Bull. Environ. Contam. Toxicol. (1994) 53:913–918

Environmental Contamination and Toxicology

Simple Technique for Estimation of Biofilm Accumulation

D. Liu, Y. L. Lau, Y. K. Chau, G. Pacepavicius

Rivers Research Branch, National Water Research Institute, 867 Lakeshore Road, P.O. Box 5050, Burlington, Ontario, Canada L7R 4A6

Received: 20 September 1993/Accepted: 1 May 1994

Bacterial biofilms are very common in the aquatic environment and account for much of the biomass and microbial activity in the system (Ong et al. 1990). Biofilm growth also takes place in wastewater treatment facilities, such as trickling filters and rotating contact disks (Williamson and McCarty 1976). Recent studies have demonstrated that the removal and degradation of chemical contaminants from the aquatic environment is largely accomplished by microbial populations in the bacterial biofilm (Carey et al. 1984; Pignatello et al. 1985). For this reason, knowledge about biofilm growth will not only enhance our ability to predict the fate of chemicals in the environment but may also lead to better designs for wastewater treatment.

It is difficult to properly characterize a biofilm because it is not a single entity. By definition, a biofilm is a complex of functional consortia made up of living microorganisms and of inorganic and organic solids (Gilbert et al. 1989). Because of this complexity in structure and components, biofilm is normally assessed by approximation. This may involve a microscopic examination of the biofilm morphology (McCoy et al. 1981) and thickness (Hoehn and Ray 1973; La Motta 1976; Nielsen 1987); determination of its dry and wet weights (Pedersen 1982); assay for the ATP level and C/N contents (Aftring and Taylor 1979); determination of its polysaccharide and protein concentration (Aftring and Taylor 1979), and its potential for dye absorption (Pedersen 1982). Various direct and indirect methods for measuring biofilm quantity have been reviewed by Characklis et al. (1982). Because biofilms contain relatively large amounts of polysaccharide, the determination of total attached polysaccharide can be used as a measure of biofilm mass. The development of a simple and rapid biochemical method for the quantitative estimation of biofilm mass based on the concentration of polysaccharide (in terms of carbohydrate) is presented.

MATERIALS AND METHODS

Biofilms were grown in an outdoor open channel which was 2 m long x 0.2 m wide with 0.2 m high sidewalls. The channel was situated beside Hamilton Harbour, a large industrial harbour located at the western tip of Lake Ontario. Water was pumped from the harbour into a large headtank from which it flowed by gravity into the channel and then back into the harbour. The water depth in the channel was approximately 8.5 cm, and the velocity was 22.0 cm/s. The bottom of the channel was lined with 14.5 mm diameter Porox porcelain balls(surface area 6.6 cm^2 per ball) on which the biofilms developed. The balls were sampled periodically for the determination of total carbohydrate content. The dry biofilm mass of the same samples was also obtained, and this provided direct comparison with

Correspondence to: Y. L. Lau

the carbohydrate measurement. As the development of biofilm on the balls was not completely homogeneous, three balls were taken each time sampling was carried out.

Biofilms were also grown on mini glass plates(1.5 x 2.5 cm) in the laboratory as well as *in situ* in the harbour. The mini plates, each individually mounted on a polycarbonate clamp, were suspended on a string in the harbour, one to two metres below the surface. In the laboratory, the plates were suspended in harbour water in a 3000 L tank into which water was fed continuously at a rate of approximately 200 L per day.

The porcelain balls, which were removed from the outdoor channel, were placed in individual aluminium trays and dried in a drying oven overnight at 80 ^{0}C. They were then allowed to cool in a desiccator and weighed. The total carbohydrate content of the biofilm on each ball was then determined by a modified phenol-sulphuric acid method (Liu et al. 1973). Each ball was placed into a PTFE cylinder, and 4 mL each of distilled water and 10 % phenol solution were added, followed by the rapid addition of 20 mL of concentrated sulphuric acid. After standing at room temperature for 10 min, the reaction mixture was scanned in a spectrophotometer (Shimadzu model UV-260) for the absorbance maximum from 650 to 350 nm against a reagent blank (1 mL each of distilled water and 10% phenol plus 5 mL of concentrated sulphuric acid). With increasing amounts of biofilm on the balls, the amount of reagents used was increased while maintaining a 1:1:5 ratio of water:phenol:sulphuric acid. After the absorbance determination, the balls, now stripped of biofilm, were washed thoroughly in distilled water, dried again in their original aluminium trays, and weighed. From the difference in mass with and without biofilm, the biofilm mass on each ball could be calculated.

For the mini glass plates, the test was performed in a test tube using 1 mL each of distilled water and phenol plus 5 mL of acid.

RESULTS AND DISCUSSION

Figure 1 presents maximum absorbance values (measured at 485 nm) obtained from the porcelain balls over a period of about 400 hours of biofilm development. The absorbance values were theoretical values calculated based on the volumes of reagents used, as described in the previous section. It can be seen that the increase was rather slow over the first 150 hours but became much more rapid after that. The same trend was observed from the dry mass measurements. Figure 2 is a plot of the absorbance measurements against the corresponding dry mass measurements. The linear relationship obtained indicates that the biofilm mass is directly related to the maximum value of absorbance. As carbohydrate concentration has been shown to be directly proportional to absorbance (Liu et al. 1973), the results show that total carbohydrate content can be used as a good indication of biofilm mass.

Microscopic examination of biofilms developed on standard microscope slides suspended in the laboratory tank and in Hamilton Harbour revealed differences in their composition. The laboratory biofilm had a simpler structure, with bacteria as the predominant organism. The biofilm developed directly in the harbour was much more complicated, with many more trophic levels and organisms (bacteria, fungi, protozoa, diatoms, and algae). This difference in community structure was also detected by our carbohydrate method (Figure 3). The significant absorbance

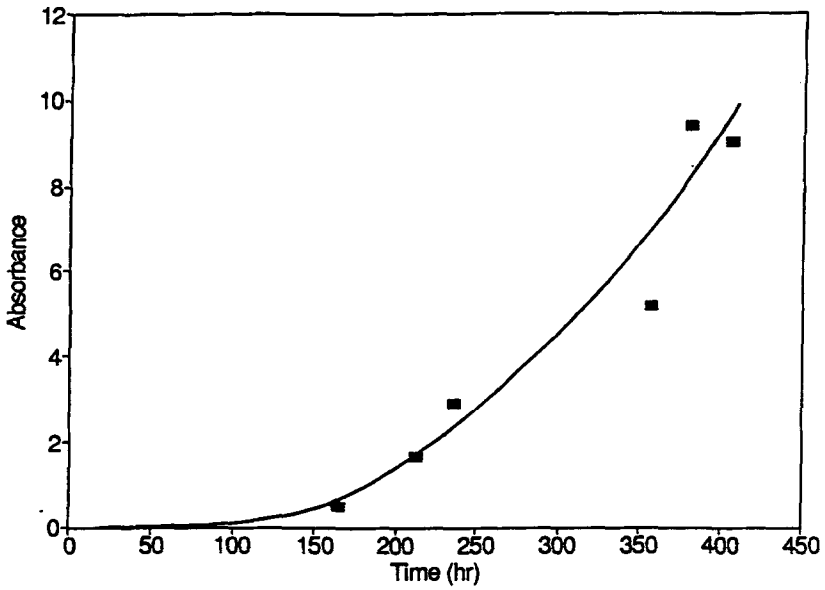

Figure 1. Absorbance measurements from biofilms developed on porcelain balls. Each datum point represents the average reading from three balls.

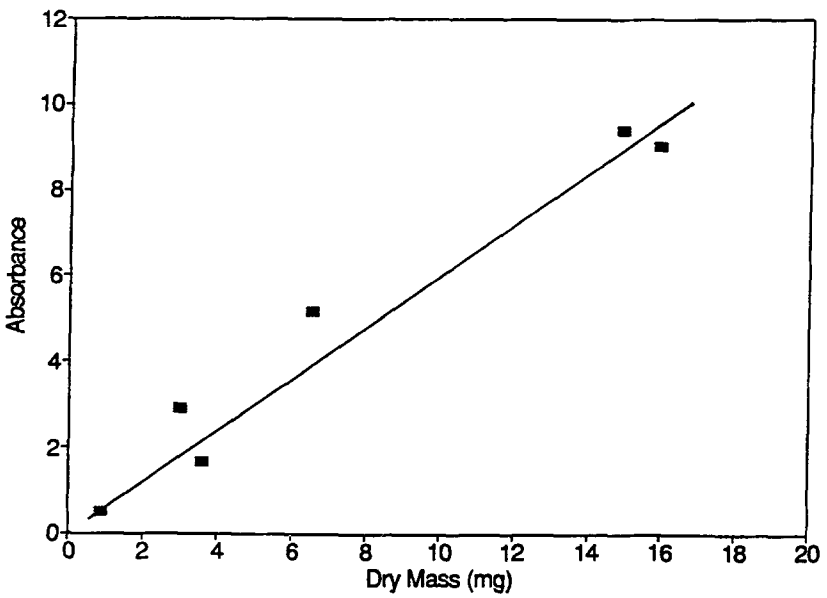

Figure 2. Relationship between absorbance and dry biofilm mass.

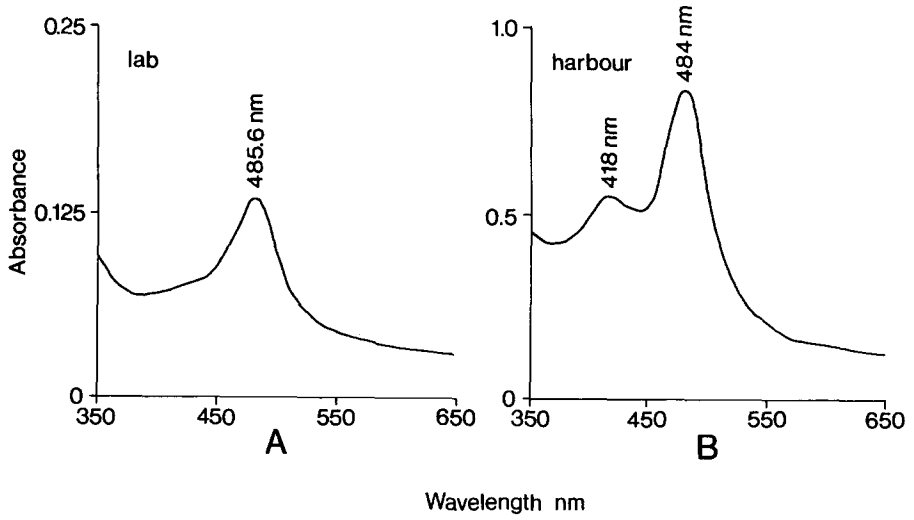

Figure 3. Absorbance spectra of biofilms developed in (A) in laboratory and (B) in situ.

maximum at 484-486 nm suggests that both the laboratory and *in situ* developed biofilms contained approximately equimolar mixtures of pentoses and hexoses, base sugar units of polysaccharide. However, the biofilm developed *in situ* in Hamilton Harbour also had a secondary absorbance peak at 418 nm, which indicated the possible presence of methyl pentose (Snell and Snell 1961), a major component of algal polysaccharide in the biofilm.

At the early stages of biofilm development, it is difficult to accurately assess the accumulated biofilm mass by dry weight determination because the quantities are usually very minute. However, with the proposed carbohydrate method, biofilm growth can be measured even at a very early stage. Figure 4 shows the absorbance spectrum from a mini glass plate after only one hour in the laboratory tank. This absorbance corresponds to approximately 1.4 µg of carbohydrate, as calculated from a standard curve given in Liu et al.(1973). Figures 3 and 4 demonstrate the sensitivity and specificity of the carbohydrate method in biofilm quantification.

In general, total carbohydrate in a sample can be estimated by the anthrone or by the phenol-sulfuric acid method (Snell and Snell 1961). Both methods have been successfully applied to the estimation of biofilm mass in the marine environment (Aftring and Taylor 1979). Based on our past experiences (Liu et al. 1973) and literature data (Snell and Snell 1961; Herbert et al. 1971), it appears that the phenol-sulfuric acid method is the method of choice for the approximation of biofilm mass, mainly because both the reagent and the method have the advantage of greater simplicity, sensitivity, and reliability. Unlike the labile anthrone reagent, the phenol reagent is stable for a long period (for at least one year) at room temperature, thus eliminating the need of fresh reagent and new standard curve each time a carbohydrate determination is made (Herbert et al. 1971). The modified phenol-sulfuric acid method used in the present study is approximately 20% more sensitive than the original phenol-sulfuric acid method (Dubois et al. 1956) in total carbohydrate determination. This increased sensitivity would facilitate the

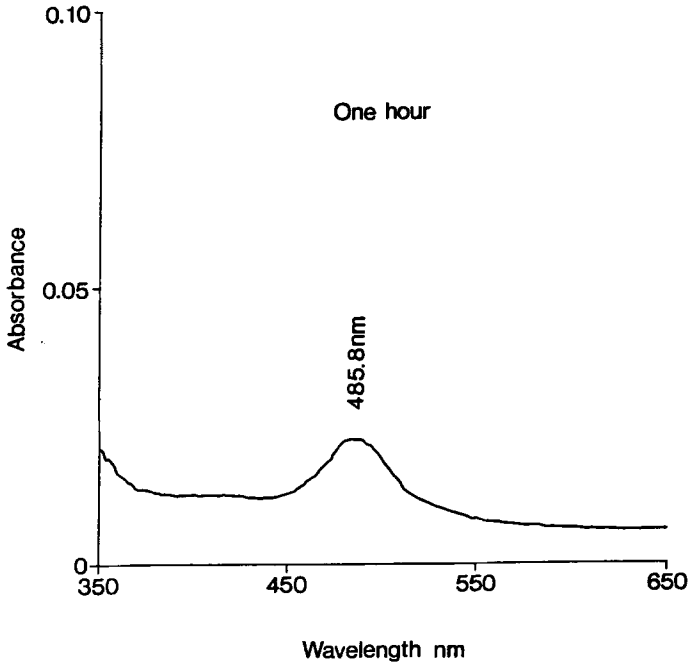

Figure 4. Absorbance spectrum of in-situ grown biofilm after one hour of development.

detection of biofilm accumulation at the early stage of development as shown in Figure 4. Moreover, literature studies indicate that the phenol-sulfuric acid method is not only more sensitive, but is also less likely to be affected by the variations in the experimental conditions when compared to the anthrone method (Herbert et al. 1971). In addition, the colour developed by the phenol reagent is stable for several hours (vs. approximately 15-30 min for the anthrone), offering a greater flexibility in the management of laboratory experiment. Furthermore, unlike the robust phenol-sulfuric acid method, determination of total carbohydrate by the anthrone method also requires that the test sample and reagent must be first cooled down to 0°C before mixing, and heated an exact time in a boiling water-bath (Herbert et al. 1971). This is not only more time consuming but may also contribute to the variation of experimental results.

Traditionally, biofilm material must be physically removed from the supporting substratum by scraping or vacuuming prior to carbohydrate determination (Aftring and Taylor 1979). This would contribute a significant variation in the calculation of the biofilm mass, because it is physically impossible to remove all the biofilm material from a substratum. With the present method, direct determination of the biofilm mass developed on the mini slides can be made using the modified phenol sulfuric acid method, thus greatly improving the accuracy in carbohydrate determination.

Biofilm is a hetrogeneous and amorphous material without any reference standard by which the recovery, accuracy and precision of the proposed method can be measured against. The present method can measure biofilms containing only several µg of carbohydrate. This is much more sensitive than quantifying biofilm by weighing, which requires at least 0.1 mg of dry biofilm material. The results have shown that the carbohydrate method is a simple, rapid and low cost method for biofilm estimation. It has been used for studying the effects of flow rate on biofilm development in open channels (Lau and Liu 1993).

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