Modeling the biotransformation of trimethoprim in biological nutrient removal system

Olumuyiwa O. Ogunlaja and Wayne J. Parker

ABSTRACT

A pilot scale biological nutrient removal (BNR) process, batch experiments and modeling exercises were employed to investigate the removal and biotransformation of trimethoprim (TMP) in a BNR activated sludge process. The concentrations of the active microbial groups – ammonia oxidizing bacteria (AOB), ordinary heterotrophic organisms (OHOs) and polyphosphate accumulating organisms (PAOs) – in the BNR bioreactor were quantified through modeling of the pilot bioreactor. The overall TMP removal efficiency for the pilot BNR process was 64 ± 14% while the TMP biotransformation efficiencies in the anaerobic, anoxic and aerobic zones were 22 ± 20%, 27 ± 8% and 36 ± 5% respectively. Batch tests with and without nitrification inhibition showed that AOB played a role in the biotransformation of TMP in BNR activated sludge. A pseudo first order model which incorporated the contributions of PAOs, OHOs and AOB to the overall biodegradation of TMP was found to describe the biodegradation of TMP in batch tests with and without nitrification inhibition. This model showed that PAOs, OHOs and AOB contributed towards the biotransformation of TMP in aerobic BNR activated sludge with the biotransformation rate constants following the trend of $k_{AOB} > k_{OHOs} > k_{PAOs}$.

Key words | ammonia oxidizing bacteria, biological nutrient removal, biotransformation, ordinary heterotrophic organism, polyphosphate accumulating organism, trimethoprim

ABBREVIATIONS

- AOB: Ammonia oxidizing bacteria
- AN: Anaerobic
- AX: Anoxic
- AO: Aerobic
- BOD: Biochemical oxygen demand
- BNR: Biological nutrient removal
- cBOD: Carbonaceous BOD
- COD: Chemical oxygen demand
- DO: Dissolved oxygen
- HRT: Hydraulic retention time
- LC-MS/MS: Liquid chromatography interfaced with a tandem mass spectrometer
- MLVSS: Mixed liquor volatile suspended solids
- N: Nitrogen
- NH$_3$: Ammonia
- NO$_3$: Nitrate
- NO$_2$: Nitrite
- NOB: Nitrite oxidizing bacteria
- NSE: Nash–Sutcliffe efficiency
- OHO: Ordinary heterotrophic organism
- PAO: Phosphorus accumulating organism
- P: Phosphorus
- PHB: Poly-β-hydroxybutyrate
- PO$_4$-P: Orthophosphate
- SRT: Solids residence time
- TKN: Total Kjeldahl nitrogen
- TMP: Trimethoprim
- TP: Total phosphorus
- TrOC: trace organic compound
- VFA: Volatile fatty acid
- WWTP: Wastewater treatment plant

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INTRODUCTION

The removal of trace organic compounds (TrOCs) in wastewater treatment plants (WWTPs) has been a major research area due to the potential risks associated with the undesirable effects of TrOCs on the ecosystem and human health (Heberer 2002; Jones et al. 2004; Fent et al. 2006). Many of these compounds are either recalcitrant or partially removed during wastewater treatment because WWTPs were not originally designed to remove TrOCs (Bueno et al. 2012; Zhang et al. 2014a, 2014b). Effluents from WWTPs have been repeatedly identified as a primary source of TrOCs in the environment (Kolpin et al. 2002; Ternes et al. 2003; Lishman et al. 2006). Consequently, the optimization of WWTPs could play a critical role in attenuating the discharge of TrOCs into the environment (Onesios et al. 2009; Pomies et al. 2013).

WWTPs that are configured for biological nutrient removal (BNR) have been reported to have higher removal efficiencies of TrOCs along with conventional pollutants such as ammonia-nitrogen (NH$_3$-N), nitrate-nitrogen (NO$_3$-N), carbonaceous biochemical oxygen demand (cBOD) and total phosphorus (TP) when compared to other activated sludge treatment systems (Kimura et al. 2007; Ogunlaja & Parker 2015). These previous studies have investigated the effects of WWTP design and operating conditions on the removal of TrOCs from wastewater. Parameters such as hydraulic retention time (HRT), solids retention time (SRT), biomass type, type and presence of growth substrate, temperature, redox conditions, pH, structure and physico-chemical properties of the TrOCs have been reported to have effects on the performance of BNR with respect to TrOCs removal (Cirja et al. 2007; Helbling et al. 2012; Phan et al. 2014). Despite all these studies, it is still unclear how these factors contribute to the more effective removal of TrOCs in BNR systems when compared to conventional activated sludge systems.

Biotransformation or biodegradation has been identified as a major removal mechanism for attenuating the discharge of TrOCs in the effluents of WWTPs. Biodegradation involves the use of microorganisms that are capable of assimilating target TrOCs either directly as growth substrate (Yang et al. 2013) or indirectly through co-metabolism. It is hypothesized that the TrOCs biotransformation pathways in BNR activated sludge systems may differ between the various microbial groups that are present in the activated sludge. However, few studies have elucidated the roles that these microbial communities play in the biotransformation of TrOCs in activated sludges (Shi et al. 2004; Yang et al. 2013; Phan et al. 2014). In addition, there is no agreement in the literature on the types of microorganism that are responsible for the biodegradation of TrOCs in activated sludge systems (Shi et al. 2004; Yang et al. 2013). Hence, further study is required to investigate the role of specific active microbial groups in the biotransformation of TrOCs in BNR activated sludge.

Trimethoprim (TMP), a hydrophilic synthetic antibiotic compound, was selected in this study as a representative TrOC because it is one of the prevalent TrOCs found in wastewater. TMP is usually prescribed for the treatment of chest or urinary tract infections (Batt et al. 2006) TMP has been found to be present in the effluent of WWTPs due to its limited biodegradability (Khunjar et al. 2011) and hydrophilic nature (Nolan et al. 1989). The reported removal of TMP in WWTPs has been below 10% (Paxeus 2004) and concentrations as high as 0.53 μg/L have been reported in the effluents of WWTPs in the United States of America (Kümmcrer et al. 2000; Aga 2008). There are limited reports on the removal of TMP in BNR systems. Therefore, the biotransformation of TMP in a BNR activated sludge was the focus of this paper.

Models of TrOC fate can be employed to analyze test data for obtaining an improved understanding of the processes involved in TrOC removal. The biotransformation of TrOCs in activated sludge systems has usually been described by either first order (Batt et al. 2005; Glassmeyer et al. 2005) or pseudo first order kinetics (Hashimoto & Murakami 2009). These kinetic expressions typically include a kinetic rate coefficient, the dissolved TrOC concentration and the biomass concentration. In previous biodegradation studies, the biomass concentration has been approximated by the total or volatile suspended solid concentration (Maurer et al. 2007; Wick et al. 2009). A limitation of this approach is that it does not consider the potential contributions of the different microbial organisms which are present in activated sludge.

Nitrification is the conversion of ammonia or ammonium to nitrite, followed by the oxidation of nitrite to nitrate. The biotransformation of ammonia to nitrite is performed by ammonia oxidizing bacteria (AOB) and the conversion of nitrite to nitrate is performed by nitrite oxidizing bacteria (NOB). The conversion of ammonia to nitrite is the rate limiting step of the nitrification reaction. Heterotrophic organisms are organisms that absorb organic carbon to produce energy and synthesize other essential compounds to maintain their life. Heterotrophs that are unable to absorb polyphosphate are called ordinary heterotrophic organisms.
(OHOs) while those that can absorb polyphosphate are called polyphosphate accumulating organisms (PAOs). These groups of microorganisms represent the microbial populations that are typically considered present in BNR activated sludge (Tchobanoglous et al. 2005).

For the purpose of this study, AOB were selected to be investigated over NOB because AOB typically mediate the rate limiting step of the nitriﬁcation reaction. Hence, it was hypothesized that the integration of the fractions of AOB, OHOs and PAOs into the biotransformation models may provide an improved description of the biotransformation of TrOCs in BNR activated sludge. In addition, the current TrOC biodegradation models do not adjust the rate parameters to accommodate for the impact of the redox zones (anaerobic, anoxic and aerobic) in BNR systems.

The objectives of this study were to (1) estimate the fractions of the active biomass (PAOs, OHOs and AOB) in a BNR activated sludge process, (2) investigate the removal of TMP in pilot BNR process and batch reactors, (3) estimate the biotransformation rate constants of TMP with respect to PAOs, OHOs and AOB under different redox condition using BNR activated sludge and (4) assess the contributions of PAOs, OHOs and AOB towards the removal of TMP under different redox conditions.

METHODS

BNR pilot plant description

The pilot scale University of Cape Town (UCT)-BNR process consisted of a 0.718 m³ primary clarifier tank, 0.36 m³ bioreactor and a 0.286 m³ final clarifier for solid–liquid separation. A detailed description of the UCT-BNR pilot plant is provided elsewhere (Ogunlaja 2015) and a schematic diagram is presented in the supporting information (SI) Figure S1. The operating and design conditions of the UCT-BNR pilot plant are summarized in SI Table S1. (Figure S1 and Table S1 are available with the online version of this paper.) The pilot plant was operated on raw municipal wastewater that was augmented with sodium bicarbonate, di-potassium phosphate and sodium acetate to achieve influent chemical oxygen demand (COD), alkalinity and total phosphorus concentrations of 367 ± 48 mg/L, 268 ± 21 mg/L as CaCO₃ and 11 ± 7 mg/L respectively. The raw wastewater was augmented to stabilize the variability in the concentrations of these constituents.

The pilot plant was operated for 12 months with regular monitoring of conventional (NH₃-N, NO₃-N, NO₂-N, cBOD, TP) and operational (flow rates, SRT, HRT, waste flow rate, dissolved oxygen (DO), pH) parameters for over 6 months. Twenty-four hour composite influent and effluent samples were collected three times a week for 3 weeks (except for the first week), with two sampling campaigns for TMP analysis. Eight hour composite samples were also collected for TMP analysis from the three zones of the bioreactor.

Estimation of active biomass fractions

The active biomass fractions in the BNR bioreactor were estimated by simulating the pilot plant responses as presented in detail elsewhere (Barker & Dold 1997). The activated sludge model no. 2d was employed within the wastewater treatment modeling software BioWin 3.0.

Figure 1 | Trimethoprim concentrations along BNR pilot plant (deviation bar represents standard deviation of measurements (n = 8)).
Average influent wastewater parameters (SI Table S2, available online) and the pilot plant’s design and operating conditions (SI Table S1) were input into the simulator prior to simulating steady state performance of the pilot BNR process. The readily biodegradable fraction of the influent COD \((f_{\text{in}})\) was adjusted to reflect the sodium acetate that was added to the influent stream. Simulated and observed soluble effluent COD and total Kjeldahl nitrogen (TKN) were matched by adjusting the soluble unbiodegradable fractions of the influent COD \((f_{\text{up}})\) and influent TKN \((f_{\text{SN}})\). Simulated and observed effluent suspended solids concentrations were matched by adjusting the final clarifier solids removal efficiency. The simulated and observed bioreactor mixed liquor volatile suspended solids (MLVSS) concentrations were matched by adjusting the particulate unbiodegradable fraction of the influent COD \((f_{\text{up}})\). Aside from these calibrations, the default values were used for the kinetic and stoichiometric parameters (Barker & Dold 1997).

**Batch experiments**

A series of batch tests were performed to investigate the biotransformation of TMP in BNR activated sludge and to assess the contributions of the active biomass (PAOs, OHOs and AOB) to the biotransformation of TMP under aerobic, anoxic and anaerobic conditions. Aerobic tests with and without nitrification inhibitor (allylthiourea) were conducted to assess the roles of PAOs, OHOs and AOB in the biotransformation of TMP and the contribution of AOB to TMP biotransformation respectively. Separate batch tests were conducted under anaerobic and anoxic conditions to investigate TMP biotransformation under these conditions. A summary of the experimental conditions under which the batch tests were performed is shown in SI Table S3 (available online). The reproducibility of the tests was evaluated by assessing the aerobic tests that were conducted without nitrification inhibition.

The innocula for the batch tests were collected from the aerobic section of the pilot BNR process. The batch reactors had a working volume of 10 L and were filled with 6 L of activated sludge diluted with 4 L of settled raw wastewater to achieve an average MLVSS concentration of approximately 2,500 mgVSS/L. The initial TMP concentration in the batch tests ranged from 0.2 to 1 \(\mu\)g/L. The initial concentrations of the chemicals that were added to facilitate biomass growth in the batch tests are shown in SI Table S3. In the aerobic tests, the mixed liquor in the reactors was aerated to maintain DO concentrations of approximately 2–5 mg/L. The temperature of the reactors was maintained at 18 \(\pm\) 2°C and the pH was maintained in the range of 7.5–8.4.

Time-dependent changes in the concentration of TMP and conventional parameters in the effluent samples were characterized to assess the reactors’ performance. From each batch reactor, 250 mL of mixed liquor samples were collected in prewashed amber bottles, before the addition of TMP and 15 minutes after the addition of TMP to the reactors and subsequently every 4 hours for 5 days. Each sample was centrifuged at 4,000 rpm for 5 minutes and 60 mL of the filtered centrate was employed for analysis of the conventional pollutants, 150 mL was employed for TMP analysis and the unfiltered samples were analyzed for total suspended solids (TSS) and VSS according to Standard Methods (Eaton & Franson 2005).

**TMP analysis**

Analysis of samples collected from the pilot BNR and the batch experiments for TMP was conducted in duplicate after filtering (1.5 \(\mu\)m glass fibre) and initially involved solid phase extraction (Li et al. 2010) using Oasis HLB 60 mg cartridges. Prior to extraction, 0.1 mL of a mixture of an internal standard (500 ng/mL) containing the stable isotope labeled surrogates of the analyte was added to the samples. The eluates were evaporated to dryness under a gentle stream of nitrogen and reconstituted in 0.4 mL of methanol. An internal standard (Trimethoprim-\(^{13}\)C\(_6\)) was added to the reconstituted samples prior to instrumental analysis to improve the quantitative analysis. The extracted samples were shipped on ice to the analytical lab to be quantified using liquid chromatography interfaced with a tandem mass spectrometer (LC-MS/MS) that employed an electrospray ionization source. The LC-MS/MS was run in double polarity mode using positive and negative voltage switching as described elsewhere (Li et al. 2010). A detailed description of the sample preparation and LC-MS/MS procedure that was employed to quantify the TMP was described in detail elsewhere (Miao & Metcalfe 2003). The limit of quantification and limit of detection were calculated to be between 0.1 and 2 ng/L.

**Biotransformation model**

A pseudo first order kinetic expression is commonly used to describe the biodegradation of TrOCs in activated sludge systems (Cowan et al. 1993; Monteith et al. 1995; Schwarzenbach et al. 2003). Two different forms of the pseudo first order models were employed to investigate the use of
MLVSS concentration and estimated active biomass concentrations in determining the biotransformation rate constants for TMP in BNR activated sludge processes. Both models assumed that the MLVSS and the active biomass concentrations were constant throughout the duration of the batch tests. This assumption is typically valid for short duration batch tests, and a plot of the VSS concentrations with time (SI Figure S7, available online) for the batch tests confirmed the validity of this assumption. Model 1 did not differentiate between biomass species and hence the rate of biotransformation of TMP was assumed to be a function of the MLVSS concentration in the reactors (Equation (1)).

$$r_i = -k_iX_{mlvss}S_i$$  \hspace{1cm} (1) 

where

- \( r_i \) = rate of biotransformation of compound \( i \) \([\mu g/L\cdot hr]\)
- \( k_i \) = biotransformation rate constant for compound \( i \) \([L/gCOD\cdot hr]\)
- \( X_{mlvss} \) = mixed liquor volatile suspended solid concentration \([gCOD/L]\)
- \( S_i \) = soluble concentration of compound \( i \) \([\mu g/L]\)

By contrast, model 2 incorporated three biotransformation rates, where the rate of biotransformation of TMP was assumed to be dependent on each of the active biomass concentrations and were redox zone specific (Equation (2)).

$$r_i = \sum_{j=1}^{n-3} k_{ij}X_jS_i$$  \hspace{1cm} (2) 

where

- \( k_{ij} \) = biotransformation rate constant for compound \( i \) \([L/gCOD\cdot hr]\) with respect to biomass \( j \)
- \( X_j \) = active biomass concentration \([gCOD/L]\)
- \( S_i \) = soluble concentration of compound \( i \) \([\mu g/L]\)
- \( j \) = PAOs, OHOs, AOB

Statistical analysis

The conventional and chemical data were analyzed for outliers using Grubbs’ test. The fit between the simulated and measured TMP concentrations in the batch experiments was assessed based on \( r^2 \) values and the Nash–Sutcliffe efficiency (NSE) metric (Nash & Sutcliffe 1970) (Equation (3)). NSE values range from \(-\infty\) to 1 but are typically greater than zero. A negative value of NSE indicates that the mean of the measured value is a better predictor than the model.

Typically, NSE values greater than 0.7 are an indication of a strong predictive characteristic of the model (Nash & Sutcliffe 1970).

$$NSE = \left(1 - \frac{\sigma^2_e}{\sigma^2_o}\right)$$  \hspace{1cm} (3) 

where

- \( \sigma^2_e \) = variance of residuals
- \( \sigma^2_o \) = variance of observed values

RESULTS AND DISCUSSION

Conventional contaminants in pilot plant

The measured responses of the conventional contaminants (Table 1) in the effluent of the pilot plant were relatively consistent throughout the sampling campaign.

The values demonstrated efficient and consistent performance of the pilot plant despite the variability in the influent contaminants. The cBOD\(_3\) was consistently removed in the pilot plant with the effluent concentrations ranging from 2 to 9 mg/L. This was deemed to be indicative of good removal of biodegradable organic matter. In addition, the pilot plant was expected to have high removal efficiencies for TKN, NH\(_3\)-N, NO\(_2\)-N and TP. These patterns were consistently observed throughout the sampling period as shown in Table 1. These results suggest that the pilot plant was effectively nitrifying, denitrifying and biologically removing phosphorus. Hence, it was concluded that the operation of the BNR pilot plant was consistent with known efficiencies of typical full scale BNR treatment plants (Jeyanayagam 2005; Moriasi et al. 2007).

<table>
<thead>
<tr>
<th>Response</th>
<th>Primary clarifier (influent) mean (± SD)</th>
<th>Secondary clarifier (effluent) mean (± SD)</th>
<th>Predicted effluent</th>
<th>Removal efficiency %</th>
</tr>
</thead>
<tbody>
<tr>
<td>COD</td>
<td>367 ± 48</td>
<td>33 ± 12</td>
<td>33</td>
<td>91</td>
</tr>
<tr>
<td>TKN</td>
<td>25 ± 12</td>
<td>2.3 ± 0.2</td>
<td>2.3</td>
<td>91</td>
</tr>
<tr>
<td>NH(_3)-N</td>
<td>19 ± 8</td>
<td>0.04 ± 0.03</td>
<td>0.04</td>
<td>100</td>
</tr>
<tr>
<td>NO(_3)-N</td>
<td>0.54 ± 0.2</td>
<td>3 ± 2</td>
<td>3</td>
<td>n/a</td>
</tr>
<tr>
<td>NO(_2)-N</td>
<td>0.06 ± 0.03</td>
<td>0.03 ± 0.01</td>
<td>0.03</td>
<td>50</td>
</tr>
<tr>
<td>TP</td>
<td>11 ± 7</td>
<td>3 ± 1.7</td>
<td>3</td>
<td>73</td>
</tr>
<tr>
<td>TSS</td>
<td>32 ± 2.5</td>
<td>9 ± 1.6</td>
<td>10</td>
<td>72</td>
</tr>
</tbody>
</table>

Note: n/a, not applicable; SD, standard deviation.
Pilot plant simulation and active biomass estimation

The steady state effluent concentrations of the conventional pollutants and the MLVSS concentration in the bioreactor were simulated using the BioWin modeling software. The measured and calibrated MLVSS concentrations in the bioreactor were $3,240 \pm 11$ g COD/m$^3$ and $3,240$ g COD/m$^3$ respectively while the simulated percentages for the active biomass groups AOB, OHo's and PAOs in the pilot BNR bioreactor were 19%, 56% and 25% respectively. The relative error between the measured and steady state simulated data (Table 1).

TMP removal in BNR pilot plant

The concentrations of TMP in the pilot plant streams were measured to investigate the performance of the pilot in terms of TMP removal. The influent TMP concentration ($n = 8$) was $78 \pm 39$ ng/L while the effluent TMP concentration ($n = 8$) was $28 \pm 8$ ng/L; hence the overall TMP removal efficiency was calculated as $64 \pm 14\%$. The removal efficiencies obtained in this study was consistent with a prior study that reported the removal of TMP in a membrane bioreactor system ($57 \pm 10\%$) (Radjenovic et al. 2009) but on average, 3–6 times higher than that reported for conventional activated sludge systems ($11 \pm 31\%$) (Tchobanoglous et al. 2003; Eichhorn et al. 2005; Göbel et al. 2007). Thus, it was concluded that the performance of the pilot BNR process in terms of TMP removal was comparable to the performance of previously investigated advanced wastewater treatment processes.

The bioreactor of the BNR process had three different redox conditions. It was anticipated that the different redox zones would contribute differently to the overall biotransformation of TMP. This expectation was because the amount of energy that is captured by microorganisms in aerobic conditions is higher than that captured in anoxic and anaerobic conditions (Batt et al. 2007). This bioenergy could impact the biotransformation of TMP in some of the BNR zones. Furthermore, it has been demonstrated elsewhere that in the presence of DO, oxygenase enzyme activity can enhance the initial transformation of complex organic compounds (Batt et al. 2006). The TMP concentrations in the influent, effluent and the different zones of the pilot BNR bioreactor are presented in Figure 1.

It is apparent from Figure 1 that the TMP concentration decreased along the redox zones of the bioreactor, which suggested that each of the zones contributed to the overall TMP removal from the wastewater, with the concentration of the TMP reducing from the anaerobic zone to the downstream aerobic zone.

Recycle flows could have diluted contaminants to different extents through the BNR system. Therefore, a set of mass balances were employed to characterize the fate of TMP in the pilot BNR process (SI Figure S2, available online). In the mass balances, the biotransformation efficiency of TMP in each zone was calculated as the difference between the mass flow entering and leaving each zone, divided by the mass flow entering the zone. The difference between the mass inflow and outflow in the aqueous phase across each zone of the bioreactor was assumed to be due to microbial biotransformation within the zone of the bioreactor. This assumption was because previous studies had shown negligible TMP removal by sorption in activated sludge systems (Tchobanoglous et al. 2003; Göbel et al. 2007; Khunjar et al. 2011). The TMP biotransformation efficiencies in the anaerobic, anoxic and aerobic sections of the BNR bioreactor were calculated to be $22 \pm 20\%$, $27 \pm 8\%$ and $36 \pm 5\%$ respectively. The removal efficiencies were consistent with the energy available to the organisms in the different redox conditions (Göbel et al. 2007) and the availability of DO to act as a direct reactant in oxygenase reactions.

Conventional contaminant responses in batch reactors

Batch tests were conducted to obtain an improved understanding of the kinetics associated with the removals that were observed in the BNR pilot plant. The conditions present in the batch tests were initially ascertained by investigating the behavior of the conventional pollutants through the duration of the experiments. Figures S3 and S4 in the SI (available online) show the profiles of the conventional pollutants in the batch tests conducted under aerobic conditions without nitrification inhibition (Aerobic$^{-1}$) and with nitrification inhibition (Aerobic$^{2}$) respectively. It is apparent from Figure S3 that the AOB were active in Aerobic$^{-1}$ batch tests as evidenced by the decrease in ammonia with a simultaneous increase in nitrate. By contrast, there was no change in ammonia or nitrate concentration throughout the duration of the experiment in Aerobic$^{2}$ batch tests, which indicated the inhibition of AOB activity by the added allylthiourea (Figure S4).

Under aerobic conditions, PAOs oxidize intracellular poly-$\beta$-hydroxybutyrates (PHBs) to obtain energy for growth and maintenance requirements. The intracellular PHBs support PAO growth and soluble phosphate (PO$_4$-P)
uptake from the bulk liquid in the reactor. The accumulated polyphosphates provide the energy required for soluble volatile fatty acid (VFA) uptake in the anaerobic zone. Figures S3 and S4 show a decline in the PO₄-P concentrations that ceased after approximately 20–24 hours. The reduction in the PO₄-P concentration in the reactors signified that the PAOs were actively taking up PO₄-P to form intracellular polyphosphates. The cessation of PO₄-P uptake may have been due to the depletion of the intracellular PHBs of the PAOs. In the absence of PHBs, the PAOs have no source of energy to carry out their metabolic activities under aerobic conditions. Hence, it was concluded that the PAOs in the aerobic batch tests were active during the initial 20–24 hours of the tests and became inactive for the rest of the duration of the batch tests.

Under aerobic conditions, OHOs oxidize COD for cellular growth and maintenance. Figures S3 and S4 show a decline in the soluble COD concentrations, which ceased after approximately 20–24 hours. After the soluble COD concentration in the aerobic reactor reached a minimum, the concentrations tended to gradually increase until the end of the reaction in the aerobic batch tests. During biomass growth, decay and lysis also occur concurrently. The cell decay and lysis likely released soluble substrates and particulate substrates into the bulk liquid in the reactor. The particulate substrates can undergo hydrolysis to produce more soluble substrate for cellular consumption (Perez et al. 2005). These results suggest that the rate at which OHOs oxidized the soluble COD for growth was initially faster than cell death and lysis at the beginning of the batch tests, but after approximately 20–24 hours of reaction the rate of COD consumption became slower than the rate of release of soluble COD into the reactor by biomass death and lysis. This was demonstrated by the slightly increasing COD concentration in both batch tests. Hence, it was concluded that after 20–24 hours of reaction in the batch tests, the biomass in the reactors entered a stationary phase as depicted by the constant soluble COD concentrations.

Under anoxic condition, the AOB are typically inactive due to the absence of oxygen and OHO metabolism becomes fermentative rather than respiratory as under aerobic and anoxic conditions. The PAOs can uptake VFA to produce intracellular poly-hydroxybutyrate (PHB). During this process, soluble phosphate is released by the PAOs, thereby increasing the concentration of the soluble PO₄-P in the anaerobic batch reactor. A plot of the concentrations of PO₄-P with time (SI Figure S6, available online) showed that the PO₄-P increased as the test proceeded, with a simultaneous reduction in VFA in the anaerobic reactor. Hence, it was concluded that the PAOs were active in the anaerobic batch test.

Trimethoprim removal in batch tests

The fraction of the initial TMP remaining in the aqueous phase was plotted against time (Figure 2) to investigate the effect of AOB inhibition and redox conditions on TMP removal in the batch tests.

Figure 2 shows that the TMP concentration fractions remaining in the batch experiments with AOB inhibition and without AOB inhibition followed similar decreasing trends. However, the biotransformation of TMP appeared to be more rapid with active AOB than with inhibited AOB. The difference in trends between the two batch experiments suggests the effects of AOB on the biotransformation of TMP. This result was consistent with a previous study that reported that AOB played a role in the biotransformation of TMP in aerobic batch experiments (Batt et al. 2006).

The impact of redox conditions on the biotransformation of TMP was assessed by comparing the decline in TMP concentration fractions in the aerobic, anoxic and anaerobic batch tests (Figure 2). From Figure 2, the rate of decline in TMP fractions decreased from the aerobic to anoxic and to the anaerobic batch tests. This confirmed the importance of redox conditions on the biotransformation of TMP in activated sludge. The results of the batch tests were qualitatively consistent with the previously described removal efficiencies in the corresponding zones of the BNR pilot plant.

Model assessment

The biotransformation of TMP in the batch tests was examined using the two previously described pseudo first order kinetic models. In the application of the models, it was assumed that the MLVSS concentration was constant in model 1 and that the estimated active biomass
concentrations were constant in model 2. This was supported by the measured VSS concentrations that were relatively constant throughout the duration of the batch tests (SI Figure S7).

TMP responses under aerobic conditions were available for tests with and without nitrification inhibition. In the model calibration, it was assumed that AOB were not active in the nitrification-inhibited tests and hence only OHO and PAO rate constants were estimated with this data. All three biomass types were assumed to be active in the aerobic batch tests that were conducted without nitrification inhibitor. To model the TMP under anaerobic and anoxic conditions, select assumptions were made about the activity of the different biomass species in the differing redox conditions. It was expected that the AOB would not be active in the absence of oxygen so the rate constants for TMP removal by AOB in these tests were set to zero. OHOs ferment under anaerobic conditions while PAOs do not (Barker & Dold 1997) and hence it was assumed that only OHOs were active anaerobically. Both OHOs and PAOs are capable of facultative growth with nitrate (Barker & Dold 1997) and hence it was assumed that they were both active in the anoxic zone and hence were included in the multi-species model. An integral least square method that minimized the sum of squares of the residuals between the predicted and measured TMP data was employed to simultaneously fit the models to the TMP responses from the batch tests.

Figure 3 shows plots of the measured and predicted fractions of TMP remaining in the aerobic tests while Figure 4 shows these responses in uninhibited aerobic, anoxic and anaerobic tests. The goodness of fit of the two models were estimated using $r^2$ and NSE.

The results (Table 2) show that the $r^2$ and NSE values for both models 1 and 2 were larger than 0.98 for the tests, indicating that both models could represent the behavior of TMP in the batch tests. Hence, it was concluded that models 1 and 2 effectively described the behavior of TMP in the aerobic, anoxic and anaerobic batch tests. An additional feature of model 2, however, was the ability to estimate the contribution of each active biomass group to the overall TMP removal in the BNR activated sludge.

The estimated biotransformation rate constants for TMP with the two models are summarized in Table 2. At the time this paper was prepared, this study was the deemed to be the first to estimate the biotransformation rate constant for TMP under different redox conditions with respect to the active biomass groups or MLVSS in a BNR activated sludge; hence there was no previous study for comparison. From Table 2, the biotransformation rate constants for TMP with respect to PAOs, OHOs and AOB followed a pattern of $k_{\text{AOB}} > k_{\text{OHO}} > k_{\text{PAO}}$, which signifies the potential for PAOs, OHOs and AOB to contribute differently towards the overall biotransformation of TMP in BNR activated sludge.

**Contribution of each active biomass groups towards trimethoprim biotransformation**

To improve the understanding of TMP removal rates by each active biomass, the estimated biotransformation rate constants were employed in model 2 to predict the species-specific removal rates. Figure 5 presents the contributions of PAOs, OHOs and AOB towards the overall TMP removal rate in the batch tests.
Figure 5 shows that AOB contributed towards the overall removal of TMP under aerobic conditions but not under anoxic and anaerobic conditions. Under the anoxic and anaerobic redox conditions, the OHOs contributed the most towards the overall removal rates, followed by PAOs. Therefore, it was concluded that each of the biomass
groups in the BNR mixed liquor contributed collaboratively to achieve the observed overall TMP removal rate but at different proportions, in the order of OHOs > AOB > PAOs for aerobic conditions and OHOs > PAOs for anoxic and anaerobic conditions. Although TMP is an antibiotic that has high toxicity to algae and bacteria at low concentrations, the concentration threshold of a similar antimicrobial has been shown to be in the mg/L range (Yi et al. 2017). In this study, the TMP concentration was three orders of magnitude lower than this threshold; thus in this study, it was assumed that the presence of the TMP had no deleterious effect on the microbial population in the activated sludge.

CONCLUSIONS

Data collected from a pilot scale BNR activated sludge system and batch experiments were employed in a modeling exercise to investigate the removal and biodegradation kinetics of TMP in BNR activated sludge. The results showed that TMP can be effectively removed in a BNR activated sludge system with each of the redox zones contributing different proportions. The overall TMP removal efficiency was calculated as 64 ± 14% while the biotransformation efficiencies in the anaerobic, anoxic and aerobic sections of the BNR bioreactor were 22 ± 20%, 27 ± 8% and 36 ± 5% respectively. These results indicated that TMP removal in the BNR process was related to the redox conditions in each of the treatment zones. A comparison of TMP removal rates in the aerobic batch reactors with and without AOB inhibition showed a faster removal rate in the reactor without AOB inhibition, suggesting that AOB played a role in TMP removal in the BNR activated sludge.

The biotransformation of TMP in the batch tests containing BNR activated sludge was found to be effectively described by a modified pseudo first order model that incorporated the fractions of each active biomass group. The biotransformation rate constants for TMP with respect to PAOs, OHOs and AOB followed a pattern of \( k_{\text{AOB}} > k_{\text{OHO}} > k_{\text{PAO}} \). PAOs, OHOs and AOB contributed collaboratively to achieve the overall observed TMP removal rate but at different proportions, in the order OHOs > AOB > PAOs for aerobic conditions and OHOs > PAOs for anoxic and anaerobic conditions.

AUTHOR CONTRIBUTION

The manuscript was written through contributions of both authors. Both authors have given approval to the final version of the manuscript.
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