Factors Regulating Microbial Biofilm Development in a System with Slowly Flowing Seawater

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Microbial biofilm development was followed under growth conditions similar to those of a projected salinity power plant. Microscope glass cover slips were piled in biofilm reactors to imitate the membrane stacks in such a plant. A staining technique closely correlating absorbance values with biofilm dry weight was used for the study. Generally, the biofilms consisted of solitary and filamentous bacteria which were evenly distributed with considerable amounts of various protozoa and entrapped debris of organic origin. Protozoa predation was shown to decrease the amount of biofilm produced. The biofilm development lag phase was longer at lower temperatures. The subsequent growth phase was approximately arithmetic until stationary phase appeared. Adaptation of a hyperbolic saturation function gave curves that agreed well with the logarithm of the amount of biofilm as a function of time. Increased flow velocity, temperature, and nutrient concentration increased the biofilm production rate. An exponential relationship was shown between biofilm production rate and flow velocity within the range of 0 to 15 cm s⁻¹. Intervals in which the biofilms were exposed to fresh water decreased the biofilm production rate more than four times. If the cover slips were inoculated with untreated seawater for 24 h, subsequent UV treatment had an insignificant effect on the biofilm formation.

When a cation- and anion-exchange membrane are placed with fresh water flowing in the middle and salt water flowing on the sides, a voltage develops over the membrane pair. Multiplication of the number of membrane pairs with appropriate fresh and salt water flows creates an electrochemical concentration cell able to produce a useful voltage (14). This investigation arose from plans to use multiple groups of concentration cells to exploit the decrease in free energy (ΔG) that occurs when river water mixes with seawater (11, 18). An investigation of the possibility of building a 200-MW salinity power plant (SPP) is in progress in Sweden, considering technical, economical, and biofouling questions. The projected plant would utilize 1,500 m³ of seawater s⁻¹, 390 m³ of river water s⁻¹, and 76 km² of membranes (6). The risk of severe microfouling problems is obvious, at least on the seawater side of the membranes. Indeed, there is also a great risk for macrofouling problems, but that subject is outside the scope of this journal.

The plant has been projected on the basis of "expected commercial technological level attained 1990" (6), assuming membrane characteristics not available today. Thus, as no relevant concentration cells have been available for studies of the effect of microfouling on the membranes, the present investigation has been restricted to a study of the microfouling potential at growth conditions approximating those of the flowing seawater in a SPP.

In planning a seawater intake location, it is of interest to know how variations in water type, nutrient concentration, and temperature affect biofilm development. The ideal flow velocity through the membrane stacks in the SPP has been approximated at 5 cm s⁻¹. It can be calculated how changes in the flow affect the electricity yield of the plant, but what is the corresponding relationship between the flow rate and biofilm production rate? The ultimate question when dealing with biofilm problems is how to eliminate, or at least decrease, the problem. Sterilization of a whole plant is practically impossible, but if the feed water could be sterilized, for instance by UV light, what would the effect on developing biofilms be? Many organisms are sensitive to variations in osmotic pressure. If that is true for biofilm organisms, a convenient way to decrease the biofouling problem would be alternations of the river and seawater flows through the plant.

MATERIALS AND METHODS

Biofilm dry weight estimation. The experimental equipment has been described in detail previously by
me (15). Basically, lamellar piles of glass cover slips (60 by 24 by 0.15 mm) were placed in biofilm reactors and exposed to continuous flows of seawater. During the biofilm developments, cover slips with developing biofilms were sampled and stained with crystal violet (3). Thereafter, the absorbance at 590 nm of the biofilms on the cover slips was measured at six fixed points. The number of observations, n, given in the results section indicates the number of slips employed for each absorbance determination if divided by 6. The absorbance of stained biofilms has been shown to correlate closely with biofilm dry weight (15). The very high precision of the method was also shown. The following relationship between biofilm dry weight, \( B_{dw} \), and the absorbance at 590 nm (\( A_{590} \)) of stained biofilms was obtained:

\[
B_{dw} = \frac{A_{590}}{0.00675}
\]  

Biofilm wet mounts and stained biofilms were observed and photographed in a differential interference contrast microscope (Olympus BH-NIC with a PM-10-A camera) during the experiments.

Field station seawater system. With one exception (see below), all of the experiments were performed at Tjärnö Marine Biological Station, located in the northern part of the Swedish west coast. Three periods were used for study: 22 February to 18 April 1978, 17 July to 5 September 1978, and 26 June to 18 July 1979, henceforth identified as winter 78, summer 78, and summer 79, respectively. At the station, continuous flows of seawater from depths of 2 and 40 m were available. The 2-m water had an average salinity of 23%o and was pumped from the bay outside the station via a tiled reservoir (retention time 2 to 5 h) through the biofilm reactors. The bay received biologically treated sewage water from about 20 people during the winter and up to 50 people in the summer. The water circulation in the bay was good, and there was no visible pollution effect. The 40-m water had an average salinity of 33%o and was pumped through a conduit from the outer coastline about 1 km away from the station via another tiled reservoir (retention time 2 to 5 h). The flow velocity through the reactors was kept at 5 cm s\(^{-1}\), except in the flow velocity experiment. When samples were taken, the temperature of the waters used was recorded with a YSI model 33 S-C-T meter (Fig. 1). On its way to the station, the 40-m conduit ran through shallow water, which usually had a different temperature from the 40-m water. Thus, the summer temperature of the latter was raised at most to 19°C, and its winter temperature was decreased to as low as 2°C for part of the experimental time. Before entering the biofilm reactors, the water had to be passed through a 125-µm stainless steel filter, otherwise the edges of the cover slips became covered with fibrous material, mostly algal debris. The filter system was automatically refilled every 12 h.

Laboratory seawater system. For nutrient enrichment experiments, a tank system providing complete stirring was used at the laboratory in Göteborg. The system is fully described elsewhere (15) and consisted of a 200-liter tank from which seawater was circulated through the biofilm reactors. The seawater was collected by boat and continuously fed to the tank at a rate of 15 liters day\(^{-1}\), resulting in a dilution rate of 0.003 h\(^{-1}\). The flow was kept at 5 cm s\(^{-1}\), and the temperature was kept at about 17°C.

Nutrient, temperature, and flow velocity experiments. A nutrient-depleted biofilm was achieved when the laboratory seawater system was used with a very low dilution rate, 0.003 h\(^{-1}\) (9). When the biofilm development rate diminished, a single instantaneous addition of glucose (10 mg liter\(^{-1}\)) and nutrient broth (Difco Laboratories) (10 mg liter\(^{-1}\)) was made and

FIG. 1. Temperature in field station seawater during the test periods. Symbols: ●, 40 m, winter 78; ■, 40 m, summer 78; ▲, 40 m, summer 79; △, 2 m, summer 79.
repeated when the biofilm development rate diminished again.

Biofilm development was studied at ambient temperatures in winter 78, summer 78, and summer 79 with the 40-m water. In additional biofilm reactors, the biofilm development was followed with the same water and synchronously with the ambient temperature studies, but with the temperature raised to 15°C in winter 78 and to 25°C in summer 78. The temperature increases were achieved by passing the water through stainless steel loops immersed in a heated water bath which was regulated by a contact thermometer situated in the discharge from the reactors. When the temperature was increased, dissolved gases in the water formed small gas bubbles which could disturb the biofilm development. Therefore, the water was degassed by passage through a 200-liter water tank before entering the reactors.

The biofilm development was studied at flows of 0.5, 5, and 15 cm s\(^{-1}\) with 40-m water during summer 78. This experiment was combined with the heating experiment described above in which the ambient water temperature (Fig. 1) was raised to 25°C. On day 22, the heating system failed, and the reactors were changed over to ambient-temperature water. The flows were regulated by valves and controlled with flow meters (Rotameter, series 2000 ± 4%).

Biofilm control experiments. During summer 79, the flow of the 2-m water through two parallel reactors was shifted to a freshwater flow for 2 min every hour. The fresh water came from the local drinking well and was untreated except for pH buffering to pH 7. At the start of the summer 79 period, two other parallel biofilm reactors were connected to the 2-m water. After 24 h, it was assumed that a biofilm was induced on the cover slips. The reactors were then connected to a UV sterilizer (Raydar/Ellner, REP-12 with a 65-W 253.7-nm lamp). The volume of the sterilization chamber was 30 liters, resulting in a water retention time of 15 min and an intensity of 0.5 Ws cm\(^{-2}\). The accurate function of the sterilizer was checked with viable counts on the sterilized water. The biofilm developments were compared with the development in two reactors used with untreated 2-m water.

Mathematical and statistical treatment of data. The absorbance data obtained were transformed to dry weight by equation 1, and the natural logarithms of the dry weight values were plotted against time. The plotted data suggested the adaptation of a hyperbolic saturation function curve of the form

\[
Y = \frac{ax}{b + x}
\]

\(a > 0, \ b > 0\) \hspace{1cm} (2)

The data plots also indicated the necessity of taking into account a biofilm development lag-phase time that indicated the end of a period with no detectable growth and the start of a significant growth phase. The choice of lag phase time, \(t\), and the amount of biofilm at the end of the lag phase, \(B_0\), were standardized as follows. The standard deviation of the dry weight values at time zero was ±0.4 µg (dry weight) of biofilm cm\(^{-2}\). That variation was due to variations in the staining technique (amount of mounting medium and the thicknesses and pretreatment of the cover slips and microscope slides) and variations with the spectrophotometer (dissolving limit = 0.15 µg [dry weight] of biofilm cm\(^{-2}\)). \(B_0\) therefore was chosen as 0.4 µg (dry weight) of biofilm cm\(^{-2}\), and it was defined as the last day, \(t\), with a biofilm dry weight at day \(t\), \(B(t)\), not significantly different from zero. With a nonlinear regression program (Gauss-Newton method [8]), the following function was adapted to the growth phase:

\[
\ln \frac{B(t)}{B_0} = \frac{a(1 - \frac{t}{t})}{b + (t - t)}
\]

\(a > 0, \ b > 0, \ B(t) \geq 0.4, \ t \geq t\) \hspace{1cm} (3)

In most of the experiments, this regression function resulted in a small sum of squares (see result plots). However, in some of the plots, the data points were too few for good statistics or the time was too short for a good indication of a saturation level. The curve was then drawn subjectively, and the constants were left out of the figure.

For the analysis of how the factors studied related to the biofilm development, biofilm production rates \(P_{t-1} - t\) (in micrograms [dry weight] per square centimeter per day) were calculated according to the equation

FIG. 2. Biofilm development after single instantaneous nutrient additions in the laboratory seawater system with low dilution rate. Arrows indicate nutrient addition. Symbols: ▲, biofilm development in the first experiment, with an initial addition on day 17 (n = 24); ○, biofilm development in the repeated experiment, with initial addition on day 10 (n = 36). The bars indicate the standard deviations of \(n \ln\) (dry weight) determinations.
Fitted values used in this development response addition was observed. The bands of benthic flow-oriented weight [dry weight] cm^{-2} day^{-1} (Fig. 4A). The protozoa were able to decrease the biofilm at a rate of -7.15 \mu g [dry weight] cm^{-2} day^{-1} (p_{20-21}). The grazing protozoa caused an increase in the variance of the absorbance data. When the experiment was repeated with initial nutrient addition at day 10, the pattern of fast responses (4.27 \geq p_{17-18} \geq 0.93 \mu g [dry weight] cm^{-2} day^{-1}) and decreases in biofilm production was repeated at every new addition (Fig. 2). The protozoa also appeared in this experiment, but not with the same intensity as in the preceding experiment; at most at a rate of -2.57 \mu g (dry weight) cm^{-2} day^{-1} (p_{22-23}).

Influence of water temperature changes. The winter 78 water temperature was within the range of 2 to 7°C, resulting in a long biofilm development lag phase, 27 days, and in a low production rate; p_{0-55} was 0.19 \mu g (dry weight) cm^{-2} day^{-1} (Fig. 4A). For the biofilm development with the water temperature increased to 15°C, the lag phase decreased to 2 days and the production rate, p_{0-55} (1.06 \mu g [dry weight]
cm\(^{-2}\) day\(^{-1}\) ), was increased by 5.6 times that for the ambient-temperature biofilm.

The ambient water temperature during summer 78 was between 12 and 19\(\degree\)C and gave a lag phase of 11 days (Fig. 4B). The biofilm production rate, \(p_{0-55}\), was 0.89 µg (dry weight) cm\(^{-2}\) day\(^{-1}\), and \(p_{0-22}\) was 0.40 µg (dry weight) cm\(^{-2}\) day\(^{-1}\). A temperature increase to 25\(\degree\)C decreased the lag phase to 5 days and doubled the \(p_{0-22}\) to 0.82 µg (dry weight) cm\(^{-2}\) day\(^{-1}\).

**Relation between flow velocity and biofilm production rate.** When the flow velocity over the biofilms was increased from 0.5 to 5 and 15 cm s\(^{-1}\), the function constants \(a\) and \(b\) also increased. The lag-phase time was retained at 5 days (Fig. 5A). A plot of \(p_{0-50}\) against flow velocity (Fig. 5B) showed an increasing function with constants \(a = 5.88 (±0.31)\) and \(b = 9.60 (±1.67)\).

**FIG. 4.** (A) Biofilm development in winter 78 at ambient 40-m water temperature (2 to 7\(\degree\)C) (\(\triangle\)) \((n = 12)\) and with the temperature increased to 15\(\degree\)C (●) \((n = 12)\). The bars indicate the standard deviations of \(n \ln\) (dry weight) determinations. Numbers in parentheses indicate the asymptotic 95% confidence intervals. (B) Biofilm development in summer 78 at ambient 40-m water temperature (12 to 19\(\degree\)C) (\(\triangle\)) \((n = 24)\) and with the temperature increased to 25\(\degree\)C (●) \((n = 12)\). The bars indicate the standard deviations of \(n \ln\) (dry weight) determinations. Numbers in parentheses indicate the asymptotic 95% confidence intervals.
velocity, \( f \), suggested a nonlinear relationship of the form

\[
p = h \cdot f^{(k)}
\]

\[
f \leq 15
\]

Equation 5 then shows that \( p_{0-50} \) increased exponentially with \( f \) in such a way that the influence of the flow velocity on the biofilm production rate decreased exponentially.

**Effect of the field station seawater intake location.** There was a pronounced difference in biofilm development between the 2-m and 40-m water intakes (Fig. 6A). The 2-m water had a \( p_{0-22} \)
of 1.92 μg (dry weight) cm$^{-2}$ day$^{-1}$ and a lt of 3 days. The 40-m water $p_{0-22}$ was only 0.09 μg (dry weight) cm$^{-2}$ day$^{-1}$ and had a lt of 14 days.

**Biofilm control experiment.** An interesting indication of a possible method to control biofilm was obtained with the freshwater interval experiment. The freshwater treatment decreased the biofilm production rate, $p_{0-22}$, more than four times and increased the lag-phase time from 2 to 9 days (Fig. 6B). A similar effect was indicated in repeated experiments, once with the same freshwater intervals and the 40-m field station water and once with the laboratory seawater system and 8-min intervals of dechlorinated tap water every sixth hour.

The data in Fig. 6B do not indicate any significant effect of UV-treated seawater on the biofilm development. However, in the microscope, it was discovered that the UV-treated seawater biofilm differed in composition from that usually observed. The biofilm consisted significantly more of solitary bacteria and less of filamentous bacteria and entrapped debris. This difference must be an effect caused by the UV treatment equipment, but there were too many variables to assess the reasons for it.

**DISCUSSION**

The mathematical approach. Assuming the dry weight of a bacterium to be $5 \times 10^{-13}$ g, the $B_{0}$ of 0.4 μg (dry weight) cm$^{-2}$ would correspond to at most $10^{6}$ bacteria cm$^{-2}$; about 10 bacteria per sight field in a microscope at 400× enlargement. By the microscope observations, it could be verified that the ends of the lag phases were ends of periods with occasionally appearing bacteria and the start of periods with significant appearances of bacterial microcolonies, filamentous bacteria, protozoa, and organic debris. The lag-phase definition does not exclude eventually appearing growth during the lag phase, but such growth will not be significant in relation to that of the mature biofilms that develop dry weights 100 to 200 times larger than the lag-phase dry weight limit.

In balanced microbial growth, there is usually a linear relationship between the logarithm of the cell number and time, until various limitations create a stationary phase. The adaptations of straight lines to the results would have to include a subjective judgment of the end of exponential growth phases varying in length.
from short to almost infinite. This problem was avoided by the use of equation 3 in nonlinear regression. The generally low residual sum of squares reveals a good validity of the equation. The availability of nutrients in seawater for microbial growth is limited (5, 17). If all available nutrients transported to a biofilm in a seawater flow are utilized, the growth will be arithmetic. The high nutrient sensitivity in the nutrient addition experiment indicated nutrient-limited growth in marine biofilms. The biofilm development then will be constant at a steady state of regulating factors. As the development approaches a stationary phase, it will be retarded by increasing death, predation (protozoa), diffusion limits, and re-entrance of biofilm material to the flow (2, 7). The suggestion above would explain the applicability of equation 3 to the biofilm data obtained.

A drawback of equation 3 is the assumption of a constant state of regulating factors throughout the test periods. It is more likely that the conditions regulating the biofilm development fluctuate. In the nutrient addition experiment, the exaggerated fluctuation in nutrient availability strongly affected the biofilm development. Such effects not covered by equation 3 could also be observed in the field station seawater system. The most obvious example of this was obtained in winter 78 (Fig. 4A), when the biofilm developments jumped to new, higher stationary levels after day 27.

By the use of biofilm production rate and lag phase time in the comparison of biofilm development data, the drawback is reduced. As long as the comparisons are applied only to the time interval studied, the $l_1$ and $P_{1-2}$ deduced from equation 3 offer an attractive way to compare the results. It should be emphasized that the expressions “biofilm development” and “biofilm growth” comprise both organism growth and attachment processes. The biofilm dry weight assay used does not discriminate between these processes. However, although the microscope observations indicated organism growth and debris attachment as dominating processes in the overall buildup of the biofilms studied, the temperature experiments indicated organism growth as more dominating. This could be tested by complementing with microbial activity measurements, labeling, and Coulter Counter experiments.

Factors regulating SPP biofilm development. The 2-m water was taken from a bay that was exposed to fertilization from human activities around the bay. A 21-fold-higher production rate was observed in the 2-m water as compared with the 40-m water. The waters differed at most by about 3°C, a difference too small to explain the production rate difference. A SPP seawater intake should be carefully planned to avoid sewage plumes from town regions.

The temperature increases shortened the lag phase durations and increased the production rate. The temperature gradients in the SPP due to different temperatures of the sea and river waters will cause corresponding biofilm gradients. The biofilm problem will be reduced during the winter season and increase to a maximum sometime during late summer.

Increased flow did not affect the lag phase, but it did increase the production rate. The relation between the flow and the production rate in the flow region below 1 cm s⁻¹ indicates the influence of an adsorption phenomenon that accelerates the supply of material to the biofilm. As the flow increases, this effect will stay approximately constant. Consequently, decreases of the flow in a SPP will not result in a corresponding decrease in the biofilm problem.

SPP biofilm control. The freshwater experiments indicate that alternations of the sea and river water flows in the SPP can be an effective method for biofilm control. The optimum flow times and intervals between the alternations will have to be studied in relation to the SPP electricity yield before a definite judgment of the efficiency of the method can be given.

An effective filtration, preferably at a filter size ≤ 0.1 mm, will decrease problems with fibrous materials clogging the membrane edges. Most of the larvae from macrofouling organisms will also be eliminated by a filter of that size.

Pre-sterilization of the feed water could not be shown to affect the biofilm development. Any type of treatment intended for biofilm control has to reach the biofilm to be successful.

The results presented in this paper have shown the biofilm potential at conditions relevant to a SPP. In the future, it is hoped that the relation between membrane function and biofilm amount can be studied. By merging the results from those studies, the likelihood in a SPP can be predicted.

Biofilm characteristics in relation to regulating factors. Investigations of microbial biofilm formations in flowing water are being reported frequently, mainly as chemostat works or as studies in relation to biofilm problems in Ocean Thermal Energy Conversion (OTEC) heat exchangers (16). In comparison, OTEC studies are more closely related to SPP biofilm problems than are most chemostat works, which usually utilize nutrient concentrations that are too high to be realistic for marine biofilms. But there are crucial differences between OTEC and SPP. The flow velocity in a SPP (≈5 cm s⁻¹) is much lower than the 1 to 2 m s⁻¹ used in the OTEC studies. In addition, the seawater used for OTEC studies is from open oceans that are very
different with respect to water temperature and probably with respect to nutrient composition as compared with the near-shore seas intended for a SPP. Increasing flow increases the shear forces on a biofilm and the velocity of the transport mechanisms to and from a biofilm. Together with relatively high temperatures (\(\sim 25°C\)), this could explain why OTEC research workers report more compact, filamentous (1, 13) and productive \((p_{O_2} = 4 \text{ to } 16 \mu g \text{ [dry weight]} \text{ cm}^{-2} \text{ day}^{-1}, \text{ depending on tube material [10]}\) biofilms as compared with what is reported here. An additional reason for the difference may be the difference in the material and the structure of the solid surfaces (metal versus glass).

The early stages of biofilm formation observed with my experimental setup resemble more closely those reported by Corpe (4; personal communication) and Marszalek (12). Corpe observed both solitary and filamentous bacteria and an acid-polysaccharide matrix together with diatoms, protozoa, and debris on glass slides submerged in seawater for up to 10 days. Marszalek reported similar initial development but also a continuing development towards a two-tier biofilm on both glass and metal surfaces over a period of about 35 days. Although glass and stainless steel were fouled in the same way, biologically active copper alloys were fouled at slower rates. In conclusion, the data presented in this paper offer valuable information about the biofilm potential not only for a SPP but also for solid surfaces in other systems involving slowly flowing seawater.

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LITERATURE CITED