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Development of an artificial biofilm to study the effects of a single microcolony on mass transport

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Abstract

Alginate harvested from a mucoid strain of *Pseudomonas aeruginosa*, grown on YTG agar plates, was used to develop an artificial biofilm. The alginate was sterilized and fixed to a glass slide where it served as the biofilm matrix. High densities of *P. aeruginosa* were injected into specific locations within the alginate matrix to represent microcolonies similar to those found in natural biofilms. Dissolved oxygen microelectrodes, with tip diameters of 10 μm, were constructed and used to measure oxygen profiles through the artificial biofilm. Using mathematical models the kinetic parameters for microbial respiration were extracted from the profiles. The activity of immobilized microorganisms was monitored and the dynamics of dissolved oxygen transport to a single microcolony was evaluated.

Keywords: Artificial biofilm; Heterogeneity; Microelectrodes; Respiration kinetics

1. Introduction

When growing biofilms in the laboratory, the researcher controls only the quality of the influent into the reactor, the kind of organism under study, the substratum material, and to a certain extent the hydrodynamic conditions of the reactor. Beyond these parameters, biofilm formation is no longer under the researchers control. In addition, with natural biofilm systems, it may be difficult to interpret or obtain reproducible results as parameters such as bacterial density, bacterial distribution, and biofilm thickness can change from experiment to experiment. One way to avoid these difficulties, as well as give the researcher increased control over the experimental conditions, is through the use of artificial biofilms.

Artificial biofilms are useful laboratory tools, serving as simplified 'models' for more complex biofilm phenomena, to conduct fundamental biofilm research under controlled and reproducible conditions. These synthetic biofilms are easy to assemble, have well-defined parameters, and can provide results quickly. They usually consist of a polysaccharide matrix material (i.e. kelp alginate, agar, etc.) into which bacteria are uniformly distributed [1–4]. Although these homogeneous biofilms are representative of some natural biofilms, recent studies that utilize confocal laser microscopy have shown that the structure of some biofilms is complex and heterogeneous [5,6]. These heterogeneous biofilms

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contain bacterial aggregations (microcolonies) separated by interstitial voids. Usually artificial biofilms lack this structural heterogeneity.

Our goal was to develop an artificial biofilm that included some of the physical characteristics found in natural biofilms. The artificial biofilm developed in this work incorporated the structural heterogeneity observed in natural biofilms. This was accomplished through the immobilization of high bacterial densities in localized regions of a sterile matrix material to represent 'microcolonies'. These microcolonies, formed by microinjection of suspended organisms, were on the same scale as those found in some natural biofilms. Finally, the artificial biofilm developed in this work was compatible with microelectrode use enabling the effects of the heterogeneity on the biofilm activity and mass transport to be studied.

The usefulness of the artificial biofilm developed in this work was demonstrated by studying the effects of a single microcolony on the transport of dissolved oxygen. The biofilm was probed using a dissolved oxygen microelectrode to obtain oxygen concentration profiles through the biofilm. The microbial respiration kinetics of the biofilm were extracted from these profiles.

2. Materials and methods

2.1. Biofilm matrix preparation

The matrix material of the artificial biofilm was composed of bacterial alginate. This polymer was produced by the bacterium *Pseudomonas aeruginosa* strain 8830, a stable mucoid variant capable of producing copious amounts of polymer [7]. *Pseudomonas aeruginosa* 8830 was grown in batch culture on a defined medium containing the following (g/l): K_2HPO_4, 7.0; KH_2PO_4, 3.0; $(NH_4)_2SO_4$, 1.0; $MgSO_4 \cdot 7H_2O$, 0.1; glucose, 2.0, pH = 7.2. When the batch culture became turbid, several spread plates of this organism were made on yeast, tryptone and glucose (YTG) agar plates. YTG contained the following (g/l): yeast extract, 5.0; tryptone, 10.0; glucose, 2.0; agar, 15. These plates were incubated at room temperature for several days after which a uniform layer of alginate covered the entire plate. The alginate was removed from the agar plates using the edge of a microscope slide to scrape the surface gathering the alginate. This conglomerate of alginate and bacteria, too viscous to be directly filtered, was transferred to a test tube and diluted with distilled water (10:1, v/v). This slurry was homogenized using a tissumizer (Tekmar Company, Cincinnati, OH) and centrifuged (RC5C Sorvall Instruments, Du Pont Company, Wilmington, DE) at 47 807 × *g* for 20 min. The supernatant was transferred to another tube and the pellet of bacteria was discarded. The tissumizer/centrifugation step was repeated to remove any remaining bacteria from the supernatant. The supernatant was then filtered through a 0.2 μm filter (Cole-Parmer, Niles, IL) into a sterile test tube. Excess water was removed from the diluted alginate with an HBI standard rotary evaporator (Model 421-1655, Buchler Instruments, Lenexa, MO). The supernatant was placed into a sterile round bottom flask on the rotary evaporator. This flask contained an extension tube into which glass wool was tightly packed to prevent contamination of the sterile bacterial alginate upon equilibration of the pressure in the vacuum vessel with the surrounding ambient environment. The evaporator was run until the alginate formed a gel that had a similar consistency as originally produced by the bacteria on the agar plates. The sterilized alginate was then collected from the round bottom flask using a sterile syringe equipped with an 18 gauge needle and stored at 4°C for no more than 1 day before use. The sterile alginate was checked for microbial contamination by plating two 0.1-ml aliquot onto YTG agar plates. If microcolonies grew on the plates, the bacterial alginate was discarded. The sterile alginate was used as the matrix material of the artificial biofilm entrapping bacterial cells.

2.2. Polymer characterization

The extracellular polymer produced by *Pseudomonas aeruginosa* strain 8830 was characterized by ^{1}H-NMR. Cells were diluted in 0.9% (w/v) NaCl and centrifuged. The supernatant was divided into two parts of approximately the same volumes. The first part was dialyzed exhaustively against MQ-water. The bacterial alginate was precipitated by adding isopropanol (50% v/v). The alginate was further washed with 70% ethanol and then 96%

ethanol, and finally with diethylether before air drying. The second part of the supernatant was treated with 0.1 M NaOH at room temperature for 20 min. to remove *O*-acetate groups bound to the D-mannuronic acid residues of the alginate. Following neutralization the deacetylated alginate was precipitated as described above, except that ethanol replaced isopropanol in the first step.

Both alginates were subjected to a slight depolymerization before analysis by NMR. This involved mild acid hydrolysis at pH 5.6 for 60 min, followed by hydrolysis at pH 3.8 for 60 min. The temperature was 100°C. The pH was then adjusted to 6.8, and the material was freeze-dried. Chemical composition and sequential parameters were determined by ^{1}H-NMR analysis as described by Grasdalen [8].

Molecular weights and optionally molecular weight distributions were determined by analytical size exclusion chromatography using three serially connected columns (TSK G6000-PWXL, G5000-PWXL and G4000-PWXL) eluted at 0.5 ml/min at ambient temperature with 0.05 M Na_2SO_4 containing 0.01 M EDTA (pH 6). Injection volumes were 50 μl with polysaccharide concentrations in the range 0.4–1 mg/ml. On-line light scattering data (Chromatix KMX-6 light scattering photometer equipped with the standard HPLC flow cell) and refractive index data (Shodex RI SE-61 detector) were collected and analyzed by the PCLALLS software, which also controlled the autoinjector (Shimadzu SIL-10A). A value of 0.150 was used for the refractive index increment (dn/dc) in molecular weight calculations.

2.3. Microorganism, medium, and growth conditions

The bacterium selected for immobilization in the alginate matrix was *Pseudomonas aeruginosa* strain 8830 [7]. A chemostat with a working volume of 450 ml, operated with a dilution rate of 0.0011 min^{-1} at 25°C, was used to culture this organism. The medium used to culture the bacteria in the chemostat contained (g/l): glucose, 1.0; $(NH_4)SO_4$, 0.15; 3-phosphoglyceric acid, 0.05; $MgSO_4 \cdot 7H_2O$, 0.1; L-histidine, 0.01; $CaCl_2$, 3.68; and Tris–HCl, 0.1 *M* (pH 7.2). A chemostat was utilized so that the immobilized bacteria were always at the same physiological state. After a steady state in the chemostat had been achieved (three residence times) the entire volume of bulk fluid was centrifuged at 11 951 × *g* for 15 min to concentrate the cells. The pellet of cells (10^{10} CFUs) was then suspended uniformly into 0.5 ml of sterile bacterial alginate resulting in a known cell density.

2.4. Dissolved oxygen microelectrode

A Clark-type oxygen microelectrode as described by Jørgensen and Revsbech [9] was constructed. This probe was used to determine the concentration gradient of oxygen through the artificial biofilm. The probe consisted of an outer casing that housed both a single cathode and a silver–silver chloride (Ag–AgCl) reference electrode. The circuit was completed with a 0.5 M KCl electrolyte filling solution. The inner components of the probe were separated from the bulk aqueous environment by a silicone rubber membrane that permitted the passage of dissolved oxygen. A Hewlett Packard 4140B multimeter was used to apply a polarizing voltage and to serve as a picoammeter to measure the current. The cathode was polarized (−0.8 V) against the internal Ag–AgCl reference electrode. When oxygen diffused through the silicone membrane, it was reduced at the cathode, resulting in a flow of electrons that was detected as a current by the ammeter. This current was proportional to the concentration of dissolved oxygen present at a localized region in the biofilm. Before use, the electrode was calibrated by immersing its tip into aerated water followed by immersion into nitrogen-purged water. This determined the current associated with oxygen saturated and nitrogen saturated (zero oxygen) concentrations. These endpoint readings (zero oxygen, saturated oxygen) were then used to determine the dissolved oxygen concentrations from the recorded data collected by the picoammeter.

2.5. Experimental setup

The experimental setup is shown in Fig. 1. The custom-made, open channel reactor was constructed from polycarbonate and contained a glass window located at the bottom of the channel. The glass window allowed the biofilm to be viewed from

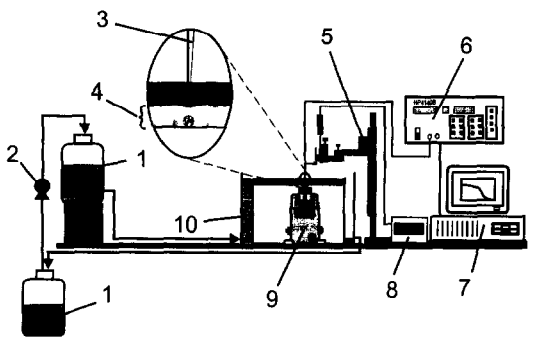

Fig. 1. Schematic diagram of experimental setup. (1) Upper and lower medium reservoirs, (2) pump, (3) dissolved oxygen microelectrodes. (4) artificial biofilm, (5) micromanipulator, (6) picoammeter, (7) data acquisition system, (8) controller, (9) inverted microscope, (10) open-channel reactor.

below using an inverted microscope, while still allowing easy access by the microelectrode from above. The nutrient medium, from the upper reservoir, was gravity fed to the reactor and had a flume depth of 1.0 cm. The flow of the bulk fluid through the reactor was laminar (Reynolds number = 112) with an average flow velocity of 2 cm/s. The temperature of the fluid was maintained at 25°C. The medium composition in grams per liter was as follows: glucose, 0.075; $(NH_4)SO_4$, 0.0075; 3-phosphoglyceric acid, 0.0025; $MgSO_4 \cdot 7H_2O$, 0.005; L-histidine, 0.01; $CaCl_2$, 3.68; and Tris–HCl, 0.1 M (pH 7.2). A background of calcium chloride (25 mM) in the nutrient medium was provided to stabilize the bacterial alginate matrix of the biofilm. Without its presence the biofilm would lose mechanical stability and wash away.

2.6. Artificial biofilm construction:

A one square centimeter layer of the sterile bacterial alginate (~1.2 mm thickness) was fixed onto the glass bottom of the reactor to serve as the matrix of the artificial biofilm. This was accomplished by spreading a thin film of sterile kelp alginate (3% w/v) (Sigma Chemical Co., St. Louis, MO) onto the glass slide in the reactor with a sterile swab and allowing it to dry. The kelp alginate-treated surface was then overlaid with the sterile bacterial alginate which was dispensed from a syringe. A microcolony was formed within the alginate matrix by injecting a high density of viable bacteria (10^{10} CFUs/ml alginate) with a micropipet. The micropipet, with a tip opening of 0.02 mm, was made from a 1-mm glass capillary tube (World Precision Instruments Inc., New Haven, CT) using a microelectrode puller (Stoelting Co., Wooddale, IL). The alginate/bacteria suspension was loaded into the micropipet using a syringe equipped with a 25 gauge needle. The tip of the micropipet was completely filled with the suspension to avoid injecting air bubbles into the matrix. The micropipet was mounted on a micromanipulator (Model M3301R, World Precision Instruments, New Haven, CT) equipped with a stepper motor (Model 18503, Oriel, Stratford, CT) and manipulated by a computer controller (Model 20010, Oriel, Stratford, CT). The tip of the micropipet was lowered into the sterile bacterial alginate matrix with the use of the micromanipulator to a depth of 0.15 mm above the substratum. The viscous bacteria/alginate slurry was forced through the micropipet by applying compressed air to its end. The entire process and drop size were monitored using bright field microscopy (10× objective) through an inverted microscope (IMT-2, Olympus, La Palma, CA) equipped with an ocular micrometer. When 1.8 nl of the bacteria/alginate slurry was injected into the bacterial alginate matrix, the air supply to the micropipet was quickly stopped and the micropipet removed from the matrix leaving a spherical microdrop similar to that in Fig. 2 located immediately above the reactor substratum (Fig. 1). The flow of the medium to the reactor was started and ran for 3 h before probing with a dissolved oxygen microsensor.

The oxygen concentration gradients in the artificial biofilm were measured as the microelectrode, mounted to the motor driven micromanipulator, was moved vertically through the biofilm in predetermined increments. The movement of the micromanipulator was controlled by the computer, controller, and stepper motor which allowed precise steps of 0.01 mm to be made through the biofilm. The computer contained a custom-made software program that recorded the magnitude of the current and the accumulated distance the probe had moved. After each step the current in the microelectrode was measured by the picoammeter and recorded by the computer presenting a profile of dissolved oxygen versus distance.

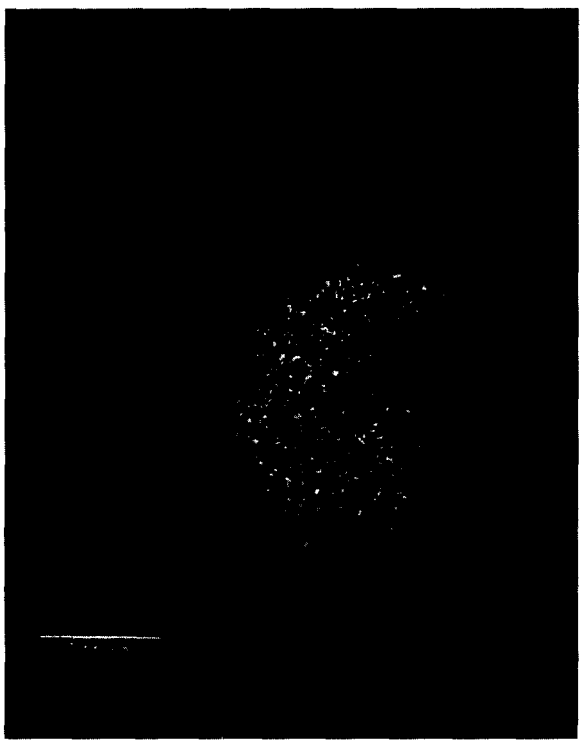

Fig. 2. A confocal microscope image (horizontal slice) of a microdrop containing viable bacteria in a sterile alginate matrix. The bacteria, which were stained using a 0.1 ppm ethidium bromide solution, appear white, while the sterile alginate matrix appears black.

2.7. Microbial respiration kinetics analysis

Fig. 3 is an outline of the procedure used to determine the microbial respiration kinetics of the biofilm from substrate concentration profiles. The details of this procedure have been described elsewhere by Lewandowski [10]. The position of the biofilm-bulk water interface was located on the dissolved oxygen concentration profile using the inverted microscope. The biofilm thickness was found by focusing on the biofilm-bulk water interface and recording the distance travelled by the probe tip until it reached the bottom of the reactor. The dissolved oxygen profile was then divided into two parts: the bulk, which contained information about substrate transport to the biofilm, and the biofilm, which contained information about the microbial activity [10]. The oxygen profile in the water phase was described by the following empirical equation.

$$\frac{C - C_s}{C_b - C_s} = 1 - Exp[-A(x - x_s)] \quad (1)$$

where C (mg/l) is the local dissolved oxygen concentration, C_s is the dissolved oxygen concentration at the surface of the biofilm; C_b is the dissolved oxygen concentration in the bulk fluid, x (cm) is the distance from bottom of the biofilm, x_s is the biofilm thickness, and A is an experimental coefficient determined graphically from the slope of a linearized form of Eq. 1.

The transport of dissolved oxygen through the biofilm (biofilm oxygen profile) was described by the following equation.

$$\left(\frac{\partial C}{\partial t}\right)_f = D_f \left(\frac{\partial^2 C}{\partial x^2}\right)_f - \frac{V_{MAX} C}{K_s + C} \quad (2)$$

where

$$V_{MAX} = \frac{\mu_{MAX} X}{Y_{x/s}}$$

where D_f (cm^2/s) is the diffusion coefficient for the dissolved oxygen in the biofilm, C (mg/l) is the dissolved oxygen concentration at the distance from bottom of the biofilm x (cm). V_{MAX} (mg/l/s) is the maximum oxygen utilization rate, K_s (mg/l) is the half-saturation coefficient, μ_{MAX} is the maximum growth rate (1/s), X is the biomass concentration (mg/l), and $Y_{x/s}$ is the biomass yield. The first term on the right side of Eq. 2 corresponds to diffusional resistance in the biofilm, the second term corresponds to the substrate utilization rate, assumed to follow Monod kinetics.

When consumption equals the rate of transport due to diffusion, a steady state concentration of dissolved oxygen within the biofilm $(dC/dt = 0)_f$ is achieved.

$$0 = D_f \left(\frac{d^2 C}{dx^2}\right) - \frac{V_{MAX} \times C}{K_S + C} \quad (3)$$

Eq. 3 was used to describe the biofilm activity. The procedure outlined in Fig. 3, along with Eq. 3 and the appropriate boundary conditions, yielded the kinetic parameters (V_{MAX} and K_S), and diffusivity of oxygen through the biofilm.

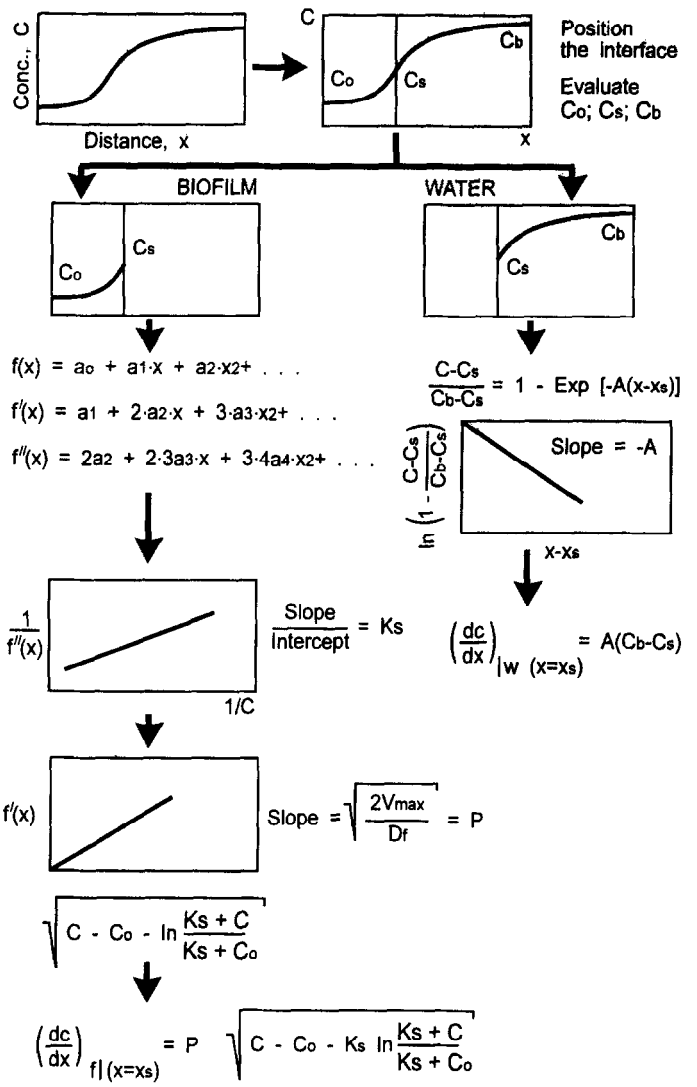

Fig. 3. Procedure for extracting kinetic parameters from a substrate concentration profile [11].

3. Results and discussion

3.1. Polymer analysis and characterization

Bacterial alginate, produced by *Pseudomonas aeruginosa* strain 8830, was chosen to serve as the matrix material for the artificial biofilm. In contrast to kelp alginate, the bacterial alginate was found to be easily penetrated with microelectrodes. The polymer characteristics of the bacterial alginate as determined by ^{1}H-NMR are given in Table 1.

Analysis of the peak areas from the 400 MHz NMR spectrum of the purified bacterial alginate, correspond well to that obtained earlier for bacterial alginate from *P. aeruginosa* strain DE27 [12]. Analysis of the peak areas from the NMR spectrum, showed the bacterial alginate consisted of 95% mannuronic acid (M) and 5% guluronic acid (G). Moreover, the G residues were always linked to M-residues, as no peak corresponding to the GG sequence was found in the spectrum. The alginate is therefore an almost pure polymannuronic acid poly-

Table 1
Characteristics of purified alginate from *P. aeruginosa* strain 8830

Property	Native	De-*O*-acetylated
Molecular weight (g/moles)	1.8×10^6	1.5×10^6
Intrinsic viscosity (η) (ml/g)	29.0	—
O-Acetyl content (moles/uronic acid residue)	0.69	—
Fraction of D-mannuronic acid (F_M)	ND	0.95
Fraction of L-guluronic acid (F_G)	ND	0.05
Fraction of G units adjacent to a second G (F_{GG})	ND	0.00
Fraction of G units adjacent to an M (F_{GM+MG})	ND	0.05

ND, not determined.

mer. Of particular importance is the absence of G-blocks, i.e. consecutive sequences of L-guluronic acid. Such G-blocks, as found in kelp alginate, are a prerequisite for the formation of firm gels in the presence of calcium ions. This explains why the bacterial alginate studied here did not form a strong gel and was therefore easily penetrated with the microelectrode.

3.2. Application of artificial biofilm

A heterogeneous artificial biofilm was constructed and used to study the effects of a single microcolony on the mass transport of dissolved oxygen. The microcolony had a volume of 1.8 nl, and a cell density of 10^{10} CFUs/ml alginate. Dissolved oxygen profiles were taken by probing directly in the artificial biofilm above and adjacent to the bacterial microcolony using a Clark-type dissolved oxygen microelectrode. The 10 profiles (A–J) shown in Fig. 4 were taken through the sterile alginate matrix at various horizontal distances adjacent to the microcolony. These profiles were similar in shape to those measured in natural biofilms [10,13]. The distance of each profile from the center of the microcolony is shown in the legend of Fig. 4. The

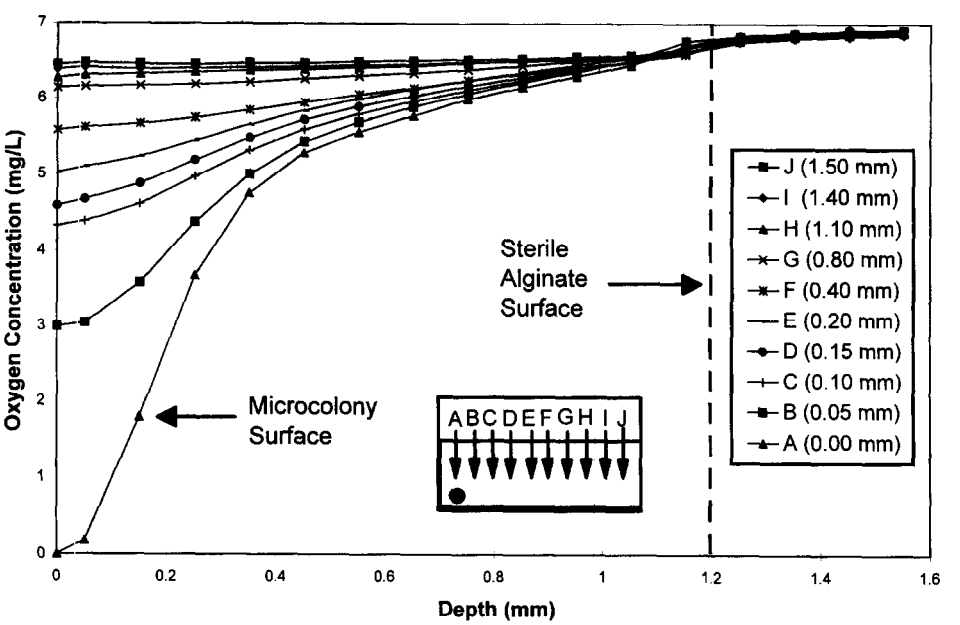

Fig. 4. Dissolved oxygen profiles through artificial biofilm containing a single microcolony. Profile (A) was taken directly through the microcolony. Profiles (B–J) were taken at various distances adjacent to the microcolony. Insert shows where each profile was made relative to the drop.

dissolved oxygen concentration in the bulk fluid at 25°C was 6.9 mg/l. The average flow velocity over the biofilm was 2.0 cm/s. The base of the biofilm is located at $x = 0$ mm, while the alginate–water interface is located at approximately 1.2 mm. The dissolved oxygen concentration above the interface was constant throughout the bulk fluid at 6.9 mg/l. Below this interface, a decrease in the oxygen concentration was observed in all profiles (A–J). These profiles indicated that the dissolved oxygen field was affected by the respiratory activity of the bacteria up to a distance of 1.4 mm away from the microcolony. However, at a distance greater than 0.4 mm from the microcolony, the decrease in the dissolved oxygen concentration due to its consumption by the microcolony was not as pronounced. The decrease observed in profiles (A–I) was due to the consumption of oxygen by the immobilized bacteria and the mass transfer resistance of polymer gel matrix. Profile J, taken a distance of 1.5 mm from the center of the bacterial drop, was not influenced by the respiring bacteria. The decrease in the oxygen concentration observed in profile J was due to the mass transfer resistance of the sterile alginate matrix.

Profile A, taken directly through the microcolony, was analyzed using the method outlined in Fig. 3 to determine the microbial respiration kinetics. The surface of the microcolony was located at approximately 0.15 mm on this profile. Using the oxygen diffusivity through water of 2.5×10^{-5} cm^2/s at 25°C [14] the following parameters were obtained. The diffusivity through the bacterial microcolony (D_f) was 2.16×10^{-5} cm^2/s, the maximum substrate utilization rate (V_{MAX}) was 0.44 mg/l/s, and the half saturation coefficient (K_S) was 0.025 mg/l. The values calculated here are similar to values reported in literature for natural biofilms [10,13].

4. Conclusions

An artificial biofilm has been developed which used bacterial alginate as the biofilm matrix polymer, incorporated microcolonies of bacteria as seen in natural biofilms, and was compatible with microelectrode use. The microbial respiration kinetics were determined by probing the biofilm with a dissolved oxygen microelectrode and analyzing the resulting dissolved oxygen profiles. This study demonstrated the applicability of the artificial biofilm, to monitor the effects of a biofilm's heterogeneity on mass transport. The artificial biofilm is a useful laboratory tool allowing some experiments to be carried out in a more controlled and reproducible environment.

Acknowledgments

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