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Studies on enhancement of biofilm formation and adherence due to mechanical treatment of titanium surfaces in cooling-water systems

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ABSTRACT

Titanium has proven to be the heat exchanger material of choice for sea-water-cooled power plants owing to its outstanding resistance to pitting and crevice corrosion in a wide range of aggressive media. However, the inertness of the titanium surface makes it highly susceptible to biofilm formation and subsequent biofouling. This can hinder the heat transfer properties and flow of water. Fouling control strategies in condensers include a combination of mechanical, chemical and thermal treatments. However, reports from various industrial situations suggest that mechanical treatment may not have long-term effects. This study aimed to find out whether mechanical cleaning eventually enhances biofilm formation and increases the adherence of biofilm. In our studies epifluorescence micrographs of biofilms on control and mechanically treated titanium surfaces clearly showed accelerated biofilm formation as well as increased adherence on the mechanically cleaned surface. Total counts of viable bacteria acquired by culturing technique, and biofilm thickness measurements made using microscopic techniques, confirmed this observation. Surface profilometry showed increased roughness of the titanium surface, facilitating adherence of biofilm. The number of microbial species was higher on mechanically cleaned and re-exposed surfaces than on fresh titanium. Thus we concluded that mechanical cleaning can increase biofilm formation and adherence of biofilm, thereby increasing the potential of biofouling in the long term.

INTRODUCTION

Titanium and its alloys are used extensively in various applications including aerospace engines and airframes, military hardware, cryogenic equipment, geothermal plants, metal extraction equipment, nuclear waste storage facilities, springs, ultracentrifuges, and vehicle armour (D. K. Peacock, 2001, personal communication from Director, European manufacturing and technology, titanium metals corporation). They are also used in the fabrication of dental and orthopedic implants (Massaro *et al.*, 2002). The advantages of titanium over other metals are its weight, strength to weight ratio, resistance to corrosion and non-magnetic properties (Williams, 1970). Titanium possesses outstanding resistance to pitting and crevice corrosion in a wide range of aggressive media. It can resist erosion in clean waters at velocities of up to 9 m/s and is totally resistant to attack by hydrogen, sulfide, nitrites, ammonia and ferrous ion compounds (Birch & Burleigh, 2000). The reason for the remarkable corrosion resistance exhibited by this material relies on the presence of the inert, strongly adherent surface oxide film, which forms almost instantly on exposure to moisture, air and oxygen (Schultz & Covington, 1981). This oxide film is self-healing and exceptionally stable over a wide range of pH and potentials in conditions ranging from highly oxidizing to mildly reducing (Schultz, 1991).

Titanium has proven to be the heat exchanger material of choice for sea-water-cooled power plants. Titanium tubing will also be used in the steam condensers of the 500 MWe fast breeder reactor, whose construction is underway at Kalpakkam, India. The thin titanium

oxide layer makes the surface very passive. As such passive surfaces do not release metallic ions, many microbes attach non-specifically. This is different than on less passive surfaces such as carbon steel. The titanium surface therefore is highly susceptible to biofilm formation and subsequent biofouling (Characklis & Marshall, 1990). Although biofouling does not cause corrosion in titanium condensers, it does cause a reduction in its heat transfer properties and enhances resistance to water flow (Melo & Pinheiro, 1992). A variety of microorganisms, including bacteria, fungi and algae, in the biofilms induce biomineralization. Biomineralization on titanium surfaces has been reported to further reduce the heat transfer properties as well as make the biofilm refractory to treatment regimens (Characklis & Robinson, 1983).

Fouling control strategies in condensers include a combination of mechanical, chemical and thermal treatments. In power plants, traveling water screens are used to prevent large particulate matter and larvae from entering the pump house. Subsequently, chlorination, sponge ball cleaning, and abrasive ball cleaning are used in the condenser pipes to remove microfoulants that can form biofilms on the condensers (Characklis, 1990). Effectiveness of chlorination decreases with time because microbes can mutate into resistant forms or may produce extracellular polymeric substances (EPS) as a protective sheath. Excessive secretion of EPS increases chlorine demand (Characklis & Dydek, 1976).

Although these cleaning methods are efficient in the case of macrofoulants, they do not guarantee complete removal of microfoulants such as bacteria and unicellular algae. It is also reported from various plants that mechanical treatment programs enhance subsequent biofouling potential (Characklis & Robinson, 1983). This study was initiated to establish whether mechanical cleaning procedures enhance biofilm formation and increase the adherence of biofilm, which can make the cleaning more difficult in cooling-water systems.

MATERIALS AND METHODS

Materials used in the study

Materials used in this study include commercially pure (CP) titanium (grade 2). Titanium (grade 2) is widely used as a heat exchanger material and also as medical implant material. The composition of this material is given in Table 1.

Specimen preparation

The titanium specimens used in the laboratory studies were obtained by cutting titanium sheets into medium-sized coupons (60 mm × 30 mm × 1 mm). Prior to subjecting the titanium surface to experiments, the coupons were pickled in an agitated acid bath (nitric acid 400 g/l and hydrofluoric acid 40.0 g/l; Williams, 1970) for 10 min to remove the oxide layer and also surface stains.

After 10 min the coupons were cleaned ultrasonically (5 min) using soap solution in an ultrasonicator. The coupons were then rinsed several times with distilled water again by ultrasonicator and then wiped dry using tissue paper.

Table 1: Composition of commercial purity (CP) titanium (grade 2)

Element	C	Fe	N_2	O	H_2	Ti
Weight%	<0.1	<0.3	<0.03	0.25	0.12	Bal

Bal, balance.

Exposure studies using sea water

Titanium frames (35 cm × 30cm × 3 mm) with sample holders were used for exposing the titanium sheets (120 mm × 90 mm × 1 mm) to the treated cooling water in a tank made of fiber-reinforced plastic with a capacity of approximately 1000 liters. The content of the tank was refreshed each day with cooling water from the forebay of Madras Atomic Power Station (containing 0.1–0.3 p.p.m. residual chlorine). A high pressure pump was used to recirculate the water to maintain a dynamic flow. The source of sea water for the cooling-water system was the coastal waters of Kalpakkam (Bay of Bengal).

The following two sets of experiments were conducted:

1. *Short-term exposure studies with a single cleaning cycle.* Newly pickled titanium specimens were exposed in sea water for 7 days. Retrieved specimens were swabbed with a big cotton plug under water, simulating sponge ball cleaning. Then they were re-exposed again for 7 days with a control set (newly pickled) to compare, by epifluorescence microscopy, the effect of the single cleaning cycle on regrowth of biofilms under short-term exposures.
2. *Long-term exposure studies with two cleaning cycles.* Newly pickled titanium specimens were exposed in sea water for 39 days. Retrieved specimens were swabbed (one time) as described above and then re-exposed for 39 days. Then these specimens were again retrieved and swabbed (second time) and re-exposed for 39 days with a control set (newly pickled) to compare the effect of two cleaning cycles on regrowth of biofilms under long-term exposures with two cleaning cycles by using total viable count (TVC) analysis, biofilm thickness measurements and epifluorescence microscopy.

Post exposure analysis

Total viable counts

Three coupons of each experimental condition (triplicate experiments) were used for TVC estimation (APHA, 1989). The coupons were washed gently to remove loosely adhering cells and the bacterial cells on the coupons were dispersed into 15 ml of sterile phosphate buffer (KH_2PO_4 0.0425 g/l, $MgCl_2$ 0.19 g/l, pH 7.2) using ultrasonification for 10 min. Plate counts of total bacteria were taken by plating in artificial sea water nutrient agar (sodium chloride 28.13 g/l, potassium chloride 0.77 g/l, calcium chloride dihydrate 1.60 g/l, magnesium chloride hexahydrate 4.80 g/l, sodium bicarbonate

0.11 g/l, magnesium sulfate heptahydrate 3.50 g/l, peptone 5 g/l, beef extract 3 g/l, pH 7.5 ± 0.2).

Microscopic observation

Two coupons of each experimental condition (duplicates) were used for direct microscopic observation. The coupons were washed gently with sterile water and air-dried in a sterile chamber. The coupon surface was flooded using acridine orange (0.1% (v/v) solution in distilled water). After 2 min, the excess stain was drained off and the coupons were washed in sterile water, dried and observed. Acridine orange, a fluorescent dye, differentially stains single-stranded RNA and double-stranded DNA, fluorescing orange when intercalated with the former and green while complexing with the latter (Mah & O'Toole, 2001) when observed under a Nikon Eclipse E600 epifluorescence microscope (excitation filter BP 490; barrier filter O 515).

Biofilm thickness measurements

The biofilm thickness on the titanium samples was measured using an optical microscope (Bakke & Olssen, 1986). One part of the sample was wiped clean using tissue paper. The thickness of the film was calculated by focusing on the wiped part and then on the biofilm. The corresponding readings on the microscope-focusing knob were noted. The difference in the reading gave the thickness in micrometers. Ten different regions were measured on each coupon and the mean thickness value calculated. This was repeated for three different titanium coupons.

Statistical analysis

Statistical analysis of the TVC and biofilm thickness data was carried out using MYSTAT INSTAT software. Data from the three replicates were analyzed for each experimental condition. An unpaired t-test was performed to assess significance in the difference in bacterial counts and biofilm thickness on control and mechanically cleaned surfaces.

Analysis of species richness on treated surfaces

Identification of bacterial species

Characterization and identification of the bacterial pure culture isolates up to genus level was carried out on the basis of morphological, physiological and biochemical tests as described in *Bergey's Manual of Determinative Bacteriology* (Holt *et al.*, 1994).

Genomic DNA isolation

Liquid culture of the isolates (24 h, log phase) was centrifuged at 10 000 revolutions/min for 10 min at room temperature and the supernatant removed to obtain the cell pellet. To the cell pellet, 500 µl of TE buffer and 300 µl of lysozyme were added and incubated at 37 °C for 30 min. Sodium dodecyl sulfate (60 µl) and proteinase K (3 µl) were mixed and incubated at 55 °C for 2 h. After incubation, an equal volume of Tris-saturated phenol (900 µl) was added and the mixture was centrifuged at 10 000 revolutions/min at 4 °C. The supernatant containing the DNA was washed with the organic solvents (phenol : chloroform : isoamylalcohol) and the aqueous phase was transferred to a fresh tube; 0.1 vol. of 3 M sodium acetate (pH 5.5) and 2.5 vol. of ice-cold ethanol were added to precipitate the DNA at –80 °C for 30 min. DNA was then washed with 70% (v/v) ethanol and the DNA pellet was dried and suspended in 100 µl of TE buffer (Wawer & Muyzer, 1995).

PCR amplification, cloning and sequencing of 16 S rRNA genes

The amplification of 16 S rRNA genes was performed with genomic DNA (100 ng) isolated primers (Sigma Genosys, India) 8F-5′-AGA GTT TGA TCC TGG CTC AG-3′, 1490R-5′, 1490R-5′-GAC TTA CCA GGG TAT CTA ATC C′-3′ (0.5 µM each), and 25 ml of Super Hot Master mix (Bioron Ltd, GmbH, Germany) in a Thermal Cycler (Thermo Hybaid, UK). The amplification cycle contained 30 cycles of denaturation at 92 °C for 30 s, primer annealing at 50 °C for 1 min and extension at 72 °C for 10 min. The polymerase chain reaction (PCR) products were purified with a Qiagen QIAquick PCR purification kit as described by the manufacturer (Qiagen Sciences, USA), and the purified PCR products were cloned using a Qiagen PCR cloning plus kit as described by the manufacturer (Qiagen Sciences, USA). The clones were selected and screened and the plasmids with the insert were sequenced using M13 sequencing primers with an Applied Biosystems automated sequencer.

The sequences obtained were analyzed using the Blast-n tool (Altschul *et al.*, 1997) and identified bacterial species were tabulated (see Table 4).

Surface profilometry studies

After removal of the biofilms, the surface roughness (R_a) of the exposed specimens was measured using a Sloan DEKTAK 3030 Profilometer. This was performed only for specimens under short-term exposure with a single cleaning cycle.

RESULTS

Short-term exposure studies with a single cleaning cycle

Fig. 1a and b are epifluorescence micrographs of control and mechanically treated surface showing more biofilm formation on the mechanically treated surface (Fig. 1b) under short-term exposures with a single cleaning cycle. Fig. 2a and b are epifluorescence micrographs of the specimens shown in Fig. 1, after removal of the biofilms. They show increasing adherence of the biofilm on mechanically treated specimens (Fig. 2b) as a response to mechanical cleaning.

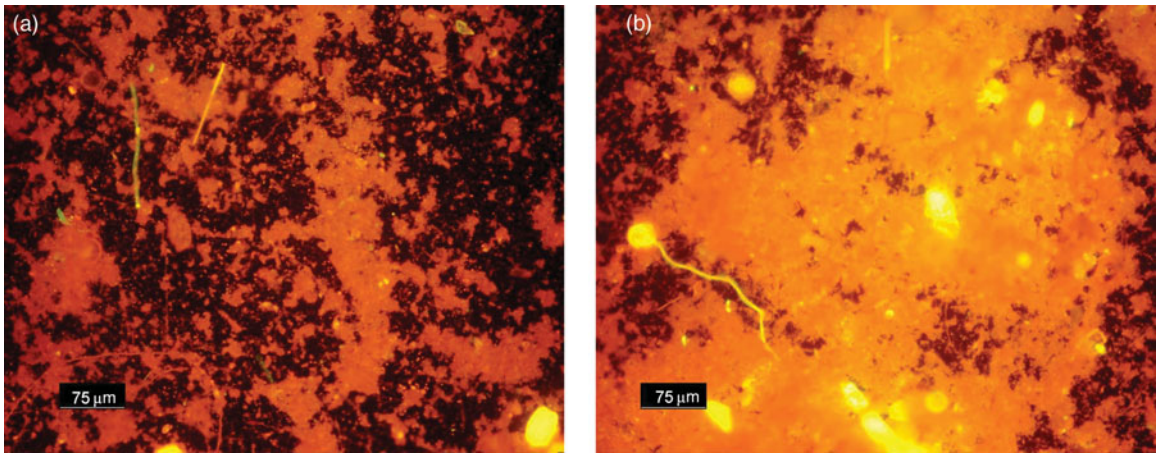

Fig. 1: Epifluorescence micrographs of control (a) and mechanically treated specimens (b) showing more biofilm formation on the mechanically cleaned surface after short-term exposure with a single cleaning cycle.

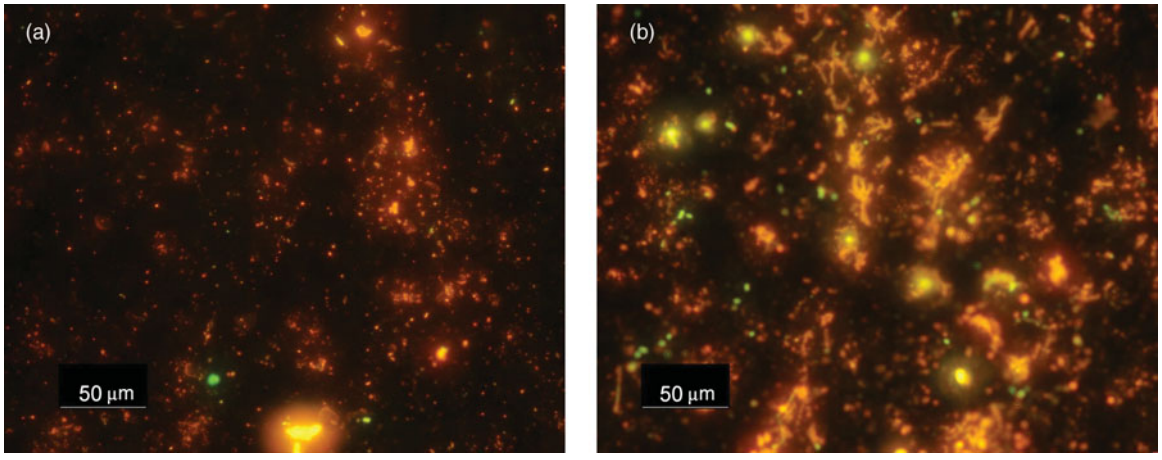

Fig. 2: Epifluorescence micrographs of the control (a) and mechanically treated specimens (b) shown in Fig. 1, after removal the biofilms, showing increasing adherence of the biofilm on the mechanically treated surface after short-term exposure with a single cleaning cycle.

Long-term exposure studies with two cleaning cycles

Fig. 3a and b are epifluorescence micrographs of control and mechanically treated surfaces showing more biofilm formation on the mechanically cleaned surface (Fig. 3b) under long-term exposure with two cleaning cycles. Fig. 4a and b are epifluorescence micrographs of the specimens shown in Fig. 3, after removal of the biofilms. They show increasing adherence of the biofilm on mechanically treated specimens (Fig. 4b) as a response to mechanical cleaning.

The biofilm thickness and bacterial TVC were compared between the control and mechanically treated specimens under long-term exposure and with two cleaning cycles (Figs. 5 and 6). Statistical analysis of triplicate specimens gave a two-tailed *P* value of 0.0014, and hence the differences between the bacterial counts and the biofilm thickness between the control (freshly immersed) and mechanically cleaned surfaces are considered to be highly significant.

Identification of bacterial species

Tables 2 and 3 show the biochemical and morphological characteristics, respectively, of the isolates from the biofilm on control and mechanically treated titanium surfaces. Isolates 1 to 4 were obtained from control titanium surfaces and isolates 5 to 12 were obtained from mechanically cleaned titanium surfaces. Table 4 shows the species name of these isolates.

Sequences submitted to GeneBank

The sequences of the organisms identified were submitted to the NCBI Gene Bank database under the accession numbers as shown in Table 3.

Surface profilometry results

Control and mechanically treated titanium specimens under short-term exposure studies with a single cleaning cycle were subjected to surface profilometry analysis after

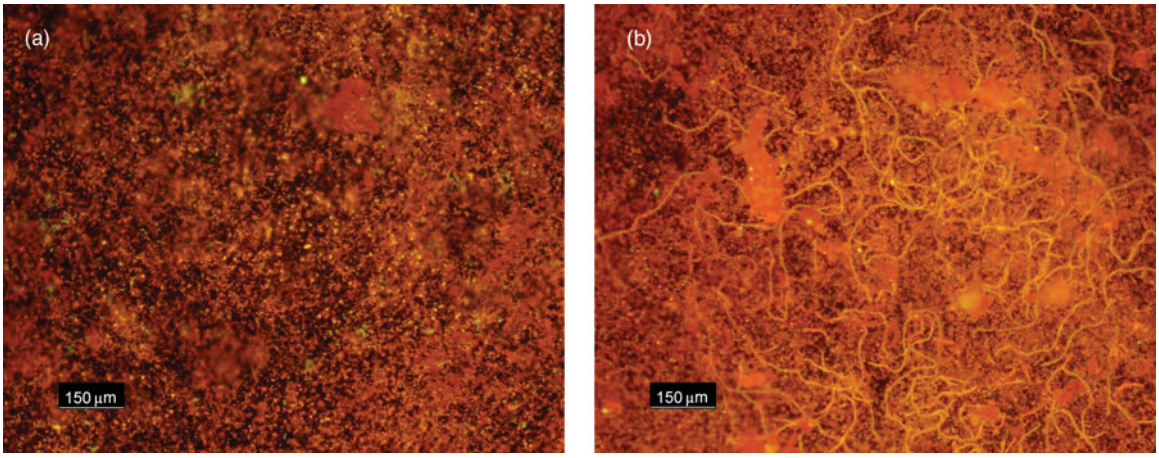

Fig. 3: Epifluorescence micrographs of control (a) and mechanically treated specimens (b) showing more biofilm formation on the mechanically treated surface after long-term exposure with two cleaning cycles.

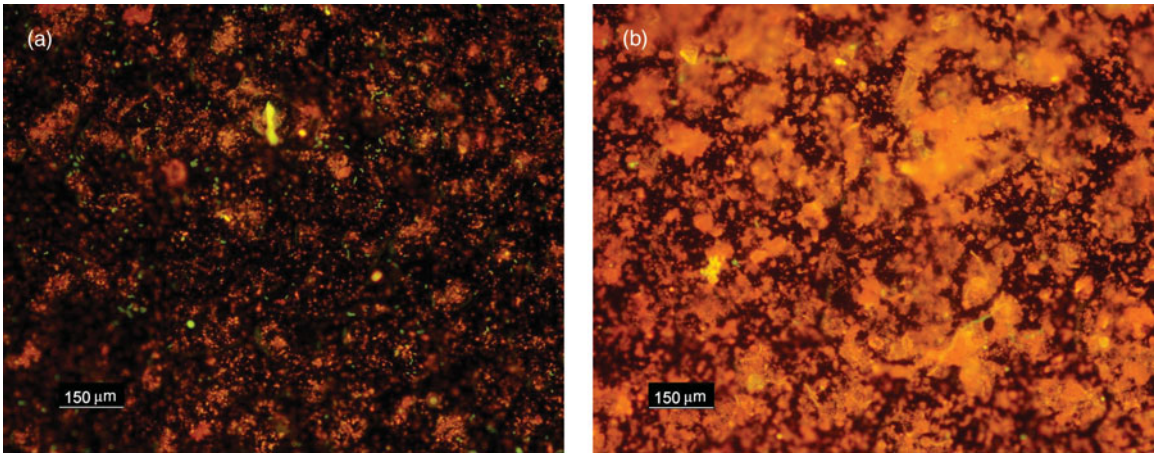

Fig. 4: Epifluorescence micrographs of control (a) and mechanically treated specimens (b) shown in Fig. 3 after removal of the biofilms, showing increasing adherence of the biofilm on the mechanically treated surface after long-term exposure with two cleaning cycles.

removal of the biofilms. The control specimens gave a R_a value of 3.70 µm and the mechanically treated specimens gave a R_a value of 4.57 µm, indicating the greater degree of roughness of the mechanically treated surface.

DISCUSSION

This study aimed to assess, by systematic short-term and long-term exposure experiments, whether mechanical cleaning procedures adopted in condensers could enhance biofilm formation and adherence of biofilms and thereby contribute to aggravation of biofouling problems. Epifluorescence micrographs of exposed specimens clearly showed the accelerated biofilm formation as well as increased adherence on mechanically cleaned surfaces. Bacterial TVC taken by culturing technique and biofilm thickness measurements made using microscopic techniques further supported this observation. Surface profilometric results provided further evidence of increased roughness on mechanically cleaned surfaces and thereby more adherence of biofilms.

The mechanically treated surface seemed to harbor different microbial species than the control surface. Also the number of species was doubled after mechanical cleaning.

According to Connell (1978), high diversity is a consequence of regularly changing conditions. Organisms are killed or damaged by disturbances that happen at various scales of frequency and intensity. Connell proposes that the highest diversity is maintained at intermediate scales of disturbance. The cleaning is performed with sponge balls that are slightly larger in diameter than the condenser tubes (Suzuki *et al.*, 2005). Once or twice a day, the balls are passed through the cooling water to clean and polish the inner surface of the tubes. Apparently, in this environment the cleaning regimen offers an intermediate scale of disturbance to the biofilm-forming microbes and hence, in agreement with Connell's (1978) hypothesis, can contribute to increase in species richness.

Table 2: Biochemical characteristics of the isolates

Isolate numbers	Oxidase	Catalase	Indole	Carbohydrate	Nitrate	Citrate	Starch	Lipid
1	−	+	−	+	+	−	+	−
2	−	+	−	+	+	+	+	−
3	−	+	−	+	+	+	+	−
4	+	+	−	+	+	+	+	−
5	−	+	−	+	+	+	+	−
6	−	+	−	+	+	+	+	−
7	−	+	−	+	+	+	+	−
8	−	+	−	+	+	+	+	−
9	−	+	−	+	+	+	+	−
10	+	+	−	+	+	+	+	−
11	+	+	−	+	+	+	+	−
12	+	+	−	+	+	+	+	−

+ signs indicate grows in the presence of the metabolite; – signs indicate no growth in the presence of the metabolite.

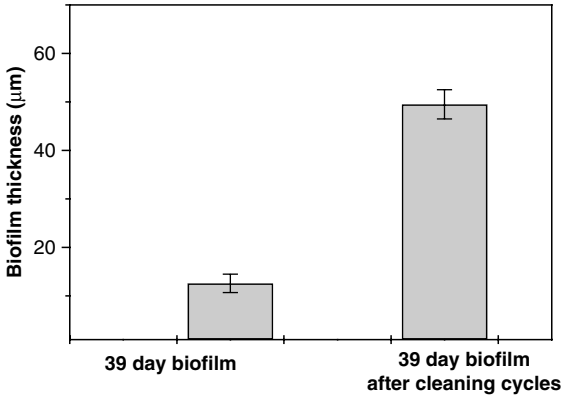

Fig. 5: Comparison of the biofilm thickness on control (39 day biofilm) and mechanically treated specimens (39 day biofilm after two cleaning cycles) after long-term exposures.

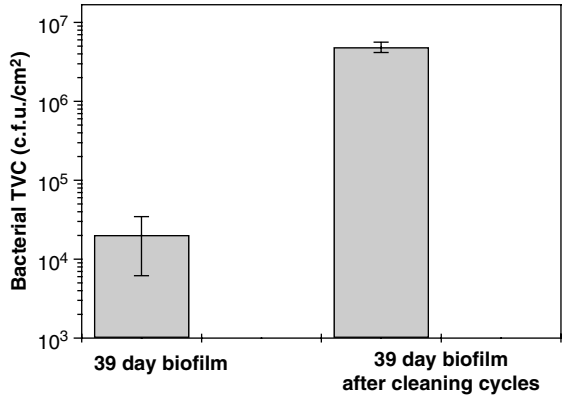

Fig. 6: Total viable count of bacteria on control (39 day biofilm) and mechanically treated specimens (39 day biofilm after two cleaning cycles) under long-term exposures.

Studies from various industrial situations have reported that mechanical treatment may not result in effective long-term control. In oil field water flood operations and in drinking water distribution systems, periodic pigging

Table 3: Morphological characters of isolates

Isolate numbers	Morphological characters
1	Gram-positive big rods, motile, spore-forming, white opaque colonies on agar
2	Gram-positive cocci, non-motile, opaque colonies
3	Gram-positive cocci, non-motile, opaque colonies
4	Gram-negative small rods, motile, translucent glistening colonies on agar
5	Gram-positive cocci, non-motile, opaque colonies
6	Gram-positive cocci, non-motile, opaque colonies
7	Gram-positive cocci, non-motile, opaque colonies
8	Gram-positive cocci, non-motile, opaque colonies
9	Gram-negative, cocco-bacilli, pairs or chain, glistening colonies, non-motile
10	Gram-negative, small rods, glistening colonies, motile
11	Gram-negative, rods, glistening clonies
12	Gram-negative, rods, glistening colonies

treatments are used to reduce biofouling. However, Galbraith & Lofgren (1987) have recommended biocide treatment immediately after pigging, as an effective method for reducing biofilm recovery.

Costerton (1984) argued that mechanical treatment may have a subtle, yet critical, effect on further biofilm accumulation. The biofilm organisms remaining after mechanical treatment may lead to accelerated biofilm accumulation, and repeated cleaning leads to anti-fouling-resistant biofilms.

At a Canadian power plant, fouling reduction on admiralty brass tubes was studied by using soft sponge balls (Characklis & Robinson, 1983). Results showed that three passes with the sponge balls was initially effective in reducing the effects of fouling. A second treatment was effective after 12 ball passes. However, subsequent treatments with the soft balls resulted in the formation of a refractory deposit.

Berger & Berger (1986) also indicated that the passage of sponge rubber balls through titanium, stainless steel (AL-6X) and aluminum (3004) tubing removes loose surface biofouling material but was inadequate for

Table 4: Species level identification of biofilm bacteria and database number

Biofilm on	Sample no.	Name of bacterium	GeneBank accession no.
Titanium – newly exposed	Sample 1	*Bacillus* sp.	EF517948
	Sample 2	*Staphylococcus* sp. IGCAR 1	EF517949
	Sample 3	*Staphylococcus* sp. IGCAR 2	EF517950
	Sample 4	*Pseudomonas* sp.	EF517951
Titanium – mechanically cleaned and re-exposed	Sample 5	*Staphylococcus* sp. IGCAR 5	EF517952
	Sample 6	*Staphylococcus* sp. IGCAR 6	EF517953
	Sample 7	*Staphylococcus* sp. IGCAR 7	EF517954
	Sample 8	*Staphylococcus* sp. IGCAR 8	EF517955
	Sample 9	*Acinetobacter johnsonii*	EF517956
	Sample 10	*Marinobacter hydrocarbonoclasticus*	EF523424
	Sample 11	*Emterobacter* sp. IGCAR 11	EF523425
	Sample 12	*Enterobacter* sp. IGCAR 12	EF523426

long-term control. However, for the first time, by means of systematic exposure studies of titanium specimens in treated cooling water from a coastal nuclear power plant, we have clearly demonstrated that mechanical treatment increases biofilm formation and adherence of biofilm thereby increasing the potential of biofouling in the long term, and furthermore increases the biodiversity in the biofilms.

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