Biofouling, 2013
Vol. 29, No. 4, 401–411, http://dx.doi.org/10.1080/08927014.2013.777046

Taylor & Francis
Taylor & Francis Group

A novel biofilm channel for evaluating the adhesion of diatoms to non-biocidal coatings

John A. Finlay[a]*, Michael P. Schultz[b], Gemma Cone[a], Maureen E. Callow[a] and James A. Callow[a]

[a]*School of Biosciences, University of Birmingham, Birmingham, UK;* [b]*Department of Naval Architecture and Ocean Engineering, United States Naval Academy, Annapolis, MD, USA*

(*Received 28 September 2012; final version received 9 February 2013*)

Laboratory assessment of the adhesion of diatoms to non-toxic fouling-release coatings has tended to focus on single cells rather than the more complex state of a biofilm. A novel culture system based on open channel flow with adjustable bed shear stress values (0–2.4 Pa) has been used to produce biofilms of *Navicula incerta*. Biofilm development on glass and polydimethylsiloxane elastomer (PDMSe) showed a biphasic relationship with bed shear stress, which was characterised by regions of biofilm stability and instability reflecting cohesion between cells relative to the adhesion to the substratum. On glass, a critical shear stress of 1.3–1.4 Pa prevented biofilm development, whereas on PDMS, biofilms continued to grow at 2.4 Pa. Studies of diatom biofilms cultured on zwitterionic coatings using a bed shear stress of 0.54 Pa showed lower biomass production and adhesion strength on poly(sulfobetaine methacrylate) compared to poly (carboxybetaine methacrylate). The dynamic biofilm approach provides additional information to supplement short duration laboratory evaluations.

Keywords: diatom biofilms; adhesion; fouling-release; zwitterionic coatings; polydimethylsiloxane; *Navicula incerta*

Downloaded by [US Naval Academy] at 05:53 11 April 2013

Introduction

In the initial stages in the design of antifouling (AF) and fouling-release (FR) coatings, the availability of coating material is usually limited, and tests to identify the potential for further development have to be made using small samples (Stafslien et al. 2007; Briand 2009). These evaluations typically begin in the laboratory, initially with a suite of rapid screening tests, followed by replicated experiments on a subgroup of candidate materials before scale-up to field panels and test patches on ships or other structures. Each stage therefore relies upon the predictive power of the preceding one to select good coatings and consequently it is important that evaluations are as relevant as possible to natural conditions. Laboratory tests are generally specific for the test organism and numerous different protocols have been developed (eg Webster et al. 2007; Briand 2009). Such tests have the advantage of being carried out using controlled conditions allowing a high degree of precision and experimental control, but the relevance to the real world is still a common concern. Two of the limitations of laboratory assays are the short duration of tests and the unnatural 'simplified' environments in which they are conducted. Studies typically concentrate on the initial stages (hours) of settlement using larvae or cells of a single species, and under conditions of static immersion. In the natural environment, attached microorganisms soon divide and become part of a biofilm (Molino & Wetherbee 2008), and in most cases surfaces are exposed to water movement, which can have profound effects on the adhesion of cells.

Diatoms are important primary colonisers of surfaces immersed in the sea along with other microorganisms including bacteria, fungi, protozoa and other algae. Diatom-dominated slimes up to several hundred microns in thickness are often formed (Schultz & Swain 2000). Diatom slimes are a problem on many types of immersed surfaces including copper-containing AF paints, and more recently on biocide-free silicone-based elastomeric FR coatings (Molino & Wetherbee 2008; Dobretsov & Thomason 2011; Landousli et al. 2011). The diatom used in the current study, *Navicula incerta* (length 13 μm), is typical of the *Navicula* genus of raphid diatoms found in such biofilms (Cassé & Swain 2006; Zargiel et al. 2011). Standard laboratory assays have tended to quantify the initial density of attached single diatom cells on pristine surfaces over a matter of a few hours and to measure the adhesion strength of these single cells to the surface (eg Holland et al. 2004; Schilp et al. 2007). To study diatoms under more natural conditions, an extension of the laboratory assays into the early stage of biofilm formation is required (Hodson et al. 2012). Hydrodynamic parameters play a large part in fashioning the characteristics of the biofilm as they determine both the bulk transfer of nutrients and local shear stress experienced by cells. It is therefore relevant to have a laboratory assay that provides for the controlled growth of diatom biofilms in flowing water and allows the adhesion properties

*Corresponding author. Email: j.a.finlay@bham.ac.uk

© 2013 Taylor & Francis

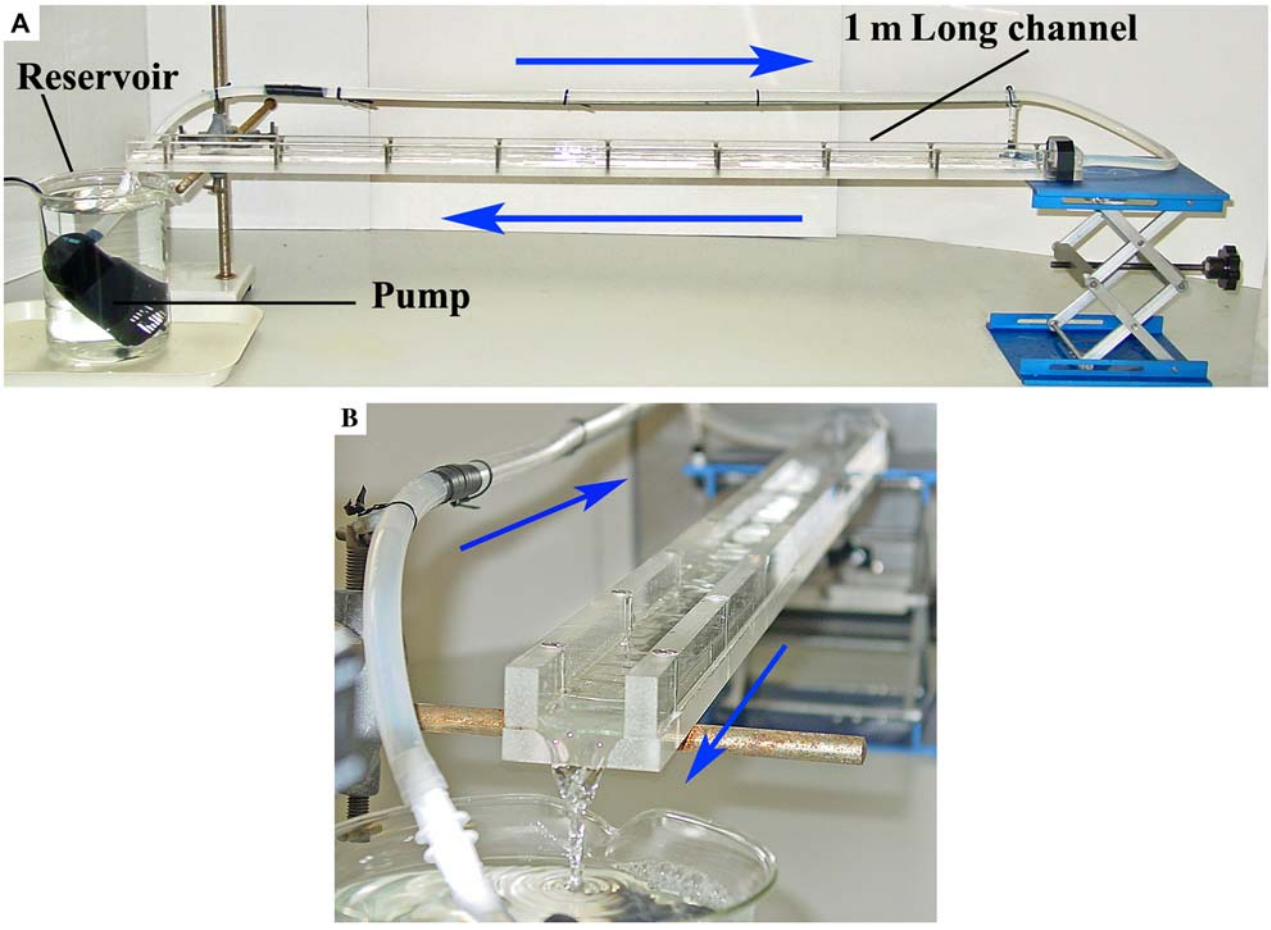


Figure 1. Images of biofilm channel labelled to show significant features. A = overview of channel set up; B = outflow of seawater from channel.

Downloaded by [US Naval Academy] at 05:53 11 April 2013

of such biofilms to be measured. In the present paper, a method of culture to develop biofilms on candidate AF/FR coatings under conditions of defined shear stress is described. The adhesion properties of the resulting biofilms were determined using glass and a polydimethylsiloxane elastomer (PDMSe) as 'model' surfaces. The methodology was then applied to compare the development and adhesion of diatom biofilms on zwitterionic coatings of poly(sulfobetaine methacrylate) (SBMA) and poly(carboxybetaine methacrylate) (CBMA), as an example of a non-biocidal coating technology with AF and FR potential. SBMA brush films have previously been shown to reduce settlement and/or adhesion of *N. incerta* (Zhang et al. 2009).

Materials and methods

Biofilm channel construction

The channel was constructed from 10 mm thick transparent acrylic sheet and had a total length of 1 m and an internal width of 27 mm (Figure 1(A)). A silicone cube securing the entrance pipe occupied the first 1.5 cm, followed by a fixed 3.5 cm section of glass microscope slide as a leader. At the downstream end, there was a 4 cm fixed section of glass slide as a block to prevent movement of the 12 glass slides (26 × 76 mm) that filled the rest of the channel. Positions 5–10 (numbered from the upstream end) were used to accommodate 6 test samples; the remaining positions were filled by blank slides of the same thickness. Artificial seawater (ASW; Tropic Marin®) and growth medium (see 'Growth of diatoms in biofilm channel' section below) was circulated through the system using either a 'Fluval 1 Plus' or 'Fluval 3 Plus' water pump immersed in a collection reservoir at the downstream end of the channel. Water exited the channel into this reservoir as a waterfall (Figure 1B). The seawater/medium was conveyed to the upstream end through 8 mm internal diameter silicone tubing opening out into an orifice of 16 mm in diameter fitted with a stainless steel mesh (1 mm^2 pores) to baffle the flow.

Downloaded by [US Naval Academy] at 05:53 11 April 2013

Characterisation of hydrodynamic flow

Calculating the properties of water flow in open channels is more complicated than in a pipe due to the presence of a free surface subject to atmospheric pressure. The flow of water is achieved by the effects of gravity along the slope of the channel rather than by a pump. Uniform flow, defined by constant water cross-section and depth, occurs at a set distance downstream of the entrance depending on the slope of the channel and the volume of water entering the channel. The flow characteristics of the channel were determined using a number of standard equations for open channel flow. Measurements of the height of the flow depth (h), the discharge per unit time and the slope of the channel were made. Examples of data for low and high flow regimes are given in Table 1. Constants used for calculations were: kinematic viscosity of seawater: 1.0×10^{-6} ($m^2\,s^{-1}$); density of seawater: 1025 ($kg\,m^{-3}$) and acceleration due to gravity: 9.8 ($m\,s^{-2}$).

It was determined that the viscosity of the seawater medium in the biofilm channel was not significantly different at the end of the experiment compared with fresh, uninoculated seawater medium.

Reynolds numbers

The Reynolds number is a measure of the ratio of inertial force to viscous force and can be used to determine whether the flow conditions are laminar or turbulent. Reynolds numbers (Re) were determined using the hydraulic radius as the representative length scale of the apparatus (Kirkgöz & Ardiçlioğlu 1997). This is the ratio between the cross-sectional area of the channel and the wetted perimeter:

$$R = A/P \tag{1}$$

where: R = the hydraulic radius (m), A = the cross-sectional area of flow (m^2) and P = the wetted perimeter (m). Reynolds numbers were calculated using the formula:

$$\mathrm{Re} = 4VR/v \tag{2}$$

where V is the water velocity and v is the kinematic viscosity. Calculated in this way, laminar flow occurs below 500, transitional or turbulent flow between 500 and 2000, and only turbulent flow when Reynolds number exceeds 2000 (Streeter 1966). Reynolds numbers for the biofilm channel, calculated under conditions of low and high flow rates, exceed 2000 indicating that the flow was turbulent under all the present test conditions. This was further verified by direct observation of dye distribution patterns at a range of flow rates: the transition from laminar to turbulent flow occurring between Reynolds numbers of 1350 and 2325.

Froude numbers

The Froude number is a measure of the ratio of inertial force to gravitational force and is important in determining the length of the flow development zone. Froude numbers (Fr) were calculated using the equation:

$$\mathrm{Fr} = V/(gh)^{1/2} \tag{3}$$

where V = velocity, g = acceleration due to gravity and h is depth of flow. Fr values for high and low flow rates are shown in Table 1.

Flow development zone

The velocity profile of water entering the biofilm channel will vary due to viscous effects created by the walls. A certain distance from the entrance the velocity profile will cease to change and the flow will be 'fully developed'. For open channel flow, the length of the flow development zone (L) can be determined using the equation of Kirkgöz and Ardiçlioğlu (1997):

$$L/h = 76 - 0.001\mathrm{Re}/\mathrm{Fr} \tag{4}$$

Consequently $L = (76 - 0.0001\ \mathrm{Re}/\mathrm{Fr})h$. Operating at a low flow rate the system gives a Re of 2778 and a Fr of 0.98, the ratio of the two being Re/Fr = 2835. When multiplied by 0.0001, the factor becomes 0.2834 which is so small that it has little effect on the outcome of the equation. Therefore, L effectively becomes $76 \times h$. The depth of flow is typically in the region of 0.0045 m giving an L value of 0.34 m. This value coincides with the beginning of the first test slide in the channel. At distances greater than this the flow is at steady state with

Table 1. Examples of flow characteristics at high and low flow regimes, created by altering seawater discharge from the pump and the slope of the channel.

Flow	Gradient (m)	Velocity ($m\,s^{-1}$)	Reynolds no.	Froude no.	Water depth (m)	L (m)	Bed shear stress (Pa)	Amended bed shear stress (Pa)
Low	0.015	0.21	2778	0.98	0.0045	0.34	0.68	0.54
High	0.037	0.41	5555	1.96	0.0045	0.34	1.67	1.32

a constant shear stress spanning the six slide positions used for testing coatings.

At the end of the channel, after the test section, the exit waterfall produced a region of altered flow in which water velocity increased and uniform flow was lost. Measurements of this region indicated a length of between 0.04 and 0.09 m from the exit point (depending on flow rate), which is well within the 0.19 m length extending beyond the end of the last test slide.

Bed shear stress

The bed shear stress in an open channel can generally be described by the 'depth-slope' product:

$$\tau = \rho g h S \tag{5}$$

where τ = bed shear stress, ρ = water density, g = acceleration due to gravity, h = water depth and S = the slope of the channel. However, this assumes a channel of infinite width and does not take into account the sidewall effects that finite width–depth ratio will have on the water flow (For the biofilm channel the width–depth ratio for conditions giving a water depth of 0.0045 m would be 0.027/0.0045 = 6). An adjustment in the bed shear stress can be made for finite width–depth ratios using the equation of Guo and Julien (2005):

$$\frac{\overline{\tau_b}}{\rho g h S} = \frac{4}{\pi}\tan^{-1}\exp\left(-\frac{\pi h}{b}\right) + \frac{\pi}{4}\frac{h}{b}\exp\left(-\frac{h}{b}\right) \tag{6}$$

For example, for a flow rate giving a water depth of 0.0045 m, the left-hand side of Equation (6) yields 0.79, signifying that the bed shear stress is 79% of that for an infinitely wide channel (Equation (5)). All the calculated shear stress values reported in this paper account for the finite width–depth ratio.

Test surfaces

Nexterion glass slides (Schott, Germany, 75 × 25 mm) were used as hydrophilic surfaces (water contact angle < 20°). Glass slides (75 × 25 mm) coated with PDMSe, Silastic® T-2 (Dow-Corning Corporation, water contact angle 109°) were made by the method of Hoipkemeier-Wilson et al. (2004). PDMSe samples were equilibrated in deionised water for 48 h followed by 2 h in 0.22 µm filtered ASW, before each experiment. SBMA and CBMA films on glass slides were provided by Professor S. Jiang, University of Washington using surface-initiated atom transfer radical polymerization to form a layer of polymer brushes as described in Aldred et al. (2010). Samples were equilibrated in 0.22 µm filtered ASW for 10 min prior to diatom settlement.

Growth of diatoms in biofilm channel

Pre-culture of cells

Cells of *N. incerta* were cultured in 50 ml of F/2 medium contained in 250 ml conical flasks (Guillard & Ryther 1962). After three days, the cells were in log phase growth. Cells were washed 3 times with fresh medium before harvesting and diluting in ASW supplemented with 50% strength F/2 nutrients (0.22 µm filtered) to give a suspension with a chlorophyll *a* content of 0.125 µg ml^{-1}, determined by the method of Jeffrey and Humphrey (1975).

Initial attachment of cells

The height of the channel was adjusted to a horizontal position at the same height as the pump outlet. The channel was plugged at both ends and 175 ml of diatom culture were added to completely cover all test surfaces. Lighting was provided by four overhead growth lamps (Arcadia, 11 watt, 230 mm lamp) giving an irradiance of 10 µmol m^{-2} s^{-1}. The cells were allowed to settle for 2 h, during which time they descended through the seawater medium to the surfaces and attached, before the flow phase was begun.

The initial density of cells on the surfaces prior to flow was measured in three separate experiments by removing slides from positions 1 to 3 in the channel and performing cell counts. The slides removed were replaced by new slides prior to the flow phase. Slides with attached cells were fixed in 2.5% glutaraldehyde in ASW, washed in deionised water and dried in air. The cells were visualised by fluorescence of chlorophyll under a Zeiss epifluorescence microscope (λ excitation and emission: 546 and 590 nm, respectively) with an AxioVision 4.8 imaging system and counts were made for 30 fields of view (each 0.15 mm^2) on each of the three slides (Callow et al. 2002).

Flow phase

The flow phase was initiated after the 2 h settlement period by unplugging the ends of the channel and switching on the circulation pump. Having the settlement phase within the channel allowed the transfer from static to circulating conditions without the need to move samples from a separate container to the channel, thereby negating disturbance or exposure to air. The angle of the channel was adjusted as described above and the biofilm allowed to develop *in situ*. Diatom biofilms were cultured on glass and PDMSe over a range of bed shear stresses (0–2.4 Pa). On zwitterionic coatings, biofilms were cultured under static conditions and under a single, calculated bed shear stress of 0.54 Pa. Previous studies (Zhang et al. 2009) showed that diatoms adhere weakly to polysulfobetaine-coated surfaces and therefore this low bed shear stress was considered to be suitable, as confirmed by a pilot study.

Downloaded by [US Naval Academy] at 05:53 11 April 2013

Downloaded by [US Naval Academy] at 05:53 11 April 2013

Assessment of biofilm growth

After three days (72 h), the slides from positions 5 to 10 along the biofilm channel, the region in which bed shear stress was uniform, were removed and the biomass on each slide quantified by measuring chlorophyll fluorescence in a fluorescence plate reader (Tecan GENios Plus) (excitation $\lambda = 430$ nm, emission $\lambda = 670$ nm) (Finlay et al. 2008). The biomass was quantified in terms of relative fluorescence units (RFU). The RFU value for each slide was the mean of 90 point fluorescence readings taken from the central area ($\sim 42 \times 12$ mm^2).

Adhesion strength measurements

Adhesion strength of biofilms cultured in channel

To accurately measure adhesion strength it was necessary to carry out cell counts before and after exposure to a known hydrodynamic force in a turbulent flow, closed water channel (Schultz et al. 2000, 2003). The six replicate sample slides were removed from the biofilm channel and transferred to individual wells of 'Quadriperm' polystyrene culture dishes (Greiner) containing 10 ml of culture medium. Cell counts were made *in situ* for five fields of view (0.083 mm^{-2}) on each of the six slides (30 counts in total). The samples were then subjected to a shear stress of 52 Pa in a turbulent flow water channel for 5 min. The density of cells remaining was counted and the percentage removal calculated to provide a measure of adhesion strength to the surface.

Adhesion strength of single cells

The adhesion strength measurements for cells at the beginning of the experiment, before a biofilm had formed, were carried out as above except that the slides were removed after the 2 h settlement period. Counts were made by the method described in the 'Initial attachment of cells' section.

Results

Initial attachment of cells

At the end of the 2 h settlement period, the density of cells in contact with all surfaces would have been approximately the same since cells reach the surface by gravity. Mean counts on glass were 232 cells mm^{-2} (± 9.9 standard error (SE)). Since the samples were not washed prior to fixation, this count represents the number of potential colonising cells available.

Biofilm development at different shear stresses on glass and PDMSe

Three successive calibration runs of the biofilm channel using glass slides, with a nominal flow rate of 1500 ml min^{-1} (Reynolds number ~2900) and a shear stress of 0.46 Pa produced diatom biofilms with a mean RFU of 6190. One-way analysis of variance showed that there was no significant difference between the biofilm density in the 6 slide positions used for the tests (F 5, 12 = 0.87, $p > 0.05$). For the assays described below, variation in biomass obtained for slides in the six positions was therefore due to surface/cell interactions rather than their position within the channel.

The effect of changing the bed shear stress in the biofilm channel on the development of the diatom biofilm generated a biphasic curve (Figure 2). Culture under static conditions (ie no applied shear stress) produced a biofilm that was similar, both in terms of biomass and appearance, on both glass and PDMSe. These biofilms were composed of single cells interspersed with small clumps of cells (Figure 3A and B). The cell density on both surfaces was ~2550 cells mm^{-2}. Therefore, assuming growth was exponential, the doubling time was 21 h.

At low flow rates within the biofilm channel, ie up to 0.54 Pa, the biofilm on glass and PDMSe remained at a similar level to that under static conditions. On both surfaces, the biofilms consisted of a monolayer of cells and small clumps (Figure 3C and D), but the frequency of clumps was greater on glass than on PDMSe.

Increasing the shear stress beyond 0.54 Pa caused a precipitous drop in biofilm biomass on both surfaces. On glass, at a shear stress of 1 Pa, the biofilm was composed mostly of single cells (Figure 3E). On PDMSe cultured at between 1 and 2.0 Pa, a large number of small clumps remained with clear zones around them, giving the impression that the cells had been swept together into clumps (Figure 3F). Some clumps were elongated with the upstream end attached to the surface similar to the

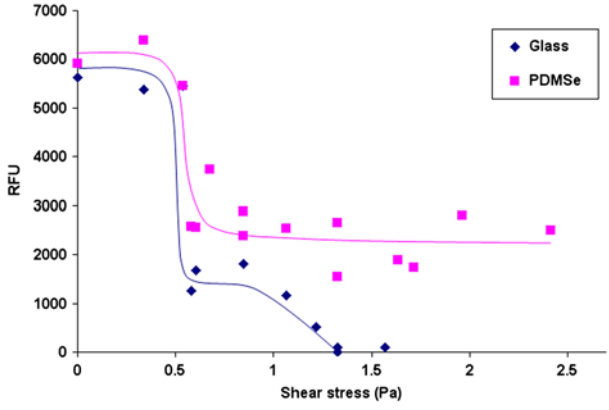


Figure 2. Biomass generation on glass and PDMSe for *N. incerta* cultured in the biofilm channel at different bed shear stresses. Each point is derived from the mean biomass accumulated over 3 days, from six replicate slides. Biomass was measured using a fluorescence plate reader (RFU = relative fluorescence units).

Downloaded by [US Naval Academy] at 05:53 11 April 2013

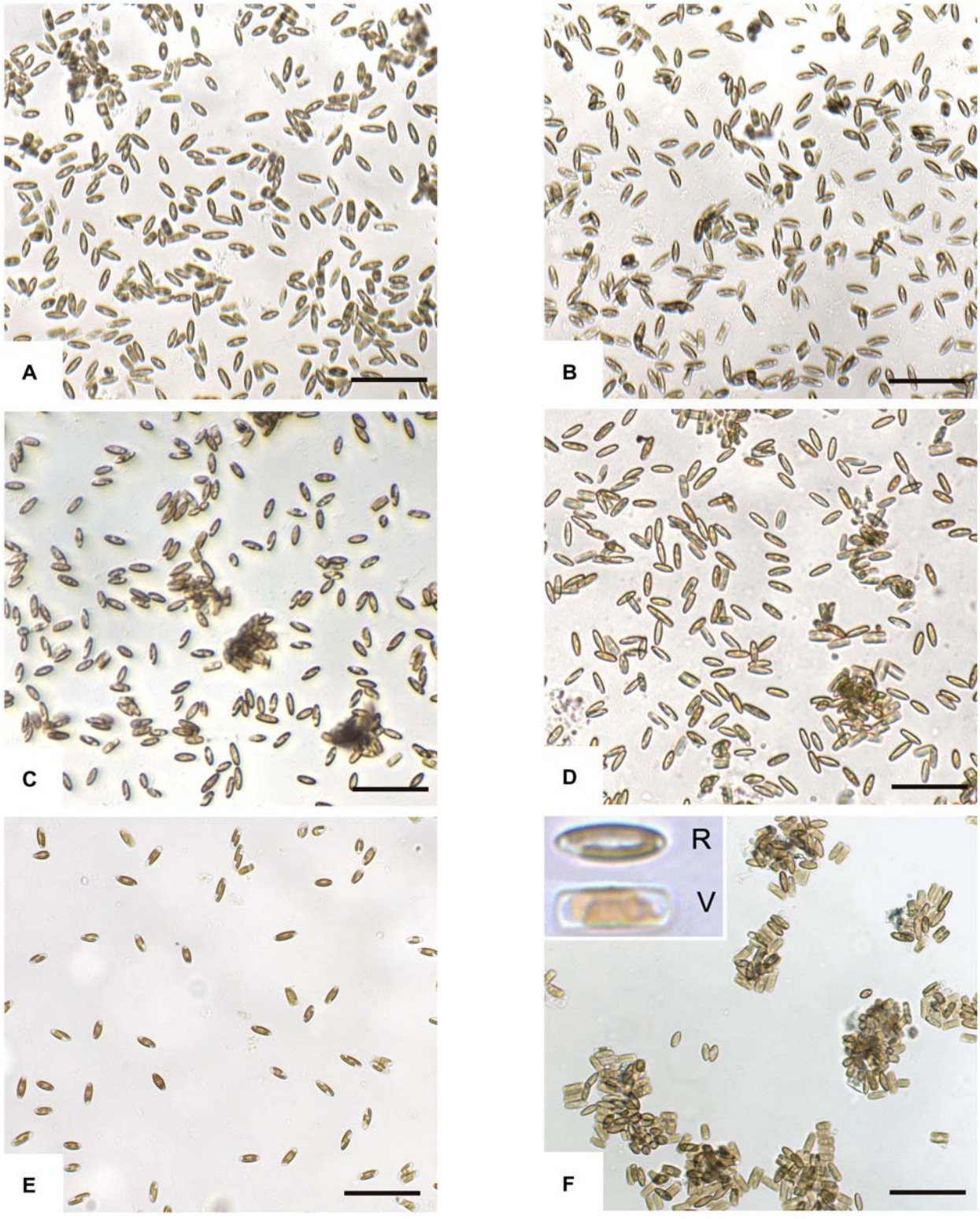


Figure 3. Typical images of diatom biofilms on glass and PDMSe surfaces. A = static biofilm on glass; B = static biofilm on PDMSe; C = biofilm at culture shear stress of 0.54 Pa on glass; D = biofilm at culture shear stress of 0.54 Pa on PDMSe; E = biofilm at shear stress of 1.0 Pa on glass; F = biofilms at shear stress of 2.0 Pa on PDMSe. Inset to F shows difference in orientation of cells, R = a cell raphe-side down, V = a cell valve-side down. Scale bars = 50 μm.

'streamers' reported for bacteria cultured under flow (eg Stoodley et al. 2001).

Culture at 1.3 Pa on glass produced a thinly populated biofilm and cell counts showed that the cell density was approximately the same as the initial starting level, ie after 2 h settlement. Above 1.3 Pa, biofilm biomass was reduced to a negligible amount. On PDMSe, the diatom biofilm persisted at a reduced level up to a shear stress of 2.4 Pa. At this shear stress, the biofilm consisted mostly of small clumps of cells interspersed by a few single cells, the frequency of cells valve-side down having increased (Figure 3F). The channel cannot be used for shear stresses >2.4 Pa without recourse to a larger delivery system as increasing the channel slope causes the water to flow in waves rather than smoothly. Consequently, the critical shear stress for the maintenance of a biofilm on PDMSe could not be identified.

After culture at 1 Pa, observations of biofilms on both surfaces were made using samples that were kept submerged in seawater without passing through the air water interface (which could affect the orientation of cells on PDMSe as the hydrophobic surface dewetted). On glass, there was a mixture of single cells and small clumps, with the majority of cells orientated raphe-side down (>95%). In contrast, on PDMSe a larger proportion of the cells were in clumps and more were valve-side down (>50%). In the latter position, a greater contact area with the PDMSe occurred and a more hydrodynamic profile was generated which should serve to reduce further removal from the surface. As the shear stress of culture increased, the tightness of the clumps and the frequency of cells attached valve-side down also increased (Figure 3F).

Strength of attachment to diatoms to glass and PDMSe

Measurements of the relative adhesion strengths of both single diatom cells (2 h static assay) and biofilms were made by exposing samples to 52 Pa wall shear stress in a turbulent flow water channel (Schultz et al. 2000, 2003). The percentage removal of single cells after a 2 h settlement period showed that the cells were much more strongly adhered to PDMSe than to glass (Figure 4; single cell adhesion).

Exposure of biofilms cultured on glass under static conditions (0 Pa) to a shear stress of 52 Pa in the closed water channel of Schultz et al. (2000), caused removal of ~25% of the cells. Microscope observations revealed that most of the cells that were in clumps were removed leaving a uniform biofilm composed almost entirely of single cells. The percentage removal from PDMSe was lower with a large proportion of the clumps persisting. The difference in adhesion strength on the two substrates was primarily due to the differential adhesion of clumps compared to single cells.

On glass, as the shear stress used for culture increased, the adhesion strength of the biofilm decreased (Figure 4).

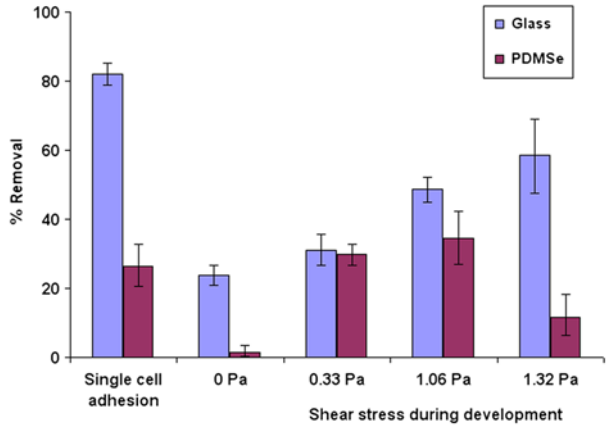


Figure 4. Effect of bed shear stress during culture on the strength of attachment of the diatom biofilm measured as percentage removal when exposed to a shear stress of 52 Pa in a closed water channel. Adhesion measurements for single cells in a 2 h assay are included for comparison. Single cell adhesion data are the means of 90 counts from three replicate slides; biofilm adhesion data is the mean from 30 counts from six slides. Bars show 95% confidence limits derived from arcsine-transformed data.

At the lower shear stresses (0.5–1 Pa), this was probably due primarily to a loss of clumps, and at the higher shear stresses (>1 Pa) due to a loss of integrity of the extracellular polymeric substances (EPS) that held the biofilm together. On PDMSe, biofilms cultured at the lower shear stresses (up to 1 Pa) were more weakly attached, ie the percentage removal was higher than for those cultured under static conditions. The percentage removal was similar to that from glass for biofilms cultured at 0.33 Pa, and larger clumps that had formed, such as the streamers described above, were lost. At the higher culture shear stresses (>1 Pa) the adhesion strength of cells increased. For biofilms cultured at 1.32 Pa, the percentage removal after exposure to 52 Pa shear stress was 58% from glass but only 12% from PDMSe.

Biofilm development and adhesion strength on zwitterionic films

Diatom biofilms were cultured on the zwitterionic surfaces by the above method using either static conditions or a bed shear stress of 0.54 Pa. The level of biomass cultured on the SBMA films was low under both static and dynamic conditions (Figure S1, Supplementary information). [Supplementary material is available via a multimedia link on the online article webpage.] In contrast, levels of biomass were approximately 4 times higher on the static cultured CBMA surfaces and this was further increased to approximately 7 times by the dynamic conditions of the flowing channel. One-way analysis of variance showed significant differences between CBMA biomass levels and SBMA (F 3, 8 = 27.2 $p < 0.05$).

Downloaded by [US Naval Academy] at 05:53 11 April 2013

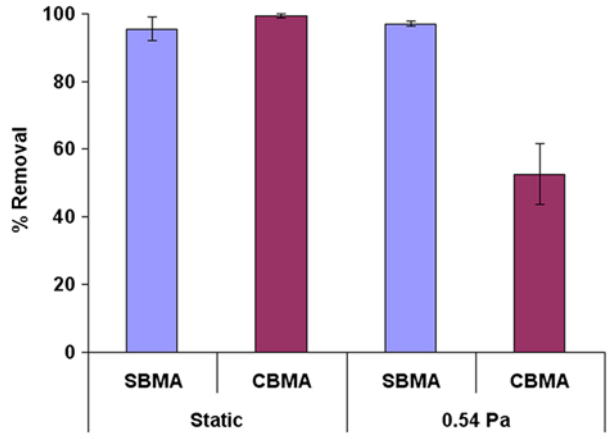


Figure 5. Effect of bed shear stress during culture on the strength of attachment of the diatom biofilm measured as percentage removal when exposed to a shear stress of 30 Pa in a closed water channel. Each point is the mean biomass from three replicate slides measured using a fluorescence plate reader (RFU = relative fluorescence units). Bars show the SE of the mean derived from arcsine-transformed data.

The biofilm cultured on the SBMA and CBMA surfaces was exposed to a shear stress of 30 Pa in a closed water channel as described above, and the percentage removal from each surface was calculated as a measure of adhesion strength (Figure 5). The biofilms on SBMA were weakly attached irrespective of culture conditions and were easily removed from the surfaces. However, on the CBMA surfaces the biofilms cultured under a low shear stress (0.54 Pa) were more strongly attached than those cultured under static conditions. Approximately 50% of the biofilm cultured under the dynamic conditions withstood a shear stress of 30 Pa in the turbulent flow water channel.

Discussion

The precise development of diatom biofilms for study in the laboratory can be achieved using continuous culture techniques, but these require the use of complex equipment, lighting conditions and holders for coated surfaces (Lebeau & Robert 2003; Buhmann et al. 2012). Enclosed flow in pipes and microfluidic chambers also work well (Arpa-Sancet et al. 2012), as the cells can be kept continually submerged during measurements of settlement and adhesion strength, thus preventing damage to the cells by drying out. This issue was resolved for larger sized samples mounted on microscope slides by Hodson et al. (2012) using an ingenious test method involving seawater filled loading tanks attached to a water channel.

A method of culture using open channel flow is presented in this paper as a simple alternative for use with microscope slide-sized samples. Previous open channel culture of algal and bacterial biofilms has generally used large channels and been used for the study of natural waters, modelling local environmental conditions, rather than as a tool to test the adhesion properties of diatoms to specific surfaces (Marker & Casey 1982; Lau & Liu 1993; Serra et al. 2009).

Biomass generation in the biofilm channel is dynamic and is dominated by 2 processes. The first is the rate of cell erosion *vs.* rate of cell division and the second is clump formation. Both of these processes are influenced by the interaction between the adhesive materials secreted by the cells and the surfaces.

Benthic diatoms produce large quantities of EPS which contribute to the stability of the surrounding environment (Lind et al. 1997). These consist largely of polysaccharide materials that are released through pores in the frustule (Smith & Underwood 1998). Adhesion and motility is achieved by the production of additional polysaccharides and glycoproteins which are secreted through the raphe to form part of the adhesion complex (Chiovitti et al. 2006; Molino & Wetherbee 2008). Over time these materials coat the substratum and the cell/EPS complex forms a continuous, coherent matrix often referred to as a 'slime layer' (Haynes et al. 2007). The adhesion strength of cells in/on this matrix is generally enhanced compared to that of individual cells on clean surfaces as demonstrated in Figure 4.

Although there was a difference between adhesion strength of the biofilms grown on glass and PDMSe under static conditions, the adhesion strength on the 2 surfaces was the same when cultured under conditions of low shear stress. In addition, the adhesion to glass was stronger than at the higher shear stresses or in the 2 h single cell experiments. Under both static and low shear stress conditions the biofilm was likely to be at its most stable consisting of cells in a continuous matrix of EPS.

Culture of the biofilms between shear stresses of 0.54 and 0.58 Pa caused a sharp reduction in biofilm biomass on both surfaces. The change in biofilm structure over this narrow range of shear stress produced a biphasic shape to the biomass generation curve. The change accounted for a decrease in biomass of between 50 and 70% (higher for glass than PDMSe). Microscopic observations indicated that the decrease was largely due to the removal of loose clumps of cells rather than the removal of single cells in close contact with the surfaces. The loose clumps of cells could only be attached to the surface by the lower cells/EPS and presented a large surface increasing form drag and consequently they were relatively easily dislodged. The mechanism of formation of the clumps is not understood, but they are probably derived from cells that only formed a weak attachment to the surface. Studies using single diatoms in a short-term assay (2 h) have shown that a proportion of cells

Downloaded by [US Naval Academy] at 05:53 11 April 2013

Downloaded by [US Naval Academy] at 05:53 11 April 2013

are often easily removed and this subpopulation may form the loose clumps (Schultz et al. 2000). These clumps were different to the tighter clumps that formed on PDMSe at the higher shear stresses (>1 Pa) in which the cells pushed up against each other, frequently in valve-side down orientation and in close contact with the surface (Figure 3F). Biofilms on glass were not sustainable when culture conditions exceeded 1.3 Pa (Figure 2). Adhesion strength data indicate that biofilm attachment to the surface became weaker as the shear stress during culture in the biofilm channel was increased. Under flowing conditions, the increase in biomass through cell division and production of EPS is opposed by hydrodynamic forces that remove weakly adhered cells and secreted polymers. The development of a biofilm therefore requires that the rate of cell division exceeds the rate of cell removal (Characklis 1981). At a culture shear stress of 1.3 Pa, the low density of the biofilm caused a lack of cohesion between cells, EPS and the surface, reducing the adhesion strength of the cells towards that of single cells in the 2 h assay, in which a coherent layer of EPS was absent. Newly attached cells on glass need to actively secrete adhesive polymers in order to attach (Wang et al. 1997) and it is possible that newly divided cells were lost at cell division as they attempted to attach in turbulent flow to a surface with a low coverage of EPS. It should also be remembered that diatoms can detach from a surface that they find unfavourable (Wetherbee et al. 1998). Studies on nitric oxide signalling (Thompson et al. 2008) have shown that diatoms are more stressed on glass than PDMSe and this could play a part in the erosion rate of cells from the glass surface by preventing or reducing the secretion of adhesive or affecting its cross-linking.

Although the biofilm on glass could not endure beyond a culture shear stress of 1.3 Pa, on PDMSe, a biofilm was still produced when cells were cultured at 2.4 Pa. The persistence of the biofilm on PDMSe was linked to the orientation of the cells and tight clump formation. In contrast to the situation on glass, many diatoms have a general affinity for hydrophobic surfaces and readily associate with the surface without the active secretion of adhesive polymers (Wang et al. 1997). This non-specific attachment probably allows the cells to be moved along the PDMSe surface by hydrodynamic forces, forming aggregates, rather than being detached from the surface as on glass. Furthermore, it is known that diatoms can adjust their EPS production to suit changes in the local environment (Abdullahi et al. 2006; Khodse & Bhosle 2010) and this may happen over the 3-day-culture period, contributing to the increase in adhesion strength on PDMSe. On glass, the cells may not be able to produce EPS materials that are compatible with the surface and so the rate of cell erosion exceeded the rate of cell division.

The valve-side down orientation of many of the diatoms cultured under the higher shear stresses on PDMSe exposed less surface area to the flow than when in a side-on raphe-down position. This reduced form drag and increased the contact area with the surface thereby increasing attachment force. The aggregation in clumps may also provide increased adhesion as individual cells benefit from the adhesion strength of their neighbours. The ability to take on a more energy favourable orientation, adpressed against the surface, may be important in facilitating their retention on PDMSe during the early stages of biofilm formation.

In the natural environment, the biofilms that form on panels immersed in the sea or on ships' hulls would not be composed only of diatoms, but also of bacteria, fungi, other algae and protozoans (eg Dobretsov & Thomason 2011; Briand et al. 2012). These organisms together make up the slime fouling community and contribute to the adhesion strength of the biofilm as a whole. The experiments in the biofilm channel were not carried out under aseptic conditions and a few bacterial cells were observed on the test surfaces. To what extent these and conditioning macromolecules (see Thomé et al. 2012) may have modified the adhesion of diatoms is impossible to tell from these experiments. Reduced adhesion of single diatom cells to bacterial biofilms developed on both glass and PDMS-based coatings compared to the same non-biofilmed coatings has been recently reported (Mieszkin et al. 2012).

In this study, zwitterionic films were used as an example of surfaces with AF and FR potential. The attachment of diatoms to SBMA films has been studied previously (Zhang et al. 2009), but their interaction with the closely related CBMA surfaces had not been examined. Biofilm formation on SBMA films had a 'lace-like' appearance which was more disrupted under conditions of flow than under static conditions (Figure S2, Supplementary information). [Supplementary material is available *via* a multimedia link on the online article webpage.] The diatom biofilms on these surfaces were unstable, presumably because the secreted EPS layer was unable to bond with the surface. On the CBMA films, the biofilms were attached more strongly and did not 'pull apart' to the same degree as on SBMA. Furthermore, culture under a low shear stress (0.54 Pa) created a more strongly attached biofilm on CBMA. This suggests that flowing seawater improved the integrity of the biofilm, perhaps by stimulating greater (or different) EPS production or by removing weakly attached material rather than allowing it to remain and pull the biofilm apart.

Overall, the study has shown that the biofilm channel can be used for the culture of diatom biofilms under conditions of defined bed shear stress. Depending on the conditions chosen for culture, the biofilm channel can be used to produce either robust biofilms or biofilms that

are subject to high levels of cell removal. This has been demonstrated using the model surfaces of glass and PDMSe, and showed that adhesion was stronger on PDMSe, a finding consistent with field observations (Terlizzi et al. 2000; Zargiel et al. 2011). Examination of biofilm development and adhesion on zwitterionic test surface has shown that the method can be used to discriminate between closely related test coatings and can show differences in coating performance that are not apparent under static culture. It is anticipated that use of the biofilm channel will provide useful information on the early stages of diatom biofilm development and can play a role in testing coatings for AF and FR efficacy.

Nomenclature

R	Hydraulic radius (m)
A	Cross sectional area of flow (m^2)
P	Wetted perimeter (m)
Re	Reynolds number
V	Water velocity ($m\,s^{-1}$)
v	Kinematic viscosity ($m^2\,s^{-1}$)
Fr	Froude number
g	Acceleration due to gravity ($m\,s^{-2}$)
h	Depth of flow (m)
L	Flow development zone (m)
τ	Bed shear stress (Pa)
ρ	Water density ($kg\,m^{-3}$)
S	Slope of channel

Acknowledgements

This research was supported by the Office of Naval Research grant N00014-08-1-0010. The PDMSe surfaces (glass slides coated with Silastic®-T2) were provided by Professor A.B. Brennan of the University of Florida. SBMA and CBMA zwitterionic films were supplied by Professor S. Jiang of the University of Washington.

References

Abdullahi AS, Underwood GJC, Gretz MR. 2006. Extracellular matrix assembly in diatoms (Bacillariophyceae). V. Environmental effects on polysaccharide synthesis in the model diatom, *Phaeodactylum tricornutum*. J Phycol. 42:363–378.

Aldred N, Li G, Gao Y, Clare AS, Jiang S. 2010. Modulation of barnacle (*Balanus amphitrite* Darwin) cyprid settlement behaviour by sulfobetaine and carboxybetaine methacrylate polymer coatings. Biofouling. 26:673–683.

Arpa-Sancet MP, Christophis C, Rosenhahn A. 2012. Microfluidic assay to quantify the adhesion of marine bacteria. Biointerphases. 7:26.

Briand JF. 2009. Marine antifouling laboratory bioassays: an overview of their diversity. Biofouling. 25:297–311.

Briand JF, Djeridi I, Jamet D, Coupé S, Bressy C, Molmeret M, Le Berre B, Rimet F, Bouchez A, Blache Y. 2012. Pioneer marine biofilms on artificial surfaces including antifouling coatings immersed in two contrasting French Mediterranean coast sites. Biofouling. 28:453–463.

Buhmann M, Kroth PG, Schleheck D. 2012. Photoautotrophic–heterotrophic biofilm communities: a laboratory incubator designed for growing axenic diatoms and bacteria in defined mixed-species biofilms. Environ Microbiol Rep. 4:133–140.

Callow ME, Jennings AR, Brennan AB, Seegert CE, Gibson A, Wilson L, Feinberg A, Baney R, Callow JA. 2002. Microtopographic cues for settlement of zoospores of the green fouling alga *Enteromorpha*. Biofouling. 18:237–245.

Cassé F, Swain GW. 2006. The development of microfouling on four commercial antifouling coatings under static and dynamic immersion. Int Biodeterior Biodegrad. 57:179–185.

Characklis WG. 1981. Fouling biofilm development: a process analysis. Biotechnol Bioeng. 23:1923–1960.

Chiovitti T, Dugdale TM, Wetherbee R. 2006. Diatom adhesives: molecular and mechanical properties. In: Smith AM, Callow JA, editors. Biological adhesives. Berlin: Springer-Verlag; p. 79–103.

Dobretsov S, Thomason J. 2011. The development of marine biofilms on two commercial non-biocidal coatings: a comparison between silicone and fluoropolymer technologies. Biofouling. 27:869–880.

Finlay JA, Fletcher BR, Callow ME, Callow JA. 2008. Effect of background colour on growth and adhesion strength of *Ulva* sporelings. Biofouling. 24:219–225.

Guillard RR, Ryther JH. 1962. Studies on marine planktonic diatoms. 1. *Cyclotella nana* Hustedt and *Detonula confervacea* (Cleve). Can J Microbiol. 8:229–239.

Guo J, Julien PY. 2005. Shear stress in smooth rectangular open-channel flows. J Hydraul Eng. 125:30–37.

Haynes K, Hofmann TA, Smith CJ, Ball AS, Underwood GJC, Osborn AM. 2007. Diatom-derived carbohydrates as factors affecting bacterial community composition in estuarine sediments. Appl Environ Microbiol. 73:6112–6124.

Hodson OM, Monty JP, Molino PJ, Wetherbee R. 2012. Novel whole cell adhesion assays of three isolates of the fouling diatom *Amphora coffeaeformis* reveal diverse responses to surfaces of different wettability. Biofouling. 28:381–393.

Hoipkemeier-Wilson L, Schumacher JF, Carman ML, Gibson AL, Feinberg AW, Callow ME, Finlay JA, Callow JA, Brennan AB. 2004. Antifouling potential of lubricious, micro-engineered, PDMSe elastomers against zoospores of the green fouling alga *Ulva* (*Enteromorpha*). Biofouling. 20:53–63.

Holland R, Dugdale TM, Wetherbee R, Brennan AB, Finlay JA, Callow JA, Callow ME. 2004. Adhesion and motility of fouling diatoms on a silicone elastomer. Biofouling. 20:323–329.

Jeffrey SW, Humphrey GF. 1975. New spectrophotometric equations for determining chlorophylls a, b, c1 and c2 in higher plants, algae and natural phytoplankton. Biochem Physiol Pflanz. 167:191–194.

Khodse VB, Bhosle NB. 2010. Differences in carbohydrate profiles in batch culture grown planktonic and biofilm cells of *Amphora rostrata* Wm. Sm. Biofouling. 26:527–537.

Kirkgöz MS, Ardiçlioğlu M. 1997. Velocity profiles of developing and developed open channel flow. J Hydraul Eng. 123:1099–1105.

Landousli J, Cooksey KE, Dupres V. 2011. Review – interactions between diatoms and stainless steel: focus on biofouling and biocorrosion. Biofouling. 27:1105–1124.

Lau YL, Liu D. 1993. Effect of flow rate on biofilm accumulation in open channels. Water Res. 27:355–360.

Downloaded by [US Naval Academy] at 05:53 11 April 2013

Lebeau T, Robert J. 2003. Mini review: diatom cultivation and biotechnologically relevant products. Part I: cultivation at various scales. Appl Microbiol Biotechnol. 60:612–623.

Lind JL, Heimann K, Miller EA, van Vliet C, Hoogenraad NJ, Wetherbee R. 1997. Substratum adhesion and gliding in a diatom are mediated by extracellular proteoglycans. Planta. 203:213–221.

Marker AFH, Casey H. 1982. The population and production dynamics of benthic algae in an artificial recirculating hard water stream. Philos Trans R Soc Lond B. 298:265–308.

Mieszkin S, Martin-Tanchereau P, Callow ME, Callow JA. 2012. Effect of bacterial biofilms formed on fouling-release coatings from natural seawater and *Cobetia marina*, on the adhesion of two marine algae. Biofouling. 28:953–968.

Molino PJ, Wetherbee R. 2008. Mini-review: the biology of biofouling diatoms and their role in the development of microbial slimes. Biofouling. 24:365–379.

Schilp S, Kueller A, Rosenhahn A, Grunze M, Pettitt ME, Callow ME, Callow JA. 2007. Settlement and adhesion of algal cells to hexa(ethylene glycol)-containing self-assembled monolayers with systematically changed wetting properties. Biointerphases. 2:143–150.

Schultz MP, Finlay JA, Callow ME, Callow JA. 2000. A turbulent channel flow apparatus for the determination of the adhesion strength of microfouling organisms. Biofouling. 15:243–251.

Schultz MP, Finlay JA, Callow ME, Callow JA. 2003. Three models to relate the detachment of low form fouling at laboratory and ship scale. Biofouling. 19(Suppl): 17–26.

Schultz MP, Swain GW. 2000. The influence of biofilms on skin friction drag. Biofouling. 15:129–139.

Serra A, Corcoll N, Guasch H. 2009. Copper accumulation and toxicity in fluvial periphyton: the influence of exposure history. Chemosphere. 74:633–641.

Smith DJ, Underwood JC. 1998. Exopolymer production by intertidal epipelic diatoms. Limnol Oceanog. 43:1578–1591.

Stafslien SJ, Bahr JA, Daniels JW, Wal LV, Nevins J, Smith J, Schiele K, Chisholm B. 2007. Combinatorial materials research applied to the development of new surface coatings VI: an automated spinning water jet apparatus for the high-throughput characterization of fouling-release marine coatings. Rev Sci Instrum. 78:072204.

Stoodley P, Wilson S, Hall-Stoodley L, Boyle JD, Lappin-Scott HM, Costerton JW. 2001. Growth and detachment of cell clusters from mature mixed species biofilms. Appl Environ Microbiol. 67:5608–5613.

Streeter VL. 1966. Fluid mechanics. 3rd ed. New York, NY: McGraw-Hill; p. 705.

Terlizzi A, Conte E, Zupo V, Mazzella L. 2000. Biological succession on silicone fouling-release surfaces: long-term exposure tests in the harbour of Ischia, Italy. Biofouling. 15:327–342.

Thomé I, Pettitt ME, Callow ME, Callow JA, Grunze M, Rosenhahn A. 2012. Conditioning of surfaces by macromolecules and its implication for the settlement of zoospores of the green alga *Ulva linza*. Biofouling. 28:501–510.

Thompson SM, Taylor AR, Brownlee C, Callow ME, Callow JA. 2008. The role of nitric oxide in diatom adhesion in relation to substratum properties. J Phycol. 44:967–976.

Wang Y, Lu J, Mollet J, Gretz MR, Hoagland KD. 1997. Extracellular matrix assembly in diatoms (Bacillariophyceae): II. 2,6-dichlorobenzonitrile inhibition of motility and stalk production in the marine diatom *Achnanthes longipes*. Plant Physiol. 113:1071–1080.

Webster DC, Chisholm BJ, Stafslie SJ. 2007. Mini-review: Combinatorial approaches for the design of novel coatings systems. Biofouling. 23:179–192.

Wetherbee R, Lind JL, Burke J, Quatrano RS. 1998. Mini-review – the first kiss: establishment and control of initial adhesion by raphid diatoms. J Phycol. 34:9–15.

Zargiel KA, Coogan JS, Swain GW. 2011. Diatom community structure on commercially available ship hull coatings. Biofouling. 27:955–965.

Zhang Z, Finlay JA, Wang LF, Gao Y, Callow JA, Callow ME, Jiang SY. 2009. Polysulfobetaine-grafted surfaces as environmentally benign ultralow fouling marine coatings. Langmuir. 25:13516–13521.

Downloaded by [US Naval Academy] at 05:53 11 April 2013