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Effect of free nitrous acid as inhibitors on nitrate reduction by a biological nutrient removal sludge

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ABSTRACT

Nitrite has been commonly thought to have a broad inhibitory effect on bacterial metabolism. Little is known about the impact of nitrite on nitrate reduction with pH considered as an important factor. This study investigates the nitrite inhibition on nitrate reduction during denitrification under various pH conditions by using a biological nutrient removal (BNR) sludge. The results showed that nitrate reduction performance had a much stronger relationship with the free nitrous acid (FNA) than that of nitrite concentration, implying that FNA, rather than nitrite, is likely the real inhibitor on nitrate reduction. The nitrate reduction activity of the biomass was observed to be inhibited about 60% in the range of 0.01–0.025 mg HNO_2-N/L and was totally inhibited when FNA level was greater than the threshold concentration (0.2 mg HNO_2-N/L). Moreover, the recovery rate from inhibitory effect was found to be dependent much more strongly on the concentration of FNA, of which the biomass was exposed to during the inhibition period, than on the duration of the inhibition and the feeding mode of inhibitor. It was also found that nitrite reduction was significantly inhibited by FNA and the nitrite reduction rate was linear to nitrate reduction rate due to the inhibitory mechanism under which FNA may react with the enzymes involved in the denitrification process.

1. Introduction

The conventional biological nitrogen removal requires a two-step process, nitrification followed by denitrification. Commonly, denitrification is the reduction of nitrate to gaseous nitrogen compounds with nitrite as an intermediate product [1]. The process is performed by heterotrophic microorganisms which use nitrate/nitrite instead of oxygen as electron acceptors, especially under anaerobic or anoxic conditions. The electron donor is usually the organic matter, which could be obtained from the feed wastewater to municipal wastewater treatment plant (WWTP). Denitrification can be described as a type of anoxic respiration and involves enzymes such as the nitrate, nitrite, nitric oxide and nitrous oxide reductase [2].

Denitrification is an important step since its efficiency has an influence on the effect of nitrogen removal in biological wastewater treatment plant. As a heterotrophic process, denitrification tends to be less sensitive to environmental parameters than nitrification; however, it is still affected by the variations in temperature, pH, alkalinity, and inhibitory compounds presence [3–5]. Decreasing wastewater temperature (T_{water}) has a negative impact on both nitrification and denitrification, nevertheless the latter was found to be affected further when T_{water} dropped from 22 to 12 °C [6]. Denitrification includes two major biological processes, namely, denitratation (i.e. nitrate reduction to nitrite) and denitritation (i.e. nitrite reduction to gaseous N_2). Furthermore, nitrate and nitrite have been found to inhibit denitrification systems [1,7–12]. Although detailed information is provided in the literature for the inhibition on each individual denitrification step, little attention has been paid to the possible inhibitory effect of sound inhibitor-nitrite with pH considered as a very important factor on denitrification. The fact that both nitrite and pH were observed to have a significant impact on nitrate reduction suggests a potential role of free nitrous acid (FNA), the protonated species of nitrite, with a concentration calculated using formula ($S_{N-NO_2}/K_a \times 10^{pH}$) and K_a value determined using formula ($e^{-2300/(273+T)}$) for a given temperature T (°C) [13]. Indeed, FNA has been reported to inhibit the growth and/or energy generation by a wide range of phylogenetic types, including ammonia, nitrite oxidizing bacteria [13–17], denitrifiers [18], and also denitrifying polyphosphate-accumulating organisms [19,20]. Partial nitrification, as one of the most cost-effective and sustainable biological nitrogen removal processes, has gained much attention lately. Shortcut nitrification and denitrification via nitrite has attracted many researchers' interests due to its potential to reduce the aeration consumption and save the carbon source dosage [21]. The accumulation of nitrite could be expected

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through optimizing operational parameters to achieve nitrogen removal via nitrite way purposely [6,22,23]. Still, nitrite has been frequently observed to accumulate during the full nitrifying process of biological nutrient removal system. FNA is determined by the level of nitrite accumulated and pH in biological nitrogen removal system. As a result, to study the impact of FNA as inhibitors on denitrification is of great significance.

Biological nutrient removal (BNR) in activated sludge has become more and more common practice in wastewater treatment during the last decades. Many BNR configurations for activated sludge process are available, but of interest in this research is to investigate the inhibition on denitrification by using the sludge from a CAST reactor where a single activated sludge culture passes through the sequence of aerobic and anoxic conditions to achieve oxidation of BOD, nitrification and denitrification, as well as biological phosphorus removal.

The objective of the present study is to experimentally analyze the inhibitory effects and gain improved understanding of the often observed "nitrite inhibition" on nitrate reduction during denitrification. This was achieved by measuring the nitrate reduction rate of an activated sludge performing BNR under different nitrite and pH levels. The recovery of nitrate reduction after being inhibited was also studied.

2. Materials and methods

2.1. Sludge source

The sludge used for this study was obtained in a 72 L lab-scale cyclic activated sludge technology (called the parent CAST) operated under alternating anoxic and aerobic conditions with low COD/TN ratio (about 3.8) municipal wastewater fed in the anoxic period for enhancing biological nutrient removal performance. A cycle time of 6 h consisted of three anoxic/oxic combinations with both of the durations fixed at 40 min, 75 min settling and decanting, and 45 min idle periods. The lab-scale CAST reactor was composed of selector and complete mix zone with a capacity ratio of 1:10. The returned activated sludge (RAS) and influent pumped to selector mixed by the ratio of 1:4. The reactor was operated with a hydraulic retention time (HRT) of 18 h and a sludge retention time (SRT) of 10 days. All batch experiments described below were carried out when the running CAST reached quasi-steady state with the sludge displaying excellent BNR efficiencies of 89% and 93% from wastewater containing approximately 79.2 mg N/L and 5.4 mg P/L, respectively.

2.2. Batch tests

Three sets of batch experiments were performed for the purpose of determining the inhibitory effects of FNA on nitrate reduction. The common methods and materials are described below.

2.2.1. Preparation of seed sludge

Before each test, sludge was withdrawn from the parent CAST during the last aerobic phase. Acetate was added to the sludge resulting in an initial COD concentration in the reactor of 300 mg/L for anaerobic phosphorus release. After 3 h, when most COD was stored in the biomass, the sludge was washed using tap water to remove any residual soluble COD and phosphate which would be available for denitrifying poly-phosphate accumulating organisms and thus compete nitrate/nitrite as the terminal electron acceptor with other ordinary denitrifiers. The pretreated sludge was then transferred to the 1 L batch reactors for the experiments. Acetate, nitrite and nitrate stock solutions were prepared by adding 48.05 g of CH_3COONa in 250 mL of Milli-Q water (resulted in the acetate concentration of 150 g COD/L), adding 4.92 g of $NaNO_2$ in 100 mL of Milli-Q water (resulted in the nitrite concentration of 10 g NO_2^--N/L), and adding 6.07 g of $NaNO_3$ in 100 mL of Milli-Q water (resulted in the nitrate concentration of 10 g NO_3^--N/L), respectively.

Table 1
Experimental conditions applied in FNA inhibition batch tests.

Test	pH	NO_2^- (mg N/L)	FNA × 10^3 (mg HNO_2-N/L)	VSS (g/L)
1	8.5	30	0.23	2.184
2	8.5	50	0.38	2.184
3	8.5	70	0.54	2.184
4	8.0	30	0.73	2.184
5	8.0	50	1.22	2.184
6	8.0	70	1.70	2.639
7	8.0	90	2.19	2.639
8	7.5	30	2.31	2.639
9	7.5	50	3.85	2.639
10	7.5	70	5.38	2.639
11	7.5	90	6.92	2.162
12	7.0	30	7.30	2.162
13	7.0	50	12.16	2.768
14	7.0	70	17.03	2.768
15	7.0	90	21.89	2.162
16	7.0	120	29.19	2.162
17	6.5	30	23.08	2.522
18	6.5	50	38.46	2.498
19	6.5	70	53.84	2.522
20	6.5	90	69.23	2.547
21	6.5	110	84.69	2.764
22	6.5	140	108.53	2.764
23	6.5	170	132.30	2.764
24	6.5	200	153.83	2.764
25	6.5	260	199.99	2.764
26	6.5	390	299.98	2.764

2.2.2. Sampling schedule and analytical methods

A 0.5 M HCl or 0.5 M NaOH solution was used to control the pH in the batch reactor, maintaining pH at ±0.05 of the pre-designed set-points (see below). Mixed liquor samples were taken every 10 min using a syringe and immediately filtered through disposable Millipore filter units (0.45 μm pore size) for the analyses of nitrate and nitrite. pH was measured online using pH sensor (WTW 340i, WTW Company). Mixed liquor volatile suspended solids (MLVSS), nitrate (NO_3^-–N) and nitrite (NO_2^-–N) were measured according to the standard methods at the end of each test [24].

The rates of nitrate reduction were determined through linear regression of the measured nitrate profiles. The nitrite reduction rates in the absence of nitrate were determined similarly from the measured nitrite profiles. In the presence of nitrate, the nitrite reduction rates were determined from the NO_x^-(=$NO_3^- \pm NO_2^-$) profile. In the latter case, nitrite accumulated and the slope of the NO_2^- profile give the nitrite accumulation rate rather than the nitrite reduction rate. The NO_x^- reduction rate gives the true nitrite reduction rate in this case. The extent of FNA inhibition on nitrate reduction was calculated and expressed as the percentage ratio between the biomass-specific nitrate reduction rate measured at different FNA levels and that measured in the absence of FNA.

2.2.3. Set 1 tests: nitrate reduction at different pH in the presence of different levels of nitrite

Twenty-six batch tests were performed, as shown in Table 1. The pH in each experiment was controlled at the pre-selected set-point and varied between 6.5 and 8.5. Acetate stock solution was added to the reactor in different volumes at the beginning of each experiment. The reactor was then sealed immediately followed by the injection of 6 mL of nitrate and various dosage of nitrite solutions described above, resulting in the initial nitrate concentration of 60 mg NO_3^--N/L and FNA concentrations varying between 0 and 0.30 mg HNO_2-N/L. The C/N ratio was established

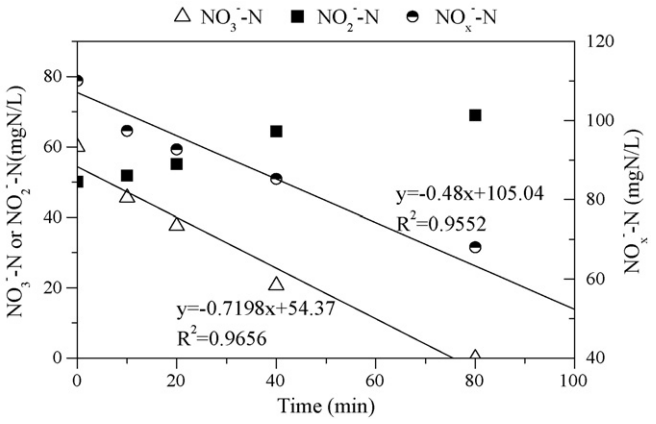

Fig. 1. Nitrate and nitrite concentration profiles measured in Set 1 Tests.

as 10, in order to assure excess in electron donor capacity in all the experiments.

2.2.4. Set 2 tests: nitrate reduction at different pH in the absence of nitrite

Five batch reactors were operated in parallel with pH controlled at 8.5, 8, 7.5, 7, and 6.5, respectively, to determine the impact of pH on nitrate reduction in the absence of nitrite. Reactions were started by the addition of the nitrate and acetate solutions prepared prior to the experiment. The test under each pH condition was performed in triplicates.

2.2.5. Set 3 tests: recovery of nitrate reduction from inhibition

Three steps were involved in each test of this type. In Step 1, fresh sludge of 1 L from the parent CAST after an anaerobic period was incubated in a batch reactor for 10-min control test in the simultaneous presence of only nitrate and acetate. The nitrite and acetate stock solution was then regularly spiked into the reactor to ensure that carbon source for both electron acceptors would be in excess (Step 2), which resulted in the total FNA concentration of 0.0008–0.006 mg HNO_2-N/L to inhibit nitrate reduction. All the nitrite and acetate solutions in Step 2, unless otherwise described, were fed by instant-feeding mode. The durations of Step 2 varied between 10 and 80 min in different tests. At the end of Step 2, the sludge was washed using tape water until no nitrite was detected. Another nitrate and acetate solutions were injected to increase the nitrate concentration to approximately 30 mg NO_3^--N/L (Step 3). The biomass-specific nitrate reduction rate in the following 60 min was determined and compared with that in Step 1. During all the experiments of this type, pH was controlled at 7.5 ± 0.05.

3. Results and discussion

3.1. FNA inhibition on nitrate reduction

Fig. 1 shows the nitrate and nitrite concentration profiles measured in Set 1 Batch Tests. Under pH 8.0 and an initial nitrite concentration of 50 mg N/L, nitrate and nitrite reduction occurred simultaneously. The nitrate reduction rate was determined as 19.77 mg N/($h \times$ gVSS) through linear regression. The values obtained here are in agreement with those reported in the literature. Dincer and Kargi [25] found values for the maximum specific nitrate consumption rate of 9.6 mg N/($h \times$ gVSS). Tchobanoglous and Burton [26] established maximum nitrate consumption rate values in the range of 9–94 mg N/($h \times$ gVSS). The slope of the NO_x^- profile gave a specific nitrite reduction rate of 12.87 mg N/(h × gVSS). Additionally, the rate of nitrite accumulation was determined as 6.91 mg N/($h \times$ gVSS) through linear regression of the measured nitrite profiles. Fig. 2(a) and (b) shows the correlation between the rates of nitrate reduction and the nitrite concentrations under different pH, respectively, measured in the 26 batch experiments.

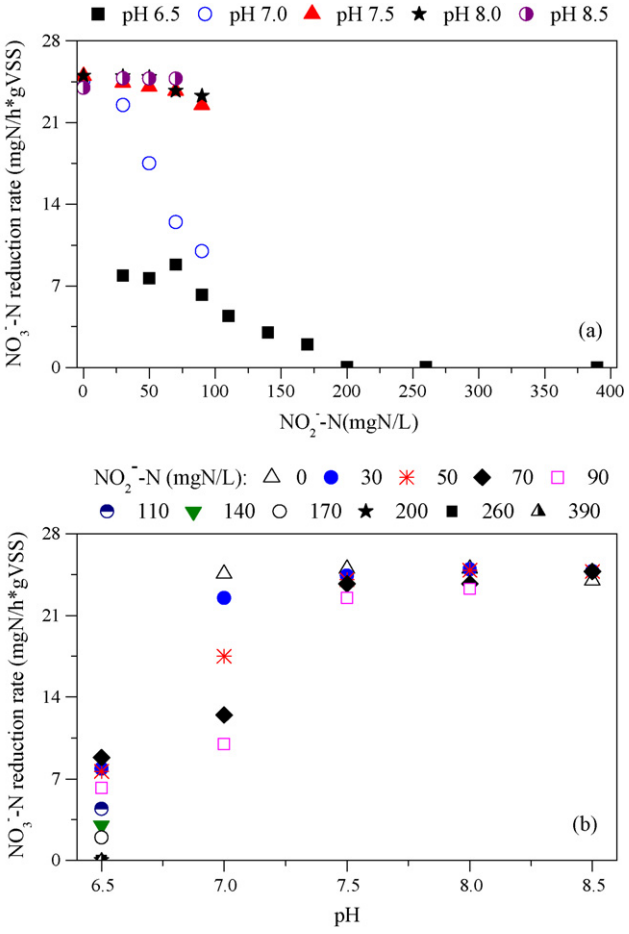

Fig. 2. Correlation between the biomass nitrate reduction rate and the nitrite concentration (a), pH (b).

As shown in Fig. 2(a), there was a general negative impact of nitrite on nitrate reduction. However, the correlation between the extent of inhibition and the concentration of nitrite was not strong. pH clearly had an impact on the extent of inhibition. At the same concentration of nitrite (70 mg NO_2^--N/L), the nitrate reduction rate of the biomass varied from 24.76 mg N/($h \times$ gVSS) to 8.82 mg N/($h \times$ gVSS) under different pH levels. The lower the pH, the stronger the inhibition. However, the correlation between nitrate reduction rate and pH suggested that pH was not the main inhibitory factor either in Fig. 2(b). The inhibition on nitrate reduction rate varied at different levels of nitrite, but under the same pH. Higher nitrite levels gave stronger inhibitory effect. These findings indicated that FNA might be more likely the actual inhibitor rather than nitrite and pH.

Fig. 3(a) confirms that the level of inhibition had a much stronger correlation with FNA concentration, suggesting that FNA may directly cause the inhibition. Also shown in Fig. 3(a) is the comparison between the experimental data and the theoretical assumptions by the exponential formula. It can be seen that the inhibition of FNA concentration on nitrate reduction could be well described by an exponential correlation. All data are presented as the percentage ratio between the measured nitrate reduction rate and the maximum reduction rate measured in these tests. The biomass nitrate reduction decreased by 10% with the increased FNA

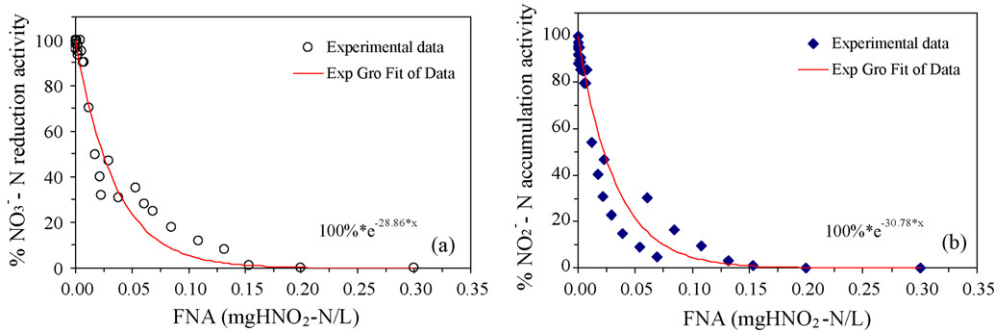

Fig. 3. Correlation between the biomass nitrate reduction activity (a), nitrite accumulation activity (b) and FNA concentration.

concentration even in the very low range of 0–0.007 mg HNO_2-N/L. The nitrate reduction activity decreased by 60% when FNA concentration was increased from 0.01 to 0.025 mg HNO_2-N/L and was completely halted when the FNA concentration was greater than 0.2 mg HNO_2-N/L (260 mg NO_2^--N/L at pH 6.5).

Fig. 3(b) shows the nitrite accumulation at different FNA concentrations. The fit between the experimental data and the theoretical assumptions by the exponential formula further confirms the results discussed above as supporting information. The lower exponent suggests the weaker inhibition of FNA on nitrite reduction which will be discussed later.

3.2. Nitrate reduction at different pH

Although pH's effect on denitrification was known quite early [27], the investigation of pH's effect on nitrate reduction was necessary in this study in order to reveal the real inhibitor. Fig. 4 shows the nitrate reduction performance at different pH levels in the absence of nitrite, measured in the Set 2 Tests. The biomass displayed relatively constant nitrate reduction performance at pH 7.0–8.0. However, the performance decreased to 80–85% at pH 6.5. The results, which were in agreement with the observations made in Glass and Silverstein [9], indicated that pH had an impact on the nitrate reduction performance. However, this impact was insignificant compared to the inhibition of FNA.

3.3. Nitrate reduction recovery from FNA inhibition

Fig. 5 shows the nitrate profile measured during one of the Set 3 Tests. The noninhibited nitrate reduction rate was determined from the first 10 min profile (Step 1). The nitrate reduction in the following 40 min (Step 2) was inhibited as confirmed by the fact that the measured nitrate reduction rate decreased. The recovery step was carried out after the residual nitrite in the mixed liquor was removed via sludge washing. Nitrate reduction resumed after immediate injection of acetate and nitrate, however, the rate of nitrate reduction did not recover to 100% of that observed in Step 1. The degree of recovery in Step 3 measured in the experiments was 24% under 40 min of nitrite exposure by step-feeding mode.

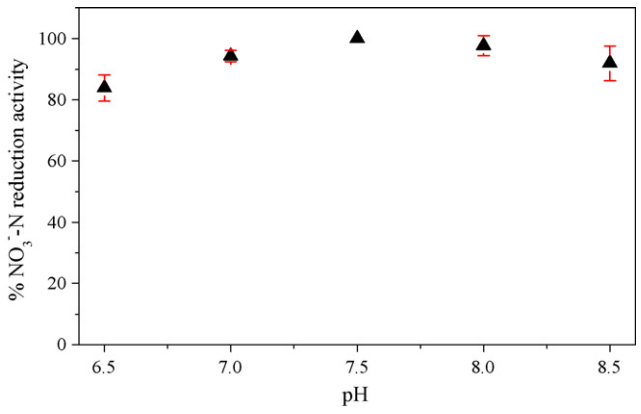

Fig. 4. Nitrate reduction activity at various pHs (where triplicates were performed).

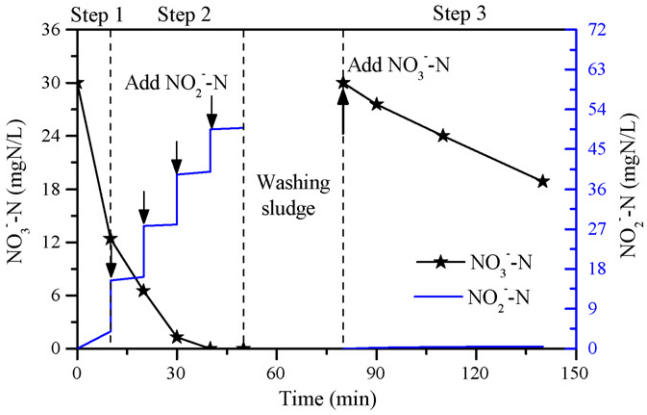

Fig. 5. Nitrate profile measured in one of the 3rd-set batch tests. The biomass was incubated in Step 2 for 40 min in a medium containing FNA at a total concentration of 0.00385 mg HNO_2-N/L.

Table 2 shows the recovery degrees of nitrate reduction from FNA inhibition under different conditions of the inhibition duration, the concentration of FNA the biomass was exposed to and the feeding mode at pH 7.5.

As shown in Table 2, the inhibition was observed to be reversible, with the recovery rate dependent on both the duration of inhibition and the FNA concentration of which the biomass was exposed to during the inhibition period. A longer duration of inhibition or a higher FNA concentration would have caused a lower recovery rate. However, the concentration of FNA had a stronger impact on the recovery rate. Additionally, the step-feeding mode was found

Table 2
Recovery degrees of denitratation from FNA inhibition at pH 7.5.

NO_2^- (mg N/L)	FNA × 10^3 (mg HNO_2-N/L)	Duration of inhibition (min)	Recovery degree (%)
10	0.77	20	72.54
30	2.31	20	69.82
50	3.85	10	37.09
50	3.85	20	22.69
50	3.85	40[a]	24.14
50	3.85	40	16.04
50	3.85	80	15.40
70	5.38	20	3.04

[a] Denotes the step-feeding mode.

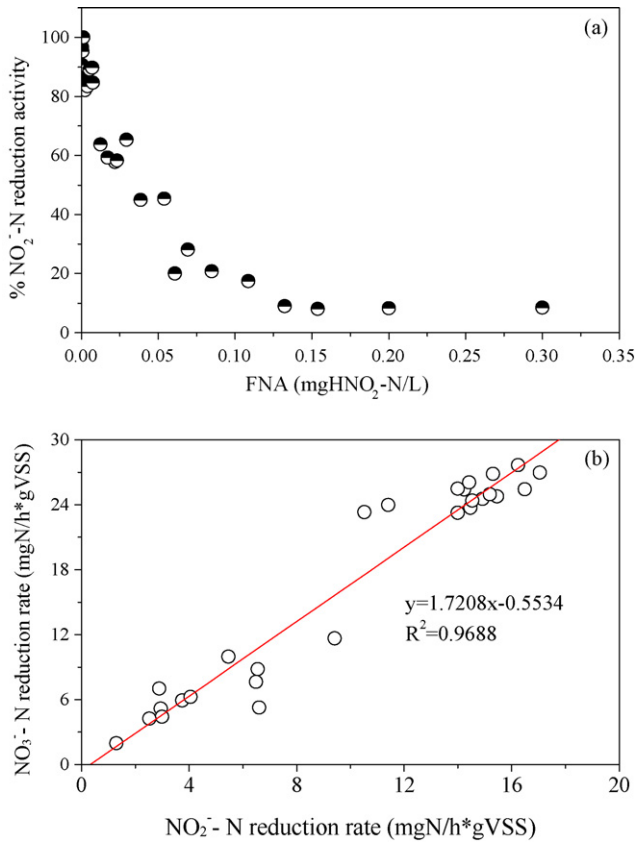

Fig. 6. Nitrite reduction activity of the biomass under various FNA concentrations (a) and the relationship between the reduction rates of the two electron acceptors (b).

to be helpful for the recovery of reduction capability compared to instant-feeding mode.

3.4. FNA inhibition on nitrite reduction

Fig. 6(a) shows that the nitrite reduction rates in these tests remained 90% of the maximum reduction rate measured at an FNA concentration of 0.0002–0.0012 mg HNO_2-N/L, suggesting that nitrite reduction rate was likely limited by substrate (HNO_2) availability when FNA concentration was lower than 0.001 mg HNO_2-N/L. It also shows that FNA concentration, in the range of 0–0.007 mg HNO_2-N/gVSS, barely inhibited nitrite reduction. The results were consistent with the observation made in Zhou et al. [19]. Moreover, the nitrite reduction was found to be affected significantly when FNA concentration exceeded 0.01 mg HNO_2-N/L. However, there was no complete inhibition of nitrite reduction even though the biomass being exposed to the highest level of FNA (up to 0.3 mg HNO_2-N/L) adopted in this study, not to mention the threshold concentration of FNA (up to 0.2 mg HNO_2-N/L) to which nitrate reduction was totally inhibited. These results showed that the nitrite reduction was similarly inhibited as the reduction of nitrate by FNA, whereas the former would not be completely inhibited within the inhibitory radius of the experiments for the reason that FNA was the real substrate of nitrite reductase. This conclusion is in agreement with what resulted from Fig. 3(b).

In Fig. 6(b), the nitrite reduction rate was found to be linear to the rate of nitrate reduction and the straight slope was larger than 1.0. This implied that there was nitrite accumulation in these tests. FNA, which is jointly determined by nitrite and pH, therefore played a significant role in the inhibition of the nitrite reduction. As shown in Fig. 6(b), FNA could inhibit nitrate consumption, however, even under these conditions, nitrite reduction was postponed in order to consume nitrate. Nitrite accumulates in the liquid media when the nitrate reduction rate was larger than the nitrite reduction rate. Once the nitrate presence was consumed, nitrite consumption began. This sequential scheme was not entirely expressed by the mathematical models existing in the literature on denitrification and would be in agreement with the physical arrangement of denitrifying enzymes in the cellular structure [28,29]. While the nitrate reductase enzyme is located at the internal side of the cytoplasmic membrane, the nitrite reductase enzyme is periplasmatic; this distribution could explain the inhibitory behavior by FNA. Since nitrite reductase is located on the external cell membrane, it would lack physical protection barriers, which makes it more sensitive to FNA than the nitrate reductase.

3.5. Inhibitory mechanisms of FNA on denitrification

Early studies have shown that both nitrite and pH influence nitrate reduction during denitrification. This study demonstrated, however, that FNA, which is jointly determined by nitrite and pH, is likely a strong inhibitor of nitrate reduction. While the nitrate reduction rate seems to be lowered at pH 6.5 compared to pH 7.0–8.5, which suggests an independent effect of pH, the inhibitory effect is much more moderate in comparison to that of FNA.

The inhibition on nitrate reduction may be due to the uncoupling effect of FNA. HNO_2 was reported to be capable of crossing the membrane and shuttling protons between the two sides without generating energy as an uncoupler, which may result in the collapse of proton motive force [13,18,30]. However, cells would be expected to increase their respiration rate to pump out more protons in order to maintain the pmf in the presence of FNA uncoupling, which would cause a higher rather than lower nitrate reduction rate due to FNA stimulation. This contradicts the experimental data shown in Fig. 3(a). The hypothesis is therefore not supported experimentally.

Alefounder et al. [31] also suggested that the nitrite reductase competing for electrons with nitrate reductase could explain the decrease in the nitrate reduction rate in the presence of nitrite, if the enzyme of nitrate reduction had a lower affinity with the electron donor than other reductases. In Fig. 6(b) of this study, however, the results showed that the addition of nitrite and the increasing nitrite reduction rate did not result in a lowered nitrate reduction rate. The results suggested that the electrons required by the nitrite and nitrate reductases, when both were functional, were adequately provided by the carbon oxidation processes. In other words, the decrease in the nitrate reduction rate observed in Fig. 2(a) when nitrite/FNA was present was not due to the mechanism of preferential electron flow to nitrite rather than nitrate reductase.

A more likely mechanism is that FNA may react with the enzymes involved in the nitrate reduction. Nitrate reductases carry a *b*-type cytochrome, encoded by the *narI* gene. Such heterotrimeric enzymes exhibit the electronic spectrum and absorbance intensities of a diheme protein [29,32]. FNA may cause serious damage to the cell wall or membrane [33], hence, *b*-type cytochrome-contained enzyme may be destroyed by FNA. Furthermore, as mentioned previously, nitrous acid, an extremely reactive molecule capable of interaction with a wide variety of substrates including myoglobin, amino groups, may denature proteins. Furthermore, the reduction of nitrite or HNO_2 produces nitric oxide (NO) and nitroxyl anion (NO^-). These molecules are deemed to react directly with heme and metal centers of proteins, forming nitrosyl complexes [34]. Because of the function of copper-sulfide proteins in electron transport and ATP generation in anaerobic bacteria, the formation of the complex and hence the destruction of the catalytic site on the enzyme would almost certainly inhibit the electron transport

and growth. Such effects could directly lead to reduction in the denitrification rates.

4. Conclusions

The inhibitory effect of free nitrous acid on nitrate reduction by denitrifiers using a biological nutrient removal sludge was investigated systematically in the study. The results showed that:

(1) Free nitrous acid rather than nitrite is likely the real inhibitor on nitrate reduction. FNA starts inhibiting nitrate reduction at low levels (<0.01 mg HNO_2-N/L), and totally halts the reduction at 0.2 mg HNO_2-N/L or above.
(2) The inhibition of nitrate reduction by FNA is found to be reversible, with the recovery rate dependent on both the duration of inhibition and concentration of FNA of which the biomass was exposed to during the inhibition period. A longer duration of the inhibition or a higher FNA concentration would cause a lower recovery rate. Moreover, step-feeding mode was observed to be helpful for the recovery of reduction capability compared to instant-feeding mode.
(3) Nitrite reduction is also inhibited by FNA. However, the inhibitory effect is weaker than that on nitrate reduction. The nitrite reduction was found to be affected significantly when FNA concentration exceeded 0.01 mg HNO_2-N/L, whereas no complete inhibition was observed even though the biomass being exposed to the highest level of FNA (up to 0.3 mg HNO_2-N/L, 0.2 mg HNO_2-N/L is the threshold concentration at which nitrate reduction was completely inhibited) adopted in this study. This could be explained that FNA was the real substrate of nitrite reductase.

Acknowledgements

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