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Changes in the process performance and microbial community by addition of the metabolic uncoupler 3,3',4',5-tetrachlorosalicylanilide in sequencing batch reactors

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Abstract

A complete study about the effects of 3,3',4',5-tetrachlorosalicylanilide (TCS) on organic matter elimination performance, sludge production and on the microbial community of a biological wastewater treatment process has been performed. For this purpose two sequencing batch reactors (SBR) worked in parallel for 43 days with 0.8 $mg \cdot L^{-1}$ of TCS (SBR-1) and without this metabolic uncoupler (SBR-2). Results indicated that 63.3% of sludge reduction was achieved in SBR-1. However, COD removal efficiency was maintained in similar values in both reactors (89.1% and 92.1% in SBR-1 and SBR-2, respectively). The exhaustive mixed liquor characterization led to know deeply the action mechanism of TCS. In this way, a 69% of adenosine triphosphate (ATP) reduction was observed in SBR-1 in comparison with values measured in SBR-2. On the contrary, an increase in soluble microbial products (SMP) and DNA concentrations occurred as a consequence of TCS addition. Thus,

it could be concluded that sludge reduction due to TCS addition was due to both uncoupling effect and cellular lysis. Also, increase in all microbial hydrolytic enzymatic activities measured was observed, which explained the stable performance achieved in SBR-1 despite to the results explained above. It should be highlighted that this uncoupler should not be used in biological treatments that require nitrogen elimination because nitrifying bacteria were affected by its addition (*Nitrosomonas* and *Nitrospira*). Finally, the 16S rRNA gene amplicon sequencing informed that an important reduction of bacterial diversity resulted in SBR-1 due to TCS addition.

Keywords: Biological wastewater treatment; metabolic uncoupler; TCS; bacterial communities; sludge reduction

1. INTRODUCTION

Different techniques to reduce sludge production in biological wastewater treatment have been studied over the years. These techniques include chemical, mechanical, thermal and electrical treatments. Some of these techniques are ozonation (Fall et al., 2018; Semblante et al., 2017), oxic-settling-anoxic (OSA) process (Ye and Li, 2010), oxidation (Zuriaga-Agustí et al., 2012)…or uncoupler substances. Russell and Cook, (1995) introduced the term "uncoupling". Under normal growth conditions, the catabolism and anabolism of bacteria are coupled. However, under uncoupled conditions the catabolism is undisturbed leading to unchanged substrate consumption, while the adenosine triphosphate (ATP) synthesis and anabolism slow down causing reduction in biomass yield. The phenomenon of uncoupled metabolism may be carried out under abnormal conditions such as the presence of inhibitory compounds or some heavy metals (Zn, Ni, Cu, Cr), not optimal temperatures, nutrient limitations, oxic-anoxic (or oxic-anaerobic) cycling conditions and chemical uncouplers

(Foladori, 2010; Velho et al., 2016). Some of the substances commonly used as chemical uncouplers are DNP (2,4-dinitrophenol) (Ding et al., 2019), TCS (3,3',4',5-tetrachlorosalicylanilide) (Ferrer-Polonio et al., 2017), pNP (para-dinitrophenol) (Zuriaga-Agustí et al., 2016) or another reagents based on the phenolic compounds such as mNP (m-nitrohenol), oNP (o-nitrophenol) or pCP (p-chlorophenol) (Wang et al., 2017). TCS has been widely adopted as an environmentally-benign uncoupler to reduce yield of activated sludge. However, its potential impact on the microbial community of SBR activated sludge is still unknown. The 16S rRNA next generation sequencing (NGS) technology provides sufficient sequencing depth to cover complex microbial communities (Xu et al., 2018; Zhang et al., 2018). This technique involves polymerase chain reaction (PCR) amplification of taxonomically informative regions of the 16S ribosomal rRNA gene (Conrad and Vlassov, 2015). In this method, DNA from the microbial community dead cells can also be extracted. To overcome false-positive bacterial relative abundance rates, due to DNA persistence after cell death, different DNA intercalating dyes are applied (Jäger et al., 2018; Lin et al., 2011; Tian et al., 2017). One of these dyes is propidium monoazide (PMA), which only penetrates into membrane-damaged cells, where a photo-induced azide group covalently binds to DNA (Nocker et al., 2006).

The use of uncouplers to reduce the sludge production in wastewater treatment has been reported in several recent research articles. Han et al., (2017) achieved 43.7% of sludge reduction using 10 $mg \cdot L^{-1}$ of 2,4-dichlorophenol, while Ding et al., (2019) obtained 37.5% with 45 $mg \cdot g\ VSS^{-1}$ of another phenol compound (2,4 dinitrophenol). Other substances like folic acid have been also employed, resulting a 44.7% of sludge reduction adding 0.8 $mg \cdot L^{-1}$ of this substance. However, this substance lost its effect after 40 days of continuous dosing (Ferrer-Polonio et al., 2019). Regarding TCS uncoupler, Chen et al., (2002) reported that low concentrations of TCS (0.2 and 0.4 $mg \cdot L^{-1}$) have not influence on sludge production. But,

40% and 48% of sludge reduction were achieved with 0.8 and 1.0 $mg \cdot L^{-1}$ of TCS, respectively. Under similar operational conditions Feng et al., (2014) reported 42.7% of sludge reduction adding 1.0 $mg \cdot L^{-1}$ of TCS. Nevertheless, in these works there are a lack of information concerning mechanism and effects in the microbial community.

Gostomski and De Vela, (2018) propose several mechanisms by which metabolic uncouplers are likely to promote biomass reduction in microbial systems in addition to the classic uncoupler explanation. A definitive explanation relating energy diversion to non-growth use, reduction in the biofilm stability, and shift in microbial diversity of the culture is yet to be established. This can be done by further clarifying the correlations of key parameters: sludge reduction, microbial respiration and ATP synthesis, soluble microbial products (SMP) production and composition, quorum signaling molecules production, change in cell surface hydrophobicity and microbial ecology (Gostomski and De Vela, 2018).

In this work, a holistic study was designed to investigate the effect of uncoupler TCS on biological wastewater treatment. For it, two SBRs working in parallel for 43 days with and without TCS were studied. Sludge reduction, microbial respiration, ATP synthesis, microbial hydrolytic enzymatic activities (MHEA), SMP production, biomass characterization and process performance were analyzed throughout experimental time. In addition, NGS technology was used for detailed analysis of bacterial community structure, with and without PMA treatment. All of these parameters contributed to the understanding of the phenomena occurring after addition of TCS in a sequencing batch reactor.

2. MATERIALS AND METHODS

2.1. Sequencing batch reactors and their operation

Biological treatment was performed in two identical SBRs (SBR-1 and SBR-2) that were operated in parallel. Figure S1 (supplementary material) shows a scheme of the laboratory SBR including the main components. Activated sludge from a municipal wastewater treatment plant from Valencia (Spain) was used to start-up the reactors. Mixed liquor suspended solids (MLSS) concentration was maintained around 2.5 $g \cdot L^{-1}$ in both SBRs during the experimental procedure (2582 ± 208 and 2524 ± 156 $mg \cdot L^{-1}$ in SBR-1 and SBR-2, respectively). To maintain this concentration periodically sludge withdrawals were carried out. SBR-1 and SBR-2 worked under the same operational conditions (Table 1) for 43 days.

Table 1. Operating parameters in SBR-1 and SBR-2.

Operating parameters SBR-1 and SBR-2		
Reaction volume (V_R)	6	L
Hydraulic retention time	16	h
F/M ratio	0.3	kg COD·kg $MLSS^{-1} \cdot d^{-1}$
MLSS	≈ 2.5	$g \cdot L^{-1}$
Operation days	43	d
Number of cycles	3	$cycle \cdot d^{-1}$
$Volume_{fed/draw}$ ($V_{feed/draw}$)	3	$L \cdot cycle^{-1}$
Cycle characteristics SBR-1 and SBR-2		
Filling+Anaerobic reaction	2	h
Aerobic reaction	4	h
Sedimentation	1.5	h
Draw	25	min
Idle	5	min
Feed characteristics		
	0-8 day	**9-43 day**
SBR-1	SWW	SWW+TCS
SBR-2		SWW

Both reactors were fed with the same synthetic wastewater (SWW) prepared with peptone and meat extract as nitrogen and organic matter sources (in equal amount; 225 $mg \cdot L^{-1}$) and K_2HPO_4 as phosphorus source (28 $mg \cdot L^{-1}$). As it can be seen in Table 1, after an initial period of 8 days, for biomass acclimation to the synthetic wastewater, a concentration of 0.8 mg of TCS was added to each liter of SWW prepared to feed SBR-1 (SWW+TCS) until the end of the experiment, while SBR-2 worked as blank with SWW. This TCS concentration was selected due to the low solubility of this compound, observing precipitation above the tested concentration. In terms of wastewater characterization both SWWs had a COD of 517 ± 34 $mg \cdot L^{-1}$ and a relationship COD:N:P of 100:12:1.

2.2. Analysis

2.2.1. SBR performance

Throughout the experimental procedure several parameters of the effluent and mixed liquor (ML) were analyzed. Three times a week the following parameters were measured: pH (GLP 21+; Crison), conductivity (GLP 31+; Crison), turbidity (D-122; Dinko) and COD of the effluent samples, and MLSS of the ML samples. Additionally, total nitrogen (N_T) and total phosphorous (P_T) in the effluent samples were analyzed once a week. To measure COD, N_T and P_T a Spectroquant NOVA 30 and reactive kits (both from Merck) were used. MLSS was measured according to APHA, (2005).

MLSS values allowed calculating the average sludge production (ΔX). For the calculation, it was obtained the sludge production in several periods of the experimental time, in which sludge withdrawal was performed (to maintain 2.5 $g \cdot L^{-1}$ into the reactors) in between the days (i and j), according Eq.(1).

$$X_{i-j} = \frac{(MLSS_j - MLSS_i) \cdot V_R}{j - i} + [SS_{ef} \cdot Q_{ef}]_i^j \qquad \text{Eq.(1)}$$

The first term of this equation takes into account the biomass growth into the reactor and the second one the biomass drained in the treated effluent (it was practically 0 in both reactors for all the time).

The ΔX and the average sludge retention time (SRT) were calculated using Eq.(2) and Eq.(3), respectively.

$$\Delta X = \frac{\sum X_{i-j}}{d} \qquad \text{Eq.(2)}$$

$$SRT = \frac{\overline{MLSS} \cdot V_R}{\Delta X} \qquad \text{Eq.(3)}$$

where "d" was the number of days in which average sludge production was calculated and $\overline{MLSS}$ was calculated as the average MLSS concentrations measured during the experimental time, which value was around 2.5 $g \cdot L^{-1}$.

2.2.2. Nitrification

Nitrification capacity of biological treatment was evaluated by Respirometer BM-Advance from Surcis. This equipment allowed obtaining the dynamic respiration rate of microorganisms (Rs; mg $O_2 \cdot L^{-1} \cdot h^{-1}$) under addition of an ammonium source. This test was performed in the last day of the experimental time of both reactors. Before the respirometric test, air was supplied until endogenous conditions of activated sludge were achieved. For the test, 1 L of the ML sample was placed in the respirometer. Then, ammonium chloride (NH_4Cl) in a concentration of 150 $mg \cdot L^{-1}$ was added to the ML maintaining the mechanical stirrer and the air diffuser connected during the test. Dissolved oxygen and pH were measured continuously during the experimental procedure in order to assess whether

nitrification occurs. The temperature was the same as in the SBR. Results were expressed in terms of the oxygen consumption rate (Rs), expressed in mg $O_2 \cdot L^{-1} \cdot h^{-1}$.

2.2.3. Biological characterization

An exhaustive description of the methodology employed to determine SMP, ATP, cellular viability, Microbial hydrolytic enzymatic activities (MHEA) and bacterial community can be found in another work of the research group (Ferrer-Polonio et al., 2019).

SMPs were characterized in terms of proteins (BCA method) and carbohydrates (anthrone method) concentrations. PhotonMaster™ Luminometer from Luminultra® and QG21Waste™ kit were used to quantify the total ATP (tATP) and the dissolved ATP (dATP), which included extracellular ATP released from dead or lysis cell. From these values the cellular ATP (cATP: corresponding to ATP of viable cells) was obtained. Cellular viability was evaluated assessing the membrane integrity of bacteria using Film Tracer™ LIVE/DEAD™ Biofilm. Additionally, lipase, acid an alkaline phosphatase, α-D-Glucosidase, dehydrogenase and protease concentrations were measured following a modified methodology reported by Gessesse et al., (2003) and Goel et al., (1998). All the parameters commented were measured once a week.

The variations of bacterial community, due to TCS addition, were evaluated using massive partial sequencing of 16S rRNA genes. This technique was applied to SBRs samples in 6th (before TCS addition and after biomass adaptation) and 41st operating days. In this work abundance of total cells and viable cells of community were assessed. For it, propidium monoazide stain (PMA) was added on the samples for evaluating the viable cells, while samples without PMA provided the total cells (viable and no viable).

2.2.8. Statistical analysis

The effect of TCS addition on the system was assessed by one-way ANOVA analysis (confidence level of 95 %) with Statgraphics Centurion XVII. All the measures carried out in the SBRs during the experimental time were compared in both reactors to determine the statistical significance between TCS addition and biological treatment. In this way, parameters related to effluent characteristics like pH, conductivity, turbidity, COD, N_T and P_T were evaluated. In the same way, parameters related to biomass were also compared, which included ΔX, ATP and SMP.

3. RESULTS AND DISCUSSION

3.1. TCS effect on the SBRs performance and sludge reduction

3.1.1. SBRs performance

Table 2 shows the average values of some effluent parameters with their standard deviations. In addition, in Table 2 the F-ration and p-value are presented when one-way ANOVA is performed for each parameter considering the SBR as factor. F-ratio provides information about the differences in the mean of the variances between the two groups studied (reactor with and without TCS addition) respect to the mean of the variance within the groups as a whole:

$$F = \frac{Mean\ square\ between\ groups}{Mean\ square\ within\ groups} \qquad \text{Eq.(4)}$$

In this way, when two means are similar, F is close to 1. On the contrary, the high values of F indicate that there is a significant difference. A p-value less than 0.05 shows that this difference is not random and there is a statistical significance.

Table 2. Mean values with their standard deviations of effluent parameters. F ratio and p-value of one-way ANOVA

	SBR-1	SBR-2	F ratio; p-value
pH	7.9 ± 0.1	7.7 ± 0.2	33.92; < 0.0001
Conductivity ($\mu S \cdot cm^{-1}$)	1285 ± 93	1126 ± 97	78.68; < 0.0001
Turbidity (NTU)	3.96 ± 5.63	0.23 ± 0.81	9.18; 0.0062
COD ($mg \cdot L^{-1}$)	54.5 ± 14.6	39.6 ± 14.7	19.14; 0.0002
N_T ($mg \cdot L^{-1}$)	47.4 ± 7.6	25.6 ± 13.8	71.80; < 0.0001
P_T ($mg \cdot L^{-1}$)	5.9 ± 4.4	3.7 ± 2.3	0.53; 0.4878

From these results, it can be concluded that TCS addition led to an increase in mean values of pH, conductivity and turbidity. Differences observed in pH and conductivity were due to presence of TCS in the feed, since this chemical caused a slightly increase of both parameters in SBR-1 feed. Turbidity differences were mainly due to higher SMP concentrations in SBR-1, which will be explained in more detail in 3.2.1 section. Regarding the organic matter degradation and nutrients assimilation, lower COD and N_T concentrations were observed in SBR-1. Although TCS addition reduced the effluent quality, it should be highlighted that effluent COD values of SBR-1 met the legal standard for this parameter established by the European Directive 91/271 for the treated urban wastewater. This loss of effluent quality was observed neither by Aragón et al., (2009) nor by Jiang and Liu, (2013), who reported that the addition of 0.8 $mg \cdot L^{-1}$ of TCS reduced sludge production without compromising the organic matter removal.

Finally, no statistical significance was observed for P_T, though the higher biomass production in SBR-2 (explained in 3.1.3 section) should result in higher phosphorus consumption used as nutrient. This fact can be explained because the less phosphorus amount required for biomass production in SBR-1 was compensated by the biological enhanced phosphorous removal in this reactor, due to anaerobic conditions. It has to be highlighted that TCS inhibited

nitrification in SBR-1, as it will be explained in more detail in section 3.1.2. Consequently, during the 2 h of reaction phase without aeration, anaerobic conditions in SBR-1 were achieved, meanwhile in SBR-2 nitrification occurred and the operating conditions were anoxic in this cycle phase.

3.1.2. Nitrification

Particular attention should be paid on the removal percentage of N_T in SBR-1, since a progressive decrease was observed in this reactor throughout the experimental time (Figure 1-left). However, a stable N_T removal efficiency value was achieved in SBR-2 (mean value of 63.1 ± 4.8%). These results indicated that nitrifying bacteria were affected by TCS addition. To confirm this behaviour, respirometric tests of MLs of both SBRs in the last experimental day were carried out as explained in section 2.2.2. In Figure 1-right, it can be seen Rs evolution when NH_4Cl was added. In SBR-2 the dissolved oxygen (DO) was consumed due to NH_4Cl degradation (increasing Rs) for 15 min, from DO consumption stopped.

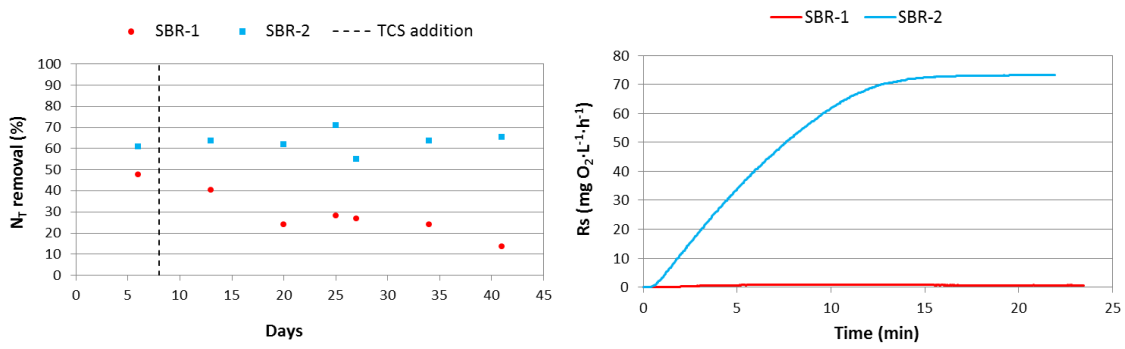

Figure 1. Left: N_T removal percentages in SBR-1 and SBR-2. Vertical line indicates TCS addition start. Right: Experiments with addition of $ClNH_4$ in Respirometer BM-Advance

This fact indicated that biomass of SBR-2 was able to oxodize the fed ammonium-nitrogen, since this was the only substrate source that can be oxidized. Nevertheless, Rs of SBR-1 was negligible throughout the entire test, which indicated that NH_4Cl was not degraded because nitrifying bacteria of biomass have been inhibited by TCS. These results were also confirmed

by the analysis of the microbial population. *Nitrosomonas* (ammonia oxidizing bacteria) and *Nitrospira* (nitrite oxidizing bacteria) are two bacterial genera related to ammonia transformation (Tian et al., 2017). The study of bacterial community indicated that the viable *Nitrosomonas* relative abundance decreased from 0.85% in 6th day to 0.03% in 41st day in the reactor with TCS, while in SBR-2 its relative abundance remained nearly constant (from 0.97% to 0.88%). The same behaviour was observed on viable *Nitrospira* relative abundance in SBR-1, decreasing from 2.57% to 0.03%, while in SBR-2 its relative abundance increased from 2.10% to 3.74% between the first and the final analysis. Thus, the influence of TCS dosage on these microbial populations can be observed through the gradual increase of NH_4^+-N and decrease of NO_3^--N in SBR-1 effluent (Figure S3). The same behaviour was observed by Yang et al., (2019), which reported a decrease in NH_4^+-N removal efficiency adding TCS. Thus, in view of these results, it seems clear that this uncoupler should not be used in biological treatments that require nitrogen elimination.

3.1.3. Sludge production

A progressive decrease of ΔX in SBR-1 was observed from the 8th to 14th day. From this day on, stable values of ΔX in both reactors were obtained until the last experimental day (Figure S2 left), resulting in a mean value of 0.32 ± 0.05 and 0.94 ± 0.15 g MLSS·d^{-1} in SBR-1 and SBR-2, respectively. Therefore, 63.3 ± 4.3% of sludge reduction was achieved when 0.8 mg·L^{-1} of TCS was added. This result was corroborated by one-way ANOVA analysis, in which statistical significance was observed between ΔX (from the 14th to 43rd day) and TCS addition (F = 26.02; p-value = 0.0001). A Tukey diagram is presented in supplementary material as Figure S2 right. Other authors reported lower reductions in the sludge productions when TCS was added. Feng et al., (2014) reported 42.7% of sludge reduction using 1 mg·L^{-1} of TCS, while Chen et al., (2002) and Aragón et al., (2009), who worked with 0.8 mg·L^{-1},

reported around 40% and 30% of sludge reduction, respectively. These disagreements in the results reported can be due to differences in the working conditions of the operated reactors.

SRT in SBR-1 was higher than in SBR-2 (from the 8^{th} to 43^{rd} days) due to decrease in the biomass growth, achieving values of 35.9 and 19.7 days, respectively. This data are important for the results interpretation since the different sludge production led to different SRTs in spite of working both reactors under the same food to microorganisms ratio (same organic load and same microorganisms mass). Additionally, the SRTs values confirmed that the lack of nitrification in SBR-1 was not due to an insufficient cellular retention time in the system.

3.2. Effect of the TCS on the mixed liquor characterization

An exhaustive ML characterization consisting of the analysis of parameters related to microorganisms behaviour like ATP, cellular viability, MHEA, SMP and to bacterial community were measured to assess the effect of TCS addition on the biomass. This thorough characterization is of paramount importance in order to explain the mechanisms of action for TCS.

3.2.1. ATP, cellular viability and microbial hydrolytic enzymatic activities (MHEA)

Specific cellular ATP (ScATP) was significantly lower in SBR-1 than in SBR-2 (Figure 2) after TCS dosage, which proved the occurrence of metabolic uncoupling due to TCS addition.

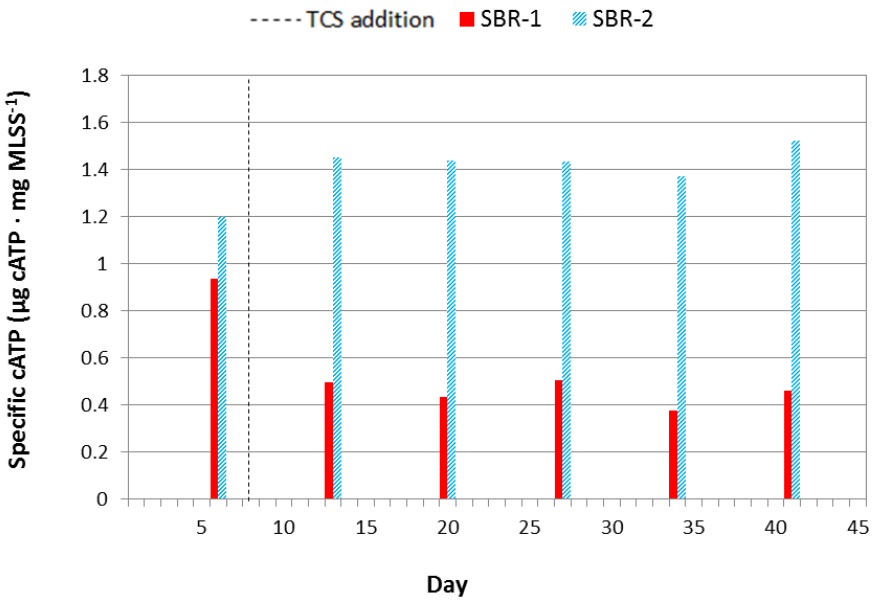

Figure 2. Specific cellular ATP of SBR-1 and SBR-2. Vertical line indicates the TCS addition start.

The same behaviour was reported by Feng et al., (2014). As it is known, uncoupling substances decrease the proton gradient across cell membrane, which reduces ATP generation. This fact implies lower anabolism activity leading to lower biomass growth (Fang et al., 2015). The mean ScATP values from the 14th to 43rd day were 0.45 ± 0.05 and 1.45 ± 0.05 µg cATP·mg MLSS^{-1} in SBR-1 and SBR-2 respectively, which drove to a 69% lower ScATP in the SBR feed with TCS. Statistical significance was proved by on-way ANOVA ($F = 876.43$; p-value < 0.0001). In addition, it is demonstrated that there is a tight relationship between the diminution in ScATP and the sludge reduction (63.3 ± 4.3% as reported in section 3.1.3).

Concerning cellular viability, TCS effect was slower than that observed on ATP concentrations and results in both reactors were very similar (77.5 ± 1.3% of viable cells) until 14th day. This value decreased in SBR-1 to achieve 51.3% in 20th day, resulting in a stable value from this day to the end of experimental time (52.4 ± 4.5%). On the contrary, an increase in cellular viability was observed in SBR-2, remaining in 85.8 ± 6.0% from the 20th day. This behaviour is contrary to that observed by Feng et al., (2014), which reported that

sludge lysis was not intensified by TCS basing this conclusion in measurements of DNA in the reactor effluent. Nevertheless, in the present work, the use of a more specific technique to assess the cellular viability revealed more lysed cells in SBR with TCS than in SBR without TCS addition. In this way, it can be concluded that uncoupling is not the only mechanism for the sludge reduction, since cell lysis also plays an important role.

This last mentioned mechanism should lead to a greater amount of cellular material in the SBR bulk. In other words, an increase in SMP and DNA concentrations should be observed. In the first 14^{th} days, similar DNA values were measured in both reactors (105.4 ± 1.1 and 90.9 ± 2.1 $ng \cdot mL^{-1}$ in SBR-1 and SBR-2, respectively). However, from 20^{th} day DNA increased in SBR-1 achieving an average value of 146.2 ± 11.0 $ng \cdot mL^{-1}$, while in SBR-2 remained practically constant (87.9 ± 12.6 $ng \cdot mL^{-1}$). Concerning SMPs, higher concentrations were achieved in the SBR-1 after TCS addition (Figure 3), resulting in average concentrations of 19.6 ± 5.7 and 15.6 ± 3.8 $mg \cdot L^{-1}$ in SBR-1 and SBR-2, respectively.

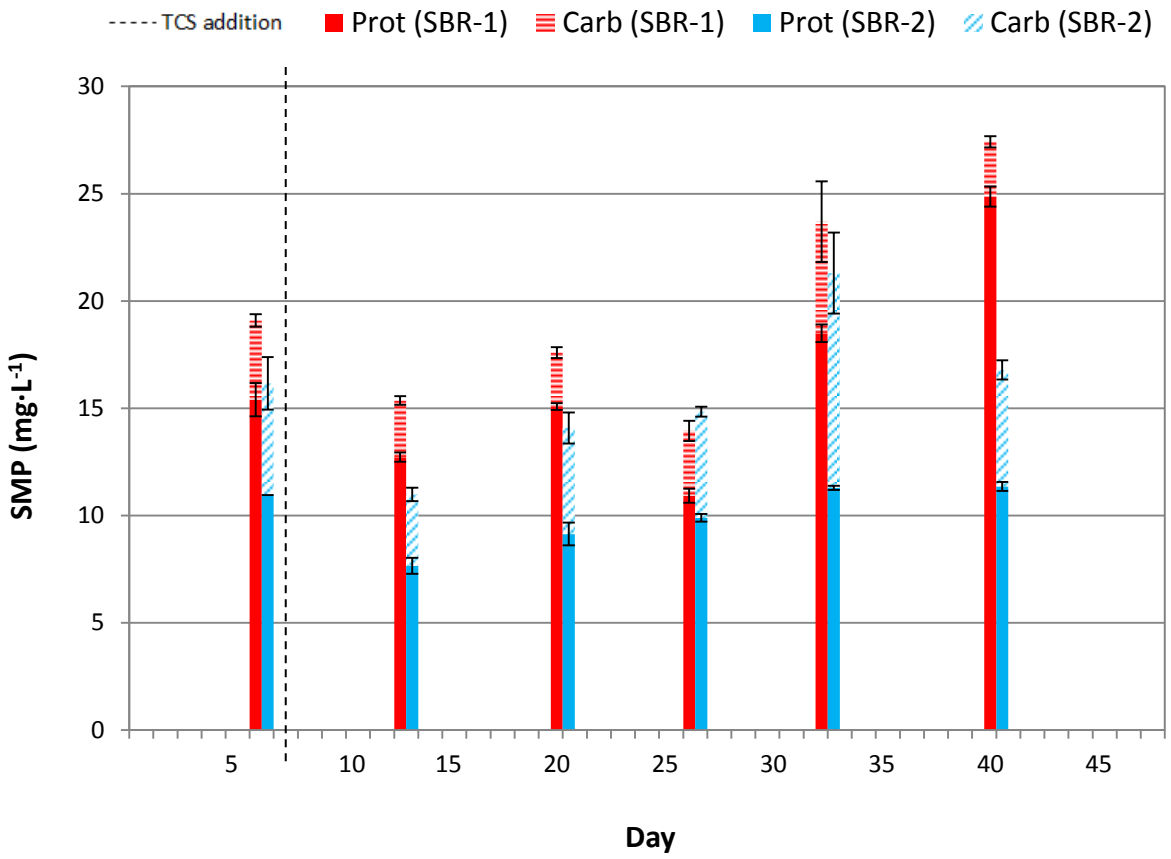

Figure 3. SMP concentrations as sum of proteins (Prot) and carbohydrates (Carb) in SBRs.

Thus, this fact together with DNA increase, confirmed that more cellular debris was present in SBR-1. In addition, more proteins/carbohydrates ratio (P/C ratio) was observed in SBR-1 comparing with those measured in SBR-2 in the same period, achieving values of 5.5 ± 2.5 and 1.9 ± 0.4, respectively. In this way, although it was not observed a statistical significance between SMP of both reactors ($F = 2.30$; p-value = 0.1607), it was found that comparing protein concentrations part ($F = 8.68$; p-value = 0.0106), while no significance was achieved comparing carbohydrates concentrations ($F = 5.44$; p-value = 0.0519). This fact is because proteins are the main components of cellular debris (Xiao et al., 2017).

According to the results explained above, more cellular lysis and less biomass growth in SBR-1, it could be expected a higher difference between performances in both reactors.

However, only a difference of 3% in the average percentage of COD removal in both reactors was observed (89.1 ± 2.8% and 92.1 ± 2.8% in SBR-1 and SBR-2, respectively). This fact can be explained by the MHEAs values. Before TCS addition, similar concentrations of each MHEA were measured in both reactors. However, an increase in all MHEA of SBR-1 from TCS dosage can be observed in Figure 4, while MHEA of SBR-2 remained more or less constant. This fact was corroborated by statistical significances found for all MHEA, which are included in Figure 4 (caption of each graphic). Thus, the inhibited proton gradient generated by TCS produced a lower concentration of sludge in SBR-1 but with a metabolically more active biomass. Other studies also reported the correlation between MHEA increase and COD consumption (Ferrer-Polonio et al., 2017) or the total organic carbon removal in the reactor (Kreutz et al., 2016).

Oxidative phosphorylation is the mechanism of the aerobic bacteria to generate ATP using ADP (adenosine diphosphate) and inorganic phosphorous. The low ScATP concentration observed in SBR-1 showed an inorganic phosphorous deficit, which average value in this reactor was below 0.03 mg $P\text{-}PO_4 \cdot L^{-1}$, meanwhile in SBR-2 was 3.9 ± 0.03 mg $P\text{-}PO_4 \cdot L^{-1}$. Thus, microbial hydrolytic phosphatase activity increased in SBR-1 to obtain this nutrient. In addition, intracellular material (mainly proteins, lipids and carbohydrates) is released into the medium due to cell lysis. The higher protease, lipase, glucosidase and glucuronidase (glucuronidase data are not shown) enzymatic activities in SBR-1 explained why an increase in intracellular material did not result in higher increase in effluent COD, what could be expected.

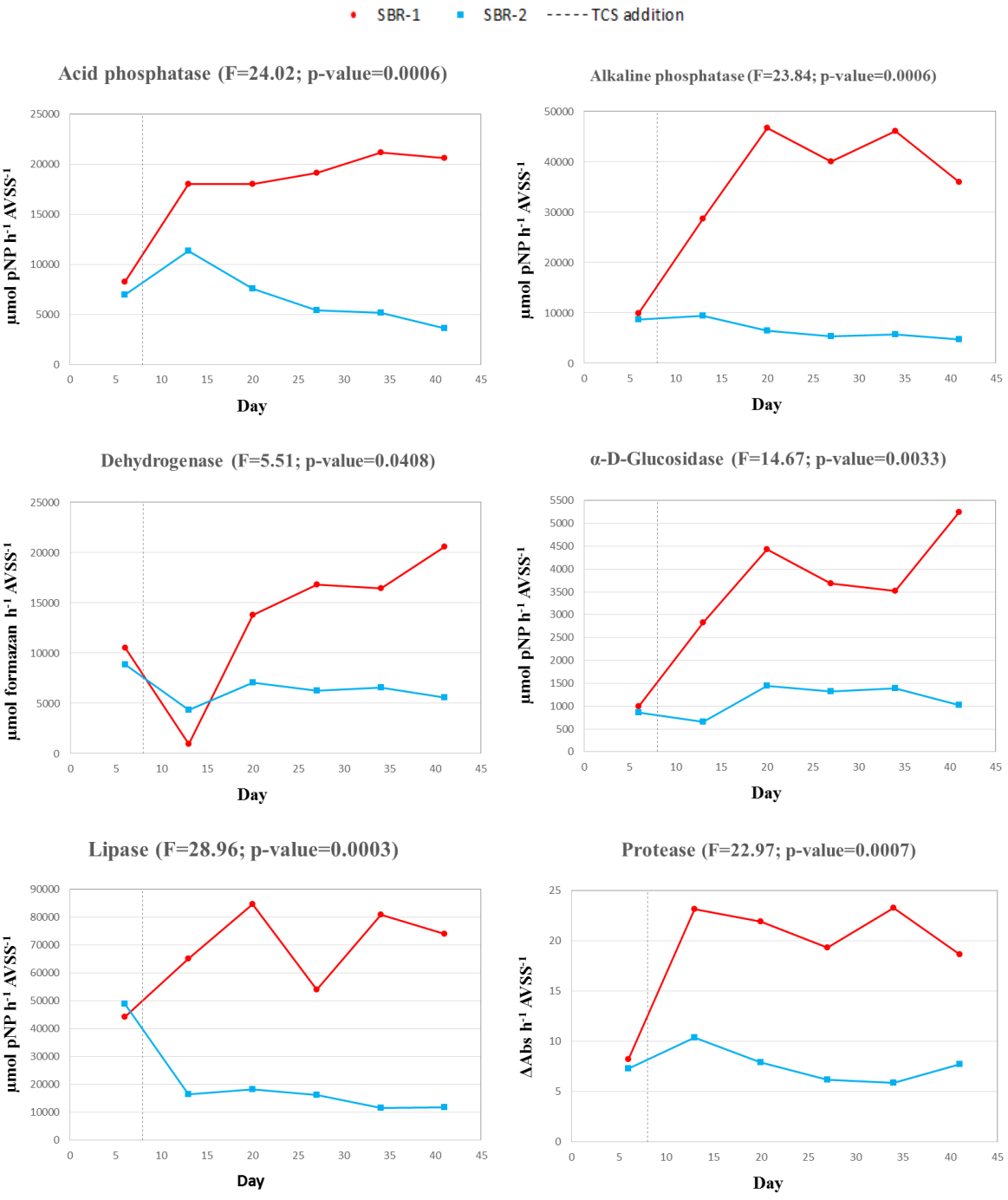

Figure 4. MHEA in SBR-1 and SBR-2.

Finally, the higher dehydrogenase enzymatic activity in SBR-1 showed an activated sludge with greater oxidation of organic matter than SBR-2, though its AVSS concentrations were lower.

Unlike this work, the only two studies in that biomass activity is reported after TCS addition were based on the measurements of other parameters to describe the cell activity, reaching contradictory results between them. Thus, Chen et al., (2002) observed that the microbial activity increased significantly in the reactors in that TCS was added. They used cell respiratory activity-detecting techniques (specific oxygen uptake rate, SOUR) in order to measure the percentage of respiring bacteria, i.e. this is a global activity measuremen. These measurements corroborated an increment of respiring cells when TCS was added. However, Feng et al., (2014) used as activity measurement two tetrazolium salts to study the effect of TCS on the electronic transported system (ETS). Thus, they used the named TTC-ETS and INT-ETS activities. Contrary to the work by Chen et al., (2002), TTC-ETS and INT-ETS activities decreased significantly after ten days from the beginning of TCS addition. In our work, dehydrogenase activity was measured with the same INT salt as that used in INT-ETS technique reported by Feng et al., (2014). Our results showed a different behavior, since activity increased with TCS addition. On the other hand, the other activities measured in this research assess the activity of the cells for degrading targeted compounds (proteins, glucose…). All of these analysis showed clearly a sharp increase of the cellular activity corroborating the results of Chen et al., (2002) obtained with other techniques as explained above.

3.2.2. Bacterial diversity and structure

The differences in the global composition of viable bacterial phyla communities (with PMA) of SBR-1 and SBR-2 between the 6^{th} and 41^{st} days are showed in Figure 5 and Figure 6. Initial composition (6^{th} day) was similar in SBR-1 and SBR-2, in which the most abundant phyla were: *Proteobacteria* (36.1% and 36.7%), *Actinobacteria* (7.3% and 9.2%), *Chloroflexi* (4.8% and 4.4%), *Firmicutes* (3.0% and 2.2%) and *Nitrospirae* (2.6% and 2.1%). In terms of the final composition of the reactors, in SBR-2 no significant variations in the phyla relative

abundances were performed, while important changes were observed in the bacterial community of SBR-1 during TCS addition. To study these changes, phyla have been classified into two groups: phyla whose relative abundance decreased in SBR-1 (Figure 5) and phyla whose relative abundance increased in SBR-1 (Figure 6). In general terms, an important reduction of bacterial diversity resulted in SBR-1 due to TCS addition. In this way, at the end of the experimental time, six of fourteen phyla presented in Figure 5 had disappeared and another six had a relative abundance below 0.07%.

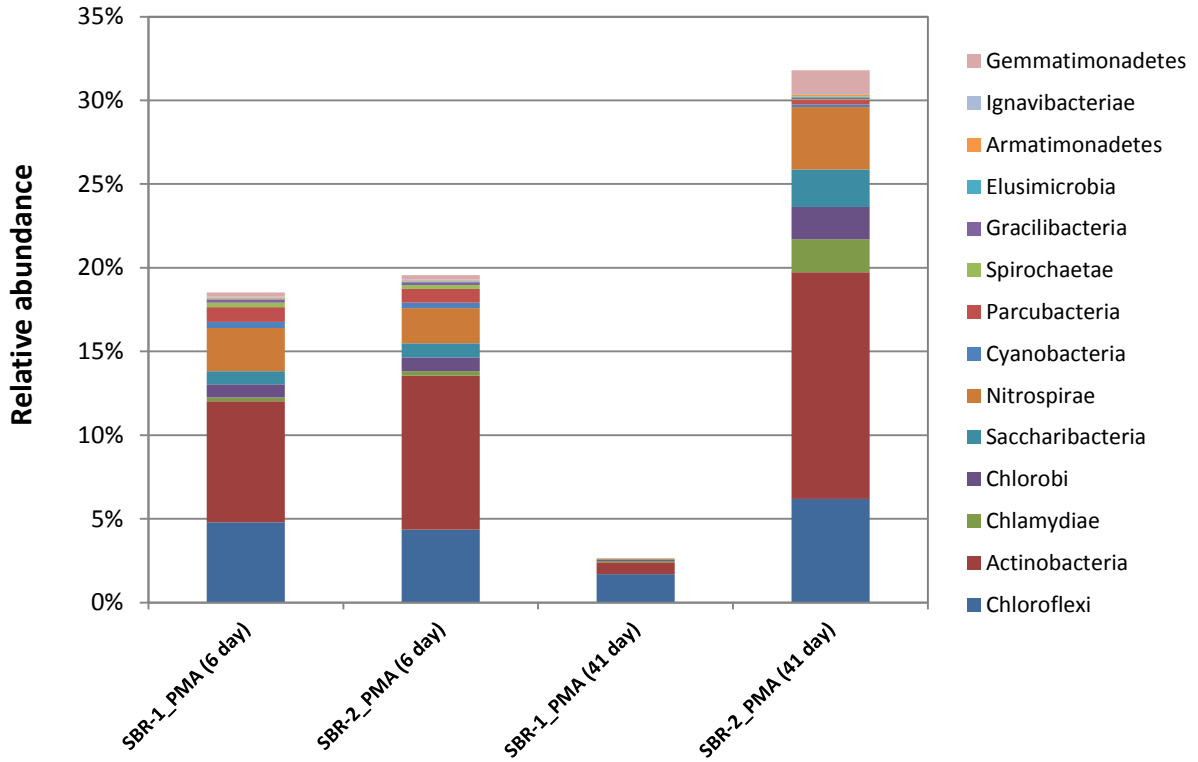

Figure 5. Relative percentage abundances of fourteen viable bacterial phyla (samples with PMA), which abundances decreased in SBR-1 during TCS addition.

Additionally, it has to be highlighted that four phyla (*Proteobacteria, Firmicutes, Fusobacteria,*and *Tenericutes*) represented 72.4% of the total bacterial community in this reactor in the 41st day (Figure 6). *Proteobacteria* was the most abundant phylum in SBR-1, increasing its relative abundance from 36.1% to 55.1%. Analyzing *Proteobacteria* class, it

should be commented that its increase was due to *Alphaproteobacteria* (from 13.5% to 24.9) and *Gammaproteobacteria* (from 7.4% to 24.1%). On the contrary, the other two classes decreased during the experiment: *Betaproteobacteria* (from 11.8% to 5.5%) and *Deltaproteobacteria* (from 3.3% to 0.7%).

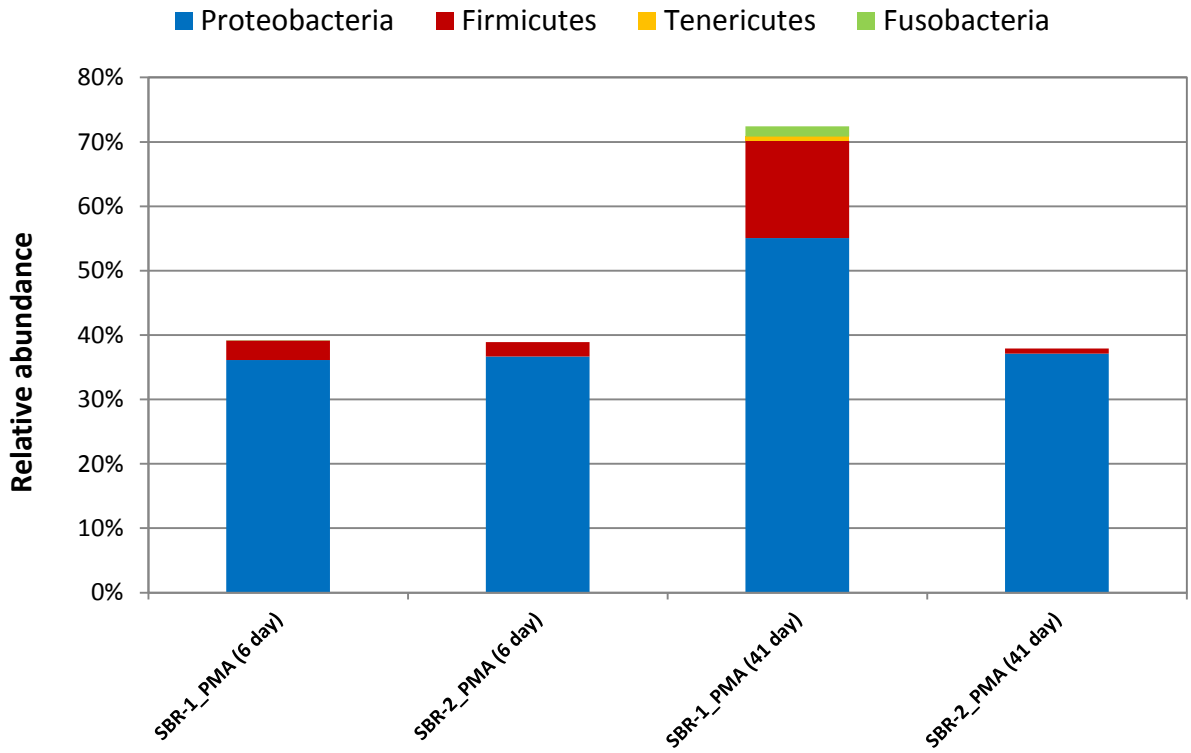

Figure 6. Relative percentage abundances of the four viable bacterial phyla (samples with PMA), which abundances increased in SBR-1 during TCS addition.

In order to go deeper on the functional change of bacterial community, the identified OTU at the genus level was ranked. Among most abundant genera (above 0.01%) identified in SBR-1 and SBR-2 samples, 36 genera reduced their relative abundance in SBR-1 throughout the experiment (Table S1), whereas 34 genera increased it (Table S2). Results are expressed both in terms of percentage of viable cells (with PMA) and in terms of percentage of total cells (without PMA). In general, the live/total ratios of genera in SBR-2 samples (without/with PMA) on 41st day was between 0.8 and 1.2 (48 genera), which indicated that no distinct

changes in their abundances within the population due to PMA-treatment occurred. In case of SBR-1 a decrease of the ratio was observed in PMA treated sample for 9 genera ranging from 0.59 (*Sphingosinicella*) to 0.79 (*Ferruginibacter*, *Nitrosomonas* and *Pseudoxanthomonas*), whereas 9 genera showed a ratio from 1.21 (*Candidatus* Competibacter) to 1.89 (*Rheinheimera*) indicating an increase in sequence abundances in the living fraction.

It is obvious that uncoupler addition enhances a population shift to microorganisms that are fully acclimated to it (Hiraishi and Kawagishi, 2002). Thus, the change pattern of viable bacterial population during TCS addition suggested that some TCS-tolerant bacteria in activated sludge were augmented: *Aeromonas* and *Stenotrophomonas* (genera of *Gammaproteobacteria*), *Ensifer*, *Bosea* and *Brevundimonas* (genera of *Alphaproteobacteria*), *Proteiniclasticum*, *Sedimentibacter* and *Erysipelothrix* (genera of *Firmicutes*) and *Macellibacteroides* (genera of *Bacteriodetes*). *Aeromonas* was the most abundant genus in SBR-1 at the end of the experiment, increasing from 0.34 to 16.24 %. This genus has been identified as core BOD-removal microorganism in the A-stage bioreactor of a two stages system (Gonzalez-Martinez et al., 2016). In addition, *Aeromonas* have been found to produce chitin-degrading enzymes (Chong et al., 2012), thus being capable of predation on cell biomass (Gonzalez-Martinez et al., 2016).

Conversely, there were several genera which relative abundances decreased after TCS addition as can be observed in Table S1, indicating that they were more vulnerable bacteria compared with other bacterial genera. Thus, *Zooglea*, *Ferruginibacter*, *Thauera*, *Nannocystis*, *Haliscomenobacter* and *Dokdonella*, which are important floc-forming bacteria and are responsible for degradation of organic compounds (Cheng et al., 2018; Gonzalez-Martinez et al., 2016; Liu et al., 2013; Thomsen et al., 2007; Tian et al., 2017), were reduced in the SBR-1 biomass.

4. CONCLUSIONS

The results of this study showed that a stable sludge reduction of 63.3% was achieved in 33 days with TCS addition in a concentration of 0.8 $mg \cdot L^{-1}$. Comparing COD removal percentages in SBRs with and without TCS, similar values were obtained (89.1% and 92.1% in SBR-1 and SBR-2, respectively). In SBR-1 an important decrease in ATP concentrations was observed due to uncoupler effect of TCS in this reactor. In this way, average ScATP value in SBR-1 was 69% lower than that measured in SBR-2. Additionally, more SMP and DNA concentrations were detected in SBR-1 than in SBR-2. Both results confirmed that SBR-1 contained more cellular debris than SBR-2. Therefore, ATP and SMP and DNA results confirmed that the sludge reduction was due to uncoupler effect of TCS together with cellular lysis resulting from the addition of this substance.

The 16S rRNA gene amplicon sequencing tests concluded that bacterial community was affected by TCS addition. Fourteen bacterial phyla decreased their abundance and only four increased it. In this way, it can be concluded that TCS addition drove to a less bacterial community diversity, since 72.4% of the total bacterial community was composed by only four phyla (*Proteobacteria*, *Firmicutes*, *Fusobacteria*,and *Tenericutes*). *Proteobacteria* was the main phylum, increasing its abundance from 36.1% to 55.1% during TCS addition. Analyzing *Proteobacteria* class, *Alphaproteobacteria* and *Gammaproteobacteria* increased their abundance since *Betaproteobacteria* and *Deltaproteobacteria* decreased it. In addition, nitrifying bacteria were affected by TCS addition. *Nitrosomonas* decreased from 0.85% to 0.03% and *Nitrospira* from 2.57% to 0.03% in SBR-1 during the experimental time, while 0.88% and 3.74% of these phyla were observed in SBR-2 in the last sampling days. This fact led to a progressive decrease in the N_T removal percentage in SBR-1 during TCS addition. Thus, this uncoupler should not be used in biological treatments that require nitrogen elimination.

Acknowledgements

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Supplementary Material

Changes in the process performance and microbial community by addition of the metabolic uncoupler 3,3',4',5-tetrachlorosalicylanilide in sequencing batch reactors

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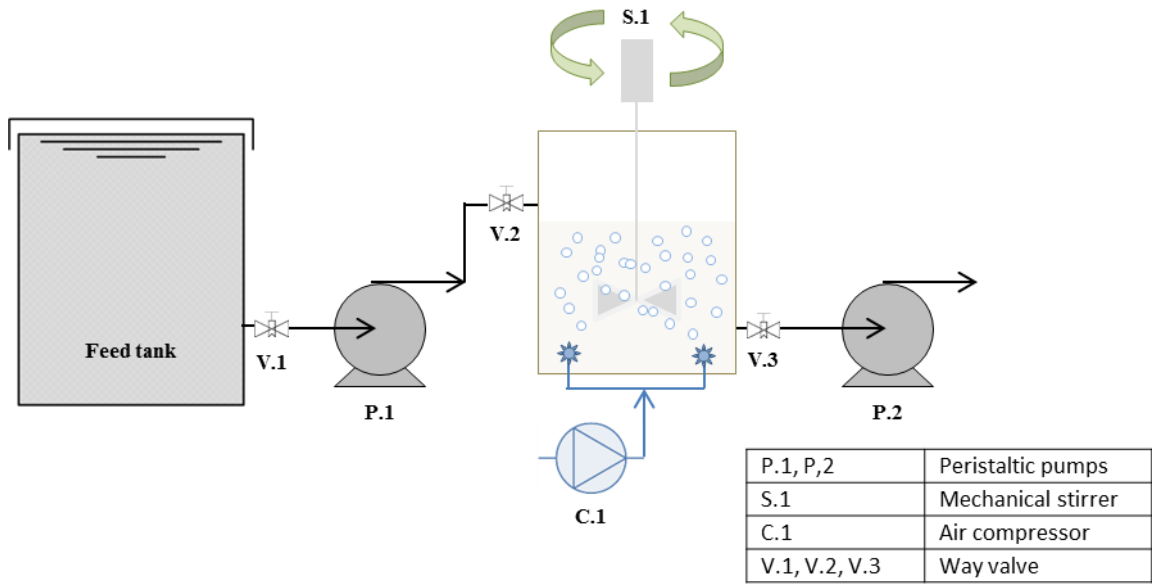

Figure S1. SBR scheme.

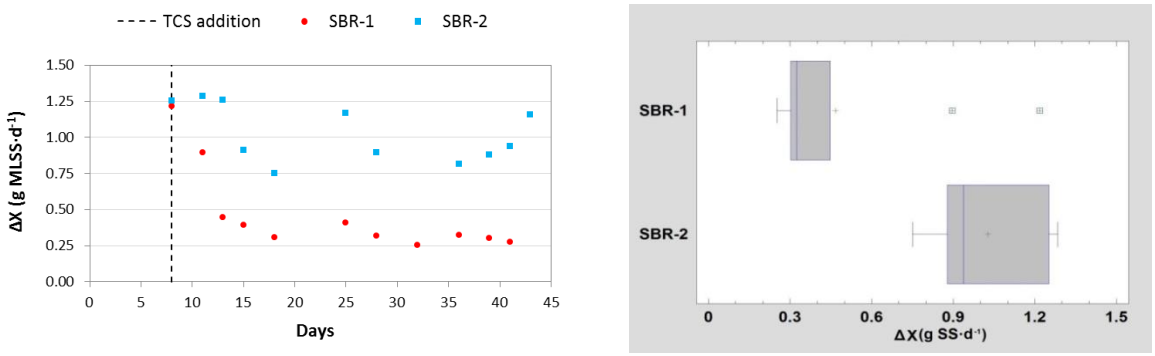

Figure S2. Evolution of sludge growth (left figure) and Tukey diagram (right figure, from 14th to 43rd days) in SBR-1 and SBR-2 between 14th to 43rd days.

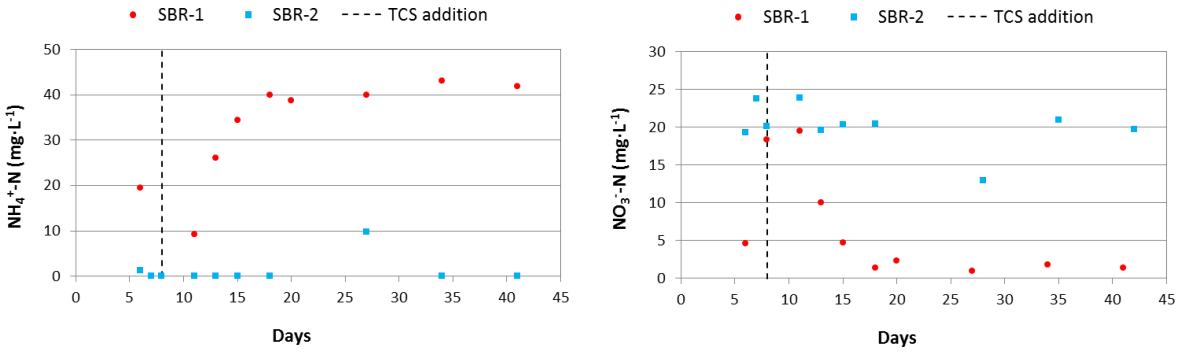

Figure S3. NH_4^+-N (left figure) and NO3$^-$-N (right figure) concentrations in effluents of SBR-1 and SBR-2. Vertical line indicates TCS addition start.

Table S1. Bacterial genera which relative percentage abundances decreased in SBR-1 during TCS addition.

Genus	SBR-1 (%)		SBR-1_PMA (%)		SBR-2 (%)		SBR-2_PMA (%)		Ratio PMA/non-PMA 41 day	
	6 day	41 day	6 day	41 day	6 day	41 day	6 day	41 day	SBR-1	SBR-2
Thauera	2.684	1.088	2.473	1.148	2.131	4.168	2.305	4.484	1.055	1.076
Ferruginibacter	2.470	0.580	3.092	0.460	2.855	2.179	3.246	2.320	0.793	1.065
Nitrospira	2.503	0.024	2.573	0.027	2.179	3.486	2.101	3.742	1.125	1.073
Dokdonella	1.974	0.102	2.461	0.186	2.305	0.772	2.873	0.899	1.823	1.165
Zooglea	1.923	0.060	2.203	0.072	1.767	1.076	2.092	1.175	1.200	1.092
Planctomyces	1.064	0.207	0.953	0.207	0.896	0.712	0.893	0.721	1.000	1.013
Tetrasphaera	0.814	0.015	0.526	0.003	0.754	3.426	0.634	2.566	-	0.749
Haliscomenobacter	0.808	0.006	0.727	0.006	0.724	0.607	0.832	0.577	-	0.951
Nitrosomonas	0.739	0.042	0.853	0.033	0.772	0.766	0.968	0.884	0.786	1.154
Runella	0.619	0.003	0.619	0.003	0.553	0.733	0.676	0.730	-	0.996
Sphaerotilus	0.568	0.003	0.547	0.000	0.553	0.144	0.685	0.135	-	0.938
Candidatus Alysiosphaera	0.213	0.150	0.147	0.162	0.228	0.850	0.171	0.893	1.080	1.051
Haliangium	0.523	0.015	0.301	0.009	0.499	0.204	0.273	0.171	-	0.838
Chryseolinea	0.403	0.030	0.472	0.003	0.466	1.094	0.376	1.064	-	0.973
Prosthecobacter	0.595	0.427	0.760	0.436	0.721	0.634	0.790	0.649	1.021	1.023
Azohydromonas	0.433	0.000	0.502	0.000	0.466	0.072	0.436	0.078	-	1.083
Bdellovibrio	0.406	0.000	0.568	0.009	0.445	0.204	0.538	0.231	-	1.132
Glutamicibacter	0.430	0.108	0.361	0.000	0.349	1.794	0.400	2.020	-	1.126
Phaeodactylibacter	0.252	0.006	0.273	0.000	0.337	0.556	0.301	0.661	-	1.189
Roseiflexus	0.273	0.003	0.231	0.006	0.307	0.261	0.231	0.243	-	0.931
Woodsholea	0.162	0.096	0.225	0.129	0.237	0.219	0.243	0.243	1.343	1.110
Nannocystis	0.295	0.003	0.174	0.006	0.225	0.096	0.195	0.090	-	0.938
Sulfuritalea	0.189	0.006	0.252	0.006	0.180	0.075	0.198	0.069	-	0.920
Filimonas	0.138	0.000	0.177	0.006	0.171	0.135	0.174	0.165	-	1.222
Crocinitomix	0.114	0.003	0.099	0.000	0.165	0.298	0.129	0.216	-	0.725
Leucobacter	0.105	0.027	0.072	0.009	0.141	3.537	0.078	3.231	-	0.913
Paeniglutamicibacter	0.120	0.012	0.105	0.003	0.135	0.096	0.105	0.099	-	1.031
Lewinella	0.141	0.000	0.108	0.006	0.123	0.120	0.096	0.129	-	1.075

Phaselicystis	0.048	0.000	0.042	0.009		0.078	0.355	0.072	0.261	-	0.735
Gemmatimonas	0.060	0.009	0.045	0.000		0.069	0.102	0.087	0.114	-	1.118
Arenimonas	0.039	0.018	0.033	0.015		0.048	0.093	0.066	0.090	0.833	0.968
Paludibaculum	0.024	0.018	0.033	0.003		0.039	0.111	0.036	0.105	-	0.946
Blastocatella	0.039	0.000	0.018	0.000		0.030	0.096	0.051	0.102	-	1.062
Rheinheimera	0.036	0.027	0.024	0.051		0.027	0.183	0.030	0.144	1.889	0.787
Chryseobacterium	0.009	0.000	0.039	0.000		0.024	0.135	0.015	0.141	-	1.044
Sediminibacterium	0.009	0.000	0.000	0.000		0.003	0.156	0.009	0.084	-	0.539

Table S2. Bacterial genera which relative percentage abundances increased in SBR-1 during TCS addition.

Genus	SBR-1 (%)		SBR-1_PMA (%)			SBR-2 (%)		SBR-2_PMA (%)			Ratio PMA/non-PMA 41 day	
	6 day	41 day	6 day	41 day		6 day	41 day	6 day	41 day		SBR-1	SBR-2
Proteiniclasticum	1.217	9.854	1.208	10.609		0.829	0.129	0.823	0.117		1.077	0.906
Ensifer	0.577	2.158	0.496	2.446		0.550	0.457	0.526	0.469		1.133	1.026
Aeromonas	0.252	15.174	0.340	16.240		0.168	0.313	0.225	0.307		1.070	0.981
Rhodovulum	0.036	0.171	0.060	0.219		0.054	0.063	0.042	0.081		1.281	1.286
Bosea	0.033	1.133	0.030	1.533		0.045	0.132	0.045	0.222		1.353	1.681
Stenotrophomonas	0.030	3.519	0.027	3.775		0.030	0.078	0.012	0.066		1.073	0.846
Comamonas	0.012	0.264	0.024	0.267		0.018	0.015	0.018	0.018		1.011	1.200
Acinetobacter	0.012	0.823	0.009	0.884		0.003	0.000	0.003	0.000		1.074	-
Macellibacteroides	0.009	11.639	0.003	11.516		0.003	0.039	0.003	0.039		0.989	1.000
Rhizobium	0.009	0.258	0.015	0.210		0.009	0.054	0.021	0.048		0.814	0.889
Sphingomonas	0.009	0.313	0.003	0.216		0.003	0.024	0.000	0.045		0.690	1.875
Citrobacter	0.009	0.192	0.015	0.222		0.012	0.000	0.021	0.000		1.156	-
Sphingopyxis	0.003	0.763	0.009	0.863		0.015	0.117	0.021	0.120		1.131	1.026
Achromobacter	0.003	0.526	0.000	0.655		0.000	0.003	0.000	0.009		1.245	-
Bacteroides	0.000	0.117	0.006	0.099		0.009	0.009	0.003	0.000		0.846	-
Dysgonomonas	0.000	0.126	0.000	0.114		0.000	0.000	0.000	0.006		0.905	-
Sedimentibacter	0.000	1.791	0.000	1.593		0.000	0.006	0.000	0.018		0.889	-
Erysipelothrix	0.000	2.083	0.000	1.677		0.000	0.009	0.000	0.003		0.805	-
Hydrogenoanaerobacterium	0.000	0.207	0.000	0.331		0.000	0.000	0.000	0.000		1.599	-
Sphingosinicella	0.000	0.132	0.000	0.078		0.000	0.006	0.000	0.006		0.591	-
Desulfomicrobium	0.000	0.445	0.006	0.478		0.000	0.000	0.000	0.000		1.074	-
Acholeplasma	0.000	0.980	0.000	0.652		0.000	0.006	0.000	0.000		0.665	-
Pseudoxanthomonas	0.000	0.111	0.006	0.087		0.003	0.042	0.006	0.021		0.784	0.500
Lachnoclostridium	0.000	0.093	0.000	0.075		0.000	0.006	0.000	0.000		0.806	-
Steoidobacter	0.000	0.036	0.003	0.063		0.000	0.003	0.000	0.021		1.750	-
Sphingobium	0.057	0.319	0.075	0.343		0.057	0.000	0.054	0.003		1.075	-
Dechloromonas	0.090	0.117	0.072	0.159		0.075	0.024	0.078	0.018		1.359	0.750

Ochrobactrum	0.144	0.231	0.153	0.252		0.156	0.096	0.108	0.126		1.091	1.313
Pirellula	0.180	0.340	0.195	0.219		0.153	0.144	0.174	0.147		0.644	1.021
Brevundimonas	0.301	1.268	0.334	1.115		0.406	0.120	0.319	0.120		0.879	1.000
Cloacibacterium	0.030	0.108	0.033	0.084		0.021	0.009	0.009	0.006		0.778	-
Paracoccus	0.529	0.541	0.562	0.454		0.448	6.536	0.547	6.780		0.839	1.037
Candidatus Accumulibacter	0.006	0.138	0.000	0.150		0.000	0.748	0.003	0.781		1.087	1.044
Candidatus Competibacter	0.027	0.117	0.030	0.141		0.054	0.754	0.039	0.784		1.205	1.040