Biofilm Dynamics and Kinetics during High-Rate Sulfate Reduction under Anaerobic Conditions

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The sulfate kinetics in an anaerobic, sulfate-reducing biofilm were investigated with an annular biofilm reactor. Biofilm growth, sulfide production, and kinetic constants ($K_m$ and $V_{ma}$) for the bacterial sulfate uptake within the biofilm were determined. These parameters were used to model the biofilm kinetics, and the experimental results were in good agreement with the model predictions. Typical zero-order volume rate constants for sulfate reduction in a biofilm without substrate limitation ranged from 56 to 93 μmol of SO$_4^{2-}$ cm$^{-3}$ h$^{-1}$ at 20°C. The temperature dependence ($Q_{10}$) of sulfate reduction was equivalent to 3.4 at between 9 and 20°C. The measured rates of sulfate reduction could explain the relatively high sulfide levels found in sewers and wastewater treatment systems. Furthermore, it has been shown that sulfate reduction in biofilms just a few hundred micrometers thick is limited by sulfate diffusion into biofilm at concentrations below 0.5 mM. This observation might, in some cases, be an explanation for the relatively poor capacity of the sulfate-reducing bacteria to compete with the methanogenic bacteria in anaerobic wastewater treatment in submerged filters.

Biofilms are used in a number of fixed-film wastewater treatment systems in which substrate removal is a complex combination of mass transfer and microbiological processes. However, during anaerobic treatment of wastewater in submerged filters, hydrogen sulfide produced by microbial sulfate reduction is often an undesirable by-product. Removal of sulfide is expensive, and the gas is highly toxic. Furthermore, in the presence of oxygen, sulfide is chemically or biologically oxidized to the very corrosive sulfuric acid (25, 31). Besides the production of sulfide, the activity of the sulfate reducers is also responsible for anaerobic metal corrosion, which is well known, e.g., in the petrochemical industry (6). Also, in sewer systems transporting wastewater under anoxic conditions over long distances, the sulfide production from “slimes” or biofilm results in health and economic problems (3, 24, 31). However, in some cases sulfate reduction in biofilm is used to remove sulfate from sulfate-rich mine water or industrial effluents (20).

Despite the fact that sulfate reduction is a widespread and well-known phenomenon, no studies of the biofilm kinetics in high-rate sulfate-reducing biofilms have been published, although biofilm kinetics in methanogenic reactors have been described (30, 33). Sulfate reduction is well described only in marine and fresh water sediments, in which the process has been shown to be very important for the anaerobic mineralization of organic matter (12, 15). Several models exist that describe the fundamental biofilm processes (e.g., see references 2, 26, and 35), but none of these models deals directly with sulfate reduction in biofilms. Some empirical models to explain the sulfide build-up in sewer systems have been published (3, 24, 31), but the biofilm kinetics are not directly taken into account. Studies with a tubular biofilm reactor have resulted in sulfide production rates comparable to those found in actual sewer systems (9).

The aim of this work was to study the dynamics and kinetics of an anaerobic, sulfate-reducing biofilm. The measurements were made by using a completely mixed rotating annular reactor (4) with biofilm growth on the outer cylinder. The substrate uptake of the biofilm was modeled and compared with experimental results at low sulfate concentrations.

MATERIALS AND METHODS

Experimental system. All experiments except the determination of $K_m$ were performed in an annular reactor system, which consisted of a drum of polyvinyl chloride enclosed in a transparent polyvinylchloride cylinder (Fig. 1). The drum rotated at 190 rpm (80 cm$^{-1}$). Two magnets in the drum and one fastened to a motor outside the reactor served for rotation. At this speed the stagnant liquid layer and, therefore, the external mass transfer resistance were insignificant (16). A circulation circuit was connected to the reactor to prevent secondary streamings in the reactor. To minimize gas transfer through the reactor, tubes, fittings and the circulation system were made from low-permeability materials including nylon, Teflon (E. I. du Pont de Nemours & Co., Inc.), and polyvinylchloride. Samples for analyses were taken with a syringe through butyl rubber stoppers placed in the lid of the reactor. Biofilm formation was restricted to the outer cylinder by cleaning the lids and drum regularly under strict anoxic conditions. The combined volume of the reactor and circulation circuit was 562 ml, and the biofilm surface-to-volume ratio was 90 m$^{-2}$.

Media and biofilm growth conditions. Bacteria were grown in a mineral salt medium with the following components (grams per liter of distilled water unless otherwise specified): KH$_2$PO$_4$ (0.5), NH$_4$Cl (1.0), CaCl$_2$ · 2H$_2$O (0.06), MgCl$_2$ · 6H$_2$O (0.05); trace elements (1 ml) (34), resazurin (0.01), sodium citrate · 2H$_2$O (0.3), and sodium lactate (2 ml of a 50% solution). The pH was adjusted to 7.2 with NaOH, and the solution was flushed with pure N$_2$ and reduced by adding Na$_2$S · 9H$_2$O from an anoxic sterile stock solution. Various concentrations of sulfate were added from an anoxic sterile stock solution, and the concentration was, in some cases, controlled by ion chromatography.

The inoculum of sulfate-reducing bacteria originated from raw sewage. A stock culture was grown as a batch and fed with anoxic domestic wastewater. During the first 3 to 4 days, the reactor was run as a batch reactor until some
surface colonization had occurred. Thereafter, substrate was continuously fed by a peristaltic pump. The sulfate concentration in the reactor was kept at around 1 mM, which is a common value for the sulfate concentration in domestic wastewater. All experiments were carried out in the dark at 15 or 20°C. Biofilm thickness was determined microscopically (4) on three plugs, each with a diameter of 6 mm, drilled out from the reactor wall. Ten measurements were made on each plug, and the thickness was determined as the mean with a standard deviation of approximately 10 to 15%. Dry weight was determined by removing the biofilm from a known area of the reactor wall. The sample was filtered onto a preweighed filter (pore size, 0.45 μm) and dried to a constant weight at 60°C.

Sulfide production in the biofilm. Sulfide production in the biofilm was calculated from the initial velocity of sulfide formation in the reactor after the flow through the reactor was stopped. The flow was stopped when the actual sulfate steady-state concentration was obtained, normally after 4 to 5 residence times. The duration of each rate measurement varied from 20 to 30 min.

The sulfide production rate was also measured as a steady-state balance in the continuously fed culture. The sulfide production rate was calculated from the difference in sulfide concentrations in the inlet and outlet and the flow rate through the reactor. Sulfide levels were determined photometrically by the methylene blue method (5).

Measurements of \( K_m \) and \( V_{\text{max}} \). The kinetic parameters for sulfate uptake were determined by using radiotracer techniques to produce a progress curve of sulfate depletion versus time in a suspended culture. A part of the biofilm from the annular reactor was gently homogenized under strict anaerobic conditions and washed in anoxic sulfate-free medium. An adequate amount of the homogenized biofilm was transferred into 120-ml serum bottles which each contained 50 ml of sterile medium. The bottles were sealed with black butyl rubber stoppers and incubated in the dark at 20°C in a shaking-water bath. After 2 h of incubation, unlabelled sulfate (final concentration, 5 to 20 μM) and 10 to 20 μCi of carrier-free \(^{35}\)SO\(_4^{2-}\) (Riso, Roskilde, Denmark) were injected into the bottles. Samples of 0.5 ml each were injected into 5-ml portions of a 1% (wt/vol) zinc acetate solution to precipitate the sulfide and terminate bacterial activity. The zinc sulfide was removed by centrifugation, and the radioactivity of the sulfate was measured in a subsample of the supernatant by using a liquid scintillation counter (Packard Instrument, Co., Inc.). At least three progress curves of sulfate depletion were used to determine the kinetic constants calculated from the linearization of the integrated Michaelis-Menten equation (27):

\[
\frac{t}{(S_0 - S)} = \frac{(K_m/V_{\text{max}})}{ln(S_0/S) + 1/V_{\text{max}}} \quad (1)
\]

where \( S_0 \) is the substrate concentration at time zero, \( S \) is the substrate concentration, \( V_{\text{max}} \) is the maximal specific rate of substrate uptake, \( K_m \) is the apparent half-saturation constant, and \( t \) is time.

Modeling biofilm kinetics. The substrate uptake of bacteria is normally described by Michaelis-Menten kinetics, and the diffusion into biofilm is normally described by Fick’s second law of diffusion (35). At steady state the mass balance for one limiting substrate with no external mass transfer resistance can be described by the following equation (35):

\[
D_f (d^2S_p/dz^2) = X V_{\text{max}}S_p/(K_m + S_p) \quad (2)
\]

where \( S_p \) is the substrate concentration at a point within the biofilm, \( D_f \) is the molecular diffusion coefficient within the biofilm, \( X \) is the biomass, and \( z \) is the distance normal to the biofilm surface.

The second-order nonlinear, ordinary differential equation does not have an explicit solution. It is, however, possible to use some analytical approximations (7, 26, 30). In this study the equation was solved by a numerical method, and the surface flux through the biofilm surface was calculated for various sulfate concentrations.

**RESULTS**

Biofilm growth. An increase in biofilm thickness in the reactor, caused by the growth of a mixed population of anaerobic bacteria without substrate limitation, is shown in Fig. 2. After an induction period for the development of a primary layer of biofilm, a nearly exponential increase in the biofilm thickness was observed. When the thickness of the biofilm was over 300 to 400 μm, it was no longer fully penetrated by sulfate at an actual concentration of 1 mM in the reactor (see Fig. 5). At higher biofilm thicknesses, increasing hydraulic stress caused the rate of accumulation of bacteria in the biofilm to decrease and finally to stop (data not shown). The activity of the sulfate reducers in the biofilm was measured as the sulfide production rate for fully penetrated biofilm. This activity showed a nearly exponential increase with time, and the doubling time for sulfide production was around 30 to 31 h. This means that the doubling time for the sulfate reducers was less than 30 h, because a part of the population was continuously washed out of the chemostat.

In other experiments in which biofilm thicknesses ranged from 100 to 300 μm, the measured zero-order volume rate constant \( k_0 \) for fully penetrated biofilm ranged from 56 to 93 μmol of SO\(_4^{2-}\) cm\(^{-3}\) h\(^{-1}\) at 20°C. The biofilm dry weights were between 40 and 91 mg cm\(^{-3}\).

The temperature dependence of the bacterial activity in the biofilm was measured at temperatures between 9 and 20°C. The sulfide production in the fully penetrated biofilm
showed a linear increase in an Arrhenius plot (Fig. 3). The activation energy of the process was found to be 84.9 kJ mol\(^{-1}\) which yields a \(Q_{10}\) of 3.4 for \(k_{\text{off}}\) in the measured temperature interval.

**Sulfate uptake kinetics of the bacteria.** A typical progress curve for sulfate use in the homogenized biofilm is shown in Fig. 4A. These data were used to calculate the kinetic constants with a linearized form of the integrated Michaelis-Menten equation (Fig. 4B).

The initial sulfate concentration in the experiments varied from 5 to 20-fold the \(K_m\). The \(K_m\) for a mixed population of sulfate reducers in the biofilm was \(1.4 \pm 0.2\) (standard error of the mean) \(\mu\)M \(\text{SO}_4^{2-}\), and the specific sulfate uptake \(V_{\text{max}}\) was \(1.4 \pm 0.1\) mmol of \(\text{SO}_4^{2-}\) g\(^{-1}\) h\(^{-1}\) (\(n = 3\)).

**Biofilm kinetics.** The sulfide production rates at different sulfate concentrations from two biofilms with different thicknesses are shown in Fig. 5. The experiments were done at 15°C to minimize the effect of biofilm growth during the experiment, which normally lasted 6 to 8 h. For each biofilm the sulfate production rate from the biofilm surface did not increase when the sulfate concentration exceeded a certain value. Consequently, beyond these values the biofilms were fully penetrated with sulfate, and all the bacteria within the biofilms were active. The addition of more lactate did not increase the sulfide production rate, which showed that lactate was always in surplus.

Since sulfate was the only source for sulfide production, the zero-order constant for sulfide production was assumed to be identical to the sulfate reduction rate. This assumption was confirmed for the thin biofilm, in which the rate of sulfate consumption was measured by radiotracer techniques. At four different sulfate concentrations the sulfate consumption and sulfide production were in good agreement (Fig. 5A). \(k_{\text{off}}\) was calculated based on the sulfide production rate in the fully penetrated biofilms and found to be \(44.7 \times 10^3\) and \(36.2 \times 10^3\) mmol m\(^{-2}\) h\(^{-1}\) at biofilm thicknesses of 112 and 239 \(\mu\)m, respectively.

Sulfate reduction in the biofilm was modeled by solving equation 2 by numerical methods with the values shown in table 1. The diffusion coefficient for sulfate in pure water (\(D_0\)) is \(8.28 \times 10^{-6}\) cm\(^2\) s\(^{-1}\) at 15°C (18), and the diffusion coefficient within the biofilm is assumed to be \(0.8 \times D_0\) (36). The predicted sulfate reduction in the biofilm based on the model showed good agreement with the measured sulfide production rates at different sulfate concentrations for both biofilm thicknesses (112 and 239 \(\mu\)m) (Fig. 5).

In these experiments sulfate reduction in rather thin biofilms (<300 \(\mu\)m) without limitations on organic matter were observed to be limited by sulfate diffusion into the biofilm at the typical sulfate concentrations (0.1 to 0.5 mM) found in both fresh water and domestic wastewater.

**DISCUSSION**

It has been shown that sulfide production from a sulfate-reducing biofilm could be described by a well-known, relatively simple model when kinetic constants were available. Furthermore, it was seen that diffusional limitation of sulfate reduction might be an important phenomenon in many high-rate anaerobic biofilm systems. The biofilm used in this study gave the same growth and activity patterns with time as have been reported for other heterotrophic biofilms (8, 32).
When the necessary kinetic constants are obtained for modeling biofilm kinetics, it is important to distinguish between the kinetic parameters for individual bacteria inside the biofilm ($K_m$ and $V_{max}$) and the biofilm kinetic parameters ($k_{of}$ and $D_f$). $k_{of}$ includes the bacterial density and the specific activity of the bacteria ($V_{max}$).

The kinetic constants for the population of sulfate-reducing bacteria in the homogenized biofilm were comparable to the constants reported in other studies (10, 22). The $K_m$ of 1.4 µM was far below normal fresh-water sulfate concentrations. This value was slightly lower than the level of 4 to 10 µM reported for different fresh-water strains. The $K_m$ (30 to 300 µM) for marine strains indicates that the sulfate reducers in nature are adapted to ambient sulfate concentrations (10, 11). This probably means that sulfate reducers (and maybe other types of bacteria) in a thick biofilm have different affinities ($K_m$) for the substrate, depending on their locations within the biofilm. In this study the biofilm was always fully penetrated with sulfate except for rather short experimental periods. Therefore, variations in $K_m$ for the sulfate reducers were probably very small. The $V_{max}$ for sulfate reduction was around 1.4 mmol SO$_4^{2-}$ g$^{-1}$ h$^{-1}$, which is in agreement with earlier findings for pure cultures (10).

The volumetric $k_{of}$ is very important for the amount of sulfate which is reduced in the biofilm and therefore is also important for modeling biofilm kinetics. A $k_{of}$ of 56 to 93 mmol SO$_4^{2-}$ cm$^{-3}$ h$^{-1}$ at 20°C was rather high compared with results from other studies of the activity of sulfate reducers in mixed microbial communities. In marine sediments, where sulfate reduction is a very important mineralization process, rates around 100- to 1,000-fold lower than our results have been found (15). The specific activity of the bacteria is probably in the same range as that found in this study, but the density of sulfate reducers is much lower. The presence of excess lactate throughout our study caused a buildup of a high density of sulfate reducers compared with other high-rate systems. Only in areas rich in easily degradable organic matter can comparably high bacterial densities and rates be expected, e.g., in connection with the discharge of wastewater or in wastewater treatment systems. Sulfide production from biofilms of unknown thicknesses has been measured in sewer systems, and reported values are as high as 7.3 to 32 mM S$^{2-}$ m$^{-2}$ h$^{-1}$ (31). Assuming an active biofilm thickness of 250 µm will give a $k_{of}$ of around 30 to 120 mmol cm$^{-3}$ h$^{-1}$, which is in the same range as the values reported in this study. To my knowledge only one other study has reported the measurement of sulfate uptake rates in biofilms (9). Using an inoculum from a creek sediment in a tubular biofilm reactor at high sulfate concentrations, these researchers found $k_{of}$ of 200 to 500 mmol SO$_4^{2-}$ cm$^{-3}$ h$^{-1}$ at 41°C.

The activity of the sulfate reducers was highly dependent on temperature, with a $Q_{10}$ of around 3.4. Comparably high values for $Q_{10}$ of 3.4 to 3.5 for sulfate reduction have been found in marine coastal sediments and in salt marsh sediments (1, 15), and a $Q_{10}$ of 2.9 was found in eutrophic fresh-water lake sediment (12). This implies that the temperature effect is rather high on a fully penetrated biofilm.

Another important parameter for the transport of sulfate into the biofilm is the molecular diffusion coefficient, which is often assumed to be about 80% of the value in pure water (36). This value was used in this study for modeling the biofilm kinetics. Recently, molecular diffusion coefficients within aerobic heterotrophic biofilms have been reported ranging from 60 to nearly 100% of the value in pure water.

![Biofilm surface sulfide production rate at various concentrations of sulfate. Temperature, 15°C. Curves: A, experimental data for sulfide production (□) and sulfate reduction (●) in 112-µm-thick biofilm; B, experimental data for sulfide production in 239-µm-thick biofilm (△). The two curves are predictions from the model for sulfate uptake in the biofilm, based on the parameters listed in Table 1.](http://aem.asm.org/)

**FIG. 5.** Biofilm surface sulfide production rate at various concentrations of sulfate. Temperature, 15°C. Curves: A, experimental data for sulfide production (□) and sulfate reduction (●) in 112-µm-thick biofilm; B, experimental data for sulfide production in 239-µm-thick biofilm (△). The two curves are predictions from the model for sulfate uptake in the biofilm, based on the parameters listed in Table 1.

**TABLE 1.** Values of kinetic parameters of the model

<table>
<thead>
<tr>
<th>Biofilm thickness (µm)</th>
<th>Value of indicated kinetic parameter (units)$^c$</th>
<th>$\kappa^a$ (g m$^{-2}$)</th>
<th>$V_{max}$ (mmol g$^{-1}$ h$^{-1}$)</th>
<th>$K_m$ (mmol m$^{-3}$)</th>
<th>$D_f$ (m$^2$ h$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>112</td>
<td>59 × 10$^3$</td>
<td>0.75</td>
<td>1.4</td>
<td>2.39 × 10$^{-6}$</td>
<td></td>
</tr>
<tr>
<td>239</td>
<td>48 × 10$^3$</td>
<td>0.75</td>
<td>1.4</td>
<td>2.39 × 10$^{-6}$</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Temperature was kept at 15°C.

$^c$ Calculated with $k_{of}$ of 44.7 × 10$^3$ and 36.2 × 10$^3$ mmol m$^{-3}$ h$^{-1}$ in biofilms of 112- and 239-µm thicknesses, respectively, and with a $V_{max}$ of 1.40 mmol g$^{-1}$ h$^{-1}$ at 20°C, assuming a $Q_{10}$ of 3.4.
(23, 29). Therefore, determination of the diffusion coefficient would improve the prediction accuracy of the model.

The effect of internal diffusional resistance is seen very clearly from the experimental results and the prediction from the model (Fig. 5). Despite the very high affinity for sulfate shown by the sulfate-reducing bacteria in the biofilms with a $K_m$ of around 1.4 $\mu$M, diffusional resistance would limit sulfate reduction in biofilms at much higher sulfate concentrations, depending on the biofilm thickness. Therefore, it is also very important to avoid any diffusional resistance when $K_m$ for single bacteria in aggregates or flocs is determined. Microscopic investigations of the homogenized biofilm in this study showed only single bacteria and aggregates below 5 to 10 $\mu$m, so the diffusional resistance was not expected to be significant in the determination of $K_m$.

The model can be used to predict sulfate reduction in biofilm under different conditions. For instance, an important parameter such as temperature often affects the processes in biofilm. The overall effect of temperature on sulfate reduction in biofilm depends on the degree of penetration of sulfate. If the biofilm is fully penetrated, a high temperature dependence with a $Q_{10}$ of 3.4 can be expected, arising only from the temperature dependence of the bacterial activity. However, if sulfate reduction in biofilms is limited by diffusion, the amount of sulfate reduced is determined partly by $k_{df}$ and partly by the diffusion coefficient. The temperature dependence of the diffusion coefficient is much lower than was observed for $k_{df}$, namely an increase of around 35% at temperatures of 10 to 20°C (18). Therefore, the apparent $Q_{10}$ for sulfate uptake and flux into the biofilm would be considerably lower than 3.4. For instance, the model predicts that the apparent $Q_{10}$ for sulfate uptake in thick biofilm (Fig. 5B) would be only 1.8 at a sulfate concentration of 50 $\mu$M.

In most natural environments and in some high-rate anaerobic systems, the methanogenic bacteria are poor competitors with the sulfate reducers (17, 19, 28). However, some authors have reported the opposite in high-rate anaerobic reactors (14, 21). Several explanations have been proposed for this observation, e.g., different substrate uptake kinetics for the bacteria, a more effective adhesion of the methanogenic bacteria to the filter carrier in the reactor (13, 14), and lack of a suitable energy-rich organic substrate for the sulfate reducers to outcompete the methanogenic bacteria (21). However, this study has shown that the mass transfer of sulfate may also be a very important factor for limiting sulfate reduction in high-rate biofilm, especially at the normally rather small sulfate concentrations found in domestic wastewater.

The data and the model presented here show that in high-rate systems sulfate reduction, like other processes such as oxygen consumption and nitrate reduction (7), very often was limited by mass transfer of the electron acceptor into a thick biofilm. The results presented here are based on laboratory experiments under optimal nutritional conditions; therefore, the sulfate reduction rates found were probably higher than in most natural high-rate systems. However, with the calibration of a given system by using an actual zero-order volume constant, this model could be used to calculate sulfate flux and penetration depth into a sulfate-reducing biofilm or, in some cases, into sediment.

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LITERATURE CITED


