Mechanisms of Unstable Nitrite Inhibition of Aerobic Phosphate Uptake

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ABSTRACT
Recently, nitrite has been recognized as one of the considerable inhibitors of biological phosphorus removal. In fact, there are several reports on inhibitory effect of nitrite. While unfortunately, the reported critical levels of nitrite widely spread. So the real effect of nitrite has not yet been well understood. In this study, several batch tests were conducted to obtain stable and quantitative relation between the size of nitrite exposure and the size of inhibition. The obtained results are as follows; 1) Nitrite inhibits aerobic phosphate uptake of PAOs, but the inhibition is not direct inhibition by nitrite but indirect inhibition caused by reduced respiration, 2) PAOs with higher anoxic activity can reduce the inhibitory effect of nitrite, possibly because of aerobic nitrite denitrification, 3) Nitrite inhibition of aerobic phosphate uptake is successfully expressed by the model including aerobic nitrite denitrification rates. These results strongly suggest that unstable nitrite inhibition of aerobic phosphate uptake is caused by widely distributed anoxic activities of PAOs.

Keywords: polyphosphate-accumulating organisms (PAOs), nitrite, inhibition, aerobic denitrification, biological phosphorus removal, anoxic phosphate uptake, activated sludge

INTRODUCTION
Eutrophication of closed water bodies has been one of the most serious water pollution problems all over the world. To solve this problem, nitrogen and phosphorus have to be removed from wastewater. Especially, phosphorus is often a limiting element for eutrophication so that stable performance of phosphorus removal is eagerly required. However, it is well known that biological phosphorus removal (BPR) process often becomes unstable. Therefore, it is an urgent subject to clarify the mechanism of BPR instability. Although several researchers have revealed the influencing factors (Hascoet and Florents, 1985; Kuba et al., 1994; Cech and Hartman, 1993; Liu et al., 1996), it is supposed to be other unknown factors.

This study focuses on nitrite that is known as a strong inhibitor for bacteria (Rowe et al., 1979; Almeida et al., 1994; Weon et al., 2002). Since nitrite is often present in full-scale wastewater treatment plants as an intermediate both of nitrification and denitrification, nitrite could be one of the factors deteriorating biological phosphorus removal. Actually, it has been reported that nitrite severely inhibits aerobic and anoxic phosphate uptake of Poly-Phosphate Accumulating Organisms (PAOs). However, the reported critical concentration of nitrite has been widely varying (Saito et al., 2004; Kuba et al., 1996;Lee et al., 2001; Meinhold et al., 1999; Ahn et al., 2001). In order to attain stable BPR performance, the mechanism of unstable response of PAOs to nitrite exposure
must be clarified.

One of the key observations is that an inhibitory effect of nitrite on anoxic phosphate uptake is less sensitive than aerobic phosphate uptake (Saito et al., 2004). This is not curious, because some of PAOs can utilize nitrite as an electron acceptor under anoxic condition (Meinhold et al., 1999; Ahn et al., 2001; Hu et al., 2003; Shoji et al., 2003). Even though nitrite has an inhibitory effect, some of denitrifying PAOs must be able to utilize and detoxify nitrite under anoxic condition. But, how about aerobic phosphate uptake? It is well known that some of ordinary denitrifiers can reduce nitrite under aerobic condition (Alefounder et al., 1980), in other words, ‘aerobic nitrite denitrification’. If some of PAOs can do that, the inhibitory effect of nitrite will be reduced. Hence, the purpose of this study is to examine the effect of anoxic activity of PAOs on reduction of inhibitory effect of nitrite.

MATERIALS AND METHODS
Cultivation of PAOs with acetate as a sole carbon source
Three sequencing batch reactors (AO SBR, AO/N SBR and AA SBR) were used to cultivate the enriched PAOs that have different anoxic phosphate uptake activities. All reactors were fed with acetate as a sole carbon source. Composition of synthetic wastewater is listed in Table 1. The cycle operations were shown in Figure 1. AO SBR and AO/N SBR were operated with 4L of cylindrical reactor under the alternating anaerobic(180min)-aerobic(130min) conditions. AO/N SBR received a small amount of nitrite that theoretically resulted in 1 mgN/l in the reactor at initial 1 minute of aerobic phase, while AO SBR did not. Allylthiourea (ATU) was periodically added to both of the reactor to suppress nitrification. AA SBR was operated with 1L of cylindrical reactor under the alternating anaerobic-anoxic conditions. 1 L of cylindrical reactor for AA SBR was operated with the alternating anaerobic(120min)-anoxic(190min) conditions. During initial 120 minutes of anoxic phase, nitrate was introduced into the reactor to theoretically result in 46 mgN/l in the reactor. Other experimental conditions were the same for all the reactors. HRT, SRT, water temperature and pH were controlled at 12hours, 10 days, 20 ± 5 °C and 7.0 ± 0.1, respectively. Seeded sludge were from a bench-scale SBR (Yoshida et al., 2005) operated with anaerobic-aerobic-anoxic-aerobic cycle to remove nitrogen and phosphorus from sewage.
Table 1 - Composition of Synthetic Wastewater

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration (g/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH2COOK</td>
<td>12.3</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>0.40</td>
</tr>
<tr>
<td>K2HPO4</td>
<td>0.97</td>
</tr>
<tr>
<td>KH2PO4</td>
<td>1.88</td>
</tr>
<tr>
<td>NH4Cl</td>
<td>3.05</td>
</tr>
<tr>
<td>MgSO4·7H2O</td>
<td>3.60</td>
</tr>
<tr>
<td>CaCl2</td>
<td>0.21</td>
</tr>
<tr>
<td>Trace Elements</td>
<td>6 (ml)</td>
</tr>
</tbody>
</table>

Methods of nitrite inhibition batch tests
21 sludge from AO SBR (7 sludge), AO/N SBR (11 sludge) and AA SBR (3 sludge) were tested with the procedure as follows. 200 to 300 ml of sludge was taken from the reactor at the end of anaerobic phase and was divided into several portions. The one was provided with air. The others were provided with air and different concentration of nitrite (0.27 to 4.9mgN·gVSS-1). They were controlled at around 7.0 ± 0.1 of pH and 20 °C of water temperature. Some sludge were tested with several concentration of nitrite so that total number of batch tests were 36. In some of batch tests, oxygen uptake rate (OUR) was also measured to evaluate nitrite effect on respiration.

Measurement methods of aerobic and anoxic phosphate uptake activities
Evaluation of aerobic and anoxic phosphate uptake activities of PAOs was conducted by the methods (Wachtmeister et. al., 19997) with some modifications. 100 to 200 ml of sludge was taken from the reactor at the end of anaerobic phase and was divided into two portions. The one was provided with air and the other was added with nitrate anaerobically. Both of pH were controlled at around 7.0 ± 0.1. The aerobic and anoxic phosphate uptake activities were calculated with the phosphate-decreasing rate during initial 20 to 30 minutes and 50 to 60 minutes, respectively.

Characteristics of the enriched PAOs in terms of anoxic activity
Since AO sludge was cultivated with the alternating anaerobic-aerobic cycle operation without exposure to oxidized nitrogen, they have no anoxic phosphate uptake activity (0 mgP/gVSS.h). AO/N sludge has a little anoxic activity (0 to 6.4 mgP/gVSS.h), because a small amount of nitrite was added under aerobic condition. This kind of operation is known to increase anoxic activity (Yoshida et. al., 2005; Saito et. al., 2005). AA sludge has the largest anoxic activity (12.6 to 25.9 mgP/gVSS.h), because they were cultivated by nitrate as an electron acceptor instead of oxygen. Thus, the enriched PAOs with different anoxic activities were obtained. Variation of aerobic phosphorus uptake activity was caused by rather unstable operation.
Analytical procedures
Phosphate, MLSS and MLVSS were analyzed in accordance with Japanese Standard Methods for Examinations of Wastewater. The concentration of nitrite and nitrate were determined by Shimadzu HPLC system equipped with a Shimadzu CDD-10A detector and a Shim-pack IC-A3 column. 0.45 m of membrane filters were used to separate dissolved and particulate matters.

RESULTS
Typical results of nitrite inhibition batch tests
Among 36 batch tests, the results of AA sludge with 4.9mgN/L of nitrite addition are shown in Figure 2 as representatives. As shown in the figure ‘(a) control’, in the case without nitrite addition, initial OUR was the highest and OUR decreased with time. This OUR curve resembles the curve reported by Smolders et al. (1994). Phosphate concentration also decreased linearly with time. Hence, the amount of oxygen respiration and the amount of phosphate uptake was not linear. This might be because the energy produced by oxygen respiration is used for several activities of PAOs, namely polyphosphate storage, glycogen restoration, cell growth and maintenance. On the other hand, in the case with nitrite addition, we observed the characteristic curves of phosphate concentration and OUR. Initially, low OUR was observed as compared to those without nitrite addition (‘control’), and later, OUR once increased and again decreased. These fluctuations are significantly corresponding to the course of phosphate concentration. Phosphate concentration initially showed the gradual decrease (corresponding to low OUR). Then, the sudden decrease was monitored (in other words, sudden increase of phosphate uptake). This behavior synchronized with increase of OUR. Moreover, this sudden increase of both phosphate uptake and OUR indeed synchronized with nitrite disappearance. These results suggest that suppression of phosphate uptake by nitrite is caused by suppression of oxygen respiration to some extent, because phosphate uptake requires energy produced by respiration. The other important observation is that both of phosphate uptake and oxygen respiration are not fully recovered. Even after nitrite completely disappeared, inhibition still remained. Moreover, nitrite disappeared under aerobic condition without nitrate production.

Figure 2 - Results of Nitrite Inhibition Batch Test (AA sludge with 4.9mgN/L of nitrite addition) (a) ‘control’ without nitrite addition, (b) with nitrite addition, Grey circle: phosphate, Empty square: OUR, Grey triangle: nitrite
Different response of PAOs with different cultivation career

The effect of cultivation career on the size of inhibition was examined by comparing the responses of AO sludge, AO/N sludge and AA sludge with the same nitrite concentration. The results are shown in Figure 3 and Table 2.

In the case of AO sludge (Figure 3(a)) with 2.3 mgN/gVSS of initial nitrite concentration, the initial phosphate uptake was completely inhibited. The phosphate uptake activity was almost 0 mg gVSS$^{-1}$ h$^{-1}$. After 60 min, the phosphate uptake activity recovered to some degree (23% of that in the absence of nitrite). Interesting observation is that nitrite concentration decreased without nitrate production (data not shown) and the recovery of phosphate uptake activity started just after nitrite disappearance. Similarly, in the case of AO/N with 2.0 mgN/gVSS of initial nitrite concentration, phosphate uptake was initially inhibited, but recovered soon at the time when nitrite disappeared. The phosphate uptake rate in the presence of nitrite was 13 mgP gVSS$^{-1}$ h$^{-1}$ and the % activity of phosphate uptake was 39%. On the other hands, in the case of AA with 1.9 mgN/gVSS of initial nitrite concentration, the size of inhibition was the smallest among them. The phosphate uptake rate in the presence of nitrite was 20 mgP gVSS$^{-1}$ h$^{-1}$ and the % activity of phosphate uptake was 60%. These results indicate that the size of inhibition is clearly different on different PAOs, even if they are exposed to the same range of nitrite concentrations. Interestingly, the rest of batch tests also indicate that the sizes of inhibition are AA sludge < AO/N sludge < AO sludge in order. And this order is the same as the order of their anoxic activity. Moreover, in all tests, nitrite disappeared without nitrate production even under aerobic condition and, the order of nitrite decreasing rates was also consistent with that of anoxic activity of PAOs. These observations imply a mutual relation among the size of inhibition, the anoxic activity and the nitrite-decreasing rate.

![Figure 3 - Comparison of Cultivation Career (AO, AO/N and AA) and Nitrite Inhibition at The Same Range of Nitrite Exposure.](image-url)

(a) AO 2.3 mgN-gVSS$^{-1}$  (b) AO/N 2.0 mgN-gVSS$^{-1}$  (c) AA 1.9 mgN-gVSS$^{-1}$

- Time (min)  - Phosphate (mgP/gVSS)  - Nitrate, Nitrite (mgN/gVSS)

Figure 3 - Comparison of Cultivation Career (AO, AO/N and AA) and Nitrite Inhibition at The Same Range of Nitrite Exposure. (a) AO with 2.3mgN/gVSS of nitrite, (b) AO/N with 2.0mgN/gVSS of nitrite, (c) AA with 1.9mgN/gVSS, Empty circle: phosphate in control, Grey circle: phosphate with nitrite addition, Triangle: nitrite
Table 2 – Summary of Comparison of Cultivation Career

<table>
<thead>
<tr>
<th>Nitrite dose (mgN/gVSS)</th>
<th>Anoxic PUA (mgPgVSS^-1-h)</th>
<th>Aerobic PUA (mgPgVSS^-1-h)</th>
<th>PUA with nitrite (mgPgVSS^-1-h)</th>
<th>% activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AO</td>
<td>2.3</td>
<td>0.0</td>
<td>30</td>
<td>0.5</td>
</tr>
<tr>
<td>AO/N</td>
<td>2.0</td>
<td>6.4</td>
<td>34</td>
<td>13</td>
</tr>
<tr>
<td>AA</td>
<td>1.9</td>
<td>13</td>
<td>33</td>
<td>20</td>
</tr>
</tbody>
</table>

### Relationship between anoxic activity of PAOs and aerobic nitrite disappearance

It is so interesting that we observed nitrite disappearance under aerobic condition without nitrate production in all batch tests, since there are no reports on aerobic nitrite denitrification by PAOs. However, our results strongly suggest that unstable response of PAOs to nitrite exposure, must come from their different anoxic phosphate uptake activities, in other words, PAOs with higher anoxic activity can reduce nitrite under aerobic condition (aerobic denitrification!) and, hence, can reduce nitrite inhibition.

To confirm that aerobic nitrite disappearance is caused by a biologically mediated reaction by PAOs, several experiments were conducted (data not shown). First, the filtrates of culture solution and sludge were aerated with nitrite. However, nitrite never disappeared. This fact suggests that the aerobic nitrite disappearance is not a chemical reaction but a biologically mediated reaction. Next, the enriched PAOs was provided with more than 4 hours of excessive aeration to deplete internally stored organic substances completely. Then, nitrite was added. However, nitrite again never disappeared. This result suggests that the observed aerobic nitrite disappearance requires an internally stored organic substance.

Next, to confirm the participation of PAOs, the relationship between anoxic phosphate uptake activity and aerobic nitrite decrease that are obtained from all batch tests was examined. The result is shown in Figure 4. As shown in the figure, AO sludge that has little anoxic activity has lower nitrite decreasing rate. AO-N sludge that has small anoxic activity has relatively higher rate and AA sludge that has the highest anoxic activity has the highest nitrite consumption rate. And interestingly even PAOs with no anoxic phosphate uptake activity have a little aerobic nitrite denitrification activity.

Finally, the possibility of ordinary denitrification inside thick floc of the enriched PAOs was examined by adding nitrate instead of nitrite. Since nitrate concentration did not change, while nitrite disappeared, it was verified that nitrite was not reduced under anoxic condition inside floc. If anoxic condition was formed, nitrate also must have been reduced. From these results, it is concluded that the enriched PAOs in this study can do aerobic nitrite denitrification.
Dependency of nitrite inhibition on aerobic nitrite denitrification by PAOs

From above, it is suggested that nitrite inhibition not only depends on nitrite concentration, but also on aerobic nitrite denitrification. In this paragraph, an appropriate expression of unstable inhibitory responses is studied. First, all tested sludge were classified into three groups in terms of aerobic nitrite denitrification rate as shown in Table 3.

<table>
<thead>
<tr>
<th>Group</th>
<th>Aerobic Nitrite Denitrification Rate (mgN·gVSS⁻¹·h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group-1</td>
<td>&lt; 1.8</td>
</tr>
<tr>
<td>Group-2</td>
<td>1.8 &lt; and &lt; 4.5</td>
</tr>
<tr>
<td>Group-3</td>
<td>4.5 &lt;</td>
</tr>
</tbody>
</table>

PUA : phosphate uptake activity

To know the effect of aerobic nitrite denitrification activity on relieving inhibitory effect quantitatively, inhibitory effect of nitrite is expressed as non-competitive inhibition as follows;

\[ r = r_{max} \cdot \frac{K_{NO2}}{K_{NO2} + (C_{NO2})^\alpha} \]

\( r \) : aerobic phosphate uptake rate (mgP·gVSS⁻¹·h⁻¹), \( r_{max} \) : aerobic phosphate uptake activity (mgP·gVSS⁻¹·h⁻¹) without nitrite addition (control), \( K_{NO2} \) : inhibition constant of nitrite on phosphate uptake, \( C_{NO2} \) : concentration of nitrite (mgN/gVSS), \( \alpha \) : inhibition coefficient
The obtained constants are listed in Table 4. As shown in the table, the inhibition constant of Group-3 is the largest among them, since they have the largest anoxic activity and aerobic nitrite denitrification activity. Next is Group-2 and the most sensitive group is Group-1 that has little anoxic activity. $K_{NO_2}$ is 0.1 and $\alpha$ is 3.6. The fact that obtained values in the case of Group-1 and 2 are more than 1.0 may indicate that there are not one but several inhibition mechanisms. Anyway, since mechanism of nitrite inhibition has not yet been clarified, the used inhibition formula is not theoretical but practical one. In order to develop the adequate expression of nitrite inhibition, further research is required especially on inhibition mechanisms of phosphate uptake.

Table 4 – Determined Inhibition Constants

<table>
<thead>
<tr>
<th></th>
<th>$K_{NO_2}$ (mgN/gVSS)</th>
<th>$\alpha$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group-1</td>
<td>0.1</td>
<td>3.6</td>
</tr>
<tr>
<td>Group-2</td>
<td>1.0</td>
<td>1.8</td>
</tr>
<tr>
<td>Group-3</td>
<td>2.8</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Figure 5 shows the responses of PAOs to nitrite exposure with the obtained curves of modified non-competitive model. Although plots are somewhat scattering, the classification of tested sludge into three groups does make clear the size of inhibition. It is concluded that the aerobic nitrite denitrification rate is the key parameter to express the size of inhibition. Plots of Group-2 are rather scattering. Although the reason of the scattering is not clear, this may be caused by fragile denitrification potential of PAOs and/or other unknown reasons.

Figure 5 - Relationship between Nitrite Exposure and Inhibition of Phosphate Uptake, Empty circle: Group-1, grey triangle: Group-2, dark triangle: Group-3.
DISCUSSION

Aerobic nitrite denitrification by PAOs
Recent developments of molecular biology techniques, e.g., fluorescent in situ hybridization (FISH) and polymerase chain reaction (PCR), revealed that Rhodocyclus-related PAOs are predominant in a lab-scale anaerobic-aerobic reactor fed with acetate (Hesselmann et al., 1999; Crocetti et al., 2000). Rhodocyclus-related PAOs are also identified as denitrifying PAOs cultivated with acetate (Zeng et al., 2003). Since, in this study, PAOs were enriched by acetate as sole carbon source, Rhodocyclus-related PAOs were probably predominant. Hence, the enriched PAOs in this study could perform aerobic nitrite denitrification. Because, it is well known that some of gram-negative heterotrophic denitrifiers can perform aerobic denitrification of nitrite, since they have nitrite reductase on the periplasmic side of cell membrane. So at least some of the PAOs enriched in this study most likely have nitrite reductase on the periplasmic side of cell membrane and can reduce nitrite under aerobic condition. So far, there are no reports on aerobic nitrite denitrification by PAOs. However, aerobic nitrite disappearance observed in this study is probably an aerobic nitrite denitrification by PAOs.

Mechanism of buffering nitrite inhibition by PAOs
Nitrite is a well-known inhibitor of aerobic metabolism of bacteria (Rowe et al., 1979). Main mechanism of inhibitory effect of nitrite is cytochrom oxidation by nitric oxide produced by aerobic nitrite reduction, and is not by a direct attack by nitrite (Kucera et al., 1986; Carr et al., 1990). Hence, nitric oxide concentration inside cell is important to control inhibitory effect of nitrite. One of the ways is to supply full amount of COD. If readily biodegradable COD is abundantly available, aerobically produced nitric oxide will be easily removed by aerobic reduction and inhibitory effect can be significantly reduced (Casey et al., 1999). In this study, PAOs with higher anoxic activity performed higher aerobic nitrite denitrification and is less sensitive to nitrite. That is because they have enough ability to remove nitric oxide by denitrification. Mechanism of buffering phosphate uptake inhibition is most likely aerobic denitrification ability of PAOs.

Another interesting point is that aerobic phosphate uptake of AO sludge was strongly inhibited, though it has no anoxic phosphate uptake activity and, hence no nitric oxide production potential. In the case of Paracoccus denitrificans without nitrite reductase, aerobic respiration was not inhibited even in the presence of nitrite (Kucera et al., 1986). While, aerobic activity of AO sludge was strongly inhibited. One of the reasons is that nitric oxide was produced with nitrite reductase. AO sludge with no anoxic phosphate uptake activity has aerobic denitrification activity of nitrite (see Figure 4). Anoxic activity of PAOs is measured by nitrate denitrification. Possible explanation is that they had nitrite reductase, but did not have nitrate reductase. Regardless of inhibition mechanism (attack by nitric oxide or direct attack by nitrite), phosphate uptake inhibition is expectedly buffered by aerobic denitrification that can reduce nitrite or nitric oxide concentration inside cell. Observations of phosphate uptake and OUR recovery from inhibition soon after nitrite disappearance (Figure 2) strongly supports the hypothesis mentioned above.
CONCLUSIONS
In order to clarify the mechanism of unstable response of PAOs to nitrite exposure, nitrite inhibition batch tests were conducted with the enriched PAOs that have different anoxic activity.

- Nitrite inhibits aerobic phosphate uptake of PAOs, but the inhibition is not direct inhibition by nitrite but indirect inhibition caused by reduced respiration.
- PAOs with higher anoxic activity can reduce the inhibitory effect of nitrite, possibly because of aerobic nitrite denitrification.
- Nitrite inhibition of aerobic phosphate uptake is successfully expressed by the model including aerobic nitrite denitrification rates.

From these results, we concluded that unstable response of PAOs to nitrite is caused by widely distributed anoxic activities of PAOs.

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REFERENCES


