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Chronic impact of tetracycline on the biodegradation of an organic substrate mixture under anaerobic conditions

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Abstract

The study evaluates the chronic impact of the antibiotic *tetracycline* on the biodegradation of organic substrate under anaerobic conditions. The experiments involved an anaerobic

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sequencing batch reactor fed with a synthetic substrate mixture including glucose, starch and volatile fatty acids, and operated in a sequence of different phases with gradually increasing tetracycline doses of 1.65 – 8.5 mg/L, for more than five months. *Tetracycline* exerted a terminal/lethal effect at 8.5 mg/L on the microbial community under anaerobic conditions, which caused the inhibition of substrate/COD utilization and biogas generation and leading to a total collapse of the reactor. The microbial activity could not be recovered and re-started within a period of more than 10 days, even after stopping t*etracycline* dosing. At lower doses, substrate utilization was not affected but a reduction of 10-20% was observed in the biogas/methane generation, suggesting that substrate utilization of *tetracycline* to the biomass was limiting their bioavailability. During the experiments, *tetracycline* was partially removed either through biodegradation or conversion into its by-products. The adverse long-term impact was quite variable for fermenting heterotrophic and methanogenic fractions of the microbial community based on changes inflicted on the composition of remaining/residual organic substrate.

Keywords: tetracycline; chronic inhibition; anaerobic biodegradation; methanogenesis; COD removal

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1. INTRODUCTION

Antibiotics, as one of the most important pharmaceutical group, have different usage areas such as human and veterinary medicine, growth promoters in livestock, and agriculture. Since these active compounds are not totally metabolized in human bodies and cannot be eliminated completely in sewage treatment systems (Ternes *et al.*, 2004) they are found in receiving water bodies. While antibiotic concentrations in raw domestic wastewater are usually reported in the range from 100 ng/L to 6 μg/L (Giger *et al.*, 2003; Santos *et al.*, 2009) their concentration in hospital and pharmaceutical industry effluents can reach 100 - 500 mg/L level (Kummerer, 2001, Larsson *et al.*, 2007), and an effective control and removal of these compounds would provide greatly beneficial stability in domestic sewage treatment. As antibiotics inhibit biological activities directly, they are likely to exert adverse/inhibitory effect on the biodegradation of organic compounds in the wastewaters and this way, they negatively affect the efficiency while by-passing conventional aerobic biological treatment processes (Joss *et al.* 2006). Anaerobic treatment is an alternative for the removal of these compounds in pharmaceutical industry waste streams because of high COD content and persistent character (Oktem *et al.*, 2008).

Tetracycline (TET) is one of the most extensively used antibiotics in human activities (Figure 1). It is generally used for the treatment of respiratory tract infections and has a reversible inhibitory effect. It is a broad-spectrum active compound, which inhibits bacterial protein synthesis by binding the 30S ribosomal subunit to prevent the association of the aminoacyl-tRNA to the ribosomal acceptor-A site (Chopra and Roberts, 2001). It causes structural change in 16S rRNA (Loftin *et al.*, 2005). The behavior of this compound on sewage treatment plants has been reported in the literature: It remains non-biodegradable, but it is easily sorbed onto sewage sludge and therefore it is mostly discharged to the environment through biosolids (Kummerer, 2001; Prado 2009). In another study, the authors found out that tetracycline presented good adsorbability with 72 mg/g of the Langmuir maximum

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adsorption capacity (Cs,max) (Prado *et al.*, 2009). The compound and its derivatives are commonly used as promoter in animal growth, and therefore most of the studies about TET degradation have focused on the anaerobic digestion of manure, which contains this compound (Arikan et al, 2006; Stone *et al.*, 2009; Wu *et al.*, 2011; Hu *et al.*, 2011). These studies showed that TET in manure could be biodegraded in a range from 70% (Wu *et al.*, 2011) to more than 90% (Hu *et al.*, 2011) under anaerobic conditions. On the contrary, Gartiser *et al.* (2007) determined TET as non-biodegradable under the anaerobic conditions in the water matrix. On the other hand, limited information was found about the effect of TET on anaerobic wastewater treatment systems: Arikan *et al.* (2008) reported a 30% inhibition in methane production with 9.8 mg/L of TET dosing while the same level of inhibition was observed with a much higher TET concentration of 28 mg/L in the study conducted by Stone *et al.* (2009).

In order to evaluate the inhibitory impact of a selected compound in a biological system two different experimental approaches are commonly applied: chronic and acute tests. The short-term, acute tests usually involve a microbial population not previously exposed to the inhibitor. Under anaerobic conditions, the methanogenic activity has been successfully interpreted to yield the magnitude of observed inhibition induced by the tested chemical (Ince *et al.*, 2009). Only a few studies have focused the removal and inhibition of antibiotics in anaerobic systems, and some of them use the enzyme analogy for the evaluation of the inhibitory action (Amin *et al.*, 2006; Fountalakis *et al.*, 2008). In a recent study, Cetecioglu *et al.* (2012) evaluated the acute inhibition impact of three antibiotics including tetracycline on the methanogenic activity of acclimated biomass fed with acetate. The significant effect was mainly on process stochiometry, preventing complete utilization of substrate removed in metabolic reactions; almost complete methane inhibition was observed for antibiotic doses above 500 mg/L. Although acute tests provide valuable information about inhibitory impact of a contaminant, they only give a partial image of inhibition, while long-term chronic experiments with continuous feeding of the inhibitor may indicate changes in the

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biodegradation pattern accounting for adaptation and/or resistance of the microbial community as argued by Kummerer (2004). Indeed, acute and chronic experiments complement on another in providing information on the full response of the microbial community in biological treatment systems under different conditions. The chronic experiments are the indispensable part of the evaluation as they reflect the continuous impact of lower antibiotic concentrations, similar to those encountered in full-scale treatment systems.

In this context, the main objective of this study was to evaluate the chronic impact of tetracycline on the biodegradation of a synthetic substrate under anaerobic conditions as well as to evaluate degradation and distribution of tetracycline itself. For this purpose, a sequencing batch reactor system operated with semi-continuous tetracycline feeding throughout the experiments enabled to interpret the chronic inhibitory impact of the selected antibiotic on process performance. Accordingly, both methane/biogas production together with the biodegradation characteristics of both, tetracycline and the synthetic substrate mixture (glucose, starch and volatile fatty acids) were used as the main evaluation parameters. The results obtained from the chronic study were compared with those from previous acute inhibition tests performed in similar experimental conditions (Cetecioglu, 2011 and Cetecioglu *et al.*, 2012).

2. MATERIALS AND METHODS

2.1. The experimental approach

The experiments were essentially designed for evaluating the chronic inhibitory impact of tetracycline on the metabolic activities of a microbial culture sustained in a reactor operated at steady state, under anaerobic conditions. An anaerobic sequencing batch reactor (ASBR) was run in a daily “fill and draw” mode using a synthetic substrate mixture including volatile fatty acids, glucose and starch. The operation of ASBR included a start-up period of around

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150 days for acclimation and establishment of steady state conditions. Then, its performance was observed during the next 154 days under steady state conditions, to make sure that these conditions prevailed before semi-continuous exposure to TET dosing, *i.e.* chronic impact of TET could be observed on an acclimated microbial community with a well-defined culture history. A sequence of five different phases were included in the experimental observation: During the first phase, *phase A*, (till day 77) ASBR was operated with feeding of just the selected synthetic substrate without TET addition whereas during the following three phases it was operated with semi-continuous feeding of the substrate/TET mixture: In *phase B* (days 78-90), the daily TET dose was maintained at 1.65 mg/L; the antibiotic dose was gradually increased to 5.7 mg/L in *phase C* (days 91-114) and to 8.5 mg/L in *phase D* (days 115-143). The TET dosing was stopped in the last phase (*phase E*) in order to observe a possible recovery of the reactor performance during the next 10 days (days 144-154). A second ASBR, which was operated in parallel for the entire period under identical conditions, but without antibiotic dosing, served as control reactor. The sequence of different phases was primarily designed to observe the tolerance and possible failure of the microbial community under semi-continuous exposure to TET; this was the reason why TET concentration was gradually increased once the expected microbial response was observed. In fact, at the highest tested TET dosing, the observed response was the metabolic collapse of the microbial culture, which did not recover after TET dosing was stopped. This approach enabled to observe different responses of the system at selected/gradually increased TET doses, which constituted the basis of the evaluation.

The evaluation of ASBR performance was mainly based on daily measurements of soluble COD and volatile fatty acid (VFA) concentrations determined both in the influent and effluent streams; they were accompanied with parallel daily measurements of biogas production and composition assessing main fractions such as CH_4, CO_2 and H_2. Specific methanogenic activity tests (SMA) were also conducted on biomass sustained under different TET feeding

regimes, for assessing the methanogenic activity of the acclimated microbial community under inhibitory conditions.

2.2. Operation of Anaerobic Sequencing Batch Reactor systems

Two Anaerobic Sequencing Batch Reactors (ASBRs) with 1 L total volume were set-up and operated at 35 ºC under dark conditions to prevent photo degradation. The reactors were operated with a 24-hour cycle consisting of fill (10 min), react (23 h), settle (45 min) and decant (5 min). The reactors were mixed continuously using a magnetic stirrer at 90 rpm. The systems were inoculated by an anaerobic sludge taken from the stock reactor treating a synthetic substrate with a total COD of 4400 mg/L including the following ingredients: starch, 2090 mg COD/L; glucose, 1350 mg COD/L; sodium acetate, 240 mg COD/L; sodium butyrate, 330 mg COD/L; sodium propionate, 490 mg COD/L. The MLVSS concentration of the reactors was 4500 mg/L. The total COD of the synthetic substrate used for the reactors was adjusted to 2250 mg/L; it was a similar mixture, mainly composed of starch and glucose: starch, 1045 mg COD/L; glucose, 675 mg COD/L; which accounted for more than 76% of the COD feeding; it also contained 120 mg/L of acetate, 165 mg/L of butyrate and 245 mg COD/L of propionate, corresponding to the remaining 24% of daily COD loading. Trace element solution which is adapted from a previous study (Amin *et al.*, 2006) as mg/L ($FeCl_2.4H_2O$, 2; $CoCl_2.6H_2O$, 2; $MnCl_2$, 0.32; $CuCl_2$, 0.024; $ZnCl_2$, 0.05; H_3BO_3, 0.05; $(NH_4)Mo_7O_{24}.4H_2O$, 0.09; Na_2SeO_3, 0.068; $NiCl_2.6H_2O$, 0.05; EDTA, 1; resazurine, 0.5; HCl (36%) 0.001 mL), vitamins as mg/L (4-aminobenzoic acid, 0.04; D(+)-biotin, 0.01; nicotinic acid, 0.1; calcium D(+)-pantothenate 0.05; pyroxidine dihydrochloride, 0.15; thiamine, 0.1 in NaP buffer (10 mM, pH 7.1) and 0.05 mg/L B12) solution were added to the wastewater. The pH of the reactors at the start of each cycle was observed to vary from 6.8 to 7.2 mainly due to the alkalinity level of around 1000 mg/L $CaCO_3$ for sustaining the operation stability of the anaerobic reactor.

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The reactors were operated with an organic loading rate (OLR) 1.4 g/L.d for the first 10 days of operation and then increased to 2.25 g/L.d in a stepwise manner. The solid retention time was approximately 50 days throughout the study for both ASBRs and was calculated based on VSS loss in the effluent and removed during sampling of the excess sludge. The hydraulic retention time of the reactors was 2.8 days.

Temperature, pH and gas production were monitored daily in situ. Duplicate samples were collected from the reactors for chemical and microbiological analysis.

2.3. Specific Methanogenic Activity Test

Methanogenic activity tests were performed using the pressure transducer technique (Colleran *et al.*, 1992) to determine the chronic effect of TET on methanogenic pathway. The pressure increase in sealed vials fed with non-gaseous substrates as acetate, propionate, butyrate was monitored. The hand-held pressure transducer (Lutron PM-9107, U.S.A.) was capable of measuring a pressure in a range of 5 to 7000 mbar, corresponding to 0.01 mol biogas in 60 mL headspace. The biomass seed was adjusted to 2000 mg/L VSS, so that each serum bottle was inoculated with 120 mg VSS at the start of operation in 60 mL active volume. The sludge taken from TET fed ASBR at the end of each period to use an inoculum in test bottles. The aim of this test was to compare the chronic effect of TET on the methanogenic activity. Acetate and VFA mixture (acetate, butyrate, propionate) concentrations in a range of 1000-5000 mg/L were initially tested in order to reach maximum potential methane production (PMP) rate during the batch tests. Among those 4000 mg/L of acetate concentration and 3000 mg/L of VFA concentration were found to be optimum. The basal medium in the batch experiments was prepared based on OECD311 protocol under strict anaerobic conditions (2006). During the 6-day test duration, the bottles were stored at 35±2 ℃ and shaken daily by hand. Headspace pressur e was measured every day by hand-held pressure transducer.

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2.4. Analytical Methods

Methane content in the biogas and VFA concentrations were measured using gas chromatograph (Perichrom, France and Agilent Technologies 6890N, USA, respectively). Suspended solids (SS), volatile suspended solids (VSS), total suspended solids (TS), total volatile suspended solids (TVS) and soluble COD were determined according to Standard Methods (APHA, 2005).

2.5. Measurement of Tetracycline in water and sludge samples

A mass balance could also be established for tetracycline through measurements in the influent, effluent and biomass samples. For the sludge samples, 20 mg of freeze-dried sludge was weighted in 15 mL centrifuge tube and 10 mL of the extraction buffer (5% (w/v) sodium acetate, 100 mM EDTA in a methanol:water (1:1) solution adjusted to pH 8 with sodium hydroxide) was added to each tube. The tube was sonicated for 15 min and then centrifuged at 1370Xg at 25ºC during 10 min. The supernatant was transferred to 60 mL glass tube. The extraction protocol was performed 3 times for each sample and the obtained supernatant was evaporated at 25ºC under nitrogen stream to remove the organic solvent and diluted with MilliQ water to 500 mL and filtered. Further sample clean-up was performed by solid phase extraction (SPE) using OASIS HLB cartridges (6mL, 200 mg, Waters, USA). Each cartridge was conditioned with 5 mL methanol followed by 5 mL HPLC grade water. Sample was loaded into the cartridge at a rate of approximately 1 mL/min. The cartridge was washed by 10 mL HPLC grade water and then dried by vacuum during 30 min. Sample was then eluted by 6 mL of methanol. The extract was evaporated to less than 50 µL under nitrogen streams and then reconstituted to 1 mL with 1:1 methanol:water mixture. Before analysis, 10 ppb of chlorotetracycline as internal standard was added. The concentration of TET in the samples was quantified by internal standard calibration curve, in order to correct for possible matrix effects.

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Wastewater samples were filtered and diluted by 1:50 according to their expected concentration in the reactor, using the methanol:water mixture. Analysis of both, wastewater and sludge extracts was performed by ultra-high-performance liquid chromatography coupled to quadrupole-linear ion trap tandem mass spectrometry following the method developed by Gros *et al.* (2012). The Waters Acquity Ultra-PerformanceTM liquid chromatograph system was equipped with two binary pumps (Milford, MA, USA) and an Acquity BEH T3 colum (50mm x 2.1mm i.d., 1.7 µm particle size) was used for chromatographic separation. Compounds were analyzed under positive ionization mode. The optimized separation conditions were as follows: solvent (A) acetonitrile, solvent (B) water with 0.1% formic acid at a flow rate of 0.5mL/min. The gradient elution was: initial conditions 5% A; 0–3 min, 70%A; 3.0–3.5 min, 100% A; 3.5–5.0 min, 100% A; from 5.0 to 5.1 return to initial conditions; 5.1 to 6, equilibration of the column. A sample volume of 5µL was injected in the UPLC instrument, coupled to a 5500 QTRAP hybrid triple quadrupole-linear ion trap mass spectrometer (Applied Biosystems, Foster City, CA, USA) with a turbo Ion Spray source. Tetracycline and the corresponding internal standard were analyzed by positive ionization mode in Multiple reaction monitoring (MRM) as indicated by Gros *et al.* (2012). Limit of detection (LOD) and limit of quantification (LOQ) of the measurement were 0.58 and 1.94 ng/mL, respectively. The recovery of the sludge sample was 117.2±29.0%.

2.6. Statistical Analysis

To determine the statistical significance of TET inhibition, COD removal efficiencies of the ASBRs were compared using ONE WAY ANOVA test, which was followed by running a Post-hoc Dunnett's test and student's T-test, respectively. Graphpad Prism 4 software was used for all statistical analysis.

3. RESULTS

3.1. COD removal

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Efficient COD removal was observed during *phase A* in the TET reactor: Soluble COD in the effluent was reduced from an initial COD concentration of 2200 mg/L at the beginning of each cycle to 73 ± 19 mg/L, corresponding to an efficiency higher than 96% (see Figure 2). Similar COD removal could be maintained in the control reactor for the entire monitoring period. It should be noted that the synthetic substrate is composed of organics compounds that are all totally biodegradable in nature; based on similar studies conducted with these compounds as single substrates or substrate mixtures, it would be acceptable to assume that under the operation conditions selected for the reactors they would be totally removed so that the low soluble COD level measured in the effluent is essentially residual soluble microbial products generated in the course of biochemical reactions (Germirli Babuna *et al.*, 1998, Amin *et al.*, 2006).

Semi-continuous TET dosing of 1.65 mg/L in *phase B* and 5.7 mg/L in *phase C* did not seem to exert a noticeable effect on the overall COD removal: As illustrated in Figure 2, the effluent soluble COD basically maintained the same level as before, with an average level of 71 ± 28 mg/L and only slightly decreased to 57 ± 3 mg/L. However, TET dosing increased to 8.5 mg/L in the following operation phase (*phase D*) resulted in a significant upset in the reactor performance: The soluble COD value in the effluent increased to more than 2000 mg/L corresponding to overall COD reduction of only 9% after 134th day (Figure 2). At the end of *phase D*, TET dosing was stopped in order to observe any possible recovery in the reactor performance in the final *phase e*. However, the metabolic activity of the biomass could not be re-activated to induce noticeable substrate utilization and the reactor operated was terminated on day 154.

3.2. Biogas generation

Biogas generation is the inherent complement of COD removal under anaerobic conditions; it is now regarded as the scientific yardstick for evaluating the magnitude of related metabolic activities. During the initial ASBR operation without TET dosing in *phase a*, complete COD

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removal was also accompanied with a biogas generation of 1046 ± 28 mL/day, corresponding to an average biogas yield, Y_{BG} of 0.46 L/g COD removed. The generated level remained quite stable throughout the phase and almost coincided with the level monitored in the control reactor (Figure 3). The methane percentage in the biogas was determined as 62.5 ± 3%, indicating an average specific methane production yield, Y_{CH4} of 0.32 L/g COD removed. This level is in conformity with the default value reported by Tchobanoglous *et al.* (2003). Analysis of the biogas composition revealed that the other main component of the biogas was CO_2 (37.5 ± 3.8%), with no detectable H_2 formation. Cetecioglu (2011) mentioned that methane percentage was 58.0± 1.7% in the short-term operation up to 250 mg/L TET fed anaerobic system. The slight difference in the level of methane generation is obviously due to different carbon sources utilized in the two studies, without noticeable impact of TET inhibition.

In *phase B* involving semi-continuous TET dosing of 1.65 mg/L, biogas generation persisted at a slightly lower level of 951 ± 12 mL/day, i.e. with a 10% decrease. A similar decrease down to around 60% was also observed in the methane content, corresponding to a methane production yield of 0.25 L/g COD removed. A further decrease in the biogas generation started in day 91, the beginning of *phase C*, with the application of a higher TET dose of 5.7 mg/L: The daily biogas level was reduced to 864 ± 21 mL/day, 82% of the level in the control reactor, while the methane content of the biogas remained approximately the same (58%). It should be noted that *phases B* and *C* were characterized with complete COD removal as in the early phase of the ASBR operation without the antibiotic addition (*phase A*). The observed decrease in the biogas/methane generation despite full COD removal confirms results obtained in the acute test with TET under anaerobic conditions, similarly preventing complete utilization of substrate removed in the corresponding metabolic reactions (Cetecioglu *et al.*, 2012). In the following *phase D* characterized by a higher semi-continuous TET dose of 8.5 mg/L, the significant adverse effect of the reactor performance was also observed for biogas generation, which dropped from 853 mL/day to 71mL/day between days

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115 and 143, tandem with a similar decrease in substrate removal. While the methane generation exhibited a parallel decrease, the methane remained in the range of 0.2 L CH_4 / g COD removed (Figure 3b).

3.3. Effluent VFA composition

Monitoring of the presence and composition of the volatile fatty acids in the process effluent provided additional information on the chronic impact of *tetracycline* on the metabolic activities under anaerobic conditions. The analysis in the effluent covered, aside the three VFAs in the influent, isobutyrate, isovalerate and n- valerate. VFAs were not detected in the effluent until *phase D* i.e during the first 116 days of operation where the semi-continuous TET dosing was started and gradually increased to 5.7 mg/L, confirming complete removal of the available substrate. It also confirms the inhibitory impact of semi-continuous TET dosing in the selected range of 1.65 – 5.7 mg/L, which partially blocked the utilization of the substrate removed in the metabolic activities, as evidenced by the observed reduction in biogas/methane generation. However TET dosing, when increased to 8.5 mg/L in *phase D*, seriously impaired and inhibited propionic and acetic acid utilization pathways as shown in Figure 4: The observations indicated that after the first day in *phase D* (day 116), acetate and propionate accumulation in the system began and levels were measured as 27 and 28 mg/L, respectively.

Acetic acid concentration in the effluent increased to 110 mg/L at the end of *Phase D* and reached to 457 mg/L at the end of the operation, *Phase E*.

Propionic acid accumulation has also similar trend however, the concentration was higher than acetic acid. Its concentration was measured as 750 mg/L at the end of *Phase D*. However propionic acid concentration decreased in the *Phase D* and it was detected as 385 mg/L at the end of operation.

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Also butyric and valeric acids were observed in the effluent of the TET reactor at *Phases D* and *E*. While butyric acid concentration varied between 4 and 20 mg/L, valeric acid concentration increased from 14 mg/L to 70 mg/L slowly until the end of operation.

3.4. Fate of tetracycline during ASBR operation

TET was measured in the effluent and in the biomass in order to ascertain its fate and possible biodegradation in the anaerobic reactor in each phase of treatment. Measurement indicated that TET concentrations in the effluent always remained significantly lower than the corresponding influent doses as seen in Figure 5a: In phase b the effluent TET concentration was 0.55, around one third of the influent level. In the following phase (*phase C*) the TET value in the effluent was slightly lowered to 0.44 mg/L, while the influent dosing was increased to 5.7 mg/L. When the influent TET concentration was increased to final level of 5.5 mg/L, the corresponding effluent level initially remained the same (0.47 mg/L), then it was reduced down to 0.06 mg/L (*phase D_2*), to finally reach a higher value of 1.36 mg/L in *phase D_3* as illustrated in Figure 5a.

One of the possible explanations for the observed discrepancy between TET influent and effluent TET levels in the anaerobic reactor is physical removal by means of sorption onto biomass. In fact, a number of similar studies on activated sludge systems reported sorption as the dominant mechanism for the removal of antibiotics (Kim *et al.*, 2005; Prado, 2009). As shown in Figure 5a, TET sorption onto biomass did not exhibit a ascending trend, i.e. a continuous increase in the TET fraction in the sludge: This level was initially 0.17 mg/L in *phase B*; it dropped down to an almost negligible level of 0.05 mg/L in the following phase and then it increased to 1.78 mg/L with a gradual descent to 1.25 mg/L by the end of *phase D*, when the influent TET dose was adjusted to 8.5 mg/L. In the following phase (*phase E*),

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where the TET dosing was stopped, TET fraction in the biomass was desorbed and the concentration was measured as 0.32 mg/L.

The results outlined above and displayed in Figure 5a cannot be directly used for mass balance, which would indicate the extent of TET biodegradation, mainly because the TET fraction sorbed onto sludge would accumulate the same way as biomass, leaving the reactor only as part of the excess sludge. Therefore, the observed TET concentration in the sludge, TET_S, should be corrected by a factor of (HRT/SRT) in order to obtain the effective TET concentration in the biomass, TET_{SE}, and the corrected value incorporated in the mass balance (Hocaoglu and Orhon, 2010):

$$TET_{SE} = TET_S \, (HRT/SRT)$$

This expression allows calculating of the extent of TET degradation efficiency (TET deg_eff), corrected for entrapment and accumulation in the biomass:

$$TET\ deg\_eff = (TET_I - TET_E - TET_{SE}) / TET_I \times 100$$

where, TET_I = influent TET dose; TET_E = measured TET concentration in the effluent.

Efficiency of TET reduction in different phases of reactor operation, using the expression defined above is illustrated in Figure 5b. It basically indicates a TET reduction pattern that started with more than 50%, increased to more than 90% in phase c, and sustained around 40% at the end of *phase D*, where the metabolic activities and the COD removal efficiency were practically stopped. The reduction profile may be attributed to total biodegradation of TET under anaerobic conditions, a novel result not previously reported, or to its partial biodegradation and conversion to its major by-products

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3.5. Assessment of specific methanogenic activity

Assessment of the specific methanogenic activity of the biomass (SMA) in batch reactors has been a useful experimental approach for the appraisal of adverse/inhibitory effects (Ince *et al.*, 2009); this approach was previously adopted evaluate the acute impact of *tetracycline* along with two other antibiotics on the biodegradation of acetate and VFA mixture under anaerobic conditions (Cetecioglu, 2011 and Cetecioglu *et al.,* 2012). The SMA test is designed differently for acute and chronic impacts: While the acute SMA test involves a series of parallel batch reactors inoculated with the same (control) biomass and the selected substrate but with increasing doses of the antibiotic, the chronic SMA test is run with biomass seeding from different phases of the reactor operation under semi-continuous impact of the antibiotic and fed with the same substrate dose.

In this study, the SMA test was similarly performed with biomass seeding taken from the end of different operation phases of the TET reactor, namely from phases A, B, C, D and E; it should be noted that semi-continuous TET dosing was adjusted to 1.65, 5.7 and 8.5 mg/L in the first three phases and stopped in the last phase. The SMA test was run twice, the first one with acetate and the second/parallel one with a VFA mixture – i.e. an acetate-butyrate-propionate mixture.

In the first SMA test, all batch reactors, each with an effective volume of 60 mL, were started with 4000mg/L of acetate as the sole carbon source so that the reactors all included the same initial acetate dose of 4250 mg COD/L or around 255 mg acetate COD. The test was run for 8 days (192 hours). The observed cumulative biogas production (CBP) and cumulative methane production (CMP) profiles in the SMA test are given in Figure 6, showing that each specific profile reached a different plateau after around 168 hours depending on the operation phase which yielded the biomass seed. The CMP value of the biomass representing the initial phase without TET dosing was determined as 77 mL, corresponding to 0.30L/g COD, a value quite in agreement with the level associated with semi-continuous operation. As shown in Figure 6, the CMP test detected a loss of activity in

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the biomass taken from phase b (1.65 mg/L TET dosing), as the collected methane volume was reduced down to 55 mL, corresponding to around 30% decrease. The CMP levels were gradually reduced to 47mL (39% decrease) and finally to 23 mL (71% decrease) in reactors seeded with biomass from phases *C* and *D*. However, a significant recovery of the methanogenic activity was observed in connection with the last phase (*phase E*) where the TET dosing was stopped, evidence with an increase in the corresponding CMP value from 23 mL to 42mL, i.e. 016L methane/g COD_removed. The methane content of the biogas was also decreased gradually depending on TET concentration. While the methane percent was 65%, 60% and 58% at *Phases B*, *C* and *D*, respectively, the value increased to 63% again at *Phase E*, in which TET addition was terminated.

In the second set of SMA tests, all batch reactors, each with an effective volume of 60 mL, were fed acetate, butyrate and propionate mixture; as 3000 mg/L of each VFA. The initial VFA dose for each reactor corresponds to 13080 mg COD/L. The CBP and CMP profiles during 8 days are given in Figure 7 and each profile reached the specific plateau at around 7^{th} day like acetate fed SMA test reactors. The CMP value of *Phase A* without TET dosing was observed as 312 mL and this value is equivalent to 0.39L/g COD, which is quite higher. As seen in Figure 7, CMP value of *Phase B* (1.65 mg/L TET dosing) decreased dramatically to 93 mL, corresponding to around 0.12L/g COD. A 70% reduction in CMP value was observed. The CMP values gradually decreased to 68 mL and 14 mL in *Phase C* and *D*, respectively. A 75% recovery was also observed at the last phase (*Phase E*) in which TET dosing was stopped and the CMP values reached to 52 mL. Differently from acetate fed SMA test bottles, the methane content of VFA fed set was quite stable as 50%.

The results explained above compares two sets of batch SMA tests, one conducted with acetate and the other with selected VFA mixture and show that the biogas methane generation in the latter test conducted with the VFA mixture always remained clearly below

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the corresponding levels obtained with acetate tests. This observation may be interpreted as lack of available acetate for acetoclastic methanogens and therefore, failure of heterotrophic fermenters to convert propionate and butyrate into acetate due to adverse impact of TET dosing. This observation supports the findings related to the semi-continuously fed ASBR that the chronic damage of TET dosing was more effective and finally lethal on heterotrophic fermenters as compared with methanogens.

4. DISCUSSION

4.1. Difference from acute impact

The chronic impact of *tetracycline* on substrate biodegradation under anaerobic conditions was severe and occurred in the range of 5.7 – 8.5 mg/L dosing level, a much lower level as compared with the acute impact: In parallel tests, an initial TET dose of 50 mg/L was observed as the threshold of a noticeable acute impact on anaerobic biodegradation; the inhibitory effect of TET addition became detrimental when the initial dose was increased to 500 mg/L (Cetecioglu *et al.*, 2012). These results indicate that while short-term assays may be useful in assessing the effect of inhibitors received as pulse discharges, they will not be sufficient to reflect the real inhibition mechanism on microbial communities in the long range (Kummerer, 2004).

4.2. Terminal/lethal effect

Tetracycline exerted a terminal/lethal effect at 8.5 mg/L on the microbial community under anaerobic conditions, stopping substrate/COD utilization and biogas generation and leading to total collapse of the reactor. The microbial activity could not be recovered and re-started within a period of more than 10 days; even after the *tetracycline* dosing was stopped. From a practical perspective, this level is obviously too high for domestic sewage, but quite relevant for pharmaceutical plants, hospitals, *etc.*, where anaerobic treatment becomes appropriate, due to high organic content of the effluents (Amin *et al.*, 2006, Larsson *et al.*, 2007).

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4.3. Nature of impact - different with variable doses

The effect of *tetracycline* at the lower dose of 5.7 mg/L was quite different: In this phase of the study (*phase B*) substrate utilization was not impaired and full COD removal was achieved but around 20% reduction was observed in the biogas/methane generation. The same mechanism with a lower decrease of biogas generation in the range of 10% was also measured when the *tetracycline* dose was 1.65 mg/L in the previous phase. These results confirm similar findings reported in the literature, where the *stoichiometric disturbance – i.e.* substrate removed but partially utilized for methane generation - was attributed to the blockage of certain enzymatic steps in related metabolic reactions (Fountoulakis *et al.*, 2008; Cetecioglu *et al.*, 2012). In a simplified way, it may be interpreted as the substrate binding effect of TET, in accordance with *uncompetitive inhibition* analogy.

4.4. Accumulation/biodegradation

Semi-continuous dosing resulted in the accumulation of *tetracycline* in the biomass with a gradual increase to around 1.5 mg/L throughout the observation period as indicated in previous studies conducted with erythromycin (Amin *et al.*, 2006) and tylosin (Shimada *et al.*, 2008). The observed *tetracycline* profile in the biomass suggested equilibrium established on the basis of simultaneous adsorption/desorption mechanisms. The interesting/novel aspect of the evaluation was that the major fraction (>80%) of the *tetracycline* introduced into the anaerobic reactor could be fully or partially biodegraded along with the organic substrate. Figure 5 shows an appreciable overall removal of TET even in *phase D*, where the COD removal efficiency was significantly dropped. Since TET is a xenobiotic much more difficult to degrade compared to the substrate mixture fed to the reactor, it is more likely that the main mechanism for the observed TET removal in this study is formation of metabolites rather than biodegradation. This issue deserves more emphasis in future studies on the subject.

4.5. Microbial dynamics

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The experimental results were quite interesting from the viewpoint of substrate/biomass interactions and microbial population dynamics. In other words, the experiments were designed in such a way to yield the impact of *tetracycline* not only on the overall anaerobic biodegradation efficiency, but also on changes inflicted on the nature of remaining/residual organic substrate and on the activity of different significant components of the microbial community. It should be remembered that the organic substrate mixture was mainly composed of starch and glucose (76%), but it also included 120 mg COD/L of acetate, 245 mg COD/L of propionate and 165 mg COD/L of butyrate corresponding to a total VFA level of 530 mg/L, i.e. only 23% of the total COD level in the influent. Latest developments in the modeling of anaerobic systems such as ADM, identify exactly similar changes and conversions betweens substrate components and correlate them with groups of microorganisms capable of performing these metabolic activities, without having to go into a detailed molecular analysis (Batstone *et al.*, 2002). Adoption of a similar basis of evaluation for the substrate mixture selected for the study would suggest that the mixed microbial culture sustained at steady-state in the reactor would inherently include regular *anaerobic heterotrophs* to hydrolyse starch into simple sugars; *fermenting heterotrophic microorganisms* converting glucose/simple sugars mainly to acetate and propionate; *propionate degraders,* utilizing propionate for the generation of acetate, and finally methanogens processing mainly acetate for the production of biogas. Interpretation of the results obtained enabled to visualize the impact of *tetracycline* on these metabolic activities and therefore, *on* related components of the microbial community throughout the observation period: (i) The initial impact of *tetracycline* when increased to 8.5 mg/L (*phase D*) was more focused on *methanogens*, evidenced by the gradual increase of VFAs in the effluent; at the end of *phase D*, the total VFA level was increased to around 820 mg COD/L, significantly higher than the corresponding influent level. (ii) The inhibitory impact was specifically pronounced for propionate degraders. (iii) A recovery of methanogenic functions was observed after the *tetracycline* dosing was stopped, with gradual depletion of accumulated VFAs, suggesting adaptation/resistence mechanisms in the corresponding fraction of the

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biomass; this result was also in conformity with a similar recovery in the specific methanogenic activity. (iv) The adverse effect of *tetracycline* dosing on regular/fermenting heterotroph gradually increased and finally inflicted lethal and non-reversible damage, stopping VFA generation and the overall COD removal. This interpretation was also fully supported by a comprehensive microbial community analysis based on DNA and RNA based molecular microbial techniques; the results of the molecular analysis will be reported in detail as the following part of the study.

5. Conclusions

In the light of evaluations presented in the previous sections, the significant findings of the study on the chronic impact of tetracycline may be outlined as follows:

The results suggested that the nature of the *adverse impact was quite variable* as a function of the inhibitor dose: At low levels, available substrate was removed but only partially utilized for biogas/methane generation, presumably due to the blockage of certain enzymatic steps in related metabolic reactions; at higher doses, it induced total collapse of the microbial activity and metabolic functions. For the selected conditions of the study the terminal dose for tetracycline inhibition was 8.5 mg/L.

The effect on microbial dynamics was *selective,* exerting markedly different inhibitory impact on various steps of substrate utilization and metabolic reactions associated with the activities of the microbial community sustained in the reactor. *A cumulative impact* was observed for the sequence of biochemical processes converting different substrate fractions into acetate,, possibly affected by adsorption and progressive accumulation of *tetracycline* on the biomass. However the adverse effect was quite different and *reversible* for the methane generation process,, upsetting the utilization of available/generated VFAs at first and with subsequent partial recovery of methanogenic activity when the inhibitor addition was stopped. This

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aspect was further investigated by means of parallel molecular studies reflecting changes on the composition and nature of the microbial community. Related results are quite comprehensive and will not reported at this stage.

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Highlights

- Chronic impact of tetracycline was lethal at 8.5 mg/L on the microbial community
- At lower doses, substrate removal was not impaired but biogas volume was reduced.
- Tetracycline was partially biodegraded.
- Impact was cumulative on fermenting heterotrophs due to TET adsorption/ accumulation.
- Impact was reversible for methanogens with partial recovery of biogas generation.

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Figure Captions

Figure 1. Chemical structure of Tetracycline

Figure 2. COD removal efficiency in the tetracycline reactor

Figure 3. (a) Effect of tetracycline on biogas generation, (b) Stoichiometry of CO_2 and CH_4 generation

Figure 4. Fate of volatile fatty acids under the inhibitory impact of tetracycline

Figure 5. (a) Tetracycline concentration in liquid/solid phases; (b) Biodegradation profile of tetracycline

Figure 6. Specific methanogenic activity induced by acetate feeding at different phases of reactor operation (a) biogas production; (b) methane production

Figure 7. Specific methanogenic activity induced by VFA feeding at different phases of reactor operation (a) biogas production; (b) methane production

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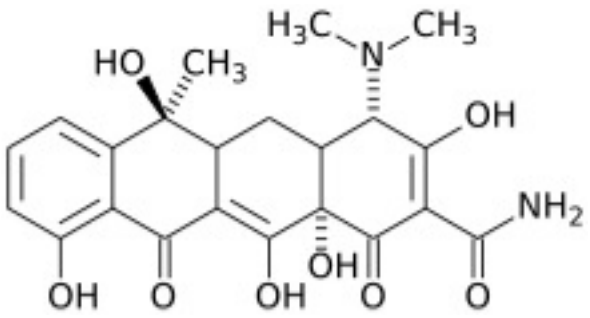

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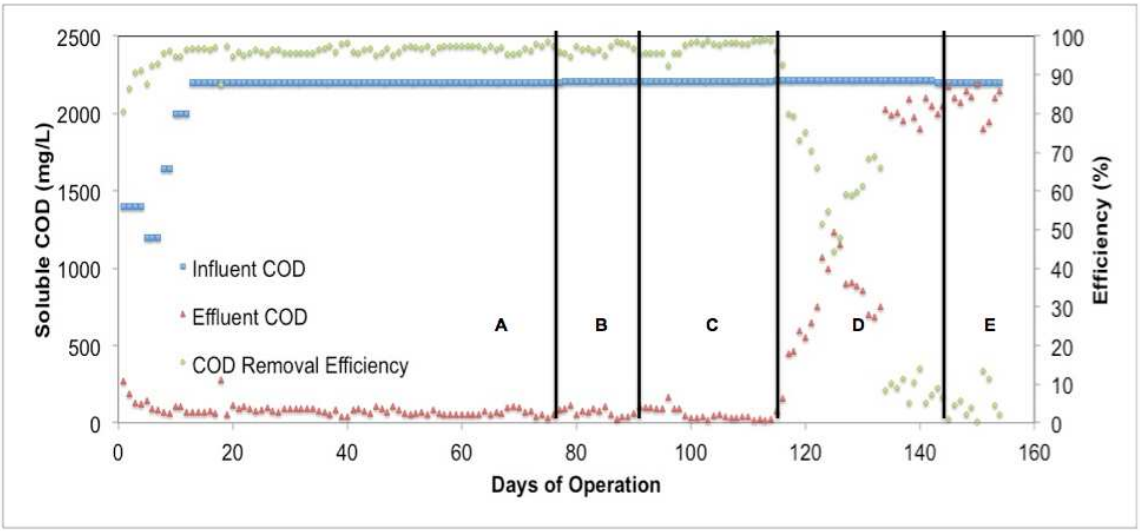

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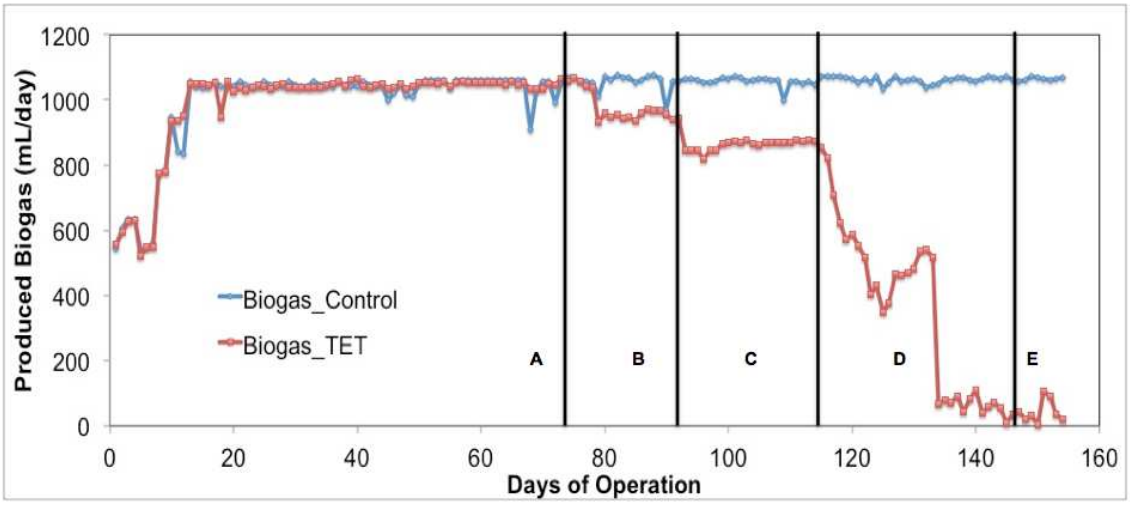

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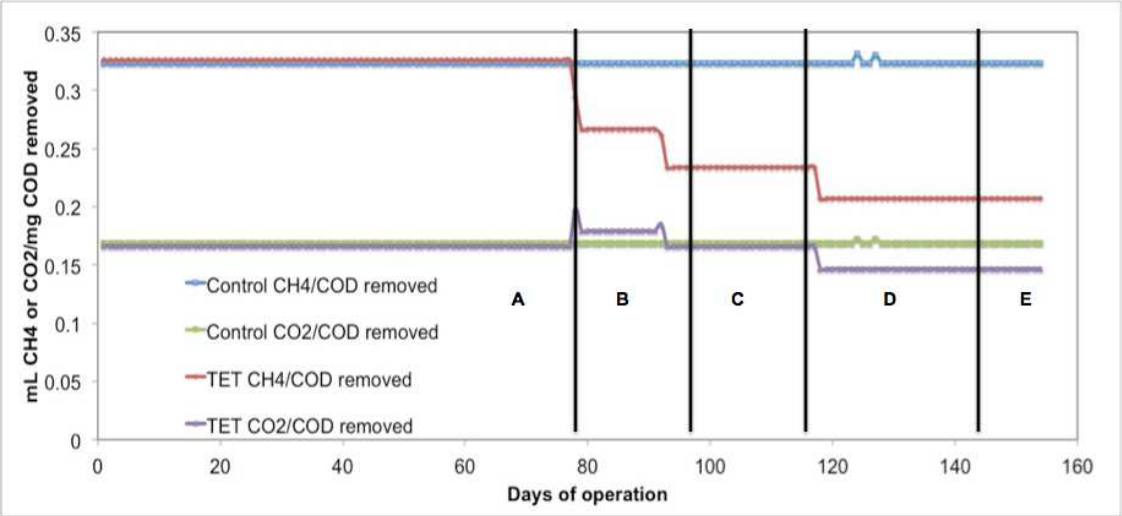

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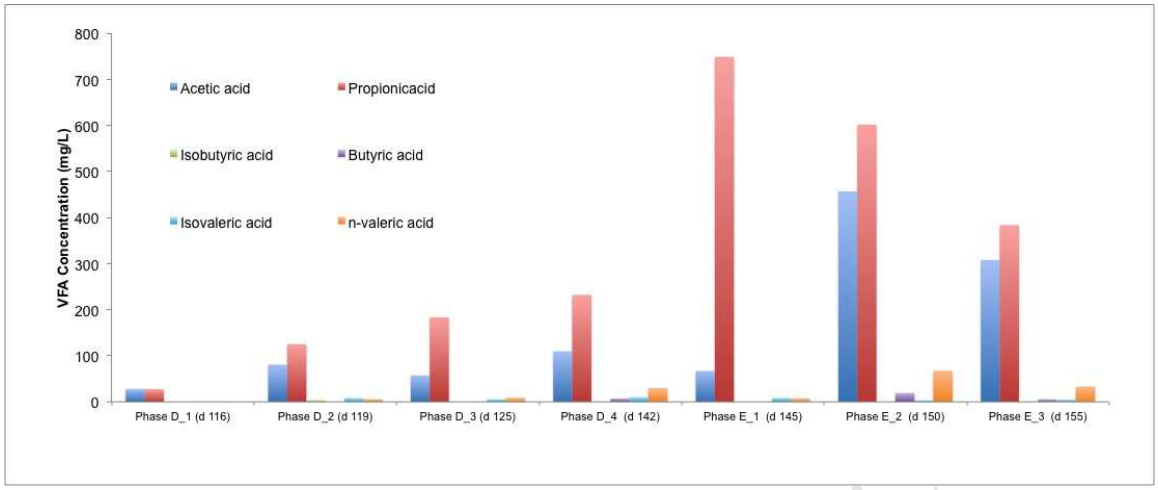

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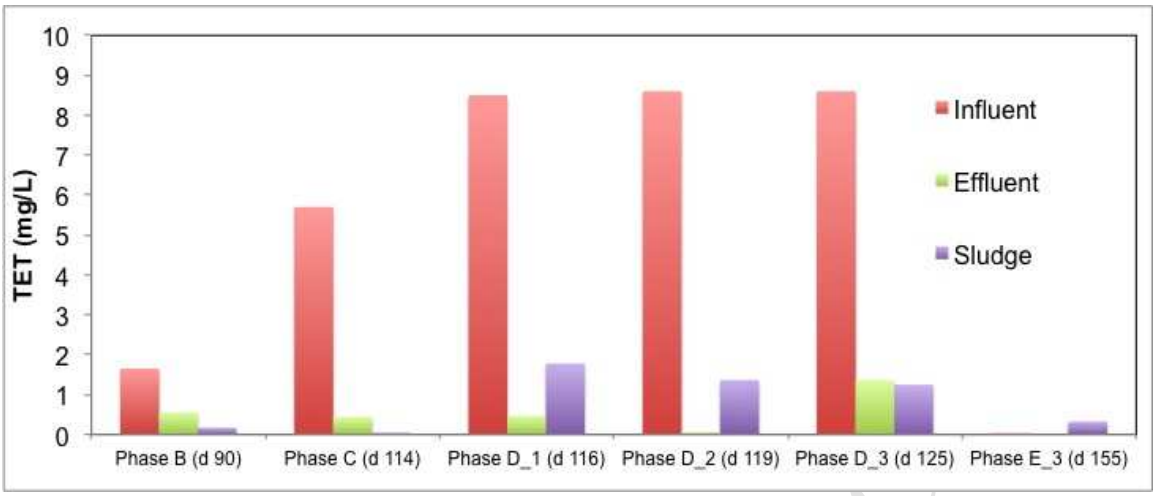

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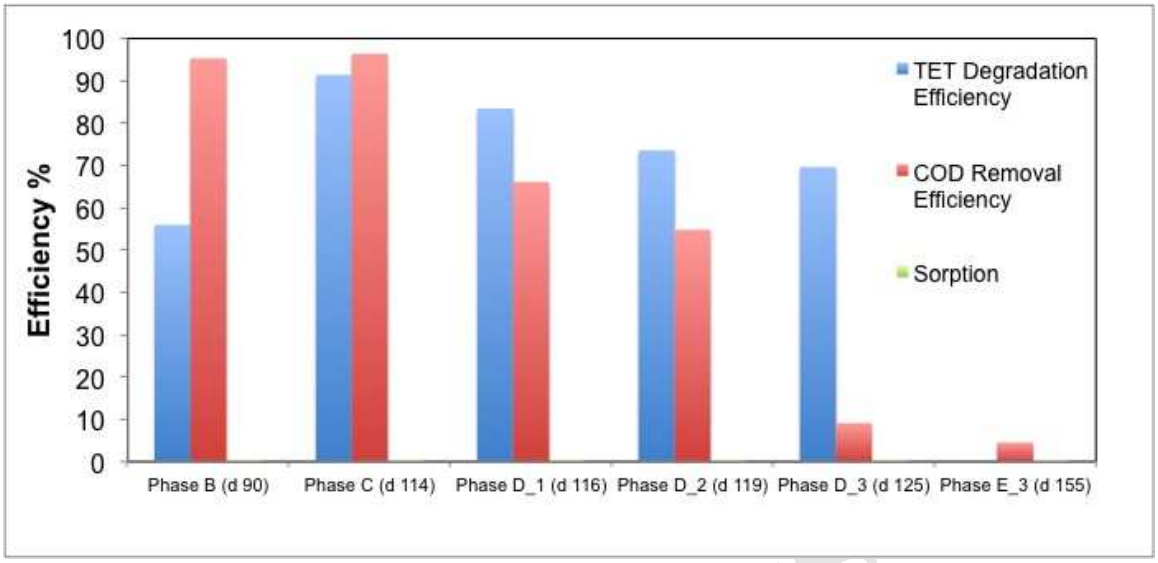

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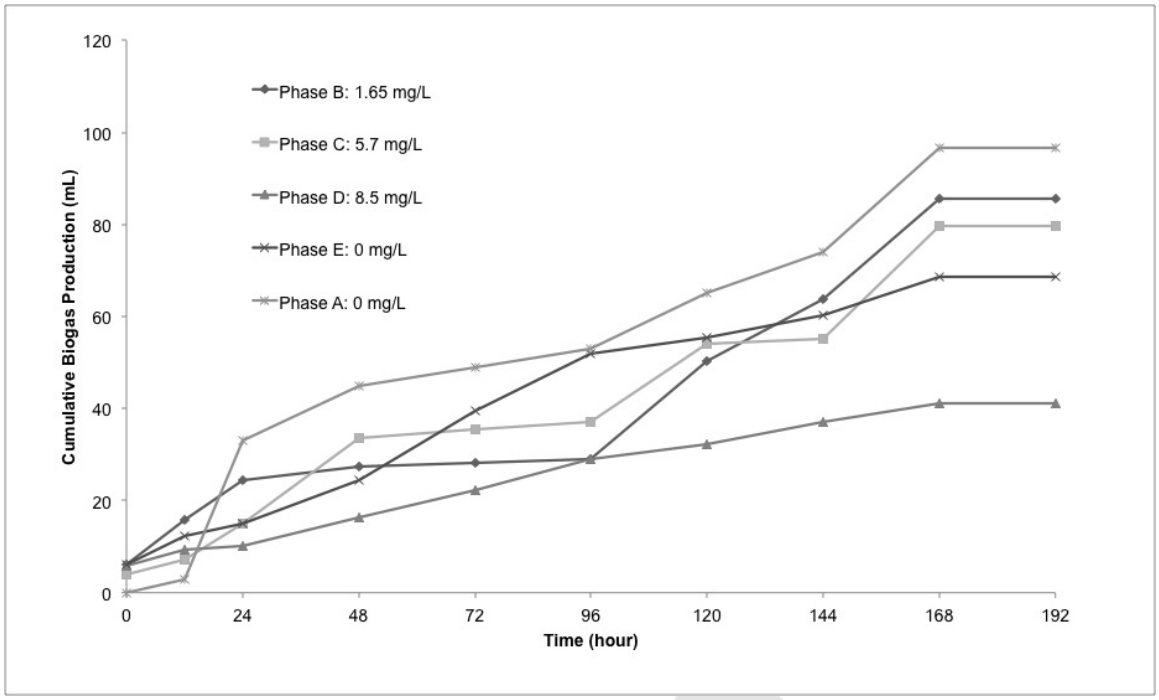

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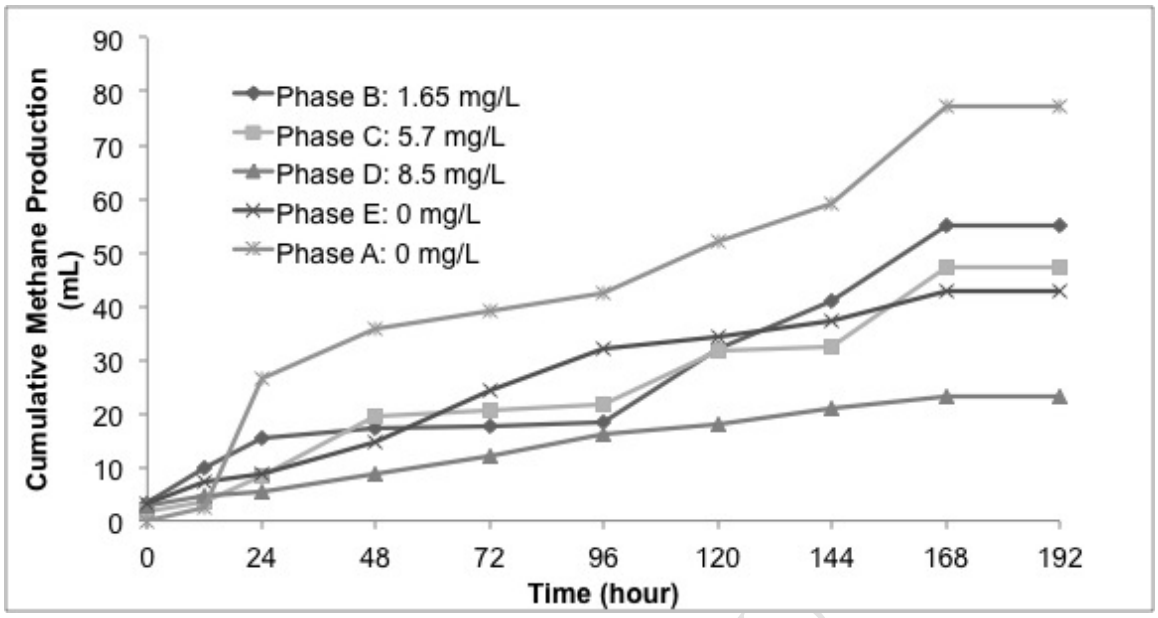

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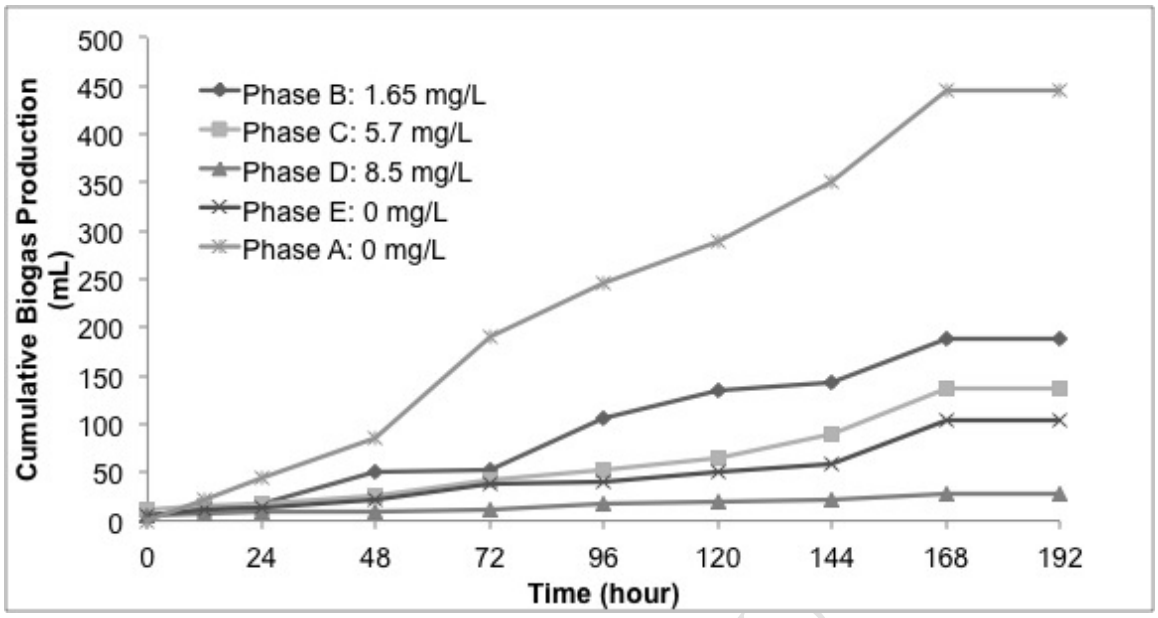

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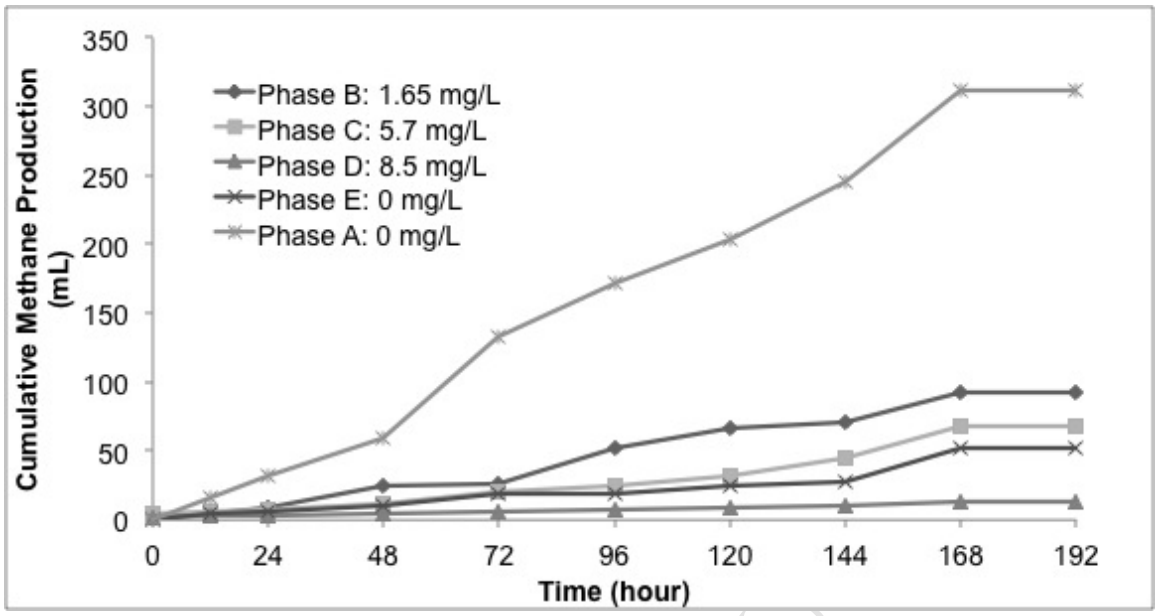

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