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Free Nitrous Acid Inhibition on Nitrous Oxide Reduction by a Denitrifying-Enhanced Biological Phosphorus Removal Sludge

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Nitrite has generally been recognized as an inhibitor of N_2O reduction during denitrification. This inhibitory effect is investigated under various pH conditions using a denitrifying-enhanced biological phosphorus removal (EBPR) sludge. The degree of inhibition was observed to correlate much more strongly with the free nitrous acid (FNA) concentration than with the nitrite concentration, suggesting that FNA, rather than nitrite, is likely the true inhibitor on N_2O reduction. Fifty percent inhibition was observed at an FNA concentration of 0.0007−0.001 mg HNO_2-N/L (equivalent to approximately 3−4 mg NO_2^--N/L at pH 7), while complete inhibition occurred when the FNA concentration was greater than 0.004 mg HNO_2-N/L. The results also suggest that the inhibition on N_2O reduction was not due to the electron competition between N_2O and NO_2^- reductases. The inhibition was found to be reversible, with the rate of recovery independent of the duration of the inhibition, but dependent on the concentration of FNA the biomass was exposed to during the inhibition period. A higher FNA concentration caused slower recovery.

Introduction

Nitrous oxide (N_2O), one of the greenhouse gases, is increasing globally at an alarming rate of 0.31% per year (*1*). Although the concentration of N_2O in the atmosphere is about a thousandth of that of CO_2, its large contribution to the green-house effect can by no means be neglected since its effect per molecule is 200−300 times stronger than that of CO_2. Human activity has been determined to be a significant source of global N_2O (*2*), with wastewater treatment systems known to be a potential anthropogenic source. N_2O is a byproduct of microbial nitrification and denitrification, which are the main mechanisms for nitrogen removal in the wastewater treatment processes (*3*). Complete denitrification from nitrate (NO_3^-) to molecular dinitrogen (N_2) involves four reduction steps with nitrite (NO_2^-), nitric oxide (NO), and nitrous oxide (N_2O) as intermediates.

N_2O is continuously produced during denitrification but is normally reduced to nitrogen gas by the enzyme nitrous oxide reductase. Several studies on denitrification in environmental systems, such as soil and sediments, have shown that the N_2O reductase is sensitive to external factors and is inhibited at high oxygen and nitrite concentrations, or low pH (*4*−*6*). These environmental factors are believed to have a stronger inhibitory effect on N_2O reductase than on the enzymes responsible for the proceeding reduction steps (*7*). Inhibition of N_2O reductase will lead to an increased fraction of N_2O as the end product of denitrification.

It has been frequently observed that the accumulation of nitrite decreases N_2O reduction and leads to an increase in the N_2O emission (*8*−*11*). Itokawa et al. (*11*) reported nitrite accumulation (10 mg NO_2^--N/L) as a possible cause of N_2O production in a denitrifying sludge. Others, using formalism of enzymatic competition, claimed that the competition between terminal electron acceptors (*9, 10*) may be responsible, although reductions of NO_2^- and N_2O do not proceed through the same reductase in respiratory chains (*12*). However, Betlach et al. (*8*) showed that the addition of nitrite does not always cause nitrous oxide accumulation.

Kinetic studies of the N_2O reductase using pure cultures indicated that the activity of this enzyme is dependent on pH. The N_2O reductase activity was observed to increase when the pH increased from 4 to 8, reaching a maximum at pH 8−9 before decreasing (*13*).

The fact that both nitrite and pH were observed to have a significant impact on N_2O reduction suggests a potential role of free nitrous acid (FNA), the protonated species of nitrite, with a concentration jointly determined by the nitrite concentration and pH. Indeed, FNA has been reported to inhibit the growth and/or energy generation by a wide range of phylogenetic types, including both ammonia and nitrite oxidizing bacteria (*14, 15*), denitrifiers (*16*), and also denitrifying polyphosphate-accumulating organisms (*17*).

Enhanced biological phosphorus removal (EBPR) is the most economical and sustainable process for removing phosphorus from wastewater. EBPR is achieved by recycling polyphosphate-accumulating organisms (PAOs) through alternating anaerobic and aerobic conditions. PAOs take up carbon sources while releasing orthophosphate in the anaerobic phase and store them in the form of polyhydroxyalkanoates (PHA), using the energy produced through the hydrolysis of intracellular polyphosphate. In the subsequent aerobic phase, PAOs grow and take up orthophosphate by using the stored PHA as the carbon and energy sources.

Phosphorus uptake also occurs under anoxic conditions (*18*). This means that the carbon source taken up by PAOs in the anaerobic phase is used for both denitrification and phosphorus removal, which is advantageous when the wastewater contains a relatively low level of organic carbon. The requirement for organic carbon can be further lowered by achieving nitrogen removal via nitritation and denitritation. This is a process where ammonium is only oxidized to nitrite, which is directly reduced to nitrogen gas. However, N_2O accumulation has previously been observed in denitrifying EBPR systems achieving nitrogen removal via nitrite (*19, 20*).

The aim of this study is to gain improved understanding of the often observed "nitrite inhibition" on N_2O reduction during denitrification. This was achieved by measuring the N_2O reduction rate of an activated sludge, performing EBPR and nitrogen removal under different nitrite and pH levels. The recovery of N_2O reduction after being inhibited was also investigated.

Materials and Methods

Sludge Source. The sludge used in this study was withdrawn from a 4 L laboratory-scale sequencing batch reactor (called the sludge SBR) working under anaerobic, anoxic, and aerobic

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conditions. Abattoir wastewater was fed to the reactor anaerobically, where most of the organic carbons were taken up, in a 2 h reaction period. This, along with the concomitant release of phosphate, showed the anaerobic metabolism of PAOs. After a short settling period, 3 L of supernatant (rich in $N\text{-}NH_4^+$ and $P\text{-}PO_4^{3-}$) was transferred into a 3.5 L aerated Biofilm SBR where ammonia was oxidized to nitrite in 6 h. The nitrified stream drained from the Biofilm SBR, containing $N\text{-}NO_2^-$ and $P\text{-}PO_4^{3-}$, was then continuously fed to the sludge SBR for 2.5 h, during which denitrification and phosphorus uptake took place simultaneously. The treated wastewater was discharged from the system after a further aerobic polishing period of 1 h. The system was designed to achieve nitrogen and phosphorus removal from the abattoir wastewater used, which contained high levels of nitrogen (approximately 250 mg N/L) and phosphorus (40 mg P/L) but relatively low levels of organic carbon (478 ± 81 mg $COD_{soluble}$/L). Details of the reactor design, operation and performance can be found in Zhou et al. (*21*). When the experiments reported below were carried out, the system was displaying excellent performance, having a nitrogen and phosphorus removal efficiency of 81% and 94%, respectively. Granules dominated the sludge SBR.

Batch Experiment. Four types of batch experiments were carried out. The common methods and materials are described below.

Sludge Source and Pretreatment. All batch experiments, unless otherwise described, were carried out in 300 mL sealed reactors (called the batch reactors). The granular sludge was withdrawn at the end of the anaerobic phase from the sludge SBR, when most COD was stored in the biomass, washed using a synthetic medium described below, and then manually smashed to convert it into floccular form (smashed with a tissue grinder-Kontes glass, 885300−0015). The washing step was in order to remove any residual soluble COD and deplete the external carbon sources which would be available for ordinary denitrifiers. The last step was required in order to avoid substrate diffusion limitation in the experiments. In general, high substrate concentrations are required in order for substrates to fully penetrate into granules, and this could interfere with the experimental design and increase uncertainties in data interpretation. In each test, 50 mL of smashed granular sludge, with an MLSS concentration of 12−13 g/L, was resuspended into a volume of 300 mL in the batch reactor with the synthetic medium, which contained 30 mg NH_4^+-N/L, 5 mg Mg^{2+}/L, 5 mg Ca^{2+}/L, and 4 mL/L of a trace element solution (composition according to Smolders et al. (*22*)). There was no headspace in the reactor. However, a 10 mL reservoir filled with the same mixed liquor was connected to the lid of the reactor to allow for liquid phase sampling.

A nitrite stock solution was prepared by adding 2.46 g of $NaNO_2$ in 100 mL of Milli-Q water (resulted in the nitrite concentration of 4991 mg NO_2^--N/L). An N_2O solution was prepared by sparging Milli-Q water with 100% N_2O gas for 5 min (N_2O concentration ranged from 360 to 660 mg N_2O-N/L).

Sampling and Batch Reactor Operation. A 0.5 M HCl solution was used to control the pH in the batch reactor, maintaining pH at ±0.05 of the predesigned set-points (see below). Mixed liquor samples were taken every 10 min using a syringe and immediately filtered through disposable Millipore filter unites (0.22 μm pore size) for the analyses of nitrite. Mixed liquor volatile suspended solids (MLVSS) was measured at the end of each test. N_2O in the liquid phase was measured online using an N_2O microsensor (N_2O25, Unisense A/S, Aarhus, Denmark).

Type I Batch Tests: N_2O Reduction at Different pH in the Presence of Different Levels of Nitrite. Twenty batch experiments were performed, as shown in Table 1. Nitrite stock solution was added to the reactor in different volumes at the beginning of each experiment, which resulted in the initial concentrations of nitrite varying between 0 and 90 mg N/L. The reactor was then closed immediately followed by the injection of 5 mL of N_2O solution described above, resulting in an N_2O concentration 6−11 mg N_2O-N/L. The pH in each experiment was controlled at the preselected set-point and varied between 7.0 and 8.5 (see Table 1). The resulting concentrations of FNA, calculated from nitrite and pH according to Anthonisen et al. (*14*), are also shown in Table 1, along with the MLVSS concentrations of the sludge. The method for calculating the rates of N_2O and NO_2^- reduction is detailed in Supporting Information (Figure S1). The biomass-specific N_2O and NO_2^- reduction rates were calculated by dividing the corresponding volumetric rates by the MLVSS concentration. The extent of FNA inhibition on N_2O reduction was calculated and expressed as the percentage ratio between the biomass-specific N_2O reduction rate measured at different FNA levels and that measured in the absence of FNA.

TABLE 1. Experimental Conditions Applied in FNA Inhibition Batch Tests

test	pH	NO_2^- (mg NO_2^--N/L)	FNA $\times 10^3$ (mg HNO_2-N/L)	MLVSS (g/L)
1	8.5	10	0.07	2.29
2	8.0	0	0	1.55
3	8.0	10	0.2	1.72
4	8.0	10	0.2	2.32
5	7.5	0	0	2.38
6	7.5	10	0.7	2.45
7	7.5	20	1.5	2.44
8	7.5	30	2.2	2.4
9	7.5	40	2.9	2.38
10	7.5	50	3.6	2.24
11	7.5	10	0.7	1.82
12	7.5	20	1.5	1.73
13	7.5	30	2.2	1.68
14	7.5	40	2.9	1.66
15	7.5	50	3.6	1.6
16	7.5	60	4.4	1.54
17	7.5	10	0.7	2.26
18	7.5	70	5.1	2.3
19	7.5	90	6.6	1.76
20	7.0	10	2.4	2.28

Type II Batch Tests: N_2O Reduction at Different pH in the Absence of Nitrite. Four batch reactors were operated in parallel with pH controlled at 8.5, 8, 7.5, and 7, respectively, to determine the impact of pH on N_2O reduction in the absence of nitrite. Reactions were started by the addition of the N_2O solution prepared prior to the experiment. The test under each pH condition was performed in triplicates.

Type III Fed-Batch Tests: Electron Competition between N_2O and NO_2^- Reduction. Five fed-batch experiments were carried out by continuously feeding a nitrite stock solution with a concentration of 185 mg NO_2^--N/L into the five parallel reactors, preloaded with N_2O at a concentration of approximately 10 mg N_2O-N/L, with a flow rate of 0, 0.001, 0.002, 0.003, 0.005 mL/s, respectively, using a continuous feed pump (TPS, MicroCHEM, pH transmitter module). These feeding rates were designed to enable different nitrite reduction rates in the reactor so that the N_2O reduction rates under different nitrite reduction rates can be determined and compared. Each test lasted for 30 min, during which the pH was kept at 8.0 ± 0.05. Mixed liquor samples were taken at the beginning and the end of each test and analyzed for nitrite.

Type IV Batch Tests: Recovery of N_2O Reduction from Inhibition. *IVa. N_2O Reduction Recovery from Inhibition and Its Dependency on the Duration of Inhibition.* Three steps were involved in each test of this type. In Step I, a 10 min

control test was performed with only the N_2O solution injected. The nitrite stock solution was then injected to the reactor (Step II), which resulted in the FNA concentration of 0.006 mg HNO_2-N/L to completely inhibit N_2O reduction. The durations of Step II varied between 10, 20, 40, and 60 min in different tests. The 10 min test was carried out in triplicate. At the end of Step II, the sludge was washed using the synthetic medium described before until no nitrite was detected with nitrite test strip (Merckoquant, Germany). Another N_2O solution pulse was injected to increase the N_2O concentration to approximately 9.0 mg N_2O-N/L (Step III). The biomass-specific N_2O reduction rate in the following 40 min was determined and compared with that in Step I. pH during all the experiments of this type was controlled at 7.5 ± 0.05.

IVb. Parent Reactor Recovery after Nitrite Shock Loading. This experiment aimed to investigate the ability of the biomass in the sludge SBR to recover its N_2O reduction capability after a shock loading of nitrite/FNA. The N_2O reduction rates were measured before the shock loading (control), during the shock loading, and in several cycles after nitrite being removed, and the parent reactor was operated under normal conditions.

The inhibition experiment was carried out at the end of an anaerobic period. A nitrite pulse was added to the reactor at the beginning of the anoxic phase, resulting in a nitrite concentration of 135 mg NO_2^--N/L, mimicking a dump load of the Biofilm SBR effluent to this reactor. This gave rise to an FNA concentration of 0.03 mg HNO_2-N/L at the pH condition in the reactor, which was expected to completely inhibit N_2O reduction based on results obtained from other tests. The biomass was incubated in the high concentration of nitrite for 40 min. At the end of the incubation, the biomass was washed with the effluent of the sludge SBR until there was no nitrite detected. The SBR was then operated under normal conditions.

The profiles of nitrite reduction and N_2O production/reduction were obtained using the batch reactor through N_2O and NO_2^- measurements as described for other batch tests. A 300 mL volume of granular sludge was withdrawn from the sludge SBR prior to the addition of nitrite, at the end of the incubation with nitrite, and at the end of the anaerobic period of the first, fourth, eighth, and twelvth cycles. The sludge was washed twice to remove residual soluble organic carbon and nitrite and suspended with the same synthetic medium as used in previous tests. The tests were carried out following the same procedure as used for type I tests at pH 8 ± 0.05. However, intact granules were used in these tests.

Analysis. The ammonia (NH_4^+) and nitrite (NO_2^-) concentrations were analyzed using a Lachat QuikChem8000 Flow Injection Analyzer (Lachat Instrument, Milwaukee). Mixed liquor suspended solid (MLSS) and MLVSS were analyzed according to the standard methods (APHA, 1995).

Parameter Estimation. The FNA inhibitory effect on N_2O reduction was modeled based on the experimental data. The MATLAB (Mathworks, Inc., Natick, MA) function "lsqcurvefit" was used to find the parameter values that resulted in the best fit between the models used and the experiment data. More details of this estimation can be found in the Supporting Information.

Results

FNA Inhibition on N_2O Reduction. As shown in Figure 1a, there was a general negative impact of nitrite on N_2O 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, the N_2O reduction activity of the biomass varied from 100% to 20% under different pH

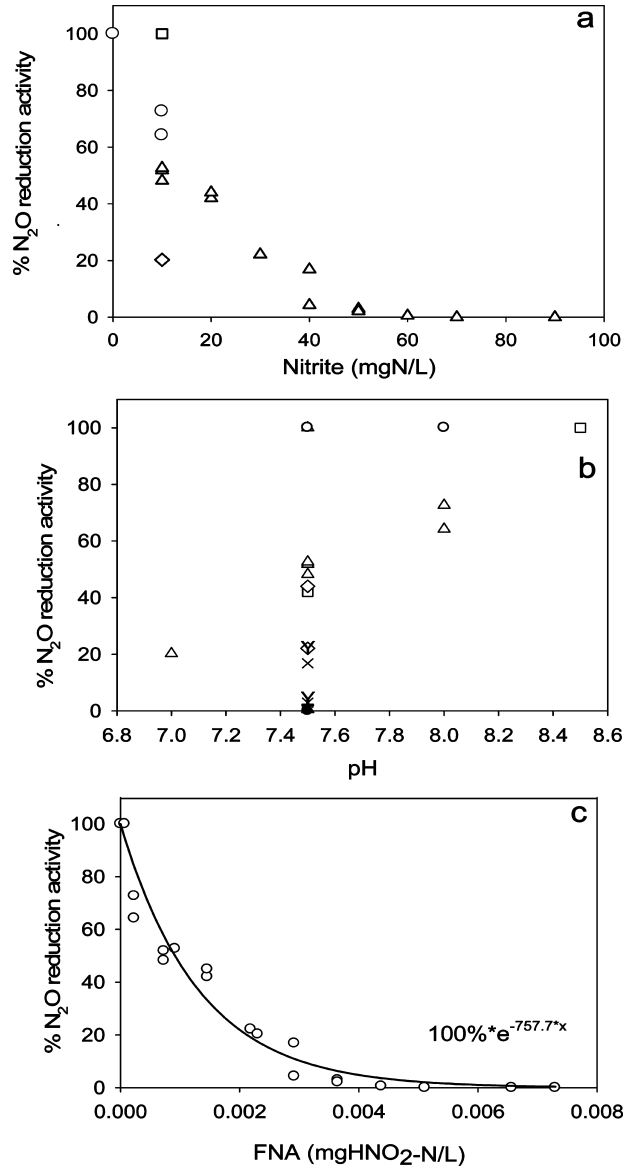

FIGURE 1. Correlation between the biomass N_2O reduction activity and the nitrite concentration (a), pH (b), and the FNA concentration. In graph a, □- pH 8.5, ○- pH 8.0, Δ- pH 7.5, ◇- pH 7.0. In graph b, nitrite concentration (mg NO_2^--N/L) ○- 0, Δ- 10, □- 20, ◇- 30, Y- 40, ×- 50, +- 60, ▲- 70, ●- 90. In graphs c, the solid line is the prediction of the inhibition model proposed. The 95% confidence interval of the model parameter is [−870, −650].

levels. The lower the pH, the stronger the inhibition. However, the correlation between N_2O reduction activity and pH suggests that pH was not the main inhibitory factor either (Figure 1b). The N_2O reduction activity was inhibited at very different levels at the same pH, but different nitrite levels. These observations suggest that FNA may more likely be the true inhibitor than nitrite and pH. Figure 1c confirms that the level of inhibition had a much stronger correlation with the FNA concentration, indicating that FNA may directly cause the inhibition. Also shown in the figure is the fit between the experimental data and the predictions by an exponential model. It is seen that the inhibitory effect of FNA on N_2O reduction could well be described by an exponential function.

The N_2O reduction activity of the biomass decreased significantly with the increased FNA concentration even in the very low range of 0−0.0002 mg HNO_2-N/L. The N_2O reduction activity decreased by 50% at FNA concentrations of 0.0007−0.001 mg HNO_2-N/L (equivalent to a nitrite

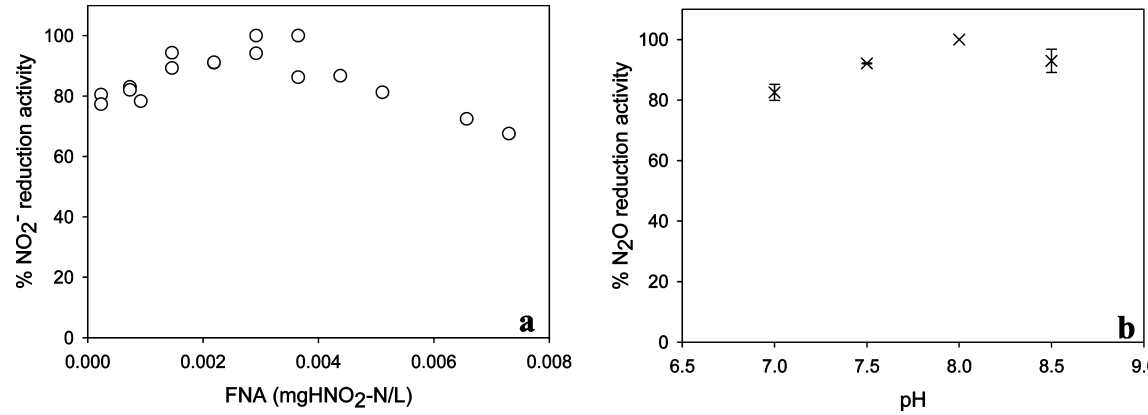

FIGURE 2. (a) Nitrite reduction activity of the biomass under various FNA concentrations; (b) N_2O reduction activity at various pH (where triplicates were performed). All the data is presented as the percentage ratio between the measured NO_2^- reduction rates (a) or N_2O reduction rates (b) and the maximum reduction rate measured in these tests.

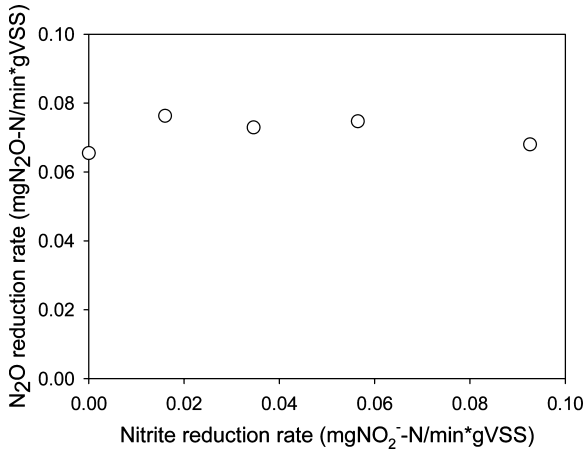

FIGURE 3. N_2O reduction rates measured in type III tests at different nitrite reduction rates.

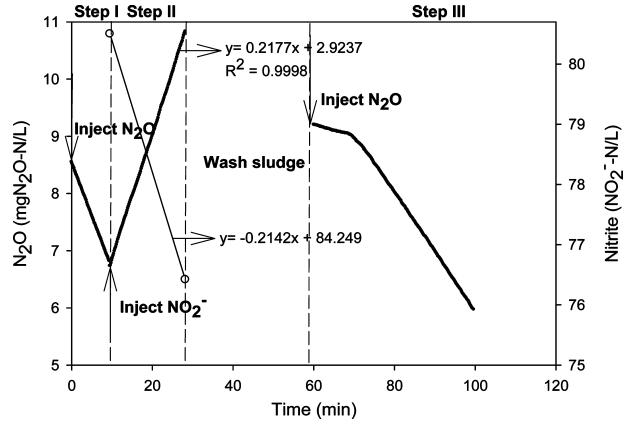

FIGURE 4. N_2O profile measured in one of the IVa-type batch tests. ○ - nitrite; ● - N_2O. The biomass was incubated in Step II for 20 min in a medium containing FNA at an initial concentration of 0.006 mg HNO_2-N/L.

concentration of approximately 3−4 mg NO_2^--N/L at pH 7) and was totally inhibited when the FNA concentration was greater than 0.004 mg HNO_2-N/L (17 mg NO_2^--N/L at pH 7).

Figure 2a shows that the nitrite reduction rates in these tests remained 70−100% of the maximum rate measured. The figure suggests that nitrite reduction rate was likely limited by substrate (HNO_2) availability when the FNA concentration was lower than 0.001 mg HNO_2-N/L. It also shows that FNA, in the range of 0−0.005 mg HNO_2-N/L, barely inhibited nitrite reduction. The results were consistent with the observation made in Zhou et al. (*17*). These results show that the nitrite reduction rate was relatively constant in majority of the tests reported in Figure 1c.

N_2O Reduction at Different pH. Figure 2b shows the N_2O reduction activity at different pH levels in the absence of nitrite, measured in the type II tests. The biomass displayed relatively constant N_2O reduction activity at pH 7.5−8.5. However, the activity decreased to 80−85% at pH 7.0. The results, which in agreement with the observations made in Ghosh et al. (*23*), indicate that pH has an impact on the N_2O reduction activity. However, this impact is insignificant compared to the inhibition of FNA.

Competition for Electrons by NO_2^- and N_2O Reduction. The N_2O reduction rates under different nitrite consumption rates, measured in type III tests, are presented in Figure 3. With the increasing nitrite reduction rates, the N_2O reduction rates remained at a relatively constant level. The results show that the varying nitrite reduction rate did not have an impact on the N_2O reduction rate. It is worthwhile to note that, in the test with the highest nitrite loading rate, the maximum nitrite reduction rate was obtained as evidenced by the nitrite accumulation during the test. When nitrite started accumulating, nitrite reduction was no longer able to keep up with the feeding. At this point, the maximum nitrite reduction rate should have been achieved.

N_2O Reduction Recovery from FNA Inhibition. *a. N_2O Reduction Recovery from Inhibition and the Dependency of Recovery on Duration of Inhibition.* Figure 4 shows the N_2O profile measured during one of the four type IVa tests. The noninhibited N_2O reduction rate was determined from the first 10 min profile (Step I). The N_2O reduction in the following 20 min (Step II) was completely inhibited as confirmed by the fact that the measured N_2O accumulation rate was identical to the nitrite reduction rate. This was as expected from the results presented in Figure 1c. The recovery step was carried out after the residual nitrite in the mixed liquor was removed through sludge washing. N_2O reduction restarted after a short lag period of approximately 10 min. However, the rate of N_2O reduction did not recover to 100% of that observed in Step I. The degrees of recovery in Step III measured in all four experiments were 69%, 70%, 67.5%, and 56.3%, respectively, under 10, 20, 40, and 60 min of nitrite exposure. It suggests that the recovery was not strongly dependent on the duration of inhibition. The slightly lower N_2O reduction rate in the test with 60 min inhibition was probably due to the limitation of PHA since more nitrite was reduced during Step II, causing more PHA consumption in this step.

b. Parent Reactor Recovery after Nitrite Shock Loading. The N_2O reduction ability of the biomass in the parent reactor was completely inhibited by the high level addition of nitrite.

The ability of the biomass to reduce N_2O recovered gradually when normal operation was resumed (Figure S2, Supporting Information). The biomass was unable to reduce N_2O in the first normal cycle. However, the N_2O reduction rate recovered to 50% within one day of normal operation, and to 100% at the end of the second day. The results indicate that the N_2O reduction capability of the biomass could recover relatively quickly.

Discussion

Mechanisms for FNA Inhibition on N_2O Reduction. Previous studies have shown that both nitrite and pH influence N_2O reduction during denitrification. This study demonstrated, however, that FNA, which is jointly determined by nitrite and pH, is likely a strong inhibitor of N_2O reduction. While the N_2O reduction rate seems to be lowered at pH 7.0 compared to pH 8−8.5, which suggests an independent effect of pH, the inhibitory effect is much more moderate in comparison to that of FNA.

Schalk-Otte et al. (*24*) suggested that the competition between the enzymes of denitrification for electrons from the cytochrome c pool could explain the decrease in the N_2O reduction rate in the presence of nitrite, if N_2O reductase had a lower affinity with the electron donor than other reductases. In the current study, however, the results show that the addition of nitrite and the increasing nitrite reduction rate did not result in a lowered N_2O reduction rate (Figure 3). The results suggest that the electrons required by the nitrite and N_2O reductases, when both were functional, were adequately provided by the carbon oxidation processes. In other words, the decrease in the N_2O reduction rate observed in Figure 1 when nitrite/FNA was present was not due to the competition for electrons between the nitrite and N_2O reductases.

The inhibition on N_2O reduction may also be due to the uncoupling effect of FNA. HNO_2 was reported to be able to passively diffuse across the cell membrane and shuttle protons between the two sides without generating energy, which may result in the collapse of proton motive force (pmf) (*14, 16*). 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 lead to a higher rather than lower N_2O reduction rate. This contradicts the experimental data shown in Figure 1c.

A more likely mechanism is that FNA may react with the enzymes involved in the N_2O reduction. N_2O reductase contains two metal centers, a binuclear copper center, Cu_A, that serves to receive electrons from soluble donors, and a tetranuclear copper-sulfide center, Cu_Z, at the active site (*25*). HNO_2 could bind to the active sites of copper-contained enzymes, causing competitive inhibition to N_2O reduction.

On the other hand, the reduction of nitrite or HNO_2 produces nitric oxide (NO) and nitroxyl anion (NO^-). These molecules are thought to react directly with heme and metal centers of proteins, forming nitrosyl complexes (*26*). Because of the function of the 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. However, in the experiments presented in Figure S1 (Supporting Information) and Figure 4, and indeed any other experiments with complete inhibition of N_2O reduction (when the FNA concentration is greater than 0.004 mg HNO_2-N/L, see Figure 1), the nitrite reduction rate was identical to the N_2O accumulation rate. This implies that there was no significant NO accumulation in these tests. NO therefore unlikely played a significant role in the inhibition of the N_2O reductase. This is further confirmed by the linearity of the N_2O profiles observed in all experiments ($R^2 > 0.99$, see Figure S1 in Supporting Information for an example). If NO accumulated during the tests and was responsible for the inhibition on the N_2O reductase, the N_2O profile should not be linear, as the NO concentration is expected to be varying during a test, accumulating at the beginning and depleting at the end causing different levels of inhibition.

The results presented in Figure 4 and in the recovery experiments suggest that the inhibition is reversible. The recovery speed appears to be independent of the duration of the inhibition, but dependent on the level of FNA to which the biomass was exposed. Even in the cases with a fast recovery due to relatively low levels of FNA applied, a 10 min lag time was always observed at the beginning of the recovery test. This time was probably required for the dissociation of the inhibitor from the binding sites.

FNA at the concentrations used in this study was not observed to have a similar inhibitory effect on nitrite reduction. For the reduction of nitrite, two entirely different enzymes, namely the tetreheme protein cytochrome cd1 and CuNIR, have been reported for denitrifying bacteria, although they have not been found within the same cells (*27*). These enzymes are different in terms of both structure and the prosthetic metal. Both CuNIR and cytochrome cd1 have been found in each of the alpha, beta, and gamma subclasses of *Proteobacteria* (*12*). The bacteria that are typically found to be responsible for EBPR have been reported to belong to *Proteobacteria* (*28−30*). It is possible that the community formed by these bacteria as well as other denitrifying bacteria possess both types of enzymes for the nitrite reductase. While FNA should bind and/or react with the copper center of CuNIR, cytochrome cd1 may continue to catalyze the redox reaction. Zhou et al. (*17*) showed that nitrite reduction by a similar EBPR sludge was inhibited by 40% at an FNA concentration of 0.01 mg HNO_2-N/L, and no further decrease was observed when the FNA concentration increased to 0.04 mg HNO_2-N/L.

While this study provides strong evidence showing that FNA has an inhibitory effect on N_2O reduction, the proof of this hypothesis requires pure culture studies. Because of the special operation of the parent reactor, the N_2O reduction processes studied in this work were primarily carried out by the organisms that were able to take up carbon sources under anaerobic conditions, namely PAOs and likely glycogen-accumulating organisms as well. Ordinary heterotrophic bacteria without the capability to take up carbon source anaerobically unlikely contributed significantly to the N_2O reduction.

Implications to the Operation of Wastewater Treatment Plants. In wastewater treatment systems, the conversion rate of the N_2O reductase is believed to be much faster than the rate of the other enzyme systems, thus preventing the formation of measurable N_2O concentrations (*31*). However, this study suggests that the rate of N_2O reduction could be lowered considerably due to FNA inhibition. Figure 1c shows that 50% inhibition occurred at an FNA concentration of 0.0007−0.001 mg HNO_2-N/L, or a nitrite concentration of approximately 3−4 mg NO_2^--N/L at pH 7. Such a low inhibition threshold constitutes a serious concern for N_2O emission from biological nitrogen removal wastewater treatment plants, particularly those designed and operated to achieve nitrogen removal via the nitrite pathway. Fux et al. (*32*) reported the advantages of a nitritation and denitritation system for ammonium-rich wastewater removal, while they also proposed the potential possibility of the production of NO and N_2O during the denitritation. N_2O emission was also reported in a full-scale reject water treatment plant via nitritation, which represented 1.7% of total nitrogen load (*33*). Given its strong radioactive forcing, N_2O emission from biological nitrogen removal wastewater treatment plants should be investigated thoroughly. The accumulation of

nitrite should be avoided in nitrogen removal systems. This could be achieved through continuous rather than pulse loading, which would regulate the concentrations of nitrite to relatively low levels.

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Supporting Information Available

Method for calculating the rates of N_2O and NO_2^- reduction, parameter estimation in Figure 1, and detailed experimental procedures and results of IVb type batch tests. This material is available free of charge via the Internet at http://pubs.acs.org.

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