Fate of tetracycline resistant bacteria as a function of activated sludge process organic loading and growth rate

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Abstract The objective of this research was to elucidate the fate of tetracycline resistant bacteria as a function of activated sludge organic loading rate and growth rate. Techniques employed to evaluate the effect of these factors on the fate of tetracycline resistant bacteria were: (1) resistant bacteria concentrations in the SBR biomass; (2) production of tetracycline resistant bacteria as measured by a combination of effluent efflux and intentional solids wasting; (3) net specific growth rates as determined by an SBR population balance; and (4) percentage of resistance as determined by normalising resistant concentrations to total concentrations. Based on these evaluation parameters, increases in organic loading and growth rate both resulted in amplification of tetracycline resistance. These trends were observed for activated sludge reactors loaded with typical municipal background tetracycline concentrations (~ 1 µg/L) and those receiving influent augmented with 250 µg/L tetracycline. Accordingly, biological wastewater treatment plants, such as the activated sludge process, may be significant sources of antibiotic resistance to the environment.

Keywords Activated sludge; growth rate; organic loading rate; tetracycline; tetracycline resistant bacteria

Introduction

The activated sludge process has been a highly effective process to remove organics from domestic wastewater. More recently, there has been concern about the fate of micro-contaminants, such as tetracyclines, in the activated sludge process. Tetracycline has been used widely in human therapy and livestock due to its low toxicity and broad-spectrum activity (Madigan et al., 1997). The tetracycline group is the second most widely used antibiotic in the world after penicillins (Col and O’Connor, 1987). Not only is the fate of influent antibiotics of concern, but their potential to amplify antibiotic resistance during treatment is also of concern. Influent domestic wastewater contains significant numbers of antibiotic resistant organisms that survive in conveyance systems (Guardabassi and Dalsgaard, 2002) and provides a continuing flux of resistance to treatment plants.

As the ultimate source of both excreted antibiotics and antibiotic resistant organisms to the environment, there is a clear need to elucidate the fate of antibiotic resistant bacteria in activated sludge as a function of typical operational parameters. Some studies have concluded that treatment plants raise antibiotic resistance because an increased portion of antibiotic resistance, normalised by total target organisms, has been observed (Anderson, 1993; Mezrioui and Baleux, 1994). However, other researchers have shown the opposite result (Kish and Lampky, 1983; Walter and Vennes, 1985). Guardabassi and Dalsgaard (2002) suggested that these conflicting results could be due to factors affecting the efficiency of removal of resistant bacteria at different plants or differences in the materials and methods used for the assessment of antimicrobial resistance. Other researchers have
suggested that differences in the treatment plants and/or their operation affect the fate of resistant bacteria or their resistance genes (Mezrioui and Baleux, 1994).

Accordingly, the objective of this research was to study the fate of tetracycline resistant organisms in the activated sludge process as a function of typical operational parameters. The organic loading rate, as dictated by influent strength and hydraulic residence time (HRT), and the growth rate, as controlled by solids retention time (SRT), are two conventional parameters typically used to operate and control activated sludge processes (Metcalf and Eddy, Inc., 2002). Based on the research objective, two hypotheses were tested: (1) increases in organic loading rate amplify tetracycline resistant bacteria; and (2) increases in growth rate amplify tetracycline resistant bacteria. Data generated as a part of this study are expected to contribute to a better understanding between common process variables and survival/amplification of tetracycline resistant bacteria.

**Methods and materials**

**Study design**

Four 4-L Plexiglas lab-scale sequencing batch reactors (SBRs) were operated in this study. The town of Amherst, New York Wastewater Treatment Plant No. 16 primary clarifier effluent was used as the influent source. Preliminary studies showed that influent wastewater could be stored for up to 5 days without significant degradation (Kim, 2005). Accordingly, wastewater was collected twice weekly (Tuesday and Friday) and stored at 4°C for later use. The initial biomass inocula for all SBRs were obtained from the Amherst, New York Stage 1 aeration basins.

The SBRs were operated with different rates of influent addition to mimic different feeding patterns employed in a variety of activated sludge processes. In two SBRs, influent was added over a 2 min period simulating a pulse load, called “slug”. The other two SBRs had influent added slowly and continuously over the appropriate feed cycle time and more closely mimicked a continuously fed complete mix reactor, called “cont”. No consistent differences in tetracycline resistance were noted between the two feed patterns employed (Kim, 2005), and the results from both are grouped in this paper. Because tetracycline has been shown to be a strong selector for antibiotic resistant populations (Kim, 2005), each set of two SBRs based on rate of feed addition was further subdivided by the amount of tetracycline it received. In one set (B), the SBRs were fed wastewater with background tetracycline which was approximately 1 μg/L (Kim et al., 2005). In the other set of two (A types), the SBRs were augmented with 250 μg/L tetracycline. The tetracycline concentration employed in the augmented reactors mimics concentrations found in agricultural wastewater (Meyer et al., 2000). Figure 1 is the schematic diagram of SBR system.

![Figure 1 Schematic diagram of SBR system](https://iwaponline.com/wst/article-pdf/55/1-2/291/430900/291.pdf)
Operation
To evaluate the effect of activated sludge operation on the fate of tetracycline resistant bacteria, three phases of operation were applied during the study. Changes in organic loading were achieved by altering the influent wastewater flux. Changes in growth rate were achieved by altering the solids retention time of the reactors and were independent of organic loading rate. Using this approach, the organic loading rates in Phases 1, 2 and 3 were 0.18, 0.77 and 0.82 kg TCOD/m³·d, respectively. In Phases 1, 2 and 3, average growth rates, calculated using a mass balance on total suspended solids, were 0.1, 0.1 and 0.33 d⁻¹, respectively. Accordingly, in Phase 1, SBRs were operated under low growth rates and low organic loading rates. In Phase 2, SBRs were operated under high organic loading rate and low growth rates. In Phase 3, SBRs were operated under high organic loading and high growth rates.

Daily SBR operation consisted of four separate periods in each cycle. These were fill, react, settle and decant. The length of each period for the experimental phase is presented in Table 1.

Sampling and analytical methods
During the study, the SBR and its effluent were sampled twice a week for pH, total and filtered chemical oxygen demand (TCOD/FCOD), total and volatile suspended solids (TSS/VSS) and dissolved oxygen (DO), according to Standard Methods (Standard Methods, 1998). Commercially available tetracycline ELISA kits (R-Biopharm GmbH, Darmstadt, Germany) were used for the detection of tetracycline concentration in feed wastewater and effluents of reactors. The ELISA technique has been described previously (Kim et al., 2005).

Tetracycline resistant heterotrophs, total enterics, and lactose fermentors in the SBR influent, biomass, effluent, and wasting were monitored twice weekly using a spread plate method as outlined in Standard Method 9215C (Standard Methods, 1998). The surviving colonies on agar with 5 mg/L tetracycline were defined as intermediate tetracycline resistant bacteria and colonies surviving on 20 mg/L tetracycline were considered as tetracycline resistant bacteria based on recommendations by the National Antimicrobial Resistance Monitoring System (NARMS, 2001). Cultures were also plated with no tetracycline for control purposes. Before plating, each biomass sample was blended for 3 min to homogenise the culture, while influent and effluent samples were blended for 2 and 1 min, respectively (Kim, 2005). For each sample, duplicate plate counts were conducted and the average used for statistical analysis.

Data analysis
Four techniques were employed to evaluate the effect of influent tetracycline on the fate of tetracycline resistant bacteria: (1) resistant concentrations in the SBR biomass;

Table 1 Operating schedule of sequencing batch reactors

<table>
<thead>
<tr>
<th>Feed</th>
<th>Phase</th>
<th>Organic loading rate (kg TCOD/m³·d)</th>
<th>Growth rate (d⁻¹)</th>
<th>Operating time in a cycle</th>
<th>Cycles/day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Fill (min)</td>
<td>React (min)</td>
<td>Settle (min)</td>
<td>Decant (min)</td>
</tr>
<tr>
<td>Slug</td>
<td>1</td>
<td>0.18</td>
<td>0.1</td>
<td>2</td>
<td>598</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.77</td>
<td>0.1</td>
<td>2</td>
<td>298</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.82</td>
<td>0.33</td>
<td>2</td>
<td>298</td>
</tr>
<tr>
<td>Cont</td>
<td>1</td>
<td>0.18</td>
<td>0.1</td>
<td>600</td>
<td>600</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.77</td>
<td>0.1</td>
<td>300</td>
<td>300</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.82</td>
<td>0.33</td>
<td>300</td>
<td>300</td>
</tr>
</tbody>
</table>
production of tetracycline resistant bacteria as measured by a combination of effluent efflux and intentional solids wasting; (3) net specific growth rates as determined by a SBR population balance; and (4) percentage of resistance as determined by normalising resistant concentrations to total concentrations. Net specific growth rates were calculated for each sampling date using a population balance for the sampled SBR, as shown in equation (1):

\[ V \frac{dX}{dt} = Q_{in}X_{in} - Q_eX_e + \mu_{net}XV - Q_wX \]  

(1)

where \( \mu_{net} \) = net specific growth rate for tetracycline resistant bacteria (d\(^{-1}\)); \( X \) = tetracycline resistant bacterial concentrations in SBR mixed liquor (CFU/mL); \( X_{in} \) = influent tetracycline resistant bacterial concentrations (CFU/mL); \( X_e \) = effluent tetracycline resistant bacterial concentrations (CFU/mL); \( X_w \) = wasted tetracycline resistant bacterial concentrations (CFU/mL); \( Q_{in} \) = daily influent wastewater flow rate (L/d); \( Q_w \) = daily wasted volume (L/d); \( Q_e = Q_{in} - Q_w (t) \); and \( V \) = Reactor volume (L).

With the exception of \( dX/dt \) and \( \mu_{net} \), all parameters in equation (1) were measured directly. The term \( dX/dt \) was calculated by measuring the difference in bacterial counts between sampling dates. Once \( dX/dt \) was known, \( \mu_{net} \) was calculated directly using equation (1). This approach provided multiple values of \( \mu_{net} \) for each phase enabling statistical comparisons.

The Wilcoxon rank sum test at significance level of 0.05 was adopted for statistical analysis because these data were not normally distributed. Details of the Wilcoxon rank sum test are provided in Kim (2005). One phase was judged as having a larger (or smaller) value than another phase when the score result was larger (or smaller) than the range. The ranges were calculated at a significance level of 0.05. Shown in Table 2 are the percentage frequencies of where Phase 2 percent intermediate resistant and resistant heterotrophs were greater than Phase 1 (P2 > P1), etc. for each condition tested. For ease of presentation, these percent frequencies are plotted as bar charts in the results section. The cases where there was no significant difference (e.g. P1 = P2) are not plotted. Accordingly, the percentages in the graphs add up to 100% only in cases where all the results favoured one phase or the other.

Results and discussion
Activated sludge process performance

All monitored items except tetracycline were within typical values during the study period. The pH, DO, MLSS, effluent TSS and FCOD data collected were typical of a

Table 2 Sample results about percentage of resistance (PR) comparisons for heterotrophic bacteria

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Intermediate resistant (% ± S.D.)</th>
<th>Resistant (% ± S.D.)</th>
<th>Wilcoxon rank sum test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Range</td>
<td>Intermediate resistant</td>
<td>Resistant</td>
</tr>
<tr>
<td>Cont A</td>
<td>P1</td>
<td>6.21 ± 3.67</td>
<td>1.76 ± 0.50</td>
</tr>
<tr>
<td></td>
<td>P2</td>
<td>11.83 ± 3.26</td>
<td>2.62 ± 0.89</td>
</tr>
<tr>
<td>Slug A</td>
<td>P1</td>
<td>6.77 ± 2.61</td>
<td>1.94 ± 0.72</td>
</tr>
<tr>
<td></td>
<td>P2</td>
<td>7.86 ± 3.11</td>
<td>2.47 ± 0.59</td>
</tr>
<tr>
<td>Cont B</td>
<td>P1</td>
<td>3.90 ± 1.79</td>
<td>2.10 ± 0.79</td>
</tr>
<tr>
<td></td>
<td>P2</td>
<td>5.67 ± 2.40</td>
<td>1.84 ± 0.85</td>
</tr>
<tr>
<td>Slug B</td>
<td>P1</td>
<td>4.55 ± 1.87</td>
<td>2.06 ± 0.59</td>
</tr>
<tr>
<td></td>
<td>P2</td>
<td>4.80 ± 2.09</td>
<td>1.59 ± 0.72</td>
</tr>
</tbody>
</table>

S.D.: standard deviation
well operated activated sludge process with little differences noted between phases. The tetracycline concentrations were different depending on influent loading and operation. These data were presented and discussed earlier (Kim, 2005; Kim et al., 2005).

**Statistical analysis results summary**

The statistical analysis results obtained by comparing the results of Phases 1 and 2, and Phases 2 and 3, represent the effect of organic loading rate change and growth rate change on tetracycline intermediate resistant and resistant bacteria, respectively. The results of these analyses are plotted in Figures 2 and 3 for organic loading rate and growth rate, respectively.

**Impact of organic loading rate.** As shown in Figure 2, tetracycline intermediate resistant and resistant heterotrophic, total enteric, and lactose-fermenting bacterial concentrations (BC) and productions (BP) were statistically higher for the higher organic loading rates of Phase 2 when compared to lower ones in Phase 1. This trend was consistent for background influent tetracycline loadings (B SBRs) and those with 250 µg/L added (A SBRs). Higher organic loading rates also resulted in higher percent resistance (PR) for all bacterial populations studied at the higher influent tetracycline concentrations. The relationship between organic loading rates and percent resistance is not as clear for SBRs receiving background tetracycline.

Higher net specific growth rates (NSG, $\mu_{\text{net}}$) of intermediate resistant and resistant bacteria were more frequently observed for the lower organic loading conditions of Phase 1. For both background tetracycline SBRs and those augmented with tetracycline, this trend is more obvious for total enteric and lactose-fermenting bacteria as compared to heterotrophic bacteria where there was little difference. These observations are consistent with SBR operation in which heterotrophic growth rates were intended to match one another and controlled through the use of total suspended solids, which are typically used

![Figure 2](https://iwaponline.com/wst/article-pdf/55/1-2/291/430900/291.pdf)

**Figure 2** The summary of organic loading rate effect on tetracycline intermediate resistant and resistant bacteria (P1: Phase 1, P2: Phase 2) [BC: bacterial concentration; BP: bacterial production; NSG: net specific growth rate ($\mu_{\text{net}}$); and PR: percentage of resistance; A: influent augmented with 250 µg/L tetracycline, B: background tetracycline concentration ~1 µg/L]
as a surrogate for heterotrophs. There are two complementary reasons for the higher net growth rates observed for total enterics and lactose fermenters in Phase 1: 1) there was little new growth of enterics or lactose fermenters in any of the SBRs during these phases; and 2) Phase 2 SBRs had higher loss rates of enterics and lactose fermenters through effluent flux ($Q_eX_e$ from equation (1)) than did their Phase 1 counterparts.

Impact of growth rate. As shown in Figure 3, the higher heterotrophic growth rate conditions applied in Phase 3 resulted in generally higher tetracycline resistant heterotroph, total enteric, and lactose fermenter concentrations (BC) in SBRs receiving influent with 250 mg/L tetracycline added as compared to their Phase 2 counterparts. For each population, half of the SBRs demonstrated higher tetracycline resistance while there was no difference between phases for the remaining half.

For SBRs receiving background tetracycline, little difference was noted for heterotrophs and lactose fermenters, but total enterics clearly favoured the higher growth rate conditions of Phase 3. It is somewhat surprising that any of the Phase 3 SBRs resulted in statistically higher concentrations of tetracycline resistant populations because of the higher imposed rates of wasting ($Q_eX_e$ component of the mass balance presented in equation (1)) needed to achieve the higher growth rate conditions of Phase 3. Accordingly, the fact that comparisons between Phase 3 and Phase 2 concentrations were found either to be neutral or favour Phase 3 suggests that increase in growth rate positively impacts the fate of tetracycline resistance.

Even stronger evidence for the positive impact growth rate plays on the fate of tetracycline resistant and resistant populations can be observed when comparing bacterial production (BP) and growth rate (NSG, $\mu_{net}$) data between Phases 2 and 3. Two possibilities exist for these results. First, resistant populations present in the influent responded favourably to the growth conditions applied and achieved net positive growth. Second, increases in tetracycline resistant gene transfer frequencies to host populations may have increased resistance. Levin et al. (1979) reported that the fastest
rate of R-plasmid conjugation is thought to occur during high growth rate. Ehlers (1997) suggests that the positive strong relationship between increased growth rates and conjugation process is because they both require replication of DNA and the fastest rate of conjugation is thought to occur during exponential growth phase.

There are no clear trends in the percent resistance data which could be used to support the hypothesis that increased growth rates amplify tetracycline resistance.

Conclusions

Based on the results of this research, the following conclusions were reached:

- increases in organic loading rate increased tetracycline resistance;
- increases in growth rate increased tetracycline resistance;
- the positive impact of organic loading rate and growth rate on tetracycline resistance was observed for tetracycline concentrations typical of domestic wastewater influent and those of agricultural wastewaters.

This study has significant ramifications on the fate of tetracycline resistance and demonstrates that the fate of resistant populations is a function of common activated sludge operational parameters. Under typical operating conditions, biological wastewater treatment plants may be significant sources of tetracycline resistance to the environment.

References


