Structural Analysis of a Biofilm Which Enhances Carbon Steel Corrosion in Nutritionally Poor Aquatic Environments

YASUNORI TANJI,* YUHKI MORONO, AYA SOEJIMA, KATSUTOSHI HORI, AND HAJIME UNNO
Department of Bioengineering, Faculty of Biotechnology, Tokyo Institute of Technology, 4259 Nagatsuda-cho, Midori-ku, Yokohama 226-8501, Japan

Received 4 June 1999/Accepted 2 August 1999

Carbon steel coupons were exposed to nutritionally-poor synthetic wastewater inoculated with activated sludge from a municipal waste water plant. Biofilm formation was observed after one day incubation, and the thickness of the film increased proportionally with the incubation period. Mass loss of the coupons was also proportional to the incubation time, and reached 70.4 (mg/cm²) after incubation for 140 d. The observed mass loss was 5 times as much as that under sterile conditions. To characterize the microbiologically influenced corrosion (MIC) of carbon steel, structural analysis of the biofilm was performed. Rapid decrease in the dissolved oxygen (DO) concentration in the zone near the surface of the biofilm was observed by a microelectrode mounted on a micromanipulator. Heterogeneous distribution of the DO concentration on the surface of the steel plate was observed after multiple analyses. The heterogeneous structure of the biofilm composed of viable cells, inanimate objects, voids and pores was elucidated by confocal scanning laser microscopy. Concentrations of both aerobic bacteria and sulphur-reducing bacteria in the biofilm decreased with the incubation time, indicating that the increase in the biofilm thickness reflected an increase in the density of dead microbial cells or in extracellular polymer accumulation by the microbes. The average roughness of the metal surface observed after 112 d of incubation was ±7.14 μm, which was 14.1% of the average thickness of the coupons. These observations indicated that uneven distribution of the DO profile and the cell concentration were critical for MIC of the carbon steel.

[Key words: corrosion, carbon steel, microbiologically influenced corrosion, biofilm, microelectrode]

Since carbon steel is cheaper than stainless steel or plastic piping, it is widely used for transporting and sealing solutions. Unfortunately, carbon steel is susceptible to microbiologically influenced corrosion (MIC). MIC has increasingly been recognized as posing a serious problem when metal surfaces are exposed to nutritionally-poor natural water (1, 2). MIC occurs as a consequence of heterogeneous biofilm formation which leads to formation of oxygen concentration gradients and differential aeration cells on the metal surface (1). These bio-degradatory effects are considerably aggravated when the growth of the aerobic heterotrophic flora is accompanied by that of anaerobic sulphate-reducing bacteria (SRB). SRB possess the ability to survive in bioflock or biofilm by that of anaerobic sulphate-reducing bacteria (SRB). Structural Analysis of a Biofilm Which Enhances Carbon Steel Corrosion

 MATERIALS AND METHODS

Experimental setup and procedure Carbon steel coupons (30 mm in diameter) weighing 1.85–1.98 g, with a thickness of 0.35 mm were cut from a sheet stock. The specified composition of the carbon steel coupons was, in wt% 99.71 Fe, 0.03 C, 0.19 Mn, 0.017 S, 0.013 P, and 0.01 Si. The surface was wet-polished with an 800-grid polishing paper. The polished coupons were cleaned ultrasonically in acetone for 15 min, weighed (W₀), air-dried, and stored in a desiccator. Eighteen coupons were immersed in the test chamber (Fig. 1A) with 3.15 L of synthetic wastewater containing (in mg/l) glucose 250, urea 43, KH₂PO₄ 15, MgSO₄·7H₂O 11.3, CaCl₂·2H₂O 1.4, and FeCl₃·6H₂O 0.1. Activated sludge (350 ml) from
DO profile in the biofilm

The DO profile in the biofilm was analyzed using the oxygen microelectrode designed by Lewandowski, et al. (8) with a tip diameter of about 200 μm. For measurement of the microprofiles in biofilms, the electrode was mounted on a micromanipulator (Shimadzu MMS-20) with a step motor, allowing a 1-μm positioning accuracy. A steel coupon covered with the biofilm was placed with the DO test vessel with continuous aeration to saturate the medium with oxygen (Fig. 1B). The tip of the electrode was inserted into the biofilm a step at a time, at 50 to 100 μm intervals. Synthetic wastewater was introduced into the vessel while the DO concentration was analyzed. To obtain a nutritionally poor condition, medium sampled from the test chamber after 5 d use was used. Measurements were started after 3 h of incubation of the steel coupon with the biofilm in the test vessel. Calibration of the DO electrode was performed using pure water saturated with air or nitrogen gas.

Aerobic bacteria and SRB counting

For the counting of aerobic bacteria in the biofilm, the biofilm was removed from the steel coupon, weighed, scraped and suspended in 100 times its volume of phosphate-buffered saline, homogenized, and spread-plated on LB agar medium in triplicate. The most probable number technique (MPN) was employed to evaluate the SRB count in the biofilm (3).

Confocal scanning laser microscopy

Surface biofilms attached on the steel coupons were stained with acridine orange (0.02% final concentration) for 4 h and washed with distilled water for 24 h. Samples were observed with a confocal scanning laser microscope (CSLM) equipped with a water immersion objective lens (FLUOVIEW-SP/ TSUH: Olympus Optical Co. Ltd., Tokyo). A 488-nm beam of argon ion laser was used for the fluorescent microbial image analysis.

Surface analysis of steel coupon

Corrosion products were selectively removed from the test coupon as described previously. The roughness of the metal surface was analyzed by the Surfcom 5A (Tokyo Seimits, Tokyo).

RESULTS

Mass loss and surface roughness of the steel coupon

The mass loss of the steel coupons in synthetic waste water was analyzed for 140 d in the test chamber at 25°C (Fig. 2). The mass loss observed was normalized relative to the total surface area (14.14 cm²) of the metal coupon. A 10-fold diluted sample of activated sludge from a municipal wastewater plant was used as the initial microbial inoculum. No bacterial source was supplied thereafter to the reactor. Brownish spots of steel rust surrounded by biofilm were observed after a 24-h incubation period. Both sides of the steel coupon were totally covered with the mixture of corrosion products and biofilm after incubation for 1 week. The mass loss observed increased with the incubation period at a constant corrosion rate of 0.51 (mg/d-cm²). No inhibitory effect of biofilm enlargement on MIC of carbon steel was observed. On the contrary, the corrosion rate increased after incubation for 12 weeks. After 140 d incubation, a part of the coupon was perforated. The metal coupon immersed in activated sludge showed a 4- to 5-fold increase in mass loss compared with the coupons exposed to sterile conditions.

Glucose (250 mg/l) was supplemented as sole carbon source.

FIG. 1. (A) Test chamber designed for the analysis of MIC of carbon steel (3500 ml working volume): (1) air sparger; (2) partition; (3) steel coupons. (B) Schematic diagram of the test vessel for DO profile measurements (200 ml working volume): (1) test coupon; (2) oxygen microelectrode; (3) micromanipulator; (4) multimeter; (5) recorder; (6) air sparger; (7) medium tank; (8) partition.

Mass loss observed (mg/cm²) was used as the indicator of the extent of corrosion. A steel coupon was removed from the test chamber periodically and the wet weight of the coupon (W_i) was measured. Corrosion products and biofilm were selectively removed from the steel coupon by scraping and incubating at 60°C for 40 min in a 10% HCl solution with 0.3 vol% 1bd (Asahi Kagaku Kogyo, Osaka), a poly cationic amine derivative which protects metal steel. The metal coupon was dried and weighed (W_f). The mass loss was estimated by subtracting W_i from W_f. The biofilm thickness was estimated based on the assumption that the density of the biofilm formed (W_i-W_f) on the steel coupon is 1.0 g/cm³.

The DO concentration in the medium was maintained at 6.5 by adding 0.1 N-NaOH at a pH-stat. Water-saturated air was continuously supplied through a sparger. The DO concentration in the reactor and biofilm was 98% ethanol were immersed in sterilized synthetic wastewater in a glass chamber with a continuous supply of filtered sterilized air. The DO concentration in the medium was periodically analyzed using a DO electrode (DO-220-PB, Oriental Biotechnology Systems) and the value was maintained at 6.8 (mg O₂/l) by controlling the air supply. Experiments were conducted at 25°C.

As a control experiment, polished steel coupons rinsed with 98% ethanol were immersed in sterilized synthetic wastewater in a glass chamber with a continuous supply of filtered sterilized air. The DO concentration in the medium also was maintained at 6–8 mg-O₂/l. The medium in the control ferment was not changed during the test period.

The mass loss observed (mg/cm²) was used as the indicator of the extent of corrosion. A steel coupon was removed from the test chamber periodically and the wet weight of the coupon (W_i) was measured. Corrosion products and biofilm were selectively removed from the steel coupon by scraping and incubating at 60°C for 40 min in a 10% HCl solution with 0.3 vol% 1bd (Asahi Kagaku Kogyo, Osaka), a poly cationic amine derivative which protects metal steel. The metal coupon was dried and weighed (W_f). The mass loss was estimated by subtracting W_i from W_f. The biofilm thickness was estimated based on the assumption that the density of the biofilm formed (W_i-W_f) on the steel coupon is 1.0 g/cm³.
source. Half of the medium in the test chamber was exchanged weekly. The concentrations of glucose and total organic carbon (TOC) in the test chamber were analyzed after the change of the medium at 48 d of incubation (Fig. 3). Glucose was rapidly consumed in the reactor and almost totally used up in 5 h. The TOC concentration also decreased with the decrease in glucose concentration. The TOC value then increased at 10 h of incubation for a short while. Since the decrease in pH was always observed in association with the increase in TOC, some organic acids were supposed to be secreted from the microbes. Until 12 h after exchange of the medium, both glucose and TOC concentrations were almost zero until the next change of the medium (data not shown). Therefore, it could be inferred that the incubation condition of the steel coupons was nutritionally very poor during the test period.

When the biofilm was removed from a steel coupon by scraping, distinct pits and grooves were observed. To analyze the corrosion quantitatively, the biofilm was selectively removed from the steel coupon, and the roughness of the surface was analyzed (Fig. 4). The initial roughness of the metal surface was ±0.5 μm. The roughness increased with incubation time, indicating that the corrosion occurred heterogeneously on the surface. The average roughness observed after 112 d of incubation was 4.27 μm. The depth of several grooves was more than 10 μm. Since 45% of the metal was corroded during by 112 d of incubation, the depth of the observed grooves was equivalent to 14% of the remaining metal thickness. Partial percolation of the metal also indicated the heterogeneous corrosion of the metal.

**Biofilm structure** The mechanism of the enhanced mass loss of the steel coupons immersed in the nutritionally poor environment was investigated from the biological and chemical points of view. The average thickness of the biofilm formed on the metal surface was estimated from the wet weight of the test coupon (Fig. 5). Since there was no liquid circulation in the test chamber, the...
biofilm formed adhered stably to the metal surface. The average thickness of the biofilm increased with the incubation period over 140 d. The biofilm could be separated into several layers consisting of blackish rust, relatively dense biofilm, and a sparsely layer opening to the bulk fluid. The average concentrations of aerobic bacteria and SRB in the biofilm removed from a steel coupon by scraping were analyzed (Fig. 6). Since the concentration of aerobic bacteria decreased gradually after 30 d incubation in inverse proportion to the increase in biofilm thickness shown in Fig. 5, the total number of aerobic bacteria in the biofilm was assumed to be almost constant. Several different kinds of protozoa such as *Vorticella* and *Philodina* were detected by microscopic observations (data not shown). The detection of SRB in the biofilm suggested formation of anaerobic pockets in the biofilm. The concentration of SRB decreased with the incubation time.

To analyze the three-dimensional structure of the biofilm, CSLM was used (Fig. 7). Microorganisms were stained with acridine orange to visualize their localization in the biofilm. The structure of the biofilm from the surface to a depth of 50 μm was observed. Initially the microorganisms formed a highly dense biofilm, the surface of which was relatively flat (1 d). However, the heterogeneity of the biofilm surface increased with incubation time. Microorganisms formed clusters, which were sparsely scattered (30 d). Thus the abiotic area in the biofilm increased, while the concentration of the bacteria decreased as described above.

**Profiles of the DO concentration in biofilm** It is of fundamental importance to an understanding of the MIC of carbon steel to have a knowledge of the environmental conditions at the site where corrosion occurs. It is especially important to know whether oxygen is present or not. An oxygen microelectrode mounted on a micromanipulator was used to analyze the vertical distribution of DO in the biofilm formed after 48 d of incubation (Fig. 8). As shown by CSLM analysis, the surface of the biofilm was heterogeneous. DO analysis by the microelectrode was initiated from the bulk liquid, and the point at which the DO concentration started to...
FIG. 8. Vertical profiles of DO concentration in the biofilm measured when supplied with the used medium sampled from the test chamber A (○, point-1, thickness=2.25 mm; ●, point-2, thickness=2.45 mm; △, point-3, thickness=1.90 mm; ■, point-4, thickness=2.35 mm), and synthetic wastewater B (○, point-1, thickness=2.30 mm; ●, point-2, thickness=2.55 mm; △, point-3, thickness=2.10 mm). Test points were selected randomly. A test coupon was taken from the test chamber after 48 d of incubation. The surface of the biofilm (depth=0) was determined at which the DO concentration started to drop.

The decrease was assumed to be the surface of the biofilm. Since the biofilm in the test vessel was swollen, the thickness of the biofilm (1.90-2.55 mm) as observed by DO analysis was higher than that estimated from the wet weight of the film as shown in Fig. 5. The oxygen microelectrode was lowered in steps at 100 μm intervals. When fresh synthetic wastewater containing glucose at 250 mg/l was supplied to the test vessel, a rapid decrease in the DO concentration was observed in the region close to the surface. DO concentration at the three test points almost reached zero at 1000 μm depth where aerobic bacteria predominantly assimilated carbon sources and consumed oxygen. On the other hand, when nutritionally poor medium obtained from the reactor was circulated in the test vessel, a gradual decrease in the DO concentration was observed. The observed DO profiles varied from point to point. At test point-3, a decrease in DO with respect to the depth was observed. A relatively rapid decrease in the DO concentration was observed at test point-1. These data clearly demonstrated that the DO profiles at biofilm were heterogeneous in both vertical and horizontal directions, as presumed from the heterogeneity of the biofilm structure.

DISCUSSION

Synthetic wastewater containing glucose at 250 mg/l as the sole carbon source was used as the medium for incubation of the test samples. Since the added glucose was rapidly consumed in the test chamber, the aquatic environment in the reactor was nutritionally very poor during most of the test period. Even in such a poor environment, the mass loss of steel coupons was 5-fold higher than that in sterile controls. Microbes in the activated sludge used as the inoculum were mainly found in the medium during the first week, however the concentration of the microbes in the medium decreased with the incubation period. After one month of incubation, the concentration of aerobic bacteria in the bulk liquid was less than 100 CFU/ml (data not shown), indicating that microbes preferentially existed in the biofilm formed on the metal surface in the nutritionally poor environment. The average concentration of the aerobic bacteria in the biofilm was approximately 1 × 10^6 CFU/ml during the first one month of incubation, and decreased to 1 × 10^5 CFU/ml after 140 d of incubation (Fig. 6). During this period, the thickness of the biofilm increased almost 10-fold. Therefore, the total number of aerobic bacteria in the biofilm did not change significantly. These observations suggest that the growth of the biofilm was mainly caused by the accumulation of dead cells and/or extracellular matrix. CLSM analysis also confirmed the presence of a biofilm consisting of live and dead cells embedded in a matrix.

Under favorable conditions, protective biofilms for the prevention of corrosion are generated with pure cultures of Pseudomonas fragi and Escherichia coli DH5α in LB medium (5). It appears that only a small layer of active, respiring cells is required to inhibit corrosion, and the corrosion inhibition observed is due to the attached biofilm which causes oxygen depletion from the metal surface (6, 7). Vertical DO profiles in the natural biofilm were non-uniform. Even in the presence of a thick biofilm, oxygen can be delivered to the metal surface through water channels. This uneven distribution of DO on the metal surface could lead to the differential aeration of cells and provide anaerobic pockets for colonization by SRB which causes an increase in corrosion by the production of hydrogen sulphide and iron sulphide (9).

The structural image of the biofilm as examined by CLSM revealed that the biofilm formed on the surface of a steel coupon was composed of abiotic areas as well as areas in which microorganisms formed clusters. The abiotic area is considered to be composed of not only corrosion products and externally secreted substances from microorganisms but also of water channels, voids or pores (10). This area has been reported to contribute to mass transfer of water and nutrients (11). Therefore, even in the presence of a thick biofilm, oxygen can be delivered to the metal surface via the abiotic area. The heterogeneous structure is considered to cause heterogeneous distribution of microorganisms and substances such as oxygen, with enhancement of corrosion. The combined testing approaches used in this study provided an insight into the complex interactions between biofilms and metal surfaces.

REFERENCES


