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Bioelectrochemical treatment of table olive brine processing wastewater for biogas production and phenolic compounds removal

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ABSTRACT

Industry of table olives is widely distributed over the Mediterranean countries and generates large volumes of processing wastewaters (TOPWs). TOPWs contain high levels of organic matter, salt, and phenolic compounds that are recalcitrant to microbial degradation. This work aims to evaluate the potential of bioelectrochemical systems to simultaneously treat real TOPWs and recover energy. The experiments were performed in potentiostatically-controlled single-chamber systems fed with real TOPW and using a moderate halophilic consortium as biocatalyst. In conventional anaerobic digestion (AD) treatment, *ie.* where no potential was applied, no CH_4 was produced. In comparison, Bio-Electrochemical Systems (BES) showed a maximum CH_4 yield of 701 ± 13 NmL $CH_4 \cdot L_{TOPW}^{-1}$ under a current density of 7.1 ± 0.4 A m^{-2} and with a coulombic efficiency of 30%. Interestingly, up to 80% of the phenolic compounds found in the raw TOPW (i.e. hydroxytyrosol and tyrosol) were removed. A new theoretical degradation pathway was proposed after identification of the metabolic by-products. Consistently, microbial community analysis at the anode revealed a clear and specific enrichment in anode-respiring bacteria (ARB) from the genera *Desulfuromonas* and *Geoalkalibacter*, supporting the key role of these

electroactive microorganisms. As a conclusion, bioelectrochemical systems represent a promising bioprocess alternative for the treatment and energy recovery of recalcitrant TOPWs.

Keywords: Bioelectrochemical Systems; Table Olive Processing Wastewater; Methane production; Phenolic compounds; Electroactive biofilm

1. INTRODUCTION

The production of table olives is an industrial sector that is increasing in importance worldwide. According to the International Olive Oil Council, the world production has increased from less than 1000 tons per year in 1990 to more than 2600 tons in 2014 ("Table olives world production," 2014). Manufacturing table olives constitutes a major economic activity in Mediterranean countries, with almost 50% of the annual world production coming from EU countries (Niaounakis and Halvadakis, 2006). Table olive processing is composed of several preparation steps operated in series of cleaning with fresh water followed by debittering in an alkaline solution (NaOH), rinsing with water and a final fermentation in brine (NaCl). Up to 6 L kg^{-1} of table olive processing wastewaters (TOPWs) are generated along the process (Cappelletti et al., 2009). Due to its high organic content (Kopsidas, 1994, 1992), TOPW cannot be directly released into municipal wastewaters treatment plants and require high amounts of water for dilution. Moreover, the corrosive nature of the salts contained in TOPW makes its disposal particularly difficult (Niaounakis and Halvadakis, 2006). TOPW exhibit also antimicrobial, ecotoxic and phytotoxic properties due to the presence of phenolic compounds (Aggelis et al., 2001; Cappelletti et al., 2009; Kyriacou et al., 2005). This, together with high NaCl and NaOH concentrations, make the TOPW unsuitable for efficient treatment through conventional aerobic (Kyriacou et al., 2005) or anaerobic (Aggelis et al., 2001) biological processes. Disposal of TOPW is a serious issue since TOPW are mainly discharged into evaporation ponds or natural receivers with subsequent contamination of soils and groundwaters (Kyriacou et al., 2005). In recent years, emphasis has been given to the use of advanced

oxidation processes to treat TOPW such as ozonation (Beltrán et al., 1999), wet air oxidation (Katsoni et al., 2008), electrochemical oxidation (Deligiorgis et al., 2008), TiO_2 photocatalysis (Chatzisymeon et al., 2008) and integration of (electro-) chemical with biological process (Kotsou et al., 2004; Kyriacou et al., 2005). Despite all these efforts, technological solutions are not yet economically and/or environmentally acceptable.

In this context, bioelectrochemical systems (BESs) must also be considered as emerging and promising technologies. BESs present a high potential of applications in many disciplines and more particularly in bioremediation, wastewater treatment, biofuels and biochemicals production, in a completely organic and environmentally compatible way (Harnisch et al., 2015). BESs are based on the ability of specific "electrogenic" or "anode-respiring" bacteria to transfer electrons out of their cells to an electrode (Lovley, 2008). In BESs, unfavourable reactions can be made possible by evacuating extra amounts of electrons by controlling the applied potential (Zhang and Angelidaki, 2014). A wide range of substrates have been tested as electron donors in BESs with high performances both in terms of organic removal and energy recovery (*e.g.* electricity or hydrogen at the cathode) (Kadier et al., 2014; Montpart et al., 2014; Pant et al., 2010). It has already been shown that inserting a BES inside an anaerobic digester can increase methane production while stabilizing the anaerobic digestion process at the same time (De Vrieze et al., 2014). In addition, BESs have been applied to remove recalcitrant chlorinated and fluorinated aromatic compounds (Feng et al., 2014; Jiang et al., 2016). Among all of the various configurations that can be used to operate a BES, a single chamber membrane-less BES is the most suitable to wastewater treatment since it presents the lowest installation and operation costs (Montpart et al., 2014).

This work is the first attempt in demonstrating the feasibility to apply a BES to treat and simultaneously recover energy from recalcitrant TOPWs. A lab-scale BES coupling TOPW oxidation and biomethane generation was performed, after having selected the most suitable applied potential.

2. MATERIALS AND METHODS

2.1 Table olive brine processing wastewater (TOPW) and inoculum

TOPW was collected from a local cooperative producing table olives (Bize Minervois, France). Initial pH was 5.4, chemical oxygen demand (COD) 23.8 g L^{-1}, and conductivity 55.3 mS cm^{-1}. TOPW characteristics are more detailed in Supplementary Information (Table S1). Since TOPW had a significant content of salt (about 18 g·L^{-1}), a saline sediment was used as inoculum (10% w/v) after collection in a lagoon receiving wastewaters from a salt factory (Gruissan, France). This inoculum was previously shown to be an effective seed for efficient growth of electroactive biofilms (Pierra et al., 2015). Characteristics of the seed sediments have been described elsewhere (Pierra et al., 2014).

2.2 Electrochemical experiments

2.2.1 General electrochemical conditions

BES experiments were conducted in hermetic potentiostatically controlled half-cell systems set up as described elsewhere (Carmona-Martínez et al., 2013) (SI Fig. S1). Working electrodes (WE) corresponded to planar graphite plates (2.5 cm x 2.5 cm x 0.25 cm). Counter electrodes (CE) grids were composed of 90% platinum–10% iridium (Heraeus PSP S.A.S., France), and could be considered as abiotic (no biofilm). The reference electrode (RE) was a standard calomel electrode (SCE) (KCl 3.0 M, +240 mV vs. SHE). Unless indicated, all potentials were reported and calculated according to the standard calomel reference electrode (SCE) (KCl 3.0 M, +240 mV *vs.* SHE). All bioelectrochemical experiments were conducted in batch-mode under potentiostatic control ensured by a multi-channel potentiostat/galvanostat VMP3 (BioLogic Science Instruments, France). Incubations were performed at 37°C under strict anaerobic conditions. The medium of the BES reactors was continuously mixed with a magnetic stirrer at 200 rpm. If not stated otherwise, MES buffer (7.6 g L^{-1}) was added to undiluted TOPW and pH was adjusted at 7.0 with KOH (0.1M). Characteristics of amended TOPW (t0-TOPW) are detailed in Supplementary Information (Table S1).

2.2.2 *Test to find the most suitable applied potential for biofilm development*

A bioelectrochemical reactor (R0) containing four planar graphite working electrodes (WEs) was used to select the most suitable applied potential for developing an electroactive biofilm and treating TOPW. A N-Stat (N) configuration was used, as previously described by Torres et al. (2009). The reference electrode

(RE) was placed in the center of the reactor. Each WE was connected to a separate potentiostat channel sharing the same RE and counter electrode (CE) according to BioLogic N-Stat connection mode protocol. In the N-Stat configuration (SI Fig. S1), the multi-channel potentiostat individually controls each anode with respect to a single RE. Tested applied potentials were +0.2; +0.4; +0.6. +0.8 (V vs. SCE) and were used to further name the samples for microbiological analysis as R0-N-0.2V, R0-N-0.4V, R0-N-0.6V and R0-N-0.8V respectively, as indicated in Table 1.

2.2.3 *Tests of energy recovery and phenolic compounds removal from TOPW*

After the development of an electroactive biofilm in N-stat, chronoamperometric (CA) experiments were conducted in independent reactors equipped with a single WE (S), by applying a potential of +0.2 V. Duplicate reactors were carried out under strict identical experimental conditions (Table 1: R1-S-0.2V and R2-S-0.2V).

Three control reactors were carried out under different conditions to investigate: (i) the feasibility of developing electroactive biofilms from unmodified TOPW with no inoculum addition (C1); and (ii) the effect of initial pH on biofilm formation (C2), biogas production and phenolic compounds removal not related to electrochemistry (C3) (Table 1). The control reactors C1 and C2 were operated with unmodified TOPW (original pH, no buffer addition), while MES buffer (7.6 g L^{-1}) was added to undiluted TOPW in reactor C3 and pH was adjusted at 7.0 with KOH (0.1M).

2.2.3 *Tests of electrochemical performances of the electroactive biofilm over three months TOPW treatment*

A reactor (Table 1: R3-S-0.2V) was operated in fed-batch mode for three cycles by replacing the TOPW plus the sediment when the current dropped to values near zero which indicated substrate exhaustion (defined as one fed-batch cycle of operation). The stability of the electrogenic biofilm and its electrochemical performance over time were here investigated. A further fourth cycle was performed with

124 reactor R3-S-0.2V with no sediments addition to corroborate the presence of an active specific biofilm on
125 the electrode (Table 1).

Table 1. Tested experimental parameters and performances of electrochemically derived biofilms grown within TOPW.

Experimental conditions*						Time/	j_{max}/	CE/	Simpson	Dominant ARB in the
Code	**Reactor**	**$E_{app.}$*/ V**	**Configuration**	**Inoculum**	**pH**	**days**	**A m^{-2}**	**%**	**diversity index**	**biofilm (%) †**
Test to find the most suitable applied potential for biofilm development in TOPW										
R0-N-0.2V	R0	+0.2	NStat	Yes	7.0	25	7.4	N.A.	0.74	*Desulfuromonas* (80)
R0-N-0.4V	R0	+0.4	NStat	Yes	7.0	25	7.8	N.A.	0.60	*Desulfuromonas* (86)
R0-N-0.6V	R0	+0.6	NStat	Yes	7.0	25	5.1	N.A.	0.82	*Desulfuromonas* (89)
R0-N-0.8V	R0	+0.8	NStat	Yes	7.0	25	1.5	N.A.	0.85	*Desulfovibrio* (67)
Tests of energy recovery and phenolic compounds removal from TOPW										
R1-S-0.2V	R1	+0.2	Single/batch	Yes	7.0	25	6.7	27	0.83	*Desulfuromonas* (55)
R2-S-0.2V	R2	+0.2	Single/batch	Yes	7.0	25	7.5	33	0.80	*Desulfuromonas* (23.5)/ *Geoalkalibacter* (22.7)
R3-S-0.2V	R3	+0.2	Single/fed batch	Yes (cycles1/3) No (cycle4)	7.0	80 17	6.6 5.0	23 to 38 59	0.70	*Desulfuromonas* (45)/ *Geoalkalibacter* (36)
Control reactors										
C1		+0.2	Single/batch	No	5.4°	40	N.A.	N.A.	N.A.	N.A.
C2		+0.2	Single/batch	Yes	5.4°	40	N.A.	N.A.	N.A.	N.A.
C3		N.A.	Not potentiostically controlled	Yes	7.0	25	N.A.	N.A.	N.A.	N.A.

*$E_{app.}$: working electrode (WE)/anodic applied potential (vs. SCE); NStat: biofilm grown in an electrochemical reactor containing up to 4 WEs simultaneously; and Single: grown biofilm in conventional three electrode arrangement set-up containing a single WE. °TOPW unmodified pH (not controlled). †Names in *italics* correspond to the closest affiliated genus, numbers in parentheses represent the relative abundance obtained from sequencing analysis. N.A. = not applicable/available.

2.3 Electrochemical data processing

Chronoamperometric (CA) maximum current densities (j_{max}) of the microbial biofilms were carried out as shown elsewhere (Carmona-Martínez et al., 2013). Coulombic efficiency (CE) was estimated in terms of electrons recovered from the COD removed from TOPW (Logan, 2008). Cyclic voltammetry (CV) was used to monitor the formation of an electroactive biofilm by comparing (i) a control CV of the bare graphite electrode immersed in TOPW with sediments before starting the CA and (ii) a turnover CV (Harnisch and Freguia, 2012) at j_{max}. Methane recovery efficiency (%), was calculated as the ratio of total Coulombs recovered in methane and the Coulombs transferred through the circuit as proposed by Siegert et al (2015).

2.4 Analytical procedures

Total biogas production was assessed by measuring the headspace pressure with a manometer. All volumes were expressed under standard temperature and pressure conditions. Biogas composition (CH_4, CO_2, H_2 and N_2) was determined using a gas chromatograph (Clarus 580, Perkin Elmer) coupled to a thermal catharometer detector as described elsewhere (Quéméneur et al., 2012). The chemical oxygen demand (COD) analysis was performed using analytical test kits for high chloride content waters (MERCK 117059.0001).

2.5 Analysis of the phenolic compounds

Analytical conditions for UPLC-DAD-MS analyses were the same as those described in Koffi et al. (2013). Identifications were achieved on the basis of retention times, UV-visible and mass spectra, and by comparison with those of reference compounds when available. All concentrations were calculated from peak areas at 280 nm and expressed in tyrosol equivalents.

2.6 Microbial community analysis

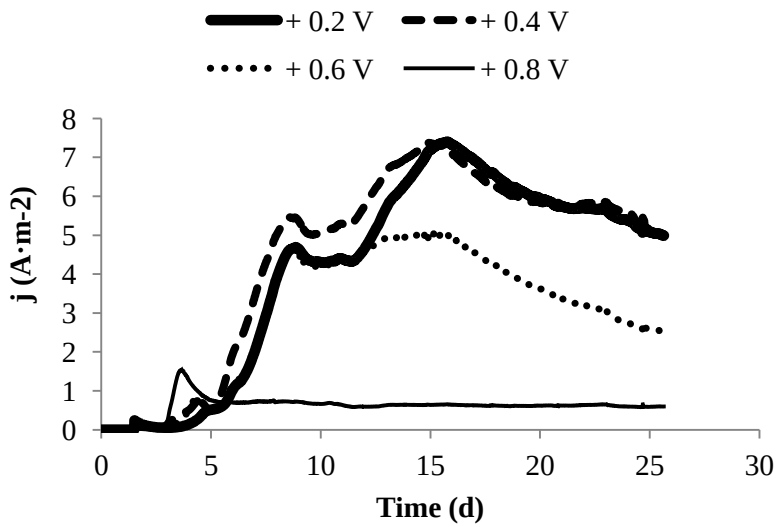
At the end of the experiments, the anodic biofilm and the bulk phase were sampled. Genomic DNA (bacteria and archaea) was extracted and purified as previously described (Milferstedt et al., 2013). DNA

155 samples of the anodic biofilm were sequenced by Illumina MiSeq (get.genotoul.fr) as described elsewhere
156 (Carmona-Martínez et al., 2015).

157 **3. RESULTS AND DISCUSSION**

158 **3.1 Choice of an optimal applied potential**

159 Fig. 1 shows the current density of the chronoamperometric growth of electrogenic biofilms on four
160 anodes operated at different applied potentials using TOPW as electron donor (N-STAT). This
161 configuration implied that all working electrodes (anodes) were operated under strict identical
162 experimental conditions against only one counter electrode and SCE. The four anodes showed a similar
163 lag phase of approximately three days before current production started. Anodes poised at +0.2, +0.4, and
164 +0.6V vs SCE showed an exponential increase in current density and reached a maximum value at day 15
165 with a clear indication of the formation of an electroactive biofilm. The anode poised at +0.8V showed a
166 faster current increase at around day 3 and reached a maximum current density of only 1.54 $A \cdot m^{-2}$,
167 followed by a sudden drastic decrease. Later, current density for R0-N-0.8V remained almost linear for
168 the rest of the experiment. This behaviour could be related to the establishment of a different electroactive
169 biofilm (Table 1, Fig. S4 in SI).



170

Fig. 1. Current densities (j/ A m^{-2}) generated with four different set anode potentials (+0.2, +0.4, +0.6 and +0.8V vs SCE) in reactor R0: N-STAT configuration experiment (R0-N-0.2V, R0-N-0.4V R0-N-0.6V and R0-N-0.8V), using TOPW as electron donors.

Table 1 shows the parameters of the reactors operated under different experimental conditions. Performances of the electrochemically derived biofilms including abundance (%) of dominant anode-respiring bacteria (ARB) found in the biofilm are also presented in Table 1. The microbial community characterization (sequencing of bacterial and archaeal 16s DNA gene) of the biofilms developed on the four anodes is presented in SI.

The main objective of growing electroactive biofilms with different working electrodes immersed in the same medium was to select the most suitable applied potential. Anodes poised at low potentials (+0.2 and +0.4 V) produced the highest current densities (>7 A m^{-2}) when compared to the anodes poised at high potentials (5.1 and 1.54 A m^{-2} for +0.6 and +0.8 V, respectively).

These results are consistent with those obtained by Torres et al. (2009), although their highest anodic potential was +0.13 V *vs.* SCE, which is near the most efficient potential applied here. Zhu et al. (2014) reported that the maximum current increased by increasing the value of anode applied potentials from −0.49 to +0.03 V, while, it decreased at anode applied potentials +0.27 and 0.57 V vs SCE (Zhu et al., 2014). In the present study, in which an alternative to treat a complex waste as TOPW is proposed, low applied potentials appear to be more suitable in recovering energy from TOPW compared to higher applied potentials. Since at more positive set anode potentials more energy was applied to the BES without really improving current density production (Call and Logan, 2008), the lowest applied anode potential was selected for further experiments (+0.2V).

3.2 Bioelectrochemical energy recovery and methane production

Two replicate reactors R1 and R2 were operated in independent reactors equipped with a single WE by applying the previously selected potential (i.e. +0.2 V vs SCE) (Table 1). R1 and R2 showed the typical

four-phase current behaviours versus time: i) lag-phase, ii) exponential current increase iii) plateau and iv) current decrease due to substrate exhaustion. From Fig. 2, one can clearly notice the formation of an electroactive biofilm. Such curve generally shows the achievement of a stable biofilm (Gimkiewicz and Harnisch, 2013). Here it also indicates the biofilm capability to immediately recover its activity and produce current after temporary interruption of CA due to CV performing. Chronoamperometric batch cycle of electrochemically derived biofilm from the two replicate reactors R1 and R2 are presented in SI (Fig S2).

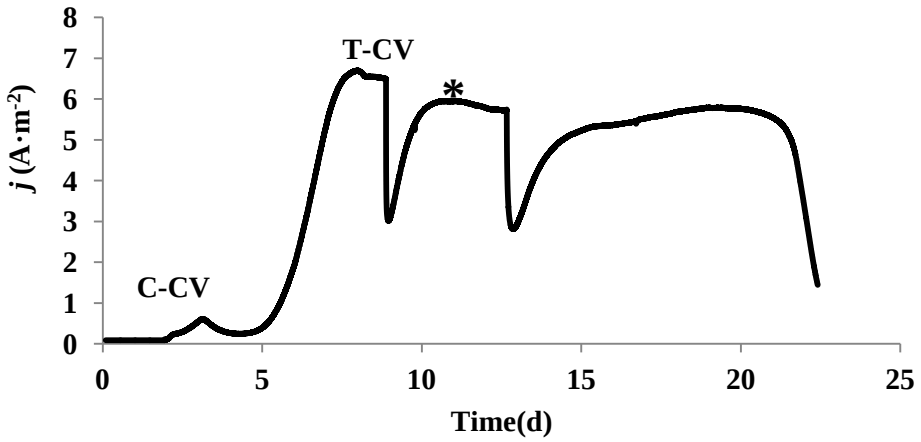


Fig. 2. Representative chronoamperometric batch cycle of an electrochemically derived biofilm. TOPW was used as substrate at a set anode potential of +0.2V vs SCE. C-CV and T-CV stands for control CV and turnover CV, respectively. "*" indicates a temporary interruption of the measurement due to an accidental power failure.

3.2.1 *Cyclic voltammetry as a tool to confirm the presence of a biofilm on the electrode surface*

Cyclic voltammetry (CV) was used to confirm (Harnisch and Freguia, 2012) the presence of an electroactive biofilm formed at the surface of the anode (Fig. 3). CV of the bare electrode (control) clearly showed the absence of an electroactive biofilm due to the flat shape of the voltammogram from -0.7 until

approximately 0.0 V. Interestingly, from 0.0 to +0.3 V a sudden and sharp increase of the current was observed very likely due to the oxidation of either hydroxytyrosol (HT) or tyrosol (TY), the main phenolic compounds detected in TOPW.

Indeed, according to Enache et al. (2013), the oxidation process of HT and TY occurs at a potential around +0.06 V vs. SCE. The discrepancies between the oxidation potential detected here and those published by Enache et al. (2013) might be explained by the different experimental conditions used. While they worked with printed electrodes on which they deposited purified samples of phenolic compounds analysed by CV in buffer solutions (Enache et al., 2013), a CV was here performed on raw TOPW, a more complex solution containing phenolic compounds (Table S1 in SI).

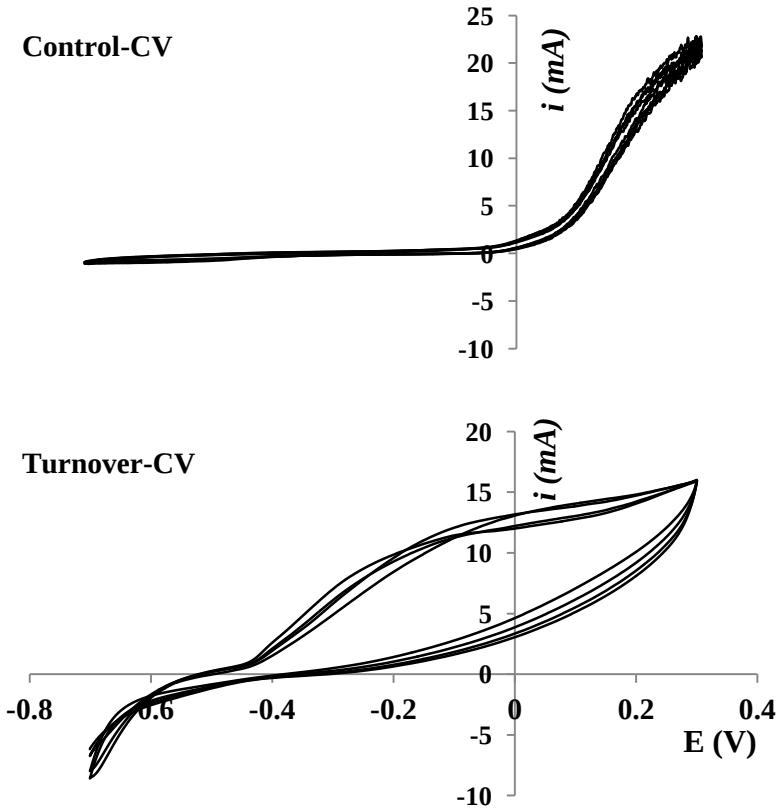


Fig. 3. CV performed in different conditions (four replicate cycles are shown): CV of bare graphite electrode immersed in TOWW (Control) and CV at maximum current density (Turnover), CV shapes are similar for all the electrodes.

The turnover CV showed a very different shape compared to the control CV with the bare graphite electrode immersed in TOPW (Fig. 3), indicating a successful enrichment in ARB (Harnisch and Freguia, 2012). Although the CV shape indicated the presence of an electroactive biofilm, a formal potential could not be calculated, very likely due to the complexity of the microbial matrix (*i.e.* electroactive biofilm composed of a mixed consortium). To further analyse the turnover-CV shape observed here, these voltammograms were compared with those obtained by Rousseau et al. (2013) who used the same saline sediments as inoculum. Although the objectives of their study differed, they used pure acetate as electron donor in a synthetic medium containing a high salinity; they developed a biofilm that depicted a similar turnover-CV shape which very likely indicates a similar electron transfer mechanism in both studies.

3.2.2 *Bioelectrochemical energy recovery, biogas production and process efficiency*

The replicate reactors R1 and R2 showed similar performances in terms of chronoamperometric current density and biogas production (Table 2).

The current density started to increase around day 3-5 until a maximum of 7.1 ± 0.4 $A \cdot m^{-2}$ at approximatively day 8-10. The experiments were considered finished when the current production dropped to values near zero indicating substrate exhaustion (day 22 ± 3), with a total transferred charge (Q_{MAX}) of 11780 ± 936 C. On average, both reactors produced 701 ± 13 NmL $CH_4 \cdot L_{TOPW}^{-1}$ which corresponds to a yield of 109 ± 21 NmL $CH_4 \cdot g\ COD_{removed}^{-1}$. Methane concentration in reactors' headspace reached 63 ± 1 %.The reactors showed satisfactory performance, both in terms of CE, and COD removal efficiency, which were about 30 ± 2.9% and 29 ± 3% respectively.

In all the three control reactors (Table 1), neither biogas evolution nor current production (in the case of C1 and C2 with poised anode potential) was observed. This result clearly indicates that all the operation parameters adopted in this study (anode applied potential, saline sediment inoculum and initial pH adjusted at 7) were crucial to efficiently treat and recover energy from raw TOPW.

By considering the experimental conditions used in this study (i.e. anode set potential of +0.2 V, anaerobiosis, 37°C, pH 7), H_2 was probably produced at the cathode leading to further CH_4 accumulation

253 (Lalaurette et al., 2009). Methane might therefore have been mainly produced by hydrogenotrophic
254 methanogens from H_2 evolved at the cathode (Clauwaert and Verstraete, 2009). Nevertheless, part of the
255 produced methane could have also derived from direct electrochemical reduction of carbon dioxide at the
256 cathode (Cheng et al., 2009). Due to the complex composition of the substrate, the mixed microbial
257 community (bacteria and archaea) and the reactor configuration, the reactions occurring in this system
258 could not be dissociated.
259 Interestingly, the methane recovery efficiency, calculated as the ratio of total Coulombs recovered in
260 methane compared to the Coulombs transferred through the circuit (Siegert et al., 2014), was about 102 ±
261 2%, showing that all transferred electrons were converted to CH_4 as end-product, likely after consumption
262 by hydrogenotrophic methanogens (Siegert et al., 2014). Furthermore, analysis of the archaeal microbial
263 community of the bulk phase suggested that methane production was mainly due to hydrogenotrophic
264 methanogens. Indeed, most of the 16S rDNA archaeal sequences found in reactors R1 and R2 belonged to
265 members of the family *Methanomicrobiaceae* (> 93%). In this family, methanogens mainly use the
266 hydrogenotrophic pathway, ie. H_2/CO_2 or formate as substrates, to produce methane (Rosenberg et al.,
267 2014). More precisely, species affiliated to the *Methanoplanus* genus were dominant in the bulk liquid of
268 reactor R1 (87%) followed by the genus of *Methanosarcina* (7%). Similar results were obtained in reactor
269 R2 with *Methanoplanus* genus and *Methanosarcina* relative abundances of 87% and4%, respectively.
270 In addition, it is worth noticing that acetate, lactate, and ethanol contained in the raw TOPW were totally
271 consumed. Interestingly, a significant amount of propionate (1.4 ± 0.08 g·L^{-1}) was also produced during
272 the process, which is not surprising since propionate is produced by fermentative degradation of organic
273 matter. Furthermore, it has been reported that propionate is not easily consumed by ARB (De Cárcer et al.,
274 2011).
275 The results presented here represent a relevant biotechnological alternative for the treatment of recalcitrant
276 TOPWs, especially when expensive and complex procedures are required for their treatment (Beltrán et
277 al., 1999; Chatzisymeon et al., 2008; Deligiorgis et al., 2008; Javier Benitez et al., 2001; Katsoni et al.,
278 2008; Kotsou et al., 2004; Kyriacou et al., 2005). Up to now, despite several efforts have been made to

propose anaerobic treatment of TOPW, only one study succeeded in methane production from TOPWs, obtaining yield between 245 and 295 $mLCH_4$ g $COD_{removed}^{-1}$ (Beltran et al., 2008). The authors worked with an initial COD concentration of 0.6 g L^{-1}, which indicates that the TOPW was highly diluted (the raw TOPW contained 49.6 gCOD L^{-1}). Therefore, the toxicity of antimicrobial compounds, such as phenolic compounds and chloride, was certainly lower in those conditions.

3.2.3 *Electrochemical performances of the electroactive biofilm during TOPW treatment*

An additional reactor (R3) was operated in fed-batch mode for 4 cycles (almost 3 months) to evaluate long-term performances of the BES at the optimum anode potential (Table 1). As Fig. 4 shows, in R3 a slight gradual increase in the maximum current density was observed from cycle 1 (5.2 A m^{-2}) to cycle 3 (6.6 A m^{-2}), corresponding to the increase of CE from 23% (cycle 1) to 38% (cycle 3). This suggests that the anode activity was enhanced by steady exposure of the anode at +0.2 V throughout the multiple cycles. Similarly to electrochemical performances, COD removal efficiency increased during the fed-batch treatment from 18% (cycle 1) to 32% (cycle 3). The overall electrical performances of reactor fed-batch R3 along with the COD removal efficiencies observed at each CA cycle are reported in Table 2.

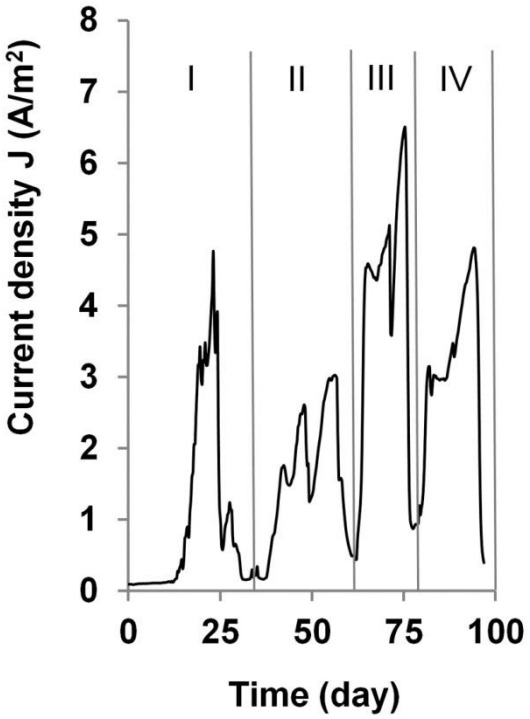


Fig. 4. Chronoamperometric batch cycle of electrochemically derived biofilm of reactor R3 (R3-S-0.2V) operated in fed-batch mode for 4 cycles (I to IV). TOPW was used as substrate at a set anode potential of +0.2V vs SCE. The last cycle (IV) was performed with no inoculum (sediments) addition to corroborate the presence of an active specific biofilm on the electrode.

Interestingly, in the last cycle (cycle 4), which was performed without adding sediment, the CE reached 59% although the maximum current density obtained was lower (5 A m^{-2}). This may be because, in this condition, no extra-COD or alternative electron acceptor from the sediment was present. However, the COD removal was lower (<15%), which suggests that microorganisms present in the sediment, other than ARB, play a key role in the anaerobic degradation of the organic content of TOPW.

Overall, such bioelectrochemical system is suitable to treat and recover energy from recalcitrant TOPW. However, the highest COD removal reached only about 32%, meaning that BES-treated TOPW would require further post-treatment step to remove the remaining COD. At this point, further investigation is required to improve the overall efficiency of the BES and avoid a second step treatment process.

Table 2. Overall electrochemical performances and COD removal efficiency obtained from reactor R3 (R3-S-0.2V) operated in fed-batch mode for 4 cycles (I to IV). TOPW was used as substrate at a set anode potential of +0.2V vs SCE.

Cycle	time (days)	Q_{MAX} (C)	J_{MAX} (A m^{-2})	CE (%)	COD removal efficiency (%)
I	20	4129	5.2	23	18
II	25	5391	3.0	25	29
III	15	8292	6.6	38	32
IV*	17	7170	4.8	59	11

*Cycle IV was performed with no inoculum (sediments) addition.

3.3 Phenolic compounds analysis in bioelectrochemical systems

Phenolic compounds were analysed before and after BES treatment of the TOPW to evaluate their behaviour during operation. UPLC-DAD-MS analysis of TOPW revealed complex chromatographic profiles (SI Fig. S3). Hydroxytyrosol (HT) and tyrosol (TY) were the major peaks detected, with concentrations in the raw TOPW of 1177 ± 94 mg L^{-1} and 178 ± 14 mg L^{-1} equivalent TY, respectively. Losses of HT and TY ranged from 40 to 88% (61-88% for HT and 40-59% for TY) in reactors R1, R2 and R3, whereas in the control reactor C3, no degradation of HT was observed and the loss of TY was limited to 18%.

At the end of the experiment (t_{END}), several peaks which were detected neither in the raw TOPW (t_0) nor in the control reactor (t_{END}) appeared in the chromatograms (SI Fig. S3). The major peak (C) was identified as 3,4-dihydroxyphenyl acetic acid (> 300 mg·L TY equivalent). A smaller peak (E), corresponding to a less polar molecule and showing a similar UV-visible spectrum was tentatively identified as p-hydroxyphenyl acetic acid. Compound C was highly unstable and was gradually converted during storage of the t_{END} sample to compound D identified as 3,4-dihydroxybenzaldehyde (SI Fig. S3). The presence of 3,4-dihydroxyphenyl acetic acid and p-hydroxyphenyl acetic acid is not commonly reported in TOPW. They have been detected in low amount in olive mill wastewater (OMWW) (Dermeche et al., 2013; El-Abbassi et al., 2012). The electrochemical formation of 3,4-dihydroxyphenyl acetic acid and p-hydroxyphenyl acetic acid is possible under certain conditions (Ingle et al., 2000; Mefford et al., 1996). 3,4-dihydroxyphenyl acetic acid was found as an intermediate in the aerobic metabolism of p-hydroxyphenyl to 3,4-dihydroxybenzaldehyde of the aerobic bacterium *Pseudomonas putida* (O'Connor and Witholt, 2001). Catalytic wet air oxidation of p-hydroxybenzoic acid has been also reported to yield p-hydroxybenzylalcohol (not detected in the present study) and p-hydroxybenzaldehyde as main oxidation products (Minh et al., 2007). Elucidation of the electro-/bio-chemical mechanism of the degradation and conversion of phenols in bioelectrochemical reactors needs further investigation.

Nevertheless a possible scheme of the biochemical conversion pathway that occurred in the electroactive system can be proposed (Fig. 5).

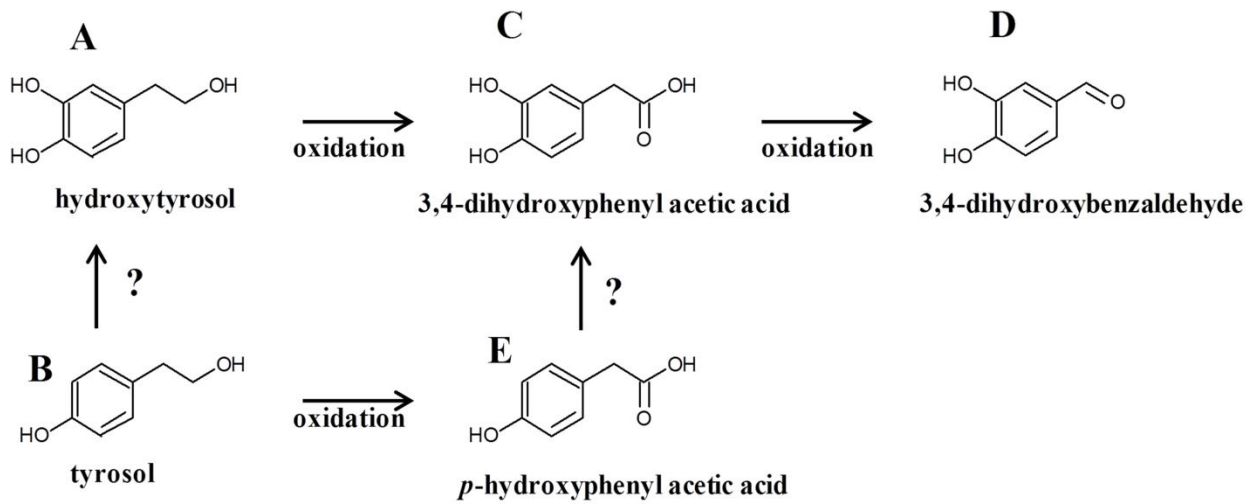


Fig. 5. Proposed scheme of the putative biochemical conversion pathways occurring with phenolic compounds contained in TOPW when treated through a BES here. A/ B and C/E indicate the start and the end of the reaction, respectively; D indicates the molecule formed after storage. Figure 5 indicates hypothetical reactions that could have occurred in the reactor or not.

Mainly, phenolic compounds contained in TOPW present interesting biological activities, and some authors proposed the use of phenolic extract (i.e. hydroxytyrosol, tyrosol and 3,4-dihydroxyphenyl acetic acid) for the stabilization of refined oils as an alternative to synthetic antioxidants (Fki et al. 2005), or for pharmaceutical purposes (Bouaziz et al., 2008; Visioli and Bernardini, 2012). However, the concentration of phenolic compounds found in TOPW (< 2 gL^{-1}) is too low to make an extraction process economically viable, especially when considering the high salt content of TOPW (about 18 gL^{-1}). The amount of phenolic compounds in TOPW is nevertheless higher than the minimal concentration to present an inhibitory effect on microorganisms, as previously reported by Bouaziz et al. (2008). This makes TOPW unsuitable for efficient treatment through conventional biological processes. In our study, current density as well as biogas production in BES were substantially higher than in conventional anaerobic treatment. A

356 part from degradation of the most inhibitory compounds initially present in raw TOPW, another plausible
357 explanation could be that the bacterial biomass within the electroactive biofilm was also protected from
358 the inhibiting components due to the thickness of the electroactive biofilm (De Vrieze et al., 2014). This
359 "protection" effect likely occurred due to the thickness of the biofilm as previously observed by others
360 when exposing electroactive biofilms to certain inhibitory concentrations of heavy metals (Heidrich et al.,
361 2014). Up to now, only a few studies reported the treatment of wastewater (mainly synthetic media)
362 containing phenolic compounds in BES.
363 Nonetheless, a literature review of phenolic compounds removal in BESs is shown in Table 3.
364
365 Table 3. Literature overview of phenolic compounds removal in BESs.

BES type	**Medium type/ carbon source**	**Main phenolic compound detected**	**Initial concentration $mg \cdot L^{-1}$**	**CE (%)**	**Phenols removal in BES system (%)**	**Phenols removal in control system^{+} (%)**	**Ref.**
MFC at 1000 Ω	Synthetic/ phenol	Phenol (synthetic)	100	n.a.	86	64(open-circuit)	(Friman et al., 2013)
MFC at 1000 Ω	Synthetic/ phenol Phenol + Glucose	Phenol (Synthetic)	400 1000+500	1.5 2.7	95 95	88(open-circuit)	(Luo et al., 2009)
MFC at 1000 Ω	Synthetic/ phenol*	Phenol (Synthetic)	600	3.7	88.9	77(open-circuit)	(Song et al., 2014)
Soil MFC at 1000 Ω	Waterlogged paddy soil	Phenol (Synthetic)	80	3.7	90	28(open-circuit) 12(no MFC)	(Huang et al., 2011)
H-type MEC/ Anode applied voltage : +0.6V	Synthetic/ mixture of two furanic and three phenolic compounds	syringic acid; vanillic acid and 4-hydroxybenzoic acid	43 to 180; 40 to 167 and 34 to 133	58 69 44	100 100 100	0	(Zeng et al., 2015)
Single MFC at 1000 Ω	Oil mill wastewater	Gallic acid /3,4-dihydroxybenzoic acid/ tyrosol /p-coumaric acid	1810	N.A.	49	N.A.	(Bermek et al., 2014)

Potentiostatically controlled reactor/ Anode applied potential: +0.2V	Table olive brine processing wastewater	Hydroxytyrosol and Tyrosol	1177 and 178	N.A.	61-88 and 40-59	0 and 18 (not electrochemical)	This study

N.A.= data not available; [+]not electrochemical; *Phenol was used as sole substrate after a gradient acclimation procedure with glucose

Interestingly, the ARB *Geobacter metallireducens* GS-15 was described as able to degrade phenol (Schleinitz et al., 2009). *Cupriavidus basilensis* can grow and produce current (0.31 A m^{-2}) in a medium containing phenol (1.06 mM) as sole carbon source (Friman et al., 2013). Luo et al. (2009) studied phenol degradation in an aqueous air cathode MFC using phenol (400 mg L^{-1}) as sole carbon source. Phenol degradation rates in the MFC were increased by 15% as compared to the open-circuit controls and the degradation efficiencies reached 95% within 60h of reactor operation. Latterly, Zeng et al. (2015) successfully demonstrated the feasibility of producing hydrogen (H_2) using a mixture of two furanic (furfural; 5-hydroxymethyl furfural) and three phenolic (syringic acid; vanillic acid; and 4-hydroxybenzoic acid) compounds as substrates in a two-chamber microbial electrolysis cell (Zeng et al., 2015). In that study, despite the long biofilm acclimatization procedure adopted (6 months enrichment plus 9 weeks start-up), current and H_2 production were inhibited at an initial substrate concentration of 1200 mg L^{-1} (Zeng et al., 2015), which is lower than the phenolic compounds concentration (1894 mg L^{-1}) noticed in the present study.

Recently, Bermek et al. (2014) proposed the treatment of olive mill wastewaters (OMWWs) in single-chamber air-cathode MFC. Although OMWWs are produced by a similar agro-industrial sector as TOPW, they present different physicochemical characteristics. Their main similarity is the high content in phenolic compounds. In the study by Bermek et al. (2014), a stable voltage of 0.35 V at the external resistance of 1 kΩ was produced, while the total phenol removal was around 50%, with more than 90% of gallic acid, tyrosol and p-coumaric acid removed. OMWW was diluted with carbon source free culture

388 medium solution at a 1:10 ratio prior to feeding the MFCs. Such a dilution very likely lowered the toxicity
389 of phenols.
390 Furthermore, Fiorentino et al. (2003) investigated the ecotoxicity of phenolic compounds extracted from
391 OMWW (including HT, T, 3,4-dihydroxyphenyl acetic acid and p-hydroxyphenyl acetic acid) on different
392 aquatic organisms. The authors found that HT had the highest toxicity amongst all tested phenolic
393 compounds. This observation suggests that the toxicity of the products obtained in our study after TOPW
394 treatment with BES should be lower when compared to the molecules of origin, rich in HT.
395

396 ## 3.4 Microbial community analysis of anodic biofilms by sequencing of bacterial 16s
397 rDNA

398 Fig. 6 shows microbial community composition of the anodic biofilms at family level, while the
399 abundance (%) of dominant well-known anode-respiring bacteria (ARB) at genus level is reported in
400 Table 1. Bacterial sequences from the anodic biofilms of the duplicate reactors (R1-S-0.2V and R2-S-
401 0.2V) showed 66.7% of similarity. The two reactors were highly similar in terms of reactor performances
402 (Table 2).
403 The phylum of *Proteobacteria* was always predominant with > 48% of the 16S rDNA bacterial sequences
404 belonging to the "δ" group, while sequences belonging to the phyla of *Bacteroidetes* and *Firmicutes* were
405 found only in minor abundance. The ability of several *Deltaproteobacteria* to transfer electrons to an
406 electrode material has been widely described (Patil et al., 2012). Interestingly, the most abundant ARB
407 species found in the electroactive biofilms belonged to the order of *Desulfuromonadales.* Sequences
408 belonging to the order of *Desulfovibrionales* were also found, but only in minor abundance. Among the
409 *Desulfuromonadales* order, the major number of sequences were representative of a unique OTU belonged
410 to the *Desulfuromonas* genus of *Desulfuromonadaceae* family (55% and 23% of sequences for R1-S-0.2V
411 and R2-S-0.2V respectively), followed in the case of reactors R2, by sequences affiliated to a unique OTU
412 belonging to the *Geoalkalibacter* genus of *Geobacteraceae* family (Table 1 and Fig. 6).

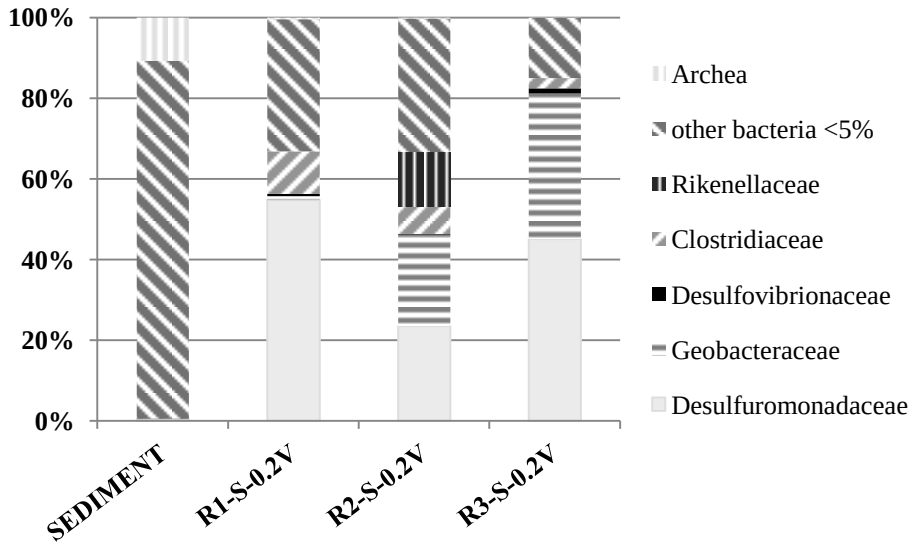


Fig. 6. Microbial community composition (OTUs with sequences abundance >5%) of derived anodic biofilm developed, at set anode potential of +0.2V vs SCE, in replicate batch reactors (R1-S-0.2V and R2-S-0.2V), fed batch reactor (R3-S-0.2V) and in the sediment of origin.

In previous BESs studies, it has already been observed that the microbial communities of anodic biofilms can diverge between replicates due to temporary fluctuation during long term operation despite very similar performances (Ishii et al., 2012). In this study, although in the electroactive biofilms from reactors R1 and R2 some variations of the bacterial community were observed at a family level, the microbial communities can still be considered very similar in terms of selection of ARB. By considering the sequences belonging to the families in which ARB have been previously identified, ARB members represent an average of 50 ± 6% of all total bacterial sequences. This could explain the high reproducibility in terms of electrochemical performance in reactors R1 and R2 (average maximum current density of 7.1 ± 0.4 A m^{-2}) and indicate a successful enrichment of electroactive bacteria at the anodes. This last finding is confirmed by the analysis of the anodic microbial community in the fed-batch reactor, R3, which was operated for over 3 months. In the R3 (R3-S-0.2V) electroactive biofilm, the percentage of

sequences affiliated to *Desulfuromonadales* increased up to 81% and they were affiliated to both the *Desulfuromonas* (45%) and *Geoalkalibacter* (36%) genera (Table 1). Therefore, such long-term operation further enriched the electroactive biofilm in ARB.

The results obtained in this study are in good agreement with previous study in which ARB species found in electroactive biofilms, grown from a saline sediment-based inoculum were related to either *Desulfuromonas acetoxidans* or *Geoalkalibacter subterraneus* with 97% DNA similarity (Pierra et al., 2015).

The fraction of *Archaea* 16S rRNA genes was very low ($\leq 0.5\%$) in all the anodic communities when compared to the 11% found in the sediment used as inoculum (see Section SI) and $15 \pm 0.02\%$ found in the bulk liquid of reactors R1 and R2. Therefore, it can be concluded that methanogenic activity was mostly attributable to the activity of planktonic methanogens.

4. CONCLUSIONS

For the first time, the use of bioelectrochemical systems was successfully applied for remediation and energy recovery from TOPW, a wastewater usually recalcitrant to biological degradation.

In this context, an anodic applied potential of +0.2V vs. SCE was the most suitable to grow an effective ARB community from a saline sediment. A maximum current density of 7.1 ± 0.4 Am^{-2} was achieved with a Coulombic Efficiency (CE) of 30%. Additionally, the employed conditions were successful to obtain a CH_4 production of 701 ± 13 NmL $CH_4 \cdot L_{TOPW}^{-1}$ equivalent to 109 ± 21 $NmLCH_4/gCOD_{removed}$.

Interestingly, up to 80% of the main phenolic compounds detected in the raw TOPW, *i.e.* hydroxytyrosol and tyrosol, were removed and a theoretical conversion pathway was proposed after identification of the metabolic by-products. The electro-/bio-chemical mechanism of the degradation and conversion of phenols in bioelectrochemical reactors remains unknown and needs further investigation.

Overall, all these results strongly support that bioelectrochemical systems might be suitable to treat and recover energy from recalcitrant TOPW. In this prospective, treatment and energy valorization of TOPW

by BES opens an attractive field of investigation and bioelectrochemical systems could become a new effective technology for remediation of TOPW. Nevertheless, at this stage, further efforts are still required to optimize the whole efficiency of the process and reach a mature treatment technology.

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