Chapter 6

Conclusions and Perspectives

6.1 Conclusions of this study

The most serious environmental problems of the 21st century have the potential to alter the course of life on this planet. Global warming, toxic waste, water and air pollution, acid rain, and shrinking energy supplies are frightening challenges that may threaten our future if we do not face up to them. Thus, the 21st century is dubbed “the century of the environment”. In the present day, the biotechnology leads up to clue these environmental problems. In particular, many biotechnologies have been introduced to solve many water pollutions, e.g. wastewater treatment process. At a wastewater treatment process, the wastewater containing nitrogen-compounds is generally treated by a combination of the two biological processes of nitrification and denitrification. In recent years, microbial ecology involved in wastewater treatment has been investigated for an enhancement of process performance. For the microorganisms involving nitrogen removal from wastewater, nitrifying bacteria have been studied more than denitrifying bacterial because nitrification is the rate-limiting step and the detection of nitrifying bacteria is easier because of a restricted phylogenetic distribution of the capacity for nitrification. However, denitrification performance is fluctuated by environmental change and the operation of denitrification process (e.g. addition of carbon substrates) strongly impacts the system performance and microbial community structure. Therefore, it needs to accumulate the information about denitrifying microbial ecology in wastewater treatment systems.

One of the important operations at wastewater treatment systems is adjustment of the ratio of carbon to nitrogen (C/N) by adding an external carbon sources. This operation affects the running costs and system performance. Thus, the objectives of this study are investigation of denitrifying microbial community in wastewater treatment systems for revealing the relationship between carbon substrate and denitrifying population.

In chapter 1, the meaning and objectives of this study were indicated. And also, the features of some molecular biological techniques, current situation of microbial community analysis using these methods and detection of denitrifying bacteria in the previous studies were described.

In chapter 2, stable-isotope probing (SIP) analysis is applied for identifying the acetate- or methanol-assimilating bacteria under nitrate-reducing conditions in activated sludge. It is found that the diversity level for methanol-assimilating bacteria is considerably lower than that for acetate–assimilating bacteria. Furthermore, SIP analysis identifies the *Comamonadaceae* and *Rhodocyclaceae* of *Betaproteobacteria* and the *Rhodobacteraceae* of *Alphaproteobacteria* as acetate-assimilating bacteria. Also, SIP analysis identifies the *Methylophilaceae* of *Betaproteobacteria* and the *Hyphomicrobiaceae* of *Alphproteobacteria* as methanol-assimilating bacteria.

In chapter 3, we further characterize nitrite reductase genes (*nirS* and *nirK*) as functional marker genes for denitrifier communities in acetate- or methanol-assimilating populations. In *nir* clone libraries, some *nir* sequences were related to the *nirS* or *nirK* sequence of some known pure cultures, such as *Alcaligenes*, *Hyphomicrobium*, and *Thauera*. However, most of the *nirS* or *nirK* sequences derived from acetate- or methanol-assimilating populations were clustered in some unidentified groups. Phylogenetic analysis of nitrite reductase genes (*nirS* and *nirK*) suggests that the denitrifying bacterial population strongly depends on the type of organic carbon source as well as the results of 16S rRNA analysis. Furthermore, it is found that the diversity of the *nirK* gene is almost equal to that of the *nirS* gene in acetate- or methanol-assimilating denitrifying bacteria in the activated sludge of a municipal sewage treatment system.

In chapter 4, DNA-SIP analysis determines the active bacterial population involved in the methane-dependent denitrification (MDD) under oxygen-limited conditions and further the fate of carbon derived from methane through an indirect labeling effect via intermediates of the active methanotrophic bacteria. Firstly, it is found that the supplementation of nitrate have a significant impact on the activity of methane consumption and the active methanotrophic population. Our 16S rRNA gene and *pmoA* gene analysis results show that type I and type X methanotrophic bacteria tend to dominate under nutrient rich conditions while type II tend to appear under nitrogen-limiting conditions. In addition, this study suggests that the *Hyphomicrobiaceae* play an important role as a major nitrate-reducer with methane-derived organic intermediates (e.g. methanol, formate) produced by methanotrophic bacteria as electron donors in MDD ecosystem. Finally, SIP also detects the transfer of ^{13}C from the primary consumer (methanotrophic bacteria) to non-methanotrophic bacterial population (heterotrophic bacteria, methylotrophic bacteria and micropredators) coexisting in the sewage sludge.

In chapter 5, acetate and methanol is compared for their performance as carbon source for denitrification and microbial community structure under various saline conditions. As a result, acetate-fed process attained stable and high nitrate removal at 0-10% NaCl, while methanol were proven beneficial electron donors at 0-3% NaCl. Changes of bacterial community structure with the increase of salinity are monitored by molecular biological analysis (i.e. cloning, T-RFLP analysis). In acetate-fed reactor, a specialized microbial population (i.e. the genera *Halomonas* and *Marinobacter*) adapts to a high saline environment. In methanol-fed reactor, the genera *Azoarcus* and *Methylophaga* increase when salinity concentration was at 1% to 3% NaCl. However, the denitrifying activity of methanol-fed sludge was drastically decreased at 4% NaCl. This result suggests that methanol-utilizing populations in sewage sludge are unable to

adapt to a high saline environments (> 4% NaCl). Therefore, it was shown that the selection of organic carbon source as an electron donor for denitrification is a matter of great important factor to trigger high nitrate removal performance in high saline environments.

6.2 Perspectives

In this study, denitrifying microbial community structure was characterized based on ^{13}C-based stable-isotope probing (^{13}C-SIP) techniques. SIP linked the phylogenetic identification of microorganisms with their function about carbon cycles (e.g. assimilation, dissmilation) in the complex community structure. However, there are following limitations for the comprehensive understanding of the truly important denitrifying bacteria in complex microbial communities although the ^{13}C-SIP application provides insights into the denitrifying bacteria that consume specific ^{13}C-labeled substrates (e.g., methanol). One is that denitrifying bacteria in natural environment utilize a wide variety of environmental organic substrates. The other is the restriction of the ^{13}C-substrates available on the market. To overcome the demerit of ^{13}C-SIP for characterizing denitrifying populations in ecosystems, currently, we try to characterize it by using the SIP application with ^{15}N-substrates (i.e. nitrate, nitrite).

Additionally, specific probes are designed based on phylogenetic information obtained by SIP analysis, and the subsequent microautoradiography-fluorescence *in situ* hybridization (MAR-FISH) analysis can directly link the phylogeny of uncultured microorganisms with those functions in complex ecosystems (Lee *et al*., 1999; Ginige *et al*., 2004). Furthermore, monitor of radioisotope substrate in bacterial cells by beta microimaging instead of MAR can be characterized it above the single-cell level (Gieseke *et al*., 2005). However, MAR-FISH limits its use to elements that have a radioisotope with a suitable half-life and excludes the study of other elements (e.g. nitrogen).

Recently, other powerful isotopic techniques have been developed: isotope-array (Adamczyk *et al*., 2003); nano-scale secondary ion mass spectrometry (nanoSIMS) (Lechene *et al*., 2006); Raman microscopy (Huang *et al*., 2007). We anticipate that these isotopic techniques will open clearly the mechanism of microbial process in natural environments.

References

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