Chapter II

DEVELOPMENT OF MICROELECTORDE

Measuring Distributions of Substrate Concentration inside Biofilm by Ion-selective Microelectrodes and an Oxygen Selective Microelectrode

2.1 INTRODUCTION

In this chapter, oxygen, ammonium-ion, and nitrate-ion concentrations and pH in the biofilms of several kinds was measured using original fabricated microelectrodes. Two different treatment systems run for organic carbon wastewater and inorganic carbon wastewater. This microelectrode method is most important in this study. Furthermore, microbial distribution inside biofilm was determined by fluorescence in situ hybridization (FISH) method.

2.2 BIOFILM FROM ORGANIC TREATMENT SYSTEM

2.2.1 MATERIALS AND METHODS

2.2.1.1 Reactor system

A complete-mixing three-phase fluidized bed reactor was used for continuous nutrient oxidation. The effective volume of the reactor was 3 L. The reactors were operated with a hydraulic retention time (HRT) of 5 h. A cement ball (CB) fabricated from coal ash was used as the carrier particle on which biofilms were formed. The particle had an average diameter of 0.23 mm and a density of 1.92 g/cm^3. The temperatures of the feed tank and reactor were maintained at 5 and 20 ºC, respectively, using a thermostat. Sufficient air was supplied using an air pump to maintain a bulk oxygen concentration of about 3-4 g/m^3. Artificial wastewater composed of the chemicals shown in Table 2.1 was used.

The quality of this wastewater corresponds to a biochemical oxygen demand (BOD_5) of 600 g/m^3, a total organic carbon (TOC) content of 400 g/m^3, a total nitrogen (T-N) content of 200 g/m^3 and an ammonium-nitrogen (NH_4^+-N) content of 100 g/m^3. For water quality analysis, TOC content was measured using a TOC meter (Shimadzu, TOC-5000),

ammonium-ion content was measured using an ion chromatograph (DIONEX, DX120), nitrite and nitrate contents were measured using a high-performance liquid chromatograph (HPLC) equipped with an anion column (Tosoh, IC-Anion-PW) and an ultraviolet detector (Tosoh, UV-8011), and dissolved oxygen content was measured using an oxygen-selective electrode potentiometer (TOA, DO-11P). T-N was analyzed in accordance with the Standard Methods for the Examination of Water and Wastewater [20]. All samples were filtered through a glass filter (Whatman, GF/C) before analysis.

Table 2.1 Composition of artificial wastewater [g/L]

Dextrin	0.122
Meat extract	0.298
Yeast extract	0.262
Bacto peptone	0.262
Sodium chloride (NaCl)	0.026
Magnesium sulfate ($MgSO_4 \cdot 7H_2O$)	0.034
Potassium dihydrogen phosphate (KH_2PO_4)	0.074
Potassium chloride (KCl)	0.054
Ammonium chloride (NH_4Cl)	0.327
Sodium bicarbonate ($NaHCO_3$)	0.766

2.2.1.2 Biofilms

Biofilms were grown in a continuously operated reactor. After one month of growth, biofilms of 200-300 μm thickness were obtained. Furthermore, biofilm thickness gradually increased, exceeding 1 mm after 2 years of operation. The biofilms had a fast growth rate because of the high loading rate and high amount of biodegradable substrate. Five experimental runs started at different times were simultaneously conducted. From these runs, biofilms of different thicknesses were obtained. Biofilm thickness was calculated from the image obtained by optical microscopy (BH-2, Olympus Co.) [3].

The biofilms of different thicknesses were randomly sampled. The number, equivalent diameter, wet volume and dry weight were measured for many biofilms. The overall biofilm dry density was calculated using each biofilm thickness.

Recently, many researchers have reported that biofilms are heterogeneous, and have conducted mathematical modeling of heterogeneous biofilms [21, 22]. Moreover, the density and porosity of biofilms change in the depth direction [23]. Therefore, two types of biofilm, of 120 and 1200 μm thicknesses were sampled. The biofilm samples were immediately fixed in freshly prepared 4% paraformaldehyde solution for 20 h. A 20-μm-thick biofilm section was prepared using a cryostat (Leica, CM-3050) at -20 ºC. Each slice was observed using a reflection light microscope with high resolution (Keyence,

VH-7000). The internal physical structure of the biofilms was visualized.

2.2.1.3 Dissolved Oxygen Microelectrode

Oxygen concentration was measured with a Clark-type microelectrode. The microelectrode was fabricated in our laboratory as described by Revsbech [24]. The tip of the microelectrode was 5 μm in diameter and its time response of 90% (t_{90}) was about 10 s. Linear calibration was carried out in an air-saturated medium and a medium containing 300 mM $NaSO_3$ and a small amount of $CoCl_2 \cdot 6H_2O$ with nearly zero oxygen concentration.

The position of the biofilm surface was determined visually under a dissection microscope. The biofilms were fixed on a glass plate filled with a solution obtained from the reactor. All measurements were performed at 20 ºC within several minutes. Then, the oxygen microelectrode was inserted into the biofilms in 10 μm steps using a micromanipulator (Narishige, MMO-203) to measure the spatial distribution of oxygen in the biofilms.

2.2.2 RESULTS AND DISCUSSION

2.2.2.1 Oxygen penetration depth

Using an oxygen microelectrode, the oxygen distribution in the biofilms was measured while maintaining the bulk oxygen concentration at about 3 g/m^3. Examples of the experimental profile and the curves fitted for the exponential equation in the case of biofilms with 425 and 980 μm thicknesses are shown in Figure 2.3. As a result, we found that oxygen was gradually eliminated by bacteria in the biofilm and the liquid film formed in the bulk near the biofilm surface by the reaction. Oxygen penetration depth was determined from the obtainable oxygen distribution in the biofilms. Oxygen penetration ratio was calculated by dividing the oxygen penetration depth by the biofilm thickness.

The results of the oxygen penetration depth and ratio are shown in Figure 3.7. Oxygen diffused completely into the bottom of the biofilm of less than 300 μm thickness and oxygen penetration depth increased with increasing biofilm thickness. Oxygen penetration ratio gradually decreased with increasing biofilm thickness. Therefore, there is a large anaerobic zone at the bottom of the thick biofilm, indicating the possibility of microbial denitrification. Pochana et al. [29] considered the denitrification activity in the anaerobic zone in a floc and simulated the distribution of oxygen in the floc. Dalsgaard et al. [30] reported that denitrification activity in the anaerobic zone in a biofilm is computed from the mass balance equation using microelectrode analysis and that the denitrification zone is specified. However, the anaerobic zone is not necessary for oxidation processes such as carbon oxidization and nitrification. The thin biofilm without an anaerobic zone is effective for oxidation processes from the standpoint of obtaining a larger aerobic zone throughout the entire biofilm.

Table 2.2 Water quality in the runs using thin or thick biofilms

	Thin biofilm (d = 120 μm)	Thick biofilm (d = 1200 μm)
TOC, g/m^3	15.6	42.1
T-N, g/m^3	156.2	73.4
NH_4^+-N, g/m^3	44.0	34.4
NO_2^--N, g/m^3	97.2	14.3
NO_3^--N, g/m^3	6.4	35.3
DO, g/m^3	3.7	3.5
Biofilm packing ratio, %	20	20
Ratio of carbon removal, %	95.5	88.0
Ratio of nitrification, %	67.4	76.4
Ratio of nitrogen removal, %	3.2	54.5

2.2.2.2 Water quality

The water treatment experiment was continuously carried out using the two reactors with the thin (representative biofilm thickness: 120 μm) and thick (representative biofilm thickness: 1200 μm) biofilms. Table 2.2 summarizes the water quality data of the effluent of each reactor in the steady state. The removal efficiency of TOC was high in the reactor with the thin biofilm because oxygen completely diffused into the bottom of the biofilm, and thus all biomass inside the biofilms contributed to the oxidation of organic compounds. In contrast, the removal efficiency of TOC in the reactor with the thick biofilm was lower than that with the thin biofilm, probably because the population of oxidizing bacteria was relatively small due to the existence of the anaerobic zone inside the biofilm, as determined based on the FISH results.

2.2.3 CONCLUSIONS

The oxygen distribution in biofilms was measured using an oxygen microelectrode that we fabricated. Oxygen penetration depth and ratio were computed based on the oxygen distribution. When the oxygen concentration in the bulk was 3 g/m^3, oxygen penetrated completely into the biofilms of less than 300 μm thicknesses. Oxygen penetration ratio gradually decreased with increasing biofilm thickness.

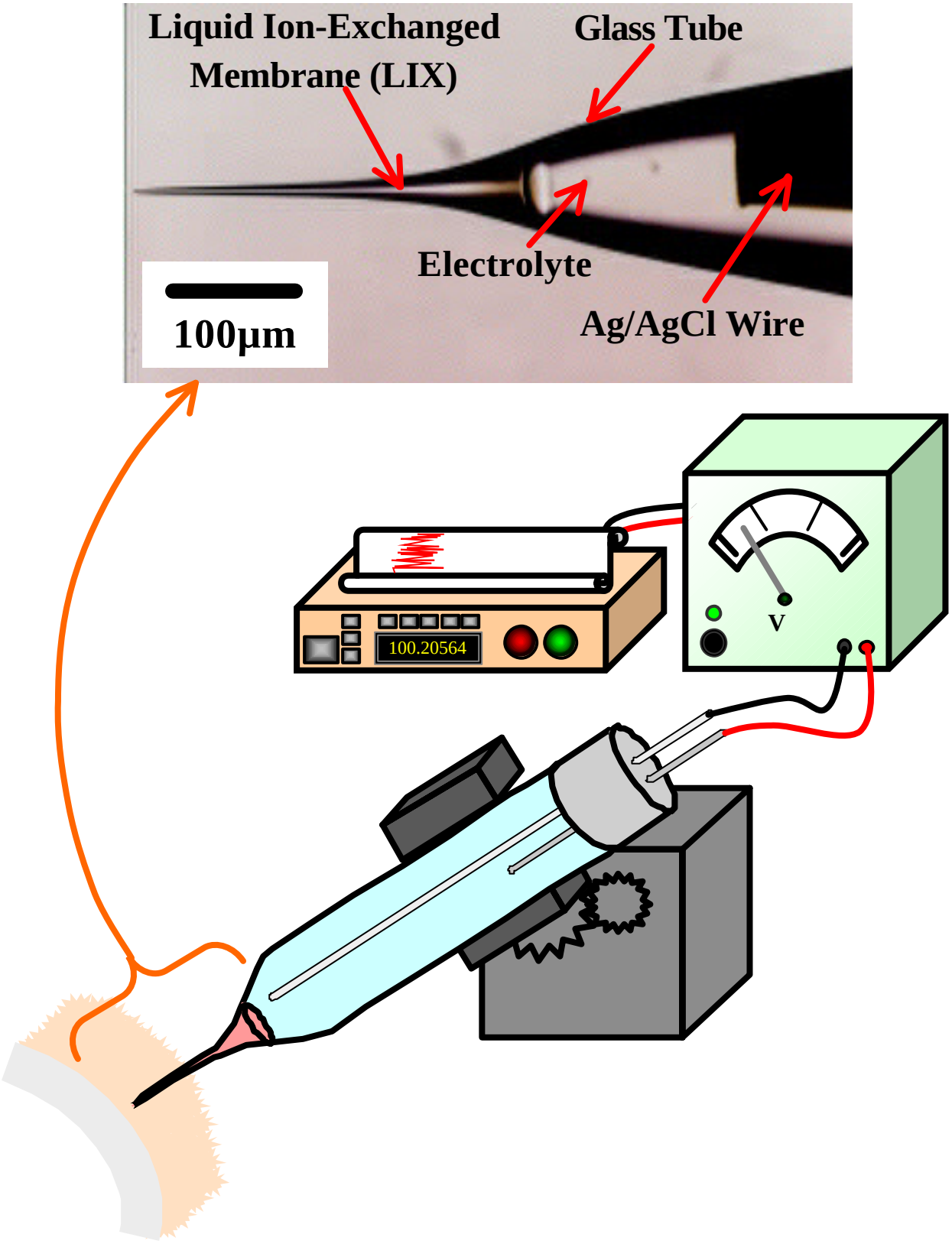
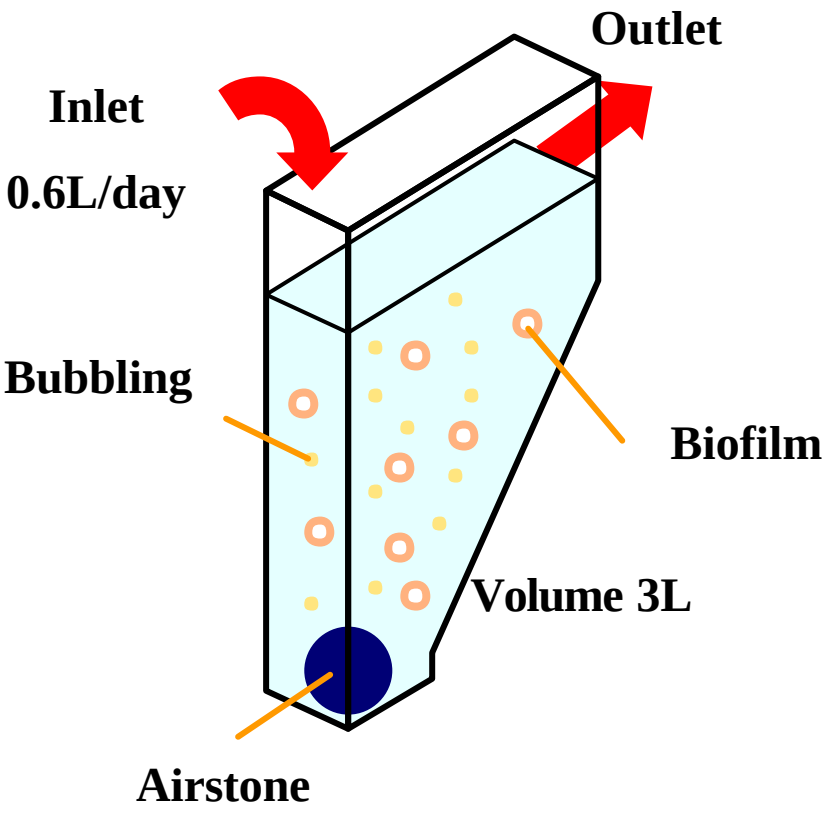
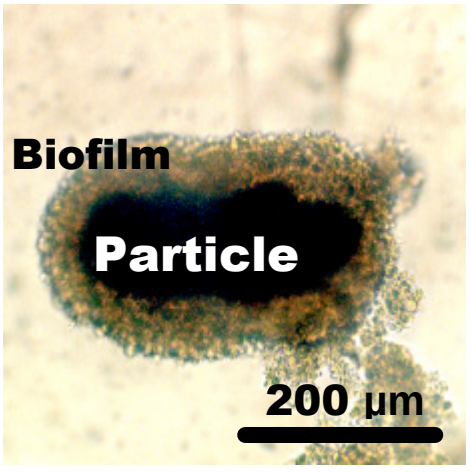


Figure 2.1 Liquid ion-selective exchanged microelectrode and concept image of microelectrode system

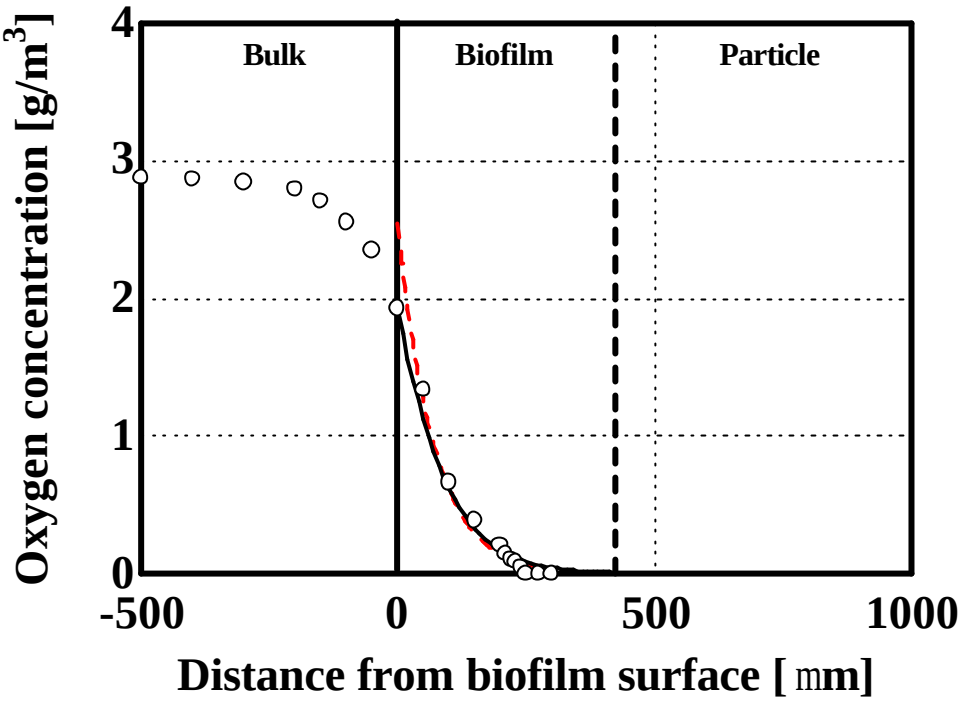


(a) Reactor system

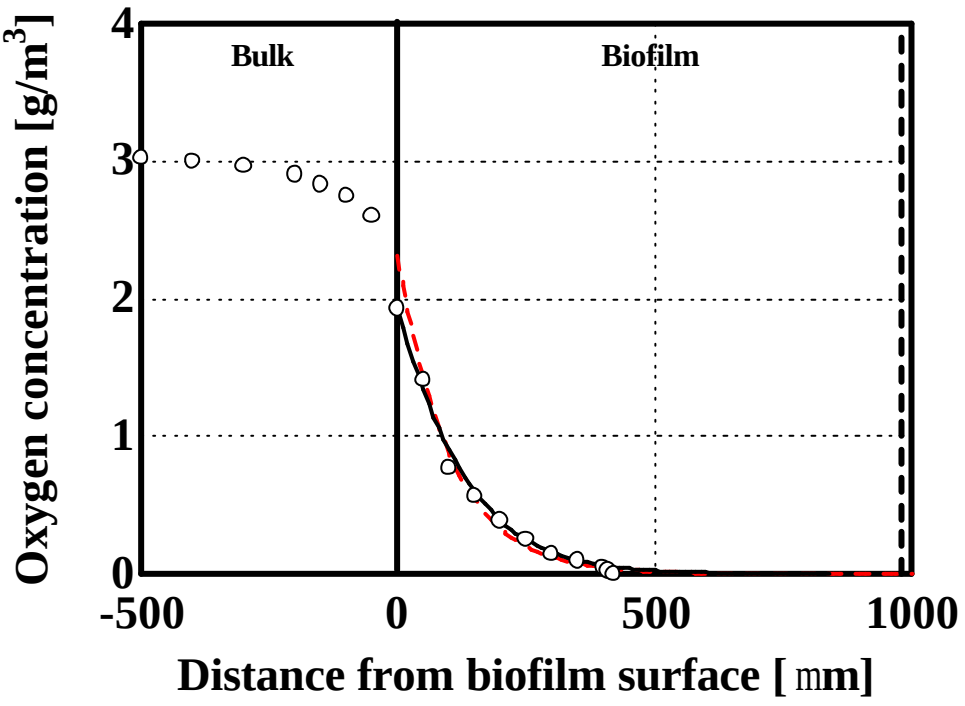


(b) Biofilm sample

Figure 2.2 Fluidized-bed biofilm reactor system and image of biofilm slice sample



(a)



(b)

Figure 2.3 Distribution of oxygen concentration inside biofilms of (a) 425 µm and (b) 980 µm thicknesses (open circles: experimental data from microelectrode analysis; broken line: fitted curve for exponential equation; continuous line: simulated curve)

2.3 BIOFILM FROM INORGANIC TREATMENT

2.3.1 MATERIALS AND METHODS

2.3.1.1 Reactor System

The above-described substrate (600 g-N/m^3) was continuously fed at 1.0 L/day to a fluidized bed bioreactor with an effective volume of 2 L. The unmodified and modified membranes were used as supporting materials on which biofilms were immobilized. The temperature was maintained at 30℃. Air was supplied to the reactor at a suitable rate to fully disperse the biofilms and to provide excess dissolved oxygen. The pH of the solution in the reactor was adjusted to 7.5-8.0 by the addition of 1 *M* NaOH 1-3 times a day.

Table 2.3 Composition of artificial wastewater (g/L)

$(NH_4)_2SO4$	2.83
KH_2PO_4	0.5
K_2HPO_4	1.0
$FeSO_4$	0.05
$MgSO_4 \cdot 7H_2O$	0.3
$CaCl_2 \cdot H_2O$	0.002
$MnCl_2 \cdot 4H_2O$	0.2
$Na_2MoO_4 \cdot 2H_2O$	0.1

All culture samples were filtered through a 0.2-μm-pore-size membrane filter (Isopore®, Millipore Co.) prior to water quality measurement. The amount of organic pollutants was evaluated as total organic carbon (TOC) with an automatic TOC analyzer (TOC-500, Shimadzu Co.). Ammonium-nitrogen (NH_4^+-N) was determined using an ammonia-selective electrode (F-203, Horiba Co., Japan). Nitrite-nitrogen (NO_2^--N) and nitrate-nitrogen (NO_3^--N) were determined using an ion-chromatograph system (IC-Anion-PW, UV-8011, Tosoh Co.). Biofilm thickness was calculated from the image obtained by optical microscopy (BH-2, Olympus Co.). Bacterial adhesion to membranes was confirmed from SEM (S-2500, Hitachi Co.) images.

2.3.1.2 Biofilms

Biofilms were grown in a continuously operated reactor. The thickness of biofilm on the grafted membrane was approximately 20 μm at day 88 and approximately 100 μm at day 150. In contrast, no biofilm was formed on the unmodified membrane. From these runs, biofilms of different thicknesses were obtained. Biofilm thickness was calculated from the image obtained by optical microscopy (BH-2, Olympus Co.) .

The biofilms of different thicknesses were randomly sampled. The number, equivalent diameter, wet volume and dry weight were measured for many biofilms. The overall biofilm

dry density was calculated using each biofilm thickness.

Recently, many researchers have reported that biofilms are heterogeneous, and have conducted mathematical modeling of heterogeneous biofilms. Moreover, the density and porosity of biofilms change in the depth direction. Therefore, two types of biofilm, of 120 and 1200 μm thicknesses were sampled. The biofilm samples were immediately fixed in freshly prepared 4% paraformaldehyde solution for 20 h. A 20-μm-thick biofilm section was prepared using a cryostat (Leica, CM-3050) at -20 ºC. Each slice was observed using a reflection light microscope with high resolution (Keyence, VH-7000). The internal physical structure of the biofilms was visualized.

2.3.1.3 Liquid Ion-Selective Membrane (LIX) Microelectrodes

Liquid ion-exchange membrane (LIX) microelectrodes for pH and NH_4^+ were prepared as described by de Beer *et al.* [7]. Using a micropipettpuller (MPT-1, Shimadzu Co.), 1-mm-diameter soda lime glass tubes (100 μL Micropipetts, Drummond Co.) were drawn into microcapillaries. The tip diameter was about 5 μm for all LIX microelectrodes and the tips of the electrodes were silanized with 20% (vol/vol) solution of trimethylchlorosilane in carbon tetrachloride to obtain a hydrophobic surface for optimal adhesion of the LIX membranes. After the tips of the microelectrodes were filled with the silanized-solution, the electrodes were baked for at least 1 h at 130℃ to remove traces of water. The liquid membrane used was 10% (wt/wt) tridodecylamine and 1% (wt/wt) sodium tetraphenylborate in 2-nitrophenyloctyl ether for pH microelectrode and 10% (wt/wt) nonactine and 1% (wt/wt) sodium tetraphenylborate in 2-nitrophenyloctyl ether for NH_4^+ microelectrode. The filling electrolytes used were 0.04 *M* KH_2PO_4, 0.023 *M* NaOH and 0.015 *M* NaCl for pH microelectrode and 0.01 *M* NH_4Cl for NH_4^+ microelectrode. Finally, Ag/AgCl wires were inserted into the capillaries and fixed using epoxy.

The pH microelectrode was calibrated in adjusting series of the substrate by 1 *M* NaOH or 1 *M* HCl. The NH_4^+ microelectrode was calibrated in dilution series of NH_4^+ in the medium for the experiment.

Nitrifying biofilms were taken out from the bioreactor at day 150 for microelectrode measurement. A biofilm was fixed on a glass plate that was filled with the solution obtained from the bioreactor. Then, pH and NH_4^+ microelectrodes were inserted into the biofilm at 10 μm steps using a micromanipulator (MMO-203, Narishige Co.) to measure the spatial distributions of pH and NH_4^+ inside the biofilms. Microprofiles were determined five times at different positions in the biofilms.

2.3.1.4 Fluorescence in situ hybridization (FISH) method

The biofilm samples for FISH, which were taken out from the bioreactor at day 150,

were immediately fixed with 4% paraformaldehyde. A biofilm section of 20 µm thickness was prepared from the frozen biofilm sample embedded in OCT-compound using cryostat (CM3050, Leica Co.) at -20℃, and placed in a hybridization well on a gelatin-coated microscopic slide.

For hybridization of the biofilm sections on the slide, the standard hybridization protocol described by Amann was used [8]. Two 16S rRNA targeted oligonucleotide probes were used for *in situ* detection of ammonia-oxidizing and heterotrophic bacteria: (1) NSO190 (labeled with TRITC): specific for the region of the 16S rRNA of all ammonia-oxidizing bacteria of the β-subclass of proteobacteria [9]; (2) EUB338 (labeled with Cy5): a probe for targeting all bacteria [10]. After probing, slides were examined with a confocal laser-scanning microscope (TCS NT, Leica Co.).

2.3.2 RESULTS AND DISCUSSION

2.3.2.1 Substrate profile inside biofilms

The pH and NH_4^+-N profiles of nitrifying biofilms on the membranes were measured using microelectrodes. The results are shown in Figure 2.5 where the plots and error bars correspond to averages and standard deviations, respectively. Since NH_4^+-N sufficiently diffused into the deep zone of the biofilms, it was thought that the rate-limiting factors were not only substrate diffusivity but also biological reaction. Therefore, both larger specific biofilm surface area and thicker biofilm increase the nitrification rate. Because pH decreased gradually with increasing depth of in the biofilm, this implied that nitrification occurred at every part of the biofilm. In addition, the present biofilm was sufficiently thick to remove nutrients from wastewater, because in the case of biofilms thicker than the present one, nitrifying bacteria might lose their activity at low pH at the deep zone inside the biofilms.

2.3.2.2 Identification of bacteria inside the biofilm by FISH method

The FISH image of the biofilm that was taken out from the bioreactor at day 150 is shown in Figure 2.7. The biofilm was found consist of mostly autotrophic ammonia-oxidizing bacteria. This result is consistent with the substrate's composition, *i.e.*, large amount of ammonium-nitrogen without any organic carbon source. However, a microorganism that differed from ammonia-oxidizing bacteria, supposedly a nitrite-oxidizing bacterium, existed in small numbers in the biofilm.

2.3.2.3 Water quality

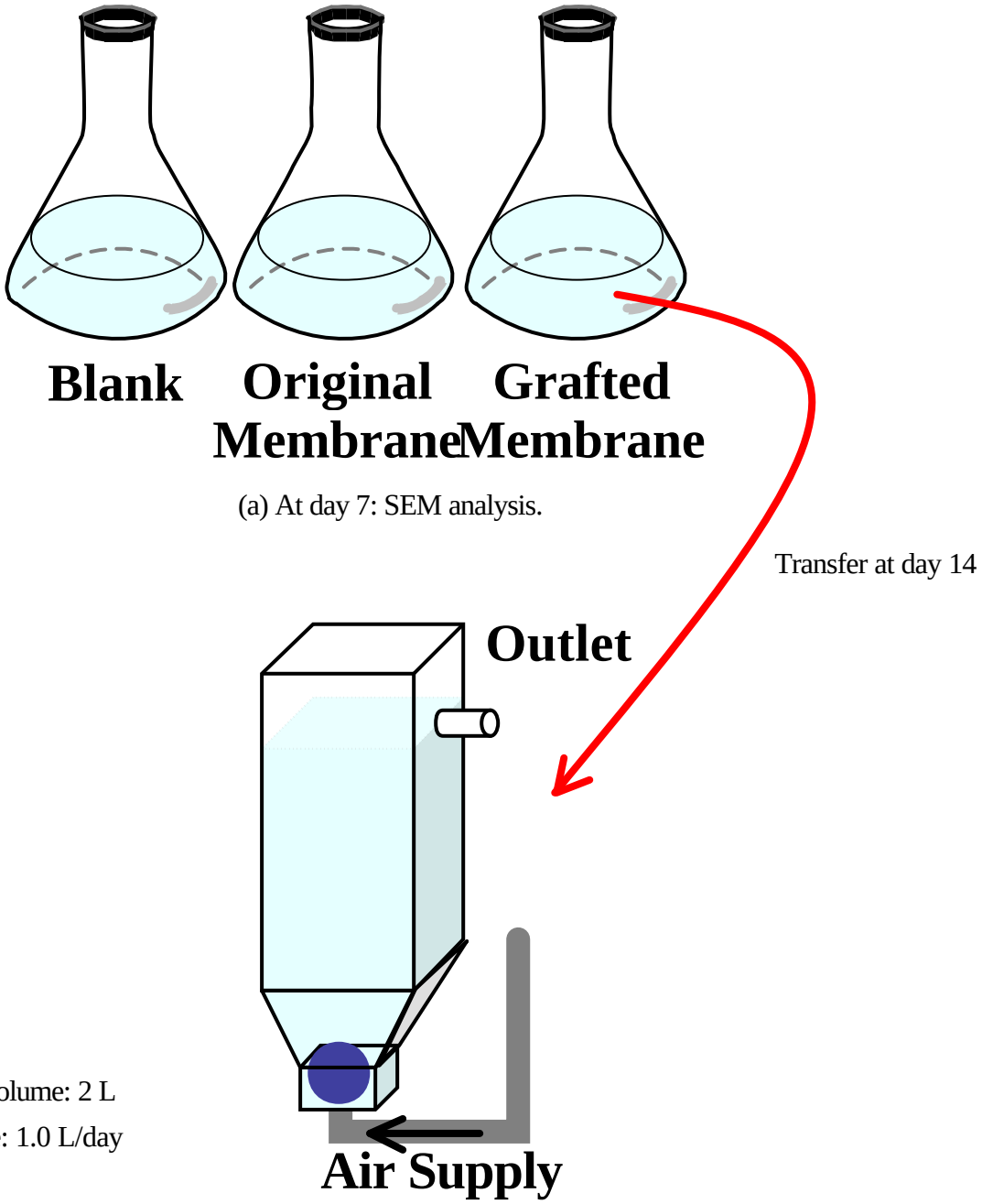
Membranes with nitrifying biofilm were introduced into a fluidized bed bioreactor where NH_4^+ as a nutrient was continuously fed. The results of water quality analysis are shown in

Figure 2.6. Biofilm thickness was observed every day by optical microscopy. Representative images of biofilms on the membranes are shown in Figure 5.3.

The thickness of biofilm on the grafted membrane was approximately 20 μm at day 88 and approximately 100 μm at day 150. In contrast, no biofilm was formed on the unmodified membrane. Therefore, a new supporting material suitable for biofilm formation was successfully developed by modification of the surface by the grafting method. Nitrification rate was very unstable until day 50 because of the small amount of attached and suspended nitrifying bacteria. However, after day 50, a thick biofilm was retained, and thus the NH_4^+-N removal rate reached as high as 0.3 kg-N/(m^3·day) and became stable.

2.3.3 CONCLUSIONS

The profiles of pH and NH_4^+ inside the biofilms were successfully measured by using the originally fabricated the microelectrodes. NH_4^+-N removal rate per unit area of biofilm was calculated from the bulk concentration change and from the concentration profile inside the biofilm. As a result, this biofilm exhibited a markedly higher NH_4^+-N removal rate than other biofilms.



(a) At day 7: SEM analysis.

(b) Water quality and at day 150: microscope analysis.

Figure 2.4 Explain of batch and continuous experiment for bacterial attachment on original and grafted membranes

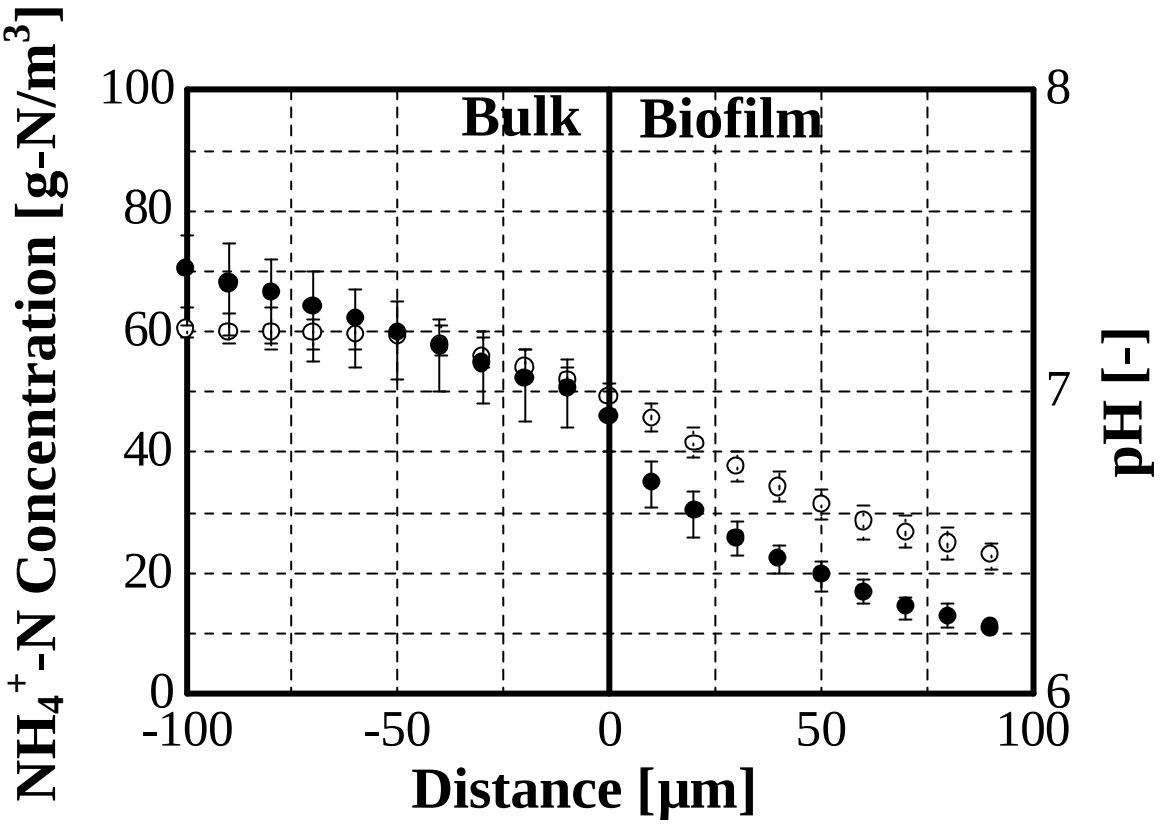


Figure 2.5 The pH and NH_4^+-N profiles inside nitrifying biofilm on grafted membrane. NH_4^+-N (?), pH (?)

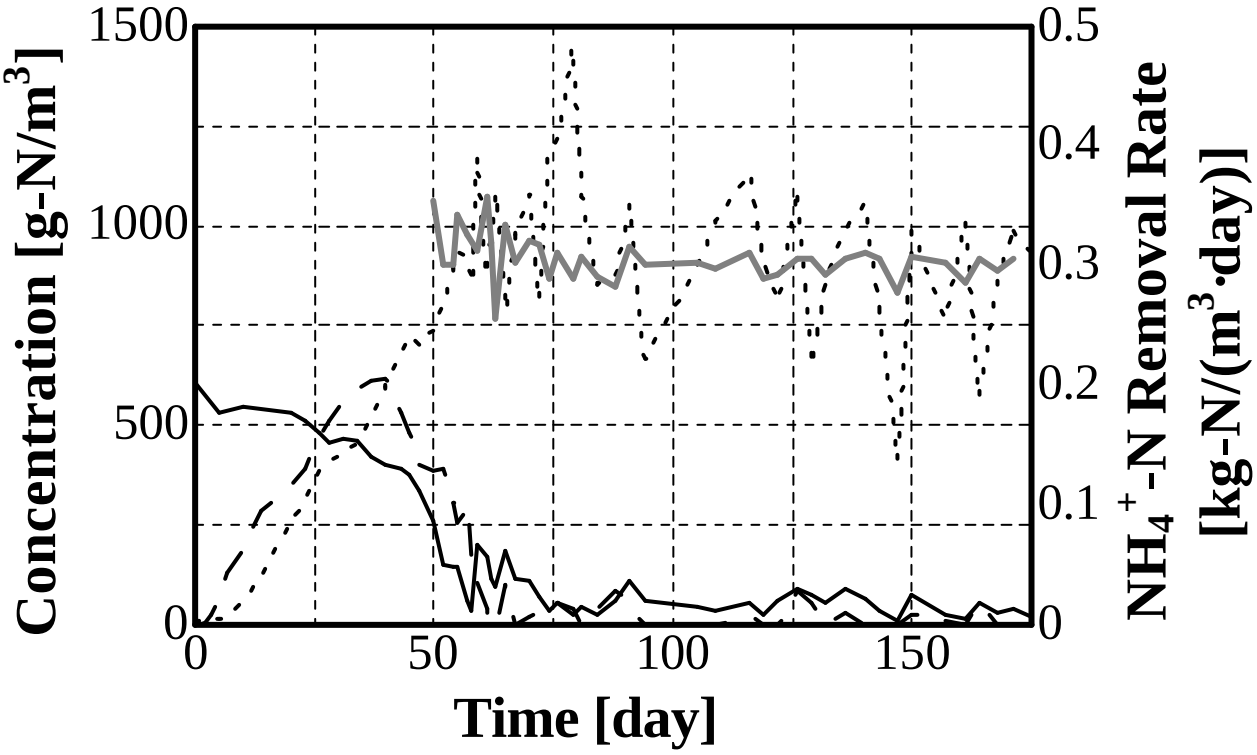


Figure 2.6 Time course of nitrogen compounds concentration in fluidized bed bioreactor containing grafted membrane with nitrifying biofilm. NH_4^+-N ( ), NO_2^--N ( ), NO_3^--N ( ), NH_4^+-N Removal Rate ( )

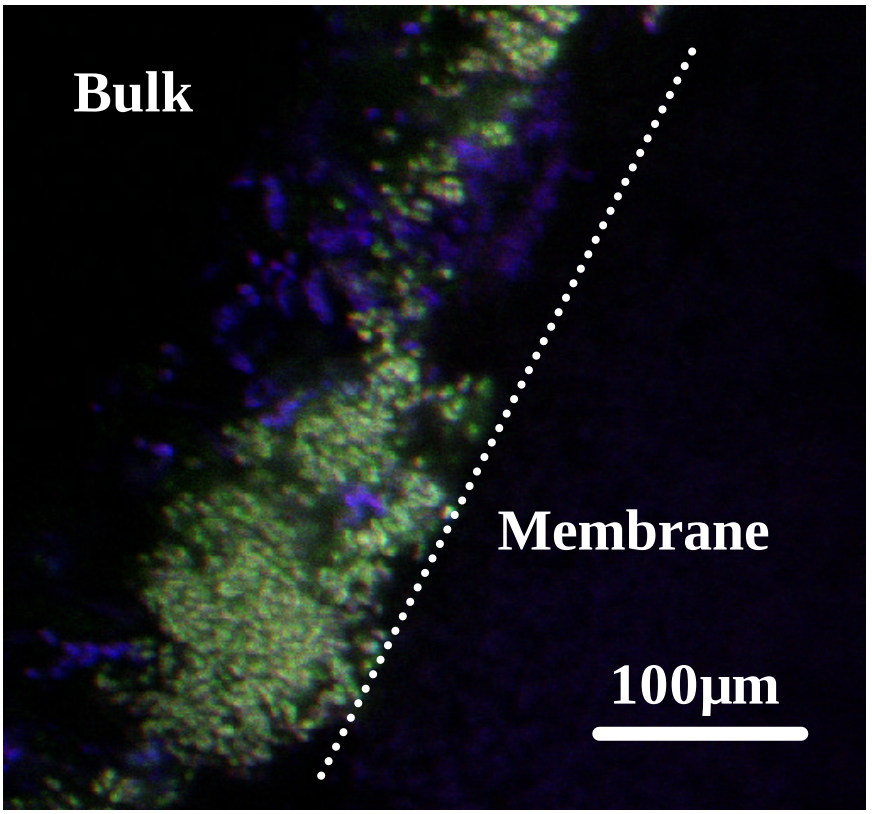


Figure 2.7 FISH image inside biofilm at day 150 (×1000). Green yellow part shows ammonia oxidizing bacteria and purple part shows other bacteria