December 16, 2013

Dr. Manhong Huang
College of Environmental Science and Engineering
Donghua University
Shanghai, 201620, People's Republic of China

Dear Dr. Huang,

RE: Removal performance and microbial communities changes of SBRs under trace tetracycline pressure of aerobic and anoxic conditions
Ms.#A-3660

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Shahamat U. Khan
Removal performance and changes in the microbial communities of SBRs under aerobic and anoxic conditions with trace tetracycline pressure

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Abstract

The reactor performance and microbial community composition of sequencing batch reactor (SBR) under aerobic and anoxic conditions were investigated in this study. The experimental results showed high chemical oxygen demand (COD) removal efficiency. The tetracycline (TC) removal efficiencies were not obviously affected by aerobic and anoxic conditions, and were 64–97 and 60–87%, respectively. Aerobic condition was observed to be more suitable for decreasing tetracycline-resistant bacteria (TRB) than anoxic condition in synthetic and real wastewater. Denaturing gradient gel electrophoresis (DGGE) and clone library analysis revealed that *Chlorobaculum thiosulfatophilum* was the dominant species in the tested SBR systems. TC significantly influenced the relative numbers of TRB- and TC-resistant genes, and the microbial community diversity changed with the addition of 250 g L\(^{-1}\) of TC. The genes of *tetA* and *tetC*, *tetM* and *tetS*, *tetA* and *tetM*, *tetS* and *tetA* showed significant correlation with each other \((p<0.05)\).

Keywords: Sequencing batch reactor (SBR); aerobic; anoxic; tetracycline; resistance characteristics.

Introduction

Antibiotics are used extensively in clinical treatment and in the livestock industry. Among them, tetracycline (TC) is the second most widely used antimicrobial in the
Wastewater treatment plants (WWTPs) receive TCs with the inflow sewage water originating from hospitals, private households, industry, and animal husbandry. WWTPs are well-known important reservoirs for various antibiotic-resistant organisms. The bacteria in the sewage generate resistance under antibiotic selection pressure. In addition, existence of antibiotic-resistant genes in wastewater effluents to streams has also been reported. Lapara et al. and Munir et al. showed that effluent of WWTPs discharged into lakes and rivers may contain higher concentrations of antibiotic-resistant organisms, and that these organisms producing antibiotic-resistant genes could lead to drug resistance. Although WWTPs have used some advanced technologies, it has been observed that they are still an important source of antibiotic-resistance genes. Auerbach et al. found that the influent and effluent of a WWTP contained a high concentration of drug-resistant strains or antibiotic resistance genes, and that these could not be effectively removed by biological treatment and ultraviolet disinfection. Although there are many reports on the removal of antibiotics and antibiotic-resistant bacteria from treated municipal wastewater, studies on the changes in microbial communities under TC pressure are rarely reported.

The sequencing activated sludge process is the most common form of secondary treatment employed in WWTPs around the world. It is well known that the activated sludge process operating conditions can have a significant effect on the transfer and transport of contaminants and microbial communities of the sludge during the treatment process. Previous studies have focused on the removal of antibiotics, which involved
different wastewater treatment techniques, such as aerobic degradation, advanced treatment,\cite{18-20} etc. Till date, few studies have investigated the influence of operating conditions on the removal efficiency of antibiotics,\cite{21} and most of them have only been focused on the development of drug resistance and prevention of increased antibiotic resistance from the activated sludge mixed liquor into the environment.

Wastewater treatment and operation involve various environmental conditions, some of which influence bacterial metabolism and resistance development. For future evaluation/control of potential health risks posed by effluent discharge, in the present study, four sequencing batch reactors (SBRs) operated at different hydraulic retention times (HRT) were employed to simulate a typical activated sludge process in the laboratory. The study aimed to (1) estimate the removal efficiencies of a laboratory-scale SBR activated sludge process with trace TC addition and (2) determine the development and changes of microbial communities and drug resistance resulting from different oxygen conditions.

Materials and methods

**Seed sludge and acclimation**

The seed sludge was obtained from the return activated sludge tank of Shanghai Song-jiang Municipal WWTP. The activated sludge was acclimated using four SBRs with two cycles every day. Aerobic conditions were maintained by aeration (DO=4.8±0.5
mgL$^{-1}$), whereas anoxic conditions were maintained by adding sodium nitrate as the nitrogen source. Furthermore, NH$_4$Cl was added to the aerobic SBR. The concentrations of NH$_4$Cl and sodium nitrate, added as the nitrogen source to the aerobic and anoxic SBRs, were about 115 and 185 mg L$^{-1}$, respectively, to maintain the same total nitrogen (TN) concentration of 30 mg L$^{-1}$. Under anoxic conditions, nitrate acted as the electron acceptor.

Aerobic SBR-1 (A1) and aerobic SBR-2 (A2) were operated in the presence and absence of TC, respectively. Similarly, anoxic SBR-1 (AN1) and anoxic SBR-2 (AN2) were operated in the presence and absence of TC, respectively. Each cycle included 1 h of filling, followed by 9 h of reaction, 1 h of settling, 30 min of drawing, and 0.5 h of idling. The working volume of each reactor was 12 L. Anoxic mixing was achieved by using a motor-driven mixer with a shaft speed of 90 rpm. The reactors were placed in a temperature-controlled room at about 20°C.

The activated sludge in A1, A2 and AN1, AN2 was acclimated using substrate solution A and solution B, respectively. Solution A included the following (per 50 L of tap water): glucose, 20g; CH$_3$COONa·3H$_2$O, 2.4118g; NaHCO$_3$, 6.25g; KCl, 0.2085 g; CaCl$_2$, 0.125g; NH$_4$Cl, 5.75g; KH$_2$PO$_4$, 1.2g; MgSO$_4$·7H$_2$O, 1.375g; and mineral salt solution, 60 mL. The mineral salt solution contained the following (mg L$^{-1}$):FeCl$_3$, 375; MnCl$_2$·4H$_2$O, 30; H$_3$BO$_3$, 37.5; ZnSO$_4$·7H$_2$O, 30; CuSO$_4$·5H$_2$O, 7.5; EDTA, 2500; and KI, 45. Solution B contained the same components as those of solution A, except for NH$_4$Cl, which was replaced with NaNO$_3$ with the same concentration of TN. TC was spiked in A1 and
AN1 with the initial concentration of 250 g L\(^{-1}\).

**Sample collection**

From each of the four SBRs, 500 mL of mixed liquor samples were collected before the end of the reaction period for denaturing gradient gel electrophoresis (DGGE) analysis, and 500 mL of liquor samples from the effluent were collected for qPCR.

**Culture method**

A total of 100 mL of aqueous samples and 50 mL of sludge samples mixed with 50 mL of phosphate-buffered saline (PBS, pH 7.2) were collected and shaken at 200 rpm for 2 h. The total heterotrophic bacteria (THB) in the samples were counted on Luria-Bertani (LB) agar plates using the spread plate method after serial dilution. TC-resistant bacteria (TRB) were enumerated by plating the samples on LB agar amended with TC. For each assay, duplicate plate counts were carried out with three different dilutions for each sample.

**DNA extraction and amplification**

The bacteria were collected after centrifugation and dissolved in 5 mL of buffer extraction solution (100 mM Tris-Cl, 100 mM EDTA-Na\(_2\), 200 mM NaCl, and 2% CTAB; pH 8.0). The samples were shaken for 45 min at 37°C and then 0.75 mL of 20% SDS was added. After immersion in a water bath for 1 h at 65°C, the samples were centrifuged
(12,000 rpm, 10 min) and the supernatant was collected. The supernatants were extracted twice with isometric phenol:chloroform:isoamyl alcohol (25:24:1), and 0.3 M NaAc (pH 5.2) was added and 2 times the volume of anhydrous ethanol and allowed to precipitate for 1 h at room temperature. The precipitate was collected by centrifugation (12,000 rpm, 4°C, 20 min) and rinsed twice with 70% alcohol, after drying in 50 μL of TE buffer solution (10mM Tris-Cl and 1mMEDTA; pH 8.0).

The variable region, V3, of the 16SrDNA was amplified using primers F357 and R518. The reaction sequence was as follows: preliminary degeneration at 94°C for 4 min, 94°C for 0.5 min, 56°C for 1 min, and72°C for 0.5 min, and 30 cycles of 72°C extension for 7 min. The first PCR product was used as an amplification template along with primers F357 and R518 to amplify the total bacterial 16SrDNA V3 area.

**PCR-DGGE analysis**

A 400-ng sample of the V3 area PCR product was evaluated using the D-Code mutation detection system for DGGE analysis. After electrophoresis, the gel was washed with ultrapure water, placed in Gold view dye solution (5%) for 30 min, and photographs were taken by using the UVI system. A typical band was selected and the target DGGE band was put into a 1.5-mL centrifuge tube and recovered by using the SK1135 kit. The PCR products were sent to Sangon Biotech Co. Ltd. (Shanghai, China) to determine the DNA sequence. The sequencing results were compared with the sequences in the NCBI gene library. The diversity and homology of the bands were analyzed, and the system
phylogenetic tree was established. To perform the diversity statistical analysis, Quantity
One software was employed. According to the quantities and intensities of the bands,
Shannon’s diversity index (SH)\textsuperscript{[22]} and Simpson’s index (SI)\textsuperscript{[23]} were used to evaluate the
diversity of the microbial community.

\section*{qPCR}

The target genes (\textit{tet}A, \textit{tet}C, \textit{tet}E, \textit{tet}M, \textit{tet}S, and 16SrRNA) were quantified by qPCR
assays with the fluorescent dye SYBR-Green I. Plasmids carrying the target genes were
used to generate calibration curves, and their concentrations were measured with a
spectrophotometer. As the lengths of the vector and target gene inserts were known (Table
1), the gene copy numbers were calculated directly from the plasmid DNA concentrations.
Ten-fold serial dilutions of a known copy number of the plasmid DNA were generated to
produce the standard curve. The PCR efficiencies (80.5–95.1\%) were examined to test for
inhibition. The $R^2$ values were more than 0.99 for all the calibration curves. The total
volume of each reaction mixture was 25\,\mu L, comprising 12.5 \, L of SYBR Green qPCR
Master Mix, 0.5 \, M of each primer, and 2 \, L of template DNA. The PCR protocol was
as follows: 2 min at 95°C, followed by 40 cycles of 95°C for 10 s and annealing
temperature for 40 s. Product specificity was confirmed by melting curve analysis
(55-95°C at 0.5°C increments and a 30-s hold per read) and visualization in agarose gels.
Five-point calibration curves ($Ct$ value vs. log of the initial \textit{tet} copy number) for qPCR
were generated using 10-fold serial dilutions of the plasmid carrying a \textit{tet} gene, from $10^3$
to $10^7$ target copies per reaction. Based on the calibration curves, the $Ct$ value of the test
sample was used to calculate the number of *tet* gene copies normalized by the mass (ng) of the extracted DNA and volume (mL) of the original samples.

**Analyses**

The values of mixed liquor suspended solids (MLSS), TN, total phosphorous (TP), and soluble chemical oxygen demand (COD) in the liquid samples were determined in accordance with the standard methods for the examination of water and wastewater.\(^{[24]}\) Dissolved oxygen (DO) was determined by using dissolved oxygen meter (Oxi330i, WTW, Germany). Commercially available tetracycline ELISA kits (R-Biopharm GmbH, Darmstadt, Germany) were used for the detection of TC concentration in the effluents of the SBRs, according to the manufacturer’s instructions (RIDASCREEN Tetracycline, RBiopharm GmbH, Darmstadt, Germany); this ELISA technique had also been described in a previous study.\(^{[1]}\) The absorbance was measured by using a plate reader (Bio-TekEL800, Winooski, VT, USA). According to the European Commission Decision 2002/657/EC, the recovery, specificity, and precision of the ELISA method were investigated to observe its reliability. The recovery values were in the range of 73-127%. The method limit and mean coefficient of variation were 0.15 g L\(^{-1}\) and 5%, respectively. A specificity test was performed for chlortetraacycline (CTC) and TC, and it was confirmed that the method has the ability to distinguish between TC and CTC.

**Results and discussion**
Characteristics of TC and TRB in anoxic and aerobic SBRs

Effect of HRT on removal characteristics

Several studies have shown that HRT can have a remarkable effect on the adsorption, biotransformation, and decomposition of pollutants in WWTPs.\cite{1}\cite{25} Fig. 1 shows the data obtained for the removal efficiency of pollutants, including TN, TP, COD, and TC, as a function of HRT. As illustrated in Fig. 1, the COD, TN, and TP removal efficiencies increased as HRT increased. However, the TC removal efficiency improved when HRT increased from 8 to 12 h, and then decreased as the HRT increased. This suggested that the COD, TN, and TP removal efficiencies were less affected by HRT than the TC removal efficiency. In AN1, the removal efficiencies of TN, TP, and COD increased from 34, 79, and 83% to 48, 85, and 86%, respectively, when the HRT was increased from 8 to 20 h. In addition, the TC removal efficiency reached a stable high level (above 80%) when the HRT of the SBRs was greater than 12 h. This may be due to the incomplete biodegradation of COD, TN, and TP at 8 h. It must be noted that with respect to COD, TN, and TP removal efficiencies, longer HRT signified longer reaction time; however, for the removal of TC at trace concentration, 12 h of HRT was sufficient to reach a balance between TRB and TC load, and beyond that, the TC removal efficiency decreased.

In AN1, the number of TRB in the sludge was $0.25\times10^9\text{CFU g}^{-1}$ when HRT was 20 h, which was the lowest, when compared with those observed at other HRTs ($1.87\times10^9\text{CFU g}^{-1}$ at 16 h, $2.83\times10^9\text{CFU g}^{-1}$ at 12 h, and $5.93\times10^9\text{CFU g}^{-1}$ at 8 h). This
indicated that the extension of HRT, to a certain level, could alleviate TRB increase in anoxic and aerobic environments, because the increase in HRT could have possibly reduced the organic load, leading to a reduction in bacterial growth rate. The TRB in A1 slowly changed when HRT was more than 12 h. On the contrary, the TRB in AN1 was significantly affected by the increase in HRT. As shown in Table 1, the TRB in A1 was about $2.4 \times 10^8$ CFU g$^{-1}$, and that in AN1 was much higher with a value of $2.83 \times 10^9$ CFU g$^{-1}$. Thus, the aerobic condition (A1) was more suitable for inhibiting TRB than the anoxic condition (AN1) under trace TC pressure.

Effect of trace TC on TRB in anoxic and aerobic SBRs

The changes in TRB under the influence of trace TC were presented in Table 1. It can be noted that in the SBR without TC, the number of TRB decreased, indicating that drug-resistant microorganisms in the water without TC gradually became non-resistant. The decreases in the number of TRB in A2 and AN2 were $0.078 \times 10^8$ and $0.049 \times 10^8$ CFU g$^{-1}$, respectively, which were very low, when compared with the increase in the number of TRB in A1 and AN1. When the TC concentration in the influent was 250 μg L$^{-1}$, the number of TRB in the SBR showed an upward trend, indicating that some microorganisms gradually became resistant at this TC concentration. Furthermore, in the water without TC, the activated sludge system contained TC-resistant microorganisms, which maybe owing to the sludge obtained from the WWTP, which always contains trace antibiotics. The number of TRB changed according to different conditions and finally stabilized after about 10 days. Table 1 shows that the number of TRB increased with time
in the presence of trace TC, and decreased with time in the absence of trace TC.

McKinney et al. \cite{26} investigated the oxidation pond treatment of livestock wastewater and examined the effect of antibiotic concentration and operating parameters on the number of TRB and resistant genes. They found that the total amount of TC-resistant genes (TRGs) increased with increasing concentrations of antibiotics, which is consistent with the results of the present study.

The removal efficiencies of COD, TN, and TP were compared with the effect of trace TC on the SBRs with and without TC addition. It was found that the addition of trace TC had no effect on the COD and TP removal efficiency (COD, \( p > 0.05 \); TP, \( p > 0.05 \)), but had significant correlation with TN removal efficiency (TN, \( p < 0.05 \)). This may be owing to the denitrifying bacteria being more sensitive to trace TC addition than other heterotrophic bacteria under anoxic condition.

Changes and comparison of microbial communities and TRGs in the anoxic and aerobic SBRs

Bio-faces of the SBR systems

The scanning electron microscope (SEM) images of the activated sludge are shown in Fig. 2. The microbial composition was obviously different in the aerobic and anoxic SBR systems. As shown in Fig. 2, the coccis-shaped bacteria were the dominant microorganisms in A1, which could be responsible for the high removal efficiency of TC.
in this SBR system. In the anoxic SBR systems, dendrite microscopic structures were found, which could have resulted from the simultaneous culture of multiple microorganisms in the activated sludge. The SEM images demonstrated that there were no significant discrepancies in the shapes of the microorganisms in the SBR systems in the absence of trace TC.

**Microbial communities**

The microbial communities of the mixed liquor in the two aerobic and anoxic SBRs were analyzed by using PCR-DGGE. According to the DGGE profile and clustering analysis (Fig. 3), the aerobic sludge communities were significantly different from the activated sludge mixed liquor communities in anoxic SBRs. The microbial population diversity and richness of the aerobic SBR sludge were higher than those of the anoxic SBR sludge. The microbial community in A1 showed 0.73 similarities with that in A2, whereas that in AN1 and AN2 indicated 0.88 similarities. Furthermore, the similarity index between A1 and AN1 was 0.60; as the SBRs operated under aerobic and anoxic conditions, the microbial population diversities decreased, whereas the brightness of dominant bacterial bands increased. Bands 1, 6, and 8 were consistently present under all conditions, although its intensities varied in different SBRs. Some bands, such as band 2, which represented dominant species in A1, became weak or even became almost invisible under other operating conditions. Based on these changes, it could be concluded that these bacterial species, represented by these bands, were accustomed to the environment of trace TC. Furthermore, while other bands acted differently, band 6 appeared or became
more intense at short anoxic conditions, indicating that the bacterial species represented by this band could have been more resistant to antibiotic loadings under anoxic conditions. Thus, the oxygen condition might have affected the capabilities and performance of the biological treatment systems by influencing the dominant microbial species. The classification of bacterial bands in the DGGE profile is presented in Table 2. The bands were excised and amplified for nucleotide sequence analysis. *Chlorobaculum thiosulfatophilum* (bands 2 and 6) was frequently detected in the nucleotide sequence analysis, which had not been detected in earlier studies on TC biodegradation. Furthermore, bands 2 and 6 of samples from A1 and AN1 in which trace TC was added were intensified. *C. thiosulfatophilum* is not known for its ability to degrade TC, and hence, further investigation concerning the effect of this bacterium on TC removal should be carried out. *Acinetobacter* sp. (band 2) became weak or invisible in A2, AN1, and AN2, and was detected in all SBRs. This bacterium has been reported to have the ability to resist and adsorb antibiotics \(^{[8]}\). Subsequent cloning library analysis also showed frequent detection of this bacterium.

Table 3 presents the number of bands formed corresponding to various reactors. It can be noted that the difference in the number of bands among the reactors was not large, and that the aerobic SBRs had an only slightly higher number of bands than the anoxic SBRs. SH was used to evaluate the abundance and evenness, and SI was used to determine some of the most familiar species (Table 3). Both SH and SI were higher in A1 and AN1 with TC than those in SBRs without TC. When the concentration of TC was 250 g L\(^{-1}\) under aerobic conditions, the SH and SI reached maximum values of 6.001 and
2.300, respectively. This illustrates the increase in the microbial community diversity with the addition of trace TC under aerobic conditions. Furthermore, the diversity of microbial community under aerobic conditions was also higher than that observed under anoxic conditions.

Characteristics of TRGs

Real-time quantitative PCR was used to examine the dynamic changes in the total bacterial 16SrRNA gene copies and TRGs (Table 4) in the SBRs. Table 4 shows the changes in the TRG copy numbers under different operating conditions. With respect to \( \text{tetC} \), the gene copy number increased from \( 2.38 \times 10^4 \) and \( 2.36 \times 10^5 \) copiesmL\(^{-1}\)(biomass) at a TC concentration of 250 g L\(^{-1}\) to \( 1.17 \times 10^4 \) and \( 9.68 \times 10^4 \) copiesmL\(^{-1}\)in controlled SBR (without TC) under aerobic and anoxic conditions, respectively. The proportion of total bacterial 16SrRNA genes under aerobic conditions was lower than that under anoxic conditions, suggesting that antibiotic loading in the influent relative to the MLSS increased under anoxic conditions, and that \( \text{tetC} \) gene carrier was slightly enriched in the SBR after addition of trace TC. Similar to \( \text{tetC} \), the genes \( \text{tetA}, \text{tetE}, \text{tetM}, \) and \( \text{tetS} \) were also appreciably affected by changes in oxygen and TC contents. All TRGs copy numbers in the SBRs with added TC under different oxygen conditions remained at a higher level, demonstrating that bacteria with \( \text{tet} \) gene were highly enriched in the SBRs with trace TC. These TRGs carrier bacteria in the SBRs ensured higher antibiotic removal efficiency, especially for TC antibiotics.
The descending order of TRGs numbers in the sludge in anoxic SBRs was as follows: 
\[tetA > tetC > tetE > tetM > tetS;\] in the aerobic SBRs, the order was almost the same, except that the number of \(tetS\) was greater than that of \(tetM\); thus the order was:
\[tetA > tetC > tetE > tetS > tetM.\] Furthermore, the correlation between the five TRGs were also examined (Table 5), and significant correlations were observed for \(tetA, tetC, tetM,\) and \(tetS\) \((p<0.05)\). However, the relationship between the number of \(tetE\) and other four detected resistance genes was found to be considerably weak \((p>0.05)\).

**Implication for wastewater treatment**

Wastewater characteristics and operational decisions can greatly influence bacterial metabolism and resistance development in wastewater treatment systems. In a previous study \([27]\), dramatic changes were observed in the microbial communities of SBRs under the pressure of O-nitrobenzaldehyde in real wastewater. Dreher et al. \([28]\) found that in swine manure slurry treatment system, anaerobic conditions of the SBR did not affect the level of biogas, but the presence of CTC antibiotics reduced the methane content. Therefore, it is possible to optimize the operation of WWTPs to reduce antibiotic resistance in the real wastewater treatment.

The present study demonstrated that trace TC could change the microbial communities of the activated sludge and cause proliferation of TRGs in the SBR systems. The increase in the number of TRGs in the sludge may potentially pose ecological risks during land application of the waste activated sludge, and hence, needs more attention. Using
synthetic wastewater, the effect of TC on the treatment procedure and microbial communities could be clearly observed.

To assess the effect of optimized SBR on real wastewater contaminated with TC, real wastewater and activated sludge from Shanghai Song-jiang Municipal WWTP were used in the optimized SBR. The chemical characteristics of real wastewater were as follows: COD, TP, and TN of 140, 1.93, and 22.9 mg L$^{-1}$, respectively, and the initial TC concentration was 0.54 g L$^{-1}$. The activated sludge was acclimated for about 2 weeks. The performance of anaerobic and anoxic SBRs is shown in Fig. 4. From the figure, it can be seen that the removal efficiencies of TN, TP, TC, and COD were lower than those noted with synthetic wastewater, which may be owing to the different TC concentrations and coexistence of pollutants other than TC in real wastewater. Furthermore, similar to that observed with synthetic wastewater, aerobic conditions was found to be more suitable for reducing TRB in real wastewater than anoxic conditions. It is in accordance with that of synthetic wastewater.

**Conclusion**

This study compared the performance and microbial community composition of SBR used for the treatment of wastewater containing trace antibiotics. The results showed that the SBRs were able to achieve good conventional pollutant removal efficiencies, and that aerobic conditions were more suitable for reducing TRB proliferation in real and synthetic wastewater. Bacterial community analyses suggested that *C. thiosulfatophilum*
was the dominant bacterial species, which played an important role in the treatment of wastewater containing TC. Furthermore, community diversity was found to be influenced by anoxic and aerobic conditions under trace TC pressure. All the detected TRGs copy numbers in SBRs with added TC under different oxygen conditions remained at a higher level than those observed in SBRs without TC. In addition, significant correlations were observed for \textit{tet}A, \textit{tet}C, \textit{tet}M, and \textit{tet}S ($p$<0.05).

**Acknowledgments**

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**References**


[3] Miège, C.; Choubert, J.M.; Ribeiro, L.; Eusèbe, M. Fate of pharmaceuticals and


[18] Huang, M.H.; Tian, S.X.; Chen, D.H.;; Zhang, W.; Wu J.; Chen, L. Removal of


[26] McKinney, C.W.; Loftin, K.A.; Meyer, M.T.; Davis, J.G.; Pruden, A. *Tet* and *sul* antibiotic resistance genes in livestock lagoons of various operation type; configuration;


LIST OF FIGURE CAPTIONS

Figure 1. Effect of HRT on the removal characteristics of TRB and TC under aerobic and anoxic conditions

Figure 2. SEM images of A1 sludge (a), A2sludge (b), AN1 sludge (c), and AN2sludge (d)

Figure 3. DGGE fingerprints and cluster analysis of mixed liquor samples at different oxygen conditions. A1, A2 – Aerobic SBR sludge samples; AN1, AN2 – Anoxic SBR sludge samples

Figure 4. Removal efficiency of optimized SBR with real wastewater
Fig. 1

TRB increases compared to the controlled SBR/×10^9 CFU·g⁻¹

Anoxic removal efficiency/%

HRT/h

Aerobic remove efficiency/%

HRT/h
Fig. 2
Fig. 4
<table>
<thead>
<tr>
<th>SBR</th>
<th>HRT/SRT</th>
<th>TC concentration gL⁻¹</th>
<th>TRB changes in sludge/×10⁸ CFUg⁻¹SS</th>
<th>COD/%</th>
<th>TN/%</th>
<th>TP/%</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>12h/8d</td>
<td>250</td>
<td>2.40</td>
<td>88±1</td>
<td>71±5</td>
<td>83±4</td>
</tr>
<tr>
<td>A2</td>
<td>0</td>
<td>0</td>
<td>-0.078</td>
<td>87±1</td>
<td>65±5</td>
<td>81±4</td>
</tr>
<tr>
<td>AN1</td>
<td>12h/8d</td>
<td>250</td>
<td>28.3</td>
<td>86±2</td>
<td>46±8</td>
<td>76±1</td>
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<tr>
<td>AN2</td>
<td>0</td>
<td>0</td>
<td>-0.049</td>
<td>85±1</td>
<td>49±9</td>
<td>73±6</td>
</tr>
</tbody>
</table>

Abbreviations: TC, tetracycline; TRB, tetracycline-resistant bacteria; HRT, hydraulic retention time; SRT, sludge retention time; COD, chemical oxygen demand; TN, total nitrogen content; TP, total phosphorus content; A1, aerobic SBR-1; A2, aerobic SBR-2; AN1, anoxic SBR-1; and AN2, anoxic SBR-2.
Table 2 Classification of bacterial bands in the DGGE profile

<table>
<thead>
<tr>
<th>Band no.</th>
<th>Clone No.</th>
<th>V3 zone</th>
<th>Bacteria species</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>&gt;2.11XD1013(DG41-2)</td>
<td>194bp</td>
<td><em>Thiovirgasulfuroxydans</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>&gt;2.11XD1013(DG41-2)</td>
<td>192bp</td>
<td><em>Acinetobacter</em> sp.</td>
</tr>
<tr>
<td>2</td>
<td>&gt;2.11XD1014(DG41-3)</td>
<td>186bp</td>
<td><em>Chlorobaculumthiosulfatophilum</em></td>
</tr>
<tr>
<td>3</td>
<td>&gt;2.11XD1015(DG41-4)</td>
<td>169bp</td>
<td>genus <em>TM7_genera_incertae_sedis</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>&gt;2.11XD1015(DG41-4)</td>
<td>186bp</td>
<td><em>Chlorobaculumthiosulfatophilum</em></td>
</tr>
<tr>
<td>4</td>
<td>&gt;2.11XD1022-5-DG41-11</td>
<td>194bp</td>
<td><em>Hydrogenophagapseudoflava</em></td>
</tr>
<tr>
<td>5</td>
<td>&gt;2.11XD1016(DG41-5)</td>
<td>194bp</td>
<td><em>Acidovorax</em> sp</td>
</tr>
<tr>
<td>6</td>
<td>&gt;2.11XD1017(DG41-6)</td>
<td>185bp</td>
<td><em>Chlorobaculumthiosulfatophilum</em></td>
</tr>
<tr>
<td>9</td>
<td>&gt;2.11XD1020(DG41-9)</td>
<td>194bp</td>
<td><em>Desulfobulbusrhabdoformis</em></td>
</tr>
<tr>
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<td></td>
</tr>
<tr>
<td></td>
<td>&gt;2.11XD1020(DG41-9)</td>
<td>189bp</td>
<td><em>Prolixibacter</em></td>
</tr>
<tr>
<td>10</td>
<td>&gt;2.11XD1021(DG41-10)</td>
<td>172bp</td>
<td><em>Spartobacteria bacterium</em></td>
</tr>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>&gt;2.11XD1021(DG41-10)</td>
<td>189bp</td>
<td><em>Actinomycesdenticolens</em></td>
</tr>
</tbody>
</table>
Table 3 Function diversity index of the sludge microbial community

<table>
<thead>
<tr>
<th>Sample</th>
<th>Simpson Index</th>
<th>Shannon Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>2.300</td>
<td>6.001</td>
</tr>
<tr>
<td>A2</td>
<td>2.226</td>
<td>4.998</td>
</tr>
<tr>
<td>AN1</td>
<td>2.117</td>
<td>3.999</td>
</tr>
<tr>
<td>AN2</td>
<td>1.948</td>
<td>3.001</td>
</tr>
</tbody>
</table>
Table 4 Changes in the target *tet* genes in the SBRs (copiesmL$^{-1}$)

<table>
<thead>
<tr>
<th>Sample</th>
<th><em>tetA</em></th>
<th><em>tetC</em></th>
<th><em>tetE</em></th>
<th><em>tetM</em></th>
<th><em>tet S</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>76539.92</td>
<td>23838.09</td>
<td>7405.58</td>
<td>1728.655</td>
<td>3190.2</td>
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<tr>
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<td>41</td>
</tr>
<tr>
<td>A2</td>
<td>72940.27</td>
<td>11659.44</td>
<td>2406.854</td>
<td>251.0435</td>
<td>2575.5</td>
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<tr>
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<td>75</td>
</tr>
<tr>
<td>AN1</td>
<td>596387.8</td>
<td>235711.4</td>
<td>13217.05</td>
<td>104621.7</td>
<td>5273.9</td>
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<td></td>
<td>2</td>
</tr>
<tr>
<td>AN2</td>
<td>192161.1</td>
<td>96810.55</td>
<td>652.0493</td>
<td>5447.726</td>
<td>3471.0</td>
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<td>56</td>
</tr>
</tbody>
</table>
Table 5 Correlation matrix of the five *tet* genes in the collected samples

(Pearson correlation coefficients)

<table>
<thead>
<tr>
<th></th>
<th><em>tet</em>A</th>
<th><em>tet</em>C</th>
<th><em>tet</em>E</th>
<th><em>tet</em>M</th>
<th><em>tet</em>S</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>tet</em>A</td>
<td>1.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>tet</em>C</td>
<td>0.989*</td>
<td>1.000</td>
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<td></td>
<td></td>
</tr>
<tr>
<td><em>tet</em>E</td>
<td>0.763</td>
<td>0.691</td>
<td>1.000</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>tet</em>M</td>
<td>0.983*</td>
<td>0.945</td>
<td>0.851</td>
<td>1.000</td>
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</tr>
<tr>
<td><em>tet</em>S</td>
<td>0.977*</td>
<td>0.978*</td>
<td>0.809</td>
<td>0.958*</td>
<td>1.000</td>
</tr>
</tbody>
</table>

*p < 0.05