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Comparison of two strategies for the start-up of a biological reactor for the treatment of hypersaline effluents from a table olive packaging industry.

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Abstract

Biological treatment of hypersaline effluents with high organic matter concentrations is difficult to carry out and it can require a long start-up phase. This is the case of the treatment of fermentation brines from the table olive packaging (FTOP) industries. These effluents are characterized by conductivity values around 90 mS/cm, COD around 15000 mg/L and total phenols concentration around 1000 mg/L. In this work, FTOP has been treated in two sequencing batch reactors (SBRs) operated in parallel. In each SBR a different start-up strategy has been carried out. In the SBR-2, biomass was previously acclimated to high salinity using simulated wastewater without phenolic

compounds, meanwhile in the SBR-1, FTOP was added from the beginning of the start-up. Results indicated more operational problems in the SBR-2 consisting in a higher deflocculation that drove to high turbidity values in the effluent. Besides, at the end of the start-up, the SBR-1 reached higher COD removal efficiencies than SBR-2 (88% and 73%, respectively). In both reactors, an increase in γ-proteobacteria in the microbial population was observed for increasing conductivities. In addition, phenols were completely removed in both reactors at the end of the start-up, what implied very low toxicity values in the effluent.

Keywords: Fermentation brines; Hypersaline effluents; Polyphenols biodegradation; SBR; Table olives; Wastewater treatment.

1. INTRODUCTION

Wastewaters from some industries are characterized by high organic matter and salts concentration. Their treatment by means of biological processes is always complicated, especially when the high wastewater conductivity is combined with some organic compounds, as phenolic compounds, that can inhibit biomass. The main types of industries that generate high salinity effluents are food processing industry (mainly pickled vegetables and fish processing industries), tanneries and petroleum industries [1].

It is well-known that salinity affects the correct performance of an activated sludge process. The effects on the sludge have been summarized in some review papers [1–3]. Salt concentrations above 1-2% may result in plasmolysis and loss of activity of cells.

In addition, the physical properties of the activated sludge are affected, decreasing their hydrophobicity, filterability, settlement and bioflocculation [4,5]. However, an acclimation of the microorganisms is possible by means of a gradual salinity increase. Acclimation will not be successful if salinity is increased too rapidly [6], what would imply the release of cellular material and consequently an increase in soluble COD. On the other hand, a sudden decrease of salinity is also damaging for biomass. This also implies that settling is affected, especially when NaCl concentration is higher than 20 g/L [7].

Some authors have reported the existence of a limiting salt concentration for the achievement of an appropriate organic matter removal with an adapted activated sludge [8]. According to them, the use of halophile microorganisms would be the key to enhance the process performance. Halophilic microorganisms are those that require salt for their survival and can be classified into moderate (3 – 15% NaCl) and extreme (15 – 30% NaCl) halophiles [9]. Other authors report slightly different NaCl ranges (5 -20% for moderate and 20-30% for extreme halophiles) [10].

One of the hypersaline effluents coming from industry is the fermentation brine from the table olive processing (FTOP). The finality of table olive processing is to make edible the olive fruit. This is performed by the following steps: 1) Debittering; treatment with sodium hydroxide solution (1–2% w/v) to remove the olive natural bitterness (in this stage, oleuropein is hydrolysed) [11]. 2) Rinsing cycles for eliminating the alkali excess. 3) Fermentation; olives are submerged in brine (4–8% w/v) of sodium chloride for several months. The wastewater volume generated in all stages is about 3.9–7.5 m^3 per ton of green olives [12]. FTOP contributes to the 80-85% of the global pollution of wastewater generated during the production in these types of agro-food industries [13].

However, it represents only 20% of the total volume. This is the reason why it is important to segregate the FTOP to treat it separately.

FTOP is characterized by high conductivities (around 90 mS/cm) combined with high organic matter content (between 7 and 20 g/L of CDO), and phenols compounds (between 700 and 1500 mg/L). These features will entail very high environmental impacts if these effluents are not correctly managed [14]. The traditional management of these effluents consisted in either their disposal in lagoons for water evaporation or their transport to large municipal wastewater treatment plants for their blending with the municipal wastewater. However, the increasing legislation strictness and environmental awareness have led to study different alternatives for the management of these effluents.

Biological treatment of olive oil mill wastewater has been reported in many research works [15,16]. These effluents are characterized by COD ranges between 35 to 200 g/L (around 10% of this organic matter corresponds to phenolic compounds), and high total solid content. If olive mill wastewaters (OMW) and FTOP are compared, COD of OMW is higher than COD of the FTOP. However, conductivity of the FTOP is considerably higher than that of the OMW. In fact, conductivity values of the FTOP are around 10 times higher than those reported for OMW. A direct biological treatment of the fermentation brines has not yet been reported in the bibliography. There are only a few works in which the removal of phenolic compounds from saline wastewater has been studied [17,18], but they are performed with simulated water. However, there are several studies that consider the treatment of other table olive packaging effluents; as the global wastewater [19], the global wastewater excluding fermentation brines [18,21], the alkaline debittering wastewaters [22,23]and olive washing water [24]. In other papers, FTOP is treated by other techniques, such as electro-coagulation [25] or

the biological treatment is combined with chemical or electrochemical processes [26,27].

The aim of this work is to perform a direct biological treatment of FTOP wastewater from a table olive packaging industry without previous dilution or physico-chemical treatment, by a gradual adaptation of activated sludge to high salinity and polyphenols. Difficulties for the treatment of these wastewaters are not only focused on high salinities but also on the eventual inhibitory effect of the polyphenols concentration. The experiments were performed in two sequential biological reactors (SBRs), and two different start-up strategies have been compared.

2. MATERIAL AND METHODS.

2.1. Analysis.

For the tests, two different samples from the table olive packaging industry (FTOP 1 and FTOP 2) were used. The characterization of fermentation brines included the analysis of pH, conductivity, soluble COD (filtered to 0.45 µm), total phenols (Folin-Ciocalteu method), phenolic profile (analysis of simple phenolic compounds with UPLC-PDA analysis), sodium, chloride, turbidity, suspended solids (SS), volatile suspended solids (VSS) and total antioxidant activity (TAA). For the characterization of the SBRs effluents, pH, conductivity, soluble COD (filtered to 0.45 µm), turbidity and total phenols were monitored. In the last days of the start-up, in order to check phenols degradation, total phenols, phenolic profile, TAA and toxicity were measured. SS, VSS

and microbial community analysis by fluorescence in situ hybridization (FISH) were measured to characterize the biomass in SBRs.

pH and conductivity measurements were carried out with pH-Meter GLP 21+ and EC-Meter GLP 31+ (CRISON), respectively. Turbidity was determined with a Turbidimeter D-112 from DINKO INSTRUMENTS. Suspended solids (SS) and volatile suspended solids (VSS) were measured according to APHA, 2005 [28]. Sodium and chloride ions and soluble COD were analyzed using kits and a Spectrophotometer DR600 (HACH LANGE).

2.1.1. Phenolic compounds and total antioxidant activity.

For phenols measurement, all samples were previously treated in order to extract them according to El-Abbassi et al. [29]. The extracts were brought to dryness in a rotary evaporator (Rotavapor R-114 from BÜCHI) at 40ºC and the residue was dissolved in methanol. The extracts obtained were used for total phenols and UPLC-PDA analysis. Total phenols (simple phenolic and polyphenolic compounds) were measured spectrophotometrically according to the Folin-Ciocalteu method [30]. Results were expressed as ppm equivalent of tyrosol (mg TY/L). Phenolic profile was measured by liquid chromatography. UPLC-PDA analysis were carried out on Waters Acquity UPLC system (Milford, MA, USA) equipped with a binary solvent manager, sample manager, column compartment, and 2996 PDA detector, connected to Waters Masslynx 4.1 software. The separation was carried out using a Waters BEH C18 column (2.1 × 100 mm, 1.7 μm) at 40ºC. The optimal chromatographic conditions were established: solvent system, phase A, 1 % formic acid in acetonitrile, and phase B, 1 % formic acid in water; gradient conditions were as follows: 100% B at 0 min for 1 min to 55% A in

25 min, then 100% A at 30 min, held for 5 min, returned to 100% B in 2 min, and equilibrated for 3 min before the next injection; flow rate of 0.4 mL min-1 and injection volume of 5 μL. Results were expressed as ppm of phenolic compound tested.

Total antioxidant activity (TAA) was determined by the modified version of ABTS assay reported by Cassano et al. [31]. Results were expressed in terms of mM trolox equivalents.

2.1.2. Toxicity

The Microtox® was used for the estimation of the toxicity [32]. The light emission reduction of microorganisms *Vibrio fischeri* in contact with FTOP was measured. The effective concentration of contaminant (mg/L) which reduces a 50% of the intensity of light bacteria emission, after 15 minutes contact, is named EC_{50}. The toxicity results have been expressed in toxicity units (TU). This parameter is the inverse of EC_{50} multiplied by 100. Emission toxicity limit values for industrial wastewater discharges into the municipal sewer system according to regional authority (EPSAR) are 15 (maximum daily average concentration) and 30 (maximum instantaneous concentration) [33].

2.1.3. Fluorescence in situ hybridization (FISH) and microscopic observation for quantification Bacteria and Archaea.

Samples were fixed in 4% paraformaldehyde at 4 ºC for Gram-negative organisms and in 50% ethanol at 4 ºC for Gram-positive [34]. The fixed biomass was washed three times with phosphate-buffered saline (PBS), and re-suspended in a 1:1 (v/v) volume of

PBS and absolute ethanol and then stored at -20 ºC. The fixed samples were immobilized on gelatin-coated glass slides, air-dried, and consecutively dehydrated in 50%, 80% and absolute ethanol. Hybridization buffer and probes were applied to the slide and incubated at 46 ºC for 1-3 hours. Excess probes were washed off by heating at 48ºC for 15 min in a washing buffer [35]. List of oligonucleotide probes [36] applied and respective formamide (FA) concentrations are shown in Table 1.

Table 1. List of oligonucleotide probes applied and respective formamide concentrations

Probe	Sequence	%FA	Organism
EUB338	GCTGCCTCCCGTAGGAGT	35	Most bacteria
EUB338II	GCAGCCACCCGTAGGTGT	35	*Planctomycetales*
EUB338III	GCTGCCACCCGTAGGTGT	35	*Verrucomicrobiales*
EUB338IV	GCAGCCTCCCGTAGGAGT	35	*Eubacteria*
ALF968	GGTAAGGTTCTGCGCGTT	35	*α-Proteobacteria*
CFX1223	CCATTGTAGCGTGTGTGTMG	35	*Chloroflexi*
GNSB941			
BET42a	GCCTTCCCACTTCGTTT	35	*β- Proteobacteria*
BET42a competitor	GCCTTCCCACATCGTT		
GAM42a	GCCTTCCC CATCGTTT	35	*γ- Proteobacteria*
GAM42a competitor	GCCTTCCCACTTCGTTT		
LGC354a	TGGAAGATTCCCTACTGC	35	*Firmicutes*
LGC354b	CGGAAGATTCCCTACTGC		
LGC354c	CCGAAGATTCCCTACTGC		
HGC69a	TATAGTTACCACCGCCGT	25	*Actinobacteria*
HGC69a competitor	TATAGTTACGGCCGCCGT		
CF319a	TGGTCCGTATCTCAGTAC	35	*Cytophaga*
ARCH915	GTGCTCCCCCGCCAATTCCT	35	*Archaea*

Microscopic observation was performed using an epifluorescence microscope (Olympus BX50 equipped with a CCD camera (Olympus DP12). A minimum of 20 images of randomly chosen microscopy fields were taken for each probe-hybridized sample. The

signals detected by FISH were quantified using automated bacteria quantification software [37] based on thresholding techniques using Matlab 7.1. The software-generated report states the percentage areas occupied by hybridized bacteria and the measurement uncertainty, i.e. the standard deviation divided by the square root of the number of fields examined.

2.1.4. Isolation and identification of saline tolerant bacteria.

Saline tolerant bacteria were isolated from SBR-1. Enrichment culture were obtained using salinity wastewater medium (SWM) containing NaCl (70 g/L). After 48 h culturing at shaking speed of 130 rpm/min and room temperature incubation, 1 mL from the SWM was transferred with pipette onto the salt medium agar [38], containing NaCl 70 g/L, and surfaces of the Petri dishes and incubated for 24-48 h at room temperature. Purified colonies were obtained by repeated streaking onto salt medium agar. The salt tolerant isolates were suspended in glycerol medium and stored at -20ºC. The 16S rRNA gene of the isolate was PCR amplified using bacterial universal primers F27 (5′–AGAGTTTGATCMTGGCTCAG–3′) [39] and R1492 (5´–TACGGYTACCTTGTTACGACTT–3´) [40]. PCR products were purified using GenElute PCR Clean-Up Kit (Sigma-Aldrich, Saint Louis, Missouri, USA), and were sent to IBCMP (Valencia, Spain) for sequencing. The 16S rRNA gene was amplified from genomic DNA, purified and sequenced, and the 16S rRNA fragment for H1 was sequenced and submitted to the National Center for Biotechnology Information for BLAST analysis [41].

2.2. Biological reactors.

The experiments were carried out in sequencing batch reactor (SBR). SBR presents many advantages in pilot-scale work: low construction and maintenance cost, robustness, single basin operation, better control of shock loads, and flexibility in operation. In fact, they have been particularly used for the treatment of saline wastewater [9,17,42] so it is validated their suitability for the purpose of this study.

Two identical laboratory SBRs were operated in parallel. In each SBR, mixing was carried out by a mechanical stirrer (VELP SCIENTIFICA). Mixing was connected during all the reaction phase. Aeration was provided by a compressor Air 550 R Plus (SERA PRECISION), air flow rate 550 L/h, through a diffuser on the bottom of the reactors. An oximeter OXI 49 (CRISON) measured the oxygen concentration in the reactor and this was regulated automatically between 1.5 and 2.5 mg/L. According to the established phase duration, one of the peristaltic pumps (AIGUAPRES) switched on either to begin the fill or the drawing phase. Pumps switched off according to the level indicator. A scheme of the each SBR is illustrated in figure 1.

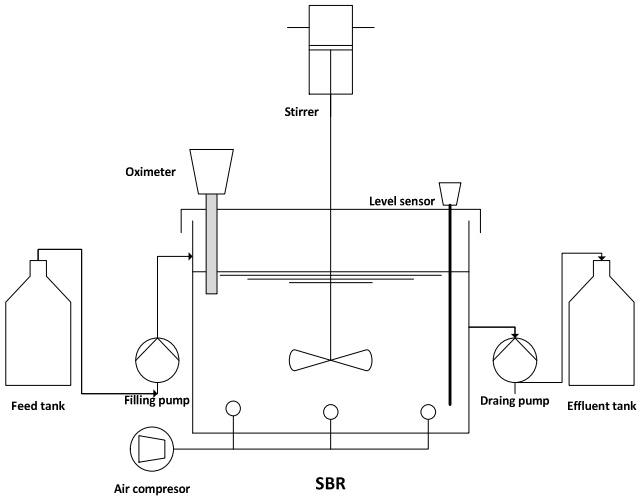


Figure 1. SBR scheme.

2.2.1. SBRs operation

Both SBRs (SBR-1 and SBR-2) were seeded with mixed liquor from a biological reactor treating landfill leachate (conductivity = 12 mS/cm). This activated sludge presents two advantages over conventional cultures. On the one hand, it was already acclimated at salinity concentration higher than that of a municipal plant. On the other hand, leachates contain slowly degradable organic matter and even inhibitory substances; thereby the biomass was also adapted to complex organic substances. These features could make possible a faster biomass adaptation under conditions of increasing salinity and phenols.

The strategy of the start-up of the SBR-1 consisted in feeding it with FTOP in order to adapt biomass increasing gradually both salinity and phenolic compounds. However, the SBR-2 was previously adapted to high salinity. The SBR-2 was fed with simulated wastewater (SWW), whose composition was: 20 g/L of casein peptone, 2.5 g/L of glucose, 2.5 g/L of dipotassium hydrogen phosphate anhydre and 100 g/L de sodium chloride. The COD of the SWW was around 10000 mg/L. Once mixed liquor conductivity reached 60 mS/cm and the COD of the SWW was removed above 85% (data not shown), FTOP was fed to the reactor. This pre-acclimation procedure was achieved in 45 days. From this moment, the comparison study of the two strategies for the start-up in both reactors began.

Table 2 shows the SBRs operation characteristics (identical operation for both SBRs once both reactors were fed with FTOP). SBRs were operated in 24 hours cycles. Each cycle consisted of the following steps: filling, reaction, sedimentation, draw, and idle. The duration of each phase through cycle is also presented in table 2.

242

243 **Table 2. SBRs operation characteristics.**

	Operation characteristics
Reaction volume	6 L
Feed volume	150 mL/d
Temperature	18-20ºC
Dissolved oxygen	1.5 – 2.5 mg/L
Hydraulic retention time	40 days
Daily number of cycles	1
Operation days	108
Cycle characteristics	
Phase	**Time**
Filling	2 min
Anoxic reaction	60 h
Aerobic reaction	21 h
Sedimentation	1.5 h
Draw	2 min
Idle	26 min

244

245

246 The feed FTOP volume was 150 mL/d in both reactors, but the initial conductivity were
247 12 mS/cm in the SBR-1 and 60 mS/cm in the SBR-2 (the difference was due to the
248 biomass pre-acclimation to salinity in SBR-2). Through the operation days, the initial
249 conditions in the mixed liquors were changing. Salinity and phenols concentrations
250 gradually increased in both reactors. The high hydraulic retention time (40 days) was
251 necessary in the starting-up period due to the biomass inhibition by the high salinity
252 content and phenolic compounds and due to the high COD levels. During the SBRs
253 operation no sludge was withdrawn.

254 During the SBRs operation time (108 days), 2 different FTOP wastewater samples were
255 treated. The first sample was fed to the reactors from the first day to the day 48th while
256 the sample 2 was used from day 49th to 108th. Table 3 shows their characteristics. The
257 parameters presented in this table were measured by triplicate.

258

259 **Table 3. FTOP characteristics.**

Characteristics	FTOP 1	FTOP 2
pH	3.9 ± 0.02	4.0 ± 0.02
Conductivity (mS/cm)	90.3 ± 0.3	94.2 ± 0.2
COD (mg/L)	14130 ± 130	17700 ± 95
N_T (mg/L)	270 ± 7	365 ± 10
P_T (mg/L)	62 ± 3	75 ± 7
Suspended solids (mg/L)	1010 ± 18	936 ± 32
Chloride concentration (mg/L)	47970 ± 215	50000 ± 325
Sodium concentration (mg/L)	81500 ± 200	81500 ± 185
Total phenols (mg tyrosol/L)	929 ± 8	1109 ± 11
Antioxidant capacity (mM trolox)	13.4 ± 0.3	11.1 ± 0.3

260

261 As it can be observed, FTOP is characterized by acidic pH (around 4). Conductivity is
262 very high (above 90 mS/cm) due mainly to the sodium chloride added for olives
263 conservation. Suspended solids concentration is also high (around 1000 mg/L), what
264 implies high turbidity values. Concerning organic matter, COD values were 14130
265 mg/L in FTOP-1 and 17700 in FTOP-2. Total phenols concentration was around 1000
266 mg tyrosol/L.

267 The necessity of nutrients in wastewater was evaluated by the relationship: COD/N/P in
268 amounts 250/5/1. According table 3, it can observed that no external nutrient addition
269 was needed in the experiments.

3. RESULTS AND DISCUSSION.

3.1. SBR-1 performance.

Figure 2 shows the evolution of the COD removal efficiency, the conductivity and the turbidity values in the effluent for 108 days of reactor operation. For this period (feeding with FTOP 1 and FTOP 2) no nutrients were required. During this time pH effluent was between 8.2 and 8.5. Temperature of the reactor was between 18 and 20ºC.

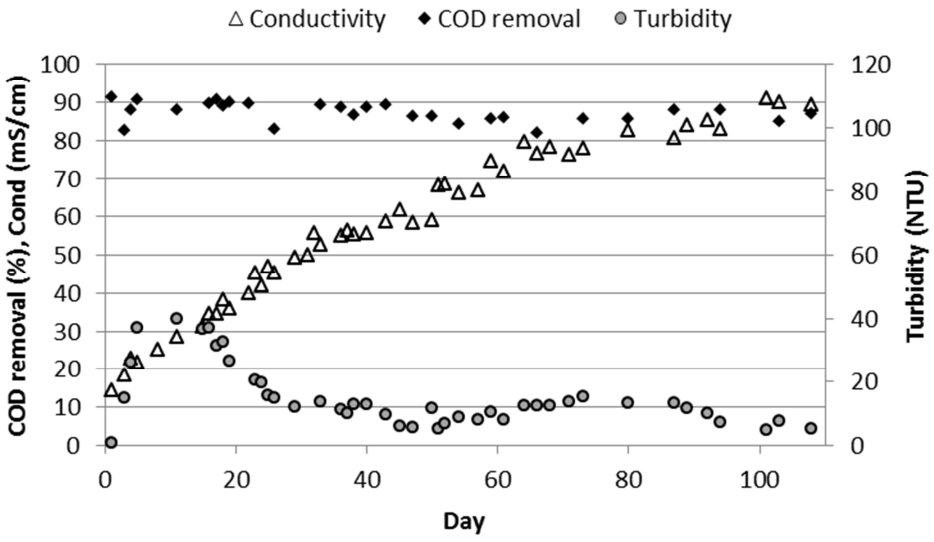


Figure 2. COD removal efficiency (%), conductivity (mS/cm) and Turbidity (NTU) of the effluent from SBR-1.

In figure 2, it can be observed that the conductivity from SBR-1 had changed from 12 to 59 mS/cm after 43 operating days. In spite of the conductivity increase, the COD removal efficiency of SBR-1 remained around 90% (COD in the SBR-1 effluent was around 1700 mg/L). From 54^{th} to 80^{th} day, conductivity of the SBR-1 went on

increasing up to 83 mS/cm. During this period, COD removal efficiency had been slightly reduced down to 85% because FTOP-2 was fed from 49th day. FTOP-2 COD was higher than FTOP-1 one. From 81th to 108th day COD removal efficiency increased to 87.1% ± 1.5. This slight increase was due to the increase of the MLSS in the reactor (Figure 3), since no sludge withdrawal was carried out. Also, SBR-1 conductivity increased to 91 mS/cm, which is very near the FTOP wastewater conductivity. In this way, it can be stated that the start-up of the reactor had finished.

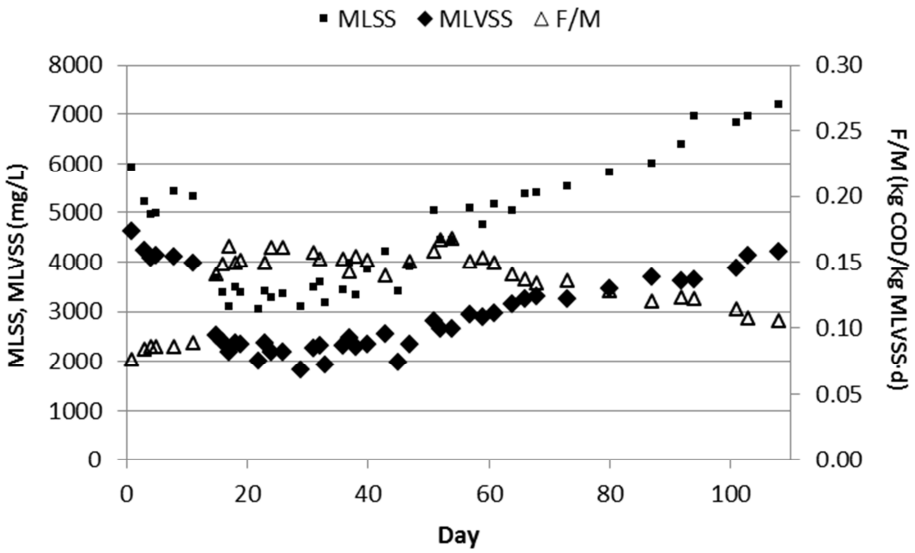


Figure 3. MLSS, MLVSS and the relation F/M in the SBR-1.

Figure 3 shows the parameters measured of the mixed liquor samples from the SBR-1: MLSS, MLVSS and the ratio food-to-microorganism (F/M). The F/M was calculated by Eq. 1 [43]:

$$F/M = \frac{COD_0 \cdot Q}{V_R \cdot MLVSS} \qquad \text{Eq.(1)}$$

where COD_0 is the initial COD in FTOP 1 or FTOP 2 (mg/L), Q is the daily wastewater volume fed to SBR (L/day), V_R was the reaction volume (L) and MLVSS is the mixed liquor volatile suspended solids in mg/L.

In the first days F/M was around 0.09 kg COD/kg MLVSS·d. This parameter increased to 0.14 kg COD/kg MLVSS·d in 15^{th} day, because there was a MLVSS drastic reduction, from 3900 to 2500 mg/L. This phenomenon can be explained because floc disaggregation occurred during the first days of the SBR-1 start-up, due to osmotic shock. In particular, an increase in the effluent turbidity was observed (turbidity reached 39 NTU) due to the presence of biomass in the effluent, which may be caused by cell lysis and reduction of the populations of protozoa and filamentous organisms required for proper flocculation [18]. As expected, MLSS also decreased from 5900 to 3100 mg/L. From 17^{th} day to 47^{th}, MLVSS remained around 2300 mg/L and ratio F/M was between 0.14-0.16 kg COD/kg MLVSS·d. Turbidity remained between 5 and 10 NTU. From 47^{th} to 108^{th} day MLSS and MLVSS increased progressively up to 7200 and 4200 mg/L, respectively. From this period on, ratio F/M decreased to 0.10 kg COD/kg MLVSS·d. This organic load value lies in the range commonly used for the design and operation of biological reactors treating wastewaters from agro-industries [43]. Thus, it can be concluded that COD removal efficiency can be as high as in non-saline wastewater.

Other authors [44] reported that COD removal efficiencies were reduced from around 90% to 63% when influent salinity was higher than 50 g/L. These authors worked with a SBR treating soaking wastewater from a tannery. Thus, the type of wastewater and, consequently, its characteristics could exert an additional influence on the SBR

performance. In the case of FTOP wastewater it can be achieved due to the presence of high concentrations of biodegrabable COD like volatile organic acids and glucose.

3.2. SBR-2 performance.

Figure 4 shows the evolution of the COD removal efficiency in the reactor operation and the conductivity and turbidity values of the reactor effluent. As in SBR-1, pH effluent was between 8.2 and 8.5. Figure 5 illustrates the parameters characterizing the mixed liquor from the SBR-2. Reactor temperature was around 20-22 ºC.

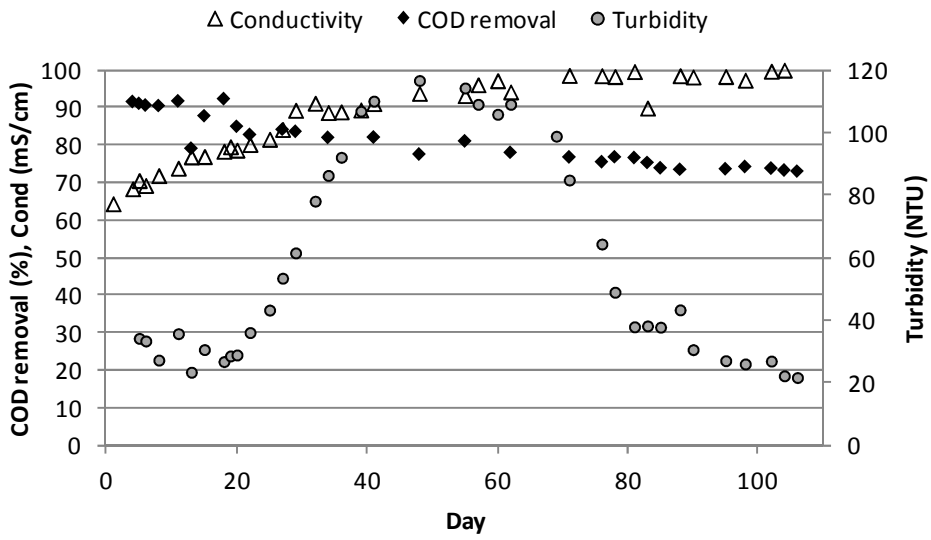
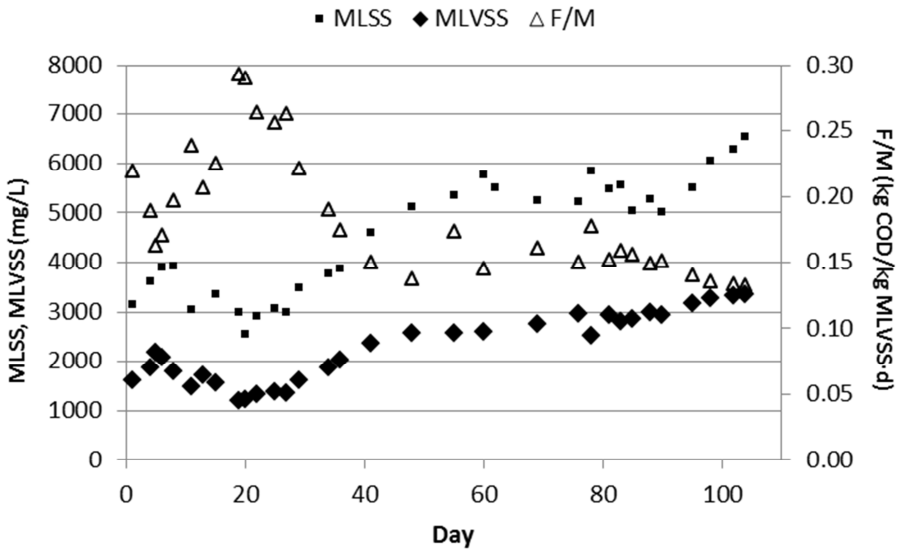


Figure 4. COD removal efficiency (%), conductivity (mS/cm) and Turbidity (NTU) of the effluent from SBR-2.



336

337 **Figure 5. MLSS, MLVSS and the relation F/M in the SBR-2.**

338

339 After 20 operating days, SBR-2 conductivity had changed from 64 to 83 mS/cm and the
340 COD removal efficiency decreased from 91 to 85% (COD in the SBR-2 effluent was
341 near 2100 mg/L). The loss in COD removal efficiency was due because in this period
342 SSLM and MLVSS decreased. This phenomenon can be explained by the negative
343 initial effect of the polyphenols on the biomass previously acclimated to high salinity
344 conditions. MLSS decreased from 3900 to 2500 mg/L, and MLVSS from 2100 to 1200
345 mg/L. Accordingly, the ratio F/M increased from 0.22 to 0.29 kg COD/kg MLVSS·d.

346 From 21th to 60th day biomass was gradually adapted to phenolic compounds and both
347 SSLM and SSVLM increased to 5500 and 2600, respectively. The ratio F/M decreased
348 to 0.14 progressively. Nevertheless COD removal efficiency decreased to 78%, because
349 the FTOP-2 was fed from 49th day, and their COD was higher than FTOP-1. As
350 expected, COD removal efficiency decreased.

From 61th to 108th MLSS and MLVSS increased slowly. MLSS increased to 6500 mg/L and MLVSS to 3400 mg/L. The ratio F/M was near 0.15 ± 0.01 kg COD/kg MLVSS·d. For this period the COD removal efficiency remained around 74.5% ± 1.4.

From 20th to the 48th day turbidity increased from 29 to 117 NTU. Unlike SBR-1 it was not associated with a MLSS diminution, but changes in biomass population drove to a release of cellular material, what implied an increase in the effluent turbidity. From 49th to 90th biomass was gradually adapted to phenolic compounds and turbidity decreased down to 30 NTU. COD removal efficiency decreased to 74%. Afterwards, turbidity went on decreasing more slowly down to 20 NTU, but COD removal remained around 75%. At the same time, MLSS increased gradually up to 6500 mg/L.

3.3. Comparison of the performance of the reactors.

The behavior of the two reactors related to pH variation was the same. It can seem surprising that pH above 8 is maintained in the reactors when they are fed with FTOP, whose pH is 4. This low pH is due to the organic acids, formic and acetic acid, which are produced in high concentrations in the fermentation process. In addition, the action of *lactobacters* convert olives sugars into lactic acid, which also contributes to pH decrease [45]. They were degraded in the biological process and pH consequently increases [46].

The ratio F/M was different in both reactors for the first 40 days of operation. However, from that day on, the ratio F/M resulted similar in the two reactors (as it can be observed in figures 3 and 5), reaching 0.14 ± 0.02 kg COD/kg MLVSS·d in SBR-1 and 0.15 ± 0.01 kg COD/kg MLVSS·d in SBR-2.

When COD removal efficiencies are compared in both reactors, it can be observed that SBR-1 performance hardly diminished with the operation time, meanwhile the pre-adapted to salinity SBR-2 yielded lower COD removal efficiencies than SBR-1. The extremely high conductivity values (near 100 mS/cm) and the different start-up strategy drove to reactor performance reduction. At the end of the start-up COD removal efficiencies were near 74.5% ± 1.4 in SBR-2 and 87.1% ± 1.5 in SBR-1.

In figure 6 the relationship MLVSS/MLSS in both reactors is presented. It can be seen that the ratio MLVSS/MLSS in the first days in SBR-1 was 0.81.

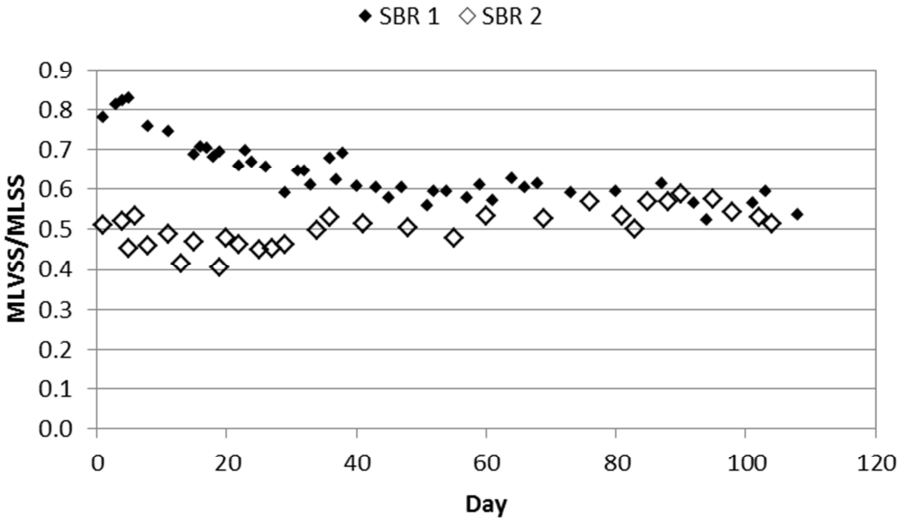


Figure 6. MLVSS/MLSS from SBR-1 and SBR-2.

From 4th to 43th day the ratio MLVSS/MLSS decreased to 0.60. This indicates accumulation of inorganic compounds inside the microbial flocs when salinity increased [47] (conductivity changed from 12 to 59 mS/cm after 43 operating days). In SBR-2, initial ratio MLVSS/MLSS was around 0.51, and conductivity was 58.7 mS/cm. This value of MLVSS/MLSS is similar to that achieved in SBR-1 for the same conductivity. This can explain that COD removal efficiency was higher in SBR-1 than in SBR-2 since

391 the amount of microorganisms that potentially could degrade the organic matter was
392 higher in SBR-1. From 44th day the values of MLVSS/MLSS were similar in both
393 reactors, and remained around 0.59 ± 0.03 in SBR-1, and 0.53 ± 0.04 in SBR-2. These
394 low values can be explained by the high sludge retention time, what enhances the cell
395 endogenous respiration, that is, bacteria oxidize their own cellular material. It has to be
396 pointed out that the sludge retention time in SBR-2 was higher than in SBR-1 since
397 sludge came from the pre-adaptation period. It justifies the lower volatile percentage of
398 the mixed liquor in SBR-2 in comparison with that measured in SBR-1.

399

400

401 **3.4. Phenolic compounds removal, TAA and toxicity of the effluents.**

402 As explained in the materials and method section, 2 different FTOP samples were used
403 as feed for the SBRs. In both samples only hydroxytyrosol (HTY) and tyrosol (TY)
404 were identified, that is, no other polyphenols compounds were detected. This agrees
405 with Brenes et al. [48] and Fendri et al. [49]. These authors reported that some phenolic
406 compounds such as caffeic acid and p-cumaric acid disappear during the fermentation
407 stage, however, HTY and TY concentrations remain practically constant. HTY is the
408 main product of the hidrolysis of oleuropein therefore its concentration is predominant
409 in FTOP wastewater [48,50]. Table 4 shows the concentrations of total phenols, HTY
410 and TY, TAA and toxicity of both FTOP samples and the SBRs effluents (SBR-1 and
411 SBR-2) in the last days of the start-up (100th day).

412

413

414 **Table 4. Total phenols, HTY and TY concentration, TAA and toxicity.**

	FTOP 1	FTOP 2	SBR-1	SBR-2
Total phenols (mg TY/L)	929 ± 8	1109 ± 11	24 ± 2	33 ± 2
HTY (mg HTY/L)	553 ± 37	613 ± 25	n.d	n.d
TY (mg TY/L)	82 ± 6	76 ± 7	n.d	0.77 ± 0.1
TAA (mM trolox)	13.4 ± 0.3	11.1 ± 0.3	1.8 ± 0.04	1.9 ± 0.01
Toxicity (UT)	38.8 ± 0.2	40.0 ± 0.8	3.6 ± 0.2	3.1 ± 0.3

415 **n.d. = not detected**

416

417 It can be stated that phenols were removed in both SBRs. In fact, 97.8% and 97.0%
418 were the total phenols removal efficiencies reached at the end of the start-up in the
419 SBR-1 and the SBR-2, respectively. This was confirmed by the HTY and TY by UPLC-
420 PDA analysis. In Table 4 it can be observed that the presence of HTY and TY was not
421 detected. In the same way, the TAA was removed at a high extent (80 %).

422 However, in a complex wastewater and with no pure bacterial cultures it is not possible
423 to confirm that polyphenols have been degraded completely to carbon dioxide and
424 water. Although phenols have been hardly detected in the SBRs effluents, formation of
425 other compounds as quinones could also occur. Toxicity analyses help confirming that
426 no dangerous intermediate products have been formed. The toxicity of FTOP 1 and
427 FTOP 2 were 38.8 and 40.0 TU, respectively. After biological treatment, the effluent
428 toxicity decreased to 3.6 TU in SBR-1 and 3.1 TU in SBR-2.

429

430

431

3.5. Microbial community analysis.

Figure 7, 8 and 9 show the evolution of the bacteria and archaea population during the tests (46th, 66th and 108th, respectively) from the active biomass in the SBR-1 and the SBR-2.

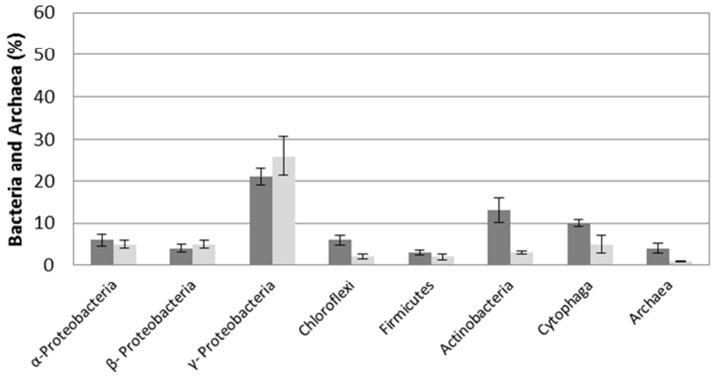


Figure 7. Quantification Bacteria and Archaea from 46th day;

SBR-1 (58.3 mS/cm), SBR-2 (77.0 mS/cm)

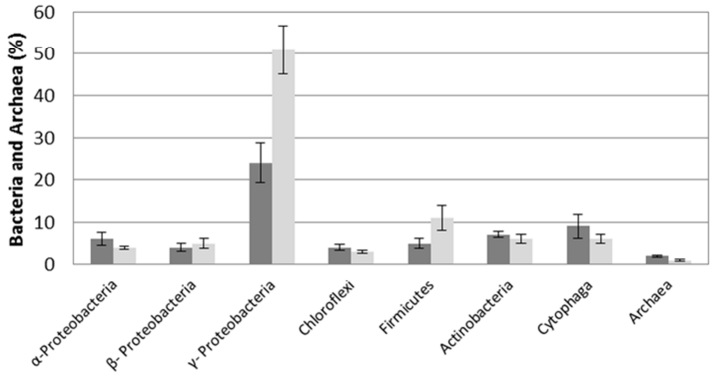


Figure 8. Quantification Bacteria and Archaea from 66th day;

SBR-1 (76.7 mS/cm), SBR-2 (88.7 mS/cm)

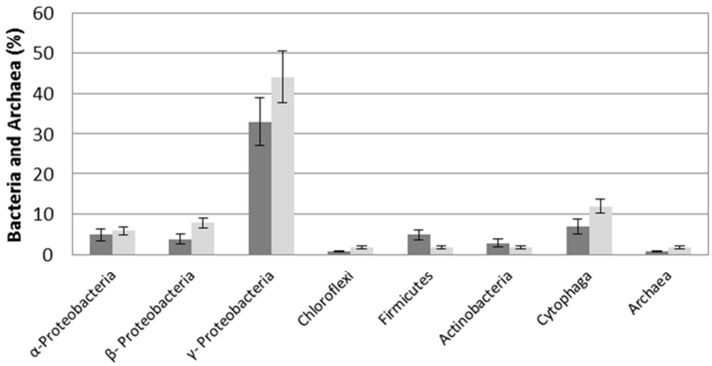


Figure 9. Quantification Bacteria and Archaea from 108th day;

SBR-1 (89.3 mS/cm), SBR-2 (98.5 mS/cm)

In these figures, the percentages and their uncertainty of the bacteria phyla and archaea isolated from the total population are represented. The conductivity values of every sample can be observed in the corresponding figure caption.

It can be observed that *Proteboacteria* are dominant in both reactors with the subclass gamma (γ) playing the main role. These results agree with those obtained by other authors for saline waters [51,52]. Thus, Park et al. [51] reported that 47% of the bacteria in Korean solar saltern were affiliated with *γ-Proteobacteria*. At the end of start-up, there were 33% and 44% of *γ-Proteobacteria* in the SBR-1 and the SBR-2, respectively. The amount of *γ-Proteobacteria* increased with the reactor salinity, though they slightly decreased in SBR-2 when conductivity was near 100 mS/cm .There is a non-negligible presence of Gram-positive bacteria. *Firmicutes* and *Actinobacteria* phylla were variable but in both reactors reached a maximum around 13%. *Archaea* population was not relevant. The amount of *Phylum Chloroflexi* decreased with the reactor salinity in the SBR-1 (from 6 to 1%), meanwhile it remained constant in the SBR-2 (around 2%).

463 Finally, it has to be commented that the average percentage of *Phylum Cytophaga* also
464 decreased slightly in the SBR-1 (from 10 to 7%), meanwhile it increased in SBR-2
465 (from 5 to 12%).

466 As *γ-Proteobacteria* was the predominant microorganisms, the following step
467 consisted in identifying the most important strain among them. Thus, the salt-tolerant
468 strain (H1) was obtained. It can survive in the salinity up to 7%. Alignment of the strain
469 indicated that the partial 16S rRNA sequence of H1 is 99% identical to *Salinicola sp.*

470

471 **4. CONCLUSIONS**

472 FTOP biological treatment is very complicated because salinity shocks cause physical
473 and biochemical changes of the activated sludge and phenolic compounds can inhibit
474 biomass, primarily by bactericidal effect. To achieve a successful treatment of this kind
475 of effluents, a gradual acclimation of the biomass is required.

476 The two studied start-up strategies led to some differences in reactors performance.
477 Thus, COD removal efficiencies were slightly lower in the SBR with a biomass pre-
478 adapted to salinity (SBR-2) than in SBR-1. Besides, deflocculation occurred at a higher
479 extent. Therefore, according to this study, the best implementation strategy is one that
480 performs the simultaneous adaptation to the presence of salt and phenolic compounds.
481 After 108 days of SBRs operation, COD removal efficiencies were 88% in the SBR-1
482 and 75% in the SBR-2, and phenols were almost completely removed in spite of the
483 high salinity (between 90 and 100 mS/cm), what implied very low toxicity values in the
484 effluent. Concerning biomass population, an increase in γ-proteobacteria in the
485 microbial population for increasing conductivities was observed in both reactors. A *γ-*

Proteobacteria strain, a salt-tolerant one, was obtained and its partial 16S rRNA sequence was 99% identical to *Salinicola sp.*

In view of these results, the augmentation of this strain has been achieved without pre-adaptation to salinity. Then, for a full-scale start-up of this type of reactors, a simultaneous adaptation to salinity and phenols is recommendable.

Although further research has to be carried out to reduce the HRT in the reactor, the process could be economically feasible since FTOPs volumes are not very high and they depend on the season; thereby reactors with appropriate sizes could be implemented in spite of the high HRTs.

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