Effects of surface charge and Gibbs surface energy on the settlement behaviour of barnacle cyprids (Balanus amphitrite)

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Gibbs surface energy has long been considered to be an important parameter in the design of fouling-resistant surfaces for marine applications. Rigorous testing of the hypothesis that settlement is related to Gibbs surface energy however has never been accomplished, due mainly to practical limitations imposed by the necessary combination of surface engineering and biological evaluation methods. In this article, the effects of surface charge and Gibbs surface energy on the settlement of cyprids of an important fouling barnacle, Balanus amphitrite, were evaluated. Settlement assays were conducted on a range of self-assembled monolayers (SAMs) (CH 3-, OH-, COOH-, N(CH 3)3 þ-, NH 2- terminated), presented in gold-coated polystyrene well plates, varying in terms of their surface charge and Gibbs surface energy. Contrary to contemporary theory, settlement was not increased by high-energy surfaces, rather the opposite was found to be the case with cyprids settling in greater numbers on a low-energy CH 3- SAM compared to a high-energy OH- SAM. Settlement was also greater on negatively-charged SAMs, compared to neutral and positively-charged SAMs. These findings are discussed in the context of data drawn from surfaces that varied in multiple characteristics simultaneously, as have been used previously for such experiments. The finding that surface charge, rather than total surface energy, may be responsible for surface selection by cyprids, will have significant implications for the design of future fouling-resistant materials.

Keywords: barnacle cyprid; self-assembled monolayer; settlement assay; Gibbs surface energy; surface charge; Balanus amphitrite

Introduction

Gibbs surface energy is defined as the work done in a reversible, isothermal process to increase the surface of a substance by a unit area (Gibbs 1928; Adamson 1990). More broadly, it can be used to describe the ‘reactivity’ of a surface, which may be an important parameter in adhesion. Surface energy comprises two independent components, viz. dispersive (Lifshitz-Van der Waals (γ L)) and polar (Lewis acid/base (γ AB)) forces (Good 1993; van Oss 1994). The former describe weak, long-range (100 Å) interactions caused by polarization and induced dipoles, and the latter (γ AB) stronger but short-range (<3 Å) electron donor–electron acceptor bonds, such as hydrogen bonds.

In the context of marine biofouling, surface energy is deemed to be an important parameter for predicting the attachment and/or adhesion of marine organisms to surfaces (Crisp 1984; Rittschof and Costlow 1989; Brady and Singer 2000). Numerous studies (eg Dexter 1979; Baier and Meyer 1992; Zhao et al. 2009) have demonstrated a zone of minimal adhesion for surfaces with surface energy values between 20 and 30 mJ m –2 and this has become known as the ‘Baier minimum’, a reference to an empirical observation of the dependence of microbial adhesion on surface energy presented by Baier and DePalma (1971). Importantly, however, most studies that have attempted to correlate adhesion to surface energy have focused on experiments employing microorganisms for their conclusions (Ista et al. 2004; Zhao et al. 2004; Katsikogianni et al. 2008; Li et al. 2010) or involve complex systems, such as natural aqueous solutions (Dexter 1979). There have also been attempts to explain observations in the context of theoretical models (Dexter 1979; Brady 1999). However, studies attempting to determine, in a controlled manner, the effects of surface energy on the settlement and adhesion of organisms at larger sizescales (hundreds of μm) are limited (Tang et al. 2005; Petrone et al. 2009; Finlay et al. 2010; Bennett et al. 2010; Gunari et al. 2011).

Balanus amphitrite (= Amphibalanus amphitrite) (Clare and Høeg 2008) is an organism of great interest in biofouling and antifouling research, being an important fouler of ships’ hulls and other immersed...
marine structures (Christie and Dalley 1987; Aldred and Clare 2008). Studies with this organism usually involve either quantification of settlement of competent larvae to test surfaces (Aldred et al. 2010a), or mechanical removal of adult organisms. The latter tend to suggest that the adhesion strength (force per unit area of attachment) of adult barnacles may be compromised on low-energy surfaces, such as elastomers of poly(dimethylsiloxane) (PDMS) (Swain and Schultz 1996; Swain et al. 1998; Berglin and Gatelenholm 1999), although there have been suggestions that in reality the system may be significantly more complex. For example, Brady (1999) stated that relative adhesion does not directly correlate with surface energy, as estimated theoretically and empirically (Baier curve) for silicones (22 mJ m\(^{-2}\)) which performed better than very low-energy fluorocarbon-based coatings (10–18 mJ m\(^{-2}\)). Importantly, however, confounding factors such as the roughness and elastic modulus of the materials concerned could have important and unquantifiable effects in these studies. Maki et al. (1994) suggested that there was no correlation between surface wettability (a proxy for surface energy) and temporary adhesion strength of cypris larvae of *B. amphitrite* to surfaces; an observation supported by recent imaging surface plasmon resonance experiments (Aldred et al. 2011) which suggest that surface charge, rather than surface energy *per se*, may determine the temporary adhesion strength of cyprids of *Semibalanus balanoides*.

Quantification of larval settlement on surfaces that vary only in terms of their surface energy is similarly challenging, and results are often equivocated by the choice of surfaces used, since it is not possible to modulate surface energy without also changing other variables. Tests on a range of hydrogels, viz. agarose, chitosan, alginate and polyvinyl alcohol substituted with light-sensitive stilbazolium groups (PVA-SbQ), cast directly in polystyrene (PS) plates, revealed that with light-sensitive stilbazolium groups (PVA-SbQ), cast directly in polystyrene (PS) plates, revealed that cyprids preferred to settle on PS, but that this effect was reduced after 3 days of experimentation. Hung et al. (2008) also found that larvae preferred to attach to glass (polar and high surface energy) as opposed to PS (non-polar and low surface energy) surfaces and Finlay et al. (2010) observed that *B. amphitrite* cyprids preferred to settle on high surface energy organosilica-based xerogel films. Importantly, however, two superhydrophilic zwitterionic polymer coatings, poly(sulfobetaine methacrylate) and poly(carboxybetaine methacrylate) possessing a strong electrostatically-induced hydration layer, inhibited cyprid settlement, implying that high surface energy materials are not always inductive to settlement of cyprids of *B. amphitrite* (Aldred et al. 2010b). In the light of all of these data, it seems increasingly unlikely that surface selection by cyprids can be explained by a simple response to surface wettability. Indeed, experiments on sugar-terminated self-assembled monolayers (SAMs) have recently demonstrated that cyprid settlement was indistinguishable (and very low) for surfaces with advancing water contact angles ranging from <10 to 76°, and with surface energies of ca 46 and 29 mJ m\(^{-2}\) respectively (Ederth et al. 2011).

Despite the varying conclusions and experimental approaches of studies aiming to clarify this issue, there remains a widely-held view that cyprids of *B. amphitrite* have a preference for ‘high-energy’ surfaces (Rittschof and Costlow 1989; Gerhart et al. 1992; Holm et al. 1997; Qian et al. 2000; Hung et al. 2008). The most well controlled experiments to date have used glass surfaces modified by silanisation as experimental substrata (Rittschof and Costlow 1989; Roberts et al. 1991; Gerhart et al. 1992; Phang et al. 2009). Gerhart et al. (1992) observed the settlement of *B. amphitrite* cyprids on a range of surfaces differing in surface chemistry and surface energy. Their conclusion was that cyprids settle in higher numbers on high-energy surfaces. However, the silanised surfaces tested varied in several facets, not least in terms of their surface functional groups, charges and chlorinated and fluorinated end-groups. Additionally, contrary to the initial prediction, no linear relationship was evident between surface energy and settlement for cyprids during experiments, casting some doubt upon the conclusion of that element of the study.

Given the importance of *B. amphitrite* as a model organism and, indeed, surface energy in the design of minimally adhesive marine coatings, a controlled study with well-characterized surfaces is long overdue (Callow and Callow 2011). The chemisorption of alkylthiol molecules on gold surfaces results in the formation of a well-ordered and densely packed SAM (Bain and Whitesides 1988) to which a multitude of chemical functionalities can be terminated. The relative ease of preparation and stability of these surfaces, combined with the ability to easily vary the thiol terminal groups make SAMs suitable for a wide range of applications (Bain et al. 1988; Bain and Whitesides 1989; Dubois and Nuzzo 1992). SAMs can thus provide a uniform set of surfaces that differ primarily in the nature of the exposed terminal group, allowing clearer interpretation of the cyprid settlement preference on a range of well-characterised and homogeneous surface chemistries.

SAMs have been used previously in investigations of the attachment of organisms to surfaces, for
instance algal spores, diatoms and marine bacteria (Callow et al. 2000; Finlay et al. 2002; Ista et al. 2004; Callow et al. 2005; Ederth et al. 2008). Aldred et al. (2006) investigated the attachment and adhesive spreading of mussel (Mytilus edulis) byssal threads on alkanethiol SAMs varying in wettability. Using this method, the adhesive plaques of adult mussels were shown to spread preferentially on high-energy surfaces. Using environmental scanning electron microscopy (ESEM), Petrone et al. (2009) demonstrated that larvae of the mussel Perna canaliculus did not adhere to Teflon, a highly hydrophobic and low-energy surface, and instead the larvae were observed to stick to each other.

In the present manuscript the influence of surface energy on the surface preference of B. amphitrite cyprids is investigated. The data demonstrate the necessity for well-controlled experimental procedures to quantify the effects. Initially, previous experiments were reproduced to observe the difference in settlement between laboratory-grade glass and PS substrata, before taking a more controlled approach towards performing experiments using SAMs that differ in surface energy. Finally, the settlement of cyprids on SAMs with terminal functional groups that varied only in terms of charge and Lewis acid/base characteristics was studied to partition the observed effects on settlement between those arising from a response to Gibbs surface energy and those resulting from changes in surface charge.

Materials and methods

Culture of cyprids of B. amphitrite

Adult barnacle brood-stock (supplied from the Duke University Marine Laboratory, North Carolina, USA) was allowed to release nauplii naturally and approximately 10,000 stage 1 nauplii were collected over a period of 2 to 3 h. The nauplii were attracted to a cold light source, according to the protocol of Elbourne et al. (2008), and transferred at intervals to a dilute solution of Tetraselmis suecica (2008), and transferred at intervals to a dilute solution of Tetraselmis suecica (2008), and transferred at intervals to a dilute solution of Tetraselmis suecica. When sufficient numbers had been collected, all nauplii were transferred to a clean plastic bucket containing 10 l of 0.7 μm filtered seawater with 36.5 mg l^−1 of streptomycin sulphate and 21.9 mg l^−1 of penicillin G. The larvae were fed an excess of Skeletonema marinoi and T. suecica for 5 days until metamorphosis to the cyprid stage. Cyprids were then filtered from the solution, transferred into 0.22 μm filtered natural seawater (FSW) and used for settlement assays after 3 days of storage at 6°C.

Statistical analysis

Settlement data are reported as means ± standard error (SE). Effects of surface treatment on settlement were investigated using one-way analysis of variance (ANOVA) when the assumptions for parametric analysis were fulfilled. When data were not normally distributed or showed dissimilar variances, Kruskal–Wallis analysis was used followed by Dunn’s test. All analyses are displayed with 95% confidence intervals. The normality of the data was assessed using normal plots of residual values and homogeneity of variance was measured using plots of standardized residual values versus fitted values.

Substrata for settlement assays

Settlement assays were conducted on a range of substrata. Clean borosilicate glass shell vials, 19 × 65 mm, of 11.1 ml capacity (Fisher Scientific Co., USA) were prepared by heating to 500°C for 4 h (Gerhart et al. 1992). Cleanroom-cleaned Nextreron® Glass B slides (Schott, Germany), 76 mm × 25 mm, 1 mm in thickness, and sterile flat-bottom 24-well PS plates for traditional tissue culture (TC Plate 24Well F) (Sarstedt, USA) (3.6 ml maximum volume per well) were used as received.

Gold-coating procedure

Glass slides, PS plates and silicon (Si) were gold (Au) coated prior to immersion in thiol solutions for SAM formation. Silicon (100) wafers (Topsil Semiconductor Material A/S, Denmark), used for ellipsometry-based SAM-stability measurements, were cut into pieces and subsequently cleaned with TL1 solution, which is a 1:1:5 solution of 25% NH₃ (Merck, Germany), 30% H₂O₂ (Merck, Germany), and 18.2 MΩ cm Milli-Q water (Millipore), for 10 min at 80°C prior to metal deposition. Metal deposition was carried out in a resistively-heated vacuum evaporation system with a base pressure of < 4 × 10⁻⁴ mbar. Glass slides and Si pieces were primed with 30 Å-thick layer of titanium (Ti) (Balzers, Liechtenstein, 99.9%), as an adhesion promoter and then coated with 300 Å thick Au layer (Nordic High Vacuum AB, Sweden, 99.9%) at a rate of 0.5 and 10 Å s⁻¹, respectively. PS plates were placed on a rotating sample holder, which was gradually inclined by a moving arm at an angle up to 30° during metal evaporation, enabling metal deposition on both the side and the bottom of the wells. A schematic of the evaporation system for metal deposition on PS plates along with a gold-coated plate is shown in Figure 1.
99.5% ethanol (Kemetyl, Sweden) for 24 h, in the dark, at room temperature. Figure 2 illustrates the chemical structure of the thiols used for the preparation of SAM surfaces, viz. HS(CH$_2$)$_{15}$CH$_3$ (1-hexadecanethiol) (Fluka Chemie, Switzerland), HS(CH$_2$)$_{16}$OH (16-hydroxy-1-hexadecanethiol) (Sigma-Aldrich, Sweden), HS(CH$_2$)$_{16}$COOH (16-mercaptohexadecanoic acid) (Sigma-Aldrich, Sweden), HS(CH$_2$)$_{11}$N(CH$_3$)$_3^+$Cl$^-$ (N,N,N-trimethyl-(11-mercaptoundecyl) ammonium chloride) (Prochimia, Poland), HS(CH$_2$)$_{16}$NH$_2$ (16-amino-1-hexadecanethiol) (Prochimia, Poland), HSCH$_2$COOH (thioglycolic acid) (Sigma-Aldrich, Sweden), and HSC$_6$H$_4$COOH (thiosalicylic acid) (Sigma-Aldrich, Sweden). After incubation, the surfaces were rinsed with ethanol, ultrasonicated in ethanol for 3 min and then dried under nitrogen flow. The Au-coated PS wells were filled by pipetting in 3 ml of 100 μM thiol solutions immediately after removal from the metal evaporation chamber.

**Infrared reflection-absorption spectroscopy – IRAS**

A Bruker IFS 66 FT-IR spectrometer equipped with a liquid nitrogen-cooled mercury-cadmium-telluride (MCT) detector was used to record IR spectra of the SAM surfaces on gold-coated Si substrata. IRAS spectra were acquired with $p$-polarized light aligned at 85° angle of incidence, and with 2000 scans at 2 cm$^{-1}$ resolution. The background spectrum was obtained using a deuterated 1-hexadecanethiol [HS(CD$_2$)$_{15}$CD$_3$] SAM on gold. The optical bench was continuously purged with N$_2$ gas during the measurements.

**Ellipsometry**

Thickness measurements of SAMs on gold-coated Si substrata were carried out using an automatic null ellipsometer (Rudolph Research AutoEL III) equipped with a He–Ne laser ($\lambda = 632.8$ nm) set at an incidence angle of 70°. SAM thicknesses were calculated from the measurement outputs ($\Delta$ and $\Psi$) using a three-layer parallel slab Au/SAM/air model. An isotropic refractive index $n = 1.50$ and $k = 0$ was assumed for the SAM. The reported values were the averages of five measurements on each surface.
Contact angle goniometry

Advancing contact angles of SAMs prepared on gold-coated Si substrata were measured with a CAM 200 Optical Contact Angle Meter (KSV Instruments Ltd, Finland) equipped with a manual liquid dispenser. SAMs were withdrawn from ethanol solution, washed in ethanol and subsequently dried under a N2 stream. The reported advancing contact angle values were the average of five measurements with each liquid at different locations.

Surface energy calculation

Gibbs surface energy ($\gamma_s$) was estimated from contact angle data for each SAM using the Good–van Oss–Chaudhury (GvOC) model (Good 1993). This is a relatively robust approach since it makes use of three different test liquids, rather than only one or two as required for other methods. Contact angles were measured with water (W), glycerol (G) and diiodomethane (DIM), and their surface energy parameters are reported in Table 1. In the GvOC model, the surface energy is divided into a dispersive Lifshitz-Van der Waals ($\gamma_{LW}$) component and a polar Lewis acid/base ($\gamma_{AB}$) component. $\gamma_{AB}$ is further divided into a Lewis base (electron donor) component $\gamma^-$ and a Lewis acid (electron acceptor) component $\gamma^+$, so that

$$\gamma_s = \gamma_{LW} + \gamma_{AB} = \gamma_{LW} + 2\sqrt{\gamma^- \gamma^+}$$

(1)

The GvOC model provides a relation between contact angle and surface energy parameters

$$\gamma_i (\cos \theta_i + 1) = 2 \left( \sqrt{\gamma_{LW} \gamma_{LW}} + \sqrt{\gamma^+ \gamma^-} + \sqrt{\gamma^- \gamma^+} \right)$$

(2)

where the subscript $S$ indicates the solid, and $li$ denotes the liquid $i$.

For an apolar liquid $\gamma_{AB} = \gamma^- = \gamma^+ = 0$, and thus $\gamma_{LW}$ can be directly obtained from

$$\gamma_{LW}^S = \gamma_{LW}^i (\cos \theta_i + 1)^2 \frac{4}{\gamma_{LW}^i}$$

(3)

The so-obtained $\gamma_{LW}$ and contact angles measured with two polar liquids can be substituted into Equation 2 to obtain $\gamma_{AB}$ (a system of two equations with two unknown parameters, $\gamma^-$ and $\gamma^+$).

Stability tests

The stability of alkanethiol SAMs on gold has been tested in previous studies following 6 months immersion in NaCl at high concentration (1 M) (Ederth et al. 1998) and also in biological media (Flynn et al. 2003). These data suggest that SAMs in aqueous solutions can be reliably employed over a time scale of several weeks. However, prior to the biological assays, the stability of the SAMs was assessed by incubation in artificial seawater (ASW) for 4 days at room temperature. ASW was prepared by dissolving sodium chloride (420 mM), magnesium chloride hexahydrate (54.6 mM), sodium sulfate (28.8 mM), calcium chloride dihydrate (10.5 mM), and potassium chloride (9.3 mM) in Milli-Q water (this is a simplified form of the ASW specified by ASTM D1141-98 (2003)). The pH was adjusted to 8.2 with 0.1 M NaOH, and the ASW was subsequently filtered with a 0.2 $\mu$m filter (Nalgene, USA). After 4 days immersion, SAMs were thoroughly rinsed with deionized water to remove salts, dried under a nitrogen stream, and their stability was subsequently investigated by contact angle goniometry, ellipsometry and IRAS.

The ellipsometric thicknesses calculated for COOH-, Ni(CH3)3+-, CH3- and OH-terminated SAMs, along with the corresponding advancing water contact angles, remained unchanged upon immersion (Table 3). IRAS spectra showed CH2 stretching vibrations to be about 2917 and 2850 cm$^{-1}$, which are indicative of crystalline-like assemblies of alkyl chains on the gold substratum. A comparison of the IRAS spectra before and after immersion in ASW showed negligible differences (see Supplementary online material [Supplementary material is available via a multimedia link on the online article webpage]).

Results

Determination of settlement assay method

Prior to the quantitative study of settlement of cyprids of B. amphitrite, from which data are reported here, various assay methods were tested to determine which was the most consistent approach to use in order to derive the effects on settlement of a single surface variable. Initially, settlement assays were conducted in drops on glass microscope slides, a surface with high wettability and high surface energy (Zisman 1972). Additionally, two SAMs, alkyl- (CH3) and hydroxyl (OH)-terminated, were also prepared on Au-coated glass slides. The advancing water contact angles ($\theta_w$) for CH3- and OH-terminated SAMs were 107° and

| Liquid          | $\gamma$ | $\gamma_{LW}$ | $\gamma_{AB}$ | $\gamma^+$ | $\gamma^-$ |
|-----------------|---------|---------------|---------------|-----------|-----------|
| Water           | 72.8    | 21.8          | 51.0          | 25.5      | 25.5      |
| Glycerol        | 64      | 34            | 30            | 3.92      | 57.4      |
| Diiodomethane   | 50.8    | 50.8          | 0             | 0         | 0         |
39°, respectively, and for the microscope slide glass a value of 16° was recorded. A volume of ~2 ml filtered seawater containing 20 cyprids was placed on each substratum, resulting in a droplet height of ~0.5 cm for the CH₃-terminated SAM at the highest point of the meniscus, and of <0.3 cm for the OH-terminated SAM with the solution spreading to the edge of the slide. In three assays performed on these three chemistries, cyprid settlement was consistently negligible after 48 h.

**Experiment 1: settlement assay using glass and polystyrene**

Cyprid settlement assays were carried out in glass vials and in PS well plates with the same number of cyprids in the same volume for each treatment, viz. 20 cyprids in 2 ml of FSW and 12 replicates for each surface. Surface energy values for glass and PS, estimated by the GvOC approach, were 48.0 and 47.3 mJ m⁻², respectively. Figure 3 shows the mean settlement percentage of cyprids after 24 and 48 h in glass vials and in PS well plates. The mean percentages of settlement for glass and PS were 43 ± 4% and 15 ± 4% at 24 h, and 57 ± 5% and 32 ± 5% at 48 h, respectively. Settlement data differed significantly between these surfaces at both 24 h (p < 0.05, F = 25.72) and 48 h (p < 0.05, F = 11.39) at 95% confidence (ANOVA).

**Experiment 2: settlement in response to varying Gibbs surface energy**

Tests were continued using plates with wells containing OH- and CH₃-terminated SAMs. These SAMs had surface energy values of ca 47.4 mJ m⁻² and 19.6 mJ m⁻², respectively. Figure 4 shows data from the settlement of cyprids onto these two surfaces after 24 and 48 h.

The results suggested that settlement of cyprids was not significantly different on the two types of SAM at 24 h (p > 0.05, F = 12.87), but became significant after 48 h (p < 0.05, F = 25.66) at 95% confidence (ANOVA).

**Experiments 3 and 4: settlement in response to surface charge**

In addition to the CH₃- and OH-terminated SAMs tested in Experiment 2, two further alkanethiols (see Figure 2) were included in Experiment 3 to determine the effects of surface charge on surface selection by cyprids. Both COOH- and N(CH₃)₃⁺-terminated SAMs have low advancing water contact angles (45° and 60°, respectively) and relatively high overall γ₆ (41.5 mJ m⁻² and 50.9 mJ m⁻², respectively). For comparative purposes, the OH-terminated SAM used in Experiment 2 was also included in Experiment 3 (Table 3) as a neutrally-charged, high-energy (γ₆ = 47.4 mJ m⁻²) control surface.

Cyprid settlement data were plotted against the nominal surface charge after 48 h exposure to surfaces in Experiment 3 (Figure 5). Three additional high-energy, charged SAM surfaces, derived from thiosaliclyclic and thioglycolic acids (negatively-charged) and an NH₂-terminated SAM (positively-charged), were included in a fourth experiment (Experiment 4). Mean settlement percent on the control PS was comparable in all the presented experiments, as also shown in Figure 5. Kruskal–Wallis analysis followed by Dunn’s test suggested that settlement was similar on all three negatively-charged surfaces and on both positively-
charged surfaces. Increased settlement was shown on the negatively charged surfaces, however, compared to the positive and neutrally-charged surfaces (see Table 2 for results of pairwise comparisons; Kruskal–Wallis $W = 28.24$, $p < 0.001$). Settlement on the second positively-charged NH$_2$-SAM was also negligible, confirming previous results on the N(CH$_3$)$_3^+$-SAM. Differences between the positively-charged SAMs and the OH-terminated SAMs were not significant (Dunn’s test).

Data for all surfaces were plotted against surface energy in Figure 6. Glass and PS (Experiment 1) were not included as these surfaces differed in more than one parameter. Mean settlement percent was also plotted against the dispersive ($\gamma^{LW}$) and polar ($\gamma^{AB}$) components of surface energy separately and additionally against $\gamma^+$ and $\gamma^-$, and still no trend emerged (data not presented). Contact angles measured with the three test liquids ($\theta_W$, $\theta_{DIM}$, $\theta_{CI}$) and surface energy parameters ($\gamma_S$, $\gamma^{LW}$, $\gamma^{AB}$, $\gamma^+$, $\gamma^-$) for all surfaces are reported in Table 3, along with the settlement assays (Exp. 1, Exp. 2, Exp. 3, Exp. 4) in which they were employed.

**Discussion**

The central aim of this study was to determine specific effects of surface wettability and Gibbs surface energy on the settlement of cyprids of *B. amphitrite*, and to this end, experiments were carried out initially on the substratum that has been used most often in the literature, ie glass microscope slides. The expectation was that settlement would be higher on high surface energy materials (Rittschof and Costlow 1989; Gerhart et al. 1992; Hung et al. 2008) and lower on those surfaces with lower surface energy, as previously reported. However, despite repeated attempts, cyprid settlement was consistently low or negligible when tested in drop-assay format (Aldred et al. 2010b) on SAM-coated microscope slides. It was concluded that the low contact angle of water on the more hydrophilic surfaces restricted cyprid movement and these data were thus considered to be unreliable. SAMs on gold-coated glass slides have been used to conduct settlement assays with microorganisms smaller than cyprids, for instance algal spores, diatoms and marine bacteria (Callow et al. 2000; Finlay et al. 2002; Ista et al. 2004; Callow et al. 2005; Ederth et al. 2008). Glass slides are used regularly for various standard biofouling assays and, while this is unproblematic when the whole slide is immersed in a solution containing the organisms under investigation, the method presents difficulties for assays such as those with cyprids. To date, experiments using cyprids confined to droplets on flat surfaces have

| Differences | Significance level |
|-------------|-------------------|
| N(CH$_3$)$_3^+$ vs COOH | $<0.01$ |
| N(CH$_3$)$_3^+$ vs thiosalicylic acid | $<0.01$ |
| N(CH$_3$)$_3^+$ vs thioglycolic acid | $<0.001$ |
| NH$_2$ vs thioglycolic acid | $<0.05$ |

**Figure 5.** Mean percent with SEs of *B. amphitrite* cyprid settlement on N(CH$_3$)$_3^+$-, OH- and COOH-terminated SAMs after 48 h (Exp. 3). Cyprid settlement on SAMs obtained from NH$_2$-terminated thiol, thioglycolic and thiosalicylic acids are also shown (Exp. 4). Settlement percentage on the PS controls after 48 h was 29 ± 3% in Exp. 2, 32 ± 3% in Exp. 3, and 28 ± 3% in Exp. 4. Data were obtained from 20 cyprids per well in 2 ml of FSW and 12 replicates.

**Figure 6.** Mean settlement percentage of *B. amphitrite* cyprids with SEs vs surface energy for all SAMs employed in this work.
Table 3. The subset of samples used in each comparative experiment is indicated, as well as SAM thicknesses, contact angles measured with the three test liquids (θW, θDIM, θG), and surface energy parameters (γ1W, γAB, γ+, γ−, γS) calculated with the GvOC model, for all surfaces.

| Experiment | Surface     | Thickness (Å) | Contact angle (°) | Surface energy parameter (mJ m⁻²) |
|------------|-------------|---------------|-------------------|----------------------------------|
| 1          | Glass       | d             | θW = 16 ± 2       | θDIM = 34 ± 3                   | θG = 31 ± 2 | γ1W = 42.5 | γAB = 5.5 | γ+ = 0.1 | γ− = 71.1 | γS = 48.0 |
| 2          | PS          | –             | 79 ± 3            | 26 ± 3                         | 54 ± 2     | 45.8       | 1.5      | 0.1     | 6.8      | 47.3     |
| 3          | –CH₃       | 18.4 ± 0.2    | 107 ± 1           | 77 ± 1                         | 83 ± 2     | 19.1       | 0.5      | 0.1     | 0.5      | 19.6     |
| 4          | –OH        | 21.3 ± 0.2    | 39 ± 2            | 38 ± 3                         | 18 ± 2     | 40.6       | 6.8      | 0.3     | 40.7     | 47.4     |
| 5          | –COOH      | 21.3 ± 0.2    | 45 ± 3            | 40 ± 2                         | 41 ± 2     | 39.6       | 1.9      | 0.0     | 43.9     | 41.5     |
| 6          | –N(CH₃)₃⁺  | 14.3 ± 0.5    | 60 ± 2            | 28 ± 3                         | 50 ± 3     | 45.0       | 5.9      | 0.3     | 26.8     | 50.9     |
| 7          | –NH₂       | 20.8 ± 0.5    | 47 ± 2            | 32 ± 3                         | 49 ± 2     | 43.4       | 10.1     | 0.6     | 46.0     | 53.5     |
| 8          | Thiosalicylic acid | 3.8 ± 0.4 | 56 ± 3            | 19 ± 2                         | 21 ± 2     | 48.1       | 3.8      | 0.2     | 19.3     | 51.9     |
| 9          | Thioglycolic acid | 3.5 ± 0.3 | 61 ± 2            | 27 ± 2                         | 53 ± 3     | 45.4       | 7.7      | 0.5     | 27.2     | 53.1     |

often been perturbed by low settlement. However, for many novel antifouling materials, particularly those involving texture modulation that cannot be applied in wells, tubes or dishes, there remains no other viable option.

Further settlement assays of *B. amphitrite* cyprids (Experiments 1–4) were thus performed in glass vials and PS well plates that offered similar environments to the microscope slides used initially, due to the difficulties associated with precisely measuring contact angles inside a glass tube. It is not only glass that shows considerable variability in reported surface energy however. PS has also been reported to possess surface energy ranging from 33 mJ m⁻², as tabulated by Zisman and Fowkes (1964), up to 42.5 mJ m⁻² (Dann 1965). The surface energies of both glass and engineered polymers also depend very much on the history of fabrication and cleaning procedures used. For instance, oxygen plasma-treatment of PS well plates for cell culture studies (as used here) and acid-washing of glass increases wettability and surface energy of the materials.

Surface energy values for the glass and PS used in experiments, estimated by the GvOC approach, were 48.0 and 47.3 mJ m⁻², respectively. Thus, the total surface energies of the two materials were estimated to be very similar, despite the clear difference in advancing water contact angle (Table 3). High-energy PS for cell culture was selected specifically to demonstrate that differences in settlement between glass and PS were due to factors other than surface energy. Had surface energy been estimated only from the water contact angles (following eg Kwok and Neumann 1999), the surface energy estimate for glass would have been far higher than for PS, thus leading towards the erroneous conclusion that differences in surface energy may be responsible for promoting cyprid settlement.

There is considerable variability in the reported surface energy value for glass surfaces, not only due to the inherent variability of the material, but also because of the numerous different approaches employed towards determining surface energy (see eg Cerne et al. 2008 for a discussion on the choice of probe liquids). The surface energy value for glass presented here is not considered to be unreasonable. It should also be noted that the glass γS value reported in Table 3 does not refer to the vials used for the cyprid settlement assay (Experiment 1), but to the microscope slides used initially, due to the difficulties associated with precisely measuring contact angles inside a glass tube. It is not only glass that shows considerable variability in reported surface energy however. PS has also been reported to possess surface energy ranging from 33 mJ m⁻², as tabulated by Zisman and Fowkes (1964), up to 42.5 mJ m⁻² (Dann 1965). The surface energies of both glass and engineered polymers also depend very much on the history of the surfaces and the fabrication and cleaning procedures used. For instance, oxygen plasma-treatment of PS well plates for cell culture studies (as used here) and acid-washing of glass increases wettability and surface energy of the materials.

One factor that varies between these surfaces, and which may explain the difference in cyprid settlement between the two materials, is surface charge. Glass surfaces acquire a negative charge density in water due to the deprotonation of terminal silanol groups (–SiO⁻) (Behrens and Grier 2001). At the pH of seawater, the surface charge of PS is not neutral, as its chemical structure would suggest, rather it possesses net negative surface charge (Norde and Lyklema 1978; Ohsawa et al. 1986; Schmitt et al. 1999). Importantly though, this charge is significantly weaker than that of
glass and it seems likely that charge, not surface energy, was the determining factor in settlement on these surfaces. The results from glass and PS suggest that a net negative surface charge may be inductive to settlement of cyprids of *B. amphitrite*, all other things being equal.

To determine the effects of surface energy in a more robust way, in the absence of charge effects, a settlement assay was conducted in PS well plates coated with either OH- or CH3-terminated SAMs. Prior to SAM formation, gold-coating by evaporation in vacuum is usually performed on substrata on a rotating sample holder to ensure homogenous deposition on the flat substratum surface, and is usually restricted to microscope slides and other ‘two-dimensional’ surfaces. With the modified system used here, however, the interior walls of the PS plates received a uniform covering of gold, thus ensuring complete SAM coverage in each well (Figure 1). In all performed tests, cyprids were observed to settle not only on the horizontal bottom of the wells, but also vertically on the side walls of the wells. OH- and CH3-terminated SAMs differ significantly in surface energy, with values of 47.4 mJ m⁻² and 19.6 mJ m⁻² respectively, and neither carries a charge at the pH of seawater (the pKₐ of organic alcohols lies in the range between 15.5–19 (Allinger et al. 1980)). Again, contrary to the predictions of previous literature and the initial experiment (Experiment 1), the settlement assay did not indicate any preference of cyprids for the surface with higher surface energy at 24 h (Figure 4). After 48 h it is noteworthy that settlement was statistically higher on the low-energy CH3 surface than on the high-energy OH SAM. The inconsistency between these findings, Experiment 1 and the historical assays with glass and PS can probably be assigned therefore to either inappropriate estimation of surface energy for the surfaces, as explained above, or the unquantified influence of other surface factors, such as surface charge. Either way, these data would seem to refute the prevailing view that cyprids of *B. amphitrite* ‘prefer’ high-energy surfaces per se.

To further investigate the influence of surface charge, settlement assays were carried out on a series of SAMs differing primarily in charge with relatively constant surface energy (Experiment 3, Table 3). Cyprid settlement was clearly dependent upon surface charge (Figure 5), with significantly higher settlement on the negatively-charged (−COO⁻) SAM, intermediate settlement on the neutral −OH SAM, and lowest settlement on the positively-charged (−N(CH3)3⁺) monolayer. To further validate the preference of cyprids for negatively-charged surfaces, SAMs of two other acidic thiols, thiosalicylic and thioglycolic acids were later tested and the results of these further tests (Experiment 4) supported the findings of Experiment 3 (Figure 5, Table 3). Additionally, another positively-charged SAM with NH3-termination, was tested and once more cyprid settlement was negligible on the surface possessing a positive charge.

When all settlement results for SAM surfaces were plotted against Gibbs surface energy, calculated using the GvOC model, no trend was evident for the relation between settlement and surface energy (Figure 6).

The data presented here run contrary to most previous findings, including those of Roberts et al. (1991) who used silanized glass surfaces to demonstrate higher settlement of cyprids of *B. amphitrite* on surfaces with higher surface energy, as well as those of Phang et al. (2009), who concluded that cyprids of the same species settled in higher number on aminosilane functionalised glass (NH₃-) than on alkyl (CH₃)-terminated glass. It should be considered, however, that in addition to the complexities of surface energy calculation and the presence of confounding chemical functionalities there are other problems associated with using silane chemistry on surfaces that are difficult to avoid. A significant number of silanol groups may be left ungrafted during a silanization reaction; Vansant et al. (1995) reported that only 30 to 50% of silanol groups were actually grafted and, additionally, although NH2 groups are positively charged at the pH of seawater (~8.2), at pH values above 7.0, aminosilane functionalized glass surfaces may exhibit a net negative charge, due to the presence of free silanol groups (SiO⁻) from the underlying glass surface or hydrolyzed alkoxy groups of the silane molecules (Carré et al. 2003). On the other hand, one of the main advantages of using SAMs resides in the fact that this procedure ensures good coverage of the substratum surface. Additionally, IRAS spectra in the CH-stretching region are indicative of the SAM structure. The bands arising from CH₂ asymmetric and symmetric stretching region are found at ~2917 and 2850 cm⁻¹, respectively, which are indicative of well-ordered, crystalline-like assemblies of the alkyl chains (see Supplementary online material [Supplementary material is available via a multimedia link on the online article webpage]) (Nuzzo et al. 1987, 1989). In conjunction with the ellipsometric thicknesses (Table 3), this further demonstrates that the gold surface is completely covered by the SAM.

As demonstrated in previous theoretical and experimental work (Marinova et al. 1996; Dicke and Hähner 2002; Chan et al. 2003; Kreuzer et al. 2003), SAMs and hydrophobic surfaces without dissociable sites will acquire a negative charge in water at pH >4 due to the ubiquitous phenomenon of preferential adsorption of hydroxyl ions (OH⁻). The top water layer of hydrophobic substrata is oriented, generating
a dipole moment and thus a potential drop which induces the OH\textsuperscript{−} adsorption (Netz 2004). Surface charge is screened beyond the double layer and at high ionic strength, such as in seawater, the potential drop occurs over a very short distance (only a few Å; the Debye screening length). However, surface charge is likely to play a critical role in the cyprid attachment process since cyprids explore and sense the substratum by making physical contact via the antennular pad (Aldred and Clare 2009; Clare and Aldred 2009; Maruzzo et al. 2011). Hence, the preference of cyprids for negatively-charged surfaces may have arisen from a natural adaptation, due to the fact that most surfaces are negatively-charged in natural aquatic environments. Additionally, pristine metal oxide surfaces become hydroxylated in water as a consequence of the dissociative coordination of water molecules (Lewis bases) to surface metal ions (Lewis acids), thus acquiring a negative charge at the pH of seawater (Stewart et al. 2011).

Another aspect that must be considered is surface contamination and conditioning, which is caused by adsorbed ions, biopolymers and microbial biofilms. Nowhere is this process more acute than in natural aquatic environments. Thus, high-energy solid/liquid interfaces are conditioned rapidly by organic layers lowering the interfacial energy (Wahl 1989), which may go some way towards explaining the preferential settlement of cyprids also on low-energy surfaces (Figure 6).

To summarise, the present data suggest that total surface energy does not directly influence cyprid settlement in the manner predicted by previous studies and the substrata used for previous studies such as PS and silanized glass differ in too many respects to be appropriate for drawing such conclusions. Further, the method of estimating surface energy may significantly affect the conclusions of any such study and should be considered carefully. Surface charge would seem to be a more important parameter for controlling settlement of cyprids in the design of barnacle-resistant marine coatings. It is worth bearing in mind when considering the low surface energy theory of minimally adhesive surfaces that most successful commercial products tend to also be soft and lubricious and, indeed, several contemporary products are amphiphilic in nature rather than super-low energy. It is also important to note that, as previously explained, quantitative measurements of SAM surface charge are both difficult and uncertain in seawater due to the Debye length being in the order of a few Å. Thus, the use of ‘surface charge’ in this article should be considered as ‘nominal surface charge’, and the accurate quantification of surface charge at high ionic strength, such as in seawater, is the focus of on-going research.

**Conclusions**

Cyprids of *B. amphitrite* were observed to settle preferentially on negatively-charged SAMs (−COOH), with neutral intermediate (−OH and −CH\textsubscript{3}), and the lowest settlement was observed on the positively-charged (−(N(CH\textsubscript{3})\textsubscript{3})\textsuperscript{+} and −NH\textsubscript{2}) monolayers. No effect of surface energy on settlement of cyprids was observed. This study thus demonstrates that increased surface energy does not necessarily increase the settlement of cyprids of *B. amphitrite*, but other factors can significantly influence the substratum choice during cyprid exploration, such as surface charge. The preference for settlement on negatively-charged surfaces may arise from a natural adaptation as negative surfaces predominate in natural aqueous systems. Additionally, surfaces become rapidly conditioned upon immersion in natural aquatic environments and also during laboratory settlement assays, thereby lowering the interfacial energy. This observation may account for the fact that cyprids did not show any preference for settling on high-energy surfaces.

In conclusion, surface energy alone cannot aid the design of an efficient surface to avoid cyprid adhesion. Surface charge proved to be a crucial parameter for surface selection by cyprids, and it needs to be taken into account for the development of novel antifouling materials.

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