Ferrer-Polonio, E.; Fernández-Navarro, J.; Alonso Molina, JL.; Mendoza Roca, JA.; Bes-Piá, M.; Amoros, I. (20-0). Towards a cleaner wastewater treatment: Influence of folic acid addition on sludge reduction and biomass characteristics. Journal of Cleaner Production. 232:858-866. https://doi.org/10.1016/j.jclepro.2019.06.021

**Total number of Words:** 6,457 words
**Total number of Tables:** 5 tables (table captions = 47 words)
**Total number of Figures:** 5 figures (figure captions = 82 words)
**Total Supplementary material:** 1 figure and 4 tables (supplementary captions = 78 words)

# Towards a cleaner wastewater treatment: influence of folic acid addition on sludge reduction and biomass characteristics

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## Abstract

Reduction of sludge production is one of the most desired goals in the wastewater treatment plants operation. Alternatives like the enhancement of metabolism uncoupling or the increase of endogenous respiration by different chemicals have been reported in the bibliography. In this work, two folic acid concentrations (0.8 and 1.6 $mg \cdot L^{-1}$) added to a laboratory sequencing batch reactor were tested in order to assess the effect of this vitamin on the biological treatment. For this purpose, in addition to study sludge reduction and organic matter removal, an exhaustive biomass characterization including soluble microbial products,

cellular viability, adenosine triphosphate (ATP), microbial hydrolytic enzymatic activities and bacterial community analysis have been performed. Results indicated that a concentration of 0.8 $mg \cdot L^{-1}$ led to the greatest sludge reduction (44.7%), due to the increase of endogenous respiration. In the reactor with folic acid addition, higher soluble microbial products, enzymatic activities, cellular viability and lower cellular ATP was observed. Regarding the microbial community, folic acid addition did not affect nitrifying bacteria although it did reduce the abundance of denitrifying, increased the amount of filamentous bacteria (*Caldilineacaeae* and *Haliscomenobacter*) and enhanced *Bacteriodetes* and *Chloroflexi* phyla. A continuous addition of folic acid drove to biomass adaptation that caused a diminution of folic acid activity after 40 days.

***Keywords:*** Bacterial community; Biological wastewater treatment; Folic acid; Sequencing batch reactor; Sludge reduction. .

## 1. Introduction

In the last years, the management of the sludge from wastewater treatment plants (WWTPs) has been deeply discussed. In Europe, the circular economy approach and the lack of consensus to approve the pending Council Directive about sludge management keep up the debate about what to do with the sludge (Mininni et al., 2015).

Reduction of the sludge production would save the management costs and would reduce the environmental problem. In this way, new technologies are proposed for the use of sludge as a substrate, for example, like a building material (Świerczek et al., 2018) or in clinker production (Liu et al., 2015), what provides different alternatives to conventional sludge

management. Focusing on sludge production reduction, many authors have worked on achieving a diminution of the secondary sludge production, since primary sludge cannot be avoided, coming from a physical separation process. Bibliography about sludge reduction was updated by some review papers from the beginning of this century (Khursheed and Kazmi, 2011; Liu, 2003; Wei et al., 2003). Foladori (2010) and Wang et al. (2017) reported the sludge reduction both in wastewater and in the sludge treatment lines processes, which were grouped in cell lysis plus cryptic growth, uncoupled metabolism, endogenous metabolism, microbial predation and hydrothermal oxidation.

Focusing on the sludge reduction processes that require the addition of chemicals, the uncoupled metabolism plays an important role. In this way, the influence of the addition of 2-4-5 trichloro-phenol (TCP), 2-4 dinitrophenol (DNP), 4 nitrophenol (pNP) and 3,3',4',5-tetrachlorosalicylanilide (TCS) on the sludge production has been studied by several authors. Results were very promising. For instance, the sludge reduction in the biological process became 62% with 3 $mg \cdot L^{-1}$ of pNP (Qiong et al., 2013) and 50% with 1 $mg \cdot L^{-1}$ of TCS (Feng et al., 2014). However, the hazardous characteristics of the chemicals used and the lack of information about eventual reduction of the organic matter removal efficiency at mid and long term and about the presence of these compounds in the treated effluent seem to stop the implementation of the uncoupled metabolism at an industrial scale.

Nevertheless, in the last years other chemicals have been assessed for the reduction of the sludge production in the biological processes. Unlike metabolic uncouplers, these chemicals aim to increase the bacterial activity in order to enhance the endogenous respiration. This is the case of the folic acid (FA). FA is a soluble compound belonging to the group of B complex vitamins. It is also named as B9 vitamin. Akerboom et al. (1994) reported that the addition of FA to the activated sludge treating wastewater from a recycling paper industry drove to an increase of the bacterial activity that let increase the organic matter elimination

and could reduce the sludge production simultaneously. It was achieved because biomass reached endogenous conditions faster, which enhanced lysis or decay of biomass (Friedrich and Takács, 2013) resulting in reduction of the produced sludge.

Only three works have been found related to the application of FA to sludge production reduction. Martins et al. (2015) reported the result of pilot scale experiments where FA was added at a concentration of 0.2 $mg \cdot L^{-1}$. The results showed a 45% of sludge production decrease comparing with a control reactor. However, these authors compared the FA addition with the oxic-settling-anaerobic (OSA) process, resulting higher sludge reduction in the OSA system. On the contrary, Velho et al., (2016) concluded that 0.2 mg/L of FA addition was the best way for sludge reduction after having compared it with other techniques at laboratory scale for sludge reduction (OSA process, uncoupling metabolism with TCS addition, lysis with chlorination and ultrasounds plus cryptic growth and FA addition). Finally, Alexandre et al. (2016) studied the effect of the addition of a bioproduct based on FA on the sludge systems treating oil refinery wastewater. The chemical was added at a concentration of 0.8 $mg \cdot L^{-1}$. These authors reported that the increase of the sludge retention time (SRT) from 20 to 29 days led to a reduction of the sludge production of 15.9%. Combining the increase of SRT with the bioproduct addition resulted in a reduction of the sludge production of 43.2%.

All of these works are focused on measuring COD removal efficiency, which is not altered by the addition of FA, and on measuring sludge production, which is lower than control reactor (though the achieved percentages are variable). However, no information about the biomass characterization is reported. In this way, the mechanisms of the sludge reduction cannot be known. Thus, in this work, a deep study of the biomass characteristics including soluble microbial products (SMP), microbial hydrolytic enzymatic activities (MHEA), adenosine triphosphate (ATP) measurement and bacterial diversity has been carried out. The objective is to find out whether the reduction of sludge production by adding FA to the process is

corroborated and to take a step towards understanding the mechanisms of the sludge reduction.

## 2. Materials and methods

### *2.1. SBR*

Two identical SBRs (SBR-1 and SBR-2) were operated during 80 days in a temperature of 21.3 ± 2.2ºC. The components of each SBR were the following: cylindrical tank (30×20 cm of height and diameter), air compressor (dissolved oxygen concentration remained above 2.5 $mg \cdot L^{-1}$), two air diffusers (both located at the bottom of the reactor), mechanical stirrer (200 rpm) and two peristaltic pumps.

Both reactors were started-up with activated sludge (2.5 $g \cdot L^{-1}$) from a municipal wastewater treatment plant from Valencia (Spain). In SBR-1 FA was added on the feed, while SBR-2 worked without the addition of this chemical as blank. The operating parameters are shown in Table 1. As it can be seen in this table, mixed liquor suspended solids (MLSS) must be maintained in 2.5 $g \cdot L^{-1}$. For it, periodical sludge withdrawals were carried out. From the experimental values, sludge retention time (SRT) and average sludge production ($\Delta X_{average}$) were calculated by Eq.(1) and Eq.(2) , respectively:

$$SRT = \frac{MLSS_{average} \cdot V_R}{\Delta X_{average}} \qquad \text{Eq.(1)}$$

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

$$\Delta X_{i-j} = \frac{(MLSS_j - MLSS_i) \cdot V_R}{j - i} + [SS_{ef} \cdot Q_{ef}]_i^j \qquad \text{Eq.(3)}$$

$MLSS_{average}$ was the average value of MLSS during the experimental procedure, which should be around 2.5 $g \cdot L^{-1}$. The sludge production was calculated between two days (i and j) in which no sludge withdrawn in between was performed (Eq.(3)). Between each period,

effluent was collected and the suspended solids concentration ($SS_{ef}$) was measured. Finally, the average sludge production was calculated (Eq.(2)).

**Table 1. Operating parameters and FA addition procedure.**

| Reactor | Operating parameters | | |
|---|---|---|---|
| SBR-1 and SBR-2 | Reaction volume | 6 | L |
| | Hydraulic retention time | 0.67 | d |
| | F/M ratio | 0.3 | kg COD·kg $MLSS^{-1}$·$d^{-1}$ |
| | MLSS [a] | ≈ 2.5 | g·$L^{-1}$ |
| | Operation days | 80 | d |
| | Number of cycles | 3 | cycles/d |
| | Volume feed/draw | 3 | L/cycle |
| | **Cycle characteristics** | | |
| | Filling+Aerobic reaction | 6 | h |
| | Sedimentation | 1.5 | h |
| | Draw | 25 | min |
| | Idle | 5 | min |

| Reactor | FA addition (mg·$L^{-1}$) | | | |
|---|---|---|---|---|
| | 0-14 day | 15-42 day | 43-63 day | 64-80 day |
| SBR-1 | 0 | 0.8 | 1.6 | 0 |
| SBR-2 | 0 | 0 | 0 | 0 |

[a] MLSS = mixed liquor suspended solids. In both SBRs the sludge withdrawals was carried out to maintain this value.

### *2.2. Wastewater*

The SBRs were fed with synthetic wastewater (SWW) prepared with peptone and meat extract (in equal mass amount) and $K_2HPO_4$ as phosphorus source. Additionally, from 15$^{th}$ day, FA was added on SBR-1 feed in the concentrations presented in Table 1.

In each reactor a volume of 9 L of SWW was fed per day ($V_{feed|draw}$) while the reaction volume ($V_R$) was 6 L. All reagents were supplied by Panreac and were diluted in tap water. This composition provided a relationship COD:N:P of 100:12:1. The amount of each

compound was calculated to obtain a COD that resulted in a food-to-microorganisms (F/M) ratio of 0.3 kg COD·kg $MLSS^{-1}$·$d^{-1}$. Calculations were done using Eq.(4):

$$\text{F/M ratio} = \frac{\text{COD} \cdot V_{\text{feed|draw}}}{V_R \cdot \text{MLSS}} \qquad \text{Eq.(4)}$$

The amounts were 225 mg·$L^{-1}$ of peptone and meat extract and 28 mg·$L^{-1}$ of $K_2HPO_4$, which resulted in a COD of 500 mg·$L^{-1}$.

### *2.3. Analysis*

#### *2.3.1. Sequencing batch reactors performance*

Effluent pH, conductivity, turbidity and total COD were measured three times a week and total nitrogen ($N_T$) and total phosphorus ($P_T$) once a week. Effluent samples were collected during the draw step of the first cycle of the day. Additionally, MLSS and volatile suspended solids (MLVSS) in the mixed liquor were analysed (three times a week). To measure COD, $N_T$ and $P_T$ a Spectroquant NOVA 30 and reactive kits, from Merck, were used. MLSS and MLVSS were measured according to APHA (2005).

#### *2.3.2. Soluble microbial products*

Soluble microbial products were characterized biweekly (samples were collected at the end of reaction time). For this purpose, proteins by BCA method (Zuriaga-Agustí et al., 2013) and carbohydrates by anthrone method (Frølund et al., 1996) were analyzed. SMP were obtained from liquid phase filtered at 0.45 µm (cellulose acetate filters) after centrifuging the mixed liquor at 12,000 g.

#### *2.3.3. Adenosine triphosphate*

In this work PhotonMaster™ Luminometer from Luminultra® was used to measure ATP of 9 mixed liquor samples of each reactor taken once a week (samples were collected at the end of

reaction time). QG21Waste™ kit was used to quantify the total-ATP (tATP) as cellular ATP (cATP) plus extracellular ATP released from dead cell named dissolved-ATP (dATP). For it, 1.0 mL and 100 μL of activated sludge were used, respectively. The results were determined in RLU (Related Luminescence Units) and were converted to ng ATP·$mL^{-1}$ using a Standard ATP solution UltraCheck™ and LumiCapture™Lite software. Active Volatile Suspended Solids (AVSS) were calculated from the ATP data as the total mass of living microorganisms contained in the sample (cATP x 0.5; he conversion factor of 0.5 is an established factor to convert from ng ATP/mL to mg Solids/L).

*2.3.4. Cellular viability*

The membrane integrity of bacteria was evaluated once a week in both reactors (9 samples for each reactor collected at the end of reaction time) by Film Tracer™ LIVE/DEAD™ Biofilm viability kit (Molecular Probes Eugene, OR, USA). In this test 1 mL of activated sludge sample was mixed with 3 μL live/dead stain mixture (SYTO9/Propidium iodide, 1:1) and incubated for 15 min at room temperature to achieve a estimation of the viable/intact (green colour) and dead/damaged (red colour) bacteria. Twenty microscopic fields of each sample were evaluated with a BX50F microscope (Olympus, Tokyo, Japan) equipped with a 100-W high-pressure mercury lamp and color micrographs were taken with a digital camera Olympus DP 10 (Olympus Optical Co., Hamburg, Germany). The image analysis software BioImageL™ v. 2.1 was used to analyze the stained samples (Chavez de Paz, 2009).

*2.3.5. Microbial hydrolytic enzymatic activities*

In the initial and final days of each FA dosage period, mixed liquor samples were taken at the end of reaction time in both SBR to measure MHEA. In Table 2 the methodology followed to perform the analysis of each MHEA can be seen. Enzymatic activities measured were normalized according to AVSS concentration.

**Table 2. MHEA methodology measurements.**

| MHEA | Method | Measure | |
|---|---|---|---|
| | | **Absorbance** | **Units** |
| Lipase [(a)] | Gessesse et al. (2003) | | |
| Acid phosphatase<br>Alkaline phosphatase<br>α-D-Glucosidase | Goel et al. (1998) | 410 | μmol p-nitrophenol·$h^{-1}$·$gAVSS^{-1}$ |
| Dehydrogenase [(a)] | | 490 | μmol formazan·$h^{-1}$·$gAVSS^{-1}$ |
| Protease | | 340 | ΔAbs·$h^{-1}$·$gAVSS^{-1}$ [(b)] |

[(a)] Adaptation of method was carried out in these MHEA.

[(b)] Abs=absorbance

To eliminate the substances from the mixed liquor that could produce interferences in the method, the samples were centrifuged at 2500 g for 2 min and wastewater was drained. Solid portion which contained the microorganisms was re-suspended in the same amount of Tris-HCl buffer (0.2 mol·$L^{-1}$, pH 7.6) to preserve AVSS concentration

In this work, some adaptations on the original method of Gessesse et al. 2003) and Goel et al. 1998 were performed: incubation step was carried out at 37ºC for 30 min to determinate lipase activity. Dehydrogenase activity was obtained using 0.75 mL of iodonitrotetrazolium chloride (0.3% w/v). Additionally, it should be commented that protease activity was analyzed after 60 min of samples incubation.

*2.3.6. Bacterial community analysis*

To evaluate the change in bacterial community due to FA addition, mixed liquor samples (5 mL) were analyzed with and without propidium monoazide stain (PMA, Biotium Inc., USA), at 15$^{th}$ and 43$^{rd}$ day. PMA enter only membrane-compromised cells, but are excluded from entry into cells with intact cell membranes (Nocker et al., 2006). Samples were disaggregated with IKA-Ultra-Turrax (IKA Labortechnik, Germany) as described by Ziglio et al. (2002). Disaggregated samples (1 mL by duplicate) without PMA (for evaluating viable/no viable cells) and with PMA (for evaluating only viable cells) were collected for DNA extraction. Samples treated with PMA were incubated in the dark for 5 min (4ºC) and were exposed for 15 min to light in the PhaST Blue-Photo activation system for tubes (GenIUL, S.L., Spain). Finally, PMA treated samples and non-PMA treated samples were centrifuged at 8,000 rpm for 4 min. Liquid was decanted and pellets were used for DNA extraction. These pellets were lysed using the FastPrep®-24 Instrument (3700 rpm, 120 s) and DNA was extracted using the FastDNA® SPIN kit for soil according to the manufacturer's instructions, both from MP Biomedicals (USA). DNA samples were eluted in a final volume of 50 µL and OneStep™ PCR Inhibitor Removal Kit (Zymo Research, USA) was added in order to remove inhibitors which could affect enzymatic reactions. DNA concentration was measured by two methods: Qubit® dsDNA BR Assay Kit and NanoDrop ND-1000 UV/Vis spectrophotometer, both from Thermo Fisher Scientific (USA). Finally, V3–V4 regions of bacterial 16S rRNA gene were amplified in all samples using Illumina primers and following Illumina protocols. Libraries were sequenced on a MiSeq Illumina platform using a 2×300pb paired-end run at Lifesequencing service (Valencia, Spain). Raw data were analyzed using QIIME™ 1.9.1 (Caporaso et al., 2010), applying additional scripts available in MicrobiomeHelper virtual box (Comeau et al., 2017). Joined forward and reverse reads were checked using VSEARCH v2.4.3 (Rognes et al., 2016). Taxonomic assignment against 16S SILVA_128 database (Quast

et al., 2012) at 97% similarity of the most abundant sequence of OUT (Operational Taxonomic Units) were achieved.

*2.3.7. Statistical analysis*

Statistical significance of FA addition was evaluated by one-way ANOVA analysis (confidence level of 95 %) with Statgraphics Centurion XVII. The following parameters were assessed in both reactors: pH, conductivity, turbidity, effluent COD, $\Delta X_{i-j}$, enzymatic activities, ATP and SMP.

## 3. Results and discussion

### *3.1. Sequencing batch reactor performance and sludge reduction*

FA addition did not affect effluent parameters like pH, conductivity, turbidity and COD, since similar values were achieved in the treated wastewater from both reactors (Table 3). Thus, one-way ANOVA analysis confirmed that not statistical significance was achieved between these parameters and FA addition.

**Table 3. Average parameters values for the 80 experimental days.**

| | | **SBR-1** | **SBR-2** |
|---|---|---|---|
| pH | | 7.9 ± 0.2 | 7.9 ± 0.2 |
| Conductivity | ($\mu S \cdot cm^{-1}$) | 1278 ± 2 | 1279 ± 1 |
| Turbidity | (NTU) | 0.03 ± 0.01 | 0.03 ± 0.01 |
| Total COD | ($mg \cdot cm^{-1}$) | 26 ± 10 | 25 ± 12 |
| $N_T$ | ($mg \cdot cm^{-1}$) | 41.2 ± 8.7 | 44.3 ± 10.9 |
| $N\text{-}NH_4^+$ | ($mg \cdot cm^{-1}$) | 2.5 ± 1.2 | 3.1 ± 0.8 |
| $NO_3^-$ | ($mg \cdot cm^{-1}$) | 35.6 ± 3.8 | 37.2 ± 5.5 |
| $P_T$ | ($mg \cdot cm^{-1}$) | 0.6 ± 0.4 | 1.4 ± 1.2 |

Other effluent parameters like $N_T$, $N\text{-}NH_4^+$, $NO_3^-$ and $P_T$ remained also similar in both reactors as can be shows in Table 3. Thus, no particular effect on nitrification was observed when FA was added in contrast to other compounds used to reduce the sludge production that inhibit nitrification (Rho et al., 2007; Tian et al., 2013). The relative abundance of viable nitrifying and denitrifying bacteria is reported in Table 4 (samples with and without PMA are presented in Supplementary Table 1S).

**Table 4. Relative abundance of viable nitrifying and denitrifying bacteria (samples with PMA).**

| Genus | Percentage of viable cells (%) | | | |
|---|---|---|---|---|
| | 15th day | | 43rd day | |
| | SBR-1 | SBR-2 | SBR-1 | SBR-2 |
| *Nitrospira* | 0.44 | 0.42 | 0.05 | 0.05 |
| *Nitrosomonas* | 0.76 | 0.88 | 0.58 | 0.78 |
| *Nitrosospira* | 0.00 | 0.00 | 0.00 | 0.01 |
| **Total nitrifying bacteria** | 1.20 | 1.30 | 0.63 | 0.84 |
| *Hyphomicrobium* | 0.05 | 0.05 | 0.09 | 0.14 |
| *Mesorhizobium* | 1.96 | 1.64 | 1.29 | 1.36 |
| *Paracoccus* | 0.59 | 0.50 | 0.23 | 0.39 |
| *Comamonas* | 0.11 | 0.10 | 0.08 | 0.08 |
| *Candidatus Accumulibacter* | 0.06 | 0.10 | 0.82 | 0.81 |
| *Methyloversatilis* | 0.14 | 0.13 | 0.02 | 0.02 |
| *Thauera* | 3.82 | 3.19 | 1.71 | 3.51 |
| *Pseudomonas* | 0.14 | 0.07 | 0.00 | 0.00 |
| **Total denitrifying bacteria** | 6.87 | 5.78 | 4.24 | 6.31 |

As it can be observed in Table 4, there was no effect of FA on nitrifying bacteria responsible for ammonia and nitrite oxidation (*Nitrospira, Nitrosomonas and Nitrosospira*), since their abundance decreased in both reactors during the experimental time. However, FA dosage affected denitrifying bacteria, whose total abundance decreased in SBR-1 while increased in SBR-2. This fact was due mainly to *Thauera* genus, whose relative abundance decreased from 3.82% to 1.71% in SBR-1 while remained constant in SBR-2 (around 3.67%). This

effect should be studied in detail, carrying out a biological treatment with anoxic reaction step, to check if whether FA dosage could affect denitrification.

Regarding filamentous population it can be commented that, FA additions increased their relative abundances (Table 5), since total filamentous abundance in SBR-1 increased in higher proportion during experimental time than in SBR-2 (52.5% and 29.2% in SBR-1 and SBR-2, respectively).It can be highlighted that FA improved Caldilineacaeae and Haliscomenobacter growth.

**Table 5. Relative abundance of viable filamentous bacteria (samples with PMA).**

| Genus | Percentage of viable cells (%) | | | |
|---|---|---|---|---|
| | 15th day | | 43rd day | |
| | SBR-1 | SBR-2 | SBR-1 | SBR-2 |
| *Caldilineacaeae* | 3.19 | 4.10 | 11.08 | 7.70 |
| *Roseiflexus* | 0.01 | 0.05 | 0.06 | 0.07 |
| *Candidatus microthrix* | 1.85 | 1.41 | 0.08 | 0.17 |
| *Tetrasphaera* | 0.50 | 0.73 | 0.64 | 0.79 |
| *Haliscomenobacter* | 0.44 | 0.45 | 1.15 | 0.58 |
| *Trichococcus* | 0.18 | 0.15 | 0.00 | 0.00 |
| *Isosphaera* | 0.05 | 0.06 | 0.12 | 0.32 |
| *Candidatus alysiosphaera* | 0.09 | 0.05 | 0.21 | 0.24 |
| *Meganema* | 0.02 | 0.03 | 0.08 | 0.08 |
| *Gordonia* | 0.00 | 0.00 | 0.00 | 0.00 |
| *Rhodococcus* | 0.00 | 0.00 | 0.03 | 0.01 |
| *Mycobacterium* | 0.06 | 0.04 | 0.01 | 0.02 |
| **Total** | 6.39 | 7.07 | 13.46 | 9.98 |

As it can be observed in Table 5, *Haliscomenobacter* abundance increased, between 15th to 43rd days, from 0.44 to 1.15%, while in SBR-2 it was maintained around 0.52%. *Haliscomenobacter* is considered as rarely responsible for bulking and foaming, but is frequently found inside the flocs of activated sludge (Kragelund et al., 2008). Concerning *Caldilineacaeae* abundance, this bacterium increased in both reactors, but this increase was

higher in SBR-1 (from 3.19 to 11.08%) than in SBR-2 (from 4.10 to 7.70%). *Caldilineacaeae* is a *Chloroflexi* filamentous bacterium. Filamentous *Chloroflexi* constituted a large fraction of the total bacterial biovolume in some full-scale activated sludge plants (Kragelund et al., 2011).

Concerning the sludge production, it can be seen in Figure 1.a that FA reduced the sludge growth per day in SBR-1 from the 15 to 55 days. Statistical significance was observed when SBR-1 and SRB-2 were compared throughout the 80 days ($F=20.92$; p-value=0.0001).

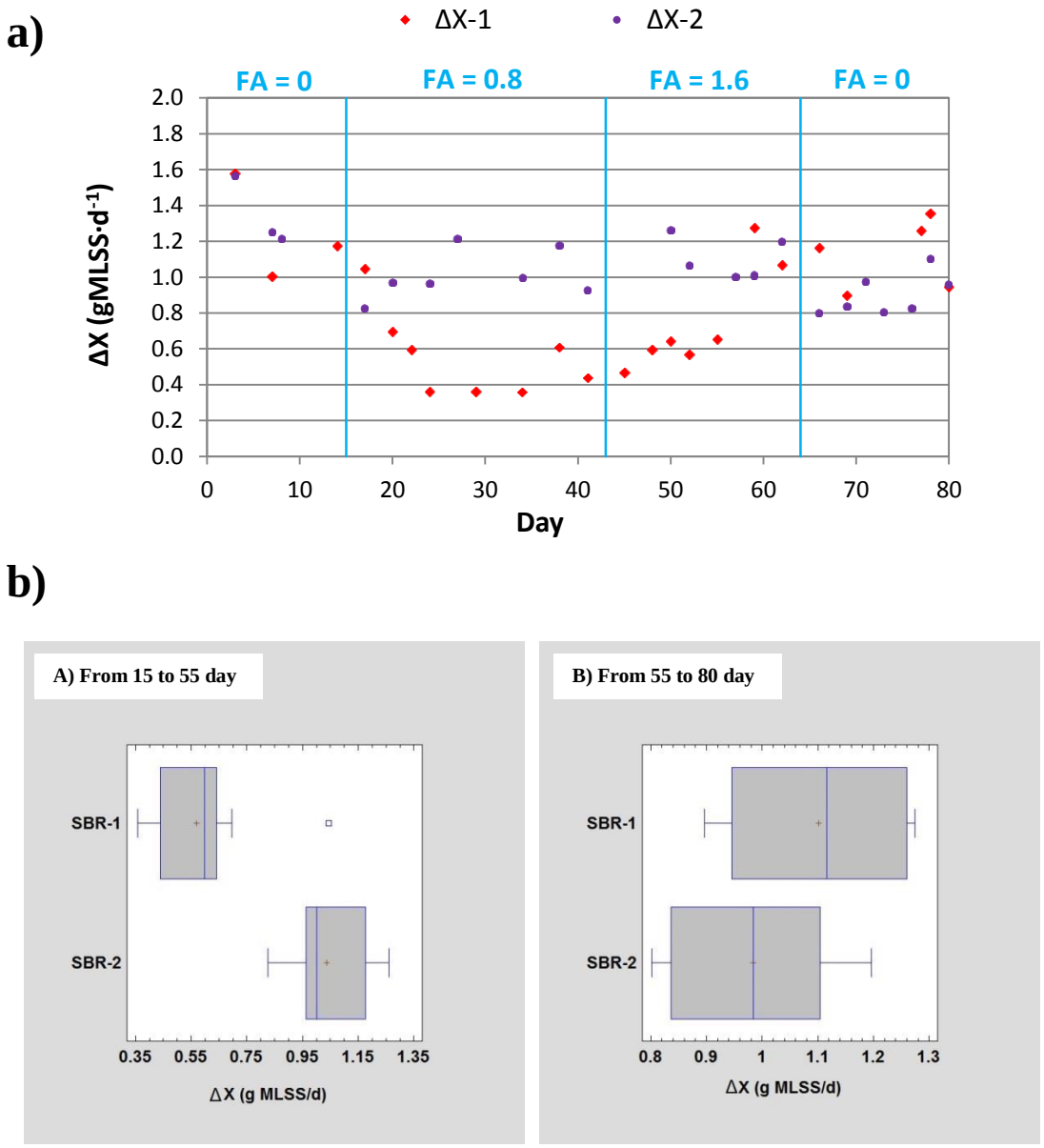


**Figure 1. a) Sludge production in SBR-1 and SBR-2 (vertical lines indicate the different FA periods); b) Tukey diagram for sludge production in SBR-1 and SBR-2.**

It has to be commented that the highest sludge reduction was achieved when FA was added in a concentration of 0.8 mg·$L^{-1}$. In this period, the average values of ΔX were 0.56 ± 0.24 g MLSS·$d^{-1}$ in SBR-1 and 1.01 ± 0.14 g MLSS·$d^{-1}$ in SBR-2, resulting in an average reduction value of 44.7% in the sludge production. In the maximum sludge reduction period (from the 24 to 43 days), this average value increased to 59.7%. These values were in the same order of magnitude reported by Senorer and Barlas (2004), who achieved for different SRT values

between 30% and 50% of sludge reduction for 0.2 $mg \cdot L^{-1}$ of FA. On the other hand, due to sludge reduction higher SRT was achieved in the reactor with lower ΔX between $15^{th}$ to $43^{rd}$ days since MLSS concentration was maintained constant. In this way, SRT values were 28 and 19 days in SBR-1 and SBR-2, respectively. Doubling the FA concentration did not improve the reduction achieved in the sludge production as it can be seen in Figure 1.a (from day 43 until 64). Additionally, results showed that the effect of FA decreased along the dosage period gradually. In this way, similar ΔX values were observed in both reactors after 40 days of FA addition. In this regard, Alexandre et al. (2016) also reported a loss of sludge reduction efficiency after 44 days of dosage of a bioproduct based on folic acid in a SBR. This fact could be explained by adaptation of microorganisms to FA. They become to have tolerance to FA, explaining the loss of its effect on their metabolism. Similar ΔX was observed in the last period without FA addition.

In Tukey diagrams (Figure 1.b), it can be observed the range of ΔX for both reactors. This figure shows two experimental periods: the first period in which FA addition had a statistical significance on sludge production (from 15 to 55 day; $F=48.99$; p-value<0.0001) and the second period in which FA lost its effect on sludge reduction or its dosage was stopped (from 56 to 80 day; $F=1.70$; p-value=0.2212).

### *3.2. Mechanism of action of folic acid*

According to Jenkins et al. (2003) and Akerboom et al. (1994), FA improves the metabolic pathways of microorganisms and activates the cells, resulting in faster substrate consumption. This fact increases the endogenous respiration time, in which cells oxidize cellular constituents to produce energy for cell maintenance requirements. This leads to sludge reduction.

SMP (sum of proteins and carbohydrates) concentration may be related by the endogenous respiration, since SMP is the sum of the utilization-associated products (UAP) and the biomass-associated product (BAP) (Lu et al., 2001). UAP include the products generated from substrate utilization and the cell growth and BAP consist of the products from the endogenous respiration of cell mass. Therefore, an increase in the SMP concentration under the same substrate and operational conditions indicates longer endogenous respiration time. It should be commented that FA addition did not alter the measured organic concentrations in the feed. Thus there were not differences among the three SWW used in this work and similar COD concentrations were measured for SWW without FA, SWW with 0.8 $mg \cdot L^{-1}$ and SWW with 1.6 $mg \cdot L^{-1}$ (570, 577 and 579 $mg \cdot L^{-1}$, respectively). In Figure 2 it can be observed that SMP concentration in SBR-1 was higher than in SBR-2 when sludge production reduction was higher, confirming this hypothesis. Between 15 to 43 day the average values of SMP were 10.0 ± 1.5 and 7.6 ± 1.7 $mg \cdot L^{-1}$ in SBR-1 and SBR-2, respectively.

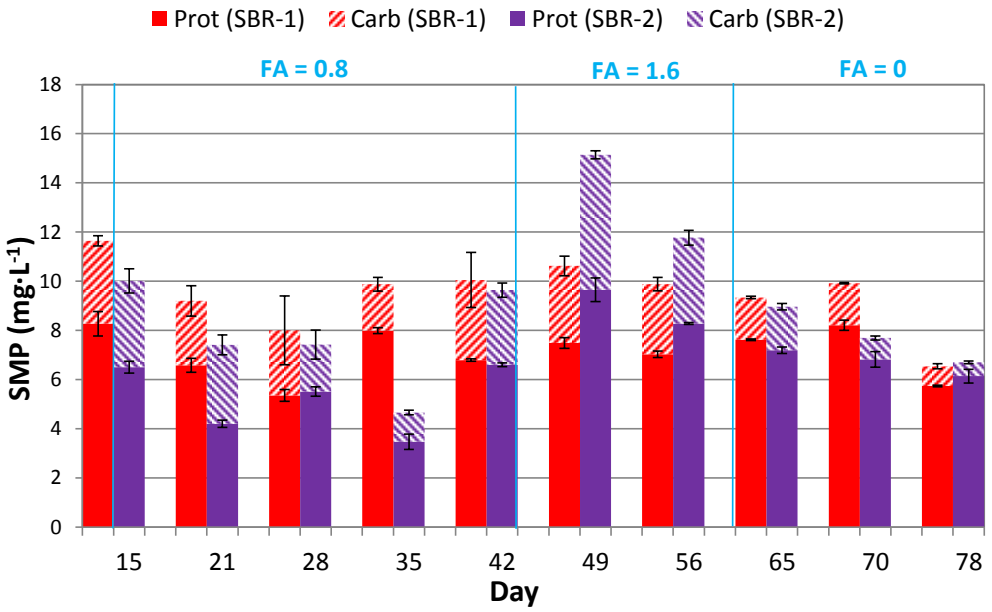


**Figure 2. SMP concentration in SBR-1 and SBR-2. Vertical lines indicate the different FA periods.**

It can be highlighted that this difference was due to proteins concentration, resulting a statistical significance (F=5.58; p-value=0.0458) between this parameter and the reactors. However, no statistical significance was achieved for carbohydrates concentration (F=0.45; p-value=0.5232).

On the other hand, in the first period of FA addition (0.8 $mg \cdot L^{-1}$) a lower cellular ATP concentration was observed in SBR-1 in comparison to SBR-2 (Figure 3).

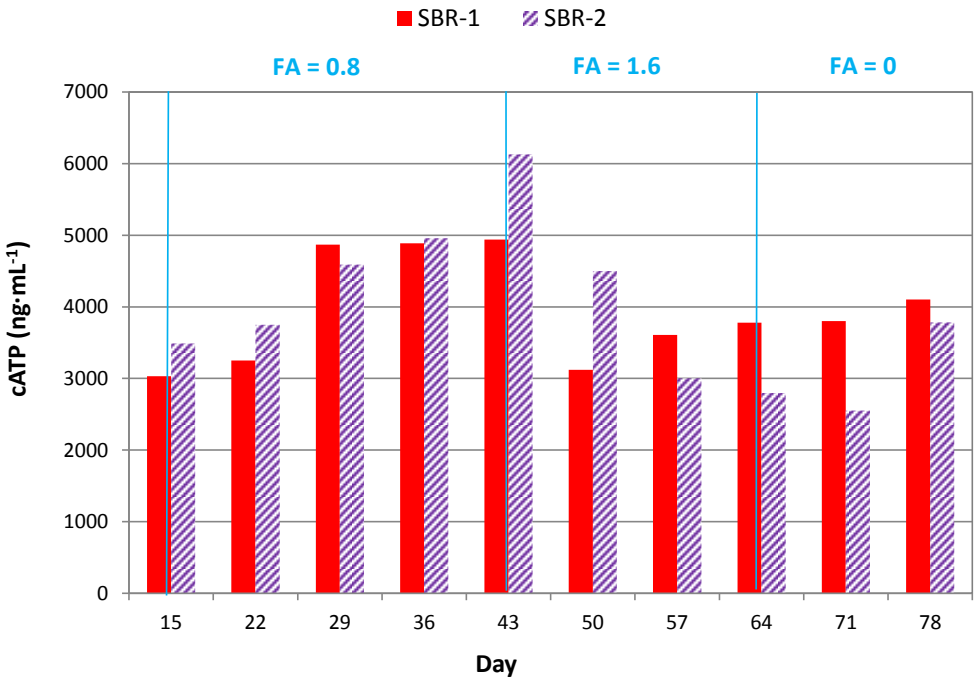


**Figure 3. Cellular ATP concentration. Vertical lines indicate the different FA periods.**

This behavior can be related with the higher SRT in SBR-1 due to lower microbial growth, which implied lower ATP consumptions. It can be highlighted that in this period (from 15$^{th}$ to 43$^{rd}$ day) a progressive increase on cATP values in both reactors was observed. This behavior can be related by the room temperature, which increased from 20 to 25ºC in this experimental time due to the seasonal period. This fact also affected on MHEA measurements as observed in Figure 4, which decreased in both reactors.

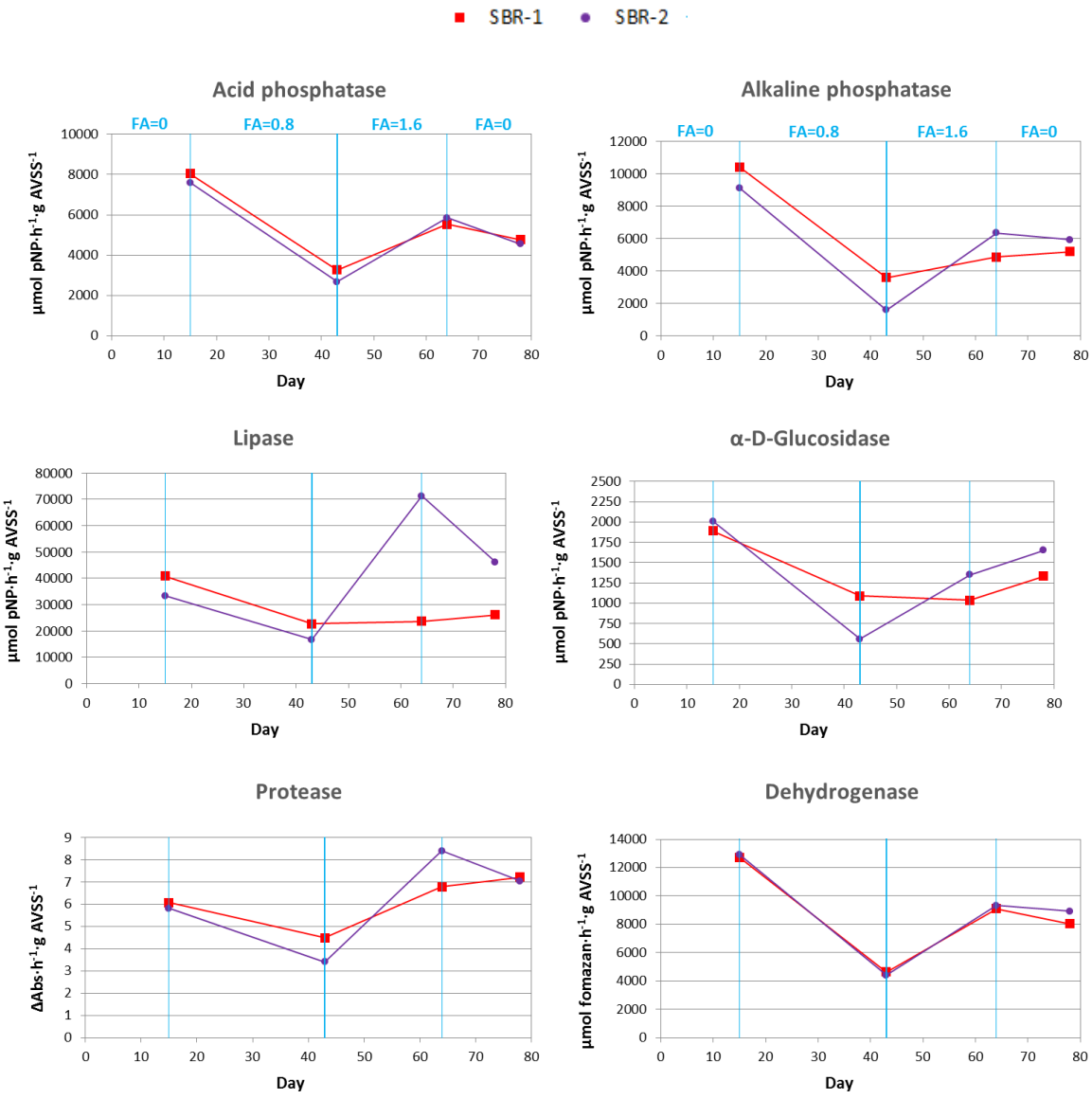


**Figure 4. MHEA in SBR-1 and SBR-2. Vertical lines indicate the different FA periods.**

It is known that temperature enhances the metabolic activities of microorganisms. Thus, under these conditions, lower enzymatic substances concentrations were required for substrate degradation. Additionally, this fact drove to low energy requirements, which implied ATP storage. Despite progressive decrease in MHEA values in period with 0.8 $mg \cdot L^{-1}$ of FA addition, higher values of all MHEA were observed in SBR-1 (except for deshydrogenase activity, which remained constant in both reactors during the 80

experimental days). These results agree with other research works (Senorer and Barlas, 2004; Velho et al., 2016), where it was reported that external addition of FA promotes a fast metabolic activity in the activated sludge process. Another indicator that endogenous respiration was achieved was the higher protease concentration in SBR-1. According to Yan et al. (2008), microorganisms auto-oxidation, due to endogenous phase, leads to a release of protease. Regarding cell viability results (Supplementary Table S3), it can be concluded that FA also entailed a slight improvement in the percentage of viable cells, achieving 96% in SBR-1 and 89% in SBR-2 on day 43. Thus, FA addition accelerates the substrate degradation due to both increases of cell viability and enzymatic activities, resulting in longer endogenous respiration and lower sludge production in SBR-1.

In the second period of FA addition (1.6 $mg \cdot L^{-1}$), cATP and MHEA had the contrary behavior to that observed in the first one. From $43^{rd}$ day the room temperature remained at lower and more stable values (22.0 ± 0.8ºC). These conditions slowed the metabolism of microorganisms, resulting in higher enzymatic activities and higher ATP requirements. Thus, an increase in MHEA and a decrease in ATP storage were observed in both reactors. Despites FA was losing its effect on the sludge reduction in this period, this substance behaved as a buffer for MHEA and ATP values since a shorter variation range was achieved for each parameter measured in SBR-1 in comparison to SBR-2.

In the last period, without FA addition, energy storage and substrates requirements for catabolism were equated in SBR-1 and SBR-2. Thus, both ATP and MHEA amounts had a trend to similar values as shown in Figure 3 and Figure 4, respectively. The same behavior was observed in viability measurements in this period (from 64 to 80 day), reaching average values of viable cells in SBR-1 and SBR-2 of 79 ± 7% and 78 ± 3%, respectively.

### *3.3. Bacterial community*

The average operational taxonomic units (OTU) in a given number of sequences of each sample were analyzed from rarefaction curves. These curves were calculated at 97% similarity for a 565,153 raw reads, resulting in 494,683 high-quality sequences after informatics filtering, which can be seen in Supplementary Figure S1. OTUs in both reactors were higher in 15$^{th}$ day than in 43$^{rd}$ day. This fact was due to synthetic feed, which led to lower biodiversity and lower abundance of bacterial community.

In Figure 5 the main phyla of bacterial community are presented. In this figure it can be seen that phyla *Bacteriodetes* and *Proteobacteria* were dominant from 15 to 43 day.

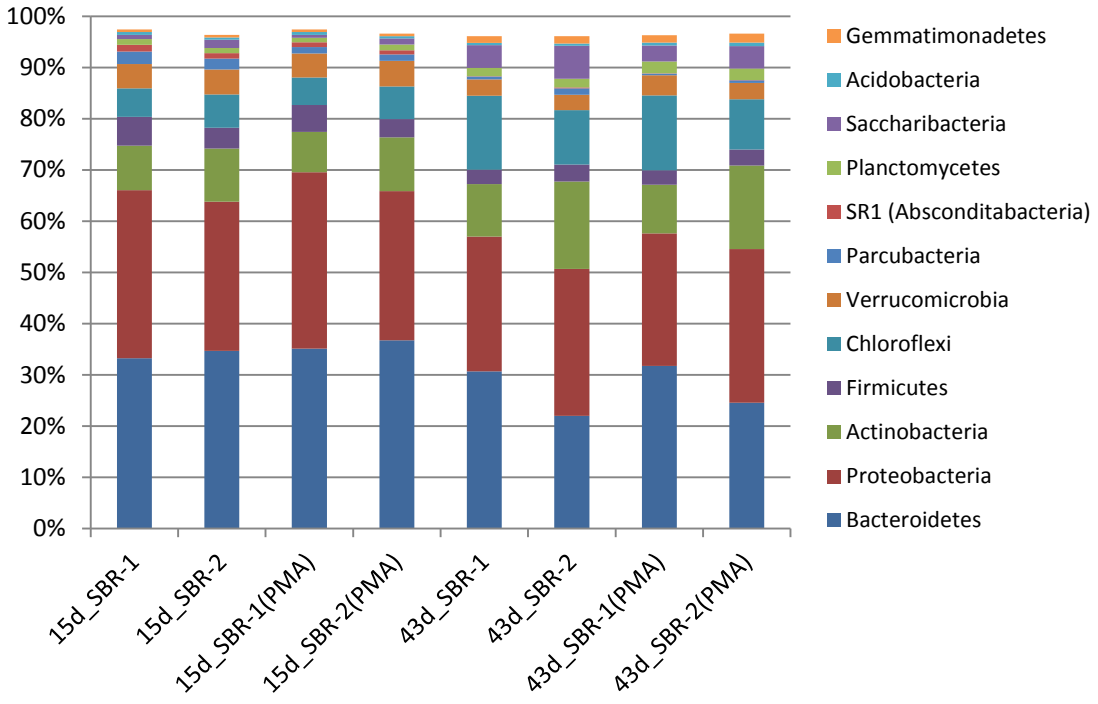


**Figure 5. Relative abundances phylum level of SBR-1 and SBR-2 in 15$^{th}$ and 43$^{rd}$ day.**

In both reactors both phyla decreased during this experimental time. In the last day of 0.8 mg·L$^{-1}$ of FA addition, viable *Bacteriodetes* abundance was higher in SBR-1 (31.7%) than in SBR-2 (24.6%), while *Proteobacteria* abundance was lower (25.9% and 30.0% in SBR-1 and SBR-2, respectively). In other studies (Hu et al., 2012) the most predominant phylum was

*Proteobacteria* followed by *Bacteroidetes* as it happens in SBR-2. Thus, according to the results of this work, FA addition improved abundance of *Bacteriodetes* phylum on bacterial community structure. Other phyla that should be taken into account are *Actinobacteria* and *Chloroflexi*, which increased their abundance in both reactors. In the 43$^{rd}$ day abundance of *Actinobacteria* was higher in SBR-2 (9.5% and 16.3% in SBR-1 and SBR-2, respectively) while *Chloroflexi* was higher in SBR-1 (14.6% and 9.8% in SBR-1 and SBR-2, respectively). The other phyla remained in similar values in both reactors. It should be highlighted that *Chloroflexi* can metabolize soluble microbial products and cell decay materials released by bacteria (Kindaichi et al., 2012; Miura et al., 2007). This fact could explain why SMP concentrations only increased by 24% whereas ΔX decreased by 44.7% due to endogenous respiration.

Microbial analysis at genus level provided a deeper understanding of the effects of FA addition on the community structure (Supplementary Table S4). In this way, assessing viable bacteria it can be commented that all the amounts of genus level were below 2.9% in 43$^{rd}$ day. The relative abundance of viable *Proteiniclasticum* and *Rhodobacter* decreased between 15 and 43 day in SBR-1, while their amounts remained constant in SBR-2. Conversely, abundance of *Opitutus* and *Leucobacter* increased in SBR-1 while decreased in SBR-2. *Planctomyces*, *Seminibacterium* and *OM27 clade* abundance also were improved by FA addition, which increased in a rate higher in SBR-1 than in SBR-2.

**4. Economic analysis**

The aim of this section is to estimate the savings that would be achieved by the use of FA in a WWTP. Results confirmed that the optimal concentration of FA was 0.8 mg·L$^{-1}$. Additionally, it has to be taken into account that this work shows that FA addition loses its effectiveness after 40 day. Thus, after this time, stopping of FA addition should be recommended,

restarting it after the time equivalent to the SRT. As SRT in SBR with FA addition was 28 days, it is proposed to vary out cycles consisting of 40 days with FA dosage and 28 days without it (equivalent to SRT so that biomass can be renewed) should be performed. In this way, FA will be added during 225 days per year while the system will work without FA in the other 140 days.

To calculate the sludge production per year in a reactor with FA addition, the following issues are assumed: 1) dry sludge production with and without FA addition are 0.56 $g \cdot d^{-1}$ (during 225 days) and 1.01 $g \cdot d^{-1}$ (during 140 days), respectively and 2) these values will be maintained constant in each period with and without FA dosage. In these conditions, the sludge production per year in a biological system working with and without FA addition will be 262.9 g and 368.7 g, respectively. Taking into account that the reactor treats 9 L per day (3285 L per year), the sludge production per each liter of treated wastewater will be 0.081 $g \cdot L^{-1}$ and 0.112 $g \cdot L^{-1}$ in SBRs working with and without FA dosage. Thus, working with FA, it is possible to reduce the sludge production by 0.031 grams of dry sludge per each liter of treated wastewater.

The cost of the sludge management varies according to the treatment applied and to final destination: use of sludge in agriculture and soils restoration, use in construction industry, incineration or raw materials recovery (Cieślik et al., 2015). In Spain, 80% of sludge production is applied to the agriculture ("Lodos de depuradora," 2013). For this purpose, sludge stabilization and reduction of water content through centrifugation (final humidity above 80%) are performed. The cost of this sludge management system can range from 3.5 to 5 $€ \cdot ton^{-1}$ of wet sludge. However, the factor with the highest contribution to the final cost of sludge management is the sludge transport to the areas where it has to be applied. This value may vary from 20 to 55 $€ \cdot ton^{-1}$ of wet sludge (after centrifugation), depending on the size of WWTP, the type of transport vehicle (container or bathtub). If an average cost of 30 $€ \cdot ton^{-1}$

of wet sludge is assumed in a WWTP that treats 40,000 $m^3 \cdot d^{-1}$, a saving of 67,890 $€ \cdot year^{-1}$ will be achieved because of the sludge reduction by FA addition.

In addition, 7,200 $kg \cdot year^{-1}$ of FA will be necessary according to the aforementioned calculations. Since the FA is not a product that is sold in large quantities, it is very difficult to find an approximate price. This working methodology would be economically viable if FA price is below 9.43 $€ \cdot kg^{-1}$.

## 4. Conclusions

In this work an exhaustive study about the effect of FA addition on wastewater biological treatment was carried out. Dosage of 0.8 $mg \cdot L^{-1}$ of FA into the feed of SBR-1 showed a decrease on sludge production around 44.7%. Sludge reduction was due to the increase on endogenous respiration, which was corroborated by higher SMP concentrations in SBR-1 than in SBR-2.

An increase in FA concentration up to 1.6 $mg \cdot L^{-1}$ did not improved this behavior and FA addition lost its effect after 40 days of dosage due to biomass adaptation. In the period with high FA influence on the wastewater biological treatment (between $15^{th}$ to $43^{rd}$ days), an increase in MHEA of SBR-1 was observed, which explained that performance in these reactors was maintained in similar values of SBR-2 despite lower sludge production. In addition, in this period lower ATP values were achieved in SBR-1 due to lower microbial growth, which resulted in lower ATP consumptions. Regarding bacterial community, *Bacteriodetes* and *Proteobacteria* were the main phyla in both reactors resulting 57.6% and 54.6% of total in SBR-1 and SBR-2, respectively. *Bacteriodetes* and *Chloroflexi* abundance was improved by FA addition.

Summarizing, FA addition has a great potential in sludge reduction. However, the addition of FA has to be stopped once microorganisms have adapted the metabolism to this vitamin. Thus a periodical dosage of FA could be of great interest to reduce the production of secondary sludge in wastewater treatment plants. This methodology can result in a saving of 67,890 €·year$^{-1}$.

**Acknowledgements**

This work was supported by the Ministry of Economy and Competitiveness (MINECO), Spain (grant no. CTM201454546-P-AR)

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## Supplementary Material

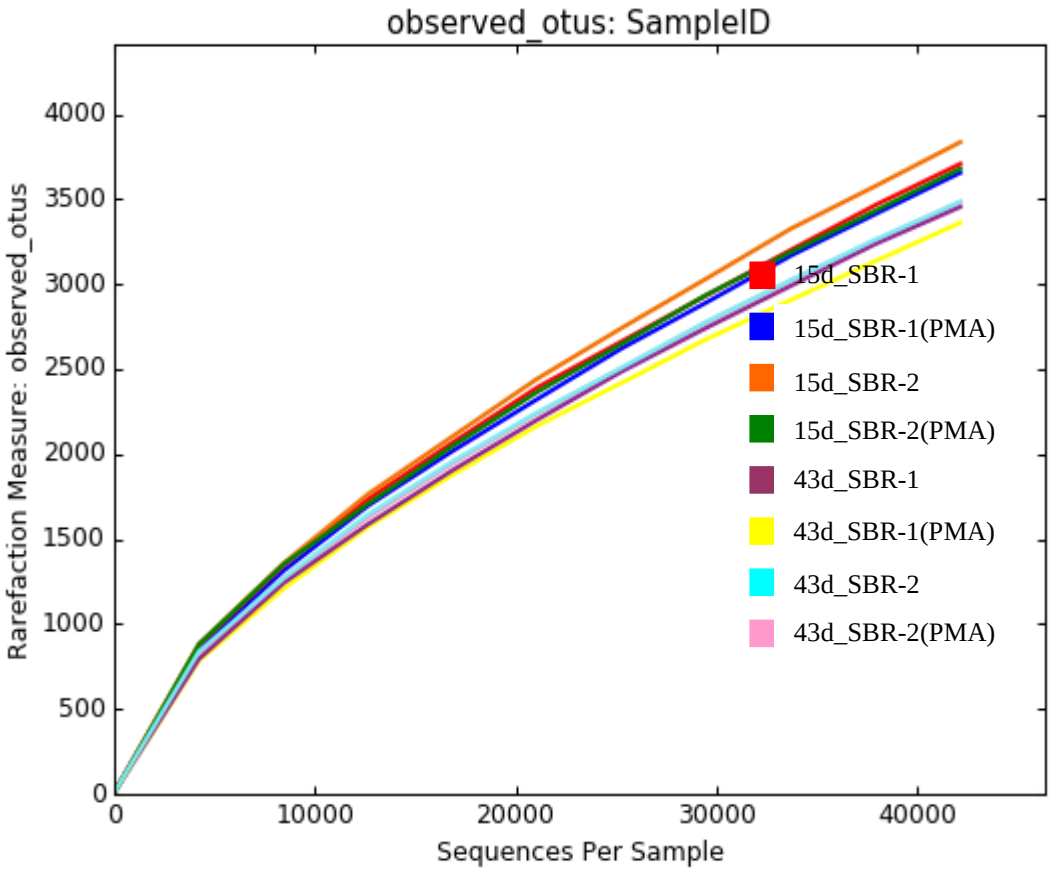


**Figure S1. Rarefaction curves of each reactor in 15$^{th}$ and 43$^{rd}$ day. The number of reads of all samples was normalized to the sample with the lowest number of reads (42,163).**

**Table S1. Relative abundance (%) of nitrifying and denitrifying bacteria.**

| Genus | 15th day | | | | 43rd day | | | |
|---|---|---|---|---|---|---|---|---|
| | SBR-1 | SBR-2 | SBR-1 PMA | SBR-2 PMA | SBR-1 | SBR-2 | SBR-1 PMA | SBR-2 PMA |
| *Nitrospira* | 0.39 | 0.39 | 0.44 | 0.42 | 0.05 | 0.04 | 0.05 | 0.05 |
| *Nitrosomonas* | 0.65 | 0.75 | 0.76 | 0.88 | 0.60 | 0.64 | 0.58 | 0.78 |
| *Nitrosospira* | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.01 |
| *Hyphomicrobium* | 0.08 | 0.05 | 0.05 | 0.05 | 0.12 | 0.12 | 0.09 | 0.14 |
| *Mesorhizobium* | 1.89 | 1.55 | 1.96 | 1.64 | 1.26 | 1.24 | 1.29 | 1.36 |
| *Paracoccus* | 0.59 | 0.57 | 0.59 | 0.50 | 0.20 | 0.43 | 0.23 | 0.39 |
| *Comamonas* | 0.07 | 0.11 | 0.11 | 0.10 | 0.07 | 0.10 | 0.08 | 0.08 |
| *Candidatus Accumulibacter* | 0.08 | 0.07 | 0.06 | 0.10 | 0.97 | 0.77 | 0.82 | 0.81 |
| *Methyloversatilis* | 0.14 | 0.12 | 0.14 | 0.13 | 0.01 | 0.03 | 0.02 | 0.02 |
| *Thauera* | 3.90 | 3.07 | 3.82 | 3.19 | 1.76 | 3.12 | 1.71 | 3.51 |
| *Pseudomonas* | 0.14 | 0.04 | 0.14 | 0.07 | 0.00 | 0.00 | 0.00 | 0.00 |

**Table S2. Relative abundance (%) of filamentous bacteria.**

| Family/Genus | 15th day | | | | 43rd day | | | |
|---|---|---|---|---|---|---|---|---|
| | SBR-1 | SBR-2 | SBR-1 PMA | SBR-2 PMA | SBR-1 | SBR-2 | SBR-1 PMA | SBR-2 PMA |
| *Caldilineacaeae* | 3.31 | 4.04 | 3.19 | 4.10 | 10.84 | 8.21 | 11.08 | 7.70 |
| *Roseiflexus* | 0.03 | 0.09 | 0.01 | 0.05 | 0.04 | 0.09 | 0.06 | 0.07 |
| *Candidatus microthrix* | 2.38 | 1.90 | 1.85 | 1.41 | 0.16 | 0.24 | 0.08 | 0.17 |
| *Tetrasphaera* | 0.50 | 0.71 | 0.50 | 0.73 | 0.74 | 0.85 | 0.64 | 0.79 |
| *Haliscomenobacter* | 0.49 | 0.42 | 0.44 | 0.45 | 1.20 | 0.67 | 1.15 | 0.58 |
| *Trichococcus* | 0.21 | 0.23 | 0.18 | 0.15 | 0.01 | 0.01 | 0.00 | 0.00 |
| *Isosphaera* | 0.04 | 0.04 | 0.05 | 0.06 | 0.08 | 0.26 | 0.12 | 0.32 |
| *Candidatus alysiosphaera* | 0.07 | 0.06 | 0.09 | 0.05 | 0.15 | 0.22 | 0.21 | 0.24 |
| *Meganema* | 0.01 | 0.02 | 0.02 | 0.03 | 0.10 | 0.11 | 0.08 | 0.08 |
| *Gordonia* | 0.00 | 0.01 | 0.00 | 0.00 | 0.01 | 0.00 | 0.00 | 0.00 |
| *Rhodococcus* | 0.00 | 0.00 | 0.00 | 0.00 | 0.04 | 0.01 | 0.03 | 0.01 |
| *Mycobacterium* | 0.04 | 0.03 | 0.06 | 0.04 | 0.03 | 0.01 | 0.01 | 0.02 |

**Table S3: Viable and dead cells (%) in SBR 1 and SBR 2.**

| Sampling day | SBR-1 | | | SBR-2 | | |
|---|---|---|---|---|---|---|
| | Viable | Dead | ND | Viable | Dead | ND |
| 15 | 91 | 8 | 1 | 85 | 11 | 4 |
| 22 | 89 | 7 | 4 | 84 | 12 | 4 |
| 29 | 84 | 10 | 6 | 74 | 17 | 9 |
| 36 | 92 | 7 | 1 | 88 | 10 | 2 |
| 43 | 96 | 3 | 1 | 89 | 10 | 1 |
| 57 | 88 | 10 | 2 | 77 | 19 | 4 |
| 64 | 80 | 19 | 1 | 74 | 24 | 2 |
| 71 | 76 | 22 | 2 | 77 | 20 | 3 |
| 78 | 72 | 24 | 4 | 82 | 15 | 3 |

ND: not detected

**Table S4. Relative abundances at genus level of SBR-1 and SBR-2 in 15th and 43rd day.**

| Genus | 15th day | | | | 43rd day | | | |
|---|---|---|---|---|---|---|---|---|
| | SBR-1 | SBR-2 | SBR-1 (PMA) | SBR-2 (PMA) | SBR-1 | SBR-2 | SBR-1 (PMA) | SBR-2 (PMA) |
| *Acidovorax* | 3.63% | 2.08% | 4.42% | 2.16% | 0.15% | 0.20% | 0.14% | 0.18% |
| *Proteiniclasticum* | 3.10% | 2.39% | 3.46% | 2.21% | 1.36% | 2.67% | 1.14% | 2.60% |
| *Terrimonas* | 2.77% | 2.73% | 2.91% | 2.83% | 0.88% | 1.14% | 0.93% | 1.01% |
| *Ferruginibacter* | 2.31% | 2.57% | 2.37% | 2.82% | 0.79% | 0.74% | 0.97% | 0.81% |
| *Bdellovibrio* | 1.96% | 2.06% | 2.45% | 1.95% | 0.43% | 0.44% | 0.48% | 0.38% |
| *Rhodobacter* | 1.35% | 1.37% | 1.35% | 1.28% | 0.55% | 1.07% | 0.49% | 1.18% |
| *Aeromonas* | 1.14% | 0.71% | 1.32% | 0.72% | 1.37% | 0.57% | 1.34% | 0.61% |
| *Opitutus* | 1.06% | 1.12% | 1.08% | 1.13% | 1.04% | 0.75% | 1.16% | 0.85% |
| *Sphingobacterium* | 1.05% | 0.48% | 1.11% | 0.48% | 0.00% | 0.00% | 0.00% | 0.00% |
| *Leucobacter* | 0.96% | 2.25% | 0.81% | 2.56% | 0.85% | 0.90% | 0.88% | 0.67% |
| *Taibaiella* | 0.95% | 0.88% | 1.13% | 0.91% | 0.04% | 0.07% | 0.04% | 0.06% |
| *Rhodovulum* | 0.93% | 1.11% | 0.89% | 0.92% | 0.21% | 0.42% | 0.16% | 0.39% |
| *Prosthecobacter* | 0.92% | 1.06% | 0.90% | 1.06% | 0.52% | 0.79% | 0.66% | 0.79% |
| *Dokdonella* | 0.90% | 1.11% | 0.93% | 1.20% | 0.29% | 0.17% | 0.27% | 0.21% |
| *Glutamicibacter* | 0.85% | 0.97% | 0.93% | 0.82% | 0.14% | 0.09% | 0.12% | 0.12% |
| *Planctomyces* | 0.79% | 0.71% | 0.57% | 0.72% | 0.90% | 0.60% | 1.31% | 0.82% |
| *Shinella* | 0.60% | 0.37% | 0.49% | 0.29% | 0.36% | 0.26% | 0.28% | 0.34% |
| *Bosea* | 0.47% | 0.41% | 0.42% | 0.47% | 0.26% | 0.58% | 0.31% | 0.58% |
| *Flavobacterium* | 0.46% | 0.25% | 0.48% | 0.25% | 0.21% | 0.08% | 0.21% | 0.16% |
| *Arenimonas* | 0.43% | 0.42% | 0.39% | 0.42% | 0.22% | 0.30% | 0.19% | 0.42% |
| *Brevundimonas* | 0.41% | 0.43% | 0.44% | 0.50% | 0.18% | 0.20% | 0.26% | 0.21% |
| *Ochrobactrum* | 0.40% | 0.16% | 0.54% | 0.17% | 0.07% | 0.01% | 0.09% | 0.02% |
| *Thermomonas* | 0.39% | 0.34% | 0.37% | 0.43% | 0.01% | 0.03% | 0.02% | 0.03% |
| *Sediminibacterium* | 0.33% | 0.29% | 0.40% | 0.31% | 0.89% | 0.28% | 1.25% | 0.36% |

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|---|---|---|---|---|---|---|---|---|
| *Phaeodactylibacter* | 0.28% | 0.30% | 0.32% | 0.41% | 0.86% | 0.92% | 0.85% | 0.87% |
| *BD1-7 clade* | 0.27% | 0.24% | 0.21% | 0.14% | 0.02% | 0.03% | 0.02% | 0.02% |
| *Brevifollis* | 0.24% | 0.22% | 0.25% | 0.22% | 0.04% | 0.08% | 0.11% | 0.06% |
| *Novosphingobium* | 0.24% | 0.28% | 0.35% | 0.23% | 0.01% | 0.02% | 0.02% | 0.02% |
| *Gemmatimonas* | 0.19% | 0.19% | 0.15% | 0.18% | 0.35% | 0.36% | 0.34% | 0.37% |
| *Lewinella* | 0.17% | 0.13% | 0.12% | 0.14% | 0.41% | 0.23% | 0.41% | 0.26% |
| *Stenotrophomonas* | 0.15% | 0.06% | 0.21% | 0.06% | 0.23% | 0.00% | 0.27% | 0.01% |
| *Sphingopyxis* | 0.14% | 0.14% | 0.19% | 0.13% | 0.06% | 0.10% | 0.05% | 0.15% |
| *Candidatus Competibacter* | 0.13% | 0.12% | 0.14% | 0.10% | 0.50% | 1.15% | 0.53% | 1.20% |
| *Pseudoxanthomonas* | 0.12% | 0.08% | 0.11% | 0.10% | 0.07% | 0.17% | 0.08% | 0.15% |
| *Woodsholea* | 0.12% | 0.11% | 0.12% | 0.11% | 0.06% | 0.07% | 0.07% | 0.11% |
| *Lactobacillus* | 0.11% | 0.05% | 0.05% | 0.03% | 0.06% | 0.03% | 0.06% | 0.03% |
| *Pirellula* | 0.09% | 0.08% | 0.07% | 0.09% | 0.15% | 0.19% | 0.21% | 0.28% |
| *Sphingomonas* | 0.09% | 0.03% | 0.09% | 0.04% | 0.02% | 0.03% | 0.02% | 0.03% |
| *Ignavibacterium* | 0.07% | 0.10% | 0.12% | 0.12% | 0.22% | 0.04% | 0.19% | 0.10% |
| *OM27 clade* | 0.07% | 0.20% | 0.06% | 0.18% | 2.61% | 1.44% | 2.15% | 0.69% |
| *Sphingobium* | 0.04% | 0.02% | 0.06% | 0.02% | 0.14% | 0.26% | 0.13% | 0.35% |
| *Gemmata* | 0.04% | 0.04% | 0.05% | 0.09% | 0.08% | 0.08% | 0.12% | 0.14% |
| *Devosia* | 0.03% | 0.07% | 0.04% | 0.05% | 0.10% | 0.23% | 0.12% | 0.24% |
| *Sandaracinobacter* | 0.03% | 0.03% | 0.05% | 0.04% | 0.04% | 0.14% | 0.06% | 0.17% |
| *Phaselicystis* | 0.03% | 0.04% | 0.01% | 0.03% | 0.25% | 0.40% | 0.13% | 0.23% |
| *Haliangium* | 0.01% | 0.02% | 0.00% | 0.02% | 0.40% | 0.13% | 0.41% | 0.13% |