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# Microbial Biofilms Facilitate Adhesion in Biofouling Invertebrates

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**Abstract.** Much interest has focused on the role of microbial layers—biofilms—in stimulating attachment of invertebrates and algae to submerged marine surfaces. We investigated the influence of biofilms on the adhesion strength of settling invertebrates. Larvae of four species of biofouling invertebrate were allowed to attach to test surfaces that were either clean or coated with a natural biofilm. Measuring larval removal under precisely controlled flow forces, we found that biofilms significantly increased adhesion strength in the ascidian *Phallusia nigra,* the polychaete tubeworm *Hydroides elegans,* and the barnacle *Balanus amphitrite* at one or more developmental stages. Attachment strength in a fourth species, the bryozoan *Bugula neritina,* was neither facilitated nor inhibited by the presence of a biofilm. These results suggest that adhesive strength and perhaps composition may vary across different invertebrate taxa at various recruitment stages, and mark a new path of inquiry for biofouling research.

## Introduction

Marine biofouling refers to the accumulation of organisms and biogenic structures on ship hulls and other submerged surfaces. Permanently fixed to a substratum, sessile marine invertebrates in particular compose the framework of many fouling assemblages. Animals such as barnacles and tubeworms also produce shells or other firm structures as they grow, allowing for attachment of additional organisms after primary substratum has been preempted, resulting in multilayered fouling communities (Scheer, 1945). This biological buildup increases frictional drag on ships, smothers oceanographic equipment, adds bulk to floating structures, clogs seawater lines to power plants, and promotes structural deterioration (Thompson *et al.*, 1988). For the global shipping industry alone, biofouling costs billions of dollars per year in prevention, maintenance, and fuel consumption (Alliance for Coastal Technologies, 2004).

Biofouling begins with adsorption of organic molecules onto newly submerged surfaces, followed by the accrual of bacteria, diatoms, and other microorganisms bound together in a film of extracellular polymeric substances (Zobell and Allen, 1935). Such biofilms develop on surfaces within hours of immersion, increasing in density and structural complexity over time (Donlan, 2002). Subsequent interactions of macrobiota with these microbial films lead, within days and weeks, to the attachment and growth of invertebrates and algae, which account for most of the hydrodynamic drag associated with biofouling (Schultz, 2007).

In colonizing a surface, many invertebrates rely on a swimming larval stage to make contact with and attach to the substratum. The larvae then metamorphose to a sedentary stage, in some taxa growing in size and in others dividing asexually to form a spreading colony. Critical links in the biofouling process include detection of appropriate substrata and secure adhesion by larvae. Chemical cues play a pivotal role in invertebrate settlement (Hadfield and Paul, 2001), and microbial biofilms in particular provide biochemical signals that larvae employ in selecting a settlement site, attaching to it, and undergoing metamorphosis (Meadows and Williams, 1963; Hadfield *et al.,* 1994; Keough and Raimondi, 1994; Carpizo-Ituarte and Hadfield, 1998; Unabia and Hadfield, 1999; Huang and Hadfield, 2003; Dahms *et al.*, 2004). Biofilms can also modulate physical surface properties such as wettability or texture (Crisp and Ryland, 1960; Gray *et al.*, 2002), which are important to

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settling larvae (Maki *et al.*, 2000). Furthermore, biofilms can provide larvae with information on the environmental regime under which a film has formed. Larval barnacles, for instance, when presented with biofilms cultured under different flow velocities, increase their exploratory behavior (Neal *et al.,* 1996) and attachment tenacity (Neal and Yule, 1994) with biofilms from higher flow treatments. Larvae contend with a dynamic flow environment, risking dislodgment and conveyance to unsuitable habitat if they cannot rapidly assess a substratum and firmly adhere to it at touchdown. Although tidal currents across fouling communities in harbors and bays, where biofouling predominates, are often slow (0.01–0.05 m/s), peak velocities of waves (wind chop and boat wake) can reach 0.1 m/s (M. Koehl and T. Cooper, University of California Berkeley, pers. comm.), sufficient to rapidly remove minute organisms that are not firmly attached.

Antifouling research has provided insight into the development, structure, and function of biofilms (Costerton *et al.,* 1995; Stoodley *et al.,* 1999), especially regarding how they adhere to surfaces (Dunne, 2002) and how specific bacterial components of biofilms induce recruitment of some larvae (*e.g.,* Huang and Hadfield, 2003). However, a large gap remains in understanding how adhesion of settling invertebrates is influenced by microbial films. Intuitively, the presence of a thick, mucous organic layer underlying the relatively small attachment organs of settling larvae would be expected to impede the ability of larvae to adhere tightly to a substratum. We assessed the influence of microbial films on adhesion of newly settled invertebrate larvae by comparing removal rates of settlers from glass surfaces with and without natural biofilm coatings after exposure to precisely controlled forces of surface shear. Four species of common marine fouling organisms —a polychaete worm, an ascidian, a barnacle, and a bryozoan—were tested, each varying in its responsiveness to biofilms at settlement. The strength of larval attachment was assessed by recording their removal—or not—in a turbulent channel flow apparatus (Schultz *et al.*, 2000) where nominal wall-shear forces could be determined.

## Materials and Methods

### *Experimental organisms*

(1) *Phallusia nigra* Savigny, 1816, is a solitary tunicate known from the Mediterranean to Micronesia (DeFelice *et al.*, 2001). It develops *via* a tadpole larva with adhesive papillae on its anterior surface by which it attaches to a substratum. Within hours of attachment, the larva metamorphoses by resorbing its tail, rotating its body, opening its siphons, and sending adhesive extensions of its body wall (ampullae) out along the substratum. Larvae of this species are behaviorally indifferent to biofilms at settlement (Hurlbut, 1991).

(2) *Hydroides elegans* (Haswell, 1883) is a polychaete tubeworm that is found worldwide in tropical and subtropical bays and harbors (ten Hove, 1974). As a larva, it initiates attachment by tethering itself to the substratum with a sticky thread and soon thereafter secretes a proteinaceous primary tube adherent to the substratum. Then within less than 12 h, it cements itself permanently in place with a secondary calcified tube. The larva requires a biofilm signal to induce metamorphosis naturally, but artificial/chemical stimuli can also be employed in the absence of natural cues (Carpizo-Ituarte and Hadfield, 1998).

(3) The barnacle *Balanus amphitrite* Darwin, 1854, occurs circumtropically and is commonly found on a wide variety of hard surfaces (DeFelice *et al.,* 2001). It passes through seven planktonic larval stages and at the terminal cyprid stage attaches by gluing its antennules (anterior sensory appendages with adhesive ducts) to a surface. The cyprid actively explores the substratum and responds positively to biofilms but will also attach and metamorphose in their absence (Holm *et al.*, 2000; Qian *et al.*, 2003). Metamorphosis begins within hours of attachment and ends with production of a shelled juvenile within about 24 h. Settling barnacles secrete several adhesives of varying tenacity at different stages: a weak temporary adhesive during surface exploration by the cyprid; a stronger permanent adhesive at definitive attachment by the cyprid; and an even stronger cement at completion of metamorphosis by the juvenile. In *Semibalanus balanoides,* the permanent adhesive of the cyprid fully cures within 1–3 h, and the juvenile cement begins to be secreted within a day (Yule and Walker, 1987).

(4) The bryozoan *Bugula neritina* (Linnaeus, 1758) is a widespread temperate and tropical organism (DeFelice *et al*, 2001) that, as an adult, forms erect bushy colonies. Each colony develops by asexual budding from a single larva that attaches by adhesive secretion to the substratum. This species prefers settling in the presence of a biofilm but, after a few hours, will do so on clean surfaces as well (Mihm *et al.*, 1981; Brancato and Woollacott, 1982; Dobretsov and Qian, 2006). On biofilms, attachment occurs rapidly, and within 2 to 3 h the newly settled individuals begin to bud asexually.

### *Laboratory procedures*

Gametes or larvae of the four invertebrate species were obtained from field-collected adults, and cultures were maintained in the laboratory following standard protocols (Costello *et al.*, 1957; Rittschof *et al.*, 1992, Wendt and Woollacott, 1995: Carpizo-Ituarte and Hadfield, 1998). Settling requirements differed among the taxa, thus various procedures were employed in cleaning slides and inducing settlement. For the experiments with ascidian and bryozoan larvae, clean microscope slides were obtained by immersing new slides for 10 min in 10% HCl, followed by 10 min in 10% $NH_4OH$, and then thoroughly rinsing them in deion-

ized water. For experiments with tubeworm and barnacle larvae, slides were cleaned by immersing them in a 10% solution of the nonionic surfactant Triton X-100 for 5 d, followed by thorough rinsing in deionized water. Biofilms were obtained by suspending new slides for about 10 days in laboratory tanks receiving a constant flow of unfiltered, ocean-drawn seawater. Once competent to settle, larvae were concentrated in a beaker and pipetted in 2–4 ml of seawater onto the surface of the slides. Each slide was placed in a covered 100-ml petri dish to prevent evaporation. In separate trials for each species, larvae were allowed to settle on the microscope slides to an early attachment stage, after which the dishes containing the slides were flooded with 0.2-$\mu$m-filtered seawater, and the attached larvae were counted.

Tadpole larvae of *P. nigra* were induced to settle with a solution of seawater containing an excess of 10 mmol $l^{-1}$ KCl in both the clean and biofilmed slide treatments. Larvae were allowed to settle for 24 h and were tested at one attachment stage only.

Larvae of *H. elegans* were left to settle on biofilmed slides for either 2-h or 12-h periods, corresponding to primary and secondary tube development, respectively. Larvae of *H. elegans* absolutely require a biofilm for natural settlement; therefore, to achieve settlement on a biofilm-free surface, the larvae were exposed to a seawater solution containing 10 mmol $l^{-1}$ excess CsCl, previously shown to induce settlement in this species (Carpizo-Ituarte and Hadfield, 1998). As a control for possible effects of CsCl on the attachment strength of *H. elegans*, CsCl was also included in a series of slides exposed to biofilm as described above.

Cyprid larvae of the barnacle were allowed to settle for 24 h, at which time three settlement stages—attached cyprid, metamorphosed juvenile, and partially metamorphosed juveniles—were counted on the slides. The three stages co-occurred on all test slides and were subjected to flow together but were analyzed separately.

Larvae of the bryozoan were tested in separate trials at two settlement intervals, 1.5 and 3.5 h, corresponding to pre- and post-budding stages, respectively. A seawater solution containing a 10 mmol $l^{-1}$ excess of KCl was used as an artificial inducer to enhance settlement (Wendt and Woollacott, 1995). The solution was applied for the full duration of the settlement period, and two treatments were compared: clean glass with KCl and biofilmed glass with KCl.

During trials in the turbulent channel flow apparatus, two or three replicates (*i.e.,* slides) from each treatment were tested together, and except for the barnacle, multiple runs (from 2 to 8) utilizing larvae from different adults were conducted with additional replicates for each species. Slides were placed in a specially designed turbulent channel flow apparatus for testing (Fig.1A). The device holds up to six microscope slides per run in a small test chamber: three slides flush with the floor facing up and three slides flush with the ceiling facing down (Fig. 1B). The chamber is open

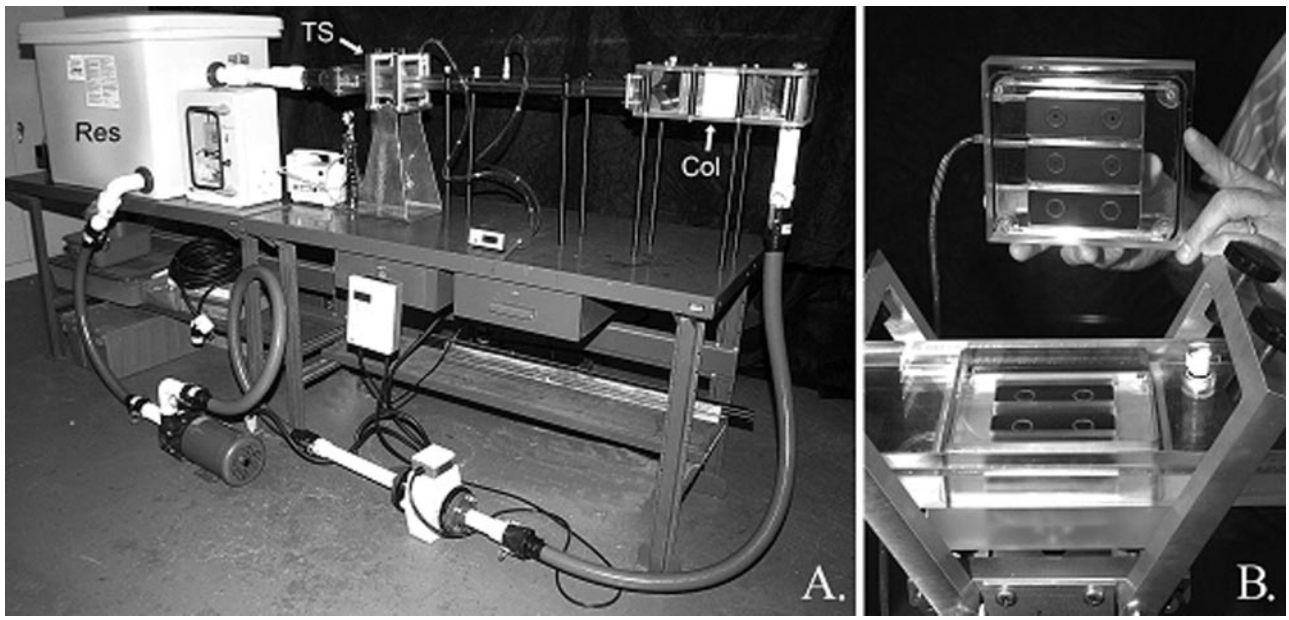


**Figure 1.** (A) Turbulent channel flow apparatus used in testing adhesion of four invertebrate species to glass microscope slides. Slides are held in the test chamber by a suction device. Charged with seawater, the device circulates water through a collimator (Col), the test chamber (TS), and into a receiving reservoir (Res) (for details of hydrodynamic flow see Schultz *et al.,* 2000). (B) Close-up of the test chamber. Photographs by Dr. M. P. Schultz, U.S. Naval Academy.

at both ends and positioned in-line in a recirculating seawater system. Engineered to direct fully developed turbulent flow across the surfaces of the test chamber, the apparatus produces precise wall-shear stresses (*i.e.,* forces parallel to the substratum) in a biologically relevant range of 0 to 120 Pa (Schultz *et al.*, 2000).

In pilot trials, the wall-shear stress required to remove 50% or more of settlers from the slides was found to vary among the species. Thus, depending on the species under observation, the experimental wall-shear stress applied was 50, 75, or 120 Pa. Actual wall-shear stresses experienced by the test organisms may have been slightly higher than the calibrated measures, which were determined using smooth test surfaces. Hence, we refer to the forces exerted as "nominal wall-shear stresses."

For each experiment, organisms were exposed to the target nominal wall-shear stress for 4 min, after which the attached larvae remaining on each slide were counted under a stereomicroscope. The proportion of larvae remaining on each slide was transformed by the arc-sine of the square-root of the variate (Sokal and Rohlf, 1981). Normally distributed data were compared statistically by ANOVA, and heteroscedastic data were assessed by the nonparametric Kruskal-Wallis statistic as implemented using the Analyse-it (ver. 2.03) statistical add-on software for Microsoft Excel (Analyse-it Software, Ltd., Leeds, England).

## Results

### *Larval settlement*

Within each species studied, average larval settlement numbers on test slides in the laboratory were mostly similar across treatments by settlement stage, but with high standard deviations (Table 1). An exception was *Hydroides elegans*, where numbers of settlers were much greater on biofilmed glass than on clean surfaces. In the experiments using *Balanus amphitrite,* three settlement stages co-occurred, with attached cyprids much less abundant on the slides than either juvenile or partially metamorphosed stages. This was most likely due to the duration of the experiment; metamorphosis in most larvae had progressed to later stages before tests were begun in the turbulent flow channel apparatus. Taken as a whole, barnacle settlement averaged 46.0 (SD ± 25.5) for clean glass and 44.3 (SD ± 31.6) for natural biofilm.

### *Larval adhesion*

For the ascidian, tubeworm, and barnacle larvae, biofilms had a positive effect on adhesion strength for at least one

**Table 1**

*Mean number of settlers of four biofouling invertebrate species at various settlement stages*

| Species and stage | *n* | Treatment | Mean (±SD) | | Range |
|---|---|---|---|---|---|
| *Phallusia nigra* | 12 | clean (A) + KCl | 17.67 | (8.86) | 4 to 35 |
| | 11 | clean (T) + KCl | 17.18 | (6.42) | 6 to 29 |
| | 10 | biofilm + KCl | 21.20 | (11.21) | 4 to 37 |
| *Hydroides elegans* | | | | | |
| primary tube | 14 | clean (T) + CsCl | 85.00 | (44.04) | 37 to 195 |
| | 14 | biofilm + CsCl | 119.07 | (115.40) | 10 to 383 |
| | 14 | biofilm | 125.86 | (156.89) | 8 to 638 |
| secondary tube | 16 | clean (T) + CsCl | 45.38 | (27.36) | 13 to 99 |
| | 16 | biofilm + CsCl | 165.25 | (69.19) | 78 to 284 |
| | 16 | biofilm | 335.38 | (176.06) | 91 to 645 |
| *Balanus amphitrite* | | | | | |
| cyprid | 3 | clean (T) | 5.67 | (5.03) | 1 to 11 |
| | 3 | biofilm | 4.00 | (1.73) | 3 to 6 |
| partially metamorphosed | 3 | clean (T) | 29.00 | (17.06) | 15 to 48 |
| | 3 | biofilm | 19.33 | (20.53) | 2 to 42 |
| juvenile | 3 | clean (T) | 11.33 | (8.50) | 5 to 21 |
| | 3 | biofilm | 21.00 | (9.54) | 15 to 32 |
| *Bugula neritina* | | | | | |
| pre-bud | 8 | KCl (A) | 48.63 | (13.79) | 26 to 63 |
| | 8 | biofilm + KCl | 34.88 | (13.74) | 16 to 58 |
| post-bud | 6 | KCl (A) | 24.17 | (3.06) | 19 to 28 |
| | 5 | KCl (T) | 25.80 | (2.28) | 22 to 28 |
| | 6 | biofilm + KCl | 31.67 | (11.48) | 22 to 54 |

Settlement numbers are averaged among replicates (*n*) by experimental treatment. Also given is the range of settlers across replicates. Slides were either acid cleaned (A) or cleaned with Triton X-100 (T). The artificial inducers of metamorphosis potassium chloride (KCl) and cesium chloride (CsCl) were included in some experimental treatments.

settlement stage. In the single stage tested for *Phallusia nigra*, adhesion was significantly greater ($F$ 17.53, $P < 0.0001$) on biofilmed slides than on cleaned slides (Fig. 2). In *Hydroides elegans*, adhesion at both the primary and secondary tube stages was significantly greater on biofilmed slides than on clean glass ($F$ 38.86, $P < 0.0001$, and Kruskal-Wallis $H$ 22.76, $P < 0.0001$, respectively), but there were stage-dependent differences with the combination of biofilm and inducer (Fig. 3). For the primary tube stage, *post hoc* orthogonal contrasts showed that adhesion was significantly greater with biofilm and inducer than with clean glass and inducer alone and that adhesion was significantly greater yet with biofilm alone ($P < 0.05$). For the secondary tube stage, the treatments of biofilm with inducer and biofilm alone did not significantly vary from each other in their effect on adhesion ($P > 0.90$).

For *Balanus amphitrite*, all of the attached cyprids were removed from clean glass, but less than 5% were removed from the biofilmed surface (Fig. 4A). This result must be interpreted cautiously owing to low sample sizes of cyprids; however, it does mirror the trend found with the partially metamorphosed individuals and the juvenile stage (Fig. 4B). For partially metamorphosed barnacles, adhesion was significantly greater on biofilmed slides than on clean glass (T 6.59, $P < 0.005$). For post-metamorphic juveniles there was no significant difference in adhesion between treatments (Fig. 4C).

*Bugula neritina* was the only species tested in which adhesion was not influenced by the presence or absence of a biofilm. For the pre-budding stage, there was no significant difference ($F$ 0.18, $P = 0.68$) among treatments employing clean glass, an ionic settlement inducer, and a biofilm (Fig. 5A). For the post-budding stage, adhesion did not significantly vary ($F$ 0.12, $P = 0.89$) among the biofilm treatment and the two wash treatments for clean glass (Fig. 5B).

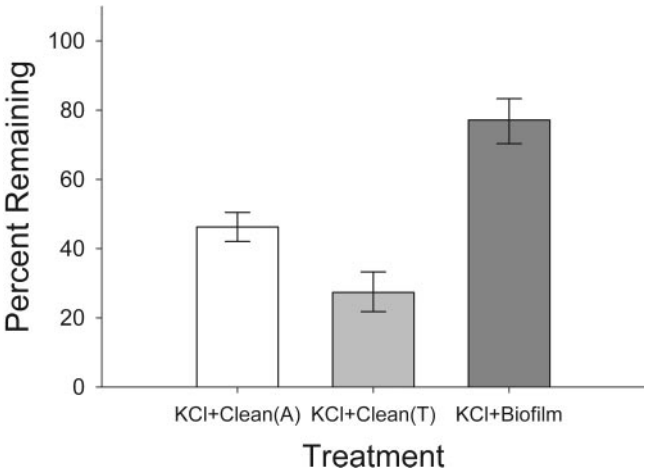


**Figure 2.** Mean percentage of *Phallusia nigra* remaining on slides after exposure to a nominal wall-shear stress of 120 Pa. Treatments consisted of glass slides cleaned with acid (A) or Triton X-100 (T), or coated with a natural biofilm. Excess KCl (10 mmol $l^{-1}$) was applied to all treatments. Error bars = 1 SE.

Across the taxa tested, *B. amphitrite* and *P. nigra* were most resistant to removal, requiring the maximum nominal wall-shear stress attainable by the channel flow apparatus to achieve substantial removal (120 Pa). The presence of a biofilm was most advantageous for the partially metamorphosed barnacle (excluding results for the cyprid stage), providing on average a 72.9% increase in the number of individuals adhering over the clean glass treatment. When compared to that on clean glass, adhesion on biofilmed surfaces increased an average of 44.8% for the ascidian; for larvae of *H. elegans*, there was a 20.1% average increase at the primary tube stage and a 17.0% increase at the secondary stage.

## Discussion

Facilitation of adhesion in larval and early juvenile invertebrates by microbial films represents a previously unidentified role of biofilms in biofouling. How such facilitation is accomplished remains unknown, but biochemical, behavioral, or physical mechanisms may all be involved. Invertebrate adhesives are primarily viscoelastic gels that vary in composition but have similar physical properties (Smith, 2002), suggesting a common mode of adhesive action. The surface energy of a substratum also influences adhesive ability, with more hydrophilic surfaces tending to provide for stronger attachment (Becker, 1993), probably due to polarity of the adhesives with similar wettability as the substratum (Wu, 1973). Thus the relative hydrophobicity of the surface material, the nature of the adhesive, and the presence of a biofilm form a complex interaction.

Differences in removal rates among settling invertebrate species raise the question, does adhesion strength vary by taxon or does it depend on the nature of the biofilm stimulus? It is unknown whether larvae initially penetrate biofilms and adhere directly to surfaces or integrate their adhesives with the biofilm to increase tenacity. Microbial films may also stimulate larvae to increase or decrease the amount of adhesive they produce or to modulate their adhesive composition and thus its strength. Another possibility is that biofilms alter the near-surface velocity gradient enough to reduce shear stresses along the substratum.

The barnacle *Balanus amphitrite* and the tubeworm *Hydroides elegans* were more susceptible to removal at their younger stages. Similarly, Eckman *et al.* (1990) had previously demonstrated for *Balanus amphitrite* that the longer the attachment duration of larvae, the greater the drag force required to detach them. Possible explanations for these age-related changes in both species include (1) older stages have larger and thus perhaps stronger adherent areas; (2) older stages may have different and stronger adhesive; and

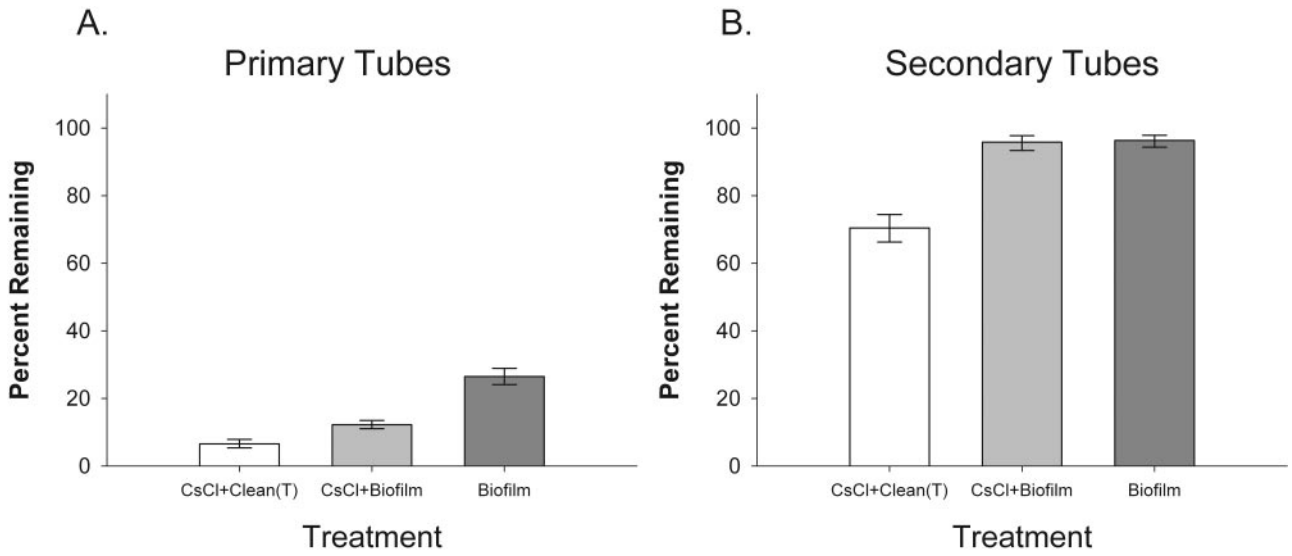


**Figure 3.** Mean percentage of *Hydroides elegans* remaining on slides after exposure to a nominal wall-shear stress of 50 Pa. Metamorphosed individuals of *H. elegans* were evaluated at the (A) primary and (B) secondary tube stages. The proteinaceous primary tube is secreted shortly after the initiation of metamorphosis. The secondary tube is calcified and secreted after metamorphosis. Treatments consisted of glass slides cleaned with Triton X-100 (T) and biofilmed slides. Excess CsCl (10 mmol $l^{-1}$) was used to induce larvae to metamorphose on clean slides, and as a control, larvae were exposed to biofilm and CsCl. Error bars = 1 SE.

(3) older stages are calcified, which may provide a firmer attachment. Additionally, increased adhesion strength may simply reflect an increase in the amount of adhesive as attachment duration increases; "curing" of the adhesive, which leads to increased strength.

The discovery that biofilms facilitate invertebrate adhesion highlights a new avenue of inquiry in mitigating biofouling at the interface of biofilm development and larval settlement. Investigations are needed into the physical architecture of the biofilm-invertebrate connection, focusing in particular on where larval adhesive is deposited relative to microbial films. Additional insight into the nature and function of invertebrate adhesives and how they are biologically modulated is also merited.

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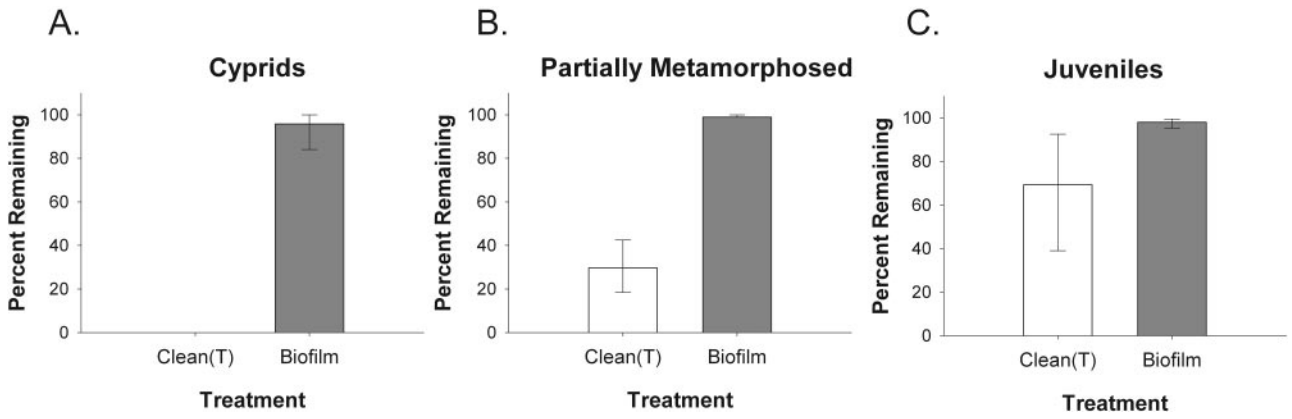


**Figure 4.** Mean percentage of *Balanus amphitrite* remaining on slides after exposure of a nominal wall-shear stress of 120 Pa. Metamorphosed individuals were evaluated at the (A) attached cyprid, (B) partially metamorphosed, and (C) post-metamorphic juvenile stages. Treatments consisted of glass slides cleaned with Triton X-100 (T) and biofilmed slides. Error bars = 1 SE.

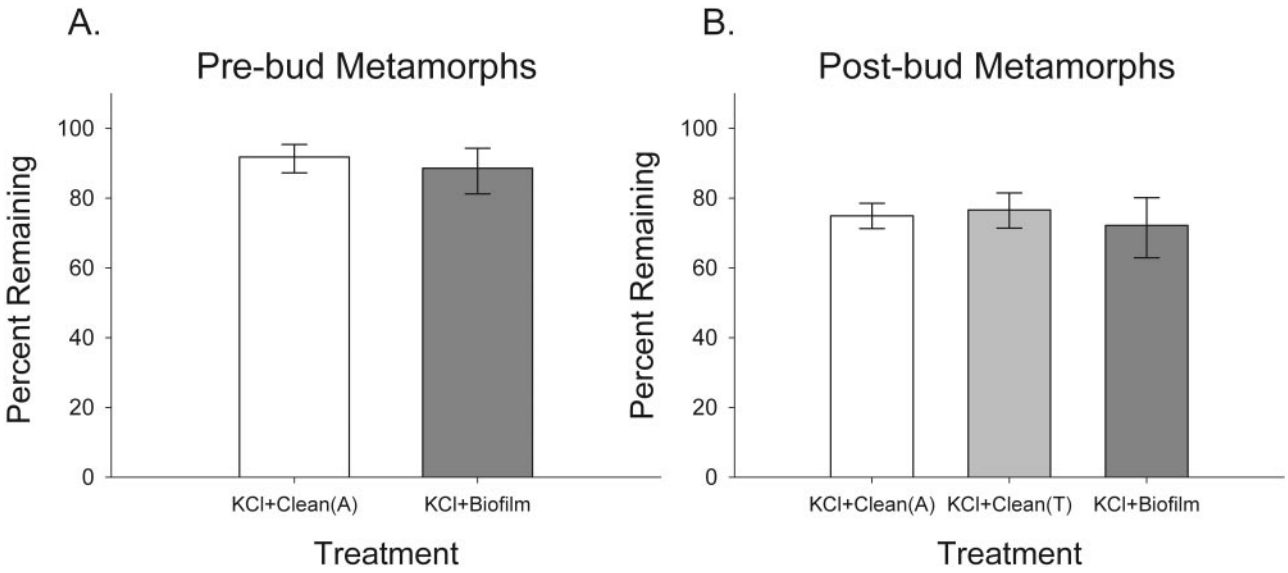


**Figure 5.** (A) Mean percentage of *Bugula neritina* at the pre-budding stage remaining on slides after exposure to 75 Pa nominal wall-shear stress. Treatments consisted of glass slides cleaned with acid (A) or biofilmed slides. (B) Mean percentage of *B. neritina* at the post-budding stage remaining on slides following exposure to 75 Pa nominal wall-shear stress. Treatments consisted of glass slides cleaned with acid (A), Triton X-100 (T), or coated with a natural biofilm. Excess KCl (10 mmol $l^{-1}$) was applied to all treatments. Error bars = 1 SE.

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