Research Article

Received: 30 June 2015 Revised: 24 August 2015 Accepted article published: 1 September 2015 Published online in Wiley Online Library: 24 September 2015

(wileyonlinelibrary.com) DOI 10.1002/jctb.4806

Fate of tetracycline at high concentrations in enriched mixed culture system: biodegradation and behavior

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Abstract

BACKGROUND: Tetracycline (TC), a group of broad-spectrum synthetic antibiotics, has been detected in wastewater at concentrations ranging from ng L^{-1} to mg L^{-1}. However, low biodegradation efficiencies of TC, especially at high concentrations, were obtained in conventional activated sludge processes. This study aimed to investigate the biodegradation and behavior of TC at high concentrations in an enriched mixed culture system.

RESULT: High TC removal efficiencies of 83.6 ± 4.3% and 93.2 ± 2.2% were obtained in two reactors with the addition of TC at 10 mg L^{-1} and 1 mg L^{-1}, respectively. In the presence of TC, activated sludge floc size kept increasing and aggregation phenomena were observed. In addition, TC exposure decreased the microbial diversity and enriched the dominant bacteria. These effects were more prominent with higher TC concentration.

CONCLUSION: TC at high concentrations could be effectively removed in enriched mixed culture, and biodegradation was the primary mechanism. TC induced the aggregation of activated sludge and the effect was more prominent under higher TC stress. The TC exposure affected the microbial communities of activated sludge, resulting in a decrease in microbial diversity and the enrichment of dominant bacteria.

Supporting information may be found in the online version of this article.

Keywords: activated sludge; tetracycline; biodegradation; microbial community; aggregation

INTRODUCTION

The frequent detection of human and veterinary pharmaceuticals in the environment has become of increasing concern due to their potential physiological toxicity effects on ecological and human health.[1] Tetracycline antibiotics are a group of broad-spectrum synthetic antibiotics which are extensively used in human therapy and the livestock industry.[2] These kinds of antibiotics could be released into the environment from different routes, and residues have been detected in sewage effluents, hospital effluent, surface water and ground water.[3,4] Prior studies found that tetracycline antibiotics discharged into the environment are usually detected at concentrations ranging from ng L^{-1} to μg L^{-1},[5] but effluents from pharmaceutical factories can reach higher levels up to mg L^{-1}.[6] Moreover, Hamscher, Sczesny[7] found that veterinary drugs can also be released directly into the environment by spreading liquid manure, and tetracycline up to 20 mg L^{-1} could still be detected after application of these drugs in recommended dosages. Therefore, the treatment of tetracycline antibiotics, especially at high concentrations, is an urgent requirement.

Activated sludge processes are one of the most common biotechnologies for wastewater treatment. However, most wastewater treatment plants are not designed for emerging contaminants, and antibiotics cannot be removed effectively.[8,9] Many studies have focused on the fate and biological removal of antibiotics in activated sludge processes, and low degradation rates were obtained for antibacterial activity of antibiotics.[10] Spongberg and Witter[11] compared concentrations of 20 pharmaceutical compounds in influent and effluent from three wastewater treatment facilities in Northwest Ohio. They found that most antibiotics could be detected in effluent although the content in influent was quite low (<0.05 μg L^{-1}); the removal efficiency of tetracycline was only 11.6%. It has been reported that tetracycline was mainly removed via adsorption with little biodegradation in activated sludge systems.[12] To enhance the biodegradation of antibiotics, other biological methods need to be employed. Recently, high antibiotics biodegradation efficiencies were achieved in nitrifying sludge, but a long lag period was needed and the influent concentrations were at low μg L^{-1} level.[13,14] Furthermore, tetracycline at

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high mg L^{-1} level strongly inhibits the microbial activity of nitrifying sludge[15] and even stop nitrite oxidation.[16] Until now, there has been a lack of information regarding the effective biodegradation of tetracycline at high concentrations using activated sludge technology. More research needs to be done to ascertain the fate and behavior of tetracycline in such systems.

In this study, activated sludge was employed as an enriched mixed culture system to enrich microorganisms capable of degrading or tolerating pharmaceutical compounds. To investigate the fate of high-concentration tetracycline in this system, long-term operation and batch experiments were carried out, and changes in the microbial activity and microbial community due to tetracycline exposure were also evaluated. The results provide useful information to understand the biodegradation and behavior of tetracycline during wastewater treatment. In addition, this kind of knowledge is also important for the development of new technology to deal with pharmaceutical contaminants.

MATERIALS AND METHODS

Reactors and operation parameters

Two 1.5 L sequencing batch reactors (designated RI and RII) with a working volume of 1 L were seeded with activated sludge collected from the secondary clarifier of a pharmaceutical wastewater treatment plant in Jinan, China. The initial mixed liquid suspended solids concentrations (MLSS) were measured according to a standard method,[17] and were 783 and 750 mg L^{-1} in RI and RII, respectively. Both reactors were run at 25 ± 2 °C and covered with tin foil to prevent photolysis of tetracycline (TC), which was selected as a model tetracycline antibiotic in this study. The two reactors were operated continuously in successive cycles of 72 h, consisting of 20 min influent feeding with peristaltic pumps at a flow rate of 25 mL min^{-1}, 71 h stirring at 90 rpm, 30 min settling and 10 min effluent withdrawal. In each cycle, half of the reactor working volume was exchanged with influent wastewater. No sludge was discharged other than for sample analysis and the MLSS was stable during the operation.

Synthetic wastewater composition

In this study, synthetic wastewater was used to feed the reactors. The organic carbon source in the synthetic wastewater was sodium acetate (640 mg L^{-1}, COD was about 500 mg L^{-1}) and the nitrogen source was ammonium chloride (76 mg L^{-1}, NH_4^+-N concentration was about 20 mg L^{-1}). Sodium bicarbonate was added to maintain pH at 7.4 ± 0.4. Other trace components were added to the synthetic wastewater according to a previous report.[14]

Tetracycline (TC) of analytical grade was purchased from Aladdin Industrial Corporation (Table S1, Supplementary material), and stock solution at 50 mg L^{-1} was prepared with ultrapure water. At the beginning of each cycle, TC at doses of 10 mg L^{-1} and 1 mg L^{-1} was added in RI and RII, respectively. The range of TC concentrations was much higher than that observed in municipal wastewater but within the level of those found in effluent from drug manufacturers and from hospitals.[6,18] It was suitable to evaluate the treatability and fate of high-concentration TC in pharmaceutical sludge system.

In order to evaluate the effects of TC on activated sludge, original sludge was inoculated in a reactor without TC as control. Two other control reactors filled with inactivated sludge were operated to estimate the removal of TC by adsorption, because the microbial activity of the sludge was inhibited by adding 0.3% sodium azide. Reactors with only TC added were used for blank experiment to check and rule out the hydrolysis and volatilization of TC. The reactors used for blank and control experiments were then kept under identical conditions to RI and RII (details described in Supplementary material).

Analytical methods

The concentration of TC was analyzed by high performance liquid chromatography (HPLC, LC-20AT, Shimadzu, Japan) equipped with a 5 μm × 4 mm × 250 mm reversed-phase ODS-C18 column and detected using a UV detector at a wavelength of 360 nm. Isocratic elution was performed at a flow rate of 1 mL min^{-1}, and the mobile phase was a mixture of 0.01 mol L^{-1} oxalic acid solution/acetonitrile/methanol 80:16:4 (v/v/v). Before measurement, a standard curve ($R^2 > 0.999$) was made using an external standard method for TC determination and the limit of detection based on a signal-to-noise ratio greater than 3 was 10 μg L^{-1} for TC.[7]

The particle size was measured using a laser particle size analysis system (Mastersizer 2000, Malvern Instruments, UK). The morphology of activated sludge was observed using a scanning electron microscope (SEM, JEOL JSM-7600 F).

Analysis of TC in the liquid and solid phases

About 0.5 mL slurry samples were collected from both reactors and filtered through a 0.22 μm filter, and then stored at −20 °C until HPLC analysis for TC concentration in the liquid phase. For solids content analysis, samples were prepared using a modified method.[19] Suspension sludge (10 mL) was centrifuged at 3500 rpm for 10 min for solid phases sampling. The supernatant was removed and the precipitate was washed twice with 10 mL normal saline solution (0.9% NaCl solution). Then, the precipitate was re-suspended by addition of 10 mL normal saline solution and treated with ultrasonication, followed by centrifugation at 8000 rpm for 10 min, and the supernatant was collected into a 50 mL volumetric flask. The previous step was repeated three times and all the supernatant was collected into the volumetric flask and diluted to 50 mL. Finally, the diluted solution was filtered through a 0.22 μm filter, and stored at −20 °C until analyzed by HPLC to detect the TC concentration in the solid phase. The TC recovery using this method was $97.3 \pm 2.6\%$.

A mass balance method was used to calculate the fraction of TC removal via adsorption and biodegradation.[14]

$$\text{Water}\% = C_{w,t} / \left(C_{w,0} + C_{s,0}\right) \quad (1)$$

$$\text{Adsorption}\% = C_{s,t} / \left(C_{w,0} + C_{s,0}\right) \quad (2)$$

$$\text{Biodegradation}\% = 1 - \text{Adsorption}\% - \text{Water}\% \quad (3)$$

where $C_{w,0}$ and $C_{w,t}$ are the concentrations of TC in liquid phase at 0 h and t h, respectively; $C_{s,0}$ and $C_{s,t}$ are the concentrations of TC in solid phase at 0 h and t h, respectively.

Assay of dehydrogenase activity

The activity of dehydrogenase (DHA) was assayed according to a method described elsewhere.[20,21] This method is based on the reduction of 2, 3, 5-triphenyl tetrazolium chloride (TTC) as an artificial electron acceptor for dehydrogenase. A 10 mL slurry sample was taken from the reactors and centrifuged at 3500 rpm

for 10 min. The supernatant was discarded and the precipitate re-suspended with normal saline solution (0.9% NaCl solution) and centrifuged to wash the impurities away. After washing three times, the remaining sludge was re-suspended by adding 10 mL normal saline solution and treated with an ultrasonic cell disruption system for 10 min. Then, the mixed liquor was transferred to 50 mL centrifuge tubes with a cap, followed by the addition of 5.0 mL Tris–HCl buffer (pH = 8.4), 2.5 mL 0.36% Na_2SO_3 solution and 5 mL 0.4% TTC aqueous solution. The tubes were closed and incubated in the dark in a thermostatic water bath oscillator at a rotating speed of 200 rpm at 37 ± 1 °C for 1 h. After incubation, 2.5 mL of formaldehyde was added to the tube to stop the reaction and 10 mL of acetone was also added to extract the triphenyl formazan (TPF) formed. Then, the tube was mixed with a vortex mixer for 30 s and shaken at 37 ± 1 °C for 30 min in the dark. Finally, the mixture was centrifuged at 3500 rpm for 10 min and the supernatant was separated to measure the absorbance at 485 nm with a spectrophotometer. Dehydrogenase activity was expressed as mg TPF g^{-1} SS h^{-1}.

DNA extraction and 16S rRNA pyrosequencing

Sludge samples were collected from RI and RII after 2 months operation, and samples of the original sludge were also taken as the control. In this study, DNA of these sludge samples was extracted using the FastDNA Spin Kit for Soil (Sangon Biotech Co, Ltd). All operations were conducted according to the kit instructions. The extracted DNA was amplified with the universal primers 27f and 1492r under standard polymerase chain reaction (PCR) conditions.[22] 16S rRNA high-throughput pyrosequencing was performed with standard 454/Roche GS-FLX Titanium protocols. Operational taxonomic units (OTUs) and other relevant parameters were analyzed with Mothur software,[23] and the Ribosomal Database Project (RDP) classifier was used for Taxonomy analysis.[24]

RESULT AND DISCUSSION

Removal of TC during long-term operation

The TC removal performance during the long-term operation of the two reactors was investigated. As shown in Fig. 1, the influent concentration of TC was stable during operation, at 9.55 ± 0.81 mg L^{-1} and 0.91 ± 0.17 mg L^{-1} in RI and RII, respectively. Stable TC removal efficiencies of 83.6 ± 4.3% and 93.2 ± 2.2% were also obtained in RI and RII, indicating that the reactors were already in steady state operation. In order to determine the elimination of TC via hydrolysis or volatilization, blank experiments with only TC added were carried out in the dark. It was found that TC was stable and the elimination can be ignored (Fig. S1, Supplementary material), suggesting that the removal of TC in RI and RII was attributed mainly to adsorption and biodegradation by activated sludge. Moreover, sodium azide was added to inactivate activated sludge and minimize the removal of TC via biodegradation in control reactors. As shown in Fig. S2, TC was removed mainly in the first cycle and the removal efficiency was 76.7 ± 5.7% and 90.7 ± 2.8%, which was similar to those in RI and RII. Therefore, it was concluded that the initial removal of TC was mainly due to adsorption with little biodegradation in RI and RII. Little removal (less than 10%) was observed in the second cycle in control reactors, suggesting that the adsorption had reached equilibrium in the first cycle. Therefore, it was assumed that TC was mainly removed via adsorption onto activated sludge in the initial cycle, followed by continual removal via biodegradation in the subsequent period in RI and RII.

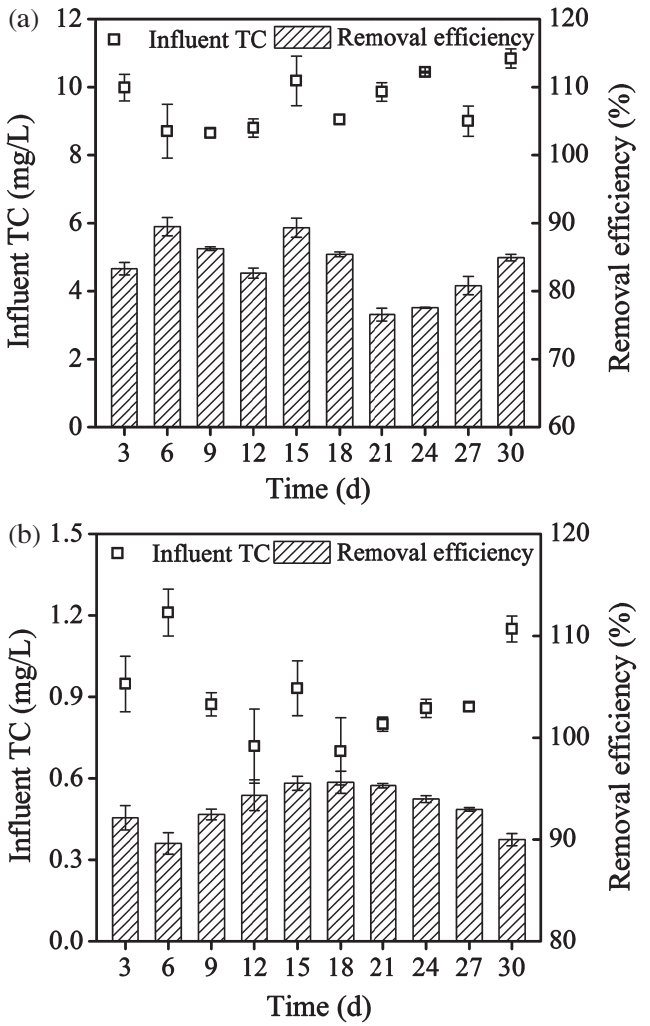

Figure 1. Removal of TC during the long-term operation of RI (a) and RII (b).

Fraction of TC removal

In this study, batch experiments were carried out to obtain further insights into the fraction of TC in the activated sludge system. The concentrations of TC in the liquid and solid phases were analyzed to estimate residual and adsorbed TC. Figure 2 shows the content changes of TC in the liquid and solid phases over 72 h in RI and RII. The proportion of adsorption decreased slightly during the initial stage of the cycle, and then stabled at 23.4 ± 4.5% and 23.9 ± 6.3% in RI and RII, respectively. These results implied that the sorption of TC by activated sludge had completely reached equilibrium, which was consistent with the results in control experiments (Fig. S2). Compared with adsorption, the biodegradation of TC occurred mainly in the initial 46 h. At the end of the experiment, the removal of TC via biodegradation increased to 35.2% and 55.7% in RI and RII, respectively. Furthermore, up to 63.3% and 67.4% of the total removed TC were biodegraded by activated sludge in RI and RII, showing that biodegradation is the primary mechanism for TC removal in both reactors. However, the efficiency of removal of TC via biodegradation in RII was improved by only 20% of that of RI, while the dosage of TC in RII was only one tenth of that in

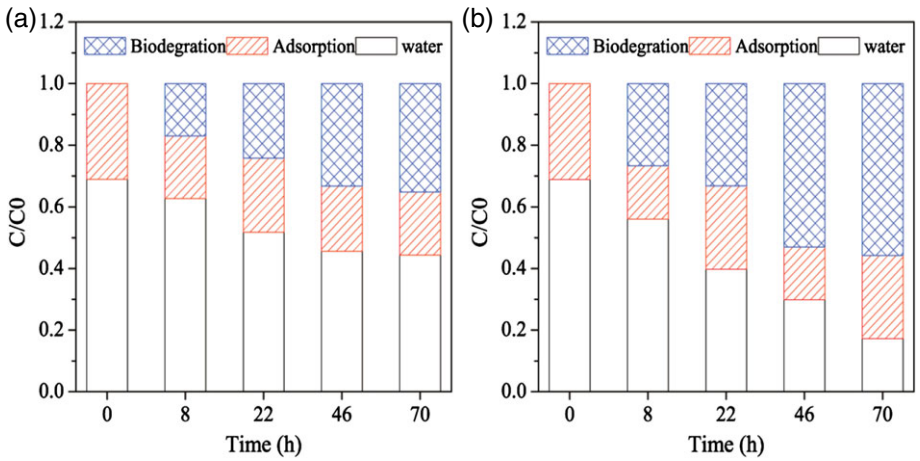

Figure 2. Fraction of TC in RI (a) and RII (b).

RI. The removal of micropollutants in the activated sludge process is affected by various factors, for example organic substrate might be related to the biodegradation of micropollutants for the co-metabolism.[25] Sodium acetate was added as organic carbon source in this study. As shown in Fig. S3, CH_3COONa was almost exhausted in the first 46 h, while about 95% of TC was removed via biodegradation at the same time. Therefore, co-metabolism might be the main method of TC biodegradation in this study.

A long lag period has often been observed before biodegradation of antibiotics in activated sludge reactors, and could be for three reasons: first, the accumulation of antibiotic in the solid phase occurs before biodegradation;[19] second, readily biodegradable substrates could cause competitive inhibition of antibiotic oxidation,[26] and last, microbial acclimation is required for bacteria to utilize micropollutants.[27] In this study no obvious lag period was found, which might be due to the original sludge and operating conditions. The original sludge was collected from a pharmaceutical factory having pharmaceutical wastewater treatment, and the two reactors were run for two months. Therefore, the microorganism had adapted to the environment with antibiotic. Besides, adsorption fully reached equilibrium during the initial period and biodegradation began at the beginning of the cycle.

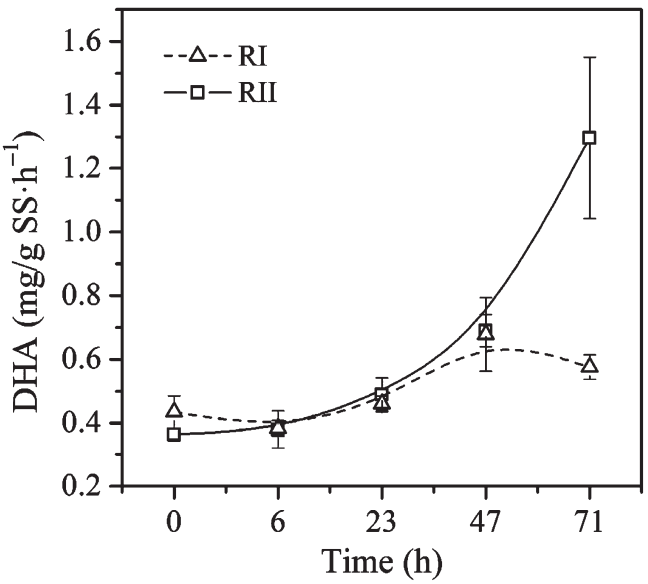

Figure 3. Variation of dehydrogenase activity during a cycle in RI and RII.

The enzyme activity of activated sludge

Dehydrogenases are enzymes that decompose organic compounds through a series of oxidation reactions involving transferring hydrogen atoms from organic compounds to an electron acceptor.[20] Therefore, the enzymatic activity of bacteria that utilize organic substrates is commonly investigated based on the dehydrogenase activity (DHA). TC is typical of many antibiotics and exerts a biostatic effect by inhibiting bacterial synthesis of proteins.[28] In this work, the DHA at different time in a cycle was monitored to evaluate the short-term exposure effects of TC on microorganism (Fig. 3). In RI, DHA changed little in the first 24 h, and then increased slightly to a stable level, while it increased rapidly from 0.36 ± 0.02 mg TPF g^{-1} SS h^{-1} to 1.29 ± 0.25 mg TPF g^{-1} SS h^{-1} in RII, which was more than twice as high as that in RI. In the initial period of each cycle, the content of TC was quite high in both reactors, which strongly inhibited microbial activity and resulted in low DHA. After that, TC was eliminated gradually via the biodegradation of activated sludge. The DHA in RII increased rapidly because of low TC concentration and weak inhibition, while the TC concentration in RI was still so high for the bacteria that DHA remained at a low level. DHA represents the substrate metabolism activity when the substrate is added.[29] However, the substrate was a mixture (sodium acetate and TC) in this study, implying that the DHA obtained might reflect a summation of activities of several different dehydrogenases.[21]

Particle size distribution of activated sludge

Aggregation phenomena were observed in both reactors during the operation, and particle size was analyzed to evaluate the macroscopical variation of activated sludge in the experiment (Fig. 4). The original sludge was a typical activated sludge with a fluffy, irregular and loose structure, and the average diameter was 260.6 ± 12.1 μm with a range of 80–300 μm, which is the typical diameter of activated sludge particles in conventional processes.[30] After 2 months operation, the size of activated sludge floc was 279.4 ± 9.2 μm in the control reactor without TC addition, which was a little higher than that of the original sludge. The sludge diameter increased to 551.8 ± 14.9 μm and 463.8 ± 5.9 μm in RI and RII, respectively. Therefore, the increase in sludge diameter might be mainly attributed to the addition of TC. These results

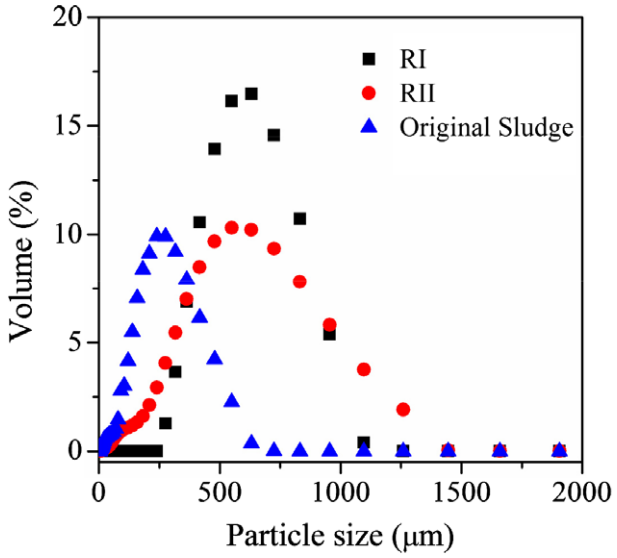

Figure 4. Particle size distribution of activated sludge.

indicated that TC might promote the aggregation of microorganisms, resulting in the increased particle size. Similar results have been observed in other studies. Fitzpatrick, Humphreys[31] reported that tetracycline at sub-inhibitory concentration had the potential to promote biofilm formation via elevated expression of the relevant gene. Some antibiotics can facilitate the aggregation of bacteria by inducing biofilm formation, and the effect would be enhanced by increasing antibiotic concentration over a certain range,[32] which is in agreement with our results. Furthermore, aggregation could provide protection for bacteria in the activated sludge against the inhibiting effects of toxic compounds.[33]

Microbial community analysis

16S rRNA pyrosequencing analysis was carried out to estimate bacterial community changes in the reactors with different TC concentrations. After screening out noise and poor-quality sequences, a total of 19 728 reads passed quality control and were used for subsequent analyses. The average number of sequence reads was 6576 per sample and the average length of the sequences was 416, 415 and 344 bp in RI, RII and original sludge, respectively. Good's coverage was more than 97.5% for all sequences (Table S3), indicating that the sequences could well represent the majority of microbial communities in the samples.[34] The phylogenetic classification of sequences by phylum and genus is summarized in Fig. 5. The most abundant phylum was *Proteobacteria* (average abundance > 90%) and *Bacteroidetes* (average abundance > 5%) in all samples, which is similar to a few studies on bacterial communities in activated sludge.[35] *Acidobacteria* (average abundance = 1.53%) and *unclassified_Bacteria* (average abundance = 1.83%) were also dominant phyla in original sludge, while only a little (average abundance < 0.5%) was observed in RI and RII. Furthermore, a few phyla, including *Chloroflexi*, *Cyanobacteria*, *Deinococcus*, *Gemmatimonadetes*, *Nitrospira*, and *Verrucomicrobia*, were also found in samples of the original sludge, and were undetected in samples of RI and RII. The reduction of microorganism phyla might be attributed to the exposure to TC in RI and RII. In order to better estimate the variation of bacterial community and to identify the most dominant microorganism under TC stress, the sequences were analyzed at the genus level (Fig. 5(b)). The genus *Thauera* constituted more than 75% of the total bacteria in the original sludge

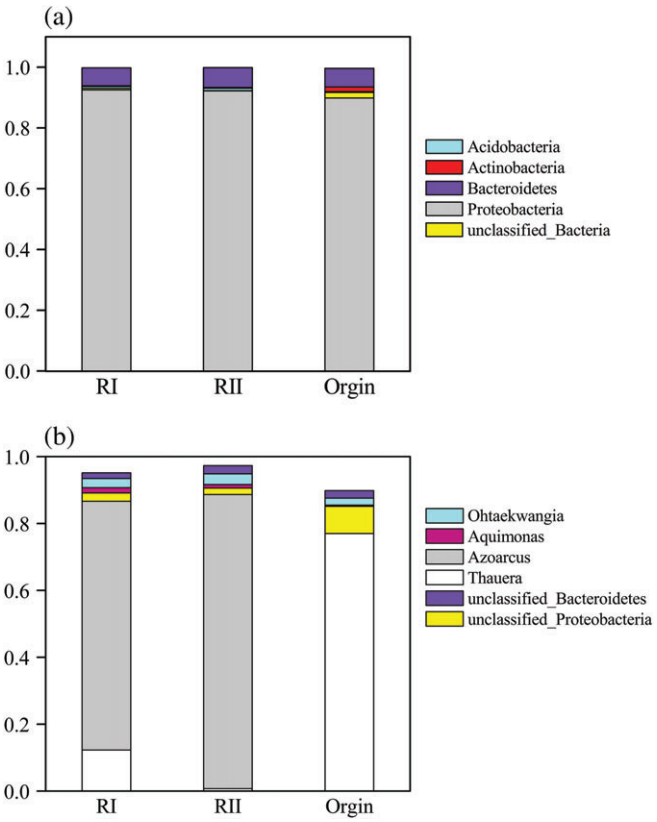

Figure 5. Taxonomic classification of bacterial reads at phylum (a) and genus (b) level based on bacterial OTUs at a dissimilarity level of 3%.

while *Azoarcus* was the most dominant genus, about 74% and 88% in RI and RII, respectively. Moreover, *Thauera* were no longer found in RII and the amount decreased to 12% in RI. *Thauera* and *Azoarcus* are a group of nitrate-reducing bacteria which can metabolize a great variety of aromatic compounds under both oxic and anoxic denitrifying conditions.[36] Besides, many strains of *Azoarcus* are related to the biodegradation of aromatic compounds via a meta-cleavage pathway of the aromatic rings.[37] Obviously, *Azoarcus* could better adapt the severe environment with TC than *Thauera* in this study. The *unclassified_Proteobacteria* decreased from 8.1% to 2.5% (RI) and 1.4% (RII) after the addition of TC, suggesting that these bacteria were susceptible to TC exposure.

There was significantly different microbial diversity and biological community in samples of the original sludge and reactors with TC added (RI and RII). The samples were divided into two clusters based on the jest algorithm (Fig. S5). The sequences in RI and RII were similar but very distinct from those in the original sludge, indicating that the microbial community changed after TC exposure. The community similarity was also investigated and the Venn diagrams at the 3% dissimilarity (Fig. S6) show that about 40% of phylotypes in RI were also present in RII whereas only 2.5% and 3.5% of total OTUs in the original sludge were observed in RI and RII. That could be explained by the selective pressure exerted by TC. It is known that TC specifically targets bacteria, and bacteria which could not tolerate TC would be gradually replaced by those adapting to TC.[38]

Community differentiation was also observed between RI and RII, especially at genus level. This could be attributed to the protection afforded by aggregation. The results of particle distribution revealed that TC could induce the formation of activated sludge

aggregation. Moreover, aggregation could protect the bacteria inside from TC exposure,[39] and the protection would be more prominent in RI having bigger particle size. Therefore, RI retained greater species diversity than RII while microorganism in RII suffered the screening of TC and most strains were eliminated, along with enrichment of the dominant bacteria.

CONCLUSIONS

In this study, we evaluated the biodegradation and behavior of high-concentration tetracycline in an enriched mixed culture. Tetracycline could be removed effectively via initial adsorption and subsequent biodegradation. The enzyme activity of activated sludge was inhibited by TC. Moreover, aggregation was induced by TC and the effect was more prominent under higher TC stress. The TC exposure affected the microbial communities of activated sludge, resulting in a decrease in microbial diversity and the enrichment of dominant bacteria.

ACKNOWLEDGEMENTS

This research was supported by the National Natural Science Foundation of China (51178254 and 51208283) and China Postdoctoral Science Foundation (2015 M570596). The authors wish to thank the Natural Science Foundation for Distinguished Young Scholars of Shandong province (JQ201116) for the support of this work.

Supporting Information

Supporting information may be found in the online version of this article.

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