Measurement of ATP, cell viability and enzymatic activities in sequence batch reactors after addition of a metabolic uncoupler, 3,3',4',5-tetrachlorosalicylanilide (TCS)

Amoros I.[1*], Fernández-Navarro J.[1], Alonso J.L.[1], Ferrer-Polonio E.[2] And Mendoza J.A.[2]

[1] Instituto de Ingeniería del Agua y Medio Ambiente, Universitat Politècnica de València, Camino de Vera 14, 46002 Valenca, Spain

[2] Instituto de Seguridad Industrial, Radiofísica y Medioambiental, Universitat Politècnica de València, Spain

*corresponding author: Amorós I.

e-mail: iamoros@ihdr.upv.es

Abstract. A substantial part of the operating costs of activated sludge process is associated with the management and treatment of the excess sludge generated during the treatment process. Among the in-situ excess sludge reduction technologies, the metabolic uncoupler addition method is promising because it can be easily fed to the aeration tank of a wastewater treatment plants. Metabolic uncouplers disrupting proton gradient could directly decrease adenosine triphosphate (ATP) production and reduce the total energy for biomass synthesis. Thereby, monitoring the ATP variation could be beneficial to directly identify the occurrence of metabolic uncoupling in microorganisms and provide a deep understanding of the uncoupling metabolism. The more commonly tested uncouplers include 3,3',4',5-tetrachlorosalicylanilide (TCS). In this work the ATP and microbial hydrolytic enzymatic activities were measured to study the influence of TCS addition on the biomass and on the process performance. Four parallel sequence batch reactors (SBR) were evaluated under different operating conditions, two F/M ratios, during 41 days with and without TCS addition. During the 41 days operation, the TCS amount added to the SBR was 1 mg/L. ATP values showed that TCS addition has decreased the ATP generation and biomass in the reactors where TCS was added.

Keywords: ATP, redox activity, enzymatic activities, TCS metabolic uncoupler

1. Introduction

The excess sludge generated from the biological treatment process is a secondary solid waste that must be disposed of in a safe and cost-effective way. So far, the ultimate disposal of excess sludge represents one of the most expensive problems faced by wastewater utilities, e.g. the treatment of the excess sludge may account for up to 65% of the total plant operation cost (Yang *et al*., 2002). In order to solve excess sludge-associated environmental and economic problems, to reduce sludge production from the wastewater biological treatment process rather than the post-treatment or disposal of the sludge generated constitutes a main objective.

Some authors (Yang *et al*., 2002; Zheng *et al*., 2008; Aragón *et al*., 2009; Feng *et al*., 2014; Zuriaga-Agustí, *et al*., 2016; Wang *et al*., 2017) have studied the use of metabolic uncouplers to reduce excess sludge production which means to disassociate the energy coupling between catabolism and anabolism. Metabolic uncouplers disrupting proton gradient could directly decrease adenosine triphosphate (ATP) production and reduce the total energy for biomass synthesis. Therefore, as Feng *et al*. (2014) have concluded monitoring the ATP variation could be beneficial to directly identify the occurrence of metabolic uncoupling in microorganisms and provide a deep understanding of the uncoupling metabolism

ATP is a measure for all active cells, including nonculturable cells, in a sample. Thus, the ATP measurement can provide a better estimate of the total active biomass in water samples. The ATP assay has proven to be applicable for measuring active biomass in various aquatic environments (Eydal and Pedersen, 2007; Aoki *et al*.,2008; Bushon *et al*., 2009).

Cell viability and microbial processes as enzymatic activities have been extensively used in engineered systems, as wastewater treatment and, metabolic uncouplers could have negative and nocive effects in the microbial cells.

The determination of cell viability can provide information about potential negative effects of TCS although TCS has been widely considered and used as a gentle and environmental benign metabolic uncoupler (Li *et al*., 2012).

Fluorescent methods for directly determining the viability of bacteria based on the enzymatic hydrolysis of the tetrazolium salts as 5-cyano-2,3-di-4-tolyl-tetrazolium chloride (CTC) to fluorescent formazan salts, when they are chemically or biologically reduced,(redox activity) have been used in several works, (Rodriguez el al., 1992: Wuertz *et al*., 1998; Bartosch *et al*., 2003)

This property has been used to microscopically determine the proportion of metabolically active cells in a sample

(Wuertz *et al*., 1998).Healthy cells respiring via the electron transport chain will absorb and reduce CTC into an insoluble, red fluorescent formazan product. Cells not respiring or respiring at slower rates will reduce less CTC and consequently produce less fluorescent product, which can be observed and measured using a fluorescence microscope.

Dehydrogenase enzyme catalizes the electron transport (ETS) wich is related whit oxidative substrate removal and viable biomass fraction (Goel *et al*., 1998).

Previous publications (Feng *et al*., 2014) reported that using an uncoupler metabolism showed the remarkable reduction of electronic transport system (ETS) activity in TCS reactor, which proved the occurrence of metabolic uncoupling. At the same time an increase of extracellular polymeric substances (EPS), with major components of extracellular proteins was observed in SBR when TCS was added (Feng *et al*., 2014).

The study of enzymatic activities as dehydrogenase, and protease provides useful information about how ETS and EPS are influenced by the TCS in activated sludge.

Thus, the main objectives of this study were to compare effectiveness of TCS as metabolic uncoupler in reducing sludge reduction as well as to investigate their effects on microbial cells.

2. Methods

2.1. Continuous operation of four sequence batch reactors

In order to identify the effect of TCS at different F/M ratios, batch tests were conducted. First, two 5-L sequence batch reactors (SBR) were operated in batch mode in parallel. Reactor (SBR-1) was operated with 1.0 mg/L TCS. Reactor 2 (SBR-2) was operated as the control reactor without TCS addition. The two SBR were operated under F/M of 0.18 kg CODs/kg MLVSS d F/M for 41 days at room temperature in 8 h cycle with 15 min for filling phase, 6 h for aeration phase, 1.5 h for settling phase and 15 min for drawing phase. The experiment was repeated at different F/M (0.35 kg CODs/kg MLVSS d) with two SBR, reactor 3 (SBR-3) with 1.0 mg/L TCS addition and reactor 4 (SBR-4) without TCS addition.

2.2. ATP measurement

Total ATP values were obtained using the 3M™ Clean-Trace Biomass Detection kit (3M™).The kit contains vials of reagent for the detection of Adenosine Tri-Phosphate (ATP) in liquid samples. 100 µl sludge sample is placed in a cuvette together with extractant to release the ATP from microorganisms in the sample. After 1 min of extraction the re-hydrated reagent is added to the vial to react with the sample containing ATP to produce light. The intensity of light is proportional to the amount of ATP. Measurement of light was carried out with a 3M™ Clean-Trace ™ luminometer using a cuvette holder. The results are displayed in Relative Light Units (RLU). The higher RLU higher ATP concentration.

2.3. Redox activity

The *Bac*Light™ Redox Sensor™ CTC Vitality Kit (Molecular Probes™, Invitrogen) was used to measure redox activity. The kit contains 5-cyano-2,3, ditolyl tetrazolium chloride (CTC) and SYTO®24 green-fluorescent nucleic acid stain as counterstain to differentiate cells and calculate total cell numbers.

25µL of the *Bac*Light™ Redox Sensor™ CTC working solution was added to 225 µL SBR sludge samples and incubated for 30 min at 37ºC. After incubation 0.25µL SYTO®24 was added and samples were maintained in agitation 15 min in darkness before microscopy examination. A control sample treated with formaldehyde (3.7%,wt/vol) prior to CTC application to detect possible abiotic formazan salts was also tested.

Cells containing CTC-formazan and SYTO®24 stained cells were examined with fluorescence objectives of an epifluorescence microscope Olympus BX-50 with appropriate filters. The quantification of the signals detected was performed by means of automated cells quantification software (Borrás, 2008) based on thresholding techniques implemented in Matlab®. The software generates a report that gives the percentage of areas occupied by target cells together with the uncertainty measurement, which is calculated as the standard deviation divided by the square root of the number of fields examined.

2.5. Dehydrogenase activity

Dehydrogenase activity was determined employing a procedure adapted from Goel et at. (1998) by mixing 1.0 mL of sample of activated sludge with 0.75 mL of 0.3% (w/v) substrate solution (Iodonitrotetrazolium chloride from Sigma-Aldrich) and 2.0 mL of Tris-HCl buffer (0.2 mol L-1, pH 7.6). Samples were incubated at 37 ºC for 30 min. After incubation 0.5 mL of 37% (v/v) formaldehyde solution was added to stop the enzymatic activity. The mixed liquid was preserved at 4ºC for 30 min, then was centrifuged at 2500 rpm for 2 min and drained the excess water. The pellet was suspended in 3 mL of methanol in darkness during 30 min. Finally, the mixed liquid was centrifuged at 2500 rpm for 2 min and the absorbance of supernatant was measured at 490 nm in Thermo Scientific™ 9423UVG1002E spectrophotometer.

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. The activity values were normalized dividing them by the MLVSS concentration.

2.4. Protease activity

Protease activity was determined employing a procedure adapted from Goel et at. (1998) by mixing 3.0 mL of sample of activated sludge with 1.0 mL of 0.5% (w/v) substrate solution (Azocasein from Sigma-Aldrich). Samples were incubated at 37 ºC for 30 min. After incubation, 2.0 mL of 10% (w/v) of trichloroacetic acid were added to stop the enzymatic activity. The mixed liquid was centrifuged at 2500 rpm for 2 min. Finally, the absorbance of supernatant was measured at 340 nm in Thermo Scientific™ 9423UVG1002E spectrophotometer.

For the protease activity the reaction products are unknown and then the enzyme unit was defined as the absorbance increase after 30 minutes. The activity values were normalized dividing them by the MLVSS concentration.

3. Results and Discussion

3.1. ATP measurement

The ATP concentrations (measured as RLU percentages) on the different SBR are showed in table 1. As observed, control SBR had similar amounts of ATP at the end of the experiment. However, in SBR with TCS addition the ATP concentration was reduced, 33% for SBR-1 and 56% for SBR-3 compared to the concentration present on day 1.

When comparing SBR with TCS addition with control reactors after 41 days in SBR-1, ATP concentration was decreased a 14%, whereas in the SBR-3 it was reduced a 46% with respect to the SBR-4.

Similar results were obtained by Feng *et al*. (2014) although in this work they observed a decrease of ATP in TCS addition reactor specially after the first day, of TCS addition revealing that TCS could effectively limit the production of ATP.

The effect of TCS in reactors with high F/M ratios is more important than low F/M ratios, due to the lower concentration of microorganisms present.

3.2. Redox activity

The study of cell viability (table 2) as redox activity in SBR control reactors (SBR-2 and SBR-4) showed similar behaviour decreasing both until, 91%. Reactors with TCS addition, SBR-1 and SBR-3, showed 9% and 20% less redox activity compared to their respective control reactors, SBR-2 and SBR-4.

High F/M ratios are more affected by the addition of metabolic uncoupler agents such as TCS, as observed with RLU.

3.3. Enzymatic activities: Dehydrogenase and Protease

TCS addition (table 3) produced an increase in the dehydrogenase activity for high F/M ratio (SBR-1 versus SBR-2), while for low F/M ratio the activity of SBR-3 decreased compared to SBR-4. Other studies studying the effects of TCS addition (Feng *et al*. 2014) found that TCS could effectively inhibit the electron transport system or respiratory activity.

The evolution of protease activity observed throughout the experiment (Table 4), indicated an increase of this activity in all of the biological reactors. This fact could be due to the assimilation of the wastewater by the microorganisms. However, at the end of the experiment it was slightly higher in the SBR-1 than in the SBR-2 due to the inhibition produced by the TCS in the production of sludge.

Table 1. ATP concentration (percentage of RLU)

	Day 0	**Day 41**
SBR-1	250535	192185
SBR-2	202840	226314
SBR-3	217819	123902
SBR-4	180831	231695

Table 2. Redox activity, (percentage).

	Day 0	**Day 41**
SBR-1	88	83
SBR-2	100	91
SBR-3	93	73
SBR-4	99	91

Table 3. Dehydrogenase activity, (µmolFormazan/hour/gVSS).

	Day 0	**Day 41**
SBR-1	285.13	334.58
SBR-2	302.35	315.22
SBR-3	254.37	228.31
SBR-4	217.86	329.28

Table 4. Protease activity, (ΔAbs/hour/gVSS).

	Day 0	Day 41
SBR-1	2.14	3.66
SBR-2	2.19	2.84
SBR-3	1.76	2.70
SBR-4	1.90	3.05

4. Conclusions

The metabolic uncoupler, TCS has showed a sludge reduction capacity at the studied concentration after 41 days.

With such TCS concentrations microbial activities as

ATP production, cell viability and enzymatic activities are not negatively influenced.

ATP decrease could be considered as an indicator of metabolic uncoupling and therefore as rapid indicator of potential sludge reduction.

F/M ratios plays an important role in enzymatic activities when TCS is added due to the concentration of microorganisms. These are more active so with low production of sludge there is equal assimilation of COD.

Acknowledgements

This work was supported by the Ministry of Economy and Competitiveness (MINECO), Spain (grant no. CTM2014-54546-P-AR).

References

Aoki Y., Fukuba, T., Yamamoto T., Fujii T. (2008), Developmente of ISSA-ATP system in deep sea environment in: Oceans 2008-MTS/IEEE. Kobe Techno Ocean Book series: OCEANS-IEEE. Vols 1-3 ,548-551.

Aragon, C., Quiroga, J., Coello, M. (2009), Comparison of four chemical uncouplers for excess sludge reduction. *Environmental Technology*, 30 (7), 707-714.

Bartosch S., Mancsch R., Knötzsch, Bock E. (2003), CTC staining and counting of actively respiring bacteria in natural stone using confocal laser scanning microscopy. *Journal of Microbiological Methods*, 52, 75-84

Borrás, L., 2008. Técnicas Microbiológicas aplicadas a la identificación y cuantificación de microorganismos presentes en sistemas EBPR. Tesis Doctoral. Valencia, España. Universidad Politécnica de Valencia.

Bushon R.B., Likridopulos C.A., Brady A.M. (2009), Comparison of Immunomagnetic separation/adenosine triphosphate rapid method to traditional culture based method for E.coli and enterococci ennumeration in waste water. *Water Research*, 43, 4940-4486.

Eydal H.S.C., Pedersen K. (2007), Use of an ATP assay to determine viable microbial biomass in Fennoscandian Shield groundwater form depths of 3-100. *Journal of Microbiological Methods*, 70, 363-373.

Feng X., Guo W., Yang S., Zheng H., Du J., WuQ., Ren N. (2014), Possible causes of excess sludge reduction adding metabolic uncoupler, 3 ,3′,4′,5- tetrachlorosalicylanilide (TCS) in sequence batch reactors. *Bioresource Technologie*, 173, 96–103.

Goel R., Mino T., Satoh H., Matsuo T. (1998), Enzyme activities under anaerobic and aerobic conditions in activated sludge sequencing batch reactor. *Water Research*, 32 (7), 2081–2088.

Li Y., Li A.M., Xu, J., Liu, B., Fu, L.C., Li, W.W., Yu, H.Q., (2012), SMP production by activated sludge in the presence of a metabolic uncoupler, 3,30,405-tetrachlorosalicylanilide (TCS). *Applied Microbiology and Biotechnology*, 95, 1313–1321.

Rodriguez G.G., Phipps D., Ishiguro K., Ridgeway H.F. (1992), Use of fluorescent redox probe for direct visualización of actively respiring bacteria. *Applied and Environmental. Microbiology*, 58, 1801-1808.

Wang Q., Wei W.,Go Y., Yu Q., Li Q., Sun J., Yuan J. (2017), in press. Technologies for reducing sludge production in wastewater treatment plants: State of the art. *Science of total Environment*, in press.

Wuertz S., Pfleiderer P., Kriebebitzsch K., Spath R., Griebe T., Coello-Oviedo D., Wilderer A.P., Flemming H.C. (1998), Extracellular redox activity in activated sludge. *Water Science and Technology*, 37, 379-384.

Yang, X., Xie, M., and Liu, Y. (2003), Metabolic uncouplers reduce excess sludge production in an activated sludge process. *Process Biochemistry*, 38, 1373–1377.

Zuriaga-Agustí, E., Mendoza-Roca, J.A., Bes-Piá, A., Alonso-Molina, J.L., Amorós-Muñoz, I., (2016.)Sludge reduction by uncoupling metabolism: SBR tests with Para-nitrophenol *and a commercial uncoupler*. *Journal of Environmental Management*, 182, 406–41.

Zheng G.H., Li M.N., Wang L.,Cheng Z.I., Qiang Y.F., Zhou Q. (2008), Feasibility of 2,4,6-Trichlorophenol and Malonic Acid as Metabolic Uncoupler for Sludge Reduction in the Sequence Batch Reactor for Treating Organic Wastewater. *Applied Biochemistry and Biotechnology*, 144, 101-109.