Title: Microbiological evaluation of combined advanced chemical-biological oxidation technologies for the treatment of cork boiling wastewater

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ABSTRACT

This paper contains a multidisciplinary approach that will contribute to design and properly evaluate a treatment line for complex biorecalcitrant wastewaters. To demonstrate this approach a specific industrial wastewater (cork boiling wastewater, CBW) has been used. A treatment line based on a coagulation-flocculation step followed by an Advanced Oxidation Process (AOP) (solar photo-Fenton) and combined with an aerobic biological system has been evaluated. Applied microbiological techniques: optical microscopy, plate count, DNA extraction and qPCR, indicated that some communities disappeared after the activated sludge adaptation period to the partially treated wastewater, while resistant population was unable to withstand changes in the feeding stream: 2-log reduction of total heterotrophic bacteria (THB) and a decrease in DNA concentration from 200 ng/μL to 65 ng/μL were observed. Therefore, chemical and microbiological results obtained along the set of experiments, suggested the inefficiency of the combined treatment option between solar photo-Fenton and advanced aerobic biological systems for CBW, which lead to the necessity of applying chemical oxidation technologies until the complete mineralization of the pre-treated wastewater with the objective of improving the effluent quality enough for being reused in the own industry. Toxicity tests based on different organisms showed increase on acute toxicity (from 46% to 71% after CBW treatment by means of respirometric assays) and the development of chronic toxicity (from 21-29% to 83-90% at the end of the treatment also measured by respirometric assays) along the treatment line, made evident the incompatibility of this type of wastewater with a biological treatment even after the application of an AOP.

Keywords: Biological treatment, Cork boiling wastewater, qPCR, solar photo-Fenton, Toxicity

1. INTRODUCTION

Cork is a natural renewable material extracted from the outer bark of the cork oak tree (*Quercussuber* L.) that traditionally has been used in the production of stoppers for wine industry. The initial processing operation in cork industries is a boiling process. This procedure improves the elasticity and homogeneity of the material, generating high volumes of wastewater (140-1200 L/ton of cork).The boiled effluent, named Cork Boiling Wastewater (CBW) is characterized by a dark color, acid pH, low biodegradability and high acute toxicity (Mendonça et al., 2007). Considering CBW recalcitrant nature, conventional biological treatments are not efficient as some microorganisms are particularly sensible to the organics present, especially to high concentration of polyphenolic compounds (Benitez et al., 2003). Therefore, it is not possible an adaptation of a conventional biological system to CBW (Ponce-Robles et al., 2018), demonstrating the necessity to search and propose alternative solutions to their remediation (Dias-Machado et al., 2006) mainly based on the application of chemical oxidative technologies as pretreatment for attaining CBW biocompatibility improvement.

The requirement of more acute information for the design of new treatment concepts to tackle with the remediation and possible reuse of industrial wastewater, makes highly interesting and necessary the evaluation of the impact of target wastewater (even partially oxidized) to potentially adapt biological systems (always highly efficient) from the economic point of view.

Advanced oxidation processes (AOPs) have been widely demonstrated to be efficient when combined with other advanced technologies after defining specific treatment strategies (Malato et al., 2016). In this sense, high efficiency in removal of large loads

of organic contaminants has been shown in those specific cases in which combination of AOPs and biological treatment succeed. However, the choice of a suitable treatment option for complex wastewaters treatment depends on its effectiveness, so, it is necessary to select adecuate parameters that allow to control and monitor the selected processes (Oller et al., 2011). Physicochemical parameters including dissolved oxygen, pH, DOC, COD, TSS, or VSS are generally considered as fundamental criteria to define the potential use of a treated effluent, however, this information results insufficient. A good complementary option is the application of bioassays in order to evaluate toxic effects of complex mixtures commonly found in industrial wastewater dischargers. The importance of the use of this strategy has been well documented in literature (Isidori et al., 2004; Hemming et al., 2002; Middaugh et al., 1997). More specifically and in the case of aerobic biological treatments based on activated sludge, the evaluation of micro-fauna community evolution along the purificacion processes is crucial to correctly interpret the results but also to modify the operation parameters when neccesary. The species present in the system are responsible for the stability and efficiency of the whole treatment (Cydzik-Kwiatkowska and Zielińska, 2016). These studies are based in traditional culture methods and optical microscopy, identifying predominant microbial populations present in Wastewater Treatment Plants (WWTPs). In addition, Polymerase Chain Reaction (qPCR) has been demostrated to be a useful tool for quantititative analysis of specific microorganisms present in environmental samples (Fortunato et al., 2018).

The main goal of this work was to assess the efficiency of a specific treatment line based on an aerobic biological treatment for the remediation of a complex industrial wastewater. With this aim, a previous step based on an optimized physic-chemical pre-treatment (coagulation-flocculation) combined with an AOP (solar photo-Fenton

process) were evaluated. Specific microbiological techniques (optical microscopy, plate count, DNA extraction and qPCR) were used to monitor and evaluate the microbial adaptation of activated sludge to the partially treated CBW.

2. MATERIALS AND METHODS

2.1. Industrial wastewater and reagents

Cork boiling wastewater used in this study was received from a cork processing plant located in San Vicente de Alcántara (Extremadura, Spain). Conventional activated sludge was provided by the municipal wastewater treatment plant (MWWTP) of El Toyo (Almería). The chemical characterization of both waters is shown in table 1.

Ferric chloride anhydrous ($FeCl_3$) was supplied by Panreac for coagulation-flocculation pre-treatment step. Reagent-grade hydrogen peroxide (30% w/v), sulphuric acid and sodium hydroxide (for physic-chemical pre-treatment and pH adjustment) were provided by Panreac and Merck, respectively. Sodium acetate was supplied by Merck.

2.2. Analytical determinations

Organic matter was measured as chemical oxygen demand (COD) using Merck®Spectroquant kits and dissolved organic carbon (DOC) was measured in a Shimadzu TC-TOC-TN analyzer, model TOC-V-CSN. Total dissolved nitrogen was measured in the same TC-TOC-TN analyzer coupled to a TNM-1 unit. Total suspended solids (TSS) and volatile suspended solids (VSS) were determined according to American Standard Methods. Anions were quantified by ion chromatography using Metrohm 872 Extension Modules 1 and 2 configured for gradient analysis. Cations were determined using a Metrohm 850 Professional IC configured for isocratic analysis.

Total iron concentration was determined using the 1,10-phenantroline method following ISO 6332, and hydrogen peroxide was measured using titanium (IV) oxysulfate according to DIN 38402H15. Total phenolic content was determined spectrophotometrically using the Folin-Ciocalteu reagent with Gallic acid as the reference standard for plotting calibration curve, both provided by Merck (Germany).

2.3. Coagulation-flocculation-filtration step

A coagulation-flocculation-filtration (C/F) step was performed in a pilot plant available at Plataforma Solar de Almeria (Spain) with a filtration in a PEVASA silex filter (75 µm) followed by two AMETEK cartridge micro-filters (25 and 5 µm, respectively). This plant is designed to treat 1m^3/h of water and it has two centrifugal pumps (EPSA) that can operate in manual or automatic mode. The coagulant used was $FeCl_3$, under the experimental conditions already optimized in a previous published work (Ponce-Robles et al., 2017): 3 min of rapid mixing at 100 r.p.m, 30 min of slow mixing at 30 r.p.m to promote flocculation and 30 min of settling solids separation. Such conditions gave high efficiency in turbidity and DOC removal: 86% and 29%, respectively.

2.4. Solar photo-Fenton process at pilot plant scale

Solar photo-Fenton experiments were carried out using a Compound Parabolic Collector (CPC) pilot plant operating in batch mode specially designed for solar photocatalytic applications (Gernjak et al., 2006). This pilot plant was operated at 25 L/min and has a total volume of 75 L (V_T) and a total illuminated volume of 44.6 L (V_i). CBW (after physic-chemical pre-treatment) was introduced into the CPC pilot plant and homogenized in darkness during 15 min. Then, pH was adjusted to 2.8-3.0. Addition of iron was not necessary because enough dissolved Fe (III) remained in

146 solution after the C/F step (between 46 and 80 mg/L). Finally, the CPC was uncovered
147 and photo-Fenton process started. Two different photo-Fenton experiments (p-F1 and p-
148 F2) were performed with the pre-treated CBW by adding two different initial dosages of
149 H_2O_2 (1g/L and 2.5 g/L), according to previously reported experience with CBW treated
150 by solar photo-Fenton (Ponce-Robles et al., 2017).

151

152 **2.5. Aerobic biological system**

153 A Sequencing Batch Bioreactor at laboratory scale was used for biological assays,
154 performed in a 5 L stirred flask reactor provided with a porous air diffuser placed at the
155 bottom of the reactor, keeping dissolved oxygen concentration close to saturation
156 values. The average temperature was 25ºC.

157 Activated sludge taken from the MWWTP was kept in aeration for 24 hours for
158 attaining endogenous phase. Partially treated CBW was studied after adjusting the pH to
159 7. In order to avoid an extremely high organic load of this complex wastewater in the
160 first stages of the adaptation phase, one liter effluent from each photo-Fenton
161 experiment (p-F1 and p-F2) were added in the bio-reactor (B1 and B2 experiments),
162 completing a total volume of 5 L with mixed liquor from the secondary treatment of a
163 MWWTP (see figure 1). Final diluted DOC from the mixture was 63 mg DOC/L and
164 23 mg DOC/L, respectively for B1 and B2 experiments (p-F1 and p-F2, respectively).

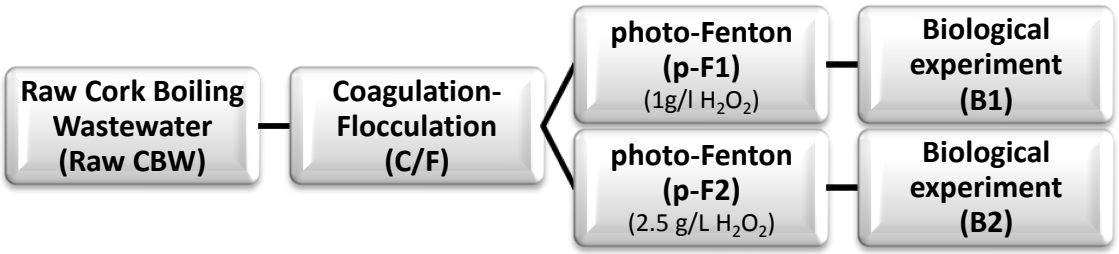

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Figure 1.Scheme of the proposed treatments.

Aerobic biological experiments lasted 17 days (408 hours). Five additions of pre-treated CBW were done along the experiment at different contact times (0, 3, 7, 10, and 14 days) to avoid inhibition effects provoked by a lack of organic feeding. Finally, and with the aim of assessing the viability of the adapted new microbial communities to the partially oxidized CBW, a last feeding with sodium acetate (highly biodegradable) was done after 16 days of experiment. Biological systems were tested by duplicate (B1 and B2). Total Suspended Solids (TSS), Volatile Suspended Solids (VSS), optical microscopy, plate counting, DNA extraction and Polymerase Chain Reaction (qPCR) were monitored throughout the experimental time.

2.6. Microbiological analyses

Microbial enumeration was done by Heterotrophic Plate Count (HPC) or standard plate count technique in Tryptone Glucose Yeast Agar (TGYE Agar) nutrient medium according to standard methods (Gtreenberg et al., 1992). TGYE Agar was prepared by using 5 g/L of Tryptone (Oxoid, UK), 1 g/L of Glucose (JT Baker, USA), 2.5 g/L of yeast extract and 15 g/L of bacteriological agar (Panreac, Spain). The pH of the medium was 7. The method was done through 10-fold serial dilutions of samples (from d1 to d6) in phosphate-buffered saline (PBS) solution. Then, 500µL of each dilution was dropped onto the TGYE Agar nutrient medium. Finally, colonies were counted after incubation for 7 days at 25ºC.

Microscopic observation of mixed liquor samples was done by a Nikon Eclipse 50i Microscope coupled with a Nikon DS camera. A drop of each sample was carefully deposited on a glass slide, and then, samples were covered with a cover slip.

DNA of samples was extracted using a Fast DNA™ SPIN Kit for Soil (MP Biomedicals, Solon, OH 44139 USA) according to manufacturer´s kit instructions with a FastPrep® FP24 Classic Instrument. For that, 1 mL of each sample was centrifuged at 12000 r.p.m. during 2 min. Later, manufacturer´s specifications were followed. DNA concentration was quantified using a NanoDrop spectrophotometer (NanoDrop Lite, Thermo scientific).

The quantification of bacterial 16S rDNA and ammonia oxidizing bacteria (AOB) 16S, extracted DNA samples were amplified using a 7500 Fast Real Time PCR System (Applied Biosystems, USA) instrument with 96-barcode well plates. Commercial primers and probes used were supplied by Sigma-Aldrich (USA). Due to complexity of samples, ten-fold dilution of DNA extracts was used to prevent PCR inhibition. Negative control was performed to validate DNA-amplification results. Bacterial 16S rDNA and AOB concentration was estimated using an external qPCR calibration curve previously reported (Polo-López et al, 2017),which correlates qPCR Cycle threshold (Ct) with bacterial concentration in terms of colony forming units (CFU/mL).

2.7. Biodegradability and toxicity assays

The biocompatibility of pre/treated CBW and the acute and chronic toxicity were evaluated employing different bioassays.

2.7.1 Biodegradabilty measurements

Short term biodegradability analysis were carried out in a BM-T respirometer (Surcis S.L.), which consists of a 1L capacity vessel, equipped with temperature and pH control systems. It also has a dissolved oxygen probe (Protos 3400, Knick ElektronischeMessgeräte GmbH & Co. KG) for measurement of the oxygen uptake rate (OUR). The respirometer was loaded with 700 mL of activated sludge in endogenous

phase. Temperature was controlled at 20ºC and the system was continuously aerated and agitated. Then, 300 mL of oxygen-saturated pre-treated CBW were added. The ratio COD/CODb (total chemical oxygen demand/easily biodegradable chemical oxygen demand) allows establishing the biodegradable character of the sample. A sample is considered to be biodegradable when COD/CODb is greater than 0.3; slightly biodegradable, when it is comprised between 0.1 and 0.3; and non-biodegradable when it is lower than 0.1 (according to instructions given by the respirometer´s manufacturer, SURCIS S.L.).

2.7.2 Toxicity assays

Accurate bioassays for checking toxicity must include representatives from different trophic levels. Acute and chronic toxicity evaluation of the samples was performed using conventional activated sludge and a battery of four commercial tests (BioFixLumi, Protoxkit, Daphtoxkit M and Artoxkit M). Commercial toxicity tests, Protoxkit F and Artoxkit M were purchased by Microbio Test Inc. (Belgium). BioFix® Lumitest was supply by GmbH & Co. KG (Düren, Germany). Each bioassay was repeated at least two times. For the ecotoxicological analysis with the commercial tests, a dilution series of 50, 25, 12.5 and 6.25 % v/v of each sample were carried out after pH adjustment to around 7. Samples from solar photo-Fenton treatment did not contained hydrogen peroxide as they were collected after complete consumption of this reagent.

Toxicity results were expressed as a percentage of toxic effect (inhibition, mortality or immobilization). In each bioassay the toxic effect was calculated by the following equation:

$$\%\, Toxic\ effect = \left[\frac{response\ of\ control - response\ of\ sample}{response\ of\ control}\right] * 100$$

235

236 2.7.2.1. Acute toxicity analysis

237 *Conventional activate sludge*: Analysis were carried out in the same BMT respirometer
238 as biodegradable assays. The respirometer was loaded with 1L of endogenous activated
239 sludge. Temperature was controlled at 20ºC and the system was continuously aerated
240 and agitated. Toxicity was evaluated by comparing the maximum bacterial oxygen
241 uptake rate ($OUR_{max\text{-}ref}$) in a control test made by the addition of sodium acetate (as
242 highly biodegradable compound) with the obtained when the same amount of the
243 partially treated CBW sample (by solar photo-Fenton process) was added ($OUR_{max\text{-}}$
244 $_{sample}$). The control was made by 30 mL of distilled water with 0.5 g of sodium acetate/g
245 VSS. Moreover, 30 mL of sample was added to obtain the $OUR_{max\text{-}sample}$. OUR values
246 were also measured along B1 and B2 experiments for assessing possible acute toxicity
247 effects.

248 *Biofix Lumitest (*30 min bioluminescence test): The reagent was a freeze dried
249 preparation of *Vibrio fischeri* (formerly known as *Photobacterumphosphoreum*, NRRL
250 number B-11177). The test was performed according to manufacturer (Biofix®Lumi-10)
251 specifications following UNE_EN_ISO-11348_1998. With this purpose, the sample
252 was incubated in contact with the bacteria for 30 minutes and the luminescence intensity
253 after the incubation time was measured with a luminometer (BioFix® Lumi-10,
254 MACHEREY-NAGEL®). The luminescence intensity of samples was compared with a
255 reference sample containing the bacteria in isotonic solution. This reference sample was
256 used as a negative control, while K_2CrO_7 (Panreac, Barcelona, Spain; purity 99 %,
257 18.7 mg/L) was used as positive control. Reduction in light emission by bacteria
258 referred a toxic effect which was finally expressed as an inhibition percentage.

Artoxkit M.: This test is based on the use of instar larvae of the brine shrimp *Artemia franciscana* hatched from cysts that were incubated in synthetic seawater under artificial light at 25ºC. Reconstituted water of normal seawater salinity (35%) was used to prepare the hatching medium for the cysts and the dilution medium for the toxicant dilution series. The standard seawater (35% salinity) was also used as negative control and $K_2Cr_2O_7$ (Panreac, Barcelona, Spain; purity 99 %, 100 mg/L) was used as positive control. After 24 hours of contact with samples, viable population was measured using a dissection microscope to calculate the % of mortality.

Daphtoxkit F.: The 48 h immobilization test with the crustacean *Daphnia magna* was carried out according to the operational procedure of the kit, in adherence to standard guidelines (OECD Guidelines for the Testing of Chemicals, Section 2, Test No. 202: Daphnia sp. Acute Immobilisation Test). Standard Freshwater was used as negative control and K_2CrO_7 (3.2 mg/L) was used as positive control. The percentage of immobilitazion was calculated recording the number of immobile organisms after 48 h of exposure.

2.7.2.2. Chronic toxicity analysis

Conventional activate sludge: Tests were performed in a BM-T respirometer (Surcis S.L.) by recording the slope of OUR (and so the consumption of dissolved oxygen) when the aeration is stopped and comparing such values obtained for the activated sludge coming from the MWWTP before adding the pre-treated CBW samples and after 1, 48, 144, 312 and 408 hours of contact in B1 and B2 experiments. With this aim, one liter of samples from biological treatments were added into the system and OUR was measured again along the experimental time. A decrease in the slope of OUR mean chronic toxicity.

Biofix Lumitest: Chronic toxicity test were evaluated at the same conditions of acute toxicity test described above but samples were incubated during 24 hours following an adaptation of protocols previously published by other authors (Westlund et al., 2018). The effective concentration which provoked the 50% of inhibition of *Vibrio fischeri* for a solution of Phenol was determined as reference after 30 minutes and after 24 hours of exposure, observing an increase from 35 mg/L to 91 mg/L, showing the absence of chronic toxicity for this solution.

Protoxkit F. test: The inhibition in the growth of the ciliated protozoan *Tetrahymena thermophila* was evaluated after 24 hours exposure according to the Protoxkit F (1998) standard operational procedure. In brief, the test is based on the optical density measurement of the food substrate provided to the ciliates at 440 nm. The growth inhibition is reflected by higher turbidity in the tested samples, in comparison to the negative controls (Standard Freshwater). K_2CrO_7 (56 mg/L) was used as positive control.

3. RESULTS AND DISCUSSION

3.1. Physic-chemical pre-treatment for wastewater remediation

Flocculation/coagulation pre-treatment based in the addition of $FeCl_3$ at 0.5 g/L after adjusting pH of CBW to 5 was selected according to previous studies reported by Ponce-Robles et al.(Ponce-Robles et al., 2017). A first stage based on physic-chemical pre-treatment is usually required in the remediation of wastewater with the main objective of reducing suspended solids and turbidity, color and DOC. Pre-treatment stages normally increase the efficiency of the subsequently applied chemical oxidation process for the wastewater complete treatment (Wang et al., 2011; Papaphilippou et al., 2013). As it can be observed in table 2, more than 60% of DOC removal was attained

after the flocculation/coagulation of CBW. Following the pre-treatment step, a solar photo-Fenton process was applied. Photo-Fenton process has been widely demonstrated to be a good option for industrial wastewater treatment, generating non-selective oxidizing species (mainly hydroxyl radicals) that degrade a wide variety of compounds (Chong et al., 2010; Comninellis et al., 2008; Munter et al., 2001). Two different solar photo-Fenton strategies (p-F 1 and p-F 2, considering different initial doses of H_2O_2) were performed with the pre-treated CBW, showing a final short-term biodegradability of 0.2 and 0.3, respectively, while the percentage of inhibition remained constant. A reduction of 31% of DOC and 34% of COD were measured after a total consumption of 1g/L of H_2O_2 (required accumulative UV energy of 0.27 kJ/L) while the consumption of 2.5 g/L of H_2O_2 showed a reduction of 82 % of DOC and 82 % of COD (required accumulative UV energy of 1.02 kJ/L) (see table 2). It should be noted that after the coagulation-flocculation step, CBW samples showed total dissolved iron of 70 mg/L and pH decreased from 5 to 2.8-3, both parameters at the optimum values for the subsequent solar photo-Fenton process.

No significant changes were observed in terms of biodegradability in the raw CBW (0.1) compared to the obtained after the C/F step (0.1) and both solar photo-Fenton strategies (0.2-0.3). Despite the chemical oxidation (solar photo-Fenton process) hardly improved the biodegradability, in all cases the ratio COD/CODb remained in the range of 0.1-0.3 considered as slightly biodegradable. In like manner the percentage of inhibition remained also constant. However, the possible adaptation of activated sludge from MWWTP to the partially treated and slightly biodegradable samples after solar photo-Fenton process was tested at laboratory scale.

3.2. Aerobic biological treatment at laboratory scale

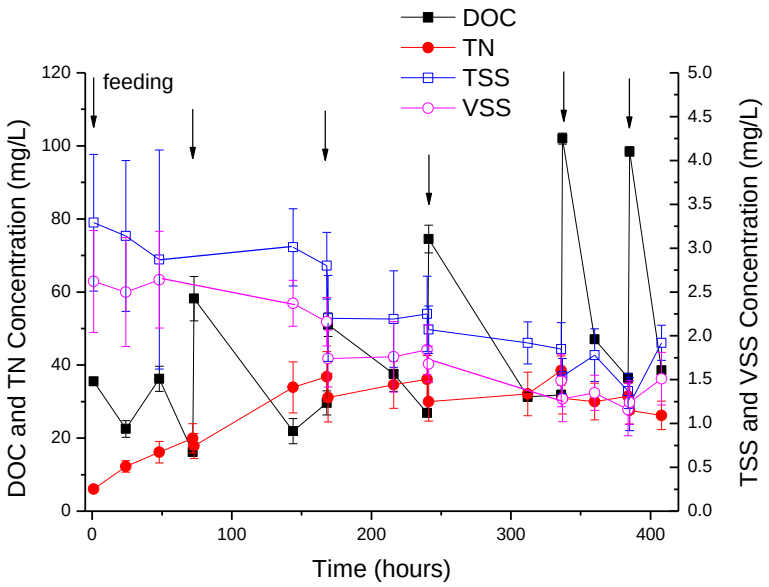
A laboratory scale study was performed to determine if the effluents from the solar photo-Fenton process could be successfully treated in an aerobic biological system including an initial adaptation step. Chemical parameters combined with microbiological techniques were used in order to evaluate the changes in bacterial population of a conventional activated sludge in contact with the two solar photo-Fenton effluents.

As first step, mixed liquor taken from the secondary treatment of a MWWTP from El Toyo (Almería, Spain) was characterized (table 1). It was left with continuous aeration during 24 hours in order to obtain an endogenous phase. After that, one liter of each sample was placed in the 5L-biological reactor containing 4 L of mixed liquor (Experiments B1 and B2, respectively). The system was operating under continuous aeration and agitation during 408 hours (17days). After attaining a stable residual DOC value, new additions (five in total) of the same effluents were done to the biological reactors after stopping aeration, letting biomass settled down and eliminating supernatant already bio-treated (one liter, as initially added). Finally, a sixth feeding of sodium acetate (substrate of easy assimilation) was carried out in order to check the possible recovery of activated sludge.

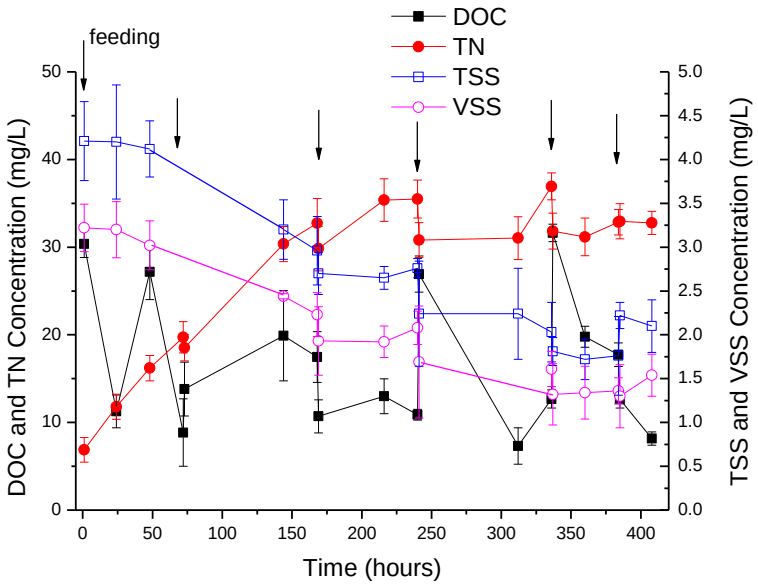
Figure 2a and 2b show the evolution of DOC, TN, TSS and VSS along the aerobic biological treatment. DOC values increased after each feeding, followed by a subsequent decrease as a result of the degradation of its biodegradable fraction. DOC value after each addition was related with the added quantity (63 mg DOC/L and 23 mg DOC/L, for B1 and B2 experiments, respectively) and with the residual DOC before addition. The decrease tendency of DOC values measured after each feeding though

along the treatment days an accumulated DOC was starting to be measured probably coming from the inactivated microorganisms. This effect was much more evident in B1 experiment (Figure 2a).

In both cases, TSS and VSS values decreased along the contact time, showing approximately 42% of reduction in experiment B1 and about 51% of reduction in experiment B2. TN values showed a significant increase just after the initial feeding, attaining stable values around 26 and 33 mg/L in B1 and B2, respectively. Such increase in TN gives an initial signal of malfunction of nitrification processes and probably a reduction in the amount of amino-oxidizing bacteria that must be checked by more sensitive microbiological techniques.



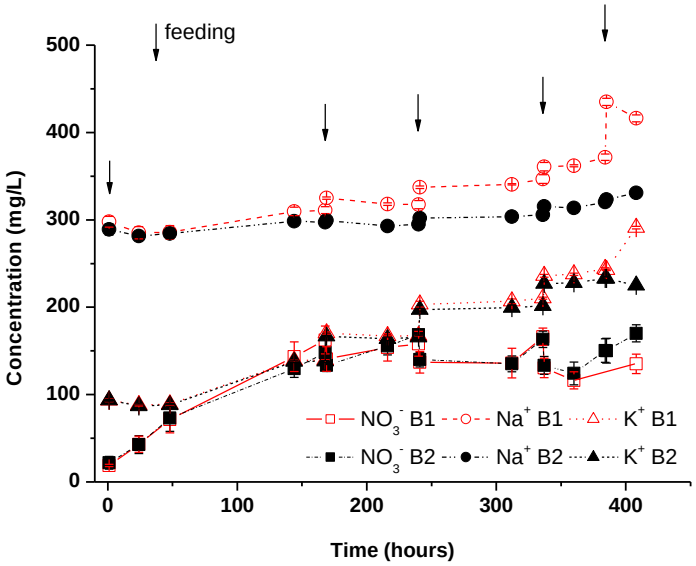
a)



b)

Figure 2. DOC, TN, TSS and VSS evolution along: a) B1 experiment and b) B2experiment.

Ions that substantially changed in both experiments during the biological treatment are shown in figure 3. It is important to stress an increase of more than 300 % for nitrate, sodium and potassium in the twocases, showing final concentrations of 135 mg/L, 416 mg/L and 291 mg/L for B1 and 170 mg/L, 330 mg/L and 224 mg/L for B2, respectively at the end of contact time. The increase of nitrate was the consequence of the successful nitrification process along the experiment; nevertheless, the elimination of nitrate via denitrification process did not occurred because anoxic cycles were not programmed. Sodium and potassium are present at high concentrations in the intracellular fluid of cells, so the increasing of the concentration clearly revealed a significant bacteria damage and breakdown of the cytoplasmic membrane, releasing sodium and potassium to the medium.



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383 **Figure 3.**Concentration of main ions for B1 and B2 experiments.

384 The decrease of TSS and VSS, jointly with a significant release of ions evidenced the
385 bacterial flocs broke-up and the destabilization of the biological system. In addition, as
386 it will be discussed along this work, some new microbial species appeared in the
387 activated sludge, which were, however, unable to deal with the recalcitrant organic
388 carbon degradation and the nitrification process.

389

390 **3.3. Plate count technique for evaluation of aerobic biological treatment**

391 Total heterotrophic bacteria (THB) were followed during B1 and B2 experimental tests
392 at different contact times. Results are shown in figure 4. Initial concentration of THB
393 was around 10^7 CFU/mL. According to figure 4, it was observed fluctuations on THB
394 concentration (between 10^4-10^7 CFU/mL) along the biological treatment with a decrease
395 at the end of the contact time (2-log reduction of THB concentration). However, no
396 substantial changes were found regarding the typology-morphology of the colonies

during both experiments, with three predominant types of colonies observed during the contact time; orange, yellow and white. Some filamentous bacteria appeared in B1 and B2 experiments at 24 and 48 hours respectively and were maintained until the end of both experiments.

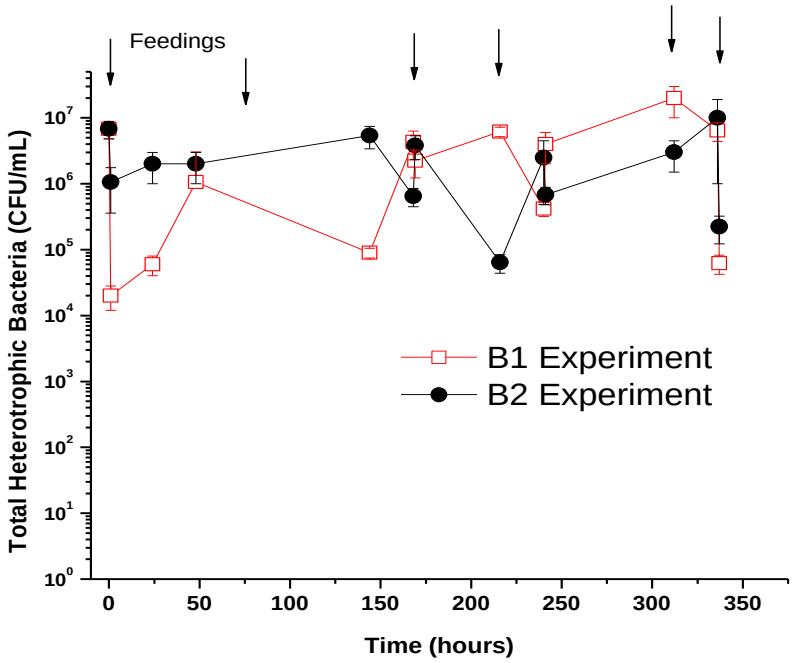


Figure 4. Total heterotrophic bacteria detected by plate count along B1 and B2 experiments.

3.4. Identification of microbial species by optical microscopy

Traditionally, activated sludge present in MWWTPs contains diverse ecosystems normally exposed to extreme conditions (Madoni et al., 1994). Observation in the optical microscope is a good procedureto evaluate the status and performance of the activated sludge and best operating conditions in a MWWTP. The effect of selected partially oxidized CBW on the microbial population during the biological tests (B1 and B2) was studied "in vivo". Prior to the experiments, activated sludge taken from the

411 MWWTP was analyzed by optical microscopy, showing highly compact flocs with
412 medium size, covering approximately 60-70 % of the glass slide surface. In general,
413 microfauna living in activated sludge have a precise composition that depends on the
414 specific nature of the MWWTP. Microfauna werecomposed mainly by protozoa: free-
415 living flagellates as *Peranema* and a variety of ciliate species (*Tetrahymena*, *Acineria*,
416 *Colepshirtus*, *Aspidisca* or *Thuricola*). Metazoa (rotifers), testate amoeba (*Arcella*) and
417 naked amoeba were also present in the selected sludge. These species are commonly
418 present in activated sludge from MWWTPs (Eikelboom, 2000). Substantial variations
419 were observed along B1 and B2 experiments. After one hour of contact time, similar
420 population as the reference was observed for B1 and B2, detecting only a slight increase
421 in ciliates.Traditionally, the presence of ciliated species in activated sludge is frequently
422 reported (Al-Shahwani and Horan, 1991; Curds, 1982; Madoni, 1994) and reflects an
423 increase in the effluent quality due to the predatory activities of ciliates upon the
424 dispersed growth of bacteria which contribute to the clarification and the reduction of
425 coliform bacteria during activated sludge processes (Salvado et al., 1995). At the same
426 time, fragmentation of some *Arcellas* was observed only in B2 experiment. After 48
427 hours, the total number and variability of microorganisms for B1 experiment remained
428 constant. However, some changes were detected in B2 experiment, presenting a
429 significant decrease in rotifers (approximately 50%).

430 After 144 hours, a clear decrease of ciliated species was observed in both cases; it must
431 be highlighted that some dead *Thuricola*sp in B1were observed (figure 5 a.2).
432 Traditionally, presence of *Thuricola* sp indicated good purification performance,
433 associated with low organic mass loads and high cell retention times in the reactor
434 (Isacet al., 2003). In addition, the disappearance of ciliated and rotifers has been also
435 associated to the presence of toxic organic compounds in influents of MWWTPs

436 (Eikelboom, 2000). After feeding the system at 169 hours, total number of died ciliated
437 and rotifers increased (figure 5c.2) although the number of general living species was
438 greater for B2 than for B1. At this time, *Linotonus lamella* appeared in B1suggesting a
439 poor sedimentation of activated sludge (Lee et al., 2004).

440 In B1, after 216 hours of contact time, *Thuricola* species disappeared and *Arcella*
441 species started broken-up (figure 5b.2). According to Mara et al. (2003),
442 *Arcellatestaeamoeba* is commonly present in good quality activated sludge. Free
443 swimming ciliates disappeared and the predominant specie was rotifers, although they
444 were in small number. *Aspidisca* were found as resistant specie, which according to Lee
445 et al., (Lee et al., 2004) is indicative of a bad effluent quality, showing an old activated
446 sludge and high organic loading rate. At the same time, in B2, some broken *Arcella*
447 were observed. After 312 hours of contact time, filamentous bacteria significantly grew
448 in both systems, remaining until the end of the experiments. The excessive growth of
449 these bacteria reduces the efficiency of the wastewater treatment, producing very often
450 bulking and foaming (Kragelund et al., 2007). Total number of species decreased and
451 some *Nematodes* appeared. Nematodes are usually observed in sludge containing low
452 organic loading levels (Eikelboom, 2000).

453 From 336 hours the flocs were damaged, and complete deflocculation was
454 observed.Almost all microbial species died and disappeared. Only a few units of
455 *Rotifers* and *Amoebas* were detected at the end of B1, though not detected in B2, after
456 384 hours of contact time.

457 When sodium acetate was fed after 384 hours, stable flocs were not formed again and
458 the development of new species was not observed. In consequence, the adaptation of

activated sludgecoming from a conventional MWWTP was not successful. Optical microscopy assays confirmed, then, the results presented in former sections.

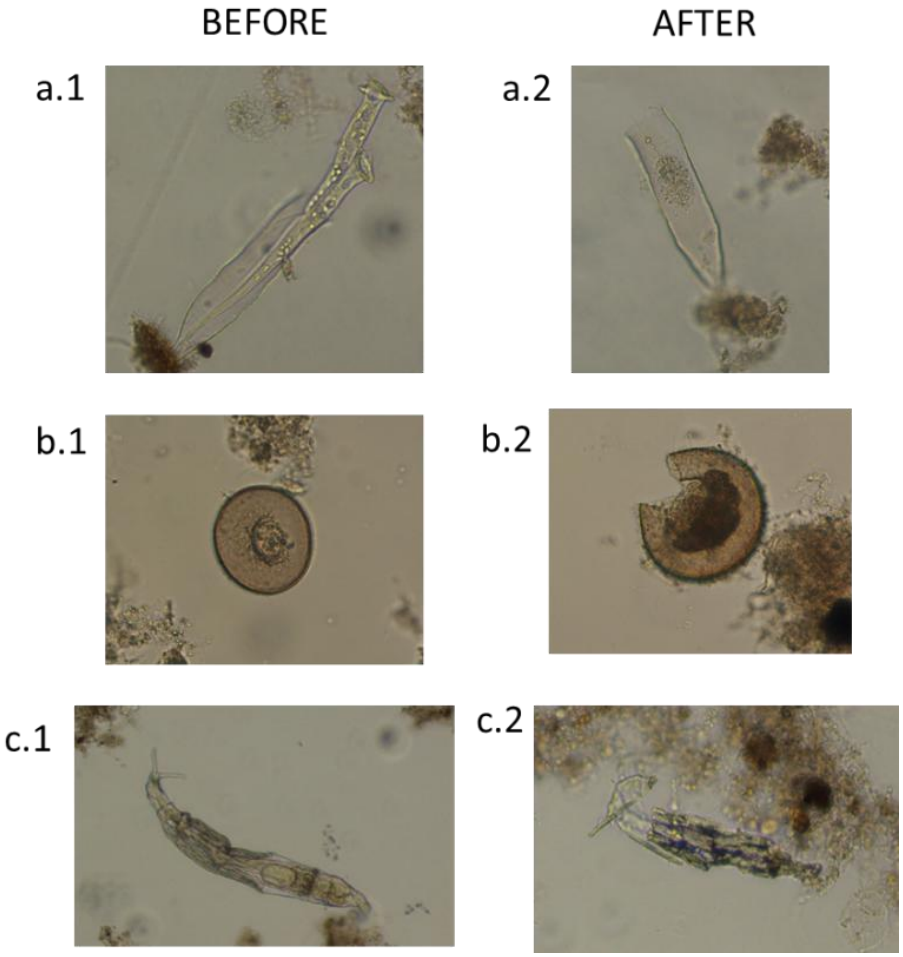


Figure 5. Evolution of microbiological communities during B1 experiment.a.1) *Thuricola* in sludge a.2) Broken *Thuricola* after 144 hours of contact; b.1) *Arcella* in sludge; b.2) Broken *Arcella* after 216 hours of contact; c.1) *Rotifer* in sludge; c.2) Broken *Rotifer*after 169 hours of contact. Views obtained with phase contrast optical microscope (40X).

3.5. DNA extraction and qPCR evaluation

DNA concentration, evaluated along experiments B1and B2, ranged between 60 and 200 ng/μL. Figure 6 shows the evolution of DNA. Initial concentration of DNA present

in activated sludge used in the inoculation of the reactor (83 ng/µL), increased to 189 and 194 ng/µL after the first feeding in B1 and B2, respectively. However, after the rest of feedings, DNA concentration decreased until attaining a minimum concentration around 65 ng/µL for both bioreactors. This behavior indicates the reduction of the microbial load along the biological test, which supports the previously results found in the quantification of THB (Figure 4) and evidence also the inhibition of the biological treatment.

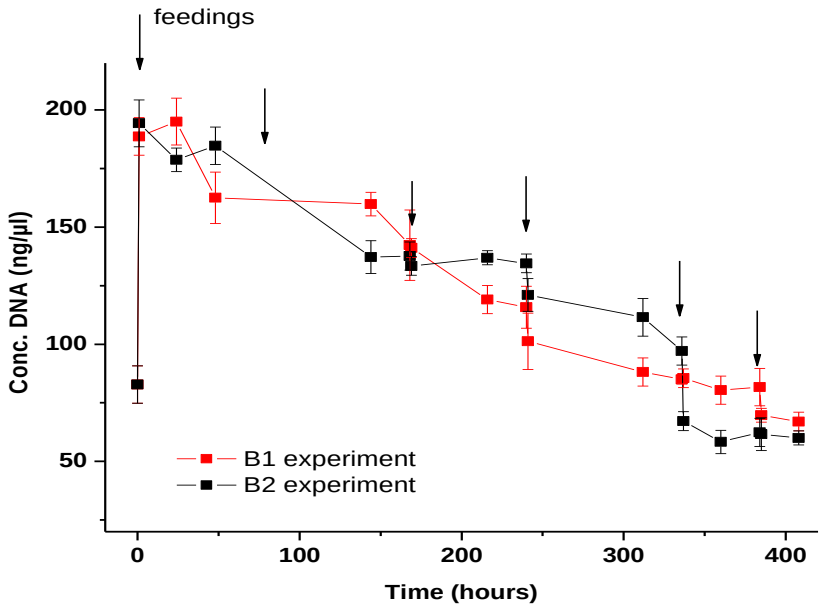


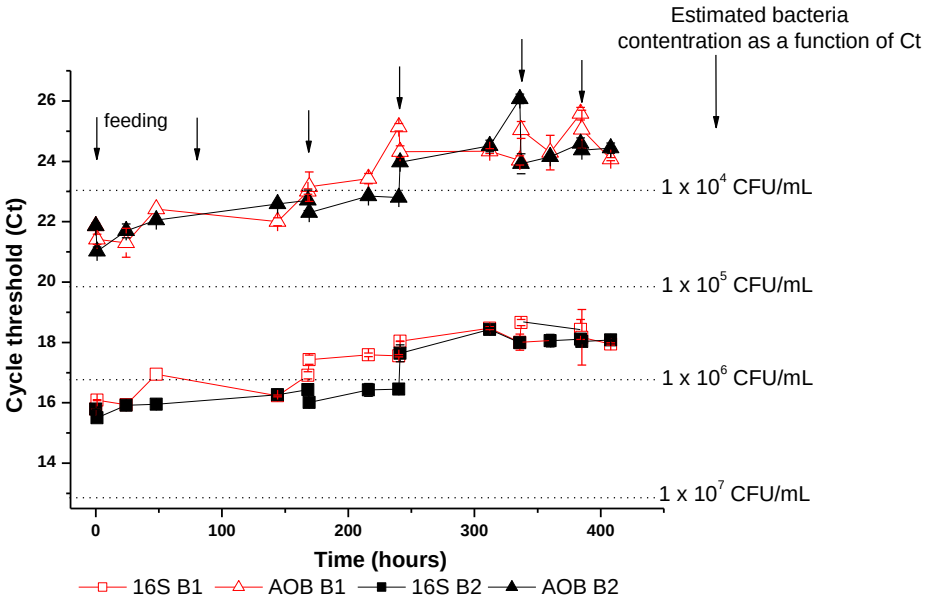
Figure 6. DNA concentration during B1 and B2 experiments at lab-scale aerobic biological treatments.

Apart from general DNA results, it was also necessary to identify those species that could be damaged along the experiment, such as bacteria associated with nitrification processes. With this purpose, real time qPCR assays were also performed. Evolution of total bacteria (16S) and ammonia oxidizing bacteria (AOB) concentration was investigated and results are show in figure 7. The profiles for both bioreactors (B1 and

485 B2) were similar, showing a difference of approximately 2-log between total bacteria
486 and AOB. These results are in concordance with Harms et al. 2003, which indicated a
487 variation of ca. 1.5 log between total bacteria and AOB in a mixed liquor collected from
488 a conventional MWWTP during one year.

489 On the other hand, results showed in figure 7, indicated a total reduction of
490 approximately 1 log for total bacteria and AOB in both experiments (B1 and B2),
491 suggesting an alteration of the equilibrium of the sludge system, including the
492 nitrification process.

493



494

495 **Figure 7.**Total bacteria (16S) and ammonia oxidizing bacteria (AOB) along B1 and
496 B2experiments.

497 In both experiments, the reduction of AOB concentration affected negatively to
498 biological system, worsening the efficiency of nitrification processes. Therefore, it has

been demonstrated that in the case of CBW, an aerobic biological system would be inhibited.

Finally, activate sludge respirometry test was also used to check the toxic effect of the pre-treated CBW on the bacterial activity in the sludge along both biological treatments (B1 and B2). For this purpose, a group of selected samples along B1 and B2 experiments (initial, final and some samples during the treatment) were used to evaluate a possible chronic effect on the activated sludge, measured as a reduction on OUR rate (table 3). Results for B1 showed an increase on inhibition from 21 % after one hour of contact to 83 % after 408 hours. Similar results were observed for B2 experiment, chronic toxicity varied from 29 % after 48 hours to 90 % at the end of the experiment.

In addition, and taking into account the results observed, it is also important to evaluate the possible development of chronic toxicity along the adaptation stage and biological treatment of the partially oxidized samples from CBW after solar photo-Fenton process.

3.6. Toxicity analysis

Considering the highly complex nature of CBW, severalecotoxicity tests were carried out after physic-chemical pre-treatment and solar photo-Fenton process as they can provide useful information onthe hazard potential of such wastewater.Toxicity measurements in complex samples can include synergistic, additive and antagonistic interactions, so, it is important to evaluate toxic effects in organisms representing different trophic levels.

Several acute and chronic toxicity tests on different microorganisms were only carried out in samples partially oxidized by solar photo-Fenton treatment p-F1, (with an initial H_2O_2 dosage of 1g/L) as better biodegradability results were observed compared to p-F2 accompanied with a lower consumption of reagent, what can be translated in lower

operating costs. In order to compare all tests, toxicity results were presented as % of toxic effect calculated following the equation described in section 2.6and shown in table 4.

Different sensitivity was observed in terms of acute toxicity for the different organisms tested. While the raw CBW exhibited an appreciable toxic effectfor the bacterium *V. fisheri* (46.5 ± 14.8 % of luminescence inhibition) and the activated sludge (48 ± 6.1 % inhibition), no noticeable toxic effect was observed for the crustacean *D. Magna* and *A. franciscana* (17 ± 0.2 % mortality and 7.5 ± 3.5 % immobilisation, respectively). These results confirm the findings reported in other ecotoxicological evaluation carried out in raw CBW samples, indicating that the bacterium *V. fischeri* is more sensitive than the crustacean *D. Magna* (Mendoza et al. 2007). In a like manner, after the pre-treatment process (C/F followed by p-F1) a decrease in the acute toxic effect was observed only for both crustacean tested. Indeed, no toxic effects were observed neither for the freshwater crustacean *D.magna* nor for the marine *A. franciscana*. Conversely, for the activated sludge and *V.fischeri,* the observed toxic effectsdid not change significantly after eachpre-treatment step. More specifically, only a slight increase in the acute toxicity was observed (71 ± 6.4 % and 66.5 ± 0.7% inhibition, respectively). These results are consistent with previously reported works which found also an increase in the inhibition percentages for *V.fischeri* in partially treated CBW by solar photo-Fenton, indicating the formation of more toxic compounds (Vilar et al 2009).

Regarding chronic toxicity, CBW caused 100 % growth inhibitory effect to the protozoa *T. Termophila*. Nevertheless, this inhibitory effect was significantly reduced during the pre-treatment line, from 70.4 ± 6.9 % after the C/F stepto 23 ± 1.1% after the solar photo-Fenton process. These results match those observed in previousstudies indicating as well a decrease in the growth inhibition after the applicationof a solar photo-Fenton

548 process (Freitas and Esteban, 2017). In contrast, although the CBW also resulted
549 initially toxic, inhibition percentageincreased during the treatment line from 27 ± 3.6 %
550 to 46 ± 6.3% for the activated sludge by respirometryand from72 ± 3.5 up to 100% for
551 *V.fischeri*.

552 As a summary, the assessment of the potential impact of CBW discharge into the
553 environment showed a decrease in the toxic effect for the crustacean and protozoa
554 species tested after its partial oxidation by solar photo-Fenton treatment. In contrast, the
555 physic-chemical pre-treatment followed by a solar photo-Fenton process did not
556 improve CBW toxicity for the bacteria *V. fischeri* denoting its high sensitivity to this
557 specific wastewater. Similarly, C/F pre-treatment followed by solar photo-Fenton did
558 not show any significant improvement in the toxic effect observed by respirometry
559 (acute and chronic toxicity).

560 Results evidenced that pre-treated CBW is harmful to diverse organisms belonging to
561 different trophic levels, even after its partial oxidation by solar photo-Fenton treatment.
562 In addition, this was also demonstrated with the increase in chronic toxicity in activated
563 sludge along B1 and B2.

564

565 **4. Conclusions**

566 Different advanced analytical and microbiological assays have been applied to the
567 evaluation of the treatment of especially complex industrial wastewater. The impact of
568 these wastewaters in the microbial population contained in the activated sludge gave
569 substantial information for the design of an efficient integrated remediation system.

As demonstrated in this work, analytical and microbiological assays gave concordance results. For instance,DNA concentrations were always in concordance with the decrease of TSS and VSS, main ions evolution and optical microscopy findings along the experiments, evidencing the reduction of microorganisms concentration through the biological treatment due to chronic inhibition effect.

It has been also demonstrated the impossibility of applying a combined treatment based on AOPs and conventional biological system to CBW, suggesting the necessity to apply only chemical oxidative technologies until complete mineralization for this industrial wastewater,which would suppose dealing with higher operating costs.

This multidisciplinary approachwill contribute to find an appropriate treatment line for certain complex industrial wastewaters.

Acknowledgements

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*Graphical Abstract

PRE-TREATMENT
Coagulation/flocculation

CBW
Cork boiling wastewater
Industrial effluent

PROPOSED
TREATMENT LINE

PHOTO-FENTON
process

AEROBIC BIOLOGICAL
TREATMENT

EVALUATION:
analytical and microbiological assays

DESTABILIZATION OF BIOLOGICAL SYSTEM:
Deflocculation, Reduction of
microorganisms (DNA), Chronic Toxicity

*Highlights (for review : 3 to 5 bullet points (maximum 85 characters including spaces per bullet point)

Highlights

- Combination of coagulation-flocculation/solar photo-Fenton/aerobic bio-treatment was not successful.
- Advanced microbiological analysis showed breakage on specific species after 6 days.
- DNA concentration analysis clearly indicated the biotreatment non-feasibility.
- Chronic toxicity was generated after partial oxidation of cork boiling wastewater.

Title: Microbiological evaluation of combined advanced chemical-biological oxidation technologies for the treatment of cork boiling wastewater

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ABSTRACT

This paper contains a multidisciplinary approach that will contribute to design and properly evaluate a treatment line for complex biorecalcitrant wastewaters. To demonstrate this approach a specific industrial wastewater (cork boiling wastewater, CBW) has been used. A treatment line based on a coagulation-flocculation step followed by an Advanced Oxidation Process (AOP) (solar photo-Fenton) and combined with an aerobic biological system has been evaluated. Applied microbiological techniques: optical microscopy, plate count, DNA extraction and qPCR, indicated that some communities disappeared after the activated sludge adaptation period to the partially treated wastewater, while resistant population was unable to withstand changes in the feeding stream: 2-log reduction of total heterotrophic bacteria (THB) and a decrease in DNA concentration from 200 ng/μL to 65 ng/μL were observed. Therefore, chemical and microbiological results obtained along the set of experiments, suggested the inefficiency of the combined treatment option between solar photo-Fenton and advanced aerobic biological systems for CBW, which lead to the necessity of applying chemical oxidation technologies until the complete mineralization of the pre-treated wastewater with the objective of improving the effluent quality enough for being reused in the own industry. Toxicity tests based on different organisms showed increase on acute toxicity (from 46% to 71% after CBW treatment by means of respirometric assays) and the development of chronic toxicity (from 21-29% to 83-90% at the end of the treatment also measured by respirometric assays) along the treatment line, made evident the incompatibility of this type of wastewater with a biological treatment even after the application of an AOP.

Keywords: Biological treatment, Cork boiling wastewater, qPCR, solar photo-Fenton, Toxicity

1. INTRODUCTION

Cork is a natural renewable material extracted from the outer bark of the cork oak tree (*Quercussuber* L.) that traditionally has been used in the production of stoppers for wine industry. The initial processing operation in cork industries is a boiling process. This procedure improves the elasticity and homogeneity of the material, generating high volumes of wastewater (140-1200 L/ton of cork).The boiled effluent, named Cork Boiling Wastewater (CBW) is characterized by a dark color, acid pH, low biodegradability and high acute toxicity (Mendonça et al., 2007). Considering CBW recalcitrant nature, conventional biological treatments are not efficient as some microorganisms are particularly sensible to the organics present, especially to high concentration of polyphenolic compounds (Benitez et al., 2003). Therefore, it is not possible an adaptation of a conventional biological system to CBW (Ponce-Robles et al., 2018), demonstrating the necessity to search and propose alternative solutions to their remediation (Dias-Machado et al., 2006) mainly based on the application of chemical oxidative technologies as pretreatment for attaining CBW biocompatibility improvement.

The requirement of more acute information for the design of new treatment concepts to tackle with the remediation and possible reuse of industrial wastewater, makes highly interesting and necessary the evaluation of the impact of target wastewater (even partially oxidized) to potentially adapt biological systems (always highly efficient) from the economic point of view.

Advanced oxidation processes (AOPs) have been widely demonstrated to be efficient when combined with other advanced technologies after defining specific treatment strategies (Malato et al., 2016). In this sense, high efficiency in removal of large loads

of organic contaminants has been shown in those specific cases in which combination of AOPs and biological treatment succeed. However, the choice of a suitable treatment option for complex wastewaters treatment depends on its effectiveness, so, it is necessary to select adecuate parameters that allow to control and monitor the selected processes (Oller et al., 2011). Physicochemical parameters including dissolved oxygen, pH, DOC, COD, TSS, or VSS are generally considered as fundamental criteria to define the potential use of a treated effluent, however, this information results insufficient. A good complementary option is the application of bioassays in order to evaluate toxic effects of complex mixtures commonly found in industrial wastewater dischargers. The importance of the use of this strategy has been well documented in literature (Isidori et al., 2004; Hemming et al., 2002; Middaugh et al., 1997). More specifically and in the case of aerobic biological treatments based on activated sludge, the evaluation of micro-fauna community evolution along the purificacion processes is crucial to correctly interpret the results but also to modify the operation parameters when neccesary. The species present in the system are responsible for the stability and efficiency of the whole treatment (Cydzik-Kwiatkowska and Zielińska, 2016). These studies are based in traditional culture methods and optical microscopy, identifying predominant microbial populations present in Wastewater Treatment Plants (WWTPs). In addition, Polymerase Chain Reaction (qPCR) has been demostrated to be a useful tool for quantititative analysis of specific microorganisms present in environmental samples (Fortunato et al., 2018).

The main goal of this work was to assess the efficiency of a specific treatment line based on an aerobic biological treatment for the remediation of a complex industrial wastewater. With this aim, a previous step based on an optimized physic-chemical pre-treatment (coagulation-flocculation) combined with an AOP (solar photo-Fenton

process) were evaluated. Specific microbiological techniques (optical microscopy, plate count, DNA extraction and qPCR) were used to monitor and evaluate the microbial adaptation of activated sludge to the partially treated CBW.

2. MATERIALS AND METHODS

2.1. Industrial wastewater and reagents

Cork boiling wastewater used in this study was received from a cork processing plant located in San Vicente de Alcántara (Extremadura, Spain). Conventional activated sludge was provided by the municipal wastewater treatment plant (MWWTP) of El Toyo (Almería). The chemical characterization of both waters is shown in table 1.

Ferric chloride anhydrous ($FeCl_3$) was supplied by Panreac for coagulation-flocculation pre-treatment step. Reagent-grade hydrogen peroxide (30% w/v), sulphuric acid and sodium hydroxide (for physic-chemical pre-treatment and pH adjustment) were provided by Panreac and Merck, respectively. Sodium acetate was supplied by Merck.

2.2. Analytical determinations

Organic matter was measured as chemical oxygen demand (COD) using Merck®Spectroquant kits and dissolved organic carbon (DOC) was measured in a Shimadzu TC-TOC-TN analyzer, model TOC-V-CSN. Total dissolved nitrogen was measured in the same TC-TOC-TN analyzer coupled to a TNM-1 unit. Total suspended solids (TSS) and volatile suspended solids (VSS) were determined according to American Standard Methods. Anions were quantified by ion chromatography using Metrohm 872 Extension Modules 1 and 2 configured for gradient analysis. Cations were determined using a Metrohm 850 Professional IC configured for isocratic analysis.

Total iron concentration was determined using the 1,10-phenantroline method following ISO 6332, and hydrogen peroxide was measured using titanium (IV) oxysulfate according to DIN 38402H15. Total phenolic content was determined spectrophotometrically using the Folin-Ciocalteu reagent with Gallic acid as the reference standard for plotting calibration curve, both provided by Merck (Germany).

2.3. Coagulation-flocculation-filtration step

A coagulation-flocculation-filtration (C/F) step was performed in a pilot plant available at Plataforma Solar de Almeria (Spain) with a filtration in a PEVASA silex filter (75 µm) followed by two AMETEK cartridge micro-filters (25 and 5 µm, respectively). This plant is designed to treat $1 m^3/h$ of water and it has two centrifugal pumps (EPSA) that can operate in manual or automatic mode. The coagulant used was $FeCl_3$, under the experimental conditions already optimized in a previous published work (Ponce-Robles et al., 2017): 3 min of rapid mixing at 100 r.p.m, 30 min of slow mixing at 30 r.p.m to promote flocculation and 30 min of settling solids separation. Such conditions gave high efficiency in turbidity and DOC removal: 86% and 29%, respectively.

2.4. Solar photo-Fenton process at pilot plant scale

Solar photo-Fenton experiments were carried out using a Compound Parabolic Collector (CPC) pilot plant operating in batch mode specially designed for solar photocatalytic applications (Gernjak et al., 2006). This pilot plant was operated at 25 L/min and has a total volume of 75 L (V_T) and a total illuminated volume of 44.6 L (V_i). CBW (after physic-chemical pre-treatment) was introduced into the CPC pilot plant and homogenized in darkness during 15 min. Then, pH was adjusted to 2.8-3.0. Addition of iron was not necessary because enough dissolved Fe (III) remained in

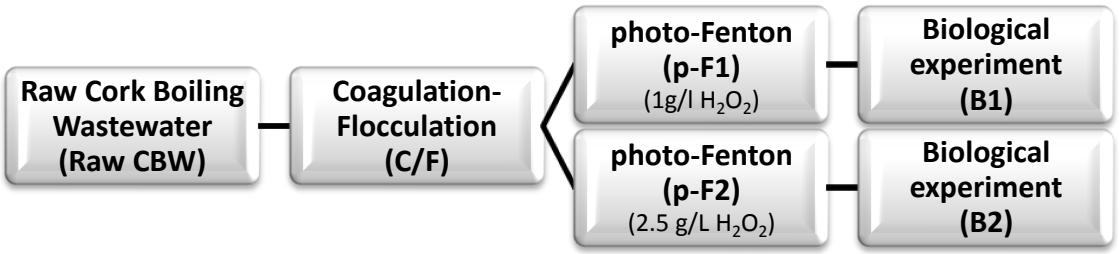
146 solution after the C/F step (between 46 and 80 mg/L). Finally, the CPC was uncovered
147 and photo-Fenton process started. Two different photo-Fenton experiments (p-F1 and p-
148 F2) were performed with the pre-treated CBW by adding two different initial dosages of
149 H_2O_2 (1g/L and 2.5 g/L), according to previously reported experience with CBW treated
150 by solar photo-Fenton (Ponce-Robles et al., 2017).

151

152 **2.5. Aerobic biological system**

153 A Sequencing Batch Bioreactor at laboratory scale was used for biological assays,
154 performed in a 5 L stirred flask reactor provided with a porous air diffuser placed at the
155 bottom of the reactor, keeping dissolved oxygen concentration close to saturation
156 values. The average temperature was 25ºC.

157 Activated sludge taken from the MWWTP was kept in aeration for 24 hours for
158 attaining endogenous phase. Partially treated CBW was studied after adjusting the pH to
159 7. In order to avoid an extremely high organic load of this complex wastewater in the
160 first stages of the adaptation phase, one liter effluent from each photo-Fenton
161 experiment (p-F1 and p-F2) were added in the bio-reactor (B1 and B2 experiments),
162 completing a total volume of 5 L with mixed liquor from the secondary treatment of a
163 MWWTP (see figure 1). Final diluted DOC from the mixture was 63 mg DOC/L and
164 23 mg DOC/L, respectively for B1 and B2 experiments (p-F1 and p-F2, respectively).



165

Figure 1. Scheme of the proposed treatments.

Aerobic biological experiments lasted 17 days (408 hours). Five additions of pre-treated CBW were done along the experiment at different contact times (0, 3, 7, 10, and 14 days) to avoid inhibition effects provoked by a lack of organic feeding. Finally, and with the aim of assessing the viability of the adapted new microbial communities to the partially oxidized CBW, a last feeding with sodium acetate (highly biodegradable) was done after 16 days of experiment. Biological systems were tested by duplicate (B1 and B2). Total Suspended Solids (TSS), Volatile Suspended Solids (VSS), optical microscopy, plate counting, DNA extraction and Polymerase Chain Reaction (qPCR) were monitored throughout the experimental time.

2.6. Microbiological analyses

Microbial enumeration was done by Heterotrophic Plate Count (HPC) or standard plate count technique in Tryptone Glucose Yeast Agar (TGYE Agar) nutrient medium according to standard methods (Gtreenberg et al., 1992). TGYE Agar was prepared by using 5 g/L of Tryptone (Oxoid, UK), 1 g/L of Glucose (JT Baker, USA), 2.5 g/L of yeast extract and 15 g/L of bacteriological agar (Panreac, Spain). The pH of the medium was 7. The method was done through 10-fold serial dilutions of samples (from d1 to d6) in phosphate-buffered saline (PBS) solution. Then, 500µL of each dilution was dropped onto the TGYE Agar nutrient medium. Finally, colonies were counted after incubation for 7 days at 25ºC.

Microscopic observation of mixed liquor samples was done by a Nikon Eclipse 50i Microscope coupled with a Nikon DS camera. A drop of each sample was carefully deposited on a glass slide, and then, samples were covered with a cover slip.

DNA of samples was extracted using a Fast DNA™ SPIN Kit for Soil (MP Biomedicals, Solon, OH 44139 USA) according to manufacturer´s kit instructions with a FastPrep® FP24 Classic Instrument. For that, 1 mL of each sample was centrifuged at 12000 r.p.m. during 2 min. Later, manufacturer´s specifications were followed. DNA concentration was quantified using a NanoDrop spectrophotometer (NanoDrop Lite, Thermo scientific).

The quantification of bacterial 16S rDNA and ammonia oxidizing bacteria (AOB) 16S, extracted DNA samples were amplified using a 7500 Fast Real Time PCR System (Applied Biosystems, USA) instrument with 96-barcode well plates. Commercial primers and probes used were supplied by Sigma-Aldrich (USA). Due to complexity of samples, ten-fold dilution of DNA extracts was used to prevent PCR inhibition. Negative control was performed to validate DNA-amplification results. Bacterial 16S rDNA and AOB concentration was estimated using an external qPCR calibration curve previously reported (Polo-López et al, 2017),which correlates qPCR Cycle threshold (Ct) with bacterial concentration in terms of colony forming units (CFU/mL).

2.7. Biodegradability and toxicity assays

The biocompatibility of pre/treated CBW and the acute and chronic toxicity were evaluated employing different bioassays.

2.7.1 Biodegradabilty measurements

Short term biodegradability analysis were carried out in a BM-T respirometer (Surcis S.L.), which consists of a 1L capacity vessel, equipped with temperature and pH control systems. It also has a dissolved oxygen probe (Protos 3400, Knick ElektronischeMessgeräte GmbH & Co. KG) for measurement of the oxygen uptake rate (OUR). The respirometer was loaded with 700 mL of activated sludge in endogenous

phase. Temperature was controlled at 20ºC and the system was continuously aerated and agitated. Then, 300 mL of oxygen-saturated pre-treated CBW were added. The ratio COD/CODb (total chemical oxygen demand/easily biodegradable chemical oxygen demand) allows establishing the biodegradable character of the sample. A sample is considered to be biodegradable when COD/CODb is greater than 0.3; slightly biodegradable, when it is comprised between 0.1 and 0.3; and non-biodegradable when it is lower than 0.1 (according to instructions given by the respirometer´s manufacturer, SURCIS S.L.).

2.7.2 Toxicity assays

Accurate bioassays for checking toxicity must include representatives from different trophic levels. Acute and chronic toxicity evaluation of the samples was performed using conventional activated sludge and a battery of four commercial tests (BioFixLumi, Protoxkit, Daphtoxkit M and Artoxkit M). Commercial toxicity tests, Protoxkit F and Artoxkit M were purchased by Microbio Test Inc. (Belgium). BioFix® Lumitest was supply by GmbH & Co. KG (Düren, Germany). Each bioassay was repeated at least two times. For the ecotoxicological analysis with the commercial tests, a dilution series of 50, 25, 12.5 and 6.25 % v/v of each sample were carried out after pH adjustment to around 7. Samples from solar photo-Fenton treatment did not contained hydrogen peroxide as they were collected after complete consumption of this reagent.

Toxicity results were expressed as a percentage of toxic effect (inhibition, mortality or immobilization). In each bioassay the toxic effect was calculated by the following equation:

$$\%\, Toxic\ effect = \left[\frac{response\ of\ control - response\ of\ sample}{response\ of\ control}\right] * 100$$

2.7.2.1. Acute toxicity analysis

Conventional activate sludge: Analysis were carried out in the same BMT respirometer as biodegradable assays. The respirometer was loaded with 1L of endogenous activated sludge. Temperature was controlled at 20ºC and the system was continuously aerated and agitated. Toxicity was evaluated by comparing the maximum bacterial oxygen uptake rate ($OUR_{max\text{-}ref}$) in a control test made by the addition of sodium acetate (as highly biodegradable compound) with the obtained when the same amount of the partially treated CBW sample (by solar photo-Fenton process) was added ($OUR_{max\text{-}sample}$). The control was made by 30 mL of distilled water with 0.5 g of sodium acetate/g VSS. Moreover, 30 mL of sample was added to obtain the $OUR_{max\text{-}sample}$. OUR values were also measured along B1 and B2 experiments for assessing possible acute toxicity effects.

*Biofix Lumitest (*30 min bioluminescence test): The reagent was a freeze dried preparation of *Vibrio fischeri* (formerly known as *Photobacterumphosphoreum*, NRRL number B-11177). The test was performed according to manufacturer (Biofix®Lumi-10) specifications following UNE_EN_ISO-11348_1998. With this purpose, the sample was incubated in contact with the bacteria for 30 minutes and the luminescence intensity after the incubation time was measured with a luminometer (BioFix® Lumi-10, MACHEREY-NAGEL®). The luminescence intensity of samples was compared with a reference sample containing the bacteria in isotonic solution. This reference sample was used as a negative control, while K_2CrO_7 (Panreac, Barcelona, Spain; purity 99 %, 18.7 mg/L) was used as positive control. Reduction in light emission by bacteria referred a toxic effect which was finally expressed as an inhibition percentage.

Artoxkit M.: This test is based on the use of instar larvae of the brine shrimp *Artemia franciscana* hatched from cysts that were incubated in synthetic seawater under artificial light at 25ºC. Reconstituted water of normal seawater salinity (35%) was used to prepare the hatching medium for the cysts and the dilution medium for the toxicant dilution series. The standard seawater (35% salinity) was also used as negative control and $K_2Cr_2O_7$ (Panreac, Barcelona, Spain; purity 99 %, 100 mg/L) was used as positive control. After 24 hours of contact with samples, viable population was measured using a dissection microscope to calculate the % of mortality.

Daphtoxkit F.: The 48 h immobilization test with the crustacean *Daphnia magna* was carried out according to the operational procedure of the kit, in adherence to standard guidelines (OECD Guidelines for the Testing of Chemicals, Section 2, Test No. 202: Daphnia sp. Acute Immobilisation Test). Standard Freshwater was used as negative control and K_2CrO_7 (3.2 mg/L) was used as positive control. The percentage of immobilitazion was calculated recording the number of immobile organisms after 48 h of exposure.

2.7.2.2. Chronic toxicity analysis

Conventional activate sludge: Tests were performed in a BM-T respirometer (Surcis S.L.) by recording the slope of OUR (and so the consumption of dissolved oxygen) when the aeration is stopped and comparing such values obtained for the activated sludge coming from the MWWTP before adding the pre-treated CBW samples and after 1, 48, 144, 312 and 408 hours of contact in B1 and B2 experiments. With this aim, one liter of samples from biological treatments were added into the system and OUR was measured again along the experimental time. A decrease in the slope of OUR mean chronic toxicity.

283 *Biofix Lumitest:* Chronic toxicity test were evaluated at the same conditions of acute
284 toxicity test described above but samples were incubated during 24 hours following an
285 adaptation of protocols previously published by other authors (Westlund et al., 2018).
286 The effective concentration which provoked the 50% of inhibition of *Vibrio fischeri* for
287 a solution of Phenol was determined as reference after 30 minutes and after 24 hours of
288 exposure, observing an increase from 35 mg/L to 91 mg/L, showing the absence of
289 chronic toxicity for this solution.

290 *Protoxkit F. test:* The inhibition in the growth of the ciliated protozoan *Tetrahymena*
291 *thermophila* was evaluated after 24 hours exposure according to the Protoxkit F (1998)
292 standard operational procedure. In brief, the test is based on the optical density
293 measurement of the food substrate provided to the ciliates at 440 nm. The growth
294 inhibition is reflected by higher turbidity in the tested samples, in comparison to the
295 negative controls (Standard Freshwater). K_2CrO_7 (56 mg/L) was used as positive
296 control.

297 **3. RESULTS AND DISCUSSION**

298 **3.1. Physic-chemical pre-treatment for wastewater remediation**

299 Flocculation/coagulation pre-treatment based in the addition of $FeCl_3$ at 0.5 g/L after
300 adjusting pH of CBW to 5 was selected according to previous studies reported by
301 Ponce-Robles et al.(Ponce-Robles et al., 2017). A first stage based on physic-chemical
302 pre-treatment is usually required in the remediation of wastewater with the main
303 objective of reducing suspended solids and turbidity, color and DOC. Pre-treatment
304 stages normally increase the efficiency of the subsequently applied chemical oxidation
305 process for the wastewater complete treatment (Wang et al., 2011; Papaphilippou et al.,
306 2013). As it can be observed in table 2, more than 60% of DOC removal was attained

after the flocculation/coagulation of CBW. Following the pre-treatment step, a solar photo-Fenton process was applied. Photo-Fenton process has been widely demonstrated to be a good option for industrial wastewater treatment, generating non-selective oxidizing species (mainly hydroxyl radicals) that degrade a wide variety of compounds (Chong et al., 2010; Comninellis et al., 2008; Munter et al., 2001). Two different solar photo-Fenton strategies (p-F 1 and p-F 2, considering different initial doses of H_2O_2) were performed with the pre-treated CBW, showing a final short-term biodegradability of 0.2 and 0.3, respectively, while the percentage of inhibition remained constant. A reduction of 31% of DOC and 34% of COD were measured after a total consumption of 1g/L of H_2O_2 (required accumulative UV energy of 0.27 kJ/L) while the consumption of 2.5 g/L of H_2O_2 showed a reduction of 82 % of DOC and 82 % of COD (required accumulative UV energy of 1.02 kJ/L) (see table 2). It should be noted that after the coagulation-flocculation step, CBW samples showed total dissolved iron of 70 mg/L and pH decreased from 5 to 2.8-3, both parameters at the optimum values for the subsequent solar photo-Fenton process.

No significant changes were observed in terms of biodegradability in the raw CBW (0.1) compared to the obtained after the C/F step (0.1) and both solar photo-Fenton strategies (0.2-0.3). Despite the chemical oxidation (solar photo-Fenton process) hardly improved the biodegradability, in all cases the ratio COD/CODb remained in the range of 0.1-0.3 considered as slightly biodegradable. In like manner the percentage of inhibition remained also constant. However, the possible adaptation of activated sludge from MWWTP to the partially treated and slightly biodegradable samples after solar photo-Fenton process was tested at laboratory scale.

3.2. Aerobic biological treatment at laboratory scale

A laboratory scale study was performed to determine if the effluents from the solar photo-Fenton process could be successfully treated in an aerobic biological system including an initial adaptation step. Chemical parameters combined with microbiological techniques were used in order to evaluate the changes in bacterial population of a conventional activated sludge in contact with the two solar photo-Fenton effluents.

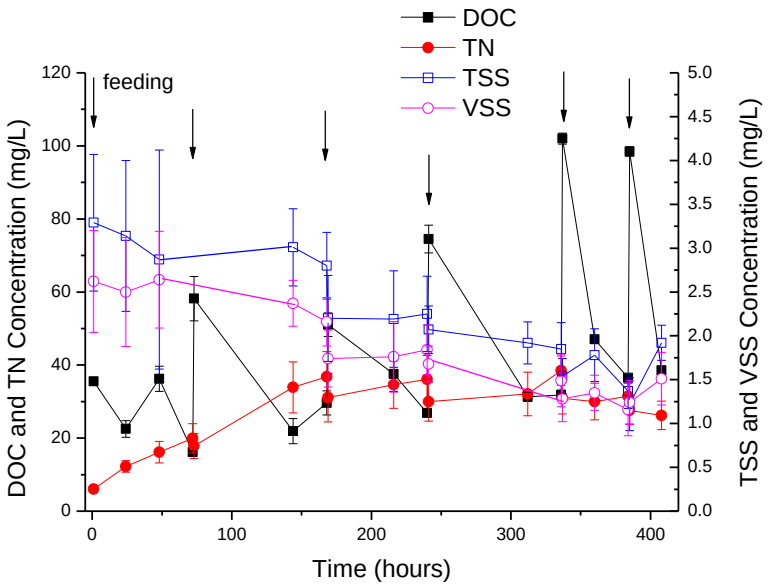
As first step, mixed liquor taken from the secondary treatment of a MWWTP from El Toyo (Almería, Spain) was characterized (table 1). It was left with continuous aeration during 24 hours in order to obtain an endogenous phase. After that, one liter of each sample was placed in the 5L-biological reactor containing 4 L of mixed liquor (Experiments B1 and B2, respectively). The system was operating under continuous aeration and agitation during 408 hours (17days). After attaining a stable residual DOC value, new additions (five in total) of the same effluents were done to the biological reactors after stopping aeration, letting biomass settled down and eliminating supernatant already bio-treated (one liter, as initially added). Finally, a sixth feeding of sodium acetate (substrate of easy assimilation) was carried out in order to check the possible recovery of activated sludge.

Figure 2a and 2b show the evolution of DOC, TN, TSS and VSS along the aerobic biological treatment. DOC values increased after each feeding, followed by a subsequent decrease as a result of the degradation of its biodegradable fraction. DOC value after each addition was related with the added quantity (63 mg DOC/L and 23 mg DOC/L, for B1 and B2 experiments, respectively) and with the residual DOC before addition. The decrease tendency of DOC values measured after each feeding though

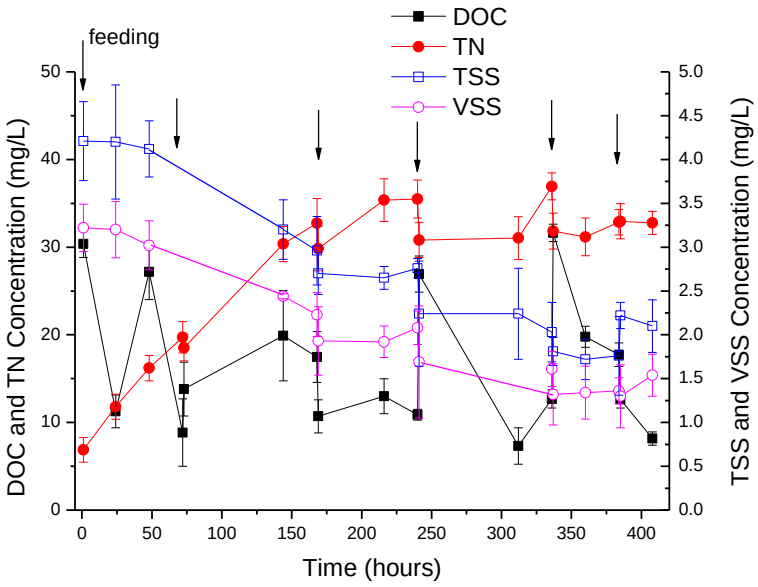
355 along the treatment days an accumulated DOC was starting to be measured probably
356 coming from the inactivated microorganisms. This effect was much more evident in B1
357 experiment (Figure 2a).

358 In both cases, TSS and VSS values decreased along the contact time, showing
359 approximately 42% of reduction in experiment B1 and about 51% of reduction in
360 experiment B2. TN values showed a significant increase just after the initial feeding,
361 attaining stable values around 26 and 33 mg/L in B1 and B2, respectively. Such
362 increase in TN gives an initial signal of malfunction of nitrification processes and
363 probably a reduction in the amount of amino-oxidizing bacteria that must be checked by
364 more sensitive microbiological techniques.

365



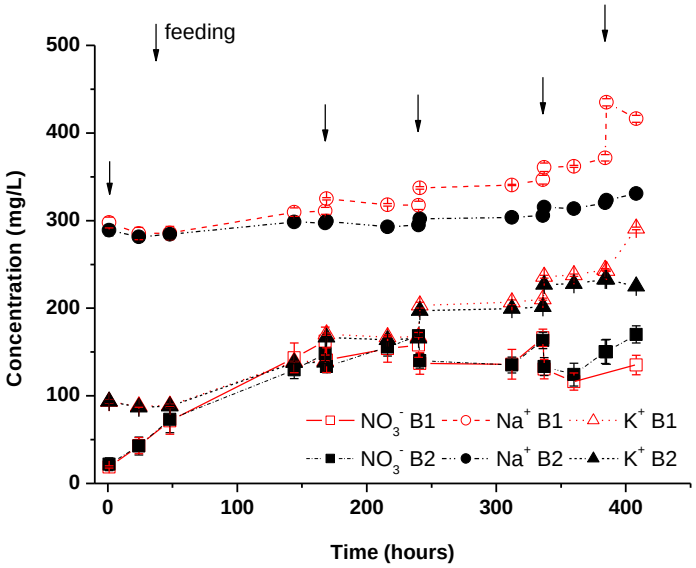
366 a)



b)

Figure 2. DOC, TN, TSS and VSS evolution along: a) B1 experiment and b) B2experiment.

Ions that substantially changed in both experiments during the biological treatment are shown in figure 3. It is important to stress an increase of more than 300 % for nitrate, sodium and potassium in the twocases, showing final concentrations of 135 mg/L, 416 mg/L and 291 mg/L for B1 and 170 mg/L, 330 mg/L and 224 mg/L for B2, respectively at the end of contact time. The increase of nitrate was the consequence of the successful nitrification process along the experiment; nevertheless, the elimination of nitrate via denitrification process did not occurred because anoxic cycles were not programmed. Sodium and potassium are present at high concentrations in the intracellular fluid of cells, so the increasing of the concentration clearly revealed a significant bacteria damage and breakdown of the cytoplasmic membrane, releasing sodium and potassium to the medium.



382

383 **Figure 3.**Concentration of main ions for B1 and B2 experiments.

384 The decrease of TSS and VSS, jointly with a significant release of ions evidenced the
385 bacterial flocs broke-up and the destabilization of the biological system. In addition, as
386 it will be discussed along this work, some new microbial species appeared in the
387 activated sludge, which were, however, unable to deal with the recalcitrant organic
388 carbon degradation and the nitrification process.

389

390 **3.3.Plate count technique for evaluation of aerobic biological treatment**

391 Total heterotrophic bacteria (THB) were followed during B1 and B2 experimental tests
392 at different contact times. Results are shown in figure 4. Initial concentration of THB
393 was around 10^7 CFU/mL. According to figure 4, it was observed fluctuations on THB
394 concentration (between 10^4-10^7 CFU/mL) along the biological treatment with a decrease
395 at the end of the contact time (2-log reduction of THB concentration). However, no
396 substantial changes were found regarding the typology-morphology of the colonies

during both experiments, with three predominant types of colonies observed during the contact time; orange, yellow and white. Some filamentous bacteria appeared in B1 and B2 experiments at 24 and 48 hours respectively and were maintained until the end of both experiments.

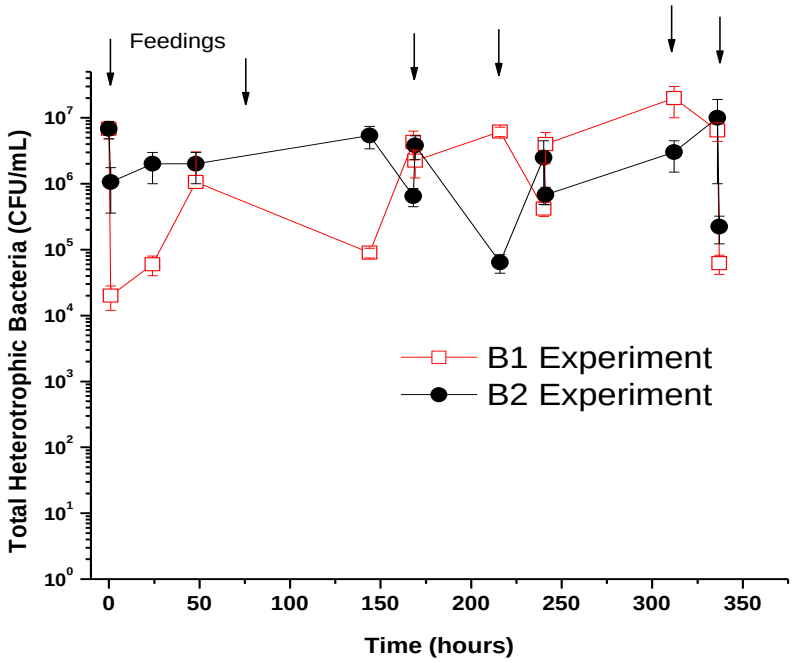


Figure 4. Total heterotrophic bacteria detected by plate count along B1 and B2 experiments.

3.4.Identification of microbial species by optical microscopy

Traditionally, activated sludge present in MWWTPs contains diverse ecosystems normally exposed to extreme conditions (Madoni et al., 1994). Observation in the optical microscope is a good procedureto evaluate the status and performance of the activated sludge and best operating conditions in a MWWTP. The effect of selected partially oxidized CBW on the microbial population during the biological tests (B1 and B2) was studied "in vivo". Prior to the experiments, activated sludge taken from the

411 MWWTP was analyzed by optical microscopy, showing highly compact flocs with
412 medium size, covering approximately 60-70 % of the glass slide surface. In general,
413 microfauna living in activated sludge have a precise composition that depends on the
414 specific nature of the MWWTP. Microfauna werecomposed mainly by protozoa: free-
415 living flagellates as *Peranema* and a variety of ciliate species (*Tetrahymena*, *Acineria*,
416 *Colepshirtus*, *Aspidisca* or *Thuricola*). Metazoa (rotifers), testate amoeba (*Arcella*) and
417 naked amoeba were also present in the selected sludge. These species are commonly
418 present in activated sludge from MWWTPs (Eikelboom, 2000). Substantial variations
419 were observed along B1 and B2 experiments. After one hour of contact time, similar
420 population as the reference was observed for B1 and B2, detecting only a slight increase
421 in ciliates.Traditionally, the presence of ciliated species in activated sludge is frequently
422 reported (Al-Shahwani and Horan, 1991; Curds, 1982; Madoni, 1994) and reflects an
423 increase in the effluent quality due to the predatory activities of ciliates upon the
424 dispersed growth of bacteria which contribute to the clarification and the reduction of
425 coliform bacteria during activated sludge processes (Salvado et al., 1995). At the same
426 time, fragmentation of some *Arcellas* was observed only in B2 experiment. After 48
427 hours, the total number and variability of microorganisms for B1 experiment remained
428 constant. However, some changes were detected in B2 experiment, presenting a
429 significant decrease in rotifers (approximately 50%).

430 After 144 hours, a clear decrease of ciliated species was observed in both cases; it must
431 be highlighted that some dead *Thuricola*sp in B1were observed (figure 5 a.2).
432 Traditionally, presence of *Thuricola* sp indicated good purification performance,
433 associated with low organic mass loads and high cell retention times in the reactor
434 (Isacet al., 2003). In addition, the disappearance of ciliated and rotifers has been also
435 associated to the presence of toxic organic compounds in influents of MWWTPs

436 (Eikelboom, 2000). After feeding the system at 169 hours, total number of died ciliated
437 and rotifers increased (figure 5c.2) although the number of general living species was
438 greater for B2 than for B1. At this time, *Linotonus lamella* appeared in B1suggesting a
439 poor sedimentation of activated sludge (Lee et al., 2004).

440 In B1, after 216 hours of contact time, *Thuricola* species disappeared and *Arcella*
441 species started broken-up (figure 5b.2). According to Mara et al. (2003),
442 *Arcellatestaeamoeba* is commonly present in good quality activated sludge. Free
443 swimming ciliates disappeared and the predominant specie was rotifers, although they
444 were in small number. *Aspidisca* were found as resistant specie, which according to Lee
445 et al., (Lee et al., 2004) is indicative of a bad effluent quality, showing an old activated
446 sludge and high organic loading rate. At the same time, in B2, some broken *Arcella*
447 were observed. After 312 hours of contact time, filamentous bacteria significantly grew
448 in both systems, remaining until the end of the experiments. The excessive growth of
449 these bacteria reduces the efficiency of the wastewater treatment, producing very often
450 bulking and foaming (Kragelund et al., 2007). Total number of species decreased and
451 some *Nematodes* appeared. Nematodes are usually observed in sludge containing low
452 organic loading levels (Eikelboom, 2000).

453 From 336 hours the flocs were damaged, and complete deflocculation was
454 observed.Almost all microbial species died and disappeared. Only a few units of
455 *Rotifers* and *Amoebas* were detected at the end of B1, though not detected in B2, after
456 384 hours of contact time.

457 When sodium acetate was fed after 384 hours, stable flocs were not formed again and
458 the development of new species was not observed. In consequence, the adaptation of

activated sludgecoming from a conventional MWWTP was not successful. Optical microscopy assays confirmed, then, the results presented in former sections.

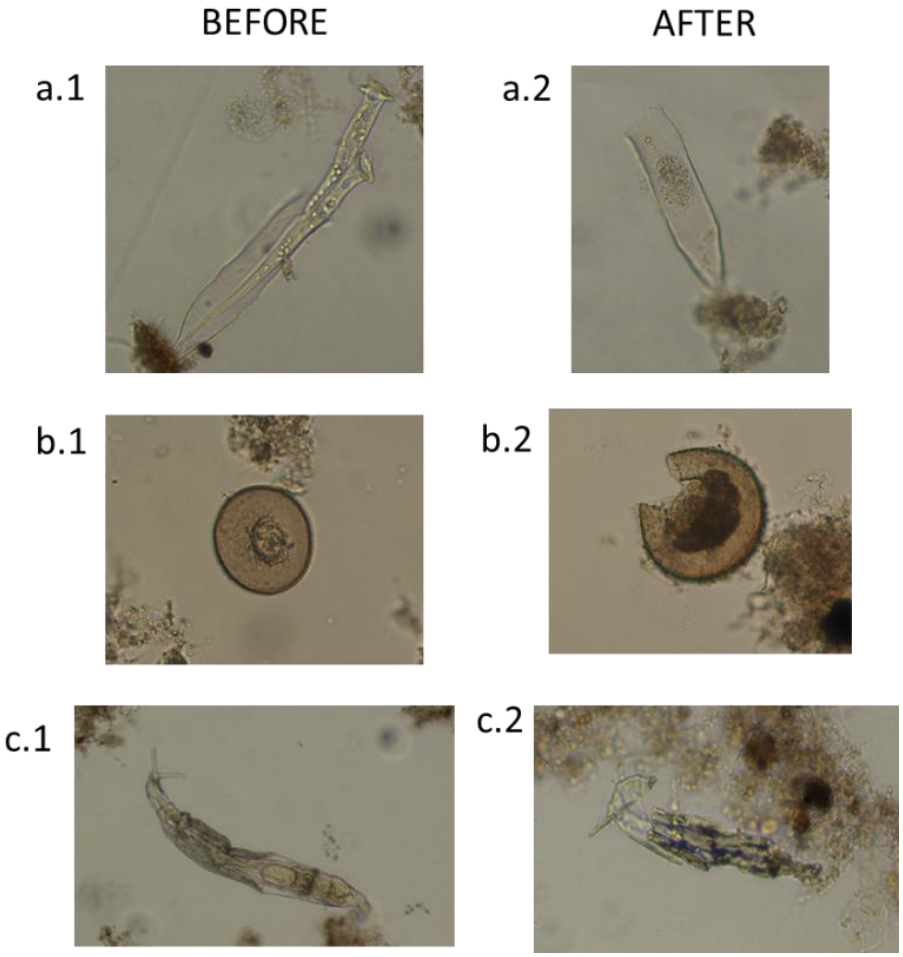


Figure 5. Evolution of microbiological communities during B1 experiment.a.1) *Thuricola* in sludge a.2) Broken *Thuricola* after 144 hours of contact; b.1) *Arcella* in sludge; b.2) Broken *Arcella* after 216 hours of contact; c.1) *Rotifer* in sludge; c.2) Broken *Rotifer*after 169 hours of contact. Views obtained with phase contrast optical microscope (40X).

3.5. DNA extraction and qPCR evaluation

DNA concentration, evaluated along experiments B1and B2, ranged between 60 and 200 ng/μL. Figure 6 shows the evolution of DNA. Initial concentration of DNA present

in activated sludge used in the inoculation of the reactor (83 ng/µL), increased to 189 and 194 ng/µL after the first feeding in B1 and B2, respectively. However, after the rest of feedings, DNA concentration decreased until attaining a minimum concentration around 65 ng/µL for both bioreactors. This behavior indicates the reduction of the microbial load along the biological test, which supports the previously results found in the quantification of THB (Figure 4) and evidence also the inhibition of the biological treatment.

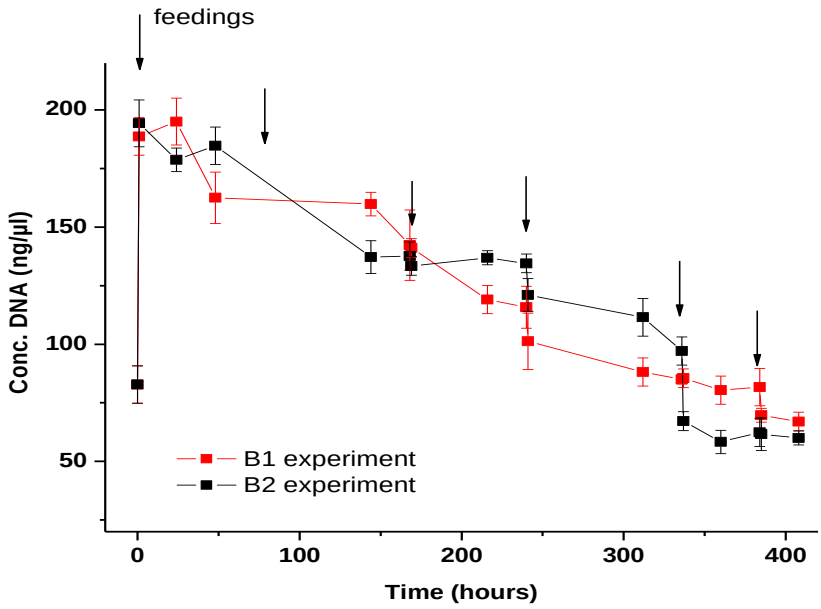


Figure 6. DNA concentration during B1 and B2 experiments at lab-scale aerobic biological treatments.

Apart from general DNA results, it was also necessary to identify those species that could be damaged along the experiment, such as bacteria associated with nitrification processes. With this purpose, real time qPCR assays were also performed. Evolution of total bacteria (16S) and ammonia oxidizing bacteria (AOB) concentration was investigated and results are show in figure 7. The profiles for both bioreactors (B1 and

B2) were similar, showing a difference of approximately 2-log between total bacteria and AOB. These results are in concordance with Harms et al. 2003, which indicated a variation of ca. 1.5 log between total bacteria and AOB in a mixed liquor collected from a conventional MWWTP during one year.

On the other hand, results showed in figure 7, indicated a total reduction of approximately 1 log for total bacteria and AOB in both experiments (B1 and B2), suggesting an alteration of the equilibrium of the sludge system, including the nitrification process.

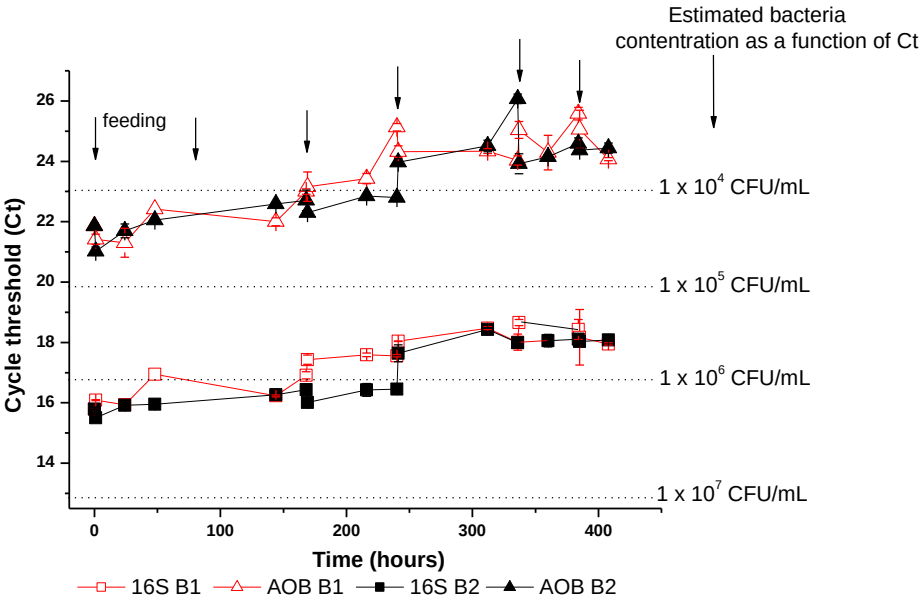


Figure 7. Total bacteria (16S) and ammonia oxidizing bacteria (AOB) along B1 and B2experiments.

In both experiments, the reduction of AOB concentration affected negatively to biological system, worsening the efficiency of nitrification processes. Therefore, it has

been demonstrated that in the case of CBW, an aerobic biological system would be inhibited.

Finally, activate sludge respirometry test was also used to check the toxic effect of the pre-treated CBW on the bacterial activity in the sludge along both biological treatments (B1 and B2). For this purpose, a group of selected samples along B1 and B2 experiments (initial, final and some samples during the treatment) were used to evaluate a possible chronic effect on the activated sludge, measured as a reduction on OUR rate (table 3). Results for B1 showed an increase on inhibition from 21 % after one hour of contact to 83 % after 408 hours. Similar results were observed for B2 experiment, chronic toxicity varied from 29 % after 48 hours to 90 % at the end of the experiment.

In addition, and taking into account the results observed, it is also important to evaluate the possible development of chronic toxicity along the adaptation stage and biological treatment of the partially oxidized samples from CBW after solar photo-Fenton process.

3.6. Toxicity analysis

Considering the highly complex nature of CBW, severalecotoxicity tests were carried out after physic-chemical pre-treatment and solar photo-Fenton process as they can provide useful information onthe hazard potential of such wastewater.Toxicity measurements in complex samples can include synergistic, additive and antagonistic interactions, so, it is important to evaluate toxic effects in organisms representing different trophic levels.

Several acute and chronic toxicity tests on different microorganisms were only carried out in samples partially oxidized by solar photo-Fenton treatment p-F1, (with an initial H_2O_2 dosage of 1g/L) as better biodegradability results were observed compared to p-F2 accompanied with a lower consumption of reagent, what can be translated in lower

operating costs.In order to compare all tests, toxicity results were presented as % of toxic effect calculated following the equation described in section 2.6and shown in table 4.

Different sensitivity was observed in terms of acute toxicity for the different organisms tested. While the raw CBW exhibited an appreciable toxic effectfor the bacterium *V. fisheri* (46.5 ± 14.8 % of luminescence inhibition) and the activated sludge (48 ± 6.1 % inhibition), no noticeable toxic effect was observed for the crustacean *D. Magna* and *A. franciscana* (17 ± 0.2 % mortality and 7.5 ± 3.5 % immobilisation, respectively). These results confirm the findings reported in other ecotoxicological evaluation carried out in raw CBW samples, indicating that the bacterium *V. fischeri* is more sensitive than the crustacean *D. Magna* (Mendoza et al. 2007). In a like manner, after the pre-treatment process (C/F followed by p-F1) a decrease in the acute toxic effect was observed only for both crustacean tested. Indeed, no toxic effects were observed neither for the freshwater crustacean *D.magna* nor for the marine *A. franciscana*. Conversely, for the activated sludge and *V.fischeri,* the observed toxic effectsdid not change significantly after eachpre-treatment step. More specifically, only a slight increase in the acute toxicity was observed (71 ± 6.4 % and 66.5 ± 0.7% inhibition, respectively). These results are consistent with previously reported works which found also an increase in the inhibition percentages for *V.fischeri* in partially treated CBW by solar photo-Fenton, indicating the formation of more toxic compounds (Vilar et al 2009).

Regarding chronic toxicity, CBW caused 100 % growth inhibitory effect to the protozoa *T. Termophila*. Nevertheless, this inhibitory effect was significantly reduced during the pre-treatment line, from 70.4 ± 6.9 % after the C/F stepto 23 ± 1.1% after the solar photo-Fenton process. These results match those observed in previousstudies indicating as well a decrease in the growth inhibition after the applicationof a solar photo-Fenton

548 process (Freitas and Esteban, 2017). In contrast, although the CBW also resulted
549 initially toxic, inhibition percentageincreased during the treatment line from 27 ± 3.6 %
550 to 46 ± 6.3% for the activated sludge by respirometryand from72 ± 3.5 up to 100% for
551 *V.fischeri.*

552 As a summary, the assessment of the potential impact of CBW discharge into the
553 environment showed a decrease in the toxic effect for the crustacean and protozoa
554 species tested after its partial oxidation by solar photo-Fenton treatment. In contrast, the
555 physic-chemical pre-treatment followed by a solar photo-Fenton process did not
556 improve CBW toxicity for the bacteria *V. fischeri* denoting its high sensitivity to this
557 specific wastewater. Similarly, C/F pre-treatment followed by solar photo-Fenton did
558 not show any significant improvement in the toxic effect observed by respirometry
559 (acute and chronic toxicity).

560 Results evidenced that pre-treated CBW is harmful to diverse organisms belonging to
561 different trophic levels, even after its partial oxidation by solar photo-Fenton treatment.
562 In addition, this was also demonstrated with the increase in chronic toxicity in activated
563 sludge along B1 and B2.

564

565 **4. Conclusions**

566 Different advanced analytical and microbiological assays have been applied to the
567 evaluation of the treatment of especially complex industrial wastewater. The impact of
568 these wastewaters in the microbial population contained in the activated sludge gave
569 substantial information for the design of an efficient integrated remediation system.

As demonstrated in this work, analytical and microbiological assays gave concordance results. For instance, DNA concentrations were always in concordance with the decrease of TSS and VSS, main ions evolution and optical microscopy findings along the experiments, evidencing the reduction of microorganisms concentration through the biological treatment due to chronic inhibition effect.

It has been also demonstrated the impossibility of applying a combined treatment based on AOPs and conventional biological system to CBW, suggesting the necessity to apply only chemical oxidative technologies until complete mineralization for this industrial wastewater,which would suppose dealing with higher operating costs.

This multidisciplinary approachwill contribute to find an appropriate treatment line for certain complex industrial wastewaters.

Acknowledgements

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Table

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Table 1. Cork boiling wastewater and activated sludge (mixed liquour) characterization.

Parameters ~~(mg/L)~~	Mixed liquour	CBW
pH	7.1	5.0
Conductivity (µS/cm)	-	1684
Dissolved Organic Carbon (DOC) (mg/L)	11	1060
Chemical Oxygen Demand (COD) (mg/L)	35	3000
Total Suspended solids (TSS) (mg/L)	8	-
Volatile Suspended Solids (VSS) (mg/L)	6	-
Total polyphenol content (mg/L)	-	455
Total nitrogen (mg/L)	5	10
Na^+ (mg/L)	480	30
K^+ (mg/L)	46	350
Cl^- (mg/L)	730	90
NO_3^- (mg/L)	0.6	3
NO_2^- (mg/L)	n.d.	n.d.
SO_4^{2-} (mg/L)	250	2.5
PO_4^{2-} (mg/L)	20	40
NH_4^+ (mg/L)	n.d.	n.d.
Mg^{2+} (mg/L)	60	13
Ca^{2+} (mg/L)	100	70
Short term biodegradability (COD/CODb)	-	0.1 (non-biodegradable)
Acute toxicity	-	48%

n.d.: non detected

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n.d.: non detected

Table 2. Characterization of CBW after both physic-chemical and solar photo-Fenton treatments.

	Raw CBW	**C/F**	**p-F 1**	**p-F 2**
pH	5.0	2.9	2.8	2.9
Conductivity (µS/cm)	1324	3210	2940	2410
DOC (mg/L)	1060	390	270	70
COD (mg/L)	2970	1220	810	215
Total polyphenol content (mg/L)	455	189	88	25
H_2O_2consumption (g/L)	-	-	1.0	2.5
Short-term biodegradability	0.1	0.1	0.2	0.3

* Short-term biodegradability values: 0.1 (non-biodegradable), 0.2-0.3 (slightly biodegradable)

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* Short-term biodegradability values: 0.1 (non-biodegradable), 0.2-0.3 (slightly biodegradable)

Table 3. Activated sludge inhibition during B1 and B2 experimental set.

Time (hours)	% Inhibition	
	B1	**B2**
1	21	0
48	38	29
144	58	54
312	81	88
408	83	90

Table

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Table 4. Toxicity tests results for a group of selected samples.

	Acute Toxicity			**Chronic Toxicity**		
Test Organism	Respirometry Activate Sludge	BioFix® Lumi *V. fischeri*	Artoxkit M *A.salina*	Respirometry Activate Sludge	BioFix® Lumi *V. fischeri*	Protoxkit F *T. thermophyla*
Sample concentration	*100% (v/v)*	*100% (v/v)*	*100% (v/v)*	*100% (v/v)*	*100% (v/v)*	*100% (v/v)*
Endpoint /Toxic effect	*%inhibition*	*% luminiscence inhibition*	*% immobilization*	*% inhibition*	*% luminiscence inhibition*	*% growth inhibition*
Raw CBW	48 ± 6.1	46.5 ± 14.8	17 ± 0.2	27 ± 3.6	72 ± 3.5	52.3 ± 6.5
Coagulated samples	65 ± 4.7	70 ± 4.2	0	59 ± 8.5	99 ± 0.7	70.4 ± 6.9
Photo-Fenton samples (p-F1)	71 ± 6.4	66.5 ± 0.7	0	46 ± 6.3	100	23 ± 1.1