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# Humic like substances extracted from oil mill wastes in photo-Fenton processes: Characterization, performance and toxicity assesment

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ABSTRACT

Olive mill waste has been used as sourcing materials for the isolation of humic like substances (OMW-HLS) which have demonstrated its capacity to expand the range of applicability of photo-Fenton process to pH= 5. During the isolation process, membranes of three different pore sizes (300 kDa, 150 kDa and 50 kDa) were employed in order to obtain three batches of OMW-HLS. Four pollutants contained in 2013/39/EC were used as target substances: terbutryn (TBT), diclofenac (DCF), chlorfenvinphos (CVF) and pentachlorophenol (PCP). Results showed that OMW-HLS was able to enhance photo-Fenton at pH = 5, but differences were not significant, either among fractions or with commercial humic substances. Reactions were scaled-up and driven under real sunlight and pollutants removal was faster in the presence of OMW-HLS. Toxicity was monitored according to bioassays based on different organisms or cell lines. Detoxification was observed with and without OMW-HLS, although higher toxicity was detected in the presence of humic acids, most probably due to the surfactant effect, that allows a better contact between pollutant and organism.

## 1. Introduction

Advanced oxidation processes (AOP) have been successfully employed for decontamination of wastewater; in particular removal of pollutants of emerging concern, such as pesticides or drugs has been achieved [12,26]. Photo-Fenton, one of the most efficient AOP, consists in the use of iron salts to catalyse decomposition of hydrogen peroxide into highly reactive species, such as hydroxyl radicals, in a process that is accelerated upon irradiation [25]. One of the major drawbacks of photo-Fenton process is the highly acidic media required to avoid the formation of oxides or hydroxides which inhibit the catalytic role of iron [27]. This can be prevented by adding chemical auxiliaries able to form photoactive complexes with iron.

Dissolved organic matter (DOM), such as humic or fulvic acids, is known to generate, under solar radiation, highly reactive species able to oxidize pollutants [17,33], and interestingly, they are good iron complexing agents [13]. Other macromolecules with similar characteristics (humic-like substances, HLS) have been demonstrated to be useful for this purpose [15]. HLS can be isolated from different sources, such as solid wastes ([14,19,32]), what is aligned with the objectives of European Union towards circular economy (COM/2017/033) [7]. In this context, a paper has been recently published on the use of olive mill wastes as starting material for the isolation of humic like substances (OMW-HLS) [14]. This is an interesting result for the Mediterranean countries, as olive oil production industry has an important impact because of the high amounts of waste that generates. However, only very preliminary results on their application in photo-Fenton process were given, and some aspects such as the effect of the size of HLS or the aqueous matrix remains unexplored.

Monitoring the effect of the photochemical process on the effluent composition is another important issue that deserves investigation, as mineralization is a goal that cannot be achieved in AOPs, or the irradiation time required is too long, making the process uneconomic [22]. For this reason, gaining further insight into the nature of the by-products that have been released, as well as achieving a reliable monitoring of the toxicity of the treated sample is advisable according to European requirements (Council Directive 91/689/EC) [10]. However, monitoring changes in toxicity is not easy, as results strongly depend on the bioassay

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that is employed and on the kind of organism that is involved. For this reason, it is not convenient to rely in just one assay but employing a battery of them involving organisms from different levels from trophic chain [2,12]. As far as we know, this study remains to be performed for iron-complexed photo-Fenton process.

With this background, the aim of this work is to determine the performance of photo-Fenton process using OMW-HLS as iron complexing agent at mild pH conditions, paying special attention to the toxicity according to the battery of bioassays. This study is of interest in order to determine if HLS from other sourcing materials than urban wastes or activated sludge can be used for this purpose. Also, dialyzed and non-dialyzed fractions obtained with different cut-off membranes have been characterized and tested as auxiliaries for photo-Fenton, and results have been scaled-up to solar plant and run with real sunlight for the first time. In order to carry out this research, four pollutants listed as priority substances in European Commission directives 2013/39/EC and 2008/105/EC [8,9] were selected as target pollutants belonging to different families: terbutryn (TBT), a widely used herbicide; diclofenac (DCF), anti-inflammatory drug; chlorfenvinphos (CFV), an insecticide; and pentachlorophenol (PCP), a disinfectant commonly used in wood treatment and textile industry [26]. These emerging pollutants could affect organisms at very low concentrations; due to this reason several toxicity assays at different treatment times of photo-Fenton process could help to elucidate if the process is able to provide water enough level of quality be discharged into the environment.

## 2. Material and methods

### 2.1. Materials

High purity TBT, DCF, CVF and PCP, employed as target pollutants, were purchased from Sigma-Aldrich. Sulfuric acid (96%), Iron (III) chloride hexahydrate, hydrogen peroxide (33% w/v) and acetonitrile (HPLC grade) were supplied by Panreac. Water employed in all the experiments was Milli-Q grade. The OMW-HLS employed in this study were isolated from the olive mill waste taken from Millena, located in Valencian Community (Spain).

Organisms employed in the bioassays were obtained from different sources: *A. fischerii* was purchased from Fisher Scientific (Madrid, Spain), *P. subcapitata* and *D. magna* ephippia were obtained from ECOtest S.L. (Valencia, Spain), and. Cell lines were kindly provided by Dr. Adelaida García Gimeno, from Institute of Biomedicine (CSIC; Valencia, Spain).

### 2.2. Isolation and characterization of OMW-HLS

Fresh OMW, not subjected to any aging process, was submitted to a basic digestion. 0.125 kg (dried weight) of waste was digested in 0.5 L of KOH 0.3 M during 24 h. Then, the suspension was filtered using 100 μm filter to eliminate all the insoluble fraction still present (e.g. big fragments of olive bones). Then, an ultrafiltration process was carried out using, consecutively, three ceramic membrane with different pore sizes.: 300 kDa, 150 kDa and 50 kDa. Membranes were supplied by TAMI Industries and were operated in a tangential flux of 4 L/$m^2$ h at a 2 bar pressure and 25 °C. The retentate of each membrane was collected and dried to eliminate all the humidity in an air oven.

Spectroscopic characterization of these substances was performed with UV-Vis spectrophotometer UH5300 model from Hitachi. Fluorescence emission-excitation matrices were measured with a modular fluorimeter QuantaMaster (PTI). Hitachi Chromaster chromatograph (VRW) equipped with a Shodex OHpak SB-805 HQ methacrylate column was used to estimate the distribution of molecular weights of the humic substances. The eluent consisted in an isocratic mixture of acetonitrile (30%) and a phosphate buffer at pH 7.2 (70%). The flow was 0.8 mL $min^{-1}$ and the detection wavelength was 260 nm. Alternatively, dynamic light scattering (DLS) was used to determine the size distribution. A ZETASIZER Nano series (Nano-ZS) apparatus from Malvern was employed and suspensions containing from 3 g $L^{-1}$ to 20 mg $L^{-1}$ were measured.

### 2.3. Photochemical reactions

Photochemical experiments were carried out in a cylindrical Pyrex vessel (55 mm internal diameter). The reactor was loaded with 250 mL of the target solution containing 1 mg $L^{-1}$ of each pollutant, 20 mg $L^{-1}$ of OMW-HLS, iron (5 mg $L^{-1}$) and hydrogen peroxide (stoichiometric amount, 10 mM). These conditions were chosen as a result of optimization experiments based on experiment design methodology performed with a similar HLS [16]. The pH was adjusted to 5 in all cases by adding diluted sulphuric acid or sodium hydroxide when was required. Solar simulator (Oriel Instrument 81160) equipped with 300 W xenon short arc lamp was used as irradiation source for the experiments at laboratory scale.

Selected experiments were scaled up using a pilot plant Solardetox Acadus-2001 (Ecosystem), based on CPC technology, which has been described in detail elsewhere [31]. The reactor was loaded with 5 L of aqueous solution containing the emerging pollutants. The surface and the volume irradiated was 0.26 $m^2$ and 1.83 L respectively, and the photoreactor was tilted 30° with the horizon. A radiometer ACADUS 85 was used to monitor the irradiance and the accumulated UVA energy. To normalize changing solar conditions, $t_{30w}$ was calculated according to Eq. 1, where $UV_{ac}$ was the accumulated UVA irradiation, $V_i$ and $V_t$ were the irradiate and total volume used respectively, and I the average light intensity (30 W $m^2$ in Southern Spain).

$$t_{30w} = \frac{UV_{ac} * V_i}{\mathrm{I} * V_t} \quad (1)$$

Samples were periodically taken during photo-Fenton process, in presence and absence of OMW-HLS, in the solar photoreactor at times: 0, 30, 60 and 180 min to be submitted to toxicity bioassays and chemical analyses.

### 2.4. Chemical analysis

Pollutants concentration was determined by high performance liquid chromatography (LaChrom from Merck-Hitachi) equipped with diode array detector. A reverse phase column LiChrospher® 100RP-18 (5 μm) was used, and the eluent was a mixture of Acetonitrile (A) and 0.25% sulphuric acid aqueous solution (B). The eluents percentage was changed in a linear gradient from 50% A to 90% A in 14 min with a flow rate of 1 mL $min^{-1}$. For detection, the wavelengths employed were 220 nm (PCP), 230 nm (TBT), 240 (CVF) and 280 (DCF). Before analysis, 0.3 mL of methanol were added to 1.6 mL of sample. This is a common procedure when sampling in (photo)-Fenton experiments, as the presence of iron and hydrogen peroxide in samples might keep the reaction after sampling, thus modifying pollutants concentrations. In order to avoid it, an excess of organic solvent (e.g. methanol) is added in order to quench the reactive species and to stop the reaction. Finally, samples were filtered through Chromafil® Xtra PTFE-45/25 before injection.

Iron concentration species in water was measured according to the o-phenanthroline standardized spectrometric method (ISO 6332). Hydrogen peroxide consumption was checked according to metavanadate method [20].

### 2.5. Toxicity bioassays

A set of toxicity assays were carried out: bioluminescence inhibition assay with *A. fischeri*, growth inhibition assay with Chlorophyceae algae *P. subcapitata*, inhibition of mobility of Cladocera *D. magna*, and cytotoxicity assay with mammalian cell lines.

#### 2.5.1. Bioluminescence inhibition assay

The assay is based on the measurement of the bioluminescence inhibition of marine bacterium *A. fischeri* (strain NRRL B-11177) exposed to serial dilutions of samples (UNE-EN ISO 11348–3:2007 adapted) [35]. Conditioned samples were placed in a white flat bottom microplate using the injector module of the Tecan Infinite M200 microplate reader. In parallel, the lyophilized bacteria just reconstituted were dispensed manually in another microplate and after 15 min, bioluminescence was measured. Then, the content of the first plate was transferred to the second plate and after 30 min of exposure, bioluminescence was monitored again. The assay was carried out at 15 °C. Data obtained were treated according to control values.

#### 2.5.2. Growth inhibition assay

A miniaturized growth inhibition test with *P. subcapitata* was carried out according to the standardized method (UNE-EN ISO 8692:2004, adapted) [37]. The algae were exposed to serial dilutions of samples during 72 h. Assays were carried out in 96-well transparent flat bottom microplates. Briefly, the microplates were filled with samples and controls using the injector module (Tecan Infinite M200). A three-day algal culture was used as inoculum at an initial concentration of $10^4$ cells $mL^{-1}$. The plates were incubated at 22 ± 1 °C under continuous illumination and 2% $CO_2$ atmosphere. Growth of cultures was measured every 24 h by the fluorescence of the in vivo content of chlorophyll, at 430 nm and 663 nm as wavelengths of excitation and emission, respectively.

#### 2.5.3. Acute immobilization test

A bioassay based on the inhibition of the mobility of *D. magna* (UNE-EN ISO 6341:2012) [36], using 24-h old organisms hatched from the ephippia. Five neonates were placed in appropriate vessels with 10 mL of different sample dilutions. The assay was conducted in a climate chamber in the dark at 21 ± 1 °C. After 24 h and 48 h period, mobile individuals were counted in each vessel. Sample dilutions and controls were prepared with the standard freshwater according guideline.

#### 2.5.4. Cytotoxicity

Cytotoxicity was assessed as cell viability according to standardized procedure (UNE-EN ISO 10993–5:2009 adapted) [34]. The method is based in the reduction of resazurin, a redox indicator, by metabolic active cells, giving resorufin, a highly fluorescent compound. Two mammalian cell lines were used: N2a (Mouse Neuroblastoma) and HEK (Human Embryonic Kidney). Both cell lines are well characterized test systems given their widespread use in cell biology and biotechnology research, and they have been frequently used to evaluate the cytotoxic effects of chemicals for its relevance to the toxicity models in human [21]. Also, their ease of growth and laboratory maintenance is well known. N2a cell line has been regularly used in neurotoxicity and pesticide research for mechanistic and screening studies [28]. On the other hand, HEK293 is widely used as in vitro model for cytotoxicity assays to probe oxidative stress effects [29].

The cells were maintained in tissue culture plates using Dulbecco's Modified Eagle Medium (DMEM) supplemented with 1% L-glutamine, 2% penicillin-streptomycin and 10% foetal bovine serum. Cell incubation was performed at 37 °C, and 5% $CO_2$ in a humidified atmosphere. Cellular viability and density were assessed by trypan blue with a haemocytometer. The assay was carried out in a 96-well tissue culture plate; each well was filled with 100 μL of a cellular suspension ($1 \times 10^5$ cells/mL) in DMEM supplemented. Next day, cells were treated with serial sample dilutions and incubated during 24 h; then, the medium was removed and 100 μL of 15 μM resazurin solution was added. After 4 h incubation, the fluorescence (excitation 560 nm and emission 590 nm) was read on the multifunctional plate reader. Negative controls and blanks were run simultaneously.

#### 2.5.5. Statistical analysis

Toxicity data were used to calculate the MID value (sample dilution required to produce a 50% response) by Probit analysis, using the Statistical Analysis System SPSS (v. 16.0). Toxicity data were analyzed with one-way analysis of variance (ANOVA) to inspect the effect of photo-Fenton treatment time over assay organisms and to calculate the LID values (Lowest Ineffective Dilution). A $p < 0.05$ was taken to indicate statistical significance. When assessing the toxicity of each pollutant, $EC_{50}$ (half maximal effective concentration) was used instead of LID and MID.

## 3. Results and discussion

### 3.1. Isolation and characterization of OMW-HLS

The OMW-HLS were isolated according to the procedure reported in the experimental section. Three different batches were obtained by using, in the ultrafiltration process, membranes with different cut-off: > 300 kDa (A), 300–150 kDa (B) and 150–50 kDa (C) All three batches were submitted to further purification by dialysis, using cellulose membranes with 12 000 Da pore size, in order to remove the salts that are incorporated in the isolation process, and three new samples were obtained: D-A, D-B and D-C.

All six samples were analysed by means of size exclusion chromatography, in order to estimate their molecular weight distribution. Fig. 1 shows the chromatograms that were obtained in each case, together with that of commercial Sigma-Aldrich humic substances (SAHS). Very similar weight distributions were obtained in all cases, with only very slight variations (e.g. a tail with higher molecular weights in sample A). All the substances exhibit maximum weights around 6000 Da, calculated according to the calibration performed with PSS, including the SAHS substances. All the values agreed with those obtained for aquatic humic substances, according to literature [24].

Alternatively, dynamic light scattering (DLS) was used to determine the hydrodynamic radius of the substances, which accounts not for the size of the macromolecules, but for that of their aggregates. Values in Fig. 2 show that although there is a significant dispersion in the size, they agree with the values of ca. 400 nm previously measured for this type of materials (OMW-HLS), and that can be found in literature [14]; this is especially true in for values obtained from dialyzed samples, where the effect of sample matrix that might affect aggregation (e.g. presence of salts) is minimized. Sizes of OMW.HLS aggregates are above those reported for HLS isolated from urban wastes, which was 135 nm [3]. Hence, radii seem to depend on the sourcing materials that are employed.

In order to gain further insight into the chemical composition of the macromolecules, excitation emission matrixes (EEMs) were employed, as they are good tools for the analysis of complex organic samples [18], as these fluorescence techniques exhibits high sensibility and sensitivity, and they are not highly time or money consuming [5,23].

Fig. 3 shows EEMs of fractions A, B, and C, the mixture of all three OMW-HLS and SAHS. Interestingly, matrices obtained for all OMW-HLS samples were very similar, while significant differences were observed for the SAHS. However, in all cases a strong signal can be found in the region 250–400 nm (excitation wavelength) and 380–550 nm (emission wavelength) that falls within the region corresponding to the humic acids [13]. Interestingly, SAHS did not show fluorescence in the region 250–320 nm (excitation) and 300–400 nm (emission) in contrast with OMW-HLS, that showed an intense signal in this region, which is commonly assigned to protein/amino acid components [40]. This is a logical result, as being olive mill wastes the sourcing material for OMW-HLS, important amount of substances of protein origin released from the tissues of olives are expectable in OMW-HLS.

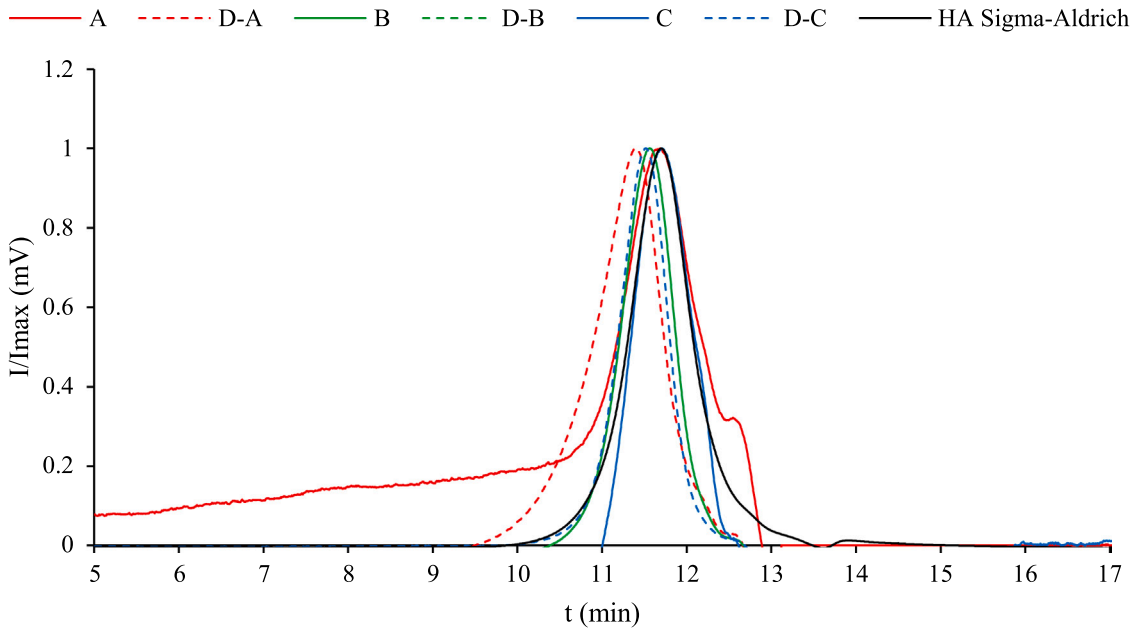


**Fig. 1.** Size exclusion chromatograms obtained for the different fractions of OMW-HLS and for SAHS.

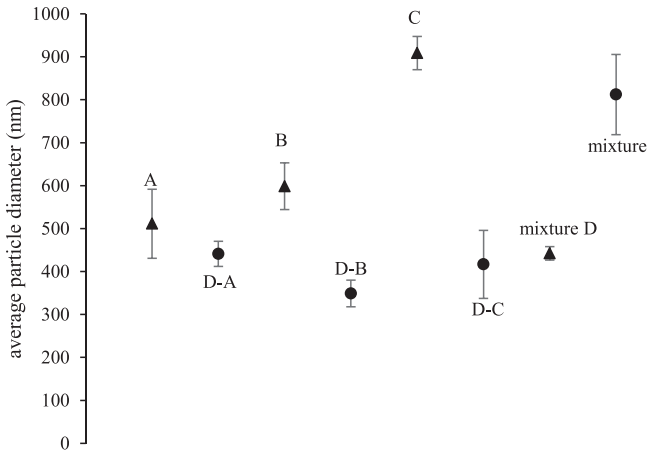


**Fig. 2.** Average particle diameter obtained by DLS measurements for the different OMW-HLS that have been isolated:.

### 3.2. OMW-HLS as auxiliary for photo-Fenton process

All the fractions obtained in the isolation of OMW-HLS, both dialyzed and non-dialyzed, were used as auxiliaries to drive photo-Fenton process at circumneutral pH. In all cases, the percentage of degradation of the pollutants was very similar (**see** Fig. S1). These results, together with their analyses reported in Section 3.1, seem to indicate that the OMW-HLS fractions are indeed, very similar. The membranes with different cut-off are not able to reach a good differentiation of HLS according to their molecular weight, most probably because they are in form of aggregates, whose size might depend on many factors that are difficult to control, rather than on the molecular weight of the individual macromolecules. For this reason, throughout the rest of the paper, a mixture of non-dialyzed OMW-HLS will be employed.

Results obtained with OMW-HLS were compared with those reached in the absence of humic-(like) substances and with SAHS (see Fig. 4). The remaining amount of pollutants ($\Sigma EC/(\Sigma EC_0)$ was plotted versus time. Comparison between SAHS and OMW-HLS cannot be done by adding the same amount of product (e.g. 20 mg $L^{-1}$) as they have actually different amount of inorganic species (e.g. salts). Hence two pairs of experiments were done: a) 20 mg $L^{-1}$ of SAHS and 53.1 mg $L^{-1}$ of OMW-HLS; and b) 20 mg $L^{-1}$ of OMW-HLS and 7.6 mg $L^{-1}$ of SAHS; each pairs of experiments correspond to equivalent amounts of carbon sourced from OMW-HLS or SAHS. It can be observed that in all cases, the performance of photo-Fenton with OMW-HLS or SAHS was well above that of photo-Fenton without complexing agent, conforming the ability of this substances to preserve the catalytic role of iron. In contrast, similar behaviours were obtained in all reactions containing the humic substances, independently of its origin and amount in the studied range.

To complete the information on how these OMW-HLS were able to complex iron during photo-Fenton process at circumneutral pH, experiments were performed in tap water and real wastewater treatment plant effluents (WWTP) with and without OMW-HLS (Fig. 5). The efficiency was always lower than when distilled water was used, but interesting, differences can be found when both matrices are compared. In the case of tap water, OMW-HLS were able to enhance the degradation achieved after 120 min (40% with OMW-HLS vs. 25% without this substances); this is probably due to ability of the HLS to complex iron, that partly overcome the interference caused by some anions (e.g. chlorides) that decrease photo-Fenton efficiency by forming less active complexes (e.g. $FeCl^{2+}$): the higher ability of HLS to complex iron, together with the enhanced photoreactivity of Fe-HLS when compared with $FeCl^{2+}$might explain this difference [38]. In contrast, similar removal (40%) is observed with and without HLS in WWTP effluent. In this case, dissolved organic matter that can complex iron, at least partly, is already present in the matrix; thus, HLS does not represent significant changes in iron availability, and their potential beneficial effect is compensated by the competitive role for the oxidizing agent, namely hydrogen peroxide.

Iron was determined using the o-phenanthroline method (ISO 6332) for all the experiments at 30, 60 and 120 min to determine ability of the studied substances to prevent precipitation of iron oxides or hydroxides, which constitutes the major pathway for iron deactivation (Fig. 6).

In all the cases where HLS were used, more iron remained at the end of the process than in the absence of these substances, what demonstrates that HLS were able to complex iron maintaining it in solution during photo-Fenton process. Interestingly, same concentration of organic macromolecules resulted in equivalent amounts of dissolved iron, independently on the type substance (OMW-HLS or SAHS). When WWTP was used as matrix, higher amounts of iron remain in solutions after the treatment, what agrees with the ability of the organics present

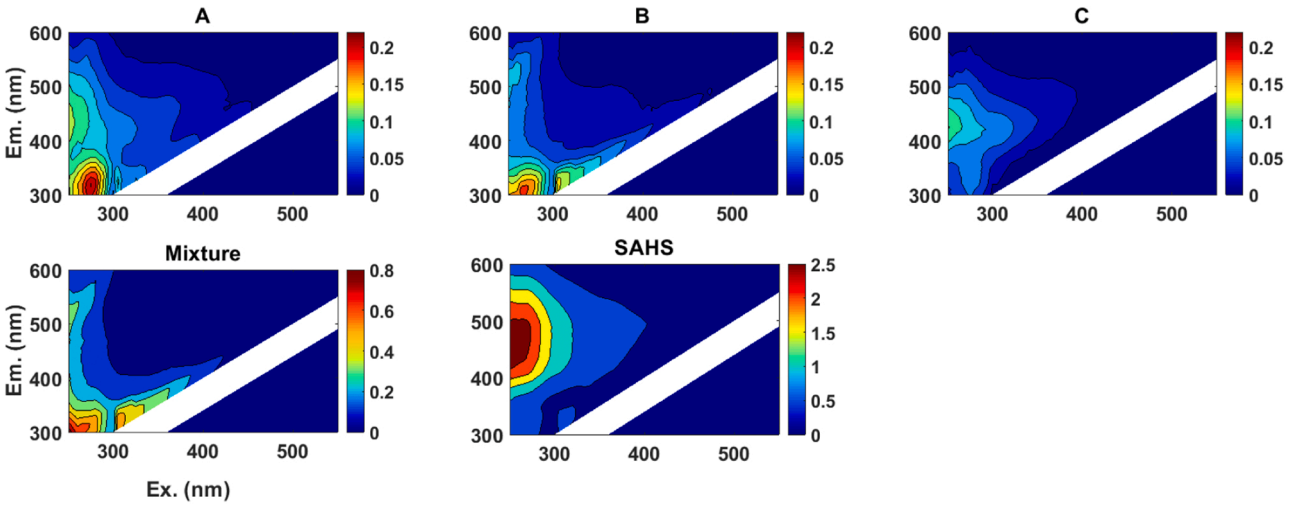


**Fig. 3.** Excitation Emission matrices for the three fractions non-dialyzed fractions: A (above, left), B (above, center), C (above, right), the mixture of them (below, left) and SAHS (below, centre). X- axis correspond to the excitation wavelength (250–500 nm), whereas Y-axis shows the emission wavelength (300–600 nm).

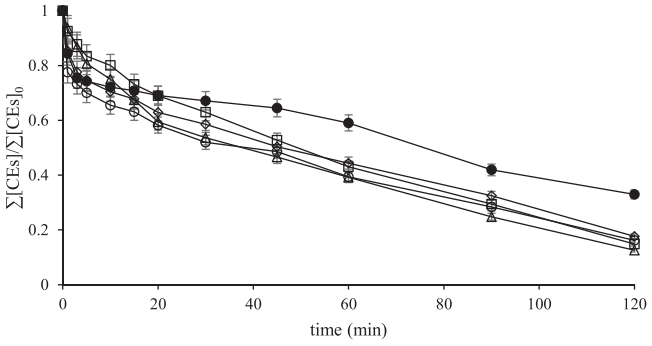


**Fig. 4.** Plot of ΣCEC/$\Sigma CEC_o$ vs time during a photo-Fenton process the solar photoreactor with: [EC] = 1 mg $L^{-1}$ each, [Fe(III)] = 5 mg $L^{-1}$, [$H_2O_2$] 10 mM. Absence of HLS (•), [OMW-HLS] = 20 mg $L^{-1}$ (□), [SAHS] = 20 mg $L^{-1}{}_0$ (Δ), [OMW-HLS] = 53.08 mg $L^{-1}$ (○), [SAHS] = 7.54 mg $L^{-1}$ (◇).

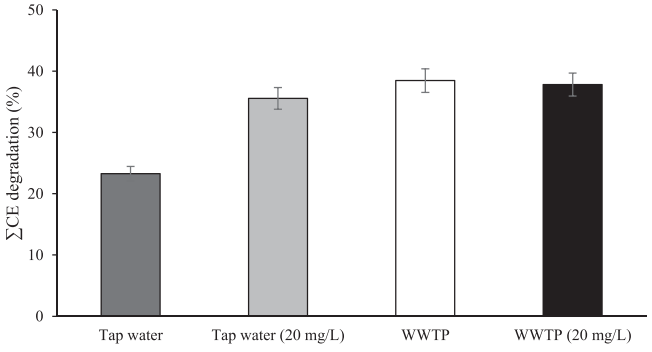


**Fig. 5.** Removal of the ΣCEC after 120 min of photo-Fenton process in solar simulator ([ECs] = 1 mg $L^{-1}$ each, [Fe(III)] = 5 mg $L^{-1}$, [$H_2O_2$] = 10 mM.) Tap water without OMW-HLS (dark grey); Tap water with OMW-HLS (light grey); WWTP effluent without OMW-HLS (white); WWTP effluent with OMW-HLS (black).

in WWTP to complex iron, as hypothesised above.

### 3.3. Pilot plant experiments and toxicity bioassays

#### 3.3.1. Pilot plant degradation

Experiments were scaled up to solar photoreactor pilot plant at pH = 5 with and without OMW-HLS (mixture of fractions). Results shown in Fig. 7 indicates that the degradation of all four pollutants was enhanced in the presence of HLS, although the relative order DCF > PCP ≥ TBT > CVF is kept. As a matter of fact, when $t_{30w}$ was ca. 90 min, only CVF remained in the solution with 20% of its initial concentration, in sharp contrast with the experiment without HLS, in which after the same irradiation, more than 50% of the initial CVF and 20% of TBT was detected.

This improved performance can be attributed to stabilization of iron as species able to drive photo-Fenton-like processes. This is confirmed by the amount of dissolved iron profiles, as non-soluble iron oxides or hydroxides are mainly formed in the absence of HLS, but also by hydrogen peroxide consumption, as this reagent is more rapidly consumed in the HLS process, indicating an improved photocatalytic role of iron. As a matter of fact, a nearly complete exhaustion of this reagent occurs at $t_{30\,W}$ = 30 min, that also proves the competitive effect of HLS for the hydrogen peroxide vs. the pollutants. Finally, it is interesting to remark that even when hydrogen peroxide was already consumed, some reaction was kept in the experiment carried in the presence of HLS; this could be attributed to the ability of photoactive HLS to catalyse photodegradation of pollutants, in a process that is less efficient that photo-Fenton.

#### 3.3.2. Toxicity bioassays

Toxicity assays were first carried out for each pollutant, involving different microorganisms: a) cytotoxicity according to mammalian cells, b) bacterial toxicity on *A. fischeri*, c) algae toxicity vs. *P. subcapitata*, d) Cladocera toxicity using *D. magna* (see Fig. 8).

Cellular viability was endangered by all pollutants, being the HEK cell line more sensitive to CVF and N2a to TBT. Interestingly, in HEK cell lines, DCF shows a very high $EC_{50}$ (551 mg $L^{-1}$), which represents a low toxicity, expectable for a compound that is used as pharmaceutical for humans. Regarding to bacteria *A. fischeri*, which is an excellent tool to evaluate toxicity related with cellular metabolism [1], PCP was the most toxic compound, although a loss of bioluminescence has been revealed for all compounds. The alga *P. subcapitata* has shown a high sensitivity to PCP followed by TBT, while the effect is lower for the other two compounds. Different authors [6,11,30] have highlight the high sensibility to xenobiotics exposure for this organism, that might have adverse effects on the food chain. TBT is an herbicide, what makes sense the high toxicity revealed in case of algae organism. In the case of the Cladocera *D. magna*, CVF was the most toxic pollutant causing the highest effect by far compared to the other three compounds, which was expected as CVF is an organophosphorus pesticide, which inhibits of the enzyme

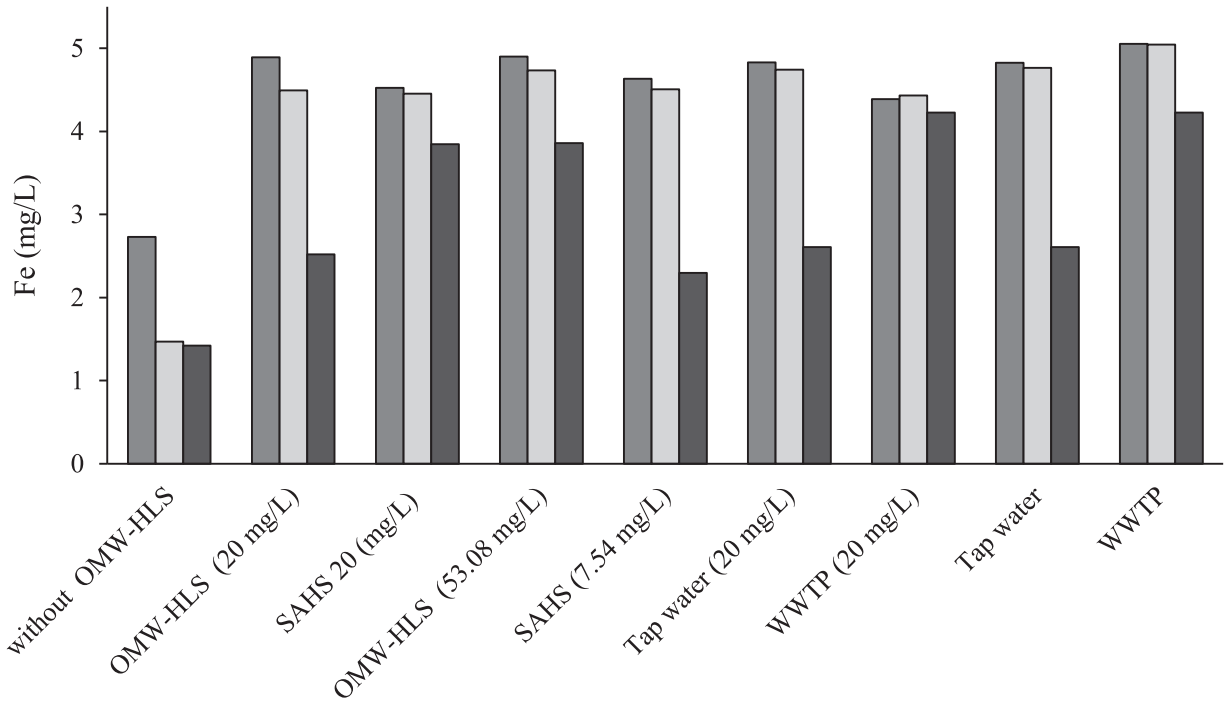


**Fig. 6.** Remaining amount of iron in solution during a photo-Fenton process in simulated sunlight at pH = 5 after different times of treatment: 30 min (dark grey), 60 min (light grey), and 120 min (black).

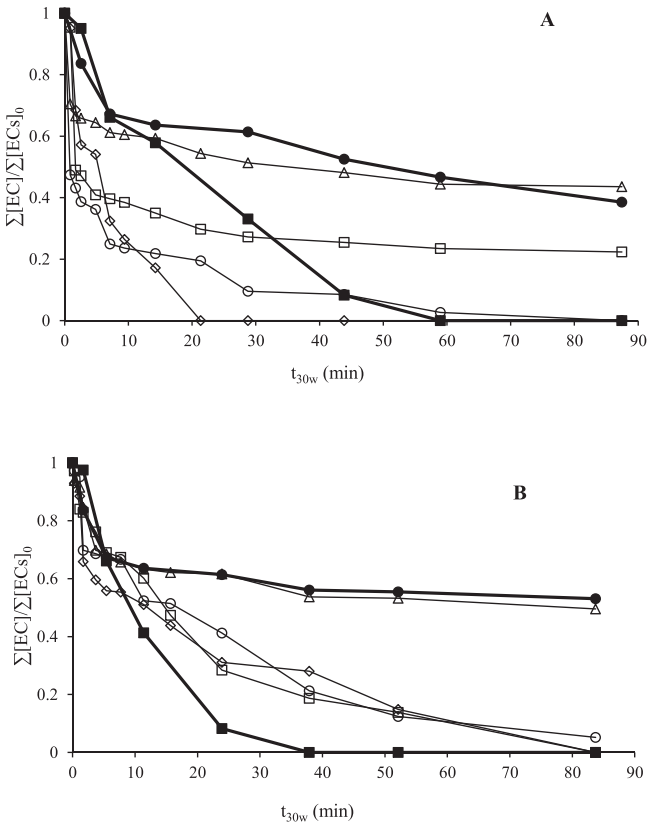


**Fig. 7.** Kinetics for photo-Fenton process at pH = 5 in pilot plant without OMW-HLS addition (**A**) and with OMW-HLS (20 mg $L^{-1}$) (**B**). ([EC] = 1 mg $L^{-1}$ each, [Fe(III)] = 5 mg $L^{-1}$, [$H_2O_2$] 10 mM.) Plots of the relative concentrations of pollutants vs time: TBT (□), DCF (◇), CVF (Δ) and PCP (○) Remaining iron concentration (●) and $H_2O_2$ (■) are also given.

acetylcholinesterase [39]; among all the organisms studied, only daphnia has this enzyme.

Changes in the toxicity of the sample containing all four pollutants when submitted to photo-Fenton with and without OMW-HLS was determined according to all bioassays. Table 1 shows the result of each bioassay at different treatment times of photo-Fenton process, expressed as LID and MID. When comparing the toxicity of the initial samples, it can be observed that they were higher in the samples containing OMW-HLS. This effect might be due to the surfactant role of these substances, that allows a better contact between the microorganism and the pollutants [4].

Regarding the treatment process, a remarkable detoxification can be observed in both cases but, also now samples containing OMW-HLS were slightly more toxic, despite the higher removal of pollutants that was reached. At the end of the process carried out without the humic substances, only toxicity was detected for *D. magna* assay, which is in line with the low degradation of CVF, which was the compound showing the highest effect towards this organism. For the OMW-HLS driven process, in addition to *D. magna*, some toxicity was still detected for all bioassays except for cell lines. This apparent contradiction can be attributed to two possible factors: a) primary removal of the parent pollutant does not ensure complete detoxification, as toxic by-products can be released; this is more true in the case of OMW-HLS, as this substance competes for the $H_2O_2$ for the pollutants and a lack of this reagent might result in by-products accumulation, b) the surfactant role of HLS might enhance the toxicity of the remaining pollutants, in line with observation with the untreated sample.

## 4. Conclusions

OMW-HLS have demonstrated its ability to drive photo-Fenton process at mild pH, under simulated and real solar irradiation. No noticeable differences have been observed among samples isolated with using membranes with different pore sizes, neither with commercial humic substances. Dissolved iron, hydrogen peroxide consumption and the role of other species present in the sample (organic matter, inorganic salts) are compatible with the complex formed between iron and OMW-HLS as

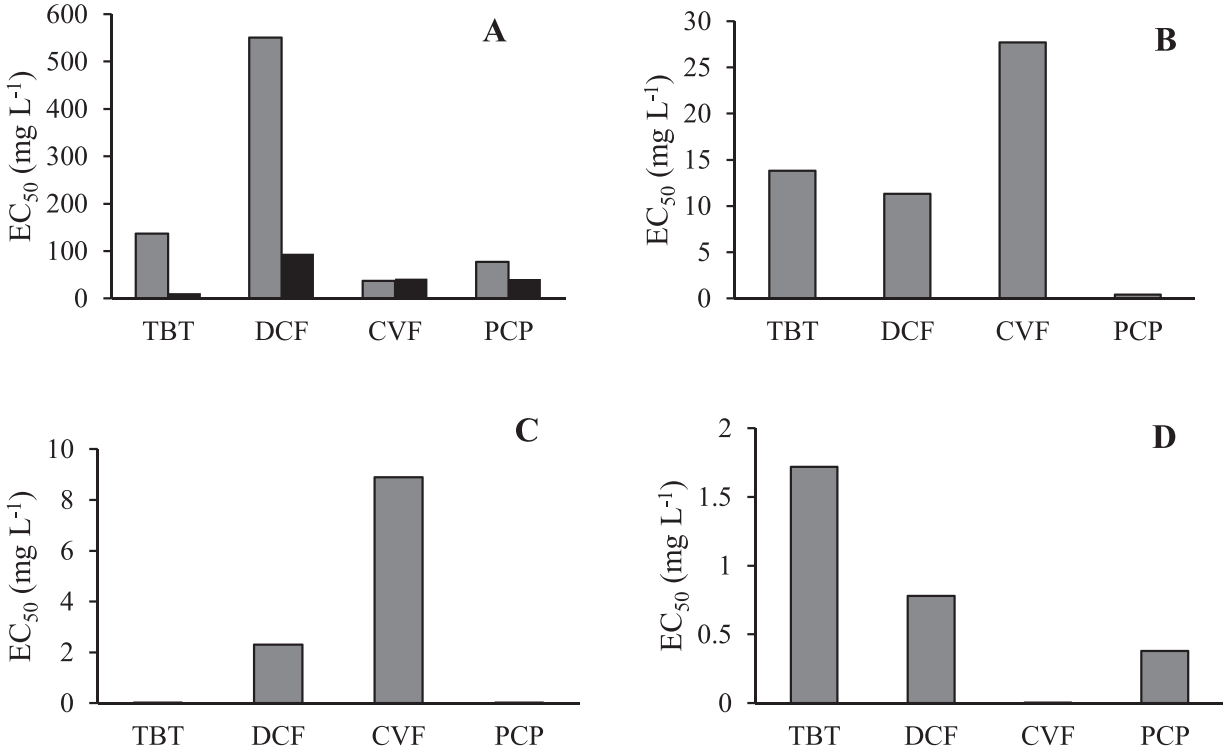


**Fig. 8.** $EC_{50}$ (mg $L^{-1}$) values calculated for each pollutant according to different bioassays. **A**: cytotoxicity. HEK (grey) and N2a (black); **B**: *A. fischeri*; **C**: *P. subcapitata*; **D**: *D. magna* (48 h).

**Table 1**
LID and MID values at different treatment times (0, 30, 60 and 180 min) for all the organisms tested using solar photo-reactor. [Fe(III)] = 5 mg $L^{-1}$, [$H_2O_2$] = 10 mM, [OMW-HLS] = 20 mg $L^{-1}$. Initial concentration of TBT, DCF, CVF and PCP 1 mg $L^{-1}$ each.

| | | Samples treated in absence of OMW-HLS | | | | Samples treated in presence of OMW-HLS | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 0 | 30 | 60 | 180 | 0 | 30 | 60 | 180 |
| HEK | **LID** | < 1:1 | < 1:1 | < 1:1 | < 1:1 | 1:3 | 1:3 | < 1:1 | < 1:1 |
| | **MID** | < 1:1 | < 1:1 | < 1:1 | < 1:1 | < 1:1 | < 1:1 | < 1:1 | < 1:1 |
| N2a | **LID** | < 1:1 | < 1:1 | < 1:1 | < 1:1 | 1:6 | 1:6 | 1:2 | < 1:1 |
| | **MID** | < 1:1 | < 1:1 | < 1:1 | < 1:1 | < 1:1 | < 1:1 | < 1:1 | < 1:1 |
| *A. fischeri* | **LID** | 1:2 | < 1:1 | < 1:1 | < 1:1 | 1:5 | 1:8 | 1:3 | 1:3 |
| | **MID** | 1:2 | < 1:1 | < 1:1 | < 1:1 | 1:1.8 | 1:0.8 | < 1:1 | < 1:1 |
| *P. subcapitata* | **LID** | 1:32 | 1:8 | < 1:1 | < 1:1 | 1:360 | 1:240 | 1:240 | 1:180 |
| | **MID** | 1:10 | 1:2 | < 1:1 | < 1:1 | 1:125 | 1:100 | 1:100 | 1:91 |
| *D. magna* (24 h) | **LID** | 1:512 | 1:128 | 1:128 | 1:54 | 1:512 | 1:256 | 1:128 | 1:128 |
| | **MID** | 1:38 | < 1:1 | < 1:1 | < 1:1 | 1:56 | 1:43 | 1:32 | 1:17 |
| *D. magna* (48 h) | **LID** | 1:512 | 1:256 | 1:128 | 1:128 | 1:512 | 1:256 | 1:256 | 1:128 |
| | **MID** | 1:90 | < 1:1 | < 1:1 | < 1:1 | 1:83 | 1:32 | 1:14 | 1:7 |

the key species to extend the applicability of photo-Fenton. In this line, further research is required to understand the nature of this complex and to investigate the reactive species that are involved.

On the other hand, a battery of bioassays has demonstrated that removal of parent pollutants does not guarantee detoxification of the solution. Most probably, the surfactant effect of the humic substances enhances the toxicity of the substances present in the solution; this fact, together with the competitive role of OMW-HLS for the reactive species, make it necessary addition of further amounts of hydrogen peroxide.

Studying other solid wastes as sources of HLS substances is meaningful and requires further research, as many organic substances are candidates for generating HLS. Also determining the effect on the HLS composition and behavior of the pretreatment of the solid wastes (e.g. aging, composting or fermentation) seems an interesting task to be carried out in the future.

Finally, in order to assess the real applicability of this procedure, further research is needed in order to optimize and scale up, both the isolation process and the photo-Fenton treatment. Life cycle assessment studied would be necessary to investigate if the OMW-HLS driven process results in an enhanced sustainability of photo-Fenton, and operation costs of should also be estimated in view of implementation in a real scenario.

## CRediT authorship contribution statement

**P. García-Negueroles**: Investigation, writing original; **S. García-Ballesteros**: investigation, data curation, conceptualization; **L. Santos-Juanes**: conceptualization, writing, Review and editing; **C. Sabater**: investigation, conceptualization; **M.A. Castillo**: investigation, conceptualization, **M.F. López-Pérez**: investigation, conceptualization, **R. Vicente**: data curation, conceptualization **A.M. Amat**: supervision, funding acquisition, **A. Arques**: supervision, review and editing.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jece.2021.106862.

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