REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE (DD-MM-YYYY)	2. REPORT TYPE	3. DATES COVERED
23/10/2002	FINAL REPORT	1/12/1995-30/11/2001

4. TITLE AND SUBTITLE

1. Primary adhesion in *Enteromorpha*.
2. Cue detection and surface selection in the settlement and adhesion of *Enteromorpha* spores

5a. CONTRACT NUMBER

5b. GRANT NUMBER
N00014-96-0373

5c. PROGRAM ELEMENT NUMBER

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

6. AUTHOR(S)

Callow, James A. Prof
Callow, Maureen E. Dr

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

School of Biosciences
University of Birmingham
Birmingham, B15 2TT, UK

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)

Office of Naval Research
800 N. Quincy St.
Arlington
VA 22217-5000, USA

10. SPONSOR/MONITOR'S ACRONYM(S)
ONR

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AILABILITY STATEMENT

Distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

Enteromorpha is the most important alga fouling hulls, whether coated with conventional Cu-based paints or foul-release silicones. Since fouling by *Enteromorpha* is increasing as a consequence of the increased deployment of TBT-free coatings, it is important to understand the processes involved in the initiation of fouling, i.e. spore settlement and adhesion. Our results provide the most comprehensive characterisation of the settlement and adhesion processes and the roles of surface-associated cues, of any soft-fouling species to date. We have shown that spores respond to chemical, physico-chemical, biological (microbial biofilm) and topographic cues. Novel insights have been gained on communication processes involved in the development of a micro-fouling community. Thirteen papers in refereed journals and several abstracts were published.

15. SUBJECT TERMS

biofouling, adhesion, algal spores, *Enteromorpha*, anti-fouling, inter-species communication, biofilms, topography, chemical cues

16. SECURITY CLASSIFICATION OF: a. REPORT	b. ABSTRACT	c. THIS PAGE	17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
Unclass	Unclass	Unclass	UL	4	Prof. JA Callow
					19b. TELEPHONE NUMBER (Include area code) +44 121 414 5559

Standard Form 298 (Rev. 8/98)
Prescribed by ANSI Std Z39.18

20021129 044

FINAL REPORT

Grant #: N00014-96-0373

PRINCIPAL INVESTIGATORS: Prof. J A Callow and Dr Maureen E Callow

INSTITUTION: University of Birmingham, UK

GRANT TITLE: 1) Primary adhesion in *Enteromorpha*. 2) Cue detection and surface selection in the settlement and adhesion of *Enteromorpha* spores.

AWARD PERIOD: 1 December 1995 - 30 November 2001

OBJECTIVES: Our long-term goal is to prevent fouling of marine underwater structures, specifically ships and submarines, by the green alga *Enteromorpha*. Towards this goal this project aimed to 1) characterize settlement and adhesive processes used by *Enteromorpha*; 2) develop adhesion microassays and use these to explore adhesion phenomena quantitatively; 3)examine the detailed behaviour of zoospores during settlement and attachment to various substrata using video microscopy; 4)identify and evaluate the role of diffusible chemical and topographical settlement cues; 5)evaluate the importance of the microbial biofilm as a source of such signals; 6) evaluate the impact of different surface chemistries and physical properties on spore settlement and attachment; 7)extend the analyses of the influence of these chemical, physical and biological factors on primary adhesion, to their impact on subsequent development and establishment of sporelings.

APPROACH: Quantitative assays of zoospore settlement and adhesion were developed and used to analyse the kinetics of adhesion to test surfaces and to test the influence of exogenous compounds. Assays were supported by video microscopy observations of spore behaviour. The bioassays were used to ascertain the importance of cues (chemical, physico-chemical, biological (microbial biofilms) and topographical) in settlement and adhesion of *Enteromorpha* spores. Microtopographies were fabricated in PDMS by collaborators.

ACCOMPLISHMENTS: Standardised protocols were developed for a reliable quantitative bioassay for zoospore settlement and adhesion (Callow *et al.*, 1997). The assays formed the basis of subsequent extensive studies (13 primary publications) during which many novel features of the settlement and adhesion processes were demonstrated. This includes their use in parallel molecular programmes funded by other agencies including the production of anti-adhesive antibody probes to characterise the spore adhesive (Callow *et al.*, 2000a, Callow *et al.*, 2001, Stanley *et al.*, 1999).

Scatchard-type analyses of adhesion data revealed interesting spore density-dependent affects with positive co-operativity being observed at low densities and negative co-operativity at high densities. The significance of these results is that they suggest the possibility of inter-spore or spore-surface communication (Callow *et al.*, 1997).

The most critical event in surface colonisation is the selection of a suitable surface on which to settle by a motile spore since commitment to adhesion results in permanent attachment. High resolution video microscopy identified a number of novel features of the spore attachment process (Callow *et al.*, 1997). Surface sensing appears to proceed via the apical papilla, not the flagella. The spore spins on the apical papilla during a phase of 'surface sensing' and temporary adhesion. If the surface is 'inhospitable', it swims away to seek a more favourable attachment site. Permanent adhesion is moderated by the discharge of adhesive from vesicles present in the swimming spore. During and immediately after adhesive discharge, the spore undergoes 'spacefilling' amoeboid-like movements in order to maximise adhesion to the substratum. Gregarious settlement (settlement in groups) is frequently observed. Subsequent detachment studies showed that spore groups are more strongly attached than single spores, thereby enhancing survival in a turbulent environment (Finlay *et al.*, 2002).

To investigate the influence of surface physico-chemical properties on spore settlement we have made extensive use of model surfaces in the form of alkanethiol self-assembled monolayers (SAMs) (collaboration with Prof G Lopez, UNM)in preference to diverse substrates commonly used. SAMs of different wettability but uniform charge (uncharged) revealed a positive correlation between the number of spores that attached and increasing hydrophobicity (Callow *et al.*, 2000b). However, a negative correlation was found between the strength of spore attachment and increasing wettability (Finlay *et al.*, 2002). Spores responded differently to (COOH-)SAMs compared to (OH-SAMs) of equivalent wettability suggesting the involvement of parameters such as charge in spore attachment (Ista et al., in preparation).

We have shown that *Enteromorpha* spores respond to a number of external chemical cues. They are strongly attracted to trace quantities of certain solvents and to dibutyltin dilaurate, the curing agent used in commercial foul-release silicones. The cue appears to be the fatty acid (lauric acid) component of the molecule and we subsequently showed that spore attraction increased with the number of carbon atoms up to a maximum at C_{14}/C_{15} (Callow & Callow, 1998b). This study laid the foundation for subsequent detailed explorations of natural chemical cues produced by *Enteromorpha* and by biofilm organisms (see below).

In the search for natural, settlement-modifying chemical cues produced by *Enteromorpha* lipophilic fractions of vegetative plants and swimming spores were investigated by LC-MS and bioassay methods. We showed that methanol and acetone extracts promoted and inhibited spore settlement respectively. Separation of both extracts by HPLC revealed many peaks some of which either inhibited or stimulated spore settlement. A number of closely related peaks with inhibitory activity were analysed by MS and NMR. Molecular masses ranged from 737.5 - 769.5 which is consistent with structures based on MGDG (monogalactosyldiacylglycerol) with differing acyl chains. In parallel studies on high molecular weight, water-soluble cues, 2 protein fractions with inhibitory and stimulatory effects on spore settlement were identified by SDS-PAGE and FPLC. The 180 kDa (native) inhibitor protein runs at 58 kDa on denaturing gels. Tryptic fragments were analysed by MALDI-QTOF but peptide sequence data were equivocal, hence the protein awaits further characterisation.

The response of spores to topographic cues was investigated via collaboration with Prof. A B Brennan (Florida). Microtextures fabricated in polydimethyl siloxane (PDMS) showed that spores settle preferentially in depressions (pits & grooves) and against features protruding from the surface (Callow *et al.*, 2002, Wilkerson *et al.*, 2001). Settlement of spores was much greater on patterns that were based on valley/ridge designs compared to pillars and that were 5 µm deep/high compared to ones 1.5 µm deep/high. Most spores attached in the angle between the valley floor and wall, an area of minimum energy. The addition of oils reduced settlement in the 5 µm wide valleys but the effect of oils were less marked on the patterns with lower profile (1.5 µm) features and no combinations of features and oil reduced spore settlement below that on the equivalent smooth, flat surface.

In studies on the role of cues presented by biofilm bacteria, we showed that natural mixed species biofilms promoted spore settlement and spores were preferentially associated with microcolonies (Joint *et al.*, 2000). To examine the specificity of this reponse individual strains of bacteria were isolated from rocks and *Enteromorpha* plants and identified by 16S rDNA sequencing (Patel *et al.*, 2003). Biofilms of all strains were examined for their effect on spore settlement. Results showed that the effect of bacterial strains on spore settlement was strain- but not taxon-specific and activity varied with the age and density of the biofilm. The results suggest the possibility of prokaryotic-eukaryotic signalling and in associated work we have subsequently shown that one type of cue is the acylated homoserine autoinducer compounds that many bacteria produce in the 'quorum-sensing' process involved in biofilm formation (Joint *et al.*, 2002). This is the first reported example of molecular cross-talk between prokaryotes and eukaryotes based on this particular type of molecule.

CONCLUSIONS: These studies provide the most comprehensive description and quantitative analysis of the settlement and adhesion processes and the roles of surface-associated cues, of any soft-fouling species to date. We have shown that *Enteromorpha* spores respond to a variety or cues including chemical, physico-chemical, biological (microbial biofilm) and topographic. Major new insights have been gained on communication processes that may be involved in the early stages of developing a micro-fouling community. Compounds that inhibit spore settlement could be employed in coatings to deter fouling. Understanding which factors promote settlement will inform the development of novel control strategies. Of immediate practical importance, our work has shown that in order to minimise fouling by *Enteromorpha,* surfaces should be smooth, since surface defects of a few microns amplitude will attract and shelter swimming algal spores. Coatings that contain known spore settlement promoters should also be avoided. The assays developed during this programme form the basis of test methods being used in a parallel ONR-funded programme to screen coatings for inhibition of algal settlement and foul-release potential.

SIGNIFICANCE: *Enteromorpha* is the most important macroalga that fouls ships and submarines whether coated with conventional copper-based paints or foul-release silicones. Since the occurrence of ship hulls fouled by *Enteromorpha* is currently increasing as a consequence of the increased deployment of TBT-free coatings, it is important to understand the processes that control and moderate spore settlement and adhesion. The most critical event in surface colonisation is the selection of a suitable surface by a motile spore since commitment to adhesion results in permanent attachment. Understanding these processes is fundamental to the development of novel coatings designed to prevent or minimise attachment.

PATENT INFORMATION: None

AWARD INFORMATION: MEC was promoted to Senior Research Fellow

PUBLICATIONS AND ABSTRACTS:

Callow J A, Stanley M S, Wetherbee R, Callow M E (2000a) Cellular and molecular approaches to understanding primary adhesion in *Enteromorpha*: an overview. *Biofouling* **16**: 141-150

Callow M E, Callow J A (1998a) Attachment of zoospores of the fouling alga *Enteromorpha* in the presence of zosteric acid. *Biofouling* **13**: 87-95

Callow M E, Callow J A (1998b) Enhanced adhesion and chemoattraction of zoospores of the fouling alga *Enteromorpha* to some foul-release silicone elastomers. *Biofouling* **13**: 157-172

Callow M E, Callow J A, Ista L K, Coleman S E, Nolasco A C, Lopez G P (2000b) The use of self-assembled monolayers of different wettabilities to study surface selection and primary adhesion processes of green algal (*Enteromorpha*) zoospores. *Applied and Environmental Microbiology* **66**: 3249-3254

Callow M E, Callow J A, Pickett-Heaps J D, Wetherbee R (1997) Primary adhesion of *Enteromorpha* (Chlorophyta, Ulvales) propagules: quantitative settlement studies and video microscopy. *Journal of Phycology* **33**: 938-947

Callow M E, Crawford S, Wetherbee R, Taylor K, Finlay J A, Callow J A (2001) Brefeldin A affects adhesion of zoospores of the green alga *Enteromorpha*. *J Exp Bot* **52**: 1409-1415

Callow M E, Jennings A R, Brennan A B, Seegert C E, Gibson A, Wilson L, Feinberg A, Baney R, Callow J A (2002) Microtopographic cues for settlement of zoospores of the green fouling alga *Enteromorpha*. *Biofouling* **18**: 237-245

Finlay J A, Callow M E, Ista L K, Lopez G P, Callow J A (2002) The influence of surface wettability on the adhesion strength of settled spores of the green alga *Enteromorpha* and the diatom *Amphora*. *Integrated Comparative Biology* **(In Press)**

Joint I, Callow M E, Callow J A, Clarke K R (2000) The attachment of *Enteromorpha* zoospores to a bacterial biofilm assemblage. *Biofouling* **16**: 151-158

Joint I, Tait K, Callow M E, Callow J A, Milton M, Williams P, Cámara M (2002) Cell-to-cell communication across the procaryote / eucaryote boundary. *Science* **(in press)**

Patel P, Callow M E, Joint I, Callow J A (2003) Specificity in the settlement - modifying response of bacterial biofilms towards zoospores of the marine alga Enteromorpha. *Submitted*

Stanley M S, Callow M E, Callow J A (1999) Monoclonal antibodies to adhesive cell coat glycoproteins secreted by zoospores of the green alga *Enteromorpha*. *Planta* **210**: 61-71

Wilkerson W R, Seegert C A, Feinberg A W, Zhao L C, Callow J A, Callow M E, Brennan A B (2001) Bioadhesion studies on microtextured siloxane elastomers. *Polymer Preprints* **42**: 147-148

OTHER:

Callow M E, Callow J A, Pickett-Heaps J D, Wetherbee R (1997) Primary adhesion of Enteromorpha propagules. 6th International Phycological Congress, Leiden 9-5 August (Abstract)

Callow M E, Callow J A (2000) Some seaweed surprises. *BBSRC Business*. April 2000. 16-18

Callow M E, Callow J A, Ista L K, Lopez G P, Brennan A B (2000)Chemical and surface-associated cues for attachment of spores of the green alga Enteromorpha. Polymillennial 2000, Waikoloa, Hawaii. (Abstract)

Feinberg A W, Gibson A L, Wilkerson W R, Seegert C A, Wilson L H, Zhao L C, Baney R H, Callow J A, Callow M E, Brennan A B (2001) Investigating the energetics of bioadhesion on micro-engineered siloxane elastomers. ACS symposium on silicones. San Diego April 2001.(Chapter)

Callow M E, Callow J A (2002) Marine Biofouling: a sticky problem. *Biologist* **49**: 10-14

Callow M E, Callow J A, Finlay J A, Jennings A R, Patel P, Brennan A B, Seegert C E, Gibson A, Feinberg A, Baney R, Ista L K, Lopez G P (2002) Cue detection and surface selection in the settlement of spores of the green alga *Enteromorpha*. 11th ICMCF, San Diego 21 - 26 July 2002. (Abstract)