Chapter 1

General Introduction

Microbial ecology involved in denitrification and Biological nitrogen removal process in wastewater treatment systems

1.1 Introduction and objectives

Recently, the achieved levels of total nitrogen and phosphorus have not been satisfactory in enclosed aquatic environments (e.g. estuary, inland harbors, inland seas, lake, marshes, and rivers). The elimination of nutrient (nitrogen- or phosphorus-containing compounds) discharged from wastewater treatment facilities is of increasing importance because the influx of nutrient compounds contributed to the eutrophication (i.e. red tide) and the subsequent depletion of dissolved oxygen (i.e. blue tide) in aquatic environments. Therefore, there is urgent need to develop highly advanced biological wastewater treatment process.

Normally, the wastewater containing nitrogen-compounds is treated by a combination of the two biological processes of nitrification and denitrification. In recent years, a biological reactor regarded as “black box” has been investigated for understanding microbial ecology involved in wastewater treatment by using various approaches. Microorganisms involved in nitrification, especially ammonia-oxdizing bacteria, have been studied in wastewater microbiology. This results from the fact that the nitrification process is generally a rate-limiting step in the biological nitrogen removal system at wastewater treatment facilities. Also, this is attributed to a restricted phylogenetic distribution of the capacity for nitrification (e.g. *Nitrosomonas*, *Nitrospira*). Therefore, molecular biological approaches based on the 16S rRNA sequences are powerful tools for a specific characterization of nitrifying populations. In contrast to nitrifying populations, denitrifying ability is widespread among *Archaea*, *Bacteria*, *Fungi*. Thus, the investigation about denitrifying bacteria has depended on culture techniques because 16S rRNA-based approaches are unable to detect denitrifying bacteria specifically. Therefore, there remains not to identify microorganisms truly involved in denitrification in wastewater treatment systems. To determine the activity, identification, and diversity of denitrifying population, other approaches are needed. Some such approaches are the isotope-labeling or functional gene-based analysis.

In the biological nitrogen removal systems, most important factors are the composition of the electron acceptor and donor in wastewater. The denitrification process involves the conversion of nitrate or nitrite to gaseous nitrogen, i.e. N_2, N_2O. At present wastewater treatment plants, the denitrification process is operated based on heterotrophic denitrification, which requires a biodegradable organic carbon source as electron donor. For wastewater that has a low C/N ratio or lacks readily biodegradable carbon sources, various organic compounds as external carbon sources may be added to achieve a satisfactory degree of denitrification (Akunna *et al.*, 1993; Christensson *et al*., 1994). Recently, methane has been proposed as an alternative, inexpensive, and effective carbon source because it is non-toxic, is produced as a biogas by anaerobic treatment, and is available in numerous existing treatment plants (Werner and Kayser, 1991). However, the use of such compounds incurs significant costs in an industrial-scale plant. Furthermore, the select of carbon substrates should be carefully evaluated because the operation for adding an external carbon substrate strongly impacts the system performance and microbial community structure. Therefore, it needs to reveal the relationship between carbon substrate and denitrifying population for controlling the denitrifying microbial ecology in wastewater treatment systems.

In this study, we focused on stable-isotope probing techniques (Radajewski et al., 2000) and denitrifying functional gene-based analysis (Braker et al., 1998) to reveal the relationship between carbon substrate and denitrifying population. Furthermore, we think that these approaches provide insight into the metabolic capabilities of the microorganisms and the interactions between microorganisms in denitrification process.

1.2 Nitrogen removal process in wastewater treatment systems

Nitrogen removal process in wastewater treatment systems has been taken advantage of microbial nitrogen cycle, i.e. aerobic nitrification, denitrification, anammox (**Fig. 1.1**).

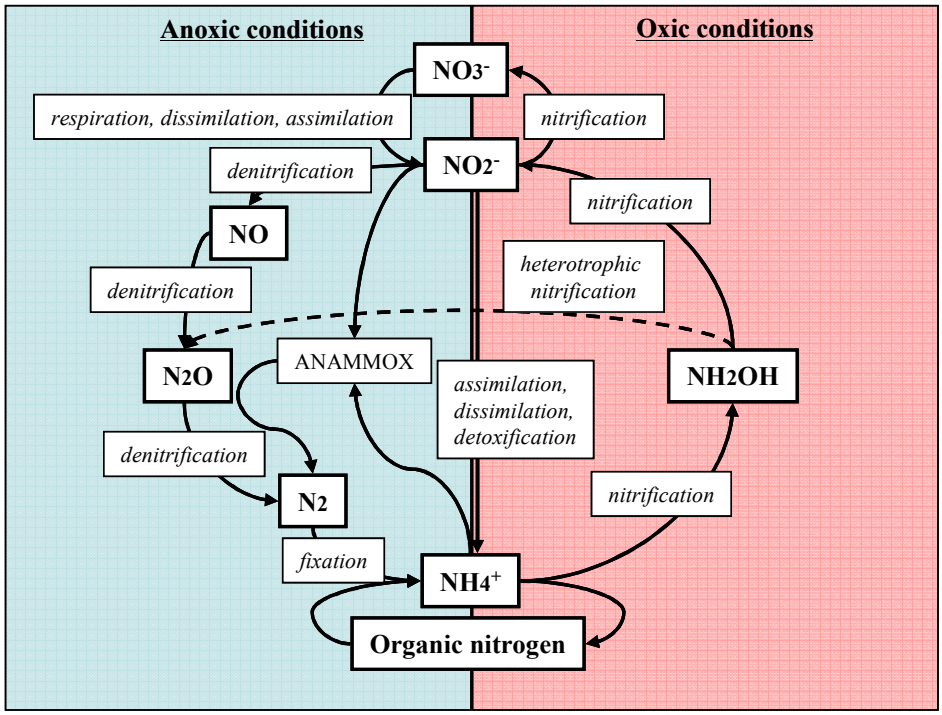

Fig. 1.1 Biological nitrogen cycle

1.2.1 Aerobic nitrification

Aerobic nitrification, which is a two-step process of the oxidation of ammonium (NH_4^+) to nitrite (NO_2^-) and nitrite to nitrate (NO_3^-), is carried out by two types of the slow-growing autotrophic bacteria, i.e. ammonia-oxidizing bacteria and nitrite-oxidizing bacteria. Although the nitrification process has been intensively investigated during the past several years, it is also still difficult to keep stable nitrification performance. This is mainly due to the low growth rates of these microorganisms and their sensitivity to many heavy metals, organic compounds, pH, and temperature. The inhibition by organic compounds can be indirect and related to oxygen depletion by heterotrophs coexisting in the system. In general, process monitoring (e.g. nitrifiers number, activity) is necessary for the improvement of nitrification process.

1.2.2 Denitrification

Denitrification is a process of converting nitrate to gaseous nitrogen (i.e. N_2, N_2O) under anoxic conditions (Zumft, 1997). Denitrifying microorganisms gain energy by using nitrate or nitrite as terminal electron acceptors and using hydrogen, organic compounds, and sulfur as electron donors. Denitrification ability is widespread among the eubacteria, and almost exclusively in those strains that are capable of aerobic growth. One of the operating parameters in denitrifying process is the ratio of carbon to nitrogen (C/N). For wastewater that has a low C/N ratio or lacks readily biodegradable carbon sources, various organic compounds as external carbon sources, such as acetate, ethanol, glucose, and methanol, are added to achieve a satisfactory degree of denitrification. Methanol has been often selected because of its relatively low cost and the small sludge production compared with other organic compounds, although only some bacteria can utilize methanol as the carbon source (Nyberg *et al*., 1992).

1.2.3 Anaerobic ammonium oxidation (anammox)

Anammox is a newly discovered microbial pathway in which ammonium is converted into nitrogen gas with nitrite as the electron acceptor by the some microorganisms belonging to the *Planctomycetes* (Mulder *et al*., 1995; Van de Graff *et al*., 1997; Strous *et al*., 1999). In contrast to the widespread nitrogen removal process (i.e. nitrification-denitrification process), anammox process can achieved significant high nitrogen removal rates (Sliekers *et al*., 2003). Other merits of anammox process are large reduction of doses of hydrogen donor for denitrification (e.g. methanol) and low productions of the amount of excess sludge. Therefore, anammox process is anticipated future nitrogen removal process. However, it is also still difficult to keep stable nitrification performance as well as aerobic nitrification. This is mainly due to the low growth rates of these microorganisms and their sensitivity to environmental conditions (e.g. organic compounds, pH, and temperature).

1.3 Molecular biological approach for understanding microbial ecology

The cultured microorganisms represent a small fraction of microbial communities in natural environments (e.g. sea, soil, wastewater treatment plant), and most of all microorganisms (>99%) in nature can not also still be isolated in pure cultures due to ignorance of the culture conditions which these microorganisms live in their natural environments (Amann *et al*., 1995). Therefore, culture-dependent techniques are insufficient for understanding microbial diversity. The accomplishment of polymerase chain reaction methods using Taq DNA polymerase brings the development of molecular biological techniques. Molecular biological approaches have allowed us to explore microbial diversity at the genetic level because ribosomal RNAs and their genes reflect their evolutionary relationship (Woese, 1987). In order to determine microbial diversity, PCR-amplified genes have to be separated prior to subsequent sequencing because they constitute a heterogeneous mixture of sequence.

1.3.1 Cloning in *Escherichia coli*

There are some strategies for cloning PCR products (Felske and Weller, 2004). In particular, “TA cloning” in *E. coli* is the most widely used approach to obtain individual sequences from a heterogeneous mixture of sequence generated by PCR amplification. TA cloning takes advantage of the terminal transferase activity of *Taq* polymerase and other non-proofreading DNA polymerases which adds a single 3'-deoxyadenosine overhang to each end of the PCR product (Clark, 1988). The resulting PCR product is then ligated into a linear vector with single, 3' deoxythymidine (T) overhangs at both ends, under the action of T4 DNA ligase. Other cloning strategies are “restriction stick end cloning” (Scharf *et al*., 1986) and “blunt-end cloning”. In restriction stick end cloning, a restriction enzyme target site is introduced into each of the PCR primers. The resulting PCR product and cloning vector are digested with the restriction enzymes to generate complementary ends at the PCR product and the vector which are then ligated.

In blunt-end cloning, blunt-end PCR product generated by proof-reading polymerase such as the *Pfu* DNA Polymerase can also be cloned into a blunt-end vector.

1.3.2 PCR-based fingerprinting techniques of the microbial community

The so-called full-cycle rRNA analysis approach involves cloning of amplified products of 16S rRNA gene, comparative sequence analysis of individual clones, and, subsequently, probe design, and application of probes to environmental samples. However, the cloning approach has disadvantage that a statistically significant number of clones is required for understanding the complex microbial communities, i.e. the cloning analysis is very time-consuming, expensive, laborious methods. Therefore, the cloning approach is insufficient for exploring of microbial diversity or a successional population change under various conditions or environments. Consequently, some PCR-based genetic fingerprinting techniques have so far been developed in order to determine the diversity and monitor the microbial community structure. The fundamental principle of a fingerprinting technique (**Fig. 1.2**) is the electrophoresis of PCR products (Marsh, 1999; Muyzer, 1999).

In denaturing gradient gel electrophoresis (DGGE) or temperature gradient gel electrophoresis (TGGE), separation of DNA fragments based on the decreased electrophoretic mobility of partially melted double-strand DNA molecules in polyacrylamide gels containing a linearly increasing gradient of denaturant (i.e. formamide and urea) or a linear temperature gradient. To prevent the complete melting of double-stranded DNA, a stretch of GC-rich sequences (GC-clamp) is frequently added to one-end of the DNA sequences. DGGE and TGGE allow the simultaneous analysis of multiple samples making it possible to follow the change of community structure over time. Additionally, these techniques make it possible to identify the community members by sequencing of the excited bands or by hybridization analysis with specific probes, which is not possible with other fingerprinting techniques.

Terminal restriction fragment length polymorphism (T-RFLP) analysis have recently been focused as a highthroughput fingerprinting technology by the development of automated capillary electrophoresis systems that provided digital output. In PCR for T-RFLP analysis, 5' end of the primer is labeled with fluorescence material (e.g. FAM, HEX). Subsequently, PCR products are digested with restriction enzymes (usually teteranucleotide recognizing) and fragments are size separated with automated sequencers, whereby only the labeled terminal fragments (T-RFs) are detected and quantified. Individual T-RFs can be assigned presumptively to operational taxonomic units (OTUs), which ideally correspond to phylogenetically related microorganism, based on the sequences database or *in silico* analysis of sequences from clone libraries established in parallel from the same sample. T-RFLP analysis provides insight into the statistical data of the structure and function within microbial communities for comparative analyses.

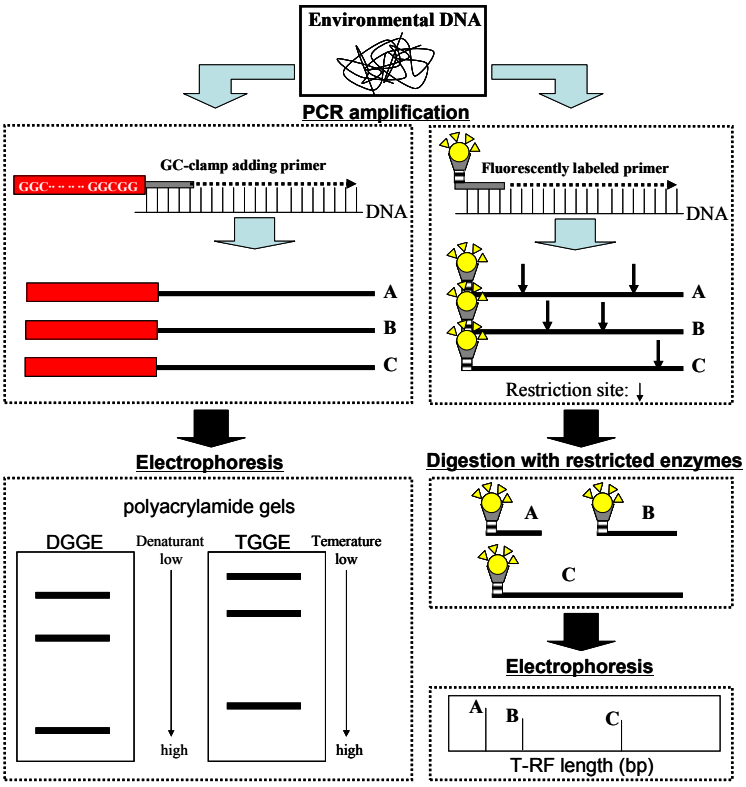

Fig. 1.2 Genetic fingerprinting techniques (DGGE, TGGE, T-RFLP)

1.3.3 Isotope-labeling based molecular biological techniques

Phylogenetic analysis based on ribosomal RNA sequence generally provides few direct information about the metabolic functions and interactions of microorganisms in natural environments. To link the identification of microorganisms with their function in the complex communities, isotope-labeling techniques have developed in the last few years: microautoradiography-fluorescence *in situ* hybridization (MAR-FISH); stable-isotope probing (SIP).

MAR-FISH involves short-term incubation of environmental samples with specific radioisotope labeled substrates, fixation of thin sections of these samples to glass slides and subsequent analysis by FISH and inverse confocal laser scanning microscopy. MAR-FISH allows the detection of active bacteria utilizing a specific substrate by using specific 16S rRNA-targeted FISH probes (Lee, *et al*., 1999). MAR-FISH also provided insight into a information about spatial structure and physical interactions in highly aggregated environments (e.g. biofilms). However, one of demerits in MAR-FISH is a requirement for some prior phylogenetic information of the microorganisms that are likely to utilize a substrate of interest. A parallel SIP analysis is helpful to get a information of key-players.

SIP is increasingly being applied in order to link the identity of microorganisms to specific biochemical processes. When samples incubate with the substrate labeled with stable isotopes (e.g. ^{13}C, ^{15}N), DNA of microorganisms involved in metabolism of the substrate can be labeled with the heavy stable isotope. The incorporation of the heavy stable isotope into DNA greatly enhances the buoyant density difference between labeled and non-labeled fractions, although the density of DNA varies with it guanine-cytcine (G+C) content. After separation of labeled DNA and non-labeled DNA by the CsCl density gradient centrifugation, the identity of microorganisms involved in a process of interest can be revealed by PCR amplification and subsequent comparative sequence analysis of their 16S rRNA genes or of other genes encoding key enzymes for

specific metabolic pathway (e.g. methane-oxidation, denitrification). Furthermore, labeled DNA can also be used to generate large-insert genomic libraries (Dumont and Murrell, 2005).

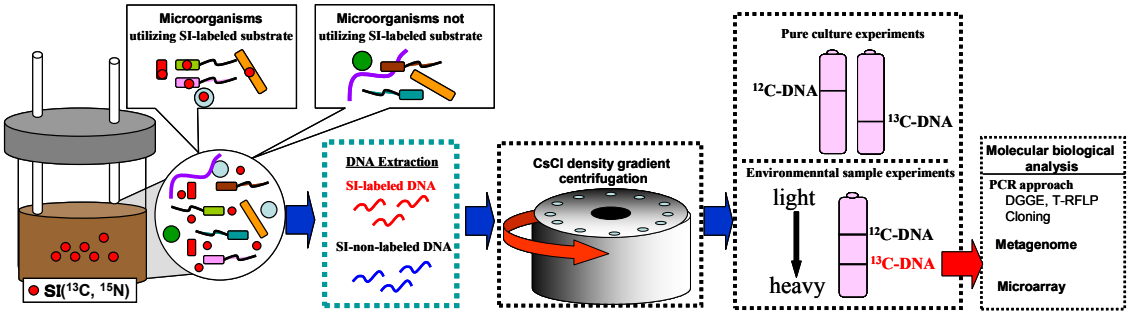

Fig. 1.3 Stable-isotope probing

1.4 Microbial community analysis targeted as denitrifying bacteria

Denitrification ability is widespread among the eubacteria, and almost exclusively in those strains that are capable of aerobic growth. Most of the characterized denitrifiers belong to the *Proteobacteria*. Although the majority of denitrifiers are gram-negative, denitrifying bacteria are well represented among gram-positive bacteria (e.g. *Bacillus*). Furthermore, a number of denitrifiers are in other eubacterial genera (e.g. *Archaea*, *Fugi*). However, close relatives of known denitrifiers will not necessarily have the denitrification ability. Therefore, 16S rRNA-based approaches are unable to detect denitrifying bacteria specifically.

Recently, functional genes, which encode for the enzyme involved in denitrification pathway, have been used as a marker for denitrifying bacteria in natural environments. The reduction of nitrite (NO_2^-) to nitric oxide (NO) distinguishes denitrifying bacteria from nitrate-respiring bacteria (Zumft, 1997). Nitrite reduction is central to denitrification and is two different types of nitrite reductases (Nir), either a cytchrome *cd1* enzyme encoded by *nirS* gene or a Cu-containing enzyme encoded by *nirK* gene (Braker *et al*., 1998). Furthermore, *norB* gene encoding nitric oxide reductase (Braker *et al*., 2003) and *nosZ* gene encoding nitrous oxide reductase (Scala and Kerkhof, 1998) have also been used as a marker for denitrifiers.

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