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Laboratory studies on adhesion of microalgae to hard substrates

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Abstract

Adhesion of *Chlorella vulgaris* (chlorophyceae), *Nitzschia amphibia* (bacillariophceae) and *Chroococcus minutus* (cyanobacteria) to hydrophobic (perspex, titanium and stainless steel 316-L), hydrophilic (glass) and toxic (copper, aluminium brass and admiralty brass) substrata were studied in the laboratory. The influence of surface wettability, surface roughness, pH of the medium, culture age, culture density, cell viability and presence of organic and bacterial films on the adhesion of *Nitzschia amphibia* was also studied using titanium, stainless steel and glass surfaces. All three organisms attached more on titanium and stainless steel and less on copper and its alloys. The attachment varied significantly with respect to exposure time and different materials. The attachment was higher on rough surfaces when compared to smooth surfaces. Attachment was higher on pH 7 and above. The presence of organic film increased the attachment significantly when compared to control. The number of attached cells was found to be directly proportional to the culture density. Attachment by log phase cells was significantly higher when compared to stationary phase cells. Live cells attached more when compared to heat killed and formalin killed cells. Bacterial films of *Pseudomonas putida* increased the algal attachment significantly.

Introduction

Microorganisms attach on all submerged surfaces in the aquatic environment which leads to formation of 'biofilm' (Characklis & Cooksey, 1983). Biofilms cause damage to all water distribution systems including the cooling system of chemical, fertilizer and power plants. In cooling systems, biofilms reduce the thermal efficiency and increase the pressure drop in heat exchangers (Bott, 1990). Microalgae are one of the major components in the biofilms and problems due to algae in many industrial cooling systems have been reported (Ludyansky, 1991; Callow, 1993).

In cooling water systems, materials such as titanium, stainless steel 316-L, aluminum brass, admiralty brass are used as condenser tube materials. These materials are highly affected by the biofilm formation which leads to their corrosion. The cooling systems of Fast Breeder Test Reactor (FBTR) at Kalpakkam, India faced many operational problems due to fouling and corrosion in the system. Preliminary studies showed that fouling and corrosion of construction material of the FBTR were mainly due to microoganisms present in the cooling water (Rao et al., 1993). Since microalgae are one of the major components in the biofilm it was felt necessary to study their attachment to surfaces and also influencing factors on their attachment.

Chlorella vulgaris (chlorophyceae), *Nitzschia amphibia* (bacillariophyceae) and *Chroococcus minutus* (cyanobacteria) were dominant forms during the early development of biofilms. In the present study, the attachment of these three organisms on perspex, glass, titanium, stainless steel, copper, aluminum brass and admiralty brass were studied. In addition, the influence of surface roughness, pH of the medium, culture density, age, cell viability and the presence of organic and bacterial films on the attachment of *Nitzschia am-*

phibia was studied to find out factors which influence the attachment of this diatom on the surfaces.

Materials and methods

Preparation of coupons

All the materials of size 2 × 2 × 0.5 cm (coupons) were used in the experiment. The metal coupons were polished up to 400 grit, degreased with acetone and air dried.

Isolation, purification and maintenance of algae

Chlorella vulgaris, *Nitzschia amphibia* and *Chroococcus minutus* were isolated from the biofilms developed on perspex panels in the freshwater cooling system at Kalpakkam, India. The organisms were axenised by antibiotic treatment using Benzyl Penicillin, Streptomycin and Chloromphenicol following the method of Droop (1967). The cultures were maintained in modified Chu 10 medium (Gerloff et al., 1950) at 24 ± 1 °C in a thermostatically controlled room illuminated with white fluorescent lamps (Philips 40 W) at an irradiance of 60 μE m^{-2} s^{-1} in 12:12 hours L:D regime.

Adhesion assay

The organisms were grown in 500 ml conical flasks using Chu 10 medium (Gerloff et al., 1950). During log phase, the algae suspended in flasks were discarded and the remaining cells firmly attached on the flask walls were scraped with a soft brush (Sharma et al., 1990). The cells were suspended in half strength Chu 10 medium and the culture density was adjusted to 1–1.5 × 10^5 cells ml^{-1} using the same medium. The coupons were placed in replicate large Petri dishes (15 cm diameter) and the medium (75 ml) was poured into the dishes. The dishes were then placed on an orbital shaker at 40 rpm and maintained at 12:12 L:D regime. Replicate coupons were retrieved after 2, 6, 12, 24 and 48 h and the attached cells in ten random fields were counted under an epiflourescence microscope after staining with 0.1% acridine orange (Holmes, 1986; Fukami et al., 1989).

Effect of surface wettability and roughness on adhesion

The wettability of the perspex, glass, titanium, stainless steel, aluminum brass, admiralty brass and copper coupons was studied by the drop spread method (Burchard et al., 1990). Replicate coupons were used for the experiment. The effect of surface roughness on adhesion was studied by using titanium and stainless steel coupons. Surfaces of different roughness were obtained by polishing the coupons using 120, 220, 320, 400, 600 and 800 grit of polishing papers. Log phase culture of *N. amphibia* was used and the experiment was conducted as mentioned earlier. The attached cells were counted after 24 h.

Effect of pH and organic film on adhesion

The half strength Chu 10 medium was adjusted to different pH's such as 6, 7, 8 and 9 using IN HCl or 1N NaOH. Log phase culture of *N. amphibia* was harvested and suspended in the media with different pH's. Replicate titanium (hydrophobic) and glass (hydrophilic) coupons were used for this experiment and the attachment was compared after 24 h. A suspension of gum arabic was used to test whether an organic film could influence the adhesion of algae to surfaces. Titanium and glass coupons were placed in the gum arabic solution (30 g 100 ml^{-1} distilled water) for 30 min and then dried at 30 °C according to the method of Kirchman et al. (1982). The adhesion of *N. amphibia* to organic film coated and control coupons (without film) was studied for 24 h.

Effect of culture density, age and viability on adhesion

Different culture densities of *N. amphibia* such as 2 × 10^2, 2 × 10^3, 2 × 10^4, 2.3 × 10^5 ml^{-1} were prepared in half strength Chu 10 medium. The effect of log and stationary phase cultures of *N. amphibia* on the adhesion was studied using titanium and glass coupons. The attachment of live and dead cells (heat killed and formalin killed) of *N. amphibia* on titanium and glass coupons was also studied. The cells were killed by high temperature (100 °C) or by treatment with formalin (5% solution) for 1 h, and their adhesion was compared with that of control (live cells).

Effect of bacterial film

The influence of the bacterial film (two strains of *Pseudomonas putida)* on the adhesion of *N. amphibia* was studied by using titanium and glass coupons. The bacteria were cultured in Nutrient broth (Peptone 10 g, Beef extract 10 g, Sodium chloride 5 g and Distilled water 1000 ml) and log phase cells were used for the experiment. Titanium and glass coupons were placed in Petri dishes containing *P. putida* suspension of 10^7 cells ml^{-1} for 12 h. Then the coupons were rinsed with sterile distilled water to remove the loosely adhered cells and placed in *N. amphibia* suspension. The density of the *P. putida* biofilm on coupons was 3×10^6 cells cm^{-2}. Coupons without the bacterial film served as the control. The attached cells on both the control and the bacterial film coated coupons were counted after 24 h.

Statistical analysis

The differential attachment of organisms on various materials and different incubation times was analyzed by one way and two way ANOVA (Sokal & Rohlf, 1987). The differential attachment of organisms on materials of different roughness, pH and cell density, cell viability and bacterial film coating was analyzed by one way ANOVA. Student's *t*-test was used to test differences in attachment in treatments such as organic film coating and culture age with their respective controls. The correlation between the attachment and the wettability of different materials was ascertained using Pearson's correlation test.

Results

Influence of wettability and material composition on adhesion

The wettability coefficients (W_c) of different materials used in the present study are given in Table 1. The results showed that glass is relatively hydrophilic whereas titanium, stainless steel 316-L and perspex are hydrophobic in nature.

The adhesion of *C. vulgaris* showed significant variation among the materials (one way ANOVA, $F_{145} = 6$; $p < 0.0001$) and with time (one way ANOVA, $F_{254.7} = 4$; $p < 0.0001$). The interaction between both the materials and time was also significant (two way ANOVA, $F_{17.2} = 24$; $p < 0.0001$). At the end of the 48 h the attachment of *C. vulgaris* was maximum on stainless steel, followed by titanium, perspex and glass (Fig. 1a). The colonization was poor on aluminum brass, admiralty brass and copper. The attachment was not correlated with the surface wettability of the materials ($r = -0.1997$; $P = 0.3986$).

Table 1. Wettability coefficient (W_c) of different materials used in the present study

Material	Wettability coefficient (W_c)
Glass	66.6
Copper	34.1
Stainless steel	29.9
Admiralty brass	29.2
Aluminum brass	28.5
Perspex	27.6
Titanium	22.5

The adhesion of *N. amphibia* also varied significantly with materials (one way ANOVA, $F_{192.4} = 6$; $p < 0.0001$) and with time (one way ANOVA, $F_{142.6} = 4$; $p < 0.0001$). The interaction between the materials and time was also significant (two way ANOVA, $F_{6.5} = 24$; $p < 0.001$). Maximum colonization of *N. amphibia* occurred on titanium, followed by stainless steel, perspex and glass (Fig. 1b). The colonization was poor on copper and its alloys. Colonization increased rapidly up to 24 h and then stabilized/decreased. The rate of attachment was higher when compared to *C. vulgaris*. The attachment was significantly higher ($p < 0.01$) on titanium and stainless steel coupons when compared to glass and copper alloys. A low but significant negative correlation between the attachment and the wettability of the materials was observed ($r = -0.5581$; $p < 0.002$).

The adhesion of *C. minutus* varied significantly with materials (one way ANOVA, $F_{114} = 6$; $p < 0.0001$) and with time (one way ANOVA, $F_{101.5}$; $p < 0.0001$). The interaction between the materials and exposure time was also significant (two way ANOVA, $F_{2.2} = 24$; $p < 0.0001$). *C. minutus* also colonized titanium panels better than stainless steel, perspex and glass (Fig. 1c). Its colonization was poor on copper and its alloys. The attachment was significantly higher ($p < 0.01$) on titanium panels when compared to glass and copper alloys. As in the earlier case, a significant negative correlation between the attachment and the wettability of the materials was observed ($r = -0.4940$; $p < 0.0075$).

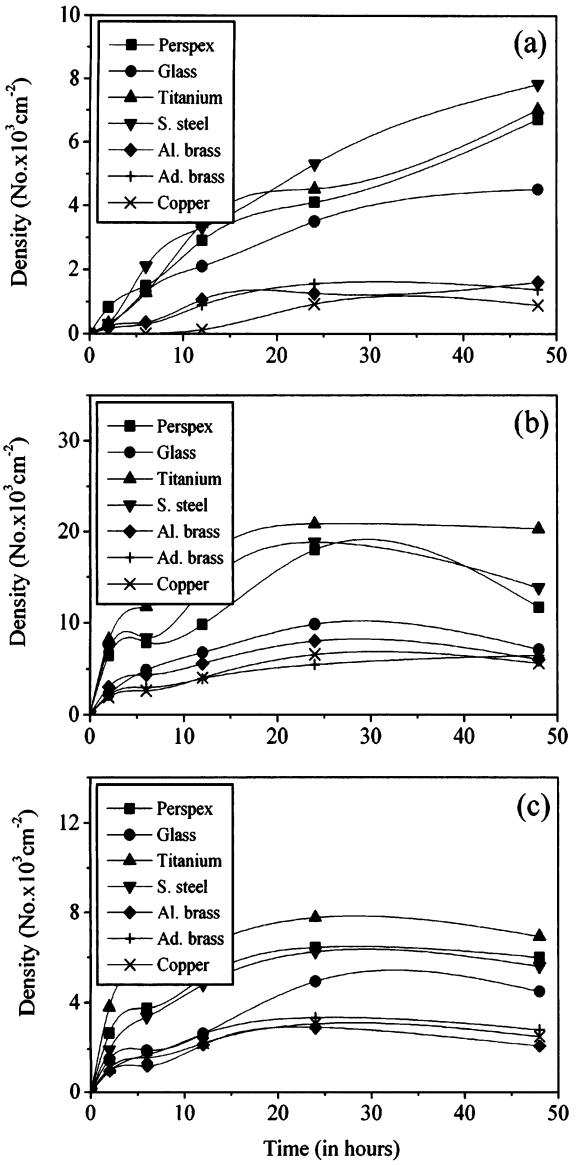

Figure 1. Adhesion of *Chlorella vulgaris* (a), *Nitzschia amphibia* (b) and *Chroococcus minutus* (c) to different hard substrata over time.

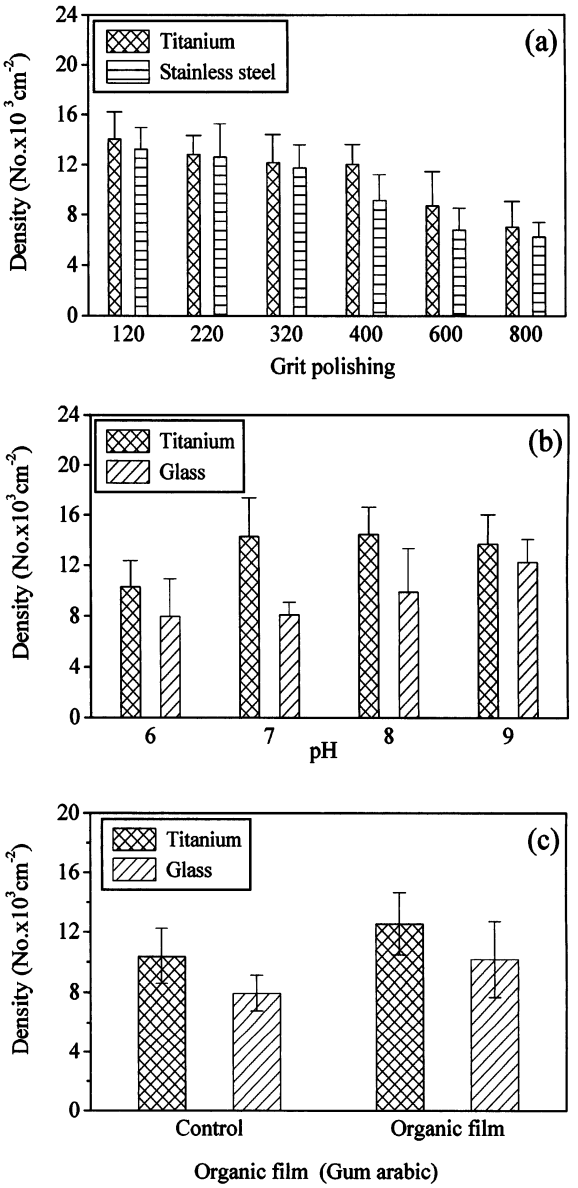

Figure 2. Influence of substratum roughness (a), pH (b) and organic film (c) on the mean (± S.D.) adhesion density of *Nitzschia amphibia*.

Influence of surface roughness on adhesion

The attachment was higher on 120 grit polished coupons, and the attachment decreased progressively with increasing smoothness (Fig. 2a). The results showed higher attachment on rough surfaces when compared to smooth surfaces in the case of both titanium and stainless steel coupons. The attachment varied significantly different between different roughnesses on both titanium (one way ANOVA, $F_{15.5} = 5$; $p < 0.0001$) and stainless steel (one way ANOVA, $F_{23.3} = 5$; $p < 0.0001$) coupons.

Influence of pH and organic film on adhesion

Adhesion of *Nitzschia amphibia* was studied on titanium and glass coupons immersed in media of different pH's (6, 7, 8 and 9). The results of a one way ANOVA showed that the attachment on both titanium (one way ANOVA, $F_{5.7} = 3$; $p < 0.05$) and glass (one way ANOVA, $F_{5.9} = 3$; $p < 0.0002$) varied significantly between different pH's. The attachment was significantly higher ($p < 0.01$) at pH 7, 8 and 9 when compared to pH 6 on titanium coupons (Fig. 2b). On

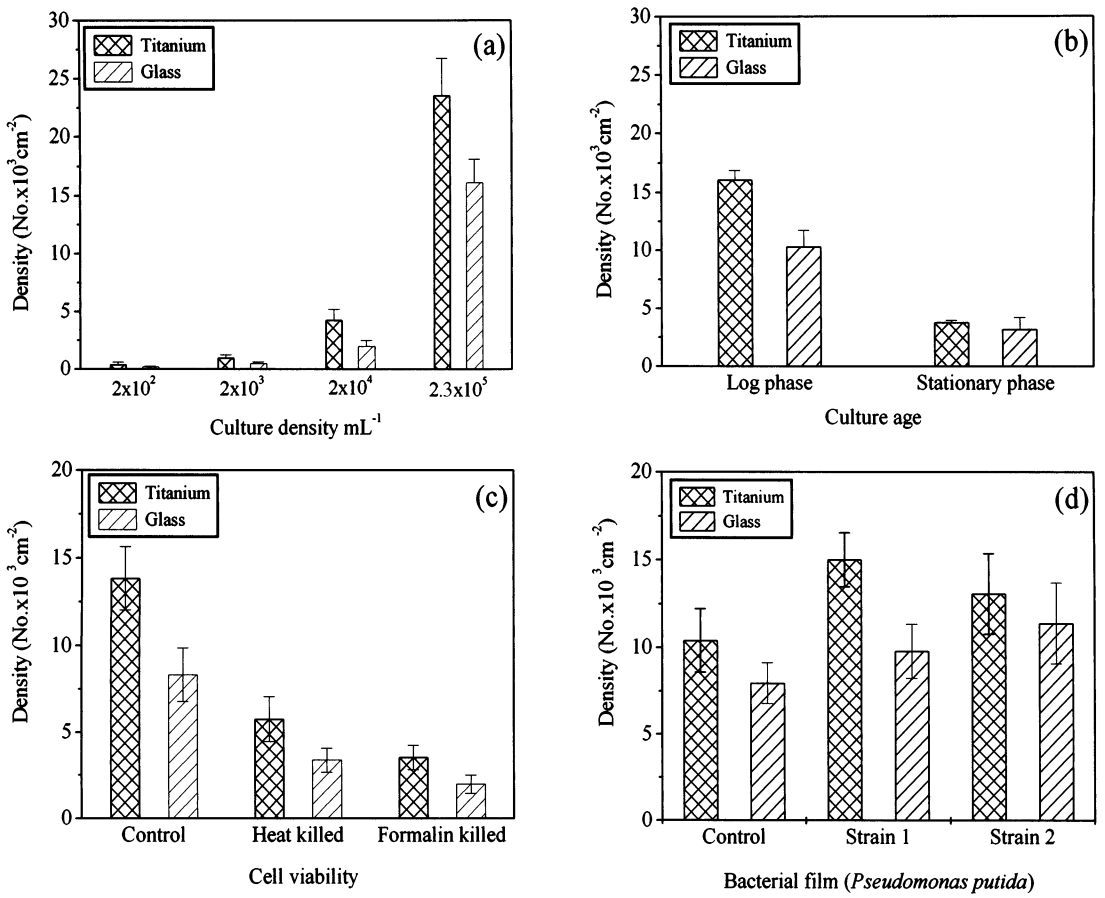

Figure 3. Influence of culture density (a), age (b), viability (c) and bacterial film (d) on the mean (± S.D.) adhesion density of *Nitzschia amphibia.*

glass, the attachment was significantly higher at pH 9 when compared to pH 7 ($p < 0.01$) and 6 ($p < 0.01$).

The adhesion of *N. amphibia* on titanium and glass coupons coated with organic films showed that the presence of organic films increased the algal attachment significantly over control on both titanium (Student's *t*-test, $t = 2.356$; $p < 0.05$) and glass ($t = 2.451$; $p < 0.05$) coupons (Fig. 2c).

Influences of culture density, culture age and cell viability on adhesion

Different culture densities of *Nitzschia amphibia* (2×10^2, 2×10^3, 2×10^4, 2.3×10^5 cells ml^{-1}) were used to study adhesion of the diatom on titanium and glass coupons. The adhesion increased with increase in the culture density. A maximum attachment was at 2.3×10^5 cells ml^{-1} followed by 2×10^4, 2×10^3 and minimum at 2×10^2 cells ml^{-1}, on both titanium and glass coupons. The results of one way ANOVA showed that the attachment was significantly different among various densities on both titanium (one way ANOVA, $F_{293} = 3$; $p < 0.0001$) and glass ($F_{113} = 3$; $p < 0.0001$) coupons (Fig. 3a).

The attachment of log phase cultures on titanium and glass coupons was higher than that of stationary phase cultures (Fig. 3b). The differences were significant for both titanium (Student's *t*-test, $t = 18.6$; $p < 0.0001$) and glass ($t = 12.3$; $p < 0.0001$) coupons.

The adhesion of live, heat killed and formalin killed cells of *N. amphibia* on titanium and glass coupons showed significantly low attachment of heat killed and formalin killed cells on both titanium ($F_{147.5} = 2$; $p < 0.0001$) and glass ($F_{95.2} = 2$; $p < 0.0001$) coupons when compared to control (Fig. 3c).

Influence of bacterial films on adhesion

Surfaces coated with the bacterial film induced greater attachment of *N. amphibia* on both titanium and glass coupons, when compared to control. The attachment was more on biofilm coated titanium coupons than on

glass. The results of one way ANOVA showed significant differences in attachment on coated coupons of titanium ($F_{13.2} = 2$; $p < 0.0001$) and glass ($F_9 = 2$; $p < 0.0001$) when compared to their respective controls (Fig. 3d).

Discussion

The development of microbial films on surfaces has recently received considerable attention. Ford et al. (1989) emphasized the importance of microbial film formation on industrial metals under natural conditions. Fattom & Shilo (1984) reported that cell surface hydrophobicity was a property aiding adhesion of cyanobacteria, similar to bacterial hydrophobicity. Becker & Wahl (1991) and Becker (1996), while studying the colonization of fouling organisms on materials of varying surface tensions, found that the attachment was more on hydrophobic surfaces when compared to hydrophilic surfaces (glass). While studying the bacterial attachment to surfaces Wrangstadeth et al. (1996) found that the higher attachment on hydrophobic surfaces is mediated by the water exclusion mechanism, whereas in hydrophilic substrata water is poorly excluded resulting in less attachment (Burchard et al., 1990). In the present study, the increased attachment observed by all three algae on hydrophobic surfaces (titanium, perspex and stainless steel) may be due to water exclusion mechanism. While studying the biofilm formation on various metals under natural conditions (Ford et al. 1989) found that the attachment was more on titanium and stainless steel, whereas it was poor on the copper alloys. In the present study, the attachment by all the organisms were also poor on copper and its alloys. Though copper, aluminum brass and admiralty brass are relatively hydrophobic, the attachment on those substrata was poor, possibly due to their toxic nature. Hence, both substratum wettability and material composition may be playing important role in the attachment of microalgae.

The density of attached algal cells varied with time among the three organisms. The attachment was highest in *N. amphibia,* followed by *C. minutus* and least in *C. vulgaris*. While studying the attachment strength and adhesion of four marine fouling diatoms *Amphora, Navicula* (procumbent species), *Achnanthes* and *Lichmophora* (stalked species), Woods & Fletcher (1991) found that the attachment rate varied with different species. The attachment of *Amphora* sp. was more whereas the attachment of *Lichmophora* sp. (stalked) was less and they concluded that the variation was due to the cells' ability to produce EPS in a short time. Tosteson & Corpe (1975) reported that the adhesion of algal cells to solid surfaces depended on the ability of the organisms to secrete adhesive materials. The variation of attachment observed in the present study may also be related to attachment mechanism and their differential ability to produce EPS. Microalgal attachment mechanism varies with different groups of organisms. Most of the diatoms attach to the substrata by the production of EPS in the form of stalks, apical pads, mucilage pads and cell coatings (Hoagland et al., 1993) whereas in the case of filamentous green algae the attachment is mainly by the holdfast. Most of the cyanobacteria attach to the substrata by the production of EPS similar to that of bacteria and diatoms (Scott et al., 1996).

Woods & Fletcher (1991), while studying the adhesion of *Achnanthes minutissima*, *Amphora coffeaeformis* and *Navicula corymbosa* on smooth (mean amplitude Ra < 0.025 μm); fine (Ra = 5.75 μm) and coarse (Ra = 35 μm) glass surfaces, found that *A. minutissima* attached more on smooth surfaces, when compared to rough surfaces, whereas *Amphora coffeaeformis* and *Navicula corymbosa* attached more on coarse (rough) surfaces. Hunt & Parry (1998) studied the effect of surface roughness on biofilm development using P280 (rough) and P800 (smooth) grit sand papers for a period of 14 days and found that the bacterial attachment showed significant differences between rough and smooth surfaces, whereas the attachment of algae did not show much variation because of the formation of a uniform bacterial layer over both surfaces. In the present study, axenic cultures of *N. amphibia* were used for experiments on adhesion and hence bacterial film formation may not have preceded the algal attachment. Characklis et al. (1990) found increased bacterial attachment on rough surfaces when compared to smooth surfaces and explained that it was due to the increased convection associated with such surfaces. The rough surfaces provide more surface area for attachment when compared to smooth surfaces. In the present study, the increased attachment of *N. amphibia* observed on rough surfaces may also be due to this reason.

pH is an important factor in natural biofilms and may vary in different layers of the biofilm. It plays an important role in establishing the community (Liehr et al., 1988; Keithan & Barnese, 1989). Microalgae grow well in the pH range between 6 and 9. The effect

of pH on the adhesion of *N. amphibia* showed that the attachment was favoured at pH 7 and above. There was a preferential attachment of diatoms in the alkaline range.

Nitzschia amphibia showed significant differences in attachment with variations in culture density. The attachment was directly proportional to the culture density, indicating that density of the organisms in the bulk water would be a factor influencing the rate of colonization by microalgae. The culture age of alga is also very important in its attachment. Log phase cells attached in more numbers when compared to stationary phase cells. Zaidi & Tosteson (1972) observed differential adhesion of *Chlorella* cells during different stages of the life cycle. They found that log phase cells attached vigorously when compared to cells in the other phases. Live cells of *N. amphibia* attached in greater numbers when compared to heat killed and formalin killed cells, showing the role of active process in its attachment.

Peterson & Stevenson (1989), while studying the effect of naturally 'conditioned' and 'unconditioned' ceramic tiles on adhesion of diatoms, found that the conditioning film did not have much influence on the attachment of both *Nitzschia* and *Synedra* sp. in both fast and slow current regimes. In contrast, Steinman & Parker (1990) observed a significant difference in biomass on conditioned ceramic tiles when compared to unconditioned ceramic tiles, for up to 9 days. They concluded that the influence of substratum conditioning on algal attachment was relatively a short-term effect. In the present study, organic film increased the attachment of *N. amphibia* significantly over control, indicating the role of organic polymers on algal attachment process.

Tosteson & Corpe (1975) found that the presence of bacterial films enhanced the attachment of *Chlorella vulgaris* to glass surfaces. In contrast, Kawamura et al. (1988) reported that a film of *Alcaligens* sp. had no effect on the attachment of *Synedra* sp. and concluded that a bacterial film was not always necessary for the attachment of diatoms to substrata. Fukami *et al.* (1989) found that film of *Alcaligens* sp. had a promoting effect on the attachment of *Nitzschia* sp. to surfaces and concluded that an ethanol-insoluble fraction of the bacterial culture (mainly polysaccharides) was most effective in promoting both the attachment and growth of diatoms on surfaces, especially when they were grown under unfavourable conditions. In the present study, both the strains of *Pseudomonas putida* promoted the attachment of *N. amphibia* on both titanium and glass coupons. Interestingly, titanium collected more cells than glass when a bacterial film was present. The results would suggest that substratum properties exerted an influence on algal attachment even in the presence of bacterial films.

In conclusion, the surface property (wettability and roughness) and composition of the material play an important role in microalgal attachment to its surface. The attachment was also found to be influenced by pH, organic film, culture age, culture density, cell viability and bacterial films. Though all the above mentioned factors were found to influence the algal attachment, further experiments are required to find out the mechanisms involved in the attachment process.

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