Anaerobic/oxic/anoxic granular sludge process as an effective nutrient removal process utilizing denitrifying polyphosphate-accumulating organisms

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\textbf{ABSTRACT}

In a biological nutrient removal (BNR) process, the utilization of denitrifying polyphosphate-accumulating organisms (DNPAOs) has many advantages such as effective use of organic carbon substrates and low sludge production. As a suitable process for the utilization of DNPAOs in BNR, an anaerobic/oxic/anoxic granular sludge (AOAGS) process was proposed in this study. In spite of performing aeration for nitrifying bacteria, the AOAGS process can create anaerobic/anoxic conditions suitable for the cultivation of DNPAOs because anoxic zones exist inside the granular sludge in the oxic phase. Thus, DNPAOs can coexist with nitrifying bacteria in a single reactor. In addition, the usability of DNPAOs in the reactor can be improved by adding the anoxic phase after the oxic phase. These characteristics enable the AOAGS process to attain effective removal of both nitrogen and phosphorus. When acetate-based synthetic wastewater (COD: 600 mg/L, NH\textsubscript{4}-N: 60 mg/L, PO\textsubscript{4}-P: 10 mg/L) was supplied to a laboratory-scale sequencing batch reactor under the operation of anaerobic/oxic/anoxic cycles, granular sludge with a diameter of 500 \textmu m was successfully formed within 1 month. Although the removal of both nitrogen and phosphorus was almost complete at the end of the oxic phase, a short anoxic period subsequent to the oxic phase was necessary for further removal of nitrogen and phosphorus. As a result, effluent concentrations of NH\textsubscript{4}-N, NO\textsubscript{x}-N and PO\textsubscript{4}-P were always lower than 1 mg/L. It was found that penetration depth of oxygen inside the granular sludge was approximately 100 \textmu m by microsensor measurements. In addition, from the micro-biological analysis by fluorescence in situ hybridization, existence depth of polyphosphate-accumulating organisms was further than the maximum oxygen penetration depth. The water quality data, oxygen profiles and microbial community structure demonstrated that DNPAOs inside the granular sludge may be responsible for denitrification in the oxic phase, which enables effective nutrient removal in the AOAGS process.

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1. Introduction

In conventional biological nutrient removal (BNR) processes, nitrogen removal is achieved by aerobic nitrification and anoxic denitrification using nitrifying and denitrifying bacteria, respectively, while phosphorus removal is accomplished under anaerobic–aerobic conditions using polyphosphate-accumulating organisms (PAOs) (Zeng et al., 2004). Thus,
three organisms are necessary for simultaneous nitrogen and phosphorus removal. However, PAOs compete with denitrifying bacteria for organic carbon in the influent because both denitrification and phosphate release require organic carbon. Therefore, the phosphorus removal efficiency often decreases when the available organic carbon content is low (Morling, 2001). To solve this problem, denitrifying polyphosphate-accumulating organisms (DNPAOs) have recently received much attention.

The use of DNPAOs can relieve the competition for organic carbon because they can treat nitrate/nitrite and phosphate using the same carbon sources (Mino et al., 1998; Shoji et al., 2003). In the same manner as PAOs, DNPAOs take up external carbon substrates and store them as polyhydroxyalkanoates in the cell under anaerobic conditions. However, they can utilize nitrate or nitrite instead of oxygen as an electron acceptor to remove phosphorus under anoxic conditions (Ahn et al., 2002). Moreover, DNPAOs are 40% less efficient in generating energy and thus have a 20–30% lower cell yield (Mumeleitner et al., 1997). Consequently, the utilization of DNPAOs affords many advantages in BNR. Although external nitrification processes such as DEPHANOX and A2N have already been developed for the effective use of DNPAOs (Kuba et al., 1996; Bortone et al., 1999; Shoji et al., 2003), they are very complicated processes that require many reactors and mixed liquor recycling streams. Therefore, it is necessary to develop a simple BNR process that can utilize DNPAOs. To simplify the process, the use of a sequencing batch reactor (SBR) is one of the effective methods because an SBR makes it possible to remove nutrient in a single reactor without mixed liquor recycling streams. Moreover, it has been verified, in full-scale studies, that this BNR process of using an SBR is cost-effective as compared with continuous flow processes (Peters et al., 2004). Although an anaerobic/oxic (with low dissolved oxygen (DO)) process and anaerobic/oxic/anoxic process have been proposed for the utilization of DNPAOs in SBRs (Tsuneda et al., 2006; Zeng et al., 2004), these processes have some disadvantages. In the former, denitrification is mainly responsible for denitrifying glycogen-accumulating organisms (DNGAOs), competitors of PAOs. In the latter, a large amount of external carbon must be added at the beginning of the oxic phase to prevent aerobic uptake of phosphate by PAOs. The common problem in these processes is that DNPAOs are exposed to oxygen. Although necessary for nitrification, aeration creates hostile conditions for DNPAOs. To solve this problem and enable the use of DNPAOs, we propose an anaerobic/oxic/anoxic granular sludge (AOAGS) process in this study.

It has recently been reported that granular sludge can be formed using an SBR without any carrier material (Beun et al., 2002; McSwain et al., 2004). Under an aeration condition, there are not only oxic zones but also anoxic zones in the granular sludge because the oxygen penetration depth inside the granular sludge is limited. Therefore, if the reactor is operated under alternate anaerobic/oxic conditions, it is expected that anaerobic/anoxic conditions suitable for the cultivation of DNPAOs (Ng et al., 2001) can be created inside the granular sludge. Reportedly, PAOs existed in the granular sludge, and simultaneous nitrification, denitrification and phosphorus uptake were observed under the aeration condition in the anaerobic/oxic granular sludge process without control of sludge retention time (SRT) (de Kreuk and van Loosdrecht, 2004; de Kreuk et al., 2005). In the AOAGS process, the usability of DNPAOs would be improved by adding the anoxic phase subsequent to the oxic phase because their effective action under aeration conditions is limited to the depth of the granular sludge. In addition, the SRT is exactly controlled in the AOAGS process by discharge of excess sludge to stabilize the nutrient removal.

In this study, the nutrient removal performance of the AOAGS process was investigated. Additionally, the inside of the granular sludge was inspected by molecular and microsensor techniques. A microsensor was used for the measurement of the microscale distribution of DO inside the granular sludge. Fluorescence in situ hybridization (FISH) was also performed for microbiological analysis of the granular sludge.

2. Materials and methods

2.1. Reactor setup and operation

Granular sludge was cultivated in a laboratory-scale SBR with an effective volume of 9 L. The hydraulic retention time was set at 18 h. Three liters of the influent wastewater was fed into the reactor every cycle. Stirring speed was set relatively low (300 rpm) to avoid breaking the granules. Air was introduced by a fine-bubble aerator at the bottom of the reactor. The airflow rate was set at 3.0 L/min. The water temperature of the reactor was maintained at 20±2 °C. DO and pH were continuously monitored by the sensors inserted in the reactor.

The reactor was operated on 6-h cycles, consisting of 20-min influent feeding (no mixing, no aeration), 90-min anaerobic phase (mixing), 120-min oxic phase (mixing, aeration), 120-min anoxic phase (mixing), 0.5-min sludge settling and 9.5-min effluent discharge periods. The sludge settling time was chosen such that only particles with a settling velocity higher than 8.4 m/h were retained in the reactor.

Synthetic wastewater with the following composition was used in this study: 769 mg of sodium acetate (600 mg/L as COD basis), 43.9 mg of KH2PO4 (10 mg/L as PO4-P basis), 229.3 mg of NH4Cl (60 mg/L as NH4-N basis), 90 mg of MgSO4·7H2O, 14 mg of CaCl2·2H2O and 0.3 mL of trace solution per liter. The trace solution consisted of the following compounds per liter: 1.5 g of FeCl3·6H2O, 0.15 g of H3BO3, 0.15 g of CuCl2·5H2O, 0.18 g of CuSO4·5H2O, 0.06 g of Na2MoO4·2H2O, 0.12 g of MnCl2·2H2O, 0.15 g of CoCl2·6H2O and 10 g of EDTA (Smolders et al., 1994b).

Seed sludge was obtained from an urban nutrient removing wastewater treatment plant. There would be nutrient removal organisms such as DNPAOs and nitrifying bacteria in the seed sludge because this plant was operated with an A2O process configuration. Initial concentration of total suspended solids (TSS) in the reactor was approximately 3350 mg/L. In the start-up period (day 0–day 21), discharge of excess sludge from the reactor was not performed because the concentration of TSS in the effluent solution was extremely high. Sludge discharge was started from day 22
NO3-N and NO2-N were analyzed with an ion chromatograph determined with dextran blue (Beun et al., 2002). Sludge taken from the reactor. The biomass density was measured by timing the settling time of individual granular sludge (Japan). The settling velocity of the granular sludge was obtained with an attached digital camera (HC-300Zi; FUJIFILM, Japan), and images were obtained with a total organic carbon (DOC) was detected with a total organic carbon analyzer (TOC-5000; Shimadzu, Japan). The development of granular sludge was observed using an inverted microscope (ECLIPSE TE300; NIKON, Japan), and images were obtained with an attached digital camera (HC-300Zi; FUJIFILM, Japan). The settling velocity of the granular sludge was measured by timing the settling time of individual granular sludge taken from the reactor. The biomass density was determined with dextran blue (Beun et al., 2002).

2.2. Analytical methods

TSS and sludge volume index (SVI) were analyzed in accordance with standard methods (APHA, 1995). NH4-N, NO3-N and NO2-N were analyzed with an ion chromatograph (IC 7000; Yokogawa Analytical Systems, Japan). Dissolved organic carbon (DOC) was detected with a total organic carbon analyzer (TOC-5000; Shimadzu, Japan). Organic carbon (DOC) was measured using a total organic carbon analyzer (TOC-5000; Shimadzu, Japan). The settling velocity of the granular sludge was obtained with an attached digital camera (HC-300Zi; FUJIFILM, Japan). The settling velocity of the granular sludge was measured by timing the settling time of individual granular sludge taken from the reactor. The biomass density was determined with dextran blue (Beun et al., 2002).

2.3. Microsensor measurements

An oxygen microsensor (OX25; Unisense, Denmark) connected to a picoameter (PA2000; Unisense, Denmark) was used to measure oxygen concentration profiles in the granule. DO profiles were measured by moving the microsensor stepwise into a granule fixed by needles in a flow cell (Fig. 1), using a micromanipulator (MEO-223; Narishige, Japan). Granular samples were taken from the reactor at the end of the anaerobic phase. The flow cell was filled with the liquid obtained from the reactor at the end of the anaerobic phase. Mixed liquor was filtered through a glass filter (GF/C; Whatman, UK) before pouring it into the flow cell. This measurement was performed three times to confirm reproducibility of the results.

2.4. FISH analysis

Sample preparation for FISH analysis was performed according to the reported protocol (Aoi et al., 2000). Granule samples were immediately fixed in freshly prepared paraformaldehyde solution (4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.2) at 4 °C for 18 h and subsequently washed in PBS. A 20-μm-thick granule section was prepared from a frozen granule sample embedded in OCT compound (Miles; Elkhart, USA) using a cryostat (CM 3050; Leica, Germany) at −20 °C. Each slice was placed in hybridization wells on a gelatin-coated microscopic slide and immobilized by air drying and dehydrating in a graded series of 50%, 80% and 98% ethanol.

Two 16S rRNA-targeted oligonucleotide probes were used for in situ detection of PAOs and glycogen-accumulating organisms (GAOs): 6-FAM-labeled PAOMIX probes (PAO462, PAO651 and PAO846) specific for Candidatus Accumulibacter phosphatis (Crocetti et al., 2000) and Cy3-labeled GAOQMIX probes (GAOQ431 and GAOQ989) specific for Candidatus competibacter phosphatis (Crocetti et al., 2002). In this study, both probes were used at a formamide concentration of 35%.

Hybridization of the granule sections on the slide was performed according to the standard hybridization protocol (Amann, 1995). Subsequently, the slides were examined by fluorescence microscopy (Axio skop2 plus; Carl Zeiss, Germany) to visualize the microbial population. Image combining, processing and analysis were performed using Jasc Paint Shop Pro Version 6J (Jasc Software Inc.).

2.5. Calculation procedure

The penetration depth of oxygen inside the granular sludge was calculated according to the following equation (Beun et al., 2002):

$$\delta = \sqrt{\frac{2C_l D}{\bar{\gamma}_m C_{x,lim}}}$$

in which $\delta$ is the penetration depth (m), $C_l$ is the concentration of oxygen in the liquid (mol/m³), $D$ is the diffusion coefficient ($= 7.2 \times 10^{-6} m^2$/h), $\bar{\gamma}_m$ is the maximal conversion rate of oxygen (mol/(Cmol h)) and $C_{x,lim}$ is the biomass concentration in the biofilm ($= biomass$ density/molecular weight biomass (Cmol/m³)); $\bar{\gamma}_m$ was determined using the sludge in the effluent.

3. Results and discussion

3.1. Formation of granular sludge

Fig. 2 shows variations of TSS in the reactor and in the effluent solution during the start-up period. For a first few days, TSS in the effluent solution was extremely high because of very short sludge settling time. Therefore, TSS in the reactor decreased despite the lack of discharge of excess sludge. However, TSS in the effluent solution gradually decreased, and TSS in the reactor increased after 1 week. This is because the settleability of sludge markedly increased due to the evolution of granular sludge in the reactor, as shown in Figs. 3 and 4. SVI decreased with increasing average diameters of the granular sludge. After day 43, SVI after 5 min and SVI after 30 min were almost the same because of well-settling characteristics. On day 61, there were many rigid granules with diameters of approximately 1 mm. The settling velocity and density of the granular sludge were 24 m/h and
85 g/L granules, respectively. Reportedly, short settling time in the SBR cycle favors the fast-settling granules (McSwain et al., 2004). Because sludge settling time was very short (0.5 min), granular sludge was successfully formed in this study.

3.2. Nutrient removal performance

Fig. 5 shows track analysis data on day 61. In the anaerobic phase, DOC consumption and phosphate release occurred in the same manner as most conventional BNR processes. In the oxic phase, ammonia and phosphate decreased due to the nitrification and polyphosphate uptake by nitrifying bacteria and PAOs, respectively. The stoichiometric formula of nitrification was defined as (US Environmental Protection Agency, 1975)

\[
55\text{NH}_4^+ + 76\text{O}_2 + 109\text{HCO}_3^- \rightarrow \text{C}_6\text{H}_7\text{NO}_2^- + 54\text{NO}_2^- + 57\text{H}_2\text{O} + 104\text{H}_2\text{CO}_3^-, \tag{2}
\]

\[
400\text{NO}_3^- + \text{NH}_4^+ + 4\text{H}_2\text{CO}_3 + \text{HCO}_3^- + 195\text{O}_2 \rightarrow \text{C}_6\text{H}_7\text{NO}_2^- + 3\text{H}_2\text{O} + 400\text{NO}_3^- \tag{3}
\]

However, the amount of increase of NO\textsubscript{3}-N inside the reactor (17.1 mg) was much less than the amount of decrease of NH\textsubscript{4}-N (151.2 mg). According to the Eqs. (2) and (3), the amount of increase of NO\textsubscript{3}-N should be 148.1 mg. Therefore, it was considered that 131.0 mg of NO\textsubscript{3}-N was removed by denitrification in spite of aeration. Due to this specific phenomenon, nitrogen and phosphorus removal were almost complete at the end of the oxic phase. Therefore, the length of the anoxic phase can be considerably reduced.

Although denitrification generally occurs under the anoxic condition, the DO concentration in the bulk solution during the oxic phase was more than 2.5 mg/L except in the first 10 min, as shown in Fig. 5. Therefore, anoxic zones might exist inside the granules. Moreover, as we expected, DNPAOs might act inside the granular sludge and carry out denitrification using internal carbon sources such as polyhydroxybutyrate. According to the reported ratio of phosphate uptake to NO\textsubscript{3}-N consumption by DNPAOs (2.10 g P/g NO\textsubscript{3}-N) (Kuba et al.,...
DNGAOs were also involved in the denitrification during the anoxic phase. Because 558.9 mg of PO₄-P was removed inside the reactor, approximately 50% of PO₄-P was removed by DNPAOs while the other 50% of PO₄-P was removed by PAOs if DNPAOs were fully responsible for the denitrification. However, it has been reported that DNGAOs also have the denitrification ability using internal carbon sources (Zeng et al., 2003a). Therefore, it was suggested that only DNPAOs inside the granules were not responsible for denitrification. We shall return to this inference later.

Phosphorus release and uptake rates of this study were 0.72 and 0.12 mmol P/g VSS/h, respectively. Compared with the activated sludge process, these rates of this study were low. The previous study has reported that phosphorus release and uptake rates of the activated sludge process were 1.55 and 0.56 mmol P/g VSS/h, respectively (Zeng et al., 2003c). This is because diffusion resistance of substrate and nutrients exists in the granular sludge. It was well known that these resistances exist in the biofilm and affect nutrient removal (Ettouney et al., 1996). In addition, in the oxic phase, all of the PAOs would not act due to lack of oxygen inside the granular sludge. Although DNPAOs might act in anoxic zones inside the granular sludge, the anoxic phosphorus uptake rate is generally lower than the oxic uptake rate (Meinhold et al., 1999). This is why the phosphorus uptake rate of AOAGS process was particularly low.

Meanwhile, pH of the bulk solution was relatively high (7.5–8.1) as shown in Fig. 5. Therefore, precipitation of phosphorus may occur in this process. Because phosphate concentration at the end of the anaerobic phase was very high (approximately 65 mg P/L), the precipitation would occur at this period. The previous study has reported that the precipitation can occur under this condition (water temperature: 20 °C; PO₄-P: 65 mg/L; pH: 7.6) (Maurer et al., 1999). Therefore, it was considered that phosphorus was partly removed by this chemical reaction.

Average effluent concentrations of NH₄-N, NO₃-N and PO₄-P during 70 days after the start of sludge discharge were <0.1, <0.1 and 0.3 mg/L, respectively. Although phosphorus removal was not stable compared with nitrogen removal, concentrations of NH₄-N, NO₃-N and PO₄-P were always lower than 1 mg/L. Thus, good nitrogen and phosphorus removal was attained using granular sludge in this study.

3.3. Necessity and control of the anoxic phase

As mentioned above, nitrogen and phosphorus removal were almost complete because nitrification, denitrification and phosphate uptake occurred simultaneously in the oxic phase. However, small amounts of NO₃-N and PO₄-P (approximately 2 mg/L) remained at the end of the oxic phase, as shown in Fig. 5. After approximately 30 min from the start of the anoxic phase, denitrification and phosphate uptake were complete. The amount of decrease of NO₃-N (1.9 mg/L) during the anoxic phase was greater than calculated value (1.0 mg/L) based on the reported ratio of phosphate uptake to NO₃-N consumption by DNPAOs (2.10 g P/g NO₃-N) (Kuba et al., 1993). Therefore, it was considered that other microorganisms such as DNGAOs were also involved in the denitrification during the anoxic phase. Thus, a short anoxic period enhanced nitrogen and phosphorus removal in this process. However, the anoxic phase would not always be necessary in this experimental condition because nitrogen and phosphorus remaining at the end of the oxic phase were sufficiently small as shown in Fig. 5. In addition, it was considered that extension of the oxic phase length instead of adding the anoxic phase would also enhance nitrogen and phosphorus removal because the denitrification still continued at the end of the oxic phase as shown in Fig. 5.

Meanwhile, slight phosphate release was observed after 30 min, as shown in Fig. 5. This is because the anaerobic condition was created in the reactor due to the completion of denitrification. Therefore, the length of the anoxic phase must be exactly controlled in this process. To solve this problem, we propose to use pH profiles. Several investigators have identified the “nitrate apex point” in pH profiles, which indicates the end of denitrification by general heterotrophic bacteria (Al-Ghusain and Hao, 1995; Kishida et al., 2003). Although DNPAOs and DNGAOs are considered to be responsible for denitrification in this study, the “nitrate apex point” also appeared. After the completion of denitrification, pH profiles rapidly dropped, as shown in Fig. 5. Therefore, it is possible to use pH profiles in the control of the anoxic phase.

Meanwhile, it took approximately 30 min to complete the DO consumption as shown in Fig. 5. If DO remained in the reactor at the end of the cycle, aerobic heterotrophic bacteria can consume the DOC and grow at the beginning of the next cycle. This is unfavorable for the nitrogen and phosphorus removal, and the cultivation of PAOs. Therefore, the anoxic phase would also contribute to the prevention of the growth of aerobic heterotrophic bacteria.

3.4. Nutrient removal mechanism in the oxic phase

The water quality data in terms of nutrient removal indicated that DNPAOs and DNGAOs inside the granular sludge were responsible for denitrification, as mentioned above. As the next step, the denitrification mechanism in the oxic phase
was elucidated by investigating the microenvironment inside the granular sludge.

Fig. 6 shows oxygen concentration profiles inside the granule measured using the microsensor. The penetration depth of oxygen was approximately 100 μm when the DO concentration in bulk water was 5.5 mg/L. Since the DO concentration in the oxic phase varied from 0 to 5.5 mg/L, as shown in Fig. 5, it was considered that the penetration depth was, at most, 100 μm. On day 61, there were many granules with diameters of approximately 1 mm, as mentioned above. Therefore, there were sufficient anoxic zones for denitrification inside the granules.

Meanwhile, if only nitrification occurred in the reactor during the oxic phase, the calculated penetration depth of oxygen becomes approximately 138 μm when the DO concentration of the bulk water was 5.5 mg/L. This value is a little further than the value (100 μm) measured by the microsensor. Therefore, it was considered that other microorganisms such as PAOs partly consumed the oxygen during the oxic phase.

From the microbiological analysis by FISH, it was found that there were PAOs and GAOs in the granules (Fig. 7). GAOs mainly existed near the granule surface, whereas PAOs existed not only near the granule surface but also in the inner part of the granule. The existence of PAOs in this study was similar to results of a previous study. Reportedly, PAOs existed not only in the outer layers of granular sludge but also in the inner layers in the anaerobic/oxic granular sludge process (de Kreuk et al., 2005). Moreover, the existence depth of PAOs was further than the maximum oxygen penetration depth (100 μm), as shown in Fig. 7. This trend was also observed in other granules (data not shown). In addition, the results of our previous study suggested that some PAOs (Candidatus Accumulibacter phosphatis) have denitrification ability (Tsuneda et al., 2005). Therefore, it was considered that the denitrification in the oxic phase may be due to the activity of DNPAOs. However, there may be many other microorganisms with the ability of denitrification which were not identified by the FISH analysis. Hence, these unidentified...
organisms living in proximity to the anoxic layer would be possibly responsible for the denitrification.

Meanwhile, the question as to why the habitats of PAOs and GAOs were different remains unsettled. Although the anaerobic/oxic condition was created near the surface of the granule, the anaerobic/anoxic condition was created in the inner part of the granule due to the limited diffusion of oxygen. Therefore, it was inferred that GAOs prefer the anaerobic/oxic condition to the anaerobic/anoxic condition as compared with PAOs. It was reported that polyhydroxylate (PHV) production under the anaerobic/oxic condition was greater than under the anaerobic/anoxic condition (Zeng et al., 2003b). Because PHV production is mainly due to GAOs (Mino et al., 1998), it is reasonable to conclude that GAOs prefer the anaerobic/oxic condition to the anaerobic/anoxic condition. For this reason, it was considered that GAOs existed near the granule surface as compared with PAOs. Thus, DNPAOs could effectively remove nitrogen due to the formation of granular sludge in this process.

4. Conclusions

In this study, the effectiveness of the AOAGS process for nitrogen and phosphorus removal was investigated. The following are the main outcomes.

(1) Granular sludge could be easily formed using a laboratory-scale SBR. Rigid granules with diameters of approximately 1 mm were obtained after 60 days.

(2) In the oxic phase, simultaneous nitrification, denitrification and phosphate uptake were observed when the granular sludge was formed. In the anoxic phase, slight denitrification and phosphate uptake were observed.

(3) In the proposed AOAGS process, effective nitrogen and phosphorus removal was achieved. Effluent concentrations of NH$_4$-N, NO$_2$-N and PO$_4$-P were always lower than 1 mg/L.

(4) Results of microsensor measurements and FISH analysis confirmed that some PAOs existed further than oxygen penetration depth inside the granular sludge. These PAOs may be responsible for denitrification.

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