Chapter 1

General Introduction

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Biological nitrogen removal, mechanisms of bacterial adhesion and subsequent biofilm formation, and development of novel biofilm reactor

Summary

Nitrogenous compounds cause eutrophication, leading to a significant negative impact to aqueous environment. Essentially, removal of nitrogenous compounds is required for preservation of the aqueous environments. Nitrogen removal in wastewater not only from sewage plant but also industrial plant, *e.g.*, wastewaters from food processing, brewery, power plant, photo processing, livestock and so on, would be necessary. Considering the fact that some industrial plants do not have enough space for wastewater treatment process, small system applicable to nitrogen removal is required. Application of biofilms for biological nitrogen removal is very useful in that biofilm itself is very compact and robust, yielding high bacterial density in a reactor. Furthermore, most natural biofilms exhibit redox stratification and the presence of strong concentration gradients of both

electron donors and acceptors, resulting in simultaneous nitrification and denitrification. An engineering challenge is how we can control biofilm stiffness and maintain such redox stratification. Essentially, we need to chase the mechanism of biofilm formation and to make robust biofilms. In this chapter, biological nitrogen removal, basics of biofilm, initial bacterial adhesion and subsequent biofilm formation, and development of novel biofilm reactor with use of a gas-permeable membrane are described.

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1.1 Origin of nitrogen pollution

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 atomospheric 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).

1.2 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.

1.2.1 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).

1.2.2 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 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).

1.2.3 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.

1.2.3.1 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:

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

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

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

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):

$$FA as NH_3 (g/m^3) = \frac{17}{14} \times \frac{(NH_4^+ - N) \times 10^{pH}}{K_b / K_w + 10^{pH}} \qquad (1.5)$$

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

$$FNA as HNO_2 (g/m^3) = \frac{46}{14} \times \frac{(NO_2^- - N)}{K_a \times 10^{pH}} \qquad (1.7)$$

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

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 (Figure 1.1), 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.

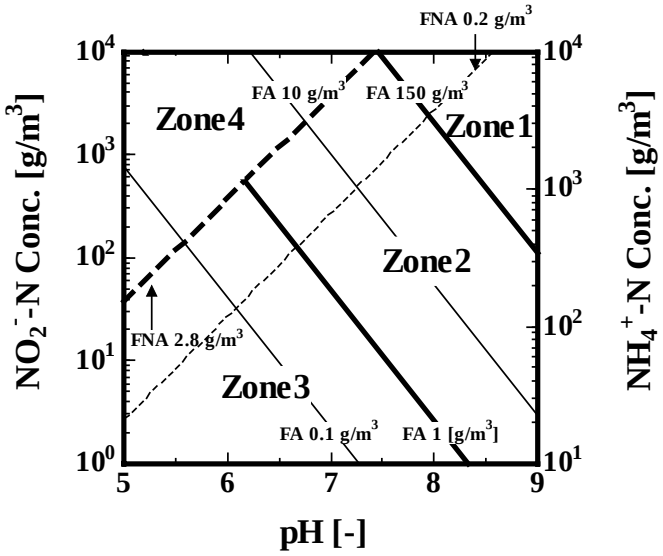

Figure 1.1 Dependence of free ammonia (FA) and free nitrous acid (FNA) on pH in the solution proposed by Anthonisen *et al.* (1976). Zone 1 shows FA inhibition of *Nitrobacter* and *Nitrosomonas*, Zone 2 shows FA inhibition of only *Nitrobacter*, Zone 3 shows complete nitrification, and Zone 4 shows 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}.

1.2.3.2 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.

1.2.3.3 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.

1.2.3.4 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).

1.2.4 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).

1.2.5 Application to novel nitrogen removal

1.2.5.1 Nitrogen removal via nitrite

As already mentioned in the previous chapters, nitrite is an intermediate in both nitrification and denitrification (Figure 1.2). 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.

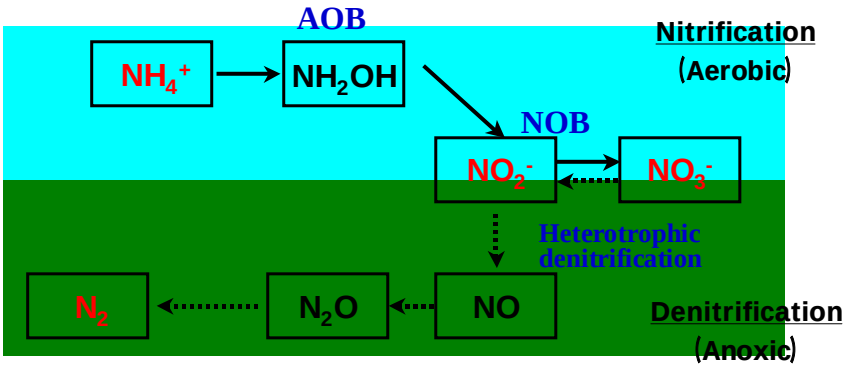

Figure 1.2 Schematic diagram of biological nitrogen removal pathway.

1.2.5.1 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.

1.3 Basics of biofilms and application to biofilm reactor

1.3.1 Why biofilms?

Bacteria tend to adhere onto surfaces. Once bacteria attach to a substratum surface, a multistep process starts leading to the formation of a complex and heterogeneous biofilm

(Bos *et al.*, 1999). Biofilms are layer-like aggregations of bacteria and their extracellular polymeric substances (EPSs) attached to a solid surface (Rittmann and MaCarty, 2001). Biofilms occur ubiquitously in nature and are increasingly important in engineered processes used in pollution control, *e.g.*, trickling filters, rotating biological contactors, membrane-aerated biofilm reactor and anaerobic filters. Biofilm processes are simple, reliable and stable because natural immobilization allows excellent biomass retention and accumulation without the need for separate solids-separation devices.

A biofilm can be sometimes formed by a single bacterial species, but more often biofilms consist of many species of bacteria, for instance, fungi, algae, protozoa, debris and corrosion products. The relative ratio of bacterial cells and EPS has been reported to vary significantly, from 10 to 90% of the organic matter (Nielsen *et al.*, 1997). EPS is mainly represented by polysaccharides (up to 65%) and is for this reason also known as the glycocalyx matrix. However, other substances are present, such as proteins (10-15%), nucleic acids, lipids, DNA and humic acids (Wingender *et al.*, 1999).

Here one fundamental issue about biofilm (actually, all aggregated systems) emerges: why do bacteria preferably attach to surfaces and belong to part of biofilm? Considering the issue, there are some answers as follows (Rittmann and MaCarty, 2001):

1. The biofilm create an internal environment (*e.g.*, pH, O_2, or products), which is more hospitable than the bulk liquid. In other words, the biofilm generates unique, self-created microenvironments, for example, aerobic and anoxic conditions in the same biofilm that could achieve simultaneous nitrification and denitrification (de Beer *et al.*, 1997)
2. Different bacterial species must live together in obligate consortia for substrate transport or some other synergistic relationships; the close juxtaposition of cells in a biofilm, *e.g.*, AOB and NOB, is necessary for the

exchanges (James *et al*., 1995).

3. The surface itself creates a unique microenvironment, such as by adsorption of toxins or corrosive release of Fe^{2+}, which is an electron donor.
4. The surface triggers a physiological change in the bacterial.
5. The tight packing of cells in the aggregate alters the cells' physiology.

Possibilities 1-3 involve microenvironment effects and seem to occur in specific instances. They are forms of ecological selection, and biofilm formation is one tool for ecological control of a process. Evidence to support possibility 4 is spare for systems of relevance to environmental biotechnology, although it may by important for specific interactions between bacteria and surfaces. Possibility 5 is often called "quorum sensing", and evidence of its role in biofilms and other aggregates is just emerging (Miller and Bassler, 2001).

1.3.2 Mechanisms of biofilm formation

The formation of a biofilm in an aqueous environment is generally illustrated to proceed as follows (Bos *et al.*, 1999; Bryers, 2000) (Figure 1.3):

1. A conditioning film of adsorbed components is formed on the surface prior to the arrival of the first coming bacteria.
2. Bacteria are transported to the surface through diffusion, convection, sedimentation or active movement.
3. Initial microbial adhesion occurs From the physicochemical aspects of the bacterial adhesion phenomenon, since individual cell size is more or less 1 μm, cell surface characteristics, such as surface electric potential, hydrophobicity and surface polymer structure, play significant roles in bacterial adhesion (Bos *et al.*, 1999; Hayashi *et al.*, 2001; Hayashi, 2002;

Rutter *et al.*, 1984). This phenomenon is a reversible step.

4. Attachment of adhering microorganisms is strengthened through secretion of EPSs and unfolding of cell surface structures. This step is an irreversible step.
5. Surface growth of attached bacteria and continued secretion of EPSs.
6. Localized detachment of biofilm organisms caused by occasionally high fluid shear or other detachment forces.

Localized detachment of biofilm organisms commences after initial adhesion, although adhesion of individual bacteria is often considered irreversible. The ratio of detachment event could be dependent of interaction between bacterial cells and physicochemical surface characteristics, flow rate in the bulk and so on. The importance of the studied of the initial bacterial adhesion in biofilm formation has been questioned because the number of cells in a mature biofilm after the growth phase is several times higher that involved in the initial bacterial adhesion (Fox *et al*., 1990; Petrozzi *et al*., 1993). However, Busscher *et al.* (1995) proposed the significance of bacteria that initially adhere as the link between the colonized surface and the biofilm. Regarding initial bacterial adhesion onto a substratum, electrostatic force is considered to be the most important, because it is markedly influenced by the surface potentials of both bacterial cells and the substratum or the chemical properties of the solution, that is, ionic concentration and valence of ions (Terada *et al.*, 2005).

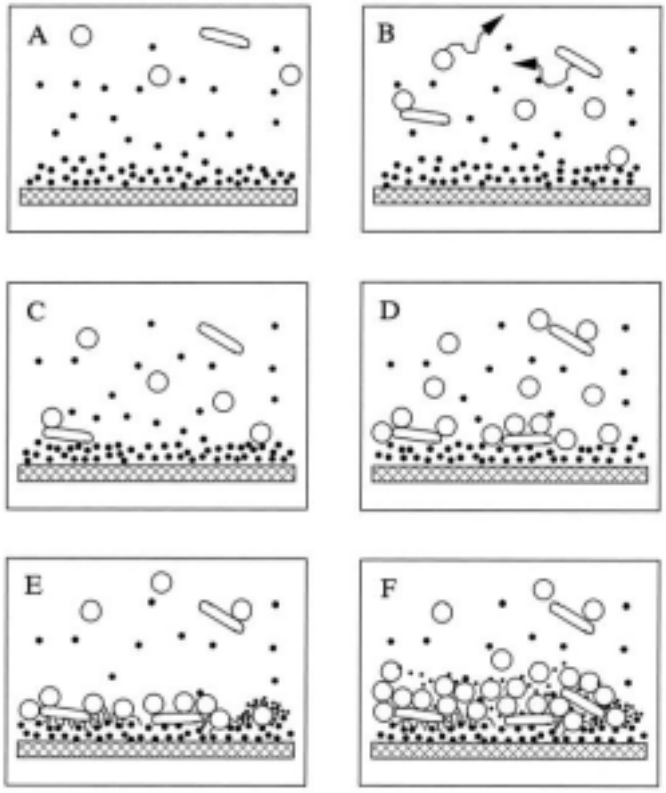

Figure 1.3 sequential illustrations of the initial steps in biofilm formation. A: Adsorption of conditioning film components; B: microbial transport and coaggregation; C: adhesion of single bacterial cells and of microbial coaggregates; D: co-adhesion between microbial pairs; E: anchoring or the establishment of firm, irreversible adhesion through secretion of EPSs; F: biofilm growth and detachment (From Bos *et al.* 1999).

1.3.3 Biofilm detachment

Detachment is an event by definition balancing the growth of a biofilm in steady state (van Loosdrecht *et al.*, 1995). It can be defined as the transport of particles from the attached solid matrix into the fluid phase. Attachment can be considered as a separate process or included in the detachment, leading to a net detachment rate. There are four different types of detachment as follows:

1. Erosion: a continuous process by which relatively small pieces of biofilm are removed from the biofilm's surface.

2. Abrasion: removal of small groups of bacterial cells from the surface as a result of collision with particles, *e.g.*, in fluidized bed or biofilm airlift reactors, or during backwash of fixed beds.
3. Sloughing: an abrupt, intermittent loss of a large section of biofilm..
4. Grazing: protozoa prey bacterial cells in a biofilm

Sloughing can result in drastic changes in the local biofilm accumulation (Rittmann and MaCarty, 2001). Considering that biofilm should be robust in a reactor for wastewater treatment, we should set a carrier, which would help bacterial cells to attach very firmly and robustly.

1.3.4 Methodology for biofilm monitoring

1.3.4.1 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).

1.3.4.2 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.

1.3.5 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 (Hibiya *et al.*, 2003; 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 Figure 1.4 (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).

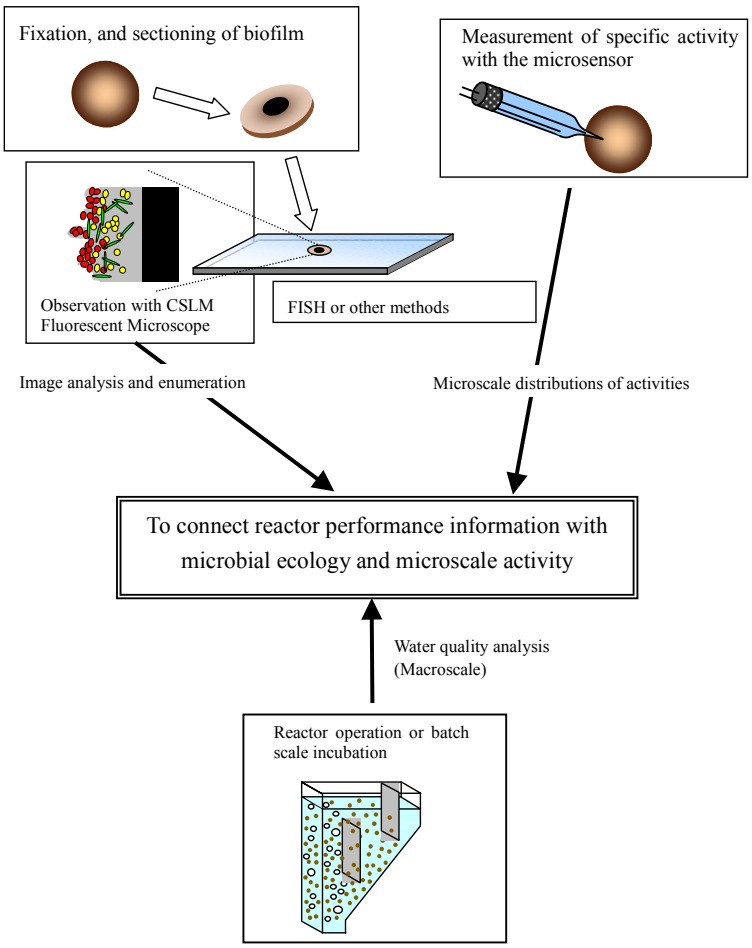

Figure 1.4 Schematic illustration of the analysis of the *in situ* organization of a biofilm community using various methods and the connection with reactor performance (partly from Aoi, 2002).

1.3.6 Surface modification by grafting method -toward enhancement of biofilm formation-

As mentioned in the chapter *1.3.3*, the sloughing from the base biofilm would be a serious problem in a biofilm reactor because almost all of the biofilm is removed from a substratum. When the biofilm has redox stratification, *i.e.*, aerobic and anaerobic part, the problem itself would become serious; all biofilm function would lose completely. Busscher *et al*. (1995) proposed that the strength between adhering bacteria and a substratum surface is determinant on biofilm strength and resistance against sloughing.

The author think consider surface modification that makes bacteria easy to attach onto the surface swiftly and robustly since initial bacterial adhesion is one of significant factor to determine biofilm property, *e.g.*, biofilm density, thickness and activity. In this study, the author is focusing on radiation induced graft polymerization (RIGP) (Figure 1.5). RIGP is one of the techniques for modifying polymeric materials with new properties. Irradiation with electron beam or gamma-rays onto a polymeric material (trunk polymer) produces radicals (reaction sites) in the polymer, Using radicals as starting sites for polymerization, vinyl monomers that come into contact are polymerized to form polymeric brushes (polymer chains or branch polymers) (Kawai *et al.*, 2003). Although other excitation sources such as plasma and UV light can also produce radicals on the trunk polymer, RIGP is powerful in that it can introduce a high density of polymeric brushes bearing functional groups into the entire volume of the trunk polymer uniformly (Lee, 1997). The advantages of RIGP are:

1. A selectivity and chemically physically stable polymeric materials;
2. A wide application various types and shapes of polymeric materials;
3. A wide range of applicable reaction temperature; and
4. An easiness of controlling the distribution of reaction sites in the trunk

polymer.

This technique can be used to maintain the physical strength and chemical stability of the trunk polymer while appending various functionalities to the trunk polymer, for instance, ion-exchange function, microbial-cell-capturing function, metal-ion-chelating function and catalytic function.

Application this method to enhancement of bacterial adhesion would be promising because of two reasons:

1. Bacterial cell surface has originally negative charges at neutral pH range.
2. By using RIGP, trunk polymer can be changed into the surface with positively charged.

Lee *et al*. (1996) reported that application of RIGP to polyethylene (PE) fiber, concretely PE was modified in order to have positively charged surface, can make *Staphylococcus aureus* cells-capturing ability increase up to 1000-fold faster than the original trunk polymer, PE. In addition, they suggested that polymer chains on the PE capture a bacterial cell softly, which remains bacterial activity (Figure 1.6). On the other hand, Hibiya et al. (2000) reported that a nitrifying biofilm forms successfully on the surface modified sheets, which are originally made of PE, under high hydrodynamic conditions. However, there are some issues to be clarified: is there the link between initial bacterial adhesion and subsequent biofilm formation?; how is the activity of the adhering bacteria?; and can we generalize bacterial adhesion behavior statistically?.

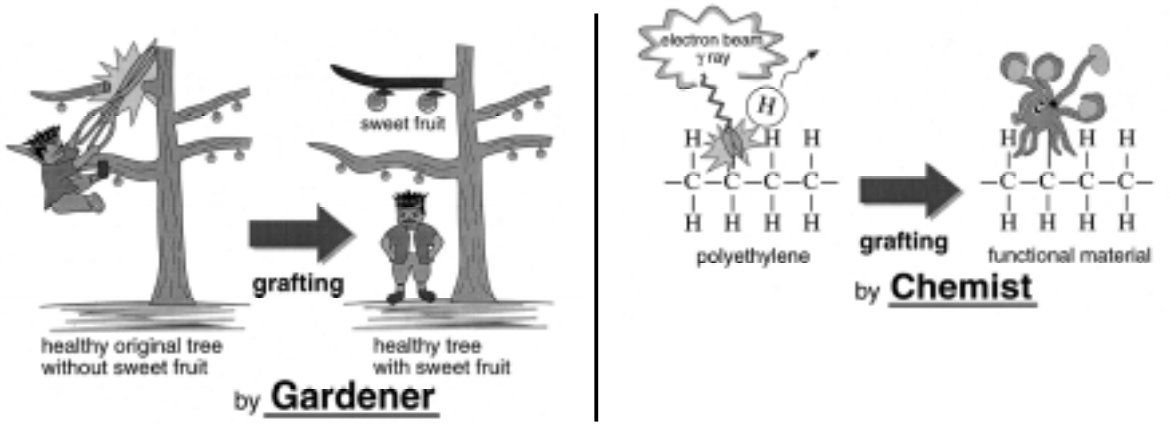

Figure 1.5 An illustration of radiation-induced graft polymerization (From Saito *et al.* 1999).

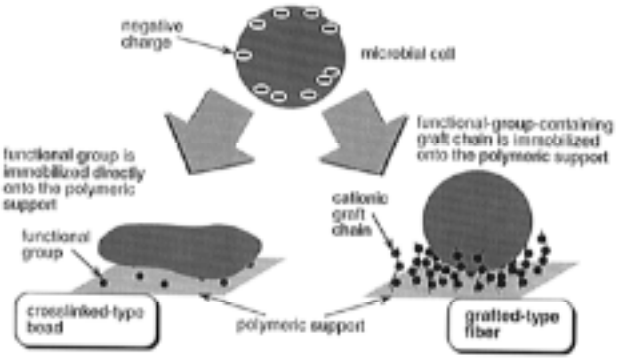

Figure 1.6 Suggested images for the adsorption of bacterial cell onto two kinds of polymers (from Lee *et al.*, 1996).

1.3.7 Novel biofilm reactor applicable to simultaneous nitrification and denitrification -with use of membrane-aerated biofilm-

Interestingly, most natural biofilms exhibit redox stratification and the presence of strong concentration gradients of both electron donors and acceptors (Amann and Kühl, 1998; Schramm 2003). The oxygen level in most natural biofilms is modulated by external factors such as flow, light or organic loading, and consequently the redox conditions often show a pronounced spatial heterogeneity over timescales ranging from hours to days. While redox gradients can, thus, establish in natural biofilms and traditional biofilm reactors, they lack the system control measures required for precise manipulation of

multiple biochemical environments within a single bioreactor unit.

Membrane-aerated biofilm reactors (MABRs) are an emerging technology that could permit a more rigorous oxygen control (Brindle et al., 1996, 1998; Casey et al., 1999; Pankhania *et al.*, 1994; Suzuki *et al.*, 1993, 2000; Yamagiwa *et al.*, 1994, 1998). Gas permeable membranes, which can be fabricated from a variety of polymers (poly (dimethylsiloxane), polyethylene and polypropylene in single or multiple layer) with different functional properties, permit bubbleless aeration and very high oxygen transfer rates (Ahmed et al., 2004; Cote et al., 1989). There are two types of aeration: flow thorough and dead-end (Figure 1.7). Such aeration type is dependent on what kind of membrane is used. Characterization of the aeration types is summarized in Table 1.1.

By growing the desired microorganisms on gas-permeable membranes, with oxygen delivered through the membrane at the base of the biofilm, and the other nutrients provided to the surface of the biofilm from the water within which the membranes are suspended, redox-stratified biofilms can be attained. Such counter diffusing fluxes of oxygen and other nutrients are, additionally, amenable to separate manipulation, yielding unprecedented opportunities for control. It is expected that MABRs would be suitable to create the redox stratification in the biofilms, leading to simultaneous nitrification and denitrification from organic carbon-containing wastewater (Figure 1.8). Some researchers clarified that such simultaneous reaction would be feasible from mathematical modeling (Bell *et al.* 2005; Shanahan and Semmens, 2004) and from experimental work (Cole *et al.*, 2003; Hibiya *et al*., 2003; Satoh *et al.* 2004; Semmens *et al*., 2003; Timberlake *et al.* 1988; Terada *et al.*, 2003). Therefore, we need to verify some issues regarding MABRs, *e.g.*, whether biofilm forms on a membrane surface; whether we need to modify membrane surface to enhance biofilm formation; whether redox stratification is properly created by appropriate oxygen transfer and whether shortcut

nitrogen removal via nitrite (nitrate) is feasible or not.

Table 1.1 Variation of membrane material and its characterization

	Non-porous membrane	Porous-membrane	Composite membrane
Property	Normally hydrophobic (it depends on time)		
Oxygen transfer	Dissolved	Through pores	Through pores and dissoved
Driving force of oxygen transfer	Diffusion	Diffusion or convection (it depends on operation)	Diffusion and convection
Bubbling point	High	Low	Extremely high
Cost	High	Low	High
Biofilm formation	Difficult	Easy	Easy
Specific membrane surface area	Small	Large	Large
Membrane wall	Thick	Thin	Thin
Aeration type	Cross-flow	Cross-flow/dead-end	Dead end

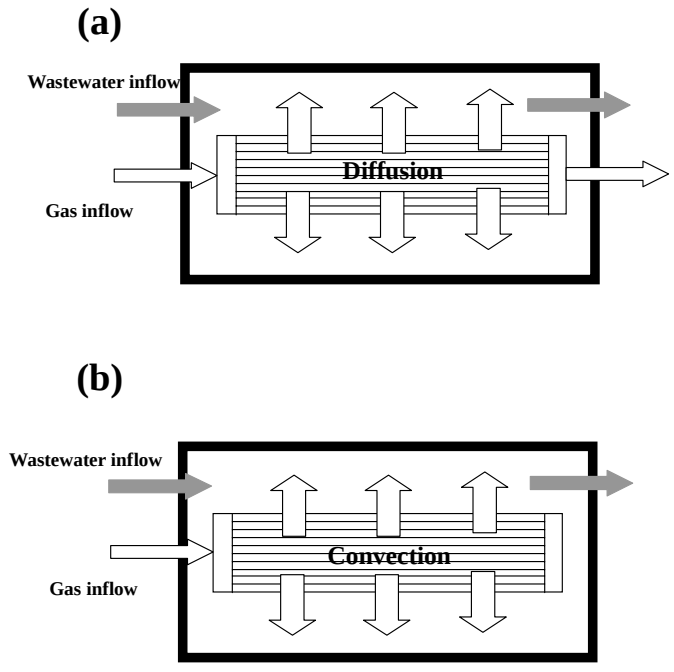

Figure 1.7 Composition of membrane module: (a) cross-flow system; (b) dead-end system.

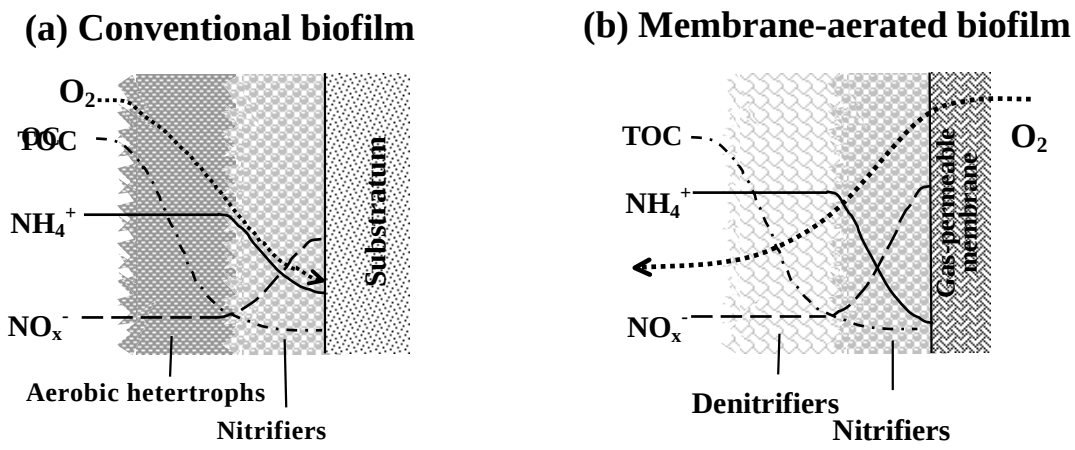

Figure 1.8 Comparison of biofilm structures for organic carbon and nitrogen removal: (a) conventional biofilm on an impermeable support; (b) proposed biofilm grown on an oxygen-permeable membrane

1.4 Objective of this study

The final objective of this study is to create stable redox stratification in a biofilm and to develop novel biofilm reactor applicable to simultaneous nitrification and denitrification. For achieving these goals, a challenge is how we can make swift biofilm formation and robust biofilms. Therefore, the author conducted these experiments:

1. Initial bacterial adhesion experiment: the author clarified the determinant factor on initial bacterial adhesion. Since RIGP is a very powerful method to prepare the sheets with different physicochemical properties, *e.g.*, surface roughness and surface potential, for the adhesion experiment, various membrane sheets with different properties were prepared and subsequently the initial bacterial adhesion onto the prepared surfaces were carried out (Chapter 2).

2. Bacterial adhesion and activity experiment: the relationship bacterial adhesion rates onto the surface modified sheets and their activity were investigated. And discussion whether or not the surface modified sheets would be useful for enhancement of biofilm formation is summarized in Chapter 3.
3. Biofilm formation test: the relationship between initial bacterial adhesion and the subsequent biofilm formation is linked with use of a flow cell. And some corroborative experiments were conducted to support the result of the flow cell. Through these experiment, the effectiveness of surface modified sheets was evaluated (Chapter 4).
4. Primary nitrification test: Initial adhesion of nitrifying bacteria and biofilm formation were investigated to clarify the effectiveness of the surface modified hollow-fibers for rapid biofilm formation. Moreover, MABR, which employed the modified hollow-fiber module, was constructed and operated for nitrification test. Especially, controllability, *i.e.*, the relationship supplied oxygen and ammonia oxidation, was evaluated (Chapter 5).
5. Simultaneous nitrification and denitrification test with MABR: the MABR has been operated for one year to chase process performance from macroscale and microscale analyses. Regarding the microscale evaluation, the combination of FISH and microsensor was employed to verify the concept of membrane-aerated biofilm. Furthermore, the feasibility of simultaneous nitrification and denitrification via nitrite was discussed (Chapter 6).

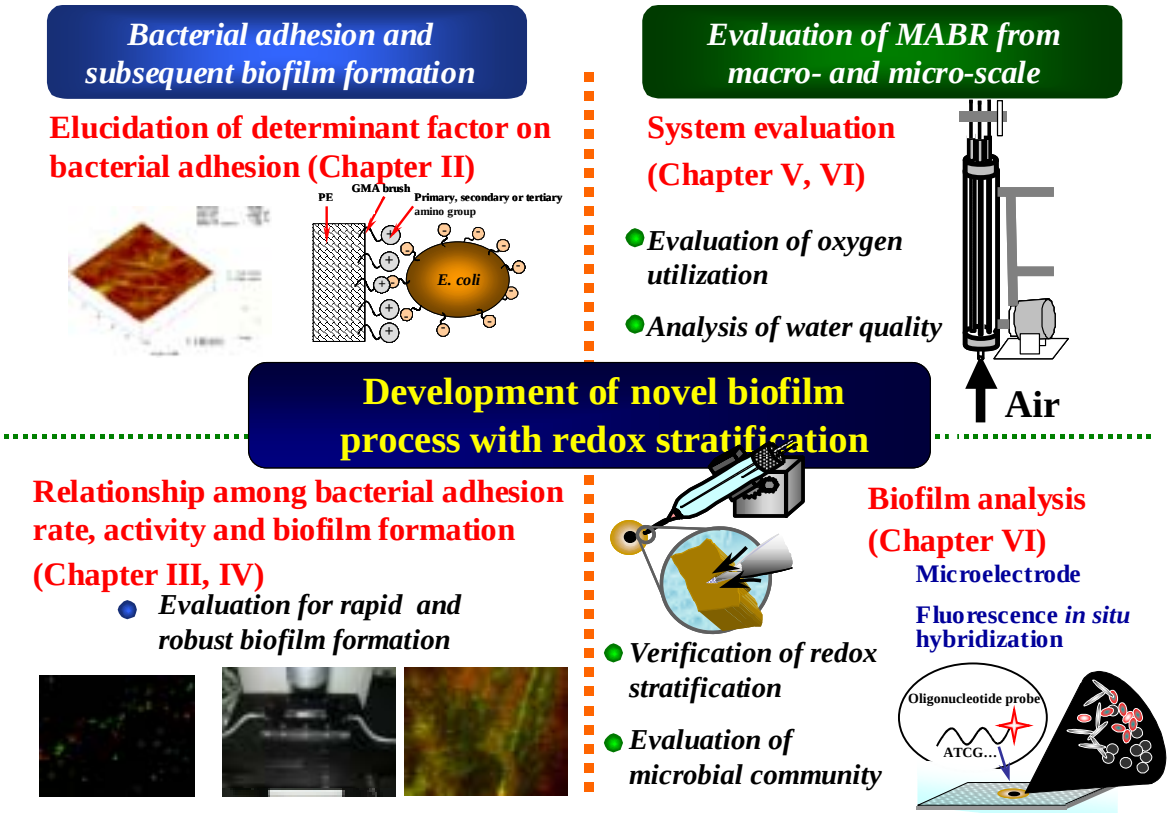

Figure 1.9 Outline of this thesis.

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