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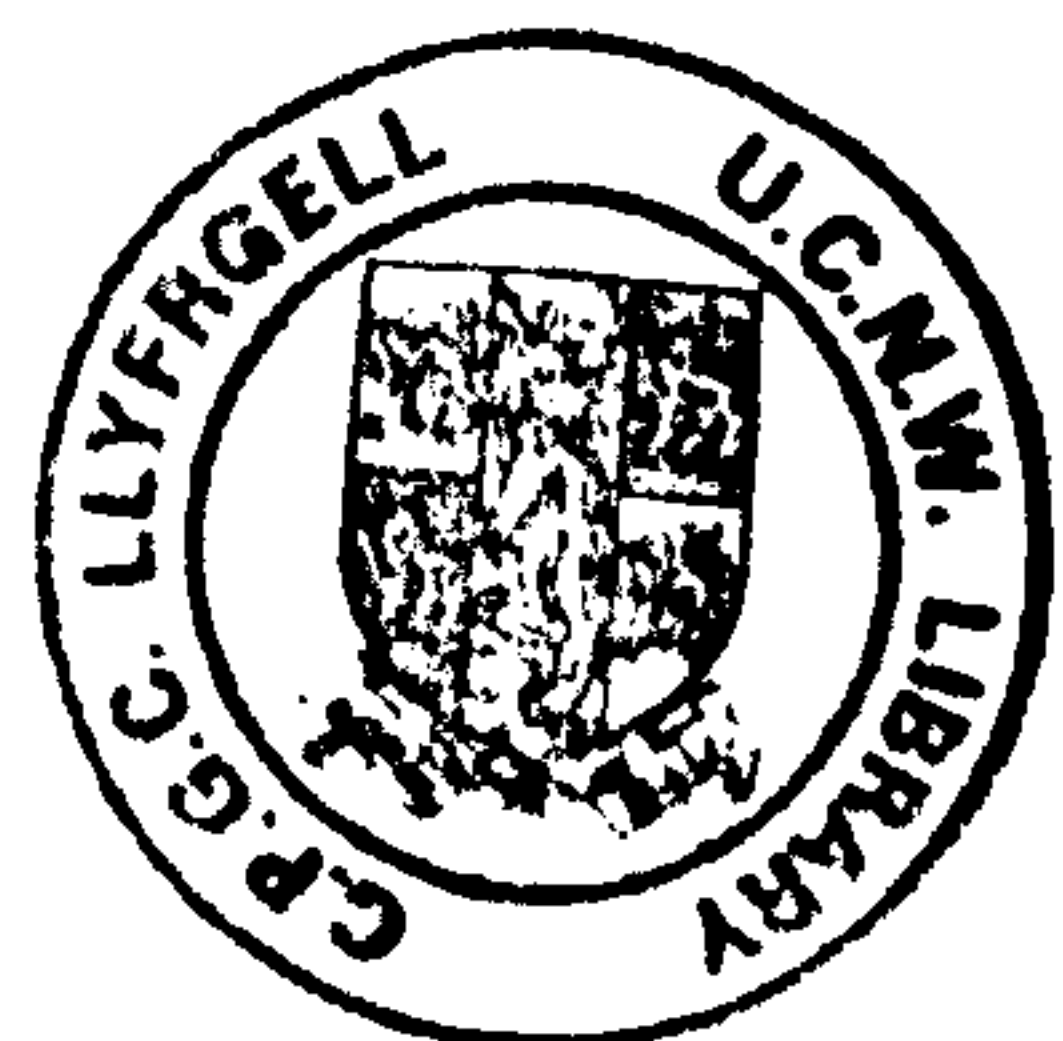
The influence of bacterial films
upon barnacle cypris temporary
adhesion

A thesis submitted to the University of Wales, Bangor for the
degree of *philosophiae doctor* in the School of Ocean Sciences

by

A.L.Neal B.Sc (London), M.Sc (Wales)

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Chapter 3, pages 11-18

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SUMMARY

The thesis describes experiments investigating interactions between barnacle cypris larvae and bacterial films covering substrata. The work begins with a confirmation of the link between temporary adhesion of cypris larvae and subsequent settlement. The following chapters then investigate, in detail, barnacle/biofilm interactions by measuring cypris temporary adhesion to films. Initially, bacterial strains were isolated from solid surfaces in the intertidal areas of the Menai Strait in an attempt to correlate bacterial zonation on the shore with barnacle zonation. However, the results of bacterial collection were inconclusive and a more in-depth study would have proved too onerous to be completed in this body of work. Instead the bacterial strains isolated were used to study the effect of monospecific films and their products on the larvae of *Verruca ströemia* larvae. The results show that individual biofilms differ in their likelihood to encourage settlement and that exopolysaccharides from one of the isolates (*Pseudomonas* W1⁺) have a concentration dependent inhibitory effect upon temporary adhesion. The next chapter deals with the effects of shear upon biofilms and the subsequent effect upon the temporary adhesion of *Elminius modestus* and *Balanus perforatus* cyprids. The work demonstrates that the two species may appreciate differences in bacterial communities grown under contrasting shear régimes. Finally, the effect of individual polysaccharide components of bacterial films upon temporary adhesion of five barnacle species is studied showing that monosaccharides have an inhibitory effect upon temporary adhesion, but that pentoses have the least effect, whilst hexoses have a more marked effect. The greatest inhibition is caused by uronic acids, probably because of their more polar nature. The overall conclusions suggest that the physico-chemical nature of bacterial films has a more profound effect upon cypris adhesion and settlement than species composition and that bacterial films do have a rôle to play in settlement and possibly in zonation of barnacles.

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1.0 General Introduction

Intertidal zonation of sessile marine invertebrates is classically regarded as the product of interspecific competition for food and space, and the varying extent to which different species are sensitive to predation and desiccation and other environmental factors (Yonge, 1966; Newell, 1979; Foster, 1987). However, recruitment into the population is closely allied to larval settlement and recent research (Holm, 1990; Bertness *et al.*, 1992) suggests that the maintenance of intertidal zonation is more dependent upon initial settlement rather than differential mortality.

At kilometre scales larval settlement is stochastic, determined by larval density in nearshore waters (Strathmann & Branscomb, 1979; Grosberg, 1982; Gaines *et al.*, 1985; Roughgarden *et al.*, 1988; Gaines & Bertness, 1992). To some extent, larval behaviour may play a limited rôle in on-shore transport, directing larvae towards hydrodynamic discontinuities, such as zones of convergence over tidally forced internal waves (Shanks, 1983; 1986; Shanks & Wright, 1987), internal tidal bores (Pineda, 1991) or salinity/temperature fronts, *etc.*, which may facilitate their transport to likely shores (Crisp, 1985). Such behaviour, employing light and gravity as vectors (Crisp, 1984), in itself can only be effective over a few metres (small, slow moving larvae have Reynolds' Numbers <10), but it may allow larvae to control their position in the water column and take advantage of prevailing currents over much larger distances (*eg.* Le Fèvre &

Bourget, 1991; Hill, 1991).

It is only at the centimetre scale that larvae are able to exercise a degree of determinism, and then, only when the larva is in the boundary layer above, or in contact with a surface. Once in contact with a surface, barnacle larvae explore surfaces with tracks as long as 3cm (see Crisp & Austin, 1960; also Walters, 1992). A cyprid's adhesion to that surface plays a significant rôle in determining whether settlement will follow. Whilst searching for a suitable settlement site larvae are sensitive to numerous stimuli, both positive and negative, which control the progression of a behavioural cascade leading to settlement. The capacity for discrimination on the part of the larvae has only recently been questioned. Crisp *et al.*, (1985) have suggested that barnacle larvae may not readily settle on, or permanently attach to, a substratum to which their temporary adhesive does not bind strongly, suggesting that adhesion perception could be a primary sense in surface assessment. As a cyprid ages, adhesion to a particular surface rises (Yule & Crisp, 1984), and as the urge to settle increases, so discrimination between surfaces is reduced (Crisp, 1985). Artificial ageing of *Balanus amphitrite* cyprids in the laboratory, using the nonsteroidal ecdysone mimic RH-5849, similarly results in increased settlement (Clare *et al.*, 1992). Yule & Walker (1984, 1987) have suggested that although behavioural responses to factors not associated with adhesion do play a rôle in determining the maximum tenacity shown by a cyprid to a particular surface, more profound limitations are probably related to the texture and

molecular nature of the surface. Thus, tangible properties, such as surface roughness and polarity have a greater rôle to play in settlement, contributing to any subjective appraisal of attractiveness. The barnacle cyprid does not necessarily choose between surfaces when offered a number simultaneously, as in the classical discriminatory experiments of Crisp and Meadows (1962). The probability of a cyprid's settlement on any surface on which it alights is determined by physico-chemical properties of the surface and the prevailing conditions. It follows from this argument, that in measuring the temporary adhesion of cyprids to experimental surfaces we are not simply gauging a surface's total attractiveness, but are reflecting the physical and chemical nature of the surface and its interaction with the temporary adhesive, combined with a measure of the individual's willingness to remain on that surface (behaviour). Following Crisp *et al.*, (1985) we can assume that stronger temporary adhesion reflects a greater propensity for settlement in barnacle cyprids. Factors influencing larval behaviour can be physical (Crisp, 1984; Wethey, 1986; Chabot & Bourget, 1988), or biological (Scheltema, 1974; Crisp, 1984; Chabot & Bourget, 1988; Pawlik, 1992).

If the maintenance of zonation patterns on a shore is in any way dependent upon initial settlement, then larvae should not settle throughout the intertidal, but in distinct zones, neglecting much, if not most, of the available space. Larvae would require criteria of position on the shore, concomitant with the prevailing conditions. Ambient conditions at the time of settlement are not necessarily indicative of long-term prospects, but the presence of conspecific adults is a sure indication of

habitat suitability, including intertidal position (see Knight-Jones, 1953). However, adult barnacles with relatively long life spans will not immediately reflect any changes in a surface's suitability. Biofilms, however, responding over much shorter time scales will be more sensitive to environmental change and will therefore provide an instantaneous appreciation of a surface's suitability. Interactions between bacteria and barnacle cypris larvae have been described (Maki *et al.*, 1988, 1990; Neal & Yule, 1994), and the possibility exists for elegant interactions between cyprids and the biofilm, with obvious ecological significance.

The behavioural state of a particular cyprid, governed by cypris age, is thus a second important factor in cypris settlement. Chemically identical surfaces, differing only in colour, elicited significantly different levels of temporary adhesion (Yule & Walker, 1984), a subtle but important finding highlighting the behavioural control that a larva has over temporary adhesion. Settlement is thus the product of temporary adhesion and behavioural state, which is ultimately dependent upon the nutritional state of the cyprid (see Lucas *et al.*, 1979). A cyprid's appreciation of a surface is thus based upon the strength of the adhesive bond generated between the cyprid's temporary adhesive and the substratum, mediated *via* a behavioural cascade and transduced into either settlement or quitting. As the cyprid ages, so the threshold force of adhesion required to precipitate settlement behaviour is reduced, and as a consequence, so is cyprid discrimination.

For students of larval invertebrate settlement, there is what is fast becoming a bewildering amount of review material, (fifteen reviews in the last twenty years) much of it repetitive. Topics under review have been the effect of con- and allospecifics on the settlement of larvae (Scheltema, 1974; Crisp, 1974, 1984, 1985; Gabbott & Larman, 1987; Yule & Walker, 1987; Bourget, 1988; Morse, 1991; Richmond & Seed, 1991; Pawlik, 1992; Clare *et al.*, 1992; Rodríguez *et al.*, 1993), the effect of bacterial films (Weiner, 1985; Bonar *et al.*, 1986), physical effects (Crisp, 1984; Wethey, 1986; Bourget, 1988), and toxic and nontoxic methods of antifouling (Dalley & Christie, 1987; Clare *et al.*, 1992; Evans & Clarkson, 1993). To add further to this list would be unproductive. It will be sufficient here to say that the arrival of a larva at a surface is a largely stochastic event, larvae having only limited control over their movement within the water column. Once at a surface, the larva is sensitive to both positive and negative biological and physical stimuli which acting on a behavioural cascade, determine whether settlement proceeds or the larva quits the surface. Not all chosen settlement sites are ideal. Larvae will often settle in unsuitable areas solely because they do so in large numbers and ideal sites are then a limited resource. The success of intertidal barnacles arises, however, not from blanket settlement in seasons of high larval settlement, but from their ability to locate, in seasons of relatively low recruitment, suitable areas for settlement, maximising the reproductive potential of the relatively few settlers.

Numerical modelling of larval settlement suggests interaction between larval behaviour and hydrodynamic forces

(Gross *et al.*, 1992). When potentially settling larvae are relatively old (with a strong urge to settle), the rate of settlement is dependent upon the delivery of larvae into the boundary layer by hydrodynamic forcing and larval advection, or water column processes. When settling larvae are relatively young (with a reduced urge to settle), the rate of settlement is less dependent on water column processes and dependent upon settlement behaviour itself, or boundary layer processes. Therefore, hydrodynamic processes are only important when larval urge to settle is relatively strong. By combining this form of settlement model with our understanding of larval ageing and the threshold stimulus to settlement, we can predict two phases of settlement during any one season. Early on in the season, when larval behaviour is retarded, the threshold stimulus eliciting settlement will be high, but at this stage of the model settlement is dependent upon larval behaviour. Hydrodynamic delivery of larvae onto the shore will only result in settlement once stringent requirements are met. These requirements are only met for each species in distinct zones within the inter- and subtidal, hence the zonation of species within the intertidal is maintained. As the season progresses, the larvae arriving at the shore will be behaviourally older, requiring less of a stimulus to settle. At such a stage, settlement is dependent upon larval delivery upon the shore and so blanket settlement will occur in years of good larval survival. The settlement of these later arrivals allows incursion into areas less suitable for growth of a particular species but blanket settlement increases the chance of survival and so species' range on small, and possibly large scales, may be extended by this opportunistic settlement behaviour. It is in this

light that the interactions between cypris larvae and bacterial films must be considered.

2.0 Common Materials and Methods¹

All adult barnacles were maintained in the laboratory under natural light conditions, at appropriate temperatures and fed *Artemia* sp. nauplii (Artemia 90, Sanofi Aquaculture, Paris) daily. Larvae were routinely collected at a point light source and removed to 5l culture vessels containing fine-filtered (0.2 μ m), UV-irradiated seawater.

Larval cultures were maintained according to methods described by Moyse (1960) and Yule (1984). All algal species were grown in semi-continuous batch culture (Fay & Kulasooriya, 1973). Algal species used were the diatom *Skeletonema costatum* (Greville) Cleve, the flagellates *Pavlova lutherii* (Droop) Green and *Rhinomonas reticulata* (Lucas) Novarino, and the eustigmatophyte *Nannochloropsis oculata* (Droop) Hibberd². Each larval culture was fed daily with final algal concentrations in excess of 100 cells. μ l⁻¹ and the water changed every 48h. Following their appearance in the cultures, cypris larvae were removed to poly(propylene) beakers and stored at <7°C in the dark. Under such conditions cypris activity is limited and very little settlement occurs.

¹ The methods described here are common to all of the following chapters, in that all nauplii were reared using similar methods and all cyprids were prepared for experimentation in the same way. Where different algal diets were provided, attention has been drawn to this fact in the relevant chapter. Surface preparation and the bacteriology involved are not included here, because of the diverse surfaces and bacterial species used in the whole study.

² All algal species were cultured at Menai Bridge by Mr. Malcolm Budd.

The force of adhesion of cypris larvae to test surfaces was measured using methods detailed by Yule & Crisp (1983), except that a precision torsion balance (White Electrical Instrument Co. Ltd.) was used instead of a modified Cahn micro-balance. Cyprids were individually placed on absorbent paper under a dissecting microscope (x25 mag.). Each cyprid was cemented, using a small amount of cyanoacrylate adhesive ('Superglue 3', Loctite), to a stainless steel wire 2-3cm. in length and 0.5mm. in diameter. The wires were attached to the carapace behind a compound eye, either on the dorsal ridge or flank of the cyprids. Successfully prepared cyprids were suspended in seawater for a minimum of 30min. at room temperature before use. Tethered cyprids were suspended from the balance arm over the test surface held in a rectangular glass dish filled with filtered seawater. The dish sat on an adjustable stage which was raised until the cyprid was almost in contact with the test surface. The balance was zeroed to compensate for the weight of the cyprid and attached wire, then the cyprid was allowed to attach its antennules to the test surface. Cyprids were viewed using a binocular microscope (x20 mag.) which could be swung into position in front of the dish. Tension upon the cyprid was increased at a slow, constant rate and the force required to remove the cyprid from the test surface was recorded. Where antennular disc areas are given, they were calculated by measuring the disc diameter of cyprids, anaesthetised using MS-222 (Sandoz) and then fixed in a 5% formaldehyde solution, using a binocular microscope, under x400 magnification.

Seven barnacle species were used in the study. *Semibalanus*

Seven barnacle species were used in the study. *Semibalanus* (= *Balanus*) *balanoides* (L.) cyprids were collected from the plankton in the Menai Strait in April and May using a medium-mesh plankton net. All other larvae were raised from adult cultures maintained in the laboratory; *Elminius modestus* Darwin and *Verruca ströemia* (O.F. Müller) adult cultures were collected from the Menai Strait; *Balanus perforatus* (Bruguière) adults were collected from Broad Ledge, Lyme Regis, Dorset; *Balanus improvisus* (Darwin) adults were collected from the Conwy estuary; *Euraphia withersi* (Pilsbury) were collected from the Swire Marine Laboratory, Hong Kong; and *Pollicipes pollicipes* (Gmelin) were collected from Castelejo, Portugal.

**3.0 The Link Between Cypris
Temporary Adhesion and Settlement
of *Semibalanus balanoides*.**

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§ This chapter was published under the same title in 1992; *Biofouling* Vol. 6, pp. 33-38.

4.0 Settlement of *Verruca* *ströemia* cyprids: effect of bacterial zonation

4.1 Introduction

Until recently, the interaction between barnacle cypris larvae at settlement and the biofilm covering the substratum was rarely considered. Extensive advances have been made on the effects of conspecifics (see Gabbott & Larman, 1987 for review) or physical phenomena such as surface contour and flow (Crisp, 1984; Wethey, 1984). However, the rôle of the resident biofilm (although first proposed by Zobell and Allen, 1935) has only recently attracted attention, largely inspired by the search for bacterially derived anti-fouling agents as the marine biotechnology industry has come of age (Weiner, 1985; Bonar *et al.*, 1986; Holmström *et al.*, 1992; Evans & Clarkson, 1993).

Despite clear evidence that biofilms nearly always enhance settlement (see Crisp, 1974 for review), monospecific biofilms vary widely in acceptability to cypris larvae. Settlement of *Balanus amphitrite* cyprids has been enhanced on a film of IM32, discouraged by a film of ID16 but completely unaffected by a *Pseudomonas atlantica* film (Maki *et al.*, 1988). The actual community structure of any biofilm is thus likely to have a significant influence on cypris settlement.

It is now evident that different barnacle species behave differently on the same bacterial film. Both *B.amphitrite* (Maki *et al.*, 1990) and *Elminius modestus* (Neal & Yule, 1994) show reduced settlement on films of *Deleya marina*. However, inferring settlement potential from the force of cypris tenacity, Neal & Yule (1994) have demonstrated that only relatively older films have a negative effect upon settlement. Younger films increase the likelihood of settlement on a surface. In contrast, whilst both ages of film are inhibitory to settlement of *B.amphitrite*, the older film is more inhibitory than the younger film. Two contrasting interactions can therefore be inferred; irrespective of the chemistry involved, the inhibitory factor responsible for reduced settlement of *B.amphitrite* accumulates with the age of the film, whilst the factor responsible for *E.modestus* reduced settlement is either present or absent from the film according to film age. The fact that *Deleya marina* elicits such differing behavioural responses from two barnacle species suggests that biofilms may play a rôle in the maintenance of zonation of intertidal barnacle species.

Until now, all our knowledge of barnacle/biofilm interactions is based upon the Balanomorpha. Since the group constitutes the major contributors to marine biofouling problems, this is not surprising. The effect of monospecific biofilms upon the tenacity of the verrucomorph *Verruca ströemia* has therefore been studied to widen the taxonomic range of our knowledge. *V.ströemia* is a poorly studied barnacle found in the low and sublittoral under stones, or associated with the shells of bivalves such as *Pecten* spp. or *Chlamys* spp. or even other barnacles such as *Chirona hameri* and *B.balanus* (Stone & Barnes, 1973). Around

Britain, *V.ströemia* has a depth distribution of 5-90 fathoms (10-180m) (Darwin, 1854), and relies largely upon detritus swept up from the substratum as a food source (Anderson, 1980).

4.2 Materials and Methods.

4.2.1 Adult Collection and Larval Rearing

Adult *Verruca ströemia* specimens were collected in March 1992 from an area between Llandysilio and Ynys Welltrog, Menai Strait, North Wales. The adults were removed from the substratum and brought into the laboratory where ripe or ripening egg masses were removed and placed in UV-irradiated, cartridge-filtered (0.2µm) sea water (UVFS) at 10°C. Any larvae that had hatched were attracted to a point light source, removed and placed in a 5l. culture vessel containing UVFS and kept at 10°C with slow aeration. Unhatched egg masses were placed in a sieve (mesh size 80µm) and suspended in an aquarium of UVFS with vigorous aeration. Hatching larvae were again attracted to a point light source and placed in culture. Larvae were reared on cultures of *Skeletonema costatum* and *Rhinomonas reticulata*.

All cyprids were used 8d. after they metamorphosed from the metanaupliar stage and were removed from the larval culture, an age at which discriminatory behaviour was discernible. Preparations for adhesion measurements were made following the methods detailed in section 2.0. The average area of the antennular disc of *V.ströemia* was measured at 348.4 µm² (N=14,

SE=29.6 μm^2).

4.2.2 Bacterial Collection and Culture

Initially, the aim of the bacterial collection was to isolate suites of bacteria found associated with zones on the Menai Strait shore. These zones approximated the zones occupied by the five barnacle species present in the Strait. It was hoped that such a collection would allow the investigation of whether particular bacterial strains, or suites of bacteria, were utilised by settling cyprids to identify position on the shore. It very quickly became abundantly clear, however, that the methods employed in this study were far too crude to provide any meaningful results; although ten different bacterial strains were isolated, the same ten were isolated from all areas of the shore and were found on every sampling visit. Despite OZR medium being a general purpose isolating medium (Sieburth, 1967), it was only isolating a fraction of the total bacterial strains present. A far more comprehensive and time consuming study would be required, employing a battery of isolating media and lengthy biochemical identification, to provide an accurate appreciation of the true bacterial ecology of any shore. Consequently, the original aims of this part of the study were not followed up. Instead, the isolated strains were used in the work reported here. Collections were made using pre-sterilised cotton wool swabs and grown on OZR medium, a variant of 2216E medium (Zobell, 1941; Zobell & Upham, 1944).

The bacteria collected were incubated at 27°C. They were separated and identified according to colour, growth form, and

response to specific growth media (*Pseudomonas* isolating media and TCBS medium for the isolation of *Vibrio* spp.).

Cells of each isolated strain were grown to mid-exponential phase at 27°C in 200ml of a nutrient broth containing 10g tryptone, 5g yeast extract and 10g NaCl in 1l distilled deionised water, adjusted to pH 7.5 (LB). The cells were harvested by centrifugation and resuspended in 200ml of UVFS. Cell concentration was estimated by measuring absorbance at 600nm. A 200ml suspension of each isolate was prepared to equal cell concentration, and then 10ml of each isolate suspension was added to a petri dish containing an autoclaved coverslip (22 x 22mm, No.1½ ARH Ltd.) for film production. Three different biofilm preparations were used; films were either aged for 1d, or 10d with or without the addition every 24h of a 10% LB broth.

A pure culture of *Pseudomonas* W1⁺ was grown on LB agar for 5d at 27°C until a thick confluent growth had formed. The colonies were removed from the agar surface with a sterile glass rod and placed in distilled deionised water. The cells were removed by centrifugation and the exopolysaccharides (EPS) recovered from the culture supernatant by the addition of 3 vols. of acetone followed by centrifugation of the supernatant alone (Williams & Wimpenny, 1977). After being redissolved in distilled deionised water the EPS was purified by rotor-evaporation at 20mmHg. The pure EPS was redissolved a second time in distilled deionised water. Colorimetric, quantitative estimation of EPS concentration was

TABLE 4.1. Tenacity of *Verruca stroemia* cypris larvae to single species biofilms collected from the Menai Strait intertidal. Tenacity measurements are given as 10^5 Nm^{-2}

Biofilm	N	Mean (\pmSE)
Control	30	1.706 (0.049)
Dy 1 ⁻	30	1.383 (0.045)
Purple	30	1.843 (0.069)
<i>Vibrio</i> M 1 ⁺	30	1.736 (0.054)
W 2 ⁻	30	0.870 (0.024)
WBr 3 ⁻	30	0.877 (0.025)
<i>Pseudomonas</i> Br 4 ⁺	30	1.547 (0.073)
<i>Vibrio</i> WBr 2 ⁺	30	1.418 (0.044)
<i>Pseudomonas</i> W 3 ⁺	30	1.475 (0.050)

Bartlett's statistic= 57.99; df= 8; $p < 0.001$

Following log_e transformation

Bartlett's statistic= 7.82; df= 8; $p = 0.349$

ANOVA table for log_e biofilm data

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	F	p
Biofilm	8	18.318	2.290	67.93	<0.001
Error	261	8.798	0.034		
(Total	269	27.115)			

Q for N=30, 3.39

SE=0.010; Q(SE)=0.034

made using the phenol-sulphuric acid method of Dubois *et al* (1956) compared with glucose standards.

4.3 Results

Of the ten bacterial strains isolated, one would not grow on LB, and By6⁻ would not adhere to the glass surface provided. The remaining eight strains were allowed to attach to glass coverslips for 3h. Tenacity measurements were then made (Table 4.1), the variances proved to be significantly heterogeneous (Bartlett's Statistic=57.99; df=8; $p<0.001$) but a \log_e transformation stabilised the variance (Bartlett's Statistic=7.82; df=8; $p=0.349$) and the resulting analysis of variance table is also shown in Table 4.1. There was a highly significant effect of bacterial strain ($F_{8, 261}=67.93$; $p<0.001$) on temporary adhesion. Pair-wise differences between \log_e of mean tenacity on the surfaces was analysed using Tukey's method. The 95% confidence interval of the difference between any two means was $0.034 \times 10^5 \text{ Nm}^{-2}$. Of the films, two, the purple strain and *Vibrio* M1⁺ elicited increased levels of tenacity compared to control surfaces, $1.843 \times 10^5 \text{ Nm}^{-2}$ for the purple bacteria compared to a control value of $1.706 \times 10^5 \text{ Nm}^{-2}$, but these differences were not significant at the 5% level. All other biofilms resulted in reduced tenacity compared to the control surface, the Tukey method dividing the six remaining biofilms into two groups; the first containing *Pseudomonas* W1⁺, *Pseudomonas* Br4⁺, *Vibrio* WBr2⁺ and Dy1⁻, the second containing WBr3⁻ and W2⁻.

TABLE 4.2. Tenacity of *Verruca stroemia* cypris larvae to aged single species biofilms; unaged films were grown in fine filtered UV-irradiated seawater, aged films were grown for 10d., either with or without the addition of 10% strength LB broth. Tenacity measurements are given as 10^6 Nm^{-2} .

Biofilm	N	Mean (\pm SE)
<u>Unaged</u>		
Control	30	1.706 (0.049)
<i>Pseudomonas</i> W 1 ⁺	30	1.383 (0.045)
<i>Pseudomonas</i> Br 4 ⁺	30	1.843 (0.069)
Purple	30	1.738 (0.054)
<u>10d. LB⁻</u>		
Control	30	1.587 (0.069)
<i>Pseudomonas</i> W 1 ⁺	30	1.071 (0.051)
<i>Pseudomonas</i> Br 4 ⁺	30	1.347 (0.054)
Purple	30	1.488 (0.055)
<u>10d. LB⁺</u>		
Control	30	1.723 (0.067)
<i>Pseudomonas</i> W 1 ⁺	30	1.175 (0.065)
<i>Pseudomonas</i> Br 4 ⁺	30	1.237 (0.057)
Purple	30	1.458 (0.058)

Bartlett's statistic= 8.055; df= 11; $p = 0.572$

ANOVA table for controls

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	F	p
Date	2	4.37×10^9	2.19×10^9	1.87	0.160
Error	87	1.02×10^{11}	1.17×10^9		
(Total	89	1.06×10^{11})			

Two-way ANOVA table for aged biofilms

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	F	p
Strain	2	5.99×10^{10}	2.99×10^{10}	28.81	<0.001
Treatment	2	6.51×10^{10}	3.25×10^{10}	31.10	<0.001
Interaction	4	4.50×10^9	1.12×10^9	1.07	0.370
Error	261	2.73×10^{11}	1.05×10^9		
(Total	269	4.03×10^{11})			

Q for N=30, 3.39

SE= 1.743×10^4 ; Q(SE)= 0.591×10^9

Following the finding that the tenacity of *V.stroemia* cyprids is significantly affected by the presence of biofilms, and that different biofilms elicit different behaviour from the larvae, three bacterial strains were used to study the effect of ageing and nutrient limitation upon adhesion. Table 4.2 shows the mean cypris tenacity to the purple bacterium, *Pseudomonas* W1⁺ and *Pseudomonas* Br4⁺ biofilms, unaged and aged, with and without the addition of a 10% LB solution. A comparison of the three control surfaces from the three trials indicates that there is no significant difference ($F_{2, 87}=1.87$; $p=0.160$) between trials, and the resulting data concerning aging and nutrient limitation can be directly compared. The variances associated with the data resulting from adhesion measurements on the aged biofilms proved homogeneous (Bartlett's Statistic=8.055; $df=11$; $p=0.572$) and the resultant two-way analysis of variance again showed a significant main effect due to different bacterial films ($F_{2, 264}=28.61$; $p<0.001$), and also a significant main effect due to biofilm age and/or nutrient status ($F_{2, 264}=31.10$; $p<0.001$). However, there was no significant interaction term ($F_{4, 264}=1.07$; $p=0.370$). Further analysis of the data using Tukey's method (95% confidence interval for difference between any two means= $0.591 \times 10^5 \text{ Nm}^{-2}$) showed that ageing of the films caused a significant reduction of tenacity on all three strains. In addition, differing nutrient status affected tenacity on the two 10d old *Pseudomonad* films. There was no significant effect of nutrient status upon the purple bacterium.

TABLE 4.3. The effect of concentration of *Pseudomonas* W 1⁺ derived exopolysaccharide upon the temporary adhesion of *Verruca stroemia* cypris larvae.

Tenacity measurements are given as 10^5 Nm^{-2}

<u>EPS concentration</u>	<u>N</u>	<u>Mean (\pmSE)</u>
Control	30	1.266 (0.032)
6.65 μM Glucose equiv.	30	1.214 (0.025)
12.30 μM Glucose equiv.	30	1.060 (0.027)

Bartlett's statistic= 2.30; df= 2; p= 0.683

ANOVA table for *Pseudomonas* W 1⁺ EPS concentration

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	F	p
Concentration	2	8.11×10^9	4.06×10^9	16.99	<0.001
Error	87	2.08×10^{10}	2.39×10^8		
(Total	89	2.89×10^{10})			

Q for N=30, 3.39

SE= 0.028×10^5 ; Q(SE)= 0.095×10^5

Pseudomonas W1⁺ elicited reduced tenacity compared to the control surface (Table 4.1). *Pseudomonas* W1⁺ derived exopolysaccharides (EPS) were therefore used to test whether this reduced tenacity might be due to the presence of EPS within the slime layer, and to see if tenacity was in any way related to EPS concentration. The variances of the means were again found to be homogeneous (Bartlett's Statistic=2.30; df=2; $p=0.683$) and the resultant analysis is presented in Table 4.3. The analysis of variance (Table 4.3) demonstrates that the presence of EPS on a surface has a significant effect ($F_{2, 87}=16.99$; $p<0.001$) upon tenacity. Tenacity dropped from $1.288 \times 10^5 \text{ Nm}^{-2}$ on the control surface to $1.214 \times 10^5 \text{ Nm}^{-2}$ on a surface to which a $6.65 \mu\text{M}$ glucose equivalent EPS solution had been allowed to adsorb for 10 minutes, but this difference was not significant when tested using Tukey's Method of pair-wise comparisons (95% confidence interval for difference between any two means= $0.095 \times 10^5 \text{ Nm}^{-2}$). At a higher concentration however, ($12.30 \mu\text{M}$ glucose equiv.), tenacity was reduced to $1.060 \times 10^5 \text{ Nm}^{-2}$, a significant reduction when compared to the control surface. *Pseudomonas* W1⁺ EPS therefore has a concentration dependent negative effect upon tenacity.

4.4 Discussion

At present we have only a very basic understanding of the interaction between biofilms and cypris larvae at the point of settlement. Cypris larvae settle differentially upon a wide range of monospecific biofilms (Maki *et al.*, 1988), and in a manner which is dependent upon biofilm growth stage (Maki *et al.*, 1990; Neal & Yule, 1994). Additionally, different species of cyprids

behave very differently to the same biofilm, for example, *Balanus amphitrite* cyprids (Maki *et al.*, 1990) behave in a markedly different way from *Elminius modestus* cyprids (Neal & Yule, 1994) on *Deleya marina* biofilms. The present study shows that cypris larvae of *Verruca stroemia* also behave differentially, in a manner similar to that of *B.amphitrite* cyprids, to a range of biofilms isolated from the intertidal areas within the Menai Strait. Bacteria such as *Vibrio* M1⁺ and the purple, unidentified, bacterium elicit increased forces of adhesion compared with a control surface. Others, for example, *Pseudomonas* W3⁺ and WBr3⁻ cause reduced cypris tenacity, again emphasising the importance of biofilm community structure first proposed by Maki *et al* (1988).

The ageing of a bacterial film would appear to result in reduced cypris tenacity to that film since significant reductions in tenacity were observed on all aged films. Other barnacle species exhibited the same general trend with aged films of *Deleya marina* reducing the tenacity of both *Balanus amphitrite* and *Elminius modestus* larvae (Maki *et al.*, 1990; Neal & Yule, 1994). The phenomenon was interpreted by the latter authors as either showing growth related production of different exopolymers, or by a reduction of either adhesive or cohesive forces associated with the film. Maki *et al* (1990) suggest the accumulation of an inhibitory factor within the film as it aged. Such an explanation seems unlikely in the case of a film which initially promotes tenacity and then becomes inhibitory with age, as is the case for films of the unidentified purple bacterium. The changes in tenacity towards films which were nutrient limited would tend to support the hypothesis that different exopolymers are involved

rather than the accumulation of an inhibitory factor.

The Gram-negative *Klebsiella aerogenes* NCTC418 produces a cell envelope containing four major proteins when grown under optimal conditions (Sterkenberg *et al.*, 1984). However, the chemical composition of the cell envelope is altered by nutrient limitation and is specific to the type of limitation imposed. A second marine isolate, S14, exhibits both quantitative and qualitative changes in lipid, poly- β -hydroxybutyrate and cell wall peptidoglycan content under imposed nutrient limitation (Malmcrona-Friberg *et al.*, 1986). Nutrient limitation also results in changes in the cell wall hydrophobicity of *Spirillum*, *Flavobacterium* and *Vibrio* species (Kjelleberg & Hermansson, 1984). *Pseudomonas* S9 is stimulated to produce an extracellular polysaccharide containing *N*-acetylgalactosamine (37%), *N*-acetylglucosamine (35%) and glucose (28%) which is associated with a reduction in cell-substratum adhesion (Wrangstadh *et al.*, 1986). There exists, therefore, ample evidence to show that chemical changes within a biofilm can result from nutrient limitation. In the present study, nutrient limitation resulted in no change in tenacity on the unidentified purple bacterium, but on the two *Pseudomonad* films changes do occur, one an increase, the other a reduction in tenacity. The evidence does not support the accumulation of an inhibitory chemical and suggests that both ageing of films and the imposition of nutrient limitation results in chemical changes within the biofilm which consequently affects tenacity to those films. However, the possibility of reduced bacterium/substratum adhesion as described for *Pseudomonas* S9, being misinterpreted as behaviourally mediated reduced cypris

tenacity must not be overlooked.

The effect of exopolysaccharides from *Pseudomonas* W1⁺ upon the tenacity of *V.ströemia* cypris larvae is to reduce the force of tenacity. However, the minimum effective concentration lies somewhere between 6.65 and 12.3 μM glucose equivalents. A small, but insignificant, reduction in adhesion is brought about by a concentration of 6.65 μM suggesting that a chemical interruption of the adhesion process is taking place and, presumably, at a much higher EPS concentration the adhesive process would be completely interrupted. Sugars, specifically D-glucose at a concentration of 250 μM , have also been shown to reduce the settlement of the spirorbid polychaete *Janua brasiliensis* (Kirchman *et al.*, 1982). Work on *J.brasiliensis* has identified a lectin on the larval surface which binds to polysaccharides and glycoproteins produced by bacterial films. Our understanding of barnacle settlement induction suggests that lectins are not involved in the settlement process, but sugars need not require lectin binding sites to interrupt cypris adhesion. Polysaccharides are inherently sticky molecules, which should coat the surface of an already sticky surface quite freely. Once an antennular glue becomes covered with polysaccharide it seems likely that the cyprid would not be able to maintain previous levels of tenacity. Presumably, adhesive potential could only be restored once the surface of the antennule is covered with fresh temporary adhesive. Such logic suggests that natural biofilms which possess high concentrations of polysaccharide in their exopolymers will prevent proper temporary adhesion, and therefore larval settlement (Yule & Crisp, 1983; Yule & Walker, 1984; Section 3 *this volume*).

5.0 The Effect of Shear-Induced Physico-Chemical Differences between Biofilms upon Adhesion of Cyprids to those Films.

5.1 INTRODUCTION

Bacterial films are extremely dynamic phenomena, a fact which causes major problems for microbiologists, but suggests many potential rôles for natural biofilms in chemical ecology. The physico-chemical nature of a film is transient, dependent upon such influences as the nature of the sources of carbon and nitrogen and electron donors. A film provided with differing nutrient sources will produce differing exo-polymers, thus altering the whole chemical identity of the film. Other differences between films will also arise from physical influences. Hydrodynamic shear stress has profound effects upon a range of biofilm properties, firstly on the initial colonisation of a film, and secondly, upon the established film.

Increased shear stresses affect the initial adsorption of cells onto a new surface; both the surface-particle capture factor (ϵ_{sp}) (Escher & Characklis, 1990) and sticking efficiency (α_e) of *Bacillus cereus* (Powell & Slater, 1983) and *Pseudomonas aeruginosa*

§ A version of this chapter is published in 1994; *Journal of the Marine Biological Association of the United Kingdom* Vol., 74, pp., 251-257 under the title "A comparison of the tenacity of *Elminius modestus* and *Balanus perforatus* cyprids to bacterial films grown under contrasting shear regimes."

(Escher & Characklis, 1990) are affected detrimentally by increased shear stress. ϵ_{sp} describes the affinity for adsorptive interactions between cells and a substratum, whilst α_e describes the probability that a cell transported to the substratum surface will adsorb to that surface. The establishment of a pioneer population from which a biofilm can flourish will therefore occur in a shorter time at reduced shear stress.

Shear stress will also affect established biofilms in other ways. Studies have shown that biofilm formation in potable water systems is increased at higher flow rates (Sly *et al.*, 1988) and in glass capillaries films of *Streptococcus aureus* were augmented at higher flow rates (Rutter & Leach, 1980). This increased accumulation of biomass is probably associated with the increased delivery of nutrients and electron donors to the biofilm surface. As shear stresses increase, cell detachment increases in importance (Dudderidge *et al.*, 1982; Rittman, 1982; Trulear & Characklis, 1982; Lau & Liu, 1993). At shear stresses as high as 130 Nm^{-2} *P. fluorescens* cells are removed from films (Duddderidge *et al.*, 1982) and at a greater rate from thick films than from thinner films (Rittman, 1982). In planktonic cultures of *Brevibacterium flavum* increased shear stresses again are associated with increased growth and production rates (Toma *et al.*, 1991) up to a critical stress, above which an increase in stress results in decreased growth rates due to decreased ATP generation and lower O_2 uptake. This phenomenon is termed turbohypobiosis and is likely to be relevant to biofilms as well as planktonic cultures. An established biofilm will exist therefore in a state of quasi-equilibrium and be the product of

cell growth, cell removal, and turbohypobiosis, all of which are directly related to shear stress. There exists, therefore, an optimal shear stress, at which nutrient and electron donor delivery is at a maximum, whilst cell removal is at a minimum, which will greatly promote the growth of a biofilm.

The three-dimensional structure of a *P. fluorescens* biofilm is allied to current velocity. Biofilm density is increased at higher shear stresses (Christensen & Characklis, 1990), similarly, films developed in a 2.5 ms^{-1} current were more compact and thinner than films developed at 0.5 ms^{-1} (Santos *et al.*, 1991).

Perhaps the most comprehensive study to date, of the effects of shear upon established films has been conducted by Mittleman *et al* (1990). Using *P. atlantica* monocultures, they were able to show that biofilm development was increased with shear stress up to a critical value ($\approx 120 \text{ Nm}^{-2}$). The composition of the film was also affected by shear stress: biomass, measured as protein and carbohydrate *per unit area*, increased with shear, but metabolic rate and cell size decreased. Most of the critical effects of shear occurred at stresses $\leq 30 \text{ Nm}^{-2}$.

In summary, increased shear stresses will hamper the initial adsorption of cells onto a surface, but once established, biofilms thrive in areas of increased shear, probably due to the increased delivery of nutrients at the film surface. At a critical shear, determined by the adhesive ability of the particular bacteria associated with a particular surface, cell removal will outweigh growth and biofilm depletion will begin to occur. At intermediate

levels of shear, the three-dimensional structure of the film is affected as the film increases in density as it is thinned by removal of cells and/or cell products. The production of exopolysaccharides that aid in adhesion is probably stimulated (Mittleman *et al.*, 1991) and so the chemical identity of the film will also change. Irrespective of individual species components therefore, biofilms established in high shear environments will share common physical (three-dimensional structure) and chemical (production of adhesive exopolymers) properties which contrast tangibly (to a cyprid) with films from relatively lower shear environments.

The present work investigates whether the cypris larvae of two barnacle species could differentiate between multispecies biofilms which were developed under contrasting shear stresses. The barnacle species studied occupy different ecological niches, although both can be found on the south-western shores of England and Wales. *Balanus perforatus* inhabits the low intertidal to sublittoral zones, generally of the more exposed shores. *Elminius modestus* predominates on the middle to upper reaches of the shore, but is readily found in the lower intertidal. Although often described as an inhabitant of sheltered shores (eg Rainbow, 1984), *E.modestus* can survive and grow in exposed locations (Crisp, 1958). The terms "exposed" and "sheltered" refer to the severity of wave action experienced on the shore, and are not terms which directly describe the predominant shear stresses associated with the shore. Many shores, such as those of the Menai Strait, experience little wave action and yet tidal currents create considerable shear stresses across the substratum.

5.2 MATERIALS AND METHODS

Balanus perforatus nauplii grew best when fed on a mixture of the flagellates *Pavlova lutherii* and *Rhinomonas reticulata* up until the metanaupliar stage, after which the diatom *Skeletonema costatum* (chain length <20 μm) was included. *Elminius modestus* nauplii however, grew very well when fed on a *S.costatum* and *R.reticulata* mixture. Both species were cultured at 25°C. Average antennular disc areas were measured at 537.9 μm^2 (N=30, SE=17.6 μm^2) for *E.modestus* and 1089.8 μm^2 (N=30, SE=24.6 μm^2) for *B.perforatus*.

All glass surfaces on which control measurements were to be made and biofilms were allowed to develop were previously organically cleaned by heating in a muffle furnace for 4h at 500°C. Natural biofilms were allowed to develop on glass coverslips in the absence of light (to keep diatomaceous fouling to a minimum) at 23°C. Coverslips (22 mm x 25 mm, Weber Scientific International Ltd., Lancing) were held in place on glass slides with a little petroleum jelly and the slide placed in a flume. Three cover glasses were affixed to each slide. One slide was placed on the flume floor, the other on a raised stage. Natural seawater, filtered to <10 μm , was pumped through the flume and flow rates 5 mm above the two slide surfaces were measured using an impeller flow meter (Novonik Streamflow). Flow was 0.075 ms^{-1} above the bottom coverslips and 0.415 ms^{-1} above those on the raised stage, providing shears of 15 s^{-1} and 83 s^{-1} respectively. The surfaces were left in the flume for two months, allowing the development of a well established biofilm.

TABLE 5.1. Force of temporary adhesion (10^4 Nm^{-2}) of *Elminius modestus* and *Balanus perforatus* cypris larvae to natural biofilms developed at high (83s^{-1}) and low (15s^{-1}) shear rates.

<u><i>Elminius modestus</i></u>				<u><i>Balanus perforatus</i></u>		
<u>Surface</u>	N	Mean	\pm SE	N	Mean	\pm SE
No biofilm	30	8.19	0.258	30	6.75	0.179
Low Shear	30	7.59	0.274	30	4.94	0.136
High Shear	30	9.20	0.219	30	6.50	0.162

Bartlett's Statistic=1.44; $p=0.486$

Bartlett's Statistic=2.79; $p=0.248$

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE	F	p	SUM OF SQUARES	MEAN SQUARE	F	p
Cypris	4	1.152×10^9	2.881×10^8	0.687	0.621	1.912×10^9	4.779×10^7	0.575	0.682
Biofilm	2	3.972×10^9	1.986×10^9	12.522	<0.001	5.822×10^9	2.911×10^9	35.029	<0.001
Interaction	8	3.357×10^8	4.196×10^8	2.646	0.013	5.051×10^8	6.314×10^7	0.760	0.639
Error	75	1.189×10^{10}	1.586×10^8			6.233×10^9	8.311×10^7		
(Total	89	2.038×10^{10}				1.275×10^{10}			

Q for N=30, 3.39

SE= 0.230×10^4 ; Q(SE)= 0.779×10^4

SE= 0.166×10^4 ; Q(SE)= 0.564×10^4

5.3 RESULTS

After two months in the flume, both experimental surfaces developed confluent films. Under x20 magnification it could be seen that the high shear film formed a very thin but dense cover, whereas the low shear film showed greater three-dimensional heterogeneity with a much looser structure. When plated onto 2216E medium, *Pseudomonad* and *Vibrio* species were isolated from both surfaces with no striking visual differences between plates inoculated from either surface.

Table 5.1 shows the results for the tenacity of cypris larvae of both species to the various surfaces. Despite their overall greater size, the tenacity of *B.perforatus* cyprids tended to be lower (ca. $6 \times 10^4 \text{ Nm}^{-2}$) than tenacity for *E.modestus* (ca. $8 \times 10^4 \text{ Nm}^{-2}$). Even so, it would require a force of $6.5 \times 10^{-5} \text{ N}$ to remove a *B.perforatus* cyprid from a surface, but only $4.3 \times 10^{-5} \text{ N}$ for the smaller *E.modestus*.

The results for both *E.modestus* and *B.perforatus* cypris tenacity exhibited homogeneity of variance (Bartlett's statistic 1.444, $p=0.486$ and 2.788, $p=0.248$ respectively). The results were, therefore, analysed by separate two-factor analyses of variance using the origin of the biofilm and individual cyprid as factors. The result for *E.modestus* showed a significant ($F_{2,75}=12.522$; $p<0.001$) main effect of biofilms but no significant effect due to individual cyprids ($F_{4,8}=0.687$; $p=0.621$). However, there was a significant ($F_{8,75}=2.646$; $p=0.013$) interaction term which on

inspection of the data for individual cyprids could be accounted for by two of the five cyprids having elevated levels of tenacity on the control surface, but having similar levels of tenacity on the two filmed surfaces. The presence of such interaction would suggest that the group of *Elminius modestus* cyprids used were not behaviourally identical, as emphasised by the control levels of tenacity (see Section 3.4 *this volume*), but once on the filmed surfaces this difference between behaviour types was lost. Pairwise differences between mean tenacity on the surfaces were analysed by Tukey's method (Table 5.1). The 95% confidence interval of the difference between any two means was $0.783 \times 10^4 \text{ Nm}^{-2}$ demonstrating that tenacity to the low shear surface was not significantly different from that to the control surface (difference = $0.592 \times 10^4 \text{ Nm}^{-2}$). The high shear biofilm, however, elicited a significantly elevated tenacity than either of the other two surfaces (differences = $1.013 \times 10^4 \text{ Nm}^{-2}$ & $1.605 \times 10^4 \text{ Nm}^{-2}$).

Table 5.1 also details a similar analysis for *B.perforatus* cyprids. The analysis of variance once more showed the main effect of the biofilms to be significant ($F_{2,75}=35.026$; $p<0.001$) and neither the effect of individual cyprids nor the interaction proved significant ($F_{4,75}=0.575$; $p=0.682$ & $F_{8,75}=0.760$; $p=0.639$ respectively). The 95% confidence interval of the difference between any two means was $0.559 \times 10^4 \text{ Nm}^{-2}$ (Tukey's method). There was no significant difference between the tenacity to the control and high shear surfaces (difference = $0.233 \times 10^4 \text{ Nm}^{-2}$). In contrast to the results for *E.modestus* cyprids, the low shear surface elicited significantly reduced tenacity for *B.perforatus* cyprids than the

other two surfaces (differences= 1.338×10^4 & $1.571 \times 10^4 \text{ Nm}^{-2}$).

5.4 DISCUSSION

The characteristics of a biofilm on a given substratum are subject to multifarious influences and, in certain ways, must reflect prevailing conditions (over the short or even long term). If biofilms are to be utilised by a searching larva, geographically distant but environmentally equivalent films must share some essential characteristics, for example, as in the relationship between *Semibalanus balanoides* and the intertidal alga *Achnantes parvula* (Le Tourneaux & Bourget, 1988; Bourget, 1988) on New Brunswick coasts. Such 'indicator' biofilm communities need not consist of identical species assemblages, but must be analogous in some physico-chemical sense and, moreover, must be appreciable to the larva.

The behaviour of the two species of cyprids (*ie*, their voluntary ability to detach), or the physical adhesion of the temporary glue (see Yule & Walker, 1984), towards the two biofilms differs significantly. In both cases, tenacity to the high shear film was significantly greater than to the low shear film. Biofilms developed in high shear environments are likely to be relatively thin (Santos *et al.*, 1991) and dense (Christensen & Characklis, 1990) resulting from a greater nutrient availability and exchange afforded by faster flow. Relatively thick films, with reduced relative density, are characteristic of biofilms developed in low shear environments due to reduced hydrodynamic removal of cells and exopolymers from the film (Dudderidge *et al.*,

1982; Rittman, 1982; Trulear & Characklis, 1982; Characklis *et al.*, 1984; Lau & Liu, 1993). The primary difference between the two barnacle species however, is in the way the cyprids behave towards low shear films. Tenacity of *E.modestus* cyprids was not significantly reduced compared to the control surface. However, *B.perforatus* cyprids exhibited significantly reduced tenacity. This reduction cannot be a result of either adhesive failure of the film from the coverslip surface, or a lack of physical adhesion between the film and the cypris antennule since *E.modestus* cyprids, on the same surfaces with potentially the same proteinaceous glue, showed no such reduction of tenacity between control and low shear surfaces. The surface and glue combination was therefore capable of maintaining a higher bond strength than that ultimately measured for *B.perforatus* cyprids on the low shear surface. *B.perforatus* cyprids exhibited voluntary detachment from low shear films in much the same manner as shown for *S.balanoides* cyprids on light coloured perspex surfaces (Yule & Walker, 1984). Such a behavioural response (voluntary detachment) measured as reduced tenacity, would lead to a reduced settlement of *B.perforatus* in areas which produced biofilms with characteristics equivalent to those developed in low shear during the course of the current experiments, whereas no such settlement reduction should be evident for *E.modestus* cyprids.

The biofilm produced under high shear gave no greater tenacity than control surfaces for *B.perforatus* cyprids, yet encouraged greater than control tenacity for *E.modestus* cyprids. The results of the present study show clear species specific differences in the relationship between bacterial films and

potentially settling barnacle larvae and thus indicate that the larvae can appreciate differences in the bacterial films and highlight the potential for natural biofilm involvement in the patterns of barnacle settlement.

The mechanism by which *B.perforatus* discriminates low shear films has not been investigated, but since *E.modestus* cyprids stuck equally well to the biofilm as they did to an unfilmed surface, it is unlikely to involve a tenacity measurement process as postulated by Yule & Crisp (1983) for the positive effect of arthropodins on cypris behaviour. The difference in tenacity between the two species of cyprid may be indicative of a slightly different adhesive composition, but we have no evidence to suggest such differences. Barnacle cyprids appear to be capable of sensing subtle differences in arthropodins showing greater settlement in the presence of their own, or, closely related species than to more distant relatives (Knight-Jones, 1953; Whillis *et al.*, 1990; Crisp, 1990). Such differences in behaviour between species, as shown by *E.modestus* and *B.perforatus* towards the same biofilm, are essential findings in advancing the argument for differential larval settlement playing a more substantial rôle in the distribution/zonation of certain sessile animals than may post-settlement mortality.

6.0 The Effect of Individual Exopolysaccharide Components upon Temporary Adhesion.

6.1 INTRODUCTION

Surfaces in the marine environment can develop adsorbed macromolecular 'conditioning' layers within an hour of immersion (Goupil *et al.*, 1980; Baier, 1984; Meyer *et al.*, 1988). Through the use of techniques such as internal reflection infra-red spectroscopy the nature of this so called 'conditioning' layer has been investigated. Irrespective of the type of substratum or flow conditions, the initial macromolecular layer is predominantly proteinaceous in all natural, aqueous systems (Baier, 1984) and exists in a highly hydrated state (Goupil *et al.*, 1980). The rate of accumulation of the layer is dependent upon the prevailing flow conditions and solute concentration in the surrounding medium (Baier, 1984). Surfaces suspended in Biscayne Bay, Florida were covered within 10 minutes of immersion and protein accumulation continued for at least 21 hours (Goupil *et al.*, 1980) after which carbohydrates were detected, probably due to the colonisation of the surface by bacteria. One major effect of the accumulation of such proteinaceous films is that differences in critical surface tension between surfaces are lost (Goupil *et al.*, 1980; Meyer *et al.*, 1988). Surfaces immersed in the Gulf of Mexico for 144 hours (Goupil *et al.*, 1980) accumulated macromolecular layers which were characterised as being both low energy, and low polarity in nature, having a critical surface tension of 32 mNm^{-2} . Similarly,

suspending surfaces of differing initial critical surface tensions for 12 days in Biscayne Bay resulted in the surfaces having a common critical surface tension of $\approx 36 \text{ mNm}^{-2}$ (Meyer *et al.*, 1988).

The formation of a proteinaceous layer on a newly immersed surface is followed by bacterial colonisation of the surface (Goupil *et al.*, 1980; Meyer *et al.*, 1988). Initial colonising bacteria are small rod-shaped chemoautotrophs (Marszalek *et al.*, 1979; Baier, 1984; Little, 1984). Despite early bacterial colonisation, maximum bacterial numbers ($>5 \times 10^6 \text{ cm}^{-2}$) are not reached until as much as 5 days later (Meyer *et al.*, 1988). Bacteria respond to a surface by producing exopolymers which are mostly polysaccharide in nature, highly hydrated (Sutherland, 1980; Costerton *et al.*, 1985), promoting both cell adhesion and biofilm formation (Allison & Sutherland, 1987). However, the appearance of bacterial exopolymers, observable as slimes, lags bacterial colonisation by approximately 24 hours (Meyer *et al.*, 1988), when stalked or flagellated bacteria begin to replace the rod-shaped initial colonisers (Baier, 1984; Little 1984). Three neutral sugars; *D*-mannose, *D*-glucose and *D*-galactose, have been found to occur most frequently among bacterial exopolysaccharides from both the marine and freshwater environments (Sutherland, 1980).

The predominant chemical characters of a recently immersed surface thus changes from proteinaceous to polysaccharide as a function of time. The time at which a larva encounters a surface is, therefore, potentially extremely important. There is a wealth of research (reviewed by Gabbott & Larman, 1987) detailing

interactions between cyprids and proteins, specifically in relation to conspecific settlement inducers. Until now, interactions between cyprids and sugars has not been considered. Insight gained from such studies will not only increase our limited knowledge of how marine bioadhesives function, but will also help to identify possible uses outside of the marine environment as well as aiding in the search for environmentally acceptable antifouling coatings (Evans & Clarkson, 1993).

6.2 MATERIALS AND METHODS

The effect of biofilm-associated sugars upon the temporary adhesion of cypris larvae was studied in five barnacle species; the balanids *Balanus perforatus* and *B.improvisus*, the archaeobalanid *Semibalanus balanoides*, the chthamalid *Euraphia withersi* and the scalpellid *Pollicipes pollicipes*[§]. Larvae of *B.improvisus* and *P.pollicipes* were reared on a diet of *Rhinomonas reticulata* and *Skeletonema costatum*, whilst *B.perforatus* larvae were reared on *R.reticulata* and *Pavlova lutherii* up until the metanaupliar stages, after which *S.costatum* (chain length <20 µm) was added. In common with other chthamalid larvae (Moyse, 1960), *E.withersi* larvae were reared solely on the flagellates *P.lutherii* and *R.reticulata* and *Nannochloropsis oculata*. *S.balanoides* cyprids were collected from the plankton in the Menai Strait, in April 1993.

[§] *Pollicipes pollicipes* cyprids were reared at Menai Bridge by Mr Michael Kugele.

Initially, measurements of temporary adhesion to organically-clean glass slides were made in the presence of a range of glucose concentrations (10^{-10}M - 10^{-4}M) and compared with measurements made in seawater with no added glucose (*ie* background glucose levels). In addition, the effect of soaking *B.perforatus* cypris larvae in a 10^{-6}M added glucose solution for 5 minutes was also studied, adhesion measurements being made in natural seawater with no added glucose.

Once a model of the effect of *D*-glucose concentration upon temporary adhesion had been described, the effect of increasing concentrations of other sugars, the hexoses *D*-galactose and *D*-mannose, the pentose *D*-arabinose and the uronic acid *D*-glucuronic acid, upon temporary adhesion was compared to the glucose model.

Whilst cyprids of equal age could be obtained for each species (excepting *S.balanoides*), reducing intraspecies behavioural differences, interspecies differences could not be avoided because of the different metabolic rates of the five species (for an explanation of the link between cypris age and behaviour see section 3.4 *this volume*). In each case, six cyprids were used to make measurements of temporary adhesion, six measurements being made with each of the cyprids.

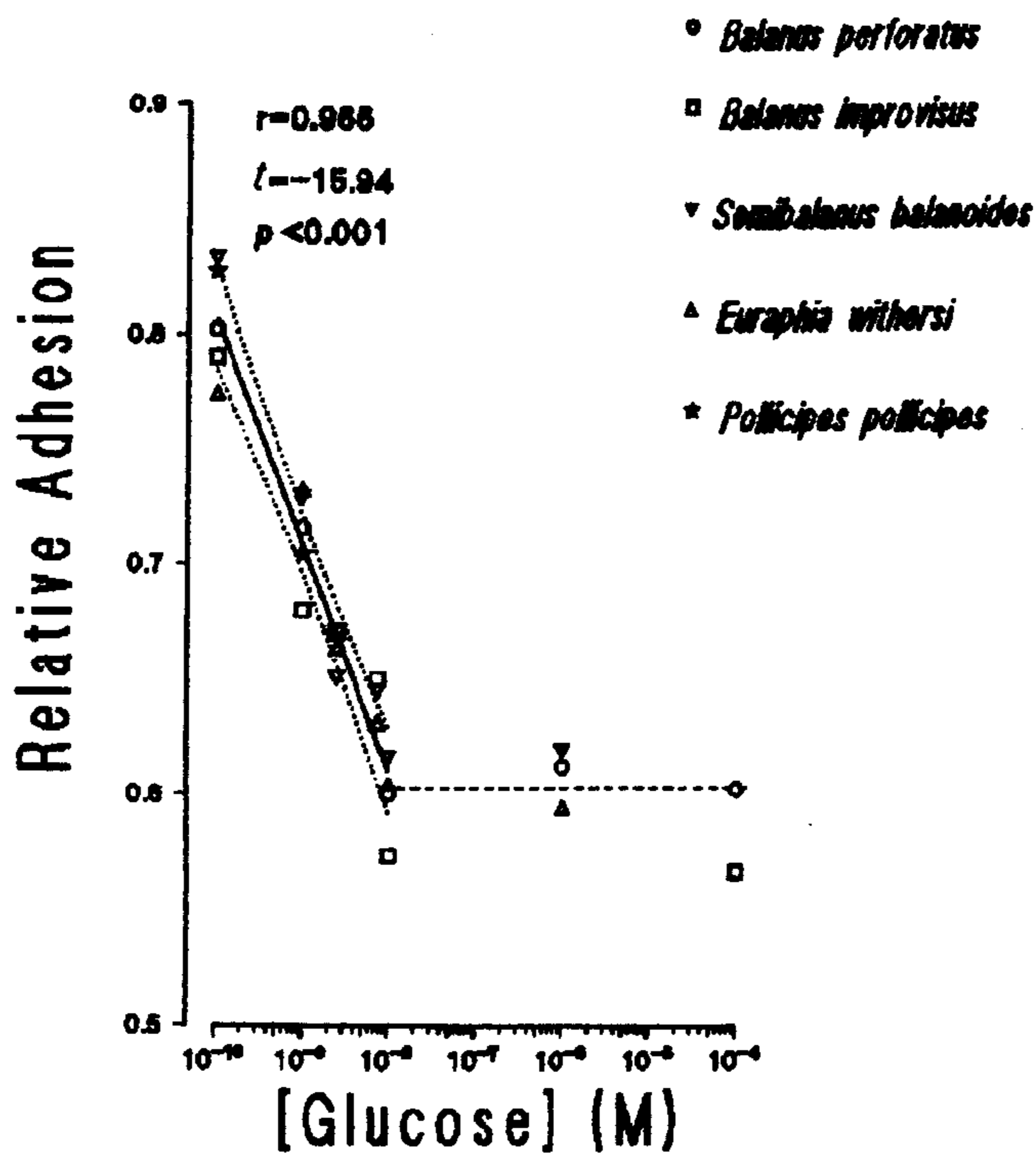
All sugar solutions were made up in cartridge filtered ($0.2\mu\text{m}$), UV-irradiated seawater. Background concentrations of glucose in coastal waters are of the order of 10^{-6}M - 10^{-7}M (Meyer-Reil *et al.*, 1979; Billen *et al.*, 1980; Gocke *et al.*, 1981; Lochte, 1985), and therefore all sugar concentrations are, in

Fig. 6.1 Effects of sugars upon cypris temporary adhesion:

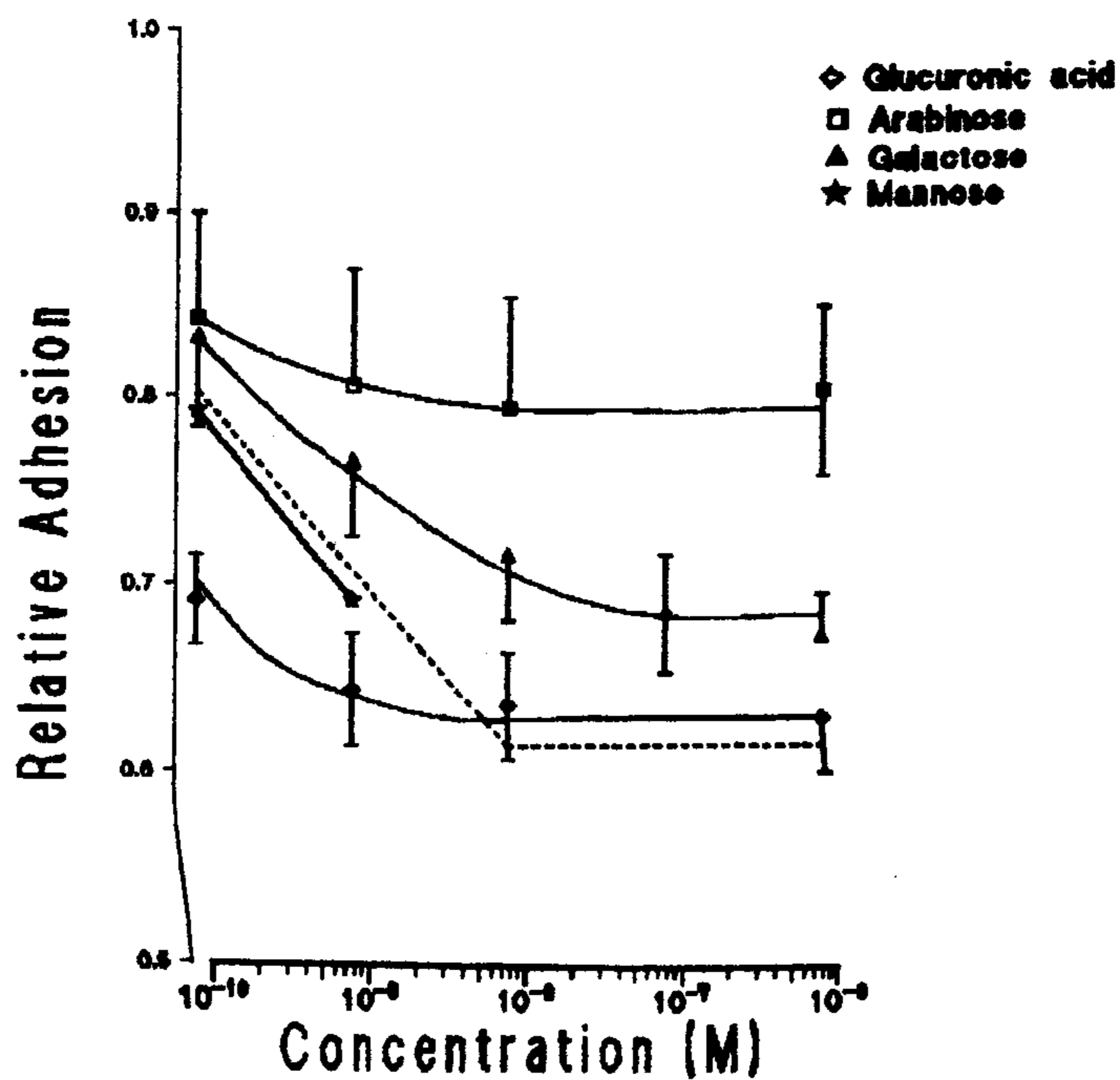
a) effect of *D*-glucose upon adhesion of five species of barnacle cyprid, the fit and 95% confidence intervals of the fit are shown,

b) a comparison of the effects of polar and non-polar sugars upon the adhesion of *Balanus perforatus* cyprids. Temporary adhesion is expressed as relative adhesion (mean adhesion/ mean control adhesion) \pm 95% confidence intervals. The dotted line represents the glucose model from Fig 6.1a.

a)



b)



effect, added concentrations.

6.3 RESULTS

For each barnacle species, glucose had a concentration dependent, inhibitory effect on cypris temporary adhesion to which a monoexponential decay model was fitted by a least-square method ($r=0.966$; $t=-15.94$; $p<0.001$; Fig. 6.1a), maximum inhibition (to $\approx 60\%$ of control values) was achieved by a 10^{-6}M *D*-glucose concentration. Greater concentrations also resulted in the maximum level of inhibition.

The effect of soaking *Balanus perforatus* cyprids in a 10^{-6}M glucose solution for 5 minutes was to reduce temporary adhesion to the glass surface by 30% to $5.82 \times 10^4 \text{ Nm}^{-2}$ (95% CI = $0.27 \times 10^4 \text{ Nm}^{-2}$) from a pre-soak level of $8.26 \times 10^4 \text{ Nm}^{-2}$ (95% CI = $0.23 \times 10^4 \text{ Nm}^{-2}$), a significant reduction (t -test, $n=36$; $t=-13.03$; $p<0.001$).

The effects of the different sugars upon *B. perforatus* temporary adhesion are shown in Fig. 6.1b. The glucose inhibition model is also shown for comparison. All the sugars tested have a concentration dependent, inhibitory effect upon temporary adhesion. However, when compared to the glucose model, temporary adhesion is differentially affected by the sugars. The pentose, *D*-arabinose, is less inhibitory in its effect than the hexoses. Maximum inhibition, caused by 10^{-9}M arabinose was to $\approx 80\%$ of control levels, compared to a maximal inhibition of $\approx 60\%$ for a

10^{-8} M glucose solution. Amongst the hexoses, *D*-galactose exhibited reduced inhibition compared to either *D*-glucose or *D*-mannose. Whilst the two mannose concentrations tested fit the glucose model well, the measurements made in the presence of galactose consistently exhibit a reduced inhibition compared to the glucose model. The uronic acid of glucose, *D*-glucuronic acid, inhibits temporary adhesion to a much greater extent than any of the other sugars tested. Although the maximum inhibition is still $\approx 60\%$ of control values, this is achieved at a lower concentration (10^{-9} M) than glucose (10^{-8} M).

6.4 DISCUSSION

Marine bioadhesives when released must displace and repel surface adherent water and spread over the substratum, thus allowing the formation of adhesive bonds. Their study affords them many potential applications in both industry and medicine (Rzepecki & Waite, 1991). The source of temporary adhesive of the barnacle cyprid was first postulated to be the antennular glands which open onto the surface of the antennular disc (Nott, 1969; Nott & Foster, 1969). The presence of a proteinaceous adhesive covering the antennular disc surface was discovered by Walker & Yule (1984) who concluded that the antennular glands were likely to be modified hypodermal cells specialised for protein secretion and storage. The adhesive was expected to be modified integumentary protein thought to act as a pseudo-Stéfan adhesive (Yule & Crisp, 1983). However, the fact that surface free energy and the ratio of polar to dispersion (attraction) forces associated

with a surface influences temporary adhesion (Yule & Walker, 1987) suggests some interaction between the temporary adhesive and the surface associated polar forces.

Figure 6.1a clearly shows that *D*-glucose has a significant concentration dependent effect upon temporary adhesion of the cyprids of all five species, suggesting a common adhesive mechanism. The fact that the five species were all similarly affected, despite being of different physiological ages, and therefore, different behavioural states (section 3.4 *this volume*), suggests that the effect of glucose is not that of a stimulus influencing cypris behaviour, but is more likely to be a physical interference with the adhesive mechanism.

Small molecules may interrupt adhesion by adsorbing either to the adherend or to the adhesive. Glucose would not be expected to adsorb well to the glass surface (adherend) in our experimental set-up because of water's capacity as a solvent, and because of the strong ionic nature of seawater. This would suggest that the interference is occurring at the adhesive (cypris temporary adhesive surface). Clear evidence for such an argument derives from the reduced adhesion of *B.perforatus* cyprids suspended for 5 mins. in a 10^{-4} M glucose solution, where glucose can only interact with the adhesive.

Thus both circumstantial and experimental evidence suggests that glucose is interrupting adhesion by adsorbing to the cypris temporary adhesive. Adsorption is the product of electrostatic interaction forces and dispersion (attraction) forces between two

phases (Baier *et al.*, 1968). Electrostatic forces between the surface charges of the two adherends are of greater importance than dispersion forces, *ie* van der Waals and London forces (Birdi, 1981) which act at very small distances. It is possible that glucose in solution adheres electrostatically, *via* -OH and/or -COOH groups known to increase adhesion (Baier *et al.*, 1968), to polar groups associated with the cypris temporary adhesive (Yule & Walker, 1987). As glucose concentrations in the surrounding medium increase, so more polar groups become blocked, nullifying their contribution to the total force of temporary adhesion to the substratum. It follows that the adhesion of barnacle cypris larvae will never achieve its full potential because of the levels of free glucose in the marine environment.

Measurements made in the presence of other neutral sugars and *D*-glucuronic acid suggest that they too inhibit adhesion in a concentration dependent manner. Assuming the inhibitory mechanism is common to all the molecules certain inferences can be made. The fact that the maximum level of adhesive inhibition is comparable ($\approx 60\%$ of control values) between the hexoses and *D*-glucuronic acid suggests that they adsorb to a common polar group upon the temporary adhesive (we assume the effect of *D*-mannose to compare favourably with the glucose model over the whole concentration range). What differs between the four molecules is their affinity for this hexose binding site. As one would expect, the most polar molecule, glucuronic acid, exhibits the highest affinity, maximum inhibition occurring at 10^{-9} M. The neutral hexoses show less affinity, maximum inhibition occurring at 10^{-8} M for glucose and mannose, and at $>10^{-6}$ M for galactose. These differences in affinity

can be ascribed to the structures of the different molecules in a seawater solution. The reduced maximal inhibitory effect (to $\approx 80\%$ of control values) of the pentose, *D*-arabinose, suggests it involves binding sites different from those of the hexoses, and fewer in number.

Whilst it is not possible to identify the polar groups responsible for the adsorption of the sugar molecules, the effect of sugars upon the temporary adhesive, and the effects of different sugars are important findings in the study of bioadhesives, and have important implications for the ecology of barnacle cypris larvae. The fact that the hexoses and glucuronic acid tend to reduce temporary adhesion by roughly 40% suggests that 40% of the total adhesive force is due to polar interactions between the adhesive and the substratum. The remaining 60% of total adhesive force would be derived from van der Waals and London forces.

The finding that different sugars affect temporary adhesion to different extents has important implications for the interaction between barnacle cyprids and bacterial films on submerged surfaces in the marine environment. This study suggests that the ratios of the different sugars within the bacterial exopolysaccharide will determine the maximum potential force of temporary adhesion derived between the temporary adhesive on the antennular disc surface and the biofilm. Sutherland (1980) suggests the most common neutral sugars found in bacterial exopolysaccharides are the three hexoses used here, but the relative amounts of these differ both with growth stage and

nutrient status (Omar *et al.*, 1983; Uhlinger & White, 1983; Christensen *et al.*, 1985;). Similarly, the level of uronic acids, the most polar, and therefore the most active, molecules in the exopolysaccharide is dependent upon nutrient limitation (Wilkinson, 1972; Minnikin *et al.*, 1974; Uhlinger & White, 1983). Sutherland (1980) has shown that uronic acids usually make up 20-25% of bacterial exopolysaccharides, but Corpe (1970) has described an exopolymer where the ratio increased to parity (50% uronic acid). The relative amount of uronic acids in the exopolymer would appear to have the greatest effect upon temporary adhesion and a greater understanding of the environmental conditions eliciting their production is essential to the understanding of how bacterial films influence barnacle settlement.

7.0 General Discussion.

The study of rocky shore and fouling ecology has long (perhaps too long) been held in thrall to the old paradigm. Zonation of species has been ascribed as the product of inter- and intraspecies competition for food and substratum, predation and the varying extent to which species are susceptible to negative environmental effects; insolation, temperature fluctuation, desiccation etc. Many of these conclusions are derived from the study of the quasi-equilibrium confronted on any shore; the end result of myriad actions and reactions.

More recently, larval input, not adult-adult interactions, has been proposed as the driving force behind the observable zonation of species on a shore (Caffey, 1985; Gaines *et al.*, 1985; Roughgarden *et al.*, 1988; Holm, 1990; Bertness *et al.*, 1992) placing greater emphasis upon the settlement behaviour of larvae than the physiological tolerances of the various life history stages. The hydrodynamic input of larvae into discrete zones on the shore would allow for rigorous microscale differentiation and requires that larvae are sensitive to habitat 'indicators' which identify suitable settlement sites. A well documented example is the interaction between *Semibalanus balanoides* cyprids and the intertidal diatom *Achnantes parvula* (Bourget, 1988).

A keystone of the old paradigm is the specious concept of succession where microfouling follows the development of a molecular conditioning film, and is in turn succeeded by algal fouling, and ultimately, macrofouling, finally resulting in a

"stable" community (Wahl, 1989). Verisimilitude is no proof. Whilst there are, indeed, studies which support the succession thesis (Zobell, 1938; Cole & Knight-Jones, 1949; Miller *et al.*, 1948), studies which dispute the veracity of the phenomenon also abound (Miller, 1946; Ferguson-Wood, 1950; Daniel, 1955; Crisp & Ryland, 1960). In his review of biological cues to invertebrate settlement, Scheltema (1974) states

"Although bacterial films are certainly conducive to the attachment of many marine organisms, Zobell's original hypothesis that slime films might prove to be a prerequisite in the ecological succession of fouling communities has not been unequivocally substantiated."

Twenty years on, and we still have yet to provide unequivocal evidence. Studies using monospecific films prove even less conclusive; Meadows & Williams (1963), Maki *et al.* (1988) and section 4 of this volume, have shown that the constituents of a film are important, some bacteria promoting settlement, whilst others prohibit it. Even between barnacles, the same bacterium can have significantly different effects (*cf.* Maki *et al.*, 1990 and Neal & Yule, 1994). Additionally, the fact that settlement of invertebrate larvae is largely dependent upon the distribution (in time and space) of larvae in the water column (Holm, 1990; Bertness *et al.*, 1992) helps undermine the attractiveness of the succession hypothesis, and all but very general predictions of outcomes appear both impossible and unhelpful.

A more pliant dynamic model has been suggested by Roberts *et al.*, (1991). The dynamic model proposed suggests that availability of settlers or "foulers" (bacterial, microalgal or macroinvertebrate), is the driving force behind the establishment

of a fouling community and that, therefore, it is not necessarily a successional process (Clare *et al.*, 1992). It becomes clear, therefore, that fouling on a marine surface cannot be prevented simply by arresting biofilm development. Evidence is plentiful, with many of the classical settlement studies reported in the literature having been conducted in such ways as to preclude the development of substantial biofilms, and yet settlement has continued fruitfully.

For a pelagic larva, such as a barnacle cyprid, the location of a suitable site for settlement is essentially a two step process. Macrodistribution of an individual is governed by external, hydrodynamic processes (Shanks 1983, 1986; Shanks & Wright, 1987; Hill, 1991; Pineda, 1991). These large scale processes determine on which shore, and at which point in time, the larva comes ashore. Microdistribution is governed, in part, by turbulent hydrodynamic processes delivering a larva onto the substratum surface (Denny, 1988; Gross *et al.*, 1992), and in part by larval behavioural processes evidenced by movement across the substratum (Crisp & Austin, 1960; Walters, 1992). Crisp (1974) identifies two mechanisms by which a larva can evaluate a potential settlement site; gregarious settlement, where conspecifics or close relatives influence settlement, and associative settlement, where allospecifics, including bacteria, influence settlement.

Studies have shown (Maki *et al.*, 1988; Avelin *et al.*, 1993; section 4 *this volume*) that individual bacterial strains have significantly different effects upon cypris larvae. The

possibility that biofilm species composition is important in determining whether a film encourages or discourages settlement is thus established. Unfortunately, the current attempt to study qualitatively the bacteria present at different levels of the shore at different times of the year proved unsuccessful. The task would require a large-scale sampling programme, utilising many selective media and hours of laborious identification. It is therefore difficult to assess whether differences between individual bacterial strains in 'attractiveness' to cyprids, measured in the laboratory, are of any importance when encountered as constituents of a dynamic biofilm in the field.

In contrast to differences in species composition between bacterial films, differences in the films' physical properties may have more environmental relevance, particularly as the results of the shore survey of bacteria (section 4 *this volume*) found the same suite of bacteria at all levels of the shore. It has been demonstrated that two biofilms, which are likely to have very similar, if not identical, species compositions, but were grown in either high or low shear, influence cypris behaviour in differing ways (section 5 *this volume*). Both cyprids of *Elminius modestus* and *Balanus perforatus* exhibited greater adhesion upon the thin, dense film established in a high shear régime than on the thick, less dense film from low shear. The primary difference between the two films is the amount of exopolysaccharide present; high shear tends to remove extracellular products, so that films grown at low shear contain higher levels of exopolysaccharides. The situation is analogous to the aging of individual bacterial films in section 4, where exopolysaccharides accumulate with age. Again, adhesion

of *Verruca ströemia* cyprids to *Pseudomonas* W3⁺, *Pseudomonas* Br4⁺ and the purple bacterium films tends to decrease with age. The exopolysaccharides derived from *Pseudomonas* W1⁺ (section 4), were shown to have concentration dependent inhibitory effects upon adhesion of *V.ströemia* cyprids.

All the current evidence suggests that species composition, although potentially significant, plays only a minor rôle (compared with the physico-chemical nature of the film) in determining a cyprid's behaviour to a bacterial film. Areas which favour the accumulation of exopolysaccharides; low shear environments, eutrophic areas and areas with reduced levels of physical disturbance, will be places where unfavourable biofilms are likely to develop. Such places are likely to experience low energetic input in an ecological sense, and are therefore likely to be unfavourable habitats for filter feeders such as barnacles.

The concentration dependent effect of bacterial exopolysaccharides is emphasised by the findings described in section 6 where components of bacterial polysaccharides, the hexoses; *D*-glucose, *D*-mannose and *D*-galactose appeared to inhibit cypris temporary adhesion. There are minor differences in the level of inhibition among the hexoses, but between the neutral hexoses and polar *D*-glucuronic acid, there is a substantial difference, the more polar molecule having increased inhibitory activity.

The chemistry of the film is thus a second property likely to influence a cyprid's behaviour substantially. Whilst the ratios

of hexoses within the polymer will have an effect, the ratio of hexoses to uronic acids will have a much greater effect, probably one of substantive ecological significance. Although uronic acids are common components of bacterial exopolymers, generally constituting 20-25% of a film (Sutherland, 1980), the imposition of nutrient limitation upon a film results in the increased cellular production of uronic acids (White, 1984) and their concentration in the exopolymer increases. Biofilms developed in areas of relative oligotrophy (having a relatively greater proportion of polar sugars) will tend to elicit lower levels of temporary adhesion than films developed at intermediate nutrient levels.

The arguments elaborated above suggest that, whilst bacterial films are not a prerequisite to settlement, cypris larvae are capable of differential behaviour in response to physico-chemical differences between biofilms representative of particular ecological niches. The behaviour of *Elminius modestus* and *Balanus perforatus* cyprids to the shear related films (Section 5 *this volume*) suggests that species' responses to a particular biofilm property differ, resulting, *ipso facto*, in "zonation". Such "zonation" could occur on an extremely small scale: shear rates on a boulder surface may differ significantly over distances of less than a centimetre. The accumulation of evidence begins to suggest that larval site selection, generally considered an important, but somewhat "blunt instrument", is in fact perhaps more finely tuned. The "blunt" view of barnacle settlement explained the general blanket covering of a shore in terms of a gregarious response (Crisp & Meadows, 1963) given the presence of

other barnacles. Interspecies interaction or, more exactly, competition, is left to explain species zonation upon a shore. With the description of interactions between *Semibalanus balanoides* and *Achnantes parvula* (Bourget, 1988) and those investigated in this volume we are approaching a more refined view of zonation (which reduces the necessity to invoke competitive interaction in explanation) through selective settlement in discrete niches using microflora (and, no doubt, fauna too) as indicators of the suitability of the niche, hence less emphasis need be placed on competitive interactions and the obvious energetic waste to species that this implies.

Competition is a strong modulating force in the environment when resources such as space are limiting. When larval supply is greatly in excess of the available space, for example, as would happen in a year of good recruitment, then wastage of larvae is of little consequence. In years of poor larval supply, wastage is a more serious issue and the ability of barnacles to locate optimum settlement sites must confer survival advantage under those conditions.

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