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A Pilot Study Combining Ultrafiltration with Ozonation for the Treatment of Secondary Urban Wastewater: Organic Micropollutants, Microbial Load and Biological Effects

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Abstract: Ozonation followed by ultrafiltration (O_3 + UF) was employed at pilot scale for the treatment of secondary urban wastewater, envisaging its safe reuse for crop irrigation. Chemical contaminants of emerging concern (CECs) and priority substances (PSs), microbial load, estrogenic activity, cell viability and cellular metabolic activity were measured before and immediately after O_3 + UF treatment. The microbial load was also evaluated after one-week storage of the treated water to assess potential bacteria regrowth. Among the organic micropollutants detected, only citalopram and isoproturon were not removed below the limit of quantification. The treatment was also effective in the reduction in the bacterial loads considering current legislation in water quality for irrigation (i.e., in terms of enterobacteria and nematode eggs). However, after seven days of storage, total heterotrophs regrew to levels close to the initial, with the concomitant increase in the genes 16S rRNA and *intI*1. The assessment of biological effects revealed similar water quality before and after treatment, meaning that O_3 + UF did not produce detectable toxic by-products. Thus, the findings of this study indicate that the wastewater treated with this technology comply with the water quality standards for irrigation, even when stored up to one week, although improvements must be made to minimise microbial overgrowth.

Keywords: advanced oxidation; membrane technology; micropollutants; biological contaminants; cytotoxicity; wastewater reuse

1. Introduction

Urban wastewater reuse is considered an important strategy when addressing water scarcity issues [1]. This is a common practice in some countries, where the treated wastewater is mostly directed for agricultural irrigation [2]; however, urban wastewater often contains a variety of contaminants, such as salts, metals, metalloids, pathogens, and organic micropollutants, such as residual drugs, endocrine-disrupting chemicals, and residues from personal care products, among others [3,4]. Moreover, there is growing evidence that conventional urban wastewater treatment plants (UWWTPs) are not completely effective in eliminating bacteria and chemical micropollutants [5,6], rendering the effluent unsuitable for crops irrigation. Failure to properly treat and manage wastewater can generate adverse health effects, accumulation of heavy metals in crops, and the production of low-quality agricultural goods [3]. A new regulation on minimum preconditions for water reuse for agricultural irrigation has entered into force in the EU, which encompasses coordinated water-quality monitoring requisites for the safe reuse of treated urban wastewater [7]. These new rules will be put into practice in 2023 and are expected to promote water reuse. This regulation also demands an established water reuse risk management plan that should consider the environmental quality standards for priority substances and certain other pollutants, as well as additional requirements, such as heavy metals, pesticides, disinfection by-products, pharmaceuticals, and other substances of emerging concern, including micropollutants and microplastics. It also addressed the identification of some preventive measures that can be taken to limit risks, namely additional disinfection or pollutant removal measures.

Advanced oxidation processes (AOPs) and technologies (AOTs), such as ozonation, have emerged as effective tertiary treatments for the removal of both chemical and biological contaminants in UWWTPs [8,9]. Ozonation is among the few AOTs that have been applied to large-scale water treatment, due to its strong oxidation ability and broad-spectrum disinfection [10]. Ozone can react either by direct oxidation of organic pollutants (mostly at acidic conditions), or via hydroxyl radical formation (mainly produced under alkaline conditions) [10]. Studies employing ozone-based AOTs in UWWTP effluents have yielded remarkable results regarding the simultaneous removal of CECs and the reduction in the microbial load at different ozone doses and contact times [11–16]; however, bacterial regrowth in stored treated wastewater has been observed [14–16], which might be the result of the bacteria's ability to repair injuries, promoting fast regrowth, when stress levels are lowered. This may jeopardize water quality in the long term, thus prompting its immediate reuse rather than storing this water. Additionally, the use of chlorine as the traditional disinfection agent in stored water may not ensure its safety, because injured bacteria can also survive and regrow at low chlorine doses [17]. A suitable approach would be a physical separation step, using membrane-like technology. Although ozone may damage cell components, such as lipids, proteins and DNA, membrane filtration acts via size exclusion and adsorption, retaining microorganisms [18]. Among the available options in the market for full-scale applications, ultrafiltration (UF) membranes are favourable alternatives for bacteria removal due to their small pore size (0.01 to 0.1 µm). Moreover, studies have shown that UF is preferred to other filtration alternatives to avoid the regrowth of antibiotic-resistant bacteria (ARB) [19,20]. For example, Hembach et al., 2019 [18] reported the efficiency of UF in the disinfection of a secondary effluent of a UWWTP, and the results were compared with those obtained with single ozonation. The authors reported that UF (using a membrane pore size of 20 nm) was not able to remove the entire bacterial community, whereas ozonation presented limited effectiveness on the reduction in the same contaminants when using an ozone concentration optimised for micropollutant removal. Thus, these authors suggested further investigations coupling both technologies to achieve both micropollutant removal and bacteria mitigation, which was the target of the present study.

Thus, the present study investigated the potential of using UF in combination with ozonation, operating in continuous mode at a pilot scale, for the treatment of the secondary effluent of a UWWTP. Parameters commonly legislated in different countries were considered when assessing the suitability of treated wastewater for reuse in irrigation (Portuguese laws, US EPA, FAO guidelines and WHO). Moreover, envisaging higher quality criteria, the following parameters were also included in this work:

(i) priority substances and CECs identified in Directive 2013/39/EU and Decision 495/2015/EU [21,22], respectively; (ii) load of selected microbial groups; and (iii) potential estrogenic activity, cytotoxicity, and cell viability (biological effects). All these parameters were analysed in both freshly collected and O_3 + UF treated wastewater to assess treatment efficiency. Biological effects are particularly important to evaluate, due to the possibility of formation of toxic by-products after ozonation. Moreover, microbiological indicators were re-examined after a 7-day storage period to assess potential bacteria regrowth. Regarding other studies coupling O_3 to UF, only a few evaluate the feasibility of this system for urban wastewater reclamation [23–26] and, as far as it is known, none of those comprise the simultaneous evaluation of physico-chemical parameters, removal of priority substances and CECs, microbial inactivation and regrowth, and investigation of biological effects, which are important parameters for safe wastewater reuse, this work bringing a valuable contribution to the knowledge on this field.

2. Materials and Methods

2.1. Chemicals and Materials

All reference and isotopically labelled internal standards for liquid chromatography (>98% purity) were acquired from Sigma-Aldrich (Steinhein, Germany). Ethanol 99.5% (HPLC grade) was obtained from Fisher Scientific U.K. Ltd. (Loughborough, UK). Acetonitrile (MS grade) was purchased from VWR International (Fontenay-sous-Bois, France), whereas formic and sulphuric acid were obtained from Merck (Darmstadt, Germany). Multichannel tubular ceramic membranes with a selective layer of α-Al_2O_3 (nominal pore size of 10 nm) were provided by Rauschert Distribution GmbH, Inopor® (Schesslitz, Germany). Membrane dimensions were 305 mm in length with 15 mm glazed ends. The external diameter was 25 mm, and it contained 19 internal channels of 3.5 mm diameter each.

For microbial culture analyses, water samples were filtered through cellulose nitrate membranes (0.22 µm pore size, 47 mm diameter), provided by Sartorius (Gottingen, Germany). For DNA-based analyses, water samples were filtered through track-etched polycarbonate membranes (0.22 µm pore size, 47 mm diameter) from Whatman® Nuclepore™, provided by VWR (Alfragide, Portugal).

For cell culture experiments, dimethyl sulfoxide (DMSO; ≥99.9%), Triton™ X-100, and thiazolyl blue tetrazolium (MTT) were purchased from Sigma-Aldrich (Steinhein, Germany). Dulbecco's modified Eagle medium (DMEM; ref: 31966-021), heat-inactivated foetal bovine serum (FBS), penicillin-streptomycin (PenStrep), and trypsin-EDTA (1X) were purchased from Gibco® through Life Technologies™ (Warrington, UK). Murine fibroblasts L929 were obtained from the American Type Culture Collection (ATCC, Wesel, Germany). Caco-2 cell line was also purchased from ATCC and used between passage number 35 and 42. LDH Cytotoxicity Detection Kit was acquired from Takara Bio Inc. (Shiga, Japan). The XenoScreen YES/YAS assay kit for estrogenic activity assessment was acquired from Xenometrix® (Allschwil, Switzerland).

The ultrapure water used in the experiments and analytical methods was supplied by a Milli-Q water system (18.2 MΩ cm).

2.2. Secondary Effluent and Treated Samples

The secondary effluent used in the advanced treatment assays was collected at three different dates (between September and October 2019) from a full-scale UWWTP located in northern Portugal. In this UWWTP, the water line treatment includes a preliminary step (trash racking and dredging) followed by decantation, biological treatment with activated sludge, and a final decantation stage before discharging the effluent to the river. In this study, freshly collected samples of this UWWTP secondary effluent were divided into two aliquots, one of which was immediately analysed (WW) and another was directed to the O_3 + UF treatment unit. Details of the analytical methods employed to characterise the UWWTP secondary effluent (WW) are given in Section 2.4, and its chemical and biological characterisation can be found in Tables 1 and 2. Samples collected after O_3 + UF treatment (TWW_0) were also immediately

processed for microbiological analyses and DNA extraction. In addition, aliquots of TWW_0 were stored for seven days in sterile glass bottles under dark conditions and at room temperature (herein named as TWW_7) to assess possible bacterial regrowth in a hypothetical storage scenario for wastewater reuse.

Table 1. Characterisation of the urban wastewater treatment plant (UWWTP) secondary effluent, before (WW) and immediately after treatment (TWW_0), and standards of water for irrigation (Decree-Law 236/98) and wastewater reuse in irrigation without restriction, for urban wastewaters which treatment includes a disinfection step (Decree-Law 119/2019) and for wastewater reuse in the Eastern Mediterranean Region—WHO, 2016.

			Decree-Law		WHO
Parameters	**UWWTP Secondary Effluent (WW)**	**After O_3 + UF Treatment (TWW_0)**	**236/98 [27] MVR**	**119/2019 [28] PV**	**2016 [29] MVR**
Al (mg/L)	9.55×10^{-5}	6.10×10^{-5}	5.0	5	5.0
As (mg/L)	1.12×10^{-5}	$<5 \times 10^{-6}$	0.1	n.a	0.1
Ba (mg/L)	4.25×10^{-5}	1.52×10^{-5}	1.0	n.a	n.a
Be (mg/L)	$<5 \times 10^{-6}$	$<5 \times 10^{-6}$	0.5	0.1	0.1
B (mg/L)	1.29×10^{-4}	1.06×10^{-4}	0.3	variable	n.a
Cd (mg/L)	$<5 \times 10^{-6}$	$<5 \times 10^{-6}$	0.01	n.a	0.1
Pb (mg/L)	6.73×10^{-6}	6.55×10^{-6}	5.0	n.a	5.0
Cl^- (mg/L)	80.8	79.5	70	n.a	142 [b]
Co (mg/L)	$<5 \times 10^{-6}$	$<5 \times 10^{-6}$	0.05	0.05	0.05
Cu (mg/L)	1.26×10^{-5}	6.28×10^{-5}	0.2	n.a	0.2
Total Cr (mg/L)	$<5 \times 10^{-6}$	$<5 \times 10^{-6}$	0.1	n.a	0.1
Sn (mg/L)	$<5 \times 10^{-6}$	$<5 \times 10^{-6}$	2.0	n.a	n.a
Fe (mg/L)	1.06×10^{-4}	2.36×10^{-5}	5.0	2.0	5.0
F^- (mg/L)	<DL	<DL	1.0	2.0	1.0
Li (mg/L)	1.98×10^{-5}	1.96×10^{-5}	2.5	2.5	2.5
Mn (mg/L)	4.56×10^{-5}	3.77×10^{-5}	0.2	0.2	0.2
Mo (mg/L)	2.45×10^{-5}	8.60×10^{-5}	0.005	0.01	0.01
Ni (mg/L)	$<5 \times 10^{-6}$	$<5 \times 10^{-6}$	0.5	n.a	0.2
NO_3^- (mg/L)	0.9 ± 0.4	7.70	50	n.a	9.5 [b]
Salinity (µS/cm)	848	782	1000	variable	700 [b]
TDS (mg/L)	335	191	640	n.a	450 [b]
SAR (meq/L)	2.49	1.50	8	variable	3.0
Se (mg/L)	$<5 \times 10^{-6}$	$<5 \times 10^{-6}$	0.02	0.02	0.02
TSS (mg/L)	24.50	0.00	60	≤10	20 [c]
SO_4^{2-} (mg/L)	45.2	50.0	575	n.a	n.a
V (mg/L)	$<5 \times 10^{-6}$	$<5 \times 10^{-6}$	0.1	n.a	0.1
Zn (mg/L)	4.70×10^{-5}	2.61×10^{-5}	2	n.a	2.0
pH	7.0 ± 1.0	8.0 ± 0.2	6.5–8.4	n.a	6.5–8.4
E. coli (log CFU/100 mL)	6.67	<DL	2.0	≤10	2.3 [c]
Intestinal parasite eggs [a]	0.00	0.00	n.a	≤1	n.a

DL stands for detection limit; MVR stands for maximum value recommended; n.a stands for not applicable/available; SAR stands for sodium adsorption ratio; PV stands for parametric value; TDS stands for total dissolved solids; TSS stands for total suspended solids. [a] Analysed by an external laboratory—the maximum value allowed (MVA) for this parameter in the Decree-Law 236/98 is 1. [b] Value up to which there is no restriction to use in irrigation. [c] Permitted limit for greywater reuse in irrigation of vegetables likely to be eaten uncooked.

Table 2. Additional analyses made to the UWWTP secondary effluent, before (WW) and immediately after treatment (TWW_0).

			Becerra et al., 2015 [30]		Decree-Law 119/2019 [28]
Additional Analyses	**UWWTP Secondary Effluent (WW)**	**After O_3 + UF Treatment (TWW_0)**	**MVA**	**MVR**	**PV**
Dissolved organic carbon (DOC, mg/L)	11.0 ± 0.8	9.6 ± 0.8	n.a.	n.a.	n.a.
Biological oxygen demand (BOD_5, mg/L)	15.1 ± 1.1	0	10 [b]	n.a	≤10 [c]
Chemical oxygen demand (COD, mg/L)	22.7 ± 0.7	5.4 ± 0.8	60–200	n.a	n.a.
Turbidity (NTU)	3.25 ± 0.15	0.28 ± 0.02	2	n.a	≤5
NH_4^+	<DL	0.59	n.a	n.a	10
PO_4^{3-}	<DL	<DL	n.a	n.a	n.a

DL stands for detection limit; MVA stands for maximum value allowed; MVR stands for maximum recommended value; PV stands for parametric value; n.a stands for not applicable/available.

2.3. Experimental Setup and Procedure

A scheme of the experimental apparatus is depicted in Figure 1. Ozonation was performed in a packed-bed column (2.2 I.D × 70 cm height) with a useful volume of approximately 0.35 L and containing glass Raschig rings (6 mm I.D × 6 mm height), because the water–ozone mass transfer achieved in the column packed with these Raschig rings was up to 3 times higher than that in a bubble column [31]. Firstly, the reactor was filled with ultrapure water (through a peristaltic pump) to regulate the desired concentration of ozone in the liquid phase. Ozone was produced from pure oxygen in a BMT 802X ozone generator and bubbled at the bottom of the column. The ozone concentration in the gas inlet was regulated by adjusting the oxygen gas flow rate with a mass flow controller and the electric intensity of the ozone generator (BMT 802X). The concentration of ozone in the liquid phase (dissolved ozone) was measured with an ATI model Q45H dissolved ozone analyser placed at the exit of the column. High ozone doses and contact time increase the capital and operating costs, therefore a low ozone dose (0.9 ± 0.1 gO_3/gDOC) and a short hydraulic retention time (HRT: 8 min obtained with a liquid flow rate of 46 mL min^{-1}) were investigated. These experimental conditions were selected in preliminary tests and fixed for all the subsequent experiments.

After a period, ultrapure water in the inlet liquid stream was replaced by the UWWTP effluent to start the ozonation experiments. Samples of ozonised wastewater were only collected after a period of two residence times (~16 min), in order to ensure that the steady state was achieved (i.e., when the outlet wastewater achieved a constant concentration of pollutants in two subsequent measurements). Then, the ozonised effluent was directed to the feed tank of the UF pilot reactor, aiming for the physical removal of microbial cells. Fifteen litres of ozonised effluent was pumped to the UF pilot through a peristaltic pump (Varmec®) and filtered through the 10 nm α-Al_2O_3 membrane operating in cross-flow mode (1 bar of transmembrane pressure). The UF pilot was designed in a way that the liquid flow of ozonised wastewater was automatically regulated to maintain the pressure constant inside the membrane housing compartment. The concentrate was recirculated to the feed tank [32], while a composite sample of the permeate was collected and split for microbiological and chemical analysis (TWW_0 immediately after O_3 + UF treatment and TWW_7 after being stored for seven days). UF was performed after O_3 and not the other way around, because by doing so, the membrane fouling is minimised [33,34]. At the end of the treatment, the membrane was left with H_2O_2 (30% *w/v*) overnight, followed by abundant washing with boiling water and autoclaved before starting another experiment. This cleaning procedure was defined to restore the membrane permeance and sterility.

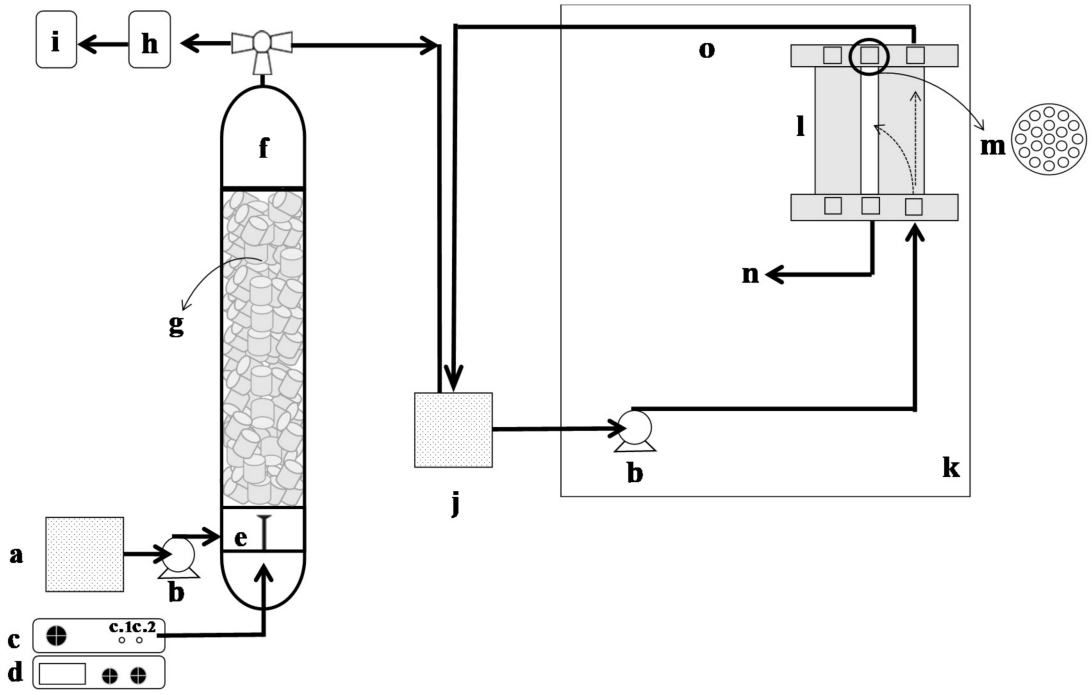

Figure 1. Scheme of the experimental apparatus. (**a**) feed tank containing deionised water or UWWTP effluent; (**b**) peristaltic pump; (**c**) ozone generator (c.1—O_2 entrance; c.2—O_3 exit); (**d**) mass flow controller; (**e**) ozone diffuser; (**f**) packed-bed column; (**g**) Raschig rings; (**h**) ozone analyser; (**i**) ozone destroyer; (**j**) feed to the ultrafiltration (UF) pilot; (**k**) UF pilot system; (**l**) membrane housing; (**m**) 19 channel ceramic membrane (top view); (**n**) permeate stream; (**o**) concentrate stream.

2.4. Chemical Analyses

The anionic and cationic contents (Cl^-, NO_3^-, SO_4^{2-}, Na^+, K^+) in water samples were determined by ion chromatography, as, using a Metrohm 881 Compact IC Pro apparatus equipped with a Metrosep C4 Cationic Exchange Column (250 mm × 4.0 mm) for the quantification of cations and a Metrosep A Supp 7 Anionic Exchange Column (250 mm × 4.0 mm) for quantification of anions. The content of metals was determined by using an inductively coupled plasma-optical emission spectrometer (ICP-OES, thermo scientific, model iCAP 7000 Series). The pH and conductivity of water were measured with pHenomenal® pH 1100L apparatus (VWR, Germany) and a conductivity meter (Crison GLP 31), respectively. Other relevant parameters (referred to as "additional analyses" in Table 2) were considered to assess the quality of water for irrigation: dissolved organic carbon (DOC) determined in a TOC-L analyser (Shimadzu TOC-5000A); turbidity measured with a turbidimeter (Hanna instruments, model HI88703); chemical oxygen demand (COD) determined by the closed reflux method (EPA standard method 5220D); and biochemical oxygen demand measured according to the EPA standard method 5210B (respirometric method) for a 5 day period (BOD_5). These analyses were performed as recommended in the standard methods for the examination of water and wastewater [35].

Moreover, the concentration of target organic micropollutants was determined using ultra-high performance liquid chromatography with tandem mass spectrometry (UHPLC-MS/MS) with Shimadzu Corporation apparatus (Tokyo, Japan) consisting of a triple quadrupole mass spectrometer detector (Ultra-Fast Mass Spectrometry series LCMS-8040) with an ESI (Electrospray Ionisation) source operating in both positive and negative ionisation modes. The mobile phase and operating conditions of the UHPLC-MS/MS system for the detection and quantification of the target pollutants are described elsewhere [16,36]. Prior to UHPLC-MS/MS analysis, WW and TWW_0 samples were pre-concentrated and cleaned up by solid-phase extraction (SPE) using Oasis® HLB (Hydrophilic-Lipophilic-Balanced sorbent, 150 mg, 6 mL) cartridges (Waters, Milford, Massachusetts, USA), according to the methodology

described elsewhere [37]. For internal calibration, isotopically labelled internal standards were added to the samples before SPE. The preconcentration procedure was performed in duplicate for all the samples. This methodology allows to determine a total of 14 organic micropollutants.

2.5. Microbial Culture Analyses

Volumes ranging from 100 mL to 1 mL of WW, TWW_0 or TWW_7 samples or of serial 10-fold dilutions thereof were filtered in triplicate and placed onto the appropriate culture media of the target microbial group: Plate Count Agar (PCA, VWR International (Pennsylvania, USA)) (30 °C, 48 h) for culturable heterothrops, m-Faecal Coliform Agar (mFC, Thermo Fisher Scientific, Massachusetts, USA) (37 °C, 24 h) for enterobacteria, Slanetz Bartley Agar (Thermo Fisher Scientific, Massachusetts, USA) (37 °C, 48 h) for enterococci, and Rose Bengal Chloramphenicol Agar (Thermo Fisher Scientific, Massachusetts, U.S.A.) (25 °C, 5 days) for fungi. Results were expressed as colony forming units per 100 mL of sample (CFU/ 100 mL).

2.6. DNA Extraction, 16S rRNA and Inti1 Genes Quantification

Volumes of 100 mL of WW, 2 L of TWW_0, and 800 mL to 1 L of TWW_7 were vacuum-filtrated and processed in three independent samplings as biological replicates. DNA extraction was performed using the DNeasy® PowerWater® Kit (QIAGEN, Hilden, Germany) according to Rocha et al., 2020 [38] and with two additional steps suggested in the manufacturer's troubleshooting guide: after adding the lysis solution, a heating step at 65 °C for 10 min was included in the protocol; and to ensure the removal of residual ethanol before DNA elution, the centrifugation step was conducted in a clean collection tube for an additional minute. DNA samples were stored at −20 °C until quantitative PCR (qPCR) analysis.

The 16S rRNA gene (a marker for total bacteria) and the *intI*1 gene encoding a class 1 integron-integrase (a marker of anthropogenic impact) were quantified based on qPCR to assess the removal efficiency of bacteria after treatment [39,40]. Gene-specific primer sequences are listed in previous studies [41,42] and provided as supplementary information in Table S1. Gene quantification was based on SYBR Green qPCR assays in a StepOnePlus™ Real-Time PCR System (Life Technologies, USA) and interpolation to the standard curve run in each assay, as described elsewhere [39,43].

The data that met the quality criteria described in Rocha et al., 2018 [44] were expressed as the ratio of gene copy number per 100 mL of water sample (WW, TWW_0, and TWW_7). The secondary wastewater effluent (WW) was used as reference to assess the removal efficiency of both 16S rRNA and *intI*1 genes in treated samples, immediately after treatment (TWW_0) and after storage for 7 days (TWW_7). The duration of 7 days was selected to allow enough time for eventual injured cells surviving the treatment to fully recover, as we have verified in previous works with other treatment solutions [14,15].

2.7. Biological Effect Assays

2.7.1. Cell Culture and Incubation with Water Samples

Murine fibroblasts L929 and Caco-2 cells were cultured in Dulbecco's modified Eagle medium (DMEM) with D-glucose (4.5 g L^{-1}), sodium pyruvate (0.11 g L^{-1}), L-alanyl-L-glutamine (0.86 g L^{-1}) and further supplemented with 10% (*v/v*) heat inactivated foetal bovine serum (FBS), and 5% (*v/v*) of PenStrep (37 °C, 5% CO_2 and 95% of humidity). For cell viability and cytotoxicity assessment, the cells were detached from the culture flask as described elsewhere [45]. After cell counting in Neubauer chamber (Boeco, Germany), the suspension was centrifuged at 300 *g* for 5 min, and the cell pellet was suspended in culture medium to a final concentration of 5×10^4 cells per well. Cells were then seeded in a 96-well microplate (100 µL per well) and cultured for 24 h at 37 °C (5% CO_2 and 95% humidity).

2.7.2. Thiazolyl Blue Tetrazolium Reduction (MTT) and Lactate Dehydrogenase (LDH) Assays

Cellular metabolic activity was evaluated as indicator of cytotoxicity by the thiazolyl blue tetrazolium reduction (MTT) assay, whereas cell membrane integrity was evaluated through the lactate dehydrogenase (LDH) assay, providing information about cell viability. Briefly, test water samples were filtered using Corning® syringe filters (Sigma-Aldrich®, St. Louis, MO, USA) with 0.20 µm pore diameter and diluted 1:10 and 1:5 in DMEM. After discarding culture supernatant, 100 µL of diluted samples were added to cell layers and incubated at 37 °C (5% CO_2 and 95% humidity). After 24 h, the supernatant was removed for LDH assay, while the remaining content of the wells was used for MTT assay. For MTT assay, absence of cytotoxicity (100%) was estimated by replacing water test sample by culture medium. For LDH assay, the absence of cell viability (100%) was estimated by replacing water test sample by 1% (*v/v*) Triton X-100 solution prepared in culture medium.

2.7.3. Yeast Estrogen Screen (YES) Assay for Estrogenic Activity Assessment

WW and TWW_0 samples were filtered through 0.21 µm hydrophilic membranes and analysed directly, without any preconcentration. The YES assay and data analysis were performed according to the kit manufacturer's instructions. Calibration was established using standard solutions of the natural estrogen 17-β-estradiol (E2), at concentrations between 10^{-6}–10^{-9} mol L^{-1}. E2 also worked as positive control while ultrapure water was used as negative control. E2 standard solutions were prepared in DMSO (<1% in the assay medium), therefore a solvent blank was also assayed. Samples, standards, and control solutions were transferred to a 96-well microplate, mixed with assay medium, and inoculated with the transformed yeast cells. The mixture was then incubated for 48 h at 31 °C under orbital shaking. Spectrophotometric measurements at 570 nm (β-galactosidase expression) and 690 nm (yeast growth) were carried out using a Cytation3® microplate reader (Bio-Tek Instruments, Winooski, USA). The potential estrogen agonistic activity was estimated through the calculation of the parameters growth factor (G) and induction ratio (IR). The G parameter was calculated as the ratio of absorbance values measured at 690 nm for the sample and for the solvent $(A_{690})_{sample}/(A_{690})_{solvent}$. The IR parameter was calculated as $(1/G) \times ((A_{570} - A_{690})_{sample}/(A_{570} - A_{690})_{solvent})$.

3. Results and Discussion

3.1. Micropollutant Removal, Mineralisation, and Other Physico-Chemical Parameters

Under the regulation on minimum requirements for water reuse in agricultural irrigation, the environmental quality standards for priority substances and certain other pollutants should be targeted [7,21]. Moreover, the same regulation refers to additional requirements for risk assessment, including micropollutants. From the chemical organic micropollutants analysed in fresh (WW) and O_3+UF treated water samples (TWW_0), only 9 out of 14 were detected. The antiplatelet clopidogrel, the herbicide isoproturon, the anti-inflammatory diclofenac, the industrial compound PFOS (perfluorooctanesulfonic acid), and the lipid regulator bezafibrate were detected with a frequency of 100% in WW samples during the sampling campaign (Figure 2). Alachlor was also detected in all WW samples but below the limit of quantification ($LOQ_{alachlor}$ < 25 ng L^{-1}), whereas warfarin, citalopram, and clofibric acid were detected only in some samples. According to the Directive 2013/39/EU and Decision 495/2015/EU [21,22], alachlor, isoproturon and PFOS are considered PSs, whereas the others are considered CECs. After treatment, most micropollutants presented values below LOD—Limit Of Detection. Only alachlor, clopidogrel, citalopram and isoproturon were detected: the first two were below the LOQ—Limit Of Quantification (25 and 5 ng L^{-1}, respectively), whereas the latter two were found at concentrations up to 529 and 10.6 ng L^{-1}, respectively. In fact, isoproturon was the micropollutant with the lowest removal percentage (i.e., 80% of maximum removal). All priority substances (alachlor, isoproturon and PFOS) were below their environmental quality standards defined in the EU Directive 2013/39 [21], complying with the requirements of the EU Regulation 2020/741 [7].

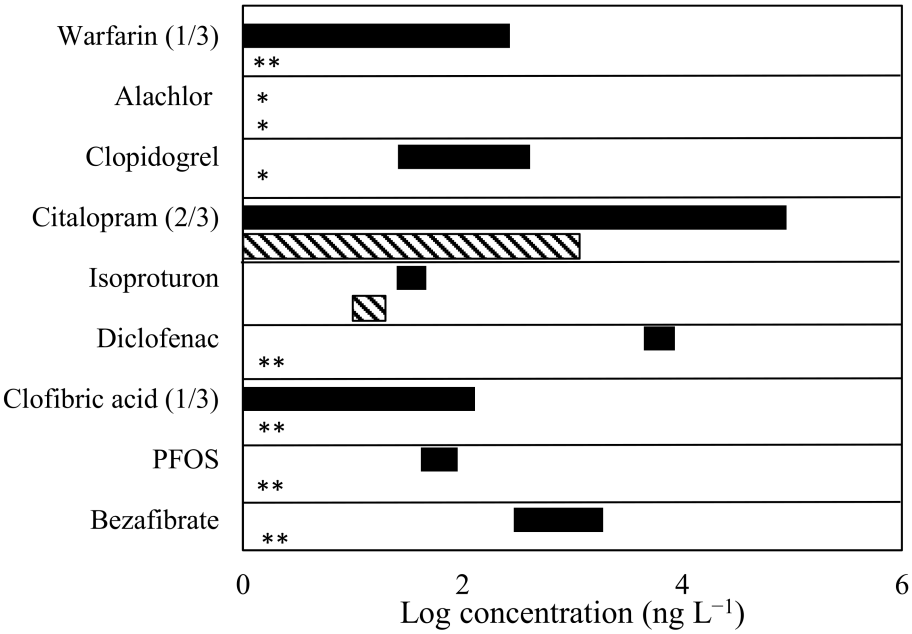

Figure 2. Logarithmic range of concentrations (ng L^{-1}) of the detected micropollutants in WW (black bar) and TWW_0 (striped bar) for samples with concentrations above LOQ. The frequency of occurrence was 100% (3/3) for all compounds, except when indicated in brackets after the compound name. * <LOQ and ** <LOD (compounds with concentrations < LOD before treatment are not shown in this figure for the sake of simplicity).

DOC and pH values did not remarkably vary after treatment (Tables 1 and 2, respectively). Values for DOC are not regulated and both pHs (before and after treatment) comply with the maximum value allowed (MVA). Thus, considering that regulations of water quality for irrigation often do not inform about adequate levels of organic matter, it can be assumed that the achieved values of DOC and micropollutants in treated water do not invalidate its use for irrigation. Moreover, the available literature mentioning the monitoring of DOC in water for irrigation recommends the evaluation of DOC when COD and BOD_5 are at the so-called alarming levels (>60 mgO_2 L^{-1} and >10 mgO_2 L^{-1}, respectively) [28,30,46], which is not the case of TWW_0 (Table 2).

In the combined process, ozonation was expected to be mainly responsible for the removal of micropollutants and dissolved organic matter rather than UF [18]. These results are coherent with other studies performing solely ozonation, in which the authors attributed the low yield of mineralisation to the formation of recalcitrant organic intermediates deriving from the organic micropollutants or, more likely, from the oxidation of dissolved organic matter naturally present in the wastewater [14,16]. For instance, using a similar experimental apparatus for the continuous ozonation of a secondary-UWWTP effluent (without UF), Moreira et al., 2016 [14] reported a DOC removal of ~30% (retention time of 26 min), whereas Iakovides et al., 2019 [16] obtained a DOC removal of ~10% (with similar ozone dosage and retention time).

Regarding other physico-chemical parameters, TWW_0 presents values below the maximum recommended in the Portuguese Laws of (i) water for irrigation [31] and (ii) treated wastewater for reuse [28,30]. The only exception is for the concentration of chloride in Table 1 (ca. 80 mg L^{-1} before and after treatment) which is slightly higher than the maximum value recommended (MVR) of 70 mg L^{-1} in the oldest law [27], which is not included in the newest one [28]. It is worth mentioning that this maximum value recommended for chloride was stipulated considering the sensitivity of tobacco crops; therefore, TWW_0 might not be appropriate for irrigation of this specific crop, but not necessarily inappropriate in the case of crops tolerant to these concentrations of chloride. For instance, some crops of fruits and vegetables are highly tolerant to chloride, such as Rangpur lime and cauliflower, for which

the water for irrigation can contain up to 600 and 710 mg L^{-1} of chloride, respectively [47]. In fact, TWW_0 can be applied for irrigation according to the WHO (World Health Organization) and FAO (Food and Agriculture Organization of the United Nations) guidelines of water quality for surface irrigation, where the allowed chloride concentration is up to 142 mg L^{-1} (Table 1) [29], i.e., well above the value determined for the wastewater in this study (ca. 80 mg L^{-1}). The value of salinity (782 µS/cm) is slightly higher than that recommended by FAO and WHO [28] for the use of water for irrigation with no restriction (<700 µS/cm), but this value is not defined in Portuguese guidelines. Another interesting observation is the increase in the nitrate concentration after treatment, although still below the maximum value recommended [27], which can be attributed to the oxidation of nitrogen-containing substances that are likely to be present in the secondary effluent of UWWTPs [48]. Sulphate and copper contents also suffer a slight increase after treatment, which can be due to their release from sediments/soil particles after ozonation [49,50].

Future work must consider the energy demand of these processes [51] and life cycle assessment (LCA) [52–54] for the elimination of micropollutants from urban wastewater—these studies being particularly scarce with data at full scale. For instance, it has been concluded that ozonation has a lower energy demand compared to the use of membranes or UV/H_2O_2 [9]. Conversely, the electrical energy demand of ozonation is higher than those determined for powdered activated carbon (PAC) addition or granular activated carbon (GAC) filtration, but always being a plant-specific issue [51]. Performing LCA, it was suggested that ozonation has a better overall environmental performance than the photo-Fenton process [53], whereas reverse osmosis causes higher environmental burdens than ozonation due to the high energy and material consumption [52]. In these processes, generated impacts result mainly from the production of energy needed (and the respective energy mix) and from the use of some specific reagents [54].

3.2. Microbial Inactivation and Regrowth

As expected, a reduction in the load of the microbiological groups analysed was observed immediately after treatment (Figure 3). Reductions of nearly 3.5 log-units of 16S rRNA gene (indicative of the abundance of total bacteria) and 3.7 log-units of culturable heterotrophs occurred. The abundance of *intI*1 followed a similar trend, with a reduction of ~4.6 log-units immediately after treatment, whereas enterobacteria, enterococci, and fungi, with reductions higher than five log-units, reached values below the detection limit (0.33 CFU per 100 mL). Microbial inactivation can be transient [14,55,56], therefore further assays testing the regrowth capacity after seven days of storage of the treated wastewater were performed (TWW_7 samples). It is known that bacterial reactivation is influenced by factors such as storage conditions, in particular temperature, availability of nutrients, ultraviolet light, and assimilable organic carbon content, among others [57,58]. Therefore, the conditions to perform this assessment were selected to mimic the most common real storage conditions, i.e., room temperature (25 ± 2 °C) and absence of light to minimise DNA repair mechanisms [59]. The abundance of the 16S rRNA and *intI*1 genes, as well as the heterotrophic counts, recovered to values close to those observed in WW samples. The same pattern was observed for fungi, although with a lower regrowth extent (~1.6 log-units). The transient effect of single ozone-based processes for the treatment of UWWTP effluents was reported before [14–16]. In fact, even when operating with close ozone doses (0.75 gO_3/gDOC) and higher HRT (10–60 min) to those used here (0.9 gO_3/gDOC, HRT 8 min), reactivation of all the microbial groups analysed in the current study has been described in the literature [14,15]. In contrast, in the present study, regrowth of faecal indicators (enterobacteria and enterococci) was not observed in TWW_7 samples. Notwithstanding, from a microbiological quality point of view, both TWW_0 and TWW_7 comply with the biological parameters included in the quality standards of water for crops irrigation, both in Portugal [27] and United States [60], or the Portuguese/European Union quality standards of wastewater reuse in irrigation without restriction [28,61]. In fact, faecal coliforms or *Escherichia coli* (enterobacteria) and nematode eggs are the only biological parameters included in these quality standards, for which values were found below the stipulated thresholds (Table 1).

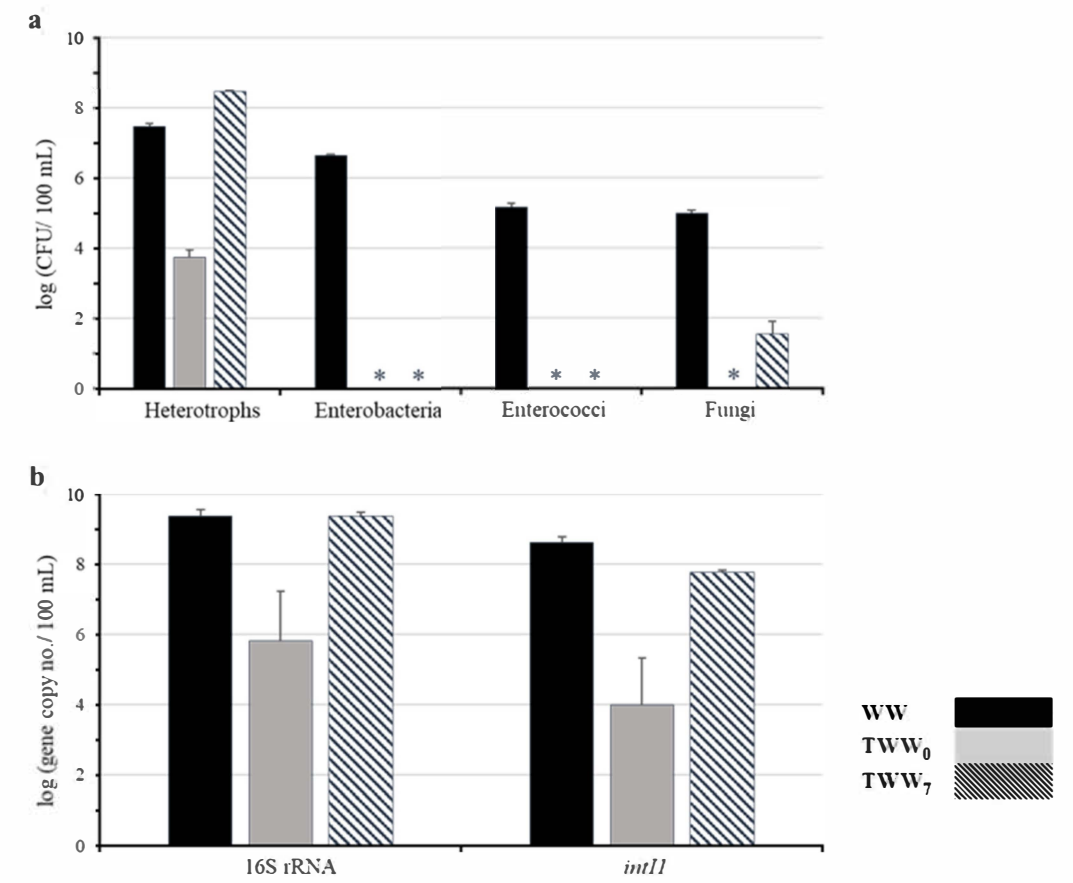

Figure 3. Microbiological water quality. (**a**) Culturable heterotrophs, enterobacteria, enterococci, and fungi, expressed as log (CFU/100 mL of sample); and (**b**) qPCR-based quantification of 16S rRNA and intI1 genes, expressed as log (gene copy number/ 100 mL of sample). * below the detection limit (0.33 CFU/100 mL).

Based on the abundance of enterobacteria in wastewater immediately after ozonation (10^2–10^3 CFU 100 mL $^{-1}$) or after 3 day storage (10^3–10^4 CFU 100 mL^{-1}) reported by Moreira et al. (2016) [14] and Iakovides et al., 2019 [16], the utilisation of ozonation alone would not produce wastewater compatible with its further use in irrigation. In contrast, the combination of UF with O_3 utilised here improved the efficiency of the treatment. The membrane fouling observed during the filtration process, which was evidenced by the permeate flow decrease from ~60 mL min^{-1} to ~16 mL min^{-1}, was most likely derived from bacteria that survived ozonation, cell debris and undissolved (in)organic matter. Nevertheless, the total suspended solid (TSS) value after O_3 was unquantifiable. In spite of the considerable improvements demonstrated in this study, the post-storage increase in total heterotrophs and genes shows that there is still room for additional tuning of the process to prevent the possible contamination of the permeate tank with spores of heterotrophic bacteria or fungi.

3.3. Evaluation of Biological Effects

Cytotoxic and cell viability effects of wastewater collected before (WW) and after treatment with O_3 + UF (TWW_0) were evaluated for skin (L929) and digestive epithelium (Caco-2) cell models by performing complementary MTT and LDH assays (Table 3). Considering that cell viability upon exposure to water samples depends on the final composition of the growth medium [62], test samples were diluted 5 and 10 times in culture medium before incubation with cell layers. Similar cytotoxicity (MTT) and cell viability (LDH) values were obtained for both dilution levels (Table 3). Moreover, cell viability was equivalent to that obtained for cell incubation with a plain culture medium. For both cell

lines, no difference in cytotoxicity was observed for water samples collected before and after treatment (Table 3, MTT assay). Cell viability was also maintained after treatment (Table 3, LDH assay), providing similar or even higher values than those obtained for plain culture media or tap water. Additionally, samples analysed right after ozonation (i.e., before UF) rendered percentages of 91 ± 6% and 23 ± 8% in the MTT and LDH assays for L929 cells, respectively, indicating that no cytotoxic compounds were produced during this step.

Table 3. Results (percentage) from MTT [a] and LDH [b] assays obtained for urban wastewater before (WW) and after treatment (TWW_0).

Cell Line	MTT Assay		LDH Assay [c]	
	WW	**TWW_0**	**WW**	**TWW_0**
L929	102 ± 13	112 ± 15	20.7 ± 2.0 (28.3 ± 3.2)	19.7 ± 1.6 (32.6 ± 4.5)
Caco-2	116 ± 8	96 ± 9	58.6 ± 4.4 (59.1 ± 6.8)	53.2 ± 7.7 (59.5 ± 5.6)

[a] Values for culture media were 100% (Relative Standard Deviation—RSD < 20%) and between 1 and 9% for Triton X-100 (total disruption of cells). Samples were diluted 5 times in culture media before incubation with cells. [b] Values for Triton X-100 (total disruption of cells) were 100% (RSD < 10%). Values for tap water were 111 ± 5 for L929 cells and 110 ± 12 for Caco-2 cells. [c] Values between brackets correspond to blank values obtained in culture media only (intact cells). Values for tap water were 26.3 ± 5.6 for L929 cells and 55.9 ± 4.7 for Caco-2 cells.

The presence of estrogenic activity was also evaluated using the YES assay for WW and TWW_0 samples. Yeast growth inhibition was not observed for any of the tested samples. Induction ratios (IR) were 1.02 ± 0.09 for WW, and 0.74 ± 0.02 for TWW_0. These values were below the kit threshold value IR10 (corresponding to 10% of the maximum IR, value of 2.82, obtained for E2 standards), which indicated no estrogenic activity.

Work on toxicity assessment of effluents treated by ozonation has provided contradictory evidence. The biological toxicity of the influent of sewage treatment plants was significantly decreased after applying different advanced treatment processes, including ozone combined with UV, using *Daphnia magna*, zebrafish (*Danio rerio*), and *Vibrio fischeri* [63] as target organisms. However, when ozone and hydrogen peroxide were used together, a slight acute toxicity was perceived for *V. fischeri* while acute toxicity was observed for *D. magna* [64]. Other work, also applying the algae *Desmodesmus quadricauda*, indicated that the toxicity class of treated wastewater may change from completely non-toxic to very high hazard category, with a clear relationship between the time of ozonation and the increase in ecotoxicity [65]. This compound-dependent behaviour was also observed in a study with zebrafish embryos where different pharmaceutical compounds were tested [66]. Therefore, our results with cell lines are in agreement with previous works, where no toxic effect was observed after treatment, particularly when low doses of ozone are applied.

4. Conclusions

The results of this study indicate that UF performed after ozonation can be a suitable approach to allow the safe reuse of urban wastewater for irrigation. The combined process resulted in an effective treatment, especially against micropollutants detected in the UWWTP secondary effluent, and in the reduction in the microbial load. Treated wastewater stored for seven days maintained the quality required for irrigation, with the physico-chemical parameters, and enterobacteria and nematode egg counts below the maximum values recommended in water quality standards. In addition, no harmful biological effects were detected concerning the viability and estrogenicity tests. However, the fact that total bacterial cells, total cultivable heterotrophs as well as the *intI*1 gene reactivated to values close to those observed for untreated wastewater, shows that there is still room for additional improvement of this process.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4441/12/12/3458/s1, Table S1: Quantitative PCR conditions used in the present study for absolute gene quantification in all WW, TWW_0, and TWW_7 samples.

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