Article

Simultaneous Removal of Nitrate and Tetracycline by an Up-Flow Immobilized Biofilter

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Abstract: The removal of nitrate (NO_3^--N) and antibiotics in aquaculture tail water is urgent and necessary. A lab-scale up-flow immobilized biofilter (I-BF) filled with polyurethane foam (PUF) carriers and a microbial consortium was developed for simultaneous removal of nitrate and tetracycline (TC). The denitrification and TC removal performance of the I-BF reactor was investigated under different TC concentrations (0, 10, 50, 100 mg·L^{-1}), carbon/nitrogen (C/N) ratio (2, 4, 5, 6) and hydraulic retention times (HRT) (4, 8, 12 h). Simultaneous removal of nitrogen and TC was achieved by the I-BF reactor. Low TC concentration ($\leq$50 mg·L^{-1}) had little effect on nitrogen removal. The denitrification performance of the I-BF reactor was inhibited at high TC load, which may be attributed to the damage of cell membranes and the inhibition of the intracellular denitrification enzymes' activities. The optimal C/N ratio and HRT were 5 h and 8 h with almost complete denitrification and high TC removal efficiency (73.46%) at influent NO_3^--N and TC concentrations of 100 mg·L^{-1} and 50 mg·L^{-1}, respectively. The I-BF reactor proposed in this study has promising applications such as the treatment of piggery wastewater, aquaculture tail water and pharmaceutical wastewater co-contaminated with nitrate and antibiotics.

Keywords: denitrification; tetracycline; immobilized biofilter reactor

1. Introduction

Aquaculture production in China reached 70.48 million tons per year in 2020, accounting for the largest share of the world's fishery production [1]. The aquaculture industry, characterized by high density, intensification and scale has resulted in high bioburden and high input farming models. The main inputs in aquaculture systems are feed and seston; only a small percentage is transformed to fish biomass, and most of it is released to water as feed wastes and excreta [2]. The nitrogen use efficiency is only about 11.7% to 27.7%. Large amounts of nitrogen discharge into aquatic ecosystems, are leading to nutrient enrichment of water and sediments [3] and acute health problems. In recirculating aquaculture systems, accumulation of NO_3^--N may result from the low rate of replacement of culture water [4]. At the same time, antibiotics have been widely used in aquaculture due to their effectiveness in preventing and controlling infectious diseases, promoting growth and reducing nutrients consumption [5,6]. The overuse of antibiotics in aquaculture has brought serious threats to the environment and human health [7]. Both nitrate and large amounts of unconsumed antibiotics end up in outlet waters [8], thus there is a need to develop methods to remove the nitrate and antibiotics simultaneously.

Advanced oxidation processes (AOPs), such as photocatalytic degradation [9] and ozonation [10], have been successfully used to remove antibiotics in water environments. However, the main drawback of AOPs is the formation of by-products which might have similar or even higher toxicity than the parent compounds [11]. Biological removal of antibiotics is a promising and environmentally friendly approach. Meanwhile, biological

denitrification process as a crucial step in nitrogen removal is also being widely applied due to its effectiveness and economic efficiency [12]. Hence, simultaneous denitrification and removal of antibiotics through a biological approach needs more attention.

TC is one of the most commonly-applied antibiotics in aquaculture for treating infections and promoting animal growth [13]. The consumption of TC shows a trend of rapid growth. TC has negative effects on denitrification [4]; however, simultaneous removal of TCs during denitrification by microorganisms can be achieved. Shao et al. [14] suggested that 49.95% of tetracycline and 60.45% of NO_3^--N were removed by *Klebsiella* sp. SQY5 in aerobic conditions. Simultaneous removal of NO_3^--N (91.97%), manganese (Mn (II)) (71.25%) and TC (57.39%) were achieved at HRT of 9 h, Mn (II) and TC concentration of 20 mg·L^{-1} and 1 mg·L^{-1} by a novel loofah immobilized bioreactor [15]. The TC degradation and nitrate removal process can be influenced by various factors, such as reactor type, carbon source, TC concentration and environmental conditions, resulting in differences of removal efficiency [16,17]. More research needs to be done to develop a simple and efficient reactor and to investigate the optimal operation conditions in order to achieve a more stable and highly-effective removal of nitrate and TC. In our previous researches, I-BF reactors based on polyurethane foams (PUFs) and functional microbial consortium, with advantages of high biological load, high degradation capacity, strong resistance to loading fluctuation, and low construction and operating cost, have been successfully used in the treatment of high ammonia-nitrogen wastewater, explosive wastewater [18,19], oil field wastewater [20] and nitrate micro-polluted water [21]. Based on these previous experiences, a lab-scale up-flow I-BF was developed for simultaneous removal of nitrate and TC in this study.

The objectives of this study were (i) to assess the feasibility and performance of I-BF in removing nitrate and TC, (ii) to investigate the effects of different TC concentrations, C/N ratio and HRT on simultaneous removal efficiencies of nitrate and TC, and (iii) to reveal the inhibition mechanism of TC on denitrification by the I-BF.

2. Materials and Methods

2.1. Materials

The microbial consortium (stored in the form of microbial freeze-dried powder) which contained various strains of microorganisms and enzymes associated with pollutant biodegradation was obtained from BIONETIX Co. (Montreal, QC, Canada). The self-made patent functional PUFs carriers with specific surface area between 80 and 120 m^2·g^{-1} were selected to immobilize the above microbial consortium. The carriers with porosity of 98% and humidity density of 1 g·cm^{-3} were trimmed into 1 cm × 1 cm × 1 cm cubes before use. TC and Chromatogram class acetonitrile were purchased from Aladdin (Shanghai, China) and Anaqua Chemicals Supply (Wilmington, NC, USA), respectively. Other chemicals used were analytical grade and purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China).

2.2. Experimental Apparatus

A lab-scale up-flow immobilized biofilter (I-BF) using an organic glass cylindrical column (diameter 8 cm, height 65 cm), with a working volume of 3L was set up to carry out the experiments as shown in Figure 1. PUFs carriers with filling rate of 30% (*v/v*) and 3 g microbial freeze-dried powder were added into the reactor. Synthetic wastewater was continuously pumped from the influent bucket into the I-BF reactor through the inlet at the bottom of the reactor by a peristaltic pump. The composition of the synthetic wastewater was as follows: 0.304 g·L^{-1} sodium nitrate (NO_3^--N concentration of 50 mg·L^{-1}), 0.320 g·L^{-1} sodium acetate (C/N = 5:1), 1.21 g·L^{-1} disodium hydrogen phosphate, 0.743 g·L^{-1} monopotassium phosphate, 40 mg·L^{-1} calcium chloride, 10 mg·L^{-1} magnesium chloride and 1 mL·L^{-1} trace element solution [22]. Nitrogen gas was filled to maintain the dissolved oxygen (DO) of the reactor below 0.2 mg·L^{-1} and fluidize PUFs carriers. During the set-up stage, HRT was 12 h.
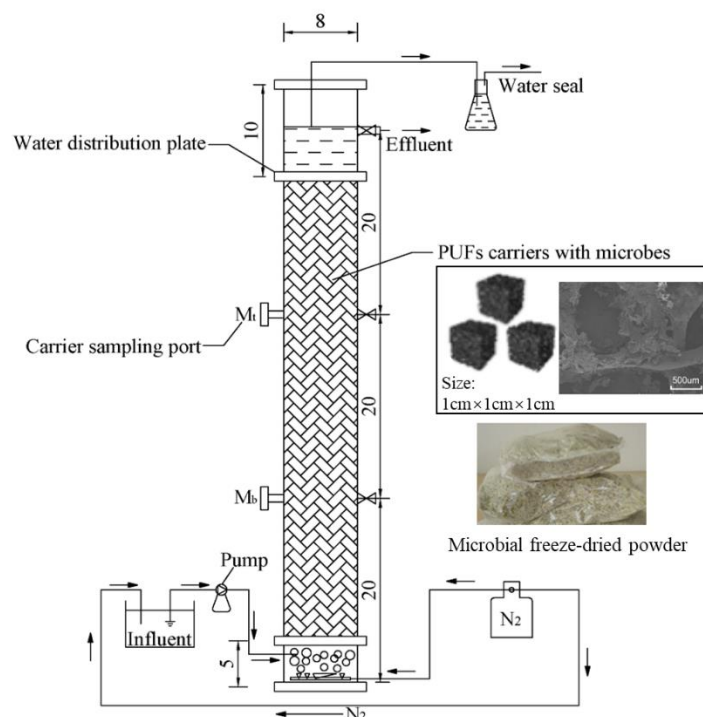
Figure 1. Lab-scale immobilized biofilter.

2.3. Experiment Procedure

After about 15 days' incubation, visible biofilms had formed on the carriers and the effluent water quality was stable. The biomass concentration on the surface of the carrier was up to 40 g·L^{-1}. The reactor started up successfully and batch assays were carried out. The I-BF reactor was operated at 25–28 °C during the operating periods and DO was controlled below 0.2 mg·L^{-1}.

In order to investigate the effect of initial TC concentration, C/N ration and HRT, the I-BF reactor was operated for 90 days under the above three different factors as shown in Table 1. The initial concentration of NO_3^--N was controlled 100 ± 10 mg·L^{-1}. During the first period, the influence of initial TC concentrations (0, 10, 50 and 100 mg·L^{-1}) on denitrification and TC removal was investigated. HRT was controlled at 12 h. During the second period, C/N ratio of the influent was adjusted to 6, 4 and 2 by adding different amount of carbon source to investigate the influence of C/N ratio. The influent TC concentration was 50 mg·L^{-1} and HRT was 12 h. During the third period, HRT was conducted successively at 12 h, 8 h and 4 h with C/N ratio and TC concentration maintained at 5 and 50 mg·L^{-1}, respectively. Each operating condition was run for 9 d until the effluent nitrogen concentration maintained a stable level. Water samples were taken from the inlet and outlet sampling ports every 24 h.

Table 1. Operating procedure of I-BF reactor.

Periods	1	2	3
Operating Time (d)	1–36	37–63	64–90
NO_3^--N (mg·L^{-1})	100	100	100
TC (mg·L^{-1})	0, 10, 50, 100	50	50
C/N ratio	5	6, 4, 2	5
HRT (h)	12	12	12, 8, 4

2.4. Enzyme Activities Assays

To investigate the inhibition mechanism of TC on denitrification, lactate dehydrogenase (LDH), nitrate reductase (NAR) and nitrite reductase (NIR) were measured during the

first operating period. Five pieces of carrier were taken out from the reactor at 9 d, 18 d, 27 d and 36 d, respectively. When the carrier was taken out, new carrier with biofilm was supplemented. Bacteria were collected by washing the PUFs carrier and centrifugation. Crude enzyme extract was prepared for enzyme activity determination according to the procedure described by Luo et al. [23]. In brief, the collected bacteria were resuspended in 100 mM Phosphate Buffer Saline (PBS) buffer followed by ultrasonicated at 20 KHz for 5 min. The crude enzyme extract was obtained by centrifuging (12,000 rpm, 4 °C) for 10 min and collection of the supernatant.

2.5. Analytical Methods

NO_3^--N and nitrite (NO_2^--N) concentrations were measured by spectrophotometric methods in accordance with Chinese Environment Standards. TC concentration was measured by HPLC (Agilent Co., Santa Clara, CA, USA) as described in a previous study [24]. NAR and NIR were measured as described in a previous study [25]. The increased or decreased nitrite concentration was measured to calculate NAR and NIR activities. LDH was measured using lactate dehydrogenase assay kit purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China) according to the manufacturer's instructions.

3. Results and Discussion

3.1. Denitrification Performance under Different TC Concentrations

Different initial TC concentrations (0, 10, 50, 100 mg·L^{-1}) were applied during the first period (1–36 d) to investigate the TC resistance of the I-BF system. Each condition was operated for 9 d. The effluent concentrations of NO_3^--N and NO_2^--N and TC are shown in Figure 2.
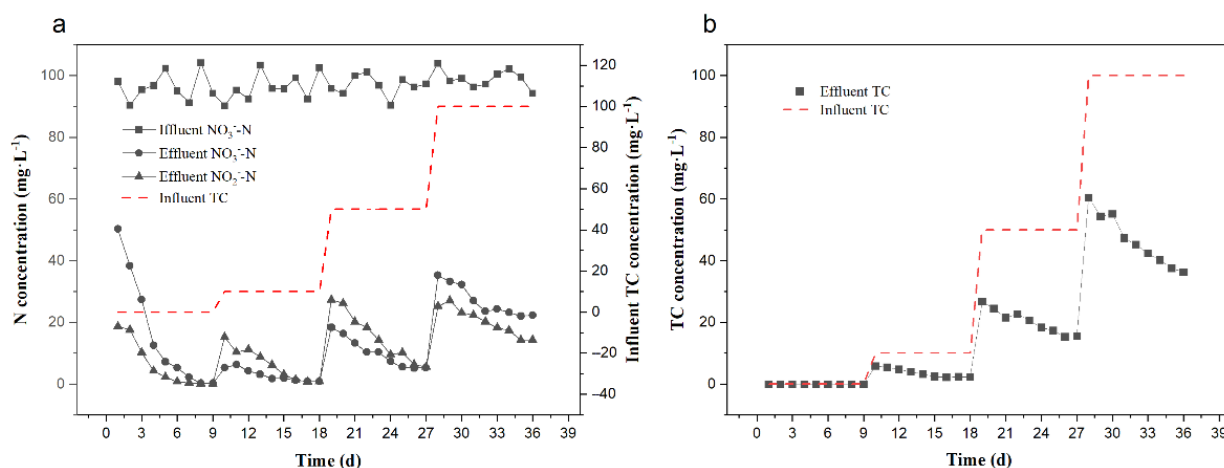
Figure 2. (**a**) Denitrification performance and (**b**) TC removal under different initial TC concentrations.

The denitrification performance of the I-BF reactor is shown in Figure 2a. When no TC was added, almost complete denitrification was achieved with the effluent NO_3^--N and NO_2^--N concentrations of 0.14 mg·L^{-1} and 0.08 mg·L^{-1}, respectively. When TC concentration increased to 10 mg·L^{-1}, the removal of NO_2^--N was inhibited with effluent concentration of 15.22 mg·L^{-1} (10 d). However, no obviously negative effects can be observed at 18 d with the effluent NO_3^--N and NO_2^--N concentrations of 0.82 mg·L^{-1} and 0.78 mg·L^{-1}, respectively. Increasing TC concentration to 50 mg·L^{-1}, the effluent NO_3^--N and NO_2^--N concentrations both significantly increased to 18.32 mg·L^{-1} and 27.25 mg·L^{-1} (19 d) and recovered during the following day with the final effluent concentrations of 5.34 mg·L^{-1} and 5.54 mg·L^{-1}. Upon further increasing TC concentration to 100 mg·L^{-1}, an obvious inhibition effect on denitrification was shown, the effluent NO_3^--N and NO_2^--N concentrations significantly increased to 35.26 mg·L^{-1} and 25.2 mg·L^{-1} (28 d) and partially recovered to 22.32 mg·L^{-1} and 14.27 mg·L^{-1}. It can be seen that the microorganisms of the I-BF reactor may gradually produce tolerance to some degree to a higher TC load through

adaptation and domestication after about 8 days operation. The influence of TC on the denitrification process may depend on operating time and reactor type. Compared to the results of the short-term TC exposure tests in our precious study [22], it can be noted that the inhibiting effect of TC on denitrification was decreased after long-term exposure. After long-term exposure to antibiotics, the denitrification performance can recover to some degree due to functional redundancy and long-term acclimation [26]. Research by Shu and Liang [27] showed the denitrification of the moving bed biofilm reactor (MBBR) system was inhibited when the TC concentration reached 10 mg·L^{-1}. These inconsistent results might be due to the different sensitivities of denitrifying bacteria to antibiotics. The immobilized microorganisms used in the present study showed stronger resistance to TC load.

The variations of TC concentration are shown in Figure 2b. The effluent TC concentration increased with increasing TC load. When TC load was 10 mg·L^{-1}, the effluent TC concentration was 2.27 mg·L^{-1} with a removal efficiency of 77.30%. When TC load increased to 50 mg·L^{-1}, the effluent TC concentration increased to 15.47 mg·L^{-1} with a removal efficiency of 69.06%. With the further increasing of TC load to 100 mg·L^{-1}, both denitrification and TC removal efficiency decreased and effluent TC concentration reached around 36.33 mg·L^{-1} with a removal efficiency of 63.67%. Although a significant increase of effluent TC concentration occurred at each changed TC load, effluent TC concentration finally showed a downward trend because of the good adaption ability of the I-BF reactor. Biodegradation and biosorption were regarded as the main mechanisms accounting for the removal of antibiotics during biological treatment processes. Previous studies on TC removal by microorganisms showed that biosorption plays a pivotal role in the biological removal of TC [28,29]. Extracellular polymeric substances (EPS) were proved to play an important role in TC removal and resisting the TC stresses of the biofilm reactor [24]. Moreover, microbial degradation of TC is also one of the important factors for the decrease of TC concentration [14].

3.2. Effect of C/N Ratio on Denitrification and TC Removal

Sodium acetate was used as carbon source due to advantages of high denitrification rate and short sludge acclimation period [30]. Different C/N ratios (2, 4 and 6) were applied through changing sodium acetate dosage during the second period (37–63 d) to investigate the effect of C/N ratio on denitrification and TC removal. Each condition was operated for 9 d. The effluent concentrations of NO$_3{}^-$-N and NO$_2{}^-$-N and TC under different C/N ratio are shown in Figure 3.

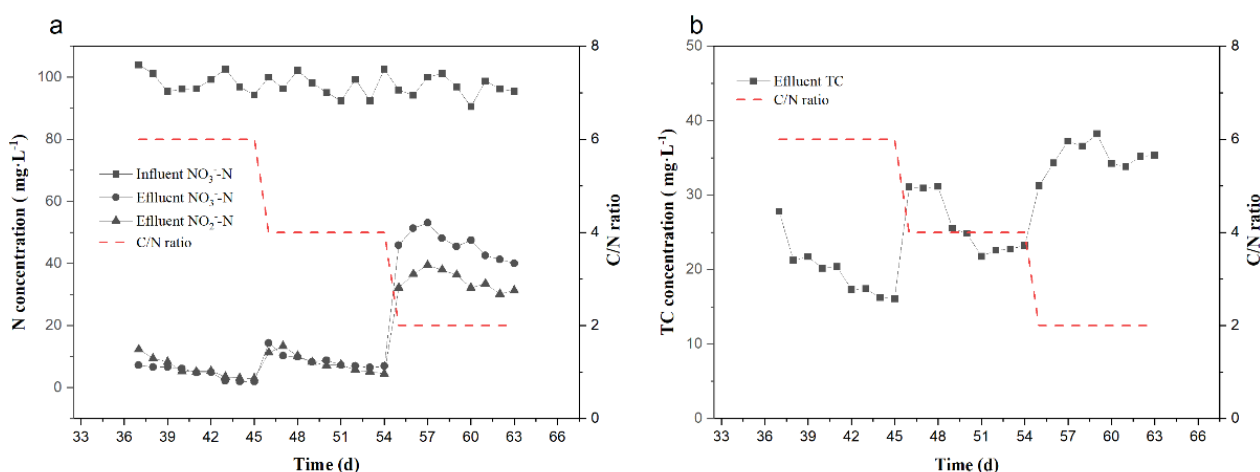**Figure 3.** (**a**) Denitrification performance and (**b**) TC removal under different C/N ratio.

As shown in Figure 3a, the NO$_3{}^-$-N and NO$_2{}^-$-N concentrations in the effluent were around 2.01 mg·L^{-1} and 2.93 mg·L^{-1} when the reactor reached a steady state, and almost complete denitrification was achieved at C/N ratio of 6. When C/N was 4, denitrification performance of the I-BF reactor did not change much, with effluent NO$_3{}^-$-N and NO$_2{}^-$-

N concentrations of 6.92 mg·L^{-1} and 4.4 mg·L^{-1}. When the C/N ratio decreased to 2, the effluent nitrate and nitrite concentrations significantly increased to 40.02 mg·L^{-1} and 31.22 mg·L^{-1}, respectively. The denitrification performance of the I-BF reactor was inhibited due to the limited amount of carbon source. Biological denitrification can be affected by the availability of electron donor, conveniently expressed as C/N ratio. Low C/N ratio usually leads to the accumulation of denitrification intermediates due to the limitation of electron supply [31], while high C/N might result in high organic concentration in the effluent. Therefore, it is particularly important to find a suitable C/N for wastewater denitrification. Synthesizing the results of the first period (19–27 d, C/N = 5, influent NO$_3$$^-$-N 100 mg·L^{-1}, TC 50 mg·L^{-1}), the optimal C/N ratio for denitrification of the present I-BF reactor was 5.

TC removal also responds to changes of C/N ratio, as shown in Figure 3b. TC removal efficiencies decreased from 67.80% to 56.48% and 29.22% when the C/N ratio decreased from 6 to 4 and 2, respectively. Synthesizing the results of the first period (19–27 d, C/N = 5, influent NO$_3$$^-$-N 100 mg·L^{-1}, TC 50 mg·L^{-1}), the optimal C/N ratio was 5 with almost complete denitrification and high TC removal efficiency (69.06%) at influent NO$_3$$^-$-N and TC concentrations of 100 mg·L^{-1} and 50 mg·L^{-1}, respectively. Degradation of most antibiotics might be a co-mechanism process; carbon source is not only vital for the growth of heterotrophic bacteria but also crucial for the degradation of antibiotics [32]. TC and sodium acetate used as co-carbon sources for bacterial growth could stimulate and induce TC biodegradation and N conversion processes [14]. Therefore, sufficient carbon sources are needed in order to maintain relative high TC removal efficiency during the degradation process.

3.3. Effect of HRT on Denitrification and TC Removal

HRT is a key operating parameter, determining the contaminants removal performance and the efficiency of the bioreactor. Thus, the impact of the HRT (4, 8, 12 h) on denitrification and TC removal of the I-BF reactor was also investigated during the third period (64–90 d). Each condition was operated for 9 d. The effluent concentrations of NO$_3$$^-$-N and NO$_2$$^-$-N and TC under different HRT are shown in Figure 4.

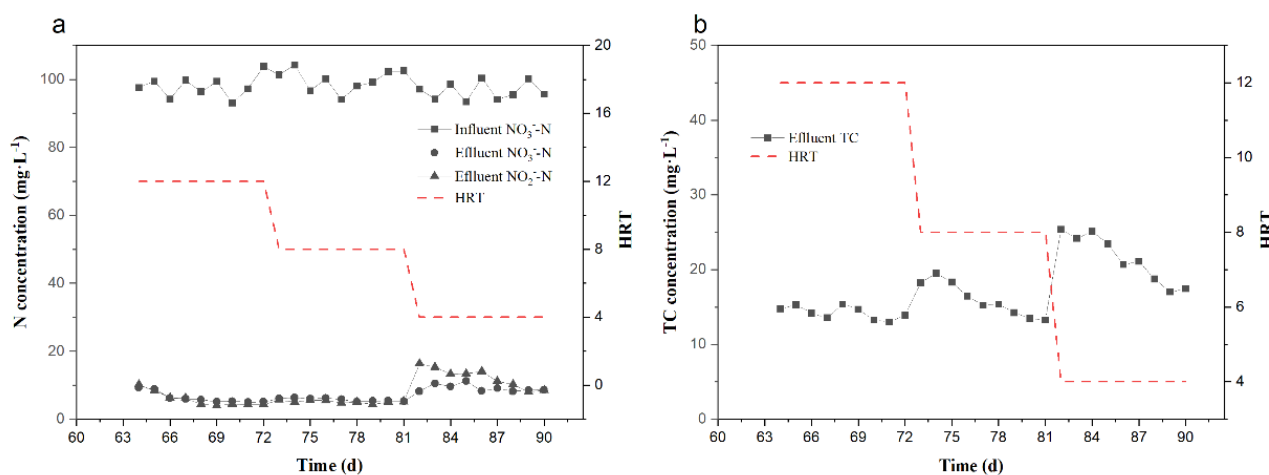**Figure 4.** (**a**) Denitrification performance and (**b**) TC removal under different HRT.

As shown in Figure 4a, the effluent NO$_3$$^-$-N and NO$_2$$^-$-N concentrations were maintained at about 5.13 mg·L^{-1} and 4.31 mg·L^{-1}, respectively, with NO$_3$$^-$-N removal efficiency of 95.1%. As HRT decreased from 12 h to 8 h, the effluent NO$_3$$^-$-N and NO$_2$$^-$-N concentrations were maintained at about 5.23 mg·L^{-1} and 5.20 mg·L^{-1}, respectively. There were no significant differences of denitrification performance when HRT decreased from 12 h to 8h. When HRT further decreased to 4 h, the effluent NO$_3$$^-$-N and NO$_2$$^-$-N concentrations increased and finally stabilized around 8.66 mg·L^{-1} and 8.52 mg·L^{-1}, respectively. The denitrification performance was inhibited to some degree with HRT of 4 h. Studies have

shown that HRT is directly related to nitrate removal efficiency, and the decrease of HRT will increase effluent nitrate and nitrite concentrations [30].

The variations of TC concentration under different HRT are shown in Figure 4b. When HRT was 12 h, the effluent TC concentration was about 13.32 mg·L^{-1}, with a removal efficiency of 73.36%. The removal efficiency of TC was 73.46% when HRT was reduced to 8 h, and no significant difference was shown. However, upon further decrease of HRT to 4 h, the TC removal efficiency significantly decreased to 65.10%. HRT influenced the performance of biological treatment systems by influencing the contact time among contaminants and the hydraulic shear force, and even affecting the components of EPS and microbial community [33]. Short HRT might lead to the washout of microbes and finally inhibit the denitrification and pollutants removal performance due to intense hydraulic shear force [21]. Long HRT promotes bacterial growth and the interactions between influent and microbes. However, it would reduce the processing load and increase costs [34]. Therefore, based on the above results, HRT of 8 h may be recommended for denitrification and TC removal of the used I-BF reactor.

3.4. Inhibition Mechanism of TC on Denitrification

In order to investigate the inhibition mechanism of TC on denitrification, relative LDH release level and key enzymes related to denitrification were studied as shown in Figure 5. LDH is a type of cytoplasm substance released by damaged cells and often selected as the indicator of membrane integrity. As shown in Figure 5a, the relative LDH release level at TC concentration of 10 mg·L^{-1} showed no significant increase; however, it was elevated greatly to 119.84% and 135.01% of the control when TC concentration was 50 mg·L^{-1} and 100 mg·L^{-1}, respectively. High TC concentration affected the bacterial membrane integrity and caused membrane damage, leading to the outflow of intracellular substance [35]. Subsequently, the intracellular enzymes' activities relating to denitrification might have been affected. NAR and NIR activities were tested to reveal the inhibition mechanism of TC stress on the denitrification performance of the I-BF reactor as shown in Figure 5b. When TC concentration was 10 mg·L^{-1}, the NAR and NIR activities were similar to that of control, which was consistent with the relatively high denitrification performance. When TC concentrations were 50 and 100 mg·L^{-1}, NAR and NIR activities were reduced, which might lead to the increase of NO$_3$$^{-}$-N and NO$_2$$^{-}$-N concentrations in the effluent. Similarly, Deng et al. [36] reported that TC had obvious inhibition impacts on denitrifying enzymatic activity in the sequencing batch reactor. It can be concluded that the decrease of denitrification efficiency under high TC load may be attributed to the damage of cell membranes and the inhibition of the intracellular denitrification enzymes activities. Compared to the results of short-term TC exposure [25], it can be found that the NAR and NIR activities were less inhibited under the long-term TC exposure. After long-term exposure, the microbes adapted to TC load and the degradation performance recovered to some degree. The above results imply that the microorganisms in I-BF reactor have a great potential for denitrification.
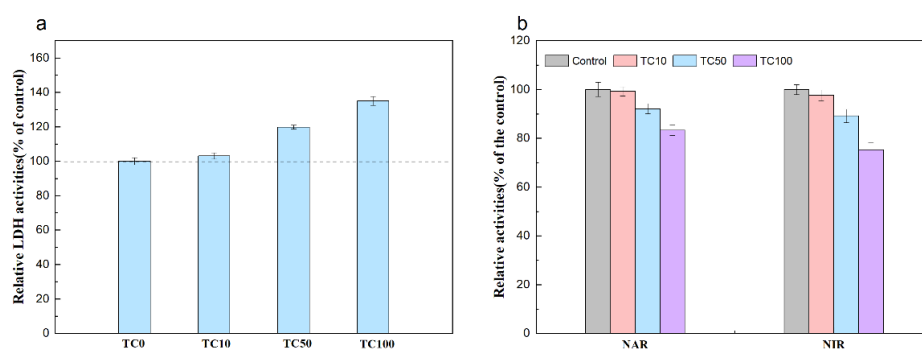
Figure 5. Effect of TC exposure on (**a**) relative LDH release level and (**b**) key enzymes related to denitrification.

4. Conclusions

Simultaneous removal of nitrogen and TC was achieved by the I-BF reactor. Low TC concentration ($\leq$50 mg·L^{-1}) had little effect on nitrogen removal. The denitrification performance of the I-BF reactor was be inhibited at high TC load, which may be attributed to the damage to cell membranes and the inhibition of the intracellular denitrification enzymes' activities. The optimal C/N ratio and HRT were 5 and 8 h with almost complete denitrification and high TC removal efficiency (73.46%) at influent NO$_3^-$-N and TC concentrations of 100 mg·L^{-1} and 50 mg·L^{-1}, respectively. The results indicated that the I-BF reactor has promising applications such as the treatment of piggery wastewater, aquaculture tail water and pharmaceutical wastewater co-contaminated with nitrate and antibiotics. For future research, it will be of vital importance to investigate the role of a functional microbial community, the distribution of antibiotic resistance genes (ARG) in the I-BF reactor and pilot or larger scale applications of I-BF reactors in actual wastewater treatment.

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Conflicts of Interest: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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