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Ecotoxicity evaluation of a WWTP effluent treated by solar photo-Fenton at neutral pH in a raceway pond reactor

A. M. Freitas[1] · **G. Rivas**[2] · **M. C. Campos-Mañas**[2] · **J. L. Casas López**[2] · **A. Agüera**[2] · **J. A. Sánchez Pérez**[2]

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Abstract Some pollutants can be resistant to wastewater treatment, hence becoming a risk to aquatic and terrestrial biota even at the very low concentrations (ng L^{-1}–μg L^{-1}) they are commonly found at. Tertiary treatments are used for micropollutant removal but little is known about the ecotoxicity of the treated effluent. In this study, a municipal secondary effluent was treated by a solar photo-Fenton reactor at initial neutral pH in a raceway pond reactor, and ecotoxicity was evaluated before and after micropollutant removal. Thirty-nine micropollutants were identified in the secondary effluent, mainly pharmaceuticals, with a total concentration of ≈80 μg L^{-1}. After treatment, 99 % microcontaminant degradation was reached. As for ecotoxicity reduction, the assayed organisms showed the following sensitivity levels: *Tetrahymena thermophila* > *Daphnia magna* > *Lactuca sativa* > *Spirodela polyrhiza* ≈ *Vibrio fischeri*. The initial effluent showed an inhibitory effect of 40 % for *T. thermophila* and 20 % for *D. magna*. After 20 min of photo-Fenton treatment, no toxic effect was observed for *T. thermophila* and toxicity dropped to 5 % for *D. magna*.

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✉ J. A. Sánchez Pérez
jsanchez@ual.es

[1] Department of Chemistry and Biology, Federal University of Technology-Paraná (UTFPR), Curitiba, Brazil

[2] Solar Energy Research Centre (CIESOL), University of Almería-CIEMAT, Almería, Spain

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Introduction

Some organic compounds may resist biological oxidation by activated sludge, and thus, they can remain in secondary effluents of wastewater treatment plants (WWTP). Even at low concentrations, these micropollutants, usually pharmaceuticals, pesticides, and personal care products, among others, represent a major environmental risk, especially to aquatic biota (Petrie et al. 2015). Powerful analytical techniques, enabling detection limits within the nanogram per liter to microgram per liter range, now allow for a large number of these so-called emerging contaminants (ECs) to be quantified in the environment, thus compelling the scientific community to consider this contamination type as a potential issue meriting concern (Santos et al. 2010). As such, both the development of more efficient tertiary treatments and the ecotoxicological monitoring of these compounds in the environment are strongly recommended (Klamerth et al. 2013).

Regarding alternative treatment options, advanced oxidation processes (AOPs) such as ozone or homogeneous and heterogeneous photocatalysis have been investigated for the removal of emerging contaminants from urban wastewater effluents (Altmann et al. 2014). Among AOPs, the solar photo-Fenton process has proved to be efficient in the removal of micropollutants from municipal effluents (Klamerth et al. 2013). Prieto-Rodríguez et al. (2013) evaluated the effectiveness of ozonation, solar heterogeneous photocatalysis with TiO_2, and solar photo-Fenton as tertiary treatments for the remediation of micropollutants in municipal wastewater treatment plant effluents at a pilot-plant scale. They concluded that the solar photo-Fenton process was the most efficient for

micropollutant removal and economically viable for applications in wastewater treatment plants. This process is based on the generation of hydroxyl radicals, which are highly oxidative and nonselective; thus, the organic pollutants can be mineralized into CO_2 and H_2O. In particular, the generation of hydroxyl radicals in the photo-Fenton process relies on the cyclical oxidation and reduction of Fe (catalyst) in aqueous solution through Reactions 1 and 2 with H_2O_2 consumption. Additionally, the reduction of Fe^{3+} can take place with H_2O_2 (Reaction 3) in the dark, the Fenton process, although Reaction 3 is much slower than Reaction 2.

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^- + HO^{\bullet} \quad (1)$$

$$Fe^{3+} + H_2O + h\nu \rightarrow Fe^{2+} + HO^{\bullet} + H^+ \quad (2)$$

$$Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + HOO^{\bullet} + H^+ \quad (3)$$

Although the photo-Fenton process is commonly carried out at acidic pH, its application at circumneutral pH is recommended to remove micropollutants. As the common wastewater pH is neutral or slightly basic, ferric ions precipitate as ferric hydroxides reducing the catalyst availability for the redox cycle (Reaction 4).

$$Fe^{3+} + 3\,H_2O \rightarrow Fe(OH)_3 + 3H^+ \quad (4)$$

In this regard, the sequential iron dosage strategy proposed by Carra et al. (2013) to maintain enough iron dissolved during the process appears to be a good choice for working at the natural pH of the effluent. As such, the reaction is run with ferrous iron additions at given time intervals to distribute the catalyst evenly throughout the process. To make the most of the iron added, it is necessary to distribute it during the reaction time, not adding it all at once since precipitation is favored at neutral pH.

Over the last few years, the photo-Fenton process has also been studied for inactivation of different bacteria and wastewater disinfection for water reuse (Klamerth et al. 2012; Rodríguez-Chueca et al. 2014). Depending on the needs of water reuse, the disinfection treatment must ensure the water quality follows the guidelines of each country, with *Escherichia coli* concentrations being one of the most important microbiological parameters used as a fecal indicator. As part of this, the Spanish legislation (RD 1620 2007) stipulates a reduction in *E. coli* concentration up to 1 CFU mL^{-1}. Most of the research on water disinfection by solar photo-Fenton was carried out with tubular reactors equipped with compound parabolic collectors (CPC) (Ortega-Gómez et al. 2014), and as far as the authors know, there are no papers dealing with wastewater disinfection in raceway pond reactors.

In general, micropollutants usually do not cause acute effects in most known test organisms. This is because their low concentrations are often not able to sensitize the organisms in short exposure times. Therefore, it is important to evaluate not only acute toxicity but particularly chronic toxicity in relation to microcontaminants. Regarding the toxicity of wastewater containing micropollutants, especially pharmaceuticals, there are few studies evaluating sublethal and chronic effects on aquatic biota, such as reproductive, growth, feeding, physiological, histopathological, and genotoxic ones along with behavioral alterations (Santos et al. 2010). WWTP discharges represent the main way that human pharmaceuticals and their metabolites are brought into the environment. This means that the aquatic wildlife is continuously exposed to these compounds, even at low concentrations (Fent et al. 2006). Because of this, environmental agencies have adopted chronic toxicity tests in the most recent draft environmental risk assessment guidance document for human pharmaceuticals (EMEA 2006). In the case of pharmaceuticals, they are designed to have a specific mode of action and many of these compounds have some persistence in the body. However, this particular effect from a medical perspective makes these compounds more persistent in the environment, with high potential for toxicity and bioaccumulation in aquatic and terrestrial biota (Fent et al. 2006). Moreover, AOPs do not always achieve total mineralization of pollutants and can generate more toxic intermediates (Klamerth et al. 2013; Carra et al. 2015). Since the toxicity is based on a biological response, different organisms from various taxonomic levels should be used to improve the reliability of results. Organisms normally used in bioassays for toxicity include representative samples of marine, freshwater, or terrestrial ecosystems, such as microorganisms, plants, invertebrates, and fish (Tothill and Turner 1996). Thus, the toxicity evaluation of advanced oxidation treatments using bioassays with various organisms is an effective way of monitoring processes and choosing between different AOPs being comparatively investigated (Rizzo 2011).

The most common organisms used for acute ecotoxicity assessment are the standardized tests with daphnids and luminescent bacteria (Zhao et al. 2007). Generally, chronic ecotoxicity tests are time consuming and require a lot of reagents and a large sample volume. In this regard, *Tetrahymena thermophila* assay is a good option since it is a short chronic test (24 h), is simple to implement, and requires only a small quantity of reagents and samples. *Tetrahymena* sp. is a nonpathogenic and free-living protozoa ubiquitously found throughout the natural world. Its presence may indicate healthy aquatic environments and represents an important trophic level in the aquatic chain food (Gerhardt et al. 2010). Regarding phytotoxicity evaluation, aquatic duckweed plants of the *Lemna* and *Spirodella* genus have been used to evaluate the effluent toxicity. The principle behind the assay for both genera is the same, the growth inhibition of germinated turions (young leaves) after exposure to contaminants. The main advantage of the proposed assay with *Spirodela* is the short execution time (3 days) (*Spirodela* duckweed Toxkit, 1998) in relation to *Lemna* sp. bioassay (7 days). In addition, the *Spirodela*

duckweed Toxkit proposes a miniaturized design, where the assay is performed in microtiter plates instead of beakers. This implies a much smaller sample volume and the possibility of evaluating multiple replicates. For phytotoxicity evaluation with terrestrial plants, *Lactuca sativa* seeds (lettuce) bioassay is a static acute toxicity test where the phytotoxic effects of pure compounds or complex mixtures in the process of seed germination and seedling development during the first days of growth are evaluated (Sobrero and Ronco 2004). Germination and early plant development is a growth period of great sensitivity where various physiological processes are taking place. Moreover, many of the reactions and processes involved are general for most of the seeds, meaning that the response of this species is largely representative of the effects on general seeds or seedlings (Sobrero and Ronco 2004).

In line with cost reduction for micropollutant removal, raceway pond reactors have recently been proposed for solar photo-Fenton treatment due to their low cost and high treatment capacity (Carra et al. 2014). They are extensive nonconcentrating photoreactors and consist of channels where water is set in motion by a paddlewheel system. Their main feature is the capability of changing the liquid depth and iron concentration as a function of irradiance to take advantage of most of the photons reaching the reactor surface (Rivas et al. 2015).

The main aim of this work was to evaluate the acute and chronic ecotoxicity of a municipal secondary effluent before and after tertiary treatment by solar photo-Fenton at neutral pH in a raceway pond reactor. Micropollutant removal, disinfection, and the sensitivities of different toxicity assays at very low pollutant concentrations were evaluated.

Materials and methods

Chemicals

High purity analytical standards (purity >90 %) used in this study were obtained from Sigma-Aldrich (Steinheim, Germany). 13C-caffeine and cyclophosphamide-d4, used as surrogate standards for the solid phase extraction, were purchased from Sigma-Aldrich. Individual stock standard solutions were prepared in methanol at a concentration of 1–2 mg/mL and stored in amber glass vials at −20 °C. Working solutions were prepared with the appropriate mixture and dilution of the stock solutions.

Acetonitrile (AcN) and methanol (MeOH) HPLC grade and formic acid (purity, 98 %) were supplied by Fluka (Buchs, Germany). Water used for LC-MS analysis was generated from a Direct-Q Ultrapure Water System from Millipore (Bedford, MA) with a specific resistance of 18.2 MΩ cm.

Sulfuric acid (95–97 %) was obtained from J.T. Baker (Deventer, Holland). Hydrogen peroxide (33 % *w*/*v*) and ferrous sulfate (99 %) were obtained from Panreac (Barcelona, Spain). Bovine catalase (Sigma-Aldrich, MO, USA; 0.1 g L^{-1}) was used to decompose residual H_2O_2 and consequently interrupt the Fenton reaction.

Experimental setup

Experiments were carried out with a municipal secondary effluent (Table 1) collected from a municipal wastewater treatment plant (MWWTP) located in the province of Almería (El Ejido, southeastern Spain). This plant treats wastewater from a population equivalent to 100,000 with a treatment capacity of 12,459 m^3 day^{-1} and is characterized by significant inputs from different sources such as greenhouses, plastics industry, and hospital wastewater. The MWWTP employs a pretreatment for solids removal, a primary treatment to remove suspended materials, an activated sludge biological treatment, and a final clarification.

As in any process driven by photons, the presence of suspended solids and turbidity reduce the efficiency of the photo-Fenton process. Therefore, before the tests, 25 L of the MWWTP effluent went through a filtration system comprising two cartridge filters. The first one had a pore diameter of 100 μm and it was connected to a second filter with a pore diameter of 20 μm. The filtered sample was stored at 4 °C in the dark until it was ready to be used.

Experiments were carried out in a PVC raceway pond reactor (RPR) of 0.98 m length and 0.36 m width. It is separated by a central wall, forming two channels. The reactor was filled with 18 L of the filtered effluent, resulting in 5-cm liquid depth as this is the common diameter used in tubular reactors such as compound parabolic collector photoreactors (CPCs). A paddlewheel connected to an engine was set at 200 rpm to obtain a well-mixed, homogeneous flow during the process and to ensure a turbulent regime with a Reynolds number of $6 \cdot 10^5$. The mixing time was experimentally measured by means of a pulse test with a tracer, resulting in ~1.52 min. A schematic of the RPR is shown in Fig. 1.

UV radiation was measured by means of a global UV radiometer (DeltaOhm, LP UVA 02 AV; spectral response 327–384 nm) mounted on a horizontal platform, providing data in terms of incident UV (W m^{-2}). The reactor was equipped with temperature (Crison 60 50) and pH (Crison 53 35) probes as well as a turbidity sensor (Hach Lange Ultraturb Plus SC100). During the course of the experiment, UV radiation, temperature, and pH were monitored online by means of a LabJack USB/Ethernet data acquisition device connected to a computer. The average UV irradiance was 26 W m^{-2} and the average temperature of wastewater was 20 ± 0.3 °C.

The solar photo-Fenton process was carried out at initial circumneutral pH with three iron additions (3 × 20 mg Fe^{2+} L^{-1}) and an initial concentration of 50 mg L^{-1} of H_2O_2. Prior to starting the experiments, the reactor was covered and the pH was adjusted to pH 6.5 ± 0.05 using 55 mL of sulfuric acid

Table 1 Main physical, chemical, and microbiological properties of the secondary and tertiary (solar photo-Fenton) effluents

Parameter	Secondary effluent	Tertiary effluent	Removal (%)
pH	7.6	7.5	–
Conductivity (mS cm^{-1})	1.2	1.3	–
Turbidity (NTU)	1.1	0.32	71
Dissolved organic carbon (mg L^{-1})	20.64	9.1	56
Bicarbonate (mg L^{-1})	429	6.5	98
Chloride (mg L^{-1})	418	426	–
Nitrate (mg L^{-1})	5.8	8.7	–
Phosphate (mg L^{-1})	6.1	0	100
Sulfate (mg L^{-1})	142.5	775	–
E. coli (CFU mL^{-1})	1.8×10^3	1	99.4
Total coliforms	7.3×10^4	1	99.9

2 N to partially remove bicarbonates until a final concentration of 5 mg L^{-1} (expressed as total inorganic carbon, TIC). The presence of carbonate (CO_3^{2-})/bicarbonate (HCO_3^-) slows AOPs down because these anions may react with $HO^•$, resulting in $HO^•$ scavenging, which inhibits the oxidative attack of hydroxyl radical (Fernández-Ibáñez et al. 2009). Then, 50 mg H_2O_2 L^{-1} was added and a recirculation time of 3 min was allowed for homogenization, corresponding to twice the mixing time in the photoreactor. Subsequently, the reactor was uncovered and the first iron salt addition was made (20 mg Fe L^{-1} as $FeSO_4$-$7H_2O$) starting the reaction. Two more Fe^{2+} additions were made 5 and 15 min later for the second and third iron additions, respectively. At the end of the process, as the final pH decreased due to iron hydrolysis, the wastewater was neutralized using NaOH 1 N (Sigma-Aldrich, Steinheim, Germany). Neutralization gave rise to ferric hydroxide precipitation, which was easily removed by decantation.

Bacterial quantification

The inactivation of wild enteric bacteria (*E. coli* and total coliforms) from the municipal effluent was determined before and after photo-Fenton treatment. Total coliforms (TC) and *E. coli* colonies were isolated from the secondary wastewater studied. Samples were enumerated using a standard plate count method with serial dilutions in a selective medium (Chromocult, Merck KGaA, Darmstadt, Germany; 26.5 g L^{-1}). Colonies were counted after 24 h of incubation at 37 °C and the colony-forming units per milliliter (CFU mL^{-1}) were calculated. In order to test cell recovery posttreatment, the samples were kept in the dark after the treatment for predetermined exposure times. Samples were plated for colony counting after a 24-h period. No regrowth was observed. The detection limit was 1 CFU mL^{-1}. All procedures were carried out in triplicate for each sample.

Analytical determinations

Before chemical analysis, all samples (10 mL) were immediately filtered (nylon filters with pores of 0.20 μm diameter, Millipore®, Milford, MA, USA) and the filter was washed with acetonitrile in an acetonitrile/sample ratio of 1:10 and mixed with the filtered water sample.

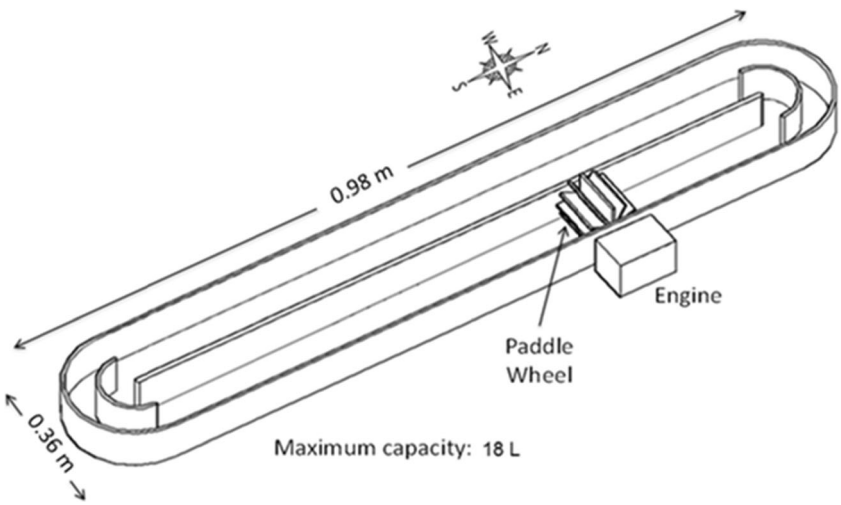

Fig. 1 Raceway pond reactor (RPR) scheme

Hydrogen peroxide was measured by a colorimetric method using ammonium metavanadate, measuring the absorbance at 450 nm (Nogueira et al. 2005). The iron concentration was determined according to the o-phenantroline standardized procedure (ISO 6332), and the red complex formed was determined spectrophotometrically at 510 nm. Dissolved organic carbon (DOC) determinations were carried out in a Shimadzu-V CPH TOC analyzer, anion concentrations were determined using ion chromatography (Metrohm 881 Compact IC pro), and the eluent used was a solution of Na_2CO_3.

Determination of micropollutants in wastewater samples

Analytes included in this study comprise a group of 77 organic pollutants, mainly pharmaceuticals, belonging to different therapeutic groups, including some of their metabolites and pesticides (see Table S.1 in Online resource 1).

A preconcentration procedure was applied to the samples based on a previously reported method (Gómez et al. 2007) by solid phase extraction (SPE). Samples were concentrated 100-fold in the case of wastewater treated by photo-Fenton and 50-fold in the case of untreated wastewater, by SPE with OasisTM HLB (6 cc/200 mg; Waters, Milford, MA) cartridges previously conditioned with 6 mL of MeOH and 5 mL of Milli-Q water at pH 8. Before extraction, samples were spiked with 0.1 μL of each surrogate standard at a concentration of 1 mg L^{-1}, and the pH was adjusted to 8 with 20 % NH_4OH (Sigma-Aldrich, Steinheim, Germany). Analyte elution was performed with 2 × 4 mL of MeOH, and the eluted volume was dried under a gentle N_2 stream and reconstituted to 1 mL of AcN. Prior to injection, appropriated dilution was applied in AcN/H_2O (10:90, *v*/*v*). Table S.2 in Online resource 1 summarizes all recoveries obtained for each target compound in both matrices, treated by photo-Fenton and untreated wastewater.

The analytical method used for the detection and quantification of the microcontaminants in raw and treated wastewater samples was developed by liquid chromatography coupled with hybrid quadrupole/linear ion trap mass spectrometry (LC-QqLIT-MS/MS) consisting of an Agilent 1200 LC system (Agilent Technologies, USA) and a 5500 QTRAP LC/MS/MS from AB Sciex Instruments (USA). Chromatographic separation took place in a ZORBAX Eclipse XDB C18 analytical column of 50 mm × 4.6 mm I.D. (1.8 μm particle size) from Agilent. Mobile phases A and B were Milli-Q water (containing 0.1 % formic acid, Sigma-Aldrich, Steinheim, Germany) and acetonitrile, respectively. The gradient program was set as follows: 10 % B (initial conditions), constant for 1 min, followed by an increase to 50 % within 4 min, an increase to 100 % within 10 min, then kept constant for 4 min, and finally reduced to 10 % in 0.1 min. Total run time was 14.1 min and postrun equilibrium time was 4 min. The injection volume was 10 μL and the flow rate was kept constant at 0.4 mL min^{-1}. The LC system was connected to the MS by a TurboIonSpray source operated in positive and negative ionization modes. Source settings were as follows: IonSpray voltage (IS), 5000 V in positive and −4500 V in negative mode; source temperature, 500 °C; curtain gas, 25 (arbitrary units); collision gas, high; ion source gas 1, 50 psi, and ion source gas 2, 40 psi. Nitrogen was used as the nebulizer gas, curtain gas, and collision gas. The MS-MS parameters (declustering potential (DP), collision energy (CE), and cell exit potential (CXP)) were optimized for maximum sensitivity by direct infusion of the standards of each compound into the MS (see Table S.3 in Online resource 1).

Analyses were carried out in the selected reaction monitoring (SRM) mode. Confirmation of analytes in the samples was performed by the presence of two SMR transitions (SRM1 and SRM2) at the correct retention time (Rt) and presenting the correct SMR2/SRM1 ratio, according to the EU guidelines for LC-MS/MS analysis (European Commission 2002). Table S.3 shows the values of the optimized MS parameters, as well as the two selected SRM transitions (SRM1 and SRM2) and the identification criteria for the analyzed compounds.

The most intense SRM transition (SRM1) of each compound was selected for quantification purposes. Two matrix-matched calibration curves prepared by spiking wastewater extracts, before and after the photo-Fenton treatment, at nine concentration levels were used for the quantification procedure (from 0.02 to 10 μg L^{-1}). The limits of detection (LOD), limits of quantification (LOQ), matrix effect (ME), and r^2 of the matrix-matched calibration curves are shown in Table S.4 in Online resource 1.

Ecotoxicity tests

For ecotoxicity evaluation, samples were collected at initial (0 min), intermediate (20 min), and final photo-Fenton treatment times (90 min) in previously washed glass flasks. The pH was adjusted to 6~7, and when necessary, catalase was added for residual H_2O_2 removal. Samples were analyzed immediately or, when this was not possible, they were kept frozen at −20 °C for a maximum of 20 days until the tests were performed. Five standardized tests were selected: *Daphnia magna* and *Vibrio fischeri* (acute toxicity), *T. thermophila* (chronic toxicity), and *Spirodela polyrhiza* and *Lactuca sativa* seeds (phytotoxicity). For all the assays described below, the initial (0 min) and final (90 min) samples were tested at 100, 50, 25, 12.5, and 6.25 % (*v*/*v*). Samples from the intermediate time (20 min) were tested without dilution (100 %).

D. magna acute tests

The 48 h immobilization test with *D. magna* was performed according to the standard operational procedure of the Daphtoxkit FTM magna (1998), in accordance with standard procedures (OECD 2004). Five neonates (6–24 h) were used

for each of the four replicates ($n = 20$). Samples were diluted in standard freshwater medium as described above. The negative control was standard freshwater medium and the positive control was K_2CrO_7 (Panreac, Barcelona, Spain; purity 99 %, 3.2 mg L^{-1} Cr^{6+}). All tests were maintained at 20 °C. After 48 h of exposure, the number of immobile organisms was recorded and the % inhibition with standard deviation (±) was calculated.

V. fischeri acute tests

The 30-min luminescence inhibition test with *V. fischeri* was performed following the standard ISO 11348-3:1998 method (ISO 1998), using the commercial kit BioFix® Lumi-10 of freeze-dried bacteria. The bacteria was stored at −20 °C and activated by hydration according to the standard operation procedure of the kit. All the samples including the negative and positive controls were tested in triplicate and kept on a thermostatic plate at 15 °C throughout the entire test. Solution of NaCl (Sigma-Aldrich, Steinheim, Germany; purity 99.9 %, 20 g L^{-1}) was used as negative control and K_2CrO_7 (Panreac, Barcelona, Spain; purity 99 %, 18.7 mg L^{-1} Cr^{6+}) was used as positive control. NaCl was used for salinity correction of the samples (final concentration 20 g L^{-1}). Bioluminescence was measured after 30 min of exposure at the sample concentrations described above. Samples were considered toxic when, for a given concentration of the sample, 50 % reduction (EC_{50}) of the light output, measured in a Macherey-Nagen Biofix® illuminometer (Macherey-Nagel GmbH & Co. KG, Duren, Germany) was obtained.

T. thermophila chronic tests

The 24-h growth inhibition tests with *T. thermophila* were performed according to the standard operational procedure of the Protoxkit F (1998). The test is based on the turnover of substrate into ciliate biomass. While normal proliferating cell cultures turn the substrate suspension clear in 24 h, inhibited culture growth is reflected by the remaining turbidity. Protozoa inoculum (initial concentration 100 cells mL^{-1}) was exposed to the samples, and the optical density was measured at 440 nm in a spectrophotometer at the beginning (OD T0) and end (OD T24) of the test. Deionized water was used as negative control and K_2CrO_7 (56 mg L^{-1} Cr^{6+}) was used as positive control. The difference between the mean OD at T0 and T24 for each sample (ΔOD_S) and for the control (ΔOD_C) was calculated. All samples, including the negative and positive controls, were tested in duplicate. The % inhibition with standard deviation (±) was calculated by the following equation: $\%\ \text{Inhibition} = (1 - \Delta OD_S/\Delta OD_C) \times 100$, according to standard operation procedure (Protoxkit F 1998).

Phytotoxicity tests with S. polyrhiza

The 72-h growth inhibition test with *S. polyrhiza* was performed according to the standard operational procedure of the *Spirodela* duckweed Toxkit (1998). The endpoint measured was the growth inhibition (%) of the germinated turions (new leaves), after 3 days of exposure to samples, in comparison with the negative control. Steinberg medium was used as negative control and KCl (Sigma-Aldrich, Steinheim, Germany; 99 % purity, 18 g L^{-1}) was used as positive control. After germination, one germinated turion was transferred to each well on a 48-multiwell test plate. Sample dilutions and the control solutions were added to the rows, in an octuplicate. Digital photos of the multiwell plates were taken at the start (T0) and at the end (T 72 h) of the test. The area measurements of the turions were taken with the aid of an image analysis program (Image-Pro Plus 6.0). The initial and final area values were used to calculate the growth inhibition (%) with standard deviation (±) according to the standard operational procedure (*Spirodela* duckweed Toxkit, 1998).

Phytotoxicity tests with L. sativa seeds

The seed germination/root elongation phytotoxicity test was conducted according to standardized protocols (US EPA 1989; Young et al. 2012) using *L. sativa* (lettuce) seeds (cv. Boston) acquired at a local market. Tests were carried out in Petri dishes lined with filter paper and with 13 seeds each, containing 4 mL of sample dilution or negative/positive control (distilled water and $ZnSO_4{\cdot}7H_2O$, Panreac, Barcelona, Spain; 100 mg L^{-1}). The assay was done in triplicate. Seeds were incubated at 22 ± 2 °C, in the dark, for 120 h. At the end of the test, the number of germinated seeds and the root elongation data were used to calculate the germination index (GI) and the relative growth index (RGI), respectively (Young et al. 2012). The RGI values were divided into three categories according to the observed toxicity effects: (a) inhibition of the root elongation: $0 < RGI < 0.8$; (b) no significant effects: $0.8 \leq RGI \leq 1.2$; and (c) stimulation of the root elongation: $RGI > 1.2$ (Young et al. 2012).

Statistical analysis

Statistical data evaluation was performed with the BioEstat 5.3 software (BioEstat Software, Belém, Brazil). For *D. magna*, the effective concentrations 50 % (EC_{50}) were calculated using the Probit method. For *T. thermophila* and *S. polyrhiza*, the calculations were done by linear regression in an Excel® spreadsheet provided by the bioassays kit manufacturer (MicroBioTests Inc., Belgium). For *V. fischeri*, the EC_{50} values were calculated by linear regression using BioFix® Lumi-10 luminometer software (Macherey-Nagel GmbH & Co. KG, Duren, Germany). When the EC_{50} was not reached

by the tested effluent samples, toxicity was expressed as % of toxic effect. For *L. sativa*, data were subject to Kolmogorov-Smirnov normality test. As data were normally distributed, they were submitted to one-way analysis of variance (ANOVA) and Tukey test ($p < 0.05$). Significance values are indicated as follows: $*p < 0.05$ and $**p < 0.01$.

Results and discussion

Photo-Fenton treatment

The chemical analysis of the secondary effluent revealed the presence of at least 39 micropollutants. Table 2 includes those detected at higher concentration levels. Concentration values ranged from 0.1 ng L^{-1} to 31 μg L^{-1} and represented a total concentration of 80 μg L^{-1}. Most of them were pharmaceutical products (antibiotics, psychiatric drugs) and pesticides, as the WWTP is characterized by significant inputs from different sources such as greenhouses, plastics industry, and hospital wastewater. Pharmaceuticals are frequently detected in effluents at levels from below 1 ng L^{-1} up to a few micrograms per liter (Schaider et al. 2014).

Analyses performed after photo-Fenton treatment demonstrated high removal efficiency (Table 2); 99 % removal of the total load of contaminants was obtained after 90 min of treatment. However, some compounds still showed resistance to oxidation, such as salicylic acid, as reported elsewhere (Rodríguez-Gil et al. 2010).

Furthermore, 56 % of the DOC was removed during the treatment, decreasing from 20.6 to 9.1 mg L^{-1} (Table 1). Oxidation and partial mineralization of the dissolved organic matter found in the secondary effluent was thought to be the main reaction taking place due to unselective attack of hydroxyl radicals caused by the photo-Fenton reaction. The degradation of micropollutants is only part of the oxidation process, although this is one goal of the tertiary treatment. Another goal was water disinfection. Complete bacterial inactivation was achieved, as the total coliforms and *E. coli* concentrations dropped from 7.3×10^4 and 1.8×10^3 CFU mL^{-1} to below the detection limit in 90 min, respectively (Table 1). These results are consistent with those reported by Ortega-Gómez et al. (2014) who also demonstrated the capability of the photo-Fenton process at neutral pH for enteric bacteria inactivation in CPCs. The present work is the first time that wastewater disinfection by photo-Fenton in RPR has been reported.

Table 2 Initial and residual micropollutant concentrations after the solar photo-Fenton treatment at natural pH

Compound	Initial concentration (ng L^{-1})	Residual concentration (ng L^{-1})	% removal
4-AAA	3203	<LOQ	100
4-FAA	10,153	<LOQ	100
Salicylic acid	620	391	37
Antipyrin	4550	<LOQ	100
Atenolol	2453	18	99
Azithromycin	31,089	<LOQ	100
Caffeine	4256	8	100
Carbamazepine	533	<LOQ	100
Clarithromycin	584	<LOQ	100
Citalopram	7525	<LOQ	100
Cotinine	519	34	93
Diuron	690	<LOQ	100
Erythromycin	543	62	89
Fluoxetine	224	<LOQ	100
Gemfibrozil	4010	<LOQ	100
Hydrochlorothiazide	2220	<LOQ	100
Lincomycin	204	<LOQ	100
Naproxen	839	<LOQ	99
Paraxanthine	2663	<LOQ	100
Sulfamethoxazole	518	<LOQ	100
Thiabendazole	457	18	96
Velaflaxime	1946	<LOQ	100
Sum of the rest	1073	<LOQ	100
Total	80,872	531	99

After iron addition, pH became acidic due to iron hydrolysis (Reaction 4), which gave rise to the precipitation of most of the iron added as ferric hydroxide (Fig. 2). However, significant concentrations of dissolved iron remained in the reactor after the third addition (9 mg Fe L^{-1}). To accomplish with the emission limit values for effluent discharges into superficial watercourses of the Andalusian Regional Government (pH in the range of 9.5–5.5 and iron concentration below 2.4 mg L^{-1}, Decree 109/2015), water neutralization was the final stage of the process. Consequently, the dissolved iron concentration dropped to 0.1 mg L^{-1}, and the precipitated iron was removed by decantation, decreasing the final wastewater turbidity from 1.1 NTU in the secondary effluent to 0.32 in the tertiary effluent. The 50 mg L^{-1} of H_2O_2 added was consumed in its entirety during the treatment process.

Overall, these results indicate that the photo-Fenton process applied at initial neutral pH in the RPR was able not only to remove micropollutants but also to disinfect this wastewater complying with Spanish legislation (RD 1620 2007) for reclaimed wastewater, where 1 CFU mL^{-1} of *E. coli* concentration is required. Thus, the final tertiary effluent quality was highly improved for industrial or agricultural reuse.

Ecotoxicity assessment

Five standard bioassays were used in the ecotoxicity evaluation of secondary effluent before and after photo-Fenton tertiary treatment. Aquatic and terrestrial organisms were tested in acute, chronic, and phytotoxicity assays. Species representing different trophic levels (primary producers, consumers, filter feeders, and decomposers) were used in this study. The organisms showed different sensitivities to effluent before and after photo-Fenton treatment, but the protozoan *T. thermophila* was the organism that showed the highest sensitivity to the effluent before the tertiary treatment, followed by the microcrustacean *D. magna* and *L. sativa* seeds.

Regarding acute ecotoxicity, the secondary effluent caused an inhibitory effect of 20 % for *D. magna* (Fig. 3 and see Table S.7 in Online resource 1). After 20 min of photo-Fenton treatment, the toxicity dropped to 5 %. These results were confirmed when samples taken at the end of the treatment (90 min) were analyzed (Fig. 3). This is an important finding, since similar studies observed an increase of toxicity after application of an advanced oxidation process, probably caused by partial mineralization of organic compounds and the appearance of transformation products more toxic than the parent compounds (Dantas et al. 2008; Pereira et al. 2009). *D. magna* has been widely used as an experimental animal in aquatic ecotoxicity. Several recent studies have demonstrated the sensitivity of the genus *Daphnia* in the evaluation of effluent ecotoxicity before and after secondary and tertiary treatments. Maselli et al. (2015) compared the sensitivity of different bioassays standardized to raw and treated effluents generated by a veterinary pharmaceutical company. The acute toxicity test with *Daphnia similis* proved to be the most sensitive for raw and treated effluents. Rizzo et al. (2009) evaluated the ecotoxicity of a municipal wastewater contaminated with pharmaceuticals before and after heterogeneous photocatalysis. Both *D. magna* and the microalgae *Pseudokirchneriella subcapitata* were sensitive to drugs and their transformation products. Acute toxicity of many pharmaceuticals has been evaluated with *D. magna*. Although variable, the EC_{50} values found are in the milligram per liter range when the compounds are tested separately (Fent et al. 2006). However, this toxicity can increase significantly when there is a mixture of pharmaceuticals. Cleuvers (2003) evaluated the ecotoxicological potential of ten prescription drugs using *D. magna*, *Desmodesmus subspicatus*, and *Lemna minor*. For most of the substances, EC_{50} values were in the

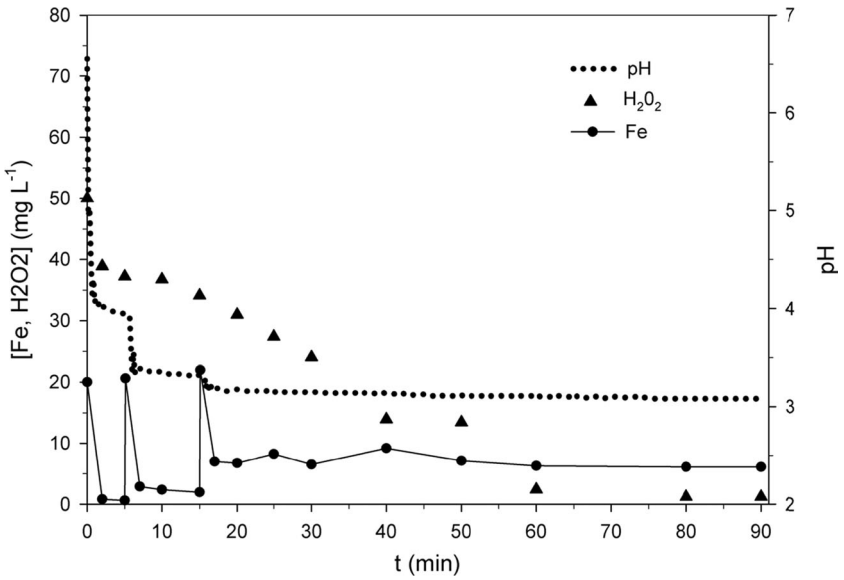

Fig. 2 Profiles of Fe, H_2O_2, and pH during the solar photo-Fenton process at natural pH with iron dosage (3 × 20 Fe mg L^{-1}) and 50 mg H_2O_2 L^{-1}

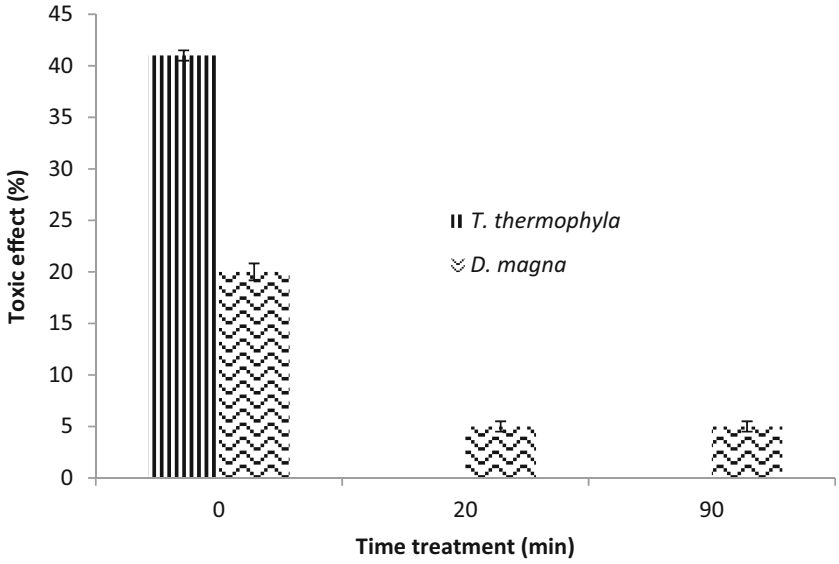

Fig. 3 Changes in effluent toxicity to *T. thermophila* and *D. magna* after the solar photo-Fenton process

range from 10 to 100 mg L^{-1}. However, tests with combinations of various pharmaceuticals revealed more pronounced effects than expected from the effects measured separately, indicating that there was a synergistic effect. For example, the effects of clofibrinic acid and carbamazepine measured individually were 1 and 16 % immobilized daphnids, respectively. The mixture of both pollutants at the same concentration caused a stronger effect of about 95 % immobilization in the daphnids. These results reinforce what was observed in our experiments. All microcontaminants were identified in the effluent at concentrations far below their EC_{50} values (acute toxicity) reported in the literature. Due to the fact that they were present in a mixture, the observed ecotoxicity may be the result of a synergistic effect among them. However, as it is a complex matrix, it is not possible to assign the toxicity to a particular compound, even to the group of microcontaminants (mainly pharmaceuticals) detected in the effluent since the observed effects might be a result of other substances not targeted in the chemical analysis. When evaluating the toxicity of mixtures and complex samples such as wastewater, two different approaches may be used: the direct toxicity assessment (DTA), which is also called whole effluent toxicity (WET), and toxicity identification and evaluation (TIE) (SETAC 2004). Methods that use a whole mixture approach or whole effluent testing (WET) are based on the direct ecotoxicological assessment of a given complex chemical mixture, such as the effluent from a WWTP (US EPA 1991). The main purpose of such analysis is to assess whether the mixture causes adverse effects and, if so, to quantify the magnitude of these effects (Kortenkamp et al. 2009). The acute test with luminescent marine bacterium *V. fischeri* showed no sensitivity to the sample tested, either before or after treatment by the photo-Fenton process. Although *V. fischeri* is often reported to be sensitive to organic pollutants in the effluent, toxicity occurs in the parts per million range concentration (Larsson et al. 2007).

For the chronic ecotoxicity assay with the protozoan *T. thermophila*, the secondary effluent caused an inhibitory effect of 40.9 % (Fig. 3 and see Tables S.5 and S.6 in Online resource 1). After 20 and 90 min of photo-Fenton treatment, no toxic effect was observed (Fig. 3). Although there are few reports in the literature, toxicity tests with this organism have shown good correlation with other eukaryotic organisms regarding sensitivity to various compounds (Gerhardt et al. 2010; Schultz et al. 2003). This was observed when the effects of 14 pharmaceuticals including NSAIDs, antibiotics, and β-blockers on the *Tetrahymena pyriformis* growth were evaluated by Láng and Köhidai (2012). Results showed that the sensitivity of *Tetrahymena* to NSAIDs and β-blockers (EC_{50} ranged from 4.8 to 308.1 mg L^{-1}) was comparable to standard bioassays, such as microalgae or *Daphnia* tests. Despite the concentrations of pharmaceuticals and other compounds being very low in the tested effluent when compared to the reported effective concentration, the inhibitory effect observed (40.9 %) might be a result of the synergetic effect by the pollutant mixture present.

Evaluating the phytotoxicity on *L. sativa* seeds, root elongation was used to calculate the relative growth index (RGI) (see Table S.8 in Online resource 2). It was observed that before treatment (0 min), the effluent stimulated root elongation with an average value of 3.7 ± 0.5 cm (RGI = 1.9) higher than the negative control mean (2.0 ± 0.6 cm). After 20 min of treatment, there was statistically significant inhibition of the root elongation mean compared to the control (1.4 ± 0.4 cm, RGI = 0.7, $p < 0.01$, Table 3). At the end of the treatment (90 min), the root elongation mean (1.6 ± 0.4 cm, RGI = 0.85) showed no significant effect (Table 3). There was no statistically significant difference between the averages of root elongation after 20 and 90 min of treatment. One aspect to consider is the increase in radicle elongation in effluent before photo-Fenton treatment (0 min) relative to the control. The stimulation

Table 3 *Lactuca sativa* seed endpoints observed after effluent exposure

	GI (%)	Mean (cm)	RGI	Toxicity category
Control	–	2.0 ± 0.3^{a}	–	–
0 min	100	$3.7 \pm 0.5^{**b}$	1.9	S
20 min	97.7	$1.4 \pm 0.2^{**c}$	0.7	I
90 min	100	$1.6 \pm 0.4^{*c}$	0.85	NSE

Mean values with different letters (a, b, c) are significantly different (Tukey's test, $p < 0.05$)

GI germination index, *RGI* relative growth index, *S* stimulation, *I* inhibition, *NSE* no significant effect

$^{*}p < 0.05$; $^{**}p < 0.01$

in this endpoint should not be interpreted as a favorable effect. Many compounds in low concentrations can act as micronutrients, leading to the stimulation of radicle growth, but this response should be evaluated in the context of the bioassay and the other endpoints analyzed (Sobrero and Ronco 2004). Before treatment, probably due to the higher organic load and nutrients, the average elongation was above the control mean. The slight inhibition after 20 min of treatment could be related to both the reduction of the organic load by oxidation and the formation of more toxic transformation products. Rizzo et al. (2009) observed similar results when evaluating a municipal effluent spiked with a pharmaceutical mixture, before and after heterogeneous photocatalysis in *Lepidium sativum* seeds. The results showed that although the germination rate was not affected (100 % germination in all of samples), the complete opposite could be said for the root elongation endpoint. Germination inhibition could be considered an acute or lethal effect, with radicle growth inhibition as sublethal. One of the objectives of the tertiary effluent treatment is to improve the quality of municipal wastewater effluent by removing residual xenobiotics in order to decrease final toxicity and make treated wastewater reusable (Rizzo 2011). In summary, this assay showed a stimulatory effect at T0 that was probably due to increased nutrient load. After 20 and 90 min with Fenton treatment, there was a slight but significant effect on mean shoot length but not on growth rate, indicating weak toxicity. In this regard, the use of *L. sativa* provides data about the possible effect of pollutants on plant communities near the banks of polluted water bodies and is also an interesting species considering its importance from the point of view of horticulture and water reuse (Sobrero and Ronco 2004).

The macrophyte *S. polyrhiza* showed no growth inhibition when exposed to the effluent before and after treatment (see Tables S.9, S.10, and S.11 in Online resource 2). However, under both conditions (treated and untreated samples), the growth of leaves was stimulated, i.e., they increased more than with the negative control. Furthermore, the growth stimulation rate was higher with the effluent prior to photo-Fenton (2.25 times higher than control), showing a possible correlation with the amount of initial nutrient load. After 90 min of treatment, the effluent was also conducive to an increase in larger leaves than in the control, but lower than in the untreated effluent (1.89 times higher than control). These results can be correlated with the decrease of DOC, which presented 56 % removal after the photo-Fenton process (Table 1). Deleterious effects such as chlorosis and necrosis were not observed in any sample. Regarding the use of duckweed plants in effluent toxicity tests, few studies have been done with *Spirodela* sp. until now. This species has been used mainly for evaluation of toxicity of metals and nanoparticles (Jiang et al. 2012). Recent studies demonstrated that *L. minor* was the most sensitive test species in the majority of the ten pharmaceuticals tested in comparison with *D. magna* and *D. subspicatus*. However, the concentrations of tested drugs were much higher than those found in our effluent samples (EC_{50} in the ppm range) (Cleuvers 2003). Finally, aquatic macrophytes, such as *S. polyrhiza*, are important to assess the impact of treated effluent after its release into water bodies. Moreover, the use of terrestrial species such as *L. sativa* intended to evaluate the possible effects of reuse of treated wastewater for irrigation purposes. Additionally, both tests were chosen because they are easy to implement and allow the assessment of toxicity through different endpoints.

In our work, the effluent toxicity before treatment by photo-Fenton could be associated with the predominant presence of pharmaceuticals. Compounds such as antibiotics (azithromycin), psychiatric drugs, and psychostimulants (citalopram, caffeine, and metabolites), anti-inflammatories and metabolites (4-FAA and 4-AAA), antilipidemic agents (gemfibrozil), β-blockers (atenolol), and diuretics (hydrochlorothiazide) accounted for about 89 % of the total concentration of the detected microcontaminants (around 72 $\mu g\ L^{-1}$). The presence of these contaminants, even after a biological secondary treatment, is probably due to the proximity of the WWTP to a major hospital in the area. However, the ecotoxicity observed, especially for *T. thermophila* and *D. magna*, could not be attributed only to the pharmaceuticals present in the effluent. The matrix complexity and the possible synergistic effects between the analytically detected and undetected substances should be considered. Recent studies have shown that pharmaceuticals in wastewater, even at trace levels, display toxicity to different organisms, both before and after secondary treatment. Maselli et al. (2015) evaluated the ecotoxicity of raw and treated effluents generated by a veterinary pharmaceutical company and compared the sensitivities of different bioassays. The acute toxicity test with *D. similis* proved to be the most sensitive for raw and treated effluents. Rizzo et al. (2009) evaluated the degradation kinetics, mineralization, and ecotoxicity of a municipal wastewater treatment plant effluent contaminated with a mixture of pharmaceutical compounds like amoxicillin, carbamazepine, and diclofenac by TiO_2 photocatalysis. In this study, the authors compared the ecotoxicity on *D. magna*, *P. subcapitata*,

and *L. sativum* of the single pharmaceuticals solutions, their mixture, and the spiked wastewater, before and after photocatalytic degradation. The initial toxicity of the spiked wastewater was higher than that of the effluent without the pharmaceutical mixture. This data may indicate that even showing a toxic effect on organisms, the main contribution was the mixture of pharmaceuticals and not of the effluent matrix. In the case of our study, there is no way to conclude that the ecotoxicity (or lack thereof) is related to the pharmaceuticals and other microcontaminants or the effluent matrix effect. However, it became clear that there was not an increase in ecotoxicity for any of the organisms tested after the photo-Fenton process.

Conclusions

Solar photo-Fenton treatment at neutral pH in a raceway pond reactor led to 99 % removal of microcontaminants after 90 min. The concentration of most of the studied compounds was successfully reduced to levels below the detection limits of the analytical method, although a few refractory compounds, such as salicylic acid, still remained at low concentrations. Under these conditions, the enteric bacterial population was completely inactivated and acute and chronic toxicity was removed. Different organisms were evaluated for their sensitivity to the effluent. Before solar photo-Fenton treatment, the protozoan *T. thermophila* was the most sensitive, followed by *D. magna* and *L. sativa.* After the tertiary treatment, *D. magna* was the most sensitive organism. Based on our results, we recommend that *D. magna* and *T. thermophila* tests should be included when assessing the ecotoxicity of effluent with a significant presence of pharmaceuticals. Finally, the treated water became suitable for irrigation use, highlighting the potential of the photo-Fenton process as an alternative tertiary treatment.

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