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Characterization of microfouling and corrosive bacterial community of a firewater distribution system

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This investigation provides generic information on the culturable corrosive and the microfouling bacterial community in a firewater distribution system that uses freshwater. Conventional microbiological methods were used for the selective isolation of the major microfouling bacteria. The isolates were characterized by 16S rRNA gene sequencing and the biofilm as well as the corrosion characteristics of the isolates were evaluated. *Pseudomonas aeruginosa* and *Bacillus cereus* were predominantly observed in all the samples analysed. Denaturing gradient gel electrophoresis (DGGE) was carried out for the various samples of firewater system (FWS) and the high intensity bands were sequenced to identify the predominant bacteria. Bacterial groups such as *Cyanobacteria*, *Proteobacteria*, *Actinobacteria*, *Bacteroidetes* and *Firmicutes* were identified. Biofilm thickness was recorded using confocal scanning laser microscopy (CSLM). This was the first study to report *Lysinibacillus fusiformis* in a firewater system and its role in iron corrosion. Sulphidogenic bacteria *Tissierella* sp. and *Clostridium bifermentans* generated sulphides in the range of 400–900 ppm. Significant corrosion rates of carbon steel (CS) coupons were observed up to 4.3 mpy. *C. bifermentans* induced more localized corrosion in CS with a pit diameter of 50 μm. Overall, the data on the characterization of the fouling bacteria, their biofilm forming potential and subsequent metal deterioration studies supported in designing an effective water treatment program.

[**Key words:** Bacteria; Freshwater; Fouling community; Corrosion; Denaturing gradient gel electrophoresis; Confocal scanning laser microscopy]

Microorganisms inhabiting artificial environments such as cooling water circuits and other freshwater pipeline distribution systems are mostly benign, but some can be detrimental to the structural material. Normally some locations in the distribution system of large industrial processes provide ideal conditions for the microorganisms to grow, either in the form of sessile biofilms on surfaces or as planktonic cells in the circulating water. In most natural and industrial aquatic systems microorganisms grow as multispecies communities called biofilms (1). Microorganisms in industrial freshwater systems are distributed unevenly and there is a great variation in cell density and composition of microbial population over space and time. Transitions in these managed environments cause continuous qualitative and quantitative variation in the composition of the microbial community.

Industrial freshwater distribution systems are mostly made of iron pipelines that are normally laid as submerged lines or aerial in erection. Firewater system (FWS) is one such freshwater system which constitutes a vital part of an industrial plant and the water used is always maintained in stagnant condition. This condition is considered as one of the main reasons for the deterioration of the metal pipelines (2). Commonly, the feed water for FWS is untreated river or lake water. Microorganisms present in these waters can readily foster adhesion and form a primary microbial film, which later transforms to a mature biofilm through a multistep process (3). The extracellular polymeric substance (EPS) produced by these bacteria will complex with the corrosion deposits of the pipeline and blocks the narrow branches of the distribution system. This reduces the inner diameter of the pipes and also the water flow velocity, which is vital as per design specifications. Heterogeneity in microbial biofilm growth and the formation of ionic metabolites concentration by microbes results in generation of corrosion cells. This initiates the corrosion process and ultimately deteriorates the metal which leads to a phenomenon often termed as microbiologically influenced corrosion (MIC) (4). In the last two decades, MIC has been recognized as an important mechanism of material degradation and significant damage in various industries has been well documented (5–8).

In the present study, the FWS of a nuclear test reactor plant at Kalpakkam, India was monitored, wherein the water pipelines were distributed over a large area. The three decades old distribution system had experienced a few pipe punctures and water leaks in its operating history. The corroded areas resembled shallow hemispherical pits which are localized in few areas with pinhole damage, which is typical of MIC. Although guidelines for suitable material selection are in place for most of FWS, there are not any stipulations regarding biological and chemical quality of firewater. To design an effective microbiological control strategy a comprehensive analysis of the microbial fouling community thriving in the system has to be assessed. The data thus obtained could aid in

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designing an effective water treatment program (9). Thus the main objective of this study was to identify the resident microbial biofilm community of the FWS and describe their role in biofilm formation and metal deterioration.

MATERIALS AND METHODS

Study site The study was carried out in the firewater system (FWS) at a nuclear test reactor plant located at Kalpakkam, Tamil Nadu State, India. The FWS of the plant obtains water from an open reservoir, which has a spread area of 130 m^2 with a volume of 7.5×10^6 gallons and maximum depth of 2.13 m. The open reservoir receives subsoil water from a riverbed and also from bore holes. The FWS system includes (i) an underground concrete sump where the water received from the open reservoir which is temporarily stored and (ii) iron pipelines that are submerged beneath the ground at a depth of 4.92 feet bearing outlet hydrants at necessary places (Fig. 1). The pipelines are pressurized by both motor pump and diesel pump at 1 kg/cm^2 pressure under stagnant conditions and 10 kg/cm^2 pressure under dynamic conditions. The structural material of the pipelines is carbon steel (50–200 NB; IS 1239, ASTM A53 Grade B) with outer diameter 220 mm, thickness 6.35 mm, length 6.0 m. The water quality data of the firewater system are given in Table S1.

Isolation of heterotrophic bacteria, iron bacteria and sulphidogenic bacteria Culturable heterotrophic bacteria were isolated from the water samples of the open reservoir, sump, pipelines and the biofilm sample of the carbon steel (CS) metal coupons that are exposed in sump for 30 days by plating on Luria Bertani (LB) agar supplemented with 0.1% glucose. Iron bacteria isolation was carried out using the iron bacteria isolation media (M622-Hi-Media) (10). Sulphidogenic bacteria were isolated with modified Postgate B media and modified iron sulphite agar media under anaerobic conditions (6). The colonies obtained were then morphologically scored and sub-cultured to obtain pure cultures. The pure cultures obtained were preserved as glycerol stocks at −80°C. Culturable heterotrophic bacteria were isolated from FWS over a period of six months, to check for repetition in isolation of the bacteria since the system is replenished every month.

Screening of biofilm forming bacteria The preserved glycerol stocks were streaked on LB agar plates and subsequently inoculated in Tris minimal media containing 0.1% glucose. The cultures were incubated at 30°C under shaking (120 rpm) overnight. This culture was taken to screen the biofilm forming organisms using the modified crystal violet method as described by O'Toole and Kolter (11). Five μl of the overnight grown cultures were inoculated in triplicates into 96 well microtitre plates containing 200 μl of tris minimal media containing 0.1% glucose. The plates were incubated at 30°C in the dark for 24 h under stationary condition. After incubation, the biofilm mass was quantified by crystal violet staining method. Briefly, the methodology is as follows; after the prescribed time the cultures were discarded and the microtitre plates were rinsed three times with 200 μl of 1× PBS to remove non-adherent bacteria. The plates were then air dried for 1 h in a laminar hood flow chamber. To each well of the microtitre plate, 200 μl of 0.2% crystal violet was then added and incubated for 20 min. The stain was removed and the wells were rinsed three times to remove the excess stain. The plates were air dried for 15 min and the bound dye was extracted in 200 μl of 96% ethanol. Absorbance at 570 nm was recorded after 5 min in a 96 well microtitre plate reader (Thermo Labsystems, Finland). Bacteria were categorized as poor biofilm formers when the OD was <0.4 and biofilm forming microorganisms were selected beyond ≥0.4 OD.

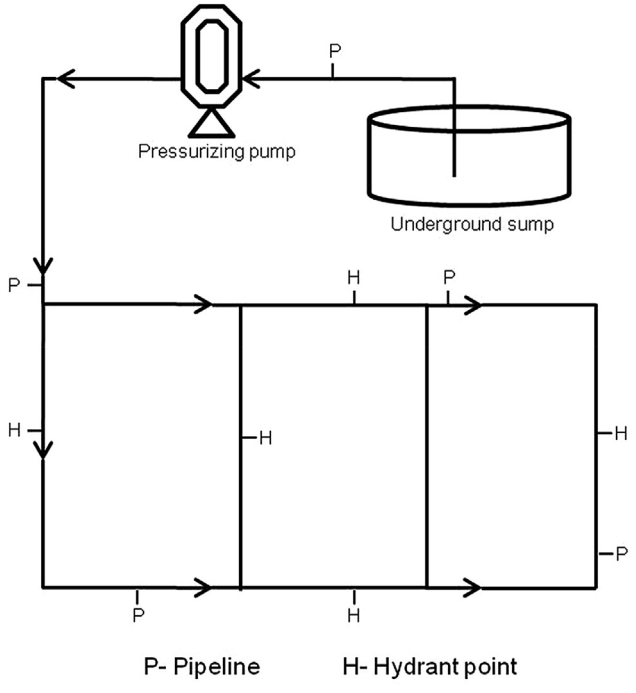

FIG. 1. Schematic of the firewater distribution system.

DNA extraction and DGGE Genomic DNA was extracted from the pure cultures that were morphologically scored; iron bacteria (18 isolates), sulphidogenic bacteria (36 isolates) and biofilm bacteria (17 isolates) using the Qiagen kit (QIAamp DNA Mini Kit, Invitrogen) according to the manufacturer's protocol. The DNA extracted was analysed electrophoretically on 0.8% (w/v) agarose gel. Denaturing gradient gel electrophoresis was used as a tool to eliminate the repeats in bacterial isolates that were isolated over a period of six months.

Denaturing gradient gel electrophoresis (DGGE) is used for identifying the bacterial diversity of various ecological niches and also as a tool to distinguish different bacterial strains. In the present study the diversity of the FWS was studied with DGGE and the bacterial primers used in this study are given in Table S2. A two step nested PCR was carried out on the total DNA extracted from the water samples of FWS to generate DGGE specific PCR product. DGGE was carried out in a linear 30–80% denaturant agent gradient (100% denaturant agent was defined as 7 M urea and 40% deionized formamide) to document the diversity profile of total bacteria as described by Balamurugan et al. (12). The number of bands in the gel reflects the species richness of a particular sample and the band intensities reflects the species abundance. The predominant bacterial population was represented by the high intensity bands in the gel and the band intensities were used to calculate the Shannon diversity index, H (13).

16S rRNA gene amplification and sequencing Full-length 16S rRNA gene amplification was done for the biofilm bacteria (6 isolates), iron bacteria (3 isolates) and sulphidogenic bacteria (2 isolates) (selected after elimination of bacterial repeats by DGGE method) using 8F/1525R universal primers. The PCR reaction conditions carried out were as follows: 5 min initial denaturation of DNA at 95°C, followed by 30 cycles of 1 min denaturation at 94°C, 1 min primer annealing at 59°C and 2 min extension at 72°C. Amplification was completed by a final extension step at 72°C for 5 min (14). The amplified products were partial sequenced using forward primer 8F using the facility at Eurofins Genomics Pvt. Ltd., Bangalore, India. Similarly, the excised DGGE bands were also sequenced using the forward primer 338F.

Biofilm imaging by confocal scanning laser microscopy Biofilm formation of the bacterial isolates was studied on carbon steel (CS) coupons. The adhesion and biofilm formation was analysed after incubation for 4 and 24 h. Aliquots (200 μl) of overnight culture were inoculated in sterile petriplates containing 20 ml of tris minimal medium containing 0.1% glucose. Pre-sterilized CS coupons were immersed into the medium as substratum for biofilm formation. The petriplates were incubated at 30°C for 4 and 24 h. The coupons were removed after the incubation period, rinsed twice with 1× PBS and stained with 0.01% acridine orange for 3 min. The coupons were then rinsed twice with 1× PBS and observed under a confocal laser scanning microscope (TCS SP2 AOBS) equipped with DM IRE 2-inverted microscope (Leica Microsystems, Germany). A 63×, 1.25 numerical aperture, water immersion objective lens was used. The 488 nm Ar laser and a 500–640 nm emission detection bandwidth was used to observe the stained biofilm. Image stacks were collected from 24 h old biofilms and x–z sagittal sections of biofilms were obtained using the Leica confocal software as described by Dusane et al. (15).

Corrosion of carbon steel by biofilm forming isolates Corrosion of CS of the selected isolates of biofilm bacteria (6 isolates), iron bacteria (3 isolates) and sulphidogenic bacteria (2 isolates) was determined. The CS coupons were finely polished up to 600-grit in a silicon carbide metallurgical paper, washed with distilled water, degreased using acetone and finally dried in a desiccator. Overnight bacterial culture of 1% was inoculated in conical flasks containing sterilized media. For anaerobic bacteria anaerobic serum bottles were used. Corrosion caused by multispecies bacteria was experimented by inoculating 1% of each culture (10 times diluted). The pre-weighed and pre-sterilized CS coupons were then aseptically transferred to the flasks containing media, plugged with cotton and incubated at 30°C in an incubator for 5 days. CS coupons (triplicate) in the respective media for each bacterial group served as control. After the incubation period, the coupons were retrieved and washed with distilled water. To measure the weight loss, the coupons were processed as described by Batista et al. (16). Initially the coupons were washed in a SDS detergent solution (6 g/L) at 70°C to remove the organic matter. Then rinsed twice with distilled water and washed in hexamine/hydrochloric acid solution (30 g/L in 50% v/v hydrochloric acid) to remove the corrosion products. The coupons were then rinsed with distilled water twice, wiped with ethanol and dried. The weight loss was measured in an electronic weighing balance with sensitivity up to 4 decimals (AUX220, Shimadzu, Japan). Corrosion rate was calculated using the formula;

$$\text{mm/y} = 87.6 \times (\text{W/DAT});\ 1\ \text{mpy} = 0.0254\ \text{mm/y} = 25.4\ \text{micron/y} \quad (1)$$