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Marine biofouling field tests, settlement assay and footprint micromorphology of cyprid larvae of *Balanus amphitrite* on model surfaces

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Atomic force microscopy (AFM), laboratory settlement assays and field tests were used to correlate cyprid footprint (FP) morphology with the behaviour of cyprids on different substrata. AFM imaging under laboratory conditions revealed more porous and larger FPs on glass exposing a CH_3-surface than on aminosilane functionalised (NH_2-) surfaces. The secreted FP volume was found to be similar on both substrata (2.1–2.6 μm^3). Laboratory settlement assays and marine field tests were performed on three substrata, *viz*. untreated clean glass, NH_2-glass, and CH_3-glass. The results distinguished settlement preferences for NH_2-glass and untreated glass over CH_3-terminated surfaces, suggesting that cyprids favour settling on hydrophilic over hydrophobic surfaces. On combining observations from different length scales, it is speculated that the confined FP size on NH_2-glass may induce a higher concentration of the settlement inducing protein complex. Settlement may be further facilitated by a stronger adherence of FP adhesives to the NH_2-surface via Coulombic interactions.

Keywords: barnacle; cyprid; biofouling; atomic force microscopy; settlement assay; field test

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Introduction

Marine biofouling is a long-standing issue with economic and environmental impact. Biofouling on ships' hulls costs billions of dollars in fuel consumption and maintenance. This increased fuel consumption contributes to greenhouse gas emissions (Yebra et al. 2004). The undesirable attachment of marine organisms, such as barnacles, green algae, diatoms and mussels compromises the functioning of man-made structures immersed in seawater. This fouling degrades the performance of high added-value marine structures, such as buoy sensors and harbour installations. Among fouling organisms, barnacles represent a significant nuisance, due to their size and gregarious nature (Crisp et al. 1985). A barnacle evolves through planktotrophic nauplius stages, leading to a non-feeding cypris stage, which metamorphoses into adulthood. Cyprids have the sole mission to explore and select a permanent settling site, where the adult barnacle will remain fixed (Aldred and Clare 2008). Cyprids use a temporary adhesive, generated by a protein extract, to facilitate their reversible attachment to surfaces (Yule and Walker 1985; Matsumura et al. 1998; Dreanno et al. 2006a,b). This material, referred to as a 'footprint,' (FP) is left on the explored surface. The exact physiochemical nature of the FP adhesive material remains largely unknown. However, a settlement-inducing protein complex (SIPC), which functions as a conspecies settlement cue, has been found in FP adhesive (Matsumura et al. 1998; Dreanno et al. 2006a,b).

Significant efforts are being made to design new, environmentally benign solutions that prevent and tackle marine fouling. Concerted action is needed by materials scientists, biologists, chemists and coatings specialists to deliver significant improvements. Despite the need to understand the settlement process to implement coating design, the relevant behaviour of marine organisms remains poorly understood (Fusetani 2004; Chaudhury et al. 2005; Kavanagh et al. 2005; Aldred et al. 2006; Callow and Callow 2006; Holm et al. 2006; Krishnan et al. 2006; Fratzl 2007; Schumacher et al. 2007; Aldred and Clare 2008; Finlay et al. 2008; Hennebert et al. 2008; Kamino 2008).

The antifouling (AF) performance characteristics of current coatings are generally evaluated by settlement assays of bacteria or macrofoulers. These assays are corroborated by field tests that evaluate the performance of the coatings in marine environments. While the majority of barnacle biofouling studies focus on the settlement of adult barnacles from field tests,

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studies on the exploration stage of cyprid larvae remain elusive. Permanent, cured proteinaceous adhesives (barnacle 'cement') used by barnacle cyprids for attachment are intriguing materials. They exhibit very high adhesion strength and consist of initially water-soluble proteins, which cure underwater (Yule and Walker 1987). It is generally considered that cyprid FP proteins are different from those in the permanent cement (Odling et al. 2006; Phang et al. 2006) and that their adhesive performance is also different. Although they may be inferior adhesives, the role they play in the settlement process is crucial. If progress is to be made in designing and preparing new AF surfaces, the first steps of the fouling process, including the deposition of FP proteins at the microscopic larval stage, must be understood. This understanding would then help to devise AF strategies as opposed to making empirical efforts to optimise AF coating formulations by 'hit-or-miss' or 'empirical' approaches.

Previously, the morphology of the FPs of barnacle cyprid larvae was studied by atomic force microscopy (AFM) at the micro- and nanoscopic length scales (Aldred et al. 2008; Phang et al. 2008). The studies found that FP adhesives deposited *in situ* at hydrophobic and hydrophilic surfaces showed significant differences in FP size and morphology, consisting of thin nanofibrils and protein fibres. Studies of FP morphology and settlement behaviour of cyprids may provide information on the relationship between the spreading and adhesion of FP adhesive and settlement behaviour.

In the study reported here, the morphology of cyprid FPs was examined using AFM, comparing it with laboratory cyprid settlement tests and marine biofouling field tests. AFM provides detailed morphological information on individual FPs with micron- and nano-scale resolution. Settlement assays determine the settlement preferences of single species cyprid larvae. Field tests generate quantitative species population data in a marine environment. Comparing AFM-based studies with larval settlement and marine biofouling may provide an indication of how FP adhesive interactions with model surfaces may be used to regulate the recruitment of barnacles and other macrofouling organisms.

Materials and methods

Surface preparation

Glass microscopy coverslips (24 mm × 24 mm, Menzel-Glaser) were sonicated in ethanol for 5 min and then immersed in piranha solution (a mixture of concentrated sulphuric acid and 33% hydrogen peroxide in a 3:1 ratio) for 15 min. Surfaces were then rinsed with nanopure water and dried under a stream of compressed nitrogen gas. Amino (NH_2) and alkyl (CH_3)-terminated surfaces were obtained by gas-phase evaporation of 3-aminopropyl triethoxysilane (APTES) and dodecyltriethoxysilane in a desiccator under vacuum, respectively (Ling et al. 2006). These glass coverslips were used for FP deposition in AFM microscopy studies. APTES was obtained from Sigma Aldrich and used as received. Surfaces were incubated for several hours and then carefully rinsed with 99% ethanol and nanopure water.

Microscope slides (75 mm × 25 mm, Sail Brand reference 7101) were stripped of adsorbed organic contaminants with pyrolysis, by heating them to 500°C followed by cooling to room temperature. Vapour deposition of aminosilane (Fluka reference 09324) was performed as described in the preceding paragraph. *n*-Octadecyltriethoxy silane, OTE, (Alfa Aesar reference 230-995-9) was deposited in solution using a procedure adapted from Peanasky et al. (1995). A pre-hydrolysis solution of OTE was prepared by mixing 0.42 g OTE and 0.25 g of 1.31 N aqueous hydrochloric acid into 50 ml tetrahydrofuran (Sigma). This solution was left at room temperature for 4 h and then stored in a refrigerator at 4°C. Silanisation was achieved by immersing the clean slides in cyclohexane (Aldrich, reference 34855) and then adding the pre-hydrolysis solution in a ratio of 1.11 g per 18.6 g of cyclohexane. The slides were incubated overnight in the silanisation solution and then rinsed by ultrasonication in cyclohexane.

To check the surface properties of these model surfaces, one glass slide from every 10 was subjected to a basic quality control. For the bare glass, cleanliness was verified by the wetting and spreading, including 0° receding contact angle, of a 2 μl water drop. Aminosilane-coated glass was stained overnight using colloidal gold (Bio-Rad reference 170–6527). After rinsing, blow-drying, and cleaning off the lower surface, the presence and uniformity of the stain indicated an aminosilane coating on the glass surface. The wettability of these slides gave sessile water drop contact angles in the range 21–23°, measured over at least three sessile drops. For OTE, 10 sessile water drops were measured. The criteria for acceptance were an average in the range 107–110° and a standard deviation (SD) of 1° across each slide.

Cyprid culture

Larvae of the barnacle *Balanus amphitrite* from field-collected adults were reared on an algal mixture of 1:1 *v*/*v* of *Tetraselmis suecica* and *Chaetoceros muelleri* at 25°C, at a density of $\sim 5 \times 10^5$ cells ml^{-1}. On this regime, larvae metamorphosed to cyprids in 5 days. These cyprids were aged at 4°C for 2–3 days prior to

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use and 45–70% settlement was observed after 24 h (Willemsen et al. 1998).

Cyprid settlement assay

The settlement assay for cypris larvae of *B. amphitrite*, referred to as cyprids, was conducted in laboratory conditions. Glass slides with NH_2-terminated and CH_3-terminated chemical functionalities were used for this assay. The glass slides were cut in half, yielding pieces of 2.5 cm × 3.5 cm, which were suspended vertically using steel paper clip in small trough (15 cm × 15 cm × 3 cm) containing filtered seawater. The area immersed in the filter seawater was ~2.5 cm × 2 cm. Glass slides were arranged in rows, spaced by 1 cm, with five slides per row. Five replicates were used for each surface. Approximately 700 cyprids were introduced into the trough containing 1 l of filtered seawater and incubated for 24 h in the dark. The cyprids attached to each slide were counted, classifying them as exploring or settled/metamorphosed. The latter were counted and subjected to a Wilcoxon Sum of Rank (Wilcoxon–Mann–Whitney) statistical analysis. This is a non-parametric test for assessing whether two statistical samples of observations originate from the same distribution (Sokal and Rohlf 1981). The parameter output from this calculation, p, represents the probability of overlap between two data sets. Its value ranges from 0 to 1, with a value of 0.05 representing the cutoff point, above which the data sets are not considered as distinguishable.

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AFM

AFM measurements were carried out using a Dimension D3100 atomic force microscope equipped with a NanoScope IVa controller and a hybrid scanner (H-153) with *x*-, *y*- and *z*- feedbacks, housed at the University of Twente. A Dimension D3100 with NanoScope IV controller and a 188CL scanner in NUS-SNI, were used in Singapore. Both instruments are manufactured by Veeco (Veeco/Digital Instruments (DI), Santa Barbara, CA). Triangular-shaped silicon nitride cantilevers (Veeco/Digital Instruments (DI), Santa Barbara, CA) were used throughout the study and cantilever spring constants were calibrated using the thermal noise method. The cantilevers used had spring constants ranging from 48 to 54 pN nm^{-1}. Cyprids used in experiments were stored in 33 ppt artificial seawater (ASW, Tropic Marin). They were deposited onto prepared silanised glass surfaces by micro-pipette. Glass samples with different surface functionalities (CH_3-glasses and NH_2-glasses) were fixed to the bottom of polystyrene Petri dishes with double-sided carbon tape prior to experimentation. Cyprid larvae were introduced into a Petri dish containing ASW, with glass substrata at the bottom, facing up. The larval exploration was monitored visually by an optical stereo microscope. When an FP was deposited, its location was marked. The cyprid was subsequently removed and the surface was transferred to AFM for measurement in air or under ASW. Typically, cyprids would attach and begin exploration when stimulated by small water currents. Explored areas of the glass were marked on the base of the coverslip and cyprids were then removed from the Petri dishes. The surfaces used were rinsed with filtered ASW to minimise surface contamination. The Petri dishes were transferred to the AFM and FPs were searched within the marked regions. Once the FP was located, imaging was performed in contact mode, using minimal force. FP morphologies measured in air and under ASW showed no differences. AFM measurements in air provide a slightly higher resolution and were thus used to generate the reported data. FP samples were rinsed with ASW and dried under a stream of nitrogen. AFM images were taken in air using the intermittent-contact mode with silicon cantilevers (PointProbe®Plus Non-Contact High resonance frequency (PPP-NCH) from Nanosensors, Wetzlar, Germany). The porosity of deposited FP adhesive material was estimated using a filling-box construct. A grid is superposed onto the AFM FP and each grid box is evaluated. If the grid box is covered by $<50\%$ FP adhesives, it is marked as a void. The porosity was estimated by summing all the void boxes and dividing by total number of boxes.

Field test by panel immersion

The marine biofouling field test was run by placing samples at constant depth on a raft moored at a test site on the West Coast of Singapore, situated in a tropical estuarine marine coastal environment. The area is relatively protected, with waves primarily generated by ferries accessing a nearby harbour and recreational boats. Test samples were immersed from 20 July 2007 to 14 September 2007. The water temperature, pH, salinity and oxygen level were monitored every fortnight. The water temperature varied from 27.9 to 31.7°C and the pH fluctuated between pH 7.81 and 8.15. Five glass slides of each type, *viz*. bare glass (B), amino-silane (N) coated glass and hydrophobic (C) glass were held with their surfaces horizontal in a PVC frame, laid out in sequence (BCNBCN, etc) in two parallel rows (Figure 1). All test samples were accessed and recorded by photography twice per week. After the field test, the slides were rinsed in fresh water, dried and then photographed. Evaluation of surfaces was done by the

commercial software, Photogrid 1.0. Images obtained from the panel immersion test were imported to Photogrid. To avoid edge effects, a 0.5-cm wide border was excluded for each slide. The central portion was subjected to analysis with PhotoGrid software using 100 points per slide. From 100 points placed at random over the image, the fouling species present at each of these points was identified visually by the operator. At each point, if a macrofouler was present, it was scored into one of the following categories: barnacle, tubeworm (serpulid and spirorbid) or bryozoan (arborescent and encrusting). The raw data were processed using the Student's *t*-test, determining the statistical probability of differentiating between surface chemistries. For the Wilcoxon Sum of Rank and the Student's *t*-test, the analysis compares two sets of data, providing the probability that the two sets are independent. Following convention, $p < 0.05$ was considered to indicate that the two data sets were distinguishable. Conversely, $p > 0.05$ indicated that the two data sets were not statistically differentiated by this analysis.

Figure 1. Sample holders for glass slides.

Results

FP morphology by AFM imaging

The morphology of barnacle FPs was studied by AFM on different substrata, including hydrophobic and hydrophilic surfaces (CH_3-glass NH_2-glass, respectively). Figure 2 shows representative AFM height images of FPs obtained from different surfaces (CH_3- and NH_2-glass) in air by tapping mode AFM (TM-AFM). Entire

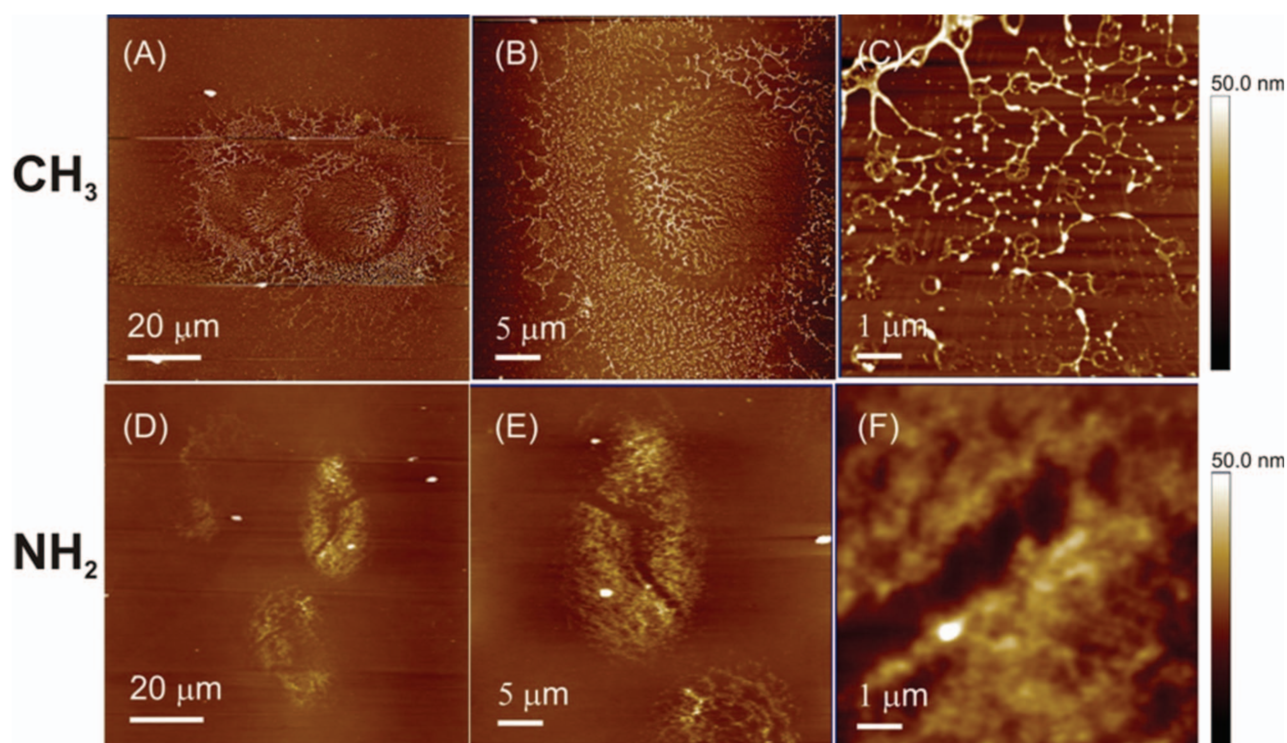

Figure 2. The morphology of footprints secreted by barnacle cyprid larvae on CH_3- (A–C) and NH_2- (D–F) functionalised glass surfaces.

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FPs and sections of FPs at higher magnification were imaged. The morphology and size of FPs deposited on the CH_3- and NH_2-terminated surfaces exhibited significant differences. FPs on CH_3-glass were generally larger in size, had a broader shape variation and exhibited an oval shape and a porous structure (Figure 2). At the edge of the FPs, fibre-like structures were seen. These were spread in a radial pattern and probably represented adhesive material, as shown in Figure 2B, C. The size of a typical FP on CH_3-glass (CH_3-FP) was ~ 60 μm by 50 μm. In contrast, FPs deposited on NH_2-glass (NH_2-FP) were smaller (30 μm by 20 μm) and had a well-defined size and shape. The three NH_2-FPs shown in Figure 2D were imaged with lower magnification. The NH_2-FPs were less porous than the CH_3-FPs. Large areas of the NH_2-FPs were densely covered with a homogeneous layer of adhesive, composed of fibres. Their layer thickness (Figure 2E,F), was much thinner than on CH_3-glass and their shape was more oval.

The outlines of all FPs collected over an 18-month period on CH_3- and NH_2-glass are compiled in Figure 3. More FPs were obtained on NH_2-glass (19 FPs) than on CH_3-glass (6 FPs). Table 1 shows the geometrical data extracted from the FP contours on NH_2- and CH_3-glass. The average surface area of CH_3-FPs was 5 times larger than NH_2-FPs. The size of the NH_2-FPs was similar to that of the attachment pad, reported to be about 20 μm in diameter (Clare et al. 1994). The thickness and volume of the FP adhesive layer deposited on the two surfaces also differed, with FP adhesive spread on CH_3-glass consisting of thick nano-fibres, with a height of 15–40 nm. On NH_2-glass, the thickness ranged from 5 to 15 nm. However, the thickness averaged over the FPs was similar if the porous area was factored into the total volume (6.5 nm for CH_3-FPs and 8.4 nm for NH_2-FPs, respectively). The porosity of CH_3-FPs was 80%, and that of NH_2-FPs was 40%. Taking this porosity into account, the FPs deposited on CH_3-glass and NH_2-glass had similar volumes, of 2.6 μm^3 and 2.0 μm^3, respectively.

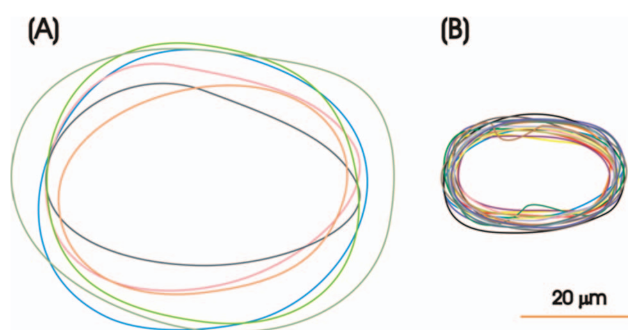

Figure 3. The outline of all FPs collected over a period of 18 months on (A) CH_3- and (B) NH_2-terminated glass surfaces.

Cyprid larval settlement assay

In addition to morphological observations of FPs, a laboratory-scale settlement choice assay was performed using *B. amphitrite* larvae on CH_3-glass and NH_2-glass, respectively. The settlement assay provided a preliminary study of the settlement preferences of barnacle cyprid larva under seawater in a single fouling species environment. Cyprid larvae were incubated in the trough containing NH_2-glass, CH_3-glass and clean (bare) glass. They were allowed to explore freely for 24 h, after which the number of cyprids settled on each surface was counted. The resulting statistics are shown in Table 2.

Wilcoxon Sum of Rank analysis performed on the CH_3-glass and NH_2-glass data indicates a clear distinction ($p < 0.008$) in cyprid settlement preference between these surfaces. In addition, CH_3-glass and bare glass also led to differences in settlement

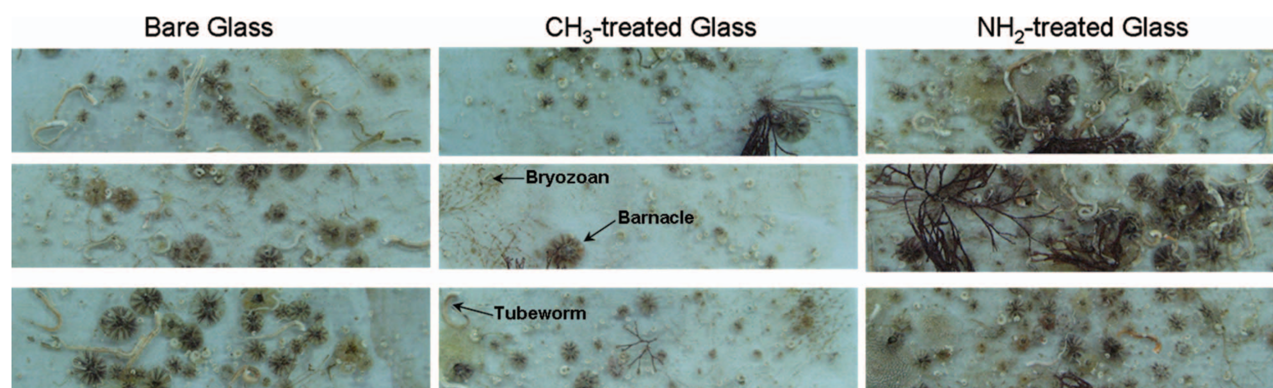

Figure 4. Representative photographs of three microscope slides from each of the model surfaces, *viz*. glass, CH_3-glass and NH_2-glass. Model surfaces were exposed to a tropical marine environment for 25 days.

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Table 1. Morphological information for footprints from data obtained by AFM.

Glass surface functionalisation	Mean footprint area (μm^2)	Average thickness (nm)	Porosity	Volume (μm^3)
CH_3 ($\theta_{Adv} = 106°$)	1980 $\pm$ 500	6.5	0.8	2.6 $\pm$ 0.7
NH_2 ($\theta_{Adv} = 60°$)	410 $\pm$ 60	8.4	0.4	2.1 $\pm$ 0.3

Table 2. Raw data for total number of settled cyprids on bare glass, CH_3-glass and NH_2-glass.

Samples	Number of settlement		
	Bare glass	CH_3-glass	NH_2-glass
1	10	0	4
2	12	0	16
3	5	0	6
4	2	0	7
5	43	2	6

($p < 0.02$). The settlement of cyprids on bare glass *versus* NH_2-glass was not distinguishable ($p < 0.81$), implying that these two hydrophilic surfaces were equally attractive for settlement. These results indicate a preference for cyprid settlement on hydrophilic surfaces (glass, NH_2-glass) over hydrophobic surfaces (CH_3-glass).

Field assessment

Panel immersion tests in a marine environment were carried out to assess the fouling conditions on CH_3-NH_2-, and bare glass. They compared marine biofouling and its associated macrofouler recruitment with a settlement assay of a single species. The objective was to correlate these observations with the AFM data. The same model surfaces were prepared, *viz*. bare glass, NH_2-glass and CH_3-glass. Five replicates were used for each type of surface.

The glass slides exposed to marine environment for 25 days recruited several macrofouler species: barnacles, soft and hard tubeworms, cnidaria (hydroids) and bryozoans. From the optical micrographs presented in Figure 4, the field test results show a similar trend in macrofouler recruitment, with less fouling observed on hydrophobic CH_3-glass than on hydrophilic bare glass and NH_2-glass. Statistical analysis of the data obtained using Photogrid provides a quantitative foundation to differentiate the behaviour on the model functional surfaces. To facilitate comparison with the laboratory settlement assay, barnacle numbers are quoted separately from the total number of macrofoulers. Table 3 shows the scores for fouling organisms obtained from the respective fouled glass surfaces. NH_2-glass had the highest barnacle score, followed by bare glass and then CH_3-glass with the lowest score. Similar trends were observed for other macrofouler organisms, with NH_2-glass attracting the highest surface coverage of macrofoulers, followed by bare glass and CH_3-glass. Statistical analysis of the entire data sets for barnacle settlement behaviour in the marine environment indicated a clear differentiation between NH_2-glass and CH_3-glass ($p < 0.0001$). Glass and CH_3-glass were barely distinguished ($p < 0.04$) whereas bare glass and NH_2-glass were not distinguished ($p < 0.11$). Tubeworms (not shown) did not show differences in their recruitment on bare glass, CH_3-glass and NH_2-glass surfaces. Bryozoan settlement was low on all but NH_2-glass. Total macrofouler settlement data were distinguishable for NH_2-glass with respect to CH_3-glass ($p < 0.0001$) and bare glass ($p < 0.007$).

Discussion

The observed differences in FP morphology on model hydrophobic and hydrophilic surfaces may explain the decreased recruitment of macrofoulers to hydrophobic surfaces in a marine environment. The AFM morphological study of cyprid FPs provides insight into the interaction of the FP adhesive material with model surface chemistries. The size of the FPs found on CH_3-glass is systematically larger than on NH_2-glass, which have dimensions comparable to those of the cyprid's attachment pad (Yule and Walker 1984a,b, 1987; Berglin and Gratenholm 2003; Wiegemann and Watermann 2003, 2004). The larger size observed on CH_3-glass indicates an additional spreading of this material or a possible sliding of the antennule while in contact with the substratum. The estimated volume of the material composing an FP on NH_2-glass and CH_3-glass is approximately the same, implying that the larger surface area generates a spreading of the FP adhesive. This observation is in agreement with Crisp et al. (1985), who suggest that a highly charged bioadhesive can displace water and spread more easily on a hydrophobic surface (Crisp et al. 1985; Callow et al. 2005; Aldred et al. 2006). Moreover, the thick micrometre adhesive fibre observed on CH_3-glass (Figure 2) may indicate a different conformation, adopted from reorganisation

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Table 3. Settled barnacles and total numbers of macrofoulers on five slides for bare glass, CH_3-glass and NH_2-glass.

Samples	Barnacle			Total macrofoulers		
	Bare glass	CH_3-glass	NH_2-glass	Bare glass	CH_3-glass	NH_2-glass
1	4	3	37	8	6	47
2	14	8	34	19	19	64
3	18	3	31	26	11	46
4	32	5	26	41	9	45
5	29	12	22	35	20	49

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and self-assembly of the molecules in the FP adhesive material. FP proteins are expected to interact with the hydrophobic surface by excluding water between their hydrophobic segments and the substratum. Studies have shown that the configuration of the adsorbed protein molecules can be altered by their interactions with the substratum (Meadows and Walker 2005; Luong-Van et al. 2007).

The morphology of the adsorbed FP protein and the size of the FPs combine to suggest enhanced protein adsorption to the NH_2-glass surface, as compared with hydrophobic glass. This surface has been shown to expose a high density of both positively charged $-NH_3^+$ and negatively charged $-SiO^-$ moieties in the vicinity of neutral pH (Carre et al. 2003). The surface charge present on the target surface is important, leading to a strong adsorption from the aggregated influence of multiple Coulombic interactions with the substratum (Arima and Iwata 2007).

It is important to note that not all proteins induce the conspecies settlement of barnacle larva (Crisp and Meadows 1962). It is generally accepted that conspecies surface-bound chemical cues, as isolated from barnacle adult extract, are responsible for inducing and mediating the gregarious settlement of cyprids. These cues are referred to as the SIPC (Knightjones 1953; Crisp and Meadows 1962; Matsumura et al. 1998; Dreanno et al. 2006 a,b). The SIPC consists of glycoprotein complexes (α_2-macroglobulin-like protein), found in the cyprid adhesive material. Studies have shown SIPC to be present in FP proteins adsorbed to the substratum following cyprid exploration (Matsumura et al. 1998). It is surmised that the spreading of FPs on CH_3-glass contributes to a lower concentration of settlement cue (SIPC) per unit area than in FPs found on NH_2-glass. This, in turn, may reduce the ability of cyprid larva to settle on hydrophobic surfaces, accounting for the observed trends in the settlement assay and field test.

Settlement behaviour distinguishes surfaces of different wettabilities, as demonstrated in the settlement assay and marine field test. Results showed a clear preference for NH_2-glass, as compared with a hydrophobic glass surface. Similar settlement preferences were observed for other macrofouling species, with a higher macrofouler colonisation of NH_2-glass *versus* hydrophobic CH_3-glass.

The settlement assay and field test observations suggest that barnacle cyprid larva settlement behaviour may correlate with the difference in FP morphology observed by AFM. These morphologies may give rise to differences in the surface density of conspecies settlement cues (SIPC). It has been established that FPs function as a settlement cue for conspecies settlement. Thus, the preferred settlement of barnacle cyprids on NH_2-glass could be explained by the higher concentration of SIPC, facilitated by an enhanced adsorption of FP proteins on NH_2-glass. This would attract other cyprid larvae for settlement. In contrast, a hydrophobic surface with its reduced SIPC surface density may be less attractive to cyprid settlement. The lower concentration of settlement cues may also contribute to lower recruitment in the settlement assay and generate slower fouling in the field test. The combined AFM, settlement assay and field test results indicate a direct correlation of surface wettability with the settlement behaviour of barnacle cyprid larvae.

Conclusions

The microscopic morphology of *B. amphitrite* cyprid FPs on surfaces with different wettabilities was examined. These data were compared with settlement behaviour. The results showed a correlation between the bioadhesive interface, which is determined by surface characteristics, and the settlement behaviour of cyprid larvae, both in a laboratory assay and in a marine field test. The FP morphology on CH_3-glass obtained by AFM was larger in size and porous, with thick microsized fibres spreading across the surface. The FPs on NH_2-glass were found to be more confined and densely packed with proteinaceous fibres at the micrometre and nanometre length scales. This morphological difference may result in a difference in concentration of the chemical cues on differing surface chemistries. The distinguishable settlement

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behaviour from the laboratory settlement assays and panel immersion tests showed that the barnacle cyprids prefer to settle on hydrophilic surfaces than on hydrophobic substrata. By combining the observations from all experiments at different length scales, it is speculated that higher surface concentrations of SIPC present on the NH_2-surface may contribute to barnacle fouling behaviour on this surface. Charge adsorption enhances the adhesion of FP proteins on a hydrophilic surface. Thus, the limited settlement behaviour found on CH_3-glass might be induced by a lower adhesion of FP materials to the substratum or by a lower concentration of SIPC per unit area. It is anticipated that by studying the bioadhesive morphology of cyprids (or of other macrofoulers), the physiochemical properties of the adhesive could be deduced and a better understanding obtained of settlement behaviour on different chemically functionalised surfaces. In the future, a chemical force microscope with different, chemically functionalised tips will be applied to probe specific protein-surface interactions (Frisbie et al. 1994; Noy et al. 1997; Schonherr et al. 2000). A library of these physiochemical properties of macrofoulers adhesives should be established and utilised in the design of new AF surfaces.

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