

Activity of *Pseudomonas aeruginosa* in Biofilms: Effect of Calcium

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Aerobic glucose metabolism by *Pseudomonas aeruginosa* biofilms at various calcium loading rates was investigated. The influence of calcium on specific growth rate, extracellular polymeric substance (EPS) formation rate, biofilm detachment rate, and biofilm calcium concentrations was determined. Calcium accumulated in the biofilm in proportion to the liquid phase concentration. Increasing calcium concentration increased the cohesiveness of the biofilm as indicated by a lower relative detachment rate. Specific activity in the biofilm was the same as that measured in a chemostat and was not influenced by changing calcium concentration. EPS formation rate in the biofilm was unaffected by calcium concentration but was higher than that observed in a chemostat.

INTRODUCTION

Microorganisms, primarily bacteria, exhibit a tendency for adsorbing to and colonizing surfaces submerged in aquatic environments. The immobilized cells grow, reproduce, and produce extracellular polymeric substance (EPS) that frequently extend from the cell forming a tangled mass of fibers lending structure to the entire assemblage, which shall be termed a *biofilm*.

Most studies on the effect of dissolved constituents on biofilm formation have been limited to the effect of organic constituents. There is little or no information on the effect of dissolved inorganic constituents. Although there are numerous inorganic components that may affect biofilm processes, the scope of this research was limited to the effect of calcium. Understanding the role of calcium in biofilm processes may be useful in ecosystem analysis, control of biofouling in heat exchangers and/or pipelines, operation of fixed-film biological wastewater treatment processes, and increasing the rate of strength of cell immobilization in a biofilm reactor for biotechnology applications.

The major emphasis in this article is the influence of calcium on cellular reproduction and EPS formation by *Pseudomonas aeruginosa* in a biofilm and its consequent effect on biofilm accumulation. *Pseudomonas aeruginosa*

was used as a test organism because this organism has been extensively studied in continuous-flow stirred-tank reactors, i.e., chemostats,¹ and in biofilm reactors.^{2,3} Specific cellular growth rates and EPS formation rates have been measured under defined experimental conditions in chemostat and biofilm environments.^{1,2}

MATHEMATICAL DESCRIPTION OF REACTOR SYSTEM

The RotoTorque reactor (RT) is a continuous-flow stirred-tank reactor (CFSTR); i.e., bulk liquid concentration gradients are negligible. The RotoTorque reactor consists of two concentric cylinders, a stationary outer and a rotating inner cylinder. The RT provides a large wetted surface-area-to-volume ratio and a turbulent liquid phase. The residence time in the RT was maintained at 10 min so that microbial processes in the bulk liquid were minimized. More details regarding RotoTorque reactor system can be found elsewhere.^{3,4} Accumulation of compounds in the RT bulk liquid can be described by material balances of the general form

$$\text{net rate of accumulation} = \text{net rate of transport} + \text{net rate of transformation} \quad (1)$$

Biofilm accumulates on the walls of the RT as a result of growth and EPS formation at the expense of substrate (Fig. 1). Some of the biofilm cells and EPS detach and are washed out in the RT effluent. Accumulation of attached biofilm components can be described by constitutive equations of the same general form as equation (1) but with no transport term. Material balances and constitutive equations for this system have been presented in detail elsewhere organized in matrix notation.² The resulting steady-state equations for the system relevant to this study are summarized in Table I. Water phase accumulation rate of substrate cells and polymer are negligible because of the high dilution rate ($D = 6 \text{ h}^{-1}$) used in the experiments. Production rate of cells and polymer in the water phase are negligible for the same reason.

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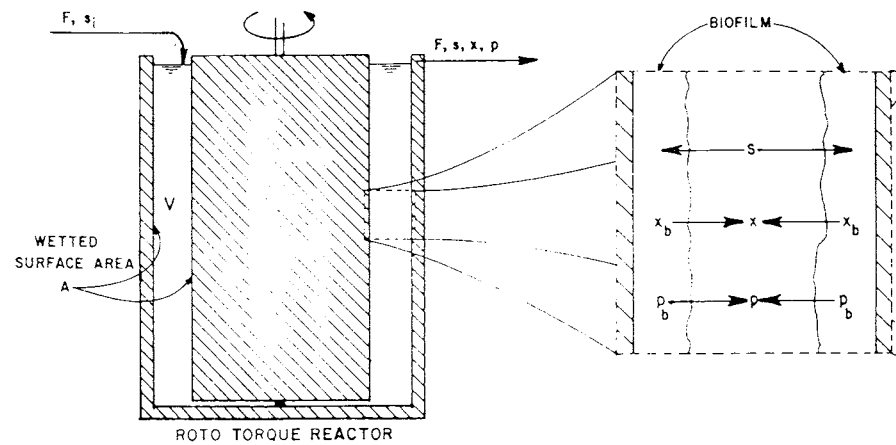


Figure 1. RotoTorque reactor (RT) operated as a continuous-flow stirred-tank reactor (CFSTR). Insert describes local environment at substratum in reactor. Mathematical model based on this physical system.

Table I. Equations describing experimental reactor system.^a

1. Material balance for substrate in water phase (specific substrate (carbon) removal rate in biofilm, q_{sb}):

$$q_{sb} = \frac{D(S_i - S)V}{X_b A} = \mu_b \left[\frac{1}{Y_{X/S}} + \frac{k}{Y_{P/S}} \right] + \frac{k'}{Y_{P/S}} \quad (2)$$

2. Material balance for cellular carbon in water phase (specific cellular detachment rate, q_{dx}):

$$q_{dx} = \frac{QX}{X_b A} \quad (3)$$

3. Material balance for polymer carbon in water phase (specific polymer detachment rate, q_{dp}):

$$q_{dp} = \frac{QP}{P_b A} \quad (4)$$

4. Accumulation rate of cellular carbon in biofilm:

$$A \frac{dX_b}{dt} = \mu_b X_b A - q_{dx} X_b A \quad (5)$$

5. Accumulation rate of polymer carbon in biofilm:

$$A \frac{dP_b}{dt} = (k\mu_b X_b + k'X_b)A - q_{dp} P_b A \quad (6)$$

6. Specific cellular growth rate in biofilm μ_b as function of substrate (carbon) concentration^b, S :

$$\mu_b = \left[\frac{\mu_m S}{K_S + S} \right] \quad (7)$$

7. Specific polymer (carbon) formation rate in biofilm q_{pb} as function of specific cellular growth rate in biofilm^c:

$$q_{pb} = k\mu_b + k' \quad (8)$$

^aFrom ref. 2. Water phase accumulation rate of substrate, cells, and polymer are negligible because of the high dilution rate used in experiments. Production rate of cells and polymer in water phase are negligible for the same reason.

^bFrom refs. 2, 3, and 6.

^cFrom refs. 2-4.

MATERIALS AND METHODS

Experimental System

All experiments were conducted in a RotoTorque reactor (RT) system consisting of two RTs, sterile dilution water, and sterile substrate feed apparatus (Fig. 2). The RotoTorque reactor consists of two concentric cylinders, a stationary outer and a rotating inner cylinder, containing four removable slides for biofilm sampling and/or biofilm mass measurements (Fig. 3). These slides formed an integral fit with the inside wall of the outer cylinder. The rotational

speed of the inner cylinder was maintained at 250 rpm. The RT provided a wetted surface-area-to-volume ratio, $A/V = 275 \text{ m}^{-1}$, and a turbulent liquid phase (residence time 10 min). More details regarding the RotoTorque reactor system can be found elsewhere.^{4,8}

Experimental Procedures

The RTs were cleaned, assembled, and then sterilized with sodium hypochlorite solution prior to all experiments. Then, dilution water, continuously sterilized by filtration

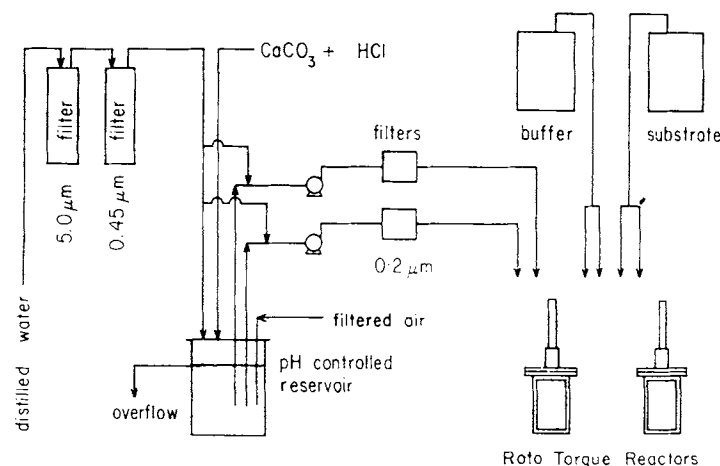


Figure 2. Flow diagram of RotoTorque reactor system.

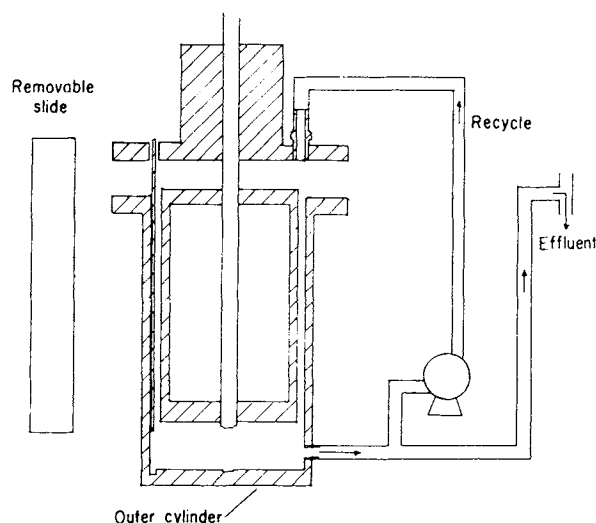


Figure 3. Simplified diagram of RotoTorque reactor.

(Fig. 2), was pumped into the RT at the desired flow rate. A minimum of 50 residence times was allowed for residual chlorine removal before inoculation. The calcium content of the dilution water was controlled by the addition of $\text{CaCO}_3 + \text{HCl}$ stock solution. Experiments were started by pumping *P. aeruginosa* ($\sim 10^8$ cells/mL) from a steady-state chemostat (dilution rate $D = 24 \text{ h}^{-1}$) into the RT for a period of 8–10 h, thus providing a defined condition for initial attachment and colonization. Sterile substrate (glucose) and nutrient solutions were fed into the RT throughout each experiment, including the inoculation period. A loopful of reactor effluent was streaked on a trypticase glucose agar extract (TGEA) plate regularly during each experiment to check for contamination using colony morphology as an indicator.

Microbial Inoculum

The strain of *Pseudomonas aeruginosa* used in this study was obtained from the culture collection of the De-

partment of Microbiology, Montana State University (Bozeman, MT). The organism was stored initially in a refrigerator of TGEA plates and replated every 2–3 weeks to maintain viability. Later, the organism was stored at approximately -10°C in a 15% glycerol medium.

Analytical Methods

The hierarchy of the analytical procedures performed on biofilm or reactor liquid samples is shown in Figure 4.

Glucose

Glucose concentration was determined with a modified version of the Sigma 510 glucose analysis procedure (Sigma Chemical Co., St. Louis, MO). Trulear³ observed no significant soluble organic carbon other than glucose carbon in this identical system. Therefore, no SOC measurements were conducted. Hence, the reported value of S represents only the glucose carbon concentration.

Cellular Carbon

Total number of cells in the effluent, X , or in the biofilm sample, X_b , was determined by enumerating cells stained with acridine orange using epifluorescence microscopy according to the methods of Hobbie and co-workers.⁹ Cellular carbon was determined by multiplying the acridine orange cell count and the average volume of the cell by the following factors: $1.0^7 \text{ g cell/cm}^3$,^{10,11} $0.22 \text{ dry cell weight/wet cell weight}$,^{10,11} and $0.5 \text{ g cell carbon/g cell dry weight}$.^{10,12}

Liquid Phase EPS Concentration

The concentration of liquid phase EPS carbon, P , was calculated by measuring the total organic carbon (TOC) of the reactor liquid sample, TOC_{soln} , and performing the following calculations:

$$P = \text{TOC}_{\text{soln}} - S - X \quad (9)$$

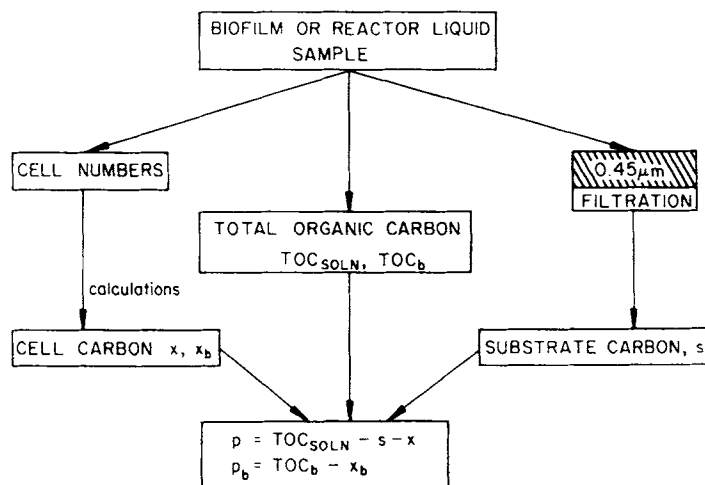


Figure 4. Hierarchy of analytical procedures.

TOC was determined using the ampule analysis module of the Oceanography International carbon analyzer (Oceanography International Corp., College Station, TX).

Biofilm EPS Carbon Density

The density of the biofilm EPS carbon, P_b , was determined by measuring the TOC of a known volume of biofilm suspension, TOC_b , and performing the following calculation:

$$P_b = TOC_b - X_b \quad (10)$$

Dry Biofilm Mass

Dry biofilm mass was determined by filtering 15–25 mL biofilm suspension through predried (103°C), preweighed, Nuclepore membranes (0.45 μm). After filtration, the filter was dried at 103°C for 1 h and weighed.

Total Calcium

Total calcium in the influent and effluent samples was determined by titration with ethylenediamine tetra-acetic acid (EDTA). Total calcium in the dry biofilm sample was determined by atomic adsorption.

C, H, and N Analysis

Carbon, hydrogen, and nitrogen content of the lyophilized biofilm samples were determined using a Carlo-Erba elemental analyzer.

RESULTS AND DISCUSSION

The effect of calcium on cellular reproduction and EPS formation was determined by varying influent calcium concentration and measuring changes in (i) biofilm cellular carbon areal density X_b , (ii) biofilm EPS carbon areal density P_b , (iii) biofilm mass, (iv) immobilized calcium, (v) liquid phase cellular carbon concentration X , (vi) liquid

phase EPS carbon concentration P , and (vii) glucose carbon concentration S . The effect of calcium on microbial transformation rate or process rate was determined from the preceding measurements using the material balances equations in Table I.

All measurements, including substrate, cells, and EPS, are reported in terms of carbon equivalents. The Roto-Torque reactor was operated at three different input calcium concentrations: 0.4 (no added calcium), 25, and 50 mg/L.

Biofilm Mass

The progression of biofilm mass for three different calcium concentrations in the dilution water is presented in Figure 5. The presence of calcium in the dilution water increases the rate of biofilm accumulation. However, in the early stages of the experiments (<48 h), there was no detectable difference in biofilm mass due to variation in dilution water calcium concentration. Accumulation was

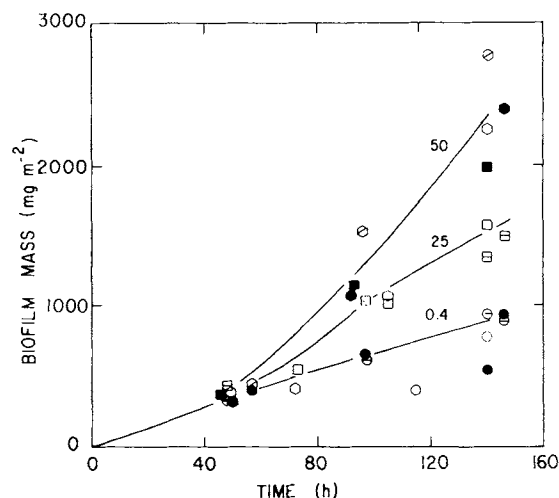


Figure 5. Progression of biofilm mass as function of time for three different calcium concentrations: (●, ○, ●) 50 mg/L (□, □, ■) 25 mg/L; and (○, ○, ●) 0.4 mg/L calcium.

highest for experiments with 50 mg/L calcium (Fig. 6). Therefore, the rate and extent of biofilm accumulation increases with increasing calcium concentration.

Biofilm Carbon

Increasing dilution water calcium concentration increases the rate of accumulation of biofilm *cellular* carbon (Fig. 7). The extent of biofilm cellular carbon accumulation after 6 days increased with increasing calcium concentration in the dilution water (Fig. 8). The amount of EPS carbon accumulated within the biofilm after 6 days was not influenced by dilution water calcium concentration

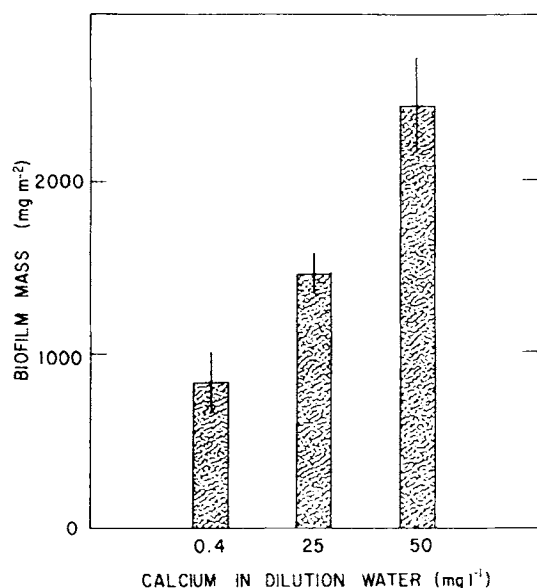


Figure 6. Biofilm mass (140 h) as function of calcium in dilution water.

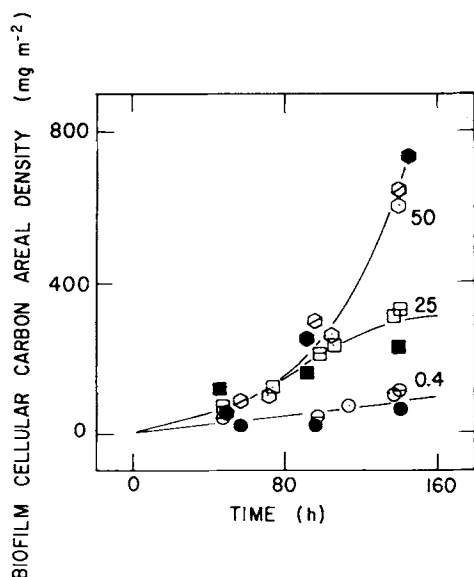


Figure 7. Progression of biofilm cellular carbon for three calcium concentrations: (○, ◊, ●) 50 mg/L; (◻, ◻, ◻), 25 mg/L; and (◊, ◊, ◊) 0.4 mg/L calcium.

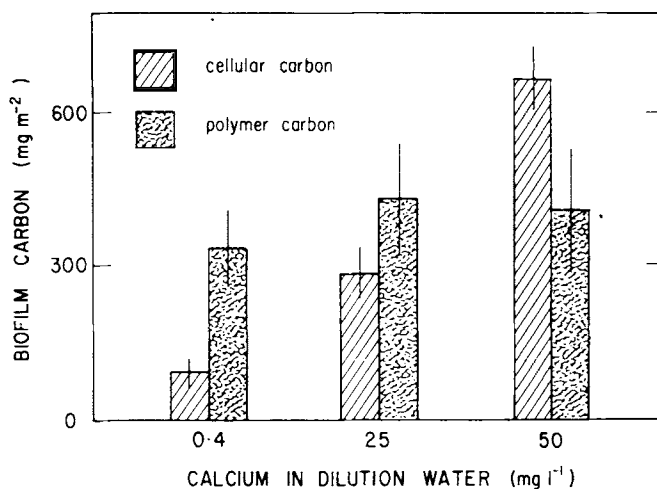


Figure 8. Biofilm cellular and polymer carbon (140 h) as function of calcium in dilution water.

(Fig. 8). There was no significant difference in biofilm EPS carbon due to changes in calcium concentration at any time during biofilm development.

Biofilm Calcium

Biofilm calcium concentration increases with increasing calcium in the dilution water (Fig. 9). Twelve residence times before sampling biofilm for calcium analysis, the flow of $\text{CaCO}_3 + \text{HCl}$ stock solution was stopped. Hence, there was no added calcium in the dilution water during the sampling period. The reported biofilm calcium does not discriminate between intracellular calcium and calcium immobilized within the extracellular matrix. Calcium is not generally accumulated in the cytoplasm by "normal" growing cells and appears to be exclusively extracellular.^{13,14} Campbell¹⁵ reported that the concentration of total calcium in the cytoplasm is usually in the range of 1–10 mmol/L

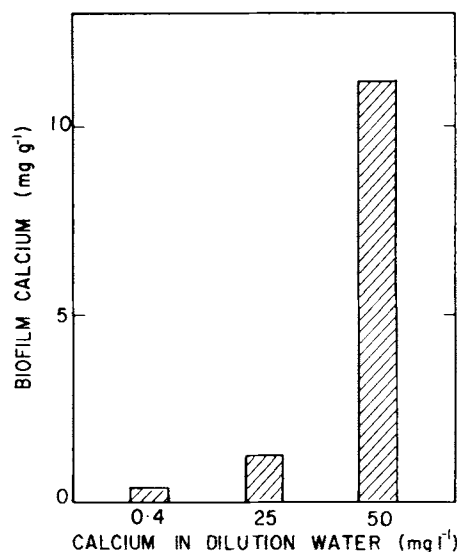


Figure 9. Biofilm calcium as function of calcium in dilution water.

cell water. On this basis, the maximum intracellular concentration of calcium in the biofilm cells developed under 50, 25, and 0.4 mg/L calcium was estimated to be 0.95, 0.78, and 0.45 mg calcium/g dry biofilm mass, respectively. The estimated values of intracellular calcium at dilution water calcium at 25 and 50 mg/L are insignificant compared to the biofilm calcium measured (Fig. 9). Hence, a major portion of the biofilm calcium is immobilized in the extracellular components of the biofilm matrix when dilution water calcium is 25 or 50 mg/L.

Biofilm Elemental Analysis

Carbon, hydrogen, and nitrogen content of lyophilized biofilm was unaffected by changing dilution water calcium concentration (Table II). The results obtained are consistent with C, H, and N content of microbial cells reported in the literature, i.e., approximately 50% carbon, 20% oxygen, 10–15% nitrogen, and 8–10 hydrogen on a dry-weight basis.

Specific Substrate Removal Rate

Metabolic activity of the biofilm cells was determined by specific substrate removal rate [eq. (2)]. The specific substrate removal rate, q_{sb} , was higher for biofilm accumulating in 0.4 mg/L calcium in the dilution water (Fig. 10), but there was no significant difference in specific substrate removal rate for biofilm accumulating at 25 and 50 mg/L calcium in the dilution water. Since lower biofilm cellular mass accumulated at low calcium, more substrate was available per cell, perhaps leading to enhanced uptake. Alternatively, the adsorbed cells at low calcium may be more active for unexplainable reasons.

A linear relationship was observed between specific substrate removal rate, q_s , and specific cellular growth rate in the biofilm, μ_b , as predicted by equation (2) (Fig. 11). The y intercept of the best-fit line is 0.165 (SE 0.175) and the slope equals 3.04 (SE 0.63). Robinson et al.¹ also obtained a linear relationship between specific substrate removal rate and specific cellular growth rate μ for *P. aeruginosa* in suspended cultures (chemostat). A *t*-test was performed to statistically determine the correlation between the biofilm data at different calcium concentrations (this study) and the chemostat (suspended culture) data reported by Robinson et al.¹ At the 5% level of statistical significance, there was no difference between the chemostat data and the biofilm data from this study. Similar results

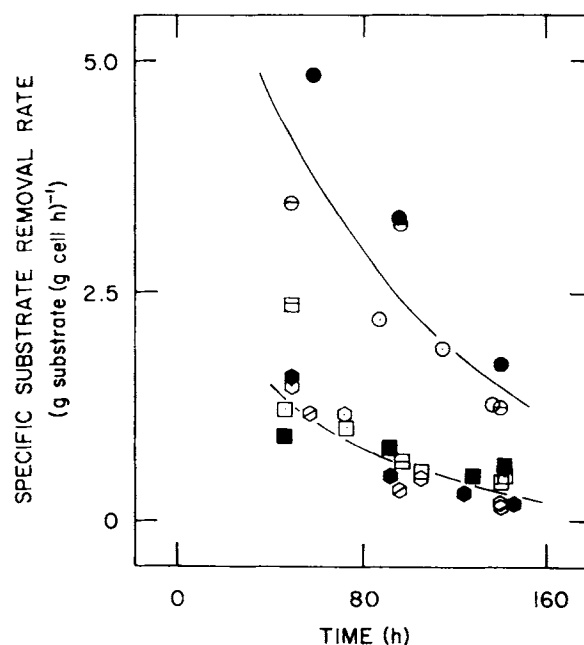


Figure 10. Progression of specific substrate removal rate as function of calcium in dilution water: (○, ●, ●) 50 mg/L; (□, □, ■) 25 mg/L; and (○, ○, ●) 0.4 mg/L calcium.

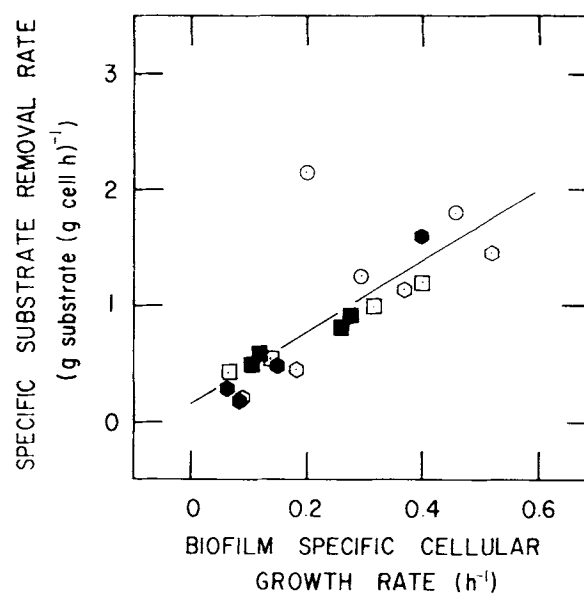


Figure 11. Variation in specific substrate removal rate with biofilm specific cellular growth rate. The straight line represents best fit described by eq. (2): (○, ●), 50 mg/L; (□, ■) 25 mg/L; and (○) 0.4 mg/L calcium.

were also reported by Bakke et al.² for *P. aeruginosa* biofilm in the RotoTorque reactor.

Results from this study and those reported by Bakke et al.² indicate that specific activity of *P. aeruginosa* in biofilms is not different than its specific activity in suspended cultures if no significant diffusion resistance exists in the biofilm. Varying calcium concentration did not influence specific activity. Hence, the specific cellular growth rate of *P. aeruginosa* in biofilms and/or suspended cultures can be estimated by monitoring the changes in specific substrate removal rate.

Table II. Composition of biofilm (dry-weight basis).

Calcium (mg/l)	Carbon (%)	Hydrogen (%)	Nitrogen (%)	Fixed solids (%)	Volatile solids (%)
0.4	47.5	6.9	12.4	13.6	86.4
25.0	48.0	7.0	12.3	13.4	86.6
50.0	48.6	6.9	13.1	13.5	86.5

Specific Cellular Growth Rate

The mean specific cellular growth rate of *P. aeruginosa* in the biofilm decreases with time, which is not surprising since the same amount of substrate is being consumed by an increasing number of cells. Robinson et al.¹ report that specific cellular growth rate of *P. aeruginosa* in suspended culture depends on substrate concentration as described by equation (7) and determined μ_m and K_s for *P. aeruginosa* in suspended culture using a linearized form of equation (7) (Lineweaver-Burk equation). The maximum specific cellular growth rate μ_m was reported to be 0.4 h^{-1} (SE 0.2) and the saturation constant K_s was 2.0 mg/L (SE 0.78). In Figure 12, biofilm specific cellular growth rate μ_b is plotted as a function of reactor substrate concentration S . The shaded portion in Figure 12 represents the 95% confidence interval of the saturation equation based on the parameters μ_m and K_s estimated by Robinson et al.¹ for *P. aeruginosa* in suspended culture. The data points outside the shaded region were obtained during the initial period (48–70 h) of the experiments in this study. The specific cellular growth rate of *P. aeruginosa* in the biofilm calculated for this initial period was greater than or equal to the maximum cellular growth rate of *P. aeruginosa* reported for other studies in suspended and biofilm reactors.^{3,16} During this initial period, however, accurate measurements of biofilm cellular carbon concentration is difficult because less than 80 mg/m^2 biofilm had accumulated on the substratum. Alternatively, cells adsorbed to the substratum during this initial period may be more "active." The increased activity may be due to a higher substrate flux than that experienced by suspended cells due to mass transfer effects or may indicate that the initial colonizers are cells with higher growth rates.

Calcium is required for growth of many, and probably all, bacterial species.^{15,17} Results from a separate study⁴ indicate that the maximum specific cellular growth rate μ_m

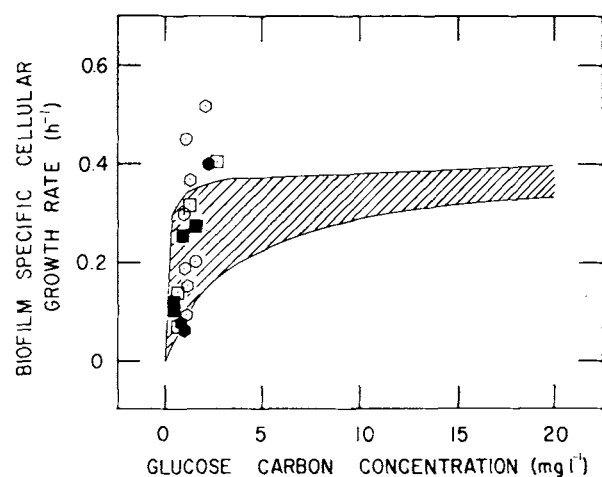


Figure 12. Biofilm specific cellular growth rate as function of glucose carbon concentration. Shaded portion represents 95% confidence interval of Monod equation based on parameters (H_a and K_s) estimated by *P. aeruginosa* in suspended culture: (○, ●) 50 mg/L; (□, ■) 25 mg/L; and (○) 0.4 mg/L calcium.

for *P. aeruginosa* (suspended culture) in the presence of 25 mg/L was approximately 16% higher than that with calcium at 3.2 mg/L. The cellular growth and reproduction were monitored by measuring cumulative oxygen uptake in a respirometer. Therefore, higher biofilm cellular accumulation in presence of 25 and 50 mg/L calcium in the dilution water, as compared to 0.4 mg/L calcium, may be at least partially due to a difference in μ_m for *P. aeruginosa* in the biofilm.

Cellular Detachment Rate

In Figure 13, the ratio of specific cellular detachment rate to specific cellular reproduction rate is plotted as a function of time for three different calcium concentrations in the dilution water. This ratio represents the fraction of cells produced in the biofilm that detach. At steady state, specific cellular detachment rate equals specific cellular reproduction rate, and the ratio will be unity. The data indicate that a major portion (>92%) of the attached cells growing under 0.4 and 25 mg/L calcium are detaching and the system is approaching steady state. The cellular carbon accumulation rate (dX_b/dt) at 0.4 and 25 mg/L calcium also seems to be approaching zero (Fig. 7). A lesser fraction of cells at 50 mg/L detach (as little as 72%). The reduced detachment at calcium concentration of 50 mg/L may be explained as follows: (a) cellular accumulation rate (within the experimental period) at 50 mg/L calcium was increasing exponentially with time (Fig. 7) and (b) specific cellular growth rate was significantly greater than specific cellular detachment rate. Thus, cells accumulating at 50 mg/L calcium tend to stay, or "stick," to a greater extent. Cellular detachment appears to be influenced by calcium concentration and may be controlling the biofilm accumulation rate. Understanding biofilm detachment is therefore not only necessary to predict biofilm

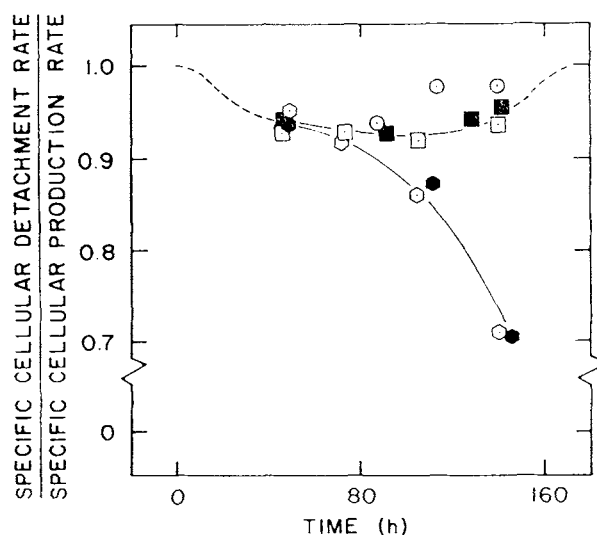


Figure 13 Ratio of specific cellular detachment rate to specific cellular production rate as function of time: (○, ●) 50 mg/L; (□, ■) 25 mg/L; and (○) 0.4 mg/L calcium.

behavior, but may also serve as a means to control biofilm activity or accumulation.

Turakhia⁴ observed immediate detachment of *P. aeruginosa* biofilm when the free calcium in the biofilm was altered by the addition of the calcium-specific chelant. Detachment was presumably caused by the removal of calcium important to biofilm structure.

Specific EPS Production Rate

Specific EPS production rate is a linear function of specific cellular growth rate in the biofilm (Fig. 14) as described by equation (8). The growth-associated EPS rate coefficient k is 2.07 mg EPS carbon/mg glucose carbon (SE 0.39), and the non-growth-associated EPS coefficient k' is -0.02 mg EPS carbon/mg glucose carbon/h (SE 0.11). The non-growth-associated EPS formation coefficient k' can be assumed to be negligible as compared to the growth-associated EPS coefficient k .

Robinson et al.¹ reported that EPS production by *P. aeruginosa* in a suspended culture was growth- and non-growth-associated. The growth-associated EPS formation coefficient (0.27 mg EPS carbon/mg glucose carbon) for *P. aeruginosa* in suspended culture was much smaller than that obtained for the biofilm organisms for this study (2.07 mg EPS carbon/mg glucose carbon). The result suggests that *P. aeruginosa* may increase its EPS production rate when immobilized in a biofilm.

CONCLUSIONS

The results of this study indicate that increasing calcium concentration in the growth medium significantly increases the rate and extent of *P. aeruginosa* biofilm accumulation. Calcium accumulated in the biofilm increased in proportion to the calcium concentration in the water phase. The

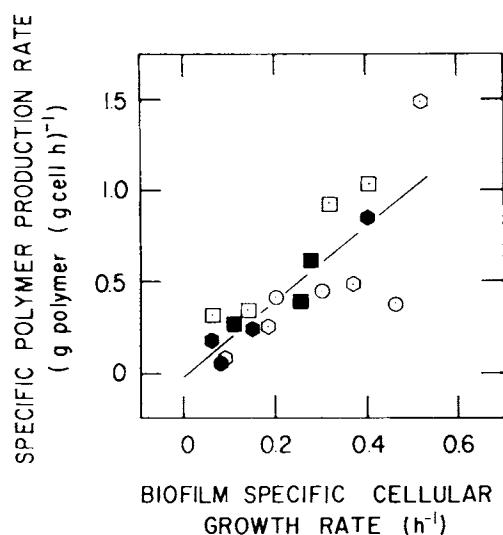


Figure 14. Specific polymer production rate as function of biofilm specific cellular growth rate. Straight line represents best-fit described by eq. (8): (○, ●), 50 mg/L; (□, ■), 25 mg/L; and (○) 0.4 mg/L calcium.

immobilized calcium increases the cohesiveness of the biofilm as reflected by the decreased detachment rate at higher calcium concentrations. The activity of *P. aeruginosa*, as indicated by specific growth rate and yield determinations, is the same as measured in a chemostat regardless of the calcium concentration. *Pseudomonas aeruginosa* in the biofilm, however, produced EPS at a higher rate than determined in a chemostat. There was no significant difference in EPS production rate with calcium concentration.

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NOMENCLATURE

A	surface area
RT	RotoTorque reactor
D	dilution rate
Q	volumetric flow rate
k	biofilm growth-associated polymer formation rate coefficient
k'	biofilm non-growth-associated polymer formation rate coefficient
K_s	saturation coefficient
P	polymer carbon concentration
P_b	biofilm polymer carbon areal density
q_{sb}	specific substrate removal rate in biofilm
q_{dp}	specific polymer carbon detachment rate from biofilm
q_{ax}	specific cellular carbon detachment rate from biofilm
q_{pb}	specific polymer carbon formation rate in biofilm
S_i	influent glucose carbon concentration
S	reactor glucose carbon concentration
t	time
TOC_b	biofilm total organic carbon concentration
TOC_{soln}	liquid solution total organic carbon concentration
V	volume of system
X	cellular carbon concentration
X_b	biofilm cellular carbon areal density
$Y_{p/s}$	yield coefficient of polymer carbon from substrate
$Y_{x/s}$	yield coefficient of cellular carbon from substrate
μ	specific cellular growth rate
μ_b	biofilm specific cellular growth rate
μ_m	maximum specific cellular growth rate

References

1. J. A. Robinson, M. G. Trulear, and W. G. Characklis, *Biotechnol. Bioeng.*, **26**, 1409 (1984).
2. R. Bakke, M. G. Trulear, J. A. Robinson, and W. G. Characklis, *Biotechnol. Bioeng.*, **26**, 1418 (1984).
3. M. G. Trulear, "Cellular reproduction and extracellular polymer formation in the development of biofilms," Ph.D. Thesis, Montana State University, Bozeman, 1983.
4. M. H. Turakhia, "Influence of Calcium on Biofilm Processes," Ph.D. Thesis, Montana State University, Bozeman, 1986.
5. J. A. Roels, *Biotechnol. Bioeng.*, **22**, 2547 (1980).
6. J. Monod, *Ann. Rev. Microbiol.*, **3**, 418 (1949).
7. R. Luedeking and E. L. Piret, *J. Biochem. Microbiol.*, Tech. Eng., **1**, 393 (1959).

8. M. G. Trulear and W. G. Characklis, *J. Wat. Pollut. Contr. Fed.*, **54**, 1288 (1982).
9. J. E. Hobbie, R. J. Daley, and S. Jasper, *Appl. Environ. Microbiol.*, **35**, 858 (1977).
10. R. N. Doetsch and T. M. Cook, *Introduction to Bacteria and Their Ecobiology*, (University Park Press, Baltimore, MD, 1973), p. 371.
11. L. R. Bakken and R. A. Olsen, *Appl. Environ. Microbiol.*, **45**, 188 (1983).
12. S. E. Luria, in *The Bacteria*, Vol. 1, *Structure*, I. C. Gunsalus and R. Y. Stainer, Ed. (Academic, New York, 1960).
13. J. W. Belliveau and J. S. Lanyi, *Arch. Biochem. Biophys.*, **186**, 381 (1978).
14. S. Silver, in *Microorganisms and Minerals*, E. D. Weinberg, Ed. (Marcel Dekker, New York, 1977), p. 49.
15. A. K. Campbell, *Intracellular Calcium: Its Universal Role as Regulator*, (Wiley, New York, 1983), p. 556.
16. D. Jenkins, National Science Foundation, Final Report, Washington, DC, Project No. CME-768442, 1980.
17. E. D. Weinberg, *Microorganisms and Minerals*, (Marcel Dekker, New York, 1977), p. 491.