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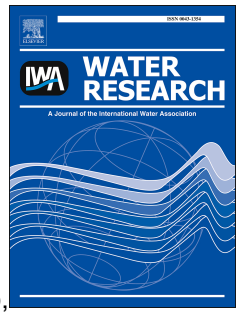

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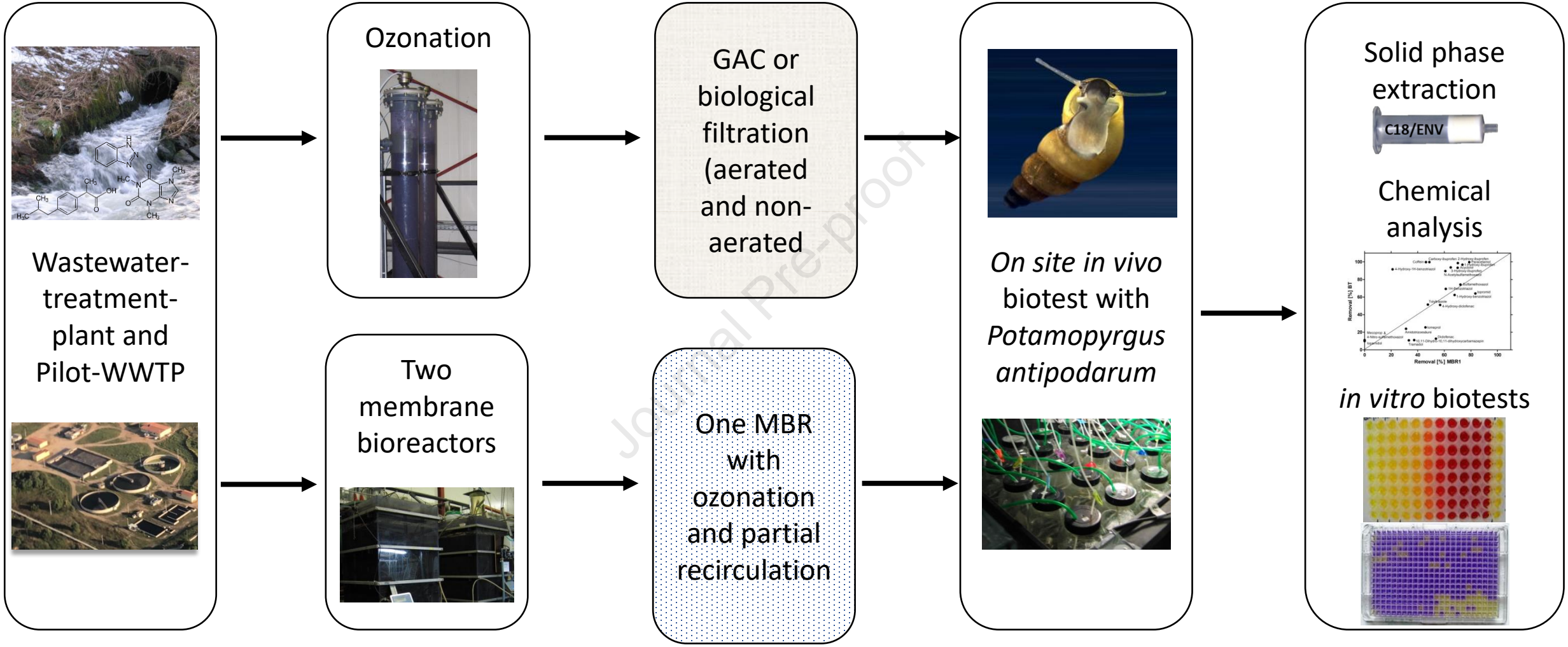

Post-treatment of ozonated wastewater with activated carbon and biofiltration compared to membrane bioreactors: Toxicity removal *in vitro* and in *Potamopyrgus antipodarum*

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Abstract

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

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

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

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

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

Keywords

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

47 **Abbreviations**

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

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

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

1 Introduction

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

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

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

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

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

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

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

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

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

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

2.2 Optimal ozone dose and hydraulic retention time

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

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

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

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

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

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

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

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

2.4 Wastewater sample preparation: Solid phase extraction (SPE)

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

2.5 *In vitro* bioassays for endocrine activities and mutagenicity

2.5.1 Recombinant yeast screens for endocrine activities

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

2.5.2 Recombinant bacterial test for mutagenicity (Ames fluctuation test)

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

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

2.6 Chemical analysis

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

2.7 Measurement of physical-chemical wastewater parameters

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

organic carbon (DOC), nitrite (NO_2-N), nitrate (NO_3-N), ammonium (NH_4-N), total phosphor (P_{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% to 79.6 ± 1.37% (Figure 2A, Table S12). No androgenic activity was detected in the BT and at all ozone doses (Figure 2B). In contrast, the anti-androgenic activity in the BT was 76.1 ± 0.72%. With increasing ozone dose, the anti-androgenic activity decreased by 35.1% to 49.3 ± 0.73% at the highest ozone dose (Figure 2B, Table S12).

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

3.1.2 Optimal hydraulic retention time

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

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

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

3.2.1 Mortality

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

3.2.2 Growth and reproduction

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

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

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

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

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

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

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

3.2.4 *In vitro* bioassays for endocrine and mutagenic activity

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

Recombinant yeast screens for endocrine activity

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

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

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

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

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

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

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

Ames fluctuation test for mutagenicity

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

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 (Figure 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 (Figures 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 to 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 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 (Figures 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 (Figure 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 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 (Figure 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 (Figure 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 (Figure 2D). The anti-estrogenic activity decreased at the lowest HRT but remained at the level of BT at higher HRTs. The results support the idea of a generation of anti-estrogenic TPs during ozonation because the estrogenic activity was on a comparable low level at all HRTs.

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

4.2 *In vivo* effects in *Potamopyrgus antipodarum*

4.2.1 Growth and reproduction

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

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

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

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

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

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

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

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

4.2.3 *In vitro* endocrine activity and mutagenicity

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

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

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

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

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

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

4.3 Removal of micropollutants

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

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

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

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

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

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

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

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

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

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.

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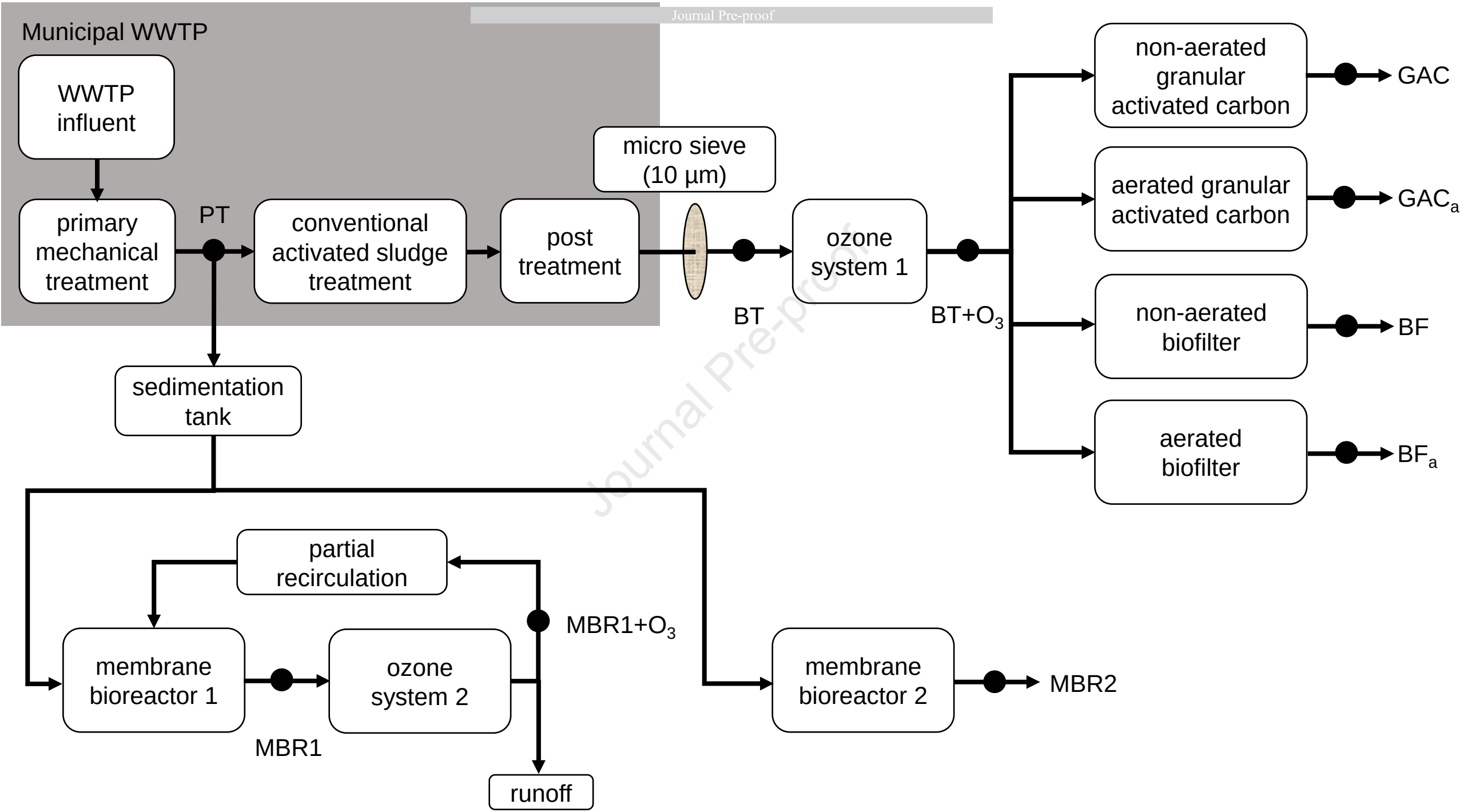


Figure 1: Process design of the WWTP and AWWT. Process design of the municipal wastewater treatment plant (WWTP) and the pilot-scale advanced wastewater treatment technologies (AWWT). Sampling points are marked with black dots. PT: after primary treatment, BT: after conventional biological treatment,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

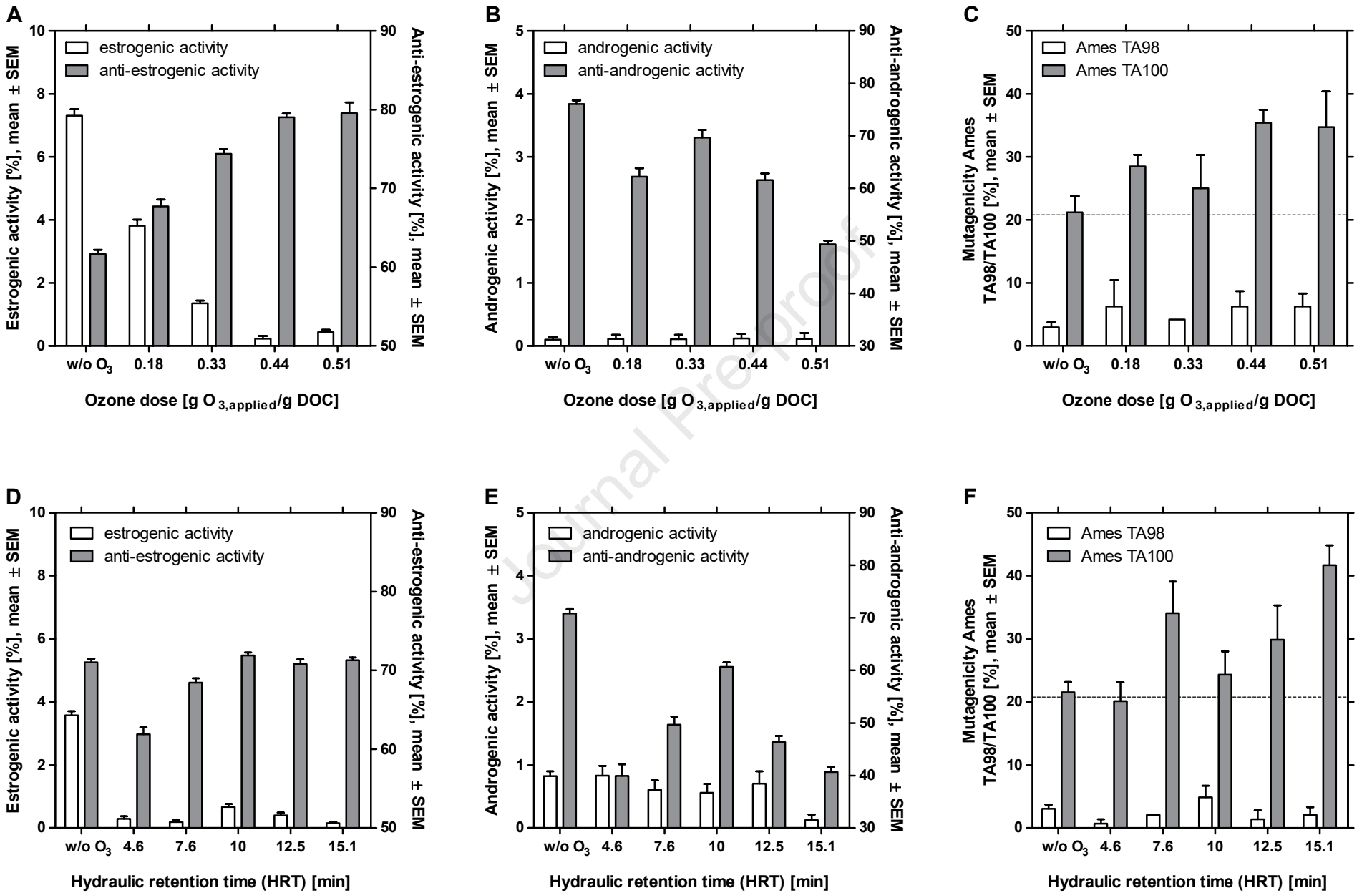
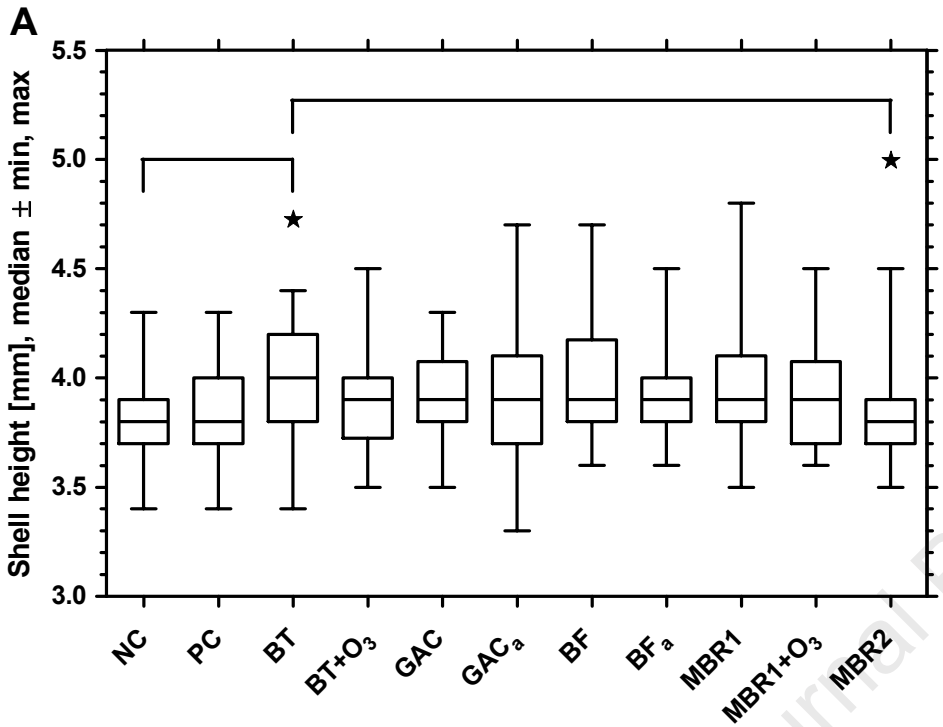


Figure 2: Optimal ozone dose and hydraulic retention time. Estrogenic and anti-estrogenic activity (A, D), androgenic and anti-androgenic activity (B, E) and mutagenicity (C, F) in % (mean ± SEM) of conventional biological treated wastewater (without ozone; A, B: n = 93–96; D, E: n = 117–120; C, F: n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



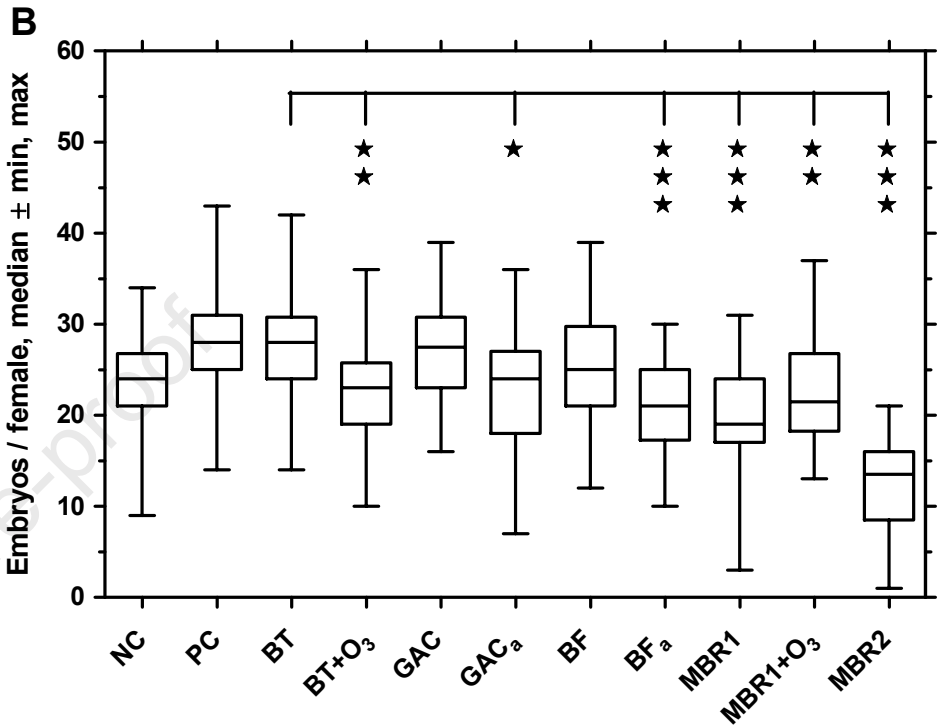


Figure 3: Growth and reproduction. Size (A) and reproduction (B) of *Potamopyrgus antipodarum* after 28 days of exposure to the negative control (NC), the positive control (PC), the conventional biological treatment (BT) and the eight advanced treatment technologies. BT+O_3: after ozone system 1, GAC: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

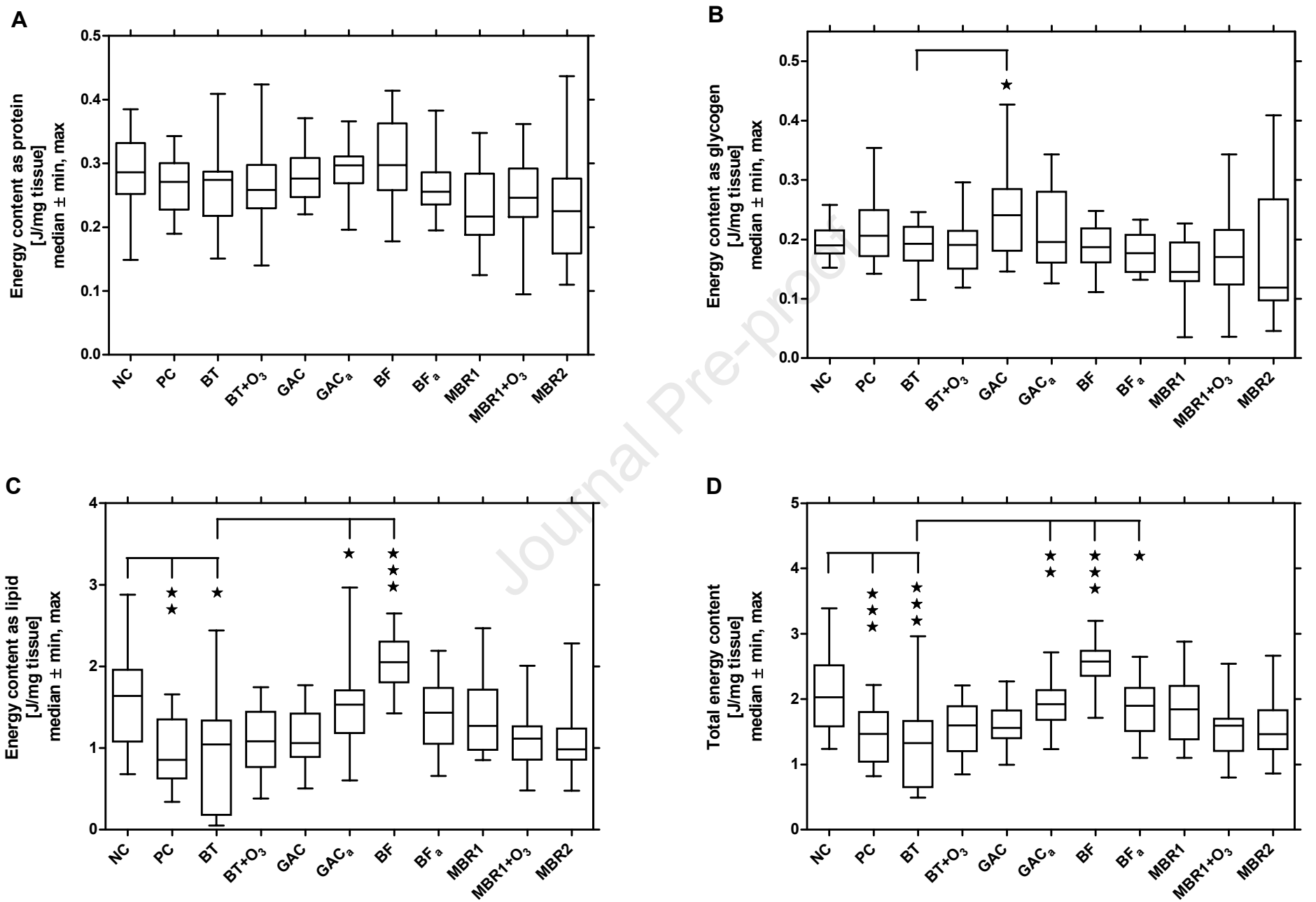


Figure 4: Biomarkers for energy reserves. Energy content as protein (A), glycogen (B), lipid (C) and total energy content (D) in J/mg tissue of *Potamopyrgus antipodarum* after 28 days exposure to water from the negative control (NC), the positive control (PC), the conventional biological treatment (BT)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

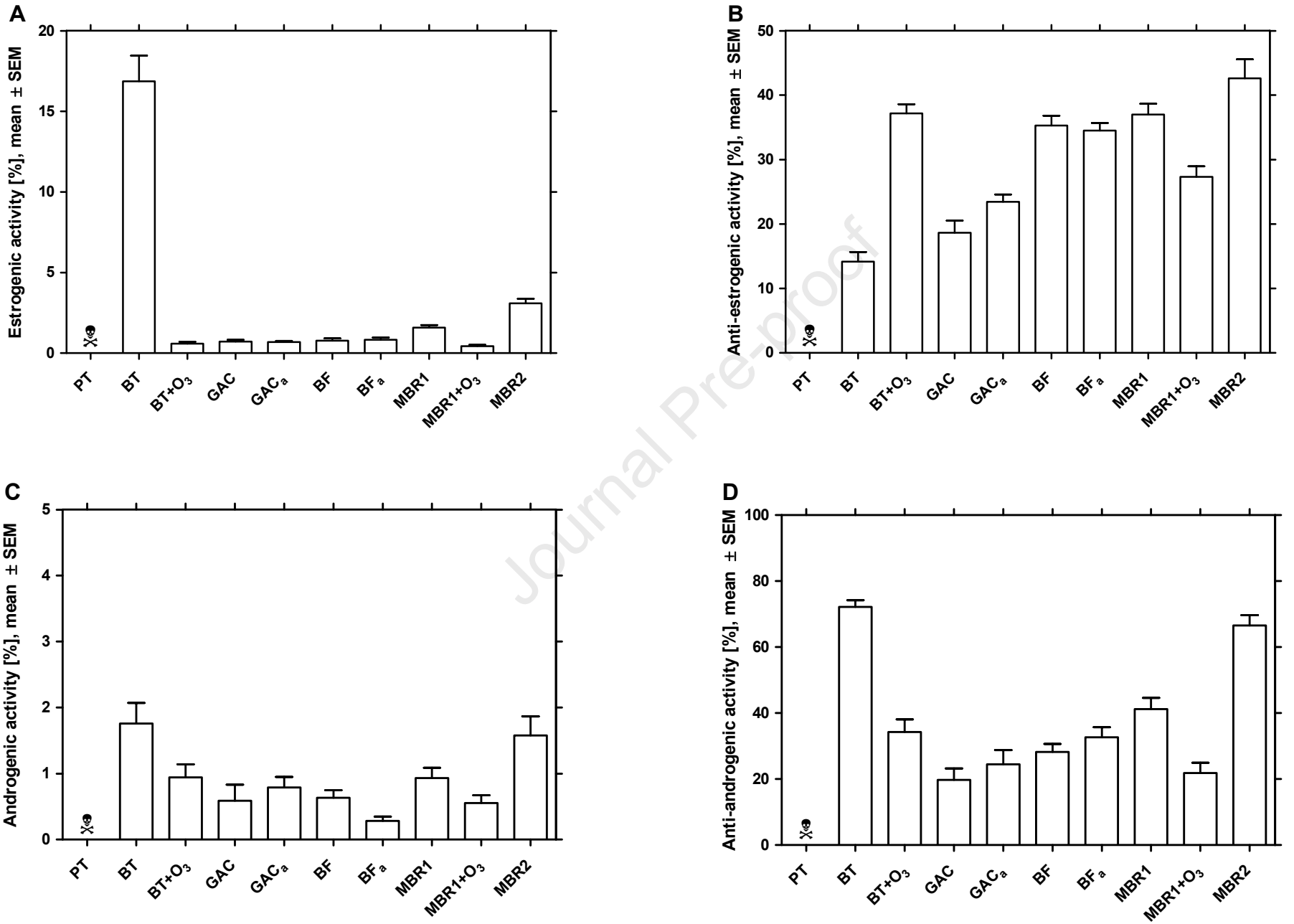


Figure 5: Endocrine activities of the on-site biotest. Estrogenic (A), anti-estrogenic (B), androgenic (C) and anti-androgenic activity (D) in SPE extracts produced from 24 h composite samples taken in parallel to the *in vivo* experiment. PT: after primary treatment, BT: after conventional biological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

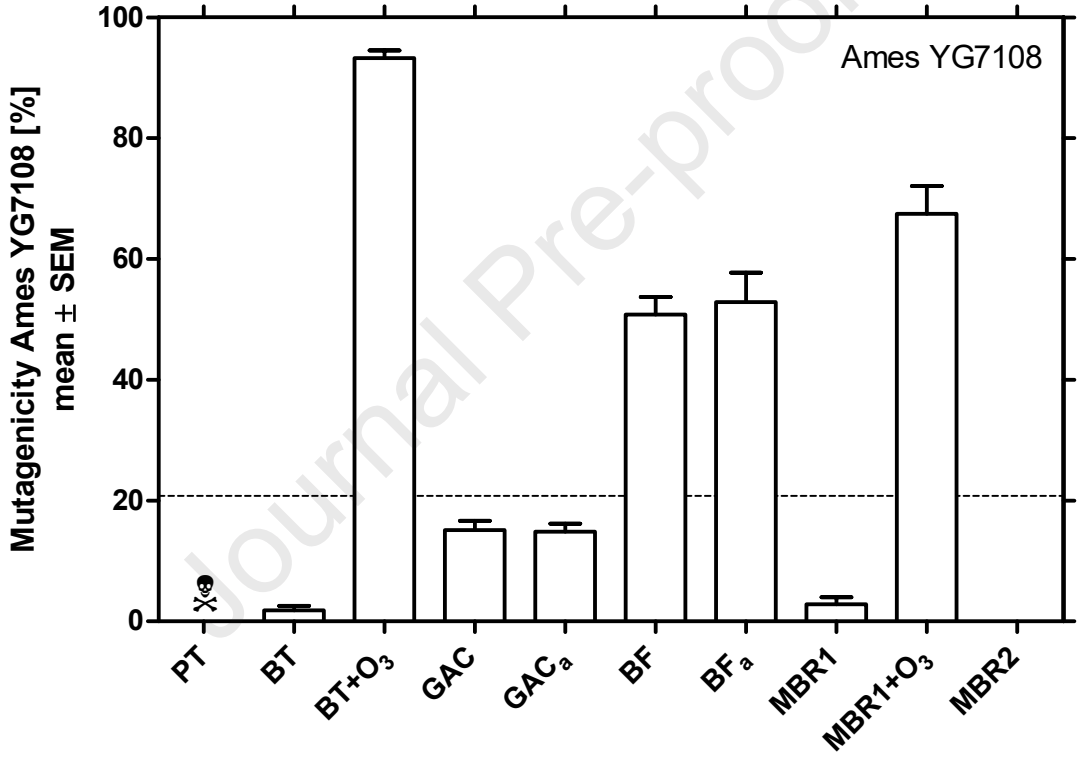
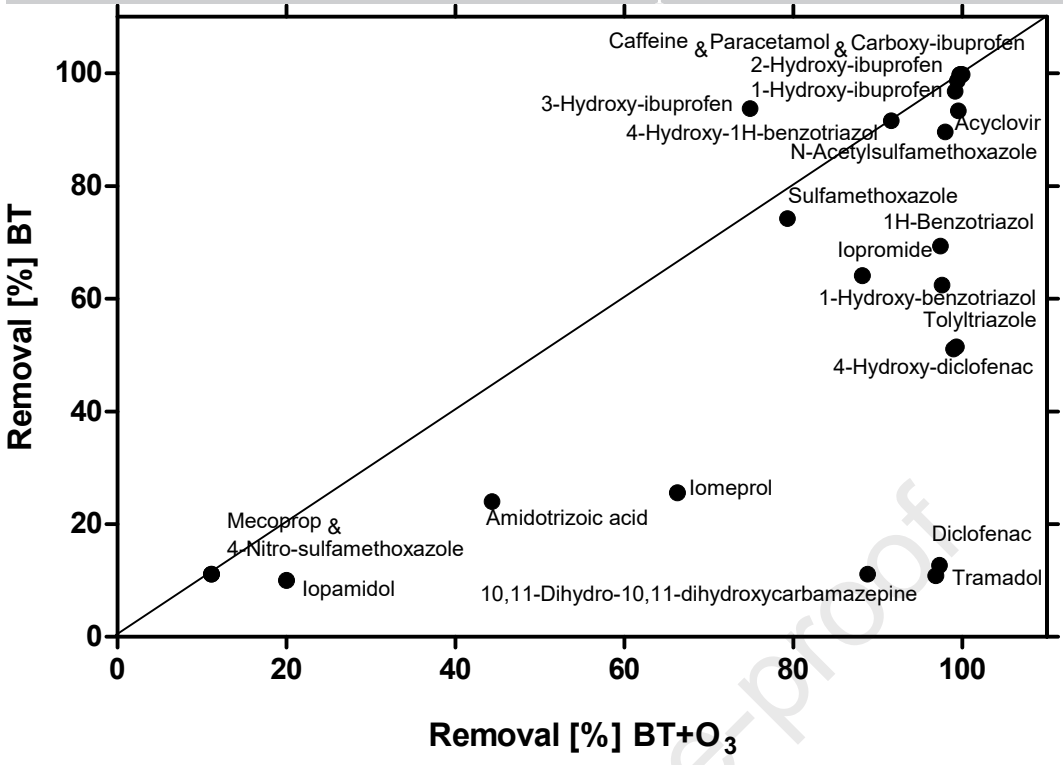


Figure 6: Mutagenicity of the on-site biotest. Mutagenicity in the Ames strain YG7108 in SPE extracts produced from 24 h composite samples taken in parallel to the *in vivo* experiment. PT: after primary treatment, BT: after conventional biological treatment, BT+O_3: after ozone system 1, GAC: after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



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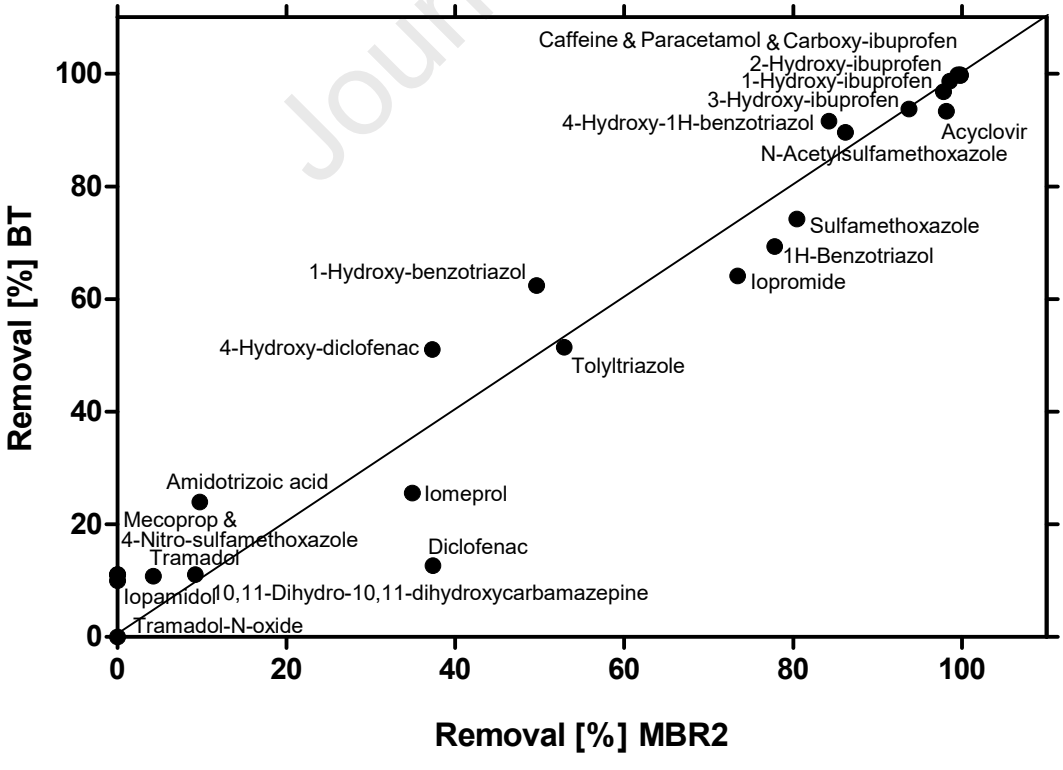

Figure 7: Chemical analysis. Removal of micropollutants by the conventional biological treatment (BT), by the ozonation (BT+O_3, A) and by the membrane bioreactor 2 (MBR2, B) compared to the primary treatment. n = 1–4.

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

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HIGHLIGHTS

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

Declaration of interests

☒ 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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: