The operation of two EGSB reactors under the application of different loads of oxytetracycline and florfenicol

Yudy Andrea Londoño, Diana Catalina Rodríguez and Gustavo Peñuela

ABSTRACT

This study evaluated the effect of the antibiotics oxytetracycline (OTC) and florfenicol (FLO) on the operation of two EGSB (expanded granular sludge bed) reactors. The experiment was conducted for 210 d in reactor R1 and 245 d in reactor R2. The reactors were inoculated with granular sludge from an upflow anaerobic sludge blanket (UASB) reactor on a local dairy farm. The sludge had an average pellet size of 2.35 mm, good sedimentability and a high percentage of organic material. The antibiotic tolerance and the inhibitory action on the bacterial population were different for each antibiotic studied. The results showed a more severe inhibitory effect on microorganisms that were in contact with increases in loads of FLO than those that were in contact with increasing loads of OTC, a condition reflected in the chemical oxygen demand (COD) removal efficiency.

Key words | anaerobic treatment, antibiotics, expanded granular sludge bed (EGSB), florfenicol (FLO), oxytetracycline (OTC)

INTRODUCTION

Concern about the effects of pharmaceuticals on aquatic environments has increased in recent years (Gonçalves et al. 2007). Examples of such pharmaceuticals are antibiotics employed in human and veterinary medicine. Antibiotics are used in the prescription of subtherapeutic doses to animals as additives to improve their growth without additional feeding (Prabhakaran et al. 2009). They are also used in the treatment of plant infections (Martinez 2009), and in aquaculture to inhibit the growth of fungi (Prabhakaran et al. 2009). They can reach aquatic environments via various sources, such as the pharmaceutical industry, hospitals and human and animal feces (Elmolla & Chaudhuri 2009). Pharmaceuticals are partially metabolized by organisms and are excreted as the same compound or a number of its metabolites before being discharged into wastewater (Prabhakaran et al. 2009). When in wastewater, these compounds become hazardous pollutants to the aquatic environment and humans due to their adverse effects on aquatic life (Elmolla & Chaudhuri 2009). Several authors have reported that their presence even at low concentrations represents a risk as a result of the promotion and development of resistance mechanisms of strains of bacteria that come into contact with these substances (Prabhakaran et al. 2009). Also, depending on the degree of microbial toxicity, these compounds may play an important role in reducing chemical oxygen demand (COD), affecting the removal efficiency of wastewater treatment systems. Chelliapan et al. (2006) reported the anaerobic treatment of wastewater containing antibiotics, although experimentation was somewhat limited.

The UASB (upflow anaerobic sludge blanket) reactor has been widely used in the treatment of industrial and domestic wastewater. Developed in the 1970s, it is a design that allows the separation of the three phases, removing the solid particles before the liquid and the gas leave the system separately (Cortesi et al. 2009). Studies showed that the internal mixing in this reactor was not optimal due to the existence of dead zones and reduced operating efficiency. To improve these conditions the EGSB (expanded granular sludge bed) systems appeared, which improved contact between the biomass and more efficiently used the volume of the reactor (Seghezzo et al. 1998). The EGSB system is characterized by its height/diameter ratio and recirculation of the effluent. It gives a rate of climb of >4 m h⁻¹ and also dilutes the concentration of the effluent, which allows the toxic compounds to be treated (Seghezzo et al. 1998). However, the degree of tolerance that anaerobic microorganisms present in the granular sludge may develop as a result of the...
application of loads of antibiotics, such as oxytetracycline (OTC) and florfenicol (FLO), is not exactly known.

OTC is an antibacterial agent belonging to the tetracycline antimicrobial group (Gonçalves et al. 2007). It is an antibiotic with a wide spectrum of activity used in the treatment of infectious diseases in humans and animals and as a growth promoter in modern livestock (Yang et al. 2009). In aquaculture it has been substantially used for decades for the treatment of bacterial diseases, due to its efficacy and low cost. However, this compound persists in the tissue of fish, has immunosuppressive effects and can cause liver damage (Gonçalves et al. 2007). Moreover, FLO is an antibacterial specially developed for veterinary use. It is used in several countries due to its broad spectrum and effectiveness in the control of various bacterial infections in fish (Gonçalves et al. 2007) and in the respiratory tract in cattle and pigs (Park et al. 2008). In animals intended for consumption, FLO has proven to be an effective antibiotic against bacteria such as Pasteurella spp., Actinobacillus pleuropneumoniae, the Mycoplasma mycoides, Staphylococcus aureus, Salmonella typhimurium and Escherichia coli and is characterized by having a high bioavailability, good tissue penetration and rapid elimination (Park et al. 2008).

This study aims to determine the effect caused by the antibiotics OTC and FLO on the operation of two EGSB reactors, measuring the removal efficiency of COD and monitoring the system control parameters.

**MATERIALS AND METHODS**

**EGSB reactor**

Two acrylic EGSB reactors were used with a working volume of 15.2 L (Figure 1). Each reactor was composed of three units. The upper unit was 23.5 cm in diameter and equipped with an exhaust gas collector, which suited solid–liquid–gas separation (López et al. 2011). The next unit was a thin cylinder with a diameter of 6.4 cm where expansion of the granular sludge took place. Finally, the bottom unit was a chamber formed by a perforated plate that allowed the flow to enter evenly. The total height of each system was 191 cm. Each reactor was equipped with two Masterflex system model (No. 7553-70) (600-600 RPM) peristaltic pumps, responsible for controlling the feed and recirculation rates. The hydraulic retention time (HRT) was 0.79 d for R1 and 0.83 d for R2.

**Synthetic water**

Twenty litres of synthetic water containing 20 g of dextrose, 20 g sodium bicarbonate and 1 g of urea was prepared daily. Twenty millilitres of macronutrients and 20 mL of micronutrients were added. In the first stage only, synthetic water was prepared using 10 g of dextrose, 20 g sodium bicarbonate, 0.5 urea and 20 mL of macronutrients and micronutrients respectively, while stabilization was achieved. The solution was prepared with the macronutrients (g/L): 1.4NH₄Cl, 1.25KH₂PO₄, 0.5MgSO₄·7H₂O, 0.05CaCl₂·2H₂O, 2NaHCO₃ and 0.5 yeast extract. The micronutrient solution was prepared with (mg/L): 50H₃BO₃, 2,000FeCl₂·4H₂O, 50ZnCl₂, 500MnCl₂·4H₂O, 30CuCl₂·2H₂O, 50(NH₄)₆Mo₇O₂₄·4H₂O, 90Al₂(SO₄)₃, 2,000CoCl₂·6H₂O, 100NaSeO₃·5H₂O, 100 EDTA, 0.1 g of Resazurin, 1 mL HCl (37%) and 50NiCl₂·6H₂O.

**Source of biomass**

The reactors were inoculated with anaerobic granular sludge from a UASB reactor used by a wastewater treatment plant on a dairy farm. It contained 35.9 g/L of total suspended solids (TSS) and 28.4 g/L of volatile suspended solids (VSS), where the VSS were approximately 80% of the TSS, thus revealing a high percentage of organic material. The sludge volume index (SVI) was 16.7 mL/g, which indicated good sedimentability characteristics. The average pellet diameter was 2.35 mm.
EGSB system operation

Samples were collected over 210 d for reactor R1 and 245 d for reactor R2, which was the time required for the stabilization and operation of the system. The experiments were divided into four different stages for R1 and five for R2; which were defined according to the removals reached in each stage, consisting of the startup and antibiotic load variation stages (Tables 1 and 2).

Analytical methods

Tests for COD, TSS, VSS, total phosphorus (TP), total Kjeldahl nitrogen (TKN-N) and the SVI were done in the diagnostics and pollution control group (GDCON) laboratory in accordance with protocols established in the Standard Methods (APHA 2005). The GDCON group laboratory is accredited by the Institute of Hydrology, Meteorology and Environmental Studies (IDEAM) to perform such analyses. OTC and FLO were determined using a high performance liquid chromatograph (HPLC, model 1100-1200) equipped with a diode array detector (Agilent Technologies). Separation was performed in an IBD 150 × 4.6 mm ultra analytical column with 5 μm of film thickness (Restek). The analytical column was maintained at 30 °C. The mobile phase was: solvent A (acetonitrile: methanol (9:1) at 0.1% in trichloroacetic acid) and solvent B (HPLC grade water at 0.1% in trichloroacetic acid). The gradient started with 80% of solvent B and 20% solvent A, and after 15 min it was 20% of solvent B and 80% solvent A, at a rate of 1.5 mL/min. One hundred microlitres of the sample was injected and monitored by the diode array detector at 356 and 230 nm.

Antimicrobial activity

The inhibition test was performed by seeding the bacteria S. aureus ATCC 6538 (F3) on nutrient agar and incubating it at 35 °C ± 0.5 for 24 h. In a phosphate buffer solution, turbidity with the bacteria was sought of up to 0.5 on the McFarland scale. Muller Hinton Agar was then seeded on the culture medium, ensuring that the bacteria were uniformly distributed on the agar. Using a sterile punch, wells were formed in which the previously 100 μL filtered sample was deposited (Giraldo et al. 2010).

An S. aureus growth control was carried out on the Muller Hinton and an environmental control was performed by opening a Petri dish with the culture medium in the laminar flow chamber for the duration of the procedure.

To test the antimicrobial activity of the isolated strain of the reactor, the following procedure was carried out: 1 mL of sludge was taken and seeded in Brewer and Bile Esculin Agar in duplicate and incubated in anaerobic containers at 25 °C for 7 d. After this time, colonies were isolated in the same media and under the same incubation conditions. This procedure was repeated until pure colonies were acquired, which were determined by observing the culture

| Table 1 | Organic loading rate (OLR), nitrogen loading rate (NLR), phosphorus loading rate (PLR) and florfenicol loading rate (FLOLR), in the four stages of operation of Reactor 1
<table>
<thead>
<tr>
<th>Stages</th>
<th>Days of operation (d)</th>
<th>OLR (g COD/L d)</th>
<th>NLR (mg TKN-N/L d)</th>
<th>PLR (mg PO43−/L d)</th>
<th>FLOLR (mg/L d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>0–107</td>
<td>0.69 ± 0.16</td>
<td>1.18 ± 0.37</td>
<td>0.34 ± 0.08</td>
<td>–</td>
</tr>
<tr>
<td>II</td>
<td>108–134</td>
<td>1.47 ± 0.15</td>
<td>10.17 ± 0.90</td>
<td>0.33 ± 0.13</td>
<td>1.09 ± 0.15</td>
</tr>
<tr>
<td>III</td>
<td>135–168</td>
<td>1.47 ± 0.08</td>
<td>10.46 ± 1.95</td>
<td>0.44 ± 0.01</td>
<td>5.39 ± 0.23</td>
</tr>
<tr>
<td>IV</td>
<td>169–210</td>
<td>1.45 ± 0.09</td>
<td>27.53 ± 4.38</td>
<td>0.42 ± 0.08</td>
<td>11.60 ± 0.52</td>
</tr>
</tbody>
</table>

| Table 2 | Organic loading rate (OLR), nitrogen loading rate (NLR), phosphorus loading rate (PLR) and oxytetracycline loading rate (OTCLR), in the five stages of operation of Reactor 2
<table>
<thead>
<tr>
<th>Stages</th>
<th>Days of operation (d)</th>
<th>OLR (g COD/L d)</th>
<th>ALR (mg TKN-N/L d)</th>
<th>PLR (mg PO43−/L d)</th>
<th>OTCLR (mg/L d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>0–107</td>
<td>0.69 ± 0.16</td>
<td>1.67 ± 0.57</td>
<td>0.32 ± 0.05</td>
<td>–</td>
</tr>
<tr>
<td>II</td>
<td>108–134</td>
<td>1.55 ± 0.12</td>
<td>10.38 ± 2.66</td>
<td>0.33 ± 0.13</td>
<td>0.48 ± 0.06</td>
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<tr>
<td>III</td>
<td>135–168</td>
<td>1.52 ± 0.10</td>
<td>19.01 ± 1.72</td>
<td>0.41 ± 0.02</td>
<td>4.54 ± 0.50</td>
</tr>
<tr>
<td>IV</td>
<td>169–210</td>
<td>1.48 ± 0.08</td>
<td>31.80 ± 4.51</td>
<td>0.39 ± 0.19</td>
<td>3.98 ± 0.70</td>
</tr>
<tr>
<td>V</td>
<td>211–245</td>
<td>1.56 ± 0.04</td>
<td>35.62 ± 2.21</td>
<td>0.40 ± 0.02</td>
<td>9.08 ± 1.77</td>
</tr>
</tbody>
</table>
and Gram staining. The colonies obtained were used for inhibition assays following the above procedure.

SEM images

Before taking the SEM images, the biomass was washed with a 0.1 mol/L phosphate-buffered saline solution at pH 7.0. Sludge granules were taken and adjusted with 2.5% glutaraldehyde for 2 h and then washed with water for 5 min. Dehydration was carried out using ethanol at concentrations of 25, 50, 70, 80, 90 and 95%, until the critical drying point was obtained.

RESULTS AND DISCUSSION

Operation and stabilization: stage I

During operation of the EGSB system, the pH at the inlet and outlet of the reactor and the effluent alkalinity were continuously analyzed, determining the contribution provided by bicarbonates and VFA. The results of the variation of these parameters over time are shown in Figure 2, (a) for R1 and (b) for R2. In R1, the recorded pH values reported a mean of 7.0 ± 0.3 and IA/PA ratio (alkalinity contribution by volatile fatty acids/bicarbonate alkalinity contribution) average of 0.29. In R2, the mean pH was 7.2 ± 0.5 and the IA/PA ratio was 0.24.

pH values for both reactors were found to be in the optimal range for anaerobic digestion reported in the literature (6.8–7.2) (Rajeshwari et al. 2000). The IA/PA ratio for both reactors was found in the range of 0.1–0.3, indicating a sufficient alkalinity to neutralize the organic acids from the hydrolysis and fermentation processes (Zhang et al. 2008; Rodriguez et al. 2011). The results showed conditions of stability for both EGSB systems, which can be observed from the behavior of the COD represented in Figures 3(a) and 3(b) for R1 and R2, respectively. Removal percentages were 86% for R1 and 91% for R2 (Figure 4). In the study by Zhang et al. (2008) for the treatment of effluents of EGSB palm oil mills, COD removal reached 85% with a granular sludge that had regained its anaerobic activity. In another study by Scully et al. (2006) for the biological treatment of phenol in an EGSB system, conditions of stability were reported with removal percentages above 90%.

Operation and performance: stage II

Once the stabilization seen in stage I was obtained for each EGSB system, the application of antibiotic load was implemented for each reactor. R1 was given a mean concentration of FLO of 0.8 ± 0.12 mg/L and R2 was given a mean concentration of OTC of 0.38 ± 0.05 mg/L. pH values of 7.8 ± 0.4 for R1 and 6.9 ± 0.2 for R2 and IA/PA ratios of 0.36 and 0.29 for R1 and R2 respectively were recorded. Instability was evident in R1 due to the onset of the application of FLO load. In contrast, after experiencing OTC load, R2 remained stable, thereby indicating less inhibitory action on the microbial population. The behavior of COD showed irregularities for both systems (Figure 3). Removal efficiency was 49% in R1 and 67% in R2. During this stage the FLO removal efficiency was 68% and the removal efficiency of OTC was 99%.

Operation and performance: stage III

A mean concentration of FLO of 4.3 ± 0.2 mg/L was given to R1 and R2 was given a mean concentration of OTC of 4.1 ± 0.4 mg/L. The mean pH values recorded were 7.0 ± 0.4 for R1 and 7.3 ± 0.2 for R2, and the IA/PA ratios were 0.32 and 0.31 for R1 and R2 respectively (Figure 2). The results showed an improvement in stability of R1, with a
COD removal percentage of 52%, slightly higher than that observed for the previous step which had a lower antibiotic burden (Figure 4(a)). This proves that there was a slight acclimatization of the biomass when coming into contact with FLO. System instability was presented for R2, clearly reflected by the decline in COD removal efficiency of 59% (Figure 4(b)). Such instability proves that there was an inhibitory effect on the microbial population. The removal percentage of FLO was 86%; greater than that presented in stage II where the adaptation of microorganisms coming into contact with the antibiotic was demonstrated. OTC removal was 84%, which was lower compared with the previous stage, mainly due to unstable conditions presented by the reactor with increasing loads of antibiotics and the action exerted by it on the bacterial population.

**Operation and performance: stage IV**

During this stage of operation, R1 was given a mean concentration of FLO of 9.2 mg/L ± 0.4 whereas 4.6 mg/L ± 2.4 of OTC was applied to R2. The mean pH was 7.2 ± 0.4 in R1 and 7.7 ± 0.2 in R2, and the ratio of IA/PA was 0.35 and 0.36 for R1 and R2, respectively. The pH results are acceptable values for the smooth operation of the reactor. However, the alkalinity relationships for both reactors showed instability, which may indicate the accumulation of VFA. The removal percentage of COD in R1 (Figure 4(a)) was 38%, the least efficient of all the stages of operation. This result reflected the state of instability of the population of microorganisms when coming into contact with the greatest burden of applied antibiotic. In the case of R2 (Figure 4(b)), the COD removal percentage was 60%, showing a similar efficiency to the previous step. Although the efficiency demonstrates a slight tendency to increase, the conditions reflected in the reactor show a degree of instability. The FLO removal percentage was 66%, which indicated a drop in efficiency compared with the previous stage, and would have been due to the increase in the concentration of the antibiotic. In R2, the removal percentage of OTC remained stable compared with the previous stage at 83%.

**Operation and performance: stage V**

This stage only took place in the R2 reactor and involved the application of an increased load of OTC to ensure its entry into the reactor before turning into its degradation products,
which can be formed by hydrolysis in the aqueous medium. A mean concentration of OTC of 7.2 ± 1.4 mg/L was applied. The pH value recorded was 7.4 ± 0.2 and the IA/PA ratio was 0.51. It was found that although the average value of pH was in the optimal range for the production of methane, the IA/PA ratio was the highest recorded of all stages of operation, a factor which showed instability in the system. However, the percentage of registered COD removal was 68%, thereby revealing a significant improvement in system efficiency compared with the previous two stages (Figure 4(b)). The removal percentage of the antibiotic was 82%, which showed stability in the efficiency of the system regarding the removal of OTC.

**Microbial inhibition tests**

In the microbial inhibition test conducted for the strain isolated from the reactor, vulnerability was displayed by the microbial population in the trials in which the antibiotic used was FLO. Figure 5(a) shows the inhibitory behavior of two antibiotic concentrations. A greater inhibitory action can clearly be observed at higher concentrations of the antibiotic. In the tests performed for OTC, using the same concentrations of the antibiotic and the same isolate, there were slight inhibitory effects on the bacterial population (Figure 5(b)). These effects can be seen by the presence of a small shadow around each well, which was greater for the highest concentration of the antibiotic. The results showed a greater tolerance of anaerobic microorganisms when in contact with OTC and a lower tolerance when in contact with FLO.

The inhibitory action generated by each antibiotic in contact with the biomass significantly affected the initial condition of the sludge, as shown in Figures 6(a) and 6(b). However, the biomass that operated under loads of FLO was more severely affected (Figure 6(c)) than that which operated under loads of OTC (Figure 6(d)).

Microbial activity assays performed using strains of *S. aureus* for the influent and effluent of each reactor showed significant inhibition conditions (Figure 7). The R1 test had a FLO concentration of 7.69 mg/L in the influent and 3.92 mg/L (Figure 7(a) and 7(b)) in the effluent, with inhibition halos of 200 and 140 mm, respectively. The R2 test had an OTC concentration of 3.3 mg/L in the influent and 0.45 mg/L (Figure 7(c) and 7(d)) in the effluent, and the inhibition halos were 200 and 120 mm respectively. The results

![Figure 5](https://iwaponline.com/wst/article-pdf/66/12/2578/441168/2578.pdf)
Figure 6 | SEM images. (a, b) Initial granular sludge micrograph. (c) Granular sludge exposed to loads of FLO. (d) Granular sludge exposed to loads of OTC.

Figure 7 | Inhibition assay using strains of S. aureus for samples taken from the influent and effluent. (a) FLO concentration in the influent of 7.69 mg/L. (b) FLO concentration in the effluent of 3.92 mg/L. (c) OTC concentration in the influent of 3.3 mg/L. (d) OTC concentration in the effluent of 0.45 mg/L.
showed that for both reactors, antibiotic concentrations at the effluent of each system generate significant adverse biological inhibition effects on the microbial population.

CONCLUSIONS

The antibiotic tolerance and inhibitory action on the bacterial population was different for each antibiotic studied. It was demonstrated that the effect of FLO on anaerobic microorganisms had a higher incidence than that generated by OTC, a situation which was reflected in the size of the wells. The increase in the concentration of FLO significantly reduced COD removal rates over time, revealing conditions of reactor instability. Adding OTC resulted in only a slight impairment to the microbial population. This was mainly characterized by a drop in COD removal efficiency and a subsequent recovery, which reflected conditions of tolerance to the antibiotic.

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REFERENCES

American Public Health Association (APHA), American Water Works Association (AWWA), Pollution Control Federation (WPCF) 2005 Standard Methods for Examination of Water and Wastewater, 16th edition. APHA/AWWA/WPCF, Washington DC.


Martinez, J. L. 2009 Environmental pollution by antibiotics and by antibiotic resistance determinants. Environmental Pollution 157, 2893–2902.


