Nitrite Dosage Strategy On N₂O Production For Denitrifying Phosphorus Accumulating Organisms Acclimated With Different Electron Acceptors

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Abstract
Simultaneous nitrogen and phosphorus removal by denitrifying polyphosphate accumulating organisms (DNPAOs) is a promising process for wastewater treatment. However, high potential of nitrous oxide (N₂O) emission may occur in this process, which would limit its sustainable application. Effect of nitrite dosage strategy on N₂O production for DNPAOs acclimated with different electron acceptors was examined. N₂O emission by the enriched DNPAOs was mainly affected by electron acceptor rather than intracellular organic carbon, and a high emission potential occurred with nitrite as the electron acceptor. By applying pulse dosage of nitrite, N₂O emission was reduced significantly. In addition, a high N₂O emission potential was observed for DNPAOs acclimated with nitrite rather than nitrate, indicating that the acclimation history had a high effect on system performance. Under all conditions, adequate anoxic duration should be maintained to denitrify the dissolved N₂O, which was another mitigating strategy for N₂O emission.

Keywords: Denitrifying phosphorus removal; DNPAOs; N₂O; Electron acceptor

1. Introduction
Simultaneous nitrogen and phosphorus removal can be achieved in the anaerobic and anoxic denitrifying phosphorus removal process by activities of denitrifying polyphosphate accumulating organisms (DNPAOs). In the anaerobic phase, DNPAOs take up volatile fatty acids (VFAs) from the wastewater with the energy supplied by polyphosphate degradation and store them in the form of poly-β-hydroxyalkanoates (PHAs). In the anoxic phase, PHAs are oxidized to provide energy for phosphate uptake with nitrate and/or nitrite as electron acceptors to achieve denitrifying phosphorus removal (Meinhold et al. 1999, Oehmen et al. 2007). During denitrifying phosphorus removal, nitrous oxide (N₂O) may be released, preventing its sustainable application. N₂O is a potent greenhouse gas, with the global warming potential of 300 times greater than that of CO₂ and its annually global increasing rate of 0.31%. N₂O not only contributes to the greenhouse effect, but also destructs the ozone layer (Ravishankara et al. 2009).

During denitrifying phosphorus removal, as typical denitrification, nitrate nitrogen (NO₃⁻-N) is reduced sequentially to nitrite nitrogen (NO₂⁻-N), nitric oxide (NO), N₂O and molecular nitrogen (N₂) by DNPAOs. The final product of denitrifying phosphorus removal might be N₂O rather than N₂ under certain conditions (Zeng et al. 2003). The utilization of PHAs and high concentration of NO₂⁻-N were considered to be two main factors inducing high N₂O emission during denitrifying phosphorus removal (Zeng et al. 2003). During denitrifying phosphorus removal with PHAs as the organic carbon, the slow degradation of PHAs led to NO₂⁻-N accumulation, consequently inducing a high N₂O accumulation and emission (Lemaire et al. 2006). In addition, a high NO₂⁻-N concentration might also inhibit denitrifying of NO₂⁻-N and N₂O, contributing to a high N₂O emission. Furthermore, Goretski and Hollocher (1988) demonstrated that a high NO₂⁻-N concentrations promoted NO generation during denitrification, which was more toxic than that of NO₂⁻-N, and this promoted N₂O accumulation. Zhou et al. (2008) and Pijuan et al. (2010) found that free nitrous acid (FNA) inhibited aerobic and anoxic phosphorus uptake, and 50% inhibition was observed at the FNA concentration of 0.0007-0.001 mg HNO₂-N/L (approximately 3-4 mg NO₂⁻-N/L, pH=7), while complete inhibition occurred at the FNA concentration of 0.004 mg HNO₂-N/L. FNA reacts with N₂O reductase (Nos) (copper-containing enzymes) and causes competitive inhibition of N₂O reduction. Further studies are still required to clarify key factors inducing N₂O emission during denitrifying phosphorus removal.

The aims of this study was to examine N₂O emission characteristics by DNPAOs acclimated under different electron acceptors and the NO₂⁻-N dosage strategy for controlling N₂O emission.

2. Materials and methods
2.1. SBRs
Two 6-L sequencing batch reactors (SBRs) were operated at 27°C to acclimate DNPAOs with NO₂⁻-N (DNPAO₅₃) or NO₃⁻-N (DNPAO₆₃) as the electron acceptor, respectively. Both SBRs were operated by alternating anaerobic/anoxic/aerobic conditions with a reaction cycle of 8 h, including 10 min filling period, 110 min anaerobic
period, 180 min anoxic period, 120 min aerobic period and 60 min sludge setting and decanting period. 150 mL of NaNO₂ (DNPAOₙ) or NaNO₃ (DNPAOₙ) was added into both SBRs at the beginning of the anoxic phase with the dosed final concentration of both 30 mg/L to induce denitrifying phosphorus removal, respectively. In each cycle, 3 L treated wastewater was pumped out and 3 L synthetic wastewater was fed into the reactors, resulting in a hydraulic retention time (HRT) of 16 h. 300 mL activated sludge was removed at the end of the aerobic phase with an applied solids retention time (SRT) of approximately 20 d. The synthetic wastewater contained CH₂COONa of 513 mg/L (COD around 400 mg/L), MgSO₄ of 90 mg/L, CaCl₂ of 14 mg/L, Na₂HPO₄ of 74 mg/L, (16 mg/L PO₄-P), NH₄Cl of 76 mg/L (NH₄-N 20 mg/L), yeast of 10 mg/L, and trace elements of 0.4 mL/L.

2.2. Batch experiments

For determination of DNPAOs fraction in PAOs, activated sludge (900 mL) was withdrawn from both SBRs at the end of the anaerobic phase and divided into three batch reactors. One was dosed with NaNO₂ at an initial NO₃-N concentration of 20 mg/L, the other was dosed with NaNO₃ at an initial NO₃-N concentration of 30 mg/L, and the last one was only aerated without dosing of NaNO₂ or NaNO₃. The batch reaction lasted for 90 min. For the effect of types of electron acceptors on N₂O emission during denitrifying phosphorus removal, mixed liquor of 600 mL was withdrawn from both SBRs at the end of the anaerobic phase and divided into two batch reactors. NaNO₂ or NaNO₃ solution was added to both reactors, achieving an initial NO₃-N and NO₂-N concentration of 20 and 30 mg/L, respectively. Both reactors were sealed and the denitrification reaction lasted for 90 min. For the effect of NO₂-N dosage strategy on N₂O emission during denitrifying phosphorus removal, mixed liquor of 1200 mL was withdrawn from both SBRs at the end of the anaerobic phase and divided into two batch reactors. NaNO₂ was dosed by two or three pulses, respectively. The initial NO₂-N concentration of each pulse addition for two and three pulses was approximately 10 and 6.7 mg/L, respectively.

During all batch experiments, samples were taken from each batch reactor at intervals and N₂ was introduced to balance the atmospheric pressure. Gas samples were collected using syringes and analyzed for N₂O. Solid and liquid samples were analyzed for NH₄-N, NO₂-N, NO₃-N, PO₄-P, PHB, SS and VSS.

2.3. Analytical methods

The liquid samples were immediately filtered for analysis. NH₄-N, NO₂-N, NO₃-N, PO₄-P, PHB, SS and VSS were measured according to Standard Methods. PHB was measured in accordance with Rodgers and Wu (2010). The concentration of N₂O in gas samples were analyzed by gas chromatograph (GC) (Agilent Technologies 6820, USA) equipped with an electron capture detector (ECD). The dissolved N₂O was measured by Microsensor Multimeter (Unisense, Denmark). FNA was calculated according to the method described by Anthonisen et al. (1976). The N₂O emission factor was calculated as the ratio of the produced N₂O-N to the removed oxidized nitrogen. The reaction rates for denitrification were calculated by linear regression of the related parameters.

3. Results and discussion

3.1. System long-term operation and performance

After a long-term acclimation, both reactors operated under steady state. In DNPAOₙ, SS and VSS concentrations were 2470±80 and 1990±30 mg/L, with the VSS/SS ratio of 0.81. The concentrations of PO₄-P at the end of the anaerobic, anoxic and aerobic phases were 58.9, 39.9 and 8.0 mg/L, respectively. The NO₃-N concentration at the end of the anoxic phase was 1.2 mg/L. The Effluent NO₂-N concentration was 2.6 mg/L, and the effluent NO₂-N was not detected. In DNPAOₙ, SS and VSS concentrations were 1980±60 and 1740±30 mg/L, with the VSS/SS ratio of 0.87. The concentration of PO₄-P at the end of the anaerobic, anoxic and aerobic phases were 83.3, 43.6 and 9.0 mg/L, respectively. The concentration of NO₂-N at the end of the anoxic phase and in the effluent was 3.2 and 11.4 mg/L, and the effluent NO₂-N was not detected. The influent PO₄-P concentration was similar in both reactors, PO₄-P release in the anaerobic phase was less in DNPAOₙ than in DNPAOₙ, indicating a high PO₄-P release capacity in DNPAOₙ. While the capacity of PO₄-P removal in both reactors were similar, owing to the similar effluent PO₄-P concentration. Dynamics of N, P, PHB, DO and pH in typical cycles of DNPAOₙ and DNPAOₙ are shown in Figure 1. In DNPAOₙ during the anoxic phase, the concentration of NO₂-N and PO₄-P decreased from 31.97 to 0.06 mg/L and 60.34 to 35.81 mg/L, and the denitrification rate and the PO₄-P uptake rate were 6.36 and 4.30 mg/g VSS·h, respectively. In DNPAOₙ, during the anoxic phase, the concentration of NO₂-N and PO₄-P declined from 29.72 to 12.43 mg/L and 88.75 to 46.91 mg/L, and the denitrification rate and the PO₄-P uptake rate were 4.19 and 8.64 mg/g VSS·h, respectively. The rate of PO₄-P uptake during the anoxic phase in DNPAOₙ was higher than in DNPAOₙ, and the denitrification rate (6.36 mg/g VSS·h) in DNPAOₙ was higher than that in DNPAOₙ. The ratios of PO₄-P uptake/NO₂-N reduction were 1.72 and 0.74 g P/g NO₂-N in DNPAOₙ and DNPAOₙ, respectively. For DNPAOₙ, PO₄-P uptake rates were 8.2, 4.2 and 17.1 mg P/g VSS·h with NO₂-N, NO₃-N and O₂ as electron acceptors, and the fractions of DNPAOs with nitrite or nitrate as the electron acceptor to the total PAOs were 60.0% and 30.8%. For DNPAOₙ, the PO₄-P uptake rates were 12.0, 4.2 and 21.2 mg P/g VSS·h with NO₂-N, NO₃-N and O₂ as electron acceptors, and the fractions of DNPAOs with nitrite or nitrate as the electron acceptor to the total PAOs were 60.1% and 64.4%. These indicated that DNPAOs enriched in both reactors were able to use NO₂-N. The rates of PO₄-P uptake and denitrification in DNPAOₙ were higher than those in DNPAOₙ with the same type of electron acceptor, indicating higher PAOs activities in DNPAOₙ than in DNPAOₙ. For DNPAOₙ and DNPAOₙ, the rate of PO₄-P uptake was higher with NO₂-N as the electron acceptor than that with NO₃-N, confirming that no NO₂-N accumulated in typical cycles.

3.2. Effect of types of electron acceptors on N₂O emission

Dynamics of N and P with NO₂-N or NO₃-N as the electron acceptor during denitrifying phosphorus removal in DNPAOₙ and DNPAOₙ are shown in Figure 2. With NO₂-N as the electron acceptor, no NO₂-N accumulation occurred and N₂O emission was relatively low during
Figure 1. Dynamics of N, P, PHB, DO and pH in typical cycles of DNPAO_N3 (A) and DNPAO_N2 (B).

With NO\textsubscript{2}-N as the electron acceptor, N\textsubscript{2}O emission was relatively high during denitrifying phosphorus removal and it was high in DNPAO_N2 compared to that in DNPAO_N3, with the N\textsubscript{2}O emission factor of 7.5% in DNPAO_N2 and 0.1% in DNPAO_N3. During the anoxic uptake of PO\textsubscript{4}-P, denitrification of NO\textsubscript{3}-N was observed without the accumulation of NO\textsubscript{2}-N. In the study of Oehmen et al. (2010), similar phenomenon was also reported but was not emphasized. However, the phenomenon was different from other previous studies, where under anoxic phase, denitrification of NO\textsubscript{2}-N was accompanied with the NO\textsubscript{2}-N accumulation in DNPAO systems, such as Wang et al. (2011). N\textsubscript{2}O generation was apparently higher with NO\textsubscript{2}-N as the electron acceptor than that with NO\textsubscript{3}-N, showing that NO\textsubscript{2}-N was an important inducer for N\textsubscript{2}O generation. The production of N\textsubscript{2}O was comparatively low with NO\textsubscript{3}-N as the electron acceptor and PHB as the carbon source, indicating that electron acceptor rather than PHB was the key factor causing N\textsubscript{2}O generation during denitrifying phosphorus removal. Denitrifying phosphorus removal, while the dissolved N\textsubscript{2}O concentration rose initially and then decreased thereafter. The rates of PO\textsubscript{4}-P uptake and denitrification in DNPAO_N3 were higher than those in DNPAO_N2. The emission factor of N\textsubscript{2}O was 0.06% in DNPAO_N2 and 0.05% in DNPAO_N3.

3.3. Effect of two pulses of NO\textsubscript{2}-N dosage on N\textsubscript{2}O emission

Effect of two pulses of NO\textsubscript{2}-N dosage on N\textsubscript{2}O emission during denitrifying phosphorus removal is shown in Figure 3. For microbial activities, the rates of PO\textsubscript{4}-P uptake and denitrification after the second NO\textsubscript{2}-N dosage were lower than those of the first dosage, indicating that denitrifying phosphorus removal activities were inhibited. Meinhold et al. (1999) showed that the PO\textsubscript{4}-P uptake was inhibited completely with the addition of 10 mg/L NO\textsubscript{2}-N during denitrifying phosphorus removal; after nitrite was completely consumed, NO\textsubscript{2}-N (5 mg/L) was further added, but this did not lead to PO\textsubscript{4}-P uptake and NO\textsubscript{2}-N was consumed at a relatively low rate. This demonstrated that NO\textsubscript{2}-N inhibition could last for a period of time even after NO\textsubscript{2}-N was completely utilized.

Figure 2. Dynamics of N and P with NO\textsubscript{3}-N (A, C) or NO\textsubscript{2}-N (B, D) as the electron acceptor for DNPAOs from DNPAO_N3 (A, B) and DNPAO_N2 (C, D), respectively.

In both DNPAO_N2 and DNPAO_N3, the dissolved N\textsubscript{2}O concentration increased after each dosage of NO\textsubscript{2}-N and then decreased after reaching a peak; the off-gas N\textsubscript{2}O concentration gradually rose during denitrifying phosphorus removal. The concentration of N\textsubscript{2}O generated in DNPAO_N3 was high and the N\textsubscript{2}O emission factor during the first and second pulse was 3.3% and 5.0%, respectively, while those were 0.2% and 0.1% in DNPAO_N2. The maximum dissolved N\textsubscript{2}O concentration during the first and second pulse was 0.07 and 0.08 mg/L in DNPAO_N3, respectively, which were lower than those of 0.98 and 2.14 mg/L in DNPAO_N2. The maximum accumulated N\textsubscript{2}O concentration in the secondary pulse was higher than that in the first pulse in both reactors. The possible reasons were as follows. Firstly, the detrimental effect of NO\textsubscript{2}-N still remained after the first dosage, resulting in inhibition of denitrifying activities. Secondly, the degradation of PHA were slow during denitrifying...
phosphorus removal, resulting in the competition between denitrifying enzymes. In addition, Nos enzyme was weak at competing for the electrons and N\textsubscript{2}O reduction was the last step in denitrification, which inhibited the reduction of N\textsubscript{2}O. Finally, considering that PHAs was consumed during the first pulse dosage, less would be available during the second pulse dosage, intensifying the competition for electrons and stimulating more N\textsubscript{2}O accumulation.

**Figure 3.** Dynamics of N and P under conditions with two dosages of NO\textsubscript{2}-N for DNPAOs from DNPAO\textsubscript{N3} (A) and DNPAO\textsubscript{N2} (B), respectively.

3.4. **Effect of three pulses of NO\textsubscript{2}-N dosage on N\textsubscript{2}O emission**

Effect of three pulses of NO\textsubscript{2}-N dosage on N\textsubscript{2}O emission during denitrifying phosphorus removal is shown in Figure 4. In DNPAO\textsubscript{N3}, the dissolved N\textsubscript{2}O concentration increased after each pulse dosage of NO\textsubscript{2}-N, and then decreased after reaching the peak, while the off-gas N\textsubscript{2}O concentration constantly increased during denitrifying phosphorus removal with the emission factor below 0.07%. In DNPAO\textsubscript{N2}, the characteristics of dissolved N\textsubscript{2}O production was similar to that of DNPAO\textsubscript{N3}, but the dissolved N\textsubscript{2}O accumulation and reduction rates were high; the off-gas N\textsubscript{2}O kept increasing during the whole process. The N\textsubscript{2}O emission factor was 0.8% during the first pulse, and then it increased rapidly in the following two pulses with values of 3.3% and 6.6%, respectively. The potential of N\textsubscript{2}O production by DNPAO\textsubscript{N2} was high, especially during the second and third pulses of NO\textsubscript{2}-N addition. The presence of NO\textsubscript{2}-N promoted the N\textsubscript{2}O generation since it was the substrate for denitrification. Inhibitory effect of initial NO\textsubscript{2}-N concentration was not the reason for N\textsubscript{2}O accumulation considering that the NO\textsubscript{2}-N concentration after each addition was the same, and it might be the long period of NO\textsubscript{2}-N presence inhibited the activity of Nos rather than NO\textsubscript{2}-N reductase (Nir) (Meinhold et al. 1999), resulting in a high N\textsubscript{2}O generation in the following two pulses. The dissolved N\textsubscript{2}O concentration in DNPAO\textsubscript{N2} was also relatively high, but it could be reduced after the depletion of NO\textsubscript{2}-N by denitrification. Based on the above analysis, the inhibition of N\textsubscript{2}O reductase might be the key factor inducing N\textsubscript{2}O accumulation.

**Figure 4.** Dynamics of N and P under conditions with three dosages of NO\textsubscript{2}-N for DNPAOs from DNPAO\textsubscript{N3} (A) and DNPAO\textsubscript{N2} (B), respectively.

3.5. **Effect of NO\textsubscript{2}-N dosage strategy on N\textsubscript{2}O emission and its control**

Several times pulse dosage of NO\textsubscript{2}-N could reduce N\textsubscript{2}O emission, especially for the NO\textsubscript{2}-N acclimated DNPAOs. Therefore, pulse addition of NO\textsubscript{2}-N could reduce the N\textsubscript{2}O emission. In addition, the above results showed that the acclimation history had a high effect on N\textsubscript{2}O emission during denitrifying phosphorus removal, with a high N\textsubscript{2}O emission potential for DNPAOs acclimated with NO\textsubscript{2}-N. Under all conditions, the dissolved N\textsubscript{2}O was initially produced and then consumed thereafter. For example, in DNPAO\textsubscript{N2}, the maximum dissolved N\textsubscript{2}O concentration under three dosage strategy conditions was 0.83, 2.14 and 2.17 mg/L, respectively. The produced dissolved N\textsubscript{2}O increased as increasing the dosage times, owing to the inhibitory effect of NO\textsubscript{2}-N. Therefore, the effect of long period presence of NO\textsubscript{2}-N on N\textsubscript{2}O emission during denitrifying phosphorus removal should be further investigated so as to provide effective N\textsubscript{2}O emission control strategy. However, special attention should be paid to the dissolved N\textsubscript{2}O that adequate reaction time could minimize the N\textsubscript{2}O production and consequently decrease N\textsubscript{2}O emission. Therefore, for controlling N\textsubscript{2}O emission under anoxic conditions, adequate anoxic reaction duration should be maintained.

4. **Conclusions**

The main conclusions were obtained as follows: (1) N\textsubscript{2}O emission was mainly affected by the electron acceptor rather than the intracellular organic carbon during denitrifying phosphorus removal, (2) N\textsubscript{2}O emission potential was high for DNPAOs acclimated with NO\textsubscript{2}-N rather than NO\textsubscript{2}-N as the electron acceptor, and (3)
adequate anoxic duration should be maintained as a controlling strategy for mitigating N\textsubscript{2}O emission.

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References


