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# Selection and enrichment of denitrifying phosphorus accumulating organisms in activated sludge

Qiuyan Yuan*, Jan Oleszkiewicz

*E1-368, 15 Gilson St., Department of Engineering, University of Manitoba, Winnipeg, Manitoba R3T 5V6, Canada*
*Tel. +1 204 4749320; email: umyuan3@cc.umanitoba.ca*

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**ABSTRACT**

Activated sludge from a local non-biological nutrient removal wastewater treatment plant was inoculated into a sequencing batch reactor (SBR) to investigate the techniques and required conditions for the selection and enrichment of denitrifying phosphorus accumulating organisms (DNPAOs). The SBR was operated under varying anaerobic, anoxic and aerobic conditions for 3 mon. In this study, it was demonstrated that Enhanced biological phosphorus removal (EBPR) was established rapidly by the application of the anaerobic/aerobic (A/O) process and the selection of DNPAOs was achieved by introducing an anoxic phase. With an extension of the anoxic period and increasing nitrate dosage, the community shift between aerobic phosphorus accumulating organisms (PAOs) and DNPAOs was observed. An approximate 60% DNPAO fraction was obtained from this experiment.

*Keywords:* Biological phosphorus removal; Denitrifying phosphorus accumulating organisms; Anoxic phosphate uptake; Microbial selection; Microbial enrichment

## 1. Introduction

Eutrophication caused by nutrient pollution of nitrogen and phosphorus into water bodies is becoming a global issue. Due to the fact that phosphorus is a limiting nutrient in aquatic environments, it is considered to be the most critical factor in many situations. Increasing evidence shows that wastewater discharge is an important point source for both nitrogen and phosphorus causing eutrophication [3]. Many wastewater treatment plants all over the world are mandated to remove nutrients to meet discharge standards and there is a current trend toward more stringent discharge limitations. In the case of domestic wastewater, biological removal is the least expensive approach when nitrogen removal is studied, and often also in the case of phosphorus removal. The luxury phosphate uptake phenomenon by phosphorus accumulating organisms (PAOs) ultimately led to the development of the enhanced biological phosphorus removal (EBPR) [9].

In a conventional biological nutrient removal (BNR) wastewater treatment plant, nitrogen removal is achieved through nitrification and denitrification. Although organic carbon is not required for nitrifying bacteria to complete nitrification, sufficient organic carbon is necessary for denitrifying bacteria to carry out denitrification. To achieve phosphorus removal, it is crucial to provide PAOs with anaerobic conditions to promote their consumption of readily bio-degradable COD. Thus, there is a competition for organic carbon between PAOs and denitrifying bacteria. The availability of biodegradable organic carbon is often the key factor for the BNR process where simultaneous nitrogen and phosphorus removal is required. This is especially important for a municipal wastewater treatment plant where organic carbon content is low. Morling [15] points out that the EBPR process often deteriorates

*Corresponding author.

when the availability of organic carbon is at a low level. At the same time, denitrification also requires a significant amount of organic carbon, often resulting in the addition of an external carbon source [6]. To tackle the problem of competition for the limited organic substrate, denitrifying phosphorus accumulating organisms (DNPAOs) have received considerable attention [1,16,17,19].

DNPAOs have been distinguished from aerobic PAOs due to their unique metabolic characteristic [10]. The mechanism of anaerobic phosphate release of DNPAOs is the same as aerobic PAOs; specifically, external organic substrate is taken up and converted to polyhydroxyalkanoate as a cell energy source. However, DNPAOs are different from aerobic PAOs in the manner of phosphate uptake. Aerobic PAOs can only use oxygen as an electron acceptor for cell respiration which promotes phosphorus removal; whereas DNPAOs can use nitrite or nitrate as an electron acceptor instead of oxygen [14]. In other words, DNPAOs can combine phosphorus removal and de-nitrification into one process using the same amount of organic substrate. In addition, less aeration is needed which translates into lower energy requirement. Research of Kuba and his team shows also that DNPAOs can reduce sludge generation by 30%, due to their low cell yield [12]. The advantage of DNPAOs has driven the improvement of biological nutrient removal systems. Single sludge systems of BCFS (Biological-chemical phosphorus and nitrogen removal) as well as two sludge systems such as DEPHANOX (De-nitrification and Phosphate accumulation in Anoxic) were developed for extensive utilization of DNPAOs. Therefore, selection and enrichment of DNPAOs is the key factor for effective operation of those processes.

Although denitrifying phosphate uptake was reported by Gerber and Comeau [7,8] in 1987, DNPAOs did not receive sufficient attention till Kerrn-Jesperson and Henze [10] distinguished them from aerobic PAOs in 1993. Later research of DNPAOs focused more on metabolic characteristics and biochemical mechanisms [5,11,18]; however, little attention was paid to its selection and enrichment. This experiment aims to investigate the required conditions and the techniques for the selection and enrichment of DNPAOs in the EBPR system in order to improve its application.

## 2. Materials and methods

### 2.1. Reactor setup and operation

A sequencing batch reactor with a 3l working volume was operated under anaerobic/aerobic conditions (phase I) and anaerobic/anoxic/aerobic conditions (phase II) for about two and half months. The biomass was the activated sludge from a local domestic wastewater treatment plant, which operates an aeration process to remove COD. The SBR was operated with an 8 h cycle at the end of which 2l of effluent was decanted. A new cycle started with filling of the same volume of influent thereby maintaining a hydraulic retention time of 12 h. Solid retention time was operated at 10 d by removing 100 ml of mixed liquor each cycle. The pH was controlled between 7.0–7.5. The reactor was operated under room temperature which is controlled between 20–22 °C.

Due to the issue of transportation of municipal wastewater to the lab where experiment was conducted, synthetic wastewater was used in this experiment. The composition of synthetic wastewater is shown in Table 1. To minimize nitrate residue in the system, ammonium was controlled in the feed to diminish nitrification. Nitrogen is supplied (from beef extract and yeast extract) in organic form.

Anaerobic conditions were achieved by bubbling nitrogen gas into the bulk liquid. Aeration was accomplished by pumping air into the reactor, and dissolved oxygen was regularly measured to ensure DO levels above 2 mg/l. At the beginning of the anoxic phase nitrate was added to the reactor in the form of $KNO_3$.

Table 1
Synthetic wastewater composition

| Synthetic wastewater | | Mineral solution | |
|---|---|---|---|
| Ingredients | Concentration (mg/l) | Ingredients | Concentration (g/l) |
| COD (NaAc) | 170 | $FeCl_3 \cdot 6H_2O$ | 1.5 |
| COD (Beef extract) | 75 | $H_3BO_3$ | 0.15 |
| COD (Yeast extract) | 80 | $CuSO_4 \cdot 5H_2O$ | 0.03 |
| $MgSO_4 \cdot 7H_2O$ | 170 | KI | 0.03 |
| $CaCl_2 \cdot 2H_2O$ | 14 | $MnCl_2 \cdot 4H_2O$ | 0.12 |
| $P/PO_4$ ($K_2HPO_4$) | 10 | $Na_2MoO_4 \cdot 2H_2O$ | 0.06 |
| TN (organic) | 14–15 | $ZnSO_4 \cdot 7H_2O$ | 0.12 |
| | | $CoCl_2 \cdot 2H_2O$ | 0.15 |
| Mineral solution | 0.3 ml | EDTA | 10 |

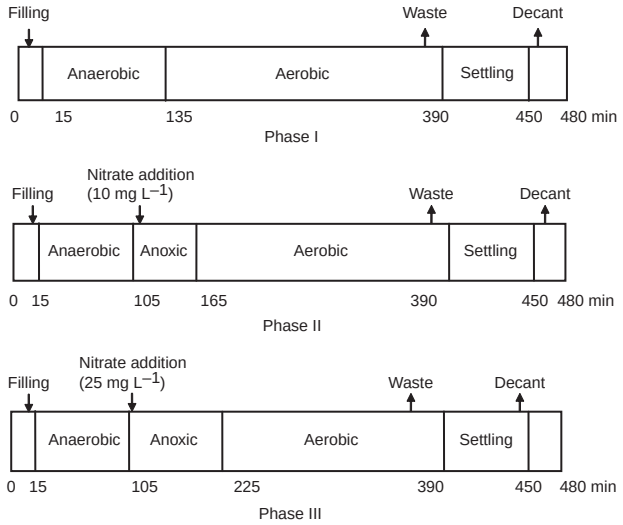


Fig. 1. SBR operational conditions.

### 2.2. Experiment operation conditions

SBR was operated at three different conditions, show as Fig. 1.

In order to study the PAOs anaerobic behaviour, short filling time of 15 min was used. This filling time was fairly short compare to the full scale application. However, our later study on the extension filling time up to 90 min which covered the entire anaerobic period showed that filling time (up to 90 min) had no significant effect on anaerobic phosphorus release.

### 2.3. Kinetic studies

Kinetic studies were carried out with 30 min sampling intervals for a total study time of 420 min. During these studies filling was performed manually to limit reactor filling time to less than 1 min.

### 2.4. Analytical methods

Carbon content of the substrates was measured as dissolved organic carbon (DOC). A Phoenix 8000 TOC Analyzer (Tekmar Kohrmann) was used for determination of DOC. Mixed liquor suspended solids (MLSS) and mixed liquor volatile suspended solids (MLVSS) measurements were performed according to Standard Methods [2]. Dissolved phosphate, nitrite and nitrate was measured by Lachat Instrument Quik Chem 8500, following Quik Chem method for orthophosphate 10-115-01-1-O and nitrate/nitrite 10-107-01-1-A respectively.

### 2.5. Microscopic analysis

Polyphosphate granule staining was processed following the Manual of Methods for General Bacteriology, and was observed under a phase contrast microscope (Leitz Wetzler, Germany) [4].

## 3. Results and discussion

### 3.1. Phase I: A/O configuration for the selection and acclimation of PAOs

Since the activated sludge came from a non-biological nutrient removal WWTP, the reactor was operated under A/O configuration for 30 d to establish biological phosphorus removal. The MLVSS concentration of the biomass started at 3.6 g/l and gradually decreased to 1.8 g/l where it remained stable. In the first 5 d, the effluent was milky and biomass was dark brown. The effluent quality started to improve from day 6 and by day 10 the MLVSS concentration in the effluent decreased to 80 mg/l and the sludge Volume Index (SVI) was 67 ml/g. This relatively high level of suspended solid in the effluent with low SVI may be due to the small reactor size, which is one of the limitation of the bench scale operation. Complex microorganism communities such as filamentous bacteria, stalked ciliates, and loose flocs, as well as some dispersed pin flocs were observed under a microscope. Neisser staining was performed and most bacteria were gram negative. Few polyphosphate granules were observed from the sludge sample taken after the aeration process. It was noticed that during the anaerobic condition, sludge gave off a strong malodour however, this did not occur in the following aerobic condition. This odour suggests fermentation which points to the strong growth of facultative or anaerobic organisms in the system during the anaerobic condition.

A shift in the microorganism community was observed through a microscopic examination of the sludge on day 28. The population of filamentous bacteria and protozoa was significantly decreased and Neisser staining showed that the majority of microorganisms in the population contained large polyphosphate granules. The presence of polyphosphate granules indicates that PAOs have become the dominant microorganisms in the system.

Fig. 2a demonstrates phosphate and nitrate concentration changes in the effluent during Phase 1. It can

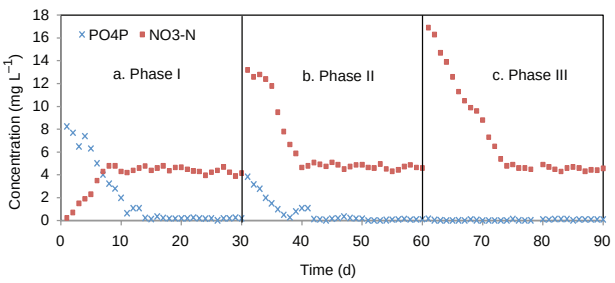


Fig. 2. Effluent nitrate and phosphate concentration.

be seen that phosphorus removal in the system was gradually achieved. About 0.2 mg/l P in the effluent was detected at day 14, and phosphorus removal efficiency stayed at 95–98% until the end of Phase 1. It is also observed that in the first 8 day, nitrate concentration in the effluent increased, but stayed constant thereafter.

About 4.5–5.0 mg/l of nitrate was detected in the effluent. This nitrate comes from organic nitrogen in the feed that is broken down and utilized by the microorganisms. Excess nitrogen in the form of ammonium is oxidized to nitrate by nitrifying bacteria. The initial very low concentration of nitrate in the effluent is probably due to the high biomass concentration (the experiment started with biomass concentration of 3.6 g/l) which resulted in high assimilation of nitrogen into the biomass. The limited organic substrate caused excess microorganisms to wash out of the system (biomass concentration decreased to 1.8 g/l). Small reactor size could be another possible reason which caused the biomass washed out. Thus, less nitrogen was assimilated by the biomass and nitrate concentration in the effluent stabilized as the biomass concentration stabilized.

Fig. 3 is the typical phosphate, nitrogen and carbon profile on day 28. It should be emphasized that it is critical to inhibit the growth of regular denitrifiers so as to eliminate the competition for carbon with DNPAOs. Therefore, the depletion of carbon during the anaerobic phase is an essential step for the subsequent selection of DNPAOs in Phase 2.

### *3.2. Phase 2: An/A/O configuration for the selection of DNPAOs*

Phase 2 lasted for 30 d (from day 31–60). At the end of Phase 1, maximum phosphate release from PAOs was reached at 90 min which is probably due to the depletion of the available carbon source (Fig. 3). Therefore, the anaerobic phase was shortened to 1.5 h in Phase 2. Anoxic conditions were achieved by 30 mg nitrate addition to obtain 10 mg/l nitrate of the bulk solution. The anoxic phase follows the anaerobic phase and lasts for 1 h.

Fig. 2b shows phosphate and nitrate concentrations in the effluent during Phase 2. It was noticed that phos phorus removal was deteriorated by the nitrate addition in the first 8 day. One reason for the deterioration might be that the aeration time was shortened due to the introduction of an anoxic phase. From Fig. 3, it can be seen that during the last half hour of aeration about 4.5 mg/l phosphate was taken up by the PAOs. Therefore, a decrease of aeration time results in insufficient time for aerobic PAOs to uptake phosphate. It can be seen however, that phosphate removal in the system gradually improved to 99% removal efficiency and stabilized at day 42 indicating that DNPAOs, which can grow under both anoxic and aerobic condition, were selected and remained in the system.

Nitrate removal was stabilized at day 41 with significant improvement from day 35 to day 40. The low nitrate removal during the anoxic phase in the first 5 day might indicate that the population of DNPAOs in the system was quite small. However, the population started to rise from day 35 and this was indicated by the increasing nitrate removal.

Although nitrate removal steadily improved and stabilized, it can be seen that on day 39, 40 and 41, about 0.8–1.1 mg/l phosphate was detected in the effluent. This is probably due to the low dissolved oxygen caused by a blocked air diffuser, which affects the aerobic phosphate uptake by PAOs. The air diffuser was replaced on day 42 and successful phosphate removal was obtained again.

The profile of nitrogen, phosphate and carbon on day 56 is shown in Fig. 4. Compared to day 28 of Phase 1 (Fig. 3, Table 2), there was no significant change in the amount of anaerobic phosphate release as well as carbon uptake. However, the phosphate uptake rate was increased considerably. During the 1h anoxic period, DNPAOs removed 10 mg/l nitrate by taking up 13.3 mg/l phosphate. This reveals that 0.75 mg phosphate was removed by DNPAOs using 1 mg $NO_3$ as an electron

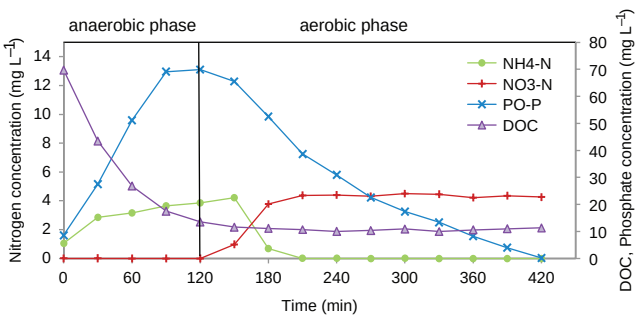


Fig. 3. Profile of Nitrogen, phosphate and DOC on day 28 (Phase 1).

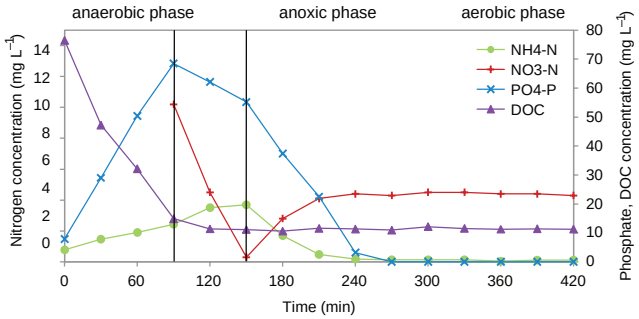


Fig. 4. Profile of Nitrogen, phosphate and DOC on day 56 (Phase 2).

Table 2
Summary of reactor performance

| | C/N/P | $PRR_{anaerobic}$ (mg/l h) | $PUR_{anoxic}$ (mg/l h) | $PUR_{aerobic}$ (mg/l h) | $PUR_{anoxic}$: $PUR_{aerobic}$ |
|---|---|---|---|---|---|
| Phase 1 | 30:1.5:1 | 40.3 | – | 9 | – |
| Phase 2 | 30:3:1 | 40.4 | 13.8 | 34.7 | 0.39 |
| Phase 3 | 30:5:1 | 51.9 | 17.4 | 24 | 0.72 |

acceptor. The aerobic phosphate uptake was also increased during Phase 2. At day 56, aerobic phosphate uptake rate (PUR) was 34.7mg/l h; whereas, on day 28, aerobic PUR was only 9 mg/l. This suggests that PAOs in the system were not fully acclimated in Phase I. An acclimation period longer than three times the SRT may be needed. (The PUR is determined based on the initial linear part of the phosphate decrease curve.)

### *3.3. Phase 3: Enrichment of DNPAOs by increasing nitrate addition and extending anoxic phase duration*

In order to increase the population of DNPAOs in the system, nitrate addition was increased to 75 mg (25 mg/l in the bulk solution) and anoxic time increased from 60 min to 120 min in Phase 3. Phase 3 continued for 30 d (from day 61–90).

As can be seen from Fig. 2c, phosphate removal efficiency remained at 95–99% and was not affected by the increased nitrate addition. Nitrate concentration in the effluent gradually decreased and stabilized at day 74. Compared to Phase 2 (Fig. 2b), the nitrate removal trend in Phase 3 is smooth and steady suggesting that the population of DNPAOs in the system is gradually increasing. It was observed that the color of biomass changed slightly from light to dark. The colour change might be caused by the extension of the anoxic phase.

Fig. 5 shows the profile of carbon, nitrogen and phosphate on day 75. As can be seen, about 87% of dissolved carbon consumption occurred in the first 60 min of the anaerobic phase. Compared to day 56 in Phase 2 the DOC consumption in Phase 3 is about 20% greater in the same amount of time. One reason might be the proliferation of fermenting bacteria. Fermenting bacteria in the system rapidly break down the complex carbon (in beef and yeast extract) to simple carbon which can be utilized by PAOs. During the 120 min anoxic phase, the added 25.2 mg/l nitrate was completely consumed by DNPAOs to uptake 34.8 mg/l phosphate. This consumption translates into 0.72 mg $NO_3^-$–N/ mg $PO_4^{3-}$–P removal. This result was very close to the result from Phase 2. The anoxic and aerobic phosphate uptake rates on day 75 were calculated to be 17.4 mg/l $h^{-1}$ and 24 mg/l $h^{-1}$ respectively (Table 2). While on day 56 they were only 13.8 and 34.7 mg/l $h^{-1}$. Thus, the $PUR_{anoxic}$:

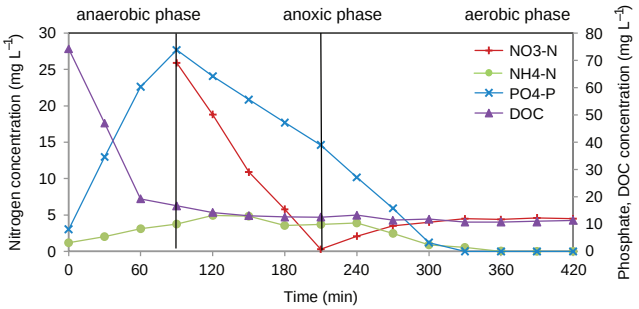


Fig. 5. Profile of Nitrogen, Phosphate and DOC on d 78 (Phase 3).

$PUR_{aerobic}$ on day 75 was 0.72 which was higher than 0.39 from day 56. This suggests that the competition between the populations of PAOs and DNPAOs resulted in the shift of microbial community to DNPAOs in the system.

### *3.4. Batch test to evaluate the DNPAOs fraction of PAOs*

Wachtmeister [20] proposed a method to estimate the DNPAO fraction of total PAOs based on the phosphate uptake rate under different conditions. The equation Wachemeister proposed is shown below:

$$\frac{XDNPAO}{XPAO} = \frac{Panoxic}{Paerobic} \times \eta G$$

where $\eta G$ is a reduction factor for the lower energy gain in the anoxic condition compared to the aerobic energy gain, a typical value of 0.8 was used in this experiment.

A batch test of anoxic and aerobic phosphate uptake rate was conducted at the end of Phase 3 to evaluate the DNPAO fraction. The system was operated under the same anaerobic/anoxic/aerobic conditions as previous batch tests. Right after the anaerobic phase, the mixed liquor was divided evenly into two parts. One part was spiked with an adequate amount of nitrate to ensure nitrate was not the limiting factor. The other part was aerated, and the DO level was maintained above 2 mg/l. Fig. 6 illustrates that the total PAOs phosphate uptake under anoxic and aerobic condition was 17.7 mg/l h and 36.7 mg/l h respectively. Thus, the

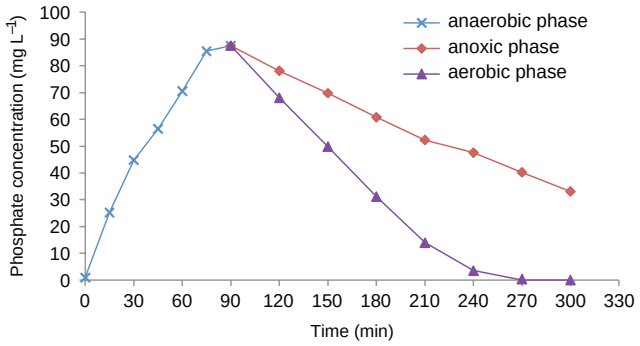


Fig. 6. Aerobic and anoxic phosphate uptake rate.

DNPAO fraction is calculated to be 60% according to Wachemeister's method.

It should be noted that the aerobic PUR of this batch test was very comparable to the aerobic PUR from day 56, where the population of DNPAOs were quite low. This suggests that the DNPAOs can perform in a similar way as aerobic PAOs when they are exposed to aerobic conditions.

It was also observed that during Phase 2 and Phase 3, no nitrite accumulation occurred in the anoxic phase. Although small amounts of nitrite (1–1.5 mg/l) were detected during the initial 30 minutes of the anoxic phase, they disappeared well before the end of the phase. This implies that some DNPAOs can use both nitrate and nitrite as an electron acceptor; low concentration of nitrite would not inhibit the DNPAO performance.

## 4. Conclusion

This experiment demonstrates the possibility to achieve EBPR in a system by acclimation of the biomass from a non-EBPR system. Using A/O configuration is an effective approach to obtain EBPR.

The coexistence of organic carbon and nitrate is unfavourable for the selection of DNPAOs. Therefore, in this experiment, limited carbon was supplied to ensure that it was fully utilized during the anaerobic phase before nitrate addition. The selection of DNPAOs was successfully achieved by using an anaerobic/anoxic/aerobic process.

A proper anoxic condition is the key factor for the enrichment of the DNPAO population. By extending the anoxic time and increasing the nitrate dose, anoxic phosphate uptake rate was increased. A 60% DNPAO fraction of total PAOs was obtained in the experiment.

The modification of the microbial community results in the change of anoxic and aerobic phosphate uptake rate, which also confirms the presence of two types of PAOs, namely aerobic PAOs and denitrifying PAOs.

No nitrite build up in the anoxic phase suggests that some DNPAOs can use both nitrate and nitrite as an electron acceptor and therefore a low dose of nitrite would not deteriorated the EBPR process.

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