Influence of biofilms on the larval settlement of *Balanus reticulatus* Utinomi (Cirripedia: Crustacea)

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ABSTRACT

Microbial films, which develop on submerged artificial surfaces, elicit variable responses in settling invertebrate larvae. There is no information on the response of the larvae of the tropical fouling barnacle, *Balanus reticulatus* to biofilms. Therefore, the influence of biofilms and their components (such as bacteria, diatoms and bacterial exopolymer) on settlement of cyprid larvae of *B. reticulatus* has been studied. Biofilms significantly reduced larval settlement when compared to clean polystyrene surfaces (control). No significant correlation was found between
percentage settlement and biofilm age. Inhibition or induction of settlement was observed, depending on the cell
density and growth phase of the bacterial films. Diatom films, regardless of cell density, were inhibitory to cypris
settlement. Bacterial exopolymers did not influence settlement at low concentrations (0.001 to 0.0001 %), but
inhibited settlement at higher concentrations (0.1 to 0.01%). Our study indicates that presence of natural biofilms
and their components such as bacteria, diatoms and bacterial exopolymers on polystyrene render an otherwise
attractive surface unsuitable for settlement by larvae of *B. reticulatus*.

*Keywords:* biofilm; bacteria; diatoms; exopolymer; *Balanus reticulatus*; cyprid settlement

**INTRODUCTION**

Artificial surfaces immersed in seawater are soon coated by organic molecules, leading to the formation of a
c conditioning film (Loeb and Neihof, 1975). Subsequently, bacteria, diatoms and other organisms colonize the
surface, ultimately resulting in the formation of a complex biofilm (Keough and Raimondi, 1995). Biofilms in
costal marine habitats are generally dominated by bacteria, fungi, microalgae and exopolymeric substances of
microbial origin (Holmstrom and Kjelleberg, 1994). Invertebrate larvae which settle on immersed surfaces generally
encounter and interact with such biofilms (Keough and Raimondi, 1995).

Biofilms have been reported to influence the settlement and metamorphosis of a wide range of marine invertebrate
larvae (see review of Holmstrom and Kjelleberg, 1994; Wieczorek and Todd, 1998). The effects of biofilms on
invertebrate larval settlement depend on their composition (Roberts *et al*., 1991), age (Maki *et al*., 1988, 1990), film
volume (Tsurumi and Fusetani, 1998) and underlying substratum (Maki *et al*., 1988). Anderson and Underwood
(1994) suggested that the pattern of the fouling community in a habitat is influenced by the nature of the biofilm.

Knowledge about the cues provided by biofilms to settling larvae would be useful in understanding spatial
variations in larval settlement and developing control strategies for biofouling (Richmond and Seed, 1991).
Moreover, any broad interpretation of settlement dynamics needs knowledge of the responses to biofilms of all or
most fouling species (Keough and Raimondi, 1995). Earlier studies have examined barnacle larval settlement
(henceforth "settlement") response to biofilms in the field (Roberts *et al*., 1991; Keough and Raimondi, 1995;
Wieczorek *et al*., 1996) and, in the laboratory, to individual films of bacteria and their exopolymers (Maki *et al*.,
indicate a lack of any uniformity of species response to biofilms. Biofilms were found to elicit a facilitatory
response in *B. amphitrite* cyprids in the laboratory until the film volume grew to 0.1-1 µm³m⁻², and the effect decreased thereafter (Tsurumi and Fusetani, 1998). Biofilms developed in the aquarium inhibit barnacle settlement in the laboratory (Maki *et al.*, 1988), while they enhance the settlement in field conditions (Meenakumari and Nair, 1994). Settlement of *B. variegatus* and *Elminius modestus* cyprids was found to be greater on unfilme (or less filmed) surfaces, when compared to filmed surfaces, in field conditions (Keough and Raimondi, 1995). *B. improvisus* larvae preferred to settle on bacterial films which were coated on hydrophilic surfaces and the same bacterial film inhibited larval settlement when it was coated on hydrophobic surfaces (O'Connor and Richardson, 1996).

The objective of this study was to assess the influence of natural biofilm and its components (such as bacteria, diatoms and bacterial exopolymers) on barnacle larval settlement. In this study, cyprids of *Balanus reticulatus* were chosen as test material for the following reasons, i) *Balanus reticulatus* is a dominant fouling species in the east and west coasts of India (Thiyagarajan *et al.*, 1997a), and ii) the larval settlement behaviour of this species is not known yet (Thiyagarajan *et al.*, 1997b).

**MATERIALS AND METHODS**

**Larval culture**

Adult barnacles were collected (using a chisel) from the jetty piers of Madras Atomic Power Station. First (sometimes second) - stage nauplii were obtained by immersing the adult barnacles in clean seawater, after a few hours of desiccation. Nauplii were reared up to the cypris stage, using the procedure of Thiyagarajan *et al.* (1996). *Chaetoceros wighamii*, a commonly available diatom, was used as larval food. Cyprids were harvested from the culture container using 240 µm plankton net. The larvae were used immediately or stored at 6°C in the dark for later use (Rittschof *et al.*, 1992).

**Settlement assay procedure**

The assay procedure outlined by Rittschof *et al.* (1992) was followed with some modifications. Sterile polystyrene petri dishes (35mm diameter) (Tarson, India) were used as the substratum for settlement. Five ml of aged (in the dark for 10 days) and membrane filtered (Millipore, 0.22 µm) seawater (30 ppt) in an assay petri dish was inoculated with 25-50 cyprids. After incubation for 24 hours in complete darkness at room temperature (28± 2°C),
Preparation of biofilm

Biofilms were developed in polystyrene petri dishes by suspending sterile dishes in the coastal waters (at Kalpakkam, East Coast of India) at 3 m depth for 1 to 5 days. The dishes were placed inside a metal cage and covered with 60 µm plankton net, to avoid larval settlement (Keough and Raimondi, 1995). The number of bacteria attached on the dish was counted using epifluorescence microscopy after staining the dish with acridine orange (Daley and Hobbie, 1975). The bacterial numbers are represented as a mean of ten random field counts.

Preparation of bacterial films

Three different strains of bacteria were isolated from biofilms developed in coastal waters, following the procedure of Mary et al. (1993). Films of the bacteria on polystyrene petri dishes were prepared by following the method of Maki et al. (1988). Individual films were developed using cells harvested from logarithmic (3-6 h old culture) and stationary growth phases (24 h old culture). Petri dishes were filled with 5 ml of bacterial suspension and incubated for 2 hours. Films with different bacterial density were developed by incubating serially diluted bacterial suspensions.

Preparation of diatom films

Two pure cultures of marine fouling diatoms, *Nitzschia* sp. and *Amphora* sp. were maintained in f/2 medium (Guillard and Ryther, 1962). Late logarithmic phase cultures (5 to 7 day old culture) were taken from the flask wall using a bottlebrush. Diatom films were developed on sterile polystyrene dishes by incubating a diatom suspension under light. Films of different diatom densities were obtained by incubating the suspension for 1, 3, 6, 12 and 24 hours. The number of attached diatoms was counted using light microscopy. The diatom numbers are represented as a mean of ten random field counts.

Preparation of bacterial exopolymeric film

A film of bacterial exopolymer was prepared according to the procedure of Maki et al. (1990). Five ml of 0.1 to 0.0001 % solution (dissolved in double distilled water) of exopolysaccharide isolated from *Pseudomonas* sp.
(supplied by T. Subba Rao) was placed in a polystyrene dish for 2 hours. After incubation, the dishes were rinsed with filtered seawater.

**Statistical Analysis**

The differences between treatments were tested using one-way ANOVA (Sokal and Rohlf, 1987). Data were tested for homogeneity using Bartlett's test. Differences between individual treatments were tested by the SNK test. Student's t-test was used to test the difference between treated and control. The differences were considered significant at a level of P<0.05.

**RESULTS**

**Effect of biofilm**

Natural biofilms developed in the coastal waters comprised 1.6 to 3.1x10^6 bacteria/cm^2 in 1 to 5 days. In addition to bacteria, diatoms and detritus were also observed under the microscope. Vibrio spp. were dominant in the biofilms. The bacterial density was relatively high in the 2-day old film (3.1x10^6 bacteria/cm^2) and low in the 4-day old biofilm (1.6x10^6 bacteria/cm^2). Cypris settlement was significantly reduced by biofilms, irrespective of the film age, when compared to unfilmed control dishes (Fig. 1). There was no significant relationship between percentage settlement and either biofilm age or bacterial density in the biofilm (P>0.05).

![Figure 1](http://www.bioline.org.br/request?bf99001) Influence of biofilm on *Balanus reticulatus* settlement

**Effect of bacterial films**

Logarithmic phase cells of strain I reduced larval settlement at cell densities ranging from 4.4x10^5 to 7.1x10^6 cells/cm^2, when compared to the control (Fig. 2). Films of strain I derived from stationary phase cells showed both inhibition and induction of larval settlement depending on its density in the film (Fig. 3). Settlement was significantly different between the films derived from logarithmic and stationery phase cells of strain II at all densities tested (Figs 4 and 5). Settlement was not strongly induced by logarithmic phase cells of stain III at 1.5x10^5 cells/cm^2, whereas at a density of 7.1x10^5 to 2x10^6 cell/cm^2 the stationary phase cells inhibited settlement (Figs. 6
and 7). Even though, the films of stain-III developed from logarithmic phase cells at the densities ranging from 2.1-3.1x10^6 cell/cm^2 led to reduced settlement values, the values were not significantly different from the control (P>0.05).

**Figure 2** Effect of bacterial film (logarithmic phase cells) (strain I) on *Balanus reticulatus* settlement

**Figure 3** Effect of bacterial film (stationary phase) (strain I) on *Balanus reticulatus* settlement

**Figure 4** Effect of bacterial film (logarithmic phase) (strain II) on *Balanus reticulatus* settlement

**Figure 5** Effect of bacterial film (stationary phase) (strain II) on *Balanus reticulatus* settlement

**Figure 6** Effect of bacterial film (logarithmic phase) (strain III) on *Balanus reticulatus* settlement

**Figure 7** Effect of bacterial film (stationary phase) (strain III) on *Balanus reticulatus* settlement

Inhibition/induction of larval settlement by bacterial films, in general, depends on the growth phase of the cells. All the bacterial films, irrespective of the strain and growth phase, inhibited settlement at low cell density and had no effect at higher densities except in a few instances (Figs. 2-7).

**Effect of diatom films**

Diatom films, irrespective of cell density, inhibited settlement (Figs. 8 and 9). In contrast to bacterial films, the inhibition of cypris settlement was positively correlated with diatom density (r = 0.96 - *Nitzschia* sp; r = 0.86 - *Amphora* sp).

**Figure 8** Effect of diatom film (*Nitzschia* sp) on *Balanus reticulatus*

**Figure 9** Effect of diatom film (*Amphora* sp) on *Balanus reticulatus*
Effect of exopolymer

Data showed that exopolymeric films adsorbed on polystyrene dishes at the concentration of 0.001% to 0.0001% (w/v), did not have any effect on settlement, when compared to the control, whereas films developed at 0.1 to 0.01% (w/v) strongly inhibited settlement (Fig. 10).

**Figure 10** Effect of exopolymer of bacteria on *Balanus reticulatus*

DISCUSSION

Physical factors such as vibration, light, substratum type, colour of the surface, surface energy of the substratum, salinity, water current, chemical cues originating from biofilms and cypris age have significant influence on barnacle larval settlement (Branscomb and Rittschof, 1984; O'Connor and Richardson, 1994; Holm et al., 1997; Rittschof et al., 1998; Wieczorek and Todd, 1998). Barnacle larvae receive both negative and positive cues (Holmstrom and Kjelleberg, 1994) from the environment. Laboratory static experiments demonstrated that the presence of natural biofilms, or films composed of single species of marine bacteria, could stimulate or inhibit the settlement of *B. amphitrite* (Maki et al., 1990) and *B. reticulatus* (present study) larvae.

Polystyrene dishes immersed in the coastal waters were soon coated by organic detritus, bacteria and diatoms. This is the generally reported pattern of microfouling in coastal waters (Roberts et al., 1991; Tsurumi and Fusetani, 1998). In the biofilms, the bacterial density reached $10^6$ cells/cm$^2$ within a day and remained almost constant for several days (Maki et al., 1988; Wieczorek et al., 1995). Our studies showed that biofilms which developed on polystyrene dishes constitute a surface which is not preferable for barnacle settlement. The presence of biofilms consisting of bacteria, diatoms and organic polymers render the surfaces more wettable (Mihm and Banta, 1981). Since *B. reticulatus* larvae do not prefer a wettable surface (Thiyagarajan, unpublished), the presence of biofilms on a hydrophobic (polystyrene) surface might have reduced the cypris settlement when compared to clean, untreated hydrophobic surfaces, due to a change in surface wettability. O'Connor and Richardson (1996) reported that the settlement of *B. improvisus* cyprids decreased in the presence of bacterial cells when filmed on a hydrophobic (polystyrene) surface whereas settlement was facilitated when bacteria were coated on hydrophilic (glass) surface. Types of surface (surface energy of a substratum) and cues (positive/negative) associated with biofilms, determine the strength of adhesion of barnacle larvae (tenacity) (Neal and Yule, 1994). That is, in the absence of a suitable (hydrophobic surface in the case of *B. reticulatus*) substratum and a strong positive cue for settlement and
metamorphosis, cyprids undergo voluntary detachment which leads to reduced settlement (Neal and Yule, 1994), as in this case of *B. reticulatus* (present study).

In the present study, Vibrio-type bacteria were dominant in the biofilm. Among the bacterial types, vibrios have been reported to be the most potent inhibitors of cypris settlement (Mary *et al.*, 1993). The inhibition of settlement by biofilms, therefore, may not only be due to change in surface energy and production of negative cues but also be related to the species composition of the biofilm.

A large number of studies on the effect of biofilms on barnacle larval settlement have used single-species cultures of microorganisms (Maki *et al.*, 1988; 1990; 1994; Mary *et al.*, 1993; O'Connor and Richardson, 1996), which is inappropriate if inferences on larval responses to biofilms under natural condition are to be drawn. Nonetheless, it is clear that only by utilizing a single species film in the laboratory can one identify the specific components of a biofilm that may be an important cue to larval settlement in the field (Wieczorek and Todd, 1998). Therefore, we studied the effect of single species bacterial/diatom films on *B. reticulatus* larval settlement. Our data show that settlement of *B. reticulatus* cyprids on bacteria adsorbed onto a polystyrene surface can vary with the growth phase of the cells (inhibition, no difference and induction). This observation is similar to that reported by O'Connor and Richardson (1996) for *B. amphitrite* and *B. improvisus* larvae. Since exopolymers are involved in bacterial adhesion (Maki *et al.*, 1990), the production of qualitatively different exopolymers may be attributed to the effect of growth phase on settlement.

Results of the present study revealed that bacterial films at higher densities (<10^6 cells/cm^2) were not inhibitory to settling cyprids. These results are in accordance to those reported by Maki *et al.* (1990) for *B. amphitrite* larvae. However, the nature of bacterial density and barnacle larval interaction is not clear.

Diatom cells secrete glue (usually acidic polysaccharides) for firm attachment to surfaces (Webster *et al.*, 1985). Therefore, settling cyprids are not only exposed to bacterial exopolymers but also to biomolecules of diatom origin. Results of this study show that barnacle larvae are strongly inhibited by fouling diatoms, probably due to their surface bound molecules. However, this point has to be studied in detail.

Finally, this study demonstrates that biofilms and their components (bacteria and diatoms), which cover surfaces immersed in coastal waters, may act as a negative cue or make a surface unsuitable for *B. reticulatus* cyprid settlement in the laboratory. Even though large number of studies have emphasized the interaction between barnacle...
larvae and biofilms, still it is not known how bacteria and diatoms interact with settling cyprid larvae and influence their attachment.

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