity especially in ozonated wastewater should undergo further research. Overall, antagonistic (anti-estrogenic as well as anti-androgenic) activities and the related compounds are of interest and high importance because they are not significantly reduced compared to the respective agonistic activity during ozonation, MBR treatment and SF. The assessment of these persistent antagonistic effects for their ecotoxicological relevance is difficult because until now little attention has been paid to antagonistic substances of steroid receptors. Thus, the observed high activities of antagonistic substances probably are masking and diminishing the respective agonistic activity and demonstrate the relevance of these compounds for the wastewater treatment. Therefore, the investigation of antagonistic activity is advisable in parallel to the agonistic activity although chemical analysis was conducted. The performed studies show that additional research is essential specifically on the antagonistic activities and their elimination in WWTPs (Gehrmann et al. 2018; Itzel et al. 2018).

Overall, the AWWT technologies led to a decreased toxicity (generally endocrine activity) of the wastewater on the base of *in vitro* bioassays that could be a population relevant benefit for sensitive species in contaminated surface waters (Stalter et al. 2010a).

3.3.2.5 Sensitivities of different *in vitro* bioassays

Another important fact for the assessment of the wastewater is the investigation of its endocrine activity, mutagenicity and genotoxicity with different *in vitro* bioassays because they have different sensitivities. For example, yeast-based bioassays indicated no estrogenic or androgenic activity in aqueous wastewater samples

whereas the more sensitive ER-CALUX and androgen receptor (AR)-CALUX using a human breast cancer cell line detected a respectively estrogenic and androgenic activity in the identical wastewater samples. Although these bioassays rely on the same principle for the detection of estrogenic effects and utilise the same receptor, differences between the cell lines and their physiology (such as the existence of a cell wall functioning as a barrier, the ability to metabolise estrogenic active substances, the possible downregulation of the expression of the estrogen receptor, or substances acting as agonists and as antagonists), differences in the used media and reference substances, and partly in data analysis affect the results of the bioassays (Gehrmann et al. 2018).

In this context, estrogenic activity was detected in almost all investigated wastewater samples using two yeast two-hybrid bioassays. One bioassay included the human estrogen receptor α (hERα) and the other one included the estrogen receptor α from the medaka (*O. latipes*) (medERα). The estrogenic activity determined in the medERα bioassay was consistently higher in comparison to the hERα bioassay that is a typical behaviour of these assays. The hERα is most sensitive to natural and synthetic steroid hormones but shows a relatively limited response range whereas the medERα is more sensitive to non-steroidal natural and synthetic estrogenic substances and has a much wider response range (Mispagel et al. 2009).

Furthermore, the Ames test showed no mutagenicity in the investigated wastewater whereas the *umuC* assay indicated a high genotoxicity in the samples. In addition, the mutagenicity increased with increasing ozone dose whereas the genotoxicity decreased with increasing ozone concentrations (Magdeburg et al. 2014).

Overall, the results confirm that substances with non-specific toxicity as well as compounds with a receptor-mediated mode of action are effectively removed or

transformed throughout the ozonation process and the treatment with activated carbon (Stalter et al. 2011).

Furthermore, Maletz et al. (2013) pointed out that most of the performed *in vitro* bioassays for the detection of endocrine disruption focus on the receptors for the steroid hormones themselves. But the results of their study indicated that an objective assessment of the endocrine activity of the wastewater requires the inclusion of other endpoints than the specific binding activity to the relevant receptor such as the interaction with the endogenous synthesis pathway of the sex steroids.

In the end, each *in vitro* bioassay has its own benefits and limitations. Furthermore, another factor affecting the results of the bioassays is the sample handling and the preparation of the samples (compare above chapter 3.1) as long as there are no standard operating protocols for the determination of for example hormonal activities (Mispagel et al. 2009).

3.4 Removal of *in vivo* toxicity during conventional and advanced wastewater treatment

3.4.1 Conventional wastewater treatment

The survival of the rainbow trout *O. mykiss* exposed to conventional treated wastewater in the fish early life stage test (FELST) was significantly lower compared to the control group and the development of the fish was affected. At the beginning, a high mortality of the larvae was already detected, the hatching progress was delayed, and the hatching success of the larvae was also significantly lower. The swim up of the juvenile fish was delayed as well and less than the half of the fish reached the juvenile stage at the end of the exposure. Furthermore, the length and weight of an individual fish was again significantly lower in comparison to the control. Thus, the conventional

treated wastewater still contained substances with toxic sub-lethal and lethal effects on diverse developmental stages of the rainbow trout (Margot et al. 2013).

In a comparable experimental setup, unfiltered conventional treated wastewater caused a complete coagulation of the eggs of the rainbow trout *O. mykiss* in the FELST as a possible result of a microbial contamination with fungi mycelia and vorticellas. The membrane filtration of the wastewater aiming the minimisation of microbial impacts reduced the coagulation of the eggs possibly due to the additional removal of suspended particular matter and consequently all particle bound pollutants. But macromolecules and organic compounds were not retained by the membrane filtration. Thus, the filtered conventional treated wastewater as well impaired the survival and the development of the rainbow trout by a still significantly increased coagulation of the eggs, a significant decrease in the hatching success of the larvae, a considerable delay of the swim up of the juvenile fish, and a significant reduction of the body length and biomass of the fish. Solely the mortality was not significantly increased (Stalter et al. 2010b). However, Magdeburg et al. (2014) reported an increased mortality of rainbow trout embryos and larvae in the conventional treated wastewater in comparison to the control group.

The observed effects of the conventional treated wastewater on the rainbow trout are presumably due to ammonia (NH_3) which is the unionised form of ammonium (NH_4^+). Embryos and larvae of the fish are very sensitive to ammonia (Kienle et al. 2022; Margot et al. 2013). Even low concentrations (0.006-0.18 mg N-NH_3/L) are able to cause sub-lethal effects such as a decrease of the weight of the larvae. A delay in the development to the swim up stage was detected at concentrations above 0.01 mg N-NH_3/L. Lethal effects were attributed to concentrations in the range of 0.022 mg and 0.13 mg N-NH_3/L. The calculated concentrations of unionised ammonia in conventional treated wastewater were relatively high and varied between 0.02 and

0.06 mg N-NH_3/L and therefore be responsible for the demonstrated sub-lethal effects and even mortality of the fish. In addition, rainbow trouts are sensitive to nitrite (NO_2^-) inducing decreased growth rates at 0.3 mg N-NO_2^-/L and an increased mortality at 0.91 mg N-NO_2^-/L The concentrations of nitrite in the conventional treated wastewater ranged between 0.04 mg and 0.55 mg N-NO_2^-/L and thus, were improbably accountable for the detected sub-lethal and lethal effects on the fish. Furthermore, the toxicity caused by nitrite can be inhibited by chloride ions. The chloride concentrations in the investigated wastewater were relatively high (80-170 mg/L) and consequently have the ability to reduce the toxic effect of nitrite (Margot et al. 2013).

However, the explicit impact of the conventional treated wastewater on the rainbow trout such as the high mortality and the delayed development cannot be explained by the toxicity of ammonia solely, because comparable ammonia concentrations were measured in the ozonated wastewater. But the ozonated wastewater induced much lower mortality and developmental delay. Hence, the observed effects of the conventional treated wastewater are possibly due to substances like pharmaceuticals and pesticides that are oxidised during the ozonation process (Margot et al. 2013). Also, Kienle et al. (2022) reported on a significantly lower overall survival as well as post-hatch survival of the rainbow trout exposed to conventional treated wastewater.

Finally, such effects of the conventional treated wastewater on the development and the fitness of the rainbow trout in natural systems, especially in small rivers with a low dilution of the effluent, is able to increase the risk for predation since larvae are unable to escape before the swim-up. Furthermore, the fish may have an increased sensitivity towards environmental and anthropogenic stressors leading to an increased mortality. Thus, conventional treated wastewater probably has a significant impact on salmonid fish populations in natural environments (Margot et al. 2013; Stalter et al. 2010b).

Furthermore, the concentration of vitellogenin in the juvenile fish exposed to the conventional treated wastewater was significantly increased compared to the control group (Margot et al. 2013; Stalter et al. 2010b).

Vitellogenin is an egg yolk precursor naturally generated in mature female fish and can serve as a biomarker for the exposure to exogenous substances with estrogenic activity in juvenile and male fish. Thus, an increased vitellogenin concentration in the juvenile fish exposed to the conventional wastewater can be attributed to the presence of environmentally relevant levels of estrogenic active compounds and can be an indicator for an effect on their reproduction system (Margot et al. 2013; Stalter et al. 2010b). Besides, the mortality of two fish species (the Nile Tilapia (*O. niloticus*) and the common carp (*C. carpio*)) each reached around 90% even in diluted wastewater samples with effluent fractions of 70% of conventional treated wastewater. However, the mortality rates of the fish decreased to around 75% and 70%, respectively, under elevated nitrogen concentration conditions that is possibly due to the advancement of the bacterial abundance and diversity especially of the (heterotrophic) nitrifying bacteria. Both bacterial groups produce enzymes that are crucial to the degradation of toxic compounds and thus, effected the decreased toxicity of the wastewater (Boonnorat et al. 2017).

Next to the adverse effects on fish, conventional treated wastewater also increased the reproduction of the mudsnail *P. antipodarum*, possibly due to substances in the wastewater with an estrogenic activity, as well as the genotoxicity detected with the comet assay using the haemolymph of the zebra mussel *D. polymorpha* (Stalter et al. 2010a). Adverse effects of conventional treated wastewater were also reported by Magdeburg et al. (2012). The survival rate of the large water flea *D. magna* as well as the reproduction of the mudsnail *P. antipodarum* were significantly reduced. However, the growth rate of the *D. magna* population and the offspring of the female water fleas

were distinctly increased that could be explained by an additional nutrition such as suspended particular matter, algae, and bacteria.

The reproduction toxicity of the mudsnail *P. antipodarum* was reduced due to the conventional activated sludge treatment but it was still on a high level indicating a not sufficient cleaning capacity of the conventional wastewater treatment (Giebner et al. 2018).

The reproduction of the annelid *L. variegatus* was significantly reduced in conventional treated wastewater possibly due to MPs in the wastewater. For example, carboxy-acyclovir, a biological TP of the antiviral drug acyclovir (Prasse et al. 2012, 2011), was found to significantly reduce the reproduction level of the large water flea *D. magna* (Schlüter-Vorberg et al. 2017, 2015).

Altmann et al. (2012) reported a significantly increased number of egg-carrying females as well as the number of eggs after the exposure to conventional treated wastewater of the Japanese medaka *O. latipes* in comparison to the negative control and even the control group that was exposed to E_2. These increased numbers are possibly due to feeding effects because the wastewater included suspended particular matter that was ingested and digested by the fish. However, bioassays with the green algae *P. subcapitata*, with the large water flea *D. magna* and with eggs of the zebrafish *Danio rerio* showed no adverse effects of the wastewater.

The wastewater of 13 representative WWTPs originating from different countries were investigated using toxicity tests with the yeast *S. cerevisiae* and a marine diatom. The aim of the study was the analysis of the potential hazard to the growth of the organisms caused by complex mixtures of chemical substances even if they are present at low concentration when they are considered individually. The results indicated that most of the effluents showed no adverse effects on the test organisms but a few effluents caused cytotoxicity in both organisms. Some effluents even induced an increase of the

growth of the yeasts and diatoms. This impact also pointed to a higher concentration of nutrients in the wastewater and indicated the eutrophication potential of the wastewater that is a relevant aspect especially for autotrophic organisms like diatoms (Loos et al. 2013). An increased growth of the algae in conventional treated wastewater also indicated that potential adverse effects of (micro)pollutants might be overcompensated by the presence of nutrients or other stimulating compounds in the wastewater that enhance the algal growth (Itzel et al. 2017).

Our results (Schneider et al. 2020, Annex A.2) showed an increased shell height of the mudsnail *P. antipodarum* as well as an increased total number of embryos in the conventional treated wastewater possibly due to an additional nutrient supply and/or MPs with an estrogenic activity. However, the total energy content of the mudsnails decreased at the same time arguing against an additive nutrition. Furthermore, the mortality of the amphipod *G. fossarum* was increased in the conventional treated wastewater (unpublished data: compare 2.2.1 and Annex A.5) indicating the occurrence of toxic substances in the wastewater. However, the increased total energy content of the amphipods after the conventional treatment (compare 2.2.3 and Annex A.5) could be attributed to a better nutrition of the test organisms.

A decrease in the survival of *G. fossarum* was detected in conventional treated wastewater as well (Rothe et al. 2022). Adverse effects on the shredding amphipod *G. fossarum* were also reported by Bundschuh et al. (2011a). The amphipods were exposed to conventional treated wastewater in a semi-static test system under laboratory conditions and showed significant reductions in the feeding rate (–25%), the absolute consumption of leaf material (–35%), the food assimilation (–50%), the dry weight (–18%), and the lipid content (–22%) whereas the glycogen content was not significantly affected by the wastewater. These impairments are most probably caused by a complex mixture of MPs detected in the wastewater such as herbicides, beta

blockers, and analgetics. Also, the presence of mixtures of antibiotics in wastewater altered the feeding behaviour of amphipods (Bundschuh et al. 2009). The feeding rate of *G. fossarum* was as well significantly reduced in conventional treated wastewater in comparison to the control group exposed to river water (Bundschuh et al. 2011b). Besides, also other processes requiring energy such as the reproduction may be impaired resulting in a decreased breeding activity and reproductive output and thus, negatively affected population dynamics. In addition, freshwater populations of amphipods may become more susceptible to chemical stressors because it was shown that a reduced lipid content in a marine *Gammarus* species increased its sensitivity to cadmium by 40%. Overall, adverse effects on amphipods in streams and rivers resulting in a reduced leaf breakdown may become more relevant for ecosystem functioning in the future (Bundschuh et al. 2011a).

Furthermore, fitness traits of *G. fossarum* were negatively affected by conventional treated wastewater indicated by retarded moult cycles of the female amphipods and a reduced reproduction (fecundity and fertility) additionally to the induction of embryonic malformations of more than 90% of the embryos and to a lesser extent sperm genotoxicity. However, a direct correlation between the observed toxic effects and the quantified concentrations of MPs could not be evidenced (Wigh et al. 2017).

The investigation of the effects of conventional treated wastewater on a range of behaviours (such as feeding rate, phototaxis, activity, velocity and precopula pairing) in adult freshwater amphipods *G. pulex* showed an intensified and significant reduction in the overall activity. Furthermore, male amphipods exposed to the wastewater re-paired with non-exposed females four to six times faster in comparison to the control amphipods. The consequences of the detected behavioural changes are not known to date and highlight the need for varying sets of tools in the assessment of behavioural changes in wildlife (Love et al. 2020).

The feeding rate of the amphipod *G. pulex* was slightly but not significantly increased downstream of a discharge of a WWTP compared to amphipods collected upstream of the WWTP using *in situ* experiments. This effect was possibly due to some compounds that were detected in the wastewater with the ability to alter the microbial community on the leaves serving as food for the amphipods (Könemann et al. 2019).

In situ experiments were also conducted by Ganser et al. (2019) and Bundschuh et al. (2011c) indicating that the feeding rate of caged *G. fossarum* was significantly reduced up to 90% 50 m and 150 m downstream of a WWTP effluent with conventional treatment in comparison to the reference site upstream of the WWTP and even in a distance of 400 m downstream of the WWTP effluent the feeding rate of the amphipods was significantly reduced. However, the production of vitellogenin in male amphipods was not induced although a significantly increased estrogenic activity was detected using the YES downstream of the WWTP compared to the upstream reference site. Laboratory experiments supported these results suggesting that treated wastewater released into aquatic ecosystems impairs the function of the ecosystem regarding the decomposition of leaf litter.

In the end, these results indicate that the detected increases in growth and/or reproduction of the test organisms in conventional treated wastewater could not distinctly be attributed to an additional nutrition or the occurrence of (micro)pollutants (with potential endocrine activity) in the wastewater that may be a general challenge in *in vivo* test systems (Burdon et al. 2022). But the results also show that the conventional treated wastewater still show adverse impacts on diverse test organisms. Furthermore, field studies showed that common organic pollutants affected benthic invertebrates at current exposure levels. In addition, the benthic invertebrate taxa that were sensitive in the field were not used in regulatory ecotoxicology. These taxa were

affected at concentrations that were lower than expected from laboratory tests (Berger et al. 2016).

3.4.2 Advanced wastewater treatment

3.4.2.1 Ozonation

The ozonation of the conventional treated wastewater significantly decreased the toxicity of the wastewater on the development of the rainbow trout *O. mykiss* compared to the conventional treatment investigating the endpoints overall survival of the fish, hatching progress and hatching success of the larvae, swim-up of the juvenile fish, and the individual development (precisely size and weight of the fish). However, in the ozonated wastewater were still low lethal and sub-lethal effects (delays in the hatching progress and the swim-up and a reduction of the length and weight of the fish) detectable that could be attributed to high concentrations of unionised ammonia (NH_3) varying between 0.02 mg and 0.06 mg N-NH_3/L and improbably to the nitrite concentrations ranging from 0.04 mg to 0.55 mg N-NO_2^-/L (compare above chapter 3.4.1), but the toxicity was at a level close to the control. The ozonation process also reduced the induction of estrogenic effects (compare 3.3.2). In this context, the juvenile fish exposed to the ozonated wastewater indicated no increase of the vitellogenin concentration that was on a comparable level to the control group whereas the estrogenic activity in the extracts at the same time further decreased by 89% (Margot et al. 2013).

In the end, the treatment with ozone reduced the effects of the MPs on the fish in the conventional treated wastewater efficiently and thus, removed diverse compounds influencing the development and survival of the rainbow trout (Margot et al. 2013). In this context, the overall survival of the rainbow trout was significantly improved in the ozonated wastewater (Kienle et al. 2022).

In contrast, in a study by Stalter et al. (2010b) the ozonation process of membrane filtered wastewater indicated an increased toxicity on the rainbow trout *O. mykiss* in a comparable experimental setup that resulted in a substantial retardation in the development of the fish, particularly a higher egg coagulation, a delay in the hatching success and the swim-up process, a decreased body length and biomass of the fish and a high mortality. Indeed, the egg coagulation in the ozonated wastewater was delayed compared to the conventional treated wastewater presumably due to the disinfectant effect of ozone. But the egg coagulation was still increased compared to the control that may be the result of a fast regrowth of microorganisms (compare above chapter 3.4.1) because ozonation is known to produce high amounts of AOC (Zimmermann et al. 2011). The further detected adverse impacts of the ozonated wastewater are possibly due to a conversion of chemicals into more complex and more toxic metabolites and/or the formation of more toxic TPs compared to the chemical precursors such as PAHs. The impeded embryonic and/or larval development of the fish could be explained by those oxidative by-products such as aldehydes, carboxylic acids, ketones, brominated organic compounds, and other oxygen-rich compounds that resulted from the oxidation of the DOM (Zimmermann et al. 2011). In turn, the delay in the development of the embryos and the larvae could be the reason for the significant decrease in the body length and the body weight of the fish suffering from the developmental disadvantages. On the other hand, the contrary results of both studies could be explained by different ozone reactor configurations (for example the number of contact chambers and the HRT) and/or different wastewater compositions (for example residual ozone in the effluent) (Margot et al. 2013). However, Stalter et al. (2010 a,b) reported that the detected adverse effects of the ozonated wastewater on the investigated test organisms are most probably generated by toxic oxidation by-products. Residual ozone as a reason for the adverse effects can be excluded because

no ozone residuals could be detected after a retention time of 45 minutes. Therefore, a retention time of 60 minutes was chosen before the ozonated wastewater reached the test vessels to ensure a complete degradation of the ozone residuals.

But again, the vitellogenin concentration of the juvenile fish exposed to the ozonated wastewater decreased even below the level of the control group supporting previous findings of the high efficiency of the ozonation process to eliminate the estrogenic contamination in the wastewater rather than the formation of compounds with an anti-estrogenic activity (Stalter et al. 2010b).

However, the exposure to pure effluent before and after the ozonation indicated no mortality or any signs of disease on the wild-type Japanese medaka *O. latipes*. Neither the morphological examination of the fish nor the reproductive output pointed to estrogenic or androgenic endocrine effects. But ozonation reduced the number of egg-carrying females compared to the conventional treatment but it was still slightly higher in comparison to the negative control group and a group exposed to E_2. This impact could again be attributed to feeding effects caused by suspended particular matter enclosed in the wastewater (Altmann et al. 2012).

Besides, the ozonation of the wastewater increased the toxicity resulting in a significant inhibition of the reproduction and a significant reduction of the biomass of the annelid *L. variegatus* as well as to a decreased reproduction of the mudsnail *P. antipodarum* and an increased mortality of the zebra mussel *D. polymorpha*. Furthermore, the genotoxicity was significantly increased detected with the comet assay using the haemolymph of the zebra mussel. These increases in toxicity after the ozonation process are again possibly due to the formation of reactive and toxic oxidation by-products. However, the decreased reproduction of the mudsnail is not only a possible result of oxidative by-products. The reduction of the number of the embryos could also

be explained by the decreased estrogenic activity after the AWWTs (Stalter et al. 2010a).

Our results (Schneider et al. 2020, Annex A.2) also showed a decreased reproduction of the mudsnail *P. antipodarum* that could be attributed to an increase of the concentration of toxic compounds and/or a decrease of the estrogenic activity. In contrast, the mortality of the amphipod *G. fossarum* decreased and the body length of male and female amphipods as well as the part of brooding females and the total egg number increased after the ozonation process (unpublished data: compare 2.2.1, 2.2.2, and Annex A.5) indicating an improved wastewater quality compared to the conventional treatment.

The feeding rate of the leaf-shredding invertebrate *G. fossarum* also significantly increased in the ozonated wastewater with subsequent SF in comparison to the conventional treated wastewater and reached the level of the control group that was exposed to river water. However, the results of the bioassays also revealed that the application of ozone may result in the formation of toxic TPs that presumably mask the positive effects that were caused by the oxidation of the parent compounds (Bundschuh et al. 2011b).

An application of ozone to conventional treated wastewater of a municipal WWTP with following SF also significantly increased the feeding activity of *G. fossarum* exposed in outdoor flow-through stream microcosms compared to the non-ozonated wastewater. In addition, the population size in the ozonated wastewater was significantly increased as well at the end of the experiment indicating that the ozonation process may improve the quality of the wastewater by the reduction of the loads of MPs (Bundschuh & Schulz 2011a). These results were confirmed by further experiments as well resulting in significantly higher feeding rates of *G. fossarum* in ozonated wastewater compared to conventional treated wastewater suggesting again that the detected effects are most

likely triggered by a reduction of the concentration of the sum of MPs and not by an alteration in the organic matrix (Bundschuh & Schulz 2011b). In addition, *in situ* experiments with caged *G. fossarum* upstream and downstream of a WWTP effluent discharging ozonated wastewater showed no significant alterations of the feeding rate of the amphipods (Bundschuh et al. 2011c).

On the contrary, several sub-lethal effects and fitness traits of *G. fossarum* were impaired by the ozonated wastewater indicated by retarded moult cycles of the female amphipods and a reduced reproduction (fecundity and fertility) in addition to the induction of malformations of more than 90% of the analysed embryos and also, but to a lesser extent sperm genotoxicity. However, a direct correlation between the observed toxic effects and the quantified concentrations of MPs could not be evidenced. The toxic effects of the ozonated wastewater on the investigated endpoints were on a comparable level to the conventional treated wastewater and showed no significant differences although the concentrations of the quantified MPs differed and were commonly lower in the ozonated wastewater (Wigh et al. 2017).

Furthermore, Magdeburg et al. (2014, 2012) reported on adverse or toxic effects of the ozonated wastewater on the annelid *L. variegatus* and on the rainbow trout *O. mykiss* in terms of a significantly reduced biomass and number of the worms as well as an increased mortality of the fish embryos and larvae next to a delay in the development of the fish. The genotoxicity in the blood cell deoxyribonucleic acid (DNA) of the fish was also increased. Furthermore, the growth inhibition of the common duckweed *L. minor* was increased, the offspring of the female large water fleas *D. magna* increased as well whereas the reproduction of the mudsnail *P. antipodarum* slightly decreased that was also reported by Giebner et al. (2018). These results indicate that the oxidation products that were induced by the ozonation revealed a higher toxicological impact than the precursor compounds before their degradation within the ozonation

process and are in contrary to an expected reduction of the toxicity as a result of an effective elimination of organic trace pollutants. Regarding the water fleas, the higher reproductive output is presumably not an indication for a reduced toxicity because single substances and effluents have shown hormetic effects meaning that an increased number of neonates is induced at lower concentrations whereas at higher concentrations a decreased number of the offspring could be observed. Furthermore, a high reproductive output does not exclude a potentially attendant high mortality of the neonates. In case of the mudsnails the lower reproduction was attributed to distinct non-specific toxic effects and additionally to a reduction of the estrogenic activity caused by the reduction of the sum of (xeno-)estrogens that were able to mask the general toxicity on the reproduction (Magdeburg et al. 2012).

The ozonation of the wastewater indicated minimal effects on the common duckweed *L. minor* (decreased growth rate), on the annelid *L. variegatus* (increased reproduction and decreased biomass), and on the large water flea *D. magna* (reduction of the reproduction and the growth rate, but in part with significant differences). These effects could not be explained definitely and are presumably due to impaired nutrient conditions or the presence of toxic components. As an example, N-(4-carbamoyl-2-imino-5-oxo-imidazolidin)-formamido-N-methoxyacetetic acid (COFA), a TP of carboxy-acyclovir after an ozonation process (Prasse et al. 2012) inhibited the growth of a green algae (Schlüter-Vorberg et al. 2017, 2015).

The reproduction of the water flea *Ceriodaphnia dubia* was also impaired in the ozonated wastewater that could be attributed to heavy metals or other contaminants that were not effectively removed by the ozonation process (Kienle et al. 2022). Furthermore, the growth of oligochaete worms was significantly reduced after ozonation (Kienle et al. 2015).

Depending on the percentaged fractions of 50% and 80%, the ozonated wastewater on the one hand indicated a significant growth reduction of the green algae *D. subspicatus* whereas on the other hand a numerically increased growth was detected. However, altogether the ozonation process led to a reduction of the impeding effects indicating that the formation of more toxic compounds in terms of algae growth may be excluded but needed further investigations (Itzel et al. 2017).

Bioassays with the green algae *P. subcapitata*, with the daphnid *D. magna* and with eggs of the zebrafish *D. rerio* showed no adverse effects of the wastewater after the ozonation process (Altmann et al. 2012).

Finally, the diverse effects of the ozonation process that were detected among the different studies could be a result of specific differences between the species (Altmann et al. 2012), for example the Nile Tilapia (*O. niloticus*) is more sensitive to contaminants in the wastewater than the common carp (*C. carpio*) (Boonnorat et al. 2017), but as well regarding the variable sensitivity of several developmental stages within one species. Furthermore, differences in the wastewater characteristics and the methodology of the ozonation is of relevance for the interpretation of the results (Altmann et al. 2012).

3.4.2.2 Activated carbon

The addition of PAC to the conventional treated wastewater and a subsequent UF significantly reduced the toxicity of the wastewater on the development of the rainbow trout *O. mykiss* compared to the conventional treatment. The investigated endpoints overall survival of the fish, hatching progress and success of the larvae, swim-up of the juvenile fish, and the individual development (precisely size and weight) of the fish were distinctly enhanced and were collectively in the range of the control group that could be explained by low unionised ammonia concentrations (< 0.01 mg N-NH_3/L)

during the whole exposure due to a further nitrification in the reactor. An increase of the vitellogenin concentration in the juvenile fish as it was observed after the conventional treatment was not detected after the treatment with PAC and was on a comparable level to the control as well contaminant with a significantly reduced estrogenic activity (Margot et al. 2013). The treatment of the wastewater with PAC achieved a significant reduction of the genotoxicity but the PAC treatment also significantly reduced the reproduction of the mudsnail *P. antipodarum*. Furthermore, a slightly increased toxicity on the annelid *L. variegatus* and the common duckweed *L. minor* was detected after the treatment with PAC (Stalter et al. 2010a).

Magdeburg et al. (2014) reported on a lower mortality of the embryos and larvae of the rainbow trout *O. mykiss* after a PAC treatment of the wastewater in comparison to the conventional treatment indicating that the PAC treatment improved the quality of the wastewater. The application of PAC to conventional treated wastewater showed no significant effects on the feeding rate of the amphipod *G. fossarum*. However, the addition of PAC to the wastewater also reduced the bioavailability of nutrients that had to be considered in the evaluation of the detoxification potential of this AWWT method conducted with whole effluent samples (Bundschuh et al. 2011b).

The egg production of the fathead minnow *P. promelas* was significantly reduced after the treatment with GAC (Filby et al. 2010).

The reproduction of the mudsnail *P. antipodarum* was significantly decreased after the filtration of the conventional treated wastewater through a filter with GAC (Giebner et al. 2018). In a further experiment, the filtration of ozonated wastewater through a GAC filter showed that the reproduction toxicity on the mudsnail was on a comparable high level as after the ozonation (Giebner et al. 2018). These results indicate that the induced reproductive toxicity was partially reduced but not eliminated by the GAC filtration.

The reproduction of the water flea *C. dubia* was also impaired after the filtration of ozonated wastewater with a GAC filter indicating that this post-treatment did not effectively remove the contaminants that were responsible for the observed effects. However, a filtration with GAC further increased the overall survival of the rainbow trout (Kienle et al. 2022).

Ozonated wastewater passed through a non-aerated and aerated (with ambient air) GAC filter and showed no further relevant toxic effects or other impacts on the common duckweed *L. minor*, the annelid *L. variegatus*, and the large water flea *D. magna* indicating that possible toxic compounds after the ozonation process were efficiently removed by the GAC filtration (Schlüter-Vorberg et al. 2017).

3.4.2.3 Biofiltration

A SF of the ozonated wastewater did not further affect the residual toxicity in terms of an improvement of the low lethal and sub-lethal effects that were detected on the development of the rainbow trout *O. mykiss* after the ozonation process. The comparable impacts of the sand filtered wastewater on the investigated endpoints overall survival of the fish, hatching progress and success of the larvae, swim-up of the juvenile fish, and the individual development (precisely size and weight of the fish) could be explained by high concentrations of unionised ammonia (NH_3) as the sand filtered wastewater indicated as high NH_3 concentrations as the ozonated wastewater. The vitellogenin concentration of the juvenile fish after the SF was as low as in the ozonated wastewater attended by a very low-level estrogenic activity. In one case, the estrogenic activity as well as the vitellogenin concentration in juvenile fish showed a minor increase after the SF that was presumably due to a contamination of the new sand with estrogenic compounds such as bisphenol A (Margot et al. 2013).

The toxic effects of the ozonated wastewater on the annelid *L. variegatus* as well as the increased genotoxicity detected with the haemolymph of the zebra mussel *D. polymorpha* were reduced after the SF step to levels previous to the ozonation indicating that SF obviously is an effective barrier to toxic oxidation by-products. A sand filter is known for the reduction of aldehyde concentrations in the wastewater, for an effectively removal of the cancerogenic TP NDMA formed throughout the ozonation process, and for a highly reduction of the content of AOC. These reductions are mainly an effect of biological degradation. However, the genotoxicity of the wastewater after the SF was not reduced compared to the wastewater after the conventional treatment indicating that the sand filter is not capable to remove genotoxic oxidation by-products below environmental relevance. In addition, the reproduction of the mudsnail *P. antipodarum* was further decreased compared to the ozonated wastewater (Gerrity et al. 2014; Zimmermann et al. 2011; Stalter et al. 2010a; Hollender et al. 2009). In this context, Giebner et al. (2018) also reported on an increased reproduction toxicity of the mudsnail after a SF step in comparison to the ozonated wastewater whereas the toxicity after a combination of a GAC filter and a sand filter was on a comparable level to the ozonation.

The following SF of non-filtered and ozonated wastewater reduced the egg coagulation of the rainbow trout *O. mykiss* that could be attributed to the reduction of the amount of suspended particular matter (SPM) and of AOCs which may have reduced the microbial development (Stalter et al. 2010b). However, a subsequent SF of membrane filtered and ozonated wastewater showed slightly increased egg coagulations of the rainbow trout but the hatching of the fish was slightly earlier. In addition, the swim up was considerable earlier and the body length and weight of the fish was significantly increased after the SF. These improvements after the sand filter could be attributed to a removal of the ozonation metabolites by a spontaneous degradation, by biological

degradation or by adsorbable properties of the oxidation by-products responsible for the adverse effects (Stalter et al. 2010b). Thereby, oxygen-rich compounds are typically removed during SF (Zimmermann et al. 2011). Also, Kienle et al. (2022) reported on a further increased overall survival of the rainbow trout after a SF of ozonated wastewater.

In comparison to the ozonated wastewater the number of the annelid *L. variegatus* and the biomass of the worms were significantly increased whereas the reproduction of the mudsnail *P. antipodarum* was significantly decreased after the SF. The embryos and the larvae of the rainbow trout *O. mykiss* showed a decreased mortality and the genotoxicity was slightly reduced as well due to the SF. These results show that a SF effectively reduced the toxicity of oxidation products that are most likely due to the biodegradation processes in the bio-layer of the sand filter and/or the rapid decomposition of fractions of the causing oxidation products (Magdeburg et al. 2014, 2012).

No relevant toxic effects or other significant adverse or beneficial impacts on the common duckweed *L. minor*, the annelid *L. variegatus*, and the large water flea *D. magna* were detected in the ozonated wastewater that was treated by a subsequent non-aerated and aerated (with ambient air) BF. Thus, substances with potentially adverse impacts on the test organisms were successfully removed by the BF (Schlüter-Vorberg et al. 2017).

However, the reproduction of the water flea *C. dubia* was impaired after the SF of the ozonated wastewater possibly due to compounds that were not effectively removed. In contrast, the reduced growth of oligochaete worms after the ozonation was eliminated after the sand filter suggesting that labile and biologically active reaction products presumably generated by the ozonation process were effectively removed depending on the composition of the wastewater (Kienle et al. 2022, 2015).

3.4.2.4 Membrane bioreactors

The MBR treatment of wastewater indicated a slightly decreased growth rate of the common duck weed *L. minor* whereas the reproduction of the annelid *L. variegatus* was significantly increased. Furthermore, the reproduction was obviously and the population growth rate of the large water flea *D. magna* was significantly decreased in the MBR treated wastewater. These effects are possibly due to increased ammonium and nitrite concentrations that could occur if the denitrification process in the MBR is insufficient (Schlüter-Vorberg et al. 2017).

The MBR treated wastewater induced a mortality in two fish species (the Nile Tilapia (*O. niloticus*) and the common carp (*C. carpio*)) of around 25% and 20%, respectively, in diluted samples with a wastewater fraction of 70%. The mortality of both fish species decreased by an increased nitrogen concentration below 5%. Assimilable to the results of the conventional treatment the reduced mortality could be explained by the advantage in growth and diversity of particularly (heterotrophic) nitrifying bacteria degrading toxic compounds and therefore, decreasing the mortality of the fish (Boonnorat et al. 2017).

Our results (Schneider et al. 2020, Annex A.2) also showed a decreased shell height of the mudsnail *P. antipodarum* in the MBR treated wastewater. Furthermore, both the singly MBR treated as well as the MBR treated wastewater after the ozonation induced a decreased total number of embryos per female indicating a remaining of toxic substances with adverse effects on the growth and the reproduction of the mudsnail. In contrast, the mortality of the amphipod *G. fossarum* decreased in all MBR treated wastewater compared to the conventional treatment pointing to a reduction of toxic substances (unpublished data: compare 2.2.1 and Annex A.5).

Finally, some of these studies demonstrate that the treatment of wastewater with ozone implies the immanent hazard of the toxification of compounds present in the wastewater. However, a SF with its biological active biofilm installed subsequent to the ozonation of the wastewater is an appropriate treatment to counteract the adverse effects of the ozonation process.

Consequently, the application of an ozonation process to the already existing wastewater treatment should only be established together with a biological active post treatment capable for the removal of the oxidation by-products.

But it has to be considered that the above-mentioned studies were conducted under different conditions such as the treatment level of the wastewater before the ozonation process, the used filter media (for the post-treatment), the wastewater matrices and the overall process operations that presumably have an impact on the efficiency of the ozone-BF process. Besides, the mechanisms that are responsible for the performance of the BFs with adsorptive and non-adsorptive media are not completely comprehended. Thus, there is a need for the further investigation of the ozonation process of conventional treated municipal wastewater with following BF (Reaume et al. 2015).

In the end, *in vivo* bioassays are an effective tool to assess the overall and chronic toxicity of the wastewater effluents of the conventional treatment as well as the AWWT technologies and the biological effect of their constituents. The results distinctly show that the above-mentioned organisms, including (luminescent) bacteria, aquatic invertebrates as well as aquatic vertebrates, and test systems are suited for the detection of adverse effects of different treatment technologies. But it has to be considered that some investigated test organisms are not suitable for such an assessment because they are presumably not sensitive enough to detect the toxicity of the MPs or for example of the oxidation by-products (Stalter et al. 2010b). In fact,

considerable discrepancies in the sensitivities of different test organisms have been observed. For example, Alonso et al. (2010) contrasted the sensitivities of the freshwater amphipods *G. pulex* and *G. fossarum* to toxicants suggesting that the risk assessment of toxicants to freshwater amphipods should include bioassays with the most sensitive species and life stage. Thus, results of *in vivo* bioassays with no noticeable differences between the single treatment steps could be interpreted as false negative results. In addition, there is the possibility that the applied test organisms are afflicted by microorganisms such as bacteria and fungi or by endoparasitic organisms. In such a case, an assessment of the results of the *in vivo* bioassay is not feasible (Boonnorat et al. 2017; Stalter et al. 2010b). In this context, the results of a study by Rothe et al. (2022) indicated that the response of the amphipod *G. fossarum* exposed to conventional treated wastewater in flow channel experiments was mainly affected by an acanthocephalan parasite infection implying that parasites may represent an additional biotic stressor in multiple stressor experiments. However, Schlüter-Vorberg et al. (2017) reported on a significantly reduced parasite susceptibility of the large water flea *D. magna* in conventional treated wastewater as well as in ozonated wastewater. In addition, the long-term survival of *D. magna* was synergistically impacted by the co-exposure to a chemical and pathogen (Schlüter-Vorberg & Coors 2019). Therefore, parasites might play an important role within the measurement of the response of organisms to chemical stressors. Thus, a parasite infection and the analysis of immunotoxicity as accessory test parameters should be considered in prospective ecotoxicological studies and environmental risk assessments (Rothe et al. 2022; Schlüter-Vorberg & Coors 2019).

3.5 Comparison of advanced wastewater treatment technologies

3.5.1 Micropollutants

Several fate studies have investigated the occurrence, the behaviour and the remaining of personal care products, pharmaceuticals, endocrine disrupting substances, drugs, and other industrial chemicals in the aquatic environment. Thereby, it was figured out that the removal efficiency of these compounds strongly depended on the technology that was implemented in the WWTP (Loos et al. 2013; Hollender et al. 2009; Kaspryzyk-Hordern et al. 2009).

The performance of ozonation and PAC adsorption to remove organic MPs from conventional treated wastewater depends on the chemical properties of the substances (for example charge, hydrophobicity, and the presence of electron-rich moieties). However, both AWWT treatments showed similar removal efficiencies whereas specific substances were removed more efficiently by ozonation and the adsorption to PAC efficiently removed a wider range of MPs but to a lower degree than ozone. For example, both treatments removed carbamazepine by more than 90% whereas the ozonation performed better in the reduction of diclofenac and sulfamethoxazole whereas the treatment with PAC was more efficient for the removal of benzotriazole and iomeprol. However, a non-efficient removal of MPs might be attributed to short PAC contact times (Altmann et al. 2014; Margot et al. 2013; Kovalova et al. 2013). Furthermore, the removal efficiency of PAC was in general less predictable than for ozone. Besides, ozone and PAC have different removal mechanisms. The applied ozone dosage seemed not to be sufficient for the mineralisation of the MPs to CO_2 and thus the MPs were presumably transformed to predominantly unknown oxidation products on the one hand with the expectation to lose their biological activity but on the other hand with the potential of the genesis of a

higher toxicity of the TPs. But also, for the known oxidation by-products, no information is available on the extent and kinetics of their formation during the ozonation of the wastewater (Zimmermann et al. 2011). Dissimilar to ozone, the main advantage of the treatment with PAC is the physical removal of the MPs from the (waste)water avoiding the generation and the release of unknown TPs. However, the main disadvantage of the PAC treatment might be that it has to be renewed regularly and consequently the used and contaminated PAC has to be regenerated or disposed/incinerated, generally off site (Margot et al. 2013; Reungoat et al. 2011; Stalter et al. 2010a).

Also, Reaume et al. (2015) reported on ozonation being an effective process in the transformation of several chemicals of emerging concern that were not removed within the conventional wastewater treatment. Again, the potential formation of toxic TPs such as the cancerogenic compounds NDMA or bromate from the precursor substances *N,N*-dimethylsulfamide (DMS) and bromide, respectively, is the major insecurity of the ozonation process since little mineralisation of organic carbon is expected. Thus, the solely analysis of the disappearance of the parent compound is not sufficient because the TPs are presumably of more concern than their parent compounds (Wu et al. 2019). For example, the fungicide tolyfluanide is degraded by microorganisms to DMS that already was detected in surface water (50-90 ng/L) as well as in groundwater (100-1000 ng/L) and that could not be removed by diverse filtration, oxidation, or disinfection procedures. But 30-50% of the DMS are converted to the cancerogenic NDMA during ozonation. However, NDMA is biodegradable and can partially be removed by SF or filtration with activated carbon (Schmidt & Brauch 2008). Furthermore, the concentrations of NDMA and bromate produced within the ozonation process were below or in the range of the drinking water standards. However, in contrast to NDMA the concentration of bromate was not reduced by a subsequent biological SF (Hollender et al. 2009). However, a filtration with BAC is

suggested to perform better in the removal of chemicals of emerging concern in comparison to a sand BF (Reaume et al. 2015).

In bench scale experiments Altmann et al. (2014) compared typical ozone dose for practical applications and showed that ozonation proved to be more efficient for the abatement of sulfamethoxazole, whereas the removal of benzotriazole and iomeprol was comparatively more efficient with PAC.

Some compounds showed a comparable removal efficiency between the ozonation process and the treatment with PAC. But the removal of the selectd pharmaceuticals such as carbamazepine and sulfamethoxazole was significantly lower with PAC addition in comparison to the ozone treatment (Magdeburg et al. 2014).

The comparison of ozonation and MBR treatment showed that both processes are efficient methods for the removal of estrogenic pharmaceuticals from wastewater but again there is concern that the treatment with ozone can form toxic TPs and endocrine active metabolites (Maletz et al. 2013).

Regarding the removal of MPs the addition of PAC to the wastewater is on a comparable level as the ozonation of the wastewater. Thereby, an advantage of the PAC application is the possibility to use the PAC twice by recycling the waste PAC from for example the adsorption reactor. This double-stage usage of the PAC significantly increased the overall removal of MPs (> 80%) in comparison to the single-effluent applications (Boehler et al. 2012).

A disadvantage of wastewater treatment with GAC or PAC is that the efficiency of the adsorption processes is significantly influenced by the content and the composition of the DOM in the effluent. The DOM competes with the target organic MPs for the adsorption sides, blocks the outer pores and hence, inhibits the access to the inner micropores of the activated carbon leading to a saturation of the active pores. Thus, the sorption efficiency of GAC/PAC decreased with increasing DOM concentrations

resulting in an unsatisfactory filtration efficiency. Hence, larger amounts of the activated carbon and/or a frequent backwashing of the GAC filter are required. Furthermore, high DOC concentrations cause long-term carbon fouling also reducing the adsorption capacity and finally leading to an accelerated breakthrough of the filter. However, an adjustment of the PAC dose to the DOM concentration in the wastewater could be an appropriate strategy to improve the prediction of the removal of the organic MPs as well and as it is already known for ozone (Altmann et al. 2016; 2014; Boehler et al. 2012; Serrano et al. 2011). Furthermore, the adsorption to PAC has been proposed to be a more efficient alternative in comparison to the GAC treatment. But the release of low amounts of loaded PAC into the effluent and also into the environment even after a separation by SF cannot be excluded and thus, membrane systems represent a safer technology (Margot et al. 2013). Also, Boehler et al. (2012) highlighted that an effective separation of the used PAC from the treated wastewater is necessary to prevent the loss of the fine PAC fraction into the receiving water. Nowotny et al. (2007) as well argued that technical applications using fixed-bed GAC adsorbers may be of advantage in comparison to the usage of PAC.

Furthermore, comparing the applicability of the diverse advanced treatment options for the WWTPs that ought to be improved, advantageous for the ozonation process is the possibility of an easily testing of its efficiency in laboratory experiments whereas experiments for the suitability of GAC filter and BF are more challenging due to longer required time periods. In addition, a specification of the bed volumes of the investigated test columns of both filter systems is mandatory for the removal of MPs and for the comparability of the results from different studies. Thereby, increasing bed volumes are a disadvantage for the used GAC filters because of long-term carbon fouling resulting in decreasing sorption capacities whereas the efficiency of BF systems

improved with increasing bed volumes based on the growth of the biofilm (Ternes et al. 2017).

The conventional treatment as well as a MBR treatment showed similar performances in the removal efficiency of the investigated target compounds (Bertanza et al. 2011). Also, Camacho-Muñoz et al. (2012) demonstrated that MBR systems are an acceptable alternative to provide high-quality water for reuse purposes. Furthermore, the MBR treatment outperformed the CAS treatment in the elimination of most target antibiotics, antibiotic resistant bacteria, and antibiotic resistance genes (Le et al. 2018). However, next to the oxidation of emerging chemicals the ozonation process can as well be designed for typical disinfection targets (Reaume et al. 2015). Bertanza et al. (2011) reported on a very high disinfection performance of tertiary ozonation. Also, Altmann et al. (2014) highlighted that ozonation provided disinfection capabilities in addition to the degradation of organic MPs. Furthermore, the disinfection step is an advantage of the ozonation process that is characterised as a feature required for reuse applications that include direct human contact for example in household reuse applications (Hernandez-Leal et al. 2011). Besides, ozonation is mainly used in the drinking water production because ozone is an affective disinfectant for viruses, bacteria, and protozoa (Zimmermann et al. 2011).

3.5.2 *In vitro* bioassays

Regarding the *in vitro* bioassays, both AWWT technologies ozonation and PAC adsorption followed by UF led to an in parts significantly reduction of the residual growth inhibition (non-specific toxicity) and the residual photosynthesis inhibition (specific toxicity) of a green alga that were detected in the effluent of a conventional WWTP. In addition, a sand filter after the ozonation process further (slightly) reduced the growth inhibition and the photosynthesis inhibition. Altogether, the combination of

PAC adsorption with UF performed slightly better compared to the ozonation process followed by SF concerning the mean reduction of the non-specific toxicity (by 97% and 96%, respectively) as well as the mean reduction of the specific toxicity (by 92% and 87%, respectively) compared to the raw (untreated) wastewater (Margot et al. 2013).

The BAC BF significantly improved the quality of pilot-scale ozonated wastewater of conventional secondary treated municipal wastewater and performed much better concerning the reduction of genotoxicity and organic matter compared to the sand BF operating under the same conditions (Reaume et al. 2015).

Furthermore, the MBR treatment indicated a higher reduction of estrogenic activity in comparison to the conventional treatment (Bertanza et al. 2011).

3.5.3 *In vivo* bioassays

In the case of *in vivo* bioassays, both AWWT technologies reduced the toxicity on the development of a fish (survival, hatching success, swim-up, and length and weight of the rainbow trout) with a comparable efficiency whereas the combination of PAC adsorption with subsequent UF also performed slightly better compared to the ozonation followed by SF. On the other hand, ozonation was marginally better in the reduction of the estrogenic activity in comparison to the PAC treatment. Finally, the treatment combination of PAC with UF was recommended for sensitive receiving waters (for example drinking water resources) because of a good removal of most macropollutants and MPs without forming problematic TPs, the strongest decrease in toxicity and a total disinfection of the effluent. Furthermore, PAC offers the ability to eliminate further kinds of MPs such as dissolved heavy metals (for example chrome (Cr), iron (Fe), zinc (Zn), and lead (Pb)) due to its non-specific removal mechanisms that were not eliminated by ozonation even in combination with a subsequent SF step (Margot et al. 2013; Ruel et al. 2011; Renman et al. 2009).

Thus, the ozonation (with following SF) of the wastewater just as its treatment with activated carbon (with subsequent UF) provided an efficient degradation or removal of MPs as well as an efficient reduction of toxic effects and therefore, both AWWTs were able to improve the quality of the wastewater and might be beneficial for the health of the ecosystem (Margot et al. 2013; Stalter et al. 2010a; Escher et al. 2009; Hollender et al. 2009; Nowotny et al. 2007).

The effectiveness of a full-scale ozone treatment in combination with a fluidised moving-bed reactor as a biological post treatment was demonstrated by Itzel et al. (2017). The majority of the investigated MPs were reduced (removal rates > 80% for pharmaceuticals and industrial chemicals) without the release of relevant toxic TPs assessed by chemical analysis and toxicity-based bioassays.

Our results (Schneider et al. 2020, Annex A.2; unpublished data: compare 2. and Annex A.5) indicated that ozonation is an effective treatment for the reduction of the concentrations of MPs. Furthermore, the overall ecotoxicological toxicity decreased as well with the exception of for example increased mutagenic and anti-estrogenic activities accenting that a post-treatment after the ozonation process is required. In these cases, a filtration with GAC was successful to reduce the increased activities. Thus, ozonation with subsequent GAC filtration seemed to be the most promising AWWT processes (Table 1, 2, 3 and 4).

Table 1: Summary of the results of the *in vivo* on-site test with *Potamopyrgus antipodarum*. PC: positive control, NC: negative control, BT: conventional biological treatment, 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; arrows display differences to NC/BT: ⇧: increase, ⇔: no difference, ⇩: decrease; n.s.: not significant, ★ $p < 0.05$, ★★ $p < 0.01$, ★★★ $p < 0.001$; colours display on the one hand the real mortality of PC, BT, BT+O_3, GAC, GAC_a, BF, BF_a, MBR1, MBR1+O_3 and MBR2 and on the other hand the differences to NC/BT: green: 0-20%, yellow: 20-40%, orange: 40-60%, red: 60-80%, dark red: > 80%, *: colours display real mortality.

	PC ΔNC	BT ΔNC	BT+O_3 ΔBT	GAC ΔBT	GAC_a ΔBT	BF ΔBT	BF_a ΔBT	MBR1 ΔBT	MBR1+O_3 ΔBT	MBR2 ΔBT
mortality	⇧ n.s. *	⇧ n.s. *	⇩ n.s. *	⇧ n.s. *	⇩ n.s. *	⇔ *	⇩ n.s. *	⇩ n.s. *	⇩ n.s. *	⇩ n.s. *
shell height	⇧ n.s.	⇧ ★	⇩ n.s.	⇩ n.s.	⇩ n.s.	⇩ n.s.	⇩ n.s.	⇩ n.s.	⇩ n.s.	⇩ ★
total number of embryos per female	⇧ n.s.	⇧ n.s.	⇩ ★★	⇩ n.s.	⇩ ★	⇩ n.s.	⇩ ★★★	⇩ ★★★	⇩ ★★	⇩ ★★★
fecundity index	⇧ n.s.	⇧ n.s.	⇩ ★★★	⇩ n.s.	⇩ ★★	⇩ n.s.	⇩ ★★★	⇩ ★★★	⇩ ★★	⇩ ★★★
energy as protein content	⇩ n.s.	⇩ n.s.	⇧ n.s.	⇧ n.s.	⇧ n.s.	⇧ n.s.	⇩ n.s.	⇩ n.s.	⇩ n.s.	⇩ n.s.

Table 1: (continued)

	PC ΔNC	BT ΔNC	BT+O_3 ΔBT	GAC ΔBT	GAC_a ΔBT	BF ΔBT	BF_a ΔBT	MBR1 ΔBT	MBR1+O_3 ΔBT	MBR2 ΔBT
energy as glycogen content	⇧ n.s.	⇩ n.s.	⇩ n.s.	⇧ ★	⇧ n.s.	⇩ n.s.	⇩ n.s.	⇩ n.s.	⇩ n.s.	⇩ n.s.
energy as lipid content	⇩ ★★	⇩ ★	⇧ n.s.	⇧ n.s.	⇧ ★	⇧ ★★★	⇧ n.s.	⇧ n.s.	⇧ n.s.	⇧ n.s.
total energy content	⇩ ★★★	⇩ ★★★	⇧ n.s.	⇧ n.s.	⇧ ★★	⇧ ★★★	⇧ ★	⇧ n.s.	⇧ n.s.	⇧ n.s.

Table 2: Summary of the results of the *in vitro* bioassays of native 24 h composite samples and their SPE extracts taken in parallel to the on-site test with *Potamopyrgus antipodarum*. PT: primary treatment, BT: conventional biological treatment, 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; arrows display differences to PT/BT: ⇧: increase, ⇔: no difference, ⇩: decrease; colours display on the one hand the activity in the PT/BT and the real activity/mutagenicity of PC, BT, BT+O_3, GAC, GAC_a, BF, BF_a, MBR1, MBR1+O_3 and MBR2 in the YAAS and the Ames YG7108 and on the other hand the differences to PT/BT: green: 0-20%, yellow: 20-40%, orange: 40-60%, red: 60-80%, dark red: > 80%; n.a.: no activity; n.c.: not calculable, *: colours display real activity; ☠: cytotoxic.

	activity in		BT ΔPT	BT+O_3 ΔBT	GAC ΔBT	GAC_a ΔBT	BF ΔBT	BF_a ΔBT	MBR1 ΔBT	MBR1+O_3 ΔBT	MBR2 ΔBT
	PT	BT									
YES (native)			⇩	⇩	⇩	⇩	⇩	⇩	⇩	⇩	⇩
YAES (native)			⇩	⇧	⇧	⇧	⇧	⇧	⇧	⇧	⇧
YAS (native)			⇩	⇩	⇩	⇩	⇩	⇩	⇧	⇧	⇩
YAAS (native)		n.a.	⇩	n.c. ⇧ *	n.c. ⇔ *	n.c. ⇔ *	n.c. ⇧ *	n.c. ⇔ *	n.c. ⇧ *	n.c. ⇧ *	n.c. ⇧ *
YES (extracts)	☠		n.c.	⇩	⇩	⇩	⇩	⇩	⇩	⇩	⇩

Table 2: (continued)

	activity in		BT ΔPT	BT+O_3 ΔBT	GAC ΔBT	GAC_a ΔBT	BF ΔBT	BF_a ΔBT	MBR1 ΔBT	MBR1+O_3 ΔBT	MBR2 ΔBT
	PT	BT									
YAES (extracts)	☠		n.c.	⇧	⇧	⇧	⇧	⇧	⇧	⇧	⇧
YAS (extracts)	☠		n.c.	⇩	⇩	⇩	⇩	⇩	⇩	⇩	⇩
YAAS (extracts)	☠		n.c.	⇩	⇩	⇩	⇩	⇩	⇩	⇩	⇩
Ames YG7108 (extracts)	☠		n.c.	⇧*	⇧*	⇧*	⇧*	⇧*	⇧*	⇧*	⇩*

Table 3: Summary of the results of the *in vivo* on-site test with *Gammarus fossarum*. PC: positive control, NC: negative control, BT: conventional biological treatment, 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; arrows display differences to NC/BT: ⇧: increase, ⇔: no difference, ⇩: decrease; n.c.: not calculable, n.s.: not significant, ★ $p < 0.05$, ★★ $p < 0.01$, ★★★ $p < 0.001$; colours display on the one hand the real mortality of PC, BT, BT+O_3, GAC, GAC_a, BF, BF_a, MBR1, MBR1+O_3 and MBR2 and on the other hand the differences to NC/BT: green: 0-20%, yellow: 20-40%, orange: 40-60%, red: 60-80%, dark red: > 80%, *: colours display real mortality.

	PC ΔNC	BT ΔNC	BT+O_3 ΔBT	GAC ΔBT	GAC_a ΔBT	BF ΔBT	BF_a ΔBT	MBR1 ΔBT	MBR1+O_3 ΔBT	MBR2 ΔBT
mortality	⇩ n.s. *	⇧ ★★★*	⇩ ★★★*	⇩ ★★★*	⇩ ★★*	⇩ ★★★*	⇩ ★★★*	⇩ ★★★*	⇩ ★★★*	⇩ ★★★*
body length males	⇧ n.s.	⇩ n.s.	⇧ ★★★	⇩ n.s.	⇧ ★	⇧ n.s.	⇧ n.s.	⇧ n.s.	⇧ n.s.	⇧ ★★
body length females	⇧ n.s.	⇩ n.s.	⇧ ★★★	⇩ n.s.	⇧ n.s.	⇧ n.s.	⇧ n.s.	⇧ n.s.	⇧ n.s.	⇧ n.s.
egg number per female	⇧ n.s.	⇩ n.s.	⇧ n.s.	⇧ n.s.	⇩ n.s.	⇧ n.s.	⇧ n.s.	⇧ n.s.	⇧ n.s.	⇧ n.s.
total egg number	⇧ n.c.	⇩ n.c.	⇧ n.c.	⇧ n.c.	⇧ n.c.	⇧ n.c.	⇧ n.c.	⇧ n.c.	⇧ n.c.	⇧ n.c.
fecundity index	⇧ n.s.	⇩ n.s.	⇧ n.s.	⇧ n.s.	⇩ n.s.	⇧ n.s.	⇧ n.s.	⇧ n.s.	⇧ n.s.	⇧ n.s.

Table 3: (continued)

	PC ΔNC	BT ΔNC	BT+O_3 ΔBT	GAC ΔBT	GAC_a ΔBT	BF ΔBT	BF_a ΔBT	MBR1 ΔBT	MBR1+O_3 ΔBT	MBR2 ΔBT
energy as protein content	⇩ n.s.	⇩ ★	⇧ n.s.	⇧ ★★★	⇧ ★★★	⇧ ★★★	⇧ ★★★	⇧ ★★	⇧ ★★★	⇧ ★★★
energy as glycogen content	⇩ n.s.	⇧ ★★★	⇩ n.s.	⇩ ★★★	⇩ ★★★	⇩ ★★★	⇩ ★★	⇩ n.s.	⇩ n.s.	⇩ n.s.
energy as lipid content	⇩ n.s.	⇧ ★★★	⇩ n.s.	⇩ ★★★	⇩ ★★	⇩ ★★★	⇩ ★★★	⇩ ★★★	⇩ ★	⇩ ★★
total energy content	⇩ n.s.	⇧ ★★★	⇩ n.s.	⇩ ★★★	⇩ ★★	⇩ ★★★	⇩ ★★★	⇩ ★★★	⇩ n.s.	⇩ ★★

Table 4: Summary of the results of the *in vitro* bioassays of native 24 h composite samples and their SPE extracts taken in parallel to the on-site test with *Gammarus fossarum*. PT: primary treatment, BT: conventional biological treatment, 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; arrows display differences to PT/BT: ⇧: increase, ⇔: no difference, ⇩: decrease; colours display on the one hand the activity in the PT/BT and the real activity/mutagenicity of PC, BT, BT+O_3, GAC, GAC_a, BF, BF_a, MBR1, MBR1+O_3 and MBR2 in the YAAS and the Ames YG7108 and on the other hand the differences to PT/BT:: green: 0-20%, yellow: 20-40%, orange: 40-60%, red: 60-80%, dark red: > 80%; n.a.: no activity; n.c.: differences not calculable, *: colours display real activity; ☠: cytotoxic.

	activity in		BT ΔPT	BT+O_3 ΔBT	GAC ΔBT	GAC_a ΔBT	BF ΔBT	BF_a ΔBT	MBR1 ΔBT	MBR1+O_3 ΔBT	MBR2 ΔBT
	PT	BT									
YES (native)			⇩	⇩	⇩	⇩	⇧	⇩	⇧	⇧	⇧
YAES (native)			⇩	⇩	⇧	⇧	⇧	⇧	⇔	⇩	⇩
YAS (native)			⇩	⇧	⇧	⇧	⇧	⇧	⇩	⇩	⇧
YAAS (native)	n.a.	n.a.	n.c. ⇔ *	n.c. ⇔ *	n.c. ⇔ *	n.c. ⇔ *	n.c. ⇔ *	n.c. ⇔ *	n.c. ⇧ *	n.c. ⇧ *	n.c. ⇔ *
YES (extracts)	☠		n.c.	⇩	⇩	⇩	⇩	⇩	⇩	⇩	⇩

Table 4: (continued)

	activity in		BT ΔPT	BT+O_3 ΔBT	GAC ΔBT	GAC_a ΔBT	BF ΔBT	BF_a ΔBT	MBR1 ΔBT	MBR1+O_3 ΔBT	MBR2 ΔBT
	PT	BT									
YAES (extracts)	☠		n.c.	⇩	⇩	⇩	⇩	⇧	⇩	⇩	⇧
YAS (extracts)	☠		n.c.	⇧	⇩	⇩	⇧	⇩	⇧	⇧	⇧
YAAS (extracts)	☠		n.c.	⇩	⇩	⇩	⇩	⇩	⇩	⇩	⇩
Ames YG7108 (extracts)	☠		n.c.	⇧*	⇧*	⇧*	⇧*	⇧*	⇔*	⇧*	⇩*

In this context, a detailed systematic review paper was published by Völker et al. (2019) about the assessment of the removal of *in vitro* and *in vivo* toxicity due to ozonation and treatment with activated carbon. In general, a similar performance of ozonation and activated carbon treatment was observed regarding the significantly increased removal of the toxicity. Also, Hernandez-Leal et al. (2011) noted that both, ozonation and adsorption to GAC are suitable and effective techniques for the removal of organic MPs from aerobically treated greywater and showed predominantly similar removal efficiencies regarding the investigated MPs.

Advantages of MBRs are the excellent removal efficiencies in terms of chemical oxygen demand (COD; up to 99%) and TSS (up to 100%) that means the absence of TSS in the effluent and furthermore its disinfection properties with a complete removal of pathogens (Bertanza et al. 2017; Skouteris et al. 2012). Angelakis and Snyder (2015) mentioned that a well-designed and operated MBR can consistently achieve efficient removals of TSS, total nitrogen, total phosphor, and pathogens. In a MBR system a final sedimentation, secondary clarifiers and tertiary filtration processes are not needed and a higher content in the MBR results in smaller construction volumes and higher sludge ages that may positively affect MP removal (Besha et al. 2017; Chapman et al. 2004). However, a disadvantage and a key problem of the MBR systems is the fouling of the membranes. But different studies showed that MBRs are less sensitive to seasonal temperature change and the effluent quality regarding conventional pollutants (for example biological oxygen demand (BOD) and COD, ammonium concentration, total nitrogen, total phosphorus and TSS) was better compared to the conventional treatment (Bertanza et al. 2017; Besha et al. 2017; Angelakis & Snyder 2015; Skouteris et al. 2012).

Furthermore, MBR systems indicated a higher efficiency in the reduction of pathogens such as bacteria strains, bacteriophages, bacterial faecal indicators, and diverse virus strains compared to the conventional treatment (Francy et al. 2012).

The increased removal efficiency of some compounds in a MBR system compared to the conventional treatment could be due to the high sludge ages MBRs are operating with. The high sludge ages allow the development of a high diversity of microbial populations in the MBR that are adapted and able to degrade compounds with specific characteristics such as aromatic rings. The direct dosing of PAC into the MBR mixed liquor give beneficial synergistic effects such as the reduction of membrane fouling, a higher removal of conventional pollutants due to the formation of a biofilm on the particles of the activated carbon, and the decrease of the toxicity caused by specific inhibitors of the nitrification process (Alvarino et al. 2017; Serrano et al. 2011).

Cirja et al. (2008) noted, that there is no real difference between the conventional wastewater treatment and the MBR treatment concerning the removal of different classes of MPs. Furthermore, the MBR technology is promising concerning the removal of MPs because of the compactness of the MBR plant and the high organic load that can be applied resulting in high sludge retention times and biomass concentrations.

MBRs generally have been found to achieve comparable or even better results in the removal of pharmaceuticals and personal care products and other trace organic chemicals compared to the conventional activated sludge treatment (Lee at al. 2012).

MBRs as a stand-alone technology or as an additional step for the CAS treatment seem to be a useful option for the removal of organic pollutants due to several advantages of MBRs. Firstly, MBR systems allow a complete retention of the biomass in the system and thus provide a solid-free effluent. Secondly, the low sludge load can be expected to force bacteria also to mineralise poorly degradable organic compounds.

And thirdly, the high solid retention times that are achieved in the membrane process. For instance, the concentrations of selected pharmaceuticals and priority organic compounds in the effluent of MBR systems were lower in comparison to the effluent of the conventional wastewater treatment (Camacho-Muñoz et al. 2012).

Especially regarding the removal of the EDCs for example with estrogenic activity the advantages of a MBR treatment are (a) the ability to act as a barrier to the solids that usually bind a large number of EDCs, (b) the retention capacity of the membrane itself, and (c) the extended sludge retention time also provides time for additional biological degradation (Maletz et al. 2013).

The MBR technology itself is a BT opportunity with a high removal capability of diverse compounds. In comparison to the conventional wastewater treatment the MBR treatment indicated a higher removal efficiency of MPs and toxic effects on fish (Boonnorat et al. 2017).

Finally, the ozonation of the wastewater and the treatment with activated carbon seem to be the most promising processes for a significant decrease of the concentration of the MPs in the wastewater. Furthermore, ozone is an effective disinfectant for viruses, bacteria, and protozoa and thus, ozonation also provides a disinfection as an additional benefit. Though, the main uncertainty of the ozonation process is related to the transformation of unknown TPs of the MPs and the formation of oxidation by-products from matrix components with unknown and presumably toxic properties. But ozonation is capable to convert refractory organic matter into more biodegradable fraction and thus, a biological post-filtration step after the ozonation process may be effective in removing possible toxic TPs (Reaume et al. 2015; Zimmermann et al. 2011; Hollender et al. 2009).

In the end, the assessment and the comparison of the AWWT technologies for the further treatment of conventional treated wastewater is complex because the observed

effects of a single AWWT process (such as ozonation and filtration with activated carbon) varied between the investigated chemical compounds and the used bioassays. But the combination of the treatments ozonation and adsorption to activated carbon was almost responsible for the overall observed reduction of the detected effects. Thus, not a single AWWT achieved the optimal results but combined processes were successful (Reungoat et al. 2010).

An integrated evaluation concept was developed by Ternes et al. (2017) to asses and compare the efficiency of AWWT technologies. This multidisciplinary concept included chemical analysis (removal and/or formation of selected indicator substances and their TPs), ecotoxicological researches (*in vitro* tests for agonistic and antagonistic endocrine activities, mutagenic and genotoxic activities, cytotoxic effects, and neurotoxicity) as well as microbiological investigations (removal of pathogens (indicated by taxonomic gene markers) and bacteria that are resistant to antibiotics (indicated by antibiotic resistance genes)). The results indicated that ozonation with following GAC filtration is the most promising combination of AWWT technologies.

3.6 Do we need to upgrade wastewater treatment plants?

Effluents from 90 European WWTPs were analysed by Loos et al. 2013 for 156 polar organic chemical contaminants. They found 125 substances (≙ 80% of the target compounds) in the effluents. The most relevant chemical compounds in the effluents were artificial sweeteners, benzotriazoles, several flame retardants, pharmaceutical compounds, plasticizers, antibiotics, insecticides, and pesticides. Already in 2009 Loos et al. investigated the occurrence of polar organic persistent pollutants in over 100 individual water samples from more than 100 European river waters from 27 European countries and analysed 35 organic compounds belonging to pharmaceuticals,

pesticides, benzotriazoles, hormones, and endocrine disruptors. Only about 10% of the analysed river water samples could be classified as “very clean” regarding the chemical pollution. Another study by Loos et al. (2010) analysed 164 individual groundwater samples from 23 European countries for the occurrence of 59 polar organic pollutants and detected the same organic compounds as in the river waters. The concentrations of several MPs exceeded the European ground water quality standard for pesticides of 0.1 g/L. However, the ground water was in general less contaminated compared to river surface water. Consequently, from this point of view there is a need to upgrade WWTPs.

Studies show that most of the recalcitrant organic MPs that are detected in urban wastewater only indicate an incomplete removal during the conventional treatment process. Thus, there is the need to design new treatment concepts to address this problem adequately (Serrano et al. 2011). Also, Boonnorat et al. (2017) reported that the conventional wastewater treatment technologies are less efficient in the removal of recalcitrant MPs and toxic compounds such as phenols, phthalates, and pharmaceuticals in comparison to the AWWT systems like ozonation or the high technology BT systems such as MBRs.

Less than 50% removal was observed for 21 out of 43 compounds with an average reduction of only –50% even for the most efficient BT with complete nitrification (Margot et al. 2013). Reemtsma et al. (2010) reported on two corrosion inhibitors that are proved to be omnipresent in the surface waters of the rivers Elbe and Rhine with increasing concentrations along the course of the river. Even after a residence time of several month both substances were detected in bank filtration water whereas bank filtration is an important process to generate raw water for the production of drinking water from surface water. These results confirm the need for AWWTs as well.

In this context, the combination of ozonation and following filtration with BAC for example achieved a removal of 50% for DOC, a reduction of 70% of the non-specific toxicity, a decrease of the concentration of a wide range of trace organic chemicals of more than 90% as well as a reduced estrogenic activity of more than 95%. Therefore, this process combination is recommended as an effective impediment to minimise the discharge of trace organic chemicals into the environment or their presence in water recycling schemes (Reungoat et al. 2012). Another study reported that ozonation and the treatment with activated carbon were both able to reduce the concentration of the majority of the investigated MPs by 80% or more (Margot et al. 2013). However, field studies showed that invertebrates are affected by common organic pollutants at current exposure levels. Furthermore, benthic invertebrate taxa are lost at concentrations that were lower than expected basing on laboratory tests (Berger et al. 2016). Thus, an upgrade of WWTPs is necessary.

But one should not only focus on the results of chemical and ecotoxicological studies to find an answer to the question if there is a need to upgrade the already existing WWTPs. Further areas such as microbiology and socio-economic aspects for example investment costs, implementation, or energy consumption should also be contemplated. An advantage of the ozonation process is that the removal efficiency of MPs by ozonation is predictable with an acceptable accuracy considering the hydraulics of the reactor, the reaction kinetics, and the measurement of the ozone and the hydroxyl radical exposures in laboratory scale experiments. Based on these laboratory results the direct upscale to a full-scale treatment is feasible without the performance of costly and labour-intensive pilot studies. In addition, the operational costs can be estimated adapted from the required ozone dose determined in the laboratory experiments (Hollender et al. 2009). But a potential disadvantage of the ozonation process remaines the formation of unknown reactive by-products due to the

partial oxidation of the compounds and the reaction with matrix components. These oxidation products are usually more easily degradable and could be partially removed during a biological post-filtration. However, undesirable and toxic oxidation TPs such as NDMA, bromate, or formaldehyde can be formed (Margot et al. 2013). Thus, the largely not identified TPs after the ozonation process raise concerns regarding their potential impact on the environment and human health (Reungoat et al. 2011).

However, both AWWT treatments, ozonation with following SF and treatment with PAC (with subsequent SF), are technically and economically feasible for (large-scale) applications in municipal WWTPs in terms of efficiency, costs, and energy requirements. Both treatments had a similar cost with a similar averaged removal rate of the investigated MPs but compared to the existing wastewater treatment these two AWWT technologies increased the costs and the consumption of energy by about 30% (Margot et al. 2013; Reungoat et al. 2011; Hollender et al. 2009). Furthermore, the application of PAC in WWTPs seems to be an adequate and feasible technology for an efficient removal of MPs from wastewater (Boehler et al. 2012). But other studies referred that according to cost estimations the PAC treatment of the wastewater is expected to be about 30% more expensive in comparison to the ozonation process. In addition, the generation and disposal of the PAC requires energy and the broad scale application in WWTPs would need huge amounts of PAC. Besides, contaminated PAC presumably ended up in the aquatic environment because even a following SF is not sufficient to retain the PAC from the wastewater and a subsequent membrane filtration might not be feasible due to higher requirements for technical equipment and energy demand. Thus, the habitat of benthic organisms in the receiving water body might be impacted by the introduction of the PAC over a long period of time. Finally, a widespread use of PAC application is not realistic or worthwhile but may be an adequate alternative for small WWTPs or industrial effluents with a high load of toxic

compounds (Stalter et al. 2010a). Also, the application of GAC filter systems for the removal of organic MPs represents a promising alternative to the ozonation process or the PAC treatment of the wastewater and is also an energy- and space saving option in the AWWT technologies (Altmann et al. 2016). However, an implementation of a BAC filtration without pre-ozonation could be a low-cost AWWT option to improve the chemical quality of the wastewater (Reungoat et al. 2011).

The combination of ozone and BF may be a preferred method as well and offers advantages for the removal of pharmaceuticals and personal care products and other trace organic chemicals from treated wastewater when wider environmental impacts such as energy consumption, water recovery, and waste production are considered besides the removal efficiency (Lee et al. 2012).

The filtration of the wastewater with membranes is suitable for the retention of the MPs but the substantial higher effort for the technical equipment and the required energy is not competitive with the ozonation process or the treatment with activated carbon (Stalter et al. 2010a).

In comparison to the conventional activated sludge treatment the main advantage of BF systems are the high biomass content and their high volumetric reaction rates leading to a smaller size (–70%) of the reactor. Thus, BF systems are an alternative treatment process to the reactors of the conventional treatment and they are highly suitable to WWTPs in large urbanised regions where available land is rare (Rocher et al. 2012). Other advantages are the lower temperature dependency of the biological conversion rates and the easy shut-down and the fast start-up procedures of the system resulting in an operation of the BF on demand. Thus, BF in an adequate process for wastewater treatment with seasonally varied operation modes. Biological aerated BFs provide a wide range of options for the adjustment of the quality of the effluent to varying requirements. As the case may be, only a few or the whole BF units

could be operated, by-passed, or shut down enabling the production of wastewater with variable and controllable ammonia, nitrite, and/or phosphorus concentrations. The nitrifying BF units may stay out of operation for weeks or even several months but the biologically activity of the BF has to be maintained to allow a quick restart and if a full treatment efficiency is required. But the stand-by mode does not cause a higher energy consumption because an aeration of the BF is not needed. A further advantage of such a modular installation is the continuously operation of the residual BF if one filter unit has a malfunction or stays out of order. However, to control the water temperature and to prevent the growth of algae it is advisable to place the BF unit into a dark climate chamber (Meda & Cornel 2010a,b). Biological aerated filter systems are also a compact and suitable alternative for the treatment of greywater and they are capable to be effectively integrated in intra-urban water reuse schemes (Meda & Cornel 2010b). Thus, BF systems presumably represent an interesting alternative AWWT for the removal of organic MPs from the wastewater because they are generally robust, simple to construct and have low energy requirements. Thereby, the most usual technologies are SF, filtration with BAC, riverbank filtration, and managed aquifer recharge (Reungoat et al. 2011).

In comparison to the conventional wastewater treatment mainly disadvantages of MBR systems are related to higher costs and more complex operative procedures caused by a high energy consumption, a slightly higher production of sludge (because of the retention of the whole TSS) and the membrane surface fouling as the key problem. Thereby, the higher energy demand is due to the aeration of the MBR to reduce the fouling of the membranes. Altogether, the comparative assessment pointed to a slightly advantage of the conventional treatment whereas the higher energy consumption of the MBR is easily compensated by the reduction of the aquatic toxicity and the eutrophication in the receiving water body (Bertanza et al. 2017; Lazarova et al. 2012).

Also, Chapman et al. (2004) reported on a global trend for an increased number of MBR installations that are attributed to the declining membrane costs and the increasing demand for (high quality reuse) water. In addition, the authors assessed a low sludge production as an advantage of MBR systems because they can be designed with a long sludge age.

However, the comparison of the investment costs and the energy consumption of WWTPs with conventional sludge treatment and a MBR system both with and without tertiary treatment showed that the specific investment costs of the WWTPs with MBR treatment were lower than those of comparable conventional WWTPs with tertiary treatment. In addition, small-sized and medium-sized MBR systems indicated no disadvantage in the actual specific energy consumption compared to equal-sized conventional plants with tertiary treatment. In addition, the MBR technology has a better social acceptance (for example buildings with MBR plants fit in with its surrounding landscape reducing the buffer distance required between the plant and the nearest neighbourhood) and a similar overall environmental footprint that can be further reduced (Bertanza et al. 2017; Brepols et al. 2010; Chapman et al. 2004).

Another important factor of the wastewater treatment is the minimisation of the exposure of bacterial, viral, and protozoan pathogens to the human population. A MBR treatment of the wastewater with both microfiltration (pore sizes: 0.4 µm to 0.1 µm) and ultrafiltration (pore sizes: 0.1 µm to 0.02 µm) effectively remove protozoa (15 µm to 4 µm) and bacteria (3 µm to 0.5 µm) from the wastewater. Simply regarding the size of the pathogens, viruses are obviously smaller (0.08 µm to 0.02 µm) and presumably have the ability to pass through the membranes of the MBR. However, some full-scale WWTP with MBR technology removed viruses from the wastewater. Furthermore, pilot studies at three WWTP with MBR treatment reported on a more effectively removal of diverse microorganisms including enteric viruses (such as *Escherichia coli*,

enterococci, faecal coliforms, several coliphages, adenovirus, enterovirus, and norovirus) in comparison to two WWTP with conventional treatment (Francy et al. 2012).

However, the advantages of the conventional treatment are that the technology is comparatively simple, inexpensive (lower investment costs), and the application is user-optimised. Thus, this technology is more exercisable to treatment conditions that are restrained by financial factors, manpower, and technological aspects than the MBR technology. Furthermore, the performance of the conventional treatment could be improved by the adjustment (more precisely an increase) of the nitrogen concentration allowing the treatment of high strength wastewater that is contaminated with simple structured toxic compounds especially in the developing countries confronted with financial, personal, and technological limitations where the implementation of AWWT technologies such as the MBR are not applicable (Boonnorat et al. 2017).

Besides, the efficiency of single MBRs for the degradation of persistent MPs such as carbamazepine, sulfamethoxazole, diclofenac, and erythromycin is not yet satisfying. The use of integrated systems in which a MBR served as a pre-treatment with nanofiltration (MBR-NF) or reversed osmosis (MBR-RO) or the combination of a MBR with a strong adsorbent such as particular or granular activated carbon (MBR-PAC and MBR-GAC) are effective to remove recalcitrant organic MPs but these systems are energy intensive and induce high costs. An alternative to the present technologies could be the application of specific native (immobilised) enzymes such as fungal laccase on the membranes of the reactor instead of microorganisms for the removal of the persistent MPs (Becker et al. 2017, 2016; Besha et al. 2017; Krah et al. 2016). The use of specific enzymes on the one hand also reduced the risk of the development of resistant bacteria to chemicals but on the other hand the major limitation of the enzymatic treatment is the generation of toxic TPs that requires a post-treatment to

remove the toxicity generated by the enzymes (Becker et al. 2017, 2016; Besha et al. 2017).

Finally, pharmaceutical compounds are detected in conventional treated wastewater up to milligram per litre levels and hence, are released into surface waters. Therefore, the implementation of AWWT technologies is intended to reduce their discharge because the long-term effects of these substances on the environment as well as on human health are largely unknown up to date. For instance, the results of different studies document that AWWTs are necessary to minimize for example the estrogenic burden of highly charged sewages such as hospital wastewaters (Maletz et al. 2013). Even low levels of estrogenic activity were found in a river 3.5-35 km downstream the outlet of a municipal WWTP (Svenson et al. 2003). These aspects are of particular importance when surface water serves as drinking water sources and when indirect potable reuse is considered (Reungoat et al. 2010).

In the end, environmental relevant conclusions and consequential assessments regarding the benefits and the risks of AWWT technologies in a long run could only be reached with long-term on-site observations of the aquatic flora and fauna and field studies at the wastewater receiving water bodies both, before as well as after the establishment of the AWWT technologies. For this purpose, *in vitro* bioassays and *in vivo* tests with model organisms such as microorganism communities, macroinvertebrates, plants, and fish as well as biomarker responses and histo-pathological endpoints in the applied test organisms are appropriate instruments. In conclusion, the advantages as well as the disadvantages of the AWWT technologies have to be assessed thoroughly to prevent that a higher environmental impact will be induced than removed by these technologies (Stalter et al. 2011, 2010a; Wenzel et al. 2008).

But not only freshwater ecosystems with their flora and fauna including bacteria, algae, fungi, plants, invertebrates, and vertebrate species are directly impacted by the discharges of WWTP and chemicals applied in agriculture and so on. Indeed, MPs reach marine ecosystems. For example, phthalate metabolites (monoethyl phthalate (MEP) and mono-(2-ethylhexyl) phthalate (MEHP)) were already detected in the urine of common bottlenose dolphins (*Tursiops truncates*) in Sarasota Bay in Florida demonstrating the exposure to two of the most commonly used phthalates in commercial manufacturing (diethyl phthalate (DEP) and di-2-ethylhexyl phthalate (DEHP) (Hart et al. 2018).

In addition, human beings are also affected by chemicals detected in the environment. An average of 56 environmental organic acids (EOAs) were identified in maternal blood serum in a diverse population of pregnant woman implying several confirmed EOAs that are presumably of high priority for future biomonitoring among pregnant woman because these compounds represent high-production-volume chemicals (Wang et al. 2018).

Finally, there is need for action to reduce the exposure of MPs to ecosystems and human beings for example by the upgrade of WWTPs with AWWT technologies.

4 Conclusions and outlook

One of the major factors leading to the unsatisfactory ecological status of many rivers is the worldwide increasing contamination of the aquatic environment with thousands of industrial and natural chemical compounds and toxic substances representing one of the key environmental problems facing humanity (Busch et al. 2016; Loos et al. 2013). The presumably adverse impacts of relevant organic MPs on freshwater systems and their aquatic organisms as well as on human health have not been studied sufficiently and more attention should be paid on these issues. Although, most of these MPs are present in low concentrations diverse substances are of considerable toxicological concern, especially when they occur in complex mixtures. Thus, methods for the detection of the toxicity of these MPs as single substance or as a mixture of compounds ought to be developed (Rubio et al. 2020; Li et al. 2019; Palli et al. 2019; Loos et al. 2013).

The implementation of additional (advanced) steps during the wastewater treatment is one of the best options for the reduction of the release of MPs such as pharmaceuticals and personal care products into the surface waters since it is not realistic to limit the consumption of these compounds. Acute effects on human health are not expected but the impacts of a long-term exposure are so far not known. Several MPs even were resistant to both ozonation and treatment with PAC although they could mostly be removed with a higher ozone and PAC dosage. Therefore, the release of these substances into the sewer system and accordingly into the (aquatic) environment should be avoided that also represents a more economically feasible alternative. For example, the release of the persistent iodinated contrast media into the wastewater could be impeded by the collection of the urine of the patient within the 24 h after the

X-ray examinations and the treatment (such as incineration) of this urine in a separate system (Margot et al. 2013).

Also, Stalter et al. (2010b) mentioned that these end of pipe techniques are presumably an adequate solution for the reduction of the toxicity in the wastewater but only in a medium-term perspective. Therefore, affordable options that are additionally more beneficial for the environment could be long term source control strategies such as wastewater separation (for example urine separation, compare above), ecologically correct disposal of drugs by the end users, recycling or reuse by the pharmaceutical industry or alternative medical treatments to drug therapies.

Further improvements of the quality of the effluents at urban WWTPs can be realised using technologies such as ozonation, MBR treatment, or SF by reducing the concentration of MPs. But in parallel, the consequences of AWWT technologies will be an increased resource- and energy consumption that is in contrast with the nowadays efforts to reduce the environmental impacts from the use of energy regarding the emissions of CO_2 and other greenhouse gases. Thus, on the one hand the wastewater sector aspires for a further reduction of toxic MPs at the costs of an increased energy consumption whereas other sectors in the society aim for a CO_2 reduction presumably at the expense of an increased emission of toxic compounds. Furthermore, in some studies it was found that more environmental impact may be induced than removed by the AWWT technologies. SF had the best balance between prevented and inducted impacts that was not always the case for ozonation and MBRs. In the end, in a lot of cases there will be a net environmental benefit of the AWWT. But in a few cases, the environmental impact that is caused by the operation of the WWTP itself may supersede the impacts that are avoided by the further removal of the MPs from the wastewater (Wenzel et al. 2008).

Finally, the EU Directive 2000/60/EC determined strict quality standards for water bodies for the protection of inland surface wate, groundwater, transitional water, and coastal water. The list of priority substances included (hazardous) pollutants whose discharges, emissions, and losses should phase-out or end within 20 years. Consequently, efforts to adopt feasible and reliable techniques for wastewater treatment should be and will be made in the future. To this end, two important tasks should be pursued: firstly, the assessment of the actual removal capacity of WWTPs with conventional treatment processes and thus, secondly the evaluation of possible requirements for additional treatment steps (Bertanza et al. 2011). In the meantime, the EU directives were amended and replaced (EU Directive 2013/39/EU) and the list of priority (hazardous) pollutants was extended from 33 to 45 (groups of) compounds. In October 2022 the European Commission adopted a proposal to revise the list of priority (hazardous) substances in surface water (EU Proposal 2022). The new list even contained 70 (groups of) compounds. For the identification of these emerging contaminants a more systematic and integrated monitoring-modelling risk assessment approach is required because the proposed substances pose well-documented risks to nature and human health (Loos et al. 2009). In addition, contrary to pesticides there are no threshold limit values existing in Europe for organic chemicals. Hence, the member states of the EU ought to develop such threshold limit values in the near future (Loos et al. 2010). Furthermore, diverse substances such as bisphenol A and nonylphenol were detected in several ground water at even higher concentration levels than in surface water. Thus, the performance of the routine ground water monitoring should be enhanced to identify possible “hot spot” areas of pollution aiming for the protection of the health of the human and the ecosystem (Loos et al. 2010).

In the end, although the impact of (pharmaceutically active organic) MPs on the environment and on human health is not completely clarified and long-term effects are

largely unknown to this day, there are initiatives for AWWT on scientific, technological, and political levels in progress. Therefore, AWWT technologies should be investigated in bench scale experiments as well as in pilot and full-scale operations at WWTPs (Altmann et al. 2014).

In this context, it would be recommended if several academic fields put their heads together and work interdisciplinary (Petrie et al. 2015). The number of substances, compounds and MPs and their TPs within the wastewater that are detected by the chemical (target) analysis is continuously growing. But the chemical analytical methods are limited to a qualitative and quantitative detection of the MPs and a conclusion regarding their biological effects is not possible. The results of (eco)toxicological *in vitro* bioassays provide beneficial additional information of supposable adverse effects caused by the MPs. The diversity of *in vitro* bioassays covering different (eco)toxicological endpoints is large and constantly growing and improving. Nonspecific toxicity tests such as cell proliferation assays allow a general conclusion with regard to the overall toxicity of a (waste)water sample also including mixture effects. The implementation of more specific cell based *in vitro* bioassays provide the investigation of reporter gene activities (such as endocrine activity) and DNA damaging potentials (for example genotoxicity and mutagenicity) at the molecular level that besides might be useful for the establishment of a human risk assessment. Supplementary to the *in vitro* bioassays *in vivo* tests allow the detection of the toxicity and combined effects not only on the molecular level but on the whole test organism. This *in vivo* toxicity assessment investigating diverse endpoints provides the complete response of the test organism or cell culture to all compounds present in the (waste)water sample the test organisms are sensitive to.

Thus, the combination of chemical analysis and biological aquatic studies may then provide a complete overview beginning with the detection of a substance, followed by

the determination of its concentration, furthermore the study of molecular and systemic effects of a single substance or effects of a mixture of compounds and finally the investigation of the effects on whole populations (Dopp et al. 2021).

In the future the challenges for the securing of water resources and the disposal of wastewater will become an increasing challenge because the human population continues to grow and urbanize. Under this view of an escalating growth of the population and additionally an increased water stress in many regions of the world, the reuse of treated wastewater and wastewater recycling are becoming more important options for the water supply. Nowadays, centralised WWTP usually receive wastewater transported through collecting sewage pipes and are located near to the point of the disposal site to the environment. As a result, there is a lack of multiple distribution systems and water reuse in urban aeras is often impeded. Therefore, wastewater management systems should be decentralised in the future and should be more seriously incorporated to treat wastewater at or near the points of wastewater generation (Angelakis & Snyder 2015; Loos et al. 2013).

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Annex

A.1 to A.4 Publications as part of the thesis

A.5 Supplementary information to the *in vivo* on-site experiment with *Gammarus fossarum*

A.6 Zusammenfassung (German summary)

A.7 Acknowledgement

A.8 Curriculum Vitae

A.9 List of publications and conference contributions

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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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.

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

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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	polytetrafluoroethylene
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

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 originated from surface water, groundwater, hospital wastewater, raw (untreated), conventionally-treated and ozonated wastewater. The 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, 19) or 24 h composite samples (samples 2–5, 7, 15–18, 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.

Table 1
Overview of the investigated samples; WWTP: wastewater treatment plant. Details 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 influent of a filtration basin)	FB-IN	grab
7	conventionally treated wastewater (WTTP 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 4)	EFF-4	composite
16	ozonated conventionally treated wastewater (before microsieve, pilot WWTP 4)	EFF-4-O_3	composite
17	conventionally treated wastewater (after microsieve, pilot WWTP 4)	EFF-4-MS	composite
18	ozonated microfiltered conventionally-treated wastewater (pilot WWTP 4)	EFF-4-MS-O_3	composite
19	tap water (pilot WWTP 4)	TAP	grab

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 × 2 mL heptane, 1 × 2 mL acetone, 3 × 2 mL methanol (LC-MS Optigrade) and 4 × 2 mL ultrapure water. Supelco ENVI-Carb + cartridges were turned (top to bottom) before they were conditioned with 1 × 2 mL acetone and 1 × 2 mL methanol. Afterwards, the columns were turned again (loading direction) and conditioned with 1 × 2 mL acetone, 3 × 2 mL methanol and 4 × 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 × 2 mL acidified methanol and 5 × 2 mL acetone, each containing 0.2% formic acid. Acidified samples were consecutively eluted with 5 × 2 mL methanol and 5 × 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, 2002) 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 conducted as described by Magdeburg et al. (2014). In brief, aqueous samples were analysed after sterile filtration (injection filter with PTFE membrane: pore size 0.2 µm, neoLab, Germany) with a dilution factor of 1.7 and SPE extracts in a 20-fold final sample concentration (0.4% v/v solvent) in eight replicates. Ten concentrations between 5 and 2000 µg/L final concentration in the well of 4-nitroquinoline N-oxide (4-NQO; CAS 56-57-5, Sigma-Aldrich, Germany) were used as genotoxic reference compound (Table S1). Cytotoxicity (OD_{595}) and genotoxicity (OD_{414}) were determined photometrically. The OD_{414} was corrected for the respective cell density (OD_{595}) if no cytotoxicity occurred (see 2.5). A linear regression line was generated using the corrected OD_{414} of the reference compound (Figure S6). The induction rate (IR) was calculated using the corrected OD_{414} of the samples. An IR ≥ 1.5 is considered potentially genotoxic.

2.4.3. Mutagenicity assay (Ames fluctuation test)

Mutagenic effects (i.e., irreversible DNA damage) were analysed using the Ames fluctuation test (ISO/DIN 11350, 2012) with two genetically modified strains of *Salmonella typhimurium* (TA98 and TA100). The assay detects the induction of point mutations in special marker genes coding for enzymes involved in histidine biosynthesis as frameshift mutations (TA98) and base pair substitutions (TA100). To increase sensitivity, the strains TA98 and TA100 have a mutation in the *uvrB* DNA repair gene. In the absence of mutagens, the strains do not grow in histidine-free medium and a reverse mutation in the marker genes enables histidine synthesis and thus growth. This leads to a pH change in the assay medium that is determined photometrically at a wavelength of 414 nm.

The Ames test was conducted as described by Magdeburg et al. (2014). In brief, aqueous samples were tested after sterile filtration (see 2.4.2) with a dilution factor of 1.25 and SPE extracts in a 10-fold final sample concentration (0.2% v/v solvent). Mutagenic reference 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 $\leq$ 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 $\leq$ 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 excluded from the list leading to a ranked set of Pareto-optimal sample preparation methods. 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 (Fig. 1, full data sets in Table S2). Focusing on a change of the endocrine activities or mutagenicity of $\geq$ 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 (Fig. 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 (Fig. 1C, Table S4). Acidification caused the highest decrease of mutagenicity in the Ames TA98 with −94% followed by the RAR assay with −88% (Fig. 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 acidification significantly affects the outcomes of bioassays. Two hypotheses may explain the changes in toxicity: 1) In acidified samples, acids may interfere with active chemicals or 2) in neutral samples, microbial activity may degrade or transform the active chemicals.

Basically, the key question is whether the neutral (hypothesis 1) or the acidified sample (hypothesis 2) represent the "true" toxicity. For chemical analysis, there is consensus that acidification stabilises most compounds and prevents microbial degradation (Baker and Kasprzyk-Hordern, 2011; Vanderford et al., 2011; US EPA, 2010). However, our data implies that besides few exceptions the *in vitro* activity is lower at acidic compared to neutral pH (Fig. 1, Table S2). Accordingly, samples at a neutral pH may better represent the actual toxicity. If this hypothesis holds true, an acidification of samples would either reduce the concentration of active chemicals by increasing adsorption to suspended matter (Baker and Kasprzyk-Hordern, 2011) or by increasing hydrolysis (Prasse et al., 2015).

Alternatively, it can be assumed that the higher activity in neutral samples is an artefact caused by a change in sample composition. Here, continuous microbial activity may deconjugate compounds resulting in a higher biological activity. This occurs during biological wastewater treatment (Andersen et al., 2003; Koh et al., 2008; Wu et al., 2017). However, an on-going microbial degradation of active compounds would counteract this process (Giebner et al., 2018).

In reality, the toxicity of an aqueous sample may change at either neutral or acidic pH. As this depends on the chemical and biological composition of a sample, it is difficult to generalise which condition best represents the actual toxicity. Based on the present data, we argue that a neutral pH comes closest to reality, as the sample is minimally processed. In addition, a higher biological activity will result in a more protective water quality assessment if one accepts that the risks of false-positives outweighs the risk of false-negatives.

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).

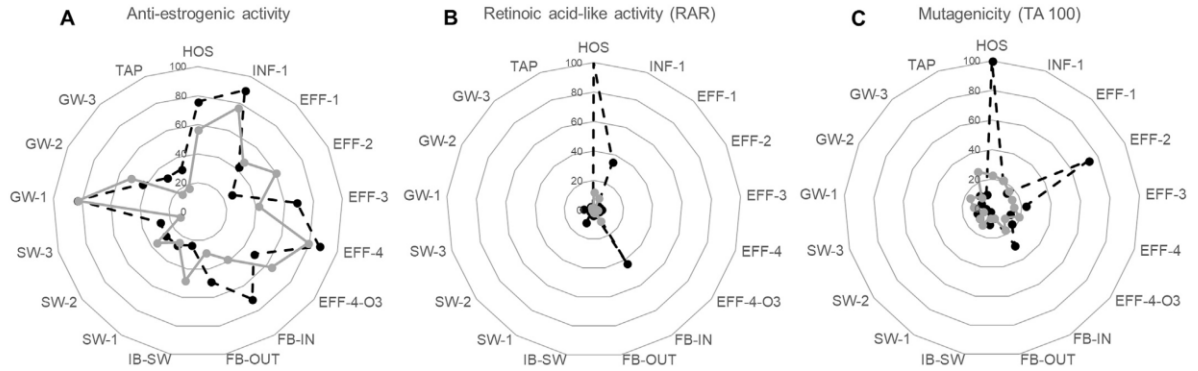

Fig. 1. Impact of acidification. Anti-estrogenic activity (A), retinoic acid-like activity (RAR, B) and mutagenicity (Ames TA 100, C) of neutral (black) and acidified (grey) aqueous water and wastewater samples (mean in %). Corresponding samples (INF-1/EFF-1, EFF-4/EFF-4-O_3 and FB-IN/FB-OUT) were taken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

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, Fig. 2A and B). 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 (Fig. 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 (Fig. 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 to 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

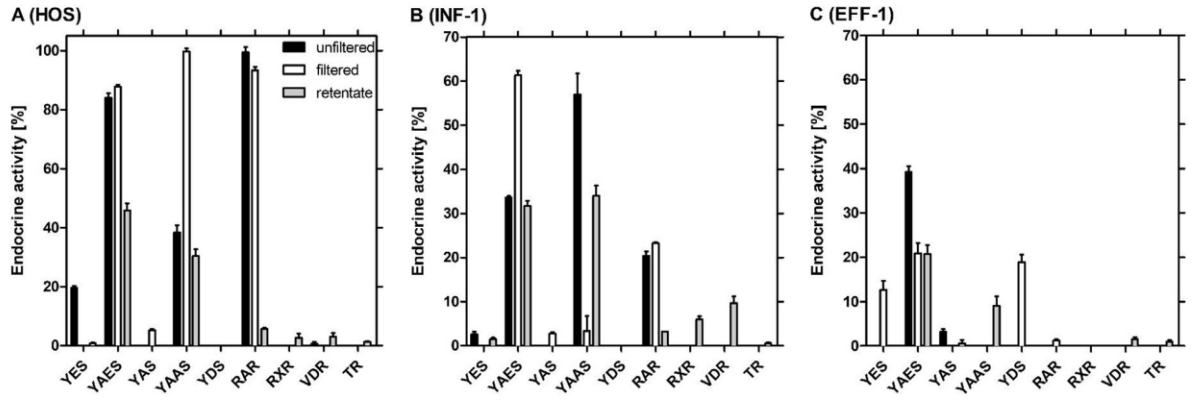

Fig. 2. Impact of filtration. Endocrine activity (%, mean ± SEM) of unfiltered (black bars) and filtered (white bars) wastewater samples and the aqueous suspensions of the filter retentate (grey bars). A: untreated hospital wastewater (HOS), B: untreated municipal wastewater of WWTP 1 (INF-1), C: conventionally 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.

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). In the present study, this was amongst others observed when evaluating the removal of (anti-)estrogenic and dioxin-like activities at WWTP 1 (Fig. 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

Comparing the toxicity of aqueous samples and corresponding SPE extracts is rarely done but has a number of advantages, such as the possibility to calculate recovery rates and evaluate the environmental relevance of obtained results (Giebner et al., 2018; Muschket et al., 2017; Tousova et al., 2017; Wangmo et al., 2018).

In the present case, most aqueous samples induced minimal estrogenic, anti-androgenic and retinoic acid-like activities (Fig. 3, Tables S8, S9, S10). However, anti-estrogenic activities between 21 and 91% were detected in all aqueous samples (Fig. 3B). The activities were <19% in the other bioassays (Fig. 3D and S9; Table S8). In extracted samples, the estrogenic activity ($\leq$8%, n = 35) was generally as low as in the corresponding aqueous samples ($\leq$13%, n = 8; Figs. 3 and 4, Table S9). The minor estrogenic activity detected in most samples in this study is in line with other studies on biological (Jalova et al., 2013; Keiter et al., 2006; Metcalfe et al., 2013) and advanced wastewater treatment (Ma et al., 2005; Maletz et al., 2013).

The anti-estrogenic activity of the extracts was variable and, depending on the SPE method, in parts very high (13–89%, n = 35) and comparable to the corresponding aqueous samples (Figs. 3B and 4). This indicated that the causative compounds were either only partially recovered or that the anti-estrogenicity of the aqueous samples is caused by the matrix (Neale et al., 2015). Interestingly, the high anti-estrogenic activities in the extracts point towards potential masking effects, whereby receptor antagonists reduce the detection of agonistic activity in water sample. This phenomenon has also been discussed by other authors (Giebner et al., 2018; Gehrmann et al., 2018; Ihara et al., 2014; Rao et al., 2014; Stalter et al., 2011). In addition, groundwater was significantly anti-estrogenic (Fig. 3B, Table S8 and S9). This calls for further clarification regarding the presence of EDCs in groundwater.

In contrast, the anti-androgenic activity was low in most aqueous samples ($\leq$5%, n = 7) but higher in the extracts (9–89%, n = 30, Figs. 3C and 4, Table S9) indicating a successful 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 (Fig. 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%; Figs. 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 (Fig. 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 (Allinson et al., 2011; Loos et al., 2012; Stalter et al., 2011) but not all studies (Jia et al., 2015; Rao et al., 2014; Reungoat et al., 2010) supporting its detection in the present WWTP effluents.

While none of the aqueous samples (n = 6) was active in the umu assay, 33% (n = 27) of the extracts were potentially genotoxic (Fig. 3F, Tables S8 and S9). Low to moderate genotoxicity was detected in extracted hospital, raw and treated wastewater but in none of the other samples. Other studies observed genotoxicity in extracted WWTP effluents (Macova et al., 2011; Keiter et al., 2006; Escher et al., 2014). These potentials generally decreased upon ozonation (Cao et al., 2009; Misik et al., 2011).

3.4. Identifying the optimal SPE method

Similar to analytical chemistry (Baker and Kasprzyk-Hordern, 2011; Maruya et al., 2016; Polo et al., 2005), SPE of (waste)water samples is advantageous for *in vitro* bioassays. Extraction prevents the microbial degradation of untreated samples and improves the detection of toxicological effects caused by low (micro)pollutant concentrations (Escher et al., 2005; Janošek et al., 2007; Macova et al., 2011; Neale et al., 2015, 2018). SPE can also minimise matrix interferences by reducing natural organic matter and excluding ions, nutrients or acids (Neale and Escher, 2014; Prasse et al., 2015; Escher et al., 2018).

In contrast to chemical analysis of target compounds, the recovery of toxicity by SPE cannot be evaluated because the causative chemicals and mixture effects remain unknown. Thus, this study aimed at maximising the extraction of toxicity by comparing two mixed-mode hydrophilic/hydrophobic (Oasis HLB and Supelco ENVI-Carb+) and one composite (Telos C18/ENV) SPE sorbents. These SPE sorbents enrich a broad and heterogeneous spectrum of chemicals (Köke et al., 2018; Leusch et al., 2012; Neale et al., 2018). Extracting both neutral and acidified samples, six different SPE methods were evaluated by a semi-quantitative (3.4.1–3.4.4) approach followed by multivariate statistics (3.4.5).

3.4.1. Blanks

In parallel to the extraction of the samples, a SPE blank was prepared to control for potential contaminants in reference waters and used materials (Kolkman et al., 2013; Neale et al., 2018; Schulze et al., 2017). Each cartridge type was loaded with ultrapure water and extracted as described in 2.3.3. The extracts of the 60 SPE blanks were negative in all bioassays except in two cases (3%):

Supelco ENVI-Carb + at pH 7 and pH 2.5 in the YAAS. Here, the activities were 2% and 3% higher than the limit of quantification. In addition, a DMSO sample was included in parallel to the SPE extracts in each *in vitro* bioassay as a solvent control. These solvent controls did not induce an effect in the bioassays.

3.4.2. Cytotoxicity

Cytotoxicity is often used as indicator of the reactive toxicity of environmental samples and their overall (micro)pollutant load. It, thus, represents an important endpoint which is integrated into several water quality assessments (Escher et al., 2014, 2018; Leusch et al., 2014; Välitalo et al., 2017). However, depending on the investigated endpoint, cytotoxicity can also prevent or mask the detection of specific toxicity (see 4).

In the present study, none of the aqueous samples induced cytotoxic effects (Fig. 4, Tables S8 and S9). Cytotoxicity was, however, frequently detected in SPE extracts (Fig. 4). Untreated wastewater induced cytotoxicity in 50% (HOS) and 38% (INF-1) of sample extracts (n = 60, each) tested in ten *in vitro* bioassays (Table 2). For conventionally treated wastewater (EFF-1, EFF-4, EFF-4-MS, n = 54–60) cytotoxicity was observed in ≤25% of extracts (Table 2). The occurrence of cytotoxicity in extracted ozonated wastewater (sample EFF-4-MS-O_3, n = 54) and groundwater (sample GW-1, n = 60) was 35 and 2%, respectively (Table 2).

The choice of the SPE method had a substantial influence on the detection of cytotoxicity: the 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 (Fig. 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 (Fig. 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 the formation of toxic transformation products (TPs) during ozonation (Jia et al., 2015; Lundström et al., 2010; Magdeburg et al., 2014). In contrast to the WWTP samples, only 2% of groundwater extracts were cytotoxic. This is in agreement with the high water quality monitored at GW sampling sites 1–3 (Seitz and Winzenbacher, 2017) as well as the rare detection of cytotoxicity in groundwater, unless influenced by landfill leachates, industrial or other contaminated sites (Baumstark-Khan et al., 2005; Baun et al., 2000).

3.4.3. Endocrine endpoints

Pooling the results according to water sample type, the highest mean estrogenic activity was found in conventionally treated wastewater (EFF-1, EFF-4, EFF-4-MS) extracted with Telos C18/ENV (pH 2.5) with 5% (n = 4) relative activity and Oasis HLB (pH 2.5) with 5% (n = 4) relative activity (Table S11; Figure S10). Samples extracted at neutral pH with the same SPE sorbents induced lower estrogenic activities (3%, n = 2; 2%, n = 3). Extracts produced with Supelco ENVI-Carb + showed low estrogenic activity regardless of the adjusted pH.

With regard to the anti-estrogenic activity of conventionally treated (EFF) and ozonated (EFF-O_3) wastewater as well as groundwater (GW) both sorbents, Oasis HLB and Telos C18/ENV showed similar effectivity when samples were extracted at pH 2.5 (Fig. 4 and S10, Tables S8 and S11). For conventionally treated wastewater (EFF) and groundwater (GW) extracted at neutral pH with the same sorbents the mean anti-estrogenic activity was higher. The highest mean anti-estrogenic activity was found in samples extracted with Supelco ENVI-Carb+ at neutral pH (62–87%, n = 1–2).

In case of the anti-androgenic activity of all sample types, acidified samples extracted with Oasis HLB and Telos C18/ENV produced similar results again (Fig. 4 and S11). Because of high cytotoxicity, the activities of neutrally extracted samples could not be analysed. Treated wastewater and groundwater extracted with Supelco ENVI-Carb + at both pH values induced lower anti-androgenic activities than the other SPE methods. As the activity

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

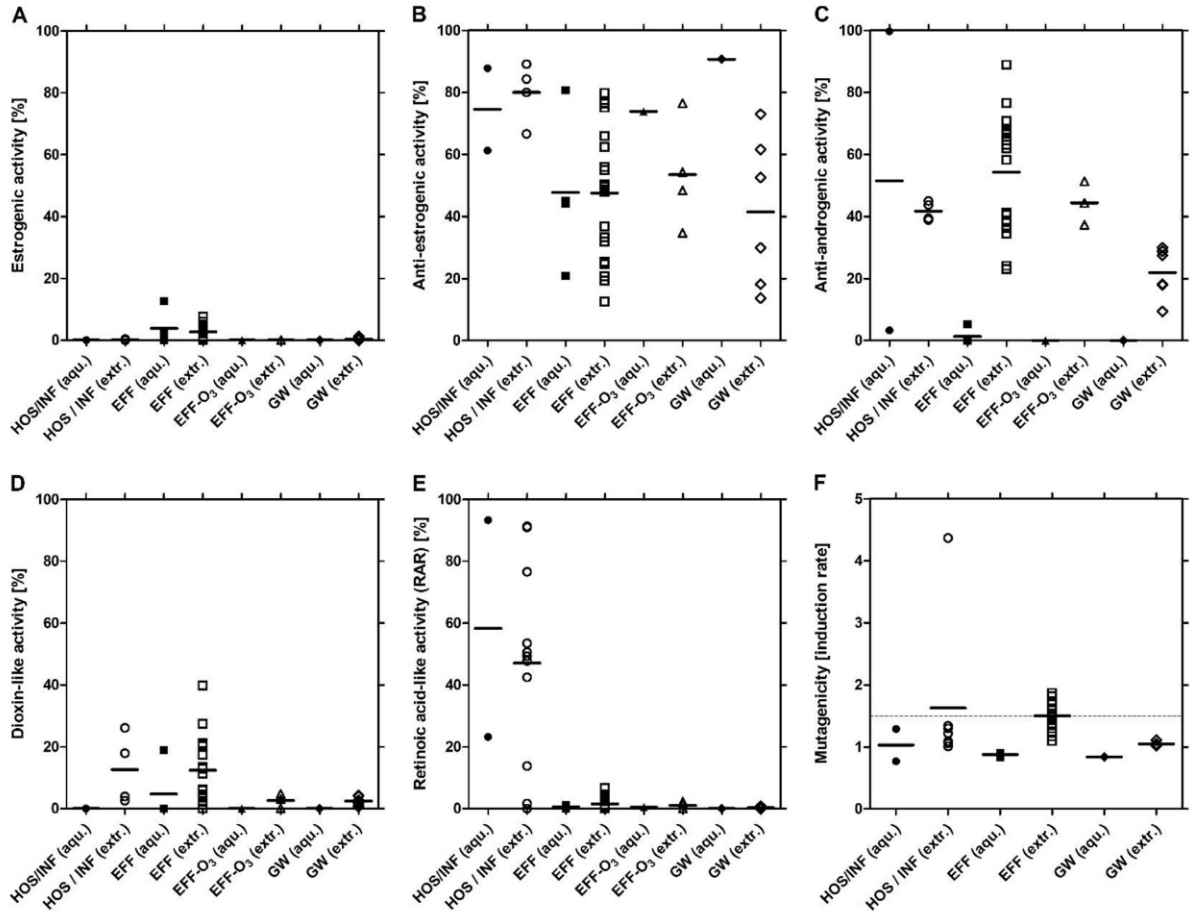

Fig. 3. Comparison of aqueous and extracted samples. Estrogenic (A), anti-estrogenic (B), anti-androgenic (C), dioxin-like (D) and retinoic acid-like (RAR, E) activity in % and genotoxicity as induction rate (umu, F) of the pooled data of aqueous (aqu.) water and wastewater samples (0.63-fold final concentration) 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.

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 (Fig. 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 genotoxicity. All extracts produced with Supelco ENVI-Carb+ (pH 7 and pH 2.5) were not active, either.

Genotoxicity was enriched from four out of six sampling sites (Figure S14, Tables S8 and S9) but IRs remained only moderately increased compared to the corresponding aqueous samples (except for hospital wastewater). One reason for this could be that genotoxicity of (waste)water samples is generally detected at higher sample enrichment factors (e.g., 100-fold, Keiter et al., 2006;

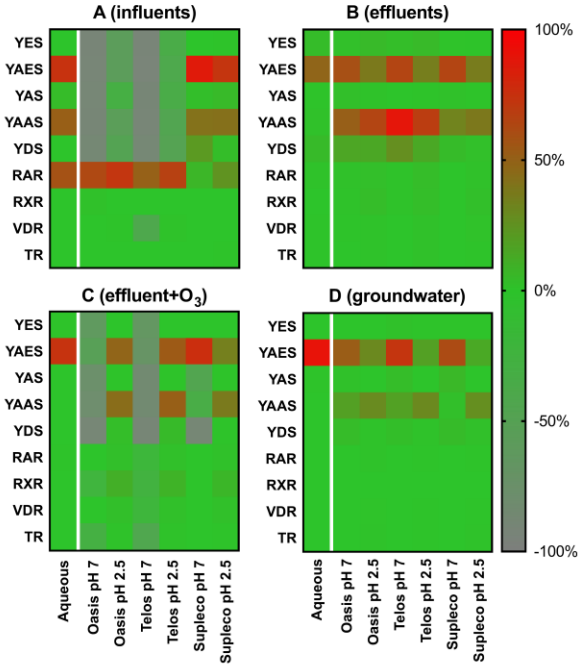

Fig. 4. Comparison of the six SPE methods. Endocrine activity (0%−100%) and cytotoxicity (0% to −100%) of aqueous samples and the corresponding SPE extracts (0.63 and 10.4-fold final concentration, respectively) of wastewater treatment plant influents (A), effluents (B), ozonated effluent (C) and groundwater (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. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Schulze et al., 2017; Stalter et al., 2016) or at contamination hotspots (Baumstark-Khan et al., 2005; Baun et al., 2000).

In line with the efficiency of the Telos C18/ENV pH 2.5 method, Magdeburg et al. (2014) extracted genotoxicity and mutagenicity from wastewater (biological and advanced treatment) using the Oasis HLB at pH 2. Although the authors did not compare different SPE methods, their results seem in agreement with the present results. Mutagenicity and cytotoxicity were also higher in biologically-treated and ozonated wastewater extracted at pH 2 (instead of pH 7) using a C18 sorbent (Misik et al., 2011). For the other investigated *in vitro* endpoints, no SPE optimisation study was found in the literature.

3.4.5. What is the best SPE method?

Regarding the results of five types of water samples tested with five *in vitro* bioassays the most effective SPE method for the extraction of endocrine activities was Telos C18/ENV pH 7 (7x), followed by Telos C18/ENV pH 2.5 and Supelco ENVI-Carb+ pH 7 (each 5x), Oasis HLB pH 7 (4x), Oasis HLB pH 2.5 (2x) and Supelco ENVI-Carb+ pH 2.5 (1x, Table 3). To statistically distinguish between optimal (and non-optimal) SPE methods a multivariate optimisation based on Pareto was implemented (Durmaz et al., 2015; Ehrgott, 2000). Pareto computed sample type and bioassay specific "Pareto optimal" methods.

The Pareto results are exemplified for conventionally treated wastewater (EFF-4) in five *in vitro* bioassays, whereby Pareto is based on the activity percentiles (Table S12) for ranking the SPE methods (Table S13). The best extraction methods ("Pareto best") were Telos C18/ENV pH 7 followed by Oasis HLB pH 7 and Telos C18/ENV pH 2.5 (see Table S13 for detailed results). The ranking of these methods was computed as follow: Instead of looking at the "best" extraction results within a certain matrix, the "worst" results were classified as "false negative responders". The Supelco ENVI-Carb+ method at pH 2.5 was three times "Pareto-worst" as it extracted the lowest activity in a maximal number of bioassays. All other methods performed better. When an extract was cytotoxic, the result was marked with the label "cytotoxic" instead of providing a value. The Pareto algorithm is capable of evaluating data sets with a limited number of such results. In case of an excessive degree of cytotoxicity (HOS and INF-1), the corresponding SPE method was, however, not listed in the respective ranking matrix and the level of relevance decreases for this parameter. This means that the ranking for this parameter is not reaching the "worst" class anymore. This evaluation procedure was performed for all data sets referring to the different samples, SPE methods and *in vitro* bioassays to obtain the following overall ranking of "Pareto optimal" SPE methods: Regarding the five sample types, the method Telos C18/ENV at pH 7 was four times "Pareto best", followed by Oasis HLB pH 7 and pH 2.5 (each 2x, Table 3 and S14). In terms of the five bioassays, the methods Telos C18/ENV at pH 2.5 and Supelco ENVI-Carb + at pH 7 were two times "Pareto best", respectively (details in Table S14).

Accordingly, the method Telos C18/ENV at pH 7 was "Pareto best" regarding the effectivity in extracting different types of water and wastewater samples with respect to the highest endocrine activities (Table 3). Higher recoveries at neutral pH (over acidic and basic pH) were also observed by Tousova et al. (2017) for several endpoints also investigated in this study. The authors, however, used other sorbents for large volume SPE of surface waters. Summing up the results of the *in vitro* bioassays and Pareto optimisation, the methods Telos C18/ENV pH 7 and Oasis HLB pH 7 were optimal to enrich endocrine activities but also the highest cytotoxicity (Table 2). The corresponding methods at pH 2.5 showed good results as well as lower cytotoxicity (Table 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

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

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 extraction is acceptable. One way of addressing this is to compare the toxicity of extracts to aqueous samples (Dagnino et al., 2010). Another way is to optimise the recovery of toxicity. Both strategies were adopted in this study to identify the best extraction method.

The forth challenge arises from cytotoxicity masking the effect under investigation, which is often the case at high concentration factors. While cytotoxicity can be considered an important toxicological endpoint by itself outweighing the specific effect is masks, it is most commonly rather regarded an obstacle that needs to be removed. This can be achieved by diluting a sample to a non-cytotoxic concentration (Inoue et al., 2009, 2011; Leusch et al., 2017; Neale et al., 2018; Välitalo et al., 2017). However, this also dilutes the effect of interest. Alternative approaches, such as minimising the dilution of aqueous samples (Niss et al., 2018) or reducing exposure times in the bioassay as well as cleaning up the cytotoxicity (e.g., by fractionation), have so far not been widely adopted.

These challenges are connected to a range of SPE parameters. Thus, the sorbent (Chang et al., 2009; Escher et al., 2005; Stalter et al., 2016), sample volumes (Macova et al., 2011; Schulze et al., 2017), eluting solvents (Lu et al., 2010; Välitalo et al., 2017; Yang et al., 2014), fractionation steps (Leusch et al., 2017; Välitalo et al., 2017) and operating modes such as large volume or multilayer SPE (Köke et al., 2018; Schulze et al., 2017) can be optimised.

Acknowledging that it is impractical to perform an optimisation for every sample and every bioassay, a range of case studies for different matrices can be used to evaluate whether specific sample preparation methods perform generally better than others. We have taken such approach in the present study and conclude that the Telos C18/ENV method at neutral sample pH performs best in recovering multiple endocrine activities and cytotoxicity from aqueous 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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.watres.2018.12.049.

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Supplementary information (Publication A.1)

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	-

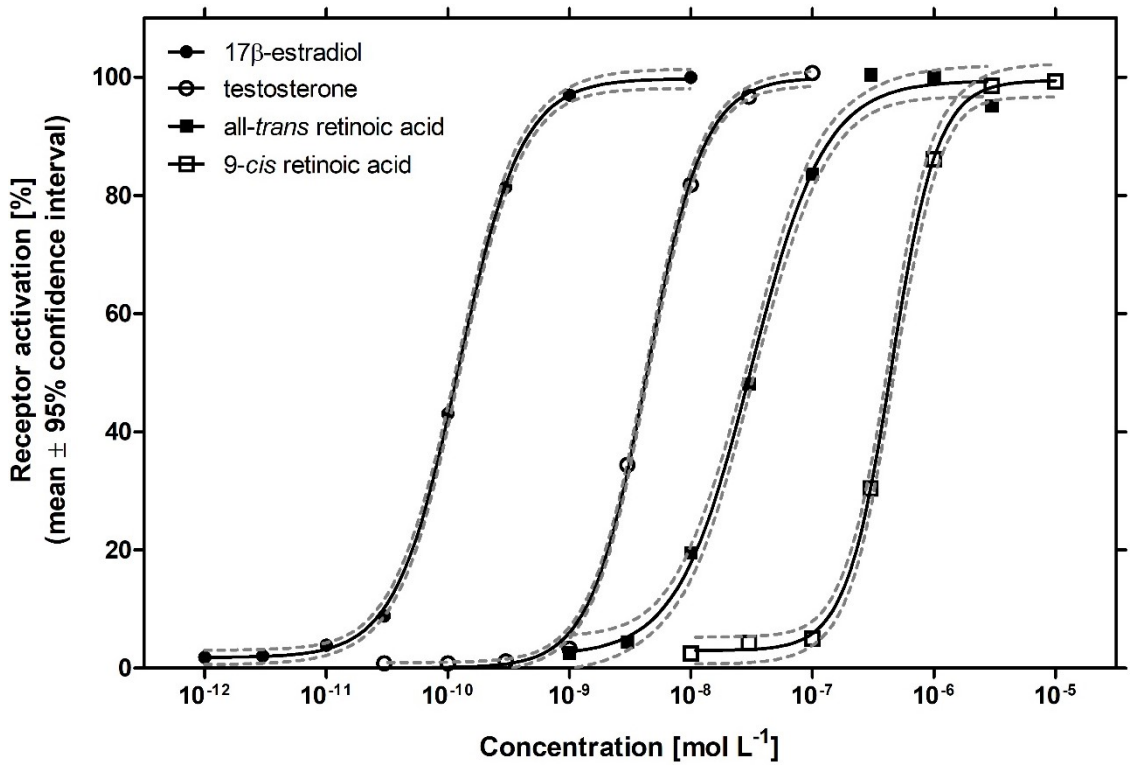

Figure S1: Receptor activation (%, mean ± 95% confidence interval) as concentration-response relationships of six (RAR, RXR) and seven (YES, YAS) experiments at the human estrogen (left), androgen (second left), retinoic acid receptor (third left) and retinoid X (right) receptor (YES: 17β-estradiol;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

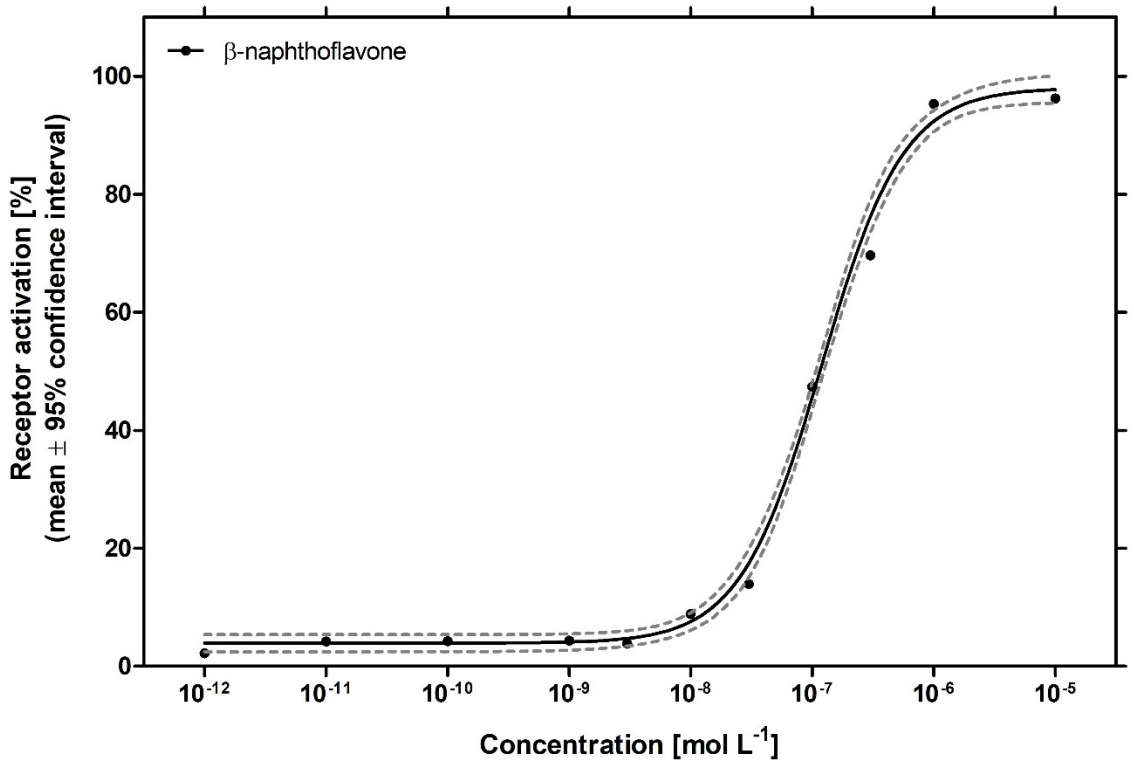

Figure S2: Receptor activation (%, mean ± 95% confidence interval) as concentration-response relationship of seven experiments at the human aryl-hydrocarbon receptor (YDS: β-naphthoflavone).

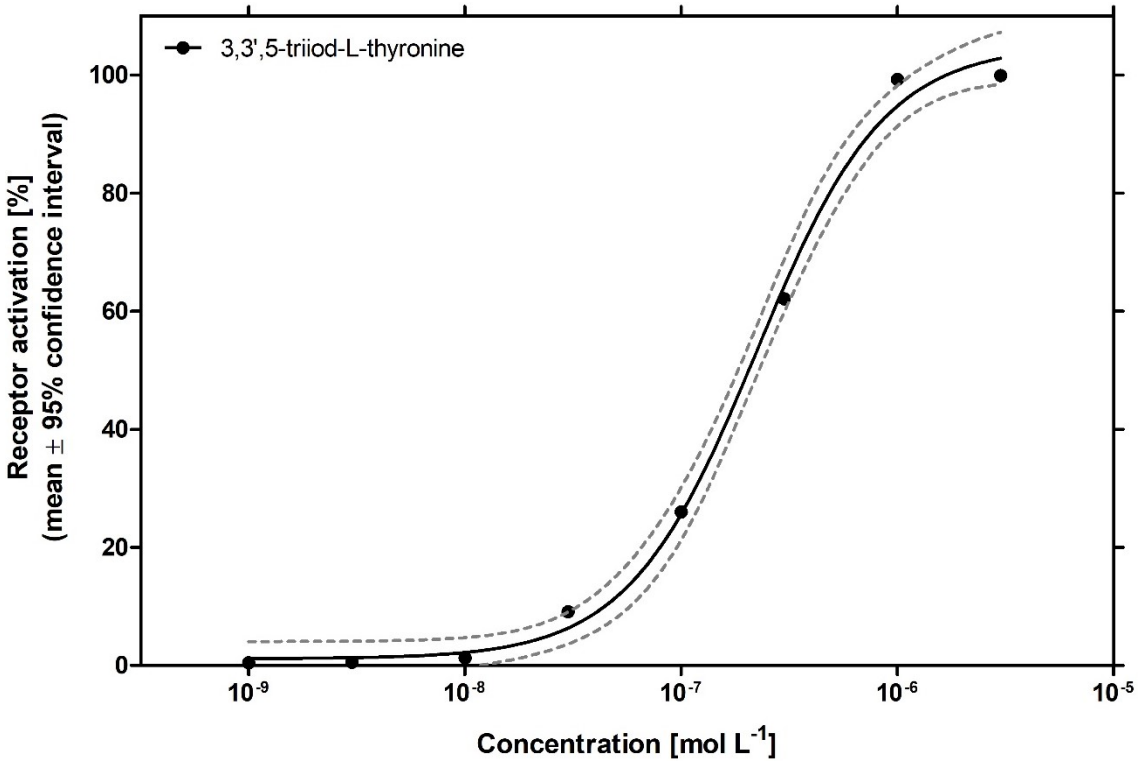

Figure S3: Receptor activation (%, mean ± 95% confidence interval) as concentration-response relationship of six experiments at the human thyronine receptor (TR: 3,3',5-triiod-L-thyronine).

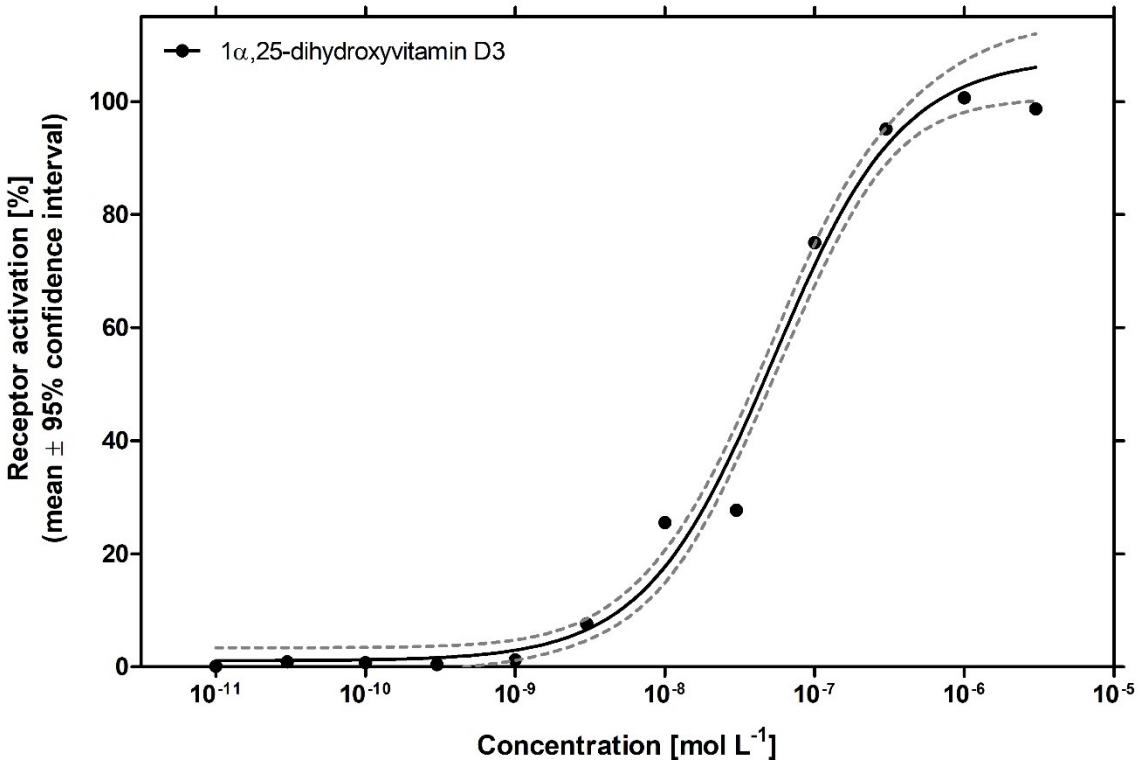

Figure S4: Receptor activation (%, mean ± 95% confidence interval) as concentration-response relationship of six experiments at the human vitamin D receptor (VDR: 1α,25-dihydroxyvitamin D3).

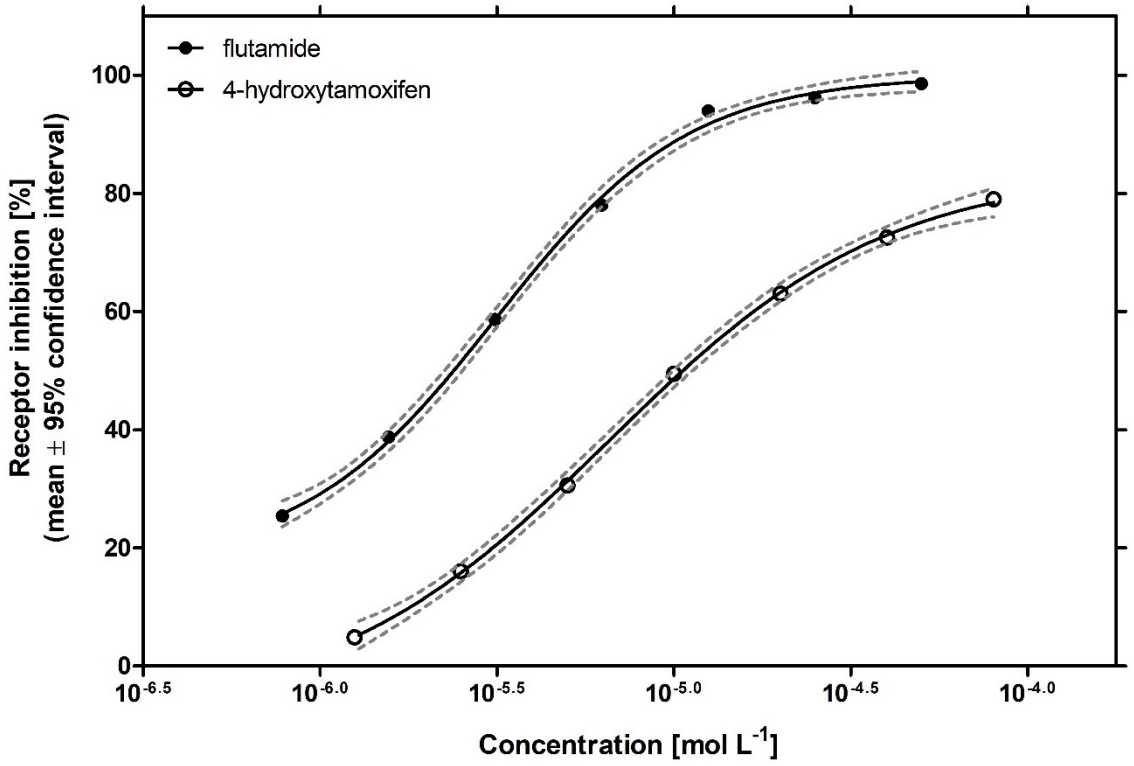

Figure S5: Receptor inhibition (%, mean ± 95% confidence interval) as concentration-response relationships of seven experiments, each, at the human androgen (left) and estrogen (right) receptor (YAAS: flutamide; YAES: 4-hydroxytamoxifen)).

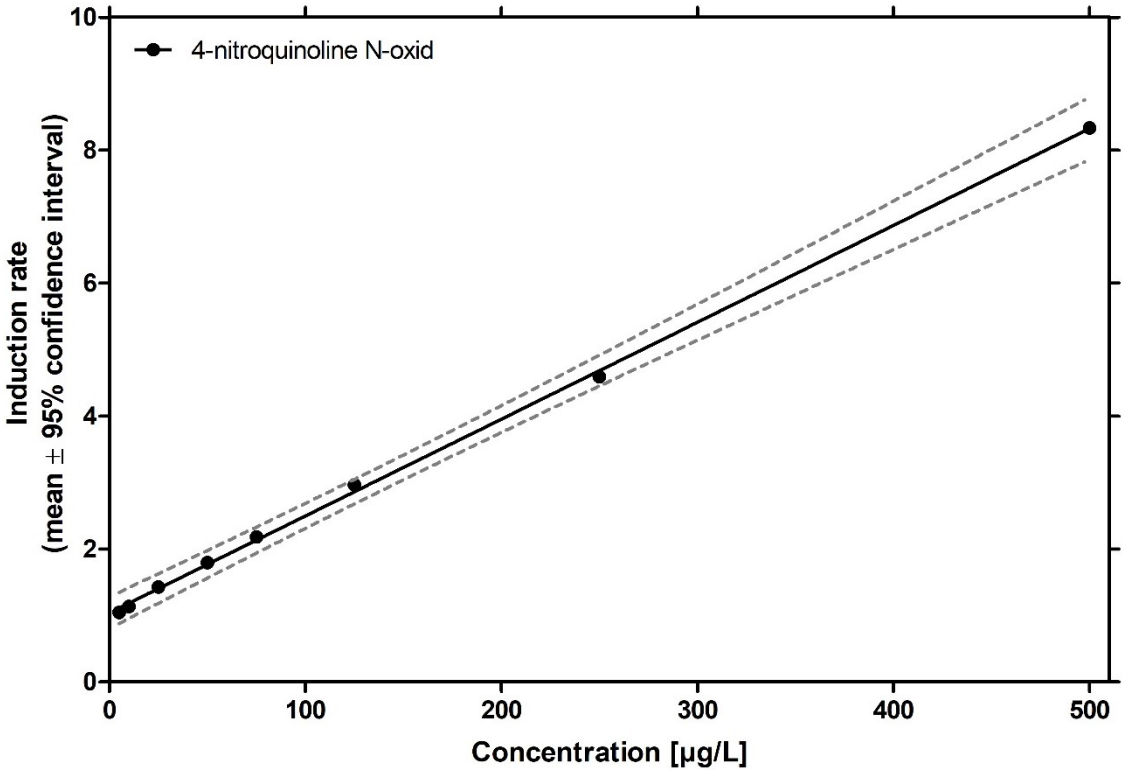

Figure S6: Induction rate (mean ± 95% confidence interval) of the positive control as linear regression of seven experiments of the umu test (4-nitroquinoline N-oxid)

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

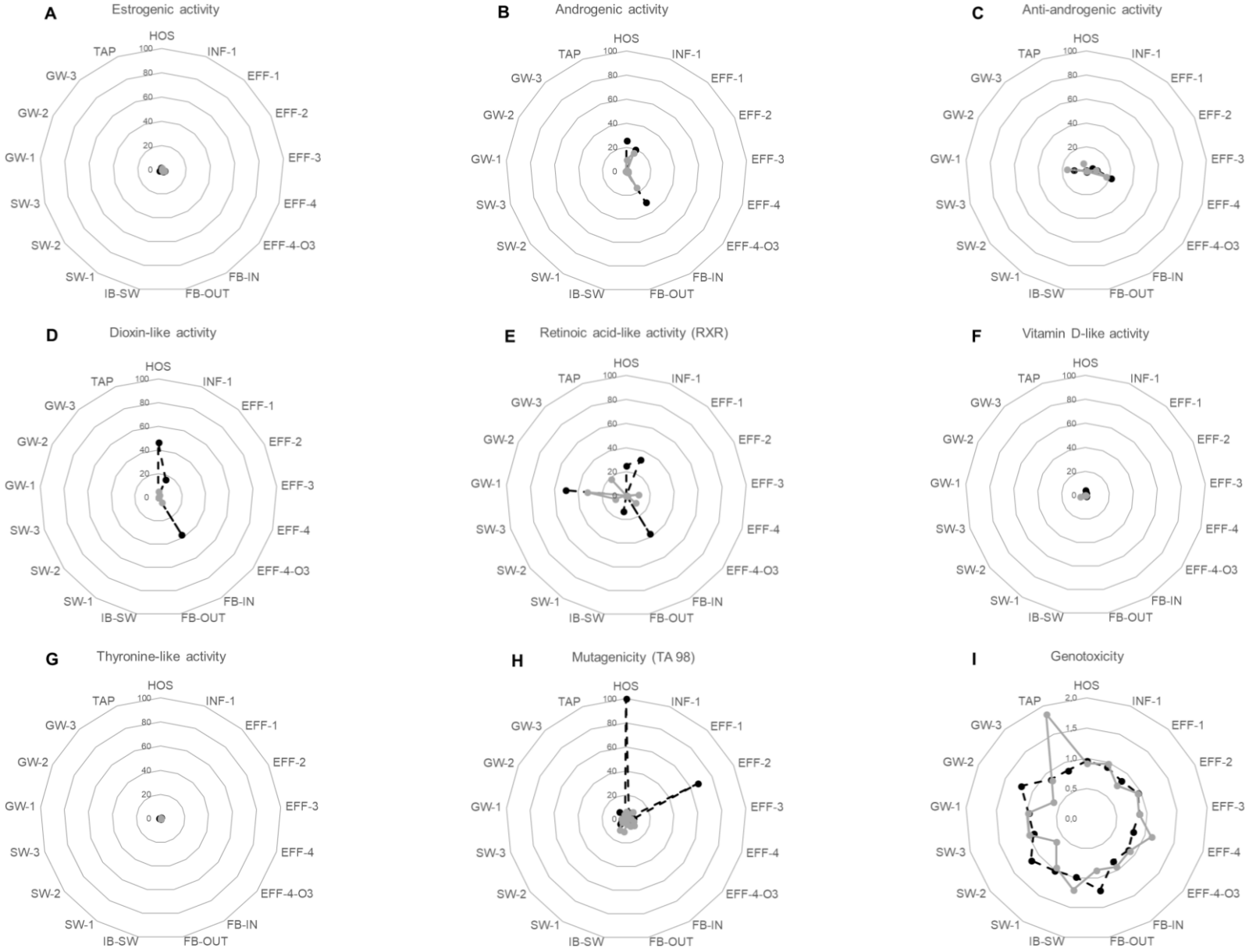
A
Estrogenic activity
B
Androgenic activity
C
Anti-androgenic activity
D
Dioxin-like activity
E
Retinoic acid-like activity (RXR)
F
Vitamin D-like activity
G
Thyronine-like activity
H
Mutagenicity (TA 98)
I
Genotoxicity
HOS
INF-1
EFF-1
EFF-2
EFF-3
EFF-4
EFF-4-O3
FB-IN
FB-OUT
IB-SW
SW-1
SW-2
SW-3
GW-1
GW-2
GW-3
TAP

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

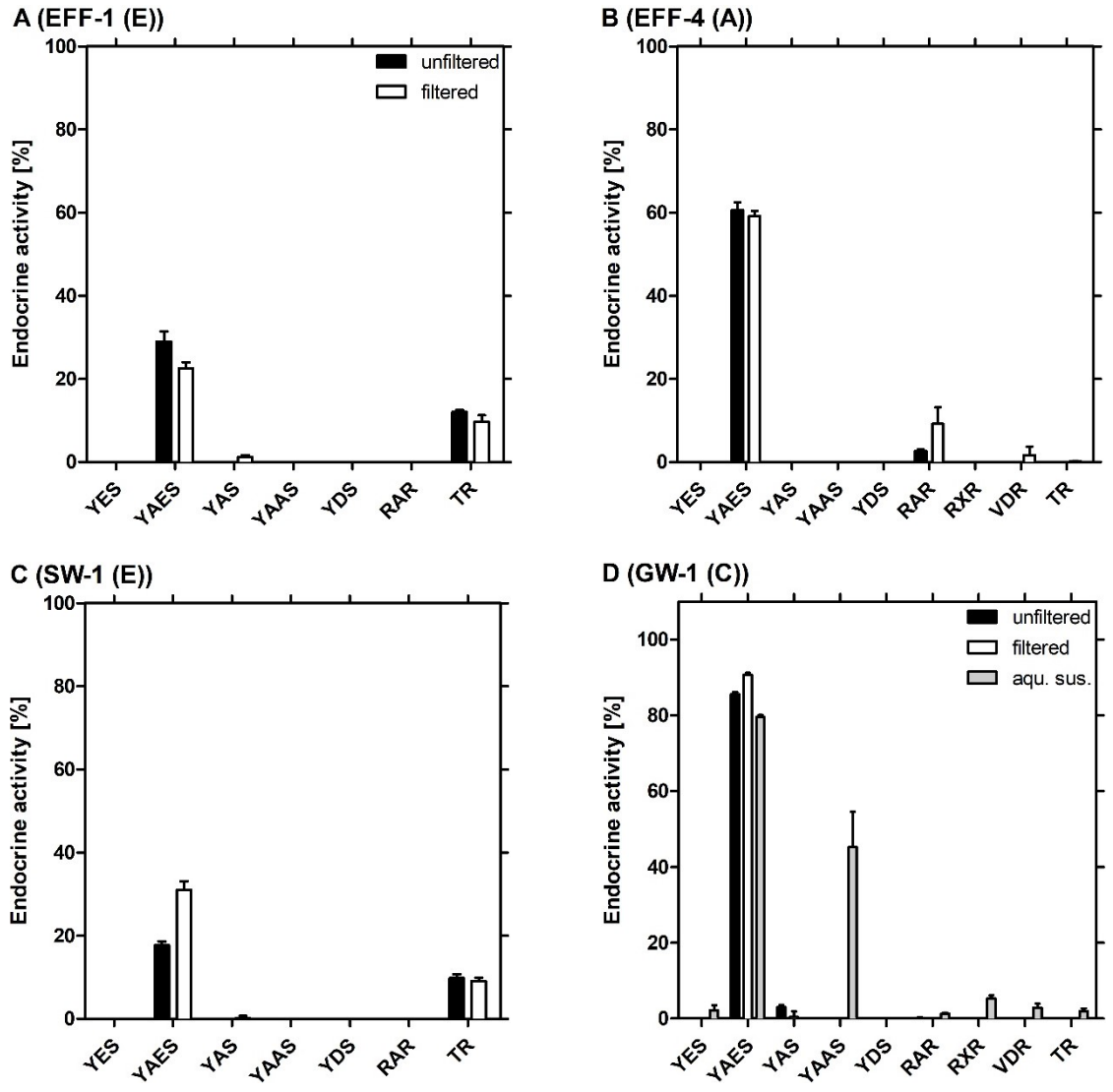

Figure S8: Endocrine activity (%, mean ± SEM) of unfiltered (black bars) and filtered (white bars) water and wastewater samples and the aqueous suspensions of the filter retentate (grey bars) of conventionally treated wastewater (A and B: effluents of two WWTPs (EFF-1 and EFF-4)), surface water (C: SW-1) 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)

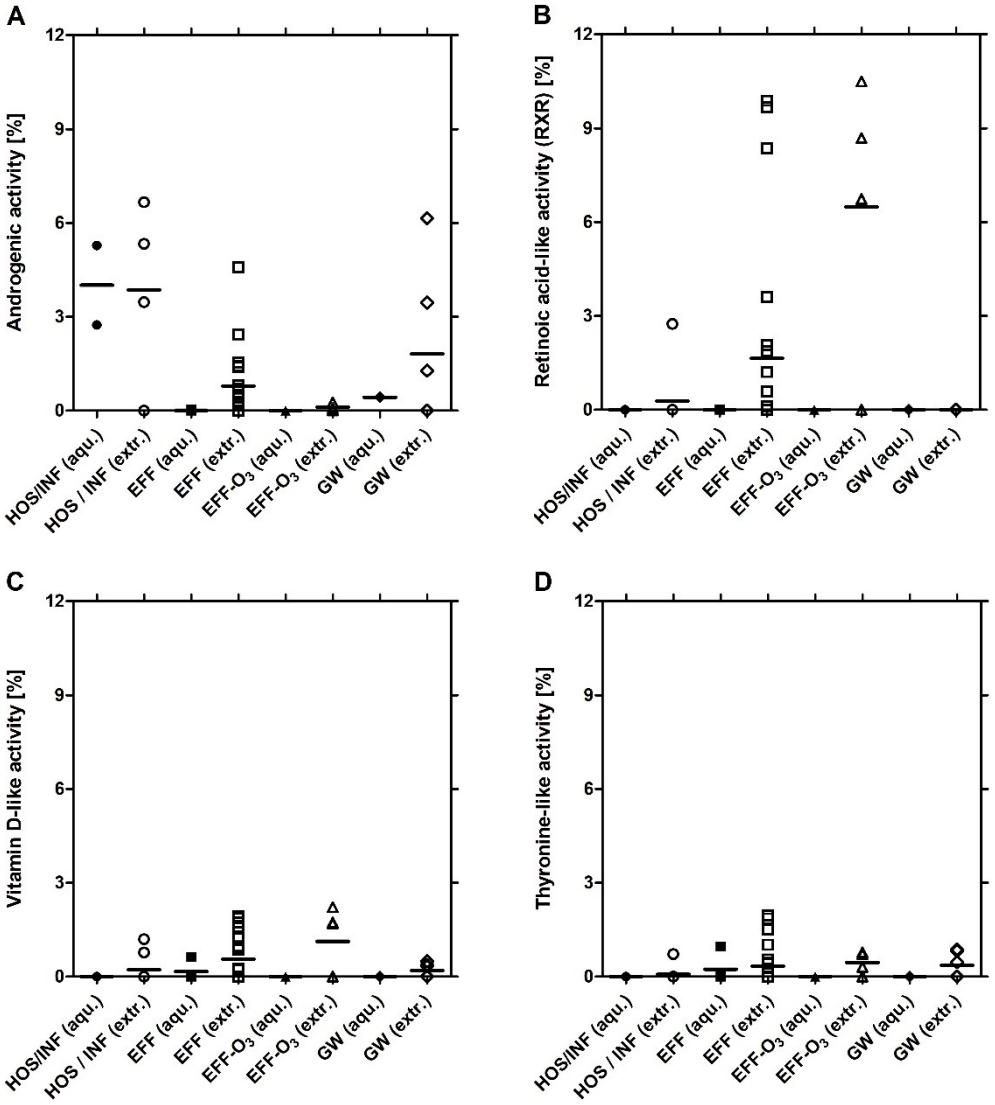

Figure S9: Androgenic (A), retinoid X-like (RXR, B), vitamin D-like (C) and thyronine-like (D) activity in % of the pooled data of aqueous (aqu.) water and wastewater samples and of the corresponding pooled 10-fold SPE extracts (extr). Symbols: activity of the individual sample, line: mean of all samples 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)

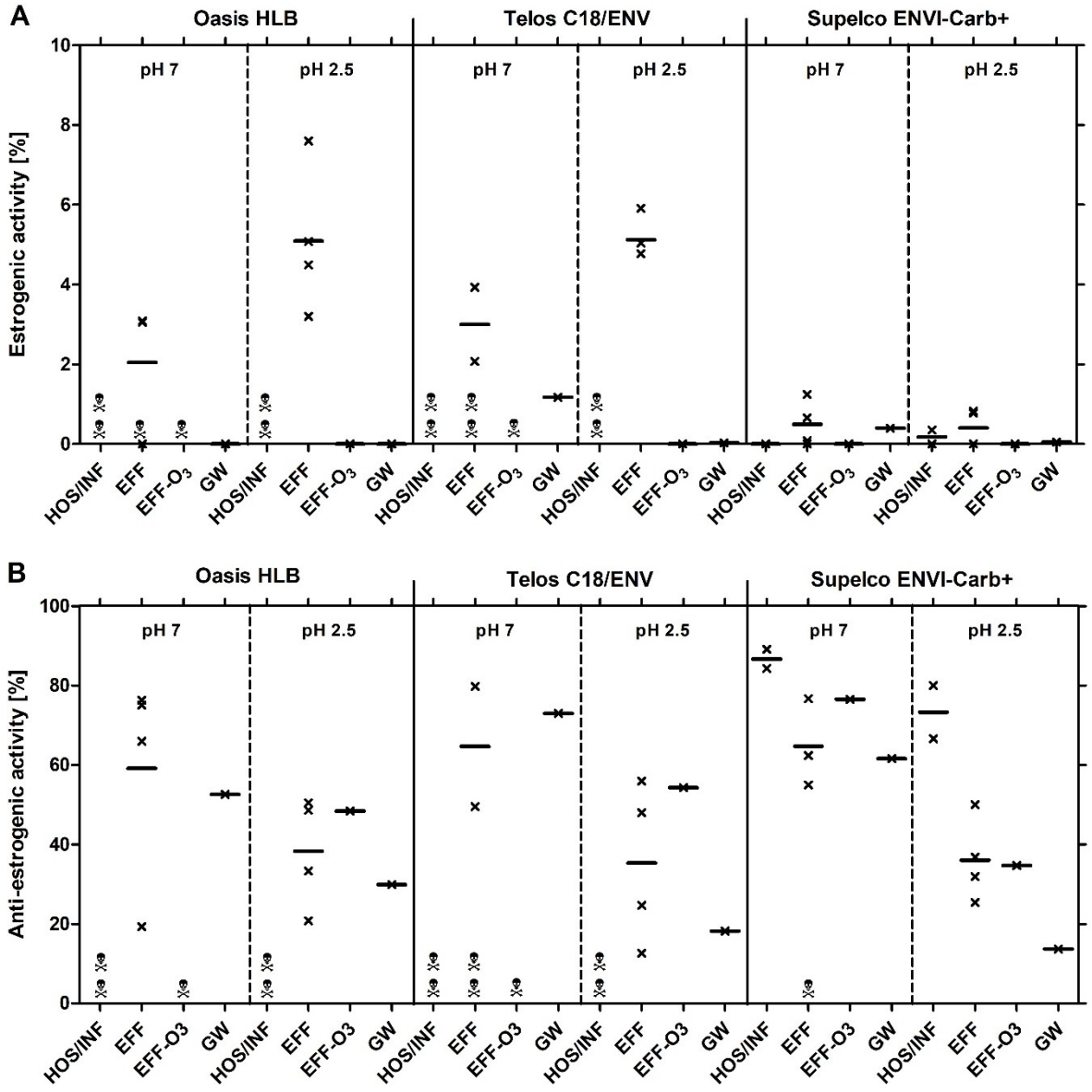

Figure S10: Estrogenic (A) and anti-estrogenic (B) activity in % of SPE extracts of water and wastewater samples using three different SPE columns and two different pH values. The results were pooled from the different samples according to water type. Symbols: activity of the individual sample, line: 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.

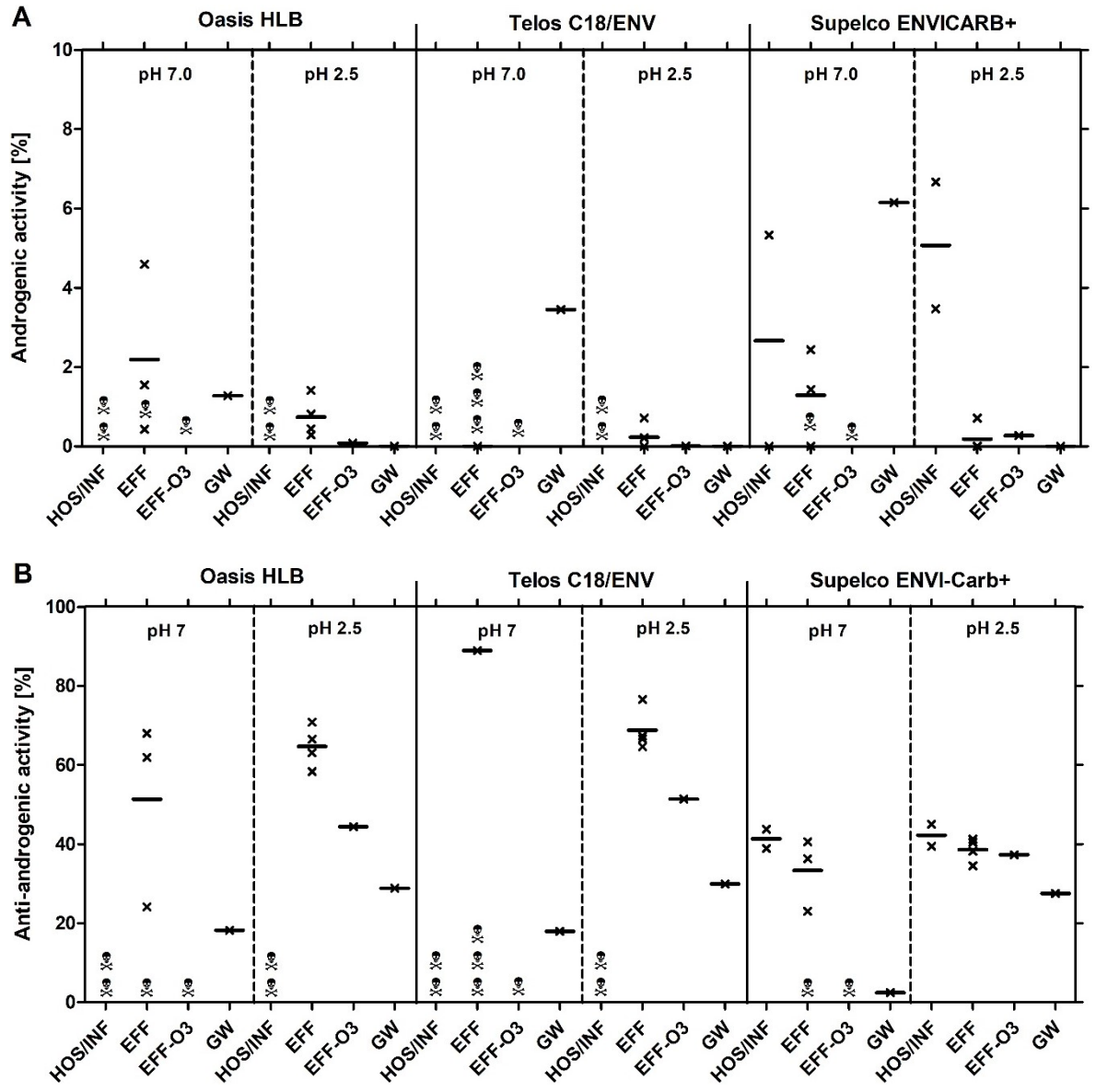

Figure S11: Androgenic (A) and anti-androgenic (B) activity in % of SPE extracts of water and wastewater samples using three different SPE columns and two different pH values. The results were pooled from the different samples according to water type. Symbols: activity of the individual sample, line: 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.

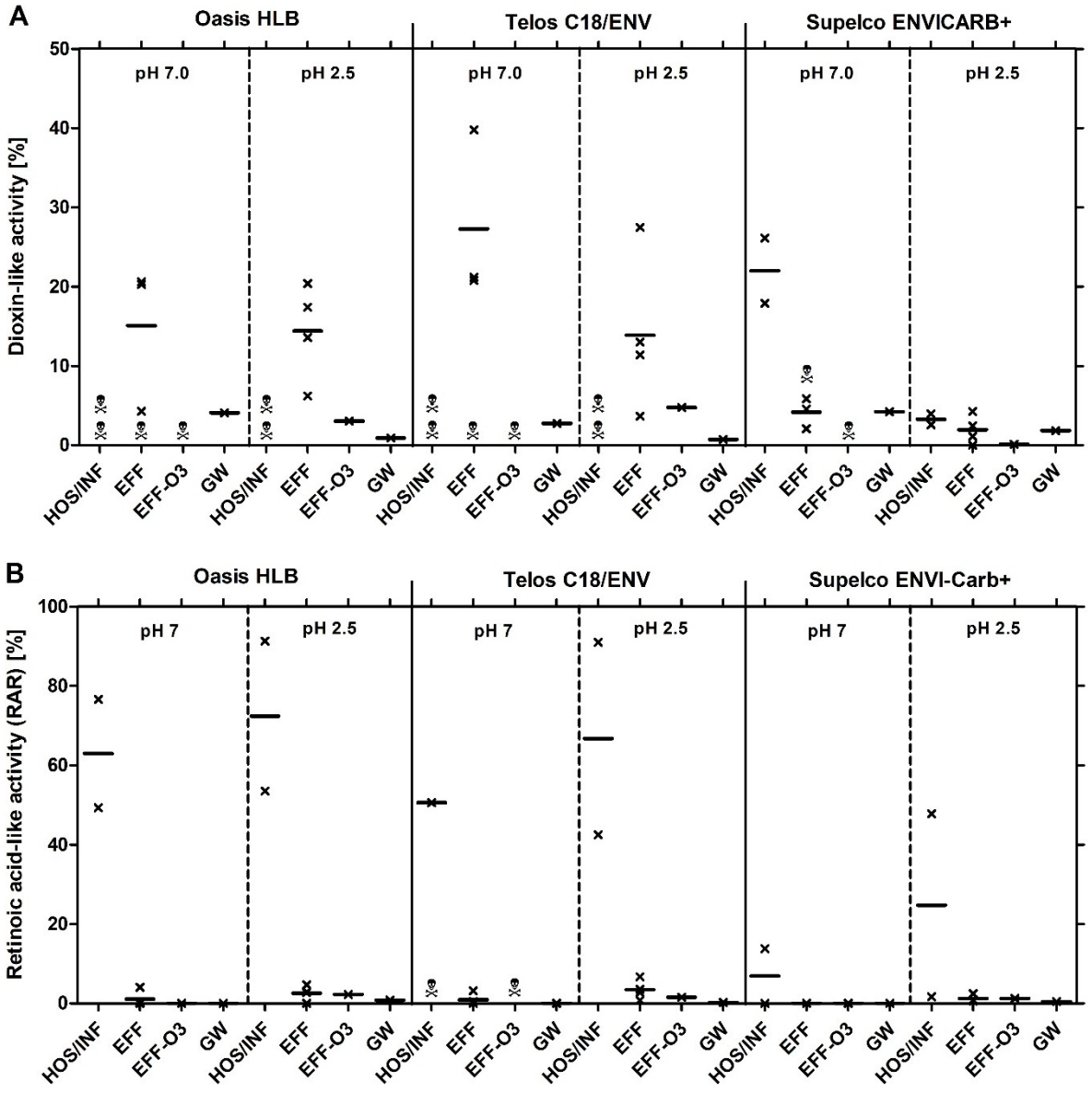

Figure S12: Dioxin-like (A) and retinoic acid-like (RAR, B) activity in % of SPE extracts of water and wastewater samples using three different SPE columns and two different pH values. The results were pooled from the different samples according to water type. Symbols: activity of the individual sample, 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.

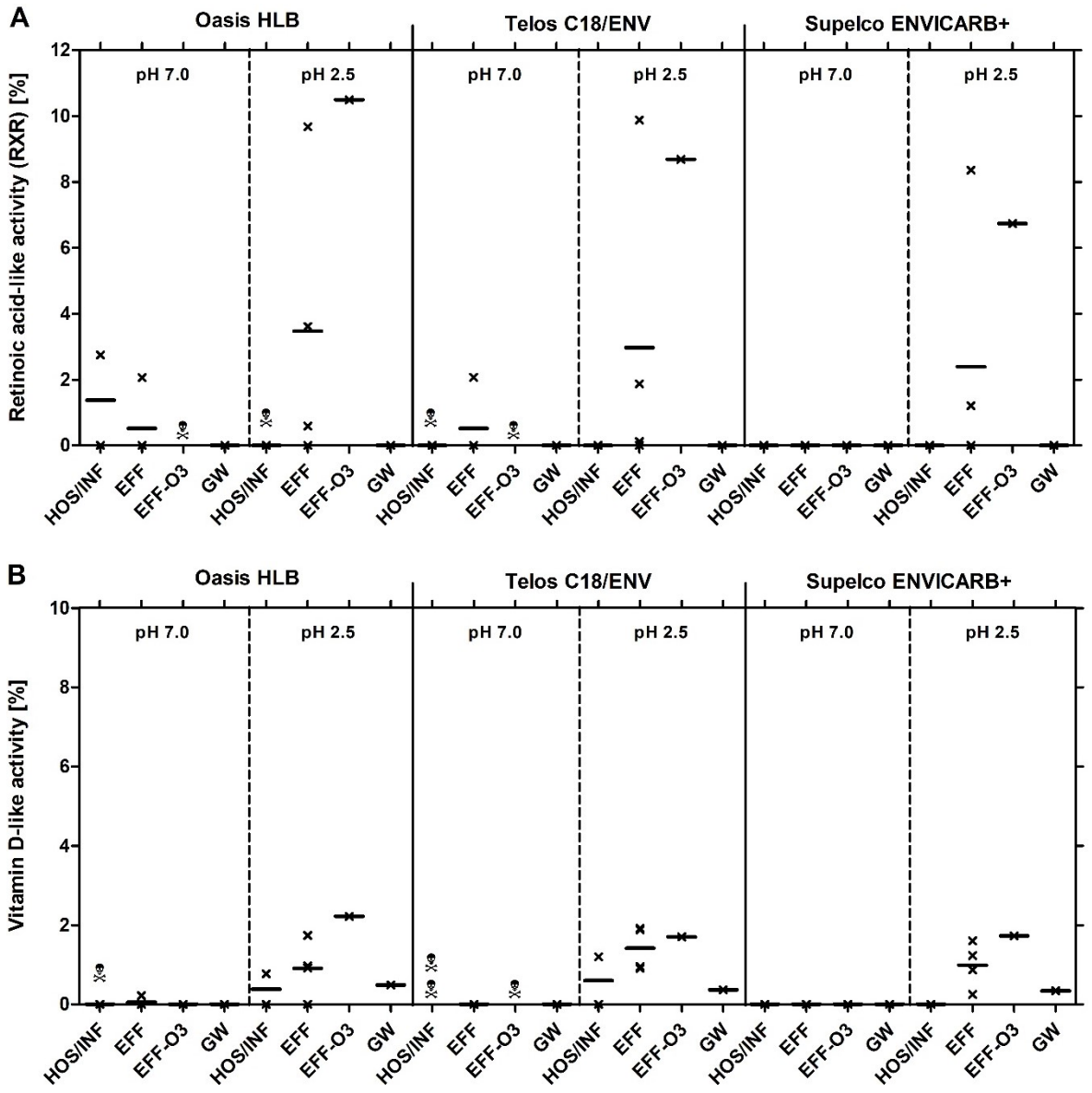

Figure S13: Retinoid X-like (RXR, A) and vitamin D-like (B) activity in % of SPE extracts of water and wastewater samples using three different SPE columns and two different pH values. The results were pooled from the different samples according to water type. Symbols: activity of the individual sample, 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.

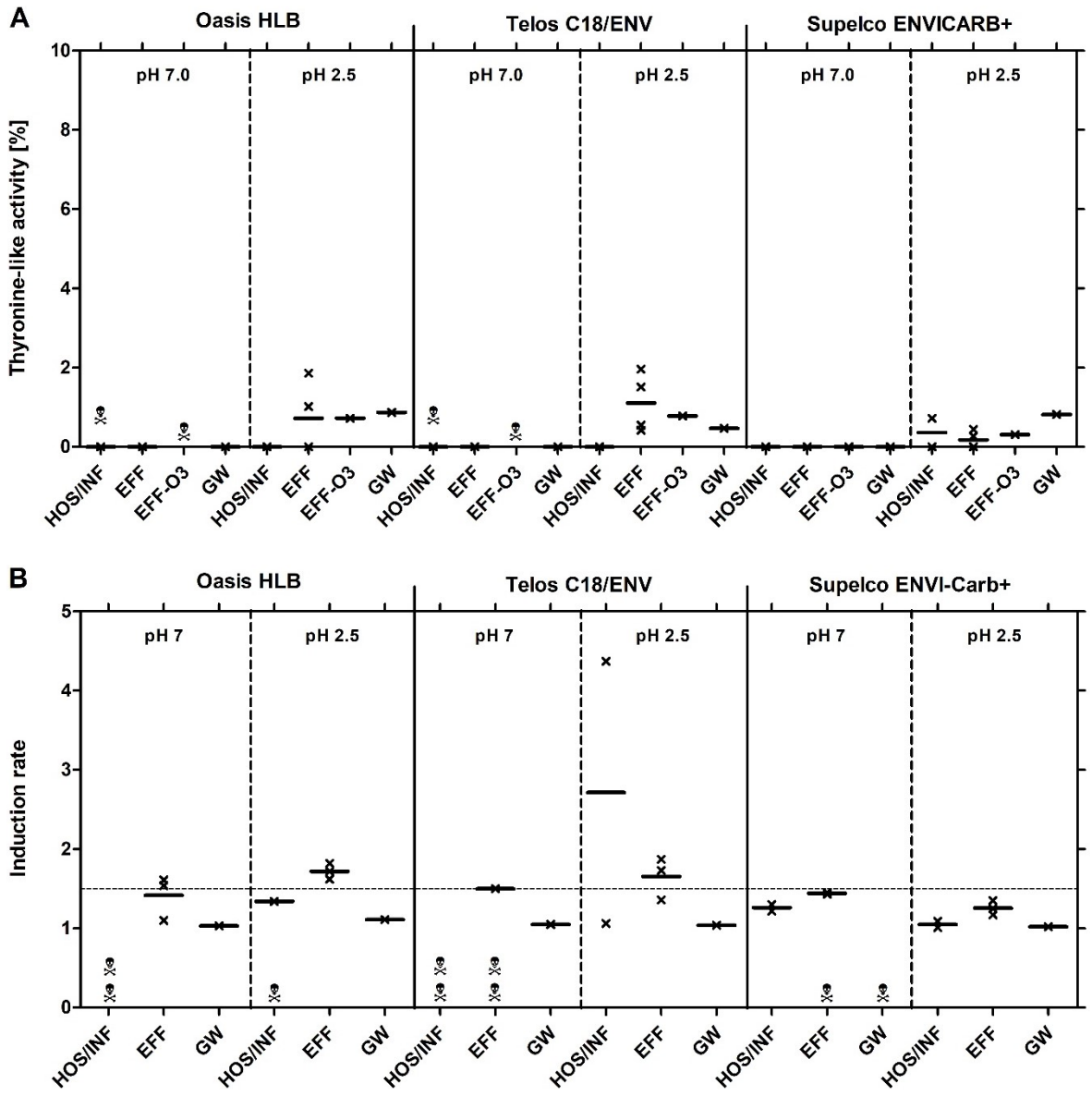

Figure S14: Thyronine-like (A) activity in % and induction rate (umu, B) of SPE extracts of water and wastewater samples using three different SPE columns and two different pH values. The results were pooled from the different samples according to water type. Symbols: activity of the individual sample, 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 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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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.

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

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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 Normung)
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

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) (Fig. 1). Ozonation was chosen because the chemical oxidation induces a transformation of (micro)pollutants in the wastewater and,

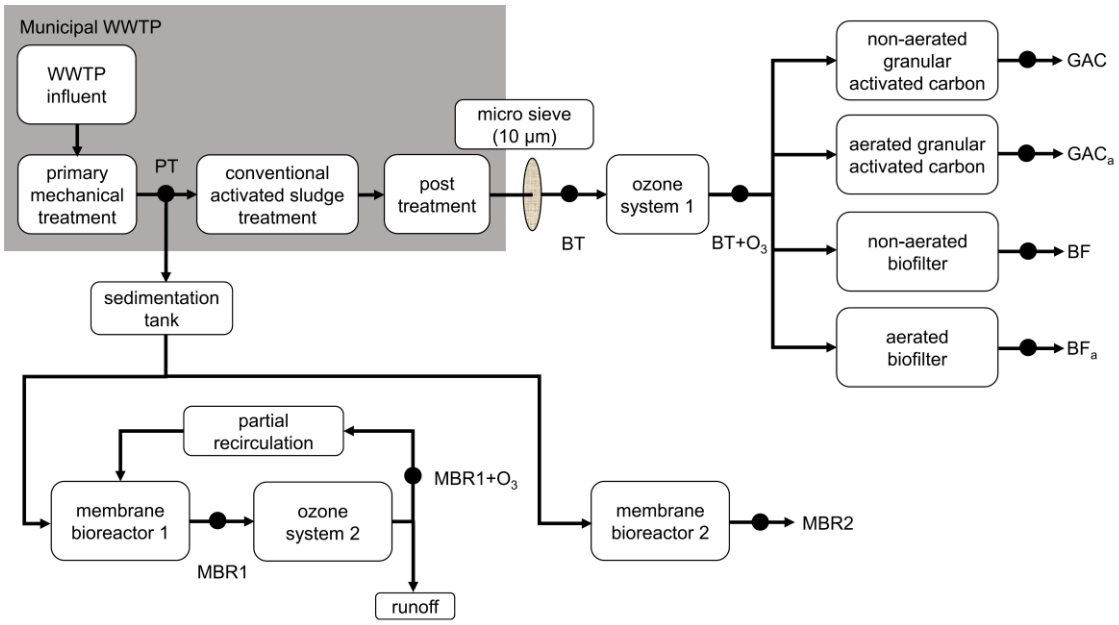

Fig. 1. Process design of the WWTP and AWWT. Process design of the municipal wastewater treatment plant (WWTP) and the pilot-scale advanced wastewater treatment technologies (AWWT). Sampling points are marked with black dots. PT: after primary treatment, BT: after conventional biological treatment, BT+O_3: 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.

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 (Fig. 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. Material and methods

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 6400 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 microsieve (10 μm, Rodisc, Huber SE, Berching, Germany) to further reduce total suspended solids before complete treatment in ozone system 1 (Fig. 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 1200 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 3.33–4.96 m/h (Table S4) achieving a net specific throughput of approximately 7500–10,000 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 (Fig. 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 (Fig. 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, Figs. 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 3 min 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 × 2.0 mL heptane, 1 × 2.0 mL acetone, 3 × 2.0 mL methanol and 4 × 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 × 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 (5000-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 Figs. 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 (Fig. 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_{total}) 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%–79.6 ± 1.37% (Fig. 2A, Table S12). No androgenic activity was detected in the BT and at all ozone doses (Fig. 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%–49.3 ± 0.73% at the highest ozone dose (Fig. 2B, Table S12).

None of the treatments was mutagenic in the Ames TA98 strain (Fig. 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 81.3–95.7% independent of the HRT (Fig. 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 (Fig. 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 (Fig. 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

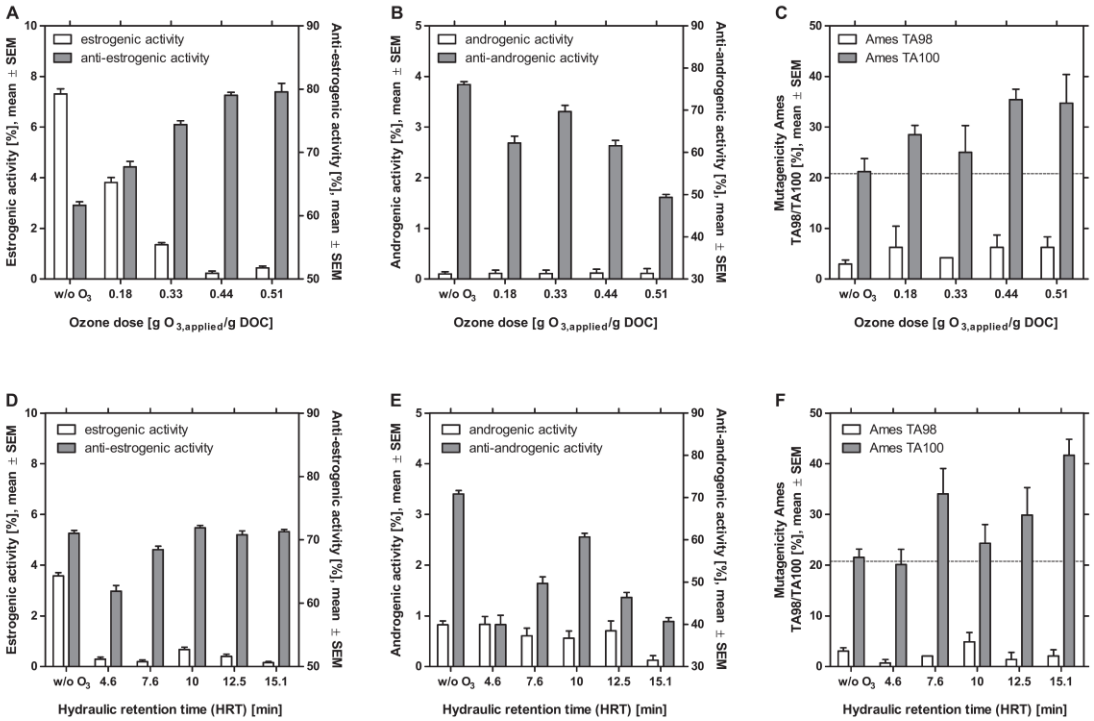

Fig. 2. Optimal ozone dose and hydraulic retention time. Estrogenic and anti-estrogenic activity (A, D), androgenic and anti-androgenic activity (B, E) and mutagenicity (C, F) in % (mean ± SEM) of conventional biological treated wastewater (without ozone; A, B: n = 93–96; D, E: n = 117–120; C, F: n = 12–15) and ozonated wastewater (three SPE extracts perozone 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.

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, Fig. 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, Fig. 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)

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, Fig. 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%, $p < 0.05$, Fig. 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, Fig. 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, Fig. 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 (Figs. 6 and 7) and, thus, not considered.

3.2.4.1. 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 (Fig. 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% (Fig. 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 95.1–95.9% 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% (Fig. 5B, Table S18). Post-filtration reduced this anti-estrogenic activity by 5.03–49.9% 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 (Fig. 5C, Table S18) of the BT extracts was 1.76 ± 0.31% and was reduced by 10.1–84.0% in all

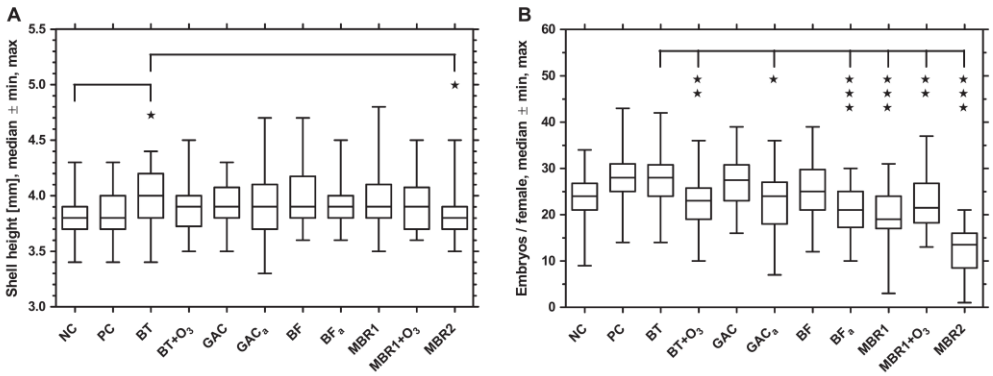

Fig. 3. Growth and reproduction. Size (A) and reproduction (B) of *Potamopyrgus antipodarum* after 28 days of exposure to the negative control (NC), the positive control (PC), the conventional biological treatment (BT) and the eight advanced treatment technologies. BT+O_3: after ozone system 1, GAC: 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.

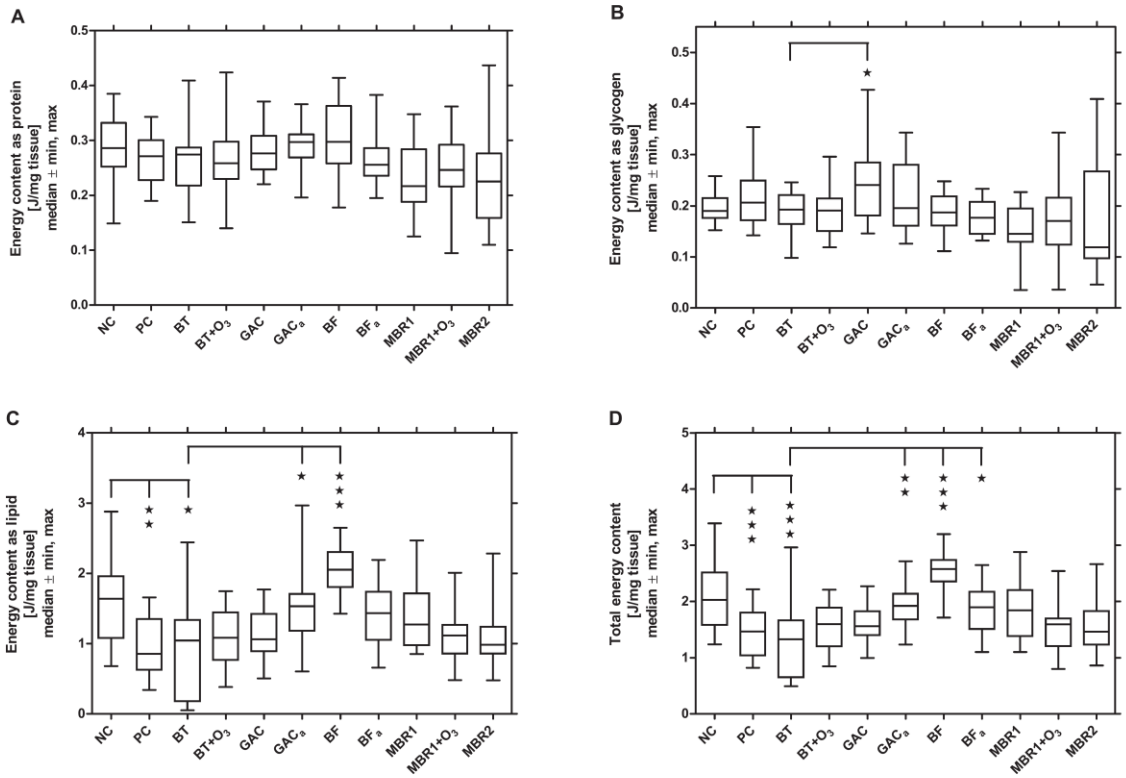

Fig. 4. Biomarkers for energy reserves. Energy content as protein (A), glycogen (B), lipid (C) and total energy content (D) in J/mg tissue of *Potamopyrgus antipodarum* after 28 days exposure to water from the negative control (NC), the positive control (PC), the conventional biological treatment (BT) 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)), $n = 17–20$.

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 (Fig. 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 7.68–72.6%.

3.2.4.2. Ames fluctuation test for mutagenicity. No mutagenic activity was detectable in the BT in the Ames strain YG7108 (Fig. 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%, respectively. No mutagenicity was detected in MBR1 and MBR2 whereas MBR1+O_3 showed a mutagenicity of 67.5 ± 4.62%.

3.3. Chemical analysis

The chemical analysis was conducted in parallel to the ecotoxicological investigations and included 28 micropollutants mainly belonging to the group of pharmaceuticals such as radio-opaque substances, anticonvulsants, antibiotics (including metabolites such as of carbamazepine, diclofenac or ibuprofen) as well as nutrition-related chemicals (caffeine), herbicides (mecoprop) and industrial chemicals (benzotriazole and tolyltriazole). In the PT, caffeine was detected at the highest concentration of 162 ± 23.2 µg/L followed by carboxy-ibuprofen (74.7 ± 6.27 µg/L), 2-hydroxy-ibuprofen (47.3 ± 4.97 µg/L) and 1H-benzotriazole (25.0 ± 0.71 µg/L). The concentrations of the other substances were between 0.025 and 14.4 µg/L (Table S19). The BT reduced the concentrations of 15 out of 28 chemicals by more than 50% (highest reduction, −99.8% for caffeine and carboxy-ibuprofen). For nine chemicals, the reduction was low (<−25%). For carbamazepine and carboxy-acyclovir a concentration increase was detected.

Ozonation led to a further reduction of 21 substances ranging from −11.1% (iopamidol) and −99.1% (carboxy-acyclovir)) compared to the BT (Fig. 7A, Table S19). The concentrations of 18 substances decreased by more than 50%. For another three compounds, the concentrations decreased by between 10 and 50%. Two TPs (3-hydroxy-ibuprofen and tramadol-N-oxide) indicated higher concentrations in the BT+O_3 than in the BT.

The post-treatments further reduced the concentrations of most target substances (Figs. S9 and S10, Tables S19 and S20). For certain compounds for which ozonation did not achieve a complete removal (e.g., 3-hydroxy-ibuprofen, diclofenac, sulfamethoxazole), a post filtration led to an overall removal of 75.0–90.7% compared to the BT+O_3. For a small set of compounds (2-hydroxy-ibuprofen, 4-hydroxy-1H-benzotriazole, carboxy-acyclovir, paracetamol), a moderate additional removal between 31.1 and 42.9% occurred in the GAC filters and BFs compared to the BT+O_3. GAC filters showed

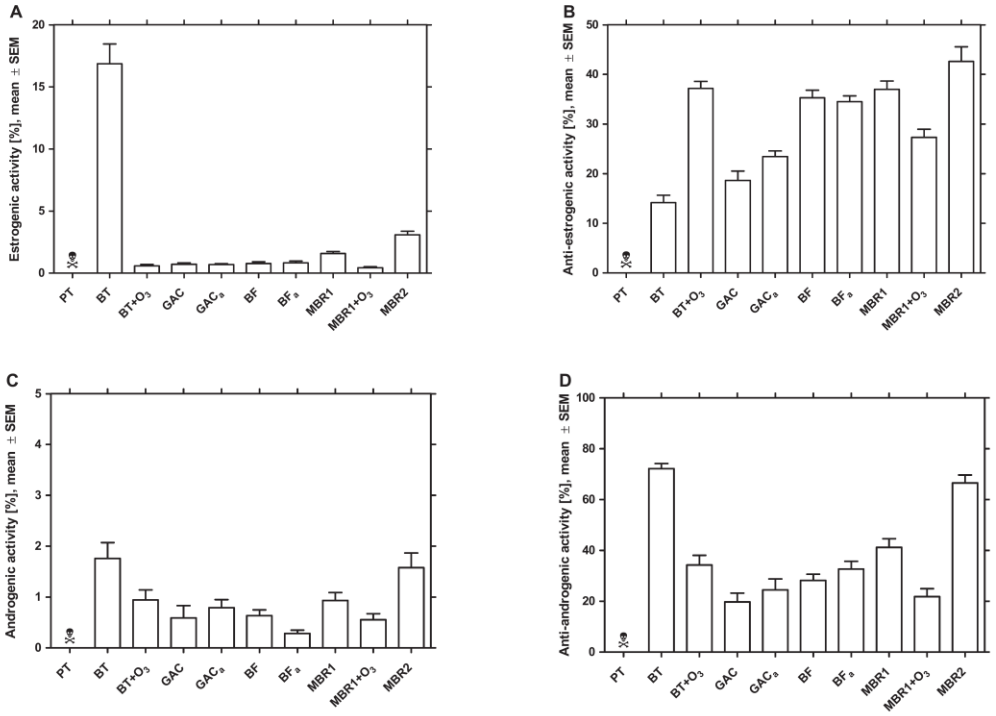

Fig. 5. Endocrine activities of the on-site biotest. Estrogenic (A), anti-estrogenic (B), androgenic (C) and anti-androgenic activity (D) in SPE extracts produced from 24 h composite samples taken in parallel to the *in vivo* experiment. PT: after primary treatment, BT: after conventional biological 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.

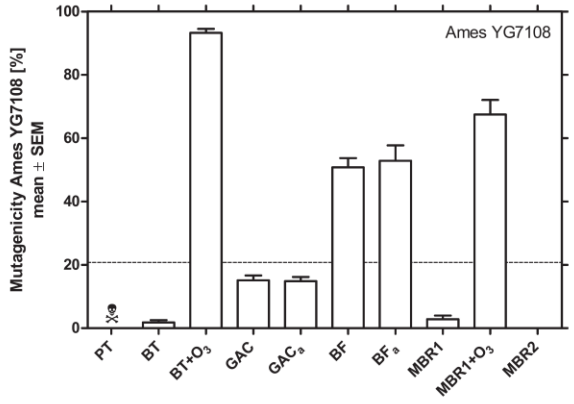

Fig. 6. Mutagenicity of the on-site biotest. Mutagenicity in the Ames strain YG7108 in SPE extracts produced from 24 h composite samples taken in parallel to the *in vivo* experiment. PT: after primary treatment, BT: after conventional biological treatment, BT+O_3: after ozone system 1, GAC: after non-aerated 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.

a higher removal rate for seven compounds including 1H-benzotrialzole, amidotrizoic acid, iomeprole, iopromide, tolytriazole and tramadol-N-oxide compared to the BFs. Certain compounds such as caffeine or mecoprop could however not be further removed by the GAC filters and BFs compared to the BT+O_3.

MBR1 and MBR2 had slightly lower removal efficacies regarding the 28 chemicals than the BT (Figs. 7B and S11, Tables S20–S21). The ozonation increased the removal in the MBR1 with efficiencies comparable to the BT+O_3. However, the concentration of carboxy-acyclovir increased in the BT (+367%), MBR1 and MBR2 (+146 and + 343%, respectively) as well as MBR1+O_3 (+39.3%).

The results for the water parameters can be found in the Supplementary Information (S2.4, Tables S22–S29).

4. Discussion

4.1. Optimal ozone dose and hydraulic retention time

4.1.1. Optimal ozone dose

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

In contrast to previous studies that reported an effective removal of anti-androgenic activity in biologically treated (Rao

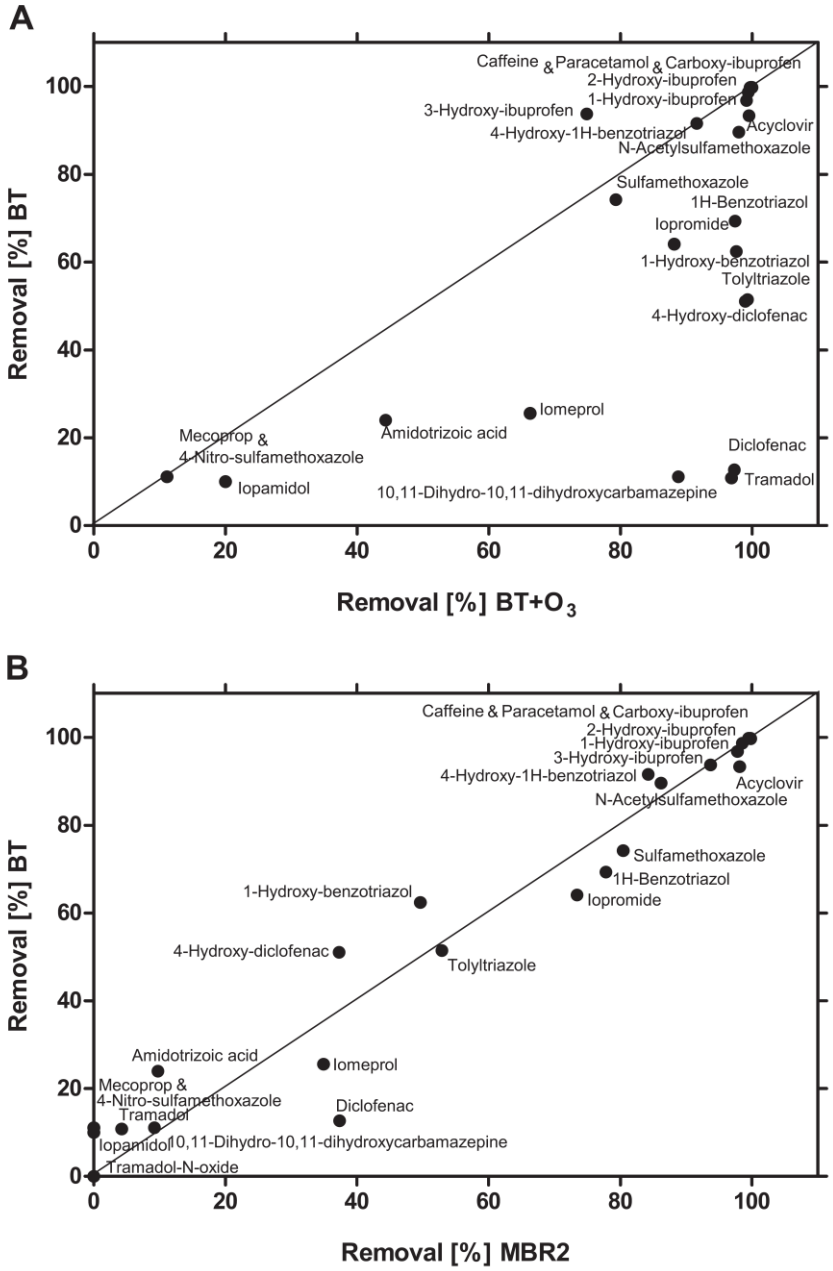

Fig. 7. Chemical analysis. Removal of micropollutants by the conventional biological treatment (BT), by the ozonation ($BT+O_3$, A) and by the membrane bioreactor 2 (MBR2, B) compared to the primary treatment. n = 1–4.

et al., 2014) and ozonated (Stalter et al., 2011) wastewater, we detected a high anti-androgenicity in the BT as well as the $BT+O_3$ samples (Fig. 2B) that was not fully removed by the applied ozone doses. Treatment with the highest dose (0.51 g $O_{3\ applied}$/g DOC) led to a 35.1% reduction. This indicated the presence of relatively stable anti-androgenic substances (Itzel et al., 2020).

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

With regards to determining the optimal ozone dose, it becomes obvious that a balance needs to be 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 (Fig. 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 (Fig. 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 (Fig. 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 (Fig. 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 (Fig. 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 and 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 (Fig. 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 (Fig. 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 (Fig. 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 (Fig. 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 (Fig. 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 (Fig. 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 (Fig. 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 a 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 (Fig. 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 (Fig. 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 (Fig. 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.

5. Conclusions

- To determine optimal ozone doses and HRTs, maximum removal rates and generation of *in vitro* toxicity have to be balanced. An ozone dose of 0.33 g O_3/g DOC and an HRT of 12.6 min seemed optimal.
- While ozonation was effective in further reducing toxicity and micropollutants it also formed toxic TPs. Thus, post-treatment is needed. Activated carbon and biological post-filtration (further) reduced most of the effect with GAC being more effective than BF.
- MBR systems as alternatives to an activated sludge treatment were similarly effective like the BT and even performed better (e.g., removal of estrogenicity). MBR+O_3 improved the removal performance but also generated mutagenicity. The latter was reduced by recirculation to the MBR which might represent a promising option.
- A significant anti-estrogenic activity remained in all AWWTs which should be further investigated.
- Conventionally treated wastewater affected growth and reproduction of *P. antipodarum* (better nutrient supply or exposure of estrogenic chemicals). Ozonation reduced the reproduction indicating a potential formation of toxic TPs. In the post-treatments these effects were compensated or remained unaffected. All MBR treatments induced reproductive toxicity.
- Ozonation of conventionally treated wastewater reduced micropollutants and improved wastewater parameters. Post-treatment with GAC/BF resulted in an additional reduction. MBRs were comparable to BT while MBR+O_3 was similarly effective like BT+O_3.
- For an optimised effect-based assessment of wastewater quality of conventional and AWWT sensitive and environmentally relevant *in vitro* and *in vivo* endpoints as well as an adapted chemical analysis are needed. In addition, further parameters (e.g., energy demand, carbon emission), alternative technical options (e.g., optimising activated sludge treatments) and socioeconomic factors (i.e., source control) have to be considered.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.watres.2020.116104.

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Supplementary information (Publication A.2)

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. MBR: membrane bioreactor.

	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	-

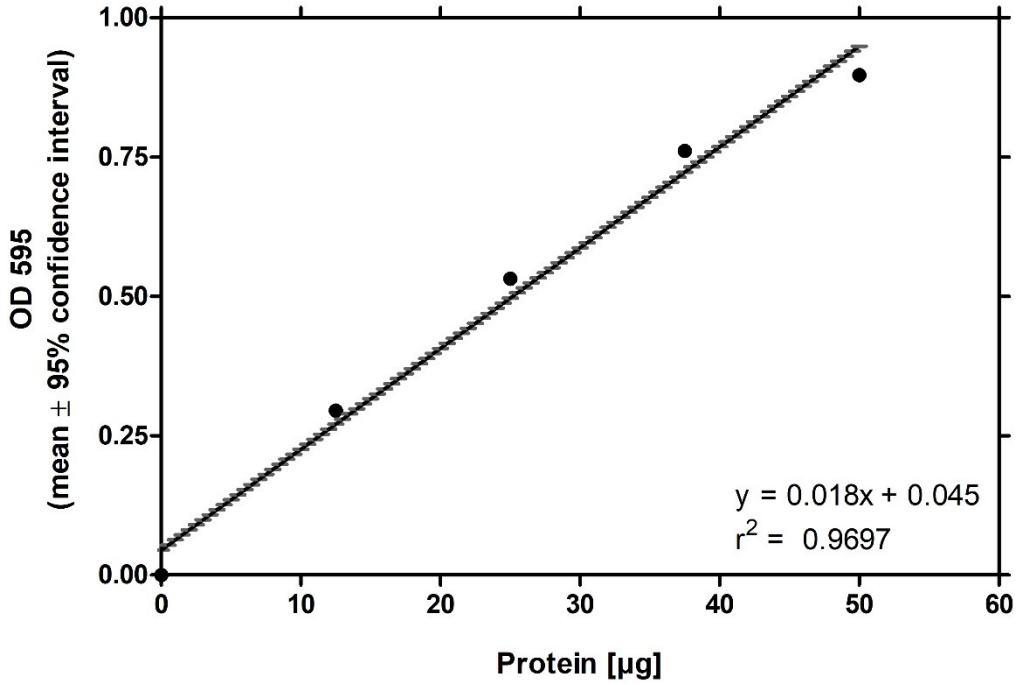

Figure S1: Optical density at a wavelength of 595 nm (OD 595) of five protein concentrations [µg] as linear regression (mean ± 95% confidence interval) of seven experiments.

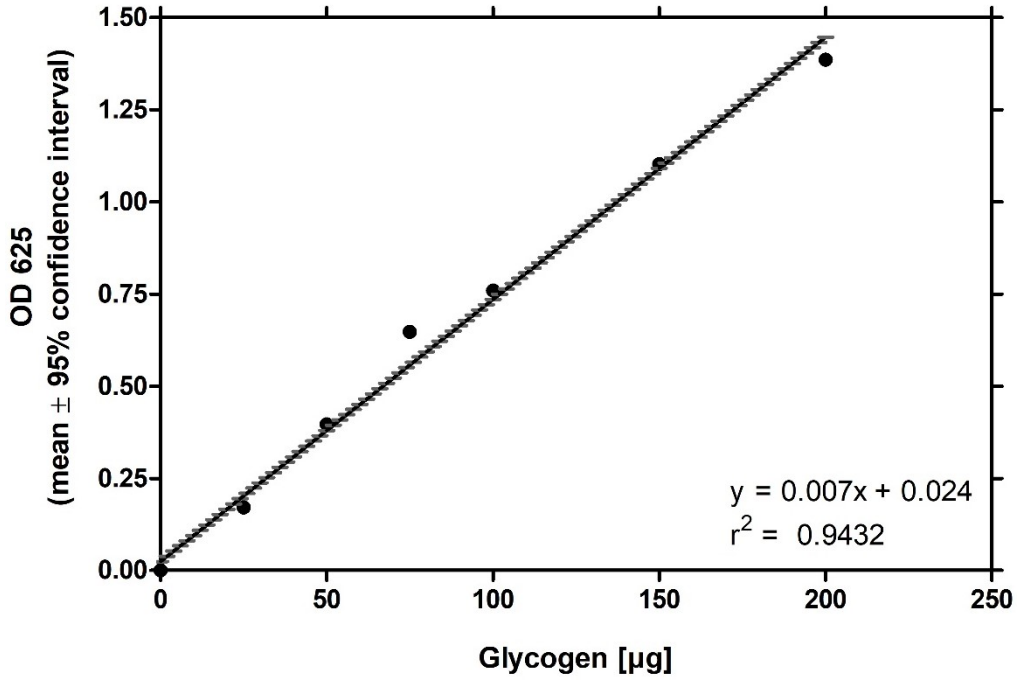

Figure S2: Optical density at a wavelength of 625 nm (OD 625) of seven glycogen concentrations [µg] as linear regression (mean ± 95% confidence interval) of seven experiments.

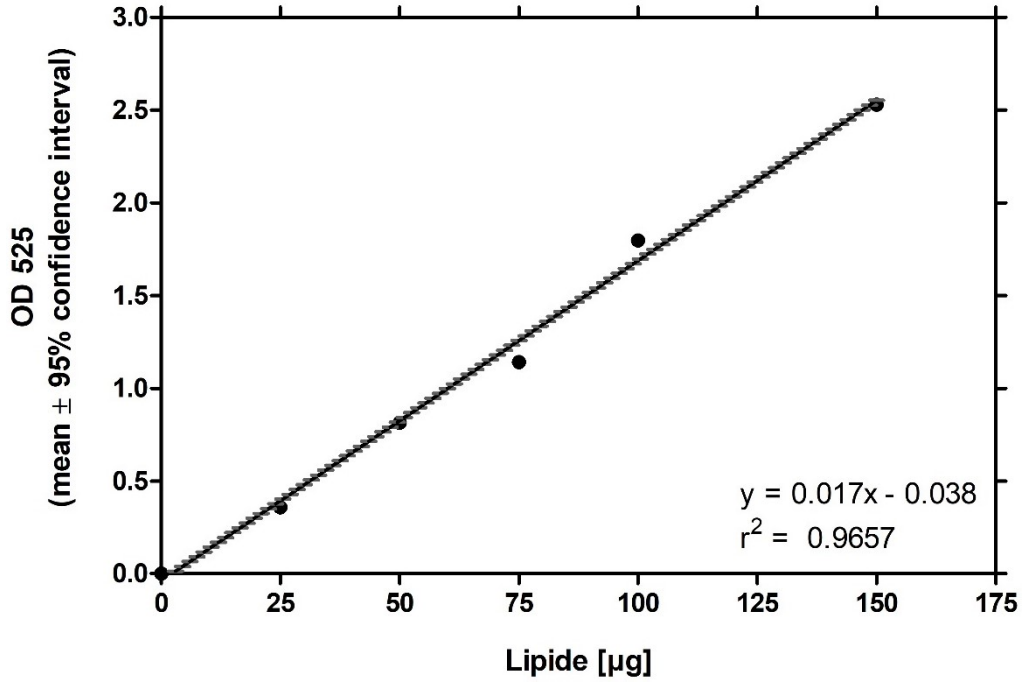

Figure S3: Optical density at a wavelength of 525 nm (OD 525) of six lipid concentrations [µg] as linear regression (mean ± 95% confidence interval) of seven experiments.

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%	-

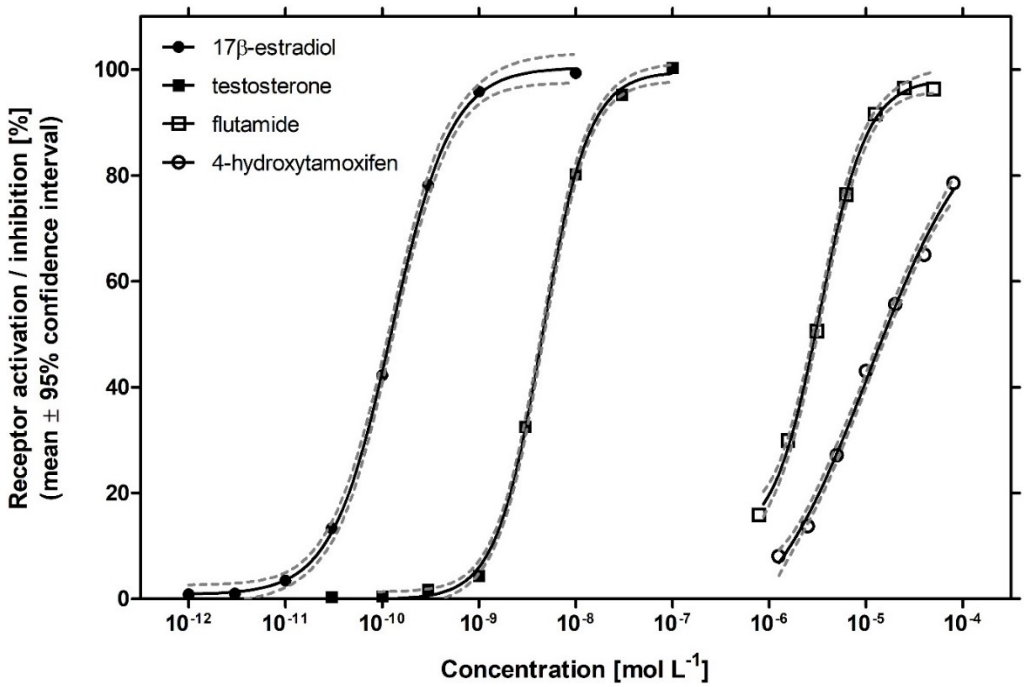

Figure S4: Receptor activation (YES, YAS) and inhibition (YAES, YAAS) as concentration-response relationships of six (YAES, YAAS) and seven (YES, YAS) experiments at the human estrogen and androgen receptor (YES: 17β-estradiol; YAS: testosterone; YAAS: flutamide; YAES: 4-hydroxytamoxifen).

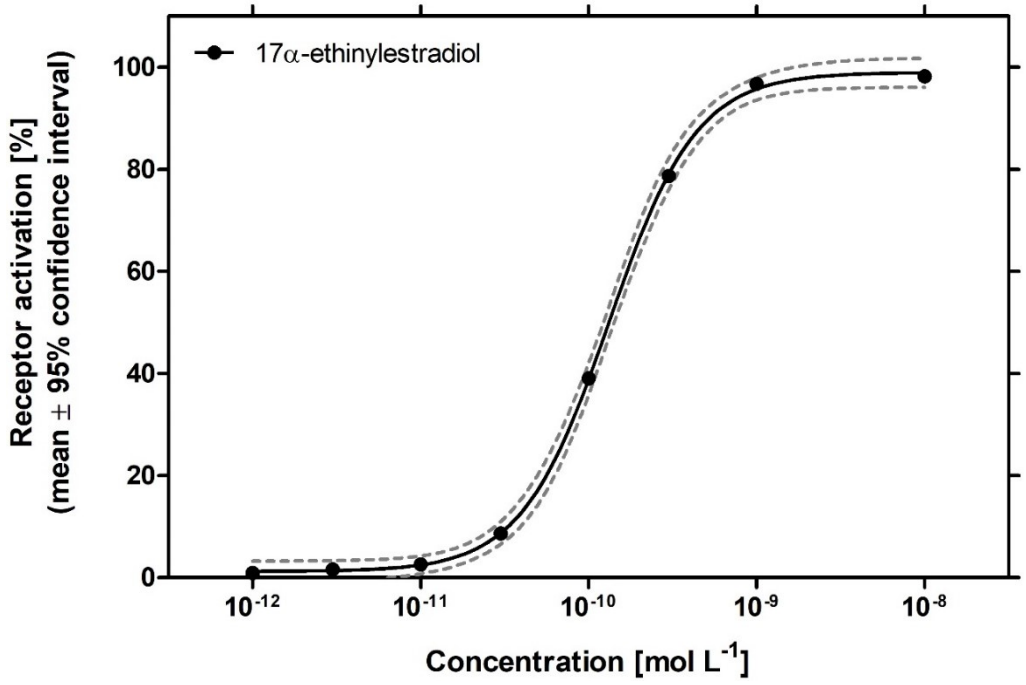

Figure S5: Concentration-response relationships of 17α-ethinylestradiol in four YES experiments.

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	measurement 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	measurement 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 (∆NC) or the conventional biological treatment (∆BT) is given in %. Significant differences compared to ∆NC and ∆BT 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]	∆ [%]	total number of embryos	∆ [%]	fecundity index	∆ [%]
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)	∆NC +1.31 (n.s.)	27.7 ± 5.36 (n = 40)	∆NC +17.0 (n.s.)	7.07 ± 1.23 (n = 37)	∆NC +14.6 (n.s.)
BT	2.00 ± 1.16 (n = 100)	3.98 ± 0.23 (n = 39)	∆NC +4.30 (★)	28.1 ± 6.00 (n = 40)	∆NC +18.7 (n.s.)	7.02 ± 1.25 (n = 39)	∆NC +13.8 (n.s.)
BT+O_3	1.00 ± 1.00 (n = 100)	3.90 ± 0.21 (n = 40)	∆BT –1.98 (n.s.)	21.9 ± 5.94 (n = 40)	∆BT –21.9 (★★)	5.61 ± 1.43 (n = 40)	∆BT –20.1 (★★★)
GAC	3.00 ± 3.00 (n = 100)	3.90 ± 0.20 (n = 40)	∆BT –1.98 (n.s.)	27.5 ± 5.30 (n = 40)	∆BT –2.07 (n.s.)	7.01 ± 1.10 (n = 40)	∆BT –0.11 (n.s.)
GAC_a	1.33 ± 1.33 (n = 100)	3.92 ± 0.28 (n = 35)	∆BT –1.56 (n.s.)	22.8 ± 6.19 (n = 35)	∆BT –18.7 (★)	5.79 ± 1.36 (n = 35)	∆BT –17.5 (★★)

Table S14 (continued)

treatment	mortality [%]	shell height [mm]	Δ [%]	total number of embryos	Δ [%]	fecundity index	Δ [%]
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 (★★★)

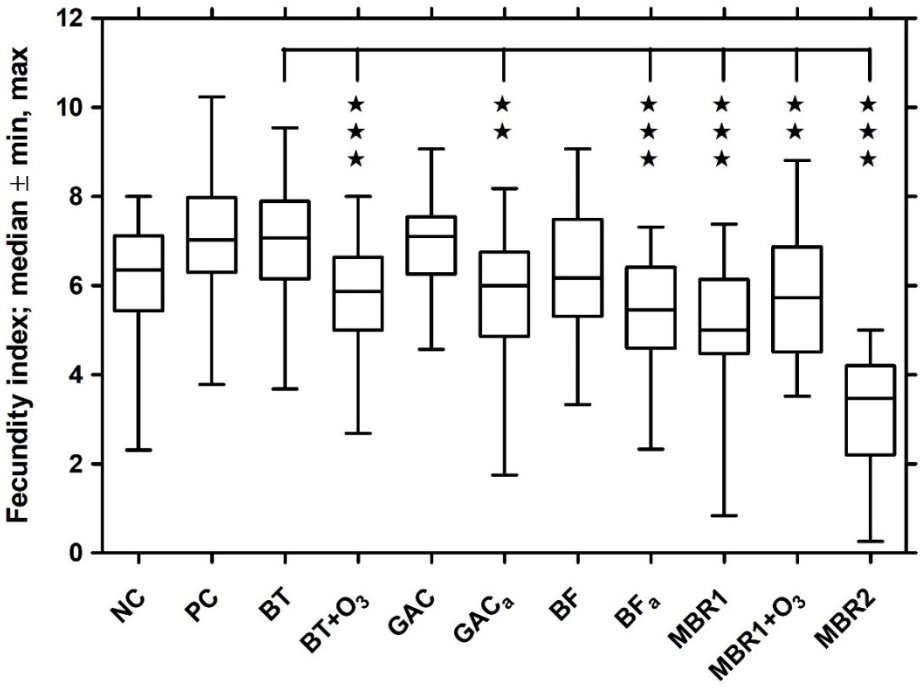

Figure S6: Fecundity index of *Potamopyrgus antipodarum* after 28 days of exposure to the negative control (NC), the positive control (PC), the conventional biological treatment (BT) and the eight advanced treatment technologies. BT+O_3: after ozone system 1, GAC: after non-aerated granular activated 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.

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.)

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 (∆NC) or the conventional biological treatment (∆BT) is given in %. Significant differences compared to ∆NC and ∆BT 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]	∆ [%]
NC	2.07 ± 0.56 (n = 19)	-
PC	1.44 ± 0.43 (n = 19)	∆NC –30.6 (★★★)
BT	1.38 ± 0.77 (n = 19)	∆NC –33.2 (★★★)
BT+O_3	1.55 ± 0.42 (n = 19)	∆BT +12.1 (n.s.)
GAC	1.61 ± 0.33 (n = 19)	∆BT +16.4 (n.s.)
GAC_a	1.94 ± 0.36 (n = 19)	∆BT +40.2 (★★)
BF	2.54 ± 0.35 (n = 20)	∆BT +83.7 (★★★)
BF_a	1.87 ± 0.47 (n = 20)	∆BT +35.2 (★)
MBR1	1.82 ± 0.53 (n = 17)	∆BT +31.6 (n.s.)
MBR1+O_3	1.52 ± 0.42 (n = 18)	∆BT +9.47 (n.s.)
MBR2	1.55 ± 0.47 (n = 19)	∆BT +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 (∆BT) is given in %. n.c.: not calculable.

	YES	∆ BT [%]	YAES	∆ BT [%]	YAS	∆ BT [%]	YAAS	∆ 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.

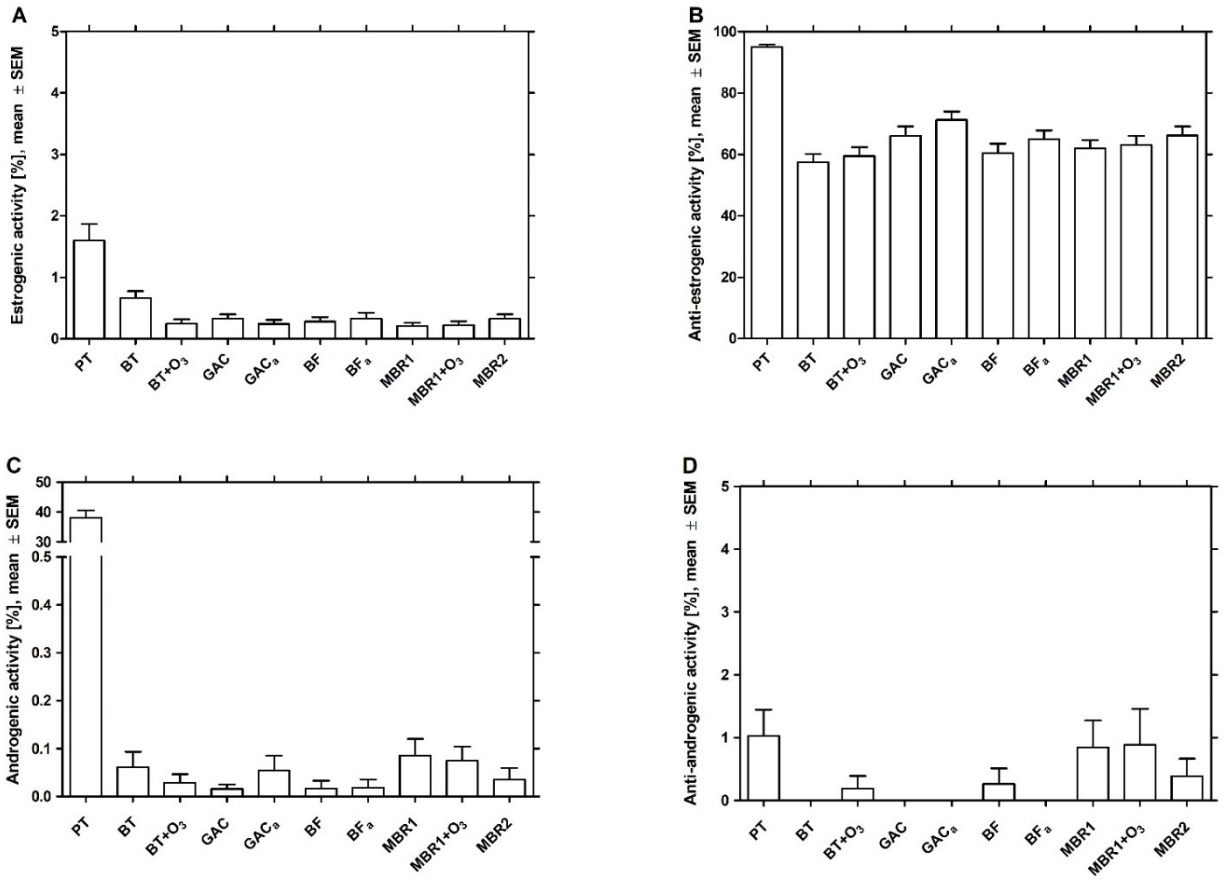

Figure S7: Estrogenic activity (A), anti-estrogenic activity (B), androgenic activity (C) and anti-androgenic activity (D) of the aqueous samples from four 24 h composite samples per treatment. PT: after primary treatment, BT: after conventional biological treatment, BT+O_3: after ozone system 1, GAC: 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).

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 (∆BT) is given in %. ☠: cytotoxic.

	YES	∆ BT [%]	YAES	∆ BT [%]	YAS	∆ BT [%]	YAAS	∆ 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)

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

Table S19 (continued)

	PT	BT	Δ PT [%]	BT+O_3	Δ PT [%]	GAC	Δ PT [%]	GAC_a	Δ PT [%]
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

Table S19 (continued)

	PT	BT	Δ PT [%]	BT+O_3	Δ PT [%]	GAC	Δ PT [%]	GAC_a	Δ PT [%]
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

Table S20 (continued)

	PT	BF	Δ PT [%]	BF_a	Δ PT [%]	MBR1	Δ PT [%]	MBR1+O_3	Δ PT [%]
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

Table S20 (continued)

	PT	BF	Δ PT [%]	BF_a	Δ PT [%]	MBR1	Δ PT [%]	MBR1+O_3	Δ PT [%]
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

Table S21 (continued)

	PT	BT (for comparison)	MBR2	Δ PT [%]
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

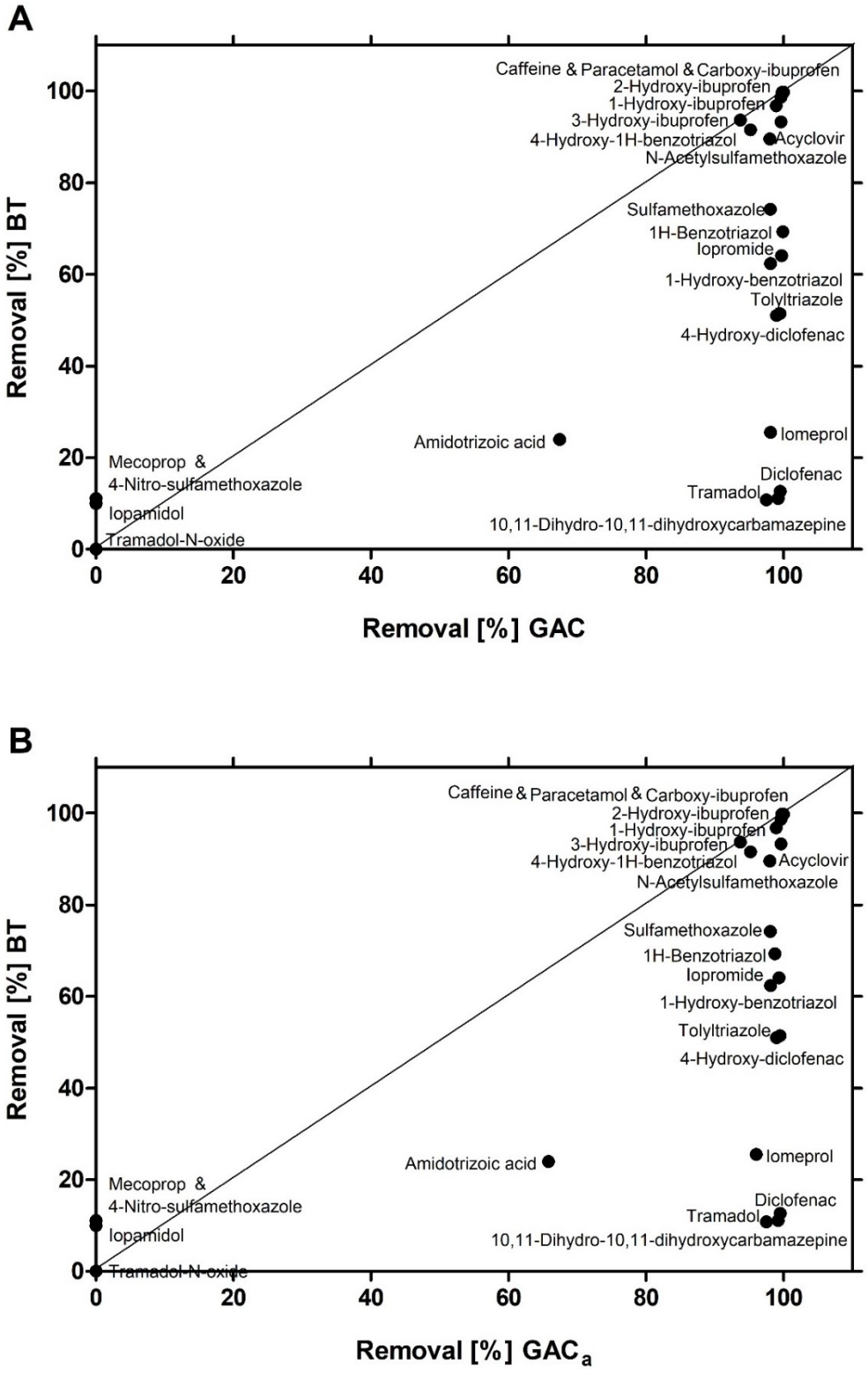

Figure S8: Removal of chemicals in the conventional biological treatment (BT) compared to the non-aerated granular activated carbon filter treatment (GAC, A) and the aerated granular activated carbon filter treatment (GAC_a, B). n = 1–4.

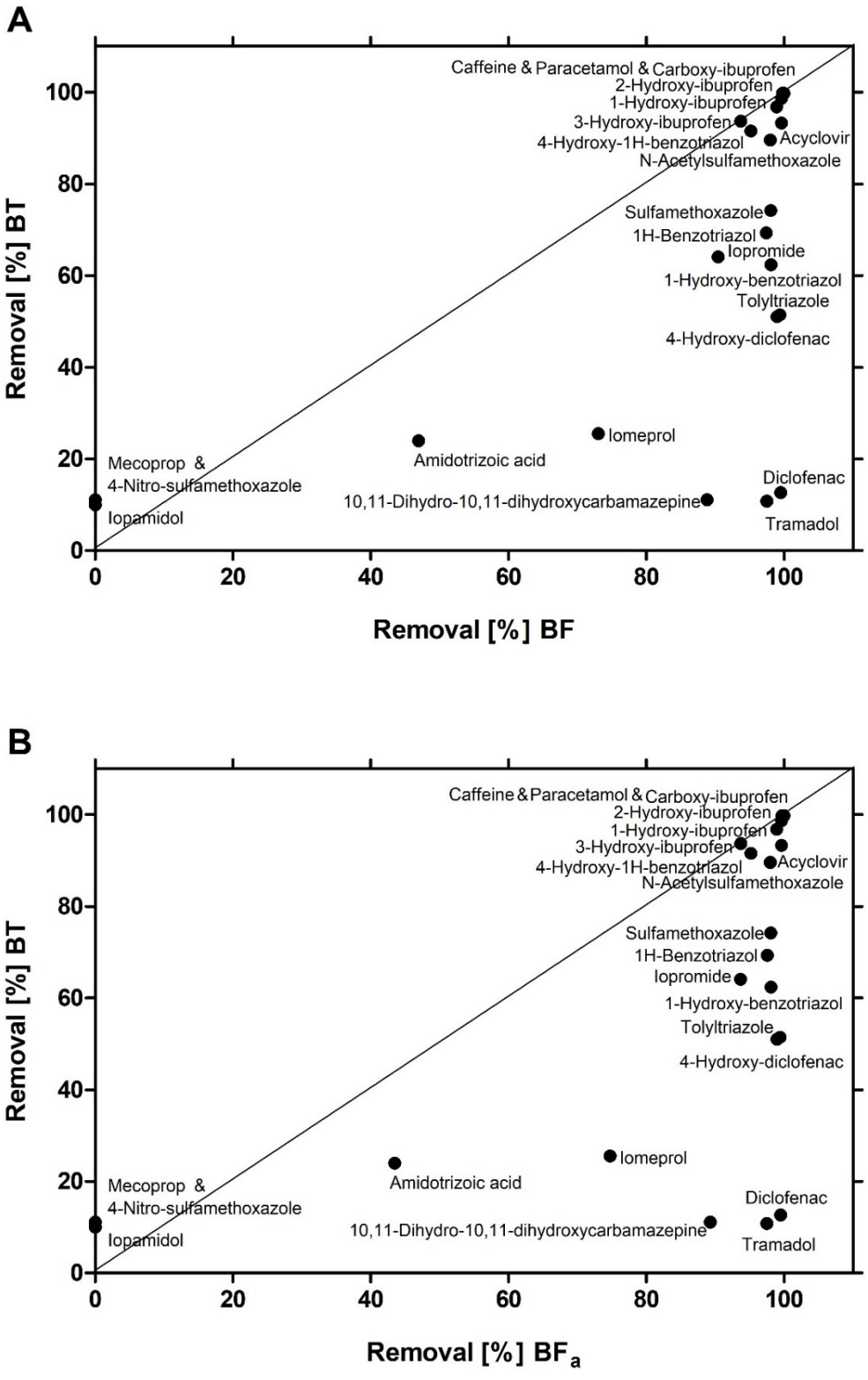

Figure S9: Removal of chemicals in the conventional biological treatment (BT) compared to the non-aerated biofilter treatment (BF, A) and the aerated biofilter treatment (BF_a, B). n = 1–4.

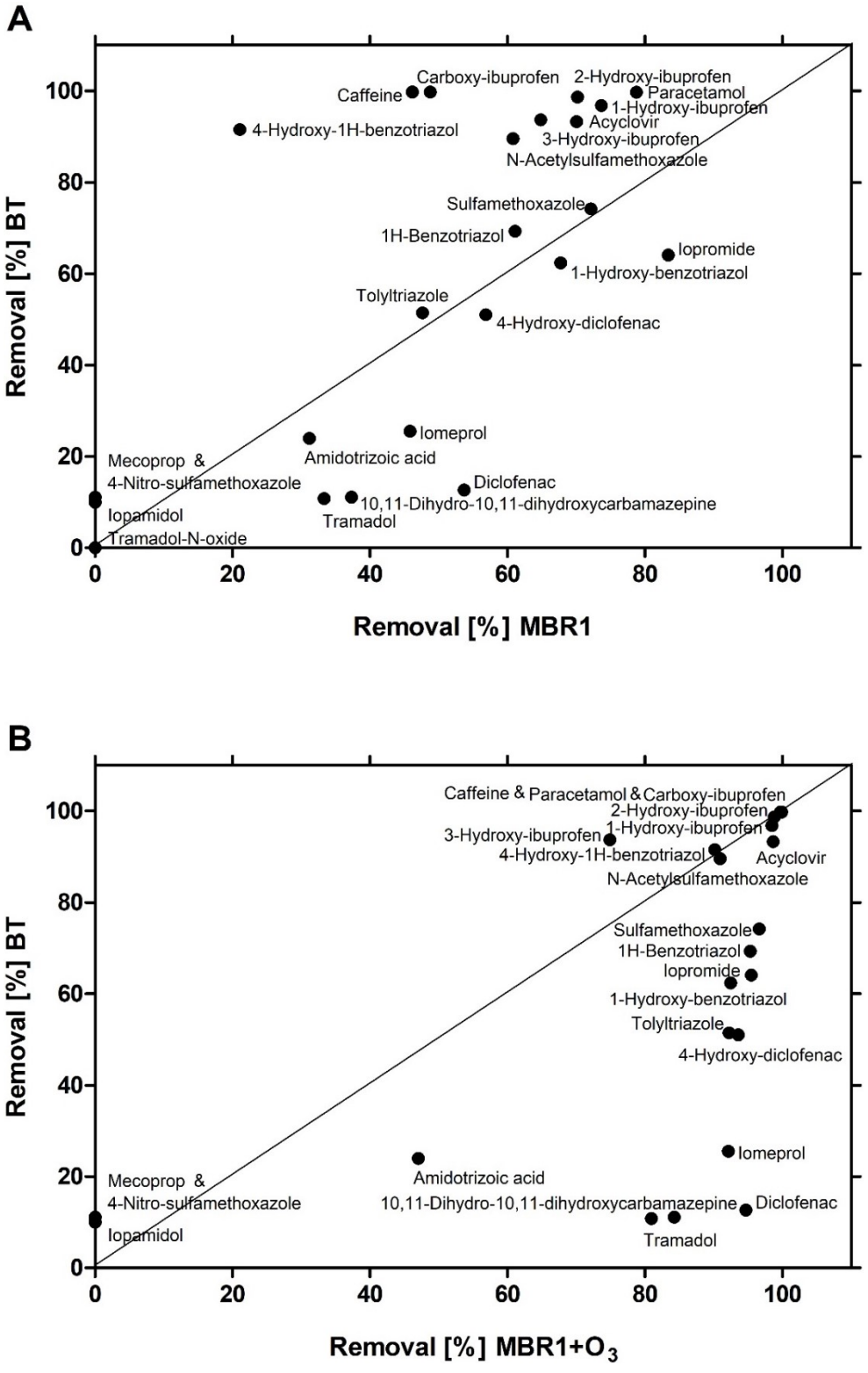

Figure S10: Removal of chemicals in the conventional biological treatment (BT) compared to the membrane bioreactor 1 (MBR1, A) and the membrane bioreactor 1 after ozone system 2 (MBR1+O_3, B). n = 1–4.

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	-

Table S29 (continued)

	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 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

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A.3 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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RESEARCH ARTICLE

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

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

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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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 days), fast reproductive cycle (3 days 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); and 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, and 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 sand filtration (e.g., Magdeburg et al. 2012). From the research on wastewater treatment processes, it also became apparent that the removal of target compounds does not necessarily result in a removal of toxicity.

The present study aimed at extending on this knowledge by assessing the removal of (micro)pollutants and toxicity (xenobiotic metabolism) by conventional and advanced wastewater treatment. Samples were collected at four WWTPs of different size classes (small, medium, and large) equipped with conventional activated sludge and different advanced treatments. The

latter were installed at one WWTP and comprised of an ozonation of the WWTP effluent and sequential GAC filtration or biofiltration (BF). In addition, surface water was sampled downstream of one of the investigated WWTPs. For the analysis of these samples, an established *C. elegans* bioassay was adapted from the International Organization for Standardization (ISO) guideline 10872 (Höss et al. 2012). Lab-scale in vivo bioassays such as ISO 10872 are valuable tools in assessing the toxicity and biological activity of environmental samples. Their outcome thereby provides valuable indications on the quality of (waste)water and can serve as proxy of potential biological impacts of chemicals. This standardized bioassay has also been used to examine the impacts of various chemicals with different modes of action in other studies (e.g., Ristau et al. 2015; Haegerbaeumer et al. 2018). The guideline comprises the apical endpoint reproduction and growth that respond sensitively to testing environmental samples (Wilson and Khakouli-Duarte 2009). A main objective of this study was to integrate molecular endpoints for xenobiotic metabolism into the assay, which may be more sensitive. *Cyp-35A3*::GFP (Menzel et al. 2007) was selected as biomarker for CYP-35A3-related xenobiotic metabolism in transgenic *C. elegans* (e.g., Min et al. 2015; Roh et al. 2014). Using the PAH and potent *cyp-35A3* inducer β-NF, proof of principle experiments were carried out on surface water and wastewater prepared by different techniques. These experiments aimed at determining the assay sensitivity and characterizing the impact of the sample matrix, such as from total suspended solids (TSS) content or background (micro)pollutant concentrations. Based on these results, 15 relevant sampling points, representative for the urban water cycle, were analyzed. Special focus was put on the comparison of conventional and advanced treatments, the respective micropollutant removal efficacies and the occurrence of residual micropollutants and/or toxicity in WWTP discharges and receiving surface water. Two main hypotheses were tested: (1) Advanced wastewater treatment is more effective in removing (micro)pollutants and toxicity. (2) The removal of target compounds does not per se translate to a removal of toxicity. For quantification of (micro)pollutants and TPs, the concentrations of 92 chemical indicator substances (Seitz and Winzenbacher 2017) and 19 WWTP parameters (Knopp et al. 2016) were determined.

Materials and methods

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 the "Sampling and sample preparation" section.

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 the "Sampling and sample preparation" section from the influent (INF-4), after activated sludge treatment (EFF-4), after the ozonation (EFF + O_3), GAC (O_3 + GAC), and BF (O_3 + BF). GAC and BF were operated in parallel in an unaerated (O_3 + GAC, and O_3 + BF) and aerated (O_3 + GAC_a and O_3 + BF_a) mode using compressed ambient air. Details on process parameters can be found in Online Resource 2.

Sampling and sample preparation

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

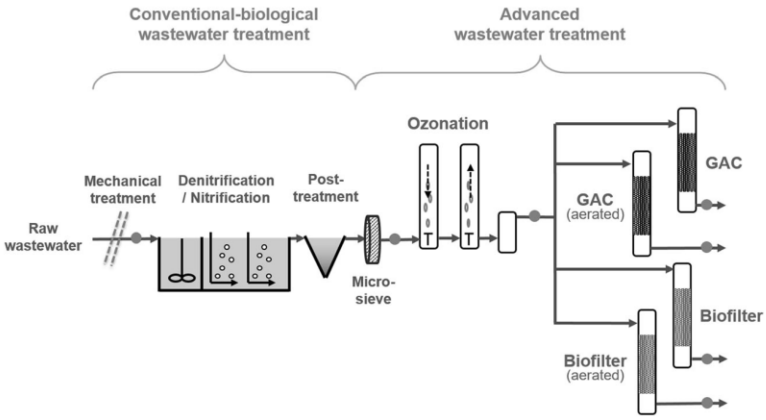

Fig. 1 Process scheme of WWTP-4. The first part of the WWTP (left) operates a conventional biological treatment process. The second part (right) is a pilot WWTP with advanced wastewater treatment technologies: ozonation connected to aerated and non-aerated granular activated carbon (GAC) filtration or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

concentration (5000×). SPE extracts were kept at −20 °C until bioassay analysis.

Spiking of samples with β-naphthoflavone

Aqueous SW and EFF-1 from December 2012 were spiked to 1 mg/L β-NF (CAS 6051-87-2, Alfa Aesar, > 98%). Ultrapure water (UPW) was used as blank sample (TKA 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 the "Sampling and sample preparation" section).

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 days prior to bioassay analysis.

Adapted *C. elegans* bioassay

ISO 10872 was adapted as follows: For the endpoint brood size and larval length, synchronized L1 larvae were transferred into 24-well microtiter plates ($n = 5$–10 per replicate, compare "Endpoint brood size and larval length" and "Endpoint *cyp-35A3*::GFP expression" sections). 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. One hundred microliters of an OP50 suspension (500 FAU, final concentration) in M9 including cholesterol (CAS 57-88-5, Sigma-Aldrich, > 92.5% GC, 0.1% final concentration) was supplemented to all wells. The resulting bacterial suspension was used as negative control (NC). For testing SPE extracts: depending on the final concentration factor, 10× or 25×, an extract volume of 1 µL (1:500) or 2.5 µL (1:200) of the 5000× SPE extracts ("Sampling and sample preparation" section) was added, respectively. For testing aqueous samples: 0.5 mL of sample was added (1:2). Addition of samples/extracts represented the starting point (t_0) of the bioassays. Microtiter plates were incubated at 20 °C in the dark for 1–96 h depending on the endpoint ("Endpoint brood size and larval length" and "Endpoint *cyp-35A3*::GFP expression" sections). Highest final SPE enrichment factor tested (25×) represented a DMSO concentration of 0.5% (*v*/*v*). At this solvent concentration, no adverse effects on *C. elegans* were reported (Boyd et al. 2010). In prescreening experiments, 10× concentrated samples were tested ("Aqueous and β-naphthoflavone spiked surface water and wastewater"). For samples from WWTP-1–3 a 25× concentration factor was applied ("Conventional wastewater treatment"). However, for these samples, mortality occurred in the INF-1–3 (data not shown); thus, 1:2 dilutions were prepared. Accordingly, WWTPs 1–3 were tested in 12.5× concentrations. Samples from WWTP-4 were tested in 25× concentrations ("Advanced wastewater treatment technologies").

Endpoint brood size and larval length

Benzylcetyldimethylammonium chloride (BAC-C16, 5 mg/L, CAS 122-18-9, Alfa Aesar, 95%) was used as additional

positive control (PC) for reprotoxicity and inhibition of growth (Höss et al. 2012). The duration of the respective bioassays was 96 h. At their termination (t_{end} = 96 h), adult and larval nematodes were sacrificed by heat shock (15 min at 80 °C) and stained with rose bengal (CAS 632-69-9, AppliChem) for microscopic evaluation (30×). For the endpoint brood size (reproduction), 10 individuals were exposed in three replicates each per experiment. Total *n* per treatment group are indicated in figure captions. For a comparative analysis in selected experiments, five individuals in five 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.

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 the "Endpoint brood size and larval length" section except for 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 100× 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.

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).

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 and 1–48 h of exposure, respectively. Logistic regression models were used to derive the median effective concentrations EC_{50} (Online Resource 5–6).

Results

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 10× concentrated SW extract. In contrast, the aqueous EFF-1 significantly reduced reproduction by 83% compared to the control. The 10× 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 aqueous spiked samples. Despite a 10× concentration factor, the spiked WWTP effluent sample induced lower reprotoxicity than the aqueous $EFF\text{-}1^s$.

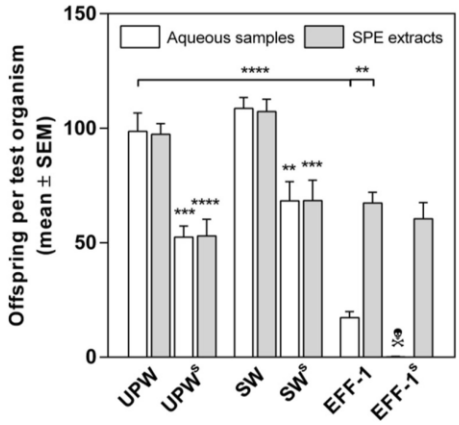

Fig. 2 Impacts of aqueous and extracted ultrapure water (UPW), surface water (SW), and wastewater treatment plant effluent (EFF-1) on the brood size of *C. elegans*. Aqueous (white bars) and extracted (gray bars) samples were analyzed in 0.5× and 10× concentrations, respectively. Spiked aqueous samples (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

Conventional wastewater treatment

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

Advanced wastewater treatment technologies

The samples from the conventional and subsequent advanced wastewater treatments at WWTP-4 were analyzed for their effects on brood size and larval lengths. These samples were tested as 25× concentrated extracts as no significant mortality occurred (compare with the "Adapted *C. elegans* bioassay" section). 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 (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

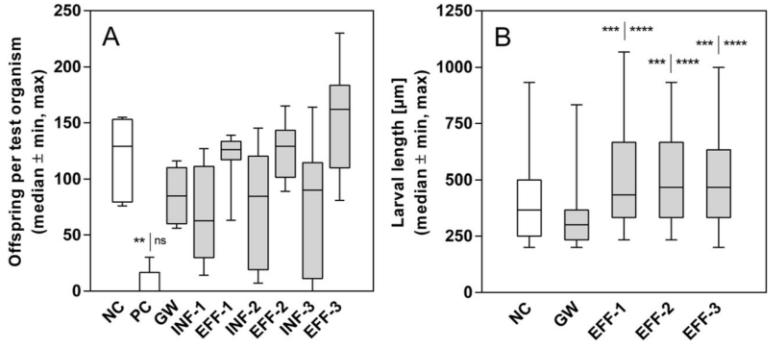

Fig. 3 Impacts of extracted groundwater (GW, SPE blank), wastewater treatment plant influent (INF-1–3) and effluent (EFF-1–3) on the brood size (**a**) and length of larvae (**b**) of *C. elegans*. Samples (gray bars) were analyzed in 12.5× concentrations. Results pooled from three experiments for brood 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 the Kruskal-Wallis test with Dunn's post-test. NC (white bar) = M9 medium. PC (white bar) = BAC (5 mg/L). ns = not significant

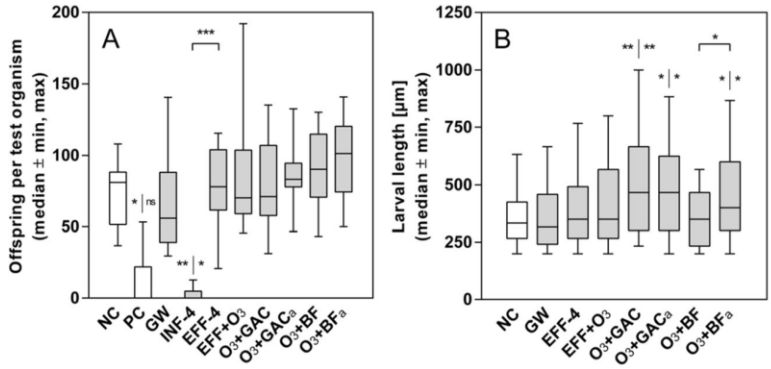

Fig. 4 Impacts of extracted groundwater (GW, SPE blank), wastewater treatment plant influent (INF-4), effluent (EFF-4), and advanced treatments on the brood size (**a**) and length of larvae (**b**) of *C. elegans*. Advanced treatments comprised of ozonation (EFF + O_3) and ozonation followed by aerated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

of GW. These results were qualitatively confirmed throughout multiple experiments ($n = 6$).

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

Based on these results, the sensitivity of *cyp-35A3*::GFP expression towards different aqueous, spiked, and enriched water and wastewater samples was compared (Fig. 5 and Online Resource 7). None of the aqueous samples (UPW,

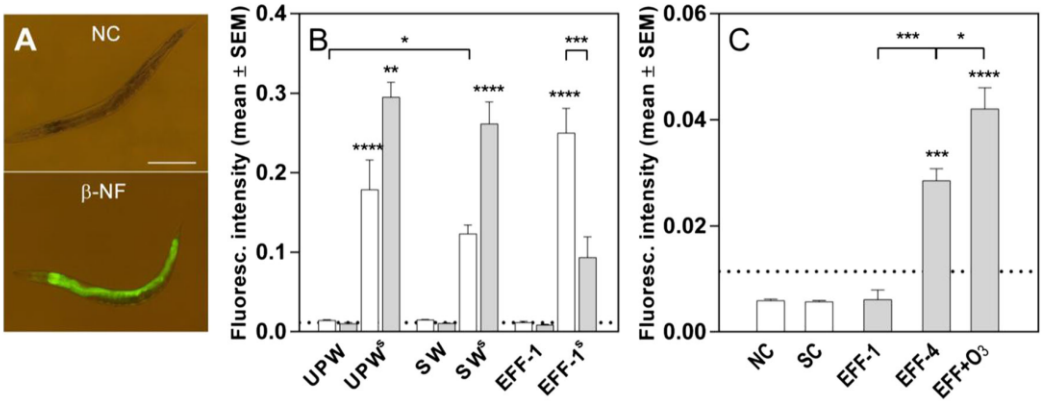

Fig. 5 **a** *cyp-35A3*::GFP expression in transgenic *C. elegans* after 8 h exposure to 1 mg/L β-naphthoflavone (β-NF). Exposed adult hermaphrodites showed a strong GFP signal along their intestine, as detected by fluorescence microscopy (100×). Images (NC, β-NF) show an overlay of differential interference contrast microscopy (DIC) and GFP channel. NC = M9 medium. Bar = 200 µm. **b**, **c** Impacts of aqueous and extracted ultrapure water (UPW), surface water (SW), wastewater treatment plant effluent (EFF-1, EFF-4), and ozonated effluent (EFF + O_3) on *cyp-35A3*::GFP expression. Aqueous (white bars) and extracted (gray bars) samples were analyzed in 0.5× and 10× concentrations respectively after 24 h exposure. Spiked aqueous samples (marked by superscript s) contained 0.5 mg/L β-NF. Results pooled from two experiments ($n = 10$ per treatment group, respectively). Significant differences ($^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$) tested unspiked against spiked samples (**b**) and against controls (**b**, **c**) by one-way ANOVA with Tukey's post hoc analysis. Dashed lines = limit of quantification. **c** NC (white bar) = M9 medium. Solvent control (SC, white bar) = 0.2% DMSO in M9 medium. Fluorescence intensity of PC (1 mg/L β-NF) = 0.185 (result not shown)

SW, EFF-1) significantly induced *cyp-35A3*::GFP. Similar to their aqueous equivalents, exposure to 10× concentrated extracts of these samples did not significantly induce *cyp-35A3*::GFP at any exposure time. In contrast, the β-NF-spiked aqueous samples (UPW^s, SW^s, and $EFF\text{-}1^s$) significantly induced the expression. Similar to β-NF, this increase was time-dependent (1–48 h) and maximal expression levels were reached after 24–48 h. The earliest significantly increased expression was detected after 1 h of exposure to $EFF\text{-}1^s$. The exposure to the extracted spiked samples UPW^s and SW^s led to slightly higher CYP-35A3::GFP levels compared to the aqueous spiked samples. Interestingly, *cyp-35A3* expression induced by $EFF\text{-}1^s$ extracts was significantly lower than for the aqueous $EFF\text{-}1^s$ sample (Fig. 5b).

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

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, and 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, and 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 3–4). 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 EFF + O_3) was determined. Diatrizoate had the highest concentrations after post-treatment (5.6–6.1 μg/L), followed by acesulfame (4.1–5.1 μg/L) and sucralose (2–4.4 μg/L).

Discussion

β-Naphthoflavone and spiked environmental samples

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

The potential impact of the sample matrix on the β-NF effects was examined by spiking surface water and wastewater samples. Spiked surface water induced a high reprotoxicity similar to the spiked ultrapure water control. For the unspiked surface water sample, no reprotoxicity was detected. This indicated that no reprotoxicity is present and that the surface water matrix does not interfere with the β-NF toxicity. This is further supported by the detected low micropollutant concentrations (Online Resource 3; Seitz and Winzenbacher 2017). The effluent of WWTP-1 decreased the brood size by 83% and spiking further increased this effect to 100% (Fig. 2). This suggests a joint effect of β-NF and other reprotoxic wastewater constituents including natural factors that may affect these toxicities. Mixture toxicity was previously

suggested for wastewater contaminants in *C. elegans* (Hitchcock et al. 1997). The fact that there was no difference in the reprotoxicity induced by the spiked aqueous and extracted ultrapure water and surface water (Fig. 2) suggested a low recovery rate towards β-NF, which may not effectively elute from the SPE sorbent due to its hydrophobicity. In contrast, the extracted effluent sample (EFF-1) induced toxicity indicating that other reprotoxic compounds than β-NF were extractable. However, the reprotoxicity in the extracted EFF-1 and EFF-1^S was lower than in their aqueous equivalents, which may attribute to particle associated reprotoxicity filtered out during SPE pre-filtration (compare below) and/or the absence of non-extractable natural factors (compare above).

Unspiked surface water and effluent of WWTP-1 did not cause any significant *cyp-35A3*::GFP induction (Fig. 5 and Online Resource 7). Spiking with β-NF, however, resulted in an effective induction, which was higher in the aqueous effluent compared to the surface water sample. This is in accordance with the results observed for reproduction and might be explained by joint effects caused by low concentrations of multiple CYP inducers in the effluent, which do not induce expression without β-NF and/or natural factors affecting the latter. Another factor might have contributed: β-NF has a log K_{ow} of 4.7 (estimated using US EPA's EPI Suite) and will adsorb to particles, such as from TSS in wastewater. Higher TSS can thus partition more bioavailable β-NF into the particulate phase of wastewater compared to surface water. As ingestion of contaminated food particles is the main exposure route for several pollutants in *C. elegans* (Offermann et al. 2009), the interaction of β-NF and wastewater-borne particles may thus explain the higher toxicity observed in the aqueous sample. In addition, this was not the case for extracted samples in which particulate matter larger than 1 μm and sample impurities were generally removed prior to or during extraction, respectively. These results underline the importance to consider contaminated suspended solids in ecotoxicological evaluations of WWTP discharges (Burton et al. 2000) for which particle-feeding species such as *C. elegans* may offer several advantages.

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 25× 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 with "Advanced wastewater treatment technologies" section).

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. 2007; 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 ("*Cyp-35A3*::GFP induction in transgenic *C. elegans*" section) 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 2009). This indicated that the particulate phase of environmental samples should be considered when estimating realistic exposure concentrations of these compounds.

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 of ozonation. The effect was attributed to the generation of toxic TPs during ozonation. This hypothesis has been corroborated using several aquatic species exposed to ozonated wastewater (Magdeburg et al. 2012; Giebner et al. 2016). In contrast to these studies, neither conventionally nor advanced treated wastewater at WWTP-4 negatively affected the reproduction of *C. elegans* (Fig. 4a). Accordingly, the removal of toxicity by the post-treatments (such as postulated

in hypothesis 1) in the "Introduction" section) could not be assessed. This is in accordance with other model species, which were not sufficiently sensitive for the evaluation of advanced wastewater treatment (Völker et al. 2017). Mutant and transgenic *C. elegans* strains, such as the mentioned hypersensitive mutant (e.g., Xiong et al. 2017), may thus represent promising alternative tools for assessing the toxicity of (highly) treated wastewaters and micropollutant effects at (very) low concentrations. Another explanation for the observation at WWTP-4 might be the general variability of the wastewater matrix. (Micro)pollutants and natural compounds in WWTP influents and effluents can vary significantly depending on the catchment area and WWTP characteristics, respectively (e.g., WWTP-1 and WWTP-4; Online Resource 1). Moreover, toxic oxidation products amongst (highly) polar compounds may be lost during SPE of ozonated (waste)water samples (Stalter et al. 2016).

In comparison, the endpoint larval growth was affected by the advanced wastewater treatment stages with a significantly increased larvae length in the activated charcoal treatments and the aerated biofilter (Fig. 4b). The largest increase was observed for the O_3 + GAC. Different anthropogenic compounds (Höss and Weltje 2007) and natural organic matter (NOM) constituents (Höss et al. 2001) demonstrated to affect *C. elegans* 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.

Micropollutant removal

The concentrations of most target compounds, DOC, and other relevant wastewater parameters decreased in the conventional biological and the advanced treatment stages ("Chemical analysis and WWTP parameters" section). This confirmed the additional reduction capacity of ozonation and the GAC/BF post-treatments such as postulated in hypothesis (1) in the "Introduction" section. The causes of the observed effects of the respective wastewater samples on *C. elegans* ("Aqueous and β-naphthoflavone spiked surface water and wastewater," "Conventional wastewater treatment," "Advanced wastewater treatment technologies," and "*Cyp-35A3*::GFP induction in transgenic *C. elegans*" sections) 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). Certain of the chemical indicators indicated (repro)toxicity, including 1-adamantylamine (Kao et al. 2016), 2-(thiocyanomethylthio)-benzothiazol (Allard et al. 2013), caffeine (Boyd et al. 2010), carbamazepine (Olga Kolychalow, personal communication), DEET (Hartman and Freedman 2005) and depressed fertility, such as saccharin (Sofia Allison, personal communication), or growth promotion, such as sulfamethoxazole (Liu et al. 2013). Nonetheless, none of these compounds seemed individually responsible for the effects observed in this study, because their concentrations (Online Resource 3) were lower than their reported ECs. A few chemical indicators were tested positively for biochemical or molecular endpoints in *C. elegans* which occurred in the microgram per liter range in the wastewater samples from conventional treatment, such as diclofenac or sotalol (Petersen et al. 2004) and the advanced wastewater treatment stages, such as acesulfame or gabapentin (Caylor et al. 2013). It should also be considered that the concentrations of chemical indicators measured in this and most of the cited studies referred to the aqueous phase of the respective wastewater samples. In contrast, their accumulation to sludge particles (Chalew and Halden 2009; McLaggan et al. 2012) and potential mixture toxicity effects (e.g., additive or synergistic) have rarely been compared. However, it is also likely, that the chemical analysis of target micropollutants did not cover the toxicologically relevant compounds (e.g., Tang et al. 2014), supporting hypothesis (2) postulated in the "Introduction" section. This further highlights the need to combine biological and chemical methods to assess the effectiveness of (advanced) wastewater treatment.

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.

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Supplementary information (Publication A.3)

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.)	38.8×10^6	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)	1.5×10^6	9–16	14	~ 50 % wastewater	Receiving water body with high groundwater infiltration.
3	16,600 (14,000)	2.5×10^6	9–15	11	~ 18 % wastewater	Periodically high proportion of external WW (from a hospital).
4	50,000 (42,000)	2.3×10^6	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)

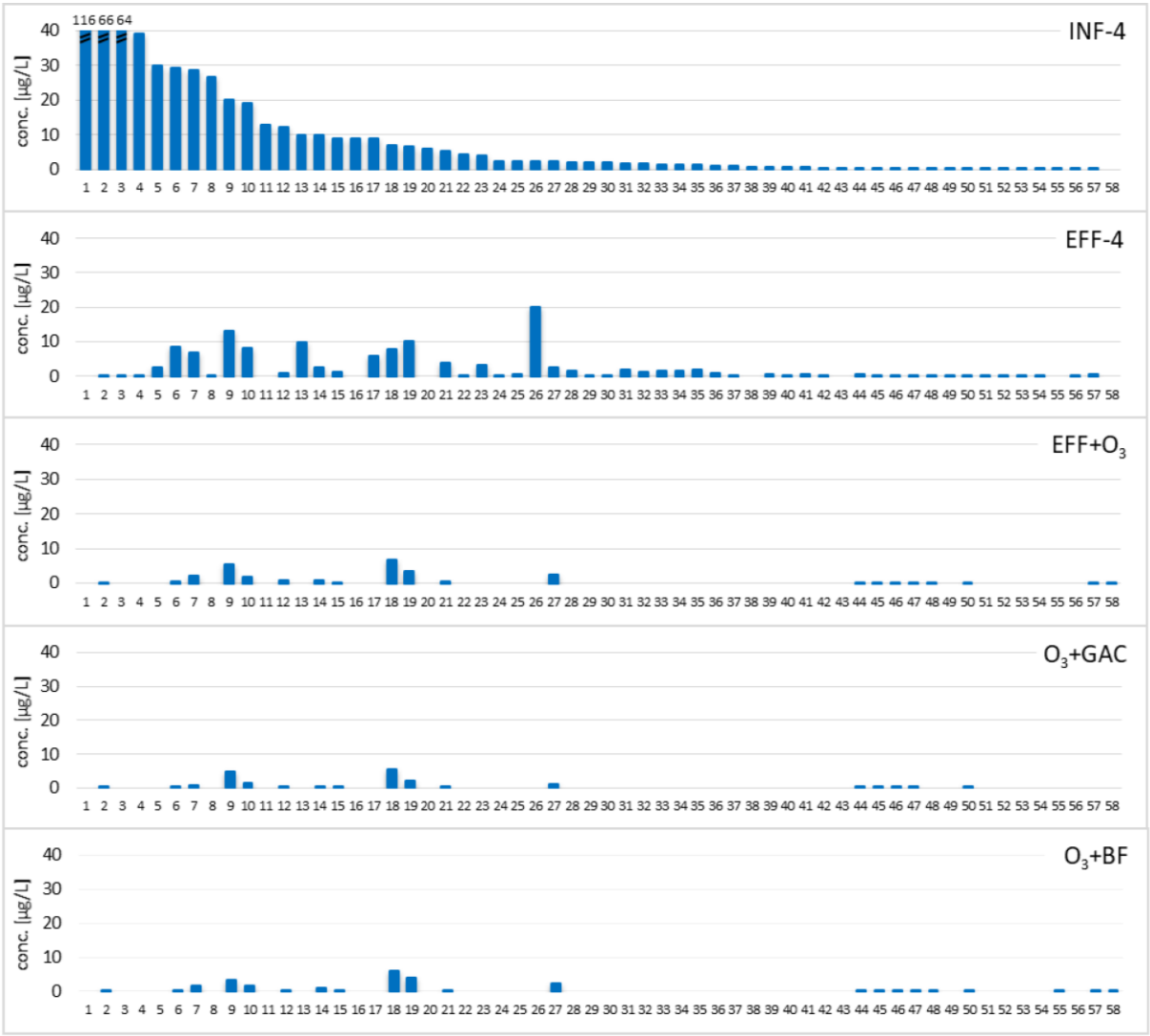
Sampling point: / Compound/Group:	SW	INF-1	EFF-1	INF-2	EFF-2	INF-3	EFF-3	INF-4	EFF-4	EFF+O_3	O_3+GAC	O_3+GAC$_a$	O_3+BF	O3+BF$_a$
Pharmaceuticals and contrast media														
Iohexol	0.11	29	11	0.07	< 0.025	5.9	5.5	8.9	1.2	0.3	0.09	0.14	0.2	0.22
Iomeprol	0.4	76	48	1.6	1.2	22	3.1	29	6.9	2.3	0.61	0.93	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

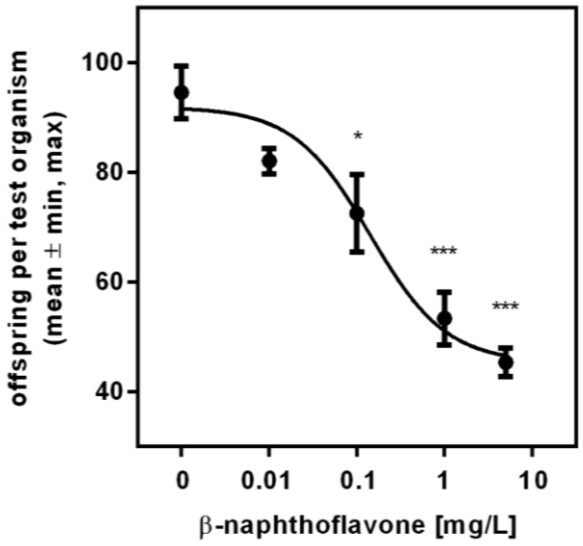
Compounds detected below the LOQ at all sampling points	Atenolol, aspartame, betaxolol, chloramphenicol, clenbuterol, *clofibric acid*, crotamiton, dapsone, diazepam, doxicyclin, etofibrate, fenofibrate, fenoprofen, gemfibrozil, indometacin, iotalamic acid, ketoprofen, metronidazol, oxytetracycline, 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)

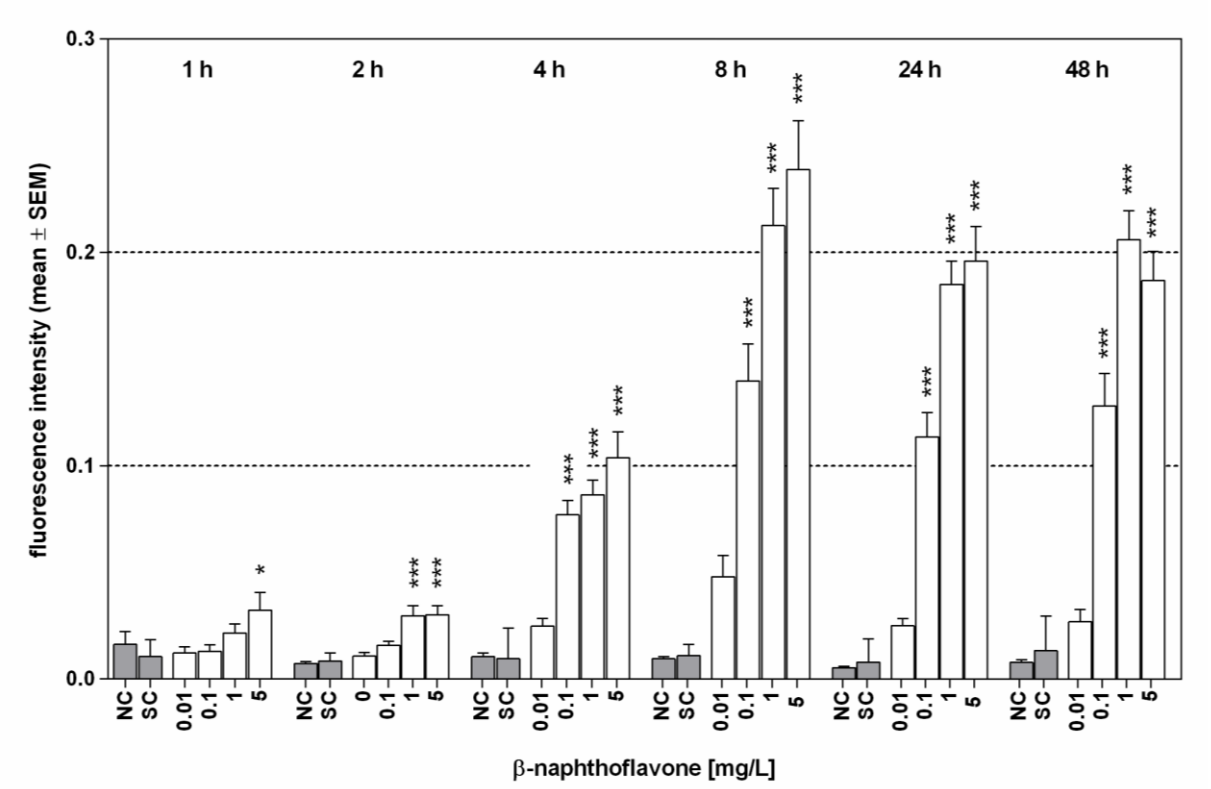


Online Resource 4: Fig. Micropollutant removal during conventional and advanced wastewater treatment at WWTP-4. Concentrations (conc., in [µg/L], y-axes) of micropollutants and transformation products (n = 58) were quantified for samples from April 2015. WWTP stages are WWTP influent (INF-4), WWTP effluent (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-*

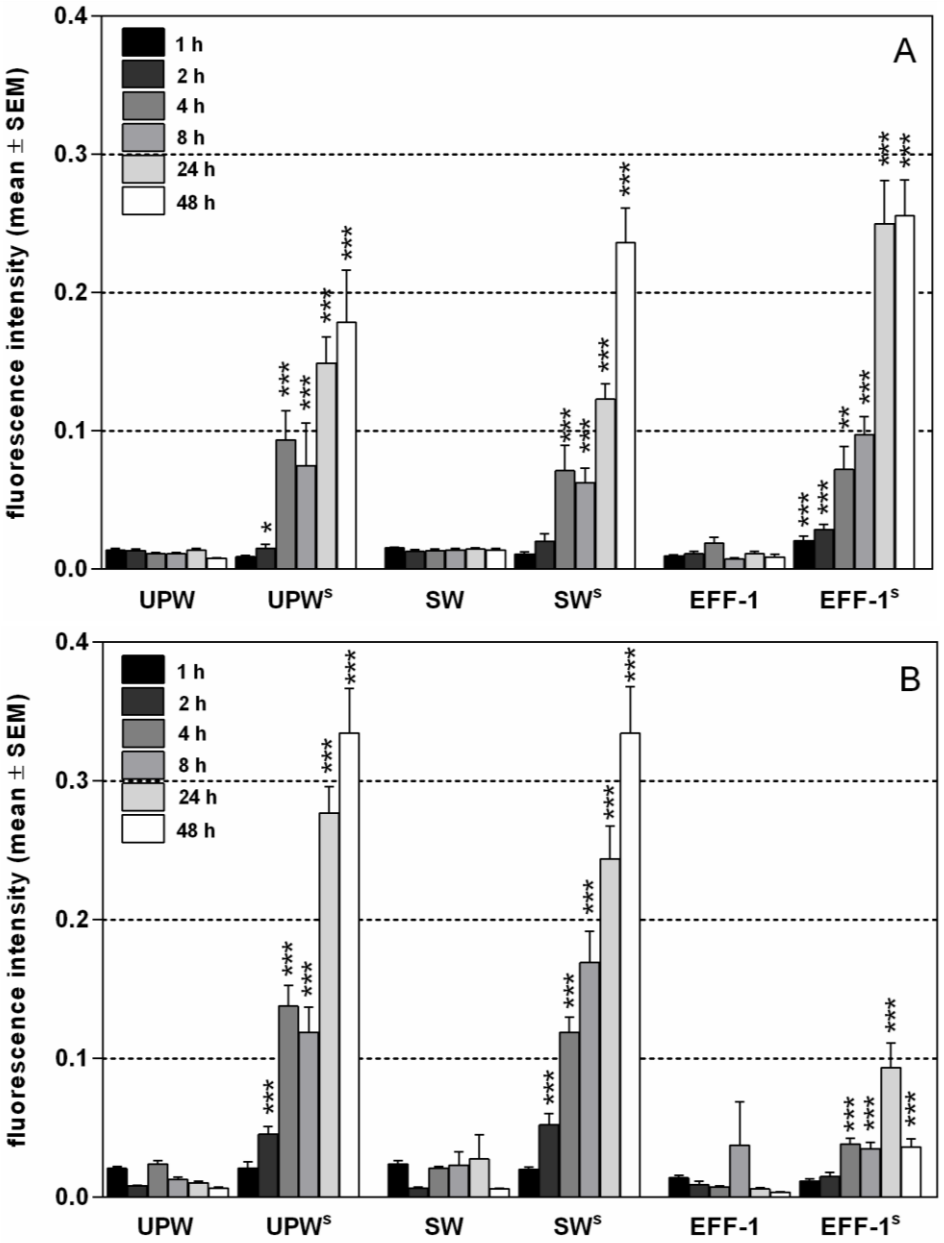
formylaminoantipyrin (13), iopromide (14), iohexol (15), *3-hydroxyibuprofen* (16), diclofenac (17), diatrizoic acid (18), sucralose (19), *1-hydroxyibuprofen* (20), *10,11-dihydro-10,11-dihydroxycarbamazepin* (21), naproxen (22), tolyltriazole (23), acyclovir (24), *1-hydroxybenzotriazol* (25), *carboxyacyclovir* (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)-benzothiazole* (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)



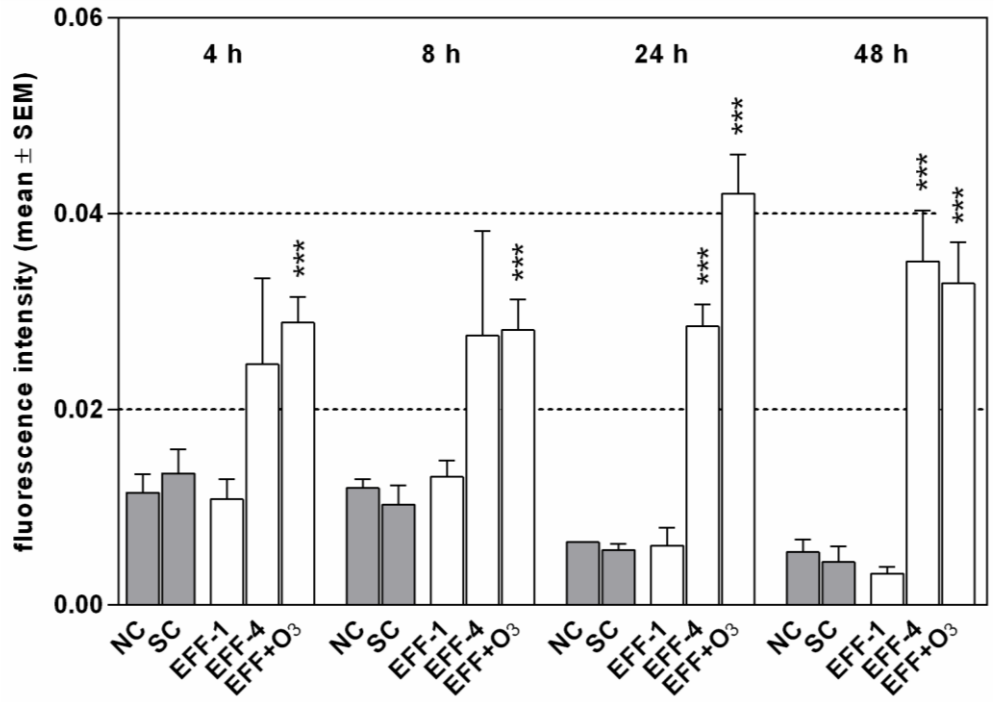
Online Resource 5: Fig. Concentration-response-curve of β-naphthoflavone for the endpoint brood size of *C. elegans*. The mean number of offspring per adult was quantified after 96 h of exposure to β-NF (0.01–5.0 mg/L) commencing at the L1 stage. Significant differences (* = $p < 0.05$, *** = $p < 0.001$) 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 ($y = 45.3 + 46.6 / (1+10^{lg(x)+0.85})$, $R^2 = 0.74$)



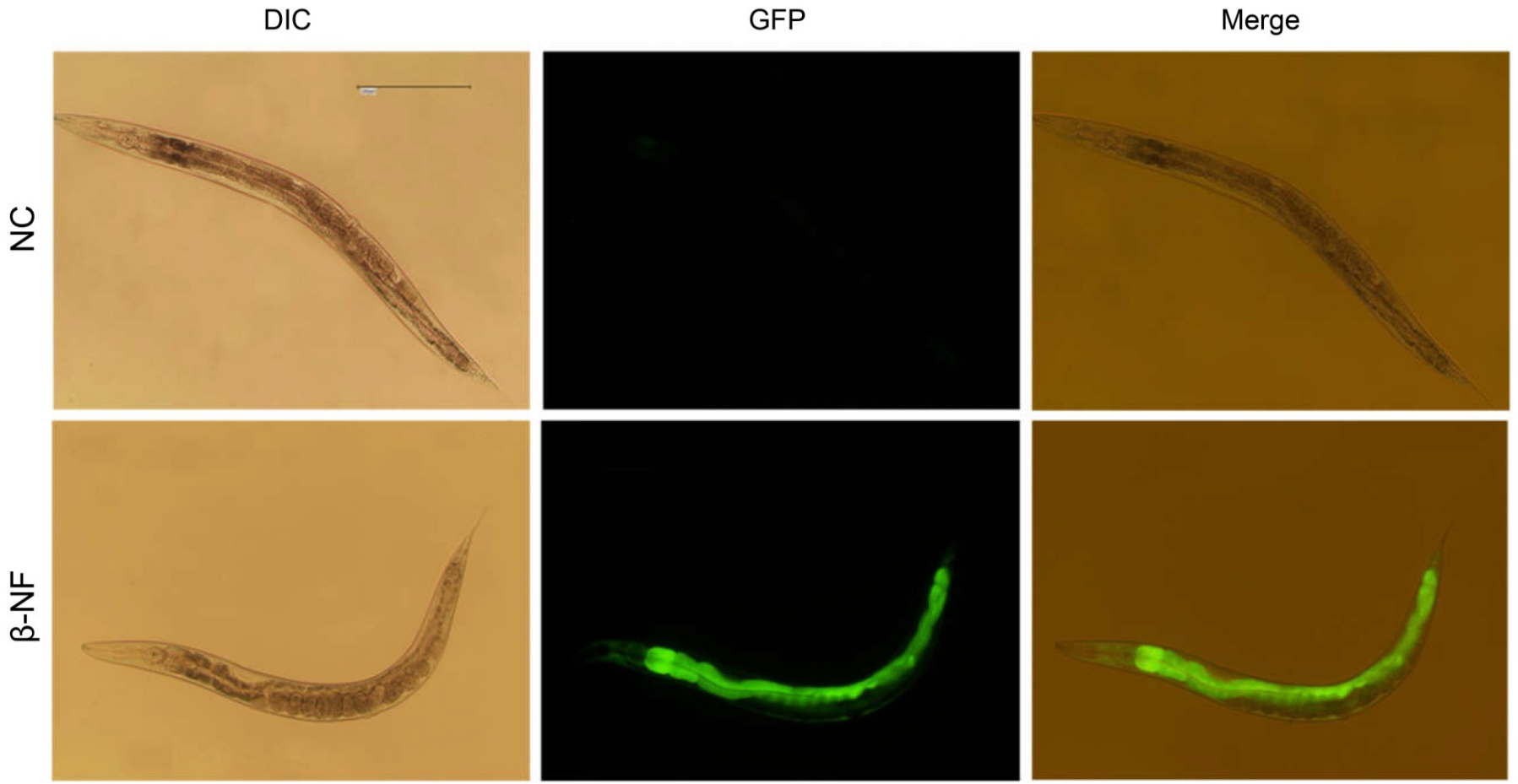
Online Resource 6: Fig. Impacts of β-naphthoflavone on *cyp-35A3*::GFP expression in transgenic *C. elegans.* Samples were analyzed after 1–48 h exposure of adult hermaphrodites to 0.01–5.0 mg/L β-naphthoflavone. Results pooled from two experiments (n = 25 per treatment). Significant differences (* $p < 0.05$, *** $p < 0.001$) 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.4 Impact of an estrogenic sewage treatment plant effluent on life-history traits of the freshwater amphipod *Gammarus pulex*

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2. Performance of experiments and bioassays

Doctoral candidate and first author (IS): 95%
Coauthors (JO, MO): 5%

3. Compilation of data sets and preparation of tables and figures:

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4. Data analyses and interpretation:

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Impact of an estrogenic sewage treatment plant effluent on life-history traits of the freshwater amphipod *Gammarus pulex*

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Despite efforts to upgrade sewage treatment plants (STPs) in the last decades, STPs are still a major source for the contamination of surface waters, including emerging pollutants such as pesticides, pharmaceuticals, personal care products and endocrine disrupting chemicals (EDCs). Because many of these substances are not completely removed in conventional STPs they are regularly detected in surface waters where they have the potential to affect local macroinvertebrate communities. The objective of the current work was to investigate the impact of an estrogenic wastewater effluent on the key life-history traits of the freshwater amphipod *Gammarus pulex*. *G. pulex* was exposed in artificial indoor flow-channels under constant conditions to different wastewater concentrations (0%, 33%, 66%, 100%). In parallel the estrogenic activity of wastewater samples was determined using the yeast estrogen screen (YES). Estrogenic activities in the STP effluent were up to 38.6 ng/L estradiol equivalents (EEQ). Amphipods exhibited an increasing body length with increasing wastewater concentrations. Furthermore, we observed a shift of the sex ratio in favour of females, a significantly increased fraction of brooding females and increased fecundity indices with increasing wastewater concentrations. The increased body length is likely to be attributed to the additional nutrient supply while the occurrence of EDCs in the wastewater is the probable cause for the altered sex ratio and fecundity in exposed *Gammarus* cohorts.

Keywords: Artificial indoor channels, endocrine disruptor, population dynamics, sex ratio, wastewater contaminants, Yeast Estrogen Screen (YES).

Introduction

Biodiversity decreases in freshwater ecosystems worldwide because of human activities despite of efforts to revitalise deteriorated surface water bodies and to upgrade existing sewage treatment plants (STPs).[1,2] Recently, Stalter et al.[2] have shown that state-of-the-art treated wastewater affects the ecological quality of surface waters and causes inter alia a reduced biodiversity in rivers. Although wastewater can provide an additional nutrient supply for heterotrophic organisms (e.g., dissolved organic carbon) and plants (e.g., nitrate, ammonium, phosphate),[3] the incomplete degradation of anthropogenic chemicals in STPs is likely more relevant for most animal species when conventional treatment is used.[4,5] STP effluents contain different organic and inorganic pollutants, including emerging contaminants such as pesticides, flame retardants, pharmaceuticals and personal care products.[6–8] Some of these pollutants are endocrine disruptors that have the ability to interfere with the normal function of the endocrine system, for example by binding to and activating or blocking hormone receptors, particularly receptors for sex hormones.[9,10]

Substances with estrogenic activity that are found in wastewater are for example the natural estrogens 17β-estradiol (E_2) and estrone (E_1) and the synthetic steroid 17α-ethinylestradiol (EE_2).[11,12] Furthermore, estrogenic active chemicals from plastic materials (e.g., phthalates and bisphenol A) and UV-filters used in cosmetics end up in the wastewater and have been reported to cause a feminization of fish and molluscs.[13–15] Accordingly, fish living downstream of STPs are feminized to various degrees ranging from high plasma vitellogenin levels to the development of intersex.[16] Beside estrogen active substances various other endocrine disrupting chemicals (EDCs) may occur in wastewater such as androgenic, anti-estrogenic and anti-androgenic chemicals.[17–24]

In comparison to vertebrates only few studies investigated the influence of EDCs on invertebrates, notably on amphipods [25,26] and molluscs.[13,27–32] Considering their ecology and taxonomy amphipods are a diverse group. Gammarids are omnivorous benthic organisms feeding

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mainly on organic material like fallen leaves with its natural cover of bacteria and fungi,[33] but also on living plants, animals and even cannibalism has been reported.[34]

Gammarus pulex is a widespread amphipod in streams in Central Europe[35,36] and a key species in the food web and for ecosystem function because of its high abundance, as an important food source for predatory fish and its efficiency in shredding coarse particulate organic matter (CPOM).[33–35] Furthermore, *G. pulex* is a sensitive indicator for environmental pollution.[37] Although estrogen or testosterone receptors have not been discovered in arthropods.[38] Schirling[39] found first evidence for a protein in *G. fossarum* with structural similarities to the vertebrate estrogen receptor α (ERα). Thus, EDCs may affect sexual differentiation and reproduction in *G. pulex* with comparable consequences for the population structure as has been shown for fish.[16] Amphipods are dioecious (separate sexed), however, intersex is an abundant phenomenon which has been reported for 20 marine and freshwater species.[40] The factors causing intersex are widely unknown yet,[41] but parasite infestation[42–44] and exposure to metals,[45] organic chemicals[46] and specifically EDCs[25–26] are discussed in the literature.

As benthic organisms gammarids are exposed to various chemicals, including estrogenic compounds, via food and sediments.[9] Peck et al.[11] suggest that riverine sediments are a major sink and a potential source of persistent estrogenic contaminants. Furthermore, the direct uptake of chemicals from the water phase is another important route of exposure.[47] Ashauer et al.[48] studied the bioaccumulation and biotransformation of 15 organic xenobiotics in *G. pulex* and showed that many metabolites reached higher internal concentrations than the initial substance.

The objective of the current work is to investigate the impact of a municipal STP effluent with known estrogenic activity on the population structure of the freshwater amphipod *Gammarus pulex* under controlled laboratory conditions. The estrogen potential of the wastewater was analysed using the yeast estrogen screen (YES) in parallel.

Materials and methods

Test organisms

Gammarus pulex was obtained from the river Schwalm in June 2010. The Schwalm with its length of 97.2 km and a catchment area of 1,299 km^2 origins in the Vogelsberg area northeast of Frankfurt am Main and flows into the river Eder. The sampling site was 3.6 km downstream the source (coordinate: 50°38′09.37″ N, 9°15′05.80″ E) and exhibits a good ecological status.[49] *G. pulex* was kick-sampled using a Surber-sampler.[50] The sampling area of the Surber-sampler was 500 cm^2 (20 × 25 cm) with an attached net (mesh size: 500 μm). Organisms were transported to the laboratory on the same day and identified by the length of the inner ramus of the third uropod, which is only around half as long as the outer ramus in *G. pulex*.[51] Amphipods were kept in a 100-L glass aquarium filled with aerated original stream water at a temperature of 6.5°C (± 0.6°C) and a light/dark period of 12:12 h.

Experimental design

Experiments were performed in four aluminium flow-channel systems (length x width x height: 56.5 × 7.5 × 5.0 cm) with a capacity of 5.3 L (Fig. 1). In each flow-channel six aluminium tubes (length: 13 cm, inner diameter: 6 cm, wall thickness: 3 mm) were placed as replicates, each containing 40 juvenile gammarids with a body length of around 6 mm. The body length of the juveniles was measured under a microscope (Olympus SZ 61) using image processing (DISKUS 4.5, Hilgers, Königswinter, Germany). Furthermore, 1 g (w.w.) alder leaves (*Alnus glutinosa*, collected at the upper region of the Schwalm) was added to each tube as food source as well as a bunch of moss (*Taxiphyllum barbieri*) as a hiding place for amphipods to avoid cannibalism. Each side of a tube was sealed with a mesh sieve (mesh size: 500 μm, SEFAR, Edling). The flow-channels were then filled with different types of water up to a level of 4 cm (resulting in a total water volume of 4 L per channel). The water of each flow-channel (closed circuit) was aerated using a commercial air compressor and a continuous circulation (0.2 m s^{-1}) was ensured by a pump (Pro Flow t600, JBL, Neuhofen, Germany). The experiments were performed in a temperature-controlled room (6.5 ± 0.6°C) with a 12:12-h light: dark regime over a period of 30 days.

One flow-channel served as a control and was filled with ISO-medium.[52] The other channels were filled with 33%, 66% (filled up to 100% with ISO-medium) and 100% effluent of the sewage treatment plant (STP) Grävenwiesbach located at the river Weil (coordinates: 50°23′33.71″ N, 8°23′24.80″ E). The STP comprises three treatment processes (mechanical, biological (nitrification, denitrification) and chemical (phosphor elimination)) and was built

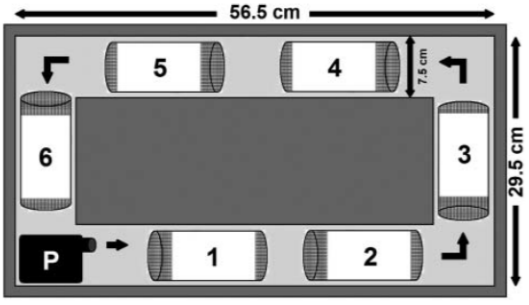

Fig. 1. Flow-channel with six exposure vessels sealed at both ends with mesh sieves. Water circulation was ensured using a pump (P).

for a population equivalent of 25,000 inhabitants.[53] The mean water level of the river Weil was 22.4 cm between 1 February and 31 December 2010 with a minimum of 7.0 cm in July and a maximum of 87 cm in February.[49] The mean flow rate of the river Weil was 0.753 $m^3 s^{-1}$ in the same period with a minimum of 0.015 $m^3 s^{-1}$ in July and a maximum of 12.0 $m^3 s^{-1}$ in February.[49]

Gammarus pulex was exposed up to 100% of wastewater in the experiment because the wastewater content in the river Weil ranged from 19% at medium water levels up to 90% at low water levels.[49] The wastewater was retrieved on June 9, 2010 and kept in darkness at a temperature of 6.5°C (± 0.6°C) overnight. On the next day the estrogenic activity of the wastewater was evaluated using the yeast estrogen screen (YES). Mortality was inspected on days 5, 8, 12, 15, 19, 23, 27 and 30 and dead gammarids were removed from the tubes. At the end of the study all surviving gammarids were preserved in 70% ethanol. To determine the egg number per female, egg carrying individuals were individually preserved in 0.5-mL Eppendorf tubes.

Yeast estrogen screen (YES)

The estrogenic activity of the wastewater was determined by the Yeast Estrogen Screen (YES) as described in Routledge and Sumpter[54] with modifications according to Wagner and Oehlmann.[55] Cell density was measured optically using a photometer (Multiskan Ascent, Thermo Labsystems, Waltham, MA USA) at a wavelength of 595 nm. By giving a buffer solution containing the yellow substrate chlorophenolred-β-D-galactopyranoside (CPRG; Roche Diagnostics, Mannheim, Germany) to the lysed yeast cells, the β-galactosidase would cleave the CPRG into a red pigment. The intensity of the red colour is directly correlated to the estrogenic activity in the sample and is measured with a photometer (Multiskan Ascent, Thermo Labsystems) at a wavelength of 540 nm. Eight concentrations (1 pM to 10 nM) of 17β-estradiol (E_2; CAS 50-28-2, SIGMA) were used as positive control. A nonlinear dose-response curve using a four parametric logistic model was determined and the estrogenic activity of the native samples (dilution factor: 1.6-fold) is provided as estradiol equivalents (EEQ) in ng /L^{-1}.[56] The 96-well microtiter plates (f-form, VWR Darmstadt, Germany) were used and sealed with gas permeable membranes (Breath-easy, Diversified Biotech, Boston, MA, USA) to reduce the risk of cross-contamination via volatile substances.

Life-history traits of Gammarus pulex

The body length of each individual was measured from the basis of the first antenna to the beginning of the third uropod along the curve of the dorsal surface[57–59] under a microscope (Olympus SZ 61) using image processing (DISKUS 4.5, Hilgers, Königswinter, Germany) at the end of the experiment. Adult gammarids with a body length ≥ 6 mm were sexed morphologically and the sex ratio calculated. Female gammarids have four pairs of brood plates (oostegites) on the ventral thorax whereas males have two penial papillae between the fifth pair of walking legs.[60,61] Additionally, the length of the second antennae and the length and width of the propodus of the first gnathopods were measured as sexual dimorphic organs.[62] Furthermore, the proportion of brooding females per replicate and the number of eggs per female were determined. The fecundity of a female was assessed via the fecundity index (ratio of number of eggs and body length) according to Ladewig et al.[10]

Statistical analysis

The statistical analysis was carried out using Excel 2007 (Microsoft Corporation, Redmond, WA, USA) and GraphPad Prism (Version 5.03, GraphPad Software Inc., San Diego, CA, USA). Significant differences for mortality, sex ratio and number of brooding female were analysed using Fisher's exact test. Weighted means according to Taylor[63] were calculated for continuous morphometric parameters such as body length or length and width of organs which were all normally distributed (D'Agostino and Pearson omnibus normality test) and characterised by nonsignificant differences of variances (Bartlett's test for equal variances).

Significant differences between treatments were analysed using one-way analysis of variance (ANOVA) with Dunnett's posttest (differences between control and wastewater treatments) or Bonferroni's post test (differences between males and females in one treatment). The relationship between body length and length of the second antennae and the relationship between wastewater concentration and total offspring were analysed using Pearson correlations. Significant differences of the estrogenic activity of wastewater samples were analysed using the nonparametric Kruskal–Wallis with Dunn's posttest because this data was not normally distributed.

Results

Estrogenic activity

Water samples from the river Schwalm, where *Gammarus pulex* was sampled for the experiment, showed no estrogenic activity in the YES. On the contrary, the effluent of the STP Grävenwiesbach that was used for the experiment regularly showed estrogenic activities ranging from 3.89 to 38.6 ng L^{-1} EEQ (Table 1). Estrogenic activity was also detectable in the river Weil up to 50 m downstream the effluent (Table 1).

At the start of the flow-channel experiment the estrogenic activity of the wastewater was 4.59 (± 0.23) ng L^{-1} EEQ while the ISO-medium used for the control did not

Table 1. Estrogenic activity [ng EEQ/L] (mean ± SEM) in the effluent of the STP Grävenwiesbach and 50 m downstream of the effluent in the river Weil.

Sampling Date	*Effluent*	*Downstream (50 m)*
March 10th	21.1 (± 2.04)	0.79 (± 1.75)
March 24th	38.6 (± 4.01)	2.80 (± 0.81) – 11.3 (± 2.42) (4 samples)
May 10th	3.89 (± 0.41)	2.07 (± 0.55)
June 09th	4.59 (± 0.23)	2.01 (± 0.18)
June 11th	5.47 (± 0.12)	0.76 (± 0.33)
June 28th	9.74 (± 0.91) – 15.5 (± 1.21) (2 samples)	no sampling

EEQ: estradiol equivalent; STP: sewage treatment plant.

show any estrogenic activity. The estrogenic activity in the wastewater treatments (33%, 66% and 100%) decreased constantly. On day eight the estrogenic activity was not detectable using the YES assay in all wastewater treatment groups.

Mortality

Mortality was very low in all treatment groups over the 30 days of the experiment with values between 3.5% (66% wastewater) and 7.1% (control) and no significant differences between treatments (Table 2).

Morphometric parameters

The mean body length increased significantly in the wastewater treatments compared to the control in both sexes (Table 2). Males were 5.48% (33% wastewater), 17.5% (66% wastewater) and 22.4% (100% wastewater) larger than in the control. Also female gammarids were 5.61% (33% wastewater), 15.8% (66% wastewater) and 17.1% (100% wastewater) larger than in the control. The increase of the mean body length was higher in males compared with females, especially in the 100% wastewater treatment. The same result was achieved for the length of the second antennae, which is highly correlated with the body length. Here, the antennae of the males exposed to 100% wastewater were 31.3% longer than the antennae of males in the control. Contrarily, the increase in length of the second antennae of females in 100% wastewater was only 12.5% compared to the control. Data analyses of the length of the propodus of the first gnathopods revealed that male gammarids always had significantly longer gnathopods than females. The results also showed that the length of the propodus of the first gnathopods of male and female gammarids increased with increasing wastewater concentrations. The differences were significant for all treatment groups and both sexes compared to the control (Table 2).

Sex ratio

The sex ratio in the control was 57.3% (± 2.97, $n = 128$) male and 42.7% (± 2.97, n = 95) female gammarids at the end of the experiment (day 30) (Fig. 2A). On the contrary we found significantly more females in every flow-channel

Table 2. Mean mortality (± SEM) [%], percentage of males and females (± SEM) and morphometrical parameters (weighted means (± SEM)) of *Gammarus pulex* reared in ISO-medium (control) and different amounts of wastewater effluent for 30 days.

			Wastewater [%]		
Parameter	*Gender*	*Control*	*33*	*66*	*100*
Mortality [%]		7.08 (± 1.76)	3.75 (± 1.41) n.s.	3.50 (± 1.28) n.s.	5.42 (± 1.19) n.s.
Percentage of individuals (> 6 mm) [%]	♂	57.3 (± 2.97)	38.5 (± 3.03) ***	30.6 (± 2.00) ***	31.1 (± 3.25) ***
	♀	42.7 (± 2.97)	61.5 (± 3.03) ***	69.4 (± 2.00) ***	68.9 (± 3.25) ***
Body length [mm]	♂	7.67 (± 0.071)	8.09 (± 0.100) *	9.01 (± 0.122) ***	9.39 (± 0.113) ***
	♀	7.66 (± 0.068)	8.09 (± 0.055) ***	8.87 (± 0.070) ***	8.97 (± 0.066) ***
2. Antenna [mm]	♂	1.76 (± 0.022)	1.88 (± 0.034) n.s.	2.17 (± 0.050) ***	2.31 (± 0.043) ***
	♀	1.60 (± 0.013)	1.69 (± 0.012) ***	1.82 (± 0.015) ***	1.80 (± 0.015) ***
Propodus of first gnathopod (length) [mm]	♂	0.517 (± 0.007)	0.563 (± 0.011) *	0.662 (± 0.016) ***	0.712 (± 0.013) ***
	♀	0.423 (± 0.003)	0.446 (± 0.003) ***	0.477 (± 0.004) ***	0.485 (± 0.003) ***
Differences in body length between male and female [mm]		0.005 n.s.	0.005 n.s.	0.138 n.s.	0.419 **

Significant differences between control and wastewater treatments are marked with asterisks: * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$; n.s. = not significant (Fisher's exact test; one-way-ANOVA with Dunnett's or Bonferroni's posttest, respectively).

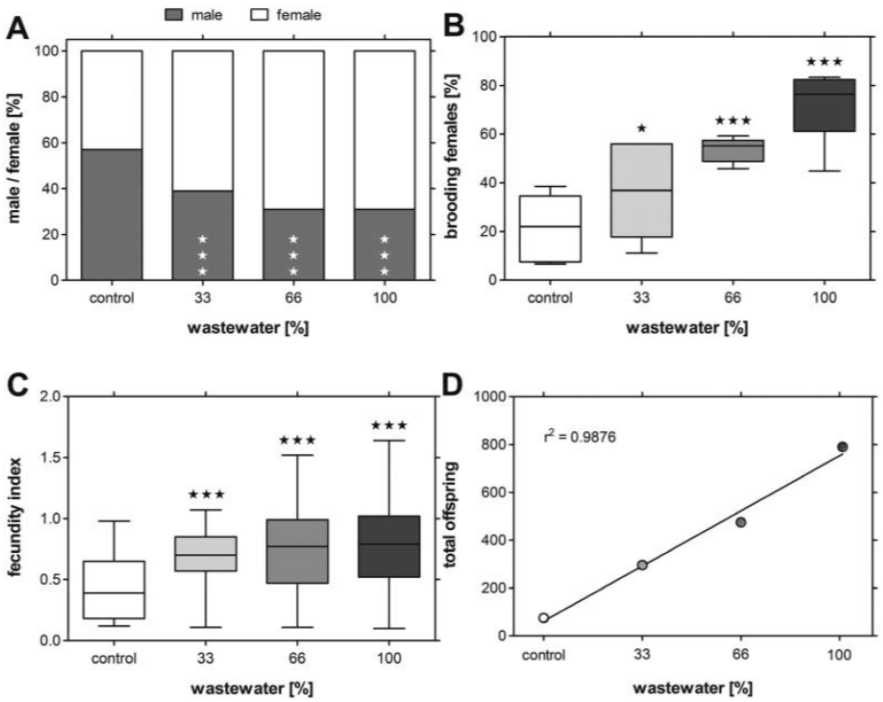

Fig. 2. Effects of wastewater exposure on life-history traits of *Gammarus pulex* on day 30. Six replicate groups with 40 gammarids each were exposed to different wastewater amounts [%] in a circulating flow-channel system. Sex ratio (A), proportion of brooding females (median ± min to max, B), fecundity index of females (median ± min to max, C) and total offspring per flow-channel correlated to the wastewater concentration (D). Significant differences between control (ISO-medium) and wastewater treatments are marked with asterisks: * = $P < 0.05$; *** = $P < 0.001$ (Fisher's exact test in A and B; one-way-ANOVA with Dunnett's post test in C).

containing wastewater compared to the control. The amount of females increased significantly ($P < 0.001$; Fisher's exact test) with increasing amount of wastewater in the flow-channels. 61.5% (± 3.03) females were found in the flow-channel with 33% wastewater, 69.4% (± 2.00) females in the flow-channel with 66% wastewater and 68.9% (± 3.25) females in the flow-channel filled with 100% wastewater.

Reproduction

At the end of the experiment a mean proportion of 21.7% (± 5.40, $n = 21$) brooding females was found in the control (Fig. 2B). With increasing wastewater fractions the share of brooding females increased significantly compared to the control. In the flow-channels with 33% and 66% wastewater mean values of 36.1% (± 7.58, $n = 53$) and 53.5% (± 2.26, $n = 72$) brooding females were found. A mean value of 71.6% (± 5.89, $n = 111$) was reached in the flow-channel with 100% wastewater. Also the mean number of eggs per female found at the end of the study was significantly affected by the exposure to wastewater.

In the control females had a mean of 2.97 (± 0.32) eggs in the brood pouch. In the 33% wastewater treatment this number increased significantly to 5.68 (± 0.24) while in 66% and 100% wastewater the mean egg numbers per female were 6.38 (± 0.36) and 7.04 (± 0.29), respectively. Furthermore, the total number of eggs produced by all females in a flow-channel was highly correlated (Pearson $r = 0.9938$) with the wastewater concentration (Fig. 2D) and increased clearly from 75 eggs in the control to 296 and 475 eggs in 33% and 66% wastewater, respectively, to a maximum of 791 eggs in 100% wastewater. The analysis of the fecundity indices (Fig. 2C) supports the results for the mean number of eggs per female and the total number of eggs per flow-channel. The fecundity index, calculated as the ratio of egg numbers in the brood pouch and body length of a given female, considers that larger females can carry more eggs in their marsupium. Fecundity indices in 33% (0.682 ± 0.026), 66% (0.725 ± 0.037) and 100% wastewater (0.765 ± 0.030) were significantly higher than in the control (0.377 ± 0.037) (Fig. 2C). These results show that female gammarids carried a significantly higher number of eggs in the brood pouch with

increasing wastewater concentrations in comparison to the control.

Discussion

The results of the flow-channel experiment clearly show that exposure to wastewater affects sex ratio, fecundity and a number of sexual dimorphic morphometrical parameters in *Gammarus pulex*, which is likely to alter also the population structure in this species. For the interpretation of the results a key aspect is that wastewater effluents contain additional nutrients, for example dissolved organic carbon (DOC). A higher DOC concentration is likely to facilitate growth of bacteria and fungi providing a better food supply for the gammarids. At the end of the experiment body length and other morphological parameters of both male and female gammarids had increased significantly in the flow-channels with an increased proportion of wastewater. Ladewig et al.[10] studied the population structure of *G. fossarum* upstream and downstream of STP effluents in two German streams. Although in one stream adult gammarids were larger downstream compared to the upstream site it was the other way round for the second stream.

An additional nutrient supply by the wastewater does not automatically result in a better energy budget for gammarids. Bundschuh et al.[64] investigated the impact of STP effluents on leaf breakdown by *G. fossarum* in laboratory experiments over 4 weeks. Wastewater-exposed specimens showed significant reductions in feeding rate (25%), absolute consumption (35%), food assimilation (50%), dry weight (18%) and lipid content (22%). In another study the authors concluded that micropollutants could abolish the effects of an additional nutrient supply by affecting the breakdown rate of leaf material and hence the energy budget for gammarids.[65] Further investigations on the feeding rates of *G. fossarum* under laboratory and field conditions showed similar results.[66,67]

The additional nutrient supply via wastewater cannot explain the significant shift of the sex ratio in favour of females and the altered reproduction. Although the increase of the body length in both sexes was similar in the flow-channel with 33% wastewater compared to the control, the body length of the females increased less than the body length of male gammarids in treatments with higher wastewater content. This indicates that the increasing wastewater content in the flow-channels with 66% and 100% wastewater urged females to allocate their energy more into reproduction instead of somatic growth.

Furthermore, sexual dimorphic parameters such as length of the second antennae, length and width of the propodus of the first gnathopods[68,69] attained significantly higher values in males compared to females in the control and in the flow-channels with 33% and 66% wastewater while there was no significant difference between both sexes regarding body length. However, in the flow-channel with 100% wastewater male gammarids were significantly larger than females. This difference in body length is an important fact concerning the reproductive success of the mate guarding amphipod *G. pulex*.[62] Only large male individuals are able to carry smaller females on their ventral side with the help of their first gnathopods during the precopula stage.[69–71]

The effluent of the STP Grävenwiesbach used in the experiments exhibits a comparatively high estrogenic activity using the YES assay. In addition water samples from the river Weil taken 50 m downstream of the STP effluent showed a relatively high estrogenic activity ranging from 2.01 to 15.5 ng L^{-1} EEQ.[49] Adler et al.[72] analysed natural and synthetic estrogen steroids in German streams and quantified 0.3 ng L^{-1} 17α-ethinylestradiol (EE_2), 0.2 ng L^{-1} 17β-estradiol (E_2) and 2.5 ng L^{-1} estrone (E_1). Water samples of the Körsch near Stuttgart (Germany) contained up to 1.7 ng/L EE_2 downstream a discharger and up to 5.0 ng L^{-1} EE_2 in the STP effluent itself.[41] Concentrations of typical estrogenic substances found in effluents of STPs are for example 7.7 ng L^{-1} E_2, 5.0 ng/L EE_2, 35.3 ng L^{-1} 4-n-nonylphenol and 33.9 ng L^{-1} 4-tert-octylphenol, 1445 ng L^{-1} bisphenol A (BPA) and 2475 ng L^{-1} dibutylphthalate.[41]

In our study the results of YES conducted daily during the first eight days of the flow-channel experiment showed that the estrogenic activity was already halved after the first day of the experiment. The following days the activities decrease continuously so that the gammarids were probably no longer exposed to estrogen active substances close to the end of the experiment. However, the results concerning the estrogenic activity of this flow-channel experiment are not transferable to the situation in the field with a continuous discharge of wastewater effluent into the river exists.

In spite of the fast reduction of its estrogenic activity, the wastewater used in our experiment had considerable effects on sex ratio and reproduction of *G. pulex*. The sex ratio of the gammarid population from the river Schwalm, which provided the test organisms for the experiment, was balanced with a ratio of male to female of 1.01 : 1 (n = 274).[49] Hynes[60] described a sex ratio for *G. pulex* in springtime that was slightly shifted to females (male: female = 0.8: 1), the sex ratio was balanced in summer before it shifted in favour of male gammarids in autumn (3 : 1). Welton[73] reported a slightly fluctuating sex ratio for *G. pulex* throughout the year that was almost balanced as well. The results of the present study show that the sex ratio in the control was slightly shifted towards male gammarids (1.34 : 1) but in all flow-channels with wastewater the sex ratio was significantly shifted in favour of females compared to the control after four weeks of exposure (0.44 : 1 to 0.63 : 1) (Fig. 2A).

Although this significant shift is correlated with the wastewater concentration and thus the estrogenic activity

of ambient water in the flow-channels, our experiment does not provide causal evidence that (xeno-)estrogens are responsible for the altered sex ratio. Other wastewater ingredients may have also contributed to the observed "feminization" of the exposed gammarid cohorts. On the other hand a contribution of EDCs to the observed effect cannot be ruled out. The increase of the mean proportion of brooding females with increasing wastewater exposure could also be linked to the estrogenic activity. The fecundity indices (Fig. 2C) underpin this assumption. Female gammarids with equal body lengths carried a significantly higher number of eggs in their brood pouches in all wastewater treatments compared to the control.

Investigations of the population structure of *G. pulex* at the river Schwalm reference site where gammarids were sampled for the experiment illustrate that this significant increase of the fecundity indices is not a natural phenomenon. Although in March we found only 11% of brooding females this proportion increased up to 78% in July.[49] However, the fecundity indices showed no significant differences at the reference site with values of 1.06 (± 0.33) in March and 1.19 (± 0.40) in July.[49] Therefore, the increased fecundity indices in the wastewater-exposed groups of our experiment indicate an impact of the tested wastewater with its estrogenic activity on the reproduction of *G. pulex*. The hypothesis that estrogenic chemicals alter the sex ratio towards females and enhance reproduction in *Gammarus* is also supported by the study of Watts et al.[68] The authors exposed individuals of *G. pulex* to three concentrations of EE_2 (100 ng L^{-1}, 1 μg L^{-1} and 10 μg L^{-1}) in a flow-through system for 100 days.

Although the sex ratio was balanced in the control, it shifted in EE_2 exposed cohorts significantly in favour of females. The population size in the two groups exposed to the highest EE_2 concentrations was significantly increased compared to the control. Juveniles represented the major part of the population.[68] Regarding the low temperature of 6.5°C (± 0.6°C) in the present study, the time of exposure was too short for the development of juveniles and to hatch from the eggs. However, the continuously increasing total number of eggs in the brood pouch of females (75 in the control, 791 in 100% wastewater) (Fig. 2D) suggest a strongly increasing population size although an increased fecundity index does not automatically indicate an increased fertility. Taken together the results from the study by Watts et al.[68] and our experiment show, that EE_2 and an estrogenic STP effluent have an impact on the population structure of *G. pulex*.

Indeed the specific effects of EDCs on the hormone system of amphipods are yet insufficiently characterised and further investigations are needed. Schirling[39] studied the inducibility of a structurally similar protein to the estrogen receptor α in *G. fossarum* following exposure to 10 μg L^{-1} EE_2 for 5 days. In adolescent females the level of this protein rose to the level in adult females. There were no effects on adult males or females. Gross et al.[9] investigated the impact of estrogenic wastewater on sexual development of *G. pulex* downstream of two STPs. The results showed that the estrogenic effluents had no effects on the gonadal structure of male gammarids but the number of females with abnormal oocyte development increased significantly. Sexual development and reproduction in amphipods are not regulated by the steroid hormones estrogen and testosterone but mainly by the peptide hormones that stimulate vitellogenesis.[9] Ecdysone is required for full vitellogenesis as well. Gross et al.[9] attributed the oocyte alterations to an interaction between the xenobiotics and the ecdysone receptor leading to a disruption of vitellogenesis.

A field study in the river Körsch in Germany also showed that estrogenic wastewater had, amongst others, an influence on the maturation and the size of oocytes in the amphipod *G. fossarum*.[74] However, Schirling et al.[74] concluded that these effects could not be attributed exclusively to estrogenic substances because the wastewater contained also many other pollutants, a fact that is clearly also relevant when interpreting the results in our study. Brown et al.[75] investigated the effects of nonylphenol on juvenile individuals of the marine species *Corophium volutator*. A concentration of 10 μg L^{-1} caused an increased mortality and a delayed growth already. There was no influence on the sex ratio, however, the fertility of the females was increased.

Furthermore, there are not only substances with estrogenic activity in the wastewater but also with androgenic, anti-estrogenic and anti-androgenic activity.[23] These substances could interact with each other to weaken or intensify their effects, and it is not known yet how these substances may affect gammarids.

Special attention deserves the increasing reproduction rate in STP effluent-exposed *G. pulex* in the experiments. An increased reproduction does not automatically imply a positive effect for the population or the ecosystem. Female gammarids pass through a rest period of two to three weeks after each reproduction cycle and reproduction is completely stopped in winter.[60] If chemicals in the wastewater suppress the rest periods and entail the females to carry more eggs in their brood pouch than it would correspond to their body length, this may compromise the fitness of the exposed females and of their progeny in a way that they are more susceptible for other environmental stressors. But even if the fitness of the effluent-exposed gammarids is not affected, an increased reproduction rate will have negative impacts on the balance of the ecosystem. Organisms with comparable food resources could be displaced by gammarids. Fish populations could increase simultaneously in the streams because gammarids are an important food source of predatory fish.

Further investigations are needed to analyse the impact of EDCs in STP effluents on the population structure and the reproduction system of gammarids and related amphipods more precisely.

Conclusion

Gammarids are sensitive biological indicators for the assessment of water quality in surface waters. Municipal wastewater contains plenty of chemical pollutants that are not fully degraded in sewage treatment plants (STPs). These chemicals are discharged into surface waters and it is difficult to predict or to conclude the impacts of these single substances and their mixtures on the environment and macroinvertebrate communities.

Flow-channel experiments showed that an estrogenic STP effluent affected the population structure of *Gammarus pulex* but in the end underlying causes for the observed effects could not be clarified. On the one hand the body length of gammarids increased significantly with increasing amount of wastewater possibly due to an additional nutrient supply. On the other hand sex ratios were shifted in favour of female gammarids, the number of brooding females, the fecundity index and the total offspring number increased with increasing wastewater exposure, probably due to effects of wastewater-borne contaminants on the hormone system of *G. pulex*.

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Nomenclature

ANOVA =	analysis of variance
BPA =	bisphenol A
CAS =	Chemical Abstracts Service
CPOM =	coarse particulate organic matter
CPRG =	chlorophenolred-β-D-galactopyranoside
DOC =	dissolved organic carbon
E_1 =	estrone
E_2 =	17 β-estradiol
EDC =	endocrine disrupting chemical
EEQ =	estradiol equivalent
EE_2 =	17α-ethinylestradiol
ERα =	estrogen receptor α
HLUG =	Hessian Agency for the Environment and Geology (Hessisches Landesamt für Umwelt und Geologie)
ISO =	International Organisation for Standardisation
n =	number
OECD =	Organisation for Economic Co-operation and Development
P =	pump
p =	probability
SEM =	standard error of the mean
STP =	sewage treatment plant
UV =	ultraviolet
YES =	yeast estrogen screen
w.w. =	wet weight

A.5 Supplementary information

S1 *In vivo* on-site experiment with *Gammarus fossarum*

S1.1 Material and methods

G. fossarum was collected in the stream Nidder in Hesse, Germany (50°28'56.4" N, 9°14'54.6" E) using a Surber sampler and kick-sampling (Schneider et al. 2015, Annex A.4). Twenty-five juvenile amphipods with a body length of 6 mm ± 1 mm were carefully placed into each of the 44 exposure vessels that were filled with 600 mL stream water of the Nidder up to the passive overflows of the exposure vessels and transported to the pilot WWTP. The endpoints body length, sex-ratio, egg-carrying females, fecundity index 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. Undiluted wastewater from points representing nine different treatment stages and degrees were tested (four replicates per treatment): after conventional biological treatment (BT), after ozone system 1 (BT+O_3), after non-aerated GAC filtration, after aerated GAC filtration (GAC_a), after non-aerated biofilter (BF), after aerated BF (BF_a), after MBR1, after ozone system 2 (MBR1+O_3) and after MBR2 (Figure 1). Like in other studies (Giebner et al. 2018, Smital et al. 2011) the primary treatment (PT) was not investigated because of high mortality upon exposure to raw wastewater. In addition, a negative control group (NC) with culture medium (OECD 2016) and a positive control group (PC) consisting of culture medium spiked with 50 ng/L EE_2 ran in parallel to the wastewater treatments in a flow-through system. The undiluted wastewater was constantly pumped by peristaltic pumps (Otto Huber, Böttingen, Germany) through polytetrafluoroethylene (PTFE) tubes from the nine treatment stages to 10 L high-grade stainless-steel reservoirs that allowed residual ozone to gas out. Smaller

peristaltic pumps (IPC 24, Ismatec, Wertheim-Mondfeld, Germany) pumped the wastewater from these reservoirs constantly through PTFE tubes into the exposure vessels containing the test organisms. Thus, the stream water of the Nidder was steadily replaced by the NC, the PC and the wastewater, respectively. Each exposure vessel (1 L) had a 6-fold volume water exchange rate per day and was filled with 600 mL medium or wastewater. In random order the exposure vessels were placed in a tank that was filled with water nearly up to the passive overflows of the exposure vessels. Water temperature was adjusted to 10°C using an external cooling unit (Julabo, Seelbach, Germany). Regularly fresh culture medium of the C and PC was prepared. All exposure vessels were aerated with ambient air filtered with a 0.2 µm laboratory injection filter. Once in a week the amphipods were fed with stamped circles (1.7 cm in diameter) of leaves of alder (*Alnus glutinosa*) collected at the Nidder. Furthermore, a piece (5 x 10 cm) of a black anti-static grid-glass PTFE-mesh (mesh size: 2 x 2 mm) (PTFE-Spezialvertrieb GmbH, 28816 Stuhr) was added as a hiding place to each exposure vessel to avoid cannibalism.

The amphipods were frozen in liquid nitrogen after 30 days of exposure and a light:dark regime of 16:8 h and stored at –80°C until analyses. Egg-carrying individuals were individually preserved in 0.5 mL Eppendorf tubes to determine the egg number per female.

For the analyses, the amphipods were defrosted and body length, sex ratio, proportion of brooding females, number of eggs per female and the fecundity index was determined as described in Schneider et al. (2015, Annex A.4).

Furthermore, protein, glycogen and lipid content of not egg-carrying females were determined as biomarkers for energy reserves. A detailed procedure is given in Schneider et al. (2020, Annex A.2).

In addition, aqueous 24 h composite samples and 5000-fold enriched SPE-samples of the nine different wastewaters were tested in *in vitro* bioassays for endocrine activity and mutagenicity. A detailed description of the SPE enrichment and the *in vitro* testing is specified in Schneider et al. (2020, Annex A.2).

Besides, the wastewater samples were analysed chemically for 28 selected MPs as tracer substances once in a week (four times) during the 30-days on-site experiment using 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. Detailed information of the selection criteria of the 28 MPs and the performance of the chemical analyses is given in Schneider et al. (2020, Annex A.2) and Seitz and Winzenbacher (2017).

S1.2 Results

S1.2.1 Mortality, growth, and sex ratio

Table S1: Mortality in % (mean ± SEM), body length of male and female individuals in mm (mean ± SD), and sex ratio in % of *Gammarus fossarum* after 30 days of on-site 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 mortality and sex ratio compared to the negative control (∆NC) or the conventional biological treatment (∆BT) is given and additional in % for the body length. Significant differences compared to ∆NC and ∆BT are marked with asterisks: ★ $p \leq 0.05$, ★★ $p \leq 0.01$, ★★★ $p \leq 0.001$ (Fisher's exact test (mortality and sex ratio) or Kruskal-Wallis with Dunn's post-test (body length)), n.s.: not significant.

treatment	mortality [%]		body length [mm]		body length [mm]		sex ratio	
		∆	male	∆ [%]	female	∆ [%]	male : female	∆
NC	18.0 ± 6.83	-	7.45 ± 1.46	-	7.54 ± 1.18	-	40.2 : 59.8	-
	(n = 100)		(n = 33)		(n = 49)		(n = 82)	
PC	11.0 ± 5.51	∆NC	7.69 ± 1.18	∆NC +3.17	7.69 ± 1.18	∆NC +1.99	47.8 : 52.2	∆NC
	(n = 100)	(n.s.)	(n = 43)	(n.s.)	(n = 47)	(n.s.)	(n = 90)	(n.s.)
BT	47.0 ± 22.3	∆NC	7.16 ± 1.04	∆NC –3.91	7.21 ± 0.93	∆NC –4.40	42.2 : 57.8	∆NC
	(n = 100)	(★★★)	(n = 19)	(n.s)	(n = 26)	(n.s.)	(n = 45)	(n.s.)

Table S1: (continued)

treatment	mortality [%]		body length [mm]		body length [mm]		sex ratio	
		Δ	male	Δ [%]	female	Δ [%]	male : female	Δ
BT+O_3	22.0 ± 10.1	ΔBT	9.73 ± 1.07	ΔBT +35.9	9.13 ± 1.24	ΔBT +26.7	56.9 : 43.1	ΔBT
	(n = 100)	(★★★)	(n = 37)	(★★★)	(n = 28)	(★★★)	(n = 65)	(n.s.)
GAC	19.0 ± 3.00	ΔBT	6.86 ± 1.16	ΔBT –4.23	7.00 ± 1.20	ΔBT –2.84	41.8 : 58.2	ΔBT
	(n = 100)	(★★★)	(n = 33)	(n.s.)	(n = 46)	(n.s.)	(n = 79)	(n.s.)
GAC_a	24.0 ± 2.83	ΔBT	8.41 ± 1.47	ΔBT +17.5	7.47 ± 0.98	ΔBT +3.66	52.6 : 47.4	ΔBT
	(n = 100)	(★★)	(n = 40)	(★)	(n = 36)	(n.s.)	(n = 76)	(n.s.)
BF	19.0 ± 4.12	ΔBT	7.63 ± 1.08	ΔBT +6.62	7.57 ± 0.97	ΔBT +5.01	49.4 : 50.6	ΔBT
	(n = 100)	(★★★)	(n = 40)	(n.s.)	(n = 41)	(n.s.)	(n = 81)	(n.s.)
BF_a	16.0 ± 2.83	ΔBT	7.82 ± 1.20	ΔBT +9.22	7.46 ± 1.14	ΔBT +3.52	48.2 : 51.8	ΔBT
	(n = 100)	(★★★)	(n = 40)	(n.s.)	(n = 43)	(n.s.)	(n = 83)	(n.s.)
MBR1	21.0 ± 4.73	ΔBT	7.61 ± 0.80	ΔBT +6.34	7.42 ± 0.68	ΔBT +2.97	54.4 : 45.6	ΔBT
	(n = 100)	(★★★)	(n = 43)	(n.s.)	(n = 36)	(n.s.)	(n = 79)	(n.s.)
MBR1+O_3	21.0 ± 4.44	ΔBT	7.16 ± 1.03	ΔBT +0.03	7.24 ± 0.82	ΔBT +0.49	53.2 : 46.8	ΔBT
	(n = 100)	(★★★)	(n = 42)	(n.s.)	(n = 37)	(n.s.)	(n = 79)	(n.s.)
MBR2	19.0 ± 1.92	ΔBT	8.32 ± 1.16	ΔBT +16.2	7.43 ± 1.11	ΔBT +3.08	56.8 : 43.2	ΔBT
	(n = 100)	(★★★)	(n = 46)	(★★)	(n = 35)	(n.s.)	(n = 81)	(n.s.)

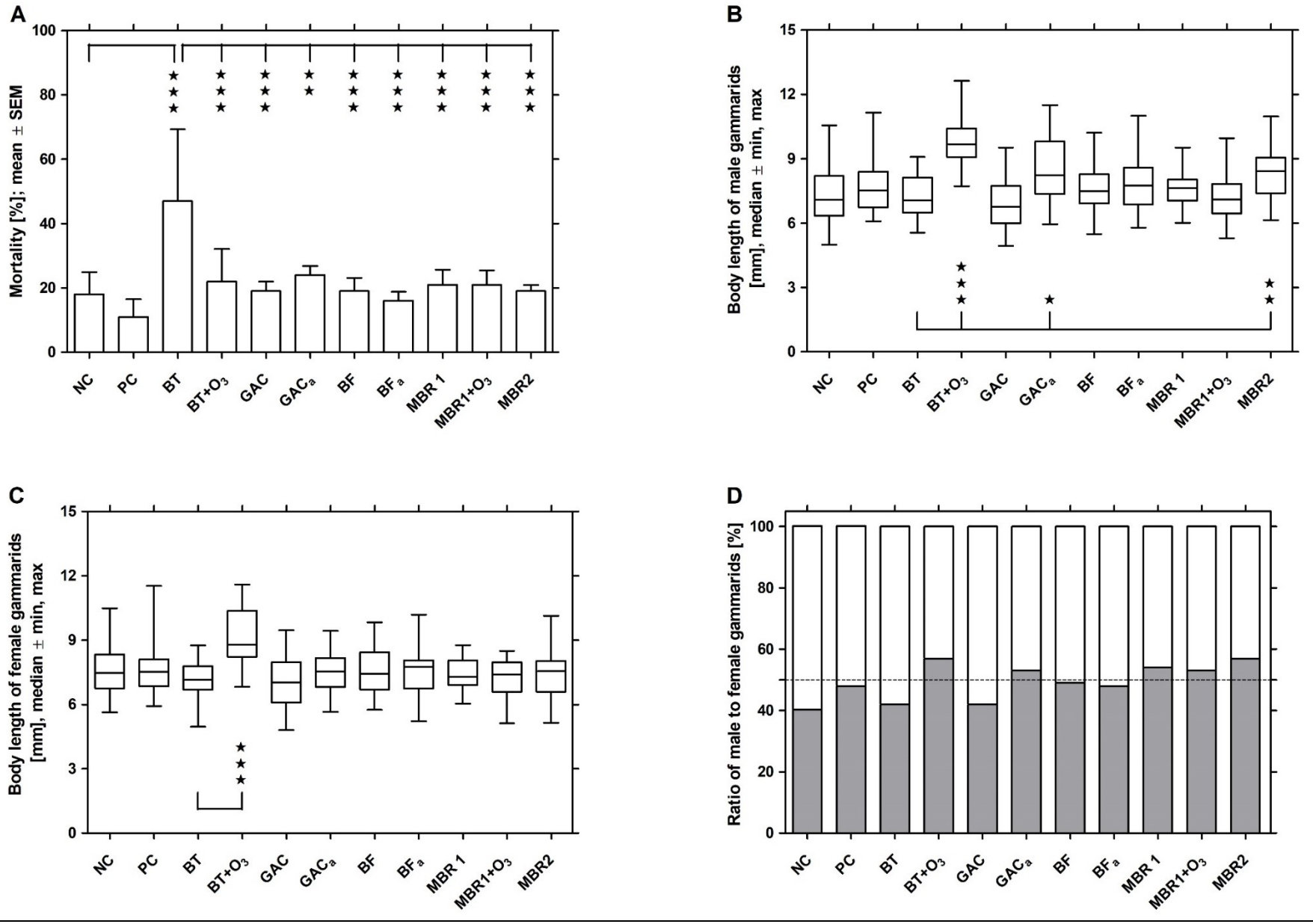
A
Mortality [%]; mean ± SEM
0
20
40
60
80
100
NC
PC
BT
BT+O3
GAC
GACa
BF
BFa
MBR 1
MBR1+O3
MBR2
B
Body length of male gammarids [mm], median ± min, max
0
3
6
9
12
15
C
Body length of female gammarids [mm], median ± min, max
D
Ratio of male to female gammarids [%]
0
20
40
60
80
100

Figure S1: Mortality (A), body length of male (B) and female (C) amphipods, and sex ratio of male (grey) to female (white) individuals of *Gammarus fossarum* after 30 days of on-site exposure to 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 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 NC/BT are indicated with asterisks: ★ $p < 0.05$, ★★ $p < 0.01$, ★★★ $p < 0.001$ (A: Fisher's exact test; B, C: Kruskal-Wallis with Dunn's post-test). n = 100 (A), n = 19–46 (B), n = 26–49 (C), n = 45–90 (D).

S1.2.2 Ratio of brooding and non-brooding females, fecundity index, egg number per female and total egg number

Table S2: Ratio of brooding and non-brooding females in %, fecundity index (mean ± SD), egg number per female (mean ± SD), and total egg number of *Gammarus fossarum* after 30 days of on-site 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 ratio of brooding and non-brooding females compared to the negative control (∆NC) or the conventional biological treatment (∆BT) is given and additional in % for the fecundity index and the egg number per female. Significant differences compared to ∆NC and ∆BT are marked with asterisks: ★ $p \leq 0.05$, ★★ $p \leq 0.01$, ★★★ $p \leq 0.001$ (Fisher's exact test (ratio of brooding and non-brooding females) or Kruskal-Wallis with Dunn's post-test (fecundity index and egg number per female), n.s.: not significant.

treatment	ratio brooding : non-brooding	∆	fecundity index	∆ [%]	egg number per female	∆ [%]	total egg number	∆ [%]
NC	30.6 : 69.4 (n = 49)	-	0.65 ± 0.32 (n = 15)	-	5.67 ± 3.13 (n = 15)	-	85	-
PC	27.7 : 72.3 (n = 47)	∆NC (n.s.)	0.87 ± 0.24 (n = 13)	∆NC +35.3 (n.s.)	7.92 ± 2.57 (n = 13)	∆NC +39.8 (n.s.)	103	∆NC +21.2
BT	7.70 : 92.3 (n = 26)	∆NC (★)	0.63 ± 0.08 (n = 2)	∆NC –2.49 (n.s)	5.50 ± 0.71 (n = 2)	∆NC –2.95 (n.s.)	11	∆NC –87.1

Table S2: (continued)

treatment	ratio brooding : non-brooding	Δ	fecundity index	Δ [%]	egg number per female	Δ [%]	total egg number	Δ [%]
BT+O_3	57.1 : 42.9 (n = 28)	ΔBT (★★★)	0.87 ± 0.28 (n = 16)	ΔBT +38.7 (n.s.)	8.31 ± 3.07 (n = 16)	ΔBT +51.1 (n.s.)	133	ΔBT +1109
GAC	10.9 : 89.1 (n = 46)	ΔBT (n.s.)	0.67 ± 0.36 (n = 5)	ΔBT +6.69 (n.s.)	5.80 ± 3.11 (n = 5)	ΔBT +5.45 (n.s.)	29	ΔBT +164
GAC_a	22.2 : 77.8 (n = 36)	ΔBT (n.s.)	0.52 ± 0.29 (n = 8)	ΔBT –17.1 (n.s.)	4.38 ± 2.50 (n = 8)	ΔBT –20.5 (n.s.)	35	ΔBT +218
BF	29.3 : 70.7 (n = 41)	ΔBT (n.s.)	0.71 ± 0.34 (n = 12)	ΔBT +12.9 (n.s.)	6.08 ± 3.06 (n = 12)	ΔBT +10.6 (n.s.)	73	ΔBT +564
BF_a	18.6 : 81.4 (n = 43)	ΔBT (n.s.)	0.71 ± 0.26 (n = 8)	ΔBT +12.2 (n.s.)	5.75 ± 1.98 (n = 8)	ΔBT +4.55 (n.s.)	46	ΔBT +318
MBR1	11.1 : 88.9 (n = 36)	ΔBT (n.s.)	0.99 ± 0.19 (n = 4)	ΔBT +57.2 (n.s.)	8.25 ± 1.89 (n = 4)	ΔBT +50.0 (n.s.)	33	ΔBT +200
MBR1+O_3	10.8 : 89.2 (n = 37)	ΔBT (n.s.)	0.75 ± 0.51 (n = 4)	ΔBT +19.0 (n.s.)	6.00 ± 4.08 (n = 4)	ΔBT +9.09 (n.s.)	24	ΔBT +118
MBR2	17.1 : 82.9 (n = 35)	ΔBT (n.s.)	1.08 ± 0.20 (n = 6)	ΔBT +71.2 (n.s.)	9.67 ± 2.66 (n = 6)	ΔBT +75.8 (n.s.)	58	ΔBT +427

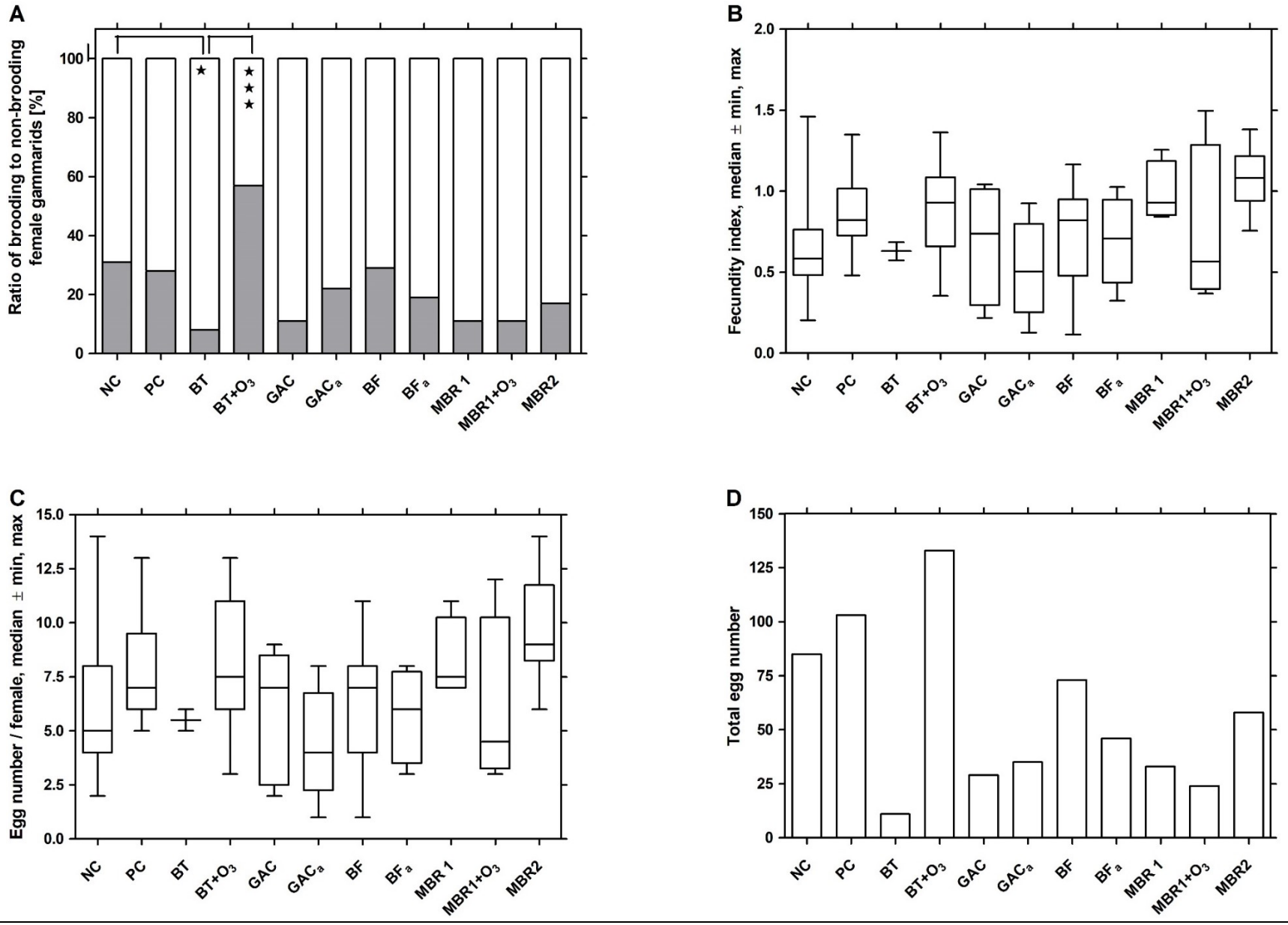
A
Ratio of brooding to non-brooding female gammarids [%]
0
20
40
60
80
100
NC
PC
BT
BT+O3
GAC
GACa
BF
BFa
MBR 1
MBR1+O3
MBR2
B
Fecundity index, median ± min, max
0.0
0.5
1.0
1.5
2.0
C
Egg number / female, median ± min, max
0.0
2.5
5.0
7.5
10.0
12.5
15.0
D
Total egg number
0
25
50
75
100
125
150

Figure S2: Ratio of brooding (grey) to non-brooding (white) female amphipods (A), fecundity index (B), egg number per female (C), and total egg number (D) of individuals of *Gammarus fossarum* after 30 days of on-site exposure to 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 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 NC/BT are indicated with asterisks: ★ $p < 0.05$, ★★★ $p < 0.001$ (Fisher's exact test). n = 26–49 (A), n = 2–16 (B, C).

S1.2.3 Biomarkers for energy reserves (glycogen, protein and lipid content)

Table S3: Energy content as protein, glycogen, and lipid, and total energy in J/mg tissue (mean ± SD) of *Gammarus fossarum* after 30 days of on-site 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, lipid and total energy 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: ★ $p \leq 0.05$, ★★ $p \leq 0.01$, ★★★ $p \leq 0.001$ (Kruskal-Wallis with Dunn's post-test), n.s.: not significant.

treatment	protein [J/mg]	Δ [%]	glycogen [J/mg]	Δ [%]	lipid [J/mg]	Δ [%]	total energy [J/mg]	Δ [%]
NC	0.21 ± 0.06 (n = 20)	-	0.003 ± 0.005 (n = 20)	-	0.47 ± 0.18 (n = 20)	-	0.68 ± 0.16 (n = 20)	-
PC	0.20 ± 0.05 (n = 20)	ΔNC –6.48 (n.s.)	0.002 ± 0.004 (n = 18)	ΔNC –44.4 (n.s.)	0.42 ± 0.15 (n = 19)	ΔNC –10.7 (n.s.)	0.61 ± 0.12 (n = 17)	ΔNC –11.1 (n.s.)
BT	0.13 ± 0.04 (n = 20)	ΔNC –38.3 (★)	0.22 ± 0.12 (n = 20)	ΔNC +6465 (★★★)	3.60 ± 1.24 (n = 19)	ΔNC +663 (★★★)	3.94 ± 1.24 (n = 19)	ΔNC +475 (★★★)
BT+O_3	0.18 ± 0.04 (n = 20)	ΔBT +40.0 (n.s.)	0.11 ± 0.04 (n = 18)	ΔBT –52.8 (n.s.)	1.90 ± 1.14 (n = 18)	ΔBT –47.3 (n.s.)	2.18 ± 1.16 (n = 18)	ΔBT –44.7 (n.s.)
GAC	0.30 ± 0.07 (n = 20)	ΔBT +129 (★★★)	0.01 ± 0.01 (n = 20)	ΔBT –93.6 (★★★)	1.14 ± 0.54 (n = 20)	ΔBT –68.2 (★★★)	1.46 ± 0.56 (n = 20)	ΔBT –63.1 (★★★)

Table S3: (continued)

treatment	protein [J/mg]	Δ [%]	glycogen [J/mg]	Δ [%]	lipid [J/mg]	Δ [%]	total energy [J/mg]	Δ [%]
GAC_a	0.32 ± 0.09	ΔBT +148	0.03 ± 0.01	ΔBT –85.8	1.38 ± 0.80	ΔBT –61.6	1.73 ± 0.83	ΔBT –56.0
	(n = 20)	(★★★)	(n = 20)	(★★★)	(n = 20)	(★★)	(n = 20)	(★★)
BF	0.26 ± 0.08	ΔBT +100	0.04 ± 0.02	ΔBT –84.2	1.38 ± 1.09	ΔBT –61.5	1.68 ± 1.10	ΔBT –57.3
	(n = 20)	(★★★)	(n = 19)	(★★★)	(n = 20)	(★★★)	(n = 19)	(★★★)
BF_a	0.33 ± 0.11	ΔBT +156	0.05 ± 0.02	ΔBT –76.0	0.63 ± 0.29	ΔBT –82.6	1.00 ± 0.35	ΔBT –74.6
	(n = 20)	(★★★)	(n = 20)	(★★)	(n = 19)	(★★★)	(n = 19)	(★★★)
MBR1	0.23 ± 0.07	ΔBT +79.5	0.10 ± 0.08	ΔBT –53.9	0.85 ± 0.54	ΔBT –76.3	1.19 ± 0.62	ΔBT –69.8
	(n = 20)	(★★)	(n = 20)	(n.s.)	(n = 20)	(★★★)	(n = 20)	(★★★)
$MBR1+O_3$	0.28 ± 0.08	ΔBT +117	0.11 ± 0.04	ΔBT –52.4	1.57 ± 0.64	ΔBT –56.3	1.98 ± 0.67	ΔBT –49.7
	(n = 20)	(★★★)	(n = 19)	(n.s.)	(n = 20)	(★)	(n = 19)	(n.s.)
MBR2	0.25 ± 0.05	ΔBT +96.4	0.10 ± 0.06	ΔBT –54.3	1.25 ± 0.43	ΔBT –65.4	1.58 ± 0.47	ΔBT –59.9
	(n = 20)	(★★★)	(n = 17)	(n.s.)	(n = 19)	(★★)	(n = 17)	(★★)

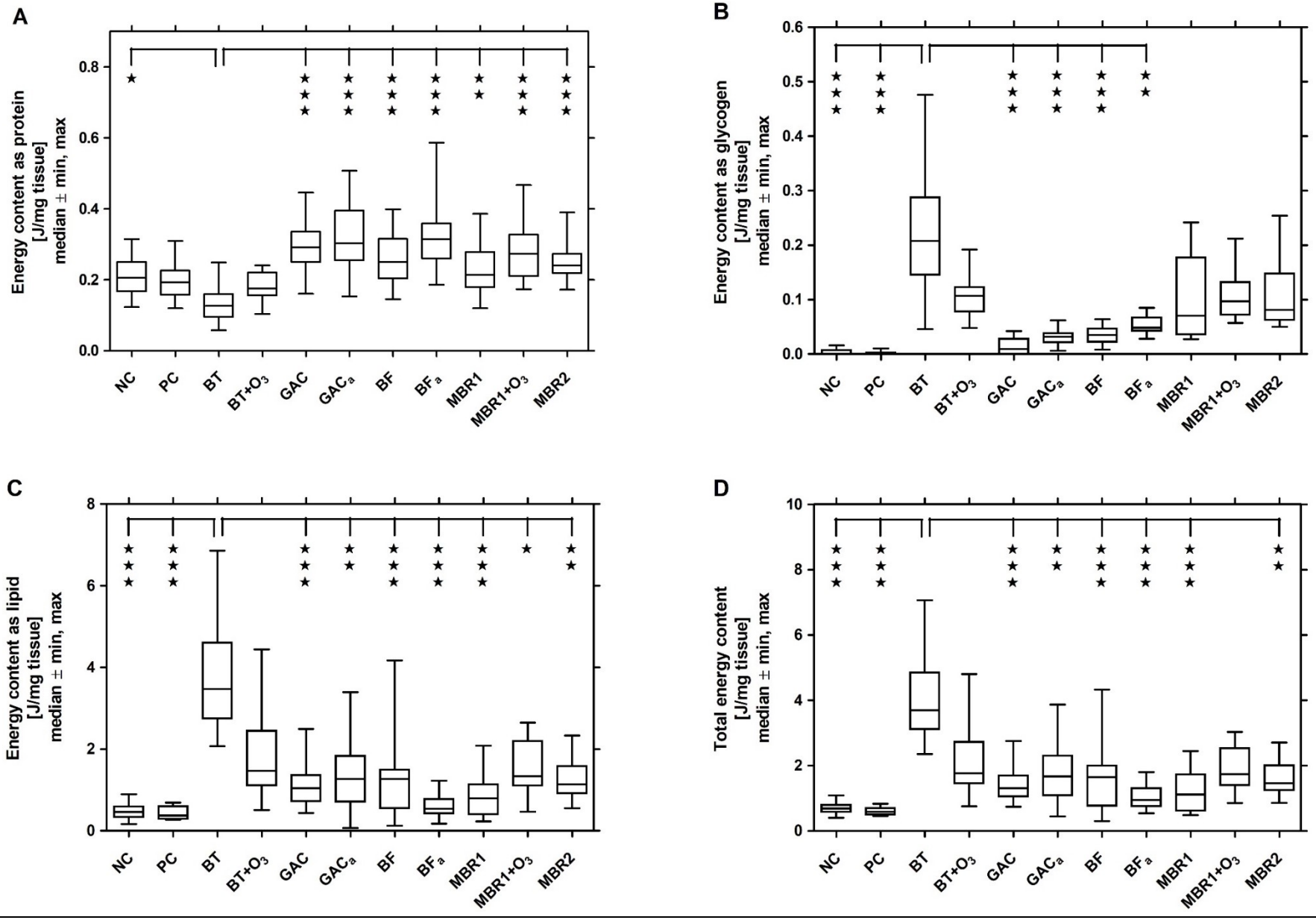
A
Energy content as protein
[J/mg tissue]
median ± min, max
0.0
0.2
0.4
0.6
0.8
NC
PC
BT
BT+O3
GAC
GACa
BF
BFa
MBR1
MBR1+O3
MBR2
B
Energy content as glycogen
[J/mg tissue]
median ± min, max
0.0
0.1
0.2
0.3
0.4
0.5
0.6
NC
PC
BT
BT+O3
GAC
GACa
BF
BFa
MBR1
MBR1+O3
MBR2
C
Energy content as lipid
[J/mg tissue]
median ± min, max
0
2
4
6
8
NC
PC
BT
BT+O3
GAC
GACa
BF
BFa
MBR1
MBR1+O3
MBR2
D
Total energy content
[J/mg tissue]
median ± min, max
0
2
4
6
8
10
NC
PC
BT
BT+O3
GAC
GACa
BF
BFa
MBR1
MBR1+O3
MBR2

Figure S3: Energy content as protein (A), glycogen (B), lipid (C), and total energy content (D) in J/mg tissue of *Gammarus fossarum* after 30 days of on-site 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/BT, are indicated with asterisks: ★ $p < 0.05$, ★★ $p < 0.01$, ★★★ $p < 0.001$ (Kruskal-Wallis with Dunn's post-test). n = 17–20.

S1.2.4 *In vitro* bioassays for endocrine activity and mutagenicity

Table S4: Estrogenic (YES), anti-estrogenic (YAES), androgenic (YAS), and anti-androgenic (YAAS) activity in [%] (mean ± SEM) of the aqueous samples from four 24 h composite samples per treatment taken in parallel to the *in vivo* on-site experiment with *Gammarus fossarum*. 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	2.40 ± 0.32 (n = 8)	–84.4	95.0 ± 0.94 (n = 24)	–43.7	19.0 ± 2.64 (n = 32)	–90.6	0.00 ± 0.00 (n = 32)	n.c.
BT	0.37 ± 0.08 (n = 39)	-	53.5 ± 0.76 (n = 40)	-	1.78 ± 0.14 (n = 31)	-	0.00 ± 0.00 (n = 24)	-
BT+O_3	0.25 ± 0.09 (n = 37)	–32.6	53.1 ± 0.61 (n = 38)	–0.60	2.56 ± 0.14 (n = 39)	+43.6	0.00 ± 0.00 (n = 32)	n.c.
GAC	0.36 ± 0.11 (n = 39)	–2.96	58.9 ± 1.17 (n = 40)	+10.2	2.28 ± 0.16 (n = 32)	+27.7	0.00 ± 0.00 (n = 32)	n.c.
GAC_a	0.13 ± 0.05 (n = 38)	–64.0	62.4 ± 0.50 (n = 40)	+16.7	2.12 ± 0.18 (n = 31)	+19.0	0.00 ± 0.00 (n = 32)	n.c.
BF	0.38 ± 0.12 (n = 32)	+0.19	55.1 ± 0.81 (n = 32)	+3.16	2.02 ± 0.16 (n = 32)	+13.1	0.00 ± 0.00 (n = 32)	n.c.
BF_a	0.25 ± 0.06 (n = 30)	–33.0	69.8 ± 1.37 (n = 32)	+30.6	2.56 ± 0.17 (n = 32)	+43.6	0.00 ± 0.00 (n = 32)	n.c.
MBR1	1.18 ± 0.21 (n = 24)	+216	53.5 ± 0.82 (n = 24)	+0.17	1.09 ± 0.14 (n = 24)	–38.7	2.81 ± 0.94 (n = 24)	n.c.
MBR1+O_3	0.90 ± 0.22 (n = 24)	+140	52.2 ± 0.83 (n = 24)	–2.34	0.88 ± 0.05 (n = 24)	–50.5	1.36 ± 0.88 (n = 16)	n.c.
MBR2	1.09 ± 0.33 (n = 24)	+190	52.3 ± 0.93 (n = 24)	–2.21	2.20 ± 0.13 (n = 24)	+23.3	0.00 ± 0.00 (n = 16)	n.c.

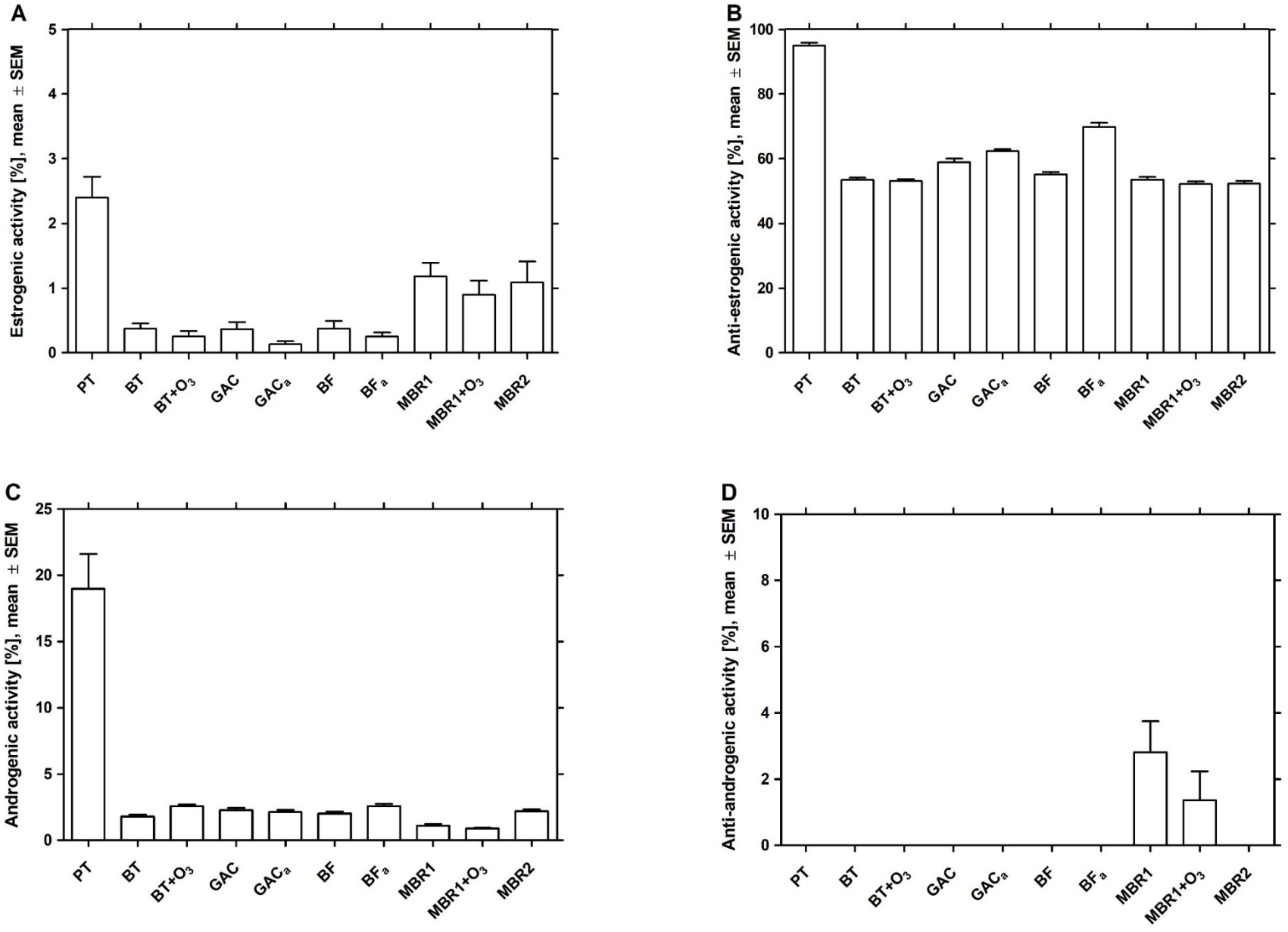
A
Estrogenic activity [%], mean ± SEM
0
1
2
3
4
5
PT
BT
BT+O3
GAC
GACa
BF
BFa
MBR1
MBR1+O3
MBR2
B
Anti-estrogenic activity [%], mean ± SEM
0
20
40
60
80
100
PT
BT
BT+O3
GAC
GACa
BF
BFa
MBR1
MBR1+O3
MBR2
C
Androgenic activity [%], mean ± SEM
0
5
10
15
20
25
PT
BT
BT+O3
GAC
GACa
BF
BFa
MBR1
MBR1+O3
MBR2
D
Anti-androgenic activity [%], mean ± SEM
0
2
4
6
8
10
PT
BT
BT+O3
GAC
GACa
BF
BFa
MBR1
MBR1+O3
MBR2

Figure S4: Estrogenic activity (A), anti-estrogenic activity (B), androgenic activity (C), and anti-androgenic activity (D) of the aqueous samples in four 24 h composite samples per treatment taken in parallel to the *in vivo* on-site experiment *with* Gammarus fossarum. 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 = 8–39 (A), n = 24–40 (B), n = 24–39 (C), n = 16–32 (D).

Table S5: Estrogenic (YES), anti-estrogenic (YAES), androgenic (YAS), and anti-androgenic (YAAS) activity, and mutagenicity (Ames YG7108) in % (mean ± SEM) in three SPE extracts each produced from 24 h composite samples per treatment taken in parallel to the *in vivo* on-site experiment with *Gammarus fossarum*. 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 (∆BT) is given in %. ☠: cytotoxic.

	YES	**∆ BT [%]**	**YAES**	**∆ BT [%]**	**YAS**	**∆ BT [%]**	**YAAS**	**∆ BT [%]**	**Ames YG7108**
PT	☠	-	☠	-	☠	-	☠	-	☠
BT	6.47 ± 0.45 (n = 24)	-	41.0 ± 1.49 (n = 24)	-	0.42 ± 0.09 (n = 24)	-	69.2 ± 2.51 (n = 24)	-	1.39 ± 0.69 (n = 3)
BT+O_3	0.21 ± 0.05 (n = 24)	–96.8	25.8 ± 1.31 (n = 24)	–37.0	0.74 ± 0.07 (n = 24)	+76.1	31.0 ± 2.56 (n = 24)	–55.2	89.6 ± 7.32 (n = 3)
GAC	0.23 ± 0.07 (n = 24)	–96.4	18.3 ± 1.65 (n = 24)	–55.3	0.19 ± 0.06 (n = 24)	–54.1	18.6 ± 2.83 (n = 24)	–73.1	15.3 ± 5.42 (n = 3)
GAC_a	0.40 ± 0.08 (n = 24)	–93.8	27.8 ± 2.42 (n = 24)	–32.2	0.36 ± 0.07 (n = 24)	–14.2	32.4 ± 4.49 (n = 24)	–53.2	13.2 ± 4.22 (n = 3)
BF	0.19 ± 0.05 (n = 24)	–97.1	34.9 ± 2.05 (n = 24)	–14.8	0.51 ± 0.07 (n = 24)	+22.2	37.2 ± 3.06 (n = 24)	–46.2	56.9 ± 6.63 (n = 3)
BF_a	0.19 ± 0.07 (n = 24)	–97.1	43.7 ± 2.57 (n = 24)	+6.64	0.07 ± 0.03 (n = 24)	–82.6	53.5 ± 2.71 (n = 24)	–22.7	52.1 ± 7.89 (n = 3)

Table S5: (continued)

	YES	Δ BT [%]	YAES	Δ BT [%]	YAS	Δ BT [%]	YAAS	Δ BT [%]	Ames YG7108
MBR1	1.50 ± 0.17 (n = 24)	–76.8	36.9 ± 2.63 (n = 24)	–10.1	0.72 ± 0.10 (n = 24)	+70.5	41.6 ± 2.54 (n = 24)	–39.8	1.39 ± 0.69 (n = 3)
MBR1+O_3	0.10 ± 0.03 (n = 24)	–98.4	6.95 ± 1.23 (n = 24)	–83.0	0.62 ± 0.05 (n = 24)	+46.2	16.9 ± 2.70 (n = 24)	–75.6	63.2 ± 10.9 (n = 3)
MBR2	1.80 ± 0.31 (n = 24)	–72.1	65.7 ± 2.17 (n = 24)	+60.2	0.48 ± 0.09 (n = 24)	+14.3	67.2 ± 2.55 (n = 24)	–2.91	0.00 ± 0.00 (n = 3)

S1.2.5 Chemical analysis

Table S6: 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 %. Wastewater was sampled in parallel to the *in vivo* on-site experiment with *Gammarus fossarum*.

	PT	BT	Δ PT [%]	$BT+O_3$	Δ PT [%]	GAC	Δ PT [%]	GAC_a	Δ PT [%]
10,11-Dihydro-10,11-dihydroxycarbamazepine	3.08 ± 0.27 (n = 5)	3.44 ± 0.35 (n = 4)	+11.8	0.30 ± 0.04 (n = 4)	–90.4	0.025 ± 0.000 (n = 4)	–99.2	0.053 ± 0.006 (n = 4)	–98.3
1H-Benzotriazol	25.5 ± 5.81 (n = 5)	7.56 ± 0.95 (n = 4)	–70.3	0.43 ± 0.08 (n = 4)	–98.3	0.044 ± 0.006 (n = 4)	–99.8	0.081 ± 0.039 (n = 4)	–99.7
1-Hydroxy-benzotriazol	0.90 ± 0.13 (n = 5)	0.42 ± 0.05 (n = 4)	–53.2	0.031 ± 0.006 (n = 4)	–96.5	0.031 ± 0.006 (n = 4)	–96.5	0.031 ± 0.006 (n = 4)	–96.5
1-Hydroxy-ibuprofen	5.92 ± 0.59 (n = 5)	0.12 ± 0.04 (n = 4)	–97.9	0.063 ± 0.013 (n = 4)	–98.9	0.063 ± 0.013 (n = 4)	–98.9	0.063 ± 0.013 (n = 4)	–98.9
2-Hydroxy-ibuprofen	39.4 ± 7.71 (n = 5)	0.40 ± 0.12 (n = 4)	–99.0	0.16 ± 0.04 (n = 4)	–99.6	0.10 ± 0.00 (n = 4)	–99.7	0.10 ± 0.00 (n = 4)	–99.7
3-Hydroxy-ibuprofen	3.80 ± 0.58 (n = 4)	0.33 ± 0.08 (n = 3)	–91.2	0.25 ± 0.00 (n = 3)	–93.4	0.25 ± 0.00 (n = 3)	–93.4	0.33 ± 0.08 (n = 3)	–91.2
4-Hydroxy-1H-benzotriazol	0.56 ± 0.13 (n = 5)	0.14 ± 0.06 (n = 4)	–75.6	0.14 ± 0.06 (n = 4)	–75.6	0.14 ± 0.06 (n = 4)	–75.6	0.14 ± 0.06 (n = 4)	–74.5

Table S6: (continued)

	PT	BT	Δ PT [%]	BT+O_3	Δ PT [%]	GAC	Δ PT [%]	GAC_a	Δ PT [%]
4-Hydroxy-diclofenac	2.54 ± 0.33 (n = 5)	1.11 ± 0.04 (n = 4)	–56.4	0.14 ± 0.06 (n = 4)	–94.6	0.14 ± 0.06 (n = 4)	–94.6	0.14 ± 0.06 (n = 4)	–94.6
4-Nitro-sulfamethoxazole	0.21 ± 0.05 (n = 5)	0.16 ± 0.06 (n = 4)	–23.8	0.19 ± 0.06 (n = 4)	–5.46	0.19 ± 0.06 (n = 4)	–5.46	0.19 ± 0.06 (n = 4)	–5.46
Acyclovir	5.67 ± 0.95 (n = 5)	0.41 ± 0.07 (n = 4)	–92.8	0.14 ± 0.06 (n = 4)	–97.6	0.14 ± 0.06 (n = 4)	–97.6	0.14 ± 0.06 (n = 4)	–97.6
Amidotrizoic acid	1.73 ± 0.66 (n = 5)	1.35 ± 0.20 (n = 4)	–21.9	0.98 ± 0.16 (n = 4)	–43.6	1.21 ± 0.10 (n = 4)	–30.2	1.22 ± 0.04 (n = 3)	–29.8
Carbamazepine	1.27 ± 0.08 (n = 5)	1.55 ± 0.12 (n = 4)	+21.7	0.14 ± 0.06 (n = 4)	–89.2	0.14 ± 0.06 (n = 4)	–89.2	0.14 ± 0.06 (n = 4)	–89.2
Carboxy-acyclovir	0.97 ± 0.11 (n = 5)	4.83 ± 0.67 (n = 4)	+399	0.14 ± 0.06 (n = 4)	–85.8	0.14 ± 0.06 (n = 4)	–85.8	0.082 ± 0.056 (n = 4)	–91.5
Carboxy-ibuprofen	75.7 ± 13.6 (n = 5)	0.10 ± 0.08 (n = 4)	–99.9	0.029 ± 0.004 (n = 4)	–100	0.025 ± 0.000 (n = 4)	–100	0.025 ± 0.000 (n = 4)	–100
Caffeine	147 ± 39.3 (n = 5)	0.50 ± 0.00 (n = 4)	–99.7	0.50 ± 0.00 (n = 4)	–99.7	0.50 ± 0.00 (n = 4)	–99.7	0.50 ± 0.00 (n = 4)	–99.7
Dehydrato-erythromycin	0.27 ± 0.11 (n = 4)	0.26 ± 0.12 (n = 3)	–4.29	0.35 ± 0.15 (n = 3)	+30.7	0.35 ± 0.15 (n = 3)	+30.7	0.35 ± 0.15 (n = 3)	+30.7

Table S6: (continued)

	PT	BT	Δ PT [%]	BT+O_3	Δ PT [%]	GAC	Δ PT [%]	GAC_a	Δ PT [%]
Diclofenac	4.48 ± 0.57 (n = 5)	4.48 ± 0.49 (n = 4)	–0.07	0.27 ± 0.13 (n = 4)	–94.0	0.27 ± 0.13 (n = 4)	–94.0	0.27 ± 0.13 (n = 4)	–94.0
Erythromycin	0.25 ± 0.05 (n = 4)	0.27 ± 0.08 (n = 3)	+9.21	0.025 ± 0.000 (n = 3)	–90.0	0.025 ± 0.000 (n = 3)	–90.0	0.025 ± 0.000 (n = 3)	-90.0
Iomeprol	16.8 ± 7.71 (n = 5)	4.81 ± 2.14 (n = 4)	–71.4	1.37 ± 0.30 (n = 4)	–91.9	0.12 ± 0.01 (n = 4)	–99.3	0.38 ± 0.05 (n = 4)	–97.8
Iopamidol	0.41 ± 0.09 (n = 5)	0.50 ± 0.00 (n = 4)	+20.6	0.44 ± 0.06 (n = 4)	+6.59	0.39 ± 0.11 (n = 4)	–5.26	0.39 ± 0.11 (n = 4)	–5.67
Iopromide	3.62 ± 3.01 (n = 5)	0.81 ± 0.29 (n = 4)	–77.6	0.21 ± 0.07 (n = 4)	–94.3	0.27 ± 0.13 (n = 4)	–92.6	0.28 ± 0.13 (n = 4)	–92.4
Mecoprop	0.025 ± 0.000 (n = 5)	0.043 ± 0.018 (n = 4)	+70.4	0.025 ± 0.000 (n = 4)	–0.56	0.025 ± 0.000 (n = 4)	–0.56	0.025 ± 0.000 (n = 4)	–0.56
N-Acetyl-sulfamethoxazole	1.41 ± 0.21 (n = 5)	0.19 ± 0.02 (n = 4)	–86.3	0.025 ± 0.000 (n = 4)	–98.2	0.025 ± 0.000 (n = 4)	–98.2	0.025 ± 0.000 (n = 4)	–98.2
Paracetamol	13.6 ± 2.38 (n = 5)	0.025 ± 0.000 (n = 4)	–99.8	0.025 ± 0.000 (n = 4)	–99.8	0.025 ± 0.000 (n = 4)	–99.8	0.025 ± 0.000 (n = 4)	–99.8
Sulfamethoxazole	0.86 ± 0.09 (n = 5)	0.39 ± 0.05 (n = 4)	–54.5	0.031 ± 0.006 (n = 4)	–96.4	0.031 ± 0.006 (n = 4)	–96.4	0.031 ± 0.006 (n = 4)	–96.4

Table S6: (continued)

	PT	BT	Δ PT [%]	BT+O_3	Δ PT [%]	GAC	Δ PT [%]	GAC_a	Δ PT [%]
Tolyltriazole	5.36 ± 0.39 (n = 5)	2.08 ± 0.16 (n = 4)	–61.2	0.26 ± 0.14 (n = 4)	–95.1	0.26 ± 0.14 (n = 4)	–95.1	0.26 ± 0.14 (n = 4)	–95.1
Tramadol	0.99 ± 0.16 (n = 5)	0.93 ± 0.11 (n = 4)	–5.93	0.025 ± 0.000 (n = 4)	–97.5	0.025 ± 0.000 (n = 4)	–97.5	0.025 ± 0.000 (n = 4)	–97.5
Tramadol-N-oxide	0.030 ± 0.005 (n = 5)	0.025 ± 0.000 (n = 4)	–16.7	0.033 ± 0.003 (n = 4)	+10.8	0.025 ± 0.000 (n = 4)	–16.7	0.025 ± 0.000 (n = 4)	–16.7

Table S7: 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 %. Wastewater was sampled in parallel to the *in vivo* on-site experiment with *Gammarus fossarum*.

	PT	**BF**	**Δ PT [%]**	**BF_a**	**Δ PT [%]**	**MBR1**	**Δ PT [%]**	**$MBR1+O_3$**	**Δ PT [%]**
10,11-Dihydro-10,11-dihydroxycarbamazepine	3.08 ± 0.27 (n = 5)	0.26 ± 0.03 (n = 4)	–91.4	0.64 ± 0.37 (n = 4)	–79.2	1.47 ± 0.12 (n = 4)	–52.3	0.060 ± 0.024 (n = 3)	–98.1
1H-Benzotriazol	25.5 ± 5.81 (n = 5)	0.38 ± 0.06 (n = 4)	–98.5	1.41 ± 0.98 (n = 4)	–94.5	3.01 ± 0.41 (n = 4)	–88.2	0.10 ± 0.05 (n = 3)	–99.6
1-Hydroxy-benzotriazol	0.90 ± 0.13 (n = 5)	0.031 ± 0.006 (n = 4)	–96.5	0.082 ± 0.049 (n = 4)	–90.9	0.080 ± 0.008 (n = 4)	–91.1	0.033 ± 0.008 (n = 3)	–96.3
1-Hydroxy-ibuprofen	5.92 ± 0.59 (n = 5)	0.063 ± 0.013 (n = 4)	–98.9	0.063 ± 0.013 (n = 4)	–98.9	0.063 ± 0.013 (n = 4)	–98.9	0.067 ± 0.017 (n = 3)	–98.9
2-Hydroxy-ibuprofen	39.4 ± 7.71 (n = 5)	0.11 ± 0.00 (n = 4)	–99.7	0.20 ± 0.09 (n = 4)	–99.5	0.15 ± 0.04 (n = 4)	–99.6	0.10 ± 0.00 (n = 3)	–99.7
3-Hydroxy-ibuprofen	3.80 ± 0.58 (n = 4)	0.33 ± 0.08 (n = 3)	–91.2	0.25 ± 0.00 (n = 3)	–93.4	0.33 ± 0.08 (n = 3)	–91.2	0.30 ± 0.05 (n = 2)	–92.1
4-Hydroxy-1H-benzotriazol	0.56 ± 0.13 (n = 5)	0.14 ± 0.06 (n = 4)	–75.6	0.19 ± 0.06 (n = 4)	–65.6	0.030 ± 0.003 (n = 4)	–94.7	0.18 ± 0.08 (n = 3)	–69.0

Table S7: (continued)

	PT	BF	Δ PT [%]	BF_a	Δ PT [%]	MBR1	Δ PT [%]	MBR1+O_3	Δ PT [%]
4-Hydroxy-diclofenac	2.54 ± 0.33 (n = 5)	0.14 ± 0.06 (n = 4)	–94.6	0.29 ± 0.12 (n = 4)	–88.7	0.61 ± 0.05 (n = 4)	–76.1	0.18 ± 0.08 (n = 3)	–93.1
4-Nitro-sulfamethoxazole	0.21 ± 0.05 (n = 5)	0.19 ± 0.06 (n = 4)	–5.46	0.19 ± 0.06 (n = 4)	–5.46	0.19 ± 0.06 (n = 4)	–5.46	0.18 ± 0.08 (n = 3)	–14.6
Acyclovir	5.67 ± 0.95 (n = 5)	0.14 ± 0.06 (n = 4)	–97.6	0.16 ± 0.05 (n = 4)	–97.1	0.073 ± 0.025 (n = 4)	–98.7	0.18 ± 0.08 (n = 3)	–96.9
Amidotrizoic acid	1.73 ± 0.66 (n = 5)	0.98 ± 0.20 (n = 4)	–43.4	0.93 ± 0.19 (n = 4)	–46.4	0.69 ± 0.15 (n = 4)	–60.5	0.37 ± 0.13 (n = 3)	–78.6
Carbamazepine	1.27 ± 0.08 (n = 5)	0.14 ± 0.06 (n = 4)	–89.2	0.36 ± 0.19 (n = 4)	–71.8	0.65 ± 0.04 (n = 4)	–48.7	0.18 ± 0.08 (n = 3)	–86.2
Carboxy-acyclovir	0.97 ± 0.11 (n = 5)	0.14 ± 0.06 (n = 4)	–85.8	0.69 ± 0.52 (n = 4)	–28.7	1.09 ± 0.10 (n = 4)	+12.7	0.18 ± 0.08 (n = 3)	–81.9
Carboxy-ibuprofen	75.7 ± 13.6 (n = 5)	0.025 ± 0.000 (n = 4)	–100	0.025 ± 0.000 (n = 4)	–100	0.032 ± 0.007 (n = 4)	–100	0.025 ± 0.000 (n = 3)	–100
Caffeine	147 ± 39.3 (n = 5)	0.50 ± 0. 00 (n = 4)	–99.7	0.50 ± 0.00 (n = 4)	–99.7	0.50 ± 0.00 (n = 4)	–99.7	0.50 ± 0.00 (n = 3)	–99.7
Dehydrato-erythromycin	0.27 ± 0.11 (n = 4)	0.35 ± 0.15 (n = 3)	+30.7	0. 50 ± 0.00 (n = 3)	+86.7	0.21 ± 0.14 (n = 3)	–20.5	0.50 ± 0.00 (n = 2)	+86.7

Table S7: (continued)

	PT	BF	Δ PT [%]	BF_a	Δ PT [%]	MBR1	Δ PT [%]	MBR1+O_3	Δ PT [%]
Diclofenac	4.48 ± 0.57 (n = 5)	0.27 ± 0.13 (n = 4)	–94.0	0.84 ± 0.51 (n = 4)	–81.2	1.65 ± 0.16 (n = 4)	–63.3	0.34 ± 0.16 (n = 3)	–92.4
Erythromycin	0.25 ± 0.05 (n = 4)	0.025 ± 0.000 (n = 3)	–90.0	0.059 ± 0.034 (n = 3)	–76.4	0.058 ± 0.016 (n = 3)	–76.7	0.025 ± 0.000 (n = 2)	–90.0
Iomeprol	16.8 ± 7.71 (n = 5)	1.47 ± 0.84 (n = 4)	–91.3	1.43 ± 0.66 (n = 4)	–91.5	1.55 ± 0.76 (n = 4)	–90.8	0.25 ± 0.15 (n = 3)	–98.5
Iopamidol	0.41 ± 0.09 (n = 5)	0.43 ± 0.07 (n = 3)	+2.70	0.50 ± 0.00 (n = 3)	+20.7	0.39 ± 0.11 (n = 3)	–5.43	0.50 ± 0.00 (n = 3)	+20.7
Iopromide	3.62 ± 3.01 (n = 5)	0.23 ± 0.09 (n = 4)	–93.8	0.32 ± 0.09 (n = 4)	–91.2	0.57 ± 0.16 (n = 4)	–84.2	0.36 ± 0.14 (n = 3)	–90.0
Mecoprop	0.025 ± 0.000 (n = 5)	0.025 ± 0.000 (n = 4)	–0.56	0.025 ± 0.000 (n = 4)	–0.56	0.025 ± 0.000 (n = 4)	–0.56	0.025 ± 0.000 (n = 3)	–0.56
N-Acetyl-sulfamethoxazole	1.41 ± 0.21 (n = 5)	0.025 ± 0.000 (n = 4)	–98.2	0.048 ± 0.023 (n = 4)	–96.6	0.046 ± 0.012 (n = 4)	–96.8	0.025 ± 0.000 (n = 3)	–98.2
Paracetamol	13.6 ± 2.38 (n = 5)	0.025 ± 0.000 (n = 4)	–99.8	0.025 ± 0.000 (n = 4)	–99.8	0.034 ± 0.009 (n = 4)	–99.8	0.025 ± 0.000 (n = 3)	–99.8
Sulfamethoxazole	0.86 ± 0.09 (n = 5)	0.031 ± 0.006 (n = 4)	–96.4	0.068 ± 0.043 (n = 4)	–92.1	0.25 ± 0.06 (n = 4)	–71.2	0.025 ± 0.000 (n = 3)	–97.1

Table S7: (continued)

	PT	BF	Δ PT [%]	BF_a	Δ PT [%]	MBR1	Δ PT [%]	MBR1+O_3	Δ PT [%]
Tolyltriazole	5.36 ± 0.39 (n = 5)	0.26 ± 0.14 (n = 4)	–95.1	0.66 ± 0.38 (n = 4)	–87.6	0.92 ± 0.11 (n = 4)	–82.9	0.34 ± 0.16 (n = 3)	–93.6
Tramadol	0.99 ± 0.16 (n = 5)	0.025 ± 0.000 (n = 4)	–97.5	0.15 ± 0.13 (n = 4)	–84.4	0.44 ± 0.04 (n = 4)	–55.3	0.025 ± 0.000 (n = 3)	–97.5
Tramadol-N-oxide	0.030 ± 0.005 (n = 5)	0.034 ± 0.005 (n = 4)	+14.1	0.035 ± 0.008 (n = 4)	+15.3	0.025 ± 0.000 (n = 4)	–16.7	0.025 ± 0.000 (n = 3)	–16.7

Table S8: 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 %. Wastewater was sampled in parallel to the *in vivo* on-site experiment with *Gammarus fossarum*.

	PT	BT (for comparison)	MBR2	Δ PT [%]
10,11-Dihydro-10,11-dihydroxycarbamazepine	3.08 ± 0.27 (n = 5)	3.44 ± 0.35 (n = 4)	3.16 ± 0.21 (n = 4)	+2.76
1H-Benzotriazol	25.5 ± 5.81 (n = 5)	7.56 ± 0.95 (n = 4)	3.96 ± 1.14 (n = 4)	–84.5
1-Hydroxy-benzotriazol	0.90 ± 0.13 (n = 5)	0.42 ± 0.05 (n = 4)	0.20 ± 0.05 (n = 4)	–77.4
1-Hydroxy-ibuprofen	5.92 ± 0.59 (n = 5)	0.12 ± 0.04 (n = 4)	0.25 ± 0.18 (n = 4)	–95.8
2-Hydroxy-ibuprofen	39.4 ± 7.71 (n = 5)	0.40 ± 0.12 (n = 4)	1.57 ± 1.36 (n = 4)	–96.0
3-Hydroxy-ibuprofen	3.80 ± 0.58 (n = 4)	0.33 ± 0.08 (n = 3)	0.45 ± 0.10 (n = 3)	–88.1
4-Hydroxy-1H-benzotriazol	0.56 ± 0.13 (n = 5)	0.14 ± 0.06 (n = 4)	0.045 ± 0.004 (n = 4)	–92.1
4-Hydroxy-diclofenac	2.54 ± 0.33 (n = 5)	1.11 ± 0.04 (n = 4)	1.22 ± 0.10 (n = 4)	–51.8
4-Nitro-sulfamethoxazole	0.21 ± 0.05 (n = 5)	0.16 ± 0.06 (n = 4)	0.19 ± 0.06 (n = 4)	–5.46
Acyclovir	5.67 ± 0.95 (n = 5)	0.41 ± 0.07 (n = 4)	0.26 ± 0.16 (n = 4)	–95.5
Amidotrizoic acid	1.73 ± 0.66 (n = 5)	1.35 ± 0.20 (n = 4)	1.28 ± 0.23 (n = 4)	–26.4
Carbamazepine	1.27 ± 0.08 (n = 5)	1.55 ± 0.12 (n = 4)	1.36 ± 0.17 (n = 4)	+7.00
Carboxy-acyclovir	0.97 ± 0.11 (n = 5)	4.83 ± 0.67 (n = 4)	3.60 ± 0.53 (n = 4)	+271
Carboxy-ibuprofen	75.7 ± 13.6 (n = 5)	0.10 ± 0.08 (n = 4)	2.12 ± 2.10 (n = 4)	–97.2
Caffeine	147 ± 39.3 (n = 5)	0.50 ± 0.00 (n = 4)	1.44 ± 0.94 (n = 4)	–99.0
Dehydrato-erythromycin	0.27 ± 0.11 (n = 4)	0.26 ± 0.12 (n = 3)	0.13 ± 0.06 (n = 3)	–51.8

Table S8: (continued)

	PT	BT (for comparison)	MBR2	Δ PT [%]
Diclofenac	4.48 ± 0.57 (n = 5)	4.48 ± 0.49 (n = 4)	4.38 ± 0.73 (n = 4)	–2.21
Erythromycin	0.25 ± 0.05 (n = 4)	0.27 ± 0.08 (n = 3)	0.15 ± 0.01 (n = 3)	–40.5
Iomeprol	16.8 ± 7.71 (n = 5)	4.81 ± 2.14 (n = 4)	2.79 ± 1.11 (n = 4)	–83.4
Iopamidol	0.41 ± 0.09 (n = 5)	0.50 ± 0.00 (n = 4)	0.43 ± 0.07 (n = 4)	+4.40
Iopromide	3.62 ± 3.01 (n = 5)	0.81 ± 0.29 (n = 4)	0.82 ± 0.33 (n = 4)	–77.3
Mecoprop	0.025 ± 0.000 (n = 5)	0.043 ± 0.018 (n = 4)	0.029 ± 0.004 (n = 4)	+15.8
N-Acetyl-sulfamethoxazole	1.41 ± 0.21 (n = 5)	0.19 ± 0.02 (n = 4)	0.13 ± 0.09 (n = 4)	–91.0
Paracetamol	13.6 ± 2.38 (n = 5)	0.025 ± 0.000 (n = 4)	0.025 ± 0.000 (n = 4)	–99.8
Sulfamethoxazole	0.86 ± 0.09 (n = 5)	0.39 ± 0.05 (n = 4)	0.70 ± 0.17 (n = 4)	–18.8
Tolyltriazole	5.36 ± 0.39 (n = 5)	2.08 ± 0.16 (n = 4)	1.97 ± 0.22 (n = 4)	–63.2
Tramadol	0.99 ± 0.16 (n = 5)	0.93 ± 0.11 (n = 4)	1.20 ± 0.06 (n = 4)	+21.2
Tramadol-N-oxide	0.030 ± 0.005 (n = 5)	0.025 ± 0.000 (n = 4)	0.025 ± 0.000 (n = 4)	–16.7

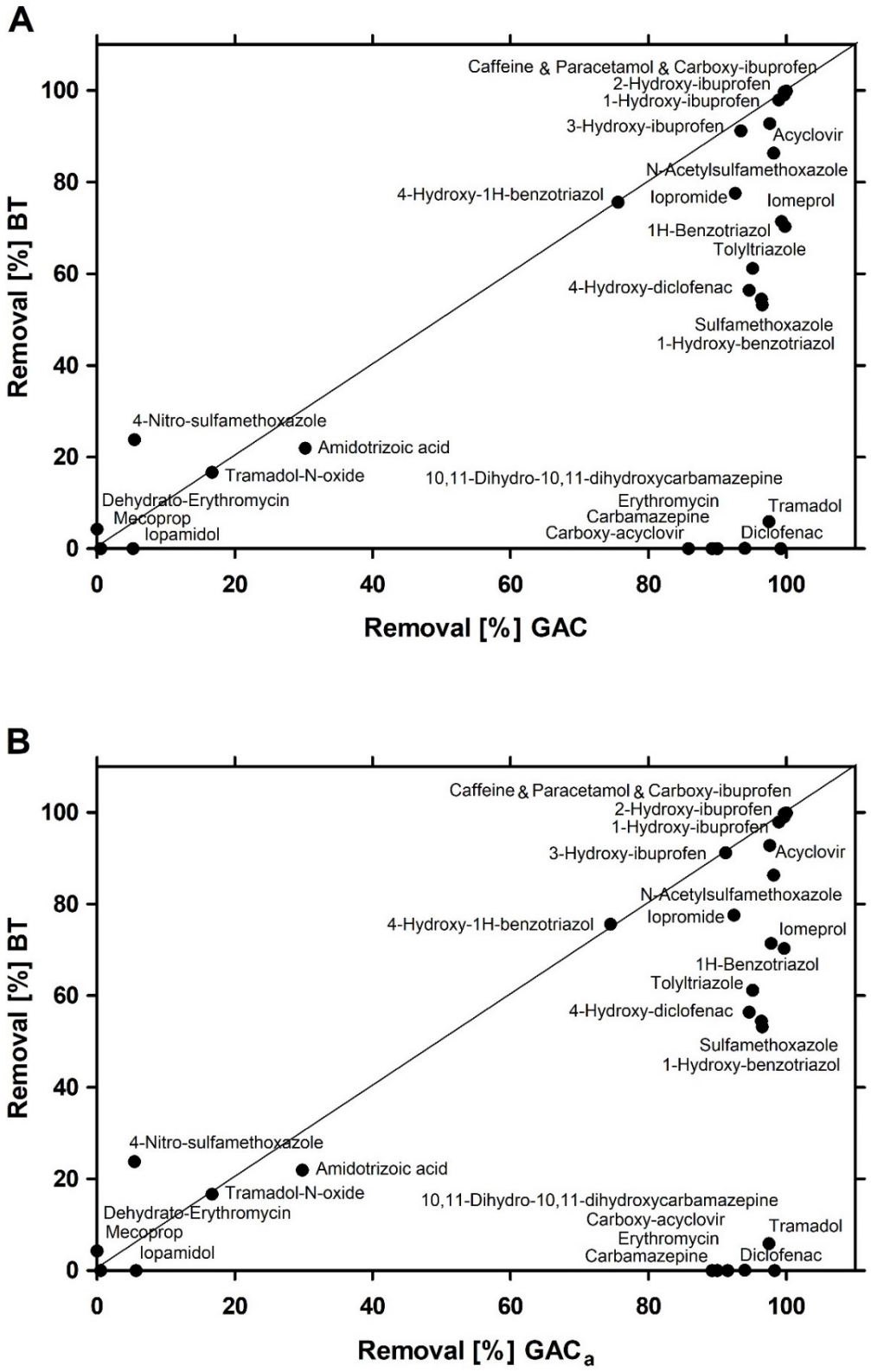

Figure S5: Removal of micropollutants in the conventional biological treatment (BT) compared to the non-aerated granular activated carbon filter treatment (GAC, A) and the aerated granular activated carbon filter treatment (GAC_a, B). Wastewater was sampled in parallel to the *in vivo* on-site experiment with *Gammarus fossarum*.

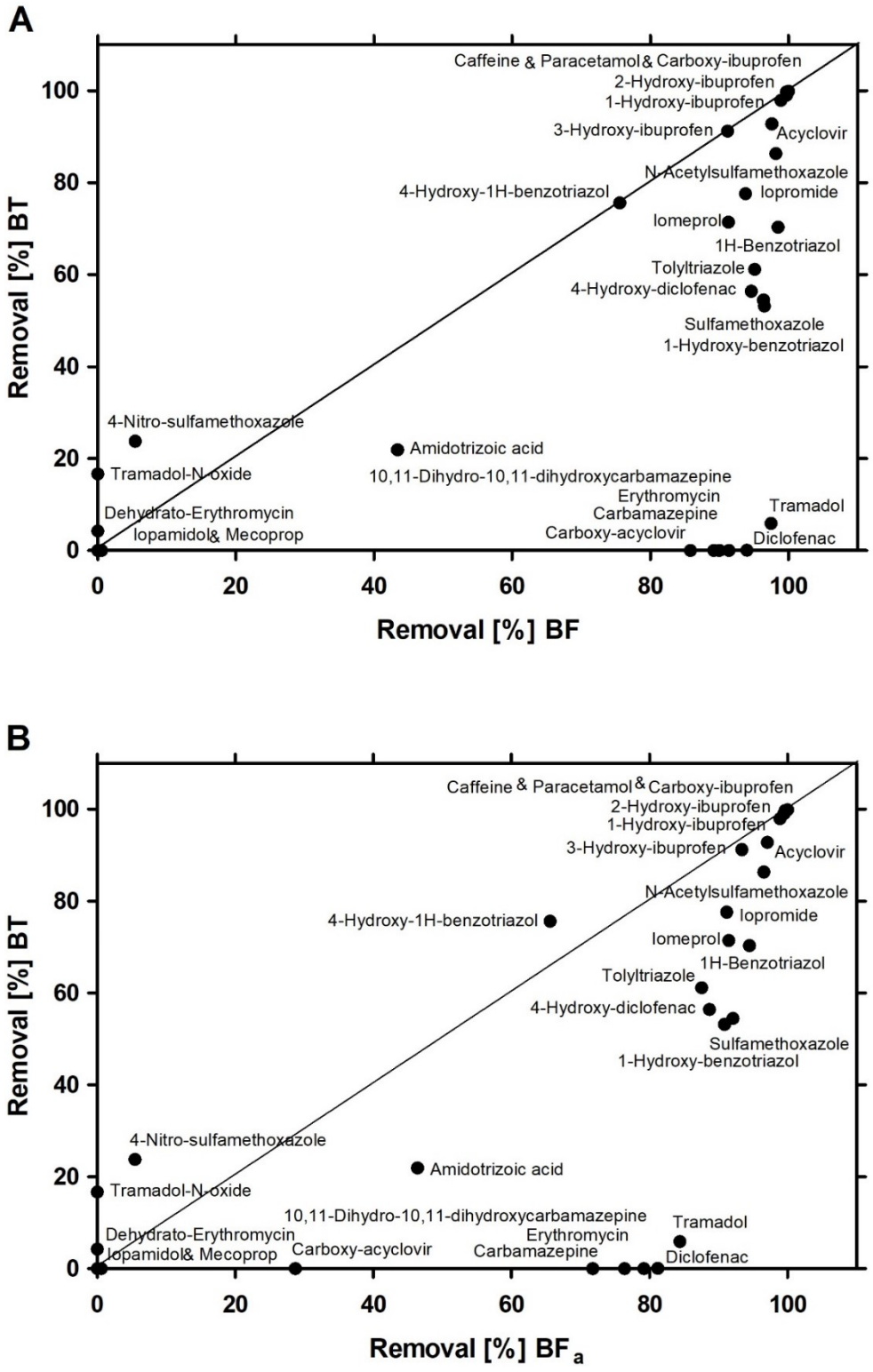

Figure S6: Removal of micropollutants in the conventional biological treatment (BT) compared to the non-aerated biofilter treatment (BF, A) and the aerated biofilter treatment (BF_a, B). Wastewater was sampled in parallel to the *in vivo* on-site experiment with *Gammarus fossarum*.

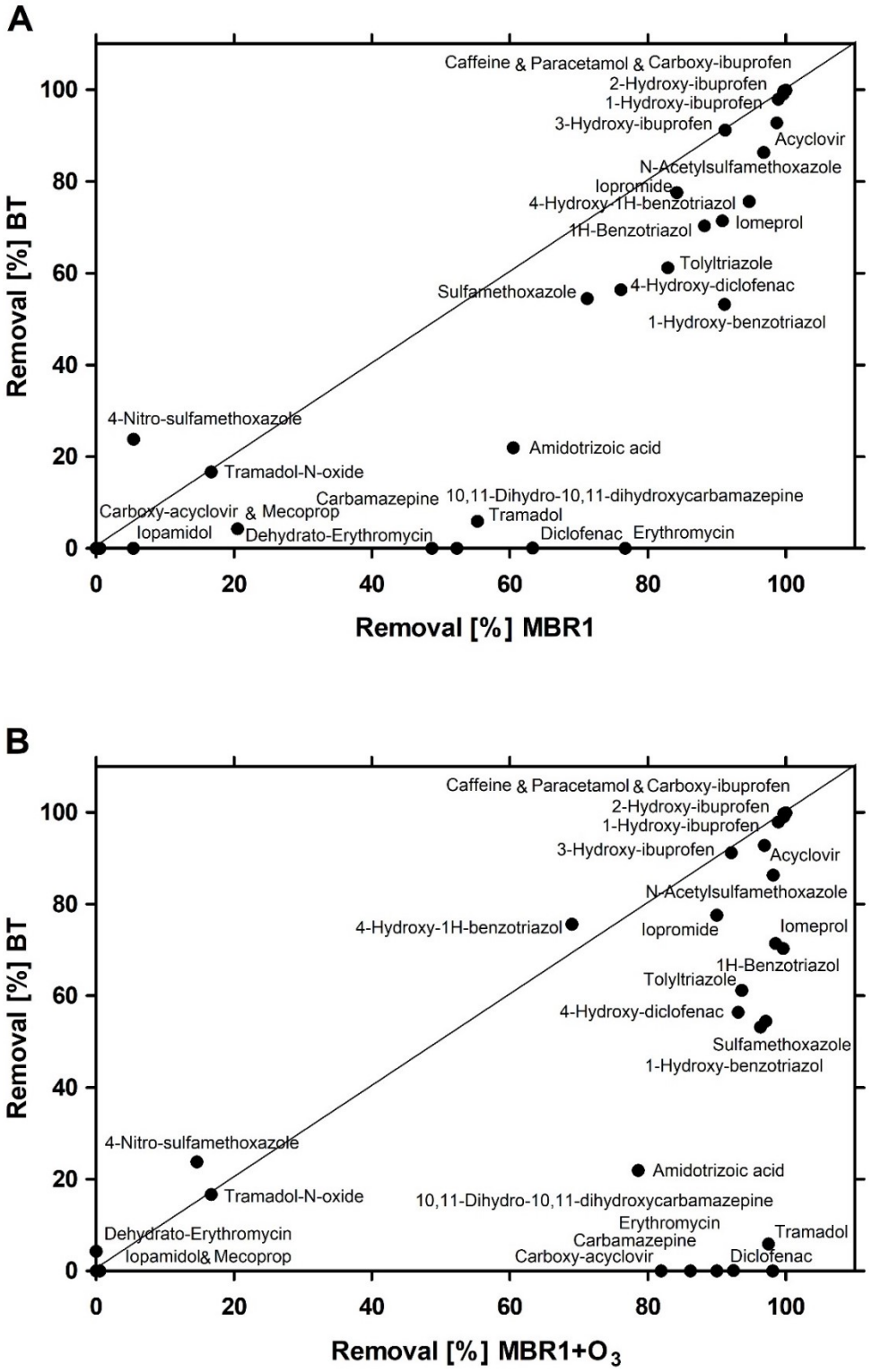

Figure S7: Removal of micropollutants in the conventional biological treatment (BT) compared to the membrane bioreactor 1 (MBR1, A) and the membrane bioreactor 1 after ozone system 2 (MBR1+O_3, B). Wastewater was sampled in parallel to the *in vivo* on-site experiment with *Gammarus fossarum*.

A.6 Zusammenfassung (German summary)

Weltweit wächst die Industrialisierung kontinuierlich und folglich werden neue Chemikalien hergestellt, die in unterschiedlichen Bereichen des täglichen Lebens verwendet werden. Dadurch ist die zunehmende Verschmutzung von natürlichen Gewässern mit chemischen Substanzen zu einem öffentlichen Hauptanliegen geworden und stellt heutzutage ein besorgniserregendes, ökologisches Problem dar. Chemische Substanzen sind allgegenwärtig und sie werden weltweit, und nicht nur speziell in industrialisierten Regionen, im Abwasser, Oberflächenwasser (Flusswasser), Grundwasser und Trinkwasser detektiert. Eine Hauptquelle für diese Wasserverschmutzung, die Freisetzung und die Verbreitung solcher synthetischen organischen Substanzen menschlichen Ursprungs, auch Mikroschadstoffe genannt, sind Kläranlagen. Studien zeigen, dass Mikroschadstoffe, wie zum Beispiel Arzneimittel, Körperpflegeprodukte, Desinfektionsmittel, Industrie- und Haushaltschemikalien, Verhütungsmittel, Hormone, Nahrungsmittelzusatzstoffe, Süßstoffe, Biozide (inklusive Insektizide und Fungizide), Pestizide und viele andere, zu den „Neuen Umweltschadstoffen“ zählende Substanzen mit den existierenden konventionellen Abwasserreinigungstechniken nur unvollständig abgebaut und unzureichend aus dem Abwasser entfernt werden. Diese Mikroschadstoffe gelangen in den Wasserkreislauf und zeigen dort bereits in sehr geringen Konzentrationen potentielle sowie nachgewiesene schädliche Auswirkungen, einschließlich Langzeiteffekten, auf aquatische Ökosysteme und die menschliche Gesundheit. Jedoch selbst wenn einzelne Mikroschadstoffe nur in geringen Konzentrationen in natürlichen Gewässern vorkommen, sind sie allein aufgrund ihrer großen Menge von relevantem (öko)toxikologischen Bedenken. Weiterhin besteht die Schwierigkeit, die Effekte von Mikroschadstoffen zu beurteilen, wenn sie in komplexen Mischungen in

der aquatischen Umwelt auftreten. Viele dieser Mikroschadstoffe sind endokrine Disruptoren (Substanzen, die störend auf das Hormonsystem einwirken und die Gesundheit schädigen können), deren Vorkommen in den letzten Jahrzehnten weltweit rapide angestiegen ist und die regelmäßig in den Abwässern von konventionellen Kläranlagen nachgewiesen werden. Endokrine Effekte auf Muscheln und Fische, wie zum Beispiel Intersex, Störungen der Reproduktion oder Verweiblichung von Männchen, wurden in Flüssen unterhalb von kommunalen Kläranlageneinleitern beobachtet und werden auf die Freisetzung von natürlichen Östrogenen (z.B. Östron (E_1) und 17β-Östradiol (E_2)), synthetischen Östrogenen (z.B. 17α-Ethinylöstradiol (EE_2)) oder der chemischen Verbindungen der Alkylphenole zurückgeführt.

Demzufolge sollten konventionelle Kläranlagen mit fortgeschrittenen und weiter entwickelten Abwasserreinigungstechnologien ausgestattet und ertüchtigt werden. Diese Technologien stellen zum Beispiel die Ozonung, die Behandlung mit Aktivkohle, die Biofiltration, Membranbioreaktoren oder die Bestrahlung mit ultraviolettem Licht dar. Dabei können die Technologien als eigenständige Systeme oder als Kombinationen verwendet werden. Jedoch zeigen einige chemische und ökotoxikologische Studien, dass besonders die Ozonung von Abwasser die Entstehung von diversen Transformationsprodukten (TPs) mit überwiegend unbekannten Eigenschaften verursacht, die eventuell toxischer sind als die ursprüngliche Substanz. Daher sind Nachbehandlungen des ozonierten Abwassers nötig und empfehlenswert.

Die vorliegende Dissertation war im Forschungsprojekt TransRisk integriert, das innerhalb des Förderschwerpunkts „NaWaM – Nachhaltiges Wassermanagement“ und der Fördermaßnahme „RiSKWa – Risikomanagement von neuen Schadstoffen und

Krankheitserregern im Wasserkreislauf“ vom Bundesministerium für Bildung und Forschung (BMBF; FKZ: 02WRS1275A) gefördert wurde. TransRisk befasste sich mit der „Charakterisierung, Kommunikation und Minimierung von Risiken durch neue Schadstoffe und Krankheitserreger im Wasserkreislauf“. Die Zielsetzung von TransRisk war eine Kombination von (öko)toxikologischen, chemischen und technischen Ansätzen zur Entwicklung neuer Strategien, um Risiken zu charakterisieren und zu minimieren, die mit Effekten von organischen Mikroschadstoffen und Krankheitserregern in kommunalen Wasserkreisläufen verbunden sind. Anschließend sollten die resultierenden Ergebnisse in ein wirkungsmäßiges Risikomanagementkonzept integriert werden.

Ein Hauptziel war die Untersuchung des Abwassers einer kommunalen konventionellen Kläranlage mit mehreren nachgeschalteten erweiterten Behandlungstechnologien innerhalb einer Pilotkläranlage, um eine weitere Reduktion von Mikroschadstoffen, neu entstandenen Transformationsprodukten und der Toxizität zu untersuchen und um eine optimale Abwasserbehandlung zu ermitteln. In bisherigen Studien wurden vor allem einzelne erweiterte Behandlungstechnologien bewertet, kaum jedoch mehrere kombinierte Behandlungstechnologien vergleichend untersucht. Aus diesem Grund wurde das konventionell gereinigte Abwasser vollständig ozoniert, wobei vier verschiedene Ozondosen und fünf verschiedene hydraulische Retentionszeiten getestet wurden. Direkt nach der Ozonung wurde das Abwasser mit vier verschiedenen Filtersystemen nachbehandelt: unbelüftete und belüftete Filtration durch granulierte Aktivkohle (GAK) sowie unbelüftete und belüftete Biofiltration (BF). Zudem wurde nur mechanisch behandeltes Rohabwasser in zwei alleinstehende Membranbioreaktoren (MBR) geleitet, von denen ein Reaktor mit einer Ozonungsanlage und einer partiellen Rückführung des Abwassers ausgestattet war. Sowohl wässrige Proben als auch Extrakte des Abwassers von zehn verschiedenen

Probenahmestellen wurden in diversen *in vitro* Biotests analysiert, um endokrine ((anti)östrogene und (anti)androgene) Aktivitäten und gentoxische sowie mutagene Potentiale zu untersuchen. Zusätzlich wurde das Abwasser von neun Probenahmestellen (abzüglich des Rohabwassers) *in vivo* vor Ort auf dem Gelände der Pilotkläranlage in einem Durchflussverfahren mit der Neuseeländischen Zwergdeckelschnecke *Potamopyrgus antipodarum* und dem Bachflohkrebs *Gammarus fossarum* hinsichtlich der Mortalität, dem Wachstum, der Reproduktion, der Energiereserven sowie der Entwicklung der Testorganismen analysiert. Parallel zu den *in vitro* und *in vivo* Biotests wurden chemische Analysen der Abwasserproben durchgeführt, um die Entfernung von 40 ausgewählten Mikroschadstoffen, sogenannten Indikatorsubstanzen, zu untersuchen. Die Ergebnisse der *in vitro* Biotests zeigten, dass östrogene Aktivitäten durch die konventionelle Reinigung reduziert wurden und durch eine Ozonung (mit steigender Ozondosis) weiter abnahmen. Im Gegensatz dazu nahmen die anti-östrogene Aktivität sowie die Mutagenität aufgrund der Ozonung (mit steigender Ozondosis) deutlich zu. Diese Zunahmen sind möglicherweise mit der Entstehung von TPs zu erklären, die durch die Ozonung verursacht wurden und eine Nachbehandlung erfordern. Eine weitere mögliche Ursache für den Anstieg der anti-östrogenen Aktivität ist die gleichzeitige Abnahme der östrogenen Aktivität, da sowohl östrogen-artig als auch anti-östrogen-artig wirkende Substanzen um denselben Rezeptor konkurrieren. Die Ozonung des Abwassers bewirkte ebenfalls eine Abnahme der anti-androgenen Aktivität. Die Nachbehandlungen des ozonierten Abwassers bewirkten eine weitere Abnahme der endokrinen Aktivitäten und der Mutagenität, wobei die Filtersysteme mit GAK im Vergleich zu den Biofiltern eine bessere Leistung erzielten. Die Ergebnisse der *in vivo* Biotests ergaben, dass konventionell gereinigtes und ozoniertes Abwasser sowie das Abwasser der MBRs den größten Einfluss auf die Zwergdeckelschnecken und die

Gammariden hatten. Die Mortalität der Gammariden war im konventionell gereinigten Abwasser maximal, während die Schnecken das größte Wachstum zeigten. Im ozonierten Abwasser war das Wachstum sowohl der männlichen als auch der weiblichen Gammariden maximal, wohingegen eine deutliche Reproduktionstoxizität bei den Schnecken festgestellt wurde. Die Behandlung des Abwassers in den MBRs bewirkte bei den Schnecken ein vermindertes Wachstum und eine starke Reproduktionstoxizität. Die Ergebnisse der Biotests zusammenfassend, scheint eine Ozonung des konventionell gereinigten Abwassers mit anschließender GAK-Filtration die vielversprechendste Option zu sein. Die Ergebnisse der chemischen Analytik unterstützen diese Vermutung. Die verbleibende hohe anti-östrogene Aktivität in allen erweiterten Behandlungstechnologien bedarf jedoch weiterer Aufklärung.

Weiterhin wurden Laborexperimente in selbst gebauten Fließrinnen mit dem Gewöhnlichen Flohkrebs *Gammarus pulex* durchgeführt, um den Einfluss verschiedener Anteile (0%, 33%, 66% und 100%) eines Abwassers mit bekannter östrogener Aktivität auf die Mortalität, das Wachstum und die Reproduktion der Testorganismen zu untersuchen. Die Ergebnisse zeigten einen erheblichen Einfluss des Abwassers auf die Populationsstruktur der Gammariden. Einerseits nahm die Körperlänge zu, was möglicherweise auf ein zusätzliches Nahrungsangebot zurückzuführen ist. Andererseits verschob sich das Geschlechterverhältnis zugunsten der Weibchen und der Anteil brütender Weibchen, der Fekunditätsindex sowie die Gesamtzahl an Nachkommen stieg stetig mit zunehmendem Abwasseranteil an. Diese Beobachtungen können mit im Abwasser vorhandenen Schadstoffen, die eventuell auf das Hormonsystem der Gammariden einwirkten, erklärt werden. Diese Ergebnisse zeigen ebenfalls, dass weitere Untersuchungen hinsichtlich der endokrinen Aktivität von Abwasser nötig sind.

Ein weiteres Hauptziel war die Identifizierung einer optimalen Aufbereitungsmethode zur Stabilisierung von Wasser und Abwasserproben für (öko)toxikologische *in vitro* Biotests, da diese Aufbereitungsmethoden bisher einzig für chemische Analysen optimiert wurden. Dazu wurden 18 verschiedene Proben, darunter Rohabwasser, Abwasser von Krankenhäusern, konventionell gereinigtem Abwasser, ozoniertem Abwasser, Oberflächenwasser von Flüssen, Grundwasser sowie Trinkwasser, verwendet. Die Proben wurden zum einen filtriert und zum anderen für 24 h angesäuert und neutralisiert, bevor sie in den Biotests getestet wurden. Zudem wurden Festphasenextraktionen mit drei verschiedenen Sorptionsmitteln (Oasis HLB, Supelco ENVI-Carb+ und Telos C18/ENV) und zwei pH-Werten (neutral und kurzzeitig angesäuert) durchgeführt, um die beste Extraktionsmethode für (öko)toxikologische Effekte zu finden. Die Wasser- und Abwasserproben sowie die Extrakte wurden in neun verschiedenen *in vitro* Biotests (hefebasierte rekombinante Reportergentests) auf endokrine Aktivitäten ((anti)östrogen, (anti)androgen, Aryl-Hydrocarbon-Rezeptor (Dioxin)-artig, zwei Retinsäure-artige, Vitamin D-artig und Thyroid-Rezeptor-artig) und in zwei *in vitro* Biotests unter Verwendung von gentechnisch veränderten Bakterienstämmen auf Gentoxizität und Mutagenität untersucht. Die Ergebnisse zeigten, dass eine Filtration und eine Ansäuerung/Neutralisierung der Proben einen zum Teil erheblichen Einfluss auf die Resultate der *in vitro* Biotests besaßen. Das unbehandelte Rohabwasser wurde durch die Ansäuerung am meisten beeinflusst, wohingegen das ozonierte Abwasser, das Oberflächenwasser und das Grundwasser am wenigsten betroffen waren. Die Ansäuerung bewirkte die größten Veränderungen innerhalb der Biotests auf anti-östrogene Aktivität und Mutagenität. Eine Filtration hatte ebenfalls den größten Einfluss auf das unbehandelte Rohabwasser, wobei das konventionell behandelte Abwasser und das Grundwasser am wenigsten oder nicht beeinflusst wurden. Die anti-östrogene Aktivität zeigte gleichfalls die größte

Veränderung aufgrund des Filtrationsprozesses, gefolgt von der östrogenen und der anti-androgenen Aktivität. Insgesamt betrachtet hatte die Filtration der Proben einen geringeren Einfluss auf die Ergebnisse der Biotests als die Ansäuerung/Neutralisierung. Es ist daher zu empfehlen, die Wasser und Abwasserproben möglichst unbehandelt zu testen, da sie am wenigsten bearbeitet wurden. Die Extrakte der Proben zeigten zum Teil eine sehr starke Zytotoxizität, wodurch keine Rückschlüsse auf Aktivitäten in den Biotest möglich waren. Trotzdem war eine Anreicherung der endokrinen Aktivität und der Mutagenität möglich, die jedoch von dem Sorptionsmittel und dem pH-Wert der Probe abhängig war. Auf der Grundlage der Ergebnisse ist eine Verwendung der Kartusche Telos C18/ENV mit angesäuerten Wasserproben zu empfehlen, da diese Kombination die geringsten zytotoxischen Effekte und gleichzeitig die größte Anreicherung von endokriner Aktivität und Mutagenität zeigte. Abschließend ist festzuhalten, dass eine Optimierung der Probenaufbereitung zur Stabilisierung von Wasser und Abwasserproben für (öko)toxikologische *in vitro* Biotest nötig ist, um maximale Ergebnisse zu erzielen, damit eine optimale Bewertung der Wasserqualität möglich ist.

A.7 Acknowledgement

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A.8 Curriculum Vitae

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A.9 List of publications and conference contributions

Journal articles (peer-reviewed)

Schneider, I., Abbas, A., Bollmann, A., Dombrowski, A., Knopp, G., Schulte-Oehlmann, U., Seitz, W., Wagner, M. & Oehlmann, J. (2020). Post-treatment of ozonated wastewater with activated carbon and biofiltration compared to membrane bioreactors: Toxicity removal *in vitro* and in *Potamopyrgus antipodarum*. *Water Research*, *185*, 116104. https://doi.org/10.1016/j.watres.2020.116104

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Conference attendance

09/2014 Conference of the German Association for Water Management, Wastewater and Waste (DWA) on the topic “Relevance of transformation products in the urban water cycle”, Koblenz, Germany.

01/2014 RiSKWa – status seminar, DECHEMA, Frankfurt am Main, Germany.

06/2013 International Conference "Micropol & Ecohazard 2013", Zurich, Switzerland.

04/2013 Congress and trade fair „Water Berlin 2013“, Berlin, Germany.

11/2012 13th EMBL/EMBO Science and Society Conference: Biodiversity in the balance - causes and consequences, EMBL Advanced Training Centre, Heidelberg, Germany.

04/2012 Conference of the German Association for Water Management, Wastewater and Waste (DWA) on the topic “Relevance of transformation products in the urban water cycle”, Koblenz, Germany.

02/2012 Kick-off RiSKWa, Frankfurt am Main, Germany

TransRisk project meetings

03/2015 Regional water supply association (LW), Langenau (Donauried), Germany.

10/2014 Federal Institute of Hydrology (BfG), Koblenz, Germany.

05/2014 Senckenberg Biodiversity and Climate Research Centre (SBiK-F), Frankfurt am Main, Germany.

03/2014 Ludwig-Maximilian-University, Munich, Germany.
Oral presentation.

10/2013 Regional water supply association (LW), Langenau (Donauried), Germany.
Oral presentation.

07/2013 DECHEMA, Frankfurt am Main, Germany.

03/2013 Technical University, Darmstadt, Germany.
Oral presentation.

12/2012 Goethe University, Frankfurt am Main, Germany.

10/2012 Regional water supply association (LW), Langenau (Donauried), Germany.

09/2012 Federal Institute of Hydrology (BfG), Koblenz, Germany.
Oral presentation.

06/2012 Technical University, Darmstadt, Germany.
Oral presentation.

02/2012 Goethe University, Frankfurt am Main, Germany.

01/2012 Kick-off meeting, Federal Institute of Hydrology (BfG), Koblenz, Germany.
Oral presentation.