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Mussel (*Mytilus edulis*) byssus deposition in response to variations in surface wettability

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Mussels (*Mytilus edulis*) are economically important in their role as an aquaculture species and also with regard to marine biofouling. They attach tenaciously to a wide variety of submerged surfaces by virtue of collagenous attachment threads termed 'byssi'. The aim of this study was to characterize the spreading of the byssal attachment plaque, which mediates attachment to the surface, on a range of surfaces in response to changes in wettability. To achieve this, well characterized self-assembled monolayers of ω-terminated alkanethiolates on gold were used, allowing correlation of byssal plaque spreading with a single surface characteristic—wettability. The present results were inconsistent with those from previous studies, in that there was a positive correlation between plaque size and surface wettability; a trend which is not explained by conventional wetting theory for a three-phase system. A recent extension to wetting theory with regard to hydrophilic proteins is discussed and the results of settlement assays are used to attempt reconciliation of these results with those of similar previous studies and, also, with recent data presented for the spreading of *Ulva linza* spore adhesive.

1. INTRODUCTION

Mussels of the genera *Mytilus*, *Dreissena* and *Perna* cause a serious and persistent fouling problem affecting, e.g. aquaculture nets, off-shore rigs and industrial coolant outflows (Kingsbury 1981; Forteath *et al.* 1984; Edyvean *et al.* 1985; Southgate & Myers 1985; Relini & Monranari 1999; Nishida *et al.* 2003). Their large size and accessibility of the attachment apparatus make mussels highly suitable model organisms for adhesion (Crisp *et al.* 1984; Waite & Qin 2001) and antifouling studies (da Gama *et al.* 2004).

Mytilus edulis is a wide-spread, temperate water species that inhabits rocky intertidal coastlines around northern Europe and north-eastern USA. Although they have the appearance of sessile organisms, the juveniles of this species are highly motile by virtue of their muscular foot. The foot has a sensory role (Mahéo 1970), but functions primarily to attach mussels to surfaces. The method of attachment by multiple, collagenous, attachment threads, or byssi (Waite *et al.* 1998) is unique to its class (Yonge 1962). The byssi attach with great tenacity to almost any submerged surface (Young & Crisp 1981) and ensure

that the organisms can remain anchored on wave-beaten shores.

The byssal thread is a complex structure that can be divided into three main areas: (i) the root that is embedded in the base of the muscular foot; (ii) the threads that are produced along a ventral groove that runs the length of the foot; and (iii) the attachment disc, or plaque, as it will be referred to here, which mediates adhesion to the substratum. The byssus is composed primarily of five proteins (termed Mefp 1–5) as well as collagen. The relatively small protein, Mefp-3, is of particular interest to this study as Mefp-3 is believed to contribute to adhesion of the byssal plaque to the substratum (Yu *et al.* 1999) and has also been referred to as an adhesive primer (Warner & Waite 1999). The basis for these hypotheses lies in the high concentration (20 mol%) of the amino acid 3,4-dihydroxyphenyl-L-alanine (DOPA; Warner & Waite 1999; Floriolli *et al.* 2000; Waite & Qin 2001) in Mefp-3, as well as the observation that multiple forms (at least 10 electrophoretic variants) of the protein may be expressed by individual mussels in response to different surface compositions. It is suggested that protein expression may be determined by the physical properties of the attachment surface (Vreeland *et al.* 1998;

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Warner & Waite 1999), i.e. the adhesive could be matched to the surface for maximal adhesive force, although this has not been shown conclusively.

Despite their status as model organisms for adhesion studies, direct measures of mussel adhesion are surprisingly rare. There is also insufficient information to construct a clear picture of the preference of mussels for different substrata (Hansen *et al.* 1994; Yamamoto *et al.* 1997; Nishida *et al.* 2003). Young & Crisp (1981) investigated the deposition and spreading of the byssal plaque of *M. edulis* on surfaces differing in free energy and polarity, namely slate, glass, paraffin wax and PTFE (polytetrafluoroethylene). Their results demonstrated that the byssal plaque spread significantly further and tenacity was lower on the low-energy surfaces (PTFE and paraffin wax, which were also non-polar). They concluded that a simple balance of thermodynamic forces governs the deposition and spreading of the plaque and a combination of Young–Dupré equations (see §4) was invoked to describe the competition between the adhesive and the liquid medium (seawater) for the test surface. In reality, however, their test surfaces differed in many other properties, including rugosity and bulk/elastic modulus. Therefore, their findings must be viewed as equivocal.

Recently, a similar study was carried out on the spreading of the secreted adhesive of spores of the green alga *Ulva linza*. This used surfaces of different wettability in the form of self-assembled monolayers (SAMS) of CH₃- or OH-terminated alkanethiolates on gold, or mixtures of the two (Callow *et al.* 2005). The use of SAMs as model surfaces with which to study the adhesion processes of marine organisms (e.g. Ista *et al.* 1996, 2004; Callow *et al.* 2000*a*; Finlay *et al.* 2002) is advantageous compared with heterogeneous substrates like slate, glass, etc. since they are chemically defined, uniform in surface topography and modulus, and in the case of CH₃- and OH-terminations, uncharged at the pH of seawater.

The results of Callow *et al.* (2005) were not consistent with those reported for mussel adhesive spreading by Young & Crisp (1981) since spreading of the spore adhesive was greatest on hydrophilic surfaces and least on hydrophobic surfaces. They were also not consistent with standard thermodynamic wetting theory based on the Young-Dupré equations for a 3-phase system (surface, fluid adhesive, liquid water) since standard theory would predict that a fluid adhesive should more easily 'wet', i.e. spread more, on a less wettable surface. In a novel extension of thermodynamic wetting theory, it was shown that this apparent contradiction can be explained on the basis that a very polar adhesive protein could indeed effectively compete with water to wet a hydrophilic surface.

In view of the results of Callow *et al.* (2005) it was decided to re-evaluate the findings of Young & Crisp (1981) on the spreading of the adhesive plaques of blue mussel byssi, using as substrates a range of SAMs differing in wettability. Assays were also performed to identify any preference mussels may have for specific surfaces.

2. MATERIAL AND METHODS

2.1. Treatment of mussels

Juvenile mussels were collected from the shore at Seaton Sluice (NZ 335 765 GB Grid ref.), Northeast England two weeks prior to experimentation. They were maintained at 10 °C under constant light in aquaria containing 50 l aerated natural seawater. The seawater was changed weekly after the mussels had been fed *Tetraselmis chui* (500 ml, culture strength *ca* 10^5 cells ml^{-1}).

Prior to experimentation mussels of the desired size $(15 \pm 1$ mm), chosen for their more extensive exploratory behaviour in comparison with fully grown adults, were separated from the culture and allowed to explore for 5 h in a shallow tray containing natural seawater. Only those mussels showing exploratory behaviour during this interval were selected for testing.

2.2. Production of SAM surfaces

SAM surfaces were produced by the method of Bain *et al.* (1989), having been used in similar studies previously (Ista *et al.* 1996; 2004; Callow *et al.* 2000*a*). Briefly, the SAMs were produced on standard glass microscope slides coated with thin layers of chromium and gold using a metal evaporator evacuated to 10^{-6} torr. Resulting surfaces were immersed in 1 mM ethanolic solutions of dodecanethiol, mercapto-undecanol, or mixtures of the two to produce surfaces with either CH₃ or OH groups or combinations thereof at the surface. The surfaces ranged in wettability as defined using advancing water contact angles $[\theta_{AW}]$ from θ_{AW} 15° (hydrophilic) to 107° (hydrophobic).

SAMs were sealed in Coplin jars containing de-oxygenated nanopure water and delivered to Newcastle overnight. SAMs were used on the day of delivery following a preparation procedure which comprised a rinse in ethanol followed by nanopure water. The slides were then dried under nitrogen before use.

2.3. Experimental set-up

Two batches of SAMs were used during this investigation. Batch 1 comprised five different SAMs with θ_{AW} values of 103°, 80°, 63°, 30° and 17° with six replicates of each. Batch 2 comprised two different SAMs with θ_{AW} values of 15° and 107° and 36 replicates of each. Batch 1 was used for a deposition study and spreading measurements, while batch 2 was used to replicate spreading measurements and for a 'choice' experiment.

2.3.1. Byssal deposition. Polypropylene containers were leached with distilled water for two weeks prior to use. One valve of each mussel was stuck to polystyrene rods using cyanoacrylate adhesive. This immobilized the mussels during the assay, while still allowing free movement of the foot over the test surfaces (figure 1). Polystyrene rods were, in turn, attached to the base of the tubs using high modulus clear silicone sealant (B&Q plc. UK.). The containers were filled with 1 l of
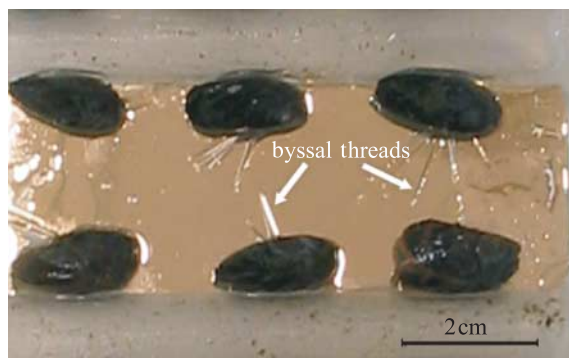
Figure 1. Juvenile *Mytilus* immobilized for deposition experiments such that they deposit byssi primarily onto the test surfaces. Gold surface is an ω-substituted alkane thiolate SAM.

natural seawater and incubated for 24 h at 10 °C under constant dim light and static water conditions.

For the first batch of SAMs, 36 mussels were used for each treatment (six mussels per slide) with all five treatments (θ_{AW} 103°, 80°, 63°, 30° and 17°) tested simultaneously. The number of byssi deposited by all mussels was counted every hour for the first 8 h of the experiment and then again after 24 h. At the end of the 24 h period the byssi deposited by individual mussels were marked by scratching the gold surface of the SAMs and then cut with fine scissors. SAM-coated slides bearing byssal plaques were removed and fixed with 3% glutaraldehyde in 0.45 μm filtered seawater, for 1 h at 5 °C. They were then stored prior to measurement (over a 5 day period) at 5 °C in 0.45 μm filtered seawater. No change in plaque size was observed over this period and measurements were conducted in a random order to negate any possible effect of storage.

2.3.2. Choice assay. Most of the second batch of SAMs was used in a choice assay. Thirty mussels were allowed to 'choose' between OH-terminated (θ_{AW} 15°) and CH$_3$-terminated (θ_{AW} 107°) SAMs in a chamber designed to restrict deposition to one of these two surfaces. This was achieved by coating all other surfaces with nylon plankton mesh (150 μm pore size); a surface inhibitory to mussel settlement. The assay was conducted over 8 h.

The assay was considered to be unbiased. As a single SAM can only be used once, the chamber could not be calibrated and the surfaces were assumed to be equally likely to be selected, with no significant influence of surface treatment. This allowed comparison of the resultant binomial data with a 50% cumulative frequency distribution.

2.4. Data analysis

Images of the byssi were captured onto an IBM-compatible PC using an analogue CCTV camera attached to an Olympus BH-2 compound microscope. The areas of the byssi were calculated in MATROX INSPECTOR software for Windows. Mean areas were taken for each mussel to reduce the skewing factor introduced for those mussels that habitually produced
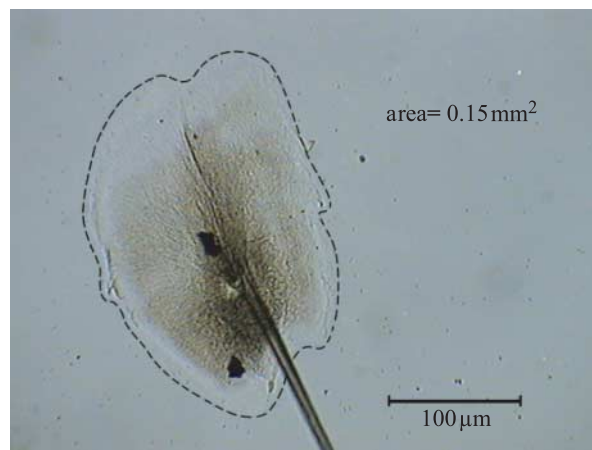

Figure 2. An image of byssal plaque, similar to those used for measurement.

more byssi. A mean spreading area per surface could then be calculated to allow inter-comparison of surfaces.

Due to the variability in byssal plaque shape, size and irregularities highlighted by transmitted light, it was necessary to manually define the edges of the adhesive plaque. A macro designed for this purpose may have been less time-consuming; however, it would have been less accurate. The use of the natural autofluorescence of the structures did not yield any increase in accuracy of measurements and in fact did not demonstrate the full extent of spreading. For these reasons, the measurements were taken from captured images of byssal adhesive plaques (greater than 500 in total) in their native state (figure 2).

3. RESULTS

3.1. Attachment of mussels

In general, mussels attached more rapidly to high wettability surfaces than to lower wettability surfaces. Attachment was defined as the deposition of one or more byssi on a surface and the time that this initial attachment occurred was recorded for each mussel. After a comparatively slow initial rate of attachment to the 103° surface, mussels attached at a high rate. In comparison, the mussels on the 17° surface attached in higher numbers immediately. Nevertheless, although attachment rates were measured throughout the investigation, no significant trend of increased attachment rate with increased wettability was detected (data not shown).

3.2. Number of byssi produced and rate of deposition on SAMs

The mean number of byssi produced, per individual, on each of the 5 surfaces was determined at all time intervals. The values for 24 h are shown in figure 3. A strong correlation coefficient and significant regression ($F=20.3$, $p=0.02$) suggested that byssal production increased significantly with decreasing wettability. The rate of byssus deposition on each surface is presented in figure 4. For clarity only two regressions, 17° and 103°, representing byssus
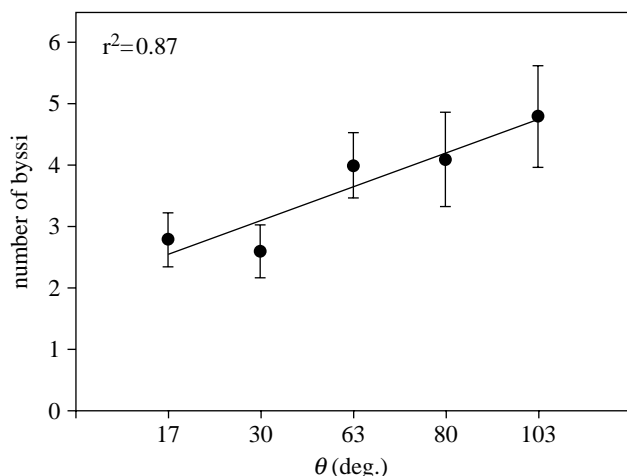
Figure 3. The mean number of byssi deposited per individual plotted against internal θ_{AW} on all test surfaces after 24 h.

Figure 4. Regressions of the rate of deposition of byssi on 100% OH (hydrophilic) terminated (17° θ_{AW}) and 100% CH$_3$ (hydrophobic) terminated (103° θ_{AW}) SAMs over 8 h.

deposition at the extremes of the wettability spectrum, are shown. The 24 h data points were removed from the regression, but in all cases were similar to the 8 h reading where deposition was maximal.

There was a significant linear increase of byssal deposition with time on the 103° SAM ($F = 113.28$, $p = <0.001$), but not on the 17° SAM ($F = 2.94$, $p = 0.174$). On the 17° SAM, most byssi were produced within the first hour, whereas byssi were continually deposited until 8 h on the 103° SAM.

3.3. Comparison of byssus plaque areas on different surfaces

The measurement of byssal areas on each of the surfaces after 24 h revealed a significant ($F = 99.39$, $p = 0.002$) positive linear relationship between plaque area and increasing wettability (figure 5). This trend was validated by a repeat experiment that also showed a significant difference ($F = 10.9$, $p = 0.002$) between plaque areas deposited on the θ_{AW} 15° and 107° SAMs. Mean plaque areas ranged from $0.16 \pm$ s.e. 0.0077 mm^2 for the 17° SAMs to $0.14 \pm 0.006 \text{ mm}^2$ for the 103° SAMs.

3.4. Choice assay

Out of 30 mussels tested, 24 selected a surface by 8 h. Eighteen of these deposited the majority of their byssi onto the 15° SAM in preference to the 107° SAM. The probability of this occurring by chance alone, according to the 50% cumulative frequency plot, is less than 0.001 and can therefore be attributed to the introduction of a significant bias by the surfaces. The 15° SAM can thus be considered significantly more attractive to settling mussels than the 107° SAM.

4. DISCUSSION

The results of this study were not consistent with previous findings (Young & Crisp 1981) since a positive correlation was observed between adhesive plaque spreading and wettability. The most obvious explanation for the different results is that while the present
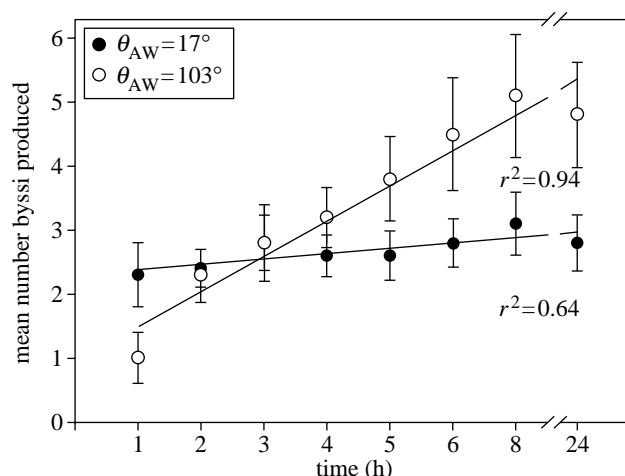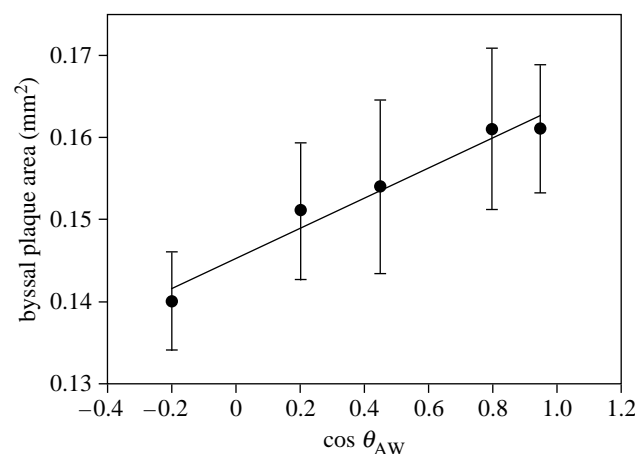

Figure 5. Mean byssal plaque area plotted against the cosine of internal θ_{AW}. As wettability increases, θ_{AW} measured internally decreases and cos θ_{AW} becomes larger.

study used SAMs, Young & Crisp (1981) used highly heterogeneous materials for their test surfaces (slate, glass, PTFE and paraffin wax). These would have differed in wettability, but also in chemistry, topography, polarity and modulus and it is therefore possible that their results were influenced by one or more of these properties, other than, or in combination with, wettability. Surface roughness in particular is known to affect adhesion of marine invertebrates (Berntsson *et al.* 2000*a,b*, 2004). Further, the number of measurements taken here (greater than 500 and replication) in comparison to nine individuals used by Young & Crisp (1981) allows confidence to be placed in the present results.

Given the discrepancies between studies, the applicability of conventional wetting should be examined. From the Dupré equation

$$\cos \theta = \frac{\gamma_{AS} - \gamma_{SL}}{\gamma_{LA}},$$

where θ is the adhesive contact angle, AS describes the adhesive/solid interface, SL the solid/liquid interface and LA the liquid/liquid interface, Crisp *et al.* (1984)

derived the equivalent of

$$W_{SL} - W_{SA} = \gamma_L - \gamma_A + \gamma_{LA} \cos \theta_{LA},$$

where W_{SA} and W_{SL} are the changes in adhesive energy, or work of adhesion, of the solid/adhesive and solid/liquid interfaces, respectively. γ_{LA} remains constant. (θ, here, is an external contact angle that is used to describe the spreading of the adhesive on a surface, under water. This should not be confused with the internal contact angle formed by a water droplet on a surface, which is used as a measure of surface wettability; typically as its cosine. To avoid confusion the latter is referred to as θ_{AW} throughout this paper). This demonstrates that as W_{SL} increases from low to high wettability, the external contact angle θ will decrease, leading to restricted spreading of the adhesive on highly wettable surfaces. This trend has also been demonstrated empirically using inert probe liquids (Clint & Wicks 2001). The fact that mussel plaques did not behave in this way in the present study suggests that the adhesive has a higher surface tension than the ambient medium—seawater.

In Callow *et al.* (2005), attention was drawn to the fact that proteins are highly complex molecules containing both polar and non-polar domains. There is potential then for them to show quite different spreading characteristics compared to inert probe liquids. Mefp-3, for example, is extensively hydroxylated and can, therefore, engage readily in hydrogen bonding (Waite & Qin 2001). Further, catechol, a DOPA side chain, is able to form strong hydrogen bonds with hydrophilic polymers (Wiegemann 2005). This hydrophilic character, however, would lead logically to the conclusion that de-wetting of the adhesive for successful contact with the surface would be difficult. Thus, in order for adhesion to be successful, the work of adhesion between the adhesive and the surface (W_{SA}) must also be greater than the energy needed to dehydrate the liquid adhesive (W_{LA}). Callow *et al.* (2005) suggested that for a highly polar proteinaceous adhesive, W_{SA} can be sufficiently large to allow further spreading of adhesive on high-energy surfaces than low-energy surfaces. The highly hydrophilic character of mussel byssal proteins may, therefore, be crucially important in their ability to spread well on high-energy surfaces.

Briefly, the conclusion of the Callow *et al.* (2005) study was that two different components of surface wettability affected the spreading of *Ulva* adhesive. Namely, dispersion forces in relation to the CH_3-terminated regions of the SAMs and hydrogen bonding in relation to the OH regions.

It is well established that high-energy surfaces allow the formation of stronger adhesive bonds than low-energy surfaces (Baier *et al.* 1968; Lindner 1992) and marine organisms, especially fouling species, may have evolved mechanisms of attachment that yield strong adhesion to low-energy surfaces when necessary; or, conversely, methods of de-wetting high-energy surfaces in order to facilitate attachment. In the case of mussels, this adaptation could be an adhesive with properties that efficiently displace water from high-energy surfaces and/or the mussel may perceive the surface

properties (Mahéo 1970) and alter the composition of the adhesive to effect maximal adhesion (Vreeland *et al.* 1998; Warner & Waite 1999).

Although further study is clearly needed to help explain the observed trend of increasing byssal plaque size with increasing surface wettability, a detailed examination of the results here is illuminating. Mussels on the hydrophobic ($\theta_{AW} = \sim 103°$) SAMs attached differently to those on the more hydrophilic surfaces: initially, deposition was slow, with the rate increasing after the first hour. Mussels ultimately produced fewer byssi on the 17° surface than on the 103° surface (figure 3), with most attachments occurring in the first hour (figure 4) on the former. This is also contrary to the findings of Young & Crisp (1981). Mussels deposited byssi at a comparatively slow rate for the first hour on the 103° SAM but then continued to deposit byssi for up to 8 h with no appreciable increase thereafter. Nishida *et al.* (2003), likewise found that the mussel, *Perna viridis*, deposited more byssi onto nylon, rubber, silicone and PTFE (low-energy surfaces) than onto glass (high-energy). However, no statistical analyses were presented.

Although the data can be interpreted in several ways, one plausible sequence of events when a mussel is presented with a high-energy surface is as follows:

  (i) high-energy surface is detected;
 (ii) identified as a good attachment site and the mussel attaches quickly;
(iii) byssi are deposited immediately;
 (iv) byssal plaque spreads well, resulting in high tenacity attachment;
  (v) few byssi are required, so byssal production stops, conferring an energetic advantage (energy that would be used to produce byssi can be used for other purposes) to the mussel.

The opposite might apply for low-energy surfaces. This scheme of events is favoured because it relies upon a physical process mediated by either the SAM surface, or an adhesive that overcomes the natural tendency of water to coat high-energy surfaces, making adhesive wetting difficult. Some marine organisms adhere preferentially to low-energy surfaces when the liquid medium is of high surface tension, e.g. water (Absolom *et al.* 1983; van Loosdrecht *et al.* 1987; Otto *et al.* 1999; Callow *et al.* 2000*a*; Finlay *et al.* 2002), so it is possible that the mussel can detect the electrostatic properties of the surface and spread its adhesive accordingly. If mussels prefer to attach to high-energy surfaces, a selection that might have reflected an increased strength of adhesion, then this hypothesis could be tested experimentally. However, if this adaptive compensation occurred in response to a physical restriction on byssal plaque spreading, similar spreading of the adhesive on all surfaces would be anticipated. This is not the case here (figure 5).

In theory at least, the *Ulva* case is made easier to explain with the knowledge that there can be no deliberate spreading of the adhesive, therefore excluding a behavioural explanation. Spores contain a finite

quantity of pre-formed adhesive, which is expressed externally and spreads passively (Callow *et al.* 2000*b*).

This work represents the first step towards investigating the adhesion of *M. edulis* systematically and moves beyond the usual application of this organism as an attachment bioassay, towards gaining a fuller understanding of why these surface preferences exist in *M. edulis*, as well as other fouling organisms. Greater knowledge of how these adhesives work would seem to be fundamental to the development of effective, minimally adhesive marine coatings. Indeed, from this study alone it is made clear that although we may know certain surfaces to be deleterious to adhesion, our explanations for this characteristic may be far from complete.

Following directly from this work, an important future step is to test the strengths of adhesion of byssi deposited onto SAMs, and to extend the test protocol further to studies of other marine fouling organisms.

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