Chapter I

GENERAL INTRODUCTION

In Situ Identification of Microorganisms in Biofilm Communities

SUMMARY

Diverse microorganisms form complex microbial communities and usually exist in biofilm communities in both natural environments and engineered systems such as a wastewater treatment process. However, the conventional approach to investigate microbial ecology has not contributed to the understanding and clarification of the structure and function of biofilm communities. Some effective methods have been developed to investigate phylogenetic affiliations, metabolic activities and genetic activities in biofilm communities at the single-cell level. These techniques have been contributing to a better understanding of the spatial organization of biofilm communities and activities in engineered systems. However, further effort is needed to set out the general rules governing community development in biofilm communities and to advance the process performance of engineered systems. This chapter describes advances and limitations in methodology, particularly focusing on fluorescence in situ hybridization (FISH) and related techniques and the application of these methods to nitrifying biofilms in wastewater treatment processes.

1.1 INTRODUCTION

Diverse microorganisms form complex microbial communities and commonly attach to solid surfaces as biofilms in natural environments. In engineered systems such as wastewater treatment systems, various types of microorganisms usually exist as biofilms on supporting materials, flocs in an activated sludge and granules. However, the conventional approach for the investigation of microbial ecology in biofilms, which does not allow exact determination of the localization of specific bacterial cells, has not contributed to the understanding of actual microbial ecology. Therefore, a bioreactor for a wastewater treatment system has to date been regarded as a "black-box", although wastewater treatment systems are the most common bioengineering processes.

The recent use of new approaches, such as the application of molecular techniques, has enabled *in situ* monitoring of microbial biofilm communities. The monitoring involves the identification of phylogenetic affiliations, determination of spatial distribution, elucidating functions and activities and establishing coordination in microbial biofilm communities. The next challenge in this field is to elucidate the mechanism of treatment activities based on microbial ecology and applying this to the development of a new wastewater treatment system or the improvement of process performance in terms of treatment activity and process stability.

This review introduces the development of and advances in new methods for the investigation of the spatial organization of biofilm communities and describes application to the analysis of biofilm communities in engineered systems using nitrifying biofilms in wastewater treatment processes as an example. The major purpose of this review is to present and discuss the effectiveness and limitations of the use of these techniques for

advancing environmental biotechnology processes such as wastewater treatment systems based on the understanding of the spatial organization and the activity of biofilm communities.

1.2 ADVANCES IN METHODOLOGY

1.2.1 Fluorescence *in situ* hybridization (FISH)

FISH is highly effective for detecting specific bacteria and analyzing the spatial organization of a complex microbial community, due to the possibility of detecting specific bacterial cells at the single-cell level by *in situ* hybridization using phylogenetic markers (16S-rRNA-targeted oligonucleotide probes) labeled with a fluorescent compound (1). rRNA is an ideal target for *in situ* hybridization with oligonucleotide probes because: (i) it is present in all organisms and the identification of natural populations is based on the phylogenetic classification of 16S rRNA sequences, (ii) a large number of sequences of different organisms are stored in databases, (iii) the high copy number per cell greatly increases detection sensitivity and enables the direct detection of a single cell by using an epifluorescence microscope or a confocal scanning laser microscope (CSLM).

FISH-dependent techniques have contributed to the clarification of the *in situ* microbial community structure in various types of biofilm communities, including those in natural environments and engineered systems. FISH is one of the most powerful tools and has become a reliable and commonly used method. Furthermore, the spatial organization of unknown and unculturable bacteria has been analyzed by the combined use of denaturing gradient gel electrophoresis (PCR-DGGE) which enables the design of an oligonucleotide

probe for FISH following the determination of target bacterial species and their 16S rDNA sequences. Detailed schemes for analyzing complex microbial communities targeting specific but unknown and unculturable bacteria have been described by Watanabe and Baker (2) and Amann *et al.* (3).

1.2.2 Microautoradiography combined with FISH (MAR-FISH)

The uptake of specific substrates by microorganisms under *in situ* conditions can be directly determined using substrates radiolabeled with [^{3}H] or [^{14}C] in combination with microautoradiography. This technique enables investigation of the microscale distribution of radiolabeled compounds in a complex microbial community at the single-cell level and identification of metabolically active bacterial cells. Microautoradiography was successfully combined with FISH (MAR-FISH) and applied for the characterization of bacterial communities. The development of MAR-FISH represents a significant advances in the investigation of microbial activities and diversity because the uptake of specific substrates can be directly correlated with the phylogenetic identity of organisms (4). Some studies investigating substrate uptake in biofilm communities combined with phylogenetic identification have been reported. Ito *et al.* investigated a sulfate-reducing sewer biofilm to identify the substrate uptake patterns and the phylogenetic affiliation of sulfate-reducing bacteria in the biofilm under different electron acceptor conditions (5). Daims *et al.* reported the substrate uptake patterns of *Nitrospira*-like nitrite-oxidizing bacteria in a nitrifying biofilm (6).

1.2.3 Microsensors combined with FISH

Microsensors employing microelectrodes facilitate the measurement of the concentrations of substrates or products inside biofilms and are powerful tools for the

estimation of the spatial distribution of *in situ* metabolic activity in biofilms. Microsensors for various chemical compounds such as N_2O, NH_4^+, NO_2^-, NO_3^-, O_2, H_2, H_2S, and glucose and for pH have been developed and used to investigate chemical gradients in various types of biofilms on a micrometer scale. FISH has recently been combined successfully with microsensor measurements to investigate sulfate reduction (7), the nitrification in trickling filter biofilms (8), and the nitrification in microbial aggregates (9, 10), and fixed bed biofilms (11). The combination of the two methods provides reliable and direct information on the relationship between *in situ* microbial activity and the occurrence of specific microorganisms in a biofilm community. Furthermore, the spatial distribution of metabolically active areas and active species in the biofilm can be simultaneously estimated.

1.2.4 Other techniques which can be combined with FISH

Some other methods have been proposed as indicators of the "cell activity" at the single-cell level. They include (i) detection of membrane potential by the use of rhodamine 123 (12) or propidium iodide (13), (ii) detection of esterase activity by a substrate such as fluorescein diacetate which is converted to fluorescent compounds by the activity of internal cellular esterase (14), (iii) use of the cyanoditoryl tetrazolium chloride (CTC) method which generates the fluorescent formazan crystals by the activity of internal cellular dehydrogenase and is used to detect activity respiring bacteria (15) and (iv) use of the direct viable count (DVC) method which has implication for cell growth (16). Some of these methods have already been successfully combined with FISH to simultaneously determine phylogenetic affiliations and cell activities.

1.2.5 *In situ* PCR

FISH targeting rRNA is a highly useful method for the phylogenetic identification of bacteria. However, FISH requires multiple targets in a bacterial cell to provide a detectable signal. Therefore, the detection of individual genes present in a single copy or low copy numbers in intact bacterial cells, such as chromosomal functional genes and mRNA, at the single-cell level by *in situ* hybridization is almost impossible. Furthermore, while FISH data indicate phylogenetic classification, they are not always correlated with bacterial function and characteristics. However, the analysis of the spatial organization of a functional gene and its activity at the single-cell level is extremely important for the further understanding of microbial ecology.

In situ PCR was developed to amplify and detect functional genes and their expression inside a single cell. It is one potential approach to characterizing the microscale genetic and phylogenetic properties of natural bacterial communities at the single-cell level. Chromosomal DNA, mRNA, and rRNA are all candidate targets for *in situ* PCR, so that genetic capabilities, expression of those capabilities, and phylogenetic information are all potentially accessible at the single-cell level. This method was first developed for eukaryotic cells in biomedical applications. In the field of environmental microbiology, Hodson *et al*. developed an *in situ* PCR method for prokaryotic cells (bacteria) and gave examples of its use for single-cell detection of a specific gene (*nahA*) and its transcripts in a model microbial community (17). Some effective methods for the detection of amplified products, which exhibit greatly increased sensitivity, have recently been developed. Tani *et al.* developed a direct *in situ* PCR method (18), to improve detection sensitivity. The combined use of a DIG-labeled primer set and an Alexa-labeled anti-DIG antibody improved detection sensitivity further, reduced non-specific signals and successfully

detected *amoA*-positive bacterial cells in the biofilm community of a wastewater treatment system (19).

However, the *in situ* PCR protocol has been predominantly applied to dispersed samples of model microbial communities. A few studies have to date been reported concerning the analysis of biofilm communities. *In situ* PCR is still less commonly used than FISH, because of too complicated protocol and the requirement for case-by-case optimization of the reaction conditions to achieve high sensitivity

1.2.6 Detection of Gene Expression

To investigate a specific gene activity in individual cells, some methods to monitor gene expression in single cells have been developed. The reporter protein assay is one such technique for the detection of gene expression in both eukaryotic and prokaryotic cells at the single-cell level, and green fluorescent protein (20) is often used as an indicator. Using this approach, *in situ* monitoring of gene expression at the single-cell level is possible in model biofilm systems. Although this is a valuable approach, it is applicable only to genetically engineered organisms, and is thus not applicable for the investigation of natural environmental microbial communities.

Nucleic acid-based methods can be applied to natural microbial communities because only the target sequence has to be known. Two methods based on molecular techniques have been developed to investigate gene activities at the single-cell level. *In situ* PCR combined with *in situ* reverse transcription (*in situ* RT-PCR) was successfully applied to detect mRNA transcribed from the *nahA* gene in a *Pseudomonas* cell in a model community (17), *lac* in *Salmonella typhimurium* (21), and *dnaK* in *Methanosarcina mazei* S-6 cells (22). Another technique is *in situ* reverse transcription (ISRT) (23) which does not require amplification (PCR) but carries out RNA-targeted primer extension (RPE) in a

bacterial cell. Fluorescently labeled nucleotides are incorporated into each transcribed cDNA inside cells, and thus the gene activity can be detected at the single-cell level.

These techniques are theoretically applicable to the investigation of bacterial communities in natural environments. However, to date, success has been achieved only in the case of pure cultured cells or model microbial communities. Therefore, the investigation of gene expression in complex microbial communities remains a challenge and the localization of gene expressions in biofilm communities in which various types of bacterial cells agglutinate is even more difficult.

1.3 APPLICATION OF ABBOVE TECHNIQUES TO A NITRIFYING BIOFILM

In this section, the application of these techniques to the investigation of biofilm communities in bioreactor systems is described using a nitrifying biofilm as an example.

Nitrification, which converts ammonia to nitrite and nitrate, is the initial step of biological nitrogen removal processes carried out by two phylogenetically independent groups of autotrophic aerobic bacteria, namely, ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB). Although nitrification is one of the most important and common steps in both domestic and industrial wastewater treatment processes, it is the rate-determining step in most nitrogen removal processes. To achieve a sufficient nitrification rate, nitrifying bacteria must be immobilized in a nitrogen removal system. Some effective methods for the immobilization of nitrifying bacteria have been established, such as the use of biofilms on supporting materials (24), entrapment in polymer gels (25), and using fibrous net-works (26). Therefore, a better understanding of

the spatial organization, and activities of immobilized nitrifying bacteria is important to improve treatment performance and process stability.

FISH dependent techniques provide reliable information regarding dominant species of nitrifying bacteria, their spatial distribution and activities in biofilms. *Nitrosomonas* (AOB) was present throughout the biofilm whereas *Nitrospira* sp. (NOB) was restricted to the inner part of the sewage wastewater biofilm as determined by combined analysis with a microelectrode (11). Combined use of a microelectrode also made it possible to estimate the *in situ* cell-specific activity of uncultured nitrifying bacteria in the biofilm-like aggregate after the determination of the volumetric reaction rate calculated from microprofiles measured by microsensors and cell numbers of nitrifying bacteria measured by FISH (10).

A schematic image of the *in situ* monitoring of a spatial biofilm community and its activity using various methods and the connection with reactor performance is shown in Fig. 1. The combined information from various approaches will contribute to the further clarification of the mechanism underlying treatment activities and highlight unfavorable fluctuations. Furthermore, the information will be used to construct a novel and reliable mathematical model for the biofilm reaction based on the microscale activities and spatial organization of biofilm communities that have previously been regarded as a black-box.

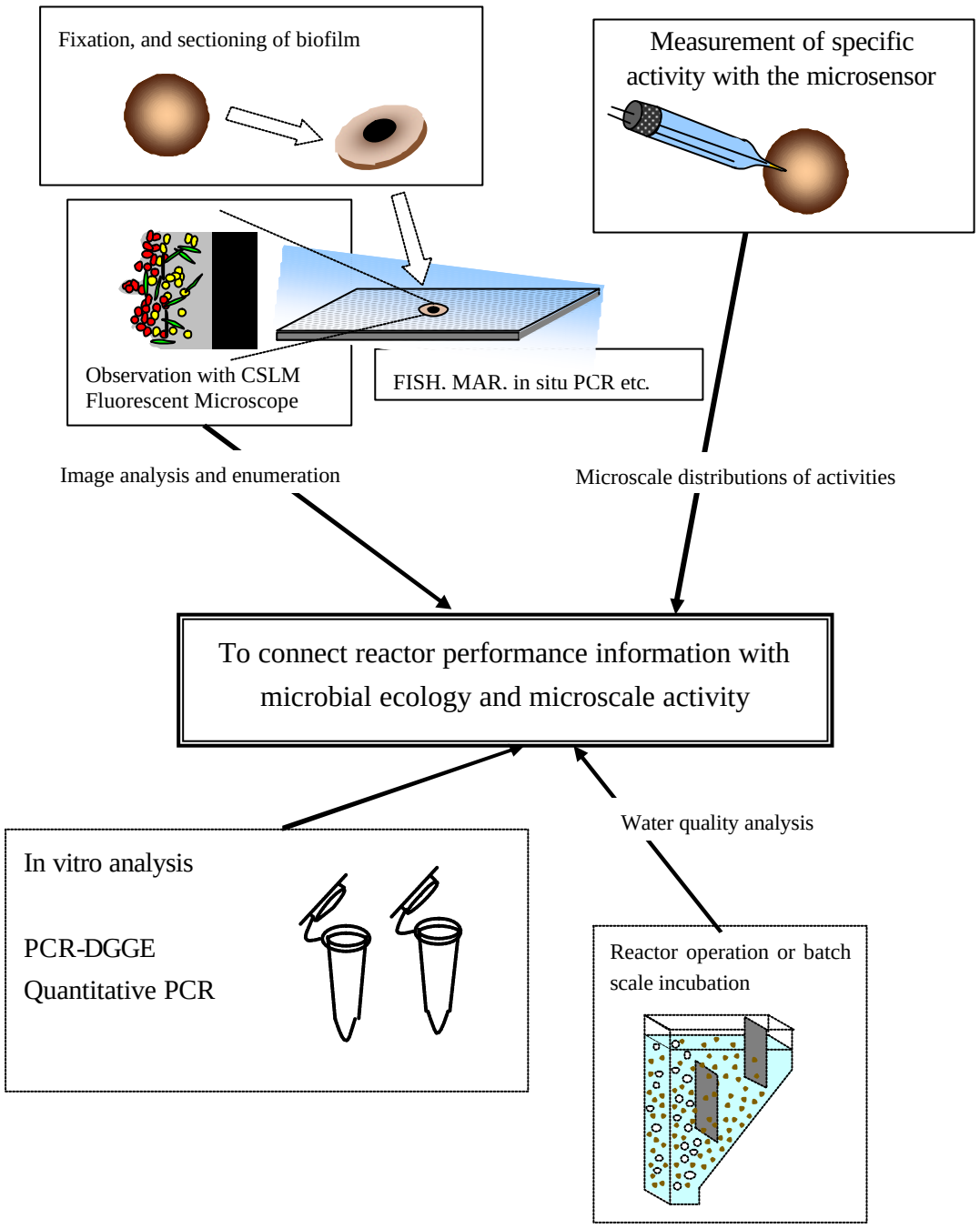


FIG. 1. Schematic for the analysis of the *in situ* organization of a biofilm community using various methods and the connection with reactor performance.

1.4 CONCLUSION

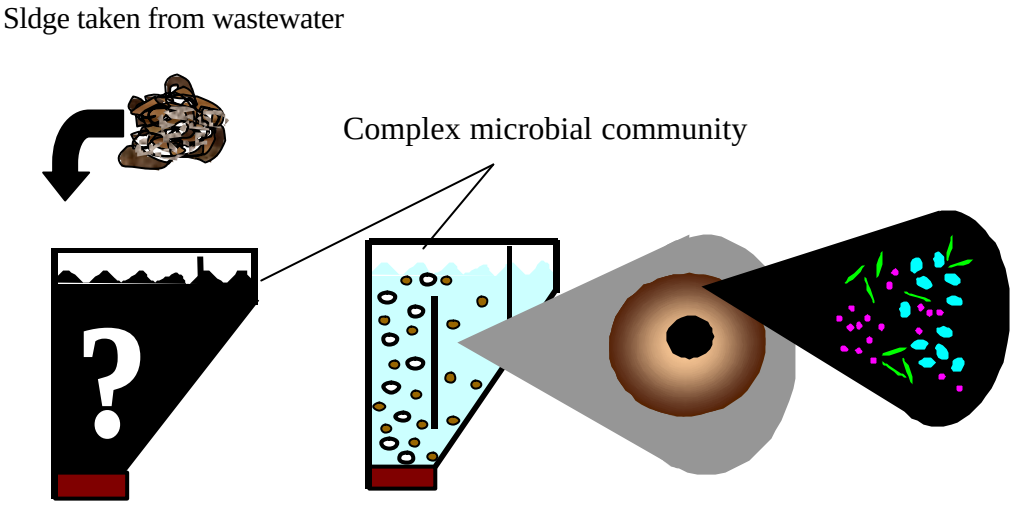
The tools or approaches for *in situ* phylogenetic identification of bacteria in biofilm communities have been established due to the advances, in the FISH method and combining other methods with FISH. On the other hand, techniques for *in situ* identification of single-copy functional genes and their expression at the single-cell level have not been well established as reliable methods. Advances (the development of new methods or improvement of existing techniques) in the *in situ* monitoring of functional genes and gene expression in biofilm communities are required because such monitoring may supply direct and important information concerning not only phylogeny but also function, activity and physiology in biofilms at the single-cell level.

The application of molecular-based high-resolution methods has changed the scale of investigation and has contributed to the better understanding of the microbial ecology and phenomena in biofilms. The target for the next decade is to set out the general rules governing community development in biofilm communities, such as those governing the relationship between operating conditions, dominant species and spatial organization, and interpretation of these rules using a mathematical model.

1.4 OBJECTIVE OF THE STUDY

The main purpose of this study is to clarify the microbial ecology in biofilm communities, especially nitrifying bacteria in the wastewater treatment processes and applied above

information to the improvement of treatment activity and development of new processes.



The detael mechanism of biological wastewater treatment system has been regarded as a BLACK- BOX.

- What kinds of bacteria? (Species)
- How many? (Number)
- Where? How? (distribution)

Molecular biological techniques → To open the BLACK-BOX

Applying to wastewater treatment

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