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Original Research Paper

Effect of Free Nitrous Acid as Inhibitor on Denitrification Process

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ABSTRACT

Denitrification process is an important step of the global nitrogen cycle. As an intermediate of this process, nitrite always can be found accumulated when the nitrite reduction rate falling behind the rate of nitrate reduction. This study investigated the influence of nitrite/free nitrous acid (FNA) on denitrification process. The nitrate reduction rate, nitrite accumulation rate, nitrite reduction rate and chemical oxygen demand (COD) removal rate were measured under different FNA concentrations, and different levels of inhibition were observed in the nitrate reduction, nitrite reduction, nitrite accumulation and COD removal process. The nitrite accumulation rate decreased by 82% when initial FNA concentration was increased from 0.022 mg/L to 0.045 mg/L, and no nitrite accumulation was observed when the initial FNA concentration was greater than 0.072 mg/L. The nitrate reduction process in this experiment was almost completely inhibited and COD removal process was totally halted when the initial FNA concentration was increased to 0.18 mg/L; in the meantime, the phenomenon of bacteriolysis might occur in this test, which led to a slight upward tendency of COD concentration. The impact of FNA on nitrate reduction process was greater than that on nitrite reduction process, though nitrite reduction was also inhibited by FNA, it could not be completely halted. The amended Monod model was chosen to describe the inhibitory effect and the inhibition of FNA concentration on nitrate reduction, nitrite reduction and COD removal process could all be well described by this model.

INTRODUCTION

Biological nitrogen removal (BNR) is widely used in wastewater treatment plant (Zhu et al. 2013) to reduce nitrogen discharge into natural water bodies, through the nitrification and denitrification processes. Denitrification is carried out by heterotrophic bacteria, which use available organic carbon as electron donor and both nitrate and nitrite as electron acceptors, under anoxic conditions (Xie et al. 2012). This process is an important step of the global nitrogen cycle.

When begins with nitrate, denitrification process includes two major biological processes, nitrate reduction to nitrite and nitrite reduction to nitrogen gas (N_2). Nitrite is produced as an intermediate, it will be usually formed transiently during the beginning of denitrification, but disappear once the process is stable equilibrium (Baumann et al. 1997). However, when the nitrite reduction rate falls behind the rate of nitrate reduction, the accumulation of nitrite is observed during the process of denitrification (Wilderer et al. 1987). Several studies have shown, high nitrite concentration in the biological wastewater treatment system, which can negatively affect various kinds of microbes (Anthonisen et al. 1976, Vadivelu et al. 2006a). Francis & Mankin (1977) reported the inhibition of denitrification by high concentrations of nitrite. Glass (1997) reported that at near-neutral pH values of 6 and 7, nitrite concentrations of 30 and 250 mg/L NO_2^--N, respectively, could inhibit denitrification. The phenomenon of the nitrite accumulation is always undesirable.

It is now known that free nitrous acid (FNA), which is the protonated species of nitrite, is likely the real inhibitory compound for denitrification (Abeling & Seyfried 1992), rather than nitrite. Abeling & Seyfried (1992) reported that when FNA reached 0.13 mg/L, the inhibition of denitrification occurred. Recent study (Ma et al. 2010) has shown that nitrate reduction started to be inhibited when FNA was at low level (< 0.01 mg/L) and was totally halted at the concentration of 0.2 mg/L. Not only denitrifiers have been reported to be inhibit by FNA, but also many other phylogenetic types, for example, ammonia, nitrite oxidizing bacteria (Almeida et al. 1995, Fux et al. 2006, Jenicek et al. 2004, Vadivelu et al. 2006a) and denitrifying polyphosphate-accumulating organisms (Zhou et al. 2007) can affect them. Now, it is fully affirmed that the concentration of FNA is decided by both the concentration of nitrite and pH, and it can be calculated using the formula $S_{N-NO_2}/K_\alpha \times 10^{pH}$ with the K_α value determined using the formula $K_\alpha = e^{-2300/(273+T)}$ for a given temperature T (°C) (Anthonisen et al. 1976). However, most predecessors' studies solely focused on the correlation between FNA, pH and nitrite concentration (Anthonisen et al. 1976, Baumann et al. 1997), and the FNA inhibition on nitrate reduction. Very few studies have looked at the effect of FNA on nitrite reduction. Ma et al. (2010) observed that FNA barely inhibited nitrite reduction when in the concentration range of 0-0.007 mg/L, and this result consist with the observation made in Zhou et al. (2007). When FNA concentration was higher than 0.01 mg/L, the nitrite reduction was affected significantly, and no complete inhibition of nitrite reduction was observed (Ma et al. 2010). Meanwhile, little attention has been given to the effect of FNA on COD removal process.

In this study, the effect of nitrite/FNA on denitrification process was investigated to validate the inhibition of nitrate reduction process by FNA, and further research was done to gain improved understanding of the FNA effect on nitrite reduction process and COD removal process. A domesticated sludge which was performing BNR was used in batch tests, and the nitrate reduction rate, nitrite reduction rate and COD removal rate were measured under different FNA levels to achieve the objective of the present study.

MATERIALS AND METHODS

Inoculum and parent reactor: The inoculum was the digested sludge from an anaerobic digester of Qige wastewater treatment plant (Hangzhou, China). Two lab-scale anoxic sequencing batch reactors (SBR) were used as parent reactors to enrich microorganisms capable of denitrification. The working volume of vessel was 10 L. Both the reactors, which were operated at temperature 23±2°C and pH 7.0-7.5, had four sequences with a cycle time of 6 h, 5 min feeding, 4 h anoxic phase, 2 h settling and 5 min extraction of 2.5 L of supernatant. The sludge concentration in the reactors (mixed liquor suspended solids, MLSS) was controlled to about 5000 mg/L. The reactors were fed with synthetic wastewater and operated under anoxic condition with four cycles each day.

Synthetic medium: In this study, methanol (CH_3OH) was used as the sole carbon source resulting in an initial COD concentration in two parent reactors of 260 mg/L for denitrification. $NaNO_3$ and KH_2PO_4 were used to provide the N and P sources in the synthetic wastewater. At the beginning of each cycle, they were quickly added into both reactors to reach initial NO_3^--N concentration of 60 mg/L. Synthetic wastewater fed into the parent SBRs contained (per liter water): 0.2 g $MgSO_4{\cdot}7H_2O$; 0.02 g $CaCl_2{\cdot}2H_2O$; 0.18 g KH_2PO_4 and 0.6 mL trace elements solution. Moreover, nitrate and methanol were added to the synthetic wastewater in different weights and volumes according to the dosage needed. $NaClO_3$ was proved to inhibit the oxidation of NO_2^--N by *Nitrobacter* at the concentration of 20 mM (Wang et al. 1999), so it was added to inhibit the oxidation of NO_2^--N selectively (Liu et al. 2011). The trace elements solution contained (per liter water): 1.5 g $FeCl_3{\cdot}6H_2O$; 0.15 g H_3BO_3; 0.03 g $CuSO_4{\cdot}5H_2O$; 0.18 g KI; 0.12 g $MnCl_2{\cdot}4H_2O$; 0.06 g $Na_2MoO_4{\cdot}2H_2O$; 0.12 g $ZnSO_4{\cdot}7H_2O$; 0.15 g $CoCl_2{\cdot}6H_2O$; 10 g ethylene diamine tetra acetic acid (EDTA). A 1.0 M HCl or 1.0 M NaOH solution was used to control the pH maintaining between 7.0 and 7.5 in the SBRs.

Batch tests: After 60 days operation, two parent SBRs achieved steady state, with > 99 % removal of both nitrate and COD; the activated sludge in reactors was used for batch tests. Before the tests, the activated sludge in parent SBRs was first washed three times with a synthetic medium to remove the COD, NO_3^--N and NO_2^--N of the previous cycle, then filled up again. The constituents of synthetic medium were the same as those of the synthetic wastewater fed into the parent SBRs (except $NaNO_3$ and CH_3OH).

When sludge and synthetic wastewater were well mixed, the mixed liquor were trickled into 1 L beakers which would be planted on the magnetic stirrers set at 150 rpm to make sure the complete mixing of the nutrients, and all batch tests were conducted in these beakers in a temperature controlled at 23±2°C. Dissolved oxygen (DO) was controlled at < 0.5 mg/L with the aeration of N_2 during the batch tests. The pH was adjusted to 6.7-6.85 with 1.0 M NaOH and 1.0 M HCl during the operation.

CH_3OH, $NaNO_3$ and $NaNO_2$ were added into the beakers at the beginning of each experiment, which resulted in initial COD concentrations of 260 mg/L, NO_3^--N concentrations of 60 mg/L, and initial concentrations of FNA varying between 0 and 0.22 mg/L.

Each experiment lasted for 30 min. Mixed liquor samples were taken every 5 min for COD, nitrate and nitrite analysis. Before analysing, mixed liquor samples were filtered through disposable millipore filter units (0.22 µm pore size) using a syringe, and stored at 4°C until the experiment finished.

Chemical analysis: When the experiment was finished, samples stored at 4°C were analysed immediately. NO_3^--N, NO_2^--N, COD and MLSS were determined according to the standard methods (APHA 1998).

RESULTS AND DISCUSSION

The FNA inhibition on nitrate reduction: The removal process of nitrate affected by different levels of initial FNA concentrations is displayed in Fig. 1a. When the initial FNA concentration was < 0.18 mg/L, nitrate was removed gradually, but when the initial FNA concentration was increased to 0.18 mg/L, nitrate reduction was ceased, and there was a gradual upward tendency of nitrate concentrations at the end of the batch experiments along with the raising of FNA concentration.

The nitrate reduction rate was observed to decrease when the initial FNA concentration further increased. As Fig. 1b illustrates, under an initial FNA concentration of 0 mg/L, the nitrate reduction rate was observed to reach its maximum at 25.2 mg/L/h/gMLSS. When the FNA inhibitor was added into the batch reactor for the first time with the concentration of 0.022 mg/L, the nitrate reduction rate decreased to about 70% of the maximum rate immediately. Then a slight decrease was observed until the initial FNA concentration was 0.090 mg/L, the rate of nitrate reduction became 50 % of the maximum. After that, the nitrate reduction rate dropped fast. When initial FNA concentration was greater than 0.18 mg/L, the nitrate reduction rate was left only 6% of the maximum and was almost completely inhibited.

Since, the change rule of nitrate reduction rate conformed to the variation trend of the amended Monod equation (Hellinga et al. 1999), which was rewritten in eq. (1), it was chosen to describe the inhibitory effect (Vadivelu et al. 2006b) in this study:

$$q = q_{\max} K_{I,HNO2} / \left(K_{I,HNO2} + S_{HNO2} \right) \quad ...(1)$$

Where, q is the specific uptake rate of a particular substrate, while $q_{\max}$ is the maximum specific uptake rate of this substrate. S_{HNO2} is the concentration of FNA, and $K_{I,HNO2}$ is FNA inhibition constant.

The equation of this system about the FNA inhibition on nitrate reduction was obtained by fitting the experimental data and eq. (1) was rewritten as eq. (2).

$$q = 0.0548 q_{\max} / \left(0.0548 + S_{HNO2} \right) \quad ...(2)$$

All data measured in these tests presented that the inhibition of FNA concentration on nitrate reduction could be well described by this amended Monod model. The FNA inhibition constant of 0.0548 mgHNO_2-N/L was obtained from the fitting model. Hellinga et al. (1999) combined the Monod kinetic model and the inhibitor of activated sludge in SHARON system and got the FNA inhibition constant of 0.21 mgHNO_2-N/L. The FNA inhibition constant obtained in this experiment was smaller than that in the study of Hellinga et al. (1999), this meant that the FNA inhibition was stronger in this SBR system.

Moreover, the nitrate reduction efficiency was also declined when initial FNA concentration was increased from 0.022 mg/L to 0.22 mg/L (Fig. 1c), and was observed to be only 2.4 % at last. These findings indicated that higher the FNA, the stronger the inhibition.

The FNA inhibition on nitrite removal: Not only the nitrate reduction was inhibited by FNA, but also the nitrite removal process was affected. Fig. 2a showed the nitrite accumulation rate at different FNA concentrations. When the initial FNA concentration was 0 mg/L, the maximum nitrite accumulation rate was found to be 5.35 mg/L/h/gMLSS. And the accumulation rate decreased by 82 % when initial FNA concentration was increased from 0.022 mg/L to 0.045 mg/L. But when the initial FNA concentration was greater than 0.072 mg/L, there was no nitrite accumulated in the whole process.

In Fig. 2b, the inhibition of nitrite reduction process was observed when the initial FNA concentration was increased from 0.022 mg/L to 0.22 mg/L. The nitrite reduction rate was 16.7 mg/L/h/gMLSS when the initial FNA concentration was 0 mg/L, while it was 13.7 mg/L/h/gMLSS when the initial FNA concentration was 0.072 mg/L, indicating that the nitrite reduction rate decreased by 18 %, and was still lower than nitrate reduction rate. With further increasing of initial FNA concentration, the nitrite reduction rate still declined but became greater than nitrate reduction rate, meanwhile the phenomenon of nitrite accumulation disappeared. The nitrite reduction rate decreased slowly to 11.4 mg/L/h/gMLSS when the initial FNA concentration was 0.11 mg/L, then dropped fast to 2.0 mg/L/h/gMLSS when the initial FNA concentration was increased to 0.18 mg/L and then remained stable. This stable nitrite reduction rate was only 12 % of the rate at the initial FNA concentration of 0 mg/L. That was, the nitrite reduction rate decreased by approximately 88% when the initial FNA concentration was increased from 0 mg/L to 0.18 mg/L, and then kept stable. Nitrite reduction was not totally inhibited even when the FNA concentration was high enough to completely halt the nitrate reduction process, at the initial FNA concentration of 0.18 mg/L.

The phenomenon of the temporary nitrite accumulation was caused when the rate of conversion process from nitrite to N_2 was less than the rate of reduction from nitrate to nitrite, and the accumulated nitrite would gradually disappear once the nitrate was used up (Baumann et al. 1997, Wilderer et al. 1987). So, when the nitrate reduction was inhibited, nitrate reduction rate was slower, nitrite accumulation rate also became slower. And the highest nitrite concentration always appeared when the nitrate was just exhausted in the experiment. The disappearance of nitrite accumulation phenomenon may be due to that the high FNA concentration inhibited nitrate reduction so that the nitrate reduction rate was too low and much lower than the nitrite reduction rate, in spite of the FNA inhibition on nitrite reduction. This conclusion is in agreement with what resulted from Fig. 2b. In other words, the impact of FNA on nitrate reduction was greater than that on nitrite reduction process. The result from Fig. 2b showed that, though nitrite reduction was also inhibited by FNA, it could not be completely halted for the reason that FNA was the real substrate of nitrite reductase. This conclusion was also supported by the result made in Ma et al. (2010).

Fig. 2c showed the fit between the experimental data and the theoretical assumptions by the amended Monod model, and the inhibition of FNA concentration on nitrite reduction could be well described by this amended Monod equation. The obtained FNA inhibition constant on nitrite removal was 0.0986 mgHNO_2-N/L, which was greater than 0.0548 mgHNO_2-N/L obtained in the analysis of the FNA inhibition on nitrate reduction. This phenomenon provided evidence again for the result of Fig. 2b, that the impact of FNA on nitrate reduction was greater than that on nitrite reduction process. And the equation of this system about the FNA inhibition on nitrite removal was rewritten in eq. (3) by fitting the experimental data and eq. (1):

$$q = 0.0986 q_{\max} / \left(0.0986 + S_{HNO2}\right) \quad ...(3)$$

The FNA inhibition on COD removal: The influence of FNA on COD removal of denitrification process was emerged in Fig. 3. Fig. 3a showed the COD concentration profiles measured in batch tests. COD concentration at the end of each batch test revealed a trend of rise when initial FNA concentration was increased, just as the upward tendency of nitrate final concentration. This may because of that the COD removal process was also inhibited by FNA. In spite of this, most of the removal curves showed a downward trend in this picture. But, instead of decline, two COD removal curve lines at the initial FNA concentrations of 0.18 mg/L and 0.22 mg/L showed a slight upward tendency. The appearance of this slight upward tendency may be because of the occurrence of bacteriolysis, which made a part of the carbon of the microbial cells discharged into the system.

The inhibition of FNA on COD removal rate and the comparison between the experimental data and the theoretical assumptions by the amended Monod model are shown in Fig. 3b. In the control group with initial FNA concentration of 0 mg/L, the maximum COD removal rate was 63.6 mg/L/h/gMLSS. When the nitrite factor was added to the batch reactor for the first time to make initial FNA concentration be 0.022 mg/L, there was a rapid decline of COD removal rate. The rate dropped to 32.4 mg/L/h/gMLSS, which was only 50 % of the maximum COD removal rate. And then the rate of COD removal descended gently. Until the initial FNA concentration was increased to 0.11 mg/L, COD removal rate decreased to 20.4 mg/L/h/gMLSS, which decreased by approximately 70 % of the maximum. When the initial FNA concentration was greater than 0.11 mg/L, COD removal rate dropped very fast again, and the rate could not be detected after initial FNA concentration was greater than 0.18 mg/L. The inhibition of FNA concentration on COD removal could be well described by this amended Monod equation, and the fitted equation was rewritten in eq. (4):

$$q = 0.0347 q_{\max} / \left(0.0347 + S_{HNO2}\right) \quad ...(4)$$

The obtained FNA inhibition constant on nitrite removal was 0.0347 mgHNO_2-N/L, which was greater than that obtained in the analysis of the FNA inhibition on nitrate and nitrite reduction. This phenomenon indicted that the impact of FNA on COD removal process was greater than that on both nitrate and nitrite reduction processes.

Fig. 3c showed the change of COD removal efficiency along with the change of initial FNA concentrations in batch tests. Methanol (CH_3OH) was added into batch reactors to reach the initial COD concentration of 260 mg/L. The efficiency was 64% in blank group and it decreased to 32% immediately when FNA inhibitor was added into the system for the first time to reach the initial FNA concentration of 0.022 mg/L. Then it decreased slowly between the initial FNA concentration of 0.022 mg/L and 0.11 mg/L, and declined to about 30% of the blank test. After that, a rapid decline of COD removal efficiency was observed again, this time, efficiency decreased to 0 when the initial FNA concentration was greater than 0.18 mg/L.

Fig. 3 confirmed that, except nitrate and nitrite reduction process, COD removal process was also inhibited by FNA. But why a rapid decline was found both of COD removal rate and efficiency when FNA inhibitor was the first time added into the system, and was followed by a slow decline when the initial FNA concentration continue increasing from 0.022 mg/L and 0.11 mg/L? The reason for this phenomenon may be that, after the first time FNA inhibitor was added into the system, the microorganisms in the reactors may be adapted to the environment contained FNA, though FNA still inhibited the COD removal process, the inhibition was not so strong as the first time. This phenomenon was also found in nitrate reduction process. Until the initial FNA concentration was increased to 0.18 mg/L, bacteriolysis happened, COD removal rate and efficiency both decreased to 0, and COD removal process was totally inhibited.

The mechanism of FNA inhibition on denitrification: Nitrate reductase might have a lower affinity with the electron donor than nitrite reductase, and the competition of electrons between nitrite and nitrate reductase might be the mechanism of FNA inhibition on nitrate reduction rate. Fig. 4 describes the relationship between the reduction rates of nitrite and nitrate. The conclusion resulted from Fig. 4 confirmed that, with the increase of nitrite reduction rate, the nitrite reduction rate did not decrease.

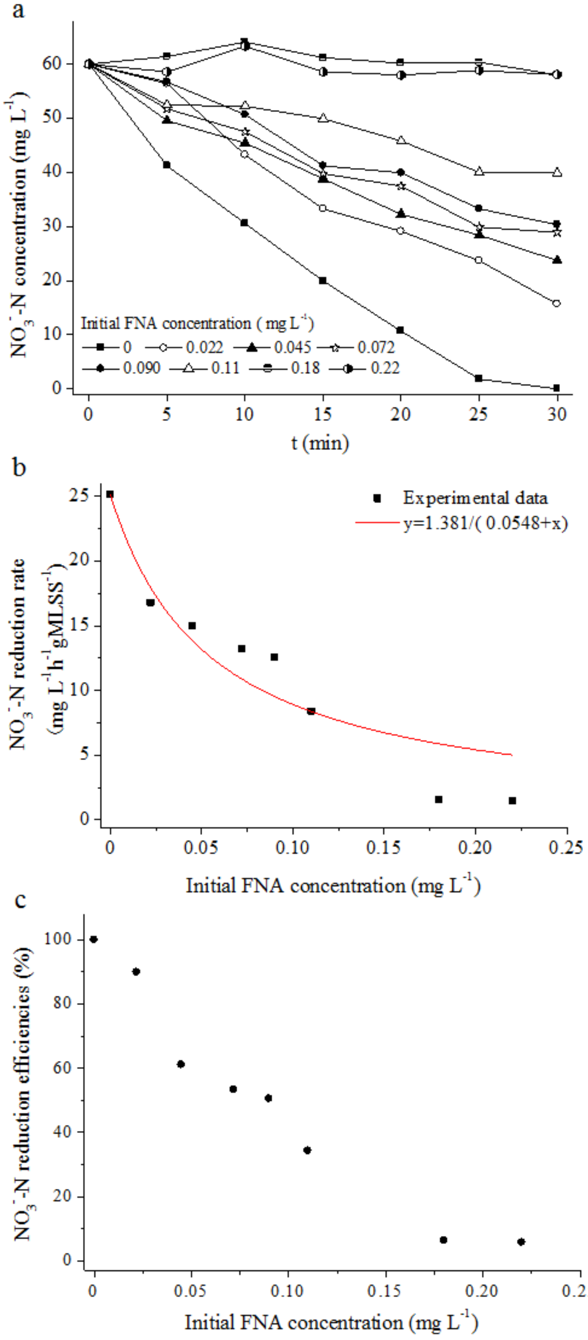

Fig. 1. (a) Nitrate concentration profiles measure in batch tests; (b) Nitrate reduction rates at different initial FNA concentrations; (c) Nitrate reduction efficiencies at different initial FNA concentrations.

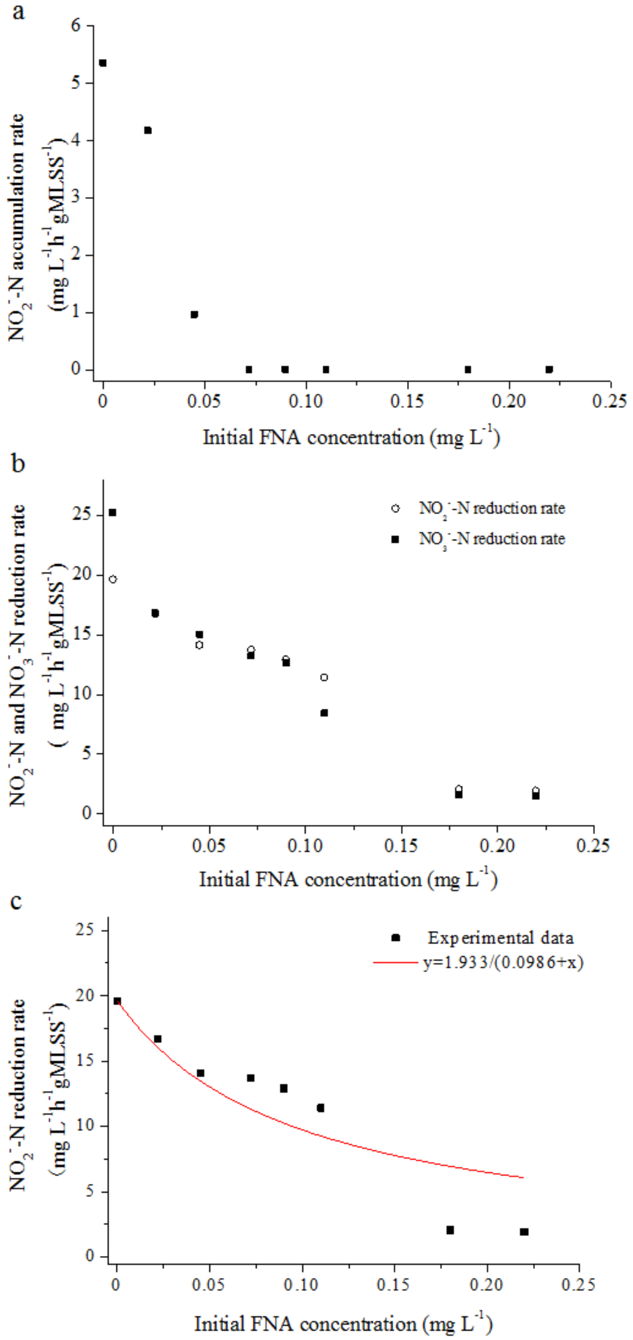

Fig. 2. (a) Nitrite accumulation rates at different initial FNA concentrations; (b) Correlation between the nitrate reduction rate and the nitrite reduction rate at different initial FNA concentrations; (c) Nitrite reduction rates at different initial FNA concentrations.

FNA might act as an uncoupler to inhibit nitrate reduction, which was an agent that caused the stimulation of basal electron transport and ATP hydrolysis, inhibition of various exchange reactions catalysed by the ATPase and ATP synthesis, but without inhibition of electron transfer (Rottenberg 1990). According to this hypothesis, cells would increase the respiration rate to release protons to maintain the pmf in existing of FNA uncoupling (Ma et al. 2010), which would cause the increasing of nitrate consumption rate instead of keeping constant or decreasing (Sijbesma et al. 1996). But Fig. 1b showed the opposite phenomenon.

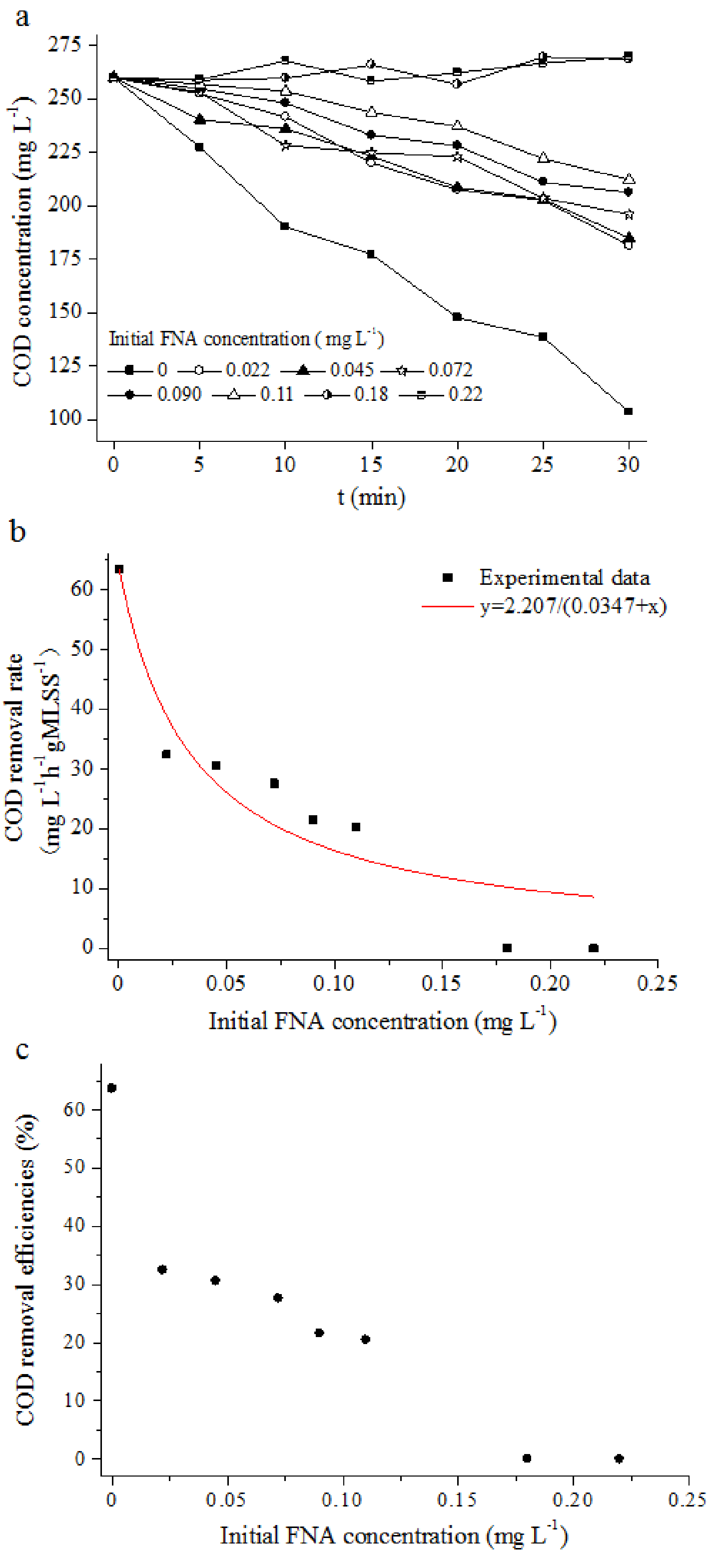

Fig. 3: (a) COD concentration profiles measure in batch tests; (b) COD removal rates at different initial FNA concentrations; (c) COD removal efficiencies at different initial FNA concentrations.

The hypothesis is, therefore, not supported experimentally.

The reaction between FNA and the enzymes involved in the nitrate reduction might explain the mechanism of the inhibition of FNA on nitrate reduction. FNA was able to damage the cell wall or membrane (O'Leary & Solberg 1976), and nitrate reductases might be destroyed by it. Moreover, the reactive intermediates, such as nitric oxide (NO) and nitroxyl anion (NO^-) were produced from the easily happened dismutation reaction of nitrite or FNA, which could react directly with haem and metal centres of proteins and forming nitrosyl complexes (Reddy et al. 1983). These effects could get a direct result of the nitrate reduction rate decreasing.

FNA, the proton form of nitrite was proved as a toxic substance (Almeida et al. 1995, Kono et al. 1994), which was able to diffuse rapidly and passively across the outer and inner cell membrane (Parsonage et al. 1985) and react with enzymes to destroy nitrite reductases causing the inhibition of nitrite reduction.

CONCLUSIONS

The denitrification process was significantly deteriorated by FNA in this study. The results showed that:

1. Approximately 30% and 50% inhibitions of nitrate reduction rate were observed at the initial FNA concentrations of 0.022 mg/L and 0.090 mg/L, respectively. And the nitrate reduction process was almost completely halted when the initial FNA concentration reached 0.18 mg/L.
2. Not only the nitrate reduction process, but also nitrite removal process was found to be inhibited by FNA. The nitrite accumulation rate decreased by 82% when initial FNA concentration was increased from 0.022 mg/L to 0.045 mg/L, and the accumulation was no longer detected when the initial FNA concentration was greater than 0.075 mg/L. The nitrite reduction rate decreased with the increasing of the initial FNA concentration, and kept stable with the rate which was only 12% of the maximum. The impact of FNA on nitrite reduction was weaker than that on nitrate reduction process, and the nitrite reduction process could not be completely halted.
3. The phenomenon of bacteriolysis might occur in batch test, and a slight upward tendency of COD concentration was observed when the initial FNA concentration was increased to 0.18 mg/L. The COD removal process in this experiment was completely inhibited at the initial FNA concentration of 0.18 mg/L.
4. The microorganisms in the system were found with the adaptive capacity of FNA after the first time FNA was added into the system, the prerequisite was that FNA concentration was not too high.
5. The inhibition of FNA concentration on nitrate reduction, nitrite reduction and COD removal process could all be well described by the amended Monod model. Different systems had different dynamic characteristics and mechanisms; this study derived the constants of the inhibition kinetics model aimed at the SBR system, which had a more helpful consulting meaning.

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