Chapter 6

CONCLUSIONS AND PERSPECTIVES

6.1 SUMMARY OF THIS STUDY

In enclosed water bodies such as lakes and inland seas, water bloom and red tide have occurred with the increase in nutrient salts such as nitrogen and phosphorus. These nutrients cause the growth of toxic algae and affect the suitability of the water for a drinking use. Recently, the achieved levels for total nitrogen and phosphorus that are important causes of eutrophication were not yet satisfactory in lakes, marshes and coastal waters. Clearly, a result of slow progress in efforts to improve the state of pollution of lakes and marshes, inland harbors, inland seas, and other closed bodies of water as well as rivers in cities that receive discharged domestic wastewater has caused water pollution in Japan. Therefore, advanced wastewater treatment plants (advanced WWTPs), which attain both the nitrogen and phosphorus removal, are very important.

In Japan, the adoption rate of advanced WWTPs, which have high nutrients (including nitrogen and phosphorus) removal capability, is quite low. Moreover, the increase in the introduction of WWTPs (including advanced WWTPs) causes the increase in the amount of excess sludge. Considering these pressing social problems, the sludge reduction processes should be introduced with the introduction or improvement of advanced WWTPs. On the other hand, the phosphorus recovery from wastewater is also important.

To date, denitrifying polyphosphate accumulating organisms (DNPAOs) have been adopted in some processes to achieve the reduction of the excess sludge reduction. One of the DNPAOs employing processes, the Anaerobic/Oxic/Anoxic (AOA) process combined with the ozonation process for sludge reduction and the phosphorus adsorption process for phosphorus recovery, have been proposed to meet these increasingly stringent requirements. This process achieved both effective sludge reduction and phosphorus recovery; however, effluent water quality was deteriorated by slowly biodegradable materials derived from the ozonated sludge. Additionally, the ozonated sludge could not inhibit the oxic phosphorus uptake by PAOs. Therefore, some modification in the ozonation system is necessary to establish the A/O/A process with sludge

ozonation and phosphorus recovery.

The microbiological characterization of the process is also necessary to understand the mechanisms of nutrient removal and to improve the process. However, especially for PAOs and DNPAOs, the phylogenetic affiliation and physiological characteristic of organisms which play important roles in the process are still unknown. Some major candidates of PAOs and DNPAOs had been reported by using culture-independent molecular techniques; however, characterization of these known PAOs and identification of unidentified other candidates have not been clarified yet. Therefore, the relations among the process operations, the wastewater characteristics and the dominant PAOs species are still not clear.

In this study, first, the A/O/A process combined with the sludge ozonation process and the phosphorus adsorption process was improved by using the fine-bubbled ozonation system, which can achieve both high sludge solubilization efficiency and high biodegradability. To understand the microbial function in the process, microbial community analysis was performed and discussed. Then, the method for selective concentration of organisms, which play important roles in the process, was also developed. Microautoradiography-fluorescence *in situ* hybridization (MAR-FISH) analysis was performed to determine *in situ* activity of organisms which play important roles for nutrient removal. Summaries of each chapter are described as follows:

In Chapter 1, the outlines of the excess sludge reduction and the phosphorus recovery from wastewater and excess sludge features were described. The mechanisms of enhanced biological phosphorus removal (EBPR) process and current studies of microbial community analysis using molecular techniques were also described. The meaning and objectives of this study were indicated.

In Chapter 2, the fine-bubbled ozonation system was introduced to a continuous A/O/A process and the continuous process was operated under various ozonation conditions. In the process operation, adequate sludge reduction was achieved when the sludge amount for

ozonation was set at 16 % of total MLSS per day. However, in this ozonation condition, nitrogen removal efficiency was deteriorated because the population density of nitrifying bacteria decreased. The decrease in the sludge amount for ozonation (9.4% of total MLSS per day) caused slight increase in MLSS concentration; however, efficient nutrient removal was achieved. The accumulated phosphorus in sludge was solubilized by ozonation and a large part of the solubilized phosphorus consisted of PO_4-P. Over 90% of PO_4-P was adsorbed by zirconium-ferrite adsorbent in the phosphorus adsorption column. Phosphorus uptake rate indicated that phosphorus was removed not only by normal PAOs but also by DNPAOs. Interestingly, not only DNPAOs but also endogenous denitrifying bacteria contributed to nitrogen removal.

In Chapter 3, organisms playing important roles for nutrient removal in the A/O/A process with no sludge production and phosphorus recovery were characterized by a PCR-cloning method and fluorescence *in situ* hybridization (FISH) method. PCR-cloning demonstrated that the organisms in the process belonged to very different phylogenetic groups. Most clone sequences were affiliated with the *Bacteroidetes*, followed by the *Betaproteobacteria* and the *Alphaproteobacteria*. These results were well accordance with the results in FISH analysis: the *Betaproteobacteria* and *Alphaproteobacteria* were prominent in the process. The family *Comamonadaceae*, which have been reported as the extracellular poly-β-hydroxybutyrate (PHB) depolymerase producing denitrifying bacteria, was detected. Intercellular PHB accumulating bacteria (the genus *Amaricoccus*, *Beijerinckia*, *Hyphomicrobium* and *Paracoccus*) were also detected in the process. Some of intercellular PHB accumulating bacteria are capable to reduce nitrite to nitrogen. Denitrifying bacteria (the genus *Dechloromonas* and *Zoogloea*) that the ability to accumulate PHB has not been reported, existed in the process. These results suggested that three types of denitrification were included in the denitrification in the A/O/A process: 1) denitrification by intercellular PHB accumulating bacteria, which accumulate PHB in the anaerobic and/or oxic tank by assimilating organic carbon derived form influent wastewater and

ozonated sludge, and then reduce nitrate to nitrogen; 2) denitrification by extracellular PHB depolymerase producing denitrifying bacteria, which depolymerize extracellular PHB derived form the ozonated sludge; 3) denitrifying bacteria which use extracellular organic carbon derived from the depolymerized PHB. In this study, PHB in both liquid and solid phase was not determined but PHB is a key substrate in the process. The sequence related to *Candidatus* 'Accumulibacter phosphatis' and *Actinobacterial* PAOs, which are the major candidates of PAOs, was not obtained in this study and it indicated that unidentified PAOs/DNPAOs (denitrifying PAOs) accumulate phosphorus in the oxic/anoxic tank of the process.

In Chapter 4, to collect PAOs and glycogen accumulating organism (GAOs)/G-bacteria selectively from the phylogenetically high-diverse activated sludge, the buoyant density separation method was proposed. The buoyant density separation method achieved the selective concentration of *Cand.* 'Accumulibacter phosphatis' and the group GB, which has been reported as major GAOs; however, the *Defluvicoccus vanus*-relative G-bacteria were failed to concentrate. Even though some bacteria related to phosphorus removal were eliminated, most of bacterial strains which do not play an important role for phosphorus removal could be eliminated by the buoyant density separation. Then, the buoyant density separation method should be useful to screening organisms which accumulate high-density intercellular compounds such as PAOs and intercellular PHB accumulating bacteria.

In Chapter 5, PAOs indicators in EBPR process evaluated by conventional analyses and that by microautoradiography-fluorescence *in situ* hybridization (MAR-FISH) analysis were compared and discussed to demonstrate the superiority of MAR-FISH analysis in the functional analysis of organisms in EBPR processes. Two types of activated sludge, EBPR sludge and non-EBPR sludge, were mixed in the different ratios and operated in the acetate-fed sequencing batch reactors (SBRs). During the start-up phase, three conventional indicators, the phosphorus content in biomass, the population density of *Cand.* 'Accumulibacter phosphatis' and the

anaerobic phosphorus release exhibited different behavior because 1) each indicator is based on the each activity of PAOs, such as acetate assimilation, phosphorus uptake and growth activity, and 2) the activities of other organisms should contribute each indicator. MAR-FISH analysis demonstrated that both the acetate assimilation by *Alphaproteobacterial* G-bacteria and no acetate assimilation by some *Cand.* 'Accumulibacter phosphatis' in SBRs with efficient phosphorus removal, suggested that MAR-FISH analysis is more appropriate for the determination of the actual substrate uptake activities.

6.2 PERSPECTIVES

In this study, A/O/A process with sludge ozonation and phosphorus recovery process was established in only the controlled experimental conditions. In these conditions, the sustainability to the seasonal change (water quality, water quantity, pH, temperature and so on) was not evaluated. These changes should cause the change in the sludge production and should require the operational changes especially for ozonation and recirculation patterns of the ozonated sludge. Unfortunately, the estimation of material balance for phosphorus was failed because unidentified accumulation of phosphorus in the reactor in this study. In future study, material balance (COD, nitrogen and phosphorus) should be also required to apply this process to full-scale WWTPs. A part of phosphorus was removed by DNPAOs; however, normal PAOs also took the other part in phosphorus removal. To inhibit this phosphorus accumulation by PAOs, the reduction of retention time in the oxic tank is necessary. However, the short retention time should cause the deterioration in nitrification efficiency. As shown in Chapter 2, ammonia was remained in the effluent, suggesting that the retention time in the oxic tank was not enough to oxidize ammonia to nitrate. Therefore, the immobilization of nitrifying bacteria such as polyethylene glycol (PEG) might be suitable to reduce the retention time in the oxic tank.

For microbiological aspects, denitrification in this process was interesting. In the A/O/A process, several types of denitrifying bacteria such as extracellular PHB depolymerase producing denitrifying bacteria played important roles for nitrogen removal. The soluble and solid PHB should be the key material in the process. In this study, both soluble and solid PHB were not determined. Therefore, the determination of those PHB is necessary. On the other hand, it is difficult to estimate each type of denitrification efficiency at present; however, the phylogenetic and functional molecular analysis such as MAR-FISH and stable-isotope probing (SIP) might clarify this phenomenon.

In Chapter 4 and 5, the buoyant density separation and MAR-FISH analysis were applied to

a model EBPR system and the results indicated that these methods should be useful tools to both the phylogenetic identification of PAOs and GAOs and the determination of their ecophysiology. However, these techniques were not applied to full-scale reactors in this study. In future study, these techniques should contribute to understand the EBPR processes.