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Impact of hydraulic retention time on the performance and archaea populations of an anaerobic reactor treating synthetic Tylosin wastewater

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ABSTRACT

Generally, the macrolide antibiotic Tylosin has been considered inhibiting the chemical oxygen demand (COD) removal in anaerobic digestion. In addition, some studies also demonstrated that the Tylosin affected the microbial populations involved during the degradation of organic compounds. Accordingly, the present study investigates the impact of Tylosin to an up-flow anaerobic stage reactor (UASR) performance and archaea populations at reduced hydraulic retention time (HRT), (4–1 day). Results showed only a minor reduction in COD removal efficiency (15%) was observed when the reactor was operated at high feed flow rate (1 day HRT). The minimal effect of the antibiotic on overall reactor performance confirms that the bacteria were adapted to Tylosin at low HRT (1 day). The fluorescent *in situ* hybridisation analysis revealed that the decrease in HRT from 4 to 1 day did not appear to cause any change in the archaeal community structure, in any of the UASR reactor stages. The predominance of archaeal cells (68–90%) was obvious in all stages of UASR at each investigated HRT with *Methanosaeta* (65–99%) dominated Stages 1–4 of UASR for all the HRT investigated.

Keywords: Archaeal cells; Fluorescent *in situ* hybridisation; Hydraulic retention time; Tylosin; Up-flow anaerobic stage reactor (UASR)

1. Introduction

There are very few published studies investigating the effect of hydraulic retention time (HRT) in staged reactors or anaerobic baffled reactors (ABR). Xing and Tilche [1] assessed the effect of HRT on the hybrid ABR at a constant loading of 10 kg COD m^{-3} d^{-1} and found that chemical oxygen demand (COD) reduction dropped from 75 to 40% when the HRT was changed from 1 to 6 day. Nachaiyasit and Stuckey [2] evaluated the performance of an ABR at HRT 20, 10 and 5 h at a constant feed COD of 4,000 mg L^{-1} (using synthetic carbohydrate-protein substrate) and showed a decrease in COD removal rate to 98, 90 and 52%, respectively. Moreover, they also investigated the effect of shock loads at constant organic loading rate, OLR (4.8 kg COD m^{-3} d^{-1}) with decreasing HRT and

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concluded that COD removal efficiency was affected only slightly (98, 97 and 96% at HRT 20, 10 and 6.6 h, respectively). Baloch and Akunna [3] studied the effect of rapid hydraulic shock loads on the performance of a granular bed baffled reactor using synthetic wastewater containing glucose as the main organic compound and showed high COD removal (94–97%) for all shock loadings (2.5–20 kg COD m^{-3} d^{-1}) upon decreasing HRT (48–6 h). Kuscu and Sponza [4] demonstrated that, as the HRT decreased from 10.38 to 2.5 day, the COD removal efficiencies decreased slightly from 94 to 92% in an ABR treating nitrobenzene. Recently, Thanwised [5] reported a study on the effects of varying HRT (24, 18, 12, 6 and 3 h) on COD removal in a continuous ABR treating tapioca wastewater. COD removal increased with reduction in HRT from around 14 (24 h HRT) to 29% (6 h HRT) and then decreased to 22% (3 h HRT). In summary, the literature above indicates that it is impossible to predict the effect of HRT on anaerobic treatment systems, since it depends on reactor configuration, type of feed and characteristics, OLR, type of biomass and method used to evaluate performance. Accordingly, each new system therefore requires specific investigation.

The novel up-flow anaerobic stage reactor (UASR) was developed according to the concept of the ABR, where each stage of the reactor represents a separate compartment. A stage reactor can provide high treatment efficiency since recalcitrant substrates will be in an environment more conducive to degradation [6]. The innovative design of the UASR results in the separation of acidogenesis and methanogenesis, which has potential benefits for reactor performance. With no moving parts or mechanical mixing, no requirement for biomass with unusual settling properties, and a high degree of stability to hydraulic and organic shock loads, the stage reactor has the potential to be applied economically as a pre-treatment system for many trade effluents [7].

The current study focused on the macrolide antibiotic Tylosin which is produced by a strain of *Streptomyces fradiae*. The main component of the mixture is Tylosin A, Tylosin B, Tylosin C and Tylosin D [8]. Tylosin has been widely used for the treatment of pneumonia, arthritis and dysentery caused by *Mycoplasma* and *Bacillus pastorianus* [9]. It has good anti-bacterial activity against most pathogenic organisms such as gram-positive bacteria, some gram-negative bacteria, vibrio, spirochetes and coccidian [10]. In addition, Tylosin is also incorporated into animal feed to improve growth rate and feed efficiency [11,12].

The aim of this study was to investigate the effects of HRT on an UASR system performances and the archaeal community structure during the treatment of synthetic wastewater containing Tylosin. The more specific objectives of this study were to confirm Tylosin can be tolerated by methanogenic sludge at reduced HRT and asses the stability of reactor for measured parameters (e.g. COD removal, pH profile, volatile fatty acids (VFAs) and methane production).

2. Materials and methods

2.1. Up-flow anaerobic stage reactor

The UASR system comprise four identical cylindrical Plexiglas compartments (Stages), 80 mm internal diameter by 640 mm height, linked in series, was constructed for the present study. The active volume of the UASR system was 11 L (4 Stages of 2.75 L). The operational set-up, flow diagram and the reactor design are presented in Fig. 1(a). Each stage of the reactor had a 3-phase separator baffle, angled at 45° and placed 50 mm below the effluent ports, to prevent floating granules from washing out with the effluent (Fig. 1(b)). Each stage was equipped with sampling ports at 100 mm intervals (lowest being 30 mm from the base) that allowed biological solids and liquid samples to be withdrawn from the sludge bed. The influent wastewater entered through a 12 mm internal diameter downcomer tube in the headplate that extended to within 15 mm of the reactor base and allowed feed to flow upward through the sludge bed. Effluent from each stage of the reactor flowed by gravity to the next, as each stage was placed on stepped platform having a 150 mm step height. The walls of the reactors were wrapped with a tubular poly vinyl chloride (PVC) water-jacket, 15 mm internal diameter, to maintain the reactor temperature at 37°C. Peristaltic pumps (Watson Marlow 100 series) were used to control the influent feed rate to the first stage of the UASR. Gas production was monitored separately for each stage using an optical gas-bubble counter having a measurement range of 0–1.5 L h^{-1} and precision within ±1%.

2.2. Reactor operation

In the first phase, the start-up of the reactor was established with a brewery wastewater feed and the reactor was operated with an OLR of 0.43–1.88 kg COD m^{-3} d^{-1} and a HRT of 4 days, which achieved the necessary acclimatisation of the sludge and stable operation required for the second phase of the investigation. A brewery wastewater was used in this study due to its ease of degradation and high COD value [13]. The brewery wastewater comprised mainly waste beer, i.e. out-of-date product, returned to the brewery for biological treatment which was then mixed

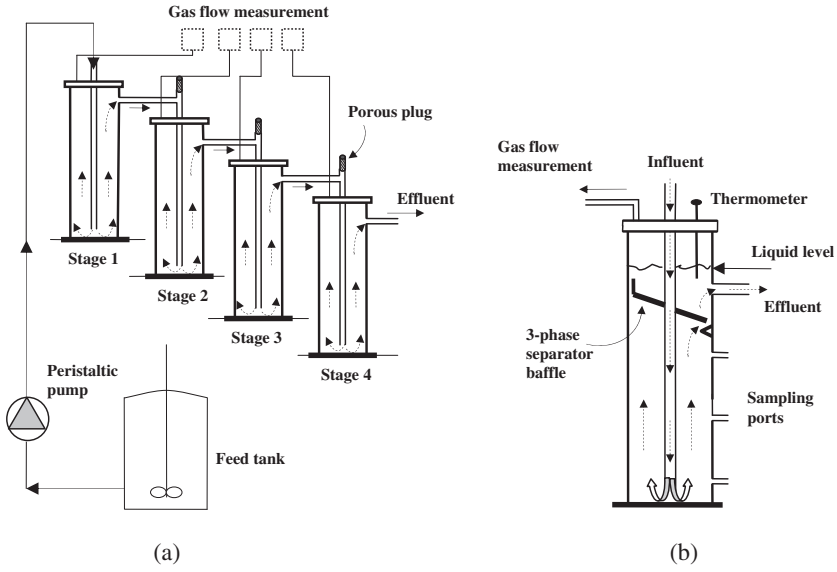

Fig. 1. (a) UASR system and flow chart; (b) details of an individual UASR stage.

with process wastewater in a balancing tank before treatment. Once the reactor had reached the steady state (93% COD removal from brewery wastewater), the feed to the reactor was supplemented with Tylosin in the form of Tylosin phosphate concentrate (supplied by Eli Lilly & Company Ltd., Liverpool, UK). The concentration of Tylosin was maintained at 200 mg L^{-1} at a constant OLR of 1.88 kg COD m^{-3} d^{-1} and the HRT was decreased gradually from 4 to 1 day by varying feed substrate concentration to the UASR.

2.3. Sampling and analysis

Supernatant liquor, gas and sludge samples were taken separately from Stage 1 to 4 for analysis. In addition, gas production rate was determined separately for each stage. Sample analysis included COD, pH and alkalinity, all according to standard methods [14]. Available phosphorous (PO_4–P) was determined by ion-chromatography (Dionex, DX-100 Ion Chromatograph), VFAs by gas–liquid chromatography (Unicam 610 Series Gas Chromatograph with auto-injector and PU 4811 computing integrator). Reactor gas composition (carbon dioxide, CO_2 and methane, CH_4) was determined by gas chromatography (Becker model 403 Gas Chromatograph with Unicam 4815 computing integrator).

2.4. Microbial community analysis

Samples of sludge from each stage of the UASR were collected through reactor sampling ports located nearest to the vertical mid-point of the sludge bed. An aliquot of these samples was fixed for *in situ* hybridisation using 4% paraformaldehyde and ethanol. Total cell counts using 4′, 6 diamidino-2-phenylindole (DAPI) were performed and means were calculated from 20 randomly chosen fields of view (FOV) for each sample. DAPI-staining [15,16] was used to quantify the relative proportion of the bacterial (EUB338) and archaeal cells (ARC915). Further insight into the archaeal community structure was also gained by using two family-genus probes, MX825 probe for *Methanosaeta* and MS821 probe for *Methanosarcina* [17]. The number of cells for each group specific probe was determined and means were calculated from 5 to 10 randomly chosen FOV for each sample. This data was used to calculate percentages of one specific group cells, relative to the total DAPI stained cells. Cells were visualised using a Zeiss standard microscope 14 (Carl Zeiss) or confocal laser scanning microscope.

2.5. Statistical analysis

Statistical analysis for valid cell counting was determined according to Davenport and Curtis [18]. In general, the following procedures were followed when analysing the cells: (1) dispersion ratio (variance/mean) was calculated for each of the counting, (2) checking the data for normality and homogeneity of variances, (3) nested ANOVA for determining the level of the most variation, (4) determination of the sample size, and (5) significant test, e.g. *t*-test or

ANOVA. For DAPI-stained counting, the number of cells per FOV was determined for 20 random observations. The variance within these levels was determined using nested analysis of variance with the MINITAB V14 program (Minitab Inc., Philadelphia, USA).

3. Results and discussion

3.1. UASR performance

Table 1 depicts the UASR performance in terms of COD removal, total VFA, methane production and pH profile in each stage of the reactor system at different HRT. From the table, it can be seen that the COD removal efficiency became less efficient (91.83–76.79%) and more variable with the HRT reduction (4–1 day). The results show that a dramatic effect on the reactor performance did not occur; however, partial inhibition by the macrolide antibiotic may have caused the COD to decrease further at HRT 1 day (76.79%) than would have been the case if Tylosin had not been present. The drop in treatment efficiency in this study at HRT 1 day might be due to high applied Tylosin load, and not due to short HRT in the UASR. Partial inhibition of bacterial biomass by Tylosin (especially in Stage 1) may have resulted in lower methanogenic activity to such an extent that the VFAs were not well metabolised, resulting in the increased effluent COD. Moreover, the difference in COD removal between HRT 4 and 1 day (91.83–76.79%) may be due to more recalcitrant molecules needing longer time for bacterial degradation in the anaerobic biomass. However, the minimal effect on reactor performance confirms that the UASR reactor was efficient at low HRTs and therefore, short HRT was not responsible for the large drop in treatment efficiency that was seen when high OLR had been achieved with real pharmaceutical wastewater by decreasing HRT to 2 day rather than increasing the COD of the feed [19]. Since stable reactor performance in an ABR or compartmentalized anaerobic reactor is indicated by an effluent total VFA concentration below 500 mg L^{-1} [20,21], it can be concluded that UASR operation was stable since the effluent VFA was less than 260 mg L^{-1} (Stage 4) throughout the experimental period (HRT 4–1 day). Methane production in each stage differed, with Stage 2 generally producing more methane (8097.49 mL d^{-1}) than the other stages. Methane production in all the stages of UASR regardless of the HRT, confirms high methanogenic activity in the reactor system. These results show that bacteria were readily adapted to wastewater containing Tylosin at lower HRTs and did not affect the reactor performance substantially.

Table 1
Typical COD removal, methane production, total VFA and pH in UASR stages at different HRT (average values when reactor approached steady-state)

		HRT steps (day)			
UASR stages	Parameter	1	2	3	4
Stage 1	COD removal (%)	38.73	42.99	63.19	82.45
	Methane (mL d^{-1})	3246.67	3172.67	2853.21	5625.17
	Total VFA (mg L^{-1})	758.26	776.77	714.33	580.00
	pH	6.50	6.57	6.74	7.16
Stage 2	COD removal (%)	19.32	19.15	8.85	2.55
	Methane (mL d^{-1})	2642.79	3195.17	1871.98	737.66
	Total VFA (mg L^{-1})	299.29	498.25	517.02	396.48
	pH	6.99	7.04	7.37	7.46
Stage 3	COD removal (%)	14.04	16.05	8.55	3.95
	Methane (mL d^{-1})	762.15	1274.30	2157.77	794.48
	Total VFA (mg L^{-1})	200.21	272.80	276.11	284.66
	pH	7.36	7.25	7.59	7.68
Stage 4	COD removal (%)	4.70	4.59	2.25	2.88
	Methane (mL d^{-1})	136.21	455.35	585.76	216.39
	Total VFA (mg L^{-1})	79.82	127.73	260.63	183.96
	pH	7.78	7.89	7.74	7.81
	Total COD removal (%)	76.79	82.78	82.84	91.83
	Total methane (mL d^{-1})	6787.82	8097.49	7468.72	7373.70

3.2. Archaeal population

Fig. 2 shows the total number of cells in each stage of the UASR system at various HRT investigated. The total number of cells counted in all stages for all the HRT investigated did not appear to change substantially, with the mean number in each stage of the reactor falling within the range of 1.12×10^9–1.32×10^9 over the entire experimental period. This result signifies that the bacteria were able to withstand Tylosin at reduced HRT and the antibiotic did not reduce the total number of cells in the reactor.

Hybridisation using universal bacteria probe EUB338 and archaea probe ARC915 revealed the distribution of both these phyla throughout the reactor, for each investigated HRT. Results in Fig. 3 are given as percentages from the comparison of probe-specific cell numbers with those of DAPI-stained cells. Generally, the predominance of archaeal cells was obvious in all the stages of UASR at each investigated HRT. Furthermore, the results did not show any definite population shift through the reactor, with predominance of archaeal in all the stages of the

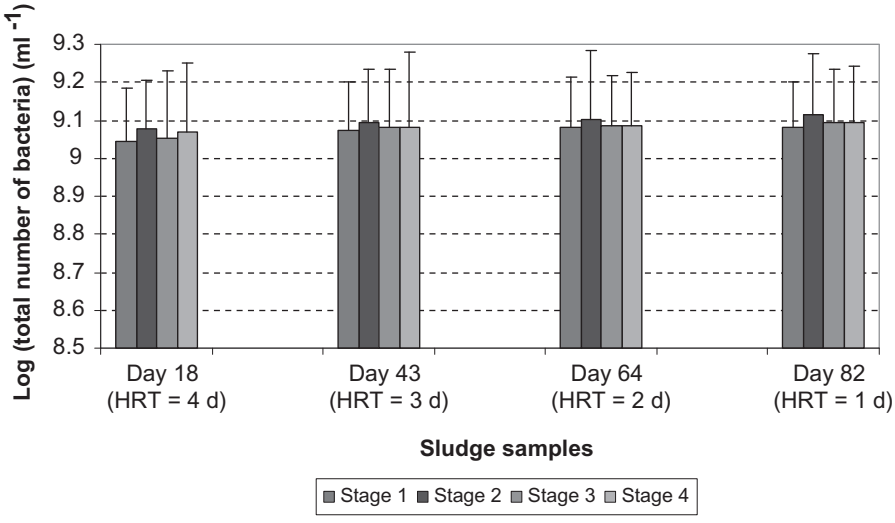

Fig. 2. Total number of cells counted using DAPI in all the stages of UASR at reduced HRT.

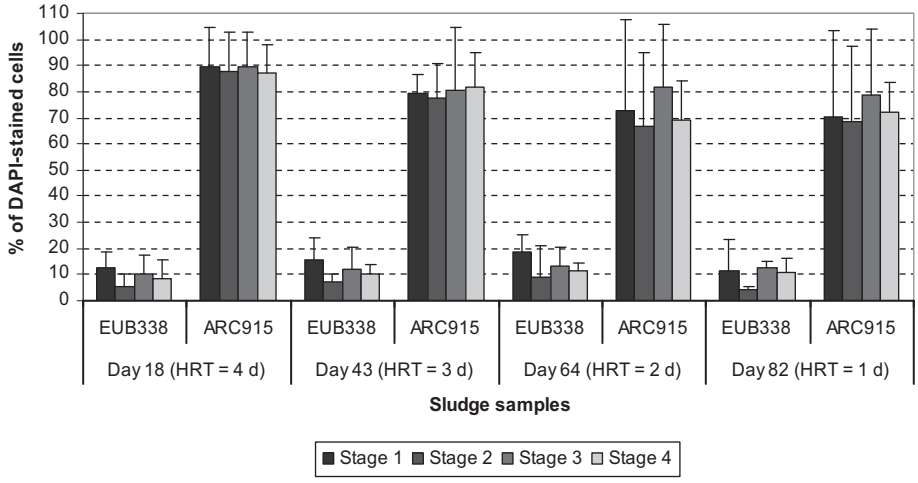

Fig. 3. Percentages of DAPI-stained cells detected by FISH with probes for archaea (ARC915) and bacteria (EUB338) in each stage of UASR for each investigated HRT. Bars indicate standard deviation (*n* ranged from 5 to 10 microscopic fields).

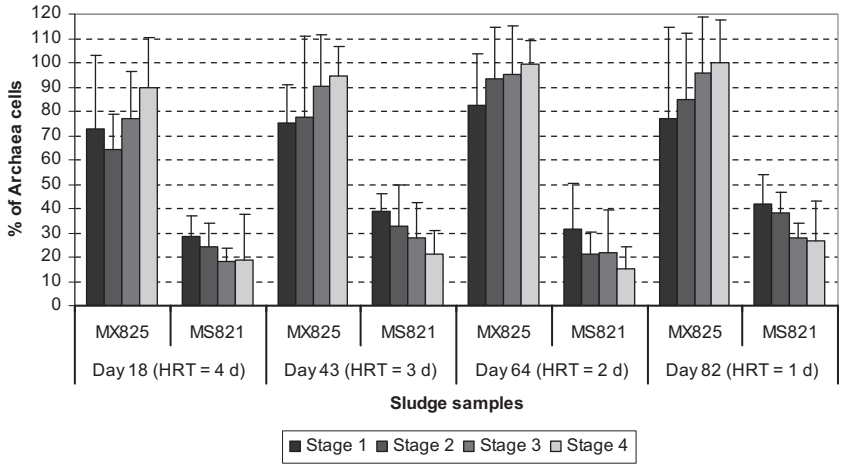

Fig. 4. Archaeal community analysis of UASR stages, sampled at each investigated HRT, showing counts obtained using probes MX825 and MS821 expressed as percentage of total archaeal counts using probe ARC915. Bars indicate standard deviation (*n* ranged from 5 to 10 microscopic fields).

reactor. These results were supported by the pH data and the methane production (Table 1), as there was no substantial change in either parameter. It can be concluded that Tylosin did not affect the microbial populations adversely in response to changes in the HRT, or affect the process performance of the reactor. Furthermore, there appeared to be no particular shift in the microbial community structure across the stages of UASR, with a predominance of archaeal cells being evident in all the stages regardless of HRT.

Further insight into the archaeal community structure was also gained by using two family-genus probes, MX825 probe for *Methanosaeta* and MS821 probe for *Methanosarcina*. It is well documented that acetoclastic methanogens which utilise acetate as a substrate, play an important role in anaerobic processes especially in the terminal step of methanogenesis [17]. Consequently, the samples were hybridised with these two family and genus specific rRNA oligonucleotide probes. In a study by Raskin [22], it was concluded that MS821 was the preferred probe to detect *Methanosarcina* cells and MX825 for *Methanosaeta* cells [23]. The changes in these populations were determined and Fig. 4 shows the plots of the archaeal community analysis of UASR Stages 1–4, sampled at each investigated HRT and shows that cells hybridised with *Methanosaeta* probe (MX825) dominated Stages 1–4 of UASR for all the HRT investigated. This result supports the evidence seen with reactor performance (Table 1) where there was a low level of acetic acid production (in total VFA) in the reactor stages, which should favour *Methanosaeta*. It is generally accepted that *Methanosaeta* with a high affinity for acetate is dominant in systems with low acetate concentrations, while *Methanosarcina* are more competitive in systems with high acetate concentrations [6].

4. Conclusion

Although Tylosin degradation rate was not monitored in this study, results showed stable performance in terms of pH, methane production and VFA when the reactor was operated at high feed flow rate (1 day HRT). In general, the minimal effect of the antibiotic on overall reactor performance confirms that the bacteria were adapted to Tylosin at low HRTs. Decrease in HRT from 4 to 1 day at constant Tylosin concentration did not appear to cause any change in the archaeal community structure in any of the UASR reactor stages. These results showed that the UASR reactor was efficient at low HRTs and therefore, short HRT was not responsible for the large drop in treatment efficiency that was seen in our previous study [16] when high OLR had been achieved with real pharmaceutical wastewater by decreasing HRT to 2 day.

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