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Additional Information

Biological treatment of hypersaline wastewater from table olive processing:

process performance and protist population under different operating conditions

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Abstract

Biological treatment of fermentation brines from table olive processing (FTOP) entails many difficulties due to their high salinity and COD concentration, which include phenolic compounds. These conditions limit the biodiversity of microbial population. Experiments treating FTOP were performed in sequencing batch reactors (SBR)

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changing operating conditions in order to study the effects on the SBR performance and on the protist population. The statistical study showed that the SBRs with high influent COD, pH and volatile solids and low influent phenol concentration, hydraulic retention time and temperature achieved the highest COD removal efficiencies and ciliate population and the lowest flagellate presence.

1. INTRODUCTION

Management of hypersaline wastewaters containing also organic matter is very complicated. This type of effluents can be found in some industries such as food-processing, leather and petroleum industries [1]. One particular case is the fermentation brine from the table olive processing (FTOP). Spanish style table olive processing consists of three steps: debittering, washing and fermentation. The wastewater volume generated in this process is about 3.9–7.5 m^3 per tonne of green olives, corresponding the 20% of this volume to FTOP [2]. The olive harvesting is mainly carried out in autumn; therefore the main wastewater volume of the olive processing is generated in this period (debittering and washing effluents). Nevertheless, the FTOP and the packing solutions are produced during all the year [3]. The main FTOP characteristics are their high salinity (conductivity around 80 mS·cm^{-1} and NaCl concentration around 80 g·L^{-1}) and high COD concentration due to oil and greases, remaining sugars after fermentation, phenolic compounds and volatile organic acids [4]. The COD and the phenolic compounds concentrations in the FTOP depend on the olive maturity [5],

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cultivar [6] and fermentation time [7]. Consequently, it can be stated that these effluents are very difficult to manage and traditionally they have been either disposed in evaporation ponds, which generate unpleasant odours and in addition, it implies potential risk of soil and aquifers pollution. Another way of management for these effluents is their transport to large wastewater treatment plants for dilution, but this practice is very expensive and it is not environmentally friendly. Nevertheless, nowadays more stringent environmental legislation compels to look for environmental-friendly solutions. One of them is the biological treatment.

The aerobic biological treatment of the organic matter in the industrial saline wastewater is very complex [8,9]. The negative effects of high salt concentrations on conventional activated sludge are well known. Under these conditions, the floc stability (hydrophobicity, filterability, settlement…) [10] and the COD removal efficiency [1] decrease. Uygur and Kargi [11] reported the deterioration of the biological process when salinity increased up to 6%. This deterioration could be observed by the increase of the COD in the treated wastewater due to loss of bacterial activity and plasmolysis. Additionally, the bactericidal effects of the phenolic compounds on the microorganisms further hampers the biological process [12,13]. Thus, in order to perform a successfully FTOP biological treatment, a slow and gradual process start-up (around 100 days) with a high initial hydraulic retention time (40 days) to overcome the loss of enzymatic activity would be needed [14,15].

The biomass of biological reactors treating hypersaline wastewaters is formed mainly by bacteria. Salvadó et al. [16] studied the effect of salt addition on the protozoan and metazoan population of a conventional activated sludge. These authors concluded that neither metazoa nor protozoa were found in the activated sludge after 24 h from the addition of 40 g·L^{-1} of NaCl. The same effect was reported by Bassin et al. [17], who

decreased the limiting salinity to 20 g·L^{-1}. Thus, the existence of ciliate in the microbial community of a biological reactor treating hypersaline wastewater is not frequent.

In this work an experimental setup of five laboratory sequencing batch reactors (SBRs) treating FTOP was used to assess the best working conditions. During this research, populations of protists were followed almost for ten months to assess possible relationships of the protist population dynamics in the plant with the physical-chemical or operational conditions of the SBRs. Also, statistical methods were used to achieve the better operational conditions in order to obtain the maximum COD removal efficiency.

2. MATERIAL AND METHODS

2.1. Wastewater samples processed in the SBRs

Four different FTOP samples provided by a table olive processing company located in Comunitat Valenciana (Spain) were used as feed for the SBRs in the sampling period. Each sample was stored at 4ºC until its use. It was checked that wastewater characteristics had not been changed before its use. Table 1 summarizes the average values of the measured parameters (by triplicate) of each FTOP sample.

Table 1. FTOP characteristics

Characteristics (*)	FTOP-1	FTOP-2	FTOP-3	FTOP-4
Sampling	1-2	3-6	7-9	10-12
pH	3.7 ± 0.1	4.2 ± 0.1	4.4 ± 0.2	4.5 ± 0.2
Cond (mS·cm^{-1})	94.1 ± 0.1	74.2 ± 0.3	78.8 ± 0.3	79.0 ± 0.1
COD (g·L^{-1})	21.50 ± 0.08	14.15 ± 0.06	7.60 ± 0.02	6.23 ± 0.03
N$_T$ (mg·L^{-1})	352 ± 2	301 ± 8	205 ± 12	247 ± 7
P$_T$ (mg·L^{-1})	76 ± 2	43 ± 2	35 ± 5	23 ± 2
Cl$^-$ (g·L^{-1})	44.93 ± 0.32	33.41 ± 0.24	38.48 ± 0.09	40.17 ± 0.11
T.Ph (mg TY·L^{-1})	1550 ± 34	885 ± 12	567 ± 13	425 ± 4
SS (mg·L^{-1})	1237 ± 43	971 ± 26	762 ± 9	1236 ± 11
VSS (mg·L^{-1})	511 ± 22	523 ± 15	402 ± 13	598 ± 9

(*) The parameters are explained in the Analysis subsection. Values represent
mean $\pm$ standard deviation

It can be observed that the FTOPs are acidic wastewaters, which is due to the volatile acids such as lactic, formic, malic and acetic acid [4]. The high conductivity values are due to the NaCl used in the fermentation brine preparation, which contain around 8% of salt [3,18]. The high total phenols content is explained by the polyphenols transfer, mainly ligstroside and oleuropein [19], from the olive to the wastewater. These compounds become tyrosol (TY) and hydroxytyrosol (HTY) after their hydrolysis (during debittering process), which are the main phenolic compounds in the FTOP [3,19]. Thus, the inhibition of the biological process and the characteristics of the biomass would not depend only on the hypersaline conditions but on the presence of high concentration of phenols, whose bactericidal effects are known [20,21]. The variability of the FTOP characteristics has already been described in the introduction section. Summarizing, the SBRs treated an industrial effluent characterized by high organic matter (including polyphenols) and suspended solids concentration that were combined with very high salinity.

Table 1 also shows which FTOP was fed to the SBRs in the different samplings carried out to identify and quantify the protist population. Samplings were performed every three weeks to a total of 12.

In SBR-3, SBR-3A and SBR-3B, the used FTOPs were the same FTOPs reported in Table 1 after pre-treatment with powder activated carbon (PAC), in order to reduce by adsorption the COD and the total phenols (T.Ph) concentration. The adsorption treatment was performed to obtain a T.Ph concentration of 400 mg $TY \cdot L^{-1}$ in FTOP-1 and FTOP-2 and 200 mg $TY \cdot L^{-1}$ in FTOP-3 and FTOP-4. The mean COD reduction percentage achieved in the four pre-treated FTOP was $15.9 \pm 4.8\%$.

2.2. Biological reactors

The SBRs were operated during 293 days. The first samples to perform the protist analysis were taken after 48 days from the beginning of their planned operation. It is important to mention that the biomass was previously adapted to the FTOP as described in Ferrer-Polonio et al. [14]. Thus, adapted drawn sludge was seeded in the reactors for these experiments. In a first experimental period (Step-1, 173 days), three SBRs (named SBR-1, SBR-2 and SBR-3) were operated in parallel (until sampling 6). From this day on, SBR-3 was split into two new reactors (SBR-3A and SBR-3B) and a new experimental period (Step-2) was started. In Step-2, four SBRs (SBR-1, SBR-2, SBR-3A and SBR-3B) were operated in parallel.

All the bioreactors were identically equipped [14]. Nevertheless, SBR-1, SBR-2 and SBR-3 worked with a reaction volume of 6L while SBR-3A and SBR-3B had a reaction volume of 3 L. All the SBRs were operated with 24 hours cycles including the

following phases: filling (2 min), anoxic reaction (1 h), aerobic reaction (21 h), settling (1.5 h), drawing (2 min) and idle (26 min).

The variables selected in the operation of the reactors, to study their effect on the protist population and the reactors performance, were the hydraulic retention time (HRT), the temperature, the food-to-microorganism ratio (F/M ratio) and the pre-treatment of the FTOP. Table 2 summarizes the operational characteristics of the SBRs used. The temperature was maintained constant in SBR-2 using an external heating system, but it was not controlled in the rest of the reactors. SBR-1 and SBR-2 were fed with the FTOPs reported in Table 1, while the other SBRs used pre-treated FTOP, according to the method explained in subsection 2.1.

Table 2. Operational conditions in the SBRs

	SBR-1	SBR-2	SBR-3	SBR-3A	SBR-3B
STEP-1 (0-173 days; sampling 1-6; FTOP-1 and FTOP-2)					
HRT (days)	40	40	30	-	-
Tª (ºC)	ambient	30±1	ambient	-	-
F/M (kg COD·kg VSS^{-1}·d^{-1})	≈ 0.13	≈ 0.13	≈ 0.13	-	-
Adsorption treatment	non	non	yes	-	-
STEP-2 (174-293 days; sampling 7-12; FTOP-3 and FTOP-4)					
HRT (days)	19	32	-	30	15
Tª (ºC)	ambient	30±1	-	ambient	ambient
F/M (kg COD·kg VSS^{-1}·d^{-1})	≈ 0.13	≈ 0.08	-	≈ 0.08	≈ 0.13
Adsorption treatment	non	non	-	yes	yes

In Step-1, the HRT was adjusted to 30 days in SBR-3 to keep a similar F/M ratio in three reactors (pretreated feed used in SBR-3 had lower COD). The HRT values were lowered by feeding higher FTOP volumes. In Step-2, the same F/M ratio as Step-1 was maintained in SBR-1 and SBR-3B. The reduction in the HRT in SBR-1 to 19 days was

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possible without reducing the process performance, due to the reduction of the COD in FTOP-3 and FTOP-4 (Table 1). In SBR-3B, the HRT was 15 days. On the other hand, the F/M ratio in SBR-2 and SBR-3A was reduced by 40% varying the HRT.

2.3. Analysis

2.3.1. Physical-Chemical analysis

The FTOP parameters reported in Table 1 were measured after reception. On the other hand, the effluent and the mixed liquor of each SBR were characterized twice a week. The parameters measured were: pH, conductivity, soluble COD (filtered to 0.45 µm), mixed liquor suspended solids (MLSS) and mixed liquor volatile suspended solids (MLVSS). The F/M ratio was calculated using the following equation [22]:

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

where COD_0 was the chemical oxygen demand of the FTOP used (mg $O_2 \cdot L^{-1}$), Q was the flow rate fed into the SBR (L·d^{-1}), V_R was the reaction volume (L) and the MLVSS was measured as mg·L^{-1}.

Furthermore, the observed sludge yield (Y_{obs}) was calculated to evaluate the biomass growth (Eq.2). This parameter is the amount of the biomass formed per mass unit of substrate used in the biochemical process [23,24].

$$Y_{obs} = \frac{MLVSS_{produced}}{COD_{removed}} = \frac{(MLVSS_j - MLVSS_i) \times (V_R/t) + X_e \times (V_d/t)}{(COD_0 - COD_e) \times (V_d/t)} \qquad (Eq.2)$$

where t is the time interval between two days "i" and "j"(no sludge was withdrawn in between), X_e was the mean volatile suspended solids concentrations in the effluent ($mg·L^{-1}$) in this time span, V_d was the influent/effluent volume (L) and COD_e was the mean chemical oxygen demand measured in the effluent at the time interval t.

The pH and conductivity measurements were carried out with pH-Meter GLP 21+ and EC-Meter GLP 31+ (both from Crison), respectively. The SS, VSS, MLSS and MLVSS were measured according to APHA, 2005 [25]. The N_T, P_T, Cl^- and soluble COD concentration were analyzed using kits and a Spectrophotometer DR600 (Hach Lange), after the appropriate dilutions to avoid interferences.

2.3.2. Biological analysis

Samples of the mixed liquors (20 mL) were taken every three weeks in each SBR for the biological analysis. These samples were preserved straight away after collection with acidified Lugol solution (20% v/v) to enumerate protists. Fixed samples were kept refrigerated (4ºC) until counting procedure was carried out. Some of the samples collected were kept alive to identify protists; these samples were maintained in the lab at room temperature.

Ciliates enumeration was carried out on 1 mL aliquots of the fixed samples in a Sedgewick-Rafter counting chamber when abundance was low. When ciliate abundances were high counting procedures were carried out on 25 µL aliquots. In this case, coverslips were sealed to the slide with Vaseline to set a smaller counting chamber and to avoid sample evaporation during the enumeration. Triplicate counting procedures were carried out for every sample. Flagellates appearance was recorded during the ciliates counting procedures when observed since they only appeared in just a few dates.

Ciliates identification was done on live observation of mixed liquor samples or on cultures from these samples. Large quantities of ciliates appeared to survive in the samples where high numbers of bacteria and organic matter was present. Culturing procedures were attempted with the unfixed original samples using a culture media prepared with sodium chloride solutions on mineral water or with filtered mixed liquor. These cultures were prepared at the same conductivity measured in the original samples. Cultures were inoculated with the original sample plus a wheat grain or wheat media, which were added to promote bacterial growth. However, no stability or long term maintenance was possible within these cultures and ciliates eventually disappeared. Live observations were carried out in these samples to assess characteristics of the protists found. Measurements of the live cells were carried out on photographs taken at x40 under phase contrast or DIC microscopy (in an Olympus BX50 and a Nikon Eclipse 80i microscopes respectively). During these procedures, ciliates were also stained with silver impregnation protocols to resolve ciliary and infraciliary patterns necessary for proper ciliates identification. Cells were collected directly from samples or cultures with a micropipette and then these were suspended in a small volume (0.5 mL) of 0.2 µm filtered wastewater in an excavated slide. Fixation was then carried out with two drops of commercial formalin (37%). Fixed ciliates were washed with distilled water at least three times. Final volume was reduced to a small drop with fixed ciliates and then the silver carbonate staining procedure [26], modified for small volumes, was developed adding at least 1 mL of the silver carbonate mix in the same excavated slide. The excavated slide was set in a water bath at 65ºC until the staining mixture changed color as in the ordinary silver carbonate methodology [26]. Stained ciliates were collected with a micropipette and observed under a Nikon Eclipse 80i microscope for proper species identification.

Flagellates identification was done on fixed samples taking into account cell shape, size, flagella number, flagella insertion and karymastigont position.

2.3.3. Statistical analysis

A matrix with raw data of physical-chemical variables (influent, mixed liquor and effluent), biological variables (ciliate counts and presence/absence of flagellates) and operational characteristics (HRT, temperature and F/M ratio) was arranged for statistical analysis. The included influent parameters were the COD and T.Ph masses fed per day and liter of reactor volume ($mg \cdot L^{-1} \cdot day^{-1}$). These influent parameters were referred to the reaction volume since experiments were performed with SBRs of different reaction volume (6 and 3 L). The MLVSS concentration was selected as parameter of the mixed liquor. Finally, the effluent variables considered were: pH, conductivity ($mS \cdot cm^{-1}$), COD concentration ($mg \cdot L^{-1}$) in the effluent and the COD removal efficiency (%). The Y_{obs} was not included in the statistical analysis because there were insufficient data coinciding with sampling days.

Analysis was carried out with Statgraphics Centurion XVII v.17.1.12 and SPAD v8.0 software packages. ANOVA, Multiple Linear Regression Analysis, Logistic Regression and Multivariate Analysis (PCA and Hierarchical Cluster Analysis) were carried out. Principal Component Analysis (PCA) was used for the ordination of samplings based only on physical-chemical variables, then factors from this analysis accounting for at least an explained 88% cumulative percentage were selected for Hierarchical Cluster Analysis (RECIP module in SPAD v8.0) using then physical-chemical variables to explain cluster partitions; description and characterization of sampling clusters were

assessed with a v-test to define biological and/or physical-chemical variables significantly associated to them.

3. RESULTS AND DISCUSSION

3.1. Identification of the protist populations

A large population of the ciliate *Pseudocohnilembus* sp. was found in the mixed liquor of the different laboratory SBRs treating the FTOP. *Pseudocohnilembus* is a genus of free-living scuticociliates described mainly in marine or brackish environmental conditions [27–34]. Table 3 summarizes *Pseudocohnilembus* species described to date and their environmental locations. Furthermore, some of these species have been recently described as pathogenic in mollusks and fish [35]. Only two species of this genus have been cited or found in fresh-waters (*P. putrinus* and *P. pusillus*) [30,36] and from these only *P. pusillus* has been found in wastewater [37–39].

Table 3. *Pseudocohnilembus* species descriptions, environmental locations and cyst presence reported to date

Species	Environment	Cysts	Publication
P. persalinus	Salt Lake	Yes	
P. hargisi	Marine	-	[27]
P. longisetus	(Shore)	Yes	
P. marinus	Marine	-	[28]
P. hargisi	Tidal marsh	-	[29]
P. putrinus (Kahl, 1928) nov.comb.	Soil	-	
P. pusillus I (Quennerstedt, 1869) nov. comb.	Freshwater	-	
P. pusillus II (Quennerstedt, 1869) nov. comb.	Tide pool	-	[30]
P. marinus (Thompson, 1966)	Salt Lake	-	
P. cantabricus	Marine	-	[31]

P. pusillus	Salt Lake	Yes	[32]
P. hargisi	Marine	-	[33]
P. hargisi	Marine	-	[34]
P. longisetus	Fish farm	Yes	
P. persalinus	Marine (pathogenic)	Yes	[35]
P. putrinus	Freshwater		[36]

Initially, this ciliate was the dominant and only protist population present reaching over 50,000 cells·mL^{-1} in some of the sampling dates of Step-1. Eventually a heterotrophic flagellate appeared sometimes with minimal numbers, which in turn became the dominant population just on one sampling date when the ciliate disappeared completely in SBR-2 (sampling 6). The limited presence of eukaryotes in the autochthonous microbial community of industrial hypersaline wastewater treatments has also been noted previously by other authors [40,41]. The absence of protists in the biological wastewater treatments, especially ciliates, produces low quality effluents with higher turbidity. This is due to the fact that these microorganisms are important bacterial predators and also promote flocculation [42,43]. Therefore it was interesting to investigate the dynamics and function of these protists and their relationship to other physical-chemical factors as well as performance in the SBR.

A threefold approach was used to study this ciliate and its variability within the SBRs. Firstly, the taxonomic identity of the ciliate and the flagellate appearing as main protist populations in the systems was assessed; secondly, the population development and variability in the SBRs were followed to find significant differences among them and finally, the relationships of the protist population dynamics and the physical-chemical and/or operational conditions in the plants were evaluated.

The ciliates observed in live samples from the SBRs were tentatively identified as *Pseudocohnilembus* sp. This ciliate during the study showed two different life stages: trophic and cyst stages (Figure 1).

Figure 1. Live observation of vegetative (trophic) and cyst stages under DIC microscopy (x40). (a-c): Trophic stage; the ciliates fed on bacteria predating actively on the surface of the flocs. Contractile vacuoles (CV) and caudal cilium (CC) in the posterior pole. Bacteria can be observed in the digestive vacuoles (arrow in c). (d-g): The cyst stages. Contractile vacuole (CV) visible (g). Sacale Bars: 10 µm

The trophic forms of this species had spindle to pyriform shape with a narrow anterior end and a rounded posterior end. As it has been observed previously in other species of this genus [32,44], the cells became smaller and more elongated with starvation, with the anterior end bending to the right. A single caudal cilium could be observed in the posterior pole. Contractile vacuole was located terminally also in the posterior pole of the cell. The cyst was rounded to ovoid with a thick cover very refractile under DIC; contractile vacuole was usually visible within many cysts, which allowed locating the posterior pole of the early cyst morphogenesis. The ciliates contributed to the appearance of large flocs in the mixed liquor due to the gregarious behavior of this ciliate species during the cyst formation. Large numbers of cysts usually appeared

associated in flocs as it has been observed by other authors [32] and flocs in this case were usually packed with cysts.

The mean size of the trophic stage had 26.1 x 9.5 μm (lugol´s fixed; n=130), while cysts had 15.0 x 10.8 μm (lugol´s fixed; n=4). After silver carbonate impregnation (Figure 2), the ciliates showed nine bipolar somatic kineties, with dikinetids in the anterior end of the cell and monokinetids in the posterior end of the cell. The contractile vacuole pore was located at the end of the third somatic kinety. The oral infraciliation with a mean length of 12.6 μm (lugol´s fixed; n=33) was composed by a haplokinety, almost straight, with a paroral membrane and three polykineties: a polykinety 1 (Pk1) long with a posterior end which is continuous with the haplokinety. Pk1 is also running parallel to the polykinety 2 (Pk2) and it is almost perpendicular to the polykinety 3 (Pk3). A large rounded macronuclei is located over the equatorial plane of the cell and a small micronuclei is generally attached on the anterior area of the macronuclei.
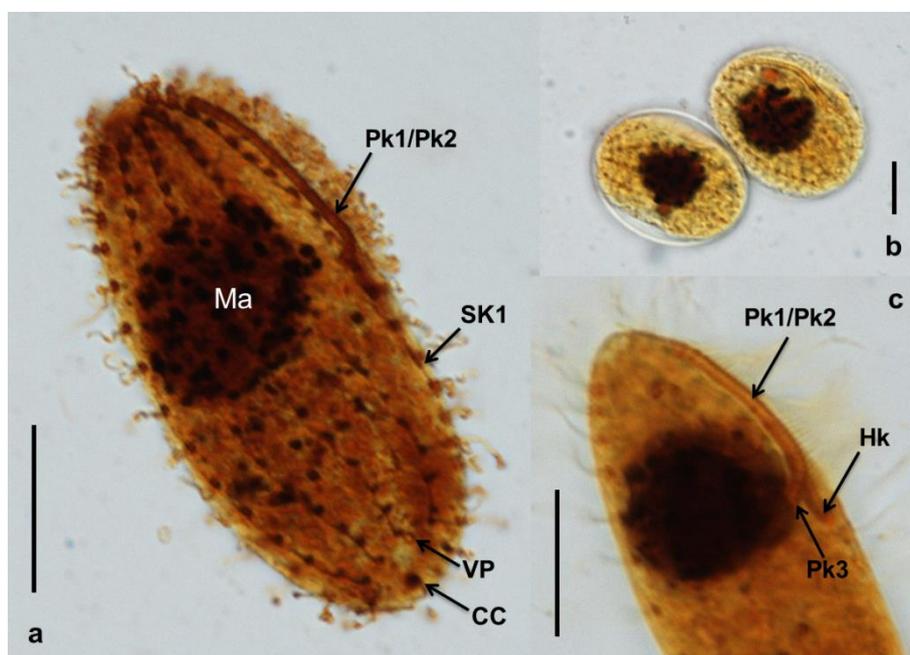
Figure 2. Silver carbonate staining of the trophic and cyst stages. Bright field (x100 and x40). (a). Trophic stage right side of the cell and oral area; arrow shows the position of the contractile vacuole pore (VP), the caudal complex (CC), the somatic kinety 1 (Sk1), the polikineties 1 and 2 (Pk1/Pk2) from the oral area and the macronuclei (Ma) (x100). (b). Cyst stages with cells already

showing infraciliary patterns on the surface of the cell; macronuclei and micronuclei are visible (x40). (c). Detail of the infraciliature from the oral area with the posterior end of the haplokunety and the oral polikineties 1, 2 and 3. Scale Bars: 10 µm

Several works have noted previously the difficulties to identify species in this genus, mainly because of the structure of the oral area, which is difficult to discern and also because there are great variations in the number of kineties and basal bodies and in the number and position of contractile vacuole pores [45]. The comparison of the ciliate found in these SBRs with previously described species of the genus *Pseudocohnilembus* (Table 3) allows finding closer similarities to two different species described in the literature: *P. persalinus* and *P. pusillus*. Foissner and Wilbert [30] sinonymized *P. persalinus* and *P. longiseta* [27] with the previously described species *P. pusillus*. After the work carried out by Song [33,45] however, Foissner et al. [46] agreed to keep *P. persalinus* as a different species. The lower number of basal bodies in all somatic kineties in *P. persalinus* compared to *P. pusillus* (e.g. ca. 12 vs.19 on average respectively) was cited as one of the differential characteristics [33].

P. persalinus shows a high morphological variability with many morphotypes that seem to represent populations of the same species [33,45]. However, there are not clear morphological comparisons in the literature of *P. persalinus* and *P. pusillus* to recognize the differences between them, except the already cited number of basal bodies in the somatic kineties, which is higher in *P. pusillus*. Nowadays, *P. persalinus* is cited as a different species even in recent literature [47] while the most recent citation of *P. pusillus* [32] do not include a detailed morphological account of this species.

Given the similarities we have found with the detailed descriptions of species of *P. persalinus*, especially considering the prominent polykinety 3 cited by Song [45] and

also observed in our species even though this is a larger one (Table 4), this brackish ciliate species from the SBRs is identified as *P. persalinus*. However, it has to be pointed out that most literature references of *P. persalinus* do not include the presence of cysts as a characteristic of this species. However, the original description of this species by Evans and Thompson [27] from a brackish environment, indicated that cysts were present as in the ciliate found in these SBRs. The presence of cyst is also a characteristic in *P. pusillus* [32] although previous authors did not record this characteristic either. Further molecular work would be necessary to confirm this identification, comparing this species with cysts in their life cycle with those species already identified as *P. persalinus* without cysts or undescribed cysts in the molecular databases. In Table 4 some characteristics of *P. pusillus*, *P. persalinus* described in the literature and the *Pseudocohnilembus* species found in the SBRs are compared.

Table 4. Morphometric data for *Pseudocohnilembus* species (dimensions in µm)

Species	Length Range Mean	Width Range Mean	Cyst	Macronuclear nodules	Environ.	Publication
P. persalinus [a]	20.0-38.0 -	10.0-18.0 -	Yes	1	Salt Lake	[27]
P. pusillus I [b]	29.0-42.0 34.5	15.0-26.0 20.8	-	1	Freshwater (dungheap)	[30]
P. pusillus II [a]	25.0-39.0 31.3	12.0-20.0 15.4	-	1	Marine (tide pool)	
P. pusillus [c]	24.6-32.3 27.7	7.7-12.3 10.0	Yes 15.5	1	Salt Lake	[32]
P. persalinus I [c]	25.0-37.0 29.9	15.0-26.0 20.3	-	1	Marine	[45]
P. persalinus II [c]	29.0-43.0	16.0-23.0	-	1	Marine	

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	33.7	18.9				
P. persalinus a	22.0-32.0	11.0-16.0	Yes	-	Fish farm (pathogenic)	[35]
	-	-				
P. persalinus d	21.7-32.6	8.6-14.9	Yes	1	SBR	This work
	27.5	10.4	15.0			

Staining methodologies: a.- Silver nitrate (wet); b.- Silver nitrate (dry); c.-Protargol; d.- In vivo

This is therefore the first citation of *P. persalinus* in a wastewater environment with hypersaline conditions. This ciliate was originally the only protist population present and survived the extreme conditions found in the reactors reaching extremely high abundances (over 58,000 cells·mL^{-1}). This is an indication of the high adaptability to the extreme environment where it was surviving.

The other protist found in the SBRs was a heterotrophic flagellate with a pyriform shape and four anterior flagella (Figure 3).
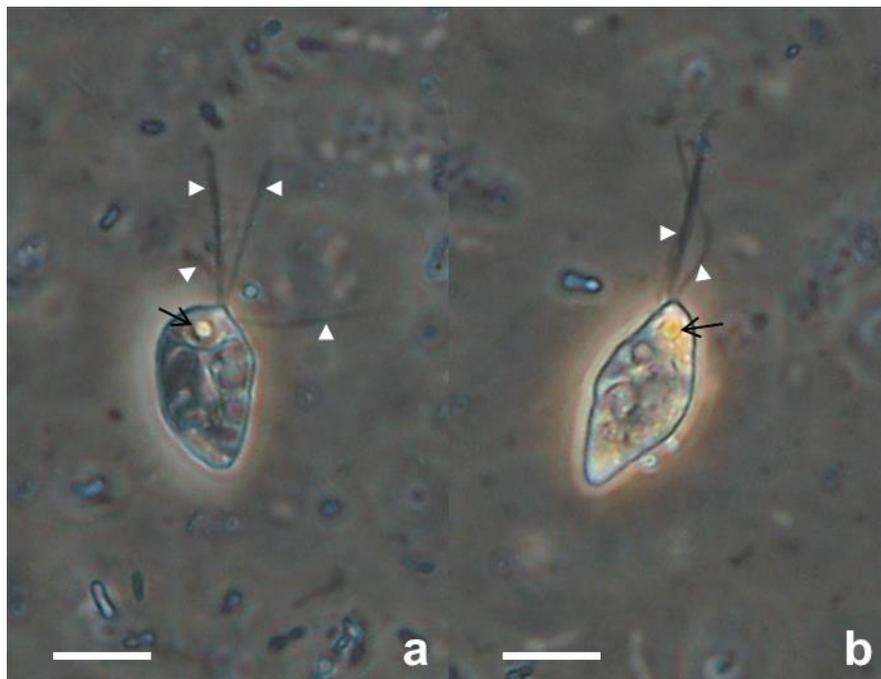
Figure 3. Lugol´s fixed flagellate. Phase contrast microscopy (x100 and x40). Three of the four flagella are clearly visible (a and b: white arrow heads) as well as the karyomastigont with the nucleus (a and b: black arrows), under the flagellar insertion. Scale Bars: 10 µm

This flagellate also showed a feeding groove and in some cases it could also be observed an anterior papilla next to the flagellar insertions. This flagellate had as well a cyst stage. The mean size of the trophic stage cell was 17.0 x 8.8 µm (lugol´s fixed; n=57) and the size of the cyst stage was between 7.5-8.0 µm. The flagella were all about the same length, with a mean length of 17 µm (lugol´s fixed; n=47). A single nucleus could be observed under the flagellar bases (karyomastigont). This flagellate resembled a *Retortamonadida* species in the shape of the cell, the anterior single nucleus, the presence of a single ventral feeding groove. However, it was clearly different in the presence of four flagella instead of two. The other genus included in this group has only two flagella, therefore we identified this flagellates as a *Chilomastix* sp. This genus is included within the *Fornicata* clade [48] and the *Excavata* supergroup. These flagellates are all heterotrophic flagellates with one or two karyomastigonts per cell. In this case, the flagellate observed in the SBRs had a trophic stage with one karyomastigont, four flagella and a cyst stage as in the case of *Chilomastix*. The observations so far would not allow fulfilling species identification any further, but this is a free-living flagellate, unlike what it is found in most species from *Chilomastix* which are in general endobionts. However, free-living *Chilomastix* species have been encountered ranging from freshwater to marine habitats from sites with little or no oxygen and/or rich in hydrogen sulphide [49–51]. The presence of this flagellate in these high salinity SBRs is also, as in the case of the ciliate, a special environmental characteristics to point out. This flagellate was predating on bacteria.

3.2. Influence of the studied parameters on the SBR performance and on the protist population

Table 5 shows the average value and standard deviations for the parameters chosen for the statistical analysis of the sampling days, for each SBR in Step-1 and Step-2. The COD and T.Ph values reported in the influent row are the amount of these parameters fed in each reactor per day of reactor volume, which were calculated with the following equation:

$$\text{COD or T.Ph } (mg \cdot L^{-1} \cdot d^{-1}) = \frac{(COD_0 \text{ or T.Ph}_0) \times V_d}{V_R \times 1\text{day}} \qquad (Eq.3)$$

Table 5. Average values ± standard deviations of some parameters of influent, mixed liquor, effluent and operational process during Step-1 and Step-2 in the sampling days

		STEP-1 (0-173 days)		
		SBR-1	SBR-2	SBR-3
Operation	HRT (days)	40	40	30
	Tª (ºC)	23.8±4.0	31.1±1.2	23.8±4.0
	F/M (gCOD·gVSS⁻¹·d⁻¹)	0.12±0.02	0.15±0.04	0.12±0.02
Influent	COD (mg·L⁻¹·d⁻¹)	415±95	415±95	445±87
	T.Ph (mg·L⁻¹·d⁻¹)	28±9	28±9	11±2
ML	MLVSS (g·L⁻¹)	3.3±0.5	2.8±0.3	3.6±0.4
Effluent	pH	8.3±0.1	8.7±0.1	8.9±0.9
	Cond (mS·cm⁻¹)	89.0±4.7	101.0±1.9	96.0±2.6
	COD (mg·L⁻¹)	3411±411	4716±1267	2434±274
		STEP-2 (174-293 days)		
		SBR-1	SBR-2	SBR-3A	SBR-3B
Operation	HRT (days)	19	32	30	15
	Tª (ºC)	20.8±2.3	30.4±0.6	20.8± 2.3	20.8±2.3
	F/M (gCOD·gVSS⁻¹·d⁻¹)	0.12±0.01	0.08±0.01	0.08±0.01	0.14±0.01
Influent	COD (mg·L⁻¹·d⁻¹)	368±8	221±65	203±23	404±45
	T.Ph (mg·L⁻¹·d⁻¹)	25±2	15±3	7±0	13±0

ML	MLVSS (g·L^{-1})	3.1±0.3	2.4±0.1	2.5±0.3	3.0±0.5
	pH	8.2±0.3	8.6±0.1	8.7±0.1	8.7±0.1
Effluent	Cond (mS·cm^{-1})	88.9±0.5	91.9±4.7	90.8±3.5	92.3±4.2
	COD (mg·L^{-1})	1661±422	2340±870	1585±10	1483±368

As it can be observed, in SBR-2 the mixed liquor temperature was maintained around 30ºC throughout the experiment, while the other SBRs worked at room temperature. In Step-1, the average F/M ratio was around 0.13 kg COD·kg MLVSS^{-1}·d^{-1}. Nevertheless, in SBR-2 this parameter was higher because the average MLVSS was lower than those achieved in the other two reactors. In Step-2, the same value of F/M ratio was maintained in SBR-1 and SBR-3B, while in SBR-2 and SBR-3A decreased to 0.08 kg COD·kg MLVSS^{-1}·d^{-1}. To achieve these F/M ratios, the HRT was varied in each reactor.

3.2.1. SBR performance

In this section, the reactors performances are analyzed through the COD removal percentage, whose evolution in the SBRs can be observed in Figure 4. In this figure, the vertical lines indicate the FTOP change and the grey area is the experimental time for Step-1.
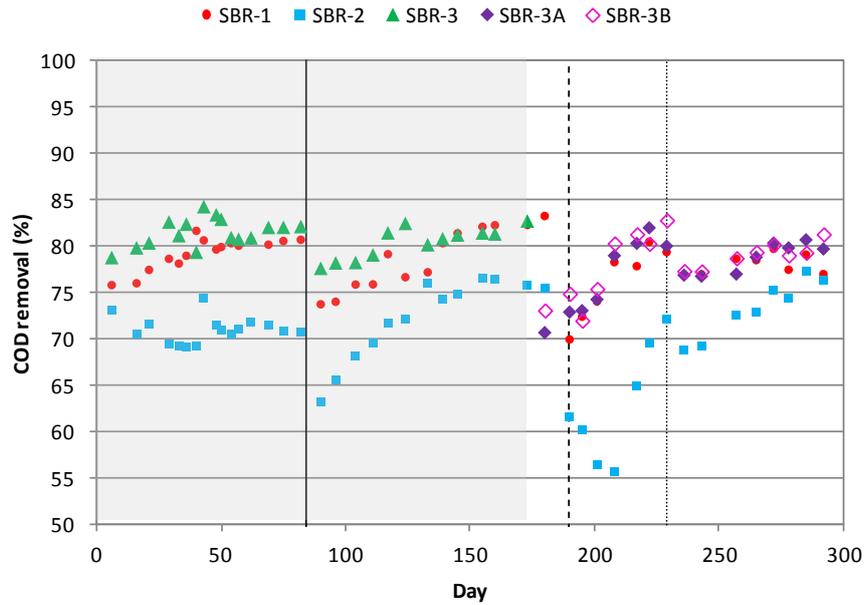
Figure 4. Evolution of COD removal percentage in the SBRs. Vertical lines: [—] FTOP-2, [- - -] FTOP-3 and [······] FTOP-4. Grey area = experimental time for Step-1

In all the reactors the COD removal percentage decreased when a new FTOP was fed, however this behavior was not due to a decrease in the reactors performance. The COD of the FTOP used in this work was decreasing from FTOP-1 to FTOP-4; therefore the effluent COD also diminished according to it. Nevertheless, the system required several days to achieve the stationary conditions for the new wastewater fed.

In Step-1, the average COD removal percentages in SBR-1, SBR-2 and SBR-3 were $78.9 \pm 2.5\%$, 71.4 ± 3.1 and $81.9 \pm 1.7\%$, respectively. Some authors have reported that the optimum bacterial activity is between 30-35ºC [52,53]. However, SBR-2 had the lowest performance surprisingly. This reactor had the highest conductivity indicating an accumulation of inorganic salt, which decreased the MLVSS/MLSS ratio to 0.45 ± 0.06, while in SBR-1 and SBR-3 this parameter was 0.51 ± 0.05 and 0.54 ± 0.06, respectively. This increase of the inorganic fraction in the activated sludge could explain the lower performance in SBR-2. On the other hand, although the COD removal

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percentage in SBR-1 and SBR-3 was similar during Step-1, SBR-3 performance can be considered higher since its HRT was the lowest and consequently the FTOP volume treated per day was higher than in the case of SBR-1.

In Step-2, the average COD removal percentages were 77.5 ± 3.2, 68.9 ± 7.1, 77.6 ± 3.3 and 78.3 ± 3.1 in SBR-1, SBR-2, SBR-3A and SBR-3B, respectively. SBR-2 showed the lowest COD removal efficiency as in Step-1. The other three reactors had similar average values for the organic matter removal, which were similar to those achieved in Step-1. This behavior confirms that the biodegradable organic matter in the FTOPs was around 80%.

It can be concluded that the optimal conditions for the FTOP biological treatment were the following: room temperature and F/M ratio around 0.13 $gCOD \cdot gVSS^{-1} \cdot d^{-1}$ (adapting the HRT). The adsorption pre-treatment enhanced slightly the reactors performance.

To further assess the relationship of the COD removal efficiency to the other physical-chemical and operational conditions, data were analyzed with Stepwise Multiple Linear Regression (S-MLR). The predictive model included only two of the variables studied: influent COD (COD_{inf}; p=0.021) and T (p<0.001). This model was the following:

$$COD\ removal\ (\%) = 83.9185 + 0.0149 \cdot COD_{inf} - 0.5375 \cdot T \qquad (Eq.4)$$

where COD_{inf} was measured in $mg \cdot L^{-1} \cdot d^{-1}$ and T in ºC. This model ANOVA was very significant; F=10.44 and p<0.001.

3.2.2. Protist populations

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The relationship between the protist populations and several operational and physical-chemical parameters is evaluated in this section by statistical analysis. As it has already pointed out in subsection 2.3.2, insufficient Y_{obs} values in sampling days did not allowed carrying out the statistical analysis of this parameter. However, this relationship can be observed in figure 5 a), b) and c), in which the Y_{obs} evolution in each SBR and the ciliates numbers are shown.

As in Figure 4, the vertical lines indicate the FTOP change and the gray area is the experimental time for Step-1. As it is shown in the graph, the ciliate population variations were related to the Y_{obs}. When this parameter decreased or increased, the same behavior was observed in the ciliates populations. If it is considered that the Y_{obs} is related to the microorganisms growth, and that the MLVSS in the SBRs were maintained around 3.0 $g \cdot L^{-1}$ (with periodical sludge withdrawal), variations in the Y_{obs} would have the same effect in the ciliates abundance.

One-way ANOVA was carried out for ciliates as dependent variable. Results showed that mean ciliates abundance was reduced in the presence of flagellates (F= 10.43; p=0.0025). This effect was very significant during Step-1 (F=7.38; p=0.015) but no significance was observed in Step-2 (F=3.41; p>0.05). This effect of flagellates on ciliates abundance reflects the possible competitive effects on the same trophic bacterial niche between both protists or might imply other interactions not included in this analysis. On the other hand, flagellates were less affected by higher temperatures and conductivities, but their presence or activity did not produce higher COD removal efficiencies.
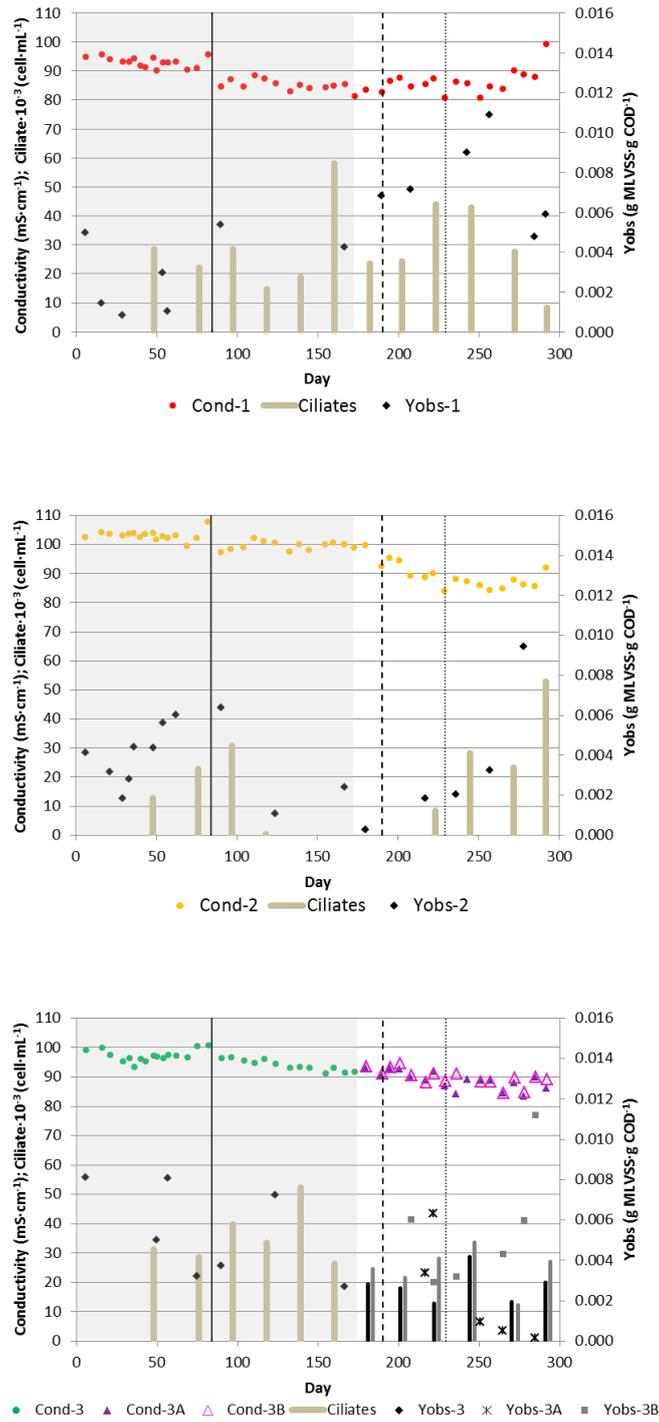
Figure 5. Relationship between conductivity, ciliate numbers and Y_{obs} in a) SBR-1, b) SBR-2, c) SBR-3, SBR-3A and SBR-3B. Vertical lines: [——] FTOP-2, [- - -] FTOP-3 and [······] FTOP-4. Grey area = experimental time for Step-1

In the case of ciliates, the Multivariate Logistic Regression (MLR) produced a predictive model, in which only two independent variables were significant (COD removal p=0.018 and Flagellate p=0.003) in the explanation of these populations variability:

$$\text{Ciliates} = -39671.3 + 868.7 \times \text{COD}_{removal} - 13714.3 \times \text{Flagellate} \qquad \text{(Eq.5)}$$

where COD removal was expressed in percentage and flagellate was a dichotomous variable (coded as 0 and 1 for absence/presence). This model ANOVA was as well very significant (F= 8.91 and p = 0.0006).

A stepwise Logistic regression protocol was used to explain the appearance of the flagellates population and its significant relationships to other variables. The Eq.6 explaining the presence/absence of flagellates retained only two explanatory variables that contributed significantly to the model (Ciliates p=0.001 and pH p=0.04):

$$\text{Flagellate} = \frac{e^{\eta}}{1 + e^{\eta}} \qquad \text{(Eq.6)}$$

where η = -36.264 – 0.00013 × ciliates + 4.344 × pH

The deviation analysis p value for this model was lower than 0.05, therefore this means that there was a statistical significant relationship between variables with at 95% confidence level. The test results for the goodness of fit indicated this was satisfactory ($\chi 2$ = 0.711, 3 degrees of freedom, p = 0.871).

The cluster analysis pinpointed which operational conditions, physical-chemical and biological characteristics were involved in the performance, independently of the reactor considered. Four clusters with similar number of samples (10 or 12 samples)

were obtained. Significant variables were selected according to their values, lower or higher than the mean value. Table 6 shows what variables characterized each of the four clusters obtained. In figure 6 the mean COD removal (%), mean ciliate numbers and flagellate frequencies are presented. These results showed that in Cluster-4 the highest mean COD removal percentages (79.9 ± 2.9%) occurred when the COD fed to the reactors per day, pH and MLVSS were high and the T.Ph fed to the reactors per day, HRT and temperature values were low (fed COD = 425.6 ± 66.2 mg·L^{-1}·d^{-1}, pH = 8.8, MLVSS = 3292 ± 532 mg·L^{-1}, fed T.Ph. = 12.2 ± 1.6 mg·L^{-1}·d^{-1}, HRT = 22.5 ± 7.5 d, T = 20.9 ± 3.1ºC). Under these conditions the mean ciliate numbers were the highest observed in the four clusters and the frequency of flagellates presence was the lowest.

Cluster 1, characterized by the lowest COD removal and ciliate populations, happened coincidentally with the highest values of the COD in the effluent, temperature, conductivity, T.Ph fed to the reactors per day and HRT, and with the highest frequencies on the flagellate presence.

Table 6. Cluster characteristic variables

CLUSTER		CHARACTERISTICS	p-value
		10 samples (80% SBR-2+20%SBR-1)	
Cluster-1	High	COD$_{efl}$	< 0.001
		T^a	< 0.001
		Conductivity	< 0.001
		T.Ph$_{inf}$	< 0.001
		HRT	< 0.001
		Presence of flagellate (frequency)	0.014
	Low	COD removal	0.002
		Ciliate number	0.001
		10 samples (40%SBR-2 + 60% SBR-3A)	
Cluster-2	Low	T.Ph$_{inf}$	< 0.001
		COD$_{inf}$	< 0.001
		COD$_{efl}$	0.009
		F/M	0.001
		MLVSS	< 0.001

		10 samples (100%SBR-1)	
Cluster-3	High	T.Ph$_{inf}$	0.010
	Low	T^a	0.006
		pH	< 0.001
		Conductivity	0.006
		Presence of flagellate (frequency)	0.023
		10 samples (100%SBR-3+SBR3B)	
Cluster-4	High	COD$_{inf}$	0.004
		COD removal	0.012
		MLVSS	0.015
		pH	0.001
	Low	T.Ph$_{inf}$	0.004
		T^a	0.021
		HRT	< 0.001
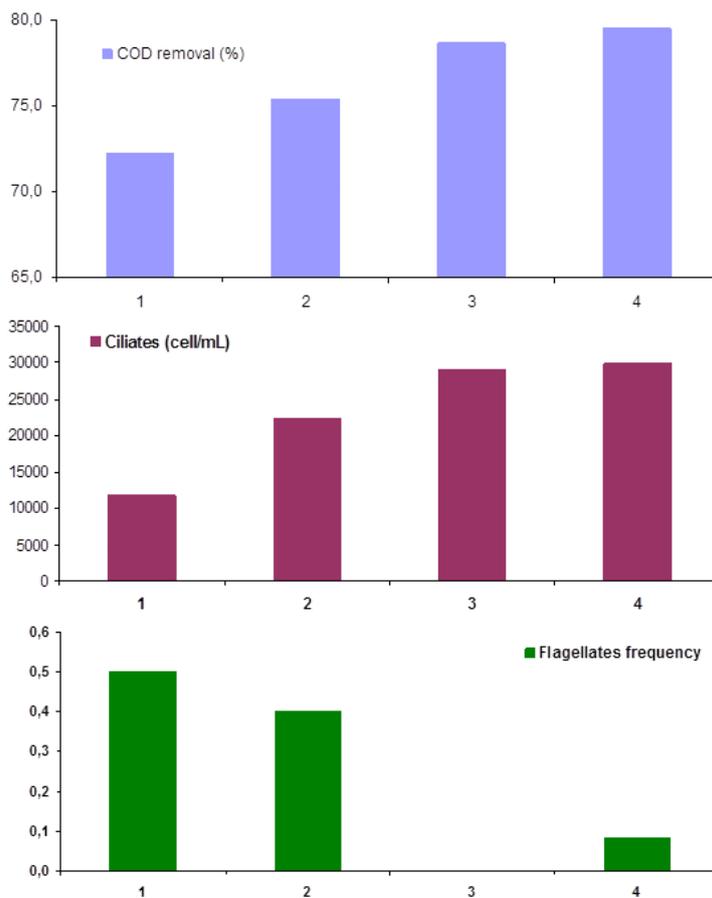
Figure 6. Mean COD removal, ciliates abundance and flagellates frequency within the four clusters obtained

4. CONCLUSIONS

FTOP biological treatment was studied under different operating conditions. The protist population characterization showed that the only ciliate specie was *P. persalinus*, whose presence in wastewater has not been reported until now. The ciliate population was reduced in the presence of a flagellate species of the genus *Chilomastix*, which coincide with the lowest COD removal. For the highest values of HRT, temperature, conductivity and T.Ph fed into the reactors, flagellate population increased. On the contrary, the highest COD removal percentages and ciliate amount were achieved when HRT, temperature and T.Ph fed into the reactors were low and pH, MLVSS and feeding COD were high.

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