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Effects of tetracycline on simultaneous biological wastewater nitrogen and phosphorus removal†

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The impact of tetracycline on simultaneous biological wastewater nitrogen and phosphorus removal and its fundamental mechanisms were investigated in this study. Compared with the control, lower concentration of tetracycline (0.2 mg/L) did not exert adverse effects on biological nutrient removal; however, the presence of 2 and 5 mg/L of tetracycline decreased the total nitrogen removal efficiency from 80.2% to 69.2% and 65.1% respectively, but they showed marginal influence on phosphorus removal. The mechanism studies showed that most of the influent tetracycline was adsorbed by sludge, which induced the release of extracellular polymeric substances (EPS) from sludge matrix, decreased the protective role of EPS on bacterial cells, declined the viability of sludge, increased the sludge volume index, and caused the detachment of denitrifying bacteria from sludge. Thus, the denitrifiers were more easily contacted with tetracycline. Further investigation revealed that it was the denitrifiers instead of nitrifiers being negatively affected by tetracycline, and the generation of electron donor for denitrification via intracellular polyhydroxyalkanoates (PHA) decomposition was depressed. In addition, tetracycline inhibited the activities of nitrate reductase and nitrite reductase as it was a strong chelating agent which reduced the free copper ions.

Introduction

Antibiotics represent one of the few classes of agents that can be widely used in the prophylaxis and therapy of human and animal infections and as animal growth promoters [1]. However, researchers found that these drugs were only partially metabolized by humans and animals after administration, and 50 - 80% of a given doses were recoverable from the urine as parent compounds [2]. Thus, some antibiotic agents can reach the environment inevitably [3, 4].

Recently, some types of antibiotics and their residues were reported in sewage and wastewater treatment plants (WWTPs) [5]. For example, high numbers of antibiotic agents were detected in Germany urban sewage treatment plants [6]. Tetracyclines and sulfonamides were commonly detected in pig farm wastewater with concentrations of 66 - 1000 and 15 - 275 μg/L respectively, in USA [7]. It was reported that 31% and 67% of water samples collected respectively near pig farms and poultry farms were antibiotics detectable [7].

The appearance of some antibacterial agents in WWTPs was observed to alter the capacity of activated sludge, and their influences on chemical oxygen demand (COD) removal and nitrifying bacterial growth were studied. For example, tetracycline inhibited COD removal and sludge yield [8], chlorotetracycline could decrease the growth of Nitrosomonas europaea [9], and ciprofloxacin reduced substrate utilization rate in biofilms [10]. Recently, it was reported that the presence of tetracycline in biofilms system had no significant effects on biodegradation of organic matter and activity of nitrifying biofilms [11]. However, the possible effects of antibiotic agents on simultaneous biological nitrogen and phosphorus removal performance and the fundamental mechanisms in wastewater treatment system have seldom been reported.

Tetracycline, one of the cheapest classes of antibacterial drugs with a broad spectrum of activity, has been used widely in developing countries [1]. In this paper, the effects of tetracycline on biological nitrogen and phosphorus removal were firstly investigated. Then, the mechanisms for tetracycline affecting biological nutrient removal were investigated from the aspects of sludge floc extracellular polymeric substances (EPS), sludge volume index (SVI), cell membrane integrity, sludge viability, transformations of influent carbon source and intracellular polyhydroxyalkanoates (PHA) and glycogen, electron donor generation for denitrification, and the activities of key enzymes.

Materials and Methods

Tetracycline and synthetic wastewater

Commercial tetracycline (Grade: UPS) used in this study was purchased from Aladdin (Shanghai, China). Before the experiments
the tetracycline stock solution (100 mg/L) was prepared by dispersing 0.1 g of tetracycline in 1 L Milli-Q water. The synthetic wastewater (3 L) was composed of 1.1 mL of acetic acid, 2.9 mL of nitrogen stock solution, 1.4 mL of phosphorus stock solution, 10 mL of concentrated solution, and 2 mL of trace-element solution. The nitrogen stock solution contained 133.75 NH₄Cl g/L. The phosphorus stock solution consisted of (g/L): 97.26 K₂HPO₄·3H₂O and 65.13 KH₂PO₄. The concentrated solution contained (g/L): 25.88 peptone, 4.24 yeast extract, 33.94 MgCl₂·6H₂O, 20 MgSO₄·7H₂O, and 9 CaCl₂·2H₂O. The trace-element solution contained (g/L): 10 EDTA, 1.50 FeCl₃·6H₂O, 0.18 KI, 0.15 CoCl₂·6H₂O, 0.15 H₂BO₃. 0.12 MnCl₂·4H₂O, 0.12 ZnSO₄·7H₂O, 0.06 Na₂MoO₄·2H₂O, and 0.03 CuSO₄·5H₂O. The final pH was adjusted to 7.5 by adding 4 M NaOH.

**Operation of sequencing batch reactor for simultaneous biological wastewater nitrogen and phosphorus removal**

The lab-scale sequencing batch reactor (SBR), with working volume of 4.0 L, was seeded with biomass from a biological nutrient removal wastewater treatment plant in Yancheng, China, and was operated in a temperature-controlled (21 ± 2 °C) room. The collection of the biomass for lab study is applicable and does not need licenses from the government of China. The SBR was operated with three cycles each day, with each cycle (8 h) consisting of 105 min anaerobic, 60 min aerobic, 60 min anoxic, 45 min aerobic, 45 min anoxic and 15 min aerobic period, followed by 60 min settling, 5 min decanting and 85 min for the remaining idle phase. In the first 10 min of the anaerobic stage, the reactor was fed with 3 L of synthetic wastewater to result in the initial concentrations of COD, ammonianitrogen (NH₄⁺-N) and soluble ortho-phosphorus (SOP) in the SBR of approximately 300, 25 and 10 mg/L, respectively. In the aerobic time, air was provided intermittently using an on/off control system with an on-line dissolved oxygen (DO) detector to keep the DO concentration in the reactor around 2.0 mg/L. Before the start of settling, sludge was wasted to maintain the solids retention time (SRT) of approximately 22 d. After the settling period, 3 L of supernatant was discharged. The reactor was constantly mixed with a magnetic stirrer except for the settling, decanting, and idle periods. After cultivation for 92 d, the stable removal efficiencies of nitrogen and phosphorus were observed in the SBR. Fluorescence in situ hybridization (FISH) analysis showed that the SBR was dominated by polyphosphate-accumulating organisms (PAO) (represented 47 ± 3% of the biomass) and glycogen-accumulating organisms (GAO) (represented 20 ± 4% of the biomass).

**Exposure experiments**

To conduct the experiments, 2400 mL of mixture was withdrawn from the above SBR at the end of the third aerobic stage, centrifuged at 100 g for 5 min, washed with 0.9% NaCl for 3 times, and resuspended in 400 mL of Milli-Q water. In this study 0.2 mg/L was chosen as the environmentally relevant concentration of tetracycline, and the higher concentrations (2 and 5 mg/L) were also considered. Then, 50 mL of resuspended sludge, 3 mL of 10% NaN₃, 225 mL of synthetic wastewater, and tetracycline stock suspension were added to 12 reactors according to Table S1 (Electronic supplementary information, ESI), and Milli-Q water was supplemented to make the final mixture volume in each reactor to be 300 mL. The initial concentrations of COD, NH₄⁺-N and SOP in each reactor were approximately 300, 25, and 10 mg/L, respectively. All reactors were bubbled with nitrogen gas for 10 min and covered with aluminum to avoid the possible light-induced effects. All other operations were the same as those described in the section of “Operation of sequencing batch reactor (SBR)”.

**Analytic methods**

In order to reveal the fate of tetracycline in reactors, water samples were filtered through 0.45 μm membrane, and then extracted using preconditioned Oasis HLB cartridges (Water, Milford, MA, USA). A high-performance liquid chromatography (LC) coupled to a triple quadrupole mass spectrometer (MS, Wasters Micromass Quattro Premier XE) equipped with electrospay ionization (ESI) was used to quantify tetracycline in the samples. LC separation was performed using a Waters Symmetry C18 column (150 mm×2.1 mm, 5μm). The chromatographic mobile phase consisted of 40% acetonitrile (A) and 60% formic acid solution (0.2%, v/v) (B). For operation in MS mode, capillary voltage of 3.5 kV was used in positive ionization mode. The gas nebulizer gas flow was set to be approximately 50 L/h and the desolvation gas flow was 450 L/h. The interface and source temperature was 300 °C and 120 °C, respectively. Dwell time was 2 s/scan. Quantification of target analytes was based on the external calibration curves, and the correlation coefficients (R²) of the calibration curves were exceeded 0.993 for all analytes. Recoveries of the tetracycline in samples were determined at different concentration levels in triplicate, and calculated as the percentages of the measured concentrations relative to the spiked concentrations. Limits of quantification (LOQ) of the tetracycline were calculated with signal/noise ratios of 10.

The extraction of effluent EPS was conducted according to the literature [12]. Briefly, at the end of the removal exposure experiments, 50 mL of the effluent was sampled and filtered by the 1.5 μm membrane. The analyses of soluble proteins, humic acids, polysaccharides, and DNA in the extracted EPS were detailed in the references [13, 14].

The relative density of denitrifying bacteria in the effluent was assayed as follows. The mineral medium was prepared according to the reference with minor modification (g/L): 5.0 glucose, 7.0 K₂HPO₄, 3.0 KH₂PO₄, 0.5 sodium citrate·2H₂O, 0.1 MgSO₄·7H₂O, 1.0 (NH₄)₂SO₄, 2.16 KNO₃ and trace elements solution of 50 μL/L [15]. The trace elements contained (g/L): 7.4 CaCl₂·2H₂O, 1.0 MnSO₄·H₂O, 3.6 ZnSO₄·7 H₂O, 0.4 CoCl₂·6H₂O, 0.96 FeCl₃·6H₂O, 0.03 CuCl₂·2H₂O, 0.3 H₃BO₃, 0.01 NiCl₂·6H₂O, 3.7 EDTA·Na₂. 100 mL mineral medium was added to each serum bottle. The nitrogen source was 300 mg NO₃⁻-N/L. Then 1 mL of effluent was added, and gas argon was purged into each bottle for 10 min to ensure the anaerobic condition. After sealing, all bottles were placed in a shaker (200 rpm) with constant temperature of 30 °C for 5 d, and the OD₆₀₀ values were tested to calculate the relative density of denitrifying bacteria in the effluents.

The cell membrane integrity and cell viability of activated sludge were respectively measured by the cytotoxicity detection kit (lactate dehydrogenase (LDH) release assay) (Roche Molecular Biochemicals) and cell counting kit-8 (cell proliferation assay) (Dojindo) according to the manufacturer’s instructions. The determinations of SVI, mixed liquor suspended solids (MLSS) and mixed liquor volatile suspended solids (MLVSS) were conducted in...
accordance with the Standard Methods [16]. The analyses of intracellular PHA, including poly-3-hydroxybutyrate (PHB), poly-3-hydroxyvalerate (PHV) and poly-3-hydroxy-2-methylvalerate (PH2MV), and glycogen were carried out by the method reported previously [17]. The detailed procedures of FISH assay and the measurements of NH$_4^+$ -N, NO$_3^-$ -N, NO$_2^-$ -N, TN (total nitrogen), SOP, copper ion, AMO (ammonia monoxygenase), NOR (nitrite oxidoreductase), NAR (nitrate reductase) and NIR (nitrite reductase) activities were carried out according to our previous publications [18, 19].

**Statistical Analysis**

All tests were performed in triplicate and the results were expressed as mean ± standard deviation. An analysis of variance (ANOVA) was used to test the significance of results and $p < 0.05$ was considered to be statistically significant.

**Results and Discussion**

**Effects of tetracycline on nitrogen and phosphorus removal**

From Fig. 1 it can be seen that the total nitrogen removal efficiency in the control test was 80.2%, and it was 82.0 % in the test of 0.2 mg/L of tetracycline. However, the TN removal was decreased respectively to 69.2% and 65.1% at 2 and 5 mg/L of tetracycline. Apparently, the increase of tetracycline to 2 mg/L in biological nutrient removal system inhibited total nitrogen removal. As to the SOP, the data in Fig. 1 showed that the removal efficiency was respectively 97.1%, 98.1%, 96.5%, and 96.8% in the control, 0.2, 2, and 5 mg/L of tetracycline tests, which indicated that the presence of tetracycline did not significantly affect phosphorus removal ($p > 0.05$). It is well established that tetracyclines inhibit bacterial protein synthesis by preventing the association of aminoacyl-tRNA with the bacterial ribosomal acceptor (A) site [1]. Therefore, to interact with their targets these molecules need to traverse one or more membrane systems depending on whether the susceptible organism is attachable or un-attachable. Hence, a discussion of the differentiated effects on nitrogen and phosphorus removal requires consideration of extracellular attachment and intracellular inhibition mechanisms. In the coming text the mechanisms for tetracycline negatively affecting nitrogen removal and marginally affecting phosphorus removal were investigated.

**Fig. 1. Effects of tetracycline on the removal of total nitrogen and soluble ortho-phosphorus during one cycle of biological wastewater treatment.** Asterisks indicate statistical differences ($p < 0.05$) from the control. Error bars represent standard deviations of triplicate tests.

**The fate of tetracycline in simultaneous biological wastewater treatment system**

The antibiotic removal function in activated sludge wastewater treatment systems includes a variety of processes, namely biodegradation, adsorption onto biomass, hydrolytic action, volatilization, and potodegradation [20, 21]. In the literature it was reported that the removal of tetracycline in wastewater treatment facilities was achieved mainly by adsorption [20, 22] or biodegradation [11]. In this study, all reactors were covered with aluminum. We, therefore, assumed that there was no light-induced degradation of tetracycline in the current biological wastewater treatment reactors. As shown in Fig. 2, when the initial tetracycline was 0.2, 2 and 5 mg/L, its effluent concentrations were respectively 14.7, 214.8 and 612.5 μg/L, and the corresponding tetracycline removal efficiencies were 92.65%, 89.26% and 87.73%.

**Fig. 2. The effluent concentration and the removal efficiency of tetracycline during one cycle of biological wastewater treatment.** Error bars represent standard deviations of triplicate tests.

According to the experimental design of Table S1 (ESI), it was found that more than 83% of the initial tetracycline was removed from wastewater by adsorption onto activated sludge, and less than 5% was hydrolyzed and volatilized (see Fig. S1, ESI). The data in Fig. S1 (ESI) showed that the biodegraded tetracycline, however, was only accounted for 1.3% -1.88%. Apparently, most of the tetracycline was removed by adsorption in the current study, which indicated that the influent tetracycline could easily contact the nitrogen and phosphorus removal microbes in activated sludge flocs.

**Effects of tetracycline on EPS release and sludge bacterail viability**

EPS play a major role in aggregation and protection of bacterial cells [23]. It is well-known that EPS, which are mainly composed of protein, polysaccharide polymers and DNA, form a gel-like matrix in which microcolonies are embedded through both electrostatic and hydrophobic interactions to impede access of chemicals to bacterial cells [12]. In the current study, the data in Fig. 3a showed that the effluent EPS concentrations were increased to 23.03 and 29.31 mg/L at tetracycline of 2 and 5 mg/L, respectively, which were higher than that in the control (19.81mg/L). This suggests that the tetracycline binding to activated sludge flocs is important to the release of EPS. Hydroxyl, carboxyl, and amino groups were the domain chemical groups involved in the interaction between tetracycline and EPS, and the binding of tetracycline onto EPS changed the structure of these chemical groups [24], which might cause the shifts in the composition of the released EPS. Further investigation on the composition of EPS indicated that all four reactors had almost the same contents of DNA and humic acids, but proteins and carbohydrates contents were increased with the increase of tetracycline (Fig. 3b). Thus, higher effluent content of EPS at tetracycline of 2 or 5 mg/L was due to the increase of proteins and carbohydrates released from activated sludge.

**Fig. 3. Effects of tetracycline on the effluent total (a) and composition (b) of ESP.** Asterisks indicate statistical differences ($p < 0.05$) from the control. Error bars represent standard deviations of triplicate tests.

The major part of the EPS is bounded with cells mainly through ion bridging with multivalent metals and cations, which neutralizes the negatively charged particles, stabilizes the negative surface charge of polymers, and acts as binding agent in forming bridges between particles and polymers [25]. The EPS matrix of sludge surrounding the attached cells also provides an effective barrier...
that restricts penetration of chemically reactive biocides inside the cells [26]. Thus, sludge viability and its flocculation are associated with EPS. Tetracycline is a strong chelating agent [27, 28], which may establish strong interactions with metal ions (e.g., Ca" and Mg") and results in the destroy of the structure of sludge flocc matrix. Destroy of the structure of sludge flocc matrix, which increased the binding chance of tetracycline with mirobes, were correlated with increasing in the susceptibility to tetracycline in isolates [1], thus causing the biological deficiency of microbes.

It was reported that the biological function of cells could be affected after cell membrane integrity had been damaged [29]. In this study the cell membrane integrity was measured by LDH release. As seen in Fig. 4 the presence of 0.2 or 2 mg/L of tetracycline induced the similar LDH release to the control, suggesting that tetracycline did not negatively affect sludge membrane integrity at these concentrations. However, with the increase of tetracycline to 5 mg/L, the LDH release was increased to almost 120% of the control. The results of cell viability assay in Fig. 4 confirmed that the adverse effect of tetracycline on sludge bacterial viability was significant at the concentration of 5 mg/L.

**Fig. 4. Effects of tetracycline on LHD release and sludge viability.** Asterisks indicate statistical differences (p < 0.05) from the control test. Error bars represent standard deviations of triplicate tests.

In the literature it was reported that killing properties of antibiotics are increasing while all possible binding sites in the EPS matrix are becoming saturated [30]. The presence of 3.33 μg/L of ciprofloxacin affected the structure and activity of biofilms [10]. However, the presence of 50 μg/L of tetracycline was observed to give no obvious impact on the removal of carbon and nitrogen in a lab-scale sequencing batch biofilm reactor [11]. The studies of Deng et al. found a significant decrease of COD removal appeared in a full-scale bio-system fed with wastewater containing high concentration of streptomycin (39.556 mg/L) [31]. The current study showed that 0.2 and 2 mg/L of tetracycline did not affect the bacterial viability of activated sludge, while 5 mg/L of tetracycline negatively affected the denitrification. It seems the influence of antibiotics on activated sludge was type and concentration dependant.

Some researchers found that the bacteria living in biofilms can be up to 1000 times more resistant to antibacterial compounds than planktonic cell [32, 33]. The changes of sludge EPS has been reported to influence its settleability, which can be expressed by the data of sludge SVI [34]. Form Fig. 5a it can be seen that the SVI value of sludge in the control reactor was 99.4 mL/g-MLSS, and it was slightly increased to 105.3 and 108.4 mL/g-MLSS in the reactor of 0.2 and 2 mg/L of tetracycline, respectively. Nevertheless, the sludge SVI was increased to 121.1 mL/g-MLSS as the concentration of tetracycline was 5 mg/L. High SVI data indicate that the sludge is loose, and the cells are easily exposed to the environment. Thus, if the environment is not conducive to the growth and metabolism of microorganism, the activity of bacteria would be decreased. The data in Fig. 5b revealed that with the increase of tetracycline more dispersed denitrifying bacteria were observed in the effluent, suggesting higher concentration of tetracycline caused the detachment of denitrifying bacteria from sludge matrix, which would lead them to be exposed directly to the toxic tetracycline.

**Fig. 5. Effects of tetracycline on the SVI of activated sludge (a) and the abundance of denitrifying bacteria in the effluent (b).** Asterisks indicate statistical differences (p < 0.05) from the control test. Error bars represent standard deviations of triplicate tests.

**Effects of tetracycline on transformations of nitrogen and phosphorus**

It is well known that biological nitrogen removal depends on successful nitrification (ammonia oxidation to nitrate) and the subsequent denitrification (nitrate reduction to nitrogen gas). As seen in Fig. 6a, there was no significant difference in the variations of ammonia nitrogen among four reactors during one cycle, which suggested that the influence of 0.2, 2, or 5 mg/L of tetracycline on nitrification was marginal. From Figs. 6b and 6c it can be seen that the average effluent NO$_3^-$-N and NO$_2^-$-N in the control (0.10 and 4.89 mg/L) were almost the same as those in the 0.2 mg/L of tetracycline test (0.09 and 4.42 mg/L). It seems that the presence of 0.2 mg/L of tetracycline did not significantly affect denitrification (p > 0.05). However, with the increase of tetracycline to 2 and 5 mg/L, the effluent NO$_3^-$-N and NO$_2^-$-N were respectively increased to 1.95 and 5.71 mg/L, and 2.30 and 6.45 mg/L, indicating that the denitrification process was negatively influenced when the concentration of tetracycline was greater than 2 mg/L. The data in Fig. 6d showed that the variations of SOP among four reactors were almost the same, which was in correspondence with the above observation of tetracycline giving no significant influence on phosphorus removal. It can be concluded that denitrifying bacteria were more sensitive to tetracycline, but other bacteria, such as ammonia-oxidizing bacteria (AOB), nitrite-oxidizing bacteria (NOB) and phosphorus accumulating organisms (PAO), showed less sensitive, and the decrease of biological nitrogen removal induced by higher concentration of tetracycline was due to its inhibition to denitrification instead of nitrification.

**Effects of tetracycline on transformations of influent carbon source and intracellular PHA and glycogen**

During the anaerobic phase the external carbon substrate (acetic acid in this study) is taken up and stored as PHA by using the energy (ATP) and reducing power respectively from poly-phosphorus and glycogen degradation. As shown in Fig. 7a, although the uptake rate of acetic acid was influenced by the increase of tetracycline, all acetic acid was consumed by the end of anaerobic phase in each of the four reactors. According to the data in Fig. 7b, however, it can be seen that the anaerobic synthesis of PHA was influenced by tetracycline as its concentrations were 2 and 5 mg/L (p < 0.05). The analysis of PHA composition showed that the reason for greater PHA synthesis at higher tetracycline concentration was due to more PHB instead of PHB and PH2MV produced (Fig. 7c). Usually, most of the influent acetic acid was bio-converted to intracellular PHB.
with very little PHV and PH2MV under anaerobic conditions of biological nutrient removal system [35]. In this study, since all of the influent acetic acid in four reactors was consumed, the same amount of anaerobic PHB accumulation was therefore observed. From the data in Fig. 7d it can be seen that, compared with the control, the anaerobic glycogen degradation was higher at tetracycline concentrations of 2 and 5 mg/L. It was reported that the degradation of glycogen under anaerobic conditions could generate propionyl-CoA (the precursor for PHV synthesis) through the succinate-propionate pathway [36]. Thus, more PHV synthesis appeared in the reactors of 2 and 5 mg/L of tetracycline.

Fig. 7. Effects of tetracycline on the uptake of influent carbon source (a), and the transformations of intracellular total PHA (b), individual composition of PHA (c) and glycogen (d) during one cycle. Error bars represent standard deviations of triplicate tests.

The PHA synthesized in the anaerobic stage was then decomposed to provide energy for aerobic and/or anoxic SOP uptake and electron donor for anoxic denitrification. The data in Fig. 7b indicated that the amount of total PHA degraded in the aerobic and anoxic phases was respectively 23.11, 23.26, 20.62, and 20.3 mg-C/g-MLVSS in the reactors of control, 0.2, 2 and 5 mg/L of tetracycline. Obviously, the presence of 2 or 5 mg/L of tetracycline decreased the degradation of PHA. The above discussion showed that there were no significant differences in the phosphorus anaerobic release, aerobic and anoxic uptake, and net removal in four reactors, which indicated that the amount of PHA being oxidized for phosphorus uptake was similar among four reactors. Thus, less PHA was utilized in anoxic stage for denitrification at tetracycline dosage of 2 or 5 mg/L, which was an important reason for higher tetracycline decreasing the total nitrogen removal in the current study.

Effects of tetracycline on activities of key nitrification and denitrification enzymes

Biological nitrification is mainly catalyzed by AMO and NOR [37], whereas denitrification is related to NAR and NIR [38, 39]. As shown in Table 1, among four nitrogen metabolism enzymes (AMO, NOR, NAR, and NIR), higher concentrations (2 and 5 mg/L) of tetracycline decreased the specific activities of NAR and NIR (p < 0.05), but showed no measurable influence on AMO and NOR (p > 0.05). These observations were in correspondence with higher effluent concentrations of NO₃⁻ -N and NO₂⁻ -N, and lower TN removal efficiencies at tetracycline concentrations of 2 and 5 mg/L (Figs. 1 and 6). It seems that the decreased biological nitrogen removal at higher concentration of tetracycline was attributed to the inhibition of NAR and NIR.

Table 1. Activities of the key enzymes related to biological nitrogen removal in the presence of tetracycline.a

<table>
<thead>
<tr>
<th></th>
<th>NOR</th>
<th>AMO</th>
<th>NAR</th>
<th>NIR</th>
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</thead>
<tbody>
<tr>
<td>control</td>
<td>0.086</td>
<td>0.022</td>
<td>0.044</td>
<td>0.292</td>
</tr>
<tr>
<td>±0.004</td>
<td>±0.002</td>
<td>±0.005</td>
<td>±0.003</td>
<td></td>
</tr>
<tr>
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<td>0.25</td>
<td>0.048</td>
</tr>
<tr>
<td>(mg/L)</td>
<td>±0.005</td>
<td>±0.002</td>
<td>±0.003</td>
<td>±0.003</td>
</tr>
<tr>
<td>2</td>
<td>0.089</td>
<td>0.020</td>
<td>0.034</td>
<td>0.176</td>
</tr>
</tbody>
</table>

a The unit is mol nitrite/(min·mg protein). The data reported are the averages and their standard deviations of triplicate tests.

Conclusions

Compared with the absence of tetracycline, the presence of 0.2 mg/L of tetracycline did not exert any influence on biological nitrogen and phosphorus removal; however, the presence of 2 and 5 mg/L of tetracycline decreased the total nitrogen removal efficiency from 80.2% to 69.2% and 65.1%, respectively, but these concentrations of tetracycline had no adverse effects on phosphorus removal. It was observed that most of the influent tetracycline was adsorbed by activated sludge in biological wastewater treatment system. Higher concentration of tetracycline caused the release of EPS from sludge matrix, the decrease of sludge viability, the increase of sludge SVI, and the detachment of denitrifying bacteria from activated sludge. Also, it was found that the inhibition of nitrogen removal by higher concentration of tetracycline was caused by the declined denitrification, rather than its inhibition to ammonia oxidation. Further investigation revealed that the decomposition of microbial intracellular PHA for electron donor generation in denitrification was depressed, and the activities of NAR and NIR were inhibited under conditions of higher tetracycline.

Acknowledgements

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Fig 1. Effects of tetracycline on the removal of total nitrogen and soluble ortho-phosphorus during one cycle of biological wastewater treatment. Asterisks indicate statistical differences ($p < 0.05$) from the control. Error bars represent standard deviations of triplicate tests.
Fig. 2. The effluent concentration and the removal efficiency of tetracycline during one cycle of biological wastewater treatment. Error bars represent standard deviations of triplicate tests.
Fig. 3. Effects of tetracycline on the effluent total (a) and composition (b) of ESP. Asterisks indicate statistical differences ($p < 0.05$) from the control. Error bars represent standard deviations of triplicate tests.
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Fig. 5. Effects of tetracycline on the SVI of activated sludge (a) and the abundance of denitrifying bacteria in the effluent (b). Asterisks indicate statistical differences ($p < 0.05$) from the control test. Error bars represent standard deviations of triplicate tests.
Fig. 6. Effects of tetracycline on the variations of \( \text{NH}_4^+ \)-N (a), \( \text{NO}_3^- \)-N (b), \( \text{NO}_2^- \)-N (c), and soluble phosphorus (d) during one cycle. Error bars represent standard deviations of triplicate tests.
Fig 7. Effects of tetracycline on the uptake of influent carbon source (a), and the transformations of intracellular total PHA (b), individual composition of PHA (c) and glycogen (d) during one cycle. Error bars represent standard deviations of triplicate tests.
**Highlights:**

- The Presence of 2 and 5 mg/L of tetracycline decreased total nitrogen removal.
- Tetracycline induced EPS release and decreased its protective role on cells.
- Denitrifiers instead of nitrifiers were negatively affected by tetracycline.
- Tetracycline depressed PHA decomposition for electron donor generation.
- Tetracycline inhibited the activities of key denitrification enzymes.