Chapter 1

Introduction

1.1 BIOFILMS

A *biofilm* is “microorganisms attached to a surface embedded in an organic matrix of biological origin”. Biofilms are found in almost every environment faced with surfaces, sufficient nutrient and some water. They range from growth on the leads of cardiac pacemakers, thorough biofilm attached to the inner surface of water distribution pipes, to the epilimnion of rocks in streams and accumulated plaque on the surface of teeth.

During biofilm development, a large number of phenomena occur simultaneously and interact over a wide range of length and time scales. As a result of nutrient conversions, the biofilm expands on the basis of bacterial growth and production of extracellular polymeric substances (EPS). Chemical species need to be continuously transported to and from the biofilm system by physical processes such as molecular diffusion and convection. Fluid flow influences biofilm growth by determining the concentrations of available substrates and products. On the other hand, the flow also shears the biofilm surface, and determines biofilm detachment processes. In the case of multi-species systems, microorganisms of different species interact in complex relationships of competition or cooperation. All these linked phenomena create a dynamic picture of the biofilm three-dimensional (3D) structure. The large number of localized interactions poses an important challenge for experimentalists. Mathematical models can prove useful because they allow testing of hypotheses and, in addition, can direct experimental efforts to complex regions of operation that can easily confound the general intuition. Although the word “modeling” is used for different purposes, the final result is invariably the same: models are no more than a simplified representation of reality based on hypotheses and equations used to rationalize observations. By providing a rational environment, models can lead to deeper and more general understanding. Ultimately, understanding the underlying principles becomes refined to such a state that it is possible to make accurate predictions.

1.2 HETEROGENEITY OF BIOFILM STRUCTURE

1.2.1 Heterogeneity of biofilm

The general view on microenvironemt in biofilm has dramatically changed during the last decade. It has been previously assumed that most of the biofilms are more or less homogeneous layers of microorganisms in a slime matrix. The use of Confocal Scanning Laser Microscopy (CSLM), and computerized image analysis tools revealed a more complex picture of biofilm morphology (Lawrence et al., 1991; Caldwell et al., 1993). CSLM and other optical investigations have shown that some biofilms possess a heterogeneous structure (Costerton et al., 1994; Gjaltema et al., 1994). Cell clusters may be separated by interstitial voids and channels, that create a characteristic porous structure. In some particular cases, biofilms grow in the form of microbial clusters taking a "mushroom" shape (Figure 1.1a, b – reproduced from Stoodley et al., 1999b). In other cases, more compact and homogeneous biofilm layers can be observed.

In many biofilms the reported nonuniformaties are other than gradients in only one direction, perpendicular to the substratum. Three-dimensional variation of microbial species, biofilm porosity, substrate concentration or diffusivities has been repeatedly reported. It is becoming clear that there are many forms of heterogeneity in biofilms, and a definition of biofilm heterogeneity is needed. According to Bishop and Rittmann (1995), heterogeneity may be defined as "spatial differences in any parameters we think is important". An adapted list from Bishop and Rittmann (1995), summarizes a few examples of possible biofilm heterogeneity:

1. Biological heterogeneity: microbial diversity of species and their spatial distribution, differences in activity (growing cells, EPS producing, dead cells, etc.).
2. Chemical heterogeneity: diversity of chemical solutes (nutrients, metabolic products, inhibitors), pH variations, diversity of reactions (aerobic/anaerobic, etc.).
3. Geometrical heterogeneity: biofilm thickness, biofilm surface roughness, biofilm porosity, substratum surface coverage with microbial biofilms.
4. Physical heterogeneity: biofilm density, biofilm permeability, biofilm visco-elasticity, viscosity, EPS properties, biofilm strength, solute concentration, solute diffusivity, presence of abiotic solids.

It appears that biological heterogeneity causes in a great measure the other kinds of heterogeneity, therefore our attention was primarily focused on this type of heterogeneity.

1.2.2 Importance of microenvironment in biofilm

Activity of microbes in biofilms is often notably different from that observed when they are in the suspended planktonic phase. Microbial cells enclosed in a biofilm matrix show significant advantages in relation to their planktonic counterparts, namely in the resistance to aggressive agents, such as increased resistance to disinfectants and antibiotics (Ceri et al., 2001) and to ultraviolet radiation (Elasri &Miller, 1999), drying (EPS are highly hydrated) and protection from grazing by predators such as protozoa.

Conversely, there are also notable disadvantages for bacteria when growing in a biofilm, such as an increased competition for limiting resources and increased mass transfer resistance, interference competition by production of antibiotics, overgrowth, and increased pressure from parasites. Most of such observed characteristics of microbial growth in biofilms can be explained by invoking transport phenomena, i.e. the physical implications of growth in densely packed environments where fluid flow is reduced. In sufficiently thick biomass clusters, as are generally the case in biofilms, diffusional distances are long enough that solute transport to inner bacterial cells becomes slow in comparison with the bioconversion kinetics of the microorganisms. In such situations, solute gradients are formed throughout the biofilm and mass transport becomes the rate-limiting process of the various biotransformations occurring (Characklis et al., 1990). In these environments, solute gradients provide favorable conditions for the creation of functional micro-niches. For example, the depletion of oxygen in proportion to depth observed in activated sludge flocs (Schramm et al., 1999; Meyer et al., 2003) and biofilms (de Beer et al., 1993) can create microenvironments suitable for the proliferation of anaerobic organisms, despite the presence of dissolved oxygen in the surrounding liquid phase. Solute gradients in oral biofilms also account for the local acidity that causes caries. In dental plaque, acidogenic and aciduric (acid-tolerating) bacteria rapidly metabolize dietary sugars to acids, which gradually accumulate, creating acidic microenvironments that are at the same time responsible for enamel demineralization (tooth decay) and for inhibiting competition from species associated with enamel health (Marsh, 2003). Mass transport limitations that impede efficient antibiotic penetration in biofilm matrices are frequently appointed as possible mechanisms responsible for the mentioned resistance to antibiotics (for references, see Mah & O'Toole, 2001). In light of these facts, an interpretation of the biofilm behavior from the extrapolation of the planktonic cell is not possible without knowledge of the mass transfer processes, in this complex morphology, responsible for the creation of the microenvironments (de Beer & Schramm, 1999).

1.3 BIOFILM MODELING

1.3.1 Why biofilm modeling?

To study the complex interaction between many of the factors acting simultaneously, we need a mathematical model. Besides experimentation, mathematical abstraction of the reality can help understanding interdependence of biofilm processes. The IAWQ International Specialty Conference on Microbial Ecology of Biofilms (Lake Bluff, IL, October 1998) provided updated information on current issues in biofilm research. According to their destination, biofilm models can be broadly classified into two categories:

1. Biofilm models for practical engineering applications, such as design, troubleshooting,

real-time operation, and education.
2. Advanced models used as research tools to investigate specific processes occurring within microbial biofilms. The application of these models is primarily intended to fill gaps in our knowledge of biofilm dynamics.

Regardless of the type of application, biofilm models should be realistic. That is, a model should not attempt to include all possible phenomena occurring within a biofilm, but should be able to accurately represent the specific facts that it is intended to simulate.

In relation to practical engineering applications, the current objectives of biofilm modeling include biofilm engineering, real-time control, and applications in education. These objectives are briefly described in the following section:

1. Biofilm Engineering. If more insight is gained on the interactions between processes involved in biofilm formation, then it would be possible to "engineer" the biofilm structure and its function. For example, we may envision the manipulation of the environmental conditions to generate dense biofilm structures that will be easily separated from a liquid phase (e.g., granules in UASB reactors, in fluidized bed or airlift reactors), or rough biofilm structures with high capacity for removal of particulate material.
2. Real-Time Control. The ability to control biofilm systems on-line requires mathematical models that incorporate both the activity of the biofilms and the stochastic behavior of system inputs.
3. Education. Biofilm models are also learning tools. If mathematical models of biofilms are to be used as design and simulation tools, it is essential to teach the fundamentals of these models to scientists and engineers. Moreover, a better understanding of basic physical and computational principles, as well as of the benefits and limitations of existing models, would contribute to an increased appreciation of the mathematical model as a basic tool for research and practical applications.

The current use of biofilm models as research tools has broader objectives, most of them related to gaining a better understanding of 3-D biofilm structure, of population dynamics, and of mechanical factors affecting biofilm formation:

1. Relevance of 3-D heterogeneity. With the abundant experimental evidence showing that biofilm structures are heterogeneous, the simplifying assumptions of 1-D models are in question. As important as the development of useful models for biofilm engineering is the critical evaluation of these original assumptions. It is fundamental to propose and develop unifying parameters to describe biofilm structure and to investigate trends within the biofilms. It is equally significant to evaluate the importance of biofilm heterogeneity on overall biofilm reactor performance.
2. Microbial Ecology. Novel experimental methods are continuously producing more evidence of the heterogeneous nature of multispecies biofilms. Even though it is possible to develop hypotheses on the ecological interactions among different

microorganisms based on the experimental observations, mathematical modeling is a key tool to evaluate the adequacy of the hypotheses.

3. Microorganisms as producers. Microbial products of interest include chemical transformation products, soluble organic compounds from autotrophic bacteria, as well as gases, EPS substances, and perhaps quorum sensing factors.
4. Analysis of potential detachment mechanisms. Advanced mathematical modeling of biofilms can be used to understand the effect of hydrodynamic flow and shear forces on the erosion and sloughing mechanisms in biofilms. These mathematical efforts need to be complemented with experimental information on mechanical properties of biofilms, such as elasticity and tensile resistance as a function of EPS and cell content.

1.3.2 Past and present in biofilm modeling

Mathematical models have been used for the last three decades as tools to simulate the behavior of microbial biofilms. The initial models described biofilms as uniform steady-state films containing a single type of organism (Fig. 1.1a), governed exclusively by one-dimensional (1-D) mass transport and biochemical transformations (Atkinson and Davies, 1974; Rittmann and McCarty, 1980). Later, stratified dynamic models (Fig. 1.1b) able to represent multisubstrate-multispecies biofilms (Wanner and Gujer, 1986; Wanner and Reichert, 1996) were developed. Although these 1-D models were advanced descriptions of multispecies interactions within the biofilm, they were not able to provide the characteristic biofilm morphology. Biofilm morphology is an input in these models, not and output. Structural heterogeneity in biofilms was already known at that time, but it has been recently underlined through numerous experimental observations. New biofilm models are needed now, to provide more complex two- and three-dimensional descriptions of the microbial biofilm (Fig. 1.1c), and incorporate solutes mass transport and transformation, population dynamics and hydrodynamics. This evolution in model complexity has paralleled the advances in computational tools. While hand calculators were the tools used in the 1970's, the biofilm models of today reflect the availability of fast personal computers and advanced parallel processing.

The amount of experimental evidence describing some biofilms as heterogeneous entities in structure and composition contradicts the simplifying assumptions of the original 1-D models. This has challenged engineers to create a more accurate mathematical description of biofilms. The challenge has resulted in an increasing model complexity, derived from the inclusion of an ever increasing number of parameters to explain the biofilm structure. The new generation of structural biofilm models should describe/predict the formation of microcolonies, the development of heterogeneous colonization patterns, the sloughing of large biofilm sections. They could be further expanded to simulate experimentally observed phenomena such as formation of streamers and advective flux through microchannels. Nevertheless, the real challenge to the modeler is not to create models that include as many parameters as possible, but rather, to determine the level of significance of these parameters in the description of the

different biofilm processes. Moreover, the mathematical evaluation of parameter significance is essential to define the required level of accuracy of experimental measurements.

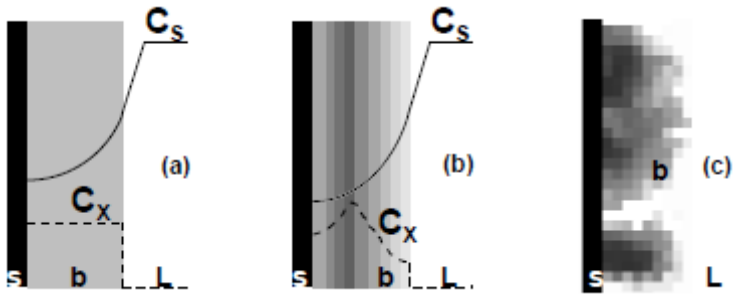

Figure 1.1 Evolution of biofilm models from (a) uniform biomass distribution and one-dimensional substrate gradient in the 1970's, to (b) one-dimensional stratified biomass and multispecies biofilms in the 1980's, to (c) multidimensional distribution of biomass and substrate at the end of 1990's.

1.3.3 Biofilm modeling with AQUASIM (Wanner et al., 2004)

AQUASIM is a computer program for the identification and simulation of aquatic systems. The program includes a 1-D multisubstrate and multispecies biofilm model and represents a suitable tool for biofilm simulation. The program can be used to calculate substrate removal in biofilm reactors for any user specified microbial systems. 1-D spatial profiles of substrates and microbial species in the biofilm can be predicted. The program also caiculates the development of the biofilm thickness and of the substrates and microbial species in the biofilm and in the bulk fluid over time. Detachment and attachment of microbial cells at the biofilm surface and in the biofilm interior can be considered, and simulations of sloughing events can be performed. Futhermore, AQUASIM allows pseudo 2-D modeling of plug flow biofilm reactors by a series of biofilm reactor compartments. The most significant limitation of the model is that it only considers spatial gradients of substrates and microbial species in the biofilm in the direction perpendicular to the substratum.

1.3.3.1 Features of the biofilm model implemented in AQUASIM

For biofilm modeling and simulation, AQUASIM offers a biofilm reactor compartment consisting of three zones: "bulk fluid," "biofilm solid matrix," and "biofilm pore water" (Fig. 1.2). For all three zones, AQUASIM calculates the development over time of microbial species and substrates, as well as the biofilm thickness. In the biofilm, spatial gradients perpendicular to the substratum are calculated for microbial species and substrates. The bulk fluid is assumed to be completely mixed, and a liquid boundary layer between the biofilm and the bulk fluid can be considered. The AQUASIM biofilm reactor compartment can be connected to other compartments. Solid arrows in Fig. 1.2 indicate possible mass fluxes across the compartment boundaries. These fluxes include influent, effluent, exchange between the bulk fluid and the atmosphere, and transport

across a permeable substratum. Shared arrows indicate mass fluxes between the various zones in the compartment. These fluxes account for detachment and attachment of microbial cells in the biofilm and at the biofilm surfaces and diffusion of soluble and suspended particulate compounds through the liquid boundary layer.

In the AQUASIM dialog box "Edit Biofilm Reactor Compartment", the properties of the biofilm system tobe modeled are specified. The reactor type is chosen to be "confined" if the volume of the biofilm plus the bulk fluid is constant, as is the case in a closed reactor, and to be "unconfined" if the biofilm can grow freely, as may be the case in a trickling filter. The pore volume can be specified to contain only a liquid phase and dissolved substrate, or it can also contain suspended solids. The biofilm matrix can be assumed to be rigid, *i.e.*, to change its volume due to microbial growth and decay only, or it can be assumed to be diffusive, which means that microbial cells can move within the biofilm matrix also by diffusion. Detachment at the biofilm surface can be described by rates, which are properties of individual microbial species and are specified via the button "Particulate Variables." Otherwise, it can be described by a global velocity, which means that all species are detached at the same rate.

The option "Variables" serves to activate or inactivate variables, which denote concentrations of substrates and microbial species. For each activated variable, AQUASIM automatically calculates mass balance equations for the substrates and microbial species in both the biofilm and the bulk fluid. The option "Processes" serves to activate or inactivate processes. Only activates processes are included in the calculation, while the value of the rates of inactivated processes is set to zero. This feature makes it possible to easily modify a model and to readily test alternative models. In AQUASIM, the term "Processes" refers to biotic or abiotic conversion reactions. There have to be specified by the user, while the equations describing transport processes are intrinsic parts of AQUASIM. The example shows the rate law and the stoichiometric coefficients of the process "heterotrophic growth." The options "Initial Conditions" and "Input" serve to provide initial and influent values for the microbial species and substrates, as well as for the water flow rate.

The properties of the microbial species considered are specified via the button "Particulate Variables." The density, defined as cell mass per unit cell volume, is the only properties that must be specified at all times. AQUASIM is set up such that additional features of the model are omitted if their parameters have a value of zero. These features include attachment of cells to the biofilm surface and to the solid matrix within the biofilm, individual detachment of cells from the biofilm surface or solid matrix, and cell diffusion in the pore water and in the solid matrix. Furthermore, the implementation of the model considers a liquid boundary layer at the biofilm surface that is omitted if the value of its resistance is set to zero. The button "Dissolved Variables" leads to a dialog box in which the properties of the dissolved substrates can be specified. The diffusivity of the substrate in the pore water of the biofilm must be specified, while the boundary layer resistance can be set to zero.

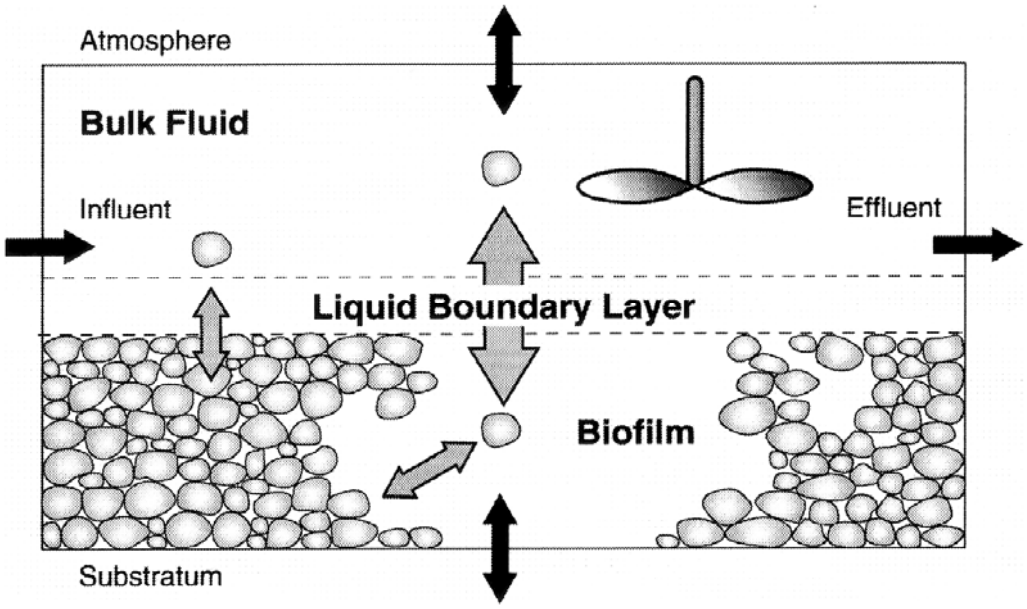

Figure 1.2 Setup of the AQUASIM biofilm reactor compartment. Solid arrows indicate possible mass fluxes across compartment boundaries, and shaded arrows indicate mass fluxes within the compartment. Taken from Wanner *et al*. (2004).

1.3.4 Particle-based multidimensional multispecies biofilm model (Picioreanu et al., 2004)

In general, a quantitative representation of the system studies is superior to a merely qualitative picture. This is one of the reasons for expressing hypotheses in mathematical form. A mathematical model consists of the full set of equations abstracting the information required to simulate a system. A rigorous and mechanistic representation of the real system must be based on the fundamental laws of physics, chemistry, and biology, which area also called first principles. The development of models based on first principles enforces systematic and imposes rational methods for approaching a problem. For example, biofilm models based on reaction and transport principles (Wanner *et al*., 1986) have proven to be useful not only for testing the soundness of different scientific concepts but also for establishing rational strategies for designing bioflm systems. Models based on first principles provide a unified view of microbial growth system, including biofilms. They promote lateral transfer of insight between various scientific domains. Diffusion-reaction models routinely used in chemical engineering are now widely used to simulate biofilm systems (Wanner et al., 1986 and 1996). Fluid mechanics methods have been used to study biofilm theology (Dockery et al., 2001; Stoodley et al., 1999) and hydrodynamic conditions in the liquid environment surrounding a biofilm

matrix (Dillon et al, 2000 and 2001; Dupin et al., 2001; Eberl et al, 2001; Picioreanu et al., 2000a, 2000b and 2001). Laws of structural mechanics and finite element analysis method of civil engineering, have been used to study biofilm growth and detachment (Dupin et al., 2001; Picioreanu et al., 2001).

When a quantitative mathematical model of biofilm structure and function is constructed, it is advantageous to construct the model from submodels, each of which describes one of the various ongoing processes in a biofilm, including (1) biomass growth and decay, (2) biomass division and spreading, (3) substrate transport and reactions, (4) biomass detachment, (5) liquid flow past the biofilm, and (6) biomass attachment. Attachment is an important process because it determines the initial pattern of colonization of the substratum and the possible immigration of any type of cell from the liquid phase to various locations in the existing biofilm, The advantages of a modular biofilm model are manifold and include a better understanding of specific phenomena, better validation of individual model components, the possibility of exchanging routines or submodels with other biofilm models, more flexibility in solving decoupled model equations, and reflection of the modular structure of biofilm communities and processes.

For the processes described above, proper representation of biomass division and spreading is one of the most controversial topics. The difficulty in modeling the spreading of microbial cells inside colonies is that there must be a mechanism to release the pressure generated by the growing bacteria. Different solutions have been proposed, but given the lack of experimental evidence, none can claim to be correct. Current models for biofilm structure deal with bacteria in two different ways, depending mostly on the intended spatial scale of the model. One approach, individual-based modeling (IbM), attempts to model the biofilm community by describing the actions and properties of individual bacteria (Kreft *et al*., 1998 and 2001). IbM allows individual variability and treats bacterial cells as the fundamental entities. Essential state variables are, for example, the cell biomass (m), the cell volume (V) etc. The other approach treats biofilms as multiphase systems and uses volume averaging to develop macroscopic equations for biomass dynamics. These models can be called biomass-based models because they use the mass of cells per unit of volume (density or concentration $[C_X]$) as the state variables for biomass. A comprehensive analysis of conditional tool has been performed (Wood *et al*., 1998 and 1999). The biomass-based models can be further divided into two classes on the basis of the mechanism used for biomass spreading. One subclass includes discrete biofilm models (*i.e*., cellular automata), in which biomass can be shifted only stepwise along a finite number of directions according to a set of discrete rules (Hermanowics et al., 1998 and 2001; Noguera et al., 1999; Picioreanu et al., 1998a and 1998b; Pizarro et al., 2001; Wimpenny et al., 1997). The other subclass of biomass-based models treats biomass as a continuum, and biomass spreading is generally modeled by differential equations widely used in physics (Dockery et al., 2001; Dupin et al., 2001; Eberl et al., 2001)

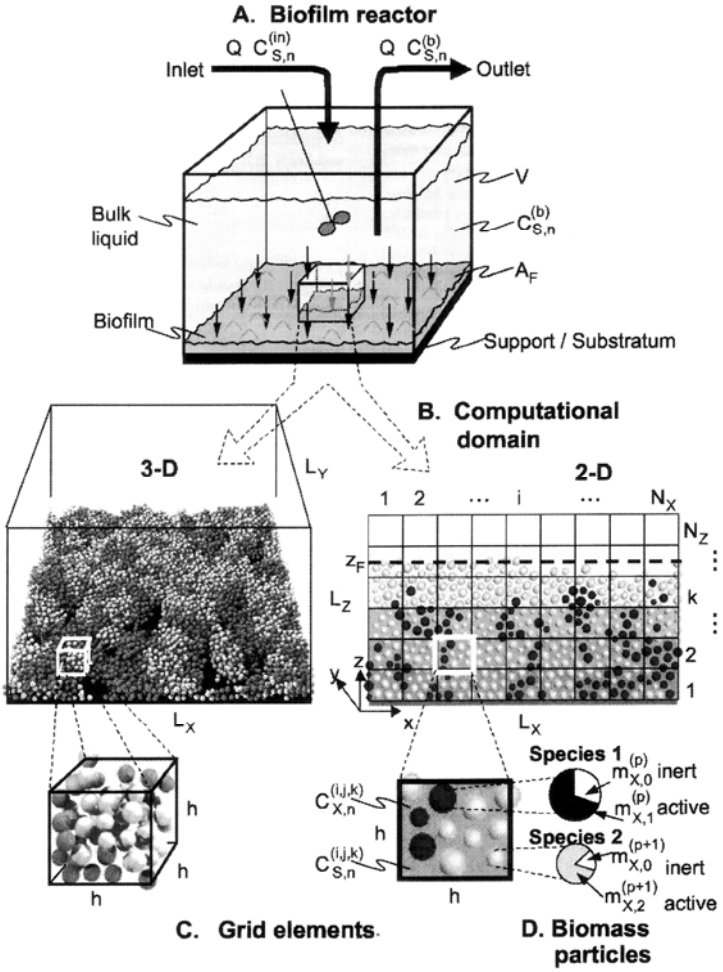

Figure 1.3 Model biofilm system. (A) Continuously stirred tank rector containing a liquid phase in contact with a biofilm growing on a planar surface. (B) Rectanglar computational domain (2-D or 3-D) enclosing a small part of the whole biofilm. (C) Rectangular uniform grid used for solution of the partial differential equations for substrate diffusion and reaction. (D) Biofilm biomass contained in spherical particles holding one type of active biomass, as well as inactive biomass. Taken from Picioreanu *et al*. (2004)

1.4 APPLICATION OF BIOFILM TO REACTOR

1.4.1 Origin of nitrogen pollusion

Nitrogen compounds are essential for all living organisms since it is a necessary element of DNA, RNA and proteins. Although it is composed of 78% of the earth's atmosphere as nitrogen gas, almost all bacteria except a few organisms cannot utilize this form of nitrogen directly. In many situations, fixed nitrogen is the limiting nutrient because its availability is usually much smaller than the potential uptake by, for example, plants (Pynaert, 2003). Hence, the supply of protein food for the global population by

agriculture is recently dependent on the use of synthetic nitrogen fertilizer generated from atmospheric N_2 by the Haber-Bosch process. The global estimation for biological nitrogen fixation is in the range of 200-240 Mt nitrogen, which indicates that the mass flows for nitrogen have a major impact on the global nitrogen cycle (Gijzen and Mulder, 2001).

The consumption of protein will yield the discharge of organic nitrogenous compounds in wastewater (Van Hulle, 2005). Some nitrogenous compounds derived from fertilizer accumulate and end up in wastewater in the form of ammonium or organic nitrogen. Other polluting nitrogenous compounds are nitrite and nitrate. Nitrate is originally used to make fertilizers, even though it is also used to make glass, explosives and so on. Nitrite is manufactured mainly for use as a food preservative. These nitrogenous compounds, *i.e.*, organic nitrogen, ammonia, nitrite and nitrate, exist ubiquitously.

The discharge of these nitrogenous compounds into water environment results in several environmental and health problems. Essentially, ammonia is a nutrient for plants and it is responsible for eutrophication, *i.e.*, undesirable and excessive growth of aquatic plants and algae. Such excessive growth of the aquatic vegetables would cause a depletion of oxygen since they consumes oxygen in the water, which has a significant impact on viability of fish. Additionally, the growth of the vegetables determines oxygen and pH of the surrounding water. The greater the growth of algae, the wider the fluctuation in levels of dissolved oxygen (DO) and pH will be. This affects metabolic processes in organisms seriously, leading to their death. Besides that, some blue-green algae have a potential to produce algal toxins, which fatally kill fish and livestock that drink the water (Antia *et al*., 1991). Ammonia itself is also toxic to water environmental organisms at concentration below 0.03 g-NH_3-N/L (Solbe and Shurben, 1989). Nitrate pollution impeded the production of drinking water critically. Nitrite and nitrate in drinking water can result in oxygen shortage of newly born, which is alternatively called ‘blue baby syndrome’ (Knobeloch *et al.*, 2001) and, during chlorination of drinking water, carcinogenic nitrosamines may be formed by the interaction of nitrite with compounds containing organic nitrogen. Therefore, nitrogenous compounds need to be removed from wastewater. For the removal of nitrogen, a wide variety of biological removal systems are available (Henze *et al.*, 1995).

1.4.2 Biological nitrogen removal

Inorganic nitrogen, which comes from domestic and industrial wastewater, is normally found in most reduced form, ammonia. In wastewater treatment, nitrogen removal with microorganisms (bacteria) is most widely applied in wastewater treatment plant because biological nitrogen removal is less costly and less harmful to water environment than physicochemical counterpart. In the biological nitrogen removal, complete nitrogen removal is achieved by two successive processes: nitrification and denitrification.

Nitrification process

Nitrification is the aerobic oxidation of ammonia to nitrate (Rittmann and MaCarty, 2001). It is an essential process prior to the actual nitrogen removal by denitrification. The process consists of two sequential steps that are performed by tow phylogenetically unrelated groups of aerobic chemolithoautotrophic bacteria and, to a minor extent, some heterotrophic bacteria. In the first step, ammonia is oxidized to nitrite by ammonia-oxidizing bacterial (AOB) and, in the second step, nitrite-oxidizing bacteria (NOB). Sometimes AOB and NOB are summarized as nitrifiers. The stoichiometry for both reactions is given in equations 1.1 and 1.2, respectively (U.S. Environmental Protection Agency, 1975). In these cases, typical values for AOB and NOB biomass yield are used as follows:

Ammonia oxidation (nitritation)

$$55NH_4^+ + 76O_2 + 109HCO_3^- \rightarrow C_5H_7NO_2 + 54NO_2^- + 57H_2O + 104H_2CO_3 \quad (1.1)$$

Nitrite oxidation (nitrataion)

$$400NO_2^- + NH_4^+ + 4H_2CO_3 + HCO_3^- + 195O_2 \rightarrow C_5H_7NO_2 + 3H_2O + 400NO_3^- \quad (1.2)$$

Both groups of bacteria are chemolithoautotrophic and obligatory aerobic. Autotrophic means that they definitely fix and reduce inorganic carbon dioxide (CO_2) for biosynthesis, which is an energy-expensive process. Such very unique characteristic of nitrifiers makes their yield values lower than that of aerobic heterotrophic bacteria. The fact that they utilize a nitrogen electron donor even lowers their cell yield due to less energy release per electron equivalent compared to organic electron donors. As a consequence, both AOB and NOB are considered slow growing bacteria. Molecular oxygen is utilized for endogenous respiration and conversion of reactant ***i.e.***, ammonia or nitrite. It is generally known that nitrifies grow well at slightly alkaline pH (7.2-8.2) and temperature between 25-35°C (Sharma and Ahlert, 1977). At a pH below 6.5, no growth of AOB is observed probably due to limited ammonia availability at such low pH value (Burton and Prosser, 2001). The optimal DO for AOB and NOB is normally 3-4 g-O_2/m^3 (Barnes and Bliss, 1983), although levels of 0.5 g-O_2/m^3 (Hanaki *et al.*, 1990) and even 0.05 g-O_2/m^3 (Abeliovich, 1987) supported significant rates of ammonia oxidation but not nitrite oxidation (Bernet *et al.*, 2001).

1.4.3 Phylogeny of nitrifying bacteria

Nitrifying bacteria (nitrifiers) have minimal nutrient requirements owing to their true chemolithotrophic nature. Nitrifiers are obligate aerobes, and they use oxygen for respiration and as a direct reactant for the initial monooxygenation of ammonia (NH_4^+) to hydroxylamine (NH_2OH). The most commonly known genus of bacteria that carries out ammonia oxidation is *Nitrosomonas*; however, *Nitrosococcus, Nitrosopira, Nitrosovibrio,* and *Nitrosolobus* are also able to oxidize ammonia to nitrite. The AOB, which all have the genus prefix *Nitroso*, are genetically diverse, but are related to each other in the

β-subdivision of the proteobacteria (Teske *et al.*, 1994). This diversity suggests that neither the *Nitrosomonas* genus nor any particular species within it (*e.g.*, *N. europaea*) necessarily is dominant in a given system.

Although *Nitrospira, Nitrospina, Nitrococcus*, and *Nitrocystis* are recognized as NOB to sustain themselves from nitrite oxidation, *Nitrobacter* is the most famous genus of the NOB. Within the *Nitrobacter* genus, several subspecies are distinct, but closely related genetically within the α-subdivision of the proteobacteria (Teske *et al*. 1994). Recent findings using oligonucleotide probes targeted to the 16S rRNA of *Nitrobacter*, which indicates that *Nitrobacter* is not the most important nitrite-oxidizing genus in most wastewater treatment processes. *Nitrospira* more often is identified as the dominant NOB (Aoi *et al.,* 2000). Since nitrifiers exist in water environment and wastewater treatment plants where organic compounds are present, such as in wastewater treatment plants, it might seem curious that they have not evolved to use organic molecules as their carbon source. While the biochemical reason that organic-carbon sources are excluded is not known, the persistence of their autotrophic dependence probably is related to their evolutionary link to photosynthetic microorganism (Teske *et al.*, 1994).

1.4.4 Differential behavior of AOB and NOB

Several environment conditions affects the activity AOB and NOB. Generally, the amount of nitrate defines NOB activity under aerobic conditions. By setting optimal conditions, we can theoretically achieve not nitrite but ammonia oxidation since NOB are more sensitive to detrimental environmental conditions, *e.g.*, unusual pH, low DO, temperature, solid retention time and so on, than AOB. Among the most important environmental parameters influencing ammonia and nitrite oxidation are the free ammonia (FA) and free nitrous acid (FNA) concentration, temperature, pH and DO concentration. Engineering challenge is how we can differentiate the activity of AOB with NOB critically.

FA and FNA inhibition of nitrifiers

The uncharged nitrogen forms are considered to be the actual substrate/inhibitor for ammonia and nitrite oxidation. The amount of FA and FNA can be calculated form temperature and pH using following equilibrium equations:

$$NH_4^+ \overset{K_b}{\rightleftharpoons} NH_3 + H^+ \qquad (1.3)$$

With a typical K_b value of 5.68×10^{-10} at 25ºC and pH 7

$$HNO_2 \overset{K_a}{\rightleftharpoons} NO_2^- + H^+ \qquad (1.4)$$

With a typical K_a value of 4.6×10^{-4} at 25ºC and pH 7

where K_b and K_a are ionization constants of ammonia and nitrous acid, respectively.
The NH_3 and HNO_2 concentrations can be calculated from equations 1.5 - 1.8 proposed by Anthonisen *et al.* (1976):

$$FAasNH_3(g/m^3) = \frac{17}{14} \times \frac{(NH_4^+ - N) \times 10^{pH}}{K_b / K_w + 10^{pH}} \quad (1.5)$$

$$K_b / K_w = e^{(6344/273+T)} \quad (1.6)$$

$$FNAasHNO_2(g/m^3) = \frac{46}{14} \times \frac{(NO_2^- - N)}{K_a \times 10^{pH}} \quad (1.7)$$

$$K_a = e^{(-2300+273+T)} \quad (1.8)$$

where NH_4^+-N and NO_2^--N are ammonia- and nitrite-nitrogen concentrations, T is temperature in ºC, respectively. For these equilibriums 1.5 and 1.7, T and pH of the solution will determine the concentrations of FA and FNA. The toxicity effect of this FA and FNA on the two groups of nitrifiers has been described regarding a diagram proposed by Anthonisen *et al.* (1976). The diagram (Fig. 1.4), where the AOB are represented by *Nitrosomonas* and the NOB by *Nitrobacter*, indicates that inhibition of AOB by FA is likely in the rage of 10 to 150 g-N/m^3 while NOB are likely inhibited at significant lower concentrations of 0.1 to 1 g-N/m^3. In case of NOB, the key enzyme, a nitrite oxidoreductase (NOR), loses activity (Yang and Alleman, 1992). This difference in NH_3 sensitivity could give rise to nitrite accumulation when wastewater with high ammonia concentration is treated. However, adaptation of NOB to high FA levels is observed by Turk and Mavinic (1989). They reported that NOB appeared capable of tolerating ever-increasing levels of FA concentrations up to 40 g NH_3-N/m^3. At low pH less than 7, FNA affect the activity of AOB and NOB. According to Figure 1.1, a FNA concentration of 0.2-2.8 g HNO_3-N/m^3 inhibits NOB.

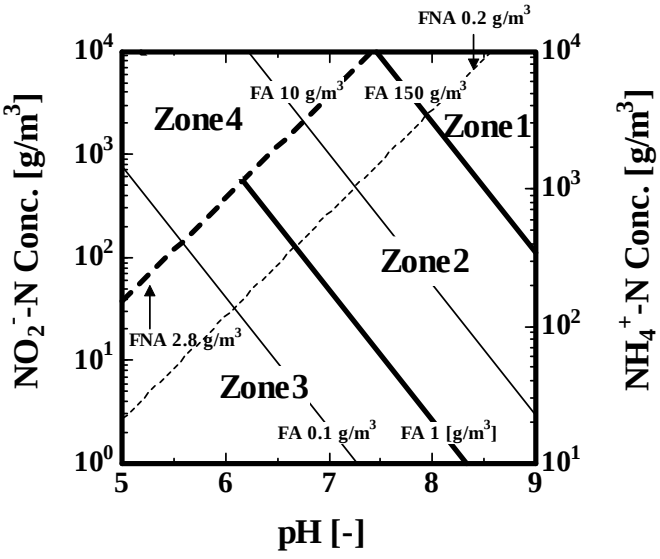

Figure 1.4 Dependence of free ammonia (FA) and free nitrous acid (FNA) on pH in the solution proposed by Anthonisen *et al.* (1976). Zone 1 shows FA inhibition of *Nitrobacter* and *Nitrosomonas*, Zone 2 shows FA inhibition of only *Nitrobacter*, Zone 3 shows complete nitrification, and Zone 4 shows FNA inhibition of *Nitrobacter*. Symbols: solid lines, FA of 0.1, 1, 10 and 150 mg l^{-1}, respectively; dotted lines, FNA of 0.2 and 2.8 mg l^{-1}.

Effect of oxygen

Both AOB and NOB require oxygen for their normal anabolism and catabolism. Low DO concentration will disrupt rates of ammonia and nitrite oxidation, leading to imbalance between the growth of AOB and NOB. The effect of DO on the specific growth rate of nitrifiers is generally governed by the Monod equation, where affinity constant of oxygen K_{O2} is a determining parameter. Considering the report that the constant for AOB and NOB are 0.6 and 2.2 g-O_2/m^3, respectively (Wiesmann, 1994), K_{O2} value for AOB are lower than that for NOB, indicating a higher oxygen affinity of AOB than that NOB at low DO concentrations. In such oxygen-limited systems, this feature could lead to a decrease in the amount of nitrite oxidation and therefore accumulation of nitrite (Bernet *et al*., 2001; Garrido *et al.*, 1997; Pollice *et al.*, 2002; Terada *et al.*, 2004).

Besides the direct inhibitory effect of low DO, there is also an indirect effect. AOB exposed to low DO levels have been shown to generate higher amounts of the intermediate hydroxylamine, which might be the determinant compound of nitrite build-up (Yang and Alleman, 1992). Kindaichi *et al*. (2004) clarified that the addition of hydroxylamine decreases the activity of NOB, which alternatively lead to an increase of AOB activity and changes of microbial community in an autotrophic nitrifying biofilm**.**

Effect of temperature

Temperature is a key parameter in the nitrification process; however, the exact influence has not been clarified because of the interaction between mass transfer, chemical equilibrium and growth rate dependency. Normally, both AOB and NOB have similar temperature ranges for their activities. Both organisms have maximum growth rates at a temperature of 35ºC (Grunditz and Dalhammar, 2001); however, they prefer moderate temperature (20-30ºC). The activities significantly decrease at temperatures below 20ºC and above 40-45ºC because of enzyme disruptions. Generally, AOB grow faster than NOB at temperatures of more than 25ºC, whereas this is reversed at lower temperatures around 15ºC. The SHARON process (Single reactor High activity Ammonia Removal Over Nitrite) employs such principle. In this process, nitritation, oxidation of ammonia to nitrite, is established in chemostat by operating under high temperature conditions (above 25ºC) and maintaining an appropriate sludge retention time (SRT), which is also a selection pressure between AOB and NOB. Such selective operation keeps AOB in the reactor, while NOB are washed out and further nitratation, oxidation of nitrite to nitrate, can be prevented. Nitrite build-up would be very useful when treating low carbon/nitrogen-containing wastewater because subsequent denitrification requires less organic carbon in case via nitrite than in that via nitrate.

Furthermore, considering the influence of temperature on microbial community between AOB and NOB, increased temperature will increase the ratio of NH_3/NH_4^+, possibly causing inhibitory effects on the NOB. Additionally, an increase of temperature decrease saturated DO concentration, leading to oxygen-limited conditions disrupting the imbalance of AOB and NOB with possible nitrite accumulation.

Effect of pH

In spite of a wide divergence of the reported effects of pH on nitrification, it is generally known that the optimum pH range for both AOB and NOB is from 7.2 to 8.2 (Pynaert, 2003). The range is also related to NH_3/NH_4^+ and NO_2^-/HNO_2 ratios, where FA and FNA can exhibit inhibitory effects starting from certain pH. Ammonia oxidation brings acidifying conditions when it occurs (see equation 1.1). If buffer capacity of this environment is too low, the pH will decrease rapidly. Below pH 7, nitrification rate decrease even though there are some reports of nitrifying activity in acidic environments (Burton and Prosser, 2001; Tarre *et al.*, 2004a, b).

Denitrification process

Denitrification is the dissimilatory reduction of nitrate or nitrite to mainly nitrogen gas. In other words, nitrate or nitrite is the electron acceptor used in energy generation. Denitrification normally occurs among heterotrophic and autotrophic bacteria, many of which can shift between oxygen respiration and nitrogen respiration. Denitrifying bacteria (denitrifiers) are common among the Gram-negative *Proteobacteria*, such as *Pseudomonas*, *Alcaligenes*, *Paracoccus* and *Thiobacillus*. Some Gram-positive bacteria, including *Bacillus*, can also denitrify. Even a few halophilic Archaea, such as *Halobacterium*, are able to denitrify. The denitrifiers used in environmental biotechnology are chemotrophs that can use organic or inorganic electron donors. Those that utilize organic electron donors are heterotrophs and are widespread among the *Proteobacteria*. Inorganic electron donors also can be used and gaining popularity (Rittmann and MaCarty, 2001). Hydrogen (H_2) is an excellent electron donor for autotrophic denitrification. Its advantages include lower cost per electron equivalent compared to organic compounds, less biomass production than with heterotrophs, and absolutely no reduced nitrogen added. The main disadvantage of H2 in the past has been lack of a safe and efficient H_2 transfer system. The recent development of membrane-dissolution devices overcomes the explosion hazard of conventional gas transfer and makes H_2 a viable alternative (Lee and Rittmann, 2000, 2002). Reduced sulfur also can drive autotrophic denitrification. The most common source of reduced S is elemental sulfur, S(s), which is oxidized to SO_4^{2-}. The S normally is embedded in a solid matrix that includes a solid base, such as $CaCO_3$, because the oxidation of S(s) generates strong acid.

During biological heterotrophic denitrification, oxidized nitrogen forms are reduced and an organic electron donor is oxidized for energy conservation. This electron donor can be the organic material present in wastewater, or, in case of shortage, an externally added carbon source, *e.g.*, acetate. An example of a denitrification reaction is given in equation 1.9, where nitrate is denitrified to nitrogen gas with acetate as an electron donor.

$$CH_3COOH + 8/5\ NO_3^- + 4/5\ H_2O \rightarrow 4/5\ N_2 + 2\ H_2CO_3 + 8/5\ OH^- \quad (1.9)$$

The pathways of denitrification are composed of four steps (equation 1.10). Each of the reduction steps is catalyzed by respective enzymes, *i.e.,* nitrate reductase, nitrite

reductase, nitric oxide reductase and nitrous oxide reductase.

$$NO_3^- \rightarrow NO_2^- \rightarrow NO\ (gas) \rightarrow N_2O\ (gas) \rightarrow N_2\ (gas) \qquad (1.10)$$

NO and N_2O are gaseous intermediates, which have to be avoided. Since the greenhouse effect of N_2O is reported to be 300 times higher than that of CO_2 (IPCC, 2001), emission of N_2O should be reduced from wastewater (Tsuneda *et al.*, 2005).

1.4.5 Application to novel nitrogen removal

Nitrogen removal via nitrite

As already mentioned in the previous chapters, nitrite is an intermediate in both nitrification and denitrification (Fig. 1.5). Accumulation or discharge of nitrite should be harmful to aqueous environment; hence, nitrite should be removed properly. Normally, ammonia is converted into nitrate by AOB and NOB under aerobic conditions; subsequently the nitrate is converted into nitrogen gas by denitrifiers under anoxic conditions. Such pathway via nitrate requires more oxygen for nitrification and organic carbon for denitrification than that via nitrite. Numerous environmental engineers have been focusing on biological nitrogen removal via not nitrate but nitrite because of economical advantages. Concretely, the nitrification-denitrification via nitrite saves around 25% on oxygen input for nitrification and 40% of organic carbon for denitrification (Abeling and Seyfried, 1992; Bernet *et al.*, 1996; Eum and Choi, 2002; Oh and Silverstein, 1999; Turk and Mavinic, 1986). It also enables required hydraulic retention time (HRT) to decrease, which could achieve a small reactor volume.

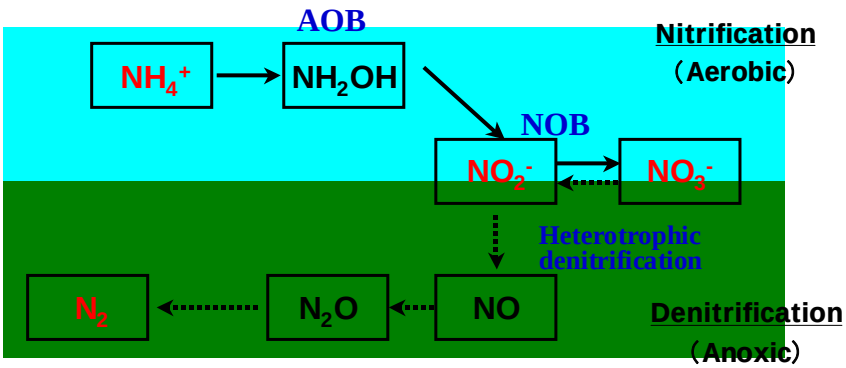

Figure 1.5 Schematic diagram of biological nitrogen removal pathway.

Simultaneous nitrification and denitrification with biofilm

Nitrification and denitrification are complementary in many ways: (1) nitrification produces nitrite or nitrate that is a reactant in denitrification; (2) nitrification reduces the pH that is raised in denitrification; and (3) denitrification generates the alkalinity that is required in nitrification (Rittmann and MaCarty, 2001). Therefore, there exists obvious advantage to carry out simultaneous nitrification and denitrification in a single reactor. In that case, it is essential to make redox stratification, *i.e.* reaction sites for aerobic and anoxic part in a single reactor. In this thesis, the author is focusing on bacterial

aggregated layer on surfaces, biofilm. The Biofilms have chemical gradients because of its thickness, leading to creation aerobic and anoxic part inside; therefore, they can theoretically provide such stratification. Engineering challenges are how we can create such redox stratification in biofilms and how we can make robust biofilm. Biofilm itself and its potential toward biofilm reactor will be described in the next chapter.

1.5 METHODOLOGY OF BIOFILM MONITORING

1.5.1 Fluorescence in situ hybridization (FISH)

FISH is highly effective for detecting specific bacteria and analyzing the spatial distribution 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 (Amann *et al.*, 1990). rRNA is an ideal target for *in situ* hybridization with oligonucleotide probes because: (i) it is present in all bacteria 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 and observation of a single cell by using an epifluorescence microscope or a confocal scanning laser microscope (CSLM).

FISH-dependent techniques have enabled the observation of the *in situ* microbial community structure in various types of biofilm communities, including those in natural environments and engineered systems. Generally, 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 Amann *et al.* (1995).

1.5.2 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. The principle of microsensor mostly relies on diffusion-dependent electrode reactions, scaling down the active surface area and diffusion distances lead to better signal stability, faster response, and practical independence of the microsensor signal on stirring of the external medium (Kühl and Revsbech, 2001). 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 (Ramsing *et al.*, 1993), the nitrification in trickling filter biofilms (Schramm *et al.*, 1996), and the nitrification in microbial aggregates (Schramm *et al.*, 1998; 1999), fixed bed biofilms (Okabe *et al.*, 1999), membrane-aerated biofilms (Hibiya *et al.*, 2003; Schramm *et al.*, 2000; Terada *et al.*, 2003). 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 (Schramm *et al.*, 2003). Furthermore, the spatial distribution of metabolically active areas and active species in the biofilm can be simultaneously estimated.

1.5.3 In situ observation of nitrifying biofilms

Nitrifiers, AOB and NOB, are chemoautotrophs. Although nitrification is one of the most significant steps in biological nitrogen removal processes, this process is rate-limiting in both domestic and industrial wastewater treatment especially after some fluctuations of water quality and temperature. To accomplish high nitrification rate in the process, high concentrations of nitrifiers should be accumulated and retained for stable nitrification. Immobilization of nitrifiers is a quite important strategy to keep nitrification rate high. Effective methods for the immobilization of nitrifiers have been developed, such as the use of biofilms on supporting materials (Tsuneda *et al.*, 2000), entrapment in polymer gels (Sumino *et al.*, 1992), using fibrous net-works (Hayashi *et al*, 2002) and hollow-fiber membrane which is gas permeable (Semmens *et al*., 2003). Therefore, a better understanding of the spatial organization, and activities of immobilized nitrifying bacteria is necessary to improve removal performance and process stability.

FISH dependent techniques provide reliable information regarding dominant species of nitrifying bacteria, their spatial distribution and activities in biofilms. Numerous researchers revealed that *Nitrosomonas* exists throughout the biofilm whereas location of *Nitrospira* sp. (NOB) is restricted to the inner part of the sewage wastewater biofilm as determined by combined analysis with a microelectrode (Okabe *et al*., 1999; Satoh *et al.*, 2003; Schramm *et al.*, 2000). Combination of a microelectrode with FISH has 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 (Schramm *et al.*, 1999). Illustration for the analysis of the *in situ* organization of a biofilm community is shown in Fig. 1.6 (partly from Aoi, 2002). The combined information from various approaches would lead to the further clarification of the mechanism underlying treatment activities and highlight unfavorable fluctuations. Moreover, 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 (Aoi, 2002).

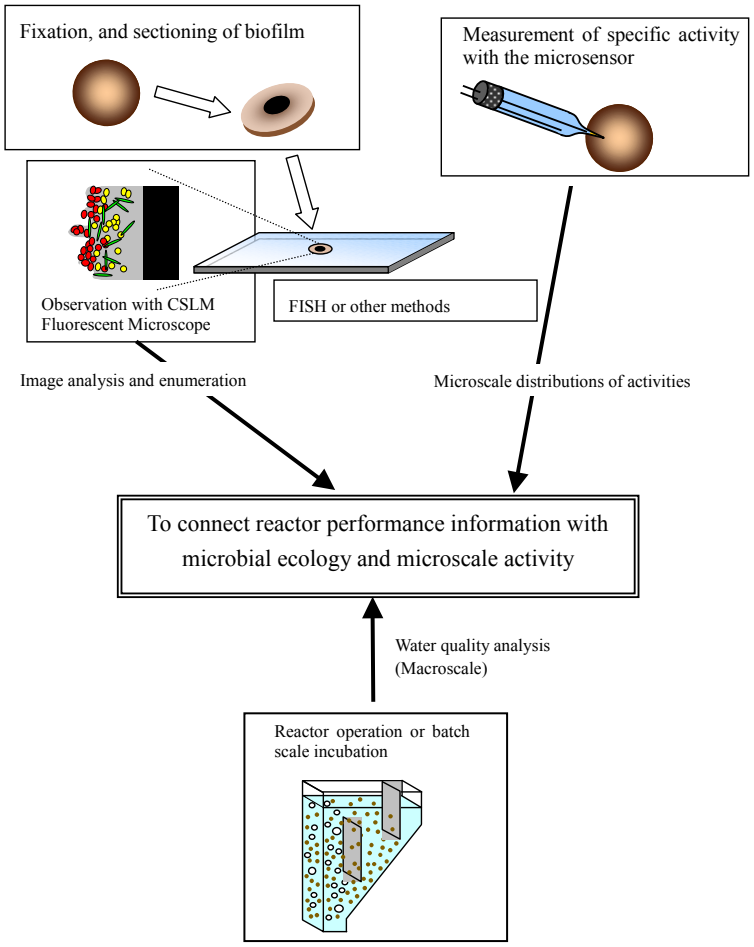

Figure 1.6 Schematic illustration of the analysis of the *in situ* organization of a biofilm community using various methods and the connection with reactor performance (partly from Aoi, 2002).

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