

Effects of environmental and surface factors on settlement and adhesion of the barnacle *Balanus amphitrite*.

Sheelagh Louise Conlan

Thesis submitted for the fulfilment of
a Doctor of Philosophy

October 2013

School of Marine Science and
Technology
Newcastle University

This thesis is dedicated to

Philip and Barbara Conlan

The parents most can only dream of

I was lucky enough to be born to

Abstract

Biofouling is a substantial problem for the maritime industry. Organisms growing on underwater surfaces increase drag on ships' hulls and damage coastal machinery. Billions of dollars are spent annually on mitigating fouling. The tropical barnacle (*Balanus amphitrite*) is a major fouling species. This thesis investigates the effects of some of the major environmental controls for this benthic species, (surface chemistry, temperature and food availability), on the adhesion and adhesives of two of its life cycle stages, the settlement-stage cypris larva (the final larval stage of barnacles) and the adult.

An existing test for fouling-release (FR) coatings measures how strongly adult barnacles attach. Chapter 2 analysed and developed this method. Two novel FR testing methods are then established and described, examining how hydrodynamics can be exploited to test earlier life stage attachment. By using settled cyprids instead of adults, testing time can be reduced by up to 12 weeks. These time savings have the potential to lead to significant cost reductions for the industry, and allow higher throughput at initial screening for more efficient down selection in the development process.

Further chapters assess the nature of the cyprid permanent cement. A method was developed to visualise the cement plaque using Congo Red staining. Using this method it was possible to determine that the cyprid cement area was highly conserved with age (up to nine-days-old) and batch, and that it was not affected by the addition of the settlement inducer isobutylmethylxanthine. Using controlled surfaces (self-assembled monolayers), cement area was found to be negatively correlated with wettability, suggesting that the adhesive spreads better on hydrophobic surfaces from which water is more easily displaced. The adhesive also spread more on negatively charged surfaces. When settlement data, obtained by Petrone et al. (2011), were compared to cyprid cement area, a strong positive correlation was found. Adhesive spreading could therefore provide a quick measure of the antifouling potential of a surface.

Chapter 5 determines the effect of temperature and feeding on growth and moulting of *B. amphitrite* from settlement to adult, and how this relates to adhesive production and adhesion strength. Each moult was found to correlate to a ring of adult cement. Adult cement was measured in terms of proportion of the basal area covered, number of rings of adhesive and mean width of each adhesive ring. Adhesive strength reduced with increasing temperature of culture. Conversely, the percentage cover of the base with adhesive generally increased with increasing temperature. This effect of temperature on adhesion and adhesive production has not been examined previously and points to the importance of the use of a range of field sites for testing fouling-release surfaces.

Finally, in Chapter 6 an experimental surface is described that utilises a bound serine protease (Subtilisin A) to prevent settlement by acting on the proteins in barnacle adhesives. This is the first time the enzyme has been shown to prevent settlement of barnacles when tethered to a surface. The effect of the tethered enzyme on cyprid behaviour, settlement and adhesion is described.

The increase in understanding of the adhesives of *B. amphitrite* provided by this research should assist in targeting the FR surfaces against the adhesive and along with the improved methodologies presented should aid in the discovery of novel non-toxic alternatives to the mostly biocidal antifouling paints utilised by the maritime industry today.

Acknowledgments

I'd like to express my gratitude first to my supervisor, ex-boss and mentor Prof. Tony Clare without whose encouragement I would never have had the confidence to begin this PhD and without whose support and assistance throughout I would not have completed it.

Thanks also to the collaborators Dr Linea Ista, Dr Luigi Petrone and Dr Mariana Tassos. A massive vote of thanks also has to go to all the people at International Paint (IP) Ltd. in Felling, who allowed me free use of, and taught me how to produce silicone-based coatings and introduced me to the techniques of Dynamic Mechanical Analysis, microindentation, goniometry and laser profilometry. In particular, I would like to thank David Williams and Cait Davies who put up with my endless questions about the chemistry of silicones. Thanks also to Jennifer Longyear, from IP, whose enthusiasm and great ideas for my work helped me at a difficult time.

Thanks to my co-workers and co-PhD students at Newcastle who made my time there so great and created such useful scientific discussions over cake, or beer. The list could take the page but in particular Gary, Nick, Helen Graham, Rebecca Herdman, Supernut, Hillary, Thea, Susan, Alessio, Sophia, Rebecca Taylor, Tristano and too many more to name.

Appreciation to the technicians in the Armstrong Building who helped with the flow cell and the water jet when it really mattered: Peter Bowes, Bob Hindhaugh, Ian Patterson. In the Ridley Building, Dave Whitaker and Alice Trowsdale helped with algae and seawater problems and talking through general lab issues. In the Dove Marine Lab John Knowles always had a smile when things were bad.

Thanks to the admin staff, Carol and Amanda, in the Ridley building and to the administration team in the Armstrong building for all the assistance, especially with submitting while away from Newcastle.

My appreciation to all my friends who put up with my constant working on the PhD through holidays and in particular those who kept me motivated towards the end special thanks are owed to Dr Simone Dürr and Dr Erica Bower who also helped with editing.

A huge vote of thanks to my family who have been supportive and full of fun throughout.

Last, but far from least, my dog Shadow who was happy and cheered me up through the worst times. I owe you so many walks and biscuits pup!

Table of Contents

Abstract.....	iii
Acknowledgments.....	v
Table of Contents.....	vii
Abbreviations.....	xii
Figures.....	xiv
Tables.....	xxiii
Chapter 1: Examining the importance of environment and life stage on biofouling using a common fouling species, the barnacle <i>Balanus amphitrite</i>	1
1.1. Introduction	1
1.2. The test species; the barnacle <i>Balanus amphitrite</i>	4
1.2.1. Barnacle biology.....	4
1.2.2. Environmental factors affecting growth of barnacles	7
1.3. Adhesion in the marine environment.....	8
1.3.1. Adhesion Theory	9
1.3.1.1. 'Dry' adhesion	14
1.3.2. Marine Bioadhesives.....	15
1.3.2.1. Barnacle adhesive systems	20
1.4. The problem of biofouling	25
1.5. Fouling-release (FR) coatings and factors affecting their performance	25
1.6. Testing biofouling solutions in the laboratory.....	26
Chapter 2: Evaluation of a fully automated method to measure the critical removal stress of adult barnacles.....	30
2.1. Abstract.....	30
2.2. Introduction	31
2.3. Materials and methods.....	33
2.3.1. Preparation of coatings.....	33
2.3.2. Barnacle culture	34
2.3.3. Preparation of epoxy replicas of barnacles for testing.....	34
2.3.4. Measures of critical removal stress	35
2.3.5. Speed related to force applied.....	40
2.3.6. Effect of rate of force development on removal using replica and real barnacles ..	40
2.3.7. Direct comparison of the two measures of adhesive force.....	41
2.3.8. The effect of size on critical removal stress.....	41

2.4. Results.....	42
2.4.1. Measurements of barnacle basal area.....	42
2.4.2. Speed related to force applied.....	43
2.4.3. The effect of rate of force development on removal using real and replica barnacles	44
2.4.4. Direct comparison of the two measures of adhesive force.....	45
2.4.5. The effect of size on critical removal stress.....	47
2.5. Discussion.....	47
Chapter 3: A critical assessment of methods of testing adhesion of early settlement and juvenile stages of <i>Balanus amphitrite</i>	52
3.1. Abstract.....	52
3.2. Introduction	53
3.3. Methods.....	55
3.3.1. Coating production	55
3.3.2. Cyprid culture.....	56
3.3.3. Adult CRS measurement	57
3.3.4. Water jet	58
3.3.5. Flow cell	59
3.3.6. Metamorphosis development over 31 h post settlement period	62
3.3.6. Analysis of data	64
3.4. Results.....	64
3.4.1. Adult CRS.....	64
3.4.2. Water-jet.....	65
3.4.3. Flow cell	68
3.4.4. Adhesive staining after metamorphosis	71
3.5. Discussion and Conclusions	72
Chapter 4: Effect of surface chemistry on cyprid permanent cement spreading, and surface selection.....	76
4.1. Abstract.....	76
4.2. Introduction	77
4.3. Methods.....	85
4.3.1. Surface preparation	85
4.3.2. Cyprid culture and settlement experiments.....	85
4.3.3. Staining of adhesive plaques.....	86

4.3.4. Imaging and measuring of cyprid cement	88
4.3.5. Description and production of self-assembled monolayers	88
4.3.6. Measurement of contact angles.	90
4.3.7. Cyprid age, batch differences and effect of an inducer	90
4.3.8. Analysis of data	91
4.4. Results.	92
4.4.1. Staining of adhesive plaques (stain types, duration and preservation).	92
4.4.2. Effect of cyprid age, batch differences and IBMX on cyprid cement.....	95
4.4.3. Effect of SAMs on cyprid cement area.....	97
4.4.4. Do cyprids select surfaces to settle on where their cement will spread further and adhere better?.....	102
4.5. Discussion and Conclusions	102
Chapter 5: The effects of feeding levels and temperature on growth, moulting and adhesive production in <i>Balanus amphitrite</i>	108
5.1. Abstract	108
5.2. Introduction	109
5.3. Methods	112
5.3.1. Effect of temperature and feeding level on adhesive production, growth and moulting	112
5.3.2. Effect of temperature on adhesion strength	114
5.3.3. Analysis of data	115
5.4. Results.....	115
5.4.1. Method development	115
5.4.2. The effect of temperature and feeding on growth.....	118
5.4.3. The effect of temperature and feeding on moulting	121
5.4.4. The relationship between moulting and growth	126
5.4.5. The relationship between moulting and adhesive ring production	128
5.4.6. The effect of temperature and feeding regime on percentage adhesive cover of basis.....	129
5.4.7. Average width of adhesive rings measured	130
5.4.8. The effect of the growth temperature of a barnacle on its CRS.....	130
5.5. Discussion and Conclusions	131
5.5.1. The effect of temperature and feeding on growth of basal area	132
5.5.2. The effect of temperature and feeding on moulting	133

5.5.3. The relationship between moulting and growth	135
5.5.4. The relationship between moulting and adhesive production.....	136
5.5.5. The effect of temperature and feeding on the percentage cover of the basis by adhesive.....	137
5.5.6. Average width of adhesive rings measured.....	138
5.5.7. Temperature of growth and CRS	138
Chapter 6: Active enzyme nanocoatings affect settlement of <i>Balanus amphitrite</i> barnacle cyprids.....	141
6.1. Abstract.....	141
6.2. Introduction	142
6.3. Methods.....	144
6.3.1. Preparation of bioactive nanocoatings.....	144
6.3.2. Characterization of the bioactive nanocoatings	145
6.3.3. <i>Balanus amphitrite</i> (syn. <i>Amphibalanus amphitrite</i> (Clare and Hoeg 2008)) cyprids	146
6.3.4. Settlement assay.....	146
6.3.5. Tracking assay	147
6.4. Results.....	148
6.4.1. Characterization of the bioactive nanocoatings (Carried out by M Tasso)	148
6.4.2. Cyprid settlement assay.....	151
6.4.2.1. Inhibitory and antifouling effect of Subtilisin A in solution	151
6.4.2.2. Antifouling potential of immobilized Subtilisin A	152
6.4.2.3. Comparison between immobilized enzyme and equivalent amount of enzyme in solution.....	154
6.4.2.4. Tracking.....	155
6.5. Discussion.....	156
6.6. Conclusions	162
Chapter 7: Final conclusions	163
7.1 Summary of findings	163
7.2 Limitations of the work.....	165
7.3 Further research	166
7.3.1. Testing other species	166
7.3.2. Modifying environmental factors	169
7.3.3. Hydrodynamic testing.....	170
7.3.4. Adhesive analysis	172

7.4 Conclusion.....	174
References	176

Abbreviations

AWG	Acid washed glass
ASTM	American Society for Testing and Materials
ASW	Artificial seawater
AFM	Atomic force microscopy
CaJ	Calcified juvenile
CP - 100K	Cement protein - (size in K) barnacle
Cm	Centimetre
CFD	Computational fluid dynamics
CBBR	Coomassie Brilliant Blue R250
CRS	Critical removal stress
DOPA	Dihydroxyphenylalanine
EC	European Commission
EU	European Union
e.g.	For example (exempli gratia)
FR	Fouling release
GDP	Gross domestic product
H	Hour
IBMX	3-isobutyl-1-methylxanthine
i.e.	That is (id est)
IMO	International Maritime Organisation
kHz	Kilo Hertz
KJ	Kilo Joules
KPa	Kilo Pascal
LDV	Laser doppler velocimetry
MPa	Mega Pascal
μl	Microliter
μm	Micrometre
Mg	Milligrams
ml	Millilitres
Mm	Millimetre
Min	Minutes

Mol	Mole
Mefp	<i>Mytilus edulis</i> foot protein
NM	Newly metamorphosed
N	Newton
OAR	Open Area Ratio
Pa	Pascal
PA	Permanently attached
PDMS	Polydimethylsiloxane
PTFE	Polytetrafluoroethylene
RO water	Reverse osmosis water
RPM or rpm	Revolutions per minute
SAM	Self-assembled monolayer
SIPC	Settlement inducing protein complex
SFE	Surface free energy
TBT	Tributyltin

Figures

Figure number and title (shortened where necessary)	Page
Figure 1.1. The life cycle of <i>Balanus amphitrite</i> .	5
Figure 1.2. Adhesive filling irregularities in the substrate and thus creating a mechanical interlocking adherence to the substrate.	10
Figure 1.3. Schematic to show the action of adsorption between polymer chains that make up the adhesive and molecules within the substrate.	12
Figure 1.4. Schematic to show the bonds formed across the adhesive/substrate interface in chemisorptions.	12
Fig. 1.5. Schematic representation of diffusional adhesion theory.	13
Fig. 1.6. Schematic showing charge-based adhesion explained by electrostatic adhesion theory.	14
Figure 1.7. The gecko foot at different magnifications showing the setae and spatula tips that create the high surface area for van de Waals adhesion to function (taken from Biomimetic Millisystems Lab, UC Berkley website).	15
Figure 1.8. Schematic illustration of the modes of attachment of mussel, barnacle, and tubeworm (adapted from Kamino 2010).	19
Figure 1.9. a) Cyprid permanent adhesive, or cement, stained using Congo Red; b) adult adhesive, in rings on the basis of an adult removed from silastic T2 and stained using Coomassie Brilliant Blue R250 stain.	21
Figure 1.10. <i>B. amphitrite</i> cyprid antennules, imaged at increasing magnification to show cuticular villi on the antenular disc (from Phang et al. 2010).	22
Figure 1.11. SEM of a fly (<i>Calliphora vicina</i>) foot showing the pulvilli, images increasing in magnification from A to C (adapted from Gorb et al. 2012).	22
Figure 2.1. Epoxy replicas, coloured with Coomassie Brilliant Blue, of two sizes of barnacle attached with cyanoacrylate adhesive to T2-coated glass slides.	34

Figure number and title (shortened where necessary)	Page
<p>Figure 2.2. The computer controlled motorised force gauge. C, camera; L, lights; Lc, load cell; M, motor; P, platform; Pb, push bar. Arrow indicates the direction of movement of the platform which houses the slide with attached test specimens.</p>	36
<p>Figure 2.3. Schematic of the computer controlled system.</p>	37
<p>Figure 2.4. Screen grabs of A) barnacles that have been scanned and their basal areas measured using the automated method's software and B) force traces produced, with each peak corresponding to a single barnacle removed, in this case from T2.</p>	38
<p>Figure 2.5. Mean basal area (+ 95% CI) for six different barnacles (A-F) measured using the automated method's software and by manual delineation using Image J.</p>	41
<p>Figure 2.6. The percentage error associated with calibrated area measurement by Image J and the automated method (+ 95 %CI). Arrow shows the lower size limit suggested by the ASTM (5 mm diameter).</p>	42
<p>Figure 2.7. Speed of movement of the push-off bar in both methods relative to the rate of force development (\pm 95% CI). Lines fitted to show second order polynomial relationship with intercept fitted through zero.</p>	43
<p>Figure 2.8. The effect of force development rate on the mean critical removal stress of real and replica barnacles attached to T2 (\pm 95% CI). ■ Replica barnacles, automated method; □ replica barnacles, manual method; ▲ real barnacles, automated method; Δ real barnacles, manual method.</p>	43
<p>Figure 2.9. Comparison of the force for removal of barnacles obtained by the manual and automated methods (+ 95% CI).</p>	44
<p>Figure 2.10. Power analysis graph for the automated method. The difference that can be discerned to 80% power and 95% confidence, is plotted against the sample size (log scale) required.</p>	45
<p>Figure 2.11. The effect of size of barnacle (basal area) on the removal stress when using (a) the automated method and (b) the manual method. The vertical line denotes the ASTM's suggested cut-off size (5mm diameter).</p>	46

Figure number and title (shortened where necessary)	Page
<p>Figure 3.1. A) Diagram of the original water jet device that the new system was based on (image from Swain and Schultz (1996)); B) motorised water jet of custom-built water jet apparatus.</p>	57
<p>Figure 3.2. Individuals not cleanly removed using water jetting. A) Cement plaque remaining after water jetting a PA individual (the antennules are clearly visible). B) Shell of a CaJ post testing.</p>	58
<p>Figure 3.3. A) Photograph of the flow cell with B) close up of the slide test area. Seawater is pumped (1) through baffles (2) and a contraction (3) into the Perspex test section (4) where fully turbulent flow is reached at the slide test area (B). Water then passes to the holding tank (5) ready to recycle around the system. The process is controlled by the computer system (6).</p>	59
<p>Figure 3.4. Comparison of CFD data from Newcastle's flow cell with that of Schultz et al. (2000) and Dean (1978) (adapted from Politis et al. 2009).</p>	60
<p>Figure 3.5. Relation of measured wall shear stress (Pa) in the test section of the flow cell with varying pump speed (rpm).</p>	61
<p>Figure 3.6. Congo Red staining of the bases of newly metamorphosed juvenile barnacles over time: A 4h; B 9h; C 15h; D 18h; E 24h; and F 31h.</p>	63
<p>Figure 3.7. The mean CRS (+ 95% CI) of adult barnacles grown on three FR surfaces.</p>	64
<p>Figure 3.8. The mean percentage removal (\pm 95% CI) of PA individuals; A) shows individuals removed with no visible cement or antennules left behind (adhesive failure), B) shows all individuals that were removed but a visible area of cement and/or one or both antennules left behind (cohesive/antennular failure).</p>	65
<p>Figure 3.9. The mean percentage removal (\pm 95% CI) of NM individuals. A) shows cleanly removed individuals, B) shows all metamorphosed individuals that were removed but with a visible area of cement and/or one or both antennules left behind (cohesive/antennular failure).</p>	66
<p>Figure 3.10. The mean percentage removal (\pm 95% CI) of calcified, 6-day-old juvenile barnacles. Only cleanly removed individuals were counted.</p>	67

Figure number and title (shortened where necessary)	Page
Figure 3.11. The mean percentage removal (\pm 95% CI) of PA individuals using the flow cell. A) shows cleanly removed individuals, B) shows individuals that showed cohesive/antennular failure.	68
Figure 3.12. The mean percentage removal (\pm 95% CI) of NM settled individuals using the flow cell. A) shows cleanly removed individuals and B) shows individuals that showed cohesive/antennular failure	69
Figure 3.13. The mean percentage removal (\pm 95% CI) of calcified, 6-day-old juvenile barnacles. Only cleanly removed individuals were counted.	70
Figure 3.14. Individuals showing staining of the basis over time from metamorphosis.	71
Figure 4.1. The <i>B. amphitrite</i> cyprid; A) free swimming, B) permanently settled, C) magnified view of the stained cyprid cement.	77
Figure 4.2. Schematic representation of the proposed mechanism of cementation during the permanent attachment of barnacle cyprids. Reproduced from Okano et al. (1996).	79
Figure 4.3. Explanation of the use of contact angle measurement to calculate values for Young's equation to determine the surface free energy of a solid (from http://www.ramehart.com/contactangle.htm).	80
Figure 4.4. Schematic diagram of the basic structure of gold alkylthiolate SAMs.	83
Figure 4.5. Schematic showing how gold-coated slides were 'printed' using different thiols to result in a similar test area for seawater drop coverage independent of the wettability of the final SAM.	88
Figure 4.6. Imaged bases of settled barnacles (cyprids (C) and juveniles (J)) stained with different stains.	92
Figure 4.7. The outer and inner areas of the cyprid cement plaque.	93
Figure 4.8. Effect of 10^{-5} M IBMX on the cyprid cement plaque area (mean + 95% CI).	94
Figure 4.9. The effect of cyprid age on the cyprid cement disc area (mean area \pm 95% CI). The linear relationships for all the data (d 0 to 14, hatched line, lower equation and R^2 value) and first 9 days of data (solid line, upper equation and R^2 value) are shown on the graph.	95

Figure number and title (shortened where necessary)	Page
Figure 4.10. Change in cyprid cement area with age and cyprid batch (Mean + 95% CI, solid bars are batch 1 cyprids, hatched bars are batch 2 cyprids).	96
Figure 4.11. The relationship of water contact angle (mean \pm 95% CI) and proportion of R-OH thiol added to the thiol mix used to prepare the SAMs.	97
Figure 4.12. A) Percentage settlement (mean \pm 95% CI) and B) percentage mortality (mean \pm 95% CI) on SAMs with different wettabilities.	97
Figure 4.13. Cyprid cement area (mean \pm 95% CI) and the water contact angle of the SAMs with varying wettabilities (mean \pm 95% CI) for assay 1 (not micro-patterned) and 2 (micro-printed).	98
Figure 4.14. Settlement levels on SAMs of different wettabilities (mean + 95% CI) with IBMX inducer with (Assay 1) and without (Assay 2) micro-printing.	99
Figure 4.15. The effect of charge on the cyprid cement adhesive area (mean + 95% CI). Bars labelled with overall charge.	100
Figure 4.16. The relationship between cyprid cement area (mean \pm 95% CI) and the SFE of various SAMs (labelled).	100
Figure 4.17. The relationship between cyprid cement area (mean \pm 95%CI) and settlement on 5 SAMs (mean \pm 95%CI). Settlement data from Petrone et al. (2011).	101
Figure 5.1. A stained barnacle basis showing the rings of cement and the measurements taken for comparison between treatments.	113
Figure 5.2. Image of a stained barnacle basis and an ESEM scan.	115
Figure 5.3. Direct comparison of light microscopy/staining results and ESEM results for A) ring number and B) adhesive percent area calculations for the same barnacles, with linear regression lines, equations and R ² values on the graphs.	116
Figure 5.4. Digital images of a) & c) two barnacle bases and their linked plastic coverslips b) & d) respectively, stained with CBBR to show the rings of adhesive.	117
Figure 5.5. Relationship between growth of overfed (A) and underfed (B) barnacle growth in terms of mean basal area (\pm 95% CI) over time from metamorphosis.	118

Figure number and title (shortened where necessary)	Page
<p>Figure 5.6. The comparison of the relationship of log age and log basal area with the predicted log age from the model (Equation 5.2) with increasing log basal areas.</p>	119
<p>Figure 5.7. Relationships between areas of bases of both overfed and underfed individual barnacles and elapsed time with temperature treatment converted to degree days (linear relationships shown; solid line for overfed barnacles; broken line for underfed barnacles).</p>	120
<p>Figure 5.8. Cumulative number of moults of barnacles (mean \pm 95% CI) exposed to different temperature regimes with increasing age of overfed (A) and underfed (B) individuals. Second order polynomial relationships fitted, (equations and r^2 values in table 5.1.).</p>	121
<p>Figure 5.9. Mean cumulative number of moults (log transformed) for overfed (A) and underfed (B) barnacles over the log transformed age range of the experiment. Linear regressions fitted; regression equations, r^2 and significance shown in table 5.2.</p>	122
<p>Figure 5.10. Cumulative number of moults of barnacles exposed to the two feeding regimes compared with the elapsed time/ temperature expressed as degree days. Mean cumulative number of moults \pm 95% CI, solid line = linear relationship for overfed individuals (equation and r^2 shown above the line); broken line = linear relationship for underfed individuals (equation and r^2 shown below the line).</p>	124
<p>Figure 5.11. Moult rates for each treatment expressed in terms of exuviae per 100 individual barnacles per day. Lines denote 2nd order polynomial relationships; solid line = overfed barnacles (equation and r^2 shown above the line); broken line = underfed barnacles (equation and r^2 shown below the line).</p>	125
<p>Figure 5.12. The relationship between moulting rate per treatment and basal area for <i>B. amphitrite</i> at different temperatures and feeding levels (mean, n=20).</p>	126
<p>Figure 5.13. The relationship between growth rate and moult rate for A) overfed and B) underfed barnacles.</p>	127
<p>Figure 5.14. Relationship between the number of moults and number of adhesive rings for each individual barnacle in each treatment.</p>	127

Figure number and title (shortened where necessary)	Page
<p>Figure 5.15. Percentage adhesive cover of base (mean \pm 95% CI) between treatments with a 2nd order polynomial relationship plotted, solid line = overfed (equation and R² shown above the line) and broken line = underfed (equation and R² shown below the line).</p>	128
<p>Figure 5.16. The relationship between adhesive ring width (mean \pm 95% CI) and increasing temperature, at two different feeding regimes.</p>	129
<p>Figure 5.17. The effect of temperature on the mean CRS (\pm 95% CI) of <i>B. amphitrite</i>.</p>	130
<p>Figure 5.18. The effect of changing temperature from 28 to 22 °C on <i>B. amphitrite</i> growth over time from metamorphosis (mean \pm 95% CI).</p>	136
<p>Figure 6.1. <u>Right panel:</u> Schematic representation of the bioactive surfaces utilized in this work showing the enzyme surface immobilization strategy. <u>Left panel:</u> thickness of Subtilisin A bound to PEMA films as a function of the enzyme concentration in solution used during immobilization.</p>	148
<p>Figure 6.2. Activity (filled symbol) and surface concentration (unfilled symbol) of Subtilisin A bound to PEMA films as a function of the enzyme concentration in solution used during immobilization. Values (mean \pm SD) with and without incubation in artificial seawater for 48 h are presented.</p>	149
<p>Figure 6.3. Mean settlement of <i>B. amphitrite</i> cyprids to POMA and PEMA films in the presence of 0 (control), 0.5, 1, and 1.5 $\mu\text{g ml}^{-1}$ Subtilisin A in 'Tropic Marin' artificial seawater. Settlement is expressed as a percentage of the total number of cyprids dispensed on each sample. White bars show settlement after 24 h, dark bars after 48 h. N = 6; error bars = + 95% confidence error intervals.</p>	150

Figure number and title (shortened where necessary)	Page
<p>Figure 6.4. A) Mean settlement of barnacle cyprids onto PEMA coatings of increasing activity and the corresponding denatured controls. Settlement is expressed as a percentage of the total number of cyprids dispensed on each sample. Settlement on the control PEMA coating was $43 \pm 9.5\%$ after 24 h and $63.6 \pm 7.5\%$ after 48 h. B) Percentage of cyprids metamorphosed and thereafter detached for PEMA active coatings of increasing activity and the corresponding denatured controls. The percentage of detached cyprids is calculated as the relative fraction of detached individuals to the total number of settled on each surface. The percentage of detached cyprids onto the PEMA control was 0% after 24 and 48 h. White bars show settlement after 24 h, dark bars after 48 h. N = 12 (active coatings) or 6 (denatured coatings); error bars = + 95% confidence error intervals.</p>	152
<p>Figure 6.5. Mean settlement of barnacle cyprids onto PEMA coatings exposed to 0 and $1 \mu\text{g ml}^{-1}$ Subtilisin A in solution and onto PEMA coatings with bound active and denatured (-D) enzyme. The amount of bound enzyme is equivalent to $1 \mu\text{g ml}^{-1}$. Settlement is expressed as a percentage of the total number of cyprids dispensed onto each sample. White bars show settlement after 24 h, dark bars after 48 h. N = 6; error bars = + 95% confidence error intervals.</p>	153
<p>Figure 6.6. A) Total distance moved, B) velocity, C) meander, and D) angular velocity for barnacle cyprids exposed to active and denatured enzyme coatings of different activities. Bars show means + 95% confidence error. The line connects medians.</p>	154
<p>Figure 6.7. Mean settlement (A) and percentage of detached cyprids (B) onto PEMA bioactive coatings of increasing activity and their corresponding denatured controls. Settlement is expressed as a percentage of the total number of cyprids dispensed on each sample. The percentage of detached cyprids is calculated as the relative fraction of detached individuals to the total number of settled on each surface. White bars show settlement after 24 h, dark bars after 48 h. N = 6, error bars = + 95% confidence error intervals.</p>	156
<p>Figure 6.8. Mean settlement of barnacle cyprids onto conditioned POMA, POMA + bound Subtilisin A with activity $59.2 \pm 11 [\text{pNa}] \text{ min}^{-1} 10^3$ (Active), its denatured control (Denatured), and acid-washed glass. Settlement is expressed as a percentage of the total number of cyprids dispensed on each sample. White bars show settlement after 24 h, dark bars after 48 h. N = 6, error bars = + 95% confidence error intervals.</p>	157

Figure number and title (shortened where necessary)	Page
<p>Figure 7.1. Diagram indicating the variability in shape of tubeworms such as <i>F. enigmatus</i> and the possible directions that force, using a hand held or machine controlled force gauge, can be applied (A1 and A2), and the shear force, using the flow cell, may act (B1 and B2). The number of arrows gives an estimate of the number of points of contact for force application using a force gauge.</p>	167
<p>Figure 7.2. A) Adult tubeworms on a rock surface with some damaged individuals showing the central base section without calcareous tube, B) schematic of tubeworm growing on a glass slide showing visual from underside.</p>	168

Tables

Table heading	Page
Table 1.1. Types of bond involved in the different types of chemical adhesion and their bond energy.	11
Table 4.1. Stain descriptions and protocols adapted from Horobin and Kiernan (2002).	86
Table 4.2. Effectiveness of different stains trialed.	91
Table 5.1. Equations and r^2 values for linear and 2 nd order polynomial relationships from data drawn on figures 5.4.3.2.A, B, C and D.	121
Table 5.2. The equation of regression, level of fit (r^2) and significance of the regression for power transformed cumulative number of moults over time since metamorphosis for each of the treatments tested.	122
Table 5.3. The significance levels (upper half of table and in bold) of comparisons of the linear regressions and the significance of the elevation of intercepts (not in bold) and the value of the elevation if not significantly different (in parenthesis) for the power transformed data of cumulative moults for each treatment over time from metamorphosis.	123
Table 5.4. Growth rate; area of basis ($\text{mm}^2 \text{ week}^{-1}$) compared with other <i>B. amphitrite</i> studies.	132
Table 5.5. Comparison of the moults per 100 individuals per day at different temperature and feeding regimes.	134
Table 5.6. Comparison of the present study and Johnston's (2010) CRS values in <i>B. amphitrite</i> grown at different temperatures	138
Table 7.1. Comparison of the sizes and timescales needed for testing <i>B. amphitrite</i> using the automated and manual testing methods.	163

Chapter 1: Examining the importance of environment and life stage on biofouling using a common fouling species, the barnacle *Balanus amphitrite*.

1.1. Introduction

‘Yet the evil still increased, and, like the parasite of barnacles on a ship, if it did not destroy the structure, it obstructed its fair, comfortable progress in the path of life’.

William Banting (1796-1878, Celebrity)

As the letter by William Banting to the public shows, as early as the 18th century the barnacle was known within the populace as a major problem to shipping, and while our knowledge of the Cirripedes has increased since that time, they are still a major biofouling pest and research into their biology, adhesive mechanisms and ecology continues.

Biofouling is a complex issue, both in terms of how it occurs as well as the effect it has. On natural substrata biofouling is seen as positive, ensuring a rich diversity of species and providing a link in the marine food chain (Krohling et al. 2006). In some instances this is also the case on manmade substrata (such as artificial reefs, some harbour walls and offshore structures designed for a dual function as offshore reefs (e.g. Linley et al. 2007)). However it is generally the case that biofouling on manmade structures such as on ship’s hulls and aquaculture nets, and in water cooling systems is seen as highly detrimental. The costs resulting from biofouling and consequential corrosion have been estimated to be in the billions of pounds per annum (for reviews see Hellio and Yebra 2009; Dürr and Thomason 2010; Callow and Callow 2011).

Attempts to prevent biofouling on ships were first mentioned directly in 200 B.C. when lead sheathing used to protect Archimedes ships was described (Jones 2009). Antifouling systems on wooden hulls progressed from the early use of tars, arsenic and

mercury, to copper cladding (Anon 1952, Jones 2009). Some historians speculate that Admiral Lord Horatio Nelson's victory at Trafalgar (Spain) in 1805 was, at least in part, due to the superior speed of his less fouled copper-clad wooden ships. When wood was replaced with steel, and copper cladding was ruled out due to galvanic effects, the antifouling systems again returned to toxic paints. These had varying levels of success until in the mid 1960s the highly successful (but environmentally catastrophic) tributyltin-based paints were developed (IMO 2002). By the 1970's most of the world's fleet utilised tributyltin (TBT) self polishing copolymer paints (Milne and Hails 1974). These were increasingly used during the 80's and 90's, with up to 80% of all antifouling paint manufactured at this time being TBT-based (Davies et al. 1998). With the banning of TBT in 2003 by the International Maritime Organisation (IMO 2007), antifouling systems have come full circle with a return to the use of copper, though in paints not cladding, and with additional co-biocides (Yebra et al. 2004b; Finnie and Williams 2010).

While the number of epifaunal benthic species (living on or are attached to a surface) is not known, estimates have been put forward of more than 150,000 and of these it is further estimated 10,000 species are found as fouling on manmade surfaces (Jones 2009). Many marine species, including barnacles, mussels, tube worms, sponges, bryozoans, algae (both macro- and micro-) and bacteria, attach to manmade substrata (Anon 1952; Forteath et al. 1984; Carlton 1988; Fusetani 2004; Mineur et al. 2007).

Barnacles are well represented within the group of epifaunal benthic species on manmade surfaces (Jones 2009). These sedentary crustaceans are considered to be extremely important due to their relatively large size, calcareous nature and highly tenacious attachment. In fact it has been reported that calcareous fouling of a ship's hull can result in a power penalty of 86% (Schultz 2007; Schultz et al. 2011).

Environmental factors such as temperature, food levels, flow rate, light levels, salinity and substrate can greatly affect fouling in terms of community structure, growth rates, reproduction and adhesion (Anon 1952).

Sessile marine invertebrates, such as barnacles, are frequently found in areas of high flow, and thus hydrodynamic shear. Barnacles select areas of high flow as they are filter feeders (this mode of feeding is especially suited to a static mode of life), and this is particularly successful where flow is high and thus organisms must be capable of adhering under high shear forces. Researched extensively for their strong adhesives (Tay and Pashley 2002; Nakano et al. 2007; Kamino 2008), barnacles are renowned for their tenacity.

One species of barnacle, *Balanus amphitrite* (= *Amphibalanus amphitrite*, Clare and Høeg 2008), is considered to be of particular importance in biofouling due to its cosmopolitan nature (Rainbow 1995) and ease of laboratory culture (Clare and Aldred 2009). As a result, a large body of research has been carried out using this species. Much of this research has concentrated on the surface selection and chemical settlement cues of the larval settlement stage, the cyprid (for reviews see Aldred and Clare 2008; 2009; Clare and Aldred 2009; and discussed in Chapters 3, 4 and 6). A further body of research concentrates on the structure of the adult adhesive (e.g. Kamino 2006 and Chapter 5) and the strength of adult adhesion on different surfaces (e.g. Meyer et al. 2006; Ekin and Webster 2007; Kim et al. 2007; Beigbeder et al. 2008). Indeed the testing of adult adhesion is one of the methods of novel antifouling/fouling-release coatings assessment (ASTM D-5618; Wendt et al. 2006; Rittschof et al. 2008, Conlan et al. 2008/Chapter 2). Interestingly little systematic research has been reported on the adhesives and adhesion strength of the settlement stage cypris larva (Crisp 1955; Yule and Walker 1984; Zardus et al. 2008; Larsson et al. 2010; Chapter 3 and 4) or on the effects of environmental factors such as temperature and food availability on adhesive production and tenacity of the adults (Johnston 2010; Chapter 5).

This introduction aims to describe the test species, introduce the topic of bioadhesion and biofouling research, explain the background theories and tie the varied parts of the thesis together, describing how adhesion is believed to act and the interlocking antifouling research to prevent it. The chapter introductions contain more detailed explanations of the specific areas discussed in that chapter. Each chapter is written as an individual piece of research and not linked to the others until the conclusions chapter (Chapter 7).

1.2. The test species; the barnacle *Balanus amphitrite*

1.2.1. Barnacle biology

Barnacles make up a keystone group of species in temperate rocky shores with important roles in the survival of many other species (Paine 1971; Menge 1978; Barnes 2000). They were separated from the molluscs by Lamarck (1815 and 1822) and their life history properly described and used to reinforce the barnacles' position within the Crustacea by Burmeister (1834). They were first fully studied and classified by Darwin (1851; 1854). Barnacles are marine Crustacea of the infraclass Cirripedia. This large and diverse group comprises approximately 1200 species.

The most common barnacle group, the acorn barnacles (of which *B. amphitrite* is one), is fully sessile and attaches to the substratum with an adhesive. Pedunculate, or goose barnacles, attach themselves to the substratum by means of a stalk and are capable of some small movement after attachment (Kugele and Yule 1993). Some barnacles are a highly valuable food, with the pedunculate barnacle *Pollicipes pollicipes* being a delicacy in Spain and Portugal, and the acorn barnacle *Austromegabalanus psittacus* popular in Chile (Lopez et al. 2010).

Most barnacles are suspension feeders and use modified legs, the cirri, to trap plankton and occasionally bacteria from the water column for feeding (Navarrete and Wieters 2000; Van Dover 2002). The food items can be actively brought to the cirri using rhythmic beating to create currents or, when flow rates allow, by passive extension of the cirri into the flow (Geierman and Emlet 2009). Thus the flow rate around a barnacle can be clearly seen to be important to its survival and growth, higher flows bring more food and allow more passive feeding to take place. Recent work has determined that the morphology of the cirri themselves changes in response to flow with higher flow rates resulting in shorter cirri, that may be less able to catch food, but are also less prone to damage (Marchinko 2003; Neufeld 2011; Neufeld and Rankine 2012).

While most barnacles are free-living, some show commensalism, for example with sharks, marine turtles and baleen whales, and there are a number of the group that have

diverged into parasitic forms, notably the genus *Sacculina* which parasitises decapods (Glenner and Hebsgaard 2006). Barnacles are generally shallow water animals, though there are exceptions, and many are found in the intertidal zone and are able to survive considerable periods of desiccation.

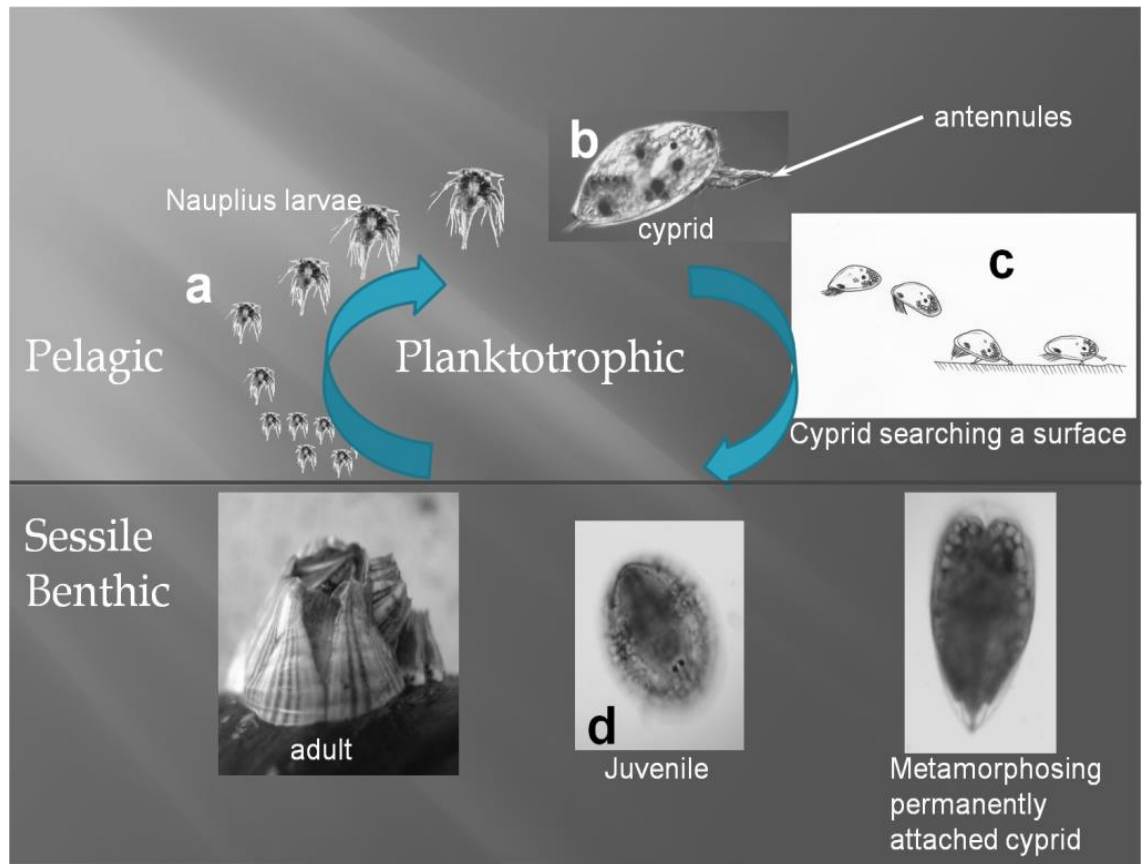


Figure 1.1. The life cycle of *Balanus amphitrite*: a) the planktotrophic nauplii are released from the adult after hatching and undergo moults until the final larval stage b) the cyprid is reached. The cyprid is able to walk in a bipedal fashion c) over the surface using its antennules and once a surface is determined to be suitable, settlement will occur and metamorphosis to a juvenile barnacle d) happens within a few hours.

The majority of sessile barnacles are hermaphrodites, though mating with nearby individuals is the normal reproductive strategy. However self-fertilisation is possible in some species (e.g. *Balanus improvises*, Furman and Yule 1990). Once eggs of the acting female are fertilised they are brooded within the mantle cavity (Anderson 1994) and hatched larvae (nauplii) are expelled into the water column. After an initial moult

the nauplii are planktotrophic (Fig. 1.1.a). The nauplii moult through four further stages to the final stage the cyprid. The cypris larva is lecithotrophic and highly specialised for travelling to, selecting and attaching to a surface (Fig. 1.1.b and c). Cyprids can swim at high speeds (e.g. 4-5 cm s⁻¹ for *Semibalanus balanoides*, Crisp 1955) allowing active selection of surfaces. Once contact with a surface is made a cyprid can either 'search' the surface to test its suitability for settlement or reject the surface and actively swim to another surface. Searching a surface involves the use of paired antennules. These highly complex organs have large numbers of receptors, in particular the on fourth antennular segments (Crisp et al. 1985). Closer study, in *B. amphitrite*, of nine setae found on the fourth segment suggested that they may each have different functions from sensing water flow and surface topography to having touch chemoreceptors and olfactory sensing capabilities (Lagersson et al. 2003). Close video observation of cyprids during searching behaviour showed that movement of these setae increases the area of a surface contacted by the antennule with each step (Maruzzo et al. 2011). If, using all of these sensory mechanisms, the surface is determined to be suitable, permanent attachment will occur.

The selection of a surface is believed to be determined by a mix of surface chemistry (including surface energy and charge; Rittschof and Costlow 1989; Graham et al. 2000; Dahlsröm et al. 2004; Aldred and Clare 2009), macro-, micro- and nano- roughness (Crisp 1974; Hills et al. 2000; Bers and Wahl 2004), biofilm (Maki et al. 2000; Burgess et al. 2003; Patil and Anil 2005), hydrodynamics (Jonsson et al. 2004), light (Walton Smith 1948; Barnes et al. 1951), colour (Dahlem et al. 1984), pheromones including those of conspecifics (Knight-Jones 1953; Clare and Matsumura 2000; Matsumura et al. 2000, Clare 2011) and from antennular secretion (Yule and Walker 1985; Clare et al. 1994; Dreanno et al. 2006a and b), as well as cypris larval health and energy reserves (Head et al. 2004; Trembley et al. 2007, Thiyagarajan 2010).

After settlement the cyprid undergoes metamorphosis to form a juvenile, uncalcified individual (Fig. 1.1.d). The juvenile barnacle is held to the surface by the cyprid attachment disc until the adult adhesive begins to be released, a process that begins 35-40 d post-settlement in *Semibalanus balanoides* (Walker 1973 and 1971). There is some argument about the existence of a juvenile adhesive that helps with the adhesion

of these young barnacles prior to adult adhesive production (Yule and Walker 1984; Crisp et al. 1985). Although there is no hard evidence for this adhesive there is a clear increase in the ‘association’ of the membrane and the substratum (Chapter 3; Crisp et al. 1985). The juvenile calcifies and grows until it reaches sexual maturity; the time required varying depending upon environmental factors such as temperature, food availability and light levels (Grave 1933; Barnes and Powell 1953; Barnes and Barnes 1956 and 1959; Bourget and Crisp 1975; Sanford and Menge 2001; Chapter 5).

1.2.2. Environmental factors affecting growth of barnacles

A large amount of work has been carried out on the nutritional needs and effects of environmental factors on adult barnacle growth and moulting (Barnes and Powell 1953; Barnes and Barnes 1956 and 1959; Grave 1933; Sanford and Menge 2001; Chapter 5). More recently, work has concentrated on the effect of environmental factors on development of larvae to the cyprid stage (Satuito et al. 1996; Jarrett and Pechenik 1997; Jarrett 2003) and the far reaching effects of larval health on juvenile barnacle growth and development (Pechenik 2006; Tremblay et al. 2007; Thyiyagarajan 2010). This research has also shown that factors which affect the growth of adult barnacles include light (Barnes 1953), immersion times (Barnes and Powell 1953), flow rates (Sanford et al. 1994), temperature (Southward 1955) and food availability (Barnes and Barnes 1959). In the field it is difficult to study these factors individually as they are frequently linked, e.g. food availability is often related to flow rate (bringing food particles to the individual) and temperature (warm equatorial zones are more productive than the mid-latitudes, and summer more than winter).

Many of the early studies into barnacle growth and moulting rates used animals at adult or near adult size taken from the field and a few used newly settled wild cyprids. As the feeding levels of the larvae affects not just the cyprid (Nasrolahi et al. 2007) but, unsurprisingly, the juvenile barnacle’s development (Pechenik 2006), using wild-collected animals introduces a high level of variability into the study. An alternative approach, using laboratory-reared individuals can minimise this variation. Using laboratory-reared cyprids, from the same larval release (from laboratory held adults) and

fed on a consistent algal food source would allow a more precise study of the effect of some of these factors on growth and moulting rates of barnacles. In Chapter 5 the effect of two important environmental factors, temperature (Southward 1958) and food availability (Crisp 1960), on growth and moulting of barnacles from settlement to adulthood are reported. The effect of temperature and food availability on adhesive production and resulting adhesive strength has not been reported in the literature and with the increasing interest in fouling-release coatings (Swain et al. 1994; Chambers et al. 2006; Sommer et al. 2010) and the concomitant increase in testing adhesion strength of adult barnacles, often grown in the field (e.g. Swain et al. 2000; Kavanagh et al. 2003; Holm et al. 2006), the usefulness of this kind of study is clear.

1.3. Adhesion in the marine environment

Many benthic marine organisms attach to surfaces using adhesives. Adhesives can be defined as the material which bonds together the surfaces of two other materials (in the case of barnacles the adhesive bonds together the base of the barnacle, or its antennules, and the substrate it has settled on). The adhesives are as varied as the organisms that utilise them and as a result our knowledge is fragmentary in nature. Many different adhesive systems have been described, some in full but most in part, and many organisms have yet to be studied in terms of adhesives. Most of the research has concentrated on a few key species in order to attempt to speculate on trends within the fouling community. This is inherently risky, as has been shown by the sudden increase in the tube worm fouling problem on coatings that have proved to be highly effective against barnacle settlement (pers. obs., Holm et al. 2000), but gaining more information is fraught with difficulties due to the very nature of the adhesives, i.e. being bound to surfaces, generally highly cross linked, insoluble, often made up of many different functionalities and relatively small in volume.

1.3.1. Adhesion Theory

There is no all-encompassing adhesion theory but rather a number of hypotheses, (termed theories within the field) to explain marine adhesion are currently proposed. Mechanical, physical and chemical forces that interact with one another are involved in bonding an adhesive to a substrate.

1.3.1.1. Mechanical interlocking adhesion

Perhaps the most intuitive of the theories, interlocking theory, states that adhesion is created when the adhesive penetrates irregularities of the substrate, locking the two surfaces together mechanically as it hardens (Fig. 1.2.). In order for this to work, the adhesive has to wet the surface and must be liquid enough to be capable of penetrating the irregularities rapidly. As well as mechanical locking, the roughness of a surface results in a larger substrate to adhesive interface allowing other interactions to take place more effectively.

This theory may explain in part the preference of some fouling organisms for particular levels of surface roughness. There are, however, hydrodynamic protection reasons behind this choice as well. A larva, or spore may derive some hydrodynamic protection from macro, or micro roughness, with the topographic features changing the hydrodynamic regime (e.g. Granhag et al. 2004). Nevertheless, as many organisms can adhere well to smooth surfaces, there must be other factors working alongside roughness.

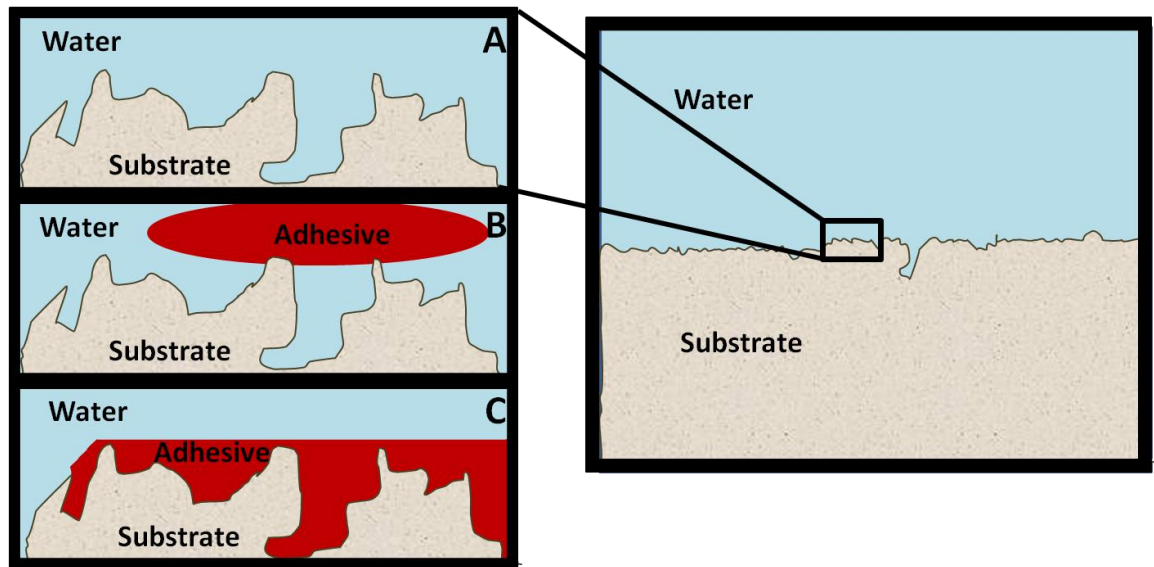


Figure 1.2. Adhesive filling irregularities of the substrate and thus creating a mechanical interlocking adherence to the substrate. A) Rough surface - scale can be nano-, micro- or macroscopic dependent on species; B) adhesive is released; and C) spreads over the surface to fill the irregularities and subsequently cures.

1.3.1.2. Chemical adhesion

Chemical adhesion theory is split into adsorption and chemisorption theory. Common forces found to act within the two types of chemical adhesion are shown in Table 1.1.

Table 1.1. Types of bond involved in the different types of chemical adhesion and their bond energy.

	Type of Bond	Bond Energy (kJ mol⁻¹)
Chemisorption	Primary	
	Ionic	600-1100
	Covalent	60-700
	Metallic	110-350
Adsorption	Donor-acceptor	
	Bronsted acid-base interactions	Up to 1000
	Lewis acid-base interactions	Up to 80
	Secondary	
	<i>Hydrogen</i>	10-25 (up to 40 with fluorine)
	<i>Van Der Waals</i>	4-20
	Permanent dipole interaction	<2
	Dipole induced dipole interaction	0.08-40
Dispersion forces		

1.3.1.3. Adsorption theory

Adsorption theory is believed to be the most important mechanism behind adhesion (Table 1.1.). Adhesion occurs due to intermolecular contact between two materials (the adhesive and substrate) resulting in surface forces developing between atoms within the two surfaces (Fig. 1.3.). Most adhesives depend on these non-covalent forces (Waite 2002). Adsorption requires the adhesive molecules and those of the substrate to be in very close proximity and to achieve this, a high degree of wetting of the substrate must occur.

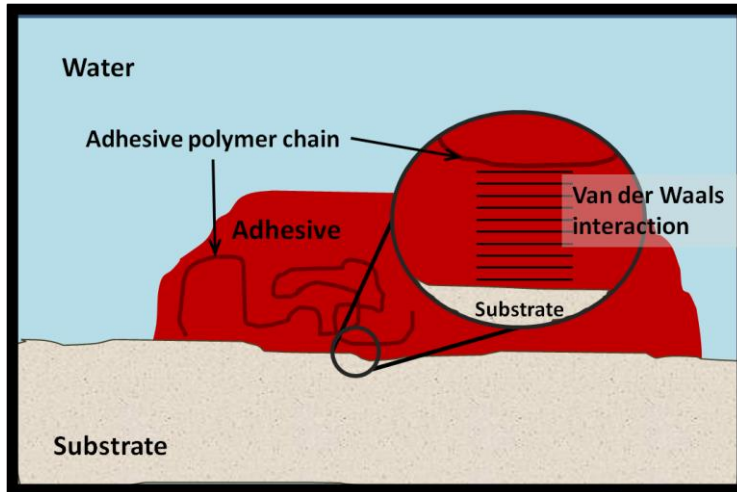


Figure 1.3. Schematic to show the action of adsorption between polymer chains that make up the adhesive and molecules within the substrate (shown as Van Der Waals interactions, but could be by any adsorption interaction in Table 1.1).

1.3.1.4. Chemisorption

Chemisorption occurs when primary chemical bonds (Table 1.1.) form across the interface of the adhesive and substrate (Fig. 1.4.). These bonds are extremely strong and contribute significantly to the strength of adhesion.

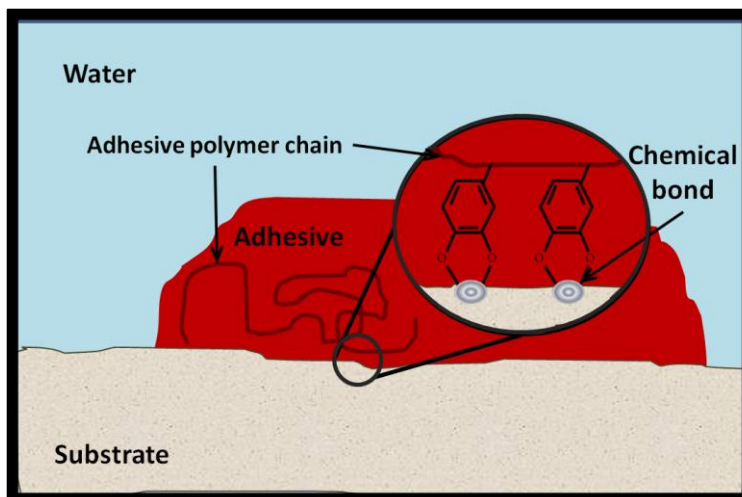


Figure 1.4. Schematic to show the bonds formed across the adhesive/substrate interface in chemisorption.

1.3.1.5. Diffusion adhesion

This theory requires both the adhesive and the substrate to be somewhat permeable to each other (generally considered only to be of relevance when two polymeric materials are involved). The molecules of each polymer diffuse across the interface and interpenetrate to create adhesion (Fig. 1.5.). This theory is of least importance in terms of natural marine adhesion as generally the substrate is not polymeric and it is often the case that the adhesive produced becomes highly cross linked, thus not allowing the polymer chains free movement and restricting diffusion.

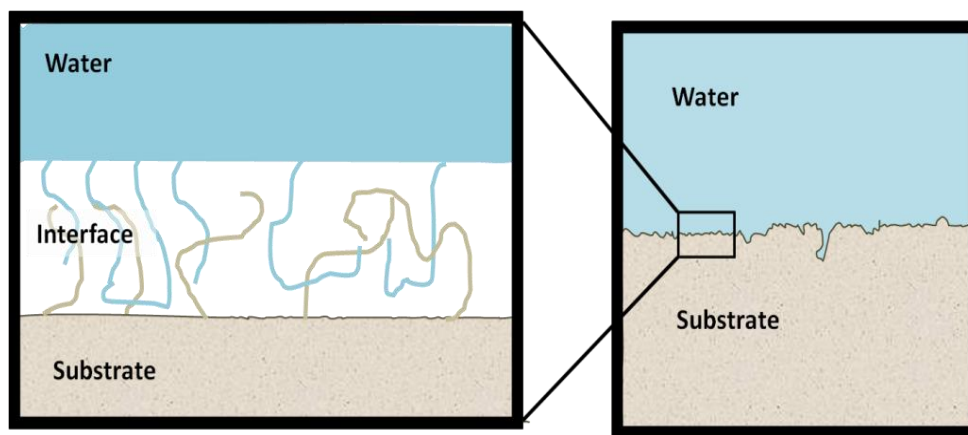


Figure 1.5. Schematic representation of diffusional adhesion theory.

1.3.1.6. Electrostatic adhesion

Electrostatic adhesion occurs when the adhesive and substrate have differing electronegativity resulting in the transfer of electrons across the interface creating different charged areas that attract each other (Fig. 1.6.).

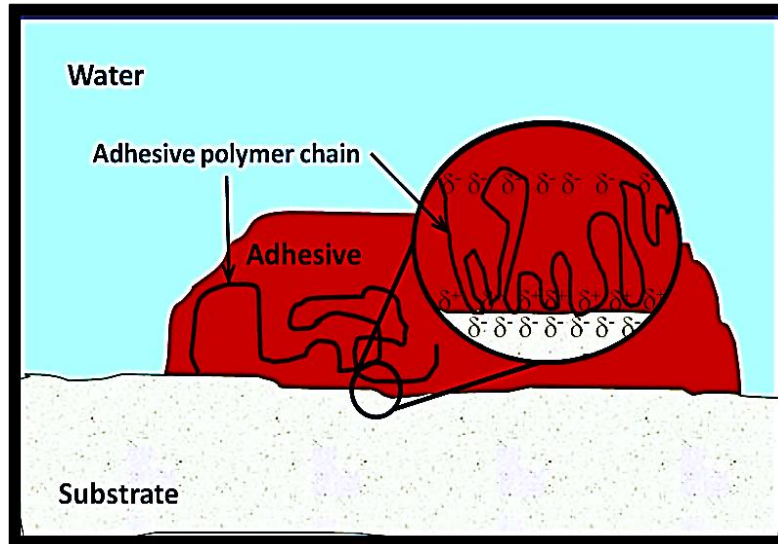


Fig. 1.6. Schematic showing charge-based adhesion explained by electrostatic adhesion theory.

With these various mechanisms in mind it becomes clear that certain criteria need to be met for a good adhesive bond to be formed. An adhesive will function best if it can spontaneously wet a substrate (and better yet be slightly soluble in the substrate although this is extremely unlikely within a natural marine adhesive system), allowing the adhesive and substrate molecules to come into rapid close contact and either adsorb or chemically bond to each other. The organism should choose a rough surface to allow mechanical interlocking, and give a large surface area for chemical adhesion. Organisms that live in adverse environments, such as marine systems with high hydrodynamic forces (e.g. the littoral zone) should use adhesives that will form covalent bonds (Fig. 1.4, Table 1.1) with surfaces as this results in the strongest bonding.

1.3.1.7. 'Dry' adhesion

Not all adhesive systems use a liquid adhesive to create an adhesive bond to a surface. Geckos, for example, do not use a liquid adhesive to affect reversible adhesion. Other animals, such as flies, use a mix of both wet (with adhesive) and dry adhesion. Some marine organisms (such as the cyprid - discussed further in section 1.3.2.5) have been hypothesised to use similar adhesive free systems (Phang et al. 2008). While these marine examples are not dry in the literal sense, as they are underwater, these are still

termed dry adhesion as they do not use a liquid adhesive and are believed to be based on the same, or similar, systems as those of true dry adhesion.

Tokay geckos are able to move rapidly up both smooth and rough surfaces and are able to walk upside down on polished glass making them an interesting model organism for adhesion studies. Indeed the gecko adhesive system has been well studied and a number of biomimetic adhesives developed from it (e.g. Lee et al. 2007). The foot of a gecko is covered in hair-like projections called setae that are tipped with spatulae (Fig. 1.7.). Each square millimetre of gecko foot is covered with around 14,000 setae and these are in turn covered with between 100 and 1000 spatulae (Hansen and Autumn 2005). These allow a very large surface area of contact for adhesion to occur. Initially it was believed that capillary adhesion was important (Huber et al. 2005) but more recent studies have rejected this in favour of van der Waals interactions (e.g. Autumn et al. 2002; Puthoff et al. 2010).

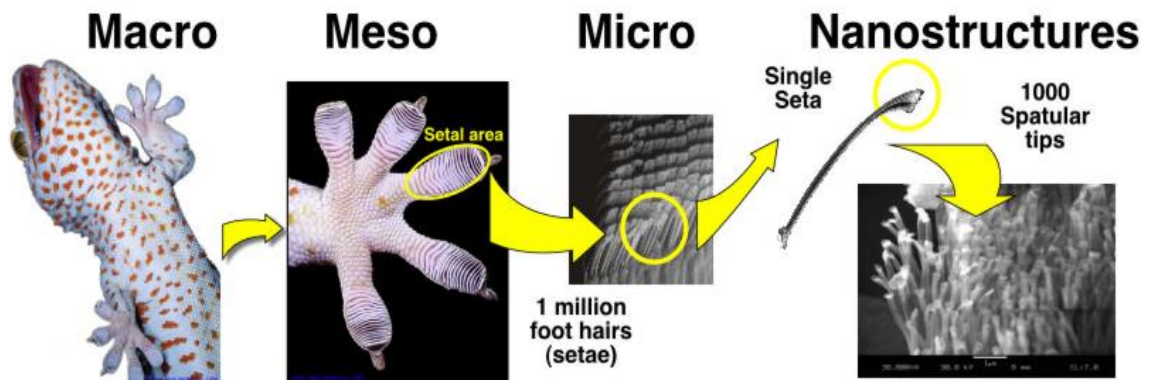


Figure 1.7. The gecko foot at different magnifications showing the setae and spatula tips that create the high surface area from van de Waals adhesion to function (taken from Biomimetic Millisystems Lab, UC Berkley website <http://robotics.eecs.berkeley.edu/~ronf/Gecko/index.html>).

1.3.2. Marine Bioadhesives

Adhesion underwater is fraught with difficulties. Water has many negative effects that must be overcome by the adhesion strategies of marine organisms. First, the difficulty in replacing the water bound to a surface with adhesive is particularly difficult for

hydrophilic compared to hydrophobic surfaces and this fact has been behind the use of hydrophilic surfaces, in the form of hydrogels, as an antifouling and FR approach (Rasmussen et al. 2002). Second, water is capable of affecting some chemical bonds as well as weakening interfacial forces such as van der Waals and acid-base interactions (Nguyen et al. 2007). Marine adhesives function exceptionally well on a huge range of surface types, despite these difficulties, and there are a tremendously diverse range of adhesives whose functionality is generally based on a mixture of the different adhesive mechanisms discussed in the previous sections.

Bacteria use three methods to make contact with a substrate: diffusional movement, active movement and convection to the boundary layer around a surface. Initially adhesion is passive but following this, active attachment occurs, either with the use of extracellular adhesives or fibrous appendages. Bacteria are known to use extracellular polysaccharides to non-specifically bind to surfaces (Costerton et al. 1985). Detailed knowledge of the polysaccharides is scarce, although it is known that primary bacterial adhesives are specialised biomolecules (Maki et al. 1990; Yun et al. 1994). However, all research to date has looked at individual isolated species and thus how the adhesives function in a biofilm is yet to be described. After attachment has occurred, individual cells become micro-colonies and these in turn grow and become a full biofilm with a highly complex nature. Bacteria can make up an important part of biofilms, which can have strong effects (both positive and negative) on the settlement and adhesion of barnacle larvae (e.g. Zardus et al. 2010).

Microalgae, along with marine bacteria and fungi form biofilms on any submerged surface. Diatoms, particularly raphid types, are one the most common marine microalgae within biofilms on new surfaces. Despite the importance of the microalgae to biofilms (particularly due to their apparent ability to colonise many of the present fouling release surfaces (Holland et al. 2004)) little is known about their adhesive mechanisms (Wetherbee et al. 1998; Domozych et al. 2007).

Diatoms are believed to make initial contact with a surface by chance, after which chemical attraction between the cell surface and substratum is involved (Lebret et al. 2009). After this initial contact, substrata can be rejected or movement to better areas on

the substratum can occur by means of secreted mucilage and gliding (Wetherbee et al. 1998) during which time individuals can be easily dislodged. However, further attachment occurs and removal is much more difficult after a period of time. This is believed to be due to the extracellular secretion of mucilage (extracellular polymeric substances, Wetherbee et al. 1998). Polysaccharides are thought to dominate, and certainly are important in many species (Hoagland et al. 1993), but the importance of proteins was implied in *Navicula perminuta* by Pettitt et al. (2004) and shown conclusively for a number of species by others (Dugdale et al. 2005; Chiovetti et al. 2006). Calcium is also important both in adhesion and motility in diatoms (Cooksey 1981). Thus the microalgae appear to be similar in their adhesive chemistry to the bacteria and the larger macro-organisms, such as barnacles (section 1.3.2.4 and 5).

In terms of macroalgae most research has been carried out using *Ulva* spp. (Lebret et al. 2009). The flagellated spore is capable of actively selecting coatings based on surface characteristics such as colour, roughness, hardness and surface energy (Callow and Callow 2002). Settlement is separated into primary (temporary) and secondary (permanent) attachment. Primary settlement in *Ulva* is expressed as a flattening of the apical region of the cell and an increase in cytoplasmic activity. Permanent adhesion, when the flagella are withdrawn and a glycoproteinaceous adhesive is secreted, occurs within one minute in *Ulva* spp. (Callow et al. 1997; Callow and Callow 2002), with the adhesive hardening about an hour later (Walker et al. 2005).

1.3.2.4. Marine invertebrate permanent adhesives

There are numerous invertebrate groups that have developed a sessile, or near sessile, lifestyle requiring the development and use of permanent adhesives. The adhesives of these sessile invertebrates are highly complex. Most of those studied to date involve a large number of different proteins (e.g. Taylor and Waite 1997; Kamino et al. 2000; Waite and Qin 2001). While the adhesive biochemical composition within taxonomic groups appears to be related, there are significant differences between groups (e.g. Flammang et al. 1998; Smith et al. 1999). Often the proportion of permanent adhesive made of protein is very high, for example more than 90% in barnacles and mussels (Waite et al. 1989; Kamino 2006). Adhesive proteins frequently show high levels of

small side chain amino acids, often polar and charged, such as in mussels (Waite et al. 1989), tubeworms (Jensen and Morse 1988), sea stars (Flammang et al. 1998) and barnacles (Kamino et al. 1996). These amino acids are thought to play an important role in the functionality of the adhesive, especially in terms of its adhesive and cohesive strength (Flammang et al. 2002).

The mussel adhesive system (Figure 1.8.) is probably the best studied. The byssus is composed of an adhesive plaque attached to the mussel by a byssal thread made up of 3 sections, the proximal (attached to the plaque) distal (central) and stem (attached to the mussel foot) sections. There are 5 foot proteins, named Mefp 1-5, with Mefp 3 thought to be most strongly involved in adhesion to a surface (Warner and Waite 1999; Yu et al. 1999). Mefp3 has particularly high levels of 3,4-dihydroxyphenylalanine (DOPA) the cross-linking of which is thought to be the driver behind mussel adhesion.

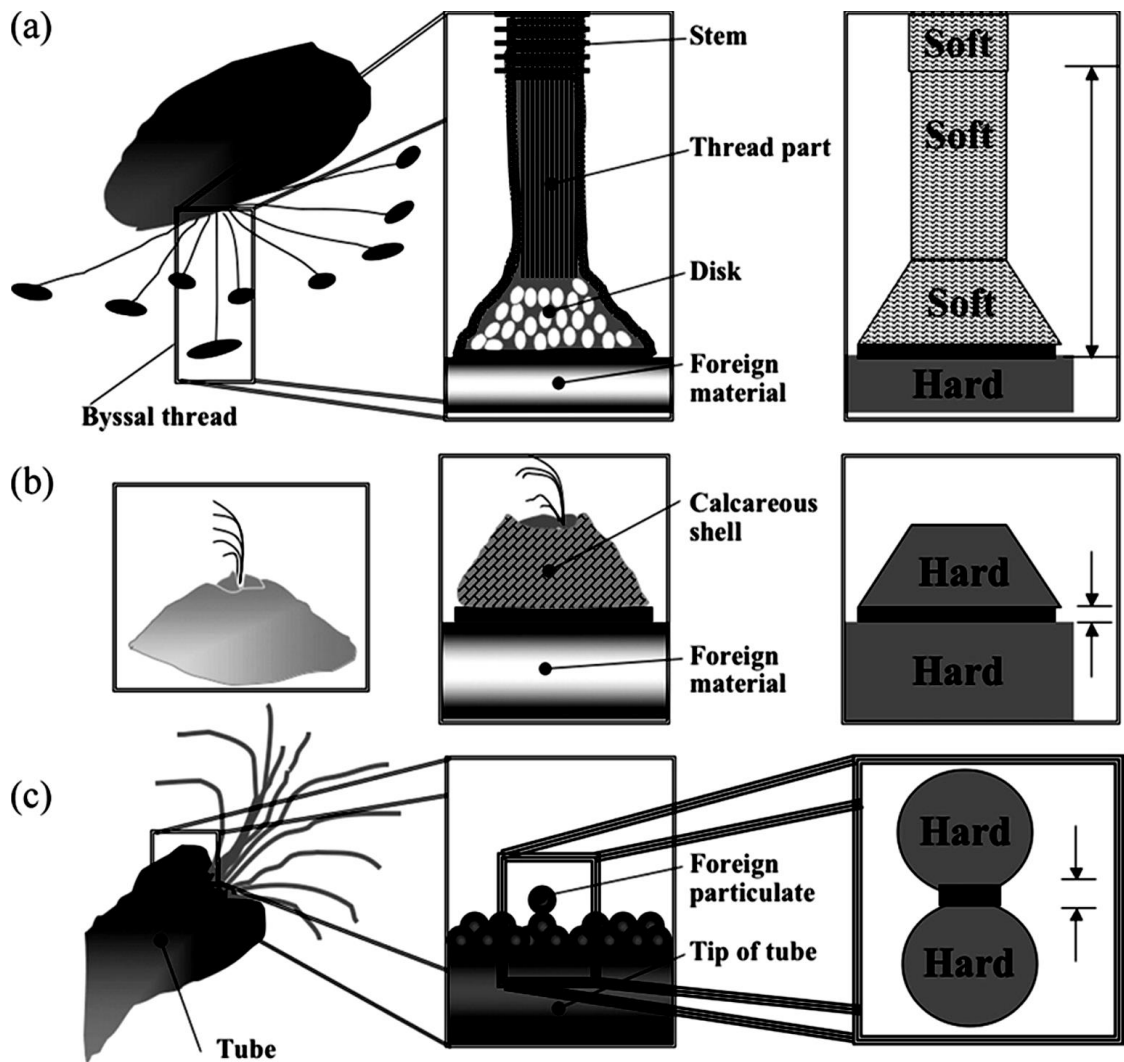


Figure 1.8. Schematic illustration of the modes of attachment of mussels, barnacles, and tubeworms. (a) Mussels make many byssal threads which act together as the holdfast of the animal, with a distance between the animal and foreign materials on the order of cm. The coupling layer at the tip of the byssal disc bonds hard matter (foreign materials) and soft matter (the mussel's own byssal thread). (b) Barnacles such as *Balanus amphitrite* attach to foreign materials by secretion of cement underneath their own calcareous basis. The cement layer on natural surfaces usually has a thickness of a few μm . Thus, the barnacle bonds two hard materials, and the distance between the two hard materials is of the order of micrometres. (c) Sandcastle tubeworms dwell in a tube made of natural particulates. The particles are bonded together via tubeworm cement to construct the tube (from Kamino 2010).

While many calcareous hard-shelled tubeworms are of increasing importance as fouling pests (e.g. *Hydroides* spp. and *Ficopomatus enigmatus*) little has been reported about their adhesives (Tanur et al. 2010). Their adhesive strategies are inferred from those of the family *Sabellarididae* which use adhesives to produce protective tube structures that protects the still fully mobile worm (Fig. 1.8.). Sabellarid cement is proteinaceous, similar in structure to a silk protein, sericin (Waite 1987; Waite et al. 1992; Zhao et al. 2005) and contains significant levels of phosphorus, calcium and magnesium (Gruet et al. 1987). The physical structure of the cement shows hollow spheroids in a matrix resulting in a microporous, foam-like structure (Stewart et al. 2004). Curing of the adhesive appears to be due to quinone cross linking (Gruet et al. 1987).

It would seem unlikely that calcareous-shelled tubeworms use the same adhesive to cement the calcareous tube to the substratum, but with no research having been published to date it would be difficult to speculate on alternatives.

Barnacles appear to use a different adhesive system to both mussels and tubeworms, as neither DOPA nor quinone tanning are implicated in adhesion.

1.3.2.5. Barnacle adhesive systems

While barnacles have been used as an experimental organism for physiologists, ecologists and evolutionary biologists (Fyhn 1974; Lopez et al. 2003), they are also frequently used as the fouling species of choice to test antifouling compounds and coatings (Swain et al. 2000; Kavanagh et al. 2003; Sommer et al. 2010) The prevention of settlement of barnacle cypris larvae, using biocides (Omae 2003; Khandeparker et al. 2005; Chambers et al. 2006) and increasingly non toxic systems (Brady et al. 1987; Swain and Schultz 1996; Swain et al. 1998; Berglin & Gatenholm 2003; Berglin et al. 2003; Sun et al. 2004), is one of the main focuses of much of the innovation and research in antifouling technology.

There are three distinct adhesives used by barnacles;

- 1) Temporary adhesive: used by cyprids to attach reversibly to a surface.
- 2) Cyprid permanent cement: released by cyprids when they select a surface to permanently attach and settle on (Fig. 1.9.a).
- 3) Adult adhesive(s) produced after metamorphosis to a juvenile and up until death (Fig. 1.9.b).

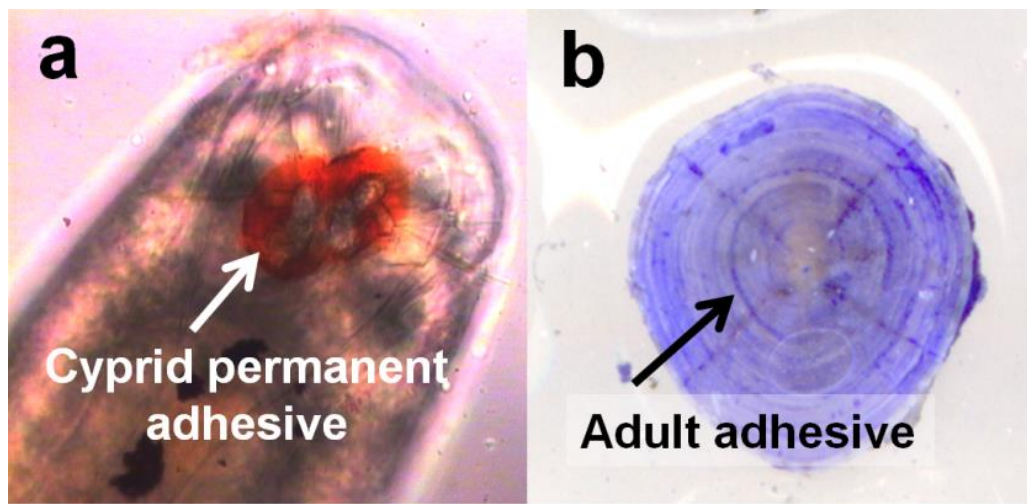


Figure 1.9. a) Cyprid permanent adhesive, or cement, stained using Congo Red; b) adult adhesive, in rings on the basis of an adult removed from silastic T2 (explained in Chapter 2, 3 and 5) and stained using Coomassie Brilliant Blue R250 stain.

The temporary adhesive (or footprint as it is known colloquially) is laid down by a cyprid when it searches a surface, by ‘walking’ in a bipedal fashion. Temporary adhesive comprises a proteinaceous secretion (Walker and Yule 1984; Clare et al. 1994a) released via two concentric rings of pores on the discs surface from a gland in the antennule (Nott and Foster 1969). This results in a trail of footprints remaining on many surfaces where a cyprid has searched. Temporary adhesion and the associated adhesive were little studied until recently. New research has begun to elucidate the features of this adhesive, notably that it is glycoproteinaceous, and either made up in part of, or entirely of the settlement-inducing protein complex (SIPC); the adult-produced settlement pheromone. Like the SIPC, temporary adhesive stimulates settlement of conspecific cyprids, lending further support to their similarity (Matsumura

et al. 1998b; Dreanno et al. 2006b). Atomic force microscopy (AFM) of the adhesive of *B. amphitrite* has revealed it to be of a fibrous nature and to adhere more strongly to hydrophobic surfaces (Phang et al. 2009). Further, the size and morphology of the footprints has been shown to vary with surface characteristics such as wettability (Phang et al. 2009). It has also been postulated that the wet adhesive for *S. balanoides* cannot explain cyprid tenacity to surfaces and that interaction between the surface and villi may also be important in temporary adhesion (Phang et al. 2008). The cyprid antennular disc is covered in cuticular villi (Nott 1969). While the role of these villi is not yet understood, the similarity of the antennular surface cuticular villi (Fig. 1.10.) and that of fly pulvilli (Fig. 1.11.) and gecko foot setae/spatulae is clear (Fig. 1.10. and Fig. 1.7.).

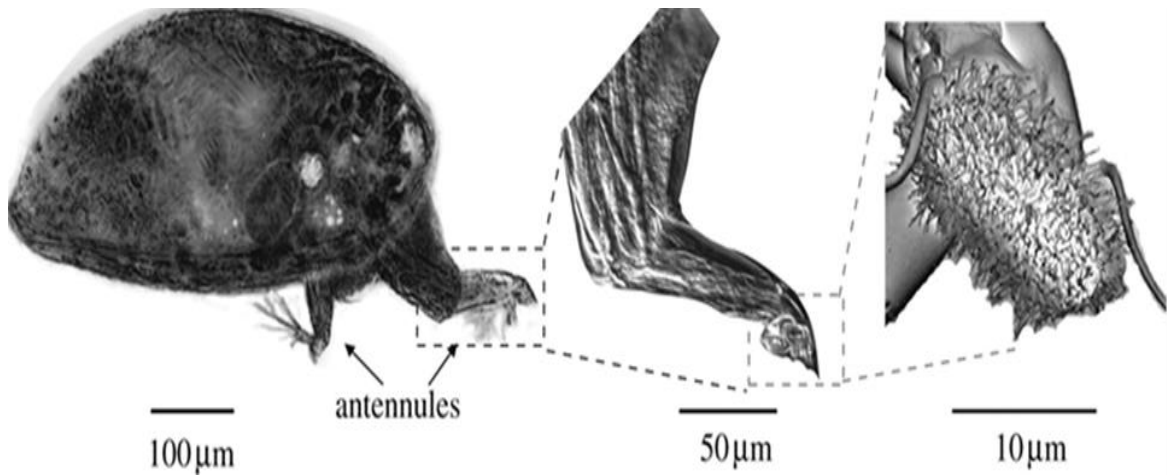


Figure 1.10. *B. amphitrite* cyprid antennules, images at increasing magnification to show cuticular villi on the antennular disc (From Phang et al. 2010).

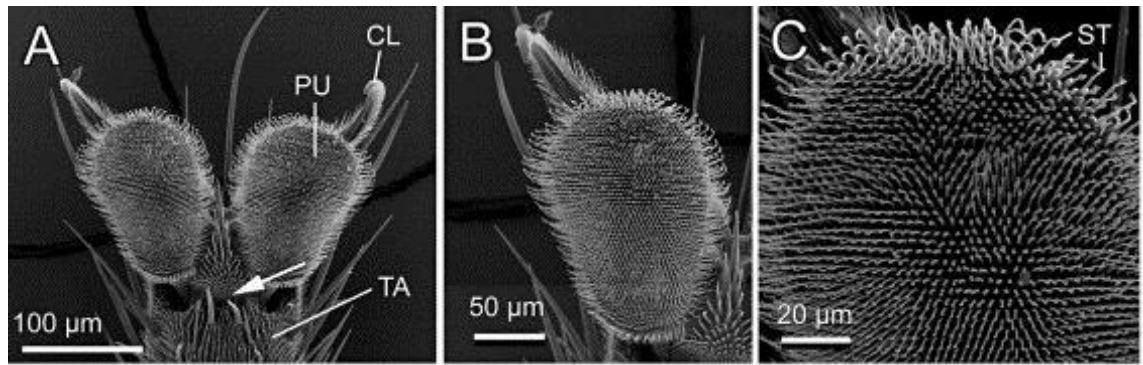


Figure 1.11. SEM of a fly (*Calliphora vicina*) foot showing the pulvilli used for dry adhesion, images increasing in magnification from A to C (adapted from Gorb et al. 2012) .

Following the searching phase, permanent attachment of the cyprid occurs if the surface is determined to be suitable. As already noted, this selection is dependent on a number of factors including light, surface chemistry, roughness, flow levels and the presence of other barnacles (Section 1.2.1.). Permanent attachment is effected by the secretion of permanent cement from the cyprids cement glands down the antennules to embed the antennules in a small globule of cement which cures over 1-3 hours (Walker 1971; 1973; 1981; Yule and Walker 1987).

The permanent cyprid cement, although initially described in the seminal work by Darwin (1853), has received little interest except in terms of the histology of cyprid cement glands within the cyprid itself (Walker 1971) and modes of release of the adhesive (Okano et al. 1996; Ödöling et al. 2006).

Walker (1971) showed histochemically that cement originates from paired cement glands, and that the final cement is likely composed of the contents of so-called α and β cells, although since questioned by Ödöling et al. (2006). The number and arrangement of these secretory cells differs significantly between species (Lacombe 1970). The two types of cell have been suggested to contain proteins, phenol and phenoloxidase in one and protein alone in the other (Anderson 1994). Anderson (1994) also reported curing occurred over 1-3 hours by means of quinone tanning. It has been shown since that the adhesive takes up to 15 hours to become resistant to a protease enzyme, suggesting the curing process could be considerably longer (Aldred et al. 2008). Walker's study

(1971) also observed that the permanent cement, once secreted, appeared stratified either due to a requirement for curing from the environment or a direct reaction with sea water. Thus the difference noted in curing time between studies could be due to extent of curing, a theory put forward by Clare and Aldred (2009) in their review of barnacle adhesives.

Work by Okano et al. (1996) has shown that the cement is produced by the cyprid by way of exocytosis controlled by catecholamine(s), the release of which is in turn controlled by the cyprid brain activating specific neurones. The release of the catecholamines results in the accumulation of cement granules in the median collecting duct of the cyprid. A study by Walley (1969) suggested expulsion of adhesive was by muscular means, and work by Nott and Foster (1969) appeared to corroborate this when they elucidated the presence of a valve at the antennular end of the cement duct. However the mechanism is not yet fully understood.

As yet little is known about the nature of the cyprid permanent cement, though it is likely to be distinct from adult adhesive, due to the lack of expression of adult cement genes at the cyprid stage (Kamino and Shizuri 1998). As most studies appear, however, to show the adult cement glands develop from the remnants of the cyprid cement gland system (Saroyan et al. 1970a) it would seem acceptable to assume the adhesive would at least be similar in mechanics. However the presence of proteins, phenols and polyphenoloxidase in the cement (Knight-Jones and Crisp 1953; Saroyan et al. 1970a; Walker 1971) has resulted in a quinone tanning mechanism being put forward (Knight-Jones and Crisp 1953).

Once permanently attached, a cyprid undergoes metamorphosis into the barnacle form and develops, grows and calcifies. After around 40 days the juvenile barnacle of *S. balanoides* begins to produce adult adhesive (Anderson 1994), which then forms a layer between the barnacle and the substrate around 5µm thick (Saroyan et al. 1970a). Barnacle adult cement is the best studied of the barnacle adhesives. DOPA, as found in mussels, is not found in barnacle adhesive which, in *Megabalanus rosa* has been shown to consist of 10 or more proteins, six of which have been characterised and are named CP (cement proteins) with a numeric addition based on their molecular weight i.e., CP-

100k, CP-25k, CP-68k, CP-20k, CP-19k and CP-16k (Kamino 2006). Three of the identified proteins, CP-100k, CP-25k and CP-68k, were shown to make up 90% of the protein complex by weight. As well as the standard adhesive compounds, the need for a trigger compound has been suggested to prevent reaction in and blockage of the antennules' cement ducts (Kamino 2006).

1.4. The problem of biofouling

Biofouling, or the bonding of biological substances or adhesion of organisms onto surfaces, is a major problem in the marine industry. It is estimated that the cost to industrialised nations due to the fouling of heat exchangers, the highest individual cost issue, is around 0.25% of their GDP (Müller-Steinhagen et al. 2005). Biofouling of offshore structures (such as oil rigs and buoys) and on the hulls of ships is together even more financially costly, estimated to be \$180-260 million per annum for the US naval fleet alone (Callow and Callow 2011). As well as this multibillion dollar annual financial cost there is an associated environmental cost due to the increase in fuel consumption and thus emissions of greenhouse gases. Thus there is an obvious economic and environmental incentive to attempt to develop novel methods to reduce, or prevent, settlement and adhesion of these organisms.

1.5. Fouling-release (FR) coatings and factors affecting their performance

The well-known, highly publicised effect of TBT paints (Beaumont and Budd 1984; Laughlin and Lindén 1985; Lee 1991) resulted in a cultural shift in the aspirations of the paint consumers, the ship owner/builders, resulting in a strong requirement for a non biocidal (non toxic), environmentally benign antifouling paint. This aim has been, at least in part, fulfilled by the development since 2000 of the FR coatings, generally based on silicones and fluoropolymers.

Fouling-release coatings work by having an ultra-smooth, low surface energy, low friction surface, as well as low modulus (or highly elastic) coating all of which inhibits strong adhesion and may reduce settlement. The polydimethylsiloxane (PDMS) that most systems are composed of is made up of an extremely flexible backbone allowing for a low surface energy configuration to form (Candries et al. 2001), as well as a low modulus. The most successful coatings utilise silicone resins with high molecular weight silicone oils, which extrude from the coating and interfere with adhesive chemical adsorption resulting in only a very weak adhesive bond being formed which easily fails, for example under hydrodynamic shear when a vessel moves through the water (Brady and Singer 2000). In fact Brady and Singer (2000) hypothesised that the best surfaces for fouling removal would be provided by thicker coatings, having lower elastic modulus and low surface energies.

One problem with these FR coatings is that they are generally prone to damage; a considerable drawback in shipping where ships' hulls are exposed to contact with piers when moored, and anchor damage if anchoring is required. Many ships' hulls also use cleaning systems to remove fouling underwater and many of these are too harsh for the FR coatings, resulting in coating damage and allowing concurrent fouling to occur. Three of the major themes running through the research behind novel FR systems are the increase in strength/hardness, the reduction in shear required to remove fouling that has accumulated during static periods, and the prevention of adhesion of species found to be resistant to the fouling release-properties of present coatings, such as micro algae (or slimes) and tube worms (e.g. Holm et al. 2000; Truby et al. 2000). As well as testing novel coatings, any change made to an existing FR coating to increase its efficacy against other species requires testing against the full range of fouling species as changes made may affect its previous FR effectiveness.

1.6. Testing biofouling solutions in the laboratory

Much of the testing of antifouling systems involves the cypris larva and employs quite simple settlement assays, utilising either coated microscope slides (e.g. Beigbeder et al. 2008), multiwell dishes (e.g. Hellio et al. 2002), small Petri dishes (e.g. Petrone et al.

2010) or large panels in the field (e.g. Bressy et al. 2010). These assays are ideal for the development of toxic and settlement preventing stratagems, as seen by their wide-scale use (for review see Briand 2009). However when novel strategies are being examined, the scale of difference in settlement required to separate two systems is large, and can hide interesting and potentially valuable results. One way to increase the ability to determine effects of novel formulations is to examine their effect on cyprid behaviour, and specifically the searching behaviour using video tracking methods (e.g. Marechal et al. 2004; Aldred et al. 2010a). This is particularly true when settlement tests utilise a number of individuals that can interact and thus affect each other's likelihood to settle (Elbourne et al. 2008).

When examining any antifouling method it would appear logical to test the initial settlement stages, i.e. the searching cyprid and the permanently attaching cyprid. However with the exception of settlement assays, this is rarely done, probably due to the higher costs associated with the purchase of equipment needed to do so. Tracking systems can cost £1000s while hydrodynamic testing equipment can cost upwards of £20,000.

While testing biocidal and antifouling stratagems using settlement assays is acceptable, this does not follow for fouling-release systems. Here surfaces are generally tested by settling cyprids onto the surface, growing them up to adult size and then measuring the adhesive force required to remove them (Swain et al. 2000; Wood et al. 2000; Kavanagh et al. 2001; Holm et al. 2006; Wendt et al. 2006; Almeida et al. 2007; Rittschof et al. 2008; Zardus et al. 2008; ASTM D-5618). With the knowledge that the adhesives and drivers for adhesion on juveniles differ from that of adults it is perhaps surprising that testing adhesion at juvenile and settlement stages has not formed the basis of the accepted testing method. As well as testing adults grown on a surface, there is an increasing amount of testing being carried out using reattached barnacles. This involves the use of adults grown on a PDMS fouling-release surface, removed intact and allowed to reattach to the test surface and the removal of these re-attached barnacles measured (Rittschof et al. 2008). There are issues with this technique in that it does not allow for long term factors, such as low level toxicity, to be observed and thus may result in the discarding of promising coatings. The technique also utilises, at least to some degree,

the ‘secondary cement’ that is believed to be slightly different than primary, normal, adult adhesive for example it may not be able to cross link fully, or contain some of the flushing compounds suggested to clear the cement ducts (Saroyan et al. 1970b). However some reports show very good correlation with results from standard adult adhesion strength tests (e.g. Rittschof et al. 2008).

This thesis attempts to answer some of the questions and issues raised within barnacle biofouling and antifouling research. It explores novel methods of testing non-toxic, and specifically fouling-release coatings and how they function when compared to existing techniques are examined in Chapters 2 and 3. Chapter 2 examines an improvement to an existing method, that of the measurement of adult critical removal stress (CRS, the force required to remove an adult from a surface). By utilising a fully mechanised system that measures the barnacle basis and the force for removal electronically it is hoped that this will remove the possibility of human error/variability. The hypothesis being that the mechanising of the method will reduce the amount of time needed to obtain data, and may allow for the more accurate measurement of CRS by removing handler introduced error. Chapter 3 examines two hydrodynamic testing systems, utilising shear and impact pressure to compare the effects of the two removal systems on different early life stages, post settlement. The aim is to determine which hydrodynamic mechanism is best suited to testing fouling release coatings, and which early post settlement life stage is most useful to discriminate between fouling release surfaces.

A novel methodology to quantify the spreading of the cyprid permanent adhesive, how it reacts to different surface chemistries and what we might infer about the adhesive from this is reported in Chapter 4. The aims of this chapter are to gain a greater understanding of the little understood cyprid permanent adhesive and to further understand the reasons behind the selection of certain surfaces by cyprids in terms of their first permanent adhesive. The hypothesis is that the adhesive cyprids use to permanently attach will determine what surfaces they select for settlement, those surfaces that the adhesive can spread and thus adhere well to will be selected in preference to those where the adhesive does not adhere well.

Chapter 5 examines the importance of environmental factors that vary so much between field and laboratory testing of coatings. The chapter aims to show the effect of temperature and food availability on the growth, moulting, adhesive production and resulting critical removal stress of adult barnacles grown under different regimes. The hypothesis being that the changing levels of food and temperature of growth will change the energy available for the production of adhesive to adhere the adult barnacles to surfaces and thus their CRS. Finally Chapter 6 reports on a possible first step towards a coating to prevent settlement and adhesion of the barnacle utilising surface-bound naturally-occurring enzyme.

Chapter 2: Evaluation of a fully automated method to measure the critical removal stress of adult barnacles.

Published: Conlan, S.L., Mutton, R.J., Aldred, N. & Clare, A.S. 2008 Evaluation of a fully automated method to measure the critical removal stress of adult barnacles *Biofouling* **24(6)** 471-481.

(all experimental work carried out by SC with assistance with determining production of force by different methods by RM)

"This is an Author's Accepted Manuscript of an article published in Biofouling [2008] [copyright Taylor & Francis], available online at <http://www.tandfonline.com/10.1080/08927010802353716> "

2.1. Abstract

A computer-controlled force gauge, designed to measure the adhesive strength of barnacles on test substrata, is described. The instrument was evaluated with adult barnacles grown *in situ* on silastic T2[®]-coated microscope slides and epoxy replicas adhered to the same substratum with synthetic adhesive. The force per unit area required to detach the barnacles (critical removal stress) using the new automated system was comparable to that obtained with ASTM D5618 (0.19 and 0.28 MPa compared to 0.18 and 0.27 MPa for two batches of barnacles). The automated method showed a faster rate of force development compared to the manual force gauge used for ASTM D5618. The new instrument was as accurate and precise at determining surface area as manual delineation used with ASTM D5618. The method provided significant advantages such as higher through-put speed, the ability to test smaller barnacles, which took less time to grow, and to control the force application angle and speed. Variability in measurements was lower than previously reported (Stein et al. 2003a), suggesting an improved ability to compare results obtained by different researchers.

Keywords: biofouling; fouling release; adhesion; barnacle; *Balanus amphitrite*

2.2. Introduction

At the end of a TBT era (IMO 2001 and 2007), developments in marine antifouling increasingly operate within regulatory frameworks that seek to prevent environmental damage and hazards to human health and well being (Bryan et al. 1986; Fent 1996; Terlizzi et al. 2001). In Europe, antifouling biocide usage is restricted by the Biocidal Products Directive (98/8/EC) and more generally falls under the 6th Environmental Action Plan (Decision N. 1600/2002/EC), and the EU Water Framework Directive (2000/60/EC). While biocide-based coatings are likely to be the mainstay of marine antifouling for the foreseeable future, there is a continued interest in developing new, non-toxic, or at least environmentally benign alternatives for the market (Anderson et al. 2003). Fouling-release (FR) coatings are important in this regard. Such coatings are not designed to prevent the settlement of fouling species, although they do have inherent antifouling activity, rather they interfere with the adhesion of organisms so that they are removed under hydrodynamic shear (Brady et al. 1987; Swain and Schultz 1996; Swain et al. 1998; Berglin and Gatenholm 1999; Schultz et al. 1999; Brady and Singer 2000; Truby et al. 2000; Wood et al. 2000; Wynne et al. 2000; Berglin & Gatenholm 2003; Berglin et al. 2003; Kavanagh et al. 2003; Sun et al. 2004).

FR coatings have been commercialised, but have a relatively small market share. A number of issues have limited their utility, including relatively high cost and ease of damage (Brady 2001; Kavanagh et al. 2001; Stein et al. 2003b; Yebra 2004; Ekin and Webster 2007). Although they can be repaired, FR coatings have a shorter in-service performance than 'traditional' biocidal coatings (Afsar et al. 2003; Yebra 2004; Weigemann and Watermann 2004). A major aim of current research is to improve the performance of FR coatings such that they are more robust and release their fouling load at lower hydrodynamic shear (Swain and Schultz 1996; Kavanagh et al. 2003; Stein et al. 2003a; Meyer et al. 2006; Ekin and Webster 2007; Kim et al. 2007; Beigbeder et al. 2008). Interest has focused on silicone and fluoropolymer coatings (Brady 2001; Brady and Aronson 2003) and in improving their FR and antifouling performance by various means including the use of silicone oils (e.g. Truby et al. 2000; Kavanagh et al. 2003; Stein et al. 2003a), tethered biocides (Al-Juhni and Newby 2006; Ruzga-Wijas et al. 2007) and surface topographies (e.g. Berntsson et al. 2000; Schumacher et al. 2007).

While larval forms are a logical target for any fouling control system, including FR coatings, the efficacy of the latter is generally determined by measuring the adhesive strength of the attached fouling organism (Swain et al. 2000; Wood et al. 2000; Kavanagh et al. 2001; Holm et al. 2006; Almeida et al. 2007; Zardus et al. 2008). Barnacles by virtue of their prominence among hard macrofoulers in the marine environment (Christie and Dalley 1987; Anderson et al. 2003) have received most attention. Early adhesion measurements on barnacles were made by applying a tensile force (Becka and Loeb 1984, Yule and Walker 1984; Swain et al. 1992) or under centrifugal shear (Dougherty 1990). The tensile method is still in use (e.g. Chisholm et al. 2007a; 2007b), but most recent studies have measured adhesion under shear (eg Swain et al. 1992; ASTM D-5618; Wendt et al. 2006; Rittschof et al. 2008).

An interest in the mechanism of adhesive failure on elastomeric coatings saw the introduction of metal or epoxy cylinders – so-called pseudobarnacles – that were used to model adhesive failure in terms of fracture mechanics (Kohl and Singer 1999; Brady and Singer 2000; Kim et al. 2007). The most obvious limitation of using pseudobarnacles is that synthetic glues are used as a substitute for barnacle cement (the natural adhesive) (Singer et al. 2000; Chisholm et al. 2007a), without a sound basis for claiming comparability. The calcified basis of real barnacles also has greater flexural rigidity than pseudobarnacles (Ramsay et al. 2008). Moreover, when grown on silicone elastomers from settlement-stage cypris larvae, barnacles may show abnormal growth and/or cement production (Berglin and Gatenholm 2003; Wiegemann and Watermann 2003; Sun et al. 2004; Wiegemann 2005; Wendt et al. 2006), which cannot be replicated with pseudobarnacles. Nevertheless, pseudobarnacles can give valuable comparative information if the limitations are understood (Chisholm et al. 2007b). Departures from pseudobarnacle release mechanics have recently been noted with comparable studies on live barnacles grown *in situ* on silicone elastomers (Singer et al. 2000; Sun et al. 2004; Wendt et al. 2006; Chisholm et al. 2007a).

Force gauges have been utilised for more practical applications, particularly field testing of FR efficacy to a standardised methodology (ASTM D5618-94; Swain and Shultz 1996; Truby et al. 2000; Kavanagh 2001; Kavanagh et al. 2003; Stein et al. 2003a;

Wiegemann and Watermann 2004; Holm et al. 2006), although departures from this method have been used (Berglin and Gatenholm 1999; Sun et al. 2004; Holm et al. 2005; Kavanagh et al. 2005; Wendt et al. 2006) ASTM D5618 determines the force to remove the barnacle and requires subsequent measurement of the basal area to determine the critical removal stress (the force normalised to the area of the barnacle's basis) (Kavanagh et al. 2001; Wendt et al. 2006). Adhesion force measurements under shear, with mechanised force gauges, have been introduced (Stein et al. 2003b; Wendt et al. 2006) but do not fully automate measurement of the critical removal stress.

High-throughput screening of FR coatings, to down select those for field testing, which is more time consuming and requires more product (Webster et al. 2007), would benefit from a fully automated system to determine critical removal stress of barnacles grown *in situ* on surfaces. In this chapter, new computer-controlled instrumentation is described (hereafter called the 'automated method') that controls the applied angle of the force (the shearing probe is kept parallel to the test surface) and the speed of advance of the shearing probe (hereafter called the push bar), measures the basal area of barnacles, and computes the force per unit area using intrinsic software. Measures of critical removal stress are made of both live barnacles and epoxy mimics and compared to those obtained with a hand-held force gauge (hereafter referred to as the 'manual method').

2.3. Materials and methods

2.3.1. Preparation of coatings

Standard glass microscope slides (76 x 26 mm, Menzel-Glaser) were coated with T2 silicone elastomer (Dow Corning). The coating was cast by weight to a thickness of 300 μm ($\pm 50 \mu\text{m}$), as determined by measurements at six points across the slide with digital callipers. These slides were used for all measurements except those aimed at examining the effect of barnacle size on critical removal stress. For these measurements, barnacles were grown on a separate batch of T2 that had been spray-coated onto glass slides to a thickness of 300 μm ($\pm 100 \mu\text{m}$).

2.3.2. Barnacle culture

Balanus amphitrite (= *Amphibalanus amphitrite*) (Clare and Høeg 2008) cypris larvae were reared according to the methodology of Hellio et al. (2004). In order to grow barnacles for testing, coatings were ‘seeded’ with day-3 cyprids (stored for 3 d at 6 °C). Approximately 50 cyprids were pipetted, in a 1-ml drop of artificial seawater (ASW, 32 parts per thousand [Tropic Marin, Tropical Marine Centre, Herts]), onto the surface of each glass microscope slide, which had been either cast or spray-coated with silastic T2 (Dow Corning). After incubating in the dark, at 28 °C, in high humidity, for 48 h, during which time a portion of the cyprids settled, the slides were rinsed gently with ASW to remove un-attached individuals. Fifteen ml of *Tetraselmis suecica* ($\sim 3 \times 10^5$ cells ml⁻¹) culture was then added to each slide, every 2 d, as food for the juveniles. After ~ 60 d, when the barnacles had reached ~ 2 mm basal diameter, they were switched to a diet of freshly hatched *Artemia* sp. (*Artemia* International LLC) and maintained at 28 °C (± 2 °C), on a 12:12 light:dark cycle, for between 6 and 13 w. By this time they had attained a basal diameter of between 3.6 and 14.3 mm. As the barnacles grew, they were thinned out to avoid contact between them, so that they had regular basal margins, and to facilitate measures of adhesion.

2.3.3. Preparation of epoxy replicas of barnacles for testing

Cyprids were allowed to settle and metamorphose on glass and then cultured to different sizes as described previously. Moulds of the barnacles were prepared using dental impression material (Extrude Type II dental medium, Kerr), ensuring that no air bubbles or deformations were present. These moulds were used to produce large numbers of replica barnacles using a two-part epoxy glass resin (International Paint Ltd); a method similar to that used by Miron et al. (1996) and Thomason et al. (1998). Approximately 0.2 mg of Coomassie Brilliant Blue (Sigma) was added to 5 ml of the resin to stain the replicas (Figure 2.1.) so that scanning and identification could be performed using the automated system’s intrinsic software. Variation in adhesive measurements was minimised by using the same batch of cyanoacrylate adhesive (Maxi Fix 9[®]) to attach

replicas to T2-coated glass slides over a short timeframe (~3 h). Care was taken to ensure that the area of adhesive matched that of the replica. This was checked after 24 h and replicas with incomplete basal coverage, or with visible defects in the adhesive, were discarded. Barnacle replicas were placed in a staggered fashion along the length of the slide (Figure 2.1.), ensuring that no two individuals would come into contact with the push-off bar at the same time.

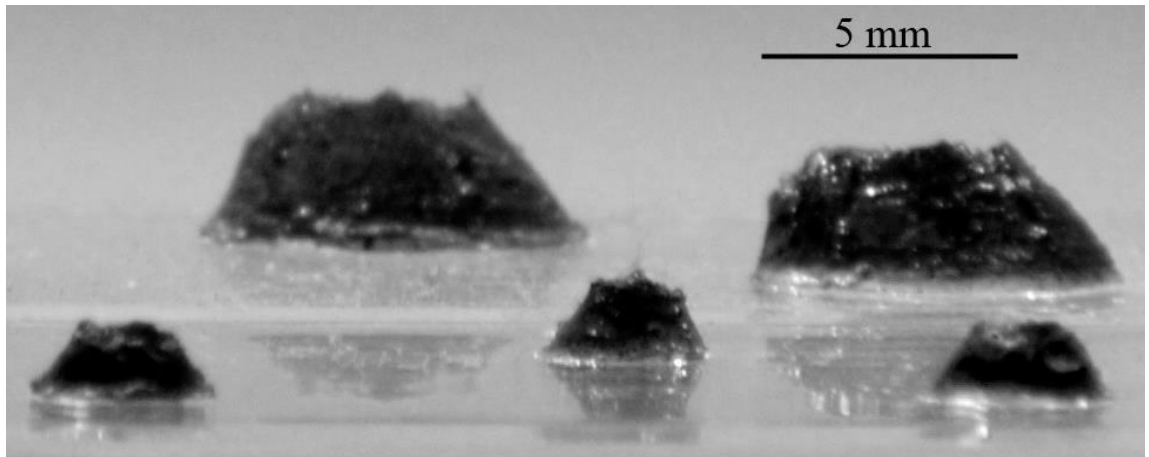


Figure 2.1. Epoxy replicas, coloured with Coomassie Brilliant Blue, of two sizes of barnacle attached with cyanoacrylate adhesive to T2-coated glass slides.

2.3.4. Measures of critical removal stress

2.3.4.1. The manual method

A hand-held spring force gauge (PSM-2K, IMADA Co Ltd 0-2 Kg F; 0-19.6N) was used to measure the force (N) required to remove a barnacle. The instrument was applied to the base of the barnacle and pushed parallel to the surface at a rate of approximately 4.5 N s^{-1} (1 lb s^{-1}). If the barnacle basis fractured so that it was incompletely removed from the coating, the reading was discarded. Barnacles were scanned (HP Scanjet 5400C) and the digital images processed using Image J software to obtain the basal area (mm^2). The precision of the basal area was determined using six barnacles and accuracy was compared using seven small to large circles of known area. Each measurement was repeated ten times. The critical removal stress (MPa) was determined by dividing the adhesion force by the basal area (Kavanagh 2001).

2.3.4.2. *The automated method*

The automated instrument (Advanced Analysis and Integration Ltd., Manchester, UK) was designed specifically for high-throughput measures of barnacle adhesive force in shear. The instrument has a camera placed above a motorised platform upon which the sample sits (Figures 2.2. and 2.3.). The camera takes an image of each barnacle, which is converted from pixels to mm² with a resolution of 0.1 mm² (Figure 2.4.A). This image can be affected by different lighting and must be carefully adapted for each surface colour to ensure accuracy, which is the time-limiting step in the method. The system ‘allows’ a maximum and a minimum image size to be set and the area of interest to be delineated, resulting in great flexibility in analysis and removal of noise. The platform moves, holding the test surface 0.1 mm above the carbon fibre push bar, the flat front surface of which (width 23 mm x height 1.87 mm) comes into contact with the barnacles and removes them. The rate of advance of the platform across the push bar can be adjusted to a constant speed of between 5 and 720 mm min⁻¹, to an accuracy of 0.1 mm min⁻¹. The force applied to remove a barnacle is passed to an electric load cell (Figure 2.3.) whose output is converted to a force (1-10 N), which is dependent upon calibrations done prior to each test.

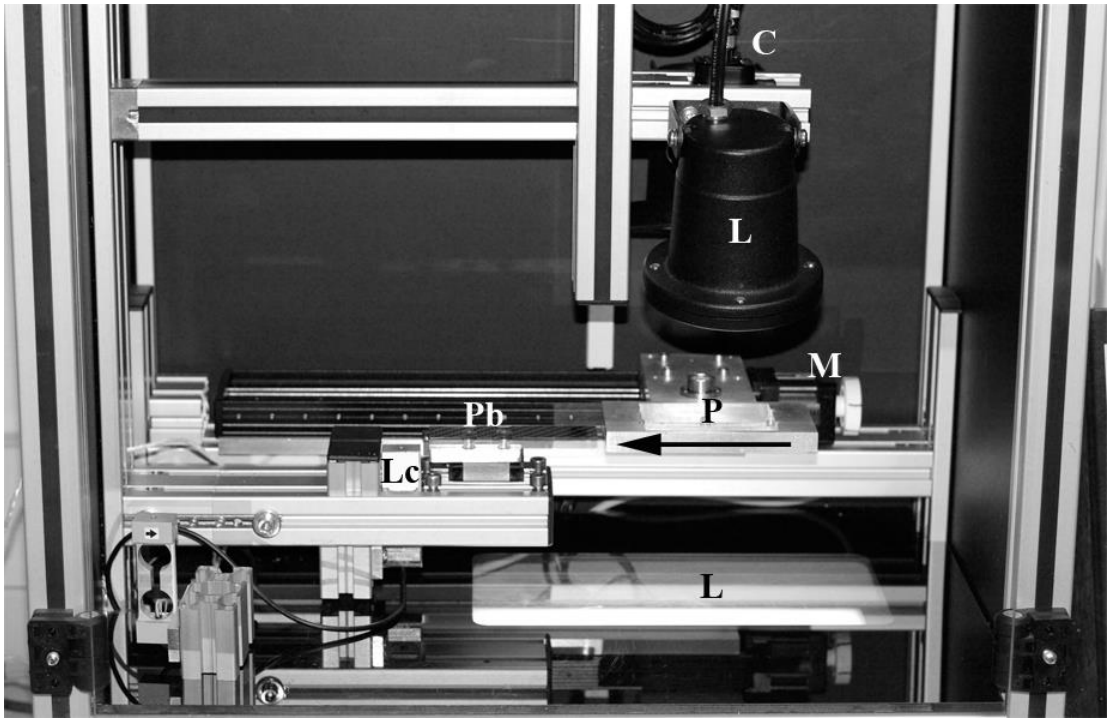


Figure 2.2. The computer controlled motorised force gauge. C, camera; L, lights; Lc, load cell; M, motor; P, platform; Pb, push bar. Arrow indicates the direction of movement of the platform which houses the slide with attached test specimens.

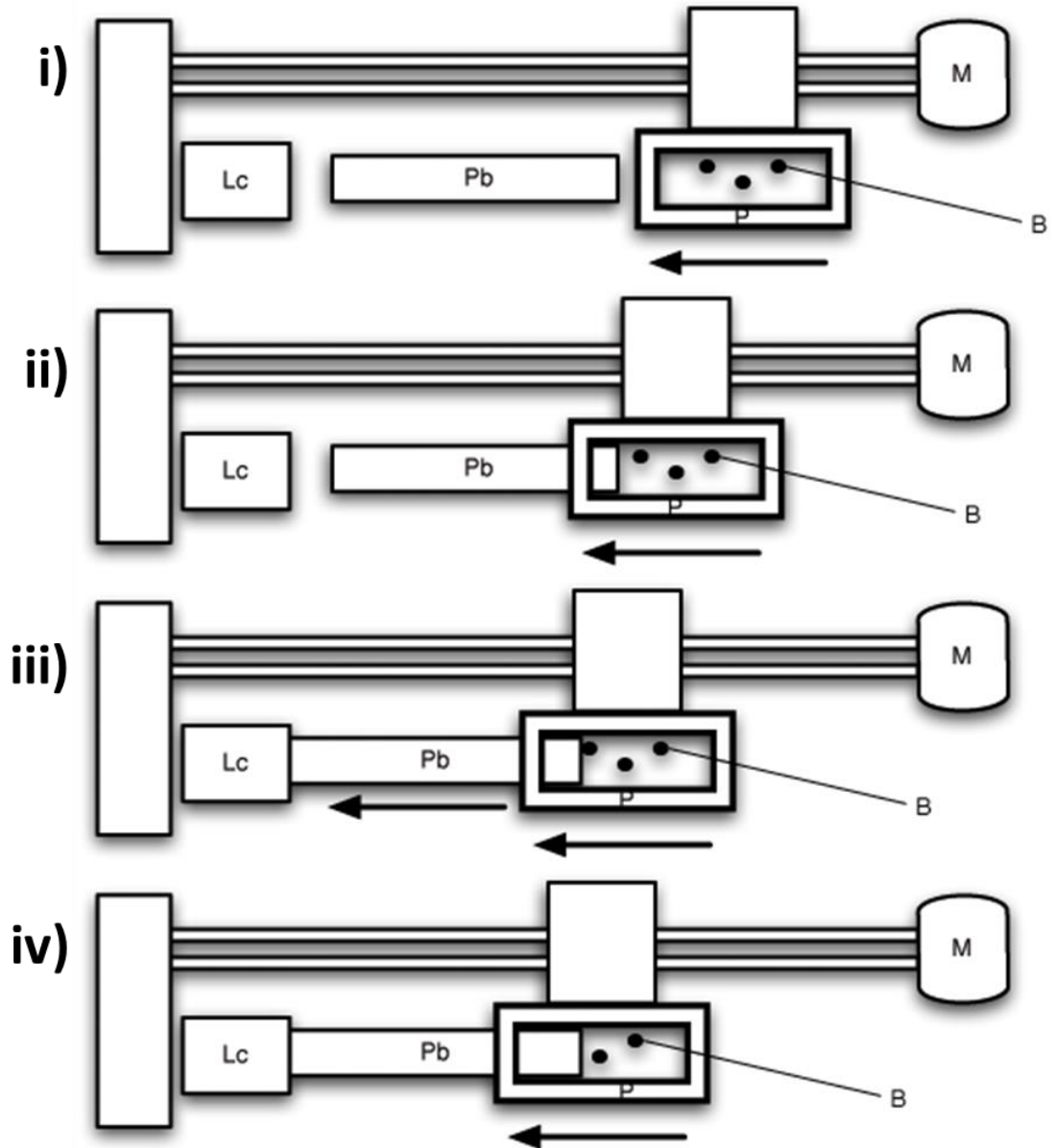


Figure 2.3. Schematic of the computer controlled system (B, barnacle; other abbreviations as in Figure 2.2.). The operational sequence is: i) the platform holding the test surface with attached barnacles advances; ii) the slide passes over the push bar, but contact has yet to be made with a barnacle; iii) the push bar comes into contact with a barnacle and is moved onto the load cell where the force is measured; iv) a barnacle is pushed off and the maximum force is measured (the push bar is no longer being pushed onto load cell).

The load cell was calibrated, prior to force measurements, using three or more standard weights (supplied by Advanced Analysis and Integration Ltd.), each measured 100 times. The mean values were used to calculate a unique offset value for every run of the machine. The computer recorded the force on the load cell in bursts of 100 readings at 10 kHz, with 40 such readings taken every second, resulting in a graphical trace of the removal of each barnacle. Differences in peak shape (Figure 2.4.B i, ii and iii) have the potential to be used for a more in-depth analysis of fracture mechanics. Automated measures of basal areas and critical removal stress employed the instrument's intrinsic software. The precision and accuracy of measures of basal area were determined as described for the manual method.

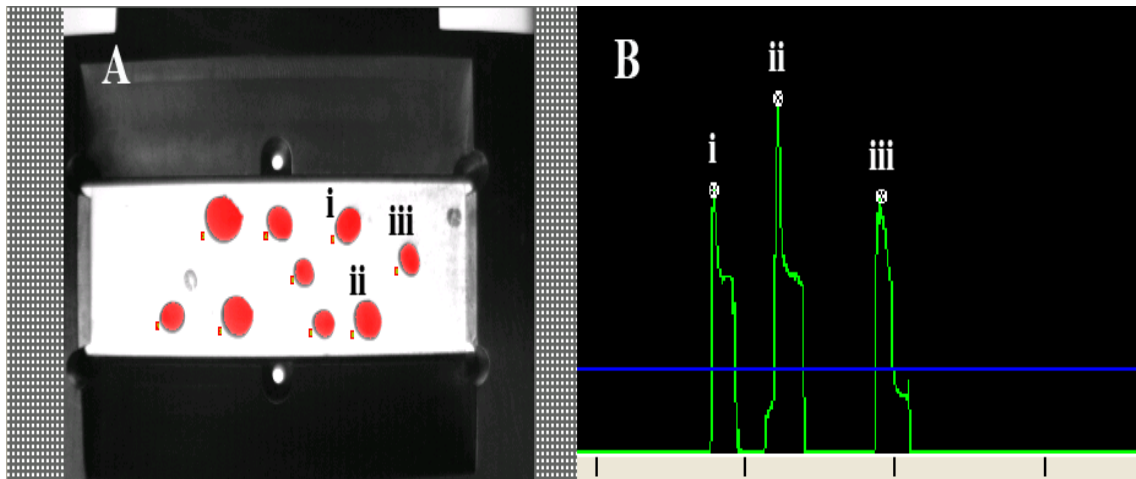


Figure 2.4. Screen grabs of A) barnacles that have been scanned and their basal areas measured using the automated methods' software and B) force traces produced, with each peak corresponding to a single barnacle removed, in this case from T2. Differences in peak pattern correspond to individual differences in release characteristics: i) barnacle was dragged a very short distance before dropping off; ii) barnacle was crushed slightly before it was fully removed; iii) barnacle was pushed off normally, but then slid across the coating before dropping off. The blue line is a minimum force (N) cut-off level set by user to allow any background noise to be removed.

2.3.5. Speed related to force applied

To relate the speed of movement of the push bar to the rate of force production, replica barnacles were attached to silastic T2-coated glass slides, as described above, and detached using the automated method. A graph of force applied over distance, produced by the machine's software, and the time taken to remove a replica barnacle, determined manually, were used to calculate the time taken to apply 1 N on the load cell. This graph only estimated the distance in mm travelled by the bar, however, and timing the rate of advance was difficult to achieve with precision. Therefore, the force generated per unit time by this method is prone to error and is expected to increase with increasing speed. In the absence of an alternative method, the measure was repeated 20 times for seven different speeds of the automated method's push off bar (5, 10, 25, 50, 100, 150 and 200 mm min⁻¹). This analysis was also attempted for the hand-held spring force gauge (manual method), but the rate of advance could not be controlled precisely by hand, especially on contact with the barnacle. Consequently only a few widely separated speeds were attempted (50, 150 and 300 mm min⁻¹), again each with 20 replicates.

2.3.6. Effect of rate of force development on removal using replica and real barnacles

Replica barnacles, of approximately 6.27 mm diameter, were produced as described and attached, using cyanoacrylate adhesive, to glass slides coated with T2. Twenty replicas were pushed off the coating using both the automated and manual methods at a range of speeds. Sixty real barnacles with a mean basal diameter of 5.01 mm were also removed from T2 using the automated method; 20 barnacles at each of three different speeds. Barnacles from the same cohort (mean basal diameter 4.80 mm) were used to test the manual method, with 15 individuals being removed at each of three approximate speeds and the average critical removal stress determined for each speed.

The automated method's rate of force development was found to be different from that of the manual method and therefore slower speeds were used – 25, 50, and 100 mm min⁻¹ – which equated to 1.18, 2.41 and 6.74 N s⁻¹. Due to the problems mentioned

previously for the manual method, only three approximate speeds could be tested, the accuracy of which was determined by repeatedly timing the bar moving over known distances. Due to the apparent slower rate of force development, however, the speeds could be faster at 50, 150 and 300 mm min⁻¹, which equated to 0.23, 1.43 and 5.1 N s⁻¹.

2.3.7. Direct comparison of the two measures of adhesive force

Two batches of cyprids, cultured from different adult populations, were each settled on a different batch of T2-coated glass slides and raised, as described above, for testing. The two barnacle groups attained mean basal diameters of 9.63 mm (test 1; n = 40) and 5.86 mm (test 2; n = 86) respectively. The force required to remove these two groups by each test method was compared, keeping the rates of force development as similar as possible to the ASTM D5618 recommendation of 4.5 N s⁻¹. All adhesive force measurements were completed within 24 h. Barnacle basal areas were determined using manual delineation (Image J) for the manual method in the first test and with the automated instrument's own software for both methods of removal in the second test. After removal, the data were converted to the critical removal stress by the operator for the manual method and using the intrinsic software for the automated method.

A *post hoc* power analysis was carried out (Cohen 1988) to determine the sample size that would be required to find different effect sizes, to 80% power, for the automated method.

2.3.8. The effect of size on critical removal stress

Cyprids from multiple cultures were settled onto different batches of T2-coated glass slides and allowed to metamorphose. The juveniles were grown on, as described previously, for varying lengths of time (1-34 w) until 500 barnacles, with basal diameters ranging from 1 mm to 8.3 mm, were obtained for testing using the automated method. For the manual method, 200 barnacles, with basal diameters ranging from 1.29mm to 7.07mm were used. Barnacles were removed using the automated method at a set speed of 90 mm min⁻¹, and at around 4.5 N s⁻¹ when using the manual method.

Data for incompletely removed barnacles were discarded. To assist in determining the testable size, all data were ranked by size and grouped into 50 individuals for the automated method and 20 individuals for the manual method. The mean removal stress and size were then calculated for each group.

2.4. Results

2.4.1. Measurements of barnacle basal area

When compared for the same barnacles, the automated method consistently delivered significantly lower estimates of basal area than by manual delineation with Image J (paired t-test $P \leq 0.001$) (Figure 2.5.). The variance of the two data sets was homogeneous (Bonferroni's test $P = 0.74$), suggesting that both methods were equally precise. The percent error for the smaller diameter barnacles was greater than for the larger barnacles and this was due to pixellation.

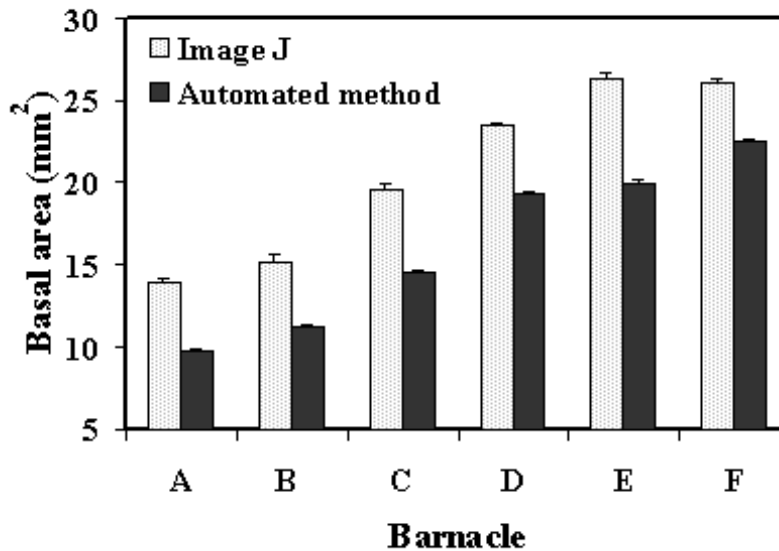


Figure 2.5. Mean basal area (+ 95% CI) for six different barnacles (A-F) measured using the automated method's software and by manual delineation using Image J.

Using precisely drawn circles of known area, the error associated with basal area estimation by both methods was quantified as a percentage of the known area (Figure 2.6.). Manual delineation introduced significantly more error than the automated method (paired t-test $P = 0.005$) and both methods lost precision with smaller areas, especially those below 12.57 mm^2 (4 mm diameter).

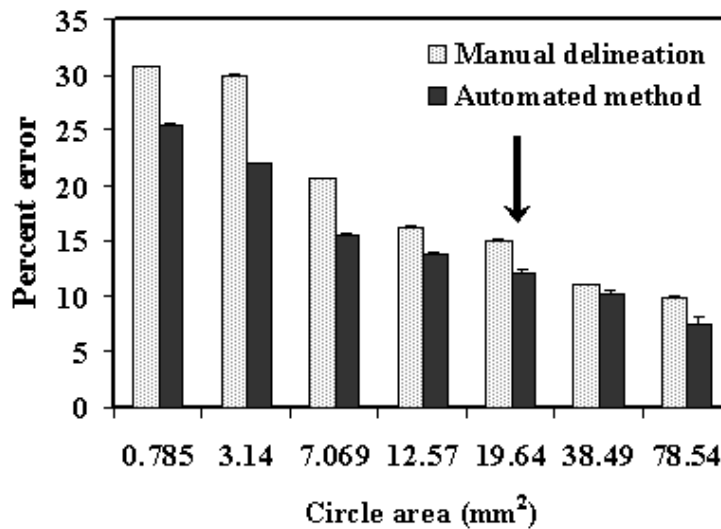


Figure 2.6. The percentage error associated with calibrated area measurement by Image J and the automated method (+ 95% CI). Arrow shows the lower size limit suggested by the ASTM (5 mm diameter).

2.4.2. Speed related to force applied

Differences were observed between the rate of force development of the manual and automated methods (Figure 2.7.) This was due to the use of a spring gauge in the former method and a load cell in the latter. The two methods resulted in trends with different slopes, with a clear second-order polynomial relationship when fitted through zero ($r^2 = 0.9948$ [automated] and 0.9931 [manual]). As previously mentioned, ASTM D5618 recommends a rate of force development of 4.5 N s^{-1} . Using the regression equation developed from the graph ($y = 0.0005x^2 + 0.0116x + 0.5825$), the automated machine reached this rate when the rate of advance of the push-off bar was 77.67 mm

min⁻¹. The corresponding rate of advance for the hand-held gauge was 280.66 mm min⁻¹ ($y = 0.00005x^2 + 0.002x$).

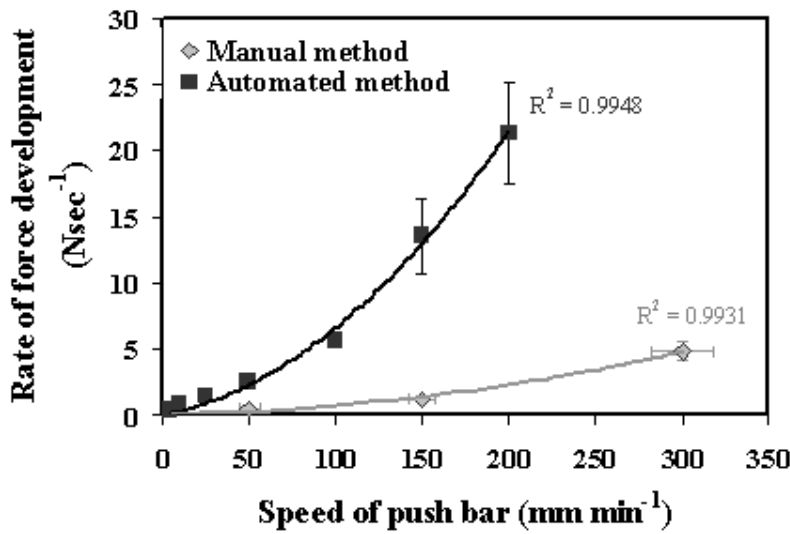


Figure 2.7. Speed of movement of the push-off bar in both methods relative to the rate of force development (\pm 95% CI). Lines fitted to show second order polynomial relationship with intercept fitted through zero.

2.4.3. The effect of rate of force development on removal using real and replica barnacles

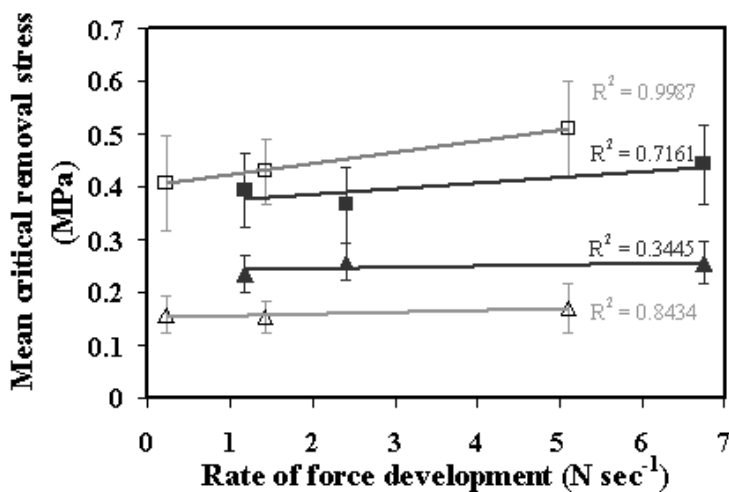


Figure 2.8. The effect of force development rate on the mean critical removal stress of real and replica barnacles attached to T2 (\pm 95% CI). ■ Replica barnacles, automated method; □ replica barnacles, manual method; ▲ real barnacles, automated method; Δ real barnacles, manual method.

Figure 2.8. suggests a positive relationship between speed and force of removal for the replica barnacle data sets. Real barnacles pushed off using either method showed a slight positive relationship (Figure 2.8.). The speed of advance did not have a significant effect for either method, whether using real or replica barnacles (one way ANOVA, $P \geq 0.23$).

The critical removal stress of both real and replica barnacles, determined by the two methods, did not differ significantly (one way ANOVA $P \geq 0.16$), and the variance of both methods was the same (Bonferroni's test, $P = 0.23$). However the replica barnacles did show significantly higher mean critical removal stress than the real barnacles, for both the automated (one way ANOVA $P = 0.05$) and the ASTM method (one way ANOVA $P = 0.03$).

2.4.4. Direct comparison of the two measures of adhesive force

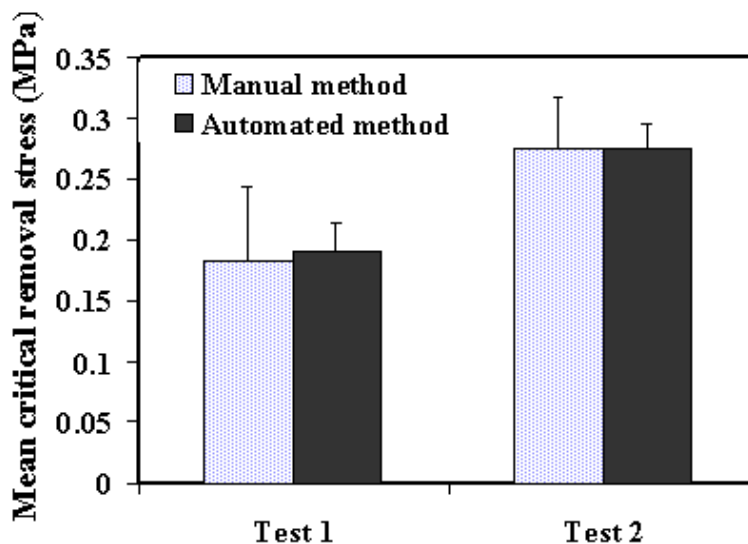


Figure 2.9. Comparison of the force for removal of barnacles obtained by the manual and automated methods (+ 95% CI).

The average critical removal stress (MPa) of barnacles obtained by the two methods did not differ significantly (Figure 2.9., ANOVA on square root transformed data $P \leq 0.007$), with the automated method recording 0.19 and 0.28 MPa and the manual

method 0.18 and 0.27 MPa for the two tests of each method. The variances of the first tests' data, however, were clearly different (Figure 2.9., Levine's test on non transformed data $P \leq 0.04$). While the variance appeared larger for the manual method in the second test, the difference was not significant (Figure 2.9., Levine's test $P \geq 0.06$). Further analysis suggested that, when tested using the automated method, the barnacles in test 2 attached with significantly greater tenacity (0.28 MPa) than those in test 1 (0.19 MPa, ANOVA $P = 0.02$); a result that was not obtained by the manual method (0.18 and 0.27 MPa, ANOVA $P = 0.06$).

The number of barnacles with an abnormal basis or cement production was not recorded in this study, although past experience in this laboratory with *B. amphitrite* has determined that the incidence on T2 is ~23 %.

Post hoc power analysis revealed that in order to obtain a significant result with 80% power (Figure 2.10.), a minimum of 11 individuals would be required for the automated method to detect a difference of 0.1 MPa between samples, while 35 individuals would be required to detect a difference of 0.05 MPa.

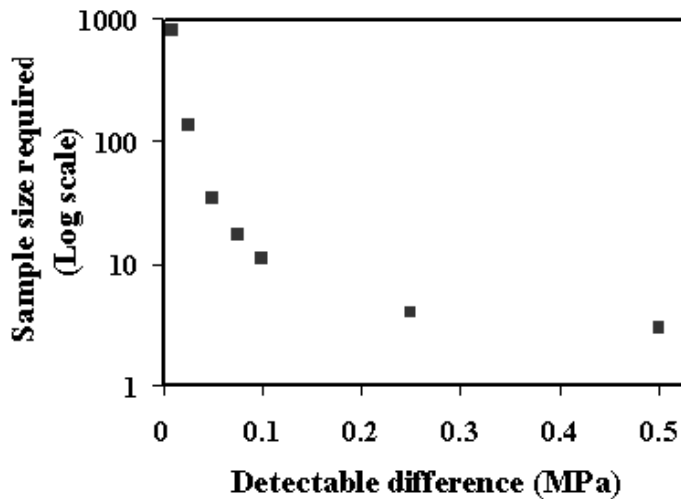


Figure 2.10. Power analysis graph for the automated method. The difference that can be discerned to 80% power and 95% confidence, is plotted against the sample size (log scale) required.

2.4.5. The effect of size on critical removal stress

Of the 500 barnacles tested using the automated method, six were removed incompletely from T2 and their data were discarded. The critical removal stress was highly variable at sizes under 6 mm^2 (diameter 2.76 mm) (Figure 2.11.a), with minimal variability between 10 and 17 mm^2 (diameter 3.57 to 4.65 mm)

Of the 200 barnacles tested using the hand-held spring gauge 11 were incompletely removed and their data discarded. The critical removal stress showed the minimal variability at a greater size, 19.5 mm^2 (diameter 4.97 mm) (Figure 2.11.b), than was found using the automated method and showed high variability at sizes under 15 mm^2 (diameter 4.37 mm). However the graphs were in all other respects broadly similar.

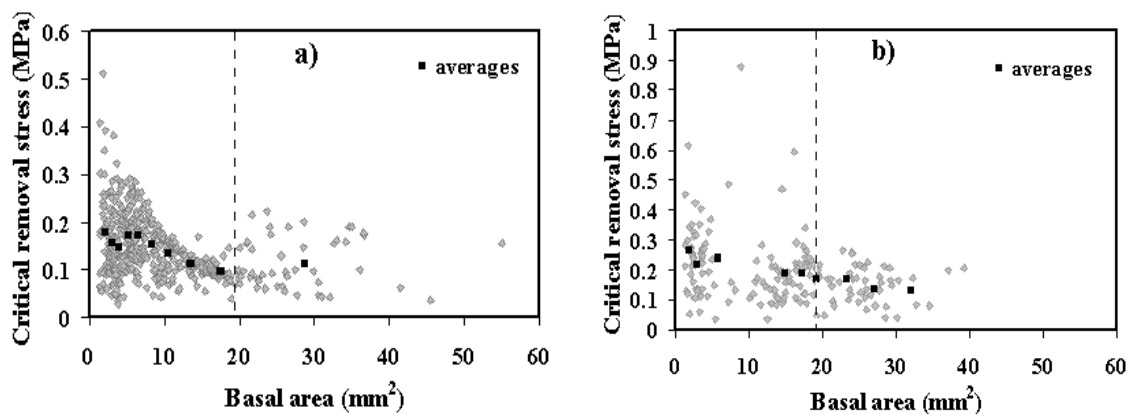


Figure 2.11. The effect of size of barnacle (basal area) on the removal stress when using (a) the automated method and (b) the manual method. The vertical line denotes the ASTM's suggested cut off size (5 mm diameter).

2.5. Discussion

This study demonstrated that the new and fully automated instrumentation developed to measure the critical removal stress of barnacles provided statistically similar data to the ASTM manual method when used in the laboratory. Furthermore, the experiments provide useful information with respect to methods used to measure basal area, the effect of rate of loading, barnacle size and accuracy.

Instruments that automate measures of the force to remove barnacles have been introduced (e.g. Stein et al. 2003b; Wendt et al. 2006) but do not automatically determine the critical removal stress, which requires that the removal force is normalised to the barnacle's basal area (Swain et al. 1992; ASTM D5618-94; Kavanagh et al. 2001). It is thus clearly important that the instrument is capable of accurate and precise measures of area. In these experiments, the automated method was found to be more precise than Image J (Figures 2.5. and 2.6.). This was especially true for relatively small barnacles because of pixellation. Therefore, when the adhesion strength was calculated there was an increase in error for the manual method. The error seen using Image J may be mitigated, in part, through the use of higher definition images to reduce pixilation in enlarged images. The method used to analyse the error of both systems may also have been biased towards the automated method, as the test involved a very clear 'black against white' simple shape measurement. Real barnacles are more complex, with shading and some barnacle species, such as *Semibalanus balanoides*, have complex basal margins, which may be delineated more effectively using the manual method. This is less of an issue with *B. amphitrite*, which has a comparatively smooth outline to the basal margin, especially if barnacles are spaced out as in this study. The instrument, of course, offers the advantage of obviating the requirement that the operator determines the basal area of barnacles, either using separate software, such as Image J used here, or from dimensions measured with callipers as detailed in ASTM D-5618, resulting in a minimum estimated time saving of 50%.

Carefully prepared epoxy replicas of adult *B. amphitrite*, removing the error associated with natural variation amongst real barnacles, were used to critically examine the operational parameters of the instrumentation. From Figure 2.7. it appears that the rate of force development increased linearly with speed of force application, at least for the automated method. The fact that this relationship was weaker for the manual method can be explained by the use of a spring scale compared to a force transducer, and to a lesser extent by the inability of the user to maintain the specified application speed exactly. In addition, the rate of force application cannot be measured by this method. In fact, the residual speed of the manual method's push-off bar, after contact with a barnacle, may be very similar for all approach speeds. Mechanising this part of the manual procedure, as has been done by several groups (Stein et al. 2003b; Holm et al.

2005; Wendt et al. 2006; Rittschof et al. 2008), would result in reduced error. The relatively large 95% confidence intervals at the higher speeds for the automated method (Figure 2.7.) are probably due to the human error in timing the advance of the gauge at these speeds; microseconds can have a large affect at speeds above 150 mm min^{-1} . It seems clear, however, that the same approach speed would manifest a much higher force development rate with the automated method (Figure 2.7.). This observation highlights the importance of precise calibration of any load cell/force gauge to ensure that its rate of force development is known prior to the commencement of testing.

It was found that the rate of force development, for ranges used in these tests, did not significantly affect the critical removal stress of real or replica barnacles, by either method. Irrespective of the rate of force development, or the method of removal used, the critical removal stress of replica barnacles was significantly higher than that of real barnacles (Figure 2.8.). This result is similar to that found by Berglin and Gatenholm (1999) and Singer et al. (2000), but the opposite of that found by Chisholm et al. (2007a). The latter study, however, used barnacles that had settled and grown in the wild and their removal was under tensile force. A comparison of the data for the automated and the manual methods, for either real or replica barnacles did not reveal significant differences (Figure 2.8.).

The difference between real and replica barnacles was most marked for the manual method (Figure 2.8.). The weaker positive relationship for real barnacles may have been due to the nine individuals that were deformed by the automated method's push bar during testing at higher speeds, presumably absorbing some of the applied force. Despite the shell damage, the barnacles were removed cleanly from the coating, with no basis remaining, so the data were not rejected. This damage was not evident with the manual method, perhaps because the bar's cross section was relatively large and accelerated more slowly. These results suggest that the rate of force development is not critical for real barnacles at the rates tested.

The direct comparison of the two methods of determining critical removal stress demonstrated their similar capability (Figure 2.9.). This was particularly true in the second test where the automated method was used to measure barnacle basal area,

removing the effect of error introduced using Image J (as is seen in test 1). Desiccation, which has been reported to impact negatively on adhesion through effects on the cement (Wiegemann and Watermann 2004), could have contributed to the differences observed between the two methods, as the manual method took longer than the automated method. However, the real barnacles were kept under ASW until just before testing and surfaces were not allowed to dry throughout the test. The larger variability with the manual method was most likely due to inconsistent operation, including speed, angle of force and height that the gauge was held off the surface.

The mean critical removal stresses for both barnacle populations and methods were similar to those reported in previous studies (Swain et al. 2000; Sun et al. 2004; Holm et al. 2005; Rittschof et al. 2008). While the variability in the critical removal stress data obtained with the manual method was similar to that reported by others (e.g. Holm et al. 2005), the automated system generally showed lower variability (e.g. Sun et al. 2004).

The effect of size on the variability of critical removal stress (Figure 2.10.) suggests that barnacles as small as 3.6 mm diameter, ie smaller than suggested by ASTM D5618, can be used for testing. This would reduce the time required to grow individuals to a testable size to between seven and nine weeks. Presently it takes at least 12 w for individuals to reach the minimum size suggested by ASTM D5618.

According to Kavanagh et al. (2005) there are a number of intrinsic problems with the manual method and different studies have offered conflicting estimates of the technique's variability. The variability can be explained by a large number of factors, including differences in growth and morphology of the test organism, mechanical factors such as force application angle and speed, and differences in the substratum. Many of the mechanical factors that introduce variability are dependent upon the technique of the handler. The computerised system can remove most of this variability and may make inter-laboratory comparisons less equivocal.

The major advantages of the manual method are its cost, relative ease of use and portability and thus great utility for field measurements of adhesion strength of fouling organisms, for which it was developed. The automated method was not designed for

field measurements but rather as a technique for high-throughput screening of coatings in the laboratory with live barnacles. The method may also be applicable to other fouling organisms, such as oysters and tubeworms (see e.g. Truby et al. 2000; Kavanagh et al. 2001). The main advantages of the automated method are the reduced time needed to test each barnacle and the ability to accurately test low numbers (Figure 2.10.) of smaller barnacles (Figure 2.11.), with additional savings in time and manpower. The method has recently been used to evaluate the effect of adding nanofillers to a silicone elastomer on its fouling-release performance (Beigbeder et al. 2008).

Chapter 3: A critical assessment of methods of testing adhesion of early settlement and juvenile stages of *Balanus amphitrite*.

3.1. Abstract

Fouling-release (FR) coatings have increasingly become an area of research and development, with the aim that an effective, non-biocidal, coating can be developed. Commercial FR coatings exist but require development in terms of robustness, efficacy at low speeds and cost. New FR coating systems are tested against one of the most economically important biofoulers, barnacles. This currently involves culturing barnacles on the surface of interest to adulthood, either in the laboratory or in the field. This process takes up to 3 months in the laboratory. Testing the early settlement stages of barnacles, i.e. permanently attached cyprids, newly metamorphosed juveniles and calcified juveniles could save considerable time and would thus be cheaper to perform.

The hydrodynamic testing methods used to date on microorganisms (i.e. water jet and flow cell) were used to compare removal rates of these early life-cycle stages with measures of the critical removal stress (CRS) of adults. The flow cell, for all early life cycle stages, was found to produce less variability amongst individuals than the water jet. Commonly used control fouling release surfaces (T2, Sylgard 184, Rhodorsil 48V750 and acid washed glass) were used to compare methods and life stages. Calcified (6-d-old) juveniles followed the same trend of removal as adult CRS tests, for both methods, suggesting that this life cycle stage would be an ideal replacement for adult barnacles. Newly metamorphosed (uncalcified) individuals showed greater variability in removal. It is hypothesised that secretion of a proteinaceous adhesive by the juvenile from ~9 h post metamorphosis, begins near the cyprid cement plaque and progressively migrates toward the periphery of the juvenile over approximately 24 h. Results thus suggest that the most reliable result can be obtained using the flow cell and calcified juveniles, although similar results can be obtained, with more variability, using the same life stage with the water jet.

3.2. Introduction

Marine biofouling, the unwanted accumulation of marine organisms on a structure or ship, has been well documented for centuries (for reviews see Hellio and Yebra 2009; and Dürr and Thomason 2010). Increasingly the economic (Champ 2000; Schultz 2007, Schultz et al. 2010 and 2011) and environmental (Townsin 2003) costs of biofouling are better understood as is the role of hull biofouling as a vector for invasive species (Molnar et al. 2008). Many different methods of preventing biofouling have been used over the centuries since shipping began, from coating with tar to self-polishing TBT coatings (Callow and Callow 2002). Antifouling technologies still primarily use toxic compounds (biocides) that generally leach slowly out of a coating to kill or deter fouling organisms as they attempt to settle (Yebra et al. 2006; Thomas et al. 1999). Settlement and toxicity assays are the methods of choice to evaluate such coatings in the laboratory (Fletcher and Callow 1992; Rittschof et al. 1992; Hellio et al. 2002 & 2004). Many of these assays are simple screens to compare biocides/coatings and reveal little about the mechanism of action. With increasing understanding of the effects some of these compounds have on the environment (e.g. Alzieu 2000; Terlizzi et al. 2001), legislation has been introduced banning some (e.g. TBT IMO 2001) and restricting the use of others (EU Biocidal Products Directive 98/8/EC; Chambers et al 2006; Srinivasan and Swain 2007). A move to non-biocidal or at least environmentally benign coatings would benefit from a better understanding of the process of biofouling, including bioadhesion, of a range of fouling species if solutions are to be broad spectrum in their efficacy.

Whilst most antifouling coatings are still dependent on biocides, including so-called booster biocides (Finnie and Williams 2010), many paint manufacturers have attempted to develop alternative non-biocidal fouling-release (FR) coatings, with some considerable success (e.g. Akzo Nobel with Intersleek 700 and 900, and Hempel with Hempasil 77500 and X3 87500). Although FR coatings have inherent antifouling activity, they are designed to interfere with adhesion of fouling organisms and to self clean when a hydrodynamic force is applied, such as when a ship is underway (Swain

and Schultz 1996; Finlay et al. 2002; Kavanagh et al. 2003; Wendt et al. 2006). Methods used to evaluate FR coatings in the development phase therefore include measures of how well adhesives, both natural and man-made, stick to the surface. A standardised method (ASTM D5618 1994) to measure adult barnacle adhesion strength, or critical removal stress (CRS) has been instrumental in this regard and has spawned a number of slight adaptations to the method to allow testing with other fouling forms and/or high-throughput measures (e.g. Swain and Shultz 1996; Schultz et al. 2000; Finlay et al. 2002; Stanley and Callow 2007; Webster et al. 2007; Chisholm et al. 2007a; Conlan et al. 2008; Zardus et al. 2008; Webster et al. 2009).

Several model organisms from different taxa, thought to be representative of fouling biodiversity (Dürr and Watson 2010; Canning-Clode et al. 2011) and modes of adhesion are used to test and develop new coatings. These include bacteria and diatoms, ‘soft’ fouling macroalgae, tunicates and hydroids, and ‘hard’ foulers such as tubeworms and barnacles. One such model organism is the cosmopolitan thoracian barnacle, *Balanus amphitrite* (Swain et al. 1998 and 1992; Holm et al. 2000; Berglin et al. 2002; Hellio et al. 2004; Wendt et al. 2006; Aldred and Clare 2008).

Barnacles are considered one of the most economically important foulers, due to their relatively large size, strong attachment and hard calcareous shell that can cut through protective coatings allowing corrosion to occur (Haderlie 1984; Schultz 2007; Phang et al. 2008). Accordingly, there has been a major effort over the past six decades to increase our understanding of the biology of barnacles and particularly how they colonise surfaces (for reviews see Barnes 1970; Walker 1995, Aldred and Clare 2008; Kamino 2008; Clare and Aldred 2009).

Testing of FR coatings against barnacles has generally measured adhesion strength of the adult barnacle after several months of growth on a surface, often in a controlled environment, e.g. the laboratory or caged area in the field. As FR coatings do not act to prevent fouling but rather release it, this is understandable, but interest in high-throughput evaluations of FR coatings and improvements in antifouling efficacy is shifting the focus to adhesion of cyprids and/or juvenile barnacles and their respective adhesives.

Measurement of cyprid and juvenile barnacle tenacity began almost 60 years ago using shear (Crisp 1955) and tensile measures (Yule and Walker 1984). The effect of biofilms on tenacity of cyprids has been recently examined (Zardus et al. 2008) and work by Larsson et al. (2010) used a 4.55 m long boat hull travelling at up to 22 knots (11.3 m s^{-1}) to generate shear to examine FR effects of three surfaces on permanently attached cyprids and juvenile barnacles. Numbers of individuals tested in both of these studies were very low compared to the present study, ≤ 27 individuals of each type were investigated. No studies have compared the effects of FR coatings on the tenacity of cyprids, newly metamorphosed and calcified juvenile barnacles using the two most common hydrodynamic testing systems, possibly due to their small size and difficulty in handling these early life-cycle stages. To date numerous methods of testing have been used for smaller organisms (bacteria and microalgae) with flow cells and water jetting apparatus appearing the most common (Swain and Schultz 1996; Holm et al. 2000; Schultz 2000; Finlay et al. 2002a and b; Holm et al. 2003; Schultz et al. 2003). However, few comparative studies on these two different methods have been carried out (Finlay et al. 2002b) and none utilising barnacles. Here the results for removal of permanently attached cyprids, fully metamorphosed but uncalcified juveniles ($\leq 36\text{h}$ post settlement) and calcified (6 day post metamorphosis) juveniles of *B. amphitrite*, on a set of standard coatings (acid-washed glass, T2, Sylgard 184 and Rhodorsil 48V750) are compared to a common adult barnacle adhesion test (critical removal stress, Wendt et al. 2006; Conlan et al. 2008) in order to determine the effectiveness of the two methods.

3.3. Methods

3.3.1. Coating production

Slides were coated with T2 (Dow Corning), Sylgard 184 (Dow Corning) and Rhodorsil 48V750 (Bluestar silicones, supplied by International Paint Ltd) using a similar method in each case. First, the slides were acid washed using a 10% nitric acid solution for 12 h and then rinsed with reverse osmosis (RO) water and air dried overnight (AWG slides

were treated in this way prior to use). Slides were then coated with a base coat of 1200OS Primer (Dow Corning; polydimethyl siloxane terapropyl orthosilicate) using a lint-free cloth. After being left overnight to dry, the slides were then coated with T2, Sylgard 184 or Rhodorsil 48V750 using a roller to thickly apply calculated weights of the coatings. Slides were left for 48 h at room temperature (~24 °C) to fully cure. Thickness measurements were then taken using digital callipers over six points on each slide. Only coatings that were at least 250 µm (\pm 25 µm) thick were used for the experiments to ensure thickness did not affect the results (Wendt et al. 2006; Kim et al. 2007). All slides were stored dry, at room temperature, in the dark in vented boxes within a fume hood for a minimum of 28 d prior to use.

After storage the slides were leached in RO water for 10-14 d, with a water change twice a week. Just prior to the settlement stage, slides were immersed in a tank of artificial seawater (ASW, 33 salinity) for 1 h and then de-wetted by being tipped to remove surface water and then placed under a cold fan for 2 min before the assay was started (this allowed water to be beaded onto the surface).

3.3.2. *Cyprid culture*

Cyprids were produced for testing from adult brood stock (sourced from Duke Marine Laboratory, North Carolina, USA) held within the laboratory in Newcastle University. Adults were cleaned and placed in clean 1 µm filtered natural seawater, kept in the dark with a cold light point source on one side of the tank. As the positively phototactic nauplii were released by the adults they swam to the light source where they were carefully pipetted, approx. every 10 min, into a beaker containing a small amount of *Tetraselmis suecica*. Once sufficient numbers were collected (approx. 10,000) they were transferred to 10 l of freshly filtered seawater with added antibiotics (penicillin and streptomycin, 21.9 and 36.5 mg l⁻¹ respectively), fed *T.suecica* at 1.25 x 10⁵ cells ml⁻¹ daily, and kept at 28 °C with a 12L:12D cycle for 4-5 days. After this time cyprids were collected using a 250 µm filter and stored at 6 °C in the dark until use. For the water jet and flow cell experiments (see below), on permanently attached individuals (PAs) and newly metamorphosed (non-calcified) individuals (NMs), the same batch of

cyprids was used in order to minimise potential genetic effects on adhesion strength (Holm et al. 2006; 2009). A second batch of cyprids was used for the calcified juvenile (CaJ) testing with water jet and flow cell and for the adult, push-off machine testing (Chapter 2; Conlan et al. 2008). Settlement on coatings occurred in the same way for all but the adult critical removal stress (CRS) tests. Around 100 cyprids (3-d-old) were placed in a 1.5 - 2 ml drop of 0.2 µm filtered ASW on the central part of each slide and incubated at 28 °C for 24 h for the water-jet and flow cell PA and NM testing, and 48 h for the CaJ testing. However, for the adult CRS testing, around 40 cyprids were allowed to settle for 48 h prior to rinsing and growing on to the adult stage.

After settlement, each slide for the PA and NM water-jet and flow cell testing was inspected and the position and stage of development (split into 3 stages, permanently attached but not metamorphosed = PA, in the final process of metamorphosing = FaM, and fully metamorphosed = NM) of each settled individual noted on each slide, hereafter referred to as localisation.

3.3.3. Adult CRS measurement

Settled cyprids on 10 replicates of each surface were grown-on post-metamorphosis for 84 d (12 weeks) at 28 °C, with a 12:12 L:D cycle. For the first 28 d juvenile barnacles were fed a dense culture of algae (~1 x 10⁴ cells l⁻¹ mainly of *T. suecica*, CCAP 66/4, but including up to 25% *Isocrysis glabana*, CCAP 927/1, and *Skeletonema* spp., Seasalter Shellfish Ltd.), after which they were fed a mix of rotifers (*Brachionus plicatilis*, CCAP 5010/4) and algae. After 42 d, barnacles were large enough to be fed on freshly hatched *Artemia* sp (Great Lakes, Artemia International LLC.). During the growing period any individuals that grew close to each other were thinned out (as per Conlan et al. 2008; Chapter 2). After 84 d adults were tested using a purpose built push off machine (see Chapter 2; Conlan et al. 2008) and the CRS of each individual measured.

3.3.4. Water jet

A water jet, based on that described by Swain and Schultz (1996, Fig. 3.1.A) and following similar adaptations to those described by Finlay et al. (2002b) was used. The new design is fully described elsewhere (Aldred et al. 2010b; Cavaco 2011). Briefly a compressed air cylinder is attached, via an inline pressure regulator, to a 20-l pressure cylinder filled with seawater. This water reservoir connects, in turn, to a motorised spray nozzle (Fig. 3.1. B) the motion of which is controlled using a computer (not shown in the figure) to spray selected areas of up to 12 slides in each run. The jet nozzle is 4.3 mm wide and is held 47 mm away from and parallel to the slide surface. The rate of movement of the jet across the slide's surface can be set (4 mm sec^{-1} used here) with the computer control system.

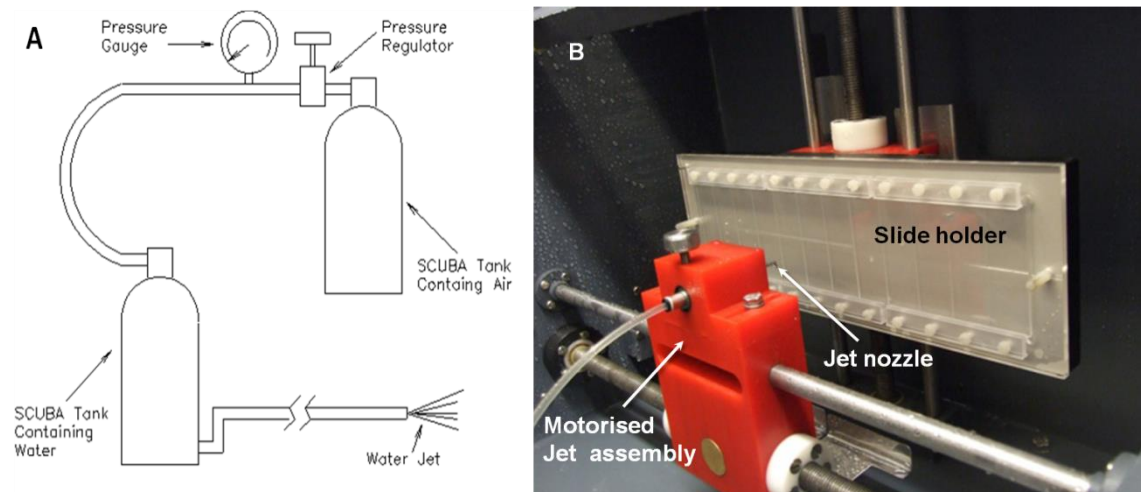


Figure 3.1. A) Diagram of primary water jet device the new system was based on (image from Swain and Schultz (1996)); B) motorised water jet of custom-built water jet apparatus (controlled by a computer not shown in the image).

The slides with settled cyprids on were tested in the water jet within 4 h of localisation (except in the case of the CaJ individuals where 24 h had passed). For PA and NM individuals, twelve replicate slides of each of the four surfaces were tested at four different impact pressures (26, 38, 50 and 62 kPa). After testing, slides were stored at 6 °C for 12 h and again localised, allowing the number of individuals removed to be determined. ‘Removal’ was separated into those that were cleanly removed and those that were removed but some, or all, of the cement plaque and/or antennules remained on

the surface (Fig. 3.2.A); often high magnification (up to x200) was needed to determine this. These individuals were termed cohesive failures for all, though it is accepted there may be adhesive failure between the individual and its adhesive, and also failure of the antennules (Fig. 3.2.A), resulting in visually similar amounts of adhesive remaining behind.

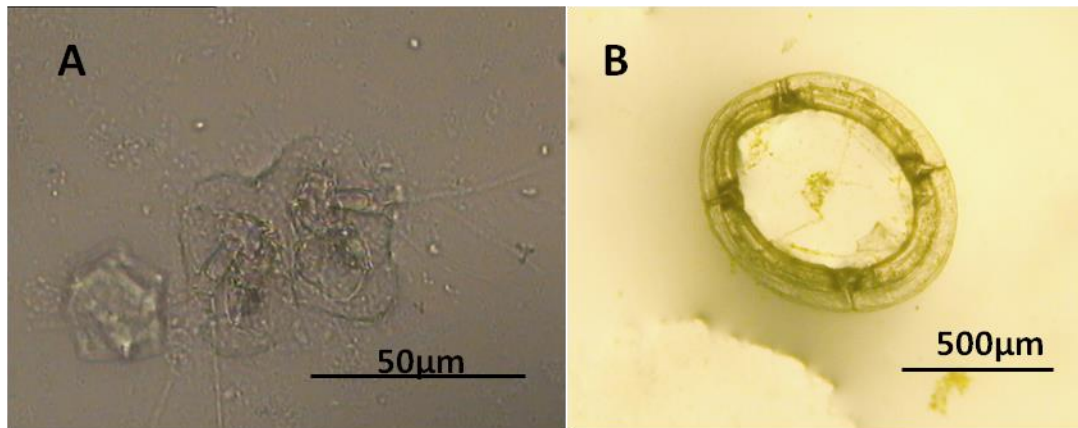


Figure 3.2. Individuals not cleanly removed using water jetting. A) Cement plaque remaining after water jetting a PA individual (the antennules are clearly visible); B) shell of a CaJ individual post testing.

Individuals for CaJ testing were allowed to settle as above but then grown for 6 d at 28 °C, during which time they were fed *T. suecica* twice at $\sim 1 \times 10^3$ cells ml⁻¹, and were calcified prior to being water jetted. Higher forces were expected to be required to remove these individuals and as a result the range of impact pressures tested was increased to 26, 50, 74 and 97 kPa. Slides were again stored at 6 °C for 12 h prior to repeat localising. Only individuals that had been cleanly removed were counted. Any that had been removed from their calcareous shell were noted (Fig. 3.2.B), but not counted as removed.

3.3.5. Flow cell

Testing was carried out using a flow cell based on that described by Schultz et al. (2000), but designed and built to generate higher wall shear stresses of up to 250 Pa. The flow cell design, calibration and computational fluid dynamics (CFD) models of the

measuring section have been reported previously (Politis et al. 2009). Briefly, the flow cell is an open water system. The inlet is attached by way of a 2-D contraction to the testing section, measuring 2300 mm long by 250 mm wide and 10 mm high, which exits to a discharge tank (Fig. 3.3.A).

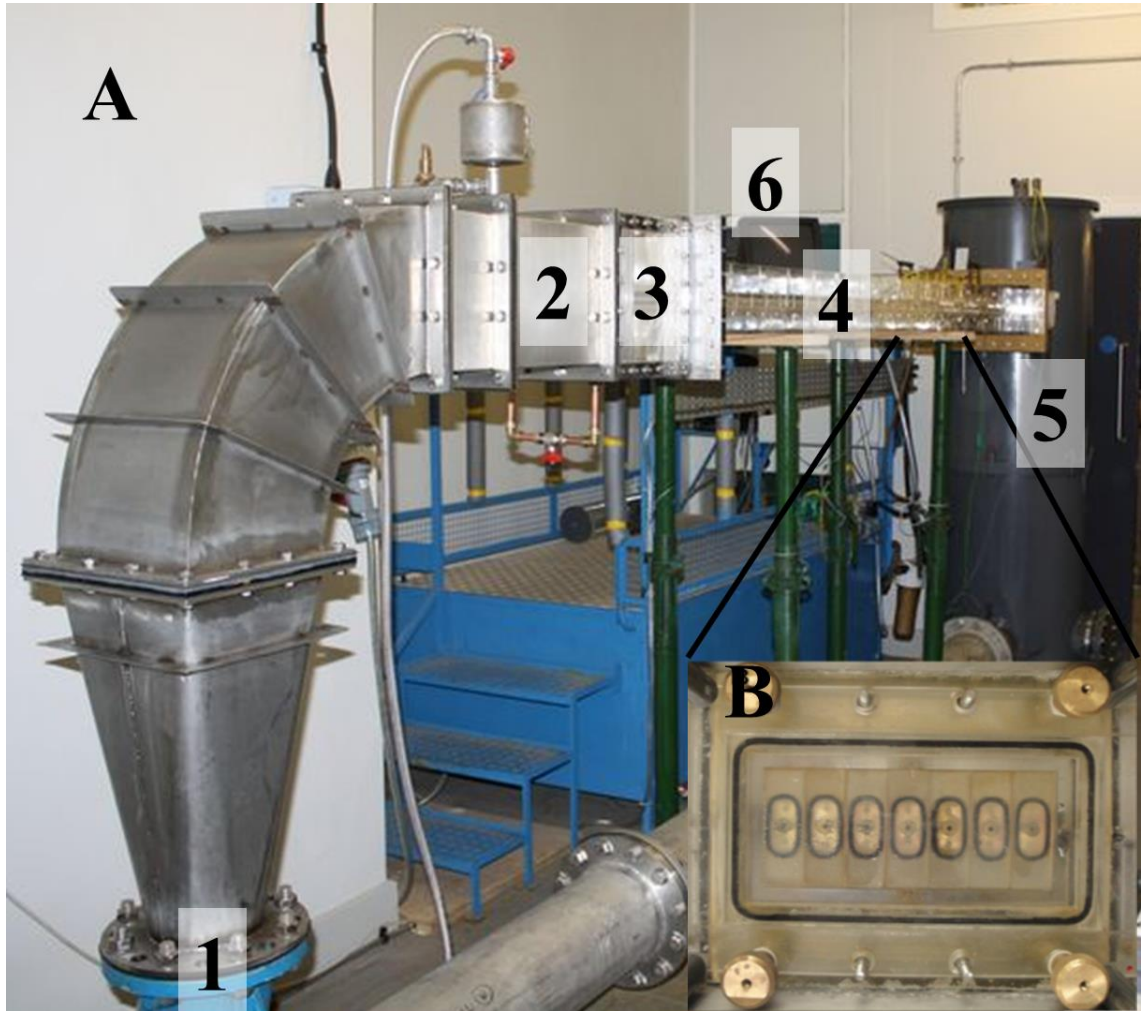


Figure 3.3. A) Photograph of the flow cell with B) close up of the slide test area. Seawater is pumped (1) through baffles (2) and a contraction (3) into the Perspex test section (4) where fully turbulent flow is reached at the slide test area (B). Water then passes to the holding tank (5) ready to recycle around the system. The process is controlled by the computer system (6).

Flow management upstream of the testing section was controlled using de-swirl plates at the pump discharge (perforated plates with 44% open area ratio (OAR) and three honeycomb screens with 60, 50 and 40% OAR). Turbulence at the inlet of the testing

section was generated using sand roughness strips (Grit #40). CFD results for the flow cell design show a satisfactory correlation with two other flow cells (Fig. 3.4., Dean 1978; Schultz et al. 2000).

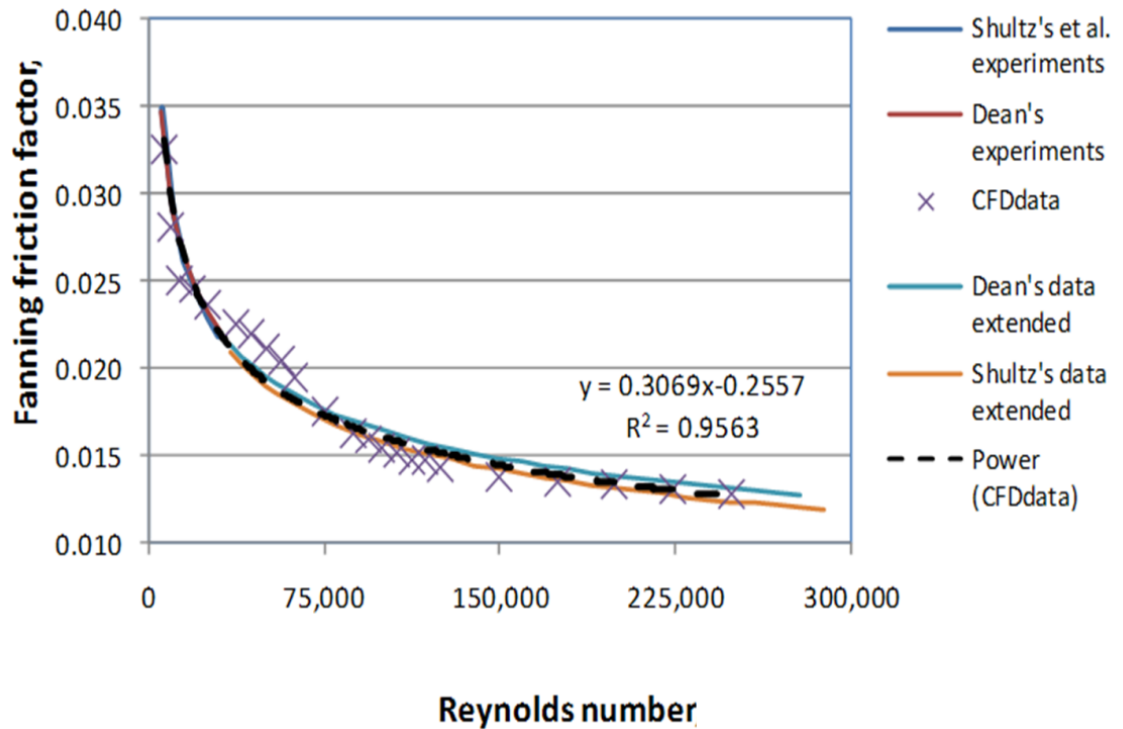


Figure 3.4. Comparison of CFD data from Newcastle University's flow cell with that of Schultz et al. (2000) and Dean (1978), (adapted from Politis et al. 2009).

Samples for testing were located 2000 mm downstream of the turbulence production (at 1600 mm flow was fully developed). The flow cell can test up to 14 microscope slides (seven on the lower surface and seven on the upper, Fig. 3.3.B) or two plates (185mm x 76mm) per run, held in place by a vacuum.

The testing section is made of transparent acrylic allowing the detachment of organisms to be observed during a run. This transparency also allows for the measurement of detailed flow characteristics using laser doppler velocimetry (LDV, Fig.3.5.A). These measurements can be used to convert to ship wall shear stress values (Fig. 3.5.B).

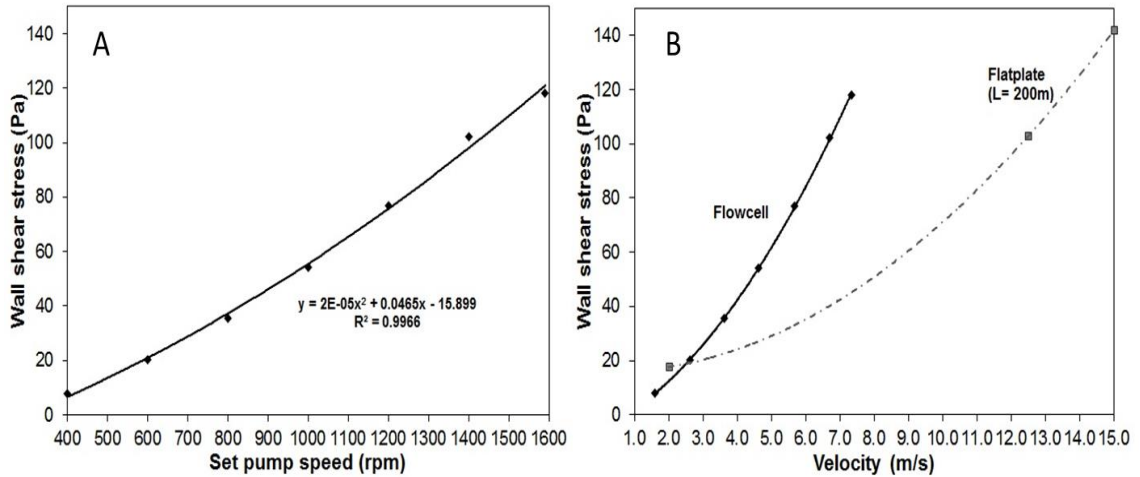


Figure 3.5. Relation of measured wall shear stress (Pa) in the test section of the flow cell with A) varying pump speed (rpm) and B) flowcell data related to CFD simulation on a 200 m flatplate (flatplate data adapted from Politis et al. 2009).

Ship wall shear stress data (Fig. 3.5.B), based on CFD simulations on a 200 m long flat plate (fully submerged in seawater), taken at mid span, show that a flow velocity of 8.6 m s⁻¹ corresponds to 13.7 m s⁻¹ at full scale (around 30 knots at 160 Pa; Politis et al. 2009). The present facility was designed to reach 250 Pa (with adjustment) so it would be relevant to vessels built or planned into the foreseeable future, including naval vessels.

Slides were treated as with the water-jet and settlement and localisation carried out in the same way (sections 3.3.1 and 3.3.2). A number of runs were carried out on 12 replicates of each coating type, at each wall shear stress (16, 48, 65, 88 and 117 Pa), exposing the different settlement stages to flow for 5 minutes.

3.3.6. Metamorphosis development over 31 h post settlement period

It was noted during testing, with the water-jet in particular, that removal rate of NM individuals was generally higher than for CaJ individuals, and also that the variability of removal was higher compared to CaJ individuals. NM individuals varied in appearance for a period after metamorphosis. They were attached by the cyprid cement disc only (Fig. 3.6.A) and, if viewed from the side, could often be seen to have space between

their bases and the substrate. After a period of time the membranous basis of the juvenile appeared to begin to adhere to the surface starting near the cyprid adhesive disc (Fig. 3.6.B); it has been suggested that this is effectively a distinct adhesive previously termed 'juvenile adhesive' (Yule and Walker 1984). Over time the area of 'attachment' increased, moving towards the outer edge of the NM (Fig. 3.6.C to E). Until the membrane is fully adhered to, or at least in contact with the surface, staining of the basis with dyes such as Congo Red used here is possible, but thereafter it is ineffective (Fig. 3.6.F). It was hypothesised this adhesion, or contact, of the membrane may, at least in part, explain the greater variability seen with NM individuals. To test this hypothesis a time-series experiment on settlement was carried out. Cyprids were allowed to settle onto 60 glass slides over 34 h, and settlement and metamorphosis of each individual was noted and localised every hour, such that the time of settlement (within 1 h) was known. Periodically a proportion of the slides were stained using Congo Red utilising the protocol below and stored at 6 °C (up to 48 h) until individuals bases could be photographed. Photographs were taken using an inverted microscope (Olympus CKX41 with Sanyo digital CCD camera, VCC-659P, mounted to obtain still images using Pico EasyGrab v5.6 software). This allowed images of individuals to be obtained with ages from settlement and metamorphosis, accurate to within 1 h, up to 31h post metamorphosis. The individuals were grouped into hourly batches (only data from time points with more than 15 individuals are presented) and the percentage showing this membrane, or adhesive, staining calculated.

Congo Red staining protocol

1 g of Congo Red dissolved in 500 ml NaCl solution (60 g NaCl dissolved in 600 ml RO water). When the stain was fully dissolved 800 ml of ethanol was added. Just prior to use 50ml of this working stock was mixed with 0.5 ml of a 1% NaOH solution and filtered through glass wool. Slides were stained for 30 min and rinsed well with seawater.

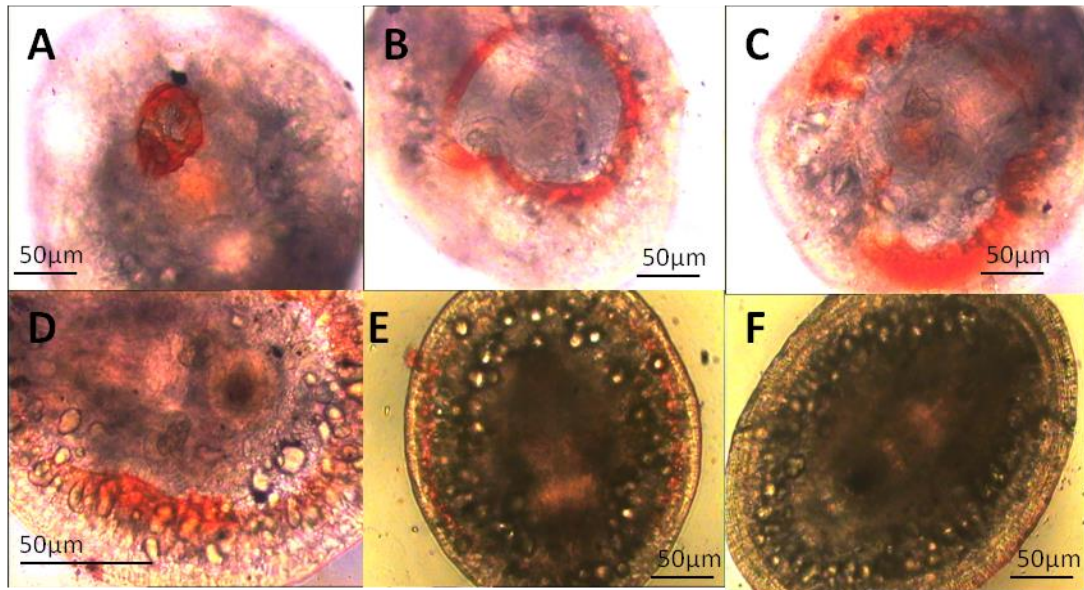


Figure 3.6. Congo red staining of the bases of newly metamorphosed juvenile barnacles over time; A 4h; B 9h; C 15h; D 18h; E 24h; and F 31h.

3.3.6. Analysis of data

All percentage data were converted to proportions and arcsine transformed prior to statistical analysis (Zar 1999). Normality and equal variance were tested using Anderson Darling and Levine's tests and ANOVA was used to compare the mean removals for each population tested, the null hypothesis being that all populations would show the same mean removals.

3.4. Results

3.4.1. Adult CRS

Large numbers of individuals survived to testing with a minimum of 40 tested on each surface. No barnacles were removed from AWG without leaving $\geq 10\%$ of their basis attached to the glass; as a result all the CRS values for AWG were discarded (chapter 2). Very few individuals showed basis failure on the FR surfaces (T2=2, Sylgard 184=2 and Rhodorsil 48V750=0). T2 showed the highest CRS value, at 0.243 MPa. Sylgard

184 was slightly lower (though significantly different $p < 0.001$) at 0.198 MPa, while Rhodorsil 48V750 showed a much lower CRS of 0.11 MPa (Fig. 3.7.).

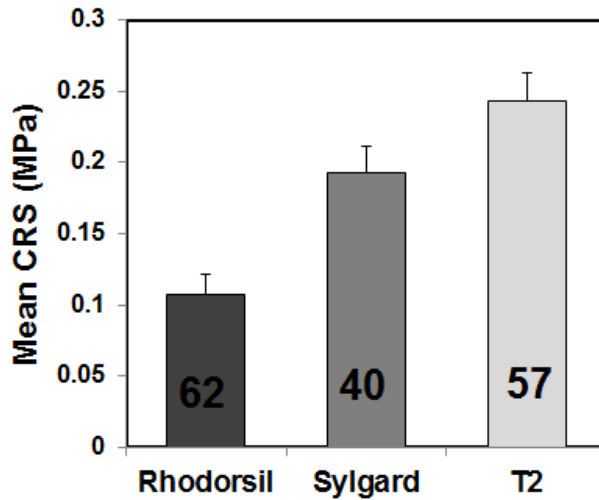


Figure 3.7. The mean CRS (+ 95% CI) of adult barnacles grown on three FR surfaces (number of replicates shown on each bar).

3.4.2. Water-jet

Settlement on all surfaces was high with a total of 1199 PAs tested: 287 on T2, 312 on Sylgard 184, 298 on Rhodorsil 48V750 and 302 on AWG. Higher numbers settled and fully metamorphosed to NM with a total of 2793 tested: 720 on T2, 697 on Sylgard 184, 598 on Rhodorsil 48V750 and 778 on AWG. Individuals still undergoing metamorphosis FaM made up fewer than 8% of the total and these data are not presented here.

Water jetting cleanly removed more PAs from the FR surfaces, T2 and Sylgard 184, and Rhodorsil 48V750, than the non FR surface AWG (Fig. 3.8.A). This method did not discriminate well, for PAs, between the three FR surfaces used. The number of individuals that showed cohesive failure (i.e. had adhesive and/or antennules remaining) was similar for all coatings and at all impact pressures, though highest for AWG (Fig. 3.8.B).

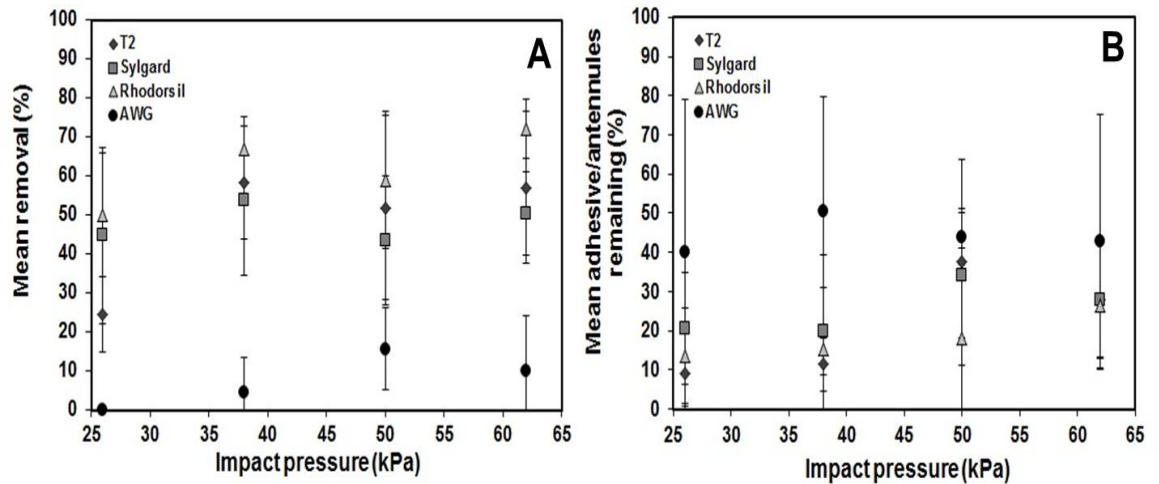


Figure 3.8. The mean percentage removal (\pm 95% CI) of PA individuals: **A)** shows individuals removed with no visible cement or antennules left behind (adhesive failure), **B)** shows all individuals that were removed but a visible area of cement and/or one or both antennules left behind (cohesive/antennular failure).

Numbers of NMs removed were generally lower than for PA with highest removal (42.32%) occurring from Rhodorsil 48V750 at the highest impact pressure, compared to the highest mean removal of PAs (72%) for the same surface and pressure (Fig. 3.8.A and 3.9.A). Variability was still high and the three different FR surfaces were not discriminated between. Moreover, at lower impact pressures, AWG was not significantly different from the FR surfaces ($p \geq 1.63$, Fig. 3.9.A). The proportion showing cohesive failure was lower, (max NM = 30.71% AWG, PA = 50.51% AWG), though the variability was less (Fig. 3.8.B and 3.9.B).

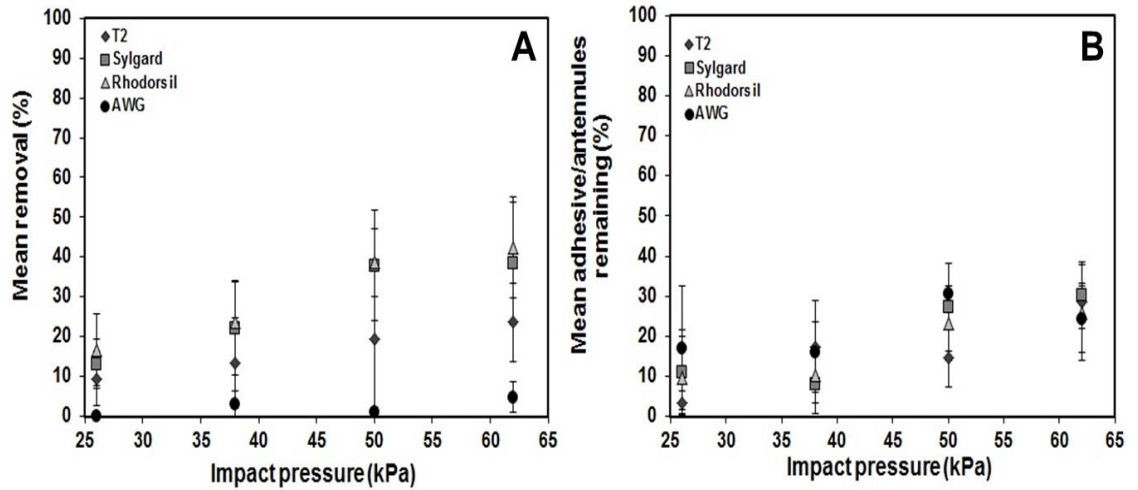


Figure 3.9. The mean percentage removal (\pm 95% CI) of NM individuals: **A)** shows cleanly removed individuals, **B)** shows all metamorphosed individuals that were removed but with a visible area of cement and/or one or both antennules left behind (cohesive/antennular failure).

The total number of CaJs tested was high at 3560 and reasonably well spread amongst the coatings (920 on T2, 1033 on Sylgard 184, 761 on Rhodorsil 48V750 and 846 on AWG). On all surfaces the waterjet damaged a small, but significant, number of individuals, in as much as the body of the juvenile had been removed totally from the calcareous shell (Fig. 3.9.B). These individuals were not counted as being ‘removed’ and were easy to spot with the naked eye (no significant differences between surfaces $p \geq 0.61$). Removal levels at the lower impact pressure (comparable to those tested for PA and NM) were similar (51% for Rhodorsil 48V750 at 50 kPa) though the variability was less (Fig. 3.8.A, 3.9.A and 3.10.). At 50 kPa the water-jet was able to discriminate well between all 4 surfaces tested but not at higher impact pressures for T2 and Sylgard 184.

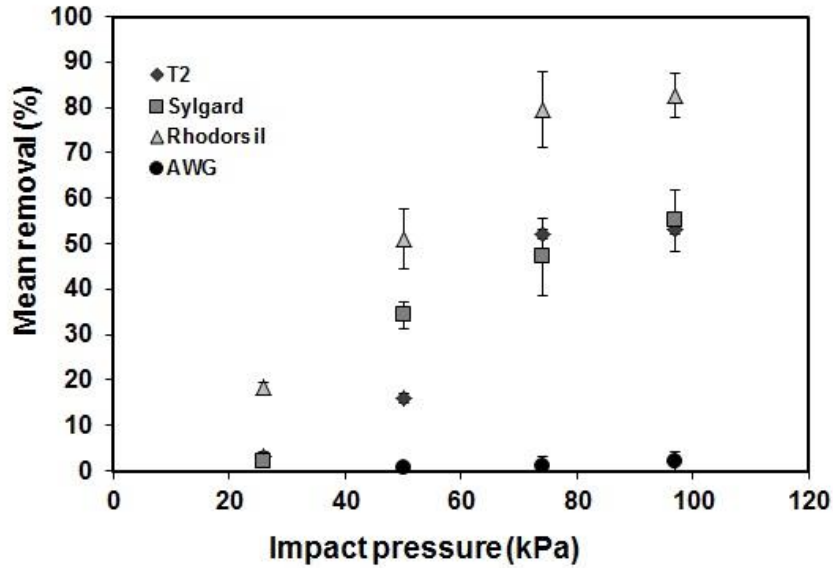


Figure 3.10. The mean percentage removal (\pm 95% CI) of calcified, 6-d-old juvenile barnacles over a range of impact pressures. Only cleanly removed individuals were counted.

3.4.3. Flow cell

Total settlement for the flow cell experiment was high with 1339 PAs tested (324 on T2, 278 on Sylgard 184, 346 on Rhodorsil 48V750 and 397 on AWG). Percentage removal at the higher wall shear stresses (88 and 117 Pa) was similar to that seen using the water-jet. Variability in the flow cell was, however, lower and a clear trend of higher removal with increasing shear stress can be seen for all the FR surfaces (Fig. 3.11.A). At the three higher wall shear stresses, removal of barnacles was consistently higher on Rhodorsil 48V750 compared to Sylgard 184, and T2 showed consistently lower removal than Sylgard 184 (Fig. 3.11.A, $p \leq 0.01$). Thus, the flow cell discriminated between all four surfaces for PAs at the highest wall shear stresses tested (65, 88 and 117 Pa). The number of individuals that showed antennular and/or cohesive failure was lower, and more consistent than when tested using the water jet for the FR coatings (Fig. 3.8.B and 3.11.B), although AWG showed similar levels at the two highest wall shear stresses. The flow cell also discriminated between FR and AWG surfaces in this respect (Fig. 3.11.B, $p \leq 0.001$).

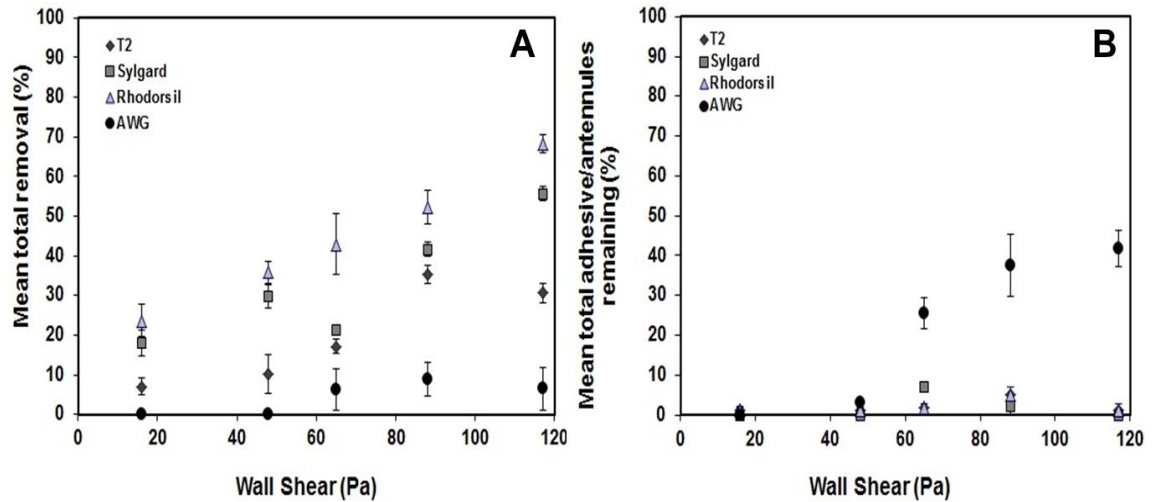


Figure 3.11. The mean percentage removal (\pm 95% CI) of PA individuals using the flow cell. A) shows cleanly removed individuals and B) shows all metamorphosed individuals that were removed but with a visible area of cement and/or one or both antennules left behind (cohesive/antennular failure).

As with the water jet, the percentage removal of NMs was much lower than that of the PAs. At wall shear stresses above 65 Pa, there was significantly more removal from FR surfaces than AWG ($p < 0.04$, Fig. 3.12.A). Coatings followed the same trend for ease of removal as adult CRS with $RV > Sylgard\ 184 > T2 > AWG$. Variability appeared slightly higher than for PAs and there was no significant difference between FR surfaces ($p \leq 0.13$). The number of individuals showing cohesive failure on FR coatings was not significantly different. AWG showed significant failures at 65 Pa, with removal values increasing with increasing wall shear stress, and showing similar levels to that found with PAs (Figs. 3.11.A and 3.12.B).

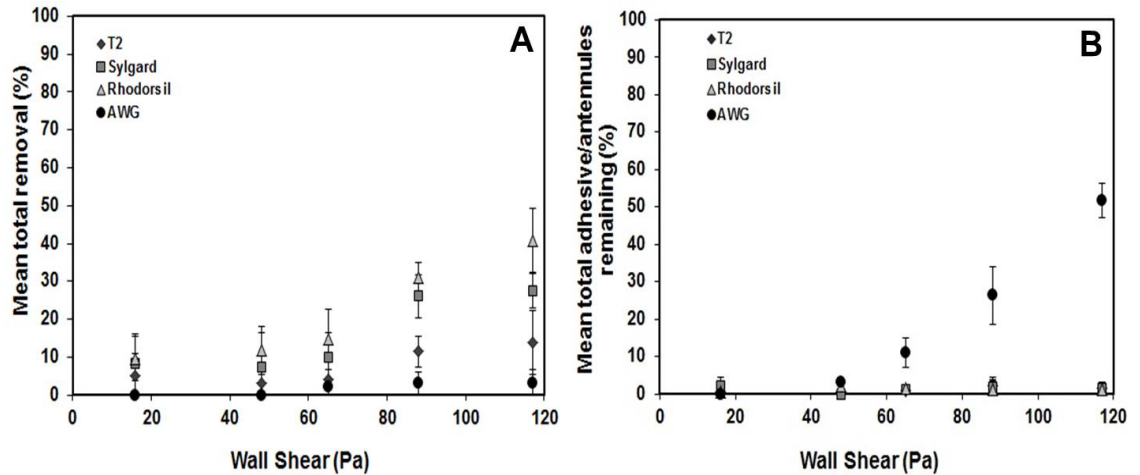


Figure 3.12. The mean percentage removal (\pm 95% CI) of NM settled individuals using the flow cell. A) shows cleanly removed individuals and B) shows all metamorphosed individuals that were removed but with a visible area of cement and/or one or both antennules left behind (cohesive/antennular failure).

A total of 3961 CaJs were tested using the flow cell (1086 on T2, 1098 on Sylgard 184, 980 on Rhodorsil 48V750 and 797 on AWG). Rates of removal were much lower for this stage of development than was found using the water-jet (Figs. 3.12. and 3.13.). No significant difference in removal was obtained at the two lowest shear stresses tested for any surface. From 65 Pa, Rhodorsil 48V750 showed significantly higher removal than the other coatings ($p = 0.003$). T2 and Sylgard 184 were not quite so well resolved only being distinct at 88 and 117 Pa shear stress ($p = 0.003$). Removal from AWG was significantly lower than all FR surfaces from 89 Pa and higher ($p = 0.003$). Very few individuals were damaged or removed from their shells by the flow cell.

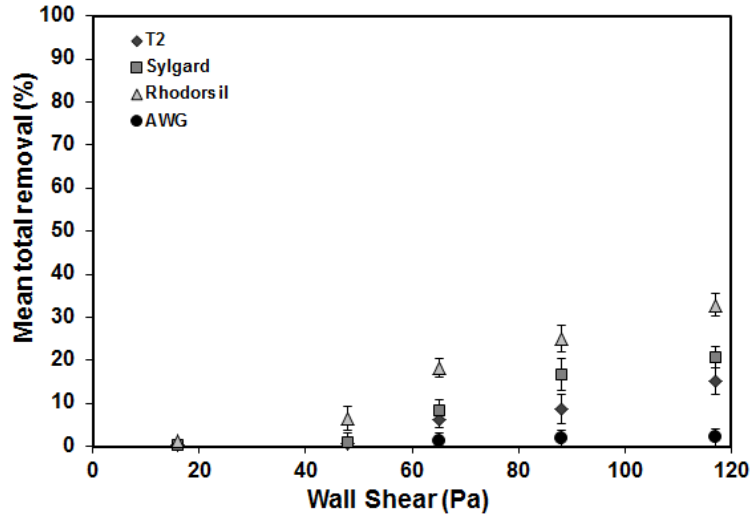


Figure 3.13. The mean percentage removal (\pm 95% CI) of calcified, 6-day-old juvenile barnacles. Only cleanly removed individuals were counted.

3.4.4. Adhesive staining after metamorphosis

For the first 8 h post metamorphosis individuals were exclusively adhered to the surface by cyprid permanent cement, as revealed by staining with Congo Red, (Figs. 3.6.A and 3.14.). From 9 h post metamorphosis a ring of stained cement was evident in some individuals (Figs. 3.6.B to E and 3.14.), which became increasingly common until 18 h post metamorphosis when a peak was reached. Subsequently fewer individuals showed stained ring on their basis, with increasing numbers of individuals show no staining at all, suggesting the basis is adhered fully to the surface, preventing the stain gaining access to the basis and the product it is staining (either amyloid protein or lipopolysaccharide). No staining of bases was observed after 31 h (Fig.3.14.).

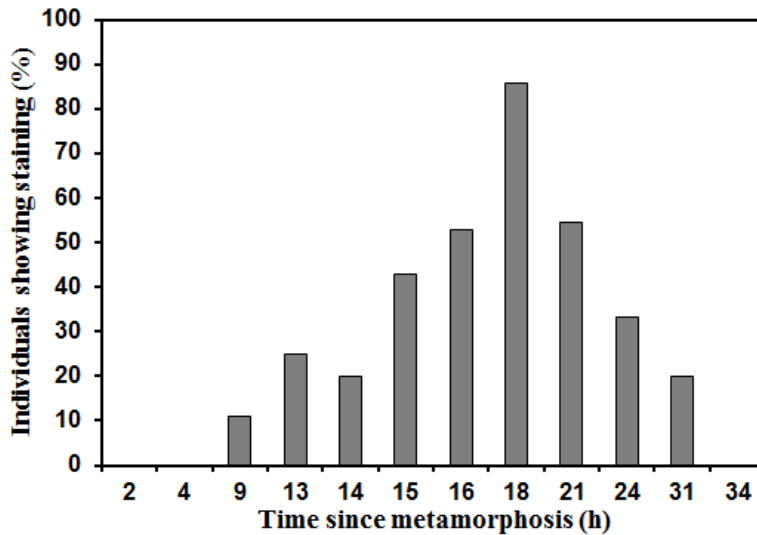


Figure 3.14. Individuals showing staining of the basis over time from metamorphosis.

3.5. Discussion and Conclusions

The adults' CRS values followed expected trends as has been seen elsewhere for T2 and Sylgard 184, and based on their modulus (T2 > Sylgard 184 > Rhodorsil 48V750).

The water jet removal values showed large variability (both with PAs and NMs) for all surfaces, resulting in large 95% confidence intervals, suggesting this method is not ideal for use with these settlement stages. It was noted that if surfaces, post testing, had not been closely examined to determine those individuals that underwent cohesive, rather than adhesive failure with the surface, removal rates would have been overestimated, especially in the case of AWG where average removal due to cohesive failures was as high as 51% in PA individuals and 31% for CaJs. Rates of removal for CaJs using the water-jet appear to be reasonably well separated for different coatings, though this varied with impact pressure used. Therefore, this method needs to be carefully assessed prior to each use to determine the ideal impact pressure needed to discriminate between coatings. Operation of the waterjet was also relatively time consuming. On average, a run of 12 slides took nearly an hour to load, test and unload. Further, as for the flow cell, barnacles had to be localised before and after the tests, which for 12 slides took 1-2 h dependent upon settlement levels. This length of time for testing may have resulted in

the PAs beginning to metamorphose, and the basal membranes of NMs becoming more closely associated with the surface. This may explain, in part, the large variability seen with this method. However, the large number of cohesive failures may also explain some of the error by reducing actual numbers available for removal from the surface. This higher level of cohesive failures is perhaps unsurprising due to the nature of the forces generated by the water-jet. The pressure is impacted immediately onto the individual and there is no time for the organism to acclimate to the force (e.g. a NM may pull its body down into contact with a surface if it is subjected to a gradual pressure increase), and the method of application obviously results in non-shear forces.

The flow cell, by comparison, results in lower variability (remembering these individuals are from the same batch of cyprids) for both PAs and NMs. As was found in work carried out by Larsson et al. (2010), removal was lower for NMs than PAs, however it was even lower for CaJs, suggesting, if we follow Larsson's argument (Larsson et al. 2010), that this life stage should be the one used to test FR surfaces for efficacy. CaJs were also more easily localised, and it may be possible to visually determine exact wall shear stresses for removal for each individual tested in the future allowing a more precise assessment of a coating's FR performance. Due to this ease of visualisation it may also be possible to measure basal area and thus obtain a CRS for these individuals allowing a real measure of adhesive strength to be obtained.

The removal of PAs from AWG was higher at 6.46% than the 0% reported by Zardus et al. (2008), for 'clean' glass. Conversely NM removal rates on clean glass were very high in the Zardus et al. (2008) study (65%) compared to those found here (3.13%). If individuals that were not cleanly removed (not mentioned by Zardus et al. 2008) are added to this figure then the numbers are much closer, increasing to 54.6% removal, suggesting that at least some of the removal seen in this earlier study may have been due to cohesive failure, or adhesive failure between the adhesive and basis of the juvenile.

The different stages of settlement show different rates of removal, and this is particularly clear for the flow cell data. PAs appear much more easily removed than NMs (for both hydrodynamic methods) and CaJs (for flow cell removal; the opposite is

true for the water-jet removal). The flow cell data is, obviously, more likely to reflect the effect seen on ships' hulls as the hydrodynamic forces experienced are similar, thus the data will be discussed in more detail for this method only. Ontogenetic differences in removal rates could be due purely to differences in the hydrodynamic shape of the organisms. The cyprid, attached by antennules, would likely be subject to more lift forces compared to the NM and CaJ individuals due to their shape and mechanism of attachment. However it could also be the case that the NM and CaJ individuals have similar adhesives (the cyprid permanent cement, and the membrane proteinaceous secretion, as described by Yule and Walker (1984) and shown here (Fig. 3.6.A-E)) while the PA individuals have only the cyprid permanent cement. It is unlikely that CaJs would have adult adhesive only 6 days from metamorphosis as, even allowing for a shorter timescale of development in *B. amphitrite*, it took ≥ 35 days for *Semibalanus balanoides* to produce adult cement (Walker 1973). Adult adhesive production would be expected to be linked to the first signs of calcification in the basis. Visual examination of bases of those individuals forcibly removed from their shells (Fig. 3.2.B) suggested basal calcification had not yet occurred and a membrane was often seen. The slight reduction in removal levels for CaJs may be due to all individuals having fully adhered membranes, while only a proportion of NMs were fully attached (this would also explain the higher variability of individuals in the NM test).

The staining of the membranous area (Fig. 3.6.B-E) showed that membranous adhesion, or close association, to the substrate began 9 h post metamorphosis. Interestingly this is very similar to the 10 h period after which cyprid cement become refractory to proteinaceous enzymes (Pettitt et al. 2004). This result suggests the ineffectiveness of the proteinaceous enzymes may not only be due to adhesive curing, but also to the membrane making contact with the substrate and the enzyme no longer being able to access the cyprid permanent cement.

While both impact pressure and wall shear stress can provide comparative information on coating efficacy, if FR coatings with similar properties are to be effectively tested (i.e. discriminated between) the flow cell is preferred. The advantage of this method over the more widely used CRS is the reduction in time needed to grow a settled individual to testing (3 months for CRS and 6 d for CaJ testing). The use of CaJs would

appear to be the most useful for testing FR surfaces as removal rate was low (on the relatively poor FR surfaces tested here) allowing for differentiation with better FR surfaces. A further advantage of CaJs is that they are easily seen and localised and non-adhesive failure is quick and easy to determine. The lack of a measure of adhesive strength, analogous to CRS in the adult, is a problem with this method at present. However, if measures were taken of the basis area of the CaJs and absolute values for force at point of removal were obtained, using video to capture the exact moment of adhesive failure this could be calculated using this method.

Chapter 4: Effect of surface chemistry on cyprid permanent cement spreading, and surface selection.

4.1. Abstract

During settlement barnacle cypris larvae undergo a non-reversible settlement step, the release of cyprid permanent cement. This could be considered to be the most important point in the life of the organism, the cost of choosing an unsuitable site being death. There have been few studies aimed directly at the study of cyprid cement. Here it is shown that cyprid cement can be visualised using a common histological stain, Congo Red. The area that this adhesive covers on a surface (such as acid-washed glass) is shown to be strongly conserved between different batches of cyprids, and is not significantly affected by cyprid age (up to 9 days post metamorphosis) or the addition of the artificial settlement inducer 3-isobutyl-1-methylxanthine (IBMX).

The effects of surface wettability, charge and surface free energy on the adhesive spreading are determined using well-characterised self-assembled monolayers (SAMs). Cyprid cement secreted onto SAMs produced using R-OH, R-CH₃ and mixes of the two thiols follows thermodynamic theories with a strong negative correlation of spreading with wettability. The adhesive spreads more on hydrophobic surfaces where the water can be more easily excluded. The spreading of this adhesive is also affected by surface charge with a greater degree of wetting occurring on negatively charged surfaces. Finally a clear link is shown between settlement levels and adhesive spreading on a number of SAMs of varying surface free energy and charge.

4.2. Introduction

Barnacles are of great importance in terms of their dominance as sessile organisms in natural marine intertidal environments (Lubchenco et al. 1978; Barnes 2000). They also have a major financial impact in terms of their prevalence as fouling organisms, the cost of which to the maritime sector is estimated to be in the billions of pounds per year (Schultz 2007; Dürr and Thomason 2010; Callow and Callow 2011; Schultz et al. 2011). Much research has been carried out into methods to reduce the impacts of biofouling including the use of biocides (Omae 2003; Yebra et al. 2004; Khandeparker 2005; Chambers et al. 2006; Finnie and Williams 2010), and non-toxic technologies such as fouling-release (FR) coatings (e.g. Brady et al. 1987; Swain and Schultz 1996; Swain et al. 1998; Berglin and Gatenholm 1999; Brady and Singer 2000; Wynne et al. 2000; Berglin & Gatenholm 2003; Berglin et al. 2003; Sun et al. 2004; Ekin and Webster 2007; Kim et al. 2007; Rittschof et al. 2008; Beigbeder et al. 2008). The logical and traditional target for antifouling and FR surfaces is the colonisation stage – the cypris larva. If an animal chooses not to settle on a surface, or if the juvenile's adhesive cannot adhere to it, there is no hydrodynamic cost in terms of fouling for this organism. This being the case, it is perhaps surprising how little is known about cyprid adhesives involved in barnacle settlement, including cyprid cement (see below).

Barnacles produce three, (perhaps four (see Chapter 3)), separate adhesives depending upon their developmental stage. Cyprids initially make contact with a surface and determine its suitability for permanent attachment by 'searching', which essentially entails 'walking' with paired antennules that engage in reversible adhesion. Reversible adhesion of cyprids is not completely understood but is thought to involve a mix of dry adhesion and wet adhesion; the latter effected by a proteinaceous secretion – often named the temporary adhesive (Aldred and Clare 2008; Clare and Aldred 2009). If the surface is deemed suitable, cyprid cement is released to fix the cyprid irreversibly to the surface (Fig. 4.1.). After permanent attachment the cyprid undergoes metamorphosis to a juvenile barnacle (Maruzzo et al. 2012). It is presently doubted as to whether there is 'juvenile adhesive' released at this time (see Chapter 3; Crisp et al. 1985), though an intermediate proteinaceous secretion, postulated to be an adhesive, was observed in *Semibalanus balanoides* (Yule and Walker 1984a). The adult cement of *S. balanoides*,

is not released until around a month after metamorphosis (Yule and Walker 1987). It follows that if no juvenile adhesive exists, the juvenile is stuck to the surface only by the cyprid permanent cement for this lengthy period, increasing the importance of understanding this adhesive more fully.

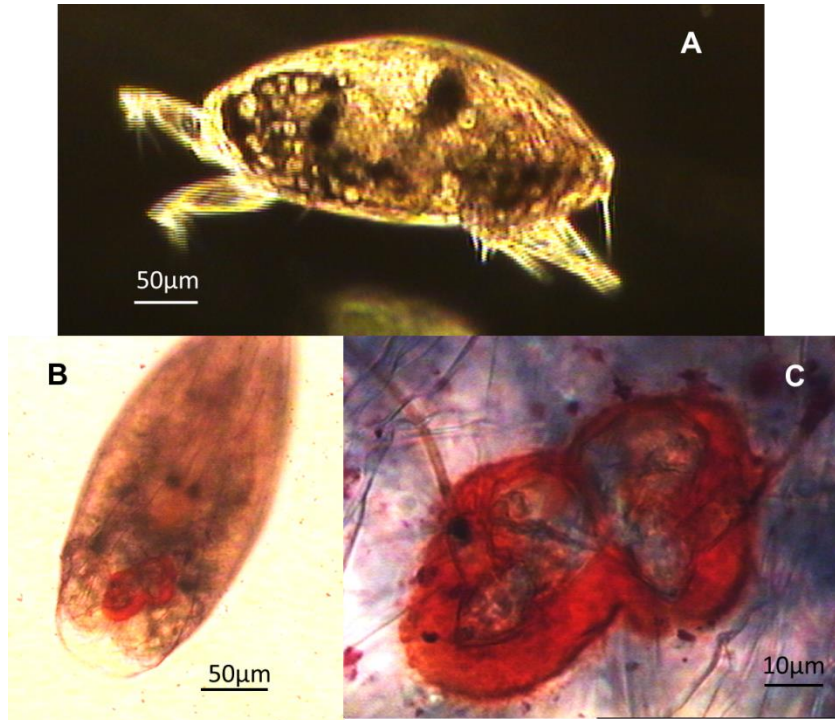


Figure 4.1. The *B. amphitrite* cyprid; A) free swimming, B) permanently settled, C) magnified view of the stained cyprid cement.

Most recent work has focussed on characterising the adult cement (e.g. Kamino 2006 and 2010). This adhesive is believed to be mostly glycoproteinaceous, containing hydrophobic components (Kamino 2008), while having a generally hydrophilic nature (Barlow et al. 2009). Raman and Kumar (2011) suggested that the adult cement of *B. amphitrite* is amorphous and contains cysteine groups, indicating the presence of disulphide bonds, in keeping with previous findings for *Megabalanus rosa* and *Balanus crenatus* by Kamino et al. (2000) and Naldrett and Kaplan (1997) respectively. Recent work has shown that the cement contains amyloid β sheets, which have been postulated to have functional roles (Barlow et al. 2010). Increasingly the importance and identification of the sugar moieties is being accepted, though this was suggested as far back as 1972 (Otness and Medcalf 1972).

The temporary adhesive (deposited as ‘footprints’) has only recently begun to be studied in depth. Walker and Yule (1984) provided evidence of the proteinaceous nature of the adhesive, and more recently immunological evidence has suggested the adhesive to be comprised entirely, or in part, of the settlement-inducing protein complex (SIPC) (Clare et al. 1994a; Matsumura et al. 1998b; Dreanno et al. 2006b).

Compared to the adult cement, cyprid cement has received little attention in terms of its adhesion to different surfaces and its structure, despite its obvious importance in settlement and thus biofouling. Cyprid cement is difficult to study as it is released in small quantities and cures rapidly (Phang et al. 2006).

While highly complex in reality (see Chapter 1), in its simplest terms release of adhesive occurs when a surface is encountered that is suitable for settlement. A large body of research has been carried out to determine where the adhesive is stored and how it is secreted. The cement was thought to be stored in two types of cell (termed α and β cells) in paired cement glands located posterior to the compound eyes (Walker 1971). However, it has more recently been postulated that there may in fact be four different types of granule within the cement gland cells rather than two cell types (Odling et al. 2006). The most detailed study of the release system for cyprid cement was carried out using *Megabalanus rosa* by Okano et al. (1996, Figure 4.2.), which showed that the adhesive was released from the cement gland by exocytosis controlled by catecholamines. The further release of the adhesive down the antennules, out of the antennular disc and onto the surface may be brought about by muscular contractions (Walley 1969), the control and stimulus of which is not yet understood.

Once released, the proteinaceous cement (Walker 1971) begins to cure with the surface, at least, having cured within 2 hours (Phang et al. 2006). The cyprid permanent cement embeds the antennules in a cement plaque completing permanent attachment (Odling et al. 2006). Walker (1971), using transmission electron microscopy, determined that the adhesive had a layered morphology, and suggested this could be due to interactions with seawater, and progressive curing to form a morphologically distinct layer, or a sequential release of differing proportions of cement products, though he did express doubt about the final possibility due to the rapidity of adhesive extrusion.

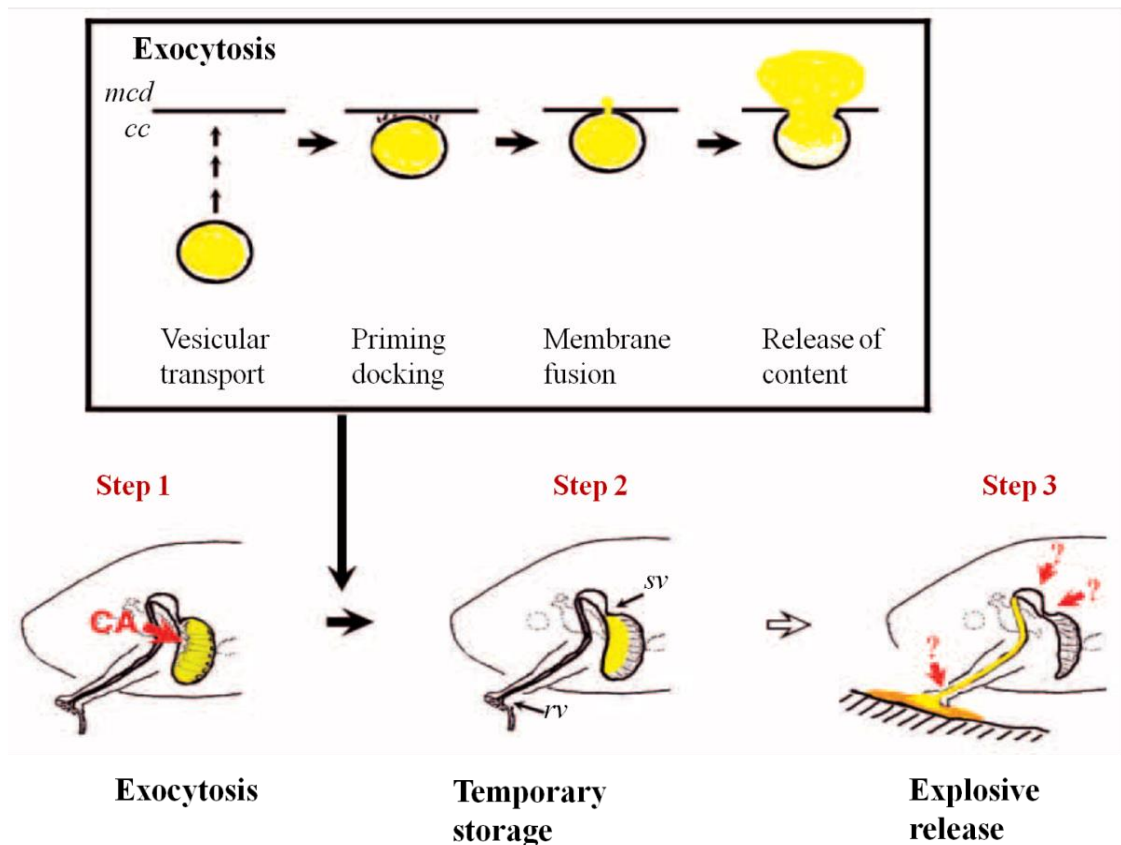


Figure 4.2. Schematic representation of the proposed mechanism of cementation during the permanent attachment of barnacle cyprids. Catecholamine (CA) released from neurones innervating the cement glands induces cement granule transport and exocytosis by cement secreting cells, resulting in cement secretion into the median collecting duct (step 1). The released cement accumulates in the median collecting duct and is stored temporarily (step 2). At the time of permanent attachment, an as yet undetermined system coordinates the actions of the valves and muscular sac contraction, resulting in explosive cement release (step 3). *mcd*, median collecting duct; *cc*, cement-secreting cells; *rv*, release valve (distal valve); *sv*, stop valve (proximal valve). Reproduced from Okano et al. (1996) with permission of The Journal of Experimental Biology.

In order to understand the adhesion of cypris larvae it is important to understand the fundamentals of adhesion (see Chapter 1). The ability of fundamental adhesive mechanisms to work is dependent on the ability of the cyprid adhesive to wet (i.e. spread onto) the substratum and displace the seawater from the surface. On a totally

smooth surface this will be determined by composition (or chemistry) of the surface and the adhesive.

Surface free energy (SFE) is defined as the excess energy available at the surface compared to the bulk of the material. If a surface has no free energy the molecules within it are fully reacted with other molecules within the bulk of the surface. They have no energy to react with a liquid resting upon it and therefore liquid will form a perfect sphere upon the surface, because of the lack of cohesive, or adhesive forces between the surface and the liquid. This free energy can be measured using a number of methods but the most commonly used is the goniometer, which measures the contact angle of different liquids on solid surfaces. This is then used to determine the surface free energy of the solid via Young's equation (Equ. 1 and Fig. 4.3.).

$$\gamma^{sv} = \gamma^{sl} + \gamma^{lv} \cos \theta$$

Equation 1: Young's equation; γ^{sv} is the solid surface free energy; γ^{sl} is the solid/liquid interfacial free energy; γ^{lv} is the liquid surface free energy; and θ is the contact angle, see Fig. 4.3.

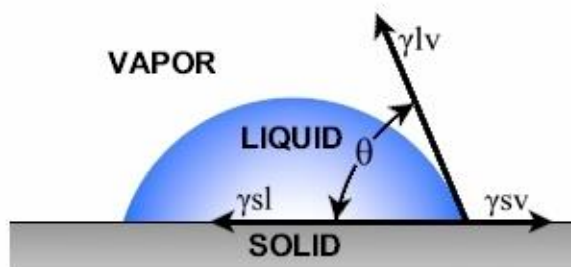


Figure 4.3. Explanation of the use of contact angle measurement to calculate values for Young's equation to determine the surface free energy of a solid (from <http://www.ramehart.com/contactangle.htm>).

Young's equation demonstrates two measurable terms, contact angle (θ) and liquid surface tension (γ^{lv}), which can be used to determine the difference between tensions of the solid and vapour and between the solid and wetting liquid ($\gamma^{sv} - \gamma^{sl}$). Thus using

Young's equation we are only able to calculate the difference between surface tensions ($\gamma^{sv} - \gamma^{sl}$) and it is impossible to calculate the SFE using this technique. There are, however, five widely used models that allow the fitting of further equations into Equ. 1 that allow the SFE to be calculated, although with some degree of error:

1. Zisman critical wetting tension
2. Girifalco, Good, Fowkes, Young combining rule
3. Owens, Wendt geometric mean
4. Wu harmonic mean
5. Lewis acid-base theory

(Hiemenz and Rajagopalan 1997)

Determining surface reactivity, SFE, is difficult and these five different methods (above) vary dramatically depending upon the type of surface being examined and the probe liquid being used. However, estimating the polar interactions of a surface is generally believed to be reliable, as non-polar/dispersive forces are thought to contribute relatively little to overall SFE when polar forces are present. The best theory at present is the Lewis acid-base theory, which requires the use of a minimum of three liquids (a Lewis acid, base and one non-polar liquid) to determine the surface energy (generally given in terms of J m^{-1} or dynes cm^{-1}). This method of measurement allows for direct comparison of SFE between surfaces, provided the same probe liquids are used (van Oss and Giese, 2004).

The surface free energy of a solid is believed to be important in bioadhesion as the liquid surface free energy (γ^{lv}) and the contact angle (θ) have been shown to be linked to the work of adhesion (W_a) through the Young-Dupré equation (Equ. 2).

$$W_a = \gamma^{lv} (1 + \cos \theta)$$

Equation 2: The Young-Dupré equation for work of adhesion (W_a) based on water contact angles.

Thus if fully characterised liquids are used, an understanding, or estimation of the SFE of a surface can be obtained. In simple terms the SFE describes how reactive a surface is and is generally defined as the work required to reversibly increase the area of a surface isothermally by a set amount. Knowledge of the surface can in turn be used to impart information about an unknown compound. In natural adhesives, however, this is complicated by the heterogeneity of the adhesive. Broad hypotheses may, however, be tested using this technique.

From research into adhesion of proteins and bacteria it has been determined that surface energy may be one of the most important physiochemical factors affecting adhesion (Fletcher and Marshall 1982). Work examining the effect of surface characteristics on marine invertebrate adhesion has been carried out, but generally on heterogeneous surfaces. Yule and Walker (1987) examined the adhesive strength (tenacity) of temporarily attached, permanently attached and adult barnacles on slate, Tufnol, glass, beeswax and polytetrafluoroethylene (PTFE) in order to attempt to determine the effects of surface tension (analogous to surface energy) on adhesion. While these surfaces clearly have differing surface tensions (from 70-18.5 dynes stated in the paper) they also vary markedly in their modulus, roughness and chemical makeup. Thus, it is uncertain if the result seen would have been due only to surface tension, however, at the time of testing this method was the best available. The highly controlled method used here, self-assembled monolayers (SAMs), while initially developed in 1946 (Bigelow et al. 1946) only began to be of interest in the study of bioadhesion in the early 1990's (Prime and Whitesides 1991).

The physical properties of SAMs allow the study of fundamental interfacial chemistry, and they have been used as model systems in many fields. SAMs allow the production of chemically well-characterised surfaces for testing the effect of a single surface chemical characteristic on settlement and adhesive behaviour (e.g. Petrone et al. 2011). SAMs have been utilised with different marine organisms to investigate surface effects on attachment (e.g. Callow et al. 2000; Ista et al. 2004; Ederth et al. 2008) and adhesive spreading (Callow et al. 2005; Aldred et al. 2006) particularly on surfaces of different wettability (Ista et al. 1996; Weincek and Fetcher 1997; Sigal et al. 1998).

SAMs are commonly produced by spontaneously adsorbing various thiols in a monolayer on a substrate (Fig. 4.4.). The thiol head groups can be varied depending upon the substrate, but in bioadhesion research a gold substrate is used most commonly. The functional group, and therefore the surface characteristics can also be varied, by changing the thiol used to bind to the gold. By changing the thiols, or the proportions of paired thiols, it is possible to produce surfaces with different controlled wettabilities/surface energy, charges and chemistries, due to the thiols having different functional groups with different reactivity (Fig. 4.4.).

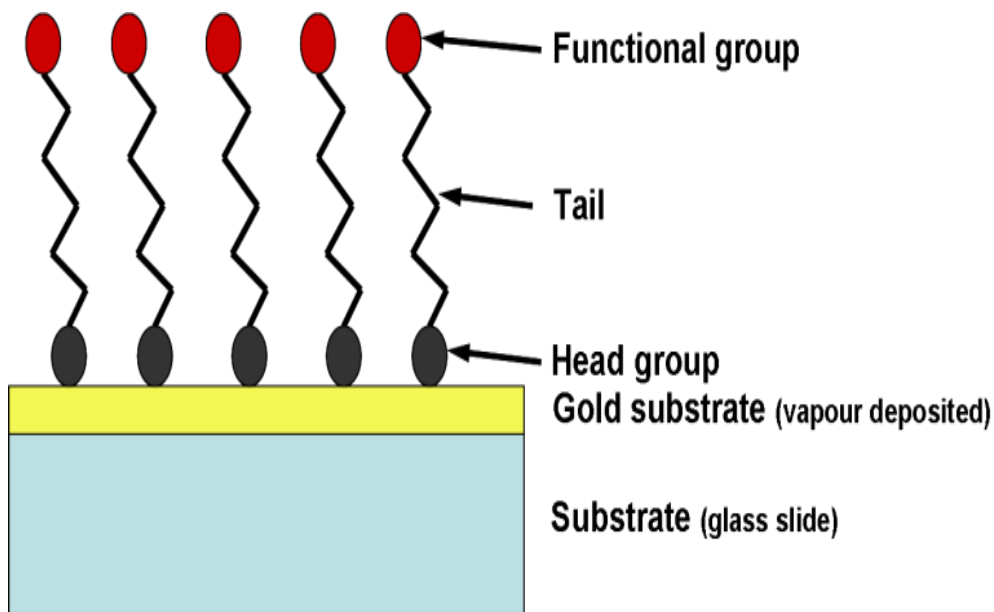


Figure 4.4. Schematic diagram of the basic structure of a gold alkylthiolate SAM.

Given a range of well characterised SAMs, cyprid adhesion on a variety of defined surfaces can be explored. By measuring the area of the cyprid cement on different SAMs it is possible to gain an understanding of how it spreads on different surfaces and this in turn gives valuable information about the adhesive. For example, an adhesive that spreads more on a hydrophilic surface than a hydrophobic one may be highly polar allowing the removal of water required to wet such a surface. Spreading of the adhesive is also clearly linked with strength of adhesion; an adhesive that spreads over a large area, i.e. wets well, allows adhesive interactions to occur over a larger area and it is generally assumed that better wetting results in better adhesion (e.g. Linder 1992).

By exploiting SAMs and the ability to control wettability and charge of surfaces this study aims to obtain information about the spreading of the cyprid cement on different surfaces. It is hypothesised that cyprids will settle more on surfaces that allow their cyprid permanent cement to best spread and adhere and thus the surfaces where highest settlement is seen will show the greatest surface area of cement discs.

4.3. Methods

4.3.1. Surface preparation

Initial experiments used acid-washed glass microscope slides (AWG). These were produced by treating standard glass microscope slides (26 x 76 mm, Menzel-gläser, Thermo Scientific Ltd), pre-washed using detergent (DECON), followed by 50% nitric acid solution for 12 h. After acid washing, slides were rinsed with RO water and stored in 100% ethanol at 6 °C until use, at which time they were rinsed using ASW prior to settlement.

4.3.2. Cyprid culture and settlement experiments

Cyprids were raised as described by Petrone et al. (2011) with the exception of naupliar feeding where only *Tetraselmis suecica* (CCAP 66/4) was used. Cyprids were collected and stored in 0.22 µm filtered artificial seawater (ASW) at a salinity of 34 at 6 °C for 3-4 d prior to settlement, unless otherwise stated. Where settlement levels were to be analysed, 20 (\pm 1) cyprids were placed in a 1.5 ml drop of ASW on the surface of the slide and maintained at 28 °C in a dark incubator for settlement enumeration at 24 and 48 h. After the initial settlement assays, where settlement was near zero in all treatments (except the acid washed glass (AWG) control), 3-isobutyl-1-methylxanthine (IBMX) was added to result in a final concentration of 10^{-5} M to induce settlement. Where cyprids were settled for analysis of adhesive spreading, in all but the first experiment (described below) around 70 cyprids were added to a 1.5 to 2 ml drop of ASW on the

slide and the settlement-inducing compound IBMX was added. Cyprids were allowed to settle for 24 h at 28 °C in the dark prior to staining.

4.3.3. Staining of adhesive plaques

An initial trial was carried out to determine if there were any effects of staining protocols on the adhesive plaques. Cyprids (stored at 6 °C for 4 d) were allowed to settle on AWG slides (24 h at 28 °C in the dark) after which slides were rinsed to remove any unsettled individuals. Slides were divided such that each of 14 treatments had between 30 and 40 individual settled cyprids within it. Each batch of cyprids was exposed for 1 h to one of the following treatments: ASW (control), RO water, RO + 10, 30 60 and 80 % ethanol, RO + 10, 30, 60 and 80% methanol, RO + 2 and 5% acetic acid and RO + 0.1 and 1% sodium hydroxide to test for all the solutions used in the staining solutions. After 1 h all treatments were well rinsed using ASW and the cyprid cement imaged and all treatments compared to that of the ASW control.

Cyprids (stored at 6 °C for 4 d) were allowed to settle on AWG (24 h at 28 °C in the dark) after which slides were rinsed to remove any unsettled individuals. Slides were split into three groups: group one were preserved immediately using 5% glutaraldehyde in ASW at 21 °C for 1 h then rinsed three times using ASW. All three groups were then stained, using a number of stains (Table 4.1.) for 1 h and either rinsed using 0.22 µm filtered ASW or destained using the appropriate destain method (as per Table 4.1.). The stains selected were (Table 4.1.):

- Neutral Red (Sigma), which is often used as a general stain in cytology
- Coomassie Brilliant Blue R250 (CBBR, Sigma), which is a general protein stain
- Nile Red (Sigma), which stains lipids
- Congo Red (Sigma), which stains amyloid and lipopolysaccharides.

(Horobin and Kiernan 2002)

As the basic components of cyprid cement are, as far as are known, proteins, lipids, and ash (including calcium) these stains were expected to stain the adhesive.

Table 4.1. Stain descriptions and protocols adapted from Horobin and Kiernan (2002)

Name of Stain	Stain contents	Method description
Neutral Red Stain	0.1g Neutral Red, 100ml RO water 1 drop glacial acetic acid	Mix NR with water and add acid, leave stirring overnight. Filter prior to use
CBBR Stain	0.25g Coomassi Blue, 75ml glacial acetic acid 500ml methanol	Mix and filter just prior to use, make up to 1l with RO water
CBBR Destain	75ml glacial acetic acid, 500ml methanol	Mix, make up to 1l with RO water
Nile Red Stain (stock sol ⁿ)	0.5mg/ml Nile red in acetone	Mix
Nile Red Stain (working sol ⁿ)	0.05ml stock sol ⁿ , 50ml 75:25 glycerol:RO water	Stir well and remove bubbles
Congo Red Stain (stock sol ⁿ)	1g Congo Red 500ml NaCl sol ⁿ (30gNaCl in 200ml RO water) 800ml ethanol	Mix stain and NaCl sol ⁿ well then add ethanol
Congo Red Stain (working sol ⁿ)	50ml Congo Red stock sol ⁿ 0.5ml 1% NaOH sol ⁿ	Mix well, filter through glass wool (use immediately)

After destaining/rinsing, group two were preserved using 5% glutaraldehyde in ASW and group three were kept in ASW and not preserved. After 24 h (during which time all samples were stored at 6 °C) settled cyprids and newly metamorphosed individuals were photographed using an inverted microscope (x200 to x400 magnification; Chapter 3). This was repeated for group two and three at approximately 24 h intervals for 6 d. The process was repeated with three separate batches of cyprids.

The quality of the staining was assessed visually and the reproducibility of staining intensity with different stages (permanently attached cyprids and newly metamorphosed individuals) and different batches of cyprids, and preservation prior to or post staining was assessed. The ability to store samples to be imaged at a later date with and without preservation was assessed by comparing measures taken each day with those taken from the initial images, as well as taking an estimation of the bacteria present.

4.3.4. Imaging and measuring of cyprid cement

All cyprid cement plaques were imaged using an inverted microscope at 200x magnification and digital images taken using Pico EasyGrab v5.6 software (Chapter 3). Measurement of plaque areas was carried out using the digital images and Image J (v1.45s) software. A stage graticule image was used to convert from pixels to μm . Plaque areas from between 200 and 300 individuals were used to obtain mean areas for analysis. Measures of areas were repeated three times and the mean value used for each plaque.

4.3.5. Description and production of self-assembled monolayers

Having determined the optimal method to visualise the cyprid cement, SAMs were used to test the effect on adhesive spreading of differences in wettability, charge and Lewis acids/bases.

4.3.5.1. SAMs to examine the effect of surface wettability on spreading on cyprid cement

SAMs to determine effect of wettability (or SFE) on cyprid cement were produced as described in previous studies (Ista et al. 1996 and 2004; Callow et al. 2000; Aldred et al. 2005). Briefly, standard glass microscope slides were coated with chromium and gold using a metal evaporator at 10^{-6} Torr, supplied by Dr Linnea Ista (University New Mexico, Albuquerque, NM, USA). Resulting surfaces were sent by airfreight to Newcastle where they were etched by exposure to piranha solution (3 parts concentrated sulphuric acid to 1 part hydrogen peroxide) for approx 1 min and placed into 1 mM ethanolic solutions of varying proportions of dodecanethiol and mercapto-undecanol resulting in surfaces with different proportions of OH and CH_3 groups. The water contact angle for each surface, with different proportions of R-OH thiol (from 0-1), was determined using a static drop measured using a goniometer (Ramé-Hart Co.), each surface was measured five times and the mean value used to determine the range of contact angles, and hence wettabilities, being tested. The contact angles of the surfaces ranged between 21° (hydrophilic) to 103° (hydrophobic). Coatings were stored for 24 h

in the appropriate thiol solutions prior to use, whereupon they were rinsed with ethanol and then ultrapure water, and allowed to dry for 1 min prior to beading a drop of seawater, for settlement, on the surface.

The initial assay showed problems with drop size variability and as a result the subsequent assays were carried out using ‘micro-printed’ surfaces (e.g. Love et al. 2005; Smith et al. 2006). This technique involved the production of a silicon stamp (or PDMS mould), which allowed the deposition of the most hydrophobic thiol onto the outer 3 - 4 mm of the slide, by simply coating the silicone mould with thiol and placing the slide onto the thiol-covered surface for 30 sec. After a 30 sec drying time the test mix of thiols was backfilled into the central section (Fig. 4.5.). This allowed the addition of similar volumes of ASW and similar surface area for exploration and settlement onto each test area, reducing the effect of wettability on drop depth and area.

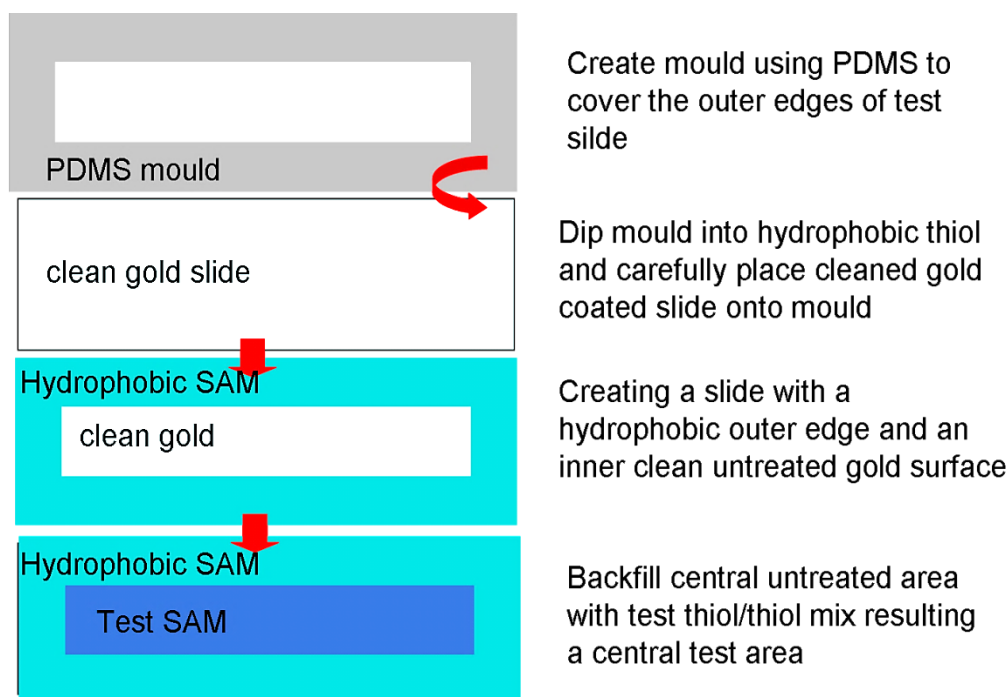


Figure 4.5. Schematic showing how gold-coated slides were ‘printed’ using different thiols to result in a similar test area for seawater drop coverage independent of the wettability of the final SAM.

4.3.5.2. The effect of SAMs with different surface charge, Lewis acid-bases and SFE effects on cyprid cement area

A further group of SAMs on glass microscope slide substrates were supplied by Luigi Petrone from Linköping University, Sweden. These surfaces were produced at the same time and exactly as described in Petrone et al. (2011). Samples were produced using 1-hexadecanethiol (CH₃), 16-hydroxy-1-hexadecanethiol (OH), 16-mercaptohexadecanoic acid (COOH), *N,N,N*-trimethyl-(11-mercaptoundecyl) ammonium chloride (NMe₃) and thiosalicylic acid (Thios). Surface free energy calculations, ellipsometry and stability tests for all surfaces were as in Petrone et al. (2011).

4.3.6. Measurement of contact angles.

Contact angles of SAMs were measured using the static drop measurement technique (Hung et al. 2010). A 20µl drop of ultrapure water (Milli Q ultrapure 18.2 Ωcm at 24 °C) was pipetted onto the surface of the SAM to be tested and allowed to equilibrate for 30 seconds. The contact angle between the drop and the surface was measured using a Rame-Hart 250 contact angle goniometer (Rame-Hart Co., USA) and imaged using DROPIImage Advanced software (Rame-Hart Co. USA). Measures were taken 5 times on each surface and the mean value used.

4.3.7. Cyprid age, batch differences and effect of an inducer

Initial settlement assays confirmed previous experience with SAMs, namely low levels of cyprid settlement (Petrone et al. 2011). Accordingly, the settlement inducer IBMX was added to increase the number of settled individuals for staining of cyprid cement and measurements of cement contact area. To test if the artificial induction of cyprid settlement with IBMX had any effect on cyprid cement area, 36 replicate glass slides were settled with ~50 cyprids on each slide over 24h. IBMX (10⁻⁵ M) was added to one set of 12 replicates and ASW was added to the remaining 24 replicates. The disparity in numbers of replicates used was to allow similar numbers of individuals to settle in each treatment so that numbers of cyprid cement areas measured would be similar.

To test the effect of cyprid age on the production of permanent cement, cyprids (d 0) were collected within hours of metamorphosis and stored at 6 °C for 3, 4, 6, 9, 12 and 14 d. Cyprids of each age (d 0 to 14) were placed onto six replicate AWG microscope slides with IBMX inducer, as described previously, and settled for 24 h prior to staining. Cyprid cement plaques for all individuals settled from each age group were digitally captured and their areas measured (Chapter 3).

To determine if cement plaques of different batches of cyprids were consistent in size, two batches of cyprids, grown up from nauplii several months apart, were induced to settle on AWG slides with IBMX. Cyprids were stored at 6 °C for intervals from 0 to 9 d, and allowed to settle for 24 h at 28 °C after which time cyprid cement plaques were stained, digital images captured and the areas of cement for each age of cyprid measured.

4.3.8. Analysis of data

All percentage data were converted to proportions and arcsine transformed prior to statistical analysis (Zar 1999). Normality and equal variance were tested using Anderson Darling and Levines tests and where the assumptions were met ANOVA was used to compare the mean cement areas and mean settlement for each SAM tested. Where multiple comparisons were used a Bonferroni correction was employed to ensure false positives were avoided due to multiple comparisons. Where the assumptions were not met the Kruskal Wallis test was utilised with a post hoc Dunn's test to determine significant differences. The null hypothesis was that the area of the plaques would be the same mean size on all surfaces and at all ages, and that the percentage settlement would be the same on all surfaces.

4.4. Results.

4.4.1. Staining of adhesive plaques (stain types, duration and preservation).

Initial trials to determine if immersing cyprid plaques for 1 h in fresh water, and fresh water with differing concentrations of ethanol, methanol, acetic acid and sodium hydroxide showed that no treatment had any significant effect on the area of the plaque that was measured, when compared to the control stored in ASW ($p < 0.87$; ANOVA).

Staining with Nile red and Neutral red stain was inconsistent but generally resulted in little staining. Staining was diffuse resulting in an inability to clearly differentiate the edges of the cyprid cement plaque. CBBR also did not stain consistently (staining two of the three batches of cyprids tested, and staining only 50% of the individuals) but when staining did occur it was highly effective resulting in darkly stained cement plaques with clear edges.

Congo red staining was effective in 75% of individuals in all three tested cyprid batches and resulted in a relatively dark stain showing the edges of the cyprid cement disc clearly. This stain also had the advantage of appearing to stain the cement plaque even when full metamorphosis had taken place allowing more individuals to be tested (Table 4.2. and Figure 4.6.).

Table 4.2. Effectiveness of the different stains trialled.

Stain type	Visualisation of cyprid cement	Reproducibility over three batches of cyprids
Neutral red stain	Staining blurred	No
CBBR stain	Clear and dark stain	No
Nile red stain	Partial staining	No
Congo red stain	Good clear staining	Yes

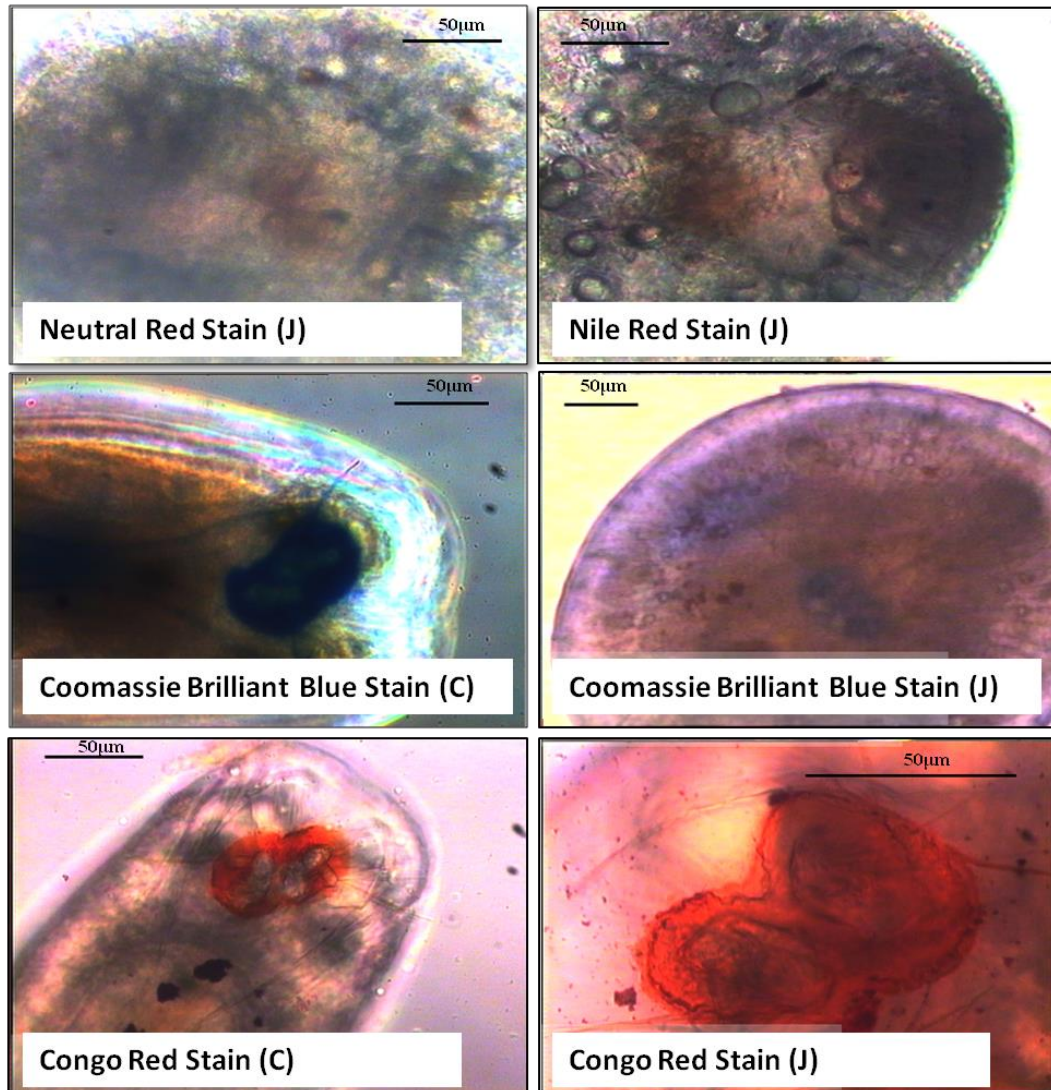


Figure 4.6. Imaged bases of settled barnacles (cyprids (C) and juveniles (J)) stained with different stains.

Two regions of adhesive plaque were noted (Figure 4.7.) but the distinction was not clear enough on most plaques to permit measurements to be made. These regions were a lightly stained outer area, which appeared thin with a smooth outline, and a thicker, more heavily stained inner area, which was more globular and irregular in outline.

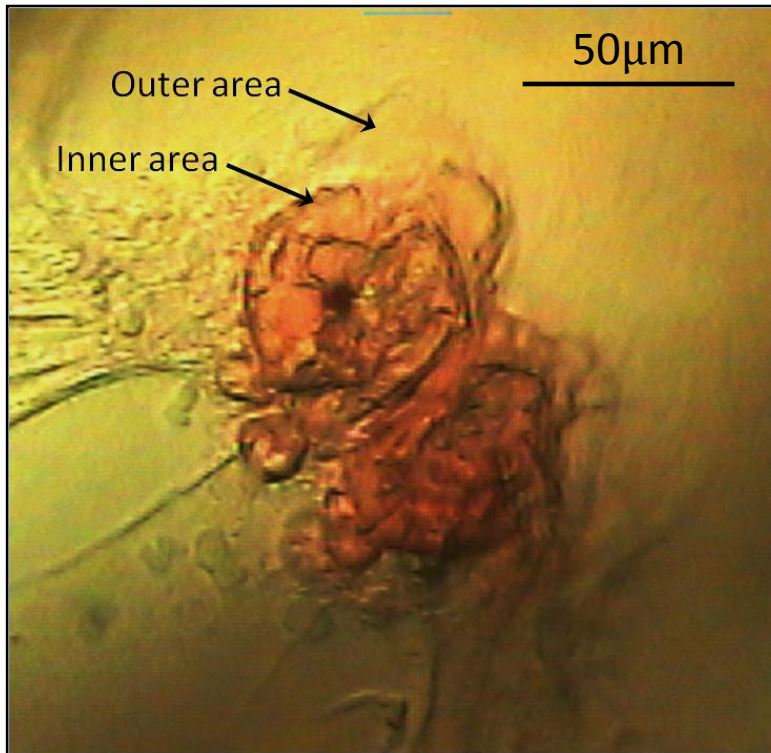


Figure 4.7. The outer and inner areas of the cyprid cement plaque (particularly clear on CH₃ SAMs stained using Congo red used here).

All samples that were preserved prior to staining showed reduced, or no staining compared to unpreserved samples. If cement was preserved after staining with Congo Red (the most successful stain), then the intensity of residual stain reduced over time and the edges became less distinct (noted at 72 h on analysis of measurement error and visible by eye at 96 h). However, unpreserved samples began to deteriorate and bacteria were visible on the surface after 48 h. As a result none of the samples examined in further experiments were preserved; they were stored at 6 °C, and imaged within 24 h of staining.

4.4.2. Effect of cyprid age, batch differences and IBMX on cyprid cement

4.4.2.1. Effect of IBMX on cyprid cement

The addition of 10^{-5} M IBMX had no effect on the cyprid cement area (Figure 4.8., ANOVA $p = 0.681$), but it did increase the proportion of cyprids that settled in a 24 h period from an average of 8.76% per slide in the ASW control to 28.83% per slide.

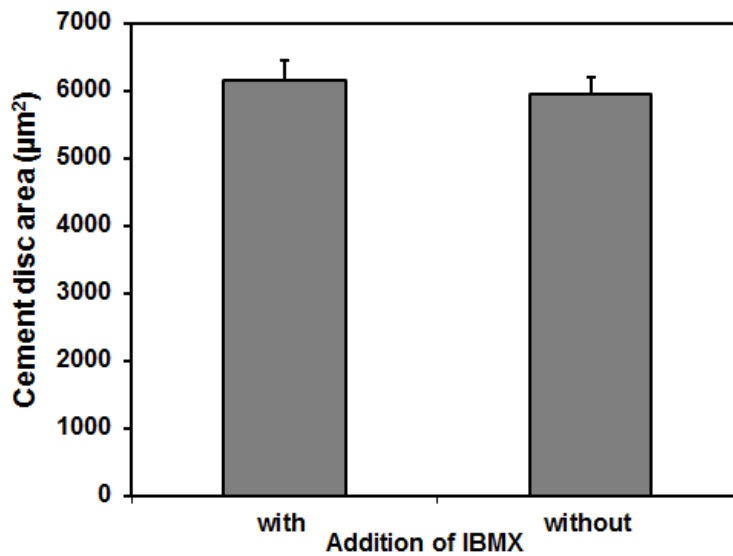


Figure 4.8. The effect of 10^{-5} M IBMX on the cyprid cement plaque area (mean + 95% CI).

4.4.2.2. Effect of cyprid age on cement plaque area

Cyprids that were stored at 6 °C for up to 9 d showed no significant differences in adhesive plaque area (ANOVA $p = 0.321$, Fig. 4.9.). While there appeared to be a negative relationship with the cement area decreasing with increasing age (Fig. 4.9) this was not found to be significant ($p \geq 0.130$, regression). However 12- and 14-d-old cement plaques were reduced in area (Kruskal-Wallis, post hoc Duns $p \leq 0.050$ Fig. 4.9.).

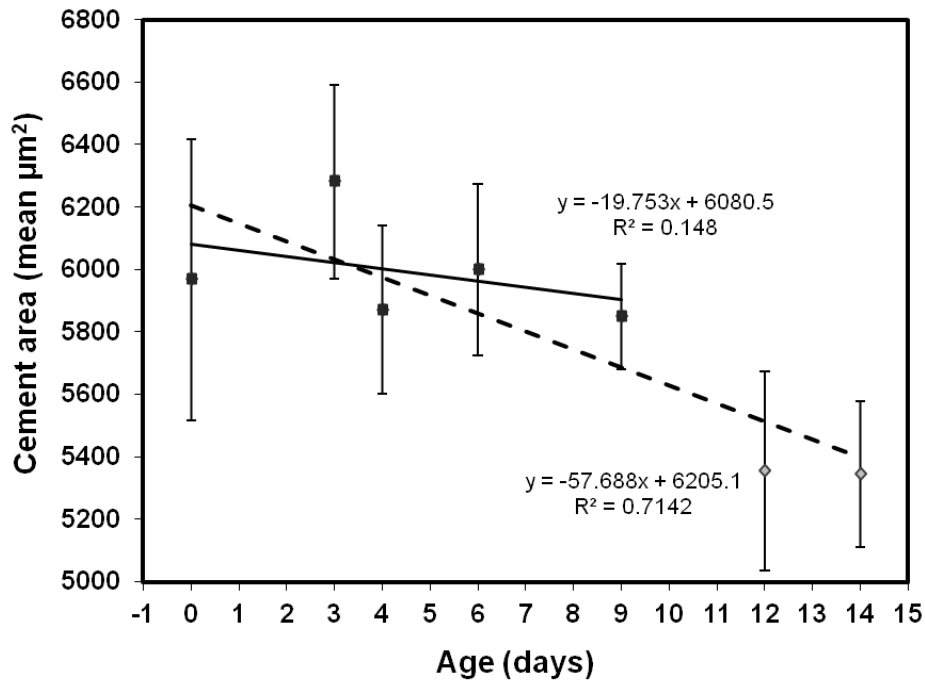


Figure 4.9. The effect of cyprid age on the cement disc area (mean area \pm 95% CI). The linear relationships for all the data (d 0 to 14, hatched line, lower equation and R^2 value) and first 9 days of data (solid line, upper equation and R^2 value) are shown on the graph.

4.4.2.3. Effect of cyprid batch on cement area

Between d 0 and d 9 there was no significant difference in cyprid cement area in relation to age or batch tested (Fig. 4.10., ANOVA $p = 0.897$)

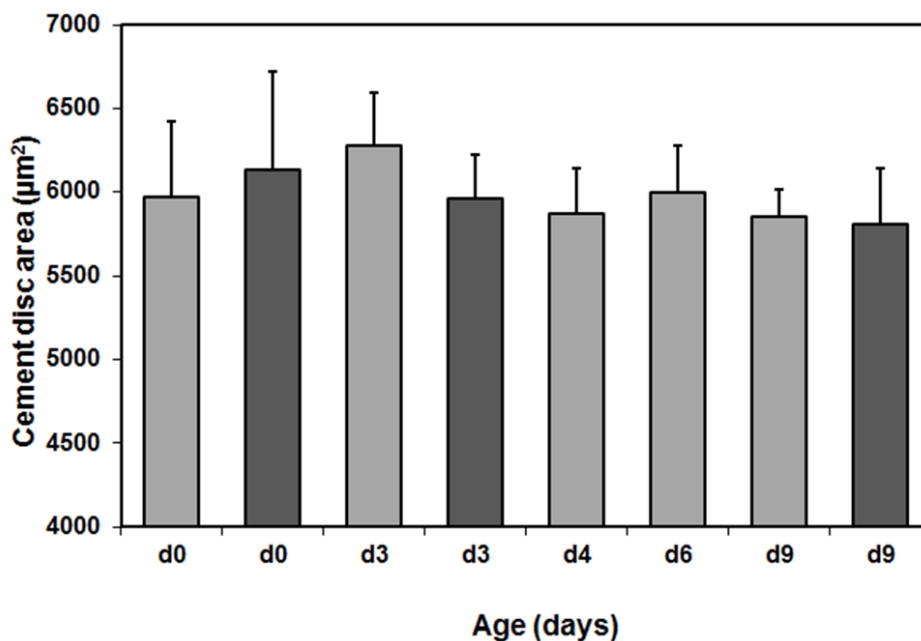


Figure 4.10. Change in cyprid cement area with age and cyprid batch (Mean + 95% CI, light bars are batch 1 cyprids, dark bars are batch 2 cyprids).

4.4.3. Effect of SAMs on cyprid cement area

4.4.3.1. The use of SAMs to test the effect of wettability on adhesive spreading.

The water contact angle decreased with increasing proportion of R-OH thiol (Fig 4.11.). SAMs made with 100 % R-OH had a water contact angle of 21° and the 100 % R-CH₃ thiol resulted in a contact angle of 102°. These values were similar to those found in other studies (e.g. Petrone et al. 2011).

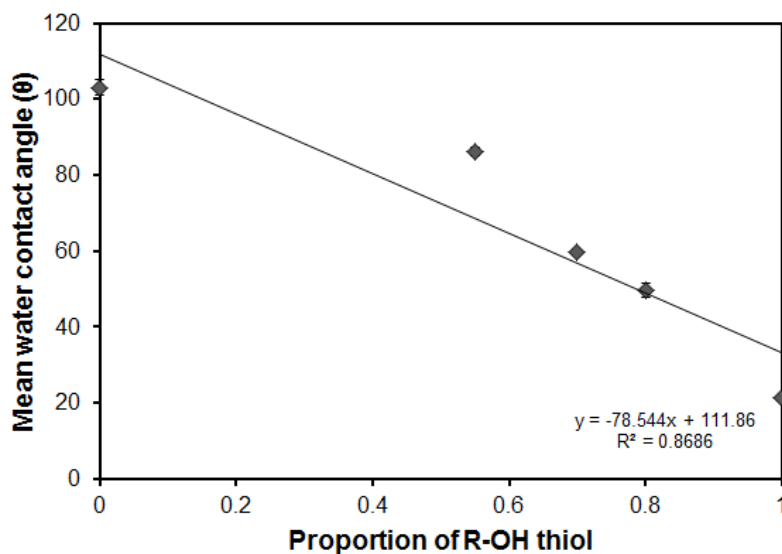


Figure 4.11. The relationship of water contact angle (mean \pm 95% CI) and proportion of R-OH thiol added to the thiol mix used to prepare the SAMs (Line = linear regression; equation and R^2 shown under the line).

4.4.3.2. The effect of SAMs with changing wettability on settlement

Without the addition of IBMX initial settlement assays obtained settlement levels close to zero across all surface wettabilities. Settlement assays using IBMX inducers showed no significant relationship between settlement and wettability over all the SAMs tested (Fig. 4.12.A., regression $p \geq 0.134$). The mortality levels were low on all SAMs (Fig. 4.12.B.).

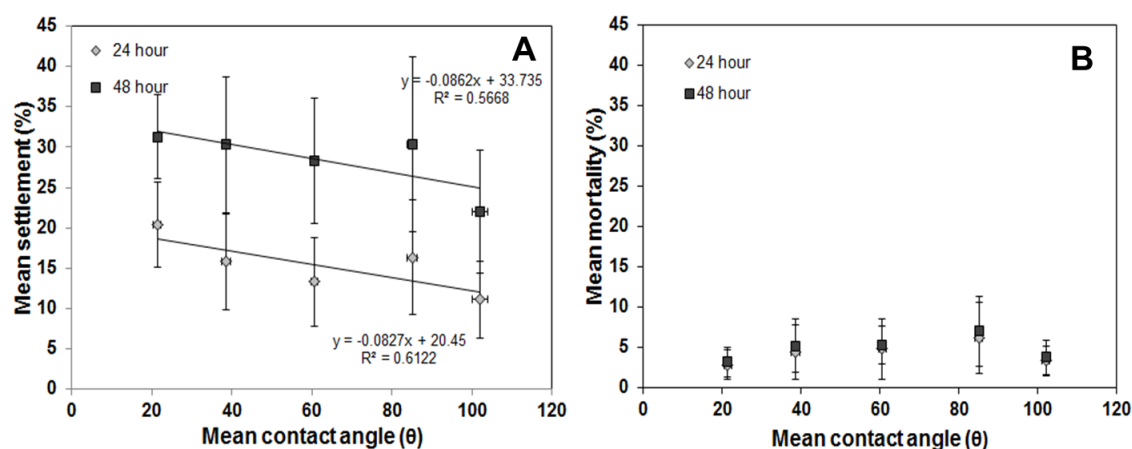


Figure 4.12. A) percentage settlement (mean \pm 95% CI) and B) percentage mortality (mean \pm 95% CI) on SAMs with different wettabilities.

4.4.3.3. The effect of wettability on cyprid cement spreading

The initial trial to determine if the adhesive spreading was affected by wettability of the SAM (Fig. 4.13. Assay 1) showed a strong correlation (0.894 Pearson coefficient), although with statistical analysis this was determined to not be significant (regression analysis, $p = 0.055$).

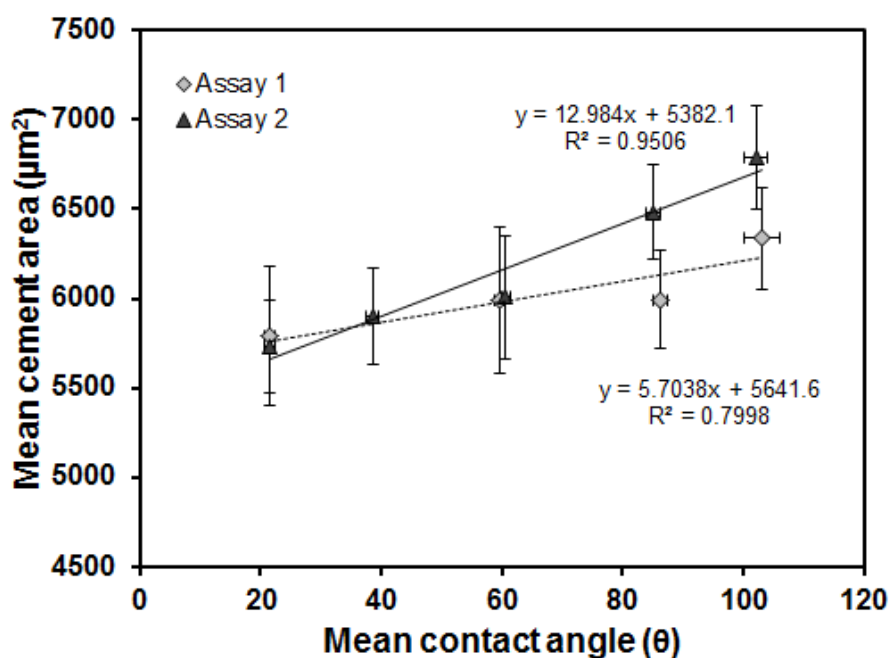


Figure 4.13. Cyprid cement area (mean \pm 95% CI) and the water contact angle of the SAMs with varying wettabilities (mean \pm 95% CI) for assay 1 (not micro-printed) and 2 (micro-printed).

It was thought that a significant relationship could be being masked due to issues of small numbers of settled cyprids on the more hydrophilic surfaces. This was probably due to the water drops spreading so widely that they were too shallow to allow adequate cyprid motility. There were also some problems with gold delamination. As a result a second set of coatings was prepared and microprinting used to ensure a consistent good depth of drops on the hydrophilic surfaces. Once the technical problems were overcome (Fig. 4.14.) a strong linear correlation was found between wettability/hydrophobicity and spreading of the cyprid cement (0.975 Pearson coefficient, Fig. 4.13. Assay 2, regression analysis $p < 0.001$)

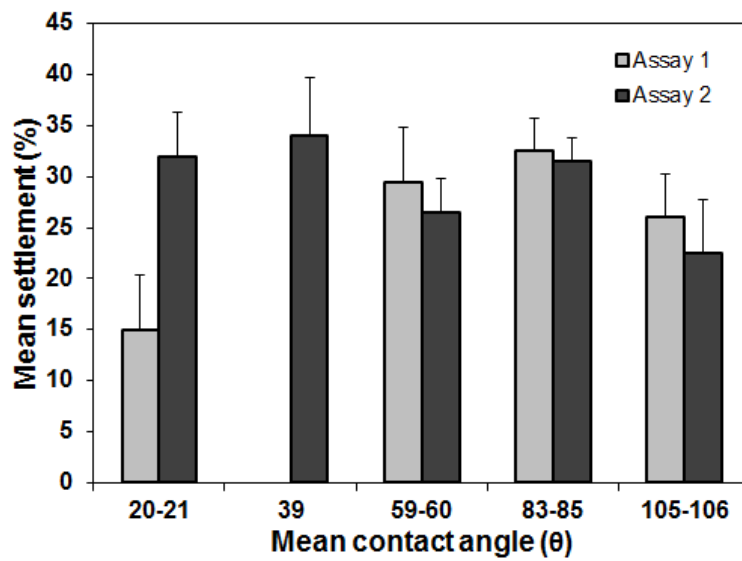


Figure 4.14. Settlement levels on SAMs of different wettabilities (mean + 95% CI) with IBMX inducer over two assays where micro-printing was used (Assay 2) or not (Assay 1).

4.4.3.4. The effect of surface charge on cyprid cement spreading

Charge had an effect on adhesive spreading with significantly more spreading occurring on negatively charged surfaces than the positively charged surfaces (Fig. 4.15.; $p \leq 0.010$, ANOVA with Bonferroni correction).

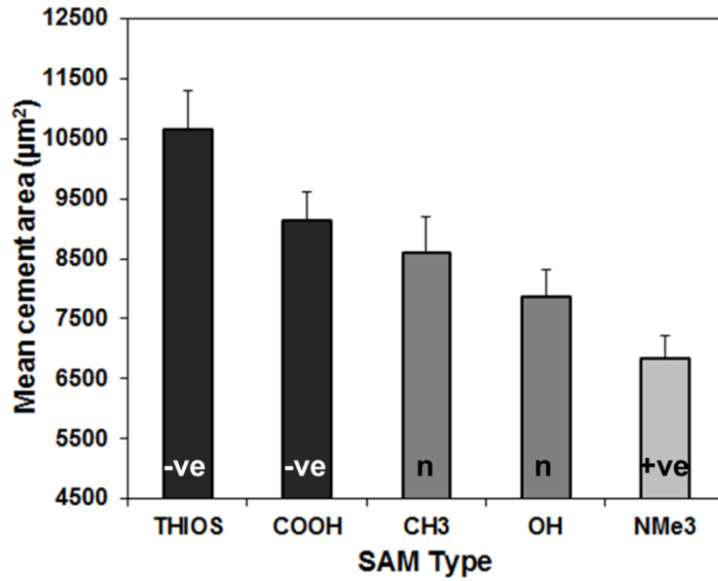


Figure 4.15. The effect of charge on the cyprid cement adhesive area (mean + 95% CI). Bars labelled with overall charge.

4.4.3.5. Surface free energy and adhesive spreading

The effect of surface energy on the spreading of cyprid cement plaques was much less clear, with no simple relationship being apparent (Fig. 4.16., $p = 0.621$)

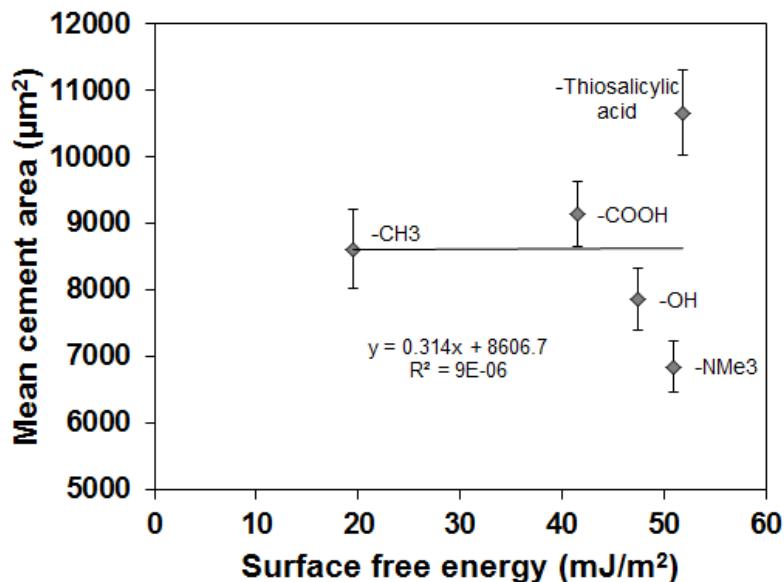


Figure 4.16. The relationship between cyprid cement area (mean \pm 95% CI) and the SFE of various SAMs.

4.4.4. Do cyprids select surfaces to settle on where their cement will spread further and adhere better?

When the areas of cement plaques on various surfaces were compared to settlement levels found on the same surfaces, as determined by Petrone et al. (2011), a clear positive, highly significant, linear correlation was found (0.909 Pearson correlation, regression analysis $p \leq 0.001$ Fig. 4.17.).

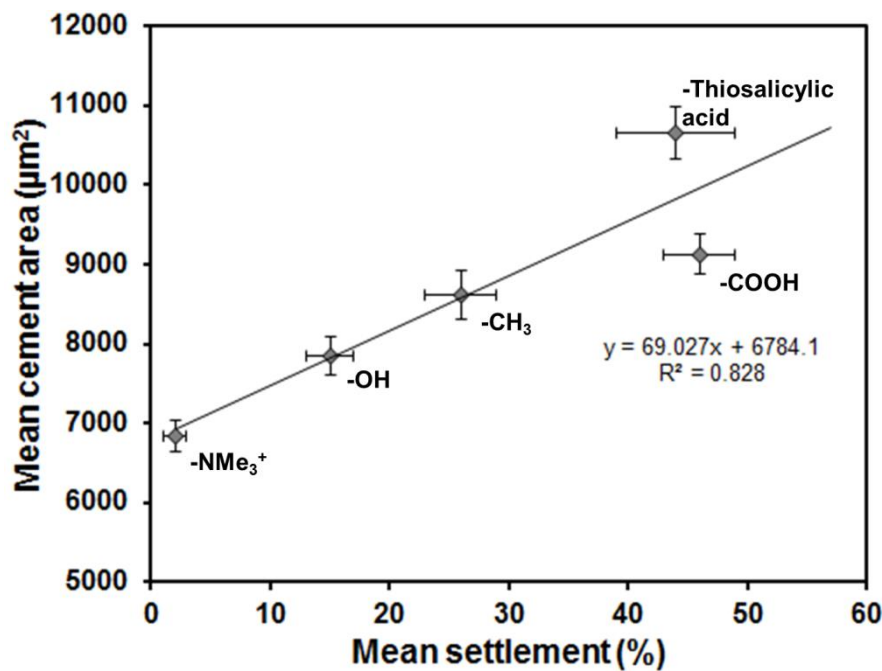


Figure 4.17. The relationship between cyprid cement area and settlement on 5 SAMs (mean \pm 95% CI), settlement data from Petrone et al. (2011).

4.5. Discussion and Conclusions

Cyprid cement was stained intensely by Congo Red, suggesting the presence of either amyloid proteins or another structured, folded protein (Khurana et al. 2001). The presence of amyloid could not be confirmed as it was not possible to carry out a birefringence test. Lipid stains did not stain the cyprid cement, suggesting that there is little or no lipid on the surface of the adhesive plaque. Perhaps unsurprisingly Neutral Red only weakly stained the cyprid cement. Neutral Red is often used to test for cell viability and requires the presence of nuclei (Horobin and Kiernan 2002); the cement

disc is not cellular and is unlikely to contain nuclei (Walker 1971). CBBR, a general protein stain, only worked intermittently, suggesting that curing of the cyprid cement may have prevented staining. The curing may have resulted in a lack of basic amino acids and/or positively charged groups in the protein for the stain to react with (Tal et al. 1984). While there are at present no studies showing definitively that cyprid cement cures, a number of researchers have suggested the likelihood using proteases (e.g. Pettitt et al. 2004, Aldred et al. 2008). The timescales suggested for such curing, based on when the enzymes became less active against the organisms, were consistent with the timing of the loss of staining seen here of around 18 h (15 h in Aldred et al. 2008).

Cyprid adhesive plaque size was strongly conserved amongst different batches of cyprids of different age, up to 9 d -old, suggesting there may be a finite amount of adhesive released by healthy cyprids settling on the same surface chemistry. After storage of cyprids for 9 d at 6 °C, the plaque size produced on settlement was smaller (Fig. 4.9.) suggesting the cyprid was either no longer capable of releasing as much adhesive, or that the adhesive had degraded in some way such that it was less fluid, perhaps cross-linking more rapidly. It may also be that the cyprids at such an advanced age showed the onset of the initial changes that result in precocious metamorphosis, this may affect the cement glands in an as yet undetermined way and result in the reduction of plaque size seen. Unfortunately it was not possible within the timescale of this research to examine this possibility but future work could examine the cement glands within aging cyprids. Perhaps it would be possible to see the vacuoles mentioned by Odling et al. (2006) that were suggested to be due to a partial release of this adhesive, a hypothesis that goes against the evidence of other research (e.g. Walker 1971; Okano et al 1996). It is uncertain if this effect would be hastened if the cyprids had been stored at more biologically realistic temperature. A temperature of 6 °C is frequently used to store *B. amphitrite* cyprids in the laboratory to enhance settlement, but they would be unlikely to experience temperatures below 18 °C in the field (Cohen 2005; Desai et al. 2006). If the results found here with laboratory-reared cyprids apply to wild cyprids, it is likely that the spreading of adhesive would be more variable due to age effects. It is also likely that differences in feeding and temperature regimes, that the larvae in the wild populations are exposed to, would result in greater variability.

Any inference drawn due to these results must be guarded as it is not certain as to what is affecting the adhesive area. The differences in the cyprid cement area on the different SAMs surfaces may have been due to different amounts of adhesive being released or differential spreading of the same amounts of released adhesive. However Walker (1971) determined histologically that after permanent attachment the cyprid cement glands were emptied, thus it is unlikely the variability in plaque size seen here was due to differences in adhesive release. It is also uncertain if what was measured here as the end of the adhesive plaque was indeed the edge. There may have been further adhesive spread so thinly as to make staining either impossible, or impossible to visualise. This would be particularly likely to occur in hydrophilic surfaces, and thus the differences between surfaces noted here may be an underestimation. This could be checked in the future utilising more complex microscopic techniques, such as confocal imaging.

The fact that there was no significant relationship between settlement and wettability (Fig. 4.12.A.) was likely due to the addition of IBMX which may have encouraged settlement on all surfaces, thus hiding preferences. Previous research has shown a clear link between wettability and settlement of cypris larvae (Aldred and Clare 2008), with more settlement being noted on hydrophilic/higher surface energy surfaces (e.g. Finlay et al. 2010; Qian et al. 2000, Gerhart et al. 1992). However much of this earlier research is less than clear, showing nonlinear relationships, and often clouded by other variables, such as surface roughness, charge and modulus. Recent work utilising SAMs (Petroni et al. 2011) showed that *B. amphitrite* cyprids settled in greater numbers on a lower SFE surface. Unfortunately when settlement assays were attempted without IBMX in the present study, too few cyprids settled for a relationship with wettability to be examined.

While the effect of the surface energy, and more recently surface charge on settlement has been examined (Aldred and Clare 2008, Finlay et al. 2010, Petroni et al. 2011), the interaction of these surface factors with cyprid cement spreading itself has not previously been elucidated. The present study has shown a strong negative relationship between spreading of the cyprid cement and wettability, suggesting that cyprids followed the expected trend i.e. hydrophobic surfaces provided better adhesion than hydrophilic, high energy surfaces. This is probably a function of the ease with which the adhesive can displace the water from the surface, hydrophobic surfaces (with large

contact angles) being easier to remove the water from than hydrophilic surfaces thus resulting in greater cyprid cement areas (Fig. 4.13.).

Adhesive staining on SAMs with varying wettabilities showed the spreading of the adhesive followed Kendall's model, seawater proving easier to displace on hydrophobic surfaces. Using a wettability series similar to that used here, Callow et al. (2000) found that settlement of spores of *Enteromorpha* (= *Ulva*) showed a positive correlation with wettability, with more spores settling on SAMs as hydrophobicity increased. However, the adhesive strength of the *Ulva* spores also decreased with increasing hydrophobicity, contrary to thermodynamic theory, which predicts that a fluid should preferentially wet a hydrophobic surface (Finlay et al. 2002b). In keeping with this result, Callow et al. (2005) found that *Ulva* spore adhesive spreads more on hydrophilic surfaces compared to hydrophobic surfaces. The explanation offered by Callow et al. (2005) for these apparent departures from predictions of thermodynamic theory was that the very polar nature of the adhesive meant that it could outcompete with water to wet a hydrophilic surface. This is not the case here, with *B. amphitrite* cyprid cement, since the adhesive follows the expected trend and so no hypothesis to the polarity of the adhesive need be put forward.

Aldred et al. (2006) found a similar trend to the present study with mussel byssus spreading. However they noted that a reduced number of byssi were produced, more rapidly, on high energy surfaces. It may be that, in the case of mussels, total byssal area is a better measurement than mean individual byssal area, and this would show a differential deposition of adhesive, not differential spreading. Indeed this cannot be totally ruled out as the factor behind the differential spreading of cyprid adhesive seen here, i.e. more adhesive being expelled and therefore spreading further, though, as discussed it is unlikely (Walker 1971).

When the spreading of adhesive on substrates with different surface energy is compared to the adhesion strength data obtained by Walker and Yule (1987) it can perhaps help to explain the increase in spreading on low surface energy (hydrophobic) surfaces. Such surfaces had the lowest adhesion strength for permanently attached cyprids (Walker and Yule 1987) and by producing more adhesive, and having a larger surface area for

adhesion, the cyprid can perhaps overcome this ‘poor’ adhesion. However, unlike the present study, Walker and Yule (1987) used disparate surfaces, and as such surfaces will have differed in modulus, roughness and colour, all characteristics known to be important to adhesion strength (Yule and Walker 1984; Hudson et al. 1983) making the comparison of strength of adhesion from Yule and Walker’s study with the spreading of adhesive measured in the present study weak. Unfortunately due to the relatively low numbers of slides available for testing it was not possible to do settlement assays, staining tests and waterjet, or flow cell tests to determine if the adhesive strength of the permanently attached individuals were dissimilar on the different SAMs.

Surface charge appears to play a role in adhesive spreading, with the adhesive spreading more on negatively charged surfaces. It is difficult to draw strong conclusions, however, due to the small sample size tested. Nevertheless, the fact that the adhesive appears to spread more in negatively charged conditions suggests that it may be positively charged.

It is more difficult to understand how surface energy affected the spreading of cyprid cement in the second experiment utilising SAMs. It appears that other factors are involved as no clear relationship with SFE could be seen. Seemingly, therefore, both charge and SFE are important to the adhesive spreading and their effect interacts in an as yet unknown manner. More interesting perhaps is that the settlement seen on the same surfaces tested here (Petroni et al. 2011) correlates very closely with the spreading of the adhesive. This begs the question: do cyprids select surfaces to settle on based on the ability of their adhesive to spread well on a surface? How would this system function? One would assume it would likely depend on the cyprid permanent adhesive being similar to the temporary and adult adhesives, as this is what a cyprid uses to make initial contact with the surface, and long term attachment respectively. Recent findings that the cyprid adhesive contains a peptide found in adult cement, suggest that this may also relate to adult adhesive spreading and strength of adhesion (Chen et al. 2011). It is also interesting to note that the cyprid temporary adhesive appears to follow a similar trend with wettability to that shown for permanent cement in the present study (Phang et al. 2006 and 2009). The increased settlement of cyprids linked to the increased spreading of their permanent adhesive suggests that the tenacity of individuals with

larger plaque areas will be greater as cyprids have been shown to select surfaces that will result in stronger adhesion (Aldred et al. 2010). However this seems to be contradicted by Maki et al. (1994) whose measurements of the tenacity of temporarily attached *B. amphitrite* cyprids showed no effect with three surfaces of different surface energy. However those surfaces were based on differently treated polystyrene and glass, so were not as controlled as SAMs in terms of roughness etc, which almost certainly affected their results.

It is interesting to speculate on the prospect of predicting antifouling/fouling-release coating performance based on a measure of the wetting of the surface by cyprid cement. However, much more research would be needed to test effects of roughness and modulus on adhesive plaque area before this could be truly entertained as a test method. The results presented here do show a clear relationship between wettability and adhesive spreading, as well as the importance of charge, and they correlate well with more recent studies utilising SAMs to examine the effects of the same characteristics on settlement of *B. amphitrite*. Thus it is clear that cypris larvae select and settle on surfaces where their adhesive will spread well allowing for a maximal interfacial area and thus adhesive strength. As yet no testing of strength of permanent adhesion on different SAMs has been carried out, generally due to the overall strong adhesion of cyprids to all SAMs and lack of a methodology to test to the accuracy required to discriminate between surfaces with the very small number of surfaces available for testing in this study. Should such a methodology become available, it should be applied to test of the hypothesis that wetting by cyprid cement is closely related to adhesive strength of the larva.

Chapter 5: The effects of feeding levels and temperature on growth, moulting and adhesive production in *Balanus amphitrite*.

5.1. Abstract

The barnacle *Balanus amphitrite* is a model species for fouling release (FR) surface testing. Adults are grown in different laboratories and at different field sites with different temperatures and feeding rates and algal food species. The effect of changing temperature and food availability on barnacle growth and moulting has long been understood. However the effect on adhesive production has not been studied, and only one small-scale temperature study has been undertaken to investigate temperature effects on critical removal stress (CRS; Johnston 2010). This study considered growth, moult production and adhesive ring formation of *B. amphitrite* at four temperatures (15, 19, 22 and 28 °C) and two feeding regimes. Barnacles were grown individually on flexible plastic coverslips to allow all the parameters to be linked to each individual. Moults and growth were noted from settlement and once a set size had been attained adults were removed from their substratum and their bases stained. The adhesive was stained and the number of rings, area of cover and width of bands evaluated.

Growth levels were found to be higher at both high and low levels of food availability than those reported in earlier investigations for this species. Moults and basal growth were closely linked, as had been found in the past. Barnacle moulting was not linked to temperature when food resources were limited.

When adult adhesive ring production was examined and compared to moults shed, it was found that a ring was formed at every moult. Moreover, average coverage of the basis with adhesive increased both with increasing temperature and increasing food availability. However the mean width of the adhesive rings did not differ between feeding treatments, but did increase with increasing temperature. Barnacles grown on an inferior FR coating (T2) showed a strong negative correlation of CRS to temperature, as in the previous study (Johnston 2010). It was hypothesised that this could be due to

gaps between adult adhesive rings in the barnacles grown under cooler conditions resulting in extra energy being required to propagate the crack over each new band of adhesive.

5.2. Introduction

Balanus amphitrite is a cosmopolitan barnacle and, as such, is able to survive in a broad range of environments. Individuals can survive in water temperatures as low as 12 °C, but are unable to reproduce at temperatures less than 15 °C (Desai et al. 2006), with an optimal temperature postulated as 23 °C (Anil et al. 1995).

Early researchers on the biology and ecology of barnacles examined environmental factors affecting growth and moulting in many species including *Semibalanus balanoides*, *Balanus glandula*, *B. crenatus*, *B. eberneus*, and *Chthamalus dalli* (Grave 1933; Barnes and Powell 1953; Barnes and Barnes 1956; 1959; Sanford and Menge 2001). Unsurprisingly clear links were observed between temperature and food availability, and average growth and moulting frequency in most species. Food availability is related to other environmental factors that are important in the field, such as exposure to current and wave action (Moore 1934; 1935; Crisp & Bourget 1985; Sanford and Menge 2001), competition (Crisp and Bourget 1985), overgrowth by algae (Barnes 1955), shore height, surface contour and macro surface texture (Crisp 1960; Crisp and Bourget 1985). Crisp (1960) postulated that growth in the field was mainly controlled by food intake and that this was most affected by current flow.

Growth and moulting in most temperate barnacles are retarded by low temperature with little or no growth or moulting occurring in the winter months for many species e.g. *Semibalanus balanoides* (Moore 1934; Crisp 1960; Barnes and Stone 1974). When examining temperature and feeding effects on moulting and growth, many researchers have utilised adult (or near adult) animals, of unknown age, collected from the field (e.g. Moore 1934; Patel and Crisp 1960; Barnes and Stone 1974). Studies of newly settled individuals of *B. trigonus* (Werner 1967), *S. balanoides* and *B. crenatus* (Barnes

and Powell 1953; Barnes 1953) showed that newly settled individuals grew at higher rates than larger adults.

Costlow and Bookhout (1953) studying *B. improvisus* found that there was no apparent correlation of growth of the barnacle shell with moulting, finding on occasions that no measurable growth occurred between moults. This result was supported by a recent study on *Austromegabalanus psittacus* (Lopez et al. 2008). However, other histological work demonstrated a direct link between moulting and adhesive ducts and shell growth in balanids (Saroyan et al. 1968).

Many inferences have been made about barnacle growth using field-based experiments. While realistic in terms of the environmental factors, these do not allow for direct analysis of individual factors on the parameters to be tested (e.g. temperature is often linked with food availability). Controlled experiments have been carried out on *B. amphitrite niveus* (Costlow and Bookhout 1956) and *B. improvisus* (Costlow and Bookhout 1953) looking at moulting and growth within the laboratory at 20 °C with changing light levels or feed respectively. However, even in these cases wild settled cyprids were used translating to large scale variations in the reserves of the newly settled juvenile barnacles.

There have been few studies on *B. amphitrite* at controlled temperatures and feeding regimes, and only one study has reported on adhesive production at different temperatures (Johnston 2010). This study examined the protein components of the adhesive and did not determine amounts of adhesive in contact with the substratum, or how the adhesive production might be linked to moults.

It would seem obvious that an organism that is under pressure in terms of its nutrients (or resources) might prioritise their use in different ways compared to one that is well supplied with nutrients, for example by laying down less adhesive per unit base area, or growing less and reproducing later. Individuals that are stressed (by temperature and/or food depletion in this case) might also be expected to behave in different ways from individuals held at ideal temperatures and with unlimited food resources. To test this hypothesis cyprids were settled and grown at different temperatures and two feeding

regimes (fed to excess and fed sparingly, termed ‘overfed’ and ‘underfed’ respectively) for over 5 months, or until they reached a size at which they could either be carefully peeled from a thin plastic, vinyl coverslip or pushed off T2-coated microscope slides. T2 is a silicone product commonly used in fouling release testing. As a relatively poor fouling release compound it is often used as an excellent standard for fouling release experiments. The bases of individuals were then examined to determine the adhesive secretion using either light microscopy on either stained or unstained individuals, or environmental scanning electron microscopy (ESEM). The aim of this experiment was to assess if food availability and temperature affected the amount of adhesive in contact with the substratum. Additionally it aimed to determine if any relationship existed between the environmental factors and the pattern of adhesive production, in terms of the number and width of adhesive rings produced

ESEM avoids many of the limitations of SEM (scanning electron microscopy) in terms of imaging (many) biological specimens, in particular it avoids the requirement for a high vacuum environment, needing clean, dry non-light emitting samples that are electrically conductive. This final point is often problematic as most biological samples are nonconductive and therefore, in order to obtain an SEM image, the sample has to be coated with a conductive element, most commonly gold. This drying and coating has the potential to introduce artefacts. By using ESEM a sample can be studied directly, even in a damp environment, thus maintaining the real environmental conditions of the sample being imaged. Comparing two methods of measuring the adhesive aimed to determine if the much cheaper method of staining and light microscopy with digital imaging was as effective as ESEM imaging.

Most studies on moulting and growth to date have used large numbers of individuals kept together and have averaged out moults and growth across the group. While this is a rapid and robust method to test the environmental effects on a broad scale it does not allow effects on individuals to be followed, such as moults and adhesive deposition, environmental effects on area of adhesive laid down and direct measures of adhesive secretion and its links to moulting. In order to examine these effects cyprids grown from laboratory broodstock were settled in the laboratory, ensuring very similar energy reserves in the cyprids and thus juvenile barnacles. The settled barnacles were all kept

individually under controlled conditions to allow individual barnacle moults to be identified and growth to be determined. This individual treatment allowed the adhesive for each barnacle to be stained and analysed to determine if there was any link to the growth and moulting frequency and what effect, if any, temperature and food availability might have on adhesive production.

5.3. Methods

5.3.1. Effect of temperature and feeding level on adhesive production, growth and moulting

B. amphitrite cyprids, obtained as previously described (Section 3.3.2) within 12 h of metamorphosis from the final naupliar stage, were settled individually onto vinyl coverslips (VWR international) over 24 h at 28 °C in the dark. The coverslips were then rinsed with 0.2 µm filtered seawater (salinity 35) and placed individually into 6-well plates. Fifteen ml of either undiluted *Tetraselmis suecica* (CCAP 66/4) algal culture ($1.5-3 \times 10^6$ cells ml⁻¹, termed ‘overfed’) or a 1000 times dilution of the same culture ($1.5-3 \times 10^3$ cells ml⁻¹, termed ‘underfed’) were added to each well. Twenty replicate barnacles of each feeding treatment were kept in incubators at four temperatures (15, 19, 22 and 28 °C all ± 1 °C) on a 12:12 light:dark cycle. Individuals were cleaned and fed the same algal concentrations as described above three times a week (Monday, Wednesday and Friday) at which time any moults in each well were noted and removed. After the first week of growth, juvenile barnacles were scanned using a flat bed scanner (HP Scanjet 5400c) twice each week and the area of each individual’s basis measured using Image J software (v1.45s).

The number of moults for each treatment was averaged over the whole experimental period and used to determine a final moult frequency in units of exuviae/100 animals/day (a commonly used unit, e.g. Barnes and Stone 1974) to compare with the results of other studies for various barnacle species.

In order to determine the effects of feeding levels, all data were converted into degree days by multiplying age (in days since metamorphosis from cyprid) by the temperature (°C) at which the animals were maintained. This allowed all the different ‘effective metabolic ages’ of the barnacles grown at different temperatures to be directly compared for growth and moult frequencies effectively removing much of the direct effects of temperature (c.f. Neuheimer and Taggart 2007).

Once individuals within a treatment were large enough ($> 8 \text{ mm}^2$ basal area), they were carefully peeled away from the plastic cover-slips. Two undamaged individuals from each treatment were randomly selected for ESEM to visualise the rings of adhesive on the bases. These individuals were rinsed using ultra-pure fresh-water (Milli Q) and dried in a 28 °C incubator for 10 min prior to being positioned on a standard SEM pin stub mount and adhered with nonconductive, double-sided carbon adhesive tabs. Samples were then stored at 6 °C until time was available for ESEM imaging (a maximum of 3 d). During imaging the adhesive rings were counted and measures of areas of rings for one individual for each treatment were taken (as far as possible in the time available; ring numbers were always counted in real time, while some measures for areas of rings were taken using the images captured to disc). After individuals were imaged using ESEM they were then stained along with the remaining individuals as follows.

Remaining individuals and their corresponding plastic coverslips were stained using Coomassie Brilliant Blue stain (CBBR; Chapter 4) for 5-6 h, destained for 30 min and rinsed in reverse osmosis (RO) water prior to being dried. Stained individuals and their coverslips were digitally imaged at 250x magnification using standard optical microscopy. The images were used to determine the number of rings present on the basis. This was possible for all individuals removed. The images of five barnacles for each treatment were used to calculate the average percentage area of the basis covered by adhesive. This was done by measuring the area of each ring of adhesive using Image J software, adding them all together to obtain the total adhesive area and dividing that value by the total area of the barnacle basis to obtain the percentage adhesive cover (Fig. 5.1.). The average width of each barnacle’s adhesive rings for each treatment was also calculated from a minimum of 20 measurements. Each ring was divided into quarters and where possible eight widths measured at roughly equal distances apart

within each quarter to make 32 measurements. When rings became too small for eight points to be measured (near the centre), five points in each quarter were used. The average of all width measurements for each ring was used to determine the average adhesive ring width for that barnacle (Fig. 5.1).

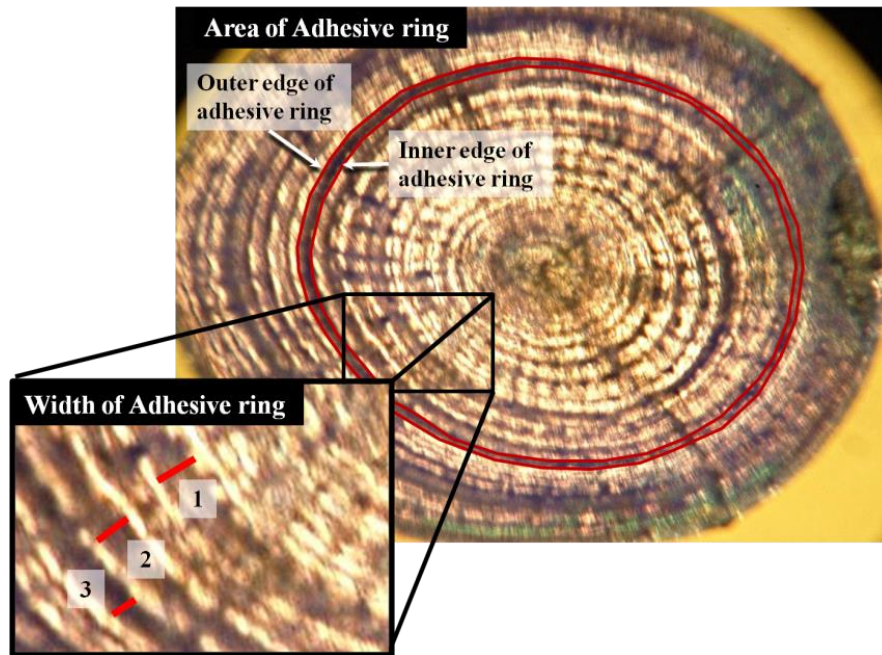


Figure 5.1. A stained barnacle (*B. amphitrite*) basis showing the rings of cement and the measurements taken for comparison between treatments.

5.3.2. Effect of temperature on adhesion strength

Cyprids (as described in section 5.3.1) were settled, after storage for 3 d at 6 °C, onto glass microscope slides coated with T2 (Dow Corning; as described in Chapter 3). After 24 h, 10 slides were rinsed and placed in slide racks, submerged in 0.2 µm filtered seawater with around 1×10^5 cells ml⁻¹ *Tetraselmis suecica* (considered to be a mid range feeding level) at each of four temperatures (15, 19, 22 and 28 °C). The seawater was changed and new algae were added twice per week. Once a week the barnacle bases were scanned during the whole growth period of 85 d. Any individuals that grew close to each other were removed to ensure that no individuals were in contact at testing for CRS (procedure as described in Chapter 2).

5.3.3. Analysis of data

The data for size, as basal area, over time from metamorphosis at the different temperatures and feeding levels was log transformed prior to all statistical analysis. The relationship between each pair of data (e.g. basal area and age) was examined using Pearson's coefficient of correlation followed by a multiple regression to obtain a model allowing an estimate of the relative importance of the factors examined to be obtained.

Similarly a log power transformation, correlation and multiple regression comparison were carried out for cumulative moult production with basal area, age, temperature and feeding regime.

Where transformed data fulfilled the assumptions ANOVA with a post hoc Tukey's test was used to determine specific differences between means, otherwise a Kruskal-Wallis test was carried out with a post hoc Dunns test to determine where the significance lay.

Data for CRS, adhesive ring width and ring numbers were not transformed prior to statistical analysis, as these data were normal and had equal variances (Anderson Darling and Levine's test) and thus ANOVA with post hoc Tukey's test was used to compare means.

Percentage coverage of the basis was arcsine transformed prior to statistical analysis. ANOVA and a post hoc Tukey's test were used to compare transformed mean proportions of bases covered with adhesive in the different treatments. (Zar 1998)

5.4. Results

5.4.1. Method development

Survival was good in all treatments with 100 % survival occurring at 22 °C and 19 °C independent of feeding level, and at 28 °C when overfed. At 15 °C 90 % of the

barnacles survived to testing age at both feeding levels. The lowest survivorship was found for underfed individuals at 28 °C, with only 70 % surviving to testing.

Removal of barnacles from the plastic coverslips without damage was quite successful with 80 % of individuals being intact after removal. Staining using CBBR and light microscopy imaging proved to be a more cost effective and time efficient method of visualising the adhesive rings compared to ESEM (Fig. 5.2.).

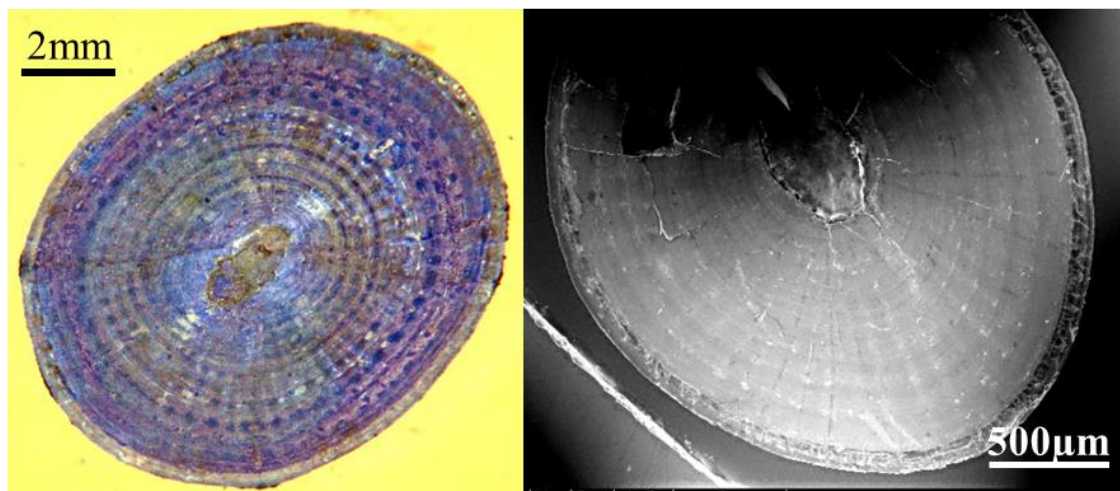


Figure 5.2. Image of a stained barnacle basis (left) and an ESEM scan (right).

Generally when using the ESEM the basis was too large to visualise in a single image and thus a montage of images had to be used to determine ring numbers and areas. When adhesive ring numbers and areas were enumerated and measured using ESEM and directly compared with the same individual following staining, light microscopy and Image J the results were very similar with linear relationships being close to 1 and highly significant (Fig. 5.3., $p \leq 0.001$).

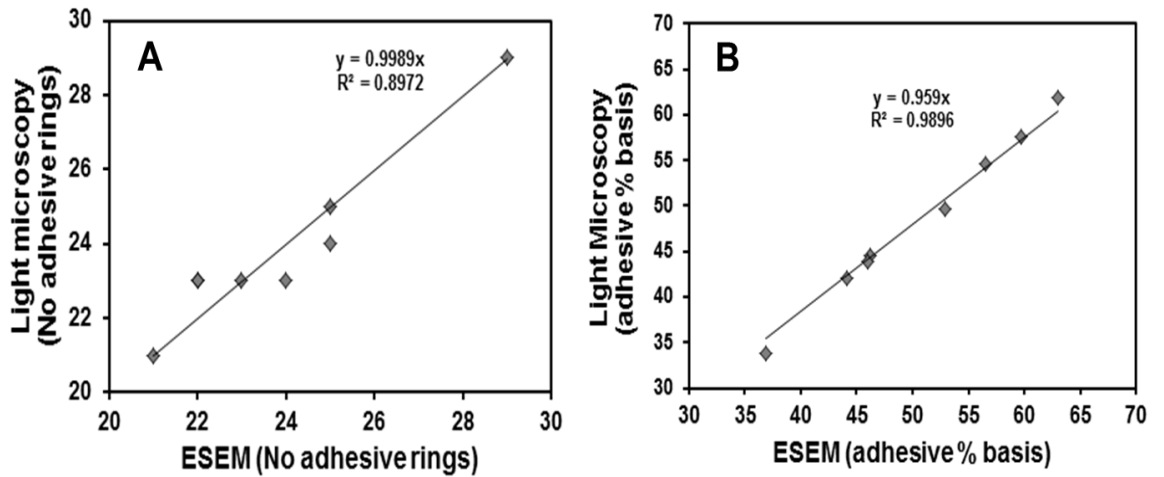


Figure 5.3. Direct comparison of light microscopy/staining results and ESEM results for A) ring number and B) adhesive percent area calculations for the same barnacles, with linear regression lines, equations and R^2 values on the graphs.

Often the adhesive rings were more easily counted by staining the plastic coverslips that the barnacles were removed from, as clear rings were left behind in over 75% of cases (Fig. 5.4.).

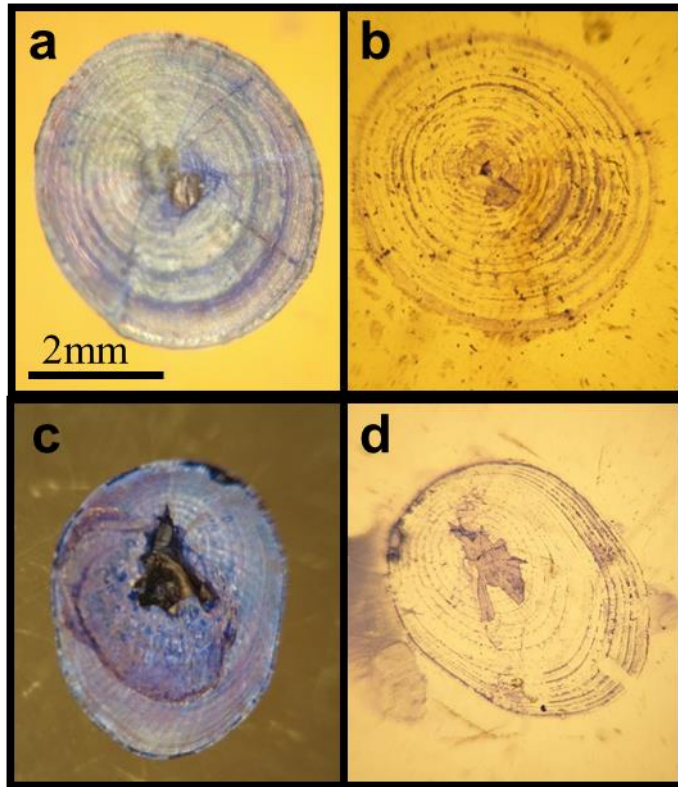


Figure 5.4. Digital images of two barnacle bases (a & c) and their coverslips (b & d respectively) stained with CBBR to show the rings of adhesive.

5.4.2. The effect of temperature and feeding on growth

Barnacles grown at all temperatures in the underfed treatments (Fig. 5.5.B) took almost twice as long to reach a specified size as the overfed individuals (Fig. 5.5.A). Overfeeding barnacles increased the variability in individual basal areas (Fig. 5.5.A and B). Barnacles at the highest temperatures and feeding levels grew at the highest rate and reached the greatest mean basal areas (means \pm 95% CI; 28 °C = 24.2 ± 2.17 mm², 22 °C = 26.4 ± 1.14 mm², 19 °C = 17.8 ± 2.62 mm²), such that at 57 d they were removed from the experiment and peeled off the coating for analysis as they were at risk of becoming too large. Barnacles at the lowest temperatures and feeding regime grew at the slowest rates; underfed individuals at 15 °C had only reached a mean basal area of 10.3 ± 1.60 mm² at the end of the experiment at d 161. Interestingly, barnacles kept at the highest temperature but underfed also grew very slowly and reached a mean basal area of only 11.3 ± 2.07 mm² at d 161.

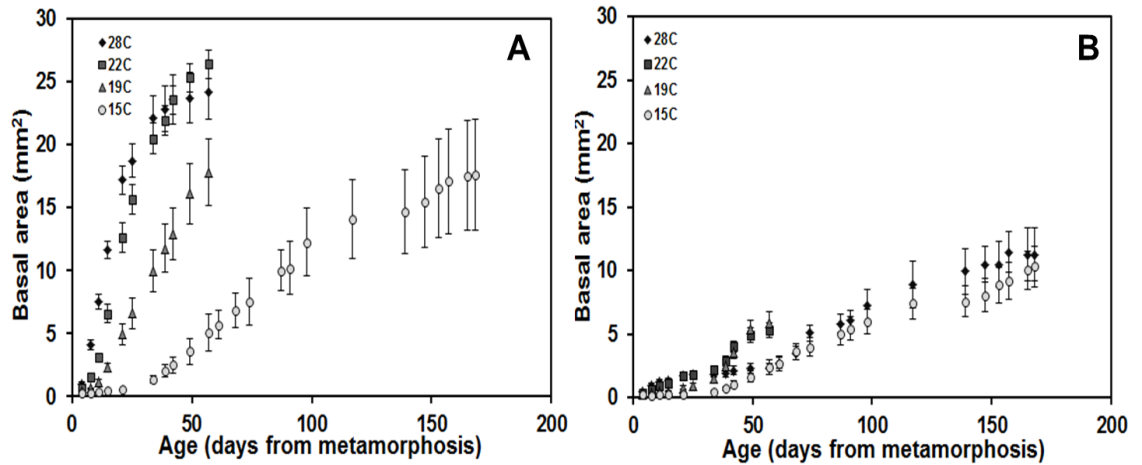


Figure 5.5. Relationship between growth of overfed (A) and underfed (B) barnacles in terms of mean basal area (\pm 95% CI) and elapsed time from metamorphosis.

The effect of temperature on the growth of barnacles from d 4-161 was affected by the feeding rate with overfed individuals (Fig. 5.5.A) showing a significant ($p < 0.01$, ANOVA) temperature effect at all but the two highest temperatures (28 °C and 22 °C, $p = 0.09$). Underfed individuals responded less to temperature in terms of growth of basal area (Fig. 5.5.B) with barnacles exposed to the highest two temperatures being similar in size ($p = 0.28$), and likewise the areas at the lowest two temperatures were not significantly different ($p = 0.22$). However barnacles cultured at the highest temperatures had significantly greater basal areas compared to the lowest temperatures ($p < 0.03$).

When the log transformed basal area data was modelled (and feeding regime was given an arbitrary numerical code of 0 for underfed and 1 for overfed) using multiple regression the resulting model (Equation 5.1) explained 78% of the variability in age and each of the factors (intercept, basal area, temperature and feeding level) had a significant impact on the model ($p < 0.0001$).

$$Age \text{ (in days)} = 108.47 + 69.90 * \log(BA) - 3.38Temp - 47.34FL$$

Equation 5.1.

BA = basal area mm²

Temp = temperature

FL = feeding level (high=1, low=0)

If the data are power log transformed (both basal area and age log transformed) then the equation can be further simplified (Equation 5.2). However this equation only explains 52% of the variability of the data and as such is a weak, though highly significant ($p > 0.0001$, Fig. 5.6), model.

$$\log \text{ Age (in days)} = 0.55 * \log \text{ Basal area (in mm}^2\text{)} + 1.25$$

Equation 5.2.

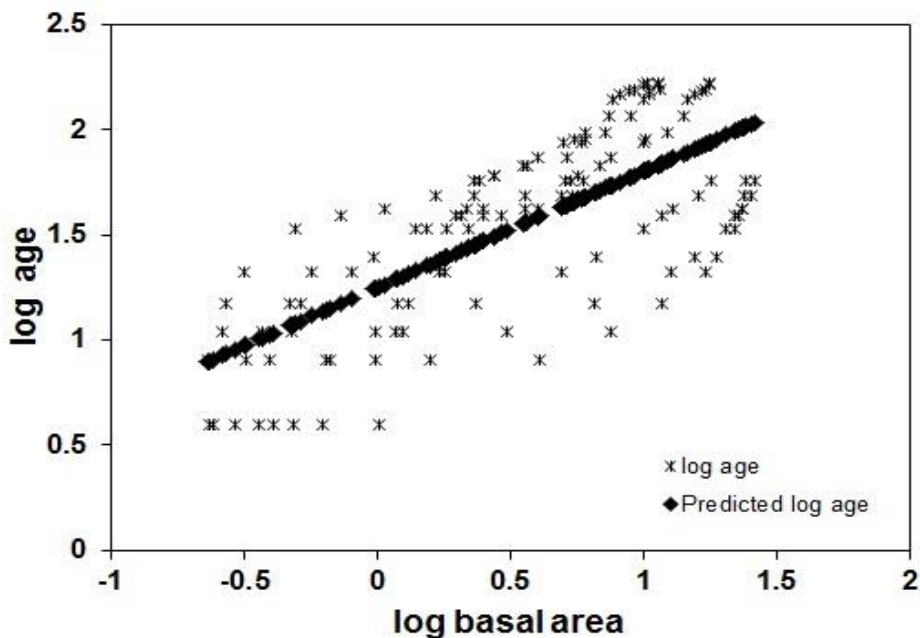


Figure 5.6. The comparison of the relationship of log age and log basal area with the predicted log age from the model (Equation 5.2) with increasing log basal areas.

The conversion of the data into degree days does not explain the overfired data, with large differences still being present within the data (Fig. 5.7., overfired $R^2 = 0.31$ and $p = 0.308$, regression), while the underfired data is well described (Fig. 5.7., underfired $R^2 = 0.86$ and $p < 0.001$, regression).

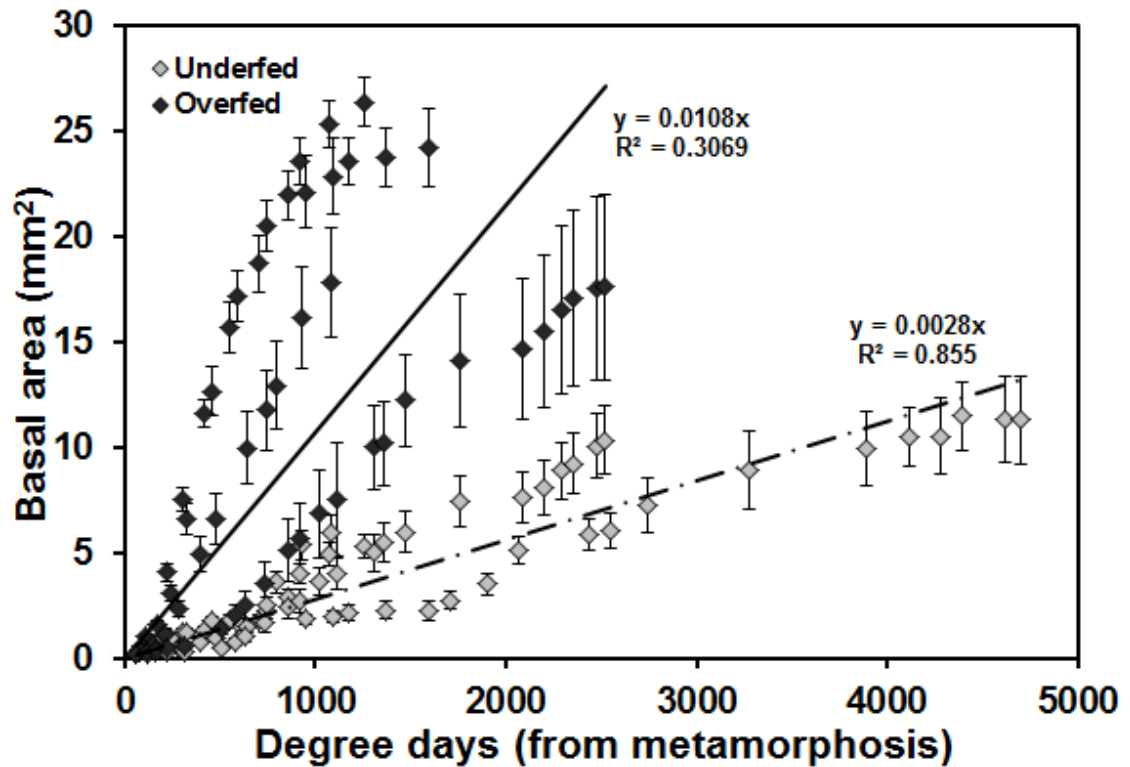


Figure 5.7. Relationships between areas of bases of both overfed and underfed individual barnacles and elapsed time with temperature treatment converted to degree days (linear relationships shown; solid line for overfed barnacles; broken line for underfed barnacles).

5.4.3. The effect of temperature and feeding on moulting

The relationship between moulting and age, for each treatment was best described using a 2nd order polynomial relationship (suggesting a peak level was reached resulting in a single curving point in the graph; Fig. 5.8.A and B; Table 5.1.). After log power transformation moulting had a significant positive linear relationship with age at all temperatures at both feeding rates (Fig. 5.9.A and B; p for regressions ≤ 0.013 , Pearson's coefficient ≥ 9.96 ; Table 5.2.). Temperature significantly affected the relationship (comparison of regressions $p < 0.01$), but the effect was less marked in the underfed individuals (Fig. 5.8.B, Fig. 5.9.B) than the overfed (Fig. 5.8.A, Fig. 5.9.A).

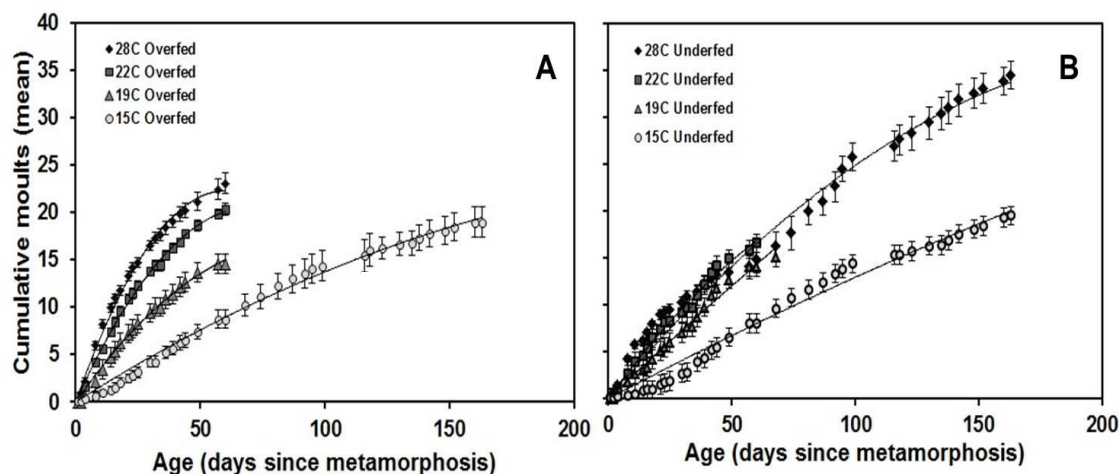


Figure 5.8. Cumulative number of moults of barnacles (mean \pm 95% CI) exposed to different temperature regimes with increasing age of overfed (A) and underfed (B) individuals. Second order polynomial relationships fitted, (equations and r^2 values in table 5.1.).

Table 5.1. Equations and r^2 values for 2nd order polynomial relationships from data shown in Figure 5.8.A and B.

Treatment	Polynomial equation (untransformed data)	Polynomial r^2
28 °C Overfed	$y = - 0.0062x^2 + 0.74x$	0.9944
28 °C Underfed	$y = - 0.0007x^2 + 0.32x$	0.9835
22 °C Overfed	$y = - 0.0041x^2 + 0.58x$	0.9966
22 °C Underfed	$y = - 0.0013x^2 + 0.36x$	0.9909
19 °C Overfed	$y = - 0.0021x^2 + 0.37x$	0.9937
19 °C Underfed	$y = - 0.0002x^2 + 0.25x$	0.9874
15 °C Overfed	$y = - 0.0003x^2 + 0.17x$	0.9930
15 °C Underfed	$y = - 0.0001x^2 + 0.14x$	0.9816

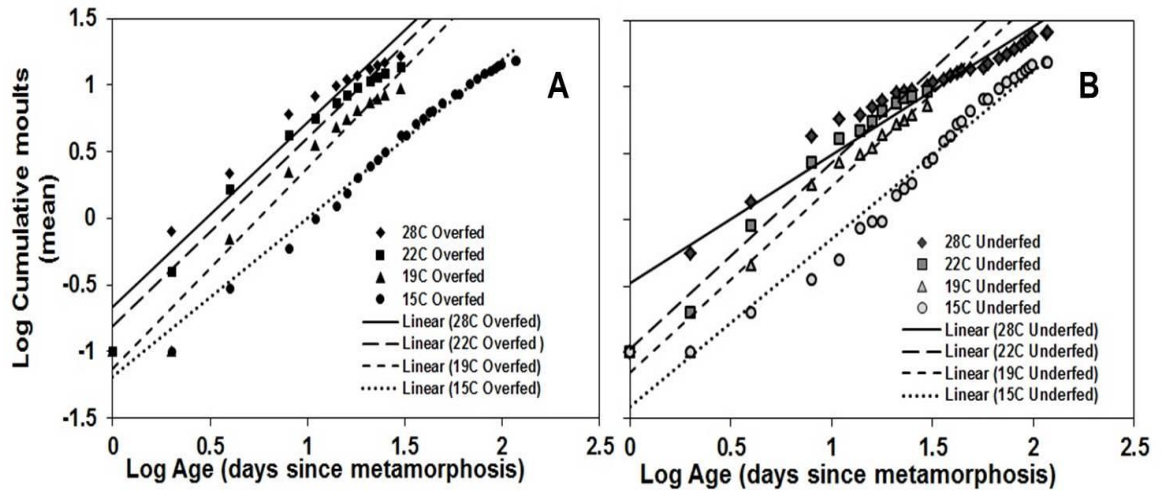


Figure 5.9. Mean cumulative number of moults (log transformed) for overfed (A) and underfed (B) barnacles over the log transformed age range of the experiment. Linear regressions fitted; regression equations, r^2 and significance shown in table 5.2.

Table 5.2. The equation of regression, level of fit (r^2) and significance of the regression for power transformed cumulative number of moults over time since metamorphosis for each of the treatments tested.

Treatment	Equation of regression	R^2 value for regression	Significance of regression
28 °C Overfed	$y = 1.39x - 0.67$	0.945	0.013
28 °C Underfed	$y = 0.97x - 0.48$	0.922	<0.0001
22 °C Overfed	$y = 1.42x - 0.81$	0.973	0.013
22 °C Underfed	$y = 1.41x - 0.97$	0.982	0.013
19 °C Overfed	$y = 1.51x - 1.13$	0.968	<0.0001
19 °C Underfed	$y = 1.41x - 1.15$	0.972	<0.0001
15 °C Overfed	$y = 1.19x - 1.19$	0.989	<0.0001
15 °C Underfed	$y = 1.26x - 1.41$	0.977	<0.0001

Only at 28 °C were the linear regressions of transformed moult and age data for overfed and underfed barnacles significantly different ($p < 0.0001$). All other temperatures showed no significant difference for moult and age regressions between feeding levels ($p \geq 0.158$, Table 5.3.). Overfed barnacles at 28 °C were significantly different to only

those grown at 15 °C when feeding levels remained the same ($p = 0.013$; Table 5.3.), while underfed barnacles at 28 °C were significantly different to all other temperatures moult rates at this feeding level ($p < 0.001$; Table 5.3.). When barnacles were kept at lower temperatures 22-15 °C their moults over time were more similar, only being significantly different to overfed barnacles at 15 °C ($p \leq 0.002$; Table 5.3.), with the exception of overfed barnacles at 19 °C which were also significantly different to underfed barnacles at 15 °C ($p = 0.012$).

Table 5.3. The significance levels (upper half of table and in bold) of comparisons of the linear regressions and the significance of the elevation of intercepts (not in bold) and the value of the elevation if not significantly different (in parenthesis) for the power transformed data of cumulative moults for each treatment over time from metamorphosis.

	28 °C Overfed	28 °C Underfed	22 °C Overfed	22 °C Underfed	19 °C Overfed	19 °C Underfed	15 °C Overfed	15 °C Underfed
28 °C Overfed		0.0006	0.8183	0.9021	0.3890	0.9010	0.0131	0.2144
28 °C Underfed	n/a		0.0001	0.0001	<0.0001	0.0002	0.0004	<0.0001
22 °C Overfed	0.0529 (-0.739)	n/a		0.8769	0.4389	0.8989	0.0007	0.0883
22 °C Underfed	<0.0001 (n/a)	n/a	0.0004 (n/a)		0.3296	0.9913	0.0005	0.1059
19 °C Overfed	<0.0001 (n/a)	n/a	0.0002 (n/a)	0.3237 (-1.050)		0.3784	<0.0001	0.0118
19 °C Underfed	<0.0001 (n/a)	n/a	<0.0001 (n/a)	0.0005 (n/a)	0.0218 (n/a)		0.0015	0.1207
15 °C Overfed	n/a	n/a	n/a	n/a	n/a	n/a		0.1578
15 °C Underfed	<0.0001 (n/a)	n/a	<0.0001 (n/a)	<0.0001 (n/a)	n/a	<0.0001 (n/a)	0.0002 (n/a)	

When the effect of temperature was removed by utilising the degree day conversion method, there was no significant effect of feeding level (Fig. 5.10., comparison of regressions $p = 0.1009$). However, while the data for the underfed barnacles using the degree day time-scale were well described using the linear regression ($r^2 = 0.9216$), only just over half the overfed barnacle moult data were described by a linear relationship ($r^2 = 0.5726$).

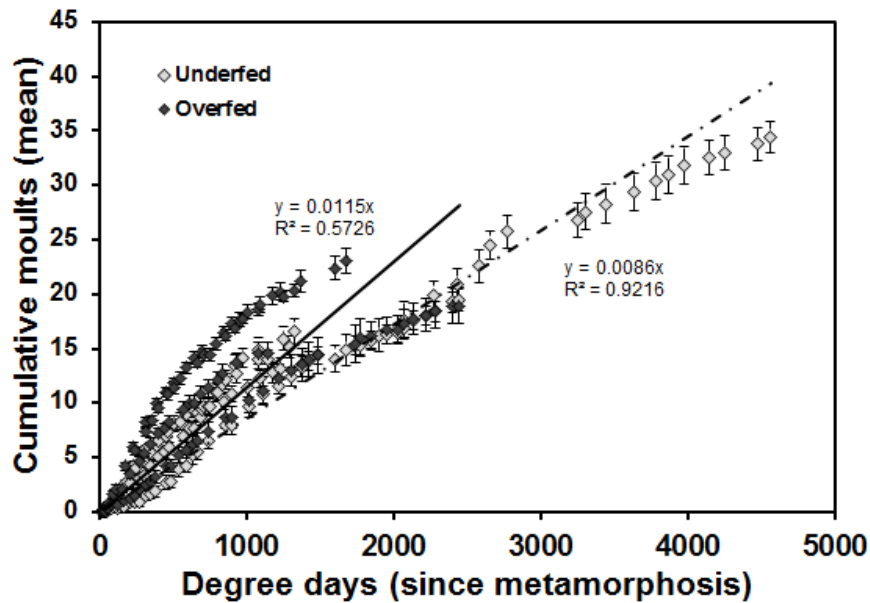


Figure 5.10. Cumulative number of moults of barnacles exposed to the two feeding regimes compared with the elapsed time/ temperature expressed as degree days. Mean cumulative number of moults \pm 95% CI, solid line = linear relationship for overfed individuals (equation and r^2 shown above the line); broken line = linear relationship for underfed individuals (equation and r^2 shown below the line).

When moults per 100 animals per day are compared for each temperature the effect of underfeeding can be clearly seen (Fig. 5.11.). At the lowest temperature, 15 °C, there is no effect of feeding level. As the growth temperature increases above 15 °C the difference becomes increasingly marked with underfed individuals at 28 °C showing lower moulting rates than all treatments above 15 °C (both over- and under- fed). Transformation did not result in significant linear relationships ($p \geq 0.061$) and as a result statistical comparison of relationships was not possible.

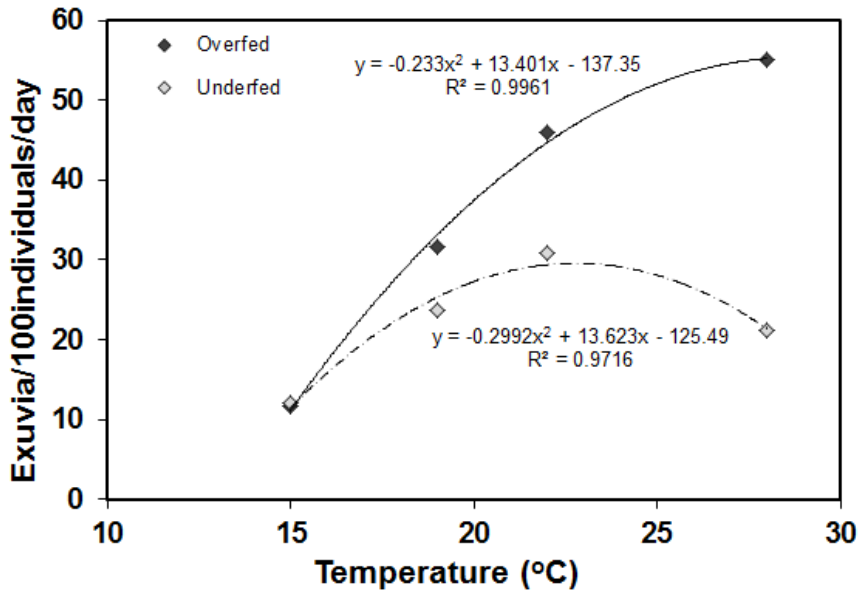


Figure 5.11. Moulting rates for each treatment expressed in terms of exuviae per 100 individual barnacles per day. Lines denote 2nd order polynomial relationships; solid line = overfed barnacles (equation and r^2 shown above the line); broken line = underfed barnacles (equation and r^2 shown below the line).

5.4.4. The relationship between moulting and growth

Over the first 60 d of experimentation (this limit was chosen as all treatments lasted for at least 60 d) the cumulative number of moults increased with increasing basal area. The positive relationship was strongly affected by temperature under both feeding regimes (Fig. 5.12.). The highest levels of moulting were found for individuals that were overfed at 22 °C, which also had some of the largest basal areas, though overfed individuals at 28 °C were similar in both moulting rate and growth. Lowest levels of moulting were found for individuals that were underfed at 28 °C. Growth in these individuals was also very low. The remaining treatments followed the trend of underfed individuals moulting and growing less than overfed individuals.

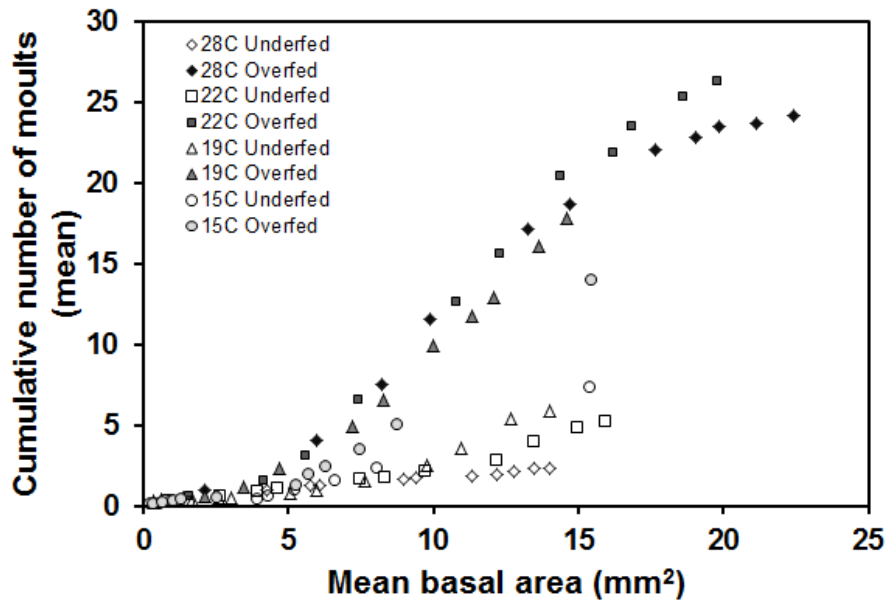


Figure 5.12. The relationship between moulting rate per treatment and basal area for *B. amphitrite* at different temperatures and feeding levels (mean, n=20).

When mean growth rate over the full experimental period (basal area increase per day; expressed as $\text{mm}^2 \text{d}^{-1}$) was compared to mean moult rate per day, a clear difference between individuals fed to excess and those underfed was seen. Underfed individuals showed a 10-fold decrease in growth but only halved their moult rate (Fig. 5.13.A and B). When individuals were underfed there was only a weak correlation ($r^2 = 0.3727$) between moult and growth rates (Fig. 5.13.B, $p = 0.04$) while when overfed there was a significant positive linear correlation of growth and moult rate (Fig. 5.13.A; $p > 0.0001$).

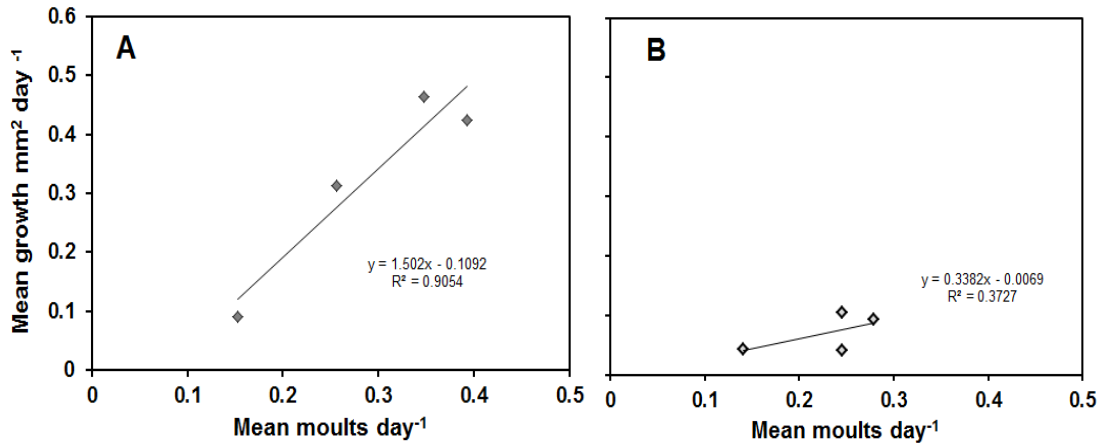


Figure 5.13. The relationship between growth rate and moult rate for A) overfed and B) underfed barnacles.

5.4.5. The relationship between moulting and adhesive ring production

The laying down of an adhesive ring in *B. amphitrite* was associated with moulting (Fig. 5.14). At all temperatures and both feeding regimes the number of moults was related to the number of adhesive rings stained in a near 1 to 1 relationship ($y = 0.9917x$, $p \leq 0.001$).

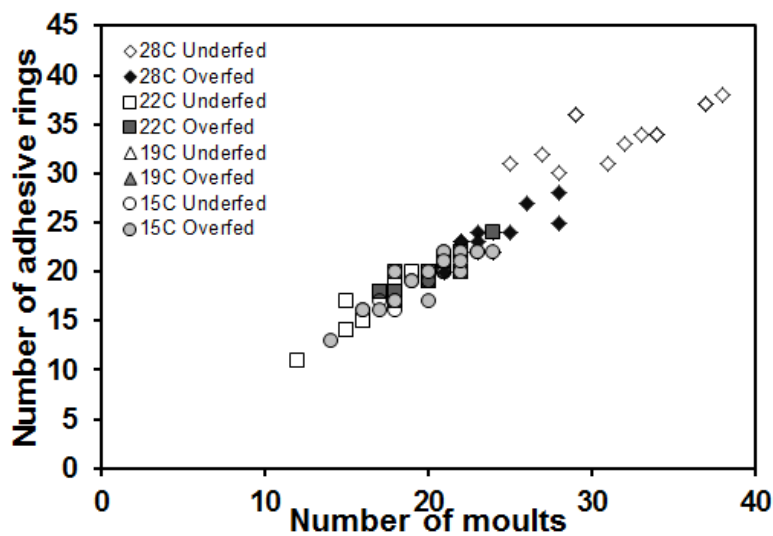


Figure 5.14. Relationship between the number of moults and number of adhesive rings for each individual barnacle in each treatment.

5.4.6. The effect of temperature and feeding regime on percentage adhesive cover of bases

When the percentage of the bases covered by adhesive for each treatment were compared there was a significant effect of feeding regime with overfed and underfed values being significantly different at all temperatures (ANOVA on transformed data $p < 0.001$, post hoc Tukey's test). There was also a clear relationship with temperature, with an increasing percentage cover of adhesive at both feeding regimes up to 22 °C followed by a drop off at 28 °C. This resulted in a poor linear fit to the untransformed data and a 2nd order polynomial relationship describing the data more accurately (Fig. 5.15.). When log power transformed the linear regressions were significant ($p \leq 0.004$). While the relationship between coverage and temperature of growth was the same for both feeding regimes (comparison of slopes $p = 0.79$ pooled slope 0.492), the barnacles showed a significant difference in percentage coverage of adhesive at each temperature dependent upon feeding regime (comparison of elevation $p < 0.0001$).

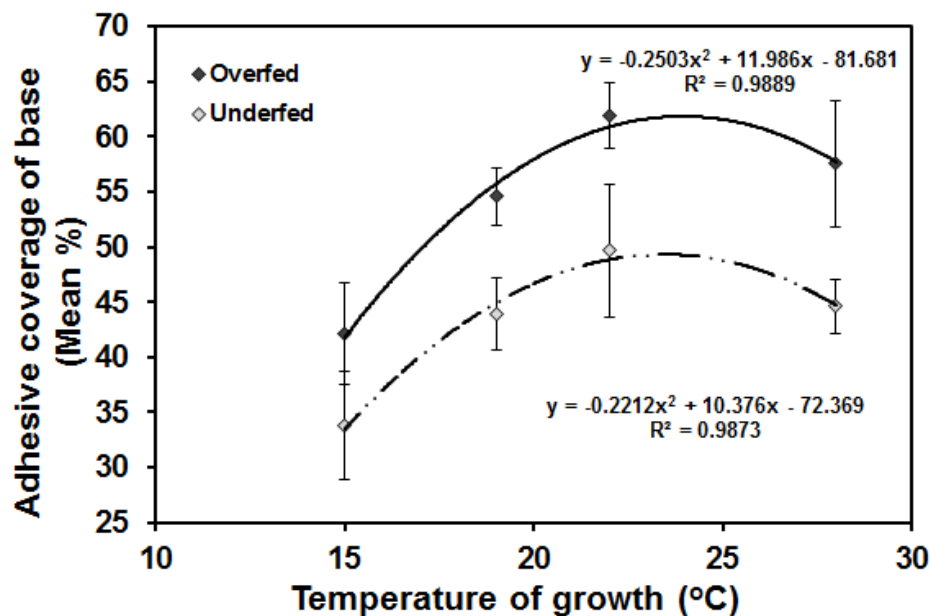


Figure 5.15. Relationships between temperature and percentage adhesive cover of bases (mean \pm 95% CI) for two feeding regimes with 2nd order polynomial curves fitted; solid line = overfed barnacles (equation and R^2 shown above the line) and broken line = underfed barnacles (equation and R^2 shown below the line).

5.4.7. Average width of adhesive rings measured

The average width of adhesive rings of barnacles grown in each treatment was not significantly different between feeding regimes at any temperature. However, the width of rings for the highest and lowest temperatures were significantly different from each other for both feeding regimes (Fig. 5.16., ANOVA, $p = 0.037$, post hoc Tukey's test). Further, the average width of an adhesive ring increased significantly with increasing temperature independent of feeding regime ($p \leq 0.02$ regression; Fig. 5.16.).

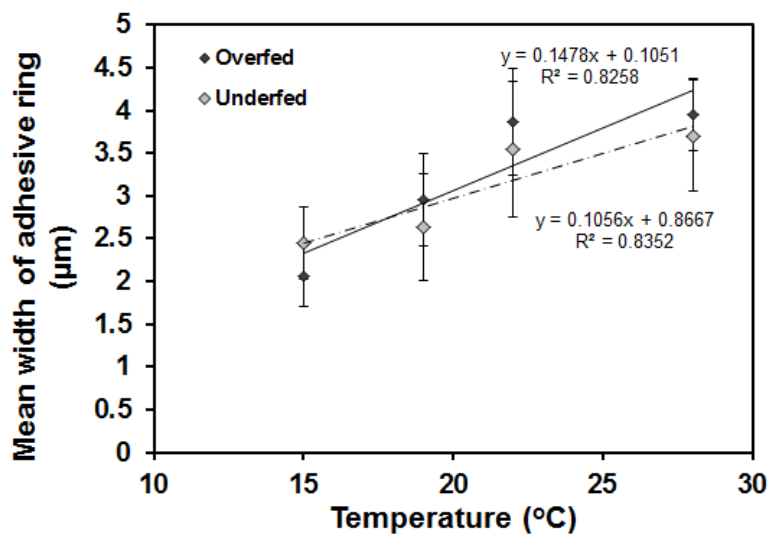


Figure 5.16. The relationship between adhesive ring width and temperature (mean \pm 95% CI) for barnacles exposed to two feeding regimes. The linear regressions are for overfed (solid line) and underfed (broken line) are plotted on the graph, with the equation and R^2 value shown above and below the line respectively.

5.4.8. The effect of the growth temperature of a barnacle on its CRS

For all temperatures tested, the numbers of bases showing cloudy abnormal cement (Berglin and Gatenholm 2003) was $\leq 5\%$. No removal data from these abnormal individuals were used for calculating average CRS. Each treatment had a minimum of 35 individuals with normal bases tested. No individuals were damaged significantly (i.e. leaving more than 10% of their basis on attached to the surface see Chapter 2) after removal.

There was a clear negative relationship between CRS and temperature (Fig. 5.17., p for regression = 0.013) with the lowest temperature resulting in a mean CRS of 0.23 (± 0.04 , 95% CI) MPa while the highest had a mean CRS of 0.18 (± 0.02 , 95% CI) MPa. The individuals at the lowest temperature also showed the greatest variability in CRS (Fig. 5.17.).

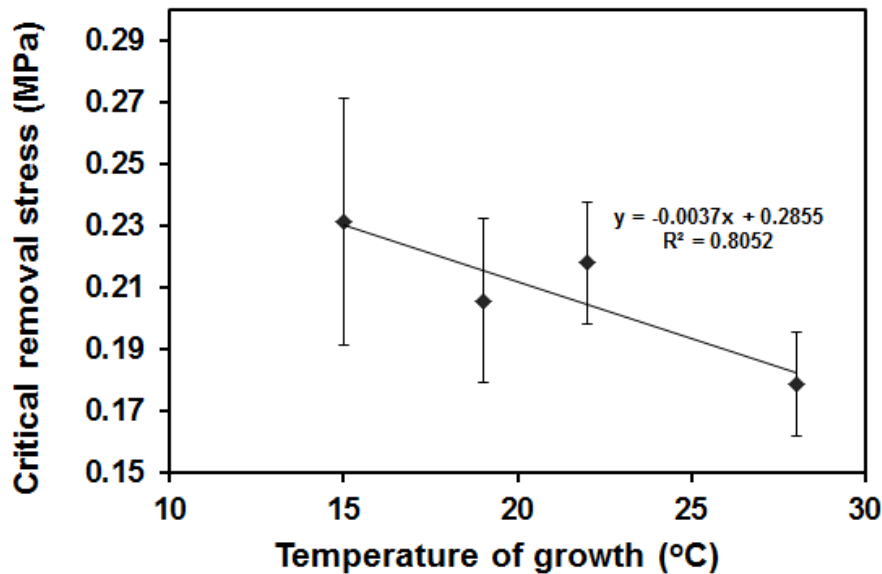


Figure 5.17. The effect of temperature on the mean CRS ($\pm 95\%$ CI) of *B. amphitrite*; linear relationship fitted; equation and R^2 shown above the line.

5.5. Discussion and Conclusions

Growing individual barnacles on plastic coverslips in individual wells allowed the control of feeding and the individual assignment of moults and as such the method was successful. Peeling barnacles off the plastic coverslips was successful in around 80% of cases; success may have been increased with the use of a more flexible plastic and allowing barnacles to reach a larger size in all cases prior to removal (all damaged barnacles tended to be the smaller individuals in a given treatment).

While ESEM resulted in more accurate measurements of adhesive ring width and area, the need to use montages of multiple images introduced error and added to the time

required for processing. The results of light microscopy and staining were very similar to those using ESEM (Fig. 5.3.). Therefore staining and light microscopy was a good technique for analysis of the adhesive bands of *B. amphitrite*.

5.5.1. The effect of temperature and feeding on growth of basal area

Unsurprisingly, overfeeding resulted in rapid growth compared to underfeeding. This was further enhanced by higher temperatures, with individuals at the two warmer temperatures growing faster than the lower temperatures (Fig. 5.5.A.). Underfeeding resulted in much slower growth and it appeared that the reduction in food was such that this became the limiting factor on growth as temperature no longer showed any effect (Fig. 5.5.B.).

Conversion of the data into degree days explains the underfed data well but does not explain the overfed data (Fig. 5.6.) suggesting there is another, unaccounted for factor affecting growth of these individuals. Possibly when fed to excess the algae were present in the wells long enough that the quality changed at different temperatures; perhaps algae produced toxins at some temperatures (though none of the species were known to be toxin producing), or the algae simply grew in the wells of the overfed individuals resulting in a variable feeding pattern with temperature. In their growth phase, algae may also have utilised micro-nutrients in the seawater required by the barnacles for growth. Algae in the overfed wells may have respired to such a level that the oxygen levels in the seawater became low enough to be stressful for the barnacles. Barnacles fed to excess would produce more excreta than those fed sparingly, although this was not measured here, and this increased level of excreta may have resulted in large levels of bacterial growth in these wells changing the growth of the barnacles due to stress, or infection.

When final sizes are used to calculate growth rates for each treatment over the course of the experiment and compared with those obtained by other researchers for *B. amphitrite* growth rates can be seen to be generally similar (Table 5.4.).

Table 5.4. Growth rates: area of bases ($\text{mm}^2 \text{ week}^{-1}$) in *B. amphitrite* , comparisons with previous studies.

Treatment	Present study	Johnston (2010)	Other studies	
15 °C starved/underfed	0.43			
15 °C fed/overfed	0.74	0.80		
19/20 °C starved/underfed	0.73			
19/20 °C fed/overfed	2.20		1.61	Costlow & Bookhout (1956)
22/25 °C starved/underfed	0.66			
22/25 °C fed/overfed	3.23	2.00	1.86	Wendt et al. (2006)
28/30 °C starved/underfed	0.51			
28/30 °C fed/overfed	2.98	1.75	3.30 1.12	Geraci et al. (2008) Simpson and Hurlbert (1998)

All treatments showed a slight curvilinear relationship between basal area and time over the period of the experiment. It is anticipated that if individuals were grown for longer periods, this curvilinear relationship would become more marked as energy is allocated to reproduction, and not just growth as has been shown in many other species (e.g. Van Rooij et al. 1995). However, due to the limitations of size for the ESEM, individuals were removed prior to this point.

5.5.2. The effect of temperature and feeding on moulting

There was a clear reduction in moulting rate with decrease in temperature and drop in feeding level (Fig. 5.8.A and B; Fig. 5.9.A and B). Comparable results have been reported in several studies on *B. amphitrite* and other species of barnacle (e.g. Costlow and Bookhout 1953, 1956; Barnes and Barnes 1959; Patel and Crisp 1960; Barnes and Stone 1974; Desai et al. 2006). When individuals were overfed, temperature significantly changed the moulting frequency (Fig. 5.8 A). However, when individuals were underfed the effect was less apparent with only the moulting level at the lowest temperature being significantly different from the others (Fig. 5.8.B). Similarly Patel and Crisp (1960) found that in starved barnacles moulting was largely independent of

temperature. Barnes et al. (1963) proposed that in *S. balanoides* carbohydrate is the primary source of energy when the animal is provided with food. During starvation (when carbohydrate is not available) the animal switches to use of lipid and protein. This results in a slower rate of metabolism and therefore moulting is little affected by temperature. They hypothesised that glucose in food was immediately metabolised and used in the formation of the exoskeleton to allow growth and increasing moult rate. Barnes et al. (1963) also determined that barnacles continued moulting even when no growth occurred (due to starvation) and suggested that the moulting cycle was linked to metabolic activity.

When the data were converted to exuviae per 100 barnacles per day (Fig. 5.11.) they showed even more clearly that underfeeding individuals at low temperatures had little effect on their moulting rate, as had been found by other researchers (e.g. Patel and Crisp 1960; Barnes et al. 1963; Barnes and Stone 1974). Rates of moulting seen in this study were much higher than those reported elsewhere for *B. amphitrite*. Patel and Crisp (1960) took adults from the field and exposed them to temperatures between 5 and 30 °C and determined their moult frequency. Desai et al. (2006) also took adults from the field and exposed them to different feeding and temperature regimes to determine the effect on moulting frequency. When the data from these earlier studies were converted to the same units as used here the increased rate of moulting seen in the present study is clear (Table 5.5.). This may well be due to the use of barnacles from settlement when the rate of growth is relatively high compared to that of adults in which growth would likely have slowed and much of the individuals' energy put toward reproduction. Young *B. amphitrite niveus*, used by Costlow and Bookhout (1956) showed a moult rate of between 47 and 50 moults per 100 individuals per day at 20 °C close to the maximum level seen in the present study (calculated from figures 5 series VI and 6 series VII in Costlow and Bookhout 1956). However, it may also suggest that the feeding level used in the present study was greater than in previous studies or that the food quality was better, although this is unlikely to have as great an impact as the use of young barnacles in the present study.

Underfed individuals in the present study showed higher moulting rates than the starved individuals in the study of Patel and Crisp (1960) probably due to the barnacles reported

here not being starved, while those in the Patel and Crisp study had to depend upon energy reserves. This is supported by the similarity shown in the moulting frequency of underfed individuals to those of the lowest feeding regime used by Desai et al. (2006, Table 5.5.).

Table 5.5. Comparison of the moults per 100 individuals per day at different temperatures and feeding regimes.

<i>Treatment</i>	<i>Present study</i>	<i>Desai et al. (2006)</i>	<i>Patel and Crisp (1960)</i>
15 °C starved/underfed	12.03		2.5
15 °C fed/overfed	12.02		7.5
19/20 °C starved/underfed	23.67	12	3.0
19/20 °C fed/overfed	31.67	28	16.0
22/25 °C starved/underfed	30.83	17	5.0
22/25 °C fed/overfed	46.00	31	24.0
28/30 °C starved/underfed	21.22	16	5.0
28/30 °C fed/overfed	55.00	37	26.0

5.5.3. The relationship between moulting and growth

While there is a clear link between moulting and growth (e.g. Bourget and Crisp 1975; Costlow and Bookhout 1956), this study was unable to ascertain if growth was directly linked to moulting due to the nature of the experiment. It is important to note however that when the growth per day and moults per day of overfed and underfed individuals were compared, the growth rate was greatly elevated in overfed individuals without such a marked increase in moult rate (Fig. 5.13. A and B). This showed that the amount of growth taking place at each moult interval was greatly increased in the overfed individuals. Variability in growth rate linked to moult cycle has been described previously amongst barnacles grown in identical conditions (Clare et al. 1994) but the difference between individuals was lower than that seen between overfed and underfed barnacles. Bourget and Crisp (1975) found *S. balanoides* that were fed moulted more often and grew more during the intermoult period with the increase in growth being

greater than the increase seen in moult rate. Due to the rapid growth of *B. amphitrite* seen here, adhesive may be produced to cover a larger area of the basis over a shorter time, perhaps resulting in poorer quality or thinner adhesive (something this study was unable to test).

5.5.4. The relationship between moulting and adhesive production

This study has shown that a ring of cement is secreted at each moult (Fig. 5.14.) in agreement with prior studies on other species (Sangeetha et al. 2010; Fernandez et al. 2002; Bourget and Crisp 1975; Bocquet-Vedrine 1963 and 1965 (translated)).

It was not possible to determine if the adhesive ring was laid down at the exact time of the moult or during the intermoult period. However what was evident was that the moult cycle in some way affects the laying down of new adhesive rings. Fyhn and Costlow (1976) using histological techniques found that the cement glands within *B. amphitrite* from the field accumulated during the intermoult cycle, strongly suggesting cement was laid down at the point of moulting. However laboratory maintained barnacles did not show such a link and Fyhn and Costlow (1976) hypothesised that there may be a seasonal element to the accumulation of cement as one batch was collected in autumn and the other in spring. This is supported by the results reported here: the quantity of adhesive produced (adhesive ring width; Fig. 5.16.) increased with increasing temperature and feeding rate (more likely in animals collected in the autumn period). Analysing the growth bands within the shell plates of barnacles, Bourget and Crisp (1975) showed that while there was a link with growth and submergence in the field there appeared to be no link to moult cycle and growth in *S. balanoides*. However, Bocquet-Vedrine (1965) showed a clear link in *Elminius modestus* with each moult linking to a shell ridge.

5.5.5. The effect of temperature and feeding on the percentage cover of the basis by adhesive

As temperature increased up to 22 °C the amount of adhesive covering the basis of the barnacle increased but after 28 °C the percentage cover again dropped. Underfed individuals always showed lower levels of adhesive coverage suggesting they may be under resource stress and unable to produce the ‘ideal’ amount of adhesive. At 28 °C both overfed and underfed individuals showed a reduced adhesive production suggesting they were also under some stress and not producing the ‘ideal’ amount of adhesive. It is uncertain why this might be and certainly it may have important implications as most laboratories use 28 °C to rear *B. amphitrite* to the adult stage for adhesion removal. While *B. amphitrite* grows at this temperature in the wild, they are only rarely exposed to this high a temperature throughout the year (NOAA 1983-1998). This result suggests that in the laboratory a lower temperature for growth of 22 °C would be more suitable for good growth and healthy levels of adhesive production. This would not require a large increase in timescales for growth as, although initially growth at 22 °C was slower by 34 d post metamorphosis, when barnacles at 28 °C reached testing size it was no longer significantly different (Fig. 5.18.). Barnacles reached testable size for CRS (5 mm diameter see Chapter 2 and ASTM D 5618. 1994) by d 34 with individuals grown at 22 °C being 5.1 mm and those grown at 28 °C having attained 5.3 mm basal diameter.

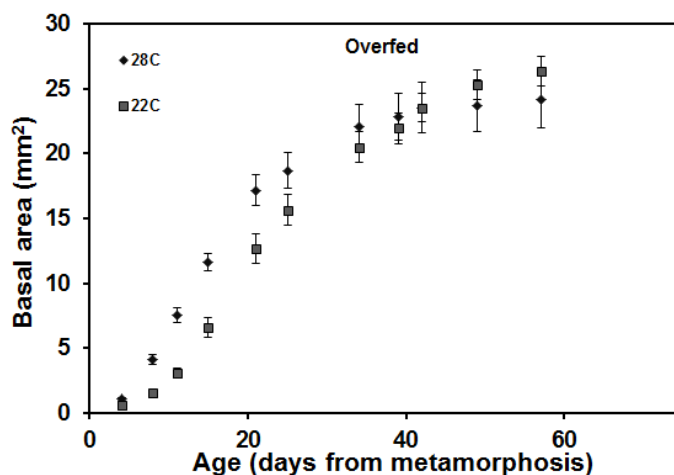


Figure 5.18. The effect of changing temperature from 28 to 22 °C on *B. amphitrite* growth over time from metamorphosis (mean \pm 95% CI)

The effects of temperature and feeding on the adhesive coverage of the barnacle basis have not previously been studied and thus it is not possible to compare these results to others.

5.5.6. Average width of adhesive rings measured

The width of the adhesive rings increased, on average, with increasing temperature (Fig. 5.16.) implying the growth of individuals was faster at higher temperatures (as previously discussed) and that the amount of adhesive laid down was therefore greater. Individuals at a higher temperature could perhaps produce more adhesive than those at lower temperatures due to increased metabolic rates. However, it would be expected that if more adhesive were being produced due to increased metabolic function (and/or energy reserves) overfed individuals would produce significantly more adhesive than underfed individuals and this did not appear to be the case (Fig. 5.16.). It may also be due to a thermodynamic effect, i.e. the viscosity of the adhesive itself being reduced at higher temperatures and therefore being able to flow more easily over the surface rather than more adhesive being released. Testing this theory was not possible in the timescale of this study, but would be relatively straightforward requiring the measurement of the thickness of the adhesive at the different temperatures, perhaps using sectioning and ESEM.

5.5.7. Temperature of growth and CRS

In a result similar to that found previously (Johnston 2010) the temperature at which a barnacle was grown significantly inversely affected the CRS (Fig. 5.17.). Johnston (2010) reported CRS values for removal from T2 close to those found in the present study (Table 5.6)

Table 5.6. Comparison of the present study and Johnston's (2010) CRS values in *B. amphitrite* grown at different temperatures

Present study		Johnston 2010	
Temperature (°C)	CRS (MPa)	Temperature (°C)	CRS (MPa)
15	0.23	15	0.22
22	0.22	25	0.16
28	0.18	30	0.13

There are a number of possible explanations for this decrease of CRS with increasing temperature of growth. The adhesive itself may have differing amounts or types of proteins expressed within in it, resulting in differing CRS as hypothesised by Johnston (2010). The makeup of the adhesive was not examined here but certainly this provides an interesting possibility.

Based on the detailed analysis of the adhesive rings, their number, width and percentage total basis coverage a fracture propagation hypothesis could explain the increase in CRS with decrease in temperature. Barnacles at colder temperatures tend to have narrower adhesive rings that cover a smaller proportion of the basis. One might reasonably expect a barnacle with less adhesive coverage to show a lower CRS, the opposite of what is found. However, a consideration of crack propagation during removal of the barnacle may help to explain the result (Kavanagh et al. 2005). Individuals grown at high temperatures have wide rings of adhesive, which often come into contact with each other, thus a crack is propagated along more than one ring of adhesive using the same energetic force. Individuals grown at low temperatures have many narrow rings of adhesive with clear gaps between the rings. Thus the crack must be re-initiated at each individual ring and energy entered into the system each time. When this is considered across a whole basis of the barnacle it may explain the counter intuitive increase in adhesive strength of *B. amphitrite* individuals grown at lower temperatures that have less adhesive.

However it must be considered that, temperature may have affected the coating itself in such a way that the adhesive was less able to form a good adhesive bond with it during development. Higher temperatures may also have adversely affected the utilisation of

the food or changed the partitioning of the resources in the higher temperature animals perhaps towards reproduction. Two individuals at the highest temperature (28 °C) and feeding regime expelled egg masses during the experiment. This re-allocation of resources may have resulted in ‘substandard’ adhesive being released, while the smaller amounts at colder temperatures may be better quality, though no measure of quality of adhesive was made, except indirectly using CRS. However, it is more likely that these individuals may partition fewer resources into growth and maintain adhesive integrity, given the importance of maintaining adhesion to the survival of a barnacle.

The fact that the CRS of such an important test species as *B. amphitrite* changes with environmental factors to such a degree should be a concern for those testing new products. Many use single field test sites often in the warm equatorial ocean to speed up growth. As a result many tests of this nature may pass coatings on to further more expensive trials when simply having a second trial site in a temperate zone may have removed the surface from consideration.

This environmental effect also explains why tests done by research laboratories using different growth parameters, controlled or uncontrolled (e.g. temperature and light regime; feeding levels), are so different and make comparisons difficult at best. This is even more the case for work carried out in the field compared to the laboratory.

It is also of concern that the standard temperature for laboratory culturing of *B. amphitrite* (28 °C) has been patently shown here to be suboptimal for both barnacle growth and adhesion. The optimal temperature range to gain highest growth and good adhesion in the laboratory is 19 °C to 22 °C. Modifying laboratory protocols to culture and test *B. amphitrite* at 22 °C, rather than 28 °C would provide more appropriate and rigorous conditions for FR studies, without significant increases in test duration.

Chapter 6: Active enzyme nanocoatings affect settlement of *Balanus amphitrite* barnacle cyprids.

Published: Mariana Tasso, Sheelagh L. Conlan, Anthony S. Clare and Carsten Werner **2012**

Active enzyme nanocoatings affect settlement of *Balanus amphitrite* barnacle cyprids

Advanced Functional Materials **22(1)** 39-47 Copyright © 2012 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

(M.T. and S.L.C contributed equally to this work; surfaces were designed and all characterisation of surfaces was carried out by M.T. Spin coated slides were provided, along with racks to allow functionalisation at Newcastle. M.T undertook initial trail assays with S.L.C. All further assays were carried out by S.L.C)

6.1. Abstract

Balanus amphitrite cyprids produce complex adhesive substances that enable their attachment to surfaces and impart a strong detachment resistance from most immersed substrata. The colonization of man-made structures by barnacle cyprids and other marine organisms is a troublesome and costly phenomenon, for which controlling strategies are actively sought. In this work, we expand previous investigations about the susceptibility of cyprid adhesives to unpurified proteases in solution by evaluating the interplay between these secreted biomolecules and a surface-confined purified protease. The strategy involved the covalent immobilization of the enzyme Subtilisin A to maleic anhydride copolymer thin films through the spontaneous reaction of anhydride moieties with lysine side chains. This enabled the production of bioactive layers of tunable enzyme surface concentration and activity, which were utilized to systematically evaluate the effect of the immobilized enzyme on cyprid settlement and exploratory behaviour. Surfaces of increasing enzyme activity displayed a gradual decrease in cyprid settlement levels (approaching inhibition) as well as an increase in post-settlement adhesion failure (evidenced by significant numbers of detached metamorphosed individuals). High activities of the bound enzyme also affected pre-settlement behaviour of cyprids, reducing the velocity and total distance moved while increasing the amount and speed of meander compared to the controls. The here-reported low enzyme surface concentrations found to be remarkably effective at

reducing cyprid settlement hold promise for the use of immobilized enzymes in the control of marine biofouling.

6.2. Introduction

Biofouling describes the undesirable accumulation of organic material and organisms, from unicellular to invertebrate species, on man-made surfaces (Yebra et al. 2004). Biofouling is a worldwide problem affecting a multitude of industrial processes and products, including food processing, medical devices, membrane separation, cooling systems, and ship hulls. In the marine environment alone, more than 4000 species of marine organisms are recognized as responsible for biofouling (Majumdar et al. 2008). Marine biofouling has been attacked from different fronts, from deterring organisms as they settle using biocides (Yebra et al. 2004; Negri and Marshall 2009) to more elaborate and environmentally-friendly options based on the principle of ‘non-stick’ or ‘fouling-release’ surfaces, which do not jeopardize marine life (Callow and Callow 2002; Anderson et al. 2003; Murthy et al. 2009; Ralston and Swain 2009).

Several marine organisms use proteinaceous adhesives to attach to surfaces (Kristensen 2008). Proteolytic enzymes are effective agents against settlement of marine bacteria, algae and invertebrates, their proposed mode-of-action being the enzymatic degradation of the proteinaceous components of the adhesives (Paul and Jeffrey 1985; Pettitt et al. 2004; Dugdale et al. 2005; Olsen et al. 2007; Aldred et al. 2008; Leroy et al. 2008). To date, research has focused on either the use of commercial preparations of proteases, which include up to 80% additives (such as sorbitol and calcium formate)(Pettitt et al. 2004) or the incorporation of pure or commercial preparations into potential antifouling paints and coatings (Kim et al. 2001; Dobretsov et al. 2007). Where commercial preparations are employed, biological results are compromised by the spurious effects of non-enzymatic components and, in general, by the limited knowledge of the actual surface concentration and activity of the enzyme. Hence, it is difficult to draw conclusions about the impact of the soluble or matrix-incorporated enzyme on the observed biological response.

Balanus amphitrite is a sub-tropical sessile crustacean considered to be a serious pest due to its rapid colonization of immersed man-made objects and its widespread geographical distribution throughout the sub-tropics (Kamino 2006; Aldred and Clare 2008). Barnacle cypris larvae explore a surface by ‘walking’ using their paired antennules bearing the attachment discs. In this exploratory phase, cyprids are capable of detaching, leaving behind deposits of temporary adhesive ‘footprints’ (Aldred and Clare 2008) that subsequently act as signaling molecules to induce the settlement of additional cyprids (Clare et al. 1994). On finding a suitable surface, the commitment to settlement is accompanied by the release of proteinaceous cement (cyprid permanent cement) that embeds the paired antennules and cures within one to three hours to form a discrete matrix (Aldred and Clare 2008). The attached individual subsequently metamorphoses and develops into the calcified adult barnacle (Aldred and Clare 2008). As an adult, a third, discrete adhesive is produced, which is renewable and has 90% protein content (Kamino 2006). The adult cement forms a thin disc between the basis of the adult barnacle and the surface to which it is attached (Kamino 2006).

Cyprid temporary adhesive, required for exploration and settlement by the larvae, is composed primarily of protein (Clare et al. 1994; Matsumura 1998) and is sensitive to hydrolysis by proteolytic serine proteases (Pettitt et al. 2004; Aldred et al. 2008). Aldred et al. (2008) demonstrated that the mode-of-action of Alcalase® (a commercial preparation containing the protease Subtilisin A) in preventing cyprid settlement was to degrade the proteinaceous temporary adhesive rather than deterring them from settling. The degradation of footprints was observed by atomic force microscopy: footprints disappeared entirely within 30 min of exposure to the enzyme. Conversely (as also observed by Pettitt et al.(2004)), cyprid permanent cement, while initially susceptible, became resistant to attack by Alcalase® within 15 h of release onto the substratum.

In this work, we aim to determine the susceptibility of the adhesives produced by *Balanus amphitrite* cyprids to the purified enzyme Subtilisin A bound to polymer nanocoatings. Subtilisin A (or Subtilisin Carlsberg; EC 3.4.21.62) is a well-studied serine protease with high specificity for the hydrolysis of proteins in aqueous media (Rawlings and Barrett 1994). The extracellular alkaline protease is produced by *Bacillus licheniformis*, has an average molecular weight of 27,280 Da, and comprises

274 amino acids (Markland and Smith 1971). The protease was covalently-bound to poly(ethylene-*alt*-maleic anhydride) copolymer films via the spontaneous reaction of anhydride moieties with the lysine amine groups of the protein (Tasso et al. 2009a). Previous work showed that these bioactive layers were effective at reducing the adhesion strength of spores of the green alga *Ulva linza* and cells of the diatom *Navicula perminuta* in a manner that correlated with the enzyme surface concentration and activity (Tasso et al. 2009b). The present study differs from the work carried out on algae in that the functional surfaces presented to barnacle, *Balanus amphitrite*, cypris larvae were of reduced enzyme content, activity and thickness. Nevertheless, the immobilized enzyme was able to interfere with settlement of cyprids and their permanent fixation to the surface, whereas algal attachment was not prevented. As for algae, a correlation between the protein content of the cyprid adhesive(s) and the efficacy of the immobilized enzyme is indicated.

6.3. Methods

6.3.1. Preparation of bioactive nanocoatings

Poly(ethylene-*alt*-maleic anhydride) (PEMA) and poly(octadecene-*alt*-maleic anhydride) (POMA) copolymer thin films were prepared as previously described (Tasso et al 2009a, Pompe 2003). Briefly, glass and silicon coverslips (for the characterization assays) or ultrasonically cleaned microscope glass slides and glass dishes (for the biological assays) were sonicated in water and ethanol for 30 min, then oxidized in a mixture of water:hydrogen peroxide:ammonia (volume ratio 5:1:1) at 70°C for 10 min, dried at 120°C for 1 h and subsequently modified with 3-aminopropyltrimethoxysilane by exposure to the compound in vapor phase overnight. The aminosilane-modified samples were thereafter rinsed in toluene, dried at 120°C for 1 h and spin-coated with PEMA (0.15 wt% in THF:acetone (weight ratio 2:1)) or POMA (0.08 wt% in THF) solutions. Stable covalent binding of the maleic anhydride copolymer film was achieved by annealing at 120°C for 2 h to generate imide bonds with the underlying aminosilane layer.

Covalent immobilization of Subtilisin A onto freshly prepared PEMA copolymer films was carried out by exposing the polymer layers to varying concentrations of the enzyme in solution ([Es]). Enzyme immobilization proceeded as disclosed in previous work (Tasso et al. 2009a and 2009b) although departing from much lower enzyme concentrations (Subtilisin A was dissolved in PBS, pH = 8.6 (adjusted with 1 and 5 M sodium hydroxide solutions) at concentrations between 0.01 and 3 mg ml⁻¹). The reduction in the enzyme concentrations used for immobilization was motivated by the lack of a settlement trend with regard to enzyme surface activity in the same activity range considered for marine algae. After overnight exposure to the enzyme solution, samples were rinsed 10 times with distilled water and were thereafter either used for assays or denatured. Denaturation of the active enzyme-containing coatings was achieved by heating the samples at 120°C for 45 min.

6.3.2. Characterization of the bioactive nanocoatings

The characterization of the bioactive coatings utilized in the biological assays with barnacle cyprids followed Tasso et al. (2009a and b) and involved the determination of immobilized protein amount, enzyme layer thickness, catalytic activity, stability upon incubation in aqueous media (aging), and surface roughness and wettability.

Briefly, the thickness of the immobilized enzyme layer was determined by single-wavelength ellipsometry (SE400, Sentech Instruments GmbH, Berlin, Germany) of freshly-prepared coatings (to avoid dehydration) using a five-layer model approximation (silicon/silicon dioxide/maleic anhydride bound to aminosilane/enzyme/air) and a refractive index for the enzyme layer of 1.375 (Tasso et al 2009a) (N = samples per condition = 4; m = measurements per sample = 6). The surface concentration (i.e. amount of immobilized enzyme per unit area) of aged and non-aged coatings was evaluated by confocal Laser Scanning Microscopy (cLSM) (TCS SP5, Leica Microsystems, Wetzlar, Germany) using immobilized TAMRA-labeled Subtilisin A and the previously-determined surface concentration of the highest surface concentration coating ($0.24 \pm 0.03 \mu\text{g}\cdot\text{cm}^{-2}$ (Tassos et al. 2009a)) as scaling factor (N = 4; m = 3). The activity of the enzyme-containing coatings was determined by following the conversion of the substrate Suc-AAPF-pNA into peptides and

phenylnitroaniline (pNa) through absorbance spectroscopy (TECAN Magellan GENios, Tecan, Austria) at 405 nm (N = 5; m = 1). The stability of the enzyme-containing coatings was assessed by evaluating the residual activity and surface concentration of the bioactive layers after incubation in artificial sea water (ASW*) for 48 h at room temperature (see Ref. (Tasso et al. 2009b) for the composition of ASW*). Atomic Force Microscopy (AFM) (MFP-3D, Asylum Research, Santa Barbara, USA; scan size = 4 μm) in tapping mode was employed to determine the surface root mean square (RMS) roughness of the coatings as freshly-prepared (N = 2; m = 2). Static water contact angles (OCA30, Dataphysics Instruments GmbH, Filderstadt, Germany) of active and control coatings were measured in air using de-ionized water and utilized as an indicator of the surface wettability (N = 4; m = 3). All characterization experiments (except for AFM) were run twice on independent sample batches.

6.3.3. *Balanus amphitrite* (syn. *Amphibalanus amphitrite* (Clare and Hoeg 2008)) cyprids

Adult, brood stock barnacles, supplied by Duke University Marine Lab, were maintained as per Hellio et al. (2004). Nauplii were cultured and grown to the cyprid stage as per Ref. (Hellio et al. 2004). Cyprids were stored for 3 days at $6 \pm 1^\circ\text{C}$ in 0.2 μm filtered natural seawater prior to all assays.

6.3.4. Settlement assay

The settlement assays with barnacle cyprids concentrated on the effects of both soluble and surface-bound enzyme on cyprid settlement. The general methodology of the ‘barnacle drop settlement assay’ was as described elsewhere (Tang et al. 2005). Enzyme immobilization was performed in situ and immediately before the assays to ensure the properties of the biolayers were as characterized.

Assays determining the effect of soluble enzyme used the base maleic anhydride (MA) coatings exposed to the enzyme buffer (PBS; pH = 8.6) overnight. Slides were then placed into Quadriperm dishes and exposed to a 1.5 ml drop of 0, 0.5, 1 or 1.5 $\mu\text{g ml}^{-1}$ of

active enzyme in 'Tropic Marin' artificial seawater (ASW) with 40 cyprids in each drop. Dishes were incubated in the dark at high humidity and at 28°C. Settlement and mortality were enumerated at 24 and 48 h.

The assays with immobilized enzyme used freshly-prepared active and denatured samples (as previously described). These slides were carefully dipped into paraffin wax along each edge of the slide to confine cyprids to the central area of enzyme immobilization. This resulted in 3 distinct areas: the central area with immobilized enzyme, the interface wax-immobilized enzyme, and the wax area itself. Twelve (active coatings) or six (denatured and PEMA control coatings) replicates of each concentration were thus produced. Surfaces were thereafter placed in Quadriperm dishes and exposed to a 1.5 ml drop of ASW containing 40 cyprids. Dishes were treated as in the previous experiment. At the end of each incubation period (24 and 48 h), the total number of settled cyprids was determined and their location noted (i.e. central, interfacial or wax area). Any individuals that had settled on the wax were excluded from data analysis. Larvae that did not settle after the 48 h experimental period were observed for signs of abnormal behaviour. During the experiments, a number of settled individuals were found to detach from the bioactive surfaces after having partially or fully metamorphosed. These detached individuals were carefully removed after the 48 h enumeration and kept for 2 weeks in ASW with two weekly changes of water and feeding (*Tetraselmis suecica*), at which time any dead individuals were noted.

Settlement results are presented as mean percent settlement (i.e. mean number of settled cyprids expressed as a percentage of the total number of dispensed cyprids) with 95% confidence intervals. Normality of the distribution was assessed by means of an Anderson Darling normality test (Minitab v15.1.0.0, Minitab Ltd., Coventry, UK). Settlement data were analyzed for statistical differences using the Kruskal-Wallis method and a post-hoc Dunn's test (GraphPad Prism v5.0, GraphPad Software, San Diego, USA).

6.3.5. Tracking assay

Tracking using EthoVision 3.1 software (www.noldus.com) was carried out as per Marechal et al. (2004). Thirty 3-day-old cyprids were tracked for 5 min each on active layers (activities 1.3 and 63 [pNa] min⁻¹ 10³) and the respective denatured and PEMA controls. Total distance moved, linear velocity, meander, total turn angle and angular velocity were analyzed. Normality of the data was evaluated using Anderson Darling (Minitab). Normal data underwent a one-way ANOVA followed by post-hoc Tukey's test (Minitab). Otherwise, a Kruskal Wallis test was carried out with a post-hoc Dunn's test (GraphPad Prism v5.0).

6.4. Results

6.4.1. Characterization of the bioactive nanocoatings (Carried out by M Tasso)

The maleic anhydride (MA) copolymer thin films that provide a platform for the immobilization of the protease Subtilisin A have been extensively characterized regarding their physicochemical properties, both in dry and swollen states (Pompe et al. 2005; Uhlmann et al. 2005; Osaki and Werner 2003; Pompe et al. 2003). The structure and properties of enzyme-containing MA copolymer films were recently investigated as a function of the polymer carrier features and of the enzyme concentration used during the immobilization process (Tasso et al. 2009 a and b).

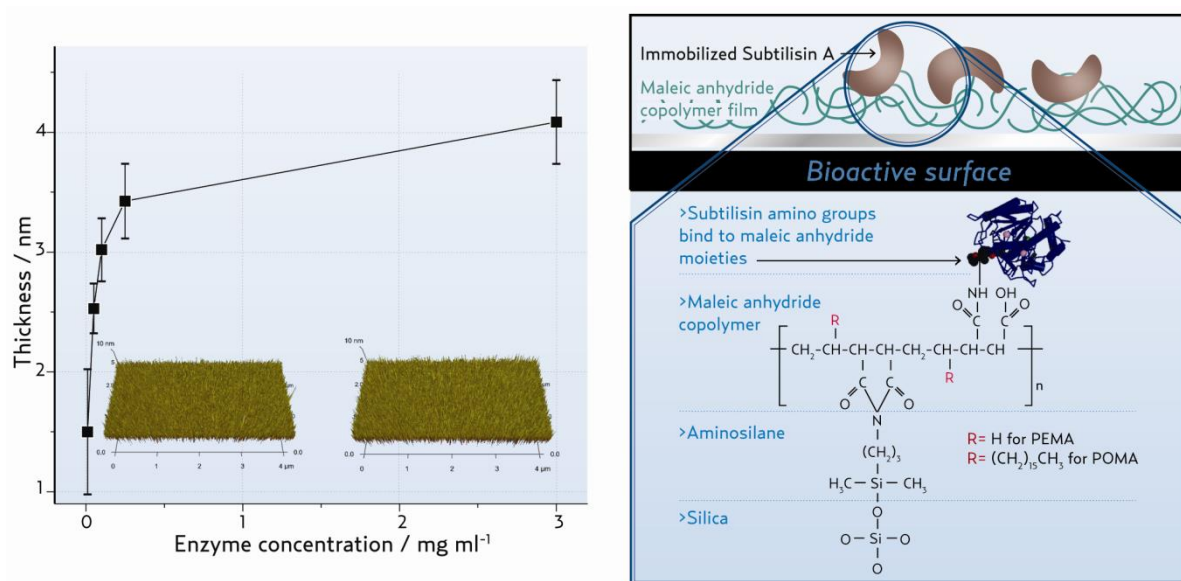


Figure 6.1. Right panel: Schematic representation of the bioactive surfaces utilized in this work showing the enzyme surface immobilization strategy. Left panel: Thickness of Subtilisin A bound to PEMA films as a function of the enzyme concentration in solution used during immobilization. Values are mean \pm SD. The two AFM amplitude images correspond to the coatings of lowest (left) and highest (right) enzyme layer thickness.

In this work, enzyme-containing coatings were developed that permitted a reduction in enzyme surface concentration and activity (compared to those described in Ref. (Tasso 2009a and b)) by using enzyme concentrations in solution from 0.01 to 3 mg ml⁻¹ for enzyme immobilization to PEMA films. Figure 6.1. depicts the layered structure of the bioactive surfaces, shows the enzyme layer thickness as a function of the enzyme concentration in solution ([Es]) used for immobilization, and includes two AFM amplitude images of the coatings of lowest and highest enzyme layer thicknesses. Figure 6.2. shows the activity and surface concentration of these bioactive layers in relation to [Es] and to the incubation time in artificial seawater (ASW*). The activity of the immobilized catalyst increased with the protein content on the surface and appeared more depleted than the latter after 48 h incubation in ASW* (eg for [Es] = 3 mg ml⁻¹, the residual activity after incubation is 14% whereas the residual protein content reaches 46% -if the means are considered-; similarly for [Es] = 0.25 mg ml⁻¹). Absorbance spectroscopy measurements of the incubation solutions after 48 h revealed the absence

of active enzyme in the media (lack of absorbance at 405 nm when in the presence of 50 vol% 0.2 mM Suc-AAPF-pNA in PBS). Furthermore, the determination of protein concentration via absorbance spectroscopy at 280 and 555 nm (back-calculation of the protein content from the fluorophore concentration) yielded values below 200 ng ml^{-1} , i.e. below the detection limit of the equipment (NanoDrop ND-1000, Wilmington, Delaware, USA), suggesting the decrease in activity is likely due to denaturation processes occurring during incubation in salty media, and not to leaching.

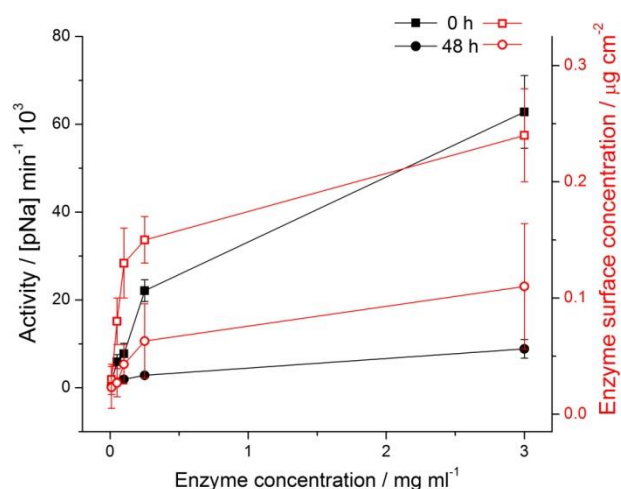


Figure 6.2. Activity (filled symbol) and surface concentration (unfilled symbol) of Subtilisin A bound to PEMA films as a function of the enzyme concentration in solution used during immobilization. Values (mean \pm SD) with and without incubation in artificial seawater for 48 h are presented.

The thickness of the enzyme layer bound to PEMA films (Figure 6.1.) displayed an increase as the [Es] increased, which is consistent with the protein content data. Thickness was in the range 1–4 nm, indicating the presence of a monolayer (mean diameter of Subtilisin A is 4.5 nm)(Neidhart and Petsko 1988).

Static water contact angle measurements of non-aged samples, confirmed the hydrophilicity of the enzyme-containing nanolayers, with water contact angles of $30^\circ \pm 5^\circ$ for the active coatings and of $45^\circ \pm 8^\circ$ for the denatured ones (PEMA control = $27^\circ \pm 5^\circ$). AFM measurements of non-aged samples yielded RMS roughness values of $0.55 \pm 0.2 \text{ nm}$ without statistical differences between active and denatured coatings (PEMA control = $0.61 \pm 0.16 \text{ nm}$).

6.4.2. Cyprid settlement assay

6.4.2.1. Inhibitory and antifouling effect of Subtilisin A in solution

The effect of 0, 0.5, 1, or 1.5 $\mu\text{g ml}^{-1}$ Subtilisin A in 'Tropic Marin' artificial seawater (ASW) on cyprid settlement onto MA surfaces after 24 and 48 h is presented in Figure 6.3. PEMA and POMA films without bound enzyme were considered for these assays. Settlement was inhibited on POMA coatings at all [Es] and incubation times tested. Settlement levels on PEMA coatings were particularly high in the absence of Subtilisin A in solution. When exposed to 1 and 1.5 $\mu\text{g ml}^{-1}$ enzyme in solution, settlement on PEMA coatings was significantly reduced at both 24 and 48 h compared to the control ($p \leq 0.001$), whereas for 0.5 $\mu\text{g ml}^{-1}$ enzyme, settlement was only significantly reduced at 24 h ($p \leq 0.01$). Based on the observed inhibitory character of the POMA films ($p = 0.82$), the further evaluation of the bioactive coatings with barnacle cyprids was restricted to the use of PEMA as platform for enzyme immobilization.

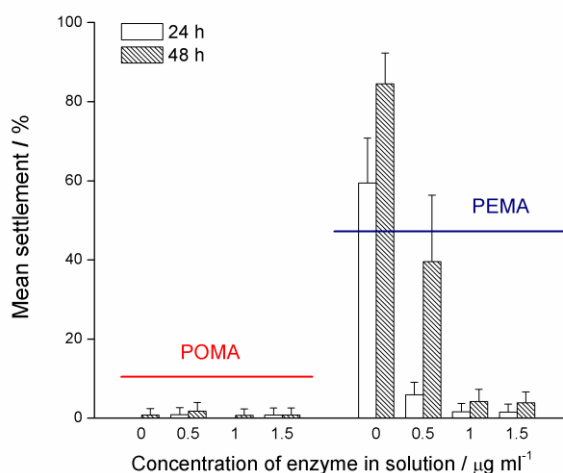


Figure 6.3. Mean settlement of *Balanus amphitrite* cyprids to POMA and PEMA films in the presence of 0 (control), 0.5, 1, and 1.5 $\mu\text{g ml}^{-1}$ Subtilisin A in 'Tropic Marin' artificial seawater. Settlement is expressed as a percentage of the total number of cyprids dispensed on each sample. White bars show settlement after 24 h, dark bars after 48 h. $N = 6$; error bars = + 95% confidence error intervals.

6.4.2.2. Antifouling potential of immobilized Subtilisin A

Barnacle cyprids of *Balanus amphitrite* were exposed to bioactive PEMA coatings of increasing activity and to the respective denatured controls for 24 and 48 h to determine settlement levels of these organisms on the test surfaces. The mean settlement of cyprids displayed a significant ($p = 0.036$) trend with respect to increasing the enzyme surface activity (Figure 6.4.A). At both incubation times, the number of settled cyprids decreased with increasing activity; the cut-off activity for action being of the order of $50 \text{ [pNa] min}^{-1} 10^3$. The denatured coatings were extensively fouled at both incubation times, with no significant differences observed ($p \geq 0.121$). Excluding the case of the coating of lowest activity, a significant difference in settlement between active and denatured coatings of the same enzyme surface concentration was observed after both incubation times ($p < 0.05$), clearly highlighting the inhibitory effect displayed by the immobilized active enzyme.

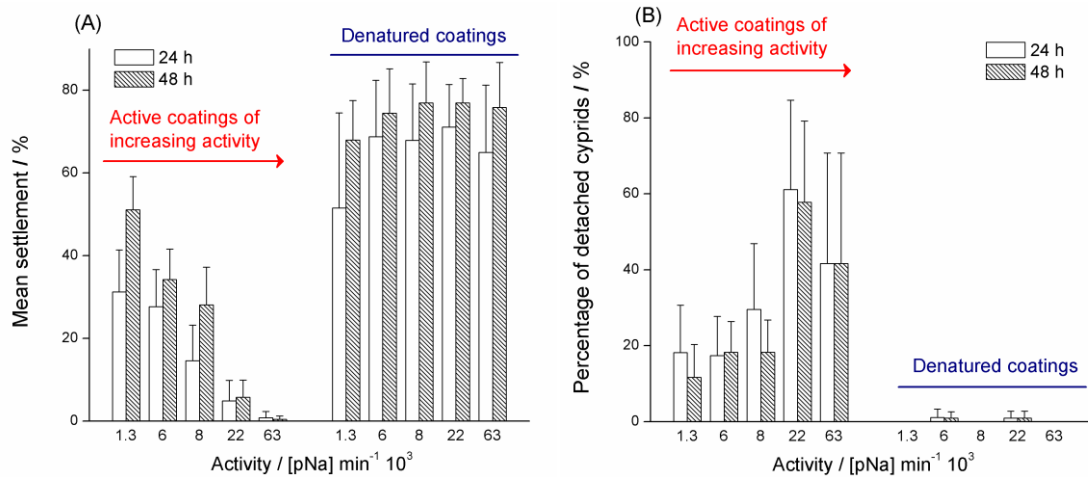


Figure 6.4. (A) Mean settlement of barnacle cyprids onto PEMA active coatings of increasing activity and the corresponding denatured controls. Settlement is expressed as a percentage of the total number of cyprids dispensed on each sample. Settlement on the control PEMA coating was $43 \pm 9.5\%$ after 24 h and $63.6 \pm 7.5\%$ after 48 h. (B) Percentage of cyprids metamorphosed and thereafter detached for PEMA active coatings of increasing activity and the corresponding denatured controls. The percentage of detached cyprids is calculated as the relative fraction of detached individuals to the total number of settled on each surface. The percentage of detached cyprids onto the PEMA control was 0% after 24 and 48 h. White bars show settlement after 24 h, dark bars after 48 h. $N = 12$ (active coatings) or 6 (denatured coatings); error bars = + 95% confidence error intervals.

The active immobilized enzyme not only inhibited settlement, but also caused a fraction of settled (and metamorphosed) cyprids to detach from the surface (Figure 6.4.B). The percentage of detached cyprids (relative to the total number of settled individuals) on the active coatings was significantly higher than on the denatured controls for the two highest activities ($p \leq 0.043$). Attachment of cyprids to the denatured coatings was strong: less than 1% of the attached and metamorphosed cyprids subsequently detached from these surfaces in clear opposition to the pattern observed for the active surfaces. The number of detached individuals from the active slides displayed an apparent increase with increasing enzyme surface activity, but the differences were not significant ($p \geq 0.05$). However, even at the lowest activity tested (i.e. $1.3 \text{ [pNa] min}^{-1} 10^3$), the relative number of detached individuals was higher than from the PEMA control (0% at both times). It was noteworthy that detached juveniles had a normal basis

and were found to behave as normal without any sign of compromise in their health and viability (95% surviving after 14 days incubation with some being able to re-attach to glass surfaces during the course of their maintenance).

6.4.2.3. Comparison between immobilized enzyme and equivalent amount of enzyme in solution

The amount of enzyme immobilized on the coating having the highest activity (i.e. 63 [pNa] min⁻¹ 10³) compares to 1 µg ml⁻¹ of enzyme in solution. Although the amount of enzyme is the same in both cases, the activity of the soluble enzyme is higher (750 [pNa] min⁻¹ 10³) than the activity of the immobilized enzyme (essentially due to the random immobilization strategy employed). Unlike previous observations with spores of the green alga *Ulva* and the diatom *Navicula*, (Tasso et al 2009b) immobilizing the enzyme did not enhance the antisettlement effect compared to the equivalent amount of enzyme in solution at any of the considered incubation times ($p \geq 0.05$) (Figure 6.5.).

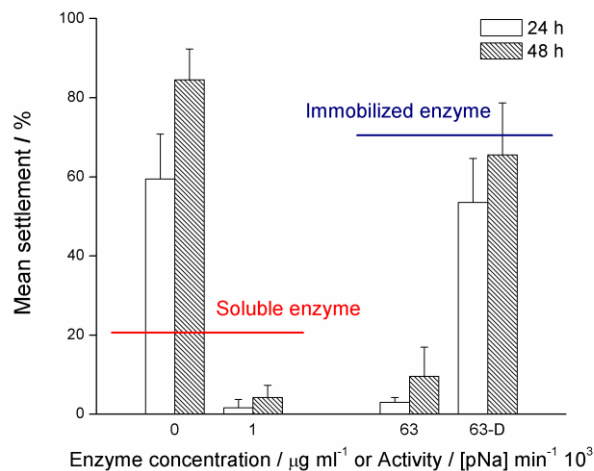


Figure 6.5. Mean settlement of barnacle cyprids onto PEMA coatings exposed to 0 and 1 µg ml⁻¹ Subtilisin A in solution and onto PEMA coatings with bound active and denatured (-D) enzyme. The amount of bound enzyme is equivalent to 1 µg ml⁻¹. Settlement is expressed as a percentage of the total number of cyprids dispensed onto each sample. White bars show settlement after 24 h, dark bars after 48 h. N = 6; error bars = + 95% confidence error intervals.

6.4.2.4. Tracking

As shown in Figure 6.6.A, surface-bound enzyme reduced the total distance moved by cyprids, although only the sample of highest activity was significantly different from the blank PEMA control ($p = 0.005$). Similar effects were observed for the mean velocity (Figure 6.6.B, $p < 0.001$). The mean angular velocity of cyprids increased significantly at the highest activity (Figure 6.6.D, $p = 0.000$), as did their meander (Figure 6.6.C, $p = 0.000$). The total turn angle, however, was not significantly different between treatments (data not shown, $p = 0.326$).

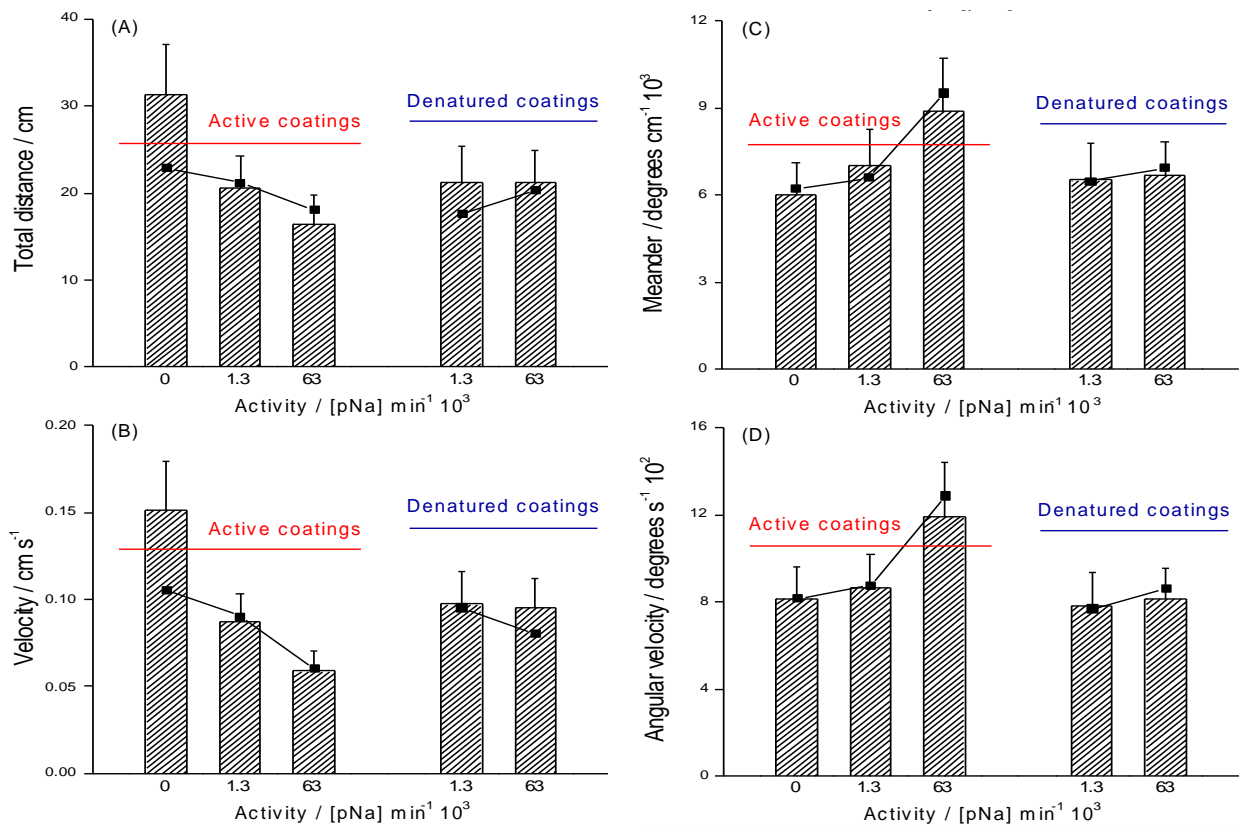


Figure 6.6. (A) Total distance moved, (B) velocity, (C) meander, and (D) angular velocity for barnacle cyprids exposed to active and denatured enzyme coatings of different activities. Bars show means + 95% confidence error. The line connects medians.

6.5. Discussion

Our reported study aimed at exploring the antifouling potential of a surface-confined purified protease against *Balanus amphitrite* barnacle cyprids. The bioactive coatings tested had a similar structure but lower enzyme surface concentration, thickness, and activity than those employed in previous investigations with marine algae (Tasso et al 2009b). Preliminary assays with barnacle cyprids revealed that changing the surface activity from 63 to 489 [pNa] min⁻¹ 10³ (same activity range as tested for algae) had no influence on cyprid settlement after 48 h (Figure 6.7.). This result prompted us to lower the surface activity of the immobilized enzyme to the range 1.3–63 [pNa] min⁻¹ 10³, hypothesizing dependence between surface activity and cyprid settlement could thus be made evident. The resulting bioactive coatings were essentially monolayers (thickness of the immobilized enzyme layer between 1 and 4 nm; values in the range of the average diameter of the enzyme molecule (4.5 nm (Neidhart and Petsko 1988)). The layers exhibited an increase in enzyme surface concentration and activity for increasing concentrations of the enzyme in solution used during immobilization. Exposure of the bioactive layers to artificial seawater (ASW*) for 48 h decreased both activity and protein concentration on the surfaces. If any enzyme was released from the surface into the supernatant, it was inactive and hence did not contribute to the digestion of the adhesive substances secreted by cyprids during exploration and settlement. Denaturation and autolysis processes might explain these findings, which stress the need to further improve the stability of the layers, eg by oriented immobilization strategies.

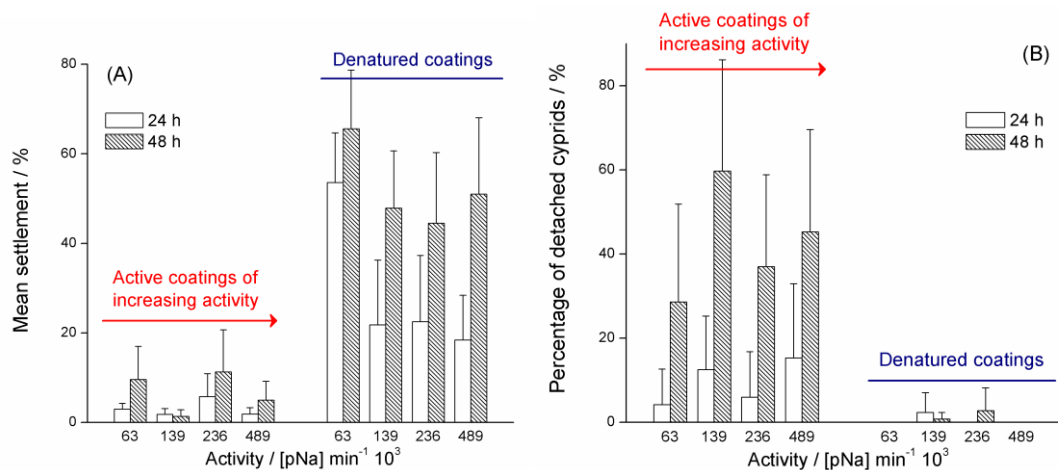


Figure 6.7. Mean settlement (A) and percentage of detached cyprids (B) onto PEMA bioactive coatings of increasing activity and their corresponding denatured controls. Settlement is expressed as a percentage of the total number of cyprids dispensed on each sample. The percentage of detached cyprids is calculated as the relative fraction of detached individuals to the total number of settled on each surface. White bars show settlement after 24 h, dark bars after 48 h. N = 6, error bars = + 95% confidence error intervals.

In the whole range of enzyme surface concentrations tested, nanorough, hydrophilic bioactive layers were obtained whose surface roughness and wettability were essentially independent of the protein content on the surface. This invariance in surface roughness and wettability over a range of enzyme surface activity is crucial to narrowing the spectrum of variables that might affect cyprid settlement and adhesion strength (Rittschof and Costlow 1989; Roberts et al. 1991; O'Connor and Richardson 1994; Schumacher et al. 2007; Aldred and Clare 2008; Aldred et al. 2010) and to analyze the cyprid-surface interaction mainly in terms of the activity of the immobilized enzyme.

The evaluation of the effect of increasing concentrations of enzyme in solution ([Es]) on cyprid settlement using MA coatings revealed the highly-inhibitory character of POMA films in contrast to the highly-inductive character of its counterpart PEMA. The inhibition of settlement observed for POMA films in the absence of enzyme seemed to have pre-determined the results found at other [Es]. As these coatings were not leached prior to the assays, the release of inhibitory compounds from POMA coatings cannot be excluded. However, the leaching of inhibitory compounds appears unlikely because the

chemicals used for the preparation of POMA surfaces were the same as for PEMA surfaces (for which no sign of inhibition was observed). The settlement inhibition found on POMA films may instead be associated with other surface-related inhibitory features, such as their more hydrophobic character and/or different surface charge density (Rittschof and Costlow 1989; Roberts et al. 1991; O'Connor and Richardson 1994). In a comparative assay using immobilized Subtilisin A on POMA films, in both active and denatured forms, plus POMA and acid-washed glass controls (Figure 6.8.), all tested POMA-based surfaces were inhibitory to settlement regardless of whether the enzyme was immobilized to the POMA surface or not, or whether the bound-enzyme was active or not. As with the enzyme in solution, the settlement-inhibitory properties of the POMA layer may still have been 'recognizable' by cyprids, even when an enzyme monolayer (thickness = 2.7 ± 0.4 nm) was immobilized onto it in either active or denatured form. These observations illustrate the 'determining' effect of the polymer carrier used for immobilization, not only on the properties of the bound catalyst but also on the resulting biological response.

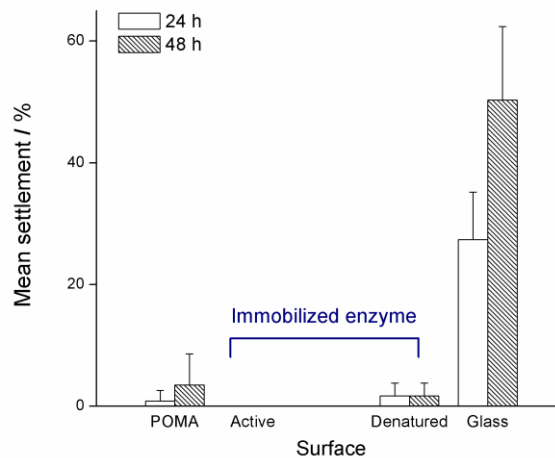


Figure 6.8. Mean settlement of barnacle cyprids onto conditioned POMA, POMA + bound Subtilisin A with activity 59.2 ± 11 [pNa] $\text{min}^{-1} 10^3$ (Active), its denatured control (Denatured), and acid-washed glass. Settlement is expressed as a percentage of the total number of cyprids dispensed on each sample. White bars show settlement after 24 h, dark bars after 48 h. N = 6, error bars = + 95% confidence error intervals.

For PEMA films, barnacle settlement was found to be dependent on [Es]. The strong inductive character of the PEMA control ([Es] = 0) vanished at [Es] $\approx 1 \mu\text{g ml}^{-1}$, revealing that relatively low [Es] are effective at deterring settlement of barnacle cyprids onto PEMA even after 48 h incubation. These findings are in agreement with previous studies by Pettitt et al.(2004) showing that Alcalase® (a commercial preparation whose active component is Subtilisin A) had a minimum inhibitory concentration of $1.1 \mu\text{g ml}^{-1}$ for barnacle cyprid settlement onto polystyrene well plates. The enzyme in solution might degrade the temporary adhesive used by cyprids during exploration, hence making it difficult for them to commit to settlement. The enzyme is also known to target the cyprid permanent cement (prior to curing), as demonstrated by Aldred et al.(2008) for Alcalase.

Compared to previous work carried out with marine algae using immobilized Subtilisin A, (Tasso et al. 2009b) enzyme surface concentrations and activities that proved effective against barnacle cyprid settlement were much lower, suggesting that cyprid adhesive(s) is more sensitive to the immobilized protease. Subtilisin A bound to PEMA films steadily decreased the number of settled cyprids on the surfaces with increasing surface concentration and activity. The denatured controls were all highly-fouled, settlement effects therefore being dependent on the presence of active enzyme. An enzyme surface activity of ca. $50 [\text{pNa}] \text{ min}^{-1} 10^3$ reduced settlement levels to ca. 5%; any further increase in surface activity had no further influence (see supporting information). The activity levels of the bound enzyme that significantly reduced cyprid settlement were substantially lower than the levels needed for *Ulva linza* zoospores (ca. $300 [\text{pNa}] \text{ min}^{-1} 10^3$)(Tasso et al. 2009b) or for *Navicula perminuta* diatom cells. No clear explanation for this difference in sensitivity can be offered based on available knowledge of cyprid adhesives. The cyprid ‘temporary adhesive’, involved in exploratory behavior (Clare et al. 1994; Matsumura et al. 1998; Aldred et al. 2008; Aldred and Clare 2008), and the cyprid cement, which fixes the larva permanently to the substratum, are proteinaceous (Aldred and Clare 2008). The temporary adhesive is thought to comprise a glycoprotein – the settlement-inducing protein complex – which has 15% *N*-linked glycans, very similar to the 17% *N*-linked glycan (Stanley et al. 1999)

composition reported for *Ulva* sp. On the other hand, the adhesive secreted by *Navicula* is largely polysaccharide with minor fractions of protein (Chiovetti et al. 2006; Molino and Wetherbee 2008). So while a higher protein content could explain a greater sensitivity of both cyprid adhesives to Subtilisin A compared to *Navicula* adhesive, and of cyprid cement compared to *Ulva* adhesive, this explanation is unlikely to hold for the comparison of cyprid temporary adhesive to *Ulva* adhesive. Furthermore, recent work from Barlow et al.(2010) on adult cement has shown around 50% of the adhesive to be made up of amyloid-like β -sheet proteins. Subtilisin A is highly effective in breaking down amyloid (Wang et al. 2005; Hsu et al. 2009) and while the available evidence suggests that the cyprid permanent cement and adult cement differ, (Kamino 2006; Aldred et al. 2008; Schmidt et al. 2009) the pronounced effect of Subtilisin A on cyprid permanent adhesion may point to amyloid content in cyprid cement.

Together with the reduction in cyprid settlement at increasing enzyme activity, an increasing number of individuals failed to permanently attach to the bioactive layers. Individuals that committed to settlement, secreted adhesives, and initiated metamorphosis most likely had their adhesion strength weakened by the surface-bound enzyme, which ultimately resulted in their detachment. This observation was supported by the near absence (< 3%) of detached cyprids from the denatured controls. Detached individuals had a shape indicative of late stage metamorphosis, suggesting that adhesive failure may have occurred during the movements associated with molting the cyprid exuvium. The lack of macroroughness or topographic features that could have strengthened the attachment of cyprids may have also facilitated adhesive failure (Aldred et al. 2010). These individuals were found to have mostly (ca. 80%) flat bases, suggesting contact with the surface during final stage metamorphosis (individuals that molt in the water column show abnormal rounded bases (SLC personal observation)). Detached individuals behaved as normal without any sign of compromise in their health or viability.

The enzyme-containing PEMA surfaces tested appear to affect the consolidation of adhesion of barnacle cyprids to the substrate without permanently interfering with earlier behaviours (i.e. commitment to settlement and metamorphosis) nor with the

viability after detachment. These facts point at the non-biocidal character of the bioactive PEMA layers, a highly-appreciated feature in environmentally-friendly strategies for fouling control.

When comparing immobilized with soluble enzyme at equal amount, a similar antifouling response was obtained although the activity of the soluble enzyme is higher (ca. one order of magnitude) than that of the immobilized enzyme. The localization of the immobilized enzyme at the interface between adhesive and surface might have counterbalanced its lower activity levels when compared to equal amount of enzyme in solution. Possibly, the time-scale of the settlement process in barnacle cyprids is long enough to allow the kinetically less-favoured immobilized enzyme to act effectively on the secreted adhesives and/or to hinder the mechanisms of recognition of substrate ligands required for successful settlement to occur.

Finally, when evaluating the behavioural pattern of cyprids exposed to the coatings of highest and lowest surface activity (and the corresponding controls), the observed reduction in total distance moved and velocity in all tested coatings compared to the blank control (PEMA) suggests that this effect was, at least in part, due to the presence of protein on the surface (i.e. enzyme in active or denatured form). Mean angular velocity and meander were significantly increased in the highest activity coating compared to all other treatments, clearly showing that cyprids' behaviour was affected by the bound enzyme. This differs from the effect of soluble Alcalase reported by Aldred et al.(2008), where relatively high concentrations of the commercial enzyme preparation had no effect on behaviour. The increase in meander is usually due to increased searching behaviour, but observations by eye suggested that some cyprids altered their swimming behaviour adopting a corkscrew-like pattern, while others were relatively inactive, which on balance may explain the reduced 'total distance moved'. In either case, cyprids experiencing natural hydrodynamic conditions rather than the static conditions of the settlement assays, would likely return to the plankton and, as future adhesion is not compromised, could attach to a more favourable surface.

6.6. Conclusions

The covalent immobilization of the protease Subtilisin A to PEMA copolymer films provided a suitable platform for the evaluation of the interplay between the bound enzyme and the adhesive substances secreted by *Balanus amphitrite* barnacle cyprids. The bound enzyme appeared to affect the consolidation of adhesion of cyprids to the bioactive layers in a manner that depends upon the surface activity. The postulated mode-of-action is based on the proteolytic degradation of the adhesive substances secreted upon attachment that eventually results in the detachment of already metamorphosed individuals without further effects on their health or viability. Changes in the swimming behavioural pattern of cyprids exposed to high activities of the bound enzyme were also demonstrated. Since the strong effects observed on settlement and adhesion strength of barnacle cyprids were gained by using low amounts of immobilized enzyme, the present study may prove valuable in the design of antifouling surfaces that seek to incorporate active, non-biocidal agents (Banerjee et al. 2011).

Chapter 7: Final conclusions

7.1 Summary of findings

Existing methods of testing barnacle adhesion in the laboratory are time consuming and expensive. Novel approaches demonstrated here allow the reduction in timescales of testing. Using methods similar to those in existence (Chapter 2), it is possible to increase throughput of samples and test the CRS of smaller adult barnacles. This reduction in size to testing could result in reduction in costs of up to one third (Table 7.1.)

Table 7.1. Comparison of necessary barnacle basal sizes and test timescales for automated and manual testing strength of adhesion of *Balanus amphitrite*.

	Minimum basal diameter of testable barnacle (mm)	Time to reach minimum testable size (weeks)
Automated testing	15	9
Manual testing	20	12

Novel techniques employing the earlier lifestages have been developed (Chapter 3) resulting in the same trends in removal as seen with traditional accepted CRS techniques, but shortening test times by 11 weeks. The flow cell was found to provide more consistent results and to better discriminate between coatings used than the water jet. Of all the life stages tested the newly calcified juveniles resulted in the lowest removal levels providing a more stringent test for improved fouling release (FR) surfaces. Using flow cells and testing newly calcified juveniles is therefore recommended as the most appropriate method for testing FR coatings in both the academic and commercial sector.

A strong conservation of cyprid cement area with cyprid age and batch was found (Chapter 4); the first study of its kind on permanent cement. Furthermore, a strong positive relationship between cyprid cement spreading and contact angle was

determined – i.e. cement spreads further on surfaces with low wettability. This result was similar to that found for cyprid temporary adhesive (Phang et al. 2006 and 2009). Cement plaque area was also linked to charge. The adhesive spread further on negatively charged surfaces. Cyprids selected surfaces that allowed greatest spreading of adhesive for settlement. This finding could theoretically be used to further reduce the timescale of testing new coatings to hours, by examining adhesive spreading rather than cyprid settlement rate. However to make this possible would require the ability to produce the adhesive artificially, or determining the most important protein in the adhesive and examining the spreading of this.

Chapter 5 showed the importance of environmental factors such as temperature and food availability on adhesion in barnacles. Moulting and secretion of cement bands in *Balanus amphitrite* were found to be linked. Each moult was linked to the production of an adhesive band. This supports prior studies on moults and growth (e.g. Sangeetha et al. 2010; Fernandez et al. 2002; Bourget and Crisp 1975). Costlow and Bookhout (1953) found no link to growth and moulting, with growth sometimes occurring in the inter-moult period. The study reported here did not determine if the formation of adhesive bands occurred at the point of moult, although with the direct 1 to 1 relationship this is likely, or during the period of intermoult. The production of cement rings was affected by both temperature and food availability; the first time this effect was studied. Lower temperatures resulted in production of fewer, narrower cement rings, while reduction in feeding levels reduced the overall coverage of the basis in adhesive, but not the average width of the adhesive bands. This resulted in the critical removal stress (CRS) of barnacles grown on T2 (a silicone often used as a poor FR standard) having an inverse relationship with temperature. This result is supported by the only previous study (Johnston 2010) that has examined the effect of temperature on CRS. Stronger adhesion at lower temperature could be caused by a number of factors, including adhesive layer thickness, quality of adhesive or differences in fracture propagations due to the patterning of the adhesive rings.

This chapter also showed that the most commonly used laboratory temperature (28 °C) for growth of *B. amphitrite* does not result in the best survival, growth and adhesion strength, and suggests that 22 °C should be used. This could have consequences on the

antifouling industry in terms of adult barnacle testing. Most testing laboratories utilise 28 °C, which may result in less challenge to the FR surface, in terms of adhesion ability of the barnacle, than a cooler growth temperature. Changing to 22 °C should have no real cost to a laboratory. The growth rates of *B. amphitrite* at the two temperatures is very similar and any slight reduction in growth would likely be covered by a small cost saving related to the use of incubators at lower temperatures. Also, as testing at 22 °C is more discriminatory, fewer sub-standard coatings would be passed on to field trials, potentially representing a very large cost saving.

The final experimental chapter (Chapter 6) showed the effectiveness of a novel enzyme coating using a protease (Subtilisin A). The protease was immobilised on a maleic anhydride copolymer film onto microscope slides for experimental purposes. The enzyme dramatically reduced settlement levels at concentrations lower than previously seen for other fouling species (Tasso et al. 2009b). Furthermore, the bound enzyme also affected cyprid behaviour on exposure. When concentrations were low enough to allow permanent attachment, a proportion of juveniles became detached, suggesting that the enzyme action at low concentration dissolved the adhesive. This did not have any toxic effect since detached juveniles survived for long periods in the laboratory. This is the first proof of principle for enzyme use in antifouling coatings against barnacles.

7.2 Limitations of the work

One of the major limitations of this study is the fact that all of the work was carried out on just one species of barnacle, *Balanus amphitrite*, and only within a controlled laboratory setting. This is unrealistic compared to natural environments where artificial surfaces are under settlement pressure from multiple species, and are exposed to a wide variety of conditions (Breitburg 1985; Wahl 1989; Jenkins et al. 1999). However in order to enable testing of environmental factors the control that only a laboratory can bring was required. Interactions between different species, while realistic (Benedetti-Cecchi 2000), were outside the scope of this study as it can make trends based on surface effects difficult to determine. At this stage in the research assays using a single model species are most appropriate. While the results are based on a single species, *B.*

amphitrite, it is reasonable to extrapolate the results in a general form to other invertebrate species due to the similarities in adhesives (Jensen and Morse 1988; Waite et al. 1989; Kamino et al. 1996; Flammang et al. 1998; Waite and Qui 2001, Flammang 2002; Kamino 2010). Differences between species will exist, however, so to gain a greater understanding of the complexities of surface effects the results should be confirmed with other species; for example barnacle species with membranous bases as well as calcified bases should be evaluated and compared in terms of adhesive responses.

Testing of the spreading of cyprid permanent cement (Chapter 4) was limited in some experiments (e.g. charge effects) by the number of coating types that could be supplied by the collaborators. These trials would be improved by increasing the number of different types of self assembled monolayers tested.

7.3 Further research

7.3.1. Testing other species

The logical next step for the research carried out utilising the push-off machine and the hand-held force gauges used in Chapter 2 would be determining the adhesive strength of other barnacle species and other hard fouling species (such as tube worms). Examining the adhesive strength of these other species, both at settlement and adult stage, with the water jet and flow cell used in chapter 3 would produce valuable information about their adhesive capabilities and ability to resist shear (in the case of the flow cell). This would require considerable initial adjustment in some cases. For the push off machine and hand held force gauges (Chapter 2) different species of barnacle should present little problem, though ensuring no adhesive failure has occurred may be harder to ensure with membranous based barnacles such as *Elminius modestus* as there would be no calcareous basal plate to clearly indicate the failure. However this should only require closer visualisation of tested surfaces for any remnant of membrane or adhesive. Testing with other organisms, such as tube worms (e.g. *Ficopomatus enigmatus*, *Hydroides elegans*) would be more difficult due to their complex irregular shape. This

could result in a point source of force application, and the area the force could be applied to would be variable between individuals (Fig. 7.1 A1 and A2). Some researchers have carried out testing using force gauges, but have described the method only as far as using ASTM D 5618 – 94 in that force is applied to the base of an adult fouling organism, no further details are given (e.g. Holm et al. 2006; Wood et al. 2000). Similar issues would arise when applying shear forces generated with a flow cell apparatus. It would be difficult to determine what the forces each individual were subjected to if there were not at the very least some record of directionality of flow with regard to the organism (Fig. 7.1. B1 and B2).

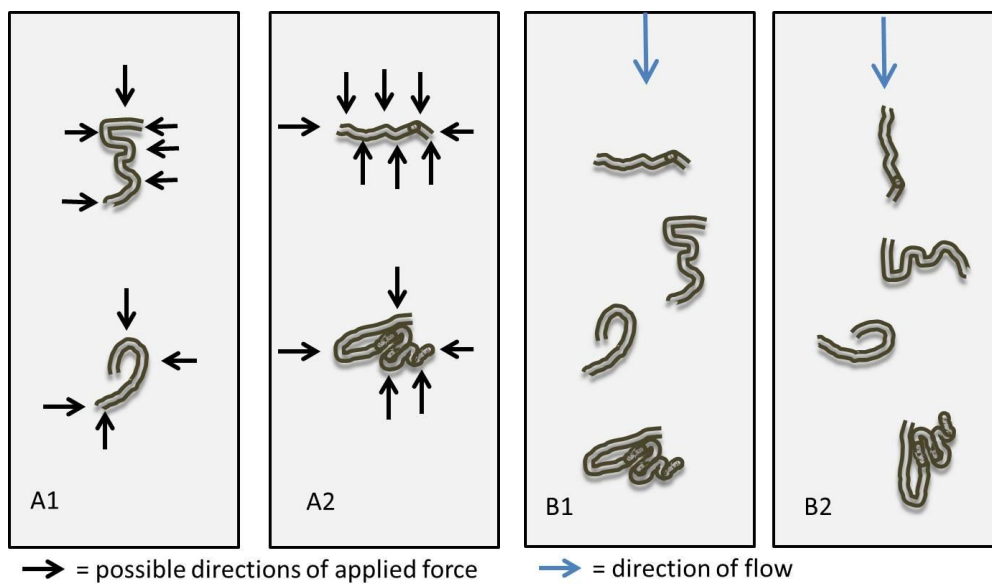


Figure 7.1. Diagram indicating the variability in shape of tubeworms such as *F. enigmatus* and the possible directions that force, using a hand held or machine controlled force gauge, can be applied (A1 and A2), and the shear force, using the flow cell, may act (B1 and B2). The number of arrows gives an estimate of the number of points of contact for force application using a force gauge.

A second concern is the lack of understanding of the adhesive mechanism in tubeworms of this type. While the general structure of the tube is well understood consisting of a calcareous tube and a membrane within it, the tube forming a complete enclosure when the worm grows vertically off a surface (in a reef; Aliani et al. 1995; Schwindt et al. 2004). Little is known about the precise mechanism of adhesion and it is unclear if the adhesive is spread over the whole area where the membrane and calcareous tube come

into contact with a surface, or purely the tube (Fig 7.2.A and B). This is particularly important when calculating the removal force as the area of attachment is used to control for differences in organism size.

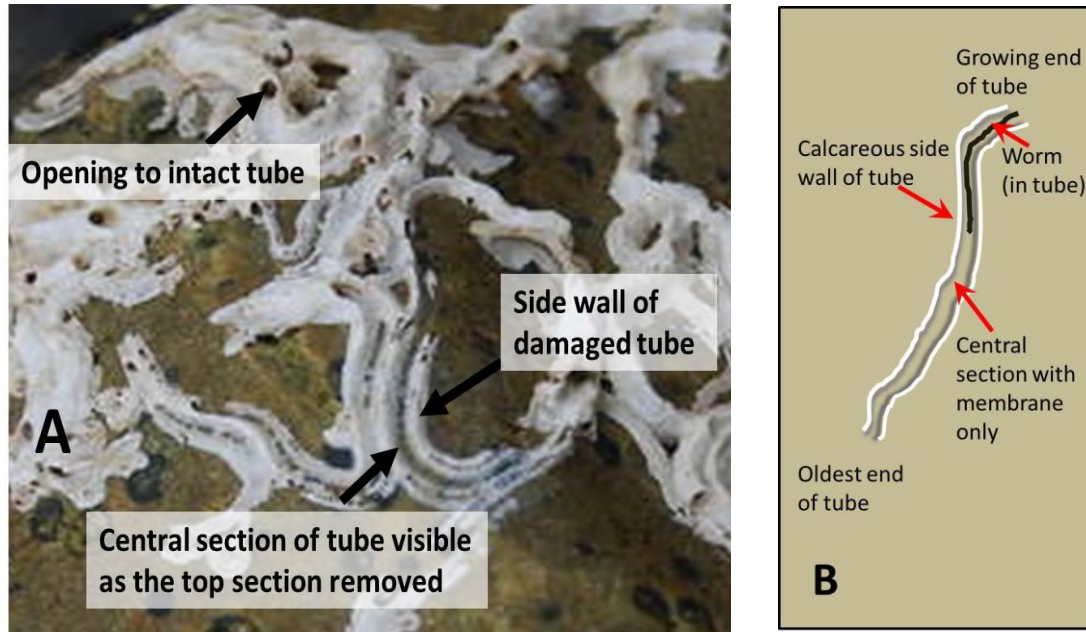


Figure 7.2. A) Adult tubeworms on a rock surface with some damaged individuals showing the central base section without calcareous tube, B) schematic of tubeworm growing on a glass slide showing visual from underside.

Chapter 5 illustrates the importance of environmental factors on adhesion in *B. amphitrite*, it is likely that this is true for many other species, not just barnacles, but other fouling organisms (e.g. tubeworms, microalgae and diatoms). Analysing the effects of environment on the adhesive production, and linking it to adhesive strength in these species merits consideration (see below).

The final chapter shows that enzyme-bound coatings work well against the settlement and adhesion of *B. amphitrite*. Looking at the effect of the tethered enzyme on another fouling organism group, the tubeworms, would provide useful information about the adhesive in these species. Although the proteinaceous nature of the adhesive is already known, tube worms have responded to coatings already in service differently to the model macro-fouling species used to date (Stein et al. 2003; Kavanagh et al. 2001).

7.3.2. *Modifying environmental factors*

As stated above, much of the suggested work on *B. amphitrite* would be very difficult, if not impossible to carry out in the field due to its need for very controlled conditions. However, more realistic ecological conditions could be introduced within the laboratory. Tests involving measurement of adhesion and cyprid cement spreading could be conducted while introducing varying temperatures and dark-light cycles, changing food provision, presence of other fouling species in a controlled manner and comparing the data with field results.

Barnacles could also be cultured in moving water, as opposed to the static set-ups tested here, to examine whether hydrodynamic stress (a 'normal' situation in the natural environment) affects adhesion strength and area (see below).

In a natural situation, cyprids are mobile and can test a variety of surfaces before selecting a settling site. Choice experiments, where cyprids are offered a choice of two or more different surfaces (within an appropriate spatial scale) could lead to some interesting insights on search and settlement behaviour, and the precise surface properties that trigger settlement.

Manipulating the balance of individual nutrients (e.g. carbon, calcium or sulphur) available to the organisms could offer insight into adhesive components and resource distribution (between growth, adhesion, defence and reproduction). This may not be easy, as minimal levels for the food organism (algae) may be higher for some nutrients than they are for the barnacles, so producing a food source with suitable ranges of nutrients may not be possible. Supplementing the sea water with minerals may offer an easier route for exploration.

In wild situations, there is likely to be a low chance of a cyprid encountering a suitable surface. Surfaces in an ocean environment are relatively rare, and are quickly colonised by competing organisms. Therefore, the majority of barnacle cyprids within the plankton are likely to be 'old and desperate' (Aldred and Clare 2008). In the laboratory, cyprids are typically aged at 6 °C for around 3 days. Extending the cyprid aging work in

Chapter 4, the effect of aging at a range of temperatures and with a range of food levels (including those likely to be encountered in open ocean situations) could be explored. An ability to reliably produce ‘desperate’ cyprids that more closely relate to the size and food reserves of those found in the wild may result in a more realistic, and possibly more discriminatory, FR testing protocol.

7.3.3. Hydrodynamic testing

Barnacles in the wild are exposed to varying and sometimes extreme hydrodynamic forces (Crisp 1955; Hoch 2011). A species adapted to settling within the intertidal zone (such as *Semibalanus balanoides*) would be expected to be more capable of coping with rapid changes than a subtidal species (such as *Balanus trigonus*). Although even subtidal species will experience continuous water movement to some degree.

It is already known that settling barnacle cyprids in light flow adjust their settlement position to take advantage of flow directions once metamorphosed (Crisp 1955). Growing already settled cyprids in the higher flow rates tested in the study reported here should give an indication of the ability of the already settled cyprid (or juvenile barnacle) to make adjustments to reduce their susceptibility to drag. At these higher rates of flow this ability to move and reduce drag may be increasingly important and the timescale that the barnacle is able to do this would also be of interest.

Experiments with the flow cell were carried out at a set rate of flow development for all tests, but if this were varied it should be possible to discover the point at which individuals no longer had time to adjust, which would be seen as an increase in removal, without increasing shear, merely speed of flow development (as long as time scales of adjustment were slower than the fastest flow development of the flowcell).

It can be expected that a barnacle must be able to cope with many shear stresses throughout its lifetime. A single exposure to shear for 5 minutes was used in this work, and it would be expected that the result would not be changed by multiple exposures to shear or longer term exposure. However it has been seen that the cirri of barnacles in

different flow regimes are different (Neufeld 2011; Neufeld and Rankine 2012), it would be interesting to establish if other physiological characteristics change under controlled flow regimes (such as shell thickness and mantle shape). If adhesion strength is increased under higher flow regimes, this may offer an improved culture method to increase the testing sensitivity for more effective FR coatings.

As settlement progresses, the settled individual changes shape and these very different shapes are likely to be exposed to different shear stresses. The permanently attached cyprid is hydrodynamically streamlined along its length and can change orientation to allow it to be parallel to the flow of the water. This modulates the forces that the settled cyprid is exposed to in flow. The newly metamorphosed individual, however, is shaped more like a juvenile barnacle, though flexible, and is held in place with the same antennules and cement plaque as the cyprid. Finally the juvenile basis is closely associated (and may be adhered) to the surface. These three different shapes would be useful to model for shear forces. This should be possible if the flow cell could be adapted and the laser doppler velocimeter used to examine the flow of the fluid around them. This could inform not just the forces that the different individuals would be exposed to, or need to be exposed to for removal, but also the drag these life stages create on settlement. Many FR coatings presently work relatively well at removing barnacles, once a certain size is reached, thus understanding the drag associated with these earlier life stages would assist in calculating the drag cost of these organisms on these surfaces.

The water jet lends itself to other work: testing the effect of impact angle on removal, and the effect of life stage on any effects seen. Will removal rates change with multiple impacts (i.e. would a cleaning system with multiple impact water hoses, at set angles be more effective than a single impact hose)? Answering these questions would have implications on the novel in service systems being developed to clean FR coatings (e.g. CleanHull Ltd; Hullbug by Maxon Motor Ltd)

Finally video imaging to capture the exact point, and hence wall shear, of removal of the individual would clarify removal results for different coatings and ease comparison of results. A shear force for 50% removal could then be used to compare different

surfaces. If it was possible to video closely enough and at high enough frame rate it may also be possible to determine the method of failure of the adhesive (Kavanagh et al. 2005).

7.3.4. Adhesive analysis

The possibility of a juvenile adhesive is intriguing (Chapter 3). It is very important to establish whether this adhesive actually exists, as well as determining when the adult adhesive is first released and cures in *B. amphitrite*. Any FR coating developed would need to function against the juvenile adhesive, as well as that of the adult. Any juvenile adhesive might also be a useful biomimetic glue. Determining when juvenile and adult adhesives are produced might also be best done using different environmental conditions, as it has been shown here that environmental conditions can greatly affect adhesive production (Chapter 5). Recent research by Burden et al (2012) speculated on a membrane based adult adhesive, this could be a continuation of the use of what has been termed juvenile adhesive in this study and by Yule and Walker (1984). Using technologies such as Fourier transform infrared spectroscopy (FTIR) and Raman spectroscopy it could be possible to elucidate the nature and timescale of production of this juvenile adhesive.

Chapter 4 raises many further questions; 1) does the size of the cyprid plaque affect the attachment strength of the cyprid and the newly metamorphosed individual (as has been shown for other species e.g. *Ulva*; Callow et al. 2005) and does plaque size scale with the size of the cyprid with consequences for adhesion strength? 2) how long would any effect last after the 'juvenile adhesive' is secreted or the close association of the juvenile basis and surface is made? 3) if different species of barnacles with cyprids of different size were used, would the plaque size vary in proportion to size of the larva? 4) cyprids of *B. amphitrite*, if produced in less than ideal conditions are smaller, and have less energy reserves than those grown at ideal conditions (personal observations). Would these smaller cyprids produce smaller cement plaques, as is suggested by the oldest cyprids forming smaller plaques (Chapter 4)?

Barnacle species show differences in preference for surface wettability (e.g. Dahlström et al. 2004; Petrone et al. 2011). As temporary adhesive is made up in part of the settlement inducing protein complex (SIPC, Dreanno et al 2006a), these differences have been explained as being due to variation in SIPC (Kato-Yoshinaga et al. 2000; Dreanno et al. 2007) between species (Clare and Aldred 2009). It would be interesting to examine if the difference in species preference were related to the spreading of the permanent cement. This might then lend weight to the suggestion put forward in Chapter 4 of some homology between the two cyprid cements. It has been suggested in the past that adult adhesive would be unlikely to be similar to the larval adhesives as it does not need such a rapid cure mechanism (Kamino 2008). However a recent study has found that at least one homologous protein is found in both adult cement and cyprid permanent cement (Chen et al. 2011). This more recent work indicates that Walker (1971) may have been correct to suggest that the cyprid cement glands have differentiated to form the adult cement system. If a link between the permanent and temporary cement were found, this would provide evidence that all three barnacle adhesive systems are linked in some way.

The fact that after cyprids had been aged for 9 days at 6 °C they showed less adhesive spreading than younger cyprids may have been a sign of the onset of morphological changes associated with metamorphosis that occur prior to settlement (Walley 1969). This may be another driver for cyprid settlement (resulting in a desperate cyprid; Aldred and Clare 2008). It would be informative to carry out an experiment to determine if cyprids choose to settle prior to this point, and if the temperature of storage has any effect on the timescale of the effect (in terms of reduced adhesive plaque area). If there is a change occurring within the cement gland, it may be possible to histologically identify this as cyprids age, and determine when cyprids reach a desperate larvae state (Aldred and Clare 2008).

Finally it is important to determine if the area of the cement plaque is related to its thickness. This could be done using confocal microscopy or histological sectioning across the plaque. Two zones were seen in the cyprid cement plaque, a central, more heavily stained inner zone, which appeared thicker and was more globular and irregular in outline than the lightly stained outer zone, which appeared thin with a smooth

outline. It was not possible to visualise them well enough to measure them accurately on different surfaces in this study. These different areas could also be examined using confocal microscopy, and/or ellipsometry to determine if the central zone is thicker than the outer zone, as appeared in this study. If they could be better differentiated it might also be possible to investigate whether these diverse parts of the plaque, with altered chemistries, react differently to disparate surface chemistries.

The environmental effects on the barnacle adult adhesive production and how it affects adult CRS raises the question of how this works. As was discussed in Chapter 5 this may be due to crack trapping; the fracture caused by the shear on the barnacle being stopped between each of the adhesive rings. High speed video could be used to investigate the removal of barnacles grown at different temperatures from surfaces (e.g. Kavanagh et al. 2005). It should then be possible to determine if the propagation of the crack is indeed stopped at the gaps between the adhesive rings. Barnacles grown at relatively high temperatures would not be expected to show this crack trapping as they had little or no gap between the adult cement rings.

The final chapter shows an initial step towards tethering enzymes in an antifouling coating and would benefit from determining the effect of the bound enzyme on adhesive strength of *B. amphitrite* at low concentrations. This study showed some settled individuals became detached after initial attachment. Applying the hydrodynamic testing methods would allow a measure of this adhesive effect to be gained. It may also be possible to visualise any effect by staining the cement plaque after time exposed to the enzyme surface. Staining would allow any enzymatic degradation of the plaques to be visualised and possibly quantified.

7.4 Conclusion

People have sought solutions to prevent biofouling in marine systems since at least 200BC. This thesis attempts to add to our body of knowledge about biofouling organisms, to improve testing systems, and to further understand the complex properties at the interface between organism and underwater surface. The likelihood of an

acceptable solution to biofouling organisms being found in the very near future that does not involve some toxic element is low. However the increase in the understanding of how the cyprid permanent adhesive of *B. amphitrite* interacts with surfaces may help further knowledge and assist in targeting non-toxic FR surfaces.

The understanding that environment effects organisms is not a new concept but evidence, provided here for the first time, that it effects the adhesive ability of macrofoulers should fuel the debate for increased ecological factors to be taken into account when analysis of natural systems is undertaken within the laboratory. It also puts forward a strong argument towards testing under a number of environmental conditions prior to down-selecting coatings. The upfront costs of extra testing may be offset by savings due to sub-standard coatings not progressing down the development stream. More appropriate, and more discriminatory, test methodologies could also result from a better understanding of the organisms interaction with their natural environment. The research into more rapid testing methodologies developed here, if taken up by industry, will assist in faster (and therefore cheaper) testing of novel technologies and should thus speed up the discovery of new non-toxic approaches to FR and antifouling.

References

- AFSAR, A., DE NYS, R. & STEINBERG, P. 2003. The effects of foul-release coatings on the settlement and behaviour of cyprid larvae of the barnacle *Balanus amphitrite* Darwin. *Biofouling*, 19, 105-110.
- AL-JUHNI, A. A. & NEWBY, B.-M. Z. 2006. Incorporation of benzoic acid and sodium benzoate into silicone coatings and subsequent leaching of the compound from the incorporated coatings. *Progress in Organic Coatings*, 56, 135-145.
- ALDRED, N. & CLARE, A. S. 2008. The adhesive strategies of cyprids and development of barnacle-resistant marine coatings. *Biofouling*, 24, 351-363.
- ALDRED, N. & CLARE, A. S. 2009. Mechanisms and principles underlying temporary adhesion, surface exploration and settlement site selection by barnacle cyprids: a short review. *Functional Surfaces in Biology: Adhesion Related Phenomena*, 2, 43-65.
- ALDRED, N., ISTA, L. K., CALLOW, M. E., CALLOW, J. A., LOPEZ, G. P. & CLARE, A. S. 2006. Mussel (*Mytilus edulis*) byssus deposition in response to variations in surface wettability. *Journal of the Royal Society Interface*, 3, 37-43.
- ALDRED, N., LI, G., GAO, Y., CLARE, A. S. & JIANG, S. 2010a. Modulation of barnacle (*Balanus amphitrite* Darwin) cyprid settlement behavior by sulfobetaine and carboxybetaine methacrylate polymer coatings. *Biofouling*, 26, 673-683.
- ALDRED, N., PHANG, I. Y., CONLAN, S. L., CLARE, A. S. & VANCOSO, G. J. 2008. The effects of a serine protease, Alcalase (R), on the adhesives of barnacle cyprids (*Balanus amphitrite*). *Biofouling*, 24, 97-107.
- ALDRED, N., SCARDINO, A., CAVACO, A., DE NYS, R. & CLARE, A. S. 2010b. Attachment strength is a key factor in the selection of surfaces by barnacle cyprids (*Balanus amphitrite*) during settlement. *Biofouling*, 26, 287-299.
- ALIANA, S., BIANCHI, C.N., DE ASMUNDIS, C. & MELONI, R. 1995. Scanning electron microscope observations on the tube of the reef forming serpulid *Ficopomatus enigmatus* (Fauvel)(Annelida, Polychaeta). *Biological Bulletin*, 62, 363-367.
- ALMEIDA, E., DIAMANTINO, T. C. & DE SOUSA, O. 2007. Marine paints: The particular case of antifouling paints. *Progress in Organic Coatings*, 59, 2-20.

- ALZIEU, C. 2000. Impact of tributyltin on marine invertebrates. *Ecotoxicology*, 9, 71-76.
- ANDERSON C., ATLAR M., CALLOW M.E., CANDRIES M., MILNE A. & TOWNSIN R.L. 2003. The development of foul release coatings for sea going vessels. Proceedings of the Institute of Marine Engineering, Science and Technology Part B: Journal of Marine Design and Operations, 4, 11-23.
- ANDERSON, D.T. 1994 Barnacles. Structure, function, development and evolution. 357pp Chapman and Hall, London.
- ANIL, A. C., CHIBA, K., OKAMOTO, K. & KUROKURA, H. 1995. Influence of temperature and salinity on larval development of *Balanus amphitrite* - implications in fouling ecology. *Marine Ecology Progress Series*, 118, 159-166.
- ANON, 1952. Marine fouling and its prevention. U.S. Naval Institute, Annapolis, Maryland, 388p.
- ASTM D-5618 (2011) Standard test method for measurement of barnacle adhesion strength in shear. Active Standard Developed by Subcommittee: D01.45 Book of Standards Volume: 06.02.
http://enterprise.astm.org/filtrexx40.cgi?+REDLINE_PAGES/D5618.htm.
- AUTUMN, K., SITTI, M., LIANG, Y. C. A., PEATTIE, A. M., HANSEN, W. R., SPONBERG, S., KENNY, T. W., FEARING, R., ISRAELACHVILI, J. N. & FULL, R. J. 2002. Evidence for van der Waals adhesion in gecko setae. *Proceedings of the National Academy of Sciences of the United States of America*, 99, 12252-12256.
- BANERJEE, I., PANGULE, R. C. & KANE, R. S. 2011. Antifouling coatings: recent developments in the design of surfaces that prevent fouling by proteins, bacteria, and marine organisms. *Advanced Materials*, 23, 690-718.
- BARLOW, D. E., DICKINSON, G. H., ORIHUELA, B., KULP, J. L., III, RITTSCHOF, D. & WAHL, K. J. 2010. Characterization of the adhesive plaque of the barnacle *Balanus amphitrite*: amyloid-like nanofibrils are a major component. *Langmuir*, 26, 6549-6556.
- BARLOW, D. E., DICKINSON, G. H., ORIHUELA, B., RITTSCHOF, D. & WAHL, K. J. 2009. In situ ATR-FTIR characterization of primary cement interfaces of the barnacle *Balanus amphitrite*. *Biofouling*, 25, 359-366.

- BARNES, H. 1963. Light, temperature and the breeding of *Balanus balanoides*. *Journal of the Marine Biological Association UK*, 43, 717-727.
- BARNES, H. 1970. A review of some factors affecting settlement and adhesion in the cyprids of some common barnacles. In Manly, R.S. (eds) *Adhesion in Biological Systems*, Academic Press, New York, 89-111.
- BARNES, H. & BARNES, M. 1956. The general biology of *Balanus glandula* Darwin. *Pacific Science*, 10, 415-422.
- BARNES, H. & BARNES, M. 1959. A comparison of the annual growth patterns of *Balanus balanoides* (L.) with particular reference to the effect of food and temperature. *Oikos*, 10, 1-18.
- BARNES, H., CRISP, D.J., & POWELL, H.T. 1951 Observations on the orientation of some species of barnacles. *Journal of Animal Ecology* 20, 227-241.
- BARNES, H., FINLAYSON, D. M. & PIATIGORSKY, J. 1963. The effect of desiccation and anaerobic conditions on the behaviour, survival and general metabolism of three common cirripedes. *Journal of Animal Ecology*, 32, 233-252.
- BARNES, H. & POWELL, H. T. 1953. The growth of *Balanus balanoides* (L.) and *B. crenatus* Brug. under varying conditions of submersion. *Journal of the Marine Biological Association UK*, 32, 107-127.
- BARNES, H. & STONE, R. L. 1974. Effect of food, temperature, and light-period (day-length) on molting frequency in *Balanus-balanoides* (l). *Journal of Experimental Marine Biology and Ecology*, 15, 275-284.
- BARNES, M. 2000. The use of intertidal barnacle shells. *Oceanography and Marine Biology, Vol 38*, 38, 157-187.
- BEAUMONT, A. R. & BUDD, M. D. 1984. High mortality of the larvae of the common mussel at low concentrations of tributyltin. *Marine Pollution Bulletin*, 15, 402-405.
- BECKA, A. & LOEB, G. 1984. Ease of removal of barnacles from various polymeric materials. *Biotechnology and Bioengineering*, 26, 1245-1251.
- BEIGBEDER, A., DEGEE, P., CONLAN, S. L., MUTTON, R. J., CLARE, A. S., PETTITT, M. E., CALLOW, M. E., CALLOW, J. A. & DUBOIS, P. 2008. Preparation and characterisation of silicone-based coatings filled with carbon

- nanotubes and natural sepiolite and their application as marine fouling-release coatings. *Biofouling*, 24, 291-302.
- BENEDETTI-CECCHI, L. 2000. Predicting direct and indirect interactions during succession in a mid-littoral rocky shore assemblage. *Ecological Monographs*, 70, 45-72.
- BERGLIN, M. & GATENHOLM, P. 1999. The nature of bioadhesive bonding between barnacles and fouling-release silicone coatings. *Journal of Adhesion Science and Technology*, 13, 713-727.
- BERGLIN, M. & GATENHOLM, P. 2003. The barnacle adhesive plaque: morphological and chemical differences as a response to substrate properties. *Colloids and Surfaces B-Biointerfaces*, 28, 107-117.
- BERGLIN, M., LARSSON, A., JONSSON, P. R. & GATENHOLM, P. 2002. The adhesion of the barnacle, *Balanus improvisus*, to poly(dimethylsiloxane) fouling-release coatings and poly(methyl methacrylate) panels: the effect of barnacle size on strength and failure mode. *Barnacle Bioadhesive Bonding: Formation, Strength and Control*, 1485-1502.
- BERGLIN, M., LONN, N. & GATENHOLM, P. 2003. Coating modulus and barnacle bioadhesion. *Biofouling*, 19, 63-69.
- BERNTSSON, K. M., ANDREASSON, H., JONSSON, P. R., LARSSON, L., RING, K., PETRONIS, S. & GATENHOLM, P. 2000. Reduction of barnacle recruitment on micro-textured surfaces: Analysis of effective topographic characteristics and evaluation of skin friction. *Biofouling*, 16, 245-261.
- BERS, A. V. & WAHL, M. 2004. The influence of natural surface microtopographies on fouling. *Biofouling*, 20, 43-51.
- BIGELOW, W.C., PICKETT, D.L. & ZISMAN, J. 1946. Oleophobic monolayers: I. Films adsorbed from solution in non-polar liquids. *Journal of Colloid and Interface Science*, 1, 513-538.
- BOCQUET-VEDRINE, J. 1963. Structure du test calcaire chez *Chthamalus stellatus* (Poli). (Translation). *Comptes Rendus de l'Académie des Sciences*, 257, 1350-1352.
- BOCQUET-VÉDRINE, J. 1965. Étude du tégument et de la mue chez le cirripède operculé *Elminius modestus* Darwin. (Translation). *Archives de Zoologie Expérimentale et Générale*, 105, 30-76.

- BOURGET, E. & CRISP, D. J. 1975a. Analysis of growth bands and ridges of barnacle shell plates. *Journal of the Marine Biological Association UK*, 55, 439-&.
- BOURGET, E. & CRISP, D. J. 1975b. Factors affecting deposition of shell in *Balanus balanoides* (L). *Journal of the Marine Biological Association UK*, 55, 231-6.
- BRADY, R. F. 2001. A fracture mechanical analysis of fouling release from nontoxic antifouling coatings. *Progress in Organic Coatings*, 43, 188-192.
- BRADY, R. F. & ARONSON, C. L. 2003. Elastomeric fluorinated polyurethane coatings for nontoxic fouling control. *Biofouling*, 19, 59-62.
- BRADY, R. F., GRIFFITH, J. R., LOVE, K. S. & FIELD, D. E. 1987. Nontoxic alternatives to antifouling paints. *Journal of Coatings Technology*, 59, 113-119.
- BRADY, R. F. & SINGER, I. L. 2000. Mechanical factors favoring release from fouling release coatings. *Biofouling*, 15, 73-81.
- BREITBURG, D.L. 1985. Development of a subtidal epibenthic community - factors affecting species composition and the mechanisms of succession. *Oecologia*, 65, 173-184.
- BRESSY, C., HELLIO, C., MARECHAL, J. P., TANGUY, B. & MARGAILLAN, A. 2010. Bioassays and field immersion tests: a comparison of the antifouling activity of copper-free poly(methacrylic)-based coatings containing tertiary amines and ammonium salt groups. *Biofouling*, 26, 769-777.
- BRIAND, J.F. 2009. Marine antifouling laboratory bioassays: an overview of their diversity. *Biofouling*, 25, 297-311.
- BRYAN, G. W., GIBBS, P. E., HUMMERSTONE, L. G. & BURT, G. R. 1986. The Decline of the gastropod *Nucella-Lapillus* around Southwest England - evidence for the effect of tributyltin from antifouling paints. *Journal of the Marine Biological Association UK*, 66, 611-640.
- BURDEN, D.K., BARLOW, D.E., SPILLMANN, C.M., ORIHUELA, B., RITTSCHOF, D., EVERETT, R.K. & WAHL, K.J. 2012, Barnacle *Balanus amphitrite* adheres by a stepwise cementing process. *Langmuir*, 28, 13364-13372.
- BURGESS, J. G., BOYD, K. G., ARMSTRONG, E., JIANG, Z., YAN, L. M., BERGGREN, M., MAY, U., PISACANE, T., GRANMO, A. & ADAMS, D. R. 2003. The development of a marine natural product-based antifouling paint. *Biofouling*, 19, 197-205.

- BURMEISTER, H. 1834. *Beiträge zur Naturgeschichte der Rankenfüßer (Cirripedia)*. Berlin: G. Reimer (translated abstract).
- CALLOW, J. A. & CALLOW, M. E. 2011. Trends in the development of environmentally friendly fouling-resistant marine coatings. *Nature Communications* 2, 244.
- CALLOW, J. A., CALLOW, M. E., ISTA, L. K., LOPEZ, G. & CHAUDHURY, M. K. 2005. The influence of surface energy on the wetting behaviour of the spore adhesive of the marine alga *Ulva linza* (synonym *Enteromorpha linza*). *Journal of the Royal Society Interface*, 2, 319-325.
- CALLOW, M. E., CALLOW, J. A., ISTA, L. K., COLEMAN, S. E., NOLASCO, A. C. & LOPEZ, G. P. 2000. Use of self-assembled monolayers of different wettabilities to study surface selection and primary adhesion processes of green algal (*Enteromorpha*) zoospores. *Applied and Environmental Microbiology*, 66, 3249-3254.
- CALLOW, M. E., CALLOW, J. A., PICKETT-HEAPS, J. D. & WETHERBEE, R. 1997. Primary adhesion of *enteromorpha* (Chlorophyta, *ulvales*) propagules: Quantitative settlement studies and video microscopy. *Journal of Phycology*, 33, 938-947.
- CALLOW, M. E. & CALLOW, J. E. 2002. Marine biofouling: a sticky problem. *Biologist (London, England)*, 49, 10-4.
- CANDRIES, M., ANDERSON, C. D. & ATLAR, M. 2001 Foul release systems and drag. Proceedings PCE 2001, 28th March 2001 Antwerp.
- CANNING-CLODE, J., FOFONOFF, P., RIEDEL, G.F., TORCHIN, M. & RUIZ, G.M. 2011. The effects of copper pollution on fouling assemblage diversity: a tropical-temperate comparison. *PLoS ONE*, 6(3): e18026.
doi:10.1371/journal.pone.0018026
- CARLTON, J. T. 1988. Biological invasions and misinterpretations of the evolution and ecology of marine communities - trans-oceanic dispersal of non-oceanic larvae and holoplankton. *American Zoologist*, 28, A140-A140.
- CASANUEVA, J. F., SANCHEZ, J., GARCIA-MORALES, J. L., CASANUEVA-ROBLES, T., LOPEZ, J. A., PORTELA, J. R., NEBOT, E. & SALES, D. 2003. Portable pilot plant for evaluating marine biofouling growth and control in heat exchangers-condensers. *Water Science and Technology*, 47, 99-104.

- CHAMBERS, L. D., STOKES, K. R., WALSH, F. C. & WOOD, R. J. K. 2006. Modern approaches to marine antifouling coatings. *Surface & Coatings Technology*, 201, 3642-3652.
- CHAMP, M. A. 2000. A review of organotin regulatory strategies, pending actions, related costs and benefits. *The Science of The Total Environment*, 258, 21-71.
- CHEN, Z.-F., MATSUMURA, K., WANG, H., ARELLANO, S. M., YAN, X., ALAM, I., ARCHER, J. A. C., BAJIC, V. B. & QIAN, P.-Y. 2011. Toward an understanding of the molecular mechanisms of barnacle larval settlement: a comparative transcriptomic approach. *PloS One*, 6, e22913.
doi: 10.1371/journal.pone.0022913
- CHIOVETTI, A., DUGDALE, T.M. & WETHERBEE, R. 2006. Diatom adhesives: molecular and mechanical properties. In SMITH, A.M.& CALLOW, J.A. (eds) *Biological Adhesives*, Berlin, Springer-Verlag, 79-103.
- CHISHOLM, B. J., STAFSLIEN, S. J., CHRISTIANSON, D. A., GALLAGHER-LEIN, C., DANIELS, J. W., RAFFERTY, C., WAL, L. V. & WEBSTER, D. C. 2007a. Combinatorial materials research applied to the development of new surface coatings - VIII: Overview of the high-throughput measurement systems developed for a marine coating workflow. *Applied Surface Science*, 254, 692-698.
- CHISHOLM B.J., WEBSTER D.C., BENNETT J.C., BERRY M., CHRISTIANSON D., KIM J., MAYO B. & GUBBINS N. 2007b. Combinatorial materials research applied to the development of new surface coatings. VII. An automated system for adhesion testing. *Review of Scientific Instruments*, 78, 072213.
- CLARE, A. S. 2011. Toward a characterization of the chemical cue to barnacle gregariousness. In Breithaupt, T. & Theil, M. (eds) *Chemical Communication in Crustaceans*, Springer, New York, 431-450.
- CLARE, A. S. & ALDRED, N. 2009. Surface colonisation by marine organisms and its impact on antifouling research. In Hellio, C. & Yebra D. (eds) *Advances in Marine Antifouling Coatings and Technologies*. Woodhead Publishing, Oxford, 46-79.
- CLARE, A. S., FREET, R. K. & MCCLARY, M. 1994a. On the antennular secretion of the cyprid of *Balanus-amphitrite-amphitrite*, and its role as a settlement pheromone. *Journal of the Marine Biological Association UK*, 74, 243-250.

- CLARE, A. S. & HOEG, J. T. 2008. *Balanus amphitrite* or *Amphibalanus amphitrite*? A note on barnacle nomenclature. *Biofouling*, 24, 55-57.
- CLARE, A. S. & MATSUMURA, K. 2000. Nature and perception of barnacle settlement pheromones. *Biofouling*, 15, 57-71.
- CLARE, A. S., WARD, S. C., RITTSCHOF, D. & WILBUR, K. M. 1994b. Growth increments of the barnacle *Balanus amphitrite amphitrite* Darwin (Cirripedia). *Journal of Crustacean Biology*, 14, 27-35.
- COHEN, A. N. 2005. Guide to the exotic species of San Francisco Bay. San Francisco Estuary Institute, Oakland, CA.
- CONLAN, S. L., MUTTON, R. J., ALDRED, N. & CLARE, A. S. 2008. Evaluation of a fully automated method to measure the critical removal stress of adult barnacles. *Biofouling*, 24, 471-481.
- COOKSEY, K. E. 1981. Requirement for calcium in adhesion of a fouling diatom to glass. *Applied and Environmental Microbiology*, 41, 1378-1382.
- COSTERTAN, J. W., LEWANDOWSKI, Z., CALDWELL, D. E., KORBER, D. R. & LAPPINSCOTT, H. M. 1995. Microbial Biofilms. *Annual Review of Microbiology*, 49, 711-745.
- COSTLOW, J. C. & BOOKHOUT, C. G. 1953. Molting and growth in *Balanus improvises*. *Biological Bulletin*, 105, 420-433.
- COSTLOW, J. C. & BOOKHOUT, C. J. 1956. Moulting and shell growth in *Balanus amphitrite niveus*. *Biological Bulletin*, 110, 107-116.
- CRISP, D. J. 1955. The behaviour of barnacle cyprids in relation to water movement over a surface. *Journal Experimental Biology*, 32, 569-590.
- CRISP, D. J., 1960. Factors influencing the growth rate of *Balanus balanoides*. *Journal of Animal Ecology*, 29, 95-110.
- CRISP, D. J. 1974. Factors influencing the settlement of marine invertebrate larvae. In Grant, P.T. & Mackie A.M. (eds) *Chemoreception in Marine Organisms*, Academic Press, London, 177-265.
- CRISP, D. J. & BOURGET, E. 1985. Growth in Barnacles. *Advances in Marine Biology*, 22, 199-244.
- CRISP, D. J., WALKER, G., YOUNG, G. A. & YULE, A. B. 1985. Adhesion and substrate choice in mussels and barnacles. *Journal of Colloid and Interface Science*, 104, 40-50.

- DAHLEM, C., MORAN, P. J. & GRANT, T. R. 1984. Larval settlement of marine sessile invertebrates on surfaces of different color and position. *Ocean Science and Engineering*, 9, 225-236.
- DAHLSTROM, M., JONSSON, H., JONSSON, P. R. & ELWING, H. 2004. Surface wettability as a determinant in the settlement of the barnacle *Balanus Improvisus* (Darwin). *Journal of Experimental Marine Biology and Ecology*, 305, 223-232.
- DARWIN, C.R. 1851. A monograph of the Cirripedia: The Lepadidae; or, pedunculated cirripedes (vol 1) *Ray Society*
- DARWIN, C.R. 1853 A monograph of the Cirripedia: The Balanidae, (or sessile cirripedes) (vol 2) *Ray Society*
- DAVIES, I. M., BAILEY, S. K. & HARDING, M. J. C. 1998. Tributyltin inputs to the North Sea from shipping activities, and potential risk of biological effects, *ICES Journal of Marine Science*, 55, 34 - 43.
- DEAN, R.B. 1978 Reynolds number dependence of skin friction and other bulk flow variables in two-dimensional rectangular duct flow. *Journal of Fluids Engineering*, 100, 215-223.
- DESAI, D. V., ANIL, A. C. & VENKAT, K. 2006. Reproduction in *Balanus amphitrite* Darwin (Cirripedia : Thoracica): influence of temperature and food concentration *Marine Biology*, 149, 1431-1441.
- DOBRETISOV, S., XIONG, H., XU, Y., LEVIN, L. A. & QIAN, P.-Y. 2007. Novel antifoulants: Inhibition of larval attachment by proteases. *Marine Biotechnology*, 9, 388-397.
- DOMOZYCH, D. S., ELLIOTT, L., KIEMLE, S. N. & GRETZ, M. R. 2007. *Pleurotaenium trabecula*, a desmid of wetland biofilms: The extracellular matrix and adhesion mechanisms. *Journal of Phycology*, 43, 1022-1038.
- DOUGHERTY, W. J. 1990. Barnacle adhesion - reattachment of the adult barnacle *Chthamalus-fragilis* Darwin to polystyrene surfaces followed by centrifugational shearing. *Journal of Crustacean Biology*, 10, 469-478.
- DREANNO, C., KIRBY, R. R. & CLARE, A. S. 2006a. Locating the barnacle settlement pheromone: spatial and ontogenetic expression of the settlement-inducing protein complex of *Balanus amphitrite*. *Proceedings of the Royal Society B-Biological Sciences*, 273, 2721-2728.

- DREANNO, C., KIRBY, R. R. & CLARE, A. S. 2006b. Smelly feet are not always a bad thing: the relationship between cyprid footprint protein and the barnacle settlement pheromone. *Biology Letters*, 2, 423-425.
- DREANNO, C., KIRBY, R. R. & CLARE, A. S. 2007. Involvement of the barnacle settlement-inducing protein complex (SIPC) in species recognition at settlement. *Journal of Experimental Marine Biology and Ecology*, 351, 276-282.
- DREANNO, C., MATSUMURA, K., DOHMAE, N., TAKIO, K., HIROTA, H., KIRBY, R. R. & CLARE, A. S. 2006c. An alpha(2)-macroglobulin-like protein is the cue to gregarious settlement of the barnacle *Balanus amphitrite*. *Proceedings of the National Academy of Sciences of the United States of America*, 103, 14396-14401.
- DUGDALE, T. M., DAGASTINE, R., CHIOVITTI, A., MULVANEY, P. & WETHERBEE, R. 2005. Single adhesive nanofibers from a live diatom have the signature fingerprint of modular proteins. *Biophysical Journal*, 89, 4252-4260.
- DÜRR, S. & THOMASON, J. C. (eds.) 2010. *Biofouling*, Wiley-Blackwell, West Sussex.
- DÜRR, S. & WATSON D.I. 2010 Fouling and antifouling in aquaculture. In DÜRR, S.T. & THOMASON, J. C. (eds.) 2010. *Biofouling*, Wiley-Blackwell, West Sussex, 267-287.
- EDERTH, T., NYGREN, P., PETTITT, M. E., OSTBLOM, M., DU, C.-X., BROO, K., CALLOW, M. E., CALLOW, J. & LIEDBERG, B. 2008. Anomalous settlement behavior of *Ulva linza* zoospores on cationic oligopeptide surfaces. *Biofouling*, 24, 303-312.
- EGUIA, E., TRUEBA, A., RIO-CALONGE, B., GIRON, A., AMIEVA, J. J. & BIELVA, C. 2008. Combined monitor for direct and indirect measurement of biofouling. *Biofouling*, 24, 75-86.
- EKIN, A. & WEBSTER, D. C. 2007. Combinatorial and high-throughput screening of the effect of siloxane composition on the surface properties of crosslinked siloxane-polyurethane coatings. *Journal of Combinatorial Chemistry*, 9, 178-188.
- ELBOURNE, P. D., VEATER, R. A. & CLARE, A. S. 2008. Interaction of conspecific cues in *Balanus amphitrite* Darwin (Cirripedia) settlement assays: Continued argument for the single-larva assay. *Biofouling*, 24, 87-96.

- FENT, K. 1996. Ecotoxicology of organotin compounds. *Critical Reviews in Toxicology*, 26, 1-117.
- FERNANDEZ, M. S., VERGARA, I., OYARZUN, A., ARIAS, J. I., RODRIGUEZ, R., WIFF, J. P., FUENZALIDA, V. M. & ARIAS, J. L. 2002. Extracellular matrix molecules involved in barnacle shell mineralization. *Materials Research Society Symposium Proceedings*, 724, 3-9.
- FINNIE, A.A. & WILLIAMS D.N. 2010. Paint and coatings technology for the control of marine fouling. In DÜRR, S.T. & THOMASON, J. (eds) *Biofouling*, Wiley and Sons Ltd, West Sussex, 185-206.
- FINLAY, J. A., BENNETT, S. M., BREWER, L. H., SOKOLOVA, A., CLAY, G., GUNARI, N., MEYER, A. E., WALKER, G. C., WENDT, D. E., CALLOW, M. E., CALLOW, J. A. & DETTY, M. R. 2010. Barnacle settlement and the adhesion of protein and diatom microfouling to xerogel films with varying surface energy and water wettability. *Biofouling*, 26, 657-666.
- FINLAY, J. A., CALLOW, M. E., ISTA, L. K., LOPEZ, G. P. & CALLOW, J. A. 2002a. The influence of surface wettability on the adhesion strength of settled spores of the green alga *Enteromorpha* and the diatom *Amphora*. *Integrative and Comparative Biology*, 42, 1116-1122.
- FINLAY, J. A., CALLOW, M. E., SCHULTZ, M. P., SWAIN, G. W. & CALLOW, J. A. 2002b. Adhesion strength of settled spores of the green alga *Enteromorpha*. *Biofouling*, 18, 251-256.
- FLAMMANG, P., MICHEL, A., VAN CAUWENBERGE, A., ALEXANDRE, H. & JANGOUX, M. 1998. A study of the temporary adhesion of the podia in the sea star *Asterias rubens* (Echinodermata, Asteroidea) through their footprints. *Journal of Experimental Biology*, 201, 2383-2395.
- FLAMMANG, P., RIBESSE, J. & JANGOUX, M. 2002. Biomechanics of adhesion in sea cucumber Cuvierian tubules (Echinodermata, Holothuroidea). *Integrative and Comparative Biology*, 42, 1107-1115.
- FLETCHER, M. & MARSHALL, K. C. 1982. Bubble contact-angle method for evaluating substratum interfacial characteristics and its relevance to bacterial attachment. *Applied and Environmental Microbiology*, 44, 184-192.
- FLETCHER, R. L. & CALLOW, M. E. 1992. The settlement, attachment and establishment of marine algal spores. *British Phycological Journal*, 27, 303-329.

- FORTEATH, G.N.R., PICKEN, G.B. & RALPH, R. 1984. Patterns of macrofouling on steel platforms in the central and northern North Sea. In Lewis J.R. & Mercer A.D (eds), *Corrosion and Marine Growth on Offshore Structures* Society of Chemical Industry and Ellis Horwood Limited, Chichester, 10-22.
- FURMAN, E. R. & YULE, A. B. 1990. Self-Fertilization in *Balanus-improvisus* Darwin. *Journal of Experimental Marine Biology and Ecology*, 144, 235-239.
- FUSETANI, N. 2004. Biofouling and antifouling. *Natural Product Reports*, 21, 94-104.
- FYHN, H. J. 1974. Proline dominance in intracellular amino-acid pool of a cirripede crustacean. *Acta Physiologica Scandinavica*, 91, A17-A18.
- FYHN, U. E. H. & COSTLOW, J. D. 1976. Histochemical study of cement secretion during intermolt cycle in barnacles *Biological Bulletin*, 150, 47-56.
- GEIERMAN, C. & EMLET, R. 2009. Feeding behavior, cirral fan anatomy, Reynolds numbers, and leakiness of *Balanus glandula*, from post-metamorphic juvenile to the adult. *Journal of Experimental Marine Biology and Ecology*, 379, 68-76.
- GERACI, J. J., AMRHEIN, C. C. & GOODSON, C. C. (2008). Barnacle growth rate on artificial substrate in the Salton Sea, California. *Hydrobiologia*, 604(1), 77-84.
- GERHART, D.J., RITTSCHOF, D., HOOPER, I.R., EISENMAN, K., MEYER, A.E., BAIER, R.E. & YOUNG, C. 1992 Rapid and inexpensive quantification of the combined polar components of surface wettability: application to biofouling. *Biofouling*, 5, 251-259.
- GLENNER, H. & HEBSGAARD, M. B. 2006. Phylogeny and evolution of life history strategies of the parasitic barnacles (Crustacea, Cirripedia, Rhizocephala). *Molecular Phylogenetics and Evolution*, 41, 528-538.
- GORB, S. N., SCHUPPERT, J., WALTHER, P. & SCHWARZ, H. 2012. Contact behaviour of setal tips in the hairy attachment system of the fly *Calliphora vicina* (Diptera, Calliphoridae): a cryo-SEM approach. *Zoology (Jena, Germany)*, 115, 142-50.
- GRAHAM, P. D., JOINT, I., NEVELL, T. G., SMITH, J. R., STONE, M. & TSIBOUKLIS, J. 2000. Bacterial colonisation and settlement of algal spores and barnacle larvae on low surface energy materials. *Biofouling*, 16, 289-299.
- GRANHAG, L. M., FINLAY, J. A., JONSSON, P. R., CALLOW, J. A. & CALLOW, M. E. 2004. Roughness-dependent removal of settled spores of the green alga

ulva (syn. *enteromorpha*) exposed to hydrodynamic forces from a water jet. *Biofouling*, 20, 117-122.

GRAVE, B. H. 1933. Rate of growth, age at sexual maturity, and duration of life of certain sessile organisms, at Woods Hole, Massachusetts. *Biological Bulletin*, 65, 375-386.

GRUET, Y., VOVELLE, J. & GRASSET, M. 1987. Biomineral components of tube cement of *Sabellaria-alveolata* (L), (Annelida Polychaeta). *Canadian Journal of Zoology-Revue Canadienne De Zoologie*, 65, 837-842.

HADERLIE, E. C. 1984. A brief overview of the effects of macrofouling. in *Marine biodeterioration: An interdisciplinary study* Costlow JD, Tipper RC (eds). US Naval Institute, Annapolis, 163–166.

HANSEN, W. R. & AUTUMN, K. 2005. Evidence for self-cleaning in gecko setae. *Proceedings of the National Academy of Sciences of the United States of America*, 102, 385-389.

HEAD, R. M., BERNTSSON, K. M., DAHLSTROM, M., OVERBEKE, K. & THOMASON, J. C. 2004. Gregarious settlement in cypris larvae: The effects of cyprid age and assay duration. *Biofouling*, 20, 123-128.

HELLIO, C., BERGE, J. P., BEAUPOIL, C., LE GAL, Y. & BOURGOUGNON, N. 2002. Screening of marine algal extracts for anti-settlement activities against microalgae and macroalgae. *Biofouling*, 18, 205-215.

HELLIO, C., MARECHAL, J. P., VERON, B., BREMER, G., CLARE, A. S. & LE GAL, Y. 2004. Seasonal variation of antifouling activities of marine algae from the Brittany coast (France). *Marine Biotechnology*, 6, 67-82.

HELLIO, C. & YEBRA, D. M. 2009. Advances in marine antifouling coatings and technologies: Introduction. *Advances in Marine Antifouling Coatings and Technologies*.

HIEMENZ P.C. & RAJAGOPALAN, R. 1997 Principles of colloid and surface chemistry. 3rd Edition Marcel Dekker, Inc.

HILLS, J. M., THOMASON, J. C., DAVIS, H., KOHLER, J. & MILLETT, E. 2000. Exploratory behaviour of barnacle larvae in field conditions. *Biofouling*, 16, 171-179.

- HOAGLAND, K. D., ROSOWSKI, J. R., GRETZ, M. R. & ROEMER, S. C. 1993. Diatom extracellular polymeric substances - function, fine-structure, chemistry, and physiology. *Journal of Phycology*, 29, 537-566.
- HOCH, J.M. 2011 Effects of crowding and wave exposure on cirrus morphology of the acorn barnacle, *Semibalanus balanoides*. *Journal of Crustacean Biology*, 31, 401-405.
- HOLLAND, R., DUGDALE, T. M., WETHERBEE, R., BRENNAN, A. B., FINLAY, J. A., CALLOW, J. A. & CALLOW, M. E. 2004. Adhesion and motility of fouling diatoms on a silicone elastomer. *Biofouling*, 20, 323-329.
- HOLM, E. R., HASLBECK, E. G. & HORINEK, A. A. 2003. Evaluation of brushes for removal of fouling from fouling-release surfaces, using a hydraulic cleaning device. *Biofouling*, 19, 297-305.
- HOLM, E. R., KAVANAGH, C. J., MEYER, A. E., WIEBE, D., NEDVED, B. T., WENDT, D., SMITH, C. M., HADFIELD, M. G., SWAIN, G., WOOD, C. D., TRUBY, K., STEIN, J. & MONTEMARANO, J. 2006. Interspecific variation in patterns of adhesion of marine fouling to silicone surfaces. *Biofouling*, 22, 233-243.
- HOLM, E. R., KAVANAGH, C. J., ORIHUELA, B. & RITTSCHOF, D. 2009. Phenotypic variation for adhesive tenacity in the barnacle *Balanus amphitrite*. *Journal of Experimental Marine Biology and Ecology*, 380, 61-67.
- HOLM, E. R., NEDVED, B. T., PHILLIPS, N., DEANGELIS, K. L., HADFIELD, M. G. & SMITH, C. M. 2000. Temporal and spatial variation in the fouling of silicone coatings in Pearl Harbor, Hawaii. *Biofouling*, 15, 95-107.
- HOLM, E. R., ORIHUELA, B., KAVANAGH, C. J. & RITTSCHOF, D. 2005. Variation among families for characteristics of the adhesive plaque in the barnacle *Balanus amphitrite*. *Biofouling*, 21, 121-126.
- HOROBIN, R.W. & KIERNAN, J.A. (Eds) 2002 Conn's biological stains. 10th Edition Taylor and Francis Oxford, pp576.
- HSU, R.-L., LEE, K.-T., WANG, J.-H., LEE, L. Y. L. & CHEN, R. P. Y. 2009. Amyloid-degrading ability of nattokinase from *Bacillus subtilis* Natto. *Journal of Agricultural and Food Chemistry*, 57, 503-508.
- HUBER, G., MANTZ, H., SPOLENAK, R., MECKE, K., JACOBS, K., GORB, S. N. & ARZT, E. 2005. Evidence for capillarity contributions to gecko adhesion from

- single spatula nanomechanical measurements. *Proceedings of the National Academy of Sciences of the United States of America*, 102, 16293-16296.
- HUDSON, C., BOURGET, E. & LEGENDRE, P. 1983. An intergrated study of the factors influencing the choice of settling site of *Balanus crenatus* cyprid larvae. *Canadian Journal of Fisheries and Aquatic Sciences*, 40, 1186-1194.
- HUNG Y.L., CHANG, Y.Y., WANG, M.J. & LIN, S.Y. 2010 A simple method for measuring the superhydrophobic contact angle with high accuracy. *Review of Scientific Instruments*, 81, 65-105.
- IMO (2001) *International convention on controlling abtifouling paints*. International Maritime Organisation. London.
- IMO (2002) *Anti-fouling systems*. International Maritime Organisation. London.
- IMO (2007) *Problems and solutions*. International Maritime Organisation. London.
- ISTA, L. K., CALLOW, M. E., FINLAY, J. A., COLEMAN, S. E., NOLASCO, A. C., SIMONS, R. H., CALLOW, J. A. & LOPEZ, G. P. 2004. Effect of substratum surface chemistry and surface energy on attachment of marine bacteria and algal spores. *Applied and Environmental Microbiology*, 70, 4151-4157.
- ISTA, L. K., FAN, H. Y., BACA, O. & LOPEZ, G. P. 1996. Attachment of bacteria to model solid surfaces: Oligo(ethylene glycol) surfaces inhibit bacterial attachment. *Fems Microbiology Letters*, 142, 59-63.
- JARRETT, J. N. 2003. Seasonal variation in larval condition and postsettlement performance of the barnacle *Semibalanus balanoides*. *Ecology*, 84, 384-390.
- JARRETT, J. N. & PECHENIK, J. A. 1997. Temporal variation in cyprid quality and juvenile growth capacity for an intertidal barnacle. *Ecology*, 78, 1262-1265.
- JENKINS, R.J., NORTON, T.A. & HAWKINS, S.J. 1999. Settlement and post settlement interactions between *Semibalanus balanoides* (L.)(Crustacea:Cirripedia) and three species of furoid canopy algae. *Journal of Experimental Marine Biology and Ecology*, 236, 49-67.
- JENSEN, R. A. & MORSE, D. E. 1988. The bioadhesive of *Phragmatopoma-californica* tubes - a silk-like cement containing L-Dopa. *Journal of Comparative Physiology B-Biochemical Systemic and Environmental Physiology*, 158, 317-324.

- JOHNSTON, L. A. 2010. *Temperature Affects Adhesion of the Acorn Barnacle (Balanus amphitrite)*. MS in Biological Sciences, California Polytechnic State University.
- JONES, G. 2009. The battle against marine biofouling: a historical review. *Advances in Marine Antifouling Coatings and Technologies*.
- JONSSON, P. R., BERNTSSON, K. M. & LARSSON, A. I. 2004. Linking larval supply to recruitment: Flow-mediated control of initial adhesion of barnacle larvae. *Ecology*, 85, 2850-2859.
- KAMINO, K. 2006. Barnacle underwater adhesive: Complexity from multi-functionality in a multi-protein complex. In Fingerman, M. & Nagabhushanam, R. (eds). *Biomaterials from Aquatic and Terrestrial Organisms*. Science Publishers, 1-610.
- KAMINO, K. 2008. Underwater adhesive of marine organisms as the vital link between biological science and material science. *Marine Biotechnology*, 10, 111-121.
- KAMINO, K. 2010. Molecular design of barnacle cement in comparison with those of mussel and tubeworm. *Journal of Adhesion*, 86, 96-110.
- KAMINO, K., INOUE, K., MARUYAMA, T., TAKAMATSU, N., HARAYAMA, S. & SHIZURI, Y. 2000. Barnacle cement proteins - Importance of disulfide bonds in their insolubility. *Journal of Biological Chemistry*, 275, 27360-27365.
- KAMINO, K., ODO, S. & MARUYAMA, T. 1996. Cement proteins of the acorn barnacle, *Megabalanus rosa*. *Biological Bulletin*, 190, 403-409.
- KAMINO, K. & SHIZURI, Y. 1998. Structure and function of barnacle cement proteins. In LeGal, Y. & Halvorson, H. (eds) *New Developments in Marine Biotechnology*. Plenum Press, New York, 77-80.
- KATO-YOSHINAGA, Y., NAGANO, M., MORI, S., CLARE, A.S., FUSETANI, N. & MATSUMURA, K. 2000. Species specificity of barnacle settlement-inducing proteins. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, 125, 511-516.
- KAVANAGH, C. J., QUINN, R. D. & SWAIN, G. W. 2005. Observations of barnacle detachment from silicones using high-speed video. *Journal of Adhesion*, 81, 843-850.
- KAVANAGH, C. J., SCHULTZ, M. P., SWAIN, G. W., STEIN, J., TRUBY, K. & WOOD, C. D. 2001. Variation in adhesion strength of *Balanus eburneus*,

Crassostrea virginica and *Hydroides dianthus* to fouling-release coatings.

Biofouling, 17, 155-167.

- KAVANAGH, C. J., SWAIN, G. W., KOVACH, B. S., STEIN, J., DARKANGELO-WOOD, C., TRUBY, K., HOLM, E., MONTEMARANO, J., MEYER, A. & WIEBE, D. 2003. The effects of silicone fluid additives and silicone elastomer matrices on barnacle adhesion strength. *Biofouling*, 19, 381-390.
- KHANDEPARKER, L., DESAI, D. & SHIRAYAMA, Y. 2005. Larval development and post-settlement metamorphosis of the barnacle *Balanus albicostatus* Pilsbry and the serpulid polychaete *Pomatoleios kraussii* Baird: Impact of a commonly used antifouling biocide, Irgarol 1051. *Biofouling*, 21, 169-180.
- KHURANA, R., UVERSKY, V. N., NIELSEN, L. & FINK, A. L. 2001. Is Congo red an amyloid-specific dye? *Journal Of Biological Chemistry*, 276, 22715-22721.
- KIM, J., CHISHOLM, B. J. & BAHR, J. 2007. Adhesion study of silicone coatings: the interaction of thickness, modulus and shear rate on adhesion force. *Biofouling*, 23, 113-120.
- KIM, Y. D., DORDICK, J. S. & CLARK, D. S. 2001. Siloxane-based biocatalytic films and paints for use as reactive coatings. *Biotechnology and Bioengineering*, 72, 475-482.
- KNIGHT-JONES, E. W. & CRISP, D. J. 1953. Gregariousness in barnacles in relation to the fouling of ships and to anti-fouling research. *Nature*, 171, 1109-1110.
- KOHL, J. G. & SINGER, I. L. 1999. Pull-off behavior of epoxy bonded to silicone duplex coatings. *Progress in Organic Coatings*, 36, 15-20.
- KRISTENSEN, J. B., MEYER, R. L., LAURSEN, B. S., SHIPOVSKOV, S., BESENBACHER, F. & POULSEN, C. H. 2008. Antifouling enzymes and the biochemistry of marine settlement. *Biotechnology Advances*, 26, 471-481.
- KROHLING, W., BROTTOT, D. S. & ZALMON, I. R. 2006. Fouling community recruitment on an artificial reef in the north coast of Rio de Janeiro state. *Journal of Coastal Research*, 39, 1118-1121.
- KUGELE, M. & YULE, A. B. 1993. Mobility in lepadomorph barnacles. *Journal of the Marine Biological Association of the United Kingdom*, 73, 719-722.
- LACOMBE, D. 1970. A comparative study of cement glands in some balanid barnacles (Cirripedia-Balanidae). *Biological Bulletin*, 139, 164-172.

- LAGERSSON, N. C., GARM, A. & HOEG, J. T. 2003. Notes on the ultrastructure of the setae on the fourth antennular segment of the *Balanus amphitrite* cyprid (Crustacea : Cirripedia : Thoracica). *Journal of the Marine Biological Association of the United Kingdom*, 83, 361-365.
- LARSSON, A. I., MATTSSON-THORNGREN, L., GRANHAG, L. M. & BERGLIN, M. 2010. Fouling-release of barnacles from a boat hull with comparison to laboratory data of attachment strength. *Journal of Experimental Marine Biology and Ecology*, 392, 107-114.
- LAUGHLIN, R. B. & LINDEN, O. 1985. Fate and effects of organotin compounds. *Ambio*, 14, 88-94.
- LEBRET, K., THABARD, M. & HELLIO, C. 2009. Algae as marine fouling organisms: adhesion damage and prevention. In Hellio, C. & Yebra, D. (eds) *Advances in Marine Antifouling Coatings and Technologies*. Woodhead Publishing Ltd, 19-45.
- LEE, H., LEE, B. P. & MESSERSMITH, P. B. 2007. A reversible wet/dry adhesive inspired by mussels and geckos. *Nature*, 448, 338-341.
- LEE, R. F. 1991. Metabolism of tributyltin by marine animals and possible linkages to effects. *Marine Environmental Research*, 32, 29-35.
- LEROY, C., DELBARRE-LADRAT, C., GHILLEBAERT, F., COMPERE, C. & COMBES, D. 2008. Effects of commercial enzymes on the adhesion of a marine biofilm-forming bacterium. *Biofouling*, 24, 11-22.
- LINDNER, E. 1992. A low surface free energy approach in the control of marine biofouling. *Biofouling*, 6, 193-205.
- LINLEY, E.A.S., WILDING, T.A., BLACK, K., HAWKINS, A.J.S. & MANGI, S. 2007. Review of the reef effects of offshore wind farm structures and their potential for enhancement and mitigation. Report to the Department for Business, Enterprise and Regulatory Reform. *RFCA/005/0029P*.
- LOPEZ, B. A., RAMIREZ, R. P., GUAITRO, S. Y. & LOPEZ, D. A. 2010. Interspecific differences in the phenotypic plasticity of intertidal barnacles in response to habitat changes. *Journal of Crustacean Biology*, 30, 357-365.
- LOPEZ, D. A., CASTRO, J. M., GONZALEZ, M. L. & SIMPFENDORFER, R. W. 2003. Physiological responses to hypoxia and anoxia in the giant barnacle, *Austromegabalanus psittacus* (Molina, 1782). *Crustaceana*, 76, 533-545.

- LOPEZ, D. A., ESPINOZA, E. A., LOPEZ, B. A. & SANTIBANEZ, A. F. 2008. Molting behavior and growth in the giant barnacle *Austromegabalanus psittacus* (Molina, 1782). *Revista De Biología Marina Y Oceanografía*, 43, 607-613.
- LOVE, J. C., ESTROFF, L. A., KRIEBEL, J. K., NUZZO, R. G. & WHITESIDES, G. M. 2005. Self-assembled monolayers of thiolates on metals as a form of nanotechnology. *Chemical Reviews*, 105, 1103-1169.
- LUBCHENCO, J., & MENGE, B.A. 1978 Community development and persistence in a low rocky intertidal zone. *Ecological Monographs*, 48, 67-94.
- MAJUMDAR, P., LEE, E., PATEL, N., WARD, K., STAFSLIEN, S. J., DANIELS, J., CHISHOLM, B. J., BOUDJOUK, P., CALLOW, M. E., CALLOW, J. A. & THOMPSON, S. E. M. 2008. Combinatorial materials research applied to the development of new surface coatings IX: An investigation of novel antifouling/fouling-release coatings containing quaternary ammonium salt groups. *Biofouling*, 24, 185-200.
- MAKI, J. S., DING, L., STOKES, J., KAVOURAS, J. H. & RITTSCHOF, D. 2000. Substratum/bacterial interactions and larval attachment: Films and exopolysaccharides of *Halomonas marina* (ATCC 25374) and their effect on barnacle cyprid larvae, *Balanus amphitrite* Darwin. *Biofouling*, 16, 159-170.
- MAKI, J. S., YULE, A. B., RITTSCHOF, D. & MITCHELL, R. 1994. The effect of bacterial films on the temporary adhesion and permanent fixation of cypris larvae, *Balanus amphitrite* Darwin. *Biofouling*, 8, 121-131.
- MAKI, J.S., RITTSCHOF, D., SAMUELSSON, M.-Q., SZEWZYK, U., YULE, A.B., KJELLEBERG, S., COSTLOW, J.D., MITCHELL, R. 1990 Effect of marine bacteria and their exopolymers on the attachment of barnacle cypris larvae. *Bulletin of Marine Science*, 46, 499-511.
- MARCHINKO, K. B. 2003. Dramatic phenotypic plasticity in barnacle legs (*Balanus glandula* Darwin): Magnitude, age dependence, and speed of response. *Evolution*, 57, 1281-1290.
- MARECHAL, J. P., HELLIO, C., SEBIRE, M. & CLARE, A. S. 2004. Settlement behaviour of marine invertebrate larvae measured by EthoVision 3.0. *Biofouling*, 20, 211-217.

- F. S. MARKLAND, F.S. & SMITH, E.L. 1971. Sub-tilisins: primary structure, chemical and physical properties. In *The Enzymes*, Vol. 3 (Ed Boyer, P.D.), Academic Press, New York and London, USA, 561-608.
- MARUZZO, D., CONLAN, S., ALDRED, N., CLARE, A. S. & HOEG, J. T. 2011. Video observation of surface exploration in cyprids of *Balanus amphitrite*: the movements of antennular sensory setae. *Biofouling*, 27, 225-239.
- MATSUMURA, K., HILLS, J. M., THOMASON, P. O., THOMASON, J. C. & CLARE, A. S. 2000. Discrimination at settlement in barnacles: Laboratory and field experiments on settlement behaviour in response to settlement-inducing protein complexes. *Biofouling*, 16, 181-190.
- MATSUMURA, K., NAGANO, M. & FUSETANI, N. 1998a. Purification of a larval settlement-inducing protein complex (SIPC) of the barnacle, *Balanus amphitrite*. *Journal of Experimental Zoology*, 281, 12-20.
- MATSUMURA, K., NAGANO, M., KATO-YOSHINAGA, Y., YAMAZAKI, M., CLARE, A. S. & FUSETANI, N. 1998b. Immunological studies on the settlement-inducing protein complex (SIPC) of the barnacle *Balanus amphitrite* and its possible involvement in larva-larva interactions. *Proceedings of the Royal Society of London Series B-Biological Sciences*, 265, 1825-1830.
- MENGE, B. A. 1978. Predation intensity in a rocky inter-tidal community - relation between predator foraging activity and environmental harshness. *Oecologia*, 34, 1-16.
- MEYER, A., BAIER, R., WOOD, C. D., STEIN, J., TRUBY, K., HOLM, E., MONTEMARANO, J., KAVANAGH, C., NEDVED, B., SMITH, C., SWAIN, G. & WIEBE, D. 2006. Contact angle anomalies indicate that surface-active eluates from silicone coatings inhibit the adhesive mechanisms of fouling organisms. *Biofouling*, 22, 411-423.
- MILNE, A. & HAILS, G. 1974. Patent GB 1 457 590 *International Paint Plc*.
- MINEUR, F., JOHNSON, M. P., MAGGS, C. A. & STEGENGA, H. 2007. Hull fouling on commercial ships as a vector of macroalgal introduction. *Marine Biology*, 151, 1299-1307.
- MIRON, G., BOURGET, E. & ARCHAMBAULT, P. 1996. Scale of observation and distribution of adult conspecifics: Their influence in assessing passive and active

- settlement mechanisms in the barnacle *Balanus crenatus* (Brugiere). *Journal of Experimental Marine Biology and Ecology*, 201, 137-158.
- MOLINO, P. J. & WETHERBEE, R. 2008. The biology of biofouling diatoms and their role in the development of microbial slimes. *Biofouling*, 24, 365-379.
- MOLNAR, J. L., GAMBOA, R. L., REVENGA, C. & SPALDING, M. D. 2008. Assessing the global threat of invasive species to marine biodiversity. *Frontiers in Ecology and the Environment*, 6, 485-492.
- MOORE, H. B. 1934. The biology of *Balanus balanoides* I. Growth rate and its relation to size, season and tidal level. *Journal of the Marine Biological Association UK*, 19, 851-868.
- MÜLLER-STEINHAGEN, H., MALAYEN, M.R., & WATKINSON, A.P. 2005. Fouling of heat exchangers - new approaches to solve an old problem. *Heat Transfer Engineering*, 26, 1-4.
- MURTHY, P.S., VENUGOPALAN, V.P., NAIR, K.V.K. & SUBRAMONIAM, T. 2009. Larval settlement and surfaces: Implications in development of antifouling strategies. In *Marine and Industrial Biofouling* (Eds: Flemming, H.C., Murthy, P.S., Venkatesan, R. & Cooksey, K.E.), Springer-Verlag, Berlin, Germany, 233-264.
- NAKANO, M., SHEN, J.-R. & KAMINO, K. 2007. Self-assembling peptide inspired by a barnacle underwater adhesive protein. *Biomacromolecules*, 8, 1830-1835.
- NALDRETT, M. J. & KAPLAN, D. L. 1997. Characterization of barnacle (*Balanus eburneus* and *B. crenatus*) adhesive proteins. *Marine Biology*, 127, 629-635.
- NASROLAHI, A., SARI, A., SAIFABADI, S. & MALEK, M. 2007. Effects of algal diet on larval survival and growth of the barnacle *Amphibalanus (=Balanus) improvisus*. *Journal of the Marine Biological Association UK*, 87, 1227-1233.
- NAVARRETE, S. A. & WIETERS, E. A. 2000. Variation in barnacle recruitment over small scales: larval predation by adults and maintenance of community pattern. *Journal of Experimental Marine Biology and Ecology*, 253, 131-148.
- NEBOT, E., CASANUEVA, J. F., SOLERA, R., PENDON, C., TARACIDO, L. J., CASANUEVA-ROBLES, T. & LOPEZ-GALINDO, C. 2010. Marine Biofouling in Heat Exchangers. In Chang, J. & Wong S. (eds) *Biofouling: Types, Impact and Anti-Fouling*, Nova Science Publishers Inc, New York, 65 - 104.

- NEGRI, A. & MARSHALL, P. 2009. TBT contamination of remote marine environments: Ship groundings and ice-breakers as sources of organotins in the Great Barrier Reef and Antarctica. *Journal of Environmental Management*, 90, S31-S40.
- NEIDHART, D. J. & PETSKO, G. A. 1988. The refined crystal-structure of subtilisin carlsberg at 2.5 Å resolution. *Protein Engineering*, 2, 271-276.
- NEUFELD, C. J. 2011. Modular phenotypic plasticity: divergent responses of barnacle penis and feeding leg form to variation in density and wave-exposure. *Journal of Experimental Zoology Part B-Molecular and Developmental Evolution*, 316B, 254-262.
- NEUFELD, C. J. & RANKINE, C. 2012. Cuticle and muscle variation underlying phenotypic plasticity in barnacle feeding leg and penis form. *Invertebrate Biology*, 131, 96-109.
- NEUHEIMER, A. B. & TAGGART, C. T. 2007. The growing degree-day and fish size-at-age: the overlooked *Canadian Journal of Fisheries & Aquatic Sciences*, 64, 375-385.
- NGUYEN, T., BYRD, W. E., ALSHED, D., CHIN, J., CLERICI, C. & MARTIN, J. 2007. Relationship between interfacial water layer adhesion loss of silicon/glass fiber-epoxy systems: A quantitative study. *Journal of Adhesion*, 83, 587-610.
- NOTT, J. A. 1969. Settlement of barnacle larvae: Surface structure of the antennular attachment disc by scanning electron microscopy. *Marine Biology*, 2, 248-251.
- NOTT, J. A. & FOSTER, B. A. 1969. On the structure of the antennular attachment organ of the cypris larva of *Balanus balanoides* (L.). *Philosophical Transactions of the Royal Society of London*, 256, 115-134.
- OCONNOR, N. J. & RICHARDSON, D. L. 1994. Comparative attachment of barnacle cyprids (*Balanus-amphitrite* Darwin, 1854, *B-improvisus* Darwin, 1854, and *B-eburneus* Gould, 1841) to polystyrene and glass substrata. *Journal of Experimental Marine Biology and Ecology*, 183, 213-225.
- ODLING, K., ALBERTSSON, C., RUSSELL, J. T. & MARTENSSON, L. G. E. 2006. An in vivo study of exocytosis of cement proteins from barnacle *Balanus improvisus* (D.) cyprid larva. *Journal of Experimental Biology*, 209, 956-964.

- OKANO, K., SHIMIZU, K., SATUITO, C. G. & FUSEYANI, N. 1996. Visualization of cement exocytosis in the cypris cement gland of the barnacle *Megabalanus rosa*. *Journal of Experimental Biology*, 199, 2131-2137.
- OLSEN, S. M., PEDERSEN, L. T., LAURSEN, M. H., KIIL, S. & DAM-JOHANSEN, K. 2007. Enzyme-based antifouling coatings: a review. *Biofouling*, 23, 369-383.
- OMAE, M. 2003. General aspects of tin-free antifouling paints. *Chemical Reviews*, 103, 3431-3448.
- OSAKI, T. & WERNER, C. 2003. Ionization characteristics and structural transitions of alternating maleic acid copolymer films. *Langmuir*, 19, 5787-5793.
- OTNESS, J.S. & MEDCALF, D.G 1972 Chemical and physical characterisation of barnacle cement. *Comparative Biochemistry and Physiology B*, 43, 443-449.
- PAINE, R. T. 1971. Short-term experimental investigation of resource partitioning in a New-Zealand rocky intertidal habitat. *Ecology*, 52, 1096-1106.
- PATEL, B. & CRISP, D. J. 1960. The influence of temperature on the breeding and moulting activities of some warm-water species of operculate barnacles. *Journal of the Marine Biological Association UK*, 39, 667-680.
- PATIL, J. S. & ANIL, A. C. 2005. Influence of diatom exopolymers and biofilms on metamorphosis in the barnacle *Balanus amphitrite*. *Marine Ecology-Progress Series*, 301, 231-245.
- PAUL, J. H. & JEFFREY, W. H. 1985. Evidence for separate adhesion mechanisms for hydrophilic and hydrophobic surfaces in *Vibrio-proteolytica*. *Applied and Environmental Microbiology*, 50, 431-437.
- PECHENIK, J. A. 2006. Larval experience and latent effects - metamorphosis is not a new beginning. *Integrative and Comparative Biology*, 46, 323-333.
- PETRONE, L., DI FINO, A., ALDRED, N., SUKKAEW, P., EDERTH, T. CLARE, A.S., LIEDBERG, B. 2011. Effects of surface charge and Gibbs surface energy on the settlement behaviour of barnacle cyprids (*Balanus amphitrite*). *Biofouling*, 27, 1043-1055.
- PETTITT, M. E., HENRY, S. L., CALLOW, M. E., CALLOW, J. A. & CLARE, A. S. 2004. Activity of commercial enzymes on settlement and adhesion of cypris larvae of the barnacle *Balanus amphitrite*, spores of the green alga *Ulva linza*, and the diatom *Navicula perminuta*. *Biofouling*, 20, 299-311.

- PHANG, I. Y., ALDRED, N., CLARE, A. S., CALLOW, J. A. & VANCOSO, G. J. 2006. An in situ study of the nanomechanical properties of barnacle (*Balanus amphitrite*) cyprid cement using atomic force microscopy (AFM). *Biofouling*, 22, 245-250.
- PHANG, I. Y., ALDRED, N., CLARE, A. S. & VANCOSO, G. J. 2008. Towards a nanomechanical basis for temporary adhesion in barnacle cyprids (*Semibalanus balanoides*). *Journal of the Royal Society Interface*, 5, 397-401.
- PHANG, I. Y., ALDRED, N., LING, X. Y., HUSKENS, J., CLARE, A. S. & VANCOSO, G. J. 2010. Atomic force microscopy of the morphology and mechanical behaviour of barnacle cyprid footprint proteins at the nanoscale. *Journal of the Royal Society Interface*, 7, 285-296.
- PHANG, I. Y., CHAW, K. C., CHOO, S. S. H., KANG, R. K. C., LEE, S. S. C., BIRCH, W. R., TEO, S. L. M. & VANCOSO, G. J. 2009. Marine biofouling field tests, settlement assay and footprint micromorphology of cyprid larvae of *Balanus amphitrite* on model surfaces. *Biofouling*, 25, 139-147.
- POLITIS, G., DOWNIE, M., ATLAR, M. 2009 Design of a turbulent channel flow facility for antifouling coating research. *Proceedings 1st Advanced Model Measurement Technology* Nantes 12-17.
- POMPE, T., RENNER, L., GRIMMER, M., HEROLD, N. & WERNER, C. 2005. Functional films of maleic anhydride copolymers under physiological conditions. *Macromolecular Bioscience*, 5, 890-895.
- POMPE, T., ZSCHOCHE, S., HEROLD, N., SALCHERT, K., GOUZY, M. F., SPERLING, C. & WERNER, C. 2003. Maleic anhydride copolymers - A versatile platform for molecular biosurface engineering. *Biomacromolecules*, 4, 1072-1079.
- PRIME, K.L. & WHITESIDES, G.M. 1991 Self-assembled organic monolayers: model systems for studying adsorption of proteins at surfaces. *Science*, 252, 1164-1167.
- PUTHOFF, J. B., PROWSE, M. S., WILKINSON, M. & AUTUMN, K. 2010. Changes in materials properties explain the effects of humidity on gecko adhesion. *Journal of Experimental Biology*, 213, 3699-3704.
- QIAN, P. Y., RITTSCHOF, D. & SREEDHAR, B. 2000. Macrofouling in unidirectional flow: miniature pipes as experimental models for studying the

- interaction of flow and surface characteristics on the attachment of barnacle, bryozoan and polychaete larvae. *Marine Ecology-Progress Series*, 207, 109-121.
- RAINBOW, P. S. 1995. Physiology, Physicochemistry and Metal Uptake - a Crustacean Perspective. *Marine Pollution Bulletin*, 31, 55-59.
- RALSTON, E. & SWAIN, G. 2009 Bioinspiration - The solution for biofouling control? *Bioinspiration and Biomimetics*, 4, 15007-15015.
- RAMAN, S. & KUMAR, R. 2011. Interfacial morphology and nanomechanics of cement of the barnacle, *Amphibalanus reticulatus* on metallic and non-metallic substrata. *Biofouling*, 27, 569-577.
- RAMSAY, D. B., DICKINSON, G. H., ORIHUELA, B., RITTSCHOF, D. & WAHL, K. J. 2008. Base plate mechanics of the barnacle *Balanus amphitrite* (= *Amphibalanus amphitrite*). *Biofouling*, 24, 109-118.
- RASMUSSEN, K., WILLEMSSEN, P. R. & OSTGAARD, K. 2002. Barnacle settlement on hydrogels. *Biofouling*, 18, 177-191.
- RAWLINGS, N. D. & BARRETT, A. J. 1994. Families of Serine Peptidases. *Proteolytic Enzymes: Serine and Cysteine Peptidases*, 244, 19-61.
- RITTSCHOF, D., CLARE, A. S., GERHART, D. J., AVELIN, S. & BONAVENTURA, J. 1992. Barnacle in vitro assays for biologically active substances: Toxicity and settlement inhibition assays using mass cultured *Balanus amphitrite amphitrite* Darwin. *Biofouling*, 6, 115-122.
- RITTSCHOF, D. & COSTLOW, J. D. 1989. Bryozoan and barnacle settlement in relation to initial surface wettability a comparison of laboratory and field studies. *Scientia Marina*, 53, 411-416.
- RITTSCHOF, D., ORIHUELA, B., STAFSLIEN, S., DANIELS, J., CHRISTIANSON, D., CHISHOLM, B. & HOLM, E. 2008. Barnacle reattachment: a tool for studying barnacle adhesion. *Biofouling*, 24, 1-9.
- ROBERTS, D., RITTSCHOF, D., HOLM, E. & SCHMIDT, A. R. 1991. Factors influencing initial larval settlement - temporal, spatial and surface molecular-components. *Journal of Experimental Marine Biology and Ecology*, 150, 203-221.
- RUZGA-WIJAS K., MIRERSKA V., FERTUNIAK W., CHOJNOWSKI J., HALSA R. & WEREL W. 2007. Quaternary ammonium salts (QAS) modified polysiloxane

- biocide supported on silica materials. *Journal of Inorganic and Organometallic Polymers and Materials*, 17, 605–613.
- SANFORD, E., BERMUDEZ, D., BERTNESS, M. D. & GAINES, S. D. 1994. Flow, food-supply and acorn barnacle population-dynamics. *Marine Ecology-Progress Series*, 104, 49-62.
- SANFORD, E. & MENGE, B. A. 2001. Spatial and temporal variation in barnacle growth in a coastal upwelling system. *Marine Ecology-Progress Series*, 209, 143-157.
- SANGEETHA, R., KUMAR, R., VENKATESAN, R., DOBLE, M., VEDAPRAKASH, L., KRUPARATNAM, LAKSHMI, K. & DINESHRAM 2010. Understanding the structure of the adhesive plaque of *Amphibalanus reticulatus*. *Materials Science & Engineering C-Materials for Biological Applications*, 30, 112-119.
- SAROYAN, J.R., LINDNER, E. & DOOLEY, C.A. 1968. Attachment mechanism of barnacles. Fouling prevention the study of the adhesion of calcareous types attaching marine organisms. *San Francisco Bay Naval Shipyard Vallejo Ca Paint Lab*, Progress report no. 1, 1-57
- SAROYAN, J. R., LINDNER, E. & DOOLEY, C. A. 1970b. Repair and reattachment in the barnacle as related to their cementing mechanism. *Biological Bulletin*, 139, 333-350.
- SAROYAN, J. R., LINDNER, E., DOOLEY, C. A. & BLEILE, H. R. 1970a. Barnacle cement - key to second generation antifouling coatings. *Industrial & Engineering Chemistry Product Research and Development*, 9, 122-129.
- SATUITO, C. G., SHIMIZU, K., NATOYAMA, K., YAMAZAKI, M. & FUSETANI, N. 1996. Age-related settlement success by cyprids of the barnacle *Balanus amphitrite*, with special reference to consumption of cyprid storage protein. *Marine Biology*, 127, 125-130.
- SCHMIDT, M., CAVACO, A., GIERLINGER, N., ALDRED, N., FRATZL, P., GRUNZE, M. & CLARE, A. S. 2009. In situ imaging of barnacle (*Balanus amphitrite*) cyprid cement using confocal raman microscopy. *Journal of Adhesion*, 85, 139-151.
- SCHULTZ, M. P. 2007. Effects of coating roughness and biofouling on ship resistance and powering. *Biofouling*, 23, 331-341.

- SCHULTZ, M. P., BENDICK, J. A., HOLM, E. R. & HERTEL, W. M. 2011. Economic impact of biofouling on a naval surface ship. *Biofouling*, 27, 87-98.
- SCHULTZ, M. P., FINLAY, J. A., CALLOW, M. E. & CALLOW, J. A. 2000. A turbulent channel flow apparatus for the determination of the adhesion strength of microfouling organisms. *Biofouling*, 15, 243-249.
- SCHULTZ, M. P., KAVANAGH, C. J. & SWAIN, G. W. 1999. Hydrodynamic forces on barnacles: Implications on detachment from fouling-release surfaces. *Biofouling*, 13, 323-335.
- SCHUMACHER, J. F., ALDRED, N., CALLOW, M. E., FINLAY, J. A., CALLOW, J. A., CLARE, A. S. & BRENNAN, A. B. 2007. Species-specific engineered antifouling topographies: correlations between the settlement of algal zoospores and barnacle cyprids. *Biofouling*, 23, 307-317.
- SCHWINDT, E., DE FRANCESCO, C.G. & IRIBARNE, O.O. 2004. Individual and reef growth of the invasive reef-building polychaete *Ficopomatus enigmaticus* in a south-western Atlantic coastal lagoon. *Journal of the Marine Biological Association UK*, 84, 987-993.
- SIGAL, G. B., MRKSICH, M. & WHITESIDES, G. M. 1998. Effect of surface wettability on the adsorption of proteins and detergents. *Journal Of The American Chemical Society*, 120, 3464-3474.
- SIMPSON, E.P. & HURLBERT, S.H. 1998. Salinity effects on the growth, mortality and shell strength of *Balanus amphitrite* from the Salton Sea, California. *Hydrobiologia*, 31, 179-190.
- SINGER, I. L., KOHL, J. G. & PATTERSON, M. 2000. Mechanical aspects of silicone coatings for hard foulant control. *Biofouling*, 16, 301-309.
- SMITH, B. L., SCHAFFER, T. E., VIANI, M., THOMPSON, J. B., FREDERICK, N. A., KINDT, J., BELCHER, A., STUCKY, G. D., MORSE, D. E. & HANSMA, P. K. 1999. Molecular mechanistic origin of the toughness of natural adhesives, fibres and composites. *Nature*, 399, 761-763.
- SMITH, M. B., TONG, J. H., GENZER, J., FISCHER, D. & KILPATRICK, P. K. 2006. Effects of synthetic amphiphilic alpha-helical peptides on the electrochemical and structural properties of supported hybrid bilayers on gold. *Langmuir*, 22, 1919-1927.

- SOMMER, S., EKIN, A., WEBSTER, D. C., STAFSLIEN, S. J., DANIELS, J., VANDERWAL, L. J., THOMPSON, S. E. M., CALLOW, M. E. & CALLOW, J. A. 2010. A preliminary study on the properties and fouling-release performance of siloxane-polyurethane coatings prepared from poly(dimethylsiloxane) (PDMS) macromers. *Biofouling*, 26, 961-972.
- SOMMER, S. A., BYROM, J. R., FISCHER, H. D., BODKHE, R. B., STAFSLIEN, S. J., DANIELS, J., YEHLE, C. & WEBSTER, D. C. 2011. Effects of pigmentation on siloxane-polyurethane coatings and their performance as fouling-release marine coatings. *Journal of Coatings Technology and Research*, 8, 661-670.
- SOUTHWARD, A.J. 1955 Feeding of barnacles. *Nature*, 175, 1124-1125
- SOUTHWARD, A.J. 1958 Note on the temperature tolerance of some intertidal animals in relation to environmental temperatures and geographical distribution. *Journal of the Marine Biological Association UK*, 37, 49-66
- SRINIVASAN, M. & SWAIN, G. W. 2007. Managing the use of copper-based antifouling paints. *Environmental Management*, 39, 423-441.
- STANLEY, M. S. & CALLOW, J. A. 2007. Whole cell adhesion strength of morphotypes and isolates of *Phaeodactylum tricornutum* (Bacillariophyceae). *European Journal of Phycology*, 42, 191-197.
- STANLEY, M. S., CALLOW, M. E. & CALLOW, J. A. 1999. Monoclonal antibodies to adhesive cell coat glycoproteins secreted by zoospores of the green alga *Enteromorpha*. *Planta*, 210, 61-71.
- STEIN, J., TRUBY, K., WOOD, C. D., TAKEMORI, M., VALLANCE, M., SWAIN, G., KAVANAGH, C., KOVACH, B., SCHULTZ, M., WIEBE, D., HOLM, E., MONTEMARANO, J., WENDT, D., SMITH, C. & MEYER, A. 2003. Structure-property relationships of silicone biofouling-release coatings: Effect of silicone network architecture on pseudobarnacle attachment strengths. *Biofouling*, 19, 87-94.
- STEWART, R.J. WEAVER, J.C., MORSE, D.E., & J. Herbert WAITE, J.H. 2004 The tube cement of *Phragmatopoma californica*: a solid foam. *Journal of Experimental Biology* 207, 4727-4734.

- SUN, Y. J., GUO, S. L., WALKER, G. C., KAVANAGH, C. J. & SWAIN, G. W. 2004. Surface elastic modulus of barnacle adhesive and release characteristics from silicone surfaces. *Biofouling*, 20, 279-289.
- SWAIN, G., ANIL, A. C., BAIER, R. E., CHIA, F. S., CONTE, E., COOK, A., HADFIELD, M., HASLBECK, E., HOLM, E., KAVANAGH, C., KOHRS, D., KOVACH, B., LEE, C., MAZZELLA, L., MEYER, A. E., QIAN, P. Y., SAWANT, S. S., SCHULTZ, M., SIGURDSSON, J., SMITH, C., SOO, L., TERLIZZI, A., WAGH, A., ZIMMERMAN, R. & ZUPO, V. 2000. Biofouling and barnacle adhesion data for fouling-release coatings subjected to static immersion at seven marine sites. *Biofouling*, 16, 331-344.
- SWAIN, G. W., GRIFFITH, J. R., BULTMAN, J. D. & VINCENT, H. L. 1992. The use of barnacle adhesion measurements for the field evaluation of non-toxic foul release surfaces. *Biofouling*, 6, 105-114.
- SWAIN, G. W., NELSON, W. G. & PREEDEEKANIT, S. 1998. The influence of biofouling adhesion and biotic disturbance on the development of fouling communities on non-toxic surfaces. *Biofouling*, 12, 257-269.
- SWAIN, G. W. & SCHULTZ, M. P. 1996. The testing and evaluation of non-toxic antifouling coatings. *Biofouling*, 10, 187-197.
- SWAIN, G. W., SCHULTZ, M. P. & VINCENT, H. L. 1994. Shear force measurements of barnacle adhesion for field-evaluation of nontoxic foul-release surfaces. In Fingerman M, Nagabhushanam R, Sarojini R & Thompson MF. (eds) *Recent Developments in Biofouling Control*, Taylor & Francis Inc., London, 335-341.
- TAL, M., SILBERSTEIN, A., & NUSSER, E. 1985 Why does Coomassie Brilliant Blue R react differently with different proteins? A partial answer. *Journal of Biological Chemistry*, 260, 9976-9980.
- TANG, Y., FINLAY, J. A., KOWALKE, G. L., MEYER, A. E., BRIGHT, F. V., CALLOW, M. E., CALLOW, J. A., WENDT, D. E. & DETTY, M. R. 2005. Hybrid xerogel films as novel coatings for antifouling and fouling release. *Biofouling*, 21, 59-71.
- TANUR, A. E., GUNARI, N., SULLAN, R. M. A., KAVANAGH, C. J. & WALKER, G. C. 2010. Insights into the composition, morphology, and formation of the

- calcareous shell of the serpulid *Hydroides dianthus*. *Journal of Structural Biology*, 169, 145-160.
- TASSO, M., CORDEIRO, A. L., SALCHERT, K. & WERNER, C. 2009a. Covalent immobilization of Subtilisin A onto thin films of maleic anhydride copolymers. *Macromolecular Bioscience*, 9, 922-929.
- TASSO, M., PETTITT, M. E., CORDEIRO, A. L., CALLOW, M. E., CALLOW, J. A. & WERNER, C. 2009b. Antifouling potential of Subtilisin A immobilized onto maleic anhydride copolymer thin films. *Biofouling*, 25, 505-516.
- TAY, F. R. & PASHLEY, D. H. 2002. Dental adhesives of the future. *The Journal Of Adhesive Dentistry*, 4, 91-103.
- TAYLOR, S. W. & WAITE, J. H. 1997. Marine adhesives: From molecular dissection to application. In McGrath K. & Kaplan D. (eds) *Bioengineering of Materials; Protein-based materials*. Birkhäuser, Boston, 217-250.
- TERLIZZI, A., FRASCHETTI, S., GIANGUZZA, P., FAIMALI, M. & BOERO, F. 2001. Environmental impact of antifouling technologies: state of the art and perspectives. *Aquatic Conservation: Marine And Freshwater Ecosystems*, 11, 311-317.
- THIYAGARAJAN, V., HARDER, T. & QIAN, P.Y. 2003 Combined effects of temperature and salinity on larval development and attachment of the subtidal barnacle *Balanus trigonus* Darwin. *Journal of Experimental Marine Biology and Ecology*, 287, 223-236.
- THIYAGARAJAN, V. 2010. A review on the role of chemical cues in habitat selection by barnacles: New insights from larval proteomics. *Journal of Experimental Marine Biology and Ecology*, 392, 22-36.
- THOMAS, K., RAYMOND, K., CHADWICK, J., & WALDOCK, M. 1999. The effects of short-term changes in environmental parameters on the release of biocides from antifouling coatings: Cuprous oxide and tributyltin. *Applied Organometallic Chemistry*, 13, 453-460.
- THOMASON, J. C., HILLS, J. M., CLARE, A. S., NEVILLE, A. & RICHARDSON, M. 1998. Hydrodynamic consequences of barnacle colonization. *Hydrobiologia*, 375/76, 191-201.
- TOWNSIN, R. L. 2003. The ship hull fouling penalty. *Biofouling*, 19, 9-15.

- TREMBLAY, R., OLIVIER, F., BOURGET, E. & RITTSCHOF, D. 2007.
Physiological condition of *Balanus amphitrite* cyprid larvae determines habitat selection success. *Marine Ecology-Progress Series*, 340, 1-8.
- TRUBY, K., WOOD, C., STEIN, J., CELLA, J., CARPENTER, J., KAVANAGH, C., SWAIN, G., WIEBE, D., LAPOTA, D., MEYER, A., HOLM, E., WENDT, D., SMITH, C. & MONTEMARANO, J. 2000. Evaluation of the performance enhancement of silicone biofouling-release coatings by oil incorporation. *Biofouling*, 15, 141-153.
- UHLMANN, P., SKORUPA, S., WERNER, C. & GRUNDKE, K. 2005.
Characterization of maleic acid/anhydride copolymer films by low-rate dynamic liquid-fluid contact angle measurements using axisymmetric drop shape analysis. *Langmuir*, 21, 6302-6307.
- VAN DOVER, C. L. 2002. Trophic relationships among invertebrates at the Kairei hydrothermal vent field (Central Indian Ridge). *Marine Biology*, 141, 761-772.
- VAN OSS, C. J. & GIESE, R. F. 2004. Role of the properties and structure of liquid water in colloidal and interfacial systems. *Journal of Dispersion Science and Technology*, 25, 631-655.
- VAN ROOIJ, J.M., BRUGGEMANN, J.H, VIDELER, J.J. & BREEMAN, A.M. 1995.
Plastic growth of the herbivorous reef fish *Sparisoma viride*: field evidence for a trade off between growth and reproduction. *Marine Ecology Progress Series*, 122, 93-105.
- VREELAND, V., WAITE, J. H. & EPSTEIN, L. 1998. Polyphenols and oxidases in substratum adhesion by marine algae and mussels. *Journal of Phycology*, 34, 1-8.
- WAHL, M. 1989. Marine epibiosis I. Fouling and antifouling: some basic aspects. *Marine Ecology Progress Series*, 58, 175-189.
- WAITE, J. H. 1987. Nature's underwater adhesive specialist. *Chemtech*, 17, 692-697.
- WAITE, J. H. 2002. Adhesion a la Moule. *Integrative and Comparative Biology*, 42, 1172-1180.
- WAITE, J. H., HANSEN, D. C. & LITTLE, K. T. 1989. The glue protein of ribbed mussels (*Geukensia-demissa*) - a natural adhesive with some features of collagen. *Journal of Comparative Physiology B-Biochemical Systemic and Environmental Physiology*, 159, 517-525.

- WAITE, J. H., JENSEN, R. A. & MORSE, D. E. 1992. Cement precursor proteins of the reef-building polychaete *Phragmatopoma-californica* (Fewkes). *Biochemistry*, 31, 5733-5738.
- WAITE, J. H. & QIN, X. X. 2001. Polyphosphoprotein from the adhesive pads of *Mytilus edulis*. *Biochemistry*, 40, 2887-2893.
- WALKER, G. 1971. A study of the cement apparatus of the cypris larva of the barnacle, *Balanus balanoides*. *Marine Biology*, 9, 205-212.
- WALKER, G. 1973. The early development of the cement apparatus in the barnacle *Balanus-balanoides* Crustacea Cirripedia. *Journal of Experimental Marine Biology and Ecology*, 12, 305-314.
- WALKER, G. 1981. The adhesion of barnacles. *Journal of Adhesion*, 12, 51-58.
- WALKER, G. 1995. Larval settlement: Historical and future perspectives. In Schram, F.R. & Hoeg, J.T. (eds) *Crustacean Issues 10; New frontiers in barnacle evolution*. AA Balkema, Rotterdam, 69-85.
- WALKER, G. & YULE, A. B. 1984. Temporary adhesion of the barnacle cyprid - the existence of an antennular adhesive secretion. *Journal of the Marine Biological Association UK*, 64, 679-686.
- WALKER, G., YULE, A. B. & NOTT, J. A. 1987. Structure and function in balanomorph larvae. In Southward, A. J. (ed) *Barnacle Biology (Crustacean Issues 5)*, Balkema, Rotterdam, 307-328.
- WALKER, G. C., SUN, Y. J., GUO, S. L., FINLAY, J. A., CALLOW, M. E. & CALLOW, J. A. 2005. Surface mechanical properties of the spore adhesive of the green alga *Ulva*. *Journal of Adhesion*, 81, 1101-1118.
- WALLEY, L. J. 1969. Studies on the larval structure and metamorphosis of *Balanus-balanoides*. *Philosophical Transactions of the Royal Society of London B Biological Sciences*, 256, 237-280.
- WALTON SMITH, F.G. 1948 Surface illumination and barnacle attachment. *Biological Bulletin*, 94, 33-39.
- WANG, J. H., BORWORNPIYO, R., ODETALLAH, N. & SHIH, J. C. H. 2005. Enzymatic degradation of a prion-like protein, Sup35NM-His6. *Enzyme and Microbial Technology*, 36, 758-765.

- WARNER, S. C. & WAITE, J. H. 1999. Expression of multiple forms of an adhesive plaque protein in an individual mussel, *Mytilus edulis*. *Marine Biology*, 134, 729-734.
- WEBSTER, D. C., CHISHOLM, B. J. & STAFSLIEN, S. J. 2007. Mini-review: Combinatorial approaches for the design of novel coating systems. *Biofouling*, 23, 179-192.
- WEBSTER, D. C., CHISHOLM, B. J. & STAFSLIEN, S. J. 2009. High throughput methods for the design of fouling control coatings. *Advances in Marine Antifouling Coatings and Technologies*, 6, 107-121.
- WENDT, D. E., KOWALKE, G. L., KIM, J. & SINGER, I. L. 2006. Factors that influence elastomeric coating performance: the effect of coating thickness on basal plate morphology, growth and critical removal stress of the barnacle *Balanus amphitrite*. *Biofouling*, 22, 1-9.
- WERNER, C., EICHHORN, K. J., GRUNDKE, K., SIMON, F., GRAHLERT, W. & JACOBASCH, H. J. 1999. Insights on structural variations of protein adsorption layers on hydrophobic fluorohydrocarbon polymers gained by spectroscopic ellipsometry (part I). *Colloids and Surfaces a-Physicochemical and Engineering Aspects*, 156, 3-17.
- WERNER, W. E. 1967. The distribution and ecology of the barnacle *Balanus trigonus*. *Bulletin of Marine Science*, 17, 64-84.
- WETHERBEE, R., LIND, J. L., BURKE, J. & QUATRANO, R. S. 1998. The first kiss: Establishment and control of initial adhesion by raphid diatoms. *Journal of Phycology*, 34, 9-15.
- WIEGEMANN, M. & WATERMANN, B. 2003. Peculiarities of barnacle adhesive cured on non-stick surfaces. *Journal of Adhesion Science and Technology*, 17, 1957-1977.
- WIEGEMANN, M. & WATERMANN, B. 2004. The impact of desiccation on the adhesion of barnacles attached to non-stick coatings. *Biofouling*, 20, 147-153.
- WIENCEK, J. M. & FETCHER, M. 1998. Effects of substratum wettability and molecular topography on the initial adhesion of bacteria to chemically defined substrata. *Biofouling*, 11, 293-311.
- WOOD, C. D., TRUBY, K., STEIN, J., WIEBE, D., HOLM, E., WENDT, D., SMITH, C., KAVANAGH, C., MONTEMARANO, J., SWAIN, G. & MEYER, A. 2000.

- Temporal and spatial variations in macrofouling of silicone fouling-release coatings. *Biofouling*, 16, 311-322.
- WYNNE, K. J., SWAIN, G. W., FOX, R. B., BULLOCK, S. & UILK, J. 2000. Two silicone nontoxic fouling release coatings: Hydrosilation cured PDMS and CaCO₃ filled, ethoxysiloxane cured RTV11. *Biofouling*, 16, 277-288.
- YANG, B. Y., GRAY, J. S. S. & MONTGOMERY, R. 1994. Extracellular polysaccharide of *Erwinia chrysanthemi* Ech6. *International Journal of Biological Macromolecules*, 16, 306-312.
- YEBRA, D. M., KIIL, S. & DAM-JOHANSEN, K. 2004a. Antifouling technology-- past, present and future steps towards efficient and environmentally friendly antifouling coatings. *Progress in Organic Coatings*, 50, 75-104.
- YEBRA, D. M., KIIL, S. & DAM-JOHANSEN, K. 2004b. Antifouling technology - past, present and future steps towards efficient and environmentally friendly antifouling coatings. *Progress in Organic Coatings*, 50, 75-104.
- YEBRA, D.M., KIIL, S., WEINELL, C.E., & DAM-JOHANSEN, K. 2006. Effects of marine microbial biofilms on the biocide release rate from antifouling paints—A model-based analysis. *Progress in Organic Coatings*, 57, 56–66.
- YU, M. E., HWANG, J. Y. & DEMING, T. J. 1999. Role of L-3,4-dihydroxyphenylalanine in mussel adhesive proteins. *Journal of the American Chemical Society*, 121, 5825-5826.
- YULE, A. B. & WALKER, G. 1984. The adhesion of the barnacle, *Balanus balanoides*, to slate surfaces. *Journal of the Marine Biological Association UK*, 64, 147-156.
- YULE, A. B. & WALKER, G. 1985. Settlement of *Balanus balanoides*: The effect of cyprid antennular secretion. *Journal of the Marine Biological Association UK*, 65, 707-712.
- YULE, A. B. & WALKER, G. 1987. Adhesion in Barnacles. In Southward, A. J. (ed) *Barnacle Biology (Crustacean Issues 5)*, Balkema, Rotterdam, 389-404.
- YUN, C., ELY, C. & SMIT, J. 1994 Identification of genes effecting the production of the adhesive holdfast of a marine caulobacter. *Journals Bacteriology*, 176, 796-803.
- ZAR, J.H. 1999. Biostatistical analysis. 4th edition. *Prentice Hall*, Upper Saddle River, New Jersey.

ZARDUS, J. D., NEDVED, B. T., HUANG, Y., TRAN, C. & HADFIELD, M. G. 2008.

Microbial biofilms facilitate adhesion in biofouling invertebrates. *Biological Bulletin*, 214, 91-98.

ZHAO, H., SUN, C. J., STEWART, R. J. & WAITE, J. H. 2005. Cement proteins of the

tube-building polychaete *Phragmatopoma californica*. *Journal of Biological Chemistry*, 280, 42938-42944.