



Evaluation of a full-scale wastewater treatment plant with ozonation and different post-treatments using a broad range of *in vitro* and *in vivo* bioassays

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ABSTRACT

Micropollutants present in the effluent of wastewater treatment plants (WWTPs) after biological treatment are largely eliminated by effective advanced technologies such as ozonation. Discharge of contaminants into freshwater ecosystems can thus be minimized, while simultaneously protecting drinking water resources. However, ozonation can lead to reactive and potentially toxic transformation products. To remove these, the Swiss Federal Office for the Environment recommends additional "post-treatment" of ozonated WWTP effluent using sand filtration, but other treatments may be similarly effective. In this study, 48 h composite wastewater samples were collected before and after full-scale ozonation, and after post-treatments (full-scale sand filtration, pilot-scale fresh and pre-loaded granular activated carbon, and fixed and moving beds). Ecotoxicological tests were performed to quantify the changes in water quality following different treatment steps. These included standard *in vitro* bioassays for the detection of endocrine, genotoxic and mutagenic effects, as well as toxicity to green algae and bacteria, and flow-through *in vivo* bioassays using oligochaetes and early life stages of rainbow trout.

Results show that ozonation reduced a number of ecotoxicological effects of biologically treated wastewater by 66 - 93%: It improved growth and photosynthesis of green algae, decreased toxicity to luminescent bacteria, reduced concentrations of hormonally active contaminants and significantly changed expression of biomarker genes in rainbow trout liver. Bioassay results showed that ozonation did not produce problematic levels of reaction products overall. Small increases in toxicity observed in a few samples were reduced or eliminated by post-treatments. However, only relatively fresh granular activated carbon (analyzed at 13,000 - 20,000 bed volumes) significantly reduced effects additionally (by up to 66%) compared to ozonation alone. Inhibition of algal photosynthesis, rainbow trout liver histopathology and biomarker gene expression proved to be sufficiently sensitive endpoints to detect the change in water quality achieved by post-treatment.

1. Introduction

Conventional wastewater treatment plants (WWTPs) discharge a wide range of organic and inorganic micropollutants into surface waters (Loos et al., 2013; Schwarzenbach et al., 2006). These include persistent pharmaceuticals, personal care products, cleaning agents, residues of pesticides as well as transformation products (Petrie et al., 2015;

Richardson and Kimura, 2016). Sensitive species and communities in aquatic ecosystems have been shown to be adversely affected by chemicals released via WWTP effluent (Jobling et al., 1998; Kidd et al., 2007; Münze et al., 2017; Stalter et al., 2013; Triebkorn et al., 2004).

In order to minimize the discharge of micropollutants from WWTPs into surface waters, Switzerland revised its water protection law in 2016. The aim is to protect flora and fauna of Swiss rivers and drinking

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water resources as well as to reduce the amount of micropollutants released to other countries (Schweizerischer Bundesrat, 1998) update: 1. January 2021). Consequently, the largest WWTPs and those discharging to lakes or watercourses with a high proportion of wastewater must be upgraded using advanced technologies such as activated carbon treatment or ozonation. Both advanced treatment methods are highly effective in removing micropollutants and their ecotoxicological effects still present in wastewater after conventional biological treatment (Escher et al., 2008b; Margot et al., 2013; Prasse et al., 2015; Stalter et al., 2013; Völker et al., 2019). Which of these advanced treatments is best suited depends on the technical design of the WWTP as well as the type of wastewater (Wunderlin et al., 2017). While activated carbon adsorbs micropollutants, ozonation oxidizes the compounds and dissolved organic matter, which produces transformation products and oxidation by-products such as aldehydes and ketones, which may be toxic (Lee and Von Gunten, 2016; Schindler Wildhaber et al., 2015; Stalter et al., 2010). Problematic substances and their associated effects were shown to be removed by post-treatment, applied to abate assimilable organic carbon and biodegradable organic carbon (Hollender et al., 2009; Kienle et al., 2011; Stalter et al., 2010). For this reason, WWTPs in Switzerland which apply ozonation for advanced wastewater treatment are required by law to evaluate the suitability of their wastewater for ozonation by a defined procedure (Wunderlin et al., 2017) and to include a biological post-treatment step before effluent can be discharged to surface waters (Schweizerischer Bundesrat, 1998; Wunderlin et al., 2017). Full-scale ozonation is also implemented internationally as an advanced wastewater treatment technique, e.g. in Germany (Dopp et al., 2021; Wolf et al., 2022) and Canada (Maya et al., 2018; Nasuhoglu et al., 2018).

Various methods are available as post-treatments. Sand filters (SF), moving bed (MB) and fixed bed (FB) systems utilize biofilms to degrade chemicals, while granular activated carbon (GAC) filters remove chemicals mainly by adsorption. Schollée et al. (2018) showed that these post-treatment methods removed 54–83% of around 700 features formed in ozonation detected by non-target screening, with GAC filters performing best. Target analysis also showed a better performance of GAC filters compared to biological systems in removing ozonation transformation products (OTP) from selected micropollutants (Bourgin et al., 2018; Gulde et al., 2021). However, ozonation effluents contain a multitude of unidentified substances, and it is not clear if post-treatment can efficiently remove the ecotoxicity of such mixtures at a specific location, or which post-treatment methods are most efficient.

Bioassays allow the detection and quantification of the combined effects of chemical mixtures in organisms or cells (Connon et al., 2012). Mode of action specific *in vitro* assays measure effects at the cellular level and are suitable for high throughput applications in the laboratory. *In vivo* bioassays measure lethal and sublethal toxicity to model organisms exposed in static or flow-through systems. Using a battery of bioassays, this study evaluated the effectiveness of full-scale ozonation for eliminating the ecotoxicity of wastewater constituents, including micropollutants, from conventionally treated wastewater, and of different post-treatment methods (full-scale and pilot-scale) in removing toxic reaction products. It complements a previous study from the same WWTP and project (Bourgin et al., 2018), which focused on the abatement of micropollutants and the formation of transformation products and oxidation by-products. Performing these studies on a full-scale plant provides the advantage that no extrapolation of results from laboratory to full-scale is necessary. Bioassays were selected based on results of (Escher et al., 2008b; Kienle et al., 2011; Margot et al., 2013), and included *in vitro* assays to assess endocrine activity, oxidative stress and mutagenicity, as well as *in vivo* bioassays using organisms at different trophic levels. Long-term tests with oligochaete worms and rainbow trout were performed in flow-through systems on site at the WWTP. In addition to standard effect endpoints, rainbow trout livers were examined for histopathological alterations and expression of selected biomarker genes. Our specific goals were three-fold: (i) to monitor the

occurrence of mutagenic potential of effluent due to ozonation, (ii) to compare the efficacy of different post-treatment technologies in removing ecotoxicity after ozonation, and (iii) to identify a set of ecotoxicological endpoints suitable for routine monitoring of ozonated wastewater.

2. Material and methods

2.1. Experimental site

In 2014, Neugut (Dübendorf, Switzerland) was the first WWTP in Switzerland to begin operation of a full-scale advanced wastewater treatment using ozonation followed by sand filtration (SF), in addition to a primary clarifier, conventional activated sludge treatment (aerobic and anaerobic including biological phosphorus removal) and a secondary clarifier (biological treatment). The WWTP has a maximum capacity of 150,000 population equivalents with an approximate industrial contribution of 50% (based on chemical oxygen demand) primarily from the food and fragrance industry. The quality of the treated secondary effluent is presented in detail in Bourgin et al. (2018) and is typical of Swiss domestic wastewater (Schindler Wildhaber et al., 2015; Soltermann et al., 2016).

Ozone was dosed to the effluent of the secondary clarifier in a full-scale multi-chamber ozonation reactor with a total volume of 530 m³ and an average retention time of 43 min during dry weather. Ozone was completely depleted under these conditions. Five post-treatment technologies were investigated: In addition to the existing sand filter (full-scale; quartz sand, 0.7 to 2.0 mm granular size with an average empty bed contact time (EBCT) of 10 min), four post-treatments (pilot scale) were installed for the purpose of this project. These were: a moving bed (300 L, filled with BWT15 carriers (14.5 × 14.5 × 5 mm), Biowater Technology AS, Norway), average EBCT 21 min), a fixed bed (220 L, filled with a stationary honeycomb pack (Wabag, Switzerland), average EBCT 25 min), a fresh GAC filter (GAC_{fresh} 77 L, filled with 32.4 kg Cyclecarb 401, density 450 g/L, 0.425 to 2.36 mm granular size, average EBCT 14.5 min) and a pre-loaded GAC filter (GAC_{loaded} 85 L, filled with 40.8 kg Norit GCN 830, density 480 g/L, 1.4 to 2.36 mm granular size, average EBCT 18 min). Additional details on technical operation and set-up are described by Bourgin et al. (2018).

2.2. Sampling and sample preparation

Samples were collected from the primary clarifier (PC), the secondary clarifier (SC), the ozonation reactor (OZ) (all full-scale) and the post-treatments described above (full-scale or pilot-scale) (Fig. 1). Flow-proportional composite samples (3 L) were collected in pre-conditioned glass bottles (rinsed three times with acetone) during three campaigns (48 h periods; February 2/3 (Campaign 1), March 9/10 (Campaign 2) and April 13/14 (Campaign 3), 2015) using automated samplers (SP5 S, MAXX, Germany and WS 316, WaterSam, Germany). Note that PC samples were always collected one day before the others to account for the hydraulic retention time (HRT) of 18 h during conventional biological treatment. The ozone dose (2.7–2.8 g O₃/m³), corresponding to a specific ozone dose in the range of 0.50–0.55 g O₃/g DOC, was maintained constant during sampling. At the sampling dates, GAC_{fresh} had run for 13,000–20,000 bed volumes and GAC_{loaded} for 35,000–43,000 bed volumes (see also SI section I). Samples were transported and stored at 2–8 °C until further analyses.

A 2.5 L aliquot of each sample was shipped (at 2–8 °C) to Solvial Santiago (Couvett, Switzerland), where the waterflea (*Ceriodaphnia dubia*) reproduction test was initiated within 24 h of sample collection. A second aliquot (0.2 L of WWTP influent (PC), 0.5 L of all other samples) was immediately filtered (1 µm, Millipore glass fiber filter, type APFD 09,050), acidified to pH 3 using 1 M HCl and stored at 4 °C. The following day, aliquots of 200 mL (PC sample) and 500 mL (all other samples and deionized water as negative control) were extracted and

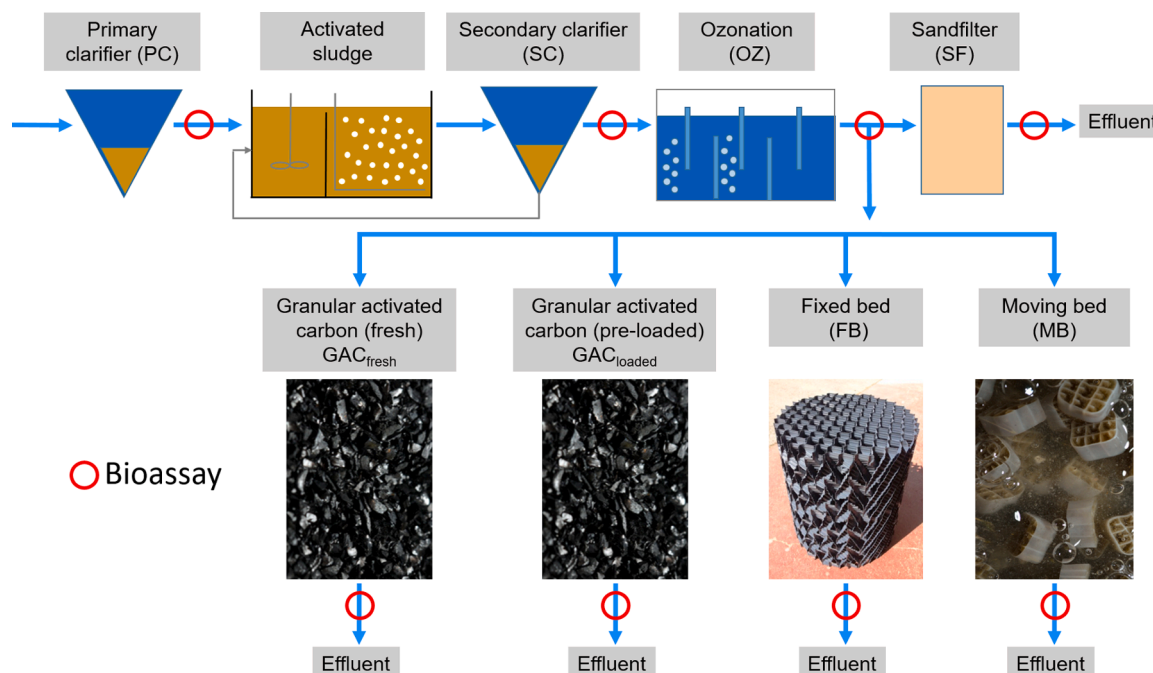


Fig. 1. Process flow diagram of the WWTP Neugut, Switzerland (modified from Böhler et al. 2017, Bourgin et al. 2018). Full-scale: primary treatment with clarifier, biological treatment with secondary clarifier, ozonation and sand filter; pilot-scale: granular activated carbon filters, fixed bed, moving bed.

concentrated using solid phase extraction (Lichrolut RP18/EN cartridges, Merck, Darmstadt, Germany) based on Escher et al. (2008a). Cartridges were eluted four times using 1 mL acetone and 1 mL MeOH. Solvent was reduced to ~0.5 mL using nitrogen, then brought to a final volume of 1 mL using EtOH. Concentrated samples (in a solvent mixture of ~50% ethanol, ~50% acetone and methanol) were stored at -20°C until *in vitro* bioassays were initiated. Further details on sample preparation are provided in SI Table S1.

2.3. Bioassays

Table 1 lists bioassays performed in this study and respective references for method details. Brief method descriptions are provided below, and enrichment factors of sample extracts are provided in SI Table S2.

2.3.1. Ames fluctuation assay

The Ames fluctuation assay was performed by Xenometrix AG (Allschwil, Switzerland) according to Gee et al. (1994) and International Organization for Standardization (2012). Two strains of *Salmonella typhimurium* with different mutations were used to detect base pair substitution (strain TA 98) or frameshift mutations (strain TA 100). The test was performed with and without metabolic activation (S9 mix) to simulate a transformation and activation of substances by metabolic enzymes. Sample extracts were tested in triplicate with DMSO as solvent and a maximum relative enrichment factor (REF) of 20 in the assay. Three substances, 2-nitrofluorene, 4-nitroquinolin-N-oxide and 2-aminoanthracene served as positive controls (see Table 1), and DMSO as a negative control. Bacteria were pre-cultivated in growth medium (Oxoid Broth, UnipathOxoid, Basingstoke, UK) for 24 h at 37°C . Subsequently, they were exposed to the samples in exposure medium with limited histidine for 90 min on a microtiter plate shaker (37°C , 250 rpm), and then mixed with histidine-free indicator medium. The mixture was distributed on 384-well plates and incubated for 48 h at 37°C . A sample was classified as mutagenic if the number of reverse mutations exceeded the threshold of double the baseline value (mean + standard deviation of reverse mutations in the negative control). Data analysis and classification of samples followed protocols described by Flückiger (2015) and are summarized in SI Table S3.

2.3.2. CALUX® assays

Chemical Activated Luciferase gene eXpression (CALUX®) assays were performed at BioDetection Systems B.V. (BDS, Amsterdam, Netherlands). Tests use a genetically modified human U2OS cell line to indicate the activation of different cellular receptors via luciferase activity. For this study, we selected assays indicative of genotoxicity (p53-CALUX®), estrogenicity (ER α -CALUX®), (anti-) androgenicity (anti-AR-CALUX®), (anti-) progesterone-like activity ((anti-)PR-CALUX®), peroxisome proliferator-like activity (PPAR γ 1-CALUX®), oxidative stress (Nrf2-CALUX®), and the activation of metabolism (pregnane X receptor, PXR-CALUX®). The p53-CALUX® assay was performed with and without metabolic activation (S9 mix). Assays were performed on 96-well microtiter plates. Sample extracts in 1% DMSO (Nrf2 and p53 (+/-S9)) or 0.1% DMSO (all other assays) were tested in triplicate at 1, 3, 10, 30 and 100-fold dilutions. Substances used as positive controls are listed in Table 1. Growth medium (DF medium supplemented with stripped (dextran-coated charcoal treated) serum) served as negative control and assay medium (DF medium supplemented with stripped (dextran-coated charcoal treated) serum and with 0.1% or 1% of solvent DMSO) served as solvent control. After pre-incubation of cells with growth medium for 24 h at 37°C and 5% CO_2 , medium was replaced with assay medium containing 0.1% or 1% sample extract in DMSO and cells were exposed for 24 h at 37°C . Afterwards, the assay medium was removed and the cells lysed in 30 μL Triton Lysis Buffer. Subsequently, luciferase activity was measured using a luminometer (Lucy 2, Anthos, Austria). Effect data are expressed as concentrations of bioanalytical equivalents (BEQ/L) (Escher et al., 2008a, 2015) of the respective reference substance (positive controls). Data were analyzed using Microsoft Excel and GraphPad Prism (Version 8.1.2). Statistical significance was evaluated by two-way repeated measures ANOVA followed by Tukey's multiple comparison test.

2.3.3. Bacterial luminescence inhibition assay

Bacteria (*Aliivibrio fischeri*) and reactivation solution were obtained from Hach Lange GmbH (Düsseldorf, Germany), and assays were performed according to ISO 11348-3 (International Organization for Standardization, 2007) adapted to 96-well microtiter plates (Escher et al., 2008a). Sample extracts, negative (EtOH) and positive controls

Table 1

Bioassays applied to monitor efficiency of advanced wastewater treatment methods (LOD limit of detection; n.a. not available).

Assay/Organism	Effect	Toxicity Endpoint(s)	Sample Type	Positive control (max. concentration tested)	LOD	Method
Ames fluctuation assay (+/- S9)	Mutagenicity	Number of wells with revertants	SPE extract	2-nitrofluorene (9.5×10^{-6} M) 4-nitroquinolin-N-oxide (5.3×10^{-7} M) 2-aminoanthracene (1.3×10^{-5} M)	n.a.	Gee et al. (1994), International Organization for Standardization (2012)
p53-CALUX® (+/- S9)	Genotoxicity	Cellular tumor antigen p53 receptor activation	SPE extract	actinomycin D (1×10^{-6} M) cyclophosphamide (3×10^{-5} M)	0.02–0.05 µg/L actinomycin D 1100–2750 µg/L cyclophosphamide	Van der Linden et al. (2014)
Nrf2-CALUX®	Oxidative stress	Nuclear factor erythroid-derived 2-like 2 (transcription factor) activation	SPE extract	curcumin (1×10^{-4} M)	26–65 µg/L curcumin	Van der Linden et al. (2014)
PPARγ-CALUX®	Disturbance of lipid metabolism	Peroxisome proliferator-activated receptor γ (PPARγ) activation	SPE extract	rosiglitazone (1×10^{-5} M)	88–220 ng/L rosiglitazone	Gijsbers et al. (2011)
ERα-CALUX®	Estrogenicity	Estrogen receptor α activation	SPE extract	17β-estradiol (1×10^{-10} M)	0.06–0.15 ng/L 17β-estradiol	Van der Linden et al. (2008)
anti-AR-CALUX®	anti-Androgenicity	Androgen receptor inhibition	SPE extract	flutamide (1×10^{-5} M)	2,920–7,300 ng/L flutamide	Van der Linden et al. (2008)
(anti-)PR-CALUX®	(anti-) Progesterone-like activity	Progesterone receptor activation or inhibition	SPE extract	Org2058 (1×10^{-8} M) (PR) Ru486 (1×10^{-9} M) (anti-PR)	0.18–0.45 ng/L Org2058 (PR) 0.38–0.95 ng/L Ru486 (anti-PR)	Van der Linden et al. (2008)
PXR-CALUX®	Xenobiotic metabolism	Pregnane X receptor activation	SPE extract	nicardipine (1×10^{-5} M)	22–55 µg/L nicardipine	BioDetection Systems (unpublished 2017)
Cytotox-CALUX®	Cell viability	Change in cell viability	SPE extract	n.a.		Van der Linden et al. (2008)
Bacteria (<i>Aliivibrio fischeri</i>)	Non-specific toxicity	Inhibition of luminescence	SPE extract	3,5-dichlorophenol, (3×10^{-4} M)	5.6 mg/L 3,5-dichlorophenol (without extraction) 0.03–0.22 mg/L 3,5-dichlorophenol (SPE extract)	Escher et al. (2008a), International Organization for Standardization (2007)
Green algae (<i>Raphidocelis subcapitata</i>)	Herbicidal activity	Inhibition of photosynthesis and growth	SPE extract	diuron (3.0×10^{-7} M)	192 ng/L diuron (without extraction) 1.5–2 ng/L diuron (SPE extract)	Escher et al. (2008a), Schreiber et al. (2007)
Waterflea (<i>Ceriodaphnia dubia</i>)	Survival and reproduction	Mortality, number of offspring	Ambient, static	n.a.	n.a.	AFNOR (2000), International Organization for Standardization (2008)
Oligochaete (<i>Lumbriculus variegatus</i>)	Survival and growth	Mortality, number of offspring	Ambient, flow-through	n.a.	n.a.	OECD (1992b)
Rainbow trout (<i>Oncorhynchus mykiss</i>)	Survival and development, biomarkers	Mortality, hatching, swim-up, growth, histo-pathological lesions, oxidative stress, expression of biomarker genes	Ambient, flow-through	n.a.	n.a.	OECD (2007)

(3,5-dichlorophenol, Table 1) were tested in triplicate in a 1:2 dilution series, with maximum REF of 67, 25, and 25 (PC), 167, 125, and 125 (SC) (Campaign 1, 2, and 3, respectively) and 167 (after ozonation and post-treatments) in the assay. The extracted samples were diluted 1:2 with ethanol on the test plate. After complete evaporation of the solvents, the samples were re-dissolved in 120 µL bacterial medium. In parallel, 100 µL freeze-dried and reactivated bacteria (for 15 min at 15 °C) were added to each well of a white 96-well plate and the initial luminescence of the bacteria measured using a plate reader (Synergy 2, Biotek, Winooski, USA). Subsequently, 100 µL of the re-dissolved samples were added and the plate incubated for 30 min at room temperature in the dark. Afterwards, luminescence was measured again. Luminescence inhibition was calculated relative to the control, and expressed as baseline-toxicity equivalent concentrations (baseline-TEQ/L) in relation to a "virtual substance" with $\log K_{ow} = 3$ and molecular weight = 300 g/mol (Escher et al., 2008a). Statistical significance was evaluated by two-way repeated measures ANOVA followed by Tukey's multiple comparison test (GraphPad Prism, version 8.1.2).

2.3.4. Combined algae assay

The bioassay with green algae (*Raphidocelis subcapitata*) was carried out according to (Escher et al., 2008a; Margot et al., 2013). Algae stocks were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Göttingen, Germany), maintained in-house and cultured in Talaquil growth medium (Le Faucheur et al., 2005) twice for at least 3 d before the test. At test initiation, the positive control diuron (30 µL/well in ethanol), sample extracts (80 µL/well) and the solvent control (ethanol: 80 µL/well, $n = 8$) were pipetted onto the plates. Samples and positive control (Table 1) were tested in triplicate as a 1:2 dilution series. Maximum REF of the sample extracts in the assay were 27, 40 or 53 (PC), 100, 117 or 133 (SC) (Campaign 1, 2 and 3, respectively), and 133 (all other treatment steps). After complete ablation of the solvent, samples were resuspended in 150 µL Talaquil assay medium. Finally, 150 µL of algae suspension (OD 0.1) were added to each well. Photosystem II (PSII) inhibition was measured as effective quantum yield after 2 h using a Maxi IPAM (imaging pulse amplitude modulation) fluorimeter (Walz, Effeltrich, Germany) as described by Escher et al. (2008a), Schreiber et al. (2007). Algal cell density was measured on a

plate reader (absorbance at $\lambda=685$ nm; Synergy 2, Biotek, Winooski, USA) at test begin (0 h) and after 24 h, plus two time points in between. PSII inhibition and growth reduction were expressed as diuron equivalent concentrations (DEQ/L) (Kienle et al., 2019). Statistical significance was evaluated by two-way repeated measures ANOVA followed by Tukey's multiple comparison test (GraphPad Prism, version 8.1.2).

2.3.5. Waterflea reproduction bioassay

Ceriodaphnia dubia reproduction assays (7 d) were performed by Soluval Santiago (Couvett, Switzerland) according to ISO 20665 (International Organization for Standardization, 2008; AFNOR, 2000) with some modifications regarding control medium and food (see below). Waterflea came from an in-house culture. Control water (moderately hard, hardness 90 ± 10 mg/L CaCO_3) consisted of a mixture of 25% Evian mineral water, 25% Elendt M4 medium (Elendt and Bias, 1990) and 50% deionized water supplemented with selenium and vitamin B12. Test organisms were fed a mixture of yeast, TetraMin® flakes and green algae (*Raphidocelis subcapitata* and *Chlorella* sp.). Neonates (< 24 h old) were exposed to a geometric dilution series of each sample (11–90% sample) with 12 replicates per dilution. Tests were performed at 25 ± 1 °C and a 16:8 h light:dark cycle (300 to 500 lux). Each day, the adult females were transferred to containers with fresh control and test solutions and survival of adults and the number of neonates was recorded. All tests met validity criteria. If toxicity was observed, results were reported as EC_{50} or EC_{20} with 95% confidence intervals (REGTOX v. 7.0.5 software (Vindimian, 2008)). The “no observed effect concentration” (NOEC) and the “lowest observed effect concentration” (LOEC) were calculated using TOXSTAT v. 3.4 Software (WEST and Gulley, 1994).

2.3.6. Flow-through toxicity assay with oligochaete worms

Freshwater oligochaete worms, *Lumbriculus variegatus*, were obtained from ECT Oekotoxikologie GmbH (Flörsheim, Germany), and synchronized for 2 weeks before test initiation according to OECD guideline 225 (OECD, 2007). The assay was performed from March 5 to April 2, 2015 in a flow-through system provided by ECT Oekotoxikologie GmbH (SI, Figs. S1 and S2). Undiluted effluent from SC, OZ and post-treatments were collected in stainless steel reservoirs and filtered (membrane cartridges, pore size: 0.4 μm , MBR Type 203, Kubota, Japan) using peristaltic pumps (Ismatec SA VARIO, and Heidolph Pumpdrive 5001, both Switzerland) and Tygon tubes (ID = 6.4 mm, IDEX Health & Science, Germany). A second peristaltic pump (Ismatec BVP and IPS, Switzerland) distributed effluents and control water (reconstituted water according to (OECD, 1992b)) to exposure chambers (250 mL glass beakers, $n = 6/\text{treatment}$) at a nominal flow rate of 10 mL/min via Teflon tubes (2 mm OD x 0.5 mm ID, Saint-Gobain, Germany). More information on the test setup is provided in SI Table S4.

One day before test initiation, quartz sand (approx. 80 mL, 3 cm deep mixed with TetraMin® at 0.4–0.5% of sediment d.w.) and effluent or control water were added to respective exposure chambers. The temperature of the water bath was adjusted to 20 ± 2 °C. Ten oligochaetes of similar size were placed into each chamber. Exposure water was gently aerated via PVC tubes with Teflon tips (6 x 4 mm [OD/ID], Rebie, Germany; and 2 x 0.5 mm [OD/ID], Saint-Gobain, Germany). After 28 d, worms were counted and weighed (wet weight) then placed on aluminum foil, dried (12 h, 100 °C) and weighed again (dry weight). Ash-free dry weight was determined after additional drying (12 h, 550 °C). Ammonium concentration was measured three times per week (range: 0.003 to 1.85 mg $\text{NH}_4\text{-N/L}$, highest concentrations measured at day 5 in all treatments) or at the beginning and end of the test (DO: 84–99%, pH: 7.6–8.6, temperature: 17.1–19.2 °C and hardness: 214–303 mg CaCO_3/L), and were within ranges specified for test acceptability (OECD, 2007). Data for reproduction (number of worms) and biomass (mg dry weight/replicate) were analyzed using one-way ANOVA (GraphPad Prism, version 8.1.2).

2.3.7. Flow-through fish early life stage toxicity (FELST) assay with rainbow trout

Unfertilized eggs and sperm of rainbow trout (*Oncorhynchus mykiss*) were obtained from an organic fish hatchery (Biofischzucht Nadler, Rohr, Switzerland; <http://www.biofischzucht.ch>). After verification of adequate sperm motility using a microscope, eggs and sperm of two females and three males were mixed by hand for 3 min at 9.1–9.6 °C then incubated for 10 min. Sixty fertilized eggs were placed into each exposure chamber.

The flow-through system provided by ECT Oekotoxikologie GmbH (Flörsheim, Germany) (SI, Fig. S1) contained three replicate exposure chambers (8 L stainless steel vessels) per treatment and four controls assigned randomly. Control water consisted of reconstituted water (deionized water supplemented with salts) (OECD, 1992a). Temperature in test chambers was maintained at 10 ± 2 °C (embryos) or 12 ± 2 °C (larvae) using water baths with two heating/cooling elements (Julabo F32-MW, Switzerland, and B400 KH DLK, Van der Heijden Labortechnik GmbH, Germany). Water was pumped to exposure chambers at a flow rate of 11 ± 1 mL/min (days 0–31), 13 ± 1 mL/min (days 32–48), and 17 ± 2 mL/min (days 49–96). Water was aerated continuously (ca. four bubbles/s) via PVC tubes with Teflon tips (6 x 4 mm [OD/ID], Rebie, Germany; and 2 x 0.5 mm [OD/ID], Saint-Gobain, Germany). Embryos were maintained in the dark. After all control embryos hatched (day 37), photoperiod was adjusted to 16:8 h D:L (see Table S5 for more details). To obtain sufficient tissue for biomarker analyses, the exposure period was extended to 96 d (January 22 - April 28, 2015), 32 d beyond the standard test period of 64 d post-hatch (OECD, 1992b).

During the test, water temperature was monitored daily (Seven Go Duo Pro, Mettler Toledo, Greifensee, Switzerland), and flow rate was verified three times a week. Conductivity (Seven Go Duo Pro), pH, dissolved oxygen (DO, Oxi 3315, WTW, Germany), water hardness (Qualipet AG, Switzerland) and ammonium/nitrate concentration (LCK 304, Hach/Lange, Germany) were measured weekly. The number of coagulated eggs, hatched, swim-up and dead larvae/juveniles was recorded daily. Coagulated eggs were removed on day 4. On day 19, unfertilized eggs were removed and the number of eggs was reduced to 30 per replicate. After swim-up, larvae were fed *ad libitum* with rainbow trout starter feed (Aller Performa, Emsland-Aller Aqua GmbH, Gölßen, Germany). On day 96, fish were euthanized (MS-222, Roth, Germany), blotted dry, weighed (PCB precision scale, Kern, Germany) and measured (standard/total length). The number of fish measured per treatment was 112 (Control), 71 (SC), 77 (OZ), 82 (SF), 78 (FB), 68 (MB), 83 ($\text{GAC}_{\text{fresh}}$) and 80 ($\text{GAC}_{\text{loaded}}$), respectively. For histopathological analyses, liver samples of five fish per test vessel (for a total of 15 per treatment) and four fish per control replicate (for a total of 16) were collected, preserved using 2% Glutaraldehyde in Sørensen's phosphate buffer (fixation solution, see SI Table S6) and stored at 4 °C. For gene expression analyses, liver and kidney samples were frozen in liquid nitrogen and stored at -80 °C (Table 2). For oxidative stress measurements, muscle (all treatments) and liver (only treatments: OZ, SF, $\text{GAC}_{\text{loaded}}$ and control) samples were frozen in liquid nitrogen and stored at -80 °C. All procedures for the FELST assay were performed in compliance with relevant laws and institutional guidelines. The appropriate institutional committee(s) have approved them.

Data were analyzed using GraphPad Prism (Version 8.1.2) and JMP 11.1.1 (SAS Institute Inc., USA). Pre- and post-hatch survival, hatch, swim up, and larval/juvenile survival (64 d, 96 d) data as well as weight and standard length data were analyzed using a one-way ANOVA followed by Tukey's multiple comparisons test. A Steel-Dwass-Test was used to analyze temperature, oxygen concentration and flow data.

2.3.8. Biomarker analyses in juvenile rainbow trout

Histopathological analysis: Liver samples were washed, placed into biopsy embedding cassettes (Langenbrick, Emmendingen, Germany), transferred into an automatic tissue processor (Leica TP1020, Germany) and embedded in paraffin (Paraplast, Leica Biosystems, Germany).

Table 2

List of selected biomarker genes and the associated cellular function.

Cellular response	Gene
Xenobiotic transport	ABCB1 (ABC-Transporter B1, P-Glycoprotein, MDR1) ABCC2 (ABC-Transporter C2, Multidrug resistance-associated protein 2 (MRP2))
Cell cycle	p53 tumor suppressor (p53) gene
Biotransformation	CYP1A (Cytochrom P450 1A) CYP3A (Cytochrom P450 3A) GST (Glutathione-S-transferase) UGT (UDP-glucuronosyltransferase)
Immune regulation and pathogen defense	TNF α (Tumor necrosis factor alpha)
Endocrine disruption	IL-1 β (Interleukin-1 β)
Metal stress	VTG (Vitellogenin)
Oxidative stress	MTb (Metallothionein B) NrF2 (Nuclear factor (erythroid-derived 2)-like 2) GR (Glutathione reductase) PGC-1 α (Peroxisome proliferator-activated receptor) CAT (Catalase) SOD (Superoxide dismutase)
Metabolism	PEPCK (Phosphoenolpyruvate carboxykinase)

Hematoxylin-Eosin and Alcian blue-periodic acid Schiff were used for staining (see SI Table S7). Tissue sections were examined using a light microscope with integrated camera (Axioscope 2 and AxioCam MRc, Zeiss, Germany). Based on the semi-quantitative evaluation of lesions, samples were assigned to five stages: 1 = control, 2 = intermediate, 3 = reaction, 4 = severe, 5 = destruction (for details see SI Table S8). Data were analyzed using JMP 11.1.1 (Mac Version 2012, SAS Institute Inc., USA) and Microsoft Excel (Mac Version, 2011, Microsoft Corp., USA). Normal distribution and homogeneity of variance were determined using the Shapiro-Wilk-Test and the nonparametric Levene's Test. Tukey-Kramer-HSD test was used to analyze differences between treatments. Nested effects of test vessels and interactions of test endpoints with abiotic parameters were analyzed using two-way nested ANOVA and ANCOVA, respectively.

Oxidative stress in muscle and liver (FOX assay): Muscle and liver samples were analyzed according to [Hermes-Lima et al. \(1995\)](#) and [Monserrat et al. \(2003\)](#) with protocols adapted for fish tissues (for details see SI section II). This assay detects lipid peroxides formed in the presence of free radicals and reactive oxygen species which oxidize Fe²⁺ at acidic pH. The subsequent formation of an Fe³⁺ complex with xylenol orange was quantified photometrically using a plate reader (Elx 8006, BioTek, Germany) and Gen5 software (BioTek instruments, Germany). The level of oxidative stress expressed as Cumene hydroperoxide equivalents was calculated according to [Monserrat et al. \(2003\)](#).

Gene expression: Biomarker genes were selected based on mechanistic information on oxidative stress, biotransformation, immune system regulation, general stress response, metal sequestration and endocrine function (Table 2). Due to concerns about potential effects of low DO levels (< 60% during limited amounts of time) during the last part of the exposure period (70–96 days), expression data of several genes which were affected by low DO (heat shock protein 70 (Hsp70), metallothionein A (MTa), heme oxygenase (Hmox), glutathione peroxidase (GPx)) were omitted from statistical analysis. Primers were designed based on available mRNA sequences (Source: GenBank, NCBI) using IDTdna software (<https://eu.idtdna.com>), and synthesized by Invitrogen (US). Functionality of primer sets on amplification of specific PCR products were verified with gel electrophoresis and sequencing (Microsynth, Switzerland). Primer sequences and qPCR efficiencies are provided in SI Table S9.

Total RNA was extracted using TRIzol Reagent (Invitrogen, US) and RNeasy MiniKit (Qiagen, Germany). Genomic DNA contamination was removed using DNase from the RNase-Free DNase kit (Qiagen, Germany). RNA quantity and quality were verified using a NanoDrop

spectrophotometer (PEQLAB Biotechnologie GMBH, Germany) and agarose gel electrophoresis. The cDNA was synthesized from total RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Germany). Levels of mRNA of selected biomarker genes were measured using the LightCycler SYBR Green PCR Master Mix (Roche, Switzerland). Samples were run in triplicate in white 384-well plates (Roche, Switzerland), using LightCycler480 (Roche, Switzerland). Transcript levels were measured as cDNAs of pooled RNA isolations from five fish of each treatment. qPCR results were calculated relative to the housekeeping gene, 18S, according to the normalization procedure of the Q-Gen Core Module (<http://www.qgene.org/>) ([Muller et al., 2002](#); [Simon, 2003](#)), which takes varying PCR amplification efficiencies into account. Significant differences between treatments were determined using one-way ANOVA and Dunnett's test (GraphPad Prism, Version 8.1.2). MayDay software (it.inf.uni-tuebingen.de, Germany) was used to perform the principal component analysis.

3. Results and discussion

3.1. Laboratory bioassays

3.1.1. Genotoxicity and mutagenicity

With a relative enrichment factor (REF) of one, the Genotox (p53)-CALUX® and the Ames fluctuation assay detected no genotoxicity or mutagenicity, respectively, in any effluent sample. When tested at higher REFs, several samples showed mutagenicity: The Ames fluctuation assay with strain TA100 without S9 showed mutagenicity at REF 10 and 20 and probable mutagenicity at REF 20 in two of 12 samples tested (OZ and SF (Campaign 1)) (SI Table S10, Fig. S3), while no mutagenicity was detected with strain TA100 with S9 (SI Table S11). Tests with strain TA98 without S9 classified samples after OZ and SF from Campaign 1 as probably mutagenic at REF 20 and 5, respectively, and one sample after SF (Campaign 3) as mutagenic (SI Table S12, Fig. S4 A, B and C). After metabolic activation with S9, the same strain indicated probable mutagenicity after SF (Campaign 3) at REF 10 (SI Table S13, Fig. S4 D). In samples from Campaign 1, post-treatments reduced (SF) or eliminated (all other treatments) the mutagenicity detected after ozonation (strain TA100 without S9) (Tables S10 and S12). In samples from Campaign 3, mutagenicity was solely observed after SF, and neither after ozonation or any of the other post-treatments (Table S12, Fig. S5).

These results suggest that ozonation at WWTP Neugut occasionally produced mutagenic compounds, which were removed by FB, MB, GAC_{fresh} and GAC_{loaded}, but not completely by SF. Similarly, a recent review on the efficacy of advanced wastewater treatment technologies ([Völker et al., 2019](#)) concluded that mutagenicity detected by the Ames fluctuation assay with strains TA98 and TA100 occurred sporadically after ozonation as well as after different post-treatments (in 3–7% of samples from 11 studies). Other studies suggest that the formation of mutagenic compounds by ozonation depends on the type of wastewater treated. [Schindler Wildhaber et al. \(2015\)](#) found an increase in mutagenicity in ozone-treated effluent from a WWTP treating wastewater with a high percentage of industrial wastewater. The bioassay used in their study employed an additional bacterial strain (YG7108) which is highly sensitive for alkylating agents ([Yamada et al., 1997](#)). Using the same assay, other studies also detected mutagenicity in ozonated wastewater, reduced significantly during post-treatments ([Magdeburg et al., 2014](#); [Mestankova et al., 2014](#)). In our study, nitrosamines, one group of alkylating agents, were chemically analyzed ([Bourgin et al., 2018](#)), therefore strain YG7108 was not included. Another factor, which might influence mutagenicity is the sample pre-treatment. Acidification of samples before SPE could lead to a decrease or an increase in mutagenicity compared to samples extracted at neutral pH, depending on the composition of the wastewater sample ([Abbas et al., 2019](#); [Magdeburg et al., 2014](#)). Chemical analytical results show that N-nitrosodimethylamine (NDMA) was formed during ozonation with a maximum concentration of 30 ng/L ([Bourgin et al., 2018](#)). This concentration can be

considered unproblematic (Wunderlin et al., 2017) and was abated during all post-treatments (by 41% (FB) to 83% (GAC_{fresh}) (Bourgin et al., 2018).

3.1.2. Endocrine disruption

ER α -CALUX®: estrogenic activity was highest in PC effluent (12, 43 and 75 ng EEQ/L) and decreased by 97% by biological treatment in SC (0.3, 1.3 and 2.4 ng EEQ/L). No estrogenicity was detected in effluent after ozonation or after post-treatments (LOD = 0.06 ng EEQ/L) (Fig. 2 A, and SI Table S14). Total elimination of estrogenic activity during all treatments was > 99%. These results confirm previous studies (for review see Völker et al. 2019): While conventional treatment (*i.e.* biological treatment) in our study removed 97% on average, ozonation removed an additional 92–93% of the remaining activity.

Anti-AR-CALUX®: No anti-androgenic activity was detected in PC effluent (LOD = 7,300 ng flutamide EQ/L), but samples from other treatment steps sporadically showed activity without a distinct pattern (Fig. 2 B and SI Table S15, LOD = 2,920 ng flutamide EQ/L). This might be related to variability in wastewater composition between the different campaigns. Previous studies have shown that ozonation and ozonation plus post-treatments can remove up to 83% of anti-androgenicity (Völker et al., 2019).

PR-CALUX®: Progesterone-like activity was detected in PC effluent (1.3, 2.3 and 4.6 ng Org2058 EQ/L) (Fig. 2 C and SI Table S16). It decreased by 36% in SC to 0.8, 1.4 and 2.3 ng Org2058 EQ/L, and was eliminated during ozonation plus post-treatments (LOD = 0.18 ng Org2058 EQ/L). A previous study (Kienle et al., 2011) showed that progesterone-like activity was up to six times higher in SC effluent than in SC influent, but reduced during ozonation and ozonation plus post-treatment by 78 and 69%, respectively.

Anti-PR CALUX®: Anti-progesterone-like activity was detected in PC effluent at 7, 23 and 23 ng Ru486 (Mifepristone) EQ/L. It decreased by 90% during SC to < 0.4, 1.1 and 1.3 ng Ru486 EQ/L (Fig. 2 D). In the first campaign, ozonation resulted in an increase of activity (by 25% to

1.3 ng Ru486 EQ/L), and there was high variability among campaigns after post-treatments (range: 0.82–4.6 ng Ru486 EQ/L). Overall, WWTP treatment reduced anti-progesterone-like activity by 80–94% (SI Table S17).

PPAR γ -CALUX®: PPAR γ -like activity was detected in PC effluents (240, 476 and 562 ng rosiglitazone EQ/L). After SC, concentrations were below the LOD (88 ng rosiglitazone EQ/L) (Fig. 2 E and SI Table S18). Similarly, Kienle et al. (2011) and Bain et al. (2014) found that 70 to >99% of PPAR γ -like activity was removed by biological treatment.

Nrf2-CALUX®: In the first campaign, high Nrf2-like activity was detected in one of three PC effluent samples (135 μ g curcumin EQ/L), which decreased in SC by 76% (to 32 μ g curcumin EQ/L) (Fig. 2 F and SI Table S19). In all campaigns, differences between the subsequent treatment steps were much smaller and not significant. Overall, elimination of Nrf2-like activity by the WWTP was 51–89%.

PXR-CALUX®: PXR-like activities detected in PC effluents collected in two campaigns were 46 and 90 μ g nicardipine EQ/L (the third sample was < LOD = 22 μ g nicardipine EQ/L). SC decreased concentrations by 33 and 43%, respectively (SI Table S20). There were no significant differences between treatment steps due to high variability between campaigns (Fig. 2 G). Overall elimination for PXR-like activity by the WWTP was between 55 and 64%.

3.1.3. Non-specific toxicity

Extracts of samples collected from PC effluent caused significant inhibition of bacterial luminescence (11, 20 and 30 mg baseline-TEQ/L). SC reduced non-specific toxicity significantly by $94 \pm 5\%$ (mean \pm SD) to 0.48, 1.2 and 1.3 mg baseline-TEQ/L (Fig. 3 and SI Table S21). Ozonation significantly reduced the remaining toxicity by $66 \pm 11\%$ to 0.14, 0.34 and 0.53 mg baseline-TEQ/L, but post-treatments had no further effect. Some values after ozonation and post-treatments were close to the detection limit (LOD = 0.03–0.11 mg/L). The total elimination efficiency of the WWTP for non-specific toxicity was 98–99%.

These results were similar to those of an earlier study conducted in

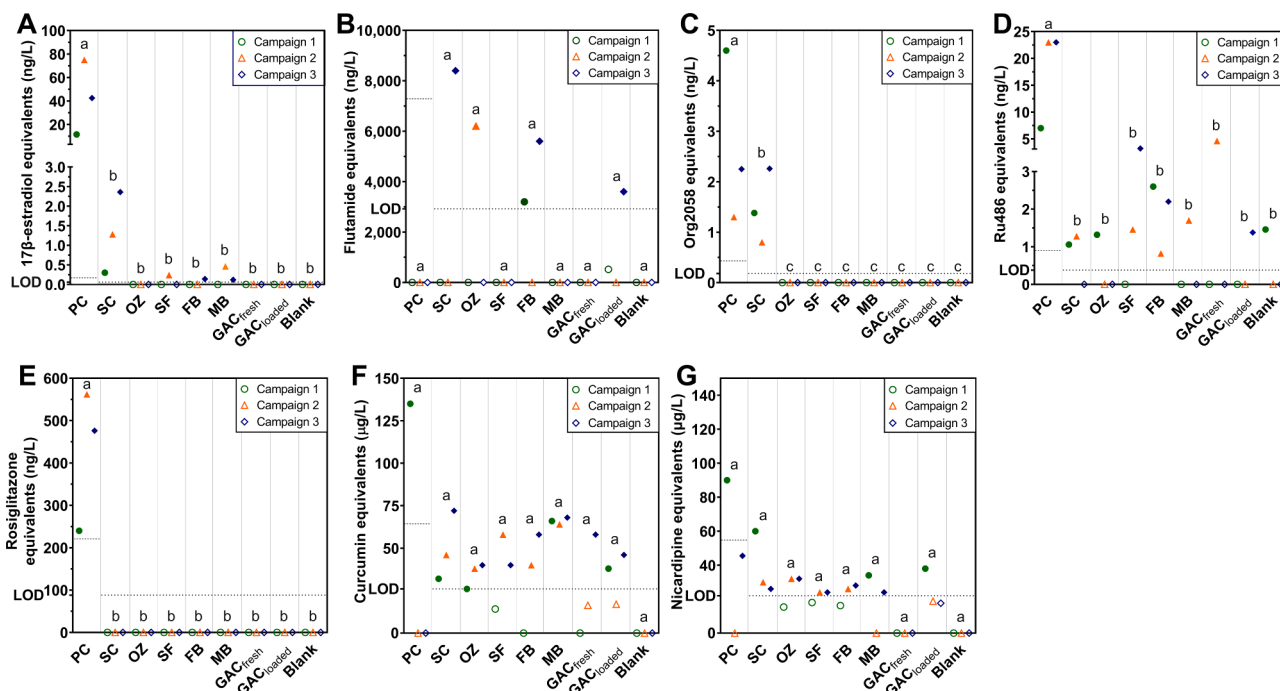


Fig. 2. CALUX® assays: (A) ER α -CALUX®, (B) Anti-AR-CALUX®, (C) PR-CALUX®, (D) Anti-PR-CALUX®, (E) PPAR γ -CALUX®, (F) Nrf2-CALUX®, (G) PXR-CALUX® in wastewater of the different treatment steps. Mean values of 48 h composite samples from three campaigns, each sample was analysed with three technical replicates. Empty symbols indicate a value below LOD (LOD = dashed line). Different letters indicate significant differences between treatments (One-way ANOVA with Dunnett's multiple comparisons test). PC = primary clarifier, SC = secondary clarifier, OZ = ozonation, SF = sand filtration, FB = fixed bed, MB = moving bed, GAC_{fresh} = fresh granular activated carbon (13,000–20,000 bed volumes), GAC_{loaded} = pre-loaded granular activated carbon (35,000–43,000 bed volumes).

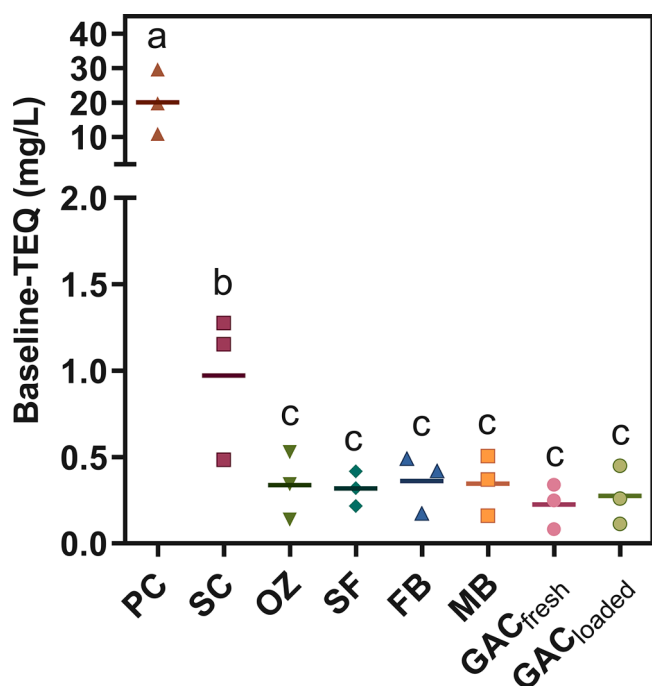


Fig. 3. Luminescent bacteria inhibition in *Aliivibrio fischeri*: baseline toxicity equivalent concentrations (baseline-TEQ in mg/L) in wastewater of the different treatment steps. Mean values of 48 h composite samples from three campaigns, each sample was analysed with three technical replicates. The line indicates the mean. Different letters indicate significant differences between treatments (two-way repeated measures ANOVA with Tukey's multiple comparisons test). PC = primary clarifier, SC = secondary clarifier, OZ = ozonation, SF = sand filtration, FB = fixed bed, MB = moving bed, GAC_{fresh} = fresh granular activated carbon (13,000–20,000 bed volumes), GAC_{loaded} = pre-loaded granular activated carbon (35,000–43,000 bed volumes).

Switzerland (Escher et al., 2009), where SC reduced non-specific toxicity from 6.8 to 0.35 mg baseline-TEQ/L. Ozonation reduced the remaining toxicity by 65% to 0.15 mg baseline-TEQ/L. In this case, however, SF removed an additional 21% to 0.12 mg baseline-TEQ/L resulting in 96% total elimination efficiency. Similarly, ozonation removed 60% of the non-specific toxicity remaining after conventional treatment at a WWTP in Australia (Macova et al., 2010).

3.1.4. Inhibition of photosynthesis and growth in green algae

Extracts of samples collected from PC effluent caused significant PSII inhibition (71, 138 and 286 ng DEQ_{PSII}/L). SC reduced toxicity by $55 \pm 22\%$ (mean \pm SD) to 43, 56 and 74 ng DEQ_{PSII}/L (Fig. 4 A and SI Table S22). Ozonation significantly reduced toxicity by $80 \pm 8\%$ to 6.4, 8.6 and 21 ng DEQ_{PSII}/L. Among post-treatments, only fresh granular activated carbon (GAC_{fresh}) reduced the remaining toxicity by another $66 \pm 8\%$ to 2.2, 3.6 and 5.4 ng DEQ_{PSII}/L. Total elimination during ozonation and post-treatments ranged from 89% (SF) to 97% (GAC_{fresh}).

Algal growth was significantly inhibited by PC effluent (1,751, 8,146 and 16,072 ng DEQ_{growth}/L). SC reduced toxicity by $85 \pm 8\%$ (mean \pm SD) to 418, 657 and 2,064 ng DEQ_{growth}/L. Ozonation reduced the remaining toxicity by $75 \pm 3\%$ to 482 ng DEQ_{growth}/L (Campaign 1) and 179 ng DEQ_{growth}/L (Campaign 3) (Fig. 4 B and SI Table S23), but the difference to SC effluent was not statistically significant. Post-treatments had little effect. Total WWTP elimination relative to influent was 97% after ozonation, and ranged from 96 to 98% after post-treatments. These results are consistent with those of previous studies (Escher et al., 2009; Kienle et al., 2013b, 2011; Schindler Wildhaber et al., 2015).

3.1.5. Survival and reproductive success of waterflea

Neither biologically treated wastewater nor samples taken after ozonation and post-treatments caused mortality of waterflea. However, reproduction was impaired (20–40% compared to control) in water samples collected after ozonation, as well as after SF, FB and GAC_{loaded} post-treatments (Campaign 2) at the highest concentration tested (90% treated wastewater + 10% control water) (Fig. 5, SI Fig. S6, SI Tables S24 and S25). Earlier studies showed that negative effects on waterflea reproduction can occur after ozonation as well as after moving bed post-treatment (Kienle et al., 2013b), but this effect is not seen consistently (Kienle et al., 2013a). It is possible that the effects observed were due to heavy metals or other contaminants not removed efficiently by ozonation or certain post-treatments. Other advanced treatment methods such as activated carbon were able to remove such contaminants (e.g. Margot et al. 2013, Martin Ruel et al. 2011).

3.2. Flow-through bioassays at WWTP Neugut

3.2.1. Survival and growth of oligochaete worms

There was no difference in 28-d survival or growth of oligochaetes between biologically treated and ozonated wastewater, nor after post-treatments (SI, Tables S26 and S27, Fig. S7). These results differ from those of previous studies performed at other WWTPs where growth was significantly reduced after ozonation (Abegglen et al., 2009; Kienle

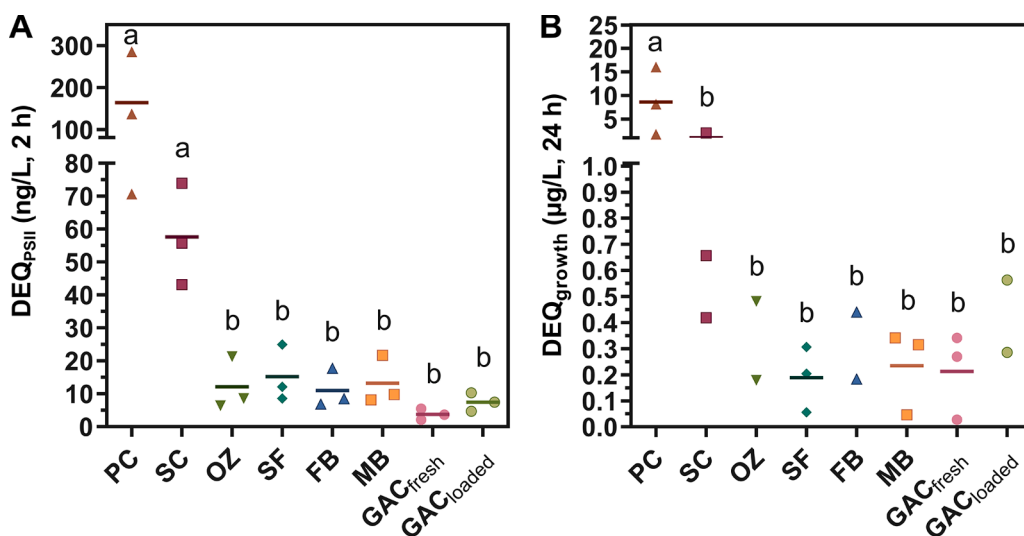


Fig. 4. Photosystem II (PSII) and growth inhibition in *Raphidocelis subcapitata*: Diuron equivalent concentrations (for (A) PSII inhibition (DEQ_{PSII} in ng/L) and (B) growth inhibition (DEQ_{growth} in µg/L) in wastewater of the different treatment steps. Mean values of 48 h composite samples from three campaigns, each sample was analysed with three technical replicates. The line indicates the mean. Different letters indicate significant differences between treatments (two-way repeated measures ANOVA with Tukey's multiple comparisons test). PC = primary clarifier, SC = secondary clarifier, OZ = ozonation, SF = sand filtration, FB = fixed bed, MB = moving bed, GAC_{fresh} = fresh granular activated carbon (13,000–20,000 bed volumes), GAC_{loaded} = pre-loaded granular activated carbon (35,000–43,000 bed volumes).

	Sampling	Control	SC	OZ	SF	FB	MB	GAC _{fresh}	GAC _{loaded}
Replicates (n)		24	12	12	12	12	12	12	12
Toxicity	Campaign 1	-							
	Campaign 2	-							
	Campaign 3	-							

Fig. 5. Reproduction assay with *Ceriodaphnia dubia*: Inhibitory effects of the tested wastewaters relative to the control. Green = no effect, orange = slight inhibitory effect (20–40% reduction compared to control), control = artificial reference medium, SC = secondary clarifier, OZ = ozonation, SF = sand filtration, FB = fixed bed, MB = moving bed, GAC_{fresh} = fresh granular activated carbon (13,000–20,000 bed volumes), GAC_{loaded} = pre-loaded granular activated carbon (35,000–43,000 bed volumes) (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

et al., 2015). Post-treatment with a sand filter eliminated this effect, which suggests that labile, biologically active reaction products can be produced by ozonation depending on the composition of the wastewater.

3.2.2. Survival and sublethal effects in early life stages of rainbow trout

Results for all biological endpoints measured in the FELST assay are summarized in Table 3. Details are provided in SI Table S28 and Figs. S8–S11. Time to hatch was significantly longer than control in the FB treatment ($p = 0.046$), and both FB ($p = 0.0034$) and GAC_{loaded} ($p = 0.020$) were significantly different from SF (Fig. S8 B). No significant differences between treatments were seen with regard to hatching success, time to swim-up, swim-up success, post-hatch survival or growth ($p > 0.05$). Hatching started after 28 d of exposure and was completed within 11 d in all treatments.

After 96 d, overall survival as well as post-hatch survival ($p < 0.05$) was significantly lower in effluent of SC than in controls, and both endpoints significantly improved following ozonation plus post-treatment with GAC_{fresh} ($p < 0.05$; Table 3, Fig. S10). Most of the post-hatch mortality in SC effluent occurred during the extended test period between 65 and 96 d (Table 3).

Our results add to findings of similar studies with rainbow trout, one of which detected detrimental effects of ozone-treated wastewater on development and growth (Stalter et al., 2010). Another showed that ozonation reduced rainbow trout toxicity (Kienle et al., 2013b). No toxicity due to ozonation was observed by Margot et al. (2013).

Data on water quality parameters during the test are provided in SI (Tables S30–S34, Figs. S12–S16). All validity criteria of OECD Guideline 210 (OECD, 1992b) were met during the standard test period (64 d post-hatch) (Table S29). During the extended exposure period (64–96 d post-hatch), dissolved oxygen saturation fell below 60% on several days (between days 70 and 96), and was lowest in SC effluent throughout this time (SI Tables S30 and S31, Fig. S12). Sublethal effects (reduced growth, liver, gill and kidney lesions) and lethality can occur if

salmonids experience dissolved oxygen levels < 5 mg/L or 47% saturation at 12 °C for prolonged periods (four months) (Larmoyeux and Piper, 1973; Matthews and Berg, 1997; Rubin, 1998). Although in our experiment low dissolved oxygen levels only occurred on a few days for maximum periods of 24 h, it may have contributed to the observed effects.

Water temperature was occasionally above the target of 12 ± 1.5 °C after 45 d of exposure but never exceeded 15.8 °C (maximum reached in SC treatment). The average temperature in experimental treatments ranged from 12.6 to 12.9 °C (SI Table S32, Fig. S13). The validity criterion (variation of not more than 1.5 °C between test chambers and successive days) was met on most days (min. 83 of 96 d). Overall, there were no significant differences in oxygen saturation, water temperature, or flow rate (SI Fig. S14) between test chambers (Steel-Dwass, $n = 25$, $p > 0.05$) during the test period.

Ammonium can be very toxic to fish, especially in its un-ionized form (NH_3) depending on pH and temperature of the water. In our experiment, concentrations ranged from 0 to 0.45 mg/L $\text{NH}_4\text{-N}$ before day 64, and from 0.4 to 1.16 mg/L $\text{NH}_4\text{-N}$ between days 84 and 96 (SI Table S34, Fig. S15), and the pH ranged from 7.1 to 8.5 (SI Fig. S16). Based on available toxicity data for rainbow trout (Brinkman et al., 2009; Thurston et al., 1984; U.S. Environmental Protection Agency, 2013) direct effects of measured ammonium concentrations on fish survival and development are unlikely. Furthermore, severity of histopathological liver lesions did not correlate with ammonium concentrations in test chambers (Spearman's ρ , $n = 5$, $\rho = 0.14$, $p = 0.13$), and there was no covariance of liver histopathology with oxygen saturation, temperature or flow rates.

An effect of the fish toxin ammonia (NH_3), which is in chemical equilibrium with ammonium, is possible. The measured values of maximum 1.16 mg $\text{NH}_4\text{-N}$ (at 12 °C) in the test correspond to about 0.003 to 0.025 mg $\text{NH}_3\text{-N}$ at pH 7–8. Ammonia values greater than 0.04 mg/L caused histopathological effects after long-term exposure over five years in rainbow trout (Thurston et al., 1984). Acute toxicity

Table 3

Fish Early Life Stage Toxicity assay with rainbow trout: Summary of the investigated biological endpoints. Mean values \pm standard deviation, $n = 4$ for control, $n = 3$ for wastewater treatments, C = control, SC = secondary clarifier, OZ = ozonation, SF = sand filter, FB = fixed bed, MB = moving bed, GAC_{fresh} = fresh granular activated carbon filter (13,000–20,000 bed volumes), GAC_{loaded} = pre-loaded granular activated carbon filter (35,000–43,000 bed volumes). *significantly different from control; a significantly different from SF, b significantly different from SC.

	Control	SC	OZ	SF	FB	MB	GAC _{fresh}	GAC _{loaded}
Hatching success								
(% of fertilized eggs)	100.0 \pm 0.0	97.8 \pm 3.9	93.3 \pm 0.0	98.9 \pm 1.9	96.7 \pm 3.4	94.5 \pm 6.9	96.7 \pm 3.4	98.9 \pm 1.9
Time to hatch (d)	35.5 \pm 0.6	36.3 \pm 1.5	35.7 \pm 0.6	34.3 \pm 0.6	*38.0 \pm 1.0	35.7 \pm 1.2	35.7 \pm 0.6	*37.3 \pm 1.2
Time to swim-up (d)	52.8 \pm 1.5	59.0 \pm 3.6	56.7 \pm 3.5	53.7 \pm 1.5	56.7 \pm 4.2	53.7 \pm 2.1	54.7 \pm 1.2	55.0 \pm 1.7
Post-hatch survival, 64 d								
(% of hatched fish)	97.5 \pm 1.7	95.5 \pm 1.9	92.6 \pm 9.8	97.8 \pm 1.9	90.8 \pm 2.2	92.8 \pm 6.3	96.6 \pm 3.45	92.1 \pm 7.0
Post-hatch survival, 96 d								
(% of hatched fish)	95.0 \pm 3.4	*81.8 \pm 4.3	92.6 \pm 9.8	97.8 \pm 1.9	89.6 \pm 3.6	88.2 \pm 1.9	^b 95.5 \pm 5.3	91.0 \pm 5.1
Overall survival, 96 d (%)	95.0 \pm 3.4	*80.0 \pm 6.7	86.7 \pm 8.8	96.7 \pm 0.0	86.6 \pm 5.8	83.3 \pm 6.7	^b 92.2 \pm 5.1	90.0 \pm 5.8
Individual weight, 96 d (g)	0.9 \pm 0.3	0.9 \pm 0.2	0.9 \pm 0.3	0.9 \pm 0.2	0.9 \pm 0.3	1.0 \pm 0.3	0.9 \pm 0.3	0.9 \pm 0.3
Standard length, 96 d (cm)	3.8 \pm 0.4	3.7 \pm 0.3	3.8 \pm 0.4	3.8 \pm 0.3	3.8 \pm 0.4	3.9 \pm 0.3	3.9 \pm 0.4	3.8 \pm 0.4
Total length, 96 d (cm)	4.4 \pm 0.5	4.4 \pm 0.4	4.4 \pm 0.5	4.5 \pm 0.4	4.4 \pm 0.5	4.5 \pm 0.4	4.5 \pm 0.4	4.4 \pm 0.4

values for $\text{NH}_4\text{-N}$ and $\text{NH}_3\text{-N}$, respectively, are 0.25 mg N/L, 2.79 mg $\text{NH}_3\text{/L}$, and 3.48 mg N/L for freshwater ecosystems, depending on pH and fish species (Ip et al., 2001; Randall and Tsui, 2002). The EC_{20} for effects on growth of early life stages of rainbow trout after 90 days was 7.72 mg/L $\text{NH}_3\text{-N}$ (Brinkman et al., 2009). Based on all available ecotoxicity data for freshwater organisms, the US EPA recommends an acute quality criterion of 7.6 mg $\text{NH}_4\text{-N/L}$ for ammonium and a chronic quality criterion of 1.3 mg $\text{NH}_4\text{-N/L}$ (at pH 8 and 12 °C) (U.S. Environmental Protection Agency, 2013). Based on these data, direct effects of periodically increased ammonium concentrations on fish survival and development in our study are unlikely. The reason for the increased ammonium concentrations is presumably the increased food requirement over time leading to an increased excretion rate of the fish. However, if regarded over the whole test period, the increased values occurred in all treatments, and thus should not influence the ability to compare between different treatments.

Histopathology: Results of histopathological examination (Fig. 6) showed that fish exposed to $\text{GAC}_{\text{fresh}}$ effluent and control water had the healthiest livers (significantly different from ozonated water (OZ), $p = 0.027$ and 0.0022, respectively). There were no significant differences between fish exposed to effluent after ozonation and other post-treatments. These data confirm that $\text{GAC}_{\text{fresh}}$ was more efficient in removing reactive substances caused by ozonation than $\text{GAC}_{\text{loaded}}$. Fish exposed to ozonated wastewater plus SF, FB and MB showed most tissue damage. These results are not entirely consistent with other test endpoints such as 96-d post-hatch or overall survival, which was lowest in fish exposed to biologically treated (SC) or ozonated (OZ) wastewater (Table 3). Based on available information (see above) it is unlikely, but cannot be fully excluded, that changes in water quality parameters, in particular oxygen saturation, temperature and ammonium concentrations affected these results.

Gene expression: Principal component analysis (PCA) of gene expression data shows a clear separation of trout exposed to SC effluent from all other treatments including controls (Fig. 7). Fish exposed to ozonated effluent separate clearly from those exposed to SC effluent, controls and post-treatments with the only exception being $\text{GAC}_{\text{loaded}}$. There was no significant correlation of oxygen saturation or temperature with expression levels of the biomarker genes included in the PCA analysis. Expression data of several genes affected by low DO (Hsp70, MTa, Hmox, GPx) were omitted.

In livers of fish exposed to SC effluent, several genes indicative of oxidative stress were significantly induced compared to the control: catalase (CAT), nuclear factor (erythroid-derived 2)-like 2 (NrF2),

superoxide dismutase (SOD), glutathione reductase (GR), and peroxisome proliferator-activated receptor (PGC-1 α) (SI Fig. S17). Expression levels of some oxidative stress biomarkers were also elevated in other treatments, but were significantly lower than levels measured in fish exposed to SC effluent. Fish exposed to ozonated effluent showed elevated levels of NrF2 and SOD (compared to controls), and expression of CAT was elevated in fish exposed to MB and $\text{GAC}_{\text{fresh}}$ effluents. No other post-treatment effluent induced oxidative stress biomarkers. No significant differences between the treatments were found in the oxidative stress analysis using the FOX assay in fish file or liver samples (Tukey-Kramer HSD, $n = 15$, $p > 0.05$ for all tested treatments) (Fig S20 A and B).

The xenobiotic transporter proteins, ABCB1a and ABCC2, and genes involved in biotransformation (cytochrome P450 1A (CYP1a), cytochrome P450 3A (CYP3a) and glutathione-S-transferase (GST)) were significantly induced in fish exposed to SC effluent (SI Fig. S18 A–E). Vitellogenin (VTG), indicative of estrogenic contaminants, was also induced by SC effluent, but not in any other treatment (SI Fig. S18 F). So were two cytokines, TNF α and IL1 β , indicative of the activation of the immune system, as well as the cell cycle/apoptosis biomarker p53 (SI Fig. S19 A–C). Ozonation eliminated the observed effects on TNF α , IL1 β and p53 expression with no further reduction due to post-treatments, however, p53 was significantly induced in effluent of $\text{GAC}_{\text{loaded}}$. Effluent from MB significantly induced CYP3a. Metallothionein b (MTb), a biomarker for heavy metal detoxification, was significantly induced in SC and OZ, but this effect was not seen after post-treatments (SI Fig. S19 D).

In summary, most biomarker genes selected for this study were significantly induced in exposed fish by conventionally (biologically) treated wastewater (SC). This overexpression was largely eliminated by ozonation. Changes were most pronounced for genes involved in biotransformation, estrogenicity, immune regulation and cell cycle processes. Post-treatments further reduced biomarker gene expression; $\text{GAC}_{\text{fresh}}$ and FB treatments were especially effective and gene expression patterns of fish exposed to these effluents were very similar to those of control fish.

3.3. Overall discussion and implications of the study

Our study demonstrates the significant beneficial effect of advanced treatment by ozonation on the water quality of wastewater effluent. Post-treatments further reduced some ecotoxicological effects. Among these, treatment with $\text{GAC}_{\text{fresh}}$ sampled at 13,000–20,000 bed volumes

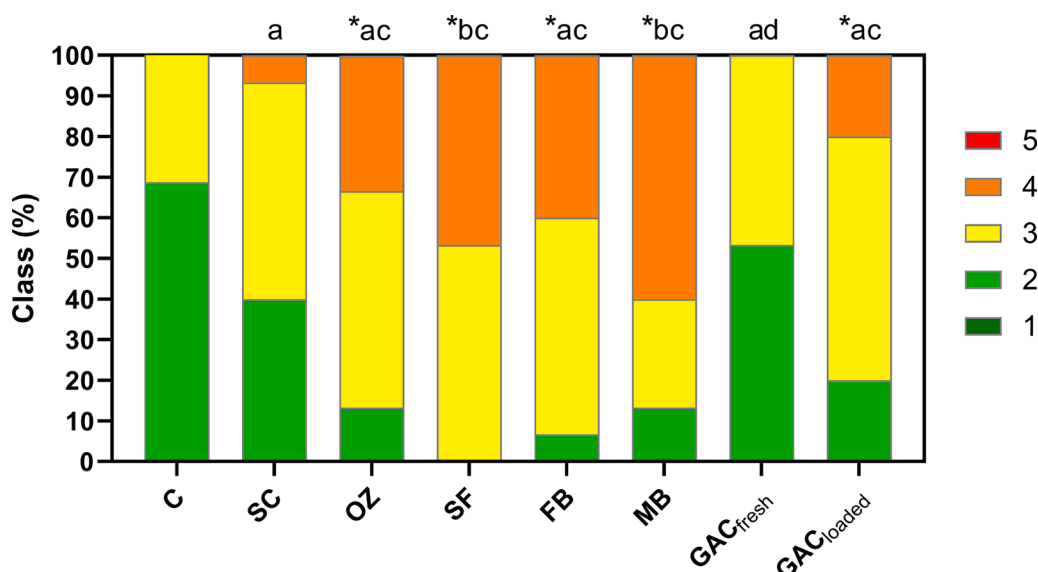


Fig. 6. Fish Early Life Stage Toxicity assay with rainbow trout: Semi-quantitative evaluation of histopathological alterations of liver for every tested treatment. $n = 4$ for control, $n = 3$ for wastewater treatments, C = control, SC = secondary clarifier, OZ = ozonation, SF = sand filter, MB = moving bed, FB = fixed bed, $\text{GAC}_{\text{fresh}}$ = granular activated carbon filter (13,000–20,000 bed volumes), $\text{GAC}_{\text{loaded}}$ = granular activated carbon filter (35,000–43,000 bed volumes). Classes: 1 = control state, 2 = intermediate state, 3 = reaction state, 4 = severe state, 5 = destruction state. Different letters indicate significant differences between treatments * significantly different from control (for details see Table S35).

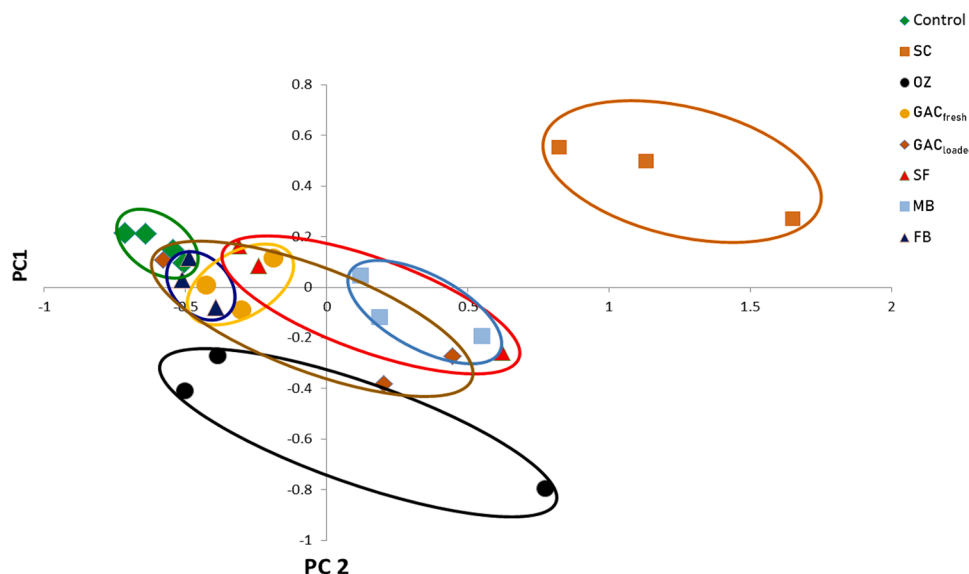


Fig. 7. Fish Early Life Stage Toxicity assay with rainbow trout: Principal component analysis of biomarker gene expression quantified in liver tissue of rainbow trout exposed to different effluents at WWTP Neugut. $n = 4$ for control, $n = 3$ for wastewater treatments. The closer the data are, the more similar the biomarker levels are in the treatments. SC = secondary clarifier, OZ = ozonation, SF = sand filter, FB = fixed bed, MB = moving bed, GAC_{fresh} = fresh granular activated carbon (13,000–20,000 bed volumes), GAC_{loaded} = pre-loaded GAC (35,000–43,000 bed volumes).

performed best.

Ozonation did not produce ecotoxicologically problematic levels of oxidation by-products in our full-scale set up. At environmental concentrations (i.e. 1-fold enrichment), no mutagenicity was detected using the Ames fluctuation assay with strains TA98 and TA100. At higher sample enrichment factors (REF > 1) in the assay, mutagenicity occurred sporadically after ozonation (Campaign 1) or after sand filtration (Campaign 3). The low-level occurrence of mutagenicity after sand filtration in one sample was unexpected, and cannot be explained by the available chemical analytical data. It should be kept in mind that the analyzed substances represent only a small fraction of those actually present.

Several bioassays demonstrated the significant improvement of wastewater quality in the treatment process: the combined algae assay (photosynthesis and growth of green algae), the bacterial luminescence inhibition assay and the ER α (estrogen receptor activation) and PR (progesterone receptor activation)-CALUX®.

The average abatement of ecotoxicological effects during biological treatment varied for the assays and ranged from 36% (PR-CALUX®) to 97% (ER α -CALUX®). Only the combined algae (*R. subcapitata*), bacterial luminescence inhibition (*A. fischeri*) and PR-CALUX® assays were able to show the significant additional benefit of ozonation. The average abatement of ecotoxicological effects during ozonation was 80% (combined algae assay: PSII inhibition), 75% (combined algae assay: growth inhibition), 66% (bacterial luminescence inhibition), and 85% (PR-CALUX®). Reproduction of water flea (*C. dubia*) was impaired in one of three campaigns after ozonation and after three post-treatments (SF, FB and GAC_{loaded}). Survival of waterflea and survival and reproduction of oligochaete worms (*L. variegatus*) was neither affected by PC effluent nor by effluent of any other treatment step.

Expression patterns of selected biomarker genes showed clear differences between control, SC effluent, ozonated effluent and some post-treatments. Among post-treatments, however, only GAC_{fresh} reduced ecotoxicological effects to the extent that multiple bioassay endpoints showed significant improvement compared to ozonation alone. Inhibition of algal photosynthesis, rainbow trout liver histopathology and biomarker gene expression proved sufficiently sensitive to detect the relatively small improvement in water quality achieved by GAC_{fresh} (sampled at 13,000–20,000 bed volumes). Post-treatment with GAC_{loaded}, which was run for a longer time than GAC_{fresh} and sampled at 35,000–43,000 bed volumes, did not show an improvement in any test. Gene expression results further suggested that the fixed bed post-

treatment was effective in improving water quality; however, neither liver histopathology nor algal PSII inhibition corroborated this finding. Histopathological results indicated that liver condition was negatively affected in fish exposed to ozonated wastewater, as well as in fish exposed to effluents from all post-treatments except GAC_{fresh}.

Our study supports the results of other international studies, which showed that wastewater ozonation removed certain ecotoxicological effects (e.g. estrogenicity, herbicidal effects), but retained or even enhanced others (e.g. certain endocrine effects) (for review see Völker et al. 2019). The study presented here is the first comparing the efficacy of various post-treatments using a broad range of *in vitro* and *in vivo* bioassays. Together with the results of the accompanying publication on micropollutant elimination, and the formation of transformation products and oxidation by-products (Bourgin et al., 2018), it provides valuable information on suitable post-treatments following ozonation. In addition, results can guide the selection of relevant bioassays for future studies evaluating wastewater treatment processes.

4. Conclusions

Based on the results of our study using a broad range of bioassays and biomarkers, we conclude that ozonation as an advanced wastewater treatment significantly reduces the ecotoxicity of treated wastewater. Increased mutagenicity due to ozonation reaction products was detected only sporadically, and was successfully reduced or eliminated by all the post-treatments applied in this study.

Further improvement of water quality could be achieved by post-treatment using a fresh activated carbon filter (sampled at 13,000–20,000 bed volumes). Other post-treatment methods such as fixed bed and moving bed could also be beneficial; however, a sand filter did not further improve wastewater quality. In general, it has to be kept in mind that water quality was already relatively good after biological treatment and ozonation, therefore it was difficult to measure an additional improvement of the water quality by post-treatments. Nevertheless, biological post-treatment after ozonation is required in Switzerland to reduce biodegradable dissolved organic carbon and potentially toxic products formed during ozonation.

This study demonstrates that the combination of ozonation and activated carbon achieved the greatest reduction of ozonation reaction products and ecotoxicity in treated wastewater. This treatment combination has received increasing attention in recent years and is already implemented at WWTP Altenrhein, Switzerland.

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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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:<https://doi.org/10.5281/zenodo.5899573>.

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