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Adhesion Strength of Settled Spores of the Green Alga *Enteromorpha*

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Short Communication

Adhesion Strength of Settled Spores of the Green Alga *Enteromorpha*

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Strengths of attachment of spores of the green fouling alga *Enteromorpha* to glass have been measured using a modified water jet apparatus. Surface pressures of ~250 kPa were required to quantitatively remove attached spores after 4 h contact with a surface. The development of adhesive and cohesive strength is highly time-dependent; after 8 h in contact with a surface spores did not detach, even at pressures in excess of 250 kPa. Spores settled in groups are more resistant to detachment than single spores, which suggests that the adaptive value of gregarious settlement behaviour may lie in the greater resistance of groups to detachment forces in a naturally turbulent environment. The interfacial forces exerted as water impinges on the surface and the derivation of adhesion strength values in terms of wall shear stress are discussed and compared with those obtained by other methods. A surface pressure of 250 kPa approximates to 325 Pa wall shear stress. Calculation using the power-law formula predicts that detachment forces of this magnitude are unlikely to be realized at operating speeds for most vessels and that most *Enteromorpha* spores would not detach from untreated hulls.

Keywords: adhesion; biofouling; green algae; *Enteromorpha*; gregarious settlement; shear stress; spore

INTRODUCTION

Marine organisms that spend their adult life attached to surfaces generally have a planktonic, dispersal stage in their life cycle. In the case of fouling species, such as barnacles and algae, larvae and spores, respectively, must find a suitable place to settle and adhere before they can complete their life cycle. Settlement is therefore considered to be the most important stage in the life cycle of fouling organisms (Hadfield, 1986; Walters *et al.*, 1999) and its prevention is an important goal in the development of effective, non-toxic antifouling strategies. A key feature of the settlement process, across all phyla, is the use of sticky materials with permanent or temporary adhesive capabilities. This adhesion process takes place rapidly, often within minutes, under water, to a wide range of substrata, over a wide range of temperatures, salinities and conditions of turbulence.

Enteromorpha is a common, green macroalga found throughout the world in the upper intertidal zone of seashores and as a fouling organism on a variety of man-made structures including ships' hulls (Callow, 1996). Dispersal is achieved mainly through asexual zoospores, quadriflagellate, pear-shaped cells, 5–7 μm in length. Colonization of substrata involves the transition from a free-swimming spore to an adhered non-motile spore (Callow *et al.*, 1997), adhesion being achieved *via* the secretion of a glycoprotein adhesive which in the settled spore forms a discrete gel-like pad on the surface (Callow & Callow, 2002). Spores often settle gregariously, *i.e.* spores settle in close proximity to previously settled spores, to form groups or rafts of cells. The cues promoting gregarious settlement in *Enteromorpha* are not well understood but the adaptive value may lie

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in the lower energy requirements for spores to attach against each other (Callow *et al.*, 2002) and the greater protection provided to cells in groups against detachment forces in a turbulent environment.

Atomic force microscopy (AFM) has been used to study the visco-elastic properties of the secreted spore adhesive (Callow *et al.*, 2000b; Callow *et al.*, 2001). However, AFM measurements of the visco-elastic properties of the adhesive *per se* do not give information on the interfacial adhesion properties of the whole system and various methodologies have been applied to the assessment of adhesion strength. For macrofouling organisms like barnacles, tube-worms and oysters a common approach (Kavanagh *et al.*, 2001; Swain & Schultz, 1996) is to apply a mechanical shear force parallel to the base of the organism, the basis of an ASTM method (ASTM, 1994). In the case of soft, microfouling species such as algae and slime layers, such an approach is impossible and hydrodynamic methods are more appropriate. Schultz *et al.* (2000) discussed the merits of various flow cell designs and reported on the development of a fully turbulent flow channel for assessment of adhesion strengths of soft fouling organisms. An alternative approach is to water jet a surface with steadily increasing impact pressures as described by Swain and Schultz (1996) for field evaluations of test panels. In this paper new data are presented on the use of a miniaturised and semi-automated version of the water jet method to characterise the baseline adhesion properties of *Enteromorpha* spores to a standard glass surface.

MATERIALS AND METHODS

Enteromorpha Settlement and Adhesion Assays

Fertile plants of *Enteromorpha linza* were collected from Wembury beach, UK (latitude 50°18′N; 4°02′W). Zoospores were released and prepared for adhesion experiments as described previously (Callow *et al.*, 1997). Zoospores were settled in individual dishes (In Vitro Systems & Services, GmbH) each containing a microscope slide to which 10 ml of zoospore suspension (1.5×10^6 spores ml^{-1}) were added. The glass microscope slides had been successively washed in a 50% methanol/50% concentrated HCl mixture, followed by 100% concentrated HCl (2 h in each). After 1 h settlement in the dark at 20°C, the slides were gently washed in filter-sterilized artificial seawater (ASW, Instant Ocean) to remove zoospores that had not properly attached. Three replicate slides were fixed in 2% (v/v) glutaraldehyde in seawater for 10 min, followed by washing as described previously (Callow *et al.*, 1997). This group of slides constitutes the 'control' treatment, *i.e.* not exposed to the water jet. The remaining slides were incubated for various periods of time in ASW in the light before exposing them to calibrated surface pressures in the water jet, followed by fixation. Adhered zoospores were counted using a Zeiss Kontron 3000 image capture analysis system attached to a Zeiss epifluorescence microscope (Callow *et al.*, 2002). Counts were made for 30 fields of view on each of 3 replicate slides. The number of spores still attached to slides exposed to flow was counted and compared with unexposed samples. Since the level of settlement in each experiment was different (*e.g.* because different spore batches were used), data are presented in terms of percentage spore removal compared with the controls.

Modified Water Jet Apparatus

The original water jet apparatus for assessment of fouled panels in the field (Swain & Schultz, 1996) employed a hand-held nozzle to produce a controlled stream of water of known pressure. This is directed at the fouling organisms to maximize fouling removal. The pressure of the water jet is increased until all the fouling is removed or until the maximum pressure of the apparatus is achieved. For the purpose of laboratory-scale, reproducible evaluations of short-term attachment strength on microscope slides, a more consistent method of applying the water jet was required. This was accomplished by building a motorised holder for 6 microscope slides. Computer-controlled stepper motors allowed the bank of slides to be moved horizontally and vertically in a raster pattern across the path of the water jet, at speeds between 2.5 and 12.5 mm s^{-1}. The pitch of movement in the vertical direction at the end of each horizontal traverse was 2 mm. The nozzle was mounted 25 mm from the bank of slides and had an internal exit diameter of 1.6 mm. The instrument is typically operated at a speed of 10 mm s^{-1} for 10 swathes, at the end of which an area of 500 mm^2 in the mid-region of each slide has been exposed to the jet of water. The water supply is housed in a pressure resistant tank and pressurised using a compressed air supply from a conventional SCUBA tank. The relationship between regulator setting and impact pressure exerted at the surface was determined as described by Swain and Schultz (1996).

RESULTS

Preliminary detachment experiments were carried out with the turbulent flow cell (Schultz *et al.*, 2000), but after a standard settlement period of 1 h, very few spores of *Enteromorpha* could be detached from the standard glass surface at the maximum flow rate of that instrument (3.4 1 s^{-1}, equivalent to a wall shear stress of 56 Pa). The much higher stresses

generated by the water jet proved to be effective. The detachment curve for spores 4 h after settlement revealed considerable variation in attachment strength within the spore population (Figure 1). Some spores could be removed by surface pressures as low as 10 kPa, but 50% removal required ~100 kPa and quantitative removal required pressures as high as 250 kPa. Detachment was also a function of contact time with the surface (Figure 2). At 62 kPa surface pressure 80% of attached spores could be removed at 40 min, the shortest time at which a measurement could practically be made. The proportion of spores removed progressively reduced with increasing contact time either through enhanced interfacial bonding, or curing reactions within the bulk of the adhesive through some form of cross-linking, or some combination of both. After a further 3 h the pressure was increased to 210 kPa. More spores were detached at this pressure but percentage removal continued to decline until by 8 h, no more spores could be removed.

To explore the influence and possible adaptive value of gregarious spore settlement on adhesion strength, spores were settled on glass slides at high cell densities to promote the formation of groups. The proportion of total spores as singles *vs* those in groups was assessed after exposure to a range of detachment forces from the automated water jet. The results showed that as the total number of attached spores declined with increased surface pressure the proportion of single spores to those in groups also declined (Figure 3).

DISCUSSION

The interaction between an adhesive material and the substrate involves wetting of the substrate by the adhesive, and the subsequent curing of the adhesive. The wetting process determines the area of contact between the adhesive and the substratum and has an important role in determining the interaction force between the adhesive and the substratum. The curing process determines the microstructure of the solid film, thus influencing both the mechanical properties and the cohesive strength. Apart from the intrinsic interest in the mechanisms of adhesion used by marine organisms, the search for non-toxic minimally adhesive surfaces for the control of marine fouling requires that comparative evaluations of novel coatings, whether by laboratory or field testing methods, be based on an understanding of the fundamentals of adhesion processes used by different fouling organisms. Comparative data on

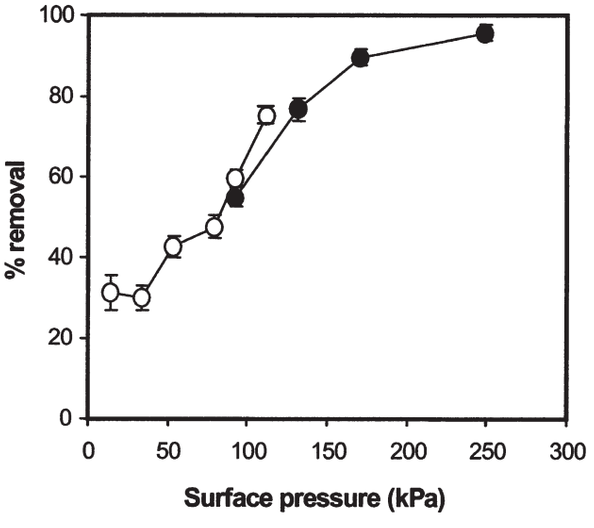

FIGURE 1 The effect of surface pressure (kPa), on the adhesion of *Enteromorpha* spores to glass, as determined in the water jet apparatus. Spores were settled for 1 h, washed gently to remove unattached spores and incubated for a further 3 h before being challenged with a range of water pressures. Percentage removal was determined from the mean of 6 replicate slides by comparison with control slides not exposed to the water jet. The two lines represent two separate experiments at different pressure ranges, each experiment having its own (unexposed) control. The mean levels of spore settlement (spores $mm^{-2} \pm 2 \times SE$) before subjecting slides to flow, were 2304.8 ± 117.2 for the low pressure range, and 3115.4 ± 128.9 for the higher pressure range. Bars = $\pm 2 \times SE$ of the mean derived from arcsine transformed data.

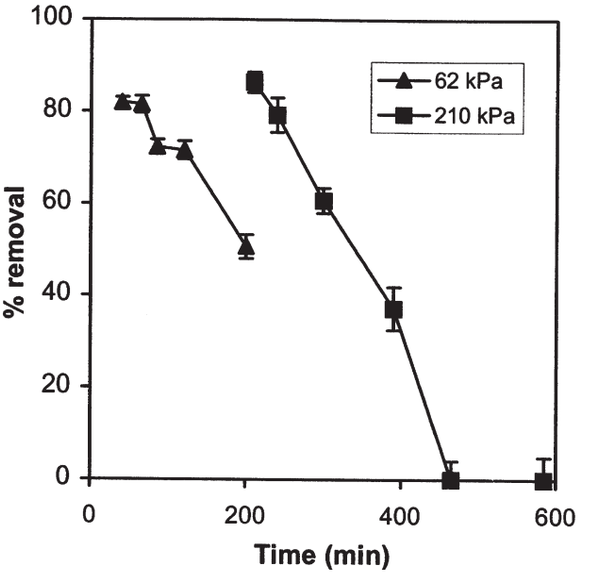

FIGURE 2 The effect of 'contact time' on the strength of adhesion of settled *Enteromorpha* spores. Cells were settled on glass slides for 1 h, unattached cells removed by rinsing, then slides were aged for various periods of time before exposure to 62 kPa (up to 3 h) (▲) or 210 kPa (3–10 h) (■) surface pressure in the water jet. All data were obtained from one experiment and show the progressive increase in attachment strength with time. Percentage removal was determined from the mean of 6 replicate slides, compared with control slides unexposed to shear stress. The mean level of spore settlement (spores $mm^{-2} \pm 2 \times SE$) before subjecting slides to flow, was 1127.6 ± 115.8. Bars = $\pm 2 \times SE$ of the mean derived from arcsine transformed data.

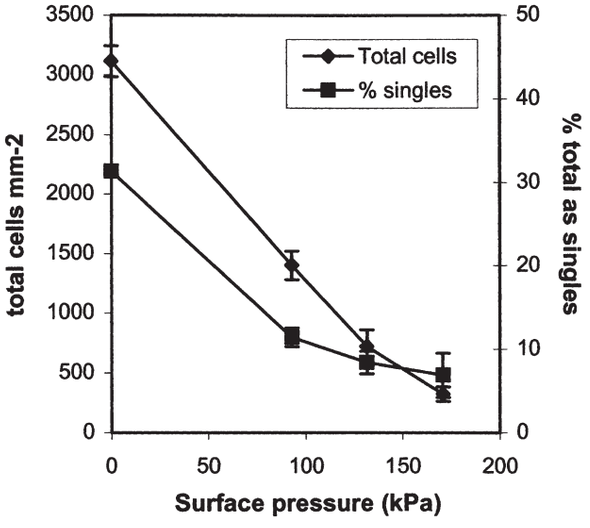

FIGURE 3 Change in the proportion of attached spores as singles *vs* those in clumps as a function of surface pressure. The graph shows the total number of spores (singles plus clumps) remaining attached to glass after exposure to water jet, and the percentage of total cells as single spores. The settlement period used was 4 h. Spores were more tenaciously attached when part of a clump than when standing alone as single spores. Each point is the mean of 90 observations on 3 replicate slides. Bars = $\pm 2 \times$ SE derived from arcsine transformed data.

tenacity of adhesion (*i.e.* adhesive force per unit of surface area) along with hydrodynamic force data for the organism can then be used to predict the velocity necessary for detachment of these organisms from a ship's hull (Schultz *et al.*, 1999).

Extensive studies have been made of the adhesion strengths of invertebrate species. For macroscopic, shelled animals there are many reports of direct tenacity measurements of shear resistance using various types of force transducer, although few authors have used glass (the authors' control surface for laboratory evaluations) as a substratum. Becker (1993) used glued tethers to measure the tensile (vertical) detachment stress of adult barnacles (*Balanus variegatus*) and tubeworms (*Pomatoleios kraussii*) on glass and obtained values of 800 and 150 kPa, respectively. Kavanagh *et al.* (2001) reported adhesion strength values within the range 90–130 kPa for adult barnacles, oysters and tubeworms attached to unmodified RTV11 silicone elastomer coatings. A more meaningful comparison with soft-fouling algae would be the extensive series of direct tensile force measurements made on cyprid larvae *via* wire tethers glued to the larva, which indicate tenacities of adhesion of *ca* 200 kPa for temporary adhesion and *ca* 900 kPa for permanent cyprid adhesion (Yule & Walker, 1987).

In comparison, there is little information on the adhesion characteristics of algae and direct measurements of tenacity of adhesion on individual cells of microscopic dimensions are not possible, hence the hydrodynamic approach adopted in this paper. These differences in experimental methods make it difficult to make quantitative comparisons between direct adhesion data for invertebrates using the force gauge and measurements on soft foulers using hydrodynamic methods; however some approximation may be made. The present paper shows that surface pressures *ca* 200–250 kPa are required to quantitatively remove settled *Enteromorpha* spores after 4 h contact time with the surface. There are no other published data for adhesion strength measurements of *Enteromorpha* using water jet methods, the most direct comparison is with the 40–100 kPa (Swain & Schultz, 1996) and >240 kPa (Terlizzi *et al.*, 2000) surface pressure required to remove mature slime films (which would have consisted of diatoms and low-form algae such as germinating *Enteromorpha* spores) from epoxy surfaces.

For meaningful quantitative comparison with adhesion strength data for other organisms, measurements of surface pressure need to be converted to shear stresses. Force gauge measurements apply a shear stress to the base of the organism to cause detachment. The water jet creates stresses that are somewhat more complex. This flow type consists of three distinct regions, *viz* a free jet region at the exit to the nozzle, an impingement region near the centre of jet impact, and a wall jet region at a greater radial distance from the centre of impact. The water jet impinging on the coating surface creates a normal stress (pressure) near the centre of impact. The normal stress is maximum at the centre of impact and becomes negligibly small at a radial distance of about 20% of the distance from the nozzle to the surface (Beltaos & Rajaratnam, 1974). The region from the centre of impact out to this radial distance is termed the impingement region. In the impingement region, there is also shear stress at the wall that is zero at the centre of impact and reaches a maximum value at a radial distance of about 15% of the distance from the nozzle to the surface. Outside the impingement region, in the wall jet region, the wall shear stress decays to less than half its maximum value at a radial distance of 50% of the distance from the nozzle to the surface (Beltaos & Rajaratnam, 1974). The maximum shear stress at the wall generated by a water jet in the impingement region as given by Beltaos and Rajaratnam (1974) is:

$$\tau_{\max} = \frac{0.32\left(\frac{1}{2}\rho U_{\text{jet}}^2\right)}{\left(\frac{H}{d}\right)^2} \approx \frac{0.32(\text{impact pressure of jet})}{\left(\frac{H}{d}\right)^2}$$

where $\tau_{\max}$ = maximum wall shear stress, ρ = density of fluid, U_{jet} = bulk mean velocity of jet at nozzle exit, H = distance from nozzle exit to surface, and d = diameter of nozzle.

This equation may be used to predict τ_{max} for water jet data. For the jet diameter and stand-off distance used in the present study, $\tau_{max} \approx 1.3 \times 10^{-3} \times$ (jet impact pressure). For instance, an impact pressure of 100 kPa (for 50% removal after 4 h surface contact) would deliver a τ_{max} of ~130 Pa. An impact pressure of 250 kPa (to quantitatively remove spores after 4 h) would give a τ_{max} of ~325 Pa. For microscopic scale fouling the shear stress at the wall is likely to be a good approximation to the actual detachment forces experienced by these cells. However, it should be noted that the impingement region is an area were both normal and shear stresses are present. It is not clear if the organisms removed by the water jet are detached through normal stress, shear stress, or combined stresses. For this reason, further research is necessary to correlate the water jet data with the other test methods and, ultimately, the fouling release on a ship. Nevertheless, it is instructive to relate these approximate shear stress resistance values for *Enteromorpha* spores, to ship operating velocities. From a known shear stress value for adhesion strength determined by laboratory experimentation it is possible to use these formulae to predict the ship velocity at which a given organism will detach. Thus, application of the power-law formula (Schlichting, 1978), predicts that at a point 30 m from the bow of a ship moving at 42 knots (21.4 m s^{-1}) flow conditions would be dynamically equivalent to wall shear adhesion strengths of up to 325 Pa. These calculations suggest that at ship operating speeds that are feasible for most vessels, most *Enteromorpha* spores should not detach from an untreated hull (assuming the 'release' properties of an untreated hull are equivalent to those of glass). The situation is further exacerbated by the development of spore adhesion strength with time; a shear stress of 325 Pa required to quantitatively remove spores is inadequate beyond the first 4 h of adhesion. Spores in groups also exhibit a greater resistance to detachment than single spores. Therefore, the pressure needed to remove all spores will depend on the distribution of spore group sizes on a surface, which is influenced by factors such as surface energy (Callow *et al.*, 2000).

The progressive, increasing tenacity of spore attachment over several hours is likely to be due to several factors including increased formation of interfacial bonds with time, changes in the viscoelastic properties of the adhesive itself (adhesive 'curing'), increased cohesiveness within the adhesive-organism continuum, and the possibility of further secretion of adhesive materials. AFM has revealed changes in the intrinsic adhesive and elastic properties of the secreted spore adhesive within 60–90 min of primary adhesion (Callow *et al.*, 2000b; Callow *et al.*, 2001). The adhesive was shown to possess the highest values for adhesive force yet recorded by AFM studies on natural, biological materials. Adhesive strength declined and compressibility increased over 60 min as the primary adhesive 'cured', presumably through some form of molecular cross-linking.

However, the continued development of adhesive strength of settled spores over a longer time period (several hours) shows that this initial rapid curing of the primary spore adhesive cannot be the sole factor in contributing to adhesive strength. The glycoprotein adhesive antigen secreted as the primary adhesive is also detectable in the developing cell wall of the settled spore (Stanley *et al.*, 1999) indicating that the antigen is continually synthesized *via* the sporeling Golgi system. It was suggested that the consolidation of adhesion after the primary adhesion event depends on the continued synthesis of the same or similar adhesive glycoproteins into the new cell wall (Callow *et al.*, 2000a). In this model, adhesion is viewed as an extension of cell wall synthesis with cross-links between these glycoproteins and other cell wall matrix components providing a cohesively strong physical continuum between the cell and the adhesive at the substratum interface.

The results reported in this paper on the baseline adhesion properties of *Enteromorpha* spores reveal their tenacious character at the scale of forces relevant to ship speeds and that considerable incentive exists for the development of effective foul-release coatings. Studies on the forces required to detach *Enteromorpha* spores from substrata with differing surface properties, including foul-release coatings, are in progress.

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