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Assessment of Ozone or Not-Treated Wastewater Ecotoxicity Using Mechanism-Based and Zebrafish Embryo Bioassays

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Abstract

Toxicity of wastewater treatment plant (WWTP) effluents is usually assessed with standardized bioassays, as e.g. the Fish Embryo Toxicity test (FET), but assessment of sub-lethal toxic effects requires to develop more adapted tests. The present work aimed to add the recording of several sub-lethal endpoints in exposed zebrafish embryo-larval stages in order to increase the sensitivity of residual toxicity evaluation of WWTP effluents using a semi-quantitative method (iFET score). This approach was complemented with the genotoxicity assessment on the exposed embryos, a sub-acute hazard particularly relevant to evaluate a potential chronic toxicity risk of low and multi-contaminated environmental matrices. Additionally, endocrine activities of effluents were quantified using human reporter cell lines. This test battery was applied to the assessment of the residual toxicity of five biological treatment effluents, further treated or not using various ozonation treatments intended to improve pharmaceutical compounds removal. Acute toxicity towards zebrafish embryos was very low. However, iFET score approach proposed was able to reveal residual toxicity through the presence of developmental abnormalities in all samples tested. Additionally, a low residual genotoxicity was measured in embryos exposed to two of the WWTP effluents when all excepted one exhibited a residual endocrine activity potential in the ng/L range providing com-

plementary information on the occurrence of endocrine active chemicals and their reduction by different processes. To sum up, such a simplified and ecologically relevant test battery was found sensitive enough to characterize and differentiate various residual effluent's ecotoxicity at contaminant levels of environmental concern.

Keywords

Wastewater, Ozonation, Genotoxicity, Endocrine Activity, Zebrafish, Early Life Stage, Pharmaceutical, Surfactant

1. Introduction

To achieve significant improvements of treated water quality, innovative advanced technologies are needed since conventional biological wastewater treatments were often found to be insufficient for the elimination of emerging contaminants such as low biodegradable pharmaceuticals, detergents and personal care products. Among the available advanced wastewater treatments, ozonation applied on biologically treated effluents is one of the most efficient and promising process for micropollutant removal regarding the balance of cost to removal efficiency [1]. However, as after any oxidative treatment inducing chemical changes, toxicity assessment is required since oxidation can result in the formation of by-products more toxic than their parent compounds [2]. Release into water bodies of treated effluents containing such toxic substances could impair aquatic organism fitness, population functioning, and subsequently the ecological equilibrium.

To ensure that discharged effluents are non-toxic to the aquatic environment, not only the chemical abatement performances but also an ecotoxicological evaluation through relevant bioassays is necessary. However, as it is the case within the European Union, there is no specific guideline for the WWTP effluent toxicity assessment taking into account the characteristics of such chemically complex mixtures with low to very low concentration levels of hazardous compounds. Several studies have underlined that several simple and cost effective standardized monospecific bioassays are non-reliable for accurate WWTP effluent toxicity evaluation, because of a lack of sensitivity and/or of interference related to the rather high organic matter and nutrient content [3] [4]. To fill this methodological gap, the rationale seems to search for suitable test organisms and endpoints targeting sub-lethal and long term effects such as reproduction and development impairment, genotoxicity and endocrine disruption as recently attempted with a long term *in situ* exposure of *Gammarus fossarum* in effluent at a WWTP [5].

During embryogenesis, a lot of molecular mechanisms can be influenced by pollutants with a possible impact on tissue differentiation and organization easily observable at the macro/microscopic level. Treated wastewaters may also con-

tain genotoxic compounds [2] [6] likely to lead to adverse effects by impairing organism fitness [7]. To evaluate effluent embryo- and geno-toxicity, fish embryo-larval stages (FELS) are of particular interest as they suit to laboratory exposure with whole effluent.

Zebrafish embryo is very popular in acute toxicity assessment though the standardized Fish Embryo Toxicity (FET) test [8]. Only lethal criteria are retained in this test that aims to evaluate the mortality rate of embryos exposed during 72 to 96 h post fertilization. This FET protocol can be regarded as a standardized basis to enlarge the panel of criteria scored in exposed embryo for sub lethality evaluation. Some attempts were already made to investigate sublethal endpoints highly relevant regarding organism fitness, namely genotoxicity and development impairment to provide an added-value for hazard assessment of complex environmental mixtures [4] [9].

Another relevant parameter that deserves to be assessed is the endocrine disrupting activity since WWTP effluents are recognized as a major source point of endocrine disruptors in the aquatic environment. A large array of compounds slow or even not abated by conventional activated sludge treatments, such as biocides, disinfectants, pesticides and pharmaceuticals, can interfere with the endocrine system of exposed organisms through multiple mechanisms of action, in particular the binding to and subsequent (in-)activation of estrogen and androgen nuclear receptors. This is considered as an important issue of environmental concern since it can lead to adverse effects in aquatic organisms living in receiving waters by impairing growth, development and reproduction [10]. In complement to estrogen and androgen receptor activity, information on glucocorticoid receptor activity could also be relevant, since hospital and urban effluents are identified sources of glucocorticoid compounds, used *e.g.* in anti-inflammatory drugs [11]. Among the various existing systems for total endocrine activity testing, *in vitro* assays based on reporter gene expression in cultured cells have generally lower limits of detection (e.g. ≤0.1 ng/L 17β-estradiol equivalent for estrogenic compounds) than chemical analyses [12]. Furthermore, it enables to determine the overall endocrine activity in effluents, which takes into account all endocrine active chemicals present in the samples.

Considering that treated wastewaters exhibit usually low or absent acute toxicity, but can contain complex mixture of micropollutants at low concentrations prone to result in sub-lethal and delayed effects [13], this study aimed to develop and implement a sensitive test battery using zebrafish embryos and *in vitro* tests evaluating relevant endpoints as survival, development, endocrine disruption and DNA integrity.

This test battery enabling to explore different biological effects was applied to the toxicity evaluation of differently treated effluents at a pilot scale fed by a mixture of hospital and urban wastewaters. In a first campaign, the raw mixed effluent was treated through a moving bed biofilm reactor (MBBR) followed by a tertiary ozonation step with a rather high ozone dose. This ozone dosage was

expected to result in significant differences in terms of residual toxicity [14]. For the second campaign, the mixed wastewater effluent was treated with conventional activated sludge (CAS) followed by tertiary ozonation and, in parallel, by CAS with ozone application into the sludge recirculation loop. For this second campaign, the ozone dosage was considered representative of the most commonly applied in urban wastewater treatment for mitigation of usually detected micropollutants.

2. Materials and Methods

2.1. Pilot Wastewater Treatment Unit and Treated Effluent Characteristics

Effluents sampled resulted from the treatment of a mixture of 50% urban and 50% hospital wastewater, at a pilot-scale. The pilot unit was installed in the Bellecombe (Haute-Savoie, France) wastewater treatment plant (WWTP) at the field observatory SIPIBEL. The pilot unit consisted of two parallel treatment lines, each of them including biological aerobic stages operated in order to achieve full nitrification of the effluent ($N\text{-}NH_4$ < 1 mg N/L and $N\text{-}NO_2$ < 1 mg N/L). Concentrations of $N\text{-}NH_4$ and $N\text{-}NO_2$ below 1 mg N/L were targeted to operate the biological treatment processes in full nitrification. These thresholds were selected consistently with the most common discharge requirements met for municipal wastewater.

During the first campaign conducted in January 2014, the influent was biologically treated through a moving bed biofilm reactor (MBBR) alone (line 1) or in the second line, with MBBR followed by an ozonation step performed using a transferred ozone dose (TOD) of 15 mg/L in a bubble column ($MBBRO_{3\,tert}$).

For the second sampling campaign carried out in June 2015, both treatment lines consisted in conventional activated sludge treatments. The first one was followed by tertiary ozonation ($CASO_{3\,tert}$) whereas the second line was operated with a simultaneous ozone application into the mixed liquor recycling loop ($CASO_{3\,loop}$). The transferred ozone doses were 5 and 9.4 mg/L, respectively for the tertiary ozonation and for the ozonation in the mixed liquor recycling loop.

Tertiary ozonation was performed in a bubble column at counter-current while ozonation of the recycled mixed liquor was applied in-pipe through direct online injection. Ozone transfer rate was above 90% for the tertiary ozonation and quantitative for the ozone application into the recycled mixed liquor. Sample name abbreviations and corresponding treatments are summarized in **Table 1**.

After at least four weeks of equilibration of each tested treatment, effluent samples were collected once during a 24 h cycle to cover the natural daily fluctuation of influent quality and to obtain a representative sample composition. Samples were cooled at 4˚C and rapidly transported in isotherm containers to the laboratory where they were aliquoted and immediately stored at -80°C in brown glass bottle to prevent sample degradation.

Table 1. Abbreviations for tested samples with description of their corresponding treatment. TOD: transferred ozone dose.

Campaign 1	
MBBR	Effluent treated with moving bed bioreactor alone
$MBBRO_{3\ tert}$	Effluent treated with moving bed bioreactor followed by tertiary ozonation [15 mg O_3/L TOD]
Campaign 2	
$CASO_{3\ loop}$	Effluent treated with conventional activated sludge ozonated in the recirculating loop [9.4 mg O_3/L TOD]
CAS	Effluent treated with conventional activated sludge alone
$CASO_{3\ tert}$	Effluent treated with conventional activated sludge followed by tertiary ozonation [5 mg O_3/L TOD]

Total organic carbon (TOC) and dissolved organic carbon (DOC) were quantified. Samples were acidified and filtered (0.45 µm) for DOC analysis. TOC and DOC analysis was based on calibration with potassium hydrogen phthalate standards. Through thermocatalytic oxidation at 850˚C, the CO_2 was detected by a non-dispersive infrared detector (NPOC Analytik Jena Multi N/C 3100).

The other physico-chemical parameters of the effluents were measured before freezing in the 24 h wastewater effluents or were measured on site at the pilot wastewater treatment plant.

Wastewater effluents stemming from the same campaign presented comparable physico-chemical parameters as described in Table 2.

After slow thawing (16 h at 4˚C), the five unfiltered and undiluted effluents were evaluated for their embryo-toxicity and genotoxicity on FELS.

Additionally, organic extract of each raw effluent (before freezing) was prepared to guarantee sterile testing conditions for cell-based assays (endocrine disrupting potential). Unfiltered effluents (2 L) were extracted using a SPE-DEX® 4790 system equipped with a HLB™ disk for the solid phase extraction of a rather broad range of hydrophilic and lipophilic compounds that were eluted with 30 ml of a methanol: dichloromethane (1:1) mixture. Extracts were dried (rotary evaporator) for 2 h at 55˚C at 245 mbar and a second time at 45 mbar. The final dry extracts were suspended in 1 mL dimethyl sulfoxide (DMSO) resulting in extracts concentrated 2000 times compared to the raw effluents. Extracts were kept in dark glass vials at −20˚C until use.

2.2. Fish Embryo Larval Stages Study

Embryo-larval development and survival were studied using an exposure protocol based on the Fish Embryo Toxicity Test [8] with some modifications.

Eight to nine months old zebrafish (*Danio rerio*) used for breeding were obtained from Aquarium Villeurbannais (France) and let to acclimatize for three weeks in an automated Zebtec aquarium device (Tecniplast®, Italy), continuously fed with reverse osmosed water automatically regulated for pH, conductivity temperature and oxygen content. Physico-chemical parameters of the Zebtec®

Table 2. Physico-chemical parameters of effluents recorded in 24 h composite samples. COD: chemical oxygen demand; TOC: total organic carbon; DOC: dissolved organic carbon.

		Campaign 1		Campaign 2		
		MBBR	$MBBRO_{3tert}$	CAS	$CASO_{3tert}$	$CASO_{3loop}$
pH		8.5	8.5	7.9	7.8	7.8
Conductivity	µS/cm	1260	1250	1670	1660	1670
Suspended matter	mg/L	16	14	13	10	7
COD	mg O_2/L	27	34	45	37	37
Ammonium	mg N/L	0.2	-	0.09	0.1	0.1
Nitrite	mg N/L	<0.1	<0.1	<0.1	<0.1	<0.1
Nitrate	mg N/L	-	-	0.14	0.04	0.14
TOC	mg/L	20	17	43	43	47
DOC	mg/L	11	12	17	14	14

water were set as followed: conductivity 550 ± 100 µS·cm^{-1}, pH 7.7 ± 0.2, dissolved oxygen 7.4 mg/L (94%), temperature 26.0˚C ± 1˚C. Photoperiodic cycle was set to 12 h light and 12 h dark. Ammonium (NH_4^+), nitrite (NO_2^-), nitrate (NO_3^-) and phosphate (PO_4^{3-}) concentrations were monitored once a week (Tests JBL®, Germany).

Male and female zebrafish were kept separately and fed on TetraRubin® flakes (Tetra GmbH, Germany) and on brine shrimp (*Artemia salina*, Ocean Nutrition™, Belgium). For breeding, two males and one female were put together in a floating breeding unit (Marina®, Hagen, France), equipped with a plastic grid to prevent parental predation on the fertilized eggs. The freshly fertilized eggs from each female were observed under a binocular microscope (Zeiss® Stemi 2000, Germany). In order to fulfil the FET test validity criteria (OECD 236, 2013), egg masses exhibiting a fertilization rate < 70% were excluded. Eggs from a minimum of three breeding groups were mixed to avoid genetic bias. Fertilized eggs without any visible abnormalities were transferred in glass jars containing 100 mL of wastewater effluent sample not later than three hours post-fertilization (hpf). One sterile polystyrene 24-well plate (Falcon®, USA) was used per sample with 20 wells devoted to the studied effluent and four wells used as negative control. Control water was prepared from reconstituted ISO water [15] adapted to the low hardness of maintenance water by dilution with deionized water to a 1:5 ratio [8]. Into another plate, eggs were exposed to the positive control 3.4-dichloroaniline (3.4-DCA) (3.7 mg/L). This concentration should lead to at least 30% mortality. Another plate was devoted to the positive control for genotoxicity evaluation with exposure to the model genotoxicant methyl methane sulfonate (MMS) at 25 µM.

One fertilized egg was placed in each well with 2 mL of either undiluted effluent, or negative control water or positive control solution. Plates were lid cov-

ered and incubated at 26˚C ± 0.5˚C for 96 hours in the dark. Three independent replicates were realized for positive controls and CAS, $CASO_{3\ loop}$, $CASO_{3\ tert}$ effluents. Five independent replicates were realized for the MBBR effluent and six for the $MBBRO_{3\ tert}$ effluents.

The standardized FET protocol [8] records four lethality parameters: coagulation of fertilized eggs, lack of somite formation, lack of detachment of the tail-bud from the yolk sac, and lack of heartbeat. Here, nine sub-lethal macroscopic defects were additionally recorded referring to [16]: abnormal eye development, abnormal pigmentation, presence of edema in various body parts, abnormal blood circulation, as well as five malformations *i.e.* head, tail, heart, spine or yolk sac. Lethal and sub-lethal criteria were noted for each egg and larvae at 24 h, 48 h and 96 h post-fertilization (hpf).

From these observations, two different variables were defined:

- Mortality: this binary variable does not account for sub-lethal criteria. If the embryo presented at least one lethal criterion, it was coded 1 (dead) and if it was alive it was coded 0.
- iFET score: this semi-quantitative variable is both based on lethal and sub-lethal criteria. This score is equal to the sum of sub-lethal criteria developed per embryo (0 to 9 possible sub-lethal criteria measurable) or to 10 if the embryo presented at least one lethal criterion.

2.3. Genotoxicity Evaluation on Danio Rerio Embryo Cells

At the end of the 96 h exposure duration, cells were isolated from whole embryos and used to assess primary DNA damage level with the Fpg (formamidopyrimidine-DNA glycosylase)-modified comet assay chosen for its improved sensitivity to detect oxidative damage and Fapy forms of bases resulting from some alkylating damages. The embryo cell suspension was prepared according to [17] with some modifications. Genotoxicity was evaluated on cells of a pool of three alive 96 h embryos without any macroscopically observable abnormality (scored 0) from each exposure plate. Embryos were anaesthetized on melting ice, minced with a razor blade and mixed with 0.5 mL of complete Leibowitz medium (L-15 medium, Sigma L5520) containing 10% fetal bovine serum, penicillin (0.1 mg/mL) and streptomycine (100 U/mL). Afterwards, 0.5 mL of a dispase II solution (12.5 U/mL) was added and the mixture was maintained at 27˚C under mild ellipsoidal agitation (300 rpm) for 15 minutes. Tubes were centrifuged at 2000 ×g for one minute at 4˚C, the cell pellet was carefully suspended with 1 mL of complete L-15 medium. After a second centrifugation (1 min, 2000 ×g, 4˚C), the cell pellet was dispersed by mild pipetting in 200 µL of complete L-15 medium. At that stage, cell viability was checked using the Trypan blue exclusion method and was always found ≥ 80%. For the comet assay, 20 µL of the cell suspension were mixed with 80 µL of agarose gel (low melting point, type VII, 0.5% final concentration). Forty µL of this mixture were laid on a pre-coated (0.8% normal agarose) and frosted microscope slide (2 duplicate gels per slide).

The assay was conducted according to [18] slightly modified as followed. The lysis solution was prepared without DMSO. Regarding the Fpg digestion step, a Fpg (New England Biolabs) solution (1.6 U Fpg/mL) was prepared in pre-warmed (37˚C) enzyme buffer (40 mM, 0.1 M KCl, 0.5 mM EDTA) containing 0.2 mg/mL bovine serum albumin. Slides placed in a tray were completely covered by the Fpg solution for 15 minutes at 37˚C in the dark and were then cooled at 4˚C for 10 minutes to stop the enzymatic reaction. Through this procedure, the enzyme solution was equally distributed within gels. After unwinding, electrophoresis, neutralization and dehydration, gels were examined through a fluorescence microscope (Axioskop 40, Zeiss Ltd.). Image analysis was performed with the Comet 4.0 software (Perceptive Instruments Ltd.). For each sample, % tail intensity (TI, % DNA in comet tail) was randomly registered on 50 cells from each replicate gel, and the entire experiment was repeated three times.

2.4. Endocrine Activities Quantification

To produce the MDA-kb2 cell line (ATCC #CRL-2713), the MDA-MB-453 human breast cancer cells were stably transfected by a MMTV promoter-luciferase plasmid construct [19]. The MDA-kb2 cell line expresses androgen (AR) and glucocorticoid (GR) receptors endogenously, as well as the luciferase reporter gene coupled to the MMTV promoter, which is activated by these two receptors. Therefore, a positive response in this assay can be mediated through AR or GR ligands or a mixture of both. To distinguish if a positive response comes from an activation of the androgen or glucocorticoid receptor, a co-exposure with specific AR (flutamide) or GR (RU486) antagonists was realized.

The MELN cell line was a kind gift from Patrick Balaguer (Montpellier, France). It was obtained by stable transfection of MCF-7 human breast cancer cells by an ERE-βGlob-Luc-SVNeo plasmid [20]. The MELN cell line stably expresses the luciferase reporter gene under transcriptional control of endogenous estrogen receptor (ER).

MELN and MDA-kb2 cells were grown at 37˚C under humidified air atmosphere (with 5% CO_2) in phenol red containing DMEM Medium (Sigma) supplemented with 10% fetal bovine serum (FBS), 1% (v/v) non-essential amino acids, penicillin/streptomycin (50 U/ml each) and 0.1 mg/ml geneticin (Invitrogen, France). Assays were conducted according to [11]. The MELN cells were seeded at a density of 1×10^4 cells per well and MDA-kb2 cells were seeded at 6×10^4 cells per well of 96-well white opaque tissue culture plates (Greiner CellStar), and left to incubate for 24 h. Serial dilutions of reference chemicals (17β-estradiol for MELN; dihydrotestosterone and dexamethasone for MDA-kb2) or of organic extracts of effluents were added in triplicates. At least two independent experiments per effluent were conducted. After 24 h exposure, the medium was removed and replaced by 50 µl of phenol red free medium containing 0.3 mM_D-luciferin (Sigma) and the luminescence signal in living cells was read after 5 min with a microtiter plate luminometer (MicroBetaWallacLu-

minometer).

For positive samples, hormone-equivalent concentrations were determined as previously described [11]. In brief, dose response curves were modeled with the Hill equation model using the macro RegTox [21] and EC_{20} was calculated. EC_{20} was then compared to that of the reference molecule, *i.e.* 17β-estradiol (E2), dexamethasone (Dex) or dihydrotestosterone (DHT), to determine hormone equivalent (E2-EQ, Dex-EQ or DHT-EQ, respectively) concentrations expressed in ng/L, as exemplified for E2-EQ by the following equation: E2-EQ = EC_{20} 17β-estradiol/EC_{20} effluent.

2.5. Chemical Analysis of Effluents

The concentration of a panel of low biodegradable pharmaceuticals and surfactants was monitored in the different treated effluents. The list of analyzed pharmaceuticals was established according to their high consumption in hospital and domestic environments, their potential for bioaccumulation and known toxic effects [22]. The most common used surfactants for industrial, hospitals and domestic activities were also monitored. Effluents were filtered through glass microfiber filter at 0.7 µm and then extracted by solid phase extraction according to [23] for pharmaceuticals and [24] for surfactants. Micropollutants in these samples were dosed by using multi-residual method coupling liquid chromatography and tandem mass spectrometry. The limit of detection (LD) and of quantification (LQ) for each compound is given in **Table 3**. LD and LQ correspond to the amounts for which signal-to-noise ratios of 3 and of 10 were obtained during the LC-MS/MS analyses, respectively.

Table 3. Limit of detection (LD) and of quantification (LQ) for pharmaceuticals [ng/L] and surfactants [µg/L].

Pharmaceuticals	LD ng/L	LQ ng/L	Surfactants	LD µg/L	LQ µg/L
Carbamazepine	0.2	0.6	Comperlan 100	0.03	0.1
Ciprofloxacin	11.8	35.3	Triton X-100	0.03	0.1
Sulfamethoxazole	2.0	5.9	Incromine SB	0.07	0.2
Salicylic acid	4.4	13.3	Stepanquat GA 90	1.67	5
Ibuprofen	0.2	0.5	BDDAC Benzyldodecyldimethyl ammonium chloride	0.08	0.24
Paracetamol	4.1	12.2	BDTAC Benzyldimethyltetradecyl ammonium chloride	0.05	0.16
Diclofenac	1.7	5	Lauryl pyridinium	0.07	0.2
Ketoprofen	3.1	9.3	Sodium 2-ethylhexyl sulfate	1.67	5
Propanolol	0.2	0.6	Sodium dodecyl sulfate (SDS)	0.17	0.5
Atenolol	1.4	4.1	LAS C10	0.67	2
Econazole	0.4	1.1	LAS C11	0.67	2
Ethinylestradiol	2.4	7.3	LAS C12	0.67	2
			LAS C13	0.67	2
			Texapon N 701 S	1.67	5
			Cetyl betain	0.03	0.1

2.6. Statistical Analysis

Statistical analysis for embryo toxicity datasets were conducted using the R free software. For binary variables (mortality), comparisons between each treatment and control or between treatments were conducted using the Fisher exact test. According to the FET guidelines [8], percentage of mortality of all replicates per sample with 20 embryos each was calculated. For the iFET score, which is a semi-quantitative variable with a non-classical distribution (normality distribution not fulfilled), comparisons were conducted using the Wilcoxon rank sum test (n = 60) with Bonferroni Holm adjustment for multiple comparison. Concerning genotoxicity evaluation, the DNA damage expressed as TI% was summed up by its median value for each of the gels obtained for each condition (n = 6). Comparisons of these median values between each treatments and control or between treatments were conducted using the non-parametric Kruskal-Wallis test followed by a Dunn's post hoc testing.

3. Results

3.1. Mortality and Developmental Abnormalities in FELS

Mortality in both control groups (1% and 2% in control 1 and 2, respectively) was below 10%, what fulfilled the validity criterion of the FET protocol. Less than 10% of embryos in each control group (6% in control 1 and 3% in control 2) showed an altered development. Hatching rate in all controls was ≥ 90%, satisfying the FET validity criterion requiring a hatching success over 80% in control groups. Mortality in positive control after 96 h exposure to 3.4-DCA was always >30% and therefore also fulfilled the validity criterion of the FET protocol (data not shown).

In the first campaign, acute toxicity (mortality) was significantly elevated in MBBR (8.8%) and $MBBRO_{3\ tert}$ (6%) groups when compared to their corresponding control 1 ($p < 0.001$ and $p = 0.005$, respectively). After integration of sub-lethal criteria (iFET score), MBBR and $MBBRO_{3\ tert}$ significantly impaired embryo development when compared to the control ($p < 0.001$) and $MBBRO_{3\ tert}$ slightly increases developmental abnormalities compared to MBBR ($p = 0.066$) (**Figure 1**). The results show that 13% of the embryos suffered at least one developmental abnormality after MBBR treatment compared to 33% of the embryos for the $MBBRO_{3tert}$.

In the second campaign, 35% of embryos exposed to CAS effluent died, what was significantly different compared to the corresponding control 2 ($p < 0.001$). Mortality of embryos exposed to $CASO_{3\ tert}$ or$CASO_{3\ loop}$ was not different from that of control 2. When taking into account developmental abnormalities (iFET score), all three effluents were significantly more toxic than the control ($p < 0.001$), with CAS effluent being significantly more toxic than $CASO_{3\ tert}$ ($p = 0.009$) and $CASO_{3\ loop}$ ($p = 0.009$) effluents. No significant difference was found between $CASO_{3\ tert}$ and $CASO_{3\ loop}$, between 30% and 35% of the embryos depicted at least one developmental abnormality after exposure to these effluents

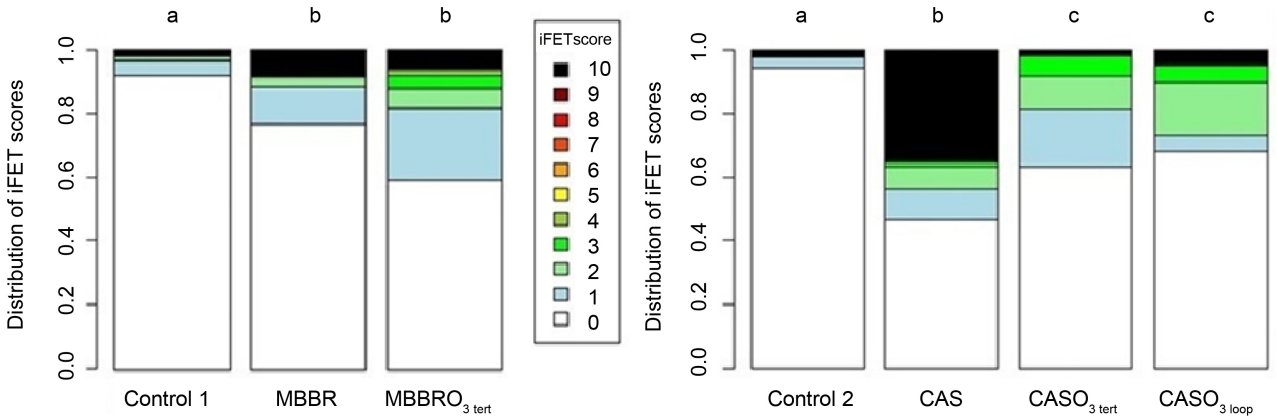

Figure 1. Distribution of iFET score in the control, and effluent samples during both campaigns. Numbers of observed sublethal criteria (0 - 9) per embryo are color-coded, whereas a value of 10 (in black) is assigned to mortality. Different letters indicate significant differences between samples with $p < 0.05$ (Wilcoxon rank sum test); Control 1 (n = 596 scored embryos) and 2 (n = 356); MBBR (n = 80); $MBBRO_{3\ tert}$ (n = 100); CAS (n = 60); $CASO_{3\ tert}$ (n = 60); $CASO_{3\ loop}$ (n = 60).

treated by both biological and ozone processes.

3.2. Genotoxicity on Danio Rerio Embryo Cells

Median primary DNA damage expressed as tail intensity was 6.63% TI and 9.15% TI in control group 1 and 2, respectively (Table 4). The positive control (MMS 25 µM) induced significantly higher DNA damage (from 35.4 to 54.6% TI) compared to the corresponding control group. Only the $CASO_{3\ loop}$ effluent induced a significantly higher DNA damage level in exposed embryos when compared to the corresponding control. No significant differences between effluent samples were found.

3.3. Detection of Endocrine Disrupting Activities

Estrogenic activity was detected in both biological MBBR (3 ng/L EEQ) and CAS effluents (5 ng/L E2-EQ) (Table 5). For both campaigns, differences between effluent samples could be evidenced; ozone-based treatments resulted in lower values than biological treatments alone, being either undetected after tertiary ozonation at TOD of 15 mg O_3/L ($MBBRO_{3\ tert}$) or at 1 ng/L E2-EQ after tertiary ozonation at 5 mg O_3/L TOD ($CASO_{3\ tert}$) and 3 ng/L E2-EQ in $CASO_{3\ loop}$ at 9.4 mg O_3/L TOD. Glucocorticoid activity was detected in all effluents excepted after tertiary ozonation at 15 mg O_3/L TOD ($MBBRO_{3\ tert}$). Androgenic activity was not detected in any of the samples but suspected in $CASO_{3\ loop}$ effluent, as the MDA-kb2 response to this sample was partly inhibited by the AR antagonist flutamide. The use of an AR specific bioassay would have been needed to confirm and quantify androgenic activity in this sample.

3.4. Chemical Analyses

Measured concentrations of pharmaceuticals in the treated effluents are detailed in Table 6. Only ciprofloxacin and ethinylestradiol concentrations remained in

Table 4. Genotoxicity evaluation on zebrafish embryos carried out at the end of the FET test. Median DNA damage is expressed as % TI (tail intensity) with minimum and maximum values (n = 6); Asterisks indicate significant difference compared to corresponding control using Kruskal-Wallis and Dunn's post hoc tests. TOD: transferred ozone dose.

Campaign (TOD mg O_3/L)	Sample	TI [%]	Min TI [%]	Max TI [%]	Significant p-value
1	Control 1	6.63	2.7	16.8	-
1	MBBR	17.11	16.6	17.8	-
1 (15)	$MBBRO_{3\ tert}$	8.45	7.1	19.6	-
1	MMS 25 µM	35.4*	19.4	64.9	<0.001
2	Control 2	9.15	8	14.8	-
2 (9.4)	$CASO_{3\ loop}$	27.46*	21.7	31.5	<0.05
2	CAS	17.75	16.8	41	-
2 (5)	$CASO_{3\ tert}$	20.34	17.4	26.3	-
2	MMS 25 µM	54.6*	40.9	76.1	<0.001

Table 5. Endocrine activity is quantified for each effluent. E2-EQ: 17β-estradiol equivalent. DHT-EQ: dihydrotestosterone equivalent. Dex-EQ: dexamethasone equivalent. LQ: limit of quantification. TOD: transferred ozone dose.

Treatment conditions		Estrogenic activity (ng/L E2-EQ)	Androgenic activity (ng/L DHT-EQ)	Glucocorticoid activity (ng/L Dex-EQ)
Campaign (TOD mg O_3/L)	Sample name			
1	MBBR	3	<LQ	250
1 (15)	$MBBRO_{3\ tert}$	<LQ	<LQ	<LQ
2	CAS	5	<LQ	509
2 (5)	$CASO_{3\ tert}$	1	<LQ	310
2 (9.4)	$CASO_{3\ loop}$	3	<LQ	701

all analysed samples below the limit of quantification. In campaign 1, the tertiary ozonation treatment at 15 mg O_3/L ($MBBRO_{3\ tert}$) resulted in much lower pharmaceutical concentrations in the effluent than after MBBR treatment alone. This trend was also observed in the second campaign where $CASO_{3\ tert}$ effluent showed the lowest concentrations of pharmaceuticals. Ozonation applied to the recycled mixed liquor also resulted in lower concentrations of monitored pharmaceuticals ($CASO_{3\ loop}$) than after CAS treatment alone, but in higher concentrations than in $CASO_{3\ tert}$ effluent.

The detailed concentrations of surfactants measured in effluent samples are presented in Table 7. Only Comperlan 100 was below LQ in all tested samples. Overall, ozonation did not lead to lower surfactant concentrations in treated effluents compared to non ozonated ones. In campaign 1, tertiary ozonation of

Table 6. Chemical analyses of pharmaceuticals (ng/L) in effluents stemming from each treatment.

	Campaign 1		Campaign 2		
	MBBR	$MBBRO_{3tert}$	CAS	$CASO_{3tert}$	$CASO_{3loop}$
Paracetamol	138.4	35.56	63.8	18.38	26.5
Carbamazepine	203.8	<LQ	3034	41.8	1476
Sulfamethoxazole	2176	<LQ	5220	700	2246
Ketoprofen	155.8	<LQ	26.46	31.24	98.6
Propanolol	307	1.288	166.4	6.56	131
Atenolol	416	<LQ	904	245.6	97.2
Diclofenac	356.2	<LQ	482	6.14	231
Ciprofloxacin	<LOQ	<LQ	<LQ	<LQ	<LQ
Econazole	9.94	2.108	30.68	4.82	29.24
Salicylic acid	101.2	31	242.2	285.6	417.4
Ibuprofen	248.8	1.398	35.44	16.58	34.06
Ethinylestradiol	<LOQ	<LQ	<LQ	<LQ	<LQ

Table 7. Chemical analyses of surfactants (µg/L) in effluents stemming from each treatment. BDDAC: Benzyldodecyldimethyl ammonium chloride, BDTAC: benzyl dimethylte tradecyl ammonium, LAS: linear alkylbenzene sulfonates, SDS: sodium dodecyl sulfate.

	Campaign 1		Campaign 2		
	MBBR	$MBBRO_{3tert}$	CAS	$CASO_{3tert}$	$CASO_{3loop}$
Comperlan 100	<LQ	<LQ	<LQ	<LQ	<LQ
Cetyl betain	<LQ	0.12	<LQ	<LQ	<LQ
Triton X-100	1.00	2.97	<LQ	<LQ	<LQ
BDDAC	0.50	0.90	0.37	0.34	0.40
BDTAC	0.62	0.44	0.21	0.24	0.23
Stepanquat GA 90	<LQ	<LQ	5.03	6.98	<LQ
Lauryl pyridinium	0.31	<LQ	0.33	0.33	0.33
Incromine SB	0.34	0.21	<LQ	<LQ	<LQ
Sodium 2-ethylhexyl sulfate	14.63	<LQ	<LQ	<LQ	<LQ
SDS	8.65	1.92	<LQ	<LQ	<LQ
LAS C10	16.46	11.48	41.95	26.35	88.15
LAS C11	15.23	34.14	23.15	16.45	45.3
LAS C12	10.32	53.14	12.8	14.5	52.7
LAS C13	8.88	90.40	5.47	2.59	21.05
Texapon N 701 S	<LQ	105.42	<LQ	<LQ	<LQ

MBBR resulted in higher concentrations of Cetylbetain, Triton X-100, BDDAC, LAS C11-13 and Texapon N 701 S than downstream MBBR treatment alone.

In campaign 2, concentrations of BDTAC, Stepanquat GA90 and LAS C12 in $CASO_{3\ tert}$ effluent were slightly higher than in CAS. The concentrations of

BDDAC, BDTAC, LAS C10-13 in $CASO_{3\ loop}$ effluent were higher than in CAS and also higher than in $CASO_{3\ tert}$ effluent apart for BDTAC.

4. Discussion

The tests performed only allow the direct comparison of the treated effluents generated during the same campaign as they resulted from the same wastewater influent. Besides, they do not permit to conclude on the impact of a given treatment configuration since reproducibility study was not carried out. Therefore, the discussion focuses on the differences observed through the measured toxicity end-points according to a given water quality rather than on the comparison between the different treatment processes.

It is indeed worth noting that the large variations in chemical composition (pharmaceuticals and surfactants) of effluents sampled at two different dates make their comparison difficult. These variations could be linked to differences in the hospital activity, as this was already pointed out in some studies where strong fluctuations in chemical composition during the day time with concomitant changes in toxicity were demonstrated [25]. It can also be assumed that wastewater exhibited higher total pharmaceutical concentrations during winter (campaign 1) than during summer (campaign 2) and can be related to the higher medicine consumption during the cold season. Moreover, the different natures of biological treatments employed here, a biofilm reactor and a conventional activated sludge, as well as the differences in effluent average temperature observed (12˚C for campaign 1, 20˚C for campaign 2) might have led to changes in microbial activity, thus possibly inducing changes in terms of treatment efficiency to remove pharmaceuticals, surfactants, hormones and personal care products as found by [26].

The effluent quality of all the samples regarding usual aggregate parameters was constant (**Table 2**). One common trend in both campaigns is the efficiency of ozonation to reduce the pharmaceutical content in effluents compared to biological treatments alone (**Table 6**). Regarding surfactants (**Table 7**) ozonation resulted in much more variable relative abatements and for several compounds, ozonation even leads to a clear-cut increase in concentrations compared to their corresponding biological treatment.

Overall, if pharmaceutical abatement was high and led to a decrease in acute toxicity (FELS mortality) in particular with tertiary ozonation, residual embryotoxicity was still found in all the ozonated effluents. During the first campaign, embryos exposed to $MBBRO_{3\ tert}$ effluent using a rather high ozone dose (15 mg/L TOD) exhibited slightly more developmental abnormalities per embryo than in the case of MBBR despite the advanced elimination of the targeted pharmaceuticals in the $MBBRO_{3\ tert}$ effluent. On the opposite, using lower TODs, contrary to the CAS sample, no significant acute toxicity was shown in $CASO_{3\ tert}$- and $CASO_{3\ loop}$-exposed zebrafish embryos but a significant impairment of embryo development was revealed through the recording of sub-lethal criteria dur-

ing the test and by applying the iFET score calculation.

The discrepancy between the reduction of pharmaceuticals resulting from tertiary ozonation and the residual developmental toxicity might be due to several phenomena. First, a part of the measured toxicity could be related to the presence of non-monitored compounds recalcitrant to the ozone chemical action, and another part related to ozonation by-products. In some previous studies, tertiary ozonation was found to result in ozonation by-products equally or more toxic than parent compounds when the latter are not totally degraded [27]. Reference [6] observed developmental retardation, significant decrease in body weight and in length of rainbow trout early life stages exposed to tertiary ozonated effluents due to by-products formed during ozonation. Hypothesis of an increase in some toxicant bioavailability after ozonation cannot be excluded. The release of toxicants, previously bound to suspended matter and thus becoming bioavailable after ozonation has been already suggested [6].

While standardized bioassays are often not sensitive enough to reveal residual toxicity of treated WWTP effluents [4], iFET score approach used in the present study seems a relevant added value to the FET test. Without the proposed iFET score approach, the overall toxicity towards FELS of ozonated effluents would have been underestimated, because on a strict ecotoxicological point of view, the most severely deformed larvae would likely not survive in their natural wildlife habitat due to reduced fitness, making them easier preys [28]. The adopted procedure is simple to perform, does not require added experimental facilities than for the classic FET and iFET score can be measured by using the same number of embryos than that required for FET.

Concerning a remaining genotoxic potential, although higher in all samples than in control, probably because of the sensitive approach chosen (Fpg-modified comet assay), it has to be stressed that very few significant differences were shown and that absolute levels found in all samples expressed as % tail intensity remained rather low with an average induction factor around 2 - 3 compared with controls. This corroborates results obtained with the Fpg-modified comet assay on the fish cell line RTL W1 showing a low genotoxic risk in hospital effluents treated in a conventional (CAS) WWTP [29]. Genotoxicity was here evaluated in macroscopically healthy hatched embryos partly to limit false positive. Base excision repair profile starts changing towards the profile of adult zebrafish as soon as embryos hatch and encounter normal oxidative stress [30]. Therefore, the genotoxicity assessment on exposed embryos 96 hpf can be considered as suitable to evaluate additional cryptic damage occurring at a molecular level enabling to complete the residual toxicity evaluation of treated wastewaters. In the present work, tertiary ozonation ($MBBRO_{3\ tert}$, $CASO_{3\ tert}$) does not cause a genotoxic potential increase but was not totally efficient to fully eliminate the residual genotoxic potential found downstream biological treatment alone, in accordance with studies on human leucocytes [3]. Following the same trend, ozonation applied in mixed liquor recirculation at higher dosage

($CASO_{3\ loop}$) even resulted in a significantly higher genotoxic potential than with tertiary ozonation $CASO_{3\ tert}$. Application of such high dose of ozone is probably able to cause sludge disintegration, releasing genotoxic compounds previously adsorbed onto suspended solids as suggested by the chemical analysis showing substantially higher concentrations for some pharmaceuticals and surfactants (**Table 6** and **Table 7**). Some surfactants such as benzalkonium chlorides and dimethyldioctadecyl-ammonium bromides were already shown to induce DNA migration in primary rat hepatocytes but at concentrations much higher (>0.33 mg/L) than those measured in the present study [31]. Nevertheless, data on toxicity of quaternary ammonium compounds is still scarce and toxic effects of mixtures need further investigation. It was shown that genotoxicity might be correlated with other relevant ecotoxicological responses such as as impairment of reproduction and growth in fish. Those defects are relevant in ecotoxicology since they provide information to relate individual effects to possible changes in population size or structure [32]. Even if one should notice in this work that the genotoxicity potential of effluents was rather low, whatever the treatment, adding genotoxicity assessment within a FELS test seems undoubtedly an added value, without the need for extra experimental facilities [33]. This work shows that DNA damage assessment on macroscopically healthy embryos using the Fpg-modified comet assay resemble a sensitive early warning system to evaluate the genotoxic potential of complex matrixes such as WWTP effluents. However, unrepaired DNA damages such as micronucleus formation, which could also be measured on an embryo cell suspension, deserved to be also considered in an improved test battery since FELS exhibit a high mitotic index and are likely to reveal micronucleus formation.

As stressed by [34], identification of an optimized fish embryo test as an available near-term alternative to FET test has to be prioritized. Our proposal to apply the iFET score concept complemented by genotoxicity evaluation on the same set of exposed embryos constitutes a step to achieve such an objective.

Moreover, impaired embryonic development could be related to endocrine disruptor compounds. For instance, some of EDCs exhibit a genotoxic potential in aquatic invertebrates, which could partly explain the observed developmental defects and genotoxicity in fish embryos in this study, but these compounds remained to be identified [35] [36]. In the present study, the use of *in vitro* assays allowed to quantify estrogenic, androgenic or glucocorticoid activities of effluents and highlighted their removal through tertiary treatment. Additionally, they exhibit a limit of quantification of hormone-mimetic compounds often lower than that of routine chemical analyses.

As an example, the limit of quantification of estrogenic activity with MELN is ≤ 0.1 ng/L E2-EQ (17β-estradiol equivalent) thus below the PNEC of 0.04 ng/L [37] when the quantification limit of the chemical analysis for 17α-ethinylestradiol is as high as 7.3 ng/L, *i.e.* 182.5 times higher

The removal of estrogenic activity here provided by ozone application to bio-

logical effluents was high with residual level even under LQ (0.1 ng/L) achieved in some cases ($MBBRO_{3\ tert}$) as already shown [10]. In this field, numerous studies have led to a guide value, to define an environmental quality threshold, of 0.4 ng E2-EQ/L. This value is already the subject of a broad scientific consensus [38] since a level of 1 ng E2-EQ/L has been related to negative effects in aquatic organisms [39]. Reference [40] showed a significant decrease in embryo production in *Potamopyrgus antipodarum* after exposure to ≥ 1 ng/L 17α-ethinylestradiol, and [41] underlined significant changes in gonad histology, alterations in mating behavior and reduction of fertilization success in zebrafish exposed to 17α-ethinylestradiol concentrations ≥ 1.67 ng/L. Recently, a PNEC value as low as 0.04 ng/L for 17α-ethinylestradiol was proposed for fish by [36]. Reference [42] confirmed that estrogenic activity measured with *in vitro* assays correlated positively with reproductive endocrine disruption observed in organisms exposed *in vivo*. So the potential of effluents of the present study with measured estrogenic activity ≥ 1 ng/L E2-EQ are still likely to induce reproduction impairment in fish through chronic *in vivo* exposure. Undoubtedly, further innovative treatment should target to reduce endocrine active compounds below a level of environmental concern.

Since the pilot wastewater treatment unit received a mixture constituted of 50% hospital and 50% urban effluent, the significant glucocorticoid activities found in all treated effluents with Dex-EQ ranging between 250 ng/L and 700 ng/L (except $MBBRO_{3\ tert}$ for which Dex-EQ was below 100 ng/L) can be partly attributed to the presence of glucocorticoid pharmaceuticals, used in high quantity in hospitals [11]. Some significant effects have been documented in fish from 100 ng/L Dex-EQ, such as a decrease in plasma vitellogenin in female, an increased expression of male secondary sexual characters and a higher susceptibility to disease [43]. Such a high glucocorticoid activity was already found in treated urban wastewater by [44] and could be in the present study responsible for a significant part of the sub-lethal defects recorded in embryos, as suggested by the work of [45] who showed that dexamethasone caused developmental abnormalities in zebrafish. The presence of a higher glucocorticoid activity in $CASO_{3\ loop}$ effluent than in CAS effluent, may argue for the release of glucocorticoid mimetic compounds, possibly due to the deconjugation of pharmaceuticals excreted as conjugated metabolites or to the elimination of strong receptor antagonists, allowing the agonist compounds to bind to the receptor, or to the desorption of parent glucocorticoids [46]. Among configurations tested, only implementation of tertiary ozonation at rather high ozone dose ($MBBRO_{3\ tert}$) was able to abate all the endocrine activity potentials below the respective levels of quantification.

5. Conclusion

The present work was an attempt to address the difficult issue of the toxicity evaluation of complex environmental matrices such as WWTP effluents down-

stream advanced treatment for emerging micropollutant control. The test battery used allowed to describe the residual toxicity towards zebrafish embryos of all tested effluents, characterized by very low acute toxicity but by significant developmental toxicity and low genotoxicity. Moreover the implemented battery allowed discriminating between biological and ozonation treatments. Thus, even if it is rightfully restrictive, this simplified test battery focusing on measurement of developmental defects and genotoxicity in FELS and including a sensitive and exhaustive measurement of residual endocrine disrupting activities constitutes an alternative to the usual combination of numerous monospecific standardized tests often lacking sensitivity to evaluate WWTP effluents toxicity towards ecosystems. This test battery represents a reasonable compromise between the relevancy of information gained regarding ecotoxicity assessment of a complex matrix, the technical facilities required, the time and money to be put in.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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