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Method for Studying Microbial Biofilms in Flowing-Water Systems

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A method for the study of microbial biofilms in flowing-water systems was developed with special reference to the flow conditions in electrochemical concentration cells. Seawater was circulated in a semiclosed flow system through biofilm reactors (3 cm s^{-1}) with microscope cover slips arranged in lamellar piles parallel with the flow. At fixed time intervals cover slips with their biofilm were removed from the pile, stained with crystal violet, and mounted on microscope slides. The absorbances of the slides were measured at 590 nm and plotted against time to give microbial biofilm development. From calibration experiments a staining time of 1 min and a rinse time of 10 min in a tap water flow (3 cm s^{-1}) were considered sufficient. When an analysis of variance was performed on biofilm development data, 78% of the total variance was found to be due to random natural effects; the rest could be explained by experimental effects. The absorbance values correlated well with protein N, dry weight, and organic weight in two biofilm experiments, one with a biofilm with a high (75%) and one with a low (~25%, normal) inorganic content. Comparisons of regression lines revealed that the absorbance of the stained biofilms was an estimate closely related to biofilm dry weight.

The development of microbial biofilms in flowing-water systems has been the subject of many investigations, usually to elucidate a large number of undesirable effects concomitant with growing biofilms. Increased flow resistance, induction and acceleration of corrosion processes, decreasing heat exchanger capacity, and clogging of filters are examples of problems caused by biofilms.

All biofilm studies require more or less complicated devices both for the biofilm cultivation and for the study of effects caused by microbial biofilms. Methods to determine biofilm mass as a function of time and regulating factors are also required. The design of these devices and methods depends very much upon the aim of the biofilm study. Those described in this paper have been designed for the study of factors regulating biofilm development under flow conditions as in electrochemical concentration cells.

A concentration cell consists of a number of anion and cation-exchange membranes. The membranes are alternately arranged in lamellar piles between an anode and a cathode with salt water and freshwater alternately flowing over the membranes. By use of such cells natural seawater and freshwater can be utilized for the production of electrical power (14).

The availability of ion-exchange membranes with the efficiency required for a useful concentration cell is very limited, so I constructed lamellary glass piles that imitate the flow conditions apparent in a concentration cell, and I used natural seawater to develop the method.

MATERIALS AND METHODS

Biofilm reactors. Microscope cover slips (60 by 24 by 0.15 mm) were fitted into acrylic plastic holders forming two parallel test piles, each with room for 19 slips (Fig. 1). The distance between the slips was 1 mm.

To get even and comparable surface energies throughout manufactured batches of slips, they were heated in a muffle furnace at 550°C for 6 h, resulting in glass surfaces on which water drops spread completely. The test piles were placed in flow cells, which, together with the diffusors and stabilizers described below, are denoted as biofilm reactors. At the flow velocity used, 3 cm s^{-1}, the flow is laminar. To separate the flow at the inlet of the reactor, three diffusors with different hole patterns were used (Fig. 1). Finally, a laminar flow between the slips was established with flow stabilizers that, except for their length of 32 mm, were identical to the test pile (60 mm). The hydrodynamic considerations are adopted from Vennard and Street (18). Since the flow direction through the reactor was alternated (see circulating water system), diffusors and stabilizers were placed on both sides of the test pile. The flow pattern through the cell was visualized by pouring Difco agar into the water pumped through the cell and observing with perpendicular illumination. No inequalities in the flow were seen. To avoid sedimentation effects and air

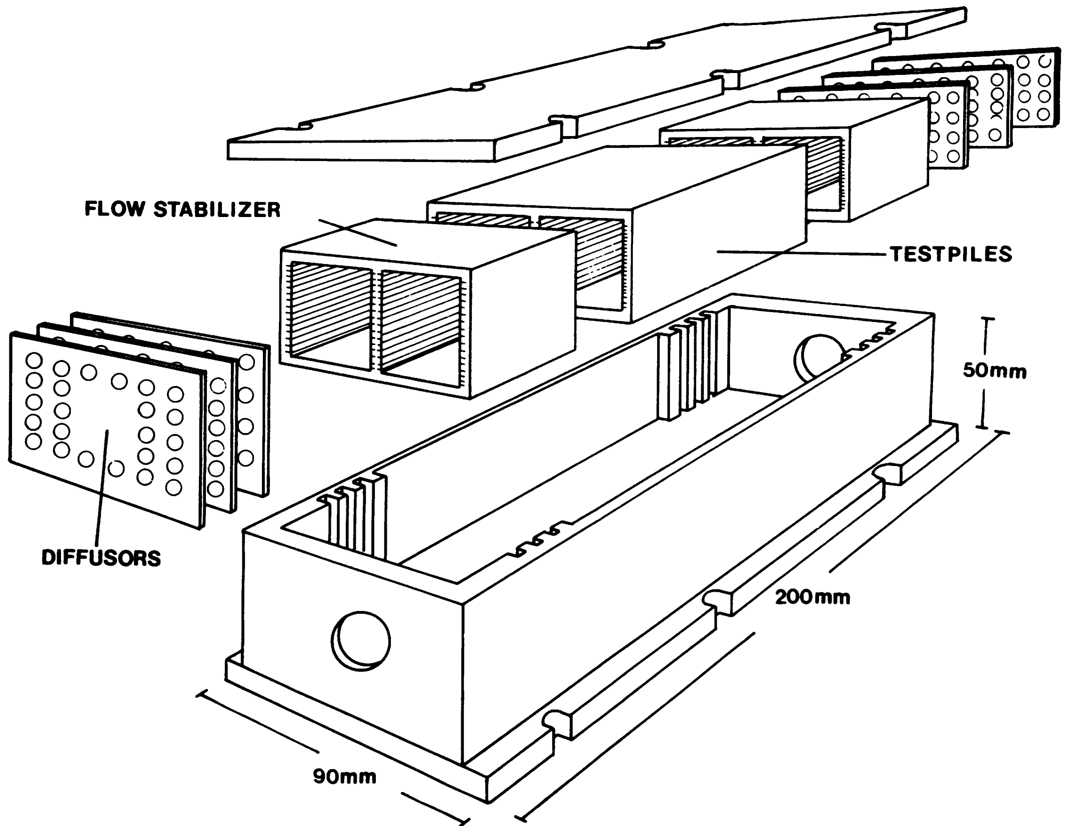

FIG. 1. The biofilm reactor, made of acrylic plastic.

bubbles on the slips, they were placed in parallel with the gravitational force during the experiments.

Sampling. At fixed times a desired number of slips were taken out for biomass determinations. Normally, one sample consisted of two slips, one from each of the two parallel piles. The sampled slips were replaced with new ones so that the flow conditions through the pile were maintained.

Circulating water system. Seawater was collected by boat from a depth of 2 m in the outer coast line of Gothenburg (57°39.0′ N, 11°36.5′ E) every 7 to 10 days. A 200-liter tank was continuously fed with 30 liters of the water a day, and a corresponding water quantity was expelled by brim drainage. From the tank a 316 stainless steel Teflon impellar pump (Liquiflo Series 86 pump) circulated the water in the water system constructed of polypropylene tubes (Ø 10-mm Eastman impolen tubing; ITE), nylon tube fittings (Jaco), and stainless steel valves (Fig. 2). Water circulation in the tank was maintained by the return flow from the system. Before entering the reactors, the water was passed through one of two parallel 125-μm stainless steel filters, otherwise the edges of the flow stabilizers became covered with fibrous material disturbing the flow. The filters were automatically reflushed every 12 h. The flow velocity through the reactors, 3 cm s^{-1}, was regulated by valves and pump speed and controlled by flow meters (Rota-meter series 2000, ±4%). The tank and the pump were kept in a cold-storage room at 8°C; the rest of the system was placed in the laboratory. The resulting water temperature in the system was 17.3 ± 0.5°C. The biofilm reactors were placed in series with the circulating system (Fig. 2). To avoid transport gradients within or between the reactors, the flow direction through the reactor series was alternated every 12 h.

Biofilms growing on the slips decrease the distance between the slips. It could be suspected that above a certain biofilm thickness, the hydraulic performance through the reactors may be impaired. The system was investigated for such effects in the following way. Manometers were connected to the inlet and outlet of the circulating water system indicating an inlet pressure of 86.66 kPa and an outlet pressure of 38.66 kPa; the resulting pressure difference of 48 kPa did not change significantly during any of the growth periods.

During one of the growth experiments a piece of iron tubing was placed in the system. The rust that developed and circulated in the system resulted in a biofilm with a high inorganic content. In this manner the analytical methods could be tested on a biofilm with a high (75%) inorganic content and on a biofilm with a low (25%, normal) inorganic content.

Biofilm experiments. Four growth experiments were completed. The first involved calibration of a staining procedure, developed as a method for biofilm mass determination. In the second growth experiment an analysis of variance was performed, and the variance

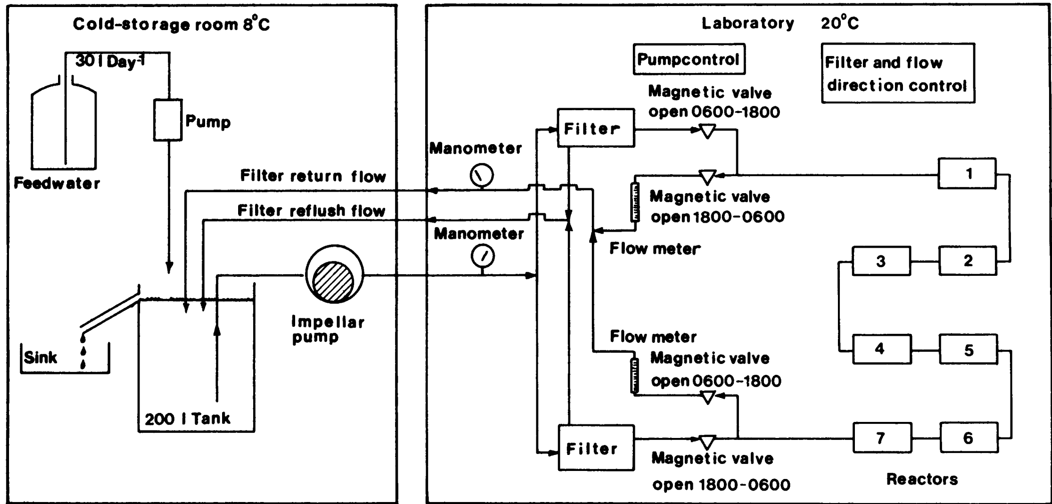

FIG. 2. Schematic diagram of the circulating water system.

components were calculated. To accelerate the biofilm development in these two growth experiments the feed water was enriched with 10 μg of glucose per ml and 10 μl of nutrient broth (Difco) per ml, resulting in mature biofilms covering the slip surfaces after 10 days.

The last two growth experiments were used to correlate growth, measured as stained and unstained biofilm on the slips, with the same growth measured as micrograms of protein N per square centimeter of slip and micrograms of dry weight and micrograms of organic weight per square centimeter of slip under growth conditions without nutrient enrichments. Pilot experiments revealed that a growth time of about 25 days then was needed to acquire a mature biofilm.

Calibration of the staining procedure. According to the calibration experiments described below, the following staining procedure was developed. The sam-

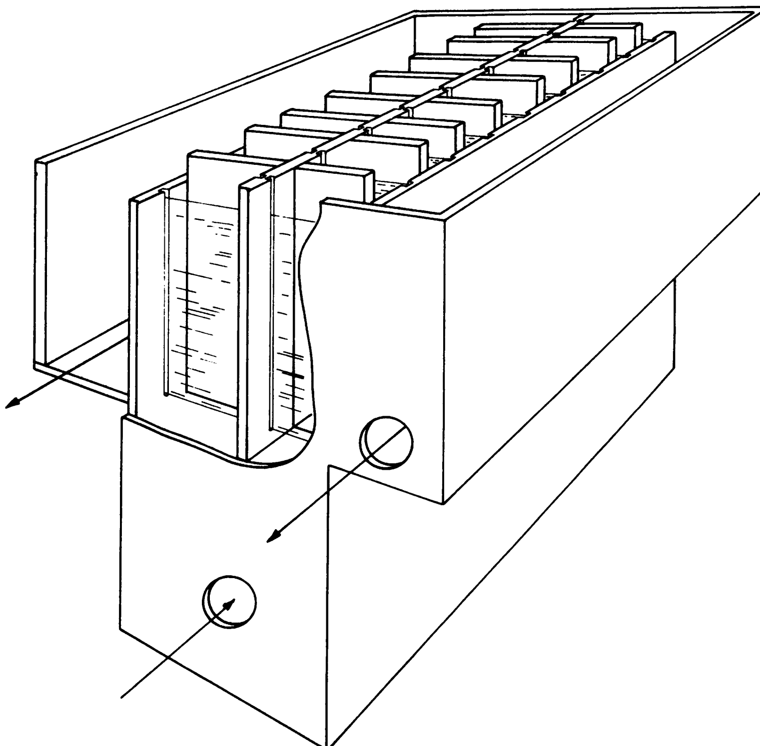

FIG. 3. Cover slip rinse, made of acrylic plastic.

pled slips with biofilm were stained for 1 min in a cuvette with Hückers crystal violet, a basic dye with maximum absorbance at 589 to 593 nm (3). The excess stain was rinsed off in a specially designed cover slip rinse (Fig. 3) at a flow of 3 cm s^{-1} for 10 min. Subsequently, each slip was dipped in distilled water, air dried (20°C), and mounted with Permount mounting medium between a microscope slide and a clean cover slip. Finally, the absorbances of the slides were measured at 590 nm on five fixed points of each slip (Beckman DU spectrophotometer completed with Multilog 311 and Multiblank 171, both from Optilab AB, Sweden).

Four reactors were used for the calibration. After 10 days, a series of 9 or 10 slips from reactors 1 and 2 were stained at different times and rinsed for 10 min; a staining time of 1 min was judged sufficient (Fig. 4). Also, a series of 9 or 10 slips from reactors 3 and 4 were stained for 1 min and rinsed at different times. The absorbance decreased exponentially with time; 10 min was considered as a sufficient rinsing time, enough for handling 18 slips with maintained accuracy (Fig. 4).

Analysis of variance experiment. After 10 days of growth all slips in five reactors were sampled and stained. A hypothesis of effects that could have affected the absorbance measurement data is summarized in the following analysis of variance model: $A_{ijkl} = m + R_i + P_{ji} + S_{ijk} + E_{ijkl}$ where A_{ijkl} is the lth measurement on the kth slip in the jth height position in the ith reactor; m is the overall mean; R_i is the effect from the ith reactor; P_{ji} is the effect from the jth height position in the ith reactor; S_{ijk} is the effect from the kth slip in the jth height position in the ith reactor; and E_{ijkl} is the residual. It is assumed that all effects are random (16). An analysis of variance and an estimation of the variance-components were executed by using the statistical analysis system (8) on the absorbance data obtained.

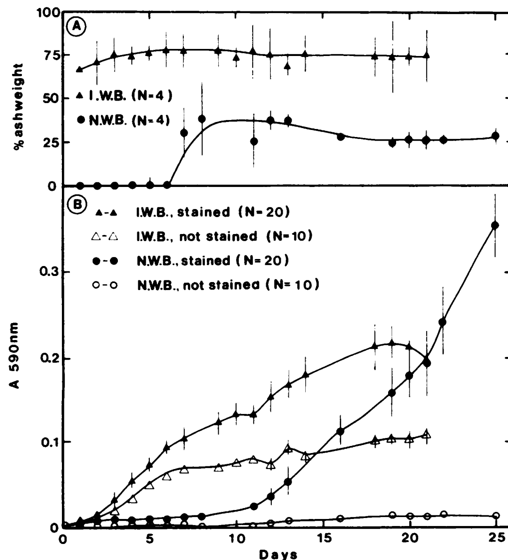

FIG. 4. Absorbance of biofilm slips stained for different times and rinsed for 10 min (●) or stained for 1 min and rinsed for different times (○). The bars indicate standard deviations for 9 or 10 slips.

Correlation experiments. The two correlation experiments lasted for 21 and 25 days. Seven reactors were used. During the first experiment an iron tube was inserted, and the developed biofilm was denoted iron water biofilm (IWB). The biofilm developed during the second experiment was denoted normal water biofilm (NWB). From each reactor, pairs of slips were sampled on each sample occasion and treated as in the staining procedure, but without the staining step. Subsequently the following treatments were performed. Biofilms from reactors 1 and 2 were analyzed for protein N content, reactors 3 and 4 were used for weight determinations, slips from reactors 5 and 6 were stained and mounted, and finally, slips from reactor 7 were mounted but without stain.

Protein assay. A heated biuret-Folin assay (5) was performed at the end of the growth periods. The slips (stored at −20°C) were thawed and crushed into the reaction tubes. To ensure that the biofilm protein came off the slips into the solution, 30 g of sodium deoxycholate (8) per liter was added to the reagent solution in the protein assay.

Weight determinations. The slips in question were weighed (Mettler ME 30 ± 1 μg) before being put in the piles. The sampled slips were dried at 70°C for 6 h and weighed to give the dry weight of the biofilm. Reweighing after 12 h in a muffle furnace at 450°C gave the ash weight. The difference between dry weight and ash weight was taken as the organic weight.

The correlation calculations included not only the correlation of the total absorbance with protein N, dry weight, and organic weight, but also the correlation between the total absorbance minus the absorbance of unstained biofilm and protein N, dry weight, and organic weight. This was done because part of the absorbance was due to the stain and the rest to the biofilm itself.

RESULTS

Characters of the biofilms. At the end of the growth experiments the four biofilms developed were studied in an interference contrast microscope. Their thickness was approximated by focusing the top and bottom of the biofilm; the difference read on the fine-adjustment knob gave the thickness. The characters for each biofilm are summarized below.

(i) Growth experiment 1. At the bottom there were bacteria and some protozoa evenly distributed and firmly linked with the surface. At the top a network of a filamentous bluegreen bacterium was spread. The thickness of the film was 20 to 40 μm.

(ii) Growth experiment 2. A dense cover of bacteria in aggregates was firmly linked with the surface and many protozoa, but few species. The thickness of the film was 50 to 70 μm.

(iii) Growth experiment 3. There were few bacteria, much deposited undifferentiated material in aggregates loosely linked with the surface, and very few protozoa. The thickness of the film was 30 to 50 μm.

TABLE 1. Results from analysis of variance with pertaining variance component estimation[a]

Source[b]	Degrees of freedom	Sum of squares	Mean square	F-test[c]	F	pr > F[d]	Expected mean square model	Variance component	Estimate
R	4	0.052026	0.013007	MS (*R*) / MS [*S*(*RP*)]	5.90	0.0001	Var(residual) + 5 var[*S*(*RP*)] + 190 var(*R*)	Var(*R*)	0.000057
P	18	0.047176	0.002621	MS (*P*) / MS [*S*(*RP*)]	1.19	0.2744	Var(residual) + 5 var[*S*(*RP*)] + 50 var(*P*)	Var(*P*)	0.000008
S(*RP*)	167	0.368349	0.002206	MS [*S*(*RP*)] / MS (residual)	2.14	0.0001	Var(residual) + 5 var[*S*(*RP*)]	Var[*S*(*RP*)]	0.000235
Residual	760	0.782367	0.001029				Var(residual)	Var(residual)	0.001029

[a] The total number of observations was 950, distributed over 190 slips in 19 height positions in five reactors. The overall mean was 0.410. Var, Variance.
[b] *R*, Effect from reactor; *P*, effect from height position; *S*(*RP*), effect from slips, nested within *R* and *P*.
[c] MS, Mean square.
[d] Probability of getting an F-value smaller than that obtained.

(iv) Growth experiment 4. The highest diversity of the four biofilms appeared, with bacteria in aggregates, solitary, stalked, and filamentous bacteria, firmly linked with the surface and many protozoa of several species. The thickness of the film was 40 to 60 μm.

All of the biofilms had at the bottom, on the slip surface, a thin film of a transparent gelatinous material easy to observe when the films were scratched. No algae were observed, except isolated algae that had stuck from the overflowing water.

Variance study experiment. The results from the analysis of variance are presented in Table 1. The effects of *R* and *S*(*RP*) were significantly different from the residual effect, whereas the effect of *P* was not. In the variance component estimation 4 and 18% of the total variance were due to effects from the reactors and slips, respectively, whereas the rest was due to natural random effects contained in the residual.

For interpretation of the variance analysis results it is necessary to expand the classification descriptions. The slips were stained in batches of 19 slips. Variations in batch treatments will affect the reactor mean and show up as a reactor effect. Variations in rinse flow velocity due to pressure differences in the tap water system, not always notified and compensated for, may then explain the small but signifi-

TABLE 2. Mean of each of the five biofilm reactors used in the variance study experiment

Reactor no.	Mean	*n*[a]	Grouping[b]
5	0.414	190	A
6	0.405	190	C
7	0.422	190	B
8	0.403	190	C
9	0.404	190	C

[a] Number of observations on which the mean is based.
[b] Means with the same letter are not significantly different. The grouping was obtained by Duncan's multiple-range test.

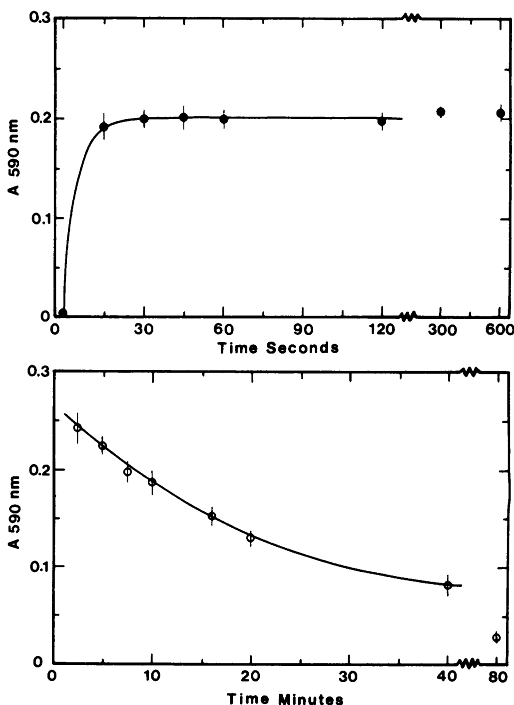

FIG. 5. A, Ash weights as percentage of the biofilms in the correlation experiments. The bars indicate standard deviations for four biofilm slips. B, Growth curves from the correlation experiments, measured as absorbance with and without the staining step. The bars indicate standard deviations from 10 to 20 absorbance value measurements.

TABLE 3. Linear regression functions with pertaining correlation coefficients (r) for the correlation between micrograms of protein N per square centimeter (P) and absorbance (A) with IWB and NWB in the correlation experiments ($n = 18$)

Correlation	Function	r
IWB P − total A	A = 1.58P − 0.015	0.965
IWB P − total A − A off unstained biofilm	A = 0.824P − 0.015	0.953
NWB P − total A	A = 0.343P − 0.004	0.997
NWB P − total A − A off unstained biofilm	A = 0.323P − 0.006	0.996

cant effect of R. When a Duncan multiple-range test was made on the biofilm reactor data, reactors 5 and 7 differed from the others (Table 2).

All treatment effects connected to separate slips, such as position in the rinse and mounting effects, are included within $S(RP)$. The major part of $S(RP)$ may be explained by an uneven flow pattern in the rinse observed when dye trace experiments were executed. By performing Duncan's multiple-range test (2), the connection between a somewhat higher absorbance value and a somewhat lower rinse column flow velocity was confirmed. A later improvement of the rinse with insertion of flow diffusers in the bottom of the rinse smoothed out the uneven flow velocities between the rinse columns.

Correlation experiments. The biofilm developments, measured as absorbance of stained and unstained biofilms, are presented in Fig. 5. The unstained NWB had a low absorbance throughout the experiment; at most all absorbance of the stained NWB was due to the stain. The IWB had a brown to yellow color caused by an iron precipitation on the slips responsible for about 50% of the absorbance of the stained IWB (Fig. 5B). The iron precipitation also constituted a considerable part of the ash weight (Fig. 5A).

There was a strong correlation between absorbance and protein (Table 3) and between absorbance and organic weight (Fig. 6) in both experiments, which means that the relationship between protein and organic weight was constant throughout the experimental periods. Assuming it is mainly the organic material that is stained, the slope of the regression line (x_2, y_2) in Fig. 6 should coincide with the slope of the (x_4, y_4) line in the same figure. This is not the case. However, when repeating the comparison for the absorbance-dry weight regression, the slopes of the lines (x_2, y_2) and (x_4, y_4) (Fig. 7) approach each other. This indicates that the

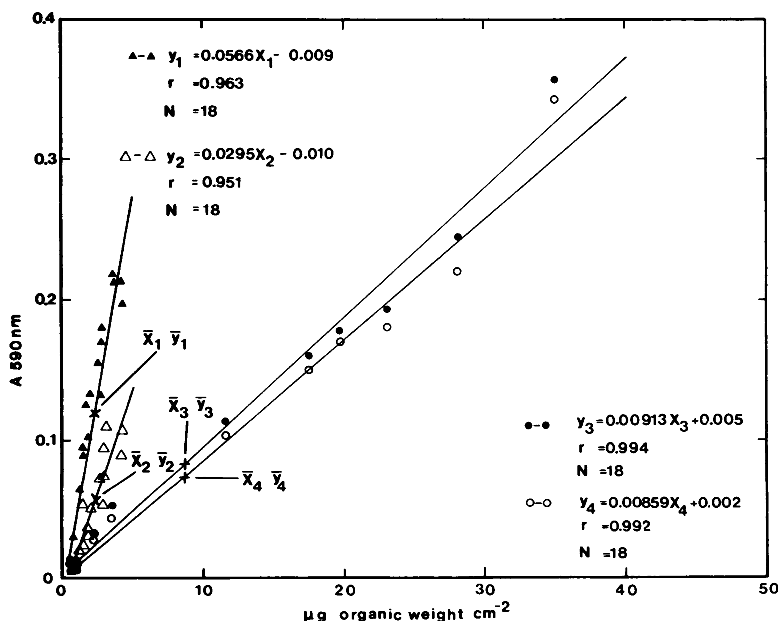

FIG. 6. Correlation of absorbance (A) and organic weight for IWB and normal water biofilm NWB. Symbols: ▲, IWB (x_1, y_1) = (micrograms of organic weight per square centimeter, total A); △, IWB (x_2, y_2) = (micrograms of organic weight per square centimeter, total A minus A of unstained biofilm); ●, NWB (x_3, y_3) = (micrograms of organic weight per square centimeter, total A); ○, NWB (x_4, y_4) = (micrograms of organic weight per square centimeter, total A minus A of unstained biofilm).

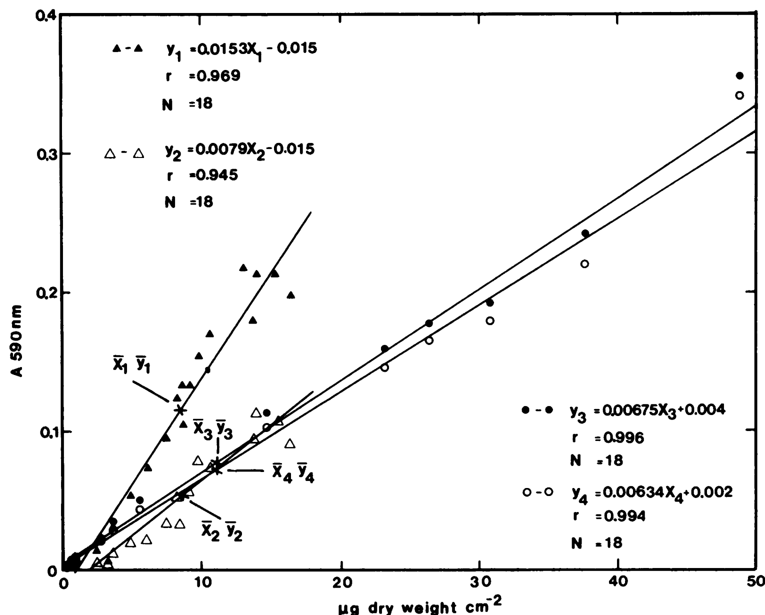

FIG. 7. Correlation of absorbance (A) and dry weight for IWB and NWB. Symbols: ▲, IWB (x_1, y_1) = (micrograms of dry weight per square centimeter, total A); △, IWB (x_2, y_2) = (micrograms of dry weight per square centimeter, total A minus A of unstained biofilm); ●, NWB (x_3, y_3) = (micrograms of dry weight per square centimeter, total A); ○, NWB (x_4, y_4) = (micrograms of dry weight per square centimeter, total A minus A of unstained biofilm).

absorbance difference between a stained and an unstained biofilm is a measure closely related to its dry weight.

DISCUSSION

Development of various biofilm types. The nature of biofilms that develop in flowing water systems depends on characteristics of the flow system and the flowing water itself. The mature biofilms differed from each other when studied under a microscope. The biofilm obtained with normal seawater resembled biofilms obtained with continuously flowing seawater (unpublished data). The IWB and the accelerated biofilms differed from natural seawater biofilms. In the study of factors regulating microbial biofilms, it is important to use water identical with the water present in the water system that the study is related to. Otherwise, the results obtained will be less applicable. When the purpose of a biofilm study is of a methodological character, then modulations of the water may give advantages such as less complicated biofilms and faster growth.

Assay of biofilm components. In the study of biofilms the purpose of the study indicates which measurement should be made. When biofilm activity is sought, an assay of ATP concentration offers an acceptable procedure for obtaining an answer (11). If the effect of biofilm formation is of interest, then the investigator has to find ways for measuring the effect as a function of biofilm mass, decreasing heat exchange capacity (12), increased flow resistance (15), and corrosion problems (10).

Biofilm mass can be assayed by various methods, i.e., by measuring thickness (9), weight, or the content of some biochemical components of the biofilm such as organic nitrogen, organic carbon and chlorophyll (1), or lipopolysaccharides (4).

Dry weight and ash weight determinations were chosen as ways to assay the total biofilm mass and its organic content. The protein assay reflects an organic part of the biofilm, excluding polysaccharides, which sometimes constitute a considerable part of biofilms (17). The staining procedure was designed to give a fast and uncomplicated method with high accuracy for the measurement of biofilm mass. Performance of protein and weight determinations at the end of a biofilm growth experiment measured by the staining procedure will make it possible to calculate protein content, dry weight, and organic weight for absorbance values within the range of good correlation.

Biofilm study equipment. In the design of biofilm study equipment two main aspects of the problem require special consideration, namely, the hydrodynamics of the equipment and the character of the surface.

A biofilm development depends completely on the transport of biofilm components to the surface. Oxygen, nutrients, particles, orga-

nisms, and, in fact, everything have to be transported to the surface in some form where they will be adsorbed, metabolized or metabolizing. When the biofilm becomes mature, reentrance of material into the water will be significant. In flowing water systems the water movement effects the major transport to the immediate vicinity of the surfaces. The transport of biofilm components over the last few micrometers to, and for some components such as oxygen and nutrients into, the biofilm (and vice versa) is completed by a variety of processes (6, 13). Because of this transport, the flow pattern through biofilm study equipment should be analyzed for inequalities which increase the variance or even give misleading results.

When the flow pattern through the reactors was visualized by pouring particles into the water, no flow inequalities could be observed. This observation was confirmed by the analysis of variance in which the position effect could not be shown to be significant. Transport gradients that appeared in spite of the alternation of the flow direction should range the means of the reactors in one way or another. When the Duncan multiple-range test was performed, reactors 5 and 7 differed a little, but significantly, from the others (Table 2). The distribution of the five reactor means cannot be explained by transport gradients. Some other random effects, probably the rinse effect suggested in the variance study, must be responsible.

If a time and a treatment effect are added to the variance analysis model, the method described above allows detection of small treatment effects on the development of biofilms. Biofilm development series with different treatments can be compared, and even small treatment effects will be significant.

If the surface can react with the liquid in the system (corrosion), if it is toxic, or if microorganisms can utilize the surface as a substrate or affect it by their activity, the interpretations of the results will be more complicated as compared with biofilm studies on inert surfaces.

The membranes in an electrochemical concentration cell are hydrophilic and charged, and at least the anion exchange membrane with high nitrogen content can theoretically be utilized by microorganisms. In addition, charge-charge interactions between the biofilm components and the membranes may have a significant influence on the biofilm formation.

To facilitate the further study of biofilms in concentration cells used with natural water, the investigation has been split into two parts. The first part is a study of how factors not related to the surface regulate biofilm development (submitted for publication). The second part will be concentrated on biofilm development on single membranes in simplified concentration cells. By putting these results together predictions about microbial biofilm problems in a salt gradient power plant can be made.

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