WATER RESEARCH 46 (2012) 3693–3702

Available online at www.sciencedirect.com

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Impact of ozonation on ecotoxicity and endocrine activity of tertiary treated wastewater effluent

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ARTICLE INFO

Article history:
Received 22 July 2011
Received in revised form
14 March 2012
Accepted 9 April 2012
Available online 17 April 2012

Keywords:
Aquatic ecotoxicity
Endocrine disruption
Enzyme immunoassay
Ozonation
Wastewater treatment plant effluent
Micropollutants

ABSTRACT

Tertiary wastewater treatment plant effluent before and after ozonation (0.6–1.1 g O_3/g DOC) was tested for aquatic ecotoxicity in a battery of standardised microbioassays with green algae, daphnids, and zebrafish eggs. In addition, unconjugated estrogen and 17β-hydroxyandrogen immunoreactive substances were quantified by means of enzyme immunoassays, and endocrine effects were analysed in a 21-day fish screening assay with adult male and female medaka (*Oryzias latipes*). Ozonation decreased estrogen-immunoreactivity by 97.7 ± 1.2% and, to a lesser extent, androgen-immunoreactivity by 56.3 ± 16.5%. None of the short-term exposure ecotoxicity tests revealed any adverse effects of the tertiary effluent, neither before nor after the ozonation step. Similarly in the fish screening assay, reproductive fitness parameters showed no effects attributed to micropollutants, and no detrimental effects of the effluents were observed. Based on the presented screening, ozonation effectively reduced steroid hormone levels in the wastewater treatment plant effluent without increasing the effluent's ecotoxicity.

Abbreviations: AM, Arithmetic mean; ANCOVA, Analysis of Covariance; DIN, Deutsches Institut für Normung (German standard methods); DOC, Dissolved organic carbon; E-Assay, EIA for estrogen-immunoreactivity; EDC, Endocrine disrupting compound; EEA, European Environment Agency; EIA, Enzyme immunoassay; E2, 17β-estradiol; HE, Haematoxylin and Eosin; ISO, International Organisation for Standardisation; MS222, Tricaine methanesulfonate; NC, Negative control; NH_4-N, Ammonium nitrogen; NO_3-N, Nitrate nitrogen; O_3, Ozone; O_3-IN, Tertiary effluent before ozonation; O_3-OUT, Tertiary effluent after ozonation; OECD, Organisation for Economic Cooperation and Development; RV, Range of variation; SD, Standard deviation; SFW, Standard freshwater; SOC, Specific ozone consumption; T-Assay, EIA for testosterone-immunoreactivity; TB, Trenbolone; TOC, Total organic carbon; VTG, Vitellogenin; WWTP, Wastewater treatment plant.

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0043-1354/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
doi:10.1016/j.watres.2012.04.017

1. Introduction

The occurrence of micropollutants in the aquatic environment, among them endocrine disrupting compounds, is a major issue for the protection of aquatic life (Moltmann et al., 2007). Therefore, a special focus is currently put on their removal during wastewater treatment. In the last decades, the best available technology for wastewater treatment improved from carbon to nitrogen removal. Nevertheless, conventional technologies are not able to completely remove such substances of concern, and even tertiary treated wastewater is considered to be one of the major point sources for micropollutants in surface waters (Schwarzenbach et al., 2006; Barcelo and Petrovic, 2008; EEA, 2011). Among micropollutants, endocrine disrupting compounds (EDCs) have the potential to seriously affect the viability of aquatic wildlife through interference with hormonally regulated functions like reproduction, development and growth or immunocompetence in organisms (Sumpter, 2008; Grillitsch et al., 2010). The group of hormonally active micropollutants comprises a wide range of substances including natural and synthetic hormones, non-hormonal pharmaceuticals as well as industrial chemicals such as bisphenol-A and alkylphenolic compounds. Most of the research on endocrine disruption has focused on estrogenic substances, and among these, hormones excreted by mammalians including humans were the predominant representatives (Damstra et al., 2002; Bursch et al., 2004; Jobling et al., 2004; Sumpter, 2008). In contrast, comparatively few data are available on the occurrence of androgenic compounds in wastewater and the aquatic environment (Svenson and Allard, 2004; Björkblom et al., 2009; Grillitsch et al., 2010). A variety of wastewater characteristics and removal mechanisms determine the efficiency of wastewater treatment (Byrns, 2001; Katsoyiannis and Samara, 2007), and plant design criteria such as sludge retention time play an important role for the elimination of contaminants including EDCs and other micropollutants (Kreuzinger et al., 2004). Schaar et al. (2010) documented the increase in removal of estrone, 17α-ethinylestradiol, bisphenol A and the alkylphenolic chemicals nonylphenol, nonylphenolmono- and nonylphenoldiethoxylate, and octylphenol for a wastewater treatment plant (WWTP) upgraded from only carbon to nitrogen removal. In order to further enhance the removal of micropollutants, advanced treatment is necessary and various technologies such as ozonation, advanced oxidation processes, activated carbon, and membrane technology have been investigated (Poseidon, 2004). Since ozonation proved to be a promising technology for micropollutant removal on laboratory scale (Huber et al., 2003) an ozonation pilot plant was set up at an Austrian municipal wastewater treatment plant for further investigations on full-scale application (Schaar et al., 2010). Due to unspecific ozone reactions with the organic wastewater matrix, the formation of toxic by-products in the effluents cannot be excluded (Von Gunten, 2003; Stalter et al., 2010a,b). Therefore, the objective of the present study was to investigate the effects of an ozonation step for tertiary treated wastewater on both aquatic ecotoxicity and endocrine activity. Based on chemical analyses of selected micropollutants (Schaar et al., 2010), the present study employed a stepwise approach starting with a basic set of short-term exposure microbioassays including green algae, daphnids and fish eggs, followed by enzyme immunoassays for estrogen and testosterone binding activity, and finally a 21-day fish screening assay for endocrine disruption (OECD, 2009).

2. Material and methods

2.1. Ozonation pilot plant and sampling campaigns

The ozonation pilot plant was situated at an Austrian municipal wastewater treatment plant with nitrification/denitrification. A side stream of the tertiary effluent ranging between 30 and 36.4 m^3/h was used as influent (O_3-IN) to the pilot-scale ozonation plant. The ozonation step comprised two 5 m^3 reactors that were operated in series. The ozone was supplied to the first reactor by fine bubble plate diffusers. Relevant information on the tertiary effluent and the ozonation parameters are listed in Table 1, more details on the WWTP and the ozonation pilot plant are given by Schaar et al. (2010). Grab samples were collected from the tertiary effluent (O_3-IN, influent to the ozonation plant) and the effluent of the second pilot plant reactor (O_3-OUT). Table 1 gives an overview of the sampling campaigns, including the specific ozone consumption (SOC) and the hydraulic retention time in the pilot plant, tertiary effluent characteristics and the applied tests to investigate ecotoxicity and endocrine activity. The pH in O_3-IN was close to neutral (6.8–6.9). Ozone in O_3-OUT was monitored with an ozone probe (Orbisphere Model 31330.15) and no residual ozone was detected during the sampling campaigns. In order to avoid detrimental effects of any residual ozone the samples were additionally stripped with air.

2.2. Basic set of aquatic ecotoxicity tests

Standardised short-term exposure bioassays with green algae (algal growth inhibition, DIN EN ISO 8692:2005–01), daphnids (daphnia acute immobilisation, DIN EN ISO 6341:1996–06), and zebrafish eggs (fish embryo toxicity, DIN 38415-6:2003–08) were employed to assess the aquatic ecotoxicity of the tertiary treated wastewater before (O_3-IN) and after ozonation (O_3-OUT) at SOC ranging from 0.8 to 1.1 g O_3/g DOC (Table 2). The microbioassays included Toxkit® MicroBioTests (Mariakerke, Belgium) with *Pseudokirchneriella subcapitata* (*Selenastrum capricornutum*, Algaltoxkit F) and *Daphnia magna* (Daphtoxkit F). Eggs for the fish egg test with *Danio rerio* were from a laboratory stock maintained at the Aquatic Ecotoxicology Unit. Upon delivery, unfiltered effluents were subject to adjustment to test temperature and dissolved oxygen concentration, preparation of dilution series with standard freshwater as specified by the respective ISO guidelines, and biotesting immediately thereafter. Numbers of conducted aquatic ecotoxicity tests and dilution steps tested are detailed in Table 2.

2.3. Enzyme immunoassays

Competitive enzyme immunoassays (EIA) using biotin linked steroids as labels and streptavidin-peroxidase as detection

Table 1 – Overview of the sampling campaigns, wastewater treatment plant effluent characteristics before ozonation (O_3-IN), ozonation parameters, and tests applied for aquatic ecotoxicity and endocrine activity.

Campaign	Sampling date	O_3-IN				Ozonation parameters		Applied tests		
		DOC[a] (mg/L)	NH_4-N[b] (mg/L)	NO_3-N[c] (mg/L)	Temperature (°C)	Specific ozone consumption (g O_3/g DOC)	Hydraulic retention time (min)	Microbioassays	Enzyme immunoassays	Fish screening assay
1	18.09.2008	7.4	0.11	8.51	17.2	0.90	18.8	x		
2	30.09.2008	7.0	0.08	12.4	19.0	1.08	18.5	x	x	
3	24.02.2009	7.6	1.08	3.96	17.1	0.65	18.5		x	
4	14.07.2009	5.8	1.39	3.26	20.8	0.81	16.9	x	x	
5	26.08.2009	7.3	1.27	2.16	23.1	0.63	17.5		x	
6	03.12.2009	8.0	0.96	3.4	15.1	0.65	16.6		x	x
7	07.12.2009	7.7	1.17	3.83	14.3	0.67	16.6		x	x
8	11.12.2009	7.0	1.18	6.07	12.8	0.69	17.1		x	x
9	14.12.2009	7.9	0.8	2.81	12.0	0.62	16.8		x	x
10	18.12.2009	8.3	0.7	5.3	11.3	0.61	16.6		x	x

a Dissolved organic carbon.
b Ammonium nitrogen.
C Nitrate nitrogen.
x test applied

system were performed to measure immunoreactive unconjugated estrogens (estrone, 17β-estradiol, 17α-ethinylestradiol and estriol, as described by Möstl et al. 1987) using estrone as standard, and immunoreactive 17β-hydroxyandrogens using testosterone as standard (Palme and Möstl, 1993). Due to the group specificity of the assays the results have to be considered as estrone or testosterone equivalents. Validation criteria for the assays are given in Supplement 1. Preparation of the water samples for EIA analysis included the following consecutive steps: filtration using glass wool; extraction of steroids from 1 L of the filtered sample using a solid-phase C18 extraction column (Sep Pak RP-18, Merck) according to the specifications of the manufacturer; elution of steroids from the columns using 4 mL methanol; evaporation of the organic phase in a stream of nitrogen; and re-dissolving of the extract in the assay buffer. All samples subjected to EIA analyses were either freshly filtered or frozen, and filtered after thawing. In accordance with literature (Ellis et al., 2004), own analyses (data not shown) indicated no difference between these two procedures.

2.4. *Fish screening assay*

Endocrine effects in fish were analysed largely following the OECD (2009) 21-day fish screening assay for estrogenic and androgenic activity, and aromatase inhibition.

Fishes treated were 5–6 months old, sexually mature, male and spawning female (gross morphometry as shown in Supplement 2) wild-type Japanese medaka (*Oryzias latipes*) bred from a laboratory stock maintained at the Aquatic Ecotoxicology Unit.

2.4.1. *Acclimation and exposure conditions*

Three weeks before the beginning of the exposure, the fish were randomly placed into the test aquaria. Each test aquarium contained five male and five female fish. The different treatments were randomly assigned to the test aquaria. The following treatments were tested in the fish screening assay: WWTP effluent before ozonation (O_3-IN; undiluted, unfiltered, aerated), WWTP effluent after ozonation (O_3-OUT; undiluted, unfiltered, aerated), negative control with dilution water (aerated, carbon filtered, tap drinking water of the City of Vienna, Austria), positive estrogenic control with 17β-estradiol (CAS number: 50-28-2, Sigma–Aldrich) and positive androgenic control with 17β-trenbolone (CAS number: 10161-33-8, Sigma–Aldrich).

During the first three days of exposure to the WWTP effluents, the proportion of O_3-IN and O_3-OUT in the test aquaria was increased gradually reaching 25% on the first day and almost 100% on the third day. Fish were continuously exposed to the test media via the ambient water for approximately three weeks (test duration was 20 days for the negative control, 19 days for the effluent treatments, and 17 days for the positive controls). The nominal concentration in both positive control groups (100 ng/L) was selected for 17β-estradiol following OECD (2009) and for trenbolone based on previous publications (Ankley et al., 2003; Seki et al., 2006; Grillitsch et al., 2010) aiming at causing detectable effects in any of the indication criteria considered in this multi-criteria approach. The actual concentration (measured as

Table 2 – Basic aquatic ecotoxicity tests. Number of replicates per test for the different dilution steps of the effluent before (O_3-IN) and after (O_3-OUT) ozonation and the negative control (NC) in standard freshwater (SFW; DIN 38415-6:2003–08, DIN EN ISO 6341:1996–06; DIN EN ISO 8692:2005–01).

	Treatments									
	NC	O_3-IN				O_3-OUT				
Dilution step (pure effluent + SFW)	0 + 1	1 + 7	1 + 3	1 + 1	1 + 0	1 + 7	1 + 3	1 + 1	1 + 0	SOC[d]
Proportion of test substance (%)	0	12.5	25	50	100	12.5	25	50	100	
Algal growth inhibition										
No. of replicates[a]	15		3	3	3		3	3	3	1.08
Daphnia acute immobilisation										
No. of replicates[b]	20	4	8	8	20				8	0.81
						4	4	4	4	0.90
							2	3	4	1.08
Fish embryo toxicity										
No. of replicates[c]	136	10	20	20	40				20	0.81
						10	10	10	10	0.90
							10	10	10	1.08

a $>10^4$/mL organisms per replicate.
b Five organisms per replicate.
c One organism per replicate.
d Specific ozone consumption (g O_3/g DOC).

immunoreactive substances) of 17β-estradiol was 109.2 ± 8.7 (94.7–117.1) ng/L (arithmetic mean (AM) ± standard deviation (SD); range of variation (RV) in parentheses). For the present study, no actual concentration of trenbolone was analysed (analytical method in progress). Every treatment was run in two simultaneous replicates (i.e., aquaria).

Handling of the WWTP effluent samples was carried out according to the following steps: for logistical reasons O_3-IN and O_3-OUT were only delivered every 3–4 days and stored at 7.1 ± 1.6 °C (AM ± SD) until use. In order to assess the stability of the analytes in the effluent samples, EIA analyses were performed immediately after delivery and at the last day of the storage period. Before transfer into the test aquaria, the effluent samples were adjusted to testing temperature and aerated. Aeration was needed to strip the over saturation of oxygen (up to 170%) in O_3-OUT. To avoid bias due to different sample preparation, also O_3-IN water was aerated ending up at an O_2 saturation of 100 ± 3% for both O_3-IN and O_3-OUT. In the test aquaria, temperature and conductivity were measured every day, whereas dissolved oxygen and pH were measured daily only during the first five days of exposure and once a week afterwards. Measured water quality characteristics (AM ± SD; treatments in parentheses) were: 24.2 ± 0.4 °C, 359 ± 17 μs/cm, 96 ± 1%, 8.5 ± 0.1 (pooled negative control and positive controls with 17β-estradiol and trenbolone); 24.2 ± 0.4 °C, 959 ± 90 μs/cm, 92 ± 1%, 8.4 ± 0.1 (O_3-IN); 24.2 ± 0.4 °C, 955 ± 92 μs/cm, 90 ± 2%, 8.3 ± 0.1 (O_3-OUT). For EIA analysis, samples from the test medium in the aquaria were taken every fourth day. Test volume was 20 L per aquarium. Medium renewal was semi-static (two times 50% exchange per day). To guarantee the required level of O_2 saturation, all aquaria were slightly aerated. Faeces and eggs were siphoned off from the bottom of the aquaria every day. Photoperiod was 16:8 h light:dark, light intensity at water surface was 800–900 lux. Fish were fed with newly hatched brine shrimp nauplii (*Artemia salina*) twice a day. At the end of exposure, fish were killed with an overdose of the anaesthetic MS222 (100 mg/L, neutral buffered tricaine methanesulfonate, Apoka). No mortalities or signs of disease were observed during the three weeks of acclimatisation and the following 17–20 days of exposure. One male fish in the 17β-estradiol treatment was accidentally wounded during the cleaning procedure and was removed from the test.

2.4.2. *Fish reproductive output and external morphology*

Female medaka carry their eggs for several hours after spawning which takes place in the morning. In order to assess the reproductive performance of the fish during the exposure period, the number of females carrying eggs was counted in each aquarium every day at the same time (before the first exchange of the test medium).

At the end of exposure, fish were killed with an overdose of the anaesthetic MS222 (see Section 2.4.1), weighed to the nearest of 0.01 g, photographed for length measurements which were taken to the nearest of 0.1 cm using ImageJ 1.42 (http://rsbweb.nih.gov/ij/), and fish condition factor (CF) was calculated [Fulton's CF = 100 × total body mass (g)/total body length (cm)^3]. At the end of exposure, the dorsal and caudal fins of all fish were examined using a stereo microscope (Wild M420; magnification up to 100-fold) for the presence or absence of the following structures which are typically present in males only: papillary processes on the anal fin rays, serration of the distal anal fin margin, caudal notch in the anal fin and caudal notch in the dorsal fin (as detailed in Grillitsch et al., 2010).

2.4.3. *Histology and immunohistochemistry*

Immediately after gross morphological examination, fish were incised ventrally and preserved in 4% buffered formaldehyde for 58–64 days at about 6 °C. After preservation, livers and gonads of fish were dissected and embedded in Paraplast® (Vogel, Giessen, Germany) by means of an automatic embedding device (Shandon Excelsior, Thermo, Cheshire, UK). For histological examination, serial sections of 2 μm thickness of

livers (one region) and gonads (three regions per fish) were cut and stained with Haematoxylin and Eosin (HE) according to Romeis (1989). Evaluation of the hepatic tissue comprised vacuolisation of the hepatocytes, alterations or degenerations of tissue sex specific structures, staining characteristics and density of the hepatocyte cytoplasm. Vacuolisation of the hepatocytes was divided into three subcategories (normal, mild, moderate). Staging of the female gonads was performed according to the OECD (2010) Guideline for Diagnosis of Endocrine-Related Histopathology of Fish Gonads. Criteria for staging male medaka gonads were modified according to the OECD (2010) staging criteria for fathead minnow and zebrafish.

In addition, livers and gonads of the fish were analysed for the presence of the egg precursor protein vitellogenin (VTG) which represents a female exclusive biomarker with the presence of VTG in the livers and gonads of male fish indicating their exposure to exogenous estrogenic compounds. For immunohistochemical examination, serial sections were cut at 4 μm and mounted on APES (3-aminopropyltriethoxysilane)/glutaraldehyde-coated slides. Endogenous peroxidase activity was blocked by incubation in 3% hydrogen peroxide (H_2O_2) in methanol for 15 min at room temperature. Afterwards, slides were washed for ten times with tap water and heat treated for 25 min in 0.01 M citric acid (pH 6.0). A protein block (1.5% goat serum) was used to minimise unspecific binding of the primary antibody. The unlabelled primary monoclonal mouse anti medaka vitellogenin CK-4B3 antibody (Biosense Laboratories, Bergen, Norway), diluted 1:50 in 1.5% goat serum, was detected with the Bright Vision secondary system (Bright Vision poly HRP-Anti-Mouse IgG, ImmunoLogic, Duiven, Netherlands) using DAB (3,3′diaminobenzidine-tetrahydrochloride) in Tris buffer pH 7.4 and 0.03% H_2O_2 as chromogen. Finally, slides were washed with distilled water, counterstained with haematoxylin, dehydrated and mounted by use of xylene-soluble medium (DPX, Fluka, Buchs, Switzerland). The specificity of the antibody used for immunohistochemical staining was verified by Western blotting using polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE), for details see Supplement 3.

VTG and HE stained sections were examined and photographed under light microscope (Reichert-Jung, Vienna, Austria, Polyvar microscope, Nikon DS-Fi1 digital camera system, Nikon NIS elements imaging software). For the present study, livers and gonads of both sexes were analysed by VTG immunohistochemistry. The detailed numbers of examined fish of each group are listed in Supplement 4.

2.4.4. Statistical analysis

For statistical analysis, PASW (SSPS) Statistics 17 was used. Square root transformation was applied to body lengths, logarithmic (ln) transformation to body mass. Duration of exposure in the fish screening assay (i.e., 17 or 19 days) had no significant effect as a covariate, and therefore the use of one-way ANOVA was justified and could be applied, when data were normally distributed (according to Kolmogorov–Smirnov, $p < 0.05$). Data sets which were not normally distributed even after square root, arcsin or logarithmic transformation (e.g., egg numbers), were analysed using Kruskal–Wallis-*H* Test. Pairwise comparisons were analysed using Sheffé post-hoc tests or Dunnett-*C* tests (when no homogeneity was found between variances). For the analysis of the ozonation effect on estrogen and testosterone immunoreactive substances Analysis of Covariance (ANCOVA) was used with the specific ozone consumption as a covariate.

3. Results and discussion

3.1. Basic aquatic ecotoxicity tests

In the present study, none of the short-term exposure ecotoxicity tests revealed any adverse effect of the WWTP effluent before and after the ozonation step. Similarly, Abegglen et al. (2009) observed no increase of short-term toxicity of effluent after ozonation for *Ceriodaphnia dubia*, green algae and fish eggs (*D. rerio*). In contrast, increased toxicity after ozonation was reported for reproduction test with *Lumbriculus variegates* and fish early-life-stage test with rainbow trout (*Oncorhynchus mykiss*) (Abegglen et al. 2009, Stalter et al. 2010a,b). These different effects of ozonation observed between the latter and the present study could be a result of species specific differences, not only beyond that the variable sensitivity of different developmental stages but also differences in effluent characteristics and the ozonation methodology. Finally, effects of effluent sample preparation before testing e.g., membrane filtration (Stalter et al., 2010a) and solid-phase extraction (Escher et al., 2009) in contrast to whole-effluent toxicity testing (as in the present study) have to be considered for the comparison of results (Escher et al., 2009).

3.2. Enzyme immunoassays

The results of the enzyme immunoassays indicated an overall decrease of immunoreactive substances in the ozonated effluent (Table 3). The concentration of the unconjugated immunoreactive estrogens decreased by 97.7 ± 1.2 (95.4–99.6)% (AM ± SD; RV in parentheses) at SOC between 0.6 and 1.1 g O_3/g DOC. This decrease was significant with the SOC as covariate (ANCOVA, $F = 6.663$, $p = 0.036$). The removal determined by the EIA analysis of filtered effluent generally parallelled the chemical analysis of the estrogens estrone and 17α-ethinylestradiol in unfiltered samples (Schaar et al., 2010). Due to its phenolic moiety the natural and synthetic steroid estrogens are readily oxidised by ozone (Huber et al., 2003). The elimination of estrogenic activity shown in Table 3 is in accordance with literature on pilot and full-scale studies reporting an estrogenicity removal (17β-estradiol equivalent concentration) of 69–98% at specific ozone doses between 0.5 and 1.2 g O_3/g DOC (Escher et al., 2009; Stalter et al., 2010b, 2011).

The testosterone-immunoreactivity declined by 56.3 ± 16.5 (32.9–90.3)% (AM ± SD; RV in parentheses) with the highest removal (>90%) at 1.1 g O_3/g DOC, see Table 3. This decline was significant with the SOC as covariate (ANCOVA, $F = 21.233$, $p = 0.002$). The lower decrease compared to estrogenicity can be attributed to the lack of a phenolic moiety in testosterone. Due to the double bond as the functional group for ozone attack Huber et al. (2003) expected it to react by approximately

Table 3 – Estrogen (E-Assay) and testosterone (T-Assay) immunoreactive substances before (O_3-IN) and after (O_3-OUT) the ozonation of tertiary effluent (general effluent characteristics as in Table 1).

Campaign	Specific ozone consumption (g O_3/g DOC)	E-Assay			T-Assay		
		O_3-IN (ng/L)	O_3-OUT (ng/L)	Reduction (%)	O_3-IN (ng/L)	O_3-OUT (ng/L)	Reduction (%)
1	0.90	–	–	–	–	–	–
2	1.08	37.9	0.2	99.6	10.0	1.0	90.3
3	0.65	17.7	0.3	98.5	3.0	1.4	53.3
4	0.81	13.6	0.2	98.3	2.2	0.9	61.2
5	0.63	20.7	0.5	97.7	3.9	1.6	59.6
6	0.65	14.3	0.7	95.4	3.5	1.7	52.0
7	0.67	16.8	0.4	97.8	3.8	1.5	58.9
8	0.69	19.5	0.5	97.4	2.8	1.9	32.9
9	0.62	21.9	0.5	97.9	3.6	1.4	61.7
10	0.61	23.9	0.9	96.4	3.4	2.1	37.0

one order of magnitude slower than phenolic steroid hormones. Corresponding to the results of the present study, Snyder et al. (2006) observed the elimination of testosterone in ozonation pilot plant experiments with wastewater (>44% removal at 0.3 and 0.5 O_3/g TOC; >99% at 1 g O_3/g TOC). In contrast, no removal of androgenicity was determined by Stalter et al. (2011), which was attributed to interactions with anti-androgens.

The variation in estrogen and testosterone immunoreactive substances within the effluent samples of a single batch during the storage period ranged at the same order of magnitude as the variation between the sampling campaigns (Table 4); EIA results showed no significant effects of storage for up to four days at 7.1 ± 1.6 °C (AM ± SD). This observation confirmed own preliminary results of previous sample stability analyses (data not shown). However, comparatively large variations were observed between the estrogen-immunoreactivity of effluent samples before and after resting in the aquaria with fish. Estrogen immunoreactive substances were slightly rising with increasing resting time of the effluent in the test aquaria (Table 4). Metabolisation effects could be responsible for the observed increase of estrogen binding activity, but sample sizes do not allow for further interpretation. Taken together, the results of the EIA analyses clearly indicated a dominance of estrogen immunoreactive substances in the effluent of the WWTP before ozonation, while testosterone immunoreactive substances dominated in the ozonated effluent.

The observed estrogen binding activities at least in the effluent before ozonation ranged in the order of magnitude of concentrations documented as effect concentrations in single compound tests with fish (Young and Borch, 2009). In contrast, regarding the androgen binding activities measured in the effluent, effective concentrations for fish known from the literature range at the μg/L level (Grillitsch et al., 2010). Therefore, based on the results of the EIA analyses, estrogenic but no androgenic effects were to be expected in the fish screening assay in the present study (see Section 3.3).

3.3. *Fish screening assay*

Comparatively few reproduction tests with fish in WWTP effluents have been conducted. The present study is one of the first to test such effluents for endocrine activity applying the fish screening assay (OECD, 2009) employing an established set of relevant indication criteria (Dang et al., 2011).

Table 4 – Estrogen (E-Assay) and testosterone (T-Assay) immunoreactive substances (ng/L) in the effluent before (O_3-IN) and after (O_3-OUT) ozonation (measured directly after sample delivery and after 3–4 days of storage) as well as in the test aquaria before and after the semi-static medium exchange during the fish screening assay.

	Effluent						Negative control		
	O_3-IN			O_3-OUT					
	AM[a]	Range	n[b]	AM[a]	Range	n[b]	AM[a]	Range	n[b]
E-Assay									
Effluent sample directly	19.3	14.3–23.9	5	0.6	0.4–0.9	5			
Effluent sample stored	18.4	14.8–27.2	4	0.6	0.4–0.7	4			
Aquarium before change	27.5	23.9–33.5	6	7.3	5.0–10.5	6	2.5	1.8–3.4	4
Aquarium after change		19.1/21.2	2		3.5/5.5	2			
T-Assay									
Effluent sample directly	3.4	2.8–3.8	5	1.7	1.4–2.1	5			
Effluent sample stored	3.6	2.7–5.5	4	2.4	1.9–3.3	4			
Aquarium before change	3.8	3.4–4.2	6	2.1	1.7–2.3	6	0.3	0.2–0.6	4
Aquarium after change		3.0/3.4	2		1.9/2.2	2			

a Arithmetic mean.
b Sample size.

3.3.1. *Fish reproductive output and external morphology*

In the fish screening assay, female reproductive performance was monitored over the whole exposure period (Fig. 1). The numbers of egg carrying females as well as the number of eggs were significantly higher in the O_3-IN group than in the negative control and the estradiol groups ($p < 0.05$, Dunnet-C). Additionally, in the O_3-OUT group, the numbers of females carrying eggs were higher than in the negative control and the estradiol groups even though the differences were not significant. WWTP effluents contained suspended particulate matter which was observed to be ingested and digested by the fish as indicated by the continuously and markedly higher amounts of fish faeces in the effluent treatment groups compared to all other control groups. Hence, the observed increase in body mass of both sexes and egg production of the female fish exposed to the WWTP effluents likely represent feeding effects which is also supported by the liver histology of male fish as described below (see Section 3.3.2).

In the fish screening assay, egg production showed higher variation in the estrogen (estradiol) and androgen (trenbolone) control groups than in O_3-IN, O_3-OUT and the negative (dilution water) control (Fig. 1) which confirms the sensitivity of this indication criteria in the fish test population. The numbers of egg carrying females in the estradiol group were smaller than in any other treatment group; numbers of carried eggs in the estradiol group were significantly lower than in the O_3-IN, and O_3-OUT groups ($p < 0.05$, Sheffé-pairwise comparisons). These results suggest, that estradiol in the concentration tested in the present study (100 ng/L) impaired egg laying in female fish. Likewise, number of eggs produced and fertility of paired medaka exposed to 463 ng/L estradiol were significantly less compared to negative control fish (Kang et al., 2002). This counter intuitive effect might be due to an estrogenic effect upon male mating behaviour, which is known to be crucial for successful spawning (Oshima et al., 2003). In the present study, no effect of trenbolone on reproductive performance other than the increased variability in egg production was observed. Because of supposed atypical concentration response relationship (Ankley et al., 2003; Seki et al. 2006) trenbolone may be replaced by 11-ketotestosterone as the androgenic reference test compound in own future studies with fish as suggested by Grillitsch et al. (2010).

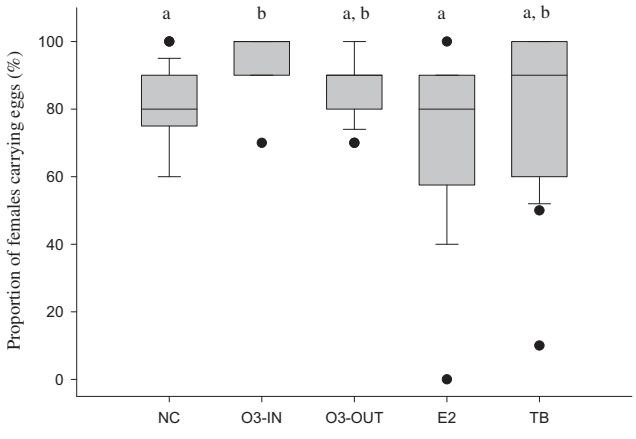

Fig. 1 – Proportion of female medaka carrying eggs in the groups exposed to the WWTP effluent before (O_3-IN) and after ozonation (O_3-OUT), dilution water (negative control, NC), 17β-estradiol (E2), and trenbolone (TB). Duration of exposure was 20 days for NC, 19 days for O_3-IN and O_3-OUT and 17 days for E2 and TB. Box Plots (median, 10th, 25th, 75th, 90th percentile, outlier dots) not sharing the same letter (a, b) are significantly different ($p < 0.05$, Dunnet-C).

After exposure to the effluents for almost three weeks, the adult male and female medaka fish were subject to detailed morphometric examination. Analysis of variance detected no statistically significant effect of the treatments for most of the parameters examined (Supplement 2). For male fish, total body length and height as well as body mass were significantly lower in the estradiol exposure group than in the O_3-OUT group ($p < 0.05$, Dunnet-C). For female fish, a trend towards comparatively increased body mass in both effluent groups was observed but was not statistically significant. In the present study, no external male sex characteristics were observed in any female fish of all treatment groups including O_3-IN and O_3-OUT.

In summary, neither the reproductive output nor the gross morphological examination of the fish exposed to the effluents before and after ozonation showed indication of estrogenic or androgenic endocrine effects. Furthermore, during almost three weeks of exposure to pure effluent before and after ozonation, no mortality or any signs of disease of the fish could be observed. These results of the present study are plausible when comparing with the scarce pertinent literature. Thorpe et al. (2009) observed no effects of WWTP effluents (UK) on survival, growth and condition (CF) in fathead minnows (*Pimephales promelas*) but a significant reduction in egg production in 50 and 100% concentration of the effluent. Similarly, no effects in survival, growth or condition of fathead minnows (*P. promelas*) exposed to effluents (UK, Derbyshire) before and after advanced treatment (granular activated carbon, ozone or chlorine dioxide) were reported by Filby et al. (2010) whereas again a significant reduction in egg production in 50 and 100% concentration of the effluent before and in 100% also after granular activated carbon treatment was observed. Growth suppression and a significant decrease of reproductive output were described by Ma et al. (2005) for secondary treated WWTP effluent (China, Beijing) above 5% concentration. In none of the mentioned studies exposure to effluent increased reproductive output.

3.3.2. *Histology and immunohistochemistry*

The results of histological examination of the livers of the fish showed no significant changes of tissue organisation and cell characterisation in the O_3-IN and O_3-OUT groups compared to the negative control group. Increase of vacuolisation of the hepatocytes was verified in all females but only in one (out of eight examined) male fish of the O_3-IN group and in two (out of ten) male fish of the O_3-OUT group (Supplement 4). In mature female fish, hepatocyte vacuolisation is known to be primarily associated with gonadal stage and VTG production (Arukwe and Goksoyr, 2003). In male fish, increased vacuolisation of the hepatocytes without any modification of the cytoplasmatic staining characteristics corresponds to the

correlation between nutritional status and vacuolisation of the hepatocytes (Boorman et al., 1997) and thus, supports the feeding effect in the effluent treatment groups described in Section 3.3.1. For this biomarker, the sensitivity of the fish tested in the present study was proved in that in all male samples of the 17β-estradiol control group, hepatocellular cytoplasmic basophilia and cytoplasma density increased, which has been reported to be correlated to VTG production (Wester et al., 2003).

In the present study, male and female gonads were assigned to three stages as indicated by the ripeness of the developing gametes (Supplement 4). In female fish, stage 2 (mid-development) dominated in the two effluent treatment groups as well as in all control groups. Only in the effluent treatment groups, the most advanced stage 3 (late) was found which parallelled the increase in body mass and egg production described above (Section 3.3.1) and likely represents feeding effects. In this case, biomarker sensitivity is more complicated to be proved because no increase in the female gonadal stage was observed in the estrogen positive control. However, this observation conforms with the observed decrease in the number of egg carrying females (see Section 3.3.1) and may result from estrogen mediated impairment of male mating behaviour known to be stimulating female spawning (Oshima et al., 2003). In the male fish, the incidence of stages 2 (mid) and 3 (late) was balanced in both effluent treatment groups and in the negative control, whereas in the estrogen positive control only stage 2 and in the androgen positive (trenbolone) control only stage 3 gonadal samples (with one exception additionally showing serious pathological alterations) were observed. Hence, also in the male fish analysed, no effluent mediated effects were observed, and the biomarker sensitivity was confirmed.

Immunohistochemical detection of VTG in the livers and gonads was positive in all female samples. Livers and gonads of female fish of all treatment groups showed the same tissue and cellular VTG. Such VTG expression patterns were only present in male livers of the estradiol treatment. Male gonads of the effluent groups and the negative control as well as the trenbolone group lacked of specific VTG staining.

In summary, neither changes of the gonadal stage nor other histological alterations indicating estrogenic or androgenic endocrine disruption in the gonads and livers of both sexes of fish exposed to the WWTP effluents before and after ozonation for almost three weeks were observed. Comparable results were rarely documented in literature (Dietrich and Krieger, 2009). Contrasting results of EDC effects of WWTP effluents (China, Beijing) were reported by Ma et al. (2005) for medaka where from concentrations of 5% effluent a significant increase of VTG plasma levels in males was observed. A significant increase in VTG plasma levels of male fathead minnows (*P. promelas*) was also documented by Thorpe et al. (2009) and Filby et al. (2010) in WWTP effluents (UK) of 50 and 100% concentration. Notably, although the estrogen binding activities of the effluents in the present study were in the range of effect concentrations of single compound tests no effects on the investigated indication criteria in the fish were observed. Yet, WWTP effluents represent complex mixtures, and different EDCs interacting in mixtures (Ankley et al., 2010) may elicit both synergistic (Young and Borch, 2009) and antagonistic effects (Kobayashi et al., 2011). Hence, further studies are needed to better understand the mechanisms of endocrine disruption of complex mixtures to aquatic organisms.

4. Conclusions

- Effluents before as well as after ozonation did not show any adverse effects in the basic set of the short-term exposure bioassays employed for aquatic ecotoxicity screening. Furthermore, adult medaka fish did not show external signs of detrimental effects during the almost three-week continuous exposure to the undiluted WWTP effluents before and after ozonation. Further research should particularly consider longer term effects in aquatic invertebrates and early life stages of fish.
- The absence of female specific VTG expression in the liver and gonads of male fish and the absence of male specific external sex characteristics in the female fish along with no changes of the gonadal tissues of both sexes and no decrease in reproductive output of female fish indicated no observable estrogenic and androgenic effects of the WWTP effluents in the fish screening assay. For EDC screening with fish, further research should consider interactions of EDCs in mixtures.
- The quantitative enzyme immunoassays showed that ozonation considerably decreased both the estrogen- and, to a lesser extent, the testosterone-immunoreactivity of the WWTP effluent. Hence, the ozonation of tertiary effluent proved to be a suitable technology for the reduction of steroid hormone levels without showing adverse effects in the present ecotoxicological screening. For EIA screening, further research should include endocrine active compounds beyond estrogen- and testosterone-immunoreactivity.

Disclosure statement

There is no conflict of interest for any of the authors.

Acknowledgements

The authors express their gratitude to S. Burger, C. Guseck, H. König, A. Kuchar, A. Tichy, and I. Walter for their valuable support. Part of this study was conducted within the framework of the Vetmeduni Vienna Initiative Doctoral College "BIOREC (Biological Responses to Environmental Challenges)". The design of the present study was discussed and approved by the institutional ethics committee. The ozonation pilot plant study was funded by the Austrian Federal Ministry of Agriculture, Forestry, Environment and Water Management, Kommunalkredit Austria AG, project number GZ A601819.

Appendix A. Supplementary material

Supplementary data related to this article can be found online at doi:10.1016/j.watres.2012.04.017.

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