Comparison of the sensitivities of fish, Microtox and Daphnia-magna bioassays to amoxycillin in anaerobic/aerobic sequential reactor systems

H. Çelebi and D. T. Sponza

ABSTRACT

In this study the anaerobic treatability of amoxycillin (AMX) was investigated in a laboratory-scale anaerobic multi-chamber bed reactor (AMCBR)/aerobic continuously stirred tank reactor (CSTR) system. The chemical oxygen demand (COD) and AMX removal efficiencies were around 94% in the AMCBR reactor at hydraulic retention times (HRTs) between 2.25 and 5.5 days. Decreasing the HRT appeared not to have a significant effect on the performance of the AMCBR up to a HRT of 1.13 days. The maximum methane production rate and methane percentage were around 1,100–1,200 mL/day and 55%, respectively, at HRTs between 2.25 and 5.5 days. The decrease in HRT to 1.5 days decreased slightly the gas productions (1,000 mL/day and 500 mL for total and methane gases) and methane percentage (45%). The AMCBR recovered back to its baseline performance within a couple of days. The acute toxicity of 150 mg/L AMX was monitored with Daphnia magna, Lepistes sp., and Vibrio fischeri acute toxicity tests. The acute toxicity removals were 98, 96 and 96% for V. fischeri, D. magna and Lepistes sp. in the effluent of the sequential system treating 150 mg/L AMX at HRTs of 2.25–5.5 days. Among the trophic organisms used in the acute toxicity tests the most sensitive organism was found to be bacteria (V. fischeri) while the most resistant organism was found to be fish (Lepistes sp.).

INTRODUCTION

Several classes of antibiotics have been detected in different environmental waters at concentrations ranging from ng/L to μg/L (Gulkowska et al. 2008; Teixeira et al. 2008; Githinji et al. 2011; Xia et al. 2012). Amoxycillin (AMX) is a chemically modified form of ampicillin and its concentrations in wastewater have also been detected in the range between 61 and 92 mg/L in pharmaceutical wastewaters (Chen et al. 2011a). AMX concentrations between 28 and 82.7 μg/L were measured in a hospital wastewater sample (Kim & Aga 2007). Due to the high chemical oxygen demand (COD) concentration in pharmaceutical wastewaters containing antibiotics, attempts have already been made to work with anaerobic processes, such as upflow anaerobic sludge blanket reactor (Chen et al. 2011b), anaerobic filter (Ince et al. 2002; Kim & Aga 2007; Snyder et al. 2007), anaerobic continuous stirred tank reactor (Oz et al. 2002) and a hybrid reactor combining upflow anaerobic sludge blanket reactor and anaerobic filter (Oktem et al. 2007). The recent studies performed by the AMX treatability are as follows: in a study performed by Deng et al. (2012) 82% AMX removal was found in a full-scale bio-system consisting of two anaerobic and four aerobic reactors at a hydraulic retention time (HRT) of 1.56 days at an influent COD and AMX concentration of 6,000 and 78 mg/L, respectively. Chen et al. (2011b) reported 87% AMX yield in a combined anaerobic/micro-aerobic two-stage process treating pharmaceutical wastewater containing 105 mg/L AMX at a HRT of 1.98 days. The other studies treating the pharmaceutical wastewaters containing AMX focused on physical and chemical treatment, such as sorption (Figueroa et al. 2004), reverse osmosis (Adams et al. 2002), Fenton and photo-Fenton process (Trovo et al. 2011), ultrasonic process (Naddeo et al. 2009) and on advanced oxidation processes (Elmolla & Chaudhuri 2009; Ay & Kargi 2010). Although high COD and AMX removal rates were observed in these experiments, they are not suitable for full-scale wastewater treatment plants (WWTPs) due to their high cost (Balcioglu & Otker 2006).
Although anaerobic treatment is an effective means for decreasing the organic content in pharmaceutical wastewaters in the absence of oxygen, high metabolites still reside in the effluent. To meet the discharge standards, an aerobic polishing step is needed after the anaerobic system to meet effluent quality standards (Kuşçu & Sponza 2009). A 5% ampicillin treatment yield was obtained when the wastewater was treated by the aerobic process alone at influent ampicillin concentration of 3.5–56 mg/L (Zhou et al. 2006). However, the removal efficiency was more than 40% in the anaerobic/aerobic process. In Fox & Venkatasubbiah (1996) 50% COD yields were found at a HRT of 1 day in a combined anaerobic baffled and an aerobic attached-film reactor treating the pharmaceutical wastewater. Similarly in the study reported by Buitron et al. (2005), a sequencing batch biofilter was operated under anaerobic and aerobic conditions to obtain high COD yields in a pharmaceutical wastewater with an influent COD of 28–72 g/L.

The main advantages of the anaerobic multi-chamber bed reactor (AMCBR) include: better resilience to hydraulic and organic shock loadings, longer biomass retention times and lower sludge yields. Probably the most significant advantage of the AMCBR is its ability to separate acidogenesis and methanogenesis longitudinally down the reactor, allowing the reactor to behave as a two-phase system without the associated control problems and high costs (Oktem et al. 2007). The compartmentalization structure of the AMCBR increases the acidogenic and methanogenic activity by a factor of up to four as acidogenic bacteria accumulate within the first compartment. The other two compartments contribute to methanogen growths. Patel & Madamwar (2001) studied the effect of increasing HRTs on the performance of AMCBR treating petrochemical wastewater. A 95% COD removal efficiency and 0.45 m3/m3 d methane yields in AMCBR were observed at a HRT of 2.5 days. Patel & Madamwar (1998) studied the performance of AMCBR treating salty cheese whey wastewater. An 83% COD removal efficiency and 68% methane content in AMCBR was observed at a HRT of 2 days. Patel & Madamwar (1998) studied the performance of AMCBR treating salty cheese whey wastewater. An 83% COD removal efficiency and 68% methane content in AMCBR was observed at a HRT of 2 days. Currently, legal limits for antibiotics in environmental waters have not been established. However, as these compounds are bioactive they could also have important toxic effects on aquatic organisms (Lindberg et al. 2007).

To assess the environmental risk, extensive data documenting the contamination of the aquatic environment by the antibiotic pollutants is needed (Taipale & Ursin 2011). Therefore toxicity measurements are often conducted to evaluate wastewater toxicity by using organisms such as invertebrate, small animals, fish and plants (Barreto-Rodrigues et al. 2009). Unwanted presence of antibiotics in pharmaceutical and antibiotic industry wastewaters is a growing concern for the ecological risk of antibiotics in the aquatic environment (Ye et al. 2007). Antibiotics may affect both primary producers and decomposers, potentially disrupting ecosystem processes (van der Grinten et al. 2010). Although WWTPs remove some pharmaceuticals during the treatment process (Hirsch et al. 1999; Carballa et al. 2004; Glassmeyer et al. 2005), the removal efficiencies vary from plant to plant. The antibiotics were measured in both the influent and treated effluent, and lower concentrations were observed in the treated wastewater, suggesting partial removal in WWTPs cause toxicity in ecological environments. The fate of antibiotics and other pharmaceuticals in WWTPs is greatly influenced by the design and operation of treatment systems. Therefore, toxicity tests should be performed in the effluents of the antibiotic industry WWTPs to different organisms and the sensitivities of the bioassay should be taken into consideration (Kim & Aga 2007).

The acute toxicity of 10 mg/L AMX to Pseudokirchneriella subcapitata and Daphnia magna (D. magna) was not reduced with photocatalytic treatment and 0.8 gTiO2/L catalyst. After 120 min irradiation time with UV (Rizzo et al. 2009). An amount of 50 mg/L AMX caused 30% inhibition of Vibrio fischeri (V. fischeri) bioluminescence and 65% inhibition of the neonates mobility in D. magna bioassays (Trovo et al. 2011). Eguchi et al. (2004) reported that the β-lactam antibiotics (ampicillin and AMX) were less toxic to V. fischeri, D. magna, Moina macrocopa, and Oryzias latipes test organisms. The toxicity of ampicillin to S. selenastrum capricornutum and C. vulgaris, was reported as 1,000 mg/L (Park & Choi 2008). In a study performed by Trovo et al. (2011) the acute toxicity of 120 mg/L AMX to D. magna decreased from 65 to 5% after 90 min of UV irradiation in the presence of FeSO4. However, it increased again to a maximum of 100% after 150 min, which indicates the generation of more toxic intermediates than AMX, reaching 45% after 240 min. The EC50 value of AMX was found as 0.0037 and 250 mg/L to algae Microcystis aeruginosa and S. capricornutum, respectively (Kim & Aga 2007). The toxicity assays of other antibiotics showed that erythromycin was found to be the less lethal antibiotic while diclofenac sodium salt, AMX and lidocaine were the most lethal pharmaceuticals throughout toxicity tests performed with Artemia salina, D. magna (van der Grinten et al. 2010). The lowest EC50 values (0.028, 0.052, 0.081 and 0.2 mg/L, respectively) with trimethoprim, sulfamethoxazole, oxytetracycline and flumequine were detected in the Microtox acute toxicity test. The overall lowest observed
EC₅₀ (0.0089 mg/L) value was found for the green algae exposed to 30 mg/L tylosin (van der Grinten et al. 2010).

Up to now, no experiment has been reported exploiting the sequential AMCBR/aerobic continuously stirred tank reactor (AMCBR/CSTR) process to treat pharmaceutical wastewaters containing AMX. No studies have been undertaken to investigate the treatment of AMX using the AMCBR in the literature. The objective of the present work was to evaluate the effect of increasing HRTs on COD and AMX removal efficiencies, total, methane gas productions and total volatile fatty acids (TVFA) in the AMCBR, as the recent literature showed that the HRT affects significantly the anaerobic reactor performances treating toxic wastewaters such as pharmaceutical and antibiotic wastewaters: pharmaceutical wastewaters containing antibiotics have been shown to require long HRT for efficient treatment (Chelliapan et al. 2006), presumably on account of their complex organic carbon content, and this probably limits the upflow anaerobic stage reactor performance at HRT below 4 days (Chelliapan et al. 2006). The COD and tylosin removal efficiencies at 4-day HRT were around 92% after which point there was a slight decrease at 3 and 2 days HRT (average 80%) and this was reduced further (average 67%) at a HRT of 1 day (Chelliapan et al. 2011). Lower HRT has caused a decrease in biomass concentration, a reduction in mean size of the granules, lowered the settling ability of the granules and the overall biomass growth rate and biomass yield (Lapara et al. 2002). Previous research has shown a correlation between short HRT and low COD yield and may control the amount of biomass that is in direct contact with the substrate at any time (Zhou et al. 2006). A short contact time between the substrate and biomass has been shown to favour acidogens which have faster growth kinetics and adapt better to reduced pH than the methanogens. Sanz et al. (2006), Zhou et al. (2006), Amin et al. (2006) and Angenent et al. (2008) reported that HRTs significantly affect the anaerobic biodegradation of pharmaceutical wastewaters containing high COD, antibiotic and diverse refractory organic materials.

Furthermore, in this study the overall COD and AMX removal efficiencies were investigated in the sequential AMCBR/CSTR system and also, the acute toxicity (V. fischeri, D. magna, Lepistes sp.) released from the sequential anaerobic/aerobic reactor system was determined.

**MATERIALS AND METHODS**

**Experimental**

A continuously fed stainless steel AMCBR and CSTR were used in sequence for the experiment. A schematic diagram of the system used in this study is presented in Figure 1. The
The effluent of the AMCBr was used as the influent of the CSTR. The AMCBr was constructed from a stainless steel rectangular container with inner dimensions of 40 cm length, 25 cm height, and 20 cm width with a volume of 4.5 L. The feed was connected to the reactor peristaltic pump. Temperature of the reactor was kept constant at 37 ± 1°C by means of a heater located inside the reactor. The outlet of the AMCBr was connected to glass U-tubing in order to control the level of wastewater and to trap the solids. The produced gas was collected via a porthole in the top of the reactor.

The AMCBRs were operated in such a way that the feed was pumped continuously in an upward direction into the first chamber. The effluent from the first chamber was allowed to flow down into the second chamber, which further moved upward into the third chamber, as shown in Figure 1. The CSTR consisted of an aerobic (volume = 9 L) and a settling chamber (volume = 1.32 L). During the aerobic phase, dissolved oxygen concentration was adjusted to around 4 mg/L with an air pump with a capacity of 2 m³/day to ensure survival of the organisms in the CSTR.

### Seed of the reactors

Partially granulated anaerobic sludge was used as seed in the AMCBr and was obtained from an anaerobic upflow sludge blanket reactor containing partially granulated biomass from the Pakmaya Yeast Beaker Factory in Izmir, Turkey. The suspended solid concentration of anaerobic sludge used as seed in the AMCBr was 65 g/L. The sludge in the CSTR was taken from the aeration tank of Pakmaya Yeast Beaker Factory in Izmir, Turkey. The suspended solid in the CSTR was 3 g/L.

### Wastewater composition

Molasses as primary substrate giving a COD concentration of 4,000 mg/L and Vanderbilt mineral medium as nutrients were used together with the AMX. In addition, a Vanderbilt mineral medium was added to the wastewater to improve the growth of methanogens in the AMCBr (Speece 1996). Sodium thioglycollate (0.50 mg/L) was added to the feed in order to maintain the anaerobic conditions necessary for operating the AMCBr under the desired reducing environment. The desired alkalinity and neutral pH were obtained by the addition of 5,000 mg/L NaHCO₃ to the feed media.

### Operational conditions

The COD concentration was kept constant at 4,000 mg/L. The values of the operating parameters for AMCBr and CSTR during 189 days of continuous operation are summarized in Table 1. The organic loading rates were increased from 0.73, 0.88, 1.78, 2.67, 3.55 to 4.44 kg/m³/day in the AMCBr by decreasing the HRTs from 5.5 to 4.5, 2.25, 1.5, 1.13 and 0.9 days. The AMCBr was operated at steady state conditions for approximately 26–37 days in every HRT. SRT in the CSTR was adjusted as 20 days by discarding a certain amount of sludge volume from the aeration of the CSTR.

<table>
<thead>
<tr>
<th>Runs</th>
<th>Period</th>
<th>HRT</th>
<th>OLR</th>
<th>AMX</th>
<th>ALR</th>
<th>SRT</th>
<th>AMCBr</th>
<th>CSTR</th>
<th>AMCBR/CSTR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Run 1</td>
<td>36</td>
<td>5.5</td>
<td>0.73</td>
<td>150</td>
<td>27.33</td>
<td>197</td>
<td>9.0</td>
<td>0.04</td>
<td>14.46</td>
</tr>
<tr>
<td>Run 2</td>
<td>37</td>
<td>4.5</td>
<td>0.88</td>
<td>150</td>
<td>33.33</td>
<td>183</td>
<td>9.0</td>
<td>0.04</td>
<td>13.5</td>
</tr>
<tr>
<td>Run 3</td>
<td>32</td>
<td>2.25</td>
<td>1.78</td>
<td>150</td>
<td>66.67</td>
<td>92</td>
<td>4.5</td>
<td>0.07</td>
<td>6.75</td>
</tr>
<tr>
<td>Run 4</td>
<td>28</td>
<td>1.5</td>
<td>2.67</td>
<td>150</td>
<td>100</td>
<td>61</td>
<td>3.0</td>
<td>0.12</td>
<td>4.5</td>
</tr>
<tr>
<td>Run 5</td>
<td>30</td>
<td>1.13</td>
<td>3.55</td>
<td>150</td>
<td>133.33</td>
<td>46</td>
<td>2.25</td>
<td>0.18</td>
<td>5.33</td>
</tr>
<tr>
<td>Run 6</td>
<td>26</td>
<td>0.9</td>
<td>4.44</td>
<td>150</td>
<td>166.67</td>
<td>37</td>
<td>1.8</td>
<td>0.44</td>
<td>11.11</td>
</tr>
</tbody>
</table>

Period: days, OLR: Organic loading rate (kgCOD/m³/day), HRT: Hydraulic retention time (day), SRT: Solid retention time (day), ALR: AMX loading rate (gAMX/m³/day), AMX: AMX concentration (mg/L).
Chemicals and reagents

All reagents and materials used were analytical grade. AMX (C16H19N3O5S) (purity higher than 97%) was provided by Sigma Aldrich Co. (St Louis, USA). High performance liquid chromatography (HPLC)-grade methanol (CH3OH) and phosphate (PO43-) were used in HPLC analyses, which were purchased from Merck Company (Darmstadt, Germany). The water used in all experiments was purified by using Millipore filtration (0.45 μm). The chemical structure and some basic characteristics of AMX are shown in Table 2.

Analytical methods

The suspended solid was measured by membrane filtration technique and COD was determined by closed reflux colorimetric method (APHA-AWWA-WEF 2005). Total and methane gas productions and methane percentage quantification were measured using the liquid displacement method (Roza-Flores et al. 1997; Beydilli et al. 1998). TVFA were measured using the titrimetric method (Anderson & Yang 1992). The pH and oxidation reduction potential (ORP) were measured using a WTW pH-ORP meter. AMX was determined by HPLC (Hsu & Hsu 1995) using an Agilent-1100 instrument with a Packing Ace 5-C18 reversed phase column (150 mm × 4.5 mm, 5 μm). All the samples were initially centrifuged (SED 5X model) to remove any particulate matter and then filtered through a 0.45 μm filter using a disposable syringe (Agilent 5835) prior to HPLC. The autosampler was set for an injection volume of 12 μL. The flow rate was 1 mL/min; the mobile phase consisted of 75% methanol and 25% phosphate. Quantification was carried out by the integration of the peak area at a wavelength of 210 nm.

*Vibrio fischeri* testing was performed according to the standard procedure recommended by the manufacturer (Lange 1994). Acute toxicity was also tested using 24-h *Lepistes* sp. and *D. magna* as described in (APHA-AWWA-WEF 2005). Acute toxicity assays were conducted using standard test organisms including a marine bacterium, *V. fischeri*; a freshwater flea, *D. magna* and a fish, *Lepistes* sp. (Choi et al. 2008). Acute effects of AMX were observed at mg/L levels in the standard aquatic ecotoxicity test mentioned above. Toxicity evaluation criteria for bioluminescent bacteria are explained by the per cent inhibition effect. If the per cent inhibitory effect changed between 0 and 5%, the effect is non-toxic. When it is between 5 and 20%, the effect is possibly toxic, and when the inhibitor effect is between 20 and 90%, the effect is toxic (Teodorovic et al. 2009).

RESULTS AND DISCUSSION

Start-up period

A start-up period led to a more complete biological degradation of the toxic substances such as antibiotics and a better adaptation of the biomass for the degradation of the antibiotic. The results of the start-up of the AMCGR are shown in Figure 2a and 2b. The AMCGR was operated through 45 days without AMX to acclimate the granular

### Table 2 | Some important characteristics of AMX

<table>
<thead>
<tr>
<th>Property</th>
<th>AMX</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular weight (g/mol)</td>
<td>365.4</td>
</tr>
<tr>
<td>Half-life in water (hour)</td>
<td>72</td>
</tr>
<tr>
<td>Water solubility (mg/L)</td>
<td>3.43E+03</td>
</tr>
<tr>
<td>Log $K_{ow}$</td>
<td>0.97</td>
</tr>
<tr>
<td>Log $K_{oc}$</td>
<td>8.65</td>
</tr>
<tr>
<td>Henry’s law constant (1/atm mol)</td>
<td>1.04E-19</td>
</tr>
<tr>
<td>Chemical structure</td>
<td></td>
</tr>
</tbody>
</table>

Log $K_{ow}$: octanol–water coefficient; log $K_{oc}$: octanol–carbon coefficient; *Raju et al. (2009).*
sludge to the AMCBR. The HRT and organic loading rate were 5.5 day and 0.73 kgCOD/m³day, respectively. The COD removal efficiency was 83% after 10 days of the operation period. The COD removal efficiency increased to 91% after 25 days of operation, then the COD yields remained stable at 94% throughout 20 days after an operation period of 45 days and the effluent COD was stabilized at 255 mg/L for 2 consecutive weeks. This showed that the AMCBR had reached steady-state conditions; the methane gas production and the methane percentage reached 358 mL/day and 46%, respectively, on day 25 and they remained stable at 520 mL/day and 55%, respectively, throughout 20 days. The ORP values observed during the studies show that relevant environmental conditions were provided for methane producing bacteria to survive in the AMCBR. During the anaerobic phase, zero dissolved oxygen was observed and the redox potential was around –360 mV and the concentration of granulated anaerobic biomass reached 72 g/L from 65 g/L after 45 days of the start-up period (data not shown).

**Effect of HRT on the COD and AMX removal efficiencies in AMCBR**

When the HRT of the AMCBR was decreased from 5.5 to 4.5, 2.25 and 1.5 days, no significant changes occurred in the effluent water quality (Figure 3). The influent COD and AMX concentrations were 4,000 and 150 mg/L, respectively, through continuous operation. Ninety three per cent COD and 94% AMX maximum removal efficiencies were obtained at HRTs of 4.5 and 5.5 days. These were the maximum COD and AMX yields obtained in the AMCBR. The COD and AMX removal efficiencies at 2.25 days HRT were around 90 and 92%, respectively, after which point there was a slight decrease at 1.5 days HRT only for COD yield (88%) while the AMX yield remained the same (92%), and the yields were reduced further (COD and AMX yields decreased to 70 and 84%) at a HRT of 1.13 days. The yields decreased to around 70% at a HRT of 0.9 day. This indicates that COD and AMX removal efficiencies became less efficient and more variable with the HRT reduction from 1.5 days to 1.13 and to 0.9 days. ANOVA test statistics showed that a significant linear relationship between COD, AMX yields and HRTs varying between 1.5 and 5.5 days was not observed ($R^2 = 0.39, p = 0.05, F = 8.67$) while the linear relationship between yields and low HRTs (0.9 and 1.13 days HRT) ($R^2 = 0.83, p = 0.05, F = 2.78$) was significant. Although the AMCBR with a high granulated sludge concentration of 89 mg/L exhibited a successful anaerobic treatment for AMX and COD at high HRTs between 1.5 and 5.5 days, these yields decreased at HRT as low as 0.9 and 1.13 days. In this study it was found that decreasing the HRT from 5.5 to 1.5 days did not have a significant effect on COD and AMX removals as the granulated sludge used in this study with high microorganism content and high activity increased the reactor performance. For the low AMX and COD yields in the

![Figure 2](image-url) (a) Methane gas variations (b) COD variations through start-up period in AMCBR.

![Figure 3](image-url) COD and AMX removal efficiencies versus HRTs for AMCBR.
AMCBR at lower HRTs such as 0.9 and 1.13 days, the granulated sludge does not have sufficient time available for mineralization of COD and AMX and their metabolites. Under these conditions the mass transfer (COD and AMX) into the granules from the AMCBR is not sufficient to remove most of the substrate. Therefore, there was an increase in the effluent COD concentration. Another possible reason for the drop in treatment efficiency (to 70%) in this study at HRTs 0.9 and 1.13 days may be the partial inhibition of granulated biomass by AMX may resulted in lower methanogenic activity to such an extent that the TVFAs were not well metabolized, resulting in the increasing effluent COD and AMX concentrations. Moreover, the difference in COD and AMX removal efficiencies of 13 and 24% (differences between HRTs 5.5, 1.13 and 0.9 days) may be due to more recalcitrant molecules in the AMX needing longer time for bacterial degradation in the anaerobic biomass resulting in toxicities in the AMCBR. However, the minimal effect on reactor performance confirms that the AMCBR was efficient at HRTs as low as 1.5 days and, therefore, a short HRT was not responsible for the drop in treatment efficiency (less than 2 and 4% for AMX and COD yields) while HRTs as low as 0.9 and 1.13 days decrease significantly the AMCBR performance.

In consequence, it appears that the performance of the AMCBR becomes virtually independent of 1.5–5.5 days HRT. For HRT lower than 1.5 days the performance of the AMCBR is dependent the decreasing HRT. When the HRT was decreased, the increasing acidogenic activity usually results in lower pH values; reduced methanogenic activity; increased COD, AMX and TVFA in the effluent of the AMCBR. Even though it was expected that the AMCBR would be stable at short HRTs, it was not able to withstand the short HRT, probably due to the complexity of synthetic pharmaceutical wastewater which contained AMX. In general, longer HRT can help the kinetics of degradation, i.e. more complex organics like recalcitrants. Simply have longer to be degraded. Nandy & Kaul (2001) have demonstrated that substrate removal efficiency increases with increase in HRT in anaerobic treatment of herbal-based pharmaceutical wastewater using fixed-bed reactor. Zhou et al. (2006) reported that when HRT of an anaerobic baffled reactor treating pharmaceutical wastewater containing antibiotics (ampicillin and aureomycin) was extended from 1.25 to 2.5 days, the COD removal efficiency increased from 77 to 85%. They also observed that the antibiotic removal efficiencies increased from 16 to 42% for ampicillin and 26 to 31% for aureomycin.

The recent literature on the anaerobic AMX treatment showed that the yields obtained in some high rate anaerobic reactors are lower than the AMCBR removals found in this study: In a study performed by Chen et al. (2011b) 60% COD and 34% AMX removal efficiencies were obtained at a HRT of 2 days in an up-flow anaerobic sludge blanket reactor at an influent AMX concentration of 61 mg/L. In the study performed by Zhou et al. (2006), the COD (67%) and AMX (40%) yields were lower than those of our data (90 and 92% for COD and AMX, respectively) at a HRT of 3 days in an anaerobic contact reactor treating 3.2 mg/L AMX.

The results obtained in this study are considerably higher than the data obtained by Sreekanth et al. (2009) in a hybrid up flow anaerobic sludge blanket reactor at an influent COD concentration of 13,000–15,000 mg/L (E = 65–75% COD removal efficiency) at a HRT of 2 days. The above results are consistent with observations made by Akbarpour & Mehrdadi (2011) in an up-flow anaerobic sludge blanket reactor, treating a chemical synthesis-based pharmaceutical wastewater. The COD yield was 54.6% at a HRT of 1.4 days. In our study, the high AMX yields in the AMCBR could be due to the reactor configuration, operational conditions, seed properties and HRTs used throughout reactor operation.

**Variation of AMX versus operation time in AMCBR**

After the start-up period, the AMCBR was operated throughout 189 days with 150 mg/L AMX at six different HRTs (Figure 4). The effluent AMX concentration was 12 mg/L for operation days of 1–15 then it decreased to 9 mg/L for 21–36 days resulting in AMX yield of 94% at a HRT of 5.5 days. The AMX yields remained as 94% on days 65–74 (the effluent AMX concentration was recorded as 9 mg/L) when the HRT was decreased to 4.5 days following the slightly decrease on days 37–48 (88%) and 50–64 (90%). On days between 99 and 103, the AMX yield slightly decreased to 92% at a HRT of 2.25 days following the slightly decrease on days between 75–87 (80%) and 90–98 (85%), respectively. The effluent AMX concentration was measured as 9 mg/L on days between 65 and 74. This showed that granulated anaerobic biomass in the AMCBR quickly acclimated to the AMX at long HRTs. The AMX yields remained as 92% and decreased to 84% on days 128 and 152 as the HRTs decreased to 1.5 and 1.13 days, respectively following the decrease on days between 104–110 and 134–145, respectively. The effluent AMX concentrations were 12 and 24 mg/L for the HRTs given above. The AMCBR reactor recovered quickly and reached steady-state conditions.
conditions for the HRTs varying between 1.5 and 5.5 days. The minimal effect of the AMX antibiotic on overall AMCBR reactor performance confirms that the bacteria were adapted to AMX at 1.5 and 5.5 days HRTs. In other words, at *P. aeruginosa* varying between 1.5 and 5.5 days the AMX have a relatively minor influence on the yield of the AMCBR and do not inhibit substantially the activity of methanogenic granular sludge microorganisms. AMX concentration remained relatively constant in the reactor effluent throughout the experiment for the HRTs given above. On days between 175 and 189 the AMX yield decreased to 70% with an effluent AMX concentration of 45 mg/L at a HRT of 0.9 day following a decrease of AMX yield (59–62%) on days 165–172. The lower AMX removal efficiency resulted from the short HRT was probably due to the incomplete degradation of the AMX at the shorter contact times.

The studies performed with the anaerobic treatability of AMX showed that the yields obtained in the present study were higher than the other studies: The AMX removal was found to be lower (82%) in the study performed by Deng *et al.* (2012) under anaerobic conditions at an influent AMX concentration of 78 mg/L at a HRT of 1.56 days. Similarly, the AMX removal efficiencies obtained by Chen *et al.* (2012b) are lower than our data. Forty seven per cent AMX yield was obtained at a HRT of 1.7 days in an up-flow anaerobic sludge blanket reactor at an influent AMX concentration of 61 mg/L. Similarly Pallavi *et al.* (2009) reported lower AMX yields 65% than those found in our study at a HRT of 1.95 days and at an influent AMX concentration of 89 mg/L.

Effects of HRT on the gas productions, methane content and TVFA in the AMCBR

The methane gas productions in the AMCBR were obtained as 619 and 618 L/d while the HRTs were 5.5 and 4.5 days, respectively (Figure 5a). The total gas productions and the methane percentage remained around 1,200 L/day and 55%, respectively, for the HRTs mentioned above. The methane gas production remained the same as is in high HRTs (599 L/day) while the total gas production decreased slightly (from 1,210 to 1,180 L/day) as the HRT decreased from 4.5 to 2.25 days. It is important to note that the decrease in HRT to half did not significantly affect the methane volume. The decrease in HRT from 2.25 to 1.5 days slightly decreased the volumetric total and methane gas productions (1,000 and 500 L/day, respectively) while the methane gas percentage was recorded as 45% (Figure 5a). At low HRTs the granulated bacteria could not have enough time to metabolize the 150 mg/L AMX. Therefore both the methane gas content which dropped to

![Figure 4](https://iwaponline.com/wst/article-pdf/66/5/1117/443256/1117.pdf)
600 and 528 L/day as well as total gas production decreased at HRTs 1.13 and 0.9 days. The methane percentage also decreased to 42 and 33% for the aforementioned HRTs. The decrease in methane content of biogas is generally observed when the rate of acid formation exceeds the rate of breakdown to methane at short HRTs (Ince et al. 2002; Kim & Aga 2001).

Another reason for decrease in methane content of the gas at low HRTs may be high carbon dioxide content resulting from TVFA accumulation. At high HRTs such as 4.5 and 5.5 days the TVFA concentrations were found to be low in all compartments compared to the short HRTs (Figure 5b). The TVFA concentrations were around 253, 110 and 90 mg/L in the 1st, 2nd and 3rd compartments while it was zero in the effluent of the AMCBR for the aforementioned high HRTs. The TVFA levels increased to 300–370, 200–270 and 120–180 mg/L at HRTs of 2.25 and 1.5 days, respectively while the TVFA concentrations were recorded as 3 and 19 mg/L in the effluent of the AMCBR (Figure 5b).

In anaerobic compartmentalized AMCBR, the 1st compartment is referred to as ‘acid fermentation’ and involves the production of TVFA, while the second and third compartments are referred to as ‘methane fermentation’ because the TVFA are converted to methane and CO₂ production (Azbar & Speece 2001). The maximum TVFA concentrations were measured as 550, 400 and 78 mg/L in the 1st, 2nd compartments and in the effluent. From the results of this study it can be concluded that the TVFAs could not be effectively transformed to methane by the sensitive methane Archae as low HRTs like 1.13 and 0.9 days did not allow enough time for this group of slowly growing bacteria. Although acidogenic bacteria could more rapidly complete the acidogenic phase, methanogenic bacteria could not produce methane at the same rate with decreasing HRT. This lead to TVFA accumulation in the 1st and 2nd compartments of the AMCBR. On the other hand at low HRTs the granulated anaerobic bacteria could not easily use the AMX as co-substrate and some toxic metabolites decrease the number of

![Figure 5](https://iwaponline.com/wst/article-pdf/66/5/1117/443256/1117.pdf)
viable methane bacteria, inhibits the methanogenic activity resulting in TVFA accumulation in the AMCBR. In this study, the methane yield can be a useful parameter to assess the performance of the AMCBR. It was observed that the methane yield decreased with decreasing of the HRTs. The methane yields decreased from 0.48 to 0.02 m³ CH₄/kg COD removed when the HRTs were decreased from 5.5 to 0.9 days (data not shown). Lower methane yields (0.26–0.34 m³ CH₄/kg COD removed) were obtained in a study performed by Nandy & Kaul (2001) throughout anaerobic treatment of fermentation-based pharmaceutical wastewaters containing 48 mg/L AMX at a HRT of 2 days. Similarly, a lower methane yield value (0.19 m³ CH₄/kg COD removed) was obtained in the anaerobic treatment of chemical synthesis-based pharmaceutical wastewater at a HRT of 1.98 days (Ince et al. 2002). The lower methane yields in the studies mentioned above could be due to the configuration of the anaerobic reactor, type of anaerobic microorganism, to the biomass concentration and to the operational conditions.

**Performance of the aerobic reactor**

Table 3 shows the effect of decreasing HRT on the COD and AMX removals in the aerobic reactor. The COD removal efficiencies were around 83% for HRTs of 5.5, 4.5 and 2.25 days, respectively in the CSTR. The AMX yields were 83, 80 and 75% for the aforementioned HRTs. The COD and AMX yields decreased to 76–65% and 70–65% at HRTs of 1.13 and 0.9 days, respectively. The COD and AMX removal efficiencies decreased at low HRTs in the aerobic reactor. As the effluent of the AMCBR was used as the feed in the influent of the CSTR the COD and the AMX remaining from the AMCBR were removed in the CSTR. In the CSTR the rest of the COD and AMX which could not be biodegraded in the AMCBR were low. This means that the COD and AMX were mainly biodegraded in the AMCBR. From 4,000 mg/L of COD and 150 mg/L AMX in the influent of the AMCBR, 3,680 mg/L of COD and 141 mg/L AMX were removed in the anaerobic reactor while the rest of the 320 mg/L COD and 9 mg/L AMX were biodegraded in the CSTR reactor with 83% yields at HRTs of 4.5 and 5.5 days. The COD removals were found to be lower (60%) in the study performed by Chen et al. (2008) under aerobic conditions treating the 78 mg/L AMX in an upflow anaerobic sludge blanket reactor, compared with the present study. Similarly, the COD removal efficiencies (38–62%) obtained by Lapara et al. (2001) are lower than those of our results.

**Performance of anaerobic/aerobic sequential reactor system**

Figure 6 shows the removal efficiencies of COD, AMX at six studied HRTs in the AMCBR/CSTR system. The COD and AMX removals in the total sequential total system were >95% as the HRTs decreasing from 5.5 to 4.5, 2.25 days. The maximum COD and AMX yields were 98 and 99%, respectively, for the HRTs given above while the lowest COD and AMX yields were 94% at a HRT of 0.9 days, in the sequential AMCBR/CSTR system. Ninety four per cent of the COD and AMX were removed in the anaerobic reactor while the remaining COD and AMX (5% of COD and AMX) were biodegraded in the aerobic reactor at HRTs between 2.25 and 5.5 days. This showed that a significant part of the AMX could be removed with high removal efficiency in the sequential AMCBR/CSTR system. In a study carried out by Chen et al. (2012b) 87% both AMX and COD yields were obtained at an influent COD and AMX concentration of 3,690 and 105 mg/L, respectively, in a combined anaerobic/micro-aerobic two-stage aerobic process at a HRT of 1.98 days. In our study the AMX and COD removal efficiencies are higher than this study.

<table>
<thead>
<tr>
<th>Table 3</th>
<th>COD and AMX yields in CSTR at six different HRTs</th>
</tr>
</thead>
<tbody>
<tr>
<td>HRT (day)</td>
<td>5.5</td>
</tr>
<tr>
<td>COD_{inf}</td>
<td>320</td>
</tr>
<tr>
<td>COD_{eff}</td>
<td>55</td>
</tr>
<tr>
<td>R_{COD}</td>
<td>83</td>
</tr>
<tr>
<td>AMX_{inf}</td>
<td>9</td>
</tr>
<tr>
<td>AMX_{eff}</td>
<td>1.50</td>
</tr>
<tr>
<td>R_{AMX}</td>
<td>83</td>
</tr>
</tbody>
</table>

*aCOD conc. in influent (mg/L); bCOD conc. in influent (mg/L); cCOD Rem. efficiency (%); dAMX conc. in influent (mg/L); eAMX conc. in influent (mg/L); fAMX Rem. efficiency (%).
although the influent AMX concentration is comparably higher than the study performed by Chen et al. (2016). The literature survey showed that the AMX yields obtained in old studies are lower than those in our data: 50% COD and antibiotic removals was obtained by Fox & Venkatassubbiah (1996) in a combined anaerobic baffled and aerobic attached-film reactor to remove pharmaceutical wastewaters containing antibiotic and sulphate at 4,000 mg/L COD and at a HRT of 1 day. Similarly, Buitron et al. (2005) found 96% COD yield in a sequencing batch bio filter operating under anaerobic and aerobic conditions to treat pharmaceutical wastewater with an influent COD of 28–72 g/L.

**Acute toxicity test results using three different trophic levels**

The acute toxicity test results performed with *D. magna* showed that the EC50 values increased from 97 mg/L in the influent of the AMCBR to 265 mg/L and to 298 mg/L in the effluent of the CSTR reactor, respectively, at a HRT of 5.5 days (Figure 7a). The maximum acute toxicity removal in sequential AMCBR/CSTR reactor system was around 97% at HRTs of 5.5 days. Santos et al. (2010) mentioned that the acute toxicity of 16 mg/L AMX is 98 mg/L based on EC50 in some industrial and anthropogenic effluents. The results of low exposure concentration range are suitable for determining the no observable adverse effect level (NOAEL). This value is the concentration at which a substance does not cause significant effect in comparison with control sample. It has an important ecological implication because it indicates the concentration above which adverse effect can be expressed. The NOAEL levels of 150 mg/L AMX were around 95 mg/L at HRTs of 5.5, 4.5 and 2.5 while the NOAEL values were calculated as 80–88 mg/L at HRTs of 1.13 and 0.9 days in the sequential AMCBR/CSTR effluents, respectively, for *D. magna*.

*V. fischeri* toxicity test showed that the EC50 values increased from 143 mg/L in the influent to 215 mg/L, and to 282 mg/L in the effluents of AMCBR at a HRT of 5.5 days (Figure 7b). The maximum total acute toxicity reduction in AMCBR/CSTR reactor effluent was 96%. The total acute toxicity reduction in sequential AMCBR/CSTR reactor effluent was 93% at a HRT of 0.9 days. Hernando et al. (2005) showed that *V. fischeri* organisms exhibited high sensitivity to pharmaceutical compounds and antibiotic discharges.

*Lepistes* sp. toxicity test results showed that the EC50 values increased from 210 mg/L in the influent to 312 mg/L, and to 353 mg/L in the effluents of AMCBR and CSTR reactors at a HRT of 5.5 days (Figure 7c). The total acute toxicity reduction in the AMCBR/CSTR reactor effluent was 92% at this HRT. The NOAEL values were around 55–78 and 67–128 mg/L for *V. fischeri* and *Lepistes* sp., respectively, at HRTs of 5.5 days. Yamamoto et al. (2007) showed that fish organisms exhibited slight sensitivity to different antibiotic groups. Eguchi et al. (2004) reported that 1,000 mg/L AMX exhibited acute toxicity to *S. capricornutum* and *C. vulgaris*. Brain et al. (2004) found a LOEC value of 1 mg/L for 56 mg/L AMX using *L. gibba*. The studies performed in recent literature showed that the beta lactam antibiotics exhibited acute toxicity to different test organisms in wastewaters. For example Andreozzi et al. (2004) found that micro-algal species (EC50 = 100 mg/L for *P. subcapitata* and EC50 = 2 mg/L for *S. leopoldensis*) are sensitive to AMX in pharmaceutical wastewaters. Holten Lutzhoft et al. (1999) found that a weak acute toxicity of 34 mg/L AMX to *M. aeruginosa* in 72-hour (EC50 = of 0.0037 mg/L). Park & Choi (2008) investigated the acute toxicity of AMX to different trophic levels. The EC50 values for *D. magna* and *O. latipes* were 42.1 and 80.8 mg/L, respectively. Furthermore, Nalecz-Jawecki et al. (2010) mentioned that Microtox bacteria were sensitive to human and veterinary antibiotics in environmental toxicity assays. The most sensitive organism among the trophic levels used was determined by the lowest EC50 values of the acute toxicity test results. A score of 1 was assigned for the most sensitive organism while a score of 3 was given to the most resistant organism. The direct comparison of sensitivity of different organisms to AMX revealed that the *V. fischeri* acute test is significantly more sensitive to AMX than the *D. magna*, *Lepistes* sp. The most resistant organism was found to be *Lepistes* sp. The degradation of pharmaceuticals (diclofenac, AMX, carbamazepine in single solutions and also in three mixtures spiked in urban wastewater
Figure 7 | Different HRT values of EC50 in AMCBR, CSTR and sequential system.
effluent) at concentrations varying between 2.5 and 10 mg/L, with ultrasound generated exhibited acute toxicity on *P. subcapitata*. *D. magna* displayed less sensitivity compared with *P. subcapitata* because it belongs in a lower taxonomic species than *D. magna*. The germination index of *L. sativum* in the presence of the AMX increased as the sonication metabolites was found to be more toxic than the parent antibiotic. The differences in toxicity responses can be explained by the differences in the treatment processes and in the operational conditions.

**CONCLUSIONS**

The results of this study showed that 150 mg/L AMX was effectively removed with high yields (94%) at HRTs varying between 1.5 and 5.5 days. Decreasing of HRT from 5.5 to 1.5 days appeared not to have a significant effect on AMX and COD yields. The maximum COD yields varied between 88 and 93% for the HRTs given above. Decreasing the HRT from 1.5 to 1.13 and 0.9 days has a relatively significant influence on the AMX, COD removal efficiencies the AMCBR reactor. The AMX yields decreased to 84 and 70% for these HRTs. Similarly the COD yields reduced to 70 and 69% for HRTs 1.13 and 0.9 days. The methane content of the gas produced in the AMCBR was around 55% for HRTs 4.5 and 5.5 days. No significant decrease in methane gas production was observed as the HRT decreased to 2.25 and 1.5 days. The methane content in the AMCBR remained around 48 and 42% as the HRT was decreased from 2.25 to 1.5 days. Low HRTs (0.9 day) decreased the methane content in the AMCBR. The maximum AMX and COD yields were 99 and 98%, in the sequential AMCBR/CSTR system. The compartmentalization structure of the AMCBR supports the effective active growth of acidogen and methanogens, separately, and reduces the AMX and the toxicity. The EC50 values decreased from 282 to 132 mg/L in bacteria, fish (353–165 mg/L) and water flea (298–121 mg/L) bioassays, respectively. The most sensitive organism was found to be bacteria (*V. fischeri*). The results of this study showed that sequential system is a very useful and feasible process for treating the AMX antibiotic and for removing the acute toxicity.

**REFERENCES**


Chelliapan, S., Wilby, T. & Sallis, P. J. 2006 Performance of an up-flow anaerobic stage reactor (UASR) in the treatment of
Lange, B. 1994 LUMISmini, Operating Manual Dr. Bruno Lange, Düsseldorf, Germany.

Downloaded from https://iwaponline.com/wst/article-pdf/66/5/1117/443256/1117.pdf by guest


First received 24 October 2011; accepted in revised form 19 April 2012