Quinone Profiles Reflecting Population Dynamics of Denitrifying Phosphate-Accumulating Organisms

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Selective enrichment of phosphate-accumulating organisms (PAOs) and denitrifying phosphate-accumulating organisms (DNPAOs) was conducted using a sequencing batch reactor (SBR). To elucidate biomarkers for DNPAOs, quinone profiles were monitored during selective enrichment. As a result, a high correlation between the mole fraction of ubiquinone with eight isoprene units (Q-8) and anoxic phosphate uptake ability was observed, indicating that Q-8 was one of the biomarkers for DNPAOs. In addition, the mole ratio of ubiquinones to menaquinoines (Q/MK) increased throughout this selective enrichment.

Key words: enhanced biological phosphorus removal (EBPR), phosphate-accumulating organisms (PAOs), denitrifying phosphate-accumulating organisms (DNPAOs), quinone profile

Phosphorus and nitrogen removal from wastewater are necessary for solving eutrophication of closed water areas. An anaerobic/anoxic/oxic process (A2O) as an enhanced biological phosphorus removal (EBPR) process combined with the biological nitrogen removal process has been developed for municipal wastewater treatment. However, it is difficult to control strictly the EBPR process because phosphate-accumulating organisms (PAOs) that can work in the EBPR process have not been isolated and thus the biochemistry and genetics of PAOs have not been elucidated(1,4). Also, the EBPR process combined with the biological nitrogen removal process has a disadvantage in that the organic substrate is a limiting factor for phosphate release and denitrification in the wastewater with a low C/N ratio. Recently the occurrence of denitrifying phosphate-accumulating organisms (DNPAOs) capable of utilizing nitrate instead of oxygen as an electron acceptor for phosphate uptake was reported(1,10,13). If DNPAOs are introduced into the biological nutrient removal (BNR) process, the problems associated with organic substrate limitation can be solved. Moreover, since the energy production efficiency by use of nitrate as an electron acceptor is lower than that by use of O2, the introduction of DNPAOs makes it possible to reduce excess sludge production. However, there has not been enough information with regard to microbial ecology and the metabolic behavior of DNPAOs as well as PAOs.

Methods based on 16S rDNA and various biomarkers have been applied to characterize microbial ecology in environmental samples. Using these molecular techniques, quinone profiles have an advantage in terms of quantification, simplicity, and reproducibility(7). For example, the detection of the class Actinobacteria is easy using quinone profiles whereas the polymerase chain reaction (PCR) has unexpected bias(15). Also, Liu et al.(12) pointed out that the quinone profiles differ from the result of PCR. Microbial quinones are components of the bacterial respiratory chain and exist in almost all bacteria. In general, one species of bacteria has only one dominant type of quinone, which means that the quinone profiles should be specific for microbial ecology(7). Therefore, in this study, to investigate biomarkers for DNPAOs, quinone profiles were monitored during selective enrichment.

Materials and Methods

Sequencing batch reactor (SBR) operation

Selective enrichment of PAOs and DNPAOs was con-
ducted using a SBR with a 2 l working volume. The SBR was operated at a cycle of 8 h, consisting of a 20 min filling phase, a 90 min anaerobic phase, a 285 min aerobic phase, a 60 min settling phase and a 25 min withdrawing phase. Both influent and effluent volumes were 1 l and thus 16 h of hydraulic retention time (HRT) was maintained. At the end of the aerobic phase, 33 ml of excess sludge (mixed liquor suspended solid, MLSS: 2000–4000 mg/l) was withdrawn and a 20 day sludge retention time (SRT) was maintained. The pH was controlled between 6.9 and 7.1. Also, the activated sludge obtained from an aerobic basin of a local municipal wastewater treatment plant where organic substrates were removed was used as inoculating sludge for the SBR operation. Firstly, the SBR was operated under anaerobic/aerobic conditions (Phase I) in which ammonium oxidation was inhibited by n-allyltiourea under aerobic phase to select PAOs capable of utilizing only O₂ as an electron acceptor for about one month. Next, 15 mg of NO₃⁻-N/l was supplemented at the start of anaerobic conditions and the SBR was operated under anoxic/anaerobic/aerobic conditions (Phase II) for selection of DNPAOs. Synthetic wastewater of the following composition was used as the feeding solution: 515 mg of CH₃COONa (BOD 400 mg/l), 65 mg of K₂HPO₄, 140 mg of (NH₄)₂SO₄, 14 mg of CaCl₂·2H₂O, 90 mg of MgSO₄·7H₂O, 4 mg of n-allyltiourea and 0.3 ml of nutrient solution⁹ per liter.

Evaluation of phosphate uptake ability

The evaluation of phosphate uptake ability of the activated sludge cultivated under the above conditions was conducted regularly in the following batch experiments. The activated sludge sample (50 ml) obtained at the end of anaerobic conditions was transferred to the flask, and then the batch experiments were conducted for a 285 min aerobic or anoxic (with the supplementation of 20 mg NO₃⁻-N/l) phase to measure amounts of phosphate uptake per cell weight. Also, during the anoxic phase, nitrogen gas was purged to secure the strictly anoxic condition.

Analytical methods

The analyses of phosphorus and MLSS were performed according to standard methods¹. Nitrate and nitrite concentrations were determined using a high-performance liquid chromatograph (HPLC) equipped with an anionic column (IC-Anion-PW, Tosoh, Japan) and an ultraviolet detector (UV-8011, Tosoh, Japan). TOC was measured using an automatic TOC analyzer (TOC-5000, Shimadzu, Japan).

Quinone analysis

Microbial quinones in the activated sludge were analyzed according to methods described by Hiraishi⁷. The suspension liquid taken from the SBR was centrifuged to obtain the activated sludge. Quinones were extracted from the sludge using a mixture of chloroform-methanol (2:1, v/v). After the quinones were extracted into hexane, menaquinone and ubiquinone were separated using Sep-Pak® Plus Silica. The types and concentrations of quinones were determined using a HPLC equipped with an ODS column (Zorbax SB-C18, 4.6 (I.D)×250 mm, Hewlett Packard, USA) and a photodiode array detector (Series 1100, Hewlett Packard, USA). In a mixture of methanol-isopropyl ether (9:2, v/v), menaquinone and ubiquinone show maximum absorption at 270 nm and 275 nm, respectively. Ubiquinone-10 and vitamin K₁ were used as the quantitative standards for ubiquinones and menaquinones, respectively.

Results and Discussion

Selection and dominance of DNPAOs

Selective enrichment of PAOs and DNPAOs was conducted and phosphate uptake ability was regularly measured in the presence of O₂ or nitrate as a respective electron acceptor. Figure 1 shows changes in phosphate uptake ability per MLSS under aerobic and anoxic conditions. At the start of the operation, neither aerobic phosphate uptake nor anoxic phosphate uptake was observed. Aerobic phosphate uptake increased after 3 days and reached 15 mg-P/g-MLSS on day 20. On the other hand, anoxic phosphate uptake was

![Fig. 1. Time course of aerobic (○) and anoxic (●) phosphate uptake of the activated sludge cultivated under each set of operational conditions.](image-url)
not observed during Phase I (anaerobic/aerobic conditions). This is probably because the activated sludge was not exposed to nitrate due to the inhibition of ammonium oxidation by n-allylthiourea under aerobic conditions. Thus, during Phase I, PAOs capable of utilizing O₂ as an electron acceptor were selectively cultivated.

From day 30, the solution containing 15 mg of NO₃⁻-N/1 was fed (anaerobic/anaerobic/aerobic conditions; Phase II). As a result, anoxic phosphate uptake gradually increased. Eventually, the amount of anoxic phosphate uptake reached about 63% of that of aerobic phosphate uptake on day 75. Typical changes of TOC, nitrate, and phosphate in Phase II on day 46 are shown in Fig. 2, which confirms that the SBR was kept under anoxic conditions during 0–30 min and under anaerobic condition during 30–90 min. In anoxic conditions, carbon substrate uptake, phosphate release and denitrification occurred simultaneously. In anaerobic conditions, carbon substrate uptake and phosphate release occurred. These results indicate two possibilities during anoxic conditions: 1) PAOs (DNPAOs) take up carbon substrate and release phosphate accompanied by denitrification under anoxic conditions; 2) PAOs take up carbon substrate and release phosphate, while denitrification is conducted by denitrifying organisms that are unable to accumulate phosphate.

During Phase II, the cultivated sludge simultaneously exhibited the following two phenomena in anoxic conditions: A) carbon substrate uptake and phosphate release accompanied by denitrification in the SBR (Fig. 2); B) an increase of anoxic phosphate uptake in the batch test (Fig. 1). Although the relationship between these two phenomena is still unclear, the overall conditions, provided by the anoxic/anaerobic/aerobic switching operation during Phase II, were more favorable for the growth of DNPAOs than for the growth of denitrifying organisms that are unable to accumulate phosphate.

In the SBR, the carbon substrate which had been accumulated in both anoxic and anaerobic conditions was consumed for phosphate uptake in the subsequent aerobic conditions. This result indicates that DNPAOs utilize carbon substrates through the TCA cycle for growth under anoxic conditions (Phase II) or DNPAOs accumulate polyhydroxyalkanoate (PHA) using the reduction equivalents and energy obtained from the oxidation of a carbon substrate through the TCA cycle under anaerobic conditions² and then grow under aerobic conditions. Thus, it is suggested that the presence of both carbon substrates (electron donor) and nitrate (electron acceptor) results in selection of DNPAOs. During Phase II, PAOs capable of utilizing O₂ and/or nitrate as an electron acceptor existed.

The change in quinone profile

Figure 3 shows the change in the quinone profile under each set of operational conditions. A drastic change was observed during the initial 7 days. On day 0 (seed sludge), menaquino-9 (MK-9)+MK-8 (H₄) (one of the 8 isoprene units is hydrogenated with 4 hydrogen atoms) was the most abundant quinone. When phosphate uptake increased (on day 7), a decrease of MK-9+MK-8 (H₂) and increase of ubiquinone-8 (Q-8) were observed. After that, Q-8 gradually increased and became the major quinone type, which suggested that Q-8-containing species belonging to β-proteobacteria were the major species of the microbial community under such operational conditions. Also, the second most

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**Fig. 2.** Typical change of TOC, nitrate and phosphate in Phase II on day 46.

**Fig. 3.** Time course of the change in quinone profiles under each set of operational conditions.
abundant quinone shifted from MK-9+MK-8 (H\textsubscript{4}) to MK-7 and MK-8 (H\textsubscript{2}) during Phase I. However, the decrease of MK-8 (H\textsubscript{2}) was observed after switching to Phase II, so that MK-7 was the second most abundant quinone during Phase II. Therefore, it is indicated that MK-8 (H\textsubscript{2})-containing species could not survive under anoxic conditions.

The mole ratio of total ubiquinones to total menaquinones (Q/MK) is shown in Fig. 4. From Fig. 4, it was demonstrated that Q/MK increased with time. In short, as the operational conditions changed and activated sludge was cultivated, the mole ratio of ubiquinone increased. These results indicated that microbial members that could not survive this selective enrichment of PAOs and DNPAOs contained MK as a dominant type of quinone. Even though ordinary heterotrophic organisms other than PAOs might exist, the fact that phosphate uptake ability increased with time suggested that the major microbial members that contribute to aerobic phosphate uptake and anoxic phosphate uptake have Q-8 as a dominant type of quinone.

Relationship between anoxic phosphate uptake ability and quinone type

The relationship between quinone profiles and anoxic phosphate uptake ability was further investigated for Phase II.

As shown in Fig. 5, there was high correlation between the mole fraction of Q-8 and anoxic phosphate uptake ability per MLSS. Although Q-8 was the major quinone type during Phase I, Fig. 5 demonstrated that Q-8 can be regarded as a biomarker showing anoxic phosphate uptake ability. Previous study demonstrated that Q-8 is the major quinone type in phosphate-removing activated sludge\textsuperscript{[5,9]} and \textit{beta-proteobacteria} (organisms related to \textit{Rhodocyclus} sp.) dominated in the EBPR\textsuperscript{[5,17]}, consistent with the results of the present study. Therefore, it was indicated that both PAOs capable of utilizing O\textsubscript{2} as an electron acceptor and PAOs capable of utilizing nitrate as an electron acceptor belong to Q-8-containing microbial species. In addition, Fig. 5 indicated that certain species of PAOs that are hard to grow during Phase I could grow more efficiently with exposure to nitrate during Phase II.

The information obtained from the quinone profiles alone is insufficient to identify organisms responsible for anoxic phosphorus uptake. Thus, a combination of molecular tools, as well as ecophysiological approaches, will be necessary to achieve a better understanding of DNPAOs and Q-8-containing organisms.

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Population Dynamics of DNPAOs

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