

Some pages of this thesis may have been removed for copyright restrictions.

If you have discovered material in AURA which is unlawful e.g. breaches copyright, (either yours or that of a third party) or any other law, including but not limited to those relating to patent, trademark, confidentiality, data protection, obscenity, defamation, libel, then please read our [Takedown Policy](#) and [contact the service](#) immediately

Acanthamoebal surface properties and the modulation of phagocytosis

Edward Alastair George Elloway

Doctor of Philosophy

Aston University

January 2002

This copy of the thesis has been supplied on condition that anyone who consults it is understood to recognise that its copyright rests with its author and that no quotation from the thesis and no information derived from it may be published without proper acknowledgement.

Thesis Summary

The surface nature of *Acanthamoeba* trophozoites and cysts was investigated with respect to cell surface charge, hydrophobicity and surface carbohydrate composition. Particulate microelectrophoresis revealed a marked negative charge for both morphological forms, though less for cyst surfaces. Hydrophobicity was determined by adhesion to n-hexadecane and indicated a relatively low hydrophobic nature of both forms, though less so for cysts. Surface carbohydrate composition was studied by the use of fluorescent lectins and flow cytometry, using a ligand-receptor approach for further in depth analysis of binding of particular lectins. These studies showed trophozoite and cyst surfaces to be rich in N-acetylglucosamine, N-acetylneuraminic acid, mannose and glucose, with the addition of N-acetylgalactosamine on cysts.

The importance of such surface properties was investigated with respect to phagocytosis of polystyrene latex microspheres, of different surface types and size. Investigations into the optimum conditions for uptake of beads indicated a preference for a medium devoid of nutrients, such as saline, though temperature was not a factor. An amoebal predilection for beads of lower charge and greater hydrophobicity was demonstrated. Furthermore, a preference for the largest bead size used (2.0 μm) was observed. The influence of either Con A or mannose or glucose on bead association was apparently limited.

The fate of foreign DNA ingested by *Acanthamoeba* appeared to indicate that such DNA was destroyed, as it could not be detected following extraction procedures and PCR amplification.

Acknowledgements

First and foremost I would like to sincerely thank my supervisor Steve Smith for his continued guidance, advice and support throughout my PhD.

On the practical side, I am grateful to John Barker for providing me with *Acanthamoeba* and instruction for its culture and to Roger Bird for analysing my numerous samples by flow cytometry.

I thank all those at CEFAS Weymouth, but particularly Gavin Barker, Richard Gardiner and Edel Chambers for water sample collection and general advice.

I would also like to thank Peter Lambert for invaluable support and being on-hand to answer questions and David Poyner for assistance with lectin binding studies. Furthermore, I am grateful to Graham Smith for his part in developing an initial sketch into a work of art!

I am, of course, indebted to those who keep or have kept the lab ticking over, specifically Rita Chohan, Lynda Birt, Roy McKenzie and Roy Tilling.

I would also like to thank all members of both the department and the lab who have helped me on various levels, whether directly with my research or just being there to listen, especially Rob Eason, Alex Foster, Lucy Harper, Conor Jamieson, Karen Jones, Sue Lang, Mike Loughlin, Sarah Loughlin, Dave Nagel and Steve Russell.

Finally, I would like to express my gratitude to my parents for everything they have done for me.

Table of contents

List of figures	9
List of tables	12
Abbreviations	13
CHAPTER 1 – Introduction.....	15
1.1 The genus <i>Acanthamoeba</i>	15
1.2 <i>Acanthamoeba</i> species as pathogens	19
1.3 <i>Acanthamoeba</i> species as reservoirs of pathogenic bacteria.....	20
1.4 Fish pathogens	23
1.5 Incidence of <i>Acanthamoeba</i> species in fish	25
1.6 Aims and Objectives.....	26
CHAPTER 2 – Culture and encystment of <i>Acanthamoeba polyphaga</i>	28
2.1 Introduction	28
2.2 Materials and Methods.....	29
2.2.1 <i>Acanthamoeba polyphaga</i> stock cultures.....	29
2.2.2 Effect of temperature on the growth of <i>Acanthamoeba polyphaga</i>	30
2.2.3 Induced encystment of <i>Acanthamoeba polyphaga</i>	30
2.3 Results.....	31
2.3.1 Effect of temperature on the growth of <i>Acanthamoeba polyphaga</i>	31
2.3.2 Induced encystment of <i>Acanthamoeba polyphaga</i>	33
2.4 Discussion	35
2.4.1 Effect of temperature on the growth of <i>Acanthamoeba polyphaga</i>	35
2.4.2 Induced encystment of <i>Acanthamoeba polyphaga</i>	37
CHAPTER 3 – Isolation of amoebae from environmental water samples.....	40
3.1 Introduction	40
3.2 Materials and Methods.....	41
3.2.1 Isolation of amoebae from pond water samples	41
3.2.1.1 Sample collection and filtration	41
3.2.1.2 Isolation of amoebae	42
3.2.2 Survey of fish farms and rivers.....	42
3.3 Results	43
3.3.1 Isolation of amoebae from pond water samples	43
3.3.2 Survey of fish farms and rivers.....	44
3.4 Discussion	46
CHAPTER 4 – Factors involved in non-specific interactions	49
4.1 Introduction	49
4.1.1 Cell surface charge.....	49
4.1.2 Cell surface hydrophobicity	52
4.2 Materials and Methods.....	53
4.2.1 Cell surface charge.....	53
4.2.1.1 Effect of pH on cell surface charge of <i>Acanthamoeba polyphaga</i> trophozoites	53
4.2.1.2 Effect of pH on cell surface charge of <i>Acanthamoeba polyphaga</i> cysts	54
4.2.1.3 Effect of culture age on cell surface charge of <i>Acanthamoeba</i> <i>polyphaga</i> trophozoites	54

4.2.1.4	Effect of age on cell surface charge of <i>Acanthamoeba polyphaga</i> cysts	55
4.2.1.5	Cell surface charge of different strains of <i>Acanthamoeba</i>	55
4.2.1.6	Surface charge of microspheres	56
4.2.2	Cell surface hydrophobicity	57
4.2.2.1	Microbial adhesion to hydrocarbons (MATH) assay	57
4.2.2.2	Effect of age on hydrophobicity of <i>Acanthamoeba polyphaga</i> trophozoites	58
4.2.2.3	Effect of age on hydrophobicity of <i>Acanthamoeba polyphaga</i> cysts	58
4.2.2.4	Hydrophobicity of different strains of <i>Acanthamoeba</i> trophozoites	59
4.2.2.5	Hydrophobicity of microspheres	59
4.2.2.6	Alternative methods for determination of cell surface hydrophobicity	60
4.2.2.6.1	Hydrophobicity interaction chromatography (HIC)	60
4.2.2.6.2	Alternative sepharose method for determination of cell surface hydrophobicity	61
4.2.2.6.3	Salt aggregation technique (SAT)	62
4.2.2.6.4	Two phase partition (TPP)	62
4.2.2.6.5	Hydrophobic microsphere attachment (HMA) assay	63
4.3	Results	64
4.3.1	Cell surface charge	64
4.3.1.1	Effect of pH on cell surface charge of <i>Acanthamoeba polyphaga</i>	64
4.3.1.2	Effect of culture age on cell surface charge of <i>Acanthamoeba polyphaga</i> trophozoites	65
4.3.1.3	Effect of age on cell surface charge of <i>Acanthamoeba polyphaga</i> cysts	67
4.3.1.4	Cell surface charge of different strains of <i>Acanthamoeba</i>	68
4.3.1.5	Surface charge of microspheres	69
4.3.2	Cell surface hydrophobicity	70
4.3.2.1	Effect of age on hydrophobicity of <i>Acanthamoeba polyphaga</i> trophozoites	70
4.3.2.2	Effect of age on hydrophobicity of <i>Acanthamoeba polyphaga</i> cysts	71
4.3.2.3	Hydrophobicity of different strains of <i>Acanthamoeba</i> trophozoites	71
4.3.2.4	Hydrophobicity of microspheres	73
4.3.2.5	Alternative methods for determination of cell surface hydrophobicity	74
4.3.2.5.1	Hydrophobicity interaction chromatography (HIC)	74
4.3.2.5.2	Alternative sepharose method for determination of cell surface hydrophobicity	74
4.3.2.5.3	Salt aggregation technique (SAT)	74
4.3.2.5.4	Two phase partition (TPP)	75
4.3.2.5.5	Hydrophobic microsphere attachment (HMA) assay	75
4.4	Discussion	75
4.4.1	Cell surface charge	75
4.4.2	Cell surface hydrophobicity	79
CHAPTER 5 – Factors involved in specific interactions		84
5.1	Introduction	84
5.2	Materials and Methods	86
5.2.1	Lectin binding to <i>Acanthamoeba</i>	86
5.2.1.1	Determination of lectin concentration for saturation of <i>Acanthamoeba polyphaga</i> trophozoite binding sites	86

5.2.1.2 Confocal microscopy of FITC-Con A binding to <i>Acanthamoeba polyphaga</i> trophozoites	87
5.2.1.3 Binding specificity of Con A to <i>Acanthamoeba polyphaga</i> trophozoites	88
5.2.1.4 Lectin binding to <i>Acanthamoeba polyphaga</i> trophozoites	89
5.2.1.5 Lectin binding to <i>Acanthamoeba polyphaga</i> cysts	90
5.2.2 Characterisation of lectin binding to <i>Acanthamoeba polyphaga</i>	91
5.3 Results.....	93
5.3.1 Lectin binding to <i>Acanthamoeba</i>	93
5.3.1.1 Determination of lectin concentration for saturation of <i>Acanthamoeba polyphaga</i> trophozoite binding sites	93
5.3.1.2 Confocal microscopy of FITC-Con A binding to <i>Acanthamoeba polyphaga</i> trophozoites	95
5.3.1.3 Binding specificity of Con A to <i>Acanthamoeba polyphaga</i> trophozoites	96
5.3.1.4 Lectin binding to <i>Acanthamoeba polyphaga</i> trophozoites and cysts....	97
5.3.2 Characterisation of lectin binding to <i>Acanthamoeba polyphaga</i>	99
5.4 Discussion	106
CHAPTER 6 – Phagocytosis of polystyrene latex microspheres	112
6.1 Introduction	112
6.2 Materials and Methods.....	113
6.2.1 Preliminary phagocytosis assay	113
6.2.2 Effect of amoebal starvation on their association with microspheres.....	114
6.2.3 Flow cytometric determination of the effect of amoebal starvation on their association with microspheres.....	115
6.2.4 Effect of microsphere size and nature on their association with amoebal trophozoites	116
6.2.4.1 Flow cytometric determination of microsphere association with amoebal trophozoites	116
6.2.4.2 Confocal microscopy of microsphere association with amoebal trophozoites	117
6.2.5 Effect of amoebal saline washing steps on amoebal association with plain microspheres.....	117
6.2.6 Effect of phagocytosis inhibitors on amoebal association with microspheres	118
6.2.7 Effect of incubation time on amoebal association with microspheres.....	119
6.2.8 Effect of temperature on amoebal association with microspheres	120
6.2.9 Effect of amoebal cell age on microsphere association	121
6.2.10 Association of <i>Acanthamoeba</i> strains with microspheres	121
6.2.11 Acanthamoebal association with protein coated microspheres	122
6.2.11.1 Adsorption of protein to microspheres	122
6.2.11.2 Quantification of protein adsorption	123
6.2.11.3 Acanthamoebal association with protein-coated microspheres.....	124
6.2.12 Effect of Con A on acanthamoebal association with microspheres.....	125
6.2.12.1 Association of microspheres with Con A bound <i>Acanthamoeba polyphaga</i>	125
6.2.12.2 Preparation of Con A-modified microspheres	126
6.2.12.3 Determination of Con A loading on microspheres.....	127
6.2.12.4 Confirmation of lectin activity	127
6.2.12.5 Acanthamoebal association with Con A-modified microspheres	128

6.2.13 Effect of carbohydrates on acanthamoebal association with microspheres	129
6.2.13.1 Pre-incubation of amoebae with carbohydrates	129
6.2.13.2 Binding of carbohydrates to microspheres	130
6.3 Results	131
6.3.1 Preliminary phagocytosis assay	131
6.3.2 Effect of amoebal starvation on their association with microspheres	131
6.3.3 Flow cytometric determination of the effect of amoebal starvation on their association with microspheres	132
6.3.4 Effect of microsphere size and nature on their association with amoebal trophozoites	134
6.3.4.1 Flow cytometric determination of microsphere association with amoebal trophozoites	134
6.3.4.2 Confocal microscopy of microsphere association with amoebal trophozoites	135
6.3.5 Effect of amoebal saline washing steps on amoebal association with plain microspheres	137
6.3.6 Effect of phagocytosis inhibitors on amoebal association with microspheres	138
6.3.7 Effect of incubation time on amoebal association with microspheres	139
6.3.8 Effect of temperature on amoebal association with microspheres	141
6.3.9 Effect of amoebal cell age on microsphere association	142
6.3.10 Association of <i>Acanthamoeba</i> strains with microspheres	144
6.3.11 Acanthamoebal association with protein coated microspheres	145
6.3.11.1 Quantification of protein adsorption	145
6.3.11.2 Acanthamoebal association with protein-coated microspheres	146
6.2.12 Effect of Con A on acanthamoebal association with microspheres	147
6.2.12.1 Association of microspheres with Con A bound <i>Acanthamoeba polyphaga</i>	147
6.3.12.2 Determination of Con A loading on microspheres	148
6.3.12.3 Confirmation of lectin activity	149
6.3.12.4 Acanthamoebal association with Con A-modified microspheres	149
6.3.13 Effect of carbohydrates on acanthamoebal association with microspheres	150
6.3.13.1 Pre-incubation of amoebae with carbohydrates	150
6.3.13.2 Binding of carbohydrates to microspheres	151
6.4 Discussion	152
CHAPTER 7 – Fate of DNA taken up by <i>Acanthamoeba polyphaga</i>	159
7.1 Introduction	159
7.2 Materials and Methods	160
7.2.1 Plasmid preparation	160
7.2.2 Quantification of plasmid L4440	160
7.2.3 Detection of L4440 by PCR	161
7.2.4 DNA adsorption to amino microspheres	162
7.2.5 PCR of <i>Acanthamoeba polyphaga</i>	163
7.2.6 Extraction and amplification of acanthamoebal DNA	165
7.2.7 Specificity test of primers and long-term viability of DNA-adsorbed microspheres	166
7.2.8 Extraction and amplification of DNA from microspheres associated with <i>Acanthamoeba</i>	167

7.2.9 Extraction and amplification of DNA from microspheres associated with <i>Acanthamoeba</i> using increased reagent concentrations.....	169
7.2.10 Extraction and amplification of DNA from <i>E. coli</i> transformed with L4440 associated with <i>Acanthamoeba</i>	170
7.3 Results.....	171
7.3.1 Quantification of plasmid L4440	171
7.3.2 Detection of L4440 by PCR	172
7.3.3 DNA adsorption to amino microspheres.....	173
7.3.4 PCR of <i>Acanthamoeba polyphaga</i>	174
7.3.5 Extraction and amplification of acanthamoebal DNA.....	175
7.3.6 Specificity test of primers and long-term viability of DNA-adsorbed microspheres.....	175
7.3.7 Extraction and amplification of DNA from microspheres associated with <i>Acanthamoeba</i>	176
7.3.8 Extraction and amplification of DNA from microspheres associated with <i>Acanthamoeba</i> using increased reagent concentrations.....	177
7.3.9 Extraction and amplification of DNA from <i>E. coli</i> transformed with L4440 associated with <i>Acanthamoeba</i>	177
7.4 Discussion	177
CHAPTER 8 – Conclusions.....	180
REFERENCES.....	183
APPENDICES	194
Appendix 1: Barbitol sodium acetate buffer formation	194
Appendix 2: Composition of gels and loading buffer for SDS-PAGE.....	194
Appendix 3: Protocol for carbodiimide binding of Con A to carboxylate microspheres	195
Appendix 4: Sigma Diagnostics glucose testing kit	195
Appendix 5: Promega Wizard [®] Plus Maxiprep DNA purification system.....	196
Appendix 6: Wizard [®] Genomic DNA Purification kit	197

List of figures

Figure 1.1. The differentiation cycle of <i>Acanthamoeba polyphaga</i> showing the two morphologically distinct stages.	17
Figure 2.1. Effect of temperature on the growth of <i>Acanthamoeba polyphaga</i> (Leeds strain) in PYG broth.	32
Figure 2.2. Induced encystment of <i>Acanthamoeba polyphaga</i> over time, showing concentrations of each cell type.	33
Figure 2.3. Induced encystment of <i>Acanthamoeba polyphaga</i> over time, showing proportions of each cell type.	34
Figure 4.1. The relationship between cell surface charge and zeta potential.	51
Figure 4.2. Representative ζ -potential profile for <i>Acanthamoeba polyphaga</i> (Leeds strain) trophozoites at pH 3 as determined by microelectrophoresis.	64
Figure 4.3. Comparison of microelectrophoresis cell surface charge determinations for <i>Acanthamoeba polyphaga</i> (Leeds strain) trophozoites and cysts suspended in barbital sodium acetate buffer at different pH values.	65
Figure 4.4. Microelectrophoresis cell surface charge determinations of <i>Acanthamoeba polyphaga</i> (Leeds strain) trophozoites with respect to trophozoite age.	66
Figure 4.5. The effect of age on cell surface charge of <i>Acanthamoeba polyphaga</i> (Leeds strain) cysts as determined by microelectrophoresis.	67
Figure 4.6. Cell surface charge of different strains of <i>Acanthamoeba</i> as determined by microelectrophoresis.	68
Figure 4.7. Determinations of ζ -potential for microspheres of two surface types and three sizes by microelectrophoresis.	69
Figure 4.8. Variation of cell surface hydrophobicity of <i>Acanthamoeba polyphaga</i> (Leeds strain) trophozoites with culture age.	70
Figure 4.9. Cell surface hydrophobicity of <i>Acanthamoeba polyphaga</i> (Leeds strain) cysts of varying age.	71
Figure 4.10. Relative cell surface hydrophobicity of different strains of <i>Acanthamoeba</i>	72
Figure 4.11. Relative surface hydrophobicity of microspheres of two surface types and three sizes.	73
Figure 5.1. Representative flow cytometry profiles of Con A binding to <i>Acanthamoeba polyphaga</i> (Leeds strain) trophozoites.	93
Figure 5.2. Effect of FITC-Con A concentration on binding to <i>Acanthamoeba polyphaga</i> (Leeds strain) trophozoites.	94
Figure 5.3. FITC-Con A binding to <i>Acanthamoeba polyphaga</i> (Leeds strain) trophozoites.	95
Figure 5.4. The effect of blocking haptens on the binding of FITC-Con A to <i>Acanthamoeba polyphaga</i> (Leeds strain) trophozoites.	96
Figure 5.5. Relative binding of a FITC-labelled lectin panel to <i>Acanthamoeba polyphaga</i> (Leeds strain) trophozoites and cysts.	97
Figure 5.6. Binding curves for FITC-Con A to <i>Acanthamoeba polyphaga</i> (Leeds strain) trophozoites and cysts.	100
Figure 5.7. Binding curves for FITC-LCA to <i>Acanthamoeba polyphaga</i> (Leeds strain) trophozoites and cysts.	102
Figure 5.8. Binding curves for FITC-PSA to <i>Acanthamoeba polyphaga</i> (Leeds strain) trophozoites and cysts.	102

Figure 5.9. Binding curves for FITC-RCA ₁₂₀ to <i>Acanthamoeba polyphaga</i> (Leeds strain) trophozoites and cysts.	103
Figure 5.10. Binding curves for FITC-SBA to <i>Acanthamoeba polyphaga</i> (Leeds strain) trophozoites and cysts.	103
Figure 5.11. Binding curves for FITC-VVA to <i>Acanthamoeba polyphaga</i> (Leeds strain) trophozoites and cysts.	104
Figure 5.12. Binding curves for FITC-WGA to <i>Acanthamoeba polyphaga</i> (Leeds strain) trophozoites and cysts.	104
Figure 6.1. The effect of incubation time in amoebal saline on association of <i>Acanthamoeba polyphaga</i> (Leeds strain) trophozoites with 2.0 µm diameter plain FITC-microspheres.	132
Figure 6.2. The effect of starvation of <i>Acanthamoeba polyphaga</i> (Leeds strain) trophozoites on association with 2.0 µm diameter plain and carboxylate FITC-microspheres.	133
Figure 6.3. Representative flow cytometry profiles of phagocytosis of 2.0 µm diameter plain FITC-microspheres by <i>Acanthamoeba polyphaga</i> (Leeds strain) trophozoites.	134
Figure 6.4. The effect of size and surface type of FITC-microspheres on association with starved <i>Acanthamoeba polyphaga</i> (Leeds strain) trophozoites.	135
Figure 6.5. Confocal micrographs of the association of 0.5 µm diameter plain and carboxylate FITC-microspheres with <i>Acanthamoeba polyphaga</i> (Leeds strain) trophozoites.	136
Figure 6.6. Confocal micrographs of the association of 1.0 µm diameter plain and carboxylate FITC-microspheres with <i>Acanthamoeba polyphaga</i> (Leeds strain) trophozoites.	136
Figure 6.7. Confocal micrographs of the association of 2.0 µm diameter plain and carboxylate FITC-microspheres with <i>Acanthamoeba polyphaga</i> (Leeds strain) trophozoites.	137
Figure 6.8. The effect of washing steps on association of different sized plain FITC-microspheres with <i>Acanthamoeba polyphaga</i> (Leeds strain) trophozoites.	138
Figure 6.9. The effect of phagocytosis inhibitors on association of 2.0 µm diameter FITC-microspheres with <i>Acanthamoeba polyphaga</i> (Leeds strain) trophozoites.	139
Figure 6.10. Association of 2.0 µm diameter plain FITC-microspheres with <i>Acanthamoeba polyphaga</i> (Leeds strain) trophozoites over time.	140
Figure 6.11. Association of 2.0 µm diameter carboxylate FITC-microspheres with <i>Acanthamoeba polyphaga</i> (Leeds strain) trophozoites over time.	141
Figure 6.12. The effect of temperature on association of plain and carboxylate 2.0 µm diameter FITC-microspheres with <i>Acanthamoeba polyphaga</i> (Leeds strain) trophozoites.	142
Figure 6.13. Association of 2.0 µm diameter plain FITC-microspheres with <i>Acanthamoeba polyphaga</i> (Leeds strain) trophozoites of increasing age.	143
Figure 6.14. Association of 2.0 µm carboxylate FITC-microspheres with <i>Acanthamoeba polyphaga</i> (Leeds strain) trophozoites of increasing age.	143
Figure 6.15. Association of 2.0 µm diameter plain FITC-microspheres with different strains of <i>Acanthamoeba</i>	144
Figure 6.16. SDS-PAGE analysis of BSA-coated microspheres.	145

Figure 6.17. The effect of BSA-adsorption to the surface of 1.0 μm and 2.0 μm diameter FITC-microspheres on association with <i>Acanthamoeba polyphaga</i> (Leeds strain) trophozoites.....	146
Figure 6.18. The effect of Con A binding to <i>Acanthamoeba polyphaga</i> (Leeds strain) trophozoites on association with plain and carboxylate 2.0 μm diameter microspheres.....	148
Figure 6.19. SDS-PAGE analysis of Con A-coated microspheres.	149
Figure 6.20. Association of Con A-modified 2.0 μm diameter plain and carboxylate FITC-microspheres with <i>Acanthamoeba polyphaga</i> (Leeds strain) trophozoites.	150
Figure 6.21. The effect of pre-incubation of <i>Acanthamoeba polyphaga</i> (Leeds strain) with carbohydrates on association with 2.0 μm diameter plain and carboxylate FITC-microspheres.	151
Figure 6.22. The effect of 2.0 μm diameter plain and carboxylate FITC-microspheres incubated with carbohydrates upon association with <i>Acanthamoeba polyphaga</i> (Leeds strain) trophozoites.....	152
Figure 7.1. Agarose gel electrophoresis analysis of L4440 plasmid preparation.	172
Figure 7.2. Agarose gel electrophoresis analysis following PCR amplification of L4440.	173
Figure 7.3. Agarose gel electrophoresis analysis of success of DNA adsorption to amino microspheres, following amplification by PCR.....	174
Figure 7.4. Agarose gel electrophoresis analysis following PCR amplification of DNA extracted from <i>Acanthamoeba polyphaga</i> (Leeds strain) trophozoites.	175
Figure 7.5. Agarose gel electrophoresis analysis following PCR amplification to determine specificity of primers directed at L4440 and long-term viability of DNA adsorbed microspheres.	176

List of tables

Table 1.1. Recognised species of <i>Acanthamoeba</i> and their morphological grouping.	16
Table 1.2. Some bacterial species capable of intra-amoebal growth in <i>Acanthamoeba</i> species, either naturally or experimentally.....	21
Table 1.3. Occurrence of <i>Acanthamoeba</i> species in fish.....	25
Table 3.1. Location and nature of sites from where water samples were taken.	43
Table 3.2. Success of amoebal isolation from fish farm and river water samples.....	44
Table 5.1. Sources of lectins used and their carbohydrate binding specificity.....	90
Table 5.2. Lectin specific activity and lectin binding to <i>Acanthamoeba polyphaga</i> (Leeds strain) trophozoites ranked in order of fluorescence intensity.....	98
Table 5.3. Lectin specific activity and lectin binding to <i>Acanthamoeba polyphaga</i> (Leeds strain) cysts ranked in order of fluorescence intensity.	99
Table 5.4. Comparison of K_d and B_{max} values (with standard errors) for lectin binding to <i>Acanthamoeba polyphaga</i> (Leeds strain) trophozoites and cysts.....	105
Table 7.1. Properties of SSF1 and SSR1 primers.	161
Table 7.2. Properties of Nelson primers, NF and NR.	164
Table 7.3. Absorbance readings of plasmid L4440 preparation, diluted 1:50.....	171
Table A1. Volumes of stock solutions required for formation of specific pH values of barbital sodium acetate buffer.	194
Table A2. Composition of separating and stacking gels, sample loading buffer and electrode buffer used in SDS-PAGE.	194

Abbreviations

2,4-dnp	2,4-dinitrophenol
ANOVA	analysis of variance
API	Analytical Profile Index
A _x	absorbance at x nm
BKD	bacterial kidney disease
bp	base pairs
BSA	bovine serum albumin
carb	carboxylate
CCAP	Culture Collection of Algae and Protozoa
Con A	Concanavalin A (<i>Canavalia ensiformis</i> agglutinin)
CSH	cell surface hydrophobicity
DBA	<i>Dolichos biflorus</i> agglutinin
ddH ₂ O	double distilled water
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphates
DSL	<i>Datura stramonium</i> lectin
dsRNA	double stranded RNA
ECL	<i>Erythrina cristagalli</i> lectin
EDTA	ethylenediamine tetraacetic acid
FISH	fluorescent <i>in situ</i> hybridisation
FITC	fluorescein isothiocyanate
FLA	free-living amoebae
fuc	fucose
GAE	granulomatous amoebic encephalitis
gal	galactose
galNAc	N-acetylgalactosamine
GFP	green fluorescent protein
glc	glucose
glcNAc	N-acetylglucosamine
GSL I	<i>Griffonia simplicifolia</i> lectin I
GSL II	<i>Griffonia simplicifolia</i> lectin II
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulphonic acid)
HIC	hydrophobic interaction chromatography
HMA	hydrophobic microsphere attachment
kb	kilobases
LB	Luria-Betani
LCA	<i>Lens culinaris</i> agglutinin
LDV	laser Doppler velocimetry
LEL	<i>Lycopersicon esculentum</i> lectin
LLAPs	<i>Legionella</i> -like amoebic pathogens
LPS	lipopolysaccharide
man	mannose
MATH	microbial adhesion to hydrocarbons
neuNAc	N-acetylneuraminic acid
OD _x	optical density at x nm
PBS	phosphate buffered saline

PCR	polymerase chain reaction
PEG	polyethylene glycol
PFGE	pulsed field gel electrophoresis
PHA-E	<i>Phaseolus vulgaris</i> erythroagglutinin
PHA-L	<i>Phaseolus vulgaris</i> leucoagglutinin
PNA	peanut agglutinin
PSA	<i>Pisum sativum</i> agglutinin
psi	pounds per square inch
PUM	phosphate urea magnesium
PYG	proteose peptone-yeast extract-glucose
RCA ₁₂₀	<i>Ricinus communis</i> agglutinin I
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RNase	ribonuclease
ROIs	reactive oxygen intermediates
SAT	salt aggregation technique
SBA	soybean agglutinin
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SJA	<i>Sophora japonica</i> agglutinin
SKDM	selective kidney disease medium
SOD	superoxide dismutase
STL	<i>Solanum tuberosum</i> lectin
SWGA	succinylated wheat germ agglutinin
TAE	tris-acetate EDTA
TE	tris-EDTA
TEMED	N,N,N',N'-tetramethylethylenediamine
TPP	two phase partition
TSA	tryptone soy agar
TSB	tryptone soy broth
UEA I	<i>Ulex europaeus</i> agglutinin I
v/v	volume for volume
VVA	<i>Vicia villosa</i> agglutinin
w/v	weight for volume
WGA	wheat germ agglutinin

CHAPTER 1 – Introduction

1.1 The genus *Acanthamoeba*

Acanthamoeba species, belonging to the subclass Gymnamoebia, are members of a group known as small free-living amoebae (FLA), distinct from large FLA such as *Amoeba proteus* and *Chaos chaos* (Byers, 1979). Once known as soil amoebae, due to their presence in soil moisture, small FLA are also found in a wide range of marine and freshwater habitats, both natural and man-made (Rodríguez-Zaragoza, 1994), as well as being a component of biofilms (Brown and Barker, 1999). Their ubiquity in such environments is widely recognised (Biddick *et al.*, 1984; De Jonckheere, 1987). Furthermore, Page (1988) suggests that *Acanthamoeba* may be the most common free-living protozoan, being the most frequently isolated genus of gymnamoeba; its survival near ensured by the difficulty in eradication of the cyst form (De Jonckheere and van de Voorde, 1976). Ecologically, phagocytosis of bacteria is one of the most important roles of amoebae and other protozoa (González *et al.*, 1990; Rodríguez-Zaragoza, 1994), as this natural feeding controls bacterial numbers in soils and aquatic environments (Sibille *et al.*, 1998), decreasing numbers by as much as 60 % (Weekers *et al.*, 1993). In addition, protozoa are also ecologically important for recycling nutrients in aquatic food chains (Barker and Brown, 1994).

The genus *Acanthamoeba* comprises more than 18 different species, over which there has historically been some confusion (Byers, 1979; Ubelaker, 1991), and even confusion at the genus level with *Hartmannella*, some species of which should correctly be attributed to *Acanthamoeba* (Visvesvara and Balamuth, 1975; Ubelaker, 1991). Despite the genus possessing distinctive morphological features, particularly in the cyst form, species of *Acanthamoeba* are not always easy to identify (Weisman, 1976), although classification of a strain at this level is usually based on distinctive features such as cell size and cyst morphology, falling into one of three groups (Ubelaker, 1991) (Table 1.1). Although morphological features can be used for species differentiation, there is often great variation in the morphology between individuals of a species (Page, 1988; Ubelaker, 1991), so clarification or further delineation of species and phylogenetic relationships are usually obtained by

immunological and molecular biology techniques. Such techniques include electrophoretic comparison of isoenzymes (Page, 1988), FISH (fluorescent *in situ* hybridisation) (Dyková *et al.*, 1999; Stothard *et al.*, 1999), PCR (polymerase chain reaction) (Walochnik *et al.*, 2000) and RFLP (restriction fragment length polymorphism) (Costas *et al.*, 1983; Byers *et al.*, 1983; Kanno *et al.*, 1998). Costas and Griffiths (1985) have even applied the API Zym system of identification, often used for bacteria, for identification of *Acanthamoeba* strains, although there is debate over the usefulness of its application (Page, 1988). Protocols associated with pulsed field gel electrophoresis (PFGE) may also show some potential in the identification of species and strains, along similar lines to those applied to other genera such as *Aeromonas* (Livesley *et al.*, 1999).

Table 1.1. Recognised species of *Acanthamoeba* and their morphological grouping (after Ubelaker (1991)).

Group I – Do not grow at 37 °C on bacteria and are difficult to grow axenically. Possess a smooth ectocyst more than 18 µm in diameter and a stellate endocyst with excystment pores at the tip of each ray.

A. astronyxis

A. comandoni

A. echimulata

Group II – Morphologically diverse. Development occurs at temperatures below 40 °C. Possess a wrinkled or irregular ectocyst less than 19 µm in diameter and a stellate to polygonal endocyst with excystment pores at the angles of the endocyst wall.

A. castellanii

A. divionensis

A. griffini

A. hatchetti

A. lugdunensis

A. mauritaniensis

A. palestinensis

A. polyphaga

A. quina

A. rhysodes

A. triangularis

A. tubiashi

Group III – Morphologically similar, usually pathogenic and development occurs at least at 40 °C. Possess thin, smooth to slightly wrinkled ectocysts less than 20 µm in diameter with rounded or slightly angular endocysts.

A. culbertsoni

A. lenticulata

A. royreba

Acanthamoeba has a two-stage differentiation cycle (Figure 1.1) existing as either of two distinct morphological forms – a vegetative trophozoite or a dormant cyst (Walker, 1996). Unlike other amoebae, there is no flagellate stage (Marshall *et al.*, 1997).

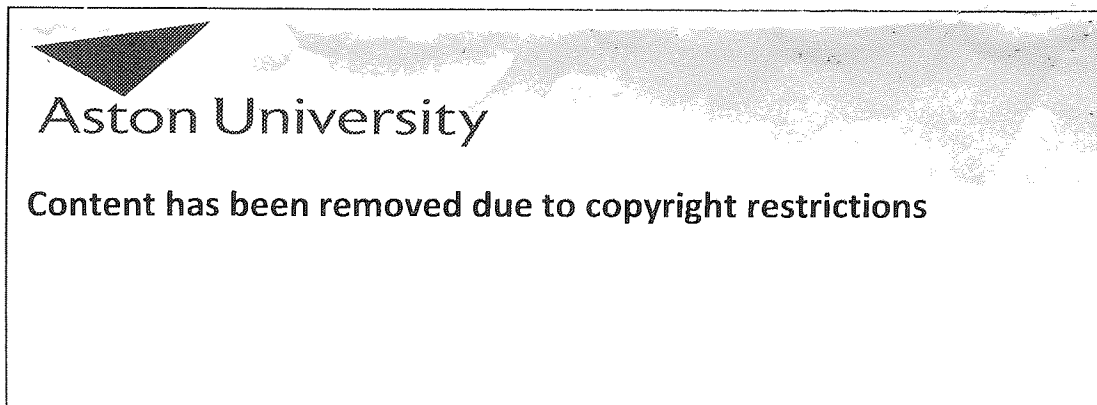


Figure 1.1. The differentiation cycle of *Acanthamoeba polyphaga* showing the two morphologically distinct stages – trophozoites and cysts. Photomicrographs are taken from Page (1988).

Measuring between 10 and 50 μm in diameter, trophozoites are mononuclear with cytoplasm containing fine granules and vacuoles (Marshall *et al.*, 1997). Active, non-rounded trophozoites possess acanthopodia (fine spine-like pseudopodia (Walker, 1996)) projecting from a broader pseudopodium (Bowers and Korn, 1968), the extension of which affords a slow, gliding means of locomotion (Marshall *et al.*, 1997). One of the most obvious internal structures observed in trophozoites is the contractile vacuole, which continuously regulates cytoplasmic osmotic potential by taking in water passively by diastole and forcibly expelling it by systole (Ubelaker, 1991). Such a mechanism has allowed the organism to take advantage of all aquatic habitats, as it can freely regulate its own internal volume and concentrations of both inorganic ions and free intracellular amino acids (Ubelaker, 1991).

Cysts are smaller, 15 to 20 μm in diameter, double-walled structures containing an ectocyst (outer wall) and endocyst (inner wall) of varying shape dependent on the species (Marshall *et al.*, 1997) (see Table 1.1). Encystment occurs naturally when encountering unfavourable conditions, such as starvation, desiccation or high salt levels, decreased oxygen and high pH (Byers, 1979), although such states, according to Yang and Villemez (1994), rarely seem to occur in nature. The encystment process involves firstly rounding up of acanthopodia before the formation of a thick cell wall, comprised mainly of lipid, protein and cellulose fibrils (Neff *et al.*, 1964a; Yang and Villemez, 1994). Conversely, excystment of an active trophozoite occurs through pores (or ostioles) occurring at the intersection of ectocyst and endocyst, when the dormant cyst encounters food, particularly Gram-negative bacteria (Larkin and Easty, 1990), leaving the empty cyst wall behind (Chambers and Thompson, 1972). Present on the cell surface are receptors thought to be involved in both inducement of encystment and suppression of excystment (Yang and Villemez, 1994). Experimentally, excystment to trophozoites occurs when cysts are placed in growth medium (Chambers and Thompson, 1972).

Members of the genus *Acanthamoeba* make ideal research organisms; most species being easily cultivated axenically (unlike many other amoebae) (Finlay, 1982; Page, 1988), with controllable differentiation (Byers, 1979) and the resemblance of trophozoites to primitive macrophages is more than superficial, with strong parallels and similarities between the two cell types in both structure and function (Bowers and Korn, 1968; Rabinovitch and De Stefano, 1971; Steinert *et al.*, 2000; Harb *et al.*, 2000).

1.2 *Acanthamoeba* species as pathogens

Acanthamoeba species, particularly *Acanthamoeba culbertsoni*, are generally regarded as opportunistic pathogens (Ubelaker, 1991; Harb *et al.*, 2000), responsible for two main conditions in humans. Firstly, acanthamoebic keratitis is a chronic sight-threatening infection of the cornea, particularly recognised in contact lens wearers (Morton *et al.*, 1991; Gordon *et al.*, 1993), accounting for 95 % of cases (Kilvington, 2000). Secondly, granulomatous amoebic encephalitis (GAE), or acanthamoebiasis (Jadin, 1987), is a chronic brain infection of immunocompromised hosts (Ubelaker, 1991; Kennett *et al.*, 1999). Both conditions are serious, can be fatal and are not always easily treated (Borazjani *et al.*, 2000). Despite this, no laboratory workers have yet been infected by any species of *Acanthamoeba* (Page, 1988) and GAE is relatively rare. However, due to the popularity of contact lenses, incidence of acanthamoebic keratitis, although still infrequent with around 400 cases in the UK between 1971 and 2000 (Kilvington, 2000), is a cause for concern (Larkin and Easty, 1990). *Acanthamoeba* species have also been implicated in other conditions including skin ulcers and lung complaints (Rondanelli and Scaglia, 1987; Marshall *et al.*, 1997). Species of *Acanthamoeba* are often present as part of the normal flora of apparently healthy individuals and, accordingly, antibodies, principally IgM and IgG isotypes, to the organism are widespread in human sera (Ferrante, 1991; Marshall *et al.*, 1997). In addition, immunological defence mechanisms such as complement activation and the respiratory burst of macrophages are important for killing *Acanthamoeba* species (Ferrante, 1991).

Whilst human infections caused by *Acanthamoeba* species are beyond the remit of this investigation, the potentially similar mechanisms by which amoebae both attach to host tissues and phagocytose bacteria requires consideration of acanthamoebal infection to support our understanding of the following experimentation. Such mechanisms include specific (lectin-adhesin) and non-specific (cell surface charge and hydrophobicity) forces. For example, although there is evidence for the role of an acanthamoebal mannose receptor in corneal attachment, Leher *et al.* (1998) point out that such a receptor is primarily involved in bacterial phagocytosis.

1.3 *Acanthamoeba* species as reservoirs of pathogenic bacteria

As noted, bacterivory by amoebae is important in natural ecosystems, causing the suppression of bacterial numbers. However, selection pressures of phagocytosis have led to the evolution of mechanisms by which bacteria can avoid digestion or subsequent killing by amoebae (Jadin, 1987). Examples of such counter-mechanisms are the production of lytic compounds by *Bacillus liqueniformis* to prevent ingestion by protozoa, the blocking of phagolysosome formation by *Legionella pneumophila*, adaptation of *Coxiella burnettii* to the harsh lysosomal environment and the escape of *Listeria monocytogenes* from the phagolysosome into the cytosol (Harb and Abu Kwaik, 2000).

In many ways, amoebae can be regarded as equivalent to macrophages (Rabinovitch and De Stefano, 1971) and, although there are differences in mechanisms of bacterial uptake and survival (Harf, 1994), there are also strong similarities at phenotypic and molecular levels (Harb *et al.*, 2000). Hence those bacteria that can infect macrophages and other phagocytic cells of *Homo sapiens* are usually also able to infect amoebal species such as those of *Acanthamoeba* (Harb *et al.*, 2000). The most widely recognised example of such an interaction is with the causative organism of Legionnaires' disease, *Legionella pneumophila* (e.g. Rowbotham, 1980; Harb *et al.*, 1998; Newsome *et al.*, 1998; Segal and Shuman, 1999). Examples of bacteria known to be able to survive within *Acanthamoeba* species, either naturally or experimentally, are shown in Table 1.2.

The relationship between bacterium and protozoan host is advantageous for the former, which is potentially provided with a protective environment, in part isolating an intracellular bacterium from adverse extracellular conditions such as high temperatures, pH extremes or biocidal agents (Silverstein, 1995). In turn bacterial longevity is often heightened and the bacterium more effectively dispersed. Thus eradication of legionellae from waterborne sources of infection and plumbing systems has proved difficult, due to the protection afforded by their amoebal hosts (Abu Kwaik *et al.*, 1998), emphasising that eradication of such pathogens will probably be impossible unless their protozoan hosts are also eradicated. However, as a

consequence of their important ecological role in controlling bacterial numbers, such action is not necessarily desirable, nor easily achieved.

Table 1.2. Some bacterial species capable of intra-amoebal growth in *Acanthamoeba* species, either naturally or experimentally.

Species	Reference
<i>Aeromonas</i> spp.	Harf (1994)
<i>Afipia felis</i>	Winiecka-Krusnell and Linder (2001)
<i>Alcaligenes denitrificans</i>	Harf (1994)
<i>Burkholderia cepacia</i>	Marolda <i>et al.</i> (1999)
<i>Burkholderia pseudomallei</i>	Harb <i>et al.</i> (2000)
<i>Caedibacter acanthamoebae</i>	Winiecka-Krusnell and Linder (2001)
<i>Campylobacter</i> spp.	Winiecka-Krusnell and Linder (2001)
'Candidatus <i>Odyssella thessalonicensis</i> '	Winiecka-Krusnell and Linder (2001)
'Candidatus <i>Paracaedibacter acanthamoebae</i> '	Winiecka-Krusnell and Linder (2001)
'Candidatus <i>Paracaedibacter symbiosus</i> '	Winiecka-Krusnell and Linder (2001)
<i>Chlamydia pneumoniae</i>	Essig <i>et al.</i> (1997)
Chlamydiales	Fritsche <i>et al.</i> (1998)
<i>Comamonas acidovorans</i>	Harf (1994)
<i>Cytophaga</i> spp.	Winiecka-Krusnell and Linder (2001)
<i>Escherichia coli</i> (0157:H7)	Barker <i>et al.</i> (1999)
<i>Flavobacterium</i> sp.	Harf (1994)
<i>Francisella tularensis</i>	Harb <i>et al.</i> (2000)
<i>Helicobacter pylori</i>	Winiecka-Krusnell and Linder (2001)
<i>Legionella pneumophila</i>	Rowbotham (1980)
<i>Listeria monocytogenes</i>	Harf (1994)
<i>Molibuncus curtissii</i>	Winiecka-Krusnell and Linder (2001)
<i>Mycobacterium avium</i>	Cirillo <i>et al.</i> (1997)
<i>Mycobacterium fortuitum</i>	Harb <i>et al.</i> (2000)
<i>Mycobacterium leprae</i>	Barker and Brown (1994)
<i>Mycobacterium marinum</i>	Harb <i>et al.</i> (2000)
<i>Parachlamydia acanthamoebae</i>	Brown and Barker (1999)
<i>Pseudomonas aeruginosa</i>	Harf (1994)
<i>Pseudomonas fluorescens</i>	Harf (1994)
<i>Pseudomonas putida</i>	Harf (1994)
<i>Ralstonia pickettii</i>	Inglis <i>et al.</i> (2000)
Rickettsiales	Fritsche <i>et al.</i> (1999)
<i>Salmonella typhimurium</i>	Barker and Brown (1994)
<i>Sarcobium lyticum</i>	Brown and Barker (1999)
<i>Shigella</i> spp.	Winiecka-Krusnell and Linder (2001)
<i>Sphingobacterium maltivorum</i>	Harf (1994)
<i>Vibrio cholerae</i>	Barker and Brown (1994)
<i>Xanthomonas maltophilia</i>	Harf (1994)
<i>Yersinia</i> spp.	Winiecka-Krusnell and Linder (2001)

Of those bacteria listed in Table 1.2, some such as *Legionella pneumophila* are endosymbionts only capable of intracellular replication, thus residence within phagocytes is essential for bacterial multiplication (Harb *et al.*, 2000). Legionellae are the only bacteria that are prolific in their intracellular replication within amoebae (Abu Kwaik *et al.*, 1998). However, in common with other bacteria, intracellular growth often enhances virulence (Cirillo *et al.*, 1994; Cirillo *et al.*, 1997) and resistance to biocidal compounds (Barker *et al.*, 1995) and often causes morphological changes in bacterial phenotype (Barker *et al.*, 1995). It is therefore quite possible that protozoa may be responsible for the emergence of pathogenic bacteria (Harb *et al.*, 2000), especially as some phenotypic variations caused by intra-amoebic growth can affect human phagocyte entry mechanisms (Cirillo *et al.*, 1999). Thus, Winiecka-Krusnell and Linder (1999) suggest that such known intra-amoebic bacteria merely represent “the tip of the iceberg” in terms of potential pathogenic bacterial species capable of intracellular survival and their impact within aquatic environments, whatever their nature.

Amongst the intra-amoebal species listed in Table 1.2 are both obligate intracellular bacteria (endosymbionts) (Fritsche *et al.*, 1993; Hall and Voelz, 1985) and facultative intracellular bacteria (Steinert *et al.*, 1998). Fritsche *et al.* (1998) report that 20 % of clinical and environmental isolates of species of *Acanthamoeba* studied had bacteria present internally. Such bacteria were capable, perhaps surprisingly, of enhancing amoebal pathogenicity, the mechanism of which has yet to be elucidated. In addition, other species known as LLAPs or *Legionella*-like amoebic pathogens, genetically related to legionellae, cannot be cultured *in vitro* on artificial media and must therefore be co-cultured with protozoa (Abu Kwaik *et al.*, 1998), thereby taking the subtlety of bacterium-amoebae interactions beyond that of a merely limited phenomenon. In line with such a standpoint, internalised bacteria, such as *Mycobacterium avium*, may even survive encystment of amoebae by residing in the walls of *Acanthamoeba polyphaga* cysts (Steinert *et al.*, 1998), thus receiving additional protection against the same unfavourable conditions that caused amoebal encystment in the first instance.

Naturally of corresponding importance with respect to a bacterial life cycle and infection of an alternate host, is the process of bacterial exit from temporary residence within an accommodating protozoan (Harb *et al.*, 2000). *Legionella pneumophila*, for example, once past log phase of growth, achieves this by killing the

protozoan host by a specific cytolytic mechanism, mediated by an ability to form pores in both the amoebal phagosomal and plasma membranes, allowing escape (Harb and Abu Kwaik, 2000). *Listeria monocytogenes*, too, kills its host, detected as a decrease in the number of viable amoebae following intracellular replication (Harb *et al.*, 2000).

1.4 Fish pathogens

Renibacterium salmoninarum is an obligate pathogen, being the causative agent of bacterial kidney disease (BKD) of salmonid fish, an economically important disease which is difficult to treat (Evenden *et al.*, 1993). The Gram-positive rods are thought to live within macrophages of infected fish, which are found in the pronephros (head kidney) (Bandín *et al.*, 1995), as well as other cell types *in vivo*, which may serve as reservoirs for subsequent infection (Ellis, 1999). The bacterium is fastidious, with only slow growth seen on selective kidney disease medium (SKDM) agar at 15 °C (Evenden *et al.*, 1993). The actual mechanisms of the disease still remain largely unknown (Evenden *et al.*, 1993; González *et al.*, 1999), although *Renibacterium salmoninarum* can enter host macrophages *in vitro* via binding to the C3b component of the complement pathway (Campos-Pérez *et al.*, 1997), following activation. Furthermore, transmission is known to occur both horizontally and vertically (Evenden *et al.*, 1993). The role of amoebae, such as small FLA, could be important in horizontal transmission in terms of survival and dispersal of the bacterium.

Aeromonas salmonicida causes furunculosis in salmonid fish. The disease, like BKD, is serious, has a vast economic impact on farmed fish (Austin and Adams, 1996), and is also difficult to control (Munro and Hastings, 1993). The bacterium is a Gram-negative rod which grows on tryptone soy agar (TSA) at 20 °C, producing a brown pigment, although non-pigment producing strains are known (Austin and Adams, 1996). *Aeromonas salmonicida* is able to infect species of *Acanthamoeba* (Harf, 1994) and infection of the ciliate protozoan *Tetrahymena pyriformis* allowed

intracellular replication of the bacterium (King and Shotts, 1988), thus providing a potential mechanism for dissemination of the bacterium.

Aeromonas hydrophila is associated with tail and fin rot and haemorrhagic septicaemia in freshwater fish (Austin and Adams, 1996), although there is debate over whether the bacterium is a primary or an opportunistic pathogen (Austin and Adams, 1996). As *Aeromonas hydrophila* is also a pathogen of humans, growth occurs at a wider range of temperatures, from 20 °C to 37 °C. Preston *et al.* (2001) recently used *Aeromonas hydrophila* as a food bacterium in studies of *Acanthamoeba* locomotion, in which case the bacterium was killed and digested.

The survival of these pathogens in water is not completely understood, although *Aeromonas salmonicida* is thought to be capable of survival in sediments for prolonged periods of time (Austin and Adams, 1996). This is precisely the aquatic habitat exploited by *Acanthamoeba* species (Rodríguez-Zaragoza, 1994), thus interactions between bacteria and amoebae are potentially likely.

The natural route of infection by *Aeromonas salmonicida* is unknown, although entry both through wounds and via the oral route is known (Austin and Adams, 1996). In addition, various workers have shown increased *Aeromonas* association with phagocytic cells mediated by the A-layer, composed of lipopolysaccharide (LPS), which also increases cytotoxicity towards such cells (Austin and Adams, 1996). Like *Renibacterium salmoninarum*, *Aeromonas salmonicida* is capable of residing within pronephros macrophages (Deere *et al.*, 1997). Activation of such macrophages for production of respiratory burst reagents, however, can lead to the killing of *Aeromonas* species (Barnes *et al.*, 1999), although the same workers also demonstrated a peroxide-inducible catalase in *Aeromonas salmonicida*, in addition to the superoxide dismutase (SOD) (Sharp and Secombes, 1993) which both act to counteract the action and efficacy of hydrogen peroxide and superoxide anions (O_2^-). Conversely, macrophage activation is limited by the intracellular presence of live *Renibacterium salmoninarum*, preventing killing (Bandín *et al.*, 1993), aided by an apparent resistance to the O_2^- anion (Campos-Pérez *et al.*, 1997) and potential release of SOD and catalase (Ellis, 1999). The ineffectiveness of the respiratory burst in killing *Renibacterium salmoninarum* is also apparently due to activation and exhaustion of respiratory burst mechanisms by *Renibacterium salmoninarum* before phagocytosis of the bacterium occurs (Ellis, 1999).

1.5 Incidence of *Acanthamoeba* species in fish

Although much is known about acanthamoebal infections of humans, few studies have positively identified any such infection in fish, though there have been quite numerous reports on the occurrence of the genus in the organs of freshwater fish (Table 1.3).

Table 1.3. Occurrence of *Acanthamoeba* species in fish.

Host	Organ	Reference
Blue tilapia (<i>Sarotherodon (Tilapia) aureus</i>)	Peritoneal fluid, intestine and gills	Taylor (1977)
Carp (<i>Cyprinus carpio</i>)	Blood	Taylor (1977)
Channel catfish (<i>Ictalurus punctatus</i>)	Gill	Taylor (1977)
Chub (<i>Leuciscus cephalus</i>)	Brain and kidney	Dyková <i>et al.</i> (1999)
Common shiners (<i>Notropis cornutus</i>)	Intestine	Franke and Mackiewicz (1982)
European catfish (<i>Silurus glanis</i>)	Spleen	Dyková <i>et al.</i> (1999)
Goldfish (<i>Carassius auratus</i>)	Gill	Taylor (1977)
Largemouth bass (<i>Micropterus salmoides</i>)	Urinary bladder and gall bladder	Taylor (1977)
Rainbow trout (<i>Oncorhynchus mykiss</i>)	Gill	Taylor (1977)
Redeye bass (<i>Micropterus coosae</i>)	Spleen	Taylor (1977)
Redfin perch (<i>Perca fluviatilis</i>)	Brain and liver	Dyková <i>et al.</i> (1999)
Roach (<i>Rutilus rutilus</i>)	Liver	Dyková <i>et al.</i> (1999)
Ruffe (<i>Gymnocephalus cernuus</i>)	Brain	Dyková <i>et al.</i> (1999)
Striped bass (<i>Morone saxatilis</i>)	Gill	Taylor (1977)
White bream (<i>Blicca bjoerkna</i>)	Spleen	Dyková <i>et al.</i> (1999)
White sucker (<i>Catostomus commersoni</i>)	Intestine	Franke and Mackiewicz (1982)

Although several of the fish species noted in Table 1.3 appeared asymptomatic, the cause of death of the blue tilapia, for example, was attributed to amoebae (Taylor, 1977). Species of *Acanthamoeba* have also been implicated as a possible agent of systematic amoebiasis in European catfish (*Silurus glanis*) and of spontaneous intracranial amoebiasis of goldfish (*Carassius auratus*). Furthermore, experimental transmission of amoebae to uninfected fish, such as goldfish (*Carassius*

auratus), by intramuscular injection showed that amoebae were capable of invading fish tissues and organs, such as brain. In a study of 833 fish caught in the Czech Republic (Dyková *et al.*, 1999), *Acanthamoeba* species were found in 14 fish (an incidence of 1.7 %). Species identification of isolates from this and other studies indicated that *A. polyphaga* was the most common, but other species identified included *A. griffini*, *A. culbertsoni* and *A. royreba*. In addition, other genera of naked amoebae, including *Naegleria*, *Hartmannella* and *Vahlkampfia*, were isolated from various fish species. Given the large numbers of protozoa that inhabit the same aquatic environments as fish, exposure of fish to amoebae is not surprising and, accordingly, infection is somewhat commonplace.

1.6 Aims and Objectives

As can be inferred from the above material, there are a number of relationships amongst *Acanthamoeba* species, microorganisms and disease in vertebrates. It is well documented that some bacteria naturally infect phagocytic cells of humans and hence potentially fish, thereby enhancing their survival within potential hosts; in turn exploitation of protozoa supports the survival and widespread dispersal of microorganisms through aquatic, aerial and terrestrial environments.

The object of this work is thus to investigate some of the elements which mark the onset of *Acanthamoeba*-microorganism interaction, through study of such aspects as specific (lectin-adhesin interactions) and non-specific (cell surface charge and hydrophobicity) forces, phagocytosis by *Acanthamoeba* species of polystyrene latex microspheres of different size and surface nature, in addition to microspheres surface modified with proteins and carbohydrates. Other investigations, including isolation of amoebae from water samples from fish farms and rivers associated with salmonid fish populations, and an investigation into the fate of DNA taken up by *Acanthamoeba polyphaga*, support such studies and further explore the subtleties of *Acanthamoeba* biology and microbial interaction. These investigations draw on diverse protocols and techniques including aseptic culture and manipulation techniques, particulate microelectrophoresis, flow cytometry, confocal microscopy and molecular biology

protocols; the contribution, value and nuances of which should be evident from the following detailed studies.

CHAPTER 2 – Culture and encystment of *Acanthamoeba polyphaga*

2.1 Introduction

As noted, *Acanthamoeba* species exist in two distinct morphological forms – a vegetative trophozoite and a dormant, but viable, cyst, which allows for various experimental manipulations. Unlike many other amoebal species, however, many species of *Acanthamoeba* are easily grown in axenic culture in broth (Neff, 1957; Byers, 1979; Page, 1988), as well as on non-nutrient agar in the presence of a food source, usually Gram-negative bacteria (Neff, 1957). Deprived of such a food source, if desiccated or in the presence of high salt concentrations or high pH (Byers, 1979), *Acanthamoeba* species will naturally encyst, albeit asynchronously (Neff *et al.*, 1964b), an occurrence observed both on agar or when placed in saline, although the exact trigger is not well defined (Chambers and Thompson, 1972). It is, however, possible to induce synchronous encystment in *Acanthamoeba* species using a defined medium (Neff *et al.*, 1964b), containing essential calcium and magnesium ions (Bowers and Korn, 1969). Interestingly, after a few hours of encystment, immature cysts apparently become committed to encystment and even if they are placed back in growth medium, they will go on to form mature cysts (Weisman, 1976). Conversely, excystment occurs when cysts are placed in growth medium (Weisman, 1976); trophozoites emerge through the ostioles in the cyst wall, often aided by the stimulation by Gram-negative food bacteria (Larkin and Easty, 1990).

The formation of cysts enables prolonged survival at a range of temperatures (Biddick *et al.*, 1984) and has also aided the ubiquity of the genus. As noted by Avery *et al.* (1994), the existence of *Acanthamoeba* species in a vast array of habitats is subject to temperature variation on both spatial and temporal levels. Thus, one would expect the organism to be capable of growth at a wide-range of temperatures.

With a generation time of six to eight hours, growth of *Acanthamoeba* species occurs by binary fission, though the mitosis stage of the cell cycle is thought to only take about ten minutes, accounting for about 2 % of the cycle (Byers, 1979).

The effect of temperature on the culture of *Acanthamoeba polyphaga* was therefore examined, in addition to induced encystment of *Acanthamoeba polyphaga*.

These studies allowed the establishment of protocols for the culture of trophozoites and formation of cysts for future experimentation.

2.2 Materials and Methods

All reagents used throughout this work, except where stated, were standard laboratory grade chemicals purchased from Sigma, USA.

All growth media used throughout this work, except where stated, were purchased from Oxoid, UK.

2.2.1 *Acanthamoeba polyphaga* stock cultures

Acanthamoeba polyphaga (designated as the Leeds strain) was provided by Dr. J. Barker, subcultured from a strain originally acquired from Leeds Public Health Laboratory, UK (Rowbotham, 1983). Each week, stock cultures of *Acanthamoeba polyphaga* were grown axenically as monolayers in 25 cm² tissue culture flasks (Sarstedt, USA) by adding 1 ml confluent culture (at least 10⁶ cells ml⁻¹) to 9 ml fresh PYG broth prepared from amoebal saline (Solution A: 1.2 % (w/v) NaCl, 0.04 % (w/v) MgSO₄·7H₂O, 1.42 % (w/v) Na₂HPO₄, 1.36 % (w/v) KH₂PO₄, 100 ml double distilled water (ddH₂O); Solution B: 0.04 % (w/v) CaCl₂, 100 ml ddH₂O). Amoebal saline was then prepared by adding 10 ml Solution A and 10 ml Solution B to 980 ml ddH₂O, before addition of 1.5 % (w/v) proteose peptone (Difco, USA), 0.5 % (w/v) yeast extract, 1 % (w/v) D-glucose, 0.0003 % (w/v) FeSO₄ for PYG broth. Following adjustment to pH 6.9 ± 0.5 with 1 M KOH, PYG broth was autoclaved at 121 °C for 15 min at 15 psi before use (Rowbotham, 1983)). Flasks were incubated horizontally at 37 °C in darkness, to prevent photoinhibition of growth (Dolphin, 1970).

2.2.2 Effect of temperature on the growth of *Acanthamoeba polyphaga*

The effect of temperature on the growth of *Acanthamoeba polyphaga* trophozoites was determined by growing replicate cultures in disposable plastic bijoux (Bibby Sterilin Ltd., UK) at a range of temperatures: 10, 15, 20, 25, 34, 37 and 40 °C. Each sample was prepared in triplicate by inoculation of 0.9 ml sterile PYG broth with 0.1 ml confluent stock culture before incubation, in darkness, at the relevant temperature. Enumeration of amoebal trophozoites was carried out daily for up to 10 days post-inoculation, with an initial count immediately after inoculation. Bijoux were briefly chilled at -20 °C, without allowing cultures to freeze, to encourage trophozoite detachment from the vessel surface by minimal mechanical disruption, before samples were aliquotted into an “Improved Neubauer” haemocytometer (Weber, UK). Any potential build-up of contamination was prevented by discarding cultures immediately post-sampling, which also served to minimise culture disturbance during sampling (Byers, 1979). Two counts were taken for each of three replicates and a mean was calculated for each replicate sample.

Results were analysed by two factor ANOVA using the data analysis package in Microsoft[®] Excel 2000 (Microsoft Corporation, USA) to determine the effect of both temperature and culture age on numbers of *Acanthamoeba* trophozoites.

2.2.3 Induced encystment of *Acanthamoeba polyphaga*

The length of incubation required for successful encystment of a significant proportion of cells was determined by inducing four-day-old cultures of *Acanthamoeba* trophozoites to encyst. Cultures were prepared in sterile plastic universals (Bibby Sterilin, UK) by the inoculation of 9 ml sterile PYG broth with 1 ml confluent stock culture before incubation horizontally in darkness at 37 °C. In order to induce synchronous encystment of *Acanthamoeba polyphaga* trophozoites, resultant cells were centrifuged at 1000 g for 10 min before washing with 10 ml encystment medium. A second 10 min centrifugation at 1000 g was performed before resuspending in 10 ml encystment medium (7.5 % (w/v) KCl, 2.5 % (w/v) Tris,

2 % (w/v) MgSO₄·7H₂O, 0.06 % (w/v) CaCl₂, 0.08 % (w/v) NaHCO₃, 1 L ddH₂O. The medium was adjusted to pH 8.9 – 9.0 with 1 M KOH before autoclaving at 121 °C for 15 min at 15 psi (Neff *et al.*, 1964b)). This washing procedure was repeated once before cultures were incubated at 37 °C in darkness. Two samples were taken from each of three replicates immediately after substitution of PYG broth for encystment medium and thereafter at hourly intervals in order to determine the relative numbers of trophozoites, young cysts and mature cysts by use of a haemocytometer. The number of each morphological form was determined by direct microscopic observation of samples aliquotted into counting chambers with classification of cell types following that outlined by Neff *et al.* (1964b): pre-encystment amoebae (trophozoites) – active amoebae with acanthopodia present; young cysts – rounded-up cells lacking double walls and mature cysts – rounded-up cells with double walls. Sampling continued for 75 hours, at which point mature cysts were present in marked proportions.

Results for each cell type were expressed as percentages of total cell counts thereby requiring arc sine transformation to ensure compliance with a normal distribution curve. Single factor ANOVA using the data analysis package in Microsoft[®] Excel 2000 was employed to determine the effect of incubation in encystment medium on the proportion of each cell type. In addition, single factor ANOVA was used to determine the effect of incubation in encystment medium on total cell counts.

2.3 Results

2.3.1 Effect of temperature on the growth of *Acanthamoeba polyphaga*

Growth curves for *Acanthamoeba polyphaga* (Leeds strain) at different temperatures are shown in Figure 2.1. This strain of *Acanthamoeba polyphaga* demonstrated an ability to grow at a wide range of temperatures between 10 °C and 37 °C, with an apparent optimum at 30 °C, attaining a maximal concentration of just under 2×10^6 cells ml⁻¹ over the full period of incubation. With the exception of

40 °C, greater amoebal concentrations were found with increasing incubation temperature for the first five days. After day 5, however, the greatest yield of trophozoites was found at an incubation temperature of 30 °C. Statistically significant differences in cell numbers were found both between days ($F = 138.46$; $P < 0.001$) and between temperatures ($F = 246.56$; $P < 0.001$). At 40 °C there was an apparent complete incapacity to grow, at least over the time period monitored. Growth at 10 °C was slow and, indeed, almost non-existent for the first week post-inoculation. For other temperatures, lag phases were not quite as pronounced, lasting only a day at most or even seemingly absent. In addition, stationary phases were not overly evident from the growth curves, though it could possibly be argued that stationary phase occurred at around seven to nine days post-inoculation at least for those cultures grown at temperatures of 15, 25 and 37 °C.

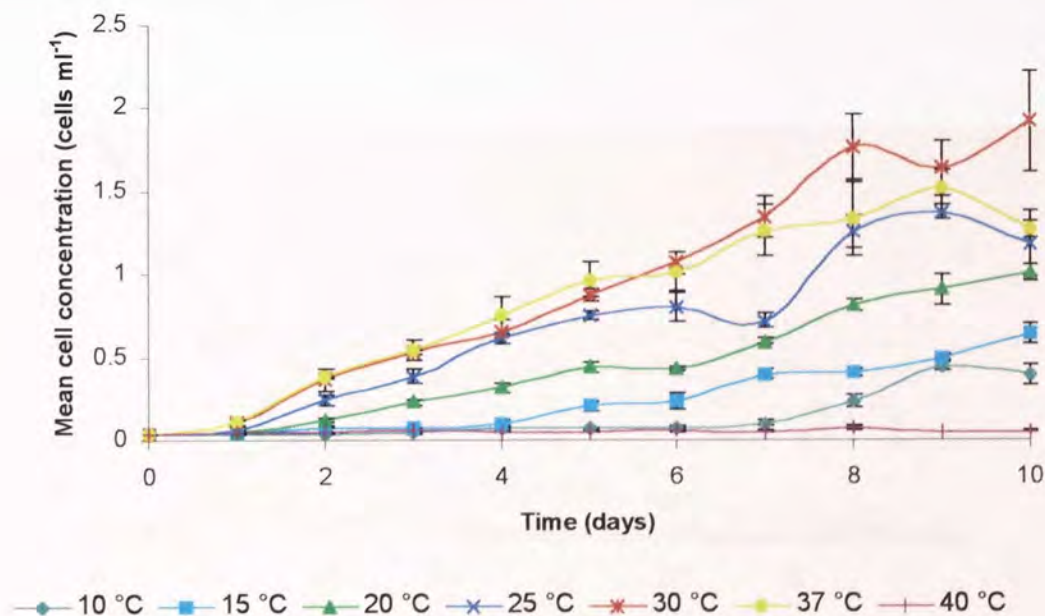


Figure 2.1. Effect of temperature on the growth of *Acanthamoeba polyphaga* (Leeds strain) in PYG broth. Cell concentrations are means calculated from total counts of three replicates, each consisting of two counts. Bars represent standard errors of the means for three replicate experiments.

2.3.2 Induced encystment of *Acanthamoeba polyphaga*

As can be seen from Figure 2.2, during the process of induced encystment the overall cell concentration diminished to approximately half the initial value determined on replacement of PYG with encystment medium (at that time consisting solely of trophozoites). Encystment commenced after a short period of seven or eight hours and, as can be seen from Figure 2.3, the proportion of cysts then increased from 0 % to around 96 % over the next three days. The proportion of young cysts remained relatively constant throughout the sampling period at just below 20 % of all cells, whereas the proportion of mature cysts steadily increased over the same time. Besides diminishing cell numbers, Figure 2.2 also shows a decrease in trophozoite numbers over time in accordance with an increasing number of cysts.

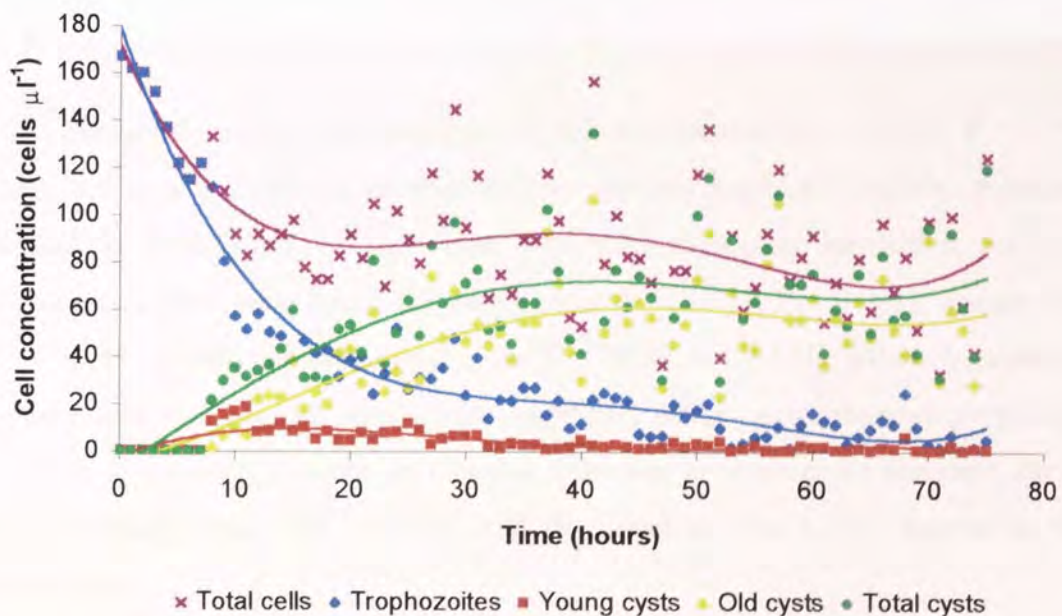


Figure 2.2. Induced encystment of *Acanthamoeba polyphaga* over time, showing concentrations of each cell type. Cell concentrations were calculated from cell counts for each form and are means of three replicates, each consisting of two counts. Lines represent fourth order polynomial best-fit curves.

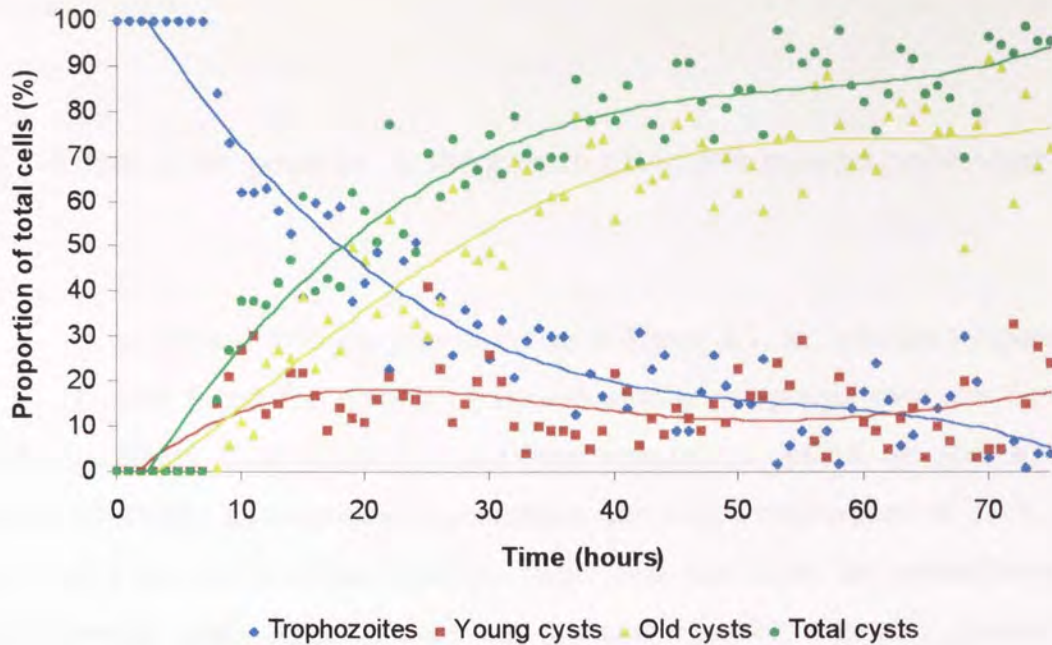


Figure 2.3. Induced encystment of *Acanthamoeba polyphaga* over time, showing proportions of each cell type. Mean percentages were calculated from total cell counts from three replicate experiments. Lines represent fourth order polynomial best-fit curves.

Statistical analysis demonstrated a significant decrease ($F = 160.56$; $P < 0.001$) in total cell counts following incubation in encystment medium. Similarly, significant increases in proportions of each cyst type with increasing incubation period in encystment medium were found for young cysts ($F = 41.51$; $P < 0.001$), mature cysts ($F = 4.03$; $P = 0.046$) and hence total cysts ($F = 20.59$; $P < 0.001$), although incubation in encystment medium had no apparent significant effect on trophozoite proportions ($F = 0.15$; $P = 0.699$), despite an obvious reduction in trophozoite numbers, due to both decreasing total cell numbers (of all amoebal forms) and increasing cyst proportions.

2.4 Discussion

2.4.1 Effect of temperature on the growth of *Acanthamoeba polyphaga*

As determined from the growth curves in Figure 2.1, an optimum temperature of 30 °C was found for growth of *Acanthamoeba polyphaga* over the ten-day incubation period. This temperature has been reported as optimal by other authors (Byers, 1979). For all subsequent experiments, however, a temperature of 37 °C was used with a four-day incubation period. Under such conditions the concentration of *Acanthamoeba* trophozoites obtained was just under 1×10^6 cells ml⁻¹, greater than that obtained at any other temperature. As reported by Chambers and Thompson (1976), a magnitude of 10^6 cells ml⁻¹ is usually the maximum cell concentration for *Acanthamoeba* at stationary phase, although some reports indicate yields of more than 3×10^7 amoebae ml⁻¹ (Jensen *et al.*, 1970). Four days' incubation was chosen as a compromise between having enough cells for experimental purposes and obtaining amoebae in as short a time as possible. This also meant that amoebae were in log phase (Przełęczka and Perkowska, 1986), potentially making them more suitable for several aspects of study. Chambers and Thompson (1976) report, for example, that stationary phase amoebae are more reluctant to phagocytose. However, for some later studies, the effect, if any, of amoebal age has been determined.

Growth of *Acanthamoeba polyphaga*, as trophozoites, was observed to occur at most temperatures studied, with the exception of 40 °C, which inhibited all growth. Even at the lowest temperature documented, 10 °C, growth was observed, but only after a much longer lag phase. Growth at 5 °C over a similar period was not observed at all (data not shown) but, given longer incubation, growth could potentially occur eventually. Such results are consistent with the natural status of *Acanthamoeba*, which has been isolated from diverse water systems, in turn associated with a wide range of temperatures. It has even been isolated from samples found in Antarctica (Brown *et al.*, 1982), although at this sub-zero temperature, amoebae were only present as cysts. Active amoebae, however, are found in various water systems, which fluctuate over a marked temperature range, dependent on such factors as climate, water depth or, if

man-made, artificial heating. Observations indicate that amoebae frequently exist as trophozoites rather than as cysts; therefore it is not surprising that growth was observed at the cooler temperatures employed in this study. Similarly, as some species of *Acanthamoeba*, including *A. polyphaga*, are known human pathogens, it is not surprising that prolific growth occurred at 37 °C. Indeed, as demonstrated by De Jonckheere (1980), the ability to grow axenically at higher temperatures is usually related to virulence, with those unable to grow even at 37 °C in axenic culture likely to be avirulent.

No cysts were observed in any of the cultures, despite reaching apparent stationary phase or population growth deceleration (Byers *et al.*, 1969). Although there is some debate over whether or not encystment automatically commences when growth ceases, several reports make a case for a delay before encystment commences (Chambers and Thompson, 1976) with Neff *et al.* (1964b) observing such events after five to ten days at stationary phase. Byers *et al.* (1969) state that only low levels of encystment occur in growing populations, amounting to no more than 0.2 % of the total populations, rising to “several percent” at late stationary phase, although such observations were not made in the present study. As in studies by Jensen *et al.* (1970), it may be that the Leeds strain of *Acanthamoeba polyphaga* had developed an inability to encyst in PYG medium, although leaving the cultures for longer periods of time may allow observable encystment, thus disproving this notion.

A further observation on growth of trophozoites is that the vessels used for culture may play a part in cell yield. This is at least partly due to a surface area phenomenon, as growth of *Acanthamoeba* trophozoites is increased following attachment to solid surfaces (Ogbunude *et al.*, 1991; Armstrong and Soothill, 1999). Other types of vessels, in comparison to the plastic bijoux used in the present study, gave different concentrations under similar conditions (data not shown). However, bijoux were not subsequently used in later experiments to culture amoebae because the maximum volume of medium for shallow culture of monolayers in a bijou is around 1 ml. Hence, although amoebal concentration in bijoux may be slightly greater, it would be impractical to use bijoux for the culture of required numbers of *Acanthamoeba polyphaga* due to insufficient volume of harvestable medium. Similar shallow monolayer cultures in universals laid horizontally, however, allow production of more trophozoites and also allow the concentration of cells by resuspension in a

smaller volume of medium, following centrifugation, making them far more suited to any application.

Harvesting amoebae, as alluded to above, relied on centrifugation, since chilling vessels such as bijoux in order to detach amoebae from the surface is both unreliable and tedious according to Ogbunude *et al.* (1991), who themselves developed a specific medium for the purpose of detachment of *Acanthamoeba* from surfaces. However, this medium was considered unnecessary and hence never actually employed in this work, as the use of universals for amoebal growth allowed centrifugation of these vessels with a relatively low force (Walker, 1996), encouraging sufficient numbers of amoebae to detach and pellet. Checks were made which confirmed that this procedure did not cause any damage to the amoebae (data not shown).

2.4.2 Induced encystment of *Acanthamoeba polyphaga*

Encystment of *Acanthamoeba polyphaga* can be viewed as a “make or break” process since during the 75 hour incubation period employed, only 50 % of cells survived the process and became cysts, either young or mature. This proportion is in accordance with that described by Neff *et al.* (1964b) for natural asynchronous encystment, unlike their experimental synchronous encystment which resulted in cultures composed of 90 % of cysts. Given that the breakage or destruction of non-encysting cells occurs at such a high rate, it is crucial that there are enough trophozoites present in the original culture before encystment is induced. Therefore, in all cases when cysts were required, trophozoites were grown for four days in PYG broth before encystment. Furthermore, in order to compensate for the potential 50 % loss in cells during the process, cultures were often combined after encystment to provide enough cysts for experimental purposes.

Avron *et al.* (1983) demonstrated through work with *Entamoeba* that not all amoebal cells can become cysts, and those that fail will fragment instead, giving rise to many “ghosts” (cellular debris) in the medium. This was clearly observed during the course of this study, with the number of fragments increasing as sampling

continued. Quite why some trophozoites are apparently incapable of forming cysts is unknown, though one mechanism by which it occurs could be apoptosis. Apoptosis was, until recently, thought to be unique to cells of multicellular organisms, though Harb *et al.* (2000) suggest apoptosis may be triggered in *Acanthamoeba* by factors that stall the normal differentiation process, thereby ensuring that cells unable to form cysts are removed from the population.

The formation of a significantly large proportion of cysts was achieved by about 40 hours, after which the percentage of mature cysts reached a plateau. In addition, there was also a lag period before any cysts were formed at all, with the first cysts being formed after three or four hours of introduction into encystment medium. The proportion of young cysts present in the cultures was steady throughout most of the latter part of the experiment. Presumably, if the incubation time had been extended, any young cysts present would either fragment or become mature cysts and suspensions would consist almost entirely of mature cysts. From this experiment, it was determined that an encystment period of three days would produce cysts in sufficient quantities for experimental purposes. Neff *et al.* (1964b) induced encystment at 30 to 32 °C, temperatures at which differentiation occurred more rapidly. However, for continuity, 37 °C was used for encystment in the present work, as this was the temperature used for initial culture of the amoebae and would in turn allow comparison of any moieties potentially expressed by trophozoites and cysts.

Encystment requires usage of trophozoite glycogen reserves for manufacture of cellulose, the main component of the cyst cell wall (Byers, 1979) and, as such, the degree of encystment is thought to depend on the state of the original trophozoite suspension, although the exact nature of such states is not well characterised (Byers *et al.*, 1969). Griffiths and Hughes (1969) induced encystment at 30 °C in *Acanthamoeba* (= *Hartmannella*) *castellanii* cultures of increasing age and found that the proportion of encysting amoebae significantly declined with increasing culture age. Four-day-old cultures in their experiments only achieved a rate of 9 % of maximum encystment, although they also indicate such cultures would have entered stationary growth phase. In addition, they found encystment did not occur at 37 °C, with culture death and abundant cell debris after 24 hours. Although a large amount of cell debris was observed in the present study, the proportion of encysting cells was much greater and, as noted, a temperature of 37 °C was used for incubation during encystment for the sake of continuity. The use of MgCl₂ for encystment was probably a key factor in

Griffiths and Hughes's (1969) observation of a lower encystment rate and earlier stationary phase, given that Neff *et al.* (1964b) state that either Ca^{2+} or Mg^{2+} ions alone support only a limited encystment. However, their observations may also potentially be attributed to the different species of *Acanthamoeba* used in their experiments or the technique they used for determining encystment (measurement of cellulose levels). In contrast, the work by Neff *et al.* (1964b) on encystment suggests that there is no difference in the ability to encyst between log and stationary phase cultures, except for a slightly delayed encystment in the older culture. Cyst proportions reached the same levels in both cultures at the end of the incubation period. Although only one age of amoebal culture was induced to encyst in the present study, the proportion of cysts obtained is clearly greater than that obtained by Griffiths and Hughes (1969) and, as such, the method used forms a suitable protocol for the formation of cysts for further experimentation.

Similarly, conditions deemed optimum for generation of *Acanthamoeba polyphaga* (Leeds strain) trophozoite cultures (i.e. incubation at 37 °C for 4 days in horizontal sterile plastic universals) formed a basis for the production of trophozoites for the remainder of the experimentation in this work, unless otherwise noted. Such studies include the examination of specific and non-specific forces that may be involved in interactions between *Acanthamoeba* and bacteria or latex microspheres, phagocytosis of latex microspheres of different size and nature and uptake of DNA by *Acanthamoeba*. Firstly, however, a survey of fish farms and rivers for environmental amoebae was carried out in order to obtain a clearer picture of the likelihood for such interactions with bacterial fish pathogens.

CHAPTER 3 – Isolation of amoebae from environmental water samples

3.1 Introduction

Amoebae, particularly *Acanthamoeba* species, are well known for their ubiquity in a wide range of natural and man-made habitats, including freshwater and marine environments; tap and bottled water; soil, dust and air; sewage; heating, ventilation and air-conditioning units; contact lens solutions and eyewash stations; dialysis machines and dental units; hot tubs and gastrointestinal washings (Marshall *et al.*, 1997) as well as ocean sediment; compost; swimming pools and frozen swimming water; medicinal pools; human nasal cavities, throats and intestines; cultured cells; fish, reptiles, birds and mammals; mushrooms and vegetables (De Jonckheere, 1987) – an astonishing range of environments, exploited mainly due to the ability to form a highly resistant cyst under adverse conditions (Avery *et al.*, 1994).

Methods have been established for isolation of amoebae from such environments usually involving either filtration or centrifugation before incubation on agar (Neff, 1957; Neff, 1958; Page, 1988; Pernin *et al.*, 1998). A filtration technique was tested on water samples collected from the lake at Aston University in order to develop a suitable protocol that could be used for amoebal isolation from water samples collected from fish farm and river water. Such habitats are also associated with a high density of salmonid fish stocking hence the likelihood of elevated bacterial numbers including pathogenic *Aeromonas* and *Renibacterium* species. Spatial co-existence of pathogen, host and potential vehicle for pathogenic spread (i.e. amoebae) would support the notion that species of *Acanthamoeba* could be potential agents for the dissemination of *Aeromonas* and/or *Renibacterium* species, affording bacterial protection, in a similar manner to that known to occur with *Legionella pneumophila*. Although circumstantial, such co-incidence of *Acanthamoeba* species and species of *Renibacterium* or *Aeromonas* would warrant further investigation into the mechanisms that may be involved in any such interaction.

3.2 Materials and Methods

3.2.1 Isolation of amoebae from pond water samples

3.2.1.1 Sample collection and filtration

The presence of amoebae in a water sample was investigated following collection of water from Aston University's lake, by scooping 500 ml water up into a sterile glass bottle. Five volumes of 100 ml were then vacuum filtered through separate 3 µm pore-size nitrocellulose filters (Millipore Corporation, USA) before inversion of filter discs and subsequent placement on non-nutrient agar supplemented with 1 % (v/v) Amphotericin B solution (1.5 % (w/v) agar (Fisons, UK), 1 L amoebal saline (Section 2.2.1). Agar was autoclaved at 121 °C for 15 min at 15 psi. Amphotericin B solution was added to the cooling agar to a final concentration of 1 % (v/v) just prior to pouring molten agar into 90 mm diameter sterile petri dishes (Sarstedt, USA)). The addition of Amphotericin B was found to have little marked effect on the growth of *Acanthamoeba* in trials where it was added to PYG broth at a final concentration of 1 % (v/v) (data not shown). Such an observation is in agreement with studies by Visvesvara and Balamuth (1975). This concentration of Amphotericin B, however, was enough to inhibit the growth of most fungal contaminants (data not shown), which could severely interfere with the successful isolation of protozoa due to competition.

A 20 ml *Escherichia coli* culture (Aston University teaching culture) in Tryptone Soy Broth (TSB) (3 % (w/v) TSB, 1 L distilled water; autoclaved at 121 °C for 15 min at 15 psi before use) was heat-killed by placing the culture flask in a 100 °C water bath for 1 hour before introduction of moribund bacteria to each agar surface, around the inverted filter disc, with a sterile inoculating loop to provide a food source for potential protozoa. Plates were then sealed with Parafilm[®] (American National Can[™], USA) to prevent moisture loss, before incubation at 37 °C in darkness for up to two weeks. After incubation for one week to allow acclimatisation

and sufficient migration, daily checks were made by light microscopy for the presence of any migrating amoebae on the agar surface.

3.2.1.2 Isolation of amoebae

Following amoebal migration, sections of agar containing surface exposed amoebae were excised with the larger bore ends of sterile glass pipettes, before being placed on further plates of non-nutrient agar supplemented with Amphotericin B solution, thereby encouraging amoebae to migrate free of contaminating organisms. After incubation for one week at 37 °C in darkness, the presence of amoebae was identified by light microscopy and agar sections containing amoebae were excised, as noted above, placed in universals containing 10 ml PYG broth, which were then incubated at 37 °C for up to two weeks in darkness, with regular checks for amoebal growth. Such cultures were extremely prone to contamination, hampering efforts to isolate amoebae in axenic culture from water samples. However, amoebae were recognisable on agar and, due to the methods used and the reported ubiquity of *Acanthamoeba* (Page, 1988), were most likely to be of that genus.

3.2.2 Survey of fish farms and rivers

Although pure isolation proved difficult, the method, as it stood, was suitable for determining the presence of amoebae, particularly *Acanthamoeba*, in water samples. It was thus applied to water samples collected from fish farms and rivers at the locations listed in Table 3.1, where salmonid fish were present and hence also the likelihood of elevated numbers of bacterial fish pathogens including *Aeromonas* and *Renibacterium* species.

Samples, in 250 ml volumes, were collected by scooping water from the surface of the farm raceway or water column into 250 ml sterile plastic bottles, chilled to 4 °C during transport to the laboratory. This volume allowed filtration of two 100 ml aliquots through separate 3 µm pore size nitrocellulose filters. Filter discs were

inverted on non-nutrient agar containing 1 % (v/v) Amphotericin B solution, as outlined in Section 3.2.1.1. One plate for each sample also had heat-killed *E. coli* spread on the agar as a food organism, which was lacking on the other plate in order to determine if the absence of the bacteria would aid isolation. Plates were sealed with Parafilm[®] and incubated at 30 °C in darkness with regular inspections for amoebae using light microscopy after one week. This temperature was used instead of 37 °C, as used for isolation of amoebae from pond water, because it encouraged the growth of a wider range of amoebae (particularly amoebae not associated with human pathogenicity), ill favoured by the higher 37 °C incubation.

Following identification of amoebae, isolation was attempted by excision of the agar block in the same manner as detailed in Section 3.2.1.2.

Table 3.1. Location and nature of sites from where water samples were taken.

Sample Number	River	Type
1	Mawddach, Wales	Fish farm
2	Dee, Wales	Fish farm
3	Dee, Wales	Fish farm
4	Dee, Wales	Fish farm
5	Dyfi, Wales	Fish farm
6	Avon, Hampshire	Fish farm
7	Stour, Dorset	Fish farm
8	Avon, Hampshire	River
9	Stour, Dorset	River
10	Frome, Dorset	River
11	Piddle, Dorset	River
12	Bere Stream, Dorset	River

3.3 Results

3.3.1 Isolation of amoebae from pond water samples

Amoebae were found on agar from each of the five samples taken from the lake at Aston University. Although the identification of amoebae is difficult (Weisman, 1976), the methods used for isolation and the high prevalence of *Acanthamoeba* would indicate that those amoebae isolated were of that genus. By way

of confirmation, some migrated amoebae had encysted, enabling further identification of the more recognisable morphology of the cyst form (Page, 1988).

Subsequent efforts to axenically culture these amoebae were hampered by the presence of contaminating organisms, principally bacteria; furthermore, despite the use of Amphotericin B, some fungi had also established themselves on agar surfaces. The presence of such organisms was perhaps inevitable given the water source employed.

3.3.2 Survey of fish farms and rivers

Table 3.2 shows that the presence of amoebae was demonstrated in samples from all sites, with the exception of those from the River Mawddach.

Table 3.2. Success of amoebal isolation from fish farm and river water samples.

Sample Number	River	Type	Presence of amoebae
1	Mawddach, Wales	Fish farm	No
2	Dee, Wales	Fish farm	Yes
3	Dee, Wales	Fish farm	Yes
4	Dee, Wales	Fish farm	Yes
5	Dyfi, Wales	Fish farm	Yes
6	Avon, Hampshire	Fish farm	Yes
7	Stour, Dorset	Fish farm	Yes
8	Avon, Hampshire	River	Yes
9	Stour, Dorset	River	Yes
10	Frome, Dorset	River	Yes
11	Piddle, Dorset	River	Yes
12	Bere Stream, Dorset	River	Yes

As with water samples from the university lake, further attempts to purify these amoebae were hindered by competition from contaminants. Such contaminating organisms were most likely those that had been filtered along with the amoebae, despite the relatively large filter pore-size used, as the contaminating organisms were also found in subcultures taken from the original samples where *E. coli* were not present as food organisms, moreover *E. coli* were heat killed, so should not have

grown. The absence of *E. coli* as a food source did not prevent either migration or survival of amoebae, although it is probable that migrating amoebae would show greater encystment due to the lack of such a food source and hence conditions would be more unfavourable. The presence of cysts, as with the pond water samples, aided identification and it would appear that the amoebae were most likely of the genus *Acanthamoeba*. Typical trophozoite and cyst forms found in each water sample are represented in Figure 3.1.

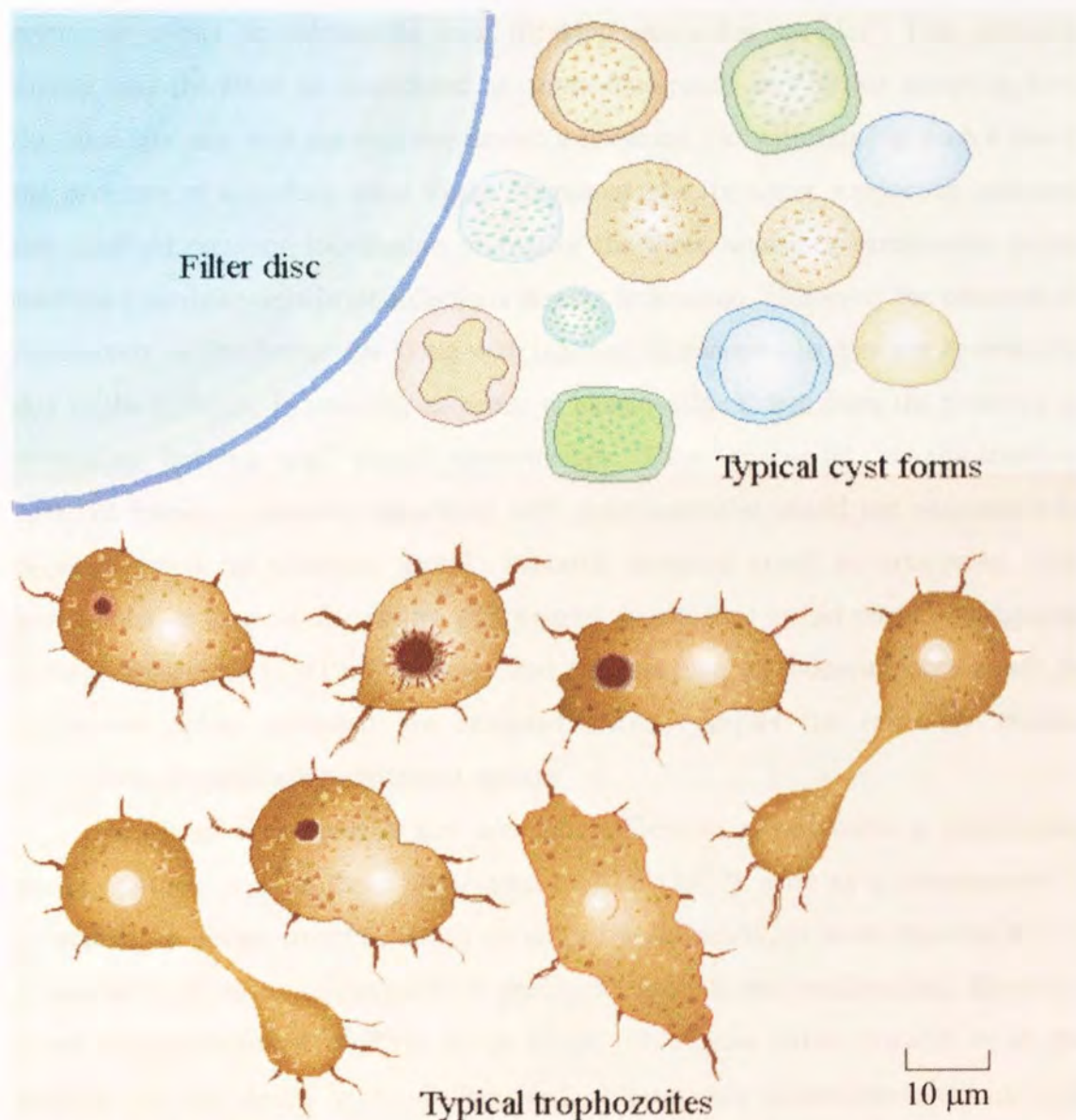


Figure 3.1. Diagrammatic representations of typical trophozoite and cyst forms isolated on non-nutrient agar following filtration of fish farm or river water samples.

3.4 Discussion

The presence of amoebae in the university lake, fish farms and river sources was not a surprise, given the reported ubiquity of protozoa, especially *Acanthamoeba* (Marshall *et al.*, 1997). In fact, the apparent absence of any protozoa in the sample taken from the fish farm on the River Mawddach was something of an unexpected result, especially as Page (1988) states that “anyone collecting freshwater or soil is certain to collect *Acanthamoeba*, even if taking only a few samples”. This particular finding may therefore be considered an anomalous result and further sampling from the same site may well demonstrate amoebal presence. Notwithstanding such a result, the presence of amoebae, most likely *Acanthamoeba*, in water supporting salmonid fish confirms common localisation of two of the three necessary participants in any microbe-protozoan-vertebrate infectious disease interaction. However, the presence of *Aeromonas* or *Renibacterium* along with isolated *Acanthamoeba* was not determined, due to the difficulty in isolating amoebae in axenic culture, free from the presence of competing bacteria and fungal contaminants. Even successful identification of bacterial species potentially associated with *Acanthamoeba* would not necessarily be of great value, as although specific bacterial presence could be confirmed, their intracellular or extracellular nature with respect to amoebae would remain ambiguous. Indeed, Fritsche *et al.* (1993) indicate that the presence of endosymbionts would be undetected unless amoebae are rendered axenic, despite the relatively marked prevalence of possible endosymbiont species.

Much of the reported and common difficulties experienced in establishing axenic cultures, such as those considered by Neff (1957), arise as a consequence of attempting to isolate amoebae which do not grow axenically, or stem from the lack of a suitable medium to support robust protozoan growth and proliferation. However, given the development of PYG broth (Page, 1988), the latter obstacle is of less concern, if the desire is to isolate and culture only *Acanthamoeba*. In such circumstances, the main challenge, as noted, is potential competition and antagonism due to contaminating bacteria and fungi. As occurrence of contaminants is not uncommon in axenicisation (Page, 1988), should more extensive isolation work be required it would also be prudent to employ antimicrobials such as penicillin or streptomycin to inhibit or suppress bacterial growth and Amphotericin B to inhibit

fungal activity. Additionally, Page (1988) recommends that such agents would probably have to be used in all subsequent subcultures to continue microbial suppression, providing, of course, they are not resistant to such antimicrobials.

Bulk recovery of amoebae from water samples can be achieved in two main ways – filtration and centrifugation. For *Naegleria fowleri* trophozoites, the study of Pernin *et al.* (1998) revealed that centrifugation was significantly better than filtration for recovery from seeded water samples. In contrast, Winiiecka-Krusnell and Linder (1998) demonstrated sharply improved recovery of *Acanthamoeba* trophozoites by filtration when compared to that by centrifugation. Pernin *et al.* (1998) considered that reduced recovery of amoebae associated with filtration might have been due to cell lysis as shown by *Naegleria* trophozoites when subjected to high filtration pressures. However, there was little evidence of cell lysis in the filtrates in the present study (data not shown), and potentially arose in the study of Pernin *et al.* (1998) due to the different amoebal genus investigated or a greater filtration pressure.

Although the present study employed a migratory method for isolation of *Acanthamoeba*, similar to that of Neff (1957), alternative methods exist involving severe chemical or physical agents, such as desiccation, pasteurisation or washing and soaking of potential isolates in caustic chemicals (Neff, 1958). Such methods are more suited to *Acanthamoeba* as their ability to form resistant cysts could enable them to survive such actions, unlike the majority of associated or contaminating organisms. The apparent lack of reported success with such methods for amoebal isolation in the scientific literature suggests either that the methods are too drastic or may simply reflect a decision to isolate a greater variety of protozoan genera than *Acanthamoeba* alone. Whatever the reasons, use of migration based protocols for isolation of amoebae appears to be the favoured technique.

Rodríguez-Zaragoza (1994) states that amoebae are more usually found in the upper reaches of water columns, encouraged by the presence of particulate matter, to which amoebae attach. Accordingly for this study, samples were collected from the top of farm raceways or water columns and all samples collected contained a variable, but small, amount of sediment. The relationship between amount of sediment and amoebal population in the water sample was not evaluated, but it was noticeable that some samples yielded greater amoebal numbers (data not shown). Vertical distribution of FLA in freshwater lakes was, however, studied by Kyle and Noblet (1985) who confirmed the presence of amoebae in association with particulate matter. The

spectrum of particle occurrence from sporadic presence throughout the water column to relative abundance near the surface was observed to alter after, for example, heavy rain with concomitant change in amoebal distribution. In the present study, which was not envisaged as prescriptive, such an effect would not be obvious, as water samples were only collected from the top of the water column and environmental conditions were not comprehensively recorded during sample collection.

Seasonal variation in distribution of amoebae is also a recognised occurrence (Kyle and Noblet, 1986), with greater numbers occurring in the summer months. All samples in the present study were collected between May and August when water temperatures are commensurate with proliferation of both protozoa and bacteria. As noted by Kyle and Noblet (1985), there is a strong correlation between bacterial abundance, the latter particularly associated with detritus, and amoebal population numbers, although other factors such as temperature, dissolved oxygen and pH play a part. Studies by O'Dell (1979) on amoebal population densities in a Nebraska lake found that there was no single correlation with pH, dissolved oxygen, water temperature, bacterial abundance, nitrate or phosphate levels, probably indicating that a combination of factors was involved in amoebal population density variation.

The present study has revealed the presence of amoebae, and potentially *Acanthamoeba* in water samples collected from fish farms and river systems, both habitats of salmonid fish and reservoirs of possible bacterial pathogens. This finding supports, albeit circumstantially, some role for species of *Acanthamoeba* as vectors for the transmission of bacterial species, particularly those such as *Renibacterium* and *Aeromonas*, whose transmission and survival outside of their hosts is largely unknown (Evenden *et al.*, 1993; Austin and Adams, 1996). The increase in recognition of amoebae as a vehicle for the spread of bacteria implicated in human disease, not least of all *Legionella pneumophila* (Rowbotham, 1980), indicates that amoebal involvement in the dispersal of a variety of vertebrate pathogens is not beyond the realms of possibility, or indeed probability. For investigation of the potential occurrence of such a role, many factors, some outlined in the following material, require consideration including possible specific (lectin-adhesin interactions) and non-specific (cell surface charge and hydrophobicity) recognition and phagocytosis initiators of *Acanthamoeba polyphaga*.

CHAPTER 4 – Factors involved in non-specific interactions

4.1 Introduction

Interactions between cells are normally subdivided into specific (lectin-adhesin interactions) and non-specific forces (cell surface charge and cell surface hydrophobicity); underpinning all such interactions, although manifest in different ways, are the same fundamental forces, including Lifshitz-van der Waals forces, electrostatic forces and Lewis acid-base interactions (Bos *et al.*, 1999).

As cells move together, such forces do not necessarily act sequentially, as they have different ranges over which they can act. Consequently, cells that are far apart will only be subject to those forces that act over long ranges. Such forces include long-range Lifshitz-van der Waals forces, which operate at a distance of approximately 50 nm and are generally attractive, in turn allowing closer contact between cells and hence other forces may come into play, such as cell surface charge and hydrophobic interactions. Specific interactions, in contrast, usually operate at a much smaller distance between cells, at around 5 nm, are more directional in their effect (Bos *et al.*, 1999) and, unlike non-specific forces, are usually considered irreversible (Smith *et al.*, 1998).

Non-specific interactions, represented by cell surface charge and hydrophobicity, were investigated with respect to the surface of *Acanthamoeba* and also polystyrene latex microspheres for consideration of the mechanisms underlying the process of phagocytosis.

4.1.1 Cell surface charge

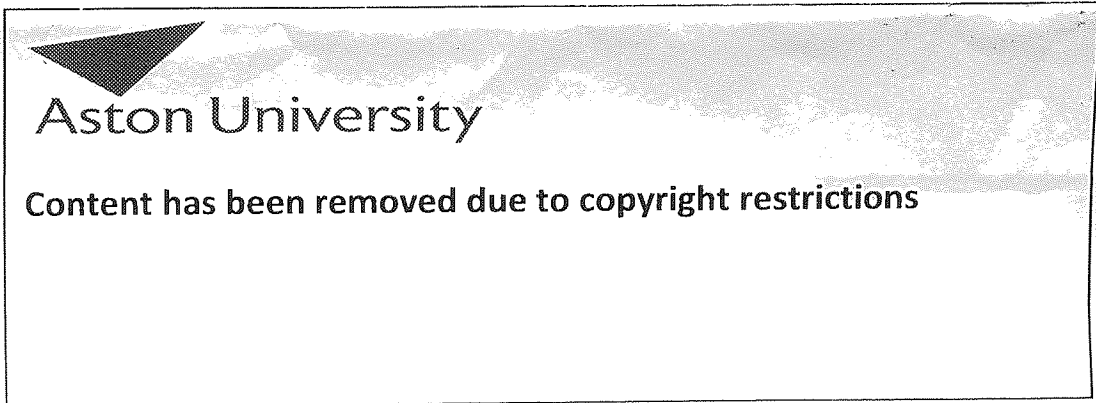
Cell surface charge is one of a number of non-specific recognition forces involved in contact between cells. This electrostatic charge occurs due to ionisation of particular moieties on a cell surface and thus its nature and extent is related to the

specific nature of the cell surface and also the pH of the surrounding medium, the temperature and ionic strength of the suspension medium (Hammer *et al.*, 1999).

As most cell surfaces are negatively charged (James, 1991), the forces involved are repellent forces, occurring at a distance between cells of approximately 2 to 20 nm. It can therefore be envisaged advantageous for cell surfaces to have a near minimal charge, so that cells can come into closer contact thereby allowing the manifestation of other recognition forces, including hydrophobic and lectin-adhesin interactions. Thus, it is apparent that there are a number of forces and other associated factors involved in the apparently simple phenomenon of contact between cells.

Cell surface charge cannot be measured directly. However, there is a measurement, known as the zeta (ζ) potential which is representative of the charge found at the actual cell surface (Figure 4.1). Surrounding the cell is a layer 0.5 nm thick known as the Stern layer, which is essentially a region of attracted ions held by either specific chemical adsorption or localised electrostatic interactions. The ζ -potential is the charge measured at the plane of shear, the point where charges associated with the cell and surrounding medium move independently, determined experimentally by electrophoretic mobility studies. Due to the effects of temperature, ionic composition and pH of the surrounding medium on cell surface charge, James (1991) recommends the measurement of ζ -potential by electrophoretic mobility studies executed at a constant 25 °C in a defined ionic strength buffer of known pH.

A range of methods can be used to measure ζ -potential, of which the most widely used is particulate microelectrophoresis (James, 1991), as used in the present study. ζ -potential was determined for *Acanthamoeba polyphaga* (Leeds strain) trophozoite and cysts, both as a function of pH and cell age. In addition, ζ -potential was determined for four further strains of *Acanthamoeba* and polystyrene latex microspheres of different size and surface chemistry. All these measurements, together with determinations of hydrophobicity, were carried out in order to elucidate some of the non-specific interactions that may be involved during phagocytosis of microspheres or microbial entities such as bacterial fish pathogens.



DISTANCE

Figure 4.1. The relationship between cell surface charge and zeta potential. Zeta potential is measured at the plane of shear found at a distance of 0.5 nm from the cell surface at the edge of the Stern layer. The plane of shear is the point at which ions associated with the cell move with the cell independently of those ions in the surrounding (bulk) solution. Taken from James (1991).

4.1.2 Cell surface hydrophobicity

If the ζ -potential of cells is not marked, then repulsion due to like charges will not be too great and further forces can progressively act, at an appropriate distance between cells. As noted, these interactions are not necessarily sequential, occurring rapidly as cells encounter each other. At a distance of 0.5 to 2 nm between cells, hydrophobic interactions are one such force, acting as a stabilising mechanism by removal of water from between cells. Cells are often defined as being either hydrophobic or hydrophilic, but, in actuality, most cells have a combination of moieties of both types on their surface, and measurement of hydrophobicity is a determination of the relative amounts of each type of moiety, termed hydrophobins and hydrophilins accordingly (Rosenberg and Kjelleberg, 1986).

The importance of surface properties, particularly hydrophobicity and charge, of phagocyte and substrate has led to the creation of the term “surface phagocytosis” and is particularly important for phagocytosis that does not involve opsonisation, such as that by amoebae (Absolom, 1986b). Bowers and Olszewski (1983) indicate that different substrates are phagocytosed by different mechanisms, with plastic microspheres attaching through hydrophobic interactions, unlike the specific lectin-like interactions involved in yeast attachment.

Several assays are available for the measurement of microbial and cell surface hydrophobicity (CSH), many based on interactions with known hydrophobic materials or chemicals (Absolom, 1986b). Success of these assays with *Acanthamoeba* was most notable with the MATH (microbial adherence to hydrocarbons) assay (Rosenberg and Doyle, 1990), a simple technique involving partition between aqueous and hydrocarbon phases, dependent on the overall CSH. Although the MATH assay was used favourably to determine the CSH of *Acanthamoeba* trophozoites and cysts and the surface hydrophobicity of microspheres, other methods for determination of hydrophobicity were attempted in the present work, to varying degrees of success, in an attempt to obtain useful comparative measurements.

Hydrophobicity determinations were carried out on *Acanthamoeba polyphaga* (Leeds strain) trophozoites and cysts of different ages, as well as on four further strains of *Acanthamoeba* and microspheres of differing size and nature. As noted, considered with measurements of ζ -potential, such hydrophobicity determinations

may provide an insight into the non-specific forces potentially involved in phagocytosis of microspheres and microbes, including bacterial fish pathogens by *Acanthamoeba* species.

4.2 Materials and Methods

4.2.1 Cell surface charge

4.2.1.1 Effect of pH on cell surface charge of *Acanthamoeba polyphaga* trophozoites

Acanthamoeba polyphaga trophozoites were prepared by inoculation of 9 ml sterile PYG broth with 1 ml confluent stock culture in sterile plastic universals that were incubated horizontally in darkness at 37 °C for 4 days. Resultant trophozoites were centrifuged at 1000 g for 10 min before resuspension in 10 ml barbital sodium acetate buffer of specific pH (Appendix 1). This procedure was repeated once before the concentration of each sample was adjusted to approximately 10^6 cells ml⁻¹.

For each pH, three replicate samples were introduced into the quartz measurement chamber of the Zetamaster PCS.V1.2 (Malvern Instruments, UK) using a 10 ml plastic syringe (Sarstedt, USA) before closure of the input valve. All measurements were made at ambient temperature. Allowance was made for movement in the suspension due to Brownian motion by a series of zero field determinations before electrophoretic mobility was determined using the technique of laser Doppler velocimetry (LDV) (or particulate microelectrophoresis) following application of a potential across the electrophoretic cell. During LDV, crossed laser beams determine the velocity of cells at a particular point, known as the stationary layer, where there is no electro-osmotic flow. Measured electrophoretic mobilities were converted into ζ -potentials by the instrument using the Smoluchowski approximation (James, 1991). The electrophoretic cell was washed through with 20 ml distilled water between each sample to flush out the previous sample.

4.2.1.2 Effect of pH on cell surface charge of *Acanthamoeba polyphaga* cysts

Four-day old *Acanthamoeba polyphaga* trophozoites were prepared in sterile plastic universals by the addition of 1 ml confluent stock culture to 9 ml sterile PYG broth before horizontal incubation in darkness at 37 °C. Cultures were centrifuged at 1000 g for 10 min before washing in 10 ml sterile encystment medium. Following a further centrifugation at 1000 g for 10 min and replacement of supernatant with 10 ml sterile encystment medium, trophozoites were induced to encyst synchronously by incubation at 37 °C for 3 days in darkness.

Resulting cysts were centrifuged at 1000 g for 10 min and washed with 10 ml barbital sodium acetate buffer, with three replicate samples for each pH. This washing procedure was repeated once before a final resuspension in 10 ml barbital sodium acetate buffer to a concentration of approximately 10^6 cells ml⁻¹. The ζ -potential of each replicate sample was then determined as detailed in Section 4.2.1.1.

Results were analysed by two factor ANOVA using the data analysis package in Microsoft® Excel 2000 to determine if there was any significant difference in *Acanthamoeba* ζ -potential associated with either morphological cell type or the pH of surrounding medium.

4.2.1.3 Effect of culture age on cell surface charge of *Acanthamoeba polyphaga* trophozoites

Cultures of *Acanthamoeba polyphaga* were set up in sterile universals by inoculation of 9 ml PYG broth with 1 ml confluent stock culture, before horizontal incubation at 37 °C in darkness for varying periods up to 12 days. After incubation, three replicate samples for each time point were centrifuged at 1000 g for 10 min and resuspended in 10 ml 1 mM KCl. This washing procedure was repeated once before resuspending in 10 ml 1 mM KCl to a cell concentration of 10^6 cells ml⁻¹, before sample pH determination. Cell surface charge of trophozoites was determined upon

three replicate samples by particulate microelectrophoresis (Section 4.2.1.1) at daily intervals for up to 12 days. Samples were discarded following measurement.

Results were analysed by single factor ANOVA using the data analysis package in Microsoft® Excel 2000 to determine whether ζ -potential of *Acanthamoeba polyphaga* trophozoites significantly differs with age.

4.2.1.4 Effect of age on cell surface charge of *Acanthamoeba polyphaga* cysts

Four-day-old cultures of *Acanthamoeba polyphaga* were prepared in the customary manner of addition of 1 ml confluent stock culture to 9 ml sterile PYG broth in sterile plastic universals, incubated horizontally at 37 °C in darkness. These cultures were centrifuged twice at 1000 g for 10 min before resuspension in 10 ml sterile encystment medium each time, before further incubation at 37 °C horizontally in darkness to induce synchronous encystment. As with trophozoites, resultant cysts were centrifuged at 1000 g for 10 min before washing in 10 ml 1 mM KCl. A second 10 min centrifugation at 1000 g allowed a final resuspension in 10 ml 1 mM KCl before pH determination of each of three replicate samples for each time point. Employing the Zetamaster as detailed previously in Section 4.2.1.1, the cell surface charge of cyst suspensions was determined from their formation (after an incubation period of 3 days) and subsequently after 1, 2, 4, 6, 8 and 12 weeks post-formation. Samples were discarded following measurement.

Results were analysed by single factor ANOVA using the data analysis package in Microsoft® Excel 2000 to determine whether ζ -potential of *Acanthamoeba polyphaga* cysts differs with age.

4.2.1.5 Cell surface charge of different strains of *Acanthamoeba*

In addition to the Leeds strain of *Acanthamoeba polyphaga* used throughout this work, four other strains of *Acanthamoeba* were purchased from the Culture

Collection of Algae and Protozoa, UK. Two strains were of the species *A. polyphaga* (CCAP 1501/3A and CCAP 1501/3B) and the other two strains were *A. castellanii* species (CCAP 1501/1A and CCAP 1501/1B).

Trophozoites from all five strains (including the Leeds strain) were cultured in sterile universals by inoculation of 9 ml PYG broth with 1 ml confluent stock culture. Universals were then incubated horizontally at 25 °C in darkness as, unlike the Leeds strain, all purchased strains failed to grow successfully at 37 °C. In addition, one set of three replicate cultures of the Leeds strain was grown at 37 °C in order to determine if growth temperature had any influence on ζ -potential. Following incubation for 4 days, amoebae were harvested by centrifugation twice at 1000 g for 10 min and resuspension in 10 ml 1 mM KCl each time. The trophozoite concentration for each strain was then adjusted to 10^6 cells ml⁻¹ by further addition of 1 mM KCl. After pH determination of each cell suspension, the ζ -potential of three replicate samples from each strain was analysed by particulate microelectrophoresis as detailed in Section 4.2.1.1.

Results were analysed by single factor ANOVA using the data analysis package in Microsoft® Excel 2000 to determine if there was any significant strain difference in *Acanthamoeba* trophozoite surface charge. In addition, single factor ANOVA was performed on ζ -potential measurements for the Leeds strain of *Acanthamoeba polyphaga* cultured at 25 °C and 37 °C in order to determine if there was any significant effect of growth temperature on trophozoite ζ -potential.

4.2.1.6 Surface charge of microspheres

As with amoebae, the surface charge of fluorescent (FITC-labelled) polystyrene latex microspheres (Polysciences, USA) was also determined. Three sizes of microspheres were used (0.5, 1.0 and 2.0 μ m) of two different surface types – plain (standard unmodified microspheres) and carboxylate-modified. For each microsphere type, a 10 ml suspension, adjusted to approximately 10^7 microspheres ml⁻¹ with sterile distilled water, was centrifuged twice at 2000 g for 10 min and resuspended in 10 ml 1 mM KCl each time, before pH determination of three replicate suspensions for each bead type and size and characterisation of ζ -potential as detailed in Section 4.2.1.1.

Results were analysed by single factor ANOVA using the data analysis package in Microsoft® Excel 2000 to determine if ζ -potential significantly differed with either size or nature of microsphere.

4.2.2 Cell surface hydrophobicity

4.2.2.1 Microbial adhesion to hydrocarbons (MATH) assay

Cultures of *Acanthamoeba* trophozoites or cysts, prepared as detailed in subsequent experimental methods, were washed and resuspended in 10 ml PUM (phosphate urea magnesium) buffer (2.22 % (w/v) K_2HPO_4 , 0.726 % (w/v) KH_2PO_4 , 0.18 % (w/v) urea, 0.02 % (w/v) $MgSO_4$, 1 L distilled water) following centrifugation twice at 1000 g for 10 min. The optical density at 470 nm (OD_{470}) of each amoebal cell suspension was adjusted to approximately 0.5 with further PUM buffer. Three replicate 1 ml aliquots of amoebal cell suspension (either trophozoites or cysts) were then transferred to small (10 × 75 mm) acid-washed glass tubes before addition of 300 μ l n-hexadecane. Samples were then left to stand for 10 min before vortexing for 45 sec, prior to a further 15 min standing period to allow aqueous and hydrocarbon phases to reappear. The bulk of the hydrocarbon phase and any associated cells were then removed by pipette before cooling to 4 °C for 15 min to allow solidification of any residual n-hexadecane. This modification, as outlined by Smith *et al.* (1998), facilitated removal, with sterile plastic inoculating loops, of all remaining hydrocarbon, thereby precluding residual hydrocarbon from compromising the optical density readings. Samples were transferred to cuvettes with sterile glass pipettes and allowed to reach room temperature before a final optical density reading was taken. Relative CSH was calculated using the following equation:

$$\text{Relative CSH (\%)} = \frac{\text{Initial } OD_{470} - \text{Final } OD_{470}}{\text{Initial } OD_{470}} \times 100$$

All recorded determinations of CSH of trophozoites and cysts of *Acanthamoeba polyphaga* in this study were carried out using the MATH assay.

4.2.2.2 Effect of age on hydrophobicity of *Acanthamoeba polyphaga* trophozoites

Amoebal trophozoites were produced in customary fashion by inoculation of 9 ml sterile PYG broth with 1 ml confluent stock culture in sterile plastic universals. Cultures were incubated horizontally at 37 °C in darkness for up to 12 days. Three replicate cultures of trophozoites were harvested daily by centrifugation at 1000 g for 10 min before resuspension in 10 ml PUM buffer. This procedure was repeated once before adjustment of the cell concentration with further PUM buffer to give an optical density of 0.5 at 470 nm. The MATH assay was then performed exactly as detailed in Section 4.2.2.1.

Results were analysed by single factor ANOVA using the data analysis package in Microsoft[®] Excel 2000 to determine whether CSH of *Acanthamoeba polyphaga* trophozoites differed significantly with age.

4.2.2.3 Effect of age on hydrophobicity of *Acanthamoeba polyphaga* cysts

Four-day-old log phase cultures of *Acanthamoeba polyphaga* were prepared by addition of 1 ml confluent stock culture to 9 ml sterile PYG broth in sterile plastic universals which were then incubated horizontally in darkness at 37 °C. Following incubation, resulting trophozoites were centrifuged at 1000 g for 10 min and resuspended in 10 ml sterile encystment medium. This procedure was performed twice before induction of synchronous encystment by subsequently re-incubating samples horizontally at 37 °C in darkness. Harvesting of cysts, with three replicates at each sample time, took place immediately after encystment was observed (following 3 days' incubation) and thereafter at intervals of 1, 2, 4, 6, 8 and 12 weeks by

centrifugation twice at 1000 g for 10 min and resuspension in 10 ml PUM buffer each time. Following adjustment to an OD₄₇₀ of 0.5 with further PUM buffer, the MATH assay (Section 4.2.2.1) was employed to determine the relative CSH of cysts.

Results were analysed by single factor ANOVA using the data analysis package in Microsoft® Excel 2000 to determine whether CSH of *Acanthamoeba polyphaga* cysts varied significantly with their age.

4.2.2.4 Hydrophobicity of different strains of *Acanthamoeba* trophozoites

The CSH of the five strains of *Acanthamoeba* (Section 4.2.1.5) was also determined. In addition to a set of three replicates of the Leeds strain grown at 37 °C in order to determine the influence, if any, of growth temperature on CSH, all five strains, also replicated three times each, were grown horizontally in darkness at 25 °C for 4 days, following an initial inoculation of 1 ml confluent stock culture to 9 ml fresh PYG broth in universals. Following incubation, trophozoites were washed in 10 ml PUM buffer following centrifugation at 1000 g for 10 min before a second 10 min centrifugation at 1000 g and resuspension in 10 ml PUM buffer. The OD₄₇₀ of the trophozoite suspension was then adjusted to 0.5 with further PUM buffer before commencing the MATH assay as detailed in Section 4.2.2.1.

Single factor ANOVA using the data analysis package in Microsoft® Excel 2000 was applied in order to determine if there was any significant difference in CSH results between *Acanthamoeba* strains. In addition, single factor ANOVA was performed on results for the Leeds strain of *Acanthamoeba polyphaga* cultured at 25 °C and at 37 °C, in order to reveal if trophozoite CSH was affected by temperature.

4.2.2.5 Hydrophobicity of microspheres

The MATH assay was also employed to determine the relative hydrophobicity of each of the different types and sizes of fluorescent polystyrene latex microspheres (Section 4.2.1.6). Three replicate 1 ml volumes of each microsphere type at a

concentration of 10^7 microspheres ml^{-1} were centrifuged at 2000 g for 10 min for washing and final resuspension in 1 ml PUM buffer, and adjusted to an OD_{470} of 0.5 with further PUM buffer, before carrying out the assay as detailed previously in Section 4.2.2.1.

Results were analysed by two factor ANOVA using the data analysis package in Microsoft® Excel 2000 to determine whether size and type of microspheres demonstrated significantly different surface hydrophobicity.

4.2.2.6 Alternative methods for determination of cell surface hydrophobicity

All the following alternative methods for the determination of cell surface hydrophobicity were performed on *Acanthamoeba polyphaga* trophozoites, cultured in the usual manner of inoculation of 9 ml sterile PYG broth with 1 ml confluent stock culture in sterile plastic universals. Experimental cultures were then incubated horizontally at 37 °C in darkness for 4 days.

4.2.2.6.1 Hydrophobicity interaction chromatography (HIC)

Four-day-old trophozoite suspensions were harvested by centrifugation twice at 1000 g for 10 min and resuspension in 10 ml PUM buffer each time. Three replicate trophozoite suspensions were enumerated by aliquotting samples into an “Improved Neubauer” haemocytometer and adjusted, if necessary, to 10^7 cells ml^{-1} with further PUM buffer.

Sepharose columns to receive trophozoite suspensions were created in sterile glass pipettes, lightly plugged with glass wool. Columns were then filled with 1 ml of either sepharose CL-4B or phenyl-modified sepharose and separately washed with four 1 ml volumes of PUM buffer before the addition of 1 ml trophozoite suspension. Samples were allowed to drain through the column and the resulting elutant collected. In order to elute physically entrapped cells, four further 1 ml volumes of PUM buffer

were introduced to each column. After collection and bulking of fractions from each column, cell concentrations eluted from each column were determined using a haemocytometer. Cell numbers were then compared before and after passing through each individual column, and also between the two column types.

4.2.2.6.2 Alternative sepharose method for determination of cell surface hydrophobicity

Due to the physical entrapment encountered with the HIC assay, an alternative sepharose method was used that dispensed with the need for columns. The method was based upon that of Mukherjee *et al.* (1993), which was used for determination of CSH of *Entamoeba*. Three replicate four-day-old trophozoite cultures were harvested by two 10 min centrifugation steps at 1000 g followed by resuspension in 10 ml PBS each time. Each sample was then adjusted to a cell concentration of approximately 2×10^4 cells ml⁻¹ with PUM buffer. Meanwhile, sepharose CL-4B and phenyl-modified sepharose were each adjusted to 10 % (v/v) concentrations with PBS before mixing of 1 ml three replicate trophozoite suspensions with 1 ml of each sepharose type in 10 × 70 mm acid-washed glass test tubes. Each sample was subsequently vortexed for 30 sec and then allowed to settle at room temperature for 5 min.

In the meantime, a discontinuous Percoll[®] density gradient was created as outlined by Avron *et al.* (1983) with stock Percoll[®] (90 % (v/v) in PBS) diluted further in PBS to concentrations of 10, 20, 30, 50, 60, 80 and 100 % (v/v) stock Percoll[®]. Each concentration was added to a 13 × 100 mm glass test tube in 1 ml volumes, with exception of 50 % (v/v) Percoll[®], of which 2 ml was added to test tubes. Each sample mixture of trophozoites with sepharose was then added to separate gradients which were then centrifuged at 2000 g for 15 min using a swing-out rotor. Those trophozoites which had attached to sepharose could be found as a band at the boundary of 10 and 20 % Percoll[®], whilst those remaining unbound were present as a band at the 40-50 % Percoll[®] boundary. An aliquot of the bands corresponding to unbound amoebae for each sepharose type was removed from each replicate sample

with a pipette and introduced to a counting chamber for enumeration thereby allowing calculation of the relative CSH according to the formula:

$$\text{Relative CSH (\%)} = \frac{\left(\text{Number of unbound amoebae in control} \right) - \left(\text{Number of unbound amoebae in treated sample} \right)}{\text{Number of unbound amoebae in control}} \times 100$$

4.2.2.6.3 Salt aggregation technique (SAT)

The salt aggregation technique (SAT) (Lindahl *et al.*, 1981) was performed on four-day-old trophozoites centrifuged twice at 1000 g for 10 min and resuspended in 10 ml 2 mM sodium phosphate buffer (2 mM Na₂HPO₄, 2 mM NaH₂PO₄, 1 L distilled water, adjusted to pH 6.8 with 1 M NH₄OH) each time, to a final concentration of 1 × 10⁷ cells ml⁻¹. A range of ammonium sulphate solutions were prepared at concentrations from 0.02 M to 0.2 M with 0.02 M intervals between each concentration and from 0.2 M to 4 M at 0.2 M intervals by dilution of a 4 M ammonium sulphate stock solution with 2 mM sodium phosphate buffer.

Three replicate 0.25 µl volumes of amoebal suspension were mixed with 0.25 µl of each respective ammonium sulphate solution on cavity slides (Superior, Germany), gently rocked for 2 min to allow precipitation. For a positive control, 0.25 µl trophozoite suspension was mixed with 0.25 µl 4 M ammonium sulphate and a negative control consisted of 0.25 µl sodium phosphate buffer with 0.25 µl trophozoite suspension. Each sample was then inspected for cellular aggregation at each concentration of ammonium sulphate, with the lowest concentration of ammonium sulphate required for aggregation being relative to a higher CSH.

4.2.2.6.4 Two phase partition (TPP)

Four-day-old trophozoites were centrifuged at 1000 g for 10 min before being resuspended in 10 ml amoebal saline to wash the cells. A further 10 min

centrifugation at 1000 g to again pellet trophozoites and allow complete removal of all residual amoebal saline was followed by uptake of trophozoites at a concentration of approximately 10^7 cells ml^{-1} in 2 ml of a polyethylene glycol (PEG) 4000 / Dextran T500 (Amersham Pharmacia Biotech, UK) phase system (7 % (w/v) PEG 4000, 7 % (w/v) Dextran T500, 0.37 % (w/v) NaCl, 0.41 % (w/v) Na_2SO_4 , 0.2 % (w/v) KH_2PO_4 , 0.0125 % (w/v) K_2HPO_4 , 100 ml distilled water). Three replicate samples were subsequently vortexed for 40 sec before phases were allowed to partition for at least 30 min before an aliquot from each phase was introduced into a counting chamber for enumeration of trophozoites present. The relative CSH was then determined by the following equation:

$$\text{Relative CSH (\%)} = \frac{\text{Cell concentration in PEG phase}}{\text{Total cell concentration in both layers}} \times 100$$

4.2.2.6.5 Hydrophobic microsphere attachment (HMA) assay

Following incubation for 4 days, trophozoites were harvested by two 1000 g centrifugation steps for 10 min and resuspension in 10 ml PUM buffer each time. Using a method based on that of Hazen and Hazen (1988), the trophozoite concentration was adjusted to 2×10^6 cells ml^{-1} before addition of the phagocytosis inhibitor cytochalasin B to a final concentration of 0.1 mM. Fluorescent (FITC-labelled) plain (unmodified) microspheres of 1 μm diameter were washed twice by centrifugation at 2000 g for 10 min and resuspension in 1 ml PUM buffer to a concentration of approximately 5×10^8 microspheres ml^{-1} before 100 μl aliquots were added to equal volumes of three replicate trophozoite suspensions in cytochalasin B in acid-washed small glass test tubes (10 \times 70 mm). After standing at room temperature for 2 min, samples were vortexed for 30 sec before immediate observation by fluorescence microscopy and enumeration using a haemocytometer. At least 100 trophozoites were counted per replicate in order to assess the proportion of trophozoites with three or more attached microspheres.

4.3 Results

4.3.1 Cell surface charge

4.3.1.1 Effect of pH on cell surface charge of *Acanthamoeba polyphaga*

Figure 4.2 shows a typical ζ -potential profile for a population of *Acanthamoeba polyphaga* (Leeds strain) trophozoites as determined using the Malvern Zetamaster. As can be seen, the population is normally distributed about a mean of approximately -5 mV and each of five replicate readings were very similar.

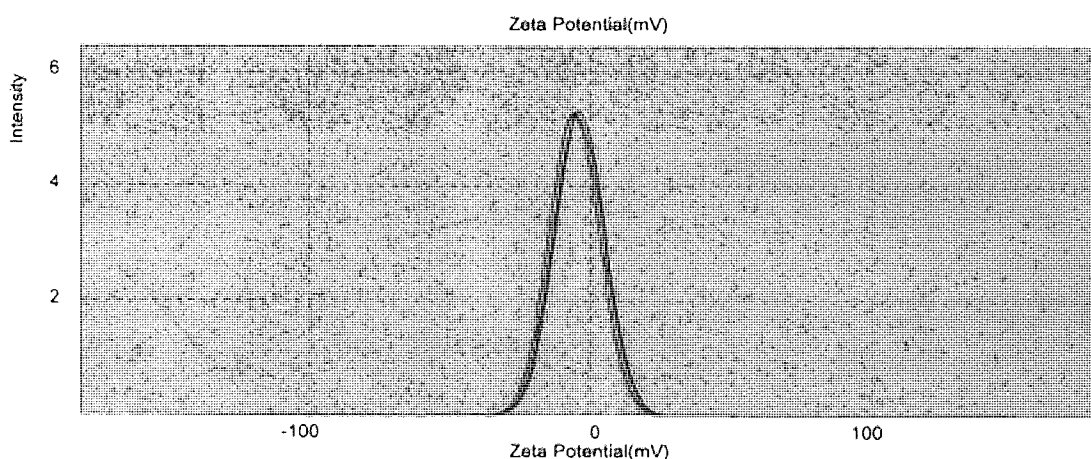


Figure 4.2. Representative ζ -potential profile for *Acanthamoeba polyphaga* (Leeds strain) trophozoites at pH 3 as determined by microelectrophoresis. Five readings can be discerned as separate, though similar, peaks of a mean ζ -potential around -5 mV.

Figure 4.3 illustrates the cell surface charge of trophozoites and cysts of the Leeds strain of *Acanthamoeba polyphaga* at different pH values. As pH increased, the ζ -potential of each of the morphological forms became more negative, due to loss of protons from the cell surface to the surrounding medium. At pH 3, the ζ -potential of both trophozoites and cysts was roughly the same at around -5 mV. However, as conditions became more basic, the ζ -potentials for trophozoites and cysts diverged. For trophozoites, the ζ -potential steadily became more negative with increasing pH, to

a value of -22 mV, whereas for cysts the increasingly negative ζ -potential trend associated with increasing pH was less marked, decreasing by only 5 mV. Two factor ANOVA indicated a significant difference between ζ -potentials of the two morphological forms ($F = 6.79$; $P = 0.012$) and also that pH had a significant effect on ζ -potential for each morphologically distinct cell type ($F = 3.49$; $P = 0.005$).

These results indicated that the isoelectric point (pI), the pH at which there is no net charge, for both trophozoites and cysts of *Acanthamoeba* was very low, falling below pH 3, although the exact pH cannot easily be determined from these results.

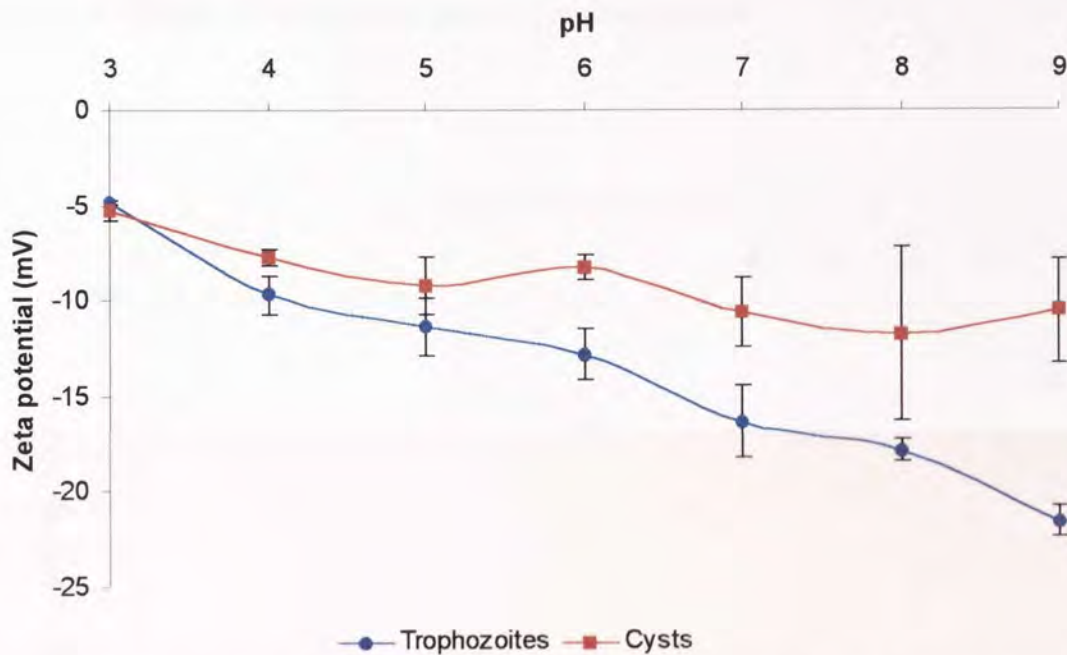


Figure 4.3. Comparison of microelectrophoresis cell surface charge determinations for *Acanthamoeba polyphaga* (Leeds strain) trophozoites and cysts suspended in barbital sodium acetate buffer at different pH values. Each point is a mean of three replicates, each themselves a mean of five microelectrophoresis determinations. Bars represent standard errors of the mean of each set of replicates.

4.3.1.2 Effect of culture age on cell surface charge of *Acanthamoeba polyphaga* trophozoites

The effect of cell age upon cell surface charge of trophozoites of the Leeds strain of *Acanthamoeba polyphaga* is shown in Figure 4.4. Determinations for time zero and the first day post-inoculation could not be made due to insufficient cell

numbers, but determinations of trophozoite ζ -potential appeared remarkably consistent over the twelve day measurement period with variation of only 1 mV about a mean of -20.9 mV, a range within the accepted normal variation of instrument performance, indicating that culture age had little effect on cell surface charge. This is supported by reference to the final measurements of -20.53 mV for stationary phase trophozoites being almost equal to the initial measurements of -20.63 mV for log phase trophozoites. Single factor ANOVA, however, indicated significant variation between results ($F = 3.67$; $P = 0.005$) and as the pH of all suspensions was around pH 5, differences in ζ -potential could not have been an effect of pH but may represent a cyclical or ill-defined biological occurrence or phenomenon.

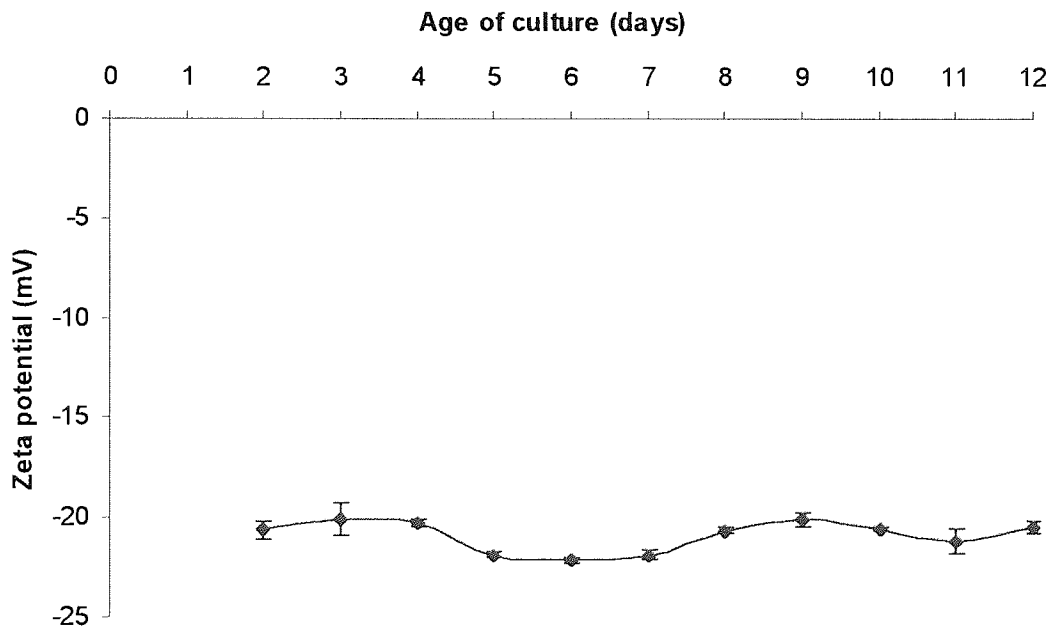


Figure 4.4. Microelectrophoresis cell surface charge determinations of *Acanthamoeba polyphaga* (Leeds strain) trophozoites suspended in 1 mM KCl, with respect to trophozoite age. Each point is a mean of three replicates, each themselves a mean of five microelectrophoresis determinations. Bars represent standard errors of the mean of each set of replicates.

4.3.1.3 Effect of age on cell surface charge of *Acanthamoeba polyphaga* cysts

Figure 4.5 represents the variation in *Acanthamoeba polyphaga* (Leeds strain) cyst ζ -potential as a function of age. There was a 4 mV range of ζ -potential measurements over the 12-week period and, in contrast to results for ζ -potential as related to pH (Section 4.3.1.1), cysts were generally found to be more negative than trophozoites with a mean of -25.9 mV, a similar value to both the initial and the final determinations. However, sample pH was determined to be around pH 6, slightly greater than that for trophozoites. Single factor ANOVA confirmed that there was no significant effect of age on ζ -potential readings ($F = 0.46$; $P = 0.836$).

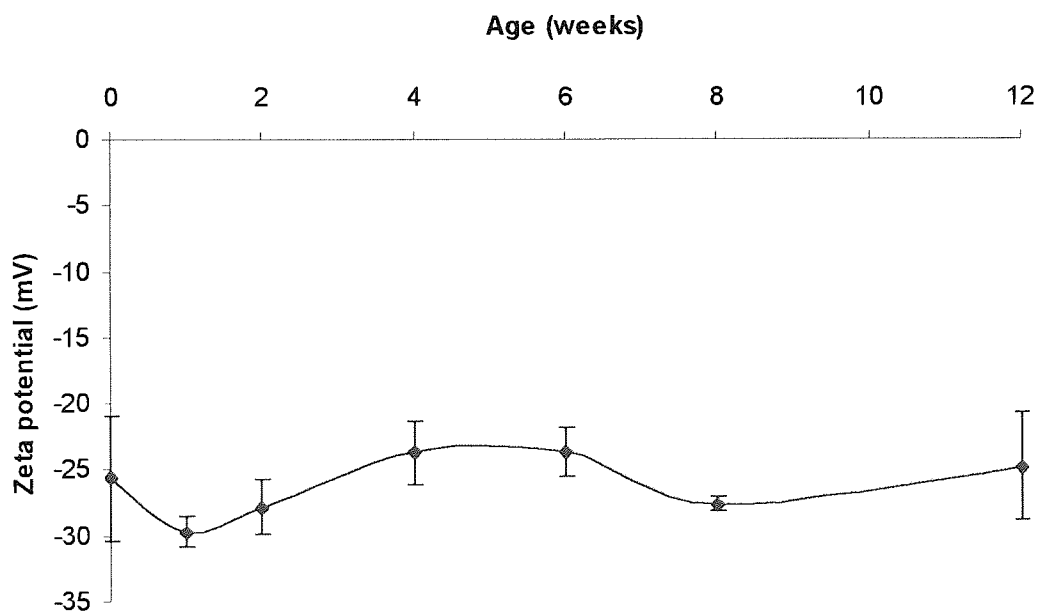


Figure 4.5. The effect of age on cell surface charge of *Acanthamoeba polyphaga* (Leeds strain) cysts, resuspended in 1 mM KCl, as determined by microelectrophoresis. Each data point is a mean of three replicates, each themselves a mean of five microelectrophoresis determinations. Bars represent standard errors of the mean of each set of replicates.

4.3.1.4 Cell surface charge of different strains of *Acanthamoeba*

Cell surface charge determination of different strains of *Acanthamoeba* can be seen in Figure 4.6. The difference between the mean ζ -potential of *A. polyphaga* (Leeds strain) grown at 25 and 37 °C was only about 2 mV, however single-factor ANOVA indicated this difference was significant ($F = 8.73$; $P = 0.042$). Comparison of the ζ -potentials for the other strains of *Acanthamoeba* at 25 °C indicated that there was also a significant difference between determinations ($F = 15.78$; $P < 0.005$), mainly resulting from the two experimental *A. castellanii* strains having more negative ζ -potentials than all three *A. polyphaga* strains measured. There was also a significant difference between the cell surface charge of the two *A. castellanii* strains ($F = 13.59$; $P = 0.021$), whereas there was no such difference between that of the three *A. polyphaga* strains ($F = 4.61$; $P = 0.061$), thus, unlike strains of *A. castellanii*, the *A. polyphaga* strains were much more uniform in their cell surface charge characteristics.

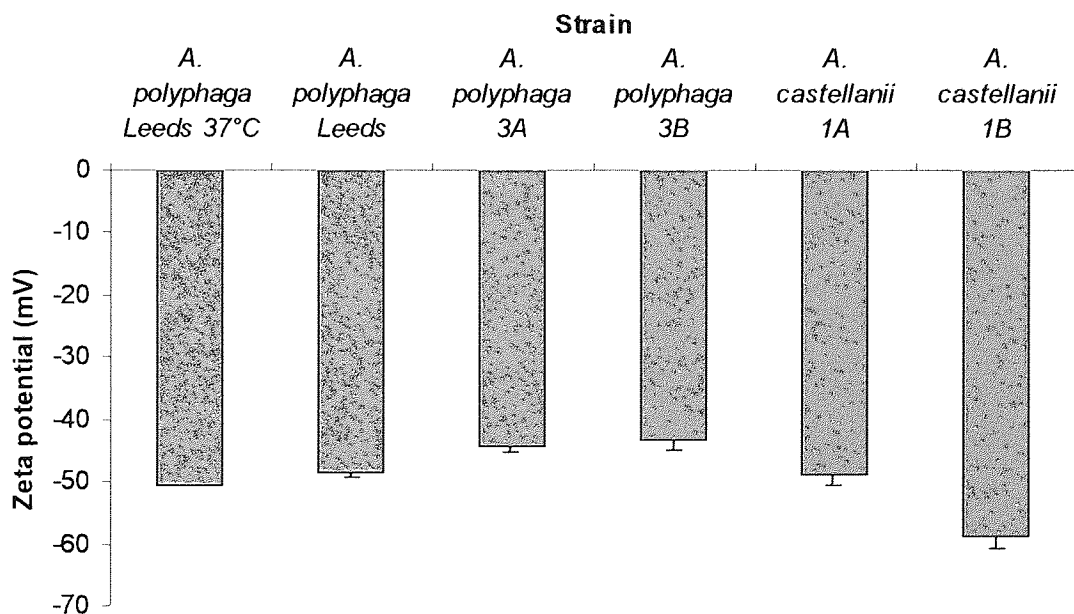


Figure 4.6. Cell surface charge of different strains of *Acanthamoeba* as determined by microelectrophoresis. All strains were grown at 25 °C, except where stated, in PYG broth, before resuspension in 1 mM KCl. Results are means of three replicates, each themselves means of five microelectrophoresis determinations with bars signifying standard errors of the mean of each set of replicates.

4.3.1.5 Surface charge of microspheres

Surface charge of polystyrene latex microspheres is shown in Figure 4.7. Generally, the charge of carboxylate-modified microspheres was, as expected, more negative than for the corresponding size of plain unmodified microspheres, though for 0.5 μm microspheres the opposite was found. Single factor ANOVA for comparison of ζ -potentials of each surface type for both 1.0 and 2.0 μm diameter microspheres revealed a significant difference ($F = 50.45$; $P = 0.002$ and $F = 13.02$; $P = 0.023$ respectively). However, for microspheres 0.5 μm in diameter, there was no significant difference in ζ -potential between the surface types ($F = 0.59$; $P = 0.485$). Statistical analysis upon the variation of ζ -potentials due to size for each type of microsphere surface revealed that determinations for plain microspheres were not significantly different ($F = 0.11$; $P = 0.892$). In contrast, those for carboxylate microspheres were significantly different ($F = 10.94$; $P = 0.010$), although there was no clear trend in microsphere ζ -potential with increasing carboxylate microsphere dimensions.

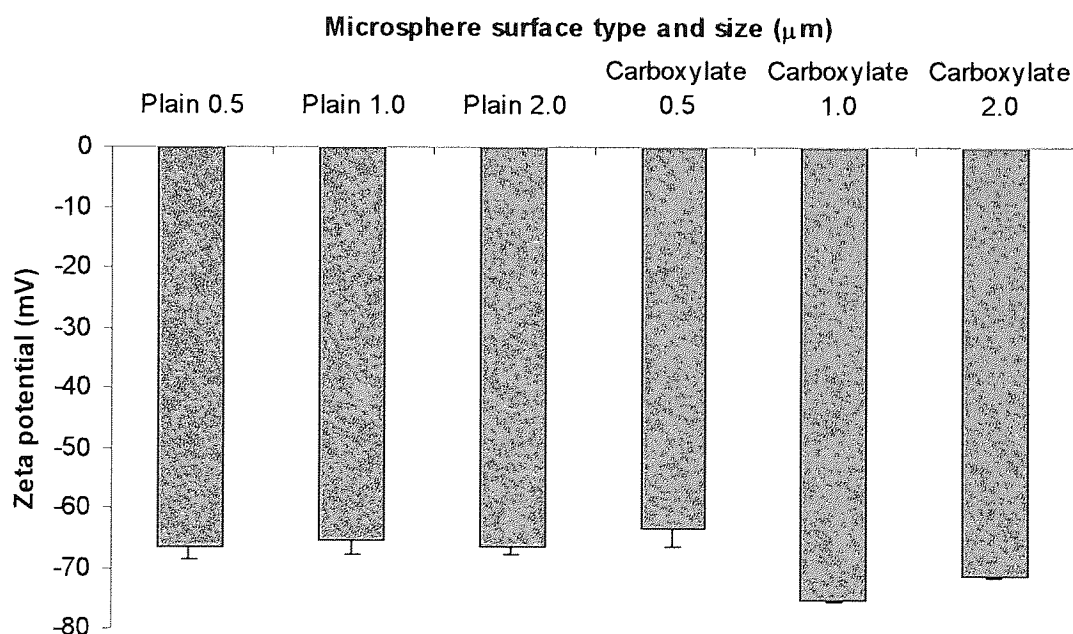


Figure 4.7. Determinations of ζ -potential for microspheres of two surface types and three sizes, suspended in 1 mM KCl, by microelectrophoresis. Results are means of three measurements with bars representing standard errors of the mean of each set of replicates.

4.3.2 Cell surface hydrophobicity

4.3.2.1 Effect of age on hydrophobicity of *Acanthamoeba polyphaga* trophozoites

Cell surface hydrophobicity measurements for trophozoites of the Leeds strain, as shown in Figure 4.8, demonstrated variation of about 7 % around a mean of 13.3 %. From these results, trophozoites could be said to possess relatively low CSH, albeit with an apparent general increase over time. No measurements were made for the first three days due to insufficient cell numbers. Single factor ANOVA indicated no significant variation of results over the measurement period ($F = 0.87$; $P = 0.566$). It must be noted, however, that relative CSH was highly variable within cell populations, as is evident from the large error bars.

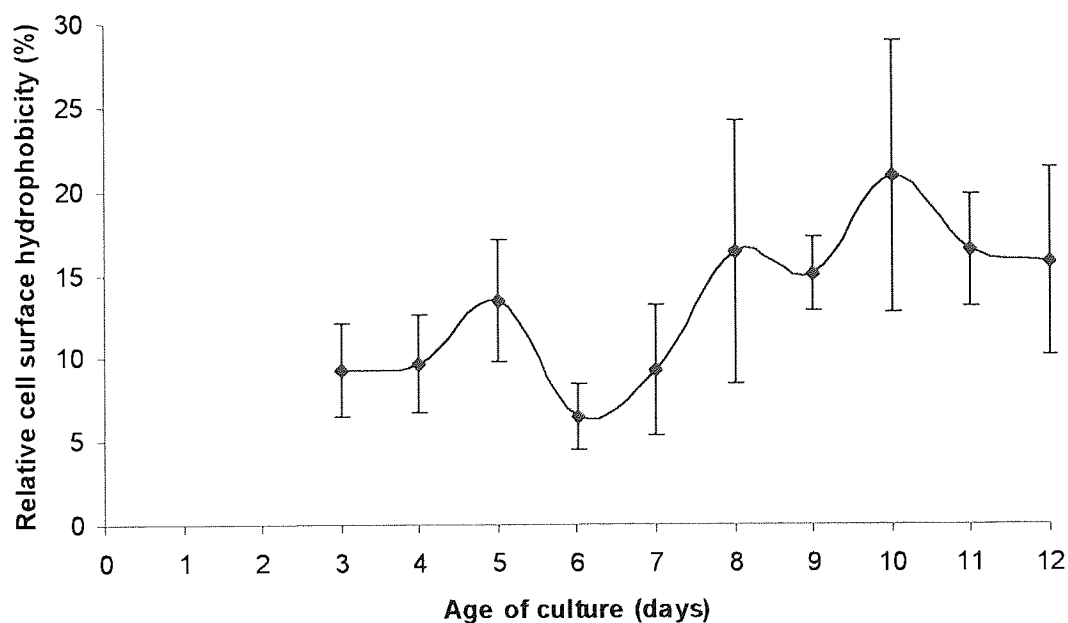


Figure 4.8. Variation of cell surface hydrophobicity of *Acanthamoeba polyphaga* (Leeds strain) trophozoites with culture age. Data points are means of three replicate determinations carried out using the MATH assay, with bars representing standard errors of the mean of each set of replicates.

4.3.2.2 Effect of age on hydrophobicity of *Acanthamoeba polyphaga* cysts

Figure 4.9 shows how CSH varied with age of cysts of *Acanthamoeba polyphaga* (Leeds strain). Differences in hydrophobicity measurements were not significant over this time span according to single factor ANOVA ($F = 2.35$; $P = 0.088$), despite an apparent increase of around 10 % over the period of study. Cyst hydrophobicity values, as for trophozoites, generally showed marked variation within replicate samples thereby, again, leading to large standard errors for all but the last sampling point.

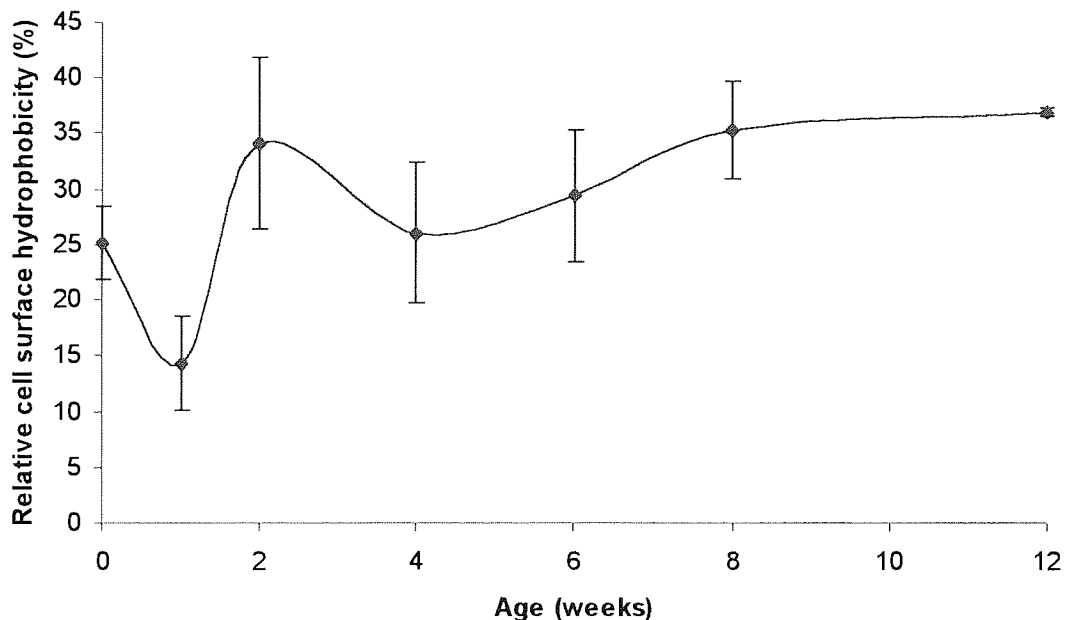


Figure 4.9. Cell surface hydrophobicity of *Acanthamoeba polyphaga* (Leeds strain) cysts of varying age, as determined by the MATH assay. Points are means of three replicate determinations, with bars representing standard errors of the mean of each set of replicates.

4.3.2.3 Hydrophobicity of different strains of *Acanthamoeba* trophozoites

The relative CSH of different strains of *Acanthamoeba* trophozoites is represented in Figure 4.10. There appeared to be little effect of temperature on the

hydrophobicity of the Leeds strain of *A. polyphaga* as very similar mean values were obtained from cultures incubated at 25 °C and 37 °C respectively. Single factor ANOVA confirmed that these results were not significant ($F = 0.01$; $P = 0.912$).

Determinations of CSH for each strain were, however, found to be significantly different from each other ($F = 17.26$; $P < 0.001$). In contrast to results for ζ -potential, CSH determinations for strains of *A. polyphaga* were significantly different ($F = 39.42$; $P < 0.001$) with these strains showing the greatest and least CSH, while CSH determinations for *A. castellanii* demonstrate no significant difference ($F = 3.57$; $P = 0.088$), due at least in part to the considerable standard error values associated with each set of replicates. Thus from these results, there seemed to be little correlation between *Acanthamoeba* trophozoite CSH and ζ -potential.

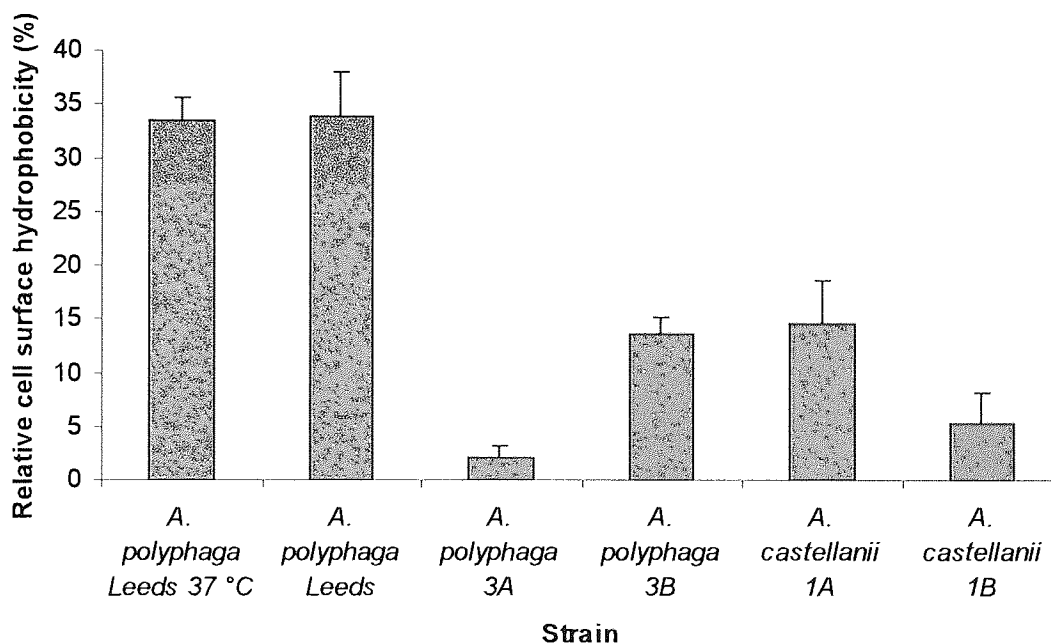


Figure 4.10. Relative cell surface hydrophobicity of different strains of *Acanthamoeba*, as determined by the MATH assay. All strains were grown at 25 °C, except where stated. Results are means of three replicate determinations with bars representing standard errors of the means of replicate samples.

4.3.2.4 Hydrophobicity of microspheres

Figure 4.11 illustrates the surface hydrophobicity of microspheres of two surface types with three sizes for each. Two factor ANOVA indicated that, generally, there was a significant difference between determinations for both microsphere size ($F = 10.31$; $P = 0.003$) and for surface type ($F = 4.04$; $P = 0.028$), with smaller microspheres generally more hydrophobic than larger microspheres and plain microspheres generally also more hydrophobic than their carboxylate counterparts. As can be seen from Figure 4.11, the hydrophobicity of plain microspheres appeared to show a trend of decreasing hydrophobicity with increasing microsphere size; in contrast such a trend is not apparent for carboxylate microspheres.

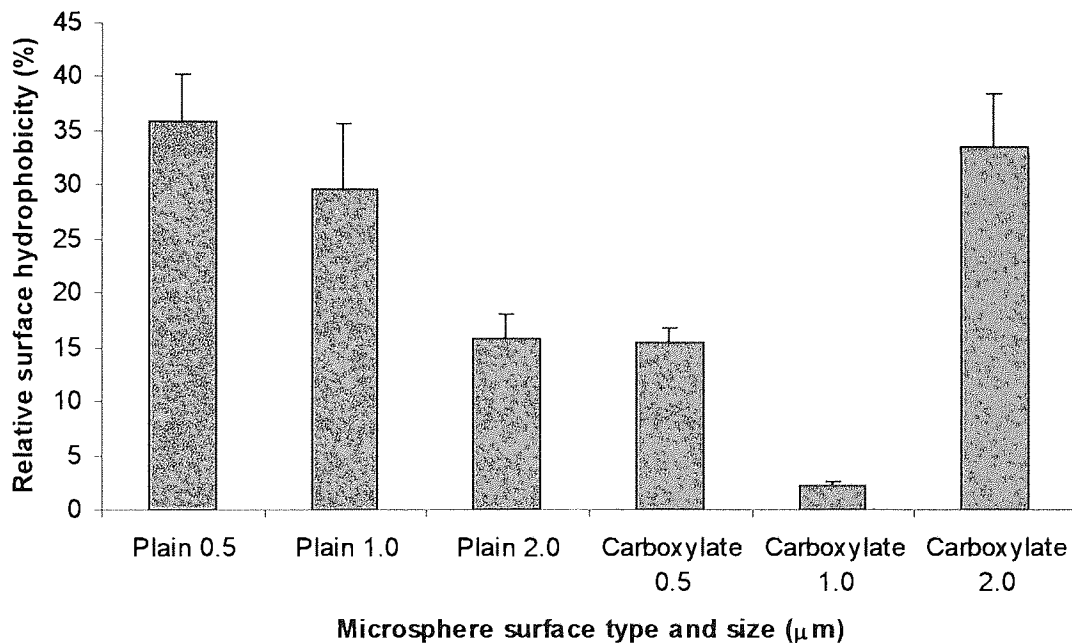


Figure 4.11. Relative surface hydrophobicity of microspheres of two surface types and three sizes, as determined by the MATH assay. Results are means of three measurements with bars representing standard errors of the mean of each set of replicates.

4.3.2.5 Alternative methods for determination of cell surface hydrophobicity

4.3.2.5.1 Hydrophobicity interaction chromatography (HIC)

Use of HIC for investigation of hydrophobicity was prevented by physical entrapment of *Acanthamoeba* trophozoites in both sepharose columns, due to the relatively large size of amoebal cells. This resulted in each suspension appearing devoid of all trophozoites following elution.

4.3.2.5.2 Alternative sepharose method for determination of cell surface hydrophobicity

Greater success was apparently achieved by the batch modification of the sepharose method. However, there was difficulty in achieving sufficient quantities of trophozoites in the bands associated with the discontinuous Percoll[®] gradient and, consequently removal of sufficient trophozoites for successful enumeration.

4.3.2.5.3 Salt aggregation technique (SAT)

The SAT was not a viable method for the determination of relative CSH of *Acanthamoeba* due to autoaggregation of trophozoites in both controls. Potentially, this could be due to a high CSH value, but this is not consistent with results from the MATH assay.

4.3.2.5.4 Two phase partition (TPP)

The main difficulty with the TPP method was that trophozoites of *Acanthamoeba* appeared to partition very poorly between the two phases, thus the identification of amoebae in either phase was subjective. There was also an issue of insufficient cell numbers, which, in turn, made any enumeration difficult.

4.3.2.5.5 Hydrophobic microsphere attachment (HMA) assay

On assessment, this final method was, again, unsuccessful. In this case, however, numbers of trophozoites were not a problem, rather that there was a difficulty in identifying any amoebae with attached microspheres, which was surprising as *Acanthamoeba* is normally avidly phagocytic (Davies *et al.*, 1991), hence the use, as noted above, of a phagocytosis inhibitor which would only interfere with uptake not attachment. All in all, the failure of these alternative methods meant that the MATH assay was the only suitable method for determination of the hydrophobicity of *Acanthamoeba*.

4.4 Discussion

4.4.1 Cell surface charge

Cell surface charge, as shown by all test strains irrespective of incubation regime, results from the presence of charged moieties, particularly amino, carboxyl and phosphate groups, on the cell surface (James, 1991) with the major determinants of a negative cell surface charge being phosphate groups (Bayer and Sloyer, 1990). The increasingly negative character of both *Acanthamoeba polyphaga* (Leeds strain) trophozoites and cysts concomitant with increasing pH is indicative that there is no

single component responsible for cell surface charge, which would be manifest as a plateau of negative determinations (James, 1991). Nor are there just two or three ionogenic groups involved, rather it is quite likely that a complex variety of surface groups exist losing protons to the medium at different pH values.

Negativity of cyst cell surfaces with increasing pH was less pronounced than for trophozoites, with cysts demonstrating ζ -potentials nearer neutrality in comparison with trophozoites, a similar finding to that of Przełęczka and Perkowska (1986), who determined the ζ -potential of *Acanthamoeba castellanii* cysts to be neutral, at pH 7.2. Furthermore, some of their determinations revealed cysts to have a positive ζ -potential, which is unusual, if not unique, for cell surfaces (James, 1991). Although species variation is a possibility, their measurements were made by direct microscopic observation of microelectrophoretic cell mobility, rather than by the use of laser Doppler velocimetry, hence were potentially less accurate.

The effect of pH on cell surface charge is an important, but also dynamic, factor as aquatic habitats, either natural or artificial, in which amoebae and microbes are found, naturally encompass varied pH ranges. In addition, the pH of soils and sediments where such entities are also found can be diverse. Therefore, environmental pH should have a bearing on cell surface charge of organisms present and consequently will influence the nature and extent of cell-cell interactions. Based upon these results, *Acanthamoeba* would rarely encounter environments acidic enough to render their surface neutral or even positively charged, hence one would expect to find them expressing varying negativity under natural circumstances, which in turn may modulate their interaction with other aquatic entities.

The variation of ζ -potential of *Acanthamoeba polyphaga* (Leeds strain) with increasing age was significant for trophozoites, but not for cysts. In the case of the latter, this was unsurprising as, being dormant structures, cysts potentially undergo little apparent change once formed. For trophozoites, however, although apparently statistically significant, there was no appreciable difference between ζ -potentials of *Acanthamoeba polyphaga* trophozoites at any age. This result indicates that although there was an apparent "normal" cell surface charge at approximately -20.5 mV, there was variation about this value, great enough to mask any trend. As noted, the measurement of cell surface charge was applied to a population of cells, so any determination was not just related to one such cell. The distribution of ζ -potentials

was, for the most part, narrow (see Figure 4.2), although replicate samples often gave rise to different determinations. One may potentially expect some change in surface composition, hence cell surface charge, with trophozoite maturation, such as observed by Przełęczka and Sobota (1982), who found a difference between log and stationary phase trophozoites with regard to binding of ruthenium red, cationised ferritin and Concanavalin A, with less binding occurring in stationary phase cells, just before encystment. However, as ζ -potential is a measurement of overall net cell surface charge, any change in the number of negatively charged surface moieties could be matched by an equivalent change in the number, distribution and strength of positively charged entities, thus although the ζ -potential has apparently remained unchanged, there may be fewer moieties to which highly charged molecules can bind.

As cell surface charge usually acts between cells in a repellent manner, cells of lower surface charge would be more likely to encounter other cells possessing similar charge. The cyst of *Acanthamoeba polyphaga*, as with other protozoa, is a dormant stage of the cell, brought on naturally when facing either nutritional deprivation or adverse environmental conditions (Yang and VILLEMEZ, 1994). In turn, excystment may occur when conditions become more favourable, such as when digestible bacteria are encountered (Larkin and Easty, 1990). Thus it would seem reasonable to speculate that a lower cell surface charge would appear advantageous for a large single-celled inert entity, promoting contact with whole prey cells or smaller, charged chemical moieties, thereby inducing excystment. Cell surface charge is inherently related to the surface composition, so any difference in cyst or trophozoite morphology and cell surface charge is related to the apparently less complex surface nature of cysts, composed largely of cellulose in addition to proteins and lipids, in contrast to lipophosphoglycan found in trophozoite plasma membranes (Bowers and Korn, 1974; Robin *et al.*, 1988). Bowers and Korn (1974) found that the majority of charged moieties on the trophozoite surface resulted from the complex lipophosphoglycan rather than from the lipids and proteins of the plasma membrane.

Despite trophozoites being relatively more negative than cysts, hence at a potential disadvantage when attempting cell contact, the importance of cell surface charge for such cells possessing appendages such as pseudopodia, may be diminished or even largely irrelevant in phagocytosis events (van Oss, 1978), as Gorlin *et al.* (1995) postulate that, although the overall surface of amoebae is usually negative, trophozoite pseudopodia may be of varying charge. Knowledge of the

physico-chemistry of such appendages is still limited (Bos *et al.*, 1999). The extension of such structures, however, can potentially overcome electrostatic repulsion, due to their smaller radius of curvature compared to that of a cell taken as a whole (Bos *et al.*, 1999). Absolom (1986b) also notes that this phenomenon may account for the fact that no correlation between ζ -potential and phagocyte-bacteria interaction has been possible.

Carboxylate-modified microspheres were, as expected, generally more negative than their equivalent sized plain counterparts. All microspheres of both surface natures, irrespective of size, were determined to have ζ -potentials that were more negative than that of any trophozoite or cyst of any *Acanthamoeba* strain. One may expect an increase in negativity with microsphere diameter, particularly with carboxylate-modified microspheres, as an increase in diameter corresponds with an increase in surface area, thus there may be a greater array of charged moieties on the surface (Müller, 1991a). However, no clear correlation was found in this respect, although determinations agree with those of Vinogradova and Yakubov (2001) who indicate that ζ -potentials of polystyrene latex spheres in 1 mM KCl are usually in the region of -80 ± 15 mV. Furthermore, studies by Hammer *et al.* (1999) found 1 μ m carboxylate microspheres to have a ζ -potential of -60.8 mV at pH 7.8, which is not too dissimilar from that obtained in the present study. In addition, they found 4 μ m carboxylate microspheres to have a ζ -potential of -107.0 mV, consistent with an effect of increasing surface area leading to increased charge. Roser *et al.* (1998) found a relationship between charge of surface-modified nanospheres and phagocytosis by macrophages *in vitro*, with charged particles, especially those positively charged, preferentially engulfed compared to particles with a zero ζ -potential. If such an observation also holds for *Acanthamoeba*, then all microsphere types used in the present study should be taken up, although one would expect other factors to be involved in phagocytosis events.

The cell surface charge of the fish pathogens *Renibacterium salmoninarum*, *Aeromonas salmonicida* and *A. hydrophila* is largely unknown, although Austin and Adams (1996) report that, for *Aeromonas salmonicida*, virulent bacteria are negatively charged and avirulent bacteria positive. The cell surface charge of such bacteria can influence the likelihood of their engulfment by amoebae, in such a way that the lower the charge the more likely it is that infection of amoebae will occur in the same way as infection of fish macrophages.

4.4.2 Cell surface hydrophobicity

Cell surface hydrophobicity, like cell surface charge, is a manifestation of cell surface nature, in turn related to cell morphology and composition. The greater CSH of *Acanthamoeba polyphaga* (Leeds strain) cysts relative to trophozoites may thus be a reflection of the high cellulose component of cyst cell walls in contrast to the lipophosphoglycan rich trophozoite plasma membrane (Bowers and Korn, 1974).

The results obtained by the MATH assay indicated that there was no marked alteration in trophozoite CSH with *Acanthamoeba polyphaga* maturation, although considerable variation was observed amongst individual age CSH determinations. Hence any foundation to a trend of gradually increasing hydrophobicity with ageing of cells (Figure 4.8) is not wholly supported. However, Przełęczka and Perkowska (1986) showed that during transition from log to stationary phase, trophozoites undergo surface changes leading to different binding properties for certain reagents, including Con A, cationised ferritin and ruthenium red. It is thus not unlikely that hydrophobicity of surfaces will change during maturation, particularly as cysts appear to have a greater cell surface hydrophobicity. Although some preparation and readjustment of trophozoites for encystment is likely, such alteration of CSH may not occur until during the rapid encystment process, when significant morphological changes occur.

In common with trophozoites, there appeared to be a trend of increasing hydrophobicity corresponding with increasing cyst age, however the evidence garnered from the MATH assay again does not fully support such a premise as the results obtained could statistically have arisen by chance, especially as cyst populations also demonstrated considerable variation. Furthermore, unlike trophozoites, CSH of cysts would not be expected to alter greatly, being dormant amoebal forms with little in the way of metabolism taking place (Yang and Villemez, 1994). It is thus unlikely that, once formed, further morphological or surface changes would occur. However the greater cyst CSH in comparison to that of trophozoites could be of some advantage to amoebae, as, with increased hydrophobicity, the potential for binding to food organisms may be elevated, in turn promoting excystment.

The MATH assay should more correctly be envisaged as a technique for measuring adhesion to a negatively charged hydrophobic surface (van der Mei *et al.*, 1997), as both hydrophobic and electrostatic interactions are involved. As such, the pH of the assay conditions becomes a factor, with the pI of most hydrocarbons between pH 2 and 4 (Bos *et al.*, 1999). Results for cysts, though not trophozoites, appeared to demonstrate some relationship between changes in ζ -potential and changes in CSH, in that a more negative ζ -potential partially corresponded with a higher cell surface hydrophobicity. Similarly, a more positive cyst ζ -potential appeared to be associated with a lower cell surface hydrophobicity. Such an inverse relationship is consistent with theoretical considerations of a greater surface charge generally increasing the potential for polar interactions with proximal water molecules (Rosenberg and Doyle, 1990), though any apparent correlations are hard to prove and may, in fact, be coincidental. Indeed, as ζ -potential is the net charge of various surface moieties, these most likely consist of both positive and negative groups, which will generally decrease the overall hydrophobicity (Rosenberg and Doyle, 1990), thus a low net negative ζ -potential could correspond with a low CSH. Hence, one should be careful before claiming any such correlation, where, in fact, there may be none.

Jones *et al.* (1996) suggest that relative CSH determinations below 30 % indicated cells to be highly hydrophilic. Employing such a criterion, few of the measurements of CSH in this study suggest cells under investigation to be hydrophobic, let alone highly hydrophobic (CSH above 70 % (Jones *et al.*, 1996)). Based purely on these results, one might therefore expect a reduced interaction with microspheres or bacterial prey, due to the very low hydrophobicity manifest amongst test strains. However the relationship of hydrophobicity, cell interaction and phagocytosis is quite complex, with van Oss (1978) demonstrating that bacteria which manifest greater CSH than an engulfing mammalian phagocyte were more easily phagocytosed and vice versa. Furthermore, Lock *et al.* (1987) found that hydrophilic *Escherichia coli* avoided uptake by human phagocytes, but not by *Acanthamoeba castellanii*, which engulfed all bacterial phenotypes investigated. However, Matz and Jürgens (2001) point out that such apparently hydrophilic bacteria may still be more hydrophobic than the amoebae, in which case engulfment would be expected to occur. Indeed, such a suggestion is not contrary to the relatively low CSH determinations of amoebal trophozoites in this study.

Although the wide variation found amongst determinations of CSH by MATH assays may represent heterogeneity in the amoebal population, such variation may arise from the use of this particular assay for CSH determination. Pembrey *et al.* (1999) recently concluded that, although use of n-hexadecane in the MATH assay was the least modifying hydrocarbon available, the MATH assay was ultimately unsuitable for measurement of cell surface hydrophobicity. Indeed, in their critique of cell surface analysis techniques, Pembrey *et al.* (1999) noted that cell preparation protocols always had an effect on the surface nature of the sample cells, regardless of the assay employed and as such, this represents a major problem with cell surface analysis techniques. Of the several methods currently available for determination of CSH, most have been developed for bacteria (Absolom, 1986b; Rosenberg and Kjelleberg, 1986; Rosenberg and Doyle, 1990), therefore, it is perhaps unsurprising that the majority have proven unsuitable for measurement of this surface property in amoebae. Despite these limitations, however, the MATH assay provided the only means by which such determinations could be made and as such is of value.

It is also important to bear in mind that all these assays only determine relative hydrophobicity, rather than actual values (Rosenberg and Kjelleberg, 1986). This means that results cannot always be directly compared with other readings (Hazen and Hazen, 1988), unless determined by the same assay, performed in a commonly defined manner. The degree of correlation between methods is not always robust, which can be problematic (Rosenberg and Doyle, 1990). One reason for this may be that biological cells, as noted, typically carry both hydrophobins and hydrophilins on their surface and some assays measure the surface as a whole, whereas others may measure hydrophobicity of particular surface regions. Determinations by the MATH assay are mediated by surface adhesion and, as such, represent CSH of surface regions. The relatively large size of *Acanthamoeba* trophozoites and cysts would potentially carry a greater number of hydrophobins and hydrophilins, such that different surface regions may carry completely different CSH properties. Depending on the orientation of each cell, adhesion to n-hexadecane may be elevated or diminished, potentially accounting for the wide variation observed in results.

Characterisation of microsphere hydrophobicity, employing similar protocols to those followed in the evaluation of biological moieties, demonstrated that plain microspheres manifest a trend of decreasing hydrophobicity with increasing size, consistent with studies of Müller (1991b). However, such a trend was not evident for

carboxylate microspheres, as the largest (2.0 μm) size demonstrated the most hydrophobic character of the three test sizes. In the main, plain microspheres appeared more hydrophobic than carboxylate-modified microspheres, which is not surprising, given that carboxylate groups are hydrophilic. Thus one could speculate, based upon hydrophobicity results, that plain microspheres are more likely to interact with phagocytic and predatory amoebae such as *Acanthamoeba*.

Reports concerning *Renibacterium salmoninarum* and *Aeromonas* species have indicated high relative CSH for these bacteria (Bandín *et al.*, 1989; Austin and Adams, 1996), which potentially has a bearing on both their uptake by amoebae and virulence (Deere *et al.*, 1997; Bruno, 1988). The high CSH of *Aeromonas salmonicida* arises from the relatively uniform A-layer which largely contains hydrophobic and uncharged amino acids (Trust *et al.*, 1983) and, unlike those that possessed the A-layer, *Aeromonas* mutants lacking the A-layer did not interact with murine macrophages *in vitro* (Austin and Adams, 1996). Bandín *et al.* (1995) indicate that *Renibacterium salmoninarum* could be phagocytosed by trout macrophages in the absence of opsonising serum factors, most likely due to hydrophobic interactions. Also of importance is the observation of Daly and Stevenson (1987) that the marked hydrophobicity of *Renibacterium* would cause the bacterium, if external to salmonid fish, to leave the water column and attach to surfaces. Such an effect, also noted for *Aeromonas salmonicida* by Deere *et al.* (1997), would also increase the potential for encounter with *Acanthamoeba* or other protozoa. Given such information and the apparent marked hydrophobicity of fish pathogens relative to *Acanthamoeba*, it seems that such phenomena would facilitate engulfment of pathogenic fish bacteria by *Acanthamoeba*. However, the attribution of phagocytic mechanisms to hydrophobic interactions alone may be something of an oversimplification as alluded to above, as a specific receptor-based interaction, as yet ill defined, may have a central role (Brattgjerd *et al.*, 1994).

Overall, only one method was suitable at least from an experimental and execution standpoint for determination of CSH of *Acanthamoeba*. Development of alternative methods for CSH measurement of *Acanthamoeba* may well therefore be useful for further CSH characterisation, especially given the apparent drawbacks of all current CSH assays whatever the biological cell type under investigation (Pembrey *et al.*, 1999). However the determinations of CSH in *Acanthamoeba polyphaga*, together with measurements of ζ -potential can be used in a predictive or speculative manner to

at least recognise some of a possible myriad particle interactions. In addition, specific surface factors, namely lectin-adhesin interactions were examined in the present study to provide a fuller picture of surface-mediated phenomena before phagocytosis investigations were made.

CHAPTER 5 – Factors involved in specific interactions

5.1 Introduction

Previous comment has noted that the interaction between cells is not necessarily a straightforward process involving a limited array of factors. When cells come into closer proximity, various forces act upon the cells in either repulsive or attractive ways. However, any such repulsion is often not too great to be overcome by attractive forces, such as interactions between hydrophobic moieties, and specific adhesive mechanisms can come into play, usually at a distance between cells of less than 0.5 nm. Such mechanisms include adhesin and lectin interactions and can be regarded as irreversible, unlike non-specific interactions (Smith *et al.*, 1998). A lectin is defined as “a carbohydrate-binding protein other than an enzyme or an antibody”, having specificity for the carbohydrate to which it binds (Doyle, 1994). These structures can be found widely on cell surfaces as they are ubiquitous in all life forms, potentially interacting with specific carbohydrate receptors found on complementary cell surfaces (Doyle, 1994), hence commercial lectins, particularly fluorescein isothiocyanate (FITC) conjugated lectins, can be used to characterise these interactions (Handley, 1991).

In order to identify the carbohydrates present on the cell surface of both trophozoites and cysts of *Acanthamoeba polyphaga*, a panel of 21 FITC-conjugated lectins of varying carbohydrate specificity was used in binding experiments. Previous studies with lectins have shown that they are useful tools for the identification of cell surface carbohydrates (Ribeiro *et al.*, 1997), but visualisation can be a problem (Mendgen *et al.*, 1985). In view of this, the technique of flow cytometry was employed to determine fluorescence. This technique is more suitable due to the quantitative nature not found with simple visualisation by microscopy alone and also the greater sample numbers that can be analysed. During flow cytometry, cells are passed, single file, through an argon laser beam to excite the FITC label attached to the lectin allowing detection. Therefore trophozoites or cysts that have bound to such lectins will have a detectable level of fluorescence that can be measured by the flow cytometer.

Before characterisation of surface carbohydrates of *Acanthamoeba polyphaga* (Leeds strain) trophozoites and cysts, however, a suitable lectin concentration was determined to ensure saturation of all available binding sites on the surface using a range of Concanavalin A (Con A) concentrations. Con A, a lectin isolated from *Canavalia ensiformis* (jack bean) (Doyle, 1994), specifically binds to α -mannose and α -glucose residues, which are ubiquitous amongst biological cell types. Thus, there was a potentially marked likelihood of Con A binding to *Acanthamoeba*, far more perhaps than other lectins. In turn a suitable lectin concentration based on that for Con A was established for use with the panel of lectins. In addition, the specificity of Con A towards *Acanthamoeba polyphaga* was investigated using blocking haptens.

Due to the different amounts of FITC conjugated to each lectin, comparison of fluorescence would only give a rough idea of the relative binding of each lectin and, consequently, also the relative amounts of carbohydrate exposed on the amoebal cell surface. Indeed, the only true comparison that could be made was between morphological forms with each lectin. In order to obtain a clearer picture of the way in which some of the different lectins bound to *Acanthamoeba*, assays were conducted based upon ligand-receptor studies in order for the calculation of K_d and B_{max} values of the lectins used with *Acanthamoeba* following construction of concentration curves. These values enable a more meaningful comparison between lectins as they reveal both the concentration of lectin required for saturation of 50 % of the available binding sites for that lectin (K_d , also known as EC50) and the maximum fluorescence obtained once full saturation has been achieved (B_{max}), from which relative B_{max} , a value relative to the amount of FITC present, was calculated. The lectins chosen for this in depth study were, firstly, those that bound to mannose residues as their primary target (Con A, LCA and PSA) and, secondly, those that were found to bind to one morphological form significantly greater than the other with a easily discernible level of fluorescence (RCA₁₂₀, SBA, VVA and WGA).

These studies allowed the identification of carbohydrates on the surface of *Acanthamoeba polyphaga* trophozoites and cysts and their relative abundance. Such information provides insight into the potential specific interactions involved in any interactions with microspheres or microorganisms during phagocytosis.

5.2 Materials and Methods

5.2.1 Lectin binding to *Acanthamoeba*

5.2.1.1 Determination of lectin concentration for saturation of *Acanthamoeba polyphaga* trophozoite binding sites

FITC-Con A (Vector Laboratories, USA) was prepared to give final concentrations of 5, 10, 20, 30 and 40 $\mu\text{g ml}^{-1}$ by dilution in supplemented sterile 10 mM HEPES buffer (10 mM HEPES, 0.15 M NaCl, 0.1 mM CaCl_2 , 0.01 mM MnCl_2 , pH 7.5, filter sterilised through a 0.2 μm pore-size nitrocellulose filter (Millipore Corporation, USA)).

Cultures of *Acanthamoeba polyphaga* (Leeds strain) were prepared in sterile plastic universals by inoculation of 9 ml fresh PYG broth with 1 ml confluent stock culture before horizontal incubation in darkness at 37 °C. After four days, each culture, consisting of log phase trophozoites, was centrifuged at 1000 g for 10 min, before resuspension in 10 ml supplemented HEPES buffer. This washing procedure was repeated twice with a final resuspension in 1 ml supplemented HEPES buffer before transfer to a sterile Eppendorf tube (Sarstedt, USA). The cell concentration was adjusted to approximately 10^7 cells ml^{-1} before three replicate 100 μl aliquots for each sample were dispensed into sterile Eppendorf tubes. A 200 μl volume of lectin was added to each replicate sample to the appropriate concentration before incubation in the dark at 4 °C for 30 min. In addition, a negative control consisting solely of amoebae in 300 μl supplemented 10 mM HEPES buffer was set up to determine background autofluorescence. Following incubation, each sample was twice centrifuged at 1000 g for 10 min and resuspended in 1 ml non-supplemented 10 mM HEPES / 0.15 M NaCl buffer each time, before a final 10 min centrifugation at 1000 g and resuspension in 300 μl non-supplemented 10 mM HEPES / 0.15 M NaCl with 1 % (w/v) paraformaldehyde to fix the sample. Samples were then stored in darkness to prevent photobleaching of the fluorochrome at 4 °C until analysis by flow cytometry using the Becton Dickinson FACS 440 housed at Birmingham University

Medical School. Fluorescence was determined by the flow cytometer using a 200 mW argon laser (with an excitation wavelength of 488 nm and emitted light detector of 530 nm (± 15 nm), adjusted to a fixed channel using standard Brite Microspheres (Coulter, USA) prior to determining fluorescence). Each sample was briefly vortexed before introduction to filtered PBS sheath fluid and fluorescence was measured from a population of 10 000 cells. The resulting measurements of each sample enabled construction of a concentration curve, from which the FITC-Con A concentration that gave the maximum fluorescence could be determined. This lectin concentration was subsequently used to assess the binding of a lectin panel.

Results were analysed by single factor ANOVA using the data analysis package of Microsoft[®] Excel 2000 to determine if increasing the concentration of FITC-Con A had any significant effect in increasing fluorescence of samples.

5.2.1.2 Confocal microscopy of FITC-Con A binding to *Acanthamoeba polyphaga* trophozoites

Acanthamoeba polyphaga (Leeds strain) trophozoites were labelled with 30 $\mu\text{g ml}^{-1}$ FITC-Con A in the same manner as detailed in Section 5.2.1.1. Instead of fixing, however, 5 μl aliquots were placed on microscope slides and immediately viewed. Images were acquired with a Zeiss Axiovert/Bio-Rad MCR 1024 OS laser scanning confocal microscope facility, utilising Laser Sharp 2000 software (Bio-Rad Laboratories, USA). A 100 mW argon laser, excitation wavelength 488 nm, was moderated by a series of neutral density filters, gain, offset and zoom functions, used to optimise image fluorescence intensity, contrast and composition. Slides were viewed with Plan-NEOFLUAR Ph 1 10 \times /0,30, Plan-NEOFLUAR Ph 2 40 \times /0,75 and Plan-NEOFLUAR Ph 3 100 \times /1,30 oil immersion objectives and subject material was subsequently captured as 512 \times 512 pixel images in turn converted from Bio-Rad PICT to TIFF format. Each sample could only be imaged once due to photobleaching of fluorescence by the laser.

5.2.1.3 Binding specificity of Con A to *Acanthamoeba polyphaga* trophozoites

The specificity of Con A binding was determined using the haptens methyl- α -D-mannopyranoside and methyl- α -D-glucopyranoside for which Con A demonstrates marked affinity. Separate 10 ml aliquots of each hapten at a final concentration of 200 mM in supplemented 10 mM HEPES were each incubated with 10 ml FITC-Con A at 20 $\mu\text{g ml}^{-1}$. In addition, both haptens together, each at a concentration of 200 mM in supplemented 10 mM HEPES, were also incubated with 10 ml FITC-Con A at 20 $\mu\text{g ml}^{-1}$. Each sample, with three replicates, was incubated in darkness at 4 °C for 30 min. Following incubation with each hapten, FITC-Con A was added at a final concentration of 10 $\mu\text{g ml}^{-1}$ to samples of four-day-old *Acanthamoeba polyphaga* (Leeds strain) trophozoites in replicates of three. These cultures were prepared in the usual manner of inoculation of 9 ml PYG broth with 1 ml confluent stock culture in sterile plastic universals, subsequently incubated horizontally in darkness at 37 °C for 4 days. Trophozoites were harvested by centrifugation at 1000 g for 10 min before resuspending in 10 ml supplemented 10 mM HEPES. This washing procedure was repeated once before a final resuspension in 1 ml supplemented 10 mM HEPES to a final concentration of 10^7 cells ml^{-1} . In addition, a negative control containing 200 μl washed *Acanthamoeba* trophozoites with 100 μl supplemented 100 mM HEPES in place of lectin and a positive control consisting of 200 μl washed *Acanthamoeba* with 100 μl FITC-Con A, which had not been exposed to any haptens, at a final concentration 10 $\mu\text{g ml}^{-1}$ were also prepared with replication. All samples were then further incubated in darkness at 4 °C for 30 min, before centrifugation at 1000 g for 10 min and washing in 1 ml non-supplemented 10 mM HEPES / 0.15 M NaCl buffer. This procedure was performed twice before finally centrifuging at 1000 g for 10 min and fixing in 300 μl non-supplemented 10 mM HEPES / 0.15 M NaCl with 1 % (w/v) paraformaldehyde. Samples were then stored in darkness at 4 °C until analysis by flow cytometry using the Becton Dickinson FACS 440 of Birmingham University Medical School.

Results were analysed by single factor ANOVA using the data analysis package of Microsoft[®] Excel 2000 to determine if there was any significant decrease in FITC-Con A binding due to each blocking hapten.

5.2.1.4 Lectin binding to *Acanthamoeba polyphaga* trophozoites

Following determination of $10 \mu\text{g ml}^{-1}$ as a suitable overall lectin concentration as derived from a concentration curve based upon results obtained by experimentation of Section 5.2.1.1, a panel of 21 lectins (Vector Laboratories, USA) with varying specificities (Table 5.1) was used to determine the presence or otherwise of interacting cell surface carbohydrate moieties on four-day-old *Acanthamoeba polyphaga* (Leeds strain) trophozoites. Amoebal cultures of 10 ml were set up in sterile plastic universals by inoculation of 9 ml fresh PYG broth with 1 ml confluent stock culture of *Acanthamoeba polyphaga*. Universals were incubated horizontally in darkness at 37°C for 4 days. The resulting log phase trophozoites were harvested by centrifugation at $1000 g$ for 10 min before resuspension in supplemented 10 mM HEPES. This washing procedure was repeated twice before a third 10 min centrifugation at $1000 g$ and resuspension in 1 ml supplemented 10 mM HEPES before adjusting trophozoite numbers to 10^7 cells ml^{-1} . Three replicate samples of amoebae for each lectin were aliquotted in 200 μl volumes before addition of 100 μl lectin in supplemented 10 mM HEPES to a final concentration of $10 \mu\text{g ml}^{-1}$. For negative controls, 100 μl supplemented 10 mM HEPES buffer was added in place of lectin in order to determine background autofluorescence. All samples were incubated at 4°C for 30 min before being centrifuged at $1000 g$ for 10 min and resuspended in 1 ml non-supplemented 10 mM HEPES / 0.15 M NaCl buffer twice before a final centrifugation at $1000 g$ for 10 min and fixing in 300 μl non-supplemented 10 mM HEPES / 0.15 M NaCl with 1 % (w/v) paraformaldehyde. Samples were then stored in darkness at 4°C until analysis by flow cytometry using the Becton Dickinson FACS 440 housed at Birmingham University Medical School.

Table 5.1. Sources of lectins used and their carbohydrate binding specificity (Vector Laboratories, 1999).

Lectin	Description / Source	Carbohydrate specificity
--------	----------------------	--------------------------



Aston University

Content has been removed due to copyright restrictions

5.2.1.5 Lectin binding to *Acanthamoeba polyphaga* cysts

Four-day-old *Acanthamoeba polyphaga* (Leeds strain) cultures were prepared in the usual manner by the inoculation of 9 ml PYG broth with 1 ml confluent stock culture in universals which were incubated horizontally in darkness at 37 °C. These cultures were then induced to undergo encystment by centrifugation at 1000 g for 10 min, washing in 10 ml sterile encystment medium, before a further 10 min centrifugation at 1000 g and resuspension in 10 ml encystment medium. Samples were re-incubated for 3 days at 37 °C in darkness. As with trophozoites, cysts were centrifuged at 1000 g for 10 min and resuspended in 10 ml supplemented HEPES buffer. This washing procedure was repeated once before a final 10 min centrifugation at 1000 g and resuspension in 1 ml supplemented 10 mM HEPES in sterile Eppendorf

tubes. Cyst numbers were then adjusted to 10^7 cells ml^{-1} before lectin binding. Each of the panel of 21 lectins was added separately in 100 μl volumes to three 200 μl replicate cyst samples to a final lectin concentration of $10 \mu\text{g ml}^{-1}$ before incubating in darkness at 4°C for 30 min. Samples were then centrifuged twice at 1000 g for 10 min and washed twice in 1 ml non-supplemented 10 mM HEPES / 0.15 M NaCl buffer. Finally, the samples were centrifuged at 1000 g for 10 min before fixing in 300 μl non-supplemented 10 mM HEPES with 1 % (w/v) paraformaldehyde. Samples were then stored at 4°C until analysis by flow cytometry using the Becton Dickinson FACS 440 of Birmingham University Medical School.

Results were analysed by single factor ANOVA using the data analysis package in Microsoft[®] Excel 2000 to determine the difference in binding between the two morphological stages of *Acanthamoeba* for each lectin.

5.2.2 Characterisation of lectin binding to *Acanthamoeba polyphaga*

Each FITC-lectin investigated (Con A, LCA, PSA, RCA₁₂₀, SBA, VVA and WGA), was diluted in supplemented 10 mM HEPES buffer to give final concentrations of 0.5, 1, 2, 2.5, 5, 10 and 20 $\mu\text{g ml}^{-1}$, in addition to a negative control with 10 mM supplemented HEPES in place of lectin for determination of background autofluorescence, for creation of concentration curves for both four-day-old log phase trophozoites and newly prepared cysts of *Acanthamoeba polyphaga* (Leeds strain). Trophozoites were prepared in the usual manner of 9 ml fresh PYG broth inoculated with 1 ml confluent stock culture in plastic universals incubated horizontally in darkness for 4 days at 37°C . Trophozoites were harvested by centrifugation of the culture vessels at 1000 g for 10 min and resuspension in 10 ml supplemented 10 mM HEPES buffer twice before a final 10 min centrifugation at 1000 g and resuspension in 1 ml supplemented 10 mM HEPES in Eppendorf tubes. Trophozoites were adjusted to 10^7 cells ml^{-1} before commencement of lectin binding assays.

Cysts were induced from four-day-old log phase trophozoites, prepared in exactly the same manner as outlined above, by replacement of PYG broth with 10 ml encystment medium following centrifugation at 1000 g for 10 min. This procedure was conducted twice before re-incubating the samples horizontally in darkness at

37 °C for a further 3 days. Cysts were then harvested in the same way as for trophozoites, as outlined above, before adjusting the concentration to 10⁷ cells ml⁻¹.

FITC-lectin binding for both trophozoites and cysts was performed by exactly the same method; addition of 100 µl lectin aliquots at the appropriate final dilution to three replicates of 200 µl *Acanthamoeba polyphaga* samples. These were then incubated at 4 °C for 30 min before centrifugation at 1000 g for 10 min twice and resuspension in 1 ml non-supplemented 10 mM HEPES / 0.15 M NaCl each time. Finally the samples were centrifuged at 1000 g for 10 min before fixing in 300 µl 1 % (w/v) paraformaldehyde in non-supplemented 10 mM HEPES / 0.15 M NaCl. Samples were then stored at 4 °C until flow cytometric analysis on the Becton Dickinson FACS 440 of Birmingham University Medical School.

Calculation of K_d and B_{max} for each individual lectin experiment was performed by GraphPad Prism[®] (v. 3.02) (GraphPad Software Inc., USA) using the standard one-site binding theoretical curve analysis tool, by the following equation:

$$\text{Bound (B)} = \frac{B_{\text{max}} \times [\text{Lectin}]}{K_d + [\text{Lectin}]}$$

where B_{max} is the maximum fluorescence upon saturation of all binding sites, K_d is the lectin concentration that produces 50 % of maximum saturation and [Lectin] is lectin concentration. However, as B_{max} values arise from fluorescence, they are still related to the amount of FITC bound to each mole of lectin (the specific activity), which is different for each lectin. Thus, to enable valid comparison of B_{max} values, they were divided by the specific activity for each lectin (Table 5.2) to give a relative B_{max}, and then normalised relative to VVA binding to *Acanthamoeba polyphaga* trophozoites.

5.3 Results

5.3.1 Lectin binding to *Acanthamoeba*

5.3.1.1 Determination of lectin concentration for saturation of *Acanthamoeba polyphaga* trophozoite binding sites

Typical representative flow cytometry profiles of FITC-lectin binding to *Acanthamoeba polyphaga* (Leeds strain) are shown in Figure 5.1. Such profiles make apparent the homogeneity or heterogeneity of acanthamoebal populations. Autofluorescence of *Acanthamoeba* trophozoites and cysts was homogeneous in nature (data not shown). However, populations were often heterogeneous for lectin binding as, typically, despite a general level of binding within a cell population, there was often a subpopulation with greater lectin avidity, seen as a shoulder on the descending tail of the peak. Mean fluorescence readings for each cell population were determined from such profiles for construction of lectin binding curves.

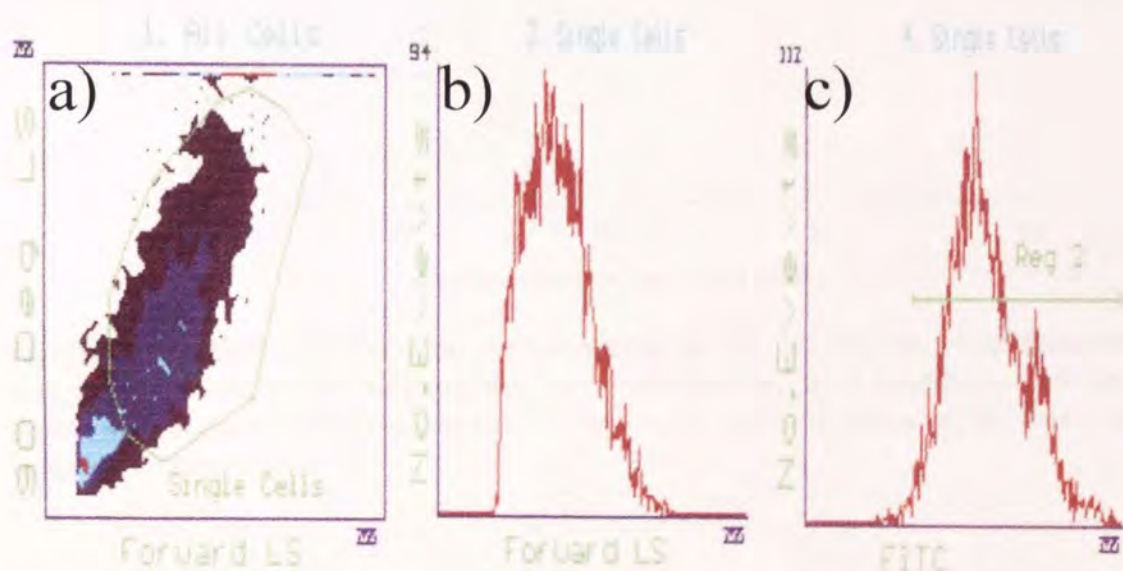


Figure 5.1. Representative flow cytometry profiles of Con A binding to *Acanthamoeba polyphaga* (Leeds strain) trophozoites. a) 2D plot of 90° light scatter against forward light scatter from which a region corresponding to single cells was selected for b) and c); b) Distribution of single cell population according to forward light scatter; c) Distribution of single cell population according to fluorescence.

The effect of increasing FITC-Con A concentration, potentially to saturate binding sites of *Acanthamoeba polyphaga* (Leeds strain) trophozoites, is represented in Figure 5.2. Increasing the concentration of Con A up to $5 \mu\text{g ml}^{-1}$ resulted in marked cell fluorescence, indicating that *Acanthamoeba* has Con A binding sites on its surface which are not fully saturated unless concentrations are increased to at least $10 \mu\text{g ml}^{-1}$ FITC-Con A. Above this Con A concentration, there were only minimal increases in fluorescence, suggesting that binding sites were saturated. Single factor ANOVA confirmed that increasing the concentration of Con A had a significant effect on fluorescence levels ($F = 35.29$; $P < 0.001$). From these results, a lectin concentration of $20 \mu\text{g ml}^{-1}$ was selected for further experimentation to encourage near saturation of potential binding sites of *Acanthamoeba polyphaga* for a panel of lectins of varying carbohydrate specificity.

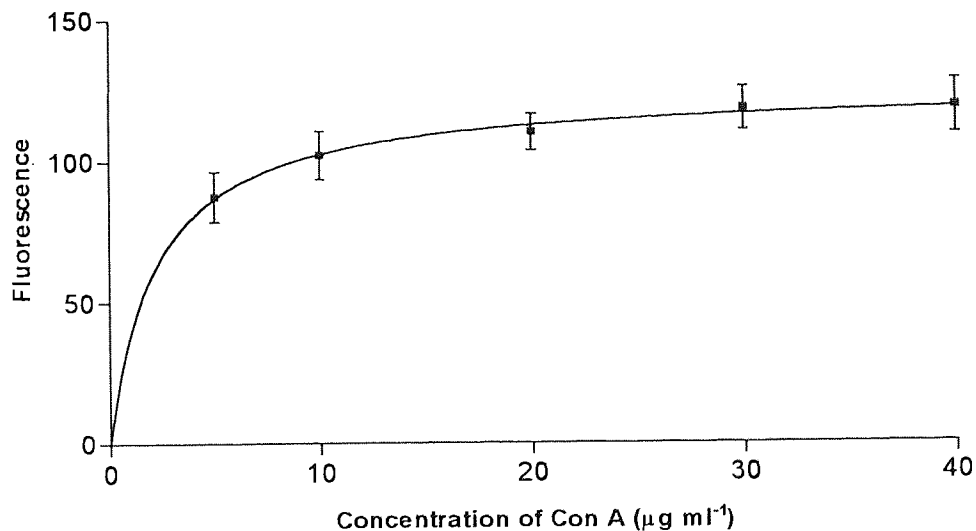


Figure 5.2. Effect of FITC-Con A concentration on binding to *Acanthamoeba polyphaga* (Leeds strain) trophozoites, as determined by flow cytometry. All data points are means of three replicates. Bars represent standard errors of the mean for triplicate measurements.

5.3.1.2 Confocal microscopy of FITC-Con A binding to *Acanthamoeba polyphaga* trophozoites

Figure 5.3 shows FITC-Con A binding (at $30 \mu\text{g ml}^{-1}$) to *Acanthamoeba polyphaga* (Leeds strain) trophozoites, as viewed by confocal microscopy. Con A can be seen to bind to much of the trophozoite surface with localisation of binding in more marked concentrations.

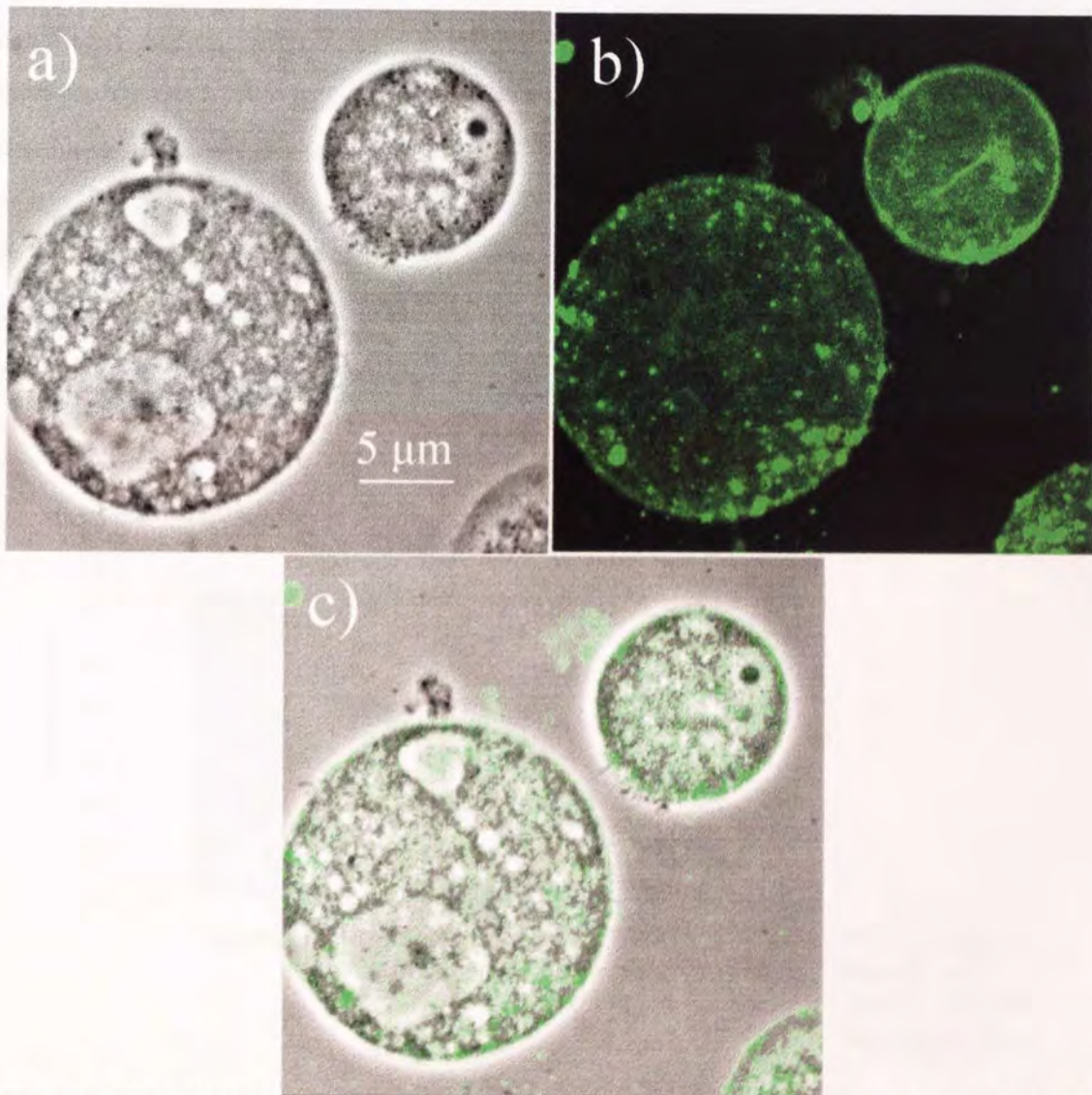


Figure 5.3. FITC-Con A binding to *Acanthamoeba polyphaga* (Leeds strain) trophozoites, as viewed by confocal microscopy. a) Transmission image, b) Fluorescence image, c) Composite of transmission and fluorescence. All images are to the same scale, as indicated in a).

5.3.1.3 Binding specificity of Con A to *Acanthamoeba polyphaga* trophozoites

As shown in Figure 5.4, the blocking haptens, methyl- α -D-mannopyranoside and methyl- α -D-glucopyranoside (mannose and glucose analogues respectively), either individually or in combination, prevented FITC-Con A binding to *Acanthamoeba polyphaga* (Leeds strain) trophozoites. In each case, sample fluorescence was only a little above that associated with trophozoite autofluorescence. Single factor ANOVA revealed, firstly, that the binding of Con A to trophozoites was significant ($F = 199.73$; $P < 0.001$) and that compared to the background autofluorescence, there was no significant increase in fluorescence in the presence of methyl- α -D-mannopyranoside ($F = 0.60$; $P = 0.481$), methyl- α -D-mannopyranoside ($F = 0.06$; $P = 0.821$) or a combination of the two blocking haptens ($F = 0.03$; $P = 0.864$), hence the blocking sugars prevented FITC-Con A binding to trophozoites, confirming the specificity of Con A to both α -mannose and α -glucose moieties.

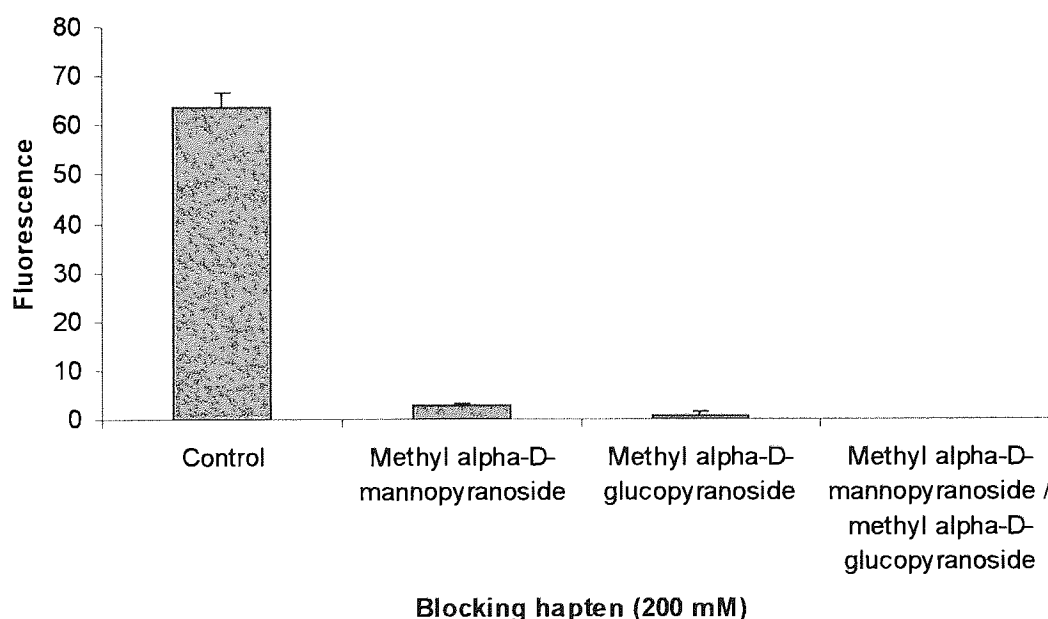


Figure 5.4. The effect of blocking haptens on the binding of FITC-Con A to *Acanthamoeba polyphaga* (Leeds strain) trophozoites, as determined by flow cytometry. Results are means of three replicates with bars representing standard errors of the mean for each set of replicates.

5.3.1.4 Lectin binding to *Acanthamoeba polyphaga* trophozoites and cysts

Binding of a FITC-labelled lectin panel to trophozoites and cysts of *Acanthamoeba polyphaga* (Leeds strain) can be seen in Figure 5.5.

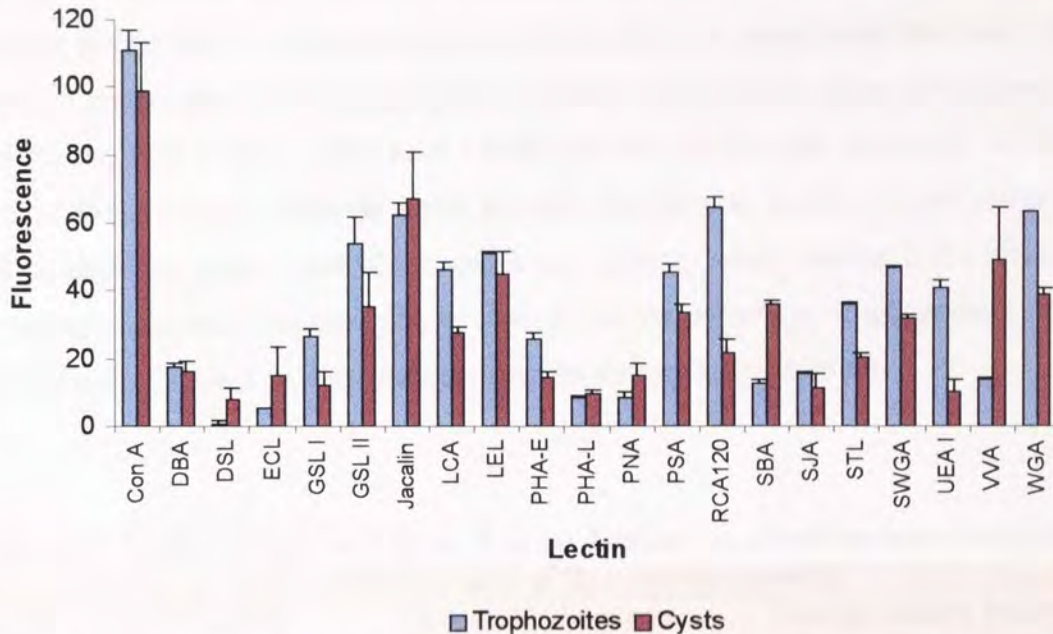


Figure 5.5. Relative binding of a FITC-labelled lectin panel to *Acanthamoeba polyphaga* (Leeds strain) trophozoites and cysts, as measured by flow cytometry. Results are means of three replicates. Bars represent standard errors of the mean of three replicates.

Comparison of individual FITC-lectin binding to trophozoites and cysts of *Acanthamoeba*, and associated single factor ANOVA revealed significant differences in trophozoite and cyst avidity for GSL I ($F = 8.17$; $P = 0.046$), LCA ($F = 32.24$; $P = 0.005$), PHA-E ($F = 19.41$; $P = 0.012$), PSA ($F = 11.70$; $P = 0.027$), RCA₁₂₀ ($F = 54.37$; $P = 0.002$), STL ($F = 217.64$; $P < 0.001$), SWGA ($F = 82.29$; $P = 0.001$), UEA I ($F = 45.72$; $P = 0.002$) and WGA ($F = 5.228$; $P = 0.084$), all with greater binding to trophozoites than to cysts. Some difference in binding with morphological form could also be attributed to SBA and VVA, appearing to bind preferentially to cysts, although single factor ANOVA values of $P = 0.060$ and $P = 0.092$, respectively, indicated no significant difference in lectin affinity between morphological forms.

However, for these and other lectins, the third replicate cyst sample was found to have a somewhat lower fluorescence, for ill-defined reasons, thus lowering mean cyst fluorescence values and enhancing replicate variation.

At first sight, it would appear that some lectins, such as Con A, Jacalin or RCA₁₂₀, bind very well to *Acanthamoeba polyphaga* in either or both morphological forms. Conversely, other lectins, such as DSL, PHA-L or PNA, appear not to bind well at all to either amoebal form. However, due to variation in specific activity amongst lectins, direct comparisons could not be made, as some lectins that bind well, such as Con A, often have a large specific activity. Furthermore, those that appear to bind poorly, such as DSL, often have a small specific activity and, conversely, a lectin such as WGA with a relatively small specific activity was found to bind markedly well to both cell types. Such observations are apparent when ranking lectin binding, according to fluorescence intensity, to *Acanthamoeba polyphaga* trophozoites (Table 5.2) and cysts (Table 5.3), alongside the specific activity for each lectin.

Table 5.2. Lectin specific activity and lectin binding to *Acanthamoeba polyphaga* (Leeds strain) trophozoites ranked in order of fluorescence intensity.

Lectin	Carbohydrate specificity	Lectin binding (mean fluorescence)	Specific activity (moles FITC: moles lectin)
Con A	α -man; α -glc	110.73	8.5
RCA ₁₂₀	β -gal	64.64	7.0
WGA	(glcNAc) ₂ ; neuNAc	63.38	2.5
Jacalin	methylated α -gal	62.28	3.1
GSL II	glcNAc	53.78	3.8
LEL	(glcNAc) ₃	50.78	5.8
SWGA	(glcNAc) ₂ ; neuNAc	46.82	2.1
LCA	α -man	46.34	5.5
PSA	α -man	45.90	3.1
UEA I	α -L-fuc	40.76	4.0
STL	(glcNAc) ₃	36.13	2.9
GSL I	α -gal; α -galNAc	26.81	7.0
PHA-E	oligosaccharide	25.74	4.6
DBA	α -galNAc	17.11	5.7
SJA	β -galNAc	15.43	8.4
VVA	galNAc	13.76	3.8
SBA	galNAc	12.4	4.1
PHA-L	oligosaccharide	8.59	4.6
PNA	β -gal(1,3)galNAc	8.55	6.8
ECL	β -gal(1,4)glcNAc	5.34	3.8
DSL	β -gal(1,4)galNAc	0.57	2.1

Table 5.3. Lectin specific activity and lectin binding to *Acanthamoeba polyphaga* (Leeds strain) cysts ranked in order of fluorescence intensity.

Lectin	Carbohydrate specificity	Lectin binding (mean fluorescence)	Specific activity (moles FITC: moles lectin)
Con A	α -man; α -glc	99.01	8.5
Jacalin	methylated α -gal	67.14	3.1
VVA	galNAc	48.98	3.8
LEL	(glcNAc) ₃	45.08	5.8
WGA	(glcNAc) ₂ ; neuNAc	39.05	2.5
SBA	galNAc	36.23	4.1
GSL II	glcNAc	35.45	3.8
PSA	α -man	33.74	3.1
SWGA	(glcNAc) ₂ ; neuNAc	31.66	2.1
LCA	α -man	27.37	5.5
RCA ₁₂₀	β -gal	21.33	7.0
STL	(glcNAc) ₃	20.69	2.9
DBA	α -galNAc	16.31	5.7
ECL	β -gal(1,4)glcNAc	14.77	3.8
PNA	β -gal(1,3)galNAc	14.74	6.8
PHA-E	oligosaccharide	14.36	4.6
GSL I	α -gal; α -galNAc	12.16	7.0
SJA	β -galNAc	11.24	8.4
UEA I	α -L-fuc	10.46	4.0
PHA-L	oligosaccharide	9.50	4.6
DSL	β -gal(1,4)glcNAc	8.07	2.1

Although direct comparisons between lectins cannot be made, it appears that both trophozoites and cysts of *Acanthamoeba* do indeed have a low affinity for some lectins, such as DSL, ECL, PHA-L, PNA and SJA particularly. Con A, conversely, as might be expected, appears to have the greatest degree of binding, although further in depth studies along the lines of ligand receptor studies can help to reveal the true nature of such postulations.

5.3.2 Characterisation of lectin binding to *Acanthamoeba polyphaga*

As noted, direct comparison of fluorescence values to contrast binding between lectins is not possible; however one way this can be achieved is by calculation of K_d and B_{max} values for the lectins used with *Acanthamoeba*. These values enable a more meaningful comparison between lectins as they reveal both the

concentration of lectin required for saturation of 50 % of the available binding sites for that lectin (K_d , also known as EC_{50}) and the maximum fluorescence obtained once full saturation has been achieved (B_{max}). Such values, with the addition of relative B_{max} (i.e. relative to the specific activity of the lectin), were calculated for all mannose-specific lectins as well as other lectins that showed marked binding to one morphological form over the other. Binding curves for Con A to *Acanthamoeba polyphaga* (Leeds strain) trophozoites and cysts are shown in Figure 5.6. As can be seen, for both morphological types, there was initially a considerable increase in fluorescence concomitant with small increases in Con A concentration, which gradually became less marked, though still related to lectin concentration. For cysts, the initial increase in fluorescence was more marked than for trophozoites, but the maximal fluorescence was roughly equal for both morphological forms.

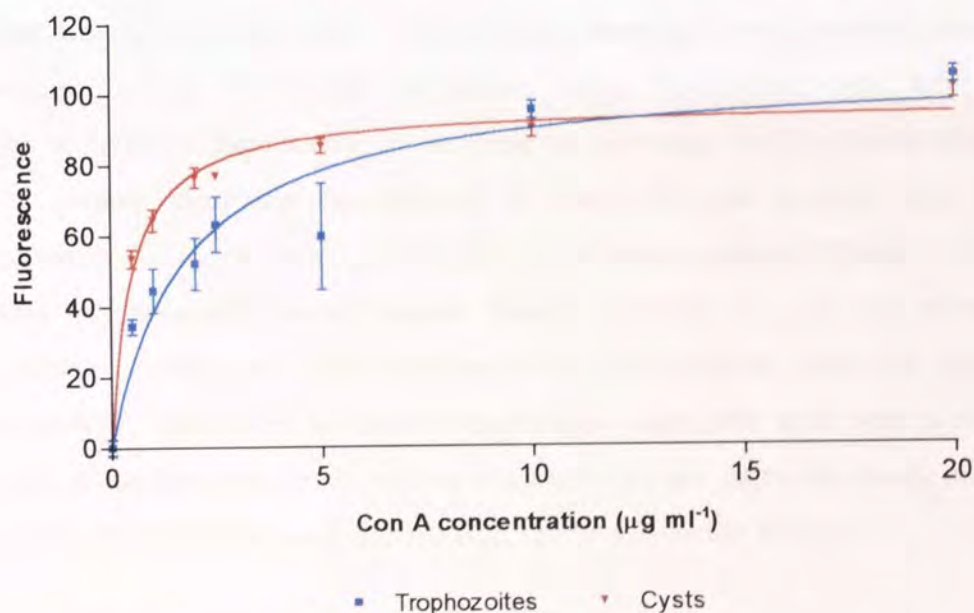


Figure 5.6. Binding curves for FITC-Con A to *Acanthamoeba polyphaga* (Leeds strain) trophozoites and cysts, as determined by flow cytometry. Data points are means of three replicate samples with bars representing standard errors of the mean of each of those replicates.

Construction of the Con A binding curve (Figure 5.6) enabled determination of K_d and B_{max} values, which are listed in Table 5.4. Concentration curves for binding to

Acanthamoeba polyphaga (Leeds strain) trophozoites and cysts are also presented for LCA (Figure 5.7), PSA (Figure 5.8), RCA₁₂₀ (Figure 5.9), SBA (Figure 5.10), VVA (Figure 5.11) and WGA (Figure 5.12). Each of these curves shows a typical increase in fluorescence concomitant with lectin concentration, marked at first, but lessening at higher lectin concentrations, although there is some variation in the form of the profiles. For LCA (Figure 5.7), there is little apparent difference between trophozoites and cysts. PSA (Figure 5.8), on the other hand, shows marked differences between the two morphological forms, with cysts exposed to low concentrations demonstrating a much sharper increase in fluorescence. In addition the plateau level of fluorescence for cysts is at a greater level compared to trophozoites. RCA₁₂₀ (Figure 5.9), too, shows different binding characteristics depending on morphological form, with a much more moderate slope for cysts and a maximum fluorescence around half that of trophozoites. There is little apparent difference in the profiles for SBA binding (Figure 5.10) to the two morphological forms. However, each curve is shallower than for other lectins, such as Con A. VVA binding (Figure 5.11) shows a marked difference between trophozoites and cysts, with the former showing a very moderate increase in fluorescence giving rise to very low plateau values. In contrast, cysts show both a steeper increase in fluorescence in response to increasing lectin concentrations and also a greater maximum fluorescence, at least two-fold greater than that of trophozoites. Finally, WGA (Figure 5.12) again shows a marked difference in binding between acanthamoebal morphological forms. Increases in cyst and trophozoite fluorescence concomitant with increasing lectin concentration takes the form of a steep gradient, though the maximum fluorescence associated with cysts is less than half that of trophozoites. Such binding characteristics are more effectively compared from the corresponding K_d and relative B_{max} values as listed in Table 5.4.

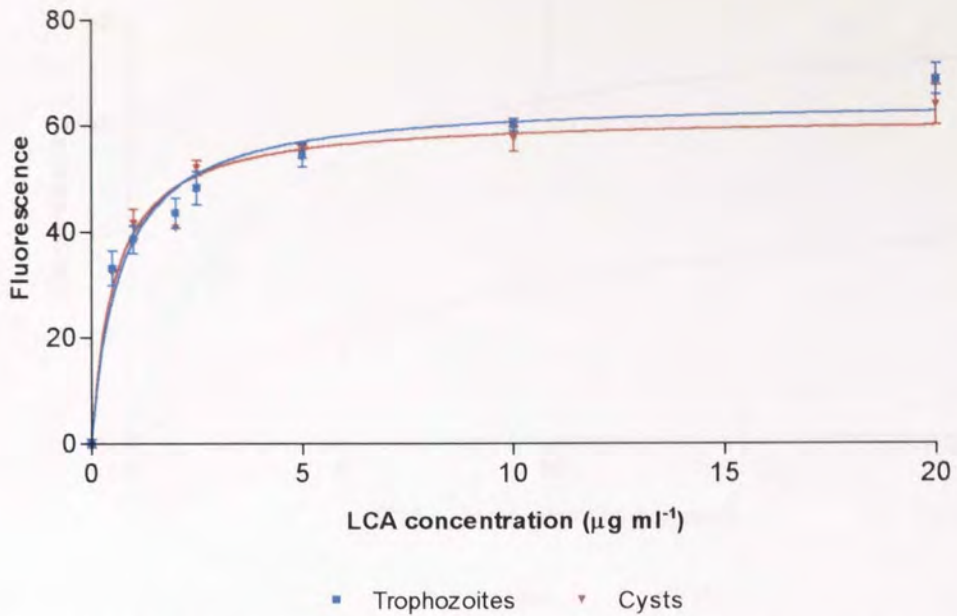


Figure 5.7. Binding curves for FITC-LCA to *Acanthamoeba polyphaga* (Leeds strain) trophozoites and cysts, as determined by flow cytometry. Data points are means of three replicate samples. Bars represent standard errors of the mean of each set of replicates.

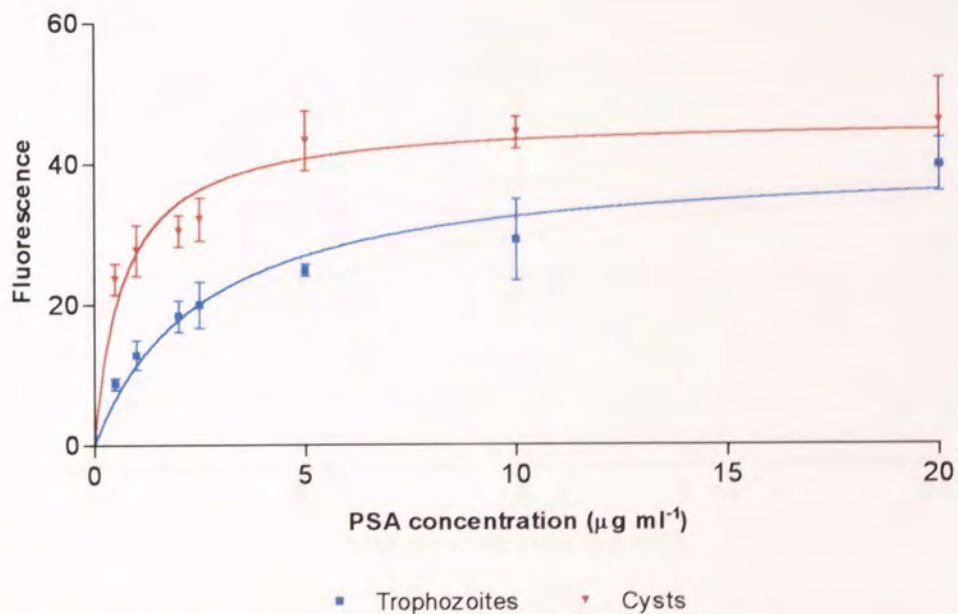


Figure 5.8. Binding curves for FITC-PSA to *Acanthamoeba polyphaga* (Leeds strain) trophozoites and cysts, as determined by flow cytometry. Results are means of three replicate samples. Bars represent standard errors of the mean of each set of results.

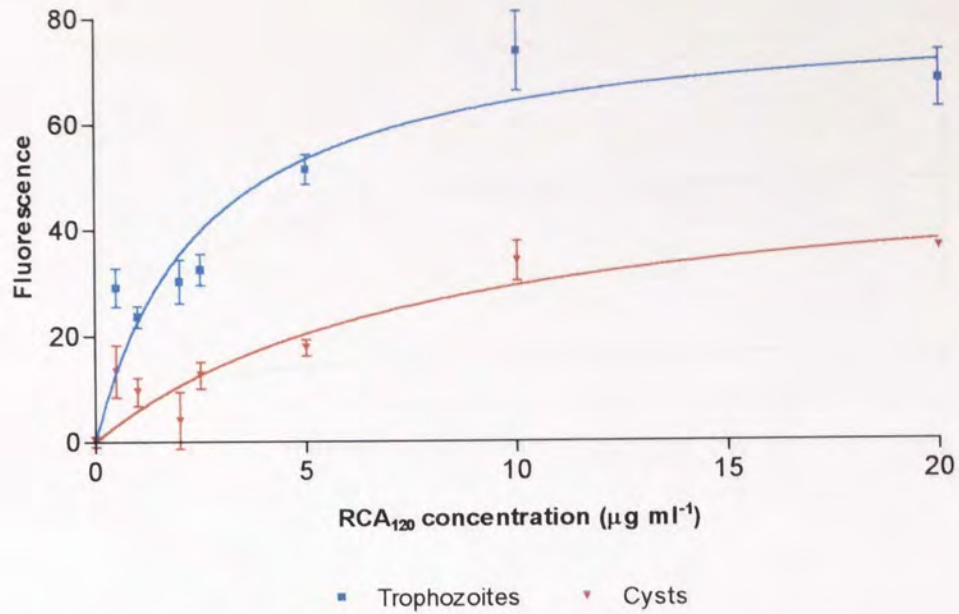


Figure 5.9. Binding curves for FITC-RCA₁₂₀ to *Acanthamoeba polyphaga* (Leeds strain) trophozoites and cysts, as determined by flow cytometry. Data points are means of three replicate samples. Bars represent standard errors of the mean of each set of replicates.

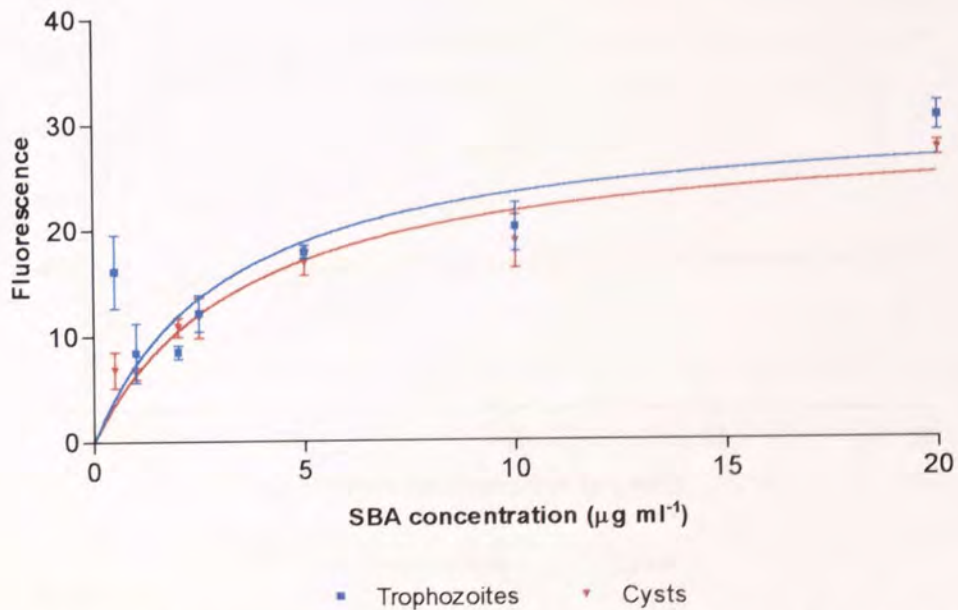


Figure 5.10. Binding curves for FITC-SBA to *Acanthamoeba polyphaga* (Leeds strain) trophozoites and cysts, as determined by flow cytometry. Data points are means of three replicate samples, with bars representing standard errors of the mean of each set of triplicate results.

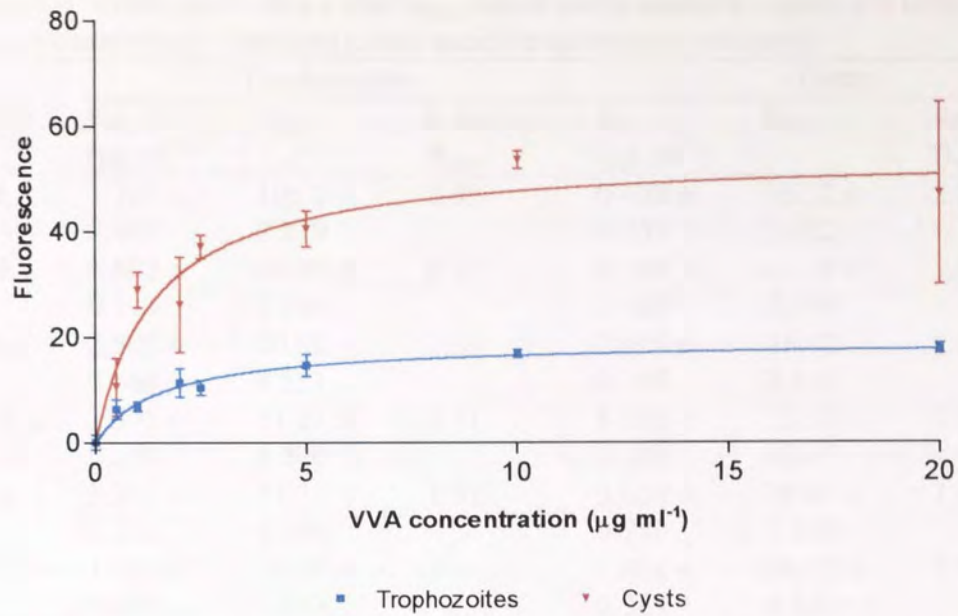


Figure 5.11. Binding curves for FITC-VVA to *Acanthamoeba polyphaga* (Leeds strain) trophozoites and cysts, as determined by flow cytometry. Data points are means of three replicate samples. Bars represent standard errors of the mean of each set of replicates.

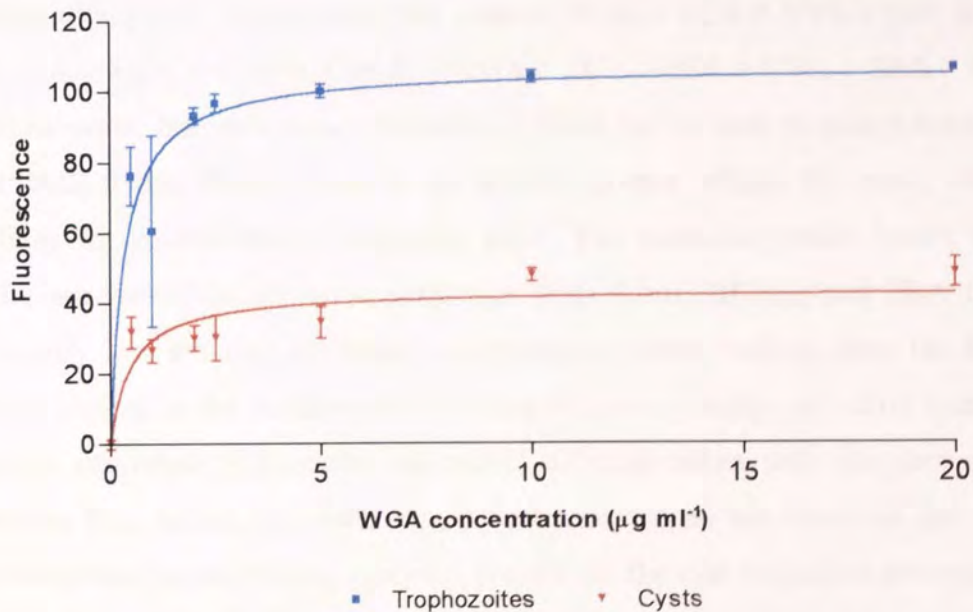


Figure 5.12. Binding curves for FITC-WGA to *Acanthamoeba polyphaga* (Leeds strain) trophozoites and cysts, as determined by flow cytometry. Results are means of three replicate samples. Bars represent standard errors of the mean of each set of results.

Table 5.4. Comparison of K_d and B_{max} values (with standard errors) for lectin binding to *Acanthamoeba polyphaga* (Leeds strain) trophozoites and cysts.

Lectin	Trophozoites			Cysts		
	K_d ($\mu\text{g ml}^{-1}$)	B_{max}	Relative B_{max}	K_d ($\mu\text{g ml}^{-1}$)	B_{max}	Relative B_{max}
Con A	1.760 \pm 0.449	106.2 \pm 8.119	2.49	0.478 \pm 0.057	96.52 \pm 2.102	2.26
LCA	0.692 \pm 0.113	64.95 \pm 2.294	2.35	0.549 \pm 0.085	61.73 \pm 0.549	2.23
PSA	2.592 \pm 0.668	40.98 \pm 3.551	2.63	0.699 \pm 0.198	46.47 \pm 2.853	2.98
RCA ₁₂₀	2.595 \pm 0.654	81.23 \pm 6.886	2.31	8.065 \pm 3.488	53.32 \pm 10.67	1.52
SBA	3.234 \pm 1.316	31.18 \pm 4.546	1.51	3.688 \pm 0.789	29.83 \pm 2.369	1.45
VVA	1.553 \pm 0.405	19.09 \pm 1.433	1	1.401 \pm 0.584	54.42 \pm 6.327	2.85
WGA	0.342 \pm 0.131	106.9 \pm 6.385	8.51	0.479 \pm 0.200	43.70 \pm 3.357	3.48

From Table 5.4, the K_d values can be ranked from the least (greatest affinity) to the greatest (lowest affinity) for each morphological form, in order to gauge relative lectin affinity. For trophozoites, this order is WGA > LCA > VVA > Con A > PSA > RCA₁₂₀ > SBA. For cysts, Con A > WGA > LCA > PSA > VVA > SBA > RCA₁₂₀; a similar order, but with some differences - WGA can be seen to have a similar affinity for both forms, though Con A has slightly greater affinity for cysts, whereas its affinity for trophozoites is relatively poor. The mannose-specific lectins LCA and PSA are ranked in the same order for both forms. RCA₁₂₀ and SBA both have relatively low affinities for either morphological form. Judging from the K_d values, lectin binding to the surface carbohydrates of cysts achieves saturation in most cases before equivalent trophozoite saturation, although taken with the generally lower relative B_{max} values for cysts, this indicates that there are fewer of the respective carbohydrate ligand binding moieties present on the cyst surface in comparison with trophozoites.

Relative B_{max} values can also be ranked in order of greatest maximum fluorescence to least. For trophozoites, this is: WGA > PSA > Con A > LCA > RCA₁₂₀ > SBA > VVA. For cysts, WGA > PSA > VVA > Con A > LCA > RCA₁₂₀ > SBA; these rankings are striking in their similarity, with the only difference being VVA being found third for cysts, but last for trophozoites, indicating the relatively

greater abundance of binding sites for this lectin on cysts in comparison to trophozoites.

From these relative B_{\max} values, one could expect greater binding to trophozoites than cysts for all lectins except PSA and VVA. The case of PSA is unexpected after the results of Section 5.3.1.4, where PSA binding was significantly greater to trophozoites in comparison to cysts, although there is no marked difference between the relative B_{\max} values for the two morphological forms. All other comparisons are in agreement with those results found earlier in Section 5.3.1.4, though the difference between relative B_{\max} values for VVA with trophozoites and cysts is markedly in favour of cysts, despite there previously being no significant difference. In the results of Section 5.3.1.4, SBA was another borderline case, apparently favouring cysts as opposed to trophozoites, though no significance was found. The relative B_{\max} values for this lectin are almost identical, upholding the statistical result. The relative B_{\max} value for WGA binding to trophozoites is perhaps the most interesting, in that it is markedly greater than that of Con A and all other lectins with trophozoites, thus underlining the value of such ligand-receptor studies, as despite the low specific activity of WGA of 2.5 moles FITC: 1 mole WGA, this lectin actually appears to bind better to trophozoites than other lectins. This finding is also true to a lesser extent for cysts.

5.4 Discussion

Saturation of Con A binding sites (α -mannose and α -glucose) of *Acanthamoeba* trophozoites occurred at relatively small concentrations, therefore a concentration of $10 \mu\text{g ml}^{-1}$ was used for all lectins. Due to its wider carbohydrate specificity than other lectins, Con A is known to bind many biological cell types (Vector Laboratories, 1999), so it was most likely that if such a concentration saturates all the binding sites of the amoebae, then a similar concentration of other lectins would be sufficient for saturation of their appropriate, potentially scarcer, binding sites of both *Acanthamoeba polyphaga* (Leeds strain) morphological forms.

Con A binding specificity for *Acanthamoeba polyphaga* (Leeds strain) trophozoites was confirmed by the use of blocking haptens, analogues for mannose

and glucose which, either independently or in conjunction, almost totally prevented any increase in fluorescence following incubation with FITC-Con A. Indeed, with both haptens together, there was no evidence of any Con A binding to trophozoites. There was also little difference between trophozoites pre-incubated with either hapten, following incubation with Con A. This indicated that, although α -glucose is often considered to be a secondary target for Con A, with respect to α -mannose, there was little apparent preference of the lectin for either carbohydrate moiety.

Despite an apparent similarity in binding of Con A to both trophozoites and cysts, K_d values indicated a much greater affinity for Con A amongst cysts, although the abundance of binding sites on each form was roughly equal. Furthermore, the relative abundance of receptors was roughly equal for all mannose-binding lectins on both trophozoites and cysts despite the apparent broader receptor specificity of Con A for α -glucose as well as α -mannose, and both LCA and PSA apparently requiring N-acetylchitobiose-linked α -fucose residues in the receptor oligosaccharides (Vector Laboratories, 1999). Although UEA I, a lectin that binds specifically to α -L-fucose residues, was found to bind better to trophozoites than cysts, the potentially fewer fucose residues in cysts would not appear to markedly compromise binding of PSA or LCA. A difference between the affinities of the three mannose lectins investigated for the two morphological forms was also determined with an order of affinity for cysts much like the reported Con A > LCA > PSA by Smith *et al.* (2001) when investigating pathogenic yeast. Somewhat in contrast, for trophozoites LCA, unexpectedly, had an apparent greater affinity than Con A, although PSA again demonstrated the lowest affinity. Overall, mannose and glucose residues appear well represented on the surface of both trophozoites and cysts of *Acanthamoeba polyphaga*. Such a finding was confirmed by confocal microscopy images (Figure 5.3), which demonstrated Con A binding to most of the trophozoite surface, with, in certain areas, regions of marked Con A binding.

Enhanced characterisation of WGA binding provided a good illustration of the benefits of determination of K_d and relative B_{max} values, as fluorescence determinations by flow cytometry alone did not suggest this lectin to have: a) the greatest affinity amongst all test lectins for trophozoites, b) second greatest affinity for cysts, or c) the highest abundance of surface receptors irrespective of acanthamoebal morphological form. WGA, unlike other lectins in the study, preferentially binds to both dimeric N-acetylglucosamine residues and sialic acid residues, potentially

revealing the presence of such residues. Fungi possess N-acetylglucosamine as part of chitin, a major structural component of cell walls (Smith *et al.*, 2001). Although fungi apparently evolved from protozoa by development of such chitinous cell walls (Cavalier-Smith, 1993), it is potentially likely that the precursors to chitin, if not chitin itself, would be present in protozoa, such as *Acanthamoeba* species.

RCA₁₂₀, a lectin specific for non-N-acetylated α - or β -galactose, was found to possess both a greater affinity for trophozoites than cysts and also a greater abundance of binding sites on the trophozoite surface. Relative to other lectins studied using the ligand-receptor studies, however, both affinity and abundance of binding sites was relatively low, indicating a relatively low exposure of galactose in both trophozoites and cysts.

VVA and SBA possess specificity for similar, yet differently linked N-acetylgalactosamine residues (Vector Laboratories, 1999). Interestingly, amongst cysts, there was a marked difference in the abundance of binding sites for VVA and SBA. Furthermore, the affinity of VVA was also greater for both forms than that of SBA. Bose *et al.* (1989) indicated that SBA did not bind to either morphological form of *Acanthamoeba castellanii* or *A. astronyxis*. Their study also often showed species variation in binding of many lectins, so the binding of SBA to *Acanthamoeba polyphaga*, but not to other species of *Acanthamoeba*, is not necessarily surprising.

Results of the present study indicated that, relative to trophozoites, cyst walls lacked marked quantities of α - and β -galactose and α -L-fucose residues, or at least had fewer such residues on the cell surface than trophozoites. Such an assumption is supported by statistical analysis indicating significantly greater binding of GSL I, RCA₁₂₀ and UEA I to trophozoites in comparison to that of cysts. Neither trophozoites nor cysts apparently demonstrated any great affinity for DSL, ECL or PNA, lectins that specifically target β -galactose residues, either linked to N-acetylglucosamine with a 1,4 glycosidic bond or to N-acetylgalactosamine with a 1,3 glycosidic bond. PHA-L, which binds to unspecified oligosaccharides (Vector Laboratories, 1999), also demonstrated little apparent affinity for either form of *Acanthamoeba*. Finally, SJA, which preferentially binds to β -linked N-acetylglucosamine, also failed to bind to either trophozoites or cysts in any marked quantity. Although in depth ligand-receptor studies were not performed for all lectins, it is likely that *Acanthamoeba polyphaga* simply does not possess many binding sites for certain lectins, particularly those that gave low fluorescence despite having considerable specific activities. Alternatively,

other factors should be considered including physical accessibility and orientation of the appropriate lectin receptor, particularly important with high molecular weight lectins, whose larger size may prevent them for accessing complex or confined sites, and the time allowed for lectin-receptor interaction (Doyle, 1994). Conversely, causes of marked binding could, in part arise, from a hydrophobic effect rather than specific binding (Doyle, 1994).

Cysts might be expected to have or expose a more restricted spectrum of carbohydrates compared to trophozoites. This is not surprising given that the polysaccharide content of the cell wall of cysts is largely constituted of cellulose (Neff *et al.*, 1964a), with glycogen scattered throughout the cyst wall (Robin *et al.*, 1988). Other carbohydrates may also be present, as speculated by Robin *et al.* (1988), which lectin binding studies can help to elucidate as their use, according to Hardham and Mitchell (1998), is highly specific and non-destructive. On the other hand, the trophozoite cell membrane is much better defined, containing a mixture of glucose (12 % of lipophosphoglycan; itself 30 % of the total membrane composition), mannose (9 %), galactose (2 %), xylose (2 %), galactosamine (2 %) and glucosamine (1 %) (Bowers and Korn, 1974). The exposure of which is, in the main, borne out by outlined lectin binding experiments. In addition to such carbohydrates present in lipophosphoglycan, Paatero *et al.* (1988) demonstrated the presence of membrane glycoproteins to which lectins could also bind.

Although the application of both flow cytometry and ligand-receptor studies to characterise and contrast surface carbohydrates of *Acanthamoeba* is considered novel, past investigations of lectin binding to *Acanthamoeba* deserve attention. For example, through visualisation of FITC-lectins, Robin *et al.* (1989) demonstrated that both Con A and WGA bind to both trophozoites and cysts giving a strong fluorescence signature, although their observations of WGA behaviour differ from those found in the present study, with greater fluorescence visualised following WGA binding to *Acanthamoeba* cysts than after WGA binding to trophozoites. The different methods used for binding determination and past lack of rigorous quantification make it difficult to realistically contrast results from other studies with those of current experimentation. However, the semi-quantitative method used by Robin *et al.* (1988) is something of a special case providing corollary support, with the exception of UEA I of which no binding was discerned to either morphological form of *Acanthamoeba*. In view of reports by Bowers and Korn (1974) on trophozoite

carbohydrates, this lack of binding by fucose-specific UEA I is not surprising. Stevens and Stein (1977) also found no reaction with fucose binding protein, determined as agglutination of *Acanthamoeba*, although they also failed to demonstrate any agglutination with WGA. However, in a further study, Bose *et al.* (1989) found that binding to UEA I was dependent on the pathogenicity of the amoeba. Although it should be noted that in all these published lectin studies Con A was found to bind with great avidity, their contradictory nature suggests that experimental approaches should ensure both quantification and qualification of lectin binding nature.

Lectinophagocytosis, as coined by Ofek and Sharon (1988), is a lectin-mediated mechanism for substrate recognition and attachment proposed for phagocytic cells, particularly amoebae, where opsonisation is not a factor. This process in *Acanthamoeba* is often thought to involve mannose (Cao *et al.*, 1998). Lectins involved in such a mechanism can be found on the surface of either phagocyte (Brown *et al.*, 1975) or substrate, such as in certain Gram-negative bacterial strains that possess a surface mannose-specific lectin that mediates attachment to phagocytes (Bar-Shavit *et al.*, 1977), or potentially both parties. Evidence for mannose as a mediator of attachment comes from findings that binding events can often be inhibited by α -methyl-mannopyranoside (Morton *et al.*, 1991; Gordon *et al.*, 1993; Garate *et al.*, 2000). Although these studies were concerned with attachment to corneal epithelium, they suggest a lectin-like or similar receptor for foreign surface residues on *Acanthamoeba* that could also be involved in uptake mechanisms for bacteria. Such a possibility was investigated by Preston and King (1984) who found that Con A could block binding of bacterial flagellae to sites on *Acanthamoeba*. Allen and Dawidowicz (1990a) also demonstrated that a mannose binding site was important in engulfment of yeast cells and, following binding, second messengers were triggered to initiate events for the completion of phagocytosis (Allen and Dawidowicz, 1990b). Alternatively, other lectins may also be implicated, such as in the uptake of *Legionella pneumophila* by *Acanthamoeba polyphaga* which can be partially blocked by galactose or N-acetyl galactosamine (Abu Kwaik *et al.*, 1998).

There is documented evidence that *Aeromonas hydrophila* has surface adhesins that bind to D-mannose and L-fucose residues on cell surfaces (Trust *et al.*, 1983), which could also mediate binding to *Acanthamoeba*, allowing uptake. In addition, Ewart *et al.* (1999) identified a lectin in Atlantic salmon serum to which mannose residues on the surface of *Aeromonas salmonicida* can bind. Similarly, Coho

salmon eggs were found to possess a galactose-specific lectin to which *Aeromonas salmonicida*, but not *A. hydrophila* or *Renibacterium salmoninarum*, could bind (Yousif *et al.*, 1994), despite the latter possessing a galactose-rich cell wall (Sørum *et al.*, 1998). *Renibacterium salmoninarum* also possesses a haemagglutinin, known as the p57 antigen, thought to enable its uptake by macrophages (Daly and Stevenson, 1987) and thus, feasibly, also *Acanthamoeba*. Studies with blocking sugars, however, have failed to identify any carbohydrate specificity of the p57 antigen, suggesting that uptake is not lectin-mediated (Evenden *et al.*, 1993). If such pathogens do interact with *Acanthamoeba*, it would appear that a lectin-mediated interaction orchestrates predation, particularly of *Aeromonas* species.

It can thus be seen that lectin-receptor interactions are implicated in the phagocytosis of bacterial entities. However, exactly which lectins are involved is yet to be determined. The presence of both mannose on *Acanthamoeba* cell surfaces as found here and the evidence provided by other workers (Preston and King, 1984; Allen and Dawidowicz, 1990a) for acanthamoebal mannose-binding lectins supports consideration of such moieties as mediators of recognition events. It may, however, also be that there are other *Acanthamoeba* lectins that recognise carbohydrates present on bacterial cell surfaces. Studies were undertaken in order to investigate the role of both specific and non-specific surface properties and other environmental conditions in phagocytosis by *Acanthamoeba* of polystyrene latex microspheres.

CHAPTER 6 – Phagocytosis of polystyrene latex microspheres

6.1 Introduction

Acanthamoeba, as a small free-living amoeba, is well recognised for its ability to phagocytose bacteria and other microbes in its natural habitat for feeding purposes, and collectively with other protozoa, this process is largely responsible for limiting bacterial populations (González *et al.*, 1990). However, as noted, some bacteria are able to subvert their amoebal hosts into providing a safe haven until conditions favour further host infection. The mechanisms by which this occurs are varied (Evenden *et al.*, 1993), and include prevention of acidification of the phagolysosome, prevention of fusion of the phagolysosome, the presence of resistant cell surface moieties, inhibition of reactive oxygen intermediates (ROIs) and escape into the cytoplasm. Several species of bacteria are able to achieve intracellular growth by these means (Table 1.2). However, the initial events involved in uptake of biologically active material are often difficult to study. Fortunately the phagocytic ability of *Acanthamoeba* is not restricted to microbial prey and it is known to be able to engulf a wide range of other entities, whether of any nutritive value or not (Bowers and Olszewski, 1983). Such apparent indiscriminate ingestion affords the use of polystyrene latex microspheres as an entity for uptake, an ideal substrate due to relatively uniform characteristics, in terms of size and surface properties. However, such attributes can also be varied with relative ease, such that microspheres are available in a range of sizes and with different surface modifications, for example the addition of carboxylate or amino charged groups. Such groups also make microspheres amenable to further modifications, such as the coupling of proteins on their surface.

The use of microspheres in phagocytosis studies with both *Acanthamoeba* and other phagocytes is relatively commonplace as a model of the uptake system without complications of either the digestive processes of amoebae or cell signalling as in other phagocytic cells (Avery *et al.*, 1995; Allen and Dawidowicz, 1990b), however there remains the possibility of novel experimentation. In this respect, investigations were performed to study the effect on phagocytosis of a number of variables. Such

variables included basic assay parameters, such as length of incubation time, temperature, presence or absence of phagocytosis inhibitors and post-assay washing; microsphere variables such as size and surface nature, protein, lectin and carbohydrate coating; and trophozoite manipulations such as starvation, culture age, strain differences, lectin coating and presence of carbohydrates. For the most part, results were obtained by flow cytometry, due to its advantages of accurate quantification of greater sample numbers than were obtained by initial microscopic observation and enumeration. However, due to the well-recognised difficulties in discrimination between internalised and surface-bound entities regardless of the technique used to quantify results (Al-Ibrahaim *et al.*, 1976), all such trophozoite-microsphere interactions in this study, whether internalised or surface-bound, were recorded as associations, expressed as the proportion of trophozoites interacting with microspheres.

Although various factors have a bearing on the phagocytic process, whilst some will differ between latex microspheres and microbes, others will be similar, thus the use of microspheres as a substrate entity can provide some insight into the process of phagocytosis.

6.2 Materials and Methods

6.2.1 Preliminary phagocytosis assay

Cultures of log phase trophozoites of *Acanthamoeba polyphaga* were prepared in sterile plastic universals by inoculation of 9 ml sterile PYG broth with 1 ml confluent stock culture, before horizontal incubation in darkness at 37 °C for 4 days. Following incubation, three replicate 0.9 ml aliquots for each microsphere type, at a concentration of approximately 10^6 cells ml⁻¹, were transferred to separate sterile Eppendorf tubes before the addition of 100 µl of either 1.0 µm diameter plain (unmodified) FITC-polystyrene latex microspheres or 1.0 µm diameter carboxylate-modified FITC-polystyrene latex microspheres (Polysciences, Inc., USA), to a final microsphere concentration of 5×10^7 microspheres ml⁻¹. Eppendorfs were incubated at

37 °C, in darkness, on a G10 Gyrotory orbital shaker (New Brunswick Scientific, USA) at 200 rpm for 1 hour.

After incubation, trophozoites were centrifuged twice at 1000 g for 10 min and resuspended in 1 ml amoebal saline each time to remove excess microspheres, before quantification of microsphere-trophozoite association using an “Improved Neubauer” haemocytometer and fluorescence microscopy. Such association was determined by the enumeration of amoebae with microspheres that were either apparently internalised or attached to the amoebal cell surface, as distinguished from those trophozoites that remained free from microspheres. Due to the marked difficulty in distinguishing between microspheres that were internalised from those that were found merely attached to the external surface, the term “associated” is potentially more accurate, hence all results were considered in terms of the percentage association of trophozoites with microspheres.

6.2.2 Effect of amoebal starvation on their association with microspheres

Four-day-old trophozoites were prepared by the addition of 1 ml confluent stock culture to 9 ml sterile PYG broth in sterile plastic universals, which were in turn incubated horizontally in darkness at 37 °C. Resultant trophozoites were harvested by centrifugation twice at 1000 g for 10 min and resuspension twice in 10 ml sterile amoebal saline before re-incubation in darkness at 37 °C. From time zero and subsequently at hourly intervals, replicate 0.9 ml samples at a concentration of approximately 10^6 cells ml⁻¹ were placed in sterile Eppendorf tubes, to which 100 µl aliquots of 2.0 µm diameter plain type FITC-microspheres were added to a final concentration of 5×10^7 microspheres ml⁻¹. Samples were then re-incubated at 37 °C in darkness on an orbital shaker at 200 rpm for 1 hour before quantification of trophozoites with associated microspheres by fluorescence microscopy. Samples were discarded following assessment.

Results were calculated as percentage of cells with microspheres associated and hence required arc sine transformation to fit data to a normal distribution curve. Subsequent analysis by two factor ANOVA using the data analysis package in

Microsoft® Excel 2000 determined whether numbers of microspheres associated with *Acanthamoeba* varied significantly with incubation periods in amoebal saline.

6.2.3 Flow cytometric determination of the effect of amoebal starvation on their association with microspheres

As flow cytometry potentially supports a more realistic characterisation of phagocytosis in comparison to the more subjective and time-intensive enumeration of experimental entities employing a haemocytometer and fluorescence microscopy, the technique was used in a corroborative manner to more effectively determine the effect of trophozoite starvation on microsphere association.

Two sets of three replicate cultures were prepared by the inoculation of 9 ml PYG broth with 1 ml confluent stock culture in sterile plastic universals before horizontal incubation at 37 °C in darkness for 4 days. One set of three replicate cultures was maintained in PYG broth whilst the other set was washed twice by centrifugation at 1000 g for 10 min and resuspension in 10 ml amoebal saline before re-incubation under starvation conditions at 37 °C in darkness for a further day. Prior to further experimentation both sets of trophozoites were adjusted to approximately 10^6 cells ml⁻¹. Subsequently, 0.9 ml aliquots from each set of replicate trophozoites in amoebal saline or PYG broth were placed in sterile Eppendorf tubes before addition of 100 µl of 2.0 µm diameter carboxylate or plain FITC-microspheres to a final concentration of 5×10^7 microspheres ml⁻¹. In addition, a sample from each set of replicates was used as a negative control, with 100 µl of either amoebal saline or PYG broth, as appropriate, in place of microspheres, in order to determine background autofluorescence when analysed by flow cytometry. All samples were incubated at 37 °C, in darkness, on an orbital shaker at 200 rpm for 1 hour before being centrifuged twice at 1000 g for 10 min and resuspended, firstly in 1 ml amoebal saline and finally in 300 µl 1 % (w/v) paraformaldehyde in amoebal saline to fix trophozoites with or without associated microspheres. Samples were then stored at 4 °C in darkness until analysis by flow cytometry using the Becton Dickinson FACS 440 housed at Birmingham University Medical School (see Section 5.2.1.1 for details). The percentage of amoebal cells associated with microspheres was determined by

identification and quantification of the subpopulation of amoebal cells with increased fluorescence over background acanthamoebal autofluorescence, arising from the presence of fluorescent microspheres.

Results were arc sine transformed before analysis by single factor ANOVA using the data analysis package on Microsoft[®] Excel 2000 in order to determine the effect of the environmental medium on amoebal association with microspheres.

6.2.4 Effect of microsphere size and nature on their association with amoebal trophozoites

6.2.4.1 Flow cytometric determination of microsphere association with amoebal trophozoites

Cultures of *Acanthamoeba polyphaga* were set up in sterile plastic universals by the inoculation of 9 ml sterile PYG broth with 1 ml confluent stock culture before incubation horizontally in darkness at 37 °C for 4 days. PYG broth was then replaced with 10 ml amoebal saline following centrifugation at 1000 g for 10 min. A second 10 min centrifugation at 1000 g allowed resuspension in 10 ml amoebal saline and samples were re-incubated at 37 °C in darkness for a further day. Both plain and carboxylate FITC-microspheres, of diameter 0.5, 1.0 and 2.0 µm, were each added in 100 µl volumes to three replicate 0.9 ml starved trophozoite suspensions in sterile Eppendorf tubes. In addition, a negative control consisting of 0.9 ml amoebal trophozoites with 100 µl amoebal saline in place of microspheres was prepared to determine background autofluorescence. Each sample was then incubated at 37 °C for 1 hour in darkness on an orbital shaker at 200 rpm, before centrifugation at 1000 g for 10 min and resuspension in 1 ml amoebal saline to remove excess microspheres. After a second 10 min centrifugation at 1000 g, samples were fixed in 300 µl 1 % (w/v) paraformaldehyde in amoebal saline. Samples were stored at 4 °C until flow cytometric analysis using the Becton Dickinson FACS 440 of Birmingham University Medical School.

Following arc sine transformation, results were analysed by two factor ANOVA using the data analysis package of Microsoft® Excel 2000 to determine if there was a significant difference in the association of *Acanthamoeba* with either different sizes or different types of polystyrene latex microspheres.

6.2.4.2 Confocal microscopy of microsphere association with amoebal trophozoites

Acanthamoeba polyphaga (Leeds strain) trophozoites were allowed to associate with FITC-microspheres of three sizes of both plain and carboxylate surface types, in the same manner as detailed in Section 6.2.4.1. Instead of fixing, however, 5 µl aliquots were placed on microscope slides and immediately viewed. Images were acquired with a Zeiss Axiovert/Bio-Rad MCR 1024 OS laser scanning confocal microscope facility, utilising Laser Sharp 2000 software (Bio-Rad Laboratories, USA). A 100 mW argon laser, excitation wavelength 488 nm was moderated by a series of neutral density filters, gain, offset and zoom functions, used to optimise image fluorescence intensity, contrast and composition. Slides were viewed with Plan-NEOFLUAR Ph 1 10×/0,30, Plan-NEOFLUAR Ph 2 40×/0,75 and Plan-NEOFLUAR Ph 3 100×/1,30 oil immersion objectives and subject material was subsequently captured as 512 × 512 pixel images in turn converted from Bio-Rad PICT to TIFF format. Each sample could only be imaged once due to photobleaching of fluorescence by the laser.

6.2.5 Effect of amoebal saline washing steps on amoebal association with plain microspheres

Four-day-old log phase cultures of *Acanthamoeba* were prepared by the addition of 1 ml confluent stock culture to 9 ml fresh PYG broth in sterile plastic universals, which were incubated horizontally at 37 °C in darkness. PYG broth was then replaced with 10 ml amoebal saline following centrifugation at 1000 g for

10 min. A second 10 min centrifugation at 1000 g was followed by resuspension in 10 ml amoebal saline before universals were re-incubated at 37 °C in darkness for a further day. Two sets of three replicate 0.9 ml aliquots at an approximate concentration of 10^6 cells ml⁻¹ were placed in sterile plastic Eppendorf tubes to which 100 µl aliquots of each of the three sizes (Section 6.2.4) of plain FITC-microspheres were added to a final concentration of 5×10^7 microspheres ml⁻¹. Background autofluorescence was determined using a negative control consisting of 100 µl amoebal saline added to 0.9 ml *Acanthamoeba* trophozoite suspension. Samples were then incubated at 37 °C in darkness for 1 hour on an orbital shaker at 200 rpm. Following incubation, one set of replicates remained untouched and the other washed as normal by centrifugation twice at 1000 g for 10 min and resuspension in amoebal saline, firstly in 1 ml, then in 300 µl. Neither set were fixed, so samples were kept at 4 °C until almost immediate analysis by flow cytometric analysis on the Becton Dickinson FACS 440 of Birmingham University Medical School.

Percentage data was normalised by arc sine transformation before single factor ANOVA using the data analysis package of Microsoft® Excel 2000 was employed to analyse the results in order to determine if washing had any significant effect on association of microspheres with amoebae.

6.2.6 Effect of phagocytosis inhibitors on amoebal association with microspheres

Acanthamoeba polyphaga cultures were prepared in sterile plastic universals by the inoculation of 9 ml sterile PYG broth with 1 ml confluent stock culture before incubation in darkness at 37 °C for 4 days. Trophozoites were then centrifuged twice at 1000 g for 10 min and resuspended in 1 ml sterile amoebal saline each time before re-incubation in darkness at 37 °C overnight. Three replicate volumes of 0.9 ml trophozoite suspension for each inhibitor, each at a concentration of 10^6 cells ml⁻¹, were transferred to sterile Eppendorf tubes to which one of each of the three inhibitors employed was added; cytochalasin B to a final concentration of 0.1 mM (determined from concentration curves, data not shown), sodium azide to a final concentration of 12.5 mM (Avery *et al.*, 1995) and 2,4-dinitrophenol (2,4-dnp) to a final concentration

of 0.2 mM (Chambers and Thompson, 1976). In addition, both positive controls were set up with no inhibitor present and negative controls (for determination of background autofluorescence) comprising 0.9 ml trophozoite suspension and 100 µl amoebal saline. Aliquots of 100 µl of 2 µm diameter plain FITC-microspheres were then added to all samples, with the exception of negative controls, to a final concentration of 5×10^7 microspheres ml⁻¹. Samples were incubated at 37 °C in darkness on an orbital shaker at 200 rpm for 1 hour before isolating trophozoites from excess microspheres by centrifugation at 1000 g for 10 min and resuspension in 1 ml amoebal saline. After a second 10 min centrifugation at 1000 g, samples were fixed in 300 µl 1 % (w/v) paraformaldehyde and stored at 4 °C until flow cytometric analysis on the Becton Dickinson FACS 440 of Birmingham University Medical School.

Following arc sine transformation, results were analysed by single factor ANOVA using the data analysis package of Microsoft® Excel 2000 to determine whether inhibitors significantly influenced the association of *Acanthamoeba* with microspheres.

6.2.7 Effect of incubation time on amoebal association with microspheres

Cultures of *Acanthamoeba polyphaga* were prepared in sterile plastic universals by the addition of 1 ml confluent stock culture to 9 ml sterile PYG broth. Universals were incubated horizontally in darkness at 37 °C for 4 days before harvesting by centrifugation twice at 1000 g for 10 min and resuspension in 10 ml amoebal saline each time. Universals were then re-incubated in darkness at 37 °C for a further day before three replicate 0.9 ml aliquots for each sample, at approximately 10^6 cells ml⁻¹, were transferred to sterile Eppendorf tubes to which separate 100 µl aliquots of 2.0 µm diameter FITC-microspheres of both plain and carboxylate types to a final concentration of 5×10^7 microspheres ml⁻¹ were added. Negative controls consisted of 0.9 ml *Acanthamoeba* trophozoite suspension with 100 µl amoebal saline in place of microspheres. Samples were incubated on an orbital shaker at 37 °C in darkness at 200 rpm for 0, 2, 5, 10, 20, 30 and 60 min, 2, 5 and 24 hours respectively. Immediately after each incubation period, cytochalasin B was added to each sample to a final concentration of 0.1 mM. Samples were then centrifuged twice at 1000 g for

10 min before resuspension, firstly in 1 ml amoebal saline and then finally in fixative of 300 μ l 1 % (w/v) paraformaldehyde in amoebal saline, before storage at 4 °C until analysis by flow cytometry on the Becton Dickinson FACS 440 of Birmingham University Medical School.

Following arc sine transformation, two factor ANOVA using the data analysis package of Microsoft® Excel 2000 was used to determine any significant effect of time on amoebal association with microspheres as well as any significant difference in association with microsphere type.

6.2.8 Effect of temperature on amoebal association with microspheres

Four-day-old trophozoites were prepared in the customary manner of inoculation of 9 ml sterile PYG broth with 1 ml confluent stock culture in sterile plastic universals, horizontally incubated at 37 °C in darkness. Following incubation, trophozoites were washed by centrifugation at 1000 g for 10 min and resuspended in 10 ml amoebal saline. This washing step was repeated once before re-incubation of cultures at 37 °C for a further day. Following starvation, 0.9 ml aliquots, in replicates of three for each sample, at approximately 10^6 cells ml^{-1} were transferred to sterile Eppendorf tubes, to which 100 μ l aliquots of either plain or carboxylate-modified 2.0 μ m diameter FITC-microspheres were added to a final concentration of 5×10^7 microspheres ml^{-1} . An additional 0.9 ml *Acanthamoeba* trophozoite suspension was placed in a sterile Eppendorf tube, to which 100 μ l amoebal saline was added as a negative control for determination of background autofluorescence. Samples were then incubated in waterbaths with rotary shaking at 200 rpm at temperatures of 10, 15, 25 and 37 °C for 1 hour in darkness. Samples were then centrifuged at 1000 g for 10 min before resuspension in 1 ml amoebal saline. A second 10 min centrifugation at 1000 g was followed by resuspension in 300 μ l 1 % (w/v) paraformaldehyde in amoebal saline, which fixed the trophozoites. Samples were stored at 4 °C until analysis by flow cytometry using the Becton Dickinson FACS 440 housed at Birmingham University Medical School.

Results were arc sine transformed before analysis by two factor ANOVA using the data analysis package of Microsoft® Excel 2000 to investigate if temperature had

any significant effect on amoebal association with microspheres and also if microsphere surface type had any effect on amoebal association.

6.2.9 Effect of amoebal cell age on microsphere association

Acanthamoeba polyphaga was cultured in sterile plastic universals by the addition of 1 ml confluent stock culture to 9 ml fresh sterile PYG broth, before incubation in darkness at 37 °C. After 2 days' incubation, three replicate cultures were removed from the incubator and subsequently each day for up to 12 days post-inoculation. Each culture was starved by washing and finally resuspending in 10 ml amoebal saline following two 10 min centrifugations at 1000 g, before re-incubation in darkness at 37 °C for a further day. Replicate 0.9 ml samples of approx 10^6 cells ml⁻¹, were then aliquotted into Eppendorf tubes, to which 0.1 ml aliquots of either plain or carboxylate 2.0 µm diameter FITC-microspheres were added to a final concentration of 5×10^7 microspheres ml⁻¹. A further sample served as a negative control for background autofluorescence determination, with 100 µl amoebal saline in place of microspheres. Following incubation in darkness for 1 hour at 37 °C on a orbital shaker at 200 rpm, samples were centrifuged at 1000 g for 10 min and washed in 1 ml amoebal saline, before a final centrifugation at 1000 g for 10 min and fixation in 300 µl 1 % (w/v) paraformaldehyde in amoebal saline. Samples were stored at 4 °C until flow cytometric analysis on the Becton Dickinson FACS 440 housed at Birmingham University.

Percentage data was normalised by arc sine transformation before analysis by single factor ANOVA using the data analysis package of Microsoft® Excel 2000 to determine if the age of *Acanthamoeba* affected its association with microspheres.

6.2.10 Association of *Acanthamoeba* strains with microspheres

Each of the five strains of *Acanthamoeba* (Section 4.2.1.5) were prepared by inoculation of 9 ml PYG broth with 1 ml confluent stock culture before horizontal

incubation in darkness at 25 °C for 4 days. Each culture was then centrifuged at 1000 g for 10 min and resuspended in 10 ml sterile amoebal saline. This process was repeated once before re-incubation for a further day at 25 °C. Three replicate 0.9 ml samples at a concentration of approximately 10^6 cells ml⁻¹ were aliquotted into sterile Eppendorf tubes for each sample before the addition of 2.0 µm diameter plain and carboxylate FITC-microspheres to a final concentration of 5×10^7 microspheres ml⁻¹. In addition, a negative control was set up to determine background autofluorescence of *Acanthamoeba* with 0.9 ml trophozoite suspension to which 100 µl amoebal saline was added. Samples were incubated in darkness at 25 °C on an orbital shaker for 1 hour, centrifuged at 1000 g for 10 min and resuspended in 1 ml amoebal saline. A further 10 min centrifugation at 1000 g was performed before fixing samples in 300 µl 1 % (w/v) paraformaldehyde in amoebal saline. Samples were stored at 4 °C before being analysed by flow cytometry on the Becton Dickinson FACS 440 of Birmingham University Medical School.

Results were analysed by single factor ANOVA using the data analysis package in Microsoft® Excel 2000, following arc sine transformation of percentage data, to determine if there was any significant difference in the association characteristics of different strains of *Acanthamoeba* with microspheres.

6.2.11 Acanthamoebal association with protein coated microspheres

6.2.11.1 Adsorption of protein to microspheres

Aliquots of 0.5 ml of plain 1.0 µm or 2.0 µm diameter FITC-microspheres, at the supplied concentrations of 4.55×10^{10} and 5.68×10^9 respectively, were centrifuged twice at 2000 g for 5 min and resuspended in 1 ml sterile distilled water each time before being transferred to a sterile plastic universal and mixed with 5 ml 1 mg ml⁻¹ bovine serum albumin (BSA) with 0.9 % (w/v) NaCl. Complete mixing of microspheres and BSA was ensured through incubation at 37 °C overnight in darkness on a Spiramix 5 roller (Denley, UK). Microspheres, potentially coated in protein, were then centrifuged at 2000 g for 5 min and washed in 5 ml fresh distilled water twice. A

final 5 min centrifugation at 2000 g was performed before resuspension in 500 μ l sterile distilled water for storage at 4 °C until use.

6.2.11.2 Quantification of protein adsorption

The nature and quantity of BSA adsorbed onto the microsphere surface was determined by the isolation and characterisation of any adherent protein by SDS-PAGE and comparison with seven standard BSA solutions, prepared in sterile distilled water at concentrations of 250, 125, 62.5, 31.3, 15.6, 7.8 and 3.9 μ g ml⁻¹, by serial two-fold dilution. A 30 μ l volume of each standard or BSA-coated microsphere sample at a concentration of approximately 10⁹ microspheres ml⁻¹ was added to separate 30 μ l volumes of sample loading buffer (Appendix 2) before heating at 100 °C for 15 min on a heating block for protein denaturation.

An 11 % (w/v) separating gel (of dimensions 80 × 70 × 1 mm) (Appendix 2) was cast using the Mini-Protean gel casting apparatus (Bio-Rad Laboratories, USA). Once set, a 5 % (w/v) stacking gel (Appendix 2) was cast on top before inserting plastic combs to form wells. When solidified, the entire gel was placed in a tank containing electrode buffer, pH 8.0 (10 ml 10 % (w/v) SDS, 0.3 % (w/v) Tris, 1.44 % (w/v) glycine, 1 L distilled water). A 20 μ l aliquot of each sample or standard was loaded on to the gel with a fine syringe before electrophoresis at 200 V for 45 min.

The resulting gel was stained by immersion in Coomassie Blue stain (20 % (v/v) methanol, 10 % (v/v) ethanoic acid, 0.1 % (w/v) Coomassie Blue) maintained overnight with gentle agitation. The gel was then destained by immersion in destaining solution (20 % (v/v) methanol, 10 % (v/v) ethanoic acid) with further gentle agitation. This solution was replaced, as necessary, until the gel was thoroughly destained and all background stain eliminated. Finally, the gel was photographed and archived using a Transilluminator (UVP Inc., USA) and camera system. The resulting digital image was analysed using Phoretix 1D Advanced Gel Analysis Software (Phoretix International, UK) to determine the protein content of the samples.

6.2.11.3 *Acanthamoeba* association with protein-coated microspheres

Cultures of log-phase trophozoites were prepared by inoculation of 9 ml sterile PYG broth with 1 ml confluent stock culture in sterile plastic universals, incubated horizontally at 37 °C in darkness for 4 days. Following incubation, cultures were centrifuged twice at 1000 g for 10 min before resuspension in 10 ml sterile amoebal saline each time. Samples were then re-incubated in darkness at 37 °C for a further day. To three 0.9 ml replicate trophozoite suspensions for each microsphere size, at a concentration of 10^6 cells ml⁻¹, 100 µl aliquots of BSA-coated plain FITC-microspheres (prepared as detailed in Section 6.2.11.1) were added to a final concentration of 5×10^7 microspheres ml⁻¹ in sterile Eppendorf tubes. In addition, a negative control (to determine background autofluorescence) consisting of 0.9 ml *Acanthamoeba* trophozoite suspension with 100 µl amoebal saline and a positive control consisting of 0.9 ml *Acanthamoeba* trophozoite suspension with 100 µl aliquots of 2.0 µm diameter plain FITC-microspheres were also prepared in sterile Eppendorf tubes. All tubes were subsequently incubated at 37 °C on an orbital shaker at 200 rpm for 1 hour in darkness before being centrifuged twice at 1000 g for 10 min and resuspended, firstly in 1 ml amoebal saline and finally in 300 µl 1 % (w/v) paraformaldehyde in amoebal saline as a fixative. Samples were stored at 4 °C until flow cytometric analysis using the Becton Dickinson FACS 440 housed at Birmingham University Medical School.

Percentage data was adjusted to a normal distribution by arc sine transformation before analysis by single factor ANOVA using the data analysis package of Microsoft® Excel 2000 to determine if there was any significant difference in microsphere association with *Acanthamoeba* due to the presence of protein coating.

6.2.12 Effect of Con A on acanthamoebal association with microspheres

6.2.12.1 Association of microspheres with Con A bound *Acanthamoeba polyphaga*

Cultures of *Acanthamoeba polyphaga* were prepared by inoculating 9 ml sterile PYG broth with 1 ml confluent culture in sterile plastic universals, incubated horizontally in darkness at 37 °C for 4 days. Following incubation, samples were centrifuged at 1000 g for 10 min before washing in 10 ml amoebal saline. A second 10 min centrifugation at 1000 g was performed before resuspending in 10 ml amoebal saline and re-incubating in darkness at 37 °C for a further day. Further trophozoite samples were harvested by centrifugation at 1000 g for 10 min and washed in 10 ml supplemented 10 mM HEPES (Section 5.2.1.1). This process was performed twice before a third 10 min centrifugation at 1000 g and resuspension of trophozoites in 1 ml supplemented 10 mM HEPES to a final trophozoite concentration of 10^7 cells ml⁻¹. Three replicate trophozoite samples were aliquotted in 0.9 ml volumes before addition of 100 µl non-conjugated Con A in supplemented 10 mM HEPES to a final concentration of 50 µg ml⁻¹. All samples were incubated at 4 °C for 30 min before centrifugation twice at 1000 g for 10 min and resuspension in 1 ml non-supplemented HEPES each time.

Three replicate 0.9 ml aliquots of Con A-bound trophozoites were then transferred to sterile Eppendorf tubes at a final concentration of approximately 10^6 cells ml⁻¹. Volumes of 100 µl of either plain or carboxylate 2.0 µm diameter FITC-microspheres were then added to each sample to a final concentration of 5×10^7 microspheres ml⁻¹ before samples were incubated in darkness at 37 °C on an orbital shaker for 1 hour. Parallel samples were also set up using three replicate 0.9 ml aliquots of trophozoites in HEPES unexposed to Con A, incubated with 100 µl volumes of each type of FITC-microsphere as a positive control. In addition three replicate 0.9 ml aliquots of trophozoites in amoebal saline at the same concentration were also placed in Eppendorf tubes and a negative control was prepared for determination of background autofluorescence consisting of 0.9 ml amoebal trophozoites incubated with 100 µl amoebal saline. After incubation, samples were

centrifuged twice at 1000 g for 10 min before resuspension, firstly in 1 ml amoebal saline and finally in 300 μ l 1 % (w/v) paraformaldehyde in amoebal saline. Samples were stored at 4 °C before analysis by flow cytometry using the Becton Dickinson FACS 440 housed at Birmingham University Medical School.

Results were arc sine transformed before analysis by single factor ANOVA using the data analysis package in Microsoft® Excel 2000 to determine, firstly, whether or not there was any difference between using saline or HEPES as the medium for acanthamoebal association with microspheres, secondly, if Con A binding to trophozoites made any significant difference on association with microspheres and lastly, if trophozoites associated significantly more with one microsphere surface type over another.

6.2.12.2 Preparation of Con A-modified microspheres

A 0.5 ml aliquot of plain type 2.0 μ m FITC-microspheres at the supplied concentration of 5.68×10^9 microspheres ml^{-1} was centrifuged twice at 2000 g for 5 min and resuspended in 1 ml sterile distilled water each time before being added to 5 ml unconjugated (i.e. non-fluorescent) Con A at a concentration of 50 $\mu\text{g ml}^{-1}$ in supplemented HEPES buffer. The sample was then incubated at 4 °C overnight in darkness on a roller to ensure thorough mixing before being twice centrifuged at 1000 g for 5 min and washed in 5 ml fresh supplemented HEPES buffer each time. A final 10 min centrifugation at 1000 g was performed before resuspension of pelleted microspheres in 500 μ l sterile supplemented HEPES and storage at 4 °C until use.

In addition, Con A was covalently bonded to 2.0 μ m diameter carboxylate FITC-microspheres using a carbodiimide reaction kit (Polysciences Inc., USA) according to the manufacturer's instructions (Appendix 3). Con A-microspheres were stored at 4 °C until use.

6.2.12.3 Determination of Con A loading on microspheres

The amount of Con A bound to plain or carboxylate FITC-microspheres was isolated and characterised using SDS-PAGE. SDS-PAGE gels were created in exactly the same manner as detailed previously (Section 6.2.11.2). In addition to 30 μl volumes of Con A standards prepared at concentrations of 15.6, 31.3, 62.5, 125, 250, 300 and 1000 $\mu\text{g ml}^{-1}$, 30 μl aliquots of Con A-modified plain and carboxylate microspheres at a concentration of approximately 10^9 microspheres ml^{-1} and 30 μl of the supernatant collected from the first wash following addition of Con A to microspheres during the carbodiimide binding reaction were all added separately to 30 μl volumes of sample loading buffer before denaturation on a heating block at 100 °C for 15 min. Sample volumes of 20 μl were introduced to separate wells of the gel using a fine syringe before electrophoresis at 200 V for 45 min.

The resulting gel was submersed in Coomassie Blue stain, maintained overnight with gentle agitation. The gel was then destained by immersion in destaining solution with further gentle agitation and periodic changes of destaining solution, as necessary, until all background stain had been eliminated. The gel was then photographed using a Transilluminator and camera system before densitometric analysis using Phoretix 1D Advanced Gel Analysis Software to determine sample protein content.

6.2.12.4 Confirmation of lectin activity

A haemagglutination assay, similar to that of Hussain *et al.* (1997), using a microtitre plate with V-shaped wells was employed to confirm Con A activity when bound to microspheres. Samples of 100 μl were added to the rows of wells in the following manner: Row 1: free unconjugated Con A (50 $\mu\text{g ml}^{-1}$) (positive control), Row 2: plain Con A-microspheres (5×10^7 microspheres ml^{-1}), Row 3: carboxylate Con A-microspheres (5×10^7 microspheres ml^{-1}), Row 4: FITC-microspheres (5×10^7 microspheres ml^{-1}) (negative control). Each sample was then diluted two-fold with PBS in the remaining wells of each row. Fresh horse blood (Oxoid, UK), diluted

in PBS to a concentration of 2 % (w/v), was added in 100 µl aliquots to each well. Samples were then incubated at 37 °C for 30 min before examination for haemagglutination (seen as the absence of an erythrocyte pellet at the bottom of the well), indicative of a positive result.

6.2.12.5 Acanthamoebal association with Con A-modified microspheres

Cultures of *Acanthamoeba* trophozoites were prepared in the customary manner of inoculating 9 ml sterile PYG broth with 1 ml confluent stock culture in sterile plastic universals before horizontal incubation in darkness at 37 °C. After 4 days of incubation, cultures were centrifuged twice at 1000 g for 10 min followed by resuspension in 10 ml amoebal saline each time and subsequent re-incubation at 37 °C in darkness for a further day. Trophozoite suspensions, at a concentration of approximately 10^6 cells ml⁻¹, were transferred in replicate 0.9 ml volumes to sterile Eppendorf tubes before the separate addition of 100 µl Con A-modified plain and carboxylate FITC-microspheres at a final concentration of 5×10^7 microspheres ml⁻¹. In addition to three replicate 0.9 ml aliquots of trophozoite suspension for each bead type, a negative control was set up with 100 µl amoebal saline in place of microspheres and three replicate positive control samples were prepared using 100 µl aliquots of unmodified 2.0 µm diameter plain or carboxylate FITC-microspheres. Samples were incubated at 37 °C in darkness on an orbital shaker for 1 hour before centrifugation at 1000 g for 10 min and resuspension in 1 ml amoebal saline. A further 10 min centrifugation at 1000 g and supernatant removal, allowed fixation of the pelleted amoebae in 300 µl 1 % (w/v) paraformaldehyde in amoebal saline. Samples were stored at 4 °C until analysis by flow cytometry using the Becton Dickinson FACS 440 housed at Birmingham University.

Results were arc sine transformed before being analysed by single factor ANOVA using the data analysis package of Microsoft® Excel 2000 to determine if the modification of microspheres with Con A had any significant effect on their association with *Acanthamoeba polyphaga* trophozoites.

6.2.13 Effect of carbohydrates on acanthamoebal association with microspheres

6.2.13.1 Pre-incubation of amoebae with carbohydrates

Four-day-old cultures of *Acanthamoeba polyphaga* were prepared by addition of 1 ml confluent culture to 9 ml sterile PYG broth in sterile plastic universals, incubated horizontally in darkness at 37 °C. Resulting trophozoites were subsequently starved following replacement of PYG broth with 10 ml amoebal saline after centrifugation twice at 1000 g for 10 min and re-incubation in darkness at 37 °C for a further day. Starved trophozoites were washed and resuspended in 1 ml of a solution of either glucose or mannose at a final concentration of 200 mM, following centrifugation twice at 1000 g for 10 min, and incubated at 37 °C for 4 hours in the presence of each carbohydrate. Three replicate 0.9 ml aliquots for each sample were transferred to sterile Eppendorf tubes at a concentration of approximately 10^6 cells ml⁻¹. Volumes of 100 µl of either plain or carboxylate 2.0 µm diameter FITC-microspheres were then added to each sample to a final concentration of 5×10^7 microspheres ml⁻¹ before samples were incubated in darkness at 37 °C on an orbital shaker for 1 hour. Parallel samples were also set up using three replicate 0.9 ml trophozoite suspensions unexposed to either mannose or glucose, incubated with 100 µl volumes of each type of microsphere as a positive control and an additional sample consisting of 0.9 ml amoebal trophozoites incubated with 100 µl amoebal saline served as a negative control for determination of background autofluorescence. After incubation, samples were centrifuged twice at 1000 g for 10 min before resuspension, firstly in 1 ml amoebal saline and finally in 300 µl 1 % (w/v) paraformaldehyde in amoebal saline. Samples were stored at 4 °C before analysis by flow cytometry using the Becton Dickinson FACS 440 of Birmingham University Medical School.

Results were converted to normally distributed data by arc sine transformation before analysis by single factor ANOVA using the data analysis package in Microsoft® Excel 2000 to determine whether either carbohydrate made any significant difference on the association of amoebal trophozoites with microspheres.

6.2.13.2 Binding of carbohydrates to microspheres

Trophozoites of *Acanthamoeba* were formed in the customary manner in sterile plastic universals by inoculation of 9 ml sterile PYG broth with 1 ml confluent stock cultures, which were incubated horizontally in darkness at 37 °C. Following incubation for 4 days, cultures were centrifuged twice at 1000 g for 10 min and resuspended in 10 ml amoebal saline each time before re-incubation at 37 °C in darkness for a further day.

Meanwhile, 0.5 ml volumes of both plain and carboxylate FITC-microspheres were transferred to sterile Eppendorfs to which either glucose or mannose was added to a concentration of 200 mM. Microspheres were incubated overnight in darkness at 37 °C on a roller to allow thorough mixing before centrifugation at 2000 g for 10 min and resuspension in 1 ml distilled water. Supernatants were retained for determination of carbohydrate concentration using a glucose testing kit (apparently suitable for any hexose sugar) (Sigma, USA) following the manufacturer's instructions (Appendix 4).

For each sample, three replicate 0.9 ml aliquots of trophozoite suspension at approximately 10^6 cells ml⁻¹ were placed in sterile Eppendorf tubes, to which 100 µl volumes of FITC-microspheres were added to a final concentration of 5×10^7 microspheres ml⁻¹. In addition to each of the four modified microsphere forms, untreated 2.0 µm diameter plain and carboxylate FITC-microspheres were used as positive controls, with three replicates. A negative control was also prepared with 100 µl amoebal saline in place of microspheres added to 0.9 ml trophozoite suspension. Samples were incubated in darkness at 37 °C on an orbital shaker for 1 hour before centrifugation at 1000 g for 10 min and resuspension in 1 ml amoebal saline. A second 10 min centrifugation at 1000 g allowed fixation of trophozoites in 300 µl 1 % (w/v) paraformaldehyde in amoebal saline. Samples were then stored at 4 °C until flow cytometry using the Becton Dickinson FACS 440 of Birmingham University Medical School.

Following arc sine transformation, results were analysed by single factor ANOVA using the data analysis package of Microsoft® Excel 2000 in order to determine if prior adsorption of carbohydrate to microspheres had any significant effect on their association with *Acanthamoeba*.

6.3 Results

6.3.1 Preliminary phagocytosis assay

Despite the reported marked phagocytic avidity of *Acanthamoeba* (Davies *et al.*, 1991), it was evident from the first few trials executed, demonstrating little apparent trophozoite-microsphere interaction, that the association and potential phagocytosis of microspheres was a more involved process. Modifications to experimental approaches were therefore required, including consideration of aspects such as the effect of trophozoite preparation and culture conditions on microsphere attachment, to evaluate more effectively the defining aspects of phagocytosis.

6.3.2 Effect of amoebal starvation on their association with microspheres

Figure 6.1 shows the effect of increasing incubation time in amoebal saline upon acanthamoebal association with 2.0 μm diameter plain FITC-microspheres. As can be seen, over the time investigated there was a general increase in association concomitant with increasing periods of incubation in amoebal saline, manifest as an increase from no amoebae with attached microspheres to around 70 % of amoebae observed having microspheres either attached or internalised. Single factor ANOVA confirmed that this increase was significant ($F = 4.54$; $P = 0.014$). The large standard errors, particularly those experienced amongst samples from extended incubation periods, were indicative of marked sample variation, a particular problem potentially associated with haemocytometer enumeration. An alternative method for evaluating the phagocytic process of microspheres was therefore sought resulting in the use of flow cytometry to define amoebal-microsphere association.

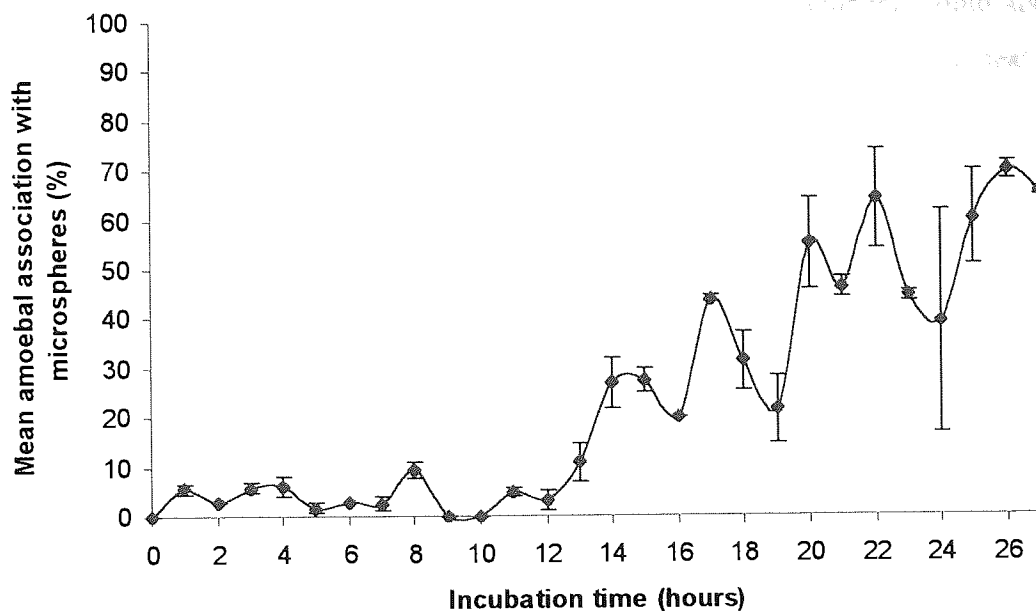


Figure 6.1. The effect of incubation time in amoebal saline on association of *Acanthamoeba polyphaga* (Leeds strain) trophozoites with 2.0 μm diameter plain FITC-microspheres, enumerated by use of a haemocytometer and fluorescence microscopy. Data points are means of three replicates and bars represent standard errors of the mean.

6.3.3 Flow cytometric determination of the effect of amoebal starvation on their association with microspheres

Figure 6.2 illustrates the effect of starvation on association of *Acanthamoeba polyphaga* (Leeds strain) trophozoites with 2.0 μm diameter microspheres of plain and carboxylate surfaces, as determined by flow cytometry. Typical flow cytometry profiles for trophozoite association with microspheres appear in Figure 6.3. Such profiles typically demonstrated two regions that corresponded to non-associated trophozoites and microsphere-associated trophozoites within the population. Results demonstrated that there was some difference both between acanthamoebal association and microsphere nature as well as the extent of microsphere association with starved and non-starved trophozoites. Two factor ANOVA indicated that both these differences were significant ($F = 146.83$; $P < 0.001$ and $F = 730.26$; $P < 0.001$, respectively). Furthermore these results also demonstrated the value of flow cytometry, which was able to detect attached plain and carboxylate microspheres in

association with amoebae whatever their status. In addition, the distribution (the number of microspheres associated with amoebal population members) could also be determined using this approach, as demonstrated in Figure 6.3, with discrete peaks for each number of associated microspheres. Figure 6.2 quite clearly shows that of the amoebae evaluated, members of a starved population in the main had more than four microspheres in association; in contrast non-starved amoebae were more usually associated with just one microsphere.

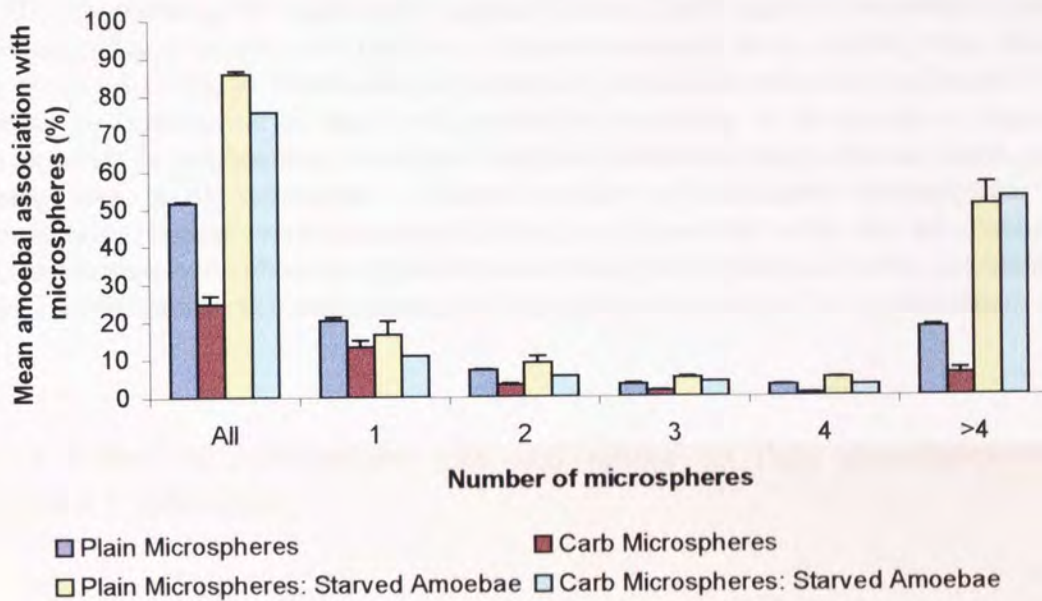


Figure 6.2. The effect of starvation of *Acanthamoeba polyphaga* (Leeds strain) trophozoites on association with 2.0 μm diameter plain and carboxylate FITC-microspheres, as determined by flow cytometry. All results are means of three replicates with standard errors of the mean represented by bars.

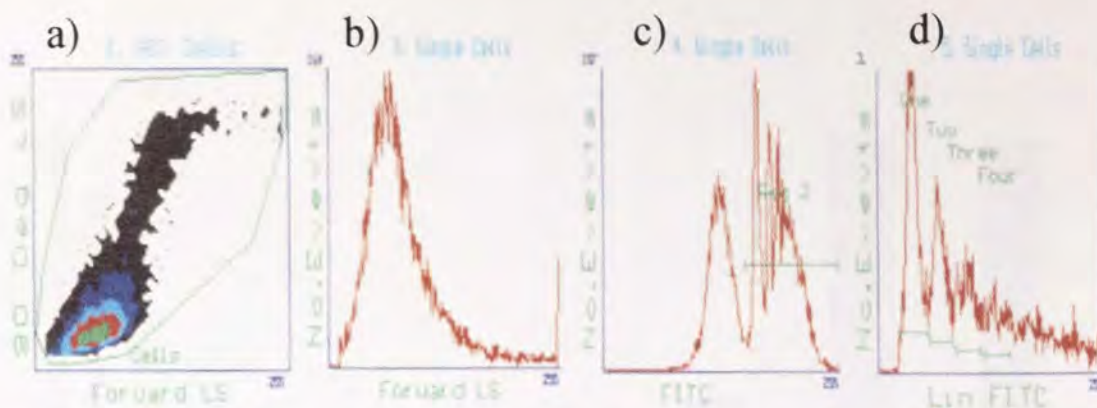


Figure 6.3. Representative flow cytometry profiles of phagocytosis of 2.0 µm diameter plain FITC-microspheres by *Acanthamoeba polyphaga* (Leeds strain) trophozoites. a) 2D plot showing 90° light scatter against forward light scatter from which a region corresponding to single cells (with or without associated microspheres) was selected for profiles b) – d); b) Distribution of single cell population according to forward light scatter; c) Distribution of single cell population according to fluorescence. Region 2 corresponds to trophozoites associated with microspheres – each of these peaks, more clearly seen in d), represents a different number of associated microspheres. The non-selected region represents trophozoites not associated with any microspheres; d) Distribution of trophozoite population according to fluorescence with, as indicated, peaks representing a different number of microspheres associated to trophozoites.

6.3.4 Effect of microsphere size and nature on their association with amoebal trophozoites

6.3.4.1 Flow cytometric determination of microsphere association with amoebal trophozoites

Figure 6.4 illustrates the effect of surface type and microsphere diameter on their association with starved *Acanthamoeba polyphaga* (Leeds strain) trophozoites. As can be seen there was a marked increase in acanthamoebal association with microspheres as microsphere diameter increased, for each microsphere surface type. Single factor ANOVA revealed that this increase in association was significant for both plain ($F = 168.46$; $P < 0.001$) and carboxylate surface types ($F = 496.27$; $P < 0.001$). Comparison of association and surface type, however, showed little marked significance, only the value obtained for 0.5 µm diameter microspheres ($F = 15.54$; $P = 0.017$) merited attention, compared to values for 1.0 µm ($F = 0.37$; $P = 0.577$) and 2.0 µm ($F = 2.10$; $P = 0.221$) diameter microspheres.

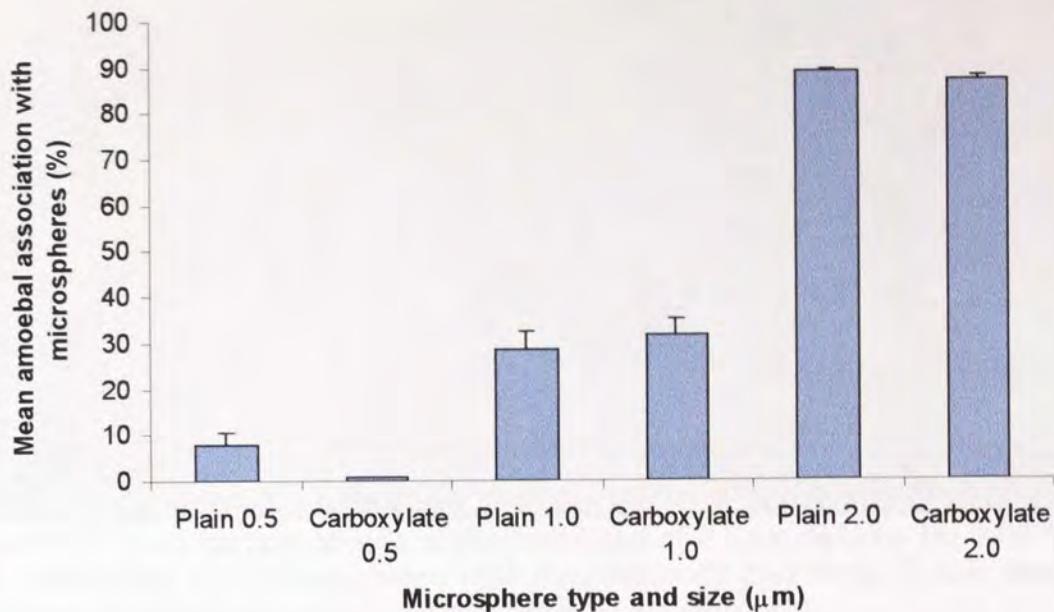


Figure 6.4. The effect of size and surface type of FITC-microspheres on association with starved *Acanthamoeba polyphaga* (Leeds strain) trophozoites, as determined by flow cytometry. Results are a mean of three replicates with bars representing standard errors of the mean for each set of replicates.

6.3.4.2 Confocal microscopy of microsphere association with amoebal trophozoites

Figures 6.5 – 6.7 show a series of confocal microscopy images of association between microspheres of different sizes and surface types and *Acanthamoeba polyphaga* (Leeds strain) trophozoites. As demonstrated, 0.5 μm diameter microspheres of both plain and carboxylate types (Figures 6.5a and 6.5b, respectively) show little association with trophozoites. The next size, 1.0 μm diameter microspheres, of each type (plain: Figure 6.6a; carboxylate: Figure 6.6b) show greater association with trophozoites, but, as in agreement with flow cytometry results of Section 6.3.4.1, the most marked association can be found with 2.0 μm diameter microspheres of both plain (Figure 6.7a) and carboxylate types (Figure 6.7b).

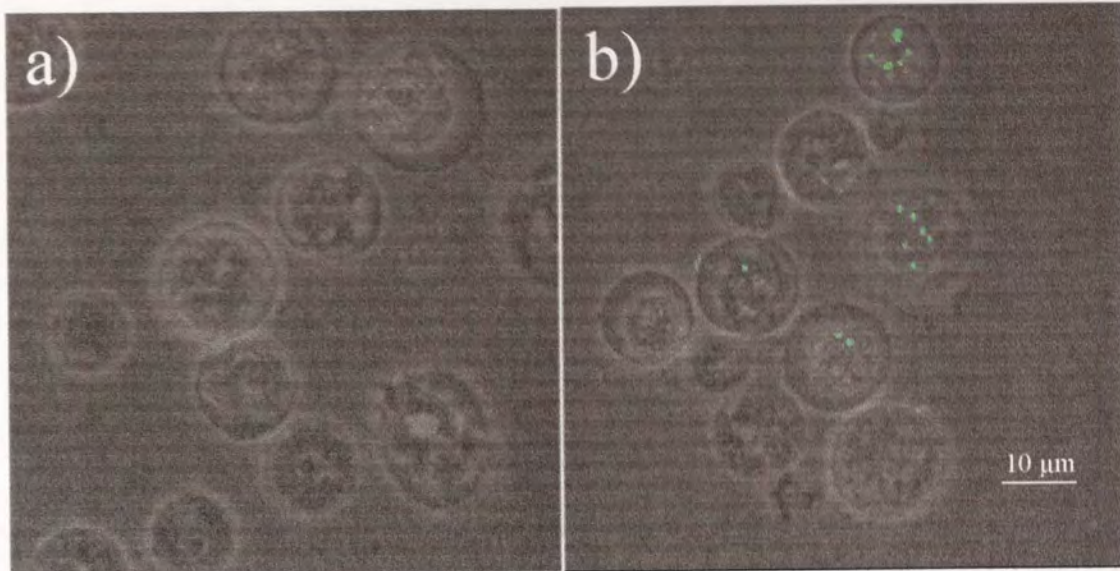


Figure 6.5. Confocal micrographs of the association of 0.5 μm diameter (a) plain and (b) carboxylate FITC-microspheres with *Acanthamoeba polyphaga* (Leeds strain) trophozoites. Both images are to the same scale, as indicated in b).

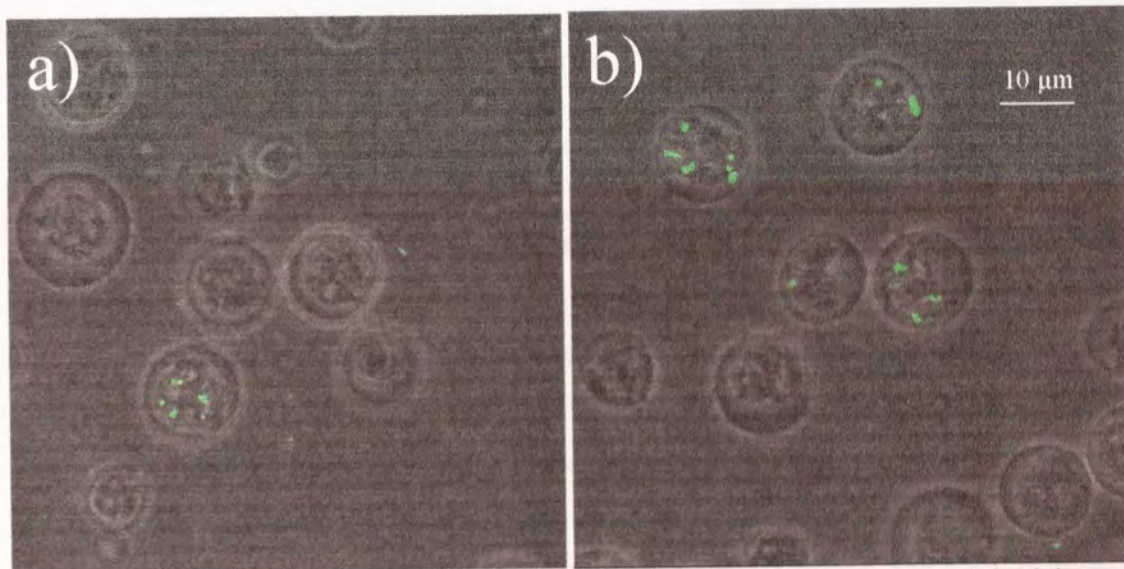


Figure 6.6. Confocal micrographs of the association of 1.0 μm diameter (a) plain and (b) carboxylate FITC-microspheres with *Acanthamoeba polyphaga* (Leeds strain) trophozoites. Both images are to the same scale, as indicated in b).

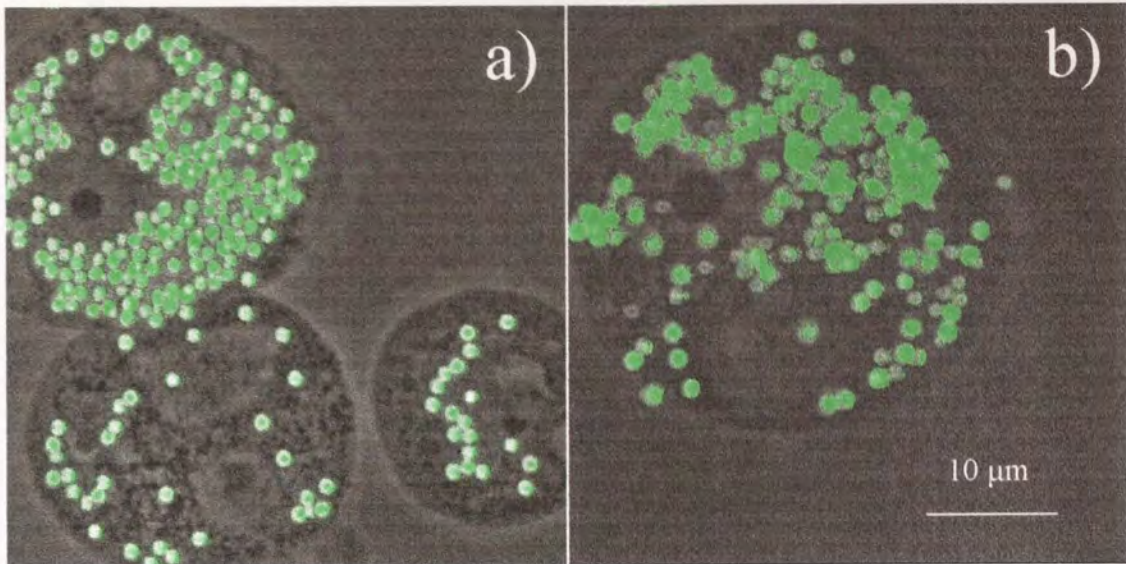


Figure 6.7. Confocal micrographs of the association of 2.0 μm diameter (a) plain and (b) carboxylate FITC-microspheres with *Acanthamoeba polyphaga* (Leeds strain) trophozoites. Both images are to the same scale, as indicated in b).

6.3.5 Effect of amoebal saline washing steps on amoebal association with plain microspheres

The effect of washing on association with microspheres by *Acanthamoeba polyphaga* (Leeds strain) trophozoites can be seen in Figure 6.8. For each microsphere size, there was an increase in association following post-incubation washing, which was significant for both 0.5 μm ($F = 43.29$; $P = 0.003$) and 2.0 μm ($F = 20.49$; $P = 0.011$) diameter microspheres, but not for those of 1.0 μm diameter ($F = 3.05$; $P = 0.156$). In addition, for reasons unknown, the association of 0.5 μm diameter microspheres was much greater than would normally be expected, greater than that of 1.0 μm diameter microspheres and even approaching that of 2.0 μm diameter microspheres.

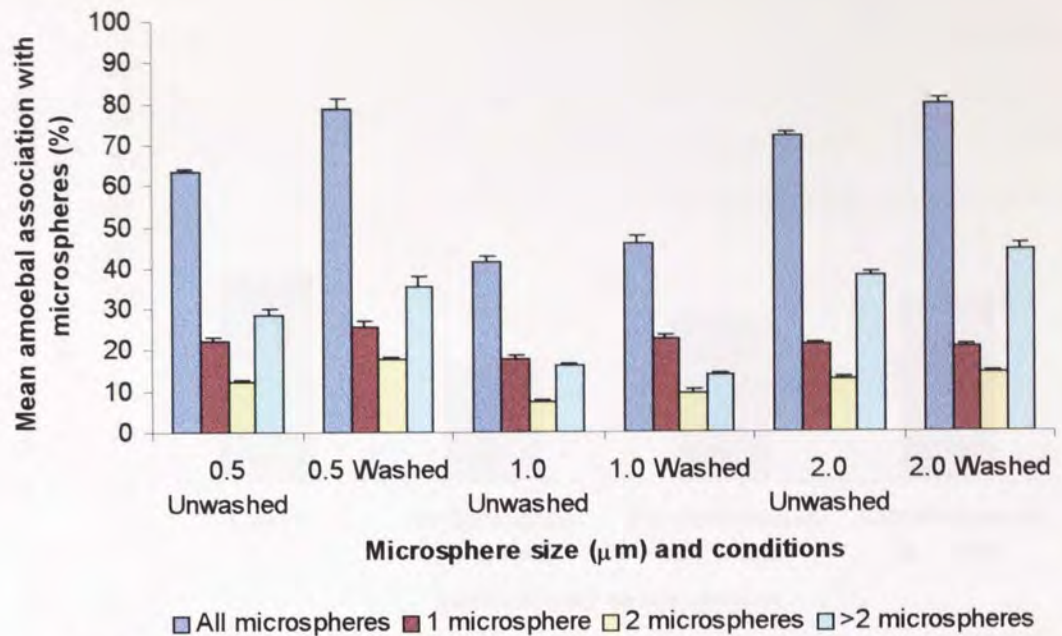


Figure 6.8. The effect of washing steps on association of different sized plain FITC-microspheres with *Acanthamoeba polyphaga* (Leeds strain) trophozoites, as determined by flow cytometry. Results are means of three replicates, with bars representing standard errors of the mean.

6.3.6 Effect of phagocytosis inhibitors on amoebal association with microspheres

Figure 6.9 shows the effect of phagocytosis inhibitors upon association of 2.0 μm diameter plain microspheres with *Acanthamoeba polyphaga* (Leeds strain) trophozoites as manifest through flow cytometry determinations. In comparison to the positive control, each inhibitor significantly reduced microsphere-trophozoite association, as significant reductions in proportions of amoebal association were obtained with inhibitor use (sodium azide: $F = 315.98$; $P < 0.001$; 2,4-dnp: $F = 46.02$; $P = 0.002$; cytochalasin B: $F = 12.69$; $P = 0.024$). However, there was also marked microsphere association with trophozoites in the presence of test inhibitors, indicative of substantial attachment of microspheres to trophozoite surfaces rather than phagocytosis. The results seen in Figure 6.9 indicate that sodium azide appeared to be the best inhibitor, though there were concerns that this agent was potentially altering the morphology of the trophozoites (data not shown), so its use was not considered further.

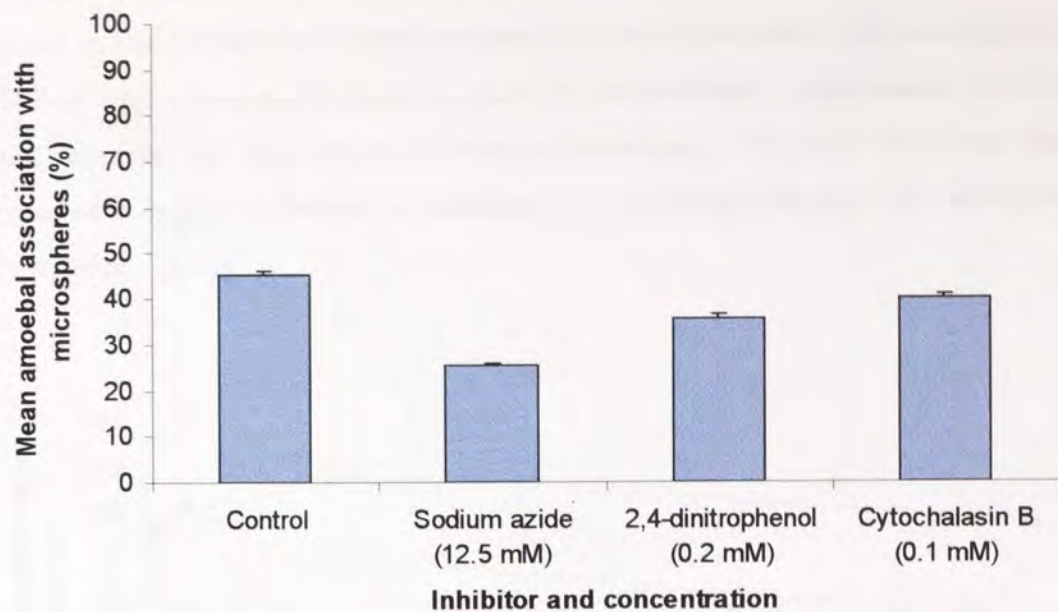


Figure 6.9. The effect of phagocytosis inhibitors on association of 2.0 μm diameter FITC-microspheres with *Acanthamoeba polyphaga* (Leeds strain) trophozoites, as determined by flow cytometry. Results are means of three replicates. Bars represent standard errors of the mean each set of replicates.

6.3.7 Effect of incubation time on amoebal association with microspheres

Figure 6.10 shows the association of 2.0 μm diameter plain microspheres with *Acanthamoeba polyphaga* (Leeds strain) trophozoites over a 24 hour time period. Figure 6.11 shows results of a similar experiment, but for 2.0 μm diameter carboxylate microspheres. In both cases, there was a general increase in acanthamoebal association with microspheres over time, with a marked and rapid increase in the first half hour of incubation, gradually increasing until five hours had passed, at which point there was a decline in the proportion of amoebae associating with plain microspheres, a phenomenon not found when investigating the carboxylate microsphere-trophozoite association. Furthermore, over the first few hours of incubation a lower percentage of trophozoites appeared to associate with carboxylate microspheres. Two factor ANOVA indicated that the increase over time was significant ($F = 40.11$; $P < 0.001$) and that there was also a significant difference between association of microsphere types with trophozoites ($F = 6.40$; $P = 0.015$). For both microsphere types there was an increase over time in the proportion of

trophozoites associated with more than two microspheres, concomitant with both a decrease in the proportion of amoebae associated with just one or two microspheres, as well as with a decrease in the proportion of non-associated trophozoites. After five hours, however, the proportion of amoebae associating with more than two plain microspheres began to decline, in contrast to a continued increase for carboxylate microspheres.

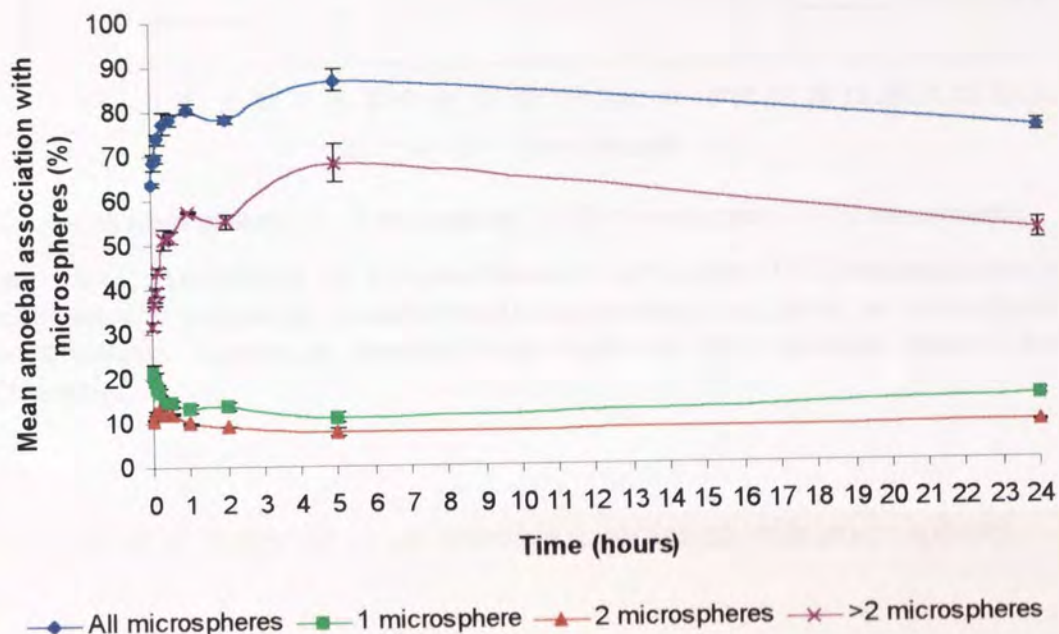


Figure 6.10. Association of 2.0 μm diameter plain FITC-microspheres with *Acanthamoeba polyphaga* (Leeds strain) trophozoites over time, as determined by flow cytometry. Results are means of three replicate samples, with standard errors of the mean represented by bars.

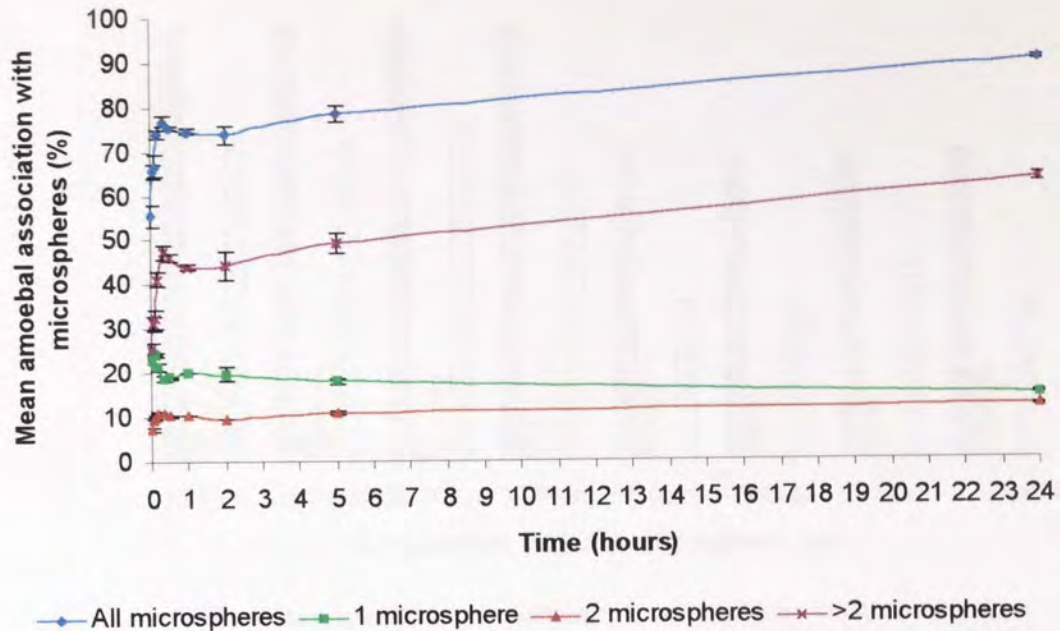


Figure 6.11. Association of 2.0 μm diameter carboxylate FITC-microspheres with *Acanthamoeba polyphaga* (Leeds strain) trophozoites over time, as determined by flow cytometry. Results are means of three replicates. Bars represent standard errors of the mean.

6.3.8 Effect of temperature on amoebal association with microspheres

Figure 6.12 shows the effect of temperature on the association of 2.0 μm diameter plain and carboxylate microspheres with *Acanthamoeba polyphaga* (Leeds strain) trophozoites. Two factor ANOVA indicated that, for each microsphere type, there was no significant effect of temperature on association ($F = 1.92$; $P = 0.167$), though carboxylate microspheres associated with amoebae significantly less than plain microspheres ($F = F = 2514.81$; $P < 0.001$).

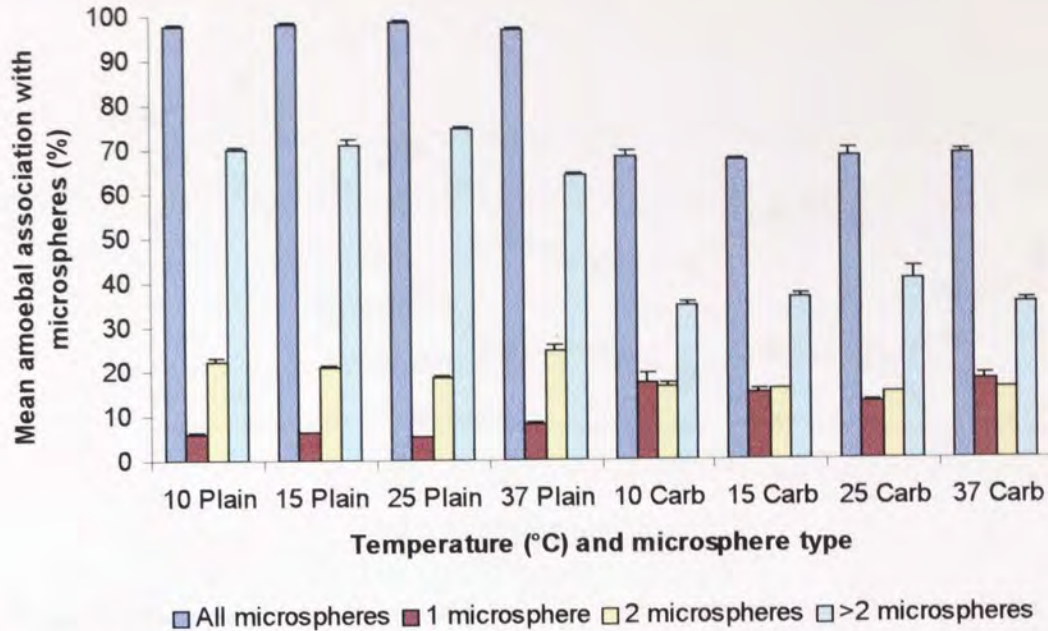


Figure 6.12. The effect of temperature on association of plain and carboxylate 2.0 μm diameter FITC-microspheres with *Acanthamoeba polyphaga* (Leeds strain) trophozoites, as determined by flow cytometry. Results are the means of three replicates, with standard errors of the means represented by bars.

6.3.9 Effect of amoebal cell age on microsphere association

Figure 6.13 shows how association of 2.0 μm diameter plain microspheres varied with *Acanthamoeba polyphaga* (Leeds strain) trophozoite age. Similarly, Figure 6.14 illustrates results obtained using 2.0 μm diameter carboxylate microspheres. With increasing age of trophozoites, for both microsphere types, association declined markedly. Single factor ANOVA indicated that this decrease was significant for both plain ($F = 194.50$; $P < 0.001$) and carboxylate microspheres ($F = 421.10$; $P < 0.001$). For both microsphere types, there was also a decline in the proportion of amoebae associated with more than two microspheres (plain: $F = 107.24$; $P < 0.001$; carboxylate: $F = 907.93$; $P < 0.001$), concomitant with an increase in the proportions of amoebae associating just one (plain: $F = 107.50$; $P < 0.001$; carboxylate: $F = 118.87$; $P < 0.001$) or two (plain: $F = 275.49$; $P < 0.001$; carboxylate: $F = 153.36$; $P < 0.001$) microspheres.

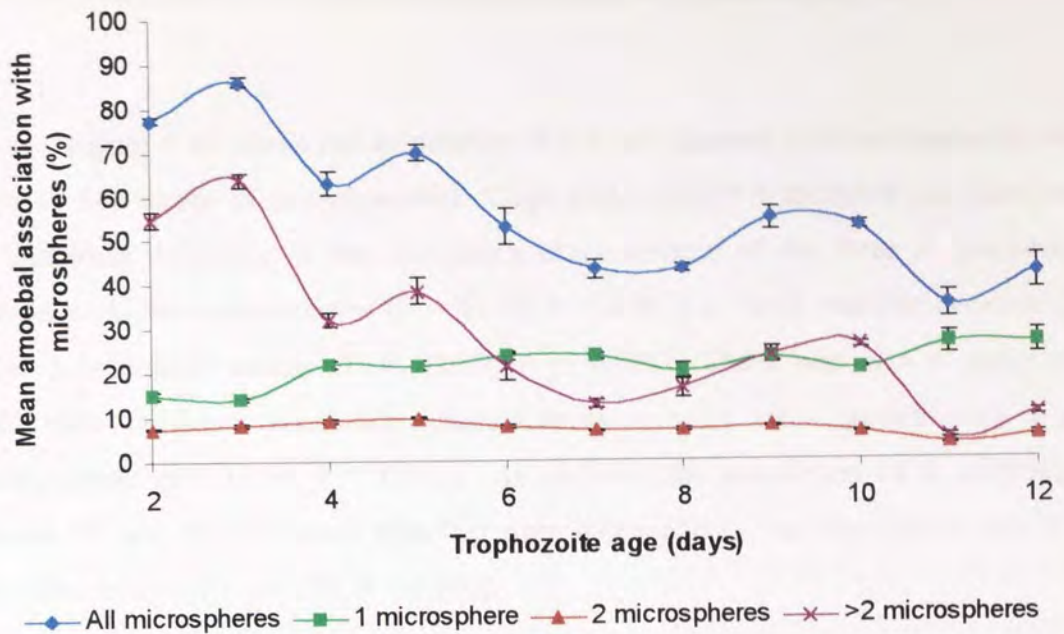


Figure 6.13. Association of 2.0 µm diameter plain FITC-microspheres with *Acanthamoeba polyphaga* (Leeds strain) trophozoites of increasing age, as determined by flow cytometry. Results are means of three replicates with bars representing standard errors of the mean.

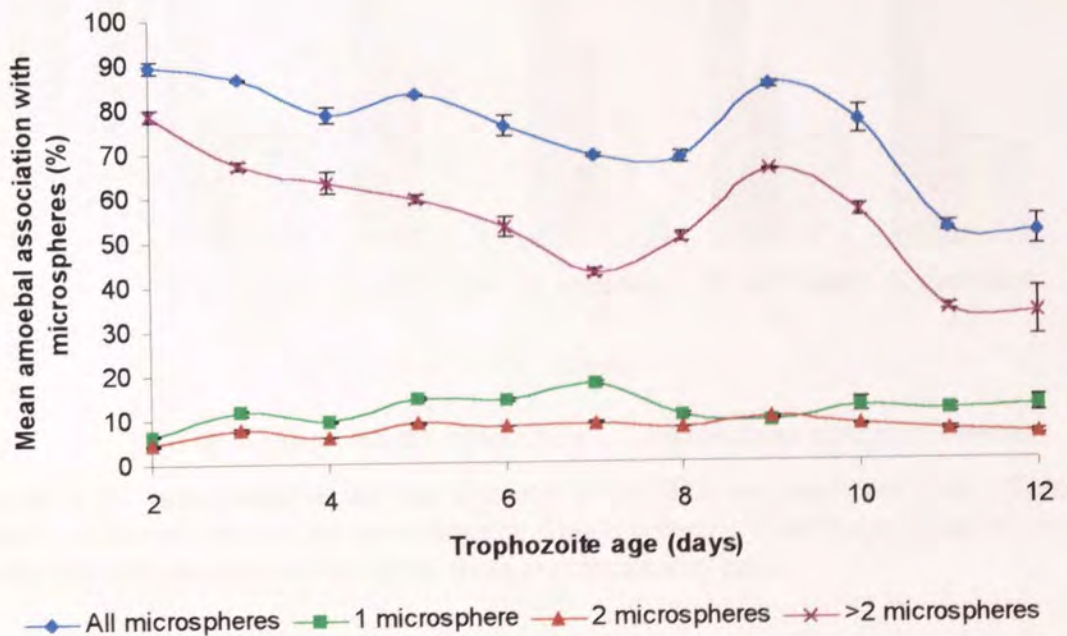


Figure 6.14. Association of 2.0 µm carboxylate FITC-microspheres with *Acanthamoeba polyphaga* (Leeds strain) trophozoites of increasing age, as determined by flow cytometry. Results are means of three replicates. Bars represent standard errors of the mean of triplicate results.

6.3.10 Association of *Acanthamoeba* strains with microspheres

Figure 6.15 shows the association of 2.0 µm diameter plain microspheres with each of five strains of *Acanthamoeba*. Single factor ANOVA indicated that there was a significant difference in the association characteristics of the three *A. polyphaga* strains with plain microspheres ($F = 44.79$; $P < 0.001$) as there was also between the two *A. castellanii* strains ($F = 42.55$; $P = 0.003$). There was also a significant difference between association characteristics of the two species with plain microspheres ($F = 35.60$; $P < 0.001$). In addition, the association of *A. polyphaga* strains 3A and 3B with more than two plain microspheres was significantly less than for other strains ($F = 488.56$; $P < 0.001$).

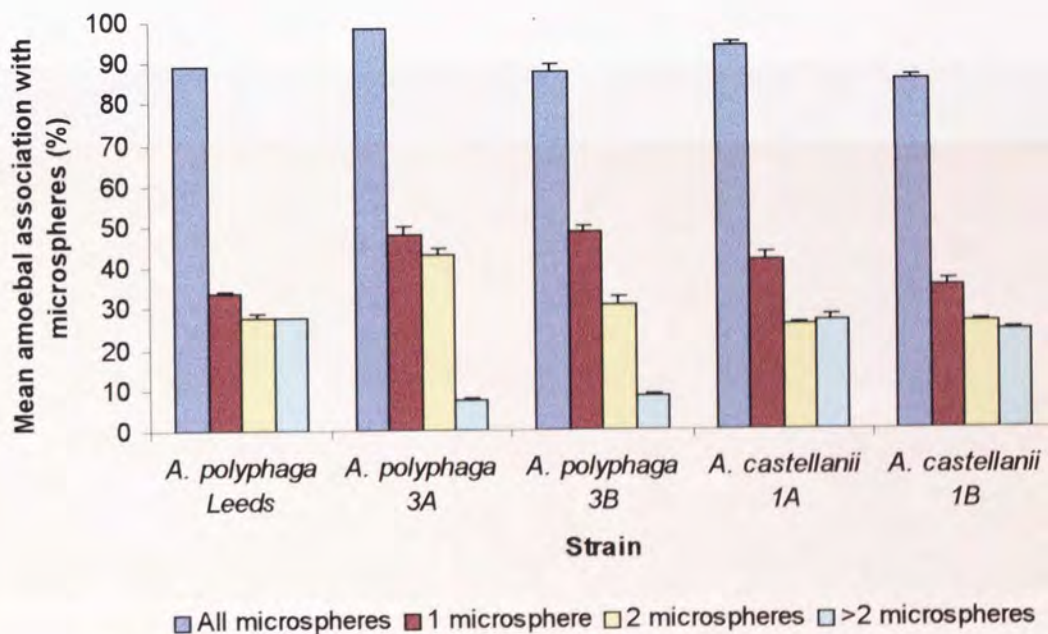


Figure 6.15. Association of 2.0 µm diameter plain FITC-microspheres with different strains of *Acanthamoeba*, as determined by flow cytometry. Results are means of three replicates with standard errors of the mean represented by bars.

6.3.11 Acanthamoebal association with protein coated microspheres

6.3.11.1 Quantification of protein adsorption

Figure 6.16 shows an SDS-PAGE characterisation of BSA-coated microspheres, as compared to seven standard concentrations of BSA. Densitometric analysis of these standards enabled construction of a standard curve (data not shown) from which the concentrations of BSA adsorbed to the surface of 1.0 μm and 2.0 μm microspheres (approximately 1.67×10^8 of each size microsphere) could be estimated at $1.09 \mu\text{g ml}^{-1}$ and $4.55 \mu\text{g ml}^{-1}$ respectively. These concentrations are roughly equal per unit of surface area, however, as a doubling of diameter (and hence also of radius) of a sphere equates with a four-fold increase in surface area.

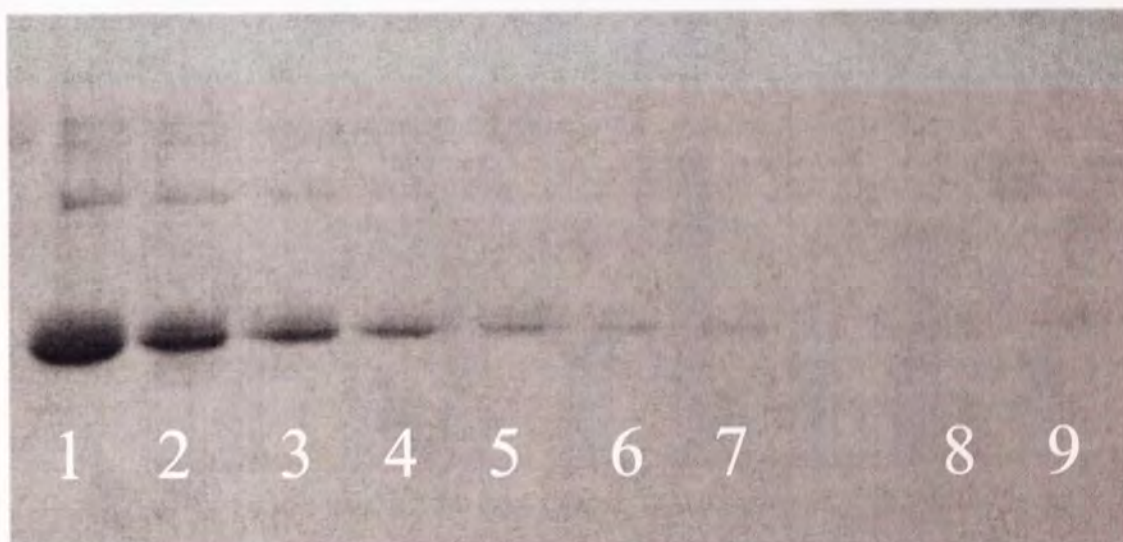


Figure 6.16. SDS-PAGE analysis of BSA-coated microspheres. Lane 1: BSA standard ($250 \mu\text{g ml}^{-1}$), 2: BSA standard ($125 \mu\text{g ml}^{-1}$), 3: BSA standard ($62.5 \mu\text{g ml}^{-1}$), 4: BSA standard ($31.3 \mu\text{g ml}^{-1}$), 5: BSA standard ($15.6 \mu\text{g ml}^{-1}$), 6: BSA standard ($7.8 \mu\text{g ml}^{-1}$), 7: BSA standard ($3.9 \mu\text{g ml}^{-1}$), 8: 1.0 μm diameter BSA-microspheres, 9: 2.0 μm diameter BSA-microspheres.

6.3.11.2 Acanthamoebal association with protein-coated microspheres

Figure 6.17 shows the difference in association of BSA-coated 1.0 μm and 2.0 μm microspheres in comparison to their uncoated plain counterparts. Single factor ANOVA indicated that there was no difference between association of uncoated and BSA-coated 1.0 μm microspheres ($F = 5.88$; $P = 0.072$), unlike the significant decrease in acanthamoebal association found on comparison of BSA exposed 2.0 μm microspheres to uncoated plain 2.0 μm microspheres ($F = 225.63$; $P < 0.001$). In addition, differences between association of 1.0 μm and 2.0 μm microspheres with trophozoites were significant for both uncoated ($F = 357.33$; $P < 0.001$) and BSA-coated ($F = 23.94$; $P = 0.008$) surface types, confirming that trophozoites showed, possibly irrespective of microsphere manipulation or nature, marked size preference for 2.0 μm microspheres as used in these investigations (Section 6.3.4).

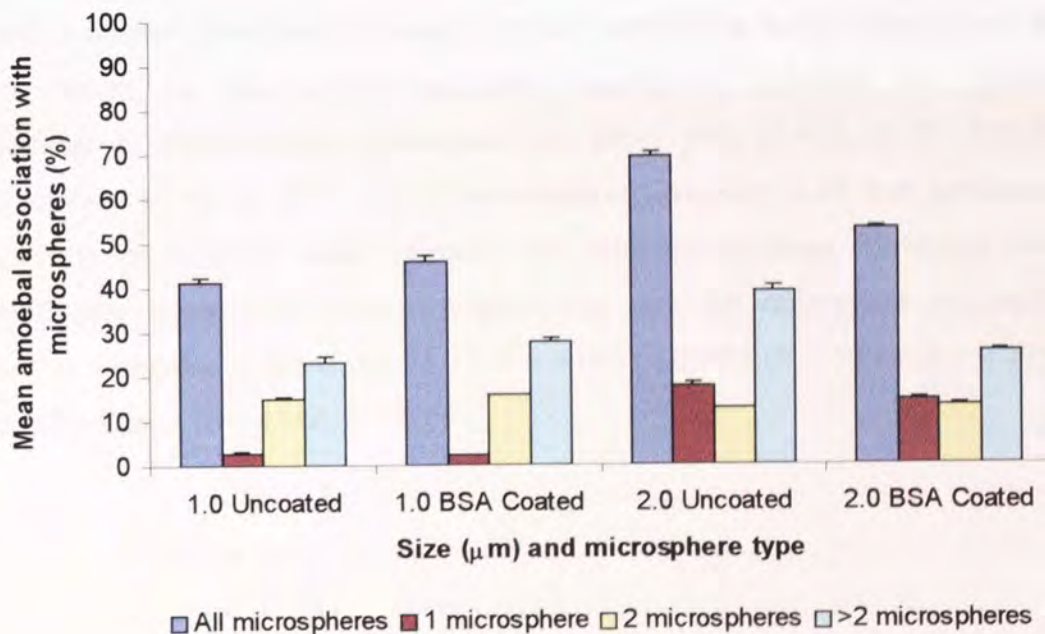


Figure 6.17. The effect of BSA-adsorption to the surface of 1.0 μm and 2.0 μm diameter FITC-microspheres on association with *Acanthamoeba polyphaga* (Leeds strain) trophozoites. Results are means of three replicates with standard errors of the mean represented by bars.

6.2.12 Effect of Con A on acanthamoebal association with microspheres

6.2.12.1 Association of microspheres with Con A bound *Acanthamoeba polyphaga*

Figure 6.18 demonstrates that Con A binding to *Acanthamoeba polyphaga* (Leeds strain) trophozoites had an effect of slightly increasing microsphere association. Single factor ANOVA, however, indicated that although Figure 6.18 shows some increase in association of plain microspheres with Con A treated trophozoites, such an observation was significant for carboxylate microspheres alone ($F = 57.21$; $P = 0.002$), and not for plain microspheres ($F = 2.49$; $P = 0.190$). Although a decrease in association could have been observed, there would have been limited potential for increasing association from the already marked proportion of around 93 % of untreated amoebae that were found associated with plain microspheres in the positive control. Comparison amongst positive controls, as to the effect of test ionic environment on microsphere-trophozoite association, indicated no significant difference in acanthamoebal association with either plain ($F = 0.51$; $P = 0.515$) or carboxylate ($F = 0.46$; $P = 0.536$) microspheres, irrespective of test environment. Single factor ANOVA also indicated that plain microspheres associated with a significantly larger proportion of trophozoites than did carboxylate microspheres whether suspended in saline ($F = 75.95$; $P = 0.001$), HEPES ($F = 59.48$; $P = 0.002$) or bound to Con A ($F = 43.06$; $P = 0.003$).

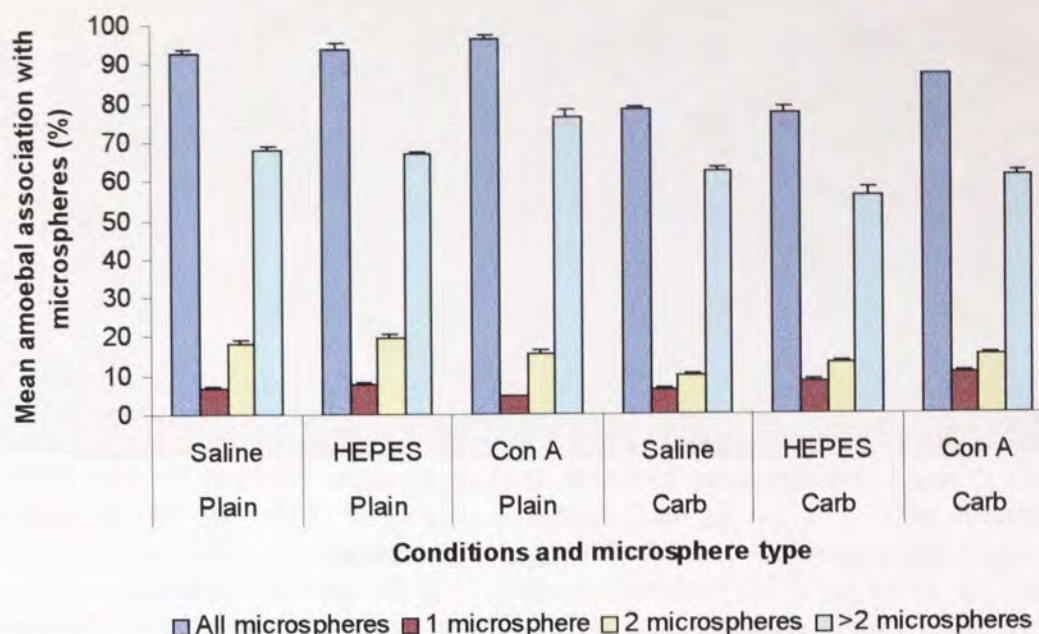


Figure 6.18. The effect of Con A binding to *Acanthamoeba polyphaga* (Leeds strain) trophozoites on association with plain and carboxylate 2.0 μm diameter microspheres, as determined by flow cytometry. Results are means of three replicates. Bars represent standard errors of the mean of three replicates.

6.3.12.2 Determination of Con A loading on microspheres

SDS-PAGE analysis of potential Con A loading onto microspheres and seven comparative Con A standards is shown in Figure 6.19. Following densitometric analysis, a standard curve was constructed (data not shown) from which concentrations of Con A bound to the surface of 2.0 μm microspheres was determined. For passive adsorption to plain microspheres, a Con A concentration equivalent to 295 $\mu\text{g ml}^{-1}$ was attained. Given that the concentration of microspheres was at approximately 10^9 microspheres ml^{-1} , this equates to 2.95×10^{-7} μg Con A on each microsphere; a very small amount. SDS-PAGE of covalently bonded Con A to carboxylate microspheres, on the other hand, could not be determined by this method, possibly on account of the robust nature of Con A-microsphere binding, in turn potentially manifest as a smeary profile. Furthermore, analysis of the supernatant collected during the carbodiimide binding procedure indicated marked Con A presence, with a band intensity not dissimilar from that of the 1 mg ml^{-1} standard. Thus, if binding had occurred, it was not to a great extent.

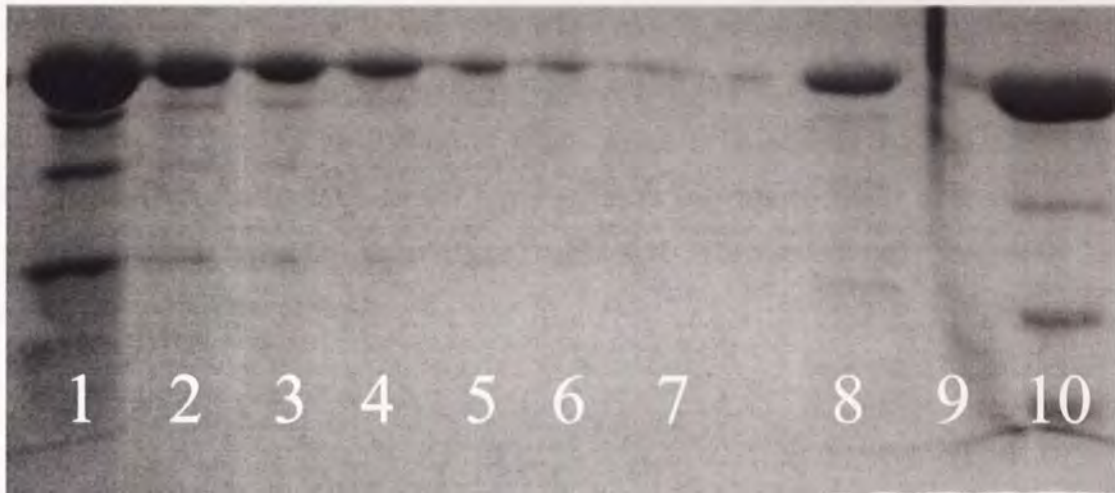


Figure 6.19. SDS-PAGE analysis of Con A-coated microspheres. Lane 1: Con A standard ($1000 \mu\text{g ml}^{-1}$), 2: Con A standard ($300 \mu\text{g ml}^{-1}$), 3: Con A standard ($250 \mu\text{g ml}^{-1}$), 4: Con A standard ($125 \mu\text{g ml}^{-1}$), 5: Con A standard ($62.5 \mu\text{g ml}^{-1}$), 6: Con A standard ($31.3 \mu\text{g ml}^{-1}$), 7: Con A standard ($15.6 \mu\text{g ml}^{-1}$), 8: $2.0 \mu\text{m}$ diameter Con A plain microspheres, 9: $2.0 \mu\text{m}$ diameter Con A carboxylate microspheres, 10: Supernatant from Con A carbodiimide binding reaction.

6.3.12.3 Confirmation of lectin activity

Lectin activity of Con A adsorbed or bound to microspheres was compared to a positive control of unbound, free Con A in a microtitre plate assay. Haemagglutination was observed in samples of both plain and even carboxylate Con A-microspheres. In turn the nature and extent of haemagglutination suggested Con A concentrations equivalent to those of the positive control, thereby indicating that lectin activity was not diminished by the action of adsorption. In addition, the negative control of unmodified microspheres showed no haemagglutination.

6.3.12.4 Acanthamoebal association with Con A-modified microspheres

Figure 6.20 demonstrates the association of *Acanthamoeba polyphaga* (Leeds strain) with $2.0 \mu\text{m}$ diameter plain and carboxylate microspheres, either unmodified or conjugated with Con A. The effect of modifying microspheres with Con A appeared to slightly increase association with trophozoites, although this was not significant for

either microsphere type (plain: $F = 5.08$; $P = 0.087$; carboxylate: $F = 5.35$; $P = 0.082$). However, in similar circumstances to those noted above in Section 6.3.12.1, both controls had a marked proportion of trophozoites in associated with microspheres, thus it was potentially unlikely for any significant increase to occur over and above these elevated baseline proportions, although, correspondingly, decreases in association would be obvious.

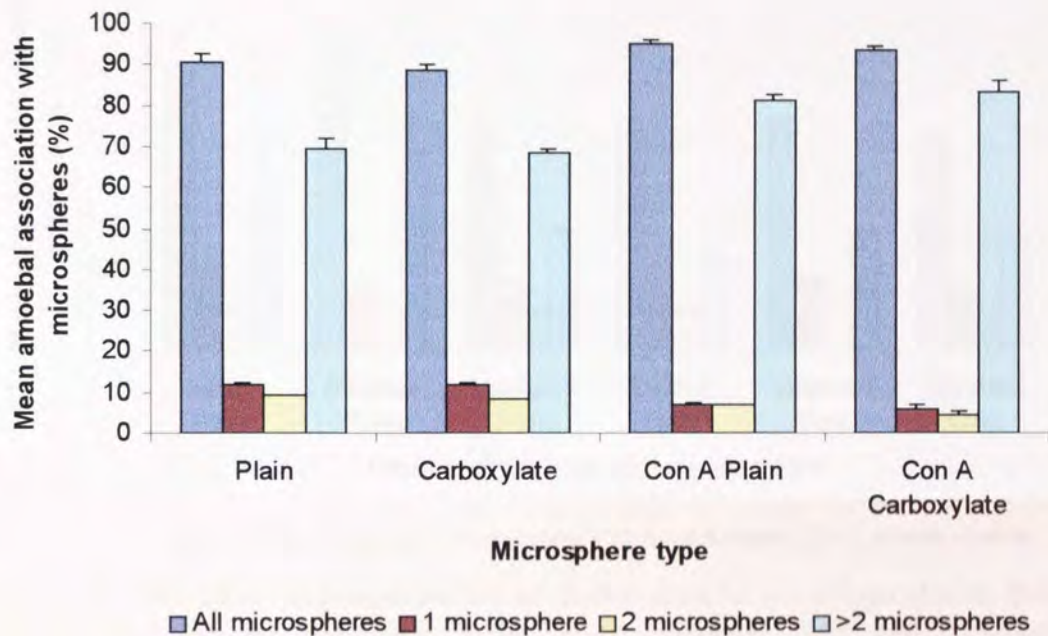


Figure 6.20. Association of Con A-modified 2.0 μm diameter plain and carboxylate FITC-microspheres with *Acanthamoeba polyphaga* (Leeds strain) trophozoites, as determined by flow cytometry. Results are means of three experiments with standard errors of the mean represented by bars.

6.3.13 Effect of carbohydrates on acanthamoebal association with microspheres

6.3.13.1 Pre-incubation of amoebae with carbohydrates

The effect of pre-incubation of *Acanthamoeba polyphaga* (Leeds strain) trophozoites with mannose and glucose upon association with 2.0 μm diameter plain and carboxylate microspheres is illustrated in Figure 6.21. No significant difference in

association was found following trophozoite pre-treatment with either carbohydrate for either plain (glucose: $F = 0.11$; $P = 0.757$; mannose: $F = 2.45$; $P = 0.193$) or carboxylate (glucose: $F = 0.56$; $P = 0.494$; mannose: $F = 0.47$; $P = 0.53$) microsphere types.

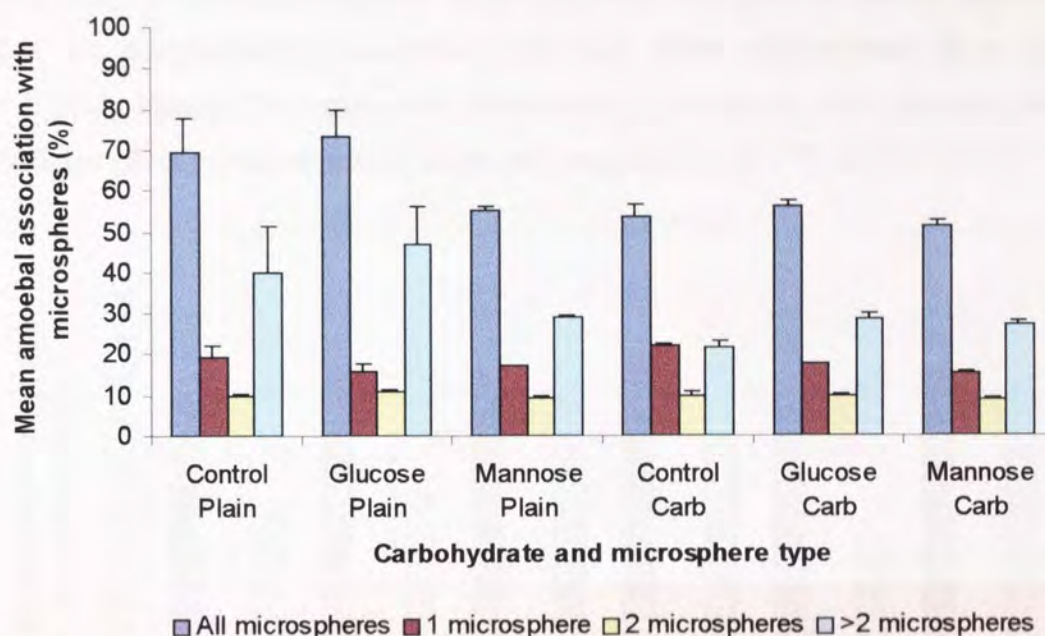


Figure 6.21. The effect of pre-incubation of *Acanthamoeba polyphaga* (Leeds strain) with carbohydrates on association with 2.0 μm diameter plain and carboxylate FITC-microspheres, as determined by flow cytometry. Results are means of three replicates with bars representing standard errors of the mean.

6.3.13.2 Binding of carbohydrates to microspheres

Determination of hexose concentration of the supernatant, collected from washing microspheres, indicated that mannose appeared to bind to both microsphere types, though glucose only appeared to bind well to carboxylate microspheres. The use of the glucose testing kit, however, was not without flaws, as the concentration of known solutions of glucose and mannose were determined by the kit as being of much lower concentration and, in addition, mannose was not detected well by the kit, despite claims of suitability for any hexose sugar.

Figure 6.22 illustrates the effect of pre-incubation of 2.0 μm diameter plain and carboxylate microspheres with glucose and mannose on association with *Acanthamoeba polyphaga* (Leeds strain) trophozoites. Statistical analysis by single factor ANOVA did not reveal any clear pattern, as plain microspheres incubated with mannose significantly increased association ($F = 9.03$; $P = 0.040$), although carboxylate microspheres incubated with mannose had no significant effect ($F = 0.41$; $P = 0.555$). Conversely, incubation of microspheres with glucose had no significant effect on acanthamoebal association with such plain microspheres ($F = 4.09$; $P = 0.113$), though for carboxylate microspheres, incubation with glucose had a significant effect on reducing association with trophozoites ($F = 75.43$; $P = 0.001$).

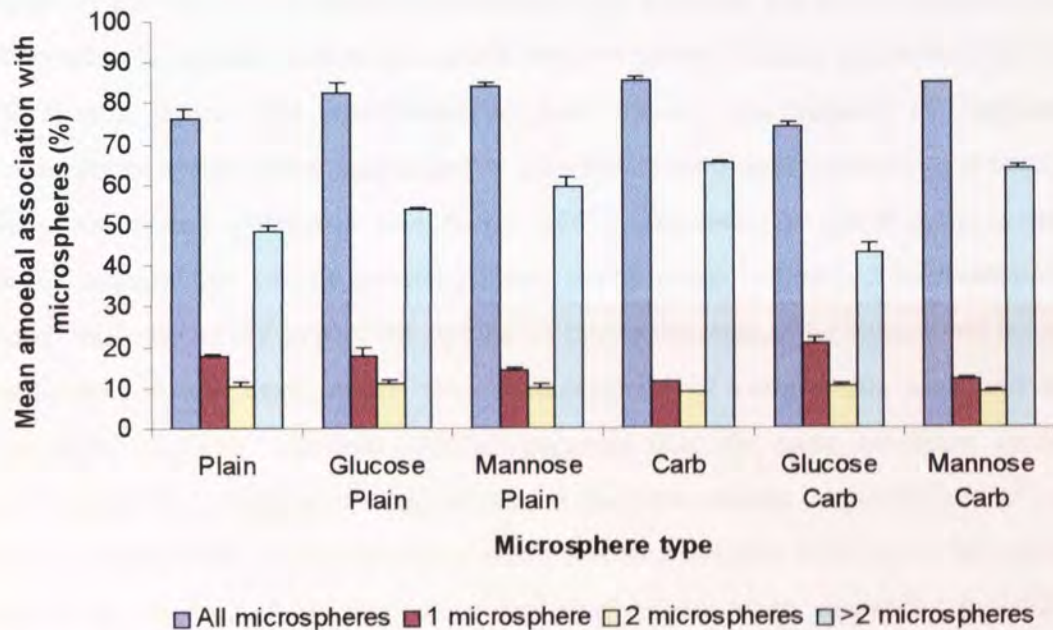


Figure 6.22. The effect of 2.0 μm diameter plain and carboxylate FITC-microspheres incubated with carbohydrates upon association with *Acanthamoeba polyphaga* (Leeds strain) trophozoites, as determined by flow cytometry. Results are means of three replicates. Bars represent standard errors of the mean of three replicates.

6.4 Discussion

When suspended in PYG broth, little, if any, uptake of fluorescent polystyrene latex microspheres was detected by direct microscopic observation. Flow cytometry, potentially due to the greater number of cells assessed, revealed a greater proportion

of broth-suspended trophozoites associated with microspheres. However, the proportion of trophozoites with microspheres was significantly lower than that for similar trophozoites incubated in amoebal saline. Such an increase in trophozoite association with microspheres following incubation in amoebal saline was thought to arise from a starvation effect – in other words, it was considered that, when suspended in broth, there was no requirement for amoebae to take up any microspheres, as sufficient nutrients could be acquired from PYG broth by pinocytosis alone. Uptake of larger substances by phagocytosis would therefore be unnecessary, even though phagocytosis may suppress pinocytosis (Bowers, 1977). This finding contrasts with observations of other workers who have conducted successful phagocytosis assays in normal growth medium (Chambers and Thompson, 1976; Weisman and Korn, 1967; Davies *et al.*, 1991). The latter also claimed that amoebae are just as phagocytically active when in growth medium. In accord with the present results, however, Harf *et al.* (1997) also found that *Acanthamoeba palestinensis* was capable of *Legionella pneumophila* uptake when suspended in amoebal saline despite reports of a required adaptation period (Weisman and Korn, 1967). However, a saline medium more closely mirrors that of any natural aquatic environment in which *Acanthamoeba* is found, thus one would expect the uptake of prey substrates to be unimpaired by such conditions. In any case, rather than a multiplicity of components as found in a “complete” medium, Absolom (1986a) suggests that the most important medium constituents for phagocytosis appear to be divalent cations, particularly Ca^{2+} and Mg^{2+} , a requirement met by the use of either amoebal saline or PYG broth. In contrast, association was not diminished when test trophozoites were suspended in HEPES buffer lacking the presence of such cations, indicative of little marked ion requirement by trophozoites participating in this investigation.

One inherent difficulty with phagocytosis assays is that of determining microsphere localisation and status, in particular whether they have been engulfed or were just attached to trophozoite surfaces (Al-Ibrahaim *et al.*, 1976). In the light of present experimentation, demonstrating considerable trophozoite association with varying numbers of microspheres, it is unlikely that all the microspheres associated with cells were present only on the outside. Furthermore, confocal micrographs (Figures 6.5 – 6.7) appear to confirm that most associated microspheres were found internally. This viewpoint is supported in that microsphere distribution was not

uniform; few were ever observed in the region of either the nucleus or contractile vacuole, an unlikely eventuality if merely surface restricted.

A significant increase in trophozoite-microsphere association with increasing microsphere diameter was noted. Post-incubation washing was found to increase trophozoite-microsphere interaction, due to the potential for increased trophozoite-microsphere contact brought about by pelleting. However, such an event was observed for all microsphere sizes, thus any artificial elevation of association with trophozoites was not size-related and could thus be disregarded. Hence, 2.0 μm diameter microspheres were chosen in the majority of experiments due to apparent amoebal preference for such microspheres thereby leading to marked cell-microsphere fluorescence and ease of detection by flow cytometry when FITC-microspheres were employed. Indeed, 0.5 μm size microspheres were often too small, relative to *Acanthamoeba* trophozoite size, to be detected efficaciously by flow cytometry, leading to potentially ambiguous results.

In addition to an increased proportion of amoebae associating with larger microspheres, confocal images (Figures 6.5 – 6.7) suggest that trophozoites engulf a greater number of the largest microspheres (Figure 6.7) compared to intermediate (Figure 6.6) and small microsphere sizes (Figure 6.5). The number of microspheres associated with trophozoite populations was often revealed by flow cytometry and, in cases where such microsphere distribution was defined, most amoebae were associated with more than two microspheres. A relationship between feeding and size has also been noted for a dinoflagellate alga, *Oxyrrhis marina*, by Hammer *et al.* (1999), who found a similar increase in particle capture with increasing particle size. Furthermore, Tabata and Ikada (1987) demonstrated an increase in the number of microspheres per macrophage corresponding to an increase in microsphere diameter to a maximum of 2 μm . Larger microspheres were found in fewer numbers, which was perhaps not surprising given physical restrictions of macrophage size. Korn and Weisman (1967) observed that larger microspheres were apparently taken up individually by *Acanthamoeba* in contrast to smaller sized microspheres, which were taken up after accumulation of a number on the surface. However, such a finding does not correlate with an increased trophozoite-microsphere association (i.e. both phagocytosed and surface-attached microspheres) with greater microsphere diameter determined in the present study, as, if true, one may expect to find little difference in the proportion of amoebae associating with microspheres of any size. Furthermore, the

study of Korn and Weisman (1967) made no apparent investigation of such proportions of *Acanthamoeba* associated with microspheres of each size.

Microsphere surface nature was often found to have an apparent bearing on their association with *Acanthamoeba polyphaga* (Leeds strain) with generally fewer trophozoites interacting with carboxylate type microspheres irrespective of size. As carboxylate microspheres were generally found to be less hydrophobic than their respective plain counterparts (true for all except for 2.0 μm size microspheres) (Section 4.3.2.4), this result may be regarded consistent with studies by Bowers and Olszewski (1983) who considered that latex microspheres primarily interacted with *Acanthamoeba* by hydrophobic interactions. In addition, the slightly more negative ζ -potential of carboxylate microspheres compared to their plain counterparts (Section 4.3.1.5) would potentially have something of a repellent effect on interactions with carboxylate microspheres. Ayhan *et al.* (1995) also found that carboxyl groups present on microspheres reduced the number of microspheres taken up by leucocytes and macrophages, in contrast to positively charged microspheres, and that hydrophobic interactions increased contact between phagocytes and microspheres. Furthermore, Tabata and Ikada (1987) indicate that in phagocytosis by macrophages, only microspheres with a zero ζ -potential were not taken up. These various observations on the effect of both electrostatic and hydrophobic interactions, taken with the present results indicate that surface nature of phagocytic substrates, relative to that of phagocytes, has an important influence on whether or not such particles will firstly attach and, secondly, be ingested.

The efficacy of phagocytosis inhibitors was investigated primarily to establish which single agent could be used to prevent further phagocytosis as part of time-course phagocytosis assessments in turn designed to ascertain any contribution from physiologically active entities to attachment or onset of phagocytosis. Experimental results revealed considerable surface microsphere attachment, which somewhat compromised experimental interpretation. However, as the action of the chosen agent is to inhibit ingestion rather than attachment, the results accentuate the importance of enduring surface determinants in the interaction of single cell and particulate moieties. From a microbial standpoint, attachment to protozoan surfaces may well suffice for transport to alternative areas or hosts resulting in further microbial colonisation or infection. Should internalisation follow the pattern of

marked attachment as observed amongst microspheres, then most entities will succumb to the destructive processes released during phagocytosis.

Bowers (1980) indicates that phagocytosis events are rapid following introduction of a substrate to a phagocyte. Such an observation is confirmed by the results of this study, with a rapid increase in association of both microsphere types with *Acanthamoeba* during the first half hour. Extending the incubation period beyond one hour did not significantly affect plain microsphere-trophozoite association, although some further trophozoite-carboxylate microsphere interaction was observed. Extended studies may be expected to reveal a decline over time, as indigestible particles are exocytosed (Bowers and Olszewski, 1983); such events potentially occurred as the proportion of trophozoites associated with plain microspheres declined after five hours.

Temperature appeared to have little effect on trophozoite-microsphere association with trophozoites interacting with similar numbers of either plain or carboxylate microspheres irrespective of incubation temperature. Such an apparent lack of phagocytosis temperature-sensitivity contradicts Avery *et al.* (1994) and Bowers (1977) who observed a decline in phagocytosis following trophozoite chilling below growth temperature. Conversely, Harf *et al.* (1997) indicates that *Acanthamoeba palestinensis* uptake of *Legionella pneumophila* is temperature-independent between 24 and 37 °C. Phagocytosis insensitivity to temperature variation would be advantageous for predatory protozoa, which as noted, can be found in a wide range of aquatic and soil habitats, encompassing a range of temperatures. Phagocytic feeding can potentially occur with equal voracity, whatever the temperature, at least in the recorded range of 10 to 37 °C.

In agreement with studies by Avery *et al.* (1995), increasing age of culture appears to reduce trophozoite association with microspheres of both plain and carboxylate types. As trophozoite cultures mature towards stationary phase onset their phagocytic ability is known to diminish (Chambers and Thompson, 1976), potentially mirrored here as a reduction in association. Interestingly, such a reduction in association was found to mainly affect the proportion of amoebae interacting with more than two microspheres, rather than those that interacted with just one or two. This could indicate that these latter amoebae had only attached, rather than internalised, microspheres to their surface, in contrast to those with a larger number of microspheres.

Coating of microspheres with the protein BSA was only found to significantly affect 2.0 μm microspheres, decreasing their association with trophozoites. Although, such a reduction could be related to the greater amount of protein required to coat larger microspheres, the observed decrease mirrors a study of Ayhan *et al.* (1995) of mammalian phagocytes. They speculated that phagocyte activity was inhibited as protein coated microspheres were recognised as self rather than foreign, thereby reducing microsphere phagocytosis. Such an occurrence is unlikely for free-living entities like *Acanthamoeba*, which do not form part of an integrated immune system, hence more subtle explanations should be sought. One such hypothesis arises from the efforts of Tabata and Ikada (1987) who indicate that albumin coating of microspheres would have the effect of lowering the hydrophobicity of such particles, therefore potentially reducing phagocytosis.

Pre-treating *Acanthamoeba polyphaga* with Con A appeared to have an effect in increasing their association with carboxylate microspheres. As noted, any equivalent increase in association with plain microspheres was probably masked, given the already marked proportion of 93 % of non-treated trophozoites in association with plain microspheres, although consideration of the surface of standard plain or carboxylate FITC-microspheres suggests that any such increase in association would be unexpected. The reciprocal binding of Con A to microspheres also appeared to marginally increase interaction with trophozoites, although this was not found to be significant. The marked proportion of trophozoites from positive controls, in association with untreated microspheres, again limited the potential for any definable increase. The effect of Con A on phagocytes, however, is not clear-cut as some studies indicate an increase in uptake of prey substrates mediated by Con A, such as that of Brown *et al.* (1975) using *Acanthamoeba*. Furthermore, Bracha *et al.* (1982) demonstrated that Con A could be used to aid binding of bacteria lacking mannose-binding lectins to *Entamoeba histolytica*. Studies by Gaziri *et al.* (1999) indicate that pre-treatment of murine macrophages with Con A increased phagocytosis of *Candida albicans* blastoconidia. Conversely, Bowers (1977) found that Con A at a concentration of 20 $\mu\text{g ml}^{-1}$ prevented phagocytosis of latex microspheres by *Acanthamoeba*, thought to be due to prevention of membrane activity, including invagination. In addition, van Oss (1978) indicates that Con A causes macrophages to be considerably more hydrophobic, thus compromising the phagocytosis of entities of lower hydrophobicity.

Pre-incubation of *Acanthamoeba polyphaga* with either mannose or glucose was found to have no effect on association with microspheres of either type. Such results are not unexpected given that both Lock *et al.* (1987) and Allen and Dawidowicz (1990a) found that, although mannose inhibited uptake of yeast, no effect was observed for latex particles. Bar-Shavit *et al.* (1977) obtained similar results with phagocytosis of *E. coli* by leucocytes, although, conversely, pre-incubation of *E. coli* with mannan was found to inhibit phagocytosis. Rabinovitch and De Stefano (1971) indicate that high concentrations of sugars can inhibit phagocytosis in *Acanthamoeba*, possibly due to hyperosmolarity, although this was discounted by van Oss (1971), due to the lack of any similar effect using ethanol to increase osmolarity. An alternative explanation proposed was that high levels of glucose caused neutrophils to round up, decreasing adhesiveness (van Oss, 1978). Incubation of microspheres with carbohydrates, on the other hand, had differing results, with significant effects on trophozoite-microsphere association recorded for mannose-plain microspheres (an increase) and glucose-carboxylate (a decrease). The lack of any consistency compromises justifiable explanation of such observations, though it seems likely that carbohydrate-coated microspheres, especially those with mannose, might be able to interact with surface lectins of *Acanthamoeba*.

It can thus be seen that phagocytosis can be influenced by a number of factors, which have a bearing on the natural activity of *Acanthamoeba* as a predator of microbial entities. Such factors will also inevitably play a part in the role of *Acanthamoeba* as a microbial vector. The diverse avidity and voracious nature of *Acanthamoeba* for a range of disparate entities, besides supporting its nutrition may have as yet ill-defined consequences. Foremost amongst which is the fate of foreign DNA taken up by *Acanthamoeba*, which in turn now receives at least preliminary consideration.

CHAPTER 7 – Fate of DNA taken up by *Acanthamoeba polyphaga*

7.1 Introduction

As indicated by the preceding text, *Acanthamoeba polyphaga*, in concert with other protozoa, is responsible for destruction of a wide-range of microbial species for nutrition purposes. However, some microorganisms are able to survive inside protozoa by a range of mechanisms and there is even consideration that such intracellular microbes have an effect on phenotypic modification of host and vice versa (Steinert *et al.*, 2000). The cause of such modification may be due to selection pressure on either host or endosymbiont by the other party or it may be that DNA of such entities has greater longevity and can interact with DNA of *Acanthamoeba* causing direct modification. Transfer of genetic information is well known amongst bacteria by three main mechanisms: transformation – uptake of free DNA following surface binding; transduction – transfer of DNA by bacteriophage viruses; and conjugation – transfer of plasmid or genomic DNA following cell-cell contact (Steinert *et al.*, 2000). Furthermore, nucleic acid transfer is also known in other species, for example in genetic interference of the nematode *Caenorhabditis elegans* by ingested double stranded RNA (dsRNA) (Timmons and Fire, 1998). Such interference was manifest by a lack of specific gene expression and indicates that nucleic acids from entirely unrelated species could interact. Introduction of dsRNA to *C. elegans* was achieved either by direct injection (Timmons and Fire, 1998) or feeding with transformed *Escherichia coli* (Kamath *et al.*, 2000). In both cases, dsRNA was produced in *E. coli* following insertion of appropriate genetic material into the double-stranded plasmid L4440 containing two T7 promoters, orientated in opposite directions.

Investigation of the fate of foreign DNA with *Acanthamoeba polyphaga* was carried out using this same plasmid. Due to the marked ability of *Acanthamoeba* to associate with polystyrene latex microspheres, amino-modified microspheres were used, coupled with L4440 prepared from transformed *E. coli* before incubation with *Acanthamoeba*. In addition, direct feeding of *Acanthamoeba* with L4440-transformed *E. coli* was carried out as an alternative means of introduction. Detection of L4440 DNA following co-incubation was carried out by PCR amplification.

7.2 Materials and Methods

7.2.1 Plasmid preparation

Escherichia coli DH5- α , transformed with the plasmid L4440, was supplied by David Lamb of Institute of Biological Sciences at Aberystwyth University on Luria-Betani (LB) agar (1 % (w/v) tryptone, 0.5 % (w/v) yeast extract, 0.5 % (w/v) NaCl, 1.5 % (w/v) agar, 1 L distilled water. Agar was autoclaved at 121 °C for 15 min at 15 psi and cooled to below 50 °C before addition of ampicillin to a final concentration of 50 $\mu\text{g ml}^{-1}$). *E. coli* were subcultured overnight at 37 °C in 500 ml LB broth (1 % (w/v) tryptone, 0.5 % (w/v) yeast extract, 0.5 % (w/v) NaCl, 1 L distilled water. Broth was autoclaved at 121 °C for 15 min at 15 psi and cooled to below 50 °C before addition of ampicillin to a final concentration of 50 $\mu\text{g ml}^{-1}$) before extraction of L4440 plasmid using a Wizard[®] Plus Maxi-Prep kit (Promega, USA), according to the manufacturer's protocol (Appendix 5). Following purification, the plasmid was diluted in sterile distilled water to 0.2 ng ml⁻¹ stored at -20 °C until use.

7.2.2 Quantification of plasmid L4440

The concentration and purity of the L4440 preparation was determined by measuring the absorbance at 260 nm (A_{260}) and 280 nm (A_{280}) of a 1:50 dilution of extract. The A_{260} value was compared to that of 1 for 50 $\mu\text{g ml}^{-1}$ pure crystal DNA in order to determine the relative concentration of DNA present. The ratio $A_{260}:A_{280}$ was calculated to give an indication of purity (pure DNA having a value of 1.8).

A 60 ml 1 % (w/v) agarose gel was prepared by dissolving 0.6 g SeaKem[®] LE agarose (BioWhittaker Molecular Applications, USA) in 1 \times TAE (Tris-acetate-EDTA: For 50 \times stock, 24.2 % (w/v) Tris, 100 ml 0.5 M EDTA (pH 8.0), 57.1 ml glacial acetic acid, made up to 1 L with distilled water). After cooling slightly, ethidium bromide was added to a final concentration of 1 $\mu\text{g ml}^{-1}$ before the molten agarose was poured into a gel caster and combs added to create wells. Once set, the

gel was submerged in an electrophoresis tank (Flowgen, UK) containing ~300 ml $1 \times$ TAE. Dilutions of 1:200, 1:100 and 1:50 in sterile distilled water of the purified L4440 plasmid were added to separate wells in 5 μ l volumes. A volume of 5 μ l containing 500 ng of a sizing ladder of λ phage DNA cut with *Hind* III restriction endonuclease (provided by Dave Nagel, Aston University) was added to a separate well. For further size comparison, undiluted L4440 preparation from a previous Wizard[®] Plus Mini-Prep (Promega, USA) as well as 500, 800 and 1000 ng of Ma3 plasmid linearised with *Hind* III (~5300 bp) (also provided by Dave Nagel, Aston University) were also added to separate wells. The gel was electrophoresed at 64 V for 1 hour before observation and archival using a Transilluminator (UVP Inc., USA) and camera with Grab-IT Annotating Grabber v. 2.59 software (UVP Inc., USA).

7.2.3 Detection of L4440 by PCR

Two primers, SSF1 and SSR1, designed for the detection of L4440 (Table 7.1), were manufactured by MWG-Biotech AG, UK. Each lyophilised primer was resuspended in sterile distilled water to a stock concentration of 100 pmol μ l⁻¹ before being stored at -20 °C in 100 μ l aliquots.

Table 7.1. Properties of SSF1 and SSR1 primers.

Property	SSF1	SSR1
Sequence	5' CCC GTC GAC ATC ATG TCC GAT GAC GGA GAT ^{3'}	5' CCC AAG CTT TTA CCA GAC ATC TTC TTG GTA ^{3'}
Length (bp)	30	30
Mass (μ g)	610	744
Melting point (°C)	70.9	65.4
GC Content (%)	56.7	43.3

To determine the primer specificity for L4440, a PCR mastermix was set up in a single tube containing (for each sample) 32 μ l sterile distilled water, 5 μ l $10 \times$ PCR buffer (containing MgCl₂) (PerkinElmer Inc., USA), 5 μ l 1 mM dNTPs (Amersham Pharmacia Biotech, UK), 1 μ l 20 pmol SSF1 primer, 1 μ l 20 pmol SSR1 primer and

1 μl BioTaq Red (Bioline, USA). Mastermix was then aliquotted in 45 μl volumes into sterile 0.5 ml PCR tubes before addition of 5 μl template to each tube, as follows: 1. Sterile distilled water (negative control 1); 2. *Acanthamoeba polyphaga* trophozoites (at 10^7 cells ml^{-1}); 3. Colony of *E. coli* transformed with L4440 plasmid; 4. 0.5 ng L4440 plasmid preparation; 5. 1.0 ng L4440 plasmid preparation; 6. Sterile distilled water (negative control 2). Each sample was then overlaid with ~ 50 μl sterile mineral oil before being subject to a PCR program on an OmniGene thermal cycler (Hybaid) consisting of 1 cycle of 94 $^{\circ}\text{C}$ (denaturation) for 2 min, 65 $^{\circ}\text{C}$ (annealing) for 1 min and 72 $^{\circ}\text{C}$ (extension) for 1 min and 29 cycles of 94 $^{\circ}\text{C}$ for 30 sec, 65 $^{\circ}\text{C}$ for 30 sec and 72 $^{\circ}\text{C}$ for 1 min. Meanwhile, a 60 ml 1 % (w/v) agarose gel was formed by dissolution of 0.6 g SeaKem[®] LE agarose in 60 ml $1 \times$ TAE. Upon cooling, ethidium bromide was added to a final concentration of 1 $\mu\text{g ml}^{-1}$ before the molten agar was poured into a gel caster and combs added to create wells. Once set, the gel was submersed in ~ 300 ml $1 \times$ TAE buffer in an electrophoresis tank. Each sample, in 15 μl volumes, was mixed with separate 3 μl aliquots of $6 \times$ PCR loading buffer (0.25 % (w/v) Bromophenol blue, 40 % (w/v) sucrose, 10 ml sterile distilled water) and loaded into separate wells. In addition, 5 μl 1 kb sizing ladder (Promega, USA) was also mixed with 3 μl $6 \times$ loading buffer before being added to a separate well. The gel was then electrophoresed for 1 hour at 64 V before observation and archival using the UV Transilluminator and camera system.

7.2.4 DNA adsorption to amino microspheres

A 0.5 ml volume of L4440 plasmid preparation, diluted to 0.2 ng ml^{-1} with sterile distilled water, was added to a 0.5 ml aliquot of 1.0 μm diameter non-fluorescent amino-modified polystyrene latex microspheres (Polysciences, USA), to a final concentration of 5×10^8 microspheres ml^{-1} , in a sterile Eppendorf tube. The sample was vortexed thoroughly, centrifuged at 2000 g for 5 min and the supernatant removed but retained. The sample was then resuspended in 1 ml sterile distilled water before further washing by centrifugation at 2000 g for 5 min and resuspension in 1 ml sterile distilled water, following collection of the supernatant. This process was repeated a further eight times.

Washed microspheres and each of the saved wash volumes were then used in a PCR amplification along with a positive control of L4440 plasmid preparation and two negative controls consisting of sterile distilled water. Each template sample, together with a positive control of 0.2 ng ml^{-1} L4440 plasmid preparation, was added in $5 \text{ }\mu\text{l}$ aliquots to separate sterile PCR tubes containing $45 \text{ }\mu\text{l}$ PCR mastermix, previously set up in a single tube containing (for each sample) $32 \text{ }\mu\text{l}$ sterile distilled water, $5 \text{ }\mu\text{l}$ $10 \times$ PCR buffer (containing MgCl_2), $5 \text{ }\mu\text{l}$ 1 mM dNTPs, $1 \text{ }\mu\text{l}$ 20 pmol SSF1 primer, $1 \text{ }\mu\text{l}$ 20 pmol SSR1 primer and $1 \text{ }\mu\text{l}$ BioTaq Red. Each sample was then overlaid with $\sim 50 \text{ }\mu\text{l}$ sterile mineral oil and was amplified by a PCR program consisting of an initial single cycle of $94 \text{ }^\circ\text{C}$ for 2 min, $65 \text{ }^\circ\text{C}$ for 1 min and $72 \text{ }^\circ\text{C}$ for 1 min before 29 cycles of $94 \text{ }^\circ\text{C}$ for 30 sec, $65 \text{ }^\circ\text{C}$ for 30 sec and $72 \text{ }^\circ\text{C}$ for 1 min. Meanwhile 0.6 g SeaKem[®] LE agarose was dissolved by heating in 60 ml $1 \times$ TAE to create a 1% (w/v) agarose gel. Ethidium bromide was added to a final concentration of $1 \text{ }\mu\text{g ml}^{-1}$ upon cooling, prior to gel casting and well formation. Following solidification, the gel was transferred to an electrophoresis tank before the addition of $\sim 300 \text{ ml}$ $1 \times$ TAE buffer in order to cover the gel. Following PCR, $15 \text{ }\mu\text{l}$ of each sample was mixed by pipetting with separate $3 \text{ }\mu\text{l}$ $6 \times$ loading buffer volumes and loaded into separate wells. The gel was then electrophoresed at 64 V for 1 hour before gel observation and archival using the UV Transilluminator and camera system.

7.2.5 PCR of *Acanthamoeba polyphaga*

An alternative set of primers was manufactured by MWG-Biotech AG for the positive amplification of *Acanthamoeba*. The properties of these primers, known as the Nelson primers, designed by Mathers *et al.* (2000) are listed in Table 7.2.

The lyophilised primers were resuspended in sterile distilled water to a stock concentration of $100 \text{ pmol }\mu\text{l}^{-1}$ before storage in $100 \text{ }\mu\text{l}$ aliquots at $-20 \text{ }^\circ\text{C}$.

Table 7.2. Properties of Nelson primers, NF and NR.

Property	NF	NR
Sequence	5' GTT TGA GGC AAT AAC AGG T ^{3'}	5' GAA TTC CTC GTT GAA GAT ^{3'}
Length (bp)	19	18
Mass (μg)	353	512
Melting point ($^{\circ}\text{C}$)	52.4	49.1
GC Content (%)	42.1	38.9

Cultures of *Acanthamoeba polyphaga* were prepared in the customary manner of inoculation of 9 ml sterile PYG broth with 1 ml confluent stock culture in sterile plastic universals which were incubated horizontally at 37 $^{\circ}\text{C}$ in darkness. After 4 days' incubation, trophozoites were harvested by centrifugation twice at 1000 g for 10 min and resuspension in 10 ml sterile distilled water each time to an approximate final concentration of 10^7 cells ml^{-1} . Dilutions were made in sterile distilled water to 1:100, 1:10³, 1:10⁴ and 1:10⁵. Aliquots of 5 μl of each sample were used as templates, together with two negative controls consisting of sterile distilled water and a further template of L4440 plasmid at 0.2 ng ml^{-1} . Each sample was added to sterile PCR tubes containing 45 μl mastermix, prepared in a single tube containing (for each sample) 32 μl sterile distilled water, 5 μl 10 \times PCR buffer (containing MgCl_2), 5 μl 1 mM dNTPs, 1 μl 20 pmol NF primer, 1 μl 20 pmol NR primer and 1 μl BioTaq Red. Each sample was overlaid with \sim 50 μl sterile mineral oil before PCR amplification on a program consisting of 1 cycle of 94 $^{\circ}\text{C}$ for 3 min, 46 $^{\circ}\text{C}$ for 1 min and 72 $^{\circ}\text{C}$ for 1 min before 29 cycles of 94 $^{\circ}\text{C}$ for 30 sec, 46 $^{\circ}\text{C}$ for 30 sec and 72 $^{\circ}\text{C}$ for 1 min. A 2% (w/v) agarose gel was created by heating 1.2 g SeaKem[®] LE agarose in 60 ml 1 \times TAE. Following cooling, ethidium bromide was added to a final concentration of 1 $\mu\text{g ml}^{-1}$ and molten agarose poured into a gel caster and combs added. Once set, the gel was transferred to an electrophoresis tank for submersion in \sim 300 ml 1 \times TAE. Aliquots of 15 μl of each sample were separately mixed with 3 μl aliquots of 6 \times PCR loading buffer before loading separate wells in the gel. In addition the marker, HyperLadder IV (Biolone, USA) was added to a separate well before the gel was run at 64 V for 1 hour. Finally, the resulting gel was observed and archived using the UV Transilluminator and camera system.

7.2.6 Extraction and amplification of acanthamoebal DNA DNA-adsorbed

Acanthamoeba polyphaga cultures were formed by the addition of 1 ml confluent stock culture to 9 ml sterile PYG broth in sterile plastic universals which were incubated horizontally at 37 °C in darkness for 4 days. Following incubation, universals were centrifuged twice at 1000 g for 10 min and the pellet was resuspended in 10 ml sterile distilled water at a concentration of approximately 10^7 cells ml⁻¹. Amoebal DNA was then extracted using the Wizard[®] Genomic DNA Purification Kit (Promega, USA) following the manufacturer's protocol (see Appendix 6).

Following extraction, four dilutions were prepared in distilled water at 1:100, 1:10³, 1:10⁴ and 1:10⁵. In addition, dilutions of trophozoite suspension were also prepared in distilled water at 1:100, 1:10³, 1:10⁴ and 1:10⁵. Templates of 5 µl of each sample were used, together with two negative controls of sterile distilled water and a further template of L4440 plasmid at 0.2 ng ml⁻¹. Each template was added to sterile PCR tubes containing 45 µl mastermix, pre-prepared in a single tube containing (for each sample) 32 µl sterile distilled water, 5 µl 10 × PCR buffer (containing MgCl₂), 5 µl 1 mM dNTPs, 1 µl 20 pmol NF primer, 1 µl 20 pmol NR primer and 1 µl BioTaq Red. Following overlay with ~50 µl sterile mineral oil, each sample was amplified by PCR on a program consisting of 1 cycle of 94 °C for 3 min, 46 °C for 1 min and 72 °C for 1 min before 29 cycles of 94 °C for 30 sec, 46 °C for 30 sec and 72 °C for 1 min. A 2 % (w/v) agarose gel was formed by heating 1.2 g SeaKem[®] LE agarose in 60 ml 1 × TAE. Following cooling, ethidium bromide was added to the molten agarose to a final concentration of 1 µg ml⁻¹ prior to it being poured into a gel caster and combs added. Once set, the gel was submersed in ~300 ml 1 × TAE in an electrophoresis tank. Aliquots of 15 µl of each amplified sample were separately mixed with 3 µl volumes of 6 × PCR loading buffer before loading separate wells in the gel. In addition HyperLadder IV (Biolone, USA) was added to a separate well for sizing purposes before the gel was run at 64 V for 1 hour. Finally, the resulting gel was observed and archived using the UV Transilluminator and camera system.

7.2.7 Specificity test of primers and long-term viability of DNA-adsorbed microspheres

Following successful extraction of DNA from *Acanthamoeba polyphaga* (Leeds strain) trophozoites, the specificity of SSF1 and SSR1 primers for L4440 plasmid could be tested. As well as a template of extracted acanthamoebal DNA, a further template of acanthamoebal DNA was combined with L4440 plasmid. In addition, DNA adsorbed amino microspheres (prepared in Section 7.2.4) that had been frozen for storage, were checked for long-term stability. Microspheres were defrosted before a further eleventh wash by centrifugation at 2000 g for 5 min and resuspension in 1 ml sterile distilled water, with the supernatant being retained for PCR amplification.

All samples, together with two negative controls of sterile distilled water and a positive control of 0.2 ng ml⁻¹ of L4440 plasmid preparation, were aliquotted in 5 µl volumes into sterile PCR tubes containing 45 µl mastermix. Mastermix for all samples was prepared in a single Eppendorf tube containing (for each sample) 32 µl sterile distilled water, 5 µl 10 × PCR buffer (containing MgCl₂), 5 µl 1 mM dNTPs, 1 µl 20 pmol SSF1 primer, 1 µl 20 pmol SSR1 primer and 1 µl BioTaq Red. Each sample was overlaid with ~50 µl sterile mineral oil before amplification by a PCR program consisting of 1 cycle of 94 °C for 2 min, 65 °C for 1 min and 72 °C for 1 min before 29 cycles of 94 °C for 30 sec, 65 °C for 30 sec and 72 °C for 1 min.

A 1 % (w/v) agarose gel was prepared by heating 0.6 g SeaKem[®] LE agarose in 60 ml 1 × TAE. Following cooling, ethidium bromide was added to a final concentration of 1 µg ml⁻¹ before the agarose was allowed to set in a gel caster with the addition of combs for well formation. The resulting gel was then immersed in ~300 ml 1 × TAE in an electrophoresis tank. Aliquots of 3 µl of 6 × PCR loading buffer were prepared in sterile Eppendorf tubes to which 15 µl of each sample were separately mixed, before loading separate wells in the gel. The gel was electrophoresed at 64 V for 1 hour before observation and archival using the UV Transilluminator and camera system.

7.2.8 Extraction and amplification of DNA from microspheres associated with *Acanthamoeba*

Four-day-old *Acanthamoeba* trophozoites were prepared by the inoculation of 9 ml sterile PYG broth with 1 ml confluent stock culture in sterile plastic universals, incubated horizontally at 37 °C in darkness. Resultant trophozoites were then starved by replacement of PYG broth with 10 ml amoebal saline following centrifugation at 1000 g for 10 min. A further 10 min centrifugation at 1000 g was performed before resuspension in 10 ml amoebal saline and re-incubation of trophozoites for a further day at 37 °C in darkness. Amoebal trophozoites at an approximate concentration of 10^6 cells ml⁻¹ were then added in 0.9 ml aliquots to sterile Eppendorf tubes at a concentration of approximately 10^6 cells ml⁻¹ to which 100 µl aliquots of DNA-microspheres were added to a final concentration of 5×10^7 microspheres ml⁻¹. These microspheres were prepared in a similar manner to those in Section 7.2.4.

One set of replicate tubes containing amoebae and DNA-microspheres were incubated at 37 °C for 1 hour, whilst another was not incubated. Both sets were then subjected to an extraction procedure using the Wizard[®] Genomic DNA Purification Kit (Promega, USA) following the manufacturer's protocol (see Appendix 6). Due to the lack of pellet formation after introduction into isopropanol at step 9, an alternative extraction using phenol:chloroform (Sambrook *et al.*, 1989) was attempted on samples of trophozoites with DNA-microspheres prepared exactly as detailed above. Each 1 ml sample was split into two equal volumes in sterile Eppendorf tubes before the addition of an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1). Each sample was then vortexed until emulsification before centrifugation at 2000 g for 15 sec. The upper (aqueous) phase of each sample was then removed and placed into a sterile Eppendorf tube before the addition of phenol:chloroform:isoamyl alcohol and mixing and separating as before. This process was repeated until protein was no longer visible at the interface between the two phases. At this point the aqueous phase of each sample was transferred to a sterile Eppendorf tube before the addition of an equal volume of chloroform:isoamyl alcohol (24:1). Each sample was again vortexed until emulsification before centrifugation at 2000 g for 15 sec. Finally the aqueous phase of each sample was transferred to a sterile Eppendorf tube for ethanol precipitation.

Firstly, sodium acetate (pH 5.2) was added to each sample to a final concentration of 0.3 M before the addition of two volumes of ice-cold ethanol. Each sample was then placed at $-20\text{ }^{\circ}\text{C}$ for 1 hour before centrifugation at 2000 g for 15 min. Following removal of the ethanol supernatant from each sample, each tube was placed, open-lidded, on a heating block at $65\text{ }^{\circ}\text{C}$ until all remaining ethanol had evaporated. Each sample was then resuspended in $100\text{ }\mu\text{l}$ sterile distilled water with additional heating at $65\text{ }^{\circ}\text{C}$, if necessary for resuspension, before storage at $-20\text{ }^{\circ}\text{C}$.

Each extracted sample was then subjected to two PCR amplifications using primers for both *Acanthamoeba* and L4440. Firstly a mastermix containing (for each sample) $32\text{ }\mu\text{l}$ sterile distilled water, $5\text{ }\mu\text{l}$ $10\times$ PCR buffer (containing MgCl_2), $5\text{ }\mu\text{l}$ 1 mM dNTPs, $1\text{ }\mu\text{l}$ 20 pmol NF primer, $1\text{ }\mu\text{l}$ 20 pmol NR primer and $1\text{ }\mu\text{l}$ BioTaq Red was prepared before dispensing $45\text{ }\mu\text{l}$ aliquots into sterile PCR tubes. Together with two negative controls of sterile distilled water and a positive control using L4440 plasmid preparation at 0.2 ng ml^{-1} , each extracted sample was then added in $5\text{ }\mu\text{l}$ volumes before overlay with $\sim 50\text{ }\mu\text{l}$ sterile mineral oil before amplification by PCR consisting of an initial single cycle of $94\text{ }^{\circ}\text{C}$ for 3 min, $46\text{ }^{\circ}\text{C}$ for 1 min and $72\text{ }^{\circ}\text{C}$ for 1 min before 29 cycles of $94\text{ }^{\circ}\text{C}$ for 30 sec, $46\text{ }^{\circ}\text{C}$ for 30 sec and $72\text{ }^{\circ}\text{C}$ for 1 min. Meanwhile a 2 % (w/v) agarose gel was created by dissolution of 1.2 g SeaKem[®] LE agarose by heating in 60 ml $1\times$ TAE. Upon cooling, ethidium bromide was added to a final concentration of $1\text{ }\mu\text{g ml}^{-1}$ before the molten agarose was poured into a gel caster to which combs were added. The solidified gel was then transferred to an electrophoresis tank to which $\sim 300\text{ ml}$ $1\times$ TAE was added in order to cover the gel entirely. Each sample, in $15\text{ }\mu\text{l}$ volumes, was mixed with separate $3\text{ }\mu\text{l}$ aliquots of $6\times$ PCR loading buffer before transferral to separate wells. The gel was electrophoresed for 1 hour at 64 V before observation with a UV Transilluminator and camera system.

For the second amplification a mastermix containing (for each sample) $32\text{ }\mu\text{l}$ sterile distilled water, $5\text{ }\mu\text{l}$ $10\times$ PCR buffer (containing MgCl_2), $5\text{ }\mu\text{l}$ 1 mM dNTPs, $1\text{ }\mu\text{l}$ 20 pmol SSF1 primer, $1\text{ }\mu\text{l}$ 20 pmol SSR1 primer and $1\text{ }\mu\text{l}$ BioTaq Red was prepared. Aliquots of $45\text{ }\mu\text{l}$ were placed into separate sterile PCR tubes, to which $5\text{ }\mu\text{l}$ of each extracted sample was added, together with two negative control samples of sterile distilled water and a positive control of 0.2 ng ml^{-1} L4440 plasmid preparation. Each sample was then overlaid with $\sim 50\text{ }\mu\text{l}$ sterile mineral oil and was amplified by a PCR program consisting of an initial single cycle of $94\text{ }^{\circ}\text{C}$ for 2 min, $65\text{ }^{\circ}\text{C}$ for 1 min

and 72 °C for 1 min before 29 cycles of 94 °C for 30 sec, 65 °C for 30 sec and 72 °C for 1 min. Meanwhile 0.6 g SeaKem® LE agarose was dissolved by heating in 60 ml 1 × TAE to create a 1 % (w/v) agarose gel. Ethidium bromide was added to a final concentration of 1 µg ml⁻¹ just prior to pouring the molten agarose into a gel caster. Following setting, the gel was submersed in ~300 ml 1 × TAE in an electrophoresis tank before the addition of 15 µl of each sample, each combined with 3 µl 6 × PCR loading buffer, into separate wells. The gel was then electrophoresed at 64 V for 1 hour before visualisation and archival using the UV Transilluminator and camera.

7.2.9 Extraction and amplification of DNA from microspheres associated with *Acanthamoeba* using increased reagent concentrations

Due to the inability to detect any plasmid DNA in the extracted samples, a similar procedure was performed, using increased quantities of all reagents. Cultures of *Acanthamoeba* were prepared in the usual manner of inoculating 9 ml sterile PYG broth with 1 ml confluent stock culture in sterile plastic universals, which were subsequently incubated horizontally at 37 °C in darkness. After 4 days' incubation, resultant trophozoites were centrifuged twice at 1000 g for 10 min and resuspended in 10 ml sterile amoebal saline each time before re-incubation at 37 °C in darkness for a further day.

A 0.5 ml aliquot of 1.0 µm diameter amino microspheres, at a concentration of 10¹⁰ microspheres ml⁻¹ was added to 0.5 ml 2 ng ml⁻¹ of L4440 plasmid preparation in a sterile Eppendorf tube. The sample was centrifuged five times at 2000 g for 5 min with replacement of the suspension medium with 1 ml sterile distilled water each time. Following washing, 100 µl aliquots of DNA-microspheres were added to 0.9 ml starved *Acanthamoeba* trophozoites at a concentration of 10⁷ cells ml⁻¹ before incubation at 37 °C in darkness for 1 hour on an orbital shaker at 200 rpm. In addition, to determine the effect of incubation, replicate samples were prepared which were unincubated. Following incubation, sample DNA was extracted using a phenol:chloroform extraction as detailed in Section 7.2.8 before amplification and electrophoresis, also as detailed in Section 7.2.8.

7.2.10 Extraction and amplification of DNA from *E. coli* transformed with L4440 associated with *Acanthamoeba*

An alternative approach to detect DNA was performed by feeding L4440-transformed *E. coli* directly to *Acanthamoeba* before extraction and PCR. *Acanthamoeba* cultures were prepared in the usual manner of inoculation of 9 ml sterile PYG broth with 1 ml confluent stock culture in sterile plastic universals, incubated horizontally at 37 °C in darkness for 4 days. Following incubation amoebal trophozoites were starved by centrifugation twice at 1000 g for 10 min and resuspension in 10 ml sterile amoebal saline each time before re-incubation at 37 °C in darkness for a further day.

Meanwhile, bacterial cultures were prepared by the inoculation of 20 ml LB broth containing ampicillin at 50 µg ml⁻¹ with a sterile loopful of *E. coli* transformed with L4440 plasmid in a sterile culture flask. After overnight incubation at 37 °C on an orbital shaker at 200 rpm, 100 µl aliquots were added to 0.9 ml volumes of *Acanthamoeba* suspension at 10⁶ cells ml⁻¹ in sterile Eppendorf tubes, subsequently incubated at 37 °C on an orbital shaker at 200 rpm for 1 hour.

Following incubation, sample DNA (potentially DNA from *Acanthamoeba*, *E. coli* and L4440) was extracted with a phenol:chloroform extraction as detailed in Section 7.2.8. Following extraction, each sample was amplified by PCR using a mastermix containing (for each sample) 32 µl sterile distilled water, 5 µl 10 × PCR buffer (containing MgCl₂), 5 µl 1 mM dNTPs, 1 µl 20 pmol SSF1 primer, 1 µl 20 pmol SSR1 primer and 1 µl BioTaq Red. Aliquots of 45 µl were placed into separate sterile PCR tubes, to which 5 µl of each extracted sample was added. In addition, two negative control samples were prepared, each with the addition of 5 µl sterile distilled water to 45 µl mastermix and also one positive control consisting of 5 µl *E. coli* culture transformed with L4440 plasmid in place of extracted sample and a second containing 5 µl 0.2 ng ml⁻¹ L4440 plasmid preparation. Each sample was then overlaid with ~50 µl sterile mineral oil and was amplified by a PCR program consisting of an initial single cycle of 94 °C for 2 min, 65 °C for 1 min and 72 °C for 1 min before 29 cycles of 94 °C for 30 sec, 65 °C for 30 sec and 72 °C for 1 min. Meanwhile 0.6 g SeaKem[®] LE agarose was dissolved in 60 ml 1 × TAE by heating to form a 1 % (w/v) agarose gel. Just prior to pouring the molten agarose into a gel

caster, ethidium bromide was added to a final concentration of $1 \mu\text{g ml}^{-1}$. Following solidification, the gel was immersed in $\sim 300 \text{ ml } 1 \times \text{TAE}$ in an electrophoresis tank before the addition of $15 \mu\text{l}$ of each amplified sample, each combined with $3 \mu\text{l}$ $6 \times \text{PCR}$ loading buffer, into separate wells. The gel was then electrophoresed at 64 V for 1 hour before visualisation and archival using the UV Transilluminator and camera.

7.3 Results

7.3.1 Quantification of plasmid L4440

Calculation of the purity of a 1:50 dilution of plasmid L4440 preparation is shown in Table 7.3. The ratio of $A_{260}:A_{280}$ was 2.026, higher than the value of 1.8 for pure DNA, indicating that the plasmid preparation was not totally pure, probably containing RNA as well.

Table 7.3. Absorbance readings of plasmid L4440 preparation, diluted 1:50.

A_{260}	A_{280}	A_{260}/A_{280}
0.462	0.228	2.026

The A_{260} value was also used to calculate the concentration of L4440 in the preparation. As $50 \mu\text{g ml}^{-1}$ of pure crystal DNA has an A_{260} of 1, an A_{260} of 0.462 is equivalent to $23.1 \mu\text{g ml}^{-1}$. For the undiluted preparation, this equated to a concentration of $1155 \mu\text{g ml}^{-1}$.

Figure 7.1 shows the plasmid (lanes 2-5) on an agarose gel following electrophoresis, in comparison to a linearised plasmid Ma3 (lanes 6-8). If of a similar size, the supercoiled L4440 plasmid would have been expected to travel further through the gel than the linearised Ma3. However, both samples migrated a similar distance indicating that L4440 is quite a large plasmid. The quoted size of L4440 is

only 2790 bp, half that of the Ma3 plasmid, therefore the L4440 plasmid must contain a large insert.

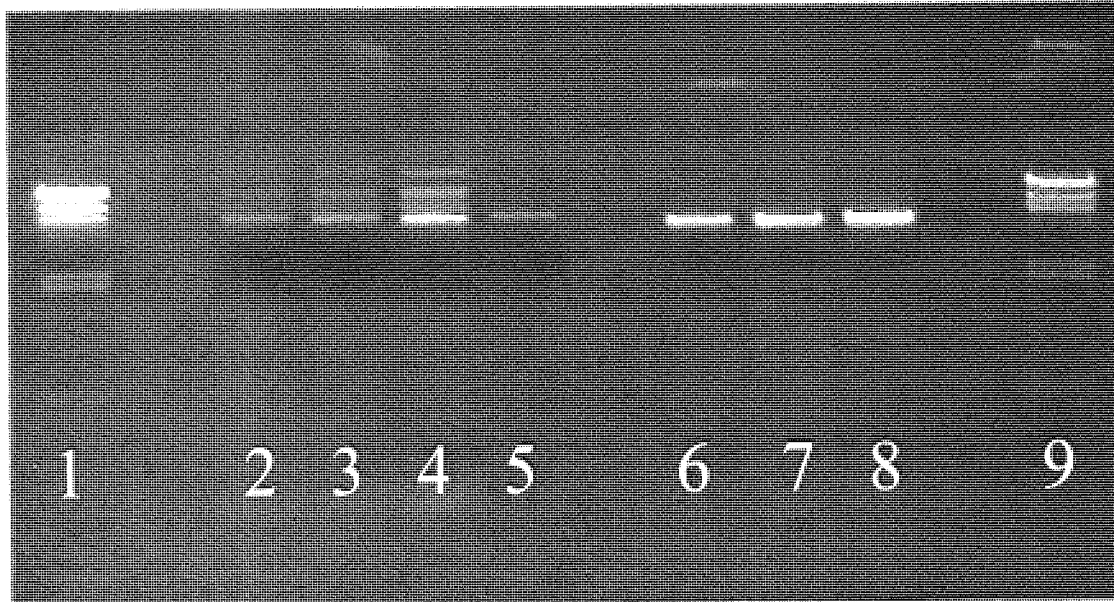


Figure 7.1. Agarose gel electrophoresis analysis of L4440 plasmid preparation. Lane 1: *Hind*III cleaved λ DNA Ladder (500 ng); 2: L4440 (1:200 dilution); 3: L4440 (1:100 dilution); 4: L4440 (1:50 dilution); 5: L4440 from mini-prep; 6: *Hind*III linearised Ma3 (500 ng); 7: *Hind*III linearised Ma3 (800 ng); 8: *Hind*III linearised Ma3 (1000 ng); 9: *Hind*III cleaved λ DNA Ladder (500 ng).

7.3.2 Detection of L4440 by PCR

As can be seen in Figure 7.2, the two primers, SSF1 and SSR1 successfully amplified the L4440 plasmid, in both the plasmid prep (lanes 5 and 6) and transformed *E. coli* DH5- α (lane 4). The 1 kb ladder allowed size estimation of the amplified region of the plasmid as being around 1800 bp. The negative controls of distilled water (lanes 2 and 7) remained negative, as did the sample of *Acanthamoeba polyphaga* (Leeds strain) trophozoites (lane 3). The latter, however, did not necessarily reflect a true negative, as there was no guarantee that the trophozoites could be directly amplified by PCR. In order to test whether PCR could be performed directly upon amoebal trophozoites, an attempt to amplify L4440 plasmid in the presence of *Acanthamoeba* trophozoites was made. No such amplification was achieved (data not shown).

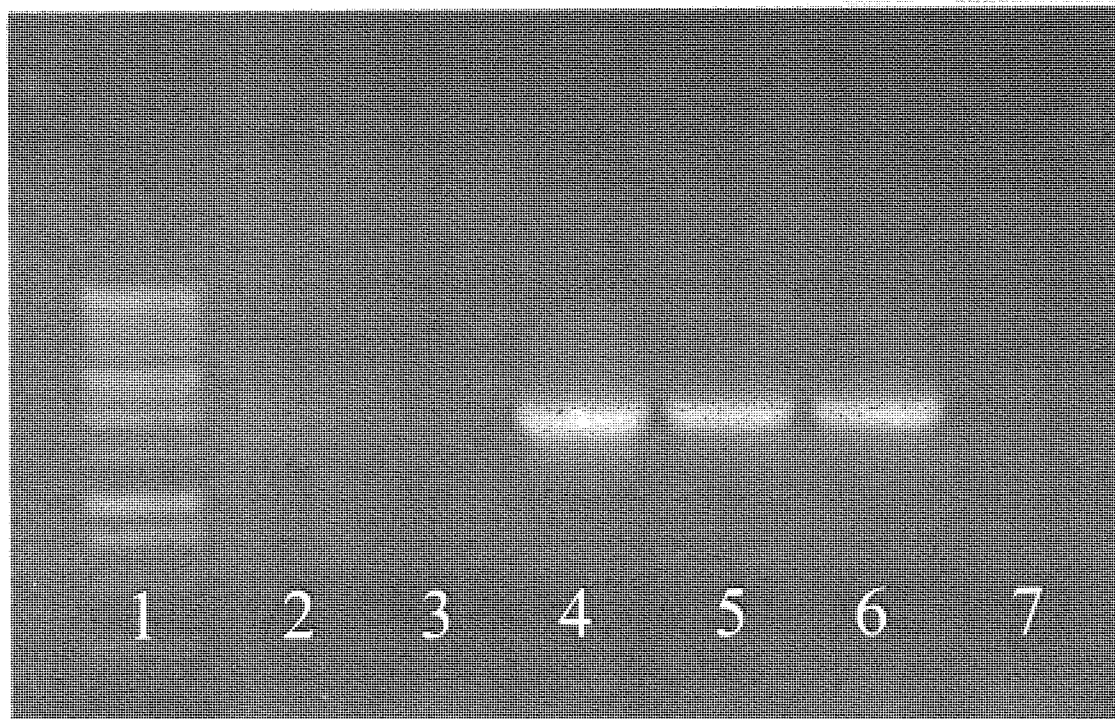


Figure 7.2. Agarose gel electrophoresis analysis following PCR amplification of L4440. Lane 1: 1 kb ladder; 2. Distilled water; 3. *Acanthamoeba polyphaga* (Leeds strain); 4. *E. coli* transformed with L4440; 5. L4440 (0.5 ng); 6. L4440 (1.0 ng); 7. Distilled water.

7.3.3 DNA adsorption to amino microspheres

As demonstrated in Figure 7.3, 1.0 μm diameter amino microspheres adsorbed L4440 plasmid onto their surface, which was stable and detectable after ten washes (lane 3). Indeed, after just five washes, no L4440 DNA was being washed off in any detectable quantity (lanes 8-13). Hence, in all further experimentation using DNA-microspheres, five washes was deemed sufficient.

Negative controls of distilled water (lanes 2 and 16) were found to be negative and a band was detected for the positive control of L4440 plasmid (lane 14). The presence of *Acanthamoeba polyphaga* (Leeds strain) trophozoites, however, with L4440 plasmid (lane 15) appeared to prevent amplification in some way, thus the sample appeared negative. In order to investigate other approaches by which either *Acanthamoeba* or DNA associated with *Acanthamoeba* could be amplified, an alternative set of primers specific to *Acanthamoeba* was developed, as detailed in Section 7.2.5.

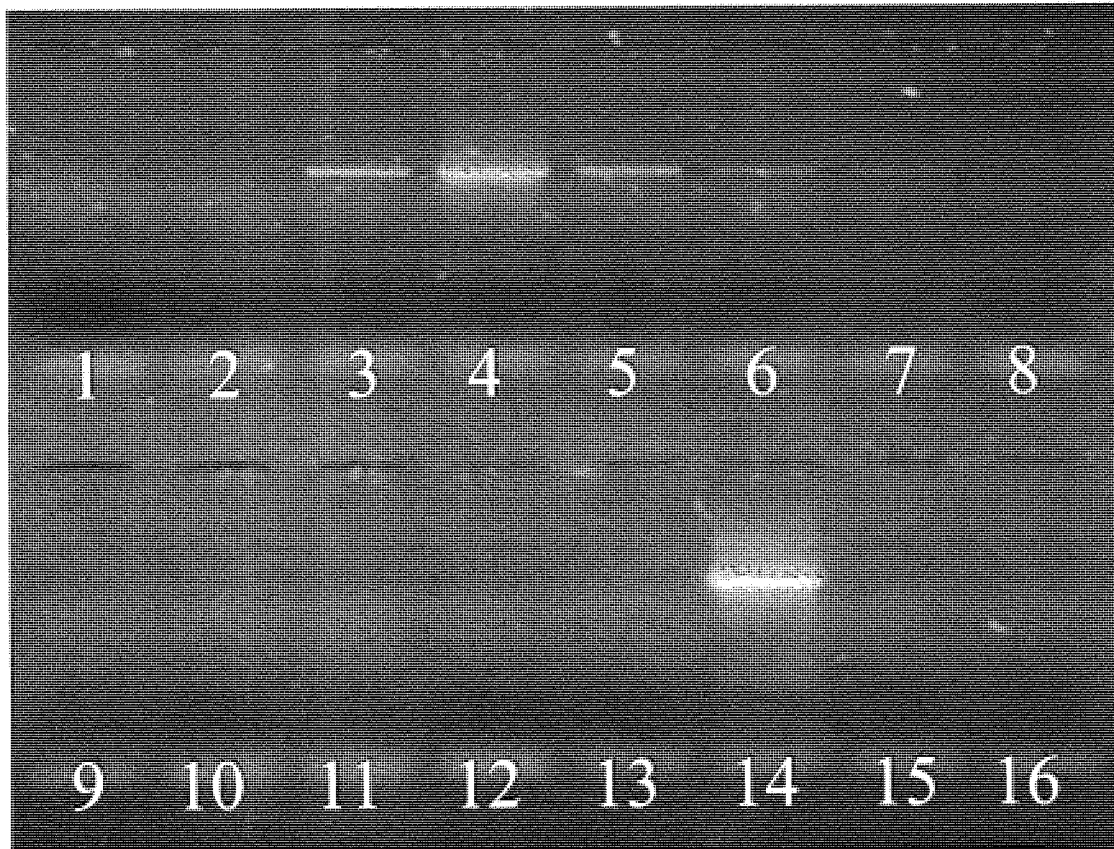


Figure 7.3. Agarose gel electrophoresis analysis of success of DNA adsorption to amino microspheres, following amplification by PCR. Lane 1: Distilled water; 2. 1.0 μm diameter amino microspheres; 3. L4440-adsorbed microspheres (post-washing); 4-13: Supernatants 1-10 from washing microspheres; 14. L4440 (1.0 ng); 15. *Acanthamoeba polyphaga* (Leeds strain) trophozoites with L4440; 16. Distilled water.

7.3.4 PCR of *Acanthamoeba polyphaga*

PCR amplification directly upon trophozoite suspensions using the *Acanthamoeba*-specific Nelson primers was not successful as no bands were detected in any of the dilutions of trophozoites, even with the modified PCR program. It was considered that this may have been due to some acanthamoebal factor inhibitory to BioTaq Red. Therefore, it was decided to try an extraction procedure to obtain acanthamoebal DNA, upon which hopefully amplification could succeed.

7.3.5 Extraction and amplification of acanthamoebal DNA

As can be seen in Figure 7.4, although PCR amplification of DNA directly from *Acanthamoeba polyphaga* (Leeds strain) trophozoites (lanes 3-6) was not achieved, prior extraction of acanthamoebal DNA did allow amplification by the Nelson primers, at least at the greater concentrations (lanes 7 and 8). Negative controls (lanes 2 and 11) remained negative in the reaction.



Figure 7.4. Agarose gel electrophoresis analysis following PCR amplification of DNA extracted from *Acanthamoeba polyphaga* (Leeds strain) trophozoites. Lane 1: Sizing ladder; 2. Distilled water; 3. Acanthamoebal trophozoites (1:100); 4. Acanthamoebal trophozoites (1:10³); 5. Acanthamoebal trophozoites (1:10⁴); 6. Acanthamoebal trophozoites (1:10⁵); 7. Acanthamoebal DNA (1:100); 8. Acanthamoebal DNA (1:10³); 9. Acanthamoebal DNA (1:10⁴); 10. Acanthamoebal DNA (1:10⁵); 11. Distilled water.

7.3.6 Specificity test of primers and long-term viability of DNA-adsorbed microspheres

As demonstrated in Figure 7.5, the primers, SSF1 and SSR1, targeted at L4440, were found to be unsuitable for amplification of *Acanthamoeba polyphaga* (Leeds strain) DNA (lane 2), indicating a true negative. Unlike whole trophozoites, however, such acanthamoebal DNA was not capable of inhibition of L4440 plasmid DNA (lane 6). The process of freezing DNA-adsorbed microspheres was found not to

affect DNA adsorption (lane 4), although it is possible that bands were not as bright as before, although the eleventh wash supernatant (lane 3) was negative, as were the negative controls (lanes 1 and 7). To err on the side of caution, however, it was deemed best to prepare DNA-adsorbed microspheres freshly each time before use.

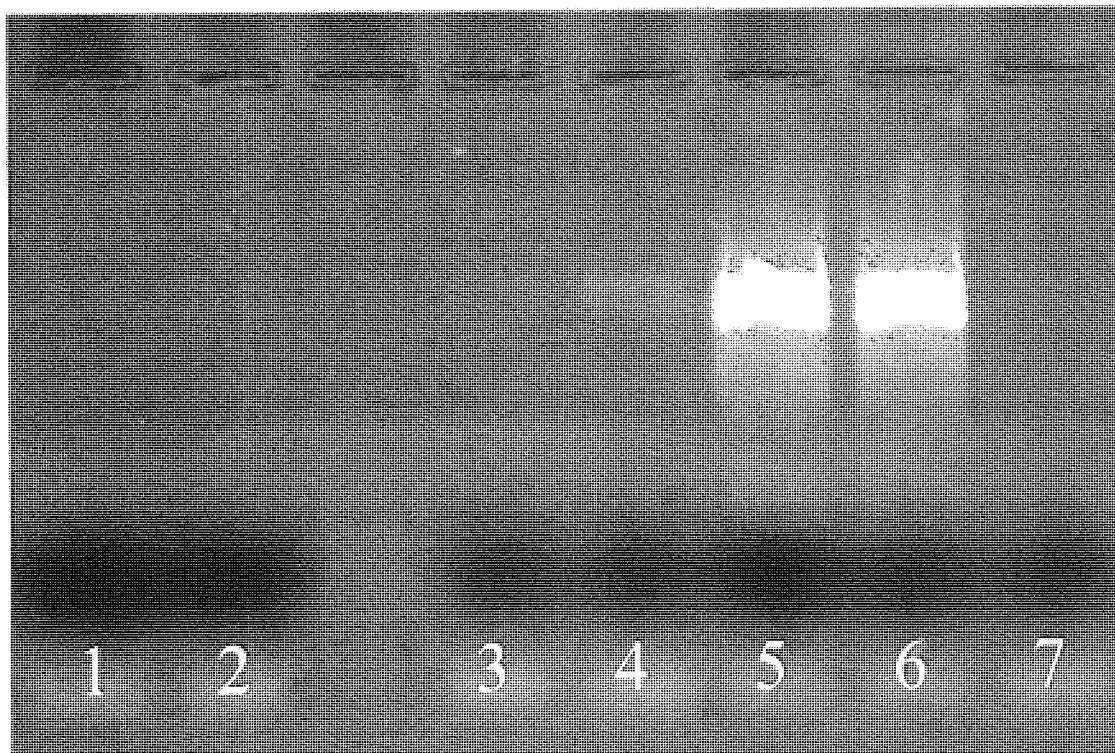


Figure 7.5. Agarose gel electrophoresis analysis following PCR amplification to determine specificity of primers directed at L4440 and long-term viability of DNA adsorbed microspheres. Lane 1: Distilled water; 2. Acanthamoeba DNA (1:100); 3. Supernatant 11 from washing microspheres; 4. L4440-adsorbed microspheres (post-washing); 5. Plasmid L4440; 6. Plasmid L4440 with acanthamoeba DNA; 7. Distilled water.

7.3.7 Extraction and amplification of DNA from microspheres associated with *Acanthamoeba*

As noted in Section 7.2.8, use of the Wizard[®] Genomic DNA Purification Kit failed to produce any DNA pellets following addition of isopropanol. Therefore a phenol:chloroform extraction was performed upon *Acanthamoeba polyphaga* (Leeds strain) trophozoites incubated with DNA-microspheres, which successfully produced

DNA pellets for all samples. PCR amplification of the samples, however, only produced bands when using the Nelson primers, specific for *Acanthamoeba*, potentially indicating that if L4440 was taken up, it could not be extracted in order to be amplified. A further adaptation of the method was made in order to increase reagent concentrations, to potentially provide enough DNA for successful PCR amplification.

7.3.8 Extraction and amplification of DNA from microspheres associated with *Acanthamoeba* using increased reagent concentrations

Despite the use of increased concentrations of all reagents, still no bands corresponding to L4440 were found in any of the amplified samples following incubation of *Acanthamoeba polyphaga* (Leeds strain) trophozoites with DNA-microspheres. Therefore, the use of microspheres was abandoned in favour of direct uptake of *E. coli* DH5- α transformed with L4440 plasmid by *Acanthamoeba polyphaga* (Leeds strain) trophozoites.

7.3.9 Extraction and amplification of DNA from *E. coli* transformed with L4440 associated with *Acanthamoeba*

Unfortunately, no conclusive answer was obtained from this last experiment as, for unknown reasons, the positive control of L4440 plasmid preparation failed to amplify either time the experiment was conducted.

7.4 Discussion

Exploration into the fate of foreign DNA taken up by *Acanthamoeba polyphaga* achieved a certain degree of success. Coupling of L4440 to microspheres, presumably by electrostatic interactions between negatively charged DNA and

positively charged amino-modified microspheres, was confirmed by PCR following a number of washes to remove unbound DNA. Efforts to introduce these DNA-microspheres to *Acanthamoeba* to determine their subsequent fate appeared to indicate that foreign DNA was destroyed by the amoebae as although a number of factors may interfere with experimental protocols, no introduced DNA was ever detected by PCR. Amplification of either introduced L4440 or acanthamoebal DNA was not achieved when PCR was applied directly to intact amoebae, possibly due to the presence of some amoebal cellular component that hindered PCR. It was considered that such a factor compromised BioTaq Red DNA polymerase function, rather than showing nuclease activity as initial 94 °C heating steps of the PCR program should destroy or disrupt nucleases unless demonstrating novel thermostability. Modifications to the experimental method were therefore executed, most notably extraction of total DNA (both acanthamoebal and plasmid) by two different methods, and also increasing initial concentrations of both plasmid DNA-microspheres and *Acanthamoeba* trophozoites. Neither of these modifications, either independently or in conjunction, resulted in amplification of L4440 that had been exposed to intact *Acanthamoeba* trophozoites. At least three scenarios can be proposed to explain this occurrence; firstly, it could simply be that *Acanthamoeba polyphaga* possesses one or even a battery of active nucleases to destroy any foreign DNA, following the engulfment and breakdown of microbial entities. Alternatively, it is possible that extraction protocols employed failed to isolate introduced L4440 DNA hence it could not be subsequently amplified. Furthermore, there is a possibility that, because L4440 DNA was thought to couple to the amino microspheres by electrostatic interactions, the resulting DNA-microspheres carried no net charge, and hence were potentially more unlikely to be taken up (Tabata and Ikada, 1987). If this were the case, however, one may still expect some amplification of DNA bound to such microspheres remaining in the sample tubes. Therefore the first scenario is probably the most likely given that extraction protocols, particularly phenol:chloroform extractions (Sambrook *et al.*, 1989), are routinely employed with success for DNA extraction from a range of biological entities. Furthermore, if such an event occurs, it appears to take place very quickly, as those samples of L4440 merely exposed to *Acanthamoeba* followed by immediate extraction failed to produce recognisable L4440 PCR products.

The present work has gone some way to determine the fate of foreign DNA within *Acanthamoeba polyphaga* and it seems that engulfed DNA is destroyed during phagocytosis by predatory amoebae. Although not proven categorically, such a situation is not particularly surprising given the harsh phagolysosomal conditions generated by *Acanthamoeba* following ingestion of microbes, including markedly acidic pH and an arsenal of degradative enzymes. However, the biological and environmental consequences, to name but two, of even limited foreign or “engineered” DNA survival and perpetuation within the protozoan community demands continued vigilance. In view of such important considerations, further modifications to the methods presented here for determination of the fate of foreign DNA within protozoa could be made such as covalent attachment of DNA to microspheres, for example employing the method of Andreadis and Chrisey (2000), or the use of expression vectors in order to see if introduced genes, such as that of green fluorescent protein (GFP) (Chalfie *et al.*, 1994), could be expressed following uptake in *Acanthamoeba*.

CHAPTER 8 – Conclusions

The work presented here was undertaken at least in part to ascertain the character and nature of potential interactions between free-living amoebae, particularly those of the genus *Acanthamoeba* and bacteria such as *Aeromonas salmonicida*, *Aeromonas hydrophila* and *Renibacterium salmoninarum*. These bacteria are responsible for diseases amongst salmonid fish, although much of their aetiology is still unknown, for example survival outside the salmonid host. Each of these bacteria is thought to be capable of intracellular growth and survival within fish macrophages, hence the potential for interaction and subsequent survival within protozoa could be envisaged. Water samples taken from fish farms and natural rivers, both contained salmonid fish and revealed the presence of free-living amoebae, most likely *Acanthamoeba*, indicating that such a notion may have foundation. However, further work with amoebae and microbial pathogens should be carried out in this regard as the direction of this work shifted as a consequence of an actively developing investigation.

Although *Acanthamoeba* is widely recognised as a vector affording protection and hence greater longevity to microbes, most notably *Legionella pneumophila*, many of the mechanisms underlying interactions between *Acanthamoeba* and microbes have yet to be elucidated. Such mechanisms, as with any contact events between cells, typically involve a variety of factors, including those of a specific and non-specific nature, principally surface hydrophobicity, surface charge and lectin-adhesin interactions. Although much is known about the organism in other respects, such as behaviour and distribution, our knowledge of the surface nature of *Acanthamoeba* is at best patchy. The present work therefore explores the surface nature of *Acanthamoeba polyphaga* trophozoites and cysts through determination of cell surface charge (or ζ -potential), hydrophobicity and lectin binding assays. These studies revealed a marked negative ζ -potential around surfaces of trophozoites, which was slightly less for cysts. Populations of both *Acanthamoeba* morphological forms, particularly cysts, manifest little hydrophobicity in relation to cells of other organisms. Lectin binding assays enable qualitative and quantitative characterisation of microbial surface carbohydrates, particularly when more in-depth analyses along the lines of ligand-receptor studies are employed. Such investigations are highly specific, non-destructive

and indicate a trophozoite surface exposure of carbohydrates such as N-acetylglucosamine, N-acetylneuraminic acid, mannose and glucose. Similar carbohydrates were manifest on cyst surfaces but, in addition, there was also marked incidence of N-acetylgalactosamine. Such carbohydrates were considered to be distributed over the entire trophozoite or cyst surface with further patches of greater lectin receptor density, as revealed by confocal microscopy of FITC-Con A binding to trophozoites.

These observations cannot be considered in isolation, and should be taken into account when investigating the likes of predation by *Acanthamoeba polyphaga* trophozoites, or other protozoa, of particulate and biological entities. This study, in investigations of the phagocytosis phenomenon, employed microspheres, rather than biological entities, due to their defined nature. Quantification of FITC-microsphere presence by flow cytometry due to its considerable accuracy and capability to evaluate many samples expeditiously ensured meaningful determinations. Through use of these techniques, trophozoite-microsphere interactions were found to be insensitive to temperature variation, unlike growth rates. Such an observation is important from the standpoint of amoebal survival in a wide range of dynamic aquatic environments subject to temperature variation, as aspects such as amoebal feeding would not be compromised by such variation in environmental conditions. Furthermore, trophozoites were found to have greater avidity for microspheres when suspended in amoebal saline rather than in nutrient broth, the former being more similar to natural conditions. This observation was thought to be a consequence of the ability amongst broth-suspended trophozoites to acquire nutrients by pinocytosis alone, potentially in preference to the energetically less favourable phagocytosis of more complex biological materials. The microspheres employed in these investigations were of three sizes (0.5, 1.0 and 2.0 μm in diameter) and two surface natures, plain (with a less negative ζ -potential and generally more hydrophobic) and carboxylate (more negative and more hydrophilic). In turn experimental flow cytometry results and confocal imaging indicated that there was generally a marked preference amongst trophozoites for both larger microspheres and also plain type microspheres, thereby demonstrating some predilection for larger phagocytic substrates of both lower ζ -potential and greater hydrophobicity.

The influence, either positive or negative, of lectins and carbohydrates on trophozoite-microsphere interactions appeared to be limited, although such findings

do not necessarily reflect microbe-trophozoite interactions, as unlike microspheres, microbes generally possess both lectin-like moieties and carbohydrates on their surface. This illustrates that whilst valuable information on the interaction between phagocyte and prey can be obtained by the use of simpler, yet defined, substrates such as microspheres, further research using bacteria should be pursued in order to establish a fuller picture of *Acanthamoeba* interaction with particulate material.

Phagocytosis assays indicated a great avidity for microspheres, mirroring the natural predation of microbes. In the survival of some bacterial species within *Acanthamoeba*, there are complex interactions between bacteria and host, potentially including genetic interactions. It is known, for example, that phenotypic variation can occur in both host and intracellular bacterium. Therefore, the fate of foreign DNA carried by microspheres within amoebal trophozoites was investigated using PCR. These studies suggested that such DNA was destroyed once taken up. However, there is potential benefit in further investigations in this area, as the persistence of foreign DNA within protozoa could be of great consequence to both the organism and the environment at large. For example, due to its own status as a pathogen, genetic variation of *Acanthamoeba* could enhance its virulence. Furthermore, endurance of foreign DNA, even if not expressed by *Acanthamoeba*, may form a reservoir of readily disseminated genetic material that could later be transferred to other biological entities in turn modifying phenotypic characteristics. Such scenarios have serious environmental and commercial implications; even our understanding of human exposure to genetic material may require re-evaluation as amoeba are both widespread and readily inhaled after droplet dispersion.

Despite the need for further work in certain areas, by employing a novel combination of approaches, this investigation presents new information and provides a basis for further explorations into potential interactions between amoebae and bacterial fish pathogens, or indeed any microbe. As demonstrated by the interaction between *Acanthamoeba* and *Legionella*, such interactions have great significance in both human disease states and those of species of immediate human concern. Due to the widespread nature of *Acanthamoeba*, its avidity as a phagocyte and the difficulty of its eradication coupled with the emergence of a greater variety of microbial species, many of them pathogenic, that can resist digestion by phagocytes, study of such interaction mechanisms has tremendous importance if we are to find a way to limit such reservoirs of disease.

REFERENCES

- Absolom, D. R.** 1986a. Basic methods for the study of phagocytosis. *Methods in Enzymology* **132**(J):95-180.
- Absolom, D. R.** 1986b. Measurement of surface properties of phagocytes, bacteria, and other particles. *Methods in Enzymology* **132**(J):16-95.
- Abu Kwaik, Y., L.-Y. Gao, B. J. Stone, C. Venkataraman, and O. S. Harb.** 1998. Invasion of protozoa by *Legionella pneumophila* and its role in bacterial ecology and pathogenesis. *Applied and Environmental Microbiology* **64**(9):3127-3133.
- Al-Ibrahaim, M. S., R. Chandra, R. Kishore, F. T. Valentine, and H. S. Lawrence.** 1976. A micromethod for evaluating the phagocytic activity of human macrophages by ingestion of radio-labelled polystyrene particles. *Journal of Immunological Methods* **10**(2-3):207-218.
- Allen, P. G. and E. A. Dawidowicz.** 1990a. Phagocytosis in *Acanthamoeba*: I. A mannose receptor is responsible for the binding and phagocytosis of yeast. *Journal of Cellular Physiology* **145**(3):508-513.
- Allen, P. G. and E. A. Dawidowicz.** 1990b. Phagocytosis in *Acanthamoeba*: II. Soluble and insoluble mannose-rich ligands stimulate phosphoinositide metabolism. *Journal of Cellular Physiology* **145**(3):514-521.
- Andreadis, J. D. and L. A. Chrisey.** 2000. Use of immobilized PCR primers to generate covalently immobilized DNAs for *in vitro* transcription/translation reactions. *Nucleic Acids Research* **28**(2):e5.
- Armstrong, M. and J. Soothill.** 1999. Enhanced growth of surface-attached *Acanthamoeba polyphaga*. *Journal of Clinical Microbiology* **37**(11):3780.
- Austin, B. and C. Adams.** 1996. Fish pathogens, 197-243. In: B. Austin, M. Altwegg, P. J. Gosling, and S. W. Joseph (eds.), *The Genus Aeromonas*. John Wiley & Sons, Chichester.
- Avery, S. V., J. L. Harwood, and D. Lloyd.** 1994. Low-temperature-induced adaptations in fatty acid metabolism of *Acanthamoeba castellanii* cultures of different ages: Relationship to changes in cell division, oxygen uptake and phagocytic activity. *Microbiology* **140**(9):2423-2431.
- Avery, S. V., J. L. Harwood, and D. Lloyd.** 1995. Quantification and characterization of phagocytosis in the soil amoeba *Acanthamoeba castellanii* by flow cytometry. *Applied and Environmental Microbiology* **61**(3):1124-1132.
- Avron, B., R. Bracha, M. R. Deutsch, and D. Mirelman.** 1983. *Entamoeba invadens* and *E. histolytica*: Separation and purification of precysts and cysts by centrifugation on discontinuous density gradients of Percoll. *Experimental Parasitology* **55**(3):265-269.
- Ayhan, H., A. Tuncel, N. Bor, and E. Pişkin.** 1995. Phagocytosis of monosize polystyrene-based microspheres having different size and surface properties. *Journal of Biomaterials Science, Polymer Edition* **7**(4):329-342.
- Bandín, I., A. E. Ellis, J. L. Barja, and C. J. Secombes.** 1993. Interaction between rainbow trout macrophages and *Renibacterium salmoninarum in vitro*. *Fish and Shellfish Immunology* **3**(1):25-33.

- Bandín, I., C. Rivas, Y. Santos, C. J. Secombes, J. L. Barja, and A. E. Ellis.** 1995. Effect of serum factors on the survival of *Renibacterium salmoninarum* within rainbow trout macrophages. *Diseases of Aquatic Organisms* **23**(3):221-227.
- Bandín, I., Y. Santos, J. L. Barja, and A. E. Toranzo.** 1989. Influence of the growth conditions on the hydrophobicity of *Renibacterium salmoninarum* evaluated by different methods. *FEMS Microbiology Letters* **60**(1):71-77.
- Bar-Shavit, Z., I. Ofek, R. Goldman, D. Mirelman, and N. Sharon.** 1977. Mannose residues on phagocytes as receptors for the attachment of *Escherichia coli* and *Salmonella typhi*. *Biochemical and Biophysical Research Communications* **78**(1):455-460.
- Barker, J. and M. R. W. Brown.** 1994. Trojan Horses of the microbial world: Protozoa and the survival of bacterial pathogens in the environment. *Microbiology* **140**(6):1253-1259.
- Barker, J., H. Scaife, and M. R. W. Brown.** 1995. Intraphagocytic growth induces an antibiotic-resistant phenotype of *Legionella pneumophila*. *Antimicrobial Agents and Chemotherapy* **39**(12):2684-2688.
- Barker, J., T. J. Humphrey, and M. W. R. Brown.** 1999. Survival of *Escherichia coli* 0157 in a soil protozoan: Implications for disease. *FEMS Microbiology Letters* **173**(2):291-295.
- Barnes, A. C., T. J. Bowden, M. T. Horne, and A. E. Ellis.** 1999. Peroxide-inducible catalase in *Aeromonas salmonicida* subsp. *salmonicida* protects exogenous hydrogen peroxide and killing by activated rainbow trout, *Oncorhynchus mykiss* L., macrophages. *Microbial Pathogenesis* **26**(3):149-158.
- Bayer, M. E. and J. L. Sloyer, Jr.** 1990. The electrophoretic mobility of Gram-negative and Gram-positive bacteria: An electrokinetic analysis. *Journal of General Microbiology* **136**(5):867-874.
- Biddick, C. J., L. H. Rogers, and T. J. Brown.** 1984. Viability of pathogenic and nonpathogenic free-living amoebae in long-term storage at a range of temperatures. *Applied and Environmental Microbiology* **48**(4):859-860.
- Borazjani, R. N., L. L. May, J. A. Noble, S. V. Avery, and D. G. Ahearn.** 2000. Flow cytometry for determination of the efficacy of contact lens disinfecting solutions against *Acanthamoeba* spp. *Applied and Environmental Microbiology* **66**(3):1057-1061.
- Bos, R., H. C. van der Mei, and H. J. Busscher.** 1999. Physico-chemistry of initial microbial adhesive interactions – Its mechanisms and methods for study. *FEMS Microbiology Reviews* **23**(2):179-230.
- Bose, K., D. K. Ghosh, and A. Bhattacharya.** 1989. Membrane carbohydrate characterization of *Acanthamoeba astronyxis*, *A. castellanii* and *Naegleria fowleri* by fluorescein-conjugated lectins. *International Journal for Parasitology* **19**(7):737-741.
- Bowers, B.** 1977. Comparison of pinocytosis and phagocytosis in *Acanthamoeba castellanii*. *Experimental Cell Research* **110**(2):409-417.
- Bowers, B.** 1980. A morphological study of plasma and phagosome membranes during endocytosis in *Acanthamoeba*. *The Journal of Cell Biology* **84**(2):246-260.
- Bowers, B. and E. D. Korn.** 1968. The fine structure of *Acanthamoeba castellanii* I. The trophozoite. *The Journal of Cell Biology* **39**(1):95-111.
- Bowers, B. and E. D. Korn.** 1969. The fine structure of *Acanthamoeba castellanii* (Neff strain) II. Encystment. *The Journal of Cell Biology* **41**(3):786-805.

- Bowers, B. and E. D. Korn. 1974. Localization of lipophosphoglycan on both sides of *Acanthamoeba* plasma membrane. *The Journal of Cell Biology* 62(2):533-540.
- Bowers, B. and T. E. Olszewski. 1983. *Acanthamoeba* discriminates internally between digestible and indigestible particles. *The Journal of Cell Biology* 97(2):317-322.
- Bracha, R., D. Kobiler, and D. Mirelman. 1982. Attachment and ingestion of bacteria by trophozoites of *Entamoeba histolytica*. *Infection and Immunity* 36(1):396-406.
- Brattgjerd, S., Ø. Evensen, and A. Lauve. 1994. Effect of injected yeast glucan on the activity of macrophages in Atlantic salmon, *Salmo salar* L., as evaluated by *in vitro* hydrogen peroxide production and phagocytic capacity. *Immunology* 83(2):288-294.
- Brown, M. R. W. and J. Barker. 1999. Unexplored reservoirs of pathogenic bacteria: Protozoa and biofilms. *Trends in Microbiology* 7(1):46-50.
- Brown, R. C., H. Bass, and J. P. Coombs. 1975. Carbohydrate binding proteins involved in phagocytosis by *Acanthamoeba*. *Nature* 254(5499):434-435.
- Brown, T. J., R. T. M. Cursons, and E. A. Keys. 1982. Amoebae from Antarctic soil and water. *Applied and Environmental Microbiology* 44(2):491-493.
- Bruno, D. W. 1988. The relationship between auto-agglutination, cell surface hydrophobicity and virulence of the fish pathogen *Renibacterium salmoninarum*. *FEMS Microbiology Letters* 51(2-3):135-140.
- Byers, T. J. 1979. Growth, reproduction, and differentiation in *Acanthamoeba*. *International Review of Cytology* 61:283-338.
- Byers, T. J., S. A. Bogler, and L. L. Burianek. 1983. Analysis of mitochondrial DNA variation as an approach to systematic relationships in the genus *Acanthamoeba*. *Journal of Protozoology* 30(2):198-203.
- Byers, T. J., V. L. Rudick, and M. J. Rudick. 1969. Cell size, macromolecule composition, nuclear number, oxygen consumption and cyst formation during two growth phases in unagitated cultures of *Acanthamoeba castellanii*. *Journal of Protozoology* 16(4):693-699.
- Campos-Pérez, J. J., A. E. Ellis, and C. J. Secombes. 1997. Investigation of factors influencing the ability of *Renibacterium salmoninarum* to stimulate rainbow trout macrophage respiratory burst activity. *Fish and Shellfish Immunology* 7(8):555-566.
- Cao, Z., D. M. Jefferson, and N. Panjwani. 1998. Role of carbohydrate-mediated adherence in cytopathogenic mechanisms of *Acanthamoeba*. *The Journal of Biological Chemistry* 273(25):15838-15845.
- Cavalier-Smith, T. 1993. Kingdom Protozoa and its 18 phyla. *Microbiological Reviews* 57(4):953-994.
- Chalfie, M., Y. Tu, G. Euskirchen, W. W. Ward, and D. C. Prasher. 1994. Green fluorescent protein as a marker for gene expression. *Science* 263(5148):802-805.
- Chambers, J. A. and J. E. Thompson. 1972. A scanning electron microscopic study of the excystment process of *Acanthamoeba castellanii*. *Experimental Cell Research* 73(2):415-421.
- Chambers, J. A. and J. E. Thompson. 1976. Phagocytosis and pinocytosis in *Acanthamoeba castellanii*. *Journal of General Microbiology* 92(2):246-250.

- Cirillo, J. D., S. Falkow, and L. S. Tompkins. 1994. Growth of *Legionella pneumophila* in *Acanthamoeba castellanii* enhances invasion. *Infection and Immunity* 62(8):3254-3261.
- Cirillo, J. D., S. Falkow, L. S. Tompkins, and L. E. Bermudez. 1997. Interaction of *Mycobacterium avium* with environmental amoebae enhances virulence. *Infection and Immunity* 65(9):3759-3767.
- Cirillo, J. D., S. L. G. Cirillo, L. Yan, L. E. Bermudez, S. Falkow, and L. S. Tompkins. 1999. Intracellular growth in *Acanthamoeba castellanii* affects monocyte entry mechanisms and enhances virulence of *Legionella pneumophila*. *Infection and Immunity* 67(9):4427-4434.
- Costas, M. and A. J. Griffiths. 1985. Enzyme composition and the taxonomy of *Acanthamoeba*. *Journal of Protozoology* 32(4):604-607.
- Costas, M., S. W. Edwards, D. Lloyd, A. J. Griffiths, and G. Turner. 1983. Restriction enzyme analysis of mitochondrial DNA of members of the genus *Acanthamoeba* as an aid in taxonomy. *FEMS Microbiology Letters* 17(1-3):231-234.
- Daly, J. G. and R. M. W. Stevenson. 1987. Hydrophobic and haemagglutinating properties of *Renibacterium salmoninarum*. *Journal of General Microbiology* 133(12):3575-3580.
- Davies, B., L. S. Chattings, and S. W. Edwards. 1991. Superoxide generation during phagocytosis by *Acanthamoeba castellanii*: Similarities to the respiratory burst of immune phagocytes. *Journal of General Microbiology* 137(3):705-710.
- De Jonckheere, J. F. 1980. Growth characteristics, cytopathic effect in cell culture, and virulence in mice of 36 type strains belonging to 19 different *Acanthamoeba* spp. *Applied and Environmental Microbiology* 39(4):681-685.
- De Jonckheere, J. F. 1987. Epidemiology, 125-147. In: E. G. Rondanelli (ed.), *Amphizoic Amoebae: Human Pathology*. Piccin, Padua.
- De Jonckheere, J. and H. van de Voorde. 1976. Differences in destruction of cysts of pathogenic and nonpathogenic *Naegleria* and *Acanthamoeba* by chlorine. *Applied and Environmental Microbiology* 31(2):294-297.
- Deere, D., J. Porter, R. W. Pickup, and C. Edwards. 1997. Measurement of cell surface hydrophobicity of *Aeromonas salmonicida*. *Journal of Fish Diseases* 20(5):393-397.
- Dolphin, W. D. 1970. Photoinhibition of growth in *Acanthamoeba castellanii* cultures. *Journal of Bacteriology* 103(3):755-760.
- Doyle, R. J. 1994. Introduction to lectins and their interactions with microorganisms, 1-65. In: R. Doyle and M. Slifkin (eds.), *Lectin-Microorganism Interactions*. Marcel Dekker, Inc., New York.
- Dyková, I., J. Lom, J. M. Schroeder-Diedrich, G. C. Booton, and T. J. Byers. 1999. *Acanthamoeba* strains isolated from organs of freshwater fishes. *Journal of Parasitology* 85(6):1106-1113.
- Ellis, A. E. 1999. Immunity to bacteria in fish. *Fish and Shellfish Immunology* 9(4):291-308.
- Essig, A., M. Heinemann, U. Simnacher, and R. Marre. 1997. Infection of *Acanthamoeba castellanii* by *Chlamydia pneumoniae*. *Applied and Environmental Microbiology* 63(4):1396-1399.
- Evenden, A. J., T. H. Grayson, M. L. Gilpin, and C. B. Munn. 1993. *Renibacterium salmoninarum* and bacterial kidney disease – The unfinished jigsaw. *Annual Review of Fish Diseases*:87-104.

- Ewart, K. V., S. C. Johnson, and N. W. Ross. 1999. Identification of a pathogen-binding lectin in salmon serum. *Comparative Biochemistry and Physiology Part C* **123**(1):9-15.
- Ferrante, A. 1991. Immunity to *Acanthamoeba*. *Reviews of Infectious Diseases* **13**(Supplement 5):S403-S409.
- Finlay, B. J. 1982. Procedures for the isolation, cultivation and identification of protozoa, 44-65. In: R. G. Burns and J. H. Slater (eds.), *Experimental Microbial Ecology*. Blackwell Scientific Publications, Oxford.
- Franke, E. D. and J. S. Mackiewicz. 1982. Isolation of *Acanthamoeba* and *Naegleria* from the intestinal contents of freshwater fishes and their potential pathogenicity. *The Journal of Parasitology* **68**(1):164-166.
- Fritsche, T. R., R. K. Gautom, S. Seyedirashti, D. L. Bergeron, and T. D. Lindquist. 1993. Occurrence of bacterial endosymbionts in *Acanthamoeba* spp. isolated from corneal and environmental specimens and contact lenses. *Journal of Clinical Microbiology* **31**(5):1122-1126.
- Fritsche, T. R., D. Sobek, and R. K. Gautom. 1998. Enhancement of in vitro cytopathogenicity by *Acanthamoeba* spp. following acquisition of bacterial endosymbionts. *FEMS Microbiology Letters* **166**(2):231-236.
- Fritsche, T. R., M. Horn, S. Seyedirashti, R. K. Gautom, K.-H. Schleifer, and M. Wagner. 1999. In situ detection of novel bacterial endosymbionts of *Acanthamoeba* spp. phylogenetically related to members of the order *Rickettsiales*. *Applied and Environmental Microbiology* **65**(1):206-212.
- Garate, M., Z. Cao, and N. Panjwani. 2000. Pathogenic mechanisms of *Acanthamoeba* keratitis: Characterization of the amoeba mannose receptor. *Glycobiology* **10**(10):59.
- Gaziri, G., L. C. J. Gaziri, R. Kikuchi, J. Scanavacca, and I. Felipe. 1999. Phagocytosis of *Candida albicans* by concanavalin-A activated peritoneal macrophages. *Medical Mycology* **37**(3):195-200.
- González, J. M., J. Iriberry, L. Egea, and I. Barcina. 1990. Differential rates of digestion of bacteria by freshwater and marine phagotrophic protozoa. *Applied and Environmental Microbiology* **56**(6):1851-1857.
- González, M., F. Sánchez, M. I. Concha, J. Figueroa, M. I. Montechinos, and G. León. 1999. Evaluation of the internalization process of the fish pathogen *Renibacterium salmoninarum* in cultured fish cells. *Journal of Fish Diseases* **22**(3):231-235.
- Gordon, V. R., E. K. Asem, M. H. Vodkin, and G. L. McLaughlin. 1993. *Acanthamoeba* binds to extracellular matrix proteins in vitro. *Investigative Ophthalmology and Visual Science* **34**(3):658-662.
- Gorlin, A. I., M. M. Gabriel, L. A. Wilson, and D. G. Ahearn. 1995. Binding of *Acanthamoeba* to hydrogel contact lenses. *Current Eye Research* **15**(2):151-155.
- Griffiths, A. J. and D. E. Hughes. 1969. The physiology of encystment of *Hartmannella castellanii*. *Journal of Protozoology* **16**(1):95-99.
- Hall, J. and H. Voelz. 1985. Bacterial endosymbionts of *Acanthamoeba* sp. *Journal of Parasitology* **71**(1):89-95.
- Hammer, A., C. Grüttner, and R. Schumann. 1999. The effect of electrostatic charge of food particles on capture efficiency by *Oxyrrhis marina* Dujardin (dinoflagellate). *Protist* **150**(4):375-382.

- Handley, P. S.** 1991. Detection of cell surface carbohydrate components, 87-107. In: N. Mozes, P. S. Handley, H. J. Busscher, and P. G. Rouxhet (eds.), *Microbial Cell Surface Analysis*. VCH, New York.
- Harb, O. S., C. Venkataraman, B. J. Haack, L.-Y. Gao, and Y. Abu Kwaik.** 1998. Heterogeneity in the attachment and uptake mechanisms of the Legionnaires' disease bacterium, *Legionella pneumophila* by protozoan hosts. *Applied and Environmental Microbiology* **64**(1):126-132.
- Harb, O. S. and Y. Abu Kwaik.** 2000. Interaction of *Legionella pneumophila* with protozoa provides lessons. *ASM News* **66**(10):609-616.
- Harb, O. S., L.-Y. Gao, and Y. Abu Kwaik.** 2000. From protozoa to mammalian cells: A new paradigm in the life cycle of intracellular bacterial pathogens. *Environmental Microbiology* **2**(3):251-265.
- Hardham, A. R. and H. J. Mitchell.** 1998. Use of molecular cytology to study the structure and biology of phytopathogenic and mycorrhizal fungi. *Fungal Genetics and Biology* **24**(1-2):252-284.
- Harf, C.** 1994. Free-living amoeba: Interactions with environmental pathogenic bacteria. *Endocytobiosis and Cell Research* **10**(3):167-183.
- Harf, C., S. Goffinet, O. Meunier, H. Monteil, and D. A. Colin.** 1997. Flow cytometric determination of endocytosis of viable labelled *Legionella pneumophila* by *Acanthamoeba palestinensis*. *Cytometry* **27**(3):269-274.
- Hazen, B. W. and K. C. Hazen.** 1988. Modification and application of a simple, surface hydrophobicity detection method to immune cells. *Journal of Immunological Methods* **107**(2):157-163.
- Hussain, N., P. U. Jani, and A. T. Florence.** 1997. Enhanced oral uptake of tomato lectin-conjugated nanoparticles in the rat. *Pharmaceutical Research* **14**(5):613-618.
- Inglis, T. J. J., P. Rigby, T. A. Robertson, N. S. Dutton, M. Henderson, and B. J. Chang.** 2000. Interaction between *Burkholderia pseudomallei* and *Acanthamoeba* species results in coiling phagocytosis, endamebic bacterial survival, and escape. *Infection and Immunity* **68**(3):1681-1686.
- Jadin, J. B.** 1987. History, 1-24. In: E. G. Rondanelli (ed.), *Amphizoic Amoebae: Human Pathology*. Piccin, Padua.
- James, A. M.** 1991. Charge properties of microbial cell surfaces, 221-262. In: N. Mozes, P. S. Handley, H. J. Busscher, and P. G. Rouxhet (eds.), *Microbial Cell Surface Analysis*. VCH, New York.
- Jensen, T., W. G. Barnes, and D. Meyers.** 1970. Axenic cultivation of large populations of *Acanthamoeba castellanii* (JBM). *The Journal of Parasitology* **56**(5):904-906.
- Jones, D. S., C. G. Adair, W. M. Mawhinney, and S. P. Gorman.** 1996. Standardisation and comparison of methods employed for microbial cell surface hydrophobicity and charge determination. *International Journal of Pharmaceutics* **131**(1):83-89.
- Kamath, R. S., M. Martinez-Campos, P. Zipperlen, A. G. Fraser, and J. Ahringer.** 2000. Effectiveness of specific RNA-mediated interference through ingested double-stranded RNA in *Caenorhabditis elegans*. *Genome Biology* **2**(1):1-10.
- Kanno, M., K. Yagita, T. Endo, K. Miyata, M. Araie, and T. Tsuru.** 1998. Restriction enzyme analysis of mitochondrial DNA of *Acanthamoeba* strains isolated from corneal lesions. *Japanese Journal of Ophthalmology* **42**(1):22-26.

- Kennett, M. J., R. R. Hook, C. L. Franklin, and L. K. Riley. 1999. *Acanthamoeba castellanii*: Characterization of an adhesin molecule. *Experimental Parasitology* **92**(3):161-169.
- Kilvington, S. 2000. Through a glass darkly – Contact lenses and personal hygiene. *Microbiology Today* **27**(3):66-69.
- King, C. H. and E. B. Shotts. 1988. Enhancement of *Edwardsiella tarda* and *Aeromonas salmonicida* through ingestion by the ciliated protozoan *Tetrahymena pyriformis*. *FEMS Microbiology Letters* **51**(2-3):95-99.
- Korn, E. D. and R. A. Weisman. 1967. Phagocytosis of latex microspheres by *Acanthamoeba* II. Electron microscopic study of the initial events. *The Journal of Cell Biology* **34**(1):219-227.
- Kyle, D. E. and G. P. Noblet. 1985. Vertical distribution of potentially pathogenic free-living amoebae in freshwater lakes. *Journal of Protozoology* **32**(1):99-105.
- Kyle, D. E. and G. P. Noblet. 1986. Seasonal distribution of thermotolerant free-living amoebae. I. Willard's Pond. *Journal of Protozoology* **33**(3):422-434.
- Larkin, D. F. P. and D. L. Easty. 1990. External eye flora as a nutrient source for *Acanthamoeba*. *Graefe's Archive for Clinical and Experimental Ophthalmology* **228**(5):458-460.
- Leher, H., R. Silvany, H. Alizadeh, J. Huang, and J. Y. Niederkorn. 1998. Mannose induces the release of cytopathic factors from *Acanthamoeba castellanii*. *Infection and Immunity* **66**(1):5-10.
- Lindahl, M., A. Faris, T. Wadström, and S. Hjertén. 1981. A new test based on 'salting out' to measure relative surface hydrophobicity of bacterial cells. *Biochimica et Biophysica Acta* **677**(3-4):471-476.
- Livesley, M. A., S. N. Smith, R. A. Armstrong and G. A. Barker. 1999. Characterisation of *Aeromonas* strains and species by pulsed field gel electrophoresis and principal components analysis. *Journal of Fish Diseases* **22**(5): 369-375.
- Lock, R., L. Öhman, and C. Dahlgren. 1987. Phagocytic recognition mechanisms in human granulocytes and *Acanthamoeba castellanii* using type 1 fimbriated *Escherichia coli* as phagocytic prey. *FEMS Microbiology Letters* **44**(1):135-140.
- Marolda, C. L., B. Hauröder, M. A. John, R. Michel, and M. A. Valvano. 1999. Intracellular survival and saprophytic growth of isolates from the *Burkholderia cepacia* complex in free-living amoebae. *Microbiology* **145**(7):1509-1517.
- Marshall, M. M., D. Naumovitz, Y. Ortega, and C. R. Sterling. 1997. Waterborne protozoan pathogens. *Clinical Microbiology Reviews* **10**(1):67-85.
- Mathers, W. D., S. E. Nelson, J. L. Lane, M. E. Wilson, R. C. Allen, and R. Folberg. 2000. Confirmation of confocal microscopy diagnosis of *Acanthamoeba* keratitis using polymerase chain reaction analysis. *Archives of Ophthalmology* **118**(2):178-183.
- Matz, C. and K. Jürgens. 2001. Effects of hydrophobic and electrostatic cell surface properties of bacteria on feeding rates of heterotrophic nanoflagellates. *Applied and Environmental Microbiology* **67**(2):814-820.
- Mendgen, K., M. Lange, and K. Bretschneider. 1985. Quantitative estimation of the surface carbohydrates on the infection structures of rust fungi with enzymes and lectins. *Archives of Microbiology* **140**(4):307-311.

- Michaelis, L.** 1962. Buffer solutions, 314-315. In: K. Diem (ed.), *Scientific Tables* (Sixth Edition). Giegy Pharmaceutical Company Limited, Manchester.
- Morton, L. D., G. L. McLaughlin, and H. E. Whiteley.** 1991. Effects of temperature, amebic strain, and carbohydrates on *Acanthamoeba* adherence to corneal epithelium in vitro. *Infection and Immunity* **59**(10):3819-3822.
- Mukherjee, R. M., T. K. Maitra, D. P. Haldar, and K. N. Jalan.** 1993. Adherence of *Entamoeba histolytica* to hydrophobic matrices: A simple method for measuring cell surface hydrophobicity. *Transactions of The Royal Society of Tropical Medicine and Hygiene* **87**(4):492-493.
- Müller, R. H.** 1991a. Characterization of carriers, 43-97. In: R. H. Müller (ed.), *Colloidal Carriers for Controlled Drug Delivery and Targetting*. Wissenschaftliche Verlagsgesellschaft, Stuttgart.
- Müller, R. H.** 1991b. Surface hydrophobicity, 99-143. In: R. H. Müller (ed.), *Colloidal Carriers for Controlled Drug Delivery and Targetting*. Wissenschaftliche Verlagsgesellschaft, Stuttgart.
- Munro, A. L. S. and T. S. Hastings.** 1993. Furunculosis, 122-142. In: V. Inglis, R. J. Roberts, and N. R. Bromage (eds.), *Bacterial Diseases of Fish*. Blackwell Scientific Publications, Oxford.
- Neff, R. J.** 1957. Purification, axenic cultivation, and description of a soil amoeba, *Acanthamoeba* sp. *Journal of Protozoology* **4**:176-182.
- Neff, R. J.** 1958. Mechanisms of purifying amoebae by migration on agar surfaces. *Journal of Protozoology* **5**(3):226-231.
- Neff, R. J., W. F. Benton, and R. H. Neff.** 1964a. The composition of the mature cyst wall of the soil ameba *Acanthamoeba* sp. *The Journal of Cell Biology* **23**(2):66A.
- Neff, R. J., S. A. Ray, W. F. Benton, and M. Wilborn.** 1964b. Induction of synchronous encystment (differentiation) in *Acanthamoeba* sp., 55-83. In: D. M. Prescott (ed.), *Methods In Cell Physiology* (vol. 1). Academic Press, New York.
- Newsome, A. L., T. M. Scott, R. F. Benson, and B. S. Fields.** 1998. Isolation of an amoeba naturally harboring a distinctive *Legionella* species. *Applied and Environmental Microbiology* **64**(5):1688-1693.
- O'Dell, W. D.** 1979. Isolation, enumeration and identification of amebae from a Nebraska Lake. *Journal of Protozoology* **26**(2):265-269.
- Ofek, I. and N. Sharon.** 1988. Lectinophagocytosis: A molecular mechanism of recognition between cell surface sugars and lectins in the phagocytosis of bacteria. *Infection and Immunity* **56**(3):539-547.
- Ogbunude, P. O. J., S. Asiri, H. P. Baer, and D. C. Warhurst.** 1991. New method for detachment and quantitation of *Acanthamoeba* trophozoites in culture. *Tropical Medicine and Parasitology* **42**(4):415-416.
- Paatero, G. I. L., L. Wikström, and B. Isomaa.** 1988. Concanavalin A-induced redistribution of surface receptors in *Acanthamoeba castellanii* at different growth phases. *European Journal of Cell Biology* **47**(1):112-120.
- Page, F. C.** 1988. *A New Key To Freshwater And Soil Gymnamoebae*. The Freshwater Biological Association, Ambleside.
- Pembrey, R. S., K. C. Marshall, and R. P. Schneider.** 1999. Cell surface analysis techniques: What do cell preparation protocols do to cell surface properties? *Applied and Environmental Microbiology* **65**(7):2877-2894.

- Pernin, P., M. Pélandakis, Y. Rouby, A. Faure, and F. Siclet. 1998. Comparative recoveries of *Naegleria fowleri* amoebae from seeded river water by filtration and centrifugation. *Applied and Environmental Microbiology* **64**(3):955-959.
- Preston, T. M. and C. A. King. 1984. Binding sites for bacterial flagella at the surface of the soil amoeba *Acanthamoeba*. *Journal of General Microbiology* **130**(6):1449-1458.
- Preston, T. M., H. Richards, and R. S. Wotton. 2001. Locomotion and feeding of *Acanthamoeba* at the water-air interface of ponds. *FEMS Microbiology Letters* **194**(2):143-147.
- Przełęcka, A. and A. Sobota. 1982. Growth phase dependent alterations in the surface coat of *Acanthamoeba castellanii*. *Acta Histochemica* **71**(2):219-229.
- Przełęcka, A. and E. Perkowska. 1986. Developmental stage dependent alterations in the net surface charge of *Acanthamoeba castellanii*. *Acta Protozoologica* **25**(3):339-344.
- Rabinovitch, M. and M. J. De Stefano. 1971. Phagocytosis of erythrocytes by *Acanthamoeba* sp. *Experimental Cell Research* **64**(2):275-284.
- Ribeiro, S., R. M. de Araujo Soares, C. S. Alviano, E. F. De Silva, W. De Souza, and J. Angluster. 1997. Cell-surface carbohydrates of *Entamoeba invadens*. *Parasitology Research* **83**(8):801-805.
- Robin, J. B., R. Chan, and B. R. Andersen. 1988. Rapid visualization of *Acanthamoeba* using fluorescein-conjugated lectins. *Archives of Ophthalmology* **106**(9):1273-1276.
- Robin, J. B., R. Chan, N. A. Rao, S. Sharma, and M. Srinivasan. 1989. Fluorescein-conjugated lectin visualization of fungi and acanthamoebae in infectious keratitis. *Ophthalmology* **96**(8):1198-1202.
- Rodríguez-Zaragoza, S. 1994. Ecology of free-living amoebae. *Critical Reviews in Microbiology* **20**(3):225-241.
- Rondanelli, E. G. and M. Scaglia. 1987. Pathogenic amphizoic amoebae (*Naegleria fowleri* - *Acanthamoeba* spp.). Morphology and cytology, 49-86. In: E. G. Rondanelli (ed.), *Amphizoic Amoebae: Human Pathology*. Piccin, Padua.
- Rosenberg, M. and S. Kjelleberg. 1986. Hydrophobic interactions: Role in bacterial adhesion. *Advances In Microbial Ecology* **9**:353-393.
- Rosenberg, M. and R. J. Doyle. 1990. Microbial cell surface hydrophobicity: History, measurement, and significance, 1-37. In: R. J. Doyle and M. Rosenberg (eds.), *Microbial Cell Surface Hydrophobicity*. American Society For Microbiology, Washington DC.
- Roser, M., D. Fischer, and T. Kissel. 1998. Surface-modified biodegradable albumin nano- and microspheres. II: Effect of surface charges on in vitro phagocytosis and biodistribution in rats. *European Journal of Pharmaceutics and Biopharmaceutics* **46**(3):255-263.
- Rowbotham, T. J. 1980. Preliminary report on the pathogenicity of *Legionella pneumophila* for freshwater and soil amoebae. *Journal of Clinical Pathology* **33**(12):1179-1183.
- Rowbotham, T. J. 1983. Isolation of *Legionella pneumophila* from clinical specimens via amoebae, and the interaction of those and other isolates with amoebae. *Journal of Clinical Pathology* **36**(9):978-986.
- Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular Cloning: A Laboratory Manual* (second edition). Cold Spring Harbor Laboratory Press, New York.

- Segal, G. and H. A. Shuman. 1999. *Legionella pneumophila* utilizes the same genes to multiply within *Acanthamoeba castellanii* and human macrophages. *Infection and Immunity* 67(5):2117-2124.
- Sharp, G. J. E. and C. J. Secombes. 1993. The role of reactive oxygen species in the killing of the bacterial fish pathogen *Aeromonas salmonicida* by rainbow trout macrophages. *Fish and Shellfish Immunology* 3(2):119-129.
- Sibille, I., T. Sime-Ngando, L. Mathieu, and J. C. Block. 1998. Protozoan bacterivory and *Escherichia coli* survival in drinking water distribution systems. *Applied and Environmental Microbiology* 64(1):197-202.
- Silverstein, S. C. 1995. Phagocytosis of microbes: Insights and prospects. *Trends in Cell Biology* 5(3):141-142.
- Smith, S. N., R. Chohan, R. A. Armstrong, and J. M. Whipps. 1998. Hydrophobicity and surface electrostatic charge of conidia of the mycoparasite *Coniothyrium minitans*. *Mycological Research* 102(2):243-249.
- Smith, S. N., R. A. Armstrong, R. A. Bird, R. Chohan, N. A. Hartell, and D. A. Poyner. 2001. Characterization of FITC-conjugated lectin binding to *Candida albicans*. *Mycologia* 93(3):422-431.
- Sørum, U., B. Robertsen, and L. Kenne. 1998. Structural studies of the major polysaccharide in the cell wall of *Renibacterium salmoninarum*. *Carbohydrate Research* 306(1-2):305-314.
- Steinert, M., K. Birkness, E. White, B. Fields, and F. Quinn. 1998. *Mycobacterium avium* bacilli grow saprozoically in coculture with *Acanthamoeba polyphaga* and survive within cyst walls. *Applied and Environmental Microbiology* 64(6):2256-2261.
- Steinert, M., U. Hentschel, and J. Hacker. 2000. Symbiosis and pathogenesis: Evolution of the microbe-host interaction. *Naturwissenschaften* 87(1):1-11.
- Stevens, A. R. and S. Stein. 1977. Analyses of pathogenic and nonpathogenic *Acanthamoeba* and *Naegleria* for lectin-induced agglutination. *Journal of Parasitology* 63(1):151-152.
- Stothard, D. R., J. Hay, J. M. Schroeder-Diedrich, D. V. Seal, and T. J. Byers. 1999. Fluorescent oligonucleotide probes for clinical and environmental detection of *Acanthamoeba* and the T4 18S rRNA gene sequence type. *Journal of Clinical Microbiology* 37(8):2687-2693.
- Tabata, Y. and Y. Ikada. 1987. Effect of the size and surface charge of polymer microspheres on their phagocytosis by macrophage. *Biomaterials* 9(4):356-362.
- Taylor, P. W. 1977. Isolation and experimental infection of free-living amoebae in freshwater fishes. *The Journal of Parasitology* 63(2):232-237.
- Timmons, L. and A. Fire. 1998. Specific interference by ingested dsRNA. *Nature* 395(6705):854.
- Trust, T. J., W. W. Kay, and E. E. Ishiguro. 1983. Cell-surface hydrophobicity and macrophage association of *Aeromonas salmonicida*. *Current Microbiology* 9(6):315-318.
- Ubelaker, J. E. 1991. *Acanthamoeba* spp.: "Opportunistic pathogens". *Transactions of The American Microscopical Society* 110(4):289-299.
- van der Mei, H. C., B. van de Belt-Gritter, G. Reid, H. Bialkowska-Hobrzanska, and H. J. Busscher. 1997. Adhesion of coagulase-negative staphylococci grouped according to physico-chemical surface properties. *Microbiology* 143(12):3861-3870.

- van Oss, C. J. 1971. Influence of glucose levels on the in vitro phagocytosis of bacteria by human neutrophils. *Infection and Immunity* 4(1):54-59.
- van Oss, C. J. 1978. Phagocytosis as a surface phenomenon. *Annual Review of Microbiology* 32:19-39.
- Vector Laboratories. 1999. Lectins. In: Vector Laboratories Catalogue 1999/2000: 63-82. Vector Laboratories, Inc., Burlingame.
- Vinogradova, O. I. and G. E. Yakubov. 2001. Forces between polystyrene surfaces in water-electrolyte solutions: Long-range attraction of two types? *Journal of Chemical Physics* 114(18):8124-8131.
- Visvesvara, G. S. and W. Balamuth. 1975. Comparative studies on related free-living and pathogenic amoebae with special reference to *Acanthamoeba*. *Journal of Protozoology* 22(2):245-256.
- Walker, C. W. B. 1996. *Acanthamoeba*: Ecology, pathogenicity and laboratory detection. *British Journal of Biomedical Science* 53(2):146-151.
- Walochnik, J., A. Obwaller, and H. Aspöck. 2000. Correlations between morphological, molecular biological, and physiological characteristics in clinical and nonclinical isolates of *Acanthamoeba* spp. *Applied and Environmental Microbiology* 66(10):4408-4413.
- Weekers, P. H. H., P. L. E. Bodelier, J. P. H. Wijen, and G. D. Vogels. 1993. Effects of grazing by the free-living soil amoebae *Acanthamoeba castellanii*, *Acanthamoeba polyphaga*, and *Hartmannella vermiformis* on various bacteria. *Applied and Environmental Microbiology* 59(7):2317-2319.
- Weisman, R. A. 1976. Differentiation in *Acanthamoeba castellanii*. *Annual Review of Microbiology* 30:189-219.
- Weisman, R. A. and E. D. Korn. 1967. Phagocytosis of latex microspheres by *Acanthamoeba*. I. Biochemical properties. *Biochemistry* 6(2):485-497.
- Winiacka-Krusnell, J. and E. Linder. 1998. *Acanthamoeba* keratitis: Increased sensitivity of the detection of parasites by modified cultivation procedure. *Scandinavian Journal of Infectious Diseases* 30(6):639-641.
- Winiacka-Krusnell, J. and E. Linder. 1999. Free-living amoebae protecting *Legionella* in water: The tip of an iceberg? *Scandinavian Journal of Infectious Diseases* 31(4):383-385.
- Winiacka-Krusnell, J. and E. Linder. 2001. Bacterial infections of free-living amoebae. *Research in Microbiology* 152(7):613-619.
- Yang, S. and C. Villemez. 1994. Cell surface control of differentiation in *Acanthamoeba*. *Journal of Cellular Biochemistry* 56(4):592-596.
- Yousif, A. N., L. J. Albright, and T. P. T. Evelyn. 1994. Purification and characterization of a galactose-specific lectin from the eggs of coho salmon *Oncorhynchus kisutch* and its interaction with bacterial fish pathogens. *Diseases of Aquatic Organisms* 20(2):127-136.

APPENDICES

Appendix A: Carboxylate

Appendix 1: Barbitol sodium acetate buffer formation

Table A1. Volumes of stock solutions required for formation of specific pH values of barbitol sodium acetate buffer. Solution A: 0.14 M sodium acetate, 0.14 M barbitol sodium, 1 L distilled water; Solution B: 0.1 N HCl, 1 L distilled water; Solution C: 8.5 % (w/v) NaCl, 1 L distilled water. Buffers were then adjusted, if necessary, to the exact pH by dropwise addition of either solution A (base) or solution B (acid), as appropriate. Adapted from Michaelis (1962).

pH	Solution A (ml)	Solution B (ml)	Solution C (ml)	Water (ml)
3.0	50	154	20	26
4.0	50	125.5	20	54.5
5.0	50	88	20	92
6.0	50	71	20	109
7.0	50	60.5	20	119.5
8.0	50	26	20	154
9.0	50	4	20	176

Appendix 2: Composition of gels and loading buffer for SDS-PAGE

Table A2. Composition of separating and stacking gels, sample loading buffer and electrode buffer used in SDS-PAGE.

Constituent	Separating Gel (11 % w/v)	Stacking Gel (5 % w/v)	Sample Loading Buffer
Acrylamide Stock I*	5.5 ml	-	-
Acrylamide Stock II†	-	2.5 ml	-
10 % (w/v) SDS	0.5 ml	0.15 ml	5 ml
1.5 M Tris-HCl (pH 8.8)	6 ml	-	-
0.5 M Tris-HCl (pH 6.8)	-	3.75 ml	2.5 ml
Distilled water	7.5 ml	8 ml	5 ml
TEMED	50 µl	40 µl	-
10 % (w/v) Ammonium persulphate	70 µl	50 µl	-
Glycerol	-	-	2.5 ml
2-mercaptoethanol	-	-	0.25 ml
5 % (w/v) Bromophenol blue	-	-	0.2 ml

* Acrylamide Stock I – 40 % (w/v) acrylamide and 0.8 % (w/v) Bis (N,N¹-methylene-bis-acrylamide) (Severn Biotech Ltd., UK)

† Acrylamide Stock II – 30 % (w/v) acrylamide and 0.8 % (w/v) Bis (N,N¹-methylene-bis-acrylamide) (Severn Biotech Ltd., UK)

Appendix 3: Protocol for carbodiimide binding of Con A to carboxylate microspheres

1. Place 0.5 ml 2.5 % carboxylate microsphere (2.0 μm diameter) suspension into an Eppendorf tube.
2. Add 1 ml carbonate buffer and cap tightly.
3. Centrifuge for 5 min at 14 000 rpm in a microcentrifuge.
4. Remove supernatant carefully with a pipette. Discard.
5. Resuspend pellet in 1.5 ml carbonate buffer and cap tightly.
6. Centrifuge and discard supernatant as before.
7. Resuspend pellet in 1.5 ml phosphate buffer and cap tightly.
8. Centrifuge and discard supernatant as before.
9. Repeat steps 7 and 8 twice more.
10. Resuspend pellet in 0.6 ml phosphate buffer until completely resuspended.
11. Make 0.75 ml 2 % (w/v) solution of carbodiimide by dissolving 15 mg carbodiimide in 0.75 ml phosphate buffer. This solution should be prepared fresh and used within 15 min. Protect stock carbodiimide from moisture by tightly capping the bottle.
12. Add 0.6 ml carbodiimide solution dropwise to the resuspended pellet and cap tightly.
13. Mix on a roller for 1 to 4 hours. Do not mix for more than 4 hours.
14. Centrifuge and discard supernatant as before.
15. Resuspend in 1.5 ml borate buffer.
16. Centrifuge and discard supernatant as before.
17. Repeat steps 15-17 twice more.
18. Resuspend in 1 ml borate buffer containing 1 mg unconjugated Con A.
19. Mix overnight on a roller
20. Centrifuge at 14 000 rpm for 10 min to allow thorough pelleting.
21. Transfer the supernatant carefully with a pipette and retain for determination of protein content.
22. Resuspend pellet in 1 ml 0.1 M ethanolamine to block unreacted binding sites on the microspheres. Mix on a roller for 30 min.
23. Centrifuge at 14 000 rpm for 5 min and discard supernatant as before.
24. Resuspend in 1 ml BSA solution to block any remaining non-specific protein binding sites. Mix on a roller for 30 min.
25. Centrifuge at 14 000 rpm for 5 min and discard supernatant as before.
26. Resuspend in 1 ml storage buffer and store at 4 °C. Do not freeze.

Appendix 4: Sigma Diagnostics glucose testing kit

1. Tube 1: Blank – add 20 μl water.
2. Tube 2: Standard – add 20 μl glucose standard.
3. Tube 3: Sample – add 20 μl sample. Set up separate tubes for each sample to be tested.

4. Add 1 ml glucose assay reagent to each tube.
5. Mix reagents by swirling before allowing tubes to stand for 5 to 10 min at room temperature.
6. Add 10 ml 0.1 N HCl to each tube and mix.
7. Transfer each sample to separate cuvettes.
8. Read absorbance at 520 nm against blank tube.
9. Calculate glucose concentration as follows:

$$\text{Glucose concentration (mg ml}^{-1}\text{)} = \frac{A_{520} \text{ of sample}}{A_{520} \text{ of standard}}$$

Appendix 5: Promega Wizard[®] Plus Maxiprep DNA purification system

1. Pellet 500 ml *E. coli* in LB broth by centrifugation at 5 000 g for 10 min at room temperature. Pour off the supernatant and resuspend in 15 ml Cell Resuspension Solution. Manually disrupt the pellet to aid resuspension.
2. Add 15 ml Cell Lysis Solution and mix gently but thoroughly, by stirring or inverting. Do not vortex.
3. Add 15 ml Neutralization Solution and immediately mix by gently inverting several times.
4. Centrifuge at 14 000 g for 15 min at room temperature.
5. Transfer the cleared supernatant by filtering through filter paper into a clean 100 ml measuring cylinder. Measure the supernatant volume then transfer to a centrifuge bottle.
6. Add a 50 % volume of room temperature isopropanol and mix by inversion.
7. Centrifuge at 14 000 g for 15 min at room temperature.
8. Discard the supernatant and resuspend the DNA pellet in 2 ml TE buffer.
9. Thoroughly wash the walls of the bottle with TE buffer to recover all of the DNA. At this point, the pellet may not be visible.
10. Add 10 ml Wizard[®] Maxipreps DNA Purification Resin to the DNA solution and swirl to mix.
11. For each Maxiprep, use one Maxicolumn. Insert the Maxicolumn tip into the vacuum manifold port.
12. Transfer the resin/DNA mix into the Maxicolumn. Apply a vacuum to pull the resin/DNA mix into the Maxicolumn.
13. Add 25 ml Column Wash Solution to the Maxicolumn and apply a vacuum to draw the solution through the Maxicolumn.
14. To rinse the resin, add 5 ml 80 % (v/v) ethanol to the Maxicolumn and apply a vacuum to draw the ethanol through the Maxicolumn. Allow the vacuum to draw for an additional 1 min.
15. Place the Maxicolumn in a 50 ml screw cap tube. Using a centrifuge with a swinging bucket rotor, centrifuge the Maxicolumn at 1300 g for 5 min. It is essential that a swinging bucket rotor be used for this step.
16. Remove the Maxicolumn and discard both the tube and the liquid. Place the Maxicolumn back on the vacuum manifold.

17. Dry the resin by applying a vacuum for 5 min. Remove the Maxicolumn from the vacuum manifold. Place the Maxicolumn in the provided Reservoir (50 ml screw cap tube).
18. Add 1.5 ml preheated (65–70°C) nuclease-free water to the Maxicolumn and wait 1 min. Elute the DNA by centrifuging the Maxicolumn/Reservoir at 1 300 g for 5 min in a centrifuge with a swinging bucket rotor
19. A white pellet of resin fines may be present in the final eluate. Whether visible or not, it is important to separate the fines from the DNA. Remove the plunger from one of the 5 ml Syringes and set it aside.
20. Attach the syringe barrel to the Luer-Lok[®] extension of a 0.2 µm Syringe Filter and pipette the eluate into the Syringe Barrel.
21. Center the filter over a 15 ml plastic tube. Carefully insert the plunger into the Syringe Barrel and gently push the liquid into the tube.
22. Transfer the eluate to a 1.5 ml centrifuge tube. Centrifuge the tube at 14 000 g for 1 min. This additional step will remove all resin fines that may be present in the final eluate.
23. Immediately transfer the supernatant to a new microcentrifuge tube for storage at –20 °C or below.

Appendix 6: Wizard[®] Genomic DNA Purification kit

1. Harvest the cells and transfer them to a 1.5 ml microcentrifuge tube.
2. Centrifuge at 14 000 rpm for 10 sec to pellet the cells.
3. Remove the supernatant, leaving behind the cell pellet plus 10 to 50 µl residual liquid.
4. Add 200 µl PBS to wash the cells. Centrifuge as in step 2 and remove the PBS. Vortex vigorously to resuspend cells.
5. Add 600 µl Nuclei Lysis Solution and pipette to lyse the cells. Pipette until no visible cell clumps remain.
6. Add 3 µl RNase Solution to the nuclear lysate and mix the sample by inverting the tube five times. Incubate the mixture for 30 min at 37 °C. Allow the sample to cool to room temperature for 5 min before proceeding.
7. To the room temperature sample, add 200 µl Protein Precipitation Solution and vortex vigorously at high speed for 20 sec. Chill sample on ice for 5 min.
8. Centrifuge for 4 min at 14 000 rpm. The precipitated protein will form a tight white pellet.
9. Carefully remove the supernatant containing the DNA (leaving the protein pellet behind) and transfer it to a clean 1.5 ml microcentrifuge tube containing 600 µl room temperature isopropanol.
10. Gently mix the solution by inversion until the white thread-like strands of DNA form a visible mass.
11. Centrifuge for 1 min at 14 000 rpm at room temperature. The DNA will be visible as a small white pellet. Carefully decant the supernatant.
12. Add 600 µl room temperature 70 % (v/v) ethanol and gently invert the tube several times to wash the DNA. Centrifuge for 1 min at 14 000 rpm at room temperature.

13. Carefully remove the ethanol using a pipette. The DNA pellet is very loose at this point and care must be used to avoid aspirating the pellet into the pipette.
14. Invert the tube on clean absorbent paper and air-dry the pellet for 10 to 15 min.
15. Add 100 μ l sterile distilled water and rehydrate the DNA by incubating at 65 °C for 1 hour. Periodically mix the solution by gently tapping the tube.
17. Store the DNA at -4 °C.