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Biodegradability enhancement and detoxification of cork processing wastewater molecular size fractions by ozone

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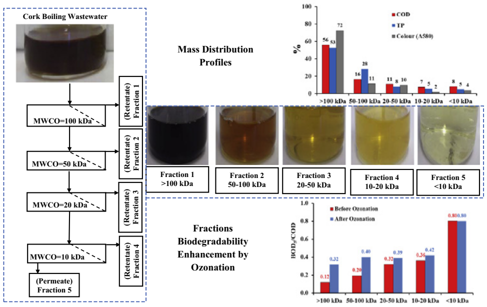
HIGHLIGHTS

- The fraction with molecular size >100 kDa corresponds to 56% of the organic load.
- Molecular size of pollutants is a major constraint to biodegradability.
- Ozone efficiency for improving biodegradability increases with molecular size.
- To raise biodegradability by ozonation the best outcome was with compounds >20 kDa.

GRAPHICAL ABSTRACT



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ABSTRACT

Cork boiling wastewater pollutants were fractionated by sequential use of four ultrafiltration membranes and five fractions were obtained: four retentates (>100, 50–100, 20–50 and 10–20 kDa) and one permeate (<10 kDa); which were used to study the correlation of molecular size with biodegradability and toxicity before and after ozonation. The results show that molecular size is correlated with organic load and restrains biodegradability. The fraction with >100 kDa corresponds to 56% of the organic load and the one with <10 kDa only 8%. The biodegradability of fractions increased 182% with fractions molecular size reduction from >100 to < 10 kDa and the chemical oxygen demand (COD) was from 3436 to 386 mg L^{-1}. For biodegradability enhancement the best outcome of ozonation was obtained with compounds having molecular size >20 kDa and range from 5% up to 175% for applied ozone doses to COD ratios between 0.15 and 0.38.

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1. Introduction

Cork is a natural, renewable and biodegradable material produced from the bark of the cork oak (*Quercussuber L.*). Close to 15% of the overall production is used to manufacture stoppers, the most valuable product of cork industry (Pereira, 2007). The acceptance of cork products can be improved if cork production and transformation are perceived as highly environmentally sustainable activities and this way contribute to the preservation of more than 2.2 million hectares of natural forest ecosystem in Mediterranean countries (APCOR, 2012). After extraction, the corkwood is dried and subsequently immersed in boiling water for 1 h. This first stage of the cork industrial processing, focused on the cleaning, disinfection and moistening of the raw material, raises some environmental concerns due to the high specific water consumption and organic load of biorecalcitrant nature of the effluent. In high quality products, it is crucial to ensure the absence of organic contaminants in corkwood, therefore the reuse of process hot

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water can be limited. Cork boiling wastewater (CBW) presents an intense dark color and contains some corkwood extracts such as phenolic acids, tannins and polyphenols; these compounds have a bio-refractory character and are also present in effluents with similar characteristics, namely those from wine distillery, olive oil extraction and wood debarking (Mendonça et al., 2007; Oller et al., 2011; Martins et al., 2012). Consequently, the biological oxygen demand for 5 days (BOD_5) and 20 days (BOD_{20}) incubation to chemical oxygen demand (COD) ratios, commonly used as biodegradability indexes (BI),range from 0.35 to 0.38 for the BOD_5/COD ratio and from 0.44 to 0.47 for the BOD_{20}/COD (Bernardo et al., 2011). Namely, the BOD_5/COD ratio is below the limit of 0.40 recommended for a successful biological depuration (Metcalf and Eddy, 2003). Besides these features, CBW has a very complex composition with more than 50 organics already identified; most of them are phenolic compounds previously reported to be toxic for the biodegradation processes (Guedes et al., 2003; Pereira, 2007; Fernandes et al., 2011). Therefore, the low biodegradability is associated with high acute toxicity (Mendonça et al., 2007). So, the COD removals achieved by conventional activated sludge treatment only ranged from 13% to 37% (Benítez et al., 2003).

These restraints have driven the study of physicochemical treatment options, namely those based on chemical oxidation, in order to improve COD removal, biodegradability enhancement and detoxification, namely Fenton reaction, ozone (O_3), photo-oxidation, TiO_2-assisted photocatalysis and solar photo-Fenton treatment, which have already been experimented and resulted in COD removals between 66% and 94% (Benítez et al., 2003; Guedes et al., 2003; Silva et al., 2004; Vilar et al., 2009; Pintor et al., 2011). However, these results were achieved by applying large amounts of oxidants and prolonged exposure to UV radiation, up to 28 days in the case of solar photo-Fenton (Pintor et al., 2011). Actually, the cost of depuration by chemical oxidation technologies is high when compared to biological treatment options for effluents with similar organic loads (Oller et al., 2011). Even so, advanced oxidation processes (AOP) are potentially attractive as a pre-treatment stage to enhance biodegradability and reduce effluent toxicity. Several examples of this strategy are reported in the literature, namely with pulp mill bleach effluents (Balcıoğlu et al., 2007), textile effluents (He et al., 2013), agro-industrial wastewaters (Martins et al., 2012) and with CBW the increase of biodegradability results reported by Benítez et al. (2003) for ozonation ranged from 13% to 55%.

Membrane technologies have been proven to be extremely efficient when applied to industrial wastewaters because they selectively concentrate pollutants, allowing frequently permeates with a quality compatible with reuse or meeting environmental requirements for discharge. A large spectrum of membrane selectivities ranging from microfiltration (MF) to nanofiltration (NF) with a molecular weight cut-off (MWCO) of 125 Da were experimented with CBW samples in continuous mode operation and the results reported for COD removal ranged from 17% to over 95% (Benítez et al., 2006; Bernardo et al., 2011). For batch mode operations with UF membranes (MWCO 5–20 kDa) and water recoveries between 50% and 92%, the COD and color removals were 60–75% and 93–99%, respectively; and above 94% with NF membranes (MWCO 150–800 Da) (Benítez et al., 2008; Teixeira et al., 2009). The major goal of these studies was to evaluate the quality (and volume) of the permeate streams, while the destination of concentrates has been ignored until recently when Teixeira et al. (2009) proposed the use of NF concentrates as a tanning agent, replacing vegetable extracts in 10% of the leather production (IPTS, 2003).

CBWs are complex aqueous solutions containing compounds within a huge range of molecular sizes up to several thousand Da for large tannins and polyphenols (Mingshu et al., 2006). Therefore, CBW fractionation by sequential use of UF membranes can cause organic compounds of different molecular sizes to end up in different fractions, allowing to establish their contribution to the overall effluent COD, color and total phenols (TP) and through their characterisation, namely for biodegradability and toxicity assessment, identify constraints to biological treatment before and after chemical oxidation.

Our study aims to evaluate the possibility of CBW depuration by an integrated treatment process including UF membranes, chemical oxidation with ozone and biological treatment. CBW was fractionated using a sequence of four UF membranes with MWCO of 100, 50, 20 and 10 kDa in batch mode operations (with 75% water recovery) and five molecular size fractions were produced. The mass distribution profiles of COD, color and TP were determined, biodegradability and toxicity were assessed by the BOD/COD ratio and the Microtox acute toxicity testing protocol, respectively, before and after ozonation using small amounts of ozone.

2. Material and Methods

2.1. Cork boiling wastewater

The raw CBW was collected in a plant located in the Portalegre district (Portugal) at the exit of the equalization and homogenization tank and kept frozen at −18 °C until being used. Prior to the first UF, the wastewater was pre-filtered using 10 and 5 μm pore size cartridge filters to remove gross suspended solids (SS) in order to minimize membrane fouling or damage. In mean terms, this pre-treatment allowed the reduction of the SS concentration by 65%, from 970 to 340 mg L^{-1}.

2.2. Experimental procedure and analysis

Four commercial UF membranes with MWCO of 100 kDa (GR40PP), 50 kDa (GR51PP), 20 kDa (GR61PP) and 10 kDa (GR81PP) supplied by Alfa Laval (Nakskov, Denmark) were used and the experiments were carried out in a Labstak M20 plate-and-frame system from DSS (Silkeborg, Denmark), described elsewhere (Gomes et al., 2005). The operational conditions used included constant temperature of 20 ± 1 °C, membranes surface areas of 0.144 m^2 (with GR61PP and GR81PP) and 0.252 m^2 (with GR40PP and GR51PP), the tangential flow velocity ($<u>$) of 0.87 ms^{-1} (the maximum value allowed by the recirculation pump, which corresponds to a Reynolds number of 1070), use of low transmembrane pressure (TMP), from 1 to 3 bar (0.1 to 0.3 MPa), and were set to minimize the impact of concentration polarization. Before use, the membranes were pressurized at 8 bar (0.8 MPa) for about 3 h by recirculation of pure water. Subsequently, membrane permeability to pure water (L_p^{water}) was measured L h^{-1} m^{-2} bar^{-1}- and in all cases the water flows correlated with TMP by straight lines. After each experiment with the CBW, the membranes were carefully washed until at least 95% of the L_p^{water} was recovered.

The procedure for the UF fractionation sequence is illustrated in Fig. 1. Before starting each fractionation stage, the TMP and temperature (20 °C) were set and after 15–20 min steady state operational conditions were achieved. The permeate was collected until a volumetric reduction factor (VRF) of 4 was achieved (VRF = V_0/V_r where V_0 corresponds to feed volume and V_r to retentate volume). The choice of the VRF of 4 was based on previously published results by Benítez et al. (2008) and Teixeira et al. (2009) for trials with CBW using UF and NF membranes. The retentate was recovered and the fractionation sequence continues successively with the permeate. The UF sequence began with 100 L of CBW to ensure enough fraction volume to complete the filtration sequence, characterisation and ozonation trials. The four retentates and the 10 kDa membrane permeate were preserved refrigerated at 4 °C

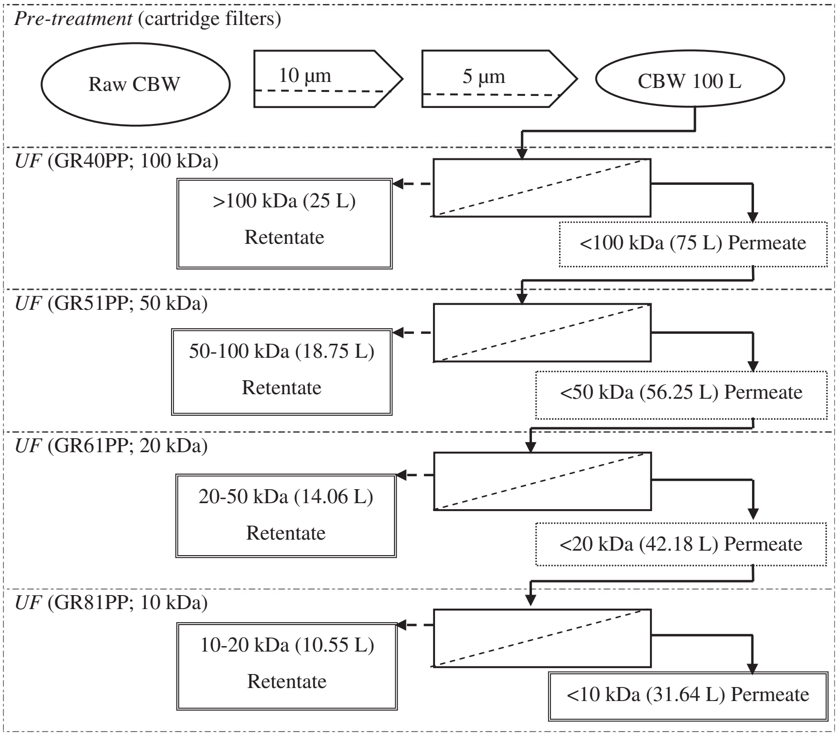

Fig. 1. Schematic diagram of the UF fractionation sequence.

until used for analysis and oxidation trials. Membrane selectivity was determined by calculating the rejection coefficient (f) according to $f = (C_f – C_p)/C_f$, where C_f and C_p are the concentration in the feed and permeate, respectively (Mulder, 1997).

A Fischer Model 502 (Bonn, Germany) ozone generator was employed to produce ozone from dry, pure oxygen. The ozone concentration in the gas phase ranged from 23 to 27 mg L^{-1} and the volumetric flow rate was set to 50 L h^{-1}, corresponding to an average production of 20.8 ± 1.2 mg O_3 min^{-1}. The ozonation of fractions was carried out in a closed vessel of 500 mL provided with a mechanical stirrer to promote the transfer of ozone gas into the solution. The oxidation trials were carried out at 20 ± 1 °C with three moderate applied ozone doses set by selecting the reaction time to achieve ratios of applied ozone dose to initial COD ($O_{3,appl}$/COD_i) in the range of 0.15–0.60, in line with previously studies (Benítez et al., 2003; He et al., 2013; Hwang et al., 2013). The ozone concentrations in feeding and exhaust gas were determined by the iodometric method and allowed us to calculate the ozone consumption.

The characterisation of CBW and fractions, before and after ozonation, included at least three determinations of pH, COD, TP, dissolved organic carbon (DOC), the absorbance measurement at 254 nm (dilution 1:50) (A254) was used to interpret the changes in the aromatic content and at 580 nm for color quantification (A580) (dilution 1:5) (Benítez et al., 2003). The results reported are for the mean values with a coefficient of variation (CV) <5%. In the case of BOD_5 and BOD_{20} the results acceptance criteria included triplicate assays with standard solution (glucose and glutamic acid) and CV <10%. Two biodegradability indexes were used: one corresponded to BOD_5/COD ratio and the other one to BOD_{20}/COD ratio. All the analytical determinations were carried out according to standard methods (APHA, 1998), except for TP content, which was quantified by methodologies adapted from Makkar et al. (1993) using a Thermo Scientific spectrophotometer, model Helios Omega (Cambridge, UK), which was also employed for absorbance measurements when necessary. The BOD determinations were carried out with an OxiTop system (WTW, Germany) according to the respirometric method (APHA, 1998). The inoculum added was originally collected from the aeration reactor of an activated sludge system treating domestic wastewater and used after a 6-month preservation period in laboratory under batch mode feeding, using a mixture of glucose and acetate (with a total COD of 2 g L^{-1}) as carbon sources; nutrients and micronutrients were provided by solutions with composition adapted from Moy et al. (2002). This procedure ensured biodegradability determination by means of no special or adapted bacteria. DOC determinations were made with samples pre-filtered (0.45 µm) using a Shimadzu TOC-V CPH analyser (Shimadzu Inc., Japan). Additionally, ecotoxicity was assessed by measuring the inhibition of *V. fischeri* exposed to dilutions of CBW and UF fractions. The bioassays were carried out with dilutions of filtered samples (0.45 µm) in a Microtox Model 500 analyser according to standard procedures (Azur Environment, 2002) including color correction. All the solutions and bacteria were purchased from Azur Environmental (Berkshine, UK). Toxicity data were computed for the EC_{50} values (percentage of sample dilution that causes a 50% reduction in bacterial light emission after 5 and 15 min exposure) and calculated according to the *gamma* method (Azur Environment, 2002). All correlation coefficients were above 0.90 (otherwise the determination was repeated) and the results of acute toxicity after 5 and 15 min expressed in toxicity units (TU) according to TU = 100/EC_{50}(Bennett and Cuttage, 1992).

The concentration of some specific pollutants was assessed by high performance liquid chromatography (HPLC). The system from Finnigan Surveyor (San Jose, USA) includes an Accela 600 pump, a diode array detector (DAD) and an electrospray ionization mass spectrometer (ESI-MS). A reverse phase C-18 Purospher Star (Merck) column (particle size 5 µm, 4.6 × 150 mm) was used thermostated at 25 °C. The identification of organic compounds was carried out using double online detection, done by a photodiode spectrophotometer for the UV–Vis spectrum with a variable working wavelength between 220 and 440 nm, to set λ_{max} used for quantification, and by mass spectrometry for the fragmentation pattern [M−H]$^-$ in mass units. The mobile phase used two eluents: A (H_2O–CH_3COOH at (999:1; v/v)) and B (CH_3OH–CH_3COOH at (999:1); v/v)). Before starting the analysis, the chromatographic column was washed with 100% B and then stabilized with an initial mobile phase up to five minutes. The gradient profile used was A:B (10:90) up to 5 min, changed to A:B (50:50) for 30 min and held for 8 min; changed to A:B (10:90) for 1 min and held for 16 min. The flow rate was 0.3 mL min^{-1} and the sample injection volume (filtered through a 0.45 μm filter) was 10 μL. The mass detector was operated as follow: capillary voltage of 4 kV and temperature set to 325 °C. Spectra were recorded in the negative ion mode between m/z 120 and 2 000. In the case of HPLC analysis up to five injections were realized if the CV was >5%. All of these solutions were prepared with water purified by a Milli-Q water system (Millipore, Billerica, USA) using reactants and solvents (HPLC grade) supplied by Sigma–Aldrich. The remaining chemicals were of analytical grade and purchased from Merck (Darmstadt, Germany) or Fulka (Buchs, Switzerland).

3. Results and discussion

3.1. CBW characterisation

The results for CBW characterisation are presented in Table 1, of which the most relevant features are the high color, the low BI of 0.26 and 0.36 for BOD_5/COD and BOD_{20}/COD ratios, respectively, and the acute toxicity ranging from 3.08 to 3.46 TU for the EC_{50} after 5 and 15 min exposure, respectively. Regarding the BOD, it is worth noticing that previous results in our laboratory with CBW samples, retentates and permeates from UF operations before and after oxidation showed that after a 5-day incubation period only a fraction of the biodegradable matter was consumed and in most cases steady values were not reached. Therefore, we increased the incubation time up to 20-days, which allowed us to reach practically constant values in all cases.

Concerning the TP concentration and COD, our values are lower than those reported by other authors, ranging from 360 to 410 mg of tannic acid L^{-1} and from 2285 to 4600 mg L^{-1}, respectively. Despite of this, values for BI are in accordance with those previously published, reporting CBW biodegradability values from 0.35 to 0.38 and from 0.44 to 0.47 for the BOD_5/COD and BOD_{20}/COD ratios, respectively (Bernardo et al., 2011). For wastewaters containing complex organics with a large range of molecular sizes, like it is the case of CBW, we may consider the BOD_5 as the rapidly biodegradable fraction of the total organic matter available, which in our case corresponded to 26%. However, by increasing the incubation time from 5 to 20 days the BOD/COD ratio increased up to 36%, meaning that after 5 days only 73% of the biodegradable organics are metabolized. The differences in pollution loads between our sample and those from the aforementioned publications may be a consequence of the higher specific water consumption, necessary to achieve stricter product quality standards, and the collection of the sample at the exit stream of the homogenization and equalization tank. Additionally, variations may also result from differences in the contamination and/or composition of the cork being processed, due to the geographical origin, climate and soil conditions, dimension and age of the tree, etc. (Pereira, 2007).

The HPLC analysis of the CBW confirmed its complexity with a chromatogram indicating the presence of close to 50 compounds, considering the absorbance in the UV–Vis region of the spectrum (220–440 nm) using the DAD. These results are a consequence of hot water being a good solvent for the extraction of compounds unbounded or loosely bounded to the cork cell. Pollutants with larger contribution to the organic load and environmentally hazardous are classified as phenols and include hydrolysable tannins (complex derivatives of gallic and ellagic acid) and non-hydrolysable tannins (proanthocyanidins) (Fernandes et al., 2011). The results for the quantification of several small molecular size phenolic compounds, using external calibration curves, are shown in Table 2. These compounds were selected among those previously quantified in CBW (Minhalma and de Pinho, 2001) and cork samples (Fernandes et al., 2011). Our results were in the range from 0.22 to 7.60 mg L^{-1} for syringic and tannic acids, respectively, below those previously published for CBW, which ranged from 2 to 35 mg L^{-1} for gallic and ellagic acids, respectively (Minhalma and de Pinho, 2001). Moreover, the concentration of specific organic compounds in our study is in line with the results for COD, both confirming the higher dilution of the effluent. In our CBW sample protocatechuic aldehyde and tannic acid were present but we were not able to detect vanillic and ferulic acids, reported for other effluents (Minhalma and de Pinho, 2001).

3.2. UF fractionation and mass distribution profiles

The values of L_p^{water} for the membranes used ranged from 13.5 to 60.0 L m^{-2} h^{-1} bar^{-1} for the GR81PP and GR40PP membranes, respectively and followed the membrane selectivity, as expected, and served us as a primary reference to set the membrane area and TMP for the fractionation stages. The UF membrane productiv-

Table 1
Volume and characteristics of CBW and molecular size fractions.

Parameter (units)		Sample/fraction				
	CBW 100 L	1 >100 kDa Retentate 25 L	2 50–100 kDa Retentate 18.75 L	3 20–50 kDa Retentate 14.06 L	4 10–20 kDa Retentate 10.55 L	5 <10 kDa Permeate 31.62 L
pH	5.42	6.18	6.55	6.61	6.45	6.86
A254[a]	0.395	1.076	0.302	0.285	0.172	0.060
A580[b]	0.118	0.342	0.072	0.082	0.020	0.014
COD (mg L^{-1})	1536	3436	1348	1175	1078	386
DOC (mg L^{-1})	595	1491	572	484	408	166
BOD_5 (mg L^{-1})	407	419	263	378	390	308
BOD_{20} (mg L^{-1})	554	867	440	524	507	359
BOD_5/COD	0.26	0.12	0.20	0.32	0.36	0.80
BOD_{20}/COD	0.36	0.25	0.33	0.45	0.47	0.93
BOD_5/BOD_{20}	0.73	0.48	0.60	0.72	0.77	0.86
TP (mg L^{-1})[c]	110.3	233.0	166.4	61.8	55.8	16.6
Toxicity (TU)[d]	3.08	5.11	1.02	1.25	1.66	0.99

[a] A254 = absorbance at 254 nm for dilutions (1:50).

[b] A580 = absorbance at 580 nm for dilutions (1:5) for color measurement.

[c] TP = total phenols expressed in terms of tannic acid concentration.

[d] For 5 min exposure and 50% inhibition.

Table 2
Concentration of some phenolic compounds in CBW and specific pollutant recovery ratios (%) after fractionation and oxidation according to ozone charge (all fractions included).

Compound	Concentration	Recovery ratios (%)			
	CBW (mg L^{-1})	After UF fractionation	After oxidation, $O_{3,appl}/COD_i$		
			0.20	0.40	0.60
Gallic acid	2.46	15	5	5	5
Protocatechuic acid	0.38	3	0	0	0
Protocatechuic aldehyde	0.56	37	69	130	52
Syringic acid	0.22	36	0	0	0
Vanillic aldehyde	0.60	199	0	0	0
Ellagic acid	2.92	442	49	104	49
Tannic acid	7.60	330	353	408	415

ity, evaluated by the variation of permeability L_p (L m^{-2} h^{-1} bar^{-1}) with elapsed time until VRF = 4, is presented in Fig. 2. The initial membrane L_p values were between 12.62 and 59.20 L m^{-2} h^{-1} bar^{-1}, and the operation time ranged from 7.30 to 15.62 h, following the membrane selectivity, the reduction of the organic load (1 536 to 520 mg L^{-1} of COD) and feed volume (100 to 42.18 L) for the UF sequence (Table 1), with the exception of the GR61PP membrane. In addition, the larger reductions of permeability during fractionation, close to 68%, were experienced with the less selective membranes. In any case, it is important to notice that our conditions were very different from those occurred during single UF operations. In our study only the GR40PP membrane directly processed the CBW sample, the following UF stages were run with permeates sequentially obtained until the 10 kDa membrane was used (Fig. 1). Additionally, the consequences of the unavoidable occurrence of polarization concentration, resulting from the concentration of rejected solutes build-up near the membrane surface, also depend on the characteristics and molecular size of the rejected organic compounds, which are also different for single and sequential operation mode. In our UF sequence, the build-up of diverse polarization layers results in different resistances to permeation that were not anticipated taking into account membrane selectivity (Mulder, 1997). Moreover, the membranes used were made of the same polymer by the same manufacturer, having only different MWCO, ensuring that the scope of interactions between solutes and membrane material remained unchanged.

The rejection coefficient (*f*) values calculated for COD, TP and A580 (color) are compiled in Table 3 for the UF sequence. Despite the variations of operational conditions and the L_p and *f* values throughout the UF sequence, the correlations between COD, TP and color of permeates and the MWCO of the membranes are close to linearity with correlation coefficients above 0.95 (data not shown). These results allow us to anticipate the potential removal that can be obtained by UF membranes with MWCO ranging from 10 to 100 kDa when processing CBW in batch mode operations (VRF = 4). At first glance, the *f* values show variations not according to the membrane selectivity, but it should be emphasized that our operation mode was sequential and as a result the effluent feed to the 50 kDa MWCO membrane was the permeate obtained by the one with 100 kDa MWCO. Therefore, the performance of the successive membranes is dependent on the volume and characteristics of the permeate obtained by the preceding one. Regarding the COD, the experimental data suggest that compounds retained by the GR40PP membrane have a strong impact on the COD concentration, while those rejected by the GR51PP membrane (50 kDa MWCO) have a smaller contribution to COD. On the contrary, the results for TP suggest that GR51PP is more effective than GR40PP. The compounds contributing to color are extensively removed by the GR40PP and GR61PP membranes, corresponding to a molecular size above 100 and 20 kDa, respectively.

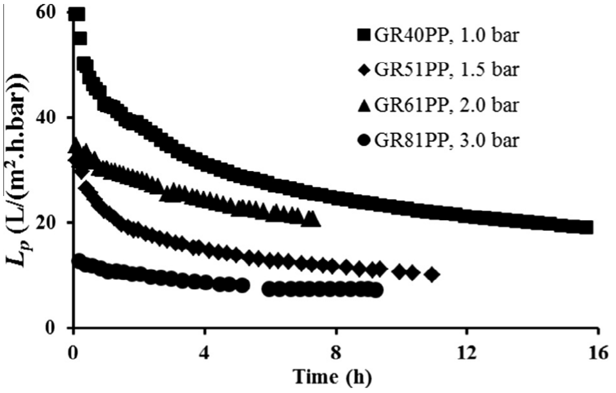

Fig. 2. Variation of the observed permeability with time required for VRF = 4 according to the UF sequence (Fig. 1).

The volume and characteristics of the five molecular size fractions produced are shown in Table 1 and were used to calculate the mass distribution profiles of COD, TP and color of the CBW through mass balance analysis, taking into account the VRF and the volumes successively ultrafiltered. These results show that compounds with molecular size above 100 kDa (fraction 1) are the major contributors to color and organic load and include high molecular weight (MW) polyphenols (ellagitannins, oligomers and polymers), tannins, among other polymers (Mingshu et al., 2006).The cumulative removal of fractions 1 and 2, which a priori should include compounds with molecular size above 50 kDa, represent 72%, 81% and 83% of the overall COD, TP and color, respectively (Fig. 3). This is a relevant result because it enable to anticipate that by using only a membrane with 50 kDa MWCO it's already possible to achieve significant pollutant and color removals and recover 75% of the wastewater volume (VRF = 4). The reliability of these conclusions is reinforced by the global recovery ratio for COD, TP, color and aromatic concentration, which are close to 100%, namely 98.5%, 99.1%, 99.2 and 103.1%, respectively. The results for the distribution profiles in Fig. 3 are in line with the previously reported correlation between COD, TP and color and the MWCO of the membranes, allowing us to conclude that large polyphenols have major contributions to the organic load and the intense brownish color of the CBW.

The biodegradability strongly increases from fraction 1 to 5 and again correlates well with molecular size reduction (Table 1), with an increment of the BOD_5/COD and BOD_{20}/COD ratios from 0.12 to 0.80 and from 0.25 to 0.93, respectively. Moreover, the fraction of easily biodegradable compounds, corresponding to the BOD_5/BOD_{20} ratio, also increases from 48% to 86%, demonstrating that smaller compounds are more rapidly and extensively available for biological oxidation. It has already been pointed out that polymers and organics with large MW tend to be more biorecalcitrant than smaller and soluble molecules due to the difficulty in gaining access to inside bacterial cells (He et al., 2013; Hong and Xiao, 2013). However, the chemistry of involved compounds cannot be completely discarded.

On the other hand, the correlation between toxicity and molecular size fractions is not so clear. Taking into account the classification proposed by Bennett and Cuttage (1992) for Microtox results, the toxicity of CBW and fractions ranges from "non-toxic" (fraction 5 with <1 TU) to "extremely toxic" (fraction 1 with >5 TU) and the intermediate size fractions present close values (between 1.02 TU and 1.66 TU) corresponding to "slightly to moderately toxic" (Table 1). However, the less clear correlation between toxicity and molecular size by comparison with BI may result from the previously mentioned sensitivity of *Vibrio fischeri* bacteria to toxicants,

Table 3
Membrane selectivity at each stage of the UF fractionation sequence for VRF = 4 (F = Feed, P = Permeate, R = Retentate).

Membrane (MWCO)	Sample	COD		TP		Colour	
		($mg L^{-1}$)	*f*	($mg L^{-1}$)	*f*	(A580 nm)	*f*
GR40PP (100 kDa)	F	1536	0.45	110.3	0.37	0.118	0.64
	P	851		69.4		0.042	
	R	3436		233.0		0.342	
GR51PP (50 kDa)	F	851	0.19	69.4	0.48	0.042	0.24
	P	686		36.1		0.032	
	R	1348		166.4		0.072	
GR61PP (20 kDa)	F	686	0.24	36.1	0.20	0.032	0.50
	P	520		28.8		0.016	
	R	1175		61.8		0.082	
GR81PP (10 kDa)	F	520	0.26	28.8	0.42	0.016	0.13
	P	386		16.6		0.014	
	R	1078		55.7		0.020	

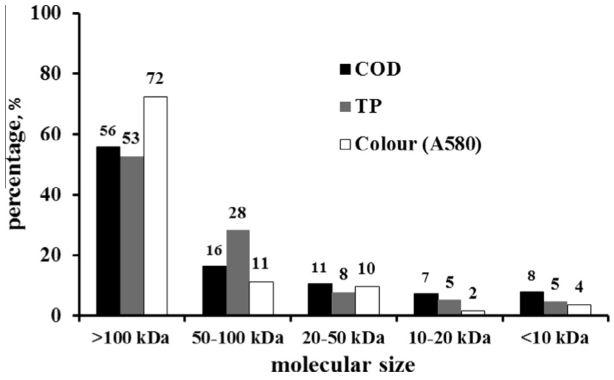

Fig. 3. Mass distribution profiles of COD, TP and colour.

but not at all representative of the effects of wastewater composition on biological treatment plants (Gutiérrez et al., 2002). This can be our case, since the results for biodegradability assessment rely on a large spectrum of bacteria collected in a wastewater treatment plant and used as seed.

Table 2 presents the recovery ratios, determined by component mass balance over all fractions, for the compounds quantified by HPLC after fractionation and ozonation. These calculations were based on the concentrations and volume of the ultrafiltered CBW (Table 1). The data compiled in Table 2 are very different from those reported for COD, TP and color overall mass distribution profiles (Fig. 3), which were close to 100%, with an unexpected variation of the recovery ratios (all fractions included) between 3% and 442%. For example, the ellagic acid recovered is more than four times the corresponding amount in the raw CBW. In addition, these compounds with molecular size ranging from 130 to 1 700 Da are present in fractions other than 5 (<10 kDa). The exception is the syringic acid, only present in fraction 5. Even so, the recovery ratio was only 36%, which points to a significant loss. Besides the occurrence of compound degradation, the results on Table 2 also suggest the possibility of inter-conversion between the monitored compounds, in addition to a concentration increase from the fragmentation of large molecular size compounds present in the CBW.

Several of the organics present in CBW, namely the phenolic class, have antioxygenic, antioxidant and radical scavenger properties, being very susceptible to undergo oxidation and hydrolysis by contact with air and in the presence of acids or bases (Santos et al., 2010). Therefore, the conditions during the UF sequence, with up to 15 h of turbulent flow for the first stage, allowed the occurrence of these complex reactions of cleavage of large organics, association or degradation of smaller compounds. Through fractionation, the recovery ratios of vanillic aldehyde, ellagic and tannic acids were clearly above 100%, with 199%, 442% and 330%, respectively. Interestingly, vanillic aldehyde was present in all fractions, which points out to a concentration increase by cleavage of larger compounds. For the remaining pollutants, the values were below 100% and consistent with the occurrence of degradation or conversion, like it is the case of the inverse correlation between the gallic and ellagic acid recovery ratios (15% and 442%, respectively), which corroborates the possibility of reaction between two gallic acids to produce ellagic acid and also indicates the occurrence of hydrolysis of ellagitannins to ellagic acid (Landete, 2011).The global recovery ratio of the tannic acid was 330% with concentrations ranging from 5.92 to 43.58 $mg L^{-1}$ for fraction 2 and 5, respectively; this may result from the degradation or cleavage of larger tannins, common metabolites of higher plants, by hydrolysis or oxidation catalyzed by different enzymes during the UF sequence (Mingshu et al., 2006). Moreover, the comparison between gallic and tannic acid molecular structures led us to successfully test the formation of tannic acid with a mixture of 10:1 of gallic acid and glucose under no special reaction conditions. The chromatographic analysis of the aqueous solution, before and after 1 h reaction, confirms the reduction of the gallic acid concentration from 600 $mg L^{-1}$ to 283 $mg L^{-1}$ and the arise of the tannic acid close to 300 $mg L^{-1}$ in agreement with the reaction stoichiometry.

3.3. Chemical oxidation of molecular size fractions

The oxidation experiments with the molecular size fractions were conducted at natural pH (Table 1) and the amount of ozone applied was set according to the initial COD in order to achieve a $O_{3,appl}/COD_i$ close to 0.20, 0.30 and 0.40. Table 4 depicts the experimental conditions and results for the ozonation trials with the effective $O_{3,appl}/COD_i$ ranging from 0.15 to 0.60 due to some variability in the ozone production rate. The amount of ozone used was intentionally limited because it's a very expensive reactant and its efficiency decreases with the amount applied, mostly due to side-reactions with non-target compounds, other than biorecalcitrant or inhibitory species (Hoigné and Bader, 1976; He et al., 2013). Before oxidation, the pH of the fractions ranged from neutral to slightly acidic (6.18–6.86) (Table 1) and decreased to 3.42–6.12 after ozonation (Table 4), indicating that strong oxidation took place, as it is the case of fraction 1, containing the larger molecular size compounds and the highest COD, with a pH reduction from 6.18 to 4.20, 3.77 and 3.42 after 18, 25 and 32 min of oxidation, respectively. The reduction of COD for the fractions can be associated with pH decay after ozonation, since these reductions were caused by the transformation of organic pollutants into oxidized carboxyl-containing derivatives and their accumulation (Hoigné and Bader, 1976). At the beginning of ozonation, with a higher concentration of organic compounds and pH close to neutrality the oxidation rate should be high and some hydroxyl

Table 4
Variation of pH, COD, TP, A254, A580, stoichiometric ratio ($COD_{rem}/O_{3,cons}$) and ozone retention for ozonation experiments with UF fractions.

Fraction (molecular size)	$O_{3,appl}/COD_i$	pH	Removals (%)				$COD_{rem}/O_{3,cons}$	Ozone retention (%)
			COD	TP	A254	A580		
1	0.20	4.20	59.4	88.3	78.4	90.6	3.75	78
>100 kDa	0.28	3.77	65.5	91.2	90.2	94.2	3.61	66
	0.38	3.42	69.4	92.0	91.1	96.5	3.40	53
2	0.15	6.12	39.3	93.4	24.1	75.0	3.26	79
50–100 kDa	0.25	6.05	43.2	95.0	43.6	83.3	2.54	69
	0.33	5.92	45.1	95.7	49.7	80.6	2.28	60
3	0.15	5.88	35.7	72.6	44.2	58.5	2.40	76
20–50 kDa	0.24	5.74	41.8	79.8	54.5	63.4	1.73	73
	0.31	5.16	48.2	81.8	58.7	61.0	1.55	71
4	0.17	5.58	28.4	77.0	38.4	27.3	2.13	76
10–20 kDa	0.29	5.39	35.9	71.7	52.0	27.3	1.84	67
	0.41	5.21	40.9	67.7	54.5	36.4	1.56	65
5	0.31	5.12	16.6	76.4	57.1	57.1	0.78	67
<10 kDa	0.60	5.01	29.0	77.5	64.9	71.4	0.73	64

radicals ($OH^{\cdot}$) are available to reinforce the contribution of molecular ozone, so it was expected that for small $O_{3,appl}/COD_i$ ratios the consumption of ozone would be high (and the COD removal fast), but as the solution pH drops the formation of radicals by ozone self-decomposition decreases and the rate of COD and the reaction yield slow down. For instance, for fraction 1 the increase of the $O_{3,appl}/COD_i$ ratio from 0.20 to 0.38 only incremented the removals from 59.4% to 69.4%, 88.3% to 92.0%, 78.4% to 91.1% and 90.6% to 96.5%, for COD, TP, A254 and A580, respectively. The ozonation of fractions 1, 2, 3 and 4 was very effective in achieving COD, TP, A254 and color reductions, which ranged from 28.4 to 69.4%, 67.7 to 95.7%, 24.1 to 91.1% and 27.3 to 96.5%, respectively (Table 4). The fraction <10 kDa presented only a residual pollutant concentration and color, so the outcome of oxidation is less effective than with previous samples.

There is a clear trend indicating that ozone effectiveness, measured by the stoichiometric ratio between COD removed and ozone consumption: $COD_{rem}/O_{3,cons}$ decreases with the decline of organic concentration. Among the pollutants present in the fractions, the phenolic compounds are easily oxidized and in all cases the TP removals were clearly above those for COD, ranging from 67.7% to 95.7% and from16.6 to 69.4, respectively.

The stoichiometric ratios were between 0.73 and 3.75, being above 1.56 for oxidation trials with fractions 1 to 4 (molecular size above 10 kDa), but only ranged from 0.73 to 0.78 with fraction 5. He et al. (2013) treating a cotton textile mill effluent with a COD in the range 83–110 mg L^{-1}, using an ozonation stage, reported a stoichiometric ratio of 2.50 at the optimal ozone dose, in good agreement with our results. The results reported for the CBW fractions also corroborate the previously mentioned possibility that organic content removal achieved by oxidation inversely follows the fractions' COD concentration and also reflect the lack of available substrates for reaction, namely due to the TP concentration reduction from 233 mg L^{-1} to 16.6 mg L^{-1} for fraction 1 and 5, respectively. Besides the stoichiometric ratios, the ozone retention yields were also calculated, corresponding to the percentage of ozone consumed in the reaction medium in relation to the applied ozone. The ozone retention yields were above 53% for the range of oxidations and clearly point to the combined effect of intense mixing and high concentration of unsaturated organic compounds, such as tannic, aromatic and phenolic compounds with large molecular sizes (for fractions 1 to 4, above 10 kDa), which are very sensitive to molecular ozone attack due to numerous groups with available electrons (Santos et al., 2010; Martins et al., 2012). As expected, ozone retention decreases with ozone charge due to the decreasing concentration of organic compounds available for reaction with ozone. Moreover, the decolourization achieved by ozonation is also very high, namely for fractions 1 to 2 ranged from 75.0% to 96.5%, as the compounds with molecular size from 50 to over 100 kDa contribute to 83% of the wastewater color. These results are in line with those previously published for ozonation of CBW (Benítez et al., 2003) and textile effluents (He et al., 2013).

However, the main goal of ozonation in our research was to boost biodegradability and detoxification of fractions without complete mineralization, allowing the formation of intermediate oxidation products with smaller MW, more oxygenated and polar, thus enhancing the chances for biological oxidation (Vilar et al., 2009; He et al., 2013; Hwang et al., 2013). The results before and after the fractions' oxidation for both BI are presented in Fig. 4. Ozonation effectiveness for improving biodegradability decreases from fraction 1 (>100 kDa) to 5 (<10 kDa), which suggests that the molecular size of pollutants is a significant constraint to biological oxidation but it can be overcome by chemical oxidation. The key role of pollutants molecular weight on biodegradation was reported by several authors (Benítez et al., 2003; Oller et al., 2011; Hwang et al., 2013). In the case of fraction 5, the initial biodegradability is already high and the oxidation outcome is not clear; on the contrary, both BI of fraction 1 more than doubled with ozonation, from 0.12 to 0.25 and from 0.25 to 0.56 for the BOD_5/COD and BOD_{20}/COD ratios, respectively. The contribution of ozonation to biodegradability enhancement can be correlated with the slope of the linear regressions in Fig. 5 and the observed reduction from fraction 1 to 5 clearly shows that the outcome of ozone reaction decreases with MW reduction.

To illustrate the potential of the cumulative system, including successively membranes separations, ozone oxidation and biodegradation, the retentate from the 100 kDa and the permeate from

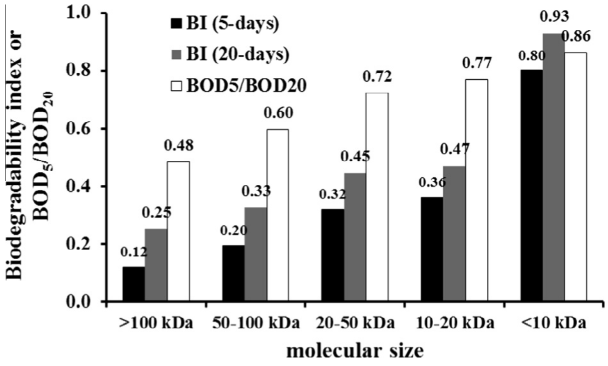

Fig. 4. Biodegradability indexes and BOD_5/BOD_{20} ratios of the UF fractions.

the 10 kDa membranes were selected. From the CBW a retentate with a COD of 3436 mg L^{-1}, TF concentration of 233 mg L^{-1} and A580 of 0.342 was obtained. By ozonation, with an ozone dose of 0.28 for the $O_{3,appl}/COD_i$ the COD, TF and A580 decrease to 1 185 mg L^{-1} (65.5% reduction), 20.5 mg L^{-1} (91.2% elimination) and to 0.020 (94.2% reduction), respectively. If we take into account the BOD_{20}/COD ratio after ozonation (0.54), the subsequent biological treatment can potentially result in a treated effluent with a COD of 545 mg L^{-1} and corresponds to an overall removal of 84%. In the case of the permeate from the 10 kDa membrane, the cumulative removal of ozonation and biodegradation is enough to ensure the environmental requirements for discharge (COD < 150 mg L^{-1}) (EU Directive, 1991). Through HPLC analysis of all fractions, it was possible to calculate the recovery ratios (in percentage) before and after each oxidation stage and the results are shown in Table 2. Even with reduced amounts of ozone, oxidation reactions resulted in the total removal of some pollutants, namely vanillic aldehyde, protocatechuic and syringic acids. However, very complex reactions continue to occur during ozonation, resulting in concentration increments for protocatechuic aldehyde and tannic acid. In this latter case, it is interesting to point out that the tannic acid recovery ratios percentage increase is correlated with oxidation, rising from 330% after the UF stages, which occurred under mild conditions and limited availability of oxygen, up to 415% when ozone was supplied. These results are possible if the CBW contains significant amounts of large molecular size compounds, which by oxidative cleavage release tannic acid molecules that are slowly oxidized. Intermediate situations, corresponding to partial removals, were observed with gallic and ellagic acids and may result from the coexistence of reactions leading to concentration increments and compound degradation, as recovery percentages after oxidation show that both compounds are susceptible to undergo oxidation.

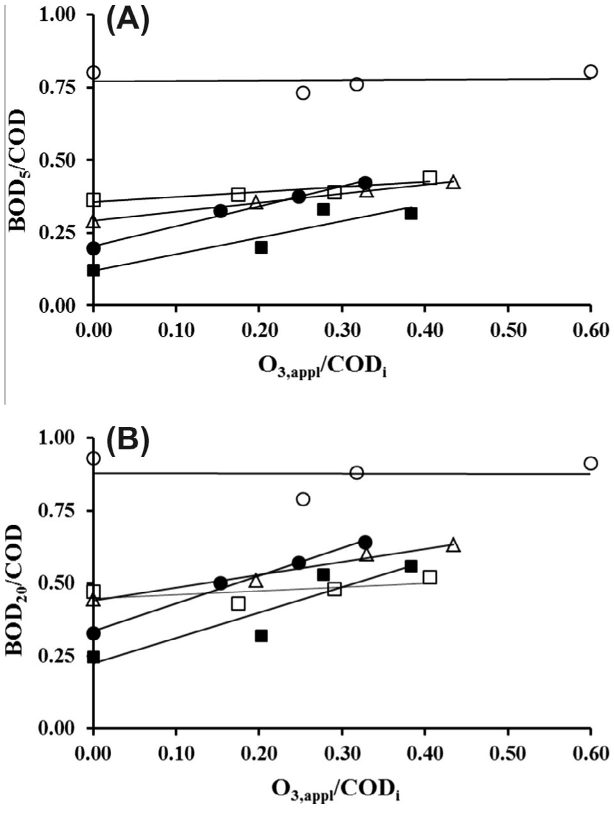

Fig. 5. Variation of biodegradability for (A) BOD_5/COD and (B) BOD_{20}/COD ratios with $O_{3,appl}/COD_i$ different molecular size fractions (■)>100 kDa, (●) 50–100 kDa, (△) 20–50 kDa, (□) 10–20 kDa and (○)<10 kDa.

4. Conclusions

Our study aimed to improve the opportunities to achieve the depuration of CBW by integration of biological treatment with UF and ozonation. The wastewater was a very complex solution with an intense brownish color and high organic load of biorecalcitrant nature. Through sequential UF with four membranes having MWCO from 10 to 100 kDa five molecular size fractions were produced and used to establish the contribution to overall COD, TF and color. The molecular size of organics in fractions was inversely correlated with biodegradability and limited amounts of ozone ($O_{3,appl}/COD_i$ ratios in the range from 0.15 to 0.38) were very effective for biodegradability enhancement (BOD_5/COD) up to 175% for fractions over 50 kDa.

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