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Changes in the process performance, sludge production and microbial activity in an activated sludge reactor with addition of a metabolic uncoupler under different operating conditions

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Abstract

Sludge production in wastewater treatment plants is nowadays a big concern due to the high produced amounts and their characteristics. Consequently, the study of techniques that reduce the sludge generation in wastewater treatment plants is becoming of great importance. In this work, four laboratory sequencing batch reactors (SBRs), which treated municipal wastewater, were operated to study the effect of adding the metabolic uncoupler 3,3',4',5-tetrachlorosalicylanilide (TCS) on the sludge reduction, the SBRs performance and the microbial hydrolytic enzymatic activities (MHEA). In addition, different operating conditions of the SBRs were tested to study the effect of the TCS on the process: two dissolved oxygen (DO) concentrations (2 and 9 $mg \cdot L^{-1}$) and two F/M ratio (0.18 and 0.35 g $COD \cdot g\ MLVSS^{-1} \cdot d^{-1}$). The sludge production decreased under high DO concentrations. At the same time, the DNA and EPS production increased in the four SBRs. After these stress conditions, the performance of the reactors were recovered when DO was around 2 $mg \cdot L^{-1}$. From that moment on, results showed that TCS addition implied a reduction of the adenosine triphosphate (ATP) production,

which implied a decrease in the sludge production. In spite of this reduction, the SBRs performances did not decay due to the increase in the global MHEA. Additionally, the sludge reduction was enhanced by the increase of the F/M ratio, achieving 28% and 60% of reduction for the low and the high F/M ratio, respectively.

Keywords: Sludge reduction; Metabolic uncoupler; microbial activity; ATP; EPS.

1. INTRODUCTION

In recent years, different techniques for the reduction of the sludge production in activated sludge processes have been studied. Thus, lysis and cryptic growth, uncoupling metabolism, reactor operation under high dissolved oxygen concentrations, oxic/settling/anaerobic (OSA) process and enzymatic hydrolysis have been reported in the literature (Khursheed and Kazmi, 2011; Liu, 2003; Low and Chase, 1999; Song and Feng, 2011). The mechanisms of most of these processes are still being discussed and contradictory results can be found. As an example, the mechanisms of sludge reduction of the OSA processes are not clear until now (Chen et al., 2003; Khursheed et al., 2015).

Focusing on uncoupled metabolism, it occurs when respiration does not control the bacteria metabolism and anabolism is the rate-limiting process, which implies energy in excess. Hao et al., 2010 discussed about its mechanisms. These authors revised both the theories supporting that uncoupling is related to maintenance energy and theories refusing it, considering that uncoupling is a separate intracellular metabolic activity. These authors supported the last one. Uncoupling can be achieved either under

substrate-sufficient conditions (this is not the case in activated sludge reactors treating municipal wastewater) or by addition of some chemicals (uncouplers).

Different uncouplers have been used successfully at laboratory and pilot plant scales for the reduction of the sludge production. The main disadvantage of the uncouplers use is the loss of process performance in terms of reduction of the organic matter removal efficiency. The first utilized uncouplers were phenolic compounds as para-nitrophenol, 2-4-5 trichloro-phenol (TCP) and 2-4 di-nitrophenol (DNP). However, the results reported about their application have been contradictory. Meanwhile Low and Chase, 1999 reported a 49% of sludge reduction working at concentrations between 100 and 120 $mg \cdot L^{-1}$ of para-nitrophenol, Qiong et al., 2013 obtained a 62% of sludge reduction with only 3 $mg \cdot L^{-1}$ of para-nitrophenol. In a recent study, Zuriaga-Agustí et al., 2016 obtained sludge reductions below 10% with 25 $mg \cdot L^{-1}$ of para-nitrophenol.

Another metabolic uncoupler, which has been studied in this work, is 3,3',4',5-tetrachlorosalicylanilide (TCS), whose use has been also successful for the reduction of the sludge production (Alexandre et al., 2016; Chen et al., 2002; Feng et al., 2014; Saini and Wood, 2008). The hazardous associated to the use of this chemical is lower than that of the phenolic uncouplers. In addition, lower concentrations of TCS are used since solubility in water at the pH of an activated sludge reactor is between 0.8 and 1 $mg \cdot L^{-1}$.

Once it is clarified that metabolic uncouplers are effective for the sludge reduction, their use at a commercial scale should be based on a deep knowledge about the consequences of their addition on the biomass and, consequently, on the process performance. Thus, the literature about metabolic uncouplers has evolved from studying the percentage of sludge reduction to wondering why it is produced (Feng et al., 2014). Important issues

like the appropriate food to microorganisms ratio (F/M) for uncoupling and the consequences of different dissolved oxygen concentrations are still to be discussed.

One of the indicators that could explain what happens in the process of sludge reduction by uncoupled metabolism is the cell activity. For its measurement, different parameters like the microbial hydrolytic enzymatic activities (MHEA) have been considered. They offer valuable information about the organic matter hydrolysis in activated sludge systems (Anupama et al., 2008; Molina-Muñoz et al., 2010). Chen et al., (2002) observed that the microbial activity and the cell respiratory activity increased significantly in the reactors where TCS was added. Feng et al., (2014) used two tetrazolium salts for the activity measurement to studying the effect of TCS on the electronic transported system (ETS). Contrary to the work by Chen et al., 2002, Feng et al., 2014 reported that ETS activities decreased significantly after ten days from the beginning of TCS addition.

Summarizing, it can be stated that contradictory results about how the addition of a metabolic uncoupler affects the biomass of an activated sludge process have been reported until now. Mechanisms are not clear and going deeper on the biomass characterization by analysis of microbial activity among other parameters is fundamental in order to know the viability of the sludge reduction by chemical uncouplers. This work has the aim of increasing the knowledge on this theme by the study of the effects of the addition of TCS under different operating conditions (two levels of dissolved oxygen concentration and two F/M ratios) on the biomass of four in parallel operated laboratory SBRs. In addition, soluble microbial products (SMP), extracted extracellular polymeric substances (eEPS), adenosine triphosphate (ATP) and MHEA were measured to study the influence of the TCS addition on the biomass and on the process performance. These parameters contributed to the understanding of the

phenomena occurring after addition of TCS under different operating conditions in a sequencing batch reactor (SBR). Moreover, COD removal efficiency and deoxyribonucleic acid (DNA) measurements were carried out to evaluate the reactors performance and cell lysis, respectively

2. MATERIALS AND METHODS

2.1. Wastewater

SBRs were fed with municipal wastewater (MWW). Samples were taken twice a week at the outlet of the primary treatment. Table 1 shows the average values and the standard deviations (SD) of several parameters for the wastewater samples used in the experiments. Analysis procedures are explained in section 2.3.

Table 1. Wastewater characterisation.

Parameter	Average ± SD
pH	7.7 ± 0.1
Conductivity ($mS \cdot cm^{-1}$)	2.2 ± 0.1
Turbidity (NTU)	70.8 ± 16.7
COD ($mg \cdot L^{-1}$)	321 ± 49
N_T ($mg \cdot L^{-1}$)	60 ± 13
P_T ($mg \cdot L^{-1}$)	49 ± 11
NH_4^+-N ($mg \cdot L^{-1}$)	6 ± 1
SS ($mg \cdot L^{-1}$)	122 ± 109
VSS ($mg \cdot L^{-1}$)	69 ± 46

2.2. Sequencing batch reactors

The experiments were carried out in four identical sequencing batch reactors, named SBR-1, SBR-2, SBR-3 and SBR-4. Figure 1 shows a scheme of each SBR.

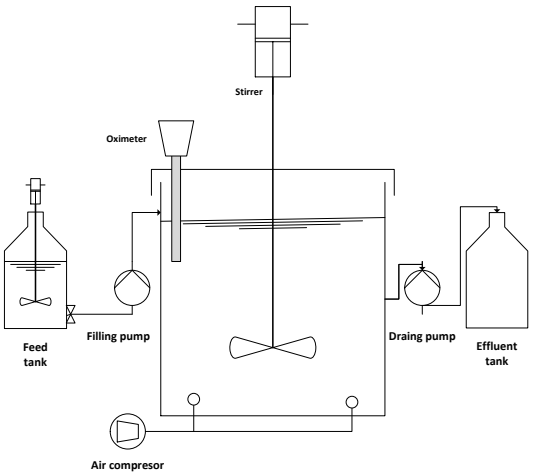

Figure 1. SBR scheme.

Each SBR consisted of a cylindrical tank (diameter of 10 cm and height of 30 cm). The main components were: mechanical stirrer (Velp Scientifica), air compressor 550 R Plus (Sera Precision), two air diffusers located on the reactor bottom, an oximeter OXI 49 (Crison) and two peristaltic pumps (Aiguapres) to fill the reactor with the aimed wastewater volume and to draw the treated effluent.

All the reactors worked with 4.5 L of volume reaction, three cycles per day and at a hydraulic retention time (HRT) of 1 day. In each cycle a wastewater volume of 1.5 L was fed into the reactor starting at this moment the aerobic reaction time. The stirrer and the air compressor worked throughout the reaction phase, stopping in the sedimentation phase. After this time, a volume of 1.5 L of treated wastewater was drawn and finally idle phase occurred. For all the reactors, the aerobic reaction and the sedimentation times were 6 and 1.5 hours, respectively.

2.3. Operation strategy

All the SBRs worked during 42 days. The effect of two F/M ratios and two dissolved oxygen (DO) concentrations in the mixed liquor on the biomass activity and process performance were studied in the reactors with addition of 1 $mg \cdot L^{-1}$ of TCS and in the reactors without uncoupler addition.

The F/M ratios were 0.35 and 0.18 g $COD \cdot g\ MLVSS^{-1} \cdot d^{-1}$. The MLVSS concentration was varied to achieve these aimed F/M ratios. Thus, MLVSS were maintained around 1000 and 1900 $mg \cdot L^{-1}$, performing the sludge withdrawals required for it. The DO tested were 2 and 9 mg $O_2 \cdot L^{-1}$. The flow supplied by the air compressor was regulated to reach these concentrations in the mixed liquor.

SBR-1 and SBR-2 worked at the highest F/M ratio, while SBR-3 and SBR-4 were operated at the lowest F/M ratio. After two weeks, once the microorganisms were acclimatized to the operating conditions, TCS was added to the feed of SBR-1 and SBR-3, while SBR-2 and SBR-4 went on operating without uncoupler. Additionally, in the four reactors, the DO concentration was increased in the mixed liquor from 2 to 9 mg $O_2 \cdot L^{-1}$ between 14th and 24th operating days. From this day on, the DO was decreased until around 2 mg $O_2 \cdot L^{-1}$. Table 2 summarizes the main operating parameters of the SBRs during their operation.

Table 2. Operational SBRs conditions.

Operating parameters	Day	SBR-1	SBR-2	SBR-3	SBR-4
TCS ($mg \cdot L^{-1}$)	1 – 13	0			
	14 – 42	1	0	1	0
F/M (g $COD \cdot g\ MLVSS^{-1} \cdot d^{-1}$)	1 – 42	0.35		0.18	
MLVSS ($mg \cdot L^{-1}$)		1000		1900	
DO ($mg \cdot L^{-1}$)	1– 13	2			
	14 – 24	9			
	25 – 42	2			

2.4. Analysis

The parameters measured for the characterization of the MWW were: pH, conductivity, turbidity, chemical oxygen demand (COD), total nitrogen (N_T), ammonium nitrogen (NH_4^+–N), total phosphorous (P_T), suspended solids (SS) and volatile suspended solids (VSS). The parameters measured in the mixed liquor were: suspended solids (MLSS) and volatile suspended solids (MLVSS) three times a week, and SMP, eEPS, DNA, ATP and MHEA, once a week.

The SBRs effluents were characterized twice a week by measuring pH, conductivity, turbidity, COD, N_T, NH_4^+-N, P_T, SS and VSS.

The pH was measured with a pH-Meter GLP 21+ and the conductivity was measured with an EC-Meter GLP 31+, both from Crison. The suspended and volatile suspended solids in the influent, effluent and mixed liquor were obtained according to APHA, 2005. Reactive kits and a Spectrophotometer DR600, both from Hach Lange, were used to measure COD, N_T, NH_4^+-N and P_T.

Furthermore, the observed sludge yield (Y_{obs}) was calculated to assess the biomass growth (Amanatidou et al., 2015; Klimiuk and Kulikowska, 2006):

$$Y_{obs} = \frac{MLVSS_{produced}}{COD_{removed}} = \frac{(MLVSS_j - MLVSS_i) \times (V_R/t) + X_e \times (V_d/t)}{(COD_0 - COD_e) \times (V_d/t)} \quad \text{(Eq.1)}$$

where t is the time interval between two days "i" and "j" (no sludge was withdrawn between "i" and "j"), X_e was the mean volatile suspended solids concentration in the effluent ($mg \cdot L^{-1}$) in this time span, V_R was the volume reaction (4.5 L), V_d was the influent/effluent volume (1.5 L) and COD_e was the mean COD measured in the effluent at the time interval t.

2.4.1. SMP and eEPS

Mixed liquor samples were collected from the reactors once a week to measure the SMP and eEPS. To obtain the SMP a volume of 25 mL of mixed liquor was centrifuged at 12,000 x g and the liquid phase was filtered at 0.45 μm. The extraction of EPS was performed using a cation exchange resin (Dowex® Marathon™ C from Sigma-Aldrich) applying the method used by Frølund et al., 1996. In both analysis, proteins by BCA method (Krieg et al., 2005; Zuriaga-Agustí et al., 2013) and carbohydrates by anthrone method (Frølund et al., 1996), were measured.

2.4.2. DNA

Quant-it™ dsDNA HS (0.2-100 ng) assay kit was used for the DNA measurement. The procedure included the filtration of 10 μL of mixed liquor (0.45 μm) and its mixing with 190 μL of Quant-it™ working solution. After incubation at room temperature for 2 minutes, the DNA concentration was measured in the Qubit™ fluorometer.

2.4.3. ATP

ATP was determined once a week by measuring the light generated in the reaction carried out between ATP and luciferin-luciferase. The light was measured by the luminometer NG Clean-Trace de 3M™ using the 3M Clean-Trace™ kit. Results were expressed in qualitative terms. Thus, the maximal amount of relative light units (RLU) in each reactor was used to indicate the highest concentration (100%) of ATP in the

reactor. In this way, the differences among values at the different conditions may relate the set conditions to the ATP production in a qualitative way.

2.4.4. MHEA

Samples of the mixed liquor were taken from the four reactors once a week to measure the MHEA. Alkaline and acid phosphatase activities were determined according to Goel et al., 1998 using 4-Nitrophenyl phosphate bis(tris) salt from Sigma-Aldrich as substrate solution. Lipase activity was determined employing a procedure adapted from Gessesse et al., 2003 using 4-Nitrophenyl palmitate (Sigma-Aldrich) as substrate solution where samples were incubated at 37 ºC for 30 min. α-D-Glucosidase activity was determined according to Goel et al., 1998 using 4-Nitrophenyl α-D-glucopyranoside from Sigma-Aldrich as substrate solution. Dehydrogenase activity was analysed employing a procedure adapted from Goel et al., 1998 using 0.75 mL of 0.3% (w/v) iodonitrotetrazolium chloride from Sigma-Aldrich as substrate solution. Protease activity was measured following a procedure adapted from Goel et al., 1998 using azocasein (Sigma-Aldrich) as substrate solution where samples were incubated for 60 minutes.

The values of the absorbances were measured at 410 nm for alkaline and acid phosphatases, lipase and α-D-glucosidase, at 490 nm for dehydrogenase and at 340 for protease activities in Thermo Scientific™ 9423UVG1002E spectrophotometer.

The reaction product of alkaline phosphatase, acid phosphatase, lipase and α-D-glucosidase activity with the substrates used in the enzymatic assays is p-nitrophenol (pNP). Hence one enzyme unit (EU) was defined to produce 1.0 mmol of p-nitrophenol in one hour. For the dehydrogenase activity the 1,3,5-Triphenyltetrazolium formazan is

the reaction product, therefore one enzyme unit was defined to produce 1.0 mmol of formazan dye in one hour. For the protease activity the reaction products are unknown and then the enzyme unit was defined as the absorbance increase after one hour. All the activity values were normalized dividing them by the MLVSS concentration.

2.5. Statistical analysis

An one-way ANOVA analysis (confidence level of 95 %) was carried out with Statgraphics Centurion XVI in order to study the statistical significance of TCS addition on the biological treatment of municipal wastewaters in the laboratory SBRs.,. The variance analysis of the parameters COD removal efficiency, SMP_{TOT} (sum of proteins and carbohydrates), $eEPS_{TOT}$ (sum of proteins and carbohydrates), ATP, Y_{obs} and MHEA have been studied.

Two levels for TCS factor were evaluated (Yes or No, for the reactors with and without TCS addition, respectively), meanwhile the corresponding units for each dependent variable were used.

3. RESULTS AND DISCUSSION

3.1. SBR performance

Figure 2 shows the evolution of the COD removal efficiencies during the experiments for the four reactors.

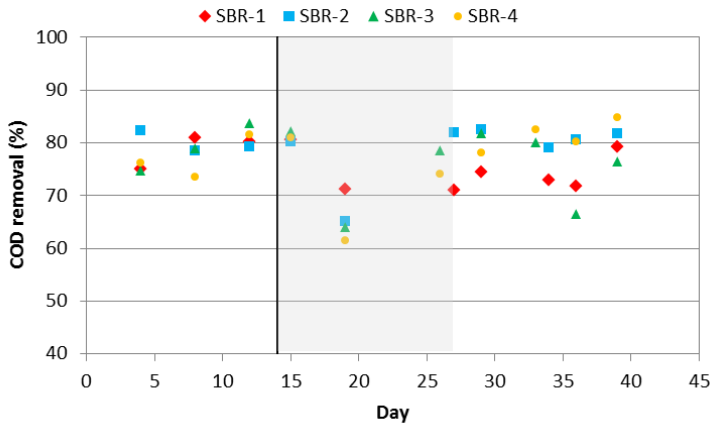

Figure 2. COD removal efficiency throughout the biological treatment.
Grey area: DO around 9 mg·L^{-1}. Vertical line: [──] TCS addition in SBR-1and SBR-3.

It can be observed that a slight increase of the COD removal efficiencies of all the reactors occurred a few days after their start-up, once biomass was acclimatized to the operating conditions of the SBRs. Thus, similar values were reached for all the reactors. The average COD removal efficiency was 76 ± 7% in the 12th day. From the 14th day on, TCS was added to SBR-1 and SBR-3 and oxygen concentration was increased up to around 9 mg·L^{-1}.

The increase of the dissolved oxygen concentration reduced the COD removal efficiencies progressively irrespective of the TCS addition. The reduction of the COD removal efficiencies was due to cell lysis (Khursheed and Kazmi, 2011), evidenced by the DNA concentrations, which increased in all the reactors. DNA average values in the four SBRs increased from 28.3 ± 7.0 on the 12th day to 147.8 ± 19.2 (ng DNA·g MLVSS^{-1}) on the 20th day. Once DO concentration below 2 mg/L was restored, the COD removal efficiencies increased again. However, the performance in the SBRs with TCS addition showed an unstable behavior (high variability) and the COD removal

efficiency remained below 80%. However, the COD removal percentage increased in the last days in SBR-2 and SBR-4 up to 85% and 82%, respectively.

Concerning the sludge production, the effects of the DO concentration, the TCS addition and the different F/M ratio on Y_{obs} in the four reactors can be observed in Figure 3 this parameter.

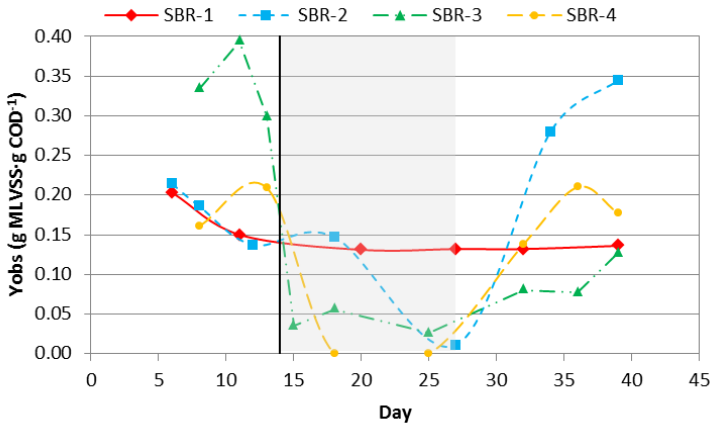

Figure 3. Y_{obs} throughout the biological treatment in four SBRs.
Grey area: DO around 9 mg·L^{-1}. Vertical line: [──] TCS addition in SBR-1and SBR-3.

High DO concentration reduced the Y_{obs} values in all the reactors, which is in concordance with the literature (McWhirter, 1978; Roques et al., 1984). Abbassi et al., 2000 reported that the increase of the oxygen concentration in the mixed liquor results in an increase to the aerobic volume inside the flocs that promote the aerobic degradation in the floc matrix, resulting in sludge reduction. In Figure 3 it can be observed that this effect had a more significant impact on the SBRs with the lowest F/M ratio, i.e. on those reactors where endogenous respiration occurs at a higher extent (SBR-3 and SBR-4).

The TCS addition caused a Y_{obs} decrease since this chemical produced the uncoupling metabolism, as stated by other authors (Chen et al., 2002; Feng et al., 2014). However,

the effect of this metabolic uncoupler on the sludge production was more pronounced in SBR-1 than in SBR-3. Thus, on the 40th day, the Y_{obs} in SBR-1 was around 60% lower than that measured in SBR-2; meanwhile the reduction percentage of Y_{obs} between SBR-3 and SBR-4 was only around 28%. This effect can be explained by the TCS/MLSS ratio. It was higher in SBR-1 (0.33 mg TCS/g MLSS) than in SBR-3 (0.17 mg TCS/g MLSS.

3.2. SMP and eEPS

Both eEPS and SMP were analyzed as explained in materials and method section. Figure 4 shows the total eEPS concentration (proteins + carbohydrates) for the 6 sampling days in the four reactors. Each bar has been divided into two: the bar at the bottom represents the protein concentration, whereas the bar at the top represents the carbohydrate concentration. The sum of the lengths of the bars gives the total eEPS concentration. It can be observed that the protein concentration was higher than the carbohydrate concentration in all the samples.

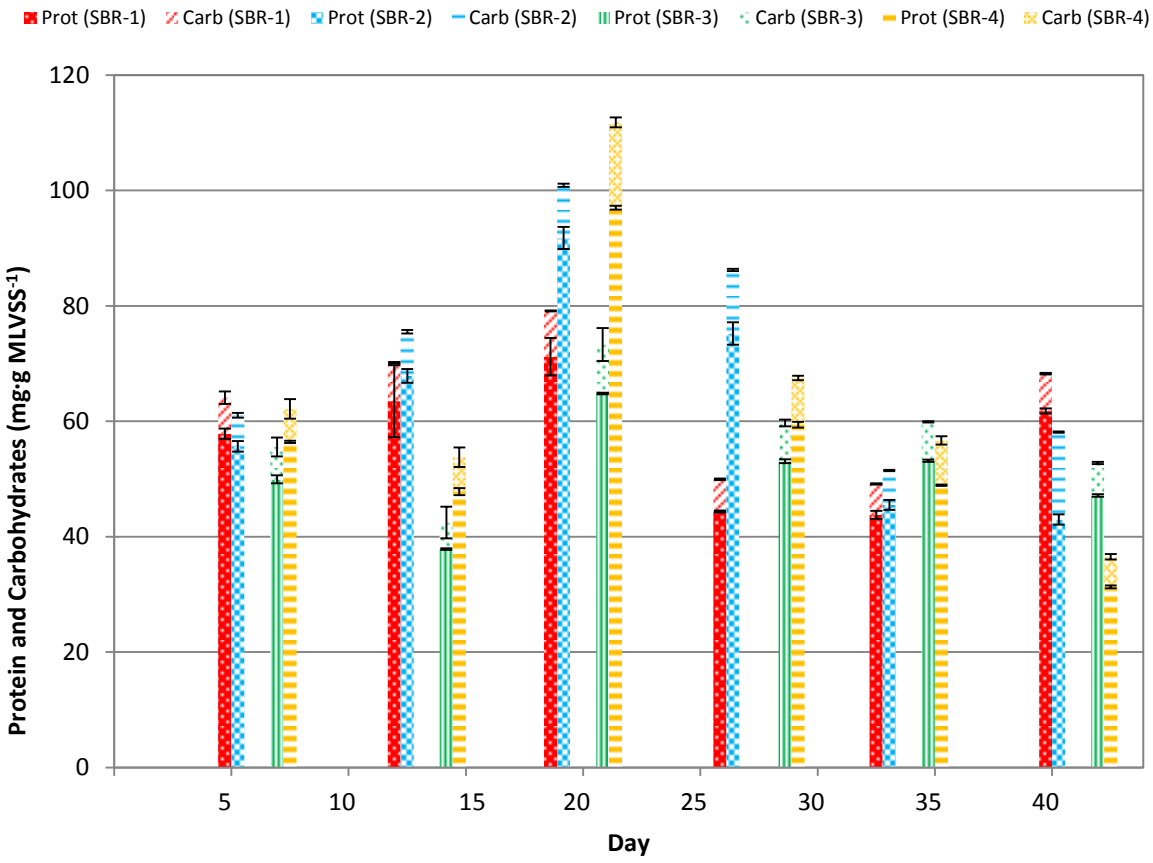

Figure 4. Proteins and carbohydrates concentrations in the eEPS.

Before the TCS addition, the eEPS concentrations were similar in all the reactors as it can be seen for the first sampling days (5^{th} and 7^{th} days). The highest eEPS concentrations were measured in the third sampling (19^{th} and 21^{st} days), i.e. in the period with the high DO concentration in the reactors. The same relation between DO and eEPS was reported by Faust et al., 2014, which worked with 4 and 1 mg $O_2 \cdot L^{-1}$. However, in our case the increase of the eEPS was considerably higher in the SBRs without TCS addition than in SBRs with uncoupler. This phenomenon was not observed at 2 $mg \cdot L^{-1}$ of DO. From that day on, when the DO concentration decreased, the eEPS decreased in all the reactors. In the last sampling (40^{th} and 42^{sd} days), the eEPS concentrations in the SBRs with TCS addition were higher than in the reactors without

TCS. This behavior was also described by other authors (Feng et al., 2014; Li et al., 2012). On the other hand, as commented above, in all the samples taken from the four reactors, proteins concentration was much higher than the carbohydrates concentration, which was always below 15 mg·g $MLVSS^{-1}$.

Concerning the SMP, the measured concentrations of proteins and carbohydrates were very similar for the four reactors in the first and in the second sampling (Figure 5).

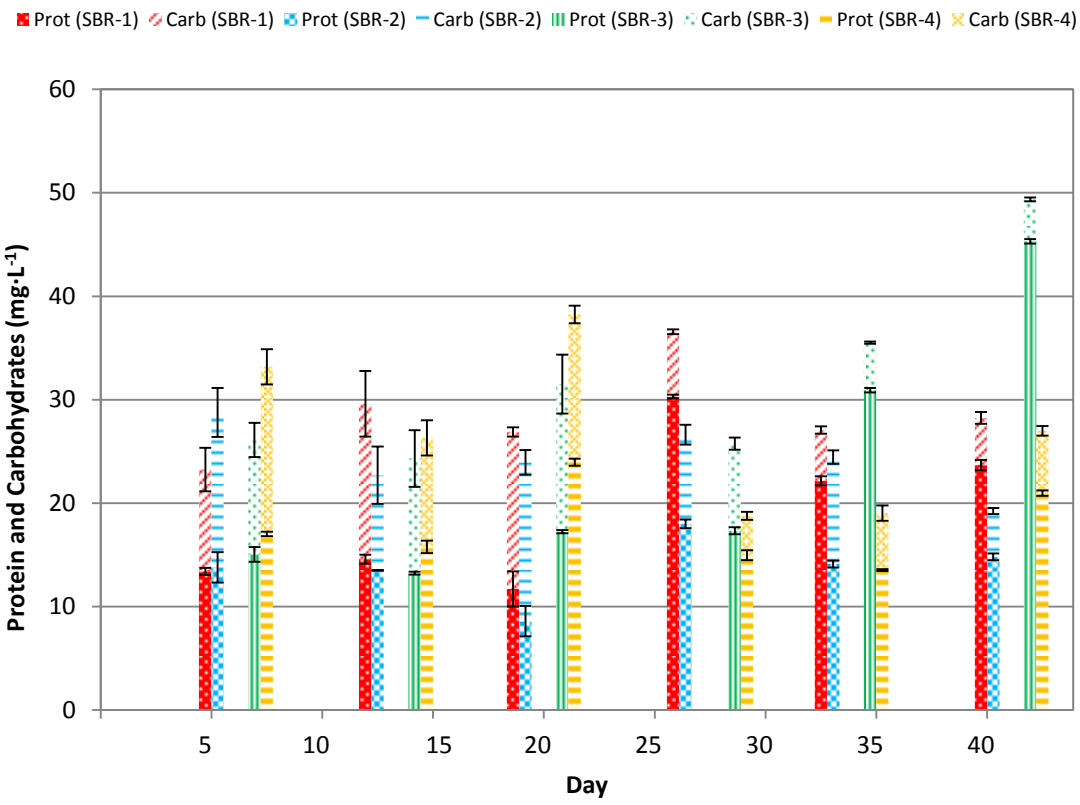

Figure 5. Proteins and carbohydrates concentrations in the SMP.

The highest DO concentration had a lower effect in the SMP than in the eEPS. Under this condition, the SMP concentrations in the SBRs with lowest F/M ratio were the highest, due to the enhancement of the endogenous processes and consequently due to the cellular debris production. Once dissolved oxygen was lowered, the

carbohydrates/proteins ratio diminished in all the reactors. In the three last samplings, the reactors where TCS was added maintained the SMP concentrations higher than SBR-2 and SBR-4. This phenomenon was also observed by some authors (Feng et al., 2014; Li et al., 2012). This can be explained by the stress effect of the TCS on the microorganisms, implying the enhancement of the SMP production.

3.4. ATP

Figure 6 shows the RLU of ATP and standard deviations in the four SBR reactors.

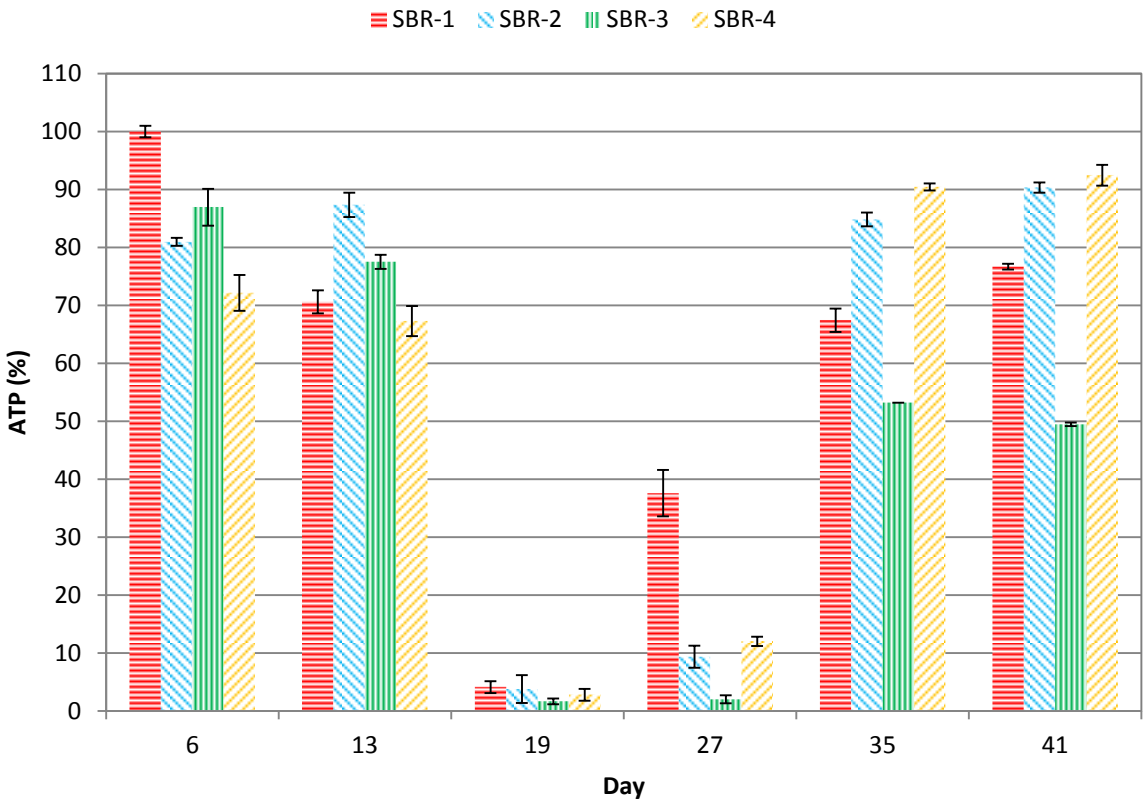

Figure 6. Variation of ATP in the SBRs.

It seems very clear that ATP was hardly detected in the third sampling (19th day) of all the reactors, when the DO concentration was around 9 mg·L^{-1}. It indicates that the high

DO concentration practically eliminated the ATP generation, implying a negligible anabolism activity. Once the DO concentration was lowered, ATP production increased gradually. In the last sampling (41st day), the ATP production in SBR-2 and SBR-4 was 90% and 92% related to the maximum value, respectively. On the contrary, inhibition of ATP production occurred in SBR-1 and SBR-3, resulting in 77% and 49% of the maximum value in the same sampling, respectively. This effect was due to the fact that the TCS addition drove to a diminution of the proton gradient across cell membrane, which decreased the ATP generation (Low and Chase, 1999; Yang et al., 2003). It implied that the biomass growth was lower in the reactors where TCS was added, what coincides with the Y_{obs} values mentioned above.

3.5. MHEA

The MHEA was evaluated through the alkaline and acid phosphatases, lipase, α-D-glucosidase, dehydrogenase and protease activities, which are represented in Figure 7 with their respective standard deviations.

When the process performance was recovered (the last sample) after stress conditions due to high DO, it can be observed that the TCS addition had different effects in the MHEA. Comparing the SBRs with and without TCS addition, there were three MHEA that increased their values in the reactors with TCS: alkaline phosphatase, lipase and α-D-glucosidase. In contrast, for acid phosphatase, dehydrogenase and protease activities the measured values remained very similar for the four reactors (except for dehydrogenase in SBR-4).

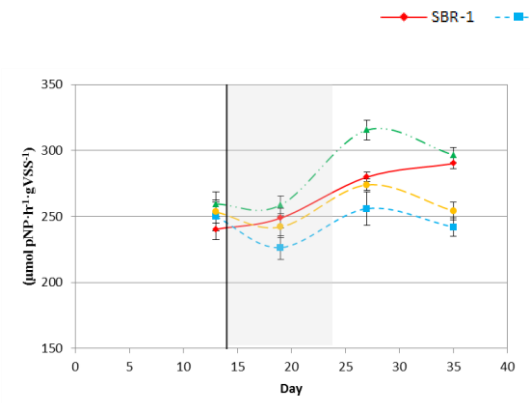

Figure 7.a) Alcaline phosphatase

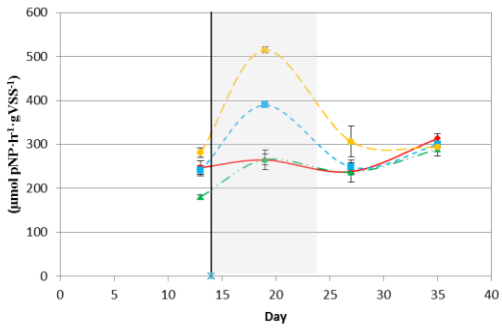

Figure 7.b) Acid phosphatase

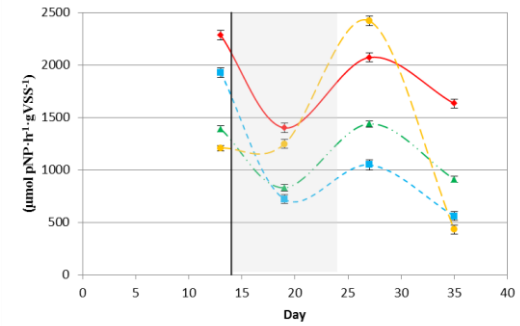

Figure 7.c) Lipase

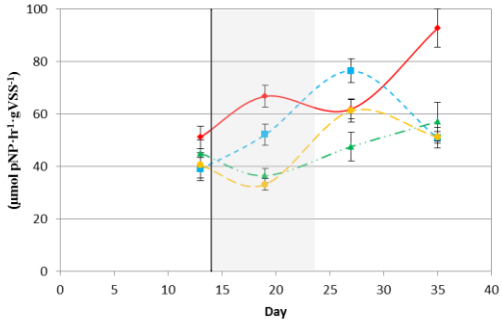

Figure 7.d) α-D-Glucosidase

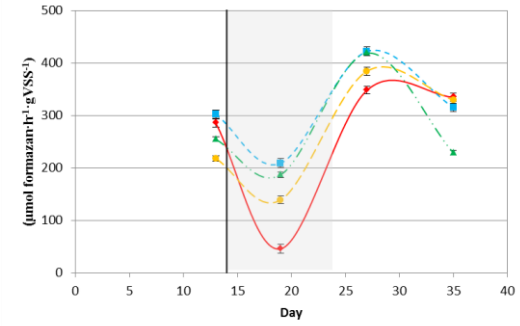

Figure 7.e) Dehidrogenase

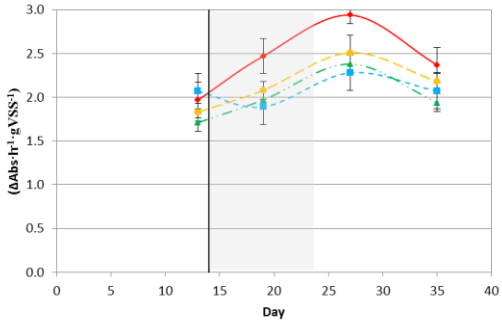

Figure 7.f) Protease

Figure 7. MHEA activities. Grey area: DO around 9 mg·L^{-1}.
Vertical line: [──] TCS addition in SBR-1and SBR-3.

Thus, it can be concluded that the lowest sludge production achieved in SBRs with TCS addition coincided with an increase in the global MHEA activities in SBR-1 and SBR-3.

This explains that the COD removal performance of these reactors achieved similar values to those obtained in SBR-2 and SBR-4, in spite of the reduction of the sludge production.

The F/M ratio showed a more significant effect on the performance of the SBRs with TCS addition in the stationary conditions. In the final sample, similar activities were measured in SBR-2 and SBR-4; meanwhile in SBR-1 and SBR-3 lipase, α-D-glucosidase, dehydrogenase and protease activities reached higher values in SBR-1, which worked with the highest F/M ratio. This is explained by the MLVSS concentration (see Table 2). Thus, the lowest concentration of MLVSS in SBR-1 exhibited the highest enzymatic activity for the degradation of the same influent COD.

On the other hand, if the effect of high DO concentration on the MHEA is discussed, it can be seen in Figure 7 (grey area) that there were three different impacts on MHEA. Lipase and dehydrogenase activities decreased in these stress conditions in the four SBRs. However, acid phosphatase and protease activities increased (except protease in SBR-2). For alkaline phosphatase and α-D-glucosidase the relationship between these activities and the effect of high DO concentration was not clear. Under these conditions, COD removal performance and Y_{obs} decreased in all the reactors due to cell lysis, which was confirmed by the DNA concentrations in the reactors (commented in section 3.1). When DO concentration decreased below 2 $mg \cdot L^{-1}$, the maximum values of the measured activities were reached in all the SBRs (27^{th} day), with the exception of the acid phosphatase that showed an opposite trend to the alkaline phosphatase. This increase in MHEA was due to the new favorable conditions in the reactors enhancing the release of enzymes by the microorganisms for the degradation of the intracellular materials released from cell lysis (Jung et al., 2006). This fact is corroborated by the diminution of eEPS (as a consequence of the increase of the enzymatic activities) after

their increment under high DO concentration. Finally, once the process performance reached the initial values (last sample), the measured activities decreased. This can be due to the fact that the cell lysis also decreased, which was substantiated for DNA measurements. This effect was more intensive in the SBRs without TCS addition, in which DNA decreased from 147.8 ± 19.2 (average value in 20th day for all the reactors) to 58.3 ± 0.7 (ng DNA·g MLVSS^{-1}), while in SBR-1 and SBR-3 decreased to 114.7 ± 9.0 (ng DNA·g MLVSS^{-1}). The differences among the reactors (according to TCS addition and F/M ratio) in this final stage have been commented at the beginning of this sub-section.

3.6. Statistical analysis

Table 3 shows F- and p-value for each dependent variable considered when the TCS addition was the factor evaluated.

Table 3. One way ANOVA results for TCS addition factor.

Dependent variable	F	p-value
Removal COD (%)	1.34	0.2656
SMP_{TOT} (mg·L^{-1})	4.83	0.0453
$eEPS_{TOT}$ (mg·g MLVSS^{-1})	0.93	0.3520
ATP (%) *	17.58	0.0057
Y_{obs} (g MLSS·g COD^{-1})	10.37	0.0062

(*) Experimental time with 2 mg·L^{-1} of DO concentration.

These results indicate a statistically significant relation between TCS addition and SMP_{TOT}, ATP and Y_{obs}, since the calculated p-values for these variables were lower than 0.05. These relationships can also be observed in the Tukey diagrams presented in figure 8, which show that TCS addition increased the total SMP in the SBRs and

decreased ATP concentration and the Y_{obs} values. On the other hand, statistically significant relation was not observed between TCS addition and COD removal efficiency in the reactors. This was due to the increase of the MHEA, especially the alkaline phosphatase ($F = 7.28$; p-value = 0.0224), whose increase when TCS was added was statistically significant.

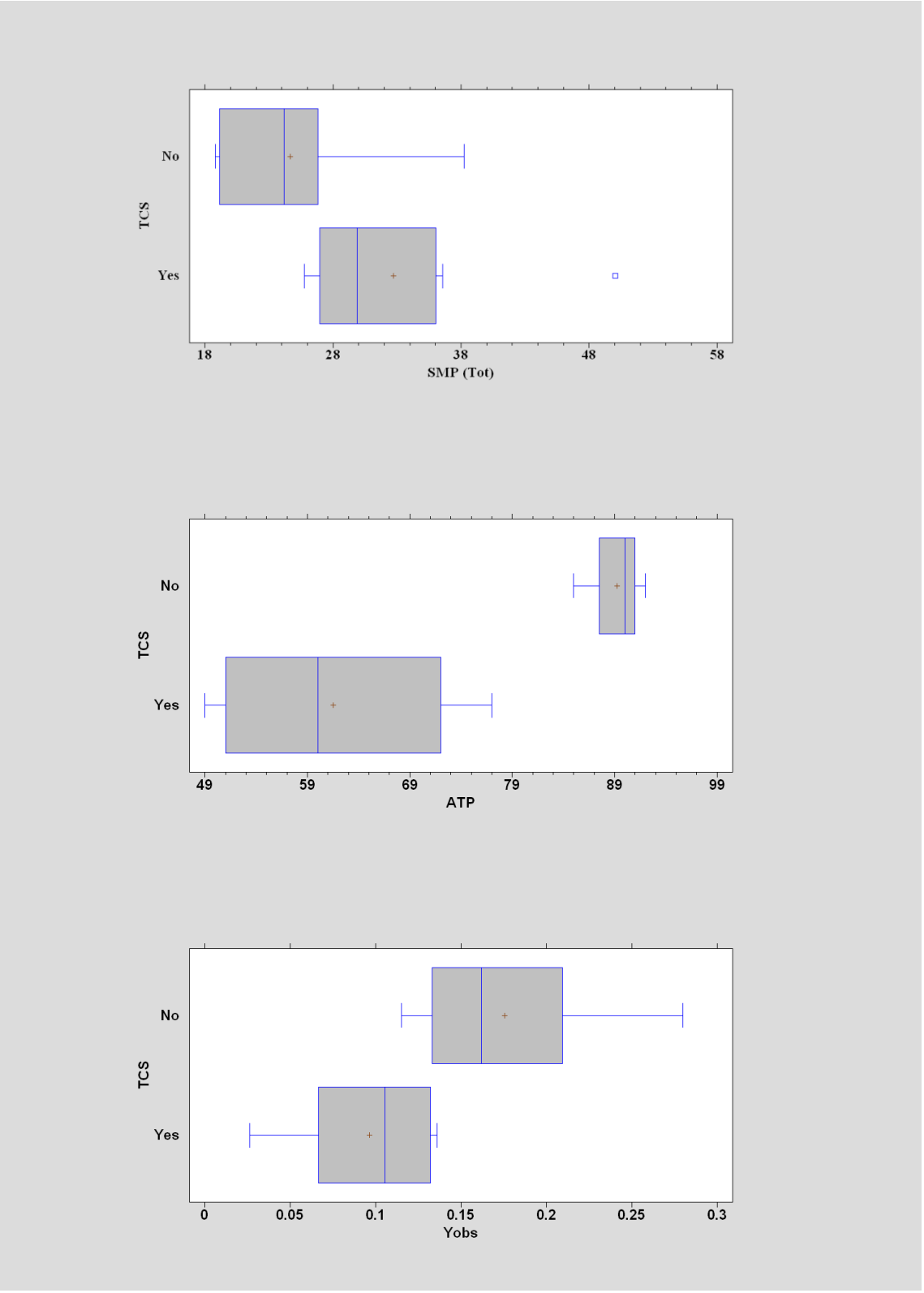

Figure 8. Tukey diagrams for TCS addition factor.

4. CONCLUSIONS

In this work, the effect of a metabolic uncoupler (TCS) on the process performance, the sludge production and the bacterial activity of the mixed liquor under different operational conditions has been studied.

The ability of the TCS for the reduction of the sludge production has been corroborated obtaining lower observed sludge yield values in the reactor with TCS addition. The highest difference was found for the highest F/M ratio tested. In addition, in the SBRs with TCS addition, an increase in the global MHEA activities was observed, which explains that the COD removal efficiencies in these reactors were similar to those achieved in the SBRs without TCS addition, in spite of the reduction of the sludge production.

Under an extreme DO concentration (9 $mg \cdot L^{-1}$) the performance and the sludge production went down in all the reactors irrespective of the TCS addition. However, among the measured MHEA, only lipase and dehydrogenase activities were reduced significantly in this period. The stress of the microorganisms under these extreme conditions has been clearly showed by the high eEPS and DNA concentrations and by the low ATP concentrations measured.

Finally, it has to be highlighted that according to our results the MHEA may be an interesting measurement to follow the effect of a metabolic uncoupler. In this work, the highest enzymatic activities were measured in the SBR with TCS addition and highest F/M ratio, under DO conditions around 2 $mg \cdot L^{-1}$.

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