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Article

Safe reuse of wastewater: Organic contaminants degradation and sanitization by ozone in a modulable continuous-flow system.

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Featured Application: The device is envisaged as a flexible tool to degrade organic compounds and sanitize different kinds of contaminated water, even on-site.

Abstract: Effective treatments improving both the chemical and microbiological quality of reclaimed wastewater are urgently requested. Ozone is a clean, economic, and environmental-friendly treatment to sanitize solutions and surfaces and to degrade organic pollutants. A simple, continuous-flow water-ozoniser system was tested to evaluate its effectiveness in batch treating various kinds of wastewater, including the effluents from small municipal plants. The degradation effects on a mixture of urban and industrial standard pollutants were investigated by HPLC-UV-MS analysis and biotoxicological assays. The results revealed that the concentration of most organic pollutants was reduced to 20-0% of the initial one within one hour. One compound resulted recalcitrant (40%. reduction only) The bioassays indicate the definitive reduction of toxic effects after treatment. Similar results were obtained when secondary, post sedimentation, wastewater treatment plant effluents were treated. Heterotrophic plate counts confirmed the good biocide activity of ozone. The developed prototype can successfully treat locally produced wastewater, secondary effluents from small-medium plants, and non-potable water resources.

Keywords: wastewater reuse; ozone; AOPs; sanitization; recirculation method

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1. Introduction

Recycling and reusing any kind and volume of wastewater (WW) seems one of the immediately feasible ways to cope with water scarcity. However, in order to provide safe water for human consumption the disinfection and removal of pollutants are mandatory to avoid undesirable ecological and human health effects. Besides, wastewater of different quality and origin, surface water, and groundwater can be equally contaminated by microorganisms and

chemical compounds, thus always requiring adequate treatments before being considered safe for human consumption [1-3].

Despite their low concentration ($\mu g \cdot L^{-1}$ to $ng \cdot L^{-1}$), micropollutants, and specifically emerging pollutants, are object of great concern because of their toxic, genotoxic, endocrine disrupting effects. Pharmaceuticals, endocrine disruptors, personal care products, pesticides, industrial dyes, and polycyclic aromatic hydrocarbons contaminate both urban and industrial wastewater since they are released mostly into the environment unchanged in their structure and activity, are resistant to biodegradation, and accumulate in water bodies, crops, and soil [4, 5]. Wastewater treatment plants (WWTPs) are not designed to remove these pollutants, thus new effective treatments are urgently needed and some of them had already been implemented.

Moreover, the chemical disinfection procedures based on chlorine compounds can give rise to disinfection by-products, some of which are carcinogenic for humans and toxic to the environment [6, 7]. In addition, some pathogenic organisms are not inactivated by these treatments [8, 9] and this can limit the reuse of the treated effluents [10].

For decades, Advanced Oxidation Processes (AOPs) [11], based on fast radical reactions, have been tested as an alternative to, or have been used in combination with, classical WW treatment methods, exploiting their disinfectant and oxidative degradation abilities [12-18]. Among them, ozone demonstrated to act as an effective parasiticide, germicide, and virucidal agent [19-21], ensuring the sanitization of several fresh and dry foods, [22-27] working places, and surfaces. As far as water is concerned, ozone treatment improves the color, smell, and taste at typical disinfection doses [28] and oxidizes inorganic compounds (arsenic, iron, manganese) to form insoluble substances, breaking down quickly without leaving residues or toxic compounds, and enhancing the biodegradability of wastewater by its oxidative action on organic pollutants [29]. These features further indicate ozone as the most appropriate approach in several particular cases [30-35].

In various countries, wastewater utilities have installed or are in the process of installing ozone generators to treat secondary effluents [36, 37] and potable water utilities do the same to remove a variety of contaminants in potable water, surface, and groundwater [1, 19].

However, wastewater not reaching the treatment plants or drinking water not coming from a suitable potable water utility are a highly common reality which requires effective and flexible solutions worldwide.

The present study aimed to investigate the performance of a new prototype of a self-assembled ozone treatment system in which the generator is coupled to a recirculation system to create a continuous flow of water and a repeated injection of ozone. We tested the rate of pollutant degradation by HPLC-UV-MS assays, the variation in toxicity by biotoxicological tests and the sanitization capacity by heterotrophic plate count.

These batch experiments should demonstrate the capability of the system in reducing biotoxicity and in sanitizing biologically contaminated water, offering a versatile tool for water reclamation, especially in locations without or far from wastewater and potable water utilities.

2. Materials and Methods

2.1 Experimental set-up.

To evaluate the capability of the system to degrade organic pollutants in complex solutions, a mixture of six compounds, which represent the most common classes of pollutants, was prepared at a concentration higher than usually detected in real wastewater in order to follow the oxidative degradation by

HPLC-UV-MS assay. 25 mg of each compound were dissolved in 250 ml of methanol and the solution was treated in an ultrasound bath (Bransonic 5, Cecchinato A. sas, Mestre, Italy) for 15 min. 100 μL were diluted 1:10 with distilled water and starting from this solution the calibration curves for the HPLC measurements were prepared. The remaining mixture was then diluted in 50 L of tap water.

Samples of WWTP secondary effluent were kindly supplied by the WW utility manager HERA SpA (Bologna, Italy), coming from the municipal WWTP of Cesena (Italy). The WWTP (about 195,000 Inhabitant Equivalent) treats domestic contributions from Cesena and Cesenatico cities, industrial discharges, and sewage trucks. A past monitoring campaign, carried out by ENEA, found a concentration of *E. coli* on the secondary effluents in the range of 2,000-25,000 Colony Forming Unit (CFU)/100mL, while at the final discharge point, the parameter was always below 5,000 CFU. A volume of 50 L was collected in a sterilized container at the outlet of the sedimentation tanks, refrigerated, and stored at 4°C until the experimental treatments. The samples appeared clear, without suspended solids.

2.2 Chemicals and reagents.

The compounds selected to prepare the solution in methanol were: Imidacloprid (insecticide), benzophenone- 4 (sunscreen in personal care products), Bisphenol-A (plasticizer), Carbamazepine (anticonvulsant), Cyprodinil (fungicide), and Ofloxacin (fluoroquinolone antibiotic). Tap water was chosen as the solvent. The compounds and the methanol were supplied by Sigma Aldrich (Milano, Italy). To perform the HPLC-UV-MS analysis ammonium acetate, formic acid, and LC-MS grade acetonitrile were purchased from Sigma-Aldrich (Milano, Italy) in the highest available purity and used without any further purification. The components of the *Vibrio fisheri* nutrient broth: (NaCl 15 g, peptone 2.5 g, yeast extract 1.5 g, glycerol 1.5 mL, HEPES 0.01 M in 500 mL, pH 7) were supplied by Sigma-Aldrich (Milano, Italy), as well as the salts to prepare the Jaworski's culture medium: $Ca(NO_3)_2{\cdot}4H_2O$ 20 g L^{-1}; KH_2PO_4 12.4 g L^{-1}; $MgSO_4{\cdot}7H_2O$ 50 g L^{-1}; $NaHCO_3$ 15.9 g L^{-1}; EDTAFeNa and $EDTANa_2$ both at 2.25 g L^{-1}; H_3BO_3 2.48 g L^{-1}; $[(NH_4)_6Mo_7O_{24}{\cdot}4H_2O]$ 1 g L^{-1}; $MnCl_2{\cdot}4H_2O$ 1.4 g L^{-1}, cyanocobalamin, biotin, and thiamine, each one 0.04 g L^{-1}, $NaNO_3$ 80 g L^{-1}; $NaH_2PO_4{\cdot}2H_2O$ 36 g L^{-1} in distilled water and the f/2 medium: $EDTANa_2$ 4.16 g L^{-1}, $FeCl_2$ $6H_2O$ 3.15 g L^{-1} , $CuSO_4$ 5 H_2O 0.01 g L^{-1} ,$ZnSO4$ $7H_2O$ 0.022 g L^{-1}, $CoCl_2$ $6H_2O$ 0.01 g L^{-1}, $MnCl_2{\cdot}4H_2O$ 0.18 g L^{-1}, Na_2MoO_4 $2H_2O$ 0.006 g L^{-1}, Cyanocobalamin and biotin, each one 0.0005 g L^{-1} ,Thiamine HCl 0.1g L^{-1} , $NaNO_3$ 0.075 g L^{-1} , $NaH_2PO_4{\cdot}2H_2O$ 0.0056g L^{-1} in artificial seawater (Instant Ocean Sea salts, from Instant Ocean, Blacksburg, VA, US) to growth the alga *Raphidocelis subcapitata* and *Dunaliella tertiolecta*, respectively. The non-selective solid medium employed in the heterotrophic plate count assay was the "Plate Count Agar" (Casein-peptone, Dextrose, Yeast Agar) from Sigma-Aldrich.

2.3 The ozone treatment system

The system is quite simple, very flexible in its structure, easy to displace, and can work on site. Currently, the generator is employed with and without a hydraulic system, to treat seasoning or storage cells, food washing lines, toilets, showers, and swimming pool water, respectively. The ozone generator "Poolsan" and the hydraulic system to perform the continuous water flow treatment were designed and assembled at the company MET SrL (Bologna, Italy). The ozone generator was available in two models, producing 2 or 4 g/h, with a power consumption of 300 or 400 Watt/h, respectively. Pure gaseous ozone was produced by an electric discharge (7,000-15,000 V) in a pure oxygen atmosphere

obtained from compressed and molecular sieve-filtered air. A Venturi jet mixer continuously fed the liquid flow with the ozone microbubbles stream. The liquid flow was conveyed to a blender tower designed to improve the water-ozone mixing: the blender is divided into two inner compartments and it functions by converse flows supported by a recirculation pump (delivery of 40L min^{-1}). The volume of the blender is defined by the volume of water under treatment, in our experiments it was 1 L to treat 50 L of water. The concentration of dissolved ozone, 4 ppm, was measured by an analytical kit based on the indigo method [38] and a photometer, both provided by Hack-Lange (Milano, Italy). The generator can be connected to a computer network for remote control.

The generator "Poolsan" and most of the hydraulic system for water treatment are shown in Figure 1.

Figure 1. The water ozone treatment system assembled at MET, based on the ozone generator "Poolsan" and hydraulic component to perform the recirculation procedure. The generator and the main component of the hydraulic system are mounted on a mobile support.

2.4 The HPLC-UV-MS analyses

The untreated and the treated mixture collected at different sampling times were analyzed by HPLC-DAD-MS. The analyses were performed on a Dyonex Ultimate 3000 HPLC (Thermo Fisher Scientific, Italy) equipped with a diode array UV and a mass spectrometer TSQ Quantum Access Max with an electrospray ionization source detector. 0.5 mL samples were used for the automated injection. The chromatographic separation was performed on a reverse phase Zorbax SB-C18 column 4.6x150 mm, 5 microns (Agilent Technologies Italia, Cernusco sul Naviglio, Italy), at a flow rate of 0.5 mL/min. Details of the HPLC and UV-MS analysis conditions were reported in Tables 1 and 2, respectively.

Table 1. Chromatographic separation details.

Time (min)	**A:** NH_4OAc 5mM in water/0.01% HCOOH	**B:** ACN	**C:** H_2O
0	98	2	0

14	5	95	0
15	0	95	5
35	0	95	5
40	98	2	0

Table 2. UV-MS analysis details.

Compound	λ_{max} (nm)	ESI Parent ion	Product ion	Collision energy
OFLOX	296	[M+1] 362.000	318.000	20
IMID	220	[M+1] 256.000	208.900	16
BP-4	282	[M-1] 307.000	226.900	30
CBZ	285	[M+1] 237.000	193.900	18
BPA	275	[M-1] 227.000	212.200	20
CPD	260	[M+1] 226.000	93.000	34

The degree of degradation was calculated by using the respective calibration curves, and the limit of detection (LOD) for each analyte was established as the first lowest calibration point. The analysis of each sample has been performed in duplicate.

2.5 The samples organic load

The organic load of the WWTP effluent samples was evaluated determining spectrophotometrically the COD by using the Hack LCK 314 Cuvette Test 15 - 150 mg/L O_2 and the TOC value by the TOC Hack-Lange test kit, following the manufacturer's instructions. Measurements were done on a DR5000 spectrophotometer. The above-mentioned kits and the spectrophotometer were from Hack-Lange, Milano, Italy.

2.6 The biotoxicity assays

2.6.1 The bioluminescent bacteria light emission inhibition assay

Lyophilized aliquots of the luminescent bacteria *V. fischeri* were prepared from fresh cultures at our laboratory starting from an original batch supplied by the Pasteur Institute (Paris, France). The 96-well "Black Cliniplate" microplates were supplied by Thermo Scientific (Vantaa, Finland) and the luminometer was the Victor Light 1420 model from Perkin-Elmer, USA.

To perform the assay, according to [39,] lyophilized aliquots of *V. fischeri* containing NaCl 3% w/v were reconstituted with 1 mL of distilled water and re-suspended in 10-30 mL of nutrient broth. Treated and untreated solutions were added with NaCl to reach the concentration of 3% w/v. 200 µL of the bacteria suspension and 100 µL of each sample were dispensed into the microplate wells. The controls consisted of 200 µL of bacteria plus 100 µL of a 3% NaCl solution in tap water. The emitted light was recorded at fixed intervals in the range of 0-48 hours. From 5 to 12 replicates were tested for each sample and the light emission intensity, reported as Relative Luminescence Units (RLU), was expressed as mean ±SD. Moreover, the bioluminescence inhibition percentage (I %) was used to express the toxicity of the samples and calculated according to:

$$I\% = \frac{L\ blank - L\ sample}{L\ blank} * 100$$

where L is the light emitted intensity of the sample or of the control (blank).

235
236 2.6.2 The algal growth inhibition assay
237 The Istituto Zooprofilattico Sperimentale of Abruzzo and Molise "G. Cap-
238 orale" (Teramo, Italy) supplied the freshwater microalga *Raphidocelis subcapitata*
239 (previously *Pseudokirchneriella subcapitata*) and the marine green alga *Dunaliella*
240 *tertiolecta* cultures. The assays were performed according to [40] with a little
241 modification concerning the evaluation of the results: we determined the effects
242 on the growth after 7-10 days from the beginning of the test.
243 The starter culture of *Raphidocelis subcapitata* was prepared by inoculating
244 in Erlenmeyer flasks 1 mL of microalgae suspension per 100 mL of the Jawor-
245 ski's culture medium. The flasks were illuminated by a white lamp/red lamp
246 Osram daylight 2 x 36W plus an Osram Gro-Lux lamp 36W (Osram, Milano,
247 Italy) following the cycle: 8 hours light/16 hours dark, at room temperature
248 (20°C). To start the tests, flasks were filled with treated or untreated samples
249 added with a suitable amount of Jaworski's medium salts and diluted algal sus-
250 pension (approximately 10^5 cells mL^{-1}), and were kept in the same conditions of
251 the starting culture. Controls were prepared by adding algae suspension to the
252 Jaworski's salts mixture. After 7-10 days the algal growth was evaluated by
253 measuring the absorbance at 663 nm, according to a standardized indirect
254 method for cell counting [41]. Aliquots of carefully hand shaken samples or
255 controls were measured, in triplicate, and then the aliquots were poured back
256 into the flask.
257 In parallel, the starter culture of *Dunaliella tertiolecta* was prepared by in-
258 oculating in Erlenmeyer flasks 1 mL of microalgae suspension per 100 mL of f/2
259 medium. The tests were performed exactly as for *R. subcapitata*.

260 *2.7 The heterotrophic plate count*

261 The Petri dishes, ∅ 9 cm, for the heterotrophic plate counts (HPC) were
262 supplied by Nuova Aptaca srl (Asti, Italy). Different volumes of ozone treated
263 and untreated effluent samples, in the range 100-1000 μL, were distributed in
264 the plates in triplicate. The solid medium solution was sterilized and poured
265 into the plates when its temperature was about 37°C. In this way, the samples
266 were perfectly included in the still liquid medium. Incubation was performed
267 at 37°C for 24-48 h and then the number of Colony Forming Units (CFU) was
268 determined. A regrowth test was carried out on specimens of the treated sam-
269 ples stored in stopped, sterile vials for 7 days at room temperature. These spec-
270 imens were plated and the CFU was counted as described above.

271 **3. Results**

272 *3.1 Samples*
273 The mixture prepared to evaluate the degradation of pollutants by HPLC-
274 UV-MS determination was stored in the dark at room temperature, and em-
275 ployed without any further manipulation after the ozone treatment. The efflu-
276 ent samples were stored refrigerated both before and after the treatment Neg-
277 ligible or no changes were detected in the pH values before, during, and after
278 the treatment ensuring ozone solution stability. This parameter was deter-
279 mined also in the case of the WWTP samples. A moderate pH increase was ob-
280 served as treatment time increased. In Table 3 the mean values of different ex-
281 periments for both kinds of the sample were reported.
282
283 **Table 3.** pH values of the pollutants' mixture and WWTP samples before and after
284 the various treatment intervals. Mean values of n=4 experiments.
285
286

Time interval	**pH value ± uncertainty**

(h)	Mixture	WWTP samples
0 (before treatment)	7.87±0.11	7.43±0.10
1	7.66±0.25	7.63±0.15
2	7.89±0.38	7.75±0.21
4	7.96±0.40	7.78±0.25
6	7.98±0.33	7.92±0.09
8	8.06±0.21	7.93±0.21
Tap water	7.80±0.21	8.02±0.05

3.2. The ozone degradation of contaminants.

The mixture of the six pollutants was bubbled with ozone continuously for 8 h. Samples were withdrawn during the treatment after 1, 2, 4, 6 hours and, again at the end of the treatment. We employed such a long treatment, with respect to the usual treatment time with ozone, because our aim at the beginning of this work was to obtain information on the performance of the system in the same conditions of a previously developed device [42]. Moreover, it is known that not all organic contaminants are easily degraded by ozone or hydroxyl radicals in a short time. As the results will show, the molecular structure of the compounds in the mixture, shown in Figure 2, was suitable to be attacked by ozone thanks to the presence of several double bonds, but this characteristic is not always a guarantee of rapid degradation.

During the treatment of the WWTP effluent samples, we followed a different sampling schedule, collecting samples after 15 min, 30 min, and 1, 2, 4, and 6 h. We estimated that the sanitizing effect should be evaluated after a time shorter than 1 h.

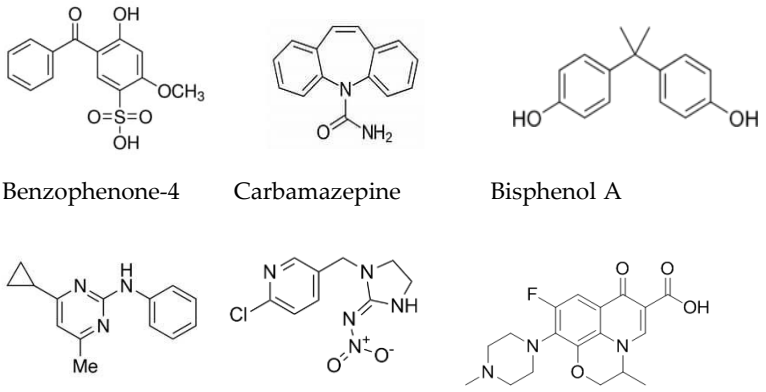


Figure 2. The chemical structure of the active principles mixed in solution to test the degradation power of the ozone treatment.

3.2.1. The HPLC-DAD-MS analysis

The mixture specimen collected at 1, 2, 4, 6, and 8 h from the beginning of the treatment were analyzed by HPLC-UV-MS to evaluate the degree of the disappearance of the parent molecule. No detailed analysis on the degradation products of each compound was performed since this procedure was out of the scope of this work. The effectiveness of the treatment from the environmental point of view was determined by the changes in toxicity it produced.

Figure 3 reports the chromatographic peak position of the single components obtained by the UV-MS analysis of the untreated mixture.

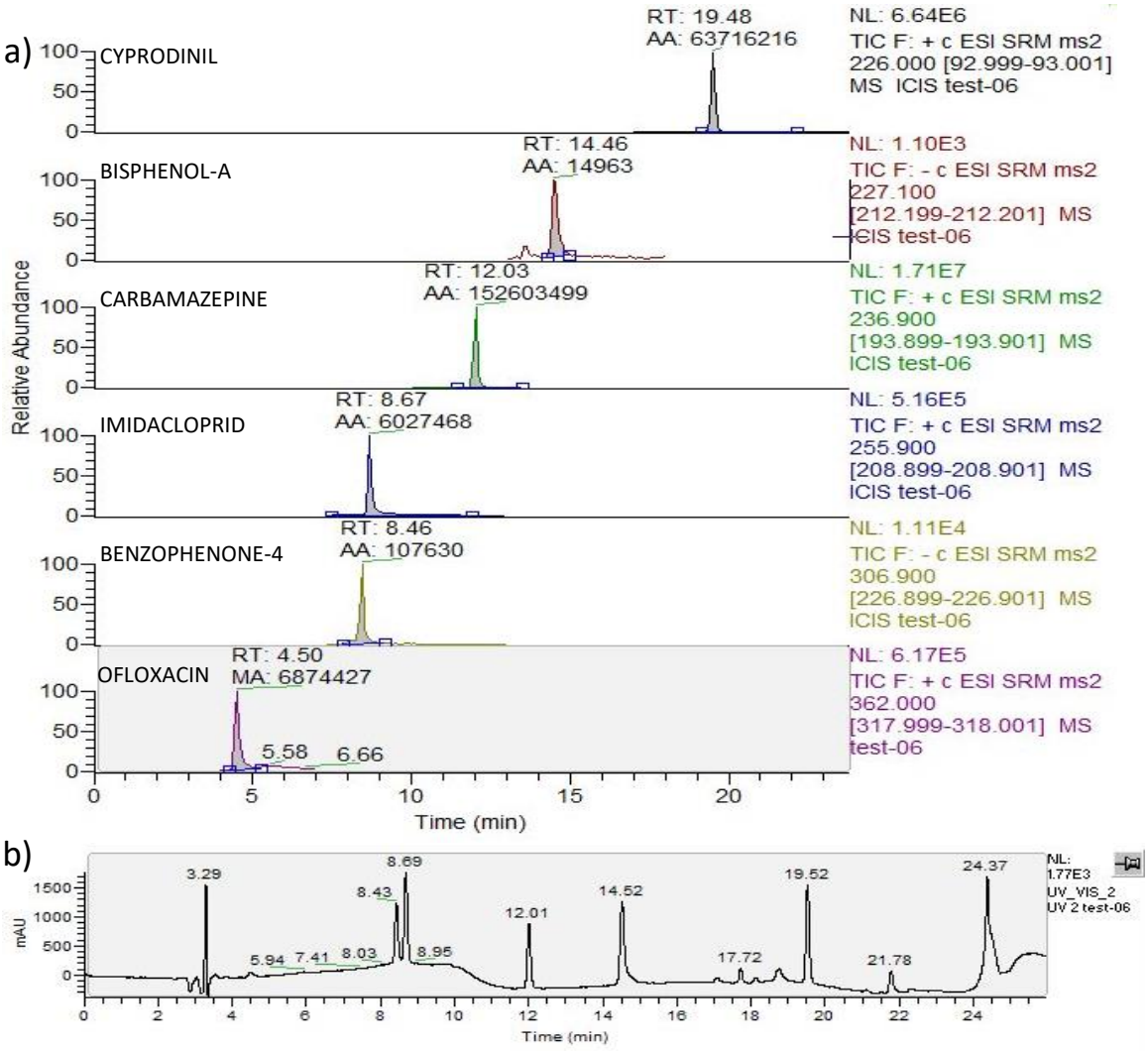


Figure 3: HPLC-UV-MS/MS analysis of the mixture at 0,1 mg/L. a) individual ion current of each contaminant; b) UV chromatogram at λ = 285 nm

In Table 4, we report the average values of the pollutants' concentration in treated and untreated mixture, compared with the respective LOD values.

Table 4. Concentration of the pollutants before (T=0) the treatment and in the treated samples.

Time (h)	Cyprodinil mg/L±SD	Bisphenol-A mg/L±SD	Carbamazepine mg/L±SD	Imidacloprid mg/L±SD	Benzophenone-4 mg/L±SD	Ofloxacin mg/L±SD
LOD→	*(25ng/L)*	*(0.1mg/L)*	*(25 ng/L)*	*(25 ng/L)*	*(5 μg/L)*	*(25 ng/L)*
0	0.225±0.003	0.535±0.014	0.561±0.015±	0.500±0.011	0.385±0.002	0.640±0.003
1	< LOD	< LOD	< LOD	0.433±0.005	< LOD	0.012±0.002
2	–	–	–	0.419±0.002	–	0.009±0.004
4	–	–	–	0.356±0.007	–	< LOD
6	–	–	–	0.303±0.005	–	–
8	–	–	–	0.284±0.005	–	–

It is possible to observe that after 1 h of treatment most of the parent molecules disappeared; the concentrations were under the limit of determination by the mass spectrometry analysis. This was an interesting information, since the concentration of each compound was about 0.5 mg/L, definitively higher

than those present in wastewater or environmental samples. Conversely, 4 h of treatment was necessary to reach this result in the case of Ofloxacin, confirming the high stability of the fluoroquinolone antibiotics and their long persistence once dispersed in the environment.

The most recalcitrant compound, however, was the insecticide Imidaclo-prid At the end of the treatment, its concentration decreased only by 40%. This insecticide was recently banned in the European Union [43] but its resistance to degradation accounts for the concern about its accumulation in the environment, soil and surface water.

3.2.2. The organic load

The concentration of the organic material was determined in the effluent samples as the COD and the TOC, which values showed in both cases little differences before and after the treatment. These data (Table 5) underline that the parent compounds disappear, but their oxidation products were still present, and negligible mineralization occurred.

Table 5. The COD and the TOC of treated and untreated samples of secondary effluent.

Samples	COD (mg/mL) (± uncertainty)	TOC (mg/L)
Untreated	19.24±5.12	21,6±2.4
Treated 6 h		14,6±1.8
Treated 8 h	15.57±1.52	
	Δ COD= 21,1±%	ΔTOC= 32,4±2.2%

3.2.3 The biotoxicity assays: the bioluminescent bacteria

The biotoxicity test based on the inhibition of the *Vibrio fisheri* light emission is widely used to determine the presence of toxic compounds in liquid or solid samples through rapid screening tests. Bacterial bioluminescence is an energy-consuming phenomenon and light is emitted only when the organisms are in their best metabolic and physiologic conditions. Any component in the environment injuring the bacterial integrity or functions will produce a reduction or the disappearance of the light emission in a way directly proportional to the intensity of the suffered damage.

As expected, the mixture of the six pollutants was highly toxic to the luminescent bacteria reducing the light emission intensity quite to zero. The ozone treatment was effective in the cleavage of the parent molecules, leading to a partial restoration of luminescence emission, as shown in Figure 4. Prolonged-time treatment seems to increase and not reduce the toxicity of the mixture. Probably, the degradation process produces smaller fragments able to interact more effectively with bacterial physiology This figure shows how the chronic toxicity assay offers more precise information with respect to the acute one: after one hour, the untreated sample looked very similar to the control. The sensitivity of the assay is underlined by the fact that even the tap water employed to prepare the solution produced a 20% of light emission inhibition with respect to the control, probably due to the sanitizing treatment residues.

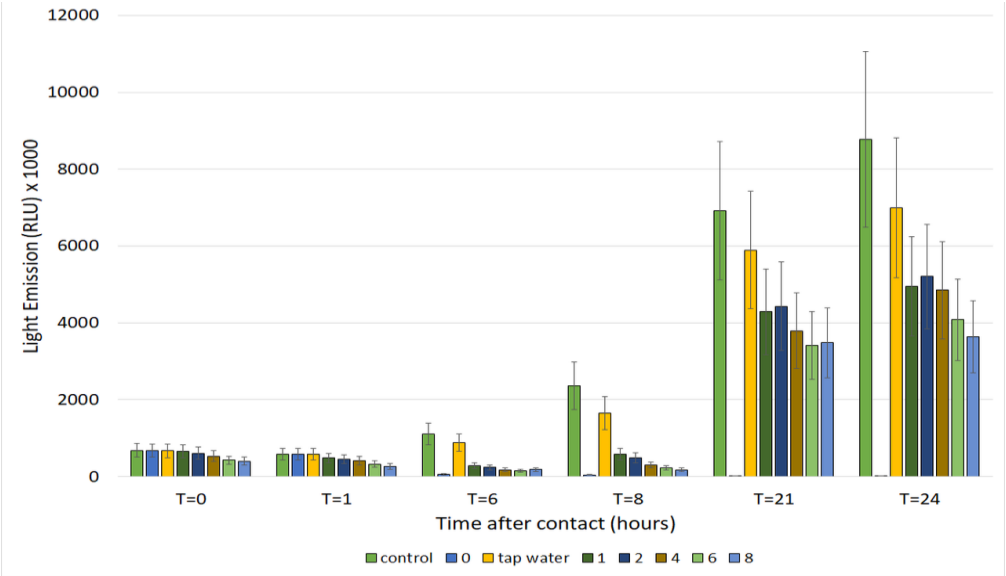
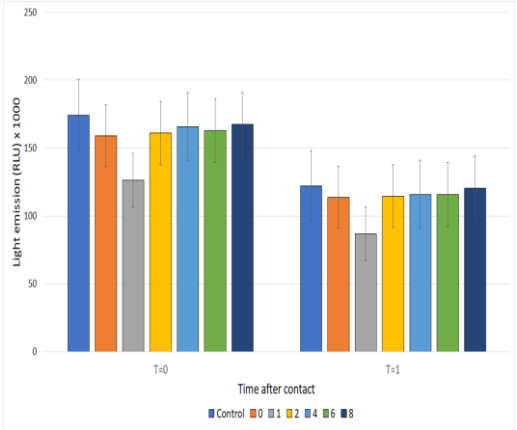
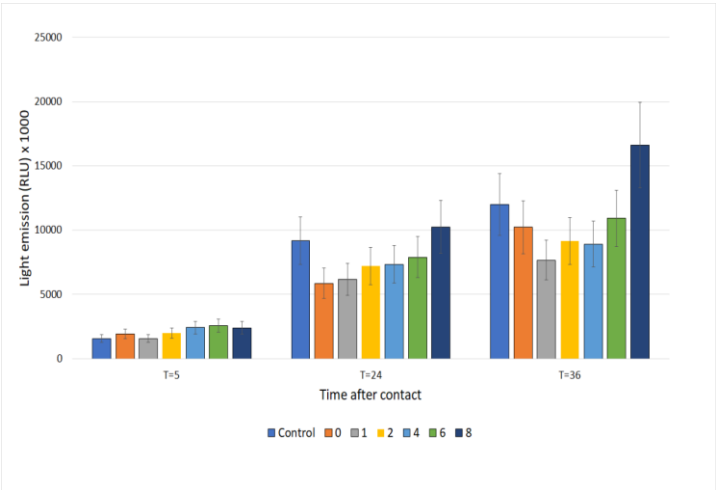


Figure 4. Light emission of luminescent bacteria in contact with the untreated (0) and the treated (1, 2, 4, 6, 8) mixture of pollutants. T=0 is the light emission immediately after the microplate was prepared. T=1 (6, 8, 21, 24) is the light emission after 1 (6, 8, 21, 24) h the microplate was prepared, or the "Time after contact" (between bacteria and sample) T=1 corresponds to the acute toxicity and T=24 to the chronic toxicity.

By testing the effluent samples, no particular toxicity of both the untreated and treated samples was revealed with respect to the acute (1 h contact time between bacteria and the sample) or the chronic toxicity assay (24 h or longer contact time). Conversely, at the chronic toxicity time, treated sample frequently showed a light emission higher than that of Control. This is not surprising since WW is rich in nutrients and the ozone degradation of the organic contaminants produces smaller molecules acting as nutrients. The stimulation degree produced by these nutrients must be carefully considered when planning the reuse of reclaimed water. In Figure 5 are reported the emissions of *Vibrio fisheri* in presence of the effluent samples.





a) b)

Figure 5. Biotoxicity assay of WWTP effluent samples treated for 0, 1, 2, 4, 6, and 8 h with ozone. (a) acute toxicity, after 1 h of contact of the samples with the bioluminescent bacteria. (b) chronic toxicity, after longer contact time (24 or 36 h). The light emission

values at T=5 were included to underline the significant growth over time of bacteria in contact with the samples, which resulted just slightly toxic.

On some occasions, it must be mentioned that the results were quite different: some of the samples were definitely more toxic than expected or greatly stimulated the light emission. Both effects seem to be present in the samples whose acute and chronic toxicity data are shown in Figure 6. These samples were treated according to the new schedule, which included sampling at 15 and 30 min. The 30- and 120-min samples resulted heavily inhibited, the untreated effluent and the other samples showed a stimulated light intensity. The assay was repeated various times to exclude a behavior artefact. The chemical composition of the effluent can be different at each sampling and its effects on living organisms can be unpredictable, but the biotoxicological data are nevertheless indispensable warning tools.

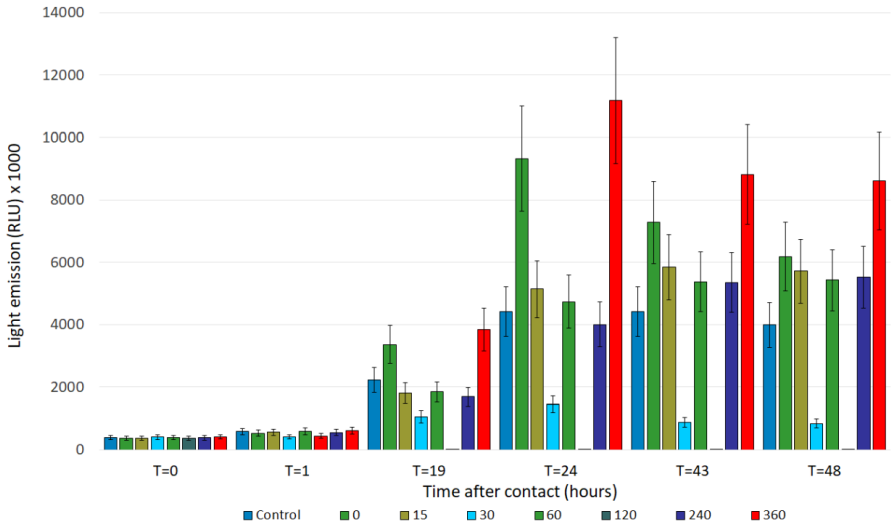


Figure 6. Light emission intensity of WWTP effluent samples collected according to the new schedule. The samples collected after 30 min and after 120 min of treatment resulted, surprisingly, highly toxic. Less surprising was the intensity of stimulation produced by the untreated sample.

3.2.4 The biotoxicity assays: the green algae

The food chain is founded on the unicellular green algae, thus knowing their response to the presence of pollutants in water bodies is of paramount importance. Nonetheless,,the sensitivity of the various strains can differ greatly: the marine strains are usually more sensitive than the freshwater ones. During the test with the pollutants' mixture, the tap water produced a 10% inhibition of the growth of *Dunaliella t*. with respect to the control, as previously observed for *Vibrio f*., another marine organism. The untreated sample produced a 97% reduction of the growth and all treated samples 100%. The effects on the growth of *Raphidocelis s*. were just a little less important. The inhibition percentage for the various samples is reported in Figure 7. These data indicate that the ozone treatment was not sufficient to remove all components responsible for negative effects on algae vitality at the high concentration considered.

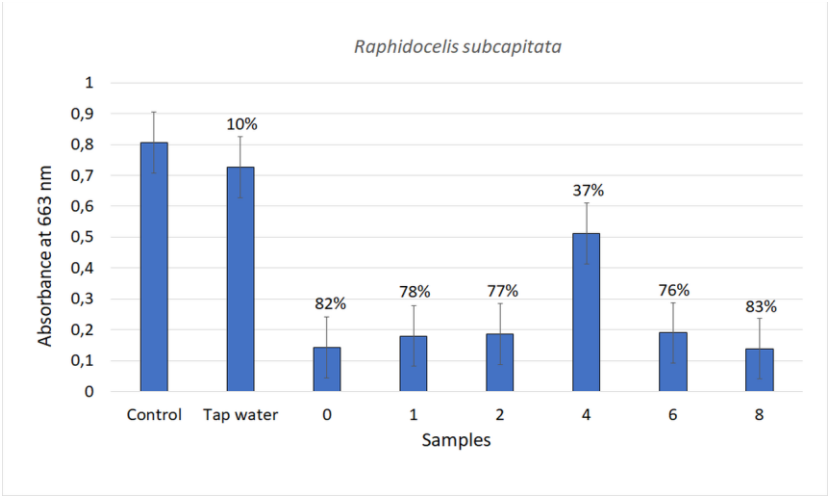


Figure 7. Chlorophyll absorbance quantifies the growth of freshwater alga in contact with the pollutant mixture treated by ozone. Above each sample is reported the % inhibition with respect to the Control.

The behavior of the two strains was different also while testing the effluent samples. The marine alga *Dunaliella t*. revealed a significant growth inhibition by the untreated sample and a slow recovery in parallel with the increase in the treatment duration. The maximum growth was produced by the 4 h treated sample (Figure 8a) and this result, independently from the effect of the untreated water, was always recorded. The growth of the freshwater alga was equally inhibited by untreated samples, but the recovery was more rapid and less regular (Figure 8b). The need for the larger possible set of biotoxicological tests to evaluate correctly the toxicity of complex samples is more than evident, since each kind of organism has a unique reaction to the presence of the same xenobiotic.

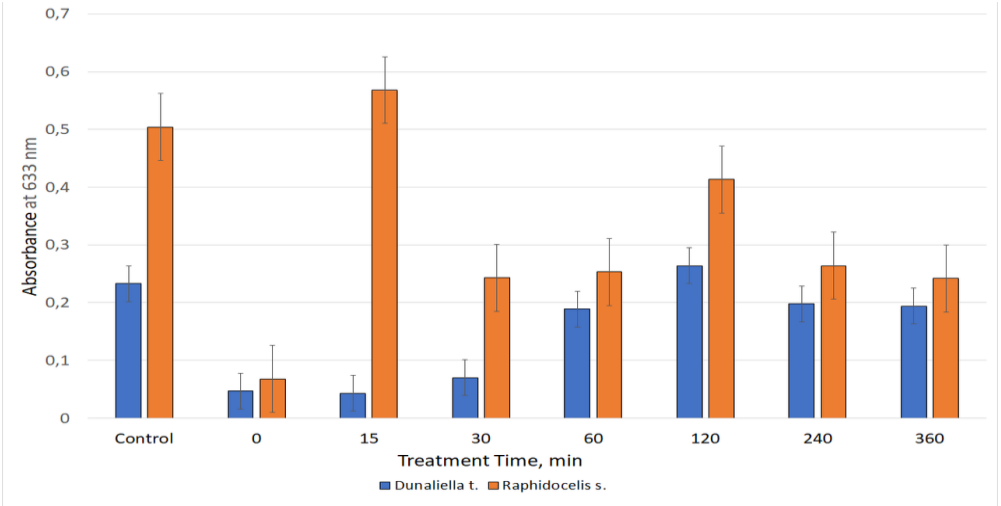


Figure 8 The histogram reports the absorbance values of chlorophyll in the cultures of *Dunaliella t.* and *Raphidocelis s*. in contact with the effluent samples, treated or not (0), and compared with the respective Control, pure culture of algae.

3.3. The heterotrophic plate counts

At present, the main application of ozone is in the sanitization of rooms, surfaces, devices, foods, or solutions. To evaluate the effectiveness of the "Poolsan" device in sanitizing the secondary effluent samples we plated both the

freshly treated samples and a specimen of the same samples 7 days after, to evaluate the effects of ozone on the number of CFU and the possible regrowth of surviving bacteria. We employed the heterotrophic plate count of thermophile bacteria as the screening test for sanitization capacity. The results are summarized in Table 6.

Table 6. The heterotrophic plate counts for freshly treated wastewater samples and for the same samples plated 7 days later. Regrowth phenomena did not occur, no colony was grown on plates of treated samples.

Samples	CFU/100 mL ±uncertainty	Regrowth CFU/100 mL
Untreated	14,600±4,000	---
O_3 treatment: 15 min	1,300±600	0
30 min	800±300	0
60 min	300±100	0
120 min	100±40	0
240 min	100±50	0
360 min	80±30	0

4. Discussion

In this study, we investigated the performance of a self-assembled device to treat different kinds of wastewater by ozone injected into the water stream. O_3 dissolved in water has been used for sterilization and detoxification of fluids in all kinds of contexts [14, 44-46], rarely alone but usually as a pre or post treatment, according to the chemical/biological content and to the final use of the reclaimed water.

Our aim was to test the possibility to employ our system to treat small-moderate volumes of wastewater produced locally during various activities, such as agriculture, small industries, isolated communities, or to sanitize surface water or groundwater not suitable for human use in areas where no potable water utilities are present, or are not available because of some kind of emergency.

Concerning the removal of chemical pollutants from WW, the experiments on a concentrated solution of six representatives of the so-called micropollutants or emerging pollutants gave positive results. The system was able to degrade completely most of the parent molecules after 1 h of treatment and probably in shorter time after optimization. Only one compound maintained more than 50% of its initial concentration. The effectiveness of our system concerning the degradation of organic pollutants at high concentrations like in industrial WW is further confirmed by the comparison with similar, already published data [2, 31, 35, 47].

The COD and TOC values indicated that the overall organic content was not greatly changed after treatment, but the known role of ozone and its radicals is just to start the molecular degradation by oxidative attacks, enabling an easier biodegradation process, not to obtain extensive mineralization of the molecules [3, 48, 49].

Identifying the degradation products was out of our interest, because several studies have been already published on the degradation of various compounds included in our solution [2, 3, 34, 50, 51] and mainly because we wished to replicate real cases, where a detailed identification of all the components and their changes is not feasible. Moreover, the treatment's effects are evaluated by

simpler tests, for example the biotoxicological or microbiological ones. We evaluated the changes in toxicity produced on the pollutants' mixture focusing on the long-term effects of samples, since short-term toxicity assays can result in not realistic information. This is confirmed, for example, by data reported in Figure 4: the real toxicity of the untreated solution can be observed only in long-term measurements. The green algae response was also evaluated as a long-term assay, to ensure the full expression of the pollutants' effects. The results can be considered consistent with those from bacteria, even though the sensitivity of these organisms was different: the marine one, more sensitive, showed no growth. The freshwater alga was able to grow, but only about 20% with respect to the control, without significant differences among the various treatment times. These differences are the rule and each research work will define a different order of sensitivity for the same organisms but different analytes [52].

Moreover, it must be taken into consideration not only the metabolic differences among the organisms we employed, but also the controversial effects of organic pollutants degradation: often the degradation products are more toxic than the parental molecule, the increased toxicity in treated samples must not be surprising.

The samples of municipal, post precipitation plant effluent produced just in a few cases toxic effects, more frequently a growth stimulation both on bacteria and algae. This is not surprising, since the municipal WWTP effluents, containing not more than few μg/L of toxic micropollutants are rich in nutrients which can help the growth of living organisms [3]. This information is important since it is not fully positive: an effluent containing a lot of nutrients will produce eutrophication phenomena in the receiving water bodies.

The second aspect of ozone treatment dealing with the microbiological content of water, was evaluated by the non-selective heterotrophic plate count assay, and although we could not identify specific strains, these preliminary results were satisfying. In the freshly treated samples, the CFUs were reduced accordingly to the treatment time, but the results from the same samples plated after 7 days were surprising: they showed no one CFU. This feature, if it will be confirmed and accompanied by specific microbiological tests, would indicate that treated wastewater can stay sanitized over time. This finding is consistent with various previous works dealing with conditions for wastewater disinfection [53-55].

5. Conclusion

The present was a preliminary work aimed to ascertain the potentialities of the continuous-flow device in improving the various aspects determining water quality. The next step concerning the sanitization aspect will be the investigation on the actual inactivation of typical bacterial strains such as Fecal Coliforms and other pathogenic and/or particularly resistant organisms. These data are necessary to confirm the efficacy of the treatment. When possible, the analytical evaluation of organic contaminants degradation in WW samples will be carried out.

Nevertheless, it is possible to affirm that the degradation rates of several very dangerous molecules at unusually high concentrations after 1 h of treatment and the zero CFU in the regrowth test were interesting and encouraging results.

Following this first test on the system, we are planning to optimize its design, obtaining the best performance in the different situations in which it can be employed. In the continuous flow system, the blender tower size and the flow speed can be tailored on the water volume to be treated, on the final use of the treated water, and on the initial organic content. The amount of ozone

and the treatment time will be recalculated according to the biological and chemical characteristics of the fluid under treatment, optimizing the costs and working time.

The development of such a simple, extremely flexible, and effective system can offer an interesting way to recycle WW produced in small communities, during limited agricultural or industrial activities, or to improve drinking water quality in developing areas, small communities, and emergency situations.

Locally sanitizing and remediating minor volumes of wastewater or not potable water sources through economic and easily conveyable systems is as important as remediating and reusing great volumes of wastewater produced by municipal and industrial treatment plants. Failing these local actions, the only alternative will be to dissipate the wastewater, to contaminate the environment, or be detrimental to human health.

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