Macrofouling in unidirectional flow: miniature pipes as experimental models for studying the interaction of flow and surface characteristics on the attachment of barnacle, bryozoan and polychaete larvae

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ABSTRACT: Tubes constructed of different materials were used to examine the role of hydrodynamics and surface characteristics, as measured by contact angles, on larval attachment of marine polychaetes, bryozoans and barnacles. Tubes (10 mm inner diameter) of 7 different materials, ranging in contact angle from 25° to 113°, were used. Laboratory and field experiments were conducted using a range of flow rates from 1.7 to 84.9 cm s^{-1}. For all 3 taxa of organisms, the results of field experiments were similar to those of the laboratory experiments. Larval settlement of the polychaete *Hydroides elegans* and the bryozoan *Bugula neritina* was the highest at a flow rate of ≤2.1 cm s^{-1}, and decreased when the flow rate was increased. *H. elegans* larvae settled within the narrowest range of flow rates (1.7 to 9.1 cm s^{-1}), *B. neritina* larvae settled over the widest range of flow rates (1.7 to 53.0 cm s^{-1}), and barnacle larvae (*Balanus* spp.) settled in an intermediate range of flow rates (4.2 to 21.2 cm s^{-1}). Barnacle larvae did not settle when the flow rate was <2.1 or >21.2 cm s^{-1}, and the highest settlement was recorded at the flow rate of 10.6 cm s^{-1}. Barnacle larvae showed the highest settlement in glass tubes (smallest contact angle) and the lowest in Teflon tubes (largest contact angle), while *B. neritina* showed the highest larval settlement in Teflon tubes and the lowest settlement in glass tubes. *H. elegans* also showed the highest larval settlement in Teflon tubes, but the lowest settlement in polyurethane and polyethylene tubes. Biofilm formation changed the contact angles of all the tubes, the degree of change varied among the 7 types of tubes. Bacterial densities of biofilm on the 7 types of tubes were significantly different and were not correlated with the initial contact angles of tubes. The bacterial densities on the Teflon tubes were the same as on glass tubes. Our results suggest a very complex interaction among substratum characteristics, flow rates, and larval settlement behavior and indicate the necessity for considering the interaction when one interprets laboratory bioassay data on larval settlement of marine invertebrates.

KEY WORDS: Fouling · Hydrodynamics · Barnacles · Bryozoans · Tubeworms · Contact angle · Flow rate

INTRODUCTION

The settlement patterns of macrofoulers in a unidirectional flow system, such as seawater intake pipes, should be determined by larval availability, flow dynamics, and physiochemical properties of the pipes. The latter strongly influences biological settlement (Rittschof & Costlow 1989a,b, Roberts et al. 1991) and adhesion and hence biofouling (Baier 1973, 1982, Burchard et al. 1991). Surface energy, a physiochemical property of all surfaces, has clear effects on larval settlement (review of Crisp 1984, Rittschof et al. 1998).

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Biofilms also directly affect the settlement of a variety of macrofouling larvae, such as barnacles, bryozoans and polychaetes (Crisp 1984, Maki et al. 1988, Holmström et al. 1992, Hadfield et al. 1994, Lau & Qian 1997, Olivier et al. 2000). Different substrata often support very different fouling communities, depending upon the time and duration of immersion (Becker & Wahl 1991, Holm et al. 1997).

Surface energy can be understood in terms of the way that fluids spread on a surface. A surface upon which water beads has low free surface energy. A surface with low free surface energy has a low wettability and is hydrophobic; organic solvents spread rather than bead on a surface with low surface energy. Examples of low surface energy surfaces are Teflon and most plastics. On the other hand, water spreads on a surface with high surface free energy. By definition, surfaces with high surface free energy are wettable and hydrophilic. Surface wettability is quantified by contact angle (the angle of a bead of fluid on a surface; Young 1805) by numerous liquids other than water and is correlated with the formation and adhesion of microbial biofilms (Baier 1973, Vrolijk et al. 1990). A surface with high surface energy has a small contact angle, while one with low surface energy has a large contact angle. When contact angles of a surface are measured with organic solvents, they are recorded as the contact angles under non-polar conditions; when measured with pure water, they are recorded as the values under polar conditions.

In seawater, surface energy is temporally variable. Interaction of molecules in the water with the surface changes the surface energy (Meyer et al. 1988, Rittschof & Costlow 1989b). Ultimately, the biological effect of surface properties on fouling depends upon a complex suite of variables that include time of year, availability of propagules in the water column, and predation (Roberts et al. 1991, Walters 1992a,b, Holm et al. 1997).

Furthermore, larval settlement in response to different cues is under the influence of many other parameters, including boundary layer flows (Butman 1986, 1987, Wethey 1986, Pawlik et al. 1991, Walters 1992a,b, Mullineaux & Garland 1993, Walters et al. 1997), chemical cues from the environment, molecular and bacterial films, and substratum conditioning (Kirchman et al. 1982a,b, Crisp 1984, Wethey 1986, Eckman et al. 1990, Pawlik 1992, Walters 1992a,b, Hadfield et al. 1994, Bryan et al. 1997, 1998, Lau & Qian 1997, Beckmann et al. 1999, Harder & Qian 1999). For instance, previous studies have documented the settlement responses to surface energy by *Balanus* spp. and *Bugula neritina* (2 of the 3 groups that are the topic of this report) in a single flow (Rittschof & Costlow 1989a), in the absence of flow (Gerhart et al. 1992), as well as in the field in

highly variable and undefined flows (Rittschof & Costlow 1989b, Roberts et al. 1991). Both groups show a clear relationship between substratum surface energy and settlement. *Balanus* spp. settle preferentially on high surface energy surfaces such as glass, while *B. neritina* settle in highest proportion on low surface energy surfaces such as fluorocarbons and polystyrene. Different surface energies can be generated which physically trap the larvae that contact them (Rittschof & Costlow 1989a, Gerhart et al. 1992). There are no reports of settlement responses of *Hydroides elegans* to surface energy.

There have been many experiments examining settlement behavior in the field, which have all, one way or another, tested for habitat selection in response to surface properties under natural flow conditions (e.g. Walters 1992a,b, Qian et al. 1999). In most field studies it is difficult to determine whether surface characteristics, flow, or a combination of the two are responsible for the observed settlement and recruitment patterns.

The objective of the present study was to determine the influence of hydrodynamics and surface properties of the substratum on larval attachment in pipes by 3 cosmopolitan fouling organisms: the tube-building polychaete *Hydroides elegans* (Haswell), the bryozoan *Bugula neritina* (L) and the barnacle *Balanus amphitrite* Darwin. We hypothesized that larval settlement is in response to surface characteristics, regardless of the flow rates being exposed. To test this hypothesis, both laboratory and field experiments for larval settlement of the 3 groups of foulers were conducted with tubes of different materials (with different contact angles), and were subjected to a wide range of flow rates.

MATERIALS AND METHODS

Studies were conducted in the laboratory with cultured larvae of *Hydroides elegans* and *Balanus amphitrite*, as well as brooded larvae of *Bugula neritina* that were released from adults that had been collected from the field. Field studies were carried out during times when competent larvae were available in the water column. In the field, *H. elegans* and *B. neritina* could be identified unequivocally, while balanoid barnacles could only be identified to genus.

The life histories and settlement biology of larvae of these 3 species have been well documented (see references listed in Qian et al. 1999).

Larval cultures for laboratory experiments. *Hydroides elegans:* Polychaete *Hydroides elegans* larvae were obtained as a laboratory culture by following the methods described in Bryan et al. (1997). Briefly, adult *H. elegans* were obtained from glass panels that were submerged at a fish farm in Port Shelter, near the Hong

Kong University of Science and Technology (HKUST), or from a laboratory broodstock. Adult worms were placed individually in Petri dishes containing filtered seawater (FSW), and their tubes were gently broken to induce the release of eggs and sperm. Eggs of several females were combined in 1 dish, and were treated with a diluted sperm suspension collected from 2 to 3 males. Eggs were exposed to the sperm suspension for 15 min, then carefully washed twice with FSW to remove excess sperm and transferred to a 4 l Nalgene beaker containing FSW. Since a fertilization rate of >95% can be easily achieved over a wide range of sperm concentrations (Pechenik & Qian 1998), sperm concentration was not monitored in the present study. Larvae were fed daily and maintained, following the method of Qiu & Qian (1997a). Larval competence to settle and metamorphose was assessed by exposing the larvae to a solution of 10^{-4} M isobutyl methylxanthine (IBMX), following the method of Qian & Pechenik (1998). Competent larvae attached within 1 to 2 h and metamorphosed within 12 h. All settlement experiments were performed with 5- to 6-d-old larvae, as counted from the time of fertilization.

Bugula neritina: Bryozoan *Bugula neritina* larvae were released from brooding adults in response to a light shock (Rittschof et al. 1988). Adult colonies utilized were collected from ropes hanging from a floating dock in a fish farm near HKUST. In the laboratory, these colonies were placed in a beaker containing aerated seawater and kept in the dark overnight. The next morning, colonies were transferred to a beaker containing FSW and placed directly under an artificial light. Adults released larvae in response to this light shock. Larvae were then attracted to another light source and within 10 min were pipetted into a clean container of FSW for use in settlement experiments. Feeding was not necessary as *B. neritina* larvae are lecithotrophic and settle within 2 to 3 h after being released (Bryan et al. 1998).

Balanus amphitrite: Barnacle larvae were cultured by a modification of the procedures of Rittschof et al. (1984). Broodstocks were collected from an intertidal mud flat in Marina Cove (22° 19′ N, 114° 16′ E), Hong Kong. These stocks were kept in the laboratory in aerated seawater. Newly hatched *Artemia* sp. were offered daily as food. The water was changed, and a light shock was used to induce release of nauplius larvae (Rittschof et al. 1984, Qiu & Qian 1997b). Nauplius I or II larvae were then attracted to a light source and transferred to a 4 l culture container using a pipette. These nauplii were separated into 1 l beakers containing FSW and kept at 28°C. Planktotrophic Nauplius II larvae were fed with the diatom *Skeletonema costatum*, maintained, and examined for the presence of cyprids after 4 d, following the method of Qiu & Qian

(1997b). Cyprids were separated from late-stage nauplii by filtration through 250 and 125 µm mesh. Cyprids retained by the 125 µm mesh were stored at 6°C in FSW. Cyprids stored in this way were inactive and did not settle on the container. Three-day-old cyprids were used in the experiments, as storage for 3 d leads to predictable larval settling rates (Rittschof et al. 1984) and has been used in many previous studies (Maki et al. 1988, 1989, 1990, 1992, 1994, Qiu & Qian 1997b, Qiu et al. 1997).

Experimental setup. Both laboratory and field experiments were conducted according to the methods described by Qian et al. (1999), with minor modifications. In the laboratory, we used a seawater flow system consisting of 3 tanks: (1) a head tank (4 l), (2) a side tank (6 l) and (3) a bottom tank (6 l) (see Fig. 1 in Qian et al. 1999). A submersible pump moved water from the bottom tank to the side tank. A second pump, in the side tank, moved seawater to the head tank where the water level (and pressure) was constant throughout the experiments.

Tubes were connected to run from the bottom of the head tank into the bottom tank. For each larval species, various flow rates were established within the tubes. There were 3 replicates of each type of tube for each flow rate. The flow rate in each tube was regulated with an adjustable valve placed at the discharge end of the tube and measured with a volumetric cylinder at the outlet to the nearest 0.1 cm s^{-1}. After adjusting the flow rate, competent larvae were released into the side tank. The initial larval concentration in the system was 1 larva ml^{-1}. The larvae that did not settle on the tubes were collected in a nylon mesh cup (90 µm) kept at the outlet of each tube. The percentage of larval population in the system that entered the tubes during the experiment varied with flow rates.

After conducting the experiments with competent larvae of the 3 different species, tubes were removed from the head tank (after 12 h for the barnacles *Balanus amphitrite* and *Balanus* spp. and for *Hydroides elegans*, and 1 h for *Bugula neritina* larvae), filled with FSW and sealed at both ends, and settled larvae were enumerated with a dissecting microscope. The percentage of larval settlement was calculated as the ratio of the number of settled larvae to the number of larvae that were collected in the nylon mesh cup plus the settled larvae.

Seven types of tubes (all with 10 mm internal diameter) were used: PVC1 (Tygon brand), PVC2 (Nalgene brand), PVC3 (Nalg-nuc.), polyethylene (PE), polyurethane (PU) (Usure brand), Teflon (Nalg-nuc.) and glass (TMS brand). All the tubes were 2 m long, except the glass tubes, which were 30 cm long.

For observing the settlement of *Hydroides elegans* larvae in the laboratory, tubes were pre-soaked in sea-

water for 48 h to allow the development of a biofilm on the tube walls. For larval settlement of *Bugula neritina* and *Balanus amphitrite*, the experiments were conducted in clean tubes. Previous studies showed that *H. elegans* larvae do not settle on unfilmed surfaces (Bryan et al. 1997, 1998, Lau & Qian 1997, Harder & Qian 1999).

Field experiments were conducted for 3 d on both *Balanus* spp. and *Bugula neritina*, and for 5 d on *Hydroides elegans* at a fish farm in Port Shelter near HKUST. A submersible pump supplied seawater to the tubes from a depth of 1 m (see Fig. 2 in Qian et al. 1999). The tubes were connected to the pump by a connector, and the same 7 types of tubes used for the laboratory experiments were employed here. Tubes were suspended horizontally from a floating raft, and aligned parallel in a single row in total darkness by fitting all the tubes in larger, black rubber tubes. The flow rate through each tube was regulated with adjustable valves at both ends of the tubes. Flow rates were checked daily by volumetric measurement at the outlet. During the experiments, the temperature and salinity of the seawater were measured daily with a thermometer and a portable refractometer (ATAGO S/Mill). Experiments were conducted during a period in which hydrobiological conditions at experimental sites were relatively stable, in order to avoid fluctuations in salinity and temperature that might affect the settlement rates of the larvae. After each deployment, tubes were removed from the experimental apparatus, and the settled larvae were enumerated under a dissecting microscope as described for the laboratory experiments. Since a fine mesh is required to collect unsettled larvae of all 3 species, but meshes were rapidly clogged in the field, we attempted to estimate the number of larvae passing through the tubes by quantifying larval density in the water column. Each day over the course of the experiments, 20 l of seawater was pumped from the experiment site with a submersible pump. The numbers of *Balanus* spp. cyprids and larvae of *H. elegans* and *B. neritina* were quantified for each sample. The average larval density in the water column over the experimental period was used to calculate the estimated total number of larvae going through the tube. This technique worked best for barnacle larvae. Larvae of *B. neritina* settled in the sampling apparatus and containers before we could count them. Estimates of *H. elegans* were very low; these larvae may be patchy in time or space. Thus, we could not determine the number of *H. elegans* and *B. neritina* larvae that settled in the tubes as a percentage. For these species, we report only the raw total number of larvae that settled in the tubes under different flows.

Surface preparation for contact angle measurement. Because the tubes were curved, it was difficult to measure the contact angle of the tube surfaces. Thin flat surfaces were required to make accurate measurements. To this end, the tubes of each type were first cleaned and washed in deionized distilled water, cut open, and cut into small pieces. These pieces were dried overnight before being pressed in a PHI compression press (Model SQ230C-XL-3A-21). The samples were pressed at different temperatures (according to their melting points), and held under pressure for 5 to 10 min before the heaters were switched off, then cooled at a rate of about 2°C min^{-1}, which is the natural cooling rate of the press. This limited heat treatment does not change the molecular structure of polymers (Li pers. comm.). To ensure that the sample surfaces were not affected by the surfaces of the press, tube samples were pressed between aluminum foil without using a mold-release agent. The samples were removed from the mold with care, avoiding contamination of their surfaces, and then stored under ambient conditions before contact angle measurement. Contact angles for both unfilmed and filmed tubes were based on flat pieces, and all of the tubes were treated in the same way. Our results are comparable to the contact angle readings of glass tubes found in earlier studies (Rittschof & Costlow 1989b), indicating that the flattening treatment did not affect contact angle measurement very much. Contact angles of each test substratum were measured with a KRUSS contact angle measuring instrument.

Water (HPLC Grade) and methylene iodide were used as test liquids for measuring the contact angle of the test substratum under polar and non-polar conditions respectively. A micromatic syringe was used to place test liquid on the sample surfaces. One drop of the test liquid was placed onto the sample surface. Contact angles were measured using volume control of the sample drops at room temperature (between 18 and 21°C). Five small pieces for each type of tube material were used to measure both the receding and advancing angles of each drop of testing liquid. The data for the receding and advancing angles were averaged to give the contact angle of each tube material.

To examine the effect of biofilm on the contact angles, natural seawater was pumped through the tubes to allow the development of biofilm on their inner walls. After 48 h, the tubes were disconnected from the pump system, and a small section of each tube (about 20 cm from the opening) was prepared for contact angle measurement following the method described above. Heating and pressing the tubes may affect the surface characteristics of the biofilm. Therefore, we only want to examine the general trend of changes in contact angles of filmed, when compared with unfilmed, surfaces. We found that the changes followed the general trend (Gerhart et al. 1992), except

for glass tubes, which appeared to be due to the effect of the high temperature required for flattening the tube. Heating glass to 500°C results in very high surface energy (Baier 1984). In the case of the lower-temperature-treated surfaces, our results should provide trends and are reported with these experimental limitations in mind.

To determine the relationship between bacterial density and the contact angles of different types of tubes, bacteria attached to the inner walls of tubes after 48 h were stained with DAPI, and their densities were determined under light microscopy at 1000× magnification (Turley 1993). The numbers of bacterial cells under 10 microscopic fields were enumerated using 5 tubes for each tube type.

Data analyses. All the statistical analyses were based on un-transformed data. Whenever the data set did not meet the normality assumptions of parametric analysis (Shapiro & Wilk test, Zar 1999), non-parametric ANOVA (Kruskal-Wallis) and non-parametric multiple comparison tests were used. Other data sets were analyzed by parametric ANOVAs (1-way or 2-way) and Tukey-*B* multiple comparison tests (Zar 1999). Specifically, 2-way ANOVA was used to test the effects of tube materials and biofilm on contact angles of 7 types of tubes under polar or non-polar conditions respectively. Due to strong interaction between tube materials and biofilm effect, 1-way ANOVA and Tukey-*B* multiple comparison tests were used to rank the tube types based on their contact angles for non-filmed tubes under either polar or non-polar conditions, as well as for filmed tubes under both conditions. The Kruskal-Wallis test and non-parametric multiple comparison test were used to test the effect of the tube type on larval settlement at each given flow rate. Non-parametric Spearman's rank correlation coefficient test was used to explore the relationship between contact angles and larval settlement at any given flow rate for each species in either laboratory or field experiments (either percentage or in number of larvae settled) (Zar 1999). The effect of the tube type on bacterial density on the inner walls of tubes was assessed using 1-way ANOVA followed by a Tukey-*B* multiple comparison test (SPSS package, Version 4.0).

RESULTS

Contact angle of 7 types of tubes

Materials for constructing tubes (tube type), and biofilm on the tubes, significantly affected the contact angles of tubes measured under either polar or non-polar conditions (Fig. 1, Table 1). However, there were also strong interactions between tube types and biofilm effects. Therefore, 1-way ANOVAs and Tukey-*B* multiple comparison tests were used to rank the 7 types of tubes (filmed or unfilmed) under polar or non-polar conditions respectively. When the tubes were unfilmed, the contact angles of the 7 types of tubes could be arranged as follows: for polar conditions, Teflon > PVC 3 > PU = PVC2 > PE > PVC 1 > Glass (1-way ANOVA, $F_{6,34} = 425.6$, p < 0.0001); for non-polar conditions, Teflon > PVC 3 > PU > PE = Glass > PVC2 > PVC 1 (1-way ANOVA, $F_{6,34} = 52.1$, p < 0.0001) (Fig. 1). When the tubes were filmed, the contact angles changed substantially. For the polar conditions, the contact angles of the filmed tubes decreased, with the largest decrease being found in PVC3 (>74%
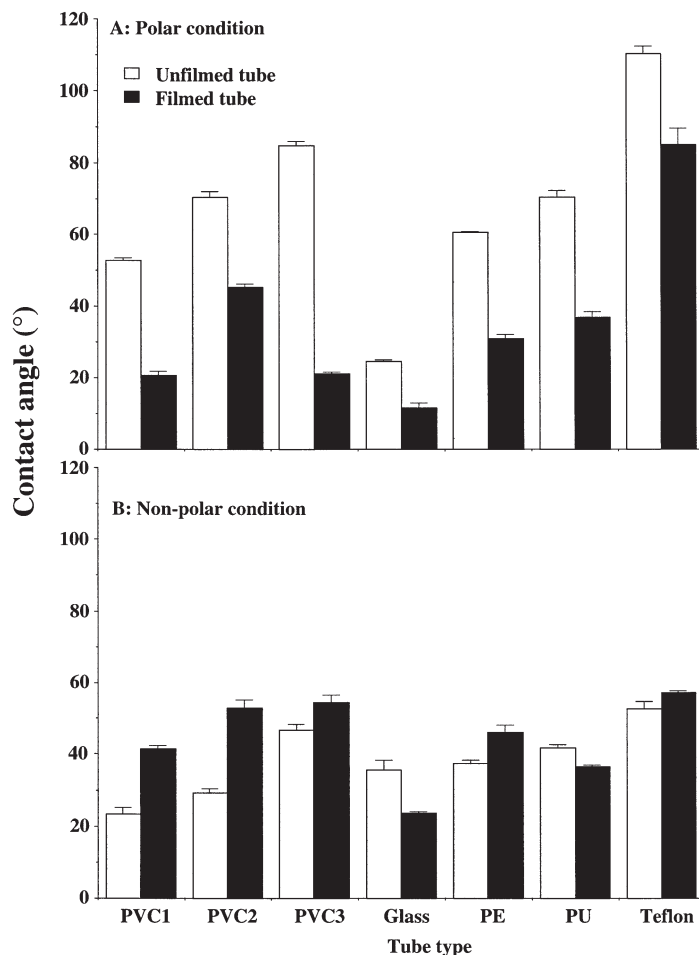

Fig. 1. Contact angle measurements of 7 types of tubes: (A) contact angles measured in polar conditions; (B) contact angles measured in non-polar conditions (open bars: unfilmed tubes; solid bars: tubes with 48-h-old natural biofilm)

Table 1. Summary of 2-way ANOVA results on effects of biofilm and types of tubes on contact angles of tubes

Factor(s)	df	SS	F	p
Polar				
Filmed/non-filmed (A)	1	7037	1240	<0.0001
Tube type (B)	6	14 116	415	<0.0001
A × B	6	1445	42	<0.0001
Error	14	80		
Non-polar				
Filmed/non-filmed (A)	1	345	84	<0.0001
Tube type (B)	6	2159	88	<0.0001
A × B	6	786	32	<0.0001
Error	14	57		

drop) (Fig. 1). This decrease might have been due to the leaching of dibutyl tin to the surface. The resulting contact angles could be arranged as follows: Teflon > PVC 2 > PU > PE > PVC3 = PVC1 > Glass (1-way ANOVA, $F_{6, 34} = 147.3$, p < 0.0001) (Fig. 1). For non-polar conditions, the contact angles of filmed tubes increased significantly, except in the case of glass tubes, for which the contact angles decreased in both the polar and non-polar conditions when the tubes were filmed. The resulting contact angles could be arranged as follows for non-polar conditions with filmed tubes: Teflon > PVC 3 = PVC 2 > PE > PVC 1 > PU > Glass (1-way ANOVA, $F_{6, 34} = 67.7$, p < 0.0001) (Fig. 1). Overall, Teflon tubes had the largest contact angles, while glass tubes had the smallest.

Larval settlement in response to flow rate in different types of tubes

Hydroides elegans

Larval settlement of *Hydroides elegans* was significantly affected by both flow rates and type of tubes in the field experiment but with strong interaction between the effects of flow rates and types of the tubes (Fig. 2A). Larval settlement decreased with increases in flow velocity for all tube types, except for the glass tube, in which the number of settled larvae increased slightly, reaching their largest number at the flow rate of 2.1 cm s^{-1} and then decreased rapidly (Fig. 2A). Among the 7 types of tubes, at a given flow rate, larval settlement was almost always the highest in the Teflon tube, second highest in the PVC3 or PVC2 as well as the glass tube, and lowest in the PU and PE or PVC 1 tubes (Table 2). There was no correlation be-

tween the number of settled larvae and the contact angle of filmed tubes (Table 3). Since larvae of this species do not settle well on surfaces without biofilm (Beckman et al. 1999), we concluded that larval settlement of *H. elegans* does not respond to contact angles of filmed tubes in field conditions. Overall, *H. elegans* only settled at very low flow rates and within a very narrow flow regime. No larvae settled in the tubes when the flow rates were at or above 12.7 cm s^{-1}.

Larval settlement of *Hydroides elegans* in the laboratory experiments (Fig. 2B) differed from that observed in the field experiments, although it was also affected by both flow rates and type of
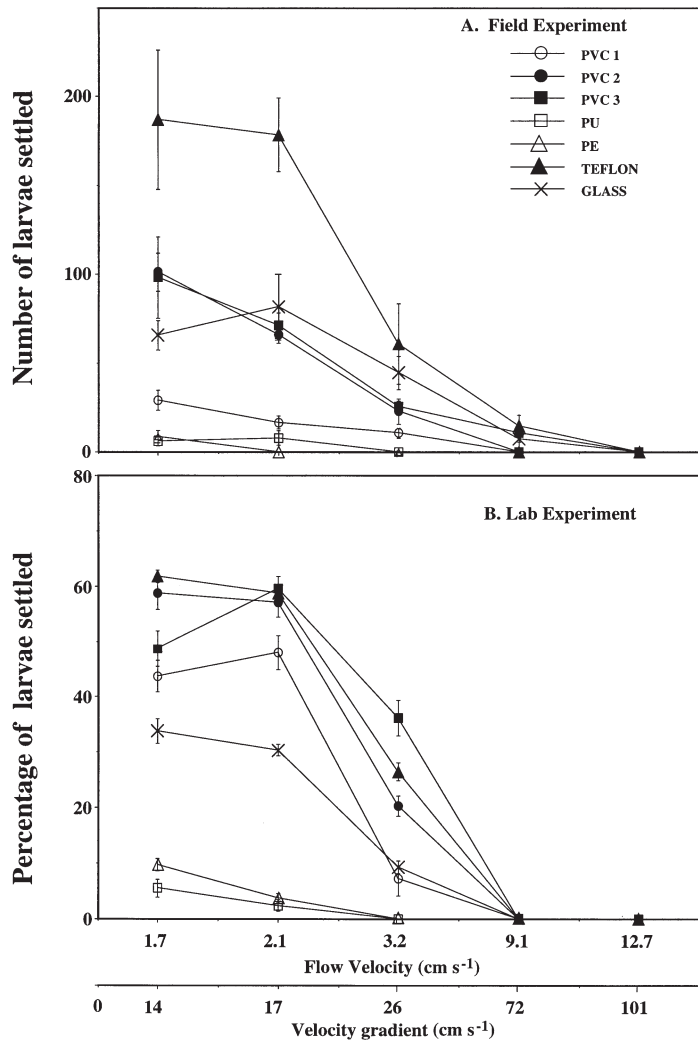

Fig. 2. Settlement of *Hydroides elegans* in 7 types of 10 mm diameter tubes: (A) in the field and (B) in the laboratory. Mean percentage of settlement or mean number of larvae settled ±SD is plotted against mean flow velocity and mean velocity gradient. The velocity gradient was calculated according to Crisp (1955) and Qian et al. (1999), and was rounded up to the nearest integrate number

tubes, and the 2 factors interacted strongly (Fig. 2B, Table 2). In both the PVC3 and PVC1 tubes, larval settlement first increased with flow rate and then dropped rapidly, while the larval settlement in other types of tubes dropped with increased flow rate. Larval settlement was the highest in the PVC3, Teflon or PVC2 tubes, and lowest in the PU and PE tubes (Fig. 2B, Table 2). Finally, larval settlement in the glass tube was higher than that in either the PE or PU tubes. In addition, the range of flow rates for larval settlement was narrower in the laboratory experiments, since no larvae settled in any types of tubes when flow velocities were at or above 9.1 cm s^{-1} (Fig. 2B). There was no correlation between the percentage of settled larvae and the contact angle of filmed tubes at any flow rate (Table 3).

Table 2. Summary of the results of the non-parametric Kruskal-Wallis test and non-parametric multiple comparison test (Zar 1999) on effects of types of tubes on larval settlement of *Hydroides elegans*, *Bugula neritina* and *Balanus* spp., in both field and laboratory experiments. Values not differing at 0.05 level are joined by underlining. Values of all parameters are shown in ascending rank order from left to right

Expt	Flow rate (cm s^{-1})	H	p	Tube types
H. elegans				
Field	1.7	18.5	<0.01	PU PE PVC1 Glass PVC3 PVC2 Teflon
	2.1	18.3	<0.01	PE PU PVC1 PVC2 PVC3 Glass Teflon
	3.2	17.9	<0.01	PE PU PVC1 Glass PVC3 PVC2 Teflon
	9.1	15.1	<0.05	PU PVC2 PE PVC1 Glass PVC3 Teflon
	12.7	No settlement		
Lab.	1.7	17.8	<0.01	PU PE Glass PVC1 PVC3 PVC2 Teflon
	2.1	19.2	<0.01	PU PE Glass PVC1 PVC2 Teflon PVC3
	3.2	19.0	<0.01	PE PU PVC1 Glass PVC2 Teflon PVC3
	9.1	No settlement		
	12.7	No settlement		
B. neritina				
Field	1.7	18.4	<0.01	Glass PVC1 PVC2 PU PVC3 PE Teflon
	8.5	18.7	<0.01	Glass PVC1 PVC2 PU PE PVC3 Teflon
	12.7	18.0	<0.01	Glass PU PVC2 PVC1 PE PVC3 Teflon
	21.2	18.1	<0.01	PVC1 Glass PVC2 PU PE PVC3 Teflon
	42.5	15.8	<0.05	PVC1 PVC2 Glass PU PE PVC3 Teflon
	53.0	12.3	>0.05	PVC1 PVC2 PE Glass PU Teflon PVC3
	74.9	No settlement		
Lab.	1.7	18.8	<0.01	Glass PU PVC1 PVC2 PE PVC3 Teflon
	8.5	18.7	<0.01	Glass PVC1 PU PVC2 PVC3 PE Teflon
	12.7	18.9	<0.01	Glass PU PVC1 PVC2 PVC3 PE Teflon
	21.2	17.9	<0.01	PU PVC1 Glass PVC2 PVC3 PE Teflon
	31.9	17.8	<0.01	PU PVC1 Glass PVC3 PVC2 PE Teflon
	42.5	No settlement		
	53.0	No settlement		
Balanus spp.				
Field	2.1	No settlement		
	4.2	15.6	<0.05	PE Teflon PU PVC3 PVC2 PVC1 Glass
	7.4	12.8	<0.05	Teflon PVC3 PE PU PVC2 PVC1 Glass
	10.6	17.8	<0.01	Teflon PVC3 PE PU PVC1 PVC2 Glass
	21.2	17.1	<0.01	Teflon PU PE PVC3 PVC1 PVC2 Glass
	31.9	No settlement		
Balanus amphitrite				
Lab.	2.1	No settlement		
	4.2	No settlement		
	7.4	15.5	<0.05	Teflon PVC3 PE PU PVC2 PVC1 Glass
	10.6	17.7	<0.01	Teflon PE PU PVC3 PVC1 PVC2 Glass
	21.2	15.8	<0.05	Teflon PVC3 PU PE PVC1 PVC2 Glass
	31.9	No settlement		

Bugula neritina

Larval settlement of *Bugula neritina* was significantly affected by both flow rates and type of tubes in the field experiment, but with strong interaction between the 2 factors (Fig. 3A, Table 2). Larval settlement of *B. neritina* decreased with increases in flow velocity for all tube types (Fig. 3A). At all the flow rates, larval settlement was the highest in the Teflon tube, second highest in the PVC3 and PE tubes, and lowest, but similar, in the other types of tubes (Table 2). At all the flow rates, there was a positive correlation between the number of settled larvae and the contact angle of filmed tubes (please see rho- and p-values in Table 3). We concluded that the contact angle of the tubes affects the larval settlement of this species.

The same trend was found in the laboratory experiment (Fig. 3B, Tables 2 & 3). There was no larval settlement in any type of tube when flow velocity reached 42.5 cm s^{-1}. In comparison, few larvae settled even at a flow rate of 53.0 cm s^{-1} in the field experiment.

Balanus spp.

Larval settlement of *Balanus* spp. was significantly affected by both flow rates and type of tubes in the field experiment, but with strong interaction between the 2 factors (Fig. 4A, Table 2). In the glass, PVC1 and PVC2 tubes, larval settlement of *Balanus* spp. first

increased with flow, reached its highest level at a flow rate of 10.6 cm s^{-1}, and then decreased with a further increase in a flow rate (Fig. 4A). The same trends were observed for the PU and PE tubes. In the Teflon and PVC3 tubes, however, the highest settlement was recorded at a flow rate of 4.2 cm s^{-1}. Overall, attachment was the highest in the glass, PVC1 and PVC2 tubes and, in general, the lowest in the Teflon tubes. However, due to the large variations observed within each tube type, the non-parametric multiple comparison tests failed to separate the other 6 types of tubes, except glass, at most of the flow rates tested (Table 2). At all the flow rates, there was a negative correlation between the percentage of settled larvae and the con-

tact angle of filmed tubes (please see rho- and p-values in Table 3), indicating that *Balanus* spp. settled mostly on materials with relatively small contact angles.

Table 3. Summary of Spearman's rank correlation coefficient between contact angles of 7 types of tubes under polar condition and larval settlement. For *Hydroides elegans*, the correlation between contact angles of filmed tubes and larval settlement was calculated for both the laboratory and field experiments; for both *Bugula neritina* and *Balanus* spp., the correlation between contact angles of filmed tubes and larval settlement was calculated for the field experiment, but the correlation between contact angles of unfilmed tubes and larval settlement was calculated for the laboratory experiments. For each test, df = 20

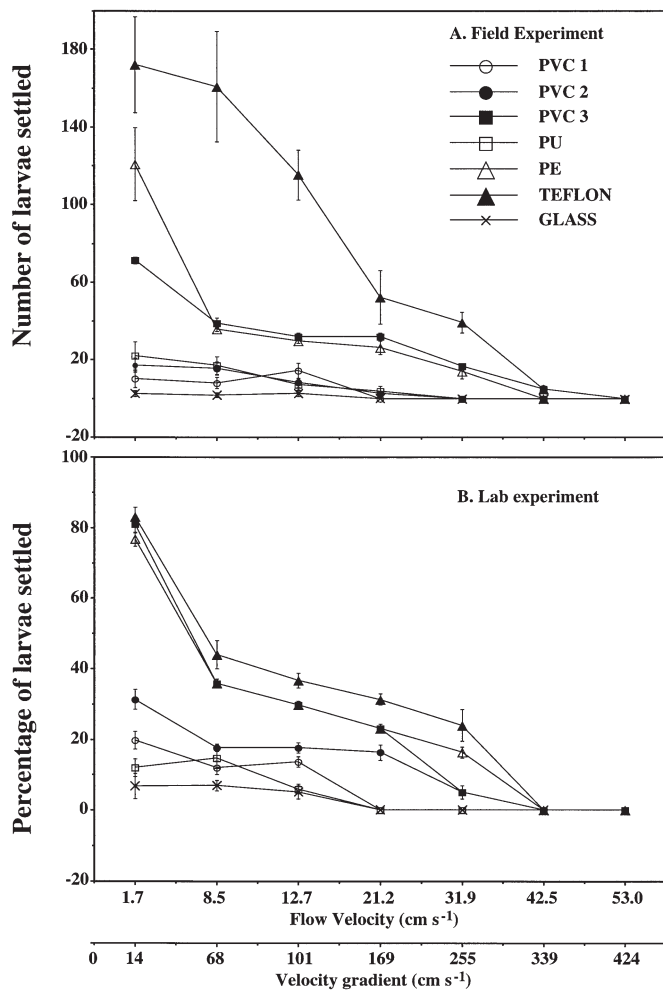| Expt	Flow rate (cm s^{-1})	Rho	Z	p
H. elegans				
Field	1.7	0.34	1.51	0.13
	2.1	0.20	0.91	0.36
	3.2	0.04	0.18	0.86
	9.1	0.085	0.38	0.71
	12.7	No settlement		
Lab.	1.7	0.42	1.86	0.060
	2.1	0.28	1.24	0.21
	3.2	0.19	0.85	0.40
	9.1	No settlement		
	12.7	No settlement		
B. neritina				
Field	1.7	0.63	2.83	0.0046
	8.5	0.63	2.83	0.0046
	12.7	0.43	1.94	0.052
	21.2	0.61	2.74	0.0062
	42.5	0.47	2.09	0.037
	53.0	0.42	1.88	0.060
	74.9	No settlement		
Lab.	1.7	0.69	3.09	0.002
	8.5	0.78	3.48	0.0005
	12.7	0.64	2.88	0.0039
	21.2	0.70	3.13	0.0017
	31.9	0.60	2.66	0.0078
	53.0	No settlement		
***Balanus* spp.**				
Field	2.1	No settlement		
	4.2	−0.53	−2.24	0.017
	7.4	−0.50	−2.24	0.025
	10.6	−0.51	−1.29	0.022
	21.2	−0.55	−2.48	0.013
	31.9	No settlement		
Balanus amphitrite				
Lab.	2.1	No settlement		
	4.2	No settlement		
	7.4	−0.76	−3.38	0.0007
	10.6	−0.78	−3.49	0.0005
	21.2	−0.58	−2.60	0.0093
	31.9	No settlement		

Fig. 3. Settlement of *Bugula neritina* in 7 types of 10 mm diameter tubes: (A) in the field and (B) in the laboratory. Mean percentage of settlement or mean number of larvae settled ±SD is plotted against mean flow velocity and mean velocity gradient. The velocity gradient was calculated according to Crisp (1955) and Qian et al. (1999), and was rounded up to the nearest integrate number
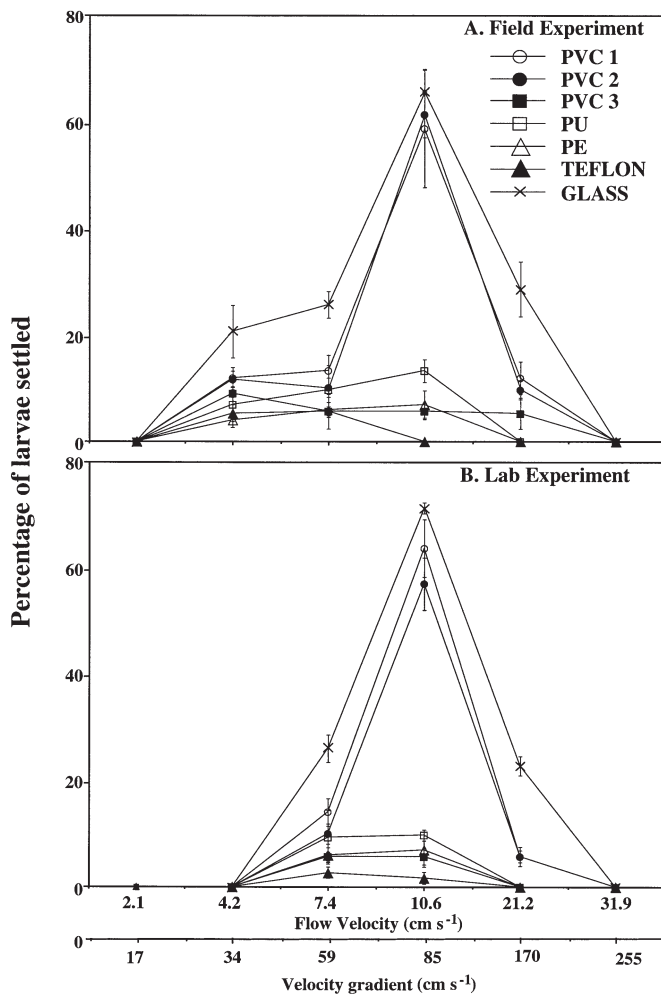
Fig. 4. Settlement of barnacle larvae in 7 types of tubes: (A) *Balanus* spp. in the field and (B) *Balanus amphitrite* in the laboratory. Mean percentage of settlement ±SD is plotted against mean flow velocity and mean velocity gradient. The velocity gradient was calculated according to Crisp (1955) and Qian et al. (1999), and was rounded up to the nearest integrate number

Larval settlement of *Balanus amphitrite* was significantly affected by both flow rates and type of tubes in the laboratory experiment, but with strong interaction between the 2 factors (Fig. 4B, Table 2). Trends in barnacle attachment in the laboratory experiments were much clearer than those in the field experiments (Fig. 4B). The 7 types of tubes could be separated into 2 distinct groups. In the glass, PVC1 and PVC2 tubes, larval settlement first increased with flow, reached the highest level at a flow rate of 10.6 cm s^{-1}, and then decreased with a further increase in flow rate. Larvae settled in flow rates ranging from 4.2 to 21.2 cm s^{-1}. In the other 4 types of tubes, larval settlement increased with flow, reached the highest level at a flow rate of 7.4 cm s^{-1}, and then dropped to 0 at a flow rate of

21.2 cm s^{-1}. Overall, the glass tube had the highest attachment at any given flow rate, the PVC1 and/or PVC2 tubes had the second highest, whereas the Teflon tube had the lowest (Table 2). At all the flow rates, again, there was a negative correlation between the number of settled larvae and the contact angle of unfilmed tubes (Table 3).

Bacterial density on the inner walls of the 7 types of tubes

The effect of tube type on bacterial density was highly significant (1-way ANOVA: $F_{6,29} = 75.2$, p < 0.0001) (Fig. 5). The Tukey-*B* multiple comparison test indicated that the bacterial density on the walls of the PVC1 tube was the highest, and those on the walls of the PVC2 and PVC3 tubes were the second highest, yet similar. The bacterial density on the PE tube walls was the third highest, while those on the walls of the glass and Teflon tubes were the lowest.

Comparisons of the responses of *Balanus* spp., *Hydroides elegans* and *Bugula neritina* to different flow rates

Larvae of the 3 organisms responded differently to the flow conditions presented. *Bugula neritina* settled maximally in low flow conditions but settled over a wide range of flows, while *Hydroides elegans* settled maximally in very low flow conditions and settled over a relatively narrow range of flows. Both *H. elegans* and *B. neritina* larvae settled maximally at a velocity of 1.7 to 2.1 cm s^{-1}. In contrast, *Balanus* spp. larvae settled over a relatively narrow range of higher flow rates, and maximally at a flow rate of 10.6 cm s^{-1} (5 to 10 times higher than the optimal rates for the other 2 species).

Comparisons of the responses of *Balanus* spp., *Hydroides elegans* and *Bugula neritina* to different types of tubes

Larval settlement of the 3 organisms in the 7 types of tubes was significantly different. *Balanus* spp. larvae showed the highest settling rate on the glass tube but the lowest settling rate on the Teflon tube. This trend was very consistent at all flows tested in the field experiments regardless of whether the tubes were filmed or not (Table 3). In comparison, there was no correlation between the type of tubes and larval settlement in *Hydroides elegans*, while there was a very good correlation in *Bugula neritina*, which had the highest settling rate on the Teflon tube (see Table 3).
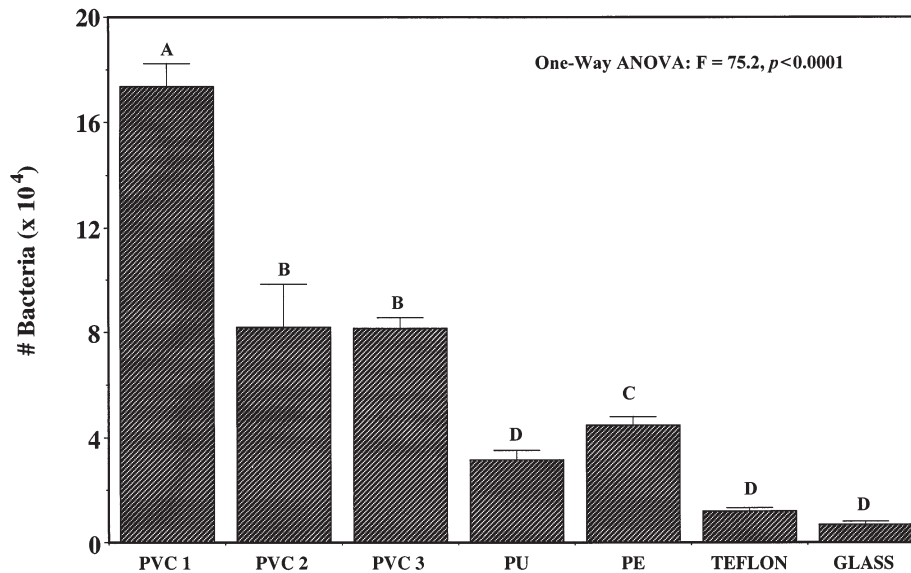
Fig. 5. Bacterial density on inner walls of 7 types of 10 mm diameter tubes developed in the laboratory over 48 h. Mean density ±SD is plotted. Different letters above the bars indicate data with significant differences

DISCUSSION

In the laboratory and field experiments, with very little variation, there were consistent negative correlations between larval settlement of barnacles and contact angles of tubes, regardless of the flow rates or of the filmed or unfilmed tubes (Table 3), indicating that barnacle larvae preferentially settled on the materials with smaller contact angles, such as glass tubes. Materials, such as Teflon tube, that have larger contact angles were clearly less attractive to barnacle larvae. In comparison, there were rather consistent positive correlations between larval settlement of *Bugula neritina* and contact angle of tubes (either filmed or unfilmed), also regardless of the flow rates (Table 3), indicating that larvae of this species preferred to settle on the materials with large contact angles. *B. neritina* larvae showed the highest settlement in Teflon tube and the lowest in glass tube (Fig. 3, Tables 2 & 3). Although *Hydroides elegans* larvae also showed the highest settlement in Teflon tube, there was no correlation between larval settlement of *H. elegans* and contact angles of tube materials (Fig. 2, Tables 2 & 3).

Why did the 3 species respond to tube materials with different contact angles differently? It has been well documented that films are a prerequisite for settlement of *Hydroides elegans* (Hadfield et al. 1994, Qian 1999, Qian et al. 1999). Larval settlement of *H. elegans* on glass slides increased linearly with microbial film of increasing age (Hadfield et al. 1994, Beckmann et al. 1999). Although numerous studies have addressed the effect of biofilms on surface properties (particularly in

terms of strength of the adhesion of molecules) (Savage & Fletcher 1985), the relationship between biofilms and the contact angles of different materials remains unclear. It is clear that the polarity of a surface will change with the nature and development of biofilm. *H. elegans* settles almost everywhere as long as the surface is filmed (see review by Qian 1999), indicating that biofilm rather than the contact angle of the surface plays an important role in larval settlement. In comparison, *Bugula neritina* larvae settled almost equally well on both filmed and unfilmed surfaces (see also Qian et al. 1999). All of the previous studies showed that the larvae of bryozoans such as *B. neritina* and *Watersipora* sp. preferred non-wettable substrata like wax and graphite (Eiben 1976, Maki et al. 1989). In the present study, we confirmed that larvae of *B. neritina* preferred to settle on the Teflon tube with the largest contact angle (low wettability), while only a few larvae settled on the glass tubes with the smallest contact angle (high wettability) (Fig. 3). Similarly, barnacle larvae do not require a biofilm for settlement, settle equally well on filmed or unfilmed tubes, and prefer to settle on glass tube (Qian et al. 1999). However, in contrast to bryozoan larvae, barnacles settled in greater numbers on substrata with small contact angles (high surface energy) (Rittschof & Costlow 1989b, Roberts et al. 1991, Qian et al. 1999). In this study, we confirmed that barnacles responded similarly in a variety of unidirectional flow conditions and settled in the greatest numbers on glass tubes and in the smallest numbers on Teflon tubes, indicating that *Balanus amphitrite* settlement in consistent flow is sim-

ilar to that in static conditions and in undefined flow (Fig. 4). Therefore, we conclude that different relationships between contact angle of tube materials and larval settlement among the 3 species are mainly due to the differences in their prerequisite for settlement.

The number of larvae settling in a pipe depends upon larval availability and the settlement responses to flow and surface type. Availability of planktonic larvae is directly related to the volume of water passing through the pipe. Thus, a flow of 2 cm s^{-1} would potentially deliver twice the number of larvae to a surface as a flow of 1 cm s^{-1}. In this study, we reported the number of larvae settled for *Hydroides elegans* and *Bugula neritina* inside tubes for the field experiment without correcting for the total water volume flowing through the tubes. If the correction is made, it decreases relative settlement at high flow rates and increases the relative settlement at low flow rates. However, the general trends would be the same, which were demonstrated by the percentage of larvae settled inside tubes plotted in Figs. 2B, 3B & 4.

Responses of *Balanus* spp. and *Balanus amphitrite* larvae to the flow in the field and laboratory experiments, respectively, show dramatic increases on wettable surfaces near an optimum flow of 10.6 cm s^{-1}. These results complement those previously reported (Rittschof et al. 1984, Rittscof & Costlow 1989a,b, Roberts et al. 1991, Qian et al. 1999) and confirmed further that barnacle larvae do not settle at very high flow. This settlement response to flow has been related to larval morphology and settling behavior (Crisp 1955). However, the optimal flow rates for *Balanus* spp. and *Balanus amphitrite* larval settlement differed from those reported in early studies for the barnacle *Balanus* (= *Semibalanus*) *balanoides* (Crisp 1955). *S. balanoides* larvae require higher velocities and hence settle in higher energy environments. *S. balanoides* have relatively large larvae and live on very high-energy, open-ocean, rocky shores, while the smaller *Balanus* spp. larvae such as *B. improvisus* and *B. amphitrite* usually occupy lower wave energy environments (O'Connor & Richardson 1996).

Larval settlement of *Bugula neritina* decreased with increase in flow rate, which is in very good agreement with previous reports (Qian et al. 1999). Decrease in settling rate with increased flow rate may indicate that larval settlement in this species is a passive process. High flow rates reduce the larval contact with surface and lead to a low settling rate. However, the relatively broad range of velocities over which *B. neritina* settle on the tubes may also suggest that the ciliated surface of *B. neritina* may enable it to gain purchase on surfaces over a wide range of flows. This may explain why this species can settle in a broader range of flow than barnacle larvae and *Hydroides elegans* larvae. As

shown in Fig. 3, although settlement of *B. neritina* was the highest at the lowest flow rate tested, some larvae settled at very high flow rates (>50 cm s^{-1}).

Hydroides elegans, with the smallest larvae of the group, had the narrowest range of settlement in relation to flow rate. Its clear propensity to settle in only very low flows suggests that swimming capability is important. Based on our observations (Qian et al. 1999, present study), one may conclude that, for both *H. elegans* and *Bugula neritina*, flow is not a requirement of larval settlement. However, all of these species (*H. elegans*, *B. neritina* and *Balanus* spp.) are found in cooling water intakes of the HKUST (Qian pers. obs.) with a continuous flow greater than 60 cm s^{-1}. This suggests that there are locations in larger pipes where turbulence may carry larvae to a surface upon which they encounter low flow conditions favorable for attachment. Growth of initial colonizers would result in new areas of favorable settlement conditions. The virtual monoculture of *H. elegans* that recently clogged the cooling water intake of HKUST attests to the fact that larvae can colonize surfaces in flows far outside the range of their initial capability in unfouled pipes.

The ability of bacteria to attach to different types of tubes varied (Fig. 5). Bacteria did not attach well to tubes that had either the largest contact angles (Teflon tube) or the smallest contact angles (glass tubes). Although it has been suggested that both surface free energy and surface charge influence bacterial attachment (Fletcher & Loeb 1979), in the present study, we found no correlation between bacterial density and contact angles of tubes. Poor correlation between contact angles and bacterial density could be caused by many parameters that were not monitored in this study. For instance, dissolved substances adsorbed by the substratum can modify the substratum's surface properties and, in turn, may play an important role in the attachment of bacteria (Neihoff & Loeb 1972, Baier 1973). Furthermore, different marine bacteria may have different preferences toward surfaces with different contact angles (Fletcher & Loeb 1979, Burchard et al. 1991). We did not study the composition of the microbial film and, thus, could not determine if there were differences in the species compositions of the biofilms found on different tube surfaces. Biofilm composition becomes increasingly complex with time and varies with the physical/chemical characteristics of the initial surface (Marszalek et al. 1979, Dempsey 1981). The composition of biofilm and its change over time, as well as its influence on larval settlement, are topics for future investigation.

It seems questionable whether our experimental design can permit full assessment of the settling process in the laboratory setup, since the tubes there were arranged vertically while those in the field experi-

ments were arranged horizontally. This concern is mainly based on the effect of gravity on larval settlement. Previous studies have indicated that larvae respond to shear forces to find surfaces (see review by Crisp 1984), and gravity plays little if any role in settlement. As discussed above, we found that larvae of the 2 groups of organisms responded to contact angles of substratum in both the laboratory and field, indicating that an active selection process was involved in larval settlement. Settlement in experiments was independent of tube orientation, and, therefore, gravity may play at best a minor role in determining the settlement patterns.

In conclusion, this study showed that larval settlement of barnacles, bryozoans and polychaetes is influenced strongly by the chemical characteristics of the substratum, as well as the flow rate. If a larva can break through the boundary layer between substratum and overlying water, its settlement is dependent on the chemical characteristics. Therefore, physical and chemical properties of the substratum should be carefully considered in the design of surfaces that come into contact with seawater. However, after a short time, this becomes less important. For this reason, a tube made of material with either a large or small contact angle will predictably promote larval settlement of these major fouling organisms until the energy of the surface is altered by adsorption of organic matter and development of biofilms.

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