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Exposure to chlorine affects the extracellular polymeric substance production and cell surface hydrophobicity in biofilm bacteria

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Abstract

Chlorination is a common antifouling method adopted by industrial units to minimize the fouling growth on cooling systems. In the present study, the effect of sodium hypochlorite on extracellular polymeric substance (EPS) production, hydrophobicity, cell adhesion and viability of marine bacteria involved in biofilm formation were assessed in laboratory condition. Two bacterial strains, tentatively identified as *Alteromonas* sp. and *Pseudomonas* sp. isolated from the surface of seaweeds were used as test organisms for the present study. The bacterial cultures were treated with sodium hypochlorite at 25% of the minimum inhibitory concentration. Results showed considerable variation in the production of EPS, viable counts, hydrophobicity and adhesion ability of bacteria treated with sodium hypochlorite. In general, the present study indicated that chlorination affects some important characteristics involved in the biofilm formation and thereby reduces the adhesion rate on surfaces.

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INTRODUCTION

Biofouling is an undesirable accumulation of organic molecules, microorganisms, plants and animals on the surfaces exposed in an aquatic environment. The fouling process takes place on both living and non-living surfaces in the marine environment and is initiated instantly once a substratum is immersed in the sea waters (Wahl et al. 1994). Formation of biofilms consisting of inorganic and organic materials, and microbes is the initial stage in the biofouling process. Biofouling affects shipping, offshore oil mining, coastal power plants, mariculture, naval applications and marine constructions (Nair et al. 1997). It is a common problem to all coastal power plant cooling systems. In cooling water circuits, the presence of biofilms can restrict the flow in pipelines and heat exchangers (Bott 1999), and reduce the heat transfer (Murthy et al. 2004). The control of biofilm and macrofouling in the industrial systems is an important component of a successful water treatment program (Ludensky 2003). Chlorination is an effective method of preventing the microbiological growth in industrial cooling water systems. Though very effective to control the biofilm formation and biofouling, chlorination has been associated with some environmentally deleterious effects. The deleterious effects of chlorination on bacterial cells include formation of chlorinated derivatives of purine and pyrimidine nucleotides, oxidative decarboxylation of amino acids, inhibition of metabolic enzymes and respiratory activity and leakage of macromolecules from cells.

Low-level continuous or intermittent chlorination is usually practiced in power plants. Generally, 0.5 to 2 mg l^{-1} of chlorine is dosed at the intake area of the cooling systems and the resultant total residual oxidant level ranges from 0.1 to 0.5 mg l^{-1} (Jenner et

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al. 1998). The most important mechanism for the action of antifoulants is through inactivation of essential enzymes (Chapman 2003, Cross et al. 2003), organic constituents in cells (Hassett et al. 1999, Chapman 2003), destabilization of cell membranes and cell lysis (Chapman 2003, Walsh et al. 2003) and uncoupling the membrane potential (Zhao et al. 1998). Chlorine belongs to the oxidizing group of biocides, which irreversibly oxidize protein groups resulting in the loss of normal enzyme activity and cause cell death. It was known that chlorine compounds destroy microbes by attacking their cell walls and interfering with essential protein formation (Anbananthan 2008). The toxicity of chlorine to the biofilm and planktonic diatoms cells was reported by Nanchariah et al. (2008). Membrane damage and microbial inactivation by chlorine in the presence and absence of a chlorine denaturing the substrate was also reported by Virto et al. (2005). However, there is no information on the effect of chlorine on other factors, such as cell hydrophobicity, cell surface charge and EPS production that are involved in bacterial adhesion to surfaces. The main objective of the present study was to assess the influence of chlorine on extracellular polymeric substance production, hydrophobicity, viability and adhesion ability of bacteria involved in biofilm formation. Proper understanding of the effect of antifoulants on biofilm forming characteristics may provide a better insight into the mechanism of inhibition of bacterial adhesion to surfaces, which will help to design appropriate antifouling strategies.

MATERIALS AND METHODS

Isolation and identification of bacteria

The bacterial strains used in the present study were isolated from the surfaces of the seaweed *Sargassum wightii* collected from the intertidal region of Kanyakumari coastal waters (8°5'81"N and 77°33'72"E), India. The *Sargassum* samples were collected from the rocky shores and kept in a polythene bag containing filter-sterilized seawater. The samples were kept in an icebox and transported to the laboratory. In the laboratory, *Sargassum* samples were rinsed with sterile seawater to remove the debris and weakly adhered microorganisms. *Sargassum* leaves were cut into small pieces and spread on Zobell Marine (HIMEDIA, India) agar plates. The plates were incubated at room temperature for 48 h for the development of bacterial colonies. The developed colonies were purified by repeatedly streaking on Zobell Marine agar plates. Biochemical and physiological characteristics were used to identify the isolates up to the genus level. The cultures were maintained on Zobell Marine agar slants at 4°C. For inoculum preparation, a loop full of culture from the slant was sub-cultured using Zobell Marine broth for 3 days at room temperature (28 ±2°C). The adhesive nature of bacterial strains maintained on agar slant was confirmed by inoculating them in the sterilized seawater in a 50 ml beaker. Clean sterile glass slides were submerged in the beaker as test surface. Bacteria that formed a slimy layer on the glass slide were selected as test organisms for the present study. Two bacterial cultures with superior film forming ability were tentatively identified as *Alteromonas* sp. and *Pseudomonas* sp.

Minimum Inhibitory Concentration (MIC) of sodium hypochlorite

The commercially available sodium hypochlorite solution (NICE chemicals, India, S10683) was used in the present study. The available chlorine in the solution was about 5%. The MIC of sodium hypochlorite was determined separately for each culture. A series of test tubes was filled with 5 ml bacterial culture. Various concentrations of sodium hypochlorite were added into each test tube prepared as above described. The tubes were incubated for 72 h in the dark at room temperature. After the incubation period, the absorbance of the culture in each tube was measured at 540 nm using a spectrophotometer (Systronics, 104). The residual chlorine in the water was estimated using sodium thiosulphate titration. In brief, 100 ml of the water sample was taken in a conical flask and 5 ml acetic acid was added to obtain the pH between 3 and 4. Approximately 1 g of potassium iodide (KI) crystals were added to the sample and mixed thoroughly with a stirring rod for about 15 min. The water sample was titrated against 0.0025 N sodium thiosulphate after adding a few drops of the starch indicator. The disappearance of blue colour was considered as the end point.

Preparation of sodium hypochlorite treated bacterial cell suspension

The bacterial cultures (100 ml in Zobell broth medium) were kept in 250 ml conical flasks along with 25% of the MIC of sodium hypochlorite.

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Cultures kept as above described without the sodium hypochlorite treatment were used as controls. All the flasks were incubated for 72 h at room temperature. After incubation, 10 ml samples were isolated to estimate a viable count and EPS production. The remaining culture broth was centrifuged at 5000 rpm for 15 min. The cell pellet obtained after centrifugation was washed with Phosphate Buffer Saline (10 mM PBS) and re-suspended in the same buffer to obtain OD_{540}=0.2 (Jain et al. 2007). This cell suspension was used for further study.

Viable cell counts

An aliquot of the 10 ml culture broth, as described above (both Sodium hypochlorite treated culture and control), was serially diluted using sterile seawater. The appropriate dilutions were spread on Zobell Marine agar plates. The plates were incubated at room temperature for 24 h, and colonies were counted manually. The experiment was replicated (n=3) and the mean values were calculated.

Estimation of the Extracellular Polymeric Substance (EPS)

The amount of EPS produced by the cultures was determined by estimating the total carbohydrate and protein. Five milliliters of the culture broth obtained as described above was centrifuged at 10,000 g for 10 min at 4°C. The supernatant was collected and filtered through a membrane filter (0.47 μm, Millipore). The filtrate was collected and the equal volume of ethanol was added for the isolation of EPS. The precipitated EPS fraction was diluted to a defined volume with distilled water and stored at 4°C. Carbohydrate was estimated by the modified Phenol Sulfuric acid method using glucose as standard (Dubois 1956). In brief, 500 μl of the sample was placed in a test tube, and 1 ml of phenol (2.5%) followed by 2.5 ml of concentrated sulfuric acid were added. The absorbance was read at 490 nm using a spectrophotometer. The total protein content of EPS, produced by the cultures, was estimated by the modified method of Lowry et al. (1951), using Bovine Serum Albumin as a standard.

Measurement of hydrophobicity

The hydrophobicity of the bacterial cell suspension was evaluated using BATHS assay following the procedure described by Rosenberg et al. (1980). In general, 100 μl of n-hexane was added to 3 ml of bacterial cell suspension (OD_{540} = 0.2) prepared as described above. The suspension was vortexed for 1 min. to ensure mixing and then left to stand for 15 min. to allow separation of the two phases. The absorbance of the aqueous phase was taken at 400 nm using a spectrophotometer. The percentage of the cells bound to n-hexane was calculated using the formula:

$$Bacterial\ cell\ adherence\ to\ n-hexane\ (\%) = \left(1 - \frac{A}{A0}\right) \times 100$$

where A0 was OD (400 nm) of the aqueous cell suspension before adding n-hexane and A was OD (400 nm) after adding n-hexane.

Bacterial adhesion assay

Three milliliters of bacterial suspension (treated with sodium hypochlorite) was added to 500 ml beaker having 300 ml of sterile seawater. Three milliliters of Zobell Marine broth was also added to the medium in order to provide nutrition. Five glass slides (microscopic slides, 7.5 × 2.5 cm) were placed inside the beakers in a slanting position. The beakers were incubated at room temperature in a sterile chamber. The slides were removed after 1,2,3,4 and 5 h of incubation (1 slide per h). Slides were then air-dried, heat fixed and stained with crystal violet. The number of bacteria adhered to the slides were counted under a binocular microscope. The experiment was repeated (n = 6) and the mean ±standard deviation values were calculated. The untreated bacterial cultures were used in the experiment as a control.

Characterization of the extracellular polymeric substance by thin layer chromatography

The EPS was characterized by thin layer chromatography. The slurry of the adsorbent was prepared using silica gel and distilled water in the ratio of 1:2 (W/V). The solvent used in this experiment were n-butanol, acetic acid, and distilled water in the ratio of 2:1:1. The distance moved by the solvent and solutions were then measured and Rf values were calculated.

Statistical Analysis

One-way ANOVA (analysis of variance) was used

to evaluate the effect of sodium hypochlorite on the adhesion of bacterial cells.

RESULTS

MIC of sodium hypochlorite

The MIC of sodium hypochlorite for *Alteromonas* sp. was 125 mg l^{-1} and in the case of *Pseudomonas* sp., it was 105 mg l^{-1}. The free residual chlorine at the MIC was 1.25 ppm immediately after the addition of sodium hypochlorite and after 72 h, it was 1.13 ppm. At 25% concentration of the MIC, residual chlorine values were found to be 0.22 ppm and 0.162 ppm, respectively, immediately after the addition of sodium hypochlorite and after 72 h of incubation.

Effect of sodium hypochlorite on bacterial cell viability

It was found that at 25% of the MIC of sodium hypochlorite, the growth of both cultures was inhibited. This was evidenced by the viable counts of both treated and control cultures. The viable counts of *Alteromonas* sp. after treated with sodium hypochlorite were 79 × 10^6 CFU ml^{-1} and 101 × 10^6 CFU ml^{-1} in the control. *Pseudomonas* sp. had 113 × 10^6 CFU ml^{-1} after treated with sodium hypochlorite while in the control, the viable counts were 260 × 10^6 CFU ml^{-1}.

Effect of sodium hypochlorite on extracellular polymeric substance production (EPS)

The carbohydrate concentration of EPS synthesized by *Alteromonas* sp. culture treated with sodium hypochlorite was 2.645 mg ml^{-1} and 4.408 mg ml^{-1} (Fig. 1) in the control. The EPS synthesized by *Pseudomonas* sp. had a carbohydrate concentration of 6.143 mg ml^{-1} in the control culture and 4.99 mg ml^{-1} when treated with sodium hypochlorite. In general, *Pseudomonas* sp. had higher concentration of carbohydrate than *Alteromonas* sp.

The protein concentration of the EPS produced by bacterial cultures in the presence and absence of sodium hypochlorite is presented in Fig. 2. The protein concentration of EPS synthesized by *Alteromonas* sp. treated with sodium hypochlorite was 3.75 mg ml^{-1} and in the control it was 5 mg ml^{-1}. The EPS synthesized by *Pseudomonas* sp. had a protein concentration of 4.85 mg ml^{-1} in the control culture and 2.9 mg ml^{-1} when treated with sodium

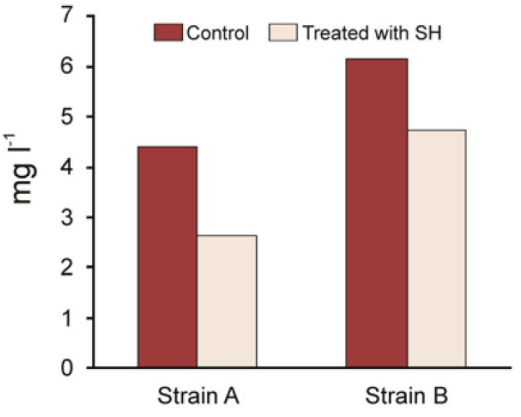


Fig. 1. Changes in the carbohydrate concentration of the Extracellular Polymeric Substance (EPS) produced by two bacterial strains after treated with sodium hypochlorite (Control = without sodium hypochlorite treatment; SH = sodium hypochlorite; Strain A = *Alteromonas* sp. Strain B = *Pseudomonas* sp.)

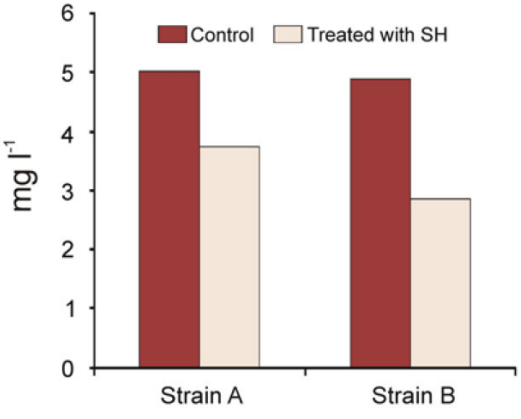


Fig. 2. Changes in the protein concentration of the Extracellular Polymeric Substance (EPS) produced by two bacterial strains after treated with sodium hypochlorite (Control = without sodium hypochlorite treatment, SH = sodium hypochlorite; Strain A = *Alteromonas* sp. Strain B = *Pseudomonas* sp.)

hypochlorite. In general, *Alteromonas* sp. had higher concentration of protein than *Pseudomonas* sp.

Characterization of the extracellular polymeric substance by thin layer chromatography

Analysis of the thin layer chromatogram revealed that there was a considerable variation in the carbohydrate composition of EPS secreted by the bacterial cultures treated with sodium hypochlorite. Three distinct carbohydrate spots were observed in

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Alteromonas sp. with the Rf values of 0.238 cm, 0.371 cm and 0.929 cm in the absence of sodium hypochlorite. When treated with sodium hypochlorite only one carbohydrate spot was observed with the Rf value of 0.734 cm (Fig. 3). Simultaneously, three distinct carbohydrate spots were observed in *Pseudomonas* sp. (0.754 cm, 0.211 cm and 0.491 cm) in the control culture and two spots with the Rf value of 0.758 cm and 0.338 cm in the EPS synthesized by the culture treated with sodium hypochlorite (Fig. 3).



Fig. 3. Thin layer chromatogram showing the changes in the composition of the extracellular polymeric substance produced by bacterial strains treated with sodium hypochlorite (A_1 = *Alteromonas* sp. control, A_2 = *Alteromonas* sp. sodium hypochlorite treated; B_1 = *Pseudomonas* sp. control, B_2 = *Pseudomonas* sp. sodium hypochlorite treated)

Hydrophobicity

The two bacterial cultures showed a considerable decrease in hydrophobicity after the sodium hypochlorite treatment (Fig. 4). The hydrophobicity of *Alteromonas* sp. decreased from 7.9 to 0.5 when treated with sodium hypochlorite. Similarly, the hydrophobicity of *Pseudomonas* sp. decreased from 36.1 to 20.5 after treated with sodium hypochlorite. The results also revealed that *Pseudomonas* sp. was more hydrophobic than *Alteromonas* sp.

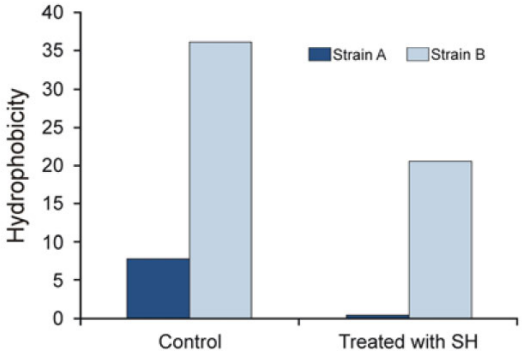


Fig 4. Hydrophobicity of bacterial strains treated with sodium hypochlorite (Control = without sodium hypochlorite treatment, SH= sodium hypochlorite; Strain A = *Alteromonas* sp. Strain B = *Pseudomonas* sp.)

Bacterial adhesion assay

The adhesion of bacterial cells to glass coupons was assessed for the duration of 5 hours. The cell attachment showed significant decrease after treated with sodium hypochlorite (Fig. 5 and 6). In the adhesion assay, the number of *Alteromonas* sp. cells attached after one hour of glass surface immersion was 4,732 cells cm^{-2} in the control culture and 1,603 cells cm^{-2} in the sodium hypochlorite treated culture. At the end of the glass surface exposure period (5 h), the number of bacteria attached was 13,265 cells cm^{-2} in the control and 6,881 cells cm^{-2} were observed in the cultures treated with sodium hypochlorite. Similarly, the biofilm adhesion assay with *Pseudomonas* sp. presented considerable variations between the control and sodium hypochlorite treated cultures. After 1 h of glass surface exposure, 2,093 cells cm^{-2} were observed in the control culture and 1,172 cells cm^{-2} in the sodium hypochlorite treated cultures. After 5 h of exposure, 9,737 cells cm^{-2} were observed in the control and 2,604 cells cm^{-2} were on coupons immersed in sodium hypochlorite treated cultures. Results from one-way ANOVA for cell adhesion showed that there was a significant variation in the adhesion of *Alteromonas* sp. treated with sodium hypochlorite and the control ($F = 6.92$, $d.f = 1.8$; $P<0.05$). However, *Pseudomonas* sp. did not show any significant variation ($F = 3.31$; $d.f = 1.8$; $P>0.05$).

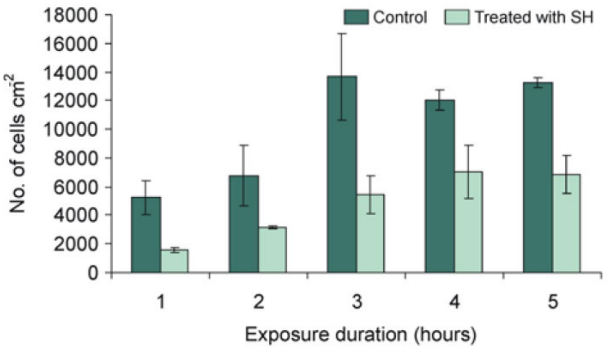


Fig. 5. Adhesion assay with the use of *Alteromonas* sp. (Control = without sodium hypochlorite treatment, SH = Sodium hypochlorite treated)

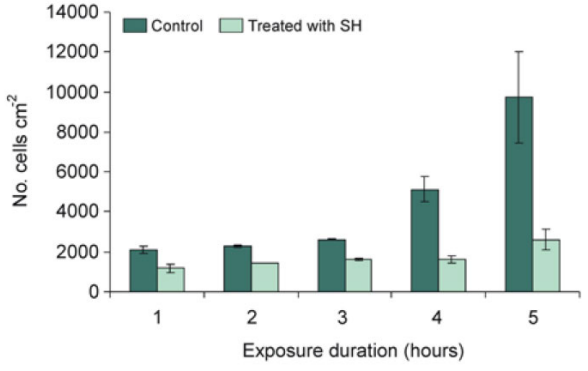


Fig. 6. Adhesion assay with the use of *Pseudomonas* sp. (Control = without sodium hypochlorite treatment, SH = Sodium hypochlorite treated)

DISCUSSION

Chlorine is one of the most common oxidizing biocides used for controlling micro- and macro-fouling in industrial cooling water systems and water distribution systems. Sodium hypochlorite is a non-specific broad-spectrum oxidizing biocide and affects metabolic and physiological processes of microorganisms by causing damage to the cell membrane, proteins and nucleic acids (Phe et al. 2004, Virto et al. 2005, Nancharaiah et al. 2008). It is highly effective against Gram- positive and Gram-negative bacteria. The broad-spectra also include organisms such as algae, protozoan and other higher invertebrates. Most bacteria have enzymes located just inside the cellular membrane. These locations are under oxidative attack from the chlorine compounds (Anbananthan 2008).

In the present study, for the two bacterial strains, the MIC varied between 105 mg l^{-1} and 125 mg l^{-1}. At 25% of the MIC, the growth inhibition for each culture was observed. This was evidenced by the viable counts of both control and sodium hypochlorite treated cultures. EPS production by the bacterial strains *Alteromonas* sp. and *Pseudomonas* sp. decreased considerably when treated with sodium hypochlorite. Microorganisms do not attach to surfaces directly. This is due to the fact that both bacteria and surfaces generally have a negative charge. In order to avoid the repulsive barrier, microorganisms produce an extracellular polymeric substance. The EPS helps the microbes to attach to surfaces and form a biofilm. Extracellular polymeric substances bind microbial cells within the substratum and permit negatively charged bacteria to adhere to both negatively and positively charged surfaces. EPS are generally rich in carbohydrates (Decho 1990, Welch et al. 1999). Production of EPS by bacteria is generally affected by various physical and chemical parameters of the surface and the aquatic medium. Hence, the low carbohydrate concentration values observed in the bacterial cultures treated with sodium hypochlorite revealed that chlorination might affect the EPS producing ability of the microorganisms.

Carbohydrates, protein, lipids and nucleic acids are generally the most abundant biochemical components in the biofilms. The relative abundance of these compounds can vary with environmental and surface properties (D'Souza & Bhosle 2003). During biofilm formation, the microbes are known to produce more carbohydrates than proteins (Vandevivere & Kirchman 1993). The results indicated a decline in protein and carbohydrate production by the bacterial cultures treated with sodium hypochlorite.

The hydrophobicity of the bacterial cultures also decreased considerably when treated with sodium hypochlorite. According to the physico-chemical theory for bacterial adhesion (Bos et al. 1999, Busscher & Van der Mei 2000), a decrease in bacterial cell surface hydrophobicity should result in a higher adhesion rate to hydrophilic surfaces. Among the factors affecting the biofilm formation, cell surface hydrophobicity is an important property determining the mechanism of bacterial attachment (Van Loosdrecht et al. 1987). As evidenced by experiments, hydrophobicity is influenced by such factors as the presence of cell appendages containing proteins (Korber et al. 1989, Zita & Hermansson 1997) and EPS (Caccavo et al. 1997). From the

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observed results, it was clear that the decrease in the EPS production might affect the cell surface hydrophobicity.

The pH of the aquatic environment is one of the important key factors determining the cell surface hydrophobicity of bacteria. Bunt et al. (1993) showed that the pH and the ionic strength of the suspending buffer influence the cell surface hydrophobicity. The hydrophobicity was found to be significantly lower at higher pH (7.4) and low ionic strength (0.5 M), while it was greater at pH 2.2 and ionic strength 1 M. The decrease in hydrophobicity of treated bacterial cultures observed in the present study may also be due to the higher pH value of sodium hypochlorite.

Adhesion of bacteria to glass surface is also reduced considerably when treated with sodium hypochlorite. According to Leroy et al. (2007), chlorine is the most effective agent in bacterial adhesion prevention with LC_{50} at about 12 mg l^{-1} and a 99% reduction rate reached at 78 mg l^{-1}. In the present study, adhesion was considerably reduced at the concentration of about 25 – 30 mg l^{-1}. Generally, the adhesion ability depends on EPS production, surface charge, hydrophobicity and the physico-chemical parameters of the environment. The EPS production and hydrophobicity changed due to the addition of sodium hypochlorite to bacterial cultures.

The present study showed that the total bacterial population could be eliminated with chlorination exposure of as high as 125 mg l^{-1}. However, coastal power plants under normal operating conditions generally adopted "low-dose chlorination" for fouling control (Jenner et al. 1988). Generally, low-level chlorination may inhibit bacterial multiplication and a higher concentration will completely kill the bacteria (Murthy et al. 2004). The data showed that MIC of chlorine varies with the bacterial species. Hence, it is necessary to identify the bacterial population present in the biofilms before choosing a biocide.

Development of control technologies for the mitigation of biofouling in industrial systems is an ongoing process (Jenner et al. 1988, Rajagopal et al. 2003). Chlorination for biofouling control in a cooling water system was initiated in 1924 (Satpathy & Nithila 2008). Even after eight and half decades, chlorine is the most widely used biocide for biofouling control. It appears that with the current knowledge and economic viability, it will still be considered a strong biocide to control biofouling. Thus, proper understanding of the mechanism of the effect exerted by chlorine compounds on various fouling species is necessary to avoid under and over chlorination (Jenner et al. 1997).

In conclusion, the results indicate that in addition to EPS production, sodium hypochlorite affects the marine bacterial cell surface hydrophobicity and thereby, reduces the adhesion to glass surface. More studies are required to analyze the other molecular damage to marine bacteria induced by chlorination. Similar studies may provide useful information regarding the optimization of chlorine doses to minimize the attack on non-target organisms.

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